UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

ACRISIO JOSÉ UCHÔA BASTOS FILHO

DETERMINAÇÃO DO EFEITO ANTIOXIDANTE E DA ESTABILIDADE ESPECTROSCÓPICA DA R-FICOERITRINA ISOLADA DA MACROALGA MARINHA *Solieria filiformis* (KÜTZING) P.W. GABRIELSON E SUA APLICAÇÃO COMO CORANTE NATURAL VERMELHO EM IOGURTE

FORTALEZA
2021

ACRISIO JOSÉ UCHÔA BASTOS FILHO

DETERMINAÇÃO DO EFEITO ANTIOXIDANTE E DA ESTABILIDADE ESPECTROSCÓPICA DA R-FICOERITRINA ISOLADA DA MACROALGA MARINHA *Solieria filiformis* (KÜTZING) P.W. GABRIELSON E SUA APLICAÇÃO COMO CORANTE NATURAL VERMELHO EM IOGURTE

Tese apresentada ao Programa de Pós-graduação em Bioquímica do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como requisito parcial para a obtenção do Título de Doutor em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientadora: Prof.ª Drª. Ana Lúcia Ponte Freitas.

FORTALEZA

2021

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

B326d Bastos Filho, Acrisio José Uchôa.
Determinação do efeito antioxidante e da estabilidade espectroscópica da R-Ficoeritrina isolada da macroalga marinha Solieria filiformis (Kützing) P.W. Gabrielson e sua aplicação como corante natural vermelho em iogurte / Acrisio José Uchôa Bastos Filho. – 2021.
99 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica , Fortaleza, 2021.
Orientação: Profa. Dra. Ana Lúcia Ponte Freitas.

1. Pigmento fluorescente. 2. Atividade antioxidante. 3. Corantes alimentícios. 4. Análise sensorial. I. Título.

CDD 572

ACRÍSIO JOSÉ UCHÔA BASTOS FILHO

DETERMINAÇÃO DO EFEITO ANTIOXIDANTE E DA ESTABILIDADE ESPECTROSCÓPICA DA R-FICOERITRINA ISOLADA DA MACROALGA MARINHA *Solieria filiformis* (KÜTZING) P.W. GABRIELSON E SUA APLICAÇÃO COMO CORANTE NATURAL VERMELHO EM IOGURTE

Tese apresentada ao Programa de Pós-graduação em Bioquímica do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como requisito parcial para a obtenção do Título de Doutor em Bioquímica. Área de concentração: Bioquímica Vegetal.

Aprovada em: 26/05/2021.

BANCA EXAMINADORA

_____

Prof ª. Dr ª. Ana Lúcia Ponte Freitas
Universidade Federal do Ceará (UFC)

_____

Prof ª. Dr ª. Marjory Lima Holanda Araújo
Universidade Federal do Ceará (UFC)

_____

Prof ª. Dr ª. Ticiana Monteiro Abreu
Universidade Federal do Ceará (UFC)

_____

Prof ª. Dr ª. Renata Line da Conceição Rivanor
Centro Universitário INTA (UNINTA)

_____

Prof ª. Dr ª. Tatiana de Oliveira Lemos
Universidade Federal do Maranhão (UFMA)

A Deus,

Aos meus pais, Acrísio e Rosângela.

AGRADECIMENTOS

A Deus, por toda saúde e força principalmente nesse momento tão devastador que está sendoessa pandemia do vírus Sars-CoV-2.

Aos meus pais, **Acrisio Bastos** e **Rosângela Bastos**, por todo apoio e ajuda de todas as formaspossíveis durante toda a minha vida.

Ao meu irmão **Átila Bastos**, por me propiciar muito dos meus melhores momentos de alegriase apoiar durante essa extensa fase da minha vida.

Ao meu irmão **Stélio Bastos,** e minha cunhada **Samantha Bastos,** por todo apoio durante meudoutorado e por ter dado a vida ao meu sobrinho-afilhado **Samuel Bastos** que nos enche de alegria todos os dias.

À Professora Drª. **Ana Lúcia Ponte Freitas**, por ter me aceitado no seu laboratório, e por todo apoio e suporte dado durante toda a minha formação, sempre me apoiando e preocupada com o meu trabalho.

À Professora Drª. **Márjory Lima Holanda Araújo**, que, como eu sempre falo para todos, ela é uma luz na minha vida, sempre disposta a ensinar com toda paciência e vibrando com todas as conquistas.

À Professora Drª. **Norma Maria Barros Benevides**, por ter aberto as portas do seu laboratóriopara que eu pudesse desenvolver esse trabalho, e sempre disposta a ajudar e tirar as dúvidas geradas durante os experimentos.

À Professora Drª. **Maria Raquel Alcântara de Miranda**, por sempre deixar às portas do Laboratório de Bioquímica e Fisiologia de Frutos (LABFRUTOS) e sempre disposta a ajudar quando fosse solicitado.

À Professora Drª. **Luciana de Siqueira Oliveira**, por todo apoio e força durante uma etapa crucial do meu doutorado e por sempre estar disponível para me ajudar e tirar minhas dúvidas.

À Ma. **Antônia Carlota de Souza Lima,** por ter se mostrado uma grande amiga e por ter se disponibilizado a ajudar mesmo estando em época de qualificação do doutorado.

À Drª. **Ticiana de Brito Lima Holanda,** por SEMPRE estar disponível para ajudar, orientandoe se disponibilizando para ajudar durante todo o doutorado.

As professoras participantes da banca examinadora a Professora Drª **Ana Lúcia Ponte Freitas,** a Professora Drª **Marjory Lima Holanda Araújo**, a Professora Drª **Ticiana Monteiro Abreu**, a Professora Drª **Renata Line da Conceição Rivanor**, e a **Prof ª. Dr ª. Tatiana de Oliveira Lemos** pelo tempo e pelas valiosas colaborações e sugestões.

Aos amigos **Áurea Brito**, **Lucas Lopes, Guilherme Peixoto e Vanessa Rodrigues** por toda ajuda nos experimentos realizados neste trabalho e por toda confiança e apoio durante todo esse período convivendo e por ter me permitido orientá-los durante seu trabalho de conclusão de curso.

A todos do **Laboratório de Aplicação Biotecnológica de Algas e Plantas (BioAP)**, **Laboratório de Carboidratos e Lectinas (CARBOLEC) e Laboratório de Algas (LabAlgas)** por todo apoio e paciência durante todos os desafios impostos a nós.

À **Rayra Melo Viana**, por todo o apoio, ensinamento e amizade durante o desenvolvimento deste trabalho.

Ao grupo **FAT FRIENDS (Camila Tauane, Marina Gabrielle, Roberta Cristiane e RobertaLaiz)** que foi criado durante a graduação e permanece até hoje, obrigado por todos os momentos de alegria e descontração e que agora foi agraciado com três integrantes grávidas, que venha com muita saúde!

Ao grupo **JUNTOSTOGETHER ♥ (Roberta Cristiane e Roberta Laiz)** por todo apoio e força durante o doutorado e por propiciar muitos momentos de descontração e alegria em todosos momentos.

Ao grupo composto por **Arthur Costa, Roberta Cristiane e Roberta Laiz** por toda diversão,alegria, amizade e por todos os momentos de apoio.

Ao grupo **CELOBRAC** criado em 2017 por alguns integrantes do laboratório e que permaneceu a amizade até hoje, obrigado por todos os momentos de descontração.

Ao grupo **CHRISTUS (Cibele Pires, Jessika Magalhães, Ryvana Mesquita e Sergiana Abreu)** fruto de uma amizade verdadeira e duradoura durante o ensino médio e que permaneceaté hoje, obrigado por todo momento de alegria e descontração.

Aos **técnicos e funcionários do Departamento de Bioquímica e Biologia Molecular** pela ajuda e amizade durante o desenvolvimento dessa pesquisa.

À **UFC**, por meio do Departamento de Bioquímica e Biologia Molecular, que disponibilizou oespaço físico para execução do referido trabalho.

A todos que contribuíram direta ou indiretamente para a realização deste trabalho, meu muito obrigado!

O presente trabalho foi realizado com o apoio da Coordenação de Aperfeioçoamento de Pessoal de Nível superior – Brasil (CAPES).

“Diga o quanto você ama a quem você ama, mas não fica só na declaração, gente. Amena prática, na ação. Amar é ação. Amar é arte (Paulo Gustavo, 2020, especial de fim de ano do programa 220 Volts).”

RESUMO

A cor é um atributo de grande impacto na decisão de consumo de um produto industrializado. A indústria utiliza uma variedade de corantes, principalmente, em alimentos e fármacos destinados ao público infantil. Entretanto, os relatos de alergenicidade de corantes artificiais têm estimulado a busca por novos corantes naturais, sobretudo os amarelos e vermelhos. As macroalgas marinhas vermelhas biossintetizam um pigmento fotossintético acessório vermelho (R-Ficoeritrina) com características físico-químicas e espectroscópicas (absorbância e fluorescência) de ampla aplicação biotecnológica e industrial. Diante do exposto, o presente trabalho teve por objetivo extrair, purificar e caracterizar a R-Ficoeritrina da macroalga marinha vermelha *Solieria filiformis*, e determinar seu potencial antioxidante e sua aplicação como corante natural em iogurte. Foram determinadas duas metodologias de purificação, atingindo índices de pureza de 4,5 e 2,4, com uma recuperação de 14,28% e 37,69%, respectivamente. R-Ficoeritrina de *S. filiformis* (R-FESf) apresentou 60% de α-hélice na sua estrutura secundária, com uma boa estabilidade espectroscópica ao calor (70 °C por 1 hora) e à variação de pH (5 a 11). As propriedades espectroscópicas da R-FESf se mantiveram estáveis nas temperaturas de -20 °C e 4 °C, ao ser armazenada por 30 dias. A R-FESf, na concentração de 500 µg/mL, atingiu elevada capacidade antioxidante em três métodos distintos, sequestro do radical ABTS, 97,98 ± 0,52%, capacidade quelante do íon ferroso, 94,75 ± 1,86%, e sequestro do radical DPPH, 79,04 ± 0,66%. A análise sensorial demonstrou que a R-FESf, ao ser utilizada como corante natural vermelho em iogurtes, obteve boa aceitação e preferência pelos consumidores. Assim, foram estabelecidos dois protocolos de purificação para R-ficoeritrina de *S. filiformis*, com índices de pureza diferentes, podendo ser aplicados industrialmente, apresentando predominância de estrutura secundária em α-hélice com propriedades espectroscópicas de elevada estabilidade, e, com um alto poder antioxidante. Além disso, R-FESf pode ser uma biomolécula promissora para substituir os corantes utilizados na indústria alimentícia.

Palavras-chave: pigmento fluorescente; atividade antioxidante; corantes alimentícios; análise sensorial.

ABSTRACT

Color is an attribute with a great impact on the decision to consume an industrialized product. The industry uses a variety of dyes, mainly in foods and drugs intended for children. However, reports of allergenicity of artificial dyes have stimulated the search for new natural dyes, especially yellows and reds. Red marine seaweed biosynthesize a red accessory photosynthetic pigment (R-Phycoerythrin) with physicochemical and spectroscopic characteristics (absorbance and fluorescence) with wide biotechnological and industrial application. Given the above, the present work aimed to extract, purify and characterize the R-Phycoerythrin from the red seaweed *Solieria filiformis*, and to determine its antioxidant potential and its application as a natural dye in yogurt. Two purification methodologies were determined, reaching purity indices of 4.5 and 2.4, with a recovery of 14.28% and 37.69%, respectively. R-Phycoerythrin from *S. filiformis* (R-FESf) showed 60% of α-helix in its secondary structure, with good spectroscopic stability to heat (70 °C for 1 hour) and pH variation (5 to 11). The spectroscopic properties of R-FESf remained stable at temperatures of -20 °C and 4 °C when stored for 30 days. R-FESf, at a concentration of 500 µg/mL, reached high antioxidant capacity in three different methods, ABTS radical scavenging, 97.98 ± 0.52%, ferrous ion chelating capacity, 94.75 ± 1.86%, and DPPH radical scavenging, 79.04 ± 0.66%. Sensory analysis showed that R-FESf when used as a natural red coloring in yogurts, obtained good acceptance and preference by consumers. Thus, two purification protocols were established for R-phycoerythrin from S. filiformis, with different levels of purity, which can be applied industrially, presenting a predominance of secondary structure in α-helix with high stability spectroscopic properties, and with a high power antioxidant. Furthermore, R-FESf may be a promising biomolecule to replace dyes used in the food industry.

Keywords: fluorescent pigment; antioxidant activity; food coloring; sensory analysis.

LISTA DE FIGURAS

Figura 1 - Classificação dos corantes alimentícios 17
Figura 2 - Estrutura do ficobilissomo 21
Figura 3 - Disposição dos ficobilissomos na membrana do tilacóide 21
Figura 4 - Diferença de coloração entre os tipos de ficobiliproteínas 22
Figura 5 - Estruturas químicas das ficobilinas 23
Figura 6 - Estruturas das ficoeritrinas contendo as bilinas 24

LISTA DE TABELAS

Tabela 1 - Corantes vermelhos alimentícios permitidos pelo Informe Técnico n.68, de 3 de setembro de 2015 da Agência Nacional de Vigilância Sanitária 19

SUMÁRIO

1	**INTRODUÇÃO**	14
2	**REVISÃO BIBLIOGRÁFICA**	16
2.1	**Corantes alimentícios**	16
2.2	**Ficobiliproteínas de macroalgas marinhas vermelhas: estrutura, caracteristicas espectroscópicas e aplicação como corante natural**	20
3	**OBJETIVOS**	26
3.1	**Geral**	26
3.2	**Específicos**	26
4	**PERMISSÃO LEGAL**	27
5	**ARTIGO 1 REFERENTE À TESE**	28
6	**ARTIGO 2 REFERENTE À TESE**	56
7	**CONCLUSÃO**	88
	REFERÊNCIAS	89

1 INTRODUÇÃO

A cor é um atributo de grande impacto na decisão de consumo de um produto. O processamento dos alimentos, geralmente, altera a cor final do produto, necessitando dos corantes alimentícios para restaurar sua coloração original (DUTCOSKY, 1996; COBUCCI, 2010). Segundo a legislação brasileira, os corantes alimentícios são substância ou mistura de substâncias que possuem a propriedade de conferir ou intensificar a coloração de alimentos, e são classificados em orgânico natural, orgânico sintético, artificial, orgânico sintético idêntico ao natural, inorgânico e caramelo (BRASIL, 1978).

Dentre os corantes disponíveis no mercado, a indústria tem priorizado o uso dos sintéticos por possuírem maior estabilidade e menor custo quando comparado com os corantes naturais. Quando se trata de corantes vermelhos a Agência Nacional de Vigilância Sanitária (ANVISA) permite o uso de 14 corantes, sendo 7 sintéticos e 7 naturais. Dentre os corantes naturais vermelhos, 6 são provenientes de frutas ou legumes, ocupando terra agricultável para produção dos mesmos, e um é proveniente de inseto, sendo atribuído a esse relato de alergenicidade (TAKEO et al., 2018). Além disso, os recentes relatos de alergenicidade atribuídos aos corantes vermelhos sintéticos têm induzido a indústria a procurar novas alternativas de corantes naturais (ANASTÁCIO et al., 2016; GIČEVIĆ; HINDIJA; KARAČIĆ, 2020; IHEANYICHUKWU et al., 2021; MOTTA et al., 2019).

As macroalgas marinhas têm se mostrado excelentes fontes de corantes naturais por biossintetizar pigmentos fotossintéticos de diferentes colorações como azul (ficocianinas), amarelo (carotenos), verde (clorofila) e vermelho (ficoeritrinas). As ficoeritrinas são ficobiliproteínas hidrossolúveis com propriedades antioxidantes e espectroscópicas (absorbância e fluorescência) de ampla aplicação na indústria, sobretudo diante da carência de corantes naturais vermelhos (HEMLATA; AFREEN; FATMA, 2018; KANNAUJIYA et al., 2021; PATEL et al., 2018; WU et al., 2020). O preço desse corante pode variar de acordo com a sua pureza, podendo ser utilizado com uma pureza menor (menor custo de produção) na indústria alimentícia e com uma pureza maior (custos de purificação elevados) na indústria farmacêutica (PAN et al., 2013).

Apesar da elevada demanda industrial por corantes naturais vermelhos, existem poucos relatos de extração de baixo custo, purificação e caracterização de ficoeritrina a partir de macroalgas (R-Ficoeritrinas), sendo necessário, ainda, um estudo aprofundado sobre as características e estabilidades química e espectroscópica dessa molécula, visando a ampliar sua aplicação na indústria alimentícia como uma alternativa aos corantes existentes atualmente.

A macroalga marinha vermelha *Solieria filiformis* é abundante nos mares tropicais, cultivável e tem sido estudada para cultivo em sistema de aquicultura multitrófica integrada e ecologicamente correta para sintese de carotenoides, ficobiliproteínas e para biofiltração (FELACO; OLVERA-NOVOA; ROBLEDO, 2020; ZEPEDA; FREILE-PELEGRÍN; ROBLEDO, 2020).

Diante da pouca disponibilidade de corante natural vermelho para aplicação em alimentos, e da escassez de dados na literatura sobre a utilização dos pigmentos das macroalgas marinhas como corante natural na indústria alimentícia, a alga *Solieria filiformis* é uma fonte sustentável do pigmento R-Ficoeritrina com um elevado poder corante, sendo capaz de substituir os corantes naturais vermelhos utilizados atualmente pela indústria alimentícia.

2 REVISÃO BIBLIOGRÁFICA

2.1 Aditivo alimentar

A Agência Nacional de Vigilância Sanitária (ANVISA), por meio da portaria nº 540, de 27 de outubro de 1997, define aditivo alimentar como:

> [...] qualquer ingrediente adicionado intencionalmente aos alimentos, sem propósito de nutrir, com o objetivo de modificar as características físicas, químicas, biológicas ou sensoriais, durante a fabricação, processamento, preparação, tratamento, embalagem, acondicionamento, armazenagem, transporte ou manipulação de um alimento. Ao agregar-se poderá resultar em que o próprio aditivo ou seus derivados se convertam em um componente de tal alimento. Esta definição não inclui os contaminantes ou substâncias nutritivas que sejam incorporadas ao alimento para manter ou melhorar suas propriedades nutricionais (BRASIL, 1997).

Os aditivos alimentares podem desempenhar diversas funções, como agente de massa, antiespumante, antiumectante, antioxidante, corante, conservador, edulcorante, espessantes, gelificante, estabilizante, aromatizante, umectante, regulador de acidez, acidulante, emulsionante/emulsificante, melhorador de farinha, realçador de sabor, fermento químico, glaceante, agente de firmeza, sequestrante, estabilizante de cor e espumante. Dentre essas funções, os corantes são um dos aditivos mais importantes em produtos industrializados, pois tem o poder de conferir, intensificar ou restaurar a cor de um alimento (BRASIL, 1997; RAMESH; MUTHURAMAN, 2018).

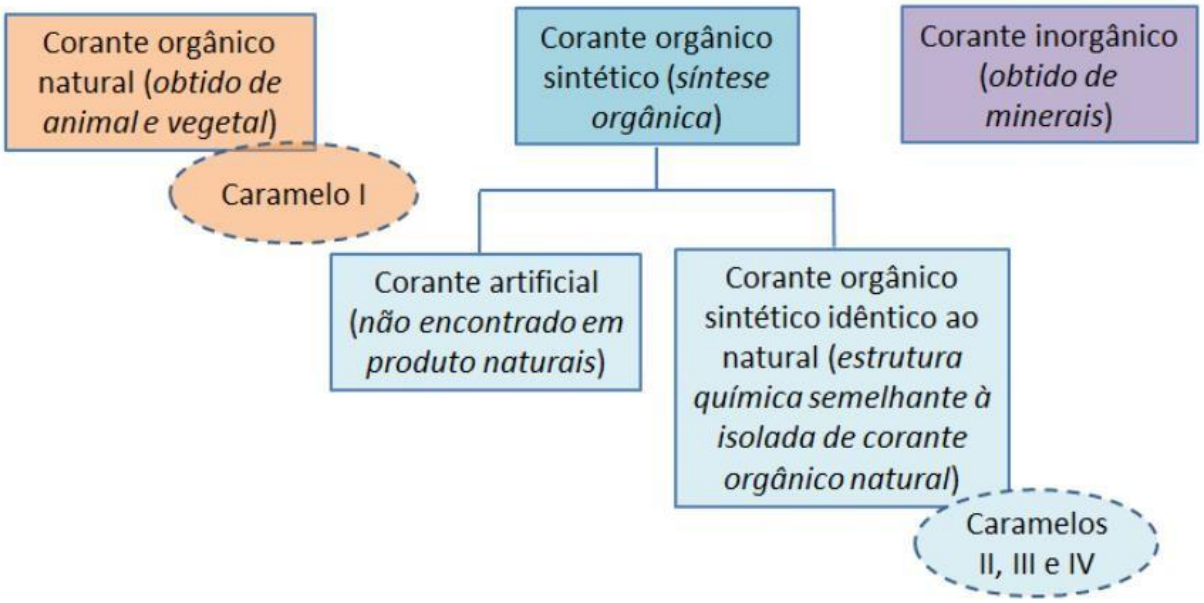
2.2 Corantes alimentícios

Corantes alimentícios são utilizados com o objetivo de atrair o consumidor por meio da sua percepção visual. Durante o processamento dos alimentos, a coloração natural do produto pode se perder, sendo necessário a utilização desses agentes de coloração para realçar a cor natural do produto, tornando-o atraente para o consumo (RAMESH; MUTHURAMAN, 2018).

Segundo a Resolução nº 44 de 1977 emitida pela Comissão Nacional de Normas e Padrões para Alimentos (CNNPA), corante é qualquer substância adicionada com a intenção de conferir ou intensificar a coloração de um alimento e que pode ser classificado como orgânico natural, orgânico sintético, artificial, orgânico sintético idêntico ao natural, inorgânico, caramelo e caramelo - processo amônia. O corante orgânico natural é aquele em que o princípio corante é extraído de vegetal ou animal, enquanto o corante orgânico sintético é obtido por síntese orgânica, podendo ser artificial (não encontrado em produtos naturais) ou idêntico ao natural (estrutura química semelhante a do princípio ativo isolado de um corante natural). Já o corante inorgânico é obtido a partir de minerais, e, por fim, o corante caramelo que é obtido

pelo aquecimento de açucares a temperatura superior ao ponto de fusão e o caramelo – processo amônia sendo classificado como um corante orgânico sintético idêntico ao natural (FIGURA 1) (BRASIL, 1978).

Figura 1 – Classificação dos corantes alimentícios.



Fonte: ANVISA, 2015.

Segundo a Portaria nº 540, de 27 de outubro de 1997 do Ministério da Saúde/Secretária de Vigilância Sanitária, os corantes alimentícios são considerados aditivos com a intenção de modificar as características espectroscópicas do produto (BRASIL, 1997).

A preferência da indústria em utilizar corantes artificiais, sobretudo em alimentos processados, é devido à estabilidade destes em relação a fatores como pH, luz, oxigênio, temperatura, poder tintorial e, principalmente, devido a um menor custo de produção em relação aos naturais. Entretanto, do ponto de vista nutricional, o uso de corantes em alimentos não traz qualquer benefício, e os aspectos toxicológicos e as quantidades empregadas em alimentos vêm preocupando a população e a comunidade científica em relação ao cumprimento da legislação e quanto à influência desses corantes na saúde humana (RAMESH; MUTHURAMAN, 2018).

Os corantes artificiais são utilizados para compensar a perda das cores dos produtos industrializados que ocorre durante o processamento e o armazenamento, devolvendo sua cor característica original com a finalidade de agradar o consumidor. Porém, eles têm sido apontados como os principais desencadeadores de reações adversas, sobretudo os dois corantes

amarelos artificiais tartrazina e amarelo de quinoleína, os quais têm sido associados à hiperatividade, asma e urticária, e os três corantes vermelhos artificiais amaranto, ponceau 4R e eritrosina, sendo descrito casos que os relacionam com a ocorrência de asma, dificuldade de aprendizagem e hiperatividade, além de ser carcinogênico em potencial (ANASTÁCIO et al., 2016; BATADA; JACOBSON, 2016; CORRADINI, 2018; GIČEVIĆ; HINDIJA; KARAČIĆ, 2020; IHEANYICHUKWU et al., 2021; MOTTA et al., 2019).

Em contrapartida, segundo a pesquisa realizada pela empresa, especialista em pesquisa de mercado, *Meticulous Market Research*®, em 2020, o mercado de corantes naturais deve chegar a US$ 3,2 bilhões em 2027 com uma taxa de crescimento anual composta de 8,5% entre o período de 2019 a 2027. Esse crescimento é devido a exigência pelo consumidor por corantes naturais, que sejam benéficos para a saúde.

Dentre os corantes vermelhos utilizados atualmente em alimentos, o Informe Técnico n. 68, de 3 de setembro de 2015 da Agência Nacional de Vigilância Sanitária (ANVISA) permite o uso de 14 corantes, sendo 7 sintéticos e 7 naturais. Na tabela 1 estão listados os corantes vermelhos permitidos atualmente, de acordo com o informe técnico citado, bem como seu código INS (*International Numbering System*), origem, solubilidade e tonalidade de cor.

