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Pricila Nass Pinheiro

ESTUDO DA BIOACESSIBILIDADE E BIODISPONIBILIDADE DE PIGMENTOS MICROALGAIS

Santa Maria, RS
2024

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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

Santa Maria, RS
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Pricila Nass Pinheiro

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Santa Maria, RS
2024

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Dedico este trabalho a minha vó Irma Nass (in memoriam), com todo o meu amor e gratidão.
Por tudo que fez por mim ao longo da vida.

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mas é o Senhor que determina os nossos passos.

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RESUMO

ESTUDO DA BIOACESSIBILIDADE E BIODISPONIBILIDADE DE PIGMENTOS MICROALGAIS

AUTORA: Pricila Nass Pinheiro
ORIENTADORA: Leila Queiroz Zepka

A biomassa microalgal é reconhecida como uma fonte promissora de compostos bioativos, destacando-se especialmente os carotenoides e clorofilas. Esses compostos possuem atributos interessantes para a manutenção da saúde. Porém, para assumirem o papel de protagonista nas funções biológicas, essas moléculas devem ser bioacessíveis para captação intestinal e posterior distribuição sistêmica. No entanto, há uma crescente preocupação em relação à influência da matriz microalgal na bioacessibilidade e biodisponibilidade dessas estruturas bioativas. Portanto, compreender as etapas que limitam a sua biodisponibilidade é obrigatório para a formulação de alimentos/ingredientes funcionais. Nesse sentido, foram empregadas diferentes estratégias envolvendo ingredientes/produtos de microalgas (biomassa microalgal liofilizada [WDB], pasta microalgal ultrassônica [WUP] e emulsão de pigmento lipossolúvel [LPE]) submetidas à digestão *in vitro*, com o objetivo de melhorar a bioacessibilidade e absorção, por células Caco-2, de carotenoides e clorofilas de duas espécies comerciais de microalgas, *Chlorella vulgaris* e *Arthrospira platensis*. Como resultado, a bioacessibilidade e absorção celular dos carotenoides e clorofilas mostrou-se aumentada de acordo com a matriz (LPE > WUP > WDB). Esses resultados geraram, 2 artigos e 1 capítulo de livro (publicados ou em processo de publicação), os quais foram organizados neste documento em capítulos. Os capítulos 1 e 2 correspondem aos artigos intitulados "*Bioaccessibility and bioavailability of bioactive compounds from microalgae*" (artigo de revisão em processo de publicação) e "*Guidance for formulating ingredients/products from Chlorella vulgaris and Arthrospira platensis considering carotenoid and chlorophyll bioaccessibility and cellular uptake*" (artigo de pesquisa/publicação concluída). Quanto ao Capítulo 3, ele está relacionado à contribuição em um livro e é intitulado "*Food Bioactive Compounds from Microalgae*" (capítulo de livro já publicado). Finalmente, é importante ressaltar que a avaliação da bioacessibilidade e biodisponibilidade revelou que a natureza do produto exerce uma influência substancial sobre os carotenoides e clorofilas presentes em microalgas, o que pode desempenhar um papel crucial na formulação de alimentos e produtos funcionais utilizando ingredientes derivados de microalgas.

Palavras-chave: Carotenoides. Clorofila. *Chlorella vulgaris. Arthrospira platensis*. Digestão *in vitro*. Células Caco-2. Produtos à base de microalgas.

ABSTRACT

STUDY OF THE BIOACCESSIBILITY AND BIOAVAILABILITY OF MICROALGAE PIGMENTS

AUTHOR: Pricila Nass Pinheiro
ADVISOR: Leila Queiroz Zepka

Microalgal biomass is acknowledged as a promising source of bioactive compounds, with carotenoids and chlorophylls being particularly highlighted. These compounds possess intriguing attributes for maintaining health. However, for them to assume a protagonist role in biological functions, they must be bioaccessible for intestinal uptake and subsequent systemic distribution. There is a growing concern regarding the influence of the microalgal matrix on the bioaccessibility and bioavailability of these bioactive structures. Therefore, comprehending the stages that limit their bioavailability is imperative for formulating functional foods/ingredients. To this end, various strategies involving microalgal ingredients/products (whole dry biomass [WDB], whole ultrasonicated paste [WUP], and liposoluble pigment emulsion [LPE]) were employed in *in vitro* digestion, aiming to enhance the bioaccessibility and absorption by Caco-2 cells of carotenoids and chlorophylls from two commercial species of microalgae, *Chlorella vulgaris* and *Arthrospira platensis*. Consequently, the bioaccessibility and cellular absorption of carotenoids and chlorophylls increased according to the matrix (LPE > WUP > WDB). These findings resulted in 2 articles and 1 book chapter (published or in the publication process), which have been structured into chapters in this document. Chapters 1 and 2 correspond to the articles titled "*Bioaccessibility and bioavailability of bioactive compounds from microalgae*" (article under review for publication) and "*Guidance for formulating ingredients/products from Chlorella vulgaris and Arthrospira platensis considering carotenoid and chlorophyll bioaccessibility and cellular uptake*" (research article/publication completed). Chapter 3 is associated with a contribution in a book and is titled "*Food Bioactive Compounds from Microalgae*" (book chapter already published). Finally, it is essential to emphasize that the evaluation of bioaccessibility and bioavailability revealed that the nature of the product exerts a substantial influence on the carotenoids and chlorophylls present in microalgae, which can play a crucial role in formulating foods and functional products using ingredients derived from microalgae. These results underscore the importance of considering the final product formulations to optimize the delivery and effectiveness of these bioactive compounds.

Palavras-chave: Carotenoids. Chlorophyll. *Chlorella vulgaris. Arthrospira platensis. In vitro* digestion. Caco-2 cells. Microalgae-based products.

APRESENTAÇÃO

Esta tese de doutorado está estruturada em seis itens principais, sendo os dois primeiros constituídos pela Introdução e Objetivos. Os demais itens estão organizados em forma de capítulos temáticos. Nesse contexto, o Capítulo 1 abrange a revisão bibliográfica dos tópicos que embasam esta pesquisa e está em processo de submissão. Nos Capítulos 2 e 3, são apresentados todos os resultados e discussões do doutorado, os quais são formatados em um artigo publicado e um capítulo de livro. Por fim, a Conclusão geral do trabalho e o item Referências apresentam apenas aquelas citadas na Introdução.

SUMÁRIO

1. INTRODUÇÃO

Compostos bioativos derivados de microalgas estão ganhando destaque como fontes altamente promissoras de insumos para a indústria de alimentos funcionais. À medida que as culturas comerciais de microalgas emergem como um mercado em expansão, a produção anual de biomassa seca é estimada em cerca de 19.000 toneladas, gerando um impacto econômico significativo de aproximadamente US $ 5,7 bilhões (HYNSTOVA et al., 2018; JACOB-LOPES et al., 2019; SHOW, 2022).

Entre elas, destacam-se a *Chlorella vulgaris* e a *Arthrospira platensis*, líderes mundiais no segmento de microalgas. Isso é apoiado fundamentalmente pelo fato de que a biomassa microalgal se tornou uma alternativa para a obtenção de pigmentos naturais, representando um modelo bem-sucedido em termos de viabilidade econômica. Com um valor de mercado global estimado em US $ 1,4 bilhões e uma taxa de crescimento anual projetada em 2,3%, esses pigmentos representam um setor em ascensão no mercado global (LIU et al., 2016; MARONEZE et al., 2019; RAZZAK, 2024).

O potencial desses pigmentos para a formulação de produtos alimentícios e funcionais tem sido amplamente explorado, impulsionando o desenvolvimento nessa área. De fato, carotenoides e clorofilas, são abundantes na biomassa microalgal. No entanto, mais pesquisas são necessárias para compreender profundamente o comportamento desses compostos no interior do corpo humano, uma vez que essas estruturas têm demonstrado atributos interessantes que podem ser associados à redução da incidência de doenças crônicas, como a diabetes tipo 2, doenças cardiometabólicas e alguns tipos de câncer (BERNAERTS et al., 2020; NASCIMENTO et al., 2021; FERNANDES et al. 2021).

Contudo, para assumirem algum protagonismo a nível biológico, os pigmentos naturais devem ser bioacessíveis para absorção intestinal e posterior distribuição sistêmica. Foi estabelecido que durante a digestão, os carotenoides e clorofilas seguem caminhos semelhantes, incluindo a liberação da matriz alimentar, interação com fluidos digestivos (gástrico e intestinal), e a inclusão em micelas lipídicas de sais biliares, facilitando o transporte através do enterócito e a subsequente distribuição para a circulação sistêmica, especialmente para os derivados lipofílicos.(FERRUZZI; FAILLA; SCHWARTZ, 2015; KOPEC; FAILLA, 2018; XAVIER; MERCADANTE, 2019).

A grande preocupação é o efeito da matriz microalgal na bioacessibilidade dos pigmentos naturais. Especificamente, em microalgas, a parede celular tem sido relatada como o principal fator limitante. Consequentemente, o processamento para a ruptura de células de microalgas por ultrassom ou obtenção de extrato de pigmentos lipofílicos pode potencializar a bioacessibilidade e subsequente biodisponibilidade desses compostos intracelulares (GRANADO-LORENCIO et al., 2009; GILLE et al. 2016; 2019; NASCIMENTO et al., 2021; FERNANDES et al. 2021).

Além disso, observou-se que a composição da matriz microalgal e as propriedades físico-químicas dos compostos pode desempenhar um papel importante na bioacessibilidade, podendo ter efeitos positivos ou negativos. Por sua vez, a presença de lipídios em conjunto com os sais biliares, são essenciais para formar as micelas que facilitam o transporte de compostos lipofílicos durante o processo de digestão. Por outro lado, acredita-se que as proteínas possam reduzir a solubilidade dos compostos bioativos, uma vez que têm a capacidade de se ligar a eles e precipitar. O efeito da fibra é controverso, variando de acordo com a fonte, composição química ou propriedades físico-químicas (TYSSANDIER et al., 2003; BOHN, 2018; KOPEC; FAILLA, 2018; XAVIER; MERCADANTE, 2019).

Associado a esses aspectos, faz-se necessária a busca constante pelo desenvolvimento de pesquisas para a otimização de ingredientes/produtos à base de microalgas. Asim estratégias adicionais, tais como protocolos de digestão, estão sendo utilizadas para orientar a formulação de novos sistemas de entrega e alimentos funcionais com melhor disponibilidade de carotenoides e clorofilas. Embora os modelos humanos sejam considerados mais precisos para estudar a bioacessibilidade, eles apresentam desvantagens, como alto custo, dificuldades técnicas, implicações éticas e complexidade na análise de múltiplas variáveis. Como alternativa, modelos *in vitro* foram desenvolvidos para simular a digestão fisiológica (FAILLA.; CHITCHUMROONCHOKCHAI, 2005; HOLST; WILLIAMSON, 2008; CARBONELL-CAPELLA et al., 2014; CARDOSO et al., 2015; KOPEC; FAILLA, 2018; GARRETT et al., 2000).

Por fim, a compreensão completa da digestão, absorção e metabolismo dos carotenoides e clorofilas pode desempenhar um papel crucial na formulação e no desenvolvimento de produtos bioativos à base de microalgas. Tal conhecimento torna-se essencial para superar os obstáculos inerentes à biodisponibilidade limitada desses compostos (GILLE et al. 2016; 2019; NASCIMENTO et al., 2021; FERNANDES et al. 2021).

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar diferentes matrizes de ingredientes e produtos derivados de microalgas, com o intuito de investigar sua bioacessibilidade e absorção por células Caco-2 de carotenoides e clorofilas.

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

Obter a biomassa microalgal de *Chlorella vulgaris* e *Arthrospira platensis* por cultivo fotossintético;

Determinar o perfil quantitativo e qualitativo de carotenoides e clorofilas de *Chlorella vulgaris* por HPLC-PDA-MS/MS;

Determinar o perfil quantitativo e qualitativo de carotenoides e clorofilas de *Arthrospira platensis* por HPLC-PDA-MS/MS;

Determinar a bioacessibilidade dos carotenoides e clorofilas de *Chlorella vulgaris* a partir da biomassa seca total, da pasta ultrassônica total e da emulsão de pigmento lipossolúvel;

Determinar a bioacessibilidade dos carotenoides e clorofilas de *Arthrospira platensis* a partir da biomassa seca total, da pasta ultrassônica total e da emulsão de pigmento lipossolúvel;

Determinar a absorção por Caco-2 células de carotenoides e clorofilas de *Chlorella vulgaris*;

Determinar a absorção por Caco-2 células de carotenoides e clorofilas de *Arthrospira platensis*.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

Bioaccessibility and bioavailability of microalgal carotenoids

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Bioaccessibility and bioavailability of microalgal carotenoids

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HIGHLIGHTS (MANDATORY)

- Microalgae offer potential as a source of bioactive carotenoids with diverse health benefits.
- Understanding the bioaccessibility and bioavailability of these compounds is crucial for their potential health effects.
- Various factors, including food matrix, processing, and individual physiology, affect the absorption and bioavailability of carotenoids.

Abstract: The large structural diversity of biomolecules synthesized from different microalgae metabolic pathways provides promising sources of bioactive molecules, such as carotenoids with specific structural features responsible for their bioactivity. Microalgae carotenoids have properties that result in biological functions beneficial to human health, which favors excellent potential for applications in the food, cosmetic, and pharmaceutical industries. Although current research focuses primarily on the structural characterization and physicochemical properties of carotenoids, studies on the human body's biological functions and possible metabolic actions are still poorly reported. To establish the real role of these compounds relating to their activities in human health, bioaccessibility and bioavailability assay should be considered. Thus, in this article, we emphasize microalgae as a rich source of carotenoids, addressing chemical structure, bioactivity,

metabolism, bioaccessibility and bioavailability, as well as general aspects related to strategies used to improve the absorption of microalgae carotenoids.

Keywords: Biocompounds; Microalgae; Bioavailability.

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INTRODUCTION

Microalgae are promising microorganisms recognized as a potential source of many metabolites with biological activities applied in the prevention, treatment and maintenance of health conditions [1, 2, 3]. Among these compounds, a significant part of this fraction of metabolites corresponds the carotenoids such as β-carotene, α-carotene, zeaxanthin, lutein, violaxanthin, echinenone, mixoxanthophyll and canthaxanthin [4, 5, 6].

These pigments have liposoluble properties and are present in addition to microalgae in other photosynthetic microorganisms, such as plants and some fungi, participate in the processes of light gathering necessary for photosynthesis, confer photo-protection and are widely recognized for their antioxidant activity [7]. However, some tetraterpenoid structures found in microalgae showed characteristics differentiated from those commonly found in higher plants.

Scientific evidence suggests that the bioactivity of the tetraisoprenoids goes beyond antioxidant activity, ranging from effects on the vision to antinflammatory and anticancer properties [8, 9, 10, 11, 12, 13, 14]. However, only the determination of the bioactivity of the carotenoids is insufficient to justify its application for health purposes because to mediate such activities, these compounds should be delivered to the target tissues after ingestion, and this verification is only possible through bioaccessibility and bioavailability tests [7]. According to [15] before concluding any potential health effect, is necessary to analyze whether the digestion process affects bioactive compounds and their stability, as this, in turn, will affect their bioavailability and their possible beneficial effects.

In general, the determination of bioaccessibility occurs by in vitro digestion procedures, simulating gastric and small intestinal digestion, sometimes followed by Caco-2 cells uptake. At the same time, bioavailability includes gastrointestinal digestion, absorption, metabolism, tissue distribution, and bioactivity [15].

Some obstacles make it difficult to integrate, thus making the bioaccessibility/bioavailability of these compounds reduced or even zero, because of these barriers, some techniques have been developed to increase the fraction available after the digestion step [16, 17, 18]. Additionally, according to [19, 20], differences in bioaccessibility values may vary depending on the natural form of the carotenoid (monoester, diester or free).

Based on the above, in this article, we emphasize microalgae as a rich source of carotenoids, addressing aspects of chemical structure, bioactivity, metabolism, bioaccessibility and bioavailability, as well as general aspects related to strategies used to improve the absorption of microalgae carotenoids.

Microalgae

Microalgae constitute a polyphyletic group of prokaryotic and eukaryotic organisms. The most abundant microalgae classes are *Cyanophyceae* (blue-green algae), *Chlorophyceae* (green algae), *Bacillariophyceae*

(including diatoms), and *Chrysophyceae* (including gold algae) [21]. Represent an unexplored source of compounds with biological activity. The chemical composition of the biomass includes proteins, carbohydrates, lipids, and pigments [1, 22].

Microalgae pigment production, unlike conventional crops, does not need to compete with food crops for agricultural land, there is also the possibility of these microorganisms being cultivated in wastewater [4, 5]. Thus, microalgae have been recognized as an excellent source of sustainable natural bioproducts applicable to food science, with an emphasis on carotenoids [1, 23]. Since, the photosynthetic efficiency of microalgae, that is, the conversion rate of solar energy into biomass production can reach 3%, whereas in plants it is only 0.2% [24, 25, 26].

In this line, the culture of microalgae in open tanks has been well developed and is still in use, but only a few species can be maintained in these systems, making production restricted. Tubular and flat plate photobioreactors allow the production of strains rich in high-value products, which in open systems is not possible [29].

Furthermore, the cultivation of microalgae in heterotrophic reactors becomes an attractive option for the production of carotenoids, since the wastewater use of food processing industries can serve as a culture medium, allowing integration into the biorefinery system [5, 30, 31].

Considering the application of biomass as a food supplement, knowing its safety is fundamental, in this sense the content of nucleic acids and the presence of toxin are considered essential parameters [2]. Nucleic acids can account for 4-6% of the dry biomass and are a source of purines that can increase uric acid in the body causing severe kidney damage [1, 32]. In addition, many species such as *Anabaena* sp., *Mycrocystis* sp., *Dynophysis* sp., and *Pseudo-nitzschia* are potential producers of toxins with a lethal impact on human health such as saxitoxins, brevetoxin, domoic acid, and ocadaic acid [1].

However, among the species of microalgae that are currently marketed, some already have the GRAS status (generally recognized as safe) granted by the Food and Drug Administration (FDA), including *Spirulina*, *Chlorella* sp., *Porphyridium cruentum*, and *Crypthecodinium cohnii* [3].

Also, 19 other species have their safety aspect classified as "no toxin known", including *Synechococcus* sp., *Tetraselmis* sp., *Chlamydomonas reinhardtii*, *Haematococcus pluvialis*, *Dunaliella* sp., *Chlorococcum* sp., *Scenedesmus*, *Desmodesmus* sp., *Parietochloris incis*, *Navicula* sp., *Nitzschia dissipata, Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Odontella aurita*, *Skeletonema* sp., *Monodus subterraneus*, *Nannochloropsis* sp., *Isochrysis* sp., *Pavlova* sp. [33].

Finally, in this state of safety (no toxins known) are included some of the main microalgae carotenoids currently marketed in the world, β-carotene (*D. salina*), and astaxanthin esters (*H. pluvialis*). The European Food Safety Authority (EFSA), the Food and Drug Administration (FDA), the Ministry of Health, Labor and Welfare of Japan (MHLW) and the Sanitary Surveillance (Anvisa) [3] already approved all these products as food ingredients.

Structural aspects of carotenoids

The total number of carotenoids found and 1,182, with 700 organisms of origin, among them is a variety of microalgae [34, 35]. These structures are lipophilic isoprenoids, with coloration usually yellow, orange, or red. The molecules play a vital role in protecting the photosynthetic mechanism, preventing photo-oxidation [36, 37].

Carotenoids participate as accessory pigments in the photosynthetic system of microorganisms, whose main function is the capture of light. The pigments are commonly divided into carotenes and xanthophylls. Carotenes are formed only by hydrocarbons (e.g. lycopene, β-carotene, and α-carotene) whereas xanthophylls contain oxygen atoms in their structure (e.g. lutein, zeaxanthin, β-cryptoxanthin, and violaxanthin). Among the xanthophylls, typical oxygen-containing substituents are the hydroxyl, epoxy, and aldehyde groups [4, 40].

The xanthophylls also have differentiated structures, such as allenic, acetylene, glycosylated, and ketocarotenoids (Figure 1). Allenic (C═C═C) is a type of structure that is present only in natural products found primarily in carotenoids, an example being fucoxanthin which may also have one side of the acetylated structure (-O-CO-CH3) and is present specifically in some divisions in algae. Acetylenic (C≡C) are found only in natural products and present only in algae (crocoxanthin). Glycosylated structures (myxoxanthophyll) are characteristic of some cyanobacteria because, during symbiosis between eukaryotic cells, carotenoids can be considerably restructured. Ketocarotenoids are produced exclusively by photosynthetic organisms (canthaxanthin and echinenone) [41, 42].

[Insert Figure 1]

Formation of xanthophyll requires specific hydroxylation reactions of the ring. In β-carotene, two sequential hydroxylations of β-rings first produce β-cryptoxanthin and then zeaxanthin. The enzyme zeaxanthin epoxidase (ZEP) introduces epoxy groups in the zeaxanthin rings, resulting in the formation of violaxanthin. The introduction of a double bond in the molecule of violaxanthin produces neoxanthin in one step catalyzed by neoxanthin synthase (NSY). Ketocarotenoids, which are produced by some algae and cyanobacteria and are rare in plants, have the ketone functional group inserted into the molecule by the enzyme β-carotene ketolase [42, 45].

Because they have double bonds in carotenoid molecules, multiple geometric isomers (*cis*/*trans* or *Z*/*E*) can be formed, which differ considerably in their chemical form. However, the vast majority of the carotenoids found in nature are in the all-*trans* (in algae mainly), that is, more stable; a small proportion of *cis* isomers is found [46].

Although the general classification includes most of the carotenoids found in nature, share the common C40 structure with different terminal groups, and is the most addressed in the literature other subclasses can be established by considering the number of carbons that constitute their structure in the carotenoids C30, C45 and C50 [43, 44].

Generally, the C30 and C50 carotenoids are synthesized by archaea, bacteria and cyanobacteria, contain a maximum of 10 C5 isoprenoid units, respectively. However, only bacteria are responsible for the synthesis of C45 carotenoids composed of nine isoprenoid units [35, 45].

All the structures mentioned above refer to the pigment in its isolated form, however the xanthophylls in their native form are most often esterified to fatty acids, as occurs in most mature fruits. The esterification occurs progressively during maturation, increasing the lipophilic character of the xanthophylls and facilitating their accumulation in the chromoplasts [49]. In addition, the esterified carotenoids appear to have greater stability [50].

In microalgae, the process of esterification of secondary carotenoids is the result of intense carotenogenesis, which activates this process and is not fully understood. This type of reaction occurs only in xanthophylls with at least one hydroxyl group, which reacts with the carboxyl group of the fatty acid, forming ester bonds [51]. [52], found in cyanobacterial esters of myristoyl-zeinoxanthin and myristoyl-β-cryptoxanthin, which are also found in various fruits.

From the point of view of the application in the food industry and nutritional value, the esters are more stable, they have a higher antioxidant capacity and, in fact, their bioavailability is higher in the organism than in the free form [53]. The esterification of microalgae carotenoids has been investigated [51]. Through metabolomic tools, [52] identified the 2'-linolenoyl-myxol ester compound, unique new identification of cyanobacteria, generating the possibility of innovations for the industry, making possible the use of microalgae as a source of carotenoids esterified.

Different carotenoid structures are found in the algae species studied, some are found only in certain divisions or classes of algae, presenting a different profile to each species [42, 55]. The composition also depends on the cellular structure. In cyanobacteria, prokaryotic, the composition is different from that present in chloroplasts in microalgae (eukaryotic), so during symbiosis between cyanobacteria and eukaryotic cells, carotenoids can be restructured [56].

In cyanobacteria majority of β-carotene, zeaxanthin, and have some specific structures of this class such as echinenone, mixoxanthophyll, and some have some additional structures, such as nostoxanthin and canthaxanthin [56]. In eukaryotic microalgae, β-carotene, zeaxanthin, lutein, neoxanthin, and violaxanthin are mainly found [57]. Carotenoids of some species are already commercialized and applied in industry, mainly in aquaculture and as a food supplement. In addition, due to the diverse biological activities of some of these compounds, new studies and applications are emerging, especially in the cosmetic and pharmaceutical sectors [3].

Lastly, bioactivity and concerns about food safety have increased the recent market for natural pigments, in particular for human consumption. However, many challenges still exist in processing, especially in the harvesting, extraction, and studies of in vivo biological activity [58].

Bioactivity

Carotenoids are bioactive compounds have properties that result in biological functions beneficial to human health, classified as phytochemicals capable of modulating metabolic processes essential to the health of the cells, due to their protective action on the cellular components against oxidative damage [59, 60, 61].

The property of exerting a specific bioactive action and all events at the metabolic level for this to occur is related to the concept of bioactivity of a compound. Among these events, the transport of the bioactive

compound, as it reaches the target tissue, interaction with biomolecules, biotransformation and metabolism that it may undergo, generation of biomarkers and the physiological response that causes are evaluated parameters. Broadly, bioactivity studies are based on what a bioactive compound can do (healthy properties or reduced risk of disease) [62, 63].

In particular, studies have reported that the biological properties of carotenoids go beyond antioxidant activity, including neuroprotective and anti-inflammatory effects, anti-cancer, cardiovascular protection, anti-obesity, photoprotective and immunomodulatory activities, and possibly protect against age-related diseases such as cataracts and macular degradation, multiple sclerosis [13, 64, 65, 66].

Continuously, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by human cellular metabolism. Moreover, they may also be produced by exogenous sources to the metabolism, e.g., radiation, tobacco smoke, and pesticides cause the release of reactive species [67, 68]. With this, concomitantly, a series of oxidative reactions occur at the metabolic level, damaging biologically important molecules like lipids, DNA, proteins, carbohydrate or lipid, which initiates further formation of ROS, e.g., hydroxyls radicals or reactive nonradical compounds like singlet oxygen and hydrogen peroxide (1O2, OH•, O2 •- ROO•, e H2O2) [64, 69]. As a defense strategy, carotenoids are potential antioxidant agents, which effectively neutralize the ROS [67, 70].

The antioxidant mechanism of carotenoids [CAR] with reactive species oxygens (ROS) can occur in at least three possible ways: (1) electron transfer system, (2) allylic hydrogen abstraction, and (3) adduct formation or addition [71, 72], represented in the following equations:

Electron transfer

$$[CAR] + ROO^{\bullet} \rightarrow [CAR]^{\bullet +} + ROO \quad (1)$$

Hydrogen abstraction

$$[CAR] + ROO^{\bullet} \rightarrow [CAR]^{\bullet} + ROOH \quad (2)$$

Addition

$$[CAR] + ROO^{\bullet} \rightarrow [ROOCAR]^{\bullet} \quad (3)$$

The important, antioxidant activity of microalgae carotenoids was documented by [6]. The peroxyl radical scavenger capacity of carotenoids extracts from microalgae *Phormidium autumnale* was determined. Considering all the fraction of characteristic carotenoids in cyanobacteria such as echinenone, and canthaxanthin shown to be potent scavengers of the reactive species studied fundamentally associated with the chemical structure with a bathochromic effect of these compounds.

Likewise, [73] evaluated the ability to remove peroxyl radicals from carotenoid extracts from three species of microalgae: *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Aphanothece microscopica Nageli*. Values of 31.1 (*Chlorella*), 14.0 (*Scenedesmus*) and 7.3 (*Aphanothece*) times more potent than α-tocopherol, were found, due to the specific compounds synthesized by microalgae with high bioactive capacity present in carotenoid extracts. These include ketocarotenoids, present mainly in *Chlorella vulgaris*, such as echinenone and canthaxanthin.

However, from the nutritional and physiological point of view, the interest in carotenoids has been focused mainly on the provitamin A activity in structures containing β-rings, as α-carotene, β-carotene e β-cryptoxanthin. Microalgae present a substantial amount of compounds with β-ionone rings in their composition. An example of this is the high concentration of β-carotene present in the biomass of *Porphyridium cruentum*, *Isochrysis galbana*, *Tetraselmis suecica*, *Phaeodactylum tricornutum* and *Nannochloropsis gaditana*. In this same work, (all-*E*)-α-carotene was identified in *T. suecica*; and (all-*E*)-β-cryptoxanthin in *P. cruentum* [29].

Recent studies with microalgae show the use of microalgae in modern cancer therapy, effectively associated with the high carotenoid content present in these microorganisms [74]. Fucoxanthin, an allenic and acetylated carotenoid present in brown microalgae and diatoms, demonstrated valuable anticancer properties by preventing the growth of malignant cells [69, 70]. At the same time, microalgae studies evaluated the bioactivity of fucoxanthin and its compounds with oxygenated functional groups (epoxy, hydroxyl, carbonyl and carboxyl) in their structure [75, 76, 77].

On the other hand, anti-inflammatory and antimicrobial properties were demonstrated in extracts of marine microalgae. Extract of *Tetraselmis* sp., *Dunaliella Salina*, *Nannochloropsis gaditana*, *Dunaliella* sp.,

Phaeodactylum tricornutum and *Isochrisis* sp. showed an inhibitory effect for *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The results obtained considered the content of bioactive compounds previously determined in the biomass of these microalgae species [78]. Among these compounds, the class of carotenoids is potentially related to these biological effects [79].

Anti-obesity effects have been proven for astaxanthin isolated the marine algae as *Haematococcus pluvialis*, *Chlorella zofingiensis*, and *Chlorococcum* sp. This carotenoid inhibited the increase in body weight and adipose tissue synoptically [80, 81].

Evidence of study with non-provitamin carotenoids A, lutein and zeaxanthin have already been extensively well documented and deserve special attention because they are preferentially accumulated in the retina and responsible for improving eye health [45, 82, 83].

Many epidemiological studies indicated a positive correlation between carotenoid intake and the prevention of cardiovascular diseases reported recently by [84, 85]. [86], investigated the antihypertensive effects of astaxanthin (50 mg/kg) in spontaneously hypertensive rodents, where in 14 days a significant reduction in blood pressure occurred along with a significant delay in the incidence of stroke. The health benefits of the major carotenoids of interest for commercialization and use as ingredients and some microalgae species that produce them are summarized in Table 1.

[Insert Table 1]

Finally, before reaching any conclusions regarding potential health effects, it is necessary to assess how the digestion process impacts bioactive compounds and their stability. This, in turn, will influence their bioavailability and potential beneficial effects.

Bioacessibility and biodisponibility

Carotenoids not synthesized by human organism and therefore must be ingested in the diet [106]. However, to perform their biological functions prominent to human health, these molecules need to be absorbed by the organism. For this to occur, carotenoids are released from the matrix after ingestion, solubilized in the lipid droplets, transferred to mixed micelles for their uptake by absorptive epithelial cells lining the small intestine, for later are incorporated into chylomicrons and secreted into the target tissues [107]. In other words, first, these compounds 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 [106, 108]. However, all prerequisites for exerting potential biological effects may be different between individuals [60]. Figure 1 shows the main steps in the absorption metabolism of β-carotene.

[Insert Figure 1]

Metabolic factors, including: the dietary release of carotenoids from the food matrix and its micellisation; its cellular uptake into the enterocytes; transport and metabolism of carotenoids; further transport and bio-distribution of carotenoids or carotenoid-metabolites; transmission and regulation of biological-mediated functions; and excretion were related as the main steps that determine the parameters of absorption, distribution, metabolism and excretion of carotenoids (ADME) - being these, essential factors to be considered in any study that evaluates the absorption metabolism of these compounds [109, 110].

Consequently, the metabolism of carotenoids starts in the oral phase with mastication, resulting in smaller particle breakage and increased surface area. Due to the minimum time that carotenoids remain in contact with the enzymes of the oral cavity (lipases), its enzymatic effects are practically null, and there is no transformation in their structures [60, 111]. However, many studies do not consider this phase in bioaccessibility analyses. Thus, the influence of enzymatic actions at this stage of carotenoid metabolism has still not been a focus of investigating.

Metabolism in the stomach in pH acid (between 2 and 5) and to the action of gastric secretions, along with enzymatic activities of pepsin, amylase, gastric lipase, among others, as phospholipids (emulsifying agent) released into the mucus, are present at this stage. In this stage of metabolism, the formation of epoxy compounds such as violaxanthin, neoxanthin is favorable due to the low pH [112].

Due to their apolar character, the carotenoids released from the matrix and transferred to the lipid phase of the bolus, its incorporation in mixed micelles of bile salts is required to be subsequently available for uptake by cells of the intestinal mucosa, which occurs predominantly in the small intestine [60]. Besides, bile salts and pancreatic enzymes are fundamental in the micellization process of carotenoids. These form micelles with free fatty acids, where the carotenoids are incorporated in the nucleus and the xanthophylls, more polar carotenoids, remain on the surface of these micelles [113]. In this way, the xanthophylls can be transferred more easily from the lipid droplets to the mixed cells. While the components associated with the core of the emulsion, carotenes require the digestion of triacylglycerols before the transfer [114]. In blood plasma and circulating chylomicrons, free xanthophylls are almost exclusively found [115].

The most crucial step of carotenoid absorption is the small intestine stage, where the micellization of these compounds occurs. This step is essential for the assimilation of lipophilic compounds, where previously formed micelles constitute a vehicle by which these compounds diffuse through the mucus and water barriers separating the mucosa from the intestinal lumen to the apical surface of enterocytes, absorptive epithelial cells which cover the intestine [7]. Thus, pancreatic lipase produces free fatty acids and monoglycerides, promoting emulsification, while bile salts assist in the process of emulsification, formation and stability of the mixed micelles [116]. In vitro, bioaccessibility studies have confirmed that the resulting micelles and bioaccessibility were severely compromised when bile salts or pancreatic lipase [117, 118].

As shown by many studies, different factors can affect the absorption of carotenoids such as the physicochemical properties of the molecules themselves, dosage of carotenoid, heat treatment, natural physical and chemical barriers of the matrix which is present, and the constitution of other compounds present in the matrix [112, 119, 120, 121, 122]. It is, therefore, opportune consider all these aspects when evaluating the absorption of these compounds.

Values between 3.5% and 90% were reported for β-carotene absorption in humans [123]. This high variability is closely related to the methodological differences used to evaluate. Therefore, among the carotenoids present in microalgae, fucoxanthin is prominent in bioactivity because it has remarkable biological properties. Thus, several research groups have evaluated the absorption of this compound. When compared to the metabolism of fucoxanthin, when compared to lutein in mice, this ketocarotenoid is more easily absorbed in the intestine [124]. Similarly, fucoxanthin metabolites such as fucoxanthinol and amarouciaxanthin. A were detected in plasma, erythrocytes, liver, lung, kidney, heart, spleen and adipose tissue of mice at a higher ratio than astaxanthin [125].

A study realised by [18], showed that the β-carotene, zeaxanthin and fucoxanthin were the main carotenoids found in the biomass of these microalgae, showing substantial bioaccessibility with values of 25%, 27% and 57%, respectively. Consequently, fucoxanthin was the most abundant carotenoid in Caco-2 cells, followed by zeaxanthin.

One of the primary current knowledge to establish the real contribution of carotenoids to human health is the bioaccessibility and bioavailability study. Although there are several definitions for the two terms, it is assumed that the bioaccessibility corresponds to results obtained using *in vitro* simulations while the bioavailability compares to *in vivo* assays [63]. However, the two conditions are co-related and are determined by various methodologies.

The term bioaccessibility refers to the observation of the entire sequence of events that occur during the digestive transformation of food into compounds that can be assimilated by the body, absorption/assimilation in cells of the intestinal epithelium and pre-systemic metabolism [106]. These steps include the transfer of carotenoids and other fat-soluble compounds from the food matrix to mixed micelles during digestion, is a necessary preliminary process by which the compound becomes accessible for apical absorption by the intestinal mucosa [108].

According to [63], bioaccessibility is generally assessed by mimicking gastric and small intestinal digestion, followed or not by Caco-2 cell uptake, while the term bioavailability can be defined as the fraction of nutrient or ingested compound that reaches the systemic circulation and is generally used includes the following steps: gastrointestinal digestion, absorption, metabolism, tissue distribution and bioactivity. According to [7], the bioavailable content is monitored for its increase in the TAG-rich fraction of the plasma containing chylomicrons after consumption of carotenoids.

Independent of the terminology the two methods of evaluation are complementary since the transfer of carotenoids into mixed micelles (measured by bioaccessibility tests) is an essential prerequisite whose efficiency is highly correlated with the extent of absorption and systemic distribution carotenoids (bioavailability), in other words, the bioavailability of carotenoids is largely dependent of the bioaccessibility of carotenoids fraction [108].

Some factors affect the bioaccessibility/bioavailability of carotenoids, which may be related to the food structure (food matrix and processing characteristics) and the physiological issues and genetic aspects related to the host, as recently mentioned by [126]. The amount and origin of fat and supplementation of the diet with other lipophilic antioxidants have been shown to affect the bioavailability of carotenoids to a limited extent, the degree of enzymatic hydrolysis achieved when carotenoids are provided in the ester form also influence [126].

Therefore, specific efforts were made to investigate the factors that influence the bioavailability of microalgal carotenoids. As can be observed in Table 2, few works have been done so far, among them very few investigations using experimental models *in vivo*. Among the few reports we have, most aim to

predict/extend the bioavailability by determining bioaccessible content, which allows to mimic the human gastrointestinal environment and investigate the influence of different factors on carotenoid absorption [127].

[Insert Table 2]

The carotenoids evaluated so far are summarized as β-carotene, lutein, zeaxanthin and fucoxanthin produced from microalgae species such as *Scenedesmus almeriensis*, *Scenedesmus bijuga, Scenedesmus obliquus, Chlorella vulgaris*, *Chlorella ellipsoidea*, *Chlorella reinhardtii*, *Chlorella sorokiniana*, *Phaeodactylum tricornutum, Arthrospira platensis* [16, 17, 18, 127, 128, 129, 130, 131,132, 134].

The [128] was the first group of researches to determine the bioaccessibility of green microalgal carotenoids, lutein, and zeaxanthin of *S. almeriensis*, noting that structural rigidity of the microalgal cell could be influencing the results. This aspect was then considered a limiting factor related to matrix and hence strategies to increase the bioaccessibility of such compounds have been used (see Table 2).

These strategies include more rudimentary techniques such as maceration and more sophisticated methods such as microfluidization and sonication. Maceration based on structural breakdown by mechanical means was used by [129] to improve the content of lutein, β-carotene fucoxanthin and zeaxanthin bioavailable from *P. tricornutum* and *C. vulgaris*. The ultrasonic method based on the propagation of ultrasonic waves [130] improved the bioaccessibility of β-carotene and fucoxanthin of *P. tricornutum*, the use of cellulase contributed to this occurrence [17, 18]. Microfluidization, which is widely used in the preparation of nanoemulsions and liposomes to create fine emulsions from high-pressure homogenization of large particles was used by [16, 127], demonstrating to be effective in increasing the bioaccessibility of lutein of *C. vulgaris* and C*. Ellipsoidea*.

Finally, designing microalgae-based ingredients and products that overcome the challenges associated with poor carotenoid bioaccessibility and bioavailability requires a broad understanding of the hotspots that potentially limit their bioaccessibility and subsequent uptake.

Conclusion

Finally, in this article, we discuss aspects related to bioaccessibility and bioavailability of microalgal carotenoids, including structure, bioactivity, absorption, evaluation techniques as well as strategies to improve bioavailable content. In short, microalgae represent a potential source for the exploration of these compounds; they have a diverse, complex and sometimes specific structure with numerous biological activities of interest in human health. In vitro and in vivo methods are used to determine the bioaccessibility and bioavailability, respectively. Some factors are limiting for its absorption but can be minimised using applied strategies on the matrix.

References

1. Matos, A. P. (2017). The impact of microalgae in food science and technology. Journal of the American Oil Chemists' Society, 94, 1333–1350.
2. Borowitzka, M. A. (2018). Chapter 9 - Microalgae in Medicine and Human Health: A historical perspective. In I. A. Levine & J. Fleurence (Eds.), Microalgae in Health and Disease Prevention (pp. 195–210). Cambridge: Academic Press.
3. Jacob-Lopes, E., Maroneze, M. M., Deprá, M. C., Sartori, R. B., Dias, R. R., & Zepka, L. Q. (2018). Bioactive food compounds from microalgae: An innovative framework on industrial biorefineries. Current Opinion in Food Science. In press. https://doi.org/10.1016/j.cofs.2018.12.003.
4. Gong, M., Bassi, A. (2016). Carotenoids from microalgae: A review of recent developments. Biotechnology Advances, 34, 1396-1412
5. Rodrigues, D. B., Flores, É. M. M., Barin, J. S., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2014). Production of carotenoids from microalgae cultivated using agroindustrial wastes. Food Research International, 65, 144–148.
6. Rodrigues, D. B., Menezes, C. R., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2015). Bioactive pigments from microalgae *Phormidium autumnale*. Food Research International, 77, 273-279.
7. Kopec, R. E., Caris-Veyrat, C., Nowicki, M., Gleize, B., Desmarchelier, C., & Borel, P., (2017).13C β-Carotene stability during digestion in healthy humans. FASEB J. 31 (1) (32.5).
8. Mohammadzadeh H., N., Saedisomeolia, A., Abdolahi, M., Shayeganrad, A., Taheri Sangsari, G., Hassanzadeh Rad, B., & Muench, G., (2017). Molecular anti-inflammatory mechanisms of retinoids and carotenoids in alzheimer's disease: a review of current evidence. Journal of Molecular Neuroscience. 61 (3), 289–304.
9. Giovannucci, E., Rimm, E. B., Liu, Y., Stampfer, M. J., & Willett, W. C., (2002). A prospective study of tomato products, lycopene, and prostate cancer risk. Journal of the National Cancer Institute 94, 391–398.
10. Sesso, H. D., & Gaziano, J. M., (2004). Heart and vascular diseases. Carotenoids in Health and Disease. Marcel Dekker, New York, pp. 491–502.

11. Sabour-Pickett, S., Nolan, J. M., Loughman, J., & Beatty, S., (2012). A review of the evidence germane to the putative protective role of the macular carotenoids for age-related macular degeneration. Molecular Nutrition & Food Research. 56, 270–286.
12. Ip, B. C., Liu, C., Lichtenstein, A. H., von Lintig, J., & Wang, X. D., (2015). Lycopene and apo-10'-lycopenoic acid have differential mechanisms of protection against hepatic steatosis in β-carotene-9', 10'-oxygenase knockout male mice. The Journal of Nutrition, 145, 268–276.
13. Kopec, R. E., Schick, J., Tober, K. L., Riedl, K. M., Francis, D. M., Young, G. S., Schwartz, S. J., & Oberyszyn, T. M. (2015). Sex differences in skin carotenoid deposition and acute UV Binduced skin damage in SKH-1 hairless mice after consumption of tangerine tomatoes. Molecular Nutrition & Food Research. 59, 2491–2501.
14. Chew, K.W., Yap, J.Y., Show, P.L., Suan, N.H., Juan, J.C., Ling, T.C., Lee, D.J., & Chang, J.S. (2017). Microalgae biorefinery: High value products perspectives. Bioresource Technology, 229, 53-62.
15. Chitchumroonchokchai, C., & Failla M.L. (2017). Bioaccessibility and intestinal cell uptake of astaxanthin from salmon and commercial supplements. Food Research International, 99, 936–943.
16. Cha, K. H., Lee, J. Y., Song, D. G., Kim, S. M., Lee, D. U., Jeon, J. Y., & Pan, C. H. (2011). Effect of microfluidization on *in vitro* micellization and intestinal cell uptake of lutein from *Chlorella vulgaris*. Journal of agricultural and food chemistry, 59(16), 8670-8674.
17. Gille, A., Trautmann, A., Posten, C., & Briviba, K. (2016). Bioaccessibility of carotenoids from *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. International Journal of Food Sciences and Nutrition, 67(5), 507–513.
18. Gille, A., Hollenbach, R., Trautmann, A., Posten, C., & Briviba, K. (2017). Effect of sonication on bioaccessibility and cellular uptake of carotenoids from preparations of photoautotrophic *Phaeodactylum tricornutum*. Food Research International, 118, 40-48.
19. Rodrigues, D.B, Mariutti., L.R.B. & Mercadante, A.Z. (2016). An *in vitro* digestion method adapted for carotenoids and carotenoid esters: moving forward towards standardization, Food & Function, 2016, 7, 4992–5001.
20. Petry, F.C., & Mercadante, A.Z. (2017). Impact of *in vitro* digestion phases on the stability and bioaccessibility of carotenoids and their esters in mandarin pulps. Food & Function, 8, 3951-3963.
21. Chacón-Lee, T.L., & González-Mariño, G.E. (2010). Microalgae for "Healthy" Foods Possibilities and Challenges. Comprehensive reviews in food science and food safety, 9, 655- 675.
22. Rizwan M, Mujtaba G, Memon SA, Lee K, & Rashid N. (2018) Exploring the potential of microalgae for new biotechnology applications and beyond: A review. Renewable Sustainable Energy Reviews, 92, 394-404.
23. Vendruscolo, R. G., Facchi, M. M. X., Maroneze, M. M., Fagundes, M. B., Cichoski, A. J., Zepka, L. Q., Barin, J. S., Jacob-Lopes, E., & Wagner, R. (2018). Polar and non-polar intracellular compounds from microalgae: Methods of simultaneous extraction, gas chromatography determination and comparative analysis. Food Research International, 109: 204-212.
24. Vonshak, A. 1990. Recent advances in microalgal biotechnology. Biotechnology Advances, 8: 709-727.
25. Raja, R., Hemaiswarya, S., & Rengasamy, R. 2007. Exploitation of *Dunaliella* for β-carotene production. Applied Microbiology and Biotechnology, 74: 517-523.
26. Sudhakar, K., & Premalatha, M. (2012) Theoretical assessment of algal biomass potential for carbon mitigation and biofuel production. International Journal of Energy and Environment, 3, 232-240.
27. Norsker, N.H., Barbosa, M. J., Vermuë, M. H., & Wijffels, R. H. (2011). Microalgal production - A close look at the economics. Biotechnology Advances, 29(1), 24–27.
28. Sierra, E., Acién, F.G., Fernández, J.M., García, J.L., González, C., & Molina, E. (2008). Characterization of a flat plate photobioreactor for the production of microalgae. Chemical Engineering Journal,138, 136–47.
29. Di Lena, G., Casini, I., Lucarini, M., & Lombardi-Boccia, G. (2018). Carotenoid profiling of five microalgae species from large-scale production. Food Research International, In Press.
30. Roso, G. R., Queiroz, M. I., Streit, N. M., Menezes, C. R., Zepka, L.Q., & Jacob- Lopes, E. 2015. The Bioeconomy of Microalgal Carotenoid-Rich Oleoresins Produced in Agroindustrial Biorefineries. Journal of Chemical Engineering & Process Technology, 6:1-7.
31. Acién, F. G., Fernández, J. M., Magán, J. J., & Molina, E. (2012). Production cost of a real microalgae production plant and strategies to reduce it. Biotechnology Advances, 30(6), 1344–1353.
32. Ravindra, A. p. 2000; Value-added food: Single cell protein. Biotechnology Advances, 18: 459-479.
33. Enzing, C., Ploeg, M., Barbosa, M., & Sijtsma, L. 2014. Microalgae-based products for food and feed sector: an outlook for Europ. JRC Scientific and Policy Reports, 1: 1-56.
34. Britton G., Liaaen-Jensen S., & Pfander H. Carotenoids: Handbook. 1st ed. Basel, Switzerland: Springer Basel AG; 2004. p. 646p.
35. Yabuzaki J. Carotenoids Database [Internet]. 2018. Available from: http://carotenoiddb.jp/ [Accessed: 22-08-2021].
36. Britton G., Liaaen-Jensen S., & Pfander H. (2017). Carotenoids: A Colourful History. 1st ed. CaroteNature; 236p.
37. Tian H, Fang H.T.L., Zhang Q., Wang M., Wang Y, Jia J. (2018). Transcriptome analysis of carotenoid biosynthesis in the *Brassica campestris L.* subsp. *chinensis* var. *rosularis Tsen*. Scientia Horticulturae, 235, 116-123.

38. Fernandes, AF, Nascimento, T.C, Jacob-Lopes, E., De Rosso, V.V. and Zepka, L.Q. Introductory Chapter: Carotenoids - A Brief Overview on Its Structure, Biosynthesis, Synthesis, and Applications. Progress in Carotenoid Research. 1st ed.
39. Rodriguez-Amaya D.B. (2016). Food Carotenoids: Chemistry, Biology, and Technology. 1st ed. John Wiley & Sons, Ltd. p. 306p.
40. Mulders, K.J.M., Lamers, P.P., Martens, D.E., & Wijffels, R.H. (2014). Phototrophic pigment production with microalgae: biological constraints and opportunities. Journal of phycology, 50, 229-242.
41. Takaichi, S., & Mochimaru, M. (2007). Carotenoids and carotenogenesis in cyanobacteria: Unique ketocarotenoids and carotenoid glycosides. Cellular and Molecular Life Sciences, 64,2607–2619.
42. Takaichi, S. (2011). Carotenoids in algae: distributions, biosyntheses and functions. Marine Drugs, 9, 1101-1118.
43. Britton G, Liaaen-Jensen S, & Pfander H. (2008). Carotenoids: Natural Functions. 4th ed. Basel, Switzerland: Birkhäuser Verlag. p. 400.
44. Rodriguez-Amaya, D.B. (2015). Carotenes and xanthophylls as antioxidants. In: Shahidi F, editor. Handbook of Antioxidants for Food Preservation. 1st ed. Newfoundland, Canada: Elsevier Ltd. pp. 17-50.
45. Rodriguez-Concepcion, M., Avalos, J., Bonet, M.L., Boronat, A., Gomez-Gomez, L., Hornero-Mendez, D., Limon, M.C., Meléndez-Martínez,A.J., Olmedilla-Alonso, B., Palou, A., Ribot, J., Rodrigo, M.J., Zacarias, L., & Zhu, C. (2018). A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. Progress in Lipid Research, 70, 63-93.
46. Saini, R.K., Nile, S.H., & Park, S.W. (2015) Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. Food Research International. 76, 735-750.
47. Beltran J.C, & Stange C. (2016). Apocarotenoids: A new carotenoid-derived pathway. Subcellular Biochemistry, 79:239-272.
48. Ikawa, M. Sasner, J.J., & James, F. (2001). Activity of cyanobacterial and algal odor compounds found in lake waters on green alga *Chlorella pyrenoidosa* growth. Hydrobiologia, 443: 19–22.
49. Gross, J., 1987. Pigments in Fruits. Academic Press, London.
50. Etzbach, L., Pfeiffer, A., Weber, F., & Schieber, A., 2018. Characterization of carotenoid profiles in goldenberry (*Physalis peruviana L.)* fruits at various ripening stages and in different plant tissues by HPLC-DAD-APCI-MS. Food Chemistry, 245: 508-517.
51. Lemoine, Y., & Schoefs, B. (2010). Secondary ketocarotenoid astaxanthin biosynthesis in algae: a multifunctional response to stress. Photosynthesis Research, 106: 155-177.
52. Maroneze, M.M., Jacob-Lopez, E., Zepka, L.Q., Roca, M., & Pérez-gálvez, A. (2019). Esterified carotenoids as new food components in cyanobacteria, Food Chemistry, 287:295-302.
53. Mariutti, L.R.B., & Mercadante, A.Z. (2018). Carotenoid esters analysis and occurrence: What do we know so far? Archives of Biochemistry and Biophysics, 648, 36–43.
54. Guedes, A., Amaro, H., & Malcata, F. (2011) Microalgae as Sources of Carotenoids. Marine drugs, 9,625–644.
55. Rowan K.S. (1989). Photosynthetic Pigments of Algae. Cambridge University Press; Cambridge, UK.
56. Takaichi, S., Maoka, T., & Masamoto, K. (2001). Myxoxanthophyll in *Synechocystis* sp. PCC 6803 is myxol 2′-dimethyl-fucoside, (3R,2′S)-myxol 2′-(2,4-di-O-methyl-α-L-fucoside), not rhamnoside. Plant Cell Physiology. 42, 756–762.
57. Schubert, N., & García-Mendoza, E. (2008). Photoinhibition in red algal species with different carotenoid profiles. Journal of Phycology. 44, 1437–1446.
58. Li, J., Zhu, D., Niu, J., Shen, S., & Wang, G. (2011). An economic assessment of astaxanthin production by large scale cultivation of *Haematococcus pluvialis* Biotechnology Advances, 29, 568-574.
59. Galanakis, C.M. (Ed.). (2016). Nutraceutical and functional food components: Effects of innovative processing techniques. Academic Press.
60. Bohn, T., Desmarchelier, C., Dragsted, L.O., Nielsen, C.S., Stahl, W., Rühl, R., Keijer, J., & Borel P. (2017). Host-related factors explaining interindividual variability of carotenoid bioavailability and tissue concentrations in humans. Molecular Nutrition & Food Research, 61.
61. Khalid, M., Bilal, M., Iqbal, H. M., & Huang, D. (2018). Biosynthesis and biomedical perspectives of carotenoids with special reference to human health-related applications. Biocatalysis and Agricultural Biotechnology, 399-407.
62. Fernández-García, E., Mínguez-Mosquera, M.I., & Pérez-Gálvez, A., 2007. Changes in composition of the lipid matrix produce a differential incorporation of carotenoids in micelles. Interaction effect of cholesterol and oil. Innovative Food Science and Emerging Technologies, 8: 379–384.
63. Carbonell-Capella, J. M., Buniowska, M., Barba, F. J., Esteve, M. J., & Frígola, A. (2014). Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. Comprehensive Reviews in Food Science and Food Safety, 13(2), 155-171.
64. Stahl, W., & Sies, H. (2005). Bioactivity and protective effects of natural carotenoids. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1740(2), 101-107.
65. Lauritano, C., Andersen, J. H., Hansen, E., Albrigtsen, M., Escalera, L., Esposito, F & Ianora, A. (2016). Bioactivity screening of microalgae for antioxidant, anti-inflammatory, anticancer, anti-diabetes, and antibacterial activities. Frontiers in Marine Science, 3, 68.
66. Eggersdorfer, M., Wyss, A. (2018). Carotenoids in human nutrition and health. Archives of Biochemistry and Biophysics, 652, 18-26.

