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Pricila Nass Pinheiro

ESTUDO DA BIOACESSIBILIDADE *IN VITRO* DE CAROTENOIDES MICROALGAIS

Santa Maria, RS
2020

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

Orientadora: Profª Dra. Leila Queiroz Zepka

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Aprovado em 13 de março de 2020:

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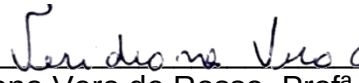

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RESUMO

ESTUDO DA BIOACESSIBILIDADE *IN VITRO* DE CAROTENOIDES MICROALGAIS

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ORIENTADORA: Leila Queiroz Zepka

A biomassa microalgal é uma fonte comprovada de compostos bioativos com destaque para os carotenoides. Entretanto a uma grande preocupação na influência da matriz microalgal na bioacessibilidade dessas estruturas bioativas. Nesse sentido, este estudo teve como objetivo investigar a bioacessibilidade de carotenoides de três produtos diferentes: biomassa total seca (WDB), biomassa ultrassônica úmida (WUB) e o extrato de carotenoide isolado (ICE) de duas espécies comercias de microalgas *Chlorella vulgaris* e *Spirulina* sp. As amostras foram submetidas ao modelo de digestão *in vitro* de acordo com o protocolo INFOGEST. Os carotenoides foram determinados por HPLC-PDA-MS/MS. Um total de vinte e dois carotenoides diferentes foram separados nos extratos controle, sendo os principais all-*trans*-luteína (48,1%) para *C. vulgaris* e all-*trans*-β-caroteno (29,3%) para *Spirulina*. Após a digestão *in vitro*, para *C. vulgaris*, seis compostos eram bioacessíveis no WDB, nove no WUB e no ICE. O 5,6:5',6'-diepoxy-β-caroteno foi o carotenoide mais bioacessível em todos os produtos (WDB 18,3%; WUB 22,6%; e ICE 29,8%). Por outro lado, para a espécie *Spirulina* seis carotenoides eram bioacessíveis no WDB, dez no WUB e no ICE. A all-*trans*-β-criptoxantina apresentou a maior bioacessibilidade (46,8%) no WDB, enquanto a all-*trans*-cantaxanthina em WUB (99,5%) e ICE (95,5%). Por fim, os resultados mostraram a influência significativa da natureza do produto na promoção da bioacessibilidade de carotenoides microalgais, uma vez que bioacessibilidade total melhorou para ambas as espécies de acordo com o tipo de produto (ICE> WUB> WDB). Desta forma, os dados sugerem que a bioacessibilidade dos carotenoides do ICE, é maior do que no WDB e WUB. Portanto, o ICE deve ser considerado um produto que fornece carotenoides biodisponíveis e pode ser a melhor escolha, como ingrediente no desenvolvimento de alimentos funcionais à base de carotenoides.

Palavras-chave: Carotenoides. *Chlorella vulgaris. Spirulina*. Digestão *in vitro*. Produtos a base de microalgas.

ABSTRACT

STUDY OF *IN VITRO* BIOACCESSIBILITY FROM MICROALGAL CAROTENOIDS

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Microalgal biomass is a proven source of bioactive compounds with an emphasis on carotenoids. However, there is great concern about the influence of the microalgal matrix on the bioactive structures bioaccessibility. In this sense, this study aimed to investigate the carotenoids bioaccessibility from three different products: whole dried biomass (WDB), wet ultrasonicated biomass (WUB); and isolated carotenoids extract (ICE) from two commercial microalgae species *Chlorella vulgaris* and *Spirulina* sp. The samples were submitted to *in vitro* digestion model according to the INFOGEST protocol. Carotenoids were determined by HPLC-PDA-MS/MS. A total of twenty-two different carotenoids were separated in the control extracts, the major ones being all-*trans*-lutein (48.1%) for *C. vulgaris* and all-*trans*-β-carotene (29.3%) in the *Spirulina*. After *in vitro* digestion, for *C. vulgaris*, six compounds were bioaccessible in WDB, nine in WUB and ICE. The 5,6:5',6'-diepoxy-β-carotene was the most bioaccessible carotenoid in all products (WDB 18.3%; WUB 22.6%; and ICE 29.8%). On the other hand, for the *Spirulina* species, six carotenoids were bioaccessible in WDB, ten in WUB and ICE. All-*trans*-β-cryptoxanthin showed the highest bioaccessibility (46.8%) in WDB, while all-*trans*-cantaxanthin in WUB (99.5%) and ICE (95.5%). Finally, the results showed the significant influence of the nature of the product in promoting the bioaccessibility of microalgal carotenoids, since total bioaccessibility improved for both species according to the type of product (ICE>WUB>WDB). Thus, the data suggest that the bioaccessibility of ICE carotenoids is greater than in WDB and WUB. Therefore, ICE should be considered a product that provides bioavailable carotenoids and could be the best choice, such as ingredients in the development of functional foods carotenoids-based.

Keywords: Carotenoids. *Chlorella vulgaris*. *Spirulina*. *In vitro* digestion. Microalgae based-products.

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APCI	Ionização química por pressão atmosférica
Caco-2	Células de adenocarcinoma do epitélico colorretal humano
CID	Dissociação induzida por colisão
CO_2	Dióxido de carbono
DMEM	Meio de cultura Dulbecco's Eagle's modificado
FBS	Soro fetal bovino
HPLC	Cromatografia líquida de alta eficiência
LC	Cromatografia liquida
MeOH	Metanol
MS/MS	Espectrometria de Massas em Tandem
MTBE	Éter metil terc-butílico
N_2	Gás nitrogênio
PBS	Salina tamponada com fosfato
PDA	Detector de arranjo de diodos
UFSM	Universidade Federal de Santa Maria
UNIFESP	Universidade Federal de São Paulo
UV/vis	Ultravioleta-visivel

SUMÁRIO

INTRODUÇÃO

Nas últimas décadas, as mudanças em hábitos alimentares promoveram alterações consideráveis na formulação dos alimentos, direcionando as tendências de consumo para produtos naturais com propriedades funcionais. Nesse contexto, as microalgas surgiram como uma alternativa para obtenção de insumos naturais (PARODI et al., 2018; NICCOLAI et al., 2019a).

Desta forma no mercado global, o maior campo de aplicação das microalgas está no setor de alimentos, onde a biomassa representa um produto promitente, seu valor de mercado é US$ 5,7 bilhões, sendo comercializada principalmente na forma de biomassa seca total (WDB), mas também biomassa úmida (WUB) ou como extratos isolados (ICE) (JACOB-LOPES et al., 2019; GLOBAL INFO RESEARCH, 2020; NASCIMENTO et al., 2021).

A indústria de alimentos se concentra principalmente na adição de *Chlorella vulgaris* e *Spirulina* como suplemento nutricional, sendo promovido a "superalimento" ou "rico em compostos bioativos" (LAFARGA, 2019). Isso é apoiado fundamentalmente pelo fato de que a biomassa microalgal se tornou uma fonte promissora para a obtenção de carotenoides naturais, como β-caroteno, zeaxantina, luteína, além de outros antioxidantes potentes, como equinenona e cantaxantina produzidos exclusivamente por microalgas (PATIAS et al., 2017; NASCIMENTO et al., 2019).

De fato, carotenoides, são abundantes na biomassa microalgal, porém o conteúdo desses compostos não serve como indicativo da quantidade que está bioacessível para a absorção intestinal e posterior atividade bioativa nos tecidos alvos. A grande preocupação é o efeito da matriz microalgal na bioacessibilidade desses compostos, uma vez que os carotenoides devem ser liberados da matriz e transferidos para a fração micelar estando acessíveis para absorção (FERRUZZI; FAILLA; SCHWARTZ, 2015; PATIAS et al., 2017; XAVIER; MERCADANTE, 2019; NASCIMENTO et al., 2021).

Diante do exposto, este estudo teve como objetivo avaliar a bioacessibilidade dos carotenoides das microalgas *Chlorella vulgais* e *Spirulina* sp. a partir da biomassa total seca, da biomassa ultrassônica úmida e do extrato de carotenoide isolado.

OBJETIVOS

Objetivo geral

Elucidar a bioacessibilidade do perfil de carotenoides da biomassa de *Chlorella vulgaris* e *Spirulina* sp., visando ampliar o conhecimento sobre o efeito da matriz na disponibilidade *in vitro* dessas biomoléculas em microalgas.

Objetivos específicos

Cultivar *Chlorella vulgaris* (CPCC 90) e *Spirulina* sp. (CPCC 695) em fotobiorreatores para obtenção da biomassa.

Determinar o perfil qualitativo de carotenoides nas espécies de microalgas por HPLC-PDA-MS/MS.

Determinar o perfil quantitativo de carotenoides nas espécies de microalgas por HPLC-PDA.

Determinar a bioacessibilidade de carotenoides de três produtos diferentes: biomassa total seca (WDB), biomassa ultrassônica úmida (WUB) e do extrato de carotenoide isolado (ICE) das duas espécies de microalgas *Chlorella vulgaris* e *Spirulina* sp.

CAPÍTULO 1
REVISÃO BIBLIOGRÁFICA

1. Microalgas

Microalgas é uma terminologia comercial sem valor taxonômico, uma vez que essas espécies não são monofiléticas e atualmente são catalogadas em quatro reinos: *Monera*, *Plantae*, *Chromista* e *Protozoa*. Do ponto de vista morfológico, a estrutura celular das microalgas é dividida em procarionte e eucarionte. Organismos procariontes são as *Cyanophyta*, já os eucariontes incluem, por exemplo, *Chlorophyta*, *Glaucophyta*, *Ochrophyta, Euglenophyta*, *Haptophyta*, *Cryptophyta.* Em termos de exploração biotecnológica os filos: *Chlorophyta*, *Cyanophyta* e *Ochrophyta* se destacam (MATA; MARTINS; CAETANO, 2010; MUTANDA et al., 2011; SUGANYA et al., 2016; BOROWITZKA, 2018).

As instalações comerciais para produção de microalgas estão espalhadas por todo o mundo, os principais produtores de biomassa microalgal e seus bioprodutos, são Taiwan, Japão, Estados Unidos, China, Brasil, Espanha, Israel, Alemanha e Mianmar com uma produção anual de biomassa de aproximadamente 19.000 toneladas, gerando um valor estimado de 5,7 bilhões de dólares (RAMIREZ-MERIDA; ZEPKA; JACOB-LOPES, 2017; JACOB-LOPES et al., 2019).