Tabela 1 – Corantes vermelhos alimentícios permitidos pelo Informe Técnico n. 68, de 3 de setembro de 2015 da Agência Nacional de Vigilância Sanitária

Classificação	Código INS	Nome	Origem	Solubilidade	Tonalidade de cor
Corantes orgânicos naturais	120	Carmim	Fêmeas de insetos cochonilha (*Dactylopius coccus* Costa)	Hidrofílico	Vermelho
	160b	Urucum	Sementes do urucum (*Bixa orellana* L)	Hidrofóbico/ Hidrofílico	Amarelo-alaranjado / Castanho-avermelhado
	160c	Páprica	Frutos secos moídos de pimentão (*Capsicum annuum*)	Hidrofóbico	Vermelho Escuro
	160d(ii)	Licopeno	Polpa de tomate vermelho (*Lycopersicon esculentum* L)	Hidrofóbico	Vermelho Escuro
	161g	Cantaxantina	Cogumelo (*Cantharellus cinnabarinus*)	Hidrofóbico	Violeta
	162	Betanina	Raízes da beterraba vermelha (*Beta vulgaris* L var rubra)	Hidrofílico	Vermelho
	163(ii)	Extrato da casca de uva (Antocianina)	Casca ou bagaço de uva após obtenção do suco	Hidrofílico	Vermelho
Corantes orgânicos sintéticos	122	Azorrubina	Artificial	Hidrofílico	Vermelho
	123	Amaranto	Artificial	Hidrofílico	Castanho-avermelhado
	124	Ponceau 4R	Artificial	Hidrofílico	Avermelhado
	127	Eritrosina	Artificial	Hidrofílico	Vermelho
	128	Vermelho 2G	Artificial	-	Rosada
	129	Vermelho Allura AC	Artificial	Hidrofílico	Vermelho Escuro
	180	Litol rubina BK	Artificial	-	Vermelho/Alaranjado

Fonte: Elaborado pelo autor a partir de dados da ANVISA (2015), CÂMARA (2017) e FAO/WHO (2021). Legenda: Traço (-) indica que não foi encontrado informação precisa

Dentre os 14 corantes permitidos, apenas os corantes naturais ISN 120, 160b e 162, e os corantes sintéticos ISN 122, 124 e 129, são permitidos para uso em leites fermentados, segundo a instrução normativa Nº 46, de 23 de outubro de 2007 (BRASIL, 2007). Os corantes orgânicos naturais aprovados para uso em leite fermentados são betanina, carmim e urucum (norbixina). Betanina é um corante hidrofílico que pode ser empregado como corante em alimentos à base de agua, entretanto, sua instabilidade a luz, pH e temperatura restringe seu uso (PÁTKAI; BARTA, 1996; SAGUY, 1979). Carmim já é usado mundialmente em alimentos, bebidas e cosméticos, entretanto esse corante é descrito na literatura como indutor de reações adversas, como reações alérgicas e choques anafiláticos (GALLO, 2018; TAKEO *et al.*, 2018). As principais cores do urucum são provenientes dos carotenoides bixina e norbixina, sendo bixina insoluvel em agua, e a norbixina soluvel em agua com pH alcalino/neutro. Essa baixa solubilidade da norbixina em pH acido dificulta sua aplicação direta nos produtos alimenticios (MØLLER et al., 2020; SCOTTER, 2009)

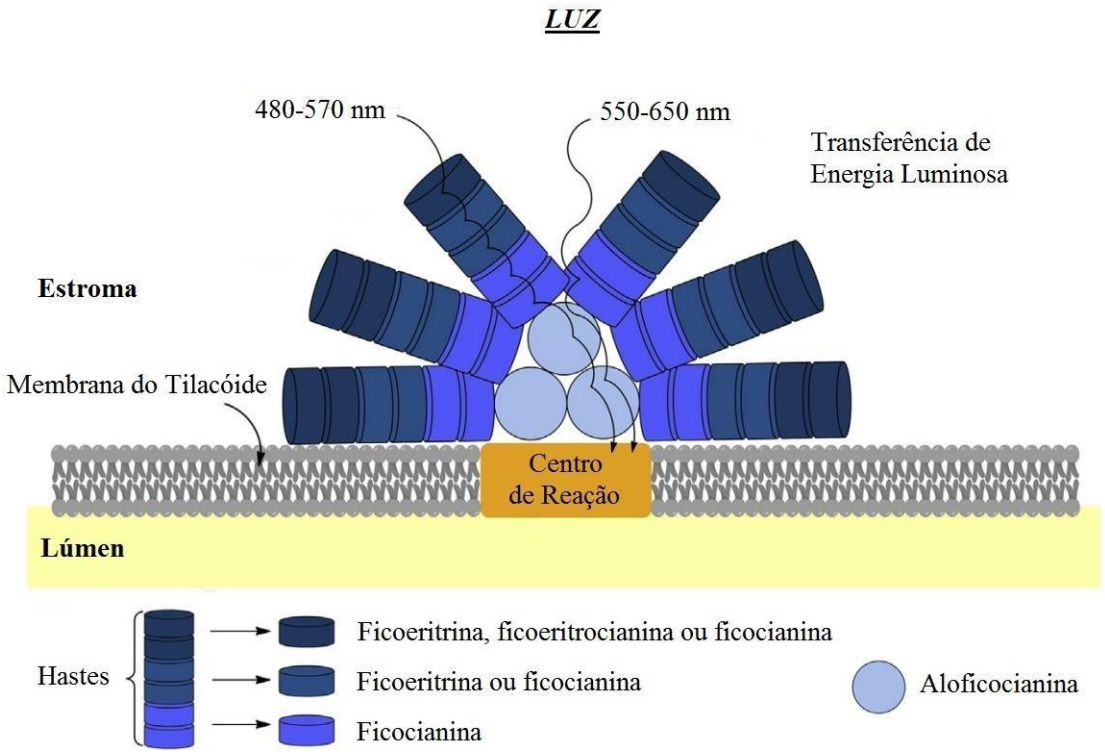
Diante do exposto, é possível perceber a necessidade de descobertas de novos corantes naturais vermelhos, sendo as macroalgas marinhas vermelhas fontes potenciais de tais corantes vermelhos, chamadas R-Ficoeritrinas, e com alto potencial biotecnológico agregado.

2.3 Ficobiliproteínas de macroalgas marinhas vermelhas: estrutura, características espectroscópicas e aplicação como corante natural

As macroalgas marinhas vermelhas são fontes de ficobiliproteínas hidrofílicas pigmentadas que possuem um ou mais grupos prostéticos tetrapirrólicos de cadeia aberta (bilinas ou ficobilinas) covalentemente ligados, por ligações do tipo tioéter, a resíduos de cisteína (GLAZER, 1984; GLAZER et al., 1976).

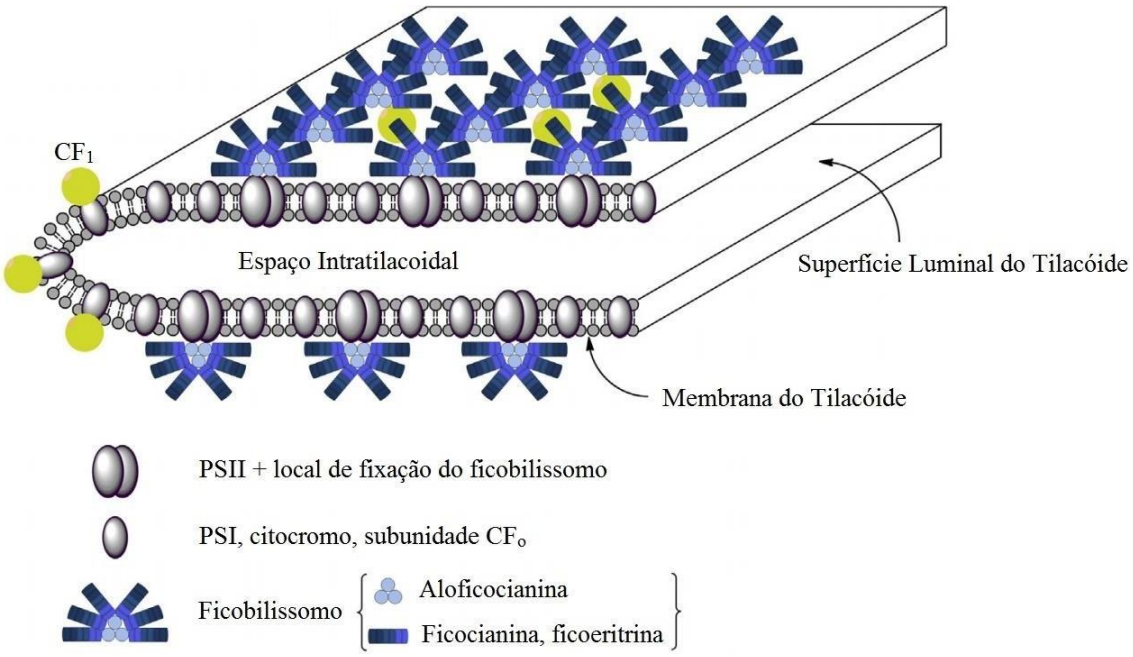
As ficobiliproteínas estão agregadas em complexos proteicos supramoleculares denominados ficobilissomos (Figura 2), onde, *in vivo*, estão dispostos na superfície externa da membrana do tilacóide, voltados para o estroma (Figura 3) (GANTT, 1980; APT; COLLIER; GROSSMAN, 1995). Essa estrutura permite qualquer um dos pigmentos absorver a luz solar, na qual a clorofila é ineficiente (450-670 nm), e transferir para o centro de reação fotossintético II com uma eficiência de captação e transferência de energia de aproximadamente 100% *in vivo*, desempenhando um papel importante para a fotossíntese (GLAZER, 1989, 1984).

Figura 2 – Estrutura do ficobilissomo.



Fonte: Adaptado de DUMAY *et al.*, 2014

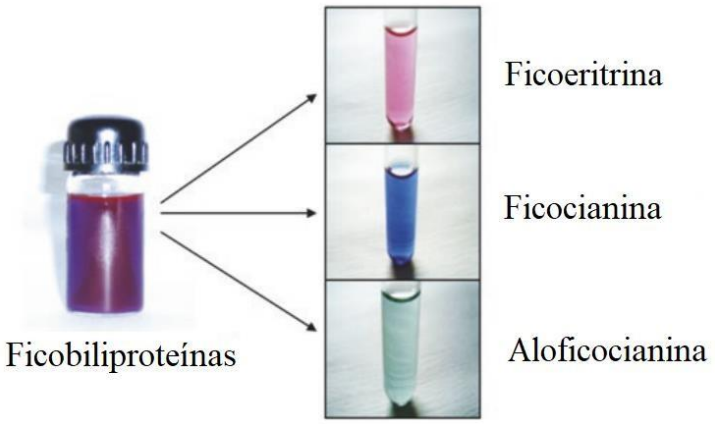
Figura 3 – Disposição dos ficobilissomos na membrana do tilacóide.



Fonte: Adaptado de DUMAY *et al.*, 2014. **Legenda:** CF – Fator de acoplamento; PS – Fotossistema

De acordo com sua coloração, as ficobiliproteínas podem ser divididas em dois grupos: o pigmento vermelho – ficoeritrina e o pigmento azul – ficocianina (O'CARRA; MURPHY; KILLILEA, 1980). Elas também podem ser dividas de acordo com suas características espectrais: ficoeritrina (λ_{max} = 495-570 nm), ficoeritrocianina (λ_{max} = 575 nm), ficocianina (λ_{max} = 610-620 nm), e aloficocianina (λ_{max} = 650-655 nm) (Figura 4) (BRYANT; GLAZER; EISERLING, 1976; GLAZER, 1985).

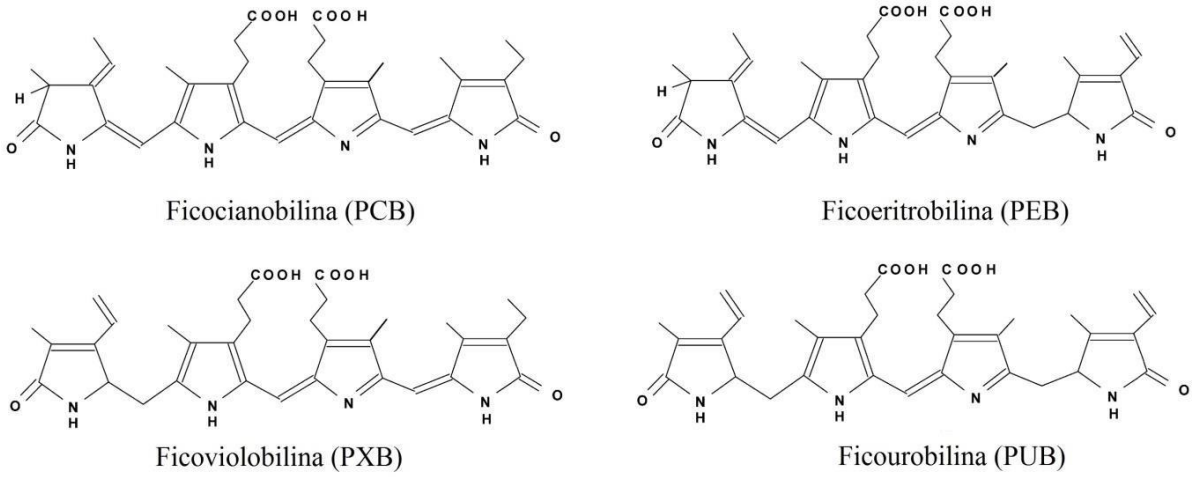
Figura 4 – Diferença de coloração entre os tipos de ficobiliproteínas.



Fonte: Adaptado de KANNAUJIYA; SUNDARAM; SINHA, 2017.

As ficobiliproteínas são constituídas de duas subunidades proteicas diferentes α e β, dispostas em forma de disco, e uma terceira subunidade diferente (γ), que é encontrada nas ficoeritrinas (FE) atuando como peptídeo de ligação (SIDLER, 1994; NIU; WANG; TSENG, 2006). Existe uma ou mais ficobilinas (bilina) ligadas em cada subunidade, podendo ser encontradas quatro tipos diferentes: ficoeritrobilina (PEB, λ_{max} = 560 nm), ficourobilina (PUB, λ_{max} = 450 nm), ficocianobilina (PCB, λ_{max} = 620-650 nm) e ficoviolobilina (Figura 5) (PXB, λ_{max} = 575 nm) (ISAILOVIC; LI; YEUNG, 2004; ZHAO *et al.*, 2015). PEB é encontrada nas C-ficoeritrinas (encontrada em cianobactérias), R-ficoeritrinas (encontrada na maioria das macroalgas vermelhas), B-ficoeritrinas (encontrada em algumas microalgas vermelhas) e ficocianinas, PUB é encontrada nas R-ficoeritrinas e B-ficoeritrinas, PCB é encontrada nas ficocianinas, aloficocianinas e ficoeritrocianinas, e PXB é encontrada nas ficoeritrocianinas, fazendo com que os tipos de ficobilinas presentes nos organismos variem de espécie para espécie, conservando os locais de ligação das ficobilinas (APT; COLLIER; GROSSMAN, 1995; ISAILOVIC; LI; YEUNG, 2004).

Figura 5 – Estruturas químicas das ficobilinas.

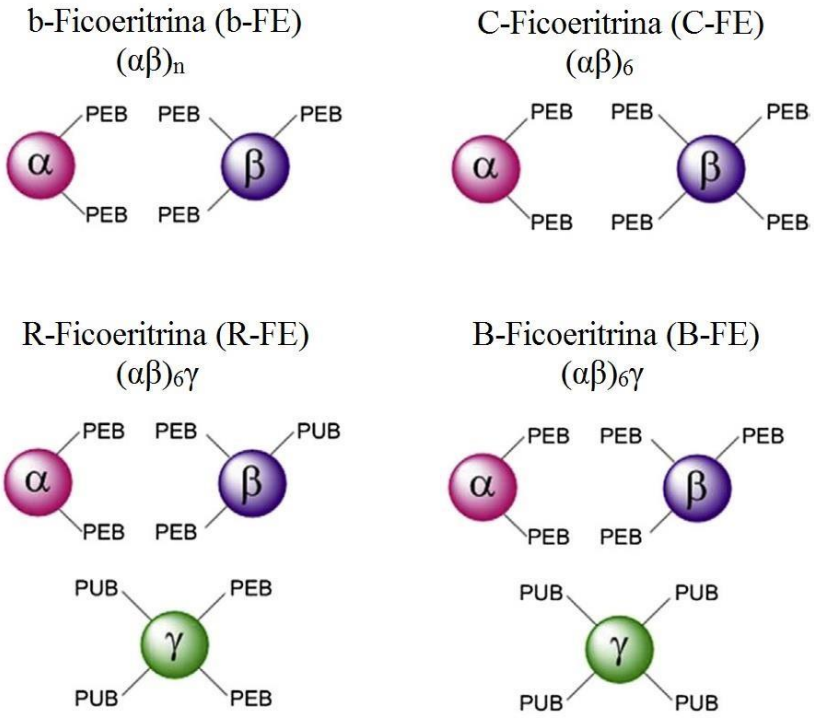


Fonte: Adaptado de ISAILOVIC; LI; YEUNG, 2004.

Por sua vez, as ficoeritrinas podem ser divididas em cinco grupos de acordo com suas características espectrais de absorbância: B-Ficoeritrina (B-FE) [(λ_{max} = 565 nm, 545 nm, e um ombro a 499 nm)], b-Ficoeritrina (b-FE) [(λ_{max} = 545 nm, e um ombro a 565 nm)], C-Ficoeritrina (C-FE) [(λ_{max} = 560 nm)] e R-Ficoeritrina (R-FE) [(λ_{max} = 565 nm, 498 nm e um ombro/pico a 545 nm)] (BOGORAD, 1975; GLAZER *et al.*, 1976; BRYANT, 1982; SIDLER, 1994).

Essa diferença espectral de absorbância entre as ficoeritrinas se deve a quantidade de ficobilinas totais presentes na sua estrutura. B-FE e R-FE são compostas por três subunidades α, β e γ e suas estruturas são descritas como complexos hexaméricos $(\alpha\beta)_6\gamma$ ou $(\alpha\beta)_3\gamma(\alpha\beta)_3$, enquanto que b-FE e C-FE também são formadas por complexo (αβ), porém não possuem a subunidade γ (Figura 6) (LUNDELL *et al.*, 1984; CHANG *et al.*, 1996).

Figura 6. Estruturas das ficoeritrinas contendo as bilinas.



Fonte: Adaptado de DUMAY *et al.*, 2014

As subunidades α, β e γ que compõem a R-FE possuem em torno de 18, 20 e 35 kDa, respectivamente, e formam complexos hexaméricos $(\alpha\beta)_6\gamma$, de 240 kDa, onde dois trímeros $(\alpha\beta)_3$ estão dispostos na forma de disco, ligados pela subunidade γ, que está localizada no centro da cavidade formada por esses trímeros, conferindo maior estabilidade (CHANG *et al.*, 1996; FLEURENCE, 2003; ROSSANO *et al.*, 2003). Elas possuem duas bilinas em sua estrutura, ficoeritrobilinas (PEB) e ficourobilinas (PUB), onde a transferência de energia de ressonância por fluorescência (FRET) da PUB para PEB resulta no pico de emissão máximo de fluorescência a 575 nm (JIANG *et al.*, 1999).

Considerando que a R-FE é um composto intracelular, vários métodos físicos, químicos e bioquímicos de rompimento da parede celular das algas foram adotados para liberar o pigmento durante a etapa de extração. Os métodos físicos são mais adotados e relatados na literatura, como os ciclos de congelamento e descongelamento (XU; WANG; HOU, 2020; ZANG et al., 2020; ZHAO et al., 2020), trituração (GANESAN; SHANMUGAM, 2020;

PEREIRA et al., 2020b; SALURI et al., 2020) e ultrasonicação (MITTAL et al., 2020; UJU et al., 2020).

O processo de purificação da R-FE é estabelecido de acordo com o grau de pureza necessário para a aplicação final. O preço final do pigmento é impactado pelo grau de purificação. Métodos como fracionamento e precipitação por sulfato de amônio, cromatografia de troca iônica, filtração em gel, hidroxiapatita e eletroforese são, geralmente, utilizados para purificação da R-FE (NGUYEN et al., 2020; ZANG et al., 2020; ZHAO et al., 2020).

Além da R-FE possuir uma coloração atrativa para a indústria, em especial a alimentícia, já foi relatado atividades antioxidantes, antibacteriana, anticancerígenas, anti-inflamatória e imunomodulatória (CIAN et al., 2012; GANESAN; SHANMUGAM, 2020; THANGAM et al., 2015). Sua propriedade fluorescente também é utilizada como sondas moleculares em várias indústrias farmacêuticas e biotecnológicas (KANNAUJIYA et al., 2021; WU et al., 2020).

3 OBJETIVOS

3.1 Geral

O presente trabalho teve por objetivo extrair, purificar e caracterizar a R-Ficoeritrina da macroalga marinha vermelha *Solieria filiformis*, e determinar seu potencial antioxidante e sua aplicação como corante natural em iogurte.

3.2 Específicos

- Extrair a R-Ficoeritrina de *S. filiformis*;
- Determinar diferentes metodologias de purificação da R-Ficoeritrina de *S. filiformis*, visando alcançar índices de pureza variáveis;
- Caracterizar a estrutura da R-Ficoeritrina de *S. filiformis*;
- Caracterizar as propriedades espectroscópicas da R-Ficoeritrina de *S. filiformis* frente a variações de temperatura e pH;
- Determinar a atividade antioxidante in vitro da R-Ficoeritrina de *S. filiformis*;
- Caracterizar os parâmetros colorimétricos de iogurtes naturais adicionados de R-Ficoeritrina de *S. filiformis*;
- Avaliar a aceitação e a preferência de iogurtes adicionados de R-Ficoeritrna de *S. filiformis*, por meio de testes afetivos.

4 PERMISSÃO LEGAL

A atividade de acesso ao Patrimônio Genético/CTA dessa tese de doutorado está cadastrada no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen), em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos, protocolado sob número A41C95F.

5 ARTIGO 1 REFERENTE À TESE

R-Phycoerythrin from *Solieria filiformis* red seaweed: Extraction, purification, stability spectroscopic and application as a natural coloring in yogurt

[1]Acrisio José Uchôa Bastos-Filho, [1*]Márjory Lima Holanda-Araújo, [1]Aurea Anette Monteiro Brito, [1]Francisco Lucas de Souza Lopes, [1]Roberta Cristiane Cavalcante Costa, [2]Antônio Augusto Lima Araújo-Filho, [1]Vanessa Rodrigues Ferreira, [1]Ticiana de Brito Lima-Holanda, [2]Maria do Carmo Passos Rodrigues, [3]Tatiana de Oliveira Lemos, [1]Norma Maria Barros Benevides, [1]Ana Lúcia Ponte Freitas.

[1] Department of Biochemistry and Molecular Biology, Federal University of Ceará, CE, Brazil
[2] Department of Food Engineering, Federal University of Ceará, CE, Brazil
[3] Department of Food Engineering, Federal University of Maranhão, MA, Brazil

[1*] Corresponding author. Phone: +55 85 3366-9826; Fax: +55 85 3366-9820.

E-mail: marjory.holanda@ufc.br

ABSTRACT

R-Phycoerythrin is a photosynthetic pigment widely applied in biotechnology as a fluorescent probe in immunology and cell biology. However, its use as a natural dye in foods such as yogurt is still underreported. The present study aimed to extract, purify and apply the R-Phycoerythrin from *Solieria filiformis* in natural yogurt. R-Phycoerythrin was extracted and purified presenting fractions with purity indexes of pigment since 1.3 at 4.5 and yields of 6.5 at 17.4 $\mu g.g^{-1}$ ws. The electrophoretic profile showed three protein bands with molecular masses of 18, 20 and 37 kDa and the spectroscopic stability of pigment was conserved at -20 °C and 4 °C for 30 days. The natural yogurt colored with R-phycoerythrin showed good acceptance and preference by consumers.

Keywords: Rhodophytes; chromatography; yield; recovery; sensory analysis; affective test.

1 INTRODUCTION

Red marine seaweeds (Rhodophyta) are an excellent source of hydrophilic natural pigments called phycobiliprotein (PBP). PBPs are a group of light-harvesting pigment protein complexed in phycobilisomes which is divided into two large groups according to their color, blue (phycocyanin) and red (phycoerythrin). The PBPs are divided into four classes according to their absorption properties: phycoerythrin (PE, λ_{max} = 490–570 nm), phycocyanin (PC, λ_{max} = 610–625 nm), phycoerythrocyanin (PEC, λ_{max} = 560–600 nm) and allophycocyanin (APC, λ_{max} = 650–660 nm) (GLAZER, 1985). More specifically, PEs are divided into four groups second your natural source: B-phycoerythrin (B-PE), b-phycoerythrin (b-PE), C-phycoerythrin (C-PE) and R-phycoerythrin (R-PE). R-PE is found in rhodophytes, B-PE in algae of the order Bangiales, and b-PE and C-PE are present in cyanobacteria (DUMAY et al., 2014a).

R-PE has a molecular mass of about 240 kDa and it's constituted of three protein subunits, assembling a structure $(\alpha\beta)_6\gamma$, and phycobilins covalently linked, such as phycoerythrobilin (PEB) and phycourobilin (PUB). The number and composition of phycobilins linked in the R-Phycoerythrins provide spectroscopic properties to these proteins (DUMAY et al., 2014a).

They have several biological activities including potent antioxidants, antibacterial, anticancer and anti-inflammatory activities (GANESAN; SHANMUGAM, 2020; THANGAM et al., 2015). The autofluorescence property of R-PE has been utilized as molecular probes in various pharmaceutical, molecular biological, and biotechnological industries (KANNAUJIYA et al., 2021). However, bright futures of commercial applications of R-PE crucially depend on the produced quantity along with a significant purity index of proteins. Considering that R-PE is an intracellular compound, several physicals, chemicals, and biochemicals methods of disrupting the algal cell wall have been adopted to release the pigment during the extraction step. The physical methods are more adopted and reported in the literature, such as freeze-thaw cycles (ZHAO et al., 2020), grinding (GANESAN; SHANMUGAM, 2020; PEREIRA et al., 2020b; SALURI et al., 2020) and ultrasonication (UJU et al., 2020). The purification process of the R-PE is established according to the purity grade necessary for the final application. Then, the price of pigment is impacted by the purification process. Methods such as ammonium sulfate fractionation and precipitation, ion-exchange chromatography, gel filtration, hydroxyapatite and electrophoresis, are recurrent (NGUYEN et al., 2020; ZHAO et al., 2020).

The low purity index R-PEs are applied in a wide variety of industrial foods, such as candies, ice cream, yogurt, puddings and drinks, because of red color and high hydrophilicity

(PAN et al., 2013; SUDHAKAR et al., 2015). However, there are few reports in the literature on the use of R-PE from species seaweeds as a natural food coloring. The majority of reports coming from agarophyte algae such as *Gracilaria gracilis*, *G. crassa*, and *G. corticata* (PEREIRA et al., 2020b; SUDHAKAR et al., 2015; SUDHAKAR; SARASWATHI; NAIR, 2014). Recently, Ganesan & Shanmugam (2020) isolated the R-PE from carragenophyte cultivated seaweed, *Kappaphycus alvarezii*, and it was applied as a natural colorant in ice cream.

Solieria filiformis is a red seaweed abundant in tropical seas. This species has been studied for biosynthesis and extraction of iota-carrageenan hydrocolloid (MURANO et al., 1997) and extraction of the lectins with antimicrobial, anticancer, antinociceptive and anti-inflammatory effects (ABREU et al., 2016; CHAVES et al., 2018; HOLANDA et al., 2005).

Thus, *Solieria filiformis* (Kützing) P. W. Gabrielson, a red tropical species seaweed abundant along the Brazilian coast and cultivated by a local fishers community from the Ceará coast was utilized to extract and purify the red pigment R-phycoerythrin and evaluate its application as a red colorant in yogurt.

2 MATERIALS AND METHODS

2.1 Seaweed

The red seaweed *Solieria filiformis* (Kützing) P. W. Gabrielson was harvested in culture strings located at 200 meters from the shore, on the Flecheiras beach (03° 13' 06" S and 39°16' 47" W), Trairí, on the west coast of State of Ceará, Brazil. After gathering, it was cleaned to remove epiphytes, salt and grains and then was washed with distilled water and stored at -20 °C (Lot 1) until use. A voucher specimen (Nº 35.682) was deposited in Herbarium Prisco Bezerra, Department of Biological Sciences, Federal University of Ceará (UFC), Brazil.

2.2 R-PE extraction

The wet seaweed was crushed in an electric mill with 0.025 M potassium phosphate buffer (PB), pH 6.5, in a ratio of 1:3 (m/v) for 3 min. The homogenate obtained was kept under constant stirring at 4 °C for 6 h protected from light and then it was filtered in nylon cloth. The liquid phase was centrifuged at 17,000 x g, 4 °C for 30 minutes and the supernatant obtained was called total extract (TE).

2.3 R-PE purification

2.3.1 Protein fractionation

The TE was submitted to protein fractionation to different ranges of ammonium sulfate saturation 0/20, 20/40, 40/60, 60/80, 80/90, 90/100 and 0/90 for 12 h, at 4 °C, after complete dissolution of the salt. The protein fractions were separated by centrifugation at 17,000 x g, 4 °C for 30 minutes, solubilized in PB and, finally, subjected to dialysis (*cutoff* 12 kDa) against PB. Each fraction was nominated with the letter F plus respective saturation (F0/20, F20/40, F40/60, F60/80, F80/90, F90/100, and F0/90).

2.3.2 Ion exchange chromatography

The ion exchange chromatography was performed in the DEAE-Sephacel matrix (GE Healthcare), previously equilibrated with PB. The not retained peak was eluted with extraction buffer and titled PI-DEAE, while the retained peaks were eluted with PB containing 0.1, 0.5 and 1.0 M NaCl and denominated PII, PIII and PIV-DEAE, respectively. The chromatography was performed under a constant flow of 1.4 $mL.min^{-1}$, collecting 3 $mL.tube^{-1}$ fractions.

2.3.3 Molecular exclusion chromatography

The molecular exclusion chromatography was performed on Sephacryl S-300 matrix previously equilibrated with PB, under a constant flow of 1 $mL.min^{-1}$, collecting 2 $mL.tube^{-1}$ fractions. Chromatography fractions containing R-PE with a purity index (PI) greater than 4.0 were collected, dialyzed against distilled water, lyophilized, and named R-PESf,

2.4 R-PE analysis

2.4.1 Protein and R-PE content

The protein content of the TE and fractions was determined the method described by Bradford (1976) using bovine serum albumin (BSA) as the standard. Already, the R-PE content ($mg.mL^{-1}$) was determined by Sampath-Wiley & Neefus (2007) and were calculated according to Equation 1:

$$R\text{-}PE = 0.1247 \times [(A_{564} - A_{730}) - 0.4583 \times (A_{618} - A_{730})] \quad (1)$$

Where A_{564}, A_{618} and A_{730} were the absorbances at 564, 618 e 730 nm, respectively, obtained in spectrophotometer Armersham Biosciences Ultrospec 2100 pro.

