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CAROTENOIDES E CLOROFILAS MICROALGAIS: ÊNFASE NA CARACTERIZAÇÃO E BIOACESSIBILIDADE

Santa Maria, RS
2021

Andrêssa Silva Fernandes

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

Orientadora: Profª Drª. Leila Queiroz Zepka

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Gratidão!

“Sempre haverá outra montanha

Eu sempre vou querer fazer isso se mover

Sempre vai ser uma batalha difícil

Às vezes vou ter que perder

Não é sobre o quão rápido eu chego lá

Não é sobre o que está esperando do outro lado

É a subida!”

(The Climb, Miley Cyrus)

RESUMO

CAROTENOIDES E CLOROFILAS MICROALGAIS: ÊNFASE NA CARACTERIZAÇÃO E BIOACESSIBILIDADE

AUTORA: Andrêssa Silva Fernandes
ORIENTADORA: Leila Queiroz Zepka

Microalgas são fábricas biometabólicas sustentáveis de produção de uma diversidade de bioprodutos como proteínas, carboidratos, ácidos graxos e principalmente moléculas bioativas que podem desempenhar efeitos benéficos na saúde humana, como os pigmentos. Assim, a biodiversidade e ecologia microalgal representam uma oportunidade para enfrentar vários dos desafios que a sociedade atual está reivindicando em relação a demandas de produtos alimentares que vão além da nutrição básica. Dentre as classes de pigmentos naturais, os carotenoides são os mais estudados, e poucos trabalhos dedicam-se ao perfil de clorofilas. Além disso, considerando o significativo número de espécies disponíveis para exploração, ainda são poucos os estudos neste campo de pesquisa, onde um número significativo de cepas permanece sub exploradas. Diante deste cenário, o presente trabalho fundamenta-se em um estudo exploratório acerca da caracterização do perfil de carotenoides e clorofilas em espécies de microalgas, bem como determinar a bioacessibilidade destes compostos a fim de orientar seu uso em produtos funcionais e nutracêuticos. Como resultado gerou-se 5 artigos e 2 capítulos de livros (publicados ou em processo de publicação) que foram organizados neste documento em capítulos. Os capítulos de 2 a 6 referem-se aos artigos e o 7 e 8 referem-se as contribuições em livros. Capítulo 2: *"An overview of microalgae carotenoids and chlorophylls: focus on bioaccessibility"* (artigo de revisão em processo de publicação); Capítulo 3: *"HPLC-PDA-MS/MS as a strategy to characterize and quantify natural pigments from microalgae"* (artigo de pesquisa/publicação concluída); Capítulo 4: *"Determination of profile of chlorophyll compounds in microalgae species"* (artigo de pesquisa/publicação concluída); Capítulo 5: *"Insights on the intestinal absorption of chlorophyll series from microalgae"* (artigo de pesquisa/publicação concluída); Capítulo 6: *"Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix"* (artigo de pesquisa/publicação concluída); Capítulo 7: *"Carotenoids: "A brief overview on its structure, biosynthesis, synthesis, and applications"* (capítulo de livro publicado); Capítulo 8: *"Chlorophylls as food additives"* (capítulo de livro publicado). Os resultados destas produções científicas indicam que as microalgas sintetizam uma diversidade de carotenoides e clorofilas, muitas destas estruturas específicas destes microrganismos e com importantes propriedades bioativas proeminentes a saúde humana. Muitos destes compostos foram identificados pela primeira vez nas espécies avaliadas. Diferenças entre as espécies de microalgas tanto no perfil de carotenoides, quanto no perfil de clorofilas foram observadas, demostrando que cada espécie possui uma constituição bioquímica única. A bioacessibilidade destes compostos também variou de acordo com a espécie de microalga e a natureza do produto digerido. Sobretudo, carotenoides e clorofilas microalgais demonstraram-se bioacessíveis tanto a partir da biomassa microalgal como a partir de extratos isolados. Clorofilas também demostraram ser captados com eficiência pelas células Caco-2. De fato, estes resultados reforçam a diversidade metabólica de microalgas, seu potencial biotecnológico e indicam estes microrganismos como fontes promissoras de uma diversidade de carotenoides e clorofilas naturais a serem fortemente explorados para fins que visem alimentação e saúde. Por fim, este estudo pode auxiliar na formulação e desenvolvimento futuro de novos alimentos naturais, funcionais e nutracêuticos e produtos a partir de ingredientes à base de microalgas.

Palavras-chave: Microalgas. Microalgas verdes. Cianobactérias. Biocompostos. Pigmentos. Compostos bioativos. Carotenoides. Clorofilas. Bioacessibilidade.

ABSTRACT

MICROALGAE CAROTENOIDS AND CHLOROPHILS: EMPHASIS ON CHARACTERIZATION AND BIOACCESSIBILITY

AUTHOR: Andrêssa Silva Fernandes
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Microalgae are sustainable biometabolic factories for the production of a variety of bioproducts such as proteins, carbohydrates, fatty acids and mainly bioactive molecules that can have beneficial effects on human health, such as pigments. Thus, biodiversity and microalgae ecology represents an opportunity to face several challenges that society today is claiming regarding the demands for food products beyond basic nutrition. Among the classes of natural pigments, carotenoids are the most studied, and few studies are devoted to the profile of chlorophylls. Moreover, considering the significant number of species available for exploration, there are still few studies in this research field where a considerable number of strains remain under-explored. Given this scenario, this work is based on an exploratory study on the characterization of the profile of carotenoids and chlorophylls in microalgae species and determining the bioaccessibility of these compounds to guide their use in functional and nutraceutical products. As a result, five articles and two book chapters (published or in the publication process) were generated, which were organized in this document into chapters. Chapters 2 to 6 refer to articles, and chapters 7 and 8 refer to book contributions. Chapter 2: *"An overview of microalgae carotenoids and chlorophylls: focus on bioaccessibility"* (review article in the process of publication); Chapter 3: *"HPLC-PDA-MS/MS as a strategy to characterize and quantify natural pigments from microalgae"* (research article/publication completed); Chapter 4: *"Determination of the profile of chlorophyll compounds in microalgae species"* (research article/publication completed); Chapter 5: *"Insights on the intestinal absorption of chlorophyll series from microalgae"* (research article/publication completed); Chapter 6: *"Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix"* (research article/publication completed); Chapter 7: *"Carotenoids: "A brief overview on its structure, biosynthesis, synthesis, and applications"* (published book chapter); Chapter 8: "Chlorophylls as food additives" (published book chapter). The results of these scientific productions indicate that microalgae synthesize a variety of carotenoids and chlorophylls, many of these structures specific to these microorganisms and with important bioactive properties prominent in human health. Many of these compounds were identified for the first time in the species evaluated. Differences in the carotenoids and chlorophylls profile were observed among microalgae species, demonstrating that each species has a unique biochemical constitution. The bioaccessibility of these compounds also varied according to the microalgae species and nature of the product digested. Above all, microalgae carotenoids and chlorophylls proved to be bioaccessible both from microalgae biomass like from isolated extracts. Chlorophylls have also been shown to be efficiently uptake by Caco-2 cells. In fact, these results reinforce the metabolic diversity of microalgae, their biotechnological potential and indicate these microorganisms as promising sources of a diversity of natural carotenoids and chlorophylls to be heavily exploited for purposes that aim at food and health. Finally, this study can assist in the formulation and future development of new natural, functional, and nutraceutical foods and products from microalgae-based ingredients.

Keywords: Microalgae. Green microalgae. Cyanobacteria. Biocompounds. Pigments. Bioactive compounds. Carotenoids. Chlorophylls. Bioaccessibility.

APRESENTAÇÃO

Esta tese de doutorado está organizada em nove capítulos principais, sendo o primeiro composto pela Introdução e Objetivos. O Capítulo 2 é composto pela revisão bibliográfica acerca dos tópicos que fundamentam esta pesquisa e está em fase de submissão. Nos Capítulos 3, 4, 5, 6, 7 e 8 encontram-se todos os resultados do doutoramento e as discussões, que são apresentados na forma de 4 artigos publicados e 2 capítulos de livros. Por fim, o Capítulo 9 contempla a conclusão geral do trabalho e o item Referências refere-se somente àquelas inseridas na Introdução. A sequência dos trabalhos segue uma ordem cronológica de execução.

SUMÁRIO

CAPÍTULO 1

INTRODUÇÃO GERAL E OBJETIVOS

1. INTRODUÇÃO

De acordo com a Organização das Nações Unidas para a Alimentação e a Agricultura (FAO), um dos maiores desafios que os sistemas alimentares globais enfrentam é nutrir e alimentar a população em crescimento por meio de sistemas alimentares sustentáveis (FAO, 2021). Por outro lado, as mudanças nas preferências alimentares estão aumentando, devido à maior conscientização da população sobre o impacto positivo das dietas saudáveis na promoção da boa saúde, melhorando o bem-estar e a qualidade de vida, o que torna os alimentos funcionais mais influentes no sistema alimentar global (KATIYAR & ARORA, 2020). Esses paradigmas levam a uma nova era de ingredientes e produtos alimentícios cada vez mais nutritivos, funcionais, inovadores e também sustentáveis (MINTEL, 2021). Em decorrência destes aspectos, um dos tópicos mais atuais de estudo na área de alimentos e saúde é o interesse em identificar novos compostos ou fontes alimentares que apresentem potencialidades bioativas que vão além da nutrição básica.

Em ambos os cenários, a biomassa de microalgas pode corroborar e se posicionar com sucesso. Microalgas são um grupo polifilético e altamente diversificado de microrganismos fotossintetizantes, considerados uma fonte sustentável promissora de bioprodutos valiosos em decorrência da ampla diversidade metabólica e alto potencial biotecnológico (BOROWITZKA, 2018; DOLGANYUK et al., 2020). O valor nutricional e funcional das microalgas utilizadas como alimento decorre de seu alto teor de nutrientes e compostos bioativos, como proteínas, ácidos graxos poliinsaturados, esteróis, polissacarídeos, vitaminas, minerais, compostos fenólicos, compostos voláteis, carotenoides e clorofilas (JACOB- LOPES et al., 2019; KUSMAYADI et al., 2021).

Nos últimos anos a caracterização de compostos bioativos a partir de microalgas surge como uma forte tendência, principalmente no desenvolvimento de novos alimentos funcionais e nutracêuticos. Dentre estes compostos ganham destaque os carotenoides e clorofilas, compostos com alto potencial de exploração como pigmentos naturais e moléculas biologicamente ativas, as quais apresentam importantes propriedades de benefícios a saúde (MATOS, 2017; CAPORGNO & MATHYS, 2018; NWOBA et al., 2020).

A possibilidade de valorização dos pigmentos gerados por bioprocessos mediados por microalgas decorre das frações significativas de carotenoides e clorofilas, com rápida taxa de biossíntese e composição única em biomassa microalgal (MULDERS, 2014; PAGELS et al., 2020). Essas são consideradas as principais classes de pigmentos fotossintéticos em microalgas, seguidos por ficobilinas. Entretanto, majoritariamente os carotenoides são as especialidades

químicas mais estudadas a partir destes microrganismos (SILVA et al., 2020). São considerados como modelo de sucesso em termos de viabilidade econômica, através do cultivo de *Dunaliella salina* e *Haematococcus pluvialis* com foco na produção bem consolidada de β-caroteno e astaxantina, respectivamente (RAMMUNI et al., 2019).

Essa classe de compostos é constituída por aproximadamente 1200 moléculas devidamente caracterizadas, as quais contemplam os carotenoides microalgais (YABUZAKI, 2021). No geral, são moléculas tetraterpênicas, consideradas importantes pigmentos acessórios no metabolismo fotossintético, pois absorvem luz nas regiões do espectro visível em que a clorofila não absorve eficientemente e atuam fotoprotegendo os sistemas e as células de danos oxidativos (AMBATI et al., 2019). Carotenoides juntamente com clorofilas, contribuem significativamente para a caracterização de novas espécies do fitoplâncton, uma vez que cada grupo de microrganismos exibe um perfil diversificado e único de pigmentos (PALIWAL et al., 2016; HUANG et al., 2017; SERIVE et al., 2017). Considerando ser necessário a exaustiva caracterização dessas biomoléculas, a composição de carotenoides em distintas espécies de microalgas tem sido investigada (RODRIGUES et al., 2015; PATIAS et al., 2017; DI LENA et al., 2019; NÖRNBERG et al., 2021). Em consequência, o específico perfil quantitativo e qualitativo dos carotenoides encontrados em diferentes biomassas microalgais confirma o seu potencial de exploração para esses compostos (NOVOVESKÁ et al., 2019).

No que se refere às clorofilas, poucos trabalhos são reportados na literatura sobre a caracterização completa do perfil destes compostos em microalgas. A maioria dos relatos disponíveis concentram esforços apenas no perfil quantitativo do total de clorofilas, ou moléculas específicas como clorofila a e b, sem analisar a composição íntegra presente no extrato (HALIM & DANQUAH, 2013; PETROVIC, ZVEZDANOVIĆ & MARKOVIĆ, 2017; SANTHAKUMARAN, KOOKAL & RAY, 2018; SARKAR et al., 2020). Além disso, um agravante nesse campo de pesquisa é o uso preferencial por detectores Uv-vis, visto que analisadores de massa (MS) tem se mostrado necessários para uma identificação confiável de clorofilas e compostos derivados (CHEN et al., 2015a; CHEN et al., 2015b; CHEN et al., 2017). Devido ao seu papel primário de captação de energia na fotossíntese, as clorofilas (principalmente clorofila a e b e feofitina a) são pigmentos onipresentes em organismos fotossintéticos. São compostos tetrapirrólicos cíclicos que compreendem um grupo de aproximadamente 100 estruturas químicas diferentes, com espécies de clorofila a, b, c, d e f e seus compostos derivadas (ROCA, CHEN & Pérez-Gálvez, 2016; ZEPKA, JACOB-LOPES & ROCA, 2019). Em decorrência de sua conformação estrutural, clorofilas juntamente com ficobilinas e ficobiliproteínas representam quase as únicas alternativas versáteis para corantes

alimentares naturais com cor esverdeada e/ou azulada, o que impulsiona investigações (PÉREZ-GÁLVEZ, VIERA & ROCA, 2017; VIERA, PÉREZ-GÁLVEZ & ROCA, 2019). Os gêneros de microalgas *Chlorella* e *Spirulina* são amplamente explorados para produção de clorofilas, sendo a primeira considerada "Alimento esmeralda" devido ao seu incrivelmente alto teor de clorofila (7 % do teor total de biomassa seca) (SAHNI et al., 2019).

Apesar dos principais interesses associados a carotenoides e clorofilas estarem relacionados à cor, muita atenção tem sido dada a estes compostos devido aos seus potenciais efeitos promocionais para a saúde humana relacionados com suas possíveis propriedades biológicas. Atividades como pró-vitamina A, antioxidante, anti-inflamatória, anti-tumor, anticarcinogênica, controle da obesidade, controle de doenças cardiovasculares e ação preventiva contra degeneração macular são alguns dos efeitos biológicos associados aos carotenoides (RODRIGUEZ-CONCEPCION et al., 2018; EGGERSDORFER & WYSS, 2018). Em adição, as clorofilas também têm apresentado importante papel na atividade anti-inflamatória, antiviral, antimicrobiana, antigenotóxica, antimutagênica e potente capacidade antioxidante para eliminar radicais livres e prevenir a oxidação lipídica (SOLYMOSI & MYSLIWA-KURDZIEL, 2017; PAREEK et al., 2017; PÉREZ-GÁLVEZ, VIERA & ROCA, 2017). Sendo, o perfil quantitativo e qualitativo destes compostos, decisivos na sua capacidade de modulação dessas ações.

Entretanto, para que esses compostos bioativos lipofílicos desempenhem suas atividades biológicas na promoção da saúde, eles devem ser liberados da matriz alimentícia após a ingestão, solubilizados em gotículas lipídicas, transferidos para micelas mistas para subsequente captação por células epiteliais absortivas no intestino delgado, para posteriormente serem incorporados em quilomícrons e secretados para os tecidos alvo (FERRUZZI & BLAKESLE, 2007; FERNÁNDEZ-GARCÍA et al., 2012; KOPEC & FAILLA, 2018). Em outras palavras, estes compostos devem estar primeiramente bioacessíveis e, portanto, o teor destes compostos na matriz alimentar não é necessariamente indicativo da quantidade que é acessível para absorção intestinal. Nesse sentido, ensaios de bioacessibilidade são estritamente necessários para estudos que visem a aplicação de biocompostos como componente funcional ou nutracêutico (DIMA et al., 2020).

Por sua vez, considerando o significativo número de espécies de microalgas, existe ainda uma ampla variedade de cepas a serem exploradas para a obtenção de compostos bioativos naturais. As microalgas pertencentes a classe das *Cyanophyceae* e *Chlorophyceae* contém espécies que possui alto potencial biotecnológico e um perfil bioquímico atrativo, tornando-a altamente utilizada em sistemas laboratoriais e pilotos (PATIAS et al., 2017;

VENDRUSCOLO et al., 2018; SEVERO et al., 2018; WANG et al., 2019; VENDRUSCOLO et al., 2019; FAGUNDES et al., 2019; MARONEZE et al., 2019; NASCIMENTO et al., 2021). No entanto, os relatos encontrados na literatura sobre caracterizações do perfil e estudo da bioacessibilidade de carotenoides e clorofilas em espécies de microalgas pertencentes a estas classes, são escassos ou referem-se apenas a alguns compostos.

Neste sentido, considerando a importância destas moléculas e visando avançar o conhecimento científico sobre fontes naturais alternativas de compostos bioativos, o presente trabalho fundamenta-se em um estudo exploratório acerca da caracterização do perfil de carotenoides e clorofilas e avaliação da bioacessibilidade destes compostos em diferentes espécies de microalgas sub exploradas para este fim.

2. OBJETIVOS

2.1 Objetivo geral

O objetivo geral deste trabalho fundamenta-se em um estudo exploratório acerca da caracterização do perfil de carotenoides e clorofilas em diferentes espécies de microalgas, bem como avaliar a bioacessibilidade destes compostos, visando ampliar o conhecimento sobre essas biomoléculas em fontes microalgais.

2.2 Objetivos específicos

Para atingir o objetivo geral foram estabelecidos os seguintes objetivos específicos:

- Explorar espécies de microalgas com perfil atrativo para fins alimentares.

- Cultivar as espécies de microalgas em fotobiorreatores para obtenção de biomassas.

- Caracterizar o perfil qualitativo de clorofilas e compostos derivados das microalgas *Scenedesmus obliquus*, *Chlorella vulgaris* e *Aphanothece microscópica Nägeli* por HPLC-PDA-MS/MS.

- Determinar o perfil quantitativo de clorofilas e compostos derivados das microalgas *Scenedesmus obliquus*, *Chlorella vulgaris* e *Aphanothece microscópica Nägeli* por HPLC-PDA.

- Caracterizar o perfil qualitativo de carotenoides e compostos clorofilados em *Scenedesmus bijuga* e *Chlorella sorokiniana* HPLC-PDA-MS/MS.

- Determinar o perfil quantitativo de carotenoides e compostos clorofilados em *Scenedesmus bijuga* e *Chlorella sorokiniana* por HPLC-PDA.

-Avaliar a bioacessibilidade das clorofilas de *Scenedesmus obliquus* a partir da biomassa seca total, biomassa úmida sonicada e extrato isolado de clorofilas.

- Avaliar a captação intestinal do extrato de clorofilas de *Scenedesmus obliquus* pelas células Caco-2.

-Avaliar a bioacessibilidade dos carotenoides de *Scenedesmus bijuga e Chlorella sorokiniana* a partir da biomassa úmida sonicada.

- Avaliar a influência da fração lipídica da biomassa de *Scenedesmus bijuga e Chlorella sorokiniana* na bioacessibilidade dos carotenoides.

CAPÍTULO 2

REVISÃO BIBLIOGRÁFICA

An overview on microalgae carotenoids and chlorophylls: focus in the bioaccessibility

Artigo publicado na revista *Brazilian Journal of Development*[1].

[1]Fernandes, A. S., Jacob-Lopes, E., & Zepka, L. Q. (2021). An overview on microalgae carotenoids and chlorophylls: focus in the bioaccessibility. Brazilian Journal of Development, 7(8), 82727-82760. https://doi.org/10.34117/bjdv7n8-470.

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RESUMO
Atualmente, a tendência mundial por hábitos alimentares cada vez mais saudáveis impulsiona consideravelmente a busca por alternativas naturais capazes de modular positivamente a saúde humana. Consequentemente, esse comportamento tem destacado produtos e ingredientes à base de microalgas. As microalgas são um grupo diversificado de microrganismos considerados uma fonte atrativa de várias moléculas biologicamente ativas. Em evidência estão os carotenoides e clorofilas, visto que potenciais funções biológicas promotoras da saúde têm sido constantemente associadas a essas biomoléculas. No entanto, para que esses compostos exerçam tais atividades, eles precisam ser bioacessíveis e absorvidos pelo corpo humano. Diante disso, essa breve revisão visa elucidar os principais aspectos relacionados aos carotenoides e clorofilas das microalgas, bem como abordar a bioacessibilidade e biodisponibilidade desses compostos.

Palavras-Chave: Microalgas, Biomassa, Biocompostos, Pigmentos Naturais, Carotenoides, Clorofilas, Bioacessibilidade, Biodisponibilidade.

ABSTRACT
Currently, the global trend towards healthier eating habits considerably boosts the search for natural alternatives capable of positively modulating human health. Consequently, this behavior has highlighted microalgae-based products and ingredients. Microalgae are a diverse group of microorganisms considered an attractive source of various biologically active molecules. In evidence are the carotenoids and chlorophylls, as potential biological health-promoting functions have been constantly associated with these biomolecules. However, for these compounds to exert such activities, they need to be bioaccessible and absorbed by the human body. Given this, this brief review aims to elucidate the main aspects related to carotenoids and chlorophylls from microalgae, as well as to approach the bioaccessibility and bioavailability of these compounds.

Keywords: Microalgae, Biomass, Biocompounds, Natural Pigments, Carotenoids, Chlorophylls, Bioaccessibility, Bioavailability.

1 INTRODUCTION

In recent decades there has been a global trend towards a lifestyle that contemplates a feed that goes beyond basic nutrition (Fanzo et al., 2020). This behavior strongly drives research and development in science and technology for new foods or food ingredients increasingly nutritious, functional, and sustainable (Tzachor et al., 2021). Consequently, it highlights microalgae, microorganisms considered potential sources for obtaining future food (Torres-Tiji et al., 2020).

This targeting is partly a consequence of metabolic versatility and microalgae biodiversity, making them an almost untapped resource of biologically active molecules (Tang et al., 2020). They are capable of biosynthesizing a wide range of phytochemicals such as carbohydrates, proteins, lipids, sterols, minerals, vitamins, and pigments (Jacob-Lopes et al., 2019).

In particular, fine chemical compounds found in microalgae are being successfully allocated to industrial sectors with increasing demands. This is the case of natural pigments such as carotenoids and chlorophylls that currently need emerging sources to consolidate the market need due to safety concerns with synthetic sources (Nwoba et al., 2020). The growing interest in this group of biomolecules stems from its effectiveness in promoting human health, which is why its use has potentially been directed to various applications in the food, pharmaceutical and cosmetic industries (Silva et al., 2020).

Microalgae are potential sources of a variety of carotenoids (Novoveska et al., 2019). They synthesize complex mixtures of these compounds, from structures found in higher plants, such as lutein, β-carotene and zeaxanthin, to microalgae-specific

carotenoids with enhanced bioactive abilities, such as echinenone, astaxanthin and canthaxanthin (Rodrigues et al., 2015; Patias et al., 2017; Nascimento et al., 2021; Nörnberg et al., 2021). Carotenoids are a vast group of terpenoid pigments in which many of these structures play a notable role as antioxidants, vitamin A precursors, and prevention and maintaining eye health (Rodriguez-Concepcion et al., 2018). In addition, scientific evidence suggests its role in reducing cardiovascular disease, cancer, obesity, immune function, diabetes, protecting neurons, among other important activities (Eggersdorfer and Wyss, 2018).

Chlorophylls are ubiquitous molecules in microalgae, which have a large and diverse content of this class of pigments, including Mg-free chlorophyll (pheophytin), dephytylated chlorophyll (pheophorbide), and its oxidized and epimer derivatives, as well as types of chlorophyll a, b, c, d and f (Zepka et al., 2019; Fernandes et al., 2021). They are widely used as a natural food coloring agent and have antioxidant, wound healing, and antimutagenic properties (Sarkar et al., 2020). In addition, other activities such as the ability to reduce the risk of some types of cancer, obesity control, anti-inflammatory properties, antimicrobial activities, antiviral activity, immunostimulatory activity, and anti-parasite activity are constantly associated with the ingestion of these biomolecules (Pérez-Gálvez et al., 2017; Saide et al., 2020).

However, for these bioactive compounds to play a role in promoting health and biological functions, they need to be bioaccessible for intestinal uptake and subsequent systemic distribution in the human body (Kopec and Failla, 2018). These parameters are monitored through bioaccessibility and bioavailability assays and are strictly necessary for studies aimed at applying biocompounds as a functional or nutraceutical component (Dima et al., 2020).

In this sense, this brief review brings together a description of the main characteristics of carotenoids and microalgae chlorophylls, with the main focus on aspects related to the bioaccessibility and bioavailability of these compounds.

2 MICROALGAE

Microalgae are a heterogeneous group of microscopic organisms with a complex taxonomy, as they designate organisms that are very different from one another in terms of origin, chemical composition and morphology. Constitute a polyphyletic group of unicellular photosynthetic microorganisms, ubiquitous, mostly present in aquatic systems, with planktonic and benthic habits (Lourenço, 2006; Sathasivam et al., 2017).

According to Guiry and Guiry (2021), under the name of microalgae, organisms with two types of cell structure are included, phylogenetically classified as prokaryotic, with representatives in the groups Cyanophyta and Prochlorophyta; eukaryotic structure, with representatives in the groups Glaucophyta, Rhodophyta, Ochrophyta, Haptophyta, Cryptophytes, Dinophyta, Euglenophyta, Chlorarachniophyta, and Chlorophyta.

Because they have high metabolic versatility, these microorganisms can be cultivated under different modes for the production of biomass, such as photoautotrophic cultivation that involves the photosynthesis process, heterotrophic cultures, where it is necessary to insert organic carbon sources and mixotrophic cultivation, which comprises both autotrophic and heterotrophic conditions (Hu et al., 2018; Pang et al., 2019). Microalgae can also grow up to 50 times faster than terrestrial plants, thus achieving a higher rate of CO_2 fixation (Mountourakis et al., 2021). Thus, due to the substantial capacity of some microalgae species bioconverts organic material and nutrients present in wastewater and mitigate atmospheric CO_2 (Khan et al., 2018), these microorganisms offer an alternative to conventional forms of agro-industrial effluent treatments, with the simultaneous generation of co-products in the form of biomass (Rodrigues et al., 2014; Fernandes et al., 2017).

In turn, they are microorganisms capable of biosynthesizing, accumulating and secreting a wide variety of metabolites in response to growing conditions, many of which are high-value substances with industrial applications and health benefits (Vieira et al., 2020). However, the biodiversity of microalgae represents an almost untapped resource, since of the possible 20,000 to 800,000 existing species, relatively about 40-50,000 species have been studied in detail from a biochemical and physiological point of view (Suganya et al., 2016).

In terms of exploration and biotechnological use, the following groups stand out: cyanobacteria (Cyanophyta), chlorophytes (Chlorophyta) and diatoms (Ochrophyta). In contrast, the most abundant classes are (Bacillariophyceae), green algae (Crysophyceae), and golden algae (Chrysophyceae) (Borowitzka, 2018; Jacob-Lopes et al., 2019).

The application of microalgae biomass or its commodities as intermediate ingredients or final products touches on several industrial sectors related to bioenergy, animal feed, biofertilizers, food, pharmaceutical, nutraceuticals and cosmetics products (Rizwan et al., 2018; Sahni et al., 2019). However, the greatest exploration and application has been in the food, pharmaceutical, nutraceuticals products. This is because the presence of a diversity of bioactive metabolites in microalgae seems to be a promising

approach for the development of healthier, more functional and sustainable products (Barkia et al., 2019; Lafarga, 2019; Tang et al., 2020).

In the nutraceutical industries, Spirulina and Chlorella are the most important species to be marketed as healthy foods and nutritional supplements with several health benefits, including increased immune system activity, antitumor effects and the promotion of animal growth, due to their abundant proteins, vitamins, active polysaccharides and other important compounds (Camacho et al., 2019). In parallel, Haematococcus pluvialis and Dunaliella salina are produced at an industrial level to obtain carotenoids, especially astaxanthin (a potent antioxidant), and β-carotene (precursor to vitamin A), respectively, while phycocyanins are commercially produced from Arthrospira platensis (Spirulina) (Silva et al., 2020). However, other species need to be scientifically explored to increase the portfolio of microalgae and their bioproducts for industrial application.

3 MICROALGAE BIOPRODUCTS

Microalgae have been considered as alternative sustainable resources for high value bioproducts, such as proteins, lipids, polyunsaturated fatty acids, carbohydrates, minerals, vitamins, polysaccharides and pigments, among other compounds in constant exploration. These biomolecules from microalgae biomass have qualitative and quantitative characteristics that consolidate the biological, nutritional, and functional value of these microorganisms (Jacob-lopes et al., 2019; Dolganyuk et al., 2020). The cellular composition of each microalgae varies according to the species and their physiological responses to growing conditions (Jabri et al., 2021).

Phycobiliproteins, carotenoids and chlorophylls are the three major classes of bioactive photosynthetic pigments in microalgae, which are responsible for the colors red/blue, yellow/orange and green, respectively (Pagels et al., 2020). Carotenoids and chlorophylls are synthesized by all microalgae species, but only the Cyanophyta (blue-green algae), Cryptophyta (Cryptomonas) and Rhodophyta (red algae) divisions synthesize phycobiliproteins (Nwoba et al., 2020).

In addition to their potential application these pigments in aquaculture and in the food and pharmaceutical industries, many of these compounds have been related to beneficial health effects, which are mainly due to their antioxidant properties, anticancer, anti-inflammatory, anti-obesity, neuroprotective activities, among other health promoting benefits (Galasso et al., 2019). Thus, natural pigments are currently one of the most

commercialized products from microalgae, renewing the interest in exploring these substances in microalgae biomass (Silva et al., 2020).

3.1 CAROTENOIDS

Carotenoids are the most abundant and widely distributed pigments on earth, second only to chlorophylls. In the last compilation by Yabuzaki (2021), it is estimated that more than 1200 natural carotenoids have been characterized in different sources, including species of the plant kingdom, animal kingdom, and microorganisms, as microalgae.

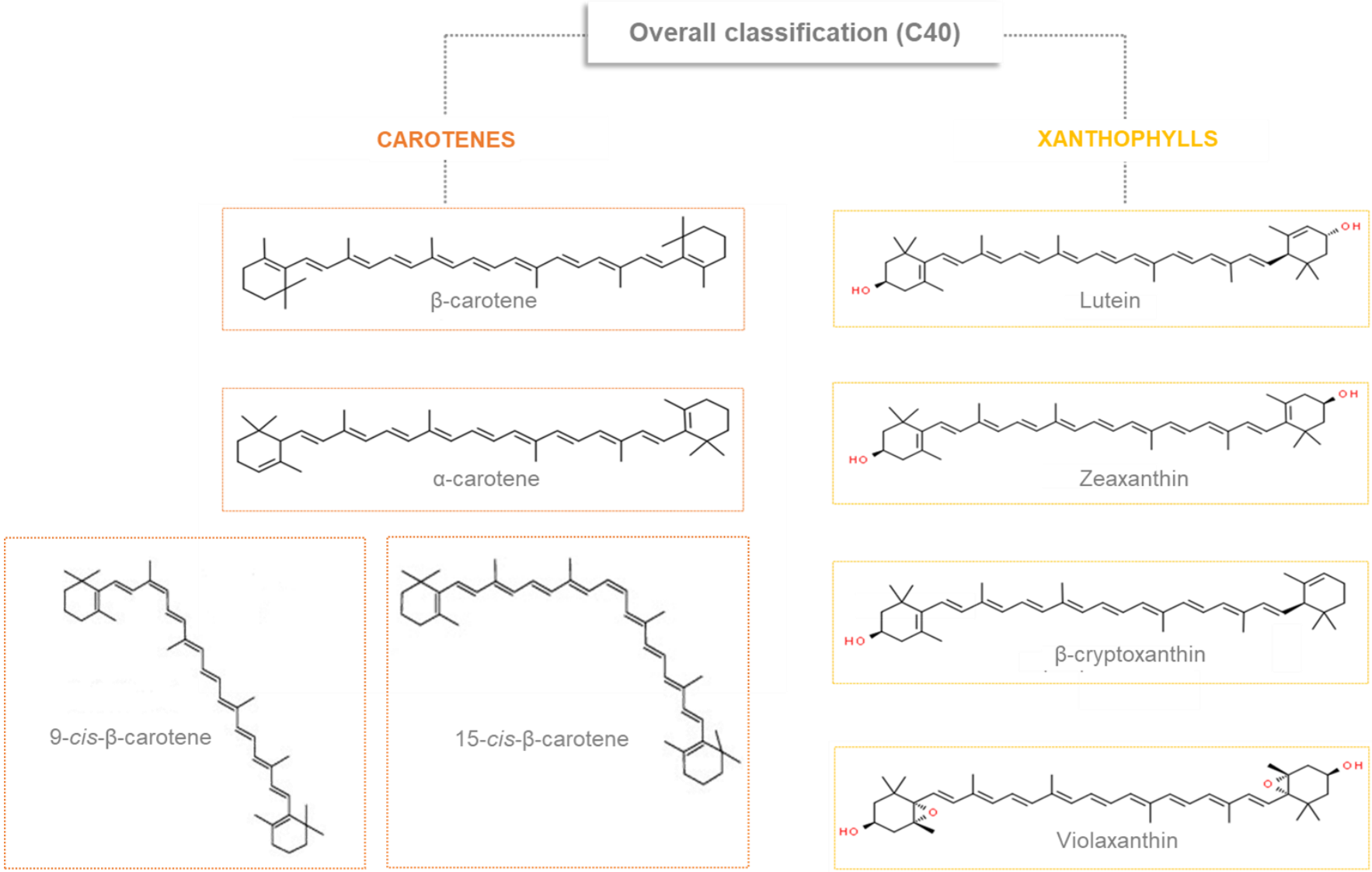
Chemically, they are classified according to the number of carbons that constitute their molecules into C30, C40, C45, and C50 carotenoid backbones. However, most carotenoids have C40-skeletons (i.e., tetraterpenes/tetraterpenoids). They are compounds fat-soluble and consist of a symmetrical structure with a set of conjugated double bonds (CDBs), which constitutes its chromophore, and which generates a π electron resonance system that moves throughout the polyene chain (Britton, 1995). Due to this structural characteristic, they absorb light in the visible region of the electromagnetic spectrum, with strong absorption in the 400 to 500 nm region (Mercadante, 2008). In turn, this part of the highly unsaturated structure is responsible for the functions, color (yellow/orange) and activities of carotenoids, such as chemical reactivity, molecular form, and activity in energy transfer processes. Also, they can be classified as acyclic (e.g., lycopene, ζ-carotene) or cyclic when they have at their ends one (monocyclic) or two (dicyclic) terminal groups in the form of rings (e.g., γ-carotene, δ-carotene) (Rodriguez-Amaya, 2015). Regarding their geometric configuration, carotenoid molecules can exist in two forms: denominated trans or cis, equivalent to E and Z. This characteristic depended exclusively on the disposition of substituent groups, specifically those that constitute a continuation of the polyene chain, about that double bond. In general, isomers (all-E)- usually have long, linear and rigid molecules, while their Z- counterparts have bent structures (Britton, 1995).

Based on their composition, carotenoids are subdivided into two groups, based on the functional groups; carotenes, which contain only the parent hydrocarbon chain without any functional group, such as α-carotene, β-carotene and lycopene, while those that contain at least one chemical function with oxygen, such as ketone (astaxanthin, canthaxanthin), hydroxyl (lutein and zeaxanthin), glycosylated (myxoxanthophyll), methoxy (spirilloxanthin) or epoxide groups (violaxanthin, neoxanthin, fucoxanthin), are

called xanthophylls, as seen in Figure 1 (Rodriguez-Concepcion et al., 2018). In addition to these important features, changes in the structure of the molecule can occur through cyclization, hydrogenation, epoxidation, dehydrogenation, the introduction of oxygen-containing groups, migration of double bonds, rearrangement, shortening or chain extension, or combinations, resulting in an arrangement of structures (Rodriguez-Amaya, 2016).

Apocarotenoids are another subclass of terpenoids that are formed from the reduction of the C40 structure by the oxidative cleavage of carotenoids. β-carotene and zeaxanthin are precursors of the main apocarotenoids described to date, including bixin, crocetin, abscisic acid, strigolactone and mycoradicin (Beltran and Stange, 2016). A recent study reported for the first

Figure 1 - Structures of carotenes and xanthophylls.



time the presence of 29 different apocarotenoids, including various apocarotenoid fatty acid esters, in different species of microalgae (Zoccali et al., 2019).

Constant advances in research and development of natural compounds from biotechnological processes, boost investigations on the composition of carotenoids in different strains of microalgae (Rodrigues et al., 2015; Patias et al., 2017; Di Lena et al., 2019; Nörnberg et al., 2021). Very recently, the occurrence of carotenoid esters in microalgae has also been reported (Maroneze et al., 2019). Carotenoid profiles vary widely between species, and the ability of microalgae to accumulate carotenoids with unique structures is well reported in the literature. Among these structures are xanthophylls with allenic (C═C═C), acetylenic (C≡C), glycosylated structures and ketocarotenoids (Takaichi, 2011). In addition, structures with chromophore more than 11 CDBs are synthesized solely from microalgae, such as echinenone and canthaxanthin, which have 12 and 13 CDBs, respectively, and have no equivalent in the plant kingdom (Rodrigues et al., 2015). Some of these structures are shown in Figure 2.

They are bioactive compounds with properties that result in biological functions beneficial to human health, classified as phytochemicals capable of modulating metabolic processes essential to cell health due to their protective action on cellular components against oxidative damage (Bohn, 2019; Khalid et al., 2019). In turn, three mechanisms have been proposed for the removal of radicals by carotenoids: electron transfer, allylic hydrogen abstraction, and addition to the conjugated double bond system (Fiedor and Burda, 2014). In addition, carotenoids also have excellent physical deactivation capabilities for singlet oxygen ($^1O_2$) through physical or chemical quenching (El-Agamey et al., 2004).

According to Rodrigues et al., (2012), the potential antioxidant effects of carotenoids are strictly related to their chemical structure, in which the number of CDBs that make up the chromophore is the most influential feature in the carotenoid's ability to reduce reactive oxygen species. Thus, it is suggested a greater antioxidant activity for microalgae carotenoids compared to conventional sources, due to the presence of exclusive carotenoids, which have a bathochromic effect, such as canthaxanthin (13 CDBs), myxoxanthophyll (12 CDBs), and echinenone (12 CDBs) (Patias et al., 2017).

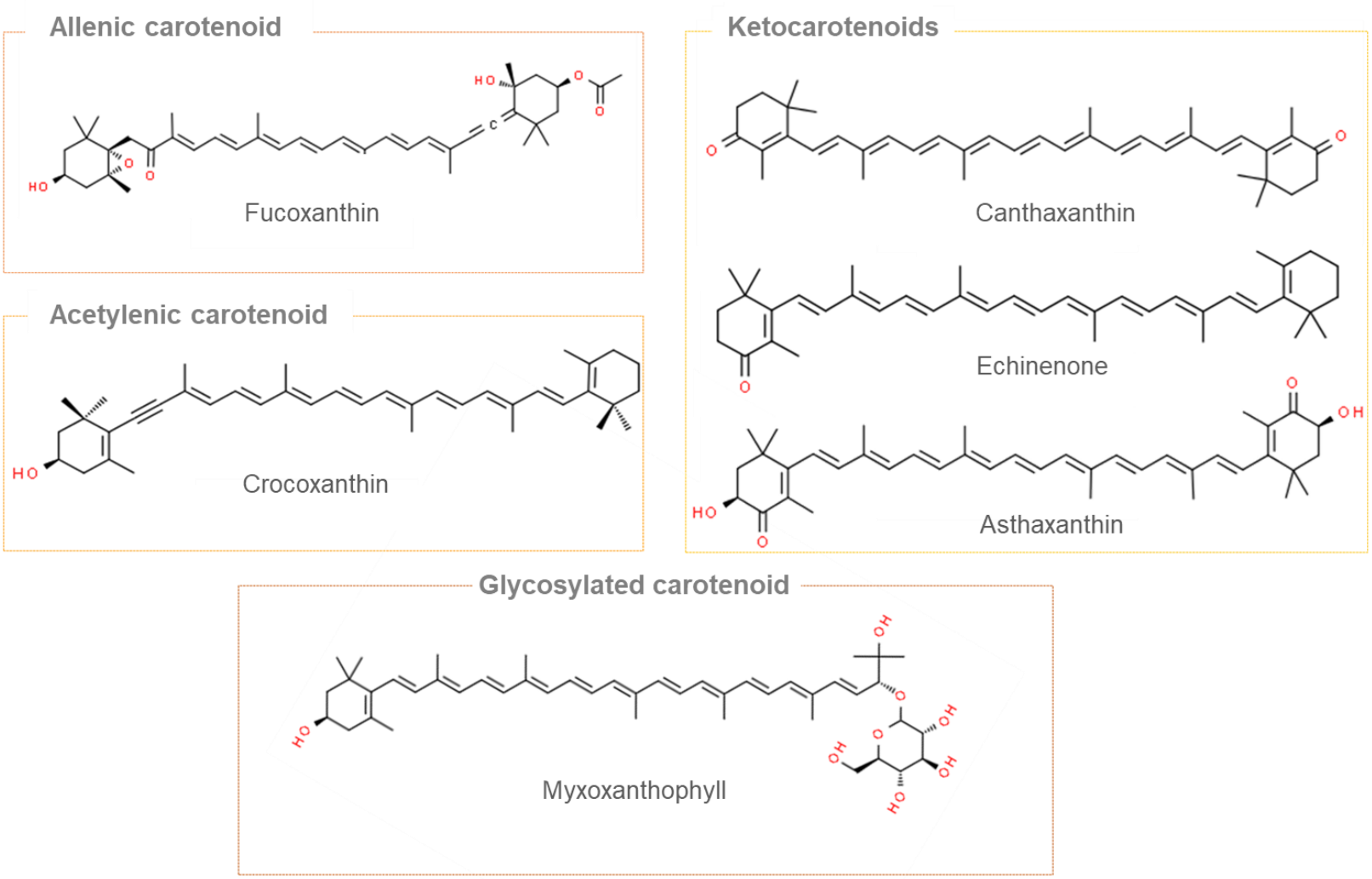
In photosynthetic organisms such as microalgae, carotenoids are associated with the light harvest photosynthesis complex, thus reducing the excess energy required in photosynthesis, transferring the absorbed energy to chlorophylls (Varela et al., 2015). Additionally, they are considered essential molecules for the survival of microalgae, as

they protect cells from reactive oxygen species generated during photosynthesis and high light intensity and also dissipate excess light as heat through the xanthophyll cycle (Hu et al., 2018).

In fact, carotenoids are essential pigments for all photosynthetic organisms; however, they also have nutritional importance related to their unique properties and potential therapeutic effects, biological activities and beneficial effects on health. A relevant property of carotenoids for human nutrition is their use as precursors of vitamin A. However, the ability of carotenoids to exert pro-vitamin is restricted to structures with at least one unsubstituted β ring with an 11-carbon polyene chain (Nascimento et al., 2019). Also, due to the protective effect exerted on the cells, these compounds are associated with the modulation of other pathologies, such as reducing the risk of developing chronic diseases such as cancer, cardiovascular diseases, cataracts and macular degeneration (Rodriguez-Concepcion et al., 2018; Eggersdorfer and Wyss, 2018).

Among the most explored carotenoids, lutein is known for its protective role against macular degeneration of the eye, making the ingestion of this xanthophyll very important, as it cannot be synthesized by humans (Becerra et al., 2020). Astaxanthin, a carotenoid found in microalgae, is a potent ketocarotenoid with well-known antioxidant properties, together with

Figure 2 – Different classes of xanthophylls.



prevention and the treatment of cancers, chronic inflammatory diseases, diabetes, obesity, cardiovascular diseases and neurodegenerative diseases (Fakhri et al., 2018; Xia et al., 2020). β-carotene is provitamin A and is also a potent antioxidant with cardioprotective effects (Grune et al., 2010).

Currently, the main application of microalgae carotenoids is in the food industry as a pigment, especially β-carotene and astaxanthin. However, due to their important bioactive properties and biologicals, pharmaceutical and nutraceutical applications are also applied (Jacob-Lopes et al., 2019). Due to this wide range of applications, the global carotenoid market is projected to reach US$2.0 billion by the year 2022. Where astaxanthin, β-carotene, lutein, fucoxanthin, zeaxanthin have the largest market shares to their wider applications. Additionally, world market projections show that in 2022 astaxanthin will reach the value of US$ 426.9 million, β-carotene US$ 572.78 million and lutein US$ 357.7 million (Gupta et al., 2021).

Along with these factors, the production of carotenoids from microalgae is continuously growing. It has become one of the most successful activities in the biotechnology sector since natural and controlled sources of carotenoid production are highly desirable due to their aspects positive economic and environmental (Khan et al., 2018). As previously mentioned, currently, Dunaliella salina and Haematococcus pluvialis are the main producers of β-carotene and astaxanthin respectively, representing 90% of the carotenoids present in the composition of these biomasses (Rammuni et al., 2019). In parallel, Muriellopsis sp., Chlorella zofingiensis, and Scenedesmus almeriensis can be explored for lutein production (Saha et al., 2020). In this context, studies are constantly being elucidated in the search for alternative microalgae species that can supplement the production of natural carotenoids.

3.2 CHLOROPHYLLS

Chlorophylls are green pigments that occur in the plastids of most plants, certain bacteria, abundantly in green algae, and also in cyanobacteria. In these organisms, chlorophylls participate in the biosynthesis of complex biomolecules ($C_6H_{12}O_6$) from simpler molecules (CO_2 and H_2O) through the process of photosynthesis . They are the most abundant and widely distributed class of pigments in nature. Due to their light energy capture and electron transfer functions in photosynthetic organisms, chlorophyll pigments are considered essential compounds in photosynthesis (Hosikian et al., 2010).

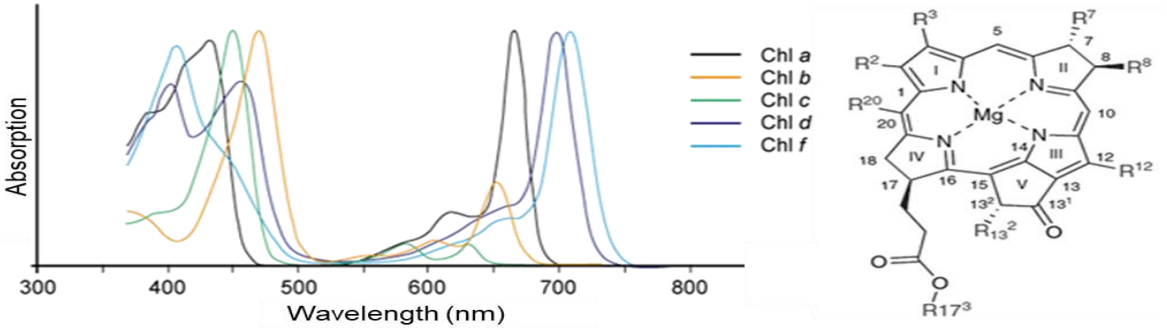
They are complex organic molecules, chemically classified within the group of porphyrins. In general, chlorophylls comprise a tetrapyrrole macrocycle system, linked by methylene bridges (-CH-) and an Mg^{2+} ion inside, coordinated to the rings by 4 nitrogen atoms. This structure also contains at C-17, a propionic acid chain esterified with phytol diterpene alcohol (a 20-carbon hydrocarbon chain), giving the molecule a hydrophobic character. However, there are exceptions to these characteristics (Roca et al., 2016).

In turn, chlorophylls constitute a large and diverse family of molecules similar to each other, called chlorophyll a, b, c (c_1, c_2 e c_3), d and f (Figure 3) (Pérez-Gálvez et al., 2017). All chlorophyll species are reported in microalgae, and except for the first two, the others are not found in higher plants (Zepka et al., 2019). Structurally, chlorophyll molecules differ from each other due to the degree of saturation of the pyrrole rings and their terminal groups, which alters the absorption of these pigments (Pareek et al., 2017). According to their degree of saturation, they are subdivided into three main classes: phytoporphyrins with a completely unsaturated macrocycle, such as chlorophylls c; bacteriochlorins present in the bacteriochlorophylls; finally the chlorins which comprise the most common chlorophylls (Roca et al., 2016).

As shown in Figure 3, chlorophyll a, chlorophyll d, and chlorophyll f have approximately equal absorption intensities in blue, red, and green. On the other hand, chlorophyll c phytoporphyrins absorb weakly in red and more intensely around 450 nm. Specifically, chlorophyll a being a blue/green pigment with maximum absorption of 660-665 nm and chlorophyll b being a green/yellow with maximum absorption of 642-652 nm (Solymosi and Mysliwa-Kurdziel, 2017).

Chlorophylls a and b are widely distributed in nature and are the best-known types. Structurally, chlorophylls a has a methyl group (CH_3) on carbon C-7, whereas chlorophyll b (Figure 3) has an aldehyde group (CHO). Chlorophyll a occurs as a ubiquitous pigment in all microalgae species, unlike chlorophyll b which is present in large concentrations in Chlorophyta and Euglenophyta which are similar to higher plants. Chlorophyll d is present in Rhodophyta and some species of Cyanophyta. While chlorophylls c are present in freshwater

Figure 3 - Chlorophyll structures and their absorption spectra.



Pigment	R2	R3	R7	R8	7-8 bond	R12	$R13^2$	17-18 bond	R20	$R\ 17^3$
Chl a	CH_3	$CH{=}CH_2$	CH_3	C_2H_5	=	CH_3	$COOCH_3$	–	H	phytol
Chl b	CH_3	$CH{=}CH_2$	CHO	C_2H_5	=	CH_3	$COOCH_3$	–	H	phytol
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	phytol
Chl f	CHO	C_2H_5	CH_3	C_2H_5	=	CH_3	$COOCH_3$	–	H	phytol

diatoms (Zepka et al., 2019). Likewise, chlorophyll f was found in a Cyanophyta (Acaryochloris ssp.) (Chen et al., 2010).

Chlorophylls are molecules are highly unstable and may undergo changes in their structures with the formation of derivative compounds. The greatest instability of chlorophyll molecules comprises the hydrocarbon side chain, which can be removed by enzymes (chlorophylases) and/or acidic conditions, thus changing the molecular polarity, which makes the molecule hydrophilic. While Mg^{2+} can be removed when exposed to acid and thermal treatment, which evidences the formation of degradation compounds with different colors such as light green, brown green and olive green (Zepka et al., 2019). Alkaline conditions, exposure to light and oxygen, also negatively affect its stability, inducing changes in its structure (Simpson et al., 2012). Furthermore, chlorophyll molecules can undergo hydroxylation reactions in their structures, in which a series of complex hydroxylated chlorophyll derivatives are formed. These compounds may be a consequence of the natural metabolism of these microorganisms, or due to the

extraction method applied to obtain these compounds (Fernandes et al., 2017; Chen et al., 2017). However, aiming its application, the stability of chlorophylls against degradation can be increased by de-esterification of chlorophyll and complexation with copper ions in place of magnesium ion (Viera et al., 2019). Great advances have been made in understanding the biological functions of chlorophylls and their derivatives, in which studies prove that these compounds are related to several biological activities, including healing, antimutagenic, anti-inflammatory, antimutagenicity, free radical scavenging capacity, nutraceutical properties and ability to inhibit calcium oxalate crystallization. These and other important biological activities of chlorophylls and their derivatives are clarified in previous works (Lanfer-Marquez et al., 2005; Pérez-Gálvez et al., 2017; Pareek et al., 2017).

Like carotenoids, chlorophylls are also of great commercial importance. For many years, they have been used as food additives, mainly as colorants, in the food industry due to the demand for natural sources of pigments (Fernandes et al., 2020). In addition to this application, these molecules are also used in various industrial sectors such as pharmaceutical, cosmetic, and especially in the food industry, with a recent appeal to produce nutraceutical and functional products due to their possible health benefits (Viera et al., 2019; Silva et al., 2020).

Commercial sources of chlorophylls include Chlorella and Arthrospira platensis (Spirulina) microalgae and plant sources such as Enteromorpha (Chlorophyta) and Ulva (Chlorophyta) (Simpson et al., 2012). In turn, chlorophylls produced on a commercial scale are currently obtained mainly from superior plants such as stinging nettle, corn, alfalfa, or spinach. However, these matrices consist of chlorophyll content often less (< 1 %) than that found in microalgae species (~ 7 %) (Sarkar et al., 2020).

