UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS FARMACÊUTICAS

DANILO COSTA GERALDES

C-ficocianina: influência da histidina e sacarídeos em sua obtenção como matéria prima e formulação gastrorresistente a base de alginato

C-phycocyanin: influence of histidine and saccharides in its obtaining as a raw material and gastro-resistant alginate based formulation

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Tese apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciências, na área de Fármacos, Medicamentos e Insumos para a Saúde.

Orientadora: Profª Drª Laura de Oliveira Nascimento

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Se vi mais longe,

foi por estar sobre o ombro de gigantes.

Sir Isaac Newton

RESUMO

O câncer de colorretal atinge mais de 40 mil pessoas por ano apenas no Brasil, que impulsiona a procura por tratamentos alternativos ou procedimentos preventivos. Entre as alternativas exploradas está a proteína C-ficocianina (CPC), proveniente da cianobactéria Spirulina. Além de suas comprovadas ações antioxidante, anti-inflamatória e imunomoduladora, estudos demonstraram sua atividade antiproliferativa em células de adenocarcinoma colorretal humano. No entanto, a CPC pode perder estrutura e função dependendo do pH, força iônica e calor, além de apresentar instabilidade em meio gástrico. Sua estabilidade pode ser favorecida por meio de excipientes como tampões para controle de pH, lioprotetores para liofilização e armazenamento em forma sólida, ou ainda adjuvantes nos processos de extração e purificação. Porém, até o momento, não encontramos estudos estruturados de formulações de CPC liofilizadas, nem sobre o efeito do tampão histidina como veículo e adjuvante de extração para esta molécula. Portanto, neste trabalho otimizamos a extração de CPC da Spirulina por diferentes tampões e modos de extração, seguida de purificação e posterior formulação com sacarídeos para liofilização sob diferentes parâmetros de ciclo. Para formulação via oral, otimizamos carreadores particulados a base de alginato, no intuito de obter liberação em meio entérico da proteína. As etapas de extração e purificação inicial foram otimizadas por um projeto fatorial 2x3, resultando em uma extração otimizada em tampão de histidina com uma pureza máxima de 0,79 (grau alimentício). A etapa de polimento por cromatografia de exclusão de tamanho e troca iônica resultou em CPC de pureza 3,41 (grau reagente). Os estudos de liofilização resultaram em uma formulação otimizada de CPC em tampão de histidina e sacarose sob ciclo de secagem abaixo da temperatura de transição vítrea (ciclo conservador). Mini-cápsulas e wafers de alginato liofilizados gastrorresistentes foram produzidos e caracterizados de acordo com parâmetros físico-químicos, teor de CPC e perfil de liberação, sem que tenha havido influência no teor da proteína, demostrando uma proteção significativa da CPC em ambiente ácido e podendo ser a base de matrizes de polissacarídeos para entregar essa proteína ao intestino. Por fim, foi desenvolvido um processo para a obtenção de ficocianina liofilizada e encapsulada capaz de ser utilizada em estudos futuros *in vivo* e *in vitro* para averiguar seu potencial no tratamento e/ou prevenção do câncer colorretal.

Palavras Chaves: C-ficocianina, purificação, liofilização, *drug delivery*, mini cápsulas de alginato, wafers de alginato

ABSTRACT

For 2022, 41,010 new colorectal cancers are expected to surge in Brazil alone. Given the high morbidity and mortality of the disease, the search for alternative treatments or preventive procedures is constant. Among the alternatives explored is the protein C-phycocyanin (CPC), from the cyanobacterium Spirulina. In addition to its proven antioxidant, anti-inflammatory and immunomodulatory actions, studies have demonstrated antiproliferative activity in human colorectal adenocarcinoma cells. However, CPC purified in solution can lose structure and function depending on pH, ionic strength and heat, and its possible therapeutic effect is hampered by its instability in the gastric environment. In order to preserve its characteristics, formulation techniques can be used: use of buffers to control pH and adjuvant in the extraction and purification processes, freeze-drying for its preservation and carriers to improve its transport. However, to date, we have not found structured studies of freeze-dried CPC formulations and process parameters nor the effect of the histidine buffer as a vehicle and extraction adjuvant for this molecule. Therefore, in this work we optimized the extraction of CPC from Spirulina by different buffers and extraction modes, followed by purification, subsequent formulation with saccharides for freeze-drying under different cycle parameters and finally the use of alginate-based carriers, pellets, as a form of transmission and release in enteric medium of the protein. The extraction and initial purification steps were optimized by a 2x3 factorial design, resulting in an optimized extraction in histidine buffer with a maximum purity of 0.79 (food grade). The additional polishing step achieved by size exclusion and ion exchange chromatography resulted in a CPC formulation of purity 3.41 (reactant grade). Freeze-drying studies resulted in an optimized formulation of CPC in histidine and sucrose buffer after a conservative freeze-drying cycle. As a form of vehicle, gastro-resistant freeze-dried pellets and alginate wafers were produced and characterized according to physicochemical parameters, CPC content and release profile, without any influence on the protein content, showing a significant protection of CPC in an acid environment and could be the basis of polysaccharide matrixes to deliver this protein to the intestine. Finally, a process was developed to obtain freeze-dried and encapsulated phycocyanin capable of being used in future studies in vivo and in vitro to investigate its potential in the treatment and/or prevention of colorectal cancer.

Keywords: C-phycocyanin, purification, freeze-drying, drug delivery, alginate pellets, alginate wafers

SUMÁRIO

INTRODUÇÃO

1. O Câncer

No Brasil, apesar de o maior número de casos ser de câncer de pele, outros tipos de tumores malignos vêm crescendo significativamente, como o câncer colorretal, que é o segundo tipo de câncer mais incidente em mulheres e o terceiro mais incidente em homens. As expectativas do Instituto Nacional do Câncer (INCA) para 2022 são de 41.010 novos casos[1].

Localizado nas camadas internas do intestino grosso (colon), no reto e/ou no ânus, seu diagnóstico precoce é dificultado pois a maioria dos acometidos não apresentam sintomas ou, quando apresentam, estes são inespecíficos, tais como dores abdominais, alteração do ritmo intestinal e presença de sangue nas fezes. Além disso, os métodos de diagnóstico precoce também são desfavoráveis, baseados em técnicas invasivas e visuais (colonoscopia e retrosigmoidoscopia), anteriores à biópsia. A relevância é tal que a OMS preconiza o rastreamento periódico desse câncer em todos os adultos acima de 50 anos[2,3].

O tratamento consiste em terapia multifatorial, ou seja, cirurgia para retirar pólipos ou tumores; retirada de seções do cólon (casos mais graves); quimioterapia em caso de metástase nos linfonodos próximos e radioterapia em metástases no fígado ou órgãos distantes[4]. Em caso de metástase, o tratamento quimioterápico vária conforme o perfil genético do paciente, sendo que em 35-40% dos casos há a presença de mutações KRAS ou NRAS, as quais terapias direcionadas eficazes ainda não estão disponíveis[5].

2. A proteína C-ficocianina e sua aplicação na terapia anti-tumoral

Considerada "*The Wonder Food of 21st Century*", a cianobactéria Spirulina (Arthrospira plantesis) tem destaque pelas suas diversas utilizações e propriedades[6]. O uso da Spirulina se estende por séculos em diversas comunidades indígenas, seja na alimentação, medicina, perfumaria, agricultura, entre outras; sua maior utilização atual é como suplemento alimentar e corante alimentício[6,7].

A proteína C-ficocianina (*C-phycocyanin*, CPC, Figura 1) é a principal responsável pela captação de luz que possibilita a fotossíntese na Spirulina[8,9]. Sua estrutura é composta por uma cadeia alfa (massa molecular de 17.601 kDa, ponto pI 5,8315) e uma cadeia beta (massa molecular de 18.093 kDa, ponto pI 4,9616) organizadas em hexâmeros (3 subunidades alfa e 3 subunidades beta) que podem agregar formando heterododecaedros[10,11]. Vale notar que seu forte tom azulado está diretamente ligado à sua conformação estrutural, com a perda de estrutura ocasionando uma perda de tonalidade em casos como mudanças de pH e desnaturação por ureia[12].

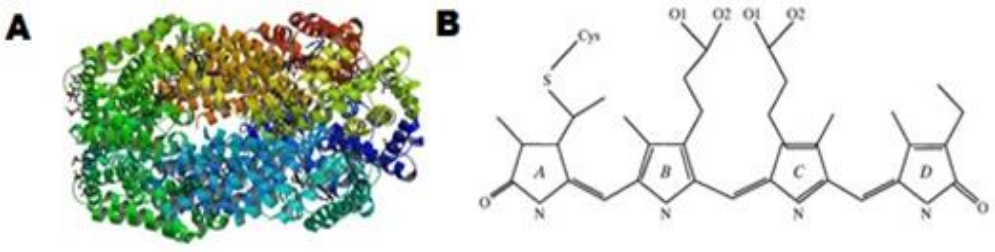

Figura 1 - A) Representação da estrutura cristalina da CPC, que se organiza em hexâmeros compostos de heterodímeros αβ[8]. B) Estrutura química do tetrapirrol linear ficocianobilina (PCB), cromóforo da CPC[9].

A CPC possui diversas ações biológicas descritas: relaxamento de vasos sanguíneos, diminuição da concentração de lipídios no sangue, proteção hepato-renal, imunomodulação, atividade anti-oxidante e anti-inflamatória[8,13–15]. Além dessas funções, estudos demonstram que a CPC possui atividade anticancerígena no câncer colorretal. Apesar do seu mecanismo de ação para tal atividade não estar claro, uma série de eventos biológicos foram observados:[16–24]

- A afinidade da CPC pelo s*cavenger receptor-A*, receptor super expresso em macrófagos associados a tumores (TAMs), propicia acumulo da proteína em ambiente tumoral [16];

- O efeito pró-apoptótico da CPC, observado no tratamento de células de câncer de mama MBA-MD-231, foi correlacionado a ativação mitocondrial/citocromo C, inibição da ciclooxigenase-2 (COX-2) e a produção de prostaglandina E(2), resultando na morte de células tumorais enquanto não apresentou toxicidade a células não malígnas[17–19];

- Seu efeito anti-proliferativo no tratamento de células de adenocarcinoma de colón humano (HT-29) foi correlacionado ao bloqueio do ciclo celular nas fases G0/G1 e consequente bloqueio da síntese de DNA[13];

- Sua ação no trato-gastrointestinal de camundongos com câncer associado a colite provocou regulação da microbiota intestinal e ativação da via de sinalização interleucina-7 (IL-7) em células epiteliais [22];

- Seu efeito em células de adenocarcinoma de colón humano e consequente inibição do câncer colorretal pela via angiogênica, com efeito anti-inflamatório [20,21]. Tal efeito foi relacionado à inibição seletiva da enzima COX-2[23] e pela indução da enzima heme-oxygenase-1 (Hmox1) via seu cromóforo tetrepirrólico[15,24].

3. Carreadores a base de alginato

Sistemas carreadores são frequentemente utilizados para o aumento na eficiência, diminuição de toxicidade e proteção de substâncias de interesse farmacológico. Quando estruturados visando um alvo farmacológico e uma rota de administração adequados, tais carreadores podem possibilitar o *drug delivery* eficaz de biofármacos[25–27].

Entre os carreadores, os *pellets* ou mini cápsulas de alginato tem se mostrado promissores devido a seu reduzido potencial alergênico, produção sustentável, livre de produtos animais e reprodutibilidade na produção[28–31]. Por serem macroscópicos, encapsulam facilmente moléculas de alto peso molecular como proteínas[28,32,33].

Os alginatos são derivados de algas marinhas da classe *Phaeophycea*[34], classificados como hidrocoloides por sua capacidade de serem solúveis em água e formarem coloides de alta viscosidade na presença de metais alcalinos tais como Mg2+, Ca2+, Sr2+ e Ba2+ [35,36]. Em seu estado desprotonado e em pH abaixo de 5,

as regiões dos alginatos concentradas em ácido gulurônico (e com baixa concentração de ácido manurônico) são capazes de se ligar a metais alcalinos formando pontes salinas divalentes e consequentemente longas cadeias poliméricas (Figura 2)[37,38]. O processo de encapsulação utilizando alginato também é considerado um processo verde, que leva em conta a sustentabilidade da cadeia produtiva. As características desse processo incluem a eliminação ou redução de solventes orgânicos e produtos químicos de alto custo ambiental, redução de resíduos de produção, entre outras medidas[39–41].

Por fim, carreadores livres de produtos de origem animal e fabricados sob processos verdes têm alta demanda de mercado e poucas opções disponíveis, o que enfatiza a importância das escolhas do projeto[42,43].

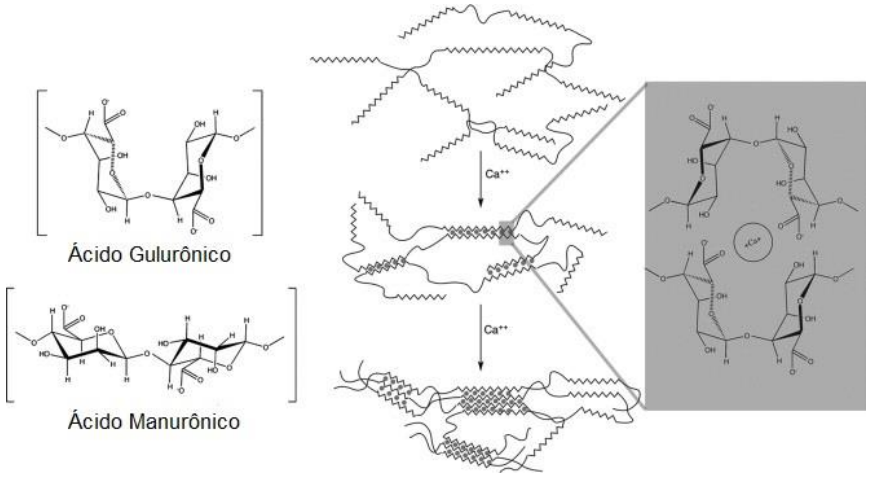

Figura 2 - Estrutura química das unidades monoméricas do alginato e representação de pontes de sal divalentes entre tais estruturas e cálcio. Adaptado de Lee e Rogers et al[38].

4. Desenvolvimento de formas farmacêuticas liofilizadas

Proteínas de interesse terapêutico são formuladas em sua maioria em diluentes aquosos, e podem ser desnaturadas por aquecimento ou resfriamento rápidos, mudanças de pH, agitação, exposições a agentes externos, hidrólise e deaminação[44]. Outras mudanças estruturais também podem ocorrer como a isomerização, formação e quebra de pontes dissulfeto e deglicosilação[45]. Para evitar os problemas mediados pelo ambiente aquoso, as estratégias mais comuns são adição de excipientes e retirada do solvente[46].

A liofilização é o processo de secagem mais comum de biofármacos, constituído de três etapas: congelamento, secagem primária e secagem secundária[47].