2.4.2 Yield, recovery, and purity

The protein yield ($\mu g.g^{-1}$) was calculated according to Equation 2:

$$Y_p = P_t/Sf \quad (2)$$

Where P_t was the total content of protein present in the TE and fractions (µg) and Sf was the mass of wet seaweed used at extraction (g).

The R-PE yield ($\mu g.g^{-1}$) was calculated according to Equation 3:

$$Y_{R\text{-}PE} = R\text{-}PE_t/Sf \quad (3)$$

Where R-PEt was the total content of R-PE present in the TE and fractions (µg) and Sf was the mass of wet seaweed used at extraction (g).

The recovery (%) was calculated according to Equation 4:

$$R_{R\text{-}PE} = (R\text{-}PE/R\text{-}PE_r) \times 100 \quad (4)$$

Where R-PE was the total content of R-PE present in the fractions and $R\text{-}PE_r$ was the total content of R-PE present in TE.

The purity index (PI) of R-PE present in TE and fractions was calculated by Equation 5:

$$PI = A_{564}/A_{280} \quad (5)$$

Where A_{280} and A_{564} were the measured of the absorbances at 280, 564 nm, respectively.

The relative purity (RP) of R-PE present in TE and fractions compared to phycocyanin was calculated by Equation 6:

$$RP = A_{564}/A_{618} \quad (6)$$

Where A_{564}, and A_{618} were measured of the absorbances at 564 e 618 nm, respectively.

2.4.3 Molecular weights

The apparent molecular weights of the R-PESf and subunits were determined by one-dimensional electrophoresis under denaturing conditions (SDS-PAGE). The electrophoresis was performed on 7.5% and 15% polyacrylamide gel in the absence and presence of β-mercaptoethanol, respectively, according to the methods described by Laemmli (1970). The apparent molecular weights of the protein bands were estimated using as molecular weight markers: phosphorylase B (97.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (45.0 kDa), bovine carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) for the 15% polyacrylamide gel.

2.4.4 Spectroscopic properties

The absorbance and fluorescence spectra were determined using a Synergy Mx microplate multi-detection spectrophotometer (BioTek, USA). The R-PESf was dissolved in PB buffer and analyzed for absorption and fluorescence emission spectra at concentrations of 0.05 $mg.mL^{-1}$ and 0.001 $mg.mL^{-1}$, respectively. The absorbance spectrum was determined at 25 ± 0.5 ° C in the range of 280 to 730 nm. The fluorescence emission spectrum was determined at 25 ± 0.5 ° C in the range 500 to 700 nm after excitation at the 495 nm wavelength.

2.5 Spectroscopic stability of R-PESf in storage

The R-PESf solution was stored at three different temperatures, -20, 4 and 25 °C for 30 days in amber microcentrifuge tubes of 2 mL. The absorbance and fluorescence analysis were realized every 5 days of storage, in triplicate. The sample was prepared and analyzed according to the methodology described in item 2.4.4. The results were expressed based on the loss of

fluorescence intensity and the variation in absorption of the three maximum peaks of the R-PESf in the initial time.

2.6 Color measurement of yogurt colored with F0/90 and PIII-DEAE

The fractions F0/90 and PIII-DEAE were applied as food coloring in natural yogurt (Danone), purchased at Pão de Açúcar supermarket, in Fortaleza, Ceará, Brazil. The F0/90 was dialyzed against water and then added to natural yogurt reaching the concentrations of 12.40 (F1); 24.80 (F2); 37.20 (F3); 49.60 (F4) and 62.00 (F5) µg R-PE.mL^{-1} of yogurt. The volumes of F0/90 added were 42, 85, 127, 169 and 212 µL.mL^{-1} of natural yogurt, respectively. The lyophilized PIII-DEAE was added directly to natural yogurt reaching the concentrations of 16.20 (P1); 20.30 (P2); 24.30 (P3); 28.40 (P4) and 32.40 (P5) µg R-PE.mL^{-1} of yogurt. The samples of yogurts were homogenized until the complete dissolution of the pigment. Commercial strawberry yogurt (Danone) artificially colored with azorubin was purchased at Pão de Açúcar supermarket, in Fortaleza, Ceará, Brazil and was used for color comparison purposes (C).

Color measurement was performed using a Colorimeter ColorQuest XE (HunterLab, USA) by determining the color value; L* (100 =white; 0 =black), a* (positive =red; negative =green), and b* (positive =yellow; negative =blue).

2.7 Affective tests of natural yogurts colored with F0/90 and PIII-DEAE

The sensorial analysis of the natural yogurts containing F0/90 and PIII-DEAE was carried out in the Sensory Analysis Laboratory of the Federal University of Ceará with 60 untrained panellists to each fraction (according to ISO 11136:2014, 60 consumers can be used in hedonic scale evaluations when there is segmentation). The panellists filled out a form, which included: sex, age group, marital status, strawberry yogurt consumption frequency, strawberry yogurt appreciation degree and the importance of color to the consumption of yogurt. Each panelist was allocated in an isolated cabin (without noise and contact with other panellists). The samples were prepared in Petri dishes, illuminated with white light for better visualization, and these were encoded with three random digits previously assigned.

In order to assess the acceptability of the color attribute of the yogurts added R-phycoerythrin from *S. filiformis* as a natural colorant, the following tests were carried out: a) Just-about-right (JAR) scale. In this test, the panellists evaluated the intensity of the color desired by them in the product. The scale ranged from 1 (Much too weak than just-about-right)

to 9 (Much too strong than just-about-right); b) Acceptance test (Hedonic scale). In this test, the panellists evaluated the acceptance of the color attribute. The test was performed using a 9-point structured hedonic scale (1 = 'disliked extremely, 5 = neither like nor dislike, and 9 = 'liked extremely'); c) Preference test. Panelists ranked the samples in order of their preference regarding the color of the sample from "least preferred" to "most preferred" (preference ranking test); d) Consumption intent. The test was performed on a 5-point structured scale (1 = "certainly would not consume" to 5 = "certainly would consume"). The consumer acceptance tests were performed according to Stone & Sidel (1973).

2.8 Statistical analysis

The statistical analysis was performed using GraphPad Prism version 6.1 for Windows, GraphPad Software (La Jolla, California, USA). The results for stability and sensory analysis were expressed as mean ± S.D. (Standard Deviation). It was used the variance analysis method (*one-way* ANOVA), followed by the Tukey test of multiple comparisons. It was considered significant values of $p < 0.05$ (LIMA et al., 2020). For the preference test, the Friedman test ($p < 0.05$) was used, according to the value established by Newell & MacFarlane (1987). All data were estimated as mean values of three repetitions, except for sensory data.

3 RESULTS AND DISCUSSION

3.1 R-PE extraction

A total soluble protein yield of TE was 145.0 $\mu g.g^{-1}$ ws and an R-PE yield was of 136.1 $\mu g.g^{-1}$ ws. The PI of the R-PE in the TE was 0.48. The red seaweed *S. filiformis* had a moisture content of 95% (data not shown), so it can be inferred that the methodology used obtained a yield of 2.72 mg of R-PE per gram of dry seaweed ($mg.g^{-1}$ ds).

The extraction of R-PE with phosphate buffer is efficient and has been used by several authors (NGUYEN et al., 2020; PEREIRA et al., 2020a). Wang et al. (2020) used the maceration for 5 h to extract the R-PE from *Pyropia yezoensis* obtaining a yield of 2.465 mg/g ds, lower than shown in this study. Using enzymatic hydrolysis with agarase and cellulase during extraction, they obtained an increase of 2.8 times on R-PE yield. However, this methodology is more expensive than maceration and depends on enzymes and seaweed species. The extraction of R-PE from the seaweed *Gelidium pusillum* using an enzyme consortium formed by agarase, cellulase and xylanase showed a yield of 0.29 mg/g ds, almost 10 times lower than the one found in this study (MITTAL; RAGHAVARAO, 2018a).

PI of R-PE, observed in the present study, was higher than obtained from *Gracilaria tenuistipitata* (0.24), *G. gracilis* (0.29) and *P. yezoensis* (0.23), even utilizing enzymatic hydrolysis (NGUYEN et al., 2020; WANG et al., 2020; ZHAO et al., 2020).

3.2 R-PE purification

3.2.1 Protein fractionation

Table 1 shows the analysis of yields, recovery, purity index, relative purity and color of the protein fractions obtained by precipitation with ammonium sulfate of the total extract from *Solieria filiformis*. These results suggest that the protein fractionation method can be used to obtain R-PE from *S. filiformis* with PI higher than 1.0 and recovery of ca. 15% if the F40/60 and F60/80 were united. Furthermore, the F0/90 can be used as a starter fraction for obtaining R-PE pure by purification process with better yield. The RP analysis showing values higher than 1.0 (1.33 at 7.78) suggests that the R-PE contents are higher than phycocianins contents in all protein fractions.

A similar result was observed in the protein fractions from *Amphiroa anceps*, where R-PE was distributed in all fractions and a wide range of ammonium sulfate saturation fraction (F0/80) was chosen as a starter to continue the pigment purification process (KAWSAR et al., 2011). A purity index higher than F0/90 was observed for the F0/85 from the *Heterosiphonia*

japonica seaweed. 0.78 (SUN et al., 2009). Given of the obtained results, and to increase the pure R-PE yield from *S. filiformis*, the F0/90 was selected to others steps of the purification process.

3.2.2 Ion exchange chromatography

The ion exchange chromatography of the F0/90 presented 4 protein peaks (Fig. S1 A), being the PI-DEAE and PII-DEAE, absent from R-PE. The PIII-DEAE, showed an intense pink color, high absorbance at 564 nm, protein and R-PE yields of 37.1 and 29.7 $\mu g.g^{-1}$ ws, respectively, and PI = 1.63, ca. of twice higher than F0/90. The PIV-DEAE showed a pink color, but of lower intensity.

Saluri et al. (2020) used ion exchange chromatography as the last step in the purification process of R-PE from *Furcellaria lumbricalis* and *Coccotylus truncates* reaching PI of 1.6 and 2.6, respectively. The value reached for seaweed *F. lumbricalis* was similar to the one reached on the present work.

3.2.3 Molecular exclusion chromatography

The molecular exclusion chromatography of the PIII-DEAE presented three protein peaks with different molecular masses as shown in Fig. S1 B. PI-Sephacryl and PIII-Sephacryl showed low absorbance at 564 nm, indicating a low content of R-PE present in these peaks. However, the chromatographic fraction referring to PII-Sephacryl was the one that showed the highest absorbance at 564 nm, reaching a yield of 20 $\mu g.g^{-1}$ ws for soluble proteins and 17.40 $\mu g.g^{-1}$ ws for R-PE. The PI increased from 1.63 in PIII-DEAE to 4.50 in PII-Sephacryl, reaching analytical grade purity.

Saluri et al. (2020) used the molecular exclusion chromatography as a purification step for R-PE from *F. lumbricalis* and *C. truncates* and obtained lower PIs of 1.2 and 1.6, respectively. Others studies related lower PI of the R-PE from *Polysiphonia urceolata* (NIU; WANG; TSENG, 2006) and *Porphyra yezoensis* (CAI et al., 2012), which reached 3.90 and 3.20, respectively.

Table 2 shows the Y_P and $Y_{R\text{-}PE}$, recovery, PI and RP of R-PE from *S. filiformis* at each stage of the purification process. The recovery of R-PESf, with PI ca. of 3.5 times higher than PI of the F40/60, *was* 14.28% and the $Y_{R\text{-}PE}$ increased 2.7 times when the F0/90 was used as started protein fraction of the purification process.

The different PIs observed for R-PE fractions shown in this study are important to define their applications. Therefore, the R-PESf can be used in assays or methods which require analytical grade pigment, while the PIII-DEAE can be used at food grade. Sudhakar et al. (2015) added R-PE from *Gracilaria Crassa* with PI of 0.91 in pudding. According to Pan et al. (2013), the R-PE must reach a PI > 0.7 to obtain the food grade and a PI > 2 for drug class purity.

The scheme of the extraction and purification of R-PE from *S. filiformis* is shown in Fig. S2.

3.2.4 Molecular Weights

The R-PESf in the absence of the reducing agent presented a single band, referring to the integral protein (Fig. 1). Before the dye solution, it is possible to observe an intense pink band (Fig. 1 A) indicating that the protein is intact. A single band was detected after the application of the dye solution, confirming its high purity (Fig. 1 B). R-PESf lost its characteristic color in the presence of the reducing agent, presenting a light pink band before staining by Coomassie and molecular weight estimated at 20 kDa (Data not shown). After coomassie staining, R-PESf showed three protein bands, with two more intense bands with an approximate molecular weight of 18 and 20 kDa, corresponding to the subunits α and β, respectively, and one band of 37 kDa, corresponding to the γ subunit, showing less intensity (Fig. 1 C).

Similar results were found by Ganesan & Shanmugam (2020) and Zhao et al., (2020) when carrying out studies with *Kappaphycus alvarezii* and *Gracilaria tenuistipitata*, respectively. In these studies, the R-phycoerythrins were composed of three subunits α, β and γ, with apparent molecular weights of 18, 20, and 30 to 38 kDa, respectively.

3.2.5 Spectroscopic properties

The absorption spectra of TE and of all R-PE purification step fractions are shown in Fig.S3. The TE presented absorption peaks at 495 nm and between 540 and 564 nm, characteristic of R-PE, however poorly defined, showing the presence of contaminating compounds capable of absorbing light at other wavelengths, corroborating with the low PI of the TE (0.48). On the others purification steps fractions, F0/90, PIII-DEAE and PII-Sephacryl (R-PESf), a gradual increase in the definition of the three maximum absorption peaks at 495, 540 and 564 nm was observed, corroborating with the increase of the PIs of these fractions.

The R-PESf absorption and fluorescence emission are shown in Fig. S4. The absorbance spectrum showed two peaks at 495 and 564 nm and one shoulder at 540 nm, and the fluorescence emission showed a defined and single peak at 575 nm when excited at 495 nm, corroborating the R-PE absorption spectra of *P. urceolata* (498, 538 and 566 nm), reported by Wang *et al.* (2015) and *Gracilaria tururu* (498, 540 and 565 nm), described by Munier *et al.* (2015).

3.3 Spectroscopic Stability of R-PESf in Storage

The spectroscopic stability of the R-PESf is shown in Fig. 2. The fluorescent capacity of the R-PESf (Fig. 2 A) maintained at 4 ºC decreases 33.3% in the first 5 days. When stored at -20 °C, the R-PESf lost 42.5% of fluorescence after the first five days, then it remained fluorescent at -20 ºC and 4 ºC for 30 days. When stored at 25 °C, it had the fluorescence emission was completely lost after the first 5 days of storage.

Similar results were observed in Rhodophytes of the Bangiales family, which maintained their fluorescent stability preferably at -20 ºC (MUNIER et al., 2014a). The literature also suggests the strong dependence of phycobiliprotein fluorescence on temperature, especially in the range of 4 to -20 ºC (GOLDSMITH; MOERNER, 2010; MAKSIMOV et al., 2013).

The light absorption spectrum of the pigment was maintained under the storage conditions at temperatures of -20 °C (Fig. 2 B) and 4 °C (Fig. 2 C). The same behavior was not observed for the storage at 25 °C (Fig. 2 D), when the 540 and 564 peaks lost 87% of the absorbance after 10 days.

The results are consistent with the thermal stability of other phycoerythrins. The freeze storage method (-20 ºC) was a better conservation method for R-PE from *Grateloupia turuturu* seaweed, when compared to storage by freeze drying at room temperature (MUNIER et al., 2013).

3.4 Color measurement of yogurt colored with F0/90 and PIII-DEAE

Table 3 shows the values of the colorimetric parameters (L*, a*, b*) of the natural yogurt colored with F0/90, PIII-DEAE and azorubine (commercial sample).

The parameter a* increased along with pigment concentration in the natural yogurt. For samples added with F0/90, a variation of the parameter was observed from 6.66 in the lowest concentration (12.4 $\mu g.mL^{-1}$ of yogurt) to 13.96 in the highest concentration (62.0 $\mu g.mL^{-1}$ of

yogurt). In the samples added with PIII-DEAE, the parameters of the lowest and highest concentration (16.2 and 32.4 μg.mL^{-1} of yogurt) ranged from 10.81 to 15.41, respectively. Both samples, in the highest concentrations, came close to the commercial sample parameter of 14.21, reaching the desired color, but the PIII-DEAE shows the highest colorant power, reaching the color of the commercial strawberry yogurt with a lower pigment concentration than F0/90.

The parameter b* was negative in the highest tested concentrations of F0/90 (62.0 μg.mL^{-1} of yogurt), indicating an increase of the blue color in the sample. This increase is directly related to the low purity of the tested sample. This can be confirmed by analyzing the absorption spectrum of F0/90, where a small peak is observed at 618 nm, indicating the presence of phycocyanin (blue pigment) in the sample. The b* factor of the samples added from the PIII-DEAE came closer to the commercial one with an increase in concentration, varying from 2.69 in the lowest concentration (16.2 μg.mL^{-1} of yogurt) to 0.81 in the highest concentration (32.4μg.mL^{-1} of yogurt). The commercial sample obtained a value of 0.09 to the parameter b*.

The clarity (L*) of all samples came close to the luminosity of the commercial sample. The samples added with F0/90 ranged from 83.23 in the lowest concentration to 76.22 in the highest concentration. Regarding the samples added with PIII-DEAE there was a variation of 83.06 to 81.11 from the lowest to the highest concentration, respectively. The commercial sample obtained an L* parameter of 78.23.

3.5 Affective tests of natural yogurts colored with F0/90 and PIII-DEAE

The demographic profile of the panelists is shown in Table S1. The highest rates were for females, aged 18 to 25 years, single and undergraduate students, reaching percentages of 75%, 68.33%, 88.33% and 65%, respectively. They like strawberry yogurt very much and consume strawberry yogurt at least once a week. The color of the yogurt is a very important attribute for 48.33% of them and extremely important for 26.67%.

The results of the affective tests for the yogurts added with F0/90 are described in Table 4. The sample scores ranged from 1.66 to 6.02 in the JAR. According to the scale, the ideal color score would be 5, with the F4 sample coming the closest to this requirement, reaching a score of 5.24, being significantly different from the other samples. With the results of the JAR, the ideal intensity of color desired by the panelists was verified on the yogurt sample added with F0/90 in the concentration of 0.0496 mg R-PE.mL^{-1}, which was not observed in the

commercial strawberry yogurt sample added with artificial coloring. This sample also obtained a good result on the hedonic scale, reaching a score of 7.05. Thus, it was observed that the F3 and F5 yogurt samples added with F0/90 obtained the same acceptance as the commercial yogurt sample by the panellists without statistical differences between them.

In the preference test, it was noticed that the F1 sample was less preferred. The yogurt samples F3, F4 and F5 colored with F0/90, obtained the same preference as the commercial yogurt sample without significant difference between and were more preferred. This demonstrates a positive consumption intention to the F3, F4, F5 and C samples by the panellists, and that these yogurt samples added by F0/90, obtained the same consumption intention as the commercial yogurt sample.

Table 4 also describes the results of affective tests for yogurts added with PIII-DEAE. The sample scores ranged from 2.80 to 5.17 in the JAR. According to the scale, the ideal color score would be 5, with samples P3 (score 4.12), P4 (score 4.80), P5 (score 5.17) and C (score 4.53) being those that came closest to the ideal color. On the hedonic scale, sample P5 (score 7.75) obtained a better score than sample C (score 6.93). The yogurt samples added with PIII-DEAE obtained hedonic scores in the region of acceptance.

In the preference test, it was observed that the P1 sample was less preferred and P5 has more preference. The results of the consumption intention showed that the samples P2, P3, P4, P5 and C obtained average scores in the positive region of consumption.

The results obtained in the sensory tests showed the possibility of applying the pigments from F0/90 and PIII-DEAE on yogurt to replace the artificial dye azorubin.

The industry prioritizes the use of synthetic dyes because it has greater stability and lower production cost compared to natural dyes. However, they have been presenting reports of allergenicity, inducing the industry to look for natural dyes that are not harmful to health (BATADA; JACOBSON, 2016; TAKEO et al., 2018). Phycoerythrins are water-soluble phycobiliproteins with antioxidant and spectroscopic properties (absorbance and fluorescence) that are widely used in industry, especially given the lack of natural red dyes (HEMLATA; AFREEN; FATMA, 2018; PATEL et al., 2018). The price of this dye can vary according to its purity, and it can be used with a lower purity (lower production cost) in the food industry and with higher purity (high purification costs) in the pharmaceutical industry. There are few reports of the application of R-PE in food, with this work being one of the few to bring this solution to use marine macroalgae pigments in the food industry. This was the first report on the application

of R-PE from *S. filiformis* as a natural yogurt colorant, requiring additional tests such as cytotoxicity, shelf-life and pigment safety for beneficial yogurt bacteria.

4 CONCLUSION

The R-Phycoerythrin red pigment from *Solieria filiformis* seaweed was extracted and purified by a scheme that included extraction with buffer solution, protein fractionation and sequential chromatographic methods, presenting fractions containing the pigment with different purity indexes and yields. The R-PESf exhibited a protein structure consisting of monomeric subunits of different molecular weights (α, β and γ), spectroscopic properties (absorbance and fluorescence) stable under storage conditions at low temperatures and fractions able to color natural yogurt with good acceptance and preference by consumers.

DECLARATION OF COMPETING INTEREST

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

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Acrisio José Uchôa Bastos-Filho: Conceptualization, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization **Márjory Lima Holanda-Araújo:** Conceptualization, Validation, Formal analysis, Resources, Writing - Review & Editing, Supervision, Project administration **Aurea Anette Monteiro Brito:** Conceptualization, Investigation **Francisco Lucas de Souza Lopes:** Conceptualization, Investigation, Writing - Original Draft **Roberta Cristiane Cavalcante Costa:** Conceptualization, Investigation, Writing - Original Draft **Antônio Augusto Lima Araújo-Filho:** Investigation, Writing - Review & Editing **Vanessa Rodrigues Ferreira:** Conceptualization, Investigation **Ticiana de Brito Lima-Holanda:** Conceptualization, Investigation **Maria do Carmo Passos Rodrigues:** Validation, Resources, Writing - Review & Editing **Tatiana de Oliveira Lemos:** Validation, Formal analysis, Writing - Review & Editing **Norma Maria Barros Benevides:** Resources, Writing - Review & Editing, Project administration **Ana Lúcia Ponte Freitas:** Resources, Writing - Review & Editing, Project administration

ACKNOWLEDGMENTS

The authors gratefully acknowledge the Associação de Produtores de Algas de Flecheiras e Guajiru (APAFG) and the Universidade Federal do Ceará (UFC).

FUNDING

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

APPENDIX A. SUPPLEMENTARY DATA

FIGURE CAPTIONS

Figure 1. SDS-PAGE (7.5%) of R-PESf. (A) Under non-reducing condition before staining by coomassie brilliant blue R250; (B) After staining by coomassie brilliant blue R250. (C) R-PESf SDS-PAGE (15%) under reducing condition after staining by coomassie brilliant blue R250. Lane 1. 4 and 8: R-PESf 16 µg; Lane 2, 5 and 9: R-PESf 20 µg; Lane 3, 6 and 10: R-PESf 30 µg; Lane 7: Standard Protein Marker: phosphorylase B (97.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (45.0 kDa), bovine carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).

Figure 2. Fluorescent capacity (A) and stability of the absorption peaks of R-PESf stored in amber glass for 30 days. (B) -20 °C, (C) 4 °C, (D) 25 °C. The results were analyzed by one-way ANOVA, followed by the Tukey test ($p < 0.05$), where letters on the same colors indicate that they don't differ statistically, and * means that it differs statistically from the initial absorbance (0 days). I.F. - Fluorescence intensity

TABLE CAPTIONS

Table 1. Yields, recovery, purity index, relative purity and color of the protein fractions obtained by ammonium sulfate of the total extract from *Solieria filiformis*.

Table 2. Extraction and Purification of R-Phycoerythrin from *Solieria filiformis* seaweed.

Table 3. Colorimetric analysis of commercial strawberry yogurt and natural yogurt colored with F0/90 and PIII-DEAE fractions containing R-PE from *Solieria filiformis* seaweed.

Table 4. Affective tests table for yogurts colored with the F0/90 and PIII-DEAE.

Figure Captions

Figure 1

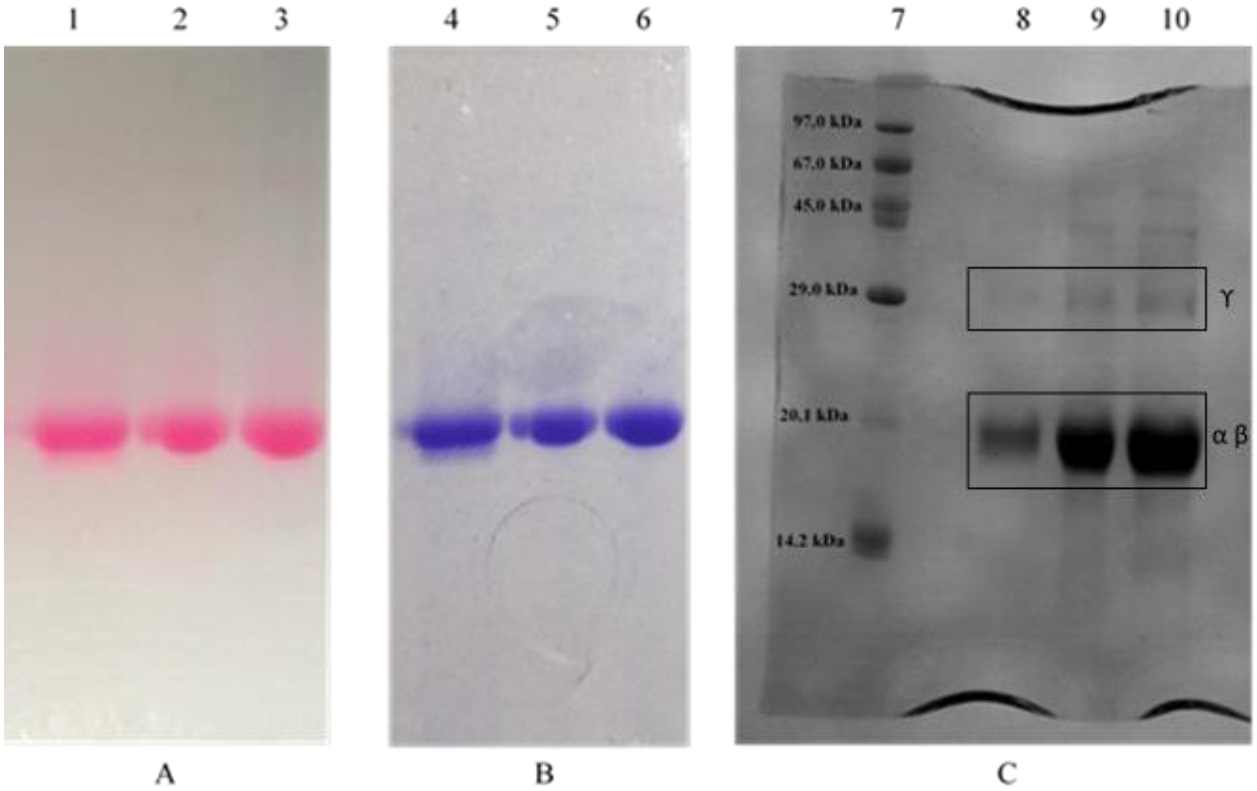


Figure 2.