67. Rodrigues, E., Mariutti, L. R., Faria, A. F., & Mercadante, A. Z. (2012). Microcapsules containing antioxidant molecules as scavengers of reactive oxygen and nitrogen species. Food chemistry, 134(2), 704-711.
68. Lerfall, J. (2016). Carotenoids: occurrence, properties and determination. Encyclopedia of Food and Health, http://dx.doi.org/10.1016/B978-0-12-384947 2.00119-7.
69. Kowluru, R. A., & Mishra, M. (2015). Oxidative stress, mitochondrial damage and diabetic retinopathy. Biochimica et Biophysica Acta, 1852(11), 24742483.
70. Young, A., & Lowe, G. (2018). Carotenoids-antioxidant properties. Antioxidants, 7(2), 28.
71. El-Agamey, A., Lowe, G.M., McGarvey, D.J., Mortensen. A., Phillip, D.M., Truscott, T.G., & Young, A.J. (2004). Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Archives of Biochemistry and Biophysics, 430, 37–48.
72. Fiedor, J., & Burda, K. (2014). Potential role of carotenoids as antioxidants in human health and disease. Nutrients, 6(2), 466-488.
73. Patias, L.D., Fernandes, A. S., Petry, F.C., Mercadante, A.Z., Jacob-Lopes, E., & Zepka, L.Q. (2017). Carotenoid profile of three microalgae/cyanobacteria species with peroxyl radical scavenger capacity. Food Research International, 100, 260-266.
74. El-Hack, M.E.A., Abdelnour, S., Alagawany, M., Abdo, M., Sakr, M. A., Khafaga, A. F., & Gebriel, M.G. (2019). Microalgae in modern cancer therapy: Current knowledge. Biomedicine & Pharmacotherapy, 111, 42-50.
75. Takahashi, K., Hosokawa, M., Kasajima, H., Hatanaka, K., Kudo, K., Shimoyama, N., & Miyashita, K. (2015). Anticancer effects of fucoxanthin and fucoxanthinol on colorectal cancer cell lines and colorectal cancer tissues. Oncology letters, 10(3), 1463-1467.
76. Foo, S. C., Yusoff, F. M., Imam, M. U., Foo, J. B., Ismail, N., Azmi, N. H., & Ismail, M. (2019). Increased fucoxanthin in *Chaetoceros calcitrans* extract exacerbates apoptosis in liver cancer cells via multiple targeted cellular pathways. Biotechnology Reports, 21, e00296.
77. Mise, T., Ueda, M., & Yasumoto, T. (2011). Production of fucoxanthin-rich powder from *Cladosiphon okamuranus*. Adv. J. Food Sci. Technol, 3, 73–76.
78. Maadane, A., Merghoub, N., El Mernissi, N., Ainane, T., Amzazi, S., Wahby, I., & Bakri, Y. (2017). Antimicrobial activity of marine microalgae isolated from *Moroccan coastlines*. The Journal of Microbiology, Biotechnology and Food Sciences, 6(6), 1257.
79. Maadane, A., Merghoub, N., Ainane, T., El Arroussi, H., Benhima, R., Amzazi, S., Bakri, Y., & Wahby, I. (2015). Antioxidant activity of some *Moroccan* marine microalgae: pufa profiles, carotenoids and phenolic content. Journal of Biotechnology, 215, 13–1.
80. Arunkumar, E., Bhuvaneswari, S., & Anuradha, C. V. (2012). An intervention study in obese mice with astaxanthin, a marine carotenoid-effects on insulin signaling and pro-inflammatory cytokines. Food & function, 3(2), 120-126.
81. Wang, H., Fu, Z., & Han, C.C. (2014). The potential applications of marine bioactives against diabetes and obesity. American Journal of Marine Science, 2, 1-8.
82. Abdel-Aal, E. S., Akhtar, H., Zaheer, K., & Ali, R. (2013). Dietary sources of lutein and zeaxanthin carotenoids and their role in eye health. Nutrients, 5(4), 1169-1185.
83. Buscemi, S., Corleo, D., Di Pace, F., Petroni, M., Satriano, A., & Marchesini, G. (2018). The effect of lutein on eye and extra-eye health. Nutrients, 10(9), 1321.
84. Kulczyński, B., Gramza-Michałowska, A., Kobus-Cisowska, J., & Kmiecik, D. (2017). The role of carotenoids in the prevention and treatment of cardiovascular disease-Current state of knowledge. Journal of functional foods, 38, 45-65.
85. Gammone, M.A., Pluchinotta, F.R., Bergante, S., Tettamanti, G., & D'orazio, N. (2017). Prevention of cardiovascular diseases with carotenoids. Frontiers in Bioscience, 9, 165-171.
86. Hussein, G., Nakamura, M., Zhao, Q., Iguchi, T., Goto, H., Sankawa, U., & Watanabe, H. (2005). Antihypertensive and neuroprotective effects of astaxanthin in experimental animals. Biological and Pharmaceutical Bulletin, 28(1), 47-52.
87. Eroglu, A., & Harrison, E.H. (2013). Carotenoid metabolism in mammals, including man: formation, occurrence, and function of apocarotenoids. Journal of Lipid Research, 54, 1719-1730.
88. Niranjana, R., Gayathri, R., Nimish Mol, S., Sugawara, T., Hirata, T., Miyashita, K., & Ganesan, P. (2015). Carotenoids modulate the hallmarks of cancer cells. Journal of Functional Foods, 18, 968-985.
89. Virtamo, J., Taylor, P.R., Kontto, J., Männistö, S., Utriainen, M., Weinstein, S.J., Huttunen, J., & Albanes, D. (2014). Effects of α-tocopherol and β-carotene supplementation on cancer incidence and mortality: 18-Year postintervention follow-up of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. International Journal of Cancer, 135, 178–185.
90. García-González, M., Moreno, J., Manzano, J.C., Florencio, F.J., & Guerrero, M.G. (2005). Production of *Dunaliella salina* biomass rich in 9-*cis*-ß-carotene and lutein in a closed tubular photobioreactor. Journal of Biotechnology, 115, 81-90.
91. Grudzinski, W., Krzeminska, I., Luchowski, R., Nosalewicz, A., & Gruszecki, W. L. (2016). Strong-light-induced yellowing of green microalgae *Chlorella*: A study on molecular mechanisms of the acclimation response. Algal Research, 16, 245-254.

92. Gayathri, S.; Rajasree, S.R.R., Kirubagaran, R., Aranganathan, L., & Suman, T.Y. (2016). Spectral characterization of ß, e-carotene-3, 3'-diol (lutein) from marine microalgae *Chlorella salina*. Renewable Energy, 98, 78-93.
93. Jaime, L., Rodríguez-Meizoso, I., Cifuentes, A., Santoyo, S., Suarez, S., Ibáñez, E., & Señorans, F. J. (2010). Pressurized liquids as an alternative process to antioxidant carotenoids' extraction from *Haematococcus pluvialis* microalgae. LWT - Food Science and Technology, 43(1): 105–112.
94. Bennedsen, M., Wang, X., Willén, R., Wadström, T., & Andersen, L. P. (2000). Treatment of H. pylori infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunology Letters, 70(3): 185–189.
95. Wu, J., Cho, E., Willett, W. C., Sastry, S. M., & Schaumberg, D. A. (2015). Intakes of Lutein, Zeaxanthin, and Other Carotenoids and Age-Related Macular Degeneration During 2 Decades of Prospective Follow-up. JAMA Ophthalmology, 133(12): 1415.
96. Fakhri, S., Dargahi, L., Abbaszadeh, F., & Jorjani, M. (2018). Astaxanthin attenuates neuroinflammation contributed to the neuropathic pain and motor dysfunctionfollowing compression spinal cord injury. Brain Research Bulletin, 143, 217, 224.
97. Cha, K., Koo, S., & Lee, D. (2008). Antiproliferative effects of carotenoids extracted from *Chlorella ellipsoidea* and *Chlorella vulgaris* on human colon cancer cells. Journal of Agricultural and Food Chemistry, 56, 10521–10526.
98. Chiu, P., Soong, K., & Chen, C.N. (2016). Cultivation of two thermotolerant microalgae under tropical conditions: Influences of carbon sources and light duration on biomass and lutein productivity in four seasons. Bioresource Technology, 212, 190-198.
99. Zhang, W., Wang, J., Wang, J., & Liu, T., 2014. Attached cultivation of *Haematococcus pluvialis* for astaxanthin production. Bioresource Technology. 158, 329-335.
100. Abrahamsson, V., Rodriguez-Meizoso, I., & Turner, C. (2012). Determination of carotenoids in microalgae using supercritical fluid extraction and chromatography. Journal of Chromatography A, 1250, 63–68.
101. Abrahamsson, I.; Rodriguez-Meizoso, I., & Turner, C. (2015). Supercritical fluid extraction of lipids from linseed with on-line evaporative light scattering detection, Analytica Chimica Acta, 853, 320-327.
102. Wang, J., Chen, S., Xu, S., Yu, X., Ma, D., Hu, X., & Cao, X. (2012). *In Vivo* Induction of Apoptosis by Fucoxanthin, a Marine Carotenoid, Associated with Down-Regulating STAT3/EGFR Signaling in Sarcoma 180 (S180) Xenografts-Bearing Mice. Marine Drugs, 10, 2055–2068.
103. Heo, S.J., Yoon, WJ., Kim, KN., Oh, C., Choi, YU., Yoon, KT., Kang, DH., Qian, ZJ., Choi, I.W., & Jung, W. (2012). Anti-inflammatory effect of fucoxanthin derivatives isolated from *Sargassum siliquastrum* in lipopolysaccharide-stimulated RAW 264.7 macrophage. Food and Chemical Toxicology, 50, 3336–42.
104. Takashima, M., Shichiri, M., Hagihara, Y., Yoshida, Y., & Niki, E. (2012). Capacity of fucoxanthin for scavenging peroxyl radicals and inhibition of lipid peroxidation in model systems. Free Radical Research, 46, 1406–1412.
105. Foo, S.C., Yusoff, F. M., Ismail, M., Basri, M., Yau, S. K., Khong, N. M. H., & Chan, K. W. (2015). Production of fucoxanthin-rich fraction (FxRF) from a diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968. Algal Research, 12, 26-32.
106. Fernández-García, E., Carvajal-Lérida, I., Jarén-Galán, M., Garrido-Fernández, J., Pérez-Gálvez, A. & Hornero-Méndez, D. (2012). Carotenoids bioavailability from foods: From plant pigments to efficient biological activities. Food Research International, 46, 438-450.
107. Furr, H.C., & Clark, R.M. (1997). Intestinal absorption and tissue distribution of carotenoids. Nutritional Biochemistry, 8, 364-377.
108. Kopec, R. E., & Failla, M. L. (2018). Recent advances in the bioaccessibility and bioavailability of carotenoids and effects of other dietary lipophiles. Journal of Food Composition and Analysis, 68, 16-30.
109. Borel P, Desmarchelier C, Nowicki M et al. (2014) Interindividual variability of lutein bioavailability in healthy men: characterization, genetic variants involved, and relation with fasting plasma lutein concentration. The American Journal of Clinical Nutrition, 100, 168–175.
110. Bohn, T., Desmarchelier, C., El, S. N., Keijer, J., van Schothorst, E., Rühl, R., & Borel, P. (2019). β-Carotene in the human body: metabolic bioactivation pathways–from digestion to tissue distribution and excretion. Proceedings of the Nutrition Society, 78(1), 68-87.
111. Kulkarni, B. V., & Mattes, R. D. 2014. Lingual lipase activity in the oro sensory detection of fat by humans. J. Physiol. Regul. Integr. Comp. Physiol, 306, R879–R885.
112. Desmarchelier, C., & Borel, P. (2017). Overview of carotenoid bioavailability determinants: From dietary factors to host genetic variations. Trends in Food Science & Technology, 69, 270-280.
113. Mutsokoti, L., Panozzo, A., Pallares, A.P., Jaiswal, S., Van Loey, A., Grauwet, T., & Hendrickx, M. (2017). Carotenoid bioaccessibility and the relation to lipid digestion: a kinetic study. Food Chemistry, 232, 124-134.
114. Parker, R.S. (1996). Absorption, metabolism, and transport of carotenoids. The FASEB Journal, 10, 542-551.
115. Coral-Hinostroza, G. N., Ytrestoyl T., Ruyter B., & Bjerkeng B. (2004). Plasma appearance of unesterified astaxanthin geometrical *E/Z* and optical *R/S* isomers in men given single doses of a mixture of optical 3 and *3'R/S* isomers of astaxanthin fatty acyl diesters. Comp. Biochem. Physiol. C Toxicol. Pharmacol, 139, 99–110.
116. Bohn T., Provitamin A Carotenoids - Occurrence, intake, and bioavailability in: Preedy V., editor. (Ed.), In Vitamin A and Carotenoids: Chemistry, Analysis, Function and Effects (Food and Nutritional Components in Focus), RSC Publishing, London: 2012.

117. Garrett, D.A., Failla, M.L., & Sarama, R.J. (1999). Development of an *in vitro* digestion method to assess carotenoid d bioavailability from meals. Journal of Agricultural and Food Chemistry, 47, 4301-4309.
118. Biehler, E., Kaulmann, A., Hoffmann, L., Krause, E., & Bohn, T. (2011). Dietary and host-related factors influencing carotenoid bioaccessibility from spinach (*Spinacia oleracea*). Food Chemistry, 125, 1328-1334.
119. Hof, K. H., West, C. E., Weststrate, J. A., & Hautvast, J. G. (2000). Dietary factors that affect the bioavailability of carotenoids. The Journal of nutrition, 130(3), 503-506.
120. Hornero-Méndez, D., & Mínguez-Mosquera, M.I. (2007). Bioaccessibility of carotenes from carrots: effect of cooking and addition of oil. Innovative Food Science and Emerging Technologies, 8, 407–412.
121. Huo, T., Ferruzzi, M.G., Schwartz, S.J., & Failla, M.L. (2007). Impact of fatty acyl composition and quantity of triglycerides on bioaccessibility of dietary carotenoids. Journal of Agricultural and Food Chemistry, 55, 8950-8957.
122. Priyadarshani, A.M.B. (2017). A review on factors influencing bioaccessibility and bioefficacy of carotenoids. Critical reviews in food science and nutrition, 57(8), 1710-1.
123. Haskell, M.J. (2012). The challenge to reach nutritional adequacy for vitamin A: betacarotene bioavailability and conversion--evidence in humans. The American Journal of Clinical Nutrition, 96, 1193s-1203s.
124. Yonekura, L., Kobayashi, M., Terasaki, M., & Nagao, A. (2010). Keto-carotenoids are the major metabolites of dietary lutein and fucoxanthin in mouse tissues. The Journal of nutrition, 140(10), 1824-1831.
125. Hashimoto, T., Ozaki, Y., Taminato, M., Das, S. K., Mizuno, M., Yoshimura, K., & Kanazawa, K. (2009). The distribution and accumulation of fucoxanthin and its metabolites after oral administration in mice. British journal of nutrition, 102(2), 242-248.
126. Viera, I., Pérez-Gálvez, A., & Roca, M. (2018). Bioaccessibility of Marine Carotenoids. Marine Drugs. 397:1-22.
127. Cha, K. H., Koo, S. Y., Song, D.G., & Pan, C. H. (2012). Effect of microfluidization on bioaccessibility of carotenoids from *Chlorella ellipsoidea* during simulated digestion. Journal of agricultural and food chemistry, 60(37), 9437-9442.
128. Granado-Lorencio, F., Herrero-Barbudo, C., Acién-Fernández, G., Molina-Grima, E., Fernández-Sevilla, J.M., Pérez-Sacristán, B. & Blanco-Navarro, I. (2009). *In vitro* bioaccessibility of lutein and zeaxanthin from the microalgae *Scenedesmus almeriensis*. Food Chemistry. 114: 747–752.
129. Gille, A., Neumann, U., Louisa, S., Bischoff, S. C., & Briviba, K. (2018). Microalgae as a potential source of carotenoids: Comparative results of an *in vitro* digestion method and a feeding experiment with C57BL/6J mice. Journal of Functional Foods, 49: 285-294.
130. Knorr, D., Zenker, M.; Heinz, V., & Lee, D. U. (2004). Applications and potential of ultrasonics in food processing. Trends in Food Science & Technology, Cambridge. 15: 261-266.
131. Akepach, P., Ribeiro-Filho, N., Wattanakul, J., Darwish, R., Gedi, M. A., Gray, D. A. (2022). Bioaccessibility of carotenoids (β-carotene and lutein) from intact and disrupted microalgae (*Chlamydomonas reinhardtii*). LWT. 160, 113292.
132. Fernandes, A. S., Nascimento, T. C., Pinheiro, P. N., Vendruscolo, R. G., Wagner, R., Rosso, V. V., Jacob-Lopes, E., Zepka, L. Q. (2021). Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix. Food Research International. 148, 110596.
133. Nascimento, T. C., Pinheiro, P. N., Fernandes, A. S., Murador, D. C., Neves, B. V., Menezes, C. R., Rosso, V. V., Jacob-Lopes, E., Zepka, L. Q. (2021). Bioaccessibility and intestinal uptake of carotenoids from microalgae *Scenedesmus obliquus.* LWT. 140, 110780.
134. Nass, P. P., Nascimento, T. C., Fernandes, A. S., Caetano, P. A., Rosso, V. V., Jacob-Lopes, E., Zepka, L. Q. Guidance for formulating ingredients/products from *Chlorella vulgaris* and *Arthrospira platensis* considering carotenoid and chlorophyll bioaccessibility and cellular uptake. Food Research International. Volume 157, July 2022, 111469.

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714 **Table 1.** Biological functions of the main carotenoids present in microalgae.

Carotenoids	Helath benefits	Microalgae	Reference
β-Carotene	Reduce cardiovascular disease risk; Reduces the occurrence of epithelial cell cancer; Anti-oxidant property.	*Dunaliella salina; Phormidium* sp.*; Chlorella protothecoides; Chlorella vulgaris; Chlorella salina; Haematococcus pluvialis*	[6, 49, 88, 89, 90, 91, 92, 93]
Astaxanthin	Anti-oxidant activity; Anti-inflammatory effects; Anti-apoptotic activity.	*Chlorella zofingiensis; Haematococcus pluvialis; Chlamydocapsa spp.*	[93, 94, 95, 96]
Lutein	Can inhibit lipid peroxidation in membranes; Slows the progression of macular degeneration; Anti-oxidant property; Prevents cardiovascular diseases.	*Dunaliella salina; Chlorella sorokiniana; Chlorella protothecoides; Chlorella vulgaris; Coelastrella sp.; Desmodesmus sp; Chlorella salina; Haematococcus pluvialis; Chlamydocapsa spp.*	[91, 92, 93, 97, 98]
Canthaxanthin	Anti-oxidant property	*Phormidium sp.; Scenedesmus sp.; Chlamydocapsa spp.*	[6, 99, 100, 101]
Fucoxanthin	Neuroprotection in traumatic brain Injury models; Antitumor effects; Anti-inflammatory effects; Promotes apoptotic effects; Radical scavenging activity.	*Isochrysis galbana; Phaeodactylum tricornutu; Chaetoceros calcitrans*	[102, 103, 104, 105]

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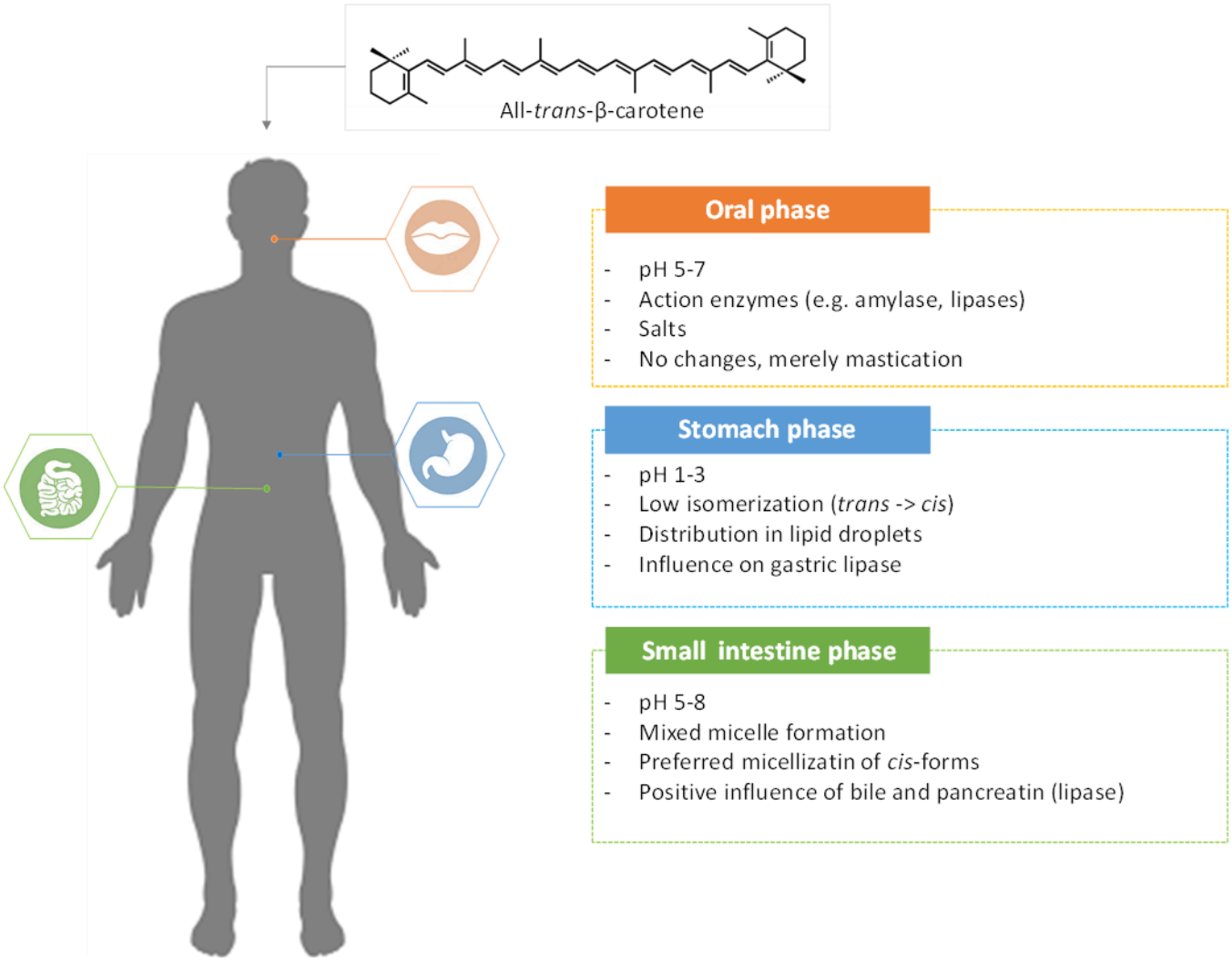

Figure 1. Simplified processing of β-carotene during absorption metabolism.

Table 2. Bioaccessibility and bioavailability assays of microalgal carotenoids.

Microalgae	Main carotenoids	Assay	Strategy	Reference
S. almeriensis	lutein, zeaxanthin	*in vitro*	nd	[128]
C. vulgaris	lutein	*in vitro* - cells caco-2	microfluidization	[16]
C. ellipsoedea	lutein	*in vitro* - and cells Caco-2	microfluidization	[127]
C. vulgaris	lutein, β-carotene	*in vitro*	sonication	[17]
C. reinhardtii	lutein, β-carotene	*in vitro*	sonication	[17]
P. tricornutum	zeaxanthin, fucoxanthin, β-carotene	*in vitro* - cells Caco-2	sonication + cellulase digestion	[18]
C. vulgaris	lutein, β-carotene	*in vitro*	maceration biomass	[129]
P. tricornutum	fucoxanthin, zeaxanthin, β-carotene	*in vitro*	maceration biomass	[129]
C. vulgaris	lutein, β-carotene	*in vivo*	maceration biomass	[129]
P. tricornutum	fucoxanthin, zeaxanthin	*in vivo*	maceration biomass	[129]
C. reinhardtii	lutein, β-carotene	*in vitro*	hydrodynamic cavitation disruption	[131]
C. sorokiniana	lutein, β-carotene	*in vitro*	sonication	[132]
S. bijuga	lutein, β-carotene	*in vitro*	sonication	[132]
S. obliquus	lutein, β-carotene	*in vitro* - cells Caco-2	whole dried biomass	[133]
S. obliquus	lutein, β-carotene	*in vitro* - cells Caco-2	wet ultrasonicated biomass	[133]
S. obliquus	lutein, β-carotene	*in vitro* - cells Caco-2	isolated carotenoid extract	[133]
C. vulgaris	lutein, β-carotene	*in vitro* - cells Caco-2	whole dried biomass	[134]
C. vulgaris	lutein, β-carotene	*in vitro* - cells Caco-2	whole ultrasonicated paste	[134]
C. vulgaris	lutein, β-carotene	*in vitro* - cells Caco-2	liposoluble pigment emulsion	[134]
A. platensis	zeaxanthin, β-carotene	*in vitro* - cells Caco-2	whole dried biomass	[134]
A. platensis	zeaxanthin, β-carotene	*in vitro* - cells Caco-2	whole ultrasonicated paste	[134]
A. platensis	zeaxanthin, β-carotene	*in vitro* - cells Caco-2	liposoluble pigment emulsion	[134]

CAPÍTULO 2

Guidance for formulating ingredients/products from *Chlorella vulgaris* and *Arthrospira platensis* considering carotenoid and chlorophyll bioaccessibility and cellular uptake

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Caco-2 cells
In vitro digestion

ABSTRACT

This study aimed to investigate the impact of different microalgal matrices on the bioaccessibility and uptake by Caco-2 cells of carotenoids and chlorophylls. In this way, the microalgal ingredients/products (whole dry biomass [WDB], whole ultrasonicated paste [WUP], and liposoluble pigment emulsion [LPE]) obtained from *Chlorella vulgaris* and *Arthrospira platensis* were submitted to *in vitro* simulated digestion. Apical uptake of pigments in micelles generated during the simulated digestion by Caco-2 human intestinal cells was determined. The influence of simulated digestion on carotenoid and chlorophyll stability and bioaccessibility was assessed by HPLC-PDA-MS/MS and the carotenoids and chlorophylls' bioaccessibility and cellular uptake were shown to be boosted according to the matrix (LPE > WUP > WDB). Our findings showed that *Chlorella vulgaris* and *Arthrospira platensis* could be considered in formulations when carotenoids and chlorophylls are the target molecules in the ingredients/products.

1. Introduction

In light of the increasing worldwide prevalence of diet-related chronic diseases, including overweight, obesity, diabetes, and cardiovascular disease, there is a growing interest in the relationship between food and health (Miller et al., 2020). Based on this understanding, microalgae-based ingredients and products are positioned firmly in the market, promoting consumers' awareness of nutrition and healthy lifestyles (Kusmayadi et al., 2021).

This global trend has expanded the commercialization of microalgae-based ingredients/products in recent years (Lafarga, 2019). Hence, the entrepreneurial sector constantly increases, corresponding to around 150 new companies each year (Deprá et al., 2020); as a result, this market is expected to grow to USD 53.43 billion by 2026 (Mehariya et al., 2021).

Most of these ingredients/products use *Chlorella vulgaris* and *Arthrospira platensis*, leading worldwide in the microalgal segment. In addition, its long history of use has assured it the GRAS status (Generally Regarded as Safe) from the Food and Drug Administration (FDA) and its marketing in the European Union (EU) without the need to comply with EU Regulation 2015/2283 on novel foods (EFSA, 2016).

Such products are often based on the whole dry biomass and commercialized as dietary supplements in the form of dried powder or capsules. Other microalgal products include capsules rich in carotenoid extracts or polyunsaturated fatty acids (PUFAs). Moreover, there is a tendency to incorporate the dry biomass, pastes, or extracts from microalgae into food formulations (milkshakes, chocolate, soups, breadsticks, crackers, juices, smoothies, snacks, pasta, yogurts, vegan meats, cakes, bread, and biscuits) as a bioactive ingredient given their nutritional and functional properties (Lafarga, 2019).

Among the bioactive compounds of microalgae, carotenoids and chlorophylls stand out, which are natural pigments that have been proposed for health maintenance. Nonetheless, in order to assume the role of the protagonist in the biological functions, these molecules must be bioaccessible for intestinal uptake and subsequent systemic distribution (Kopec & Failla, 2018). What is more, liberating these molecules from the microalgae matrix is usually a limiting step since these compounds are located within the cell, most of the time protected by a robust

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cell wall. To enhance carotenoid and chlorophyll release, disorganization/elimination of such structures must be achieved by technological processing (Bernaerts et al., 2020).

Therefore, to design a functional ingredient/product from microalgae with enhanced carotenoid and chlorophyll bioaccessibility, one of the strategies is to use process intensification techniques such as ultrasound, which is widely applied to rupture cell walls (Vasistha et al., 2021). Another alternative is to use emulsion-based administration systems that improve the stability of these compounds during the digestion process and enhance bioaccessibility (Xavier & Mercadante, 2019).

Given the above, this study aimed to investigate the impact of different types of the ingredients/products (whole dry biomass [WDB], whole ultrasonicated paste [WUP], and liposoluble pigment emulsion [LPE]) on bioaccessibility and uptake by Caco-2 cells of carotenoids and chlorophylls from *Chlorella vulgaris* and *Arthrospira platensis* in order to formulate microalgae-based foods.

2. Material and methods

2.1. Chemicals

The standards of all-*trans*-lutein, all-*trans*-β-cryptoxanthin, and all-*trans*-β-carotene (purity ≥ 98%, HPLC), were purchased from Sigma-Aldrich (Darmstadt, Germany). The α-amylase (Sigma A3176), pepsin (Sigma P7000), bile (Sigma B8631), pancreatin (Sigma P1750), lipase (Sigma L3126), Dulbecco's modified Eagle's medium–high glucose (DMEM), fetal bovine serum (FBS), non-essential amino acid solution, and penicillin–streptomycin solution were also acquired from Sigma-Aldrich (St. Louis-MO, USA). Methanol (MeOH), methyl *tert*-butyl ether (MTBE), ethanol, acetone, ethyl acetate, petroleum ether, and diethyl ether were purchased from Merck (Darmstadt, Germany). Caco-2 cell cultures were acquired from the Cell Bank of Rio de Janeiro (Rio de Janeiro, Brazil).

2.2. Microalgal culture and biomass production

Axenic cultures of *Chlorella vulgaris* (CPCC90) and *Arthrospira platensis* (UTEX3086) were used in the experiments. Stock cultures were propagated and maintained in a synthetic BG-11 medium (Braun-Grunow medium) (Rippka et al., 1979). The incubation conditions were a temperature of 26 °C, a photon flux density of 15 μmol m^{-2} s^{-1}, and a photoperiod of 12 h.

The biomass productions were made under phototrophic conditions. The cultivations were performed in a bubble column photobioreactor operating under a batch regime and fed on 2.0 L of BG-11 medium (Maroneze et al., 2019a). The experimental conditions were as follows: initial cell concentration of 100 mg L^{-1}, isothermal reactor operating at a temperature of 26 °C, a photon flux density of 150 μmol m^{-2} s^{-1}, continuous aeration of 1 VVM (volume of air per volume of culture per minute) with the injection of air enriched with 15% carbon dioxide, and a 24:0h light/dark photoperiod. The residence time for each culture was defined as the time elapsed to reach the maximum biomass density. The biomass was separated from the culture medium by centrifugation (1500 × g; 10 min; 10 °C), the supernatant was discarded, and the wet biomass (95% moisture) was stored immediately in closed containers protected from light under refrigeration until use.

2.3. Ingredient/product preparation

The wet biomass of *Chlorella vulgaris* and *Arthrospira platensis* were submitted to different preliminary operations to obtain the three ingredients/products used in the *in vitro* digestion protocol: WDB, WUP, and LPE. Before the *in vitro* digestion for WDB, the wet biomass was frozen at −18 °C for 24 h and freeze-dried (Liotop L101, São Carlos-SP, Brazil) for 24 h at −50 °C above −175 μm Hg. Subsequently, aliquots of 0.1 ± 0.02 g of freeze-dried biomass were weighed and combined with 10 mL (NaCl 120 mol L^{-1}, $CaCl_2$ 6 mmol L^{-1}, KCl 5 mmol L^{-1}). In parallel, for the WUP, 0.8 ± 0.02 g aliquots were separated from the wet biomass of *Chlorella vulgaris* and *Arthrospira platensis* (cellular concentrations of 4.8 and 4.1 g L^{-1}, respectively), equivalent to 0.1 ± 0.02 g of dry biomass, and 10 mL of saline solution were added. The resulting mixture was subjected to 15 min of an ultrasonic probe (Unique, Indaiatuba-SP, Brazil) according to Gille et al. (2016), with adaptations. The ultrasonic parameters were a probe with a 13-mm wide tip, 400 W, 40 kHz, and an ice bath to control the temperature (0 ± 2 °C). The LPE was obtained exhaustively from 0.1 ± 0.02 g of the biomass (see Section 2.6) and later emulsified, as described by Salvia-Trujillo et al. (2017), with adaptations. The LPE was 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; 4 min). A total of 5 g of emulsion per LPE was prepared to add 10 mL of saline solution.

2.4. In vitro digestion

The *in vitro* assay to estimate bioaccessibility was performed according to the protocol adapted from Murador et al. (2021). For the oral phase, 1.0 g of microalgae ingredients/products were added to 6 mL of a solution of artificial saliva containing 106 U mL^{-1} of α-amylase (Fernandes et al., 2021b), 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 (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 started with a porcine and ovine bile solution (3 mL; 40 mg mL^{-1} in 100 mM $NaHCO_3$), 4000 U mL^{-1} of porcine pancreatin and 1000 U mL^{-1} of lipase from porcine pancreas. The pH was adjusted to 6.5 and the total volume to 50 mL, and the solution was incubated (2 h; 37 °C; 7.5 × g). The solution was centrifuged after completing the *in vitro* digestion (8000 × g; 60 min; 4 °C). The supernatant containing the mixed micelles was collected, covered with nitrogen gas, frozen at −40 °C, and lyophilized for further extraction of pigments. The pigments' bioaccessibility was calculated as the ratio between pigment content in the micellar fraction (supernatant) and the initial content of the pigments (Eq. (1)).

$$Bioaccessibility\ (\%) = \frac{Pigments\ (supernatant)}{Pigments\ (initial\ content)} \times 100 \quad (1)$$

2.5. Caco-2 cell culture

The greater representativeness of the total and individual bioaccessibility of pigments was subjected to uptake by caucasian colon adenocarcinoma (Caco-2). The cells were cultivated according to Natoli et al. (2011), 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 FBS (15%), and maintained at 37 °C and 5% CO_2. After the culture acquired 80% of confluence (~10 days), FBS was reduced to 10% until monolayer formation, and the experiments were carried out. Monolayers were washed with phosphate-buffered saline (PBS) and DMEM (final volume of 5 mL) and incubated containing 25% of the micellar fraction obtained from the *in vitro* digestion step added to the monolayers, followed by incubation for 4 h (37 °C; 5% CO_2 of the atmosphere) (Nascimento et al., 2021; Fernandes et al., 2021a; Fernandes et al., 2021b). Subsequently, Caco-2 monolayers were washed with PBS, and 5 mL of pure DMEM medium were added and submitted to incubation for another 6 h. After this period, the cells were harvested with a rubber scraper, collected, and centrifuged (1500 × g; 5 min; 4 °C). Cell pellets were covered with nitrogen gas, frozen at −40 °C, and lyophilized to further extract the absorbed pigments. The cellular uptake represents the fraction of

pigments recovered in the Caco-2 cells compared to the bioaccessible content of the pigments in the micellar fraction (Eq. (2)).

$$Uptake\ (\%) = \frac{Pigments\ (Caco-2cells)}{Pigments\ (micelar\ fraction\ content)} \times 100 \quad (2)$$

2.6. Pigment analysis

The *Chlorella vulgaris* and *Arthrospira platensis* carotenoids and chlorophylls were exhaustively extracted from aliquots of 0.1 ± 0.02 g from freeze-dried biomass (see the parameters in Section 2.3.) with ethyl acetate and methanol using a mortar and pestle followed by centrifugation (Hitachi, Tokyo, Japan) (7 min; 1,500 × g). The homogenized sample suspension was filtered through a 0.22-μm polyethylene membrane and concentrated in a rotary evaporator (<30 °C); then, the extract was transferred to a mixture of petroleum ether/diethyl ether [1:1 (v/v)], and extraction solvent was removed by washing (Mandelli et al., 2012).

One aliquot of the extract obtained as described above was the denominated control extract; it represented the original content of carotenoids and chlorophyll in *Chlorella* and *Arthrospira* biomass before *in vitro* digestion and concentrated in a rotary evaporator (<30 °C), dried under N_2 flux, and stored at −40 °C until injection into the high-performance liquid chromatography photodiode array mass spectrometry (HPLC-PDA-MS/MS). Another aliquot (LPE) was subjected to the simulated digestion procedure.

The remaining pigments from *in vitro* digestion and uptake by Caco-2 cells were extracted to the protocol adapted from Ordóñez-Santos et al. (2015). The sample was exhaustively extracted by adding 15 mL of petroleum ether/diethyl ether [1:1 (v/v)] and subjected to 5 min of ultrasonic cycles (see parameters in Section 2.3.), centrifuged, and the supernatant was collected. The process was repeated until the supernatant became colorless.

The extracts intended for carotenoid analysis were saponified for 16 h with 10% (w/v) methanolic potassium hydroxide (KOH) at room temperature, and the alkali was removed by washing with distilled water. The chlorophyll extracts were transferred to diethyl petroleum ether/diethyl ether (1:1); then, the extraction solvent was removed by washing the ether phase with water. All extracts were concentrated in a rotary evaporator, placed in the N_2 atmosphere, and kept at −40 °C in the dark until chromatographic analysis.

2.7. HPLC-PDA-MS/MS analysis

The carotenoids and chlorophylls were analyzed by HPLC (Shimadzu, Kyoto, Japan) equipped with binary pumps (model LC-20AD), an online degasser, and an automatic injector (model SIL-20A HT). The chromatography with a PDA (model SPD-M20A) was connected to an atmospheric pressure chemical ionization (APCI) source (Shimadzu America, Columbia, MD, USA) and a mass spectrometer Shimadzu 8040 triple quadrupole. Pigment separation was performed on a C30 YMC column (5 μm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA); HPLC-PDA analysis was performed according to Rodrigues et al. (2015). Prior to HPLC-PDA analysis, the sample was solubilized in methanol (MeOH)/methyl *tert*-butyl ether (MTBE) (70:30) and filtered through Millipore membranes (0.22 μm). The mobile phases A (MeOH) and B (MTBE) used a linear gradient program as follows: from 0 to 30 min, 5% B; from 30 to 40 min, 5 to 30% B; from 40 to 41 min, 30 to 50% B, and 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 for carotenoids and 660 nm for chlorophylls. Carotenoids and chlorophylls were individually quantified by HPLC-PDA using six-point analytical curves ($r^2 = 0.99$) of all-*trans*-lutein (1.0–50.0 and 0.05–10 μg mL^{-1}), all-*trans*-β-cryptoxanthin (1.0–60 μg mL^{-1}), all-*trans*-β-carotene (1.0–50 and 0.05–10 μg mL^{-1}), and chlorophyll *a* (0.5–40.0 μg mL^{-1}).

The MS/MS parameters were set according to Giuffrida et al. (2017), with adaptations. The APCI interface operated in positive (+) mode; the other parameters were as follows: 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. The identification quality was improved by using MS/MS simultaneously to select the ion monitoring and multiple reaction monitoring modes.

Identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV–Vis spectrum (spectral fine structure [λmáx], ratio of the height of the longest wavelength absorption peak [III] and the middle absorption peak [II], ratio of the *cis* peak [AB], and the middle absorption peak [II]), and mass characteristics (protonated molecule ($[M+H]^+$)) and MS/MS (fragments), compared with data available in the literature (Nascimento et al., 2021; Fernandes et al., 2021b, 2020, 2017; Maroneze et al., 2020; Patias et al., 2017; Rodrigues et al., 2015, 2014).

2.8. Statistical analysis

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

3. Results and discussion

3.1. Carotenoid composition in the ingredients/products before and after digestion

A total of 28 carotenoids were separated in all assays for the ingredients/products of *Chlorella vulgaris* and *Arthrospira platensis* (Figs. S1 and S2). The separated carotenoids were identified or tentatively identified (Table 1). A description of microalgae carotenoid identification using chromatographic information HPLC-PDA-MS/MS (APCI positive mode) was previously described in detail in the literature by Fernandes et al. (2021b), Nascimento et al. (2021), Maroneze et al. (2020), Patias et al. (2017), and Rodrigues et al. (2015, 2014).

The carotenoid content quantified in the control extracts for the microalgae *Chlorella vulgaris* and cyanobacteria *Arthrospira platensis* is illustrated in Fig. 1, followed by the absolute content after *in vitro* digestion of ingredients/products. The total carotenoid levels from control extracts were 3014.20 and 1252.17 μg g^{-1} dry weight for *Chlorella vulgaris* and *Arthrospira platensis*, respectively (Table S1).

The carotenoid composition in the control extract *from Chlorella vulgaris* is shown in Fig. 1a. Among the 12 compounds identified, the major carotenoids were all-*trans*-lutein (1451.12 μg g^{-1}, peak 17) and all-*trans*-β-carotene (456.92 μg g^{-1}, peak 39), which corresponds to 63% of the total carotenoid content, followed by all-*trans*-α-carotene (177.92 μg g^{-1}, peak 36), 9-*cis*-neoxanthin (155.18 μg g^{-1}, peak 4), all-*trans*-violaxanthin (146.89 μg g^{-1}, peak 6), 9-*cis*-lutein (123.28 μg g^{-1}, peak 18), 15-*cis*-lutein (102.60 μg g^{-1}, peak 10), 9-*cis*-β-carotene (99.15 μg g^{-1}, peak 40), all-*trans*-luteoxanthin (89.27 μg g^{-1}, peak 8), 13-*cis*-lutein (82.48 μg g^{-1}, peak 14), 5,6:5′,6′-diepoxy-β-carotene (71.32 μg g^{-1}, peak 22), and 13-*cis*-neoxanthin (58.10 μg g^{-1}, peak 1), were also identified in these microalgae.

For *Arthrospira platensis*, 12 carotenoids were identified in the control extract (Fig. 1b). All-*trans*-β-carotene (366.62 μg g^{-1}, peak 39) and all-*trans*-zeaxanthin (242.92 μg g^{-1}, peak 19) were the majority (Fig. 3b), representing 48% of the total carotenoid content. Additionally, *Arthrospira platensis* presented unique microalgae carotenoids, different than *Chlorella vulgaris*, such as 2′-dehydrodeoxymyxol (163.87 μg g^{-1}, peak 25), 9-*cis*-echinenone (153.97 μg g^{-1}, peak 32), all-*trans*-echinenone

Table 1
Chromatographic, UV/Vis, and mass spectrometry characteristics, obtained by HPLC-PDA-MS/MS, of pigments found during the *in vitro* digestion and Caco-2 cell absorption of the microalgae *Chlorella vulgaris and Arthrospira platensis.*

Peak[a]	Pigments	t_R (min)[b]	UV–vis characteristics			[M+H]$^+$	Fragment ions (positive mode) (*m/z*)
			$\lambda_{máx}$ (nm)[c]	III/II[d] (%)	A_B/II[e] (%)		MS/MS
1	13-*cis*-neoxanthin	7.4	330, 414, 438, 469	81	17	601	583 [M+H − 18]$^+$, 565 [M+H − 18–18]$^+$, 547 [M+H − 18–18–18]$^+$, 509 [M+H − 92]$^+$
2	13-*cis*-neochrome	7.9	337, 400, 419, 445	66	47	601	583 [M+H − 18]$^+$, 547 [M+H − 18–18–18]$^+$, 221
3	hydroxychlorophyll *b*	8.6	467, 647	na[f]	na	923	645 [M+H − 278]$^+$
4	9-*cis*-neoxanthin	8.7	327, 415, 438, 468	78	0	601	583 [M+H − 18]$^+$, 565 [M+H − 18–18]$^+$, 547 [M+H − 18–18–18]$^+$, 509 [M+H − 92]$^+$
5	9-*cis*-neochrome	8.8	325, 400,419, 440	80	15	601	583 [M+H − 18]$^+$, 547 [M+H − 18–18–18]$^+$, 221
6	all-*trans*-violaxanthin	9.3	415, 438, 467	83	0	601	583 [M+H − 18]$^+$, 565 [M+H − 18–18]$^+$, 509 [M+H − 92]$^+$
7	9-*cis*-violaxanthin	9.9	326, 412, 435, 463	63	20	601	583 [M+H − 18]$^+$, 565 [M+H − 18–18]$^+$, 509 [M+H − 92]$^+$
8	all-*trans*-luteoxanthin	10.3	400, 420, 447	100	0	601	583 [M+H − 18]$^+$
9	13-*cis*-antheraxanthin	10.4	326, 415, 438, 467	72	13	585	567 [M+H − 18]$^+$, 549 [M+H − 18–18]$^+$, 531
10	15-*cis*-lutein	12.4	328, 415, 438, 465	14	26	569	551 [M+H − 18]$^+$, 533 [M+H − 18–18]$^+$
11	hydroxychlorophyll *a*	12.7	432, 664	na	na	909	631 [M+H − 278]$^+$
12	all-*trans*-antheraxanthin	12.8	416, 442, 473	60	0	585	567 [M+H − 18]$^+$, 549 [M+H − 18–18]$^+$, 529 [M+H − 56]$^+$, 221
13	chlorophyll *b*	13.2	469, 654	na	na	907	629 [M+H − 278]$^+$, 569 [M+H − 278–60]$^+$
14	13-*cis*-lutein	13.7	331, 415, 437, 465	37	44	569	551 [M+H − 18]$^+$, 533 [M+H − 18–18]$^+$
15	chlorophyll *b*'	14.8	468, 650	na	na	907	629 [M+H − 278]$^+$, 569 [M+H − 278–60]$^+$
16	15-*cis*-zeaxanthin	15.3	335, 418, 442, 468	0	48	569	551 [M+H − 18]$^+$, 533 [M+H − 18–18]$^+$, 477 [M+H − 92]$^+$
17	all-*trans*-lutein	15.6	419, 443, 471	57	0	569	551 [M+H − 18]$^+$, 533 [M+H − 18–18]$^+$
18	9-*cis*-lutein	18.7	326, 420, 440, 465	71	13	569	551 [M+H − 18]$^+$, 533 [M+H − 18–18]$^+$, 495, 477 [M+H − 92]$^+$, 459
19	all-*trans*-zeaxanthin	18.9	425, 449, 475	25	0	569	551 [M+H − 18]$^+$, 533 [M+H − 18–18]$^+$, 495, 477 [M+H − 92]$^+$, 459
20	chlorophyll *a*	19.6	431, 665	na	na	893	615 [M+H − 278]$^+$, 583 [M+H − 278–31]$^+$, 555 [M+H − 278–59]$^+$
21	all-*trans*-canthaxanthin	20.8	472	nc[g]	0	565	547 [M+H − 18]$^+$
22	5,6:5′,6′-diepoxy-β-carotene	21.8	419, 439, 467	100	0	569	551 [M+H − 18]$^+$, 477 [M+H − 92]$^+$, 205
23	chlorophyll *a*'	22.0	431, 665	na	na	893	615 [M+H − 278]$^+$, 583 [M+H − 278–31]$^+$, 555 [M+H − 278–59]$^+$
24	9-*cis*-zeaxanthin	23.7	338, 420, 445, 470	33	25	569	551 [M+H − 18]$^+$, 533 [M+H − 18–18]$^+$, 495, 477 [M+H − 92]$^+$, 459
25	2′-dehydrodeoxymyxol	25.4	445, 473, 504	63	0	567	549 [M+H − 18]$^+$
26	5,6-epoxy-β-carotene	27.7	420, 446, 470	50	0	553	535 [M+H − 18]$^+$, 461 [M+H − 92]$^+$, 205
27	all-*trans*-β-cryptoxanthin	28.4	420, 450, 473	25	0	553	535 [M+H − 18]$^+$
28	hydroxypheophytin *a*	29.5	408, 666	na	na	887	869 [M+H − 18]$^+$; 803 [M+H − 63]$^+$; 609 [M+H − 278]$^+$; 591 [M+H − 278–18]$^+$; 531 [M+H − 278–18 − 60]$^+$
29	all-*trans*-echinenone	30.2	462	nc	0	551	533 [M+H − 18]$^+$, 427, 203
30	15-*cis*-β-carotene	30.3	337, 420, 449, 471	5	50	537	457 [M+H − 80]$^+$, 444 [M − 92]$^+$, 399 [M − 137]$^+$, 177
31	hydroxypheophytin *a*'	30.6	400, 667	na	na	887	869 [M+H − 18]$^+$; 803 [M+H − 63]$^+$; 609 [M+H − 278]$^+$; 591 [M+H − 278–18]$^+$; 531 [M+H − 278–18 − 60]$^+$
32	9-*cis*-echinenone	32.6	342, 450	nc	20	551	533 [M+H − 18]$^+$, 427, 203
33	pheophytin *b*	34.7	436, 658	na	na	886	608 [M+H − 278]$^+$
34	pheophytin *b*'	36.5	435, 653	na	na	886	608 [M+H − 278]$^+$
35	13-*cis*-β-carotene	37.4	337, 420, 444, 470	17	50	537	444 [M+H − 92]$^+$, 399, 355
36	all-*trans*-α-carotene	37.5	420, 445, 473	62	0	537	444 [M+H − 92]$^+$, 399, 355
37	pheophytin *a*	37.9	408, 667	na	na	871	593 [M+H − 278]$^+$; 533 [M+H − 278–60]$^+$
38	pheophytin *a*'	38.7	408, 666	na	na	871	593 [M+H − 278]$^+$; 533 [M+H − 278–60]$^+$
39	all-*trans*-β-carotene	39.6	425, 451, 476	25	0	537	444 [M+H − 92]$^+$, 399, 355
40	9-*cis*-β-carotene	41.3	341, 420, 446, 472	20	14	537	444 [M+H − 92]$^+$, 399, 355

[a] Numbered according to Fig. 1, Fig. 2, Fig. 3 and Fig. 4.
[b] t_R: Retention time on the C30 column.
[c] Linear gradient MEOH: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 (A_B) and the middle absorption peak (II).
[f] Not applied.
[g] Not calculated.