O processo inicial de congelamento tem como finalidade imobilizar o produto a ser liofilizado, interrompendo ou diminuindo reações químicas/bioquímicas e interações físicas que possam ocorrer na formulação. O material previamente congelado é desidratado por sublimação de solvente em baixas temperaturas e vácuo (Figura 3). A secagem primária retira a água livre enquanto a secagem secundária sublima a água ligada às moléculas do produto ou entremeadas na matriz[48].

Figura 3 - Ilustração do Processo de Liofilização. Imagem adaptada do inglês[49].

Visando a optimização do processo de liofilização um dos parâmetros cruciais é a medida das temperaturas críticas das formulações[50]. Durante a secagem primária, a matriz congelada pode se liquefazer dependendo da temperatura utilizada, acarretando em seu colapso e possível perda de propriedades estruturais e funcionais [51]. Tais temperaturas, quando baseadas em técnicas termo analíticas, são denominadas de temperatura de transição vítrea (*prime glass transition temperature*, Tg') para matrizes amorfas e temperatura eutética (*eutetic temperature*, Te) para matrizes cristalinas[52]. Outra técnica também comumente utilizada é a microscopia de liofilização, que se baseia na simulação de um processo de liofilização para a visualização do colapso da matriz congelada e sua consequente temperatura de colapso (*collapse temperature*, Tc).[53]

Devido a energia e tempo necessários para o processo, com ciclos que podem durar dias, a liofilização pode ser um processo custoso[54,55]. No passado a prioridade máxima era a preservação da estrutura e função das proteínas liofilizadas, utilizando-se de temperaturas inferiores às Tg'/Te e maiores tempos de ciclos[56,57]. Recentemente o estudo de parâmetros visando a diminuição do tempo necessário e consequentemente o uso de maiores temperaturas durante o processo vem se

tornando comuns, tornando os ciclos mais agressivos e a escolha de excipientes visando matrizes amorfas capazes de preservar as formulações em condições mais drásticas.[54,55,58]

Entretanto, potencial problema do processo é a desnaturação de proteínas pelo congelamento e/ou pressão osmótica gerada pela desidratação[59]. Variações de pH, sobretudo pelo fenômeno de cristalização seletiva de tampões como PBS e Tris-HCl, e a velocidade inadequada no congelamento podem levar à formação de agregados[60,61]. Alguns aditivos podem cristalizar ou vitrificar durante a etapa de congelamento, acarretando na formação de uma massa liofilizada heterogênea, mudanças de pH e colapso da amostra.

5. A escolha de excipientes para o processo de liofilização

Visando o sucesso do processo e formulação, estudos de pré-formulação são realizados no desenvolvimento de formas farmacêuticas estáveis e eficazes. São estudadas as características físico e químicas dos fármacos e excipientes isolados e combinados, atendendo às exigências de qualidade de um produto inovador para a produção industrial[62,63].

Excipientes podem prevenir a desnaturação de proteínas devido ao estresse do processo e garantir uma longa vida útil[64]. Em uma formulação liofilizada os excipientes mais comumente utilizados divididos em sua função são:

- Agentes tamponantes: responsável pelo controle do pH da formulação e podem influenciar estruturas terciárias e quaternárias ligando-se a sítios específicos. Exemplos: tampão PBS, tampão histidina e tampão citrato.[65–67];

- Surfactantes: moléculas capazes de reduzir a tensão superficial podendo evitar a adsorção de proteínas aos seus receptores, agregação durante as etapas de processamento e formulação, ajudar a redobrar a proteína durante o descongelamento, inibir as interações proteína-proteína e reduzir a agregação consequente. Exemplos: polissorbatos, poloxâmeros e dodecil sulfato de sócio[66,68];

- Sais isotônicos: são usados para isotonicidade ou estabilidade/função de proteínas. Soluções isotônicas são exigidas por algumas aplicações após a reconstituição da formulação liofilizada, mas a isotonicidade pode ser alcançada com um líquido de ressuspensão, como soro fisiológico. Exemplo: cloreto de sócio[66,69];

- Diluentes: utilizados para aumentar a massa e a resistência mecânica, além de proporcionar estabilidade física, estabilização química e boa aparência para o produto. A utilização de diluentes é especialmente valiosa para formulações de baixa dosagem. Exemplos: sacarose, manitol[66,69];

- Lioprotetores: excipientes que estabilizam proteínas durante a fase de congelamento e secagem [66,70]. Três teorias foram propostas para explicar seu mecanismo de ação: a teoria da reposição de água, na qual os lioprotetores substituem a água adsorvida às proteínas[71] ; a teoria cosmotrópica, nos quais tais protetores são capazes de estabilizar a estrutura do volume de água durante o congelamento[72]; e a teoria da vitrificação, na qual tais excipientes formam uma matriz amorfa com as proteínas, impedindo a sublimação da água de ligação[73]. Vale ressaltar que nenhuma teoria exclui a outra, e um protetor pode ter mais de um mecanismo[71]. Exemplos: sacarose, trealose, dextran[66].

OBJETIVOS

Esse trabalho visou otimizar a obtenção de CPC em pó por extração da Spirulina em diferentes tampões e modos de extração, seguida de purificação e posterior liofilização sob diferentes parâmetros de ciclo e lioprotetores. A otimização também se projetou a formulação em carreadores particulados a base de alginato visando estabilidade proteica e liberação em meio entérico da proteína.

Objetivos Específicos

Os objetivos específicos deste trabalho foram divididos em dois capítulos:

Capítulo 1: *Histidine and HP-beta-cyclodextrin enhancements on extraction, thermal stability and freeze-drying parameters of C-Phycocyanin* (artigo submetido no periódico *Biotechnology Progress*).

- Avaliação da extração e purificação de CPC proveniente da cianobactéria *Arthrospira plantesis* em diferentes tampões e modos de extração;
- Realização de estudos de pré-formulação, com foco na compatibilidade excipiente-bioativo e resistência a tratamentos térmicos;
- Estudo do processo de liofilização em condições de ciclo agressivo e conservador com diferentes lioprotetores de CPC;

Capítulo 2: *Short communication: Alginate-based sustainable dosage forms for intestinal delivery of phycocyanin*

- Incorporação de CPC em sistemas particulados e monolíticos a base de alginato, incluindo a avaliação do respectivo processo de liofilização;
- Avaliação dos parâmetros físico-químicos e de manutenção da estrutura proteica pós liofilização da CPC incorporada.

CAPÍTULO 1) HISTIDINE AND HP-BETA-CYCLODEXTRIN ENHANCEMENTS ON EXTRACTION, THERMAL STABILITY AND FREEZE-DRYING PARAMETERS OF C-PHYCOCYANIN

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ABSTRACT

The protein C-phycocyanin (CPC), extracted rom the cyanobacterium Spirulina (*Arthrospira plantesis*), has several biological effects. Between them, it can modulate inflammation and exerts a pro-apoptotic effect on breast cells and anti-proliferative effect on colon adenocarcinoma cells. However, purified CPC, as other proteins, may destabilize depending on pH, ionic strength and heat. Buffers can control pH shifts and freeze-drying may preserve its characteristics but, to the present date, we found no structured studies of freeze-drying CPC formulations and process parameters nor the effect of histidine buffer as vehicle and extraction adjuvant for this molecule. Therefore, in this work we optimized CPC extraction from Spirulina by different buffers and extraction modes, followed by purification and further formulation with saccharides for freeze-drying under different cycle parameters. The extraction and initial purification steps were optimized by a 2x3 factorial design, resulting in an optimized overnight extraction in histidine buffer and purification by salting out with ammonium sulfate 40% m/w resulted in a maximum purity of 0.79 (food grade). Further polishing step achieved by size exclusion and ion exchange chromatography resulted in a CPC formulation of 3.41 purity (reagent grade). The liquid formulations could not maintain CPC proprieties under heat, and the freeze-dried studied resulted in an optimized formulation in histidine buffer and sucrose after a conservative freeze-drying cycle.

Keywords: C-phycocyanin, extraction, purification, freeze-drying, factorial-design

1. INTRODUCTION

The protein C-phycocyanin (CPC) is the main responsible for the capture of light that enables photosynthesis by the Spirulina[8,9]. Its structure is composed of an alpha chain (molecular mass of 17,601 kDa) and a beta chain (molecular mass of 18,093 kDa), organized in hexamers (3 alpha subunits and 3 beta subunits) that can aggregate forming heterododecahedrons[10,11]. Its strong bluish tone correlates to its structural conformation, with the loss of structure causing a loss of hue in cases such as pH shifts and denaturation by urea[12].

CPC has several biological actions described: relaxation of blood vessels, decrease in blood lipids, hepato-renal protection, immunomodulation, anti-oxidant and anti-inflammatory [8,13,14]. In addition to these functions, CPC has an affinity for the receptor-A scavenger, which is overexpressed in tumor-associated macrophages (TAMs) and can promote protein accumulation in tumor microenvironment [16]. Its pro-apoptotic effect was observed for MBA-MD-231 breast cancer cells by mitochondrial / cytochrome C activation and the production of reactive oxygen species that result in cell death [17–19]. The anti-proliferative effect was also observed in the treatment of human colon adenocarcinoma cells (HT-29), blocking cell cycle in the G0 / G1 phases and consequent blocking of DNA synthesis[13] and inhibition of proliferation was related to COX-2 enzyme, which participates in the angiogenic pathway[20,21].

CPC extraction procedures varies according to the condition of the raw Spirulina used, with different protocols and yields for dried and wet biomass[74,75]. Techniques include freezing and thawing, buffer extraction, organic and inorganic acid extraction, comminution by mortar and pestle followed by ultrasonic treatment and use of lysozyme [74,76,77]. The extraction procedures normally occur in distilled water or phosphate buffer. However, water may not protect the protein from pH shifts and phosphate buffer complicates further freeze drying due to problems such as partial crystallization and lower collapse temperature [56,78].

CPC is commonly purified[79–81] and formulated in PBS buffer[82], however it can undergo heterogenic crystallization during the freezing step and cause localized pH changes[56]. As an alternative, histidine based buffers can surpass other buffering systems due to its acidic-neutral pKa (6.0), providing less pain due to the subcutaneous injection (when compared to citrate), no sublimation under freeze-drying conditions (as

acetate, which leads to pH changes) and the possibility to serve as a hydrogen donor to maintain intramolecular β-sheet structures[83].

To improve its long-term stability, spirulina may be dried by an array of techniques such as freeze-drying, spray-drying, oven-drying, atmospheric drying and solar drying [84,85]. However, each method has its limitations: CPC thermal instability limits heating techniques that may cause protein loss, with freeze-drying presenting the least amount of lost material but at an increased cost for large scaling processing [77,86,87]. Purified CPC is also dried to preserve its characteristics and avoid loss of material [88,89]; however, to the present date, we found no studies on freeze-drying parameters to dry CPC.

Thermal stability can also be achieved by the use of appropriated excipients[90]. For CPC formulations, sucrose already demonstrated potential thermal protection under higher temperatures for industrial applications[91–93] besides its well-known preservation of protein's structure during freeze-drying[78,94]. Other promising excipient, specially studied for aggressive cost-effective freeze-drying process, is the cyclodextrin 2-hydroxypropyl-betacyclodextrin (HPBCD)[55].

Considering the above, this work aimed to explore CPC extraction and purification methods from dried Spirulina biomass to evaluate histidine buffer as an extraction media compared with commonly used PBS buffer and the stability of its freeze-dried formulation under different cycle conditions and excipients such as sucrose and HPBCD. This buffer is recently applied in freeze-dried protein formulations, presenting higher collapse temperature and no reported events of partial crystallization upon freezing, a common event when using PBS [56,78]. Thus, stability of CPC under heating and freeze-drying will also be studied under histidine influence.

2. METHODS

2.1 Materials

Biological material: dry extract of *Spirulina platensis* contained 60% Spirulina (Pharmanostra), reagent grade C-phycocyanin was from Taizhou Binmei Biotechnology Co., Ltd, Taozhou, China. Reagents: sodium phosphate, sodium chloride, L-histidine and sucrose were purchased from Vetec, Brazil; Hydroxypropyl-β-cyclodextrin (HP-β-CD), Comassie Blue R25, Acrylamide (99% purity) were purchased from Sigma-Aldrich.

2.2 CPC Obtention

2.2.1 Extraction and Purification

Phycocyanin was macerated and extracted with buffer (160 mg/mL), followed by ammonium sulfate purification. A factorial design 2^3 with central point in duplicate was performed to optimize these steps. The chosen inputs were buffer (histidine buffer 5 mM pH 6 or sodium phosphate buffer, PBS, 50 mM pH 7), extraction mode (freeze- thaw or overnight) and ammonium sulfate concentration (40%, 60% and 80% w/v) for initial purification by salting out. The evaluated outputs were: CPC concentration (mg/mL); purity, purification factor and yield (%).

The overnight procedure consisted of keeping the solution at 4ºC for 18h and the freeze-thaw procedure was performed by three freezing (15 minutes at -20ºC) and thawing (15 minutes at room temperature) cycles with 2h at 4ºC in the end of the procedure. After both extraction modes, cell debris were removed by centrifugation for 15 min / 10,000 g / 4ºC, the supernatant subsequently collected and submitted to purification with ammonium sulfate until saturation between 40% and 80% w/v. The mixture was kept at 25ºC (room temperature) for 18h, followed by centrifugation for 30 min/12,000 g/ room temperature. The pellet was collected, resuspended in PBS pH 7.0, and centrifuged again for 15 min / 12,300 g / 4ºC. After that, the supernatant was dialyzed overnight at 4ºC in a 14 kDa pore size membrane (Spectrum™ dialysis membrane tubing, 14,000 Dalton MWCO).

2.2.2 Polishing

The dialyzed CPC was purified by size exclusion chromatography (SEC, Superdex 200 Increase 10/300 GL) and ion exchange chromatography (IEC, 5 mL Hi-

Trap DEAE Sepharose Fast Flow anionic column, GE Health Sciences). The column was equilibrated with 35 mL (7 volumes) of Tris-HCl 20 mm buffer pH 6.5. Then, 5 mL of the sample was placed on the column, running in a linear gradient of Tris-HCl 20 mm buffer (pH 6.5) with NaCl gradient concentration (20% to 50%) and flow rate of 5 mL/min. Samples were collected in 1 mL fractions and tested for CPC concentration and total protein content. The fractions with higher purity were concentrated and buffer exchanged (5 mM Histidine buffer, pH 6,0) by ultrafiltration with a 10 KDa Amicon tube (3000 rpm / 15 min / 4ºC).

2.3 Freeze-drying Formulation

2.3.1 Optimization

A factorial design 2^3 was performed to optimize the formulation of choice. The chosen inputs were buffer (histidine buffer 5 mM pH 6 or sodium phosphate buffer, PBS, 50 mM pH 7), lyoprotector (sucrose 5% m/w or 2-Hydroxypropyl-β-cyclodextrin, HPBCD, 5% m/w) and freeze-drying cycle (aggressive or conservative). PBS and sucrose were chosen based on common choices in the freeze-drying of biological formulations[46,78,95] and the freeze-drying cycle in our past works[96,97]. Cyclodextrin came as an option to perform a more aggressive cycle for process optimization[54,55].