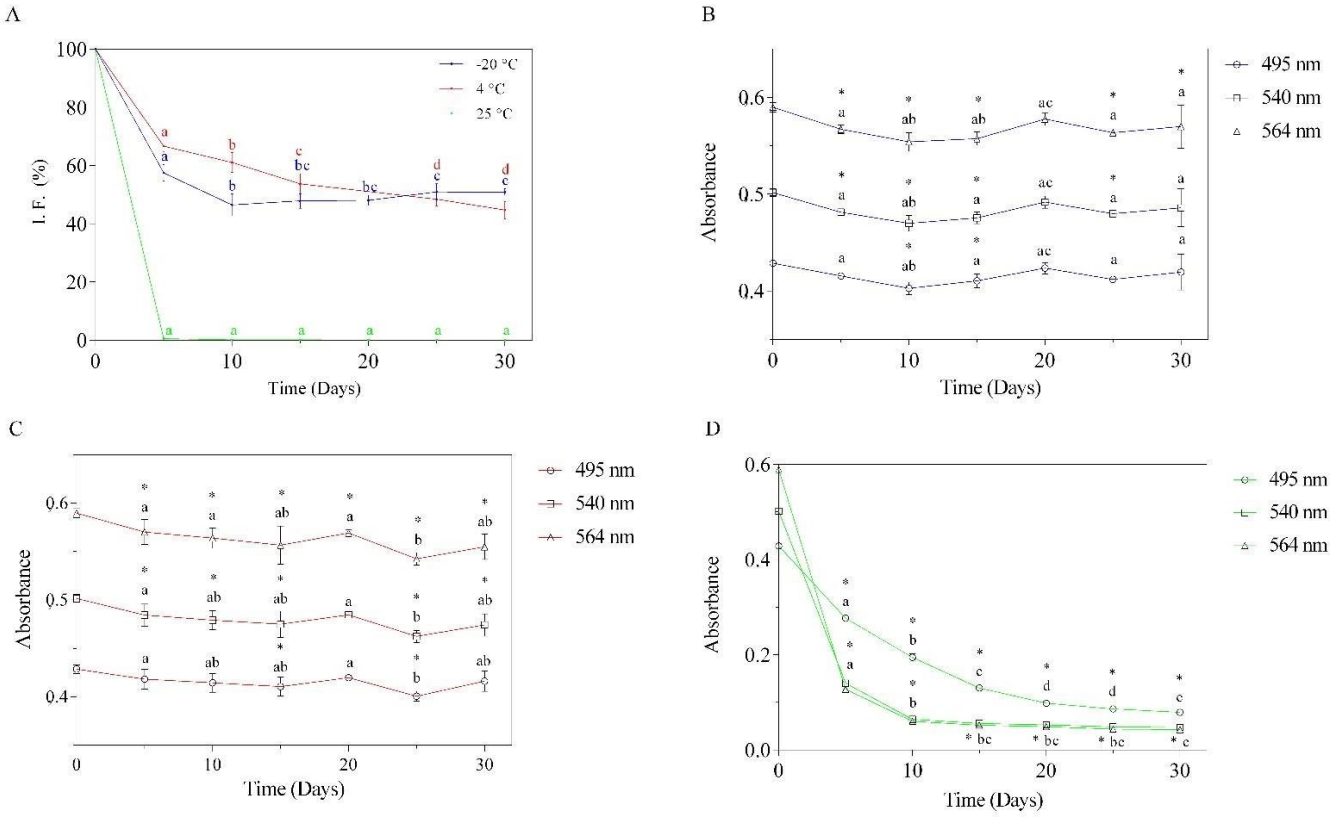


Table Captions

Table 1

Analysis	Protein Fractions						
	F0/20	**F20/40**	**F40/60**	**F60/80**	**F80/90**	**F90/100**	**F0/90**
Y_P (μg.g^{-1} ws)	8.6	5.4	8.6	6.8	3.0	2.90	88.0
$Y_{R\text{-}PE}$ (μg.g^{-1} ws)	6.0	3.6	6.5	5.3	2.4	1.70	65.7
Recovery (%)	6.62	7.41	8.54	6.63	2.42	1.35	48.2
PI	0.31	0.76	1.13	1.06	0.91	0.57	0.54
RP	1.33	2.75	1.52	1.38	1.67	2.00	7.78
Color							

Table 2

Fractions	Yield (μg.g^{-1} ws)		Recovery (%)	Purity Index	Relative Purity
	Protein	R-PE			
TE	145.00	136.10	100.00	0.48	3.47
F0/90	88.00	65.70	48.27	0.54	7.78
PIII-DEAE	37.10	29.70	21.82	1.63	30.25
PII-Sephacryl (R-PESf)	18.00	17.40	12.78	4.50	52.40

Table 3

Fractions		R-PE concentration (µg.mL^{-1} of yogurt)	Colorimetric Parameters			Color
			L*	a*	b*	
F0-90	F1	12.40	83.23	6.66	3.02	
	F2	24.80	80.37	9.16	1.60	
	F3	37.20	78.33	11.42	-0.03	
	F4	49.60	77.33	12.99	-1.02	
	F5	62.00	76.22	13.96	-1.69	
PIII-DEAE	P1	16.20	83.06	10.81	2.69	
	P2	20.30	82.83	11.73	2.27	
	P3	24.30	80.52	14.19	1.23	
	P4	28.40	80.75	14.38	1.10	
	P5	32.40	81.11	15.41	0.81	
Commercial Strawberry Yogurt		Colored with azorubine	78.23	14.21	0.09	

Table 4

Fractions	Sample	Just-about-right	Acceptance test	Preference test	Consumption intent
F0/90	**F1**	$1.66^{a} \pm 0.91$	$2.80^{a} \pm 1.96$	75^{a}	$1.80^{a} \pm 1.03$
	F2	$3.29^{b} \pm 1.07$	$4.90^{b} \pm 1.88$	150^{b}	$3.12^{b} \pm 1.10$
	F3	$4.17^{c} \pm 1.20$	$6.58^{c} \pm 1.46$	249^{c}	$4.00^{c} \pm 0.71$
	F4	$5.24^{d} \pm 1.09$	$7.05^{c} \pm 1.64$	273^{c}	$3.95^{c} \pm 0.96$
	F5	$6.02^{e} \pm 1.23$	$6.88^{c} \pm 1.65$	260^{c}	$3.95^{c} \pm 0.96$
	C	$4.54^{c} \pm 0.94$	$7.23^{c} \pm 1.32$	253^{c}	$4.10^{c} \pm 0.79$
PIII-DEAE	**P1**	$2.80^{a} \pm 1.22$	$4.82^{a} \pm 2.02$	82^{a}	$2.83^{a} \pm 1.11$
	P2	$3.45^{b} \pm 0.96$	$5.73^{b} \pm 2.00$	142^{b}	$3.50^{b} \pm 1.10$
	P3	$4.12^{c} \pm 1.08$	$6.62^{bc} \pm 1.63$	213^{c}	$3.82^{bc} \pm 0.97$
	P4	$4.80^{de} \pm 1.00$	$7.48^{cd} \pm 1.23$	270^{de}	$4.17^{cd} \pm 0.93$
	P5	$5.17^{e} \pm 0.80$	$7.75^{d} \pm 1.36$	309^{e}	$4.45^{d} \pm 0.96$
	C	$4.53^{cd} \pm 0.90$	$6.93^{cd} \pm 1.66$	244^{cd}	$4.12^{cd} \pm 0.86$

Supplementary material

R-Phycoerythrin from *Solieria filiformis* red seaweed: Extraction, purification, stability spectroscopic and application as a natural coloring in yogurt

[1]Acrisio José Uchôa Bastos-Filho, [12*]Márjory Lima Holanda-Araújo, [1]Aurea Anette Monteiro Brito, [1]Francisco Lucas de Souza Lopes, [1]Roberta Cristiane Cavalcante Costa, [1]Antônio Augusto Lima Araújo-Filho, [1]Vanessa Rodrigues Ferreira, [1]Ticiana de Brito Lima-Holanda, [2]Maria do Carmo Passos Rodrigues, [3]Tatiana de Oliveira Lemos, [1]Norma Maria Barros Benevides, [1]Ana Lúcia Ponte Freitas.

[1] Department of Biochemistry and Molecular Biology, Federal University of Ceará, CE, Brazil
[2] Department of Food Engineering, Federal University of Ceará, CE, Brazil
[3] Department of Food Engineering, Federal University of Maranhão, MA, Brazil

[12*] Corresponding author. Phone: +55 85 3366-9826; Fax: +55 85 3366-9820.

E-mail: marjory.holanda@ufc.br

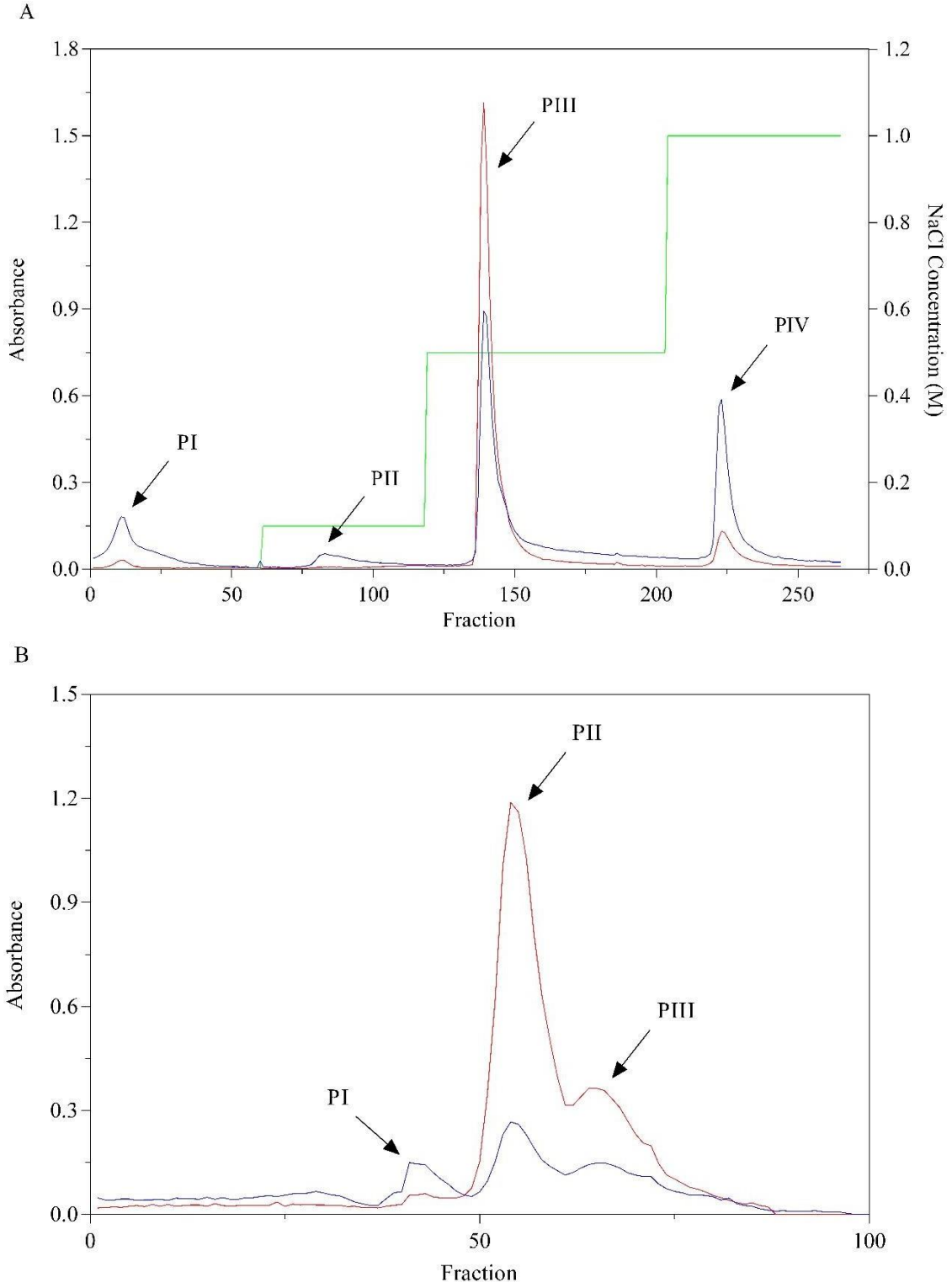


Fig. S1. Chromatographic profiles of the purification process of the R-Phycoerythrin from *Solieria filiformis* seaweed. (A): Ion exchange chromatography. Flow: 1.4 mL per min; Fraction: 3 ml per tube. (B): Molecular exclusion chromatography. Flow: 1 mL per min; Fraction: 2 ml per tube. (—) Absorbance at 280 nm. (—) Absorbance at 564 nm. (—) NaCl concentration.

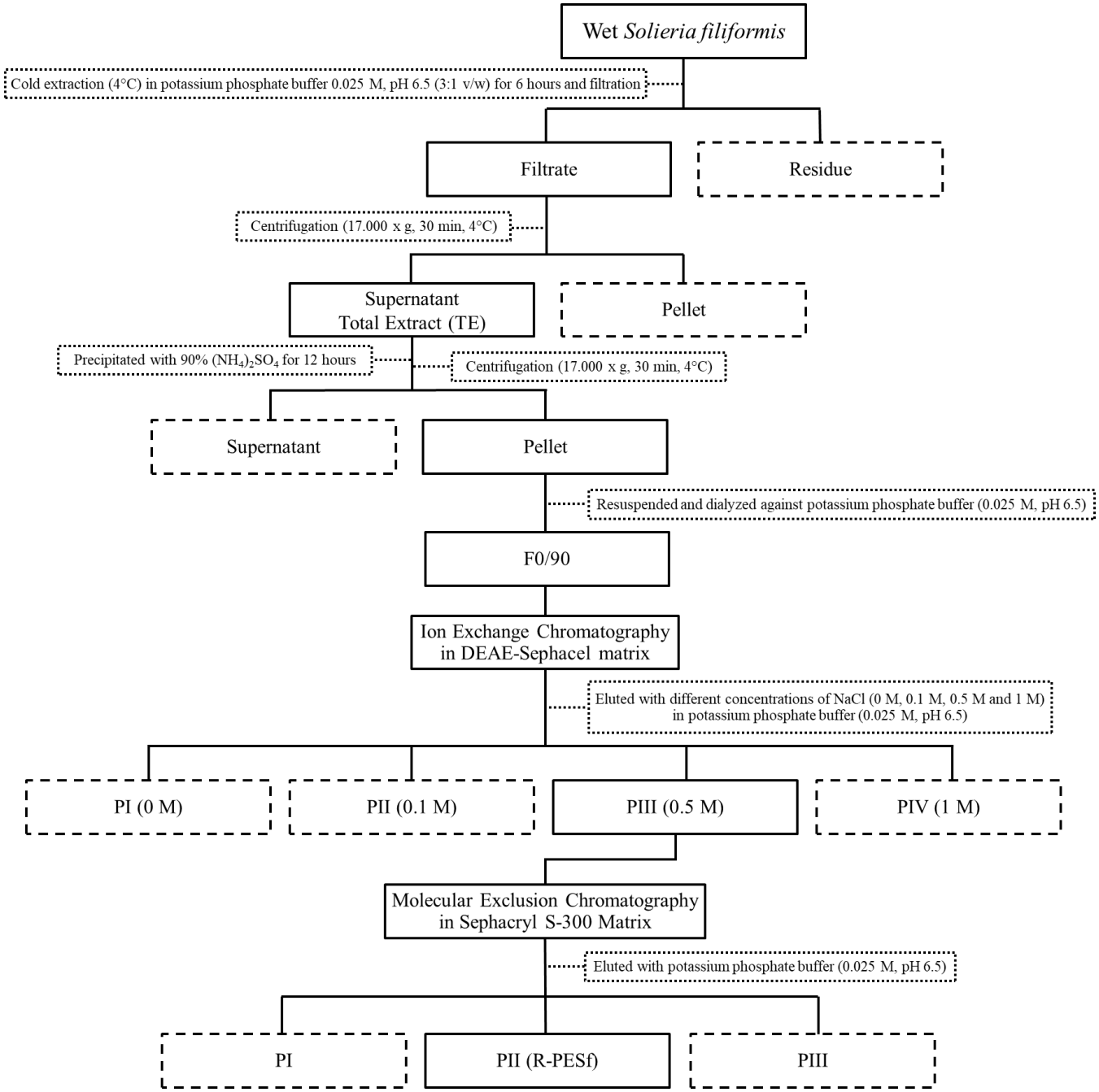


Fig. S2. Scheme for extraction and purification of the R-Phycoerythrin from *Solieria filiformis* seaweed.

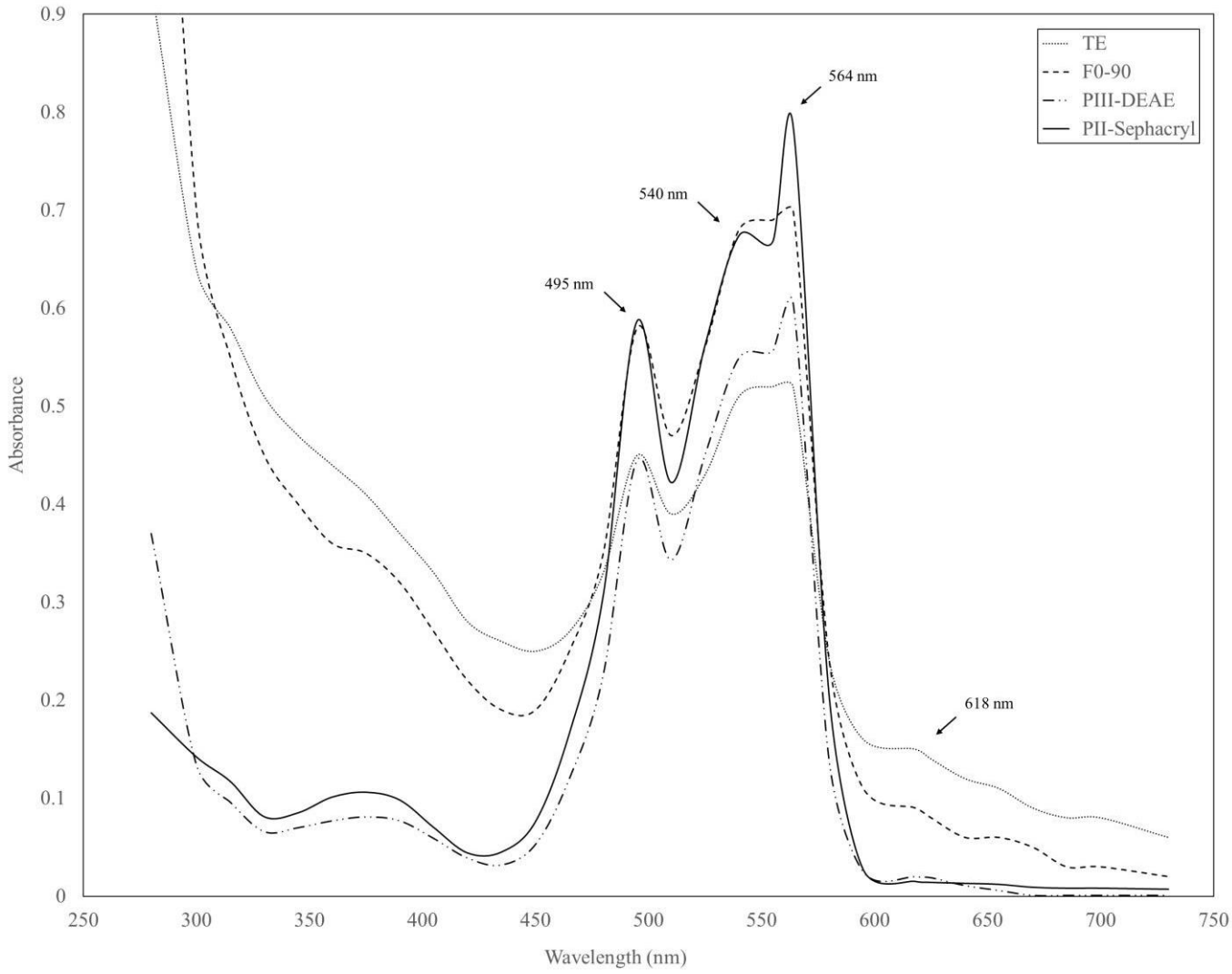


Fig. S3. Absorption spectrum of the total extract and of the R-Phycoerythrin purification process fractions from *Solieria filiformis* seaweed.

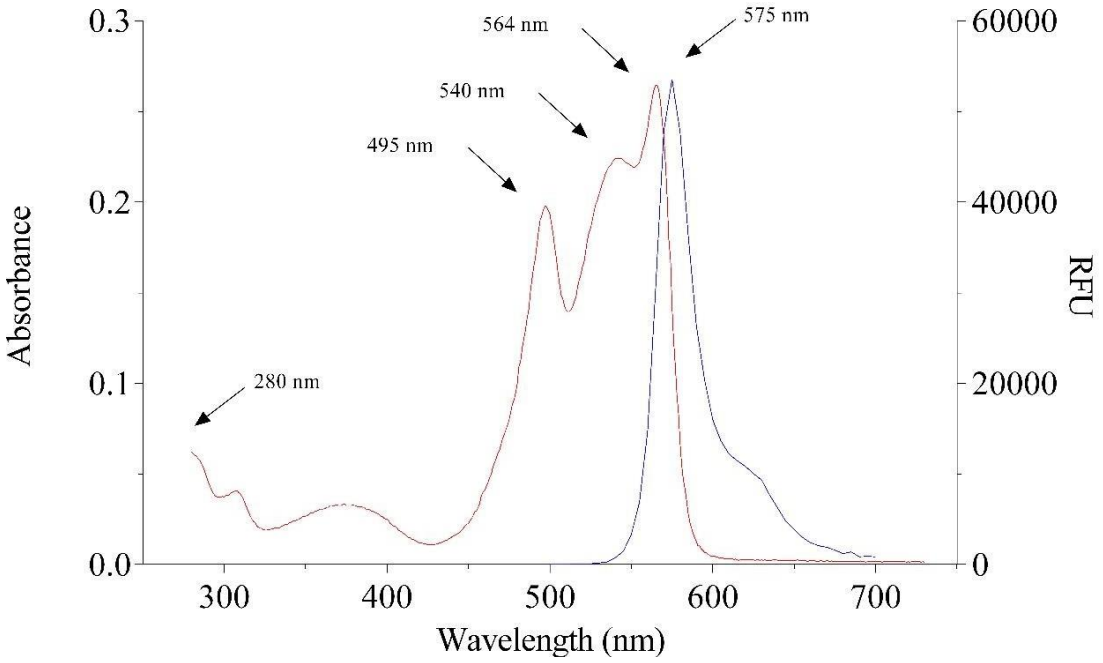


Fig. S4. Spectroscopic characteristics of R-Phycoerythrin from *Solieria filiformis* (R-PESf). RFU = Relative Fluorescence Unit. (—) Absorption spectrum. (—) Fluorescence emission spectrum.

Table S1. Demographic profile of the judges from the sensory analysis of natural yogurts added with F0-90 and PIII-DEAE containing R-PE from *S. filiformis* (n = 60).

Features	Description	Percent (%)
Sex	Female	75
	Male	25
Age	18 – 25 years	68,33
	26 – 35 years	26,67
	36 – 45 years	3,33
	45 – 55 years	0
	Over 56 years	1,67
Civil status	Married	11,67
	Not married	88,33
	Separated or divorced	0
	Others	0
Schooling	High school	0
	Higher education (ongoing)	65
	Higher education (completed)	6,67
	Graduate school	28,33
	Others	0
Degree of taste for strawberry yogurt	Like extremely	26,67
	Like very much	46,67
	Like moderately	16,67
	Like slightly	10
Frequency of consumption of strawberry yogurt	Daily	3,33
	Once a week	21,67
	2 to 3 times a week	20
	4 to 5 times a week	0
	Fortnightly	20
	Once a month	35
Importance of color in yogurt consumption	Extremely important	26,67
	Very important	48,33
	Important	23,33
	Slightly important	1,67
	Not important	0

6 ARTIGO 2 REFERENTE À TESE

R-phycoerythrin, a macromolecular phycobiliprotein from *Solieria filiformis*: Physical-chemical, structural and antioxidant characterization

[1]Acrisio José Uchôa Bastos-Filho, [1]Francisco Lucas de Souza Lopes, [1]Aurea Anette Monteiro Brito, [1]Roberta Cristiane Cavalcante Costa, [1]Antônio Augusto Lima Araújo-Filho, [2]Jéssica Roberta Pereira Martins, [1]Ticiana de Brito Lima-Holanda, [1]José Cirlânio Sousa Albuquerque, [3]Hermógenes David de Oliveira, [1]Norma Maria Barros Benevides, [1]Ana Lúcia Ponte Freitas, [1*]Márjory Lima Holanda-Araújo

[1] Department of Biochemistry and Molecular Biology, Federal University of Ceará, CE, Brazil
[2] Department of Chemical Engineering, Federal University of Ceará, CE, Brazil
[3] Drug Research and Development Center, Federal University of Ceará, CE, Brazil

[*] Corresponding author. Phone: +55 85 3366-9826; Fax: +55 85 3366-9820.

E-mail: marjory.holanda@ufc.br

ABSTRACT

R-Phycoerythrin is a macromolecular phycobiliprotein present in the photosynthetic system of red seaweeds which has industrial, medicine, food, cosmetic and biotechnological applications. R-Phycoerythrin purified from cultivated seaweed *Solieria filiformis* (R-PESf) was evaluated its spectroscopic and structural stability under temperature and pH variations and antioxidant activity. The pigment showed a purity index of 1.57 with only one purification step and 2.36 for R-PESf with two steps. The recovery of R-PESf was 35.38%. The spectroscopic stability (absorbance and fluorescence) and structural of the R-PESf were up to 70 °C and at pHs 3 to 11. It also showed antioxidant activity and maybe a promising biomolecule for application as a colorant, fluorescent probe, and natural antioxidant in product industries, control processes, and diagnostics and biotechnological methods.

Keywords: rhodophytes; thermostability; pH stability.

1 INTRODUCTION

Seaweeds are known to be rich in bioactive proteins and commercially valuable compounds, such as phycocolloids. Some species of seaweed contain a large amount of protein, up to 10–47%, w/w of dry biomass (DUMAY et al., 2013). Among this protein pool, the phycobiliproteins are accessory light-harvesting pigment complexes found in various groups of microalgae and red seaweed. They have been related as important biomolecules for use in diagnostics methods such as an immunofluorescent probe, cosmetics products and food processing such as dyes (HARNEDY; O'KEEFFE; FITZGERALD, 2017; SENTHILKUMAR et al., 2013a).

The red phycobiliprotein from Rhodophyta seaweeds is the R-Phycoerythrin (R-PE) and it is about 240–260 kDa and has a subunit structure of $(\alpha\beta)_6\gamma$. The γ subunit was identified only in R-PE and B-PE (Bangiales seaweed) and is responsible for connecting two $(\alpha\beta)_3$ trimers to form a hexameter (DUMAY et al., 2014b; NGUYEN et al., 2020).

The R-PE price is between US$ 180 and 250 per milligram, varying according to the level of purity (MITTAL; RAGHAVARAO, 2018b). Thus, protocols that optimize the extraction and isolation process of this bioactive pigment of the seaweed species have attracted attention from researchers and entrepreneurs. Many purification methods require chromatographies sequence or combinations with other purification methods, reducing the recovery of phycoerythrins with a high purification index (KAWSAR et al., 2011; ZHAO et al., 2020).

Furthermore, the poor photochemical stability of R-PE has been a bottleneck for its broad-spectrum applications. The R-PE spectroscopy stability to different light exposure times, pHs, and temperatures have also been studied to determine the best storage condition (MUNIER et al., 2014a) and the protein cohabitation also has improved the photochemical stability in the solid state (BHARMORIA et al., 2020). Therefore, the R-FE spectroscopic characterization is important to evaluate its use as commercial pigment from seaweed.