Thus, these microorganisms, especially green microalgae (Chlorophyta) and other species of cyanobacteria (Cyanophyta), become an alternative source for the biological synthesis of these compounds, as they have chlorophylls in their structures as the predominant pigments and can be cultivated in continuous systems in contrast to vegetable sources (Pagels et al., 2020).

4 BIOACCESSIBILITY AND BIOAVAILABILITY

Carotenoids and chlorophylls are not synthesized by humans and therefore are obtained from the diet or via supplementation (Pérez-Gálvez et al., 2017; Dias et al., 2018). In turn, to assume some protagonism at the biological level, these bioactive

compounds need to be absorbed by the organism. For this to occur, these biomolecules must be bioaccessible and are susceptible to absorption in the enterocytes, involved in the chylomicrons, reaching the target tissue and finally being able to exert beneficial actions to the health (Fernandez-Garcia et al., 2012; Kopec and Failla, 2018). Thus, one of the primary current knowledge to establish the real contribution of carotenoids and chlorophylls to human health is the bioaccessibility and bioavailability study.

The concept of bioaccessibility has evolved over the years and is currently the most pertinent definition refers to the sequence of events that occur during the digestive transformation of food into compounds that can be assimilated by the body (Fernández-García et al., 2012). These steps include the release and transfer of fat-soluble compounds from the food matrix to mixed micelles during digestion, which is a necessary preliminary process by which the compound becomes accessible for apical absorption by the intestinal mucosa (Kopec and Failla, 2018). The bioaccessibility of a compound added its absorption/assimilation, pre-systemic metabolism, transport, tissue distribution and bioactivity is defined as bioavailability. It represents the fraction of the ingested component available for use in physiological functions or stored in the human body (Gallardo-Guerrero et al., 2008; Saini et al., 2015). Regardless of terminology, these phases are complementary, since the ability to release biocompounds from the food matrix and transfer to mixed micelles (measured by bioaccessibility tests) is highly correlated with the absorption and your systemic distribution (bioavailability). In other words, the bioavailability of biocompounds is largely dependent on the bioaccessible fraction (Kopec and Failla, 2018).

Carotenoids and chlorophylls are lipophilic compounds and therefore are digested and absorbed in the same way as lipids. In this sense, they first need to be released from the food matrix, solubilized in particles of lipid emulsion, solubilization into pancreatic lipases and bile salts and formation of mixed micelles, movement across the microvilli, uptake by intestinal mucosal cells, incorporated into chylomicrons and enters in the lymphatic system and circulation (Ferruzzi and Blakeslee, 2007; Saini et al., 2015). As only the amount of micellarized compound is considered bioaccessible, the release of matrix and micellarization are the critical steps towards the bioavailability of carotenoids and chlorophylls.

According to Carbonell-Capella et al. (2014), bioaccessibility is generally assessed by mimicking gastric and small intestinal digestion, followed or not by Caco-2 cell uptake, one of the most widespread models for mimicking the absorption of

bioactive compounds by human intestinal epithelium (Viera et al., 2018). In parallel, the bioavailability is evaluated through the following steps: gastrointestinal digestion, absorption, metabolism, tissue distribution and bioactivity (Carbonell-Capella et al., 2014). Bioavailability is best determined by extremely controlled studies in humans. In these trials, the bioavailable content of a lipophilic compound is usually monitored for its increase in the TAG-rich fraction of blood plasma after its ingestion (Chung et al., 2004; Kopec et al., 2017). However, due to some factors such as complexity, long duration and high cost of analysis, and often inconclusive results due to the great variation in inter-individual responses, its use becomes limited. Animal models can also be used to estimate bioavailability, but they have limitations mainly related to physiological differences in relation to humans, costs and ethical considerations (Rodriguez-Amaya, 2015). In this sense, protocols that simulate in vitro digestion have been developed and have been improved and successfully applied for the initial screening of bioaccessibility and bioavailability of biocompounds from different matrices (Garrett et al., 1999; Failla and Chitchumronchokchai, 2004; Minekus et al., 2014; Rodrigues et al., 2017). Typically, these protocols are used to measure bioaccessibility and are known to mimic the physiological conditions and events that occur in the human gastrointestinal tract during the digestion process. Basically, they comprise the oral, gastric and small intestine phases. Despite not including factors related to the individual, the in vitro methodologies do not have ethical commitments, are relatively simple, rapid, inexpensive, reproducible, and valid (Rodriguez-Amaya, 2015).

The bioaccessibility/bioavailability of carotenoids and chlorophyll can be positively or negatively influenced by a complexity of factors such as food matrix interferents (e. g., constituents of the cell walls of the matrix, carbohydrates, proteins, fibers, minerals, and lipids), processing characteristics, structural physicochemical properties of molecules, and the physiological issues, genetic aspects related to the host, among others (Ferruzzi and Blakeslee 2007; Sy et al., 2012; Desmarchelier and Borel, 2017; Xavier and Mercadante, 2019). All these factors are closely related to the bioaccessibility and bioavailability of carotenoids and chlorophylls and should be considered in any study evaluating the absorption metabolism of these compounds.

The bioaccessibility and bioavailability of carotenoids are widely evaluated in different food sources, emphasizing mainly on fruits and vegetables (Reboul et al. 2006; O'Connell et al., 2007; Chitchumroonchokchai and Failla, 2017; Petry and Mercadante,

2017; Rodrigues et al., 2017; Murador et al., 2021; Nascimento et al., 2021). In parallel, chlorophylls have few studies found in the literature, although a significant advance has taken place in recent years (Ferruzzi and Blakeslee, 2007; Gallardo-Guerrero et al., 2008; Gandul-Rojas et al., 2009; Chen and Roca, 2018a; Chen and Roca, 2019). In turn, a wide range of bioaccessibility and bioavailability values can be found in the literature for the same compound. The factors mentioned above, intrinsic to the food matrix or to the compound itself, and the different methods used to assess these parameters are closely related to the high variability of the data. However, regardless of the values found in the literature, it can be considered that the absorption of carotenoids and chlorophylls by the body is considered relatively low, mainly due to factors related to the bioaccessibility of these compounds. Of these factors, it is considered that natural physical (cell wall) and chemical (interaction with other components) barriers of the food matrix and the physicochemical characteristics of the compounds are probably the main aggravating (Sy et al., 2012; Desmarchelier and Borel, 2017). Jointly to these points, the low presence of lipids in food matrices can be harmful to the absorption of these compounds since these molecules are promoters of solubilization and micellarization of lipophilic compounds such as chlorophylls and carotenoids (Failla et al., 2014; Xavier and Mercadante, 2019). Aiming at these aspects, strategies to promote increased bioaccessibility of carotenoids and chlorophylls are often being adopted, including pre-processing of foods (rupture the cell walls of the food matrix) and increase the content of lipids co-ingested with their compounds. Some of these strategies can be seen in Table 1.

To date, little is known about the bioaccessibility/bioavailability of microalgae carotenoids. As can be seen in Table 1, most of the works available so far in the literature focus only on specific carotenoids, disregarding the carotenoid profile in its entirety. Available reports are restricted to the bioaccessibility of β-carotene, α-carotene, lutein, zeaxanthin, antheraxanthin, violaxanthin, astaxanthin and fucoxanthin. In parallel, only Nascimento et al. (2021) investigated the total and individual bioaccessibility of all carotenoids present in the biomass of the microalgae Scenedesmus obliquus. Furthermore, investigations are restricted to just a few species of microalgae, which include Scenedesmus almeriensis, Chlorella ellipsoidea, Chlorella vulgaris, Chlamydomonas reinhardtii, Phaeodactylum tricornutum, Nannochloropsis sp., Spirulina platensis, Haematococcus pluvialis, Nitzschia laevis, Botryococcus braunii, and Scenedesmus obliquus. Considering the diversity and a large number of existing

microalgae species, few studies have been carried out so far, including few investigations using in vivo experimental models (Yu et al., 2012; Rao et al., 2013; Gille et al., 2018). Among the reports, most aim to determine the carotenoid bioaccessible and bioavailable content by simulating the human gastrointestinal environment through an in vitro digestion process.

The transfer of carotenoids from the matrix into micelles is a critical step that determines the extent of absorption of these compounds. In microalgae, factors influencing these aspects mainly include the structural rigidity of the microalgae cell. Therefore, strategies to increase the bioaccessibility of such biocompounds have been adopted through processes that disrupt cellular structures, promoting their dispersal in the gastrointestinal tract (Table 1). Among the

Table 1. Bioaccessibility and bioavailability of microalgae carotenoids.

Carotenoid	Microalgae	Assay	Strategy	Reference
lutein, zeaxanthin, α-carotene, β-carotene	Scenedesmus almeriensis	in vitro	extraction and dispersion in oil	Granado-Lorencio et al., 2009
lutein	Chlorella vulgaris	in vitro and Caco-2 cells	microfluidization	Cha et al., 2011
zeaxanthin, antheraxanthin, β-carotene	Chlorella ellipsoedea	in vitro	microfluidization	Cha et al., 2012
zeaxanthin	Spirulina	in vivo	addition of fat	Yu et al., 2012
β-carotene, astaxanthin, lutein	Spirulina platensis	in vivo	biomass dispersed in oil	Rao et al., 2013
β-carotene, astaxanthin, lutein	Haematococcus pluvialis	in vivo	biomass dispersed in oil	Rao et al., 2013
β-carotene, astaxanthin, lutein	Botryococcus braunii	in vivo	biomass dispersed in oil	Rao et al., 2013
lutein, β-carotene	Chlorella vulgaris	in vitro	sonication	Gille et al., 2016
lutein, β-carotene	Chlamydomonas reinhardtii	in vitro	sonication	Gille et al., 2016
lutein, β-carotene	Chlorella vulgaris	in vitro	maceration biomass	Gille et al., 2018
fucoxanthin, zeaxanthin, β-carotene	Phaeodactylum tricornutum	in vitro	maceration biomass	Gille et al., 2018
lutein, β-carotene	Chlorella vulgaris	in vivo	maceration biomass	Gille et al., 2018
fucoxanthin, zeaxanthin, β-carotene	Phaeodactylum tricornutum	in vivo	maceration biomass	Gille et al., 2018
fucoxanthin, zeaxanthin, β-Carotene	Phaeodactylum tricornutum	in vitro and Caco-2 cells	sonication and cellulase digestion	Gille et al., 2019
astaxanthin	Haematococcus pluvialis	in vitro	extraction and nanoencapsulation	Zanoni et al., 2019
fucoxanthin	Nitzschia laevis	in vitro	extraction	Guo et al., 2020
violaxanthin, antheraxanthin, zeaxanthin, β-carotene	Nannochloropsis sp	in vitro	high pressure homogenization and oil dispersion	Bernaerts et al., 2020
zeaxanthin, β-carotene	Arthrospira platensis	in vitro	biomass dispersed in oil	Tudor et al., 2021
lutein	Chlorella pyrenoidosa	in vitro	biomass dispersed in oil	Tudor et al., 2021
Total carotenoids in biomass (20 compounds)	Scenedesmus obliquus	in vitro and Caco-2 cells	ultrasonication, extraction and dispersion in oil	Nascimento et al., 2021

strategies applied, more rudimentary techniques are included, such as maceration, and more sophisticated methods, such as microfluidization, high pressure homogenization and sonication. In addition, the obtainment of isolated extract of carotenoids was also used as an alternative to enhance the bioaccessibility of these bioactive compounds. Due to the clear contribution of lipids in the micellarization process of carotenoids, the addition of oil during digestion was also adopted, in order to increase the lipid fraction present in the microalgae biomass.

The work by Granado-Lorencio et al. (2009) was the first to investigate the bioaccessibility of microalgae carotenoids. The findings of this research demonstrated that the incorporation of carotenoids in supernatants (micellarization) from S. almeriensis, specifically the compounds lutein, zeaxanthin, and β-carotene, have extremely low bioaccessibility (<1%) when administered from lyophilized biomass. However, an increase in the transfer to the micellar phase was observed when the carotenoids are extracted from the biomass and added to the olive oil used as the vehicle. The oil extracts enriched with different concentrations of carotenoids reached relative incorporation values of 35-90% for lutein, 45-80% for zeaxanthin, 50-80% for α-carotene and 25-70% for β-carotene. The authors attributed the differences in micellar incorporation to the strong effect of the microalgae matrix, mainly to the high content of fibers, components that negatively impact the bioaccessibility of carotenoids (Desmarchelier and Borel, 2017).

Maceration based on structural breakdown by mechanical means was used by Gille et al. (2018) to improve the content of lutein, β-carotene, fucoxanthin and zeaxanthin bioavailable from C. vulgaris. and P. tricornutum. The sonication method based on the propagation of ultrasonic waves significantly improved the bioaccessibility of β-carotene and lutein of C. vulgaris, reaching values of 12.5% and 18%, respectively. For C. reinhardtii, however, bioaccessibility was not influenced by sonication. Values of approximately 10% for β-carotene and 20% for lutein were found from the biomass with and without sonication (Gille et al., 2016). For unprocessed P. tricornutum biomass, bioaccessibility values of 27% for β-carotene, 29% for zeaxanthin and 52% for fucoxanthin were found after in vitro digestion, which is further improved by sonication for β-carotene (75%) and fucoxanthin (62%). The use of cellulase during the digestion process also contributes positively to bioaccessibility levels, as it aids the improved degradation of microalgae cells. Combined with the significant bioaccessibility,

fucoxanthin was the most abundant xanthophyll in Caco-2 cells, followed by zeaxanthin, while β-carotene was not detected (Gille et al., 2019).

Microfluidization, a wet milling technique that creates fine emulsions from large particles by high pressure homogenization was used by Cha et al. (2011; 2012), demonstrating to be effective in increasing the bioaccessibility of carotenoids of C. vulgaris and C. Ellipsoidea. In the first study carried out with C. vulgaris, the authors demonstrated that only 25% of the lutein presented in the untreated biomass was micellarized. Cell disruption by microfluidization processes resulted in an increase of up to 3 times more efficiency in lutein micellarization (57-73%), depending on the pressures used. As well, the final content of lutein accumulated by intestinal Caco-2 cells was also higher with microfluidization. In the second study, the results obtained for C. ellipsoedea carotenoids showed relatively low bioaccessibility values (zeaxanthin, 2.60%; β-carotene, 1.69%). Also, approximately 95% of total carotenoids were not released from the matrix and micellarized. After microfluidization of the microalgae biomass, the micelle formation efficiency increased up to 12 times more, reaching bioaccessibility values of 32.60% for zeaxanthin and 18.19% for β-carotene. The antheraxanthin content decreased with microfluidization, and this epoxy xanthophyll did not show bioaccessibility as it was not detected after in vitro digestion.

Yu et al. (2012), in an in vivo study with humans, observed an increase of zeaxanthin concentration in human serum from 0.06 to 0.15 μmol/L after ingesting a single dose of Spirulina with dietary fat. These results emphasize not only the high bioaccessibility but also the high bioavailability of zeaxanthin from this microalgae source.

The biomass dispersion strategy of S. platensis, H. pluvialis and B. braunii in oil positively impacted the bioavailability of β-carotene, astaxanthin and lutein in a healthy animal model. In this study in vivo conducted by Rao et al. (2013), it was observed that microalgae biomass could prevent lipid peroxidation by scavenging free radicals and hydroxy radicals in living cells and restoring the enzyme activity.

Nanoencapsulation, an entrapping technique of active ingredients in nanometer sized capsules, was used by Zanoni et al. (2019). In this study, the oleoresin of astaxanthin obtained from biomass of the H. pluvialis was successfully nanoencapsulated through the solvent emulsification-evaporation technique using a protein carrier, which increased the stability and bioaccessibility of astaxanthin to 76%.

The bioaccessibility of fucoxanthin varied according to the source from which it was digested in the study by Guo et al. (2020). The pure standard showed bioaccessibility values of 27.7%, while the fucoxanthin standard in emulsion with 5% oil was 27.5% bioaccessible. On the other hand, a greater total bioaccessibility for fucoxanthin (32.7%) was obtained from an extract of the microalgae Nitzschia laevis containing about 5.1% of fucoxanthin and 7% of lipids.

Bernaerts et al. (2020) reported low bioaccessibility for β-carotene, zeaxanthin, and antheraxanthin (1-6%) in the untreated biomass Nannochloropsis sp. In comparison, a threefold increase in the extent of bioaccessibility (8-16%) of the compounds was observed when cell disruption of biomass by high pressure homogenization and dispersion in oil was used. The violaxanthin present in the microalgae biomass was not detected in the bioaccessible fraction of any biomass analyzed.

Recently, the addition of organic cold-pressed coconut oil (Cocos nucifera L.) was used as a strategy to increase the bioaccessibility of carotenoids present in commercial biomass of A. platensis and C. pyrenoidosa (Tudor et al., 2021). Overall, the bioaccessibility of lutein from C. pyrenoidosa ranged from 17.77% to 19.19% associated with the type of bile source used in the in vitro digestion process (bovine bile or porcine bile) and addition or not of oil in the biomass. β-carotene from A. platensis showed bioaccessibility values from 18.94% to 20.29%, while zeaxanthin ranged from 24.68% to 42.82%. Lutein and β-carotene showed no significant difference in bioaccessibility about the bile source used; however, zeaxanthin was more bioaccessible in the presence of bovine bile. The addition of 5% coconut oil led to a significant increase in the bioaccessibility of zeaxanthin from A. platensis (from 37.2% to 42.8%); in contrast, it did not significantly alter the bioaccessibility of lutein and β-carotene.

In a study conducted with S. obliquus, it was observed that the bioaccessibility of carotenoids increases according to the degree of pre-release of the matrix to which they were submitted before the in vitro simulated digestion. Isolated extracts of carotenoids dispersed in oil showed 3 times more bioaccessible carotenoids than when digested from lyophilized biomass without treatment. At the same time, a pre-treatment with ultrasound in the wet biomass allowed an increase of up to 2 times in the bioaccessibility values. Also, this study demonstrated that carotenes and xanthophylls from S. obliquus are significantly absorbable by Caco-2 intestinal cells, reinforcing the importance of these microalgae as a potential source of carotenoids (Nascimento et al., 2021).

Although recognized as potential sources of these biocompounds, to the best of our knowledge, regrettably, there is no evidence of bioaccessibility and absorption of chlorophylls from microalgae. The available information on bioaccessibility and bioavailability of chlorophylls and their derivatives is limited and are based on in vitro assays with higher food matrix plants (Ferruzzi et al., 2001; Gallardo-Guerrero et al., 2008; Hayes et al., 2020), edible seaweeds (Chen and Roca, 2018a; Chen and Roca, 2018b; Chen and Roca, 2019) and some chlorophyll standards (Gandul-Rojas et al., 2009). In fact, these biomolecules were considered non-absorbable by our body, and only in the last few decades have studies proven the absorption of these compounds (Ferruzzi and Blakeslee, 2007). Consequently, there is currently a growing interest in expanding knowledge about the digestibility of chlorophyll pigments, which also drive studies in vivo (Fernandes et al., 2007; Hsu et al., 2014; Chao et al., 2018; Viera et al., 2018). However, there are still many gaps to be explored to increase the understanding of the metabolism and biodistribution of these phytochemicals in the human body.

5 FINAL CONSIDERATIONS

Despite numerous researches proving that microalgae are promising sources of a variety of carotenoids and chlorophylls, research focusing on the digestive behavior of these microalgae compounds is still limited. Specifically, many studies have demonstrated, mainly in vitro, the bioaccessibility and bioavailability of carotenoids from microalgae. However, studies with chlorophylls are not yet inexistent. Thus, the full exploitation of microalgae carotenoids and chlorophylls for food and health purposes requires an approach that includes the composition of these biomolecules in different microalgae species and digestive monitoring and their potential biological actions. For this, bioaccessibility and bioavailability studies are indispensable, along with strategies that can promote the accessibility and absorption of microalgae compounds. These approaches strongly contributed to the consolidation of microalgae biomass or their biocompounds in food and pharmaceutical formulations with nutritional and functional purposes.

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CAPÍTULO 3

HPLC-PDA-MS/MS as a strategy to characterize and quantify natural pigments from microalgae

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Research Paper

HPLC-PDA-MS/MS as a strategy to characterize and quantify natural pigments from microalgae

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ABSTRACT

Interest in pigment composition of microalgae species is growing as new natural pigments sources are being sought. However, we still have a limited number of species of microalgae exploited to obtain these compounds. Considering these facts, the detailed composition of carotenoids and chlorophylls of two species of green microalgae (*Chlorella sorokiniana* and *Scenedesmus bijuga*) were determined for the first time by high-performance liquid chromatography coupled to diode array and mass spectrometry detectors (HPLC-PDA-MS/MS). A total of 17 different carotenoids were separated in all the extracts. Most of the carotenoids present in the two microalgae species are xanthophylls. *C. sorokiniana* presented 11 carotenoids (1408.46 μg g^{-1}), and *S. bijuga* showed 16 carotenoids (1195.75 μg g^{-1}). The main carotenoids detected in the two microalgae were all-*trans*-lutein and all-*trans*-β-carotene. All-*trans*-lutein was substantially higher in *C. sorokiniana* (59.01%), whereas all-*trans*-β-carotene was detected in higher quantitative values in *S. bijuga* (13.88%). Seven chlorophyll compounds were identified in both strains with different proportions in each species. Concentrations of chlorophyll representing 7.6% and 10.2% of the composition of the compounds present in the biomass of *C. sorokiniana* and *S. bijuga*, respectively. Relevant chlorophyll compounds are reported for the first time in these strains. The data obtained provide significant insights for microalgae pigment composition databases.

1. Introduction

Over the last few years, changes in eating habits, and modifications in nutritional requirements have led to considerable alterations in food formulation, shifting consumption trends towards natural products with functional properties (Koyande et al., 2019). Because a consequence of these changes, the natural color of food is estimated as the largest segment of products in the food coloring market, representing more than 80% of the total revenue of this sector. So the global food colors market was set at USD 1.79 billion in 2016, with revenue growth estimated at USD 2.97 billion by 2025 (Grand, 2019). Thus, emerging technologies for obtaining these compounds are necessary to supply this high demand.

In this sense, most of microalgae biotechnology companies concentrate investments and technology in the chemical specialties, as bioactive compounds, which can be successfully allocated in industrial sectors such as the pharmaceutical, nutritional and food industries (Sudhakar et al., 2019; Jacob-Lopes et al., 2018). This is fundamentally supported by the fact that microalgae biomass has become a promising alternative for obtaining natural compounds (Sathasivam et al., 2017). Since its metabolic diversity, coupled with its high biotechnological potential, allows the production of various biocompounds such as fatty acids, amino acids, and pigments that may have beneficial effects on human health. Thus, compounds coming from microalgae can be considered for significant applications in the development of functional food products (Matos, 2017; Khanra et al., 2018). Besides, the production of microalgae biomass has the advantage of high sustainability, as they absorb CO_2 from the atmosphere, withstand extreme environmental conditions, have high productivity, and do not compete with terrestrial crops for agricultural land (Draaisma et al., 2013; Khan et al., 2018).

According to Spolaore et al. (2006), the exploration of compounds with bioactive activity has the potential to value up to 100 times the microalgae biomass when compared to the exploitation for energy or animal feed purposes. Associated with this perspective, the possibility of producing these biomolecules from biotechnological processes opens a field of exploitation with high technical-economic potential. Since the productive capacities are some orders of magnitude superior to the

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conventional systems of production of bioactive compounds currently supported in plant biomass (Yen et al., 2013; Kothari et al., 2017).

In the current state, scientific researches are continually being developed in the academic community with the primary objective of expanding knowledge about the diversity of existing microalgae species for possible commercial applications (Rodrigues et al., 2014; Fernandes et al., 2017; Patias et al., 2017; Fagundes et al., 2019; Maroneze et al., 2019; Nascimento et al., 2019; Vendruscolo et al., 2018). In this context, one of the fundamental parameters to establish the biotechnological capacity of using microalgal biomass as a source of bioproducts is to characterize the chemical composition of the biomass.

However, considering the number of species known in the world (according to some estimates 50,000 species), only 30,000 have been studied (Sathasivam et al., 2017). Among the species that are considered underexploited, the class *Chlorophyceae*, including species such as *Chlorella sorokiniana* and *Scenedesmus bijuga*, have the potential for use in bioprocesses, due to their robustness and simple nutritional requirements, rapid growth, and substantial content of compounds to be exploited (Borowitzka et al., 2018).

As photosynthetic microrganisms, microalgae are one of the most abundant and most varied producers of carotenoids and chlorophylls (Mulders et al., 2014), being the carotenoids the most exploited fraction of microalgae pigments (Gong and Bassi, 2016; Rajesh et al., 2017). In a study carried out by Patias et al. (2017), 23 different carotenoids were identified in biomass extracts of three species of microalgae. Furthermore, the relationship between function and structure was used to explain the antioxidant properties of these carotenoids. Recently, the carotenoid profiles of biomass from five eukaryotic microalgae were evaluated, and it was shown that microalgae showed species-specific carotenoid profiles and some species prevalence of xanthophylls over carotenes (Di Lena et al., 2019). Furthermore, from this promising microorganisms group, carotenoids such as β-carotene, α-carotene, zeaxanthin, lutein, violaxanthin, echinenone, mixoxantophyll, and canthaxanthin can be isolated, being some of these are produced exclusively by microalgae (Takaichi, 2011).

The microalgae represent a successful model in terms of commercial carotenoid production, through the cultivation of *Dunaliella salina* and *Haematococcus pluvialis*, with a focus on β-carotene and astaxanthin (up to 7% and 13% dry weight, respectively) (Rammuni et al., 2018). Besides, the global market for carotenoids is projected to reach US $ 1.7 billion by the year 2022. β-carotene, astaxanthin, and lutein have the largest market share. Moreover, world market projections show that in the year 2022, astaxanthin comes to reach the US $ 426.9 million, β-carotene US $ 572.78 million, and lutein US $ 357.7 million (Mcwilliams, 2018).

The main interests associated with these compounds are related to the color and the potential for sequestration of reactive nitrogen species (RNS), oxygen (ROS), especially the singlet oxygen species ($^1O_2$) and non-biological radicals, which are associated with antioxidant properties (Rodrigues et al., 2012). Additionally, these pigments have been associated with the provitamin A activity of carotenoids containing β rings, enhanced immune system functions, reduction of the risk of developing chronic diseases such as cancer, age-related macular degeneration, type 2 diabetes, cardiovascular diseases, and adipocyte function, adiposity and obesity (Rodriguez-Concepcion et al., 2018; Meléndez-Martínez, 2019; Khalid et al., 2018). Moreover, *In vivo* study in mice demonstrated the importance of the microalgae carotenoid fraction in the protective influence against tissue lipid peroxidation (Nascimento et al., 2019).

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 (Roca et al., 2016). They are reported as one of the main fractions of secondary metabolites in the constitution of the microalgae biomass, are mainly present in the species of green microalgae (Borowitzka et al., 2018).

Even if the characterization of chlorophylls has recently been reported in macroalgae (edible seaweeds) (Chen et al., 2015a, 2015b, 2017), the literature lacks information on the qualitative and quantitative profile of these compounds in microalgae species (*Chlorophyta*) (Fernandes et al., 2017; Garrido and Zapata, 1996; Garrido et al., 2011). This fact may be related to the difficulties of the analysis to characterize these compounds, mainly due to the tremendous chemical instability of these molecules and the need for specific tools, as HPLC coupled with mass spectrometry (MS/MS) for more reliable results.

As well as for carotenoids, these photochemical compounds have also been proved to possess prominent benefits to human health. Chlorophyll is a well-known detoxifying agent and a phytonutrient. It has a positive effect on human reproduction and improves the metabolism of proteins, carbohydrates, and lipids in humans (Koyande et al., 2019; Solymosi and Mysliwa-Kurdziel, 2017). Also, anticarcinogenic, antigenotoxic, antimutagenic properties, anti-inflammatory activity as well as in vitro anti-oxidant activity has been demonstrated for these compounds (Lanfer-Marquez et al., 2005; Pareek et al., 2017; Pérez-Gálvez et al., 2017).

Considering these aspects, we here present the results of a study by HPLC-PDA-MS/MS on the carotenoid and chlorophyll characterization and total content in the biomass from two microalgae species, *Chlorella sorokiniana,* and *Scenedesmus bijuga.* Furthermore, the results presented provide extra value for the composition of biomass in these species, since important functional compounds have been appropriately characterized, which may attract attention for the application of these molecules in food and nutraceutical products. As well as, the data provided are valuable in view of industrial exploitation of alternative sources for obtaining natural pigments.

2. Materials and methods

2.1. Chemicals

Standards of all-*trans*-zeaxanthin, all-*trans*-lutein, all-*trans*-β-carotene, all-*trans*-α-carotene, chlorophyll *a*, chlorophyll *b* 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., 2017). 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 *Chlorella sorokiniana* (CPCC138) were obtained from the Canadian Phycological Culture Centre (CPCC) of the University of Toronto, Canada and *Scenedesmus bijuga* (UTEX2980) was obtained from the Algae Cultures Collection (UTEX) of University of Texas. Stock cultures were propagated and maintained in synthetic BG11 medium (Braun-Grunow medium) (Rippka et al., 1979). The incubation conditions were 25 °C, photon flux density of 150 μmol $m^{-2}.s^{-1}$ and a photoperiod of 12/12 h day:night with constant agitation were used.

2.3. Microalgal biomass production

The biomass production was carried out in a bubble column photobioreactor operating on batch mode, with a total working volume of 2.0 L synthetic of BG-11 medium (Maroneze et al., 2016). The experimental conditions were as follows: initial concentration of inoculum of 100 $mg.L^{-1}$, temperature of 25 °C, continuous aeration of 1VVM (volume of air per volume of culture per minute) with the injection of air enriched with 15% carbon dioxide, a photon flux density of 150 μmol $m^{-2}.s^{-1}$, photoperiod of 12/12 h light:dark, and a residence time of 168 h. The cultivations were performed twice, and in duplicate.

2.4. Biomass concentration

The biomasses were separated from the culture medium by centrifugation. It was subsequently freeze dried (Lyophilizer Liotop L101) for

24 h at −50 °C above −175 μm Hg, and then stored under refrigeration until the time of analysis.

2.5. Dry weight determination

To determine the total dry weight (DW), the freeze-dried biomass was kept in a desiccator and subsequently weighed on an analytical balance.

2.6. Carotenoid extraction

The carotenoids were exhaustively extracted from the freeze-dried sample (0.2 ± 0.02 g) first with ethyl acetate and then with methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 1500×*g* (Mandelli et al., 2012). The extraction procedure was repeated until the supernatant becomes colorless, which was reached approximately after 11 extractions with ethyl acetate and 6 with methanol. The homogenized sample suspension was filtered through a 0.22 μm polyethylene membrane, concentrated in a rotary evaporator (T < 30 °C), suspended in a mixture of petroleum ether/diethyl ether [1:1 (v/v)], and saponified overnight (16 h) with 10% (w/v) methanolic KOH at room temperature. The alkali was removed by washing with distilled water, and each extract was once again concentrated in a rotary evaporator, flushed with N_2 and kept at −37 °C in the dark until chromatographic analysis. All extractions were performed in triplicate.

2.7. 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 10 min at 5500 rpm (Fernandes et al., 2017). 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 (50:20, 50:30, 50:40 and 50:50 v/v) and chlorophyll fraction was obtained in ethanol. To ensure the integrity of the chlorophyll pigments, the separation occurred at room temperature (T < 30 °C), in low light, for approximately 2 h. 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.

2.8. HPLC-PDA-MS/MS carotenoids and chlorophylls analysis

The carotenoids and 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 processed at 450 nm for carotenoids and at 660 nm for chlorophylls. Carotenoid and chlorophylls 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 carotenoid and chlorophyll extracts were solubilized in MeOH:MTBE (1:1) and filtered through Millipore membranes (0.22 μm). The MS parameters for carotenoids analysis were as follows: positive mode; current corona, 4000 nA; source temperature, 450 °C; dry gas, N_2, temperature, 350 °C; flow, 60 L/h; nebulizer, 5 psi; MS/MS fragmentation energy, 1.4 V. The mass spectra were acquired with scan range of m/z from 100 to 700 (de Rosso and 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. HPLC-PDA parameters for chlorophyll analysis were set as

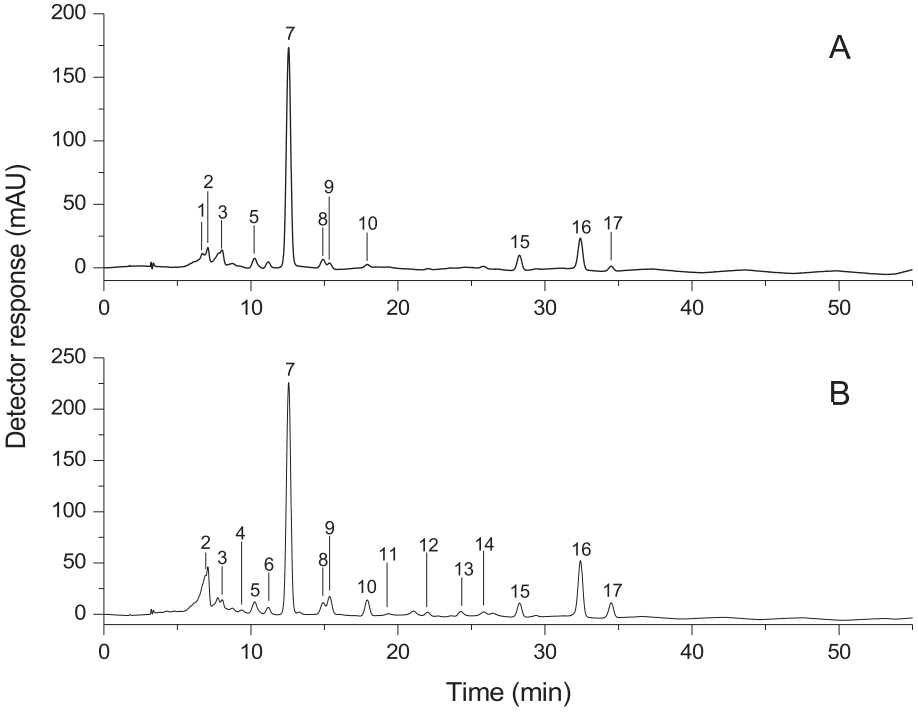

Fig. 1. Chromatogram, obtained by HPLC-PDA, of the carotenoids extract from *Chlorella sorokiniana* (A) and *Scenedesmus bijuga* (B). See text for chromatographic conditions. Peak identification and characterization are given in Table 1. Chromatogram was processed at 450 nm.

Table 1
Chromatographic, UV–vis spectrum and mass characteristics of carotenoids from *Chlorella sorokiniana* and *Scenedesmus bijuga,* obtained by HPLC-PDA-MS.

Peak[a]	Carotenoid	t_R (min)[b]	UV–Vis characteristics			Fragment ions (positive mode) (*m/z*)	
			$\lambda_{máx}$ (nm)[c]	III/II (%)[d]	AB/II (%)[e]	$[M+H]^+$	MS/MS[i]
1	All-*trans*-neoxanthin	6.7	418, 441, 469	67	0	601 (64.8)[h]	583 $[M + H\text{-}18]^+$ (100); 565 $[M + H\text{-}18]^+$ (78.3);509 $[M + H\text{-}92]^+$ (1.0); 491 $[M + H\text{-}92\text{-}18]^+$ (1.0); 221 (1.8)
2	9-*cis*-neoxanthin	6.9–7.1	421, 445, 469	11	nd[f]	601 (50.0)	583 $[M + H\text{-}18]^+$ (100); 565 $[M + H\text{-}18]^+$ (42.8); 509 $[M + H\text{-}92]^+$ (0.5); 491 $[M + H\text{-}92\text{-}18]^+$ (1.5); 221(0.4)
3	9-*cis*-violaxanthin	8.0	329, 412, 435, 463	77	12	601 (89.0)	583 $[M + H\text{-}18]^+$ (100); 565 $[M + H\text{-}18\text{-}18]^+$ (6.2); 547 $[M + H\text{-}18\text{-}18\text{-}18]^+$ (29.6); 509 $[M + H\text{-}92]^+$ (23.4);491 $[M + H\text{-}92\text{-}18]^+$ (0.7); 221 (0.6)
4	*Cis*-lutein	9.3	328, 405, 431, 448	0	31	nd[f]	551 $[M + H\text{-}18]^+$ (in source, 100); 533 $[M + H\text{-}18\text{-}18]^+$ (2.4); 495 $[M + H\text{-}18\text{-}56]^+$ (0.1)
5	15-*cis*-lutein	10.2–10.3	330, 414, 439, 465	25	39	569 (11.1)	551 $[M + H\text{-}(18]^+$ (in source, 100); 533 $[M + H\text{-}18\text{-}18]^+$ (3.1); 495 $[M + H\text{-}18\text{-}56]^+$ (1.0)
6	13-*cis*-lutein	11.2	330, 414, 437, 465	38	43	nd[f]	551 $[M + H\text{-}18]^+$ (in source, 100); 533 $[M + H\text{-}18\text{-}18]^+$ (3.0); 495 $[M + H\text{-}18\text{-}56]^+$ (1.6)
7	All-*trans*-lutein	12.6	420, 444, 472	60	0	569 (26.3)	551 $[M + H\text{-}18]^+$ (in source, 100); 533 $[M + H\text{-}18\text{-}18]^+$ (2.5); 495 $[M + H\text{-}18\text{-}56]^+$ (0.6)
8	All-*trans*-zeaxanthin	14.9	425, 450, 476	33	0	569 (100)	551 $[M + H\text{-}18]^+$ (3.3); 533 $[M + H\text{-}18\text{-}18]^+$ (0.2); 477 $[M + H\text{-}92]^+$ (1.8)
9	9-*cis*-lutein	15.3	330, 416, 439, 467	63	12	569 (25.0)	551 $[M + H\text{-}18]^+$ (in source, 100); 533 $[M + H\text{-}18\text{-}18]^+$ (3.5); 495 $[M + H\text{-}18\text{-}56]^+$ (1.2)
10	9′-*cis*-lutein	17.9	330,418, 440, 467	56	19	569 (28.5)	551 $[M + H\text{-}18]^+$ (in source, 100); 533 $[M + H\text{-}18\text{-}18]^+$ (3.4); 495 $[M + H\text{-}18\text{-}56]^+$ (1.2)
11	9-*cis*-zeaxanthin	19.3	420, 444, 471	36	nc[g]	569 (100)	551 $[M + H\text{-}18]^+$ (4.5); 533 $[M + H\text{-}18\text{-}18]^+$ (2.0); 477$[M + H\text{-}92]^+$ (0.8)
12	β-carotene-5,6-epoxide	22.0	420, 445, 472	47	0	553 (100)	535 $[M + H\text{-}18]^+$ (13.7); 461 $[M + H\text{-}92]^+$ (6.8)
13	All-*trans*-echinenone	24.3	468	0	nd[f]	551 (100)	533 $[M + H\text{-}18]^+$ (1.0); 203 (2.3)
14	13-*cis*-β-carotene	25.8	336, 418, 444, 469	7	53	537 (100)	481 $[M + H\text{-}56]^+$ (1.0); 444 $[M\text{-}92]^+$ (54)
15	All-*trans*-α-carotene	28.3	420, 445, 473	50	0	537 (100)	481 $[M + H\text{-}56]^+$ (0.5); 444 $[M\text{-}92]^+$ (36)
16	All-*trans*-β-carotene	32.4	425, 451, 477	17	0	537 (100)	481 $[M + H\text{-}56]^+$ (0.5); 444 $[M\text{-}92]^+$ (3.7)
17	9-*cis*-β-carotene	34.5	422, 445, 472	18	nc[g]	537 (100)	444 $[M\text{-}92]^+$ (4.6)

[a] Numbered according to the chromatogram shown in Fig. 1.
[b] t_R: Retention time on the C30 column.
[c] Linear gradient Methanol:MTBE.
[d] Spectral fine structure: Ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II).
[e] Ratio of the *cis* peak (AB) and the middle absorption peak (II).
[f] Not detected.
[g] Not calculated.
[h] Relatives intensities for each m/z value appear in parentheses and are expressed as a percentage of the most abundant fragment ion.
[i] Detailed data about mass fragmentation were reported in detail in the literature (Patias et al., 2017; de Rosso and Mercadante, 2007; Van Breemen et al., 2012).

previously described by Murillo et al. (2013) with some minor modifications. The mobile phase consisted in binary solvent mixture system. Solvent A consisted of MeOH:MTBE:H2O (81:15:4) and solvent B MeOH:MTBE:H2O (16:80:4), using a linear gradient program as follows: from 0 to 20 min 0% B; from 20 to 140 min, 0–100% B; from 140 to 141 min, 100 to 0% B, from 141 to 150 min, 0% B. The flow rate was set at 0.8 mL/min, the column temperature was maintained at 35 °C. The MS parameters were the same as described above for carotenoids.

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 max, spectral fine structure, peak *cis* intensity for carotenoids), and mass spectra characteristics (protonated molecule ($[M+H]^+$) and MS/MS fragments), compared with data available in the literature [14, 15, 35 45, 47, 48, 49, 50, 51].

The carotenoids were quantified by HPLC-PDA, using external calibration curves for all-*trans*-zeaxanthin, all-*trans*-lutein, all-*trans*-β-carotene, all-*trans*-α-carotene of five concentration levels. All other xanthophyll and carotene contents were estimated using the curve of all-*trans*-lutein and all-*trans*-β-carotene, respectively. The *cis*-isomers were estimated using the curve of the corresponding all-*trans*-carotenoid. Total carotenoid content was calculated as the sum of the contents of each individual carotenoid separated on the C30 column.

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*, chlorophyll *a* and chlorophyll *a*′ where quantified using the curve of chlorophyll *a*; the hydroxypheophytin *a*, pheophytin *a* using the curve of pheophytin *a*; and chlorophyll *b* and chlorophyll *b*′ where quantified using the curve of chlorophyll *b*. Total chlorophyll content was calculated considering all identified peak areas.

2.9. 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 GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla-CA, USA).

3. Results and discussion

3.1. Profile carotenoids

Although studies on carotenoids in these microalgae strains have been reported in the literature, most of these present only quantitative values of the total carotenoids profile or some specific compound by PDA without addressing in detail characterization of total carotenoid composition (Chen et al., 2016; Minhas et al., 2016; Azaman et al., 2017). Thus, to the best of our knowledge, this is the first time that this analytical approach has been applied to characterizing, both qualitatively and quantitatively, the composition of carotenoids in *C. sorokiniana* and *S. bijuga* by HPLC-PDA-MS/MS.

Table 2
Quantitative characterization of carotenoids in microalgae extracts (μg.g-1 dry weight).

Peak	Carotenoid	*Chlorella sorokiniana*	*Scenedesmus bijuga*
1	All-*trans*-neoxanthin	63.39 ± 0.09	nd
2	9-*cis*-neoxanthin	68.04[a] ± 0.08	151.52[b] ± 1.26
3	9-*cis*-violaxanthin	53.79[a] ± 0.07	32.99[b] ± 2.76
4	*Cis*-lutein	nd	13.66 ± 1.14
5	15-*cis*-lutein	36.65[a] ± 0.05	35.16[a] ± 2.94
6	13-*cis*-lutein	nd	14.75 ± 1.23
7	All-*trans*-lutein	831.18[a] ± 1.18	526.40[b] ± 4.40
8	All-*trans*-zeaxanthin	44.81[a] ± 0.06	27.59[b] ± 2.30
9	9-*cis*-lutein	24.13[a] ± 0.03	40.74[b] ± 3.41
10	9′-*cis*-lutein	16.52[a] ± 0.02	40.18[b] ± 3.36
11	9-*cis*-zeaxanthin	nd	7.52 ± 0.62
12	β-carotene-5,6-epoxide	nd	13.39 ± 1.12
13	All-*trans*-echinenone	nd	15.46 ± 1.29
14	13-*cis*-β-carotene	nd	15.95 ± 1.33
15	All-*trans*-α-carotene	71.47[a] ± 0.10	40.51[b] ± 3.39
16	All-*trans*-β-carotene	156.21[a] ± 0.22	165.95[a] ± 1.38
17	9-*cis*-β-carotene	42.27[a] ± 0.06	53.98[b] ± 4.51
Total carotenoids	1408.46[a]	1195.75[b]	

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

The carotenoids, extracted from biomass of *C. sorokiniana* and *S. bijuga*, were chromatographically separated (Fig. 1), which were identified on the based on the combined information obtained from chromatographic elution on a C30 column, co-chromatography with standards, UV–visible, mass spectra characteristics, compared with data available in the literature (Table 1). Since a detailed description of carotenoid identification using the above information was already reported in detail in the literature (Patias et al., 2017; de Rosso and Mercadante, 2007; Rodrigues et al., 2015), only the quantitative observations are discussed below.

A total of seventeen carotenoids were separated in the extracts of *C. sorokiniana* and *S. bijuga*, all of which are structure derived from α or β-carotene synthesized through hydroxylation, epoxidation, isomerization (*cis*) or ketolation reactions. Of the identified compounds, the microalgae strains show ten carotenoids in common.

Considering the quantitative profile, the highest total carotenoid content was determined in the extract of *C. sorokiniana* (1408.46 μg g^{-1}) and *S. bijuga* exhibited the lowest content (1195.75 μg g^{-1}) (Table 2).

Eleven carotenoids were identified in *C. sorokiniana* (Fig. 1A), being three epoxycarotenoid (peak 1, 2 and peak 3), five hydroxycarotenoids (peak 5, 7, 8, 9 and peak 10) and three carotenes (peak 15, 16 and peak 17) (Fig. 2). All-*trans*-lutein (831.18 μg g^{-1}) and all-*trans*-β-carotene (156.21 μg g^{-1}) were the major, as shown in Table 2, which represented 70.01% of the total carotenoid content followed by all-*trans*-α-carotene (5.07%) and 9-*cis*-neoxanthin (4.83%) as major carotenoids in this biomass. All-*trans*-neoxanthin (4.51%), all-*trans*-zeaxanthin (3.18%) have also been identified in this microalgae species, as well as the *cis* isomers 9-*cis*-violaxanthin (3.82%), 9-*cis*-β-carotene (3.01%), 15-*cis*-lutein (2.60%), 9-*cis*-lutein (1.71%) and 9′-*cis*-lutein (1.17%).

The *S. bijuga* specie exhibited the most complete profile constituted by sixteen carotenoids (Fig. 1B). The major carotenoids were the same detected *in C. sorokiniana*, all-*trans*-lutein (526.40 μg g^{-1}), and all-*trans*-β-carotene (165.95 μg g^{-1}), which corresponds to 57.91% of the fraction of carotenoids in the extract. Other major peaks were identified as 9-*cis*-neoxanthin (12.67%) and 9-*cis*-β-carotene (4.51%). In addition, 9-*cis*-lutein (3.41%), all-*trans*-α-carotene (3.38%), 9′-*cis*-lutein (3.36%), 15-*cis*-lutein (2.94%), 9-*cis*-violaxanthin (2.76%), all-*trans*-zeaxanthin (2.31%), 13-*cis*-β-carotene (1.34%), all-*trans*-echinenone (1.29%), 13-*cis*-lutein (1.24%), *cis*-lutein (1.14%), β-carotene-5,6-epoxide (1.12%) and 9-*cis*-zeaxanthin (0.62%) were detected as minor carotenoids. Different from the results described in *C. sorokiniana*, *S. bijuga* presented one ketocarotenoid (peak 13), three epoxycarotenoid (peak 2, 3 and 4), eight hydroxycarotenoids (peak 4, 5, 6, 7, 8, 9, 10 and peak 11) and four carotene (peak 14, 15, 16 and 17) (Fig. 2). In contrast, as far as we know, the literature lacks information on the profile of carotenoids in *S. bijuga*. Only one report identified two carotenoids (lutein and astaxanthin) present in this microalgae (Minhas et al., 2016).

As well as described above, the total carotenoid content was notably higher in *C. sorokiniana*. On the other hand, *S. bijuga* presented a carotenoid profile with six different compounds than *C. sorokiniana*. Of these compounds, two were mono-*cis* isomers of all-*trans*-lutein (peak 4, peak 6), mono-*cis* isomer of all-*trans*-zeaxanthin (peak 11), β-carotene-5,6-epoxy (peak12), all-*trans*-equinenone (peak 13) and 13-*cis*-β-carotene (peak 14). In contrast, all-*trans*-neoxanthin (peak 1) was only detected in the carotenoid extract of *C. sorokiniana*.

Although MS fragments have been mapped, carotenoids intermediate of the synthesis of lutein as zeinoxanthin (identified in Rodrigues et al. (2015)) and α-cryptoxanthin (identified in Di Lena et al. (Di Lena et al., 2019)) were not identified in our study. This fact may result from the high enzymatic activity of carotene β-hydroxylase (CYP97A) and carotene ε-hydroxylase (CYP97C), enzymes responsible for catalyzing the synthesis of these compounds in lutein in the α-carotene pathway (Rodriguez-Concepcion et al., 2018).

Taking as reference the same genus *Scenedesmus* and *Chlorella,* all-*trans*-lutein and β-carotene also exhibited a major profile in *Scenedesmus obliquus* presenting higher quantitative values when compared to *S. bijuga*; whereas, *Chlorella vulgaris* showed lower values for all-*trans*-lutein and higher for β-carotene when compared to *C. sorokiniana* (Patias et al., 2017). It is interesting to note that although the green microalgae compared belong to the same genus, the quantitative profile of carotenoids varies depending on species. These differences could be attributed to factors such as type of cultivation, source of nutrients, phylogenetic diversity, morphological and cytological characteristics, and the composition of genes and enzymes specific in each species of microalgae (Borowitzka et al., 2016; Begum et al., 2016).

Regarding the content of lutein, our results are in agreement with those obtained by Paliwal et al. (2016) who, after analyzing 57 strains of microalgae of different phylum, concluded that green algae (*Chlorophyta*) are a potential source this xanthophyll. Also, according to our results, Chen et al. (2016) showed that with distinct extraction methods and different culture media, such as cultures in heterotrophic systems (Chen et al., 2018) or mixotrophic (Chen and Liu, 2018; Chen et al., 2019), the production of lutein by *C. sorokiniana* is substantially significant, and may be considered as a potential source of commercial output this pigment. In addition, the obtained data by Přibyl et al. (2016) show that *Scenedesmus* sp. strain can produce high levels of carotenoids, mainly lutein, whit levels of 0.75 at 1% of dry weight biomass.

Our results are in line with those found by Cordero et al. (2011), where lutein and β-carotene predominated in biomass of the *C. sorokiniana* cultivated under mixotrophic conditions, as well as α-carotene, violaxanthin and zeaxanthin were also detected in smaller quantities. Additionally, Miazek et al. (2017) obtained a carotenoids concentration of 0.86% in dry weight for *C. sorokiniana*, when grown in a beech wood dilute-acid hydrolysate. In our study, we obtained a concentration of 0.70% in dry mass. By contrast, in a recent study, *C. sorokiniana* was cultured in photoautotrophic conditions and analyzed the profile carotenoids by spectrophotometer, showing a quantitative value of 3.8 μg mg^{-1}, higher to that found in our study (Azaman et al., 2017). In addition to these results, Matsukawa and co-workers (Matsukawa et al., 2000) submitted *C. sorokiniana* to cultivation with 10% CO_2 incorporation, in which the total carotenoids (determined spectrophotometrically) contained 0.69% dry weight, similar to the value found in our study. The lutein and β-carotene contents were 4300 and 600 μg g^{-1} dry weight, respectively. Zeaxanthin, β-cryptoxanthin, α-carotene were identified in minor amounts.

