UNIVERSIDADE FEDERAL DE SÃO PAULO

ESCOLA PAULISTA DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM NUTRIÇÃO

MONIZE BÜRCK

DESENVOLVIMENTO DE SORVETE ADICIONADO DE PIGMENTOS EXTRAÍDOS DE *SPIRULINA*: AVALIAÇÃO DOS PARÂMETROS DE COR, BIOACESSIBILIDADE E ATIVIDADE ANTIOXIDANTE

Dissertação apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina para obtenção do Título de Mestre em Ciências.

SÃO PAULO – SP

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Orientadora: Profa. Dra. Anna Rafaela Cavalcante Braga.

Coorientadora: Profa. Dra. Daniella Carisa Murador

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Chefe do Departamento:

Prof. Dr. Daniel Araki Ribeiro

Coordenador(a) do Curso de Pós-graduação:

Profa. Dra. Eliane Beraldi Ribeiro

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“Eu quase que nada não sei.

Mas desconfio de muita coisa”

Guimarães Rosa

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RESUMO

A *Spirulina* (*Arthrospira platensis*) é uma cianobactéria comumente consumida como alimento ou suplemento, que apresenta proteínas, ácidos graxos poli-insaturados e compostos bioativos denominados C-ficocianina (C-FC), aloficocianina e ficoeritrina. O composto bioativo predominante e de maior atividade antioxidante da *Spirulina* é a C-FC. Trata-se de uma ficobiliproteína hidrossolúvel e de coloração azul brilhante que pode ser usada como corante natural em alimentos. Este composto apresenta atividade antioxidante, uma vez que é capaz de interagir com espécies reativas de oxigênio, atuando assim na prevenção de vias patológicas, como inflamações, hepatopatias e câncer, dentre outras. A atividade antioxidante da C-FC depende da manutenção da sua estabilidade após a extração, pois exposição à luz, variações de temperatura e de pH aumentam sua susceptibilidade à degradação, sendo a aplicação da C-FC um desafio tecnológico para a indústria de alimentos. Tendo em vista o amplo consumo de gelados comestíveis, e sua temperatura de processamento e armazenamento, o objetivo deste trabalho foi desenvolver uma formulação de sorvete adicionada de C-FC como corante natural e adoçada com sacarose ou maltodextrina e determinar a capacidade antioxidante, estabilidade da cor e a bioacessibilidade *in vitro*. Visando o aproveitamento integral da cianobactéria, a biomassa residual (BR) após a extração de C-FC também foi adicionada em sorvete para comparação dos resultados. Ambas as formulações apresentaram estabilidade da cor durante os seis meses de análise e apresentaram atividade antioxidante. A atividade antioxidante, expressa em equivalentes de Trolox, sofreu um aumento de 2,68 vezes no sorvete contendo sacarose e C-FC, e 2,48 vezes no sorvete com sacarose e BR após digestão *in vitro* (41,3 ± 12,6 e 38,0 ±14,3) respectivamente, demonstrando seu potencial efeito biológico na saúde humana.

Palavras-chave: C-ficocianina; efeitos biológicos; estabilidade de cor, tecnologia de alimentos; desenvolvimento de produtos alimentícios.

ABSTRACT

Spirulina (*Arthrospira platensis*) is a cyanobacterium commonly consumed as food or supplement, which has proteins, polyunsaturated fatty acids and bioactive compounds called C-phycocyanin (C-PC), allophycocyanin and phycoerythrin. The predominant bioactive compound with the highest antioxidant activity in *Spirulina* is C-PC. It is a water-soluble, bright blue phycobiliprotein that can be used as a natural food coloring. This compound has antioxidant activity, since it is able to interact with reactive oxygen species, thus acting in the prevention of pathological pathways, such as inflammation, liver diseases and cancer, among others. The antioxidant activity of C-PC depends on the maintenance of its stability after extraction, as exposure to light, temperature and pH variations increase its susceptibility to degradation. As a result, the application of C-PC is a technological challenge for the food industry. Considering the great ice cream consumption and its processing and storage temperature, the objective of this work was to develop an ice cream formulation added with C-PC as a natural coloring and sweetened with sucrose or maltodextrin and to determine the antioxidant capacity, color stability and in vitro bioaccessibility. Aiming to fully utilize the cyanobacteria, the residual biomass (RB) after C-PC extraction was also added to ice cream for results comparison. Both formulations showed color stability during the six months of analysis and showed antioxidant activity. Antioxidant activity was expressed in Trolox equivalent (TE) and increased by 2.68-fold in ice cream containing sucrose and C-PC and 2.48-fold in ice cream with sucrose and RB after in vitro digestion (41.3 ± 12.6 and 38.0 ±14.3) respectively, demonstrating its potential biological effect on human health.

Keywords: Phycocyanin; biological effects; color stability, food technology; food product development.

APRESENTAÇÃO

Essa dissertação foi estruturada de modo a apresentar dois artigos gerados a partir da (I) revisão da literatura acerca da utilização da C-ficocianina (C-FC) em alimentos e (II) dos resultados obtidos através dos resultados experimentais produzidos. O primeiro foi submetido à revista *Food Chemistry*, após as considerações da banca.

Com vistas à fluidez da leitura, a dissertação foi dividida em três capítulos seguidos pela conclusão geral. No primeiro capítulo encontram-se a introdução, justificativa e objetivos da pesquisa; no segundo a revisão bibliográfica está apresentada a partir de uma ótica sustentável e que contempla as funções tecnológicas e resultados obtidos com a aplicação da C-FC em matrizes alimentares e em embalagens de alimentos nos últimos dez anos. Este artigo é intitulado "*Spirulina and phycocyanin food technological appeal: the future is green and blue*". Tal revisão proporcionou um arcabouço teórico robusto para o desenvolvimento dos sorvetes aqui propostos e criou um panorama de aplicação da C-FC para o grupo de pesquisa.

O terceiro capítulo corresponde ao artigo de resultados experimentais, onde foram organizados e discutidos os resultados obtidos a partir da elaboração de seis diferentes formulações de sorvete contendo C-FC ou biomassa residual (BR) de *Spirulina*, obtida após extração da C-FC, ambos adoçados com sacarose ou maltodextrina: sacarose controle (SC), sacarose e C-FC (SC-FC), sacarose e biomassa residual (SBR), maltodextrina controle (MC), maltodextrina e C-FC (MC-FC), e maltodextrina e biomassa residual (MBR). Após a formulação dos produtos foram realizadas análises microbiológicas, avaliação da composição em bioativos, atividade antioxidante e análise de cor no período de seis meses de todas as formulações. Também foi avaliada a bioacessibilidade dos bioativos presentes submetendo os sorvetes à digestão *in vitro* e, novamente, a determinação da atividade antioxidante após esta etapa. Esses resultados foram compilados e estão apresentados na forma de artigo de pesquisa

original e intitulado: "*Naturally colored ice-creams: antioxidant activity after in vitro digestion and color stability*" .

Por fim, há a conclusão geral seguida pelas considerações finais.

SUMÁRIO

CAPÍTULO I

Introdução e Objetivos

1 INTRODUÇÃO

As cianobactérias do gênero *Spirulina* (*Arthrospira platensis*) utilizam energia solar para capturar CO_2 da atmosfera e formar uma biomassa (MORAES *et al*., 2013) com cerca de 57,5% de proteínas, 23,9% de carboidratos e 7,72% de lipídeos em base seca (USDA, 2019). A Agência Nacional de Vigilância Sanitária possui 121 registros de alimentos contendo *Spirulina* em sua composição (ANVISA, 2022) e dosagens de 2 a 14 g são seguras inclusive para tratamento de dislipidemias (BOHÓRQUEZ-MEDINA *et al.*, 2021). O extrato de *Spirulina* é reconhecido pela *Food and Drug Administration* dos EUA como um ingrediente seguro, de uso regulamentado como corante isento de certificação e listado permanentemente para uso alimentar pelo Código de Regulamentações Federais (Título 21, Capítulo 1, Subcapítulo A, Parte 73) para aplicação em gomas de mascar, coberturas e coberturas de sobremesas, sobremesas congeladas, sorvetes, iogurtes, requeijão, entre outros usos alimentícios (CFR, 2022).

Entre as ficobiliproteínas extraídas da *Spirulina*, estão a C-FC, a aloficocianina e a ficoeritrina, sendo a C-FC (Figura 1B) o pigmento fotossintético mais abundante nesta matriz (Figura 1A) (ARASHIRO *et al.*, 2020; PATIL *et al*., 2006) e o de maior atividade antioxidante (PIÑERO ESTRADA *et al*., 2001), atuando na ativação celular de enzimas antioxidantes, inibição de danos ao DNA, inibição da peroxidação lipídica e neutralização de radicais livres (ABDELKHALEK *et al.,* 2015; BERMEJO *et al*., 2008; BERMEJO-BESCÓS *et al*., 2008) . Ademais, propriedades anticancerígenas, imunomoduladoras, anti-obesogênicas e relacionadas à saúde reprodutiva também são observadas (BOHÓRQUEZ-MEDINA *et al.*, 2021; HU *et al.*, 2019; SALGADO *et al.*, 2022; WEN *et al.*, 2020). O consumo regular de C-FC auxilia, portanto, na manutenção da saúde humana por meio da interação com espécies reativas de oxigênio (ERO) geradas a partir de processos fisiológicos, prevenindo o estresse oxidativo e

consequentes vias patológicas (BERMEJO *et al*., 2008; BERMEJO-BESCÓS et al., 2008; WU *et al.*, 2016).

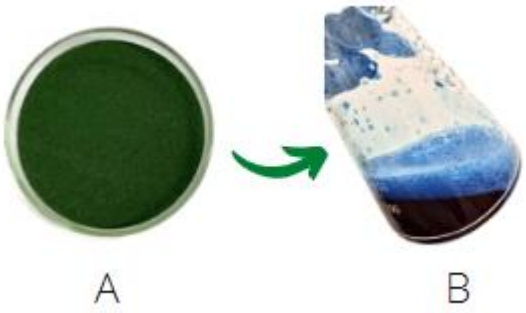

Figura 1. Elaboração própria. *Spirulina* (A) e C-ficocianina (B)

Por se tratar de um pigmento hidrossolúvel de coloração azul fluorescente (ERIKSEN, 2008), a C-FC tem aplicações na indústria farmacêutica e alimentícia, sendo descrita como biomarcador, por exemplo na elaboração de nanoprobe fluorescente ratiométrico para detecção de peroxinitrito, um tipo de ERO elevado em doenças vasculares (SHAO *et al*., 2022). Como corante natural já foi aplicada em alimentos como gelados comestíveis com preservação da atividade antioxidante (AMARANTE;*et al.*, 2020), confeitos e doces e ainda com função tecnológica emulsificante também em alimentos (CHEN, 2020; RODRIGUES et al., 2019).

Vale ressaltar que o processo de purificação da C-FC era um desafio ao seu emprego em larga escala, pois envolvia mais de uma etapa, incluindo cromatografia e costumava corresponder a 50-80% dos custos de produção, porém, atualmente, a purificação em uma única etapa é factível para a obtenção de C-FC com pureza de grau alimentar (>0,7) (MORAES *et al*., 2015), reduzindo custos para a sua incorporação em produtos alimentícios.

Ainda do ponto de vista da viabilidade econômica para a comercialização de produtos adicionados de C-FC extraída da biomassa de *Spirulina*, é importante discutir outros usos da biomassa após a extração da C-FC, a chamada biomassa residual (BR), afinal, esta mantém seu

conteúdo de proteínas, carboidratos e lipídios, que podem ser empregados, por exemplo no desenvolvimento de alimentos proteicos (SINGH *et al.*, 2015), embalagens de alimentos (LUO *et al*., 2021) e biodiesel (MOSTAFA; EL-GENDY, 2017), respectivamente. Outro fator econômico e ambiental que deve ser listado é o cultivo da biomassa de *Spirulina* independente do solo, em fotobiorreatores ou sistemas abertos com água de reuso (ARASHIRO *et al.*, 2020; LIM *et al.*, 2021) que tendem a reduzir os custos de produção e comercialização da mesma.

Diante da potencial viabilização comercial, ambiental e dos efeitos biológicos ligados à biomassa de *Spirulina* (verde) e à C-FC (azul), o estudo da aplicação destes componentes como pigmentos naturais em matrizes alimentares é atrativo e alia-se à tendência de substituição de aditivos sintéticos e corantes artificiais em produtos para consumo humano (OPLATOWSKA-STACHOWIAK; ELLIOTT, 2017), relacionados à carcinogenicidade, hiperatividade e hipersensibilidade (MOTA *et al*., 2021). No entanto, a estabilidade da cor natural da C-FC é um grande desafio para a pesquisa e para a indústria, afinal, tal molécula é sensível ao calor, à exposição luminosa e a alterações no pH (BRAGA *et al.*, 2016; CHAIKLAHAN *et al*., 2012; MARTELLI *et al.*, 2014), sendo o valor de pH ótimo entre 5,5 e 6,0 e a melhor condição de armazenamento, ao abrigo da luz (ADJALI *et al*., 2022). O esmaecimento da cor azul está relacionado à precipitação das proteínas quando a C-FC passa por tratamento térmico (CHAIKLAHAN *et al*., 2012) e à mudanças na conformação dos cromóforos ligadas às alterações no pH (FALKEBORG *et al*., 2018).

Nesse contexto, glicose, sacarose ou cloreto de sódio funcionam como agentes estabilizantes envolvendo e protegendo a superfície da molécula de C-FC, diminuindo assim perdas em sua concentração (CHAIKLAHAN *et al*., 2012). Sob refrigeração (0 ± 5 ºC), a queda na concentração de C-FC é reduzida e a presença de sacarose ou cloreto de cálcio ou ambos em associação tiveram efeito positivo na estabilidade da C-FC de grau alimentar (>0,7) em baixas temperaturas (MISHRA *et al*., 2008). Estes fatores somados à atividade biológica da C-FC e

da *Spirulina* motivaram o desenvolvimento de sorvetes naturalmente coloridos, azul e verde, respectivamente. Como a maior estabilidade da molécula de C-FC ocorre em temperaturas mais baixas e em presença de carboidratos, a escolha do sorvete foi racional. A base escolhida para o produto foi leite fermentado, uma vez que MOHAMMADI-GOURAJI *et al*., (2019) verificaram maior aceitação de iogurte contendo 4% de C-FC em comparação com o iogurte contendo 2%, sugerindo que diante da acidez, maiores concentrações de C-FC poderiam ser convenientemente aplicadas. Além disso, a diversidade de sorvetes coloridos existentes no mercado e a difusa aceitação desses produtos (SAREMNEZHAD *et al*., 2020), bem como de leites fermentados e iogurtes torna o presente trabalho atual e inovador, especialmente pela cor azul ser incomum em alimentos. O trabalho também se encontra oportunamente ligado à utilização integral da biomassa de *Spirulina*, empregando a BR para criar um sorvete de coloração verde, também incomum no mercado, salvo os sabores menta e pistache.

O objetivo do trabalho, portanto, foi elaborar diferentes formulações de sorvete contendo C-FC ou BR de *Spirulina*, adoçados com sacarose ou maltodextrina. Adicionalmente, a atividade antioxidante antes e após digestão *in vitro*, a estabilidade da cor e a bioacessibilidade dos sorvetes foram avaliadas.

2 OBJETIVOS

2.1 Objetivo geral

Desenvolver sorvetes adicionados de pigmentos extraídos de *Spirulina* e avaliar os parâmetros de estabilidade da cor, bioacessibilidade e atividade antioxidante.

2.2 Objetivos específicos

- Realizar uma revisão bibliográfica para evidenciar os resultados de aplicação de C-FC e suas funções tecnológicas em produtos alimentares;
- Extrair C-FC a partir de *Spirulina* (*Arthrospira platensis*) e purificar para atingir pureza de grau alimentício ($\geq 0,7$);
- Desenvolver sorvete adicionado de C-FC como corante alimentício azul natural e potencial efeito biológico;
- Desenvolver sorvete adicionado da BR de *Spirulina* após a extração de C-FC, visando o aproveitamento integral da matéria-prima;
- Determinar a qualidade microbiológica das formulações após a produção e após seis meses de armazenamento;
- Determinar e comparar a atividade antioxidante entre as formulações antes e após a bioacessibilidade;
- Analisar os parâmetros de cor no sorvete durante seis meses de armazenamento.

3 MATERIAL E MÉTODOS

3.1 Revisão da literatura

Foi realizada uma busca na base de dados da "*Elsevier Scopus*" para entender os principais aspectos relativos à utilização da C-FC extraída da biomassa de *Spirulina* em publicações de desenvolvimento de produtos alimentícios. Os critérios de busca foram ((("spirulina" AND "platensis" OR "phycocyanin" OR "c-phycocyanin") AND ("food product" OR "food application" OR "functional food" OR "food technology" OR "incorporation" OR "fortification" OR "enrichment" OR "packaging" OR "biotechnology"))). Foram encontrados 429 artigos desde 1971; após limitação de período para os últimos 10 anos, isto é, contemplando de 2012 a 2022, foram avaliados 310 artigos; que, após leitura de título, resumo e palavras-chave resultaram em69 estudos no escopo da pesquisa. Essencialmente, a investigação incluiu o *Scopus*, mas não se limitou a tal base, tendo o processo de leitura ampliado consistentemente para outros estudos do *PubMed* e *Web of Science.*

3.2 Experimental

3.2.1 *Spirulina*

A biomassa orgânica seca comercial Fazenda Tamanduá® de *Spirulina* (*Arthrospira platensis*), cuja composição/100 g corresponde a: 333,3 kcal; 20 g carboidratos; 53,3 g proteínas; 6,6 g gorduras totais; 2,6 g gorduras saturadas; 6 g fibras; 200 mg cálcio; 2 mg ferro; 1,26 g sódio foi obtida e armazenada em freezer (-40 ºC) para a extração de C-FC.