The *Solieria filiformis* is a red seaweed abundant in tropical seas. This species has been studied for biosynthesis and extraction of iota-carrageenan hydrocolloid (MURANO et al., 1997) and its effects on models of nociception and inflammation (DE ARAÚJO et al., 2011, 2012), gastroprotective, antioxidant (SOUSA et al., 2016), antiprotozoal (CAAMAL-FUENTES; ROBLEDO; FREILE-PELEGRÍN, 2017) and anti-herpes (ANA et al., 2021); for extraction of the lectins with antimicrobial, antinociceptive, anti-inflammatory and anticancer effects (ABREU et al., 2016; CHAVES et al., 2018; HOLANDA et al., 2005); for experimental cultivation on the sea (RODRIGUES et al., 2011); as biomass for ethanol production (DE

CASTRO et al., 2017); and for cultivation under an environmentally friendly integrated multi-trophic aquaculture system for to synthesize carotenoids and phycobiliproteins and for biofiltration (FELACO; OLVERA-NOVOA; ROBLEDO, 2020; ZEPEDA; FREILE-PELEGRÍN; ROBLEDO, 2020).

Therefore, this study aims to characterize the stability and antioxidant capacity of R-PE from *Solieria filiformis* seaweed, abundant on the Brazilian northeastern coast and with high potential for cultivation at sea.

2 MATERIALS AND METHODS

2.1 Seaweed

The *Solieria filiformis* (Kützing) P. W. Gabrielson seaweed was harvested from a cultivation area situated on Flecheiras beach (Trairí, Ceará, Brazil) (03° 13' 06" S and 39°16' 47" W) maintained by Associação de Produtores de Algas de Flecheiras e Guajiru - APAFG. The seaweed was cleaned of epiphytes, salt, and grains, washed with distilled water and stored at -20 °C until use. A voucher specimen (Nº 35.682) was deposited in Herbarium Prisco Bezerra, Department of Biological Sciences, Federal University of Ceará (UFC), Brazil.

2.2 Extraction of R-Phycoerythrin

The wet seaweed was ground in contact with 0.025 M potassium phosphate buffer (PB), pH 6.5, in the proportion of 1:3 (m/v) in an electric mill and was maintained under mechanical agitation for 6 h protected from light. Subsequently, the homogenate was filtered, the residue discarded, and the filtrate centrifuged at 17.000 x g, 4 °C for 30 min. The supernatant was called total extract (TE) and was used to continue the purification of R-Phycoerythrin from *S. filiformis*.

2.3 Purification of R-Phycoerythrin

TE was applied to ion-exchange chromatography in a DEAE Sephacel matrix (GE Healthcare), previously balanced with PB, under a flow rate of 1.0 mL/min. After eluting the non-retained fraction (PI-DEAE, the proteins adsorbed on the matrix were eluted with PB containing NaCl in concentrations 0.1, 0.5, 1.0 M and named PII-DEAE, PIII-DEAE, and PIV-DEAE, respectively. The most pigmented chromatographic fraction was subjected to ultrafiltration in a Vivaspin (GE Healthcare) sample concentrator (cutoff 100 kDa), at 1000 x g, at 4 ºC, for 20 minutes. The filtrated was named R-PESf.

2.4. Analysis

The protein content was performed second method described by Bradford (BRADFORD, 1976) using bovine serum albumin (BSA) as the standard.

The R-PE concentrations ($mg.mL^{-1}$) of the pigmented solutions were determined second Sampath-Wiley & Neefus (SAMPATH-WILEY; NEEFUS, 2007) and were calculated according to Equation 1:

$$R\text{-}PE = 0.1247 \times [(A_{564} - A_{730}) - 0.4583 \times (A_{618} - A_{730})] \quad (1)$$

Where A_{564}, A_{618} and A_{730} were the absorbances at 564, 618 e 730 nm, respectively.

The protein yield ($\mu g.g^{-1}$) was calculated according to Equation 2:

$$Y_p = P_t/Sf \quad (2)$$

Where P_t was the total content of protein present in the TE and fractions (µg) and Sf was the mass of wet seaweed used at extraction (g).

The R-PE yield ($\mu g.g^{-1}$) was calculated according to Equation 3:

$$Y_{R\text{-}PE} = R\text{-}PE_t/Sf \quad (3)$$

Where R-PEt was the total content of R-PE present in the TE and fractions (µg) and Sf was the mass of wet seaweed (ws) used at extraction (g). The yield of R-PE can also be expressed as mg per gram of dry seaweed (ds). The moisture content of the red seaweed *Solieria filiformis* was determined to be 95% (data not shown).

The recovery (%) of the R-PE from *S. filfiormis* was calculated according to Equation 4:

$$R_{R\text{-}PE} = (R\text{-}PE/R\text{-}PE_r) \times 100 \quad (4)$$

Where R-PE was the total content of R-PE present in the fractions and $R\text{-}PE_r$ was the total content of R-PE present in TE.

The purity index (PI) of the R-PE present in TE and fractions was calculated by Equation 5:

$$PI = A_{564}/A_{280} \quad (5)$$

Where A_{280} and A_{564} were the measured of the absorbances at 280, 564 nm, respectively.

2.5 Spectroscopic characterization of R-PE

The absorbance and fluorescence spectra of the R-PESf were determined using a Synergy Mx microplate multi-detection spectrophotometer (BioTek, USA). The R-PESf was dissolved in PB buffer at concentrations of 0.05 $mg.mL^{-1}$ and 0.001 $mg.mL^{-1}$ and analyzed for absorption and fluorescence emission spectra, respectively. The absorbance spectrum was determined at 25 ± 0.5 ° C in the range of 280 to 730 nm. The fluorescence emission spectrum was determined at 25 ± 0.5 ° C in the range 500 to 700 nm after excitation at the 495 nm wavelength. Fluorecence intensity (FI) was calculated at all times based on the peak at 575 nm.

2.6 Spectroscopic stability of R-PE

2.6.1 Circular dichroism

2.6.1.1 Structural Composition in native conditions

The spectrum of circular dichroism (CD) of the R-PESf was determined in the Jasco spectropolarimeter (model J-815), in the range from 190 to 260 nm under nitrogen atmosphere, with a bandwidth of 1 nm, using a cylindrical quartz cuvette of 0, 1 cm of optical path. The R-PESf 0.1 mg of protein.mL^{-1}dissolved in PB buffer was analyzed through 3 successive scans, with a scanning speed of 100 $nm.min^{-1}$ and response time of four seconds. The CD spectrum was obtained by the average of the three scans. To determine the secondary structure components, the K2D3 online server was used, according to the methodology described by Louis-Jeune; Andrade-Navarro; Perez-Iratxeta (LOUIS-JEUNE; ANDRADE-NAVARRO; PEREZ-IRATXETA, 2012).

2.6.1.2 Effect of Temperature on the Circular Dichroism Spectrum

The spectrum of circular dichroism was determined following the same methodology described in item (2.6.1.1). The R-PESf 0.05 mg of protein.mL^{-1} dissolved in PB buffer was pre-incubated at different temperatures (4, 25, 40, 50, 60, 70, 80, 90 and 100 ° C) for one hour and then your CD spectrum was analyzed. The temperature of 4 °C was used as a standard to compare with the other temperatures.

2.6.2 Effect of temperature on absorption and fluorescence emission spectra

The sample was prepared and analyzed according to the methodology described in item 2.5, then the R-PESf was distributed in test tubes containing 5 mL each, and transferred to a water bath, where they remained incubated for 1 hour at temperatures of 25, 40, 50, 60, 70, 80, 90 and 100 °C. The samples were transferred to the ice bath at the end of each incubation period, then, their absorption and fluorescence emission spectra were determined.

2.6.3 Effect of pH on absorption and fluorescence emission spectra

The sample was prepared and analyzed according to the methodology described in item **2.5.** The stability of the absorption and fluorescence spectra of R-PESf at different pHs was determined using the buffers: Glycine-HCl (20mM, pH 2.0), sodium acetate (20mM, pH 4.0 and 5.0), potassium phosphate (25mM, pH 6.5), tris-HCl (20mM, pH 7.1; 8.0 and 9.0), glycine-NaOH (20mM, pH 10.0 and 11.0) and potassium phosphate (20mM, pH 12.0). PB was used as

a control. The absorbance and fluorescence spectra were obtained at five different times with intervals of 30 min between each reading until completing 2 h. The first reading was performed as soon as the R-PESf was dissolved in the buffers. All data were estimated as mean values of three replicates.

2.7 Antioxidant activity of R-PE

2.7.1 Chelating ferrous activity

The ferrous ion chelation capacity of R-PESf was determined according to the method of Chew *et al.* (CHEW et al., 2008), with modifications. In this order, 1 ml of 0.1 mM ferrous sulphate ($FeSO_4$) and 1 ml of 0.25 mM ferrozine [3- (2-pyridyl) 5,6-diphenyl-1,2 4-triazine-p-p'-disulfonic] to 1 mL of R-PESf in different concentrations 31.25, 62.50, 125.0, 250.0 and 500.0 µg.mL^{-1}. The test tubes were vortexed and kept for 10 min at 25 °C and protected from light. Then, absorbance measures were performed at 562 nm (A_{562}). All reactions were performed in triplicate. Ethylene diaminetetraacetic acid (EDTA) was used as a positive control, being prepared in the same concentrations as the sample for further analysis and comparison of results, which were expressed as a percentage of Chelating Ferrous Activity (CFA), calculated according to Equation 6:

$$CFA\ (\%) = \{[A_0-(A-A_b)].A_0^{-1}\} \times 100 \quad (6)$$

Where $A_0 = A_{562}$ of the reagents (1.0 mL $FeSO_4$ + 1.0 mL ferrozine + 1.0 mL distilled water); $A = A_{562}$ of the reaction (1.0 mL FeSO4 + 1.0 mL ferrozine + 1.0 mL sample); A_b = A562 of the sample (2.0 mL of distilled water + 1.0 mL of sample).

2.7.2 DPPH scavenging activity

The sequestration of the free radical 2,2-diphenyl-1-picryl-hydrazylhydrate (DPPH) of the R-PESf was evaluated according to the method described by Blois (BLOIS, 1958), with modifications. First, 300.0 µL of R-PESf, in different concentrations (31.25, 62.50, 125.0, 250.0 and 500.0 µg.mL^{-1}), were vortexed with 200.0 µL of methanol (MeOH). Then, 2.5 mL of 75 µM DPPH (solubilized in MeOH) was added to these solutions. The reaction mixture was kept at 25 °C for 40 min, protected from light and the absorbance measures were performed on a spectrophotometer at 517 nm (A_{517}). All reactions were performed in triplicate. Butylated hydroxytoluene (BHT) was used as a positive control, being prepared in the same concentrations as the sample for further analysis and comparison of results, which were

expressed as a percentage of DPPH scavenging activity (DPPHSA), which was calculated according to equation 7:

$$DPPHSA\ (\%) = \{[A_0-(A-A_b)].A_0^{-1}\} \times 100 \quad (7)$$

Where $A_0 = A_{517}$ of the reagents (500 µL of methanol + 2.5 mL of DPPH); $A = A_{517}$ of the reaction (300 µL of sample + 200 µL of methanol + 2.5 mL of DPPH); $A_b = A_{517}$ of the sample (300 µL of sample + 2.7 mL of distilled water).

2.7.3 ABTS scavenging activity

The sequestration of the free radical 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by R-PESf was evaluated according to the method described by Xu *et al.* (XU et al., 2018), with modifications. First, the $ABTS^{\cdot+}$ radical was prepared from the reaction between 5.0 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The obtained mixture was kept under light for 16 h at 25 °C. Then, the $ABTS^{\cdot+}$ radical was diluted in distilled water until an absorbance of 0.70 ± 0.05 at 734 nm (A_{734}) was obtained. After that, 800 µL of the $ABTS^{\cdot+}$ radical was added to 200 µL of R-PESf in the concentrations 31.25, 62.50, 125.0, 250.0 and 500.0 $\mu g.mL^{-1}$). The reaction mixture was vortexed, kept in the dark for 6 min. After resting time, readings were performed and the absorbance measures were performed on a spectrophotometer at 734 nm. All reactions were performed in triplicate. Ascorbic acid was used as a positive control, being prepared in the same concentrations as the sample for further analysis and comparison of results, which were expressed as a percentage of ABTS Scavenging Activity (ABTSSA), which was calculated according to equation 8:

$$ABTSSA\ (\%) = \{[A_0-(A-A_b)].A_0^{-1}\} \times 100 \quad (8)$$

Where $A_0 = A_{734}$ of the reagents (200 µL of water + 800 µL of ABTS radical); $A = A_{734}$ of the reaction (200 µL of sample + 800 µL of ABTS radical); $A_b = A_{734}$ of the sample (200 µL of sample + 800 µL of distilled water).

2.8 Statistical analysis

The statistical analysis was performed using GraphPad Prism version 6.1 for Windows, GraphPad Software (La Jolla, California, USA). The results were expressed as mean ± S.D. (Standard Deviation). It was used the variance analysis method (*one-way* ANOVA), followed by the Tukey test of multiple comparisons to verification of statistical differences between groups. It was considered significant values of $p < 0.05$. All data were estimated as mean values of three replicates.

3 RESULTS AND DISCUSSION

3.1 Extraction of R-Phycoerythrin

The $Y_{R\text{-}PE}$ of the TE was 182.55 ± 21.06 μg.g^{-1} ws or 3.65 mg.g^{-1} ± 0.42 ds, and a purity index (PI) of the R-PE was 0.60. Punampalam, Khoo, Sit (PUNAMPALAM; KHOO; SIT, 2018) related a yield of R-PE 1.58 mg.g^{-1} ds from *Bangia atropurpurea*, with an IP of 0.59, achieving a yield twice as low as that observed in this study. Mittal & Raghavarao (MITTAL; RAGHAVARAO, 2018a) achieved a yield of only 0.29 mg.g^{-1} ds when using an intercrop of enzymes to extract R-PE from *Gelidium pusillum*. This yield is almost 13 times lower than observed in this study, using a more expensive extraction methodology.

3.2 Purification of R-Phycoerythrin

The yields, recovery, PI values, and visual aspect for the TE and purification fractions of R-PE from *S. filiformis* are described in Table 1. The highest yield of the R-PE was observed in the peak eluted with PB containing 0.5 M NaCl (PIII-DEAE), which showed an intense pink color. The methodology used to extract and purify the R-PE from *Solieria filiformis* is schematized in Fig. 1.

Table 1 – Purification of R-Phycoerythrin from *Solieria filiformis*.

Analysis	**Protein Fractions**		
	TE	**PIII-DEAE**	**Ultrafiltrated (R-PESf)**
Y_P (μg.g^{-1} ws)	271.16 ± 57.66	212.03 ± 15.21	87.63 ± 3.45
$Y_{R\text{-}PE}$ (μg.g^{-1} ws)	182.55 ± 21.06	107.83 ± 14.38	64.05 ± 2.94
Recovery (%)	-	59.12 ± 4.67	35.38 ± 4.09
PI	0.60 ± 0.06	1.57 ± 0.04	2.36 ± 0.15
Color under white light			
Color under blue light			

It is possible to notice in Table 1 that the R-PE was purified by two steps, saving time and increasing the final recovery by using few steps for purification. According to Pan *et al.* (PAN et al., 2013), the PIII-DEAE reached a degree of food purity and the R-PESf reached a drug class purity. This methodology was able to determine two rapid methods for purification of R-PE from *S. filiformis* with different possibilities of application in the industry. It is also possible to correlate the increase in the purity index of R-PE with the increase in color, under white light, and the emission of fluorescence intensity, under blue light.

Pigment recovery dropped 40% in the first stage of the process, this is because too much pigment is adsorbed at the end of the ion-exchange chromatography, a fact that has already been observed by Saluri *et al.* (SALURI et al., 2020) when studying phycobiliproteins from the red algae *Furcellaria lumbricalis* and *Coccotylus truncatus*. Even losing 40% of the recovery in the first stage, it remains essential, as the TE has a large amount of polysaccharides and that, if applied directly to the ultrafiltration, it could render the same unfeasible, as it would accumulate polysaccharides in the filter and could not have the same effectiveness.

Zhao *et al.* (ZHAO et al., 2020) purified R-PE from *Gracilaria tenuistipitata* by ammonium sulfate fractionation followed by chromatography of hydrophobic interaction and ion-exchange reaching a PI of 4.21, however with a low recovery of 13.83%. This recovery value is almost 3 times lower than that obtained by the present study. Saluri *et al.* (SALURI; KALDMÄE; TUVIKENE, 2019) purified the R-PE from *Furcellaria lumbricalis* also obtaining a lower PI of 1.41. The purity index of R-PE is decisive for its final application, when at low levels of purity, R-PE can be used in the food and cosmetic industries, for example. On the other hand, high levels of purity allow for applications such as biomedical, biotechnological, and pharmacological methods or molecular markers (PAN et al., 2013; SALURI et al., 2020).

3.3 Spectroscopic characterization of R-PE

3.3.1 Circular dichroism

The circular dichroism (CD) spectrum of R-PESf showed two intense negative bands at 222 and 208 nm and an intense positive band at 192 nm (Fig. 2). This spectrum corresponded at 60.19% of α-helix, 2.42% of β-sheets and 37.39% unordered structures. The dominant α-helix corroborated with the spectral characteristics of circular dichroism of R-PE from other species seaweed, such as *B. fusco-purpurea* (WU et al., 2015) and *P. urceolata* (LIU et al., 2009).

The temperature effect on the CD Spectrum of R-PESf is shown in Fig. 3. The secondary structure of the R-PESf remained stable up to 70 ° C. However, there was a drastic change in its spectrum when it reached 80 ° C, being possible to notice a change in the CD spectrum of R-PESf.

The secondary structure of R-FE changed from 60.97% α-helix and 2.26% β-sheet, when submitted to analysis at 25 °C to 3.36% α-helix and 32.91% β-sheet at 80 °C (Table 2). The α-helice structure of R-PESf was lost and converted into β-sheets, resulting in an irreversible loss of the native structure. Similar result was related for R-PE from *P. urceolata* (LIU et al., 2009).

Table 2 - Elements of secondary structure estimated from the circular dichroism spectrum of R-PESf at different temperatures.

Temperature (°C)	Secondary structure (%)		Unordered structures (%)
	α-helix	β-sheet	
4	60.19	2.42	37.39
25	60.97	2.26	36.77
40	62.38	1.87	35.75
50	61.75	2.17	36.08
60	60.21	2.39	37.40
70	55.33	5.30	39.37
80	3.36	32.91	63.73
90	5.22	31.07	63.71
100	10.51	26.71	62.78

3.3.2 Effect of temperature on absorption and fluorescence emission spectra

The influence of temperature on the absorption and concentration spectrum of R-PESf is shown in Fig. 4. Fig. 4A shows the change in the spectral profile of R-PESf with increasing temperature, maintaining stability in its profile up to 70 °C. Upon reaching 80 °C it is possible to see a complete change in the spectral profile of the pigment, corroborating with the data obtained in the CD spectrum.

The concentration of R-PESf has a significant change (p <0.05) in its concentration when reaching 70 °C, with a reduction of 15.6%, and a greater reduction of 72.1% and 86.8% is observed when reaching 80 °C and 90 °C, respectively (Fig. 4B).

The effect of temperature on the fluorescence emission of the R-PESf is shown in Fig. 5. In Fig. 5A, it is possible to note the fluorescence spectrum is more sensitive than the absorbance spectrum, visualizing spectral modification up to 70 ° C.

Fig. 5B shows the fluorescence emission capacity of R-PESf, reaching 100% capacity at 25 °C with an RFU of 53561. This fluorescence capacity remains statistically stable up to 40 ° C. When it reaches 50 °C, 60 °C and 70 °C their fluorescence capacity drops to 91.09% (48,788 RFU), 80.26% (42990 RFU) and 46.47% (24888 RFU), respectively. After 80 °C, the R-PESf loses all fluorescence capacity, reaching an RFU of 787 with a capacity of 1.47%. At 90 °C and 100 °C, the loss of R-PESf fluorescence capacity was total.

The decreases observed in the absorption and light emission profile of R-PESf were probably caused by the change in structural conformation of the protein, shown by the results of the circular dichroism spectra in different temperatures. The R-PEs purified from *G. turuturu*, *B. fusco-purpurea* and *P. palmata* were stable until 40 °C (MUNIER et al., 2014a), 55 °C (WU et al., 2015) and 60 °C (GALLAND-IRMOULI et al., 2000), respectively.

Significant reductions (p <0.05) in the secondary structure in the predominant α-helix, as well as in the ability to absorb and emit light from R-PE from *S. filiformis*, were observed only at 80 °C, suggesting a highly stable natural pigment, when compared to R-PE extracted from other species of macroalga.

3.3.3 Effect of pH on absorption and fluorescence emission spectra

The visual aspect of buffered solutions at different pH is shown in Fig. 6. The visual perception was different in the color tonality of the R-FE when dissolved in buffer solutions of different pHs. The colors tended to purple at the lowest pHs when compared to the pink color presented in the solutions of pHs from 5 to 11. A precipitate was observed after incubation for 2 hours at pH 2 and 3 (Fig. 7A). At pH 12, the R-FE completely lost its color as shown in Fig. 7B.

The effect of time on the absorbance spectra R-PESf dissolved in buffers at different pH is shown in Fig. S1. At time 0, it is possible to notice differences in the spectral profile of R-PESf dissolved in buffers at extreme pHs. At pH 6.5, the R-PESf had an absorbance of 0.409, 0.457 and 0.492 at the absorbances of 495 nm, 540 nm and 564 nm, respectively. It was noticed

a strong change in the most extreme pHs tested, decreasing its absorbance to 0.333 (495 nm), 0.340 (540 nm), 0.340 (564 nm) without pH 2 and to 0.142 (495 nm), 0.109 (540 nm) and 0.081 (564 nm) without pH 12. At pH 3 there was an increase in absorbances to 0.507 (495 nm), 0.553 (540 nm) and 0.609 (564 nm), this is because R-PESf precipitates in acidic pH, directly influencing the absorbance value.

The peaks corresponding to phycoerythrobilins (PEB) (λ = 545 nm and 564 nm) change in pH 2 and 3. The undefined peak indicated degradation of PEB and stability of ficourobilin (PUB) at 495 nm. An increase in absorbances at 280 nm was seen in the solutions that suffered degradation of the PEB-related peaks. This suggests pigment conformational changes resulting from denaturation, exposing the peptide subunits that contain aromatic amino acids, increasing absorption at 280 nm. At pH 12, the R-PESf spectrum was completely modified, decreasing both the peak for PUB and PEB in zero time. Stability in the absorbance spectrum was observed for the other pHs during the tested period (2 hours).

The variation in the concentration of R-PESf dissolved in buffers at different pHs over time is shown in Fig. 8. The maximum concentration of R-PE was observed on zero time at pHs 3, 6.5 and 7.1, with no statistical difference between them (p<0.05). At pH 3 occurred precipitate formation causing an increase in the absorbance measure. The lower concentrations of R-PESf were observed at extreme pHs (pHs 2 and 12), however, at pH 12, the pigment loss was 88.22% right after dilution. It is worth mentioning that the concentration of R-PE dissolved in buffers at pHs 3 to 11 remained above 50% by 2 h. The lowest concentrations loss of the R-PESf were at pH 6.5 and 7.1, maintaining 95.98% of content after 2 hours of incubation, with no significant difference between them (p>0.05). At pHs 2 and 12, after 2 h, the contents of pigment were 35.77 and 10.10%.

The effect of the time on the fluorescence emission spectra of R-PESf dissolved in buffers at different pHs is shown in Fig. S2. There were differences in the RFU of the solutions at different pH (zero time) and overtime. At time 0 (Fig. S2A), the R-PESF showed maximum RFU at pH 2 of 58059, and minimum at pH12 with RFU of 90. This was expected, as the sample lost all characteristic staining as soon as it came into contact with the buffer. pH12. Phs 2, 3 and 4 also had a low RFU of 1111, 3133 and 5321, respectively. At 120 min (Fig. S2E) the R-PESf showed minimum RFUs of 66, 208, 2282, 3967 for pHs 12, 2, 3 and 4, respectively. For pH 6.5, the R-PESf showed an RFU of 48,413, remaining with 83.38% of its capacity after 2 hours of dissolution.

The fluorescence intensity (FI) of the R-PESf (Fig. 9) observed at pH 6.5 at time zero was the highest among the tested pHs (58059 RFU) and was established as 100%. The FI emitted by pigment at pHs 7.5 and 9 were 90.16% and 85.72%, respectively, without statistical differences ($p>0.05$). For the pH extremes (2, 3, 4 and 12) the FIs were below 10% in zero time. After 2 h, the solution at pH 6.5 showed the best FI (83.19%). The R-PESf emitted low FI after 2 h of incubation at pHs 5 and 11, corresponding at 30.64% and 33.32% ($p>0.05$), respectively. At pH 6.5, 7.1, 8 and 9, R-PESf maintained a FI above 50% after 2 h. Then, the fluorescence emission property of R-PESF was more affected by pH and time, with a decrease in the FI observed since time zero at extreme pHs (pH 2, 3, 4 and 12). The spectroscopic properties of the R-PE from a *Solieria filiformis* were stable in a pH range of 3 to 11 for absorbance and pH of 5 to 11 for fluorescence emission.

The results obtained in the present study corroborated with Munier *et al.* (MUNIER et al., 2014b), when they evaluated the pH stability of R-PE from *Grateloupia turuturu.* The pigment was stable in a pH range of 4 to 10. They observed a color change from pink to purple at the most acidic pH (2 to 4) and precipitation, as well as the total loss of color to pH 12. Wu *et al.* (WU et al., 2015) evaluating the stability of R-PE from *Bangia fusco-purpurea*, also observed spectroscopic stability in a pH range of 5 to 9.

3.4 Antioxidant activity of R-Phycoerythrin

The antioxidant activity of R-PESf is shown in Fig. 10. The R-PE reached high Fe^{2+} chelation capacity, wherein its maximum activity of 94.75% was observed at a concentration of 500 $\mu g.mL^{-1}$, a value close to that achieved by EDTA (100%), the positive control of this assay (Fig. 10A). Besides that, in a concentration of only 125 $\mu g.mL^{-1}$ the R-PESf showed an activity of 92,93%, a value considered already high. It represents an important result because Fe^{2+} can react with hydrogen peroxide and generate oxygen species and hydrogen-free radicals, which sequentially can cause lipid peroxidation (ANDJELKOVIĆ et al., 2006).

The DPPH radical scavenging activity by the R-PESf shown a dose-dependent manner, reaching its maximum activity of 79,04% in a concentration of 500 $\mu g.mL^{-1}$, a value of only about 10% lower than that found for BHT, used as the positive control in this assay (Fig. 10B). The DPPH radical scavenging activity reached in this study was higher than that reported by Senthilkumar *et al.* (SENTHILKUMAR et al., 2013b), who found maximum antioxidant activity for the R-PE from alga *Portieria hornemannii* of only 43.55%, in a concentration ten times higher (5 $mg.mL^{-1}$) than the present study at its maximum concentration. BHT, a synthetic

antioxidant widely used in the pharmaceutical, food and cosmetic industries, has some limitations, as it can be toxic and it is potentially carcinogenic (SAITO; SAKAGAMI; FUJISAWA, 2003; SHEARN; FRITZ; THOMPSON, 2011; YU; MANDLEKAR; KONG, 2000). Thus, the R-PESf presents itself as a potential natural alternative to this synthetic antioxidant agent.

Lastly, the ABTS radical scavenging activity assay by the R-PESf was carried out. The R-PESf achieved 97,98% of its activity when 500 $\mu g.mL^{-1}$ were tested, shown a dose-dependent activity. (Fig. 10C). Wu *et al.* (WU et al., 2015) when studying the R-PE from *Bangia fusco-purpurea* found an IC_{50} for the sequestration of the ABTS radical of 769.9 $ug.ml^{-1}$. This value is higher than that obtained by this work and with lower efficiency, demonstrating the high antioxidant power of R-PESf.

4 CONCLUSION

The R-Phycoerithrin from *Solieria filiformis* seaweed was extracted and purified by two steps, ion-exchange chromatography and ultrafiltration, reaching a compatible purity index for application in the food and cosmetic industries with high recovery. The pigment showed spectroscopic stability in a wide range of temperature and pH. In addition, the R-Phycoerithrin from *Solieria filiformis* presented antioxidant activity by three tested methodologies becoming a promising biomolecule for application as a colorant, fluorescent probe and natural antioxidant in products industries, control processes and diagnostics and biotechnological methods.