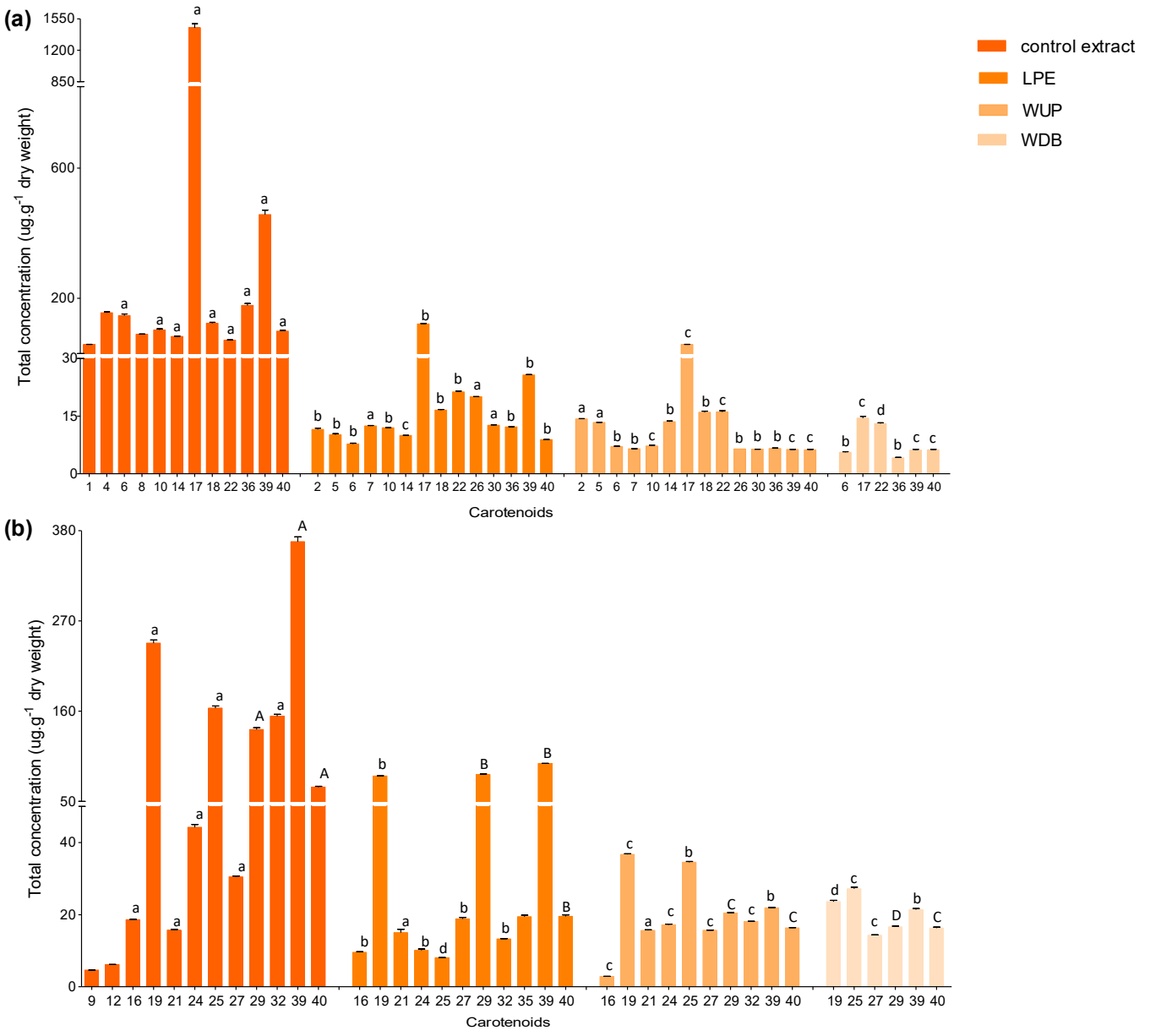

Fig. 1. The original content of *Chlorella vulgaris* (a) and *Arthrospira platensis* (b) carotenoids (control extract) before digestion and incorporation into the micelle after *in vitro* digestion from liposoluble pigments emulsion (LPE), whole ultrasonicated paste (WUP), and whole dried biomass (WDB). The numbers correspond to the compounds identified (Table 1). Significant differences are indicated by different capital and lowercase letters ($p < 0.05$).

(137.71 μg g^{-1}, peak 29), and all-*trans*-canthaxanthin (15.71 μg g^{-1}, peak 21). Other carotenoids were detected as 9-*cis*-β-carotene (67.37 μg g^{-1}, peak 40), 9-*cis*-zeaxanthin (44.20 μg g^{-1}, peak 24), all-*trans*-β-cryptoxanthin (30.47 μg g^{-1}, peak 27), 15-*cis*-zeaxanthin (18.59 μg g^{-1}, peak 16), all-*trans*-antheraxanthin (6.16 μg g^{-1}, peak 12), and 13-*cis*-antheraxanthin (4.60 μg g^{-1}, peak 9).

Considering the compounds identified, in the control extract of *Chlorella vulgaris* and *Arthrospira platensis*, only two carotenoids were common (peaks 39 and 40), providing a diverse carotenoid profile (Figs. S1a_1 and S2b_1). This is because *Chlorella vulgaris* and *Arthrospira platensis* are species from different classes (*Chlorophyceae* and *Cyanophyceae*, respectively), and the carotenoid profile differs between species according to phylogenetic diversity, morphological and cytological characteristics, and the composition of genes and enzymes that are specific in each species of microalgae (Takaichi, 2009).

As shown in Fig. 1, incorporating the compounds into the micellar fraction strongly depends on the species, ingredients/products used, and carotenoid type. Thus, after the *in vitro* digestion, there was a significant quantitative carotenoid reduction in all tested cases except for all-*trans*-canthaxanthin (peak 21) in WUP and LPE *Arthrospira platensis* (Table S1). This behavior was expected since the complexity of factors can contribute to reducing the carotenoid content at the micellar incorporation level. Among the physical aspects of interference is the breakdown of the cell wall of the matrix, that is, the intracellular release of the compounds, which is sometimes not fully effective and impairs the absolute content of the micellarized carotenoids. Another determining factor is related to the chemical structuring of the compounds, as carotenoids are known to be chemically unstable and susceptible to degradation during their passage through the gastrointestinal tract, which can alter their micellar profile at a qualitative and quantitative level (Xavier & Mercadante, 2019; Fernandes et al., 2021b). In contrast, all-*trans*-canthaxanthin behavior was surprising. Other ketocarotenoids,

such as its all-*trans*-echinenone precursor, showed reduced levels after the digestion process, demonstrating that the ketone functional group in the chemical structure does not seem to be the determining factor for the stability of the absolute content at the micellar level. However, as it is a xanthophyll with a less hydrophobic character than other carotenoids, we cannot rule out the possibility of its chemical structure contributing to greater surface uptake at the micellar level. Nonetheless, the most plausible hypothesis is that the intracellular localization of canthaxanthin in *Arthrospira platensis* may justify the stability of its absolute content in the micellar fraction. In cyanobacteria, this compound is confined to the most superficial ends of the cell membrane (Mulders et al., 2014), which may facilitate its degree of release and subsequent micellization. However, we consider that more directed studies in this regard are necessary for more effective conclusions.

The WDB of the *Chlorella vulgaris* exhibited the lowest total carotenoid incorporation content (49.69 μg g^{-1}). Only six carotenoids were incorporated into the micelle after digestion (Fig. 1a), being all-*trans*-lutein (14.51 μg g^{-1}) the major carotenoid, followed by 5,6:5′,6′-diepoxy-β-carotene (13.07 μg g^{-1}), 9-*cis*-β-carotene (6.23 μg g^{-1}), and all-*trans*-β-carotene (6.17 μg g^{-1}), which corresponds to 80% of the fraction of incorporated carotenoids. The rest (about 20%) was constituted by all-*trans*-violaxanthin (5.57 μg g^{-1}) and all-*trans*-α-carotene (4.15 μg g^{-1}).

In the WDB from *Arthrospira platensis*, the total carotenoid incorporation was also limited (118.78 μg g^{-1}), and six different carotenoids were incorporated into the micelle after digestion (Fig. 1b). 2′-Dehydrodeoxymyxol (27.20 μg g^{-1}) was quantitatively dominant, followed by all-*trans*-zeaxanthin (23.60 μg g^{-1}) and all-*trans*-β-carotene (21.37 μg g^{-1}), which represented 63% of the total incorporated carotenoid content. In addition, all-*trans*-echinenone (16.58 μg g^{-1}), 9-*cis*-β-carotene (16.25 μg g^{-1}), and all-*trans*-β-cryptoxanthin (14.28 μg g^{-1}) were detected as minor carotenoids incorporated.

The food industry mainly focuses on adding WDB as a food ingredient (Lafarga, 2019). Nevertheless, in this form, the transfer of carotenoids from the matrix to the micelles is consistently low, regardless of the species. This limitation may be associated with the constituents of the cell wall or the location of the carotenoids in the microalgae cell. Hence, increased micellar incorporation of carotenoids only can be achieved using processing operations to release these biomolecules (Bernaerts et al., 2020).

The use of ultrasound is considered a helpful processing method in disrupting the microalgae cells and has been extensively used. During this process, sound waves are applied to the microalgae paste, generating an alternative arrangement of compression and rarefaction (Vasistha et al., 2021). Microbubbles can form in the rarefaction region, leading to cytoplasmic disruption and the release of molecules. Furthermore, as ultrasound processing requires moisture, using the microalgal paste without further dehydration may reduce the cost of the drying process, albeit the shelf life remains a techno-economic bottleneck for the use of this kind of raw material (Zou et al., 2021).

The effect of WUP from *Chlorella vulgaris* increased the total content of miceralized carotenoids by 184.46 μg g^{-1}. An increase of roughly 3.7 times compared to the WDB. With a total of 14 different carotenoids incorporated into the micelles (Fig. 1a), all-*trans*-lutein (58.20 μg g^{-1}) was the major carotenoid, followed by 5,6:5′,6′-diepoxy-β-carotene (16.13 μg g^{-1}), all-*trans*-violaxanthin (7.07 μg g^{-1}), all-*trans*-α-carotene (6.60 μg g^{-1}), as well as 9-*cis*-lutein (16.04 μg g^{-1}),13-*cis*-lutein (13.54 μg g^{-1}), and 15-*cis*-lutein (7.26 μg g^{-1}), which had not been incorporated in the WDB. On the other hand, all-*trans*-β-carotene (6.33 μg g^{-1}) and 9-*cis*-β-carotene (6.25 μg g^{-1}) have the lowest micellar incorporation values. Moreover, 13-*cis*-neochrome (14.30 μg g^{-1}; peak 2), 9-*cis*-neochrome (13.36 μg g^{-1}; peak 5), 9-*cis*-violaxanthin (6.52 μg g^{-1}; peak 7), 5,6-epoxy-β-carotene (6.45 μg g^{-1}; peak 26), and 15-*cis*-β-carotene (6.41 μg g^{-1}; peak 30) were not detected in the control extract but identified after the *in vitro* digestion (Fig. 3a), and this was likely a degradation product.

For *Arthrospira platensis* (WUP), the total carotenoid content of the mixed micelles was 199.27 μg g^{-1}; therefore, total incorporation was 1.6 times higher than the WDB. Ten carotenoids were identified after the *in vitro* digestion process (Fig. 1b). The 2′-dehydrodeoxymyxol (34.53 μg g^{-1}), all-*trans*-zeaxanthin (36.70 μg g^{-1}), all-*trans*-β-carotene (21.90 μg g^{-1}), and all-*trans*-echinenone (20.47 μg g^{-1}) were the majority components of micelles, accounting for 57% of total carotenoids. The other micellarized carotenoids were 9-*cis*-β-carotene (16.31 μg g^{-1}) and all-*trans*-β-cryptoxanthin (15.65 μg g^{-1}). Positively speaking, 9-*cis*-echinenone (peak 32), 9-*cis*-zeaxanthin (peak 24), all-*trans*-canthaxanthin (peak 21), and 15-*cis*-zeaxanthin (peak 16), which had not constituted the micelle in the WDB, were effectively incorporated into the micelles with WUP at concentrations ranging from 2.85 to 18.10 μg g^{-1} (Table S1).

Even with the lightly improved incorporation of carotenoids through the WUP, the low transfer rate is probably related to the effect of other biomass constituents (Bernaerts et al., 2020). By taking this into account, we evaluated the efficiency of carotenoid micellarization from the LPE. Additionally, previous research has indicated that emulsions have proven to be an attractive option mainly due to the increased stability and incorporation of carotenoids into the micellar phase (Xavier & Mercadante, 2019).

The total carotenoid transfer efficiency from *Chlorella vulgaris* to mixed micelles from digestion of LPE (302.35 μg g^{-1}) was 6 and 1.6 times higher ($p < 0.05$) compared to the total carotenoid content incorporated into the micellar phase from WDB and WUP, respectively (Fig. 1a and Table S1).

Regarding the individual compounds, when applying *Chlorella vulgaris* LPE, carotenoid incorporation increased significantly concerning WDB and WUP in the following order (Fig. 1a2): all-*trans*-lutein (121.04 μg g^{-1}) > all-*trans*-β-carotene (25.71 μg g^{-1}) > 5,6:5′,6′-diepoxy-β-carotene (21.32 μg g^{-1}) > all-*trans*-α-carotene (12.21 μg g^{-1}) > 9-*cis*-β-carotene (8.91 μg g^{-1}). However, all-*trans*-violaxanthin (7.75 μg g^{-1}) showed no significant differences in relation WDB and WUP from *Chlorella vulgaris*.

As for carotenoid behavior detected after digesting WUP and LPE from *Chlorella vulgaris*, the concentration increased, even if not significantly, for 5,6-epoxy-β-carotene (20.05 μg g^{-1}), 9-*cis*-lutein (16.58 μg g^{-1}), 15-*cis*-β-carotene (12.65 μg g^{-1}), 9-*cis*-violaxanthin (12.45 μg g^{-1}), and 15-*cis*-lutein (11.99 μg g^{-1}). Nonetheless, concentrations decreased ($p < 0.05$) for 13-*cis*-neochrome (11.56 μg g^{-1}), 9-*cis*-neochrome (10.14 μg g^{-1}), and 13-*cis*-lutein (9.97 μg g^{-1}) (compared to WUP).

In contrast, for *Arthrospira platensis* (LPE), the total carotenoid content incorporated into the mixed micelles was 373.37 μg g^{-1}. An increase of about 3.1 and 1.8 times compared to the other ingredients/products (WDB and WUB, respectively) (Fig. 1b and Table S1). Notably, the micellar incorporation of compounds increased significantly regarding WDB and WUP. For all-*trans*-β-carotene (peak 39), all-*trans*-echinenone (peak 29), all-*trans*-zeaxanthin (peak 19), 9-*cis*-β-carotene (peak 40), 13-*cis*-β-carotene (peak 35), and all-*trans*-β-cryptoxanthin (peak 27), which were effectively micellarized at concentrations ranging from 18.81 to 96.13 μg g^{-1} Digestion from LPE led to lower 2′-dehydrodeoxymyxol levels (8.04 μg g^{-1}) compared to WDB and WUP. Additionally, peak 35 (19.41 μg g^{-1}) was only detected after *in vitro* digestion, possibly being a degradation product (Fig. S2b_2).

Considering the compounds detected after digestion of WUP and LPE from *Arthrospira platensis*, the carotenoid content significantly increased for 15-*cis*-zeaxanthin (9.55 μg g^{-1}) compared to WUP. When applying LPE, the concentration decreased, even if not significantly, for 9-*cis*-zeaxanthin (10.12 μg g^{-1}), 9-*cis*-echinenone (13.25 μg g^{-1}), and all-*trans*-canthaxanthin (15.00 μg g^{-1}) compared to WUP.

In our study, the experimental conditions of *in vitro* digestion may have contributed to changes in the carotenoid profile for ingredients/products from *Chlorella vulgaris* and *Arthrospira platensis*. Among all the carotenoids identified in the *Chlorella vulgaris* control extract, 13-*cis* and 9-*cis*-neoxanthin (peaks 1 and 4) as well as all-*trans*-luteoxanthin (peak

8) not being detected in the micellar fractions of WDB, WUP, and LPE. In contrast, in the WUP and LPE from *Chlorella vulgaris*, neochrome isomers, 9-*cis*-violaxanthin, 5,6-epoxy-β-carotene, and 15-*cis*-β-carotene (peaks 2, 5, 7, 26, and 30, respectively) were only observed after *in vitro* digestion (Fig. S1 and 1a).

As for the carotenoid profile present in the *Arthrospira platensis* control extract, the 13-*cis* and all-*trans*-antheraxanthin (peaks 9 and 12) were not identified after *in vitro* digestion of WDB, WUP, and LPE, whereas one *cis* isomer (13-*cis*-β-carotene; peak 35) was only detected in *Arthrospira platensis* LPE digestion (Figs. S2 and 1b).

Considering the carotenoid structures identified after *in vitro* digestion of WUP and LPE *Chlorella vulgaris* and *Arthrospira platensis* (LPE) that were not present in the control extract, the main reactions observed were isomerization of all-*trans* configurations for *cis* and epoxidation. These findings followed, in general, previous evidence on carotenoid transformations during their passage through *in vitro* digestion, affected by gastric acid and temperature (Kopec et al., 2017, Kopec & Failla, 2018). All-*trans*-β-carotene, for instance, may undergo isomerization during the oral phase and epoxidation during the gastric phase (Tao et al., 2021). Moreover, the epoxy-furanoid rearrangement was also observed, resulting in the formation of neochrome from neoxanthin (Biehler et al., 2012).

In that regard, stability is a major challenge in carotenoid-rich foods. In order to overcome this limitation, delivery systems through the physicochemical processes, including micro- and nano-technological encapsulation, are considered potential alternatives to improve carotenoids' chemical stability and bioaccessibility and are highly recommended in formulations of ingredients or products with greater carotenoid availability (Soukoulis & Bohn, 2018; Xavier & Mercadante, 2019).

Overall, the quantities of total carotenoids micellarized from *Chlorella vulgaris* in LPE, WUP, and WDB were 1.2, 1.0, and 2.3 times less than *Arthrospira platensis* (Fig. 1); this comparison allowed us to analyze the effect of the matrix. The most expressive differences were found for WDB, which are likely related to the physiological and morphological characteristics of the investigated microalgae. Moreover, previous trials have highlighted that *Chlorella vulgaris* had a lower digestibility (~60%) than *Arthrospira platensis* (78%) (Niccolai et al., 2019). Finally, the microalgal strain was a determining factor in incorporating carotenoids.

3.2. Chlorophyll composition in ingredients/products before and after digestion

The chromatogram obtained by HPLC-PDA revealed the presence of 11 chlorophyll species in all assays for the ingredients/products of *Chlorella vulgaris* and *Arthrospira platensis* (Figs. S3 and S4). A detailed description of chlorophyll identification using chromatographic information (HPLC-PDA-MS/MS) has been recently reported by Fernandes et al. (2021b, 2020, 2017) and Maroneze et al. (2019b); thus, only considerations regarding the unidentified chlorophylls in these previous reports were discussed below.

Peak 34 was tentatively identified as pheophytin *b*' considering the characteristic UV–visible spectra (435, 653 nm) and mass spectrum data. The protonated molecule confirmed the molecular mass at *m/z* 886 and by consecutive loss of phytol group at *m/z* 608 $[M+H-278]^+$ from the protonated molecule (Chen et al., 2015). This compound was only detected after the Caco-2 cells in the LPE *Chlorella vulgaris*, suggesting that this compound is derived from chlorophyll metabolization.

The chlorophyll content and their derivatives in the control extract of *Chlorella vulgaris* and *Arthrospira platensis* before digestion and incorporation into the micelle after *in vitro* digestion of LPE, WUP, and WDB are shown in Fig. 2. The total chlorophyll level and its derivatives in the control extracts were 39,221.42 and 26,605.78 μg g^{-1} dry weight for *Chlorella vulgaris* and *Arthrospira platensis*, respectively (Table S1).

Given the characteristic of the microalgae species belonging to the phylum *Chlorophyta*, *Chlorella vulgaris* contains both chlorophylls *a* and *b*

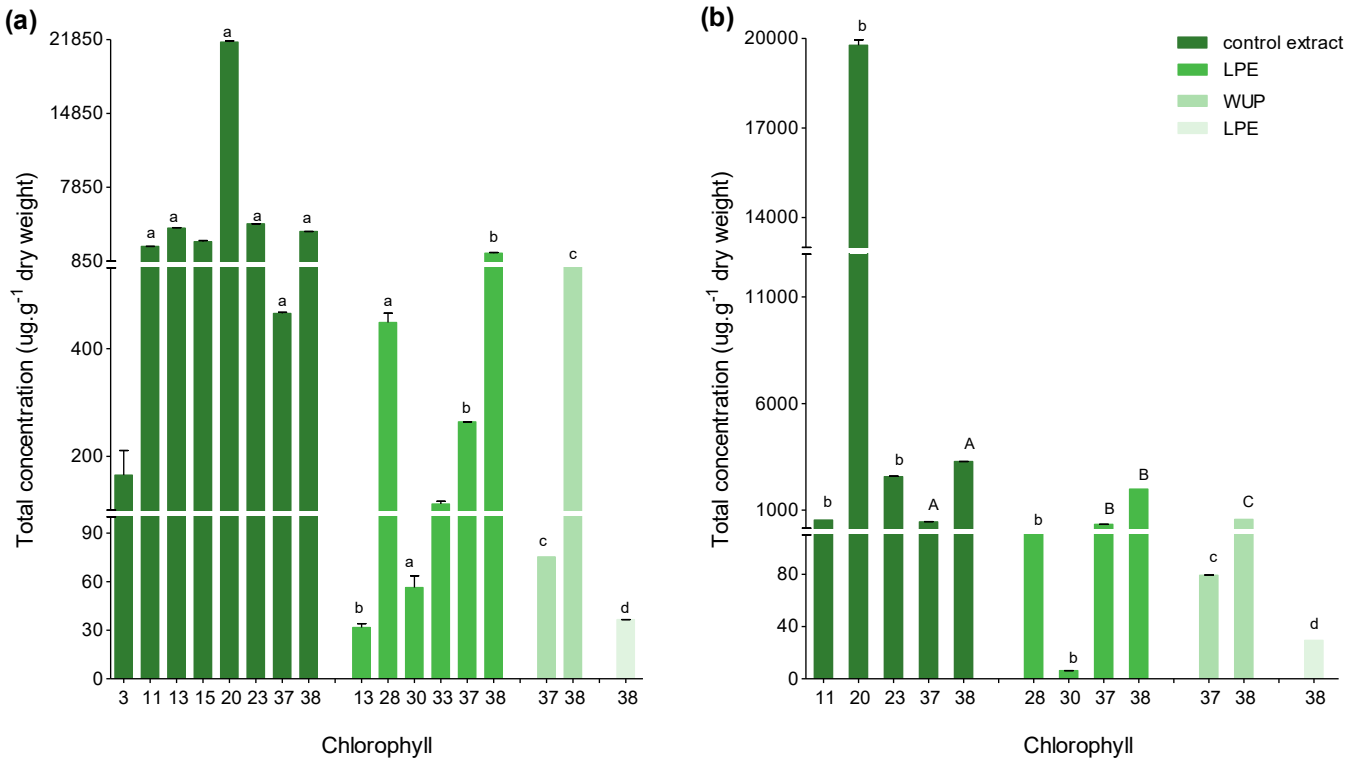

Fig. 2. The original content of *Chlorella vulgaris* (a) and *Arthrospira platensis* (b) chlorophyll and derivatives (control extract) before digestion and incorporation into the micelle after *in vitro* digestion of whole dried biomass (WDB), whole ultrasonicated paste (WUP), and liposoluble pigments emulsion (LPE). The numbers correspond to the compounds identified (Table 1). Significant differences are indicated by different capital and lowercase letters ($p < 0.05$).

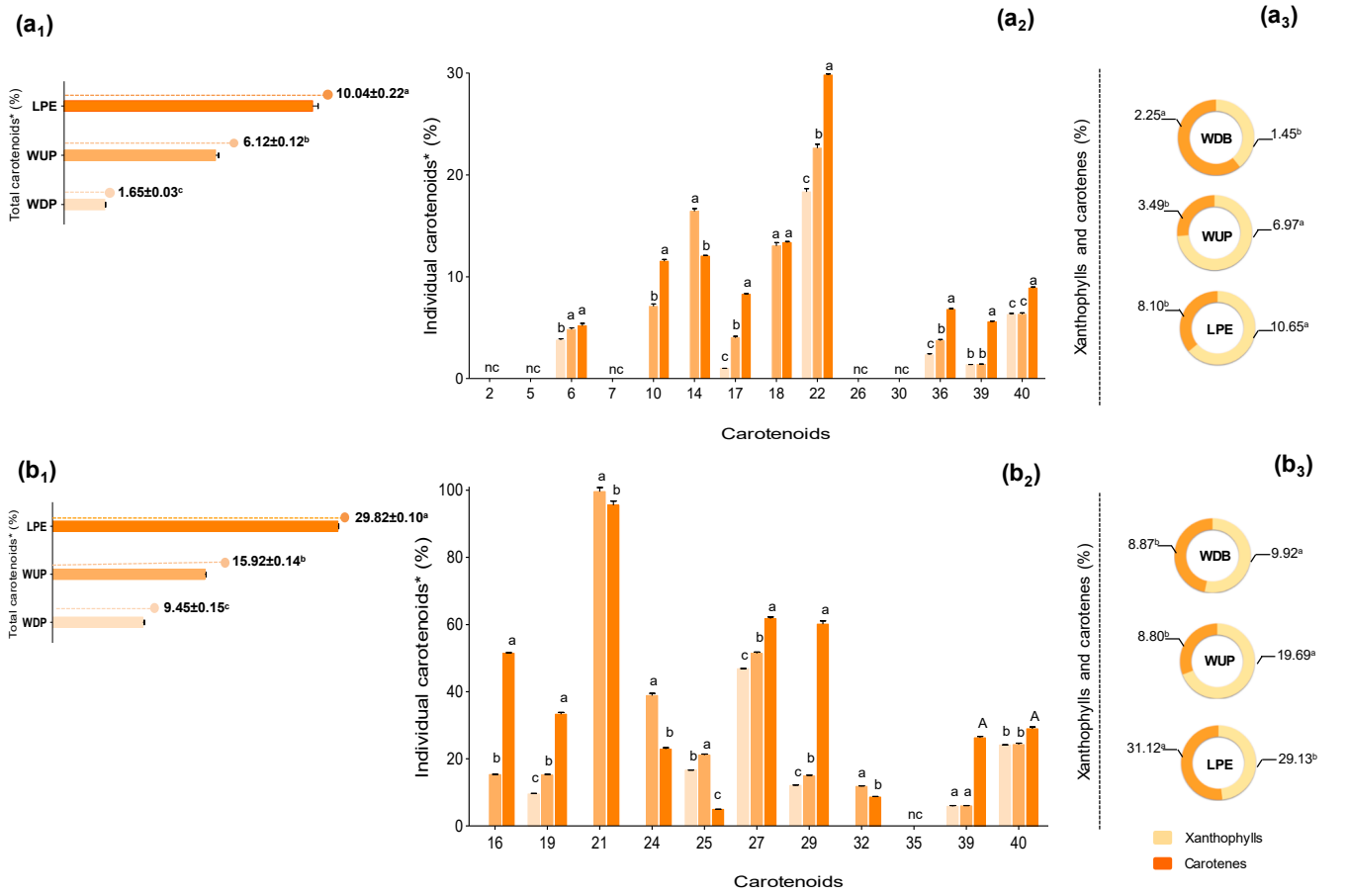

Fig. 3. Relative bioaccessibility of total carotenoids from *Chlorella vulgaris* (a_1) and *Arthrospira platensis* (b_1) and the bioaccessibility index of individual carotenoids from *Chlorella vulgaris* (a_2) and *Arthrospira platensis* (b_2). Quantitative characterization of the bioaccessibility of xanthophylls and carotenes from *Chlorella vulgaris* (a_3) and *Arthrospira platensis* (b_3) of WDB, WUP, and LPE. The numbers correspond to the compounds identified (Table 1). Significant differences are indicated by different capital and lowercase letters ($p < 0.05$); nc = not calculated.

series and its derivative compounds (Maroneze et al., 2020). A total of eight different chlorophylls and derivatives were separated and quantified in the control extract from *Chlorella vulgaris* (Figs. S3a_1 and 2a). The chlorophyll *a* (21,619.68 μg g^{-1}, peak 20) was quantitatively dominant in the profile of the control extract, followed by chlorophyll *a*' (4382.15 μg g^{-1}, peak 23), chlorophyll *b* (3980.59 μg g^{-1}, peak 13), pheophytin *a*' (3655.82 μg g^{-1}, peak 38), chlorophyll *b*' (2707.02 μg g^{-1}, peak 15), hydroxychlorophyll *a* (2245.53 μg g^{-1}, peak 11), pheophytin *a* (465.60 μg g^{-1}, peak 37), and hydroxychlorophyll *b* (165.03 μg g^{-1}, peak 3).

Among the eight chlorophylls and derivatives identified in the *Arthrospira platensis* control extract, five compounds were common to *Chlorella vulgaris* (Figs. S4a_1 and 2b). *Arthrospira platensis* belongs to the phylum *Cyanobacteria* and only contains chlorophyll *a* series (Maroneze et al., 2020). Hence, it is characterized by the prevalence of chlorophyll *a* (19,766.89 μg g^{-1}, peak 20) along with pheophytin *a*' (3279.60 μg g^{-1}, peak 38), chlorophyll *a*' (2571.01 μg g^{-1}, peak 23), representing 96% of the total chlorophyll content. Other chlorophylls were detected as hydroxychlorophyll *a* (536.91 μg g^{-1}, peak 11) and pheophytin *a* (451.37 μg g^{-1}, peak 37).

The WDB of *Chlorella vulgaris* showed the lowest total incorporation of chlorophyll derivatives. Pheophytin *a*' (peak 38) was the only chlorophyll derivative incorporated into the mixed micelles at the concentration of 36.56 μg g^{-1} (Fig. 2a). A similar situation was observed during *in vitro* digestion of WDB from *Arthrospira platensis*, where chlorophyll derivative incorporation was also restricted, and only pheophytin *a*' (29.52 μg g^{-1}) was incorporated into the micelle after digestion (Fig. 2b).

Such an effect on *Chlorella vulgaris* (WUP) allowed the total incorporation of 655.41 μg g^{-1}, being 17.8 times higher ($p < 0.05$) compared to the total content incorporated in the micellar phase from WDB (Fig. 2a). The chlorophyll derivatives micellarized were pheophytin *a*' (586.49 μg g^{-1}), the majority component of micelles, which accounted for 88% of total chlorophyll and pheophytin *a* (75.35 μg g^{-1}, peak 37). Additionally, peak 37 was previously detected in the control extract, and it had not constituted the micelle when WDB was applied.

The total chlorophyll derivative content incorporated into the mixed micelles from *Arthrospira platensis* (WUP) (646.85 μg g^{-1}) revealed a significant improvement of up to 21.9 times compared to the WDB (Fig. 2b). The main component of the micellar fraction was pheophytin *a*' (567.52 μg g^{-1}), followed by pheophytin *a* (79.33 μg g^{-1}). Thus, the behavior of *Arthrospira platensis* (WUP) was similar to *Chlorella vulgaris* (WUP), presenting the micellar incorporation of pheophytin *a* (peak 37) detected in the control extract, which had not been micellarized in the WDB.

The use of *Chlorella vulgaris* (LPE) revealed a significant increase in the total content of chlorophyll and its micellarized derivatives (2526.01 μg g^{-1}) compared to the WDB (about 70 times) and WUP (up 3.8 times) (Figs. S3a_2 and 2a). Pheophytin *a*' (1613.28 μg $g^{-1)}$ and hydroxypheophytin *a* (448.83 μg g^{-1}, peak 28) represented about 82% of the content micellar fraction. The remaining 18% is represented by pheophytin *a* (264.04 μg g^{-1}), pheophytin *b*' (111.62 μg g^{-1}, peak 34), hydroxypheophytin *a*' (56.46 μg g^{-1}, peak 31), and chlorophyll *b* (31.79 μg g^{-1}, peak 13). Peaks 28, 30, and 33 were not identified in the control

extract but detected after the simulated digestion, possibly being a degradation product. In addition, the use of LPE enabled the incorporation of chlorophyll *b* (peak 13) previously detected in the control extract, which has not been incorporated from WDB and WUP.

Nevertheless, the total incorporation of chlorophyll derivatives from *Arthrospira platensis* LPE was significantly ($p < 0.05$) higher than in WDB and WUP (from 83 to 3.7 times, respectively), totaling 2456.36 μg g^{-1} (Figs. S4b$_2$ and 2b). The majority was pheophytin *a*' (1992.39 μg g^{-1}) and pheophytin *a*' (330.82 μg g^{-1}), representing 94% of total incorporation. In addition, hydroxypheophytin *a* (126.91 μg g^{-1}, peak 28) and hydroxypheophytin *a*' (6.24 μg g^{-1}, peak 31) emerged after *in vitro* digestion. Therefore, these chlorophyll derivative fractions can be associated with the high instability of chlorophyll structure during digestion (Chen & Roca, 2018a, 2018b).

The impact of gastrointestinal conditions on the profile of chlorophyll and derivatives in microalgae ingredients/products after *in vitro* digestion can be seen in Figs. S3 and S4. For *Chlorella vulgaris*, as a consequence of the *in vitro* digestion, hydroxychlorophyll *b* (peak 3), hydroxychlorophyll *a* (peak 11), chlorophyll *b*' (peak 15), chlorophyll *a* (peak 20), and chlorophyll *a*' (peak 23) disappeared. However, after *in vitro* digestion of *Chlorella vulgaris* (LPE), the hydroxypheophytin *a*, hydroxypheophytin *a*', and pheophytin *b*' (peaks 28, 31, and 34, respectively) were observed (Figs. S3 and 2a).

Nevertheless, *Arthrospira platensis*, compounds 11, 20, and 23 were present in the control extract but not detected after digestion. In contrast, after *Arthrospira platensis* (LPE) digestion, peaks 28 and 31 were identified (Figs. S4 and 2b).

The sensitivity of natural chlorophylls to *in vitro* gastrointestinal digestion was previously reported (Chen & Roca, 2018b), and this aspect is due to the stability of chlorophyll structures being mainly affected by gastric acid and enzymes during *in vitro* digestion.

Chlorophylls under acidic conditions are prone to pheophytinisation substituting the central Mg^{2+} of the tetrapyrrole ring with two hydrogen atoms (Chen et al., 2015), thereby yielding the corresponding pheophytins. The allomerization reactions were observed during the *in vitro* digestion process since hydroxypheophytin *a* and its epimer *a*' were found only in the final digestion. These compounds are formed by the oxidation of C-13^2 of the native chlorophyll molecule (Gandul-Rojas et al., 2009). In fact, these authors showed the increased concentrations of allomerized chlorophylls and pheophytins in the micellar fraction of other food matrices during digestion due to the activity of the enzyme peroxidase or chemical reactions.

In this line, our results showed an increase of allomerized derivatives in LPE after digestion. However, in the rest of the ingredients/products, there was no presence of allomerized compounds in the micellar fraction. Thus, the origin of the allomerization reactions may be chemical. Likewise, this study also observed carotenoid oxidation reactions during the *in vitro* digestion process.

Lastly, regardless of the ingredients/products, *Chlorella vulgaris* has a slightly higher micellization rate of chlorophyll than *Arthrospira platensis* (about 1 time) (Fig. 2). Therefore, the ability of these compounds to be incorporated into micelles depended on little (or nothing) on the morphological characteristics of the species investigated.

3.3. Carotenoid bioaccessibility and cellular uptake

To complete the knowledge about the matrix effects and chemical structure in the carotenoid digestion from *Chlorella vulgaris* and *Arthrospira platensis* ingredients/products, the relative bioaccessibility (%) is illustrated in Fig. 3. Although the incorporation of some carotenoids was high, their relative bioaccessibility was low, and this is because the higher the concentrations in the control extract of the compound, the lower its bioaccessibility. Carotenoid bioaccessibility represents the relationship between the carotenoid content after simulated digestion and carotenoid content in the biomass (before *in vitro* digestion). Consequently, a high carotenoid concentration in the control extract is not required for high bioaccessibility (Gille et al., 2018).

The relative bioaccessibility of the total carotenoids in ingredients/products from *Chlorella vulgaris* was 10.04, 6.12, and 1.65% for LPE, WUP, and WDB, respectively (Fig. 3a1 and Table S2), significantly different. Notably, for *Arthrospira platensis*, LPE, WUP, and WDB were more bioaccessible (29.82, 15.92, and 9.45%, respectively) (Fig. 3b1 and Table S2).

It is well known that carotenoid bioaccessibility is often compromised by various factors, including incomplete release from the food matrix, structural and physicochemical properties, interactions with matrix interferents, and potential degradation during digestion (Sy et al., 2012; Desmarchelier & Borel, 2017). Regarding the chemical constitution of the matrix, this can positively contribute to carotenoid bioaccessibility through lipid constituents. These compounds are known to promote better carotenoid solubility in mixed micelles and consequently increase their bioaccessibility in the gastrointestinal tract (Xavier & Mercadante, 2019; Fernandes et al., 2021b); on the other hand, carbohydrate content can negatively affect carotenoid bioaccessibility (Yeum & Russell, 2002) as it is suggested that fibers and proteins also negatively affect their absorption (Desmarchelier & Borel, 2017). *Chlorella vulgaris* and *Arthrospira platensis* are species recognized as having a high protein content in their composition, with values ranging from 51 to 58 and 50 to 70% dry weight, respectively (Bleakley & Hayes, 2017); likewise, they have recognized sources of lipids. In a study by Vendruscolo et al. (2018), the lipid content of 14.23% of dry biomass was detected for *Chlorella vulgaris* and 5.07% for *Arthrospira platensis*. However, by comparing these data and our findings, one can note that the protein and lipid content are not determinant indicators of total carotenoid bioaccessibility in these species since *Chlorella vulgaris*, which has lower protein content and higher lipid content than *Arthrospira platensis*, had lower bioaccessibility values. Thus, another interference, in addition to matrix constituents, probably contributed to these bioaccessibility rates.

In the use of *Chlorella vulgaris* (WDB) (Fig. 3a2), the most bioaccessible carotenoids were 5,6:5′,6′-diepoxy-β-carotene (18.33%) and 9-*cis*-β-carotene (6.28%), whereas all-*trans*-β-carotene (1.35%) and all-*trans*-lutein (1.00%) were less bioaccessible. Nonetheless, for *Arthrospira platensis* (WDB) (Fig. 3b2), more bioaccessibility was found for all-*trans*-β-cryptoxanthin (46.85%), along with 9-*cis*-β-carotene (24.12%). In contrast, all-*trans*-zeaxanthin (9.51%) and all-*trans*-β-carotene (5.83%) were less bioaccessible.

The operating conditions related to the ultrasonication in digested *Chlorella vulgaris* (WUP) significantly increased the bioaccessibility of all-*trans*-violaxanthin (4.82%), 15-*cis*-lutein (7.09%), 13-*cis*-lutein (16.42%), all-*trans*-lutein (4.01%), 9-*cis*-lutein (13.02%), 5,6:5′,6′-diepoxy-β-carotene (22.63%), and all-*trans*-α-carotene (3.71%). While all-*trans*-β-carotene (1.39%) and 9-*cis*-β-carotene (6.31%) showed no significant differences concerning WDB (Fig. 3a2).

The use of *Arthrospira platensis* (WUP) significantly increased the bioaccessibility of 15-*cis*-zeaxanthin (15.31%), all-*trans*-zeaxanthin (15.11%), 9-*cis*-zeaxanthin (38.80%), and all-*trans*-β-cryptoxanthin (51.35%). It is worth noting that the relative bioaccessibility of unique microalgae carotenoids significantly increased up to 4.48% for 2′-dehydrodeoxymyxol (21.08%) and up to 2.83% for all-*trans*-echinenone (14.87%). In addition, all-*trans*-canthaxanthin (99.55%) and 9-*cis*-echinenone (11.76%) compounds not bioaccessible in the WDB showed bioaccessibility. In contrast, all-*trans*-β-carotene (5.98%) and 9-*cis*-β-carotene (24.21%) showed no significant differences concerning WDB (Fig. 3b2).

Applying *Chlorella vulgaris* (LPE) led to a significant increase in the bioaccessibility of most carotenoids, including 15-*cis*-lutein (11.52%), all-*trans*-lutein (8.26%), 5,6:5′,6′-diepoxy-β-carotene (29.80%), all-*trans*-α-carotene (6.77%), all-*trans*-β-carotene (5.56%), and 9-*cis*-β-carotene (8.89%) in relation to WDB and WUP. However, no significant increase for 13-*cis*-lutein bioaccessibility was observed compared with WUP. Moreover, all-*trans*-violaxanthin and 9-*cis*-lutein showed no

improvement concerning WUP (Fig. 3a2).

In the *Arthrospira platensis* (LPE), the bioaccessibility of individual carotenoids increased significantly for 15-*cis*-zeaxanthin (51.39%), all-*trans*-zeaxanthin (33.29%), all-*trans*-β-cryptoxanthin (61.74%), all-*trans*-echinenone (60.07%), all-*trans*-β-carotene (26.23%), and 9-*cis*-β-carotene (28.96%) compared to WDB and WUP. In contrast, all-*trans*-canthaxanthin, 9-*cis*-zeaxanthin, 2′-dehydrodeoxymyxol, and 9-*cis*-echinenone showed no improvement compared to WUP (Fig. 3b2).

As already mentioned, a complex set of factors interferes with carotenoid bioaccessibility. Among these factors, it is assumed that carotenoid bioaccessibility was inversely related to their hydrophobicity (Fernandes et al., 2021b). In general, our study demonstrated this trend except for *Chlorella vulgaris* (WDB) and *Arthrospira platensis* (LPE) (Fig. 3a3 and 3b3).

In addition to determining carotenoid bioaccessibility, we also investigated their uptake by Caco-2 cells, which serve as a surrogate for intestinal enterocytes. Carotenoid uptake is closely aligned with co-ingestion with lipids and quantitative content in micellar fraction (Chitchumroonchokchai & Failla, 2017). Research has shown that fatty acids in the mixed micelles can promote chylomicron secretion that can carry the hydrophobic substances through intestinal cells (Xia et al., 2020). Considering these aspects, only the LPE product was subjected to capture by Caco-2 cells.

Chlorella vulgaris presented a more diversified qualitative profile uptake by Caco-2 cells, constituted by twelve compounds. Carotenes (43.90%) were accumulated to a greater extent compared to xanthophylls (17.90%) (Fig. 4a1). The relative accumulation of total carotenoids in Caco-2 cells represented 22.71%, being all-*trans*-α-carotene (57.04%), 15-*cis*-β-carotene (51.42%), and all-*trans*-β-carotene (44.99%) the main compounds (Fig. 4a2).

Positively, all-*trans*-lutein was the compound that showed higher absolute content in the captured fraction; there was 201.26 ng/mg of cell protein of lutein after the intestinal uptake process of *Chlorella vulgaris* (LPE) that would potentially be available to be transported to the target tissue (Figs. S1a$_3$ and 4a$_3$). In addition to the well-known antioxidant potential, the uptake of this molecule plays several important metabolic roles that are mainly associated with macular protection (Arunkumar et al., 2020).

In contrast, for *Arthrospira platensis* (LPE), the relative uptake of total carotenoids by Caco-2 cells presented 23.03%. As in *Chlorella vulgaris*, carotenes (28.83%) were uptake to a greater extent compared to xanthophylls (19.74%) (Fig. 4b1). The major carotenoids accumulated were 13-*cis*-β-carotene (35.26%), 2′-dehydrodeoxymyxol (33.69%), and all-*trans*-β-cryptoxanthin (31.26%) (Fig. 4b2).

Of note, all-*trans*-β-carotene (222.82 ng/mg of cell protein) was the carotenoid that showed higher absolute content among the captured carotenoids (Figs. S2b$_3$ and 4b$_3$). This behavior may be related to the fact that *Arthrospira platensis* presents significant amounts of monounsaturated fatty acids (MUFAs) (data not shown) that may transport the hydrophobic carotenoids (Xia et al. al., 2020). Intake of this compound is the major source of provitamin A. The retinoid metabolites are important for normal growth, vision, immune health, and reproduction (Arunkumar et al., 2020).

Finally, for the level of comparison between species, the cyanobacteria *Arthrospira platensis* performed slightly better in the uptake of these compounds compared to *Chlorella vulgaris* (up to 0.32% more). This might be attributable to differences in the concentration of carotenoids in micelles, which was generated during simulated digestion. This result corroborates previous studies, which established that the amount of carotenoid uptake by Caco-2 cells is proportional to the content of

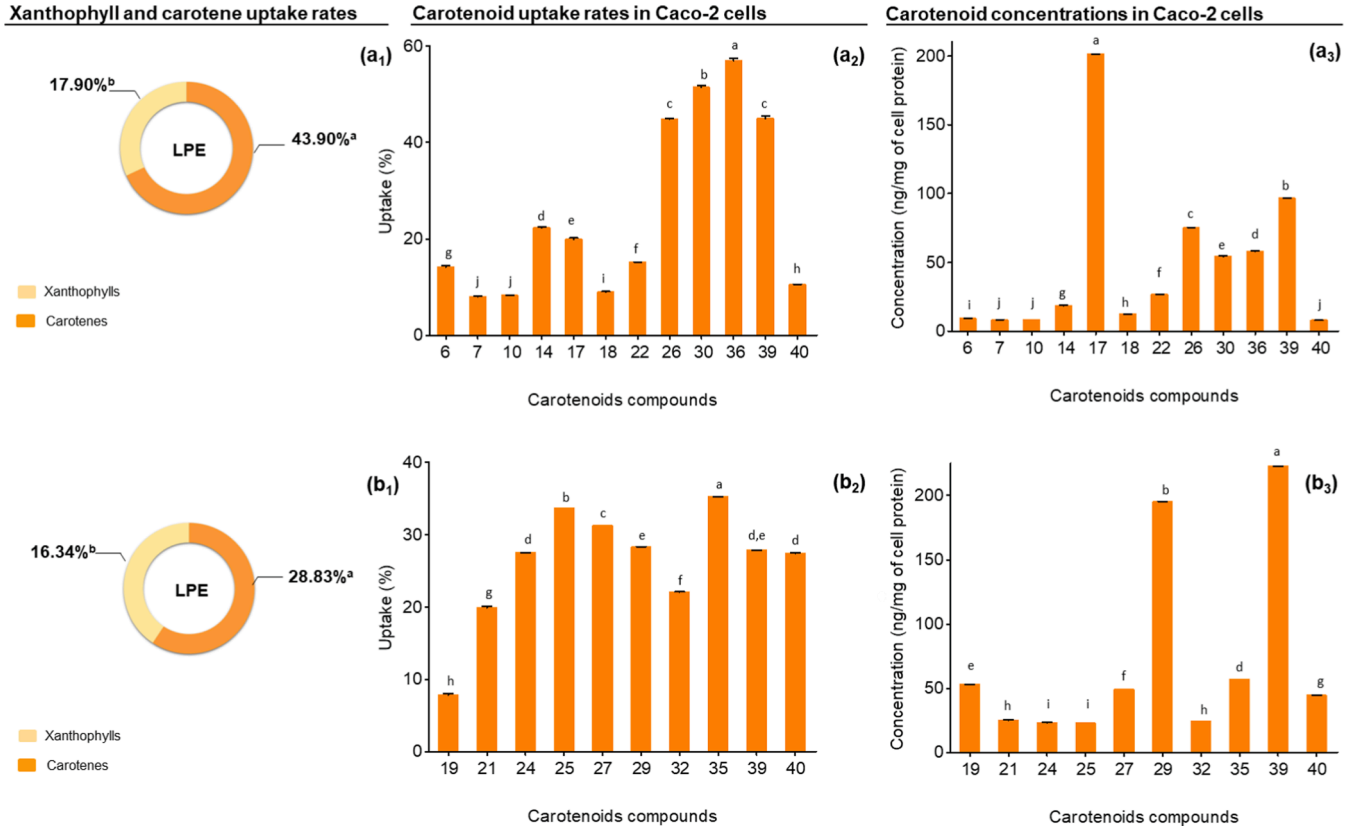

Fig. 4. Concentrations of individual carotenoids absorbed from *Chlorella vulgaris* (a_1) and *Arthrospira platensis* (b_1) and the relative uptake rate of carotenoids from *Chlorella vulgaris* (a_2) and *Arthrospira platensis* (b_2). Quantitative characterization of the relative uptake of xanthophylls and carotenes from *Chlorella vulgaris* (a_3) and *Arthrospira platensis* (b_3) by Caco-2 cells after digesting LPE (liposoluble pigments emulsion). The numbers correspond to the compounds identified (Table 1). Significant differences are indicated by different capital and lowercase letters ($p < 0.05$).

micellarized carotenoids, thereby emphasizing the importance of efficient micellization in boosting the cellular concentration of carotenoids (Gille et al., 2019).

3.4. Chlorophyll bioaccessibility and cellular uptake

The relative bioaccessibility of the chlorophylls and total derivatives in ingredients/products from *Chlorella vulgaris* was 6.44, 1.67, and 0.09% for LPE, WUP, and WDB, respectively (Fig. 5a1), thereby being significantly different. In contrast, *Arthrospira platensis* showed total bioaccessibility values of 2.43% for LPE, 1.23% for WUP, and 0.11% for WDB (Fig. 5b1). However, considering the abundance of chlorophylls in microalgae, even minimal bioaccessibility of these compounds may be physiologically significant.

The bioaccessibility of chlorophylls and derivatives from the WDB of *Chlorella vulgaris* and *Arthrospira platensis* was low after *in vitro* digestion (<1%). Specifically, pheophytin *a*' was the only chlorophyll derivative bioaccessible and showed values in the order of 1.00 and 0.90% for *Chlorella vulgaris* (WDB) and *Arthrospira platensis* (WDB), respectively (Fig. 5).

The use of WUP significantly increased the bioaccessibility of pheophytin *a* (16.58%) and pheophytin *a*' (15.87%) (Fig. 5a2). In this line, applying *Arthrospira platensis* (WUP) significantly increased the bioaccessibility of pheophytin *a* (17.59%) and pheophytin *a*' (17.30%) (Fig. 5b2).