Chlorella vulgaris é uma *Chlorophyta*, eucarionte, esférica, unicelular com um diâmetro de 2-10 µm, sua a parede celular pode atingir a espessura de 17-21 nm com uma camada microfibrilar, muito semelhante à quitosana composta por glucosamina, o que explica sua rigidez. Quanto a exploração biotecnológica apresenta rápido crescimento, um conteúdo substancial de carotenoides e um desempenho robusto em biorreatores (FRANCISCO et al., 2010; PŘIBYL et al., 2015, 2016; MARONEZE et al., 2016; ZOU et al., 2016).

Em contrapartida, *Spirulina* sp. pertence ao filo *Cyanophyta*, um microrganismo procarionte, seus filamentos são compostos por tricomas cilíndricos multicelulares, regularmente espiralada, com diâmetro de 20 a 45 µm, largura do filamento de 6 a 14 µm e comprimento total de 400 a 900 µm. Em termos biotecnológicos essa espécie é conhecida por viver em ambientes extremos, por isso possui um amplo potencial para uso em bioprocessos, devido à sua robustez e exigências nutricionais simples. Outra característica tecnológica refere-se a sua parede celular que é composta por polissacarídeos

com digestibilidade de 86% e pode ser facilmente absorvida pelo corpo humano (RICHMOND et al., 1990; YUAN et al., 2018).

A diversidade evolutiva e filogenética dessas espécies significa uma grande variabilidade na composição química, no qual, podem sintetizar estruturas químicas únicas, a maioria delas com importantes atividades biológicas, o que as torna extremamente atraentes para exploração biotecnológica como fontes comerciais de uma vasta gama de biomoléculas (HERRERO; IBÁÑEZ, 2015; RODRIGUES et al., 2015; BAJHAIYA; ZIEHE MOREIRA; PITTMAN, 2017).

2. Carotenoides

A principal função dos carotenoides nas microalgas é participar da captação da luz para a fotossíntese, os carotenoides também atuam como fotoprotetores quando as células das algas são expostas a altas irradiações, uma vez que carotenoides são antioxidantes, eles também protegem a célula da oxidação (EGELAND, 2016).

São conhecidos aproximadamente 1.183 carotenoides naturais carotenoides naturais (YABUZAKI, 2020), destes, cerca de 200 são encontrados em microalgas (EGELAND, 2011). A diversidade estrutural e os padrões de distribuição de pigmentos em espécies de microalgas são tipicamente específicos, facilitando sua aplicação como biomarcadores quimiotaxonômicos (JEFFREY; WRIGHT; ZAPATA, 2011; MC GEE et al., 2018).

As reconstruções filogenéticas atuais da evolução dos plastídios propõem que membros da linhagem das *Chlorophyta* apresentam luteína como carotenoide principal, juntamente *α*-caroteno, violaxantina, e neoxantina, sendo que o último está presente apenas nesse filo de microalgas. Os membros da linhagem das *Cyanophyta*, possuem pigmentos característicos como mixoxantofila, equinenona e cantaxantina, sendo apenas *β*-caroteno relatado em todas as espécies, frequentemente como um dos principais carotenoides (HERTZBERG; LIAAEN-JENSEN; SIEGELMAN, 1971; GOODWIN, 1980; BJØRNLAND, 1982; LIAAEN-JENSEN; EGELAND, 1999; JEFFREY; WRIGHT; ZAPATA, 2011; PALINSKA et al., 2011; TAKAICHI, 2011; MC GEE et al., 2018).

Dada a sua natureza e diversificada, a integração da produção de biomassa de microalgas com o passo de obtenção de pigmentos, estabelece uma alternativa promissora para consolidar a indústria de produtos naturais, essa afirmação baseia-se no fato de que a produção de carotenoides tendo microalgas como matéria prima se tornou um modelo de sucesso na indústria de biotecnologia. O *β*-caroteno, astaxantina e luteína apresentam as maiores participações de mercado, as projeções demonstram que no ano de 2022 a astaxantina venha atingir o valor de US$ 426,9 milhões, o *β*-caroteno US$ 572,78 milhões e a luteína US$ 357,7 milhões (MCWILLIAMS, 2018; AMBATI et al., 2019).

Além disso, a estrutura dos carotenoides determina suas ações e propriedades biológicas assim como o seu destino no trato gastrointestinal. Em outras palavras, a determinação do perfil de carotenoides é o primeiro passo para entender o comportamento destes compostos em qualquer sistema, seja ele biológico ou não (RODRIGUEZ-AMAYA, 2019).

O cromóforo poliênico conjugado apresentado na molécula de carotenoide está relacionado com potencial antioxidante (MERCADANTE, 2008), podendo ser influenciada pelo número de ligações duplas conjugadas isso sugere-se uma maior atividade antioxidante para carotenoides microalgais em relação a fontes convencionais, devido à presença de carotenoides exclusivos, os quais apresentam efeito bato crômico, como é o caso da equinenona e cantaxantina com cromóforo de 12 e 13 ligações duplas conjugadas respectivamente (NASCIMENTO et al., 2019).

A ação antioxidante dos carotenoides pode diminuir a peroxidação lipídica e, eventualmente, reduzir o estresse oxidativo consequentemente minimizar as respostas inflamatórias nas células e nos tecidos, causados pelo excesso de espécies reativas de oxigênio e nitrogênio (CHISTÉ et al., 2011; RODRIGUES et al., 2012a, 2012b; RODRIGUES; MARIUTTI; MERCADANTE, 2012).

Outra propriedade atribuída as ligações duplas conjugadas é o sistema de filtro da luz azul. Os pigmentos maculares luteína, zeaxantina e *meso*-zeaxantina, que protegem a retina contra danos causados pela luz e reduzem o impacto adverso da dispersão da luz e da diferença cromática, otimizando assim a sensibilidade ao contraste da retina. A propriedade de filtro da luz dos

carotenoides também pode fornecer proteção modesta a raios ultravioleta induzidos sob a pele (VON LINTIG et al., 2019).

A atividade da provitamina A é a capacidade dos carotenoides de formar vitamina A pela ação da caroteno dioxigenase (VON LINTIG et al., 2019). Qualquer carotenoide que contenha pelo menos um anel de *β*-ionona não modificado ligado a uma cadeia poliênica de 11 carbonos pode ser clivado para fornecer atividade da provitamina A. Aproximadamente 10% dos carotenoides são precursores de provitamina A entre eles *α*-caroteno, γ-caroteno, *β*-criptoxantina e *β*-caroteno que possui 100% de atividade da provitamina A (RODRIGUEZ-AMAYA, 2019).

Porém antes de exercer a ação bioativa, os carotenoides precisam atingir os locais de ação no corpo humano, portanto os dados de perfil e de atividade bioativa devem ser complementados com informações sobre bioacessibilidade e biodisponibilidade dessas estruturas.

3. Bioacessibilidade *in vitro*

Modelos *in vitro* baseados na fisiologia humana foram desenvolvidos buscando simular a sequência de eventos que ocorrem durante a digestão no trato gastrointestinal humano, permitindo a realização de ensaios específicos que não seriam possíveis de serem realizados *in vivo* por dificuldades práticas e éticas. Dessa forma, métodos *in vitro* possibilitam explorar questões e disponibilizar uma visão mais profunda dos fenômenos subjacentes à bioacessibilidade (HOLST; WILLIAMSON, 2008; CARBONELL-CAPELLA et al., 2014; CARDOSO et al., 2015).

No caso particular dos pigmentos, a digestão *in vitro* foi aplicada para medir a digestibilidade desses compostos a partir de fontes naturais. O estudo de Garrett, Failla e Sarama (1999) é a primeira aplicação de um modelo *in vitro* de digestão, para determinar a bioacessibilidade de carotenoides em matrizes alimentares. O procedimento experimental original segue as etapas de fase gástrica, fase intestinal e separação da fração micelar, ao longo dos anos algumas modificações ao método Garrett, Failla e Sarama (1999) foram publicadas, como por exemplo, a inclusão da fase oral (THAKKAR et al., 2007).

No modelo proposto por Garrett, Failla e Sarama (1999), a digestão foi acoplada à captação pelas células Caco-2 para simular a absorção,

confirmando que os carotenoides micelarizados são acessíveis para captação pelas células absorventes do intestino delgado. Modelos baseados em culturas Caco-2 são amplamente aceitos pela comunidade científica como referências válidas para estimar a assimilação de compostos bioativos (GARRETT et al., 1999; FAILLA; CHITCHUMROONCHOKCHAI, 2005; CHITCHUMROONCHOKCHAI; FAILLA, 2006, 2017).

Como consequência de sua natureza lipofílica, a bioacessibilidade e biodisponibilidade dos carotenoides é geralmente baixa. De fato, mais de 1000 carotenoides ocorrem na natureza, desses aproximadamente 50 carotenoides são encontrados na nossa alimentação, no entanto 34 carotenoides (incluindo isômeros geométricos, metabólitos e carotenos incíclicos acíclicos) foram identificados no soro e nos tecidos humanos. Porém, apenas 6, *α*-caroteno, *β*-caroteno, luteína, zeaxantina, *β*-criptoxantina e licopeno compreendem ~90% desse número total (OLMEDILLA et al., 2001; CHITCHUMROONCHOKCHAI; FAILLA, 2017).

Da mesma forma a bioacessibilidade e a captação de carotenoides a partir de fontes microalgais é de grande preocupação devido ao efeito da matriz, incluindo composição da parede celular e da biomassa além de questões relacionadas à estrutura do carotenoide e suas propriedades físico-químicas (GRANADO-LORENCIO et al., 2009; GILLE et al., 2016; 2019). Portanto, as operações de processamento de alimentos precisam ser consideradas e investigadas, uma vez que há indicações de que o uso de técnicas de rompimento celular resultou em uma bioacessibilidade melhorada dos carotenoides (BERNAERTS et al., 2019).

Assim o uso de tratamento com ultrassom na biomassa microalgal foi investigado e demostrou resultados promissores (GILLE et al., 2016, 2019). A técnica de sonicação visa reduzir o tamanho das partículas bem como romper as estruturas celulares melhorando a micelização dos carotenoides no trato gastrointestinal (GILLE et al., 2016; NICCOLAI et al., 2019b).

