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KARINE CRISTINE KAUFMANN

ESTABILIDADE E GELIFICAÇÃO DE EMULSÕES ÁGUA-ÁGUA INDUZIDAS POR EXTRATO DE ERVA-MATE

STABILITY AND GELATION OF WATER-WATER EMULSIONS INDUCED BY YERBA MATE EXTRACT

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Tese de Doutorado apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Engenharia de Alimentos.

Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Food Engineering.

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos”.

Isaac Newton

Dedico esse trabalho à DEUS e a minha família

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RESUMO

A incompatibilidade termodinâmica entre duas fases aquosas adicionadas de (bio)polímeros pode culminar na formação de duas fases aquosas separadas (ATPS) devido ao fenômeno denominado de exclusão estérica. A mistura dessas fases promovendo a formação de gotas é denominada emulsão água/água. Nesse estudo, a incompatibilidade termodinâmica de sistemas aquosos foi avaliada utilizando pares de proteínas-carboidratos que, em condições críticas de concentração, pH e força iônica, formaram emulsões água-água. Na primeira etapa foi avaliada a incompatibilidade termodinâmica do par caseinato de sódio (SCN) – goma jataí (LBG), induzida por adição de NaCl e extrato de erva mate. Foi possível constatar através da construção de diagramas de fase, reologia e microestrutura que o par SCN – goma jataí interagiu com compostos fenólicos presentes no extrato vegetal, promovendo comportamento de equilíbrio de fases diferente dos sistemas com adição de sal. Esse fenômeno foi verificado através da redução da inclinação das linhas de amarração para as amostras com adição do composto fenólico, indicando a redução da migração da LBG para a fase superior, que alterou a formação de gotas de carboidratos nas fases. Na segunda etapa do trabalho, diferentes concentrações de extrato de erva mate foram utilizadas na gelificação da fase superior de emulsões água/água utilizando o par SCN - goma gelana. As propriedades dos géis foram avaliadas por ensaios reológicos, mecânicos e microestrutura. A concentração de extrato influenciou na formação da rede de gel com relação à porosidade, pois em menores concentrações de extrato foi possível verificar poros de tamanhos regulares e com melhor distribuição. A configuração da rede de gel teve relação direta com as propriedades tecnológicas, como a capacidade de retenção de água (WHC) e propriedades mecânicas, uma vez que a dureza diminuiu com o aumento de WHC. A estrutura da rede foi predominantemente formada por gelana, porém estruturas proteicas foram verificadas na microscopia e modificaram as propriedades mecânicas. Maior concentração de proteína levou a géis com maior deformabilidade. Após a formação inicial da rede, o extrato de erva mate continuou interagindo com a proteína ao longo do tempo, promovendo a modificação da coloração do gel. De forma geral, os resultados demonstraram a importância de estudar a compatibilidade de sistemas aquosos formados por macromoléculas e a interação com compostos fenólicos. Esses componentes são largamente utilizados na produção de sistemas alimentares e a interação entre eles podem produzir biomateriais (emulsões e géis) com diferentes propriedades tecnológicas, aumentando o portfólio de suas aplicações.

Palavras-chave: Emulsões água/água; extrato de erva mate; compostos fenólicos; hidrogéis.

ABSTRACT

The thermodynamic incompatibility between two aqueous phases with the addition of (bio)polymers can culminate in the formation of two separate aqueous phases (ATPS) due to the phenomenon called steric exclusion. The mixture of these phases promoting the formation of droplets is called water/water emulsion. In this study, the thermodynamic incompatibility of aqueous systems was evaluated using protein-carbohydrate pairs that, under critical conditions of concentration, pH and ionic strength, formed water-water emulsions. In the first step, the thermodynamic incompatibility of the sodium caseinate (SCN)-locust bean gum (LBG) pair, induced by the addition of NaCl and yerba mate extract, was evaluated. Building phase diagram, besides evaluation of rheology and microstructure allowed to observe that the SCN-LBG pair interacted with phenolic compounds present in the plant extract, promoting phase equilibria different from systems with added salt. This phenomenon was verified by reducing the slope of the tie lines for samples with the addition of phenolic compound, indicating the reduction of the migration of LBG to the top phase, which altered the formation of carbohydrate droplets in the phases. In the second step of the study, different concentrations of yerba mate extract were used in the gelation of the top phase of water/water emulsions using the SCN - gellan gum pair. The properties of the gels were evaluated by rheological, mechanical and microstructure tests. The extract concentration influenced the formation of the gel network in relation to the porosity, since in lower extract concentrations pores of regular sizes and with better distribution were observed. The configuration of the gel network was directly related to the technological properties, such as the water holding capacity (WHC) and mechanical properties, since the hardness decreased with the increase of WHC. The structure of the network was predominantly formed by gellan, but protein structures were verified in microscopy that modified the mechanical properties. Higher protein concentration led to gels with greater deformability. After the initial formation of the network, the yerba mate extract continued to interact with the protein over time, promoting the modification of the gel color. In general, the results demonstrated the importance of studying the compatibility of aqueous systems formed by macromolecules and their interaction with phenolic compounds. These components are widely used in the production of food systems and the interaction between them can produce biomaterials (emulsions and gels) with different technological properties, increasing the portfolio of their applications.

Keywords: Water/water emulsions; yerba mate extract; phenolic compounds; hydrogels

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CAPITULO I

- ***Introdução geral***
- ***Objetivos***
- ***Estrutura da tese***

1.1 INTRODUÇÃO GERAL

A interação da água com os componentes presentes nos alimentos, principalmente as macromoléculas definem sua mobilidade dentro da mistura (LEWICKI, 2004). Em alimentos, os polímeros de origem natural, como as proteínas e os polissacarídeos, são as macromoléculas mais amplamente presentes em diversas estruturas. No entanto, a baixa entropia (ΔS) de mistura para macromoléculas quando estas são hidratadas em uma mesma solução é a principal força motriz para a produção de sistemas aquosos bifásicos (TOLSTOGUZOV, 2004). De fato, algumas combinações de soluções biopoliméricas (proteínas e carboidratos) podem alterar o equilíbrio de mistura e iniciar um processo de "*demixing"*, formando sistemas aquosos com duas fases separadas, conhecidos como ATPS (Aqueous Two Phase Systems) (KHAN; CHEONG; LIU, 2019). Esses sistemas promovem a imiscibilidade entre as fases aquosas para reestabelecer a entropia das soluções. Quando essas fases separadas formam gotas, são comumente denominados de emulsões água/água (MOSCHAKIS et al., 2018). Esse fenômeno, em geral, ocorre quando a proteína carregada negativamente interage com polissacarídeos neutros ou aniônicos (PERRECHIL; BRAGA; CUNHA, 2009).