3.2.2 Extração verde sólido-líquido e purificação de C-FC

A biomassa foi retirada do ultrafreezer (-40 ºC) 1 hora antes do início da extração, e homogeneizada em moinho analítico (IKA A11 Basic) para posterior separação das partículas em peneira de abertura 0.106 mm mesh/tyler 150. Em seguida, 4 g de amostra foram

adicionados a 25 mL de água destilada e submetidos à repouso à temperatura ambiente e ao abrigo de luz durante 1 hora (MORAES *et al*., 2010). A mistura foi centrifugada durante 60 min a 3041 *x* g, em ciclos de 15 min cada. O sobrenadante foi rapidamente separado do corpo de fundo a cada ciclo e centrifugado novamente nas mesmas condições. A fração líquida passou por filtração à vácuo e filtro de seringa de 0,22 μm e foi armazenada em ultrafreezer e ao abrigo de luz. A fração sólida também foi armazenada em ultrafreezer.

Para a purificação parcial da C-FC, foi realizada precipitação fracionada com $(NH_4)_2SO_4$ (AMARANTE et al., 2020a; SILVA et al., 2009) a fim de obter um extrato grau alimentício (pureza > 0,7). No primeiro fracionamento, foi adicionado sulfato de amônio sólido ao extrato de C-FC clarificado em uma concentração de saturação equivalente a 20% (m/v), sob agitação constante por 30 min, seguido de incubação a 4 °C por 2 h (FIGUEIRA et al., 2016). Após esse tempo, a amostra foi centrifugada (3.041 *x* g a 10°C por 20 min), e o precipitado foi descartado. No segundo fracionamento, foi adicionado sulfato de amônio sólido ao sobrenadante da etapa anterior até 50% (m/v) de saturação. A solução foi mantida nas mesmas condições do primeiro fracionamento e centrifugada. O sobrenadante foi descartado e o precipitado foi ressuspendido em tampão fosfato de sódio 0,05 mol.L-1 pH 7,0 na razão volume de ressuspensão/volume inicial de 0,52. Os precipitados ressolubilizados foram dialisados usando tampão fosfato de sódio 0,05 $mol.L^{-1}$ pH 7,0 para a remoção do sal. A concentração, pureza, fator de purificação e recuperação de C-PC foram avaliados antes e após os processos de precipitação e diálise.

3.2.3 Métodos analíticos

3.2.3.1 Concentração e pureza de C-FC

A concentração de C-FC ($mg.mL^{-1}$, Equação 1) foi obtida a partir do cálculo proposto por BENNETT e BOGORAD, (1973) com comprimento de onda modificado (MORAES e KALIL, 2009), onde A_{652} denota a presença de aloficocianina (PATIL *et al*., 2008). A pureza

do extrato (PE, Equação 2) foi calculada a partir das absorbâncias A_{620} (620 nm) e A_{280} (280 nm), cujos valores indicam concentração de C-FC e concentração de proteínas na solução, respectivamente (SALA *et al*., 2018). As leituras das absorbâncias (A) a 280, 620 e 652 nm foram realizadas em espectrofotômetro UV-visível (Shimadzu, Kyoto, Japão).

$$CFC = \frac{(A602 - (0{,}474 \times (A652))}{5{,}34} \quad (1)$$

$$PE = \frac{A620}{A280} \quad (2)$$

3.2.3.2 Rendimento de extração

O rendimento (R em $mg.g^{-1}$, Equação 3) foi calculado a partir da relação entre a concentração de C-ficocianina (C-FC em $mg.mL^{-1}$), o volume de solvente (V em mL) e a biomassa seca (BS em g).

$$R = \frac{CFC \times V}{BS} \quad (3)$$

3.2.3 Aplicação de C-FC em sorvete

Para a formulação dos produtos, foi utilizada receita de leite fermentado (Tabela 1) desenvolvida pelo grupo de pesquisas do Laboratório de Compostos Bioativos de Alimentos da Universidade Federal de São Paulo (FONSECA, 2017) e os demais ingredientes para a produção do sorvete (Tabela 2) foram combinados a partir da realização de testes preliminares para modificações na receita do fabricante de sorvetes Duas Rodas Industrial®.

3.2.4 Leite fermentado

O leite fermentado (Tabela 1) para a base do sorvete foi obtido pela combinação de leite pasteurizado desnatado, cultura láctea comercial (contendo *Lactobacillus acidophilus, Bifidobacterium* e *Streptococcus thermophilus*), sacarose ou maltodextrina, leite de vaca

integral em pó e gelatina sem sabor. O leite pasteurizado integral foi aquecido até atingir 80 ºC e os ingredientes secos adicionados gradualmente, de modo a garantir a homogeneidade da mistura. A cultura láctea comercial foi dissolvida quando a mistura atingiu 40 ºC e posteriormente, todo o conteúdo foi transferido para recipientes devidamente embalados colocados em estufa a 40 ºC durante 4 h para a fermentação (FONSECA, 2017). Os ensaios ocorreram em triplicata.

Tabela 1. Leite fermentado contendo sacarose ou maltodextrina

Ingrediente	Sacarose	Maltodextrina
	Concentração (%, m/m)	Concentração (%, m/m)
Leite integral pasteurizado	95,83	95,83
Leite em pó integral	2,87	2,87
Sacarose	0,96	-
Maltodextrina	-	0,96
Gelatina	0,24	0,24
BioRich®*	0,09	0,09

*Produzido por Chr.Hansen

3.2.5 Sorvete controle, adicionado de C-FC e adicionado BR de *Spirulina*, contendo maltodextrina ou sacarose.

Foram elaboradas duas formulações de sorvete, uma delas contendo maltodextrina e outra com sacarose. Foram divididas em: (I) controle; (II) adicionado de C-FC e (III) adicionado de BR de *Spirulina*. Com o leite fermentado base finalizado, foram adicionados sacarose ou maltodextrina, leite em pó integral, creme de leite e estabilizante em pó comercial e homogeneizados em batedeira. Transcorridos 3 min, a mistura foi congelada a -18 ºC por um período de 4 h. Foram adicionados emulsificante em pasta, liga neutra Duas Rodas Industrial® e C-FC (0,2 $mg_{C\text{-}FC}.mL_{sorvete}^{-1}$) em nova etapa de homogeneização durante 5 min e armazenamento a -18 °C. A formulação contendo biomassa e a formulação controle percorreram as mesmas etapas de produção, diferindo pelo acréscimo de biomassa de *Spirulina*

na primeira e pela não adição da C-FC em ambas. Os ensaios foram realizados em triplicata e amostras de cada sorvete foram liofilizadas e armazenadas para determinação de atividade antioxidante e bioacessibilidade.

Tabela 2. Formulações de sorvete controle, adicionado de C-FC e adicionado de BR, (adoçados com sacarose ou maltodextrina) utilizando base de leite fermentado

Ingrediente	Sorvete controle	Sorvete azul (C-FC)	Sorvete verde (BR)
	Concentração (%, m/m)	Concentração (%, m/m)	Concentração (%, m/m)
Leite fermentado	75	71.64	74.38
Sacarose ou maltodextrina	9.22	8.80	9.14
Leite em pó integral	6.57	6.28	6.51
Creme de leite fresco	7.88	7.49	7.77
Super liga neutra®*	0.65	0.62	0.64
Emustab®*	0.65	0.62	0.64
C-FC	-	4.55	-
BR	-	-	0.91

*Produzido por Duas Rodas Industrial®

3.2.6 Análise microbiológica dos sorvetes

A determinação da qualidade microbiológica dos sorvetes formulados foi realizada após a produção (tempo zero) e após 6 meses seguindo a legislação vigente para gelados comestíveis (RDC n° 331 de 23 de dezembro de 2019) (ANVISA, 2019). Com isso, foi avaliada a presença de *Staphylococcus aureus* coagulase positiva, *Salmonella*, *Enterobacteriaceae* como previstos na legislação e adicionalmente foram realizadas as análises de coliformes totais e *Escherichia coli*, e de micro-organismos mesófilos totais aeróbios. E para isso, utilizou-se a metodologia rápida Petrifilm® (3M), método validado pela *Association of Analytical Communities* (AOAC, 2005).

Para análise de *Salmonella*, foram pesados 1 g de cada amostra, as quais foram homogeneizadas individualmente em tubos de ensaio com 9 mL de água peptonada tamponada

estéril (1%) e, a partir desta, foram realizadas as diluições seriadas. A partir dessas diluições foi realizado o inóculo de 1 mL de cada diluição das referidas amostras para a quantificação de *Salmonella*. As placas de Petrifilm™ para os respectivos micro-organismos foram incubadas em estufa bacteriológica, seguindo os critérios estabelecidos pelo fabricante, por 18-24 h à 41,5 ºC e o resultado foi descrito em UFC/g.

Para análise de *Enterobacteriaceae* e coliformes à 35 ºC e *E. coli*., foram pesados 1 g de cada amostra, as quais foram homogeneizadas individualmente em tubos de ensaio com 9 mL de água peptonada tamponada estéril (1%) e, a partir desta, foram realizadas as diluições seriadas. A partir dessas diluições foi realizado o inóculo de 1 mL de cada diluição das referidas amostras para a quantificação de *Enterobacteriaceae*, coliformes à 35 ºC e *E. coli*. As placas de Petrifilm™ foram incubadas em estufa bacteriológica, seguindo os critérios estabelecidos pelo fabricante, por 24 h (quantificação de *Enterobacteriaceae* e coliformes à 35 ºC) e 48 h (quantificação de *E. coli*) e o resultado foi descrito em UFC/g.

Para as análises de *S. aureus* nas amostras de sorvetes, a quantificação desses micro-organismos seguiu o protocolo descrito pelo fabricante do sistema Petrifilm™. Inicialmente, foram pesados 1 g de cada amostra, as quais foram homogeneizadas individualmente em tubos de ensaio com 9 mL de água peptonada tamponada estéril (1%) e, a partir desta, foram realizadas as diluições seriadas. A partir dessas diluições foi realizado o inóculo de 1 mL de cada diluição das referidas amostras para a quantificação de *S. aureus*. As placas de Petrifilm™ foram incubadas em estufa bacteriológica, seguindo os critérios estabelecidos pelo fabricante, 35 °C por 48 h e o resultado foi descrito em UFC/g.

Para as análises de micro-organismos mesófilos totais aeróbios, foram pesadas 1 g de cada amostra, as quais foram homogeneizadas individualmente em tubos de ensaio com 9 mL de água peptonada tamponada estéril (1%) e, a partir desta, foram realizadas as diluições seriadas. A partir dessas diluições foi realizado o inóculo de 1 mL de cada diluição das referidas

amostras para a quantificação de micro-organismos mesófilos totais aeróbios. As placas de Petrifilm^{TM} foram incubadas em estufa bacteriológica, seguindo os critérios estabelecidos pelo fabricante, 32 °C por 48 h o resultado foi descrito em UFC/g.

3.2.7 Análise da estabilidade de cor dos sorvetes

A estabilidade da cor dos sorvetes formulados foi acompanhada semanalmente durante 182 dias (6 meses). A análise colorimétrica foi realizada através de um colorímetro espectrofotômetro portátil Konica Minolta modelo CM-25d. E os valores de L*, a* e b* foram determinados para cada formulação. Essa análise é baseada no Diagrama de cromaticidade a* b*.

O estudo da cor através desse equipamento é baseado no espaço de cor L* a* b*. As cores são expressas em termos de tonalidade, saturação e luminosidade. O valor de L* indica a luminosidade (0 – 100), as coordenadas a* e b* indicam a direção das cores, onde a* varia no espectro entre vermelho (valores positivos) e verde (valores negativos) e b* indica a quantidade de amarelo (valores positivos) ou azul (valores negativos), como mostra o diagrama de cromaticidade, Figura 2. O valor de Hue (h(º)) indica o ângulo da cor (0° vermelho; 90° amarelo; 180° verde; 270° azul), conforme a equação 4. Os valores de ΔE indicam o grau da diferença da cor e foram calculados no período de 182 dias e estão expressos na equação 5.

$$h\,(^{\circ}) = 180 + \tan^{-1}(b^*a^*) \quad (4)$$

$$\Delta E\,^*ab = \sqrt{(\Delta L\,*)^2} + (\Delta a\,*)^2 + (\Delta b\,*)^2 \quad (5)$$

3.2.8 Preparo dos extratos antioxidantes

Amostras das diferentes formulações de sorvete antes e após o ensaio de bioacessibilidade foram adicionadas de 20 mL de metanol e colocadas em agitador magnético

por 15 min. Em seguida, as amostras foram filtradas utilizando papel filtro qualitativo. O conteúdo foi lavado outras duas vezes acrescentando-se 10 mL de metanol (BRAGA *et al.*, 2018). As amostras foram filtradas em membrana de 0,22 μm previamente às análises de atividade antioxidante.

3.2.9 Determinação da atividade antioxidante empregando radical ABTS

O radical ABTS foi obtido através da reação entre 5 mL de solução estoque de ABTS (7 $mmol.L^{-1}$) e 88 μL de persulfato de potássio (140 $mmol.L^{-1}$) preparados em água e mantidos ao abrigo da luz e em temperatura ambiente durante 16 h. Esta solução foi diluída em etanol até absorbância de 0,7 ± 0,05 (734 nm) (RE *et al.*, 1999). Para determinação da capacidade antioxidante, 30 μL dos extratos metanólicos foram adicionados em 3 mL da solução de radical ABTS e a absorbância foi lida em espectrofotômetro (734 nm) após 6 min.

A determinação da atividade antioxidante total foi obtida a partir de curva padrão de Trolox em etanol, nas mesmas condições reacionais. Os resultados foram expressos em $\mu mol.g_{amostra}^{-1}$ de equivalentes de Trolox (TE).

3.2.10 Determinação da atividade antioxidante empregando método ORAC

A avaliação da atividade antioxidante foi dada pela capacidade de absorção de radicais oxigênio baseada na formação de radicais peroxila pela degradação térmica do AAPH (2,2′-azobis (2-amidinopropano) di-hidrocloreto) a 37 °C. 150 μL de fluoresceína (61 $nmol.L^{-1}$, preparada em tampão fosfato 75 $mmol.L^{-1}$ pH 7,4) foram pipetados em microplacas pretas de 96 poços, seguidos de 25 μL do extrato antioxidante em diferentes diluições (50, 100 e 1000 vezes em tampão fosfato). A microplaca foi incubada por 10 min a 37 °C, sob agitação intermitente, e após, em cada poço foram adicionados 25 μL de solução de AAPH (19 $mmol.L^{-1}$, preparado em tampão fosfato). As leituras da fluorescência (excitação 485 ± 20 nm e emissão

538 ± 20 nm) foram programadas para cada min, durante 60 min (RODRIGUES et al., 2012). Leituras do branco (solução tampão) e padrão de Trolox a 64 μM (controle positivo) também foram efetuadas. Os resultados foram expressos em $\mu mol.g_{amostra}^{-1}$ de equivalentes de Trolox (TE).

3.2.11 Quantificação de fenólicos totais

Utilizando o extrato previamente preparado de acordo com método descrito acima, o teor de compostos fenólicos totais foi determinado pelo método de Folin-Ciocalteu, segundo Singleton e Rossi, (1965) e expresso em equivalente de ácido gálico (EAG)/100g. Uma alíquota de 1 mL foi retirada de cada extrato ou das soluções padrão de ácido gálico (20, 40, 60, 80 e 100 mg/L). Os conteúdos foram transferidos para balão volumétrico de 25 mL, contendo 9 mL de água. O reagente de Folin-Ciocalteau (1 mL) foi adicionado e a mistura agitada. Após 5 min foram adicionados 10 mL de uma solução Na_2CO_3 7% e o volume completado com água. Após 90 min de incubação a 23 ºC, a absorbância foi determinada a 750 nm.

3.2.12 Avaliação da bioacessibilidade in vitro de C-FC

As amostras foram digeridas de acordo com o método proposto por Chitchumroonchokchai e Failla (2017). 2 g de sorvete liofilizado foram homogeneizados com 10 mL de solução de sais (NaCl 120 $mmol.L^{-1}$, $CaCl_2$ 6 $mmol.L^{-1}$, KCl 5 $mmol.L^{-1}$) e 6 mL de solução de saliva artificial contendo α-amilase (106 $U.mL^{-1}$) (Sigma® A3176). A fase oral foi simulada com a incubação em agitador orbital a 150 rpm, 37 °C por 10 min. A fase gástrica foi iniciada com o pH ajustado para 2,5 com HCl 1 $mol.L^{-1}$, e foram adicionados 2 mL de pepsina (Sigma® 110 P7000;40 $mg.mL^{-1}$ em 0,1 $mol.L^{-1}$ HCl). O volume foi completado para 40 mL e incubado a 37 ° C a 150 rpm durante 1 h. Para a fase intestinal e final, o pH foi ajustado para

6,0 com 1 mol.L^{-1} $NaHCO_3$ [1] e 3 mL de solução de bile suína (Sigma® 113 B8381; 40 mg.mL^{-1} em 0,1 mol.L^{-1} NaHCO3), 4.000 U.mL^{-1} de pancreatina suína (Sigma® 114 P1750) e 1.000 U.mL^{-1} de lipase de pâncreas suíno (Sigma® 115 L3126) foram dissolvidos em 1 mol.L^{-1} $NaHCO_3$ e adicionados às amostras, ajustando o pH para 6,5. O volume foi completado até 50 mL antes da incubação a 37°C, 150 rpm por 2 h. A última etapa foi centrifugar as amostras por 1 h a 3.041 *x* g a 10 °C. As amostras finais foram liofilizadas para avaliar a atividade antioxidante.