Applying *Chlorella vulgaris* (LPE) significantly increased the bioaccessibility of all compounds, including chlorophyll *b* (0.80%), pheophytin *a* (56.71%), and pheophytin *a*' (44.13%) (Fig. 5a2). In the *Arthrospira platensis* (LPE), the bioaccessibility of chlorophyll derivatives increased significantly for pheophytin *a* (73.33%) and pheophytin *a*' (60.75%) (Fig. 5b2).

Furthermore, after *in vitro* digestion, the hydroxypheophytin *a*, hydroxypheophytin *a*', and pheophytin *b* were identified in the *Chlorella vulgaris* LPE. At the same time, peaks 28 and 31 were also detected from *Arthrospira platensis* LPE (Fig. 5a2 and 5b2). These compounds were possibly formed during the digestion process since it was not detected in the control extract. Thus, we were unable to estimate the percentage value of its bioaccessibility. Fernandes et al. (2021a) supported this hypothesis and found chlorophyll derivatives after *in vitro* digestion of *Scenedesmus obliquus*. Likewise, other studies also observed chlorophyll derivatives during the simulated digestion process (Chen & Roca, 2018a, 2018b).

Comparing chlorophyll bioaccessibility within the different ingredients/products enables the bioaccessible behavior of different chlorophyll structures to be analyzed. In all ingredients/products, Mg-free chlorophyll derivatives were more bioaccessible than native chlorophylls, which corroborates sources of vegetable chlorophylls, such as spinach (Hayes et al., 2020). In addition to this, pheophytin *a*' was the only bioaccessible derivative in all ingredients/products (1–60%). It is reasonable to assume that the high bioaccessibility of these compounds in digestion media likely results from chlorophyll degradation during digestion (Cervantes-Paz et al., 2021).

Additionally, we also determined the uptake efficiency of chlorophyll and derivatives, and based on current knowledge that cellular uptake is proportional to micellar content (Ferruzzi et al., 2001), only the LPE was used in this trial due to the low efficiency of the chlorophyll derivatives' bioaccessibility of WDB and WUB.

In *Chlorella vulgaris*, the relative accumulation of total chlorophylls in Caco-2 cells represented 15.65%. Pheophytin *a*' (17.26%), hydroxypheophytin *a* (13.55%), and pheophytin *b*' (13.37%) levels were significantly higher than the other chlorophyll derivatives captured by the intestinal cells (Fig. 6a1).

As already reported, the absorption process can modify the profiles of chlorophyll derivatives (Hayes & Ferruzzi, 2020). In this study, seven compounds constituted the absorbed fraction of *Chlorella vulgaris*, of which only six were present in the LPE (Fig. S3a$_3$); pheophytin *a*' (9,269.18 ng/mg of cell protein) was the compound that showed higher absolute content captured (Fig. 6a3). In comparison, only traces of pheophytin *b* were detected.

As for *Arthrospira platensis* (LPE), the relative uptake of total chlorophyll derivatives by Caco-2 cells was 15.32%. The major chlorophyll derivatives accumulated were hydroxypheophytin *a* (30.92%) and hydroxypheophytin *a*' (16.71%). The highest accumulation rate of oxidized derivatives (including hydroxypheophytin *a* and hydroxypheophytin *a*' after Caco-2 cells of *Arthrospira platensis* (LPE) indicates that the uptake process can promote oxidative reactions. This fact was

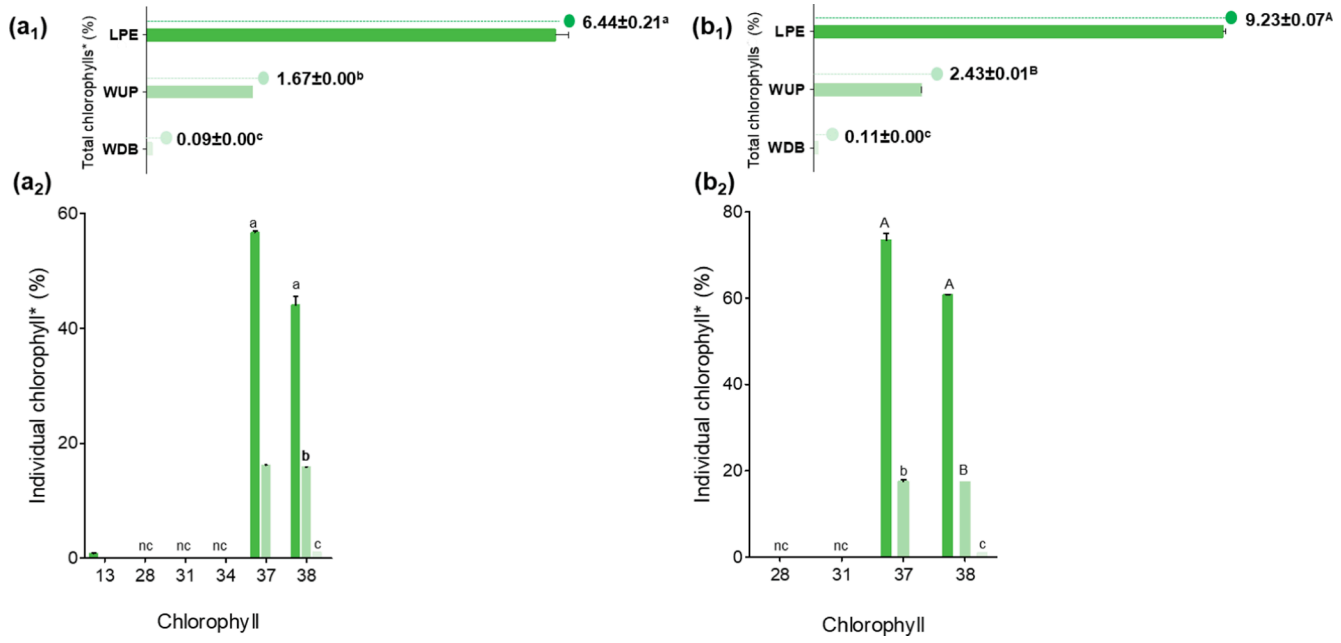

Fig. 5. Relative bioaccessibility of total chlorophylls from *Chlorella vulgaris* (a_1) and *Arthrospira platensis* (b_1) and the bioaccessibility index of individual chlorophylls from *Chlorella vulgaris* (a_2) and *Arthrospira platensis* (b_2) of WDB, WUP, and LPE. The numbers correspond to the compounds identified (Table 1). Significant differences are indicated by different capital and lowercase letters ($p < 0.05$); nc = not calculated.

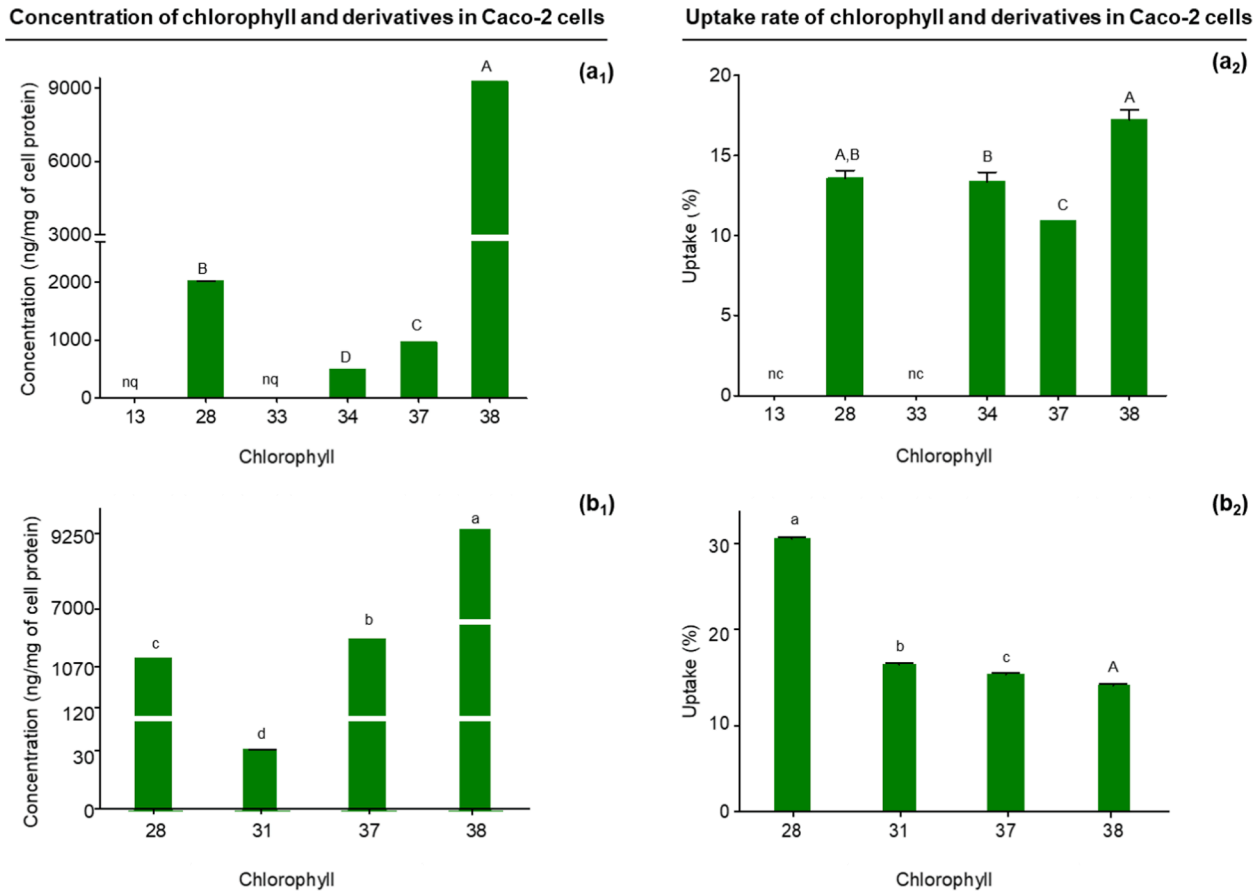

Fig. 6. Concentrations of individual chlorophyll absorbed from *Chlorella vulgaris* (a_1) and *Arthrospira platensis* (b_1) and the relative uptake rate of individual chlorophyll from *Chlorella vulgaris* (a_2) and *Arthrospira platensis* (b_2) by Caco-2 cells after digesting LPE (liposoluble pigments emulsion). The numbers correspond to the compounds identified (Table 1). Significant differences are indicated by different capital and lowercase letters ($p < 0.05$); nq = not quantified; nc = not calculated.

previously reported by Chen & Roca (2018a) for the first time. The authors evaluated the bioavailability of chlorophyll pigments from edible seaweeds and found that the uptake process by Caco-2 cells significantly increased oxidized derivatives.

Similar to the data found in the absorbed content of *Chlorella vulgaris*, pheophytin *a*' (9,483.02 ng/mg of cell protein) represents the main compound captured from the *Arthrospira platensis* (LPE) (Fig. 6b1). Chlorophyll derivatives (specifically pheophytins) also have singular functions such as antimutagenic activity, anti-inflammatory and chemopreventive (Zepka et al., 2019).

Finally, the *Chlorella vulgaris* digestion used for cell absorption has the highest chlorophyll pigment content than *Arthrospira platensis*; their sequence also reflects this in cellular absorption rate (up to 0.33% more). However, the food matrix should also influence the absorption process. The same chlorophyll derivatives (hydroxypheophytin *a*, pheophytin *a,* and pheophytin *a*') exhibited different absorption rates depending on the microalgae species.

4. Conclusions

Designing microalgae-based ingredients and products that overcome the challenges associated with poor carotenoid and chlorophyll bioaccessibility and bioavailability requires a broad understanding of the hotspots that potentially limit their bioaccessibility and subsequent uptake. Given this scenario, this study demonstrated that carotenoid and chlorophyll bioaccessibility could be improved according to the matrix employed (LPE > WUP > WDB), as the cell wall was disrupted or non-existent. Specifically, our findings also demonstrated that the LPE proved to be an efficient carrier of chlorophylls and their derivatives, in addition to carotenoids and xanthophylls. Lastly, *Chlorella vulgaris* and *Arthrospira platensis* are potential bioresources for the formulation of carotenoid and chlorophyll-based foods.

CRediT authorship contribution statement

Pricila P. Nass: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing, Data curation, Formal analysis. **Tatiele C. do Nascimento:** Investigation. **Andrèssa S. Fernandes:** Investigation. **Patrícia A. Caetano:** Investigation. **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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111469.

References

Arunkumar, R., Gorusupudi, A., & Bernstein, P. S. (2020). The macular carotenoids: A biochemical overview. Article 103770158617 *BBA - Molecular and Cell Biology of Lipids, 1865*. https://doi.org/10.1016/j.bbalip.2020.158617.

Bernaerts, T. M. M., Verstreken, H., Dejonghe, C., Gheysen, L., Foubert, I., Grauwet, T., & Van Loey, A. M. (2020). Cell disruption of *Nannochloropsis* sp. improves *in vitro* bioaccessibility of carotenoids and ω3-LC-PUFA. *Journal of Functional Foods, 65*, Article 103770. https://doi.org/10.1016/j.jff.2019.103770

Biehler, E., Alkerwi, A., Hoffmann, L., Krause, E., Guillaume, M., Lair, M. L., & Bohn, T. (2012). Contribution of violaxanthin, neoxanthin, phytoene and phytofluene to total carotenoid intake: Assessment in Luxembourg. *Journal of Food Composition and Analysis, 25*(1), 56–65. https://doi.org/10.1016/j.jfca.2011.07.005

Bleakley, S., & Hayes, M. (2017). Algal proteins: Extraction, application, and challenges concerning production. *Foods, 6*(5), 33. https://doi.org/10.3390/foods6050033

Cervantes-Paz, B., Yahia, E. M., Ornelas-Paz, J.d. J., Victoria-Campos, C. I., Pérez-Martínez, J. D., & Reyes-Hernández, J. (2021). Bioaccessibility of fat-soluble bioactive compounds (FSBC) from avocado fruit as affected by ripening and FSBC composition in the food matrix. *Food Research International, 139*. https://doi.org/10.1016/j.foodres.2020.109960

Chen, K., Ríos, J. J., Pérez-Gálvez, A., & Roca, M. (2015). Development of an accurate and high-throughput methodology for structural comprehension of chlorophylls derivatives. (I) Phytylated derivatives. *Journal of Chromatography A, 1406*, 99–108. https://doi.org/10.1016/j.chroma.2015.05.072

Chen, K., & Roca, M. (2018a). *In vitro* bioavailability of chlorophyll pigments from edible seaweeds. *Journal of Functional Foods, 41*, 25–33. https://doi.org/10.1016/j.jff.2017.12.029

Chen, K., & Roca, M. (2018b). *In vitro* digestion of chlorophyll pigments from edible seaweeds. *Journal of Functional Foods, 40*, 400–407. https://doi.org/10.1016/j.jff.2017.11.030

Chitchumroonchokchai, C., & Failla, M. L. (2017). Bioaccessibility and intestinal cell uptake of astaxanthin from salmon and commercial supplements. *Food Research International, 99*, 936–943. https://doi.org/10.1016/j.foodres.2016.10.010

Desmarchelier, C., & Borel, P. (2017). Overview of carotenoid bioavailability determinants: From dietary factors to host genetic variations. *Trends in Food Science & Technology, 69*, 270–280. https://doi.org/10.1016/j.tifs.2017.03.002

Deprá, M. C., Severo, I. A., dos Santos, A. M., Zepka, L. Q., & Jacob-Lopes, E. (2020). Environmental impacts on commercial microalgae-based products: Sustainability metrics and indicators. *Algal Research, 51*, 102056. https://doi.org/10.1016/j.algal.2020.102056

EFSA (European Food Safety Authority). (2016). Guidance on the preparation and presentation of an application for authorization of a novel food in the context of Regulation (EU) 2015/2283. Article 4594 *EFSA Journal, 14*(11). https://doi.org/10.2903/j.efsa.2016.4594.

Fernandes, A. S., Nascimento, T. C., Pinheiro, P. N., de Rosso, V. V., de Menezes, C. R., Jacob-Lopes, E., & Zepka, L. Q. (2021). Insights on the intestinal absorption of chlorophyll series from microalgae. *Food Research International, 140*, 1–13. https://doi.org/10.1016/j.foodres.2020.110031

Fernandes, A. S., Nascimento, T. C., Pinheiro, P. N., Vendruscolo, R. G., Wagner, R., de Rosso, V. V., Jacob-Lopes, E., & Zepka, L. Q. (2021). Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix. Article 148 *Food Research International*. https://doi.org/10.1016/j.foodres.2021.110596.

Fernandes, A. S., Nogara, G. P., Menezes, C. R., Cichoski, A. J., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2017). Identification of chlorophyll molecules with peroxyl radical scavenger capacity in microalgae *Phormidium autumnale* using ultrasound-assisted extraction. *Food Research International, 99*, 1036–1041. https://doi.org/10.1016/j.foodres.2016.11.011

Fernandes, A. S., Petry, F. C., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2020). HPLC-PDA-MS/MS as a strategy to characterize and quantify natural pigments from microalgae. *Current Research in Food Science, 3*, 100–112. https://doi.org/10.1016/j.crfs.2020.03.009

Ferruzzi, M. G., Failla, M. L., & Schwartz, S. J. (2001). Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an *in vitro* digestion and Caco-2 human cell model. *Journal of Agricultural and Food Chemistry, 49*(4), 2082–2089. https://doi.org/10.1021/jf000775r

Gandul-Rojas, B., Gallardo-Guerrero, L., & Mínguez-Mosquera, M. I. (2009). Influence of the chlorophyll pigment structure on its transfer from an oily food matrix to intestinal epithelium cells. *Journal of Agricultural and Food Chemistry, 57*(12), 5306–5314. https://doi.org/10.1021/jf900426h

Gille, A., Hollenbach, R., Trautmann, A., Posten, C., & Briviba, K. (2019). Effect of sonication on bioaccessibility and cellular uptake of carotenoids from preparations of photoautotrophic *Phaeodactylum tricornutum*. *Food Research International, 118*, 40–48. https://doi.org/10.1016/j.foodres.2017.12.040

Gille, A., Neumann, U., Louis, S., Bischoff, S. C., & Briviba, K. (2018). Microalgae as a potential source of carotenoids: Comparative results of an *in vitro* digestion method and a feeding experiment with C57BL/6J mice. *Journal of Functional Foods, 49*, 285–294. https://doi.org/10.1016/j.jff.2018.08.039

Gille, A., Trautmann, A., Posten, C., & Briviba, K. (2016). Bioaccessibility of carotenoids from *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. *International Journal of Food Sciences and Nutrition, 67*(5), 507–513. https://doi.org/10.1080/09637486.2016.1181158

Giuffrida, D., Zoccali, M., Giofrè, S. V., Dugo, P., & Mondello, L. (2017). Apocarotenoids determination in *Capsicum chinense* Jacq. cv. Habanero, by supercritical fluid chromatography-triple-quadrupole/mass spectrometry. *Food Chemistry, 231*, 316–323. https://doi.org/10.1016/j.foodchem.2017.03.145

Hayes, M., & Ferruzzi, M. G. (2020). Update on the bioavailability and chemopreventative mechanisms of dietary chlorophyll derivatives. *Nutrition Research, 81*, 19–37. https://doi.org/10.1016/j.nutres.2020.06.010

Kopec, R. E., & Failla, M. L. (2018). Recent advances in the bioaccessibility and bioavailability of carotenoids and effects of other dietary lipophiles. *Journal of Food Composition and Analysis, 68*, 16–30. https://doi.org/10.1016/j.jfca.2017.06.008

Kopec, R. E., Gleize, B., Borel, P., Desmarchelier, C., & Caris-Veyrat, C. (2017). Are lutein, lycopene, and β-carotene lost through the digestive process? *Food and Function, 8*(4), 1494–1503. https://doi.org/10.1039/c7fo00021a

Kusmayadi, A., Leong, Y. K., Yen, H. W., Huang, C. Y., & Chang, J. S. (2021). Microalgae as sustainable food and feed sources for animals and humans - Biotechnological and environmental aspects. *Chemosphere, 271*, 129800. https://doi.org/10.1016/j.chemosphere.2021.129800

Lafarga, T. (2019). Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products. *Algal Research, 41*, 101566. https://doi.org/10.1016/j.algal.2019.101566

Mandelli, F., Miranda, V. S., Rodrigues, E., & Mercadante, A. Z. (2012). Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. *World Journal of Microbiology and Biotechnology, 28*(4), 1781–1790. https://doi.org/10.1007/11274-011-0993-y

Maroneze, M. M., Caballero-Guerrero, B., Zepka, L. Q., Jacob-Lopes, E., Pérez-Gálvez, A., & Roca, M. (2020). Accomplished High-Resolution Metabolomic and Molecular Studies Identify New Carotenoid Biosynthetic Reactions in Cyanobacteria. *Journal of Agricultural and Food Chemistry, 68*(22), 6212–6220. https://doi.org/10.1021/acs.jafc.0c01306

Maroneze, M. M., Jacob-Lopes, E., Zepka, L. Q., Roca, M., & Pérez-Gálvez, A. (2019). Esterified carotenoids as new food components in cyanobacteria. *Food Chemistry, 287*, 295–302. https://doi.org/10.1016/j.foodchem.2019.02.102

Maroneze, M. M., Zepka, L. Q., Jacob-Lopes, E., Pérez-Gálvez, A., & Roca, M. (2019). Chlorophyll oxidative metabolism during the phototrophic and heterotrophic growth of *Scenedesmus obliquus*. *Antioxidants, 8*(12), 1–15. https://doi.org/10.3390/antiox8120600

Mehariya, S., Goswami, R. K., Karthikeysan, O. P., & Verma, P. (2021). Microalgae for high-value products: A way towards green nutraceutical and pharmaceutical compounds. *Chemosphere, 280*, 130553. https://doi.org/10.1016/j.chemosphere.2021.130553

Miller, V., Webb, P., Micha, R., & Mozaffarian, D. (2020). Defining diet quality: A synthesis of dietary quality metrics and their validity for the double burden of malnutrition. *The Lancet Planetary Health, 4*(8), 352–370. https://doi.org/10.1016/S2542-5196(20)30162-5

Mulders, K. J., Lamers, P. P., Martens, D. E., & Wijffels, R. H. (2014). Phototrophic pigment production with microalgae: Biological constraints and opportunities. *Journal of phycology, 50*(2), 229–242. https://doi.org/10.1111/jpy.12173

Murador, D. C., De Souza, L. M., Neves, B. V., Braga, A. R. C., Martins, P. L. G., Zepka, L. Q., & De Rosso, V. V. (2021). Bioaccessibility and cellular uptake by Caco-2 cells of carotenoids and chlorophylls from orange peels: A comparison between conventional and ionic liquid mediated extractions. *Food Chemistry, 339*, 127818. https://doi.org/10.1016/j.foodchem.2020.127818

Nascimento, T. C., Pinheiro, P. N., Fernandes, A. S., Murador, D. C., Neves, B. V., de Menezes, C. R., de Rosso, V. V., Jacob-Lopes, E., & Zepka, L. Q. (2021). Bioaccessibility and intestinal uptake of carotenoids from microalgae *Scenedesmus obliquus*. *LWT, 140*, 110780. https://doi.org/10.1016/j.lwt.2020.110780

Natoli, M., Leoni, B. D., D'Agnano, I., D'Onofrio, M., Brandi, R., Arisi, I., Zucco, F., & Felsani, A. (2011). Cell growing density affects the structural and functional properties of Caco-2 differentiated monolayer. *Journal of Cellular Physiology, 226*(6), 1531–1543. https://doi.org/10.1002/jcp.22487

Niccolai, A., Chini Zittelli, G., Rodolfi, L., Biondi, N., & Tredici, M. R. (2019). Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Research, 42*(101617), 1–9. https://doi.org/10.1016/j.algal.2019.101617

Ordóñez-Santos, L. E., Pinzón-Zarate, L. X., & González-Salcedo, L. O. (2015). Optimization of ultrasonic-assisted extraction of total carotenoids from peach palm fruit (*Bactris gasipaes*) by-products with sunflower oil using response surface methodology. *Ultrasonics Sonochemistry, 27*, 560–566. https://doi.org/10.1016/j.ultsonch.2015.04.010

Patias, L. D., Fernandes, A. S., Petry, F. C., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2017). Carotenoid profile of three microalgae/cyanobacteria species with peroxyl radical scavenger capacity. *Food Research International, 100*, 260–266. https://doi.org/10.1016/j.foodres.2017.06.069

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology, 111*, 1–61. https://doi.org/10.1099/00221287-111-1-1

Rodrigues, D. B., Flores, É. M. M., Barin, J. S., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2014). Production of carotenoids from microalgae cultivated using agroindustrial wastes. *Food Research International, 65*, 144–148. https://doi.org/10.1016/j.foodres.2014.06.037

Rodrigues, D. B., Menezes, C. R., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2015). Bioactive pigments from microalgae *Phormidium autumnale*. *Food Research International, 77*, 273–279. https://doi.org/10.1016/j.foodres.2015.04.027

Salvia-Trujillo, L., Verkempinck, S. H. E., Sun, L., Van Loey, A. M., Grauwet, T., & Hendrickx, M. E. (2017). Lipid digestion, micelle formation and carotenoid bioaccessibility kinetics: Influence of emulsion droplet size. *Food Chemistry, 229*, 653–662. https://doi.org/10.1016/j.foodchem.2017.02.146

Soukoulis, C., & Bohn, T. (2018). A comprehensive overview on the micro-and nano-technological encapsulation advances for enhancing the chemical stability and bioavailability of carotenoids. *Critical Reviews in Food Science and Nutrition, 58*(1), 1–36. https://doi.org/10.1080/10408398.2014.971353

Sy, C., Gleize, B., Dangles, O., Landrier, J. F., Veyrat, C. C., & Borel, P. (2012). Effects of physicochemical properties of carotenoids on their bioaccessibility, intestinal cell uptake, and blood and tissue concentrations. *Molecular Nutrition & Food Research, 56*(9), 1385–1397. https://doi.org/10.1002/mnfr.201200041

Takaichi, S. (2009). Carotenoids in Phototrophic Microalgae: Distributions and Biosynthesis. In E. Jacob-Lopes, M. I. Queiroz, & L. Q. Zepka (Eds.), *Pigments from microalgae handbook* (pp. 19–41).

Tao, W., Ye, X., & Cao, Y. (2021). Isomerization and degradation of all-*trans*-β-carotene during *in vitro* digestion. *Food Science and Human Wellness, 10*(3), 370–374. https://doi.org/10.1016/j.fshw.2021.02.028

Vasistha, S., Khanra, A., Clifford, M., & Rai, M. P. (2021). Current advances in microalgae harvesting and lipid extraction processes for improved biodiesel production: A review. *Renewable and Sustainable Energy Reviews, 137*, 110498. https://doi.org/10.1016/j.rser.2020.110498

Vendruscolo, R. G., Facchi, M. M. X., Maroneze, M. M., Fagundes, M. B., Cichoski, A. J., Zepka, L. Q., … Wagner, R. (2018). Polar and non-polar intracellular compounds from microalgae: Methods of simultaneous extraction, gas chromatography determination and comparative analysis. *Food Research International, 109*, 204–212. https://doi.org/10.1016/j.foodres.2018.04.017

Xavier, A. A. O., & Mercadante, A. Z. (2019). The bioaccessibility of carotenoids impacts the design of functional foods. *Current Opinion in Food Science, 26*, 1–8. https://doi.org/10.1016/j.cofs.2019.02.015

Xia, Z., Han, Y., Du, H., McClements, D. J., Tang, Z., & Xiao, H. (2020). Exploring the effects of carrier oil type on in vitro bioavailability of β-carotene: A cell culture study of carotenoid-enriched nanoemulsions. *LWT, 134*, 110224. https://doi.org/10.1016/j.lwt.2020.110224

Yeum, K. J., & Russell, R. M. (2002). Carotenoid bioavailability and bioconversion. *Annual review of nutrition, 22*(1), 483–504. https://doi.org/10.1146/annurev.nutr.22.010402.102834

Zou, X., Xu, K., Chang, W., Qu, Y., & Li, Y. (2021). Rapid extraction of lipid from wet microalgae biomass by a novel buoyant beads and ultrasound assisted solvent extraction method. *Algal Research, 58*, 102431. https://doi.org/10.1016/j.algal.2021.102431

Zepka, L. Q., Jacob-Lopes, E., & Roca, M. (2019). Catabolism and bioactive properties of chlorophylls. *Current Opinion in Food Science, 26*, 94–100. https://doi.org/10.1016/j.cofs.2019.04.004

CAPÍTULO 3

Food bioactive compounds from microalgae

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CHAPTER 3

FOOD BIOACTIVE COMPOUNDS FROM MICROALGAE

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ABSTRACT

The market trends, and consequently, industrial, and commercial interest, have turned to foods with natural ingredients. Thus, the richness of bioactive food biocompounds from microalgae has long inspired their exploitation, fundamentally supported by balanced composition, containing multiple molecules with myriad biological functionalities. To validate this assumption, several microalgae-based bioproducts are already commercially available, either as protein, fatty acids (FAs), and pigments. Also, divers emerging bioproducts (polysaccharides, vitamins, phycoerythrin (PE), and sterols) are yet to be fully explored. In general, these molecules are less competitive commercially than traditional sources. However, some microalgae biomolecules have advantages over synthetic molecules, due to its chemical conformation, making their use commercially attractive for the food sector, despite the higher production costs. In that context, encouraged by the growing interest in food biocompounds, this chapter provides an overview of the bioactive food compounds microalgae-based, the current status of the market of such compounds as well as to regulatory issues in this field.

3.1 INTRODUCTION

The growing demand for natural products has strengthened microalgae a promising alternative for obtaining natural compounds. Because a consequence of this trend, the worldwide market estimation of microalgae is assessed to be around US $5.7 billion, out of which about US $2.5 billion is generated by the health food ingredients (Koyande et al., 2019; Jacob-Lopes et al., 2019; Sathasivam et al., 2019; Tang et al., 2020; Katiyar and Arora, 2020).

Regarding the collection of microorganisms, we refer to as microalgae is a polyphyletic group, with an estimated number of 72,500 species consistently cataloged. However, the species considered safe and commercially consolidated as food supplements, ingredients, or additives, comprise *Arthrospira* (*Spirulina*), *Chlorella* sp., *Porphyridium cruentum*, *Crypthecodinium cohnii*, *Haematococcus pluvialis*, *Phaeodactylum tricornutum*, *Dunaliella* sp., *Nannochloropsis* sp., *Nitzschia* sp. and *Schizochytrium* sp. (Jacob-Lopes et al., 2019; Torres-Tiji et al., 2020).

In this sense, due to the metabolic and taxonomic diversity, combined with the high biotechnological potential, microalgae are excellent producers of bioactive molecules, such as proteins with essential amino acids (EAAs), fatty acids (FAs), carbohydrates, vitamins, and pigments related to many human biological activities (Guedes et al., 2011; Borowitzka, 2013; Patias et al., 2017; Fernandes et al., 2017, 2020; Niccolai et al., 2019; Nascimento et al., 2020). The main bioactive properties associated with these compounds are related to the anti-inflammatory, antioxidant, anti-aging, immunosuppressive, antihypertensive, hypocholesterolemic, photoprotective, and neurotransmitting activities (Fernandes et al., 2017; Patias et al., 2017; Sathasivam et al., 2019; Jacob-Lopes et al., 2019; Nascimento et al., 2019, 2020).

Thus, this chapter provides a comprehensive overview of the potentially valuable bioactive food compounds from the microalgae, including the biological properties of these compounds. Moreover, it is revised the current status of the market of such biocompounds as well as to regulatory milestones for your application.

3.2 MICROALGAE-BASED FOOD BIOACTIVE COMPOUNDS

The difference in morphological, physiological, and genetic characteristics between the different microalgae species provides these microorganisms with the ability to synthesize a wide range of bioactive molecules in different

proportions (Sathasivam et al., 2019). Thus, in recent decades, greater attention has been paid to studies on microalgae biochemicals with potential for application as functional food ingredients (Vaz et al., 2016; Bernaerts et al., 2018; Caporgno and Mathys, 2018).

It is known that the constitution of biomass is rich mainly in proteins, with values that can reach up over 50% of dry weight in some species (Singh et al., 2020). Thus, since the beginning of studies with microalgae, this class of biomolecules was the most explored bioactive food compounds in these matrices (Jacob-Lopes et al., 2019). However, currently, emerging bioactive compounds such as polysaccharides, FAs, pigments, and vitamins are changing their status in the field of microalgae research and gaining space in relevant research (Camacho et al., 2019; Levasseur et al., 2020).

Among the class of macromolecules, microalgae are recognized as potent and promising sources of proteins (Khanra et al., 2018). The great advantage of microalgal proteins is in the constitution of amino acids in their structures, as they present a complete profile of EAAs that are often not found in higher plants (Koyande et al., 2019). These essential compounds are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Other amino acids such as cysteine, proline, arginine, glutamine, glycine, proline, tyrosine, and aspartic acid also appear in the protein fraction of some species (Mobin et al., 2019; Torres-Tiji et al., 2020).

In particular, the presence of EAAs is very significant for the population that follows a vegetarian and vegan diet since microalgae biomass becomes an alternative to obtain these essential molecules (Koyande et al., 2019). Likewise, other significant factors such as favorable nutritional and functional properties, low allergenicity, and high protein content (~50%) drive the application of these biopolymers as functional, nutritional, and therapeutic commodities (Soto-Sierra et al., 2018). An example of this is the application of some microalgae proteins in formulations of anti-obesity functional foods, since they stimulate the production of the cholecystokinin hormone, responsible for regulating appetite suppression (Patias et al., 2018).

From the fraction of carbohydrates in microalgae, polysaccharides have become a frequent target of scientific research as compounds with bioactive properties. These compounds are complex and heterogeneous macromolecules, composed of different monosaccharide units, and some may contain some degree of neutral acids or sulfate groups (called sulfated polysaccharides) (Raposo et al., 2015).

According to the microalgae group, a diversity of structures is produced. Some species synthesize homopolysaccharides (units of monosaccharides

of the same type), and most of them have in their cellular constitution heteropolysaccharides (units of different monosaccharides). Some of these compounds are components of the cell walls, while others are secreted out of cells and are denominated exopolysaccharides (EPSs) (Raposo et al., 2015; Bernaerts et al., 2018; Li et al., 2020).

The high structural diversity reflects the bioactivity diversity of these molecules. Some polysaccharides when supplemented in diets have shown positive effects on human health. β-glucans, for example, in the human organism, act as soluble fiber and are considered great allies in the reduction of LDL cholesterol and cardiovascular diseases (Khoury et al., 2011). On the other hand, sulfated polysaccharides derived from algae have already proven to have several important properties such as cancer preventive properties, antioxidant activity, anticoagulant, immunomodulatory, antiviral activities, anti-inflammatory, antinociceptive, antihyperlipidemic, and antihepatotoxic activities (Wang et al., 2014).

Among the sulfated structures, spirulan, an algae-specific polysaccharide (*Arthrospira platensis*), also has been shown to have antiviral activity and capacity of reduction in plasma cholesterol levels (Lee et al., 2001; Samuels et al., 2002). In particular, a study realized by Guzmán et al. (2003) showed that crude polysaccharide extracts from *Chlorella stigmatophora* and *Phaeodactylum tricornutum* exhibited a high anti-inflammatory effect. Antiviral effects have been observed for polysaccharides in *Porphyridium* sp. microalgae (Huleihel et al., 2001). As well as antioxidant properties have been related to the fraction of extracellular polysaccharide from microalgae *Rhodella reticulata* (Chen et al., 2010). EPSs produced by microalgae *Chlorella pyrenoidosa*, *Chlorococcum* sp. and *Scenedesmus* sp. exhibited free radicals scavenging abilities and exhibited significant antitumor effects for colon cancer cell (Zhang et al., 2019). Considering these different biological activities, microalgae polysaccharides are suitable for application in many distinct areas, such as for food purposes as additives that improve the quality and texture of food or to obtain healthy, nutraceutical, and functional foods (Raposo et al., 2014, 2015; Bernaerts et al., 2018).

Microalgae are recognized as an important source of lipids. The most strains present levels from 20% to 50% of the dry biomass (w/w), depending on the species and cultivation conditions (Barkia et al., 2019). In particular, *Botryococcus braunii* (green microalgae), under optimal growth conditions can reach lipid concentrations of up to 86% (Tandon and Jin, 2017). The lipid profile of microalgae is basically consisting of triacylglycerols (TAG) molecules, monounsaturated fatty acids (MUFA), and polyunsaturated

et al., 2017). Given these characteristics, these compounds are currently promising candidates as food bioactive compounds.

PBPs are bioactive substances formed by a proportion of protein with an associated chromophore (called phycobilin) commonly present in cyanobacteria and red algae (D'Alessandro and Filho, 2016). Phycoerythrin (PE) and phycocyanin (PC) stemming the metabolism of *Porphyridium cruentum* and *Arthrospira platensis* (*Spirulina*), respectively, are the most well-known and studied PBPs (Raposo et al., 2013). Besides, phycobilin has antioxidant, hypolipidemic, and anti-inflammatory properties, for which it is showing great interest in the application in functional food (Fernández-Rojas et al., 2014; Baky et al., 2015; Pagels et al., 2019). In contrast, PE presents applications in the biomedical field as a fluorescent agent, tool for research and diagnosis (Pan-Utai and Lamtham, 2019; Sathasivam et al., 2019).

The important capacity for the synthesis of enzymes by microalgae has also been reported. Protease, galactosidases, amylases, phytases, lipase, laccases, cellulases, carbonic anhydrase and antioxidant enzymes are some examples used in the food industry (Brasil et al., 2017). Furthermore, it is worth mentioning the fraction of antifreeze proteins (AFPs) found in some microalgae species (Jung et al., 2014). These compounds, despite having their unknown bioactive capacity, are considered important in the food industry due to their capacity for frozen food preservation. Examples of its application are in meat products, where they were able to reduce the damage caused by freezing, loss of drip and loss of proteins and also improve the juiciness of the meat after thawed (Xiang et al., 2020).

Finally, due to the richness of microalgal biodiversity, these microorganisms have the potential to serve as a natural pool of biochemical for use in the food industry, and some have a consolidated share in the market.

3.3 CURRENT MARKET OF MICROALGAE-BASED BIOACTIVE FOOD COMPOUNDS

A few years ago, the market for bioactive compounds was dominated by synthetic molecules or animal and vegetable sources. Nowadays, the demand for natural products and the viability of industrial production of microalgae-based products appear as an opportunity for commercial expansion (Jacob-Lopes et al., 2019). The global market for microalgae products is estimated to reach approximately US $53.43 billion by 2026 (Rahman, 2020). Today, most of the microalgae biocompound market is related to the healthy food

fatty acids (PUFA) (Tang et al., 2020). Especially, PUFA, i.e., omega fatty acids (FAs), receive greater attention since they are valuable as health food supplements (Barkia et al., 2019). The nutritional importance of this class of biocompounds is associated with vital health functions, as they are considered essential for biological processes. Furthermore, the relevance of obtaining these compounds exogenously is based on the fact that human beings are unable to synthesize some of these FAs (Kaur et al., 2014).

In certain species, PUFAs are present in the constitution of biomass in concentrations between 25 and 60% of the total lipids (Vaz et al., 2016). The PUFAs profile in microalgae include ω-6 FAs as linoleic acid (LNA, 18:2n-6), γ-linolenic acid (GLA, 18:3n-6), and arachidonic acid (ARA, 20:4n-6), as well as ω-3 FAs which include α-linolenic acid (ALA, 18:3n-3), docosapentaenoic acid (DPA, 22:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and eicosapentaenoic acid (EPA, 20:5n-3) (Morais et al., 2015; Katiyar and Arora, 2020).

Among the ω-3 long-chain FAs, EPA, and DHA are well accepted as being essential components of a healthy diet, having beneficial effects on cardiovascular disease (Delgado-Lista et al., 2012), development especially of the neural system (Innis, 2007; Campoy et al., 2012), prevention, and treatment of cancer (Berquin et al., 2008), lowers the incidence of diabetes (Woodman et al., 2003) arteriosclerosis, and thrombosis (Liu et al., 2016) and arthritis (Lee et al., 2012). The cardioprotective effect of EPA and DHA FAs is related to the mechanisms of reduction of triglyceride (TG) levels, attenuation of atherosclerotic plaques, the exertion of antidysrhythmic, anti-thrombotic, and anti-inflammatory effects, lowering of systolic and diastolic blood pressures, and improvement in endothelial function (Bradberry et al., 2013). Also, DHA is considered a vital supplementary component in feeding during pregnancy and breastfeeding, as it plays an important role in the development of infants, especially the brain and retina (Echeverría et al., 2017; Sun et al., 2018).

The ω-6 fatty acid ARA, as well as EPA and DHA acids, play important roles in regulating body homeostasis. The human body is converted into eicosanoids and can regulate diverse sets of homeostatic and inflammatory processes linked to in numerous diseases, including inflammation, infection, cancer, and cardiovascular diseases (Saini and Keum, 2018). GLA, in addition to contributing to prostaglandins biosynthesis, has beneficial effects against tumor cells, dermatitis, diabetes, schizophrenia, multiple sclerosis, and rheumatoid arthritis (Raja et al., 2018). Linoleic and α-linolenic FAs are essential nutrients for the immune system and other processes related to the

tissue regeneration, as well as or the synthesis of the cell membrane prostaglandins (Raposo et al., 2013). At the same time are associated with positive effects against some pathologies. Some evidence indicates that ALA has cardiovascular-protective, neuroprotective, anti-cancer, anti-osteoporotic, antioxidative, and anti-inflammatory effects (Kim et al., 2014).

Also, epidemiological studies indicate a positive association between the intake of LNA with lower levels of plasma low-density lipoprotein-cholesterol (LDL-C) (Harris, 2008), as well as long-term glycemic control (Salas-Salvadó et al., 2011), and cardiovascular protection. Another relevant factor associated with these FAs is the ability to become more biologically more active substances. At the metabolic level, LNA is metabolized by the enzymes elongases and desaturases to generate mainly ARA (Marangoni et al., 2020). In contrast, ALA is the precursor of EPA and DHA (Kim et al., 2014).

Another class of biocompounds that can be obtained from the lipid fraction of microalgae are sterols. These compounds, considered membrane lipids, are present in higher concentrations in eukaryotic microalgae than in prokaryotic ones. However, microalgae with prokaryotic cell structure, although they do not have large concentrations of sterols, can synthesize different compositions of these molecules (Volkman, 2016). Thus, microalgae produce uncommon sterols, also known as phytosterols (C28 and C29 sterols), such as brassicasterol, clionasterol, squalene stigmasterol, campesterol, β-sitosterol, and ergosterol (Randhir et al., 2020).

Due to their bioactive properties, these uncommon compounds are being increasingly incorporated into functional foods (Luo et al., 2015). This class of compounds is associated with biological benefits, particularly due to the hypocholesterolemic properties (Devaraj et al., 2004). Also, sterols from microalgae presented potent antioxidant properties that provide protection from stroke, heart, and coronary diseases, anticarcinogenic properties, and anti-inflammatory activity (Sanjeewa et al., 2016; Matos et al., 2017).

Furthermore, sterols molecules, particularly cholesterol, are precursors to a variety of compounds important for human metabolisms, such as steroid hormones (estrogens and progesterone), vitamin D, and bile salts (Wollam and Antebi, 2011). Likewise, Blaga et al. (2018) described ergosterol as an ergocalciferol (Vitamin D_2) precursor. Another application of sterols in the nutritional area is the use of biomass from some species with significant concentrations of these compounds in formulating rations to promote the growth of juveniles, especially oysters (Raposo et al., 2013). Noteworthy, the β-sitosterol is a phytosterol that has already been marketed as a nutraceutical

component due to its ability to reduce serum cholesterol in hypercholesterolemic individuals (Luo et al., 2015).

In terms of micro-nutrients, microalgae contain high levels of essential vitamins compared to staple foods (Chew et al., 2017). They are able to synthesize hydrophilic vitamins including B-complex (thiamine-B_1, riboflavin-B_2, niacin-B_3, pantothenic acid-B_5, pyridoxine-B_6, biotin-B_7, folic acid-B_9, and cobalamin-B_{12}) and vitamin C; and lipophilic vitamins as pro-vitamin A, vitamin E and K (Nazih and Bard, 2018; Mobin et al., 2019). Although little documented, it is known that some microalgae species are also capable of synthesizing vitamin D (Ljubic et al., 2020).

These biocompounds have consolidated applications in metabolic functions essential for health, as they are precursors of important enzymatic cofactors (Galasso et al., 2019). Furthermore, vitamins play a vital role in the immune system with participation in the cell formation and blood clotting mechanism and to display strong antioxidant activity (Raposo et al., 2013). Vitamin B_{12} supplementation is of particular interest to people who follow strict vegetarian or vegan diets. This is because the main source of this vitamin is foods of animal origin, such as meat, milk, and eggs. Thus, vitamin B_{12} is often used as a food supplement (Galasso et al., 2019).

Among the biochemical components, microalgae can synthesize large quantities of pigments that act as colorant and food supplements. These compounds are classified into three classes: carotenoids, chlorophylls, and phycobiliproteins (PBPs) (Rodrigues et al., 2015; D'Alessandro and Filho, 2016). Microalgae synthesize numerous carotenoid structures, including all known xanthophylls from conventional sources (e.g., lutein, zeaxanthin, antheraxanthin). Also, are able to synthesize specific pigments such as ketocarotenoids, glycosylated carotenoids, allenic, and acetylenic carotenoids (e.g., astaxanthin, echinenone, fucoxanthin, neoxanthin, diadinoxanthin, canthaxanthin), many found only in these microorganisms (Takaichi and Mochimaru, 2007; Guedes et al., 2011; Takaichi, 2011).

These phytochemicals are associated with several important biological functions, including pro-vitamin A activities, well documented for β-carotene and other carotenoids containing β rings. However, its bioactive values go beyond the pro-vitamin activity. Many studies have reviewed the properties of these compounds and a number of other bioactivities are associated with these pigments, which include potent antioxidant activities, neurological disorders, immune system functions, prevention of degenerative chronic diseases as diabetes, obesity, certain types of cancer, macular degeneration, and cardiovascular diseases (Rodriguez-Concepcion et al., 2018; Sathasivam and Ki, 2018; Khalid et al., 2019).

In addition, due to the antioxidant properties of carotenoids can also act as protective agents of lipid peroxidation. A recent study carried out on rats demonstrated that the carotenoids of the microalgae *Scenedesmus obliquus* was able to protect against oxidative body tissues stress by their capacity to improve the activity of some antioxidant enzymes and to reduce lipid peroxidation (Nascimento et al., 2019, 2020). It is worth mentioning that these molecules represent a successful model for food components from microalgae since β-carotene and astaxanthin are produced and marketed from microalgae *Dunaliella salina* and *Haematococcus pluvialis*, respectively (Gong and Bassi, 2016). At the same time, other less established pigments as lutein and zeaxanthin are also gaining momentum in the market of natural pigments (Novoveská et al., 2019).

All photosynthetic beings, such as microalgae, have chlorophyll *a* in their composition. On the other hand, chlorophyll *b* is exclusively found in the *Chlorophyta* and their descendants. The chlorophyll fraction in microalgae may show some chlorophyll derivatives as pheophorbides, chlorophyllides, pheophytins, oxidized, and allomerized compounds (Mulders et al., 2014). In addition, other series of chlorophylls can be found in these microorganisms such as chlorophyll *c*, which is exclusively found in the descendants of the *Rhodophyta* (Jeffrey and Wright, 2005), chlorophyll *d* and *f* synthesized by cyanobacteria (Airs et al., 2014). However, knowledge about its bioactivities is lacking, since most studies only explore the series of chlorophyll *a* and *b* and their derivative compounds.

In fact, microalgae have high chlorophyll content per unit of biomass, sometimes more abundant than in higher plants (Galasso et al., 2019). Furthermore, in green microalgae (*Chlorophyta*), this class of pigments (specifically chlorophylls *a* and *b*) is predominant compared to carotenoids (Fernandes et al., 2020). Even so, considering the number of microalgae species in existence, few studies address the complete profile of chlorophylls in microalgae (Fernandes et al., 2017, 2020; Maroneze et al., 2019).

Although some microalgae biomass is commercialized with the appeal of the high content of bioactive chlorophylls, the real bioactivity of these compounds in the biomass is still unclear. Nevertheless, chlorophylls, and their derivatives compounds from other food sources or in isolated extract show a significant degree of absorption and subsequent bioavailability (Ferruzzi and Blakeslee, 2007; Gallardo-Guerrero et al., 2008; Gandul-Rojas et al., 2009; Chen and Roca, 2018) and are related to prominent biological activities as antimutagenic effect, anti-inflammatory, antigenotoxic properties and potent antioxidant capacity (Lanfer-Marquez et al., 2005; Pérez-Gálvez

segment, and large companies such as BASF, Unilever, and Dow Chemical are involved in projects related to the production of microalgae (Fernandez et al., 2017). In 2017, Taiwan, Japan, the USA, China, Brazil, Spain, Israel, Germany, and Myanmar were the leading producers of microalgae biomass and derived products (Ramirez-Merida et al., 2017). Currently, the market is dominated by the United States, Asia, and Oceania. It is believed that shortly, Europe will become one of the leaders (Rahman, 2020).

As shown in Table 3.1, microalgae-based products are widely exploited, unicellular protein (total dry biomass), and carotenoid (β-carotene, astaxanthin) PC, and polyunsaturated fatty acid have their share in the market for bioactive compounds consolidated. In contrast, other molecules such as PE, chlorophylls, sterols, vitamins, and polysaccharides are considered emerging proposals that have required efforts from the research and development sector to expand their presence and expand the portfolios of commercialized products (Jacob-Lopes et al., 2019). Additionally, the varied applications of microalgae-based products are made possible due to the plurality of their bioactive properties (Hu, 2019).

The main classes of microalgae used in the commercial production of compounds include Cyanophyceae (*Arthrospira* (*Spirulina*), *Oscillatoria*, *Nostoc*), Chlorophyceae (*Chlamydomonas*, *Dunaliella salina*, *Chlorella*), Prymnesiophyceae (*Isochrysis galbana*) and Bacillariophyceae (*Thalassiosira weissflogii* and *Cyclotella cryptica*) (Singh et al., 2020). However, *Chlorella* and *Spirulina* dominate the world market (Koyande et al., 2019).

Among microalgae-based products, dry microalgae biomass was the first product aimed at the industry. The biomass utilization as food was a traditional practice of many ancient peoples, and even today, it is consolidated worldwide as a protein supply (Ramirez-Merida et al., 2017). As a result of discoveries about its bioactivity, it has currently been frequently associated with the prophylaxis of several pathologies, such as hypolipidemic, hypoglycemic, and anti-obesity (Patias et al., 2018; Soto-Sierra et al., 2018).

Microalgae dry biomass production is equivalent to approximately 19,000 tons/year, generating an estimated annual value of 5.7 billion dollars (Jacob-Lopes et al., 2019). Of this total produced, about 12,000 and 5,000 ton/year are equivalent to Spirulina and *Chlorella* biomass, respectively (Mudliar and Shekh, 2019). According to estimates by Mudliar and Shekh (2019), Spirulina dry biomass market volume could reach US $380 million by 2027.