Da mesma forma que as emulsões convencionais, as gotas formadas em emulsões água/água (A1/A2) podem enfrentar problemas como coalescência, floculação, inversão de fases e deformação por cisalhamento. No entanto, a tensão interfacial é muito menor que em emulsões tipo água/óleo (MOSCHAKIS, 2018), tornando as emulsões A1/A2 altamente instáveis e mecanismos para melhorar sua estabilidade podem ser empregados (ESQUENA, 2023), como o uso de partículas estabilizantes na interface, alteração da composição do meio ou modificação do comportamento reológico das fases. Em especial, os compostos fenólicos podem formar complexos com biopolímeros. A interação proteínas - compostos fenólicos e carboidratos - compostos fenólicos é muito estudada no sentido de se obter informações sobre as propriedades sensoriais, nutricionais e tecnológicas originadas a partir dessas combinações, (S ECZYK et al., 2019). A associação entre esses componentes pode evitar a oxidação de compostos bioativos, além de melhorar a atuação das macromoléculas no sistema digestivo (AMOAKO; AWIKA, 2016). Já a utilização desse tipo de interação para a obtenção de estabilizantes de emulsões A1/A2, também pode ser considerada como uma rota promissora.

O extrato de erva mate é um exemplo de material com diversos compostos antioxidantes com propriedades terapêuticas, como controle da glicemia e redução da absorção de gordura. Embora a infusão da erva na forma de chás, como o popular tererê e o chimarrão, seja

bastante difundida em países da América do Sul como Brasil, Uruguai Paraguai e Argentina, a biodisponibilidade dos seus compostos e benefícios à saúde podem ser melhor explorados (ALKHATIB; ATCHESON, 2017). Nesse sentido, o carreamento do extrato de erva mate em sistemas poliméricos visando preservar suas propriedades apresenta-se como uma temática interessante. Sistemas biopoliméricos são largamente utilizados para incorporar compostos bioativos, como luteína, curcumina, antocianinas, sendo bastante estudados na literatura (MCCLEMENTS, 2017; SILVA et al., 2017; YOUSUF et al., 2016). Uma via interessante para o carreamento e entrega de compostos com atividades antioxidantes, são os géis. Hidrogéis formados a partir da adição de chás ricos em compostos fenólicos foram avaliados quanto à cor, capacidade de retenção de água, propriedades reológicas e mecânicas, e mostraram melhora nos aspectos nutricionais e tecnológicos (ZHANG et al., 2023).

Assim, esse trabalho teve como primeira etapa, o estudo da incompatibilidade termodinâmica de misturas de proteínas e polissacarídeos, avaliando a formação de emulsões A1/A2 na presença de extrato de erva mate rico em compostos fenólicos. Esse fenômeno foi estudado através da construção de diagramas de fases visual, caracterização físico-química, reológica e microscópica dos sistemas. Essa etapa possibilitou o entendimento da interação de compostos presentes no extrato de erva mate e as proteínas/carboidratos. As amostras com extrato rico em compostos fenólicos interagiram com as macromoléculas presentes no sistema, apresentando diferença no equilíbrio termodinâmico, que ficou refletido no comportamento das fases expresso nos diagramas de equilíbrio.

A segunda etapa da tese teve como foco a utilização de emulsões água/água para a produção de hidrogéis. Esses géis tiveram como agente gelificante o extrato de erva mate, que ao entrar em contato com as emulsões A1/A2, formaram géis com diferentes características, avaliadas através de diagramas de fases, caracterização físico-química dos sistemas, reologia e propriedades mecânicas. Nessa fase do trabalho, ficou evidente a natureza complexa da interação entre proteína-fenólicos e carboidratos-fenólicos. Essas interações formaram géis com diferentes propriedades mecânicas e estruturais e demonstrou a importância do entendimento da compatibilidade entre macromoléculas e compostos com atividade antioxidantes presentes em produtos alimentícios.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Formação de emulsões água em água para obter géis induzidos pela adição de extrato de erva mate.

1.2.3 Objetivos específicos

- Obter emulsões água/água, estáveis, a partir de diferentes pares proteína - polissacarídeo.
- Ajustar condições de processo para a formação das emulsões água-água utilizando extrato de erva mate.
- Avaliar as propriedades das emulsões água-água através de reologia e diagrama de fases.
- Formar géis utilizando emulsões água- água adicionadas de extrato de erva mate.
- Avaliar as propriedades dos géis através da reologia, microscopia e propriedades mecânicas.

1.3 ORGANIZAÇÃO DA TESE

Essa tese foi organizada em capítulos e o escopo de cada um deles pode ser verificado a seguir:

CAPÍTULO I

Esse capítulo apresenta os principais assuntos que serão abordados ao longo do desenvolvimento do trabalho através da introdução geral, além de serem apresentados os objetivos e a forma de organização da tese.

CAPÍTULO II

Esse capítulo contempla a revisão de literatura da tese, onde os aspectos teóricos e conceitos fundamentais para o desenvolvimento do trabalho são apresentados. Nessa revisão estão presentes tópicos como: emulsões água/água; interação proteína-fenólicos; interação carboidratos-fenólicos e formação de hidrogéis.