3.2.13 Análise estatística

Os dados foram apresentados como média e desvio padrão e para comparação de médias, os dados foram submetidos à análise de variância (ANOVA) e ao pós-teste de Tukey, considerando nível de confiança de 95% ($p < 0,05$).

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CAPÍTULO II

Spirulina and phycocyanin food technological appeal: the future is green and blue

Monize Bürck, Camilly Fratelli Pereira, Marina Campos Assumpção de Amarante, Monica Masako Nakamoto and Anna Rafaela Cavalcante Braga

Spirulina and phycocyanin food technological appeal: the future is green and blue

Monize Bürck[1,2], Camilly Fratelli Pereira[1,2,3], Marina Campos Assumpção de Amarante[4], Monica Masako Nakamoto[1,2] and Anna Rafaela Cavalcante Braga[1,2,5]

[1]Department of Biosciences, Universidade Federal de São Paulo (UNIFESP), Silva Jardim Street, 136, Vila Mathias, 11015-020, Santos, SP, Brazil.

[2]Department of Physiology, Universidade Federal de São Paulo (UNIFESP), Botucatu Street, 740, Vila Clementino, 04023-062, São Paulo, SP, Brazil.

[3]Universidade Metropolitana de Santos (UNIMES), General Francisco Glicério Avenue, 8, Encruzilhada, 11045-002, Santos, SP, Brazil.

[4]School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham, West Midlands, B15 2TT, United Kingdom.

[5]Department of Chemical Engineering, Universidade Federal de São Paulo (UNIFESP), Campus Diadema, Diadema, São Paulo, 09972-270, Brazil.

Correspondence: anna.braga@unifesp.br; Tel.: +55-13-98145-0201

Abstract

Background: *Spirulina* (*Arthrospira platensis*) is a cyanobacterium rich in bioactive compounds with antioxidant activity, predominantly C-phycocyanin (C-PC), which is related to anti-inflammatory and anti-carcinogenic potentials when frequently consumed. C-PC is a water-soluble blue pigment, that can be used as natural food dye. *Spirulina* biomass, after C-PC extraction, is rich in proteins and fatty acids with potential for developing food products, biofuels and green electricity, among other applications. **Scope and approach**: C-PC and *Spirulina* biomass as functional and technological ingredients for foods propelled this review, thus last 10 years studies were categorized and analyzed since the color stability of the molecule in these matrices is essential to meet the biological effects. The investigation was carried out under the lens of food science underpinned by circular economy concepts, addressing health and environmental demands since the interactions of food systems with economy and food industry can work synergistically. **Key findings and conclusions**: None of the food studies considered the application of the residual biomass after C-PC extraction. This potential needs to be explored, aiming at the full use of our resources. Technological challenges of preserving the color of the applied C-PC were overcome with unsophisticated procedures, such as controlling the temperature or more technologically, over nanotechnology. Besides the current lack of sensorial acceptance studies, blue foods are functional and innovative to the food industry. Residual *Spirulina* biomass are alternatively important for this and other sectors.

Keywords: Bioactive compounds; antioxidant activity; natural dye, technological ingredient; food industry; circular economy.

Highlights:

- C-PC's antioxidant activity were preserved in refrigerated food products;
- Frequent consumed foods are promising matrices to deliver health benefits from C-PC;
- Residual biomass is a potentiality that has not yet been explored
- C-PC- and Spirulina-added foods are innovative in a highly competitive market

Graphical abstract

1. Introduction

In a global scenario that encompasses the closely linked triad of climate changes, undernutrition, and obesity, as well as the synergy of these epidemics, i.e., Global Syndemic (SWINBURN *et al*., 2019), it is mandatory for the food industry to engage in strategic planning. Complex problems call for elaborated solutions. It takes technological, legal, economic, and strategic effort to reverse the Global Syndemic. To get ahead of such a joint effort, new products (mainly from vegetarian sources) and technologies for food processing are needed, where the production chains respect the environment and the food and nutrition security of the population.

Cyanobacteria of the genus *Spirulina* (*Arthrospira platensis*) use solar energy to capture CO_2 from the atmosphere, producing biomass (MORAES *et al*., 2013). The composition of the biomass (dry basis) is for about 57.5% proteins, 23.9% carbohydrates and 7,72% fatty acids (USDA, 2019). . *Spirulina* is not depended on land as conventional agriculture and livestock farming, as it can be cultivated in photobioreactors or opened systems with wastewater (ARASHIRO *et al.*, 2020; LIM *et al.*, 2021). Therefore, the recovery of promising sources of non-animal proteins and bioactive compounds such as C-phycocyanin (C-PC) and allophycocyanin are carried out respecting the environment and circular economy concepts, as soil biodiversity is protected, pollution is prevented from occurring at the outset. In addition, value added products are created, such as biofuel (MOSTAFA; EL-GENDY, 2017), green electricity (LIM *et al.*, 2021), natural food dye (AMARANTE *et al*., 2020a), high protein food products (SINGH *et al.*, 2015) and food packaging or active food packaging with *Spirulina* biomass, biopolymers and C-PC (CHENTIR *et al*., 2019; KUNTZLER *et al.*, 2020;).

Besides high aggregated value products and vast application possibilities, the C-PC purification process was a challenge to its use on a large scale, as it involved more than one step, including chromatography and used to correspond to 50-80% of the production costs. However, currently, purification in a single step is feasible to obtain food-grade purity C-PC

(>0.7) (MORAES *et al*., 2015; AMARANTE *et al*., 2020b), reducing costs for its incorporation into food products.

C-PC is the most abundant photosynthetic pigment among the *Spirulina* phycobiliproteins (ARASHIRO *et al.*, 2020; PATIL *et al*., 2006) and the one with the most expressive antioxidant activity PIÑERO ESTRADA *et al*., 2001), acting on cellular activation of antioxidant enzymes, inhibition of DNA damage, inhibition of lipid peroxidation and neutralization of free radicals (ABDELKHALEK *et al*., 2015; BERMEJO *et al*., 2008; BERMEJO-BESCÓS *et al*., 2008). Anti-inflammatory, anticarcinogenic, and immunomodulatory properties are also observed (ALADAILEH *et al.*, 2020; CZERWONKA *et al.*, 2018; SALGADO *et al.*, 2022; WU *et al.*, 2016). Therefore, bioactive compounds, including C-PC, supports human health maintenance through interaction with reactive oxygen species generated from physiological and metabolic processes, preventing oxidative stress and consequent pathological pathways (BERMEJO-BESCÓS *et al*., 2008; WU *et al.*, 2016).

Spirulina extract was recognized by the US Food and Drug Administration as a safe ingredient and had its use regulated as a colorant exempt from certification and permanently listed for food use by the Code of Federal Regulations (Title 21, Chapter 1, Subchapter A, Part 73) for application in chewing gum, dessert toppings, and coatings, frozen desserts, ice cream, yogurt, cottage cheese, among other food uses (CFR, 2022). As it is a water-soluble pigment with a strongly fluorescent blue color (ERIKSEN, 2008), C-PC has been remarkably used with a technological function in Food Science as a stable natural food dye with antioxidant properties in ice cream (AMARANTE *et al*., 2020a) and even as texturizing agents in the form of emulsifier (CHEN, 2020; RODRIGUES *et al.*, 2019). These food applications can be considered substantial, considering that color is associated with the sensory appeal of food (CAROCHO *et al*., 2015), and color stability is a great challenge for the industry.

C-PC in regularly consumed food products is in line with healthy eating trends around the world, as 31% of the global average are willing to pay for food without artificial colors (NIELSEN, 2015; OPLATOWSKA-STACHOWIAK; ELLIOTT, 2017). On the other hand, blue food is perceived as less natural since the natural blue color is infrequent compared to green, yellow and red, colors that industry has already commercialized. Consequently, natural blue food dyes can be considered the future of the food industry that seeks innovations to call consumers' attention in the face of a highly competitive market (NEVES *et al*., , 2021).

For instance, in a recent study, middle-aged volunteers (30–60 years) preferred blue potato salad (37.9%) in opposition to yellow potato salad under arguing that the dish was "new", "exceptional", "for a change" (PAAKKI *et al*., 2016). This group was more neophilic and had more disposition to try new foods compared to the others, since it has been previously reported that the aging process and its alterations in color discrimination by the lens and the blue cone mechanisms in the eyes affect the preference for blue color (DITTMAR, 2001). Thus, blue food products addressed to younger consumers constitute a strategic approach. Additionally, BORDIM *et al*., (2021) reported that the awareness of natural antioxidants positively influenced the perception and acceptance of the consumers, being an attractive claim to products with unique colors.

Correspondingly, this review aimed to present state of the art and insights regarding C-PC natural dye from *Spirulina* as a functional and technological ingredient for food products and exploitation of its biomass for food development. Nonetheless, future trends and knowledge gaps were addressed.

2. Bibliographic data analysis

An investigation was carried out in the *Elsevier Scopus* database to understand the main aspects regarding the contributions of C-PC extracted from *Spirulina* biomass on food products

development publications. The search criteria strategy was as follows: ((("spirulina" AND "platensis" OR "phycocyanin" OR "c-phycocyanin") AND ("food product" OR "food application" OR "functional food" OR "food technology" OR "incorporation" OR "fortification" OR "enrichment" OR "packaging" OR "biotechnology"))). A total of 429 articles were found since 1971; after a limitation time considering the years from 2012 to 2022, 310 articles were evaluated by reading title, abstract and keywords; among these, 69 studies were on the paper's topic.

Scopus's main functionalities (https://www.scopus.com/) have provided relevant information to analyze the bibliographic results. The top three research groups that most publish in this area are China, Brazil, and India, respectively. Until 2017, an average of 20 articles were published per year. A considerable increase occurred, reaching 57 publications in 2021, an increment of 185%. Essentially, the investigation included Scopus but was not limited to this database, due to the fact that the reading process consistently supported new studies from PubMed and Web of Science.

The VosViewer 1.6.13 software created the bibliometric network visualization map. Figure 1a shows five clusters, represented by different colors (blue, red, green, yellow, and purple), where the related terms are represented (VAN ECK; WALTMAN, 2019). The terms "*Spirulina"*, "biotechnology", and "phycocyanin" are the most relevant, characterized by larger labels (nodes) than the others. Small labels but also essential terms, in red, are related to metabolic characteristics of C-PC: "antioxidant activity", "functional food", and "diet supplementation"; in green, representing nutritional and technological food usage of *Spirulina*: "food products", "proteins", "fermentation", "color", "food storage", "quality control", "response surface methodology" and "bakeries" (Figure 1b), that identify the strongest ties (thick connectors) and weakest connections (thin connectors) that correspond to the most

frequent and least frequent co-occurrences between the terms, respectively (ECK; WALTMAN, 2019).

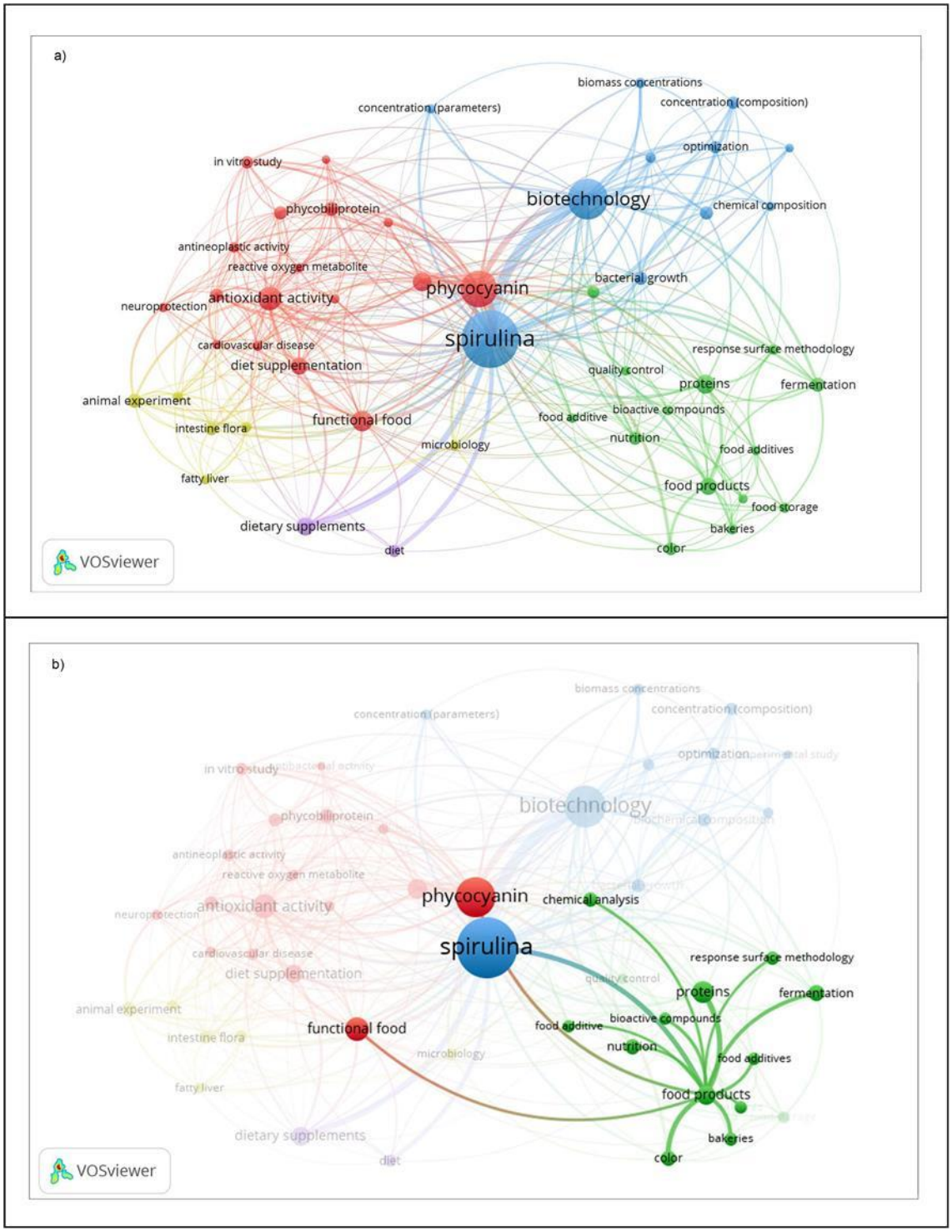

Figure 1. Bibliometric map Network visualization map resulting from Scopus keywords insertion "phycocyanin OR *Spirulina* AND food products development" (2012-2022) **(a)**;

Image representing the keywords links of nutritional and technological food usage of C-PC from *Spirulina*. Data from: VosViewer software version 1.6.13. (**b)**

Therefore, this analysis was appropriate to incite and elucidate perspectives regarding the terms highlighted and how the scientific literature has addressed C-PC in food applications. Henceforth, the most meaningful studies about this review's aims were covered.

3. Health effects

C-PC is recognized as a food colorant by ANVISA (National Health Surveillance Agency, Brazilian legislation), named as "Concentrado de alga Espirulina (*Arthrospira platensis*)" (ANVISA, 2019); and by FDA (Food and Drug Administration in Code of Federal United States Regulations), identified as "*Spirulina* extract" (FDA, 2001). In both regulations, C-PC, as a food ingredient, must be obtained exclusively by the filtered aqueous extraction of *Arthrospira platensis* dried biomass (ANVISA, 2019; FDA, 2001).

Furthermore, some differences in specifications are observed in legislation (ANVISA, 2019) has oriented the use of C-PC as a food additive (the maximum limit of use) in 1) candies and confectionery (0.38%); 2) ice cream and cold desserts (0.38%); 3) sugared coatings for sweets and confectionery products (0.38%); 4) powders for the preparation of drinks (0.035g in every 200 mL); 5) powders for the preparation of soups and sauces (0.50%); and 6) fruit preparation for yogurts (0.50%). The material published by the FDA (FDA, 2001) specifies that C-PC must be free from arsenic, mercury, and microcystin toxin impurities.

Studies reported by the literature have highlighted *Spirulina's* potential for the food and feed industrial applications (CAMACHO *et al*., 2019), which contribute to a sustainable environment (RAMÍREZ-RODRIGUES *et al.*, 2021), animals (HOLMAN; MALAU-ADULI, 2013; KHAN *et al.*, 2020) and humans' health (FERRAZZANO *et al.*, 2020).

Other scientific insights have demonstrated *Spirulina's* health effects are mainly because of its nutritional value, including macronutrients (mostly proteins and fatty acids, for about 57,5% and 7.72%, respectively (USDA, 2019), micronutrients (such as chromium, copper, and zinc), and bioactive compounds, besides carotenoids, chlorophylls and C-PC (MUYS *et al.*, 2019). According to that, ASHAOLU *et al*. (2021) have classified C-PC as a "super functional" ingredient due to its content of highly valuable substances.

FERRAZZANO *et al.* (2020) have defined functional foods as technologically developed ingredients amplifying specific actions on human health. Promising sources of functional foods, such as C-PC from cyanobacteria, are a dietary resource for preventing diseases. In the content below, in vivo and in vitro studies about *Spirulina* and C-PC health effects are described.

The utilization of probiotics *Lactobacillus plantarum* and *Bacillus subtilis* to ferment *Spirulina* was described by AN *et al*., (2020). These probiotics improved the immunomodulatory activity of *Spirulina*, enhancing splenic lymphocyte cell proliferation of Kunming mice compared with non-fermented *Spirulina*. Besides that, during *Spirulina* fermentation, molecular metabolites were accumulated characterized as peptides fermented *Spirulina* fractions as low: L-PFS (< 3 kDa); medium: M-PFS (3-5 kDa) and high: H-PFS (5-10 kDa), or non-peptides fractions (AN *et al.*, 2020).