Outro fator que pode influenciar as baixas taxas de micelarização é a localização dos carotenoides nas células microalgais. A maioria dos carotenoides nas microalgas se encontram ligados ao aparelho fotossintético nos plastídeos (MULDERS et al., 2014). Portanto, os carotenoides precisam ser transportados através da fase aquosa antes de serem incorporados nas

micelas mistas. Além disso, a presença de outras macromoléculas originárias da biomassa de microalgas (por exemplo, proteínas, polissacarídeos) podem obstruir a micelarização dos carotenoides (GRANADO-LORENCIO et al., 2009; BERNAERTS et al., 2020). Desta forma o uso do extrato, um óleo com grandes quantidades de carotenoides, tornou-se uma alternativa nos protocolos de digestão *in vitro*, uma vez que os carotenoides se encontram na fase lipídica e a presença de outras macromoléculas é evitada (ROSO et al., 2015).

Em geral, a disponibilidade de carotenos como o *β*-caroteno é relativamente menor do que a das xantofilas, como a luteína e a zeaxantina. Devido à sua natureza apolar, os carotenos se localizam profundamente dentro da micela, enquanto, as xantofilas são mais facilmente incorporadas nas porções externas das micelas no trato gastrointestinal e podem ser facilmente absorvidas aumentando assim sua biodisponibilidade (GARRETT et al., 1999; GARRETT; FAILLA; SARAMA, 2000).

O tipo de carotenoide consumido pode afetar sua distribuição no trato gastrointestinal ou sua taxa de absorção intestinal. A configuração natural dos carotenoides é a sua forma all-*trans*, no entanto, como são compostos altamente insaturados, são susceptíveis a isomerização. A isomerização modifica algumas propriedades físico-químicas da molécula, pois as conformações *cis* são menos lineares e rígidas assim influenciando a bioacessibilidade destes compostos. Entretanto, não há consenso na literatura sobre a influência da isomerização dos carotenoides na sua bioacessibilidade e biodisponibilidade (FERRUZZI et al., 2006; FAILLA; CHITCHUMROONCHOKCHAI; ISHIDA, 2008).

A condição de concorrência quando vários carotenoides são co-consumidos deve ser considerado, pois supõe que os processos de liberação de matriz, micelarização e absorção sejam os mesmos. De fato, estudos demonstraram biodisponibilidade reduzida quando dois ou mais carotenoides foram co-consumidos em comparação com a apenas um (TYSSANDIER et al., 2003; BOHN, 2018; KOPEC; FAILLA, 2018).

Além das questões relacionadas acima, durante a digestão *in vitro*, foi sugerida uma via geral típica de metabolização de carotenoides que segue a isomerização, oxidação e fragmentação, devido a influência térmica e o baixo pH que facilitam a isomerização enquanto as enzimas, metais pró-oxidativos e

lipídios insaturados resultam em oxidação. Foram relatados vários produtos formados pela oxidação como o 5,6 epoxi-*β*-caroteno, 5,8 epoxi-*β*-caroteno, 5,6:5':6' diepoxi-*β*-caroteno *cis*-*β*-caroteno, neochrome, auroxanthin, luteoxanthin. Assim pode-se supor que produtos similares possam ser formados a partir de outros carotenoides durante reações *in vivo* (BOHN, 2008; COURRAUD et al., 2013; SY et al., 2013).

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CAPITULO 2

IMPACT OF *IN VITRO* BIOACCESSIBILITY FROM CAROTENOIDS MICROALGAE

O artigo será submetido ao Journal of Functional Foods

Impact of *in vitro* bioaccessibility and cellular uptake from carotenoids microalgae

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Abstract: This study aimed to investigate the carotenoids bioaccessibility of the whole dried biomass (WDB), wet ultrasonicated biomass (WUB), and isolated carotenoid extract (ICE) from *Chlorella vulgaris* and *Spirulina* sp. using an *in vitro* digestion model. The products were submitted to *in vitro* digestion model according to the INFOGEST protocol. Carotenoids were determined by HPLC-PDA-MS/MS. A total of twenty-two different carotenoids were separated in the control extracts, the major ones being all-*trans*-lutein (48.1%) for *C. vulgaris* and all-*trans*-β-carotene (29.3%) in the *Spirulina*. After *in vitro* digestion, for *C. vulgaris*, six compounds were bioaccessible in WDB, nine in WUB and ICE. The 5,6:5',6'-diepoxy-β-carotene was the most bioaccessible carotenoid in all products (WDB 18.3%; WUB 22.6%; and ICE 29.8%). On the other hand, for the *Spirulina* species, six carotenoids were bioaccessible in WDB, ten in WUB and ICE. All-*trans*-β-cryptoxanthin showed the highest bioaccessibility (46.8%) in WDB, while all-*trans*-cantaxanthin in WUB (99.5%) and ICE (95.5%). Finally, the results showed the significant influence of the nature of the product in promoting the bioaccessibility of microalgal carotenoids, since total bioaccessibility improved for both species according to the type of product (ICE>WUB>WDB). Thus, the data suggest that the bioaccessibility of ICE carotenoids is greater than in WDB and WUB. Therefore, ICE should be

considered a product that provides bioavailable carotenoids and could be the best choice, such as ingredients in the development of functional foods carotenoids-based.

Keywords: Carotenoids. Commercial microalgae. Simulated digestion.

1. Introduction

One of the key challenges that we face in the 21st century is the need to feed an ever-increasing human population with increasingly limited natural resources. Along with macronutrients shortage, the deficiency of functional micronutrients in food, like carotenoids, exacerbated the risk of famine and global food insecurity, with an estimate that about a third of the world population suffers from critical micronutrient deficiency conditions (Ferruzzi et al., 2020). Based on this understanding, products enriched by microalgae, have the opportunity to increase the level of micronutrients in foods, to address global demands in a more efficient and environmentally sustainable way (Parodi et al., 2018; Niccolai et al., 2019a).

The microalgal biomass and products derived thereof are positioning firmly in the food health market. The food industry mainly focuses more on adding the species *Chorella vulgaris* and *Spirulina* in the form of the whole dried biomass (WDB), but also wet biomass (WUB) or as isolated extracts (ICE), with the aim of promoting food products to as “superfood” or “rich in bioactive compounds” (Parodi et al., 2018; Niccolai et al., 2019a; Lafarga, 2019).

From a nutritional point of view, enriching food products with microalgae could be a valuable strategy. This is fundamentally supported by the fact that

microalgae biomass has become a promising alternative for obtaining bioactive compounds (Jacob-Lopes et al., 2019; Bernaerts et al., 2020). Of particular interest are the carotenoids, including β-carotene, zeaxanthin, lutein, echinenone, and canthaxanthin, which ate associated with intense antioxidant activity, provitamin A activity, the reduction of some chronic diseases, such as cancer, macular degeneration, and cardiovascular and neurodegenerative diseases (Kopec & Failla, 2018; Xavier & Mercadante, 2019; Nascimento et al., 2019; Murador et al., 2020).

However, the great concern is the microalgae matrix effect on the bioaccessibility of these compounds (Gille et al., 2016; 2019; Bernaerts et al., 2020; Nascimento et al., 2021). Since in order to mediate such activities, these compounds need to be released from the food matrix and become bioaccessible and potentially available to be absorbed by the human intestine (Kopec & Failla, 2018; Xavier & Mercadante, 2019).

Given the above, this study aimed to evaluate the bioaccessibility of carotenoids from two species of commercial microalgae, *Chlorella vulgais* and *Spirulina* sp., considered the following products, whole dried biomass (WDB), wet ultrasonicated biomass (WUB), and isolated carotenoid extract (ICE).

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 (SFB), 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), both of HPLC-grade, analytical-grade solvents ethanol, acetone, ethyl acetate, petroleum ether, and diethyl ether were purchased from Merck (Darmstadt, Germany). Caco-2 cell cultures were acquired from the Bank of Cells of Rio de Janeiro (BCRJ) (Rio de Janeiro, Brazil).

2.2. Microalgae culture and biomass production

Axenic cultures of *Chlorella vulgaris* (CPCC90) and *Spirulina* sp. (CPCC 695) were used in the experiments. Stock cultures were propagated and maintained in synthetic BG-11 medium (Braun-Grunow medium) (Rippka, Deruelles, & Waterbury, 1979). The incubation conditions were temperature of 26 °C, photon flux density of 15 $\mu mol.m^{-2}.s^{-1}$ and a photoperiod of 12 h were used.

The biomass productions were made in phototrophic conditions. The cultivations were performed in a bubble column photobioreactor (Maroneze et al., 2019) operating 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, photon flux density of 25 $\mu mol.m^{-2}.s^{-1}$, continuous aeration of 1VVM (volume of air per volume of culture per minute), and continuous lighting conditions of 24:0 h light/dark. The biomass was separated from the culture medium by centrifugation (1,500 x g; 10 min; 10 °C), the supernatant was discarded and the wet biomass (95% moisture) stored

immediately in closed containers protected from light under refrigeration until use.

2.3. Samples preparation

The wet biomass of *C. vulgaris* and *Spirulina* were submitted to different preliminary operations to obtain the three types of samples used in the *in vitro* digestion protocol: whole dried biomass (WDB), wet ultrasonicated biomass (WUB), and isolated carotenoid extract (ICE). All the unit operations constituting the process are detailed the next. Before the *in vitro* digestion, for the sample WDB, the wet biomass was freezing at -18 °C for 24 hours 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 $mol.L^{-1}$, CaCl2 6 $mmol.L^{-1}$, KCl 5 $mmol.L^{-1}$). In parallel, for obtaining the WUB, 0.8 ± 0.02 g aliquots were separated from the wet biomass of *C. vulgaris* and *Spirulina* (cellular concentration 4.8 $g.L^{-1}$ and 4.1 $g.L^{-1}$, respectively), equivalent to 0.1 ± 0.02 g of dry biomass, and were added 10 mL of saline solution. The resulting mixture was subjected to 15 min of an ultrasonic probe (Ultronic, Indaiatuba-SP, Brazil) (an adaptation of Gille et al., 2016). The ultrasonic parameters were probe with 13 mm diameter, 400 W, 40 kHz, and an ice bath to control the temperature (0 ± 2 °C). The ICE 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 ICE 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 (1,860 × g; 4 min). A total of 5 g of emulsion per ICE was prepared to end added 10 mL de salina solution.