CAPÍTULO III

Nesse capítulo foram apresentados os resultados da primeira etapa do trabalho, que consistiu na produção de emulsões água/água através da adição de extrato de erva mate. Esses sistemas formados por incompatibilidade termodinâmica entre moléculas de proteínas (caseinato de sódio) e carboidratos (goma jataí) frente à adição de NaCl e extrato de erva mate foram avaliados, quanto às características reológicas e de microestrutura. A partir da construção de diagramas de fases foi possível compreender a diferença entre os sistemas quanto aos tipos de interação que ocorreram para a formação das emulsões. Este estudo foi publicado na revista **International Journal of Biological Macromolecules com o título:** *"Incompatibility between sodium caseinate - locust bean gum induced by NaCl and yerba mate extract".*

CAPÍTULO IV

Nesse capítulo estão descritos os principais resultados obtidos na segunda etapa da tese, que teve como objeto de interesse o estudo da gelificação de emulsões água/água. Foi estudada a incompatibilidade de sistemas contendo proteína (caseinato de sódio) e carboidrato (goma gelana), frente à adição de extrato de erva mate em diferentes concentrações. A partir dessa fase do trabalho foi possível a compreensão da interação entre as macromoléculas e os compostos fenólicos presentes no extrato de erva mate, através da avaliação da composição, microestrutura, propriedades reológicas e mecânicas, demonstrando que a natureza dessas interações pode afetar as características tecnológicas dos géis estudados. O estudo está anexado em formato de artigo e foi submetido na revista **Food Research International** com o título: *"Production of water-water emulsion gels by yerba mate extract"*

CAPÍTULO V

Esse capítulo apresenta a Discussão Geral, onde foram compilados os principais resultados obtidos nos dois capítulos experimentais.

CAPÍTULO VI

Estão apresentadas as impressões gerais da tese, através da Conclusão Geral.

CAPÍTULO VII

Nesse capítulo estão listadas todas as referência utilizada na escrita da tese.

CAPÍTULO VIII

Essa unidade foi destinada à descrição das atividades desenvolvidas durante o desenvolvimento da tese, e a produção científica.

CAPÍTULO IX

Esse capítulo disponibiliza o anexo I, que trata da permissão para utilização de artigos publicados, na tese.

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CAPÍTULO II

Revisão de literatura

2. REVISÃO DE LITERATURA

2.1 sistemas aquosos biopoliméricos

A água é um importante solvente biológico, apesar da sua fórmula molecular muito simples, com dois hidrogênios e um oxigênio seu arranjo espacial pode promover a hidratação tanto de compostos iônico, quanto covalentes. Os meios aquosos permitem solubilizar grande parte das biomoléculas, viabilizando reações metabólicas, químicas e enzimáticas. Apesar de simples quando estudadas de maneira isolada, as soluções aquosas apresentam comportamentos complexos que podem ser fortemente influenciados pela concentração de solutos dissolvidos, força iônica do meio, pH e temperatura (AIDER et al., 2012). Os solutos mais complexos encontrados na produção de alimentos são as proteínas e polissacarídeos.

As proteínas tem sua interação e hidratação em água assegurada dependendo do pH e equilíbrio eletrostático. Um bom exemplo é a caseína do leite, que por se apresentar na forma de sal de cálcio, é bastante solúvel em água. No entanto, quando o pH é reduzido próximo ao limite crítico de solubilidade, denominado ponto isoelétrico, as cargas que mantém a proteína ligada na forma micelar se desestabilizam e a caseína precipita tornando-se insolúvel. (DAMODARAN; PARKIN, 2018). Os polissacarídeos, por sua vez, necessitam, na maioria dos casos, de um aumento de temperatura para melhorar a hidratação das cadeias, tendo suas propriedades viscoelásticas influenciadas pela temperatura e concentração e pH (LEWICKI, 2004). Além disso, alguns polissacarídeos possuem alta tendência em formar redes de gel com o aumento da força iônica, como por exemplo, os alginatos, gelana e carragena (HILAL; FLOROWSKA; WRONIAK, 2023).

A partir do avanço da área de hidrocolóides, na última década foram disponibilizadas centenas de novas tecnologias envolvendo sistemas aquosos poliméricos que conferem produtos de maior estabilidade, qualidade e segurança ao setor de alimentos (MCCLEMENTS, 2021). Nesse sentido essa revisão aborda o comportamento de soluções aquosas sob a influência de diferentes variáveis de processo, como força iônica, pH, temperatura e concentração polimérica

2.2 Emulsões

O processamento de alimentos envolve diversos sistemas para solubilizar e estabilizar moléculas e dispersões. As soluções aquosas estão presentes nos mais variados processos, na forma de dispersões coloidais como é o caso das emulsões. Emulsões podem ser definidas como a dispersão de duas fases imiscíveis, as quais são comumente formadas por uma fase aquosa constituindo a fase polar e óleo como fase apolar. Nesse sistema uma

fase é dispersa na outra em forma de gotas esféricas. Quando a fase apolar é dispersa na fase contínua polar, denomina-se emulsão óleo/água (O/A). Por outro lado, quando a fase aquosa é dispersa na fase óleo denomina-se emulsão água/óleo (A/O) (TAN; MCCLEMENTS, 2021).

Além das emulsões tradicionais, podem ser obtidas emulsões triplas como água-óleo-água (A1 / O / A2), em que duas fases aquosas são separadas por uma fase oleosa ou óleo-água-óleo (O1 / A / O2), em que duas fases oleosas são separadas por uma fase aquosa. Arranjos com maior complexidade também podem ser aplicados, como, por exemplo, as emulsões com 4 fases intercaladas sendo A1 / O1 / A2 / O2, O1 / A1 / O2 / A2 ou O1 / O2 / A2 / O2. Outro tipo de emulsão, menos comum, é a do tipo água-água (A1/A2), que é produzida usando duas soluções aquosas imiscíveis obtidas a partir de sistemas aquosos de duas fases separadas, denominados ATPS (VLADISAVLJEVI; NUUMANI; NABAVI, 2017).