The L-PFS fraction could promote the Th1/Th2 serum balance cytokines in murine primary splenic lymphocytes in a dose-dependent immune stimulation manifested by enhanced cell proliferation and modulating anti-inflammatory interleukins (IL-2 and IL-10) secretion (BANCHEREAU *et al*., 2012). Meanwhile, L-PFS could also upregulate the mRNA expression of Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4) and inhibit the relative mRNA expression of Th1 cytokines (IL-2 and TNF-α) and Th2 cytokine (IL-10) compared with a lectin Concanavalin A -treated lymphocytes in vitro (AN *et al.*, 2020). This study demonstrated initial

insights in the potential of *Spirulina* fermented food product development and its immunomodulatory advantages. However, further studies are necessary to understand the stability of fermented *Spirulina* as a food ingredient.

Along with the immunomodulatory properties, *Spirulina* could be an adjuvant on metabolic diseases, such as diabetes mellitus type 1 and 2 (DM), which is incurable chronic disease that affects 10.5% of the worldwide population (IDF, 2021). The DM is characterized by hyperglycemia (high blood glucose levels) that damages the cell membranes, which contributes to insulin resistance of hepatic and peripheral tissue and generate reactive oxygen species (ROS) (BITAM; AISSAOUI, 2020). The AISSAOUI *et al*., (2017) study testified to *Spirulina* antidiabetic potential by administering 0.1 g of *Spirulina* powder diluted in 1 mL of distilled water by oral gavage for 50 days in alloxan-induced diabetic rats. The investigation demonstrated that *Spirulina* water extract treatment was composed of 16.54 ± 0.12 % of C-PC, showed an antihyperglycemic effect compared to the controls, and had a more powerful effect than metformin (500 $mg.kg^{-1}$ rats body weight), the primary antihyperglycemic drug used in diabetes *mellitus* regulations (AISSAOUI *et al.*, 2017).The authors believed that the antihyperglycemic effect of *Spirulina* to be due to either the presence of potent antioxidant bioactive molecules, mainly C-PC, chlorophyll, and carotenoid content, which may help the increment of insulin secretion from the islet *β*-cells or promotion of blood glucose transport to the peripheral tissues (AISSAOUI *et al.*, 2017; BITAM; AISSAOUI, 2020). The peptides and polypeptides generated by the digestion of the *Spirulina* proteins could also be responsible for the antidiabetic effect, but these anti-hyperglycemic consequences are not elucidated.

Thereby, to clarify the type 2 DM (DM2) anti-hyperglycemic gaps, HU *et al*. (2019) extracted *Spirulina* proteins using ultrasound coupled with the freeze-thaw approach (UFT), the ultrasound-assisted subcritical water technique (USW). This methodology could identify 11 anti-diabetes peptides with no cytotoxicity on liver cancer cells (HepG2). Furthermore, of these

peptides, three − (GVPMPNK (GK), RNPFVFAPTLLTVAAR (RR), and LRSELAAWSR (LR) − have demonstrated anti-diabetic activities after in silico prediction and in vitro experiments by showing potent inhibition on α-amylase, α-glucosidase, and dipeptidyl peptidase-4 (DPP-IV) enzymes.

The α-amylase and α-glucosidase enzymes are considered a prophylactic treatment for hyperglycemia because they are enzymes responsible for the carbohydrate's digestion process; their inhibition reduces carbohydrate digestion and consequently prevents the entry of glucose into the circulation (RIYAPHAN *et al.*, 2018). The DPP-IV's principal target for glycemic control decreases postprandial glucose levels by inactivating glucagon-like peptide (GLP-1), glucagon peptide, and incretin hormones (KUMAR *et al*. 2018).

Therefore, the docking of food natural compounds such as curcumin, antroquinonol, actinodaphnine, docosanol, tetracosanol, rutin, and HCD against α-glucosidase and α-amylase, have demonstrated promising results as hypoglycemic agents (RIYAPHAN *et al.*, 2018). HU*et al*., (2019) did the first study to present bioactive peptides from *Spirulina,* and more research needs to be done on its food and pharmaceutical utilization for therapeutic treatment for DM2 individuals, reducing drug side effects. Also, *Spirulina* as an adjuvant treatment to DM2, overweight or metabolic syndrome had a significant impact in lowering total cholesterol and triglycerides (BOHÓRQUEZ-MEDINA *et al.*, 2021) due to its lipid fraction (MAZOKOPAKIS *et al.*, 2014)

Spirulina has gained notoriety for its anti-obesogenic potential as a dietary supplement known as a "superfood" (CAPELLI; CYSEWSKI, 2010). MORADI *et al*., (2019) have summarized the effects of *Spirulina* supplementation in a systematic review and meta-analysis of five eligible randomized clinical trials for weight management. The research exhibited *Spirulina*, in dosages from 1 to 4.5 g/day during 6 to 12 weeks, beneficial effects on body fat percentage, body weight, and waist circumference of adults (26 to 49 years old ages) from

Poland, Mexico, Iran, and India. Still, no statistical differences were seen in body mass index (BMI) and the waist-to-hip ratio between groups that were divided into *Spirulina* and control groups, including 145 and 133 subjects in each, respectively.

The possible mechanisms involving the *Spirulina* effect in obese (lower-grade inflammation state) over than overweight individuals can be explained by *Spirulina* composition (YOUSEFI *et al*., 2019); reduction of cholesterol absorption; microbial-modulating by changing the gut microbiota composition; decreasing insulin resistance, oxidative stress and inflammatory condition, mainly since *Spirulina* bioactive compounds as C-PC and β-carotene; and appetite hormonal controlling, increasing adiponectin and decreasing leptin, with no toxic or side effects (MORADI *et al*., 2019). Further studies are necessary to figure out the mechanism by which *Spirulina* affects human body weight (YOUSEFI *et al*., 2019).

ZHAO *et al*., (2019) compared anti-obesity effects in mice fed with a high-fat diet (45% of the calories from lipids), plus 2 $g.kg^{-1}$ per diet of *Spirulina platensis* (WSP) or *Spirulina platensis* protein (SPP) or *Spirulina platensis* protein hydrolysate (SPPH), also known as *Spirulina* biomass powder, *Spirulina* extract, and *Spirulina* extract hydrolysate, concurrently. The results demonstrated that while SPP was the best for lowering glucose (39.6%), SPPH was the best for weight reduction (39.8%) and total cholesterol reduction (20.8%) among WSP, SPP, and SPPH.The authors highlighted that the difference between the effects of WSP, SPP, and SPPH maybe be explained by the compositional differences in protein fraction. WSP contains nonprotein components, SPP is a large molecule protein, and SPPH primarily consists of small molecular peptides (ZHAO *et al.*, 2019).

In addition to the compositional components, the gut-brain-liver axis has a vital role in understanding how the gene changes in brain and liver tissues are related to SPPH-treated obese mice, mainly after SPPH intervention that displayed different gene expressions that have been

shown to be associated with lipid metabolism, the most significant was Acadm (acyl-coenzyme A dehydrogenase), Retn (resistin), Fabp4 (fatty acid binding protein 4), Ppard (peroxisome proliferator activated receptor gamma) and Slc27a1 (Solute Carrier Family 27 Member 1) (ZHAO *et al.*, 2019). These inferences could also explain the results of YOUSEFI *et al*., (2019) study showed the *Spirulina* supplementation effect in obese over than overweight individuals.

Both obesity and overweight are recognized as disruptors of female fertility mainly because neuroendocrine mechanisms interfere with ovarian functions and can affect the ovulation rate and the endometrial receptivity (SILVESTRIS *et al*., 2018). WEN *et al*. (2020) conducted the first study that demonstrated that C-PC could be helpful in improving fertility in obese female mice by renewing ovary and oocyte quality. For experimental analysis, mice were divided into three groups: 1) Control (CTRL): standardized normal diet (oral) with 0.4 mL of normal saline solution (administered intragastrically); 2) High-fat diet (HFD): standardized high-fat diet (oral); 3) High-fat diet with phycocyanin (HFD+PC): HFD (oral) plus C-PC administered intragastrically each day at a dose of 500 mg/kg/day (dissolved in ultrapure water at a concentration of 50 $mg.mL^{-1}$). After C-PC administration, it was observed an increment of litter size and offspring survival rates, improvement of the level of ovarian antioxidant enzymes, and reduction in the occurrence of follicular atresia that could provide an essential strategy for clinical treatment of obesity-related infertility in females (WEN *et al*., 2020) by modulation of food intake, one of the lifestyle factors that can positively or negatively impact fertility (SILVESTRIS *et al*., 2018).

In the most recent study released by SALGADO *et al*. (2022), the C-PC, *Spirulina* majority protein content, showed anti-cancer potential through the modulation of melanoma cell proliferation, migration, and invasion. with no cytotoxic effect and maintaining cell viability of non-tumor cells in concentrations of 100, 200, and 400 $\mu g.mL^{-1}$ in 72 hours. In vitro and in silico evidence was performed to understand the main anti-melanoma components

exerted by C-PC at 4.01 analytical purity grade, 5.73 times more than the required food purity grade (0.7).

C-PC acted in the cell invasion pathway by binding to important proteins, such as N-cadherin, a cell adhesion molecule that potentiates the invasiveness of melanoma cells, decreasing cell migration and invasion (HAZAN *et al.*, 2004; MROZIK *et al.*, 2018). It is assumed that C-PC action is the responsibility of the F-chain, a β-subunit responsible for C-PC antioxidant properties, showing interaction with most of the tested targets, being more stable with N-cadherin and Bcl-2 (a cellular protein that inhibits apoptosis) (TRAN *et al.*, 2002). This C-PC ability to bind to the main cellular targets was verified by online tools (NCBI Blast, PRISM, and Protein Data Bank) and software (UCSF Chimera) (SALGADO *et al.*, 2022).

The action of C-PC is possible related to a cytostatic effect decreasing cell proliferation and not inducted by cell death, perhaps due to its interaction with proteins on the signal transduction pathway BRAF, a serine-threonine kinase which its gene is mutated in at least 50% of melanomas; MEK, a dual threonine and tyrosine recognition kinase that plays a unique role as "gatekeepers" of ERK1, inhibiting metastatic melanoma; and consequently, preventing CDK6, cyclin-dependent kinase 6, which regulates the cell cycle (SALGADO *et al.*, 2022).

Considering all the studies regarding the effect of C-PC and even the consumption of the *Spirulina* biomass on the health, it is a fact the importance of studying C-PC and *Spirulina* loaded foods for understanding synergistic health effects related to its regular consumption. These bioproducts have the potential to impact in a very positive way human health as well as the public and private Health Care Systems4. C-PC and biomass from *Spirulina* applications in food related products: a landscape

Synthetic food dyes are associated with hypersensitivity, hyperactivity and carcinogenicity (MOTA *et al*., 2021). However, they generally have better chemical properties and physical stability and are less expensive than natural additives. On the other hand, the

demand for natural foods has increased considerably in recent years, thus, using natural substances, such as C-PC, has recently gained visibility, especially for health benefits (NEVES *et al*., 2021). Besides being used as a food dye, fluorescent markers in order to monitor cells and macromolecules in biomedical research (KUDDUS *et al.*, 2013), medication development (ERIKSEN, 2008), elaboration of smart packaging (TERRA *et al.*, 2021), and nanotechnology (ASHAOLU *et al.*, 2021) are potential applications of this bioproduct that demand different purities and show the range of its utilization.

In this context, other target compounds are present (e.g., proteins and polyunsaturated fatty acids) in the same biomass that C-PC is obtained from, which makes Spirulina a significant matrix to be extensively exploited in food industry while attenuating production costs and rising above the gap of the natural additives for protein fortification, coloring and texturing or even substituting conventional plastic food packaging.

4. C-PC and biomass from *Spirulina* applications in food related products: a landscape

4.1 Food applications

The biggest obstacles to applying Spirulina more widely in foodstuff are related to the consistency of the dry biomass, its strong green color, and slight fishy odor (BECKER, 2007). On this matter, Spirulina fermentation resulted in overall smell improvement, thus, being useful for off-flavors reduction (MARTELLI *et al*., 2020); hence, it is another strategy to use for food incorporation. Furthermore, adding C-PC or isolated/concentrated *Spirulina* proteins to foods can help increase palatability and, consequently, consumer acceptance (STANIC-VUCINIC *et al*., 2018).

Most studies handle the incorporation of *Spirulina* biomass into food products, intending to improve products' nutritional profile. Bakery products, pasta, and dairy products are the main studied matrices for new product development. Table 1 summarizes the *Spirulina*

biomass incorporation into food products in the last five years with strong methodologies to analyze *Spirulina's* role in the outcomes. Studies with isolated proteins or polysaccharides from *Spirulina* were excluded from Table 1. Antioxidant activity is not always analyzed, nor is sensory acceptability, which represents a lack in Food Science searching. However, the included studies in the present review provide a landscape regarding *Spirulina* biomass applications in food, notably for increasing the protein content and/or as a texture improver.

Table 1: *Spirulina* incorporation in food products in the last five years

Food Product	Technological function	*Spirulina* ratio	Physicochemical characteristics	Antioxidant activity	Main effects	References
Vegan kefir (soy or almond milk-based)	Nutritional composition and functional properties	0.25 and 0.50% (w/w)	No significant color differences ($p > 0.05$). *WHC greater at 0.5% ratio. Improved total phenolic content.	**DPPH: 12.03 ± 4.41 was the greatest value (soy milk at 0.5%) ***ABTS: 46.90 ± 2.41 was the greatest value (soy milk at 0.25%)	*Spirulina* improved the prebiotic potential and bioactive quality of food	(ATIK *et al.*, 2021)
Crostini	Nutritional composition	6% (w/w)	-	-	C-PC were protected from thermal degradation by tocopherol	(NICCOLAI *et al.*, 2021)
Bread	Nutritional composition	2, 4, and 6% (w/w)	-	DPPH: 16.51 ± 0.85 at 6% *Spirulina* biomass ratio was the greatest value	The overall acceptability decreased as the *Spirulina* ratio increased antioxidant activity was proportional to the *Spirulina* ratio	(SAHARAN; JOOD, 2020)
Pasta	Nutritional composition	10, 30 and 50% (w/w) of *Spirulina*-soy-extrudate	-	-	Generally, pasta was accepted (5.9 on a 9-point hedonic scale) overall mean Familiarity with *Spirulina* was related	(GRAHL *et al.*, 2020)

					to acceptance, but optimization of the recipe is necessary	
Yogurt	Nutritional composition and functional properties, and coloring	0.1, 0.3, and 0.5% (w/v) oven-dried biomass and 1, 5, and 10% (v/v) wet biomass	pH decreased with biomass incorporation; WHC ranged at 53-62%; blue colors were higher for wet biomass products	-	Yogurt products showed the ability to retain water after the fermentation process	(PAN-UTAI *et al.*, 2020)
Emulsion	Low-fat oil-in-water food emulsions and coloring	1% (w/w) of water from emulsion substituted by *Dunaliella*; *Chlorella* or *Spirulina*	*Spirulina*: pH slightly decreased during storage; the emulsion were stable in texture, viscoelastic and rheological properties, as well as *Dunaliella* emulsions	-	*Spirulina*: Greenish color differences from 25 to 60 days of storage were observed	(URIBE-WANDURRAGA *et al.*, 2021)
Milk and fermented soy beverages	Boost fermentation capability	0.25 and 0.50% (w/v)	The lightness was increased at both concentration	-	*Spirulina* improved lactic acid bacteria strains and viscosity at 0.25%.	(MARTELLI *al.*, 2020)
Pasta	Nutritional composition, rheological and functional properties	0.25, 0.5, 0.75 and 1 % (w/w)	Improved mineral content; less moisture content at 0.25 and 0.5%	-	Greater color, aroma, and overall acceptability of pasta (0.25% *Spirulina*) compared to the control	(MOSTOLIZADEH *et al.*, 2020)

Gluten-free fresh pasta	Nutritional composition, rheological, functional properties, and appearance	2 and 3% (w/w)	Higher antioxidant activity; high digestibility in vitro; good mechanical properties	DPPH: 70.33% ± 4.36 and ****VCEAC: 0.77 μg.g^{-1} ± 0.02	*Spirulina* biomass enhanced the nutritional quality of pasta without affecting its cooking and texture quality properties. Sensory acceptance was greater at a 2% concentration	(FRADINHO *et al.*, 2020)
Crostini	Nutritional composition, functional properties, and appearance	2, 6 and 10% (w/w)	Improved protein, C-PC, and total phenolic content. At 6 and 10%, the crostini is considered a "source of protein."	DPPH: ranged from 57% to 61% and VCEAC: from 0.60 to 0.64μg.g^{-1}	Color and global acceptance were greater at 2% *Spirulina* concentration and lower than the control. Taste and texture at 2% were even greater than the control. Lower digestibility in vitro was found compared to control	(NICCOLAI *et al.*, 2019)
Ayran (Yogurt)	Boost on fermentation capability and probiotic bacteria	0.25 , 0.5 , and 1 % (w/v)	Improved protein at 1%. Viscosity decreased in the first seven days of storage, then it increased. The significantly ($p < 0.05$) decreased lightness	-	Enhancement in the growth of probiotic bacteria and nutritional value of ayran	(ÇELEKLI *et al.*, 2019)

			compared to the control			
Breadsticks	Nutritional composition, rheological, functional properties, and coloring	1.5% (w/w) *Spirulina* or *Chorella*	Improved mineral content and solid viscoelastic behavior was observed for both formulations	-	Brown tonality was observed due to the baking process. *Spirulina* resulted in a darker dough than *Chlorella*	(URIBE-WANDURRAGA *et al.*, 2019)
Yogurt	Nutritional composition, rheological, and coloring	0.25, 0.5, 0.75 and 1% (w/v)	Higher antioxidant activity, protein, fat, and dietary fiber contents were reported	DPPH: 52.41 ± 2.61 at 0.25% *Spirulina*	0.25% *Spirulina* concentration was the best formulation, with lower lightness values ($p < 0.01$) than the control samples. The color showed no tendency to lighten during storage (28 days) and was significantly sufficient to boost the fermentation and conserve the sensory acceptance	(BARKALLAH *et al.*, 2017)
Yogurt	Nutritional composition and functional properties	0.5; 0.75; 1; 2 and 3% (w/w)	Higher protein, viscosity and total lactic acid bacteria at 0.1% concentration with significant difference ($p<0.05$)	-	Appearance and consistency were greater at 1% concentration than the control	(AGUSTINI *et al.*, 2017)

	compared to control

*Water Holding Capacity/ **2, 2-diphenyl-1-picrylhydrazyl / ***2,2-azino-bis(ethylbenzo-thiazoline- 6-sulfonic acid) diammonium salt / ****Vitamin C Equivalent Antioxidant Capacity

Improved texture with better viscosity, reduced syneresis (BARKALLAH *et al*., 2017), and extending the products' shelf life are attributes acquainted through *Spirulina* biomass incorporation to food products as a consequence of greater water holding capacity, observed in vegan products (ATIK *et al.*, 2021), in milk-based products such as yogurt (BARKALLAH *et al.*, 2017; PAN-UTAI *et al.*, 2020), and ayran (ÇELEKLI *et al*., 2019). Both wet and oven-dried biomass were positively correlated to advantageous water holding capacity, but wet biomass is less processed (i.e., electricity-saving) and presented a higher C-PC concentration in the final product (PAN-UTAI *et al.*, 2020). These authors also obtained yogurt with stable acidity and color for both formulations; thus, the fermentation process is stimulated by *Spirulina* biomass. Corroborative results showed increased lactic acid bacteria and confirmed its potential in producing novel functional fermented dairy products (ÇELEKLI *et al*., 2019; MARTELLI *et al.*, 2021). Fermented *Spirulina* is related to immunomodulatory advantages in vivo (AN *et al.*, 2020); thus, commonly consumed products such as yogurts are interesting application matrices.