Safafar et al. (2015) identified by HPLC-PDA a profile of carotenoids similar to that found in our study, with lutein and β-carotene being the

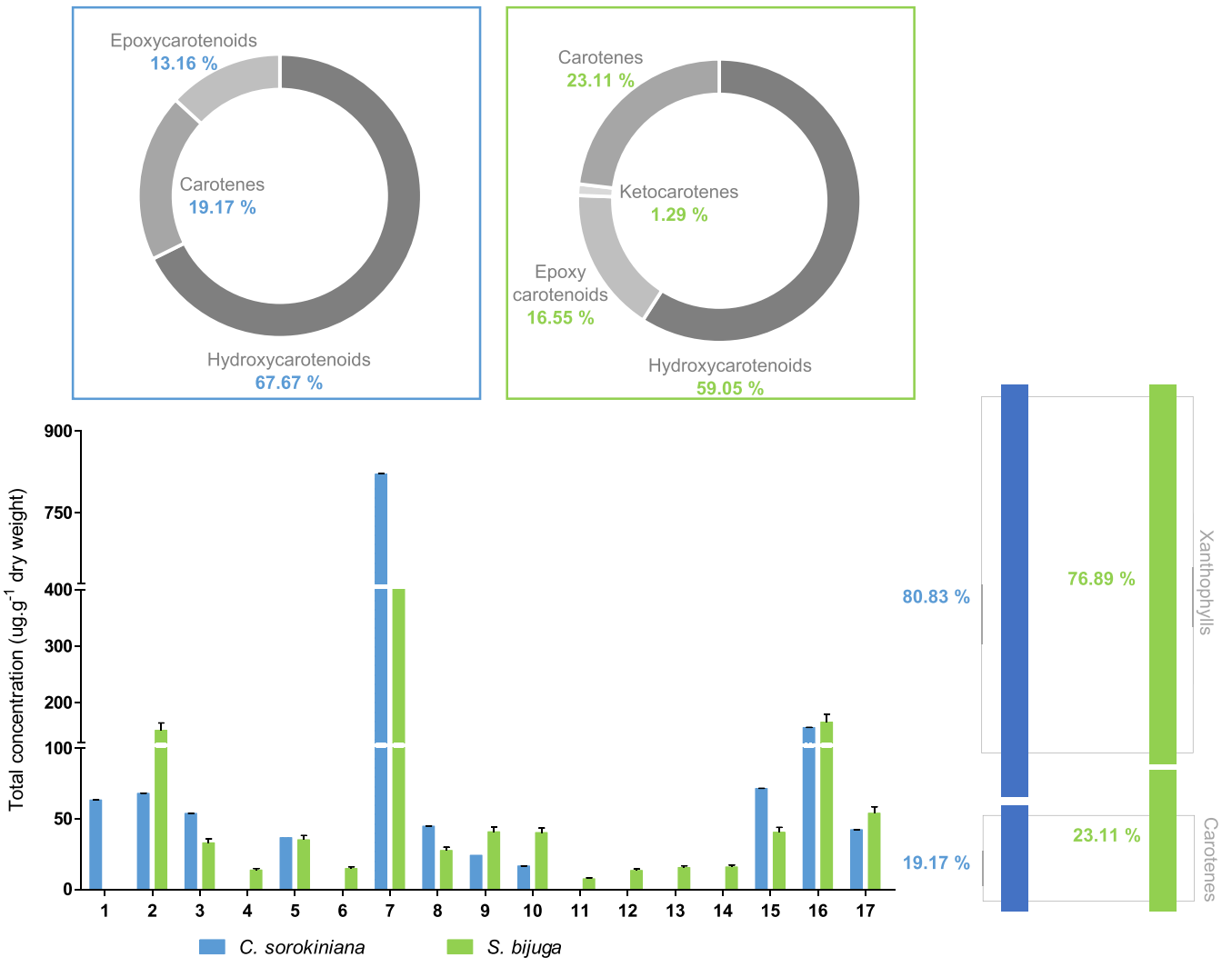

Fig. 2. Composition of the carotenoid fraction in the extracts of *Chlorella sorokiniana* and *Scenedesmus bijuga*.

main carotenoids, followed by neoxanthin, zeaxanthin, fucoxanthin and dihydro from *C. sorokiniana*, but the methodology used did not allow the identification of the fraction of *cis* isomers of these compounds. Interestingly, in this study, we did not detect the presence of fucoxanthin and dihydro lutein. Whereas in the study in Van Wagenen et al. (Van Wagenen et al., 2015), using UHPLC obtained a qualitative profile of carotenoids in *C. sorokiniana* constituted by lutein, violaxanthin, astaxanthin, fucoxanthin, zeaxanthin, and α+β-carotene. The xanthophylls astaxanthin and fucoxanthin were not identified in our study.

As can be seen in Fig. 2, most of the carotenoids present in the two microalgae species are xanthophylls, which represent a fraction of 80.83% in *C. sorokiniana* and 76.89% in *S. bijuga*, being the percentage of epoxycarotenoids more significant in *S. bijuga* and hydroxycarotenoids in *C. sorokiniana*. Thus, the remaining value of carotenoids detected corresponds to the fraction of carotenes 19.17% and 23.11%, respectively.

Among the identified xanthophylls, the epoxycarotenoid all-*trans*-neoxanthin (peak 1), detected only in *C. sorokiniana*, and its isomer 9-*cis*-neoxanthin (peak 2), detected in both microalgae, showed values which represent a fraction of 9.34% (peak 1 and peak 2) and 12.67% (peak 2), of the total xanthophyll content in *C. sorokiniana* and *S. bijuga*, respectively. The value here found is in substantial accordance with the literature, reporting low concentration of neoxanthin in *C. sorokiniana* at values of 48.29 $\mu g.g^{-1}$and 20.0 $\mu g\ g^{-1}$ when cultivated in reactors with light intensities, 2000 μmol photon $m^{-2}.s^{-1}$, and 200 μmol photon $m^{-2}.s^{-1}$, respectively (Safafar et al., 2015).

Violaxanthin, with the 5,6-epoxide group in its structure, is a xanthophyll present in microalgae belonging to the class of *Chlorophyceae* (Takaichi, 2011). In our study, we identified only its 9-*cis*-violaxanthin isomer (peak 3), although we have mapped the MS fragments of the compound in their all-*trans* form. In the literature, no reports on the presence of this compound were found in the studied species, is this the first report. It is important to emphasize that anti-inflammatory properties have already been associated with violaxanthin obtained from microalgae (Soontornchaiboon et al., 2012).

Moreover, within the class of xanthophylls, the polyhydroxylated carotenoids as lutein and its structural isomer zeaxanthin are the most studied ones. This is because they owned innumerable attributions in health promotion and, although they have no provitamin A activity in humans, both display other biological activities as antioxidant property, anti-inflammatory action, and mainly they are associated prevention of macular degeneration (Nwachukwu et al., 2016). Likewise, studies strongly report the significant potential of lutein as an antioxidant agent, which has values higher than β-carotene (Sun et al., 2015). In addition, there has been ever-increasing evidence supporting protective effects in preventing or delaying chronic diseases (Dufossé, 2006; Zhang et al., 2018). These compounds are formed by the hydroxylation of the 3 and 3' carbon atoms of β, ε-carotene or β, β-carotene, respectively, by separate hydroxylases specific for the β and ε rings (Morais et al., 2006).

At the level of industrial production, marigold flowers are the most abundant source of commercial lutein. However, its production is limited

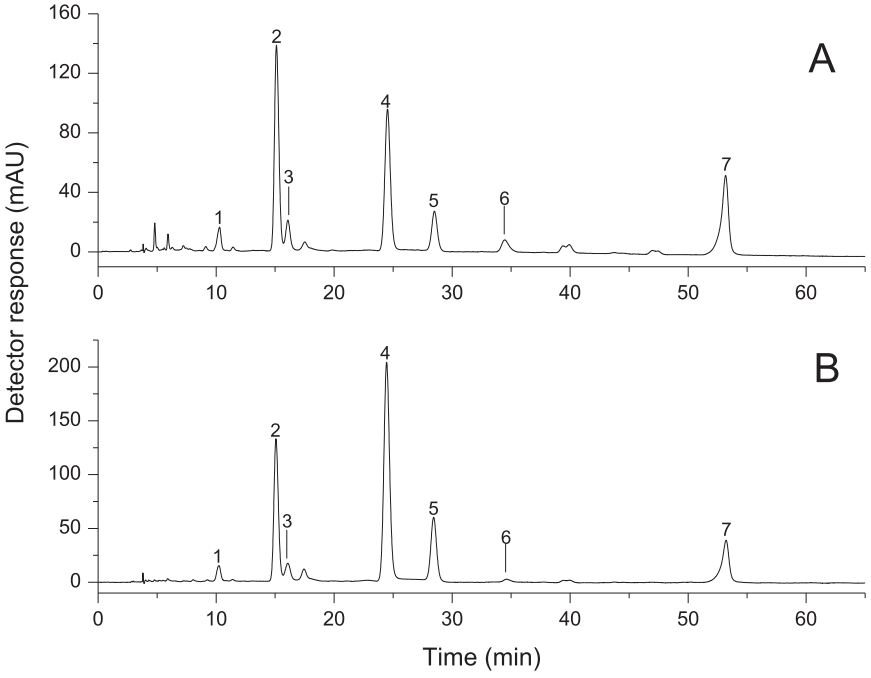

Fig. 3. Chromatogram, obtained by HPLC-PDA, of the chlorophylls extract from *Chlorella sorokiniana* (A) and *Scenedesmus bijuga* (B). See text for chromatographic conditions. Peak identification and characterization are given in Table 3. Chromatogram was processed at 660 nm.

by seasons, planting areas, and the high manpower cost. The lutein production from dried flowers of marigold *Calendula officinalis* varies from 0.04 to 0.301 mg g^{-1} (Lin et al., 2015). The lutein production rate of microalgae in study is 13–20 times higher than marigold flowers. Thus, green microalgae are considered an excellent alternative for obtaining this compound (Sun et al., 2015). One of the most studied green microalgae for lutein production is *Muriellopsis* sp. The content of carotenoid can vary from 0.4% to 0.6% per dry biomass (D'Alessandro and Antoniosi Filho, 2016). In contrast, in our study, we found values de 0.45% and 0.33% for *C. sorokiniana* and *S. bijuga*, respectively. Considering only the lutein production based on the weight of biomass, Minhas et al. (2016) mentioned that lutein productivity per day of *C. sorokiniana* and *S. bijugus* are similar under photoautotrophic conditions, with values of 0.5 and 0.47 mg $L^{-1}.d^{-1}$, respectively.

On the basis of published data, *C. sorokiniana* and *S. bijuga*, is often described as a species that has the ability to accumulate large amounts of all-*trans*-lutein (Chen et al., 2016, 2018; Minhas et al., 2016; Cordero et al., 2011; Safafar et al., 2015). This fact is in agreement with our study in which all-*trans*-lutein and their isomers *cis* represent values of 64.51% (908.48 μg g^{-1}) and 56.14% (670.89 μg g^{-1}) of the total biomass carotenoids of *C. sorokiniana* and *S. bijuga*, respectively.

In this study, we detected the presence of a carotenoid epoxide (β-carotene-5,6-epoxide) only in the *S. bijuga* extract. However, the quantitative value was relatively low, representing a fraction of 1.45% of the total xanthophylls. Exceptionally, in this microalga, all-*trans*-echinenone (peak 13) was found constituting the profile of carotenoids with the ketonic group. In a previous study, these compounds were also detected in species of green microalgae (Patias et al., 2017). However, as far as we know, β-carotene-5,6-epoxide and all-*trans*-echinenone had not yet been reported in the *S. bijuga* biomass.

Although the most significant fraction of carotenoids found consisted of xanthophylls, important carotenes were detected in this study. β-carotene, a second compound more abundant in both microalgae, is one of the most important carotenoids since it has innumerable physiological functions related to its structure, which includes the activities of pro-vitamin A and antioxidant, acting as a potent chemo-preventive agent in various disorders. Also, is a first-choice natural pigment how bioactive compound in functional food (Rodriguez-Concepcion et al., 2018; Meléndez-Martínez, 2019; Paliwal et al., 2016; Cicero and Colletti, 2017). The quantitative values of this compound were from 156.21 (*C. sorokiniana*) and 165.95 μg g^{-1} (*S. bijuga*), showing no significant difference between the two species. In the same way as β-carotene in its all-*trans* configuration, its *cis* isomers are also associated with beneficial functions for health. Previous reports have highlighted the importance of 9-*cis* isomer, which plays an important role in the suppression of oxygen free radicals, protecting human body cells from oxidative stress. 9-*cis*-β-carotene was detected in the two microalgae species studied, representing values of 3.01% and 4.52% (*C. sorokiniana* and *S. bijuga*, respectively). Besides, the isomer 13-*cis*-β-carotene, present only in *S. bijuga*, represents a fraction of 1.34% of the total carotenoids. All-*trans*-α-carotene was present at lower but significant concentrations in the two strains (71.47 and 40.51 μg g^{-1}, respectively).

3.2. Profile chlorophylls

The chromatogram obtained by HPLC-PDA of chlorophylls profile in the microalgae *C. sorokiniana* and *S. bijuga* is demonstrated in Fig. 3, while the chlorophylls compounds composition of the two species of microalgae is shown in Table 3.

As is characteristic of the two species of microalgae belonging to phylum *Chlorophyta*, only species of chlorophyll *a and b* (Borowitzka et al., 2018), and its derivative compounds, which are formed by hydroxylation, pheophytinization and decarboxymethylation reactions, were detected. Furthermore, the characterization of chlorophylls derivatives compounds was only possible through the mass spectral characteristics of the molecules (protonated molecule $[M+H]^+$ and fragments *m/z*).

A detailed description of chlorophylls identification using chromatographic information HPLC-PDA-MS/MS has already been reported in recent works (Fernandes et al., 2017; Chen et al., 2017; Kao et al., 2011). However, as far as we know, this is the first report of the detailed characterization of the quantitative and qualitative profile of

Table 3
Characterization by HPLC-PDA-MS/MS of profile of chlorophyll compounds present in biomass of *Chlorella sorokiniana* and *Scenedesmus bijuga*.

Peak[a]	Chlorophyll	t_R (min)[b]	$\lambda_{máx}$ (nm)[c]	$[M+H]^+$	MS/MS fragment ions (*m/z*)[e]
1	Chlorophyll *b*	10.2	464, 648	907 (100)[d]	875[M + H-32]$^+$ (10.9); 629[M + H-278]$^+$ (98.1); 597[M + H-278-32]$^+$ (52.7); 569[M + H-278-60]$^+$ (36.3)
2	Hydroxychlorophyll *a*	15.1	431, 662	909 (23.0)	891[M + H-18]$^+$ (30.7); 631[M + H-278]$^+$ (34.6); 613[M + H-278-18]$^+$ (42.3); 553[M + H-278-18-60]$^+$ (100), 555[M + H-278-60]$^+$ (24)
3	Chlorophyll *b*'	16.0	462, 654	907 (1.9)	875[M + H-32]$^+$ (7.6); 629[M + H-278]$^+$ (100); 597[M + H-278-32]$^+$ (13.4); 569[M + H-278-60]$^+$ (15.3)
4	Chlorophyll *a*	24.4–25.4	431, 664	893 (100)	615[M + H-278]$^+$ (44.8); 583[M + H-278-32]$^+$ (24.1); 555 [M + H-278-60]$^+$ (27.5)
5	Chlorophyll *a*'	28.4	429, 665	893 (90.9)	615[M + H-278]$^+$ (100); 583[M + H-278-32]$^+$ (31.8); 555 [M + H-278-60]$^+$ (59.0)
6	Hydroxypheophytin *a*	34.4–35.4	408, 668	887 (7.1)	869[M + H-18]$^+$ (10.7); 803[M + H-63]$^+$ (3.5); 609[M + H-278]$^+$ (100); 591[M + H-278-18]$^+$ (7.1); 533[M + H-278-60]$^+$ (18); 531[M + H-278-18-60]$^+$ (6.4)
7	Pheophytin *a*	53.1–53.2	408, 666	871 (40)	593[M + H-278]$^+$ (100); 533[M + H-278-60]$^+$ (35)

[a] Numbered according to the chromatogram shown in Fig. 3.
[b] t_R: Retention time on the C30 column.
[c] Linear gradient MeOH:MTBE:H_2O (81:15:4) and MeOH:MTBE:H_2O (16:80:4).
[d] Relatives intensities for each m/z value appear in parentheses and are expressed as a percentage of the most abundant fragment ion.
[e] Detailed data about mass fragmentation was reported in detail in the literature (Fernandes et al., 2017).

chlorophylls in microalgae species *C. sorokiniana* and *S. bijuga*.

As expected, the presence of chlorophylls in the extracts of *C. sorokiniana* and *S. bijuga* was significant, being 10–17 times more in comparison to the fraction of carotenoids. As shown in Table 4 and Fig. 4, pigment analyses provided identical chlorophylls compounds qualitative composition for the two strains tested (seven compounds). However, it showed a species-specific chlorophyll quantitative profile. The chemical structures of these compounds are shown in Fig. 5.

The contents of chlorophyll and their derivatives in chlorophyll extract from *C. sorokiniana* was 15.20 mg g^{-1}. Hydroxychlorophyll *a* was significantly higher in this microalgae strain, representing 31.47% (4.79 mg.g-^{1}) of the total chlorophyll content, followed by chlorophyll *a* 28.43% (4.32 mg g^{-1}), pheophytin *a* 21.91% (3.33 mg g^{-1}), and chlorophyll *a*' 8.15% (1.22 mg g^{-1}). The other minor chlorophylls, each <4%, summed to 10.05% of the total content. Between these compounds, chlorophyll *b* (0.49 mg g^{-1}) and chlorophyll *b*' (0.52 mg g^{-1}) contributing 6.67% to the total chlorophyll content. According to Petruk et al. (2018), molecules of chlorophyll *a* and chlorophyll *b* was also found in *C. sorokiniana* biomass at a concentration of 0.79 ± 0.11 mg and 0.66 ± 0.09 mg, respectively.

The fact that hydroxychlorophyll *a* is the most abundant chlorophyll compound in *C. sorokiniana,* also described by the first time in this microalgae, is a differentiated characteristic and which may be attributed to the high concentration of peroxidase in these strains (Loh et al., 2012). Previous work postulated that peroxidase might catalyze the oxidation of chlorophyll *a* to produce hydroxychlorophyll *a* through the intermediate phenoxy radical, formed between phenolic compounds at the para-position like *p*-coumaric acid and peroxide in the presence of peroxidase (Yamauchi et al., 2004). Nevertheless, the chlorophyll oxidation may also occur through several other mechanistic pathways as well, as elaborated by Hynninen and Sievers (1981).

Hydroxy derivative compounds have also been detected in green seaweeds. Chen et al. (2017) identified the presence of hydroxypheophytin *a* and hydroxychlorophyll *a* in *Ulva* spp. and *Enteromorpha* spp. in significant concentrations. Besides, previous studies by Steele et al. (2015), it was observed that the occurrence of hydroxychlorophyll *a* in phytoplankton was directly associated with two diatom species, the prymnesiophyte *Phaeocystis* spp. and the coccolithophorid *Emiliania huxleyi.* Also, Hussein et al. (2019) showed that hydroxypheophytin *a* can also be isolated from *Microcystis aeruginosa* cyanobacteria (21.80% of extract), demonstrating the possible marked antioxidant and anti-inflammatory activities at 500 mg/kg dose level.

The microalgae *S. bijuga* demonstrated to have the higher concentrations of the total chlorophylls contents from biomass with a value of 20.44 mg g^{-1}. The chlorophyll *a* (9.67 mg g^{-1}) (47.38%) was quantitatively dominant in chlorophyll profile of microalgae, followed by hydroxychlorophyll *a* (4.61 mg g^{-1}) (22.33%), chlorophyll *a*' (2.82 mg g^{-1}) (13.88%), pheophytin *a* (2.38 mg g^{-1}) (11.68%), chlorophyll *b* (0.44 mg g^{-1}) (2.19%), chlorophyll *b*' (0.36 mg g^{-1}) (1.82%), and hydroxypheophytin *a* (0.14 mg g^{-1}) (0.72%). Chlorophyll *b* and hydroxychlorophyll *a* (peak 1, 2) showed no significant difference between the two species of microalgae evaluated (Table 4 and Fig. 4).

The content of chlorophyll *a* is in agreement with those found by Santhakumaran et al. (2018) that demonstrated that the microalgae strain *Scenedesmus bijuga,* cultured in Bold's Basal Medium (BBM) under controlled conditions of light (8000 Lx), temperature (24 °C) and pH (7.3), can synthesize approximately 9 mg g^{-1} of chlorophyll *a* and 3.5 mg g^{-1} chlorophyll *b* (higher value than found in our study). Other valuable compounds such as carbohydrate (9.7% DW), protein (20.43%), lipid (21.08%) and carotenoids (4 mg g^{-1}) also were detected. Bhatnagar et al. (2011) analyzed the concentration of content chlorophyll (*a* + *b*) in biomass of *S. bijuga* cultivated in phototrophic, heterotrophic, and mixotrophic systems and found that the chlorophyll concentration was substantially higher under mixotrophic conditions, followed by standard phototrophic conditions. Values of 25.83 and 10.65 mg.L^{-1} were found for total chlorophyll content in mixotrophic and phototrophic conditions, respectively.

As established above, the total chlorophyll content investigated in *S. bijuga*, mainly by spectrophotometric methods, concentrates only on species of chlorophyll *a and b*, without an evaluation of its derivative compounds, such as those identified in this work including the epimers

Table 4
Quantitative characterization of chlorophyll compounds in microalgae extracts (mg.g^{-1} dry weight).

Peak	Chlorophyll	*Chlorella sorokiniana*	*Scenedesmus bijuga*
1	Chlorophyll *b*	0.49^a ± 0.03	0.44^a ± 0.06
2	Hydroxychlorophyll *a*	4.79^a ± 0.35	4.61^a ± 0.63
3	Chlorophyll *b*'	0.52^a ± 0.03	0.36^b ± 0.05
4	Chlorophyll *a*	4.32^a ± 0.32	9.67^b ± 1.32
5	Chlorophyll *a*'	1.22^a ± 0.09	2.82^b ± 0.38
6	Hydroxypheophytin *a*	0.50^a ± 0.03	0.14^b ± 0.01
7	Pheophytin *a*	3.33^a± 0.24	2.38^b ± 0.32
	Total chlorophyll	15.20^a ± 1.13	20.44^b ± 2.8

Values are average and standard deviation of triplicates.
Different letters in the same line differ significantly by Tukey test (α = 0.05).

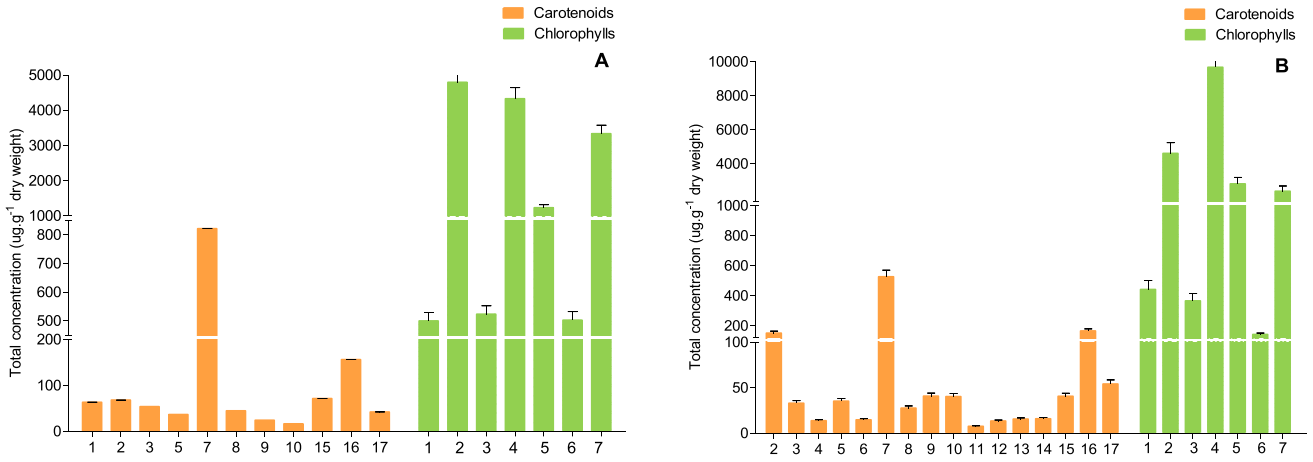

Fig. 4. The total carotenoids and chlorophyll compounds content in the extracts of *Chlorella sorokiniana* (A) and *Scenedesmus bijuga* (B).

chlorophyll *a*', chlorophyll *b*', hydroxychlorophyll *a*, hydroxypheophytin *a* and pheophytin *a*. These derivatives exist in very low concentrations in fruits and vegetables (Hosikian et al., 2010).

Sassi and co-workers (Sassi et al., 2019) reported for dried microalgae of the genus *Chlorella* and *Scenedesmus*, a chlorophyll *a* and chlorophyll *b* concentration of 58.42 and 132.32 mg g^{-1}, respectively, using controlled cultivation conditions. Additionally, in a study by Paliwal et al. (2016) the total chlorophyll content (*a* and *b*) ranged from 2.16 to 18.59 mg g^{-1} dry cell weight in fourteen microalgae strains of the genus *Chlorella*. In contrast, three microalgae strains of the genus *Scenedesmus* showed concentrations of chlorophyll in the range of 0.92–18.53 mg g^{-1} dry cell weight. Thus, it is possible determined that the concentration of chlorophylls in microalgae varies significantly in each species although they are of the same genus.

The proportion of derived compounds exceeds chlorophyll *a* and chlorophyll *b* species in the two microalgae strains. In *C. sorokiniana* represent 68.32% of total chlorophylls, while in *S. bijuga* it was 50.44%. These values represent concentrations of 10.36 and 10.31 mg.g^{-1}, respectively and not presented a significant difference between the two species. Besides, the presence of chlorophyll derivatives only of the chlorophyll *a* species in the two green microalgae species analyzed can be attributed to the higher chemical stability of the chlorophyll *b* molecule, or also due to the low concentration of this compound. Similarly, Chen et al. (2017) analyzed the chlorophylls composition in the red, green and brown seaweeds, and detected maximum content in chlorophyll derivatives (higher than 9 mg.g^{-1} dw.w.) in green seaweeds extracts (*Enteromorpha* spp. and *Ulva* spp.). Curiously, this concentration is in agreement with the values found for chlorophyll derivatives in our study. In this sense, results suggest that the synthesis of compounds derivatives from chlorophyll may be attributed to a characteristic of green microalgae species included in the phylum *Chlorophyta*.

Furthermore, a striking difference between the two species of microalgae under study is the concentration of chlorophyll *a*, which is significantly higher in *S. bijuga* (18.95% more). This may be positively related to the lowest levels of derivative compounds present in this strain, as chlorophyll *a* is the precursor of all other compounds identified in this study, as presented in Fig. 5. Zepka et al. (2019) provide an updated outline of the most recent advances that have occurred in the of chlorophyll catabolism, showing that enzymatic reactions are not involved in epimeric chlorophyll derivatives (chlorophyll *a*' and chlorophyll *b*') In contrast, oxidized derivatives such as hydroxychlorophylls, hydroxypheophytins and pheophytins can have both chemical and enzymatic origin.

Green microalgae *Chlorella* sp. is popular as a primary source of chlorophyll production and called 'Emerald food' because of its high chlorophyll content (Bewicke and Potter, 2009; Safi et al., 2014). The chlorophyll content in *Chlorella* is about 7% of the biomass, which is five times than the chlorophyll content of *Spirulina*, microalgae explored for commercial production of this compound (Khanra et al., 2018). Thus, the results found in our study contain an above-average ratio of chlorophylls per dry mass when compared those reported in the literature, since we obtained concentrations of chlorophyll representing 7.6% and 10.2% of the composition of the compounds present in the biomass of *C. sorokiniana* and *S. bijuga*, respectively. This is strongly evidenced by the fact that some authors consider that the total chlorophyll content in algae is in the range of 0.5–1.5% of dry weight (Becker and Richmond, 2004). Also, as per a recent observation by Basu et al. (2013) the chlorophyll *a and b* content of different microalgae strains (*Scenedesmus obliquus* and *Chlorella* sp.) varies within 1–6% of dry biomass. In this regard, factors such as the cultivation mode and choice of species-specific are determinants to chlorophyll accumulation in microalgae biomass, being generally assumed that sub saturating light intensities induce higher these compounds synthesis (da Silva Ferreira and Sant'Anna, 2017). Thus, a justification for the high concentration of chlorophylls found in this study may be directly related to our experimental conditions and the microalgae species explored, which have shown to be promising alternative sources for the production of these compounds.

Görs et al. (2010) showed that chlorophyll *a* can also be isolated from commercial *Chlorella* products, demonstrating concentrations ranging from 2.5 to 17.5 mg g^{-1} dry weight. In this study, the authors also reported the significant presence of pheophytin *a* in all samples. Furthermore, the axenic strain *C. vulgaris* used as reference presented percentage of pheophytin *a* of 30%.

Curiously, the biosynthesis of chlorophylls involves the synthesis of two moieties: (1) a chlorin ring, which is synthesized by a specific branch of tetrapyrrole biosynthesis, and (2) a phytol chain produced by the isoprenoid (terpenoid) biosynthesis pathway (Fig. 6) (Solymosi and Mysliwa-Kurdziel, 2017). In this sense, the fact that the chlorophyll composition is higher in the *S. bijuga* species can be associated with the lower quantitative profile of carotenoid present in this strain, since carotenoid precursor compounds can be targeted to the biosynthetic pathway of chlorophyll. The opposite was observed in *C. sorokiniana*, where the values were lower for the total chlorophyll content and higher values for the carotenoid class (Fig. 4).

In addition to its primary function in the photosynthesis, chlorophylls are in demand due to their bioactive properties. Several researchers evaluated the biological properties of chlorophyll molecules and demonstrated that chlorophyll *a*, chlorophyll *b* and pheophytin *a* (components of the photosynthetic chain identified in this study) were

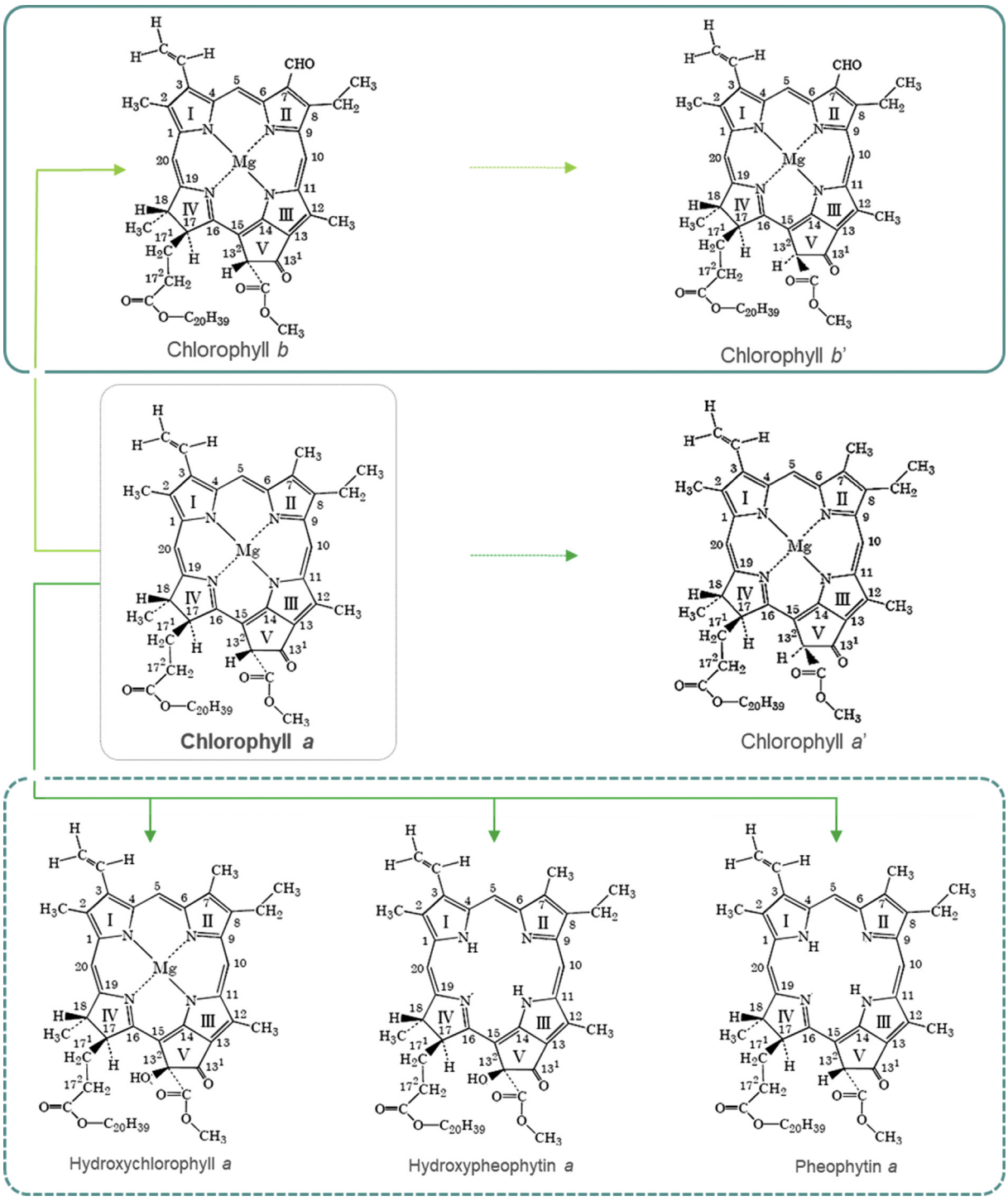

Fig. 5. Structure of the chlorophyll compounds present in extracts of *Chlorella sorokiniana* and *Scenedesmus bijuga*. Solid line: species of chlorophyll; Dotted line: derivatives compounds.

previously shown to exert antimutagenic, chemopreventive and anti-inflammatory activity as well as in vitro anti-oxidant activity (Hoshina et al., 1998; Ferruzzi and Blakeslee, 2007; Szczygieł et al., 2008; Subramoniam et al., 2012; Islam et al., 2013). Specifically, the pheophytin *a* from microalgae species macroalgae species has been identified as a potent suppressor against genotoxininduced *umu* C gene expression in *S. typhimurium* (TA 1535/pSK1002) probably associated with carcinogenesis. According to this, pheophytin *a* derivatives have been proposed to display a potent suppressive activity against chemically induced mouse skin tumorigenesis (Okai et al., 1996).

Besides, compounds chlorophylls derivatives as pheophorbide b and pheophytin b) demonstrated antioxidant activities superior to butylated hydroxytoluene (BHT). This fact demonstrated the importance of the aldehyde group and Mg-free derivatives for the functionality of these molecules (Lanfer-Marquez et al., 2005). Therefore, the presence of a mixture of chlorophyll *a* and chlorophyll *b* and their derivatives such as hydroxychlorophyll *a*, chlorophyll *b*', chlorophyll *a*', hydroxypheophytin *a*, and pheophytin *a* could represent an antioxidant defence system working with synergistic effect. Thus, considering the significant profile of chlorophyll derivatives compounds in *C. sorokiniana* and *S. bijuga*,

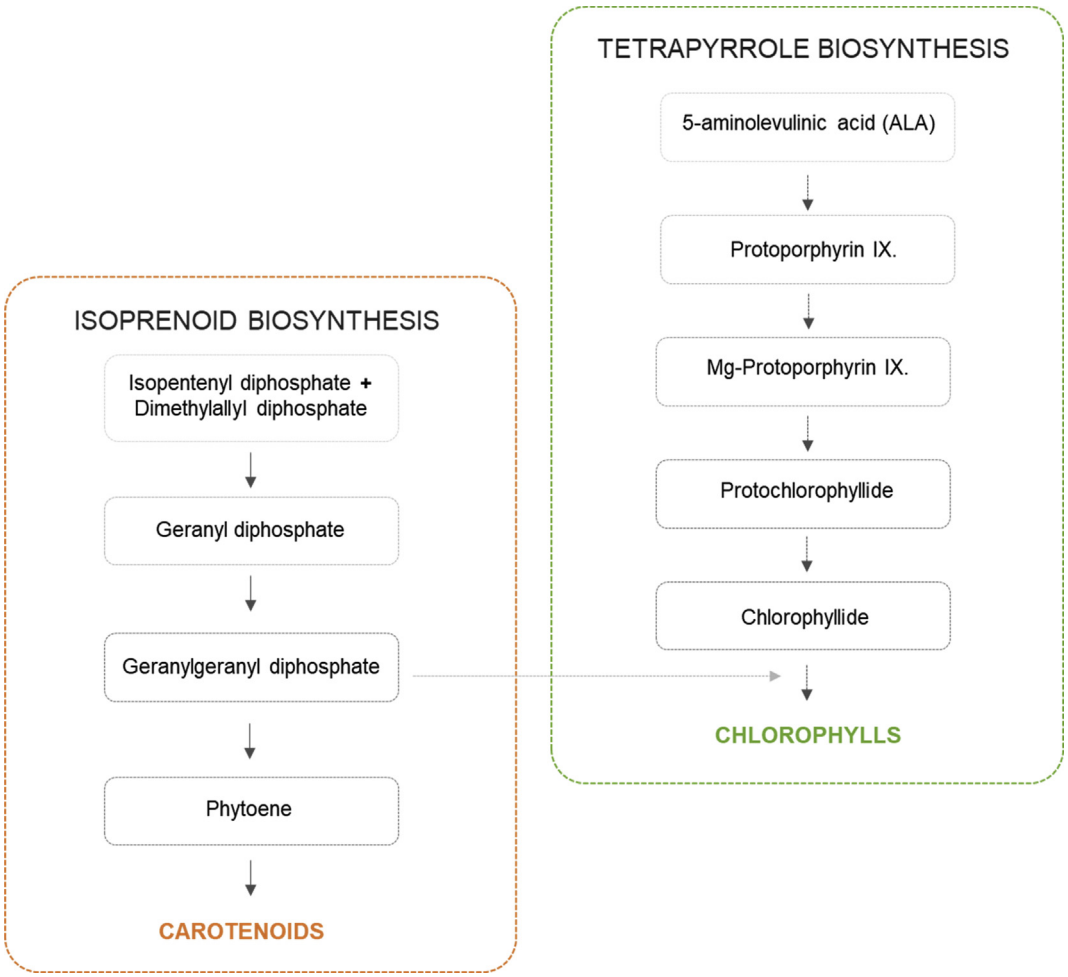

Fig. 6. Simplified scheme of the tetrapyrrole biosynthesis and its relation to isoprenoid biosynthetic pathway providing the phytol side chain for chlorophylls.

further investigations on the actual antioxidant and biological activity of these compounds, together with their bioavailability remains the next primary task in our research.

4. Conclusion

The novelty of this study was a complete characterization of the profile of carotenoids and chlorophylls and their derivatives compound by HPLC-PDA-MS/MS from biomass extracts of *C. sorokiniana* and *S. bijuga.* Specifically, the analytical characterization revealed the presence of eleven and sixteen carotenoids in *C. sorokiniana* and *S. bijuga*, as well as seven chlorophyll compounds, were characterized in both species of microalgae.

Considering the quantitative profile, the highest total carotenoid content was determined in the extract of *C. sorokiniana* (1408.46 μg g^{-1}), and *S. bijuga* exhibited the lowest content (1195.75 μg g^{-1}). Additionally, the results showed that green microalgae synthesize carotenoids with 5,6-epoxy-groups and ketocarotenes in low concentrations and that the α-branch of the carotenoid biosynthetic pathway is active preferably. In contrast to chlorophyll compounds, the highest quantitative profile was demonstrated in *S. bijuga* (20.44 mg g^{-1}); *C. sorokiniana* presented substantially lower values (15.20 mg g^{-1}). Also, the quantification of each compound was species-specific. Among the compounds identified, relevant compounds such as all-*trans*-lutein, β-carotene, and chlorophyll *a* were major compounds in both strains, with values significantly higher than the conventional sources.

When considering the profile of carotenoids/chlorophylls, *S. bijuga* stands out with 10.79% of the composition of the compounds present in the biomass, together with its diversified profile, totaling twenty-three compounds among the two class studied.

Although our study does not highlight any specific molecules, we emphasize that it is possible to increase the potential of these studied species as potential alternative sources of natural carotenoids and chlorophylls, generating new possibilities mainly for the food industry.

Credit author statement

Andrêssa S. Fernandes: Conceptualization, Investigation, Writing - original draft, Writing – review & editing, Data curation, Formal analysis.

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Eduardo Jacob-Lopes: Data curation, Formal analysis, Supervision, Writing - original draft, Writing - review & editing.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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CAPÍTULO 4

Determination of profile of chlorophyll compounds in microalgae species

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Determination of profile of chlorophyll compounds in microalgae species

Determinação do perfil de compostos de clorofila em espécies de microalgas

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RESUMO

Dentre as especialidades químicas subexploradas em microalgas, o perfil das clorofilas em diferentes espécies (*Chlorella vulgaris* e *Aphanothece microscopica Nägeli*) foi caracterizado em detalhes como o objetivo principal deste estudo. A composição das clorofilas e derivados foi determinada por HPLC-PDA-MS (APCI $^{+}$). Os padrões de

fragmentação característicos permitiram identificar oito compostos de clorofila diferentes. Compostos de relevância como espécies de clorofila *a*, clorofila *b*, moléculas derivadas de reações de feofitinização, epimerização e hidroxilação estiveram presentes nas espécies de microalgas. Valores substanciais de 10.734,19 e 9.121,89 $\mu g.g^{-1}$ de peso seco foram obtidos para *C. vulgaris* e *A. microscopica Nägeli*, respectivamente. Assim, a abordagem deste estudo contribui de forma significativa para bancos de dados de composição em constituintes bioativos das espécies avaliadas. Além disso, fornecem informações que elevam a importância desses microrganismos como alternativa para obtenção de componentes de alimentos, enfatizando-os como fontes para atender as necessidades emergentes do mercado de compostos naturais.

Palavras-chave: microalgas, cianobactérias, algas verdes, espectrometria de massa, pigmentos naturais, componentes de alimentos.

ABSTRACT

Among the specialty chemicals sub-exploited in microalgae, the profile of chlorophylls in different species (*Chlorella vulgaris* and *Aphanothece microscopica Nägeli*) was characterized in detail as the main objective of this study. The composition of the chlorophylls and derivatives were determined by HPLC-PDA-MS ($APCI^+$). The characteristic fragmentation patterns have allowed identifying eight different chlorophyll compounds. Compounds of relevance as species of chlorophyll *a*, chlorophyll *b*, molecules derivative from reactions of pheophytinization, epimerization and hydroxylation were present in the species of microalgae. Substantial values of 10,734.19 and 9,121.89 $\mu g.g^{-1}$ dry weight were obtained for *C. vulgaris* and *A. microscopica Nägeli*, respectively. Thus, the approach of this study contributes significantly to composition databases in bioactive constituents of the evaluated species. In addition, provide information that elevates the importance of these microrganisms as alternative for obtaining of food components, emphasizing them as sources to meet the emerging needs the market of natural compounds.

Keywords: Microalgae, cyanobacteria, green algae, mass spectrometry, natural pigments, food components.

1 INTRODUCTION

For decades, research involving biotechnological processes mediated by microalgae has been growing steadily. These microorganisms are considered excellent among all types of biomass sources since they can respond to the future challenges in terms of availability, high rates of growth and production, not competing for arable land. Also, they are considered ideal sources for obtaining valuable co-products for the food industry (Khan et al., 2018; Jacob-Lopes et al., 2019). Within the current market context, approximately 7000 tons of microalgae biomass is produced globally, totaling US$ 32.6 billion. The modeling projects a market of US$ 53.4 billion by 2026 (Rahman, 2020). Of these values, it is estimated that 75% of the production of microalgae-based products is destined for food formulation and obtaining bioactive ingredients (Rumin et al., 2020).

The microalgae biomass has to date found several industrial food applications, since product formulations with functional characteristics and better nutritional conditions, even applications that use pigments extracted from biomass as a dye (Lafarga, 2019). However, nowadays the microalgae biotechnology sector is eminently focused on consolidating microalgae biorefining processes strongly considering the high-value compounds, with the main objective of supporting the sustainable production of functional food compounds (Matos, 2017; Rizwan et al., 2018; Koyande et al., 2019).

Few species of microalgae have commercial importance. Those that include the genus *Chlorella* (class *Chlorophyceae* or green microalgae) constantly draw attention as commercially valuable sources of a wide spectrum of bioactive compounds (Bhalamurugan et al., 2018). Specifically, the species *C. vulgaris* is successfully used in the food industry, feed industry and also in the pharmaceutical industry (da Silva et al., 2019). Thus, considering the number of different species of microalgae existing (stipulated in more than 50,000) is still very small the number of strains that were studied (Sathasivam et al., 2017). In this respect, looking to the exploring new microalgae species for possible commercial applicability, the microalgae *Aphanothece microscopica Nägeli*, from the *Cyanophyceae* class, has been the subject of intensive research. This microalgae specie has high production rate and synthesis capacity of several valuable compounds, including fatty acids, proteins, and carotenoids (Zepka et al., 2008; Zepka et al., 2010; Patias et al., 2017; Vendruscolo et al., 2018; Maroneze et al., 2019). Nevertheless, there are other important molecules bioactive to be elucidate in the composition of microalgae biomass, one of them being class of chlorophyll pigments. These compounds still little explored in microalgae, are primary pigments in the metabolism these microrganisms due to its "light harvesting'' role in photosynthesis. Also, are considered fundamental molecules of life and probably the most important and widely distributed of all natural pigment (Mulders et al., 2014; Solymosi and Mysliwa-Kurdziel, 2017).

Consists of a class of more than 100 different structures naturally synthesized by oxygenic photosynthetic organisms such as plants, algae, microalgae and cyanobacteria, with five species characterized as chlorophyll *a*, *b*, *c*, *d* and *f* (Perez-Gálvez et al., 2017). Given his sensitivity, the chlorophyll molecules form derivatives 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. On the other hand, the formation of isomers can also occur, originating from decarbomethoxylation at position C-13_2 position (Roca et al., 2016).

Chlorophyll compounds are potentially important molecules not only as a colour pigment but also because of their health benefits. Important and prominent biological activities for human health have been demonstrated for chlorophylls and their derivatives, such as the antimutagenic effect, anti-inflammatory, antigenotoxic properties, and potent antioxidant capacity to eliminate free radicals and prevent lipid oxidation (Lanfer-Marquez et al., 2005; Ferruzzi and Blakeslee, 2007; Pérez-Gálvez et al., 2017).

These compounds are currently produced on industrial scale mainly via higher plants such as spinach, alfalfa, stinging nettle, or corn (Sarkar et al., 2020). However, the growing search for natural food components encourages research aimed at the synthesis of these compounds by alternative biological routes. Accordingly, microalgae are considered attractive sources to be explored, since these microorganisms can synthesize chlorophylls in greater proportions than higher plants (Khanra et al., 2018).

Considering the high concentration of chlorophyll and other compounds present in microalgae, several studies have shown the possibility of application of the biomass of these organisms as the functional and natural green dye in foodstuffs formulations (Gouveia et al., 2006; Gouveia et al., 2007; Gouveia et al., 2008; Fradique et al., 2010; Özyurt et al., 2015; Pool et al., 2016; Batista et al., 2017; Palabiyik et al., 2018; Lafarga, 2019). However, these studies lack exploratory analysis of chlorophyll composition in the microalgae biomass used.

In this sense, aiming to explore potential sources for obtaining natural chlorophylls, a comprehensive analysis of the chlorophylls fraction in *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* was the aim of this study.

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., 2017). Methanol, ethanol, acetone, methyl tert-

butyl ether (MTBE), ethyl acetate, petroleum ether and diethyl ether were purchased from Sigma-Aldrich (St. Louis-MO, USA).

2.2 MICRORGANISMS AND CULTURE MEDIA

Axenic cultures of *Chlorella vulgaris* (CPCC90) were obtained from the Canadian Phycological Culture Center. Axenic cultures of *Aphanothece microscopica Nägeli* (RSMan92) were obtained from the collection of the Cyanobacteria and Phycotoxins Laboratory of the Institute of Oceanography from the Federal University of Rio Grande (www.cianobacterias.furg.br). Stock cultures were propagated and maintained in synthetic BG11 medium (Braun-Grunow medium) (Rippka et al., 1979). The incubation conditions were 30 ºC, photon flux density of 15 μmol.m^{-2}.s^{-} and a photoperiod of 12 h were used.

2.3 MICROALGAE BIOMASS PRODUCTION

The biomass production was carried out in a bubble column photobioreactor (Maroneze et al., 2016) 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 1500 × g. (Mandelli et al, 2012). 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), 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 binary pumps (model LC-20AD), online degasser, and automatic injector (Rheodyne, Rohnert Park-CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometry was performed with a Shimadzu 8040 triple quadrupole mass spectrometer and atmospheric pressure chemical ionization (APCI) source (Shimadzu America, Columbia, MD, USA). The UV-vis spectra were obtained between 350 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 (70:30) and filtered through Millipore membranes (0.22 µm). HPLC-PDA-MS/MS parameters were set as previously described by De Rosso and Mercadante (2007), and Fernandes et al. (2017). 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, and the injection volume was 20 µL.

The MS/MS detection was achieved according to Giuffrida et al., (2017) with adaptations, the APCI interface operated in positive (+) mode; detector voltage: 4.5 kV; interface temperature: 350 ºC; DL temperature: 250 ºC; heat block temperature: 200 ºC; nebulizing gas flow (N_2): 3.0 L.min^{-1}; drying gas flow (N_2): 5.0 L.min^{-1}; collision induced dissociation (CID) gas: 23 kPa (argon); event time: 0.5 s. To improve the quality of identification, the MS was used simultaneously in SIM (Select Ion Monitoring) and MRM (Multiple Reaction Monitoring) modes.

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 (Chen et al., 2017; Fernandes et al., 2017; Loh et al., 2012; Gauthier-Jaques et al., 2001).

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. 13^2-hydroxy chlorophyll *a*, 13^2-hydroxy chlorophyll *a*', chlorophyll *a*, and chlorophyll *a′* where quantified using the curve of chlorophyll *a*; the 13^2-hydroxy

pheophytin *a*, pheophytin *a*, and pheophytin *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 and Student's t-test ($p < 0.05$) were applied to experimental data. The analyses were performed with the software GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla-CA, USA).

3 RESULTS AND DISCUSSION

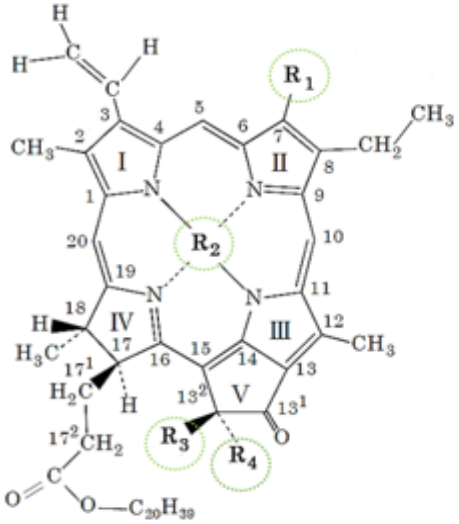
Table 1 and Figure 1 shows a qualitative profile of eight chlorophyll derivatives identified in *Chlorella vulgaris* and *Aphanothece microscopica Nägeli*. Because the chlorophyll compounds have low polarity, atmospheric pressure chemical ionization (APCI) was used to facilitate the ionization and production of $[M + H]^+$ ions.

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

Peak[a]	**Chlorophyll**	**t_R (min)**[b]	**$\lambda_{máx}$ (nm)**[c]	**$[M+H]^+$**	**MS/MS fragment ions (*m/z*)**
1	13^2-hydroxy chlorophyll *a*	9.2	421, 660	909	891$[M+H-18]^+$; 631$[M+H-278]^+$; 613$[M+H-278-18]^+$
2	13^2-hydroxy chlorophyll *a'*	10.2	422, 663	909	891$[M+H-18]^+$; 631$[M+H-278]^+$; 613$[M+H-278-18]^+$
3	Chlorophyll *b*	10.6	468, 659	907	875$[M+H-32]^+$; 629$[M+H-278]^+$; 597$[M+H-278-32]^+$; 569$[M+H-278-60]^+$
4	Chlorophyll *a*	15.1	432, 665	893	615$[M+H-278]^+$; 583$[M+H-278-32]^+$; 555$[M+H-278-60]^+$
5	Chlorophyll *a'*	17.0	431, 665	893	615$[M+H-278]^+$; 583$[M+H-278-32]^+$; 555$[M+H-278-60]^+$
6	13^2-hydroxy pheophytin *a*	24.2	409, 666	887	869$[M+H-18]^+$; 803$[M+H-63]^+$; 609$[M+H-278]^+$; 591$[M+H-278-18]^+$; 531$[M+H-278-18-60]^+$
7	Pheophytin *a*	31.7	408, 666	871	593$[M+H-278]^+$; 533$[M+H-278-60]^+$
8	Pheophytin *a'*	33.0	408, 665	871	593$[M+H-278]^+$; 533$[M+H-278-60]^+$

[a]Numbered according to the elution order on C30 HPLC column.
[b]t_R: Retention time on the C30 column.
[c]Linear gradient Methanol:MTBE.

Figure 1. Structures and nomenclature of chlorophylls and their derivatives identified by HPLC-PDA-MS/MS in *Chlorella vulgaris* and *Aphanothece microscopica nageli*.