Funding

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Acknowledgements

The authors gratefully acknowledge the Associação de Produtores de Algas de Flecheiras e Guajiru (APAFG) and the Universidade Federal do Ceará (UFC).

FIGURE CAPTIONS

Figure 1. Extraction and purification scheme for R-Phycoerythrin from *Solieria filiformis* seaweed. Full line means the methodology used in the purification; dashed line means disposal.

Figure 2. Circular dichroism spectrum of R-PESf under native conditions.

Figure 3. Effect of temperature on the circular dichroism spectrum of R-PESf.

Figure 4. Effect of the temperature on the absorption spectrum. (A) and concentration (B) of R-PESf. The results were analyzed by one-way ANOVA, followed by the Tukey test ($p < 0.05$), where equal letters indicate that they don't differ statistically.

Figure 5. Effect of the temperature on the fluorescence emission of R-PESf. RFU - Relative Fluorescence Unit. (A) Fluorescence emission spectrum; (B) Fluorescence emission peak (575 nm). The results were analyzed by one-way ANOVA, followed by the Tukey test ($p < 0.05$), where equal letters indicate that they don't differ statistically.

Figure 6. Color of R-PESf dissolved in buffer solutions at different pHs.

Figure 7. Visual appearance of R-PESF in extreme pH after two hours of incubation. A – Pigment precipitated in pH 2; B – Color ausence in pH 12

Figure 8. Effect of time on the concentration of R-PESf dissolved in buffers at different pH. (A) zero time; (B) 30 min; (C) 60 min; (D) 90 min; (E) 120 min. The results were analyzed by one-way ANOVA, followed by the Tukey test ($p < 0.05$), where equal letters indicate that they don't differ statistically.

Figura 9. Effect of time on the fluorescence emission peak (575 nm) of R-PESf dissolved in buffers at different pH. (A) zero time; (B) 30 min; (C) 60 min; (D) 90 min; (E) 120 min. The results were analyzed by one-way ANOVA, followed by the Tukey test ($p < 0.05$), where equal letters indicate that they don't differ statistically.

Figure 10. Antioxidant activity of the R-PESf. (A) Chelating ferrous activity; (B) DPPH scavenging activity; (C) ABTS scavenging activity. The results were analyzed by two-way ANOVA, followed by Tukey ($p < 0.05$), where equal letters indicate no significant difference.

Figure Captions

R-phycoerythrin from *Solieria filiformis*: An antioxidant macromolecular protein with spectroscopic and structural stability

[1]Acrisio José Uchôa Bastos-Filho, [1]Francisco Lucas de Souza Lopes, [1]Aurea Anette Monteiro Brito, [1]Roberta Cristiane Cavalcante Costa, [1]Antônio Augusto Lima Araújo-Filho, [2]Jéssica Roberta Pereira Martins, [1]Ticiana de Brito Lima-Holanda, [1]José Cirlanio Sousa Albuquerque, [3]Hermógenes David de Oliveira, [1]Norma Maria Barros Benevides, [1]Ana Lúcia Ponte Freitas, [1*]Márjory Lima Holanda-Araújo

[1] Department of Biochemistry and Molecular Biology, Federal University of Ceará, CE, Brazil

[2] Department of Chemical Engineering, Federal University of Ceará, CE, Brazil

[3] Drug Research and Development Center, Federal University of Ceará, CE, Brazil

[*] Corresponding author. Phone: +55 85 3366-9826; Fax: +55 85 3366-9820.

E-mail: marjory.holanda@ufc.br

Figure 1

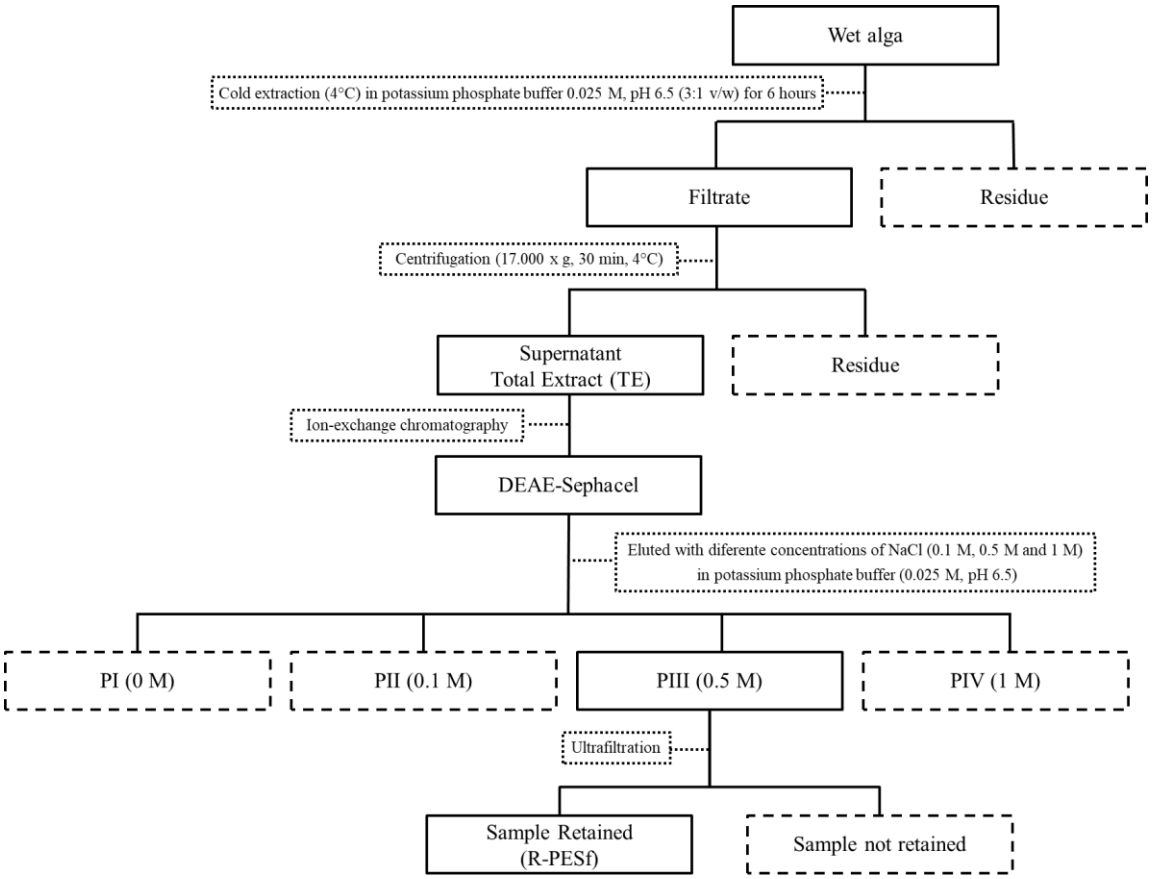

Wet alga
Cold extraction (4°C) in potassium phosphate buffer 0.025 M, pH 6.5 (3:1 v/w) for 6 hours
Filtrate
Residue
Centrifugation (17.000 x g, 30 min, 4°C)
Supernatant
Total Extract (TE)
Residue
Ion-exchange chromatography
DEAE-Sephacel
Eluted with diferente concentrations of NaCl (0.1 M, 0.5 M and 1 M)
in potassium phosphate buffer (0.025 M, pH 6.5)
PI (0 M)
PII (0.1 M)
PIII (0.5 M)
PIV (1 M)
Ultrafiltration
Sample Retained
(R-PESf)
Sample not retained

Figure 2

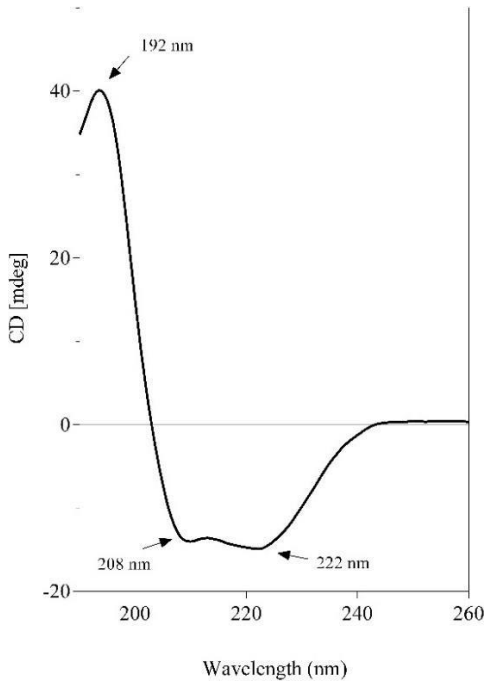

192 nm
40
20
0
-20
CD [mdeg]
208 nm
222 nm
200
220
240
260
Wavelength (nm)

Figure 3

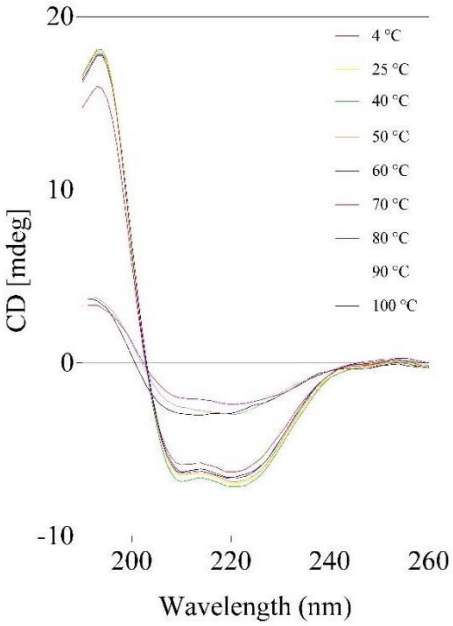
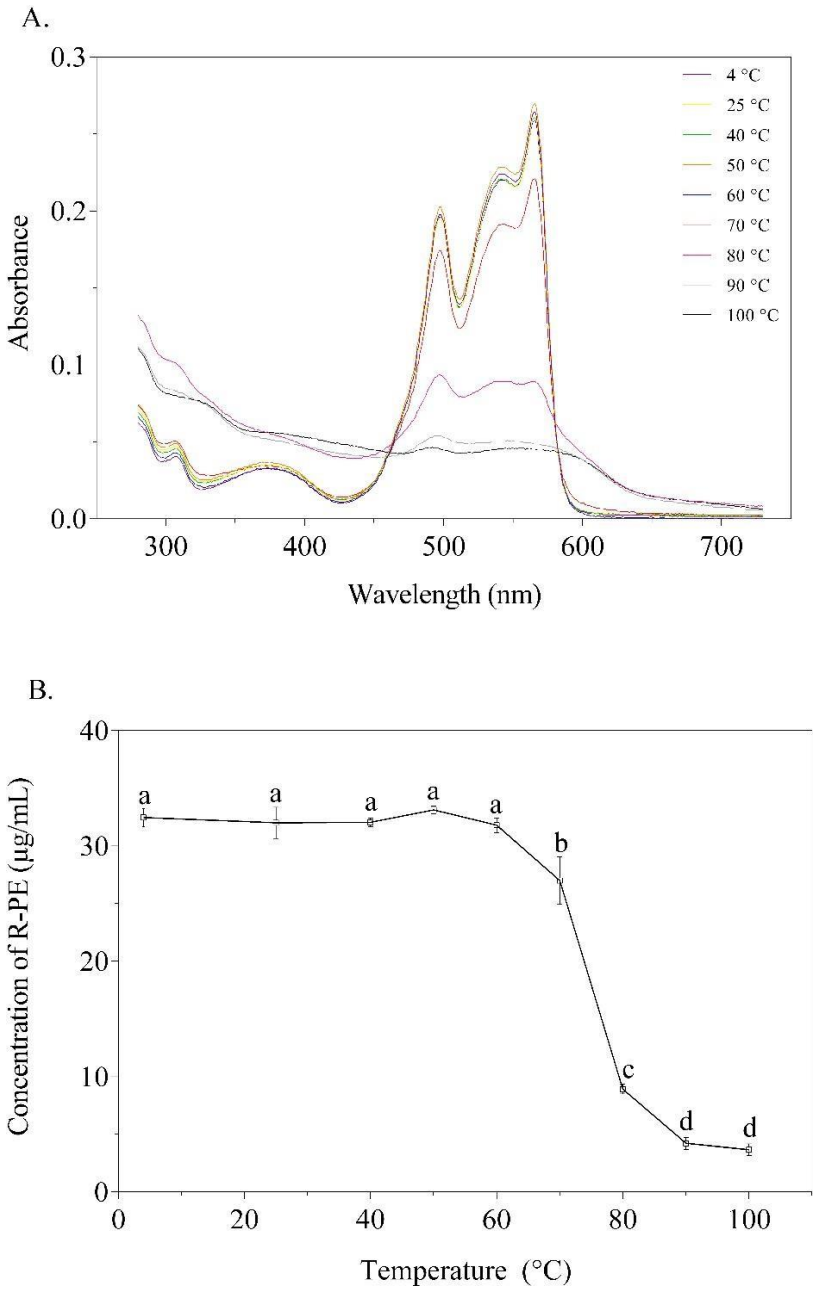
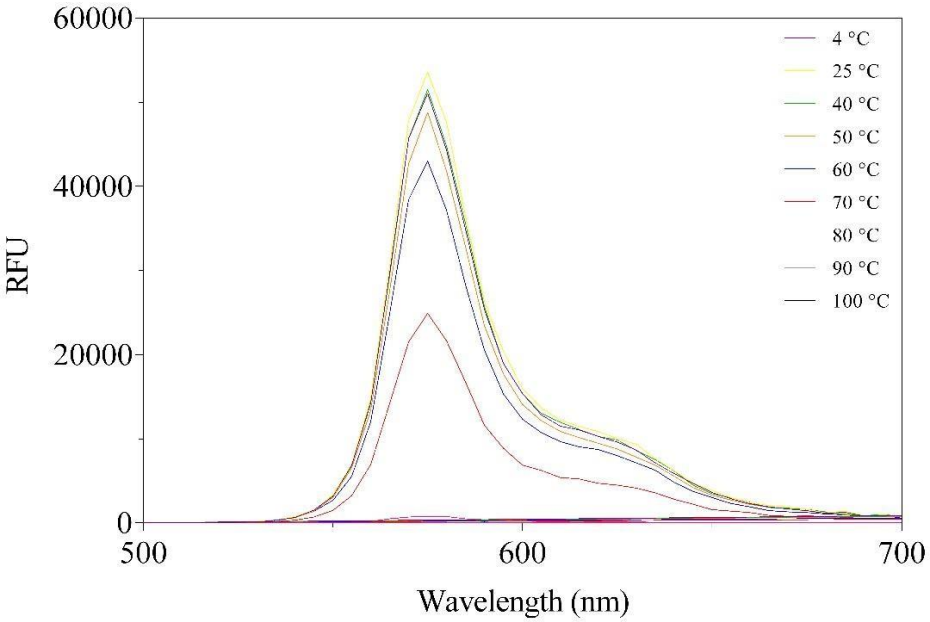

20
10
0
-10
CD [mdeg]
200
220
240
260
Wavelength (nm)
4 °C
25 °C
40 °C
50 °C
60 °C
70 °C
80 °C
90 °C
100 °C

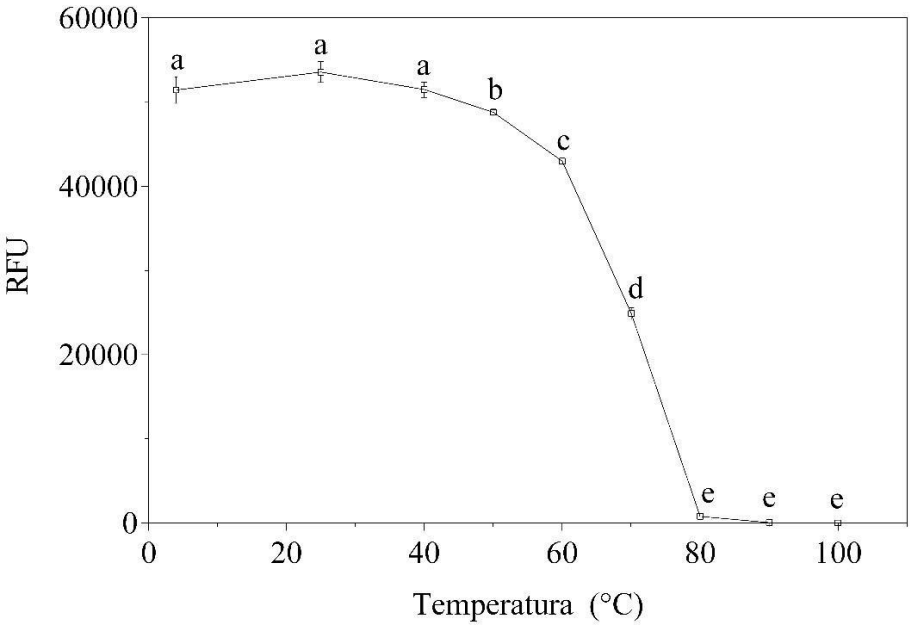
Figure 4


A.
Absorbance
Wavelength (nm)
4 °C
25 °C
40 °C
50 °C
60 °C
70 °C
80 °C
90 °C
100 °C
B.
Concentration of R-PE (μg/mL)
Temperature (°C)
a
a
a
a
a
b
c
d
d

A.


60000
40000
20000
0
RFU
500
600
700
Wavelength (nm)
4 °C
25 °C
40 °C
50 °C
60 °C
70 °C
80 °C
90 °C
100 °C

B.


60000
40000
20000
0
RFU
0
20
40
60
80
100
Temperatura (°C)
a
a
a
b
c
d
e
e
e

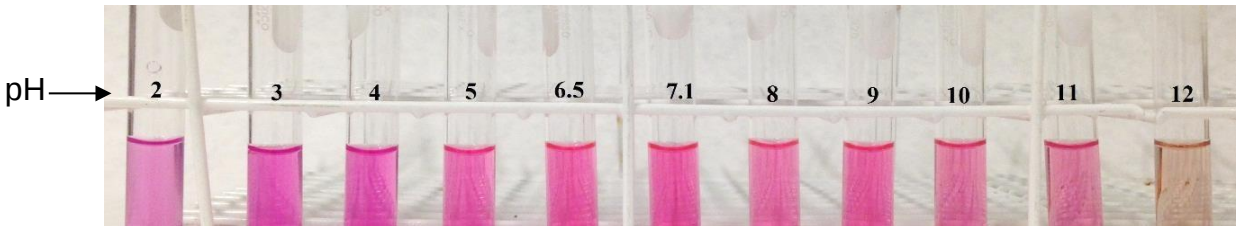

pH
2
3
4
5
6.5
7.1
8
9
10
11
12

Figure 7

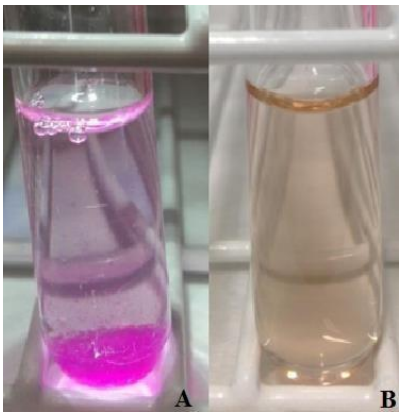


Figure 8

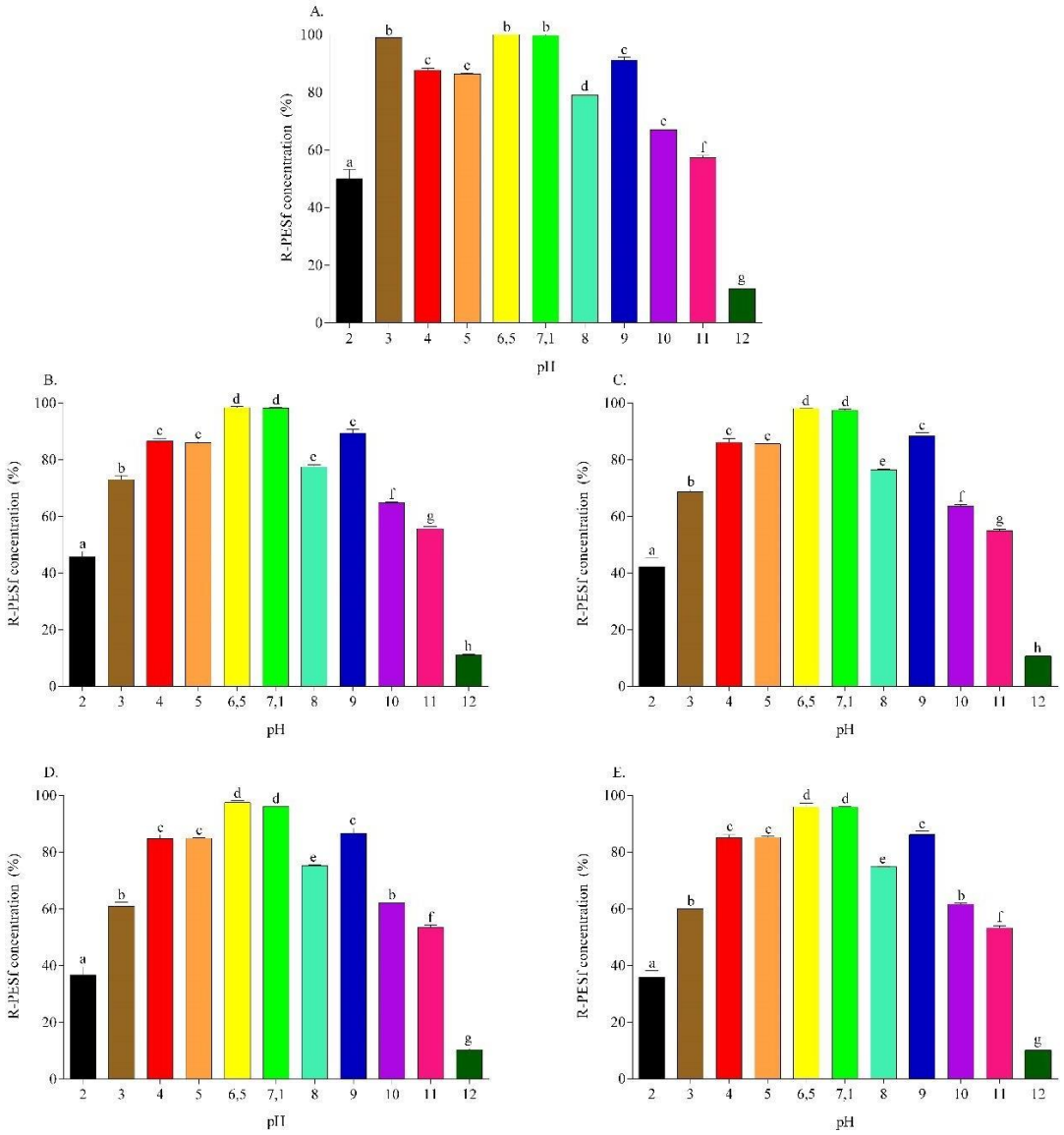


Figure 9

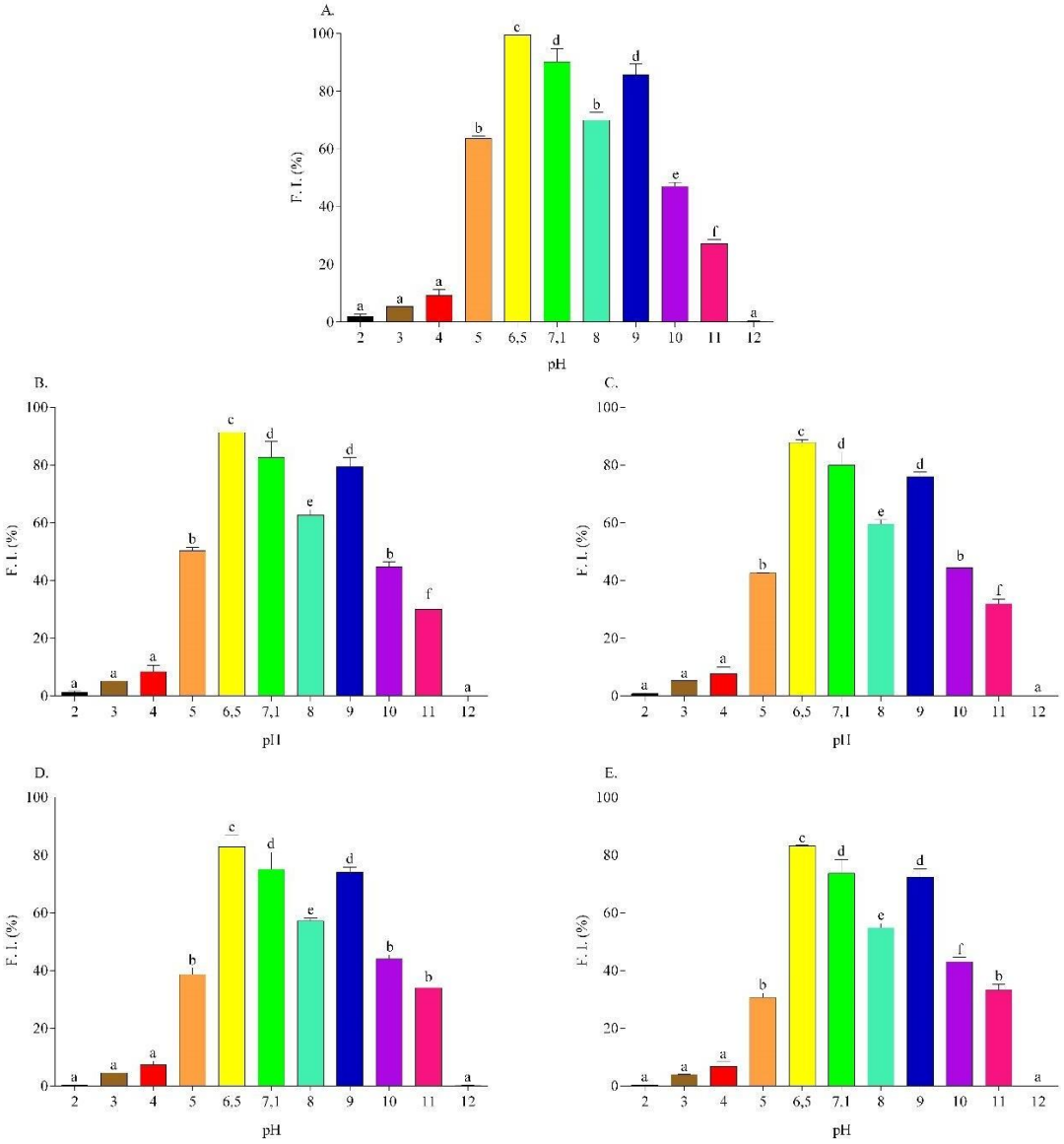
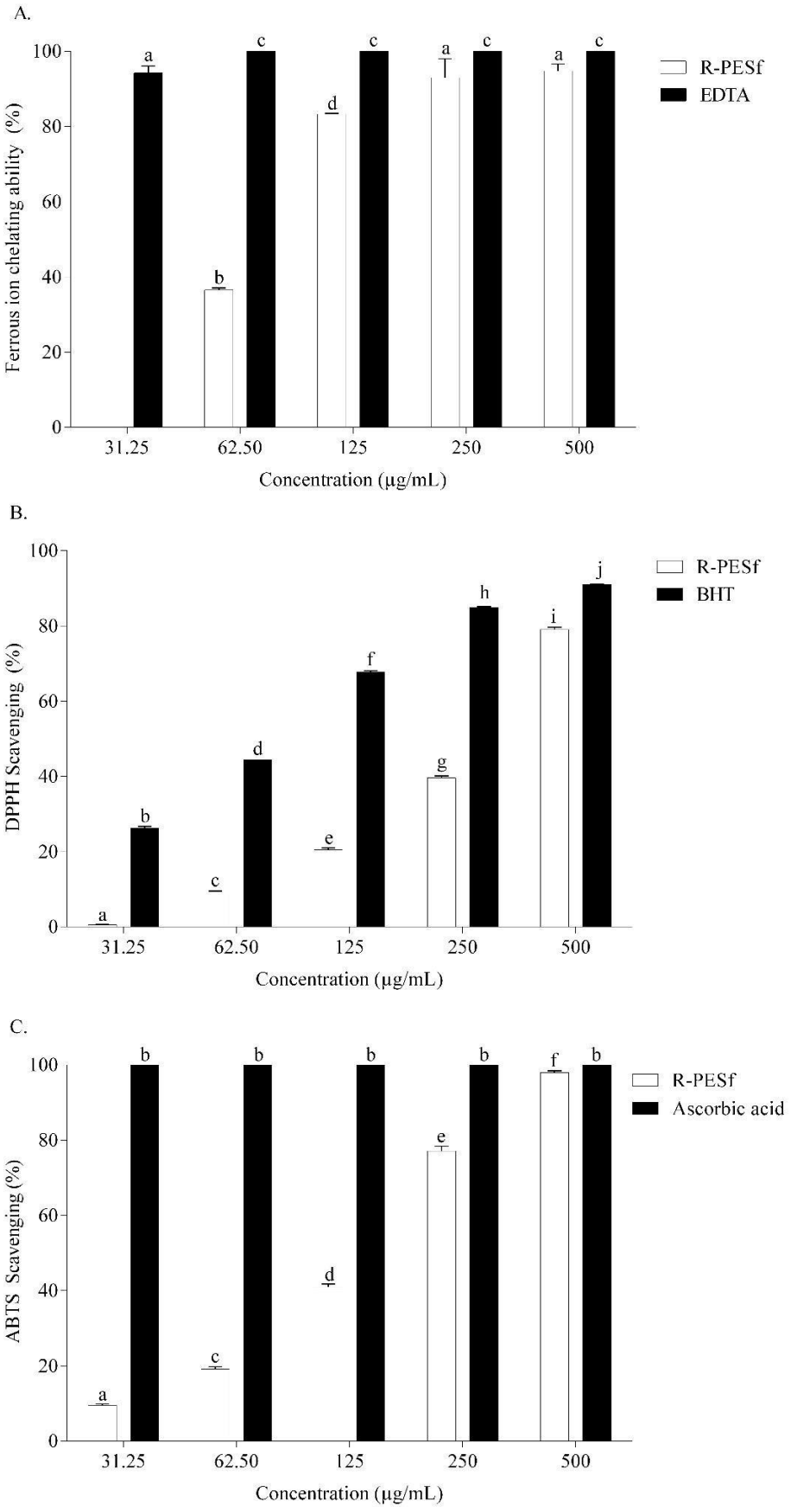


Figure 10


A.
Ferrous ion chelating ability (%)
100
80
60
40
20
0
a
b
c
d
c
a
c
a
c
31.25
62.50
125
250
500
Concentration (µg/mL)
R-PESf
EDTA
B.
DPPH Scavenging (%)
100
80
60
40
20
0
a
b
c
d
e
f
g
h
i
j
31.25
62.50
125
250
500
Concentration (µg/mL)
R-PESf
BHT
C.
ABTS Scavenging (%)
100
80
60
40
20
0
a
b
c
d
b
b
b
e
b
f
b
31.25
62.50
125
250
500
Concentration (µg/mL)
R-PESf
Ascorbic acid

Supplementary material

R-phycoerythrin from *Solieria filiformis*: An antioxidant macromolecular protein with spectroscopic and structural stability

[1]Acrisio José Uchôa Bastos-Filho, [1]Francisco Lucas de Souza Lopes, [1]Aurea Anette Monteiro Brito, [1]Roberta Cristiane Cavalcante Costa, [1]Antônio Augusto Lima Araújo-Filho, [2]Jéssica Roberta Pereira Martins, [1]Ticiana de Brito Lima-Holanda, [1]José Cirlanio Sousa Albuquerque, [3]Hermógenes David de Oliveira, [1]Norma Maria Barros Benevides, [1]Ana Lúcia Ponte Freitas, [1*]Márjory Lima Holanda-Araújo

[1] Department of Biochemistry and Molecular Biology, Federal University of Ceará, CE, Brazil

[2] Department of Chemical Engineering, Federal University of Ceará, CE, Brazil

[3] Drug Research and Development Center, Federal University of Ceará, CE, Brazil

[*] Corresponding author. Phone: +55 85 3366-9826; Fax: +55 85 3366-9820.