2.3.2 Differential Scanning Calorimetry (DSC)

The CPC formulations were weighted in an aluminum sample holder by a microanalytical balance (Mettler Toledo, model MX5, Schwerzenbach, Switzerland). The glass transition temperature (Tg' and Tg) were determined by DSC (Differential Scanning Calorimetry – Mettler Toledo, model DSC1, Schwerzenbach, Switzerland). The blank (empty sample holder) was automatically discounted in each analysis. The analyses were carried out in a nitrogen atmosphere, at a flow of 50 mL/min, in different temperature ranges.

For the Tg' of the CPC solutions, the samples were cooled up to -60 ºC from room temperature at 10 ºC/min and after 5 minutes heated back to room temperature at 5ºC/min. For the Tg of the freeze-dried solutions, the samples were heated up from -20ºC up to 70°C at 10 °C/min.

2.3.3 Freeze-drying

The formulations were freeze-dried in clear glass vials with rubber stoppers. The process was carried out in the Lyostar 3 freeze-drier (SP scientific), which contains shelves with computational pressure control and temperature ramps. Two freeze-drying cycles were proposed and named “aggressive” and “conservative”. Aggressive: the samples were frozen at 0.5ºC/min up to -35ºC, with a holding time of 360 minutes. Afterwards, the samples were heated at 0.187ºC/min up to 35ºC at 100 mTorr, with a holding time of 12h. The primary drying occurred during the heating ramp and the secondary drying during the holding time at 35ºC. Conservative: the samples were frozen at 0.375ºC/min up to their respective Tg’, with a holding time of 360 minutes. Primary drying occurred at 100 mTorr until the lost of drying signal by the equipment pirani valve. Afterwards, the samples were heated at 0.2ºC/min up to 35ºC with a holding time of 24h (secondary drying).

2.3.4 Visual Analysis

The Visual Analysis considered shape, color, uniformity, pre- and post-freeze-drying volume, texture and possible residues of the freeze-dried product.

2.3.5 Reconstitution Time

To test the time taken for the reconstitution of the freeze-dried samples, distilled water was injected into each vial with a syringe and gently agitated by hand. Complete reconstitution is considered when the samples fully dissolved, with no precipitated material detected.

2.3.6 Residual Moisture

Freeze-dried products were submitted to thermogravimetric analysis (TGA-50M, Shimadzu, Japan) to determine residual moisture. Accurately weighted dried samples (around 10 mg) were heated at 10 ºC/min from 25 up to 300 ºC, under nitrogen atmosphere at a flow rate of 50 mL/min. Residual moisture was determined by stable weight-loss (%) at temperature around 100 ºC.

2.3.7 Circular Dichroism (CD)

The formulations were analyzed by circular dichroism (CD) spectroscopy in order to confirm their secondary structure integrity. Data were collected using equipment available at Spectrometry and Calorimetry Laboratory - LNBio/CNPEM – Campinas SP (LEC). The CD spectra in the far UV region (190-260 nm) were measured on a JASCO J-815 (JASCO) spectropolarimeter equipped with a Peltier

module for temperature control, using a quartz cuvette with a 1 mm optical path. The concentration of phycocyanin protein in different forms of lyophilization was 0.1 mg/mL dissolved in ultrapure water, each spectrum obtained was the result of 20 accumulations measured at 20 °C. The measurement data were treated using the equation 1 (Eq. 1):

Eq. 1) $$[\Theta] = \frac{\Theta * 100 * M}{C * I * n}$$

Where Ɵ is the ellipticity in degrees, l is the optical path in cm, C is the concentration in mg/ml, M is the molecular mass and n is in the number of residues in the protein. The mean residue molar ellipticity [Ɵ] is given in deg.cm^2.dmol^{-1} [98].

2.4 C-Phycocyanin Characterization

2.4.1 Concentration, Purity, Purification Factor and Yield

The CPC concentration was determined by spectrophotometry at 280nm, 620nm and 652nm and the respective optical densities (O.D.) used in equation 2 (Eq. 2) by Bennet and Bogorad[99] . Purity was calculated according to equation 3 (Eq. 3), the Purification Factor according to equation 4 (Eq. 4) and yield according to equation 5 (Eq. 5):

Eq. 2) CPC concentration = (O.D. at 620nm - 0.474 x O.D. at 652nm) / 5.34

Eq. 3) Purity (%) = O.D. at 620nm / O.D. at 280nm

Eq. 4) Purification Factor = CPC Purity / Pre-Purification CPC Purity

Eq. 5) Yield (%) = Conc. CPC / Conc. Pre-Precipitation CPC

2.4.2 Native and Sodium dodecyl sulfate–polyacrylamide (SDS) gel electrophoresis

A 12% polyacrylamide gel was casted as described by Julianti et al[100]. Gels were loaded in a Biorad electrophoresis chamber coupled with a power supply, running happened under non-denaturing (native) and denaturing (SDS) conditions to confirm the purity of CPC. The bands were visualized by Coomassie blue staining. Molecular weight of proteins in the sample were estimated with Precision Plus Protein Dual Color Standard (Bio-rad).

2.4.3 Thermal Stability

To evaluate CPC thermal stability the formulations in histidine and PBS buffer added or not of sucrose or HPBCD (both at 5 % m/w) as lyoprotectors were

heated in a thermocycler (Bio-Rad T100) at 70ºC for 30, 60, 90 and 120 minutes. The CPC concentration and purity of the samples were determined by spectrophotometry.

2.5 Statistical analysis

The factorial experiments were defined, randomized and analyzed by Minitab 17, using the inputs and outputs previously described. The significance of the outputs effects caused by input variation were evaluated by Analysis of variance (ANOVA) in a stepwise regression analysis at 95% confidence level ($\alpha = 0.05$). The generated equations were evaluated for lack of fit; the residuals were checked for normal distribution and constant variance. Effects were plotted as pareto charts (standardized effects) to evaluate the significant ones and as "main effects" charts to examine differences between level means for one or more factors.

3. RESULTS AND DISCUSSION

3.1 C-phycocyanin Extraction and purification

CPC was extracted by two modes, using two buffers, followed by ammonium purification, as described in methods. Table 1 describes the design of experiments concerning these variations.

Table 1 – Factorial experiment on extraction and initial purification of C-phycocyanin (CPC). 2^3 factorial with central point. Overnight = 18h extraction at 4ºC. Freeze/thaw = three freezing (15 min/ -20ºC) and thawing (15 min/ room temperature) cycles followed by 2h at 4ºC.

	Inputs			Outputs			
	Buffer	**Extraction Procedure**	**Ammonium Sulfate (%)**	**CPC (mg/mL)**	**Purity**	**Purity Factor**	**Yeld (%)**
1	Histidine	Freeze/thaw	80	4.60	0.45	1.33	58.43
2	Histidine	Overnight	60	6.93	0.50	1.26	60.31
3	PBS	Overnight	40	7.32	0.64	1.43	74.20
4	PBS	Freeze/thaw	60	5.42	0.60	1.36	63.13
5	PBS	Overnight	80	5.71	0.58	1.28	51.60
6	Histidine	Overnight	80	5.49	0.49	1.24	45.28
7	Histidine	Freeze/thaw	40	4.67	0.57	1.84	69.81
8	PBS	Overnight	60	6.62	0.62	1.33	61.06
9	Histidine	Freeze/thaw	60	4.30	0.43	1.37	63.00
10	Histidine	Overnight	40	7.63	0.79	1.89	62.46
11	PBS	Freeze/thaw	80	5.49	0.58	1.33	62.51
12	PBS	Freeze/thaw	40	6.77	0.66	1.44	70.19

Pareto charts were plotted to evaluate the effects of input variation on the selected outputs (Figure 1). The increment of ammonium sulfate presented the highest negative effect on all outputs, except for “CPC concentration”; this output was affected by the extraction mode, recovering more protein when done as the overnight procedure. Buffer exchange influenced the “purification factor” of the samples, especially when combined with the concentration of ammonium sulfate. Since the main goal was to obtain the most purified and concentrated buffer, the chosen parameters

were histidine buffer, overnight extraction and purification at 40% ammonium sulphate concentration.

Similar results using lower ammonium sulphate saturation, in the rate between 20-50%, were demonstrated elsewhere achieving a purity factor of 0.88, 1.7 of purity factor[76], as of the use of an overnight extraction procedure[101], but to this date no study on the use of histidine buffer was found for extraction and purification of CPC.

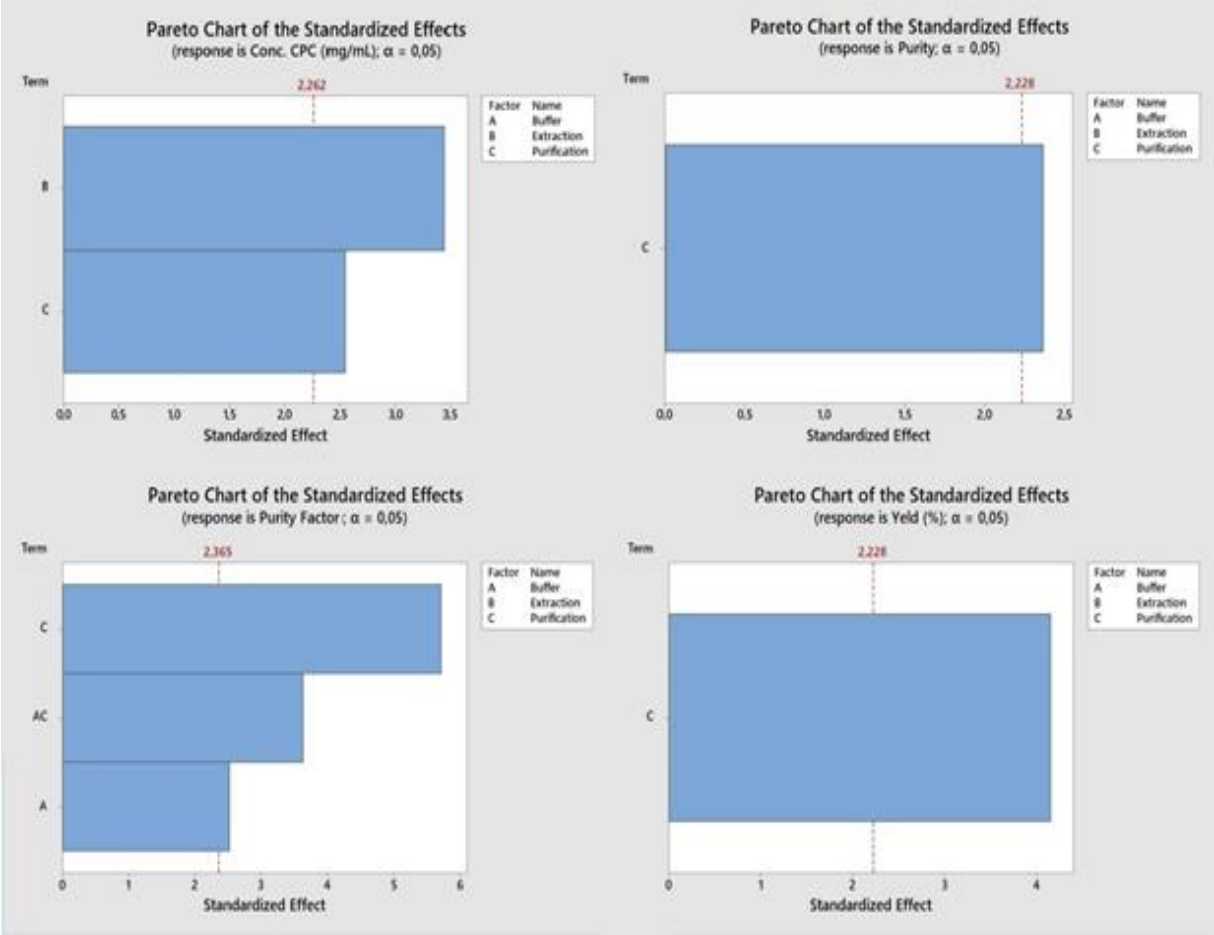


Figure 1 - Pareto chart of the factorial experiment on extraction and initial purification of C-phycocyanin (CPC) 2^3 factorial with central point. Inputs: A = Buffer (histidine x PBS); B = Extraction mode (Overnight x Freeze thaw) and C = Ammonium sulfate concentration (40-80%). Outputs: CPC concentration (mg/ml, upper left graph), Purity (nondimensional, upper right graph), Purification Factor (nondimensional, lower left graph) and Yield (%, lower right graph). The red line represents the reference line for statistical significance and the bars that cross it are statistically relevant in α = 0.05. Standardized effect is the t-statistics that test the null hypothesis that the effect is 0. Figure generated by Minitab 17 software.

The purification technique by salting out with ammonium sulfate 40% m/w resulted in a maximum purity of 0.79, a value compatible with food grade (greater than 0.7) but still lower than the levels required for reagent or pharmaceutical grade (greater

than 3.0 and 3.9, respectively)[102]. It is worth noting that CPC from fresh mass Spirulina was reported to be obtained with higher purity rates after extraction than from dried ones (as this work). Whereas fresh mass reached purity values higher than 1.0, sun/freeze-dried biomass has a reported value of 0.76 [86]. To enhance purity, a chromatography step was added to polish the protein of interest [103].

CPC polishing was done by size exclusion (SEC), ion exchange chromatography (IEC) and a combination of both. The highest purity was accomplished by the combination of IEC+SEC, resulting in a max fraction purity of 3.41, followed by SEC+IEC combination with a purity of 3.31, IEC with 3.06 and SEC with 2.11. However, the IEC+SEC and SEC+IEC combinations resulted in samples with very low concentration, inferior to 0.7 mg/mL, and a very small number of fractions with higher purity, up to 3 (Table 2).

Table 2 – Comparison between different chromatography techniques in the polishing step of C-phycocyanin (CPC) purification. SEC = Size Exclusion Chromatography, IEC = Ion Exchange Chromatography; [ ] Max = highest fraction concentration; Max Purity = highest fraction purity. Chromatography graphs available in supplementary material, figures A and B.

Sample	Volume	CPC (mg/mL)	Purity	Purity Factor	Yeld (%)
Extraction	40	7.8	0.59	XXX	XXX
Salting out	18.4	8.6	0.87	1.47	50.78
IEC (Fraction 36)	1	2.1	3.06	5.19	0.6
IEC ∑36-42	7	1.47	2.72	4.61	3.3
SEC	1	0.9	2.11	1.84	3.83
IEC+SEC	1	0.7	3.41	6.13	0.39
SEC+IEC	1	0.2	3.31	6.00	0.14

Due to the limitations in the quantity of CPC obtained after the runs with the combined techniques, the choice made for protein characterization was the use of a single IEC step for polishing, providing a greater amount of purified CPC and similar purity than the other combined techniques.