Peak	Compound	Molecular formula	R_1	R_2	R_3	R_4
1	13^2-hydroxy chlorophyll *a*	$C_{55}H_{73}MgN_4O_6$	CH_3	Mg	OH	$COOCH_3$
2	13^2-hydroxy chlorophyll *a'*	$C_{55}H_{73}MgN_4O_6$	CH_3	Mg	$COOCH_3$	OH
3	Chlorophyll *b*	$C_{55}H_{70}MgN_4O_6$	CHO	Mg	H	$COOCH_3$
4	Chlorophyll *a*	$C_{55}H_{72}MgN_4O_5$	CH_3	Mg	H	$COOCH_3$
5	Chlorophyll *a'*	$C_{55}H_{72}MgN_4O_5$	CH_3	Mg	$COOCH_3$	H
6	13^2-hydroxy pheophytin *a*	$C_{55}H_{74}N_4O_6$	CH_3	2H	OH	$COOCH_3$
7	Pheophytin *a*	$C_{55}H_{74}N_4O_5$	CH_3	2H	H	$COOCH_3$
8	Pheophytin *a'*	$C_{55}H_{74}N_4O_5$	CH_3	2H	$COOCH_3$	H

The characteristics and chemical structure of the compounds separated in the microalgae analyzed are presented in Table 1 and Figure 1, and the identification is discussed according to the elution order. Once a detailed description of chlorophylls identification using chromatographic information has already been reported by our research group (Fernandes et al., 2017), only chromatographic considerations about chlorophyll compounds not previously identified were discussed below.

Peak 1 was identified as the 13^2-hydroxy chlorophyll *a* (molecular formula $C_{55}H_{73}MgN_4O_6$) on the basis of the characteristic UV-visible spectra (421, 660), and protonated molecule at *m/z* 909, similar to the data from the literature (Kao et al., 2011; Loh et al., 2012). In this molecular structure, the subsequent loss of the hydroxyl group ($C13_2$; Fig. 1) and diterpene alcohol phytol correspond to the fragments *m/z* 891 $[M+H-18]^+$ and m/z 631 $[M+H-278]^+$, respectively.

Chlorophyll *a'* (peak 5) was identified, based on UV/visible ($\lambda_{máx}$), retention time of peak and confirmed by HPLC-MS. The maximal absorbance in the UV/visible spectrum, were located at 431 and 665 nm. The protonated molecule was identified as *m/z* 893 and the fragment ions were 615$[M+H-278]^+$, 583$[M+H-278-32]^+$, 555$[M+H-278-60]^+$. The fragment *m/z* 615 $[M+H-278]^+$ corresponds to the characteristic loss of the diterpene alcohol phytol from the C17 propionic substituent (numbering scheme in Fig.1); while the fragment at *m/z* 583 $[M+H-278-32]^+$ represent the loss of CH_3O group; and *m/z* 555 $[M+H-278-60]^+$ corresponds to loss of CH_3COO group, formed from the cleavage of the ester bond (substituent C17). As previously reported in the literature, the MS/MS fragmentation patterns of *a*/*a'* isomers are basically identical in the APCI-HPLC/MS/MS conditions (Gauthier-Jaques et al., 2001). However, a significant difference in the intensity of the main fragment ($[M+H]^+$) allows the differentiation between them. Chlorophyll *a* is higher intense in the main fragment (*m/z* 893), while in chlorophyll *a'* isomer, the intensity is lower.

In addition to the chlorophyll pigments specified above, it was possible to identify chlorophyll *b* (peak 3), chlorophyll *a* (peak 4), its Mg-free derivative pheophytin *a* (peak 7), as well as hydroxyl-containing derivatives were identified as 13^2-hydroxy chlorophyll *a'* (peak 2), 13^2-hydroxy pheophytin *a* (peak 6), and the pheophytin *a'* isomer (peak 8).

The contents of chlorophyll and their derivatives in chlorophyll extract from *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* are shown in Table 2. The total chlorophylls contents from biomass were 1,0734.19 $\mu g.g^{-1}$ and 9,121.89 $\mu g.g^{-1}$, as dry weight, respectively.

Although the use of microalgae of *Chlorella* genus is consolidated in the market of natural products, as far as we know, the literature lacks information about of the chlorophylls detailed profile in the microalgae *Chlorella vulgaris*. A total of six chlorophyll compounds were identified in the *Chlorella vulgaris* extract (Table 2). This microalgae presented a notably superior quantitative profile (10,734.19 ug.g^{-1}) when compared to another microalgae under study, considering values of 1.17 fold higher than the concentration of chlorophyll total in *Aphanothece microscopica Nägeli*. The major chlorophyll compounds in *Chlorella vulgaris* were chlorophyll *a* (57.43%), chlorophyll *a'* (15.04%), pheophytin *a'* (13.73%) and 13^2-hydroxy chlorophyll *a'* (8.22%). In parallel, pheophytin *a* and chlorophyll *b* were identified as minor compounds, representing 5.56 % of the total content of chlorophylls.

For chlorophyll *b* series, *C. vulgaris* presented a total of 277.98 ug.g^{-1}, represented only by the parental chlorophyll *b*. In contrast, we detected a higher concentration of series *a* compounds, which represents a total of 10,456.21ug.g^{-1}. The fraction of compounds without central magnesium ion in their structure (Figure 1) represents approximately 1,793.68 ug.g^{-1} of the total quantified chlorophylls and is constituted of the compounds pheophytin *a* and its

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

Peak	Compound	*Chlorella vulgaris*	*Aphanothece microscopica Nägeli*
1	13^2-hydroxy chlorophyll *a*	nd	7.14 ± 0.17
2	13^2-hydroxy chlorophyll *a'*	881.74^b ± 2.82	1,616.81^a ± 5.61
3	Chlorophyll *b*	277.98 ± 0.89	nd
4	Chlorophyll *a*	6,165.45^a ± 3.21	2,528.59^b± 4.32
5	Chlorophyll *a'*	1,615.31^a ± 5.17	723.09^b ± 2.78
6	13^2-hydroxy pheophytin *a*	nd	279.94 ± 1.24
7	Pheophytin *a*	319.33^b ± 1.02	568.34^a ± 2.08
8	Pheophytin *a'*	1,474.34^b ± 4.72	3,397.94^a ± 2.21
	Chlorophyll *a* series	10,456.21^a ± 2.72	9,121.89^b ± 6.19
	Chlorophyll *b* series	277.98 ± 1.89	nd
	Total pheophytins	1,793.68^b ± 2.74	3,966.29^a ± 3.12
	Total 13^2-hydroxy derivatives	881.74^b ± 2.82	1,903.91^a ± 1.12
	Total chlorophyll	**10,734.19^a ± 5.39**	**9,121.89^b ± 6.19**

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

pheophytin *a'* isomer. In this microalgae species, only one hydroxylated compound was detected (13^2-hydroxy chlorophyll *a'*), with a quantitative value of 881.74 ug.g^{-1}.

The composition of chlorophyll compounds in *Aphanothece microscopica Nägeli* can be seen in Table 2. A profile of seven compounds was detected in this microalgae. The pheophytin *a'* (3,397.94 µg.g^{-1}) (37.25%) was quantitatively dominant in chlorophyll profile of microalgae, followed by chlorophyll *a* (2,528.59 µg.g^{-1}) (27.72%), 13^2-hydroxy chlorophyll *a'* (1,616.81 µg.g^{-1}) (17.72%) and chlorophyll *a'* (723.09 µg.g^{-1}) (7.92%). The three minor compounds (peak 1, 6, and 7) represented about 9% of the total content.

As expected, only chlorophyll derivatives of series *a* were detected for cyanobacteria *Aphanothece microscopica Nägeli.* Hydroxylated compounds, epimers, and without the central magnesium ion constituted the profile of this species. Of these, Mg-free compounds (peak 7 and 8) represent 3,966.29 µg.g^{-1} of the total chlorophyll content. Among 13^2-hydroxy derivatives, the compounds 13^2-hydroxy chlorophyll *a* (peak 1), 13^2-hydroxy chlorophyll *a'* (peak 2), and 13^2-hydroxy pheophytin *a* (peak 6) were identified and quantified with a total of 1,903.91 µg.g^{-1}. Besides that, the 13^2-hydroxy chlorophyll *a* and 13^2-hydroxy pheophytin *a* were only identified in this species of microalgae.

The total content of pheophytins was relatively abundant in *Aphanothece microscopica Nägeli* (43.48%) which corresponds to approximately 2 times more than that found in the green microalgae under study (16.70%). This can be hypothetically explained by cell morphology, in which the synthesis and storage of chlorophylls in cyanobacteria occur dispersed in the hyaloplasm, which causes less protection of these pigments the action of enzymes or chemistry action in the displacement of the magnesium ion, formation thus, oxidized compounds. On the other hand, smaller amounts of pheophytins were evidenced in *Chlorella vulgaris* (1,793.68 µg.g^{-1}), which is probably attributed to chlorophyll being confined in chloroplasts and also protected by a hydrophobic membrane, which provides greater stability to these compounds.

Regarding the total content of 13^2-hydroxy derivatives, *Aphanothece microscopica Nägeli* also showed a higher concentration, reaching values of 1,903.91 µg.g^{-1}, while in *Chlorella vulgaris* practically half was found (881.74 µg.g^{-1}). According to literature data, the formation of these compounds is probably caused by a hydroxylation formed by the enzyme chlorophyll oxidase (Huang et al., 2008).

Additionally, the chlorophyll *a'* isomer had a relatively lower quantitative profile in the two microalgae when compared to the parental chlorophyll *a*. These results thus demonstrate a concordance with the study by Nakamura et al., (2003) that reports low concentrations of the

compound in photosynthetic microrganisms. Indeed, it has been well established that in addition to degradation, chlorophylls can be susceptible to epimerization at $C13^2$ for chlorophyll *a′* formation (Kao et al., 2011).

In terms of ratio chlorophylls *a/b*, according to Kang et al., (2018), photosynthetic microrganisms present chlorophyll *a* and chlorophyll *b* in a ratio of 3:1, however in our study we found a higher ratio, corresponding to 22:1 in *Chlorella vulgaris*. This high chlorophyll *a/b* the ratio can probably be attributed to the low enzymatic activity of oxygenase since this enzyme catalyzes the conversion of the methyl group bound to ring II (Figure 1) to aldehyde (Xu et al., 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 8.21% in *C. vulgaris*, and 20.85% in *A. microscopica Nägeli*.

Although the different microalgae phylum presents a difference in the chlorophyll fraction, five compounds (peak 2, 4, 5, 7, and 8) are common among the two microalgae investigated. However, all compounds showed a significant difference ($p<0.05$) in the quantitative profile. 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 same 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 chlorophyll synthesis in different classes of microalgae. This is because these compounds are inherently unstable and reactive in the presence of oxygen and light (Beale, 1999; Lohr et al., 2005; Larkin et al., 2016).

In relation to microalgae 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 et al., 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 5.3% (*Chlorella vulgaris*) and 4.5% (*Aphanothece microscopica Nägeli*) chlorophyll on a dry weight.

The literature reports scarce data on the complete characterization of chlorophyll pigments in the microalgae species under study (Kong et al., 2011; Plaza et al., 2012), which makes it difficult to compare them with data from the literature. However, our results demonstrate an interesting profile to be explored and considered as an alternative source for obtaining natural chlorophyll pigments.

4 CONCLUSION

The methodology used in HPLC-PDA-MS/MS allowed the complete characterization of the profile of chlorophylls and their derivatives of *Chlorella vulgaris* and *Aphanothece microscopica Nägeli*, determining a total of eight compounds.

Among the compounds identified, chlorophyll *a* was the major pigment *Chlorella vulgaris*, representing values of 57.43% in dry weight. On the other hand, *Aphanothece microscopica Nägeli* presented pheophytin *a'* as higher compound at concentrations of 37.25% in dry weight. Also, it was possible to identify molecules derivatives from chlorophyll as hydroxylated compounds, isomers, and pheophytins in the two microalgae understudy.

Although there is a constant search by the food industry for natural food components and functional, there are still few studies that explore different species of microalgae as a source of natural chlorophyll pigments. Taking these fine chemicals into account, the results obtained here generate new possibilities for the industrial sources of the presented chlorophylls, since the two species of microalgae showed a profile of chlorophylls substantially interesting to be considered for exploration to industrial level.

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CAPÍTULO 5

Insights on the intestinal absorption of chlorophyll series from microalgae

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Insights on the intestinal absorption of chlorophyll series from microalgae

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ABSTRACT

The bioaccessibility and subsequent uptake by Caco-2 human intestinal cells of chlorophyll pigments from *Scenedesmus obliquus* were determined for the first time. In order to evaluate the impact of different types of the matrix on bioaccessibility of chlorophyll from microalgae, three different products were evaluated: isolated chlorophyll extract (ICE); wet ultrasonicated biomass (WUB); and whole dried biomass (WDB). The samples were submitted to *in vitro* digestion model according to the INFOGEST protocol, and Caco-2 cells determined the intestinal uptake. Chlorophyll pigments were determined by HPLC-PDA-MS/MS. A total of ten chlorophyll pigments (8,318.48 μg g^{-1}) were separated in *S. obliquus* biomass, with chlorophyll *a* (3,507.76 μg g^{-1}) and pheophytin *a*' (1,598.09 μg g^{-1}) the major ones. After *in vitro* digestion, all tested products showed bioaccessible chlorophylls. However, the total bioaccessibility results were as follows: ICE (33.45%), WUB (2.65%), WDB (0.33%). Five compounds were bioaccessible in ICE, three in WUB, and one in WDB. The hydroxypheophytin *a* showed the highest bioaccessibility (212%) in ICE, while pheophytin *a*' in WUB (11%) and WDB (2%). As a result, bioavailability estimates of ICE using the Caco-2 cell showed hydroxypheophytin *a* (102.53%), followed by pheophytin *a*' (64.69%) as the chlorophyll pigments most abundant in intestinal cells. In summary, from a nutritional perspective, these three types of the matrix (WDB, WUB, and ICE) influence the promotion of chlorophyll bioaccessibility. In this way, the data suggest that chlorophylls bioaccessibility from ICE is greater than that in WDB and WUB. Therefore, ICE should be considered a product that provides bioavailable chlorophyll and could be the best choice, such as ingredients in the development of functional foods chlorophyll-based.

1. Introduction

Until March 2020, the global food intake information led us to believe a kind of "feeding demand paradox". On one side, the Food and Agriculture Organization of the United Nations (FAO) supported the thesis of the syndemic of undernutrition, obesity, and climate change was the greatest challenge for human and planetary health in the 21st century (Swinburn, et al., 2019; Da Silva, 2019), and the other hand the market intelligent agencies notice an increasing interest in demand for expensive, environmentally sustainable, sophisticated and innovative products, rich in bioactive compounds (Mintel, 2019). At this time, in both sceneries, the microalgae biomass could be a jack-of-all-trades.

The microalgal biomass and products derived thereof are positioning firmly in the food health market. The majority of the microalgal biomass currently commercialized for food purposes is sold mainly as whole dry biomass (WDB), but also wet biomass (WUB) or as isolated extracts (ICE). The food industry mainly focuses more on the addition of the whole dried biomass (WDB) in nutritional supplement products such as tablets, capsules, or powdered form and is promoted as "superfood", "rich in bioactive compounds" (Carpogno & Mathys, 2018; Lafarga, 2019).

Nevertheless, research on this topic is scarce, and the evidence on potential health benefits is not strong (Carpogno & Mathys, 2018). The great concern is the microalgae matrix effect on the bioactive compounds bioaccessibility. The transfer of bioactive compounds from the microalgae matrix into mixed micelles during digestion, with the release of these compounds from into the circulatory system, is happing? What is more efficient for human health, intake food with WDB, WUB, or ICE?

Another very important endpoint is about the spectrum of microalgae bioactive compounds. The chlorophyll is the less explored, even though a great bioactive potential, just a few research groups all in the world show interest in the study of intake edible seaweed chlorophyll

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(Chen & Roca, 2018a, 2018b), while the microalgae chlorophyll bioaccessibility and bioavailability has not been reported so far.

Finally, after COVID-19, a pandemic inextricably linked in cause and effect to food systems exposed the inability of systems to deliver real healthy diets. In this way, it has never been so important to obtain scientific results to understand bioactive compounds real bioavailability for improved public health. Thus, our contribution was to evaluate the possibility of the microalgae *Scenedesmus obliquus* (no toxins known) (Anvisa, 2018; EC-Regulation, 1997; FDA, 2010; HHLW, 1996), be considered a real source of bioavailable chlorophyll. Therefore the objectives of this study were: (i) to characterize the profile of chlorophylls in the biomass of *Scenedesmus obliquus*; (ii) investigate bioaccessibility and subsequent uptake by Caco-2 human intestinal cells of chlorophyll pigments; (iii) monitor the digestive stability of chlorophyll compounds; (iv) to evaluate the effect of the matrix on the bioaccessibility of chlorophylls, considered the following products, whole dried biomass (WDB), wet ultrasonicated biomass (WUB), and isolated chlorophyll extract (ICE).

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 acquired from Sigma-Aldrich (St. Louis-MO, USA). Methanol (MeOH), methyl *tert*-butyl ether (MTBE), both of chromatographic grade, ethanol, acetone, ethyl acetate, petroleum ether and diethyl ether were purchased from Merck (Darmstadt, Germany). All reagents, solvents and enzymes, α-amylase (A3176), pepsin (P7000), pancreatin (P1750), lipase (L3126) and bile (B8631), used in the *in vitro* digestion procedure assays were acquired from Sigma-Aldrich (St. Louis-MO, USA). Caco-2 cells cultures were purchased from Cell Bank of Rio de Janeiro (BCRJ). All reagents and solvents used in the experimental part were of analytical purity.

2.2. Microrganisms and culture media

Axenic cultures of *Scenedesmus obliquus* (CPCC05) used in the experiments were obtained from the Canadian Psychological Culture Collection University of Toronto, Canada. Stock cultures were propagated in solidified agar-agar (20 g L^{-1}) containing synthetic BG11 medium (Braun-Grunow medium) (Rippka, Deruelles, Waterbury, Herdman & Stanier, 1979). The maintenance conditions were 30 °C, photon flux density of 15 μmol m^{-2} s^{-1} and a photoperiod of 12 h.

2.3. Microalgal biomass production

The biomass production was carried out in a bubble column photobioreactor operating in intermittent regime, fed with 2.0 L of BG11 medium. The experimental conditions were as follows: initial concentration of inoculum of 100 mg L^{-1}, temperature of 25 °C, aeration of 1VVM (volume of air per volume of culture per minute), a photon flux density of 150 μmol m^{-2} s^{-1}, and a residence time of 168 h (Maroneze et al., 2016). The biomass was separated from the culture medium by centrifugation (Hitachi, Tokyo, Japan) for 10 min at 1500*g*. The experiments with wet biomass were carried out with the paste collected after centrifugation (95% of moisture). For experiments with whole dried biomass, the paste collected after centrifugation was subsequently freezing at −18 °C for 24 h and freeze-dried (Lyophilizer Liotop L101) for 24 h at −50 °C above −175 μm Hg, and then stored under refrigeration until the time of analysis.

2.4. Bioaccessibility and cellular uptake assay

2.4.1. Microalgae-based products preparation

An isolated chlorophyll extract from biomass before digestion, utilized as control, was obtained as reported in 2.5.1 section to represent the original content of *S. obliquus* chlorophylls. For the *in vitro* digestion simulation process was used three types of microalgae products submitted to different preliminary treatments: whole dried biomass (WDB), wet ultrasonicated biomass (WUB), and isolated chlorophyll extract (ICE) (see Figure S1 from Supplementary data). Before the *in vitro* digestion assay, the independent products were subjected to a preliminary homogenization process to ensure the desired consistency. For the WDB, aliquots of 0.1 ± 0.02 g of freeze-dried biomass from *S. obliquus* were weighed and combined with 10 mL of saline solution (NaCl 120 mol L^{-1}, CaC_{l2} 6 mmol L^{-1}, KCl 5 mmol L^{-1}). The WDB was prepared without any previous treatment of cell wall rupture. In parallel, for obtaining the WUB, aliquots of 0.8 ± 0.02 g of wet biomass (cellular concentration 4.5 g L^{-1}), equivalent to 0.1 ± 0.02 g of dry biomass, and were combined with 10 mL of saline solution. The resulting mixture was subjected to 15 min of an ultrasonic probe (Ultronic, Indaiatuba-SP, Brazil) to previously breaking the cell wall (an adaptation of Gille, Trautmann, Posten & Briviba, 2016). The ultrasonic parameters were probe with 13 mm diameter, 400 W, 40 kHz, and an ice bath to control the temperature (0 ± 2 °C).The isolated chlorophyll extract (ICE) was obtained from the exhaustive extraction from the lyophilized biomass (0.1 ± 0.02 g) by maceration with ethyl acetate and methanol in a mortar pestle followed by centrifugation (see Section 2.5.1). After extraction, an emulsification process of the extract was carried out according to adaptations by Salvia-Trujillo et al. (2017). The extracts were resuspended in 18% (w:w) of sunflower oil, 2% (w:w) of Tween 80 as a surfactant, and 80% (w:w) of distilled water, followed by ultra-disperser homogenization (1860*g*) for 4 min. A total of 5 g of emulsion per extract was prepared to end added 10 mL de saline solution.

2.4.2. Chlorophylls bioaccessibility by in vitro digestion

The samples were submitted to an *in vitro* simulated digestion model, according to the protocol adapted from INFOGEST (Minekus et al., 2014). The oral phase was started with 6 mL of a solution of artificial saliva containing 106 U mL^{-1} of α-amylase, followed by incubation at 37 °C, 10 min in an orbital shaker (7.5*g*). Before starting the gastric phase, the pH was adjusted to 2.5 with HCl 1 M followed by 2 mL of pepsin (50,000 U mL^{-1} in HCl 100 mM), the total volume was adjusted to 40 mL, and the solution was incubated for 1 h, 37 °C, 7.5*g*. After this step, the pH was changed to 6.0 with 1 M $NaHCO_3$ and the intestinal phase start with a porcine and ovine bile solution (3 mL, 40 mg mL^{-1} in 100 mM $NaHCO_3$), 4,000 U mL^{-1} of porcine pancreatin and 1,000 U mL^{-1} of lipase from porcine pancreas. The pH was adjusted to 6.5 in 50 mL and the incubation occurred for 2 h at 37 °C and 7.5*g*. After the completed *in vitro* digestion, the solution was centrifuged at 8000*g*, 60 min at 4 °C until aqueous phase separation. The supernatant containing the mixed micelles was collected, were covered with nitrogen gas, frozen at −40 °C and lyophilized for further extraction of bioaccessible pigments. The ratio between chlorophylls incorporated into mixed micelle (supernatant) and its respective concentration in the biomass of *S. obliquus* (control extract) predicts their percentage bioaccessibility, and was calculated according to Eq. (1):

$$\text{Bioaccessibility } (\%) = \left(\frac{\text{Micellar fraction content}}{\text{Total initial content}}\right) \times 100 \quad (1)$$

2.4.3. Chlorophylls uptake by Caco-2 cells culture

Considering the greater representativeness of the total and individual bioaccessibility of the ICE chlorophylls (after *in vitro* digestion), when compared to the other products (WDB and WUB), only the ICE was submitted to the uptake test by Caucasian colon adenocarcinoma cells (Caco-2) (Figure S1 from Supplementary data). The cells were cultivated according to Natoli, Leoni, D'Agnano, Zucco, and Felsani (2011), were seeded in T25 flasks at 2.5 × 105 cells/flask, containing growth medium enriched (DMEM) with non-essentials amino acids (2%),

penicillin–streptomycin (2%), and heat inactivated fetal bovine serum (FBS 15%), maintained at 37 °C and 5% CO_2. After the culture acquired 80% of confluence (~10 days), FBS was reduced to 10%, until the formation of monolayers, and then, the experiments were carried out. Monolayers were washed with PBS and DMEM (final volume of 5 mL) containing 25% of the micellar fraction obtained from the *in vitro* digestion step was added to the monolayers, followed by incubation for 4 h (at 37 °C and 5% CO_2 of atmosphere). Subsequently, Caco-2 monolayers were washed with phosphate buffered saline (PBS), and 5 mL of pure DMEM medium were added and submitted to incubation for more 6 h. After this period, the cells were harvested with rubber scrapper, collected and centrifuged at 1500*g*, 4 °C, for 5 min. Cells pellets were covered with nitrogen gas, frozen at −40 °C and lyophilized for further extraction of the absorbed pigments. Protein content of cell samples was determined by a Bradford rapid assay (BIO RAD: Quick startTM Bradford), using bovine serum albumin as standard. Once, the intestinal cellular uptake of a compound refers to the percentage content of the compound uptake by Caco-2 cells compared to the inaccessible content of the compound in the micellar fraction, the percentage uptake was calculated according to Eq. (2):

$$\text{Uptake}(\%) = \left(\frac{\text{Celular uptake content}}{\text{Micelar fraction content}}\right) \times 100 \quad (2)$$

Whereas cellular uptake content is the amount of the chlorophyll absorbed by the cells and the micellar fraction content is the amount of the chlorophyll that was incorporated into micelles.

2.5. Chlorophyll analysis

2.5.1. Chlorophyll extraction from S. obliquus biomass

The biomass of *S. obliquus* was extracted in triplicate according to the procedure described by Mandelli, Miranda, Rodrigues, and Mercadante (2012). Briefly, the chlorophylls were exhaustively extracted from the freeze-dried samples (0.1 ± 0.02 g) with ethyl acetate followed by methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 1500*g*. The extraction procedure was repeated until the supernatant becomes colorless, what happened with 11 and 6 extractions with ethyl acetate and MeOH, respectively. Then, the homogenized sample suspension was filtered through a 0.22 μm polyethylene membrane, concentrated in a rotary evaporator (T < 30 °C). The dry extracts were stored at −37 °C under a nitrogen atmosphere and kept in the dark until chromatographic analysis.

2.5.2. Chlorophyll extraction from the fraction of digesta and Caco-2 assays

The remaining pigments from *in vitro* digestion (micelles) and uptake by Caco-2 cells (cell pellets) were extracted by ultrasound-assisted extraction (UAE) using an ultrasonic processor (Ultronic, Indaiatuba-SP, Brazil) with a 13 mm diameter probe according to an adapted methodology (Ordóñez-Santos, Pinzón-Zarate & González-Salcedo, 2015). The lyophilized samples were exhaustively extracted by the addition of 15 mL of ethyl ether and petroleum ether (1:1) and subjected to 5 min ultrasonic cycles, with a frequency of 500 Hz and 80% power, were centrifuged and the supernatant was collected. The process was repeated until the supernatant became colorless. After the extracts were rotary evaporated and subjected to chromatographic analysis.

2.5.3. Separation, identification, and quantification by HPLC-PDA-MS/MS

The pigments were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with binary pumps (model LC-20AD), online degasser, and automatic injector (Rheodyne, Rohnert Park-CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometry was performed with a Shimadzu 8040 triple quadrupole mass spectrometer and atmospheric pressure chemical ionization (APCI) source (Shimadzu America, Columbia, MD, USA). The pigments separation was performed on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA). HPLC-PDA-MS/MS analysis was performed according to Fernandes et al. (2017). Prior to HPLC-PDA-MS/MS analysis, the chlorophyll extract was solubilized in MeOH:MTBE (70:30) and filtered through Millipore membranes (0.22 μm). The mobile phases A (MeOH) and phase B (MTBE), using a linear gradient program as follows: from 95:5 to 70:30 in 30 min, to 50:50 in 20 min. The flow rate was 0.9 mL min^{-1}, the column temperature set to 29 °C, and the injection volume was 20 μL. The UV–vis spectra were obtained between 450 and 660 nm, and the chromatograms were processed at 660 nm.

The MS/MS detection was achieved according to Giuffrida, Zoccali, Giofrè, Dugo, and Mondello (2017) with adaptations, the APCI interface operated in positive (+) mode; detector voltage: 4.5 kV; interface temperature: 350 °C; DL temperature: 250 °C; heat block temperature: 200 °C; nebulizing gas flow (N_2): 3.0 L min^{-1}; drying gas flow (N_2): 5.0 L min^{-1}; collision induced dissociation (CID) gas: 23 kPa (argon); event time: 0.5 s. To improve the quality of identification, the MS was used simultaneously in SIM (Select Ion Monitoring) and MRM (Multiple Reaction Monitoring) modes.

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 (Chen, Ríos, Pérez-Gálvez & Roca, 2017; Fernandes et al., 2017; Fernandes, Petry, Mercadante, Jacob-Lopes & Zepka, 2020; Gauthier-Jaques, Bortlik, Hau & Fay, 2001; Huang, Hung, Wu & Chen, 2008; Loh, Inbaraj, Liu & Chen, 2012). The chlorophylls were quantified by HPLC-PDA using analytical curves of chlorophyll *a* and chlorophyll *b*. Chlorophyll and compounds derivatives from the series *a* were quantified using the curve of chlorophyll *a*; chlorophyll and compounds derivatives from the series *b* were quantified using the curve of chlorophyll *b*. Total chlorophyll content was calculated considering all identified peak areas.

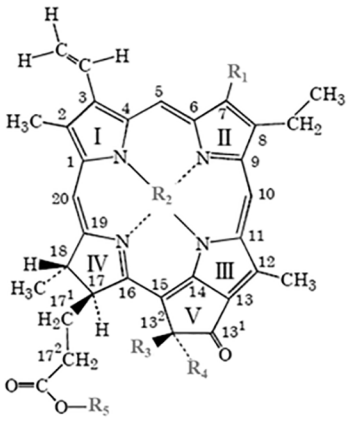
2.6. Statistical analysis

The analysis was performed using Statistica 7.0 software (Statsoft, Tulsa-OK, USA). The significance of the experimental data was determined using one-way ANOVA followed by Tukey's test ($p < 0.05$).

3. Results and discussion

3.1. Profile of chlorophylls in the biomass

Fig. 1 and Table 1 illustrate all the characteristics and chemical structure of chlorophylls and their derivative compounds found in the chlorophyll extract obtained from the biomass of *S. obliquus*. The distribution of chlorophylls series in microalgae is directly associated with the phylum to which they belong. *S. obliquus* is a species of green microalgae belonging to the Chlorophyta phylum. Due to this classification, and its morphological characteristics, *S. obliquus* synthesizes and stores in its chloroplasts the chlorophyll *a* and *b* series (Mulders, Lamers, Martens & Wijffels, 2014; Borowitzka, 2018). The structural difference between these two series of chlorophylls is the terminal group linked to C_7 (Fig. 1, radical R1). While Chl *a* has a methyl group in this position, Chl *b* owns the formyl group (Roca, Chen & Pérez-Gálvez, 2016). In our study, a diverse profile of chlorophylls derivatives was found in the *S. obliquus* biomass, including catabolites formed by oxidative allomerization, pheophytinization, de-esterification of phytol, and epimerization reactions because of natural metabolism, chemical, or enzymatic action. A reason for this is the high sensitivity of chlorophyll structures outside their natural environment, resulting from the rupture of the cell and chloroplast during obtaining the extract (Gandul-Rojas, Gallardo-Guerrero & Minguez-Mosquera, 2009).



Peak	Compound	Molecular formula	R_1	R_2	R_3	R_4	R_5
1	Pheophorbide *a*	$C_{35}H_{36}N_4O_5$	CH_3	2H	H	$COOCH_3$	H
2	Hydroxychlorophyll *a*	$C_{55}H_{73}MgN_4O_6$	CH_3	Mg	OH	$COOCH_3$	Phytyl
3	Chlorophyll *b*	$C_{55}H_{70}MgN_4O_6$	CHO	Mg	H	$COOCH_3$	Phytyl
4	Chlorophyll *b'*	$C_{55}H_{70}MgN_4O_6$	CHO	Mg	$COOCH_3$	H	Phytyl
5	Chlorophyll *a*	$C_{55}H_{72}MgN_4O_5$	CH_3	Mg	H	$COOCH_3$	Phytyl
6	Chlorophyll *a'*	$C_{55}H_{72}MgN_4O_5$	CH_3	Mg	$COOCH_3$	H	Phytyl
7	Hydroxypheophytin *a*	$C_{55}H_{74}N_4O_6$	CH_3	2H	OH	$COOCH_3$	Phytyl
8	Hydroxypheophytin *a'*	$C_{55}H_{74}N_4O_6$	CH_3	2H	$COOCH_3$	OH	Phytyl
9	Pheophytin *b*	$C_{55}H_{72}N_4O_5$	CHO	2H	H	$COOCH_3$	Phytyl
10	Pheophytin *a*	$C_{55}H_{74}N_4O_5$	CH_3	2H	H	$COOCH_3$	Phytyl
11	Pheophytin *a'*	$C_{55}H_{74}N_4O_5$	CH_3	2H	$COOCH_3$	H	Phytyl

Fig. 1. Names and structural characteristics of chlorophylls and their derivatives found in *Scenedesmus obliquus* in the different products analyzed.

Table 1
Characterization by HPLC-PDA-MS/MS of the profile of chlorophyll compounds present in the microalgae *Scenedesmus obliquus*.

Peak[a]	Chlorophyll	$\lambda_{máx}$ (nm)[b]	$[M+H]^+$	MS/MS fragment ions (*m/z*)[d]
1	Pheophorbide *a*	409, 666	595	nd[c]
2	Hydroxychlorophyll *a*	423, 663	909	891$[M+H-18]^+$; 631$[M+H-278]^+$; 613$[M+H-278–18]^+$; 553$[M+H-278–18-60]^+$
3	Chlorophyll *b*	468, 651	907	875$[M+H-32]^+$; 629$[M+H-278]^+$; 597$[M+H-278–32]^+$; 569$[M+H-278–60]^+$
4	Chlorophyll *b'*	468, 654	907	875$[M+H-32]^+$; 629$[M+H-278]^+$; 597$[M+H-278–32]^+$; 569$[M+H-278–60]^+$
5	Chlorophyll *a*	432, 665	893	615$[M+H-278]^+$; 583$[M+H-278–32]^+$; 555$[M+H-278–60]^+$
6	Chlorophyll *a'*	431, 665	893	615$[M+H-278]^+$; 583$[M+H-278–32]^+$; 555$[M+H-278–60]^+$
7	Hydroxypheophytin *a*	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	Hydroxypheophytin *a'*	399, 663	887	869$[M+H-18]^+$; 803$[M+H-63]^+$; 609$[M+H-278]^+$; 591$[M+H-278–18]^+$; 531$[M+H-278–18-60]^+$
9	Pheophytin *b*	432, 656	886	nd
10	Pheophytin *a*	408, 666	871	593$[M+H-278]^+$; 533$[M+H-278–60]^+$
11	Pheophytin *a'*	408, 665	871	593$[M+H-278]^+$; 533$[M+H-278–60]^+$

[a] Numbered according to the elution order on C30 HPLC column.
[b] Linear gradient Methanol:MTBE.
[c] Not detected.
[d] Detailed data about mass fragmentation was reported in detail in the literature (Gauthier-Jaques et al., 2001; Huang et al., 2008; Loh et al., 2012; Fernandes et al., 2017; Fernandes et al., 2020).

Our identification was entirely based on chemical evidence provided by the analysis compared with data available in the literature. The characteristics of the chromatographic and spectrometric data for the different compounds analyzed in the present study are shown in Table 1. A total of eleven compounds were separated, identified, and quantified, ten of which were identified in the control extract, and one compound characterized only after the *in vitro* digestion process (peak 9). Aspects of the identification of chlorophylls and their derivatives compound using chromatographic information were previously described in detail (Fernandes et al., 2017). However, in this study, we identified some compounds not determined in the previous report, which are discussed below.

The peak 1 presents UV–vis typical of pheophorbide *a* with λmax at 409 and 666 nm, protonated molecule at *m/z* 595, and early elution order (5.5 min) before its more hydrophobic precursor (chlorophyll *a*) due to its degree of polarity. Its chemical structure of molecular formula $C_{35}H_{36}N_4O_5$ (Fig. 1) consists of a macrocycle porphyrin ring, without diterpene alcohol phytol, and absence of the central Mg.

Peak 2 was identified as hydroxychlorophyll *a* considering their UV–visible spectrum characteristics (423 and 663 nm), mass spectra with the protonated molecule at *m/z* 909, and the most abundant fragments at *m/z* 891 $[M+H-18]^+$ and 631 $[M+H-278]^+$, attributed to losses of hydroxyl and diterpene alcohol phytol respectively, which was similar to that reported by Huang et al. (2008). Other fragments were detected, both from the MS/MS, at *m/z* 613 $[M+H-278–18]^+$, with an additional at *m/z* 553 $[M+H-278–60]^+$.

Peak 4 was identified as chlorophyll *b'*, considering the lack of fine structure in the UV–visible spectrum (468, 654 nm), protonated molecule 907 $[M+H]^+$, fragments *m/z* as well the retention time (11.7 min). The elimination of the diterpene alcohol phytol from the C17, propionic substituent (numbering scheme in Fig. 1), 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 $[M+H-278–32]^+$, 569 $[M+H-278–60]^+$, were attributed elimination of the diterpene alcohol phytol, in both fragments, together with the CH_3O group and loss of CH_3COO group, respectively (Fig. 1). The MS/MS fragmentation patterns of *b/b'* epimers are basically identical in the APCI-HPLC-MS conditions, with difference only in the intensity of the main fragment $[M+H]^+$, which showed higher intensity in chlorophyll *b* than in its epimer *b'* (Figure S2 from Supplementary data). In contrast, the epimer *b'* presented an abundance of fragment ions different, which the *m/z* 629 fragment as the most abundant, whereas the $[M+H]^+$ occur with minor intensity. These chemical evidences for the identification of these chlorophyll epimers has been well documented previously by Gauthier-Jaques et al. (2001).

Chlorophyll *a'* (peak 6) was identified, based on retention time of peak, UV–visible (λmáx), and confirmed by HPLC-PDA-MS. The protonated molecule was identified as *m/z* 893, the same value reported for chlorophyll *a* (peak 5), because it has the same chemical structure ($C_{55}H_{72}O_5N_4Mg$) and, therefore, also the same chromophore (Huang et al., 2008; Bale, Llewellyn & Airs, 2010). Moreover, the chlorophyll *a'* epimer showed practically the same characteristic UV–visible spectra

(431, 465 nm), and fragments MS/MS (615[M+H-278]$^+$, 583[M+H-278–32]$^+$, 555[M+H-278–60]$^+$) compared to the corresponding chlorophyll *a* identified by Fernandes et al. (2017) and in this study. As *a*/*a*' species are configuration epimers, their MS/MS fragmentation patterns showed the same ion fragmentation behavior for *b*/*b*' species, with the protonated molecule being the ion more intense in chlorophyll *a* and the less abundant in chlorophyll *a*' (Figure S3 from Supplementary data). 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, Chen & Chen, 2011).

Peak 9 was identified as pheophytin *b* through the characteristic UV–visible absorption spectra of this compound (432, 656 nm) and protonated molecule identified as *m/z* 886. The pheophytin *b* was detected in the ICE product after the *in vitro* digestion process, suggesting that this Mg-free derivative of the *b* series was formed from the gastrointestinal conditions. In addition to the compounds described above, it was possible to identify chlorophyll *b* (peak 3), chlorophyll *a* (peak 5), its derivative pheophytin *a* (peak 10) and the pheophytin *a*' epimer (peak 11), as well as hydroxyl-containing derivatives were identified as hydroxypheophytin *a* (peak 7), and hydroxypheophytin *a*' (peak 8).

Fig. 2 shows the concentrations of chlorophylls and their derivative compounds in *S. obliquus* biomass. A total amount of 8,318.48 μg g^{-1} of chlorophyll pigments (dry weight basis) was obtained in the extract, which corresponds to 8.31% chlorophyll in dry weight. The chlorophyll *a* (3,507.76 μg g^{-1}) (42.16%) was quantitatively dominant in chlorophyll profile of microalgae, followed by pheophytin *a*' (1,598.09 μg g^{-1}) (19.21%), pheophorbide *a* (995.77 μg g^{-1}) (12.02%) and chlorophyll *a*' (963.28 μg g^{-1}) (11.58%). The six minor compounds (peak 2, 3, 4, 7, 8 and 10) represented 15.03% of the total content. Chlorophyll *b* (peak 3), the second species of native chlorophyll present in the *S. obliquus* biomass showed a concentration of 349.22 μg g^{-1} (4.19%), while their epimer chlorophyll *a*' represents 1.61% (134.61 μg g^{-1}) of total amount chlorophyll pigments.

As previously reported, the biomass of *S. obliquus* has chlorophylls of *a* and *b* series in its constitution. In our results, derivatives of the *a* series contribute 94.19%, and derivatives of the *b* series account for 5.81% of the total fraction of chlorophyll pigments. Compounds of the *a* series showed greater instability and catabolites formed from reactions of Mg dechelation, epimerization, de-esterification of phytol, and oxidative allomerization were detected. Thus, *a* series presented phytylated and dephytylated chlorophyll pigments, which represented values of 87.98% and 12.02%, respectively, in the total composition of derivatives of *a* series. Although there is a smaller amount of substrate for the formation of *b* series derivatives, that is, smaller amounts of parental chlorophyll *b*, the *b* series showed higher stability, and only derivative compounds from epimerization reactions were found in the chlorophyll extract obtained from *S. obliquus* biomass.

Chlorophylls *a* + *b* series represent 59.54% (4,954.87 μg g^{-1}) to the total chlorophyll content (including peaks 3, 4, 5 and 6) and less than 36% of de-chelated chlorophylls (pheophorbides and pheophytins). Two pheophytins were detected (peak 10, pheophytin *a*; peak 11, pheophytin *a*') contributing with 21.67%, and only pheophorbide *a* (peak 1) was found as a dephytylated derivative. According to reports available in the literature, at the level of biochemical changes, the enzyme pheophytinase is responsible for catalyzing the conversion of chlorophyll *a* into pheophytin *a* (Schelbert et al., 2009). In contrast, for the biochemical synthesis of derivatives dephytylated and without the central of the Mg^{2+} ion, it is known that the chlorophyllase is the first enzyme responsible for catalyzing the conversion of chlorophyll *a* to chlorophyllide *a* by hydrolyzing the phytol chain, followed by a Mg dechelation by the enzyme pheophytinase that results in pheophorbide *a* (Zepka, Jacob-Lopes & Roca, 2019). However, these enzymatic reactions are only one degradation path, among others, that can occur in photosynthetic organisms for the formation of these derivative compounds.

The obtained data show that oxidative derivatives as 13^2-Hydroxy derivatives are present in the biomass in concentrations of 6.77%, between pheophytin and chlorophyll derivatives of *a* series. This result agrees with a study by Yao, Gerde, Lee, Wang, and Harrata (2015) that showed that, among the chlorophylls profile, hydroxy-compounds are present in low quantity in different microalgae species. Other studies

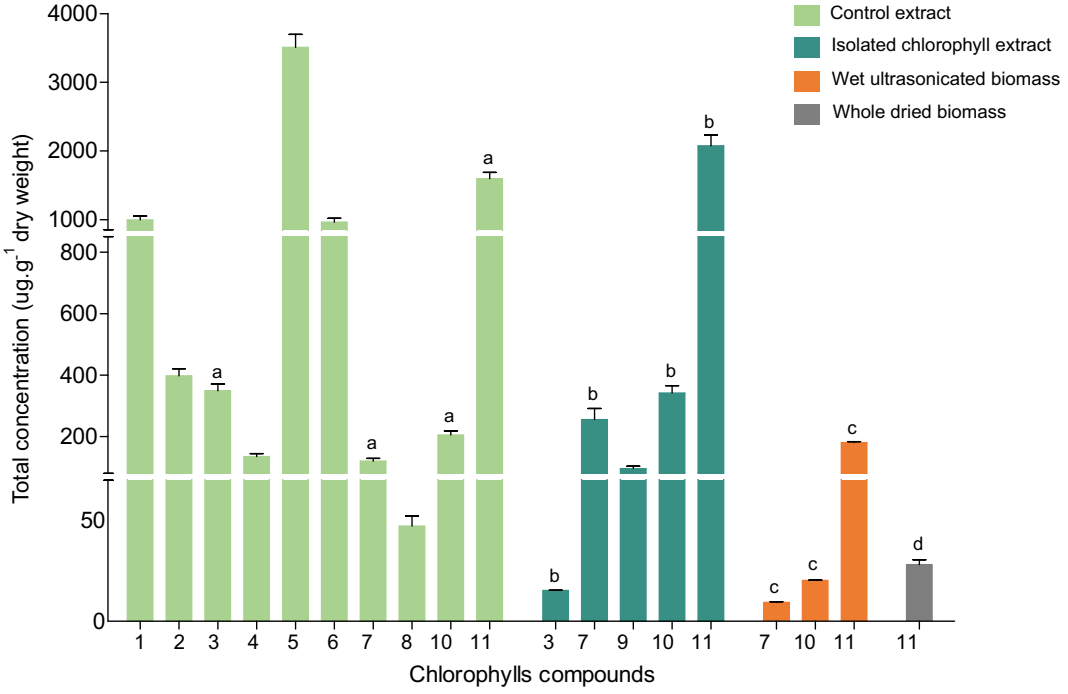


Fig. 2. Quantitative characterization of individual chlorophyll compounds from *S. obliquus* biomass before digestion and after micellar incorporation on tested samples (whole dried biomass, wet ultrasonicated biomass, and isolated chlorophyll extract). Different letters above the error bars indicate significant differences of the chlorophyll derivatives between treatments ($p < 0.05$).

reported in the literature have also demonstrated the presence of hydroxylated compounds in *Scenedesmus obliquus* and seaweed (Gilbert-López et al., 2017; Chen et al., 2017). Some authors relate the formation of these catabolites to the action of the enzyme peroxidase, since in samples of peas that received previous treatments to inactivate this enzyme, the presence of these allomerized compounds was not detected (Gallardo-Guerrero, Gandul-Rojas & Minguez-Mosquera, 2008).

A total of four chlorophyll derivatives were identified as epimers at the C13^2 position (See Fig. 1) of the parent compounds (chlorophyll *b*', chlorophyll *a*', hydroxypheophytin *a*' and pheophytin *a*') contributing 32.96% (2,743.28 μg g^{-1}) to the total chlorophyll fraction. These results do not demonstrate a concordance with the study by Nakamura, Akai, Yoshida, Taki, and Watanabe (2003) that reports minute concentrations of these catabolites in photosynthetic microrganisms. In contrast, some authors report the frequent presence of these compounds when they are isolated from natural sources or obtained in laboratories (Vergara-Domínguez, Gandul-Rojas & Roca, 2011).

Finally, after the identification of chlorophyll profile from microalgae was possible to determine the dominant polarity of compounds as lipophilic since most of the compounds identified (87.98%) have a propionic acid esterified with diterpene phytol alcohol in C_{17} (Fig. 1). However, 13^2-Hydroxy derivatives tend to the polar character. These compounds represent 4.77% (hydroxychlorophyll *a*), 1.44% (hydroxypheophytin *a*), and 0.56% (hydroxypheophytin *a*'), values that do not interfere with the dominant polarity.

3.2. Micellar incorporation and bioaccessibility of chlorophylls

Data available in the literature report that the bioaccessibility of phytochemicals present in microalgae is relatively low when the compounds are evaluated from whole biomass (Gille et al., 2016; Gille, Hollenbach, Trautmann, Posten & Briviba, 2019). This fact is mainly associated with the cell wall and/or cell membrane of these microorganisms that act as a natural barrier for lipophilic nutrients, which it becomes a critical point in the stage of release of the matrix components in the bioaccessibility process (Bernaerts et al., 2020). Hence, some authors suggest that microalgal cell disruption is a crucial step for the efficient release of the compounds present in the microalgae matrix (Bernaerts et al., 2020; Cha, Koo, Song & Pan, 2012; Cavonius, Albers & Undeland, 2016; Gille et al., 2016, 2019; Granado-Lorencio et al., 2009).

Considering these aspects, we analyzed three different products: whole dried biomass of *S. obliquus* (WDB); wet ultrasonicated biomass

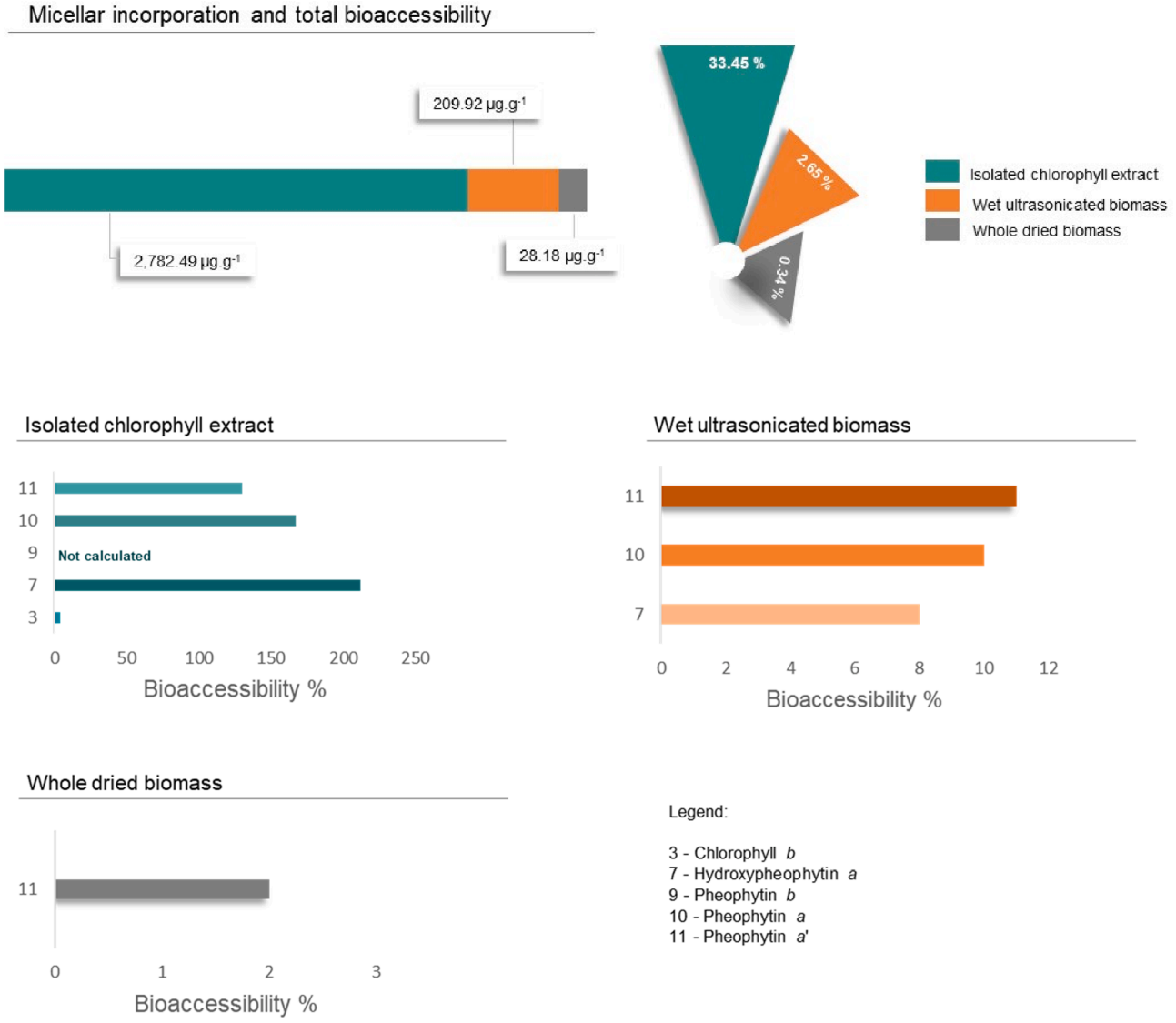


Fig. 3. Bioaccessibility index of chlorophyll derivatives of whole dried biomass, wet ultrasonicated biomass, and isolated chlorophyll extract of *Scenedesmus obliquus*. The numbers correspond to the compounds identified (Table 1 and Fig. 1). 3: Chlorophyll *b*; 7: Hydroxypheophytin *a*; 9: Pheophytin *b*; 10: Pheophytin *a*; 11: Pheophytin *a*'.

(WUB); and the isolated chlorophyll extract (ICE), to eliminate all interferences in the matrix.

Regrettably, up to now, there are no reports on the bioaccessibility of chlorophylls from microalgae, this being the first study. Thus, our discussions and comparisons with data from the literature are based on bioaccessibility analyzes of chlorophylls from other sources, such as higher plants or seaweed. Also, due to restricted data availability, some methodology applied in this work is compared with studies of carotenoids, since these compounds have physical-chemical properties similar to chlorophylls and their derivatives.

The concentration of chlorophyll derivatives bioaccessible from the products of WDB, WUB, and ICE may be observed in Fig. 3. The incorporation of the compounds into the micellar fraction is presented in Fig. 2. The incorporation into the micellar fraction is a value correlated with the bioaccessibility of the compounds, as it represents the absolute value of chlorophyll derivatives present in the aqueous micellar fraction after the *in vitro* digestion steps.

Micellar incorporation and bioaccessibility of chlorophyll derivatives differed depending on the product used. Whole dried biomass showed the lowest incorporation rate of chlorophyll derivatives in the micellar fraction. Pheophytin *a*' (peak 11) was the only chlorophyll derivative to incorporated into the mixed micelles, in the concentration of 28.18 μg g^{-1}, substantially lower when compared to the concentration present in the initial content (1,598.09 μg g^{-1}). The total bioaccessibility of chlorophylls in this product was 0.34%, specifically pheophytin *a*' showed an average bioaccessibility of 2%.