E-mail: marjory.holanda@ufc.br

Figura S1 – Effect of the time on the absorption spectrum of R-PESf dissolved in buffers at different pH.

(A) zero time; (B) 30 min; (C) 60 min; (D) 90 min; (E) 120 min.

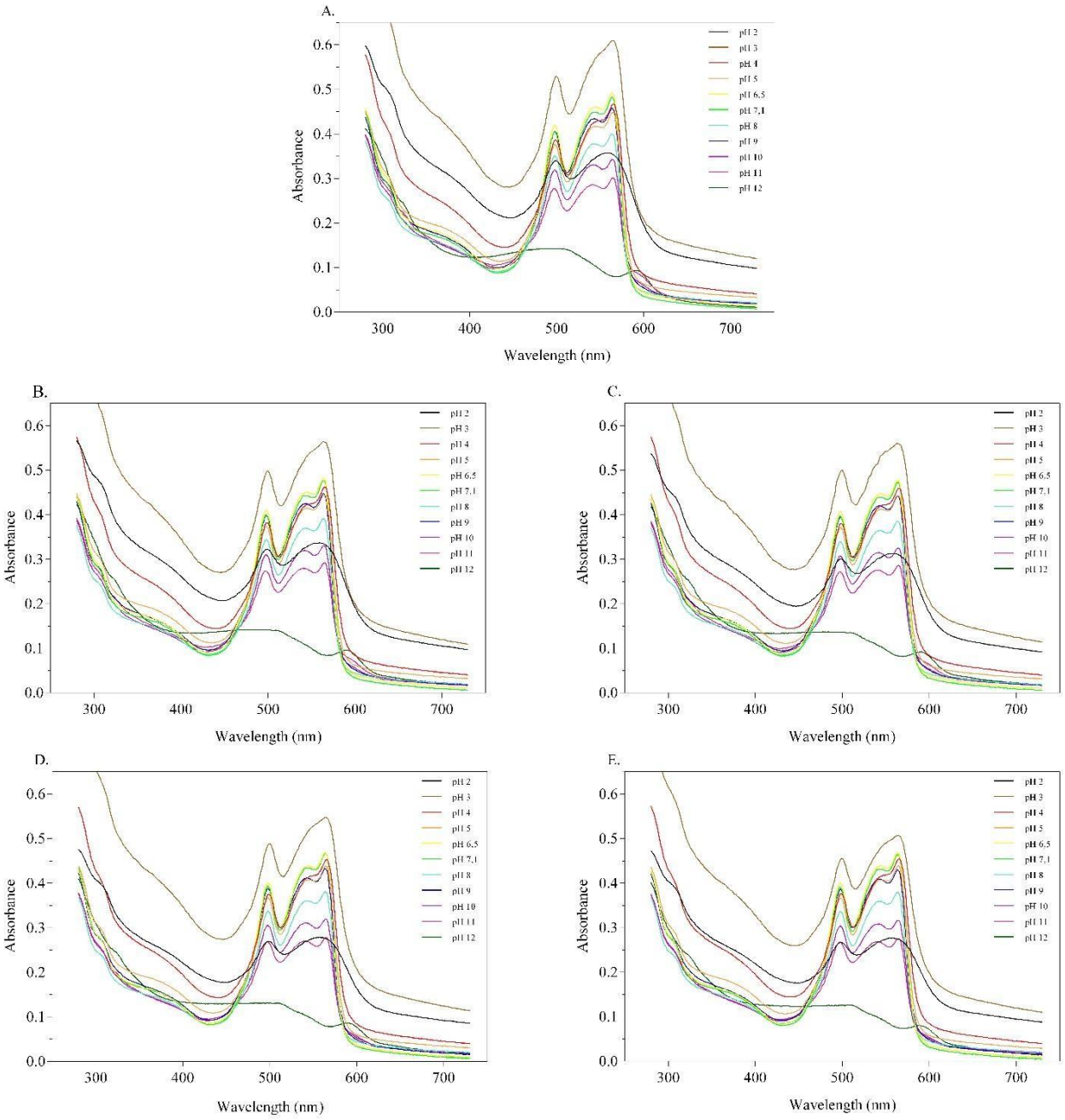
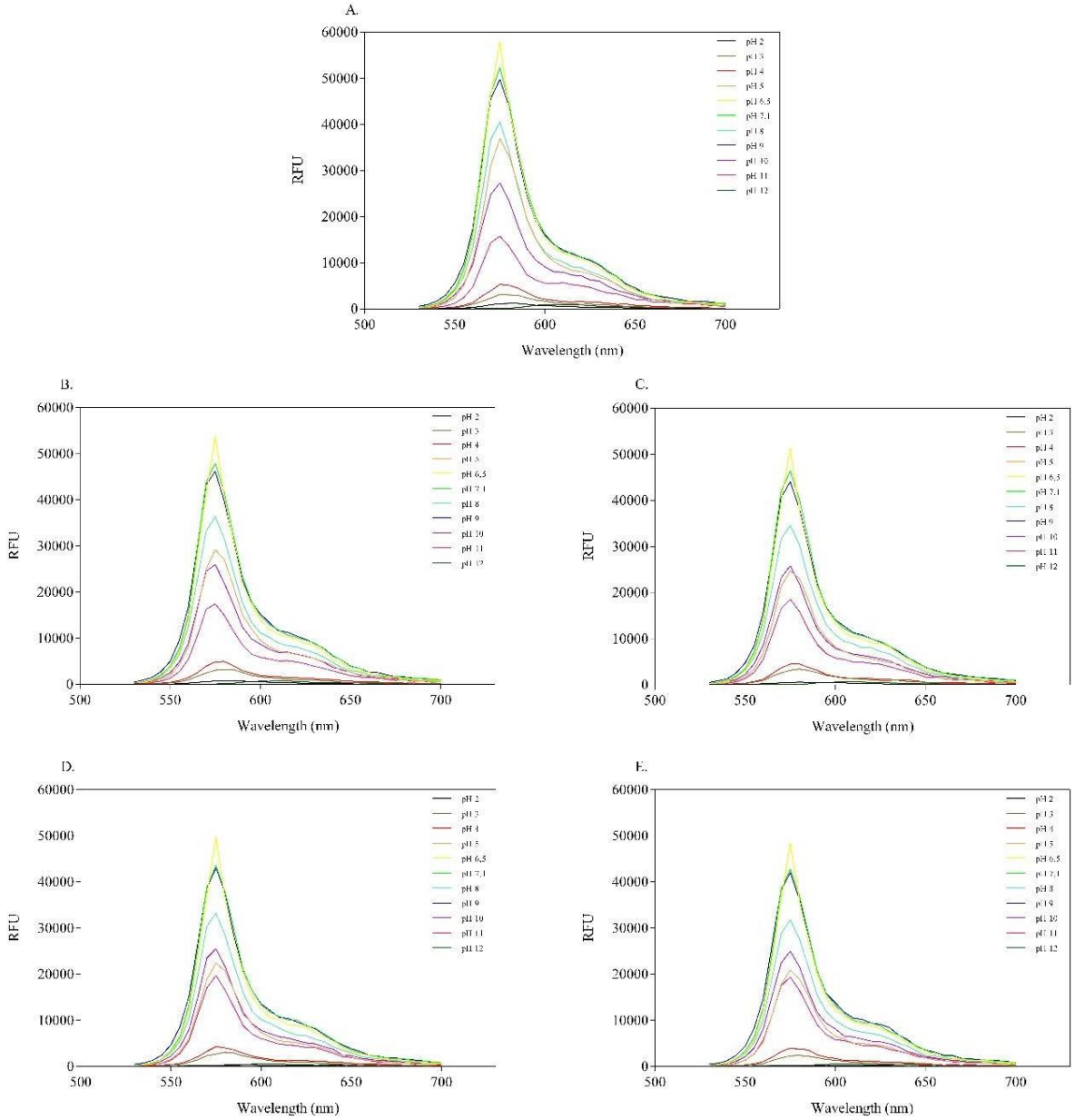


Figura S2 – Effect of time on the fluorescence emission spectrum of R-PESf dissolved at buffers at different pHs. (A) zero time; (B) 30 min; (C) 60 min; (D) 90 min; (E) 120 min.



7 CONCLUSÃO

A R-Ficoeritrina da macroalga marinha vermelha *Solieria filiformis* foi extraída e purificada por duas metodologias, atingindo um índice de pureza compatível para aplicação alimentícia e farmacológica, A R-PESf exibiu uma estrutura proteica constituída por subunidades monoméricas de diferentes pesos moleculares (α, β e γ), propriedades espectroscópicas (absorbância e fluorescência) estáveis em condições de armazenamento a baixas temperaturas, e em uma ampla faixa de temperatura e pH. Além disso, apresentou atividade antioxidante por três metodologias testadas tornando-se uma biomolécula promissora como antioxidante natural. O pigmento também foi capaz de colorir iogurte natural com boa aceitação e preferência pelos consumidores, se tornando um composto bioativo com aplicações como corante, sonda fluorescente, diagnósticos.

REFERÊNCIAS

ABREU, Ticiana Monteiro; RIBEIRO, Natássia Albuquerque; CHAVES, Hellíada Vasconcelos; JORGE, Roberta Jeane Bezerra; BEZERRA, Mirna Marques; MONTEIRO, Helena Serra Azul; VASCONCELOS, Ilka Maria; MOTA, Érika Freitas; BENEVIDES, Norma Maria Barros. Antinociceptive and Anti-inflammatory Activities of the Lectin from Marine Red Alga Solieria filiformis. **Planta Medica**, [s. l.], v. 82, n. 7, p. 596–605, 2016. DOI: 10.1055/s-0042-101762.

ANA, Peñuela; NATHALIE, Bourgougnon; GILLES, Bedoux; DANIEL, Robledo; TOMÁS, Madera Santana; YOLANDA, Freile Pelegrín. Anti-Herpes simplex virus (HSV-1) activity and antioxidant capacity of carrageenan-rich enzymatic extracts from Solieria filiformis (Gigartinales, Rhodophyta). **International Journal of Biological Macromolecules**, [s. l.], v. 168, p. 322–330, 2021. DOI: 10.1016/j.ijbiomac.2020.12.064. Disponível em: https://doi.org/10.1016/j.ijbiomac.2020.12.064.

ANASTÁCIO, Lucas Barros; OLIVEIRA, Danielle Aparecida; DELMASCHIO, Camila Rocha; ANTUNES, Lusânia Maria Greggi; CHEQUER, Farah Maria Drumond. Corantes Alimentícios Amaranto, Eritrosina B e Tartrazina, e seus possíveis Efeitos Maléficos à Saúde Humana. **Journal of Applied Pharmaceutical Sciences – JAPHAC**, [s. l.], v. 2, n. 3, p. 16–30, 2016. Disponível em: http://media.wix.com/ugd/e6f2ee_3a42125bee6e468e9660e981481eddd8.pdf.

ANDJELKOVIĆ, Mirjana; VAN CAMP, John; DE MEULENAER, Bruno; DEPAEMELAERE, Griet; SOCACIU, Carmen; VERLOO, Marc; VERHE, Roland. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. **Food Chemistry**, [s. l.], v. 98, n. 1, p. 23–31, 2006. DOI: 10.1016/j.foodchem.2005.05.044.

ANVISA. Informe Técnico nº. 68/2015 – GEARE/GGALI/ANVISA - Pag 1 de 7 Gerência de Avaliação de Risco e Eficácia para Alegações - GEARE Gerência Geral de Alimentos - GGALI. *[S. l.]*, p. 1–7, 2015.

APT, Kirk E.; COLLIER, Jackie L.; GROSSMAN, Arthur R. Evolution of the Phycobiliproteins. **Journal of Molecular Biology**, [s. l.], v. 248, p. 79–96, 1995.

BATADA, Ameena; JACOBSON, Michael F. Prevalence of Artificial Food Colors in Grocery Store Products Marketed to Children. **Clinical Pediatrics**, [s. l.], v. 55, n. 12, p. 1113–1119, 2016. DOI: 10.1177/0009922816651621.

BHARMORIA, Pankaj; CORREIA, Sandra F. H.; MARTINS, Margarida; HERNÁNDEZ-RODRÍGUEZ, Miguel A.; VENTURA, Sónia P. M.; FERREIRA, Rute A. S.; CARLOS, Luís D.; COUTINHO, Joaõ A. P. Protein Cohabitation: Improving the Photochemical Stability of R-Phycoerythrin in the Solid State. **Journal of Physical Chemistry Letters**, [s. l.], v. 11, n. 15, p. 6249–6255, 2020. DOI: 10.1021/acs.jpclett.0c01491.

BLOIS, Marsden S. **Antioxidant determinations by the use of a stable free radical [10]Nature**, 1958. DOI: 10.1038/1811199a0.

BOGORAD, Lawrence. Phycobiliprotein: Complementary Chromatic Adaptation. **Ann. Rev. Plant Physiol.**, [s. l.], v. 26, p. 369–401, 1975.

BRADFORD, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, [s. l.], v. 72, n. 1–2, p. 248–254, 1976. DOI: 10.1016/0003-2697(76)90527-3.

BRASIL. **Resolução nº 44 de 1977, de 01 de fevereiro de 1978**. Brasil: CNNPA - Comissão Nacional de Normas e Padrões para Alimentos, 1978.

BRASIL. **Portaria nº 540, de 27 de outubro de 1997**. [s.l: s.n.]. Disponível em: http://portal.anvisa.gov.br/wps/wcm/connect/d1b6da0047457b4d880fdc3fbc4c6735/PORTARIA_540_1997.pdf?MOD=AJPERES.

BRASIL. **Instrução normativa nº 46, de 23 de outubro de 2007**. *[S. l.]*, 2007. Disponível em: http://digilib.unila.ac.id/11478/16/16. BAB II.pdf.

BRYANT, Donald A. Phycoerythrocyanin and Phycoerythrin: Properties and Occurrence in Cyanobacteria. **Microbiology**, [s. l.], v. 128, n. 4, p. 835–844, 1982. DOI: 10.1099/00221287-128-4-835.

BRYANT, Donald A.; GLAZER, Alexander N.; EISERLING, Frederick A. Characterization and structural properties of the major biliproteins of Anabaena sp. **Archives of Microbiology**, [s. l.], v. 110, n. 1, p. 61–75, 1976. DOI: 10.1007/BF00416970.

CAAMAL-FUENTES, Edgar; ROBLEDO, Daniel; FREILE-PELEGRÍN, Yolanda. Physicochemical characterization and biological activities of sulfated polysaccharides from cultivated solieria filiformis rhodophyta. **Natural Product Communications**, [s. l.], v. 12, n. 6, p. 803–806, 2017. DOI: 10.1177/1934578x1701200601.

CAI, Chuner; LI, Chunxia; WU, Shuxian; WANG, Qing; GUO, Ziye; HE, Peimin. Large scale preparation of phycobiliproteins from Porphyra yezoensis using co-precipitation with ammonium sulfate. **Natural Science**, [s. l.], v. 04, n. 08, p. 536–543, 2012. DOI: 10.4236/ns.2012.48071.

CÂMARA, Arthur Medeiros. **Corantes azo: características gerais, aplicações e toxicidade**. 2017. Universidade Federal do Rio Grande do Norte, [s. l.], 2017.

CHANG, W. R.; JIANG, T.; WAN, Z. L.; ZHANG, J. P.; YANG, Z. X.; LIANG, D. C. Crystal structure of R-phycoerythrin from Polysiphonia urceolata at 2.8 A resolution. **Journal of molecular biology**, [s. l.], v. 262, p. 721–731, 1996. DOI: 10.1006/jmbi.1996.0547.

CHAVES, Renata Pinheiro et al. Structural characterization of two isolectins from the marine red alga Solieria filiformis (Kützing) P.W. Gabrielson and their anticancer effect on MCF-7 breast cancer cells. **International Journal of Biological Macromolecules**, [s. l.], v. 107, n. PartA, p. 1320–1329, 2018. DOI: 10.1016/j.ijbiomac.2017.09.116. Disponível em: http://dx.doi.org/10.1016/j.ijbiomac.2017.09.116.

CHEW, Y. L.; LIM, Y. Y.; OMAR, M.; KHOO, K. S. Antioxidant activity of three edible seaweeds from two areas in South East Asia. **LWT - Food Science and Technology**, [s. l.], v. 41, n. 6, p. 1067–1072, 2008. DOI: 10.1016/j.lwt.2007.06.013.

CIAN, Raúl E.; LÓPEZ-POSADAS, Rocío; DRAGO, Silvina R.; MEDINA, Fermín Sánchez De; MARTÍNEZ-AUGUSTIN, Olga. Immunomodulatory properties of the protein fraction from Phorphyra columbina. **Journal of Agricultural and Food Chemistry**, [s. l.], v. 60, n. 33, p. 8146–8154, 2012. DOI: 10.1021/jf300928j.

COBUCCI, R. M. A. **Analise Sensorial: Apostila do Curso**. Curso Tecnológico Superior em Gastronomia: Pontifica Universidade Católica de Goiás, 2010.

CORRADINI, M. G. Synthetic Food Colors. *In*: **Reference Module in Food Science**. [s.l.] : Elsevier Inc., 2018. DOI: 10.1016/B978-0-12-814026-0.21606-5.

DE ARAÚJO, Ianna Wivianne Fernandes et al. Effects of a sulfated polysaccharide isolated from the red seaweed Solieria filiformis on models of nociception and inflammation. **Carbohydrate Polymers**, [s. l.], v. 86, n. 3, p. 1207–1215, 2011. DOI: 10.1016/j.carbpol.2011.06.016.

DE ARAÚJO, Ianna Wivianne Fernandes; RODRIGUES, José Ariévilo Gurgel; VANDERLEI, Edfranck de Sousa Oliveira; DE PAULA, Gabriela Almeida; LIMA, Ticiana De Brito; BENEVIDES, Norma Maria Barros. Iota-carragenanas da rodofícea Solieria filiformis e seus efeitos na inflamação e coagulação. **Acta Scientiarum - Technology**, [s. l.], v. 34, n. 2, p. 127–135, 2012. DOI: 10.4025/actascitechnol.v34i2.11201.

DE CASTRO, George Meredite Cunha; BENEVIDES, Norma Maria Barros; CABRAL, Maulori Curié; MIRANDA, Rafael De Souza; FILHO, Enéas Gomes; ROCHA, Maria Valderez Ponte; ARAÚJO, Márjory Lima Holanda. Optimized acid hydrolysis of the polysaccharides from the seaweed Solieria filiformis (Kützing) P.W. Gabrielson for bioethanol production. **Acta Scientiarum - Biological Sciences**, [s. l.], v. 39, n. 4, p. 423–430, 2017. DOI: 10.4025/actascibiolsci.v39i4.37227.

DUMAY, Justine; CLÉMENT, Nathalie; MORANÇAIS, Michèle; FLEURENCE, Joël. Optimization of hydrolysis conditions of Palmaria palmata to enhance R-phycoerythrin extraction. **Bioresource Technology**, [s. l.], v. 131, p. 21–27, 2013. DOI: 10.1016/j.biortech.2012.12.146. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/S0960852412020044.

DUMAY, Justine; MORANÇAIS, Michèle; MUNIER, Mathilde; LE GUILLARD, Cécile; FLEURENCE, Joël. Chapter Eleven - Phycoerythrins: Valuable Proteinic Pigments in Red Seaweeds. *In*: **Sea Plants**. [s.l: s.n.]. v. Volume 71p. 321–343. DOI: http://dx.doi.org/10.1016/B978-0-12-408062-1.00011-1. Disponível em: http://www.sciencedirect.com/science/article/pii/B9780124080621000111.

DUMAY, Justine; MORANÇAIS, Michèle; MUNIER, Mathilde; LE GUILLARD, Cécile; FLEURENCE, Joël. Chapter Eleven - Phycoerythrins: Valuable Proteinic Pigments in Red Seaweeds. *In*: **Sea Plants**. [s.l: s.n.]. v. Volume 71p. 321–343. DOI: http://dx.doi.org/10.1016/B978-0-12-408062-1.00011-1.

DUTCOSKY, Silvia Deboni. **Análise sensorial de alimentos**. Curitiba.
FAO/WHO. **Codex General Standard for Food Additives (GSFA) Online Database**. 2021. Disponível em: http://www.fao.org/gsfaonline/additives/index.html. Acesso em: 19 abr. 2021.

FELACO, Luis; OLVERA-NOVOA, Miguel A.; ROBLEDO, Daniel. Multitrophic integration of the tropical red seaweed Solieria filiformis with sea cucumbers and fish. **Aquaculture**, [s. l.], v. 527, n. March, 2020. DOI: 10.1016/j.aquaculture.2020.735475.

FLEURENCE, Joël. R-Phycoerythrin from red macroalgae: Strategies for extraction and potential application in Biotechnology. **Applied Biotechnology, Food Science and Policy**, [s. l.], v. 1, n. 1, p. 63–68, 2003.

GALLAND-IRMOULI, A. V.; PONS, L.; LUÇON, M.; VILLAUME, C.; MRABET, N. T.; GUÉANT, J. L.; FLEURENCE, J. One-step purification of R-phycoerythrin from the red macroalga Palmaria palmata using preparative polyacrylamide gel electrophoresis. **Journal of Chromatography B: Biomedical Sciences and Applications**, [s. l.], v. 739, n. 1, p. 117–123, 2000. DOI: 10.1016/S0378-4347(99)00433-8.

GALLO, Monica. **Novel Foods: Insects - Safety Issues**. [s.l.] : Elsevier, 2018. DOI: 10.1016/B978-0-08-100596-5.22134-3. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/B9780081005965221343.

GANESAN, Abirami Ramu; SHANMUGAM, Munisamy. Isolation of phycoerythrin from Kappaphycus alvarezii: a potential natural colourant in ice cream. **Journal of Applied Phycology**, [s. l.], v. 32, n. 6, p. 4221–4233, 2020. DOI: 10.1007/s10811-020-02214-0.

GANTT, Elisabeth. Structure and Function of Phycobilisomes: Light Harvesting Pigment Complexes in Red and Blue-Green Algae. **International Review of Cytology**, [s. l.], v. 66, p. 45–80, 1980. DOI: 10.1016/S0074-7696(08)61971-3. Disponível em: http://www.sciencedirect.com/science/article/pii/S0074769608619713.

GIČEVIĆ, Armina; HINDIJA, Lamija; KARAČIĆ, Alma. Toxicity of azo dyes in pharmaceutical industry. **IFMBE Proceedings**, [s. l.], v. 73, n. January, p. 581–587, 2020. DOI: 10.1007/978-3-030-17971-7_88.

GLAZER, A. N. Light guides. Directional energy transfer in a photosynthetic antenna. **Journal of Biological Chemistry**, [s. l.], v. 264, n. 1, p. 1–4, 1989.

GLAZER, Alexander N. Phycobilisome a macromolecular complex optimized for light energy transfer. **Biochimica et Biophysica Acta (BBA) - Reviews on Bioenergetics**, [s. l.], v. 768, n. 1, p. 29–51, 1984. DOI: 10.1016/0304-4173(84)90006-5. Disponível em: http://www.sciencedirect.com/science/article/pii/0304417384900065.

GLAZER, Alexander N. Light harvesting by phycobilisomes. **Annual review of biophysics and biophysical chemistry**, [s. l.], v. 43, n. 64, p. 47–77, 1985.

GLAZER, Alexander N.; APELL, G. S.; HIXSON, C. S.; BRYANT, D. a; RIMON, S.; BROWN, D. M. Biliproteins of cyanobacteria and Rhodophyta: Homologous family of photosynthetic accessory pigments. **Proceedings of the National Academy of Sciences of the United States of America**, [s. l.], v. 73, n. 2, p. 428–431, 1976. DOI: 10.1073/pnas.73.2.428.

GOLDSMITH, Randall H.; MOERNER, W. E. Watching conformational- and photodynamics of single fluorescent proteins in solution. **Nature Chemistry**, [s. l.], v. 2, n. 3, p. 179–186, 2010. DOI: 10.1038/nchem.545. Disponível em: https://doi.org/10.1038/nchem.545.