Secondary protein structure was evaluated by circular dichroism (CD, Supplementary Material, figure C). The highest purity fraction (fraction 36A3, purity of 3.0), a lower purity fraction (Unbound, purity of 0.85) collected after IEC (DES A) resulted in the same secondary structure for these samples, showing that the protein is made up of 2 subunits formed by α-helices [9]. Primary structure and purity of CPC was qualitatively evaluated by SDS PAGE, from extraction to the polishing step, as well as its comparison with commercial reagent and food grade CPC (Supplementary Material, figure D). The results correlated with protein molecular weight of CPC monomers (alpha chain - 17.6 kDa and beta chain - 18.1 kDa) for all the samples. Higher levels of impurities were found in the samples after extraction and salting out purification compared to after the chromatography steps and the commercial food/reagent grade ones.

In the end, the purified CPC samples were considered viable for further formulation experiments after the proposed purification method resulting in a reagent grade purity. However, due to the low yield of the process and the need of high quantities for further experiments, we decided to proceed with a commercially acquired reagent grade purity CPC (described in material section).

3.2 Pre formulation studies

The Tg' data of a sample indicates the maximum temperature at which the process can occur without the collapse of the frozen matrix. Ideally, the higher the collapse temperature, the shorter the time required for freeze-drying and certain freeze dryers (mainly on an industrial scale) cannot reach temperatures below -40ºC[57]. The Tg' calculated from DSC thermograms for CPC (0.5%) and sucrose (5% m/w) was -35.74ºC in histidine buffer (5 mM, pH 6) and -23.59ºC in phosphate saline buffer (PBS, 50 mM, pH 7). CPC and HPBCD (5% m/w) presented lower Tg´ in the respective buffers: -16.25ºC in histidine buffer and -24.00ºC in PBS buffer.

CPC thermal stability was then evaluated with the same excipients. The accelerated assay (Figure 2) showed that all formulations had a high decrease in CPC concentration and purity, on average 90% and 80% loss respectively, in the first 30 minutes at 70ºC (melting temperature of CPC = 60°C). The formulations in histidine

buffer with and without excipients maintained, on average, 10% more CPC and purity levels than the ones in PBS buffer or water.

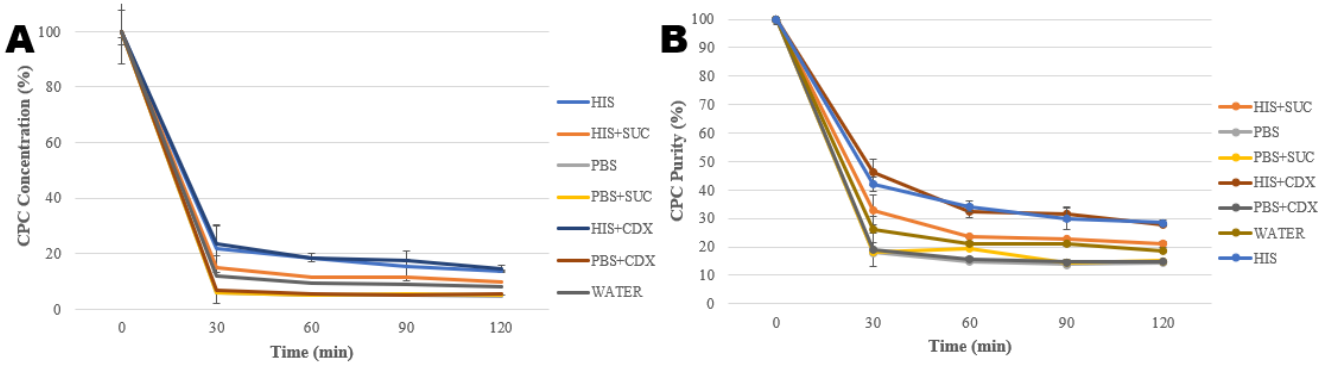


Figure 2 - C-Phycocyanin (CPC) Concentration (A) and Purity (B) after heating at 70ºC for 0, 30, 60, 90 and 120 minutes. HIS – CPC in histidine buffer; PBS – CPC in phosphate saline (PBS) buffer; SUC – sucrose; CDX – HPBCD. Figure generated by OriginLab software.

The critical temperature of CPC is 47ºC, with higher degradations hates above 62ºC[104] that may lead up to 90% loss of its concentration[105]. Many formulations variants may be used to improve such stability: pH between 5.5-6.0 and the addition of sugars were already reported to have stabilizing proprieties in CPC formulations.[93] In our test, the histidine buffer and its pH at 6 was key to higher stability in both purity and concentration, with the addition of HPBCD or of no excipient at all providing better results than the addition of sucrose.

3.3 Freeze-dried formulations

The choice to explore an aggressive and a conservative freeze-drying cycle was based on contemporary approaches regarding the freeze-drying of biological formulations. Such process normally occurs in drying temperatures below the Tg' of the formulations, with lengthy and energy-consuming primary drying steps. More recently, more "aggressive" approaches with shorter steps and higher temperatures are being applied for process optimization, with caution to avoid possible stability loss of the formulations[54,55,57]. Based on tg´ results, the conservative freeze-drying cycle with a freezing temperature of -38ºC was used for the histidine +sucrose CPC formulation and another one starting at -28ºC for the other formulations.

The resulting freeze-dried formulations (Figure 3) of samples 1C, 3C and 4C presented an elegant freeze-dried cake, whereas samples 1, 2 and 3 suffered

shinkrage, and sample 2 and 4 splashed. The cake appearance is an important quality attribute for a freeze-dried formulation, which may or may not be critical to protein stability [106].

Regarding the protein structure of the formulations, the SDS and Native gel electrophoresis results can be seen in Figure 3. The results correlated with protein molecular weight of CPC monomers (alpha chain - 17.6 kDa and beta chain - 18.1 kDa) for the denaturing gel and with a hexamer complex configuration (3 alpha and 3 beta subunits - 106.182 kDa) for the non-denaturing gel of all formulations. The secondary structure content of the formulations, the circular dichroism curves (Figure 3) indicated a predominance of structures in α-helices with troughs at 222 and 208 nm and peaks between 200 and 195 nm for all samples. This was already expected, since the crystallographic structure of c-Phycocyanin from Spirulina platensis (PDB code 1GH0) showed that the protein is made up of 2 subunits formed by α-helices, without the presence of β-sheets that could signal the presence of other contaminating proteins or random coils that are resulting of protein denaturation[9].

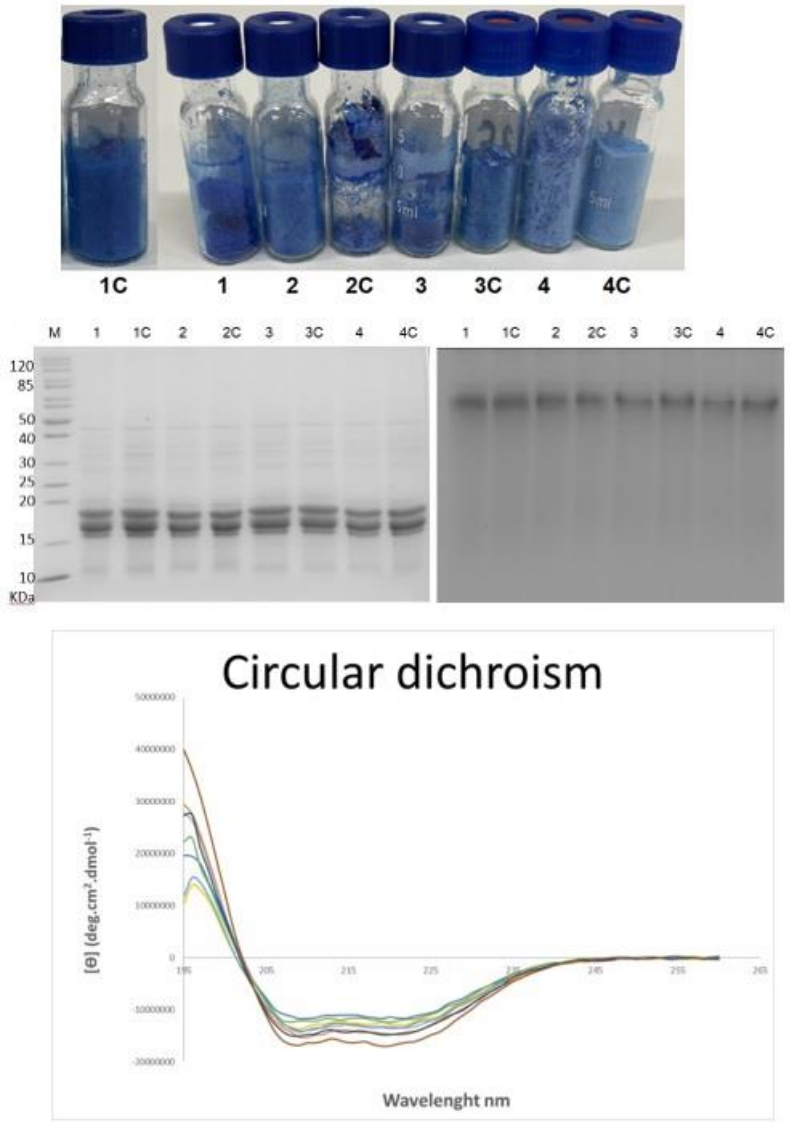


Figure 3 – Visual analysis (top), SDS (15% acrylamide, middle left) and native (15% acrylamide, middle right) gel electrophoresis and Far-UV circular dichroism (button) of the freeze-dried CPC formulations after an aggressive (no mark) and conservative (C) freeze-drying cycle. M – molecular weight mark PageRuler Unstained Protein Ladder, Thermo Fisher; 1 - CPC and sucrose in histidine buffer; 2 – CPC and HPBCD in histidine buffer; 3 - CPC and sucrose in phosphate saline (PBS) buffer; 4 CPC and HPBCD in PBS buffer. Circular dichroism graph generated by Excel software

Other characterization measurements of the freeze-dried formulations, such as residual moisture, reconstitution time, CPC concentration and purity, can be seen in Table 3. All formulations had a reconstitution time inferior to 20s, except sample 4 that was reconstituted in 40s. An ideal maximum reconstitution time is not a topic carved in stone, with some authors considering a time of less than 100s as a fast reconstitution[107].

Table 3 - Characterization of the C-Phycocyanin (CPC) freeze-dried formulation after an aggressive (no mark) and conservative (C) freeze-drying cycle. His - histidine; HPPBCD - 2-Hydroxypropyl-β-cyclodextrin; PBS: phosphate saline buffer.

Sample	Residual Moisture (%)	Reconstitution time (s)	CPC Conc. (mg/mL)	CPC Purity
1	8.6	5	2.97	1.49
1C	4.0	5	3.89	1.72
2	7.2	5	3.06	1.53
2C	6.3	5	3.27	1.71
3	7.6	20	2.80	1.31
3C	2.7	10	2.99	1.55
4	5.5	40	3.59	1.36
4C	4.4	10	3.12	1.59

A common pattern happens in residual moisture, CPC concentration and purity of the formulations, with a higher moisture and lower levels of concentration and purity in the aggressive freeze-drying cycle. The ideal moisture of a biological freeze-dried formulation varies according to its properties; it should be high enough to maintain its physical properties, but low enough to inhibit biological growth or hydrolytic reactions. For proteins, the moisture should at least shield the polar groups[108].

Finally, the results presented are not final, requiring further studies evaluating a greater number of formulations and their stability under predetermined conditions.

3.4 Statistical analysis and formulation optimization

Pareto charts were plotted to evaluate the effects of input variation on outputs (Figure 4). The freeze-drying cycle of choice had the highest impact of all inputs, influencing the visual analyses, residual moisture and purity of the sample. The buffer choice influenced both residual moisture and purity and an association of the cycle and choice of excipient influenced the residual moisture of the formulations. The choice of excipient alone had no statistical influence in any of the outputs and a model

could not be generated with the inputs results for reconstitution time and CPC concentration.

In the end, the best performance was achieved with a CPC formulation in histidine buffer and sucrose after a conservative freeze-drying cycle. Other formulations of note were with PBS buffer and sucrose/ HPBCD after a conservative freeze-drying cycle.

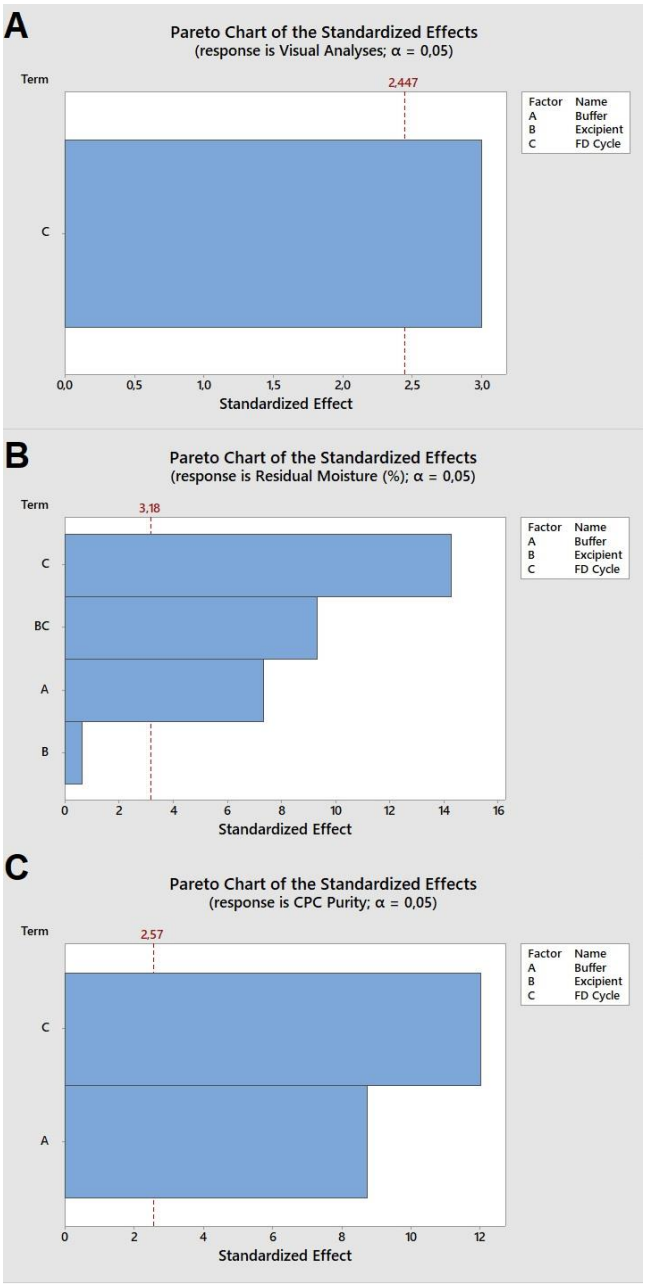


Figure 4 - Pareto chart of C-phycocyanin (CPC) freeze-dried formulations by design of experiments (2^3 factorial). Inputs: A = Buffer (histidine x PBS); B = Excipient (sucrose X HPBCD) and C = Freeze-drying (FD) cycle (aggressive x conservative). Outputs: Visual Analyses (A, nondimensional), Residual Moisture (B, %) and CPC Purity (C,

nondimensional). The red line represents the reference line for statistical significance and the bars that cross it are statistically relevant in α = 0.05. Standardized effect is the t-statistics that test the null hypothesis that the effect is 0. Figure generated by Minitab 17 software.