2.2.1 Sistemas aquosos de duas fases separadas (ATPS)

O estudo de sistemas aquosos de fases separadas remonta ao final do século XIX quando sistemas contendo amido, gelatina e água, foram combinados ao acaso e verificou-se a formação de sistemas aquosos bifásicos. A partir dessa descoberta, realizada por Martinus Willem Beijerinck em 1896, outros sistemas foram estudados e uma grande variedade de pares de polímeros que possuíam o mesmo comportamento foram identificados, além da observação da formação de fases separadas com a adição de sais inorgânicos (KHAN; CHEONG; LIU, 2019).

Sistemas aquosos de duas fases separadas (ATPS) são obtidos a partir de uma incompatibilidade termodinâmica promovida por fatores críticos como concentração, solubilidade do polímero, pH, temperatura e força iônica do meio aquoso (MOSCHAKIS et al., 2018). Quando soluções aquosas, com dois tipos de polímeros, atingem esses pontos críticos, ocorre um fenômeno conhecido como exclusão estérica, e duas fases são formadas. Para que o equilíbrio termodinâmico seja reestabelecido, forma-se uma fase de topo rica no polímero1, e uma fase de fundo com polímero 2, (YE, 2008).

Durante a bipartição desses sistemas é comum a formação de gotas dispersas, com a fase de topo apresentando gotas do polímero 2 dispersas na fase contínua rica no polímero 1 e a fase de fundo com gotas de polímero1 dispersas na fase contínua rica em polímero 2. Quando esse fenômeno ocorre, estes sistemas são denominados emulsão água-água (DUMAS et al., 2020; ESQUENA, 2016).

2.2.2 Emulsões água/água (A1/A2)

Em certas condições, algumas soluções contendo mais de um biopolímero podem produzir duas fases, onde cada uma terá uma composição polimérica, sendo esse sistema denominado emulsão água/água (A1/A2) (JONES; MCCLEMENTS, 2010). Essas emulsões podem ser obtidas a partir de soluções aquosas contendo proteínas e polissacarídeos específicos, capazes de separar em duas fases aquosas (PEDDIREDDY et al., 2016) em condições de equilíbrio devido à incompatibilidade termodinâmica das fases (GRINBERG; TOLSTOGUZOV, 1997). Soluções de proteína e polissacarídeo que mostram interações eletrostáticas de atração (apresentam cargas opostas) formarão sistemas associativos através da complexação/precipitação. No entanto, as soluções de proteínas e polissacarídeos que apresentarem interações eletrostáticas de repulsão (cargas iguais), em altas concentrações, formarão sistemas segregativos com duas fases incompatíveis (ESQUENA, 2016).

Nos sistemas segregativos, cada fase apresenta composição diferente de polímero sendo uma fase rica em um dos polímeros e a outra fase formada majoritariamente pelo outro polímero utilizado (ESQUENA, 2016). Esse efeito é obtido através da exclusão estérica, ou seja, efeito do "volume excluído" que é resultante da competição do polímero pelo solvente utilizado no sistema. Esse fenômeno ocorre devido à incapacidade de alguns materiais em compartilhar o mesmo espaço em decorrência da sua conformação ou efeito das cargas e é por isso que a partir de uma concentração crítica existe uma força motriz que promove a separação das fases (JONES; MCCLEMENTS, 2010).

A concentração crítica necessária para a formação desse sistema segregativo é obtido através de um diagrama de fases ternário (GRINBERG; TOLSTOGUZOV, 1997), como mostrado na Figura 2.1.

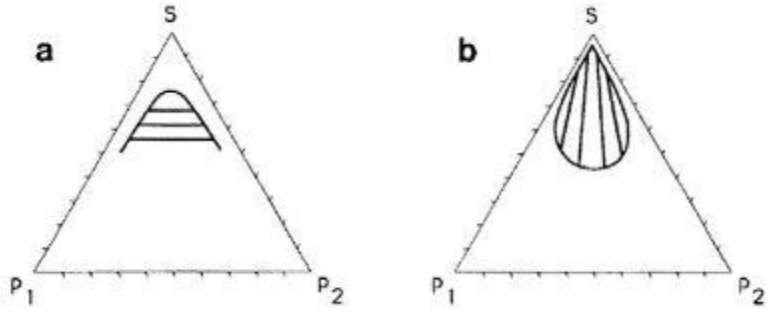


Figura 2.1. Diagramas de fases ternários, mostrando separação de fases segregativa (a) e associativa (b) em sistemas poliméricos hidrofílicos mistos. Onde S: solvente (água); P1: Polímero 1; P2: Polímero 2. **Fonte:** (ESQUENA, 2016).

A Figura 2.1a mostra o comportamento segregativo, no qual a linha binodal delimita a região imiscível e o equilíbrio de fases é representado pelas linhas de amarração convergindo para o ponto crítico onde existe apenas uma fase. A Figura 2.1b apresenta a separação associativa que mostra linhas de amarração verticais, na qual os polímeros e a água são separados com a formação de um sólido que precipita por complexação eletrostática (ESQUENA, 2016). Esses sistemas são comumente representados sem a presença do solvente como é mostrado na Figura 2.2.

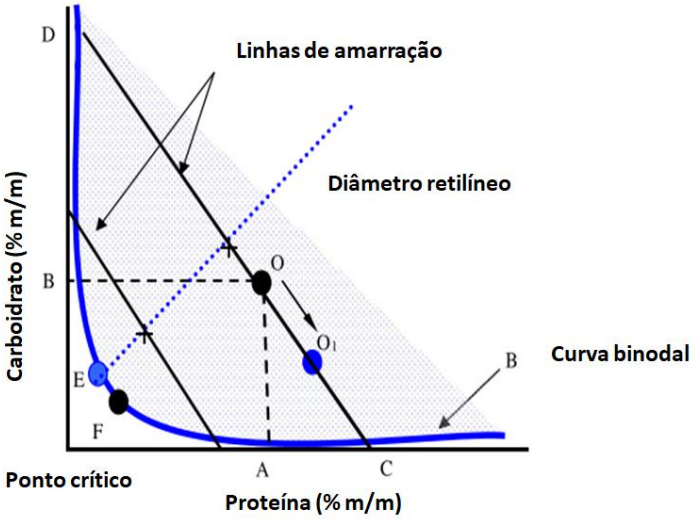


Figura 2.2. Diagrama de fases binário. **Fonte:** adaptado (GOH et al., 2019)