Because of the global trend towards healthy eating habits, the natural color of foods is considered the largest segment of the food coloring market,

TABLE 3.1 Microalgae-based Products Market, Sources, Applications, and Bioactivity

Market	Product	Microalgae Source	Applications	Bioactivity	References
Consolidated	Protein (dry biomass)	*Chlorella*, Spirulina	Food supplements, nutraceuticals, functional foods	Hypolipidemic, hypoglycemic, anti-obesity	Fernandez et al. (2017); Ramirez-Merida et al. (2017); Patias et al. (2018); Soto-Sierra et al. (2018)
	β-carotene	*D. salina*	Natural food pigments, food supplements, feed additives, pharmaceuticals, cosmetics	Antioxidant, anti-inflammatory, immunological modulation	Barkia et al. (2019); Hu (2019); Jacob-Lopes et al. (2019); Sathasivam et al. (2019); Tang et al. (2020)
	Astaxanthin	*H. pluvialis*	Natural food pigments, food supplements, pharmaceuticals, cosmetics	Antioxidant, anti-inflammatory, antitumor	Hu (2019); Jacob-Lopes et al. (2019); Tang et al. (2020)
	Phycocyanin	Spirulina	Natural food pigments, pharmaceuticals, cosmetics	Antioxidant, anti-inflammatory, neuroprotective effects	Romay et al. (2005); Hu (2019); Singh et al. (2020)
	DHA and EPA	*Schizochytrium*, *P. tricornutum*, *Ulkenia* sp., *C. cohnii*	Food supplements, feed additive	Antioxidant, anti-inflammatory, neuroprotective, hepatoprotective effects, anti-bacterial, antitumor	Echeverría et al. (2017); Jacob-Lopes et al. (2019); Singh et al. (2020); Tang et al. (2020)
Emerging	Chlorophylls	*P. autumnale*, *C. sorokiniana*, *S. bijuga*, *C. thermophila*	Natural food pigments, feed additive, nutraceuticals	Antioxidant, anti-inflammatory	Rodrigues et al. (2015); García and Galán (2017); Fernandes et al. (2017, 2020); Sarkar et al. (2020)

TABLE 3.1 *(Continued)*

Market	Product	Microalgae Source	Applications	Bioactivity	References
	Phycoerythrin	*Porphyridium*, *Rhodella*	Chemicals, natural food pigments	Antioxidant, anticarcinogenic, antigenotoxic, antimutagenic	Román et al. (2002); Borowitzka (2013); Hu (2019)
	Sterols	*H. pluvialis*, *A. solitaria*, *N. carneum*	Nutraceuticals	Anti-hypocholesterolemic, anti-inflammatory activity; antitumor	Randhir et al. (2020); Singh et al. (2020)
	Vitamins	*Chlamydomonas*, *Chlorella*, *Scenedesmus*, *Dunaliella*, *Tetraselmis*	Nutraceuticals, food supplements, functional food	Antioxidant; cell formation, blood coagulation, immunological modulation	Becker (2013); Koyande et al. (2019); Sathasivam et al. (2019); Singh et al. (2020)
	Polysaccharides	*Porphyridium*	Functional food, cosmetics, tissue engineering	Prebiotics, hypolipemic, hypoglycemic, antioxidants, antivirals	Khoury et al. (2011); Raposo et al. (2013–2015); Gaignard et al. (2019); Hu (2019)

representing more than 80% of this sector's total revenue (Fernandes et al., 2020). According to an industry report, it is estimated that the global food color market will arrive in 2025, with revenues of the US $2.97 billion (Global Natural Food Colors, 2018). The production of microalgal carotenoids arose initially through the cultivation of *Dunaliella* and *Haematococcus*, among which β-carotene and astaxanthin have consolidated economic participation in the market (Hu, 2019; Jacob-Lopes et al., 2019; Tang et al., 2020).

β-carotene is widely marketed for food supplements, natural pigment, feed additives, pharmaceuticals, and cosmetics (Hu, 2019). Along with *D. salina*, the *D. tertiolecta*, *D. bardawil*, *B. braunii*, *C. nivalis*, *C. acidophila*, *Chlorococcum* sp., *Chlamydocapsa* sp., *Tetraselmis* sp., *C. sorokiniana* and *C. striolata* dominate the production of β-carotene (Sathasivam et al., 2019). The β-carotene market is estimated to range between US $224 and 285 million in 2019 (Mudliar and Shekh, 2019).

Astaxanthin is recognized for its bioactive abilities, as well as β-carotene, its applications include food supplements, natural food pigments, pharmaceuticals, and cosmetics (Hu, 2019). The current production of microalgal astaxanthin is dominated by *H. pluvialis*, followed by *C. zofingiensis, C. nivalis*, *B. braunii*, *C. vulgaris*, *C. striolata*, *Monoraphidium* sp., *Chlamydocapsa* sp., *Neospongiococcum* sp., *Chlorococcum* sp. and *S. obliquus* (Khoo et al., 2019).

The prices of nutraceutical grade astaxanthin originating from *H. pluvialis* can reach the US $6,000/kg depending on the cultivation configuration (Dawidziuk et al., 2017), it is estimated that the market of this ketocarotenoid reaches 770 million dollars up to 2024 (Mudliar and Shekh, 2019). The global market of microalgal astaxanthin still faces some problems of competitiveness due to production costs compared to its synthetic form. While the cost of producing *H. pluvialis* astaxanthin can reach US $3600/kg (Li et al., 2011), the cost of synthetic production is the US $1000/kg (Olaizola, 2003). However, synthetic astaxanthin has its use restricted to aquaculture, making natural production necessary for human consumption and animal feed (Li et al., 2011).

Another pigment with a consolidated place in the market is PC, which is obtained from Spirulina and marketed as a natural blue pigment, being used as a natural dye for health food (beverage, dairy products, confectionery, jellies, etc.), and nutritional ingredient. The selling price of PC ranges from US $500–100,000/kg, depending on purity (Borowitzka,

2013). In 2019, the global market for this PBP was valued at US $60 million (Tang et al., 2020).

DHA and EPA are the main sources of microalgae omega-3 FAs, their market prices vary in the range of US $80–160/kg, with an estimated 2025 reaching US $898.7 million (Hu, 2019). Among the microalgal oils rich in PUFAs, authorized, and marketed for human consumption are oils extracted from *Schizochytrium* (EPA and DHA), *Ulkenia* sp. (DHA), *Crypthecodinium cohnii* (DHA), and *Phaeodactylum tricornutum* (EPA) (Echeverría et al., 2017).

While the aforementioned microalgal products include a portfolio of concrete applications from a commercial point of view, others are potentially indicated or are in the process of development (Fernandez et al., 2017; Jacob-Lopes et al., 2019). In recent years, emerging health issues, such as hypersensitivity, have emerged from synthetic compounds in food (Tang et al., 2020). Consequently, efforts were directed towards the commercial development of new microalgae products (Singh et al., 2020).

Chlorophyll is among these emerging compounds, in addition to pigmentation properties the bioactive activity has been boosting the scientific community in order to make production feasible and increase the number of microalgal pigments available for commercialization (Rodrigues et al., 2015; Fernandes et al., 2017, 2020; Sarkar et al., 2020). Although the production potential has been demonstrated for *Phormidium autumnale*, *Chlorella sorokiniana*, *Scenedesmus bijuga*, and *Chlorella thermophila* (Rodrigues et al., 2015; Fernandes et al., 2017, 2020; Sarkar et al., 2020) the commercial chlorophylls available they are produced only from plant sources, mainly spinach (Clark, 2016).

Another pigment in commercial development is PE, produced from red microalgae (*Porphyridium* and *Rhodella*) (Borowitzka, 2013; Hu, 2019). This PBP is widely used as a fluorescent probe and analytical reagent (Román et al., 2002), and to a lesser extent, in the food industry as a natural red-pink pigment (Rahman, 2020). In general, the selling price of PE ranges from US $500 to 50,000/kg (Hu, 2019).

Steroids such as β-sitosterol, campesterol, brassicasterol, stigmasterol, and ergosterol are lipid biocomposites of commercial importance due to their bioactive properties (Singh et al., 2020). The global market for these phytosterols is estimated to reach US $935 million by 2022. In this context, microalgae represent a potential commercial source, as it has been suggested that microalgae phytosterols have higher productivity than those of rapeseed plants, for example (Randhir et al., 2020). The species with the most

significant commercial relevance are *Haematococcus pluvialis*, *Anabaena solitaria* and *Nostoc carneum* (Randhir et al., 2020).

Microalgae are also considered potential sources of vitamins; from these microorganisms, it is possible to obtain substantial amounts of vitamins A, C, E, K, and B complex (Becker, 2013). The most-reported species are those of the genus *Chlamydomonas*, *Chlorella*, *Scenedesmus*, *Dunaliella*, and *Tetraselmis* (Koyande et al., 2019; Sathasivam et al., 2019). In terms of prices, the global forecast for the vitamin market by 2023 is US $7.35 billion. According to a research report, the increased demand for functional and enriched processed food products, the widespread vitamin deficiencies, and the fortification of meat and dairy products are factors that currently drive the market for vitamins.

Microalgal polysaccharides are promising, and the interest of the scientific community in these biopolymers is recent (Gaignard et al., 2019). EPSs obtained from *Porphyridium* species have been one of the most explored (Raposo et al., 2013–2015). Additionally, β-glucans, produced by many green algae, have been identified as exceptional prebiotics acting in the reduction of LDL cholesterol and the risk of cardiovascular diseases (Khoury et al., 2011). Also, the synthesis of water-soluble lubricants and thickening agents is made possible from microalgae polysaccharides (Sathasivam et al., 2019).

The bioactive properties of polysaccharides have aroused interest in several industrial sectors, such as pharmaceutical, nutraceutical, cosmetic, and food production (Gaignard et al., 2019). In particular, sulfated polysaccharides found abundantly in different microalgae species (Raposo et al., 2013–2015) have few equivalents in terrestrial plants and have chemical and biological characteristics that allow their application in the development of innovative systems for tissue engineering (Silva et al., 2012). Despite this, the commercialization of this microalgal biopolymer is still in the initial stage and therefore, the market values are not clear (Hu, 2019).

Finally, microalgal bioactive compounds have specific advantages over established alternatives, mainly due to a chemical conformation that is more effective than those of conventional competitors, making their use commercially attractive for multiple market segments (Jacob-Lopes et al., 2019). However, in order to expand the global market for microalgae products, commercial authorization is required in regional markets through various regulations that will be demonstrated in the next section.

3.4 SAFETY AND REGULATORY ISSUES OF MICROALGAE-BASED FOOD COMPOUNDS

There is a high interest in the application of microalgae, or their bioproducts, as food ingredients; therefore, it is fundamental to know their safety. However, the regulatory frameworks that control the use of microalgae like foods or food ingredients differ substantially in different regions of the world (Table 3.2) (Jacob-Lopes et al., 2019; Matos, 2019; Torres-Tiji et al., 2020; Zanella and Vianello, 2020).

TABLE 3.2 Some Quality Standards for Microalgae

Parameter	EU	USA	China	India	Japan	Brazil
Moisture (%)	<10.0	<7.0	–	<9.0	<7.0	<10.0
Ash (%)	<10.0	–	–	<9.0	–	–
Protein (%)	20–55	6–71	6–71	>55	–	–
Lipid (%)	12–60	2–40	2–18	–	–	–
Carbohydrates (%)	25–60	4–64	8–64	–	–	–
Arsenic (ppm)	<0.1	–	–	–	–	<1.0
Lead (ppm)	<3.0	<0.2	–	–	–	<0.3
Cadmium (ppm)	<3.0	<0.2	–	–	–	<0.1
Mercury (ppm)	<0.1	<0.025	–	–	–	<0.5
Microorganism	–	–	–	–	–	–
Standard plate count ($X10^6$ g^{-1})	<0.1	<0.2	–	–	<0.005	–
Mold (number/g)	<100	<100	–	–	<100	–
Coliforms (number/g)	Absent	Absent	–	–	<10	<10
Salmonella sp.	Absent	Absent	–	–	–	Absent
Staphylococcus sp. (number/g)	<100	Absent	–	–	–	<500

Source: Becker (1994); AOAC (2002); ISO (2002, 2009a, b, 2015); EN (2009); Matos (2019); Zanella and Vianello (2020); FAO (1997); Chacon-Lee and Gonzalez-Marino (2010); IS (1990); Jassby (1988); Anvisa (2001, 2013).

Specifically, in European Union (EU), the European Food and Safety Authority (EFSA) recognizes the application of *Arthrospira platensis* (Spirulina), *Chlorella pyrenoidosa*, *Chlorella luteoviridis*, and *Chlorella vulgaris* as food, because of its long history of use, can be commercialized in the EU without the need to comply with Regulation EU 2015/2283 on novel foods.

While *Odontella aurita* and *Tetraselmis chui* must follow current legislation of novel foods. As for your bioproducts from microalgae, are authorized for use β-carotene from *Dunaliella salina* (Commission Directive 2008/128/EC), DHA, and EPA extracted from *Schizochytrium* sp. (Commission Decision 2003/427/EC), oil-rich in PUFA obtained from *Ulkenia* sp. (Commission Decision 2009/777/EC), astaxanthin-rich oleoresin extracted from *Haematococcus pluvialis*, and oil-rich in EPA obtained from *Phaeodactylum tricornutum* are approved extracts and labeled as "novel food" (Matos, 2019; EFSA, 2020; Torres-Tiji et al., 2020).

Consequently, a similar standard is held in Canada, where Health Canada is the organization that supervise food safety and determine that any food that is new or has changed compared to existing food products is classified as novel foods, and its safety must be assessed by Canadian Food Inspection Agency (CFIA, 2020). In addition to these, Canada considered safe to consume *A. platensis*, *C. vulgaris*, *C. sorokiniana*, *C. regularis*, *D. salina*, and *Euglena gracilis* (CFIA, 2020; Zanella and Vianello, 2020).

In the United States (USA) jurisdiction, the Food and Drug Administration (FDA) grants GRAS status (generally recognized as safe) (US FDA, 2018). Similarly, like the EU, in the USA, *A. platensis*, *C. vulgaris*, *D. Salina*, *H. pluvialis*, and *P. tricornutum*, authorized as foods and extracts. Moreover, *Chlamydomonas reinhardtii*, *Auxenochlorella protothecoides*, *Dunaliella bardawil* and *E. gracilis*, also have GRAS status (US FDA, 2018; Jacob-Lopes et al., 2019; Matos, 2019; Torres-Tiji et al., 2020).

Regulations in India, Japan, China, and Brazil have been consulted, and the findings regarding the safety of microalgae as food are summarized, *A. platensis*, *D. salina*, and *H. pluvialis* have been found to be considered safe to consume in India, Japan, China, and Brazil. *Chlorella* is also widely assumed as safe for human consumption, but the approved species of *Chlorella* varies among countries: *C. protothecoides* is approved in Japan, *C. pyrenoidesa* is approved in China, and *C. vulgaris* is approved in Japan and Brazil. Furthermore, *Ulkenia* sp. has been found to be considered safe in Brazil. Finally, *E. gracilis* is approved by China and Japan (FSSAI, 2016; Anvisa, 2018; Torres-Tiji et al., 2020).

3.5 CONCLUSION

This chapter investigated the potential of microalgae to be exploited in the bioactive ingredients in the food industries. Various renewable bioactive

natural compounds with beneficial properties can be extracted from microalgae biomass and incorporated into industrial products to replace synthetic materials. However, new processing perspectives should be explored in order to emerge bioactive compounds not yet marketed.

KEYWORDS

- **anti-inflammatory**
- **antioxidant**
- **biological properties**
- **biomass**
- **fatty acids**
- **food ingredients**
- **food safety**
- **health-food markets**
- **microalgae**
- **phycoerythrin**
- **pigments**
- **polysaccharides**
- **protein**
- **sterols**
- **vitamins**

REFERENCES

Airs, R. L., Temperton, B., Sambles, C., Farnham, G., Skill, S. C., & Llewellyn, C. A., (2014). Chlorophyll *f* and chlorophyll *d* are produced in the cyanobacterium *Chlorogloeopsis fritschii* when cultured under natural light and near-infrared radiation. *FEBS Lett., 588*(20), 3770–3777.

Anvisa, (2001). http://portal.anvisa.gov.br/documents/33880/2568070/RDC_12_2001.pdf/15ffddf6-3767-4527-bfac-740a0400829b (accessed on 2 July 2021).

Anvisa, (2013). http://portal.anvisa.gov.br/documents/33880/2568070/rdc0042_29_08_2013.pdf/c5a17d2d-a415-4330-90db-66b3f35d9fbd (accessed on 2 July 2021).

Anvisa, (2018). http://portal.anvisa.gov.br/documents/10181/3898888/IN282018COMP.pdf/db9c7460-ae66-4f78-8576-dfd019bc9fa1 (accessed on 2 July 2021).

AOAC, (2002). http://www.aoacofficialmethod.org/index.php?mainpage=productinfo&productsid=533 (accessed on 2 July 2021).

Baky, H. H. A. E., Baroty, G. S. E., & Ibrahe, E. A., (2015). Functional characters evaluation of biscuits sublimated with pure phycocyanin isolated from Spirulina and Spirulina biomass. *Nutr. Hosp., 32*(1), 231–241.

Barkia, I., Saari, N., & Manning, S. R., (2019). Microalgae for high-value products towards human health and nutrition. *Mar. Drugs, 17*(5), 1–29.

Becker, E. W., (1994). *Microalgae: Biotechnology and Microbiology* (Vol. 10, p. 293). Cambridge University Press, Cambridge.

Becker, E. W., (2013). Microalgae for human and animal nutrition. In: Richmond, A., & Hu, Q., (eds.), *Handbook of Microalgal Culture* (pp. 461–503). John Wiley and Sons, New York.

Bernaerts, T. M., Gheysen, L., Kyomugasho, C., Kermani, Z. J., Vandionant, S., Foubert, I., & Van-Loey, A. M., (2018). Comparison of microalgal biomasses as functional food ingredients: Focus on the composition of cell wall related polysaccharides. *Algal Res., 32*, 150–161.

Berquin, I. M., Edwards, I. J., & Chen, Y. Q., (2008). Multi-targeted therapy of cancer by omega-3 fatty acids. *Cancer Lett., 269*(2), 363–377.

Blaga, A. C., Ciobanu, C., Caşcaval, D., & Galaction, A. I., (2010). Enhancement of ergosterol production by *Saccharomyces cerevisiae* in batch and fed-batch fermentation processes using n-dodecane as oxygen-vector. *Biochem. Eng. J., 131*, 70–76.

Borowitzka, M. A., (2013). High-value products from microalgae-their development and commercialization. *J. Appl. Phycol., 25*, 743–756.

Bradberry, J. C., & Hilleman, D. E., (2013). Overview of omega-3 fatty acid therapies. *Pharmacol. Ther., 38*(11), 680–691.

Brasil, B. D. S. A. F., De Siqueira, F. G., Salum, T. F. C., Zanette, C. M., & Spier, M. R., (2017). Microalgae and cyanobacteria as enzyme biofactories. *Algal Res., 25*, 76–89.

Camacho, F., Macedo, A., & Malcata, F., (2019). Potential industrial applications and commercialization of microalgae in the functional food and feed industries: A short review. *Mar. Drugs, 17*(312), 1–25.

Campoy, C., Escolano-Margarit, M. V., Anjos, T., Szajewska, H., & Uauy, R., (2012). Omega 3 fatty acids on child growth, visual acuity, and neurodevelopment. *Br. J. Nutr., 107*(2), 85–106.

Caporgno, M. P., & Mathys, A., (2018). Trends in microalgae incorporation into innovative food products with potential health benefits. *Front. Nutr., 58*(5), 1–10.

CFIA, (2020). https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods.html (accessed on 2 July 2021).

Chacon-Lee, L. T., & Gonzalez-Marino, E. G., (2010). Microalgae for "healthy" foods-possibilities and challenges. *Compr. Rev. Food. Sci. F., 9*, 655–675.

Chen, B., You, W., Huang, J., Yu, Y., & Chen, W., (2010). Isolation and antioxidant property of the extracellular polysaccharide from *Rhodella reticulata*. *World J. Microb. Biot., 26*(5), 833–840.

Chen, K., & Roca, M., (2018). *In vitro* bioavailability of chlorophyll pigments from edible seaweeds. *J. Funct. Foods, 41*, 25–33.

Chew, K. W., Yap, J. Y., Show, P. L., Suan, N. H., Juan, J. C., Ling, T. C., Lee, D. J., & Chang, J. S., (2017). Microalgae biorefinery: High value products perspectives. *Bioresour. Technol., 229*, 53–62.

Clark, M., (2016). *Handbook of Textile and Industrial Dyeing Principles, Processes and Types of Dyes* (Vol. 1, p. 680). Woodhead Publishing, Cambridge.

D'Alessandro, E. B., & Filho, N. R. A., (2016). Concepts and studies on lipid and pigments of microalgae: A review. *Renew Sust. Energ. Rev., 58*, 832–841.

Dawidziuk, A., Popiel, D., Luboinska, M., Grzebyk, M., Wisniewski, M., & Koczyk, G., (2017). Assessing contamination of microalgal astaxanthin producer *Haematococcus* cultures with high-resolution melting curve analysis. *J. Appl. Genet., 58*(2), 277–285.

Delgado-Lista, J., Perez-Martinez, P., Lopez-Miranda, J., & Perez-Jimenez, F., (2012). Long chain omega-3 fatty acids and cardiovascular disease: A systematic review. *Br. J. Nutr., 107*(2), 201–213.

Devaraj, S., Jialal, I., & Vega-López, S., (2004). Plant sterol-fortified orange juice effectively lowers cholesterol levels in mildly hypercholesterolemic healthy individuals. *Arterioscler Thromb. Vasc. Biol., 24*(3), 25–28.

Echeverría, F., Valenzuela, R., Hernandez-Rodas, M. C., & Valenzuela, A., (2017). Docosahexaenoic acid (DHA), a fundamental fatty acid for the brain: New dietary sources. *Prostaglandins Leukot. Essent. Fatty Acids, 124*, 1–10.

EFSA, (2020). https://ec.europa.eu/food/safety/novel_food/catalogue/search/public/index.cfm?ascii=N (accessed on 2 July 2021).

EN, (2009). https://cdn.standards.iteh.ai/samples/29302/799c23fe6a6a4e8cb2dd72ae1050e81f/SIST-EN-15763-2010.pdf (accessed on 2 July 2021).

FAO, (1997). http://www.fao.org/3/w7241e/w7241e0h.htm#chapter%206%20%20%20oil%20production (accessed on 2 July 2021).

Fernandes, A. S., Nogara, G. P., Menezes, C. R., Cichoski, A. J., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q., (2017). Identification of chlorophyll molecules with peroxyl radical scavenger capacity in microalgae *Phormidium autumnale* using ultrasound-assisted extraction. *Food Res. Int., 99*, 1036–1041.

Fernandes, A. S., Petry, F. C., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q., (2020). HPLC-PDA-MS/MS as a strategy to characterize and quantify natural pigments from microalgae. *Curr. Res. Nutr. Food Sci., 3*, 100–112.

Fernandez, F. G. A., Sevilla, J. M. F., & Grima, E. M., (2017). Microalgae: The basis of mankind sustainability. In: Moya, B. L., De Gracia, D. S., & Mazadiego, L. F., (eds.), *Case Study of Innovative Projects-Successful Real Cases* (pp. 123–140). IntechOpen, London.

Fernández-Rojas, B., Hernández-Juárez, J., & Pedraza-Chaverri, J., (2014). Nutraceutical properties of phycocyanin. *J. Funct. Foods, 11*, 375–392.

Ferruzzi, M. G., & Blakeslee, J., (2007). Digestion, absorption, and cancer preventative activity of dietary chlorophyll derivatives. *Nutr. Res. Rev., 27*(1), 1–12.

FSSAI, (2016). https://archive.fssai.gov.in/home/fss-legislation/notifications/gazette-notification.html (accessed on 2 July 2021).

Gaignard, C., Gargouch, N., Dubessay, P., Delattre, C., Pierre, G., Laroche, C., Fendri, I., et al., (2019). New horizons in culture and valorization of red microalgae. *Biotechnol. Adv., 37*(1), 193–222.

Galasso, C., Gentile, A., Orefice, I., Ianora, A., Bruno, A., Noonan, D. M., Sansone, C., Albini, A., & Brunet, C., (2019). Microalgal derivatives as potential nutraceutical and food supplements for human health: A focus on cancer prevention and interception. *Nutr., 11*(6), 1–22.

Gallardo-Guerrero, L., Gandul-Rojas, B., & Mínguez-Mosquera, M. I., (2008). Digestive stability, micellarization, and uptake by Caco-2 human intestinal cell of chlorophyll derivatives from different preparations of pea (*Pisum sativum* L.). *J. Agric. Food Chem., 56*(18), 8379–8386.

Gandul-Rojas, B., Gallardo-Guerrero, L., & Mínguez-Mosquera, M. I., (2009). Influence of the chlorophyll pigment structure on its transfer from an oily food matrix to intestinal epithelium cells. *J. Agric. Food Chem., 57*(12), 5306–5314.

García, J. L., Vicente, M., & Galán, B., (2017). Microalgae, old sustainable food, and fashion nutraceuticals. *Microb. Biotechnol., 10*(5), 1017–1024.

Global Natural Food Colors Market Size & Share Industry Report 2018–2025, (2018). https://www.grandviewresearch.com/industry-analysis/natural-food-colors-market (accessed on 2 July 2021).

Gong, M., & Bassi, A., (2016). Carotenoids from microalgae: A review of recent developments. *Biotechnol. Adv., 34*(8), 1396–1412.

Guedes, A. C., Amaro, H. M., & Malcata, F. X., (2011). Microalgae as sources of carotenoids. *Mar. Drugs, 9*(4), 625–644.

Guzmán, S., Gato, A., Lamela, M., Freire-Garabal, M., & Calleja, J. M., (2003). Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother. Res., 17*(6), 665–670.

Harris, W. S., (2008). Linoleic acid and coronary heart disease. *Prostaglandins Leukot. Essent. Fatty Acids, 79*(35), 169–171.

Hu, I. C., (2019). Production of potential coproducts from microalgae. In: Pandey, A., Chang, J. S., Soccol, C. R., Lee, D. J., & Chisti, Y., (eds.), *Biofuels from Algae* (pp. 345–358). Elsevier BV, Netherlands.

Huleihel, M., Ishanu, V., Tal, J., & Arad, S. M., (2001). Antiviral effect of red microalgal polysaccharides on *Herpes simplex* and *Varicella zoster* viruses. *J. Appl. Phycol., 13*(2), 127–134.

Innis, S. M., (2007). Dietary (n-3) fatty acids and brain development. *J. Nutr., 137*(4), 855–859.

IS, (1990). https://archive.org/details/gov.in.is.12895.1990 (accessed on 2 July 2021).

ISO, (2002). https://www.iso.org/standard/37272.html (accessed on 2 July 2021).

ISO, (2009a). https://www.iso.org/standard/44807.html (accessed on 2 July 2021).

ISO, (2009b). https://www.iso.org/obp/ui/#iso:std:iso:1871:ed-2:v1:en (accessed on 2 July 2021).

ISO, (2015). https://www.iso.org/standard/63542.html (accessed on 2 July 2021).

Jacob-Lopes, E., Maroneze, M. M., Deprá, M. C., Sartori, R. B., Dias, R. R., & Zepka, L. Q., (2019). Bioactive food compounds from microalgae: An innovative framework on industrial biorefineries. *Curr. Opin. Food Sci., 25*, 1–7.

Jassby, A., (1988). Some public health aspects of microalgal products. In: Lembi, C. A., & Waaland, J. R., (eds.), *Algae and Human Affair* (pp. 181–201). Cambridge University Press, Cambridge.

Jeffrey, S. W., & Wright, S. W., (2005). Photosynthetic pigments in marine microalgae: Insights from cultures and the sea. *Environ. Sci., 1*, 33–90.

Jung, W., Gwak, Y., Davies, P. L., Kim, H. J., & Jin, E., (2014). Isolation and characterization of antifreeze proteins from the Antarctic marine microalga *Pyramimonas gelidicola*. *Mar. Biotechnol., 16*(5), 502–512.

Katiyar, R., & Arora, A., (2020). Health-promoting functional lipids from microalgae pool: A review. *Algal Res., 46*, 101800.

Kaur, N., Chugh, V., & Gupta, A. K., (2014). Essential fatty acids as functional components of foods: A review. *J. Food Sci. Technol., 51*(10), 2289–2303.

Khalid, M., Bilal, M., Iqbal, H. M., & Huang, D., (2019). Biosynthesis and biomedical perspectives of carotenoids with special reference to human health-related applications. *Biocatal. Agric. Biotechnol., 17*, 399–407.

Khanra, S., Mondal, M., Halder, G., Tiwari, O. N., Gayen, K., & Bhowmick, T. K., (2018). Downstream processing of microalgae for pigments, protein, and carbohydrate in industrial application: A review. *Food Bioprod. Process., 110*, 60–84.

Khoo, K. S., Chew, K. W., Ooi, C. W., Ong, H. C., Ling, T. C., & Show, P. L., (2019). Extraction of natural astaxanthin from *Haematococcus pluvialis* using liquid biphasic flotation system. *Bioresour. Technol., 290*.

Khoury, D., Cuda, C., Luhovyy, B. L., & Anderson, G. H., (2012). Beta-glucan: Health benefits in obesity and metabolic syndrome. *J. Nutr. Metab., 2012*, 1–28.

Kim, K. B., Nam, Y. A., Kim, H. S., Hayes, A. W., & Lee, B. M., (2014). α-Linolenic acid: Nutraceutical, pharmacological and toxicological evaluation. *Food Chem. Toxicol., 70*, 163–178.

Koyande, A. K., Chew, K. W., Rambabu, K., Tao, Y., Chu, D. T., & Show, P. L., (2019). Microalgae: A potential alternative to health supplementation for humans. *FSHW, 8*(1), 16–24.

Lanfer-Marquez, U. M., Barros, R. M., & Sinnecker, P., (2005). Antioxidant activity of chlorophylls and their derivatives. *Food Res. Int., 38*(9), 885–891.

Lee, J. B., Srisomporn, P., Hayashi, K., Tanaka, T., Sankawa, U., & Hayashi, T., (2001). Effects of structural modification of calcium spirulan, a sulfated polysaccharide from *Spirulina platensis*, on antiviral activity. *Chem. Pharm. Bull., 49*(1), 108–110.

Lee, Y. H., Bae, S. C., & Song, G. G., (2012). Omega-3 polyunsaturated fatty acids and the treatment of rheumatoid arthritis: A meta-analysis. *Arch. Med. Res., 43*(5), 356–362.

Levasseur, W., Perré, P., & Pozzobon, V., (2020). A review of high value-added molecules production by microalgae in light of the classification. *Biotechnol. Adv., 107*545.

Li, J., Zhu, D., Niu, J., Shen, S., & Wang, G., (2011). An economic assessment of astaxanthin production by large scale cultivation of *Haematococcus pluvialis*. *Biotechnol. Adv., 29*, 568–574.

Li, Y., Wang, C., Liu, H., Su, J., Lan, C. Q., Zhong, M., & Hu, X., (2020). Production, isolation, and bioactive estimation of extracellular polysaccharides of green microalga *Neochloris oleoabundans*. *Algal Res., 48*, 101883.

Liu, L., Hu, Q., Wu, H., Xue, Y., Cai, L., Fang, M., Liu, Z., et al., (2016). Protective role of n6/n3 PUFA supplementation with varying DHA/EPA ratios against atherosclerosis in mice. *J. Nutr. Biochem., 32*, 171–180.

Ljubic, A., Jacobsen, C., Holdt, S. L., & Jakobsen, J., (2020). Microalgae *Nannochloropsis oceanica* as a future new natural source of vitamin D_3. *Food Chem., 320*, 126627.

Luo, X., Su, P., & Zhang, W., (2015). Advances in microalgae-derived phytosterols for functional food and pharmaceutical applications. *Mar. Drugs, 13*(7), 4231–4254.

Marangoni, F., Agostoni, C., Borghi, C., Catapano, A. L., Cena, H., Ghiselli, A., & Riccardi, G., (2020). Dietary linoleic acid and human health: Focus on cardiovascular and cardiometabolic effects. *Atherosclerosis, 292*, 90–98.

Maroneze, M. M., Zepka, L. Q., Jacob-Lopes, E., Pérez-Gálvez, A., & Roca, M., (2019). Chlorophyll oxidative metabolism during the phototrophic and heterotrophic growth of *Scenedesmus obliquus*. *Antioxidants, 8*(12), 600.

Matos, A. P., (2019). Microalgae as a potential source of proteins. In: Galanakis, C., (ed.), *Proteins: Sustainable Source, Processing and Applications* (pp. 63–93). Academic Press, Cambridge.

Matos, J., Cardoso, C., Bandarra, N. M., & Afonso, C., (2017). Microalgae as healthy ingredients for functional food: A review. *Food Funct., 8*(8), 2672–2685.

Mobin, S. M., Chowdhury, H., & Alam, F., (2019). Commercially important bioproducts from microalgae and their current applications: A review. *Energy Procedia, 160*, 752–760.

Morais, M. G., Vaz, B. D. S., Morais, E. G., & Costa, J. A. V., (2015). Biologically active metabolites synthesized by microalgae. *Biomed Res. Int., 1*–15.

Mudliar, S. N., & Shekh, A. Y., (2019). Recent advances in microalgal bioactives for food, feed, and healthcare products: Commercial potential, market space, and sustainability. *Compr. Rev. Food Sci. Food Saf., 18*(6), 1882–1897.

Mulders, K. J., Lamers, P. P., Martens, D. E., & Wijffels, R. H., (2014). Phototrophic pigment production with microalgae: Biological constraints and opportunities. *J. Phycol., 50*(2), 229–242.

Nascimento, T. C. D., Cazarin, C. B. B., Maróstica, Jr. M. R., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q., (2020). Microalgae carotenoids intake: Influence on cholesterol levels, lipid peroxidation and antioxidant enzymes. *Food Res. Int., 128*, 108770.

Nascimento, T. C., Cazarin, C. B. B., Roberto, M. M., Risso, É. M., Amaya-Farfan, J., Grimaldi, R., Mercadante, A. Z., et al., (2019). Microalgae biomass intake positively modulates serum lipid profile and antioxidant status. *J. Funct. Foods, 5*8, 11–20.

Nazih, H., & Bard, J. M., (2018). Microalgae in human health: Interest as a functional food. In: Levine, I., & Fleurence, J., (eds.), *Microalgae in Health and Disease Prevention* (pp. 211–226). Academic Press, Cambridge.

Niccolai, A., Chini, G., Rodol, L., Biondi, N., & Tredici, M. R., (2019). Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Res., 42*, 101617.

Novoveská, L., Ross, M. E., Stanley, M. S., Pradelles, R., Wasiolek, V., & Sassi, J. F., (2019). Microalgal carotenoids: A review of production, current markets, regulations, and future direction. *Mar. Drugs, 17*(11), 640.

Olaizola, M., (2003). Commercial development of microalgal biotechnology: From the test tube to the marketplace. *Biomol. Eng., 20*(4–6), 459–466.

Pagels, F., Guedes, A. C., Amaro, H. M., Kijjoa, A., & Vasconcelos, V., (2019). Phycobiliproteins from cyanobacteria: Chemistry and biotechnological applications. *Biotechnol. Adv., 37*, 422–443.

Pan-utai, W., & Lamtham, S., (2019). Extraction, purification, and antioxidant activity of phycobiliprotein from *Arthrospira platensis*. *Process Biochem., 82*, 189–198.

Patias, L. D., Fernandes, A. S., Petry, F. C., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q., (2017). Carotenoid profile of three microalgae/cyanobacteria species with peroxyl radical scavenger capacity. *Food Res. Int., 100*, 260–266.

Patias, L. D., Maroneze, M. M., Siqueira, S. F., Menezes, C. R., Zepka, L. Q., & Jacob-Lopes, E., (2018). Single-cell protein as a source of biologically active ingredients for the formulation of anti-obesity foods. In: Holban, A. M., & Grumezescu, A. M., (eds.), *Alternative and Replacement Foods* (pp. 317–353). Academic Press, Cambridge.

Pérez-Galvez, A., Viera, I., & Roca, M., (2017). Chemistry in the bioactivity of chlorophylls: An overview. *Curr. Med., 24*(40), 4515–4536.

Rahman, K. M., (2020). Food and high value products from microalgae: Market opportunities and challenges. In: Asrafu, A. M., Xu, J. L., & Wang, Z., (eds.), *Microalgae Biotechnology for Food, Health and High Value Products* (pp. 3–27). Springer, Singapore.

Raja, R., Coelho, A., Hemaiswarya, S., Kumar, P., Carvalho, I. S., & Alagarsamy, A., (2018). Applications of microalgal paste and powder as food and feed: An update using text mining tool. *BJBAS, 7*(4), 740–747.

Ramirez-Mérida, L. G., Zepka, L. Q., & Jacob-Lopes, E., (2017). Current production of microalgae at industrial scale. In: Pires, J. C. M., (ed.), *Recent Advances in Renewable Energy* (pp. 242–260). Bentham Science Publishers, Sharjah.

Randhir, A., Laird, D. W., Maker, G., Trengove, R., & Moheimani, N. R., (2020). Microalgae: A potential sustainable commercial source of sterols. *Algal Res., 46*, 101772.

Raposo, M. F. D. J., Morais, A. M. M. B., & Morais, R. M. S. C., (2014). Influence of sulphate on the composition and antibacterial and antiviral properties of the exopolysaccharide from *Porphyridium cruentum*. *Life Sci., 101*(1, 2), 56–63.

Raposo, M. F. J., Morais, A. M. B., & Morais, R. M. S. C., (2015). Marine polysaccharides from algae with potential biomedical applications. *Mar. Drugs, 13*(5), 2967–3028.

Raposo, M. F., Morais, R. M. S. C., & Morais, A. M. M. B., (2013). Bioactivity and applications of sulphated polysaccharides from marine microalgae. *Mar. Drugs, 11*, 233–252.

Rodrigues, D. B., Menezes, C. R., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q., (2015). Bioactive pigments from microalgae *Phormidium autumnale*. *Food Res. Int., 77*, 273–279.

Rodriguez-Concepcion, M., Avalos, J., Bonet, M. L., Boronat, A., Gomez-Gomez, L., Hornero-Mendez, L. M. C., Meléndez-Martínez, A. J., et al., (2018). A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Prog Lipid Res., 70*, 62–93.

Román, R. B., Alvárez-Pez, J. M., Fernández, F. G. A., & Grima, E. M., (2002). Recovery of pure B-phycoerythrin from the microalga *Porphyridium cruentum*. *J. Biotechnol., 93*, 73–85.

Romay, C., Gonzalez, R., Ledon, N., Remirez, D., & Rimbau, V., (2005). C-Phycocyanin: A biliprotein with antioxidant, anti-inflammatory, and neuroprotective effects. *Curr. Protein Pept. Sc., 4*(3), 207–216.

Saini, R. K., & Keum, Y. S., (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance: A review. *Life Sci., 203*, 255–267.

Salas-Salvadó, J., Bulló, M., Babio, N., Martínez-González, M. Á., Ibarrola-Jurado, N., Basora, J., Estruch, R., Covas, M. I., et al., (2011). Reduction in the incidence of type 2 diabetes with the Mediterranean diet: Results of the predimed-reus nutrition intervention randomized trial. *Diabetes Care, 34*(1), 14–19.

Samuels, R., Mani, U. V., Iyer, U. M., & Nayak, U. S., (2002). Hypocholesterolemic effect of spirulina in patients with hyperlipidemic nephrotic syndrome. *J. Med. Food, 5*(2), 91–96.

Sanjeewa, K. K. A., Fernando, I. P. S., Samarakoon, K. W., Lakmal, H. H. C., Kim, E. A., Kwon, O., Dilshara, M. G., et al., (2016). Anti-inflammatory and anti-cancer activities of sterol rich fraction of cultured marine microalga *Nannochloropsis oculata*. *Algae, 31*(3), 277–287.

Sarkar, S., Manna, M. S., Bhowmick, T. K., & Gayen, K., (2020). Extraction of chlorophylls and carotenoids from dry and wet biomass of isolated *Chlorella thermophila*: Optimization of process parameters and modeling by artificial neural network. *Process Biochem., 96*, 58–72.

Sathasivam, R., & Ki, J. S., (2018). A review of the biological activities of microalgal carotenoids and their potential use in healthcare and cosmetic industries. *Mar. Drugs, 16*(1), 26.

Sathasivam, R., Radhakrishnan, R., Hashem, A., & Abd-Allah, E. F., (2019). Microalgae metabolites: A rich source for food and medicine. *Saudi J. Biol. Sci., 26*(4), 709–722.

Silva, T. H., Alves, A., Popa, E. G., Reys, L. L., Gomes, M. E., Sousa, S. S. S., Mano, J. F., & Reis, R. L., (2012). Marine algae sulfated polysaccharides for tissue engineering and drug delivery approaches. *Biomatter, 2*(4), 278–289.

Singh, S. K., Kaur, R., Bansal, A., Kapur, S., & Sundaram, S., (2020). Biotechnological exploitation of cyanobacteria and microalgae for bioactive compounds. In: Verma, M. L., & Chandel, A. K., (eds.), *Biotechnological Production of Bioactive Compounds* (pp. 107–137). Elsevier, Amsterdam.

Soto-Sierra, L., Stoykova, P., & Nikolov, Z. L., (2018). Extraction and fractionation of microalgae-based protein products. *Algal Res., 36*, 175–192.

Sun, G. Y., Simonyi, A., Fritsche, K. L., Chuang, D. Y., Hannink, M., Gu, Z., Greenlief, C. M., et al., (2018). Docosahexaenoic acid (DHA): An essential nutrient and a nutraceutical for brain health and diseases. *Prostaglandins Leukot. Essent. Fatty Acids, 136*, 3–13.

Takaichi, S., & Mochimaru, M., (2007). Carotenoids and carotenogenesis in cyanobacteria: Unique ketocarotenoids and carotenoid glycosides. *Cell. Mol. Life Sci., 64*(19, 20), 2607.

Takaichi, S., (2011). Carotenoids in algae: Distributions, biosynthesis, and functions. *Mar. Drugs, 9*(6), 1101–1118.

Tandon, P., & Jin, Q., (2017). Microalgae culture enhancement through key microbial approaches. *Renew Sust. Energ Rev., 80*, 1089–1099.

Tang, D. Y. Y., Khoo, K. S., Chew, K. W., Tao, Y., Ho, S. H., & Show, P. L., (2020). Potential utilization of bioproducts from microalgae for the quality enhancement of natural products. *Bioresour. Technol., 304*, 122997.

Torres-Tiji, Y., Fields, F. J., & Mayfield, S. P., (2020). Microalgae as a future food source. *Biotechnol. Adv., 41*, 107536.

US FDA, (2018). https://www.govinfo.gov/content/pkg/FR-2018-05-07/pdf/2018-09636.pdf (accessed on 2 July 2021).

Vaz, B., Moreira, J. B., Morais, M. G., & Costa, J. A. V., (2016). Microalgae as a new source of bioactive compounds in food supplements. *Curr. Opin. Food Sci., 7*, 73–77.

Volkman, J. K., (2016). Sterols in microalgae. In: Borowitzka, M. A., Beardall, J., & Raven, J. A., (eds.), *The Physiology of Microalgae* (pp. 485–505). Springer Nature, Switzerland.

Wang, L., Wang, X., Wu, H., & Liu, R., (2014). Overview on biological activities and molecular characteristics of sulfated polysaccharides from marine green algae in recent years. *Mar. Drugs, 12*(9), 4984–5020.

Wollam, J., & Antebi, A., (2011). Sterol regulation of metabolism, homeostasis, and development. *Annu. Rev. Biochem., 80*, 885–916.

Woodman, R. J., Mori, T. A., Burke, V., Puddey, I. B., Barden, A., Watts, G. F., & Beilin, L. J., (2003). Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. *Atherosclerosis, 166*(1), 85–93.

Xiang, H., Yang, X., & Hu, Y., (2020). The properties, biotechnologies, and applications of antifreeze proteins. *Int. J. Biol. Macromol., 153*, 661–675.

Zanella, L., & Vianello, F., (2020). Microalgae of the genus *Nannochloropsis*: Chemical composition and functional implications for human nutrition. *J. Funct. Foods, 6*8, 103919.

Zhang, J., Liu, L., Ren, Y., & Chen, F., (2019). Characterization of exopolysaccharides produced by microalgae with antitumor activity on human colon cancer cells. *Int. J. Biol. Macromol., 128*, 761–767.

3. CONSIDERAÇÕES FINAIS

A elaboração de ingredientes e produtos baseados em microalgas, com o intuito de enfrentar os desafios relacionados à baixa bioacessibilidade e biodisponibilidade de carotenoides e clorofila, demanda uma compreensão profunda dos fatores críticos que podem limitar sua eficácia. Diante desse contexto, este estudo evidenciou que a capacidade de absorção dos carotenoides e da clorofila pode ser substancialmente melhorada, dependendo da composição da matriz microalgal utilizada nos ingredientes/produtos de microalgas, sendo a emulsão de pigmento lipossolúvel superior à pasta microalgal ultrassônica e biomassa microalgal liofilizada, especialmente quando a estrutura da parede celular está alterada ou ausente. Adicionalmente, nossas conclusões ressaltam que a emulsão de pigmento lipossolúvel demonstrou ser um veículo altamente eficaz não apenas para os carotenos e xantofilas, mas também para as clorofilas e seus compostos derivados. Por fim, os resultados obtidos sugerem que *Chlorella vulgaris* e *Arthrospira platensis* representam valiosos recursos biológicos na formulação de alimentos enriquecidos com carotenoides e clorofila, oferecendo uma promissora perspectiva para o desenvolvimento de produtos nutricionais mais eficazes e de maior valor agregado.

REFERÊNCIAS BIBLIOGRÁFICAS

BERNAERTS, T. M. M. et al. Cell disruption of *Nannochloropsis* sp. improves *in vitro* bioaccessibility of carotenoids and ω3-LC-PUFA. **Journal of Functional Foods**, v. 65, 2020.

BOHN, T. Bioavailability of Non-Provitamin A Carotenoids. **Current Nutrition & Food Science**, v. 4, n. 4, p. 240-258, 2008.

BOHN, T. Metabolic Fate of Bioaccessible and Non-bioaccessible Carotenoids. **Food Chemistry, Function and Analysis**, n. 5, p. 165-200, 2018.

CARBONELL-CAPELLA, J. M. et al. Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. **Comprehensive Reviews in Food Science and Food Safety**, v. 13, n. 2, p. 155-171, 2014.

CARDOSO, C. et al. Bioaccessibility assessment methodologies and their consequences for the risk-benefit evaluation of food. **Trends in Food Science and Technology**, v. 41, n. 1, p. 5-23, 2015.

FAILLA, M. L.; CHITCHUMROONCHOKCHAI, C. ***In vitro* models as tools for screening the relative bioavailabilities of provitamin A carotenoids in foods.** Washington: Harvest Plus, 2005. v. 3.

FERNANDES, A. S. et al. Insights on the intestinal absorption of chlorophyll series from microalgae. **Food Research International**, v. 140, 110031, 2021.

FERRUZZI, M. G.; FAILLA, M. L.; SCHWARTZ, S. J. Sodium copper chlorophyllin : *In vitro* digestive stability and accumulation by Caco-2 human intestinal cells. Journal of Agricultural and **Food Chemistry**, v. 50, p. 2173-2179, 2015.

GRANADO-LORENCIO, F. et al. *In vitro* bioaccesibility of lutein and zeaxanthin from the microalgae *Scenedesmus almeriensis*. **Food Chemistry**, v. 114, n. 2, p. 747-752, 2009.

GARRETT, D. A.; FAILLA, M. L.; SARAMA, R. J. Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an *in vitro* digestion/Caco-2 cell culture model. **Journal of Nutritional Biochemistry**, v. 11, n. 11-12, p. 574-580, 2000.

GILLE, A. et al. Bioaccessibility of carotenoids from *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. **International Journal of Food Sciences and Nutrition**, v. 67, n. 5, p. 507-513, 2016.

GILLE, A. et al. Effect of sonication on bioaccessibility and cellular uptake of carotenoids from

preparations of photoautotrophic *Phaeodactylum tricornutum*. **Food Research International**, v. 118, p. 40-48, 2019.

HOLST, B.; WILLIAMSON, G. Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. **Current Opinion in Biotechnology**, v. 19, n. 2, p. 73-82, 2008.

HYNSTOVA, V. et al. Separation, identification and quantification of carotenoids and chlorophylls in dietary supplements containing Chlorella vulgaris and Spirulina platensis using High Performance Thin Layer Chromatography. **Journal of Pharmaceutical and Biomedical Analysis**, v. 148, p. 108-118, 2018.

JACOB-LOPES, E. et al. Bioactive food compounds from microalgae: an innovative framework on industrial biorefineries. Current Opinion in Food Science, v. 25, p. 1-7, 2019.

KOPEC, R. E.; FAILLA, M. L. Recent advances in the bioaccessibility and bioavailability of carotenoids and effects of other dietary lipophiles. **Journal of Food Composition and Analysis**, v. 68, p. 16-30, 2018.

LIU, et al. Recent Advances in Microalgal Biotechnology, OMICS Group eBooks, Foster City, 2016.

MARONEZE, M. et al. Esterified carotenoids as new food components in cyanobacteria. **Food Chemistry**, v. 287, p. 295-302, 2019.

NASCIMENTO, T. C. et al. Bioaccessibility and intestinal uptake of carotenoids from microalgae Scenedesmus obliquus. **LWT**, v. 140, 110780, 2021.

RAZZAK, S. A. Comprehensive overview of microalgae-derived carotenoids and their applications in diverse industries. **Algal Research**, v. 78, 103422, 2024.

SHOW, P. L. Global market and economic analysis of microalgae technology: Status and perspectives. **Bioresource Technology**, v. 357, 127329, 2022.

TYSSANDIER, V. et al. Processing of vegetable-borne carotenoids in the human stomach and duodenum. **American Journal of Physiology - Gastrointestinal and Liver Physiology**, v. 284, n. 6, p. 913-923, 2003.

XAVIER, A. A. O. X.; MERCADANTE, A. Z. M. The bioaccessibility of carotenoids impacts the design of functional foods. **Current Opinion in Food Science**, v. 26, p. 1-8, 2019.

APÊNDICE

APÊNDICE A

Manuscrito: Insights on the Bioaccessibility of Natural Pigments from Diatom *Chaetoceros calcitrans*

Manuscrito puplicado no periódico

Molecules

molecules

MDPI

Article

Insights on the Bioaccessibility of Natural Pigments from Diatom *Chaetoceros calcitrans*

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Abstract: This study aimed to investigate the bioaccessibility of carotenoids and chlorophylls from the biomass of microalgae *Chaetoceros calcitrans*. The samples were submitted to an in vitro digestion protocol, and the compounds were determined by HPLC-PDA-MS/MS. A total of 13 compounds were identified in all tests. After in vitro digestion, the relative bioaccessibility of carotenoids and chlorophylls ranged from 4 to 58%. The qualitative profile of carotenoids reflected the initial sample, with all-*E*-zeaxanthin (57.2%) being the most bioaccessible compound, followed by all-*E*-neochrome (31.26%), the latter being reported for the first time in the micellar fraction. On the other hand, among the chlorophylls only pheophytin a (15.01%) was bioaccessible. Furthermore, a chlorophyll derivative (Hydroxypheophytin a') was formed after in vitro digestion. Considering all compounds, xanthophylls (12.03%) and chlorophylls (12.22%) were significantly ($p < 0.05$) more bioaccessible than carotenes (11.22%). Finally, the considerable individual bioaccessibilities found, especially for zeaxanthin, demonstrate the bioactive potential of this bioresource. However, the large reduction in the totality of compounds after in vitro digestion suggests that additional technological strategies should be explored in the future to increase the efficiency of micellarization and enhance its bioactive effects.

Keywords: brown microalgae; bioactive compounds; carotenoids; chlorophylls; in vitro digestion; bioaccessibility

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1. Introduction

As the world's population becomes more aware of health and global sustainability issues, the potential for microalgae-based processes and products to contribute to solutions to these issues is becoming evident. These microorganisms have been considered potential bioresources to meet the population's growing needs for a supply of healthier, natural, and sustainable food products, especially fine chemical compounds with potential health-promoting effects [1,2].