Another topic frequently associated with biomass was the nutritional composition booster and color stability (BARKALLAH *et al.*, 2017; PAN-UTAI *et al.*, 2020). In general, nutritional enrichment was identified, particularly in proteins and minerals (MOSTOLIZADEH *et al.*, 2020; URIBE-WANDURRAGA *et al*., 2019), and brown tonality was observed due to the baking process (URIBE-WANDURRAGA *et al.*, 2021). C-PC content in *Spirulina* enriched crostini was preserved besides the baking process (NICCOLAI *et al.*, 2019, 2021) due to the protective action of tocopherol content in extra virgin olive used as the formulation's ingredient. Hence, there are relatively simple methods to mitigate bioactive compound losses.

Meeting the consumer's demands through delivering novel products to the market must be planned. It involves sensory acceptance evaluation, which was not always carried out in the literature connected with *Spirulina* biomass or C-PC added products. Sometimes the number of

non-trained panelists was modest (e.g., 20 individuals) with few details about the methodology (BAKY *et al*., 2015). In this context, investigating the consumers' willingness to buy the novel products is a differential and was reported in just one study (NICCOLAI *et al.*, 2019). Consumers would probably buy crostini at 2% (w/w) of *Spirulina* concentration.

The technological challenges for the application of C-PC as a natural dye consist of achieving satisfactory yield and purity for an industrial scale, that is, with production costs, the number of steps, extraction and purification time viable, and also using techniques that are not harmful to the environment (HADIYANTO *et al.*, 2019; MORAES *et al*., 2010) and maintaining the stability of this biomolecule when added to the food matrix, since it is sensitive to light and heat exposure (BRAGA, *et al.*, 2016; ADJALI *et al*., 2022) and changes in pH (CHAIKLAHAN *et al*., 2012; MARTELLI *et al.*, 2014).

In this context, the addition of glucose, sucrose, or sodium chloride works as stabilizing agents involving and protecting the surface of the C-PC molecule, decreasing concentration losses and increasing the half-life at pH 7.0 and temperatures above 50 ºC when its optimal stability occurs at pH 5.5-6.0 and temperature of 47 °C (CHAIKLAHAN *et al*., 2012). Under refrigeration (0 ± 5 °C), the decline in C-PC concentration is reduced, and the presence of sucrose or calcium chloride or both in the association has a positive effect on the stability of the food-grade compound at low temperatures (BRAGA *et al.*, 2016; MISHRA *et al*., 2008).

Only a few studies have incorporated C-PC into food matrices. Table 2 outlines the C-PC incorporation in food products.

Table 2: C-PC incorporation in food products

Food product	Technological function	C-PC concentration	Physicochemical characteristics	Antioxidant activity	Main effects	Reference
Commercialized isotonic and tonic beverages	Natural blue dye	0.156 mg.mL^{-1} for tonic beverages; 11.167 mg.mL^{-1} for isotonic beverages;	No changes in viscosity. pH stability between 3.0 to 9.0	-	Color stability of cold tonic and isotonic beverages (11 days) with no significant changes	(GARCÍA *et al.*, 2021)
Ice cream	Emulsifying and stabilizing	0.13 mg.mL^{-1}	C-PC influenced emulsification and beating due to its protein content	-	The emulsifying and stabilizing activity was obtained, not influencing the overall acceptability compared to controls	(RODRIGUES *et al.*, 2020)
Ice cream	Natural blue dye	0.25 mg.mL^{-1}	Apparent density was 53.5% higher in the C-PC-added products relative to the control	*ORAC: 134.63 ± 15.68 µmolTE.g^{-1} and ABTS: 1425.19 ± 54.93 µmolTE.g^{-1}	The color was stable for 6 months. Ice creams presented antioxidant activity	(AMARANTE *et al.*, 2020a)

Yogurt	Natural blue dye	2, 4 and 8% C-PC (w/w)	Decreased syneresis increased firmness compared to control	-	Improved texture and color stability. C-PC had no adverse effects on yogurt starter cultures.	(MOHAMMADI-GOURAJI *et al.*, 2019)

*Oxygen Radical Absorbance Capacity / ** Concentration required to result in a 50% antioxidant activity (50% reducing power).

4.1.1 Refrigerated products

AMARANTE *et al*. (2020a) added C-PC obtained from *Spirulina* to ice cream and observed antioxidant activity 2 to 13 times higher than the control ice cream, indicating that the compound remained active in the final product. Another dairy product that has successfully received 2 and 4% C-PC was plain yogurt (MOHAMMADI-GOURAJI *et al*., 2019). In this matrix, C-PC maintained color stability, did not influence the starter culture, improved the texture, and decreased syneresis. Also, sensory evaluation demonstrated that 4% C-PC added yogurt had the best overall acceptability.

Besides the coloring function, C-PC presented emulsifying and stabilizing activity in ice cream (concentration of 0.13 $mg \times mL^{-1}$) without influencing the overall acceptability and contributing to better nutritional food quality (RODRIGUES *et al.*, 2019).

Blue isotonic and tonic beverages commercialized without any coloring additive (control) were studied by GARCÍA *et al*., (2021). The control received lower amounts of C-PC (<1.12 $mg \times mL^{-1}$) to reach color compatible with the blue commercialized products which contain artificial colorants (i.e., brilliant blue E-133). They presented good color stability during analysis time (11 days), in a wide range of pH (3 to 9), and C-PC did not affect the viscosity of these products.

These results are significant due to the inclusion of C-PC in products already commercialized, showing the concrete way the food industry can reinvent itself. Up to the present, refrigerated products are the most accessible options to incorporate C-PC as a natural blue dye due to the consequently color preservation under refrigerated temperatures.

4.1.2. Baked products

Referring to baked products, it was found that in 3% C-PC added biscuits and in 0.3, 0.6, and 0.9% *Spirulina* biomass biscuits have delayed oxidation during 30 days of storage time and antioxidant activity in the final products, especially at zero time (BAKY *et al*., 2015). The sensory acceptance showed no significant differences, but the number of panelists was limited. As thermal degradation of C-PC was pointed out in the literature (BRAGA, *et al.*, 2016), its incorporation in baked products seems more difficult. Perhaps the whole *Spirulina* biomass combined with tocopherol could protect more of this sort of product, as found by (NICCOLAI *et al.*, 2019, 2021).

On balance, the application of *Spirulina* biomass, as well as C-PC in food, is very discrete, and due to their remarkable potential, it is challenging to understand why so few products were developed in the last years. The use of these bioproducts to formulate food products must be fomented by academics as well as food industry decision-makers. Not only because of their nutritional characteristics as well as to equalize the ultra-processed product consumption.

4.2 Nano and microencapsulation

Natural colorants, such as blue-colored C-PC, have been preferred by consumers due to their safety when compared to their synthetic counterparts, presenting non-toxic side effects and even promoting health with antioxidant and anti-inflammatory activities (BOER *et al*., 2019; NEVES *et al*., 2021). However, C-PC has physical and chemical limitations, characterized by its instability in light, temperature variation, and acidic media, as previously described, resulting in C-PC precipitation and loss of blueness after proteolysis by thermal degradation and other denaturation processes, which limits C-PC application in food products (CHAIKLAHAN *et al*., 2012; İLTER *et al.*, 2021).

According to JACOBSEN *et al*., (2018), despite the number of commercially available food products using micro and nanotechnology is still scarce because of the: (I) limited investments; (II) low technological performance, which mainly is cost-intensive and time-consuming unit operations; and (III) mass production challenge with high reproducibility and yields (CHANDRALEKHA *et al.*, 2021), these strategical technologies appear to guarantee the stability of the bioactive compounds in food, by the incorporation of these biomolecules into micro or nanostructures to bioactive action, color, and thermal maintenance (GIACONIA *et al.*, 2020).

Therefore, the micro and nanotechnology are defined as the study, manipulation, or phenomenon of substances and devices found on the micro [ranging from 1 to 1000 micrometer (μm) then, 1 μm = 10^{-6} of meter = 0.000001 m] or nanometric scale [ranging from 1 to 100 nanometer (nm) then, 1 nm = 10^{-9} of meter = 0.000000001 m] that exhibits distinct properties with several size dependent applications (VALAMLA *et al.*, 2021).

Electrospinning (LEIDY; QUINTANILLA-CARVAJAL, 2019), electrospray (TAPIA-HERNÁNDEZ *et al.*, 2015) and emulsions (FERNÁNDEZ-LUQUEÑO *et al.*, 2021) are the most used techniques to micro or nano encapsulate different types of bioactive compounds. The first two are mainly used for hydrophilic substances (such as C-PC, anthocyanins, and others). The last one is primarily related to lipophilic compounds' micro or nanostructure development (such as carotenoids and others). To select the best methodology among these, the equipment, material, micro or nanocarriers - as carbohydrates (cellulose, guar gum, and others) (FATHI *et al*., 2014), proteins (gelatin, zein, and others) (LEIDY; QUINTANILLA-CARVAJAL, 2019) or lipids (vegetable oils and others) (FERNÁNDEZ-LUQUEÑO *et al.*, 2021), and the types and polarities of the bioactive compound must be considered. Considering these characteristics, some positive

effects can be observed to lead to different behavior in the food matrix and with other food ingredients (GIACONIA *et al.*, 2020).

In PRADEEP and NAYAK (2019) research, the authors demonstrated that extrusion technology using sodium alginate as coating material improved C-PC stability potential by microencapsulation. The morphological nature of microcapsules containing C-PC showed a homogeneous spherical appearance without any surface cracks and an average size of 1.2 ± 0.1 mm, one of the most critical parameters for the release of bioactive ingredients and for optimizing the encapsulate production (JYOTHI *et al.*, 2010; PRADEEP; NAYAK, 2019).

Besides that, encapsulating C-PC with 2.5% (w/v) sodium alginate indicates thermal stability at 60 ºC, 70 ºC, and 80 ºC, differently from native C-PC with degradation of 88.19% and 86.89% at 70°C and 80°C, respectively. The encapsulated C-PC showed stability at 6.5 (45 ºC and 55 ºC) and at pH 5.5 (55 ºC) for 120 minutes (PRADEEP; NAYAK, 2019). It is interesting to note that FTIR analysis confirms that C-PC was not degraded during microencapsulation. The process allows electrostatic interactions to protect the C-PC structure at high temperatures (80 ºC) (PRADEEP; NAYAK, 2019). In a short review, ADJALI *et al*., (2022) pointed out degradation of non-encapsulated C-PC at pH between 5,5 and 6 and temperatures below 45 ºC. These authors indicated micro or nanoencapsulation as solutions to improve C-PC's thermal stability, thus, results reported by PRADEEP; NAYAK (2019) are correspondingly.

The microencapsulation technique was also used by the OLIVEIRA *et al*. (2021) study for the development of functional chocolate milk powder formulations by the incorporation of *Spirulina* sp. (LEB-18) in two different concentrations (w/w) (F1: 5% and F2: 8.75%) into maltodextrin (15%) and soy lecithin (F1: 10% and F2: 5%), other ingredients used were sucrose (40%), cocoa powder (20%) and whey (F1: 10% and F2: 11.25). The obtained microspheres are

expected to improve functional properties of the chocolate powder due to its wall protection. Protein content was improved in microencapsulated formulation for both concentrations while water content were decreased, leading to better water retention in the dissolved form. Additionally, the sedimentation analysis showed that maltodextrin increased viscosity in the medium, preventing sedimentation and improving the texture of the chocolate powder when dissolved. Maltodextrin also was correlated with less hygroscopicity, a desirable characteristic for dehydrated products OLIVEIRA *et al*. (2021).

To obtain the biological effects of C-PC, the sustained release is the best choice to prevent acid degradation of the protein portion of the molecule. The choice of the coat materials for micro/nanoencapsulating is, therefore, fundamental. Some studies have moved in this direction. This is the case of the study by YAN et al., (2014) that identifies, from in vivo release assays, that materials such as alginate, chitosan, and carrageenan protect C-PC from gastric acidity and promote a release of the target compound under milder conditions. In vivo release assays can address physical and functional properties of the final food products.

5. Knowledge gaps and further research

As mentioned above, C-PC is already applied in a few commercialized food products, mainly refrigerated food, such as fruit and vegetable juices and smoothies, ice creams, and yogurts, as "*Spirulina* extract". However, the blue color of C-PC is one of the features that limit its application in food due to the "unnatural" aspect perceived by a portion of consumers. For this reason, the products that are currently being developed and commercialized are aimed at younger consumers, more interested in the innovative and health-improving aspects of food products. Nevertheless, marketing strategies could easily overcome this limitation, as it has in the case of

Spirulina itself, for example. Many of the food products containing *Spirulina* are commercialized, emphasizing its health benefits, and its blue-green color indicates the presence of antioxidant ingredients.

Another aspect limiting C-PC's application in food products is its poor thermal stability (BRAGA *et al.*, 2016). Protein beverages, e.g., meal-replacers or sports recovery drinks, require thermal processing to ensure consumer safety and shelf stability (WAGONER; FOEGEDING, 2017). Additionally, protein beverages are preferentially formulated at a pH ranging 4-6 to avoid astringency and off-flavors (LAN *et al*., 2018; WAGONER; FOEGEDING, 2017). This pH range encompasses the isoelectric point of C-PC extracted from *Spirulina platensis*, i.e., pH 4.8-5.8 (LIU *et al.*, 2000; PATIL; RAGHAVARAO, 2007), where it may suffer aggregation and precipitation, which is undesirable for product design, in addition to color loss (LI *et al.*, 2020). An approach that has been used to improve the thermal stability of whey and pea proteins in acidic environments is the complexation with oppositely-charged polysaccharides to form soluble complexes (GUO *et al.*, 2022; LAN *et al*., 2018; WAGONER; FOEGEDING, 2017). A single very recent study has addressed this issue by complexing C-PC with six different polysaccharides, i.e., κ-/ι-/λ-carrageenans, xanthan gum, high-methoxyl pectin, and guar gum (LI *et al.*, 2020). C-PC: ι-carrageenan complexes showed the best results, displaying minimal color loss after heat treatment at 60 and 80°C for 30 min. This report is critical because it opens possibilities to overcome the application of C-PC as a natural blue dye in baked and generally heat-treated food products. The same improvement in C-PC's stability could be achieved by the combination of vegetable oil as a supporting ingredient (NICCOLAI *et al.*, 2019, 2021).

There is also a need for more studies investigating the rheological properties of C-PC alone and when added to food systems (BATISTA *et al.*, 2006; GOUVEIA *et al.*, 2006; LOZOBER *et*

al., 2021), which are crucial to characterize the stability of these products. Moreover, we have also identified gaps regarding evaluating the antioxidant activity of C-PC in food matrices after the digestion process, either in vitro or in vivo (AMARANTE *et al.*, 2020a). This investigation is of the utmost importance in the formulation of food products with a health-benefits appeal since digestion can decrease the scavenging activities of cyanobacteria extracts (GHOSH *et al.*, 2016).