A Figura 2.2 mostra um diagrama de fases típico representando uma solução de proteína-carboidrato. Para a construção desses diagramas é necessário o preparo de uma mistura inicial representada por O e O1. Essas misturas formam duas fases, sendo cada uma delas rica em predominantemente um dos polímeros (proteína ou carboidratos). A composição das fases C e D formadas fornecem dados para que a linha de amarração seja obtida através da reta (DOC). A curva binodal (azul) separa a região monofásica do domínio bifásico (obtido pela observação direta da separação de fases em tubos de ensaio). No ponto E, os sistemas de fase separada terão 50% de proteína e 50% de polissacarídeo na mesma razão de volume de fase que pode ser obtido pelo diâmetro retilíneo. O ponto F representa o ponto crítico de separação, ou seja, a concentração mínima necessária para que os biopolímeros se separem em duas fases (GOH et al., 2019).

A separação das fases entre polissacarídeo e proteína pode ser avaliada pela energia livre de Gibbs da mistura expressa na equação (1) (TURGEON; SCHMITT; SANCHEZ, 2007).

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

Onde: ΔG é a variação da energia livre de Gibbs; ΔH é a variação de entalpia; T é a temperatura e ΔS é a variação de entropia.

A separação a uma dada temperatura acontece quando a energia de Gibbs total da mistura é positiva, ou seja, quando a variação da entalpia do sistema ocasionada pelas interações moleculares endotérmicas é maior que as variações entrópicas, favorecendo a segregação das moléculas. Quando as variações entrópicas são maiores, estas favorecem a mistura principalmente quando o sistema encontra-se diluído, impedindo a separação de fases (MOSCHAKIS et al., 2018).

Da mesma forma que as emulsões convencionais, as emulsões A1/A2 podem ser utilizadas na indústria de alimentos como via de carreamento de materiais com propriedades hidrofílicas, sendo uma alternativa às emulsões A/O para a encapsulação de vitaminas, minerais, probióticos e outros compostos funcionais (SAGIS, 2008). As emulsões A/O são mais eficientes para obtenção de encapsulados hidrofílicos, porém a fase contínua oleosa restringe sua aplicação, devido à dificuldade de separar a fase dispersa aquosa da fase oleosa contínua (PEDDIREDDY et al., 2016).

Emulsões A1/A2 tem sido objeto de estudo visando avaliar suas propriedades reológicas, propriedades interfaciais e estabilidade (SAGIS, 2008) para potenciais aplicações. No entanto, sua baixa estabilidade limita seu uso como veículo de compostos ativos. Além disso, a dificuldade mais relevante é encontrar um par específico de proteína - polissacarídeo que tenha propensão a formar emulsões A1/A2. Alguns dos pares e suas propriedades podem ser encontrados na Tabela 2.1.

Tabela 2.1. Pares de proteínas e polissacarídeos utilizados para obtenção de emulsão A1/A2.

Proteína	Polissacarídeo	pH	Temperatura	Forca iônica	Referência
Caseinato de sódio	Goma arábica	6,4	20˚C	-------	(GRINBERG; TOLSTOGUZOV, 1997); (TOLSTOGUZOV, 2004)
Proteína do leite	Amido de milho + jataí	4,0	70 ˚C	NaCl 0,05 M	(MURRAY; PHISARNCHANANAN, 2016)
Caseinato de sódio	Jataí	7,0	25 ˚C	NaCl 0,7 M	(MOSCHAKIS et al., 2018)
Gelatina	Maltodextrina	5,0	50 ˚C	--	(BELDENGRÜN et al.,

					2020)
Gelatina	Goma guar		10-60 ˚C	---	(CHEN et al., 2021)
Gelatina	Dextrana	5,0-9,0	40˚C	---	(WANG et al., 2023)

As emulsões A1/A2 podem ser empregadas na produção de microgéis com tamanho controlado de partículas. Embora as emulsões A/O sejam as mais utilizadas para obtenção de microgéis, a eliminação da fase contínua tem que ser feita por centrifugação, filtração e lavagem com solventes orgânicos (FARJAMI; MADADLOU, 2017). No entanto, mesmo com todas essas etapas para remoção do óleo podem ser encontrados resíduos que ficam impregnados nos microgéis. Nesse sentido, as emulsões A1/A2 possuem a vantagem de utilizar polímeros hidrofílicos e portanto dispersos totalmente em sistemas aquosos (ESQUENA, 2016), dispensando a dificuldade da remoção da fase externa. Essas estruturas na forma de microgéis podem ser aplicadas como carreadores de substâncias, pois apresentam características variáveis dependendo dos estímulos externos a que são submetidos como pH, temperatura e força iônica, além de possuir uma fase interna propícia para incorporação de moléculas biocompatíveis (ESQUENA, 2016). Microgéis podem ser formados tanto por métodos de auto associação (*bottom up*), como por redução de tamanho de macrogéis (*top down*). Este último método é o mais simples de se obter, tendo em vista apenas o uso de intensas forças mecânicas para a ruptura das macroestruturas.

2.3 Biopolímeros

2.3.1 Caseínas

As caseínas são proteínas extraídas do leite, comumente comercializadas na forma de sal de caseinato de sódio, bastante utilizadas como agente emulsificante e gelificante na indústria de alimentos. Essa molécula é formada por três proteínas principais, as α, β e κ - caseínas. Seus monômeros possuem baixa massa molecular e podem formar micelas, sendo as α- e β- caseínas responsáveis pela capacidade emulsificante do caseinato de sódio. Essa proteína apresenta-se na forma desordenada, e pode apresentar uma característica substancialmente hidrofóbica, viabilizando a estabilização estérica, impedindo a floculação e coalescência de emulsões (HU; MCCLEMENTS; DECKER, 2003).