2.4. *In vitro* Digestion

The *in vitro* assay to estimate bioaccessibility was performed using the protocol adapted from INFOGEST (Minekus et al., 2014). The oral step was simulated using 6 mL of a solution of artificial saliva containing 106 $U.mL^{-1}$ of α-amylase, followed by incubation at 37 °C, 10 min in an orbital shaker (7.5 × g). Before starting the gastric phase, the pH was adjusted to 2.5 with HCl 1M 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 1M $NaHCO_3$ and the intestinal phase start with a porcine and ovine bile solution (3 mL; 40 $mg.mL^{-1}$ in 100 mM $NaHCO_3$), 4,000 $U.mL^{-1}$ of porcine pancreatin and 1,000 $U.mL^{-1}$ of lipase from porcine pancreas. The pH was adjusted to 6.5 and the total volume to 50 mL, and the solution was incubated (2 h; 37 °C; 7.5 × g). After the completed *in vitro* digestion, the solution was centrifuged (8,000 × g; 60 min; 4 °C). The supernatant containing the mixed micelles was collected, were covered with nitrogen gas, frozen at -40 °C and lyophilized for further extraction of carotenoids. The carotenoids bioaccessibility was calculated as the ratio between carotenoids content in the micellar fraction (supernatant) and to the initial content of the carotenoids in the undigested (Eq. 1).

$$Bioaccessibility\ (\%) = \frac{Carotenoids\ (Supernatant)}{Carotenoids\ (Initial\ content)} \times 100 \qquad Eq.1$$

2.5. Carotenoid analysis

The carotenoids from *C. vulgaris* and *Spirulina* were exhaustively extracted of aliquots of 0.1 ± 0.02 g from freeze-dried biomass (see parameters in section 2.3.) with ethyl acetate and methanol in a mortar with a 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, concentrated in a rotary evaporator (< 30 °C); then, the extract was transferred to mixture of petroleum ether/diethyl ether [1:1 (v/v)], and extraction solvent was removed by washing.

One aliquot of the extract obtained as described above was denominated control extract, and represent the original content of carotenoids in *C. vulgaris* and *Spirulina* biomass before *in vitro* digestion, was concentrated in a rotary evaporator (<30 °C), dried under N_2 flux, and stored at -40 °C until injection into the HPLC. Another aliquot, denominated isolated carotenoid extract (ICE), was subjected to the simulated digestion procedure.

The micellarized carotenoids were extracted according to Ordóñez-Santos, Pinzón-Zarate, and González-Salcedo (2015) adaptions. The carotenoids were exhaustively extracted by the addition of 15 mL of e petroleum ether/diethyl ether [1:1 (v/v)] and subjected to 5 min 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. All extracts were

concentrated in a rotary evaporator, were placed in the N_2 atmosphere, and kept at -40 °C in the dark until chromatographic analysis.

2.6. HPLC-PDA-MS/MS analysis

The carotenoids were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with binary pumps (model LC-20AD), online degasser, and automatic injector (model SIL-20A HT). 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 carotenoids 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, Menezes, Mercadante, Jacob-Lopes, & Zepka (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 phase B (MTBE), using 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 22 ºC, the UV/vis spectra were acquired between 220 and 700 nm, and the chromatograms were processed at 450 nm for carotenoids. Carotenoids were individually quantified by HPLC-PDA using six-point analytical curves (r^2 = 0.99) of all-*trans*-lutein (1.0-50.0 $\mu g.mL^{-1}$), all-*trans*-β-cryptoxanthin (1.0-60 $\mu g.mL^{-1}$), and all-*trans*-β-carotene (1.0-50 $\mu g.mL^{-1}$), along with two more analytical curves

for low concentrations: all-*trans*-lutein and all-*trans*-β-carotene (0.05-10 μg.mL^{-1} for each).

The MS/MS analysis was achieved according to Giuffrida, Zoccali, Giofrè, Dugo, & Mondello (2017) with adaptations, the APCI interface operated in positive (+) mode; detector voltage: 4.5 kV; interface temperature: 350 ºC; DL temperature: 250 ºC; heat block temperature: 200 ºC; nebulizing gas flow (N_2): 3.0 L.min^{-1}; drying gas flow (N_2): 5.0 L.min^{-1}; collision induced dissociation (CID) gas: 23 kPa (argon); event time: 0.5 s. To improve the quality of identification, 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 (Spectral fine structure (λmáx), ratio of the height of the longest wavelength absorption peak (III) and that of the middle absorption peak (II), ratio of the *cis* peak (AB) and the middle absorption peak (II)), and mass characteristics (protonated molecule ([M+H]$^+$) and MS/MS fragments), compared with data available in the literature (Rodrigues et al., 2014; Rodrigues, Menezes, Mercadante, Jacob-Lopes, & Zepka, 2015; Patias et al., 2017; Maroneze et al., 2019).

2.7. Statistical analysis

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

3. Results and discussion

3.1. Carotenoid composition of samples before and after digestion

A total of 27 carotenoids were separated in all assay for the products of *Chlorella vulgaris* and *Spirulina* sp. (Fig. 1 and Fig. 2). The separated carotenoids were identified or tentatively identified based on the combined information obtained from the chromatographic elution, UV-visible, co-chromatography with standards, and mass spectral data (Table 1). Considering that a detailed description of carotenoid identification has already been reported for microalgae species by Rodrigues et al. (2014, 2015), Patias et al. (2017), Maroneze et al. (2019) and Nascimento et al. (2021) thus, only considerations regarding the carotenoids not identified in these previous reports were discussed below.

Peak 2 and peak 4 were tentatively identified as 13-*cis*-neochrome and 9-*cis*-neochrome, considering the elution order on C30 column, UV-visible spectra characteristics, the intensity of the *cis* peak in the range of 337 nm (%AB/II = 47) and 325 nm (%AB/II = 15), respectively, and mass spectra features similar to those reported in the literature. The mass spectra of neochrome isomers obtained in the positive ion mode showed the protonated molecule at m/z 601 and fragment ions in the MS/MS at m/z 583 $[M + H - 18]^+$ and m/z 547 $[M + H - 18 - 18 - 18]^+$, corresponding to the loss of one and three water molecule, respectively, and at m/z 221, showing the presence of an epoxy group in a hydroxylated β-ring (De Rosso & Mercadante, 2007). These two isomers were only detected after simulated digestion in the WUB and ICE products of *C. vulgaris*.

Table 2 shows the content of the carotenoids quantified in the control extracts for two microalgae species, *Chlorella vulgaris* and *Spirulina* sp., followed by the absolute contents after *in vitro* digestion process of samples whole dried biomass (WDB), wet ultrasonicated biomass (WUB), and isolated carotenoid extract (ICE). The total carotenoid contents from control extracts were 3,014.20 μg g^{-1}, and 1,252.17 μg g^{-1}, as dry weight, for *C. vulgaris* and *Spirulina*, respectively.

The composition of carotenoid in the control extract from *C. vulgaris* can be seen in Fig. 1a and Table 2. Among the 12 carotenoids identified, all-*trans*-lutein (1,451.12 μg.g^{-1}, peak 13) and all-*trans*-β-carotene (456.92 μg.g^{-1}, peak 26) were the main, which represented 63% of the total carotenoid content followed by all-*trans*-α-carotene (177.92 μg.g^{-1}, peak 25), and 9-*cis*-neoxanthin (155.18 μg.g^{-1}, peak 3) as major carotenoids in this biomass. All-*trans*-violaxanthin (146.89 μg.g^{-1}, peak 5), 9-*cis*-lutein (123.28 μg.g^{-1}, peak 14), 15-*cis*-lutein (102.60 μg.g^{-1}, peak 9), 9-*cis*-β-carotene (99.15 μg.g^{-1}, peak 27), all-*trans*-luteoxanthin (89.27 μg.g^{-1}, peak 7), 13-*cis*-lutein (82.48 μg.g^{-1}, peak 11), 5,6:5',6'-diepoxy-β-carotene (71.32 μg.g^{-1}, peak 17) and 13-*cis*-neoxanthin (58.10 μg.g^{-1}, peak 1), have also been identified in this microalgae species.

In this line, a total of 12 carotenoids were identified in the control extract of *Spirulina* (Fig. 2a and Table 2). All-*trans*-β-carotene (366.62 μg.g^{-1}, peak 26), and all-*trans*-zeaxanthin (242.92 μg.g^{-1}, peak 15) were the majority, as shown in Table 2, which represented 48% of the total carotenoid content. Additionally, different from the results described in *C. vulgaris*, *Spirulina* showed unique microalgae carotenoids, such as 2'-dehydrodeoxymyxol (163.87 μg.g^{-1}, peak 19), 9-*cis*-echinenone (153.97 μg.g^{-1}, peak 23), all-*trans*-echinenone (137.71

μg.g^{-1}, peak 22), and all-*trans*-canthaxanthin (15.71 μg.g^{-1}, peak 16). Other carotenoids were detected as 9-*cis*-β-carotene (67.37 μg.g^{-1}, peak 27), 9-*cis*-zeaxanthin (44.20 μg.g^{-1}, peak 18), all-*trans*-β-cryptoxanthin (30.47 μg.g^{-1}, peak 21), 15-*cis*-zeaxanthin (18.59 μg.g^{-1}, peak 12), all-*trans*-antheraxanthin (6.16 μg.g^{-1}, peak 10) and 13-*cis*-antheraxanthin (4.60 μg.g^{-1}, peak 8).

Considering the compounds identified, in the control extract of *C. vulgaris* and *Spirulina*, only two of the carotenoids were common, peak 26 and peak 27, providing a diverse carotenoid profile. This is because *C. vulgaris* and *Spirulina* 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 specific in each species of microalgae (Borowitzka et al., 2018; Takaichi, 2011).

Table 1. Chromatographic, UV/Vis and mass spectrometry characteristics, obtained by HPLC-PDA-MS/MS, of carotenoids found during the *in vitro* digestion from microalgae *Chlorella vulgaris* and *Spirulina* sp.