Finally, there is a lack of studies reporting the sensory acceptance of C-PC-added food products. Of the already short Table compiling reports on the application of C-PC in food products (Table 2), only three studies have included sensory evaluation analysis (BAKY *et al*., 2015; MOHAMMADI-GOURAJI *et al*., 2019; RODRIGUES *et al.*, 2019), all with a very limited number of panelists. It is essential to expand the reports on the consumer acceptance of C-PC-added foods so that it guides the formulation of these products, considering that C-PC is not the most versatile ingredient but can be very beneficial from a food design point of view, e.g., improving texture and delaying oxidation (BAKY *et al*., 2015; MOHAMMADI-GOURAJI *et al*., 2019). Another aspect worth considering lies in the extraction and purification processes for C-PC obtainment. As mentioned by BECKER (2007), the consistency of the dry biomass of microalgae can be a major drawback for its application in foods. Additionally, studies have shown that up to 50% of C-PC can be lost during its extraction from the dry biomass of *Spirulina*, possibly due to its poor thermal stability and peripheral position in the thylakoid membrane of the phycobilisome (SALA *et al*., 2018; SARADA *et al*., 1999). Therefore, it is recommended that C-PC is extracted from the wet biomass of *Spirulina*, ensuring the highest extraction yield and purity possible and minimizing the amount of biomass in the C-PC extract, which facilitates application or eventual purification processes that may be needed (AMARANTE *et al.*, 2020a). Moreover, using water or

buffer as extraction solvents is suggested in accordance with green chemistry principles (FRATELLI *et al.*, 2020).

Additionally, focusing attention on the full use of *Spirulina* is essential. It is about fully utilizing the biomass after obtaining the great value-added C-PC, after all, other nutritionally interesting compounds such as carotenoids, proteins, and fatty acids (MUYS *et al.*, 2019) are present and do not need to be isolated to contribute to the good nutrition of the population. The same fatty acids, if isolated, can be used for the production of biodiesel, for example, creating opportunities for partnerships between companies from different sectors, respecting the circular economy and, consequently, optimizing the use of natural resources and reducing the production of waste in a challenging world scenario as the preservation of the environment and food security for the population are significant concerns.

6. Conclusions

In the last period, more studies have been carried out considering *Spirulina* biomass application to food products. However, there are still gaps to overcome, such as rheological characterization, antioxidant scavenging essay, especially after digestibility in vitro, sensory evaluation, and consumers willing to buy the new products. Studies with C-PC application in food matrices and active food packaging are still extremely scarce. As blue food is uncommon, C-PC added foods are an opportunity to create novel products to innovate in a competitive market. The inclusion of C-PC in products already commercialized has already been conducted, showing the very concrete way in which, the food industry can reinvent itself. In this context, the claim of the human health benefits under environmentally responsible production would probably attract even more consideration. Initially, young consumers tend to be the most well beneficiated, as they used

to consume candies, toppings, and chewing gum artificially colored. Up to the present, refrigerated products are the most accessible options to incorporate C-PC as a natural blue dye due to the consequently color preservation under refrigerated temperatures.

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CAPÍTULO III

“Naturally colored ice-creams: antioxidant activity after in vitro digestion and color stability”

Monize Bürck, Camilly Fratelli Pereira and Anna Rafaela Cavalcante Braga

Naturally colored ice-creams: antioxidant activity after in vitro digestion and color stability"

[1,2]Monize Bürck, [1,2,3]Camilly Fratelli, and [1,4]Anna Rafaela Cavalcante Braga*

[1]Department of Biosciences, Universidade Federal de São Paulo (UNIFESP), Silva Jardim Street, 136, Vila Mathias, 11015-020, Santos, SP, Brazil.

[2]Department of Physiology, Universidade Federal de São Paulo (UNIFESP), Botucatu Street, 740, Vila Clementino, 04023-062, São Paulo, SP, Brazil.

[3]Universidade Metropolitana de Santos (UNIMES), General Francisco Glicério Avenue, 8, Encruzilhada, 11045-002, Santos, SP, Brazil.

[4]Department of Chemical Engineering, Universidade Federal de São Paulo (UNIFESP), Campus Diadema, Diadema, São Paulo, 09972-270, Brazil.

Correspondence: anna.braga@unifesp.br; Tel.: +55-13-98145-0201

Abstract

Ice cream reigns supreme over the confectionery market in Europe and the world. Due to the incredibly vast demand, ice cream manufacturers have been focusing on flavors compatible with organic ingredients for a long time, considering the generally prevailing consumer trends and introducing unusual flavors. At the same time, scientists worldwide are discussing the whole use of biomass produced globally, stimulating the circular economy in a more sustainable manufacturing path, mainly using renewable sources such as cyanobacterium, algae, and other actors from the biodiversity. This has also been occurring under increasing demand to substitute synthetic food dyes, sometimes related to carcinogenicity, hyperactivity and hypersensitivity in individuals. Considering the presented information, the present work aimed to produce four bold formulations of natural blue and green colored ice-creams applying C-phycocyanin (C-PC) and *Spirulina* residual biomass (RB) after C-PC extraction, respectively. The ice creams were sweetened by using maltodextrin or sucrose in order to compare the color stability alongside 182 days and antioxidant activity before and after bioaccessibility. All the formulated ice creams presented $ABTS^{\bullet+}$ and peroxyl radicals' scavenging capacities regarding the antioxidant activity, and the C-PC and RB sucrose ice creams had a significant improvement in antioxidant activity after in vitro digestion The results demonstrated that C-PC extract and the RB from *Spirulina* could be applied as a stable blue or green dye in ice creams and highlighted its antioxidant activity. Since both formulations have biological effects, RB with sucrose formulation is the more suitable for scaling up due to its price and human health potential.

Keywords: *Spirulina*, C-phycocyanin, residual biomass, bioactive compounds, antioxidant activity.

1 Introduction

Renewable organic matter derived from plants and animals are biomass. It contains chemical energy derived from radiant energy from sunlight, water and CO_2 (EIA, 2022). Besides its use for generating biofuels and electricity, biomass can provide nutritional and antioxidant compounds with biological effects. Along these lines, scientists worldwide are discussing the whole use of biomass produced globally, stimulating the circular economy in a more sustainable manufacturing path. It is so withcyanobacterium, algae, and other actors from the biodiversity (AMARANTE; BRAGA, 2021). This work focuses in the naturally green *Spirulina* (*Arthrospira platensis*) biomass, which contains 57.5% proteins, 23.9% carbohydrates and 7,72% fatty acids (USDA, 2019) and phycocyanin (C-PC), the major compound with antioxidant activity, followed by allophycocyanin and phycoeritrin (ARASHIRO *et al.*, 2020; PATIL *et al*., 2006). Additionally, the *Spirulina* cultivation process by using wastewater and photobioreactors (ARASHIRO *et al.*, 2020; LIM *et al.*, 2021) have gained environmental notoriety. This is also under increasing demand from the consumers to stay healthy, not only individually but alongside environmental preservation of fauna and flora, constituting the One Health concept (BANWO *et al.*, 2021). It is worth mentioning that after the C-PC's extraction, the *Spirulina* residual biomass (RB) remains nutritionally attractive and can enrich food products.

Vegetables are naturally rich in bioactive compounds, and even without knowing the nomenclature, people consume them. However, nowadays, food consumers, in particular, are familiar with the antioxidants, nutraceuticals, and designed foods loaded with functional nutrients, demanding these ingredients in processed food (AMARANTE *et al.*, 2020a). C-PC is a phycobiliprotein with a fluorescent blue color (ERIKSEN, 2008) and has a remarkable health appeal, since their regular consumption in food products is related to health benefits preventing

non transmissible chronic diseases (FRATELLI *et al*., 2021), cancer (SALGADO *et al*., 2022) and even a treatment for obese-related infertility (WEN et al., 2020). As "*Spirulina* extract" in the legislation, is recognized as a safe ingredient, regulated for use as a colorant exempt from certification and permanently listed for food use (CFR, 2022), some food products are being developed utilizing C-PC from *Spirulina*, including ice cream (AMARANTE *et al.*, 2020b). On the other hand, blue food is perceived as less natural since the natural blue color is infrequent compared to green, yellow and red, colors that industry has already commercialized. Consequently, natural blue food dyes can be considered the future of the food industry that seeks innovations to call consumers' attention in the face of a highly competitive market.

Ice cream reigns supreme over the confectionery market in Europe and the world. This cold treat has been a favorite dessert of most consumers for years. But although eating ice cream has a long tradition, the only thing left of the traditional understanding of ice cream these days is that they are cold (GREMSKI *et al.*, 2019). Experiments with flavors, consistency, and additives continue and lead to more unusual solutions. Ice cream manufacturers have been focusing on flavors compatible with organic ingredients for a long time, taking into account the generally prevailing consumer trends and introducing unusual flavors to their products (ADHIKARI *et al.*, 2020).

Considering the presented information, the present work aimed to produce six bold formulations of natural blue and green colored ice-creams applying C-PC or RB after C-PC extraction, , respectively. Two of these formulations were controls. The ice creams were based on fermented milk in order to add distinct flavor notes to the final product and were sweetened by using maltodextrin or sucrose to compare the color stability alongside 182 days and antioxidant activity before and after bioaccessibility, since previous studies relate the complex proteins-sugars

as a stabilizing agents against chemical and physical degradation due to its hydrogen bonding and better handling with native protein conformation and preventing color loss as a consequence (MENSINK *et al*., 2017;CHAIKLAHAN *et al*., 2012).

2 Material and Methods

Spirulina biomass

The commercial dry organic *Spirulina* biomass (*Arthrospira platensis*) was gently donated from Fazenda Tamanduá™. The composition/100 g corresponds to 333.3 kcal; 20 g carbohydrates; 53.3 g protein; 6.6 g total fat; 2.6 g saturated fat; 6 g fibers; 200 mg calcium; 2 mg iron; 1.26 g sodium. The biomass was stored in a ultrafreezer (-40 ºC) for C-PC extraction.

Extraction and purification of C-PC from Spirulina

Spirulina biomass was removed from the freezer (-40 °C) 1 hour before the start of extraction and was processed in an analytical knife mill (IKA A11 Basic) to separate the particles through a 0.106 mm mesh/Tyler (MORAES *et al.*, 2010). 25 mL of distilled water was added to 4 g of sample, kept at room temperature, and protected from light for 1 h. The mixture was centrifuged for 60 min at 3,040 *x* g (4 cycles of 15 min), and the supernatant was separated from the RB Once with the liquid fraction of the extract (C-PC), the samples were filtered using vacuum filtration and through a 0.22 μm hydrophilic syringe filter and kept in an ultrafreezer at -40 °C. The RB was frozen as well.

Fractional precipitation with $(NH_4)_2SO_4$ was performed (AMARANTE *et al.*, 2020a; SILVA *et al.*, 2009) to partially purify C-PC in order to obtain a food grade extract (purity > 0.7). In the first fractionation, solid ammonium sulfate was added to the clarified C-PC extract at a

saturation concentration equivalent to 20% (m/v), under constant stirring for 30 min, followed by incubation at 4 °C for 2 h (FIGUEIRA *et al.*, 2016). After this time, the sample was centrifuged (, 3,040 *x* g at 10°C for 20 min), and the precipitate was discarded. In the second fractionation, solid ammonium sulfate was added to the supernatant from the previous step until 50% (m/v) saturation. The solution was maintained in the same conditions as the first fractionation and centrifuged. The supernatant was discarded, and the precipitate was resuspended in 0.05 $mol.L^{-1}$ sodium phosphate buffer pH 7.0 at a resuspension volume/initial volume ratio of 0.52. The resolubilized precipitates were dialyzed using 0.05 $mol.L^{-1}$ sodium phosphate buffer pH 7.0 for salt removal. C-PC concentration, purity, purification factor, and recovery were evaluated before and after the precipitation and dialysis processes.

Analytical methods

C-PC concentration ($mg.mL^{-1}$) was calculated as described by BENNETT; BOGORAD (1973) with wavelength modificated by MORAES;KALIL (2009) according to Equation 1, where the absorbance at 652 nm (A_{652}) indicatesthe presence of allophycocyanin, the absorbance at 620 nm (A_{620}) indicates C-PC concentration (PATIL et al., 2008). The C-PC extract purity (EP) was calculated according to Equation 2 (ABALDE *et al*., 1998), where the absorbance at 280 nm (A_{280}) indicates protein concentration in the solution (LIU *et al*., 2005). The absorbances (A) are performed using a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). The yield (Y) was calculated using Equation 3, and expressed in mg.g-1. C-PC is the concentration of C-PC ($mg.mL^{-1}$), V corresponds to solvent volume (mL), and DB is the quantity of dry biomass (g).

$$\text{C-PC} = \frac{A_{620} - 0.474 \times A_{652}}{5.34} \quad (1)$$

$$EP = \frac{A_{620}}{A_{280}} \quad (2)$$

$$Y = \frac{C\text{-}PC \times V}{DB} \quad (3)$$

Formulation of the fermented milk

Firstly, fermented milk was fabricated in the Laboratory of Food Bioactive Compounds (UNIFESP) research group, with sweeteners variation (Table 1). The fermented milk was prepared by combining pasteurized skimmed milk, commercial dairy culture (containing *Lactobacillus acidophilus*, *Bifidobacterium* and *Streptococcus thermophilus*), sucrose or maltodextrin, whole milk powder and unflavored gelatin. The pasteurized whole milk was heated until it reached 80 ºC and the dry ingredients were added gradually, in order to guarantee the homogeneity of the mixture. The commercial dairy culture was dissolved when the mixture reached 40 °C. The mixture was transferred to properly packed containers placed in a lab oven at 40 °C for 4 h for fermentation (FONSECA, 2017). The trials were proceeded in triplicate.

Table 1. Fermented milk formulation containing sucrose or maltodextrin

Component	**Sucrose**	**Maltodextrin**
	Concentration (%, m/m)	**Concentration (%, m/m)**
Whole milk pasteurized	95.83	95.83
Whole powdered milk	2.87	2.87
Sucrose	0.96	-
Maltodextrin	-	0.96
Gelatin	0.24	0.24
BioRich™*	0.09	0.09

*Produced by Chr.Hansen

Formulation of the ice creams

Afterward, the formulated fermented milk was used as a base for developing the ice creams, according to previous studies by AMARANTE *et al*. (2020b) and adapting the Duas Rodas Industrial™ recipe (Table 2). Sucrose or maltodextrin, whole milk powder, cream and commercial stabilizer powder were added to the fermented milk and homogenized in a mixer. After 3 minutes, the mixture was frozen at -18 °C for 4 h. Then, emulsifier, neutral stabilizer Duas Rodas Industrial™ and liquid C-PC (0.25 $mg.mL^{-1}$) were added in a new homogenization step for 5 min. The mixture was stored at -18 °C. The formulation containing biomass and the control formulation went through the same production stages, differing by the addition of RB in the first and by the non-addition of C-PC in both.

Table 2. Formulation of ice creams using fermented milkt as a base (sweetened with sucrose or maltodextrin): controls, added with C-PC and RB.

Component	Control Formulation	Blue ice creams (C-PC)	Green ice creams (RB)
	Concentration (%, m/m)	Concentration (%, m/m)	Concentration (%, m/m)
Fermented milk	75	71.64	74.38
Sucrose or maltodextrin	9.22	8.80	9.14
Whole milk powder	6.57	6.28	6.51
Heavy cream	7.88	7.49	7.77
Super liga neutra™*	0.65	0.62	0.64
Emustab™*	0.65	0.62	0.64
C-PC	-	4.55	-
Residual biomass	-	-	0.91

*Produced by Duas Rodas Industrial™

In this way, six formulations were prepared: sucrose control (SC), sucrose and C-PC (SC-PC), sucrose and residual biomass (SRB), maltodextrin control (MC), maltodextrin and C-PC (MC-PC), and maltodextrin and residual biomass (MRB). Thus, in the final formulation, blue ice

creams were naturally colored with 5% of C-PC (m/m). The RB was also utilized at 1% (m/m) to create green-colored ice creams. Assays were performed in triplicate and samples of each ice cream were freeze-dried and stored for determination of antioxidant activity and bioaccessibility.

Characterization of the ice creams

The color stability of the products was monitored every 7 d for a total of 182 d using the L*a*b* color system (Figure 1). The values of L*, a*, and b* were determined for each formulation in triplicate at each point in time using a Colorimeter (Minolta, model CM25D, Japan). The Hue angle (h, Equation 4), which indicates the color angle (0°- red, 90°- yellow, 180°- green, 270°- blue, and 360°- black), was calculated by Equation 4. L* indicates brightness (0 - 100), a* indicates the amount of red (positive values) or green (negative values), and b* suggests the amount of yellow (positive values) or blue (negative values) in the samples. The color difference (ΔE, Equations 4 - 8) was also calculated from the initial (0) and final (t) values of L*, a*, and b* of each formulation (SC, SC-PC, SRB, MC, MC-PC and MRB).

$$h\,(^\circ) = 180 + \tan^{-1}\left(\frac{b^*}{a^*}\right) \qquad \text{when } (-a^*, +b^*) \text{ or } (-a^*, -b^*) \tag{4}$$

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \tag{5}$$

$$\Delta L^* = L_0^* - L_t^* \tag{6}$$

$$\Delta a^* = a_0^* - a_t^* \tag{7}$$

$$\Delta b^* = b_0^* - b_t^* \tag{8}$$

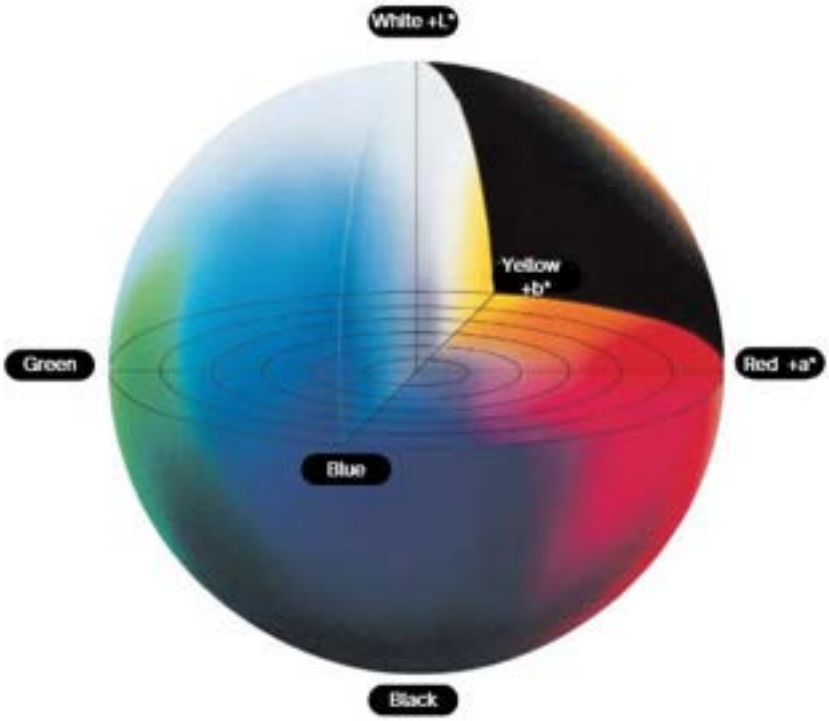


Figure 1 Representation of a color solid in the space color L* a* b*

Antioxidant activity

The freeze-dried samples were stirred with 20 mL of methanol for 15 min to extract the antioxidant compounds from the matrices. Samples were filtered using filter paper, and the retentate was washed twice with an additional 20 mL of methanol. The filtrate was concentrated using a rotary evaporator at temperatures below 40 °C (BRAGA *et al.*, 2018; ROSSO; MERCADANTE, 2007). The samples were filtered using 0.22 μm cellulose acetate membranes before analysis.