As caseínas combinadas com polissacarídeos têm sido utilizadas para a obtenção de emulsões A1/A2 (ANTONOV; VANPUYVELDE; MOLDENAERS, 2015; GRINBERG; TOLSTOGUZOV, 1997). A associação das caseínas com polissacarídeos carregados negativamente ou neutros ocorre devido à natureza eletrostática existente entre as moléculas. Desta forma, geralmente os complexos formados entre caseínas e polissacarídeos acontecem

próximos ou abaixo do ponto isoelétrico da caseína (CORREDIG; SHARAFBAFI; KRISTO, 2011).

Moschakis e colaboradores (2018) produziram emulsão A1/A2 de caseinato de sódio e goma jataí. Através da avaliação da microestrutura e reologia da emulsão foi possível verificar a formação de emulsões tanto com o caseinato na fase dispersa, quanto na fase contínua dependendo da concentração utilizada, sendo observado ainda que a adição de NaCl aumentou o tempo de estabilidade da emulsão.

2.3.2 Polissacarídeos

Os polissacarídeos são materiais com alta massa molecular, e por suas características específicas são amplamente utilizados como aditivos alimentares. Possuem alta capacidade espessante e estabilizante, pois aumentam a viscosidade e possuem a capacidade de formar géis (LE; RIOUX; TURGEON, 2017). Vários polissacarídeos já foram relatados com a capacidade de formar emulsões A1/A2 como dextrina (GONZALEZ-JORDAN; NICOLAI; BENYAHIA, 2018), goma jataí (MOSCHAKIS et al., 2018b), carragena (ESQUENA, 2016) e alginato de sódio (ANTONOV; VANPUYVELDE; MOLDENAERS, 2015).

A goma jataí, do inglês *Locust bean gum (*LBG) é um polissacarídeo extraído da semente do *Ceretonia siliqua* conhecido popularmente como alfarroba. É considerado um colóide não iônico e neutro, com cadeia formada, por ligações β(1-4) de manose ramificadas pelo monossacarídeo galactose na posição α(1-6), arranjada de maneira aleatória, na proporção 1:4 respectivamente, (GUM; APPLICATIONS, 1993). Devido a sua composição semelhante a outras gomas como a tara e guar, é considerada uma galactomanana, porém possui menor viscosidade e a sua solubilidade total ocorre apenas a 85 ˚C (CHEN et al., 2025).

A LBG possui alta massa molecular e é capaz de formar soluções viscosas devido a sua conformação altamente emaranhada, mas podem se tornar pouco viscosas em baixas concentrações (MURRAY; PHISARNCHANANAN, 2016). Devido a sua característica, espessante e estabilizante, a indústria de alimentos emprega largamente a sua utilização, principalmente na produção de derivados do leite (SCHORSCH; JONES; NORTON, 2000).

A utilização crescente de extratos de plantas rico em compostos antioxidantes se apresenta como uma alternativa biocompatível para melhorar as propriedades de materiais. A combinação de galactomananas com compostos fenólicos mostra boa compatibilidade e melhora as propriedades mecânicas dos materiais devido às pontes de hidrogênio formadas

entre os dois compostos (CHEN et al., 2025). A Figura 2.3 ilustra uma alfarrobeira, cuja vagem e semente se extrai a goma jataí.

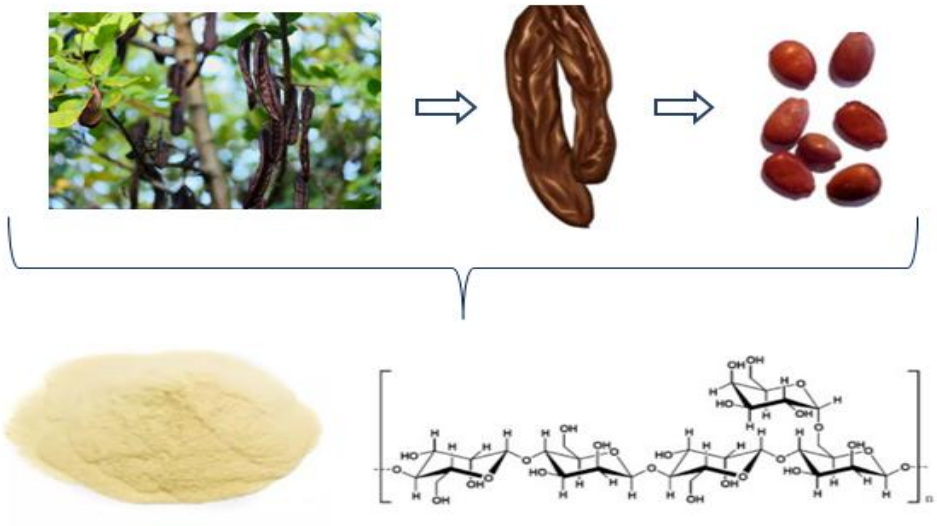
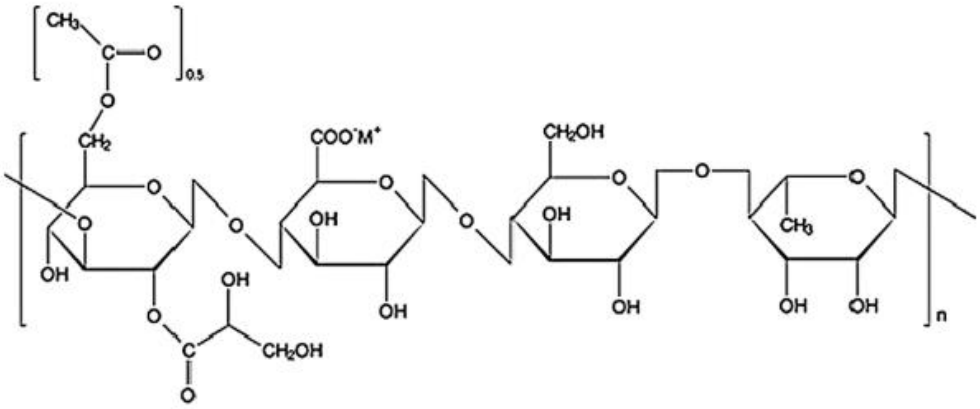


Figura 2.3. Alfarroba e a goma jataí. **Fonte:** Adaptado (CHEN et al., 2025).