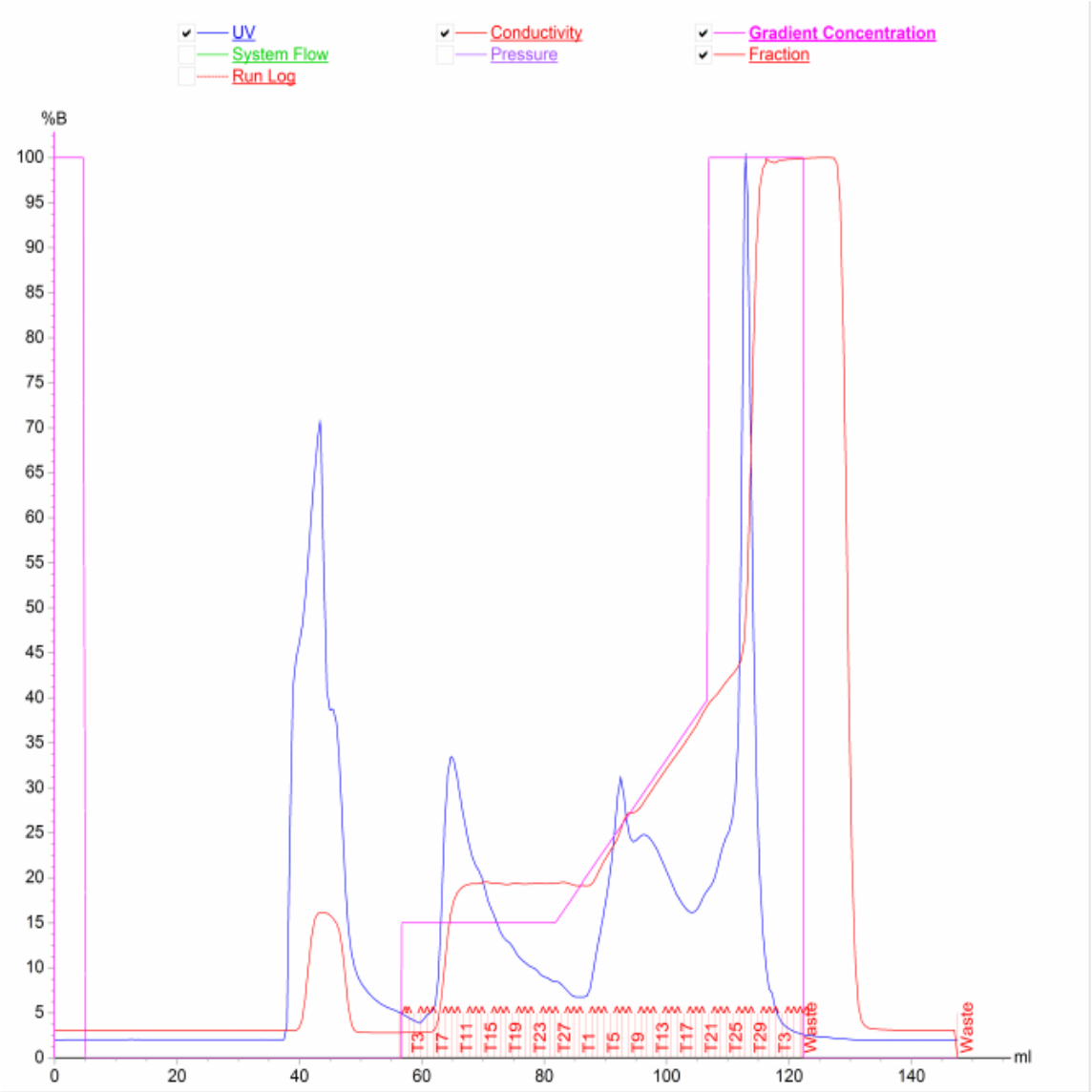
4. CONCLUSION

Extraction and purification were optimized by a design of experiments and histidine buffer, overnight extraction and ammonium sulfate extraction at 40% m/w presented the best results compatible with the ones found in the references for extraction of CPC from dried mass Spirulina. The polishing step by ion exchange chromatography followed by concentration resulted in a highly concentrated CPC solution, purity grade near to reagent grade but low yield compared to other works.

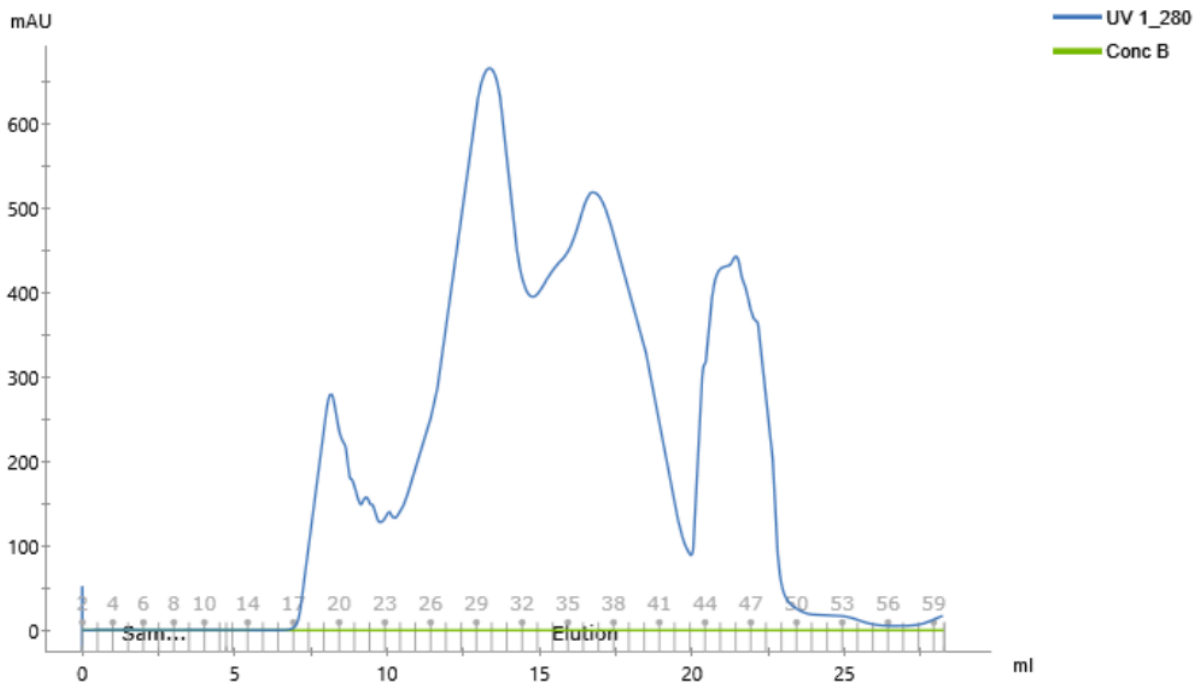
The liquid formulations could not maintain CPC proprieties under heat, and the freeze-dried studied resulted in an optimized formulation, with the one with the best performance according the outputs selected was CPC in histidine buffer and sucrose after a conservative freeze-drying cycle. Other formulations of note were with PBS buffer and sucrose/ HPBCD after a conservative freeze-drying cycle.

Future stability studies, in vitro and in vivo studies should be developed with the purified CPC freeze-dried solutions for the development of pharmaceutical forms for intestinal release for possible treatment and or therapy against colorectal cancer.

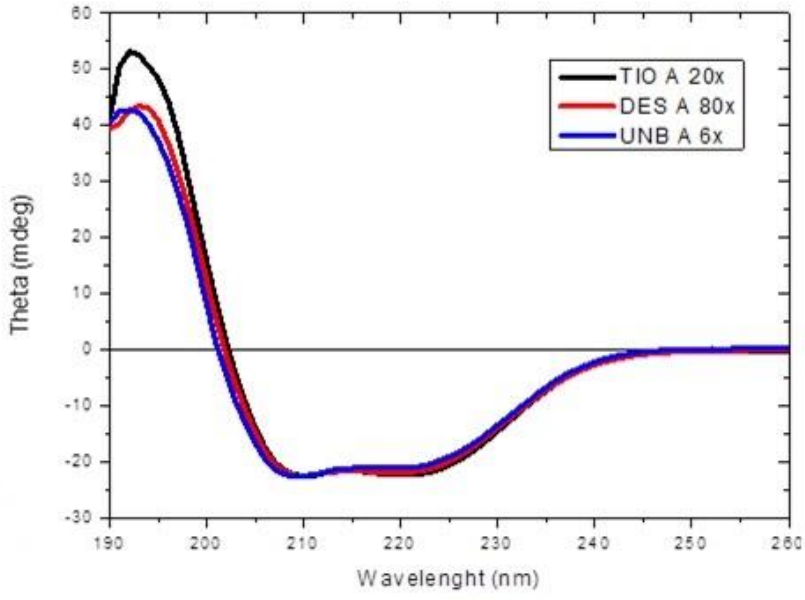
5. SUPPLEMENTARY MATERIAL



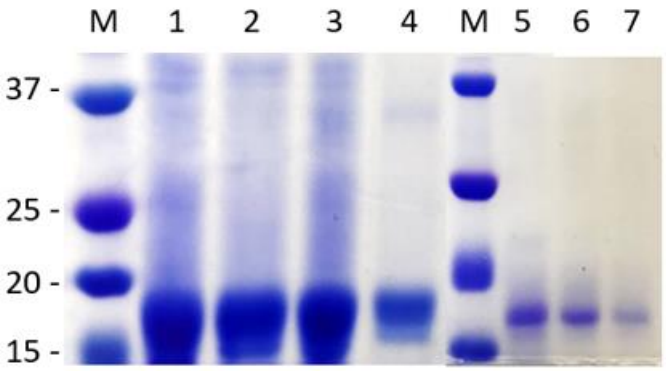
A) Size exclusion Chromatography of CPC Samples. UV – reading in 280 nm; Gradient Concentration – NaCl % concentration.



B) Ion Exchange Chromatography (IEC) of CPC Samples. UV 1 – reading in 280 nm.



C) Circular Dichroism (CD) of purified CPC Samples. Tio A – CPC purified by ion exchange chromatography; DES – CPC before chromatography; UNB – CPC impurities by ion exchange chromatography.



SDS Page
M: Molecular weight marker
1: C-phycocyanin (CPC) after extraction (0.57 purity)
2: CPC Initial purification (0.76)
3: CPC after desalting (0.89)
4: Size exclusion chromatography (1.1)
5: Ion exchange chromatography (2.84)
6: Food grade CPC (1.67)
7: Reagent grade CPC (2.87)

D) SDS page (15% acrylamide, middle left) gel electrophoresis of purified, food grade and reagent grade CPC samples. Molecular weight mark PageRuler Unstained Protein Ladder, Thermo Fisher;

CAPÍTULO 2) SHORT COMMUNICATION: ALGINATE-BASED SUSTAINABLE DOSAGE FORMS FOR INTESTINAL DELIVERY OF PHYCOCYANIN

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ABSTRACT

Colorectal cancer is one of the cancers with the highest incidence worldwide, causing approximately one million deaths in 2020. In the search for alternatives or adjuvants to chemotherapy and surgical removal, previous studies highlighted the protein C-phycocyanin (CPC) as anti-inflammatory and able to reduce tumors. As CPC degrades in acidic media and can be hydrolyzed by enzymes, its oral administration requires an enteric and sustained delivery system. Therefore, the present work studied the encapsulation and freeze-drying of alginate formulations containing C-phycocyanin for improved stability, oral administration and local enteric release. Alginate freeze-dried pellets and wafers (monolithic) were produced and characterized according to physicochemical parameters, CPC content and release profile. Pellets and wafers were gastroresistant and presented the same sustained profile under neutral media; freeze drying did not influence CPC content and represent a feasible alternative process to obtain dried phycocianin, which may enhance its shelf-life.

Keywords: C-phycocyanin, drug delivery, alginate pellets, alginate wafers

1. INTRODUCTION

Colorectal cancer calls for innovative measures: 1.9 million new cases and 0.9 million deaths in 2020 show how this disease is still deadly and widespread[109]. The relevance is such that the WHO recommends periodic screening for this cancer in all adults over 50 years of age. Treatment consists of multifactorial therapy comprising surgery to remove polyps or tumors; removal of colon sections (more severe cases); chemotherapy in case of metastases to nearby lymph nodes and radiation therapy in cases of metastases to the liver or distant organs [3].

Among the studied alternative treatments, the protein C-phycocyanin (CPC) extracted from cyanobacteria has relevant pharmacological potential to treat and prevent this type of cancer. CPC has an antioxidant effect related to tumor prevention [110,111]; anti-proliferative activity in human colon adernocarcinoma cells [13]; and anti-inflammatory action related with COX-2 inhibition, IL-17 pathway modulation and regulation of gut microbiota[22]. In vivo studies show that CPC was able to prevent cancer development in dimethylhydrazine-induced rat colon cancer[112], synergizing with piroxicam in the treatment of the same cancer[113].

As a putative drug protein, CPC is unstable under acidic media [91], light [91,114] and temperatures above 4ºC [93,114]. Therefore, its delivery must be directed to the nearly neutral intestinal environment and the formulation must provide environmental protection. Among drug carriers, alginate pellets and gels have shown promise due to their reduced allergenic potential, sustainable production, free from animal products and resistance to gastric acid [28–31]. Alginates are derived from marine algae of the Phaeophycea class, classified as hydrocolloids due to their ability to be soluble in water and form high-viscosity colloids in the presence of alkali metals such as Mg2+, Ca2+, Sr2+ and Ba2+[35,36]. In their deprotonated state (pH below 5), the regions of alginates concentrated in guluronic acid (and with a low concentration of mannuronic acid) are able to bind to alkali metals by divalent salt bridges, forming networks of polymeric chains[37,38]. Furthermore, as they are macroscopic, hydrophilic and have high water retention power, they easily encapsulate high molecular weight molecules such as proteins [28,32,33]. Noteworthy, hydrated formulations may leak drugs and dos not provide protection against hydrolysis.

Given the above, the present work aimed to study formulation parameters of dried alginate pellets containing C-phycocyanin related with improved stability, oral administration and enteric release for local action.