The reason for the low incorporation into micellar fraction content and bioaccessibility values of chlorophylls present in WDB is assumed to be based on the location of these class of pigments in *S. obliquus*. Chlorophylls in microalgae are light-harvesting pigments responsible for capturing light energy in the photosynthetic apparatus, located closest to the inner part of the photosystem (Mulders et al., 2014). Furthermore, *S. obliquus* is known for contains a multilayered cell wall exhibiting a characteristic trilaminar structure, with cellulose in the inner layers and biopolymers termed algaenans, which are localized in the trilaminar outer layers (Burczyk, 1973; Voigt, Stolarczyk, Zych, Malec & Burczyk, 2014). Resistant biopolymers of different chemical structures, such hemicellulose or cellulose-like biopolymers, glucosamine (Burczyk, Smietana, Terminska-Pabis, Zych & Kowalowski, 1999), polyamines (Burczyk, Zych, Ioannidis & Kotzabasis, 2014) and glycoproteins (Voigt et al., 2014) are also found in cell walls of this microalgae. The presence of these components contributes to significantly reduces the accessibility of the cell wall to enzymatic or physical–chemical rupture (Burczyk et al., 2014). Another important factor limiting the bioaccessibility of chlorophyll pigments in WDB is low lipid content. This strain of S. *obliquus* presented considerably low lipid content 10.01 g/100 g of dry biomass (Vendruscolo et al., 2018) which is a limiting factor for micellar incorporation and respective bioaccessibility of chlorophyll compounds. Thereby, it is hypothesized that the specific location within the cell, together with the chemical constitution of the cell, are the main barriers to the release, micellarization and possible accessibility of chlorophyll derivatives from the microalgae matrix.

The low bioaccessibility of chlorophylls using dried biomass is not surprising, being both results consistent and supporting a significant effect of the constituents of the cell wall, as previously reported for bioaccessibility of carotenoids (Granado-Lorencio et al., 2009; Gille et al., 2016; Gille et al., 2019; Bernaerts et al., 2020).

The wet ultrasonicated biomass (WUB) enabled the micellar incorporation of three compounds: hydroxypheophytin *a* (peak 7) in concentrations of 9.39, pheophytin *a* (peak 10) 20.37, and pheophytin *a*' (peak 11) 180.16 μg.g^{-1} dry weight. Thus, a total of 209.92 μg.g^{-1} of chlorophyll derivatives from WUB were incorporated into the micelles. The most incorporated compound, pheophytin *a*' represents approximately 86% of the total incorporated chlorophylls fraction that suggest preferential incorporation in the aqueous micellar fraction. The remaining 14% is represented by pheophytin *a* and hydroxypheophytin *a*. In this product, the micellar incorporation efficiency was significantly higher ($p < 0.05$) compared to the WDB. Total incorporation was 7 times higher, with pheophytin *a*' is 6 times more incorporated.

The total bioaccessibility of the compounds in this product was 2.65%. An increase of 2.30% when compared to the WDB product. The bioaccessibility range of the compounds was 8% to 11%. In WDB only 2% of the initial amount of pheophytin *a*', estimated before digesta, was bioaccessible, whereas in WUB showed bioaccessibility of 11%. On the contrary, pheophytin *a* and hydroxypheophytin *a* showed no bioaccessibility in WDB. The ultrasonication of the wet biomass increased bioaccessibility these derivatives significantly up to 8% for hydroxypheophytin *a* and up to 10% for pheophytin *a*.

Previous studies have shown that the use of sonication can be considered a useful processing method to enhance the bioaccessibility of intracellular lipophilic compounds present in algae cells. Recently, Gille et al. (2019) proved a significant increase in the bioaccessibility of carotenoids from the sonicated *Phaeodactylum tricornutum* biomass. β-carotene had a 49% increase in its bioaccessibility, while fucoxanthin in 10% and zeaxanthin remained almost unchanged. In contrast, carotenoids from microalgae *Chlorella vulgaris* were not bioaccessible without ultrasonicated. However, using the sonication treatment, increased bioaccessibility of carotenoids significantly up to 18% for lutein and up to 12.50% for β-carotene. In accordance with these results, our study also demonstrated a significant increase (Fig. 3) in chlorophyll derivatives when using the ultrasonicated treatment in the wet biomass. WUB revealed an improvement of the bioaccessibility in 2.30% (seven times more) and 9% (five times) more for pheophytin *a*', the only bioaccessible compound in WDB.

As already suggested and presented previously by some authors, in addition to the disruption of the microalgae cells, other positive points can be considered for the ultrasonicated efficiency in increasing bioaccessibility. This pre-treatment can be useful in breaking intracellular bonds (Gille et al., 2019), such as chlorophyll-protein complexes in microalgae (Mulders et al., 2014), releasing intracellular chlorophyll derivatives more easily. Also, a significant fraction of fatty acids present in the biomass can be released by ultrasonicated (Hadrich et al., 2018; Sivaramakrishnan & Incharoensakdi, 2019) and assist in the process of micellar incorporation and subsequent increase in bioaccessibility, as is typical for liposoluble compounds (Ferruzzi & Blakeslee, 2007).

Thus, considering the hypotheses mentioned above, we evaluate the bioaccessibility of chlorophylls and their derivatives from the isolated extract (ICE). As such, the extract contained chlorophyll catabolites without the presence of interfering substances of matrix composition, such as constituents of the cell wall and macromolecules (e.g., proteins and polysaccharides). It is essential to point out that, the bioaccessibility of lipophilic compounds, such as chlorophylls, is usually relatively low. Thus, one of the factors that can positively contribute and reduce this limitation is the presence and co-ingestion of fat (Ferruzzi & Blakeslee, 2007; Fernández-García et al., 2012). Considering these factors, the isolated chlorophyll extract was suspended in 18% (w:w) of sunflower oil, to assist the micellar incorporation process and subsequent bioaccessibility/uptake of the compounds.

The ICE product allowed the greater incorporation efficiency of chlorophyll derivatives in the mixed micelles in significantly concentrations ($p < 0.05$) in comparison to the other products (WDB and WUB). The total value incorporated compounds were 2,782.49 μg g^{-1}. Particularly, chlorophyll *b* (peak 3), hydroxypheophytin *a* (peak 7), pheophytin *b* (peak 9), pheophytin *a* (peak 10), and pheophytin *a*' (peak 11) were effectively incorporated into the micelles at concentrations of ranging from 15.36 to 2,074.93 μg g^{-1} (Fig. 2).

Furthermore, that pheophytins and their epimers and allomers derivatives of the *a* series are prevalent after the digestion process, which is related to the absence and strong instability of parental chlorophyll *a* during digesta. In ICE, at 74% of the pigments incorporated in the mixed micelles was pheophytin *a*', approximately 12% pheophytin *a* and 9% hydroxypheophytin *a*. In contrast, compounds of *b* series in ICE

contribute with the content of approximately 4% of the pigments incorporated into micelles.

In the ICE the bioaccessibility was significantly increased up to 212% for hydroxypheophytin *a*, up to 167% for pheophytin *a*, and up to 130% for pheophytin *a*'. Also, *b* series compounds that were not bioaccessible in the previous products showed bioaccessibility. Chlorophyll *b*, the fourth most bioaccessible compound in ICE, showed a bioaccessibility of about 4%. Pheophytin *b* was also bioaccessible. However, as this compound was possibly formed during the digestion process since it was not detected in the control, we were unable to estimate the percentage value of its bioaccessibility. Noteworthy, Mg-free chlorophyll derivatives were more bioaccessible than native chlorophylls. Besides this, pheophytin *a*' was the only bioaccessible derivative in all products (2% to 130%).

Their structural specificities can explain the difference in the incorporation rate between the a and b series compounds in the micellar fractions. As previously pointed out, the *a* series derivative (C_7:CH_3; Fig. 1) present greater hydrophobicity, which favors the incorporation in mixed micelles because of more optimal solubility. In contrast, the formyl group in C_7 (COH) increases the hydrophilicity of chlorophyll *b* molecule and their derivatives relative to chlorophyll *a* pigments (Ferruzzi, Failla & Schwartz, 2001), with this, they are incorporated in the micelles in smaller proportions. This behavior was predominant in all products and is in accordance with previous studies (Ferruzzi et al., 2001; Chen & Roca, 2018b). Among the derivatives incorporated in the micelles, the degree of hydrophobicity increasing in the following order: chlorophyll *b*, hydroxypheophytin *a*, pheophytin *b*, pheophytin *a* and pheophytin *a*'.

Specifically, the isolated chlorophyll extract was approximately 98 times more bioaccessible than whole dried biomass and 13 times higher than wet ultrasonicated biomass. This fact suggests that without physical barriers and with a greater concentration of fat, a higher number of compounds were incorporated in mixed micelles, and consequently greater was the accessibility of the compounds. Presumably, the oily fraction stimulates bile secretions and pancreatic lipase levels, which in turn increases the micellization capacity of the catabolites (Fernández-García et al., 2012).

Our study results are in accordance with those found by Bernaerts et al. (2020) where the bioaccessibility and incorporation of carotenoids in mixed micelles, from microalgae *Nannochloropsis* sp., could be significantly improved with the application the isolated oil/carotenoid extract from the microalgae. Similarly, carotenoids extracted from the *Scenedesmus almeriensis* biomass and added to olive oil, showed a significant improvement in the transfer to the micellar phase, when compared to the carotenoids present in the lyophilized biomass (Granado-Lorencio et al., 2009).

In comparison with other sources, the depicted results for WDB and WUB revealed a low bioaccessibility compared to values determined for edible seaweeds (4% to 17%), whereas ICE, showed a higher bioaccessibility (Chen & Roca, 2018b). Compared to sources of traditional chlorophylls, such as spinach (Hayes et al., 2020), the bioaccessibility index for Mg-free derivatives of the *a* series was higher in ICE (24–50%), however lower for compounds of *b* series (19–46%).

Consistently, the concentration of hydroxypheophytin *a*, pheophytin *a* and pheophytin *a*' incorporated in the micelles were higher in ICE when compared to the initial content (Fig. 2). In agreement with other authors, our results show that the content of native chlorophylls is affected during the *in vitro* digestion (Ferruzzi & Blakeslee, 2007; Scrob, Hosu & Cimpoiu, 2019), which causes an increase in the concentration of derivative compounds. Hydroxypheophytin *a* was the compound that most increased in the micellar fraction, in levels up to 2 times more than the initial fraction. In contrast, pheophytin *a* had an increase of up to 1.66 times and pheophytin *a*' was 1.30 times higher. Thus, it was achieved a bioaccessibility index over 100%, since the bioaccessibility of a compound is proportional to micellar fraction content and initial content. In addition to the increase in the bioaccessibility index, these results explain the individual bioaccessibility of the compounds in the ICE in relation to that found in WUB (Fig. 3). This behavior was only obtained in this product due to compounds being more susceptible to chemical transformations than when partially or totally confined in the cells in the biomass (WUB and WDB products). Furthermore, as in higher plants, a large part of the chlorophylls in *S. obliquus* biomass may be linked to proteins, in the configuration chlorophyll-protein complexes, which increases the stability of these compounds (Westphal, Schwarzenbolz & Böhm, 2017). However, this is highly speculative, as several factors can change the bioaccessibility of chlorophylls.

3.3. Impact of in vitro digestion on chlorophylls stability

Chlorophylls are very susceptible to undergo modifications during the digestion process. Particularly, the chelate (central Mg^{2+}), the isocyclic ring (C-13^2), the basic porphyrin structure, and the ester bond of the phytol alcohol (C-17^3) are the main parts of the molecular structure that undergo changes (Gallardo-Guerrero et al., 2008). A first metabolization step of chlorophylls is often the formation of derivatives that keep the phytol chain and are Mg-free, denominated of pheophytins (Ferruzzi & Blakeslee, 2007). This modification in the structure of photosynthetic chlorophylls occurs by pheophytinization reaction that substitution of the central Mg^{2+} of the tetrapyrrol ring by two hydrogen atoms (Chen, Ríos, Pérez-Gálvez & Roca, 2015). This structural rearrangement in the chlorophyll molecule can be seen in Fig. 4.

Acidic conditions promote this reaction, which makes the gastric phase a propitious medium for the complete disappearance of native chlorophylls in favor of Mg-free derivatives. In our study, it is likely that all chlorophyll *a* present in the *S. obliquus* biomass has been transformed into its Mg-free derivative, pheophytin *a*.

The pheophytinization reactions from native chlorophyll also can be observed for *b* series. Nevertheless, the substrate for this reaction is limited to the low content of parental chlorophyll *b* in the initial matrix. Also, chlorophyll *b* showed higher stability in digestive conditions that chlorophyll *a*, as about 4% were intact in the micellar fraction. In comparison, 27% were transformed into pheophytin *b*, and the rest was lost. This effect is attributed to the interference sterically of the formyl group at C_7, which makes the rate of this reaction always higher for chl *a* (Gallardo-Guerrero et al., 2008).

In a study with chlorophyll pigments isolated in an oily matrix, after the *in vitro* digestion process, chl *a* was almost entirely (97%) transformed to Mg-free derivatives, whereas chl *b* was around 61% (Gandul-Rojas et al., 2009). In contrast, gastric conditions caused the complete disappearance of chlorophylls *a* and *b*, in favor of the respective Mg-free derivatives (Ferruzzi et al., 2001; Gallardo-Guerrero et al., 2008).

Among all the transformations that can occur in the isocyclic ring of chlorophyll compounds, the mildest one is the epimerization of the $C13^2$ (Fig. 4). This reaction is acid- or base-catalyzed reaction and involves only a slight change in the polarity of the molecule (Mazaki, Watanabe, Takahashi, Struck & Sheer, 1992; Gandul-Rojas, Gallardo-Guerrero, Roca & Aparicio-Ruiz, 2013).

Our data showed 13^2-epimer pheophytin *a* in the micellar fraction in greater proportions than the other chlorophyll derivatives. This result is not surprising, since the gastrointestinal tract, specifically the gastric phase (pH 2.5), induce the formation of these compounds. According to our data, epimerized pheophytin was previously detected in the micellar fraction of other matrices, however in lower concentrations (Ferruzzi et al., 2001).

It is unclear whether the epimerization occurs preferably in native chlorophylls or their Mg-free derivatives. However, the low levels of 13^2-epimer chlorophyll *a* and high levels of 13^2-epimer pheophytin *a* in the initial content points to preferential epimerization in Mg-free chlorophylls. Nevertheless, as the concentrations of pheophytin *a* did not decrease and showed an increase in the micellar fraction, this was not the substrate used for the formation of epimer pheophytin *a*' (in ICE). Thus, it is evident that the proportion of pheophytin *a*' in the micellar fraction comes from the content of chlorophyll *a* our chlorophyll *a*'

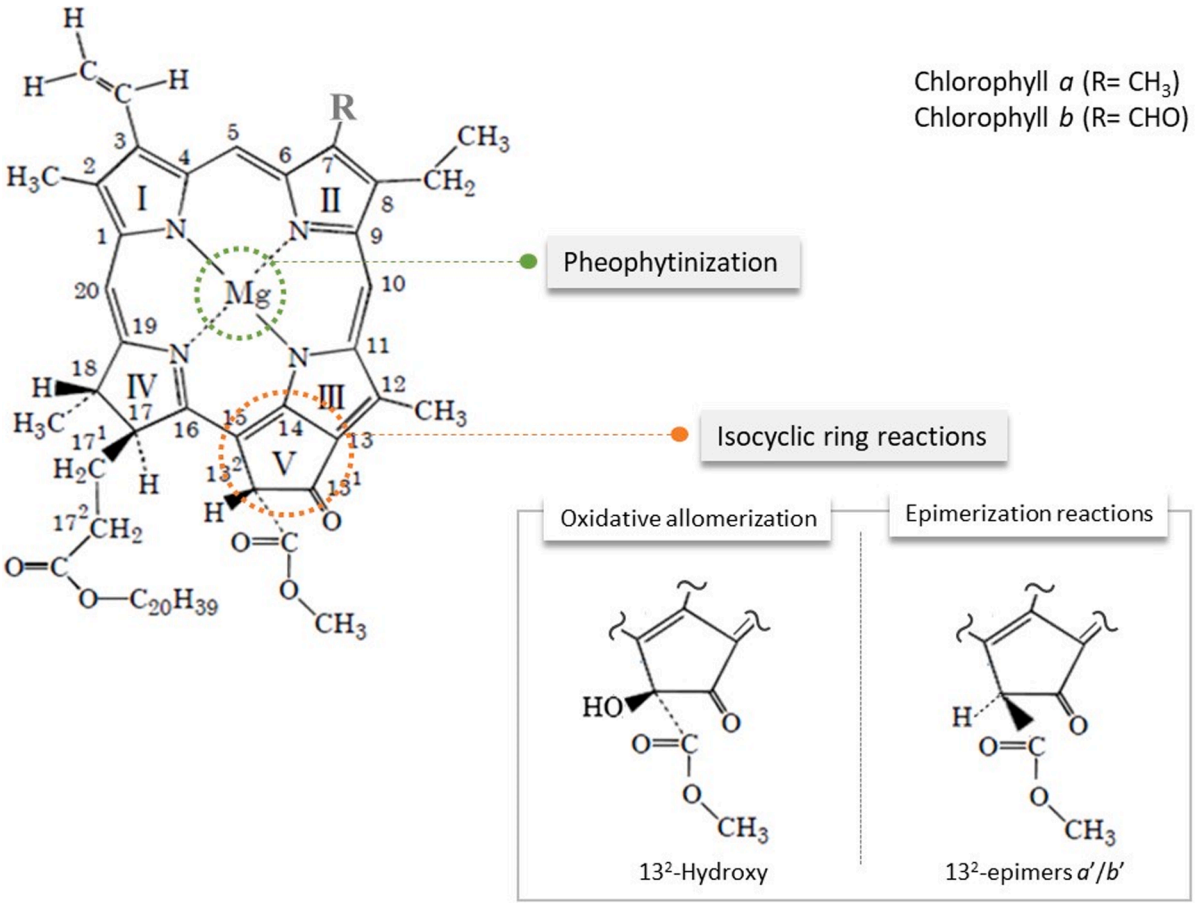


Fig. 4. Changes in the structure of chlorophyll during the *in vitro* digestion process.

presented in the initial extract, due to the successive reactions of pheophytinization and epimerization for structure native.

In WDB and WUB products, it is possible that the initial content of pheophytin *a* and 13^2-epimer pheophytin *a* remains stable during passage through the gastrointestinal tract or has been synthesized from the substrates parental chlorophyll *a*, their epimer *a*' or pheophytin *a*.

Allomerized derivatives (oxidative compounds) were also detected in relevant proportions in the micellar fraction after digesta. These compounds are formed by oxidation of the native chlorophyll molecule with the addition of oxygen in the isocyclic ring (Gross, 1991; Hynninen & Hyvärinen, 2002). In our study, we detected the presence of 13^2-hydroxy derivatives, due to substitution in the isocyclic ring of the H atom at $C13^2$ by an hydroxyl group (–OH; Fig. 4).

As previously shown, (Ferruzzi et al., 2001; Chen & Roca, 2018a) for chlorophylls containing Mg, oxidative reactions occur before pheophytinization and then yielded allomer derivatives are pheophytinizated during digestion. In this line, obvious proportional increase of hydroxypheophytin *a* is accounted for the disappearance of hydroxychlorophyll *a*. However, a second hypothesis can be considered since native chlorophyll *a* is the main substrate for the formation of this compound. It is possible that the structure of chlorophyll *a*, present in the initial content, undergoes subsequent reactions of oxidative allomerization and pheophytinization to form the hydroxychlorophyll *a* derivative. Nevertheless, considering the reaction rate and the preference for the pheophytinization reaction, it is more evidence to consider the first hypothesis.

It is worth noting that pheophytins are less susceptible to allomerization than the corresponding chlorophyll (Hynninen & Hyvärinen, 2002). This trend was observed in the initial matrix profile, where hydroxychlorophyll *a* represented 4.77% of the total chlorophyll content and hydroxypheophytin *a* contributed about 1.44%. However, when we analyze the stability of these compounds, considering the content in the micellar fraction, the allomers derivatives are represented exclusively by hydroxypheophytin *a* (from 4.47% in WUB to 9.17% in ICE). Similar results were also found in the study by Chen and Roca (2018b), where the chlorophyll profile before the digesta of *Ulva* sp. Contained 13^2-hydroxychlorophyll *a* (5.14%) and 13^2-hydroxypheophytin *a* (6.64%) and in the content of the aqueous micellar fraction only 13^2-hydroxypheophytin *a* was detected (30.09%). At the same time, when the stability of chlorophyll pigments in edible seaweed after the digestion process (before incorporation into the micelles) was assessed, these oxidative derivatives without Mg were more stable than the derivatives oxidative with central Mg (Chen & Roca, 2018a).

Enzymatic or chemical reactions can favour the degree of allomerization. Gallardo-Guerrero et al. (2008), in a study with peas, found allomerized chlorophylls after the *in vitro* digestion process and related it to the activity of the enzyme peroxidase. On the other hand, when testing pure chlorophyll pigments, the origin of the allomerization reactions was considered purely chemical. Thus, factors such as triplet molecular oxygen $^3O_2$ of the digestive environment may favour the formation of these compounds (Gandul-Rojas et al., 2009).

Specifically, the content of chlorophylls present in the micellar fractions after digesta was quite particular; however, the composition of the chlorophyll structures was similar between the products. The Mg-free compounds represent 99% of the total micellar fraction in ICE, of this, 9% are allomer derivatives, and 74% are epimer derivatives. At the same time, chlorophyll chelated with Mg represents<1% of the total composition. Meanwhile, for WDB and WUB, 100% are Mg-free compounds. With 85% in the form of epimers and 4% represented by allomers in WUB.

Another important difference is that when chlorophyll pigments are widespread in the biomass (WDB and WUB), the digestive stability of the compounds seems to be greater, since we did not observe any difference in the micellar profile with that of the initial matrix. On the other hand, a significant loss in the absolute content in the micellar fraction was observed. Thus, it is possible that the reduced release of chlorophyll pigments from the cell to the gastrointestinal tract, due to the resistance of the cell wall, may influence the stability of the compounds. However, this is only a hypothesis since we evaluated the stability of the compounds considering the micellar fraction profile.

In contrast, in ICE, the stability of the pigments was less, as we detected an increase in the absolute content of Mg-free compounds, epimer, and allomer derivatives. What shows that isolated free chlorophylls are more susceptible to structural transformations than incorporate into biomass.

In summary, with the present study, we can observe that the stability of chlorophyll pigments after *in vitro* digestion seems to be largely dependent on factors related to the type of chlorophyll and matrix.

3.4. Uptake of micellar chlorophyll derivatives by Caco-2 cells

Besides the bioaccessibility of chlorophyll compounds, we investigated their uptake in Caco-2 cell monolayers. However, due to the low efficiency of chlorophyll derivatives bioaccessibility in extracts from biomass (WDB and WUB), we consider only of the isolated chlorophyll extract (ICE) to perform this trial. The cell model used serves as a surrogate for intestinal enterocytes, and has been utilized successfully previously to analyze the uptake of chlorophyll derivatives (Chen & Roca, 2018b; Ferruzzi et al., 2001; Gallardo-Guerrero et al., 2008; Gandul-Rojas et al., 2009).

Fig. 5 shows the uptake rate by the cell cultures of chlorophyll derivatives from the aqueous micellar fraction of ICE. As in the aqueous micellar fraction of ICE, hydroxypheophytin *a* (peak 7), pheophytin *a*' (peak 11), pheophytin *a* (peak 10) and pheophytin *b* (peak 9) were the predominant chlorophyll derivatives in Caco-2 cells. These results support the findings of Ferruzzi et al. (2001) who reported cell uptake proportional to the micellar profile of chlorophyll compounds from spinach puree. This fact accentuates the relevance of the micellar incorporation efficiency for the maximization of cellular concentration of these lipophilic molecules. On the other hand, we did not detect the presence of parental chlorophyll *b* in the cell fraction, which we attribute to the low micellar incorporation from the digested (see Fig. 2). So far, the uptake of pheophytin *a*' by Caco-2 cells has not been reported. In our study, this compound stands out as the second most captured by intestinal cells.

As an average of all assays, it was estimated that the intestinal epithelial cells accumulated between 0.006 and 22.30 μg of chlorophyll derivatives per mg of cell protein, which represents a relative uptake of 0.43% up to 92%. Specifically, the relative uptake of hydroxypheophytin *a* (102.53% ± 14.42) was significantly higher than the other derivatives captured by the intestinal cells. In contrast, pheophytin *b* showed the lowest relative uptake (0.43% ± 0.03). The other compounds, pheophytin *a* and their isomer pheophytin *a*' were captured in similar proportions (55.29% ± 3.83 and 64.69% ± 3.83, respectively).

The relative content over 100% for the oxidized derivative, confirms that the uptake process can promote oxidative reactions. This fact was previously reported for the first time by Chen and Roca (2018b). The authors evaluated the bioavailability of chlorophyll pigments from edible seaweeds and found that the uptake process by Caco-2 cells promoted a large increase in oxidized derivatives. In our study, the proportion of hydroxypheophytin *a* to inoculated in Caco-2 cells was about 9%, while the proportion increases almost to 15% after the intestinal uptake process.

Although the causes of this phenomenon have not yet been explored, it is worth reaffirming that it is known that bioactive phytochemicals such as chlorophyll derivatives have strong antioxidant properties (Lanfer-Marquez, Barros & Sinnecker, 2005), and, consequently, can participate effectively in elimination reactions of free radicals in the human intestinal environment (Lobo, Patil, Phatak & Chandra, 2010; Orozco, Arriaga, Solomons & Schümann, 2012).

The accumulation of chlorophyll derivatives in Caco-2 cells represented 64% of the compounds incorporated in the micelles, while about 36% were not uptake. In addition, as well as in the micellar fraction, the profile of the compounds is predominantly made up of chlorophyll derivatives from a series (around 99%), while pheophytin *b* contributes<1%. At the same time, Caco-2 cells after absorption of the aqueous micellar fraction of the ICE product contain almost 86% pheophytins (*a* and *b* series) and 14% oxidized derivatives.

In addition to the degree of micellar incorporation, factors such as the physicochemical properties of the molecules can mediate the extent

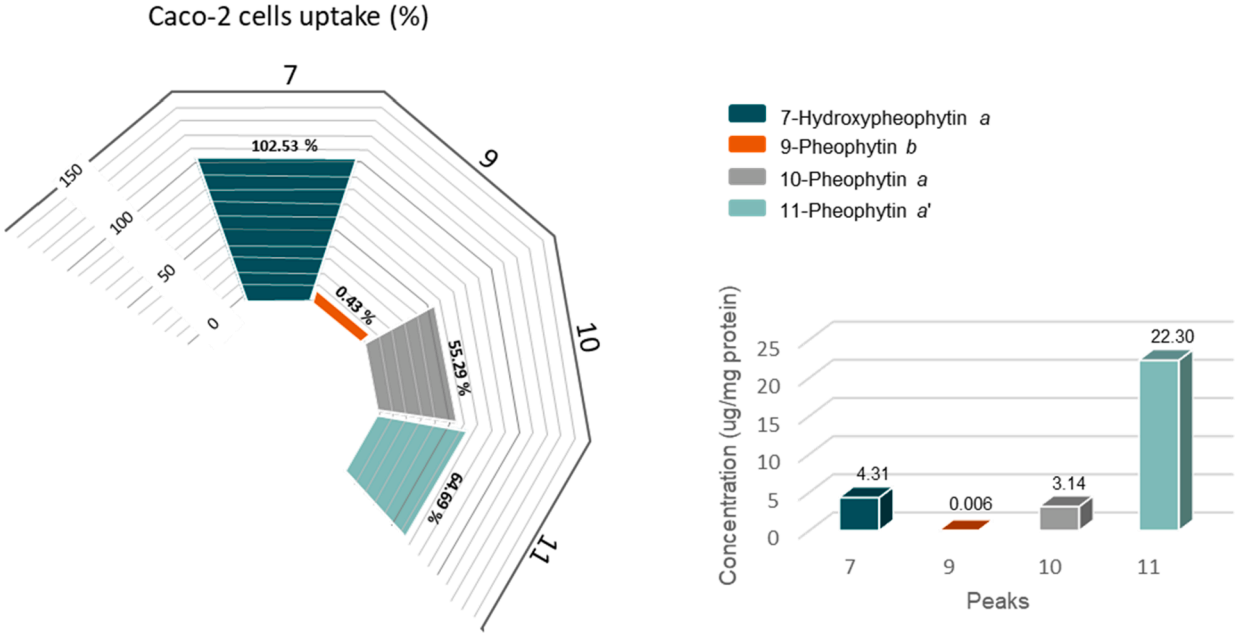


Fig. 5. Concentration and uptake rate of the micellar chlorophyll derivatives present in Caco-2 cells from isolated chlorophyll extract from *Scenedesmus obliquus*.

of uptake of chlorophyll compounds. As previously reported, the degree of molecular polarity, ionization, and molecular size highly influence the metabolism of intestinal absorption (Chan & Stewart, 1996). Furthermore, as well as for carotenoids, it is inevitable that there will be no competitive inhibition between different chlorophyll derivatives during the uptake process by intestinal cells since they have distinct structural properties (Chen & Roca, 2018b). Also, considering the hypotheses raised by Gandul-Rojas et al., (2009) it is likely that native pheophytins are absorbed by simple diffusion mechanism, while compounds more hydrophilic (as oxidized compounds and epimers identified in our study), can be transferred to cells Caco-2 by facilitated transporter mechanism. Besides, specific receptors that have the function of facilitating the delivery of the compounds to the apical membrane may be involved in the absorption preference. As recently demonstrated, the scavenger receptor SR-BI facilitates, at least partly, the absorption of derivative pheophorbide *a* (Viera et al., 2018). However, the molecular mechanisms involved in the absorption of chlorophyll derivatives are not fully understood and need further studies.

According to Ferruzzi and Blakeslee (2007), even a minimal absorption of chlorophyll derivatives can be physiologically significant. The intestinal uptake pheophytin *a*, and pheophytin *b* are of great value due to the well-known antioxidant potential these biomolecules. Although it has been captured to a lesser extent, pheophytin *b* demonstrated to be a potent natural antioxidant, whose activities can be comparable to butylated hydroxytoluene (BHT), a potent antioxidant used by the food industry (Lanfer-Marquez et al., 2005). Similarly, a study by Hsu, Chao, Hu, and Yang (2013) found that forms of Mg-free chlorophylls, such as pheophytins, exert more significant antioxidant activities compared to parental chlorophyll (Mg-chelated). However, they demonstrated that all these compounds are essential health-promoting since could prevent oxidative damage to DNA and lipid peroxidation, both by chelating reactive ions and by scavenging free radicals.

The fact that the absorption of hydroxypheophytin *a* to be biologically relevant is supported for research by Freitas et al. (2019). This study evaluated the lipid-reducing activities of hydroxypheophytin a in the zebrafish Nile red fat metabolism assay and in multicellular spheroids (MCS) of differentiated 3 T3-L1 adipocytes. The results showed that this oxidized compound is a potential candidate as a nutraceutical with significant lipid reduction activity for the control of obesity. Earlier, other important biological properties including the antiproliferative effect on cancer cells (Cheng et al., 2001; Chen, He, Tong, Tang & Liu, 2016) and anti-inflammatory activity (Shu, Appleton, Zandi & AbuBakar, 2013) have been attributed to hydroxy compounds.

Chlorophyll derivatives (specifically pheophytins and pheophorbides), also have some singular functions such as controlling obesity-associated diabetes (Semaan et al., 2018), antimutagenic activity (Ferruzzi, Böhm, Courtney & Schwartz, 2002), anti-inflammatory (Islam et al., 2013; Subramoniam et al., 2012), antimicrobial activity (Gomes et al., 2015), antiviral activity against hepatitis C virus (Wang et al., 2009), cytotoxic effects on cancer cells (Zhao, Wang, Wang, Liu & Xin, 2014), chemopreventive effect against liver and stomach carcinogenesis (Simonich et al., 2008), production of ATP (Xu, Zhang, Mihai & Washington, 2014), including the neurodifferentiation induction (Ina, Hayashi, Nozaki & Kamei, 2007).

4. Conclusion

The results of our comparison of using three different forms of ingestion of chlorophylls pigments from *S. obliquus* showed convergence with promoting the bioaccessibility. Specifically, our study showed that the compounds confined in whole dried biomass have a low bioaccessibility index, which can be improved in wet ultrasonicated biomass. Although, we observed greater efficiency of isolated extract use in promoting the higher bioaccessibility of *S. obliquus* chlorophyll pigments. The bioaccessibility assays showed that only 0.34–33.45% of the original total chlorophyll content present in microalgae biomass was bioaccessible. Same way, the individual bioaccessibility index varied among the chlorophyll-based pigments and within the different products analyzed. The study that included the bioavailability of chlorophyll pigments from the isolated extract using the Caco-2 cells assay revealed that most micellar compounds are uptake by intestinal cells, except parent chlorophylls. There is a significant preference for the uptake of *a* series compounds, with precedence by derivative oxidized, rather than derivatives Mg-free and epimer form. Besides, we demonstrated for the first time the absorption of pheophytin *a*' and detected that oxidation reactions in Mg-free derivatives occur in this process. It can be concluded from these combined *in vitro* bioaccessibility and bioavailability results that physicochemical characteristics of the matrix and compounds are significant in the process of digestion and absorption of chlorophyll pigments. As a whole, this study can assist in the formulation and future development of new natural, functional, and nutraceutical foods and products from microalgae-based ingredients.

CRediT authorship contribution statement

Andrêssa S. Fernandes: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Data curation, Formal analysis. **Tatiele C. Nascimento:** Investigation. **Pricila N. Pinheiro:** Investigation. **Veridiana V. de Rosso:** Investigation, Resources. **Cristiano R. de Menezes:** Investigation. **Eduardo Jacob-Lopes:** Data curation, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. **Leila Q. Zepka:** Conceptualization, Resources, Formal analysis, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.110031.

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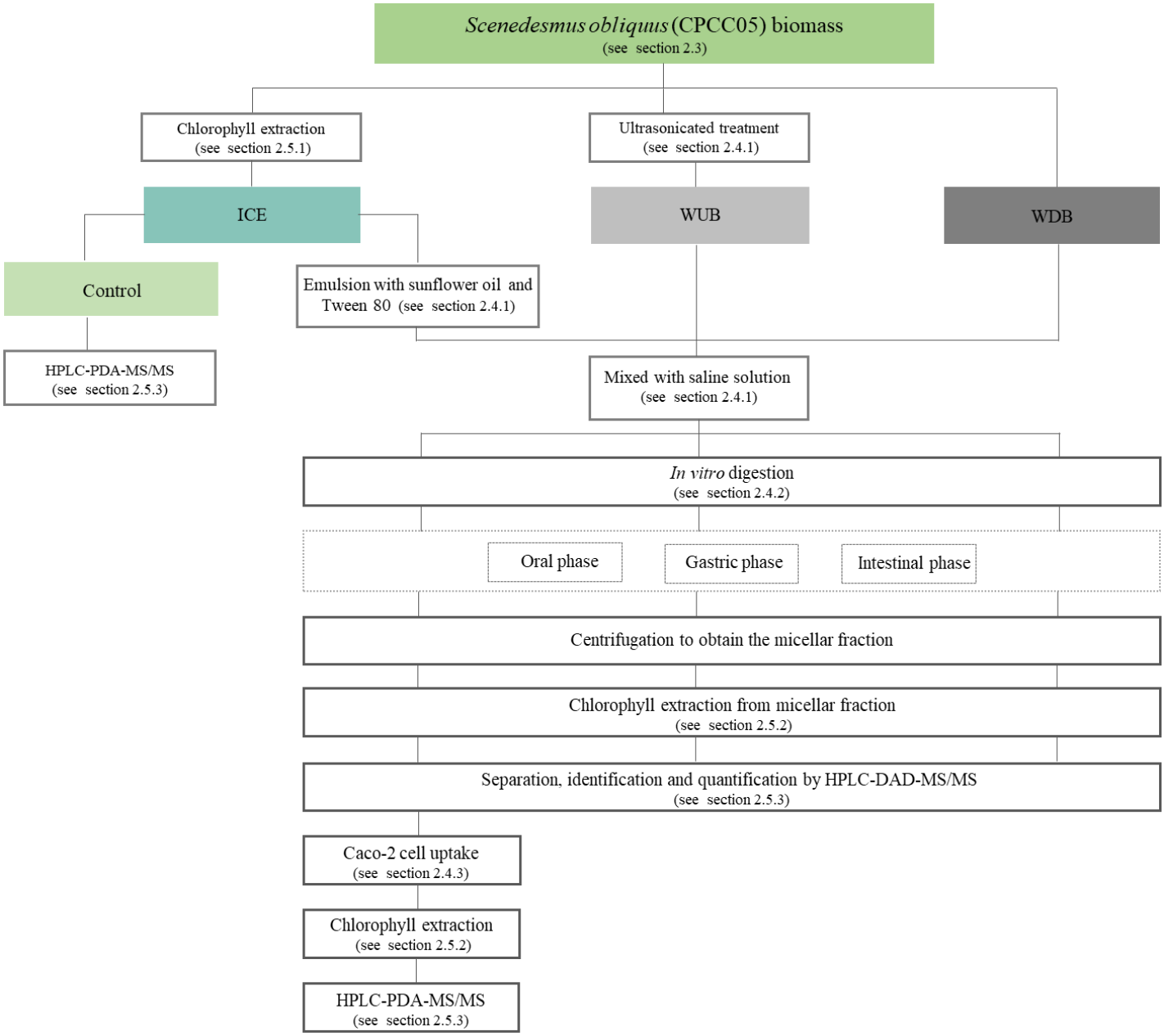
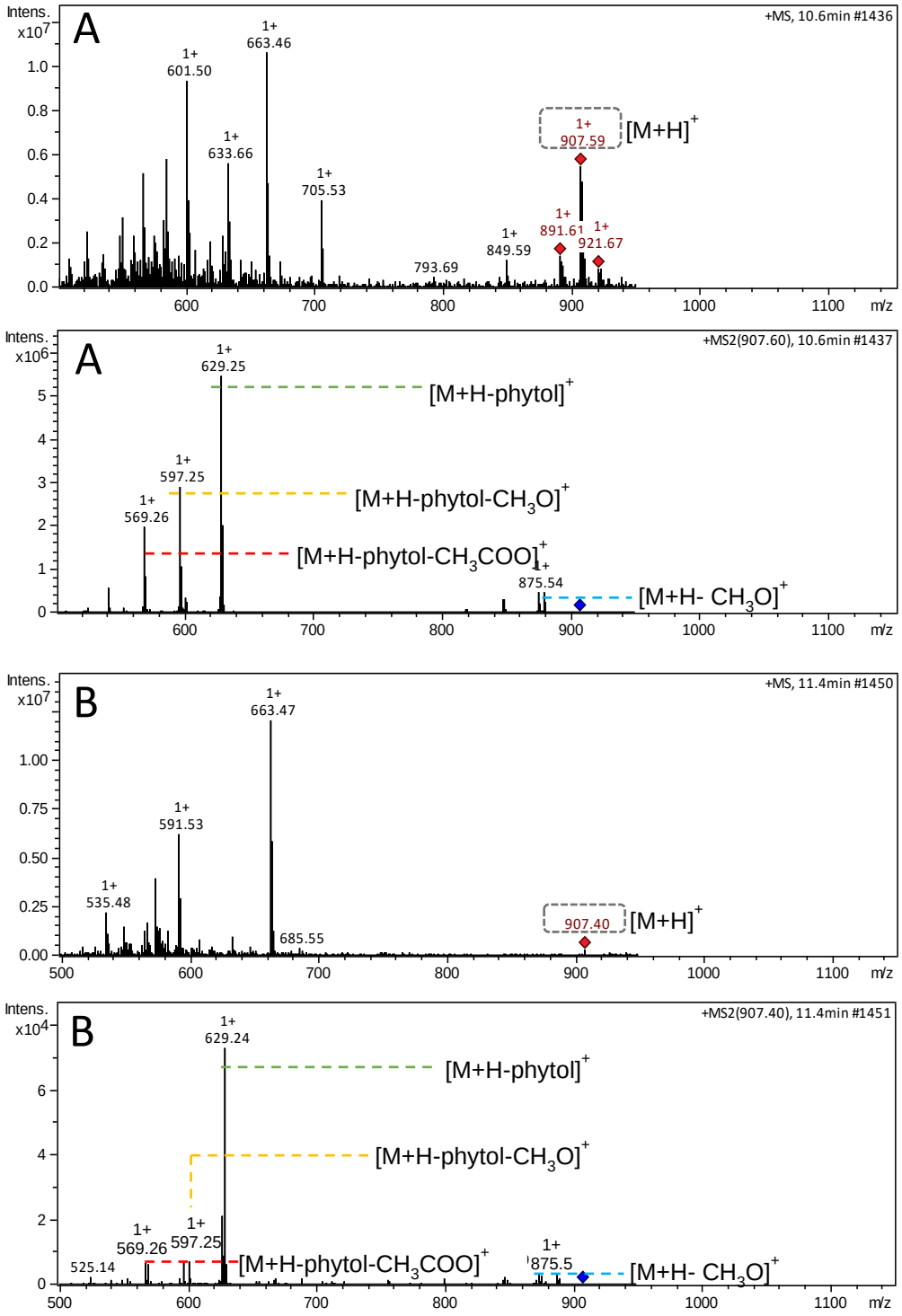
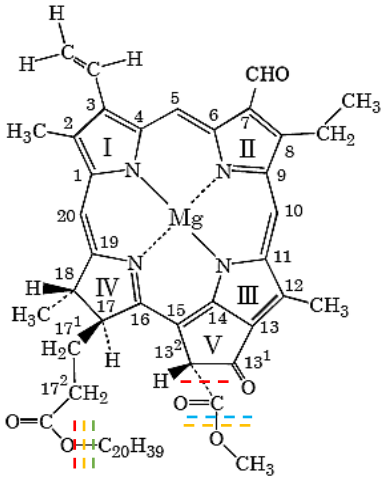
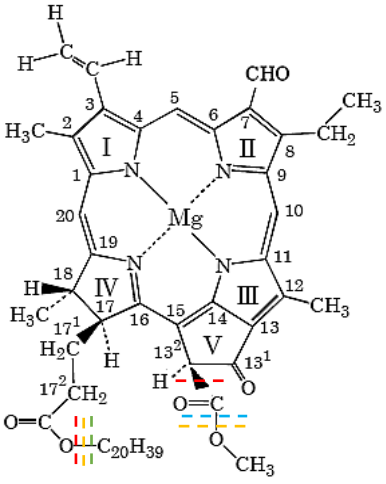


Figure S1 Summary flow chart of the experiments carried out to evaluate possible bioaccessibility and cellular uptake of chlorophylls and their derivatives from *S. obliquus*. ICE: isolated chlorophyll extract; WUB: wet ultrasonicated biomass; WDB: whole dried biomass.



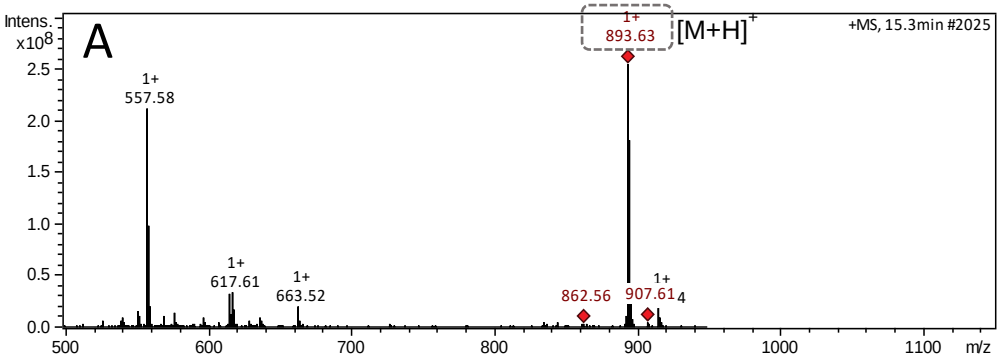


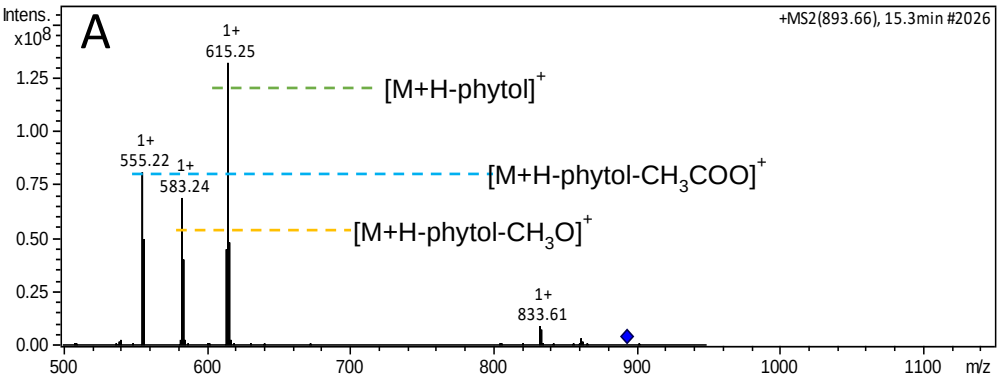
Chlorophyll *b*

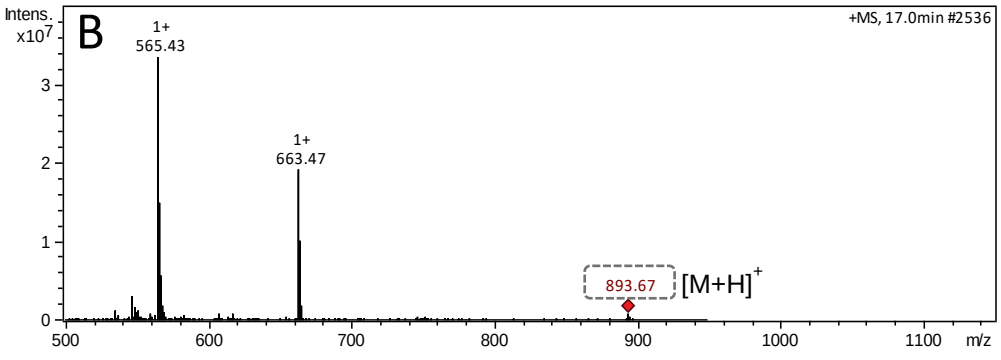


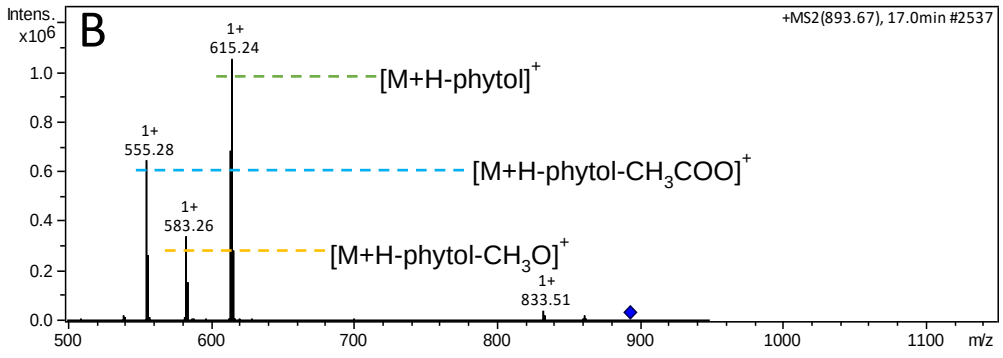
Chlorophyll *b*'

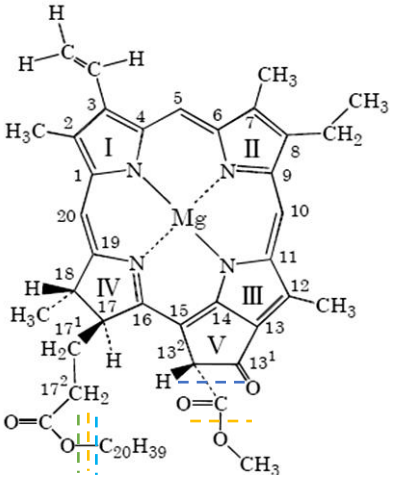
Figure S2 Mass spectrum with APCI with ion fragments characteristic of (A) chlorophyll *b* and (B) chlorophyll *b*'.











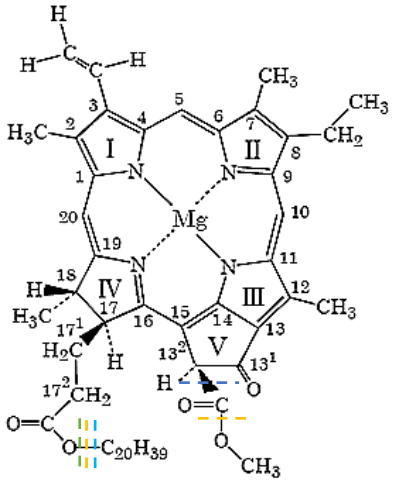


Figure S3 Mass spectra with APCI with ion fragments characteristic of (A) chlorophyll *a* and (B) chlorophyll *a*'.

CAPÍTULO 6

Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix

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Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix

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ABSTRACT

The composition of microalgae can contribute to nutritious and functional diets. Among the functional compounds, carotenoids are in focus since positive effects on human health have been established, which are in turn related to their bioaccessibility. In addition to essential nutrients, our hypothesis was that microalgae biomasses could be used as sources of bioaccessible carotenoids. Thus, this study determined for the first time the bioaccessibility of carotenoids from biomass of *Scenedesmus bijuga* and *Chlorella sorokiniana* and their possible relationship with the lipid composition of the matrix. The samples were submitted to *in vitro* digestion protocol, and carotenoids were determined by HPLC-PDA-MS/MS. Individual bioaccessibility of carotenoids was ≥ 3.25%. In general, compounds in their *cis* conformation were more bioaccessible than *trans*; and total carotenes more than total xanthophylls. Twelve compounds were bioaccessible from the biomass of *S. bijuga*, and eight in *C. sorokiniana*. In *S. bijuga,* the bioaccessibility of total carotenoids was 7.30%, and the major bioaccessible carotenoids were 9-*cis*-β-carotene (43.78%), 9-*cis*-zeaxanthin (42.30%) followed by 9-*cis*-lutein (26.73%); while in *C. sorokiniana*, the total bioaccessibility was 8.03%, and 9-*cis*-β-carotene (26.18%), all-*trans*-β-carotene (13.56%), followed by 13-*cis*-lutein (10.71%) were the major compounds. Overall, the total content of lipids does not influence the bioaccessibility of total carotenoids. Still, the lipid composition, including structural characteristics such as degree of saturation and chain length of the fatty acid, impacts the promotion of individual bioaccessibility of carotenes and xanthophylls of microalgae. Finally, the results of this study can assist the development of microalgae-based functional food ingredients and products.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), one of the biggest challenges facing global food systems is to nourish and feed the growing population through sustainable food systems (FAO, 2021). On the other hand, changes in food preferences are increasing, due to the greater awareness of the population about the positive impact of healthy diets in promoting good health, improving wellness and quality of life, which makes functional foods more influential in the global food system (Katiyar & Arora, 2020). These paradigms lead to a new era of increasingly nutritious, functional, innovative, and also sustainable ingredients and food products (Mintel, 2021). In both scenarios, microalgae biomass can successfully corroborate and position itself.

The nutritional and functional value of the microalgae used as food stems from their high content of nutrients and bioactive compounds, like proteins, polyunsaturated fatty acids, sterols, polysaccharides, vitamins, minerals, phenolic compounds, volatile compounds, carotenoids, and chlorophylls (Jacob-Lopes et al., 2019; Kusmayadi, Leong, Yen, Huang, & Chang, 2021). In contrast, high concentrations of biogenic toxins, which include nucleic acids, or compounds accumulated from the environment, such as heavy metals (non-biogenic toxins), are considered limiting factors for their application in human nutrition (Matos, 2017).

Specifically, the genus *Chlorella* has GRAS status (Generally Recognized As Safe) from the FDA (Food and Drug Administration), which guarantees its use as safe food and drug (Matos, Cardoso, Bandarra, & Afonso, 2017); and *Scenedesmus* has a safety status classified as 'no known toxin' (Anvisa, 2018; EC-Regulation, 1997; FDA, 2010; HHLW, 1996). Among these genus, *Scenedesmus bijuga* and *Chlorella sorokiniana*

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are under-exploited species for food and health purposes, although they have demonstrated the capacity to synthesize a diversity of bio-compounds (Han et al., 2014; Azaman, Nagao, Yusoff, Tan, & Yeap, 2017; Diprat, Thys, Rodrigues, & Rech, 2020; Santhakumaran, Ayyappan, & Ray, 2020; Fernandes, Petry, Mercadante, Jacob-Lopes, & Zepka, 2020).