Microalgae are acknowledged as one of the most promising renewable resources on the planet. They have some highly relevant characteristics, such as their rapid growth rate and the ability to survive in adverse conditions and substantially contribute to the capture of atmospheric CO_2 [3,4]. In addition, the countless species of microalgae already cataloged present a highly diversified biochemical composition that includes a multitude of valuable biomolecules [5]. Among these species, *Chaetoceros calcitrans*, belonging to the Chaetocerotaceae family, is a diatom that contains large amounts of natural antioxidants such as carotenoids and chlorophylls [6,7].

Carotenoids and chlorophylls constitute groups of large and complex compounds ubiquitous in microalgae species [8,9]. Many of these structures have exceptional antioxidant capabilities that are continually associated with important biological and functional properties [10,11]. The primary use of these phytochemicals is as natural pigments with wide application in the most varied industrial sectors [12]. However, due to their bioactive

properties, which are constantly being demonstrated, these natural pigments are recognized as compounds with high added value, which intensifies their application in food products for health, functional and nutraceutical purposes [13–15].

However, for bioactive compounds to exert some activity at the biological level, these molecules must be bioaccessible for intestinal uptake and subsequent systemic distribution in the human body [16,17]. Thus, the bioaccessibility of carotenoids and chlorophylls is considered an essential area of study which is fundamental to understanding their nutritional and functional values and optimizing their applications.

Bioaccessibility is dependent on the degree of release, solubilization, and incorporation of intracellular compounds in mixed-bile-salt micelles [18]. For microalgae, the step involving intracellular release of the compounds is reported as the main limiting factor for bioaccessibility due to the structural and physicochemical properties that contribute to a more rigid cell wall [19,20]. Process intensification technologies such as ultrasoundthat trigger the partial release of molecules through cell disruption have been suggested as strategies to enhance the bioaccessibility of carotenoids and chlorophylls [21,22].

Considering these aspects of bioaccessibility, the objective of this work was to evaluate the bioaccessibility of carotenoids and chlorophylls of ultrasonicated biomass of *Chaetoceros calcitrans*, following an in vitro digestion protocol.

2. Results and Discussion

2.1. Pigments Composition before and after Digestion in vitro

A total of 13 compounds were separated in all assays with the microalgae *C. calcitrans* (see Table 1). Identification was based on chemical evidence provided by chromatographic analysis such as elution order and UV-Vis characteristics and was confirmed by MS/MS experiments (The representative chromatograms HPLC-PDA and PDA-MS/MS (MRM) spectra can be found in Supplementary Materials). In addition, pigments were identified or provisionally identified based on a detailed description previously reported for different microalgae species [21,23–26].

The microalgae cell wall is the first barrier to the effective use of bioactive compounds from this promising group of microorganisms. However, previous studies have demonstrated the efficiency of using ultrasound to increase micellar incorporation of microalgae compounds such as *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Scenedesmus obliquus* [19,21,22]. Therefore, the original content of carotenoids and chlorophylls of the ultrasonicated dried biomass of *C. calcitrans* before digestion (initial content) and the micellar fraction after digestion, are shown in Table 2.

From a quantitative point of view, the initial extract of carotenoids showed seven compounds, totaling 239.88 ± 1.82 $\mu g \cdot g^{-1}$, of which all-*E*-echinenone (116.65 ± 1.52 $\mu g \cdot g^{-1}$) was the most abundant, followed by all-*E*-β-carotene (55.44 ± 0.25 $\mu g \cdot g^{-1}$). On the other hand, the initial chlorophyll extract presented five compounds, making a total of 3944.16 ± 41.56 $\mu g \cdot g^{-1}$; among these, pheophytin a (3257.52 ± 40.23 $\mu g \cdot g^{-1}$) was the most abundant.

In general, after digestion simulation, the qualitative profile of carotenoids reflected that of the initial sample. On the other hand, only one of the chlorophylls in the initial extract was identified after digestion. Among the chlorophylls, the derivated compound identified as hydroxypheophytin a' was detected only in the micellar fraction.

Possibly, this chlorophyll derivative was generated due to in vitro digestion conditions [22,27], as these conformational changes (epimerization) in chlorophyll molecules are frequent with moderate heating [28], similar to the temperature used in the present study (37 °C) to simulate biological conditions. Additionally, the appearance of hydroxypheophytin a' can occur through successive pheophytinization, allomerization and epimerization reactions from the native structure. However, no conclusion can be drawn about whether epimerization occurs preferentially in native chlorophylls or their Mg-free oxygenated derivatives [22]. Our results remain inconclusive, as hydroxypheophytin a' ap-

pears, while hydroxychlorophyll a, chlorophyll a, and hydroxypheophytin a disappear after digestion.

Table 1. Chromatographic, UV–vis spectrum, mass characteristics of *C. calcitrans* pigments obtained by HPLC-PDA-MS/MS.

Pigments	t_R [a]	UV-Vis Characteristics			Fragment Ions (Positive Mode) (*m*/*z*)	
		$\lambda_{máx}$ (nm) [b]	III/II (%) [c]	AB/II (%) [d]	$[M+H]^+$	MS/MS
All-*E*-neochrome	5.23	399, 421, 448	94	0	601	583 $[M+H-18]^+$, 491 $[M+H-92-18]^+$
Hydroxychlorophyll a	10.17	430, 664	na [e]	na	909	631 $[M+H-278]^+$ 551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$, 495 $[M+H-18-56]^+$, 477 $[M+H-92]^+$, 459 $[M+H-18-92]^+$
All-*E*-lutein	12.64	418, 444, 473	50	0	569	615 $[M+H-278]^+$, 583 $[M+H-278-31]^+$, 555 $[M+H-278-59]^+$
Chlorophyll a	15.11	432, 665	na	na	893	615 $[M+H-278]^+$, 583 $[M+H-278-31]^+$, 555 $[M+H-278-59]^+$
Chlorophyll a′	16.73	431, 665	na	na	893	551 $[M+H-18]^+$, 533 $[M+H-18-18]^+$,495, 477 $[M+H-92]^+$, 459 $[M+H-18-92]^+$
All-*E*-zeaxanthin	17.51	421, 450, 477	25	0	569	533 $[M+H-18]^+$, 427 869 $[M+H-18]^+$; 803 $[M+H-63]^+$; 609 $[M+H-278]^+$; 591 $[M+H-278-18]^+$; 531 $[M+H-278-18-60]^+$
15*Z*-echinenone	19.09	335, 447	nc [f]	20	551	533 $[M+H-18]^+$, 427 869 $[M+H-18]^+$; 803 $[M+H-63]^+$; 609 $[M+H-278]^+$; 591 $[M+H-278-18]^+$; 531 $[M+H-278-18-60]^+$
Hydroxypheophytin a	24.10	409, 666	na	na	887	481 $[M+H-56]^+$, 444 $[M-92]^+$, 413, 399, 355
All-*E*-echinenone	24.04	461	nc	nc	551	593 $[M+H-278]^+$; 533 $[M+H-278-60]^+$
Hydroxypheophytin a′	27.91	399, 660	na	na	887	481 $[M+H-56]^+$, 444 $[M-92]^+$, 413, 399
All-*E*-β-carotene	32.14	424, 450, 476	28	0	537	
Pheophytin a	32.87	408, 666	na	na	871	
9*Z*-β-carotene	34.22	353, 421, 443, 473	20	14	537	

[a]: Retention time (Linear gradient in methanol and methyl tert-butyl ether); [b]: Spectral fine structure; [c]: Ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II); [d]: Ratio of the cis peak (AB) and the middle absorption peak (II); [e]: Not applicable; [f]: Not calculated.

A significant reduction of all compounds was observed in the micellar fraction after in vitro digestion. All-*E*-β-carotene (8.45 ± 0.15 $\mu g \cdot g^{-1}$) and all-*E*-equinenone (7.91 ± 0.10 $\mu g \cdot g^{-1}$) remained the major carotenoids, followed by all-*E*-zeaxanthin (7.10 ± 0.10 $\mu g \cdot g^{-1}$), all-*E*-neochrome (2.33 ± 0.5 $\mu g \cdot g^{-1}$), all-*E*-lutein (1.72 ± 0.03 $\mu g \cdot g^{-1}$) and 15*Z*-echinenone (0.74 ± 0.03 $\mu g \cdot g^{-1}$).

Two hypotheses (i and ii)can be considered to explain the higher micellar content of all-*E*-β-carotene in detriment to other xanthophylls: (i) The high content of unsaturated fatty acids in the biomass of *C. calcitrans* is one possible cause [29], as several studies indicate that the presence of more significant fractions of fatty acids than unsaturated ones promotes the micellarization of carotenes, while the presence of saturated fatty acids promotes the micellar incorporation of xanthophylls [30–34]; (ii) Although the cell wall was partially disrupted before digestion, some conjugations between xanthophylls and proteins may remain, making it difficult to transfer these carotenoids to micelles [16,20,35].

Although many factors need to be evaluated, a convergence in the literature towards greater micellar incorporation of xanthophylls is evident [36,37]. This trend is observed for

total micellar carotenoids since the total xanthophylls (19.80 ± 0.30 μg·g^{-1}) are approximately twice the total carotenes content (8.45 ± 0.15 μg·g^{-1}).

Table 2. Pigment content of *C. calcitrans* before in vitro digestion (initial content), and the micellar fraction of carotenoids from ultrasonicated biomass after in vitro digestion. Different letters in the lines indicate a significant difference using Student's *t*-test ($p < 0.05$).

Pigments	Initial Content (μg·g^{-1})	Micelar Fraction (μg·g^{-1})
All-*E*-neochrome	7.44 ± 0.05 ^{a}	2.33 ± 0.5 ^{b}
Hydroxychlorophyll a	221.99 ± 1.00	nd
All-*E*-lutein	12.01 ± 0.10 ^{a}	1.72 ± 0.03 ^{b}
Chlorophyll a	306.03 ± 1.04	nd
Chlorophyll a'	47.77 ± 0.69	nd
All-*E*-zeaxanthin	12.40 ± 0.10 ^{a}	7.10 ± 0.10 ^{b}
15*Z*-echinenone	16.06 ± 0.15 ^{a}	0.74 ± 0.03 ^{b}
Hydroxypheophytin a	110.85 ± 0.78	nd
All-*E*-echinenone	116.65 ± 1.52 ^{a}	7.91 ± 0.10 ^{b}
Hydroxypheophytin a'	Nd	20.89 ± 0.10
All-*E*-carotene	55.44 ± 0.25 ^{a}	8.45 ± 0.15 ^{b}
Pheophytin a	3257.52 ± 40.23 ^{a}	482.10 ± 1.15 ^{b}
9*Z*-β-carotene	19.83 ± 0.15	nd
Total carotenoids	239.88 ± 1.82 ^{a}	28.24 ± 0.45 ^{b}
Total carotenes	75.28 ± 0.40 ^{a}	8.45 ± 0.15 ^{b}
Total xanthophylls	164.61 ± 1.43 ^{a}	19.80 ± 0.30 ^{b}
Total chlorophylls	3944.16 ± 41.56 ^{a}	502.15 ± 1.17 ^{b}

nd: Not detected.

Referring to the micellarized chlorophyll fraction, pheophytin a (482.10 ± 1.15 μg·g^{-1}) remained the majority compound. According to the literature [22], chlorophylls are very susceptible and can change the digestive process, especially in acidic conditions. A first step in the metabolization of chlorophylls leads to the central perfusion of Mg in the structure, giving rise to pheophytins [38], which clarifies the predominance of pheophytin in the micellar fraction. Likewise, a recent study [27], associated this micellar predominance of pheophytin with the acidic conditions of the gastric phase (pH 2.5).

2.2. Relative Bioaccessibility

In terms of relative bioaccessibility (%) of *C. calcitrans* compounds (Figure 1a,b), there was a variation ranging from 4% to 58% (Figure 1a). Among individual compounds, the most bioaccessible carotenoid was all-*E*-zeaxanthin (57.29% ± 1.27), followed by all-*E*-neochrome (31.26% ± 0.42), all-*E*-β-carotene (15.24% ± 0.20), all-*E*-lutein (14.36% ± 0.33), all-*E*-echinenone (6.78% ± 0.01) and 15*Z*-echinenone (4.59% ± 0.16), while the only bioaccessible chlorophyll was pheophytin a (15.01% ± 0.20).

These bioaccessible compounds perform essential physiological and pharmacological activities which improve human health, well-being and nutritional status. These molecules are excellent antioxidants, reduce oxidative stress, benefit cardiovascular health. They also help prevent obesity, diabetes, some types of cancer, and neurological sequelae. In addition, some compounds such as β-carotene act precisely as vitamin A precursors, and zeaxanthin and lutein act as eye health regulators [39,40].

Comparatively, the relative bioaccessibility of all-*E*-zeaxanthin (57.29%) from *C. calcitrans* was superior to the findings for sonicated biomass from *Phaeadactylum tricornutum* (29%) [41], *Nannochloropsis* sp. (<15%) [20], *S. obliquus* (9%) [21], *Scenedesmus bijuga* (6%) [23], and a diet supplemented with *P. tricornutum* (17%) [41]. In addition, the bioaccessibility of all-*E*-β-carotene (15.24%) exceeded the values found in the sonicated biomass of *Chlorella vulgaris* (12%), *Chlamydomonas reinhardtii* (<10%) [19], *S. obliquus* (3%), *S. bijuga* (8%),

and *Chlorella sorokiniana* (13%). Likewise, all-*E*-lutein (14.36%) surpassed the bioaccessibility found for sonicated biomass of *S. obliquus* (12%), *S. bijuga* (3%) and *C. sorokiniana* (6%) [21,23].

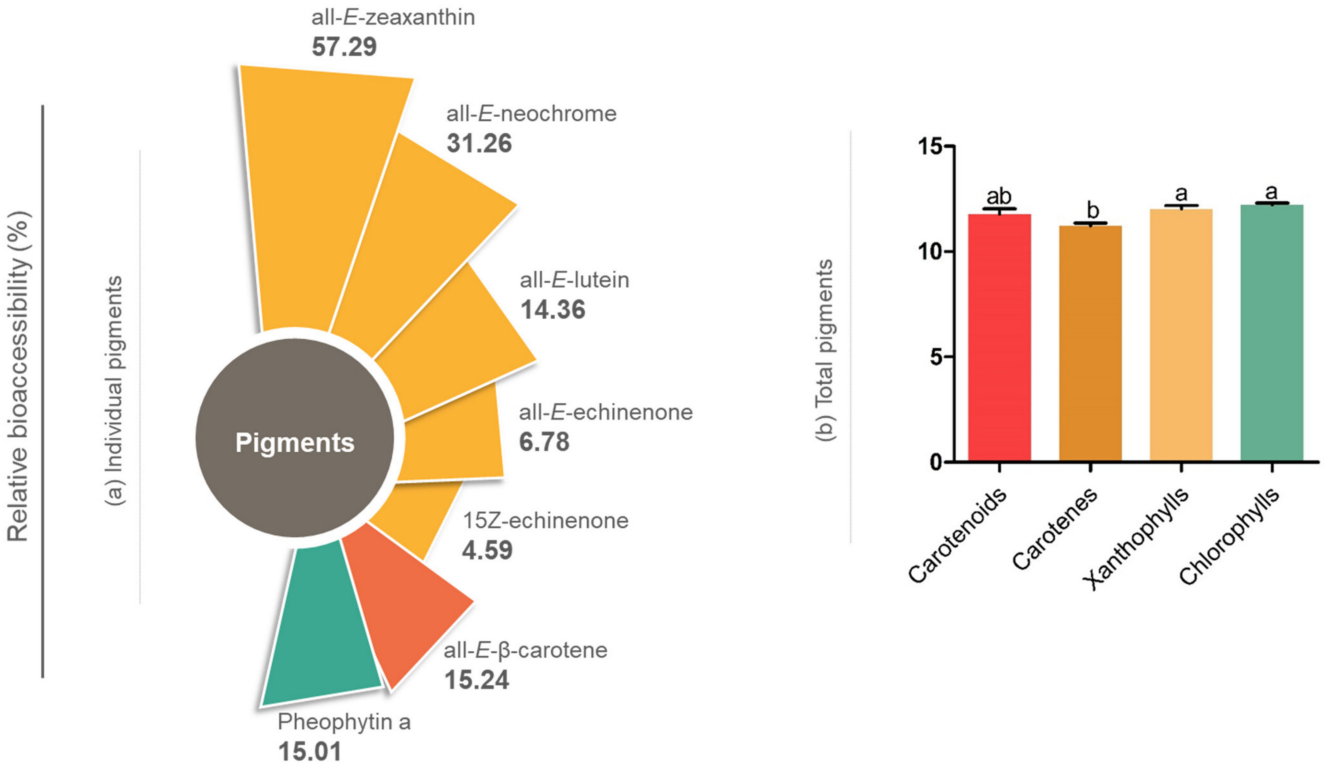

Figure 1. Relative bioaccessibility of individual (**a**) and total pigments (**b**) from *C. calcitrans*. Different letters in (**b**) indicate a significant difference using Tukey's test ($p < 0.05$).

On the other hand, a study found bioaccessibility values of β-carotene of *P. tricornutum* up to 5 times higher than those established in this work [42]. Likewise, the lutein present in the diet supplemented with *C. vulgaris* was found to be approximately 2-fold higher [19]. The bioaccessibility of all-*E*-equinenone (6.78%) was similar to that found for sonicated biomass of *S. obliquus* (6%) [21]. However, we did not find comparative data for its 15*Z* isomer (4.59%) and all-*E*-neochrome (31.26%).

It is important to highlight that, as far as we know, this is the first time that the bioaccessibility of all-*E*-neochrome has been reported, a compound whose bioactive properties remain neglected, despite its remarkable structure (See Figure 2). In addition to some oxygenated functional groups (epoxy -O- and hydroxy -OH), neochrome has an unusual allenic bond (=C=), which has been implicated in increased deactivation of radical species when present in other isoprenoid structures [43].

The relative bioaccessibility of pheophytin a (15.01%) was higher than that reported for sonicated biomass of *S. obliquus* (~10%) [22]. Comparisons with literature data are extremely limited for the bioaccessibility of this microalgae compound group. Studies to date are scarce with only one recently published report [22].

Highlighting the totality of compounds, Figure 1b shows the total relative bioaccessibility for carotenoids (11.78% ± 0.25), carotenes (11.22% ± 0.23), xanthophylls (12.02% ± 0.26) and chlorophylls (12.22% ± 0.15). Xanthophylls and chlorophylls were slightly larger and differed significantly ($p > 0.05$) from the bioaccessible total carotenes. According to the literature, xanthophylls are generally more bioaccessible than carotenes due to their lower hydrophobicity [36,37].

When compared to different sources, the total bioaccessible chlorophyll of the sonicated biomass of *C. calcitrans* (12.22%) is four times greater than that of the sonicated biomass of *S. obliquus* (3%), for example, and is within the range determined for edible

algae [22,44]. On the other hand, it is relatively low compared to experiments with isolated microalgae extracts (33%) or conventional sources (24–50%) [22,45].

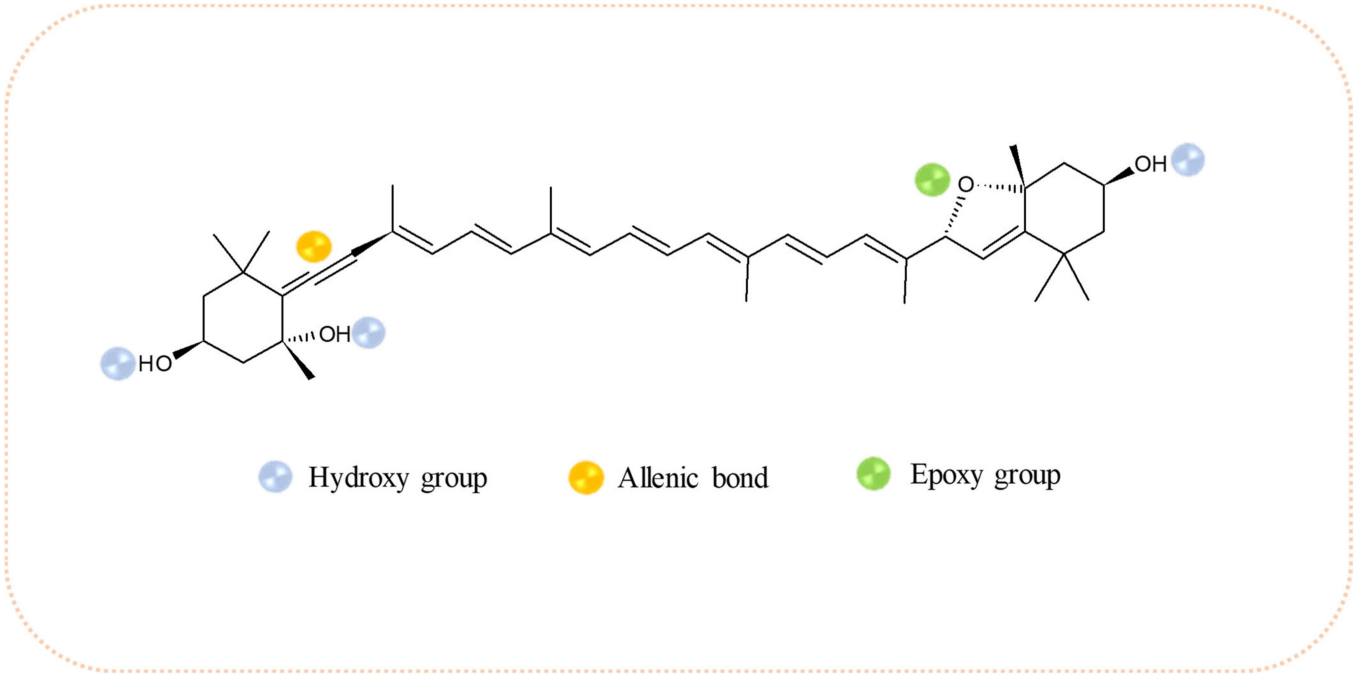

Figure 2. Chemical structure of the all-*E*-neochrome.

As already demonstrated for different matrices, including microalgae, the transfer of carotenoids and chlorophylls to the micellar fraction can be influenced by numerous factors, especially the location in the matrix and the effect of constituents such as proteins, fatty acids, soluble fibers and minerals [23,46–48]. These factors may explain the differences observed in bioaccessibility studies of microalgae compounds to date, as the metabolic diversity of microalgae is immense, varies from species to species, and is still subject to modification according to the culture conditions, making it difficult to correlate all the variables involved.

Finally, when comparing the initial totality of compounds, both classes of pigments were reduced by more than 80% after mimicking digestion. This fact leads us to consider exploring alternatives to increase the micellarization efficiency and enhance its bioactive effects in vivo. The use of emulsions as a vehicle is an attractive option, mainly due to the increase in stability and incorporation of structures with non-polar characteristics in the micellar phase [3,21,49]. In addition, the inclusion of biomass in different food preparations should also be considered since integrated consumption is a future trend [4,50,51].

3. Material and Methods

3.1. Chemicals

The standards all-*E*-β-carotene, all-*E*-lutein, and chlorophyll a (with purities ranging from 95.0% to 99.9%), were purchased from Sigma-Aldrich (Darmstadt, Germany). All solvents for extraction and chromatography analysis were purchased from Merck (Darmstadt, Germany). The α-amylase (A3176), pepsin (P7000), pancreatin (P1750), lipase (L3126) and bile (B8631) were purchased from Sigma-Aldrich (St. Louis-MO, USA).

3.2. Microalgae Culture and Biomass Production

Axenic cultures of *Chaetoceros calcitrans* (CCMP1315) were used in the experiments. Stock cultures were propagated and maintained in BG-11 medium (Braun-Grunow medium) [52].

The incubation conditions included a temperature of 26 °C, a photon flux density of 15 μmol.m^{-2}·s^{-1} and a photoperiod of 12 h.

The biomass productions were made in phototrophic conditions. The cultivations were performed in a bubble column photobioreactor under a batch regime fed on 2.0 L of BG-11 medium. The experimental conditions were as follows: initial cell concentration of 100 mg·L^{-1}, isothermal reactor operating at a temperature of 26 °C, luminous intensity of 25 μmol.m^{-2}.s^{-1}, continuous aeration of 1 VVM (with air enriched with 3% CO_2) and photoperiod of 24:0 h light/dark. The biomass was separated from the BG-11 medium by centrifugation (1500× *g*; 10 min; 10 °C), and the supernatant was discarded. The paste obtained after centrifugation was frozen at −18 °C for 24 h, and freeze-dried for 24 h at −50 °C above −175 μm Hg. The samples were stored under refrigeration until the analysis.

3.3. Sample Preparation

Before the in vitro digestion, aliquots of 100 mg of freeze-dried biomass were combined with 10 mL saline solution (NaCl 120 mol·L^{-1}, $CaCl_2$ 6 mmol·L^{-1}, KCl 5 mmol·L^{-1}) and were subjected to 15 min of an ultrasonic probe (Ultronic, Indaiatuba-SP, Brazil) to break the cell wall (an adaptation of Gille et al. [19]). The ultrasonic parameters were probe with 13 mm diameter, 400 W, 40 kHz, and an ice bath to control the temperature (0 ± 2 °C).

3.4. In Vitro Digestion

The samples were submitted to an in vitro simulated digestion model, according to the protocol adapted from INFOGEST [53] and modified by [27]. 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, 7.5× *g* in a shaking incubator (E-4200 model, Tecnal, Piracicaba, Brazil). Before starting the gastric phase, the pH was adjusted to 2.5 with HCl 1 mol L^{-1} 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* (E-4200 model, Tecnal, Piracicaba, Brazil). After this step, the pH was increased to 6.0 with 1M $NaHCO_3$ and the intestinal phase started with a bile solution (3 mL; 40 mg.mL^{-1} in 100 mM $NaHCO_3$), 4000 U.mL^{-1} of pancreatin and 1000 U.mL^{-1} of lipase. The pH was adjusted to 6.5 and the total volume to 50 mL, the incubation occurred for 2 h at 37 °C and 7.5× *g* (E-4200 model, Tecnal, Piracicaba, Brazil). After completion of the in vitro digestion, the solution was centrifuged at 8000× *g*, 60 min at 4 °C (Thermo, Langenselbold, Germany). The supernatant containing the mixed micelles was collected, covered with nitrogen gas, frozen at −40 °C and lyophilized for further extraction of pigments. The pigments bioaccessibility was calculated as the ratio between carotenoid content in the micellar fraction (supernatant) and original content in the *C. calcitrans* Equation (1).

$$Bioaccessibility\ (\%) = \frac{Pigments\ (Supernatant)}{Pigments\ (original\ content)} \times 100 \quad (1)$$

3.5. Pigments Extraction

The original content of *C. calcitrans* carotenoids and chlorophylls was extracted according to the literature [54]. The freeze-dried biomass (100 mg) was exhaustively extracted with ethyl acetate and methanol using a mortar and pestle followed by centrifugation (Thermo, Langenselbold, Germany) for 7 min at 1500× *g*. In addition, the carotenoids extract was saponified for 16 h with 10 g 100 mL^{-1} methanolic KOH at room temperature, and the alkali was removed by washing with distilled water. All extracts were concentrated in a rotary evaporator, placed in N_2 atmosphere, and kept at −40 °C in the dark until analyzed.

The micellarized pigments were extracted according to an adapted protocol [55]. The lyophilized micellarized samples were exhaustively extracted by adding 15 mL of ethyl ether: petroleum ether (1:1) and subjected to 5 min ultrasonic cycles (see parameters in Section 3.3), centrifuged, and the supernatant was collected. The process was repeated until the supernatant became colorless. Then the carotenoids and chlorophyll extracts

were rotary evaporated. The carotenoids extract underwent saponification as previously indicated. Both extracts were then in turn subjected to chromatographic analysis.

3.6. HPLC-PDA-MS/MS Pigments Analysis

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 chromatograph with photodiode array detection (PDA) (model SPD-M20A) was connected in series to an atmospheric pressure chemical ionization (APCI) source (Shimadzu America, Columbia, MD, USA), and a mass spectrometer Shimadzu 8040 triple quadrupole. The pigments separation was performed on a C30 YMC column (5 µm, 250 × 4.6 mm) (Waters, Wilmington-DE, USA). HPLC-PDA analysis was performed according to Rodrigues et al. [24]. Prior to HPLC-PDA analysis, the carotenoids extract was solubilized in methanol (MeOH): methyl tert-butyl ether (MTBE) (70:30) and filtered through Millipore membranes (0.22 µm). The mobile phases A (MeOH) and phase B (MTBE) used a linear gradient program as follows: from 0 to 30 min 5% B; from 30 to 40 min, 5 to 30% B; from 40 to 41 min, 30 to 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 29 °C, the UV-Vis spectra were acquired between 220 and 700 nm, and the chromatograms were processed at 450 nm.

The MS/MS analysis was conducted according to Giuffrida et al. [56] 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 identification quality, MS/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-Vis spectrum, and mass characteristics (protonated molecule ([M+H]$^+$) and MS/MS fragments), compared with data available in the literature [21,25,26,57–59]. The pigments were individually quantified by HPLC-PDA using five-point calibration curves. The all-*E*-lutein, all-*E*-β-carotene end chlorophyll analytical curves were used to quantify the xanthophylls, carotenes and chlorophylls, respectively.

3.7. Statistical Analysis

The statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla-CA, USA). Differences between the two variables were detected by Student's *t*-test ($p < 0.05$) and differences between more than two variables were assessed by a one-way ANOVA followed by Tukey's test ($p < 0.05$).

4. Conclusions

This study investigated the bioaccessibility of carotenoids and chlorophylls from the diatom *C. calcitrans* for the first time. The relative bioaccessibility of sonicated biomass varied over a wide range (4–58%). The qualitative profile of bioaccessible carotenoids reflected the initial sample, with all-*E*-zeaxanthin (57.29%) being the major compound, followed by all-*E*-neochrome (reported for the first time in the micellar fraction). In contrast, pheophytin a (15.01%) was the only bioaccessible chlorophyll. Additionally, a chlorophyll derivative (hydroxypheophytin a') was detected only in the micellar fraction. Considering all classes, xanthophylls (12.03%) and chlorophylls (12.22%) were significantly more bioaccessible than carotenes (11.28%). Although the considerable bioaccessibility of individual compounds is evidence for the bioactive potential of this source, the reduction of approximately 80% in the content of the compounds after in vitro digestion suggests that additional strategies to increase the micellarization efficiency are required in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27103305/s1, Figure S1: Representative chromatograms HPLC-PDA of Chaetoceros calcitrans carotenoids. Original content (control extract) before digestion (a). After in vitro digestion (b). See text for chromatographic conditions. The chromatogram was processed at 450 nm. 1. All-E-neochrome; 3. All-E-lutein; 6. All-E-zeaxanthin; 7. 15Z-echinenone; 9. All-E-echinenone; 11. All-E-β-carotene; 13. 9Z-β-carotene.; Figure S2: Representative chromatograms HPLC-PDA of Chaetoceros calcitrans chlorophylls. Original content (control extract) before digestion (a). After in vitro digestion (b). See text for chromatographic conditions. The chromatogram was processed at 660 nm. 2. Hydroxychlorophyll a; 4. Chlorophyll a; 5. Chlorophyll a'; 8. Hydroxypheophytin a; 10. Hydroxypheophytin a'; 12. Pheophytin a; Figure S3: PDA and MS-MS (MRM) spectra of some compounds identified from Chaetoceros calcitrans.

Author Contributions: Conceptualization, T.C.d.N. and L.Q.Z.; methodology, T.C.d.N.; P.N.P.; P.A.C. and A.S.F.; software, P.N.P.; validation, T.C.d.N.; P.N.P. and A.S.F.; formal analysis, T.C.d.N.; investigation, P.A.C.; resources, P.A.C. and T.C.d.N.; data curation, T.C.d.N. and E.J.-L.; writing—original draft preparation, T.C.d.N.; writing—review and editing, T.C.d.N.; P.N.P. and A.S.F.; visualization, E.J.-L. and L.Q.Z.; supervision, E.J.-L. and L.Q.Z.; project administration, E.J.-L. and L.Q.Z.; funding acquisition, L.Q.Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors have no conflict of interest to report.

Sample Availability: Samples of compounds are available from the authors.

References

1. Koyande, A.K.; Chew, K.W.; Rambabu, K.; Tao, Y.; Chu, D.-T.; Show, P.-L. Microalgae: A potential alternative to health supplementation for humans. *Food Sci. Hum. Wellness* **2019**, *8*, 16–24. [CrossRef]
2. Balasubramaniam, V.; Devi-Nair Gunasegavan, R.; Mustar, S.; Chelyn Lee, J.; Fairulnizal Mohd Noh, M.; Díaz-Marrero, A.R.; Javier Fernández Castro, J. Isolation of Industrial Important Bioactive Compounds from Microalgae. *Molecules* **2021**, *26*, 943. [CrossRef] [PubMed]
3. Ying Ying Tang, D.; Shiong Khoo, K.; Wayne Chew, K.; Tao, Y.; Ho, S.-H.; Loke Show, P. Potential Utilization of Bioproducts from Microalgae for the Quality Enhancement of Natural Products. *Bioresour. Technol.* **2020**, *304*, 122997. [CrossRef] [PubMed]
4. Tzachor, A.; Richards, C.E.; Holt, L. Future foods for risk-resilient diets. *Nat. Food* **2021**, *2*, 326–329. [CrossRef]
5. Jacob-Lopes, E.; Maroneze, M.M.; Deprá, M.C.; Sartori, R.B.; Dias, R.R.; Zepka, L.Q. Bioactive food compounds from microalgae: An innovative framework on industrial biorefineries. *Curr. Opin. Food Sci.* **2019**, *25*, 1–7. [CrossRef]
6. Azizan, A.; Bustamam, M.S.A.; Maulidiani, M.; Shaari, K.; Ismail, I.S.; Nagao, N.; Abas, F. Metabolite Profiling of the Microalgal Diatom *Chaetoceros Calcitrans* and Correlation with Antioxidant and Nitric Oxide Inhibitory Activities via 1H NMR-Based Metabolomics. *Mar. Drugs* **2018**, *16*, 154. [CrossRef]
7. Foo, S.C.; Yusoff, F.M.; Ismail, M.; Basri, M.; Khong, N.M.H.; Chan, K.W.; Yau, S.K. Efficient solvent extraction of antioxidant-rich extract from a tropical diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968. *Asian Pac. J. Trop. Biomed.* **2015**, *5*, 834–840. [CrossRef]
8. Mulders, K.J.; Lamers, P.P.; Martens, D.E.; Wijffels, R.H. Phototrophic pigment production with microalgae: Biological constraints and opportunities. *J. Phycol.* **2014**, *50*, 229–242. [CrossRef]
9. Borowitzka, M.A. Chapter 9—Microalgae in Medicine and Human Health: A Historical Perspective. In *Microalgae in Health and Disease Prevention*; Academic Press: Cambridge, MA, USA, 2018; pp. 195–210. [CrossRef]
10. Rodriguez-Concepcion, M.; Avalos, J.; Bonet, M.L.; Boronat, A.; Gomez-Gomez, L.; Hornero-Mendez, D.; Limon, M.C.; Meléndez-Martínez, A.J.; Olmedilla-Alonso, B.; Palou, A.; et al. A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Prog. Lipid Res.* **2018**, *70*, 62–93. [CrossRef]
11. Zepka, L.Q.; Jacob-Lopes, E.; Roca, M. Catabolism and bioactive properties of chlorophylls. *Curr. Opin. Food Sci.* **2019**, *26*, 94–100. [CrossRef]
12. Silva, S.C.; Ferreira, I.C.F.R.; Dias, M.M.; Filomena Barreiro, M. Microalgae-Derived Pigments: A 10-Year Bibliometric Review and Industry and Market Trend Analysis. *Molecules* **2020**, *25*, 3406. [CrossRef]

13. do Nascimento, T.C.; Jacob-Lopes, E.; .Zepka, L.Q. Microalgas e saúde: Uma breve revisão/Microalgae and health: A short-review. *Braz. J. Dev.* **2021**, *7*, 62150–62171. [CrossRef]
14. Kusmayadi, A.; Leong, Y.K.; Yen, H.-W.; Huang, C.-Y.; Chang, J.-S. Microalgae as sustainable food and feed sources for animals and humans—Biotechnological and environmental aspects. *Chemosphere* **2021**, *271*, 129800. [CrossRef]
15. Mehariya, S.; Goswami, R.K.; Karthikeysan, O.P.; Verma, P. Microalgae for high-value products: A way towards green nutraceutical and pharmaceutical compounds. *Chemosphere* **2021**, *280*, 130553. [CrossRef]
16. Kopec, R.E.; Failla, M.L. Recent advances in the bioaccessibility and bioavailability of carotenoids and effects of other dietary lipophiles. *J. Food Compos. Anal.* **2018**, *68*, 16–30. [CrossRef]
17. Fernandes, A.S.; Jacob-Lopes, E.; Zepka, L.Q. An overview on microalgae carotenoids and chlorophylls: Focus in the bioaccessibility/Uma visão geral dos carotenoides e clorofilas microalgais: Foco na bioacessibilidade. *Braz. J. Dev.* **2021**, *7*, 82727–82760. [CrossRef]
18. Carbonell-Capella, J.M.; Buniowska, M.; Barba, F.J.; Esteve, M.J.; Frígola, A. Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 155–171. [CrossRef]
19. Gille, A.; Trautmann, A.; Posten, C.; Briviba, K. Bioaccessibility of carotenoids from *Chlorella vulgaris* and *Chlamydomonas reinhardtii*. *Int. J. Food Sci. Nutr.* **2016**, *67*, 507–513. [CrossRef]
20. Bernaerts, T.M.M.; Verstreken, H.; Dejonghe, C.; Gheysen, L.; Foubert, I.; Grauwet, T.; Van Loey, A.M. Cell disruption of *Nannochloropsis* sp. improves in vitro bioaccessibility of carotenoids and ω3-LC-PUFA. *J. Funct. Foods* **2020**, *65*, 103770. [CrossRef]
21. do Nascimento, T.C.; Pinheiro, P.N.; Fernandes, A.S.; Murador, D.C.; Neves, B.V.; de Menezes, C.R.; de Rosso, V.V.; Jacob-Lopes, E.; Zepka, L.Q. Bioaccessibility and intestinal uptake of carotenoids from microalgae Scenedesmus obliquus. *LWT* **2021**, *140*, 110780. [CrossRef]
22. Fernandes, A.S.; Nascimento, T.C.; Pinheiro, P.N.; de Rosso, V.V.; de Menezes, C.R.; Jacob-Lopes, E.; Zepka, L.Q. Insights on the intestinal absorption of chlorophyll series from microalgae. *Food Res. Int.* **2021**, *140*, 110031. [CrossRef] [PubMed]
23. Fernandes, A.S.; Nascimento, T.C.; Pinheiro, P.N.; Vendruscolo, R.G.; Wagner, R.; de Rosso, V.V.; Jacob-Lopes, E.; Zepka, L.Q. Bioaccessibility of microalgae-based carotenoids and their association with the lipid matrix. *Food Res. Int.* **2021**, *148*, 110596. [CrossRef] [PubMed]
24. Rodrigues, D.B.; Menezes, C.R.; Mercadante, A.Z.; Jacob-lopes, E.; Zepka, L.Q. Bioactive pigments from microalgae *Phormidium autumnale*. *Food Res. Int.* **2015**, *77*, 273–279. [CrossRef]
25. Fernandes, A.S.; Nogara, G.P.; Menezes, C.R.; Cichoski, A.J.; Mercadante, A.Z.; Jacob-Lopes, E.; Zepka, L.Q. Identification of chlorophyll molecules with peroxyl radical scavenger capacity in microalgae *Phormidium autumnale* using ultrasound-assisted extraction. *Food Res. Int.* **2017**, *99*, 1036–1041. [CrossRef]
26. Fernandes, A.S.; Petry, F.C.; Mercadante, A.Z.; Jacob-Lopes, E.; Zepka, L.Q. HPLC-PDA-MS/MS as a strategy to characterize and quantify natural pigments from microalgae. *Curr. Res. Food Sci.* **2020**, *3*, 100–112. [CrossRef]
27. Murador, D.C.; Mesquita, L.M.D.S.; Neves, B.V.; Braga, A.R.C.; Martins, P.L.G.; Zepka, L.Q.; Rosso, V.V. De Bioaccessibility and Cellular Uptake by Caco-2 Cells of Carotenoids and Chlorophylls from Orange Peels: A Comparison Between Conventional and Ionic Liquid Mediated Extractions. *Food Chem.* **2020**, *339*, 127818. [CrossRef]
28. Gauthier-Jaques, A.; Bortlik, K.; Hau, J.; Fay, L.B. Improved Method to Track Chlorophyll Degradation. *J. Agric. Food Chem.* **2001**, *49*, 1117–1122. [CrossRef]
29. Servel, M.O.; Claire, C.; Derrien, A.; Coiffard, L.; De Roeck-Holtzhauer, Y. Fatty acid composition of some marine microalgae. *Phytochemistry* **1994**, *36*, 691–693. [CrossRef]
30. Failla, M.L.; Chitchumronchokchai, C.; Ferruzzi, M.G.; Goltz, S.R.; Campbell, W.W. Unsaturated fatty acids promote bioaccessibility and basolateral secretion of carotenoids and α-tocopherol by Caco-2 cells. *Food Funct.* **2014**, *5*, 1101–1112. [CrossRef]
31. Yuan, X.; Liu, X.; McClements, D.J.; Cao, Y.; Xiao, H. Enhancement of phytochemical bioaccessibility from plant-based foods using excipient emulsions: Impact of lipid type on carotenoid solubilization from spinach. *Food Funct.* **2018**, *9*, 4352–4365. [CrossRef]
32. Nagao, A.; Kotake-Nara, E.; Hase, M. Effects of fats and oils on the bioaccessibility of carotenoids and vitamin E in vegetables. *Biosci. Biotechnol. Biochem.* **2013**, *77*, 1055–1060. [CrossRef]
33. Zhang, R.; Zhang, Z.; Zou, L.; Xiao, H.; Zhang, G.; Decker, E.A.; McClements, D.J. Enhancing Nutraceutical Bioavailability from Raw and Cooked Vegetables Using Excipient Emulsions: Influence of Lipid Type on Carotenoid Bioaccessibility from Carrots. *J. Agric. Food Chem.* **2015**, *63*, 10508–10517. [CrossRef]
34. Han, J.R.; Gu, L.P.; Zhang, R.J.; Shang, W.H.; Yan, J.N.; McClements, D.J.; Wu, H.T.; Zhu, B.W.; Xiao, H. Bioaccessibility and cellular uptake of β-carotene in emulsion-based delivery systems using scallop (*Patinopecten yessoensis*) gonad protein isolates: Effects of carrier oil. *Food Funct.* **2019**, *10*, 49–60. [CrossRef]
35. Chitchumroonchokchai, C.; Failla, M.L. Bioaccessibility and intestinal cell uptake of astaxanthin from salmon and commercial supplements. *Food Res. Int.* **2017**, *99*, 936–943. [CrossRef]
36. Berni, P.; Campoli, S.S.; Negri, T.C.; de Toledo, N.M.V.; Canniatti-Brazaca, S.G. Non-conventional Tropical Fruits: Characterization, Antioxidant Potential and Carotenoid Bioaccessibility. *Plant Foods Hum. Nutr.* **2019**, *74*, 141–148. [CrossRef]
37. Petry, F.C.; Mercadante, A.Z. Impact of in vitro digestion phases on the stability and bioaccessibility of carotenoids and their esters in mandarin pulps. *Food Funct.* **2017**, *8*, 3951–3963. [CrossRef]

38. Huo, T.; Ferruzzi, M.G.; Schwartz, S.J.; Failla, M.L. Impact of fatty acyl composition and quantity of triglycerides on bioaccessibility of dietary carotenoids. *J. Agric. Food Chem.* **2007**, *55*, 8950–8957. [CrossRef]
39. Eggersdorfer, M.; Wyss, A. Carotenoids in human nutrition and health. *Arch. Biochem. Biophys.* **2018**, *652*, 18–26. [CrossRef]
40. Arunkumar, R.; Gorusupudi, A.; Bernstein, P.S. The macular carotenoids: A biochemical overview. *Biochim. Biophys. Acta—Mol. Cell Biol. Lipids* **2020**, *1865*, 158617. [CrossRef]
41. Gille, A.; Neumann, U.; Louis, S.; Bischoff, S.C.; Briviba, K. Microalgae as a potential source of carotenoids: Comparative results of an in vitro digestion method and a feeding experiment with C57BL/6J mice. *J. Funct. Foods* **2018**, *49*, 285–294. [CrossRef]
42. Gille, A.; Hollenbach, R.; Trautmann, A.; Posten, C.; Briviba, K. Effect of sonication on bioaccessibility and cellular uptake of carotenoids from preparations of photoautotrophic *Phaeodactylum tricornutum*. *Food Res. Int.* **2019**, *118*, 40–48. [CrossRef]
43. Sachindra, N.M.; Sato, E.; Maeda, H.; Hosokawa, M.; Niwano, Y.; Kohno, M.; Miyashita, K. Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites. *J. Agric. Food Chem.* **2007**, *55*, 8516–8522. [CrossRef]
44. Chen, K.; Roca, M. In vitro digestion of chlorophyll pigments from edible seaweeds. *J. Funct. Foods* **2018**, *40*, 400–407. [CrossRef]
45. Hayes, M.; Pottorff, M.; Kay, C.; Van Deynze, A.; Osorio-Marin, J.; Lila, M.A.; Iorrizo, M.; Ferruzzi, M.G. In Vitro Bioaccessibility of Carotenoids and Chlorophylls in a Diverse Collection of Spinach Accessions and Commercial Cultivars. *J. Agric. Food Chem.* **2020**, *68*, 3495–3505. [CrossRef]
46. O'Connell, O.F.; Ryan, L.; O'Brien, N.M. Xanthophyll carotenoids are more bioaccessible from fruits than dark green vegetables. *Nutr. Res.* **2007**, *27*, 258–264. [CrossRef]
47. Corte-Real, J.; Bohn, T. Interaction of divalent minerals with liposoluble nutrients and phytochemicals during digestion and influences on their bioavailability—A review. *Food Chem.* **2018**, *252*, 285–293. [CrossRef]
48. Granado-Lorencio, F.; Herrero-Barbudo, C.; Acién-Fernández, G.; Molina-Grima, E.; Fernández-Sevilla, J.M.; Pérez-Sacristán, B.; Blanco-Navarro, I. In vitro bioaccesibility of lutein and zeaxanthin from the microalgae *Scenedesmus almeriensis*. *Food Chem.* **2009**, *114*, 747–752. [CrossRef]
49. Xavier, A.A.O.; Mercadante, A.Z. The bioaccessibility of carotenoids impacts the design of functional foods. *Curr. Opin. Food Sci.* **2019**, *26*, 1–8. [CrossRef]
50. Lafarga, T.; Rodríguez-Bermúdez, R.; Morillas-España, A.; Villaró, S.; García-Vaquero, M.; Morán, L.; Sánchez-Zurano, A.; González-López, C.V.; Acién-Fernández, F.G. Consumer knowledge and attitudes towards microalgae as food: The case of Spain. *Algal Res.* **2021**, *54*, 102174. [CrossRef]
51. Lafarga, T. Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products. *Algal Res.* **2019**, *41*, 101566. [CrossRef]
52. Rippka, R.; Deruelles, J.; Waterbury, J.B.; Herdman, M.; Stanier, R.Y. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **1979**, *111*, 1–61. [CrossRef]
53. Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; et al. A standardised static in vitro digestion method suitable for food—An international consensus. *Food Funct.* **2014**, *5*, 1113–1124. [CrossRef] [PubMed]
54. Mandelli, F.; Miranda, V.S.; Rodrigues, E.; Mercadante, A.Z. Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. *World J. Microbiol. Biotechnol.* **2012**, *28*, 1781–1790. [CrossRef] [PubMed]
55. Ordóñez-Santos, L.E.; Pinzón-Zarate, L.X.; González-Salcedo, L.O. Optimization of ultrasonic-assisted extraction of total carotenoids from peach palm fruit (*Bactris gasipaes*) by-products with sunflower oil using response surface methodology. *Ultrason. Sonochem.* **2015**, *27*, 560–566. [CrossRef]
56. Giuffrida, D.; Zoccali, M.; Giofrè, S.V.; Dugo, P.; Mondello, L. Apocarotenoids determination in *Capsicum chinense* Jacq. cv. Habanero, by supercritical fluid chromatography-triple-quadrupole/mass spectrometry. *Food Chem.* **2017**, *231*, 316–323. [CrossRef]
57. De Rosso, V.V.; Mercadante, A.Z. HPLC-PDA-MS/MS of anthocyanins and carotenoids from dovyalis and tamarillo fruits. *J. Agric. Food Chem.* **2007**, *55*, 9135–9141. [CrossRef]
58. Rodrigues, D.B.; Flores, É.M.M.; Barin, J.S.; Mercadante, A.Z.; Jacob-Lopes, E.; Zepka, L.Q. Production of carotenoids from microalgae cultivated using agroindustrial wastes. *Food Res. Int.* **2014**, *65*, 144–148. [CrossRef]
59. Erdoğan, A.; Çağır, A.; Dalay, M.C.; Eroğlu, A.E. Composition of Carotenoids in Scenedesmus protuberans: Application of Chromatographic and Spectroscopic Methods. *Food Anal. Methods* **2015**, *8*, 1970–1978. [CrossRef]

APÊNDICE B

Capítulo: Technologies for Treatment of Emerging Contaminants

Livro: Occurrence, Distribution and Toxic Effects of Emerging Contaminants

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CHAPTER 9

Technologies for Treatment of Emerging Contaminants

Pricila Nass, Patrícia Caetano, Richard Machado, Leila Queiroz Zepka and *Eduardo Jacob-Lopes**

1. Introduction

The shortcoming of public policies and the precariousness of sanitation services, added to the uncontrolled population growth, have been identified as the main factors responsible for the decreased quality of water resources (Xu et al., 2022).

Considering these aspects, until two decades ago concerns about water quality focused on contaminants that caused color, odor, turbidity, and microorganisms that could alter water properties. Today, the preoccupation is greater because even the treated effluent can contain other harmful components, such as emerging contaminants, which have a low concentration can cause damage to the environment (Puri et al., 2023).

Emerging Contaminants (ECs) represent a wide range of compounds of natural or anthropic origin, such as pesticides, pharmaceuticals, personal care products, products from water disinfection processes, and cyanotoxins, whose effects on the environment and human health are still little understood (Wee and Ahmad, 2019).

Therefore, the input of ECs into the aquatic environment is a matter of concern, as these compounds tend to bioaccumulate in organisms, through the biomagnification process, due to their recalcitrant properties and lipophilic characteristics (Yan et al., 2014).

Thus, several studies have evaluated the toxicity of ECs, results suggest that the presence of these compounds in the environment could be related to the origin of resistant bacteria and the disruption of the endocrine system of organisms. For this reason, the occurrence of ECs in the environment, even in low concentrations, is associated with potential harm to the ecosystem and human health (Xu et al., 2020).

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The occurrence of these compounds in fresh, salty/brackish, groundwater, and even public supply waters has already been reported in several countries with different concentrations. Thus, the removal of ECs is generally not efficient by conventional processes present in water treatment plants (Chaturvedi et al., 2021; Yao et al., 2021).

Based on this understanding, microalgae have been reported for ECs removal, including *Chlamydomonas reinhardtii, Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Coelastrum* sp., *Tetraesmus dimorphus* and *Scenedesmus obliquus* (Zhou et al., 2022). Although ECs inhibit their growth, improving the removal efficiency of emerging contaminants by microalgae is a pressing issue to be solved (Alvarez-Gonz et al., 2023).