HARNEDY, Pádraigín A.; O'KEEFFE, Martina B.; FITZGERALD, Richard J. Fractionation and identification of antioxidant peptides from an enzymatically hydrolysed Palmaria palmata protein isolate. **Food Research International**, [s. l.], v. 100, n. July, p. 416–422, 2017. DOI: 10.1016/j.foodres.2017.07.037.

HEMLATA; AFREEN, Sumbul; FATMA, Tasneem. Extraction, purification and characterization of phycoerythrin from Michrochaete and its biological activities. **Biocatalysis and Agricultural Biotechnology**, [s. l.], v. 13, n. October 2017, p. 84–89, 2018. DOI: 10.1016/j.bcab.2017.11.012.

HOLANDA, M. L.; MELO, V. M. M.; SILVA, L. M. C. M.; AMORIM, R. C. N.; PEREIRA, M. G.; BENEVIDES, N. M. B. Differential activity of a lectin from Solieria filiformis against human pathogenic bacteria. **Brazilian Journal of Medical and Biological Research**, [s. l.], v. 38, n. 12, p. 1769–1773, 2005. DOI: 10.1590/S0100-879X2005001200005.

IHEANYICHUKWU, Wopara; ADEGOKE, Adebayo O.; ADEBAYO, Olusegun G.; EMMANUEL U, Modo; EGELEGE, Aziemeola Pius; GONA, Jeremiah T.; ORLUWENE, Fortune M. Combine colorants of tartrazine and erythrosine induce kidney injury: involvement of TNF-α gene, caspase-9 and KIM-1 gene expression and kidney functions indices. **Toxicology Mechanisms and Methods**, [s. l.], v. 31, n. 1, p. 67–72, 2021. DOI: 10.1080/15376516.2020.1828523. Disponível em: http://dx.doi.org/10.1080/15376516.2020.1828523.

ISAILOVIC, Dragan; LI, Hung-Wing; YEUNG, Edward S. Isolation and characterization of R-phycoerythrin subunits and enzymatic digests. **Journal of Chromatography A**, [s. l.], v. 1051, n. 1–2, p. 119–130, 2004. DOI: 10.1016/j.chroma.2004.07.038. Disponível em: http://www.sciencedirect.com/science/article/pii/S0021967304011999.

JIANG, Tao; ZHANG, Ji Ping; LIANG, Dong Cai. Structure and function of chromophores in R-Phycoerythrin at 1.9 A Resolution. **Proteins: Structure, Function and Genetics**, [s. l.], v. 34, n. 2, p. 224–231, 1999. DOI: 10.1002/(SICI)1097-0134(19990201)34:2<224::AID-PROT8>3.0.CO;2-D.

KANNAUJIYA, Vinod K.; KUMAR, Deepak; SINGH, Vidya; SINHA, Rajeshwar P. **Advances in phycobiliproteins research: innovations and commercialization**. [s.l.] : Elsevier Inc., 2021. DOI: 10.1016/b978-0-12-820655-3.00004-5. Disponível em: http://dx.doi.org/10.1016/B978-0-12-820655-3.00004-5.

KANNAUJIYA, Vinod K.; SUNDARAM, Shanthy; SINHA, Rajeshwar P. **Phycobiliproteins: Recent developments and future applications**. [s.l: s.n.]. DOI: 10.1007/978-981-10-6460-9.

KAWSAR, Sarkar M. a; FUJII, Yuki; MATSUMOTO, Ryo; YASUMITSU, Hidetaro; OZEKI, Yasuhiro. Protein R-phycoerythrin from marine red alga Amphiroa anceps : extraction , purification and characterization. **Phytologia Balcanica**, [s. l.], v. 17, n. 3, p. 347–354, 2011.

LAEMMLI, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. **Nature**, [s. l.], v. 227, n. 5259, p. 680–685, 1970. DOI: 10.1038/227680a0. Disponível em: http://www.nature.com/doifinder/10.1038/227680a0.

LIMA, Ana Carolina Viana De et al. Microfiltered red–purple pitaya colorant: UPLC-ESI-QTOF-MSE-based metabolic profile and its potential application as a natural food ingredient. **Food Chemistry**, [s. l.], v. 330, n. May, 2020. DOI: 10.1016/j.foodchem.2020.127222.

LIU, Lu-Ning; SU, Hai-Nan; YAN, Shi-Gan; SHAO, Si-Mi; XIE, Bin-Bin; CHEN, Xiu-Lan; ZHANG, Xi-Ying; ZHOU, Bai-Cheng; ZHANG, Yu-Zhong. Probing the pH sensitivity of R-phycoerythrin: Investigations of active conformational and functional variation. **Biochimica et Biophysica Acta (BBA) - Bioenergetics**, [s. l.], v. 1787, n. 7, p. 939–946, 2009. DOI: 10.1016/j.bbabio.2009.02.018. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/S0005272809000656.

LOUIS-JEUNE, Caroline; ANDRADE-NAVARRO, Miguel A.; PEREZ-IRATXETA, Carol. Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. **Proteins: Structure, Function and Bioinformatics**, [s. l.], v. 80, n. 2, p. 374–381, 2012. DOI: 10.1002/prot.23188.

LUNDELL, D. J.; GLAZER, A. N.; DELANGE, R. J.; BROWN, D. M. Bilin attachment sites in the alpha and beta subunits of B-phycoerythrin. Amino acid sequence studies. **The Journal of biological chemistry**, [s. l.], v. 259, n. 9, p. 5472–80, 1984. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/6715353.

MAKSIMOV, E. G.; SCHMITT, F. J.; HÄTTI, P.; KLEMENTIEV, K. E.; PASCHENKO, V. Z.; RENGER, G.; RUBIN, A. B. Anomalous temperature dependence of the fluorescence lifetime of phycobiliproteins. **Laser Physics Letters**, [s. l.], v. 10, n. 5, 2013. DOI: 10.1088/1612-2011/10/5/055602.

MITTAL, Rochak; LAMDANDE, Archana G.; SHARMA, Richa; RAGHAVARAO, K. S. M. S. Membrane processing for purification of R-Phycoerythrin from marine macro-alga, Gelidium pusillum and process integration. **Separation and Purification Technology**, [s. l.], v. 252, n. August, p. 117470, 2020. DOI: 10.1016/j.seppur.2020.117470. Disponível em: https://doi.org/10.1016/j.seppur.2020.117470.

MITTAL, Rochak; RAGHAVARAO, K. S. M. S. Extraction of R-Phycoerythrin from marine macro-algae, Gelidium pusillum, employing consortia of enzymes. **Algal Research**, [s. l.], v. 34, n. September 2017, p. 1–11, 2018. a. DOI: 10.1016/j.algal.2018.07.002. Disponível em: https://doi.org/10.1016/j.algal.2018.07.002.

MITTAL, Rochak; RAGHAVARAO, K. S. M. S. Extraction of R-Phycoerythrin from marine macro-algae, Gelidium pusillum, employing consortia of enzymes. **Algal Research**, [s. l.], v. 34, p. 1–11, 2018. b. DOI: https://doi.org/10.1016/j.algal.2018.07.002. Disponível em: http://www.sciencedirect.com/science/article/pii/S2211926417308548.

MØLLER, Anders Hauer; WIJAYA, Wahyu; JAHANGIRI, Amita; MADSEN, Bjoern; JOERNSGAARD, Bjarne; VAERBAK, Signe; HAMMERSHØJ, Marianne; VAN DER MEEREN, Paul; DALSGAARD, Trine Kastrup. Norbixin binding to whey protein isolate - alginate electrostatic complexes increases its solubility and stability. **Food Hydrocolloids**, [s. l.], v. 101, n. November 2019, p. 1–8, 2020. DOI: 10.1016/j.foodhyd.2019.105559.

MOTTA, Chiara Maria et al. Effects of four food dyes on development of three model species, Cucumis sativus, Artemia salina and Danio rerio: Assessment of potential risk for the environment. **Environmental Pollution**, [s. l.], v. 253, n. 1333, p. 1126–1135, 2019. DOI: 10.1016/j.envpol.2019.06.018. Disponível em: https://doi.org/10.1016/j.envpol.2019.06.018.

MUNIER, Mathilde; DUMAY, Justine; MORANÇAIS, Michèle; JAOUEN, Pascal; FLEURENCE, Joël. Variation in the biochemical composition of the edible seaweed grateloupia turuturu yamada harvested from two sampling sites on the brittany coast (France): The influence of storage method on the extraction of the seaweed pigment r-phycoerythrin. **Journal of Chemistry**, [s. l.], v. 2013, p. 568548, 2013. DOI: 10.1155/2013/568548.

MUNIER, Mathilde; JUBEAU, Sébastien; WIJAYA, Alva; MORANÇAIS, Michèle; DUMAY, Justine; MARCHAL, Luc; JAOUEN, Pascal; FLEURENCE, Joël. Physicochemical factors affecting the stability of two pigments: R-phycoerythrin of Grateloupia turuturu and B-phycoerythrin of Porphyridium cruentum. **Food Chemistry**, [s. l.], v. 150, p. 400–407, 2014. a. DOI: 10.1016/j.foodchem.2013.10.113. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/S0308814613015628.

MUNIER, Mathilde; JUBEAU, Sébastien; WIJAYA, Alva; MORANÇAIS, Michèle; DUMAY, Justine; MARCHAL, Luc; JAOUEN, Pascal; FLEURENCE, Joël. Physicochemical factors affecting the stability of two pigments: R-phycoerythrin of Grateloupia turuturu and B-phycoerythrin of Porphyridium cruentum. **Food Chemistry**, [s. l.], v. 150, p. 400–407, 2014. b. DOI: 10.1016/j.foodchem.2013.10.113.

MUNIER, Mathilde; MORANÇAIS, Michèle; DUMAY, Justine; JAOUEN, Pascal; FLEURENCE, Joël. One-step purification of R-phycoerythrin from the red edible seaweed Grateloupia turuturu. **Journal of Chromatography B**, [s. l.], v. 992, p. 23–29, 2015. DOI: 10.1016/j.jchromb.2015.04.012. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/S1570023215002159.

MURANO, Erminio; TOFFANIN, Renato; CECERE, Ester; RIZZO, Roberto; KNUTSEN, Svein Halvor. Investigation of the carrageenans extracted from Solieria filiformis and Agardhiellia subulata from Mar Piccolo, Taranto. **Marine Chemistry**, [s. l.], v. 58, n. 3–4, p. 319–325, 1997. DOI: 10.1016/S0304-4203(97)00058-3.

Natural Food Colors Market by Type (Carmine, Anthocyanins, Caramel, Annatto, Carotenoids, Chlorophyll, Spirulina), Source (Plant, Animal), Form (Liquid, Powder), Solubility (Water, Dye), Application - Global Forecast to 2027. [s.l.] : Meticulous Market

Research Pvt. Ltd., 2020.

NEWELL, G. J. ..; MACFARLANE, J. D. .. Expanded tables for multiple comparison procedures in the analysis of ranked data. **Journal of Food Science**, [s. l.], v. 52, n. 6, p. 1721–1725, 1987.

NGUYEN, Huu Phuoc Trang; MORANÇAIS, Michèle; DÉLÉRIS, Paul; FLEURENCE, Joël; NGUYEN-LE, Chau Thanh; VO, Khanh Ha; DUMAY, Justine. Purification of R-phycoerythrin from a marine macroalga Gracilaria gracilis by anion-exchange chromatography. **Journal of Applied Phycology**, [s. l.], v. 32, n. 1, p. 553–561, 2020. DOI: 10.1007/s10811-019-01947-x.

NIU, Jian-Feng; WANG, Guang-Ce; TSENG, Cheng-Kui. Method for large-scale isolation and purification of R-phycoerythrin from red alga Polysiphonia urceolata Grev. **Protein Expression and Purification**, [s. l.], v. 49, n. 1, p. 23–31, 2006. DOI: 10.1016/j.pep.2006.02.001. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/S1046592806000453.

O'CARRA, P.; MURPHY, R. F.; KILLILEA, S. D. The native forms of the phycobilin chromophores of algal biliproteins. A clarification. **Biochemical Journal**, [s. l.], v. 187, n. 2, p. 303–309, 1980. DOI: 10.1042/bj1870303. Disponível em: https://www.researchgate.net/publication/15801952_The_native_forms_of_the_phycobilin_chromophores_of_algal_biliproteins_A_clarification.

PAN, Qunwen; CHEN, Meizhen; LI, Juan; WU, Yan; ZHEN, Chao; LIANG, Bin. Antitumor function and mechanism of phycoerythrin from Porphyra haitanensis. **Biological research**, [s. l.], v. 46, n. 1, p. 87–95, 2013. DOI: 10.4067/S0716-97602013000100013. Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/23760420.

PATEL, Stuti N.; SONANI, Ravi R.; JAKHARIA, Kinnari; BHASTANA, Bela; PATEL, Hiral M.; CHAUBEY, Mukesh G.; SINGH, Niraj K.; MADAMWAR, Datta. Antioxidant activity and associated structural attributes of Halomicronema phycoerythrin. **International Journal of Biological Macromolecules**, [s. l.], v. 111, p. 359–369, 2018. DOI: 10.1016/j.ijbiomac.2017.12.170. Disponível em: https://doi.org/10.1016/j.ijbiomac.2017.12.170.

PÁTKAI, Györgyi; BARTA, J. Decomposition of betacyanins and betaxanthins by heat and pH changes. **Nahrung - Food**, [s. l.], v. 40, n. 5, p. 267–270, 1996. DOI: 10.1002/food.19960400508.

PEREIRA, Tatiana; BARROSO, Sónia; MENDES, Susana; AMARAL, Renata A.; DIAS, Juliana R.; BAPTISTA, Teresa; SARAIVA, Jorge A.; ALVES, Nuno M.; GIL, Maria M. Optimization of phycobiliprotein pigments extraction from red algae Gracilaria gracilis for substitution of synthetic food colorants. **Food Chemistry**, [s. l.], v. 321, n. March, p. 126688, 2020. a. DOI: 10.1016/j.foodchem.2020.126688. Disponível em: https://doi.org/10.1016/j.foodchem.2020.126688.

PEREIRA, Tatiana; BARROSO, Sónia; MENDES, Susana; GIL, Maria M. Stability, kinetics, and application study of phycobiliprotein pigments extracted from red algae Gracilaria gracilis. **Journal of Food Science**, [s. l.], v. 85, n. 10, p. 3400–3405, 2020. b. DOI: 10.1111/1750-3841.15422.

PUNAMPALAM, R.; KHOO, K. S.; SIT, N. W. Evaluation of antioxidant properties of phycobiliproteins and phenolic compounds extracted from Bangia atropurpurea. **Malaysian Journal of Fundamental and Applied Sciences**, [s. l.], v. 14, n. 2, p. 289–297, 2018.

RAMESH, Muthusamy; MUTHURAMAN, Arunachalam. Flavoring and Coloring Agents: Health Risks and Potential Problems. *In*: **Natural and Artificial Flavoring Agents and Food Dyes**. [s.l.] : Elsevier Inc., 2018. p. 1–30. DOI: 10.1016/B978-0-12-811518-3/00001-6. Disponível em: http://dx.doi.org/10.1016/B978-0-12-811518-3/00001-6.

RODRIGUES, José Ariévilo Gurgel; DE ARAÚJO, Ianna Wivianne Fernandes; DE PAULA, Gabriela Almeida; LIMA, Ticiana de Brito; BESSA, Érika Façanha; BENEVIDES, Norma Maria Barros. Carragenana da epífita Hypnea musciformis obtida do cultivo experimental de Solieria filiformis em Flecheiras, Estado do Ceará, Brasil. **Acta Scientiarum - Technology**, [s. l.], v. 33, n. 2, p. 137–144, 2011. DOI: 10.4025/actascitechnol.v33i2.9096.

ROSSANO, R.; UNGARO, N.; D'AMBROSIO, A.; LIUZZI, G. ..; RICCIO, P. Extracting and purifying R-phycoerythrin from Mediterranean red algae Corallina elongata Ellis & Solander. **Journal of Biotechnology**, [s. l.], v. 101, n. 3, p. 289–293, 2003. DOI: 10.1016/S0168-1656(03)00002-6. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/S0168165603000026.

SAGUY, I. THERMOSTABILITY OF RED BEET PIGMENTS (BETANINE AND VULGAXANTHIN–I): INFLUENCE OF pH AND TEMPERATURE. **Journal of Food Science**, [s. l.], v. 44, n. 5, p. 1554–1555, 1979. DOI: 10.1111/j.1365-2621.1979.tb06488.x.

SAITO, Masatoshi; SAKAGAMI, Hiroshi; FUJISAWA, Seiichiro. Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). **Anticancer research**, [s. l.], v. 23, n. 6C, p. 4693—4701, 2003. Disponível em: http://europepmc.org/abstract/MED/14981915.

SALURI, Mihkel; KALDMÄE, Margit; ROSPU, Mihkel; SIRKEL, Hannes; PAALME, Tiina; LANDREH, Michael; TUVIKENE, Rando. Spatial variation and structural characteristics of phycobiliproteins from the red algae Furcellaria lumbricalis and Coccotylus truncatus. **Algal Research**, [s. l.], v. 52, n. August, p. 102058, 2020. DOI: 10.1016/j.algal.2020.102058. Disponível em: https://doi.org/10.1016/j.algal.2020.102058.

SALURI, Mihkel; KALDMÄE, Margit; TUVIKENE, Rando. Extraction and quantification of phycobiliproteins from the red alga Furcellaria lumbricalis. **Algal Research**, [s. l.], v. 37, n. December 2018, p. 115–123, 2019. DOI: 10.1016/j.algal.2018.11.013. Disponível em: https://doi.org/10.1016/j.algal.2018.11.013.

SAMPATH-WILEY, Priya; NEEFUS, Christopher D. An improved method for estimating R-phycoerythrin and R-phycocyanin contents from crude aqueous extracts of Porphyra (Bangiales, Rhodophyta). **Journal of Applied Phycology**, [s. l.], v. 19, n. 2, p. 123–129, 2007. DOI: 10.1007/s10811-006-9118-7.

SCOTTER, M. The chemistry and analysis of annatto food colouring: A review. **Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment**, [s. l.], v. 26, n. 8, p. 1123–1145, 2009. DOI: 10.1080/02652030902942873.

SENTHILKUMAR, Namasivayam; KURINJIMALAR, Chidambaram; THANGAM, Ramar; SURESH, Veeraperumal; KAVITHA, Ganapathy; GUNASEKARAN, Palani; RENGASAMY, Ramasamy. Further studies and biological activities of macromolecular protein R-Phycoerythrin from Portieria hornemannii. **International Journal of Biological Macromolecules**, [s. l.], v. 62, p. 107–116, 2013. a. DOI: 10.1016/j.ijbiomac.2013.08.004.

SENTHILKUMAR, Namasivayam; KURINJIMALAR, Chidambaram; THANGAM, Ramar; SURESH, Veeraperumal; KAVITHA, Ganapathy; GUNASEKARAN, Palani; RENGASAMY, Ramasamy. Further studies and biological activities of macromolecular protein R-Phycoerythrin from Portieria hornemannii. **International Journal of Biological Macromolecules**, [s. l.], v. 62, p. 107–116, 2013. b. DOI: 10.1016/j.ijbiomac.2013.08.004. Disponível em: http://dx.doi.org/10.1016/j.ijbiomac.2013.08.004.

SHEARN, Colin T.; FRITZ, Kristofer S.; THOMPSON, John A. Protein damage from electrophiles and oxidants in lungs of mice chronically exposed to the tumor promoter butylated hydroxytoluene. **Chemico-Biological Interactions**, [s. l.], v. 192, n. 3, p. 278–286, 2011. DOI: 10.1016/j.cbi.2011.04.005. Disponível em: http://dx.doi.org/10.1016/j.cbi.2011.04.005.

SIDLER, Walter A. The Molecular Biology of Cyanobacteria. *In*: BRYANT, Donald A. (org.). Dordrecht: Springer Netherlands, 1994. p. 139–216. DOI: 10.1007/978-94-011-0227-8_7. Disponível em: http://dx.doi.org/10.1007/978-94-011-0227-8_7.

SOUSA, Willer M. et al. Sulfated polysaccharide fraction from marine algae Solieria filiformis: Structural characterization, gastroprotective and antioxidant effects. **Carbohydrate Polymers**, [s. l.], v. 152, p. 140–148, 2016. DOI: 10.1016/j.carbpol.2016.06.111. Disponível em: http://dx.doi.org/10.1016/j.carbpol.2016.06.111.

STONE, Herbert; SIDEL, Joel. **Sensory Evaluation Practices**. 3rd Editio ed. [s.l.] : Academic Press 2004, 1973.

SUDHAKAR, M. P.; JAGATHEESAN, A.; PERUMAL, K.; ARUNKUMAR, K. Methods of phycobiliprotein extraction from Gracilaria crassa and its applications in food colourants. **Algal Research**, [s. l.], v. 8, p. 115–120, 2015. DOI: http://dx.doi.org/10.1016/j.algal.2015.01.011. Disponível em: http://www.sciencedirect.com/science/article/pii/S2211926415000235.

SUDHAKAR, M. P.; SARASWATHI, M.; NAIR, Beena B. Extraction , purification and application study of R-Phycoerythrin from Gracilaria corticata (J . Agardh) J . Agardh var . corticata. **Indian Journal of Natural Products and Resources**, [s. l.], v. 5, n. December 2014, p. 371–374, 2014.

SUN, Li; WANG, Shumei; GONG, Xueqin; ZHAO, Mingri; FU, Xuejun; WANG, Lu. Isolation, purification and characteristics of R-phycoerythrin from a marine macroalga Heterosiphonia japonica. **Protein Expression and Purification**, [s. l.], v. 64, n. 2, p. 146–154, 2009. DOI: 10.1016/j.pep.2008.09.013. Disponível em: http://linkinghub.elsevier.com/retrieve/pii/S1046592808002490.

TAKEO, Naoko et al. Cochineal dye-induced immediate allergy: Review of Japanese cases and proposed new diagnostic chart. **Allergology International**, [s. l.], p. 1–10, 2018. DOI: 10.1016/j.alit.2018.02.012. Disponível em: https://doi.org/10.1016/j.alit.2018.02.012.

THANGAM, Ramar; SUNDARRAJ, Shenbagamoorthy; VIVEK, Raju; SURESH, Veeraperumal; SIVASUBRAMANIAN, Srinivasan; PAULPANDI, Manickam; KARTHICK, S. Vignesh; RAGAVI, A. Sri; KANNAN, Soundarapandian. Theranostic potentials of multifunctional chitosan–silver–phycoerythrin nanocomposites against triple negative breast cancer cells. **RSC Adv.**, [s. l.], v. 5, n. 16, p. 12209–12223, 2015. DOI: 10.1039/C4RA14043E. Disponível em: http://xlink.rsc.org/?DOI=C4RA14043E.

UJU; DEWI, N. P. S. U. K.; SANTOSO, J.; SETYANINGSIH, I.; HARDINGTYAS, S. D.; YOPI. Extraction of phycoerythrin from Kappaphycus alvarezii seaweed using ultrasonication. **IOP Conference Series: Earth and Environmental Science**, [s. l.], v. 414, n. 1, p. 012028, 2020. DOI: 10.1088/1755-1315/414/1/012028.

WANG, Chun; SHEN, Zhaopeng; CUI, Xin; JIANG, Yanhui; JIANG, Xiaolu. Response surface optimization of enzyme-assisted extraction of R-phycoerythrin from dry Pyropia yezoensis. **Journal of Applied Phycology**, [s. l.], v. 32, n. 2, p. 1429–1440, 2020. DOI: 10.1007/s10811-019-01963-x.

WANG, Lu; WANG, Shumei; FU, Xuejun; SUN, Li. Characteristics of an R-Phycoerythrin with Two γ Subunits Prepared from Red Macroalga Polysiphonia urceolata. **Plos One**, [s. l.], v. 10, n. 3, p. e0120333, 2015. DOI: 10.1371/journal.pone.0120333. Disponível em: http://dx.plos.org/10.1371/journal.pone.0120333.

WU, Jikui; REN, Ningna; LU, Yunfei; JIA, Min; WANG, Ruinan; ZHANG, Junling. A poly (diallyldimethylammonium chloride)-mediated R-phycoerythrin/DNA hybrid system as a fluorescent biosensor for DNA detection. **Microchemical Journal**, [s. l.], v. 152, n. April 2019, p. 104314, 2020. DOI: 10.1016/j.microc.2019.104314. Disponível em: https://doi.org/10.1016/j.microc.2019.104314.

WU, Qiang; FU, Xiao-Ping Ping; SUN, Le-Chang Chang; ZHANG, Qian; LIU, Guang-Ming Ming; CAO, Min-Jie Jie; CAI, Qiu-Feng Feng. Effects of physicochemical factors and in vitro gastrointestinal digestion on antioxidant activity of R-phycoerythrin from red algae Bangia fusco-purpurea. **International Journal of Food Science & Technology**, [s. l.], v. 50, n. 6, p. n/a-n/a, 2015. DOI: 10.1111/ijfs.12775. Disponível em: http://doi.wiley.com/10.1111/ijfs.12775.

XU, Yaoyao; SHEN, Meng; CHEN, Yidan; LOU, Yeqing; LUO, Ruichao; CHEN, Jiajia; ZHANG, Yongjun; LI, Jia; WANG, Weimin. Optimization of the polysaccharide hydrolysate from Auricularia auricula with antioxidant activity by response surface methodology. **International Journal of Biological Macromolecules**, [s. l.], v. 113, p. 543–549, 2018. DOI: 10.1016/j.ijbiomac.2018.02.059.

XU, Yifeng; WANG, Quanfu; HOU, Yanhua. Efficient Purification of R-phycoerythrin from Marine Algae (Porphyra yezoensis) Based on a Deep Eutectic Solvents Aqueous Two-Phase System. **Marine drugs**, [s. l.], v. 18, n. 12, p. 1–14, 2020. DOI: 10.3390/md18120618.

YU, Rong; MANDLEKAR, Sandhya; KONG, A. N. Ton. Molecular mechanisms of butylated hydroxylanisole-induced toxicity: Induction of apoptosis through direct release of cytochrome c. **Molecular Pharmacology**, [s. l.], v. 58, n. 2, p. 431–437, 2000. DOI: 10.1124/mol.58.2.431.

ZANG, Fan; QIN, Song; MA, Chengbo; LI, Wenjun; LIN, Jian. Preparation of high-purity R-phycoerythrin and R-phycocyanin from Pyropia yezoensis in membrane chromatography. **Journal of Applied Phycology**, [s. l.], v. 32, n. 5, p. 3411–3418, 2020. DOI: 10.1007/s10811-020-02208-y.

ZEPEDA, Enrique; FREILE-PELEGRÍN, Yolanda; ROBLEDO, Daniel. Nutraceutical assessment of Solieria filiformis and Gracilaria cornea (Rhodophyta) under light quality modulation in culture. **Journal of Applied Phycology**, [s. l.], v. 32, n. 4, p. 2363–2373, 2020. DOI: 10.1007/s10811-019-02023-0.

ZHAO, Mingri; SUN, Li; SUN, Shichun; GONG, Xueqin; FU, Xuejun; CHEN, Min. Phycoerythrins in phycobilisomes from the marine red alga Polysiphonia urceolata. **International Journal of Biological Macromolecules**, [s. l.], v. 73, n. 1, p. 58–64, 2015. DOI: 10.1016/j.ijbiomac.2014.11.004. Disponível em: http://dx.doi.org/10.1016/j.ijbiomac.2014.11.004.

ZHAO, Ping; WANG, Xulei; NIU, Jianfeng; HE, Linwen; GU, Wenhui; XIE, Xiujun; WU, Mingjiang; WANG, Guangce. Agar extraction and purification of R-phycoerythrin from Gracilaria tenuistipitata, and subsequent wastewater treatment by Ulva prolifera. **Algal Research**, [s. l.], v. 47, n. February, p. 101862, 2020. DOI: 10.1016/j.algal.2020.101862. Disponível em: https://doi.org/10.1016/j.algal.2020.101862.