The main forms of insertion of microalgae in human food are mostly raw from functional and nutritional dietary supplements (pastes, powders, capsules, tablets, syrups, and extracts) (Raja et al., 2018; Lafarga, 2019). However, incorporating microalgae biomass into processed food products of high physical–chemical, nutritional and sensory quality has actually been proven an efficient strategy (Gouveia, Batista, Miranda, Empis, & Raymundo, 2007; Fradique et al., 2010, 2013; Diprat et al., 2020).

Among the bioactive compounds of microalgae, highlight the carotenoids, lipophilic compounds that have been proposed in the prevention, maintenance, and treatment of health conditions whose discoveries are stimulating their use in functional food and nutraceutical products. As such, it was proposed that dietary intake of carotenoids, reflected in their plasma/serum and tissue levels are associated with the reduction of the incidence of chronic diseases, such as reduction of type 2 diabetes, cardiometabolic diseases and some types of cancer. In particular, lutein and zeaxanthin also appear to play a role in the amelioration of age-related macular degeneration (Bohn, 2018; Black, Boehm, Edge, & Truscott, 2020). Also, are widely known as pigments and vitamin A precursors (mainly β-carotene) (Rodriguez-Concepcion et al., 2018). As de novo synthesis of carotenoids does not occur in humans are mainly obtained from the diet through fruits, legumes, leafy vegetables, cereals, or via supplementation (Dias et al., 2018; Eggersdorfer & Wyss, 2018). Microalgae are also considered alternative sources of carotenoids and represent a successful model in the commercial production of β-carotene and astaxanthin from *Dunaliella salina* and *Haematococcus pluvialis*, respectively (Guedes, Amaro, & Malcata, 2011). In addition, some microalgae products for human supplementation are considered rich in carotenoids (Koyande et al., 2019).

Regardless of the form of ingestion, before accomplishing any of the potential health benefits, carotenoids need to be released from the food matrix, solubilized in a lipid phase and incorporated into mixed micelles to become bioaccessible for intestinal absorption by enterocytes and subsequent systemic distribution (Sy et al., 2012; Kopec & Failla, 2018). In most microalgae, carotenoids are stored inside the cell, protected by a cell wall, limiting their release/extraction, resulting in low bioaccessibility (Bernaerts et al., 2020). Consequently, processing for the disruption of microalgae cells is potential alternatives to promote the extension of the bioaccessibility and subsequent bioavailability these intracellular compounds (Gille, Trautmann, Posten, & Briviba, 2016). Ultrasonication processes proved successful for species with a very rigid cell wall, such as the genus *Scenedesmus* and *Chlorella* (Gille, Hollenbach, Trautmann, Posten, & Briviba, 2019; Bernaerts et al., 2020; Nascimento et al., 2021).

To date, little is known about the bioaccessibility of microalgae carotenoids, even though it is a key parameter to verify the nutritional and functional efficiency of any product developed to improve human health (Matos et al., 2017). Most of the works available to date in the literature focus only on specific carotenoids, disregarding the carotenoids profile in its entirety. The available reports are restricted to the bioaccessibility of β-carotene, lutein, zeaxanthin, antheraxanthin, and fucoxanthin (Granado-Lorencio et al., 2009; Cha, Koo, Song, & Pan, 2012; Gille et al., 2016, 2019; Gille, Neumann, Louis, Bischoff, & Briviba, 2018; Bernaerts et al., 2020). In parallel, only Nascimento et al. (2021) investigated the total and individual bioaccessibility of all carotenoids present in the biomass of the microalgae *Scenedesmus obliquus*. Up to now, there are few species of microalgae exploited for this purpose. As far as we know, only *Scenedesmus almeriensis*, *Chlorella ellipsoidea*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Nannochloropsis* sp., and *Scenedesmus obliquus* were investigated (Granado-Lorencio et al., 2009; Cha et al., 2012; Gille et al., 2016, 2018, 2019; Bernaerts et al., 2020; Nascimento et al., 2021).

Carotenoid bioaccessibility from different food sources is highly variable. It can be influenced by a complexity of factors, such as food matrix interferents (e. g. constituents of the cell walls of the matrix, carbohydrates, proteins, fibers, and lipids) and their structural physicochemical properties (Sy et al., 2012; Kopec & Failla, 2018). In turn, the presence of lipids during digestion is mandatory for carotenoid micellarization. They facilitate carotenoid dissolution into fat droplets of gastric emulsion, stimulate the secretion of bile salts and lipases, promoting mixed micelles formation (Desmarchelier & Borel, 2017; Xavier & Mercadante, 2019). As well as, digestive products of lipids (monoacylglycerols and free fatty acids), with bile salts, contribute to the formation of mixed micelles (Salvia-Trujillo et al., 2017).

In this new era of a global search for increasingly nutritious diets and health-enhancing, it becomes essential to expand knowledge about the bioaccessibility of food components in different matrices. Thus, our contribution to these aspects was to evaluate the bioaccessibility of the carotenoids of *Scenedesmus bijuga* and *Chlorella sorokiniana* microalgae through an *in vitro* digestion protocol. Also, we evaluated the lipid composition of the microalgae and its possible relationships with carotenoid bioaccessibility.

2. Material and methods

2.1. Chemicals

Standards of all-*trans*-lutein (purity 98.00%) and all-*trans*-β-carotene (purity 99.50%) were acquired from Sigma-Aldrich (St. Louis-MO, USA). Methanol (MeOH), methyl *tert*-butyl ether (MTBE), both of chromatographic grade, ethanol, acetone, ethyl acetate, petroleum ether and diethyl ether were purchased from Merck (Darmstadt, Germany). All reagents, solvents and enzymes, α-amylase (Sigma® A3176), pepsin (Sigma® P7000), pancreatin (Sigma® P1750), lipase (Sigma® L3126) and bile (Sigma® B8631) used in the *in vitro* digestion procedure assays were acquired from Sigma-Aldrich (St. Louis-MO, USA). All reagents and solvents used in the experimental part were of analytical purity.

2.2. Microrganisms and culture media

Axenic cultures of *Chlorella sorokiniana* (CPCC138) were obtained from the Canadian Phycological Culture Centre (CPCC) of the University of Toronto, Canada and *Scenedesmus bijuga* (UTEX2980) was obtained from the Algae Cultures Collection (UTEX) of University of Texas, USA. Stock cultures were propagated in solidified agar-agar (20 $g.L^{-1}$) containing synthetic BG11 medium (Braun-Grunow medium) (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979). The maintenance conditions were 30 °C, photon flux density of 15 $\mu mol.m^{-2}.s^{-1}$ and a photoperiod of 12 h.

2.3. Microalgae biomass production

The biomass production was carried out in a bubble column photobioreactor operating in intermittent regime, fed with 2.0 L of BG11 medium. The experimental conditions were as follows: initial concentration of inoculum of 100 $mg.L^{-1}$, temperature of 25 °C, aeration of 1VVM (volume of air per volume of culture per minute), a photon flux density of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and a residence time of 168 h (Maroneze et al., 2016). The biomass was separated from the culture medium by centrifugation (Hitachi, Tokyo, Japan) for 10 min at 1500 *g* (D-37520 model, Thermo, Langenselbold, Germany). In the experiments with dried biomass, the paste collected after centrifugation was subsequently freezing at −18 °C for 24 h and freeze-dried for 24 h at −50 °C above −175 μm Hg (Liotop L101, São Carlos-SP, Brasil). For experiments with wet biomass were carried out with the paste collected after centrifugation (95.00% of moisture). The samples were stored under refrigeration

until the time of analysis.

2.4. Sample preparation

Carotenoid extracts were obtained to identify the original carotenoid contents in *S. bijuga* and *C. sorokiniana* biomasses, as reported in the 2.6.1 section. For the analysis of *in vitro* digestion, *S. bijuga* and *C. sorokiniana* biomasses were subjected to a pre-treatment with ultrasound, using an ultrasonic processor (Ultronic, Indaiatuba-SP, Brazil) to previously rupture the cell wall (an adaptation of Gille et al., 2016). Thus, aliquots of 0.8 ± 0.02 g of wet biomass (cellular concentration 4.5 $g.L^{-1}$ and 2.7 $g.L^{-1}$, respectively), equivalent to 0.1 ± 0.02 g of dry biomass were combined with 10 mL of saline solution (NaCl 120 $mol.L^{-1}$, CaCl2 6 $mmol.L^{-1}$, KCl 5 $mmol.L^{-1}$). Then, the resulting mixture was subjected to 15 min of an ultrasonic probe with 13 mm diameter, 400 W, at a constant frequency of 40 kHz, in an ice bath to control the temperature (0 ± 2 °C).

2.5. Bioaccessibility of carotenoids by in vitro digestion

The microalgae samples were submitted to an *in vitro* simulated digestion model, according to the protocol adapted from INFOGEST (Minekus et al., 2014) and Failla, Chitchumroonchokchai, Ferruzzi, Goltz, and Campbell (2014) modified to carotenoids by Murador, Mesquita, Neves, Braga, Martins, Zepka, and De Rosso (2021). For the oral phase, to 1.0 g of wet biomass, 6 mL of a SSF (Simulated Salivary Fluid - pH 7.0) containing 106 $U.mL^{-1}$ of α-amylase were added, followed by incubation at 37 °C, 10 min in an orbital shaker (7.5 × g). For gastric phase, the pH was adjusted to 2.5 with HCl 1 M followed by SGF (Simulated Gastric Fluid) addition, with 2 mL pepsin 50,000 $U.mL^{-1}$ in HCl 100 mM, the total volume was adjusted to 40 mL, and the solution was incubated for 1 h, 37 °C, 7.5 × g. After this step, the pH was changed to 6.5 with 1 M NaOH and in the intestinal phase SIF (Simulated Intestinal Fluid) were added, 3 mL bile solution (40 $mg.mL^{-1}$ in 100 mM $NaHCO_3$), 5 mL of pancreatin 4,000 $U.mL^{-1}$ and 5 mL of 1,000 $U.mL^{-1}$ of lipase. The incubation occurred for 2 h at 37 °C and 7.5 × g. The enzyme activities were provided by the manufacturer (Sigma®). After the completed *in vitro* digestion, the solution was centrifuged at 8000 *g*, 60 min at 4 °C (Thermo, Langenselbold, Germany) to separate the supernatant fraction containing the micellarized carotenoids from the remaining solid particles. The supernatant containing the mixed micelles was collected, were covered with nitrogen gas, frozen at −40 °C and lyophilized for further extraction of bioaccessible carotenoids. The carotenoids bioaccessibility was calculated according to Eq. (1), considering the ratio between the concentration of carotenoid in the micellar aqueous phase (supernatant) and the initial content present in the microalgae biomasses.

$$\text{Bioaccessibility } (\%) = \left(\frac{\text{Micellar aqueous fraction content}}{\text{Total initial content}}\right) \times 100 \qquad (1)$$

2.6. Carotenoids analysis

2.6.1. Carotenoids extraction from S. Bijuga and C. Sorokiniana biomass

The original content of carotenoids of *S. bijuga* and *C. sorokiniana* biomass (initial extract) were obtained in triplicate according to the procedure described by Mandelli, Miranda, Rodrigues, and Mercadante (2012). Briefly, the carotenoids were exhaustively extracted from the freeze-dried samples (0.1 ± 0.02 g) with ethyl acetate followed by methanol in a mortar with a pestle followed by centrifugation (Thermo, Langenselbold, Germany) for 7 min at 1500*g*. The extraction procedure was repeated until the supernatant becomes colorless. The extract was saponified overnight, under a nitrogen atmosphere with methanolic and KOH (10:100 [w:v]) at room temperature, and the alkali was removed by washing with distilled water. Then, the sample suspension was filtered through a 0.22 μm polyethylene membrane, concentrated in a rotary evaporator (T < 30 °C). The dry extracts were stored at −40 °C under a nitrogen atmosphere and kept in the dark until chromatographic analysis.

2.6.2. Carotenoids extraction from the supernatant fraction of digesta

The carotenoids from the aqueous phase containing mixed micelles, i.e., the supernatant resulting from the centrifugation of the digesta, were extracted by ultrasound-assisted extraction using an ultrasonic processor (Ultronic, Indaiatuba-SP, Brazil) according to an adapted methodology (Ordóñez-Santos, Pinzón-Zarate, & González-Salcedo, 2015). The lyophilized samples were exhaustively extracted by the addition of 15 mL of ethyl ether and petroleum ether (1:1) and subjected to 5 min ultrasonic cycles, probe with 13 mm diameter, 400 W, at a constant frequency of 40 kHz, in an ice bath to control the temperature (0 ± 2 °C). Then, were centrifuged and the supernatant was collected. The process was repeated until the supernatant became colorless. After the extracts were rotary evaporated, saponified, and subjected to chromatographic analysis.

2.6.3. Separation, identification, and quantification by HPLC-PDA-MS/MS

The carotenoids were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with binary pumps (model LC-20AD), online degasser, and automatic injector (Rheodyne, Rohnert Park-CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometry was performed with a Shimadzu 8040 triple quadrupole mass spectrometer and atmospheric pressure chemical ionization (APCI) source (Shimadzu America, Columbia, MD, USA). The carotenoids separation was performed on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA). HPLC-PDA-MS/MS analysis was performed according to Rodrigues, Menezes, Mercadante, Jacob-Lopes, and Zepka (2015). Prior to HPLC-PDA-MS/MS analysis, the carotenoids extract was solubilized in MeOH:MTBE (70:30) and filtered through Millipore membranes (0.22 μm). The mobile phases A (MeOH) and phase B (MTBE), using a linear gradient program as follows: from 0 to 30 min 5% B; from 30 to 40 min, 5–30% B; from 40 to 41 min, 30–50% B, from 41 to 50 min, 50 to 5% B. The flow rate was set at 0.9 mL min^{-1}, the injection volume was 20 μL, the column temperature was maintained at 22 °C, the UV–Vis spectra were acquired between 220 and 700 nm, and the chromatograms were processed at 450 nm.

The MS/MS detection was achieved according to Giuffrida, Zoccali, Giofrè, Dugo, and Mondello (2017) with adaptations, the APCI interface operated in positive (+) mode; detector voltage: 4.5 kV; interface temperature: 350 °C; DL temperature: 250 °C; heat block temperature: 200 °C; nebulizing gas flow (N_2): 3.0 $L.min^{-1}$; drying gas flow (N_2): 5.0 $L.min^{-1}$; collision induced dissociation (CID) gas: 23 kPa (argon); event time: 0.5 s. To improve the quality of identification, the MS was used simultaneously in SIM (Select Ion Monitoring) and MRM (Multiple Reaction Monitoring) modes.

The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV–visible spectrum (Spectral fine structure (λmáx), ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II), ratio of the *cis* peak (AB) and the middle absorption peak (II)), and mass spectra characteristics (protonated molecule ($[M + H]^+$) and MS/MS fragments), compared with data available in the literature (Britton, 1995; De Rosso & Mercadante, 2007; Van Breemen, Dong, & Pajkovic, 2012; Rodrigues et al., 2015). The carotenoids were individually quantified by HPLC-PDA using six-point calibration curves (r^2 = 0.99) of all-*trans*-lutein (1.0–50.0 μg), and all-*trans*-β-carotene (1.0–50 μg); xanthophylls were quantified using the all-*trans-lutein* curve, and carotenes using the all-*trans*-β-carotene curve. Total carotenoids content was calculated considering all identified peak areas. The limit of detection (LOD) was calculated using the parameters of each standard curve: LOD = 3.3 × SD/*S*, where SD is the standard deviation of the response and *S* is the

slope of the curve. For all analytical curves of carotenoids, $r^2 = 0.99$, the limit of detection was 0.1 μg, and the limit of quantification was 0.5 μg.

2.7. Lipid composition

The lipids were extracted from freeze-dried biomass (0.1 ± 0.02 g) of *S. bijuga* and *C. sorokiniana* according to the method described by Vendruscolo et al. (2018) and determined by gravimetry in an air circulation oven at 105 °C. The remainder of the organic fraction containing the lipids was subjected to derivation to obtain fatty acids methyl esters (FAME) (Hartman & Lago, 1973). Then, 1 μL of hexane-diluted FAME were injected in splitless mode in a Gas Chromatograph with Flame Ionization Detector (GC-FID) (Varian, Star 3600, USA). The FAME were separated into HP-88 capillary column (Agilent Technologies, USA) (100 m × 0.25 mm; 0.20 μm of thickness film) with initial temperature of 50 °C for 1 min, increasing to 185 °C at 15 °C.min^{-1} and then at 0.5 °C.min^{-1} increased to 195 °C and finally up to 230 °C to 15 °C.min^{-1}, remaining for 10 min. Injector and detector were kept at 250 °C. Identification was performed by comparing retention times with standards of FAME Mix 37 (P/N 47885-U) (Sigma-Aldrich, USA). Results were expressed as a percentage of the total area taking into account FID correction factors for the equivalent carbon-chain length and the conversion of esters to acids (Visentainer, 2012).

2.8. Statistical analysis

The analysis was performed using Statistica 7.0 software (Statsoft, Tulsa-OK, USA). All data are results of three repetitions and expressed as means ± standard error of the mean (SEM). Normality and homoscedasticity criteria were evaluated by Shapiro-Wilk and Levene tests, respectively. For normally distributed and homoscedastic data, parametric tests were used. In turn, the means were compared by a Student's *t*-test for two independent means or by one-way ANOVA and Tukey's test ($p < 0.05$) for more than two variables. For heteroscedastic variables, the non-parametric Kruskal-Wallis post-test ($p < 0.05$) was used. In addition, carotenoid bioaccessibility and lipid composition data were submitted to Principal Component Analysis (PCA) using Pirouette 3.11 software (Infometrix Co., USA). Before this multivariate analysis, the data matrix was autoscaled for each variable so that each assumed the same weight during analysis.

Table 1
Chromatographic, UV–Vis spectrum and mass characteristics obtained by HPLC-PDA-MS/MS of *Scenedesmus bijuga* and *Chlorella sorokiniana* carotenoids.

Peak[a]	Carotenoid	t_R (min)[b]	UV–Vis characteristics			Fragment ions (positive mode) (*m/z*)	
			$\lambda_{máx}$ (nm)[c]	III/II (%)[d]	AB/II (%)[e]	[M + H]$^+$	MS/MS
1	15-*cis*-neochrome	6.2	327, 405, 428, 455	65	42	601	583 [M + H − 18]$^+$
2	15-*cis*-neoxanthin	6.8–6.9	326, 416, 438, 467	76	18	601	583 [M + H − 18]$^+$, 565 [M + H − 18–18]$^+$, 547 [M + H − 18–18–18]$^+$, 509 [M + H − 92]$^+$
3	all-*trans*-neoxanthin	7.1–7.3	418, 444, 470	82	0	601	583 [M + H − 18]$^+$, 565 [M + H − 18–18]$^+$, 547 [M + H − 18–18–18]$^+$, 509 [M + H − 92]$^+$, 491 [M + H − 92–18]$^+$
4	9-*cis*-neoxanthin	7.6	326, 415, 438, 468	81	10	601	583 [M + H − 18]$^+$, 565 [M + H − 18–18]$^+$, 547 [M + H − 18–18–18]$^+$, 509 [M + H − 92]$^+$
5	9-*cis*-neochrome	7.9	336, 398, 420, 447	85	5	601	583 [M + H − 18]$^+$
6	15-*cis*-violaxanthin	8.2	327, 412, 435, 464	86	11	601	583 [M + H − 18]$^+$, 565 [M + H − 18–18]$^+$, 509 [M + H − 92]$^+$, 491 [M + H − 92–18]$^+$
7	all-*trans*-antheraxanthin	8.8	416, 438, 466	45	0	585	567 [M + H − 18]$^+$, 549 [M + H − 18–18]$^+$, 529 [M + H − 56]$^+$, 221
8	*cis*-lutein	9.1	330, 415, 439, 466	30	50	569	551 [M + H − 18]$^+$, 533 [M + H − 18–18]$^+$
9	15-*cis*-lutein	10.3–10.6	330, 415, 440, 466	25	50	569	551 [M + H − 18]$^+$, 533 [M + H − 18–18]$^+$, 477 [M + H − 92]$^+$, 463 [M + H − 106]$^+$, 459 [M + H − 18–92]$^+$
10	13-*cis*-lutein	11.2–11.5	329, 415, 438, 464	35	45	569	551 [M + H − 18]$^+$, 533 [M + H − 18–18]$^+$, 477 [M + H − 92]$^+$, 459 [M + H − 18–92]$^+$
11	all-*trans*-lutein	12.4–12.8	418, 443, 471	58	0	569	551 [M + H − 18]$^+$, 533 [M + H − 18–18]$^+$, 495 [M + H − 18–56]$^+$, 477 [M + H − 92]$^+$, 459 [M + H − 18–92]$^+$
12	all-*trans*-zeaxanthin	14.5–15.0	422, 449, 475	30	0	569	551 [M + H − 18]$^+$, 533 [M + H − 18–18]$^+$,495, 477 [M + H − 92]$^+$, 459 [M + H − 18–92]$^+$
13	9-*cis*-lutein	15.2–15.6	330, 415, 438, 465	50	12	569	551 [M + H − 18]$^+$, 533 [M + H − 18–18]$^+$,495, 477 [M + H − 92]$^+$, 459 [M + H − 18–92]$^+$
14	9-*cis*-zeaxanthin	17.5–17.7	338,419, 438, 465	35	25	569	551 [M + H − 18]$^+$
15	9′-*cis*-lutein	17.9	332, 412, 439, 465	50	8	569	551 [M + H − 18]$^+$, 533 [M + H − 18–18]$^+$
16	5,6-epoxy-β-carotene	21.7	420, 443, 472	45	0	553	535 [M + H − 18]$^+$, 461 [M + H − 92]$^+$, 205
17	all-*trans*-echinenone	23.7	462	nc	0	551	533 [M + H − 18]$^+$, 427, 203
18	15-*cis*-β-carotene	25.6–26.0	338, 418, 441, 469	14	42	537	457, 444 [M + H − 92]$^+$, 399, 355
19	all-*trans*-α-carotene	27.8–28.4	419, 445, 472	56	0	537	481 [M + H − 56]$^+$, 444 [M + H − 92]$^+$, 413, 399
20	all-*trans*-β-carotene	31.7–32.4	424, 451, 476	27	0	537	481 [M + H − 56]$^+$, 444 [M + H − 92]$^+$, 413, 399, 355
21	9-*cis*-β-carotene	33.8–34.5	341, 423, 448, 474	20	14	537	481 [M + H − 56]$^+$, 444 [M + H − 92]$^+$, 413, 399, 355

[a]Numbered according to the elution order on C30 HPLC column.
[b]t_R: Retention time on the C_{30} column.
[c]Linear gradient Methanol:MTBE.
[d]Spectral fine structure: Ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II).
[e]Ratio of the *cis* peak (AB) and the middle absorption peak (II).
[f]Not calculated.

3. Results and discussion

3.1. Characterization of the carotenoid profile in S. Bijuga and C. Sorokiniana

The chromatographic and spectrometric characteristics of the different carotenoids separated in *S. bijuga* and *C. sorokiniana* biomass are shown in Table 1. Our identification was entirely based on chemical evidence provided by the chromatographic analysis compared with data available in the literature. The elution order, UV–Vis spectrum, and MS/MS experiments confirmed the assignment of the protonated molecule ($[M + H]^+$) of all peaks identified through the fragments expected for the carotenoid polyene. A detailed description of carotenoids identification using chromatographic information HPLC-PDA-MS/MS (APCI positive mode) has already been well established and reported in the previous literature (De Rosso & Mercadante, 2007; Rodrigues et al., 2015; Patias et al., 2017; Fernandes et al., 2020).

A total of twenty-one compounds were separated, identified, and quantified, where nineteen were identified among the initial extracts, and two compounds characterized only after the *in vitro* digestion process (peak 5 and peak 8). In this section, we focus only on considerations regarding the quantitative profiles of the identified carotenoids in the original content of *S. bijuga* and *C. sorokiniana* (initial extract), which are given below.

The quantitative profile of carotenoids in *S. bijuga* and *C. sorokiniana* before *in vitro* digestion process is presented in Table 2.

The total carotenoid content showed a significant difference ($p < 0.05$) between species, being higher in *S. bijuga* (1627.26 $\mu g.g^{-1}$). All-*trans*-lutein (909.93 $\mu g.g^{-1}$) was the compound major, followed by all-*trans*-β-carotene (182.13 $\mu g.g^{-1}$), and all-*trans*-neoxanthin (110.66 $\mu g.g^{-1}$). *C. sorokiniana* presented total carotenoid content of 1353.71 $\mu g.g^{-1}$. As in *S. bijuga*, all-*trans*-lutein (781.26 $\mu g.g^{-1}$), all-*trans*-β-carotene (106.75 $\mu g.g^{-1}$) were the largest carotenoids, followed by 9-*cis*-neoxanthin (99.82 $\mu g.g^{-1}$).

Carotenes constituted altogether 16.00 and 18.00% of total carotenoids in *C. sorokiniana* and *S. bijuga*, respectively. The most abundant fraction corresponds to xanthophylls, 82.00% in *S. bijuga* and 84.00% in *C. sorokiniana*. Ten compounds were common among species (peaks 3, 4, 6, 9, 10, 11, 12, 19, 20, and 21).With the exception of 13-*cis*-lutein and 9-*cis*-β-carotene, all showed a significant difference ($p < 0.05$). Except 9-*cis*-neoxanthin, all-*trans*-zeaxanthin, and all-*trans*-α-carotene, all other compounds were significantly ($p < 0.05$) higher in *S. bijuga*. Additionally, seven compounds were characterized only in the extract of *S. bijuga*: allenic carotenoids (peaks 1), *cis* isomers (peak 13, 14, and 18), epoxycarotenoids (peak 7 and 16), and ketocarotenes (peak 17). In contrast, 15-*cis*-neoxanthin and 9′-*cis*-lutein were detected only in *C. sorokiniana*.

3.2. Bioaccessibility of carotenoids in S. Bijuga and C. Sorokiniana

As compiled in previous studies, the bioaccessibility of microalgae carotenoids is extremely limited when ingested from whole dried biomass (Gille et al., 2016; Bernaerts et al., 2020; Nascimento et al., 2021). This fact is strongly evident for microalgae species of the genus *Scenedesmus* and *Chlorella*, mainly due to the rigid cell wall (Granado-Lorencio et al., 2009; Gille et al., 2016), which are not degraded by the digestive enzymes present in the mouth, stomach, and/or small intestine (Bernaerts et al., 2020). As such, some authors suggest that pre-treatments that promote cell disruption, like ultrasound, are necessary to promote and enhance intracellular components bioaccessibility (Gille et al., 2019; Bernaerts et al., 2020). Considering these facts, in this study, wet ultrasonicated biomass was used in all *in vitro* digestion tests.

As shown in Table 2, the incorporation of carotenoids in the micelles after the intestinal phase and relative bioaccessibility index are complementary results. The micellar aqueous fraction corresponds to the absolute carotenoids content released from the matrix and transferred to the micelles ($\mu g.g^{-1}$). Conversely, the bioaccessibility index refers to values relative (%) among of the portion of solubilized carotenoids in the micelles to the whole amount ingested.

Table 2
Individual carotenoids content in *Scenedesmus bijuga* and *Chlorella sorokiniana* biomass before digestion, after micellar incorporation, and relative bioaccessibility.

Peak	Carotenoid	Initial extract ($\mu g.g^{-1}$ dry weight)		Micellar aqueous fraction ($\mu g.g^{-1}$ dry weight)		Relative bioaccessibility (%)	
		S. bijuga	*C. sorokiniana*	*S. bijuga*	*C. sorokiniana*	*S. bijuga*	*C. sorokiniana*
1	15-*cis*-neochrome	29.82 ± 0.70[1]	nd	nd	nd	nd	nd
2	15-*cis*-neoxanthin	nd[2]	56.52 ± 0.46[a]	13.92 ± 0.35[b]	nd	nc[3]	nd
3	all-*trans*-neoxanthin	110.66 ± 1.15[a]	46.98 ± 0.44[b]	nd	nd	nd	nd
4	9-*cis*-neoxanthin	39.07 ± 0.02[b]	99.82 ± 0.07[a]	nd	nd	nd	nd
5	9-*cis*-neochrome	nd	nd	14.40 ± 0.52[a]	14.12 ± 0.18[a]	nc	nc
6	15-*cis*-violaxanthin	79.86 ± 0.87[a]	55.29 ± 0.31[b]	13.87 ± 0.19[c]	nd	17.02 ± 1.03	nd
7	all-*trans*-antheraxanthin	28.89 ± 0.69	nd	nd	nd	nd	nd
8	*cis*-lutein	nd	nd	0.98 ± 0.03[b]	13.89 ± 0.29[a]	nc	nc
9	15-*cis*-lutein	46.22 ± 0.34[a]	23.74 ± 0.97[b]	1.99 ± 0.36[c]	2.12 ± 0.10[c]	4.03 ± 0.59[b]	8.35 ± 0.58[a]
10	13-*cis*-lutein	11.60 ± 0.55[a]	12.01 ± 0.25[a]	1.36 ± 0.31[b]	1.12 ± 0.03[b]	9.40 ± 0.27[a]	10.71 ± 1.17[a]
11	all-*trans*-lutein	909.93 ± 4.40[a]	781.26 ± 5.00[b]	28.59 ± 0.50[d]	42.90 ± 0.17[c]	3.25 ± 0.43[b]	6.00 ± 0.58[a]
12	all-*trans*-zeaxanthin	13.20 ± 0.31[b]	41.68 ± 0.24[a]	0.89 ± 0.10[c]	nd	6.38 ± 0.25	nd
13	9-*cis*-lutein	6.70 ± 0.23[a]	nd	1.68 ± 0.06[c]	2.21 ± 0.30[b]	26.73 ± 1.91	nc
14	9-*cis*-zeaxanthin	5.57 ± 0.10[a]	nd	2.34 ± 0.22[b]	nd	42.30 ± 0.29	nd
15	9′-*cis*-lutein	nd	14.74 ± 0.69	nd	nd	nd	nd
16	5,6-epoxy-β-carotene	26.67 ± 1.21	nd	nd	nd	nd	nd
17	all-*trans*-echinenone	25.71 ± 0.32	nd	nd	nd	nd	nd
18	15-*cis*-β-carotene	27.46 ± 0.73	nd	nd	nd	nd	nd
19	all-*trans*-α-carotene	51.72 ± 0.47[b]	63.68 ± 0.40[a]	nd	nd	nd	nd
20	all-*trans*-β-carotene	182.13 ± 1.83[a]	106.75 ± 0.70[b]	15.26 ± 0.44[c]	14.24 ± 0.28[c]	8.45 ± 0.27[b]	13.56 ± 0.54[a]
21	9-*cis*-β-carotene	31.96 ± 0.6[a,b]	51.17 ± 0.02[a]	13.74 ± 0.21[a,b]	13.60 ± 0.09[b]	43.78 ± 0.10[a]	26.18 ± 0.96[b]
	Total carotenes	293.27 ± 2.11[a]	221.60 ± 0.80[a,b]	29.00 ± 0.64[a,b]	27.84 ± 0.36[b]	9.66 ± 0.58[b]	12.59 ± 0.59[a]
	Total xanthophylls	1333.99 ± 1.79 [a]	1132.11 ± 0.54[b]	80.06 ± 1.52[c]	76.61 ± 0.45[c]	5.39 ± 0.29[b]	6.68 ± 0.53[a]
Total		1627.26 ± 2.62[a]	1353.71 ± 4.31[b]	109.06 ± 1.59[c]	104.45 ± 1.08[c]	7.30 ± 0.61[a]	8.03 ± 0.62[a]

[1]Values are average and standard deviation of triplicates.
[2]Not detected.
[3]Not calculated.
Different lowercase letters in the same row indicate significant differences (parametric variables: Student's *t*-test for two independent samples or Tukey's test for three or more samples, $p < 0.05$; non-parametric variables: Kruskal-Wallis, $p < 0.05$).

3.2.1. Absolute carotenoids content in the micellar aqueous fraction

As expected, *in vitro* digestion process led to a pronounced reduction in the carotenoid contents in the aqueous phase containing the mixed micelles (supernatant), indicating that only a small amount of the ingested carotenoids was liberated from the matrix after the *in vitro* digestion process (Table 2). *S. bijuga* presented a more diversified qualitative profile, constitute by twelve compounds. Of these, nine were in the ingested biomass and three (peak 2, 5, and 8) were detected only after the *in vitro* digestion process. Xanthophylls (74.40%) were micellarized to a higher extent compared to carotenes (26.60%). The total carotenoid content was 109.06 $\mu g.g^{-1}$, being all-*trans*-lutein (26.21%), all-*trans*-β-carotene (13.99%), and 9-*cis*-neochrome (13.20%) the main compounds.

A total of eight carotenoids constituted the micellar aqueous fraction of *C. sorokiniana*, of which only five were present in the initial extract. Similar to that found in *S. bijuga*, 73.34% of the carotenoids were xanthophylls and 26.65% carotenes. The absolute carotenoids content was 104.45 $\mu g.g^{-1}$, contained mainly all-*trans*-lutein, all-*trans*-β-carotene, and 9-*cis*-neochrome, which summed 68.21% of the total content.

In the present study, 15-*cis*-neochrome, all-*trans*-neoxanthin, 9-*cis*-neoxanthin, all-*trans*-antheraxanthin, 9′-*cis*-lutein, 5,6-epoxy-β-carotene, all-*trans*-echinenone, 15-*cis*-β-carotene, and all-*trans*-α-carotene were detected in the initial extracts but were not found in detectable amounts in the carotenoid profile of the supernatant containing the micellarized carotenoids. At the same time, 9-*cis*-neochrome, *cis*-lutein, 15-*cis*-neoxanthin and 9-*cis*-lutein were detected only after the *in vitro* digestion process.

Due to the significant difference in starting carotenoid concentrations, we expected a varied micellar profile. However, interestingly, we observed that among the micellarized compounds in the two microalgae, only *cis*-lutein, all-*trans*-lutein, 9-*cis*-lutein showed a significant difference ($p < 0.05$) with absolute content in the micelles higher in *C sorokiniana*. These data suggest that 9-*cis*-neochrome, 15-*cis*-lutein, 13-*cis*-lutein, all-*trans*-β-carotene, and 9-*cis*-β-carotene were released and solubilized in mixed micelles in the same proportions regardless of species of microalgae. This behaviour was also observed for the total content of carotenes, xanthophylls and carotenoids, as they showed no statistically significant difference ($p < 0.05$) in the micellar aqueous phase.

3.2.2. Relative bioaccessibility of carotenoids

As shown in Table 2, the total content of bioaccessible carotenoids between microalgae showed no significant difference ($p < 0.05$). The bioaccessibility of total carotenoids ranged from 7.30% to 8.03%. In general, twelve compounds showed bioaccessibility, eight commons among species and four were bioaccessible only in *S. bijuga.* Of these, the bioaccessibility of the 9-*cis*-lutein in *C. sorokiniana*; 15-*cis*-neoxanthin in *S. bijuga*; and 9-*cis*-neochrome and *cis*-lutein in both species was not calculated as they were not present in the initial extract.

9-*cis*-β-carotene was the most bioaccessible compound in both species and showed bioaccessibility values from 26.18% to 43.78%, significantly higher in *S. bijuga* ($p < 0.05$). In contrast, all-*trans*-lutein had the lowest bioaccessibility. The % of bioaccessible lutein in *S. bijuga* was 3.25%, while for *C. sorokiniana*, it was higher 6%. However, all-*trans*-lutein was the compound that showed higher absolute content in the micellar aqueous fraction; there were 42.90 $\mu g.g^{-1}$ of lutein present in the micelles of *C. sorokiniana* that would potentially be available in the gastrointestinal tract for absorption and 28.59 $\mu g.g^{-1}$ in *S. bijuga.* 15-*cis*-lutein, 13-*cis*-lutein, 9-*cis*-lutein, and all-*trans*-β-carotene showed bioaccessibilities ranging from 4.03 to 13.56%. Of these compounds, only 13-*cis*-lutein showed no significant difference. Except for 9-*cis*-β-carotene, all compounds were significantly ($p < 0.05$) more bioaccessible in *C. sorokiniana.*

In *S. bijuga*, 15-*cis*-violaxanthin presented bioaccessibility value from 17.02%, while all-*trans*-zeaxanthin 6.38% and 9-*cis*-zeaxanthin 42.30%, this being the second most bioaccessible compound in this species.

A complex set of factors interferes with the bioaccessibility of carotenoids. Among these factors, it is assumed that the physicochemical properties of carotenoids play an important role. The isomerization of carotenoids for their *cis* configuration induces alteration in physicochemical properties of these molecules, i.e., the solubility of carotenoids dramatically improves, and they change from a "crystalline state" to an "oily (amorphous) state" (Honda et al., 2018, 2019). Thus, the higher solubility of *cis* isomers of carotenoids can be considered the key factor that leads to better micellarization of these compounds with bile salts and pancreatin in the intestinal phase, resulting in improved bioaccessibility (Yang et al., 2017a, 2018). Several reports have suggested that *cis* isomers are more bioaccessible than the all-*trans* form attributed to these characteristics (Failla, Chitchumroonchokchai, & Ishida, 2008; Mutsokoti et al., 2017). This trend was observed in our results since most of the bioaccessible compounds were in their *cis* conformation. In addition, the compounds 15-*cis*-lutein, 13-*cis*-lutein, 9-*cis*-lutein, 9-*cis*-zeaxanthin, and 9-*cis*-β-carotene, showed greater bioaccessibility when compared to their all-*trans* isomers (2.00 to 6.70 times more). Like our results, *cis* isomers of lutein and β-carotene presented higher bioaccessibility levels *in vitro* than their all-*trans* isomer (Rodrigues, Mariutti, & Mercadante, 2016; Yang et al., 2018; Nascimento et al., 2021). However, there is no consensus regarding differences in the bioaccessibility of *cis* and *trans* isomers of carotenoids since studies showing similar (Rodrigues et al., 2016; Gómez-Maqueo, Bandino, Hormaza, & Cano, 2020) and lower (Rodrigues et al., 2016; Nascimento et al., 2021) bioaccessibility of *cis* isomers compared to their respective all-*trans* form can be found in the literature.

Of particular interest, carotenoids in their *cis* configuration have received a lot of attention, as they demonstrate physicochemical properties and potential biological activities against all-*trans* forms. But, the number of carotenoids investigated in terms of these effects is still limited. As well as greater bioaccessibility, an extension of bioavailability has recently been reported for *cis* isomers of β-carotene and lutein versus their all-*trans* forms (Honda, Takasu, Nakagawa, & Tsuda, 2021). Previous studies point that the *cis* isomers of carotenoids have greater antioxidant activity than the all-*trans* isomers (Yang et al., 2017b, 2018; Honda et al., 2019). Important biological activities such as the potential to inhibit atherogenesis were also positively correlated with the 9-*cis*-isomer of β-carotene (Harari et al., 2008). Likewise, prevention of atherosclerosis progression by 9-*cis*-β-carotene was greater than its all-*trans* form (Harari et al., 2013; Relevy et al., 2015). These findings allow us to consider the relevance of the *cis* isomers of carotenoids. However, it is worth noting that the influence of the physicochemical properties of *cis/trans* isomers on the bioaccessibility, bioavailability and bioactivity varies among carotenoids and also among their isomers (Honda, 2021).

Although the influence of the constituents of the microalgae matrix on bioaccessibility, micellarization of total carotenes was consistently lower than that of total xanthophylls in both species. The bioaccessibility of xanthophylls ranged from 3.25% to 42.30% in *S. bijuga* and from 6.00 to 10.71% in *C. sorokiniana*. At the same time, carotenes ranged from 8.45% to 43.78% in *S. bijuga* and from 13.56% to 26.18% in *C. sorokiniana*. Overall, most scientific evidence points out that the bioaccessibility of lipophilic compounds was inversely related to their hydrophobicity (Reboul et al., 2006; Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012; Failla et al., 2014; Rodrigues, Chitchumroonchokchai, Mariutti, Mercadante, & Failla, 2017). In our study, we observed this behavior only for some compounds in S. *bijuga*. Specifically, the xanthophylls 15-*cis*-violaxanthin, 13-*cis*-lutein and 9-*cis*-lutein were significantly (*t*-test $p < 0.05$) more bioaccessible than all-*trans*-β-carotene. In contrast, for the other individual compounds and the total fractions in both species, the carotenes were significantly ($p < 0.05$) more bioaccessible than the xanthophylls, suggesting that factors other than hydrophobicity these hydrocarbon compounds are important for their bioaccessibility.

The interaction of carotenoids with other compounds present in the matrix determines the extension of bioaccessibility. In microalgae,

xanthophylls, for being relatively hydrophobic molecules, are typically associated with membranes and/or involved in non-covalent binding to specific proteins (Guedes et al., 2011; Mulders, Lamers, Martens, & Wijffels, 2014), which can negatively impact their bioaccessibility. In addition, the concentration of matrix minerals can interfere by increasing or decreasing the rate and extent of micellarization (Corte-Real & Bohn, 2018). The soluble fibres content also influences, as it negatively impacts the bioaccessibility of carotenoids (Yeum & Russell, 2002). In contrast, lipids are known to promote carotenoid bioaccessibility, as discussed in more detail in the next section. These and other factors have been reported previously and discussed elsewhere in detail (Tyssandier, Lyan, & Borel, 2001; Desmarchelier & Borel, 2017; Priyadarshani, 2017).

The metabolism/degradation of the compounds in the gastrointestinal tract are factors that also influence the profile of bioaccessible carotenoids. Carotenoids are known to undergo several structural transformations during their passage through the gastrointestinal tract (Petry & Mercadante, 2017). In our study, we identified the synthesis of epoxy compound (9-*cis*-neochrome) that possibly was formed from an epoxide-furanoid rearrangement from their neoxanthin intermediate. This mechanism is already known and has been previously reported in the literature for *in vitro* digestion assays (Asai, Terasaki, & Nagao, 2004).

Regarding the location of the carotenoids, in most green microalgae (*S. bijuga* and *C. sorokiniana*), carotenes and xanthophylls are synthesized within plastids and accumulate only in them. In turn, some xanthophyll structures can be synthesized in the chloroplast, exported, and, consequently, accumulate in the cytoplasm; therefore, they can be found in essentially all cell compartments (Guedes et al., 2011; Novoveská et al., 2019), which might facilitate an efficient liberation and micellarization. This evidence can explain the greater number of xanthophylls in the qualitative profile of bioaccessible compounds. In contrast, the location carotenes (embedded in plastids) possibly did not affect their bioaccessibility since total carotenes were more bioaccessible than xanthophylls.

In fact, among the numerous factors related to the matrix that affect the bio accessibility of carotenoids, the cellular structures in which they are embedded are of great relevance. Specifically, in microalgae, the rigidity of the cell wall act with a barrier that prevents the release of the intracellular compounds and is reported as the main limiting factor for the bioaccessibility of carotenoids (Gille et al., 2016; Bernaerts et al., 2020).

Unfortunately, the diversity of conditions used in *in vitro* digestion models has hampered the ability to compare the results of different studies. In addition, structural characteristics, which impact the release of the compounds, and the physical–chemical composition of the different microalgae species contribute to the discrepancy in the bioaccessibility results of microalgae carotenoids. In turn, most of our results differ from previous studies with species of the genus *Chlorella* and *Scenedesmus*.

When compared to *C. sorokiniana*, Gille et al. (2016) reported higher values for lutein bioaccessibility (18%) and lower values for β-carotene (12.50%) in *Chlorella vulgaris* ultrasonic biomass. Interestingly, in a diet supplemented with biomass of *Chlorella vulgaris* in the concentration of 25.00%, the bioaccessibility of lutein exceeded 25.00%, while β-carotene was detected in the bioaccessible fraction in traces (Gille et al., 2018).

In a study of the bioaccessibility of carotenoids from *Chlorella ellipsoidea* microfluidized, the authors reported values of 18.19% for β-carotene and 32.60% for zeaxanthin bioaccessibility. As our results, in this study with *C. ellipsoidea*, antheraxanthin did not show bioaccessibility, although it was present in considerable concentrations in the digested biomass (Cha et al., 2012).

In accordance with our results of *S. bijuga*, *cis* isomers of neoxanthin, lutein, and β-carotene also showed bioaccessibility in the ultrasonicated biomass of *Scenedesmus obliquus*. As well as the compounds in their *cis* configuration were more bioaccessible than all-*trans*. In addition, the authors found higher values for bioaccessibility of all-*trans*-lutein (12.35%) and lower for all-*trans*-β-carotene (3.32%) (Nascimento et al., 2021).

3.3. Lipid composition in S. Bijuga and C. Sorokiniana

Of particular relevance, the promotion and extension of micellar incorporation and subsequent bioaccessibility of carotenoids are enhanced by the presence of lipids (Lemmens et al., 2014). Parameters such as the concentration of matrix lipids and the nature of triacylglycerol (TAG) molecules, that is, FA chain length and unsaturation degree, can play a key role in this context since they directly influence the bioaccessibility of carotenes and xanthophylls (Xavier & Mercadante, 2019). The *S. bijuga* and *C. sorokiniana* biomasses were also characterized in terms of lipid profile, the results of which are provided in Table 3 and illustrated in Fig. 1. Twenty different fatty acids were identified between species with carbon chain lengths from 10 up to 23 carbons.

S. bijuga presented a lipid content of 14.16% with fifteen compounds in its composition, while a total lipid content of 8.94% consisting of nineteen compounds was detected for *C. sorokiniana* biomass. The dominant fatty acids in the two species were linoleic acid (C18:2n6), palmitic acid (C16:0), and α-linolenic acid (C18:3n3). Concentrations ranged from 10.35% to 44.38%. The other identified fatty acids were detected in concentrations below 6.00%.

Although *C. sorokiniana* it contains an amount of approximately 1.60 times fewer lipids than *S. bijuga*, in terms of the degree of saturation, presented the most diversified profile. Unsaturated fatty acids (59.79 ± 0.44%) was the highest fraction in this microalgae specie lipids profile, followed by saturated fatty acids (40.21 ± 0.44%) fraction. Ten saturated compounds and nine unsaturated; of these five PUFA (50.98 ± 0.39%) and four MUFA (8.81 ± 0.05%). *S. bijuga* presented nine saturated fatty acids and only six unsaturated fatty acids. However, the UFA and SFA content in *S. bijuga* (60.67 ± 1.74% and 39.33 ± 1.74%, respectively) did not differ significantly ($p < 0.05$) from those presented

Table 3
Lipid composition (%) of isolated extracts controls of species *Scenedesmus bijuga* and *Chlorella sorokiniana* before digestion.

Fatty acids	Content (%)[1]	
	Scenedesmus bijuga	*Chlorella sorokiniana*
capric acid (C10:0)	0.30 ± 0.03[a]	0.09 ± 0.00[b]
lauric acid (C12:0)	5.91 ± 0.47[a]	2.42 ± 0.05[b]
tridecyclic acid (C13:0)	1.63 ± 0.05[b]	1.76 ± 0.00[a]
myristic acid (C14:0)	2.42 ± 0.15[a]	0.45 ± 0.01[b]
tetradecenoic acid (C14:1)	nd[2]	2.27 ± 0.09[b]
pentadecylic acid (C15:0)	0.90 ± 0.06 [a]	0.15 ± 0.00[b]
palmitic acid (C16:0)	24.86 ± 1.04[b]	33.12 ± 0.62[a]
palmitoleic acid (C16:1)	0.93 ± 0.07[b]	1.16 ± 0.06[a]
margaric acid (C17:0)	0.51 ± 0.02[a]	0.30 ± 0.00[b]
heptadecenoic acid (C17:1)	0.17 ± 0.01[b]	0.18 ± 0.01[a]
stearic acid (C18:0)	2.30 ± 0.02[a]	1.56 ± 0.11[b]
oleic acid (C18:1n9)	3.30 ± 0.15[a]	5.20 ± 0.03[a]
linoleic acid (C18:2n6)	44.38 ± 1.69[a]	40.00 ± 0.40[b]
γ-linolenic acid (C18:3n6)	nd	0.33 ± 0.02
α-linolenic acid (C18:3n3)	11.78 ± 0.27[a]	10.35 ± 0.02[b]
arachidic acid (C20:0)	0.50 ± 0.02	nd
eicosadienoic acid (C20:2n6)	0.11 ± 0.01[a]	0.09 ± 0.01[b]
docosanoic acid (C22:0)	nd	0.31 ± 0.01
dihomo-γ-linolenic acid (C20:3n6)	nd	0.21 ± 0.00
tricosanoic acid (C23:0)	nd	0.05 ± 0.00
Lipid Content	14.16 ± 0.02[3,a]	8.94 ± 0.02[b]

[1]Values are average and standard deviation of triplicates.
[2]Not detected.
[3]Values expressed in % of dry biomass.
Different lowercase letters in the same row indicate significant differences (Student's *t*-test, $p < 0.05$).

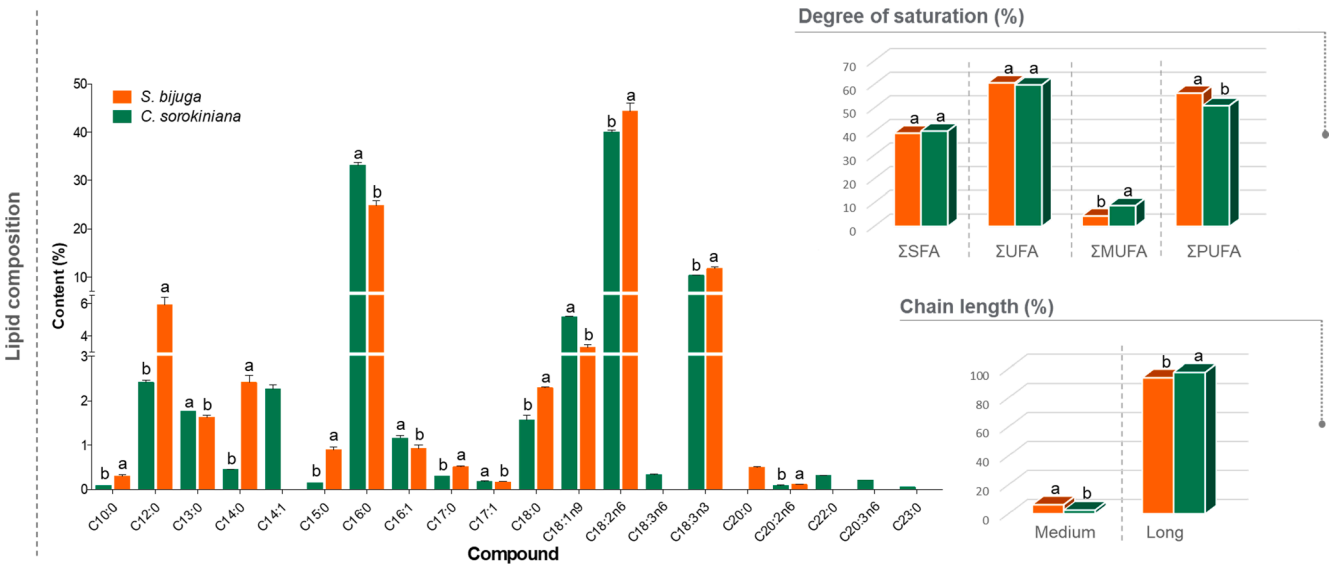

Fig. 1. Individual lipid composition of *S. bijuga* and *C. sorokiniana* biomass. The orange color corresponds to the results of *S. bijuga*, while the green color of *C. sorokiniana*. ΣSFA: Sum of saturated fatty acids; ΣUFA: Sum of unsaturated fatty acids; ΣMUFA: Sum of monounsaturated fatty acids; ΣPUFA: Sum of polyunsaturated fatty acids. Different letters above the error bars indicate significant differences of the compounds between microalgae species (*t* test, $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for *C. sorokiniana*. In contrast, the PUFA (56.28 ± 1.96%) and MUFA (4.40 ± 0.21%) fractions showed a significant difference. Briefly, five compounds were detected only in *C. sorokiniana*, being two saturated fatty acids: docosanoic acid (C22:0) and tricosanoic acid (C23:0); and three unsaturated fatty acids: tetradecenoic acid (C14:1), γ-linolenic acid (C18:3n6), and dihomo-γ-linolenic acid (C20:3n6). On the other hand, saturated fatty acid arachidic acid (C20:0) was detected only in *S. bijuga*.

Regarding the distribution of carbon chain length, microalgae have a profile with a predominance of long-chain structures and a lower content of medium-chain structures. In particular, the fatty acids capric acid (C10:0) and lauric acid (C12:0) represent the fraction of medium-chain structures in both species, which appear in concentrations of approximately 6.21 ± 0.07% and 2.51 ± 0.04% in *S. bijuga* and *C. sorokiniana*, respectively. The other compounds constitute the profile of long-chain fatty acids and present a percentage of 93.79 ± 1.36% in *S. bijuga* and 97.49 ± 1.82% in *C. sorokiniana* of the total lipid content (see Fig. 1).