Based on this, the chapter summarizes the emerging contaminants, conventional treatment processes for emerging contaminants removal, as well as the application of microalgae in bioremediation, and finally, regulatory aspects.

2. Emerging Contaminants

The occurrence of ECs in the environment is a worldwide issue. Emerging contaminants consist of a range of natural and synthetic chemical compounds. These, have physical and chemical properties such as persistent volatility, or lipophilic, and can affect both the ecosystem and the health and quality of life of living beings (Puri et al., 2023).

Present in wastewater, ECs have low concentrations ($ng.L^{-1}$ to $\mu g.L^{-1}$) and diversity of compounds, complicating detection and analysis procedures, in addition to creating challenges for water treatment processes (Rizzo et al., 2019).

Several groups of substances have been considered ECs, including pesticides, pharmaceuticals, personal care products, products from water disinfection processes, and cyanotoxins. Table 9.1 summarizes research data on ECs in the environment and their effects on humans and biota.

Pesticides are compounds intended for agriculture to prevent or reduce the effects caused by pests, diseases, or weeds. These substances are synthetic organic compounds with low molecular weight, generally with low solubility in water and high biological activity. The term includes all insecticides, fungicides, herbicides, and organic compounds used as growth regulators, defoliants, or desiccants (Degrendele et al., 2022).

Another contaminant is pharmaceuticals, biologically active chemical substances synthesized in order to produce physiological responses in humans and animals. These compounds can cause harmful effects on aquatic fauna, and cause various morphological and even metabolic damages. However, there is particular concern about antibiotics. Research has shown that these contaminants when discarded into the environment can cause biological toxicity, induction of antibiotic resistance in pathogenic bacteria, and genotoxicity, which can be defined as the ability of some chemical substances to produce genetic alterations (Nieto-Juárez et al., 2021).

Personal use products include cosmetics, fragrances, repellent insecticides, and sunscreens. Many compounds used in these products are fat-soluble and therefore have a high potential for bioaccumulation (Ebele et al., 2020).

Table 9.1 Occurrence and effects of emerging contaminants in the environment.

Class	Environmental matrice	Concentration detected ($ng.L^{-1}$)	Analytical technique	Effects on human and biota
Pesticides	Drinking water	0,2–2600	LC-MS/MS HPLC-UV HRGC-ECD GC-ECD	Deleterious effects on fish gills; feminization of aquatic organisms; reproductive and sexual systems of humans are severely affected
Pharmaceuticals	Drinking water	18,5	LC-MS/MS	Genotoxicity, neurotoxicity, and oxidative stress in molluscs; reduced algae community growth; disruption with hormones
Personal care products	Drinking water	18–135,5	LC-MS/MS GC-MS/MS	Induce vitellogenin production in juvenile rainbow trout, reduction on plasma testosterone by over 50% in goldfish, affecting follicular growth, fertilization, and implantation in females
Water disinfection products	Drinking water	100–41000	GC-ECD	Dysregulation of thyroid hormones and adverse kidney health in humans

Some compounds for industrial use are also classified as emerging contaminants. Among them bisphenol A, alkylphenols, polychlorinated biphenyls, phthalates, and perfluorinated compounds. Most have liposoluble characteristics and some of them are even classified as persistent organic pollutants (Jha et al., 2021).

Considered a contaminant of natural origin, cyanotoxins are secondary metabolites of cyanobacteria, whose occurrence is aggravated due to the release of wastewater into water bodies that increase a load of nutrients, considerably increasing the cellular reproduction of cyanobacteria. Known cyanotoxins have three main targets: hepatotoxins, neurotoxins and dermatotoxins (Mutoti et al., 2023).

Finally, water pollution with ECs represents a threat to the environment, however, the removal of these compounds is usually not efficient by conventional processes. Thus, there is a need for alternatives, linked to conventional technology for the removal of these substances.

3. Conventional Treatment Processes

In the last decade, many contaminants have been discovered in wastewater, surface water and even drinking water that should be treated to ensure the safety of the environment. Emerging contaminants include many synthetic or natural substances, detected in natural environments, from domestic, commercial, and industrial sources (Garcia-Rodríguez et al., 2014; Ahmed et al., 2017; Tran et al., 2018).

Given the current case , contaminants have received increasing attention for their ability to cause potential ecological effects, causing changes in marine environments, affecting environmental organisms and the sustainability of aquatic ecosystems.

In view of this, some treatments are used to decontaminate the ECs (Lima, 2018; Rout et al., 2021).

According to Ranjit et al. (2021), wastewater treatment allows the disposal of anthropic and industrial effluents without creating risks to human health or damage to the natural environment. The conventional treatment of effluents, carried out in Sewage Treatment Stations (STPs), occurs through a combination of physical, chemical, and biological processes, called preliminary, primary, secondary, tertiary and sludge treatment, which aim to eliminate these ECs. When applied in sequence, they increase the degree of treatment that is related to the type of contaminant to be removed (Crini and Lichtfouse, 2019).

The treatments applied in the STPs are based on the removal of pollutants such as suspended solids, oils, gases, organic materials, dissolved salts, heavy metals, dyes, nitrogen, and microorganisms. Thus, for each type of existing contaminant, there is a specific method, that is, the choice of method will depend, therefore, on the characteristics of the effluent, not only in terms of costs, but also in terms of practicality, reliability, environmental impact, production of sludge and its potential to form toxic waste. Based on this, Fig. 9.1 shows the treatments used in the STPs (Choi et al., 2022).

The levels of the steps applied in the treatment of effluents aim at the degree of removal of these pollutants. The primary treatment is responsible for the physical removal of effluents through grids and retention boxes, while the secondary removes fine solids in suspension, dispersed solids and organics that are removed by volatilization, biodegradation, and incorporation into the sludge. The purpose of

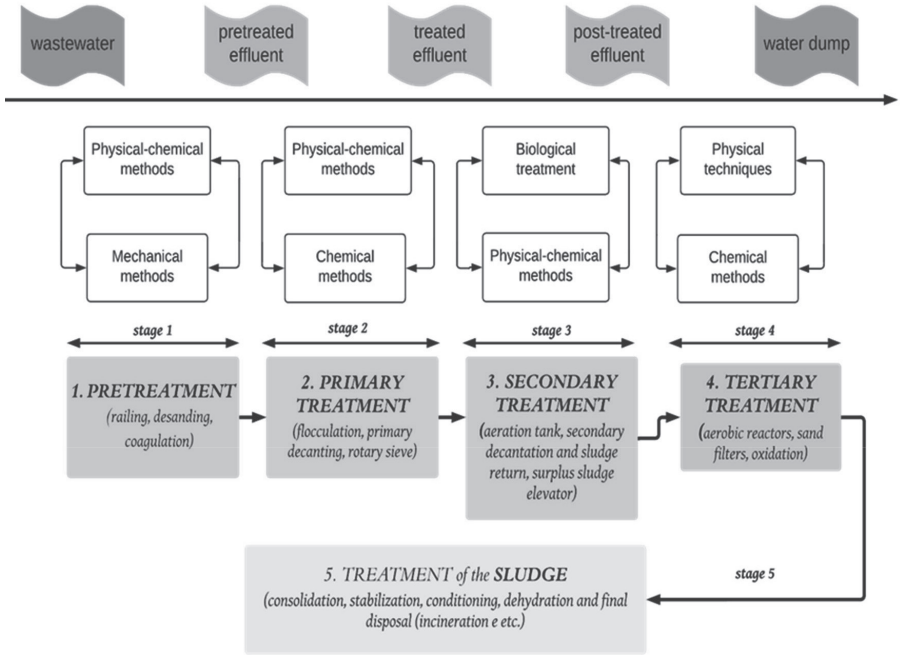

Figure 9.1 Treatment steps carried out in the STPs to control the decontamination of effluents and disposal of excess sludge.

tertiary or advanced treatment is to improve the quality of water discharged into natural waters, using a number of biological, physical, and chemical treatments (Zinicovscaia, 2016; Guillossou et al., 2019).

Sometimes, treatments applied to STPs result in a by-product with great potential for a pathogenic source, is called sludge. It is a solid or semi-solid material formed by a set of compounds such as inorganic and organic contaminants, hydrocarbons, microbial pollutants (pathogenic bacteria, etc.), which is the result of the process applied to wastewater, and can be formed by any other material dumped in the biological and chemical operation units during the treatment action (Lee et al., 2018).

Complex by nature, sludge treatment aims to reduce its volume, as it contains a large amount of water and organic materials that need stabilization. It can be classified as primary sludge formed by suspended solids, secondary sludge from waste removed from secondary decanters or settling ponds, and physical-chemical sludge generated during treatment with flocculants or coagulants (Santos and Lopes, 2022). After removing this contaminant, the sludge is reused, which can be done by different treatment methods applied in the destruction of harmful contaminants, so that it can be used as a source of energy and fertilizer (Puyol et al., 2017; Lanko et al., 2020; Chen et al., 2022).

Among the alternatives, linked to the treatment applied in the STPs for the removal of ECs are the adsorption processes with activated carbon, advanced oxidative processes and membrane filtration (Guo et al., 2021).

Among the advanced physical-chemical treatments, the adsorption processes are widely applied in the elimination of ECs. The most applied adsorbents are activated carbon, as they do not generate toxic products and have a high adsorption capacity (Guo et al., 2021).

Advanced oxidative processes are recognized as an alternative in the oxidation of ECs in the most diverse matrices, being adaptable to the most varied industrial processes. However, these oxidative processes are not selective and can lead to the formation of a wide range of by-products, which promotes the need for evaluating the toxicity of the effluent (Ajiboye et al., 2020).

Regarding membrane separation, reverse osmosis and nanofiltration processes can promote the removal of ECs. The efficiency of the application depends on the physicochemical properties of the target compounds, as well as on the operating conditions and the properties of the membranes. When comparing these two processes, it is observed that nanofiltration is less efficient than reverse osmosis, which is capable of promoting an almost complete removal of ECs, although the high energy consumption does not favor its use (Simmons et al., 2011).

Finally, while the removal effect of advanced treatment methods is satisfactory, it is difficult to apply these processes on a large scale due to their high energy consumption, uncontrollable degradation products, and low recovery rate. Thus, microalgae research on the removal of ECs from wastewater has received increasing attention, due to lower operating costs and higher bioenergy recovery (Zhou et al., 2022).

4. Microalgae in Bioremediation

There is a need for more sustainable and effective methods and microalgae have come up as a potential candidate for environment-friendly technologies. Wastewater treatment using microalgae, then assimilating high pollutant concentrations, exhibiting excellent energy-conversion efficiencies and with concomitant production of bioactive compounds (Beigbeder et al., 2021).

Photosynthetic microorganisms, but with metabolic versatility, are capable of recovering and recycling nutrients mainly inorganic Nitrogen (N) and Phosphate (P) contained in wastewater while producing biomass. Therefore, bioremediation using microalgae has been of growing interest, as it not only removes the pollutants but also purifies the water by producing oxygen (Maneechote et al., 2023). As an example, it is possible to produce about 1 and 10 $kg.m^{-3}$ of dry biomass-based waste sewage and manure, respectively. This means that microalgae have been successfully used as a waste-based nutrient cycling technology, as illustrated in Fig. 9.2 (Sartori et al., 2022), thus making wastewater treatment a circular bioeconomy (Palafox-Sola et al., 2023).

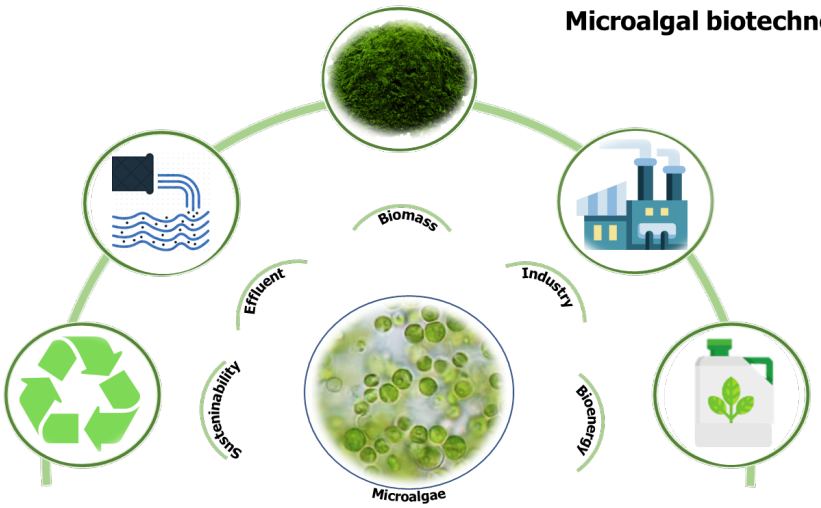

Figure 9.2 Microalgae are used as a waste-based nutrient recycling technology.

Traditional wastewater treatment methods as physical and chemical treatments are not very efficient in removing these contaminants, completely therefore a few of these pollutants are becoming persistent in the aquatic environments. Treatment processes generate several waste streams (i.e., sludge, chemical precipitates) that are difficult to dispose of and that cannot be re-circulated within the same process or revalorized. Moreover, while conventional wastewater treatment plants require costly aeration to the microbial communities, microalgae can uptake CO_2 from the atmosphere and provide aerobic microorganisms with oxygen (Nanda et al., 2021; Ajeng et al.,, 2022; Palafox-Sola et al., 2023).

Some species of microalgae more researched in the process of bioremediation, are *Chlorella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., and *Desertifilum* spp., which have been confirmed to assimilate high pollutant concentrations and exhibit excellent energy conversion efficiencies. For example, it was reported earlier that 81.7% chemical oxygen demand and 96.2% were removed, with the production of 3078 $mg.L^{-1}$ accumulating high carbohydrate content used for biobutanol production by Neochloris aquatica CL-M1 (Wang et al., 2017; Qu et al., 2019).

Microalgae are emerging as an effective bioremediation platform for heavy metals. In the study carried out by Tambat et al. (2023), between two microalgal strains, the *Chlorella sorokiniana* exhibited the highest growth rate and maximum removal of vanadium in mixotrophic cultive. The mixotrophic mode led to a higher growth rate as compared to autotrophic mode and increased removal efficiency by 292 and 66% for *Chlorella sorokiniana* and Picochlorum oklahomensis, with maximum biomass and lipid yield ranging between 2.5 and 3.0 $g.L^{-1}$ and 26.6–29.5%, respectively. These findings support the practical feasibility of combined microalgal purification and energy production systems.

Lastly, several technologies are being developed to treat effluents. However, the conditions, types, and concentrations of ECs, in parallel with current legislative restrictions should be considered.

5. Regulatory Aspect

Emerging pollutant emissions are a global environmental problem due to limited regulations and the worldwide consensus that establishes legislation in this regard. The regulatory frameworks for emerging contaminants bring measures of continuous monitoring and regulation of maximum permissible limits in environmental matrices. To this end, several countries have implemented methodologies for ECs (Salimi et al., 2017; Wang et al., 2020).

In the United States, the Environmental Protection Agency (EPA) is the body responsible for monitoring and ensuring compliance with laws and regulations. The US EPA regulates 129 priority ECs. As for the European Union, it introduced a list of 33 priority ECs for surface waters (Barbosa et al., 2016; Bopp et al., 2019; Ramírez-Malule et al., 2020).

The Australian drinking water guidelines determine priority ECs that pose a greater risk to consumers, including perfluorinated chemicals (perfluorooctanoic acid, perfluoroctane sulfonic acid, and their salts). The guidelines are developed in accordance with EU and WHO guidelines and supplementary documents (Naidu et al., 2016).

In Brazil, there are no records of official programs focused on the problem of emerging contaminants. However, discussions in different sectors of society have increased significantly in recent years and academic research has contributed significantly, providing numerous subsidies for decision-making, which has aroused the interest of regulators, sanitation companies, and government agencies (Zini and Mariliz, 2021).

Although the legislation is frequently revised, it is still limited in terms of emerging contaminants and their concentration patterns, making it possible that

water considered to be potable is contaminated by substances not yet regulated that can be harmful to human health and ecosystems.

6. Conclusion

Unquestionably alarming, is the fact that ECs are widely distributed in the environment. These include different groups of contaminants such as pesticides, pharmaceuticals, personal care products, products from water disinfection processes, and cyanotoxins. The current status of conventional methods for removing emerging contaminants is low effectiveness, requiring advanced treatment for their elimination. Among the alternatives, microalgae show good performance for the removal of ECs. Finally, the need to regulate ECs with systematic and enforceable legislation that should be implemented globally.

Reference

Ahmed, M.B., Zhou, J.L., Ngo, H.H., Guo, W., Thomaidis, N.S. and Xu, J. 2017. Progress in the biological and chemical treatment technologies for emerging contaminant removal from wastewater: a critical review. Journal of Hazardous Materials 323: 274–298.

Ajeng, A.A., Rosli, N.S.M., Abdullah, R., Yaacob, J.S., Qi, N.C. and Loke, S.P. 2022. Resource recovery from hydroponic wastewaters using microalgae-based biorefineries: A circular bioeconomy perspective. Journal of Biotechnology 360: 11–22.

Ajiboye, T.O., Kuvarega, A.T. and Onwudiwe, D.C. 2020. Recent strategies for environmental remediation of organochlorine pesticides. Applied Sciences 10(18): 6286.

Alvarez-Gonz, A., Uggetti, E., Serrano, L. and Gorchs, G. 2023. The potential of wastewater grown microalgae for agricultural purposes: Contaminants of emerging concern, heavy metals and pathogens assessment. Environ. Pollut. 1(324): 121399.

Barbosa, M.O., Moreira, N.F.F., Ribeiro, A.R., Pereira, M.F.R. and Silva, A.M.T. 2016. Occurrence and removal of organic micropollutants: An overview of the watch list of EU Decision 2015/495. Water Research 94: 257–79.

Beigbeder, J.B., Sanglier, M., de Medeiros Dantas, J.M. and Lavoie, J.M. 2021. CO_2 capture and inorganic carbon assimilation of gaseous fermentation effluents using *Parachlorella kessleri* microalgae. Journal of CO_2 Utilization 50: 101581.

Bopp, S.K., Kienzler, A., Richarz, A.N., Linden, S., Paini, A., Parissis, N. et al. 2019. Regulatory assessment and risk management of chemical mixtures: challenges and ways forward. Critical Reviews in Toxicology 49(2): 174–89.

Chaturvedi, P., Shukla, P., Giri, B., Chowdhary, P., Chandra, R., Gupta, P. et al. 2021. Prevalence and hazardous impact of pharmaceutical and personal care products and antibiotics in environment: A review on emerging contaminants. Environmental Research 194: 110664.

Chen, Y., Lin, M. and Zhuang, D. 2022. Wastewater treatment and emerging contaminants: Bibliometric analysis. Chemosphere 133932.

Choi, S., Yoom, H., Son, H., Seo, C., Kim, K., Lee, Y. 2022. Removal efficiency of organic micropollutants in successive wastewater treatment steps in a full-scale wastewater treatment plant: Bench-scale application of tertiary treatment processes to improve removal of organic micropollutants persisting after secondary treatment. Chemosphere 288: 132629.

Crini, G., and Lichtfouse, E. 2019. Advantages and disadvantages of techniques used for wastewater treatment. Environmental Chemistry Letters 17(1): 145–155.

Degrendele, C., Klánová, J., Prokeš, R., Příbylová, P., Šenk, P., Šudoma, M. et al. 2022. Current use pesticides in soil and air from two agricultural sites in south Africa: Implications for environmental fate and human exposure. Science of the Total Environment 807.

Ebele, A.J., Oluseyi, T., Drage, D., Harrad, S. and Abdallah, M. 2020. occurrence, seasonal variation and human exposure to pharmaceuticals and personal care products in surface water, groundwater and drinking water in lagos state, Nigeria. Emerging Contaminants 6: 124–32.

Elalami, D., Carrere, H., Monlau, F., Abdelouahdi, K., Oukarroum, A. and Barakat, A. 2019. Pretreatment and co-digestion of wastewater sludge for biogas production: Recent research advances and trends. Renewable and Sustainable Energy Reviews 114: 109287.

Garcia-Rodríguez, A., Matamoros, V., Fontàs, C. and Salvadó, V. 2014. The ability of biologically based wastewater treatment systems to remove emerging organic contaminants—a review. Environmental Science and Pollution Research 21(20): 11708–11728.

Guillossou, R., Le Roux, J., Mailler, R., Vulliet, E., Morlay, C., Nauleau, F. et al. 2019. Organic micropollutants in a large wastewater treatment plant: what are the benefits of an advanced treatment by activated carbon adsorption in comparison to conventional treatment? Chemosphere 218: 1050–1060.

Guo, Y., Wen, Y., Li, G. and An, T. 2021. Recent advances in voc elimination by catalytic oxidation technology onto various nanoparticles catalysts: A critical review. Applied Catalysis B: Environmental 281: 119447.

Jha, G., Kankarla, V., Lennon, E., Pal, S., Sihi, D., Dari, B. et al. 2021. Per-and Polyfluoroalkyl Substances (PFAS) in Integrated Crop–Livestock Systems: Environmental Exposure and Human Health Risks. International Journal of Environmental Research and Public Health 18: 23.

Lanko, I., Flores, L., Garfí, M., Todt, V., Posada, J.A., Jenicek, P. et al. 2020. Life cycle assessment of the mesophilic, thermophilic, and temperature-phased anaerobic digestion of sewage sludge. Water 12(11): 3140.

Lee, L.H., Wu, T.Y., Shak, K.P.Y., Lim, S.L., Ng, K.Y., Nguyen, M.N. et al. 2018. Sustainable approach to biotransform industrial sludge into organic fertilizer via vermicomposting: A mini-review. Journal of Chemical Technology and Biotechnology 93(4): 925–935.

Lima, E.C. 2018. Removal of emerging contaminants from the environment by adsorption. Ecotoxicology and Environmental Safety 150: 1–17.

Maneechote, W., Cheirsilp, B., Angelidaki, I., Suyotha, W. and Boonsawang, P. 2023. Chitosan-coated oleaginous microalgae-fungal pellets for improved bioremediation of non-sterile secondary effluent and application in carbon dioxide sequestration in bubble column photobioreactors. Bioresource Technology 372: 128675.

Mutoti, I. M., Edokpayi,J., Mutileni, N., Durowoju, O. and Munyai, F. 2023. Cyanotoxins in groundwater; occurrence, potential sources, health impacts and knowledge gap for public health. Toxicon 226: 107077.

Naidu, R., Jit, J., Kennedy, B. and Arias, V. 2016. Emerging contaminant uncertainties and policy: The chicken or the egg conundrum. Chemosphere 154: 385–90.

Nanda, M., Chand, B., Kharayat, S., Bisht, T., Nautiyal, N., Deshwal, S. et al. 2021. Integration of microalgal bioremediation and biofuel production: a 'clean up' strategy with potential for sustainable energy resources. Current Research in Green and Sustainable Chemistry 4: 100128.

Nieto-Juárez, J.I., Torres-Palma, R., Botero-Coy, A. and Hernández, F. 2021. Pharmaceuticals and environmental risk assessment in municipal wastewater treatment plants and rivers from Peru. Environment International 155.

Palafox-Sola, M.F., Yebra-Montes, C., Orozco-Nunnelly, D. A., Carrillo-Nieves, D., González-López, M. E. and Gradilla-Hernández, M.S. 2023. Modeling growth kinetics and community interactions in microalgal cultures for bioremediation of anaerobically digested swine wastewater. Algal Research 102981.

Puri, M., Gandhi, K. and Kumar, M. 2023. Emerging environmental contaminants: a global perspective on policies and regulations. Journal of Environmental Management 332: 117344.

Puyol, D., Batstone, D.J., Hülsen, T., Astals, S., Peces, M. and Krömer, J.O. 2017. Resource recovery from wastewater by biological technologies: opportunities, challenges, and prospects. Frontiers in Microbiology 7: 2106.

Qu, W., Zhang, C., Zhang, Y. and Ho, S.H. 2019. Optimizing real swine wastewater treatment with maximum carbohydrate production by a newly isolated indigenous microalga *Parachlorella kessleri* QWY28. Bioresource Technology 289: 121702.

Ramírez-Malule, H., Quiñones-Murillo, D. and Manotas-Duque, D. 2020. Emerging contaminants as global environmental hazards. A bibliometric analysis. Emerging Contaminants 6: 179–93.

Ranjit, P., Jhansi, V. and Reddy, K.V. 2021. Conventional Wastewater Treatment Processes. pp. 455–479. *In*: Advances in the Domain of Environmental Biotechnology. Springer, Singapore.

Rizzo, L., Malato, S., Antakyali, D., Beretsou, V., Đolić, M., Gernjak, W. et al. 2019. Consolidated vs new advanced treatment methods for the removal of contaminants of emerging concern from urban wastewater. Science of the Total Environment 655: 986–1008.

Rout, P.R., Zhang, T.C., Bhunia, P. and Surampalli, R.Y. 2021. Treatment technologies for emerging contaminants in wastewater treatment plants: A review. Science of the Total Environment 753: 141990.

Salimi, M., Esrafili, A., Gholami, M., Jafari, A., Kalantary, R., Farzadkia, M. et al. 2017. Contaminants of emerging concern: A review of new approach in AOP technologies. Environmental Monitoring and Assessment 189(8).

Santos, M.T. and Lopes, P.A. 2022. Sludge recovery from industrial wastewater treatment. Sustainable Chemistry and Pharmacy 29: 100803.

Sartori, R.B., Deprá, M.C., Dias, R.R., Lasta, P., Zepka, L.Q. and Jacob-Lopes, E. 2022. Microalgae-based systems applied to the dual purpose of waste bioremediation and bioenergy production. pp. 127–145. *In*: Algal Biotechnology. Elsevier.

Simmons, F.J., Kuo, D. and Xagoraraki, I. 2011. Removal of human enteric viruses by a full-scale membrane bioreactor during municipal wastewater processing. Water Research 45(9): 2739–50.

Tambat, V.S., Patel, A.K., Chen, C.W., Raj, T., Chang, J.S., Singhania, R.R. et al. 2023. A sustainable vanadium bioremediation strategy from aqueous media by two potential green microalgae. Environmental Pollution 323: 121247.

Tran, N.H., Reinhard, M. and Gin, K.Y.H. 2018. Occurrence and fate of emerging contaminants in municipal wastewater treatment plants from different geographical regions—a review. Water research, 133: 182–207.

Wang, Y., Ho, S.H., Cheng, C.L., Nagarajan, D., Guo, W.Q., Lin, C. et al. 2017. Nutrients and COD removal of swine wastewater with an isolated microalgal strain Neochloris aquatica CL-M1 accumulating high carbohydrate content used for biobutanol production. Bioresource Technology 242: 7–14.

Wang, Z., Walker, G., Muir, D. and Nagatani-Yoshida, K. 2020. Toward a global understanding of chemical pollution: A first comprehensive analysis of national and regional chemical inventories. Environmental Science and Technology 54(5): 2575–84.

Wee, S.Y. and Ahmad, Z.A. 2019. Occurrence and public-perceived risk of endocrine disrupting compounds in drinking water. Npj Clean Water 2: 1.

Xu, H., Berres, A., Liu, Y., Allen-Dumas, M. and Sanyal, J. 2022. An overview of visualization and visual analytics applications in water resources management. Environmental Modelling and Software 153: 105396.

Yan, Z., Yang, X., Lu, G., Liu, J., Xie, Z. and Wu, D. 2014. Potential environmental implications of emerging organic contaminants in Taihu Lake, China: Comparison of two ecotoxicological assessment approaches. Science of the Total Environment 470–471: 171–79.

Yang, W., Cai, C., Guo, Y., Wu, H., Guo, Y. and Dai, X. 2022. Diversity and fate of human pathogenic bacteria, fungi, protozoa, and viruses in full-scale sludge treatment plants. Journal of Cleaner Production 380: 134990.

Yao, C., Yang, H. and Li, Y. 2021. A review on organophosphate flame retardants in the environment: occurrence, accumulation, metabolism and toxicity. Science of the Total Environment 795: 148837.

Zhou, J.L., Yang, L., Huang, K., Chen, D. and Gao, F. 2022. Mechanisms and application of microalgae on removing emerging contaminants from wastewater: A review. Bioresource Technology 364: 128049.

Zini, L.B. and Mariliz G. 2021. Chemical contaminants in Brazilian drinking water: A systematic review. Journal of Water and Health 19(3): 351–69.

Zinicovscaia, I. 2016. Conventional methods of wastewater treatment. Cyanobacteria for Bioremediation of Wastewaters 17–25.

APÊNDICE C

Capítulo: Volatile organic compounds as food/feed Ingredients

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Chapter 16

Volatile organic compounds as food/feed ingredients

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1 Introduction

Volatile compounds have been regarded as an important parameter related to food acceptance. It is estimated that the relevance of the food additives market is constantly increasing. As a result, this segment is expected to increase from 13.31 to 19.72 billion USD between 2018 and 2026. Thus, emerging biotechnologies for obtaining volatile compounds are necessary to supply this demand for natural products, meeting the expectations of consumers (Hosoglu et al., 2020).

Based on this understanding, microalgae-based systems present a promising alternative to supply volatile organic compounds (VOCs) to the food industry, yet being possibly labeled as natural (EC No 1334/2008, Code of Federal Regulations Title 21/FDA) (Schempp et al., 2018).

Associated with these aspects, the biotechnological production of volatile organic compounds is considered an eco-friendly approach since these bioprocesses occur in controllable and optimizable process conditions without seasonal interference, thus reducing the use of products harmful to the environment (Berger, 2009). Moreover, microalgae-based systems may use agro-residues as alternative raw materials, which is advantageous in terms of ecological and economical sustainability. Meat processing industry wastes are good examples of how these substrates can be employed for volatile organic compound production, such as aldehydes, alcohols, terpenes, sulfurized compounds, esters, and ketones (Vieira et al., 2019).

Volatile organic compounds are the main secondary metabolites produced during microalgae cultivation. Its environmental advantages and the parameters affecting the production and recovery process are being studied (Vieira et al., 2019; Pinheiro et al., 2019).

However, a few microalgae products have a consolidated market share. Products such as volatile organic compounds are not yet competitive. Demand for these specialty chemicals is high, and at the current technology readiness level, the scale of production needs to be expanded at reasonable costs (Jacob-Lopes et al., 2020).

In that regard, the production of volatile organic compounds by microalgae meets the preferences of modern society for the consumption of natural products (Jacob-Lopes et al., 2019). This chapter describes the main volatile organic compounds produced by microalgae with applications in the food industry and presents the production and product recovery conditions.

2 Volatile organic compounds microalgae

Microalgae can synthesize several metabolites with different properties. About 90% of the carbon-based compounds released from microalgae systems constitute volatile organic compounds. This number is expected to grow with the diversity of microalgae analyzed with innovative detection methods (Jacob-Lopes and Franco, 2013).

Most volatile compounds are synthesized from enzymatic or degradation reaction pathways, comprising the isoprenoid pathway, keto acid, and fatty acid derivatives (Fig. 1). The volatile organic compounds belong to various chemical classes such as terpenes, aldehydes, alcohol, ketones, esters, and sulfurized compounds (Table 1) (Vieira et al., 2019).

Terpenes are volatile organic compounds relevant in the food industry and may be obtained by biotechnological procedures. Microalgae production of such compounds occurs through the isoprenoid pathway. To date, two routes have been reported: (i) mevalonic acid (MVA) and (ii) methylerythritol phosphate (MEP) (Lichtenthaler et al., 1997; Chappell, 2003).

Isopentenyl diphosphate and dimethylallyl diphosphate are the central intermediates from the isoprenoid pathway. In sequence, these starter structures are transformed into geranyl diphosphate (GPP), followed by farnesyl

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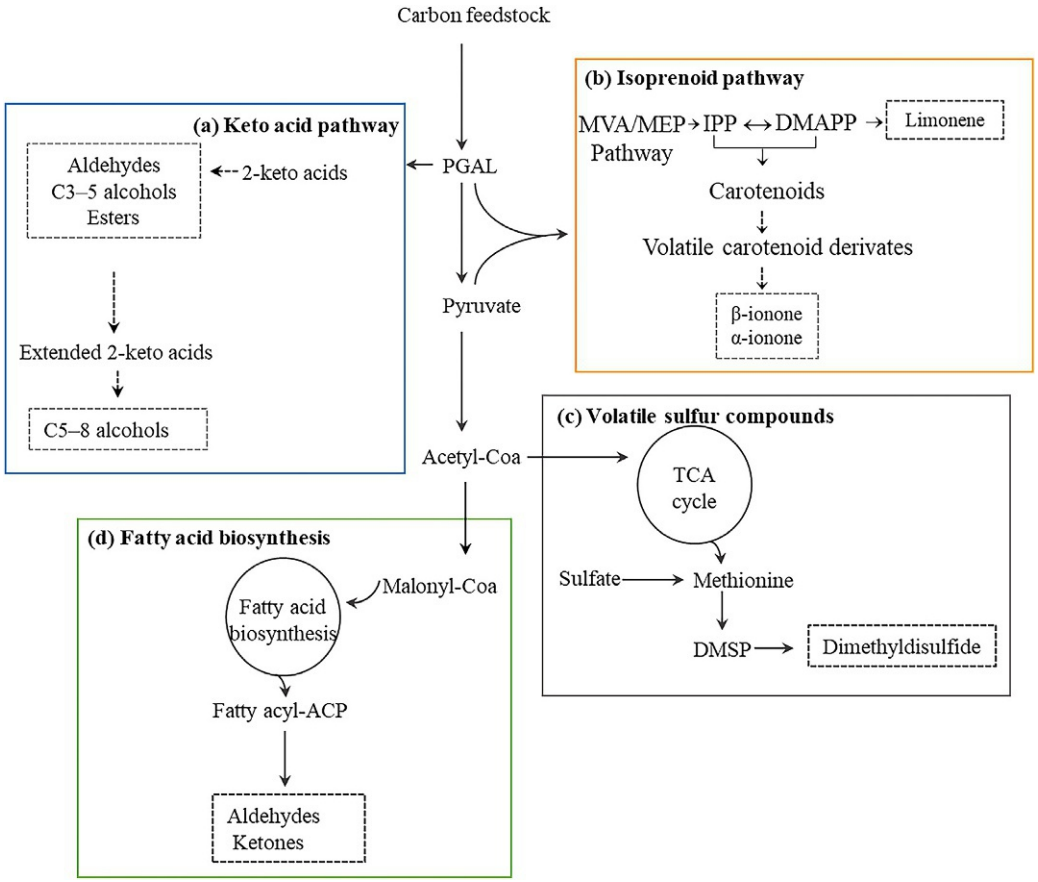

FIG. 1 General scheme of the biosynthetic pathways for producing VOCs from microalgae applicable to the food and feed industry. (A) The keto acid pathway can be used to generate aldehydes, alcohols, and esters. (B) The isoprenoid pathway synthesizes diversified terpenoids. (C) The volatile sulfur compound is derived from the amino acid methionine (arises from the TCA cycle (tricarboxylic acid cycle)), initially, decarboxylation, reduction, and finally methylation mechanism to form dimethyl sulfide. (D) From fatty acid synthesis, acids, aldehydes, and ketones are formed.

TABLE 1 Volatile organic compounds from microalgae with potential food/feed industry application.

Chemical name	Sensorial description	Species to produce the VOCs	References
Terpenes			
Limonene	Citrus, mint	*Chlorella vulgaris, Chlorella sorokiniana, Desertifilum* spp., *Scenedesmus bijuga, Scenedesmus obliquus, Spirulina* sp.	Nörnberg et al. (2022)
α-Ionone	Tropical fruity	*Botryococcus braunii, Nannochlopsis, Rhodomonas* sp., *Tetraselmis* sp.	Durme et al. (2013)
β-Ionone	Violet, flower	*Anabaena* sp., *B. braunii, Nannochlopsis oculata, Nostoc* sp., *Rhodomonas* sp., *Spirulina platensis, Tetraselmis* sp.	Durme et al. (2013), Milovanovic et al. (2015)
Aldehydes			
Hexanal	Grass, tallow	*B. braunii, C. vulgaris, Nannochloropsis oculata, Phormidium autumnale, Rhodomonas* sp., *Schizochytrium limacinum, Tetraselmis* sp.	Durme et al. (2013), Santos et al. (2016a), Hosoglu (2018)
2,4-Decadienal	Fatty, oily with a slight rancid tallow nuance	*C. vulgaris*	Lafarge and Cayot (2019)

TABLE 1 Volatile organic compounds from microalgae with potential food/feed industry application—cont'd

Chemical name	Sensorial description	Species to produce the VOCs	References
2-Pentanal	Pungent, green, apple, orange, tomato	*Arthrospira platensis, C. vulgaris, Isochrysis galbana, Nannochloropsis gaditana, Synechococcus* sp., *Tetraselmis* sp.	Moran et al. (2022)
2-Methylbutanal	Cocoa, almond	*Phormidium autumnale*	Santos et al. (2016a)
3-Methylbutanal	Malt	*C. vulgaris, Nannochloropsis oculata, Phormidium autumnale, Rhodomonas* sp., *Tetraselmis* sp.	Durme et al. (2013), Santos et al. (2016a)
Nonanal	Citrus, green	*B. braunii, Chaetoceros calcitrans, Chlorella prothecoides, C. vulgaris, Crypthecodinium cohnii, Microcystis* sp., *Nannochloropsis oculata, Nitzschia closterium, Platymonas helgolandica, Rhodomonas* sp., *Schizochytrium limacinum, Thalassiosira weissflogii*	Durme et al. (2013), Zhou et al. (2017), Hosoglu (2018)
Ketones			
2,3-Pentenedione	Toasted, caramellic	*B. braunii, C. vulgaris, Nannochloropsis oculata, N. closterium, Rhodomonas* sp., *Tetraselmis* sp.	Durme et al. (2013), Zhou et al. (2017)
Alcohols			
1-Butanol	Fruit	*Phormidium autumnale.*	Santos et al. (2016a)
1-Hexanol	Flower, green	*C. vulgaris, Nannochloropsis oculata, Phormidium autumnale, Tetraselmis* sp.	Durme et al. (2013), Santos et al. (2016a)
3-Methylbutanol	Whiskey, malt	*C. vulgaris, Microcystis aeruginosa, Nannochloropsis oculata, Phormidium autumnale, Tetraselmis* sp.	Hasegawa et al. (2012), Durme et al. (2013), Santos et al. (2016a)
1-Penten-3-ol	Green	*B. braunii, C. vulgaris, Nannochloropsis oculata, N. closterium, Rhodomonas* sp.	Durme et al. (2013), Zhou et al. (2017)
Esters			
Methyl phenylacetate	Honey	*B. braunii, Chlorella prothecoides, C. cohnii, Rhodomonas* sp., *Schizochytrium limacinum, Tetraselmis chuii*	Durme et al. (2013), Hosoglu (2018)
Methyl octanoate	Fruit, Orange	*B. braunii, Chlorella prothecoides, C. cohnii, Rhodomonas* sp., *Schizochytrium limacinum, Tetraselmis chuii*	Durme et al. (2013), Hosoglu (2018)
Sulfurized compounds			
Dimethyl sulfide	Cabbage, sulfurous	*Anacystis nidulans, Chaetoceros calcitrans, Chlorella protothecoides, C. vulgaris, C. cohnii, Nannochloropsis* sp., *Oscillatoria chalybea, Oscillatoria tenuis, Phormidium autumnale, Platymonas helgolandica, Plectonema boryanum, Schizochytrium limacinum, Synechococcus cedrorum, Tetraselmis chuii, Tetraselmis* sp., *T. weissflogii*	Durme et al. (2013), Zhou et al. (2017), Lee et al. (2017), Hosoglu (2018)
Dimethyl disulfide	Septic, garlic, putrid	*M. aeruginosa, Microcystis wesenbergii, Rhodomonas* sp., *Tetraselmis* sp.	Durme et al. (2013), Lee et al. (2017)
Dimethyl trisulfide	Alliaceous	*Rhodomonas sp., Tetraselmis chuii*	Durme et al. (2013), Lee et al. (2017)

diphosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Meena et al., 2017). These carbon precursors are converted into diversified terpenoids, such as carotenoids and their oxidative and enzymatic cleavage products β-ionone and α-ionone (Dudareva et al., 2013; Santos et al., 2016a).

Besides that, some species of microalgae produce limonene from the isoprenoid pathway by the action of the enzyme limonene synthase (LMS) from the geranyl diphosphate (GPP), which is cyclized to give limonene (Kiyota et al., 2014). For instance, the production of limonene in *Scenedesmus bijuga* is approximately two times superior to *Desertifilum* spp. In addition, other microalgae species such as *Spirulina* sp., *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Chlorella sorokiniana* also showed the production of limonene compound (Nörnberg et al., 2022).

Many aldehydes, ketones, alcohols, esters, and sulfur compounds are odor-active volatiles essential for the aroma perception in several food products. Volatile organic compounds, such as 2,4-decadienal, originate from the degradation of arachidonic or eicosapentaenoic acids by the action of the lipoxygenase/hydroperoxide lyase. The fatty acids, linoleic or linolenic acid, lead to the generation of aldehydes such as nonanal, hexanal, and 2-pentanal (Santos et al., 2016b; Jerković et al., 2018). Also, aliphatic ketones can be lipid oxidation products (Santos et al., 2016a, b).

Moreover, aldehydes, alcohols, and esters also can be synthesized through the 2-keto acid pathway. The 2-keto acid pathway covers sequential biochemical reactions such as (i) extension, (ii) decarboxylation, (iii) isomerization, (iv) reduction, (v) dehydration, and (vi) esterification of some branched-chain amino acids (e.g., leucine and valine). For example, for 1-butanol, 3-methyl-butanal, and 2-methyl-butanal subsequently reduced to 3-methyl-butanol and 2-methyl-butanol, the reaction can be extended to form 1-hexanol and other alcohols (Hasegawa et al., 2012).

The production of dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide has been described from microalgae. The dimethyl sulfide arises from the amino acid methionine, which is the forerunner of the 2-keto acid 4-methylthio-2-oxobutyrate, through transamination (see Giordano et al. (2005) and their references), followed by a reduction reaction catalyzed by enzyme 4-methylthio-2-oxobutyrate reductase, forming 4-methylthio-2-hydroxybutyrate using a nicotinamide adenine dinucleotide phosphate molecule (Giordano and Prioretti, 2016).

In the sequence, S-methylation of 4-methylthio-2-hydroxybutyrate to 4-dimethylsufonio-2-hydroxybutyrate takes place, which is lastly transformed at the dimethylsulfoniopropionate compound through oxidative decarboxylation (Giordano et al., 2005; Giordano and Prioretti, 2016). The demethylation of dimethylsulfoniopropionate produces methanethiol, which can be converted into dimethyl sulfide by methylation (Achyuthan et al., 2017).

Lastly, in order to exploit VOCs in microalgae-based systems successfully, a good understanding of biosynthesis, as well as the parameters of cultivation, is essential. This will optimize the production of volatile compounds of interest to the food/feed industry (Santos et al., 2016a).

3 Culture systems for volatile organic compound production

The production of volatile organic compounds from microalgae can be influenced by the species, light intensity, light absence, microalgae growth phase, and cultivation temperature (Achyuthan et al., 2017; Vieira et al., 2020).

Based on this understanding, the selection of a microalgae mode of cultivation is of vital importance. Three major conditions of microalgae cultivation can be used: (i) photoautotrophic, (ii) heterotrophic, and (iii) mixotrophic (Perez-Garcia and Bashan, 2015; Santos et al., 2018).

Microalgae are generally cultivated under photoautotrophic systems, converting light and carbon dioxide (CO_2) for growth (Maroneze et al., 2016). In contrast, some microalgae can grow heterotrophically (light absence), supported by an exogenous carbon source (Jacob-Lopes et al., 2020). On the other hand, in mixotrophic cultivation, microalgae use the conditions of phototrophy and heterotrophy simultaneously in the presence of light (Perez-Garcia and Bashan, 2015; Santos et al., 2018).

In photoautotrophic systems, light promotes the release of terpenoid compounds. This is fundamentally supported by the supply of energetic cofactors and carbon intermediates enhancing the availability of DMAPP, the immediate precursor of the MEP pathway. Therefore, isoprene and monoterpenes are produced via MEP and are released extracellularly into the medium after direct synthesis once there are no storage structures (Liao et al., 2016).

It was demonstrated that in heterotrophic culture, the alcohols in heterotrophic cultivation were the larger volatile group. Both 2-nonanol and 2-octanol were found in relatively high amounts, 183.1 and 124.5 $\mu g\,mg^{-1}$. Alcohols are important aroma components in many food products (Santos et al., 2016a).

Besides, applications of mixotrophic cultivation show that 2,4-decadienal was the major compound identified. This compound is helpful for savory food and, at low concentrations, has a citrus flavor (Santos et al., 2019).

The emission of volatile compounds in microalgae systems can also be affected by growth phases. As a result, aldehydes and alcohols of distinct strains did not show the same tendency and concentration between the three growth phases. Ketones in the species studied showed increasing

trends from the exponential to the stationary phase (Zhou et al., 2017).

Furthermore, elevated temperatures stimulate the release of alcohols and aldehydes, which are synthesized via oxidative degradation of fatty acids and carotenoid derivatives such as α-ionone, β-cyclocitral, β-ionone, and geranyl acetone (García-Plazaola et al., 2017).

Finally, the emission of VOCs from the microalgae system is a result of their versatile metabolism; thus, understanding the microalgae culture parameters and techniques recovery can promote the production of VOCs with industrial potential (Santos et al., 2016a, 2018).

4 Techniques for VOC recovery

Commercial production of volatile compounds obtained biotechnologically requires economic competitiveness. The synthesis of microalgae bioproducts is usually limited by the cell productivity and concentrations of target compounds. In order to achieve considerable yields, a reactor design and an adequate setting for the recovery of volatile compounds are required (Akachaa and Gargouri, 2015).

Currently, some approaches can be used for the separation and recovery of VOCs in reactors, such as (i) adsorption, (ii) condensation, (iii) absorption, and (iv) membrane-based techniques (Try et al., 2018; Saffarionpour and Ottens, 2018).

Adsorption is widely used in the recovery of VOCs from bioreactors. In this system, a solid (adsorbent) connects a gaseous component (adsorbate) to its surface (Saffarionpour and Ottens, 2018). The adsorption of volatile molecules of solid materials, as in microalgae biomass, is widely used to analyze these compounds on a laboratory scale (Lukin et al., 2018).

In a condensation system, the gas stream of the headspace of the bioreactor is driven through the vertical trap column positioned in a cryogenic bath, which enables VOCs' vapor to condense (Saffarionpour and Ottens, 2018). Condensation is applied to isolate VOCs from a plant matrix and microalgae biomass (Lukin et al., 2018).

Another technique is absorption consisting of a gas disposing into contact with a liquid to transfer the molecules into the liquid phase. The absorption structures can be applied as a single operation, in which a reactant is dissolved in the liquid phase or used with a non-reacting liquid. This device is connected to an adsorbent to recover the absorbing liquid (Wylock et al., 2015).

Membrane-based techniques, also recognized as pervaporation, are based on separating liquid mixtures through a dense membrane with the gas flow (Try et al., 2018). Pervaporation shows relevant advantages for recovering volatile molecules and hydrophobic compounds (Lukin et al., 2018).

The recovery of volatile compounds from microalgae may be a critical point because these molecules meet at low concentrations, and biomass is presented in the solid phase; moreover, the cell membrane acts as a natural barrier for volatile compounds, and the volatile compounds may be distributed in different phases, such as liquid, solid, and gas (López-perez et al., 2017; Achyuthan et al., 2017).

Finally, industrial recovery is challenging since VOCs are expected to partition between distinct phases, requiring different recovery approaches for each phase and, in some cases, additional steps.

5 Conclusion

Microalgae can produce a variety of volatile compounds for the food and feed industry. Associated with this, it is essential to know the biosynthesis of VOCs, the cultivation parameters, and their recovery techniques. However, some challenges must be overcome for these bioprocesses to be successful in the market on a large scale, including improving genetic engineering strategies to potentialize VOC production, appropriate microalgae strains, and an optimized cultivation system. Besides, volatile compound recovery technologies must be designed to follow the photobioreactor configuration, as well as the volatility of the target molecule and its partitioning between the phases under real production conditions.

References

Achyuthan, K.E., Harper, J.C., Manginell, R.P., Moorman, M.W., 2017. Volatile metabolites emission by *in vivo* microalgae - an overlooked opportunity? Meta 7 (3). https://doi.org/10.3390/metabo7030039.

Akachaa, N.B., Gargouri, M., 2015. Microbial and enzymatic technologies used for the production of natural aroma compounds: synthesis, recovery modeling, and 16/j.fbp.2014.09.011oprod. PRO 94, 675–706. https://doi.org/10.1016/j.fbp.2014.09.011.

Berger, R.G., 2009. Biotechnology of flavours- the next generation. Biotechnol. Lett. 31, 1651–1659. https://doi.org/10.1007/s10529-009-0083-5.

Chappell, J., 2003. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu. Rev. Plant Physiol. 46 (1), 521–547. https://doi.org/10.1146/annurev.pp.46.060195.002513.

Dudareva, N., Klempien, A., Muhlemann, J.K., Kaplan, I., 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. 198, 16–32. https://doi.org/10.1111/nph.12145.

Durme, J., Goiris, K., De Winne, A., De Cooman, L., Muylaert, K., 2013. Evaluation of the volatile composition and sensory properties of five species of microalgae. J. Agric. Food Chem. 61 (46), 10881–10890. https://doi.org/10.1021/jf403112k.

García-Plazaola, J.I., Portillo-Estrada, M., Fernández-Marı'n, B., Keannaste, A., Niinemets, E.U., 2017. Emissions of carotenoid cleavage products upon heat shock and mechanical wounding from a foliose

lichen. Environ. Exp. Bot. 133, 87–97. https://doi.org/10.1016/j.envexpbot.2016.10.004.

Giordano, M., Prioretti, L., 2016. Sulphur and algae: metabolism, ecology and evolution. In: Borowitzka, M.A., Beardall, J., Raven, J.A. (Eds.), Microalgal Physiology. Springer, Dordrecht, pp. 185–209, https://doi.org/10.1007/978-3-319-24945-2_9.

Giordano, M., Beardall, J., Raven, J.A., 2005. CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Annu. Rev. Plant Biol. 56 (1), 99–131. https://doi.org/10.1146/annurevarplant.56.032604.144052.

Hasegawa, M., Nishizawa, A., Tsuji, K., Kimura, S., Harada, K., 2012. Volatile organic compounds derived from 2-keto-acid decarboxylase in *Microcystis aeruginosa*. Microbes Environ. 27 (4), 525–528. https://doi.org/10.1264/jsme2.me12099.

Hosoglu, M., 2018. Aroma characterization of five microalgae species using solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry. Food Chem. 240, 1210–1218. https://doi.org/10.1016/j.foodchem.2017.08.052.

Hosoglu, M., Karagul-Yuceer, Y., Guneser, O., 2020. Aroma characterization of heterotrophic microalgae *Crypthecodinium cohnii* using solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry during different growth phases. Algal Res. 49, 101928. https://doi.org/10.1016/j.algal.2020.101928.

Jacob-Lopes, E., Franco, T.T., 2013. From oil refinery to microalgal biorefinery. J. CO_2 Util. 2, 1–7. https://doi.org/10.1016/j.jcou.2013.06.001.

Jacob-Lopes, E., Maroneze, M.M., Deprá, M.C., Sartori, R.B., Dias, R.R., Zepka, L.Q., 2019. Bioactive food compounds from microalgae: an innovative framework on industrial biorefineries. Curr. Opin. Food Sci. 25, 1–7. https://doi.org/10.1016/j.cofs.2018.12.003.