Peak[a]	Carotenoids	t_R (min)[b]	UV-vis characteristics			Fragment ions (positive mode) (*m/z*)	
			$\lambda_{máx}$ (nm)[c]	III/II[d] (%)	A_B/II[e] (%)	$[M + H]^+$	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 $[M + H - 18]^{+\cdot}$, 547 $[M + H - 18 - 18 - 18]^+$, 221
3	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 $[M + H - 92]^+$
4	9-*cis*-neochrome	8.8	325, 400,419, 440	80	15	601	583 $[M + H - 18]^{+\cdot}$, 547 $[M + H - 18 - 18 - 18]^+$, 221
5	all-*trans*-violaxanthin	9.3	415, 438, 467	83	0	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$
6	9-*cis*-violaxanthin	9.9	326, 412, 435, 463	63	20	601	583 $[M + H - 18]^+$, 565 $[M + H - 18 - 18]^+$, 509 $[M + H - 92]^+$
7	all-*trans*-luteoxanthin	10.3	400, 420, 447	100	0	601	583 $[M + H - 18]^+$

8	13-*cis*-antheraxanthin	10.4	326, 415, 438, 467	72	13	585	567 $[M + H - 18]^+$, 549 $[M + H - 18 - 18]^+$, 531
9	15-*cis*-lutein	12.4	328, 415, 438, 465	14	26	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
10	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
11	13-*cis*-lutein	13.7	331, 415, 437, 465	37	44	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
12	15-*cis*-zeaxanthin	15.3	335, 418, 442, 468	0	48	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$, 477 $[M + H - 92]^+$
13	all-*trans*-lutein	15.6	419, 443, 471	57	0	569	551 $[M + H - 18]^+$, 533 $[M + H - 18 - 18]^+$
14	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
15	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
16	all-*trans*-cantaxanthin	20.8	472	nc[g]	0	565	547 $[M + H - 18]^+$
17	5,6:5',6'-diepoxy-β-carotene	21.8	419, 439, 467	100	0	569	551 $[M + H - 18]^+$, 477 $[M + H - 92]^+$, 205
18	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
19	2'-dehydrodeoxymyxol	25.4	445, 473, 504	63	0	567	549 $[M + H - 18]^+$
20	5,6-epoxy-β-carotene	27.7	420, 446, 470	50	0	553	535 $[M + H - 18]^+$, 461 $[M + H - 92]^+$, 205

21	all-*trans*-β-cryptoxanthin	28.4	420, 450, 473	25	0	553	535 $[M + H - 18]^+$
22	all-*trans*-echinenone	30.2	462	nc	0	551	533 $[M + H - 18]^+$, 427, 203
23	9-*cis*-echinenone	32.6	342, 450	nc	20	551	533 $[M + H - 18]^+$, 427, 203
24	13-*cis*-β-carotene	37.4	337, 420, 444, 470	17	50	537	444 $[M + H - 92]^+$, 399, 355
25	all-*trans*-α-carotene	37.5	420, 445, 473	62	0	537	444 $[M + H - 92]^+$, 399, 355
26	all-*trans*-β-carotene	39.6	425, 451, 476	25	0	537	444 $[M + H - 92]^+$, 399, 355
27	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 and Fig. 2.

[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).

As shown in Table 2, the carotenoids were liberated from the microalgal matrix and micellarized; however, micellar incorporation of carotenoids showed a strong dependence on the product used (WDB, WUB and ICE). Thus, after the simulated digestion procedure, there was a significant quantitative reduction in carotenoids in all products tested, for two microalgae species, *Chlorella vulgaris* and *Spirulina* sp., except to all-*trans*-canthaxanthin in WUB and ICE sample of *Spirulina*.

The whole dry biomass *C. vulgaris* exhibited the lowest total carotenoid incorporation content (49.69 $\mu g.g^{-1}$), and only six carotenoids were incorporated into the micelle after digestion (Table 2), being all-*trans*-lutein (14.51 $\mu g.g^{-1}$), was the major carotenoid, followed by 5,6:5',6'-diepoxy-β-carotene (13.07 $\mu g.g^{-1}$), 9-*cis*-β-carotene (6.23 $\mu g.g^{-1}$), all-*trans*-β-carotene (6.17 $\mu g.g^{-1}$) which corresponds to 80% of the fraction of incorporated carotenoid. The complementary, about 20% was constituted by all-*trans*-violaxanthin (5.57 $\mu g.g^{-1}$) and all-*trans*-α-carotene (4.15 $\mu g.g^{-1}$).

A similar situation was observed during *in vitro* digestion of WDB *Spirulina*, where total carotenoid incorporation was also limited (118.78 $\mu g.g^{-1}$), and 6 different carotenoids were incorporated into the micelle after digestion (Table 2). 2'-dehydrodeoxymyxol (27.20 $\mu g.g^{-1}$) was quantitatively dominant, followed by all-*trans*-zeaxanthin (26.25 $\mu g.g^{-1}$), and all-*trans*-β-carotene (21.37 $\mu g.g^{-1}$) which represented 63% of the total incorporated carotenoid content. In addition, all-*trans*-echinenone (16.58 $\mu g.g^{-1}$), 9-*cis*-β-carotene (16.25 $\mu g.g^{-1}$), and all-*trans*-β-cryptoxanthin (14.28 $\mu g.g^{-1}$) were detected as minor carotenoids incorporated.

The use of whole microalgal biomass, for both microalgae species, *C. vulgaris* and *Spirulina*, showed low incorporation of carotenoids that may be associated with the constituents of the cell wall and the location of carotenoids in the microalgal cell (Bernaerts et al., 2020). Similar limitations in the incorporation of carotenoids from whole dry biomass have been observed by Gille et al. (2016; 2019), Bernaerts et al. (2020), and Nascimento et al. (2021). Therefore, the use of processing operations for the liberation of these structures might be desired.

The effect of wet ultrasonicated paste (WUB) from *C. vulgaris* enabled the total incorporation 184.46 $\mu g.g^{-1}$ (Table 2 and Fig. 1b). A total of 14 different carotenoids were identified, with all-*trans*-lutein (58.20 $\mu g.g^{-1}$) was the major carotenoid, followed by 5,6:5',6'-diepoxy-β-carotene (16.13 $\mu g.g^{-1}$), and 9-*cis*-lutein (16.04 $\mu g.g^{-1}$) representing 49% of the total incorporated carotenoid content. The 13-*cis*-lutein (13.54 $\mu g.g^{-1}$), 15-*cis*-lutein (7.26 $\mu g.g^{-1}$), all-*trans*-violaxanthin (7.07 $\mu g.g^{-1}$), all-*trans*-α-carotene (6.60 $\mu g.g^{-1}$), all-*trans*-β-carotene (6.33 $\mu g.g^{-1}$), 9-*cis*-β-carotene (6.25 $\mu g.g^{-1}$) have also been mixed micelles were also incorporated. Moreover, 13-*cis*-neochrome (14.30 $\mu g.g^{-1}$), 9-*cis*-neochrome (13.36 $\mu g.g^{-1}$), 9-*cis*-violaxanthin (6.52 $\mu g.g^{-1}$), 5,6-epoxy-β-carotene (6.45 $\mu g.g^{-1}$) and all-*trans*-echinenone (6.41 $\mu g.g^{-1}$) were not detected in the control extract but identified after the *in vitro* digestion, possibly being a degradation product.

For *Spirulina* WUB, the total carotenoid content incorporated in the mixed micelles was 199.27 $\mu g.g^{-1}$. Ten carotenoids were identified in *Spirulina* WUB, after the *in vitro* digestion process (Table 2 and Fig. 2b). The 2'-dehydrodeoxymyxol (34.53 $\mu g.g^{-1}$) along with all-*trans*-zeaxanthin (36.70 $\mu g.g^{-1}$), all-*trans*-β-carotene (21.37 $\mu g.g^{-1}$), and all-*trans*-echinenone (20.47 $\mu g.g^{-1}$)

were the majority components of micelles, which accounted for 57% of total carotenoid. The other micellarized carotenoids were 9-*cis*-echinenone (18.10 $\mu g.g^{-1}$), 9-*cis*-zeaxanthin (17.14 $\mu g.g^{-1}$), 9-*cis*-β-carotene (16.31 $\mu g.g^{-1}$), all-*trans*-β-cryptoxanthin (15.65 $\mu g.g^{-1}$), all-*trans*-cantaxanthin (15.63 $\mu g.g^{-1}$), 15-*cis*-zeaxanthin (2.85 $\mu g.g^{-1}$).

This effect can be attributed to rupture cell wall and/or the release of carotenoids by use ultrasonicated, assist in the process of micellar incorporation (Gille et al., 2016; 2019; Nascimento et al., 2021). However, as already suggested and presented previously by Bernaerts et al. (2020) and Nascimento et al. (2021), in addition to the disruption of the microalgae cells, the presence of other macromolecules originating from the microalgal biomass (e.g. proteins, polysaccharides) might hamper the incorporation of carotenoids in the micellar fraction. To validate the aforementioned hypotheses, we evaluate the micellarization of carotenoids from isolated carotenoid extract (ICE). As such, all carotenoids were located in the lipid phase, and the presence of other macromolecules was avoided.

When applying *C. vulgaris* ICE, the carotenoid incorporation significantly increased up to 302.35 $\mu g.g^{-1}$. Extracts after the digestion process showed fourteen different carotenoids were separated (Table 2, Fig. 1c), characterized by the prevalence of one major carotenoid corresponding to all-*trans*-lutein (121.04 $\mu g.g^{-1}$), followed by all-*trans*-β-carotene (25.71 $\mu g.g^{-1}$), 5,6:5',6'-diepoxy-β-carotene (21.32 $\mu g.g^{-1}$), 9-*cis*-lutein (16.58 $\mu g.g^{-1}$), all-*trans*-α-carotene (12.21 $\mu g.g^{-1}$). The other minority carotenoids incorporated were 13-*cis*-lutein (9.97 $\mu g.g^{-1}$), 9-*cis*-β-carotene (8.91 $\mu g.g^{-1}$), and all-*trans*-violaxanthin (7.75 $\mu g.g^{-1}$). Again, the compounds 5,6-epoxy-β-carotene (20.05 $\mu g.g^{-1}$), all-

trans-echinenone (12.65 μg.g^{-1}), 9-*cis*-violaxanthin (12.45 μg.g^{-1}), 13-*cis*-neochrome (11.56 μg.g^{-1}), 9-*cis*-neochrome (10.14 μg.g^{-1}) emerged after the simulated digestion procedure.

The *Spirulina* ICE was the product with the highest incorporation total carotenoids (373.37 μg.g^{-1}) with respect to the products studied. The carotenoid profile of *Spirulina* ICE after the digestion process showed a total of 11 different carotenoids (Table 2, Fig. 2c.). The main carotenoids incorporate were all-*trans*-β-carotene (96.13 μg.g^{-1}), followed by all-*trans*-echinenone (82.70 μg.g^{-1}), and all-*trans*-zeaxanthin (80.85 μg.g^{-1}) (Table 2, Fig. 2c.). The minor carotenoids incorporate were 9-*cis*-β-carotene (19.50 μg.g^{-1}), all-*trans*-β-cryptoxanthin (18.81 μg.g^{-1}), all-*trans*-cantaxanthin (15.00 μg.g^{-1}), 9-*cis*-echinenone (13.25 μg.g^{-1}), 9-*cis*-zeaxanthin (10.12 μg.g^{-1}), 15-*cis*-zeaxanthin (9.55 μg.g^{-1}) and 2'-dehydrodeoxymyxol (8.04 μg.g^{-1}). Moreover, 13-*cis*-β-carotene (19.41 μg.g^{-1}) was detected only after digestion *in vitro*.