2. METHODS

2.1 Material

Biological material: the reagent grade C-phycocyanin (CPC) was purchased by Taizhou Binmei Biotechnology Co., Ltd, Taozhou, China. Reagents: sodium phosphate mono and bibasic, histidine and sodium alginate (viscosity = 520 cP) were purchased from Vetec, Brazil; all other reagents were purchased from Sigma-Aldrich.

2.2 Formulation of Alginate pellets

The alginate pellets were prepared by ionic gelation with calcium chloride ($CaCl_2$) as previously described [14]. The alginate solution (2.0% w/v) with CPC (varied concentration) was added dropwise (5 mL) with a Pasteur pipette in a 2.0% calcium chloride solution (20 mL). The formed pellets were collected with sieves of average pore size of 0,020 mm. The pellets were analyzed according to their morphology, average weight, production yield (in number of beads), encapsulation rate and size distribution (average, D10/ D50/ D90 and Span). The size calculations were made by image analysis with the ImageJ software.

2.3. Formulation of lyophilized wafers

For the formation of the gel, bovine gelatin type B (0.18g) was dissolved in 9 mL of distilled water (50 °C), followed by the addition of both sodium alginate (0.072 g) dissolved in 9 mL of CPC solution in addition of 0.05% calcium chloride. After 30 min homogenization in 250 rpm the gel formed was then freeze-died to form the dried wafer form.

2.4 Phycocyanin content, purity and encapsulation rate

The Phycocyanin content and purity were determined by spectrophotometry. Content was calculated with equation 1 (Eq. 1) by Bennet and Bogorad[99] and purity was calculated according to equation 2 (Eq. 2). The encapsulation rate was determined by an indirect method. Non-encapsulated CPC of

the solution external to the pellets and washing water were analyzed by spectrophotometry (620 and 652 nm) according to the equation of Bennet and Bogorad[99]. The encapsulation efficiency was determined according to equation 3 (Eq. 3):

Eq. 1) CPC concentration = (O.D. at 620nm - 0.474 x O.D. at 652nm) / 5.34

Eq. 2) Purity = O.D. at 620nm / O.D. at 280nm

Eq. 3) ER (%) = (CPC mass weight – CPC non encapsulated mass) / CPC initial mass

2.5 Phycocyanin release profile

The release profile of the formulations was determined by dissolution in simulated gastric medium (SGM, pH 2.0 0.1 N HCl medium) and simulated intestinal medium (SIM, pH 7.4 0.2M phosphate buffer). For this, an established amount of pellets was weighed were transferred to 50 ml bequers filled with 20mL of SGM (37°C, 100 rpm, magnetic stirring) for 2h; then the pellets were washed with water and transferred to SIM, the pH was adjusted to 7.4 and formulations incubated for 4h. Samples of 1 mL of the solutions were collected at 0 and 2h for SGM and 0, 15', 30', 45', 1h, 2h, 4h for SIM and evaluated according to their phycocyanin content.

2.6 Freeze-drying studies

2.6.1 Collapse Temperature

Collapse temperatures of CPC solutions were determined by a microscope coupled to a lyophilization module, Lyostat 2, model FDCS 196 (Linkam Instruments, Surrey, UK), equipped with liquid nitrogen freezing system (LNP94 / 2) and a programmable temperature controller (TMS94, Linkam). Pressure was monitored through a Pirani valve. The equipment was calibrated with aqueous solution of NaCl (eutectic temperature of -21.1 °C). Samples were submitted to heating-cooling ramps of 5 ° C/min. Freezing, freeze drying and collapse events were filmed with a Nikon polarized light microscope, model Elipse E600 (Nikon, Japan), Data were analyzed by the Linksys 32 software.

2.6.2 Freeze Drying of the formulations

The formulations were lyophilized in a 24-well plate. The process was carried out in the Lyostar 3 lyophilizer (SP scientific), which contains shelves with computational pressure control and temperature ramps. The samples were subjected to different rates of freezing and heating, according to the previous results of the thermal analyses. From their evaluation, the formulations were lyophilized according to the following protocol: freezing at -45 °C for 5 hours, primary drying at -45 °C for 24 hours, secondary drying at -20 °C for 16 hours and at 20°C for 20 hours, at 100 mTorr.

2.6.3 Visual Analysis

The Visual Analysis considered shape, color, uniformity, pre- and post-freeze-drying volume, texture and possible residues of the freeze-dried product.

2.6.4 Residual Moisture

Freeze-dried products were submitted to thermogravimetric analysis (TGA-50M, Shimadzu, Japan) to determine residual moisture. Accurately weighted dried pellets samples (around 10 mg) were heated at 10 ºC/min from 25 up to 300 ºC, under nitrogen atmosphere at a flow rate of 50 mL/min. Residual moisture was determined by stable weight-loss (%) at temperature around 100 ºC. TGA analysis were carried in day 0 and 90 of the stability study.

2.8 In vitro evaluation of formulations

2.8.1 Antioxidant activity

Antioxidant activity was determined by the DPPH and ABTS assays. For the DPPH assay: pure CPC released from the alginate pellets (lyophilized or not) were serially diluted in distilled water and mixed 1:1 with 50 µM DPPH solution (1,1-diphenyl-2-picrylhydrazyl). The mixture was homogenized and incubated for 30 min in the dark. The antioxidant activity was calculated as a function of the necessary concentration of the sample for the reduction of the DPPH [115,116] radical (absorbance at 515 nm), according to equation 5 (Eq.5) below:

Eq. 5) Antioxidant activity (%) = [(Blank Absorbance – Sample Absorbance) / Blank Absorbance] x 100

The ABTS assay was performed according to the method of Re et al [117]. ABTS+ radicals were produced by mixing the ABTS solution (7 mM) with potassium persulfate (2.45 mM). The mixture was initially incubated overnight protected from light

at room temperature and later until it presents a constant absorbance at 734 nm, being then diluted in distilled water until it presented an absorption of 0.700 ± 0.20 at 734 nm. The CPC samples (100 µl) were mixed with ABTS+ solution (1400 µl) and the absorbance at 734 nm measured after 6 min of incubation at room temperature. Trolox was used as a standard and the antioxidant activity of the sample were expressed in antioxidant capacity equivalent to Trolox (TEAC)[118].

3. RESULTS AND DISCUSSION

3.1 Evaluation of the c-phycocyanin quantification method

The evaluation of the CPC quantification method was based on current validation guidelines in relation to linearity and specificity [119]. CPC could be reproducibly quantified in different media (potassium phosphate buffer saline, histidine buffer, CaCl2 solution), and alginate did not absorb on phycocyanin related wavelengths. Linearity was calculated with a calibration curve based on serial dilution of c-phycocyanin crude extract (8.7 mg/mL CPC) (Figure 1). The correlation coefficient (R2) was 0.997. The linear range was established at a CPC concentration between 159.77 µg/mL (upper limit) to 15.8 µg/mL (lower limit) of CPC (equation y= 6.9135x + 0.0198).

3.2 Polymeric alginate formulations containing C-Phycocyanin

3.2.1 Hydrated pellets

Pellets were successfully obtained after dropwise ionic gelation (Figure 1A), with results presented in Table 1. Weight and yield were prone to variations during its formation process. The encapsulation rate was of 75.18%, close to the ones reported for solutions between 1.5 to 2.0% of sodium alginate [120]. According to Hadiyanto et al, high levels of alginate used in the production of pellets, as used in this work, lead to an increase in the amount of crosslinking material linked to the polymeric chains, which leads to an increase in the amount of protein entrapment and consequent encapsulation rate[120]. The hydrated pellets showed an average size of 0.075 cm^2, with a circular shape and a small hollow nucleus when cut, indicated a capsule-like structure.

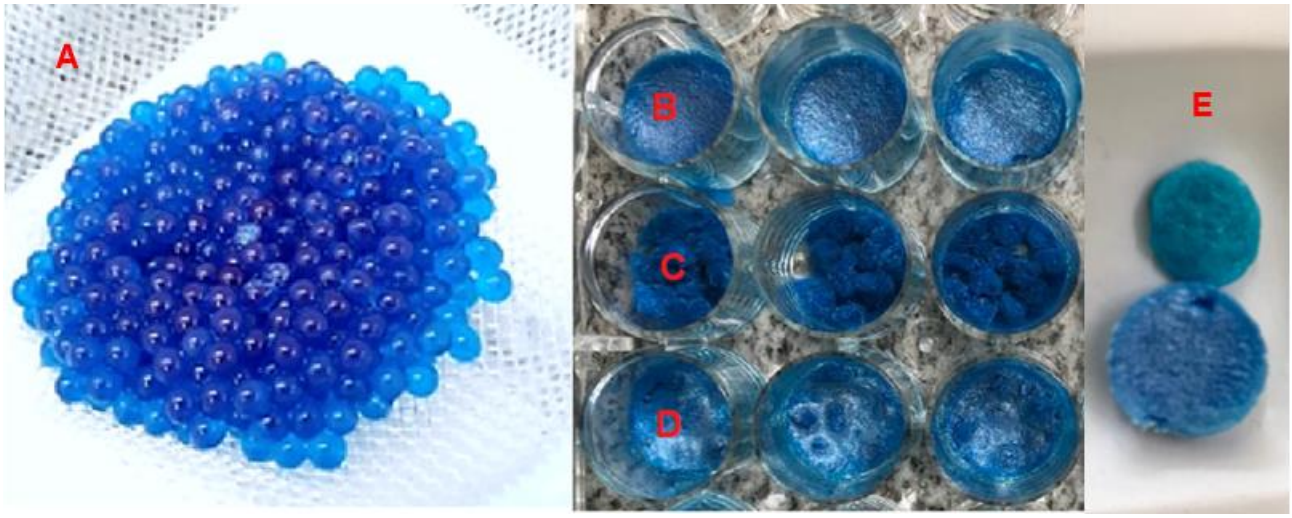

Figure 1 – Alginate formulations containing C-phycocyanin. A) Alginate pellets before freeze-drying; B) Freeze-dried wafers; C) Freeze-dried alginate pellets; D) Wafer+Pellets; E) Wafer before (lower wafer) and after (upper wafer) acid incubation for 2h.

Table 1 - Characterization of hydrated pellets containing phycocyanin. Parameters were calculated from 10 units of each batch, including 7 batchs, and compared with the initial CPC content (13,6mg, 1,36mg/mL).

Average weight (mg)	**Average Production yield (units)**	**Encapsulation rate (%)**
28.18 ± 12.06	205 ± 180	75.18 ± 5.74
Average size (cm^2)	**Smallest**	**Biggest**
0.075 ± 0.011	0.011	0.125
D10 (cm2)	**D50 (cm2)**	**D90 (cm2)**
0.065	0.076	0.086
Circularity	**Span**	**CPC (mg)/ pellet**
0.806	0.276	0.04 ± 0.3

Hydrated pellets were also obtained with alginate of low viscosity (60 cP, sigma). However, at the same concentration (2%), pellets lose circularity (drop format) and PC leaked, resulting in low encapsulation rate (32,65%).

3.2.2 Solid dosage forms

Freeze-drying microscopy was performed to predict the maximum temperature the samples can be submitted to in the primary drying stage of lyophilization without collapse (loss of structure). However, with the ramp used (10 ºC/min to 60 ºC, holding for 1 minute, pressure of 100 MTorr, heating ramp to 0 ºC of 5 ºC/min) it was not possible to visualize the collapse temperatures of the alginate formulations, which indicates that the collapse is above the ice melting temperature (0

°C), which is not detectable in the equipment. Therefore, the drying step had no limitations regarding maximum temperature of the process.

Pellets were freeze-dried as described in methodology, without diluent (Figure 1b), resulting in dried forms that ressemble the hydrated ones (Figure 1C), but with significant brittleness when added trehalose 10%. The group that described the alginate pellets dried at 4°C and detected cracks on the structures by electron microscopy, but they did not described how drying was performed. Wafers were formulated as 1.04 g of a pre-wafer gel (alginate+gelatin+$CaCl_2$) with a concentration of 4.284 mg/mL of CPC in each well of a 24 well plate and freeze-died as previously described. For total CPC quantification, the wafer was disintegrated in 50 mLs of distilled water at 4ºC for 24 h, resulting in 90.75% content.

A combination of wafer (without CPC, functioning as structural support) and pellets was also tested (Figure 1D), with a preparation of 1.0 g pre-wafer gel and 0.5 g of pellets solution (10 pellets). However, after freeze-drying the outer wafer became brittle and lost its structure.

The residual humidity by TGA analysis can be seen in Figure 4. All the formulations presented a residual moisture superior to 13%, with the solutions with trehalose presenting less than the ones without it (13% and 16.5% Vs 18% and 20%).

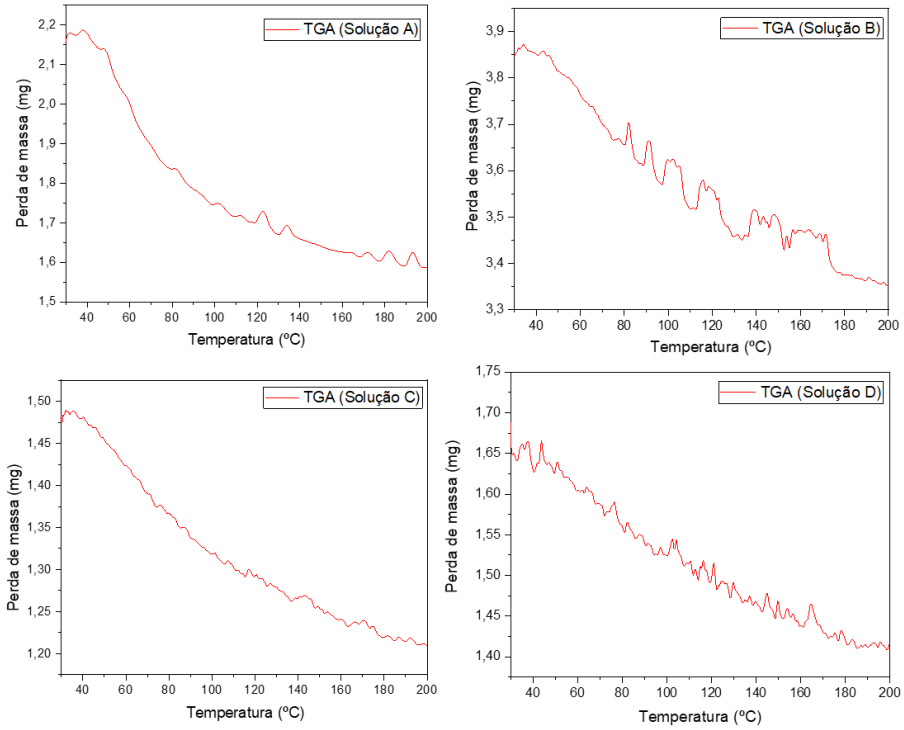

Figure 2 - *TGA analysis of freeze-dried pellets formulations containing CPC. A) Alginate cP 2000; B) Alginate cP 2000 + Trehalose; C) Alginate cP 50; D) Alginate cP 0 + Trehalose.*

3.3 Release Profiles

The release profile of the pellets, freeze-dried pellets and wafer formulations can be seen in Table 2.