Jacob-Lopes, E., Santos, A.B., Severo, I.A., Deprá, M.C., Maroneze, M.M., Zepka, L.Q., 2020. Dual production of bioenergy in heterotrophic cultures of cyanobacteria: process performance, carbon balance, biofuel quality and sustainability metrics. Biomass Bioenergy 142. https://doi.org/10.1016/j.biombioe.2020.105756.

Jerković, I., Marijanović, Z., Roje, M., Kús, P.M., Jokić, S., Čož-Rakovac, R., 2018. Phytochemical study of the headspace volatile organic compounds of fresh algae and seagrass from the *Adriatic Sea* (single point collection). PLoS One 13 (5), e0196462.

Kiyota, H., Okuda, Y., Ito, M., Hirai, M.Y., Ikeuchi, M., 2014. Engineering of cyanobacteria for the photosynthetic production of limonene from CO_2. J. Biotechnol. 185, 1–7. https://doi.org/10.1016/j.jbiotec.2014.05.025.

Lafarge, C., Cayot, N., 2019. Insight on a comprehensive profile of volatile compounds of *Chlorella vulgaris* extracted by two "green" methods. Food Sci. Nutr. 7, 918–929. https://doi.org/10.1002/fsn3.831.

Lee, J., Rai, P.K., Jeon, Y.J., Kim, K.H., Kwon, E.E., 2017. The role of algae and cyanobacteria in the production and release of odorants in water. Environ. Pollut. 227, 252–262. https://doi.org/10.1016/j.envpol.2017.04.058.

Liao, J.C., et al., 2016. Fuelling the future: microbial engineering for the production of sustainable biofuels. Nat. Rev. Microbiol. 5 (14), 288–304. https://doi.org/10.1038/nrmicro.2016.32.

Lichtenthaler, H.K., Disch, A., Rohmer, M., 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett. 400 (3), 271–274. https://doi.org/10.1016/S0014-5793(96)01404-4.

López-perez, O., Picon, A., Nunez, M., 2017. Volatile compounds and odour characteristics of seven species of dehydrated edible seaweeds. Food Res. Int. 99, 1002–1010. https://doi.org/10.1016/j.foodres.2016.12.013.

Lukin, I., Merz, J., Schembecker, G., 2018. Techniques for the recovery of volatile aroma compounds from biochemical broth: a review. Flavour Fragr. J. 33, 203–216. https://doi.org/10.1002/ffj.3447.

Maroneze, M.M., Siqueira, S.F., Vendruscolo, R.G., Wagner, R., de Menezes, C.R., Zepka, L.Q., Jacob-Lopes, E., 2016. The role of photoperiods on photobioreactors—a potential strategy to reduce costs. Bioresour. Technol. 219, 493–499. https://doi.org/10.1016/j.biortech.2016.08.003.

Meena, S., Kumar, S.R., Dwivedi, V., Kumar Singh, A.K., Chanotiya, C.S., Akhtar, Q., Kumar, K., Shasany, A.K., Nagegowda, D.A., 2017. Transcriptomic insight into terpenoid and carbazole alkaloid biosynthesis, and functional characterization of two terpene synthases in curry tree (*Murraya koenigii*). Sci. Rep. 7, 44126. https://doi.org/10.1038/srep44126.

Milovanovic, I., Mišan, A., Simeunovic, J., Kova, D., Dubravka, J.D., Anamarija, M.A., 2015. Determination of volatile organic compounds in selected strains of cyanobacteria. J. Chem. 2015, 1–6. https://doi.org/10.1155/2015/969542.

Moran, L., Bou, G., Aldai, N., Ciardi, M., Morillas-España, A., Sánchez-Zurano, A., Barron, L.J.R., Lafarga, T., 2022. Characterisation of the volatile profile of microalgae and cyanobacteria using solid-phase microextraction followed by gas chromatography coupled to mass spectrometry. Science 12, 3661. https://doi.org/10.1038/s41598-022-07677-4.

Nörnberg, M.L., Caetano, P.A., Nass, P.P., Vieira, K.R., Wagner, R., Jacob-Lopes, E., Zepka, L.Q., 2022. Limonene production in microalgal photoautotrophic cultivation. Braz. J. Dev., 10241. https://doi.org/10.34117/bjdv8n2-120.

Perez-Garcia, O., Bashan, Y., 2015. Microalgal heterotrophic and Mixotrophic culturing for bio-refining: from metabolic routes to techno-economics. In: Prokop, A., Bajpai, R., Zappi, M. (Eds.), Algal Biorefineries. Springer, Cham, pp. 61–131, https://doi.org/10.1007/978-3-319-20200-6_3.

Pinheiro, P.N., Vieira, K.R., Santos, A.B., Jacob-Lopes, E., Zepka, L.Q., 2019. Biogeneration of volatile organic compounds in microalgae-based systems. In: Ravishankar, G.A., Ambati, R.R. (Eds.), Handbook of Algal Technologies and Phytochemicals, first ed. Food, Health and Nutraceutical Applications, vol. I. CRC Press, Boca Raton, pp. 100–110.

Saffarionpour, S., Ottens, M., 2018. Recent advances in techniques for flavor recovery in liquid food processing. Food Eng. Rev. 10, 81. https://doi.org/10.1007/s12393-017-9172-8.

Santos, A.B., Fernandes, A.S., Wagner, R., Jacob-Lopes, E., Zepka, L.Q., 2016a. Biogeneration of volatile organic compounds produced by *Phormidium autumnale* in heterotrophic bioreactor. J. Appl. Phycol. 28 (3), 1561–1570. https://doi.org/10.1007/s10811-015-0740-0.

Santos, A.B., Vieira, K.R., Nogara, G.P., Wagner, R., Jacob-Lopes, E., Zepka, L.Q., 2016b. Biogeneration of volatile organic compounds by microalgae/cyanobacteria: occurrence, be-havior, ecological implications and industrial applications. In: Moore, J.P. (Ed.), Volatile Organic Compounds: Occurrence, Behavior and Ecological Implications. Nova Science Publishers, New York, pp. 1–18.

Santos, A.B., Vieira, K.R., Pinheiro, P.N., Paulino, B.N., Bicas, J.L., Jacob-Lopes, E., Zepka, L.Q., Siegmund, B., 2018. Flavour generation from microalgae in mixotrophic cultivation. In: Leitner, E. (Ed.), Proceedings of the XV Weurman Flavour Research Symposium. Flavor Science, pp. 87–90.

Santos, A.B., et al., 2019. Flavour generation from microalgae in mixotrophic cultivation. In: Leitner, E. (Ed.), Proceedings of the XV

Weurman Flavour Research Symposium. Barbara Siegmund & Erich Leitner, pp. 87–91. 01.

Schempp, F.M., Drummond, L., Buchhaupt, M., Schrader, J., 2018. Microbial cell factories for the production of terpenoid flavor and fragrance compounds. J. Agric. Food Chem. 66, 2247–2258. https://doi.org/10.1021/acs.jafc.7b00473.

Try, S., Voilley, A., Chunhieng, T., De-Coninck, J., Wache, Y., 2018. Aroma compounds production by solid state fermentation, importance of *in situ* gas-phase recovery systems. Appl. Microbiol. Biotechnol. 102, 7239–7255. https://doi.org/10.1007/s00253-018-9157-4.

Vieira, K.R., Pinheiro, P.N., Santos, A.B., Cichoski, A.J., Menezes, C.R., Wagner, R., Zepka, L.Q., Jacob-Lopes, E., 2019. The role of microalgae-based systems in the dynamics of odors compounds in the meat processing industry. Desalin. Water Treat. 150, 282–292. https://doi.org/10.5004/dwt.2019.23730.

Vieira, K.R., Nass, P., Zepka, L., 2020. Volatile organic compounds from microalgae. In: Jacob-Lopes, E. (Ed.), Handbook of Microalgae-Based Processes and Products, first ed. vol. 1. Academic Press, pp. 659–686. https://doi.org/10.1016/B978-0-12-818536-0.00024-5.

Wylock, C., Eloundou Mballa, P.P., Heilporn, C., Debaste, F., Fauconnier, M.L., 2015. Review on the potential technologies for aroma recovery from food industry flue gas. Trends Food Sci. Technol. 46, 68–74. https://doi.org/10.1016/j.tifs.2015.08.002.

Zhou, L., Chen, J., Xu, J., Li, Y., Zhou, C., Yan, X., 2017. Change of volatile components in six microalgae with different growth phases. J. Sci. Food Agric. 97 (3), 761–769. https://doi.org/10.1002/jsfa.7794.

APÊNDICE D

Capítulo: Volatile organic compounds from microalgae as an alternative for the production of bioenergy

Livro: 3rd Generation Biofuels

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Volatile organic compounds from microalgae as an alternative for the production of bioenergy

25

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25.1 Introduction

In the present scenario, given the increased demand for energy resources and the continued growth of emissions of air pollutants, ensuring energy stability is a challenge to the economy and politics worldwide. Based on this, replacing fossil energy with an energy matrix more sustainable is an inevitable trend for social and economic sustainable development (Jacob-Lopes et al., 2020).

As a result, microalgae-systems carbon capture and utilization assume some protagonism being a bioconversion strategy. This is because the versatile metabolism of microalgae allows the assimilation of different carbon forms and nutrients which result in biomass and other products of energetic interest (Severo et al., 2018, 2020a,b).

More specifically, gaseous bioproducts of the microalgae metabolism, the volatile organic compounds (VOCs) are identified as potential advanced biofuels. Considering the biosynthetic origin, these molecules belong to different chemical classes, such as alcohol, aldehydes hydrocarbons, ketones, and terpenes presenting a significant energetic value (Durme et al., 2013; Santos et al., 2016a,b; Deprá et al., 2018).

Given these aspects, the key to designing and optimizing microalgae processes is the application of advanced technologies for the recovery of volatile compounds added to the biorefinery platform (Pinheiro et al., 2019; Vieira et al., 2019, 2020).

In this sense, the purpose of the chapter was to evaluate: (i) the volatile organic compounds from microalgae, (ii) microalgae gaseous biofuels, (iii) the microalgae metabolism, as well as VOCs' biosynthesis mechanism, and, finally, (iv) environmental implications for the techniques for the separation and recovery of VOCs.

25.2 Volatile organic compounds from microalgae

Microalgae-based products are a new and valuable source of compounds for applications in several biotechnology sectors (Pinheiro et al., 2019). This prominence is attributed to the high exploratory value of the microalgae biomass, which can be

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utilized as feedstock for the generation of a variety of bioproducts (Jacob-Lopes et al., 2019). Moreover, microalgae-based systems have gained much attention between the research and industrial sectors. Since these microorganisms have the ability to biosynthesize carbon source efficiently and biotransform it into VOCs (Severo et al., 2020a).

Culture, environmental conditions, and microalgae strains can influence the production of VOCs from microalgae-based systems (Achyuthan et al., 2017; Hosoglu, 2018). Furthermore, the biosynthesis of these VOCs will occur according to the availability of carbon and nutrients, as building blocks, from the primary metabolism. Therefore, the availability of these compounds has a great impact on the composition and concentration of VOCs (Santos et al., 2016a).

Taking this into account, it is recognized that microalgae-based systems synthesize a broad spectrum of volatile organic compounds (VOCs), belong to diverse chemical classes, like alcohol, aldehydes, hydrocarbons, ketones, and terpenes (Durme et al., 2013). Many of these compounds have commercial value, and some research elucidates the biosynthesis and application of microalgae volatiles in different technological domains. But the highlight is the VOCs' application as an alternative bioenergy source (Jacob-Lopes et al., 2020).

25.3 Microalgae gaseous biofuel

Microalgae are well known by your potentiality for biodiesel production (Jacob-Lopes et al., 2018). They also have the robustness to capture greenhouse gases (GEE), especially atmospheric CO_2, and bioconvert in multiple bioproducts (Deprá et al., 2018).

In this context, depending on the production route step chosen, microalgae are a potential bioresource for the production of different types of biofuels, such as biodiesel, bioethanol, syngas, and bio-oil in addition to gaseous fuels such as biohydrogen, biomethane, and, more recently, VOCs (Damergi et al., 2017; Severo et al., 2018; Deprá et al., 2018).

Gaseous fuels, such as biohydrogen and biomethane, can be produced through a microalgae-based system, such as the use of fermentation and anaerobic digestion, respectively. These biofuels are the most efficient in terms of net energy gain among all the biofuel conversion technologies (Deprá et al., 2018; Lin et al., 2019). Biohydrogen is the biofuel with the highest energy content compared to other fuels ($142\,MJ\,kg^{-1}$) and can be used in combustion cells to produce electricity with high efficiency (Bux and Chisti, 2016; Lin et al., 2019).

Volatile organic compounds are considered gaseous bioproducts of the microalgae metabolism; these can be recovered in the form of exhaust gases. VOCs have an energy potential superior to traditional fuels (Table 25.1). Researchers reported that gaseous biofuels may have certain advantages over liquid biofuels: (i) the GHG savings in producing and using gaseous biofuels is greater than that for liquid biofuels; (ii) the pollutant emissions using gaseous biofuels as transport fuels are much less than that using liquid biofuels; and (iii) gaseous biofuels can take advantage of the existing natural gas grid system for low energy input distribution (Lin et al., 2019).

Table 25.1 Volatile organic compounds generated by microalgae and their energy potential.

Chemical name	Species	Energy potential ($MJ\,kg^{-1}$)	Reference
Alcohols			
1-Heptanol	*Phormidium autumnale*, *Scenedesmus obliquus*	4.77	Jacob-Lopes et al. (2020), Nascimento et al. (2020)
1-Hexanol	*Chlorella vulgaris*, *Nannochloropsis oculata*, *Phormidium autumnale*, *Scenedesmus obliquus*, *Tetraselmis chuii*	4.12	Durme et al. (2013), Santos et al. (2016b), Jacob-Lopes et al. (2020), Nascimento et al. (2020)
1-Nonanol	*Phormidium autumnale*	6.07	Jacob-Lopes et al. (2020), Nascimento et al. (2020)
1-Octanol	*Phormidium autumnale*	5.42	Jacob-Lopes et al. (2020), Nascimento et al. (2020)
1-Octen-5-ol	*Phormidium autumnale*	5.18	Jacob-Lopes et al. (2020)
1-Pentanol	*Botryococcus braunii*, *Chlorella vulgaris*, *Nannochloropsis oculata*, *Nitzschia closterium*, *Phormidium autumnale*, *Rhodomonas* sp., *Scenedesmus obliquus*, *Tetraselmis chuii*	3.48	Durme et al. (2013), Zhou et al. (2017), Jacob-Lopes et al. (2020), Nascimento et al. (2020)
1-Undecanol	*Phormidium autumnale*	7.36	Jacob-Lopes et al. (2020)
2-Ethyl-1-hexanol	*Chlorella vulgaris*, *Nannochloropsis oculata*, *Nitzschia closterium*, *Nostoc* sp., *Phormidium autumnale*, *Spirulina platensis*, *Tetraselmis chuii*	5.42	Milovanovic et al. (2015), Zhou et al. (2017), Nascimento et al. (2020)

Continued

Table 25.1 Volatile organic compounds generated by microalgae and their energy potential—cont'd

Chemical name	Species	Energy potential ($MJ\,kg^{-1}$)	Reference
2-Nonanol	*Phormidium autumnale*	6.07	Jacob-Lopes et al. (2020), Nascimento et al. (2020)
2-Octen-1-ol	*Phormidium autumnale*	5.18	Jacob-Lopes et al. (2020)
2-Propil-1-heptanol	*Phormidium autumnale*, *Scenedesmus obliquus*	6.72	Nascimento et al. (2020)
2,4-Decadien-1-ol	*Phormidium autumnale*	6.24	Jacob-Lopes et al. (2020)
4-Decen-1-ol	*Phormidium autumnale*	6.48	Jacob-Lopes et al. (2020)
Aldehydes			
1-Hexanal	*Botryococcus braunii*, *Chlorella vulgaris*, *Nannochloropsis oculata*, *Phormidium autumnale*, *Rhodomonas* sp., *Schizochytrium limacinum*, *Tetraselmis* sp., *Scenedesmus obliquus*	3.88	Durme et al. (2013), Santos et al. (2016b), Hosoglu (2018), Nascimento et al. (2020)
2,4-Decadienal	*Dinobryon divergens*, *Cryptomonas rostratiformis*, *Synura petersenii*, *Phormidium autumnale*	6.00	Jacob-Lopes et al. (2020)
2-Heptenal	*Phormidium autumnale*	4.29	Jacob-Lopes et al. (2020)
2-Methylbutanal	*Phormidium autumnale*, *Scenedesmus obliquus*	3.24	Nascimento et al. (2020)
2-Octenal	*Phormidium autumnale*	4.94	Jacob-Lopes et al. (2020)
Hydrocarbons			
2-Methoxy-2-methyl-propane	*Scenedesmus obliquus*	3.48	Severo et al. (2018)

Table 25.1 Volatile organic compounds generated by microalgae and their energy potential—cont'd

Chemical name	Species	Energy potential ($MJ\,kg^{-1}$)	Reference
3,3-Dimethyl-hexane	*Phormidium autumnale*	5.42	Jacob-Lopes et al. (2020)
4,7-Dimethyl-undecane	*Phormidium autumnale*	8.39	Jacob-Lopes et al. (2020)
Ketones			
2-Heptanone	*Phormidium autumnale*	4.53	Vieira et al. (2019), Jacob-Lopes et al. (2020)
2-Propanone	*Phormidium autumnale*, *Scenedesmus obliquus*	1.94	Severo et al. (2018), Nascimento et al. (2020), Jacob-Lopes et al. (2020)
Terpenes			
6-Methyl-5-hepten-2-one	*Botryococcus braunii*, *Rhodomonas* sp., *Tetraselmis chuii*, *Nannochloropsis* sp., *Phormidium autumnale*	4.94	Durme et al. (2013), Santos et al. (2016b)
β-Ionone	*Botryococcus braunii*, *Nannochloropsis oculata*, *Nostoc* sp., *Phormidium autumnale*, *Rhodomonas* sp., *Scenedesmus obliquus*, *Spirulina platensis*, *Tetraselmis* sp.	7.70	Durme et al. (2013), Milovanovic et al. (2015), Nascimento et al. (2020), Jacob-Lopes et al. (2020)

Although microalgae appear to be promising sources for the production of various known types of bioenergy, they become unattractive in the market due to the high cost of cultivation methods and processing (Severo et al., 2020a). Once the success of microalgae-based systems depends on high biomass productivity (Jacob-Lopes et al., 2020).

Considering the aforementioned fact, productivity is mediated through cultivation systems, which must be configured for an efficient downstream process. Microalgae are usually cultivated under photoautotrophic conditions, using sunlight or artificial light and inorganic carbon for growth. In the case of using an artificial light source, high energy demand makes the process more expensive and complicated (Maroneze et al., 2016).

On the contrary, heterotrophic microalgae-based systems eliminate light dependence, and the heterotrophic route is considered more straightforward to build and less onerous to maintain on a large scale (Soto-Sierra et al., 2018). Furthermore, biomass productivity in heterotrophic cultivation exceeds the yield of the photoautotrophic cultivations (Jia et al., 2014).

These systems can produce VOCs with energy potential, like 2-heptanone that presented the value of $4.53\,MJ\,kg^{-1}$ (Table 25.1) (Vieira et al., 2019). The energy potential of VOCs produced in the heterotrophic bioreactor can range from 3.48×10^{9} to $8.67 \times 10^{9}\,MJ\,kg^{-1}$, a total of energy content of $1.22 \times 10^{13}\,MJ\,kg^{-1}$. Besides, the power generation rate can reach $1.01 \times 10^{12}\,MJ\,m^{-3}\,day^{-1}$ under these cultivation conditions (Jacob-Lopes et al., 2020).

Moreover, considering chemical structures, researchers have referenced that some alcohols showed energy potential comparable to gasoline (Zhang et al., 2008; Santos et al., 2016b; Pinheiro et al., 2019). Halfmann et al. (2014) demonstrated that the terpenic compounds possessed attractive characteristics as biodiesel and jet fuel. Likewise, volatile hydrocarbons provide desirable combustion characteristics (Tong et al., 2014; Jahandideh et al., 2017; Basri et al., 2020). Additionally, aldehydes and ketones can be considered intermediate compounds of valuable alcohols and hydrocarbons (Santos et al., 2016a; Basri et al., 2020).

However, to meet the energy demands, it would be necessary to have a microalgae system that produces these compounds in high volumes, besides the systems downstream for VOCs' recovery efficiency with energy potential (Jacob-Lopes et al., 2020; Severo et al., 2020a).

Therefore, understanding the microalgae culture conditions and its metabolism can provide a piece of better understanding of the production of volatile organic compounds as an alternative renewable energy source if properly separated and collected.

25.4 Microalgae metabolism

Metabolically, microalgae species have three carbon fixation pathways: (i) photoautotrophic, (ii) heterotrophic, or (iii) mixotrophic (Perez-Garcia and Bashan, 2015; Santos et al., 2016a). Of this, the photoautotrophic is the major energetic route of the related microorganisms (Suganya et al., 2016). This mechanism involves uses CO_2 or HCO_3^- dissolved in an aqueous medium (according to pH: CO_2 ($pH < 5$); HCO_3^- ($7 < pH < 9$)) as a carbon source in the presence of light, mainly regulated by photosynthetic carbon metabolism and concentration mechanisms (CCM) (Kong et al., 2021).

In general, microalgae photosynthetic carbon metabolism occurs through the Calvin-Benson-Bassham cycle. Therefore, microalgae use light energy to generate reducing equivalents and fix CO_2 into organic molecules (via the reactions light-dependent and light-independent) (Calvin and Benson, 1948; Su, 2021). The Calvin cycle is composed of 13 steps catalyzed for about 11 different enzymes and subdivided into 3 reactions: (i) carboxylation, (ii) reduction, and (iii) regeneration (Noreña-Caro and Benton, 2018).

Alternatively, in response to the low CO_2 concentration condition to promote photosynthesis, most microalgae have different CO_2 concentrating mechanisms (CCMs) as assimilation of HCO_3^- through active transporters on the plasma membrane; or using extracellular carbonic anhydrase (CA) for enhanced converting of HCO_3^- into intracellular CO_2 (Kong et al., 2021).

On the contrary, some microalgae species also can grow heterotrophically in the absence of light, supported by an exogenous carbon source. In heterotrophic metabolism, the substrate will be converted into glucose 6-phosphate and so can start the route oxidative pentose phosphate pathway. During metabolism, there is the formation of two molecules of ATP (adenosine triphosphate). The final product is also pyruvate (Santos et al., 2016b; Pinheiro et al., 2019).

Additionally, some microalgae species are mixotrophic and can simultaneously drive phototrophy and heterotrophy. This is because the CO_2 is fixed through photosynthesis, while the organic substrates are assimilated through aerobic respiration (Perez-Garcia and Bashan, 2015; Pinheiro et al., 2019).

The bioconversion from different forms of carbon metabolizes pyruvate or acetyl-CoA. Additionally, microalgae biosynthetic and metabolic pathways can convert these substrates into VOCs with desirable combustion properties, like alcohols, aldehydes, hydrocarbons, ketones, and terpenes (Table 25.1). In general, the production of these compounds is achieved through the 2-keto acid pathways, isoprenoid, and the fatty acid derivatives (Fig. 25.1) (Zargar et al., 2017; Severo et al., 2019; Vieira et al., 2020; Jacob-Lopes et al., 2020).

The bioconversion from different forms of inorganic carbon metabolizes pyruvate or acetyl-CoA. Additionally, microalgae biosynthetic and metabolic pathways can convert these substrates into VOCs with desirable combustion properties, like alcohols, aldehydes, hydrocarbons, ketones, and terpenes (Table 25.1). In general, the production of these compounds is achieved through the 2-keto acid pathways, isoprenoid, and the fatty acid derivatives (Fig. 25.1) (Zargar et al., 2017; Vieira et al., 2020; Jacob-Lopes et al., 2020).

The 2-keto acid pathway has been leveraged to produce biofuels like alcohol and alkyl chain intermediates (Siripong et al., 2020). Fundamentally, this pathway comprises converting the corresponding aldehydes from the action of the enzyme 2-ketoacid decarboxylase. Next is the reduction into alcohols catalyzed by alcohol dehydrogenase, and then the reaction is extended to form higher alcohols (Fig. 25.1A). For example, 3-methyl-butanal and 2-methyl-butanal are reduced to 3-methyl-butanol and 2-methyl-butanol, respectively, in the sequence, the recursive elongation process occurs to form 1-hexanol and further higher alcohols (Hasegawa et al., 2012; Liao et al., 2016; Severo et al., 2019; Vieira et al., 2020).

Terpenes are synthesized from the isoprenoid pathway, which has attracted great interest as a biofuel production platform (Zargar et al., 2017). To date, three routes have been reported: (i) the mevalonic acid (MVA), (ii) methylerythritol phosphate (MEP), and (iii) modified MVA. However, for microalgae species, only the MVA/MEP pathways have been described, or both the vias in combination (Lichtenthaler et al., 1997; Chappell, 2003; Pinheiro et al., 2019) (Fig. 25.1B).

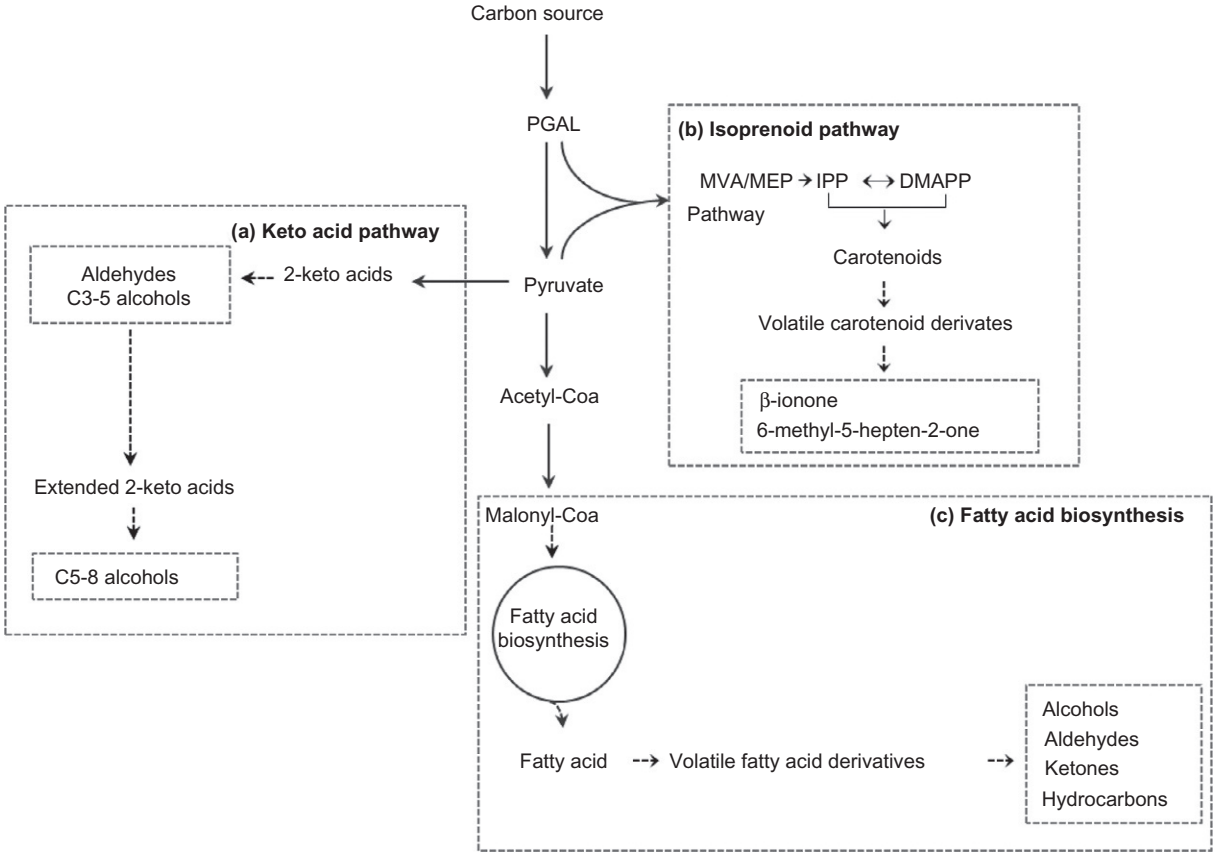

Fig. 25.1 General scheme of the biosynthetic pathways for producing VOCs with desirable combustion properties from microalgae. (A) The keto acid pathway can be used to generate aldehydes and alcohols. (B) The isoprenoid pathway uses glyceraldehyde-3-phosphate (PGAL) and pyruvate to synthesize isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the general precursors of isoprenoid synthesis, which are formed by methylerythritol phosphate (MEP) or mevalonate (MVA) routes. Posteriorly, these carbon precursors are converted into diversified terpenoids. (C) Fatty acid synthesis uses malonyl-CoA as a substrate, where compounds such as aldehydes and ketones can be derived from fatty acids, while hydrocarbons and alcohols are possibly derived from unsaturated aldehydes.

Isopentenyl diphosphate and dimethylallyl diphosphate are the central intermediates from the isoprenoid mechanism. In sequence, these starter structures are transformed into geranyl diphosphate followed by farnesyl diphosphate (Meena et al., 2017; Pinheiro et al., 2019). Posteriorly, these carbon precursors are converted into diversified terpenoids, through a reaction series catalyzed by three distinct enzymes: (i) geranyl diphosphate synthase, (ii) farnesyl diphosphate synthase, and (iii) geranylgeranyl diphosphate synthase, respectively. Finally, carotenoids and their oxidative and enzymatic cleavage products are formed, as β-ionone and 6-methyl-5-hepten-2-one are formed (Dudareva et al., 2013; Santos et al., 2016b).

Fatty acid biosynthesis is the pathway frequently used for the production of biofuels, once; fatty acid degradation generates a spectrum of volatile organic compounds, including alcohols, aldehydes, ketones, and hydrocarbons (Fig. 25.1C) (Gigot et al., 2010; Santos et al., 2016a; Zargar et al., 2017; Lin et al., 2021).

The many structurally diverse ketones are metabolic products of precursor fatty acids (Santos et al., 2016a,b; Pinheiro et al., 2019; Vieira et al., 2020). Recent examples include the production of 2-heptanone from linoleic acid oxidation (Coutron-Gambotti and Gandemer, 1999; Han et al., 2019).

Furthermore, the aldehydes produced by this mechanism are C6 and C9 aldehydes which can be rapidly metabolized to alcohols through the dehydrogenase enzyme (Santos et al., 2016a,b). For example, the fatty acids linoleic and linolenic acid are known to be biosynthetic precursors for 2,4-decadienal, 2-heptanol, 2-octenal, and 1-hexanal, which can subsequently be reduced to alcohols as 1-hexanol (Bravo-Lamas et al., 2018; Jerković et al., 2018; Pinheiro et al., 2019; Vieira et al., 2020).

Similarly, this catabolic metabolism can also form hydrocarbons, including alkanes, which can be the "drop-in" biofuels and contribute to decreasing the percentage of the greenhouse gas emissions (Basri et al., 2020). This fatty acid-to-hydrocarbon conversion proceeds using aldehydes as substrates. At least two enzymes, acyl-acyl carrier protein reductase and aldehyde deformylating oxygenase, are responsible for catalyzing the reaction (Pinheiro et al., 2019; Vieira et al., 2020; Basri et al., 2020).

Finally, in order to properly align the global environmental concerns and the metabolic versatility of these microorganisms, a more detailed understanding of potential biosynthetic pathways is necessary in order to identify VOCs with desirable combustion properties, to assist in the sustainable supply of renewable energy (Basri et al., 2020).

25.5 Final considerations

Renewable sustainable sources of biofuels are necessary to decrease the environmental burden created by the extensive use of fossil fuels. Among the different types of biofuels, advantages and disadvantages are reported; however, together they have begun to decrease the burden of global fossil fuel consumption. Due to the drawbacks associated with first- and second-generation biofuels, third- and fourth-generation biofuels have been developed (Dutta et al., 2014). Yet, microalgal-based fuels

technologies have many bottlenecks, many of which are still far from being eliminated to become viable (Severo et al., 2020b).

Fourth-generation biofuel is the term used for the production of "drop-in" biofuels directly from microalgae (Chernova et al., 2010). The benefit of using drop-in biofuels is that they can be mixed with crude derivatives without the need to develop new fuel infrastructures (Tong et al., 2014; Singh et al., 2019). With the development of fourth-generation biofuel technologies, advanced biofuels can now be produced from microalgal biomass, as well as, be recovered from the headspace of the microalgae-based systems, in the form of VOCs (Tong et al., 2014; Jacob-Lopes et al., 2020).

Considering these aspects, the VOCs generated from microalgae-based systems have been reported as promising heat sources to be integrated into a biocombustion process (Jacob-Lopes et al., 2018; Severo et al., 2020a). Among these VOCs are hydrocarbons, alcohols, ketones, and terpenes can provide the required performance characteristics when added to petroleum fuels derived (Tong et al., 2014; Jahandideh et al., 2017).

However, the successful commercial production of biofuels from VOCs obtained biotechnologically requires economic profitability. Although the biosynthesis of VOCs from microalgae-based systems has limitations by low productivity or insufficient concentrations of main compounds in the cultivation system. Therefore, in order to high yields and productivity, it is important to choose a bioreactor design and a convenient system for the recovery of volatile compounds (Pinheiro et al., 2019).

Associated with this, different techniques are exploited for the separation and recovery of VOCs from the microalgae biomass. Traditional methods most used for volatile compound extraction, include adsorption, condensation, absorption, and membrane-based techniques (Saffarionpour and Ottens, 2018). These techniques may assist microalgae-based processes when it is desired to obtain a compound or a group thereof separately (Vieira et al., 2020). Although these methods are well-established, they show many disadvantages, i.e., the loss of volatile compounds and high energy consumption (Negro et al., 2016).

Finally, from an economic standpoint, VOCs' recovery from the headspace of the photobioreactors may be preferred (Kiyota et al., 2014). However, the gas stream exiting the systems would be very dilute and present a challenge for volatile compounds recovery by simple condensation (Pinheiro et al., 2019; Severo et al., 2019; Vieira et al., 2020).

In this sense, the research evaluates new configurations for the separation and recovery systems of VOCs, as is the case Jahandideh et al. (2017), that coupled the photobioreactor output a column packed with activated carbon to absorb the volatile compound. Thus, after saturation, the adsorption column is heated and regenerates the activated carbon, and releases the compound which is then condensed via cold water. Farther, the use of microwave-assisted extraction could also be used to regenerate the activated carbon and contribute to reducing downstream expenses for VOCs' recovery (Coss and Cha, 2000).

25.6 Conclusion

The microalgae can produce a variety of volatile compounds with important energetic characteristics when subjected to different carbon forms that enable exploitation for many relevant commercial applications. Associated with this, it is crucial to know first and foremost the volatile characterization of microalgae, culture systems, and VOCs' biosynthesis. However, some challenges must be overcome for these bioprocesses to be successful in the market on a large scale, including improving genetic engineering strategies to potentialize VOCs' production, appropriate microalgae strains, and optimized cultivation system. Besides, volatile compounds' recovery technologies must be designed to follow the photobioreactor configuration, as well as the volatility of the target molecule and its partitioning between the phases under real production conditions.

References

Achyuthan, K.E., Harper, J.C., Manginell, R.P., Moorman, M.W., 2017. Volatile metabolites emission by *in vivo* microalgae—an overlooked opportunity? Meta 7 (3). https://doi.org/10.3390/metabo7030039.

Basri, R.S., Zaliha, R.N., Rahman, R.A., Kamarudin, N.H.A., Ali, M.S.M., 2020. Cyanobacterial aldehyde deformylating oxygenase: structure, function, and potential in biofuels production. Int. J. Biol. Macromol. 164, 3155–3162. https://doi.org/10.1016/j.ijbiomac.2020.08.162.

Bravo-Lamas, L., Barron, L.J., Farmer, L., Aldai, N., 2018. Fatty acid composition of intramuscular fat and odour-active compounds of lamb commercialized in northern chromatography with mass spectrometry. J. Chromatogr. A 1350, 92–101. https://doi.org/10.1016/j.meatsci.2018.02.006.

Bux, F., Chisti, Y., 2016. Algae Biotechnology-Products and Processes, first ed. Springer International Publishing, p. 344, https://doi.org/10.1007/978-3-319-12334-9.

Calvin, M., Benson, A.A., 1948. The path of carbon in photosynthesis. Science 107, 476–480. https://doi.org/10.1126/science.107.2784.476.

Chappell, J., 2003. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu. Rev. Plant Physiol. 46 (1), 521–547. https://doi.org/10.1146/annurev.pp.46.060195.002513.

Chernova, N.I., Korobkova, T.P., Kiseleva, S.V., 2010. Use of biomass for producing liquid fuel: current state and innovations. Appl. Therm. Eng. 57 (11), 937–945. https://doi.org/10.1134/S0040601510110054.

Coss, P.M., Cha, C.Y., 2000. Microwave regeneration of activated carbon used for removal of solvents from vented air. J. Air Waste Manag. Assoc. 50 (4), 529–535. https://doi.org/10.1080/10473289.2000.10464038.

Coutron-Gambotti, C., Gandemer, G., 1999. Lipolysis and oxidation in subcutaneous adipose tissue during dry-cured ham processing. Food Chem. 64 (1), 95–101. https://doi.org/10.1016/S0308-8146(98)00079-X.

Damergi, E., Schwitzguébel, J.-P., Refardt, D., Sharma, S., Holliger, C., Ludwig, C., 2017. Extraction of carotenoids from *Chlorella vulgaris* using green solvents and syngas production from residual biomass. Algal Res. 25, 488–495. https://doi.org/10.1016/j.algal.2017.05.003.

Deprá, M.C., Santos, A.M., Severo, I.A., Santos, A.B., Zepka, L.Q., Jacob-Lopes, E., 2018. Microalgal biorefineries for bioenergy production: can we move from concept to industrial reality? BioEnergy Res., 1–21. https://doi.org/10.1007/s12155-018-9934-z.

Dudareva, N., Klempien, A., Muhlemann, J.K., Kaplan, I., 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. 198, 16–32. https://doi.org/10.1111/nph.12145.

Durme, J.V., Goiris, K., De Winne, A., De Cooman, L., Muylaert, K., 2013. Evaluation of the volatile composition and sensory properties of five species of microalgae. J. Agric. Food Chem. 61 (46), 10881–10890. https://doi.org/10.1021/jf403112k.

Dutta, K., Daverey, A., Lin, J.G., 2014. Evolution retrospective for alternative fuels: first to fourth generation. Renew. Energy 69, 114–122. https://doi.org/10.1016/j.renene.2014.02.044.

Gigot, C., Ongena, M., Fauconnier, M.L., Wathelet, J.P., Jardin, P.D., Thonart, P., 2010. The lipoxygenase metabolic pathway in plants: potential for industrial production of natural green leaf volatiles. Biotechnol. Agron. Soc. Environ. 14, 451–460.

Halfmann, C., Gu, L., Zhou, R., 2014. Engineering cyanobacteria for the production of a cyclic hydrocarbon fuel from CO_2 and H_2O. Green Chem. 16 (6), 3175–3185. https://doi.org/10.1039/c3gc42591f.

Han, G., Zhang, L., Li, Q., Wang, Y., Chen, Q., Kong, B., 2019. Impacts of different altitudes and natural drying times on lipolysis, lipid oxidation and flavour profile of traditional Tibetan yak jerky. Meat Sci. 162, 108030. https://doi.org/10.1016/j.meatsci.2019.108030.

Hasegawa, M., Nishizawa, A., Tsuji, K., Kimura, S., Harada, K., 2012. Volatile organic compounds derived from 2-keto-acid decarboxylase in *Microcystis aeruginosa*. Microbes Environ. 27 (4), 525–528. https://doi.org/10.1264/jsme2.me12099.

Hosoglu, M., 2018. Aroma characterization of five microalgae species using solid-phase microextraction and gas chromatography-mass spectrometry/olfactometry. Food Chem. 240, 1210–1218. https://doi.org/10.1016/j.foodchem.2017.08.052.

Jacob-Lopes, E., Zekpa, L.Q., Queiroz, M.I., 2018. Energy from microalgae. In: Green Energy and Technology. http://link.springer.com/10.1007/978-3-319-69093-3.

Jacob-Lopes, E., Maroneze, M.M., Deprá, M.C., Sartori, R.B., Dias, R.R., Zepka, L.Q., 2019. Bioactive food compounds from microalgae: an innovative framework on industrial biorefineries. Food Sci. 25, 1–7. https://doi.org/10.1016/j.cofs.2018.12.003.

Jacob-Lopes, E., Santos, A.B., Severo, I.A., Deprá, M.C., Maroneze, M.M., Zepka, L.Q., 2020. Dual production of bioenergy in heterotrophic cultures of cyanobacteria: process performance, carbon balance, biofuel quality and sustainability metrics. Biomass Bioenergy 142. https://doi.org/10.1016/j.biombioe.2020.105756.

Jahandideh, A., Johnson, T.J., Esmaeili, N., Johnson, M.D., Richardson, J.W., Muthukumarappan, K., Anderson, G.A., Halfmann, C., Zhou, R., Gibbons, W.R., 2017. Life cycle analysis of a large-scale limonene production facility utilizing filamentous N_2-fixing cyanobacteria. Algal Res. 23, 1–11. https://doi.org/10.1016/j.algal.2017.01.001.

Jerković, I., Marijanović, Z., Roje, M., Kús, P.M., Jokić, S., Čož-Rakovac, R., 2018. Phytochemical study of the headspace volatile organic compounds of fresh algae and seagrass from the *Adriatic Sea* (single point collection). PLoS One 13 (5), e0196462.

Jia, Z., Liu, Y., Daroch, M., Geng, S., Cheng, J.J., 2014. Screening, growth medium optimisation and heterotrophic cultivation of microalgae for biodiesel production. Appl. Biotechnol. Biochem. 173 (7), 1667–1679. https://doi.org/10.1007/s12010-014-0954-7.

Kiyota, H., Okuda, Y., Ito, M., Hirai, M.Y., Ikeuchi, M., 2014. Engineering of cyanobacteria for the photosynthetic production of limonene from CO_2. J. Biotechnol. 185, 1–7. https://doi.org/10.1016/j.jbiotec.2014.05.025.

Kong, W., Shen, B., Lyu, H., Kong, J., Ma, J., Wang, Z., Feng, S., 2021. Review on carbon dioxide fixation coupled with nutrients removal from wastewater by microalgae. J. Clean. Prod. 292, 125975. https://doi.org/10.1016/j.jclepro.2021.125975.

Liao, J.C., Mi, L., Pontrelli, S., Luo, S., 2016. Fuelling the future: microbial engineering for the production of sustainable biofuels. Nat. Rev. Microbiol. 14 (5), 288–304. https://doi.org/10.1038/nrmicro.2016.32.

Lichtenthaler, H.K., Disch, A., Rohmer, M., 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett. 400 (3), 271–274. https://doi.org/10.1016/S0014-5793(96)01404-4.

Lin, R., Deng, C., Ding, L., Bose, A., Murphy, J.D., 2019. Improving gaseous biofuel production from seaweed *Saccharina latissima*: the effect of hydrothermal pretreatment on energy efficiency. Energy Convers. Manag. 196, 1385–1394. https://doi.org/10.1016/j.enconman.2019.06.044.

Lin, R., Deng, C., Zhang, W., Hollmann, F., Murphy, J.D., 2021. Production of bio-alkanes from biomass and CO_2. Trends Biotechnol. https://doi.org/10.1016/j.tibtech.2020.12.004. 2030.

Maroneze, M.M., Siqueira, S.F., Vendruscolo, R.G., Wagner, R., de Menezes, C.R., Zepka, L. Q., Jacob-Lopes, E., 2016. The role of photoperiods on photobioreactors—a potential strategy to reduce costs. Bioresour. Technol. 219, 493–499. https://doi.org/10.1016/j.biortech.2016.08.003.

Meena, S., Kumar, S.R., Dwivedi, V., Kumar Singh, A.K., Chanotiya, C.S., Akhtar, Q., Kumar, K., Shasany, A.K., Nagegowda, D.A., 2017. Transcriptomic insight into terpenoid and carbazole alkaloid biosynthesis, and functional characterization of two terpene synthases in curry tree (*Murraya koenigii*). Sci. Rep. 7, 44126. https://doi.org/10.1038/srep44126.

Milovanovic, I., Mišan, A., Simeunovic, J., Kova, D., Dubravka, J.D., Anamarija, M.A., 2015. Determination of volatile organic compounds in selected strains of cyanobacteria. J. Chem. 2015, 1–6. https://doi.org/10.1155/2015/969542.

Nascimento, T.C., Nass, P.P., Fernandes, A.S., Vieira, K.R., Wagner, R., Jacob-Lopes, E., Zepka, L.Q., 2020. Exploratory data of the microalgae compounds for food purposes. Data Brief 29, 105182. https://doi.org/10.1016/j.dib.2020.105182.

Negro, V., Mancini, G., Ruggeri, B., Fino, D., 2016. Citrus waste as feedstock for bio-based products recovery: review on limonene case study and energy valorization. Bioresour. Technol. 214, 806–815. https://doi.org/10.1016/j.biortech.2016.05.006.

Noreña-Caro, D., Benton, M.G., 2018. Cyanobacteria as photoautotrophic biofactories of high-value chemicals. J. CO_2 Util. 28, 335–366. https://doi.org/10.1016/j.jcou.2018.10.008.

Perez-Garcia, O., Bashan, Y., 2015. Microalgal heterotrophic and mixotrophic culturing for biorefining: from metabolic routes to techno-economics. In: Prokop, A., Bajpai, R., Zappi, M. (Eds.), Algal Biorefineries. Springer, Cham, pp. 61–131, https://doi.org/10.1007/978-3-319-20200-6_3.

Pinheiro, P.N., Vieira, K.R., Santos, A.B., Jacob-Lopes, E., Zepka, L.Q., 2019. Biogeneration of volatile organic compounds in microalgae-based systems. In: Ravishankar, G.A., Rao, A.R. (Eds.), Handbook of Algal Technologies and Phytochemicals: Food, Health and Nutraceutical Applications. CRC Press, New York, pp. 89–98.

Saffarionpour, S., Ottens, M., 2018. Recent advances in techniques for flavor recovery in liquid food processing. Food Eng. Rev. 10 (2), 81–94. https://doi.org/10.1007/s12393-017-9172-8.

Santos, A.B., Fernandes, A.S., Wagner, R., Jacob-Lopes, E., Zepka, L.Q., 2016a. Biogeneration of volatile organic compounds produced by *Phormidium autumnale* in heterotrophic bioreactor. J. Appl. Phycol. 28 (3), 1561–1570. https://doi.org/10.1007/s10811-015-0740-0.

Santos, A.B., Vieira, K.R., Nogara, G.P., Wagner, R., Jacob-Lopes, E., Zepka, L.Q., 2016b. Biogeneration of volatile organic compounds by microalgae/cyanobacteria: occurrence, behavior, ecological implications and industrial applications. In: Moore, J.P. (Ed.), Volatile Organic Compounds: Occurrence, Behavior and Ecological Implications. Nova Science Publishers, New York, pp. 1–18.

Severo, I.A., Deprá, M.C., Barin, J.S., Wagner, R., Zepka, L.Q., Jacob-Lopes, E., 2018. Biocombustion of petroleum coke: the process integration with photobioreactors. Chem. Eng. Sci. 177, 422–430. https://doi.org/10.1016/j.ces.2017.12.001.

Severo, I.A., Pinheiro, P.N., Vieira, K.R., Zepka, L.Q., Jacob-Lopes, E., 2019. Biological conversion of carbon dioxide into volatile organic compounds. In: Inamuddin, et al. (Eds.), Conversion of Carbon Dioxide Into Hydrocarbons. vol. 2. Springer, Cham, pp. 45–73, https://doi.org/10.1007/978-3-030-28638-5_2.

Severo, I.A., Deprá, M.C., Dias, R.R., Barin, J.S., de Menezes, C.R., Wagner, R., Zepka, L.Q., Jacob-Lopes, E., 2020a. Bio-combustion of petroleum coke: the process integration with photobioreactors. Part II—sustainability metrics and bioeconomy. Chem. Eng. Sci. 213. https://doi.org/10.1016/j.ces.2019.115412.

Severo, I.A., Deprá, M.C., Dias, R.R., Jacob-Lopes, E., 2020b. Process integration applied to microalgae-based systems. In: Handbook of Microalgae-Based Processes and Products, pp. 709–735, https://doi.org/10.1016/b978-0-12-818536-0.00026-9.

Singh, D., Sharma, D., Soni, S.L., Sharma, S., Kumari, D., 2019. Chemical compositions, properties, and standards for different generation biodiesels: a review. Fuel 253, 60–71. https://doi.org/10.1016/j.fuel.2019.04.174.

Siripong, W., Angela, C., Tanapongpipat, S., Runguphan, W., 2020. Metabolic engineering of *Pichia pastoris* for production of isopentanol (3-methyl-1-butanol). Enzym. Microb. Technol. 138, 109557. https://doi.org/10.1016/j.enzmictec.2020.109557.

Soto-Sierra, L., Stoykova, P., Nikolov, Z.L., 2018. Extraction and fractionation of microalgae-based protein products. Algal Res. 36, 175–192. https://doi.org/10.1016/j.algal.2018.10.023.

Su, Y., 2021. Revisiting carbon, nitrogen, and phosphorus metabolisms in microalgae for wastewater treatment. Sci. Total Environ. 762, 144590. https://doi.org/10.1016/j.scitotenv.2020.144590.

Suganya, T., Varman, M., Masjuki, H.H., Renganathan, S., 2016. Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: a biorefinery approach. Renew. Sustain. Energy Rev. 55, 909–941. https://doi.org/10.1016/j.rser.2015.11.026.

Tong, K., Gleeson, M.J., Rong, G., You, F., 2014. Optimal design of advanced drop-in hydrocarbon biofuel supply chain integrating with existing petroleum refineries under uncertainty. Biomass Bioenergy 60, 108–120. https://doi.org/10.1016/j.biombioe.2013.10.023.

Vieira, K.R., Pinheiro, P.N., Santos, A.B., Cichoski, A.J., Menezes, C.R., Wagner, R., Zepka, L. Q., Jacob-Lopes, E., 2019. The role of microalgae-based systems in the dynamics of odors compounds in the meat processing industry. Desalin. Water Treat. 150, 282–292. https://doi.org/10.5004/dwt.2019.23730.

Vieira, K.R., Pinheiro, P.N., Zepka, L.Q., 2020. Volatile organic compounds from microalgae. In: Handbook of Microalgae-Based Processes and Products. Elsevier Inc., https://doi.org/10.1016/b978-0-12-818536-0.00024-5.

Zargar, A., Bailey, C.B., Haushalter, R.W., Eiben, C.B., Katz, L., Keasling, J.D., 2017. Leveraging microbial biosynthetic pathways for the generation of 'drop-in' biofuels. Curr. Opin. Biotechnol. 45, 156–163. https://doi.org/10.1016/j.copbio.2017.03.004.

Zhang, K., Sawaya, M.R., Eisenberg, D.S., Liao, J.C., 2008. Expanding metabolism for biosynthesis of nonnatural alcohols. Proc. Natl. Acad. Sci. U. S. A. 105 (52), 20653–20658. https://doi.org/10.1073/pnas.0807157106.

Zhou, L., Chen, J., Xu, J., Li, Y., Zhou, C., Yan, X., 2017. Change of volatile components in six microalgae with different growth phases. J. Sci. Food Agric. 97 (3), 761–769. https://doi.org/10.1002/jsfa.7794.