When comparing *C. vulgaris* products (WDB, WUB and ICE), ICE revealed to be an efficient strategy to increase total carotenoid incorporation, whereas micellar content in the WDB and WUB were approximately 6 and 1.6 times minor respectively, then observed for ICE, hence, the absence of other macromolecules originating from the microalgal biomass and/or the location of carotenoids in the lipid phase have obviously favored the micellar incorporation.

Regarding the compounds individually, when applying *C. vulgaris* products WUB and ICE, the concentration of all carotenoids increased, even if not significantly, in comparison to the WDB. In addition, the use of *C. vulgaris* WUB and ICE allowed the micellarization of isomers as 15-*cis*-, 13-*cis*- and 9-*cis*-lutein (peaks 10, 14 and 18) present in the control extract which had not

been micellarized in the WDB (Table 2), suggesting the relevance of microalgae matrix processing to improve the incorporation of carotenoids. Additionally, the use *C. vulgaris* ICE increased the concentration of most carotenoids but decreased the incorporation of 13-*cis*- and 9-*cis*-neochrome, the low micellarization of these structures might be attributed to increased exposure of carotenoids in the liposoluble pigments extract, favoring to oxidative degradation.

Similarly, *Spirulina* products (WDB, WUB and ICE), total carotenoid incorporation in ICE, after digest, was significantly higher than in WDB and WUB (from 3.1- to 1.8-fold, respectively). Notably, all carotenoids concentrations in the WUB and ICE were higher than those observed in the WDB. Additionally, the WUB and ICE products confer the incorporation of 15-*cis*-, 9-*cis*-zeaxanthin, all-*trans*-canthaxanthin, and 9-*cis*-echinenone (peaks 16, 24, 21 and 31) detected in the control extract which had not been incorporated in the WDB (Table 2). Indicating the relevance of microalgal biomass processing since it provided the micellarization of all-*trans*-canthaxanthin, a microalgae carotenoid that is hugely revered due to its antioxidant potential provided by the 13 conjugated double bonds present in its structure (Nascimento et al., 2021). Regarding *Spirulina* product ICE, the content of all-*trans*-β-carotene, all-*trans*-echinenone, all-*trans*-zeaxanthin, 9-*cis*-β-carotene, all-*trans*-β-cryptoxanthin, and 15-*cis*-zeaxanthin, increased significantly compared with WUB.

The comparison of the carotenoids incorporation of several products (WDB, WUB and ICE) from two distinct species of microalgae, *C. vulgaris* and *Spirulina*, allowed the analysis of the behavior of the matrix. Once, each

microalga showed a different performance, the *Spirulina* products (WDB, WUB and ICE) revealed a greater incorporation of the total carotenoid content (118.78 $\mu g.g^{-1}$, 199.27 $\mu g.g^{-1}$ and 373.37 $\mu g.g^{-1}$, respectively), whereas *C. vulgaris* products WDB, WUB and ICE the incorporation reduced to 58.16%, 7.43% and 19.02%, respectively, compared to the *Spirulina* products (Table 2).

As established above, the most expressive differences were for the total microalgal biomass and these are probably related to physiological and morphological characteristics of the investigated microalgae. Moreover, this variation was previously reported for the species *Chlorella* sp. and *Chlamydomonas reinhardtii* (Gille et al., 2016). While, for the strains tested in this work, previous trials have highlighted that *C. vulgaris* had a lower digestibility (about 60%), compared to *Spirulina*, which had a higher digestibility (78%) (Niccolai et al., 2019b). Hence, the microalgal matrix was a determinant factor in the incorporation of carotenoids.

The experimental conditions of *in vitro* digestion combined with increased exposure of carotenoids due to the processing of microalgal biomass may have contributed to some changes in the carotenoid profile for the products of *C. vulgaris* WUB and ICE and *Spirulina* ICE. For *C. vulgaris*, as a consequence of the simulate digestion at WUB and ICE, 13 and 9-*cis*-neoxanthin (peaks 1 and 4), luteoxanthin (8 peak), disappeared, whereas neochrome isomers, 9-*cis*-violaxanthin, 5,6-epoxy-β-carotene, and all-*trans*-echinenone (peaks 2, 5, 7, 26 and 29, respectively) were formed (Table 2, Fig. 1c). Additionally, *Spirulina* WUB and ICE, 13-*cis* and all-*trans*-antheraxanthin (peaks 9 and 12), were not detected, in contrast, 13-*cis*-β-carotene (34 peak) was only formed ICE *Spirulina* after digesta (Table 2, Fig. 2c).

Taking into account the carotenoid structures identified after *in vitro* digestion, from microalgal products, WUB and ICE *C. vulgaris* and *Spirulina* ICE, the main reactions observed were isomerization of *trans* configurations for *cis*, epoxidation and ketolation. These findings followed, in general, previous results about the carotenoids transformations during their passage through the *in vitro* digestion procedure, which can be explained through the acidic conditions of the gastric phase and temperature (Asai, Terasaki & Nagao, 2004; Biehler et al., 2011; Kopec et al., 2017). Lycopene, for example, may undergo isomerization before absorption, since the main carotenoid found in tomato is the all-*trans*-, while in plasma and human tissues, *cis*-forms appear in higher concentrations (Kopec et al., 2017), while epoxycarotenoids have been shown to undergo a rapid expoxide-furanoid transition in the acid milieu of the stomach, resulting, e.g. in the formation of neochrome from neoxanthin and auroxanthin and luteoxanthin from violaxanthin (Asai et al., 2004, Biehler et al., 2011). However, the detection of all-*trans*-echinenone from WUB and ICE *C. vulgaris* only after digestion is surprising, considering it may act as valuable antioxidants and immunostimulants and it is not commonly available in conventional sources (Nascimento et al., 2019).

Table 2. Initial carotenoids contents from *C. vulgaris* and *Spirulina* before digestion (control extract) and micellar incorporation of WDB, WUB, and ICE after *in vitro* digestion. Different letters in the lines indicate a significant difference ($p<0.05$).

pigments	carotenoid content ($\mu g.g^{-1}$ dry weight)		micellar fraction content ($\mu g.g^{-1}$ dry weight)					
	control extract		WDB		WUB		ICE	
	C. vulgaris	*Spirulina* sp.	*C. vulgaris*	*Spirulina* sp.	*C. vulgaris*	*Spirulina* sp.	*C. vulgaris*	*Spirulina* sp.
13-*cis*-neoxanthin	58.10±0.51[1]	nd[2]	nd	nd	nd	nd	nd	nd
13-*cis*-neochrome	nd	nd	nd	nd	14.30±0.07[a]	nd	11.56±0.35[b]	nd
9-*cis*-neoxanthin	155.18±3.41	nd	nd	nd	nd	nd	nd	nd
9-*cis*-neochrome	nd	nd	nd	nd	13.36±0.07[a]	nd	10.14±0.33[b]	nd
all-*trans*-violaxanthin	146.89±4.83[a]	nd	5.57±0.18[b]	nd	7.07±0.13[b]	nd	7.75±0.21[b]	nd
9-*cis*-violaxanthin	nd	nd	nd	nd	6.52±0.02[b]	nd	12.45±0.14[a]	nd
all-*trans*-luteoxanthin	89.27±1.02	nd	nd	nd	nd	nd	nd	nd
13-*cis*-antheraxanthin	nd	4.60±0.01	nd	nd	nd	nd	nd	nd
15-*cis*-lutein	102.60±3.36[a]	nd	nd	nd	7.26±0.19[c]	nd	11.99±0.04[b]	nd
all-*trans*-antheraxanthin	nd	6.16±0.04	nd	nd	nd	nd	nd	nd
13-*cis*-lutein	82.48±1.35[a]	nd	nd	nd	13.54±0.22[b]	nd	9.97±0.04[c]	nd
15-*cis*-zeaxanthin	nd	18.59±0.08[a]	nd	nd	nd	2.85±0.05[c]	nd	9.55±0.18[b]

all-*trans*-lutein	1,451.12±46.58[a]	nd	14.51±0.47[c]	nd	58.20±0.11[c]	nd	121.04±1.43[b]	nd
9-*cis*-lutein	123.28±3.12[a]	nd	nd	nd	16.04±0.27[b]	nd	16.58±0.16[b]	nd
all-*trans*-zeaxanthin	nd	242.92±3.94[a]	nd	23.60±0.37[d]	nd	36.70±0.17[c]	nd	80.85±0.44[b]
all-*trans*-cantaxanthin	nd	15.71±0.20[a]	nd	nd	nd	15.63±0.20[a]	nd	15.00±0.97[a]
5,6:5',6'-diepoxy-β-carotene	71.32±1.19[a]	nd	13.07±0.22[d]	nd	16.13±0.31[c]	nd	21.32±0.21[b]	nd
9-*cis*-zeaxanthin	nd	44.20±0.79[a]	nd	nd	nd	17.14±0.26[c]	nd	10.12±0.33[b]
2'-dehydrodeoxymyxol	nd	163.87±2.52[a]	nd	27.20±0.42[c]	nd	34.53±0.24[b]	nd	8.04±0.06[d]
5,6-epoxy-β-carotene	nd	nd	nd	nd	6.45±0.01[b]	nd	20.05±0.04[a]	nd
all-*trans*-β-cryptoxanthin	nd	30.47±0.26[a]	nd	14.28±0.12[c]	nd	15.65±0.07[c]	nd	18.81±0.44[b]
all-*trans*-echinenone	nd	137.71±2.23[A]	nd	16.58±0.27[D]	6.41±0.02[b]	20.47±0.14[C]	12.65±0.09[a]	82.70±0.80[B]
9-*cis*-echinenone	nd	153.97±2.34[a]	nd	nd	nd	18.10±0.08[c]	nd	13.25±0.11[b]
13-*cis*-β-carotene	nd	nd	nd	nd	nd	nd	nd	19.41±0.42
all-*trans*-α-carotene	177.92±6.32[a]	nd	4.15±0.15[b]	nd	6.60±0.11[b]	nd	12.21±0.08[b]	nd
all-*trans*-β-carotene	456.92±13.67[a]	366.62±5.96[A]	6.17±0.18[c]	21.37±0.35[b]	6.33±0.03[c]	21.90±0.08[b]	25.71±0.14[b]	96.13±0.35[B]
9-*cis*-β-carotene	99.15±2.15[a]	67.37±1.20[A]	6.23±0.14[c]	16.25±0.29[C]	6.25±0.11[c]	16.31±0.10[C]	8.91±0.01[b]	19.50±0.42[B]
Total	**3,014.20±87.52[a]**	**1,252.17±15.97[A]**	**49.69±1.33[d]**	**118.78±1.82[c,d]**	**184.46±0.18[c]**	**199.27±0.14[c]**	**302.35±2.09[b]**	**373.37±1.40[b]**

[1]Values are average and standard deviation of triplicates.