For the determination of the antioxidant activity against the peroxyl radical, the ORAC (oxygen radical absorption capacity) method was used, based on peroxyl radical formation by the thermal degradation of AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) at 37 °C. 150 μL of fluorescein (61 $nmol.L^{-1}$, prepared in 75 $mmol.L^{-1}$ phosphate buffer pH 7.4) was added to 96-

well microplates, followed by 25 μL of the extract made (diluted 100, 500, and 1000 times in phosphate buffer) or buffer (blank) or Trolox standard solution (50 μmol.L^{-1}). The plate was incubated for 10 min at 37 °C under intermittent stirring, and after that, 25 μL of AAPH solution (19 mmol.L^{-1}, prepared in phosphate buffer) was added to each well. The fluorescence readings at 538 nm (excitation at 485 nm) are performed every min for 180 min (RODRIGUES *et al.*, 2012).

To determine the antioxidant activity against the $ABTS^{\bullet+}$ radical, 30 μL of the extracts were added to 3 mL of the $ABTS^{\bullet+}$ radical solution, and the absorbance of the reaction was measured in a spectrophotometer at 734 nm after 6 min. The $ABTS^{\bullet+}$ radical solution was prepared from the reaction between 5 mL of stock solution of ABTS (7 mmol.L^{-1}) and 88 μL of potassium persulfate (140 mmol.L^{-1}), both formulated in distilled water. The mixture is kept in the dark at room temperature for 16 h. This solution is diluted with ethanol to an absorbance of 0.7 ± 0.05 at 734 nm. A standard Trolox curve in ethanol, constructed under the same reaction conditions, quantifies the total antioxidant activity (RE *et al.*, 1999). Both antioxidant results were expressed in μmol.g_{sample}^{-1} of Trolox equivalents (TE).

Total phenolic compounds

Using the antioxidant extract previously described, the total phenolic compounds was determined by the Folin-Ciocalteu method, according to the procedure of SINGLETON; ROSSI, (1965) and expressed in gallic acid equivalent (GAE) /100g. An aliquot of 1 mL was withdrawn from each extract or from the standard solutions of gallic acid (20, 40, 60, 80 and 100 mg/L). The contents were transferred to a 25 mL volumetric flask containing 9 mL of water. 1 mL of Folin-Ciocalteau reagent was added and the mixture was stirred. After 5 minutes, 10 mL of a 7% Na_2CO_3

solution was added and the volume was made up with water. After 90 min of incubation at 23 ºC, the absorbance was determined at 750 nm.

Microbiological quality

For the microbiological safety analysis, 1 g of the ice cream samples were evaluated after production (at zero time) and on the 182nd d. The presence of coagulase-positive *Staphylococcus aureus* (48 h at 35 °C), *Salmonella* (18-24 h at 41,5 ºC), *Enterobacteriaceae* (24h at 35 ºC) as specified in legislation by the National Health Surveillance Agency, Brazilian legislation (ANVISA, 2019) was evaluated and additionally, analyzes of total coliforms and *Escherichia coli* (48 h at 35 ºC). Total aerobic mesophilic microorganisms (48 h at 32 ºC) were performed using the rapid methodology Petrifilm™ (3M), following the manufacturer's instructions and recommendations, a method validated by the Association of Analytical Communities (AOAC, 2005). All analyses were performed in triplicates, and the results were reported in CFU/g (FAI *et al.*, 2011; MORAES *et al.*, 2016; PEREIRA *et al.*, 2020).

Bioaccessibility

Aliquots of 2g of freeze-dried samples were digested (CHITCHUMROONCHOKCHAI; FAILLA, 2017; GIACONIA *et al.*, 2022) with 10 mL of salts solution (NaCl 120 mol.L^{-1}, $CaCl_2$ 6 mmol.L^{-1}, KCl 5 mmol. L^{-1}) and 6 mL of artificial saliva solution containing α-amylase (106 U.mL^{-1}) (Sigma® A3176). The oral phase ended with incubation in an orbital shaker at 150 rpm, 37 °C for 10 min. The gastric phase was initialized with the pH adjusted for 2.5 with HCl 1 mol.L^{-1}, to add 2 mL of pepsin (Sigma® 110 P7000; 40 mg.mL^{-1} in 0,1 mol.L^{-1} HCl). The volume was completed to 40 mL and incubated at 37 °C, 150 rpm during 1 h. For the intestinal and final phase,

the pH was adjusted to 6.0 with 1 mol.L^{-1} $NaHCO_3$, porcine bile solution (3 mL; Sigma® 113 B8381; 40 mg.mL^{-1} in 0,1 mol.L^{-1} $NaHCO_3$), 4,000 U.mL^{-1} of porcine pancreatin (Sigma® 114 P1750), and 1,000 U.mL^{-1} of porcine pancreatic lipase (Sigma® 115 L3126) was added to the samples, adjusting the pH for 6.5. The volume was completed until 50 mL before the incubation at 37 °C, 150 rpm for 2 h. The last step was to centrifugate the samples for 1 h at 3,041 *x* g and 10 °C. The final samples were freeze-dried to evaluate the antioxidant activity.

Statistical analysis

The measurements from the assays were carried out independently in triplicate and compared by applying analysis of variance (ANOVA), Tukey's post hoc test and T-test in order to compare the means, using the degree of significance of 95% ($p < 0.05$)

3 Results and Discussion

According to the FDA (Code of Federal Regulations, Title 21, Vol. 2, Part 135), ice cream is a food produced by the freezing and stirring of a pasteurized mix consisting of one or more of the optional dairy ingredients specified, such as milk, cream, and butter, among others. It may contain one or more of the optional caseinates and hydrolyzed milk proteins specified and other safe and suitable nonmilk-derived ingredients, except for other food fats. In Brazil, the Federal Legislation (ANVISA, 2005) defines what ice creams are, obtained from the emulsion of fats and proteins or the mixture of water and sugar(s), and may be added with other ice creams ingredients, provided they do not alter the product characterization.

In the present work, C-PC was extracted from the dry biomass of the cyanobacterium *Spirulina (Arthrospira platensis)*. The purified C-PC concentration was 5,81 mg.mL^{-1}, yield of

92.19 % and a purity of 0.74, considered food grade (>0,70). Blue ice creams were formulated with 5% C-PC in the final concentration (% m/m) (Table 2). Additionally, ice creams were developed containing 1% of the RB of *Spirulina* used to obtain C-PC; this way, the application of a nutritionally rich residue that usually is discarded, was utilized to enrich the food product. The use of the whole *Spirulina* biomass follows the concept of circular economy, which was one of the goals of the present work.

The formulation of the ice creams was based on the recipe of the manufacturer Duas Rodas Industrial™ (Table 2) with modification since we used fermented milk as ice creams base and evaluated maltodextrin besides the conventional sweetener, sucrose. The microbiological quality of the formulation was determined following the Brazilian legislation (ANVISA, 2019), and the results are presented in Table 3. All tests were performed in triplicate, and the results are expressed in CFU/g, and all samples were safe for consumption alongside182 d.

Table 3. Microbiological quality of the formulated ice creams (initial and final times)

Time	Sample	Coliforms at 45° C and *E. coli*	*Salmonella*/25 g	*Staphylococcus* coagulase positive	*Enterobacteriaceae*
T0 (initial)	**SC**	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	SRB	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	SC-PC	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	MC	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	MRB	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	MC-PC	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
T182 (final)	**SC**	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	SRB	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	SC-PC	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	MC	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	MRB	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g
	MC-PC	< 10 CFU/g	Undetectable	Undetectable	< 10 CFU/g

SC: sucrose control; MC: maltodextrin control; SRB: sucrose with residual biomass; MRB: maltodextrin with residual biomass; SC-PC: sucrose with C-PC; MC-PC: maltodextrin with C-PC.

3.1 Color stability of C-PC and RB added ice creams

Figure 2 shows the appearance of the formulated ice creams: the controls formulations (Figures 2a and 2b, maltodextrin and sucrose, respectively), blue C-PC-added ice creams (Figures 2c and 2d, maltodextrin and sucrose, respectively), and green RB added ice creams (Figures 2e and 2f, maltodextrin and sucrose, respectively).

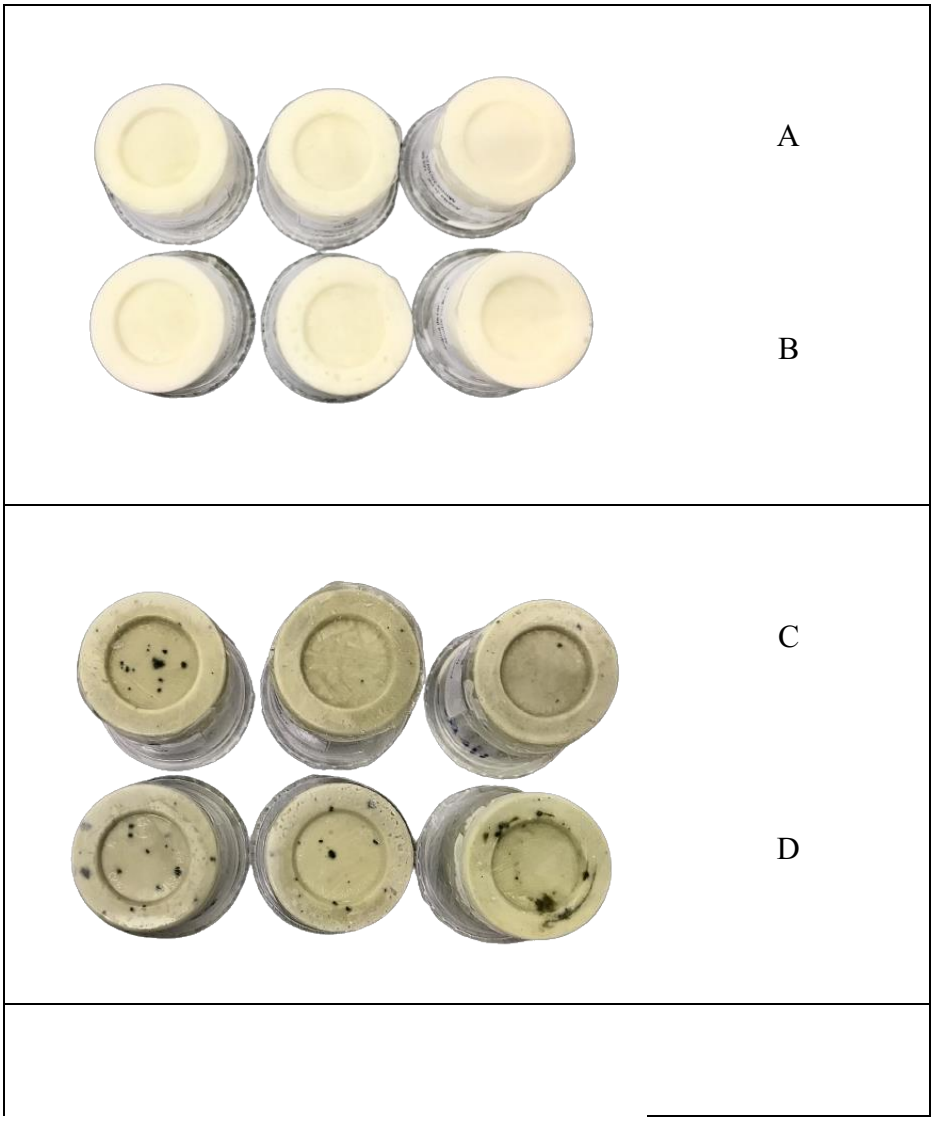


Figure 2. Control formulations (Figures 2a and 2b, maltodextrin and sucrose, respectively), green RB added ice creams (Figures 2c and 2e, maltodextrin and sucrose, respectively) and blue C-PC-added ice creams (Figures 2e and 2f, maltodextrin and sucrose, respectively).

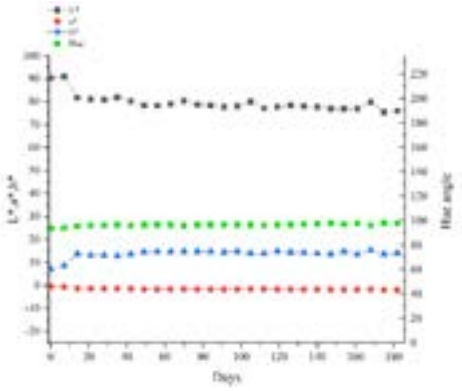
Figure 3 shows the color variation of the formulated ice creams over 182 d The Hue angle indicates the color angle (0° - red; 90° yellow; 180° green; 270° blue and 360° black), and the values of L*, a*, and b* were determined for each formulation. The value of L* corresponds to luminosity on a scale from 0 to 100; a* indicates the amount of red if positive values or green if negative values. The variable b* shows yellow when in positive values or blue when in negative values.

In all control formulations (Figure 3 a and b), b* values were positive, and the Hue angle was 93.64º ± 0.52 (SC) and 93.98º ± 0.39 (MC) on 1 d indicating slightly yellowish coloration (mean b* = 12.98 ± 1.63 and 13.69 ± 1,82, respectively), as expected due to the ingredients used to develop the ice cream, especially the heavy cream and the whole milk powdered. At 182 d, the Hue angle was 97.90º ± 0.73 (SC) and 97.61º ± 1.68 (MC), quietly changing for saturation alongside the evaluation time. For both samples, the luminosity decreased considerably from 1 d to 21 d, and was constant until 182 d, preserving its color after the 3 first weeks of maturation time.

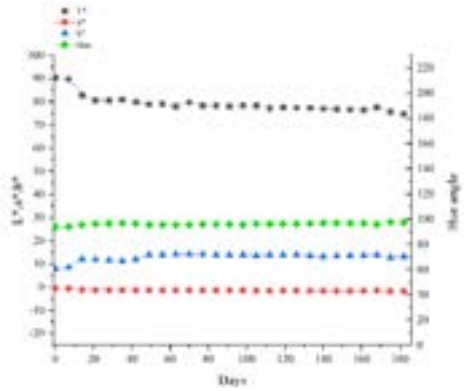
All ice cream formulations added with RB (Figure 3 c and d) showed negative a* values (mean -1.44 ± 0.16 and -0.94 ± 0.12), detecting the green color in these samples, as expected, due to the green color of *Spirulina* RB; the Hue angle at 1 d was 94.48º ± 2.44 (SRB) and 92.93º ± 0.16 (MRB). At 182 d, the Hue angle was 95.22º ± 2.43 and 93.93º ± 0.43, respectively, which, combined with positive b* values, indicated a stable color closer to yellow than green; in other words, the most detected color was that of the base ingredients. Additionally, the green spots indicate poor RB solubilization, thus, longer homogenization time for RB's formulations could improve the green color. Boosting the amount of RB could confer more greenish tonalities. However, it was shown that the sensory acceptance decreases as *Spirulina* concentration increases (FRADINHO *et al.*, 2020; MOSTOLIZADEH *et al.*, 2020). L* values changed more considerably until 49 d, and b* values started to increase. These two parameters combined revealed a fading in RB samples, independently from the sweetener.

In all ice creams containing C-PC (Figure 3 e and f), the measured b* parameter was negative. The Hue angle at 1 d for the formulation containing sucrose (SC-PC) or maltodextrin (MC-PC) was 224.23 º ± 0.53 and 223.15º ± 0.16, respectively. Combined with b* values, it scored the blue color in both formulations but less pronounced than those reported by Amarante *et al.* (2020b); they observed values close to 261.90º H in the initial analysis of milk-based ice creams added with C-PC, most likely due to the more yellowish color of the fermented milk base made with the addition of whole milk powdered to the detriment of the milk base used by the authors. At 182 d, the Hue angle was 231.80º ± 1.16 and 229.40º ± 1.62 for SC-PC and MC-PC, respectively; therefore, the saturation of the blue ice creams was stable during the 182 d of storage time.

Among the L* a* b* studied, the highest standard deviations were observed in L* (± 6.48 and ± 5.53) for C-PC added ice creams with maltodextrin (MC-PC) or sucrose (SC-PC), respectively, indicating that luminosity was the criterion that it changed more pronounced alongside the time, followed by b* (standard deviation of ± 1.97 and ±2.38, respectively), indicating maturation of the blue color in the period, that occurred more pronounced until 42 d. After that, the blue color maintained more stable b* and L* values, as indicated in Figures 3 e and f. It is worth mentioning that C-PC in ice creams (0.13 mg.mL^{-1}) also presented emulsifying and stabilizing activity without influencing the overall acceptability and contributing to better nutritional quality (RODRIGUES *et al.*, 2019).