3.4. Lipid composition of the matrix and its relationship with carotenoid bioaccessibility

Principal components analysis (PCA) was used as an exploratory analysis to visualize better the behavior of fatty acids (individual compounds, TFA, ΣSFA, ΣUFA, ΣMUFA, ΣPUFA, ΣChain-medium, ΣChain-long) and the bioaccessible carotenoids profile (individual bioaccessibility, carotene bioaccessibility, xanthophyll bioaccessibility, total bioaccessibility) from different biomasses, which helped us infer the possible relationships between these two parameters. Fig. 2A and B shows the scores (samples) and loadings (variables), respectively, from the two major components.

The first principal component of the PCA discriminates 90.00% of the total variance of the data and about 5.00% is shown by PCII. Through analysis it appears that the variables (B) were grouped in such a way that the two microalgae were clearly discriminated (A). *S. bijuga*, represented on the left side of the PCA, formed a group that differed from the *C. sorokiniana* mainly in terms of total fatty acid (TFA), chain-medium fatty acids, PUFA, UFA, and individual bioaccessible carotenoids: 15-*cis*-violaxanthin (peak 6), all-*trans*-zeaxanthin (peak 12), 9-*cis*-lutein (peak 13), 9-*cis*-zeaxanthin (peak 14), and 9-*cis*-β-carotene (peak 21). On the other hand, the *C. sorokiniana* samples, right side, showed significant differences in relation to the variables corresponding to the total concentration of SFA, MUFA, chain-long fatty acids, bioaccessibility of total carotenoids, total xanthophylls and total carotenes, 15-*cis*-lutein (peak 9), 13-*cis*-lutein (peak 10), all-*trans*-lutein (peak 11), and all-*trans*-β-carotene (peak 20).

As proven previously, several authors assume that carotenes have greater bioaccessibility in the presence of long-chain TAGs, while xanthophylls with medium-chain TAGs. Likewise, unsaturated fatty acids (UFA) promote the extension of the bioaccessibility of carotenes and saturated fatty acids (SFA) from xanthophylls (Failla et al., 2014; Nagao, Kotake-Nara, & Hase, 2014; Zhang et al., 2015; Yuan, Liu, McClements, Cao, & Xiao, 2018; Han et al., 2019). As can be seen in Fig. 2, some of these statements were confirmed in our study.

From the exploratory analysis of PCA, it is noted that through the projection of the factor loads distributed over factor I e factor II an inversely proportional relationship between the bioaccessibility of total carotenes and the total UFA concentration. However, among the correlated variables in the *C. sorokiniana* group, a correlation between total carotene bioaccessibility, all-*trans*-β-carotene, MUFA and chain-long fatty acids was observed. On the other hand, when the *S. bijuga* group is interpreted, it is evident that the bioaccessibility of 9-*cis*-β-carotene is greater in the presence of UFA and PUFA.

In line with our data, greater β-carotene bioaccessibility was proven when excipient emulsions rich in long-chain monounsaturated fatty acids (MUFAs) were used to assess the bioaccessibility of spinach carotenoids (Yuan et al., 2018). Similar observations were made for the bioaccessibility of β-carotene present in spinach when consumed with fats and oils and long-chain triacylglycerols (Nagao et al., 2014).

Among the most bioaccessible xanthophylls in this study, the lutein fraction (including *cis* and all-*trans* forms) stands out. In a study that evaluated the effects of varying the composition of the oil phase of excipient nanoemulsions on carotenoid bioaccessibility from spinach was observed that as the proportion of medium-chain triglycerides increased, the bioaccessibility of all-*trans*-lutein enhance (Yao et al., 2019). However, in our study, this relation was only observed for bioaccessibility of 9-*cis*-lutein from the *S. bijuga*. Higher concentrations of medium-chain fatty acids C10:0 and C12:0 were observed in the *S. bijuga* samples, reaching almost 2.50 times more than the content found in *C. sorokiniana*. In contrast, observing the projection of the variables in *C. sorokiniana* the promotion of bioaccessibility of all-*trans*-lutein and its

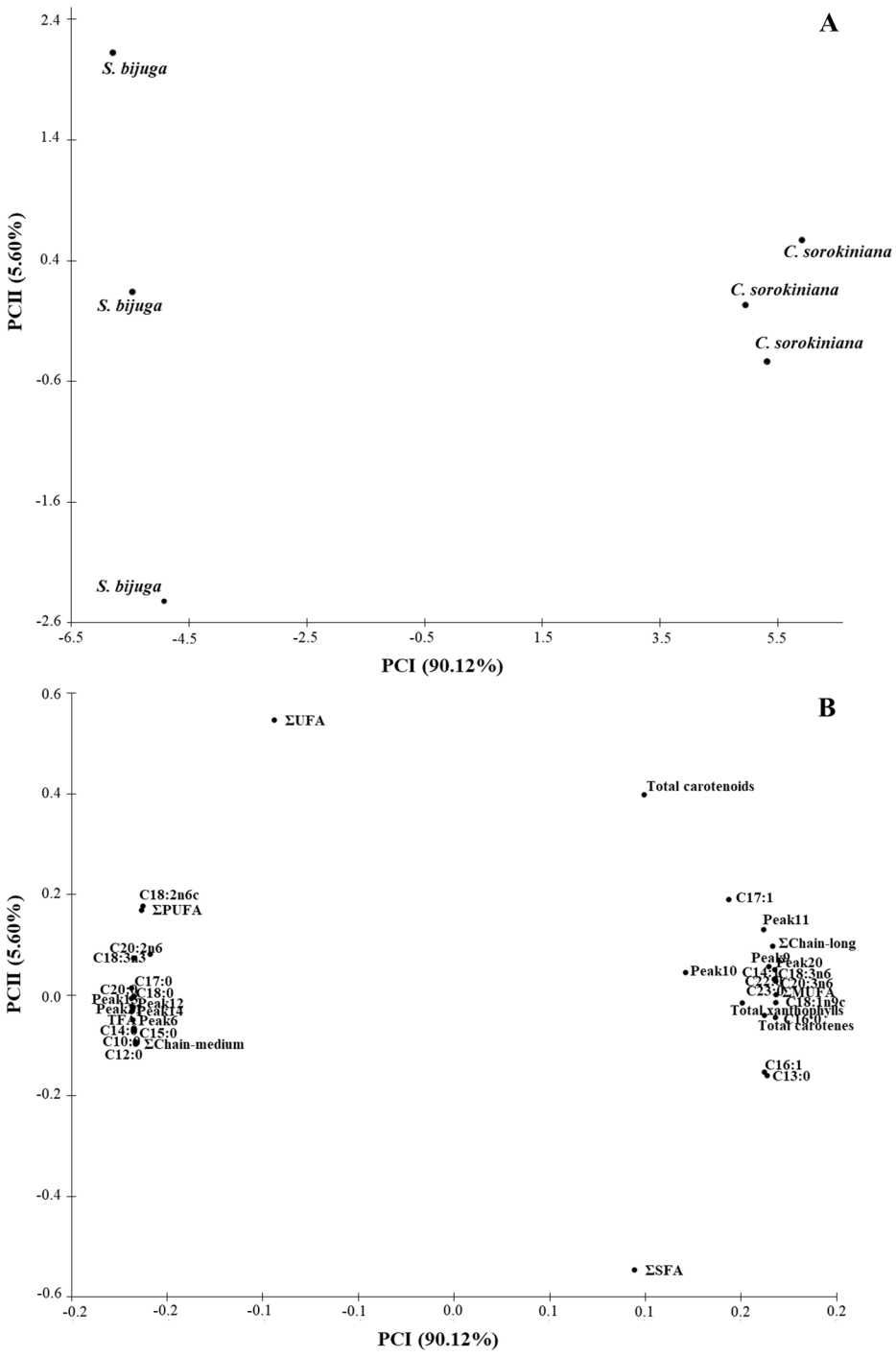

Fig. 2. Principal component analysis of fatty acid composition and the bioaccessible carotenoid profile of *S. bijuga* and *C. sorokiniana*. A - Score plots (samples), *S. bijuga* and *C. sorokiniana*; B - Loadings (variables), Composition fatty acids: TFA, ΣSFA, ΣUFA, ΣMUFA, ΣPUFA, ΣChain-medium, ΣChain-long, C10:0, C12:0, C13:0, C14:0, C14:1, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1n9, C18:2n6, C20:0, C18:3n6, C18:3n3, C20:2n6, C22:0, C20:3n6, C23:0; Bioaccessible carotenoids profile: Total carotenes, Total xanthophylls, Total carotenoids, Peak 6: 15-*cis*-violaxanthin, Peak 9: 15-*cis*-lutein, Peak 10: 13-*cis*-lutein, Peak 11: all-*trans*-lutein, Peak 12: all-*trans*-zeaxanthin, Peak 13: 9-*cis*-lutein, Peak 14: 9-*cis*-zeaxanthin, Peak 20: all-*trans*-β-carotene, Peak 21: 9-*cis*-β-carotene.

13 and 15-*cis* isomers showed a correlation with the fraction of SFA, specifically C13:0, C16:0, C22:0, and C23:0 fatty acids. Most likely, there is a greater tendency to extend the bioaccessibility of these specific xanthophylls in the presence of higher concentrations of SFAs than medium-chain fatty acids.

Interestingly, the correlation of medium-chain fatty acids with the promotion of bioaccessibility of total xanthophylls cannot be observed. However, the results indicated that there was a high correlation between the bioaccessibility of total xanthophylls and the fraction of total SFA with the *C. sorokiniana* samples. For the other xanthophylls, the total content of chain-medium fatty acids discriminated the bioaccessibility of 15-*cis*-violaxanthin, all-*trans*-zeaxanthin, and 9-*cis*-zeaxanthin in the samples of *S. bijuga*.

Furthermore, it is curious that the xanthophylls 15-*cis*-neoxanthin, 15-*cis*-violaxanthin, and all-*trans*-zeaxanthin showed bioaccessibility only from the biomass of *S. bijuga*. Although these compounds were present in the ingested biomass of *C. sorokiniana* (initial extract), they were not micellarized or bioaccessible from that biomass (see Table 2). Possibly the presence of medium-chain fatty acids is the parameter that promotes the bioaccessibility of these xanthophylls in this species of microalgae.

Although we have come a long way in understanding the bioaccessibility of microalgae carotenoids, some limitations of our study deserve consideration. In turn, our findings allow us to put some of the results into perspective and show possible directions for future research. First, to measure carotenoid bioaccessibility, after, *in vitro* digestion, a

centrifugation step was applied in this study to separate the supernatant containing the carotenoids liberated from the microalgae matrix, e.g., the micellarized ones from the rest of the solid fraction. Thus, the overestimation of bioaccessibility cannot be excluded. For future work, a complementary filtration step could be used to amplify the separation of mixed micelles from the aqueous fraction. Second, the bioaccessibility of microalgae carotenoids was evaluated considering only the intake of biomass. Future research should be carried out with microalgae biomass co-digested with different food matrices and with food products that have microalgae in their formulation to better clarify the influence of food constituents on the bioaccessibility of microalgae carotenoids. Third, we evaluated the influence of lipids on the bioaccessibility of microalgae carotenoids, considering the lipid fraction present in the biomass. However, to expand the results of the impact of fatty acid species on the bioaccessibility of microalgae carotenoids, the lipids could have been added separately to the microalgae biomass. Fifth, other factors such as the location of carotenoids in microalgae cells, cell wall interference, the interaction of carotenoids with other matrix constituents, such as protein, carbohydrate and fiber, can significantly interfere with the bioaccessibility of these compounds. In particular, studies considering these factors will be needed better to understand the bioaccessibility of carotenoids from microalgae biomass. Despite these limitations, this is the first study to show the bioaccessibility of carotenoids from the microalgae *S. bijuga* and *C. sorokiniana*. Furthermore, this was the first research to explore the influence of the lipid fraction of the microalgae matrix on the bioaccessibility of carotenoids. These findings are particularly significant, as several advances in understanding the bioaccessibility of microalgal carotenoids were achieved with this study, which may encourage even more future research.

4. Conclusion

Based on the results, *S. bijuga* (1627.26 $\mu g.g^{-1}$) presented the most attractive carotenoid profile in qualitative and quantitative terms, compared to *C. sorokiniana* (1353.71 $\mu g.g^{-1}$). Although, total micellar incorporation (109.06 and 104.45 $\mu g.g^{-1}$) and total carotenoid bioaccessibility (7.30 and 8.03%) of *S. bijuga* and *C. sorokiniana*, respectively, did not differ significantly between species. In contrast, the qualitative profile of bioaccessible carotenoids showed some differences. *S. bijuga* presented twelve bioaccessible compounds, while *C. sorokiniana* presented eight. However, all-*trans*-lutein was the most abundant carotenoid in the *S. bijuga* (28.59 $\mu g.g^{-1}$) and *C. sorokiniana* (42.90 $\mu g.g^{-1}$) micelles; and 9-*cis*-β-carotene was the most bioaccessible compound in the two microalgae (26.18–43.78%). Also, our results showed an extension of bioaccessibility for total carotenes compared to total xanthophylls. Our data also indicate that *cis* isomers were more bioaccessible than compounds in their all-*trans* conformation. In terms of the interactive effect of lipids on carotenoid bioaccessibility, it is concluded that the lipid concentration of the matrix (14.16% in *S. bijuga* and 8.94% in *C. sorokiniana*) has no direct relationship with the bioaccessibility of total carotenoids. On the other hand, the lipid composition directly interferes with the individual bioaccessibility of the compounds, both carotenes, and xanthophylls. Specifically, this study showed a relationship between the concentration of medium-chain fatty acids and the extension of the bioaccessibility of 15-*cis*-violaxanthin, 9-*cis*-lutein, all-*trans*-zeaxanthin, and 9-*cis*-zeaxanthin in *S. bijuga*. At the same time, UFAs and PUFAs appear to promote the bioaccessibility of 9-*cis*-β-carotene. In contrast, *C. sorokiniana* stands out for the greater bioaccessibility of total carotenes and total xanthophylls, possibly associated with the concentrations of MUFAs of long-chain and SFAs, respectively. Also, the bioaccessibility of the compounds 15-*cis*-lutein, 13-*cis*-lutein, all-*trans*-lutein, and all-*trans*-β-carotene was superior in this species. Finally, these results support the use of *S. bijuga* and *C. sorokiniana* microalgae as attractive bioresources of bioaccessible carotenoids to formulate microalgae-based products of interest to the functional and nutraceutical market. Likewise, our study contributions with the scientific database of bioactive compounds from different microalgae species and further encourages the exploitation of these matrices as foods that aim to benefit health and human nutrition.

CRediT authorship contribution statement

Andrêssa S. Fernandes: Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing, Data curation, Formal analysis. **Tatiele C. Nascimento:** Investigation. **Pricila N. Pinheiro:** Investigation. **Raquel G. Vendruscolo:** Investigation, Data curation. **Roger Wagner:** Investigation, Data curation. **Veridiana V. de Rosso:** Conceptualization, Resources. **Eduardo Jacob-Lopes:** Data curation, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. **Leila Q. Zepka:** Conceptualization, Resources, Formal analysis, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CAPÍTULO 7

Carotenoids: a brief overview on its structure, biosynthesis, synthesis, and applications

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Chapter 1

Carotenoids: A Brief Overview on Its Structure, Biosynthesis, Synthesis, and Applications

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Additional information is available at the end of the chapter

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Abstract

Currently, 1178 natural carotenoids have been properly characterized and reported in the literature, which present a huge structural diversity and physicochemical properties. This number comprises a wide distribution of these biomolecules in approximately 700 source organisms including plants, bacteria, fungi, and algae. Besides having a wide applicability as natural dyes, some carotenoids such as β-carotene already have another well-established application such as provitamin A activity. However, due to the structural diversity of these molecules, there are still numerous biochemical and physiological functions to be associated with this class of compounds. Accordingly, these characteristics enable a wide applicability, what drives the global carotenoid market. Thus, with the primary objective of addressing aspects regarding to basic science and applied carotenoid technology, a comprehensive description of the biology, biochemistry, and chemistry of these compounds will be described in this chapter.

Keywords: carotenoid, structure, synthesis, biosynthesis, industrial application

1. Historical aspects of carotenoids

It is often stated that without carotenoids, life in an oxygenic atmosphere would not be possible, and we would not exist. Thereby, over millions of years, the living organism chloroplasts maintained collections of carotenoids to protect the intricate and delicate photosynthetic apparatus against destruction by photooxidation [1, 2].

According to Britton et al. [1], it can be considered that the study of carotenoids exceeds 200 years of history. Was Braconnot in 1817 carried out the first investigation in paprika? The following year, Aschoff isolated from the saffron, the "crocin," apocarotenoid which we now know as bixin. In 1823, Goebel's research on crab (*Brachyura*) suggested for the first time the presence of these isoprenoids in animals. Later, after investigations with carrots (*Daucus carota* L.), from which the term carotenoids derives, Wackenroder in 1831 isolated and described for the first time carotene with structure C40, now β-carotene. Shortly thereafter in 1837, Berzelius introduced the term xanthophyll due to its presence in autumn leaves. After, Kraus and Millardet in 1843 made the first investigation of carotenoids into cyanobacteria. Only 30 years later, lycopene was isolated for the first time from fruits of *Tamus communis* by Hartsen.

However, it was in the early twentieth century that a milestone occurred in the history of carotenoids. The Russian botanist Tswett in 1906 took the first step in the chromatographic technique of separation of these pigments, which together with the introduction of mass spectrometry (MS) in 1965 and high-performance liquid chromatography (HPLC) in 1971 provided a great advance in research [1, 3]. From this and with the advent of chromatographic methods and refinements in spectroscopy, the isolation and identification of carotenoids expanded greatly.

According to the last compilation, approximately 1178 naturally occurring carotenoids have been reported with 700 source organisms [4].

2. Structure, biosynthesis, synthesis, and application

In fact, it is well known that carotenoids are naturally synthesized by all photosynthetic organisms and nonphotosynthetic some, like bacteria, archaea, and fungi, which exhibit a complex carotenogenic metabolism [5].

They are classified according to the number of carbons that constitute their structure into carotenoids C30, C40, C45 and C50, but only the C40 carotenoids are those found in nature more abundantly and consequently, are those more approached in the literature. Moreover, C40 carotenoids are biosynthesized by organism eukaryotes, archaea, and bacteria, and their chemical structures are constituted by a diverse range of terminal groups [6].

Conversely, C30 and C50 carotenoids are biosynthesized by archaea and bacteria and only contain 6 and 10 C5 isoprenoid units, respectively. By contrast, only the bacteria are responsible for synthesizing C45 carotenoids composed of nine isoprenoid units [3, 6].

More than 100 naturally occurring apocarotenoids with diverse structural and functional properties have been reported. An apocarotenoid is a carotenoid in which the normal C40 structure has been shortened by the removal of fragments from one or both ends [7, 8]. Natural examples are bixin (C25 compound), the major pigment of the food colorant annatto, and crocetin (C20 compound), the main yellow coloring component of saffron [9, 10]. Lycopene, β-carotene, and zeaxanthin are the precursors of the main apocarotenoids described to date, which include bixin, crocetin, abscisic acid, strigolactone, and mycorradicin [10]. Vitamin A

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is also considered an example of apocarotenoid, because it is the product of the symmetrical oxidative cleavage of β-carotene [7].

The formation of these carotenoid derivatives occurs via enzymatic and nonenzymatic oxidative cleavage of carotenoids [11, 12]. Carotenoid cleavage dioxygenases (CCDs) catalyze carotenoid cleavage at specific double bonds, typical act by incorporating oxygen atoms into adjacent carbon atoms along the conjugated carotenoid backbone. Some CCD cleavage reactions require isomerization to form substrate isomers favorable for cleavage [13]. On the other hand, nonenzymatic apocarotenoid formation can occur via singlet oxygen attack, primarily on β-carotene [14]. In addition, peroxidases and lipoxygenases are also reported to form apocarotenoids [15].

Regardless of metabolic origin, apocarotenoids present important biological functions, such as plant-environment interactions such as the attraction of pollinators and the defense against pathogens and herbivores. Also, include volatile aromatic compounds that act as repellents, chemoattractants, growth stimulators, and inhibitors, as well as the phytohormones abscisic acid and strigolactones [16]. Moreover, these isoprenoids are associated with other processes positively affecting human health were identified as responsible for inhibiting the lipid peroxidation and prevention of cancer and other degenerative diseases [14, 17, 18].

Nonapocarotenoid carotenoid cleavage products include norcarotenoids, which lack one, two or three carbon atoms in the central hydrocarbons skeleton (C40) [3]. The primary determinant is the number of carbon atoms formally lost from the C40 carotenoid skeleton [5]. An example is the peridinin, is one of the most complex carotenoids, a C37-norcarotenoid possessing (Z)-γ-ylidenebutenolide and allene functions. In addition, it has five chiral centers, including an epoxide ring [19].

Another subclass is that of secocarotenoids, in which a bond between two adjacent carbon atoms except between C(I) and C(6) in a ring has been broken [3, 5]. The semi-β-carotenone ($C_{40}H_{56}O_6$) is an example identified as the product of β-carotene ($C_{40}H_{56}$) oxidation in permanganate solutions [20].

In addition, isoprenoid structures with more than 40 carbon atoms are also reported. The rare C50 carotenoids are synthesized by the addition of two dimethylallyl pyrophosphate (DMAPP) molecules to C(2) and C(2′) of the respective C40 carotenoid [21]. These compounds have been mainly isolated from *Halobacteria, Halococcus*, and *Pseudomonas strain* and *Actinomycetales* [22]. The first C50 carotenoid discovered, decaprenoxanthin, was isolated from *Flavobacterium dehydrogenans* [23].

As shown in **Figure 1**, structurally, carotenoids have different terminal groups, of which there are seven: ψ, β, γ, ε, ϕ, χ, and κ, which may constitute the ends of the principal polyene chain of the structure of these molecules. In general terms, the terminal rings β, γ, and ε rings are formed from ψ ends, whereas ϕ, χ, and κ rings are formed from β end groups [6, 24].

Lycopene is the common precursor structure for the synthesis of cyclic and bicyclic carotenoids. Cyclization of this molecule is a branching point in carotenoid biosynthesis, where β-, γ- and ε-end groups are formed by proton loss from alternative positions in the

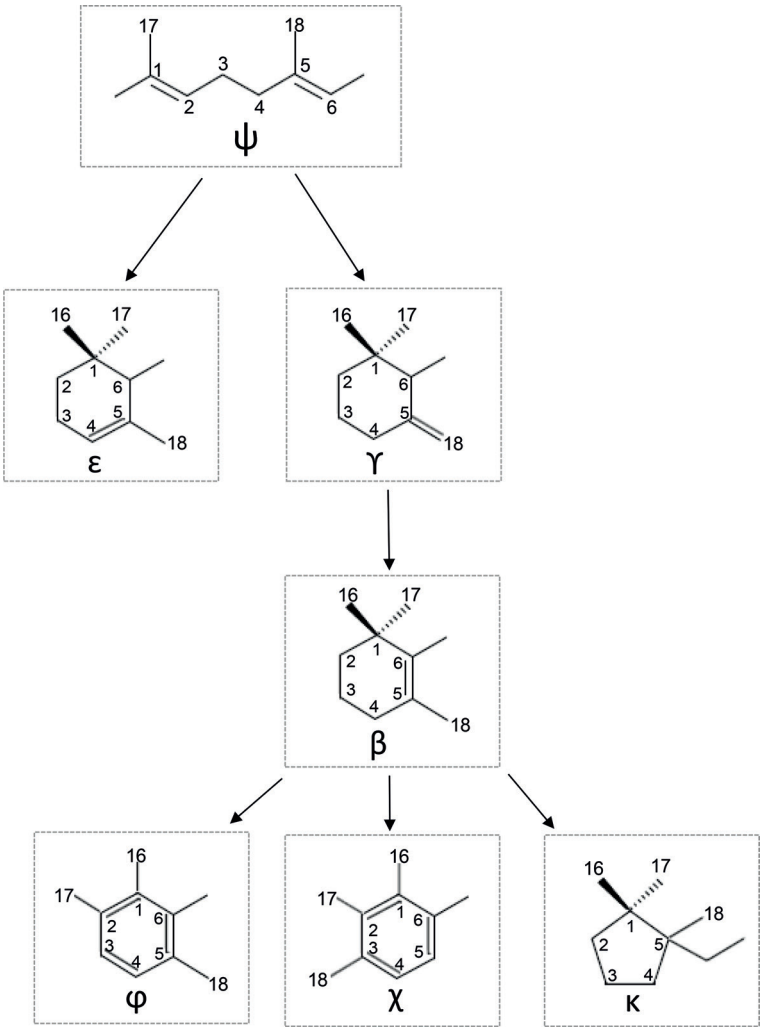
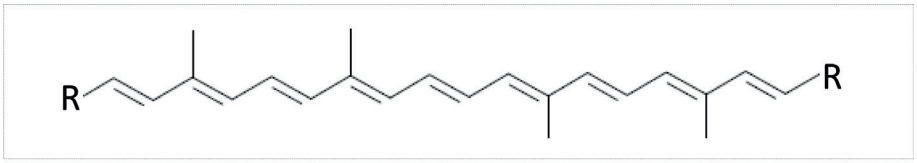


Figure 1. Different terminal rings of carotenoids.

same transient carbon ion intermediate. This cyclization process is catalyzed by the action of the enzymes lycopene cyclases [6]. The most prominent carotenoids with the ψ-, β-, γ- and ε-ends groups are licopene, β-carotene, γ,γ-carotene, and ε,ε-carotene, respectively (**Figure 2**).

The biosynthetic process of rings ϕ and χ (from ring β) occurs by the migration of methyl groups from C1 to C2 and dehydrogenation. In addition, migration of the methyl group from C5 to C3 occurs in the ring χ. Isorenieratene and renierapurpurin are representative carotenoids with the ϕ and χ end groups [24].

In addition, carotenoids with κ terminal group are biosynthesized from 3-hydroxy-5,6-epoxy-β rings found in violaxanthin and antheraxanthin. This terminal group is characteristic of capsorubin, capsanthin, and cryptocapsin, isolated from paprika (*Capsicum annuum*) [24].



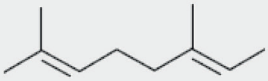
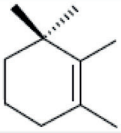
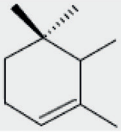
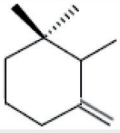
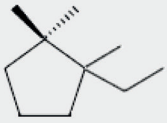
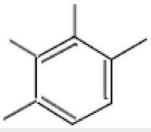
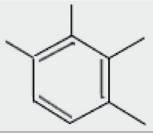
Type	End group (R)	Carotenoid
Acyclic	ψ 	Lycopene
Cyclohexene	β 	β-Carotene
Cyclohexene	ε 	ε,ε-Carotene
Methylenecyclohexane	γ 	γ,γ- Carotene
Cyclopentane	κ 	Capsorubin
Aryl	χ 	Renierapurpurin
Aryl	ϕ 	Isorenieratene

Figure 2. Carotenoids with different terminal groups.

However, even after decades of studies with carotenoids, the studies that report on the enzymatic and chemical mechanisms responsible for the constitution of the terminal moieties of these molecules are limited, despite its relevance to the full meaning of a biosynthesis of carotenoids [24].

Considering the chemical elements present in the structure of carotenoids, they can be classified into carotenes and xanthophylls as shown in **Figure 3**. The carotenes are compounds which contain only hydrocarbons in its structure (e.g., β-carotene and lycopene). On the other hand, the xanthophylls are oxygenated carotenoids, which contain different functional groups such as an epoxy (violaxanthin, neoxanthin, and fucoxanthin), hydroxy (lutein and zeaxanthin), keto (astaxanthin and canthaxanthin), and methoxy (spirilloxanthin) functional groups. In turn, xanthophylls are among the main carotenoids in photosynthetic tissues [3, 25].

According to these modifications, these functional groups containing oxygen affect the biological functions and the solubility of carotenoids, making xanthophylls more polar than carotenes, thus allowing their separation using many types of chromatographs [7].

The formation of functional groups of the xanthophylls occurs naturally by an enzymatic reaction. The hydroxy group formation occurs through ring-specific hydroxylation reactions and is normally catalyzed by carotene β-hydroxylase enzymes of the nonheme di-iron (BCH) type. In the case of β-carotene, two sequential hydroxylations of the β rings produce first β-cryptoxanthin and then zeaxanthin. The same enzymes can also participate in the synthesis of lutein [26, 27].

Zeaxanthin epoxidase (ZEP) introduces epoxy groups in the rings of zeaxanthin, resulting in the formation of violaxanthin, which undergoes the introduction of a double allenic bond in the molecule producing neoxanthin in one step catalyzed by neoxanthin synthase (NSY) [3, 26].

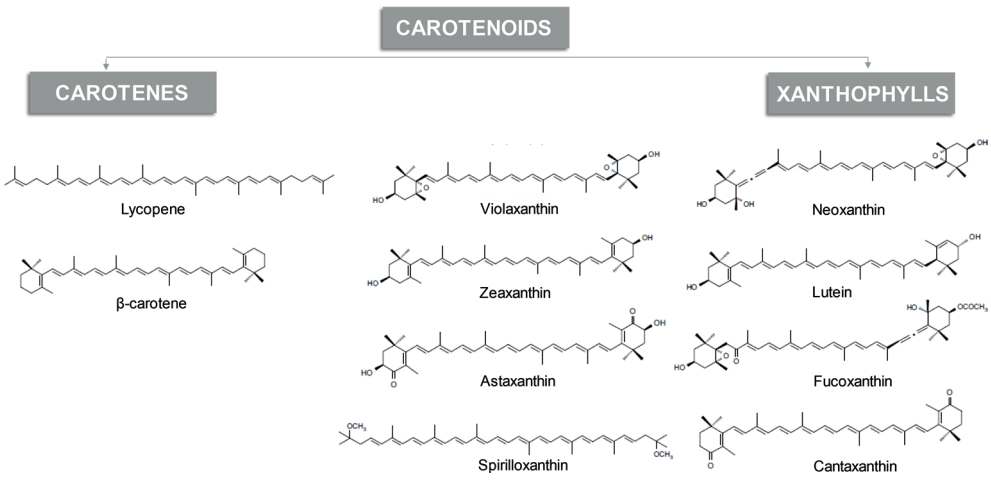


Figure 3. Examples of carotenes and xanthophylls.

Epoxy carotenoids comprise a large group of xanthophylls and are widely encountered in foods [28].

On the other hand, there are the ketocarotenoids that are produced by some algae and cyanobacteria, and are rare in plants [29]. These compounds have the ketone group inserted into the molecule by the enzyme beta-caroteneketolase. Ketocarotenoids, echinenone, and astaxanthin are the examples. Referring to astaxanthin, it still suffers action from beta-carotene hydrolase, since its structure is composed of two hydroxyls [30]. Ketocarotenoids are strong antioxidants that are chemically synthesized and used as dietary supplements and pigments in the aquaculture and nutraceutical industry [31].

Methoxy-carotenoids are previously synthesized from bacteria via enzymatic methoxylation [32], and the spirilloxanthin is an example [33]. Though the 3-methoxy-zeaxanthin has been reported in the human macula, your metabolic origins are unknown. It is suggested that methoxy xanthophyll originates from they do make metabolic changes to carotenoids acquired of the diets [32].

Besides with all the distinct conformations of carotenoids described above, these pigments may be associated to other molecules, including fatty acids (carotenoid esters), sugars (glycosylated carotenoids) or even proteins (carotenoproteins).

Carotenoids are naturally found in both free forms and esterified with fatty acids in many fruits, flowers, animals, microorganisms, and algae. For an ester to be formed, the carotenoid must have at least one hydroxyl group, since the ester linkage is formed when a carboxylic acid (fatty acid) reacts with an alcohol group (hydroxylated xanthophyll), with the elimination of a water molecule [34]. This process increases the lipophilicity of the molecule. During carotenoid biosynthesis to suggest that the xanthophyll esterification with fatty acids, it is most likely to be catalyzed by esterases or xanthophyll acyl transferases [35].

In many fruits, some plant organs, and tubers, the xanthophylls are typically found esterified with fatty acids [34, 36]. Similarly, there are carotenoids associated with sugar moieties, as it is the case of crocetin. On the other hand, some carotenoids can form complexes with proteins (carotenoproteins) that are water soluble and appear to stabilize carotenoids, as occurs with some crustaceans (astaxanthin-crustacyanine complex) [37].

Still referring to the patterns of chemical modifications, there are allene carotenoid and acetylene carotenoid structures (see **Figure 4**). In some important examples of naturally occurring carotenoids, the polyene chain is modified by the presence of one or two acetylenic (—C≡C—) or allenic (—C≡C—) groups, what is common in many marine organisms. The marine, allenic carotenoid peridinin from phytoplankton and fucoxanthin from macroalgae and phytoplankton are the carotenoids produced in largest quantity in nature [38]. Acetylenic carotenoids are synthesized de novo only in microalgae; crocoxanthin and diatoxanthin are examples of these structures [39, 40].

Given the presence of double bonds in carotenoid molecules, multiple geometrical (*cis*/*trans* or *Z*/*E*) isomers could be formed, which differ considerably in their chemical shape. However, most carotenoids found in nature are primarily in the more stable all-*trans* configurations; a small proportion of *cis* isomers is encountered [5, 12].

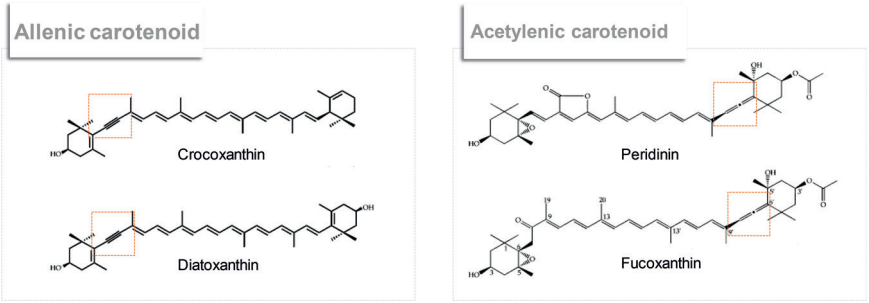


Figure 4. Carotenoids with acetylenic and allenic structure.

Theoretically, each carbon-carbon double bond in the polyene chain of carotenoids may exhibit E-Z isomerization. However, some double links like C-7,8, C-11,12, C-7′,8′, and C-11′,12′ are prevented from undergoing isomerization Z due to a steric hindrance between a hydrogen atom and a methyl group [28]. Thus, the Z-isomers of symmetrical β-carotene and zeaxanthin commonly found are the 9-*Z*-, 13-*Z*-, and the 15-*Z*-isomers, the formation of which has relatively little hindrance as it comes from two hydrogen atoms [28].

However, the C-5,6 double bond in the acyclic lycopene is unhindered, and 5-*Z*-lycopene is increasingly detected, along with the 9-*Z*-, 13-*Z*-, and the 15-*Z*-isomers [41].

Although the presence of *cis* isomers is recognized due to the isomerization caused by heat or light sources, there are some carotenoids that can occur naturally. Interestingly, they have different biological potency than their trans counterparts (e.g., lower pro vitamin A activity) [3]. Phytoene and phytofluene, which have the 15-*Z* configuration in most natural sources, are examples of carotenoids less thermodynamically stable [42]. Another example is bixin (see **Figure 5**), an apocarotenoid which occurs naturally in the *Z* form [43].

By contrast, the presence of one or more centers or axes of chirality in their molecules, some carotenoids can undergo geometric isomerization, which evidence the formation of optical (*R*/*S*) isomers. Zeaxanthin and astaxanthin are typical examples of carotenoids, in which this isomerization may occur.

Two optical isomers (3*R*-3′*R*)-zeaxanthin and (3*R*-3′*S*)-zeaxanthin, commonly referred to as *meso*-zeaxanthin and (3*S*, 3′*S*)-zeaxanthin are found in the macula lutea of the human retina [5]. Conversely, optical isomers different from astaxanthin, 3*S*, 3′*S*, 3*R*, 3′*S* (*meso*), and 3*R*, 3′*R*, in varying proportions are found in marine organisms (see **Figure 6**) [5].

At the biosynthetic level, more than 95% of all known carotenoids are formed using the same C5 building block, the isoprene (C5H8) unit, from which isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) are produced. Thus, in the route of the synthesis of isoprenoids, three molecules of IPP are sequentially added to DMAPP by prenyl transferase enzymes to yield geranylgeranyl-pyrophosphate (GGPP, C20). From this stage, the specific carotenoid biosynthetic pathway starts with condensation occurs of two molecules of GGPP, by phytoene synthase (PSY) to produce the first colorless carotenoid

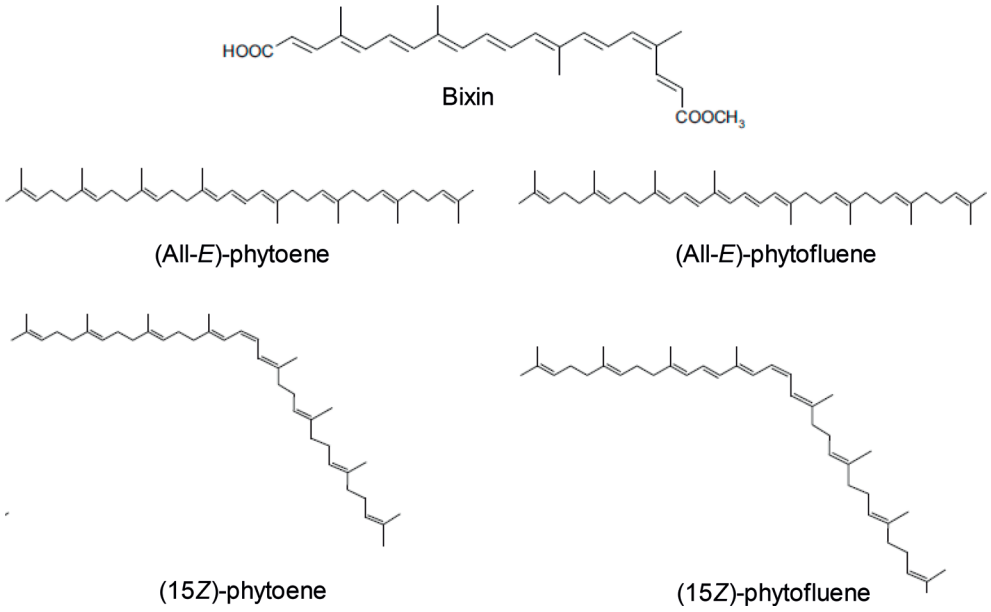


Figure 5. Geometrical isomers.

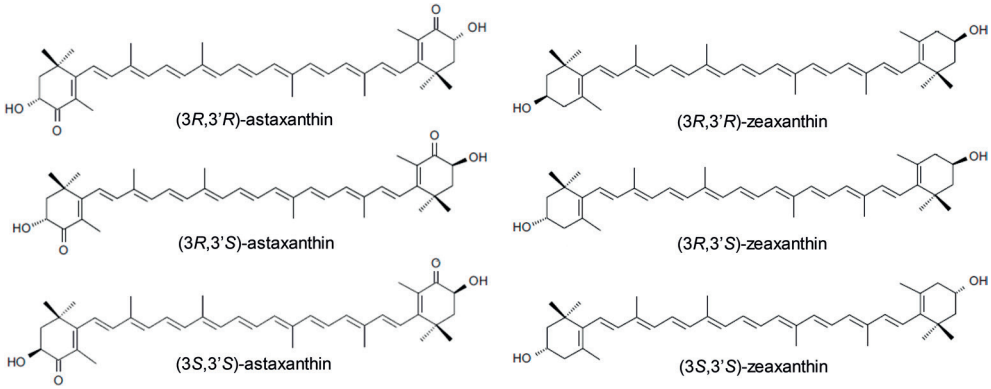


Figure 6. Optical isomers.

15-*cis*-phytoene (C40). These precursors are produced by two independent pathways in photosynthetic beings: the mevalonate (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway [3].

In contrast, approximately 5% of the biosynthesis of the other carotenoids (C30) occurs from farnesyl pyrophosphate (FPP), an intermediate precursor of geranylgeranyl-pyrophosphate (GGPP) or by the oxidative cleavage process of C40 carotenoids [21, 44].

Additionally, the carotenoids biosynthesized by different organisms are derived through a series of chemical and enzymatic modifications from the phytoene, such as reactions of desaturations, cyclizations, hydroxylations, glycosylation, oxidization, dehydrogenation, migration of double bonds, rearrangement, and epoxidations, as exemplified above [45]. These modifications are catalyzed by a number of enzymes which fall into few classes based on the type of transformation they catalyze such as geranylgeranyl pyrophosphate synthase, phytoene synthase, carotene desaturase, and lycopene cyclase. Modification of carotenes is further catalyzed by β-carotene ketolase and β-carotene hydrolase to generate various C40 carotenoids. Thus, all of these modifications contribute to yield a family of more than 1178 compounds widely distributed in nature [4, 46].

Of the total number of naturally occurring carotenoids, only eight are produced synthetically at industrial level. Between them C40 carotenoids: lycopene, β, β-carotene, (3R,3′R)-zeaxanthin, canthaxanthin, and astaxanthin; and three apocarotenoids: ß-apo-8′-carotenal, ethyl ß-apo-8′-carotenoate, and citranaxanthin [47]. For the chemical synthesis, several building concepts are possible. However, on industrial scale, only few of them have been applied successfully. The reactions of Grignard elaborated by Hoffman-La Roche in 1954 and reactions of Wittig developed by Badische Anilin- & Soda-Fabrik (BASF) in 1960, were the main reactions of syntheses employed on an industrial scale; however, Wittig reaction dominates the market currently [24, 48].

All chemically synthesized C40 carotenoids have symmetric structures, and this is explained by the fact that all structures have identical end groups at their ends. Due to these characteristics, they are efficiently produced by double Wittig condensation of a symmetrical C10-dialdehyde as the central C10-building block with two equivalents of an appropriate C15-phosphonium salt. In addition to these synthetic steps, these mixtures of isomers are thermally isomerized, in heptane or ethanol, for the full formation of all-trans/E configurations, since during the process, certain amounts of cis/Z stereoisomers are formed [24, 48]. Additionally, to use Grignard compounds, it is necessary to combine one diketone molecule and two methanol molecules, thereafter compound containing 40 carbon atoms is obtained [49].

Other methods of the synthesis of carotenoids include the hydroxylation of canthaxanthin, a C10 + C20 + C10 synthesis via dienolether condensation, and the isomerization of a lutein extracted from marigold to zeaxanthin and then oxidation to astaxanthin [48].

Apart from β, β-carotene, the other synthetically produced carotenoids are manufactured mostly by the companies Hoffmann-La Roche and BASF [47].

Furthermore, to synthetically traded carotenoids, a portion of these pigments are obtained from natural sources such as lutein (marigold flowers), β-carotene (*Dunaliella salina*), astaxanthin (*Haematococcus* spp.), and Capsorubin (*Capsicum annuum*) (see **Table** 1) [1, 50]. β-carotene followed by lutein and astaxanthin lead the carotenoid market, which is projected to reach USD 1.53 Billion until 2021 [51].

In more recent times, the major commercial use of carotenoids has been as food and feed additives for coloration. They have also found some use in cosmetics and pharmaceutical products, but the most rapidly growing market now is health supplements, which in turn, provides a stimulus growing from production [1].

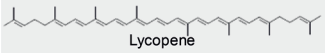
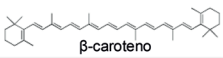
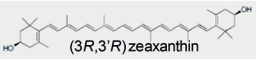
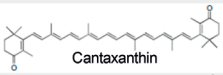
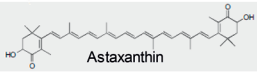
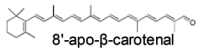
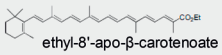
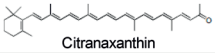
Carotenoid	Applications	Production Technology
 Lycopene	food coloration, nutritional supplement	chemical synthesis, biotechnology (Blakeslea trispora), isolation from tomato
 β-caroteno	food coloration (margarine, juice), nutritional supplement, feed additive (fertility, cattle)	chemical synthesis, biotechnology (*Dunaliella salina*)
 (3*R*,3'*R*) zeaxanthin	nutritional supplement (eye health)	chemical synthesis, isolation from natural sources
 Cantaxanthin	poultry (egg yolk and broiler pigmentation), aquaculture	chemical synthesis
 Astaxanthin	aquaculture (salmon pigmentation), dietary supplement, food coloration	chemical synthesis, biotechnology (*Haematococcus pluvialis*)
 8'-apo-β-carotenal	food coloration (cheese, dressings)	chemical synthesis
 ethyl-8'-apo-β-carotenoate	feed additive (egg yolk and broiler pigmentation)	chemical synthesis
 Citranaxanthin	feed additive (egg yolk and broiler pigmentation)	chemical synthesis

Table 1. Carotenoids industrial applications.

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CAPÍTULO 8

Chlorophylls as Food Additives

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Chapter 16
Chlorophylls as Food Additives

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Abstract There is a growing consensus in the world food industry that aims at replacing synthetic ingredients by natural ingredients. In this respect, the industrial relevance of microalgae as sources of a broad spectrum of bioproducts and as promising feedstocks for natural additives production systems is constantly increasing. Among the numerous chemical specialties present in the constitution of the biomass of these microorganisms, chlorophylls molecules, and their derivatives are emerging compounds in research and development to achieve greater commercial exploitation. Although there is a broad application of chlorophylls molecules, its strong green colour is gaining importance as food colouring. Consequently, microalgae as production systems for obtaining natural chlorophylls and derived compounds, they are highly sustainable sources, consisting of a series of unique, including chlorophylls *c*, *d,* and *f* with remarkable biological properties and relevant technological characteristics. In this sense, the present chapter describes the characteristic structures of chlorophylls and their derivatives, distribution, including aspects related the biological properties of these compounds. Finally, it presents a comprehensive overview of its participation in the food industry and the current legal regulations of different countries for its application in foodstuffs.

Keywords Chlorophyll compounds · Microalgae · Green colourant · Food colouring

16.1 Introduction

In recent decades, the changes in dietary habits and the modification of nutritional demands promoted to considerable alterations in food formulation, directing the market trends, and consequently the industrial interest for foods with the addition of natural additives in replacement and/or reduction of artificial additives (Martins et al. 2019).

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The natural colourants of food are estimated as the largest segment in the food colouring market, representing more than 80% of the total revenue of this sector. Thus, the global food colours market was set at USD 1.79 billion in 2016, with revenue growth estimated at USD 2.97 billion by 2025 (Grand View Research 2019).

In this context, to meet these requirements, the global microalgae market has emerged as a consistent alternative to gain market share in the food additive segment (Molino et al. 2018). Currently, the largest field of application of microalgae is in the food sector, where proteins, carotenoids, phycobiliproteins, and fatty acids are the products that stand out at the level of commercialization (Khan et al. 2018; Jacob-Lopes et al. 2019). However, recently, a growing proportion of promising microalgae research focuses on the production of pigments, including chlorophylls class, since these bioactive compounds are present in these microorganisms in abundance and with wide structural diversity (Begum et al. 2016; Solymosi and Mysliwa-Kurdziel 2016; Fernandes et al. 2017; Dufossé 2018).

Chlorophylls are fundamental molecules of life and probably the most important and widely distributed of all natural pigments, responsible for conferring several green hues (Sigurdson et al. 2017). Constitute a diversified group of molecules, with structures similar to each other, with five species characterized as chlorophyll *a*, *b*, *c*, *d,* and *f* (Scheer 2013; Roca et al. 2016). Conversely, one of the most significant chemical aspects of the structure of chlorophylls is their degree of instability, being susceptible to several transformations in their chemical structure in the presence of enzymes, acids, oxygen, light, and heat, giving rise to several derivative compounds. Accordingly, may present modifications in their oxidation state or degree of saturation of the aromatic system of the macrocyclic, associated with different transition metals (such as copper and zinc complexes replacing the central magnesium), epimerization in the isocyclic rings, as well the changes in the side chain substituents of these structures (Senge et al. 2014; Roca et al. 2016). Thus, are formed their respective breakdown metabolites like pheophytins, chlorophyllides, chlorophyllins, and pheophorbides, which have to present some small differences in their chemistry stability, their absorption spectra, and consequently in their tonality and bioactive potential (Pérez-Gálvez et al. 2017).

Although chlorophylls and their compounds derivative have a broad wide range of technological applications, such as in the monitoring of agricultural production and primary productivity of the oceans and use as nutraceutical and pharmaceutical area, the main economic attribute of these molecules today is their application as the food colourant. Accordingly, these molecules, deepen, or renew the food colour if it has been lost in the course of technological processing (Martins et al. 2019; Vieira et al. 2019).

Hence, your application as food colourant it is not only related to its colour attribute but their potential of the health benefits associated with their unique individual photochemical properties (Solymosi and Mysliwa-Kurdziel 2016; Pérez-Gálvez et al. 2017). Epidemiological, in cell, animal, and human intervention studies (Ferruzzi and Blaskelee 2007; Abd-Elhakim et al. 2018; Vieira et al. 2018) are in increasing constant development in order to broaden the knowledge about potential

health benefits for humans, such as antioxidant, antimutagenic, antigenotoxic, and anti-inflammatory activities (Pérez-Gálvez et al. 2017).

Consequently, the use of these molecules as an additive of colour in food is strictly regulated in the United States, the European Union, and many other countries in the world, such as China, India, Canada, Australia, New Zealand, Brazil, and Japan. Currently, commercially available chlorophyll compounds consist of structures basically related to chlorophylls *a* and *b* (Rodriguez-Amaya 2019), being emerging the exploration of no explored structures as species *c*, *d,* and *f* and their derivatives as alternatives to natural green dyes. Additionally, as a colour stabilization strategy, microalgae biomass appears to be a potential alternative as a functional ingredient with green colouring properties as secondary effect.

Thus, the purpose of this chapter is to provide a comprehensive overview of the application of chlorophylls and their compounds derived in foods, with emphasis on microalgae as potential sources for obtaining these compounds. Moreover, it is revised chemical structure of chlorophylls, distribution, biological properties of these compounds, including regulatory milestones in force in different countries for your application.

16.2 Structure and Distribution Chlorophylls

Natural chlorophylls are the most abundant pigments in nature, occurring more than 100 different structures, found in oxygenic photosynthetic organisms such as higher plants, microalgae, algae, and some bacteria (Lanfer-Marquez and Borrmann 2009; Pérez-Gálvez et al. 2017).

The structure of chlorophylls is tetrapyrrole with a system of coplanar conjugated double bonds that forms the chromophore with delocalization of electron density in π orbitals, formed from one magnesium atom as the central metal and a phytyl chain ($C_{20}H_{40}$) esterifying the propionic acid at $C17_3$, gives chlorophyll a hydrophobic character, as shown in Fig. 16.1 (Yilmaz and Gökmen 2016).

Chlorophylls *a*, *b,* and *c* were reported in the nineteenth century (Govindjee 2004), chlorophyll *d* was identified more than 70 years after the other chlorophylls (Manning and Strain 1943), and chlorophyll *f* was reported in 67 years after (Chen et al. 2010). However, the major chlorophylls in food additives are chlorophyll *a* and chlorophyll *b* (Viera et al. 2019).

Chlorophyll *a* (Fig. 16.2a, b) are different at position C_7, where chlorophyll *a* is composed of methyl group and chlorophyll *b* is composed of formyl group. The chlorophylls *d* and f are similar to chlorophyll *a*, where chlorophyll *d* (Fig. 16.2f) exhibits a formyl group at C_3 meanwhile chlorophyll *f* (Fig. 16.2g) contains a formyl group at C_2 (Chen and Blankenship 2011).

The members of the chlorophyll *c* family differ from other Chls in that they are porphyrins (i.e. they have a fully unsaturated tetrapyrrole macrocycle), which usually are non-esterified with phytol at $C17^3$. Since 1990, the number of members of the chlorophyll *c* family increased from seven (c_1, c_2, c_3, $c_{CS\text{-}170}$, c_2-like pigment

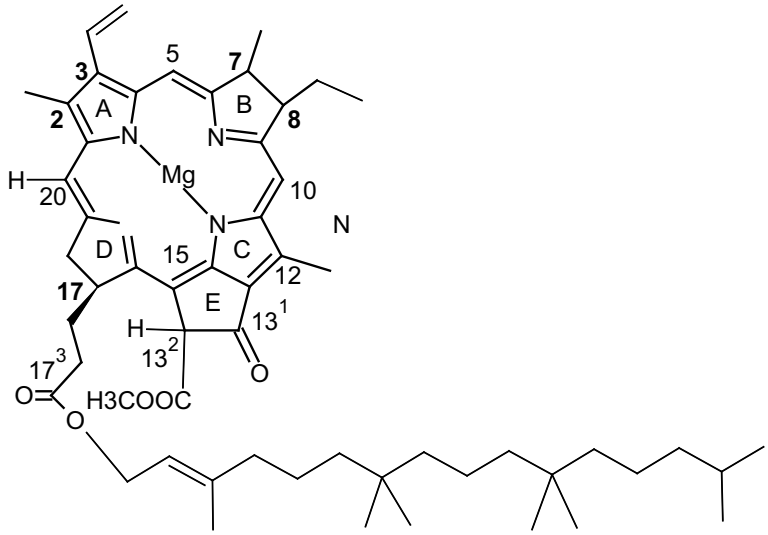


Fig. 16.1 Structure of chlorophyll *a* (a) including the IUPAC-IUB carbon-atom and rings numbering system

from Pavlova gyrans, [DV]-Pchlorophyllides, and a non-polar chlorophyll *c*-like pigment) to eleven compounds (Zapata et al. 2006). However, the most reported in the literature are the Chl c_1 (Fig. 16.2c) carries a characteristic ethyl group at C8, Chl c_2 a vinyl group at C8 (Fig. 16.2d), and Chl c_3 show of a methoxycarbonyl group at C7 (Fig. 16.2e) (Zapata et al. 2006).