[2]Not detected.

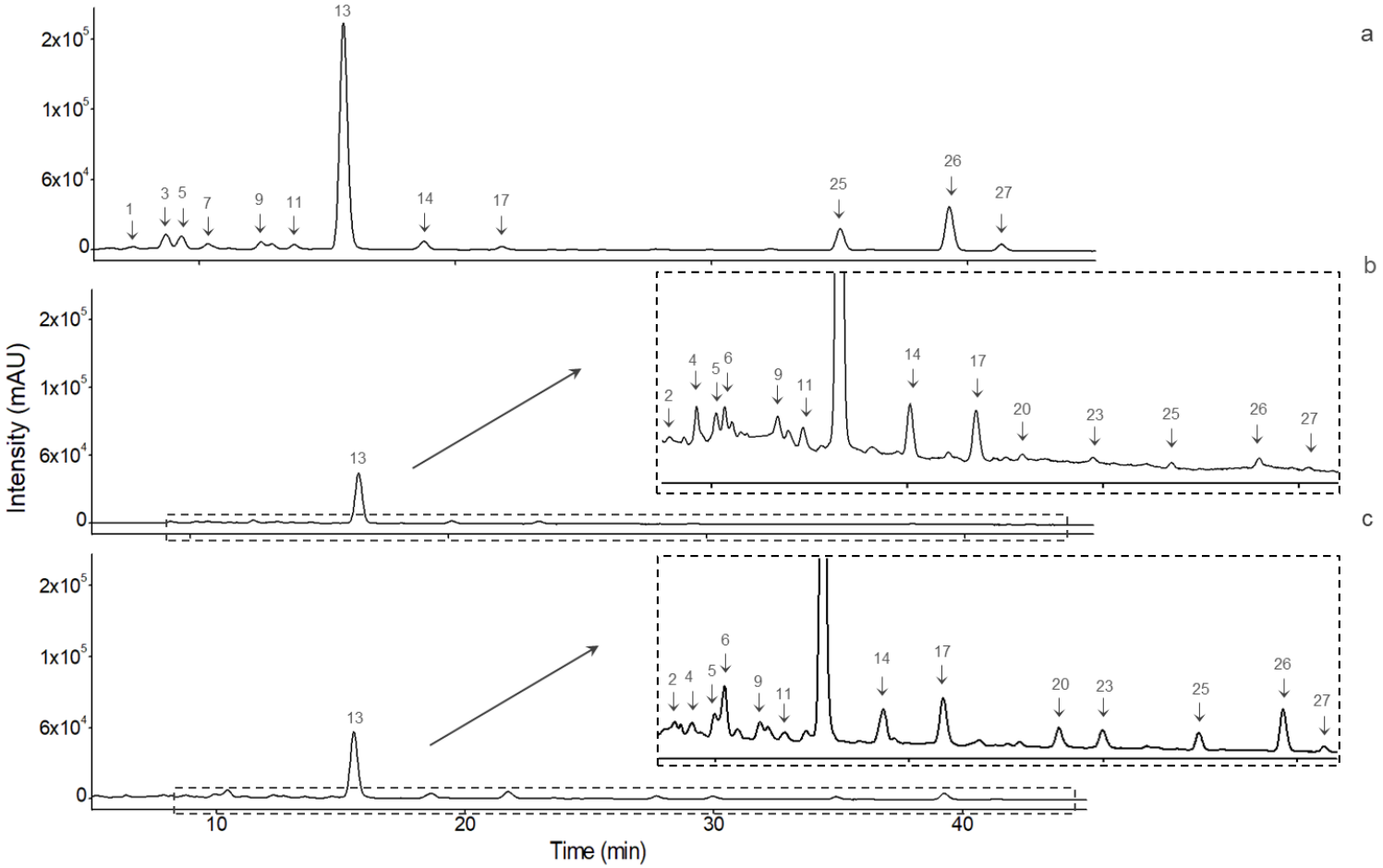

Figure 1. Representative chromatograms HPLC-PDA of *C. vulgaris* carotenoids. Control extract before digestion (a); WUB (b); ICE (c) after *in vitro* digestion. Peak identification and characterization are given in Table 1. Chromatogram was processed at 451 nm.

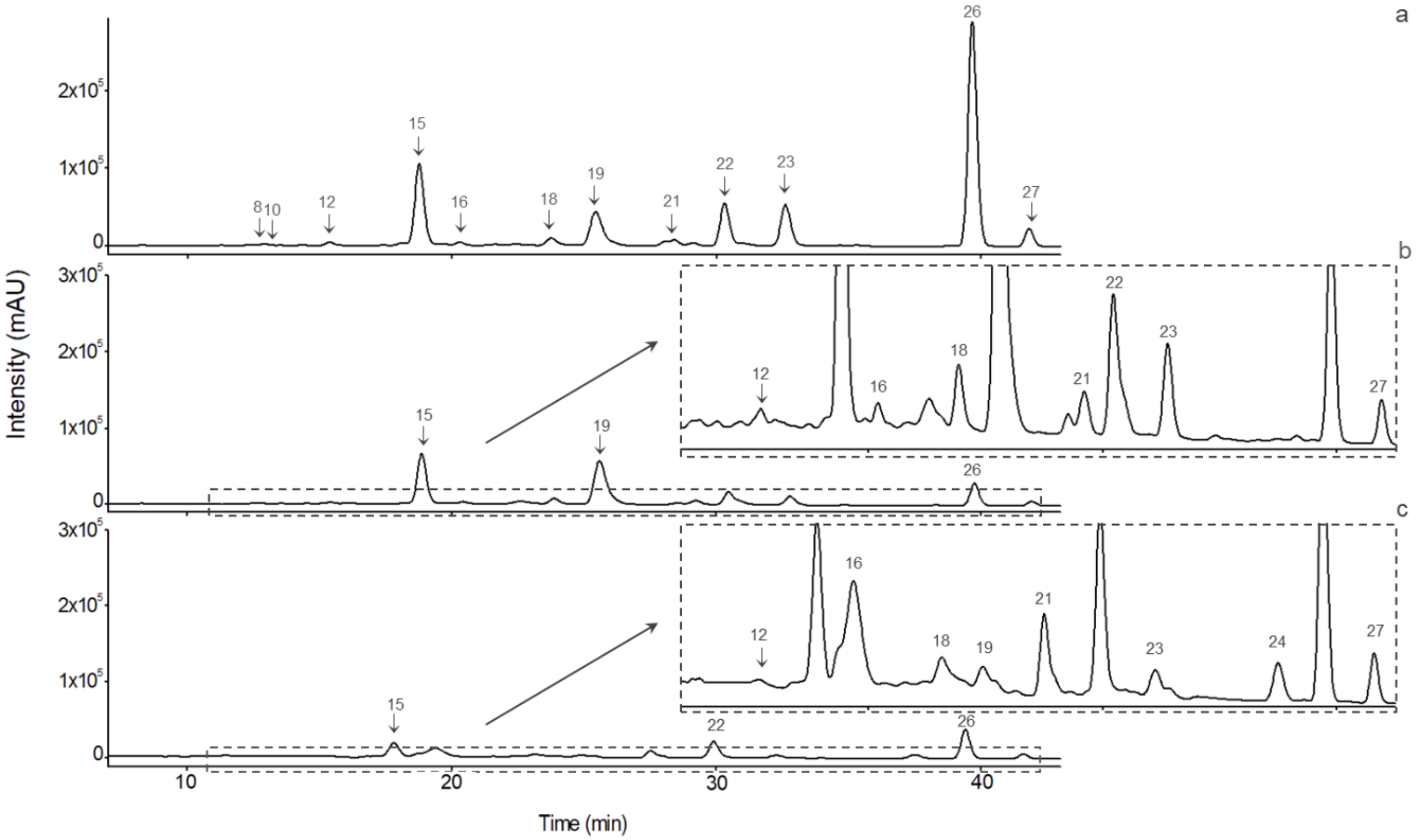

Figure 2. Representative chromatograms HPLC-PDA of *Spirulina* carotenoids. Control extract before digestion (a); WUB (b); ICE (c) after *in vitro* digestion. Peak identification and characterization are given in Table 1. Chromatogram was processed at 451 nm.

3.2. Bioaccessibility of carotenoids

To complete the knowledge about the effects of the *in vitro* digestion on carotenoids of products from *C. vulgaris* and *Spirulina* (WDB, WUB and ICE), the relative bioaccessibility (%) is reported in Table 3. This carotenoid bioaccessibility represents the relationship between the carotenoid content after simulated digestion and the content of carotenoids present in the biomass (before *in vitro* digestion).

The relative bioaccessibility of the total carotenoids in products from *C. vulgaris* was 1.65%, 6.12% and 10.04% for WDB, WUB and ICE, respectively (Table 3), which were significantly different. For WDB, the most bioaccessible carotenoids were 5,6:5',6'-diepoxy-β-carotene (18.33%), 9-*cis*-β-carotene (6.28%) and all-*trans*-violaxanthin (3.79%) while all-*trans*-β-carotene (1.35%) and all-*trans*-lutein (1.00%) were less bioaccessible. In WUB, the greatest bioaccessibility was found again for 5,6:5',6'-diepoxy-β-carotene (22.63%), along with 13-*cis*-lutein (16.42%) and 15-*cis*-lutein (7.09%). In contrast, all-*trans*-α-carotene (3.71%) and all-*trans*-β-carotene (1.39%) were less bioaccessible. In the ICE, the carotenoid with the greatest bioaccessibility was 5,6:5',6'-diepoxy-β-carotene (29.80%) followed by 9-*cis*-lutein (33.29%), and 3-*cis*-lutein (12.02%) while all-*trans*-β-carotene (5.56%) and all-*trans*-violaxanthin (5.19%) had the lowest relative values.