A goma gelana é obtida por *via* bacteriana através da secreção da *Pseudomonas elodea*. É um polissacarídeo aniônico de alta massa molecular, formado por tetrassacarídeos de unidades de α-l-ramnose e ácido β-d-glucurônico mais duas unidades de β-d-glucose. A gelana é bastante difundida na indústria de alimentos como aditivo, por suas propriedades espessante, estabilizante e formadora de gel. A gelificação ocorre devido à conformação de dupla hélice que se associa em uma estrutura tridimensional, porém são necessárias altas temperaturas para sua solubilização (BONO; ANISUZZAMAN; DING, 2014).

Comercialmente existem disponíveis dois tipos de gelana: com alto teor de acil ou acilada e baixo acil ou desacilada. A gelana alto teor de acil apresenta dois substituintes na mesma unidade de glicose, um acetato e um glicerato. Esse tipo de goma gelana, quando resfriada a 65 ˚C, apresenta géis flexíveis e macios. A gelana baixo teor de acil pode ser obtida pelo processo de alcalinização da gelana nativa. Apresenta mudanças nas propriedades mecânicas dos géis, quando resfriados a 40 ˚C, que são quebradiços e rígidos (ZIA et al., 2018). A Figura 2.4 ilustra a estrutura molecular dos tipos principais de gelana comercial.

A



B

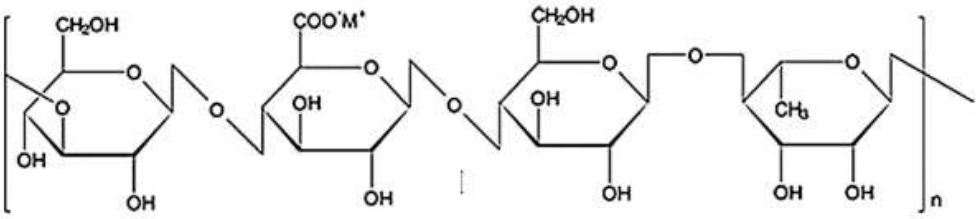


Figura 2.4. Estrutura molecular da goma gelana. **A** - Alto teor de acil e **B** - baixo teor de acil. **Fonte:** (ZIA et al., 2018)

As características comerciais mais apreciáveis da gelana são: capacidade de formar géis fortes em baixas concentrações, alta estabilidade em pH baixo, ductibilidade e propriedades mucoadesivas. Além disso, apresenta boa resistência à ação de enzimas digestivas e é capaz de formar géis com todos os íons. Na presença de íons metálicos, apresenta géis transparentes muito resistentes a altas temperaturas (ZIA et al., 2018). A formação dos géis de gelana se estabelece à frio em, basicamente, duas etapas: durante o processo de resfriamento sua configuração espiral aleatória se transforma em dupla hélice; em seguida agregados de duplas hélice são produzidos, levando à formação do gel (LI et al., 2021).

2.4 Carreamento de compostos de interesse

Nos últimos anos, muitos estudos foram realizados evidenciando a importância de consumir alimentos ricos em compostos fenólicos, como vegetais e frutas. Essas substâncias são conhecidas com antioxidantes, portanto, ativas na prevenção de doenças associadas ao estresse oxidativo do organismo como, por exemplo, diabetes, câncer e doenças cardiovasculares, tendo ainda efeito anti-inflamatório, antialérgico, antitrombótico, antimicrobiano, antiviral e anti- mutagênico (NEMLI et al., 2024).

Compostos fenólicos são produzidos como metabólitos secundários de vegetais, essas substâncias fazem parte do sistema de defesa da planta contra patógenos, degradação de luz UV e fatores de estresse ambiental. Sua capacidade antioxidante está relacionada à estrutura química formada por anéis aromáticos e muitas hidroxilas. O arranjo da composição molecular (número de hidroxilas e anéis aromáticos) origina várias substâncias que são classificadas como fenólicas: os flavonoides, ácidos fenólicos, alcoóis fenólicos, estilbenos e lignana (S ECZYK et al., 2019).

Do ponto de vista tecnológico, compostos naturais com capacidade antioxidante têm muita importância, pois podem ser uma alternativa à utilização de compostos sintéticos que são utilizados no processamento de alimentos para garantir a estabilidade oxidativa dos produtos, melhorando a vida de prateleira (RAWEL; MEIDTNER; KROLL, 2005). Além disso, esses compostos são responsáveis por diversas características sensoriais dos alimentos como amargor e adstringência (S ECZYK et al., 2019).

As características nutricionais dos macronutrientes como proteínas e carboidratos, são afetadas a partir da interação com compostos fenólicos, influenciando a absorção e disgestibilidade. No entanto, as interações macromoléculas-fenólicos, pode ser empregadas para melhorar a disponibilidade dessas substâncias, melhorando assim sua absorção, pois são considerados excelentes veículos de entrega, porque age como protetor dos compostos fenólicos frente à oxidação (BUITIMEA-CANTÚA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2018). Portanto, o estudo do carreamento de compostos fenólicos é interessante, pois garante a ampliação do portfólio de aplicação dessas substâncias em alimentos processados, levando em consideração as possíveis interações positivas e negativas com as macromoléculas presentes nas matrizes alimentares, principalmente as proteínas e carboidratos (BUITIMEA-CANTÚA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2018)

2.5 Interação proteínas - compostos fenólicos

A interação entre compostos fenólicos e proteínas está ligada a estrutura da molécula, que possui alto peso molecular e grande número de hidroxilas. A natureza da interação com as proteínas tem grande impacto na sua funcionalidade, pois afeta o equilíbrio das reações químicas e pode promover a formação de agregados que diminui a solubilidade das proteínas (BUITIMEA-CANTÚA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2018).