Table 2 – Release Profile of the polymeric alginate formulations containing C-phycocyanin; SGM = Simulated Gastric Medium, pH 2.0; SIM = Simulated Intestinal Medium, pH 7.4. Pellets a) = SIM release only; Pellets b) = SGM followed by SIM release.

Sample	SGM		SIM						
	0'	120'	0'	15'	30'	45'	60'	120'	240'
Pellets a)	X	X	4.3 %	14.8 %	20.8 %	26.6 %	30.6 %	49.5 %	83.5 %
Pellets b)	1.4 %	1.6 %	2.4 %	8.5%	26.0 %	33.5 %	34.8 %	37.8 %	37.7 %
Dried pellets	6.5 %	7.8 %	1.9 %	4.5%	6.2%	8.4%	9.3%	11.3 %	12.6 %
Wafer	0.0 %	0.0 %	0.0 %	0.1%	7.7%	13.4 %	14.8 %	26.5 %	39.4 %

In the simulated gastric medium (SGM, pH 2.0), both the pellets formulations released some CPC, with a higher release of the freeze-dried ones (7.8%), but still less than 10%, which is the borderline for gastroresistant formulations. CPC could not be detected in the medium containing the wafer formulation, however its size and weight (from 0.016 g to 0.216 g) changed after the 120 minutes period, indicating that the medium was absorbed by the formulation (Figure 1E). The release profile of Wafer + pellets presented a final release of 7.8% in SGM and 12,6% in SIM, therefore were discarded as a formulation option.

In the Simulated Intestinal Medium (SIM, pH 7.4) the pellets and wafer formulations had the same end point release (near 39% at 240 minutes), with different rates of release as shown in Figure 3. As a control, the release profile at SIM only with hydrated pellets were performed and resulted in release of 83.5%, showing that there was degradation of the protein in the previously acidic medium. The pellets release profile was very similar with the one achieved by Hadiyanto et al (2017) with a maximum release near 40% at 240 min. As for the freeze-dried pellet formulation, the release was inferior, with a maximum release of near 12% at 240 minutes. Hydrated pellets were also tested for release at pH 6.8, but no release was detected. We assume then that in vivo release would happen in the final part of the gastrointestinal tract, which favors target to colon cancer and inflammation.

3.4 Antioxidant activity

Altogether 3 tests were used: Follin, DPPH and ABTS. In the Follin test, there was a reaction between the histidine buffer and the test reagent, resulting in the precipitation of the sample (Figure 2). The CPC samples of reagent grade also precipitated in methanol and ethanol. The ABTS test could be adapted with samples in water, but not the DPPH. The ABTS adaptation is an ongoing experiment.

Figure 3 - Sample precipitation following the Folin antioxidant test.

4. CONCLUSION

Our results shows that alginate provided a significant protection of phycocyanin from acid and could be the basis of polysaccharide matrixes to deliver this protein to the intestine. We also demonstrated that a freeze-drying process can be applied to obtain solid dosage forms and, although friability increases, there is no increase in protein loss. Further studies will approach formulation improvements, such as coating and physical crosslinks to enhance protein recovery.

CONSIDERAÇÕES FINAIS

Os processos de extração e purificação da proteína C-ficocianina (CPC) foram otimizados por um delineamento experimental e o tampão histidina, processo de extração *overnight* e a utilização de sulfato de amônio a 40% m/p apresentaram os melhores resultados compatíveis com os encontrados na literatura para extração de CPC proveniente de massa seca de Spirulina. A etapa de polimento por cromatografia de troca iônica seguida de concentração resultou em uma solução de CPC altamente concentrada, grau de pureza próximo ao grau de reagente, mas baixo rendimento.

As formulações líquidas não conseguiram manter as propriedades do CPC sob calor, e os liofilizados estudados resultaram em uma formulação otimizada, sendo que a que apresentou melhor desempenho de acordo com as respostas selecionadas foi o CPC em tampão histidina e sacarose após um ciclo de liofilização conservador. Outras formulações de nota foram com tampão PBS e sacarose/HPBCD após um ciclo de liofilização conservador.

Referente a incorporação de CPC em sistemas a base de alginato, nossos resultados demostram que os mesmos forneceram uma proteção significativa do meio ácido e pode ser a base das matrizes de polissacarídeos para a entrega da proteína ao intestino. Também demonstramos que um processo de liofilização pode ser aplicado para obter formas farmacêuticas sólidas e, embora a friabilidade aumente, não há aumento na perda de proteína.

Futuros estudos de estabilidade e estudos *in vitro* e *in vivo* devem ser desenvolvidos com as soluções liofilizadas de CPC purificadas e seus respectivos sistemas de liberação para a proteção gástrica. Esperamos que tal trabalho poxa auxiliar ao futuro desenvolvimento de formas farmacêuticas de liberação intestinal para possível tratamento e/ou terapia contra o câncer colorretal.

REFERÊNCIAS

1. Bento, M. A. I. Segundo mais frequente no Brasil, câncer colorretal tem prevenção e chances de cura com diagnóstico precoce. https://www.saude.ms.gov.br/segundo-mais-frequente-no-brasil-cancer-colorretal-tem-prevencao-e-chances-de-cura-com-diagnostico-precoce/.
2. INCA - CÂNCER - Tipo - Colorretal - Detecção precoce. http://www2.inca.gov.br/wps/wcm/connect/tiposdecancer/site/home/colorretal/deteccao_precoce.
3. Colorectal Cancer: Treatment Options. *Cancer.Net* https://www.cancer.net/cancer-types/colorectal-cancer/treatment-options (2012).
4. Treatment of Colon Cancer, by Stage. https://www.cancer.org/cancer/colon-rectal-cancer/treating/by-stage-colon.html.
5. Biller, L. H. & Schrag, D. Diagnosis and Treatment of Metastatic Colorectal Cancer: A Review. *JAMA* **325**, 669–685 (2021).
6. Desai, K. & Sivakami, S. Spirulina: the wonder food of the 21st century. *Asia-Pacific Biotech News* **08**, 1298–1302 (2004).
7. Capelli, B. & Cysewski, G. R. Potential health benefits of spirulina microalgae*. *Nutrafoods* **9**, 19–26 (2010).
8. Eriksen, N. T. Production of phycocyanin—a pigment with applications in biology, biotechnology, foods and medicine. *Appl Microbiol Biotechnol* **80**, 1–14 (2008).
9. Wang, X.-Q. *et al.* Structure of C-phycocyanin from Spirulina platensis at 2.2 Å resolution: a novel monoclinic crystal form for phycobiliproteins in phycobilisomes. *Acta Cryst D, Acta Cryst Sect D, Acta Crystallogr D, Acta Crystallogr Sect D, Acta Crystallogr D Biol Crystallogr, Acta Crystallogr Sect D Biol Crystallogr* **57**, 784–792 (2001).
10. cpcA - C-phycocyanin alpha chain - Arthrospira platensis - cpcA gene & protein. https://www.uniprot.org/uniprot/P72509.
11. cpcB - C-phycocyanin beta chain - Arthrospira platensis - cpcB gene & protein. https://www.uniprot.org/uniprot/P72508.
12. Fukui, K. *et al.* Relationship between color development and protein conformation in the phycocyanin molecule. *Dyes and Pigments* **63**, 89–94 (2004).

13. Thangam, R. *et al.* C-Phycocyanin from Oscillatoria tenuis exhibited an antioxidant and in vitro antiproliferative activity through induction of apoptosis and G0/G1 cell cycle arrest. *Food Chem* **140**, 262–272 (2013).

14. Yan, M., Liu, B., Jiao, X. & Qin, S. Preparation of phycocyanin microcapsules and its properties. *Food and Bioproducts Processing* **92**, 89–97 (2014).

15. Liu, R., Qin, S. & Li, W. Phycocyanin: Anti-inflammatory effect and mechanism. *Biomedicine & Pharmacotherapy* **153**, 113362 (2022).

16. Wan, D.-H. *et al.* C-Phycocyanin as a tumour-associated macrophage-targeted photosensitiser and a vehicle of phthalocyanine for enhanced photodynamic therapy. *Chem. Commun. (Camb.)* **53**, 4112–4115 (2017).

17. Subhashini, J. *et al.* Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. *Biochemical Pharmacology* **68**, 453–462 (2004).

18. Ravi, M. *et al.* Molecular mechanism of anti-cancer activity of phycocyanin in triple-negative breast cancer cells. *BMC Cancer* **15**, 768 (2015).

19. Jiang, L. *et al.* Phycocyanin: A Potential Drug for Cancer Treatment. *J Cancer* **8**, 3416–3429 (2017).

20. Liu, Q., Huang, Y., Zhang, R., Cai, T. & Cai, Y. Medical Application of Spirulina platensis Derived C-Phycocyanin. *Evid Based Complement Alternat Med* **2016**, (2016).

21. Saini, M. K., Sanyal, S. N. & Vaiphei, K. Piroxicam and C-phycocyanin mediated apoptosis in 1,2-dimethylhydrazine dihydrochloride induced colon carcinogenesis: exploring the mitochondrial pathway. *Nutr Cancer* **64**, 409–418 (2012).

22. Pan, D. *et al.* Phycocyanin Ameliorates Colitis-Associated Colorectal Cancer by Regulating the Gut Microbiota and the IL-17 Signaling Pathway. *Mar Drugs* **20**, 260 (2022).

23. Reddy, C. M. *et al.* Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from Spirulina platensis. *Biochem Biophys Res Commun* **277**, 599–603 (2000).

24. Strasky, Z. *et al.* Spirulina platensis and phycocyanobilin activate atheroprotective heme oxygenase-1: a possible implication for atherogenesis. *Food Funct* **4**, 1586–1594 (2013).

25. Manconi, M. *et al.* Chitosomes as drug delivery systems for C-phycocyanin: Preparation and characterization. *International Journal of Pharmaceutics* **392**, 92–100 (2010).

26. Barratt, G. Colloidal drug carriers: achievements and perspectives. *CMLS, Cell. Mol. Life Sci.* **60**, 21–37 (2003).

27. Jameel, F., Hershenson, S., Khan, M. A. & Martin-Moe, S. *Quality by Design for Biopharmaceutical Drug Product Development*. (Springer, 2015).

28. Mandal, S., Kumar, S. S., Krishnamoorthy, B. & Basu, S. K. Development and evaluation of calcium alginate beads prepared by sequential and simultaneous methods. *Brazilian Journal of Pharmaceutical Sciences* **46**, 785–793 (2010).

29. Choi, B. Y., Park, H. J., Hwang, S. J. & Park, J. B. Preparation of alginate beads for floating drug delivery system: effects of CO2 gas-forming agents. *International Journal of Pharmaceutics* **239**, 81–91 (2002).

30. Whitehead, L., Collett, J. H. & Fell, J. T. Amoxycillin release from a floating dosage form based on alginates. *International Journal of Pharmaceutics* **210**, 45–49 (2000).

31. Won, Y.-W. & Kim, Y.-H. Preparation and cytotoxicity comparison of type a gelatin nanoparticles with recombinant human gelatin nanoparticles. *Macromol. Res.* **17**, 464–468 (2009).

32. Halder, A., Maiti, S. & Sa, B. Entrapment efficiency and release characteristics of polyethyleneimine-treated or -untreated calcium alginate beads loaded with propranolol-resin complex. *Int J Pharm* **302**, 84–94 (2005).

33. Bowersock, T. L. *et al.* Oral vaccination of animals with antigens encapsulated in alginate microspheres. *Vaccine* **17**, 1804–1811 (1999).

34. Mabeau, S. & Fleurence, J. Seaweed in food products: biochemical and nutritional aspects. *Trends in Food Science & Technology* **4**, 103–107 (1993).

35. Vilgis, T. A. Hydrocolloids between soft matter and taste: Culinary polymer physics. *International Journal of Gastronomy and Food Science* **1**, 46–53 (2012).

36. Kohn, R. Ion binding on polyuronates - alginate and pectin. *Pure and Applied Chemistry* **42**, 371–397 (2009).

37. Mancini, F. & McHugh, T. H. Fruit-alginate interactions in novel restructured products. *Nahrung* **44**, 152–157 (2000).

38. Lee, P. & Rogers, M. A. Effect of calcium source and exposure-time on basic caviar spherification using sodium alginate. *International Journal of Gastronomy and Food Science* **1**, 96–100 (2012).

39. Subramaniam, B., Saim, S., Rajewski, R. & Stella, V. J. Green Process Concepts for the Pharmaceutical Industry. in *Green Engineering* vol. 766 96–110 (American Chemical Society, 2000).

40. Cue, B. W. & Zhang, J. Green process chemistry in the pharmaceutical industry. *Green Chemistry Letters and Reviews* **2**, 193–211 (2009).

41. Alder, C. M. *et al.* Updating and further expanding GSK's solvent sustainability guide. *Green Chem.* **18**, 3879–3890 (2016).

42. Di Genova, T. & Guyda, H. Infants and children consuming atypical diets: Vegetarianism and macrobiotics. *Paediatr Child Health* **12**, 185–188 (2007).

43. Doke, S. K. & Dhawale, S. C. Alternatives to animal testing: A review. *Saudi Pharmaceutical Journal* **23**, 223–229 (2015).

44. Rey, L. *Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products, Third Edition*. (CRC Press, 2016).

45. Challener, C. A. Excipient Selection for Protein Stabilization. http://www.pharmtech.com/excipient-selection-protein-stabilization.

46. Costantino, H. R. & Pikal, M. J. *Lyophilization of Biopharmaceuticals*. (Springer Science & Business Media, 2004).

47. John F Carpenter, Ken-ichi Izutsu, & Theodore W Randolph. Freezing- and Drying-Induced Perturbations of Protein Structure and Mechanisms of Protein Protection by Stabilizing Additives. in *Freeze Drying/Lyophilization of Pharmaceutical and Biological Products* 167–197 (Informa Healthcare, 2010).

48. Tattini Jr, V., Parra, D. F. & Pitombo, R. N. de M. Influence of cooling rate on the structural and phase changes during lyophilization of bovine serum albumin. *Revista Brasileira de Ciências Farmacêuticas* **42**, 127–136 (2006).

49. Welcom to rotech. http://www.rotechmachinery.com/news.asp?id=56.

50. Pikal, M. J. & Shah, S. The collapse temperature in freeze drying: Dependence on measurement methodology and rate of water removal from the glassy phase. *International Journal of Pharmaceutics* **62**, 165–186 (1990).

51. Adams, G. D. J. & Irons, L. I. Some implications of structural collapse during freeze-drying using Erwinia caratovoraL-asparaginase as a model. *Journal of Chemical Technology and Biotechnology* **58**, 71–76 (1993).

52. Gabbott, P. A Practical Introduction to Differential Scanning Calorimetry. in *Principles and Applications of Thermal Analysis* 1–50 (John Wiley & Sons, Ltd, 2008). doi:10.1002/9780470697702.ch1.