In *Spirulina* products WDB, WUB and ICE, the relative bioaccessibility of the total carotenoids was 9.45%, 15.92% and 29.82%, respectively. In WDB, the greatest bioaccessibility was found for all-*trans*-β-cryptoxanthin (46.85%), along with 9-*cis*-β-carotene (24.12%) and 2'-dehydrodeoxymyxol (16.60%). On the other hand, all-*trans*-zeaxanthin (9.51%) and all-*trans*-β-carotene (5.83%)

were less bioaccessible. In the WUB the carotenoid with the greatest bioaccessibility was all-*trans*-cantaxanthin (99.55%) followed by all-*trans*-β-cryptoxanthin (51.35%), and 9-*cis*-zeaxanthin (38.80%) while 9-*cis*-echinenone (11.76%) and all-*trans*-β-carotene (5.98%) had the lowest relative values. For ICE, the most bioaccessible carotenoids were all-*trans*-cantaxanthin (95.55%), all-*trans*-β-cryptoxanthin (61.74%) and all-*trans*-echinenone (60.07%). In contrast, 9-*cis*-echinenone (8.61%) and 2'-dehydrodeoxymyxol (4.91%) were less bioaccessible.

Processing operations in the microalgae biomass of both microalgae species, *C. vulgaris* and *Spirulina*, improved significantly total bioaccessibility for WUB and ICE products, but at different extents, as previously reported for bioaccessibility of carotenoids (Nascimento et al., 2021) (Table 3).

In *C. vulgaris*, the total bioaccessibility increased by 83.56% for ICE and 73.04% in WUB in comparison to the WDB. Additionally, when compared to WDB, the WUB significantly increased the bioaccessibility of all-*trans*-violaxanthin (4.82%), all-*trans*-lutein (4.01%), 5,6:5',6'-diepoxy-β-carotene (22.63%), all-*trans*-α-carotene (3.71%). Contrastingly, it had no significant effect for all-*trans*-β-carotene, and 9-*cis*-β-carotene in relation to WDB. When applying ICE, significantly improved bioaccessibility of all carotenoids detected after simulated digestion concerning to WDB. Moreover, when compared to the use of WUB, there was an increase, even if not significant, in most carotenoids, except 13-*cis*-lutein.

In this line for *Spirulina*, the use ICE had a very significant ($p<0.05$) positive impact on bioaccessibility (about 68%) and WUB (1.69-fold) compared to WDB (Table 3). Besides, the bioaccessibility of all individual carotenoids in

WUB and ICE was higher than the bioaccessibility values determined for WDB. Moreover, when compared to WDB, the WUB significantly improved the bioaccessibility of all-*trans*-zeaxanthin (15.11%), 2'-dehydrodeoxymyxol (21.08%), all-*trans*-β-cryptoxanthin (51.35%), all-*trans*-echinenone (14.87%). While, it had no significant effect for all-*trans*-β-carotene, and 9-*cis*-β-carotene in relation to WDB.

The use ICE, significantly improved bioaccessibility of all carotenoids detected after simulated digestion concerning to WDB. Additionally, when compared to the applying of WUB, the bioaccessibility 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%). On the other hand, in the ICE, the bioaccessibility of all-*trans*-cantaxanthin, 9-*cis*-zeaxanthin, 2'-dehydrodeoxymyxol and 9-*cis*-echinenone showed in a lower bioaccessibility comparable to that found in WUB.

As observed for the WUB product, the use of sonication can be considered a useful processing method to enhance the bioaccessibility of intracellular lipophilic compounds present in microalgae cells. Recently, Nascimento et al. (2021) proved a significant increase in the bioaccessibility of carotenoids from the sonicated *Scenedesmus obliquus* biomass. The 9-*cis*-neoxanthin, 13-*cis*-lutein, all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-cryptoxanthin, all-*trans*-echinenone and all-*trans*-α-carotene increase in its bioaccessibility, while all-*trans*-neoxanthin, all-*trans*-β-carotene, and 9-*cis*-β-carotene remained almost unchanged. In accordance with these results, our study also demonstrated a significant increase (Table 3) in carotenoids when

using the ultrasonicated treatment in the wet biomass for both microalgae species.

Assessing the ICE product of the different species *C. vulgaris* and *Spirulina*, our study results are in accordance with those found by Nascimento et al. (2020) where the bioaccessibility and incorporation of carotenoids in mixed micelles, from microalgae *Scenedesmus obliquus*, significantly improved with the application of the isolated carotenoid extract from the microalgae. Similarly, carotenoids extracted from the *Nannochloropsis* sp., showed a significant improvement in the transfer to the micellar phase, when compared to the carotenoids present in unprocessed biomass (Bernaerts et al., 2020). The same behavior was observed for carotenoids extracted from the *Scenedesmus almeriensis* biomass and added to olive oil, which significantly increased bioaccessibility compared to carotenoids present in lyophilized biomass (Granado-Lorencio et al., 2009).

Table 3. Relative bioaccessibility of WDB, WUB and ICE from *C. vulgaris* and *Spirulina* carotenoids. Different letters in the lines indicate a significant difference ($p<0.05$).

carotenoids	bioaccessibility (%)					
	WDB		WUB		ICE	
	C. vulgaris	*Spirulina* sp.	*C. vulgaris*	*Spirulina* sp.	*C. vulgaris*	*Spirulina* sp.
13-*cis*-neoxanthin	nd[1]	nd	nd	nd	nd	nd
13-*cis*-neochrome	nd	nd	nc[2]	nd	nc	nd
9-*cis*-neoxanthin	nd	nd	nd	nd	nd	nd
9-*cis*-neochrome	nd	nd	nc	nd	nc	nd
all-*trans*-violaxanthin	3.79±0.13[3,b]	nd	4.82±0.16[a]	nd	5.19±0.23[a]	nd
9-*cis*-violaxanthin	nd	nd	nc	nd	nc	nd
all-*trans*-luteoxanthin	nd	nd	nd	nd	nd	nd
13-*cis*-antheraxanthin	nd	nd	nd	nd	nd	nd
15-*cis*-lutein	nd	nd	7.09±0.23[b]	nd	11.52±0.17[a]	nd
all-*trans*-antheraxanthin	nd	nd	nd	nd	nd	nd
13-*cis*-lutein	nd	nd	16.42±0.27[a]	nd	12.02±0.07[b]	nd
15-*cis*-zeaxanthin	nd	nd	nd	15.31±0.07[b]	nd	51.39±0.22[a]
all-*trans*-lutein	1.00±0.01[c]	nd	4.01±0.13[b]	nd	8.26±0.08[a]	nd

9-*cis*-lutein	nd	nd	13.02±0.33[a]	nd	13.35±0.10[a]	nd
all-*trans*-zeaxanthin	nd	9.51±0.17[c]	nd	15.11±0.24[b]	nd	33.29±0.54[a]
all-*trans*-cantaxanthin	nd	nd	nd	99.55±1.24[a]	nd	95.55±1.19[b]
5,6:5',6'-diepoxy-β-carotene	18.33±0.31[c]	nd	22.63±0.38[b]	nd	29.80±0.10[a]	nd
9-*cis*-zeaxanthin	nd	nd	nd	38.80±0.69[a]	nd	22.91±0.41[b]
2'-dehydrodeoxymyxol	nd	16.60±0.05[b]	nd	21.08±0.32[a]	nd	4.91±0.08[c]
5,6-epoxy-β-carotene	nd	nd	nc	nd	nc	nd
all-*trans*-β-cryptoxanthin	nd	46.85±0.03[c]	nd	51.35±0.43[b]	nd	61.74±0.52[a]
all-*trans*-echinenone	nd	12.04±0.15[c]	nc	14.87±0.24[b]	nc	60.07±0.97[a]
9-*cis*-echinenone	nd	nd	nd	11.76±0.18[b]	nd	8.61±0.13[a]
13-*cis*-β-carotene	nd	nd	nd	nd	nd	nc
all-*trans*-α-carotene	2.33±0.11[c]	nd	3.71±0.13[b]	nd	6.77±0.10[a]	nd
all-*trans*-β-carotene	1.35±0.03[b]	5.83±0.23[a]	1.39±0.04[b]	5.98±0.10[a]	5.56±0.07[a]	26.23±0.43[A]
9-*cis*-β-carotene	6.28±0.11[c]	24.12±0.07[b]	6.31±0.14[c]	24.21±0.43[b]	8.89±0.10[a]	28.96±0.52[A]
Total	**1.65±0.03[c]**	**9.45±0.15[C]**	**6.12±0.12[b]**	**15.92±0.14[B]**	**10.04±0.22[a]**	**29.82±0.10[A]**

[1]Not detected

[2]Not calculated.

[3]Values are average and standard deviation of triplicates.

4. Conclusion

In conclusion, the results of our comparison of using three different forms of ingestion of carotenoids pigments from two commercial microalgae species, *C. vulgaris* and *Spirulina*, showed convergence with promoting the bioaccessibility. Specifically, our study showed that the compounds confined in WDB have a low bioaccessibility index, which can be improved in WUB. Although, we observed greater efficiency of ICE use in promoting the higher carotenoids bioaccessibility.

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CONCLUSÃO GERAL

Finalmente, com base nos resultados os carotenoides das duas espécies comerciais de microalgas, *C. vulgaris* e *Spirulina*, no produto WDB apresentaram baixa bioacessibilidade. No entanto, eles foram melhorados quando os produtos WUB e ICE foram usados para ambas espécies. Para a microalga *C. vulgaris* all-*trans*-luteína foi o carotenoide mais abundante nas micelas para todos os produtos (WDB, WUB e ICE) utilizados. Enquanto para *Spirulina*, 2'-desidrodeoximixol foi o principal carotenoide incorporado dos produtos WDB e WUB, para o ICE o carotenoide dominante micelarizado foi all-*trans*-β-caroteno. Em relação à bioacessibilidade, os produtos WDB, WUB e ICE de *C. vulgaris* apresentaram 5,6:5',6'-diepoxi-β-caroteno como carotenoide mais bioacessível. Por outro lado para *Spirulina*, all-*trans*-β-criptoxantina foi o composto com maior bioacessibilidade no produto WDB enquanto para os produtos WUB e ICE all-*trans*-cantaxantina apresentou maior bioacessibilidade. Desta forma, os resultados deste estudo podem auxiliar na formulação de alimentos e produtos funcionais a partir de ingredientes de microalgas.