(a)



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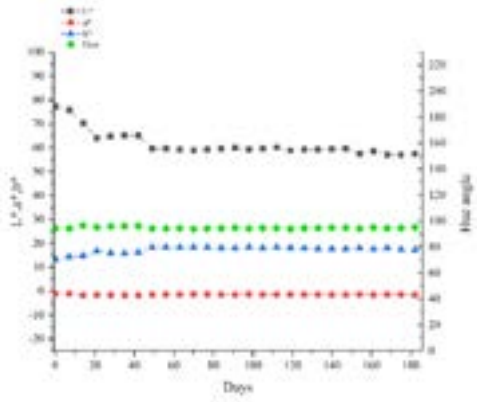

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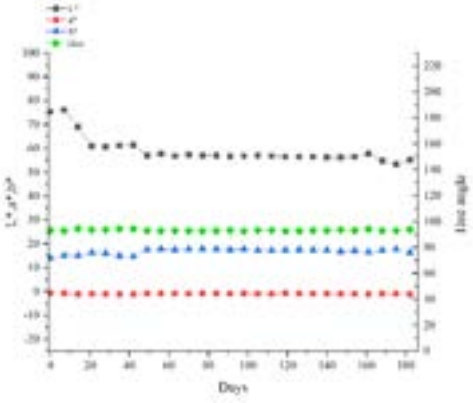
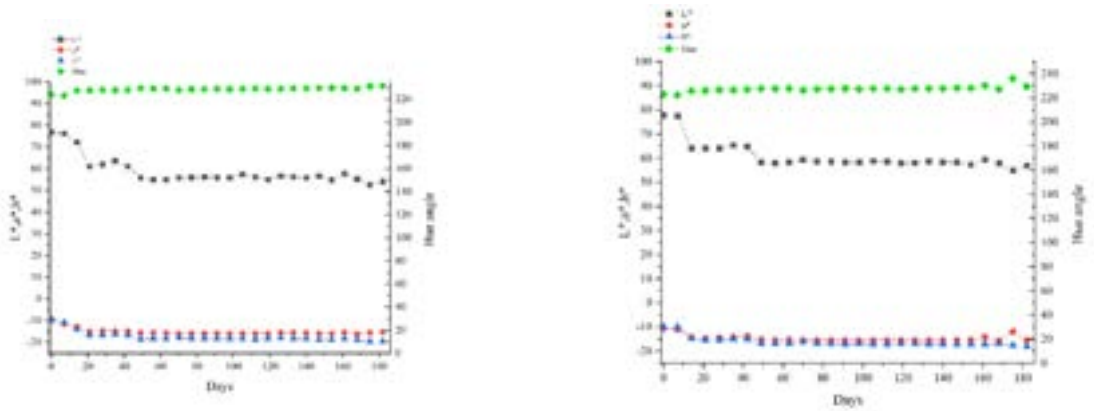


(d)



(e) (f)

Figure 3. Color variation of the formulated ice creams over the time: a - sucrose control (SC); b - maltodextrin control (MC); c - sucrose with residual biomass (SRB); d - maltodextrin with residual biomass (MRB); e - sucrose with C-PC (SC-PC); f - maltodextrin with C-PC (MC-PC).

For all the samples, the L* a* b* and Hue angle values varied little over the 182 d, even with different sweeteners. The luminosity values were the most drastically changed until 21 d for SC, MC, SC-PC, and MC-PC and until 49 d for SRB and MRB. After the maturation period, the color was stable. These results pointed out that sucrose is the best option for producing naturally colored ice creams. The challenge of C-PC's color stability was addressed, probably due to the low temperatures of storage and lack of high temperature in the production process. Another dairy product that successfully received 2 and 4% (m/v) C-PC was plain yogurt (MOHAMMADI-GOURAJI; SOLEIMANIAN-ZAD; GHIACI, 2019) C-PC maintained color stability, did not influence the starter culture, improved the texture, and decreased syneresis. The sensory evaluation demonstrated that 4% C-PC added yogurt had the best overall acceptability.

Even though it seems easier to keep the integrity of the C-PC in refrigerated products that in the baked ones, NICCOLAI *et al*. (2021) achieved well succeed results in crostini's production

due to the presence of added olive oil containing tocopherol to the recipe. It brings light to future C-PC and *Spirulina* biomass applications in food.

3.2 Antioxidant activity

The methods used in the measurement of antioxidant activity/capacity can be classified as hydrogen atom transfer (HAT)-based, electron transfer (ET)-based, and mixed-mode (HAT- and ET- based) assays (CAPANOGLU *et al.*, 2018), according to their reactive species-deactivation mechanism (BRAGA; ROCHA; HUBINGER, 2018; MURADOR *et al.*, 2018).

HAT-based assays measure the capacity of an antioxidant molecule to quench free radicals by hydrogen atom donation. In contrast, ET-based assays measure the capacity of an antioxidant to reduce an oxidant molecule, increasing or decreasing its absorbance (APAK *et al.*, 2013). Mixed mode assays are based on scavenging a radical by antioxidants using both HAT- and ET-based mechanisms (CAPANOGLU *et al.*, 2018).

The ORAC method is based on a hydrogen atom transfer (HAT) reaction and inhibits peroxyl radicals, which predominate in lipid oxidation in food, and biological systems (MUNTEANU; APETREI, 2021) is relevant to this work, while the ABTS method is usually classified as an ET-based assay, even though some authors consider it as a mixed mode assay since the $ABTS^{\bullet+}$ radical can be deactivated either by direct reduction through ET-based reactions or by radical quenching via HAT-based reactions, depending on the molecules involved (APAK *et al.*, 2013). The $ABTS^{\bullet+}$ radical determines the antioxidant capacity for hydrophilic and lipophilic compounds since it is water and organic solvent soluble (MUNTEANU; APETREI, 2021).

Considering the many variables that can influence the antioxidant activity results in food products, as they are complex samples, applying assays with different reaction mechanisms is

substantial since they work as complementary methods (CAPANOGLU *et al.*, 2018). Therefore, in this study, the antioxidant activity was determined by both the ORAC and ABTS methods to provide a complete idea of the antioxidant capacity of biomass, C-PC, and RB from *Spirulina* separately (Table 5) and in ice creams before and after bioaccessibility by using Tukey test and T-test, $p > 0.05$ (Tables 6 and 7, respectively). Also, the total phenolic compounds are presented in Table 4.

According to total phenolic content, the ice creams C-PC added (SC-PC and MC-PC) presented the highest amount of its antioxidant among the samples due to C-PC, which contains phenolic compounds on extract (purity of 0.74). Also, cow's milk used in the ice cream formulation naturally contains phenolic compounds (VÁZQUEZ *et al.*, 2015). All the samples carry phenolics; however, these combined ingredients promoted an upgraded phenolic content.

Table 4. Total phenolic content of the formulated ice creams (SC, SC-PC, SRB, MC, MC-PC, and MRB).

Formulation	Total phenolic content mg GAE/100 g of sample
SC	$2.46^{bc} \pm 0.1$
SC-PC	$2.89^{ab} \pm 0.3$
SRB	$2.20^{c} \pm 0.1$
MC	$2.44b^{c} \pm 0.1$
MC-PC	$3.19^{a} \pm 0.4$
MRB	$2.40^{bc} \pm 0.1$

Equal letters indicate no significant difference between the means (Tukey test, $p > 0.05$) in the same column. Samples - SC: sucrose control, SC-PC: sucrose and C-PC, SRB: sucrose and residual biomass, MC: maltodextrin control, MC-PC: maltodextrin and C-PC, and MRB: maltodextrin and residual biomass

One improvement in phenolic and C-PC content was observed by NICCOLAI *et al.* (2019) in crostini at 2, 6, and 10% *Spirulina* (m/m) concentration, suggesting that biomass has this

potential. Even after C-PC extraction, other biomolecules such as phenolics are reached in considering the SRB and MRB values (2.20 ± 0.1 and 2.40 ± 0.1), respectively, but the carbohydrate (maltodextrin or sucrose) did no affected the phenolic content.

Table 5 shows the antioxidant activity of the whole biomass of *Spirulina*, C-PC and RB and reinforces the functional potential of RB, as it maintains antioxidant activity, even with a statistically significant difference when compared to C-PC and *Spirulina* biomass.

Table 5 Antioxidant activity of the matrices used as ingredients to formulate the colored ice creams (*Spirulina*, RB, and C-PC) was determined by ABTS and ORAC methods.

Antioxidant Method	*Spirulina*	RB	C-PC
ORAC (μM TE/g)	$359.2^{a} \pm 39.3$	$132.5^{b} \pm 41.0$	$371.8^{a} \pm 54.2$
ABTS (μM TE/g	$9.54^{b} \pm 2.70$	$5.14^{b} \pm 1.70$	$10.06^{a} \pm 1.12$

Equal letters indicate no significant difference between the means (Tukey test, $p > 0.05$) in the same line.

The ice cream formulations presented antioxidant activities against the $ABTS^{\bullet+}$ and the peroxyl radicals (Table 6). The antioxidant capacities measured by ORAC of the ice cream prepared with RB and maltodextrin (MRB) showed the highest antioxidant activity of the other formulations before the digestion. However, after the digestion, MC-PC, SRB, and SC-PC showed no significant difference ($p > 0.05$) when compared with the initial value of MRB (37.5 ± 11.9), which implicates sucrose as a better option to produce the ice creams considering the cost.

The ABTS method detected significant differences between loaded maltodextrin ice creams (MRB and MC-PC) and the controls (SC and MC) before digestion (Table 6). The same method showed a significant difference ($p < 0.05$) through T-test only for the ice cream control (SC) (Table 7), which decreased antioxidant activity after the digestion test, suggesting that neither

maltodextrin nor sucrose changed the positive biological effects of RB and C-PC for this method; however, sucrose was the best sweetener through ORAC method.

Table 6. Antioxidant activity of the formulated ice creams (SC, SC-PC, SRB, MC, MC-PC, and MRB) was determined by ABTS and ORAC methods before and after in vitro digestion.

Formulation		Antioxidant Method	
		ORAC (µM TE/g)	ABTS (µM TE/g)
Ice cream	SC	$5.0^{a} \pm 2.4$	$0.5^{abc} \pm 0.1$
	SC-PC	$15.4^{abd} \pm 2.0$	$1.6^{abcd} \pm 0.8$
	SRB	$15.3^{ad} \pm 3.7$	$1.4^{abcd} \pm 0.5$
	MC	$9.5^{a} \pm 1.1$	$0.7^{ab} \pm 0.1$
	MC-PC	$22.6^{abd} \pm 3.4$	$2.5^{cd} \pm 1.1$
	MRB	$37.5^{bc} \pm 11.9$	$2.7^{d} \pm 0.6$
Ice cream after in vitro digestion	SC	$5.3^{a} \pm 0.7$	$0.2^{a} \pm 0.1$
	SC-PC	$41.3^{bc} \pm 12.6$	$2.1^{bcd} \pm 0.6$
	SRB	$38.0^{bcd} \pm 14.3$	$1.9^{abcd} \pm 0.5$
	MC	$8.9^{a} \pm 2.9$	$0.6^{ab} \pm 0.2$
	MC-PC	$37.1^{bc} \pm 18.3$	$2.2^{bcd} \pm 0.8$
	MRB	$49.9^{c} \pm 2.9$	$2.0^{abcd} \pm 0.8$

Equal letters indicate no significant difference between the means (Tukey test, $p > 0.05$) in the same column. Samples - SC: sucrose control, SC-PC: sucrose and C-PC, SRB: sucrose and residual biomass, MC: maltodextrin control, MC-PC: maltodextrin and C-PC, and MRB: maltodextrin and residual biomass

Furthermore, AMARANTE *et al*. (2020b) formulated an ice cream containing C-PC as a natural blue pigment. The authors added 0.07% (m/m) of C-PC obtained from *Spirulina* and observed an increase of 2 to 13 times of antioxidant activity in digested ice creams compared to the control ice cream (without the addition of C-PC) in the same conditions, indicating that the C-PC and its biological effects remained active and increased in the final product. Even with the previous literature review, we did not find another study that considered antioxidant activity after the digestion process. These results are congruent with the 2.7 improvement in antioxidant activity of the SC-PC and 2.5 in SRB ice creams after the bioaccessibility test (41.3 ± 12.6 and 38.0 ±14.3),

respectively, for the ORAC method, besides the luminosity fading in SRB evaluated by colorimetric tests (Figure 3 c). The greater results achieved by AMARANTE *et al*. (2020b) (ORAC: 134.63 ± 15.68 and ABTS: 1425.19 ± 54.93 μmolTE.g^{-1}) are probably due to C-PC's purity of 1,1.

Table 7. Antioxidant activity of the formulated ice creams (SC, SC-PC, SRB, MC, MC-PC, and MRB) was determined by ABTS and ORAC methods before and after in vitro digestion.

Formulation		Antioxidant Method	
		Ice cream	Ice cream after in vitro digestion
ORAC (μM TE/g)	SC	5.0^{A} ± 2.4	5.3^{A} ± 0.7
	SC-PC	15.4^{A} ± 2.0	41.3^{B} ± 12.6
	SRB	15.3^{A} ± 3.7	38.0^{B} ± 14.3
	MC	9.5^{A} ± 1.1	8.9^{A} ± 2.9
	MC-PC	22.6^{A} ± 3.4	37.1^{A} ± 18.3
	MRB	37.5^{A} ± 11.9	49.9^{A} ± 2.9
ABTS (μM TE/g)	SC	0.5^{A} ± 0.1	0.2^{B} ± 0.1
	SC-PC	1.6^{A} ± 0.8	2.1^{A} ± 0.6
	SRB	1.4^{A} ± 0.5	1.9^{A} ± 0.5
	MC	0.7 ^{A} ± 0.1	0.6^{A} ± 0.2
	MC-PC	2.5^{A} ± 1.1	2.2^{A} ± 0.8
	MRB	2.7^{A} ± 0.6	2.0^{A} ± 0.8

Equal letters indicate no significant difference between the means (T-test, p> 0.05) in the same line.

In this regard, LAMOTHE *et al*. (2019) demonstrated that milk and polyphenol-rich beverages act synergically to reduce polyunsaturated fatty acids oxidation during gastrointestinal digestion due to interaction between proteins from milk and polyphenols, which protects the biomolecules and consequently improves the antioxidant activity. Accordingly, higher values of antioxidant activity through ORAC in SC-CP after bioaccessibility tests are attached to the phenolic and protein content of this sample, while the higher SRB value in the same condition, despite the lowest ORAC antioxidant activity (132.5 ± 41.0) (Table 5) of RB compared with the

whole biomass and C-PC (359.2 ± 39.3 and 371.8 ± 54.2, respectively and with no significant difference), is probably due to greater access to carotenoids and allophycocyanin content in RB after C-PC extraction (TAVANANDI; VANJARI; RAGHAVARAO, 2019), once again confirming the excellent opportunity to apply the RB.

This work agrees with AMARANTE *et al*. (2020b), who observed increased health benefits of the ice creams after digestion by the human body. Additionally, both studies' color stability was reached, corroborating its reproducibility and possibility of scaling up. The results show the importance of the present work, confirming the data from the literature regarding the C-PC's great potential as a natural blue pigment as well as a functional ingredient to be used in the Food Industry.

4 Conclusion

The present work overcame the challenge of keeping C-PC active and stable for six months in a complex matrix. The results outlined that adding C-PC extract and the RB from *Spirulina* were responsible for the high scavenging capacities found in the ice creams. This work demonstrates the use of C-PC extracted from the dry biomass of *Spirulina* as a stable food dye in ice creams, and the same is valid for the RB. Since both formulations have biological effects, RB with sucrose formulation is the more suitable for scaling up due to its price and human health agent potential.

After the maturation period, the ice creams' color was stable; thus, these results associated with the antioxidant activity after the digestive test pointed out that sucrose is the best option to produce naturally colored ice creams. The presented results are significant starting points for new products containing natural pigments with functional appeal. Further research should evaluate the ice cream's quality parameters, including sensory analysis.

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CONSIDERAÇÕES FINAIS

Nos últimos três anos mais estudos foram realizados considerando a aplicação de biomassa de *Spirulina* em produtos alimentícios. No entanto, ainda existem lacunas a serem superadas, como a caracterização reológica, ensaio de atividade antioxidante, principalmente após digestão *in vitro* e avaliação sensorial. Como alimentos azuis são incomuns, os alimentos adicionados de C-PC são uma oportunidade para criar produtos para inovar em um mercado competitivo, sobretudo se analisada também a disposição dos consumidores para adquirir sorvetes funcionais naturalmente coloridos. A adição da C-FC em produtos que já são comercializados mostrou uma forma muito concreta pela qual a indústria alimentícia pode se reinventar. Inicialmente, os consumidores jovens tendem a ser os mais beneficiados pela substituição de pigmentos artificiais por naturais em balas, coberturas e gomas de mascar. Até o momento, os produtos refrigerados são as opções mais acessíveis para incorporar o C-PC como corante azul natural devido à consequente preservação da cor sob temperaturas refrigeradas. Considerando o trabalho experimental desenvolvido, o sorvete formulado com resíduo e sacarose é o que mais pode oferecer benefícios, pois em termos de efeitos biológicos não houve diferença estatística significativa comparado com a formulação com C-FC. O sorvete com biomassa residual aproveita uma matéria prima que seria descartada e ainda apresenta um menor custo relacionado com o pigmento natural utilizado.