53. Meister, E., Šaši, S. & Gieseler, H. Freeze-Dry Microscopy: Impact of Nucleation Temperature and Excipient Concentration on Collapse Temperature Data. *AAPS PharmSciTech* **10**, 582–588 (2009).

54. Bjelošević, M. *et al.* Aggressive conditions during primary drying as a contemporary approach to optimise freeze-drying cycles of biopharmaceuticals. *European Journal of Pharmaceutical Sciences* **122**, 292–302 (2018).

55. Haeuser, C., Goldbach, P., Huwyler, J., Friess, W. & Allmendinger, A. Be Aggressive! Amorphous Excipients Enabling Single-Step Freeze-Drying of Monoclonal Antibody Formulations. *Pharmaceutics* **11**, 616 (2019).

56. Carpenter, J. F., Pikal, M. J., Chang, B. S. & Randolph, T. W. Rational design of stable lyophilized protein formulations: some practical advice. *Pharm. Res.* **14**, 969–975 (1997).

57. Email, Print & (0), C. Freeze-Drying Protein Formulations above their Collapse Temperatures: Possible Issues and Concerns. http://www.americanpharmaceuticalreview.com/Featured-Articles/37021-Freeze-Drying-Protein-Formulations-above-their-Collapse-Temperatures-Possible-Issues-and-Concerns/.

58. Depaz, R. A., Pansare, S. & Patel, S. M. Freeze-Drying Above the Glass Transition Temperature in Amorphous Protein Formulations While Maintaining Product Quality and Improving Process Efficiency. *JPharmSci* **105**, 40–49 (2016).

59. Large-Scale Freezing of Biologics. *BioProcess International* http://www.bioprocessintl.com/manufacturing/fill-finish/large-scale-freezing-of-biologics-185110/ (2009).

60. Kolhe, P., Amend, E. & K. Singh, S. Impact of freezing on pH of buffered solutions and consequences for monoclonal antibody aggregation. *Biotechnol Progress* **26**, 727–733 (2010).

61. Kueltzo, L. A., Wang, W., Randolph, T. W. & Carpenter, J. F. Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze–thawing. *J. Pharm. Sci.* **97**, 1801–1812 (2008).

62. Laboratory training courses on HPLC, GC, AAS, Lab Safety, Spectroscopy. *Lab-Training.com* http://lab-training.com/landing/role-of-preformulation-studies-in-drug-development/.

63. A REVIEW ON PHARMACEUTICAL PREFORMULATION STUDIES IN FORMULATION AND DEVELOPMENT OF NEW DRUG MOLECULES | INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH. http://ijpsr.com/bft-article/a-review-on-pharmaceutical-preformulation-studies-in-formulation-and-development-of-new-drug-molecules/?view=fulltext.

64. Wang, W. Lyophilization and development of solid protein pharmaceuticals. *International journal of pharmaceutics* **203**, 1–60 (2000).

65. Rowe, R. C., Sheskey, P. J., Quinn, M. E., & American Pharmacists Association. *Handbook of pharmaceutical excipients.* (Pharmaceutical Press ; American Pharmacists Association, 2009).

66. Geraldes, D. C. *et al.* Protein drug delivery: current dosage form profile and formulation strategies. *Journal of Drug Targeting* **28**, 339–355 (2020).

67. Bouvier, E. S. P. & Koza, S. M. Advances in size-exclusion separations of proteins and polymers by UHPLC. *TrAC Trends in Analytical Chemistry* **63**, 85–94 (2014).

68. Bam, N. B., Randolph, T. W. & Cleland, J. L. Stability of Protein Formulations: Investigation of Surfactant Effects by a Novel EPR Spectroscopic Technique. *Pharm Res* **12**, 2–11 (1995).

69. Franks, F. Freeze-drying of bioproducts: putting principles into practice. *European Journal of Pharmaceutics and Biopharmaceutics* **45**, 221–229 (1998).

70. Akers, M. J. Excipient–drug interactions in parenteral formulations. *J. Pharm. Sci.* **91**, 2283–2300 (2002).

71. Crowe, J. H., Leslie, S. B. & Crowe, L. M. Is vitrification sufficient to preserve liposomes during freeze-drying? *Cryobiology* **31**, 355–366 (1994).

72. Koynova, R. D., Tenchov, B. G. & Quinn, P. J. Sugars favour formation of hexagonal (HII) phase at the expense of lamellar liquid-crystalline phase in hydrated phosphatidylethanolamines. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **980**, 377–380 (1989).

73. Wolfe, J. & Bryant, G. Freezing, drying, and/or vitrification of membrane- solute-water systems. *Cryobiology* **39**, 103–129 (1999).

74. Moraes, C. C., Sala, L., Cerveira, G. P. & Kalil, S. J. C-phycocyanin extraction from Spirulina platensis wet biomass. *Brazilian Journal of Chemical Engineering* **28**, 45–49 (2011).

75. Silveira, S. T., Burkert, J. F. M., Costa, J. A. V., Burkert, C. A. V. & Kalil, S. J. Optimization of phycocyanin extraction from Spirulina platensis using factorial design. *Bioresource Technology* **98**, 1629–1634 (2007).

76. Silva, L. A., Kuhn, K. R., Moraes, C. C., Burkert, C. A. V. & Kalil, S. J. Experimental design as a tool for optimization of C-phycocyanin purification by precipitation from Spirulina platensis. *Journal of the Brazilian Chemical Society* **20**, 5–12 (2009).

77. Sarada, R., Pillai, M. G. & Ravishankar, G. A. Phycocyanin from Spirulina sp: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin. *Process Biochemistry* **34**, 795–801 (1999).

78. Geraldes, D. C. *et al.* Protein Drug Delivery: Current Dosage Form Profile and Excipient Trends. *Journal of Drug Targeting* (2019).

79. Patel, A., Pawar, R., Mishra, S., Sonawane, S. & Ghosh, P. K. Kinetic studies on thermal denaturation of C-phycocyanin. *Indian J Biochem Biophys* **41**, 254–257 (2004).

80. Li, B., Zhang, X., Gao, M. & Chu, X. Effects of CD59 on antitumoral activities of phycocyanin from Spirulina platensis. *Biomed Pharmacother* **59**, 551–560 (2005).

81. Patel, A., Mishra, S., Pawar, R. & Ghosh, P. K. Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. *Protein Expression and Purification* **40**, 248–255 (2005).

82. Pan-utai, W. & Iamtham, S. Physical extraction and extrusion entrapment of C-phycocyanin from Arthrospira platensis. *Journal of King Saud University - Science* (2018) doi:10.1016/j.jksus.2018.05.026.

83. Breaking old habits: Moving away from commonly used buffers in pharmaceuticals. *European Pharmaceutical Review*.

84. Stramarkou, M. *et al.* Comparative Analysis of Different Drying Techniques Based on the Qualitative Characteristics of Spirulina platensis Biomass. *Journal of Aquatic Food Product Technology* **30**, 498–516 (2021).

85. Herrera, A., Boussiba, S., Napoleone, V. & Hohlberg, A. Recovery of c-phycocyanin from the cyanobacteriumSpirulina maxima. *J Appl Phycol* **1**, 325–331 (1989).

86. Chaiklahan, R., Chirasuwan, N., Loha, V., Tia, S. & Bunnag, B. Separation and purification of phycocyanin from Spirulina sp. using a membrane process. *Bioresource Technology* **102**, 7159–7164 (2011).

87. Güroy, B., Karadal, O., Mantoğlu, S. & Cebeci, O. İ. Effects of different drying methods on C-phycocyanin content of Spirulina platensis powder. *Ege Journal of Fisheries and Aquatic Sciences* **34**, 129–132 (2017).

88. Hilditch, C. M., Smith, A. J., Balding, P. & Rogers, L. J. C-Phycocyanin from the cyanobacteriumAphanothece halophytica. *Phytochemistry* **30**, 3515–3517 (1991).

89. Raja, S., Murty, V. R., Thivaharan, V., Rajasekar, V. & Ramesh, V. Aqueous Two Phase Systems for the Recovery of Biomolecules – A Review. *Science and Technology* **1**, 7–16 (2011).

90. Ohtake, S., Kita, Y. & Arakawa, T. Interactions of formulation excipients with proteins in solution and in the dried state. *Advanced Drug Delivery Reviews* **63**, 1053–1073 (2011).

91. Wu, H.-L., Wang, G.-H., Xiang, W.-Z., Li, T. & He, H. Stability and Antioxidant Activity of Food-Grade Phycocyanin Isolated from Spirulina platensis. *International Journal of Food Properties* **19**, 2349–2362 (2016).

92. Faieta, M., Neri, L., Sacchetti, G., Di Michele, A. & Pittia, P. Role of saccharides on thermal stability of phycocyanin in aqueous solutions. *Food Res Int* **132**, 109093 (2020).

93. Chaiklahan, R., Chirasuwan, N. & Bunnag, B. Stability of phycocyanin extracted from Spirulina sp.: Influence of temperature, pH and preservatives. *Process Biochemistry* **47**, 659–664 (2012).

94. Stärtzel, P. *et al.* Freeze-Drying of L-Arginine/Sucrose-Based Protein Formulations, Part 2: Optimization of Formulation Design and Freeze-Drying Process Conditions for an L-Arginine Chloride-Based Protein Formulation System. *J Pharm Sci* **104**, 4241–4256 (2015).

95. Furuishi, T. *et al.* Effect of γ-cyclodextrin as a lyoprotectant for freeze-dried actinidin. *Pharmazie* **70**, 296–299 (2015).

96. Couto, V. M. *et al.* Capsaicin-Cyclodextrin Complex Enhances Mepivacaine Targeting and Improves Local Anesthesia in Inflamed Tissues. *International Journal of Molecular Sciences* **21**, 5741 (2020).

97. Ataide, J. A. *et al.* Freeze-dried chitosan nanoparticles to stabilize and deliver bromelain. *Journal of Drug Delivery Science and Technology* **61**, 102225 (2021).

98. Correcirc, D. H. A., a & Ramos, C. H. I. The use of circular dichroism spectroscopy to study protein folding, form and function. *AJBR* **3**, 164–173 (2009).

99. Bennett, A. & Bogorad, L. Complementary chromatic adaptation in a filamentous blue-green alga. *J. Cell Biol.* **58**, 419–435 (1973).

100. Julianti, E., Susanti, S., Singgih, M. & Mulyani, L. N. Optimization of Extraction Method and Characterization of Phycocyanin Pigment from Spirulina platensis. *J. Math. Fund. Sci.* **51**, 168–176 (2019).

101. Khazi, M. I., Demirel, Z., Liaqat, F. & Dalay, M. C. Analytical Grade Purification of Phycocyanin from Cyanobacteria. in *Biofuels from Algae: Methods and Protocols* (ed. Spilling, K.) 173–179 (Springer, 2020). doi:10.1007/7651_2018_202.

102. Majdoub, H., Ben Mansour, M., Chaubet, F., Roudesli, M. S. & Maaroufi, R. M. Anticoagulant activity of a sulfated polysaccharide from the green alga Arthrospira platensis. *Biochim. Biophys. Acta* **1790**, 1377–1381 (2009).

103. Silveira, S. T., de Menezes Quines, L. K., Burkert, C. A. V. & Kalil, S. J. Separation of phycocyanin from Spirulina platensis using ion exchange chromatography. *Bioprocess Biosyst Eng* **31**, 477–482 (2008).

104. Antelo, F. S., Costa, J. A. V. & Kalil, S. J. Thermal degradation kinetics of the phycocyanin from Spirulina platensis. *Biochemical Engineering Journal* **41**, 43–47 (2008).

105. Martelli, G., Folli, C., Visai, L., Daglia, M. & Ferrari, D. Thermal stability improvement of blue colorant C-Phycocyanin from Spirulina platensis for food industry applications. *Process Biochemistry* **49**, 154–159 (2014).

106. Patel, S. M. *et al.* Lyophilized Drug Product Cake Appearance: What Is Acceptable? *Journal of Pharmaceutical Sciences* **106**, 1706–1721 (2017).

107. Horn, J., Schanda, J. & Friess, W. Impact of fast and conservative freeze-drying on product quality of protein-mannitol-sucrose-glycerol lyophilizates. *Eur J Pharm Biopharm* **127**, 342–354 (2018).

108. Hsu, C. C. *et al.* Determining the optimum residual moisture in lyophilized protein pharmaceuticals. *Dev Biol Stand* **74**, 255–270; discussion 271 (1992).

109. Xi, Y. & Xu, P. Global colorectal cancer burden in 2020 and projections to 2040. *Translational Oncology* **14**, 101174 (2021).

110. Lobo, V., Patil, A., Phatak, A. & Chandra, N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* **4**, 118–126 (2010).

111. Jensen, G. S. *et al.* Antioxidant and Anti-Inflammatory Properties of an Aqueous Cyanophyta Extract Derived from Arthrospira Platensis: Contribution to Bioactivities by the Non-Phycocyanin Aqueous Fraction. *J Med Food* **18**, 535–541 (2015).

112. Saini, M. K. & Sanyal, S. N. Targeting angiogenic pathway for chemoprevention of experimental colon cancer using C-phycocyanin as cyclooxygenase-2 inhibitor. *Biochem Cell Biol* **92**, 206–218 (2014).

113. Saini, M. K., Vaiphei, K. & Sanyal, S. N. Chemoprevention of DMH-induced rat colon carcinoma initiation by combination administration of piroxicam and C-phycocyanin. *Mol Cell Biochem* **361**, 217–228 (2012).

114. Hsieh-Lo, M., Castillo, G., Ochoa-Becerra, M. A. & Mojica, L. Phycocyanin and phycoerythrin: Strategies to improve production yield and chemical stability. *Algal Research* **42**, 101600 (2019).

115. Suzery, M., Hadiyanto, Majid, D., Setyawan, D. & Sutanto, H. Improvement of Stability and Antioxidant Activities by Using Phycocyanin - Chitosan Encapsulation Technique. *IOP Conf. Ser.: Earth Environ. Sci.* **55**, 012052 (2017).

116. Molyneux, P. The use of the stable radical Diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. **26**, (2003).

117. Re, R. *et al.* Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* **26**, 1231–1237 (1999).

118. M Valyova, S Stoyanov, Y Markovska, Y Ganeva. Evaluation of in vitro antioxidant activity and free radical scavenging potential of variety of Tagetes erecta L. flowers growing in Bulgaria. *International Journal of Applied Research in Natural Products 5 (2), 19-25*.

119. Brasil. ANVISA. RE n º899 - Guia para Validação de Métodos Analíticos e Bioanalíticos (2003).

120. Hadiyanto, Suzery, M., Setyawan, D., Majid, D. & Sutanto, H. Encapsulation of phycocyanin-alginate for high stability and antioxidant activity. *IOP Conf. Ser.: Earth Environ. Sci.* **55**, 012030 (2017).