Independently of the chlorophyll series (*a*, *b, c*, *d,* or *f*) are relatively unstable, the degradation of these compounds is a combination of enzymatic activity and chemical changes, converting as expected, chlorophylls into pheophytins, chlorophyllides, and pheophorbide (Lanfer-Marquez and Sinnecker 2008; Pérez-Gálvez et al. 2018).

The pheophytinization reaction is the substitution of the central magnesium atom of the tetrapyrrole by two hydrogen atoms. When this reaction starts from chlorophylls, it generates pheophytins; when starting from chlorophyllides, it generates pheophorbides. The de-esterification reaction is the hydrolysis of the phytol chain, chlorophyllides are produced if the reaction starts with chlorophyll, and the process yields pheophorbides if the dephytylation starts from pheophytin (Schwartz and Lorenzo 1990; Roca et al. 2016; Pérez-Gálvez et al. 2017).

In nature, higher plants contain only chlorophylls *a* and *b* and their respective catabolites like pheophytins, chlorophyllides, and pheophorbides. In addition to chlorophylls *a* and *b*, and chlorophylls *c*, *d,* and *f* show a wide occurring in aquatic photosynthetic organisms (Table 16.1), the currently known distribution patterns of chlorophylls pigments in bioresources are shown in Fig. 16.3 (Hendry 2000; Lanfer-Marquez and Borrmann 2009).

About the polar chlorophylls *c*, an overview of Chl *c* shows a major distribution in microalgae such as diatoms and brown algae. The Chl c_2 was considered the universal

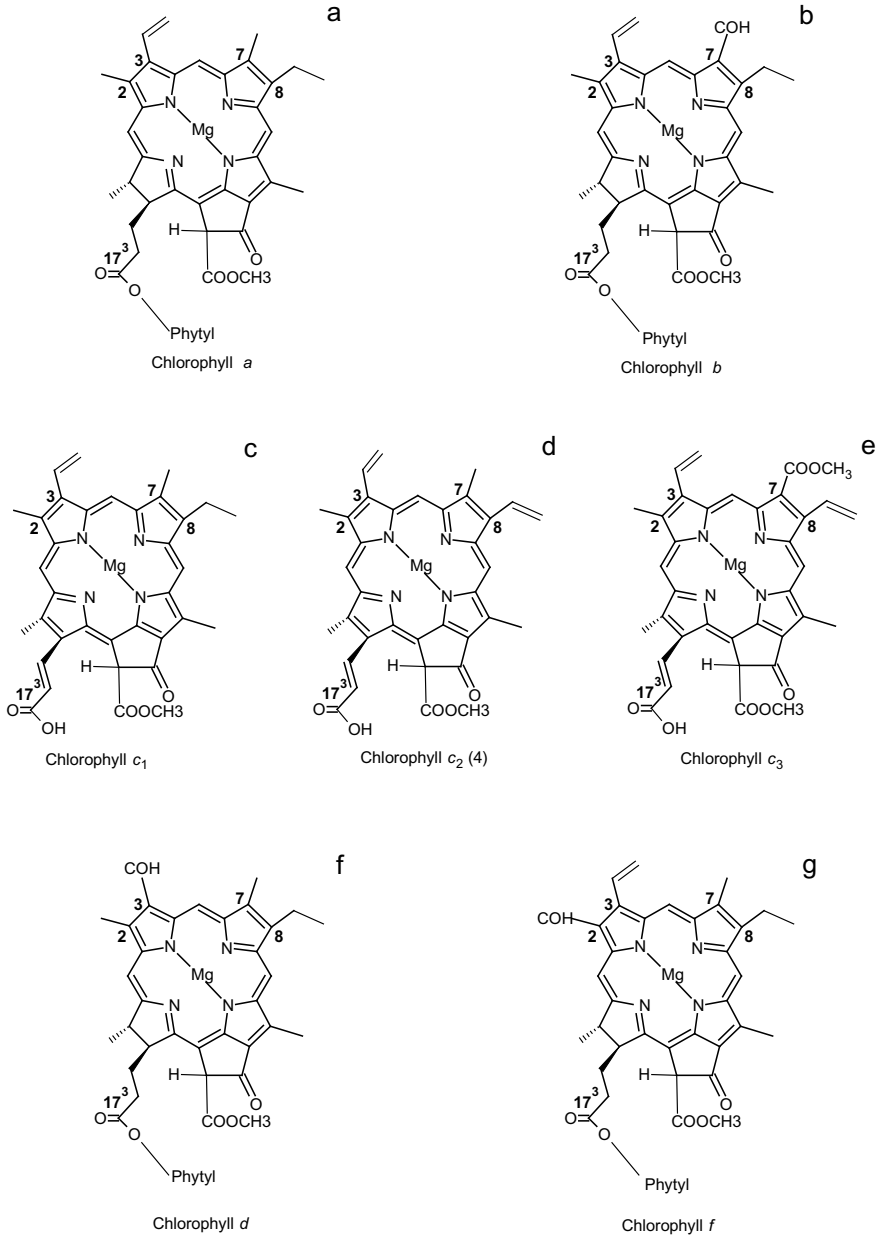


Fig. 16.2 Structures of chlorophylls *a* (**a**), *b* (**b**), c_1 (**c**), c_2 (**d**), c_3 (**e**), *d* (**f**), and *f* (**g**) including the IUPAC-IUB carbon-atom numbering system

Table 16.1 Possible phylum, classes, and species

Phylum	Macroalgae	Microalgae	Classes	Species
Rhodophyta	+	+	8	7,250
Chlorophyta	+	+	12	6,626
Charophyta	+	–	6	4,782
Dinophyta	–	+	5	3,560
Haptophyta	–	+	3	759
Euglenophyta	–	+	8	1,493
Glaucophyta	–	+	1	25
Cryptophyta	–	+	2	219
Ochrophyta	+	+	16	4,140
Cyanophyta	–	+	1	4,663
Total			67	33,511

According to Guiry and Guiry (2019): www.algaebase.org

component, since only one algae were found with Chl c_1, usually, Chl c_3 replaced Chl c_1, but the three pigments Chls c_1, c_2, and c_3 were found together in the species of haptophytes and one diatom (Stauber and Jeffrey 1988; Zapata et al. 2006).

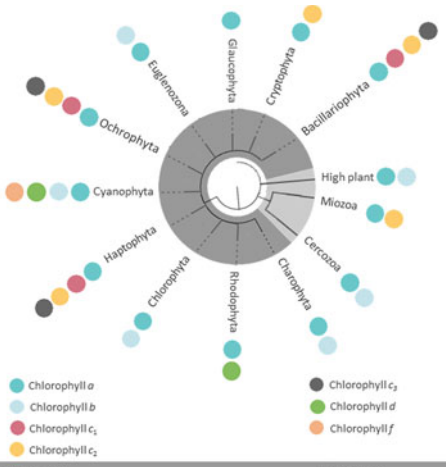
The main source of chlorophyll *d* is cyanobacterium *Acaryochloris marina*, that contains around 95% of chlorophyll *d* and only traces of chlorophyll *a*, and recently new chlorophyll *f*, was discovered in a filamentous cyanobacterium, *Halomicronema hongdechloris*, however, the proportion of Chl *f* is only 10–15% (Chen and Blankenship 2011; Zapata et al. 2006; Chen et al. 2010; Airs et al. 2014; Zepka et al. 2019).

The structural configuration of a molecule determines its mode of chemical action. In this way, it is important to know the chemical structure of chlorophylls to relate to their biological activities (Pérez-Gálvez et al. 2018).

16.3 Chlorophylls as Food Additive

With the increasing trend of the use of natural food additives, chlorophylls, and their derived compounds obtained from biological sources, is a reality that which has been gaining prominence and becoming popular in at present, as alternatives to the usual artificial chemical additives (Gárcia et al. 2017). These emerging changes are fundamentally justified about undesired and potential toxicological effects associated with artificial additives, responsible for allergic reactions, hyperactivity, and even bad taste (Oplatowska-Stachowiak and Elliott 2015).

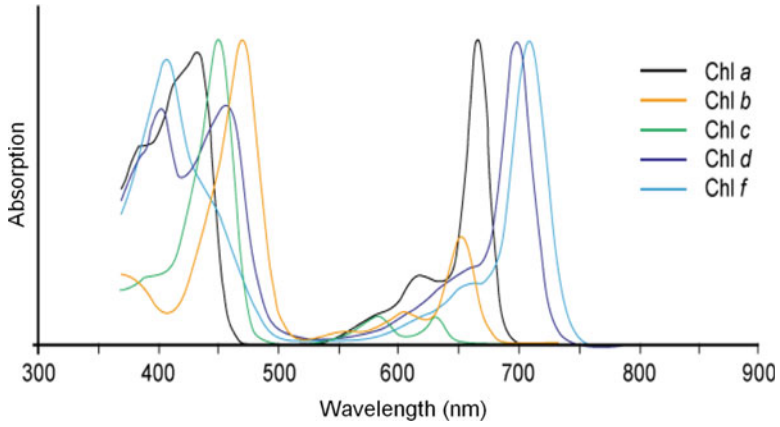
According to the *Codex Alimentarius* (FAO/WHO 2019) food additives are any substance not typically consumed as a food by itself and not normally used as an ingredient in the food but is intentionally added in the manufacture, preparation,



Representative species	Reference
Cyanophyta	
Acaryochloris marina	Miyashita, 1996; Miyashita et al., 1997; Chen et al., 2010
Rhodophyta	
Erythrophyllum delesserioides	Manning and Strain, 1943
Jania rubens	Chen et al., 2017
Chlorophyta	
Ulva spp.	Chen et al., 2017
Dunaliella Salina	El-Baz et al., 2017
Charophyta	
Chara aspera C.L.Willdenow	Blindow et al., 2003
Chara canescens	Kuster et al., 2005
Haptophyta	
Prymnesium parvum	Zapata et al., 2006
Euglenozona	
Euglena gracilis	Tolivia et al., 2013; Koziol et al., 2007
Eutreptiella gymnastica	Bjørnland, 1981
Glaucophyta	
Glaucocystis nostochinearum	Champman, 1966
Cyanophora paradoxa	Champman, 1966
Cryptophyta	
Rhodomonas báltica	Zapata et al., 2000
Ochrophyta	
Pulvinaria sp.	Zapata et al., 2006
Bacillariophyta	
Navicula jeffreyi	Zapata et al., 2006
Miozoa	
Alexandrium minutum	Zapata et al., 2000
Alexandrium tamarense	Leong and Taguchi, 2004
Cercozoa	
Bigelowiella natans	Koziol et al., 2007
Chlorarachnion reptans	Hibberd and Norris, 1984
High plant	
Lactuca sativa	Agüero et al., 2007
Brassica oleracea L. var. acephala	Kopsell et al., 2004

Fig. 16.3 Distribution of chlorophylls types in bioresources

processing, treatment, packing, packaging, transport, and holding of the food, to perform a technological function (including sensorial). In agreement with the use in the industry food, the additives are classified into 25 classes, which include about 230 different compounds. Among the different classes of food additives, chlorophylls, and their derivative compounds (chlorophyllins) are classified and are widely used as colour additives (Carocho et al. 2015; Martins et al. 2019). Thus, these molecules, may give, deepen, or renew the food colour if it has been lost in the course of technological processing, being responsible for various bluish-greenish hues (Fig. 16.4) (Zapata et al. 2011; Pareek et al. 2017; Sawicki et al. 2019).



Pigment	λ máx (nm)[a]	Colour descriptor
Chl *a*	665	blue-green
Chl *b*	652	brilliant green
Chl c_1	445	yellow-green
Chl c_2	449	yellow-green
Chl c_3	445	yellow-green
Chl *d*	696	brilliant/forest green
Chl *f*	706	emerald green

a: 100%methanol

Fig. 16.4 Chlorophyll structures and their absorption spectra

However, due to outstanding beneficial biological effects of these molecules in addition to their potential action dyes, these compounds can be used as additives in functional or nutraceuticals foods, aiming at promoting health (Humphrey 2004; Christaki et al. 2015; Janiszewska-Turak et al. 2016; Kang et al. 2018; Martins et al. 2019).

Predominantly, liposoluble chlorophyll *a* and *b* as well as their pheophytins directly extracted from natural sources are widely used in the food industry as a basis for obtaining green dyes (Rodriguez-Amaya 2019; Vieira et al. 2019). At the same time, it is known that these compounds have an inherent instability and want greater care as the physical and chemical changes during the processing (Delgado-Vargas and Paredes-López 2002; Lanfer-Marquez and Borrmann 2009). There are two key structural characteristics of chlorophyll pigments responsible for their properties, especially as it relates to their solubility and colour (Wrolstad and Culver 2012). More intense changes in the colour of chlorophylls are closely linked to the pH of the medium or enzymatic activity and can be observed when the central Mg^{2+} ion is replaced. On the other hand, the solubility of chlorophyll molecules is totally dependent on the phytol isoprenoid alcohol, since this when present in the structures gives confer the characteristic molecules more nonpolar (Galaffu et al. 2015; Pareek et al. 2017).

Consequently, to expand the applicability and reach successful application of natural chlorophylls extracted for commercial applications, some modifications are induced out in their structures and derivative compounds are formed as a strategy to increase their chemical stability and colour potential (Fig. 16.5) (Solymosi and Mysliwa-Kurdziel 2016).

Among these modifications, the copper complexes (Cu-chlorophyll), replacing the central magnesium, are frequently marketed as the green dye for the food industry, because it maintains the bright green colour and chemical stability of the molecules formed. These compounds are basically obtained by the addition of copper sulfate in acidic medium soon after extracting chlorophyll extracts (Vieira et al. 2019). More rarely, divalent cations such as Fe^{2+} and Zn^{2+} can be also used to replace the central Mg^{2+} ion during food processing (Canjura et al. 1999; Kang et al. 2018).

As well as, modifications in the hydrophobicity of the pigment molecule conferred by its long hydrophobic phytol chain and by a fifth ring (cyclopentanone) in the macrocycle are carried out starts with the alkaline hydrolization (saponification), and subsequent neutralization by K and/or Na salts, or enzymatic action. With this, are obtained chlorophyll derivatives with greater solubility in water and with similar spectral properties to those of chlorophyll, called chlorophyllins by scientists and the food colour industry. For this purpose, the term chlorophyllins refer to a complex mixture of various chlorine compounds (mainly chlorin e_6 and rhodin g_7), chlorophyllides, and pheophorbides, however its composition is not clearly defined because it is highly variable (Pérez-Gálvez et al. 2017).

Moreover, both processes of chemical transformations mentioned above (replacement of central magnesium by copper, and phytyl group removal) can be induced simultaneously in order to obtain Cu-Chlorophyllin. Several purification steps are necessary to remove interferents (Lanfer-Marquez and Sinnecker 2008). However,

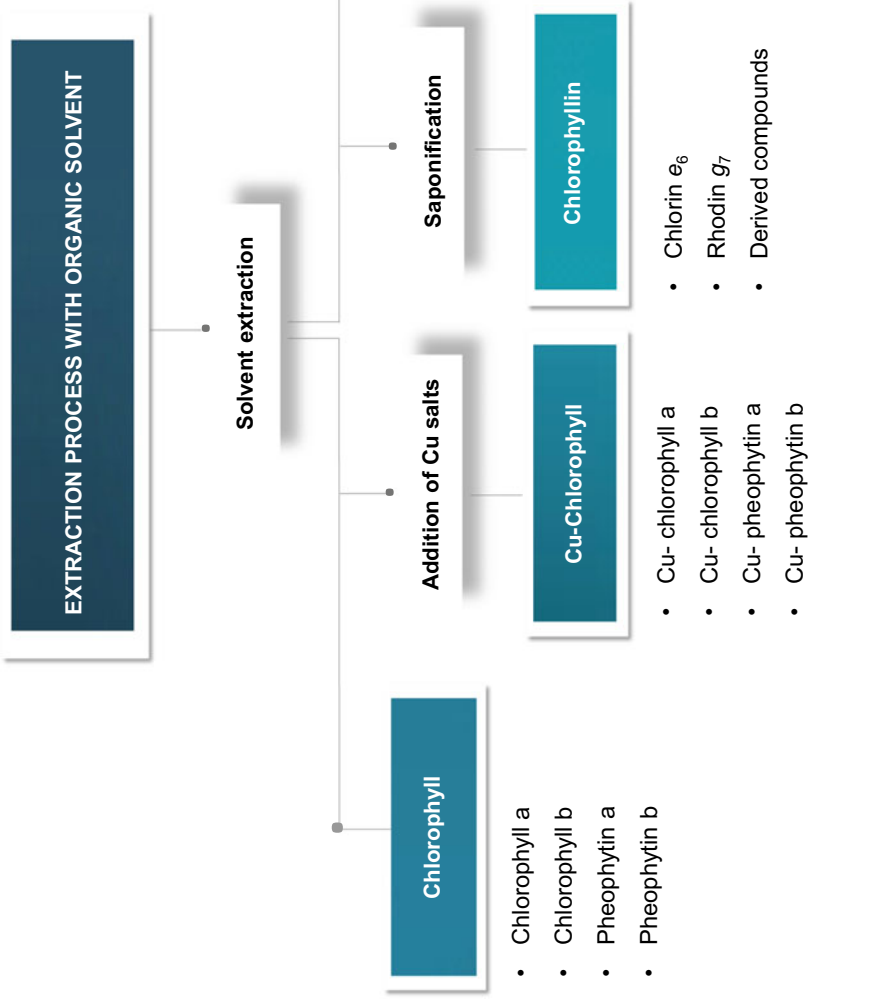


Fig. 16.5 Simplified process for obtaining chlorophylls

as with chlorophyllin synthesis, these chemical changes result in the formation of a complex mixture of various chlorine compounds including the most commonly found copper isochlorin e_4, copper chlorins e_4 (derived from chlorophyll *a*), and e_6 (derived from chlorophyll *b*), among other molecules (Mortensen and Geppel 2007; Tumolo and Lanfer-Marquez 2012).

Indeed, these chlorophyll derivatives (specifically, Cu-chlorophyll, complexes chlorophyllins, and Cu-chlorophyllin) previously mentioned not only exhibit outstanding stability to the pigment but also cause expression of more desirable green colours. Which justifies the fact that they, together with extracts of natural chlorophyll, are the approved worldwide green colourants since they present greater chemical stability to pH change, temperature, and prone to light, heat, and oxygen disintegration. In addition to these important characteristics, as a result of changes in their polarity, these formed compounds can be applied in liposoluble and hydrosoluble matrices (Shahid and Mohammad 2013; Sigurdson et al. 2017).

Although chemical methods are widely utilized to increase the stability against oxidation and aqueous solubility of green natural dyes, alternative techniques have been reported. In parallel, physical methods such as microencapsulation are currently under development (Raei et al. 2017; Ozkan et al. 2019; Kang et al. 2019). However, each type of encapsulating agent has its own specific advantages and drawbacks for encapsulation, protection as well as cost, ease of use, the biocompatibility among other aggravating factors (Ozkan et al. 2019). Recently, sodium caseinate (NaCas), a commercially available casein-rich ingredient, has been reported as a natural the chlorophyll *a* and *b* colour stabilizer the same time as increasing the solubility of these compounds in aqueous dispersion (He et al. 2019).

Regardless of these aspects, among the five different chlorophylls that exist, it is well known that green food dyes from natural sources are relatively limited to chlorophyll *a* and *b* species (Solymosi and Mysliwa-Kurdziel 2016). This is reflected by the use of higher plants as the primary source of obtaining these pigments (Wrolstad and Culver 2012). At the same time, some food industries have already explored the use of green dyes obtained from microalgae as well as other pigments widely marketed from these microorganisms (phycobilins from *Spirulina*; β-carotene from *Dunaliella*; astaxanthin from *Haematococc*us) (D'Alessandro and Filho 2016; Matos 2017). However, these extracts are obtained from microalgae that present species mostly of chlorophyll *a* and *b*, which equals, at the level of chemical constitution, the extracts obtained from higher plants.

Independent of species of chlorophylls commercially consolidated other chlorophylls (chlorophyll *c, d,* and *f*) and their derivative compounds, present exclusively in microalgae, can be implemented as alternative sources for green natural pigments considering the chemical stability of these compounds and their colour potential. Due to the structural chemical characteristics, chlorophyll *c* species, for example, indicated in the literature to be more stable and presents greater solubility in polar matrices than chlorophylls *a* and *b* (Lanfer-Marquez and Sinnecker 2008; Zapata et al. 2011, Chen et al. 2018). Considering these aspects, a greater number of molecules can be obtained to compose the green dyes class.

Although the chemical instability of these compounds is the major limitation for the use of chlorophyll as food additives, factors related to obtaining these compounds also restrict their application. In this way, sources that present high productivity, low levels of contaminants, together with simplified extraction methods are the main parameters to leverage the obtainment of natural green dyes and their possible applications (Mulders et al. 2014; Martins et al. 2016).

In addition, as an alternative to chlorophyll compounds used as food additives, the microalgae biomass is being applied as a food ingredient whose secondary effect is the green colouration. In this sense, research developed over the last years presented excellent results regarding stable green staining in all formulations, as evidenced by the high content of chlorophyll present in these microorganisms. Some examples are presented in Table 16.2.

To overcome the limitations of green dyes application, the purpose of these studies, mentioned above, is to find potential alternative sources for obtaining chlorophylls, targeting compounds that have the green colour intensity and chemical stability higher than those currently marketed. Consequently, new sources of natural chlorophylls or modified chlorophylls extracts are supported by regulations covering food additives to be marketed, which must be strictly followed.

16.4 Biological Properties

Physicochemical properties are the first resource for assuming and confirming the potential activities of a compound to be studied. Structurally, chlorophyll molecules and their derivatives are a group of compounds with different chemical structures (as can be seen in Sect. 16.2), which contemplate distinct physicochemical properties. These compounds have a significant function in the photosynthetic system, whom this versatility involves specific molecular arrangements and diversified chemical exchanges of the functional groups, serving as descriptors of the compound demonstrating its mode of action and biological activity, reaching a result that may or may not be beneficial (Agostiano et al. 2002).

Regardless of the presence of the central metal ion and the phytol chain, chemical influences and interactions also occur through other structural residues, such as the peripheral substituents and the isocyclic ring that directly influence the chromophore/absorption and the differentiated chemical and in biological behaviour (Henderson et al. 1997; Fiedor et al. 2003).

Table 16.2 Microalgae-based products that reached colouring green natural. Considering these aspects, natural and commercial chlorophylls, such as copper and sodium chlorophyllins, are widely investigated above biologically beneficial health activities, where some of these benefits are present in the healing process, antioxidant, anti-inflammatory, antimutagenic, and antimicrobial properties, as shown in Table 16.3 (Edwards 1954; Ferruzzi et al. 2002).

Accordingly, the human organism is exposed to various compounds that can damage important biological molecules such as DNA, proteins, carbohydrates, and

Table 16.2 Microalgae-based products that reached colouring green natural

Microalgae specie	Product	Application	Colouring characteristics	References
Chlorella vulgaris	Pea protein stabilized emulsions	Natural green dye and antioxidant	Stable colour for six weeks and oxidation reduction	(Gouveia et al. 2006)
Chlorella vulgaris	Butter cookies	Natural green dye and food supplement	Stable colour for three months	(Gouveia et al. 2007)
Isochrysis galbana	Biscuits	Natural green dye and food supplement	High stability of colour	(Gouveia et al. 2008)
Chlorella vulgaris and Spirulina maxima	Pasta	Increase of quality parameters and natural green dye	Stable colouring after cooking (103 °C)	(Fradique et al. 2010)
Arthrospira platensis	Pasta	Natural green dye, sensory quality, and nutraceutical potential	Stable colour during cooking	(Zouari et al. 2011)
Spirulina platensis	Pasta	Natural green dye and food supplement	Stable colour during cooking; appealing green tone	(Özyurt et al. 2015)
Spirulina platensis	Kiwifruit pastille	Natural green dye	Instability in colour over high temperatures (90 ºC)	(Pool et al. 2016)
Arthrospira platensis, Chlorella vulgaris, Tetraselmis suecica, and	Cookies	Natural green dye and food supplement	Stable colour (100ºC–120ºC)	(Batista et al. 2017)
Phaeodactylum tricornutum			and along conservation time (eight weeks); 6% microalgae seem to have more intense green colour.	
Isochrysis galbana and Nannochloropsis oculata	Chewing gum	Natural green dye	Stable and effective colour	(Palabiyik et al. 2018)

Table 16.3 Bioactive properties of different types of chlorophylls and their catabolites

Chlorophylls/catabolites	Bioactive properties	References
Chlorophyll *a*	Antimutagenic, chemopreventive, antioxidant, chemoprevention of aflatoxin B1 (AFB1), prevents DNA-mutagen intercalation, treatment of chronic ulcers and impetigo contagiosa	(Guskin 1940; Ferruzzi et al. 2001, 2002; Lanfer-Marquez et al. 2005; Castro et al. 2008; Osowski et al. 2010)
Pheophytin *a*	Antimutagenic, chemopreventive, anti-inflammatory, suppressor activity against genotoxins, inhibition of edema formation, inhibition of hepatitis C virus (HCV) proteins (NS3 protease)	(Okai and Higashi-Okai 1997; Ferruzzi et al. 2001, 2002; Wang et al. 2009; Islam et al. 2013)
Pheophorbide *a*	Antioxidant, chemopreventive, antigenotoxic, antimutagenic activity, ABCG2 inhibition, human pancreatic carcinoma in athymic mice	(Nakamura et al. 1996; Hajri et al. 1999; Ferruzzi et al. 2001; Robey et al. 2004; Ferruzzi et al. 2007)
Chlorophyll *b*	Antimutagenic, antioxidant, treatment of chronic ulcers, and impetigo contagiosa	(Ferruzzi et al. 2001; Islam et al. 2013)
Pheophytin *b*	Antimutagenic, anti-inflammatory, antioxidant, HuH-7 human hepatocellular carcinoma cells, inhibition of edema formation	(Higashi-Okai et al. 1998; Ferruzzi et al. 2001, 2002; Ferruzzi et al. 2007; Li et al. 2007)
Pheophorbide *b*	Antioxidante, HuH-7 human hepatocelular carcinoma cells	(Lanfer-Marquez et al. 2005; Li et al. 2007)

lipids. Indeed, most neurodegenerative diseases, cancer, aging is associated with the formation of excess reactive species, which results in oxidative stress and reduction of the body's antioxidant system (Ulbricht et al. 2014). Conversely, these diseases can be prevented, and the symptoms can be reduced with the use of these natural antioxidants. It is well described that chlorophyll pigments, especially pheophorbide *a* act as antioxidant compounds, acting positively under reactive oxygen species (ROS), hydrogen peroxide, free hydroxyl radicals, and lipid peroxidation, in vitro and in vivo (Nakamura et al. 1996; Kamat et al. 2000; Kumar et al. 2001; Lanfer-Marquez et al. 2005).

Among the chlorophyll compounds, Ferruzzi et al., (2002) report that chlorophyll *a* is one of the inhibitors of free radicals of greater action, this is due to the presence of Mg, Zn, Cu central ions in these structures analyzed, compared to chlorophyll *b* and pheophorbide *a* (phytol free structures), which present smaller antioxidant activity.

In addition, in vitro study of natural chlorophyll derivatives in protective action against lipid oxidation, Lanfer-Márquez et al. (2005) concluded that pheophorbide *b* and pheophytin *b*, had higher antioxidant activity than chlorine derivatives, but chlorophyllin had the highest antioxidant property all.

Likewise, pheophorbide is a potent antitumour component because of its high antioxidant mechanism. Pheophorbide *a*, present in *Capsosiphon fulvenscens*, has been shown high activity on reactive species of intracellular oxygen, inhibiting and preventing endothelial inflammation (Pangestuti and Kim 2011; Hong et al. 2016).

Al so, chlorophyllins, especially Na-Cu-chlorophyllins, are proven safe and their soluble derivatives can be applied in the wound healing process (Guskin 1940; Cady 1948; Horwitz 1951; Larato 1970; Solymosi et al. 2016). Are indicated as ideal for photodynamic therapy, photosensitization, aids in the selective destruction of tumour cells, which is obtained by activating the light phototoxicity performed by these agents, which transfer the excitation energy to produce reactive oxygen species and free radicals (Rapozzi et al. 2009; You et al. 2011).

Compounds such as Pheophorbide *a*, pheophorbide *b*, pheophytin *a* and *b*, chlorophyllin *f*, have been shown to decrease lipid peroxidation and decrease tumour cells inducing apoptosis by reactive oxygen species (ROS) (Li et al. 2007; Rapozzi et al. 2009; Du et al. 2014). Other functions of chlorophyll and its derivatives are related to the control of viral infections (Jenkins et al. 2016; Srivatsan et al. 2015; Wohellebe et al. 2011).

The chlorophyll derivatives were also tested as antimicrobials, where chlorophyllins supplemented with probiotics were ingested by dogs, where a reduction of coliforms and clostridium was evaluated, and suggesting chlorophyll additives in the canine feed to prevent bacterial infections without reducing the beneficial ones on lactic acid bacteria. These results demonstrated that chlorophyll and its derivatives are potentially beneficial in the application as supplementation and additive for functional foods (Strompfová et al. 2015).

Although the studies showed above exhibition the broad applicability of chlorophylls and their derivatives for medical and pharmaceutical purposes, the potential of these pigments with significant biological activities, including antioxidant activity, antimutagenic activity, and enzyme modulation, further stimulates the exploration of these compounds for food applications (Kephart 1955; Ferruzi and Blakeslee 2007).

Thus, interest in pursuing absorption results based on the notion that chlorophyll derivatives may be bioavailable is of paramount importance. These efforts serve to better understand the mechanism by which chlorophyll and derivatives act in the food diet, thus exploiting the real effects by absorbing foods supplemented with these biocompounds (Ferruzi and Blakeslee 2007).

Egner et al. (2000), concluded in a study with humans that Na-Cu-chlorophylls compounds are in fact absorbable and actually bioavailable in the body, but selectively. Chen and Roca (2018), found that edible algal chlorophylls *a* and *c* are better micellized than chlorophyll *b*, that is, they are more resistant to in vitro digestion of the food matrix. Also concluded that pheoforbide *c* is the most absorbable chlorophyll derivative by Caco-2 cells, which are typical research tools for nutrient absorption and demonstrate the accumulation of the compound studied by human intestinal cells and its basolateral flow by differentiated cells grown in inserts (Ferruzzi et al. 2002).

In this regard, it is assumed that chlorophylls have a high potential to be exploited as a food additive, same as today a large part of their market in food is related to

its application as a dye and not using the exploratory field of its biological activities (Humphrey 2004; Streit et al. 2005; Volp et al. 2009; Rocha and Reed 2014).

16.5 Production and World Market

The global economy is constantly changing, seeking more and more the exploitation of natural products and biorefinery products, becoming a biologically based economy, where the microalgae biomass enters with a high value and can be applied in several areas, mainly in the food industry (Barsanti & Gualtieri 2018).

Consumers' interest and the pursuit of healthy foods, including food products, functional ingredients, or dietary supplements that in some way benefit health, preventing, treating, or healing certain diseases have been increasing (Coopers 2009). Thus, the use of microalgae biomass and the exploration of derived metabolites such as chlorophylls becomes an innovative approach applied to the development of functional foods (Gouveia et al. 2008).

One of the main problems affecting the use of these pigments as food additives is the lack of globally reconciled legislation, normally the food additive regulations include the list of authorized standards, their specifications, as well as the conditions of use, but they are different rules for each country, making global production difficult (Viera et al. 2019). The main raw materials for obtaining chlorophyll are leafy foods and other green leaves and plants that produce large quantities and generally value as food or feed (Attokaran 2017).

According to Transparency Market Research (2017), the global market for chlorophylls is divided into the regions of North America, Latin America, Asia-Pacific, Western Europe, Eastern Europe, Japan, and the Middle East and Africa (MEA). Especially in China, is expected to expand, already in Latin America, it is robust due to the high demand for natural dyes. Global adoption of chlorophyll and chlorophyllin is lower in the MEA than in any other region of the world due to the low availability of raw materials in the region. In Fig. 16.6, we can observe some companies responsible for the production and commercialization of chlorophyll in the world.

Production of microalgae-based intracellular products on an industrial scale enters as a commercial opportunity that aims to cede market share in the part of bioactive molecules. Thus, in a short period of time, the microalgae biotechnology industry has grown significantly. Currently, the microalgae biomass market produces about 9000 t of dry matter/year and their global market value was US$ 2.8 billion in 2011. His market is regarded to be worth US$ 3.32 billion by 2022, with a compounded average growth rate (CAGR) growth of 6.7% (Spolaore et al. 2006; Enzing et al. 2014; García et al. 2017).

Estimates of the world production values of food colouring vary greatly with the increasing demand of this market. The global food colouring market was estimated at US$ 3.71 billion in 2017, growing at a CAGR of 5.7%, to reach US$ 5.12 billion by 2023. Estimates put the production of chlorophylls in Europe more specifically in the United Kingdom the value of approximately US$ 5.5 million per year, suggesting

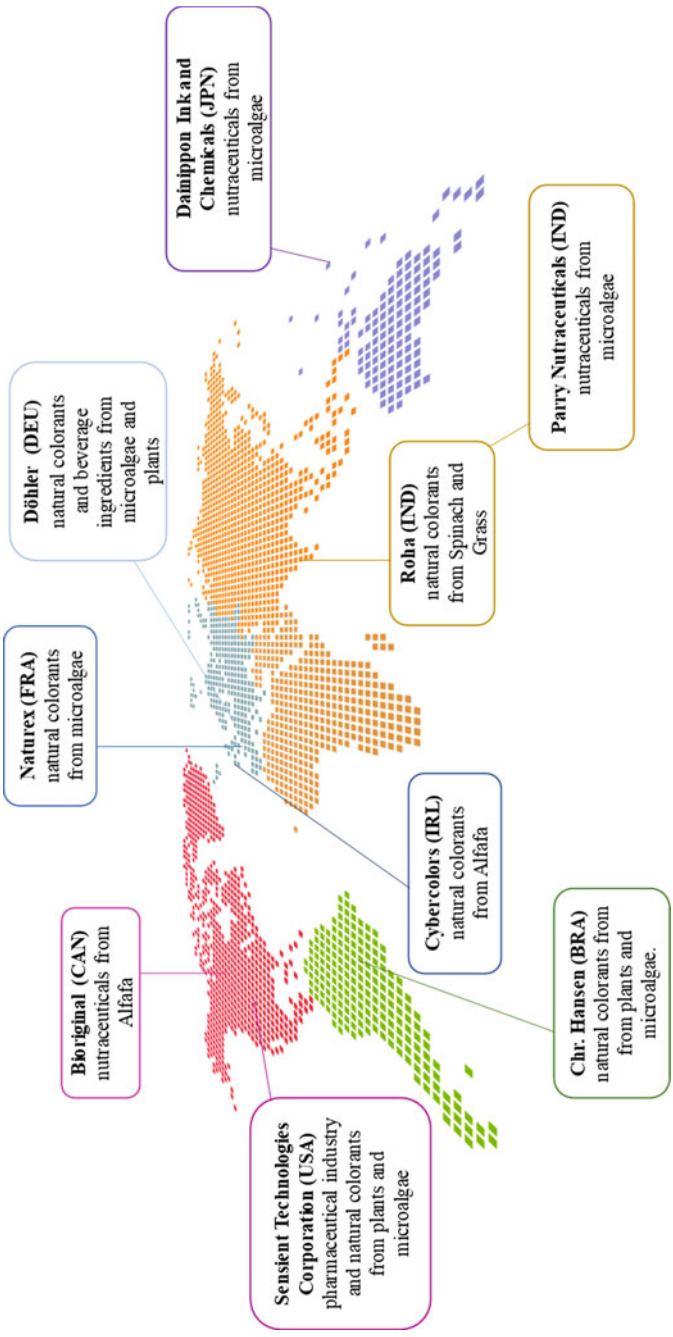


Fig. 16.6 Examples of chlorophyll and derivatives companies, applications, and sources used

that it could represent up to third of world production. In 2018, according to the Markets and Markets reports, Europe was a leader in the food colouring market, a market driven by the demand for awareness of safe products among consumers and by the inclination and pursuit of health-food natural dyes.

In order, to be considered suitable for commercial pigment production, strains must meet certain criteria such as ease of cultivation, low toxicity, nutritional value, and the presence of digestible cell walls to provide nutrients (Borowitzka 2013).

Among the species of microalgae considered to be commercially safe through the GRAS status (generally recognized as safe), which is granted by the Food and Drug Administration (FDA), are *Spirulina/Arthrospira* sp., *Chlorella* sp., *Porphyridium cruentum, Crypthecodinium cohnii*, *Haematococcus pluvialis*, *Phaeodactylum tricornutum*, *Dunaliella* sp., *Nannocloropsis* sp., *Nitzschia* sp., and *Schizochytrium* sp., some of its by-products are already authorized as ingredients by the European Union, Japan, USA, and Brazil, thus, they can be exploited as a source of natural chlorophyll (Jacob-Lopes et al. 2019).

Algae, silkworm droppings, alfalfa, pine needles, and various other types of grass are exploited for chlorophyll extraction. However, the concentration of this compound in plant sources varies, as they depend on climatic conditions and geographical position, varying the quantities over a wide range, showing one more advantage in the exploitation of these pigments in microalgae. Large-scale cultivation of microalgae has grown in recent decades, especially *Chlorella* and *Spirulina. Spirulina*'s fresh biomass is one of the largest sources of chlorophyll in nature, containing ten times more than spinach (Henrickson 1989; Lanfer-Marquez and Borrmann 2009).

Spirulina and *Chlorella* microalgae have significant benefits as potential sources of chlorophyll and are widely sold as supplements, *Spirulina* was sold at a price of US$ 20/kg and *Chlorella* at a price of US$ 44/kg in 2017, these two microalgal species gain prominence by producing the highest volumes of biomass. *Spirulina* production is widely distributed in Asia and the USA, while *Chlorella* is produced mostly in Asia. China is the country with the highest production of *Spirulina* in the world, with a total production of around 3500 t (dry weight) in 2009 (Lu et al. 2011; Voort et al. 2015; Oilgae 2016; Matos 2017).

In order to obtain chlorophyll in a given species, intracellular chlorophyll must first be extracted, and the traditional method that has been employed through extraction by organic solvents as shown in Fig. 16.7. It is a process in which it must be carried out quickly, without the presence of much light, thus avoiding the processes of photodegradation. Acetone, methanol, ethanol, hexane, chlorinated solvents, and others, are applied in the extraction (Lee 2012).

Filtration and centrifugation serve to remove the solids remaining in the solvent. After separation of the solvent, the extraction yield can reach around 20%, being chlorophylls and their derivatives.

Currently, natural pigments are the most strictly regulated additives for food applications worldwide, but there are still many obstacles to a worldwide adaptation of this type of natural product, interfering in outsourcing, import, export, and has negative economic effects. Adoption of different categories of additives is difficult, as laws

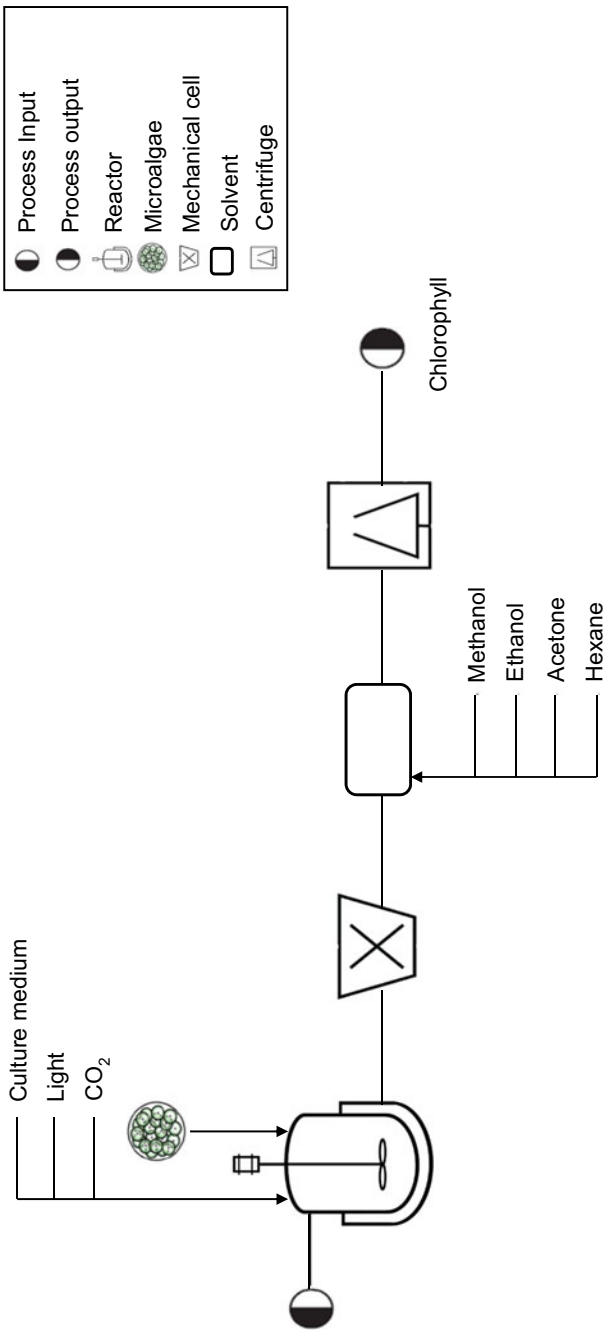


Fig. 16.7 Sketch of chlorophyll extraction process

have different limits and rules around the world, but there are also commonalities in these legislations, ensuring food safety, and ensuring procedures for risk assessment and management measures, which demonstrates that it is becoming easier to market these natural metabolites with their broad benefits to human health (Magnuson et al. 2013; Lehto et al. 2016).

16.6 Legal Aspects

The regulatory frameworks that control the application of chlorophylls as a colour additive, their specifications, as well as the conditions of use (limitations on specific foodstuff and maximum amounts) differ substantially in different regions of the world. Basically, five types of natural chlorophyll colours are authorized worldwide, although a few of them are produced by synthesis, but considered 'nature-identical' (described in Sect. 16.3). Anyhow, in each country they have a different terminology and are marketed according to their stability as to solubility and colour intensity (Table 16.4).

Based on the latest version of the commodities *Codex Alimentarius* published by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as the international body responsible for food additive safety assessment, are chlorophylls (INS 140i-140ii) and the copper chlorophylls (INS 141i-141ii) registered as dyes for application in food and beverages. This internationally recognized collection consists of indications of maximum level permissible limits of each colour additive in various food categories, guidelines, and other recommendations relating to this class of compounds (CODEX STAN 192-1995 2019).

Specifically, in European Union, the European Food and Safety Authority (EFSA) recognizes the use of four main colourants structurally related to the chlorophylls for use in foodstuff, denominate under the code E140i, E141i, E140ii, and E141ii (EFSA 2008). According to current legislation (Regulation (EC) No 1333/2008), E140i, also known as magnesium-based chlorophyll, comprises extracts obtained from plants, which are more liposoluble, since their composition consists mainly of species of chlorophyll *a*, chlorophyll *b,* and its derivative compounds. While E140ii (also known as sodium or potassium chlorophyllins) has characteristic hydrosoluble, which favours its use in water-soluble foods. In contrast, E141 products are composed of copper complexes of chlorophyll derivatives stable colouring (E141i or Cu-chlorophyll) or chlorophyllin (E141ii or Cu-chlorophyllin). As for your application, are authorized for use in a wide variety of foodstuffs with maximum limit at *quantum satis*, thus shall be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose (EFSA 2015a, b, c). Currently, as alternative sources to chlorophyll compounds, blends with *Spirulina* or their extract are considered a food ingredient with colouring properties in the European Union, can be added to all food categories without restrictions with the aim of conferring green colour (Scotter 2015; Oplatowska-Stachowiak and Elliott 2015; Lehto et al. 2017).

Table 16.4 Chlorophyll dyes for use in foodstuff authorized according to different regulations

Natural colourantes	Colouring	Description	EU	USA	India	China	Japan	Canada	Australia and New Zealand	Brazil
Liposoluble										
Chlorophyll	Olive green to dark green (depending on the content of coordinated Mg)	Waxy solid	E140i	–	6	–	177	E 140	140	140i
Cu-chlorophyll	Blue-green to dark green (depending on the source material)	Waxy solid	E141i	–	–	CNS 08.153	266	–	141	141i
Hydrosoluble										
Chlorophyllin	Green to blue/black powder	Powder	E140ii	–	–	–	116	–	–	140ii
Cu-chlorophyllin	Dark green to blue/black	Powder	E141ii	73.125	–	CNS 08.009	265	E 141	141	141ii
Na–Fe-chlorophyllin	Green	Powder	–	–	–	–	257	–	–	–

Consequently, the same chlorophyll colouring products approved in the EU are allowed in Japan, but are given denominations with numbers: 177 (Chlorophyll), 116 (Chlorophyllin), 266 (Cu-chlorophyll), and 265 (Cu-chlorophyllin). In addition to these, Japan also allows the use of a fifth natural green dye denominated Na–Fe-chlorophyllin (257), according to Ministry of Health and Welfare of Japan (MHLW). Simultaneously, the report details the target foods allowed for application these dyes, the limitation for use, maximum quantitative values, and other requirements (Japanese Food Additives Regulations 2019).

Table 16.4 Chlorophyll dyes for use in foodstuff authorized according to different regulations.While the Food and Drug Administration (FDA) in the United States authorizes the use of green natural colouring limited to Cu-chlorophyllin (classification 73.125) in mixtures of citrus-based dry beverage in an amount not exceeding 0.2 per cent in the dry mix, the Food Safety and Standards Authority of India only authorizes extracts of chlorophyll (US FDA 2019a; FSSAI 2019). Similarly as the EU, in the USA, *Spirulina* aqueous extract of the biomass of *Arthrospira platensis* (denomination 73, 530) is considered a colour additive. Thus, can be used at levels consistent with good manufacturing practice in colouring confections (including candy and chewing gum), frostings, ice cream and frozen desserts, dessert coatings and toppings, beverage mixes and powders, yoghurts, custards, puddings, cottage cheese, gelatin, breadcrumbs, and ready-to-eat cereals (excluding extruded cereals) (US FDA 2019b).

In China, current regulations are restricted only to chlorophyll copper complexes, probably due to the higher colour instability of these compounds. Thus, only the use of Cu-chlorophyll and Cu-chlorophyllin is regulated in line with China National Center for Food Safety Risk Assessment (CFSA), which designates maximum values and allowable food (CFSA 2019). In Australia and New Zealand, the FSANZ (Food Standards Australia and New Zealand) is the government body responsible for regulating the food additives that may be used as colouring agents including chlorophyll, chlorophyll copper complex, and chlorophyllin copper complex, sodium and potassium salts (FSANZ 2019). In the same way, Health Canada includes the chlorophyll and copper chlorophyllin in the list of colouring agents permitted for use in foodstuffs. The maximum level of use for chlorophyll is in accordance with good manufacturing practices and a limit of 300 ppm for sodium copper chlorophyllin is stipulated (Health Canada 2019).

In respect to Brazilian legislation, Resolution No. 44 of 1977 and its amendments published by Brazilian Health Regulatory Agency (Anvisa) classifies chlorophylls as a colour additive in foods according to the way they are obtained. Thus, in the class of the natural organic dyes of green colouration, authorizes the use only to extracts of chlorophyll. Chlorophyllin, copper chlorophyll, and copper chlorophyllin are authorized but classified as a synthetic organic dye identical to the natural one. Nonetheless, the legislation lacks food information, and maximum values allowed for application since it simply establishes the tolerated use of these compounds (Anvisa 2019). Therefore, these parameters are available in individual regulations for each foodstuff, and usually establish the use of *quantum satis,* similarly to the European Union.

Although these regulatory standards are reviewed continuously, there are still several unknowns to be clarified as to the composition of each chlorophyll-related compound. This is potentially correlated with chlorophyllins and their copper complex, compounds most used by the food industry worldwide, and lack characterization standardized. Likewise, to supply the market for natural green dyes, it is necessary news legislations in accordance to the emergence of alternatives sources to these compounds, strongly including the microalgae, microorganisms in constant exploitation for this purpose. Therefore, safety aspects should be considered for the implementation of microalgae as sources of green dyes.

16.7 Final Considerations

The market of the food colourants is evolving to more natural formulations. Thus, the development of natural alternatives for colourants formulations is necessary. Associated with this, it is indispensable to know the structural characterization of this family of pigments, its natural distribution, and information knowledge about their biological actions. The microalgae have received considerable attention in the last decades, indicating its potential as a renewable source of natural pigments. However, some hurdles must be overcome for these bioprocesses to be successful in the market, such as the selection of appropriate microalgae strains, the care for chlorophylls compounds instability, since natural green colourants are very labile and also it is not easy to reproduce green hues naturally. As well as new legislation will be necessary for the obtaining of natural chlorophylls from microalgal bioprocesses to be established in the natural pigment market. Finally, through considerable advances in research on natural pigments, in the near future, the consumer will face new green authorized food colourants.

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CAPÍTULO 9

CONCLUSÃO GERAL E REFERÊNCIAS BIBLIOGRÁFICAS

9.1 CONCLUSÃO GERAL

Foi possível caracterizar o perfil completo de carotenoides e compostos clorofilados em espécies distintas de microalgas através da metodologia analítica de cromatografia líquida de alta eficiência acoplada aos detectores de arranjo de diodos e de massas em tandem (HPLC-PDA-MS/MS). A partir dos resultados obtidos verifica-se que todas as espécies de microalgas analisadas (*Chlorella vulgaris*, *Chlorella sorokiniana*, *Scenedesmus obliquus*, *Scenedesmus bijuga*, and *Aphanothece microscopica Nägeli*) demonstraram alta capacidade de produção de pigmentos, quando comparada as fontes convencionais de produção destes compostos.

Diferenças no perfil de carotenoides e clorofilas foram detectadas de acordo com a espécie de microalga, reforçando que cada espécie possui uma composição bioquímica única. Dentro da classe das clorofilas, compostos ainda não identificados em microalgas foram encontrados, como epímeros de clorofilas e feofitinas, bem como hidroxiclorofilas e feoforbídeo. As microalgas também demonstraram a capacidade de sintetizar além de carotenos, como hidroxicarotenoides, epoxicarotenoides e cetocarotenoides. Além do mais, os resultados evidenciam que a via α-caroteno é a rota preferencial de síntese nas espécies em estudo. Logo, perfis de carotenoides e compostos clorofilados ainda não relatados na literatura para as espécies de microalgas avaliadas, foram relatos pela primeira vez.

A determinação da bioacessibilidade a partir dos ensaios de digestão *in vitro* mostrou que a natureza do produto possui influência significativa sobre as clorofilas de *Scenedesmus obliquus*. A bioacessibilidade destes compostos foi melhorada de acordo com o tipo de produto digerido (extrato isolado > biomassa úmida sonicada > biomassa seca inteira). Além disso, as clorofilas microalgais demostraram ser absorvidas pelas células Caco-2. Em relação as análises de carotenoides, os carotenos e xantofilas de *Chlorella sorokiniana* e *Scenedesmus bijuga* mostram bioacessibilidade significativas a partir da biomassa microalgal sonicada. No entanto, a bioacessibilidade de carotenos em microalgas é altamente dependente da espécie. Foi possível observar também que o conteúdo da fração lipídica da matriz influência a bioacessibilidade destes compostos.

Por fim, a caracterização destes compostos corrobora com estudos de investigação para descobrir fontes alternativas potenciais para obtenção de pigmentos naturais com propriedades bioativas, bem como contribuem significativamente para banco de dados sobre a caracterização da composição de biomassas microalgais. Além disso, os estudos de bioacessibilidade podem auxiliar na formulação e desenvolvimento futuro de alimentos e produtos funcionais e/ou nutracêuticos a partir de ingredientes à base de microalgas.

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