Substâncias antioxidantes podem interagir com proteínas através de dois tipos de ligações químicas: covalentes e não covalentes. As interações covalentes são estabelecidas através de ligações irreversíveis e são estabelecidas principalmente pela capacidade em

produzir radicais quinona (NEMLI et al., 2024). Porém, atualmente os estudos se concentram nas interações não-covalentes, devido à escassez de métodos capazes de quantificar de maneira adequada os radicais formados (BUITIMEA-CANTÚA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2018).

As interações não covalentes podem ocorrer através de forças de Van der Waals, atração eletrostática e interações hidrofóbicas, embora todas sejam conduzidas através de pontes de hidrogênio (ROCCHETTI et al., 2022). As pontes de hidrogênio são formadas através da interação dos grupos hidroxila (OH), presentes nos compostos fenólicos e que atuam como doadores de hidrogênio para os grupos C=O das proteínas. Já as interações eletrostáticas ocorrem através dos grupos NH_2 ou C-OH da proteína carregados positivamente, e um grupamento negativo do composto antioxidante (OH) (NASSARAWA et al., 2023). A Figura 2.5 representa os tipos de interações não covalentes.

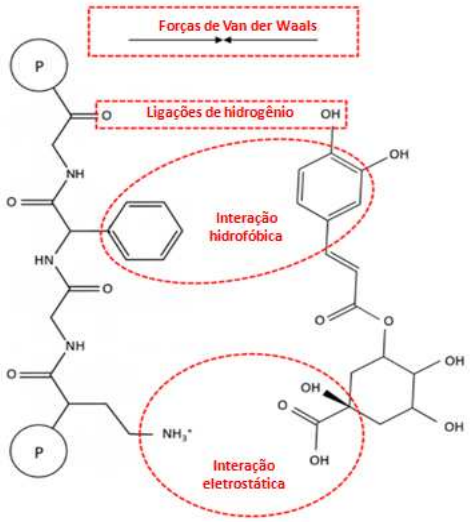


Figura 2.5. Interação não covalente entre proteína e compostos fenólicos. Forças de van der Waals, pontes de hidrogênio, interações hidrofóbicas e interações eletrostáticas.
Fonte: Adaptado de (TARAHI et al., 2024)

A associação das proteínas com fenólicos é afetada pela composição e massa molecular da proteína, concentração de compostos fenólicos, pH e temperatura. As interações, assim como as variáveis de processo, interferem no papel tecnológico da proteína como estabilidade térmica, poder emulsificante e espumante, assim como no papel nutricional como, por exemplo, na digestibilidade (TARAHI et al., 2024). Por outro lado, essas combinações podem influenciar a bioacessibilidade dos compostos fenólicos (S ECZYK et al., 2019), a associação com a proteína pode impedir a oxidação dos compostos até que eles alcancem a absorção intestinal (BUITIMEA-CANTÚA; GUTIÉRREZ-URIBE; SERNA-SALDÍVAR, 2018). Além disso, fenólicos presentes em chás, se associam com as

proteínas do leite, como a caseína, diminuindo a adstringência (KARDUM; GLIBETIC, 2018)

2.6 Interação carboidratos – compostos fenólicos

A interação de carboidratos com outras substâncias pode afetar suas propriedades tecnológicas como a capacidade de gelificação e propriedades reológicas, além das aplicações funcionais como a digestibilidade. Tais associações podem ser covalentes ou não covalentes, (SCHEFER; OEST; ROHN, 2021). As ligações entre polissacarídeo e polifenóis podem ser parcialmente reversíveis e ocorrem em um curto período de tempo e de forma não seletiva. A natureza dessa interação é fortemente influenciada pelo número de hidroxilas que estabelecerão ligações fortes e intensas (DOBSON et al., 2019).

Apesar de alguns antioxidantes não serem facilmente liberados a partir da sua ligação com os polissacarídeos, essa interação pode promover a liberação desses compostos no intestino grosso e colón, onde as enzimas e microrganismos presentes liberam as moléculas de fenólicos. Esse fenômeno disponibiliza carboidratos para a ação da microbiota e ainda viabiliza a ação antioxidante dos polifenóis que atuam na eliminação de microrganismos patogênicos (KARDUM; GLIBETIC, 2018). A presença dos carboidratos pode dificultar a interação proteína - fenólico, por mecanismos de competição devido ao caráter iônico e encapsulante de suas moléculas (JAKOBEK, 2015). Estudos estabelecem uma forte interação entre os taninos e as amiloses do amido, o que leva à formação de complexos não digeríveis e a não liberação de glicose no intestino (AMOAKO; AWIKA, 2016).

2.7 Extrato aquoso rico em compostos fenólicos: erva mate

A partir da *Ilex paraguariensis* popularmente conhecida como erva mate é possível obter uma bebida que é bastante consumida nos países sul americanos como Brasil, Argentina, Paraguai e Uruguai, mas nos últimos anos tem avançado fronteiras e conquistado vários consumidores pelo mundo, devido aos potenciais benefícios à saúde como a redução do colesterol, capacidade antioxidante, propriedade hepato-protetora, controle da obesidade e redução do índice glicêmico (HECK; DE MEJIA, 2007).

Apesar de o consumo primário ocorrer pela infusão em água do macerado das folhas, a extração e concentração em extrato dos compostos benéficos à saúde é interessante para a indústria de nutracêuticos, principalmente para a preservação da cafeína e teobromina (HECK; DE MEJIA, 2007). Na Tabela 2.2 são apresentados alguns dos componentes encontrados na erva mate e seus potenciais benefícios à saúde.

Tabela 2.2. Componentes presentes na erva mate e suas respectivas atividades biológicas.

Componentes	Atividade Biológica
Cafeína	Anti-carcinogênico, antiobesidade, antioxidante, diurético, estimulante.
Ácido clorogênico	Antioxidante, antiaterosclerótico, antibacteriano, antidiabético, analgésico, antitumoral
Colina	Antibacteriano, anticancerígeno, antidiabético, melhora as sinapses dos
Ácido nicotínico	neurônios, facilita a metabolização de gorduras
Ácido pantotênico	Antialérgico, antiartrítico, antifadiga
Rutina	Antioxidante, antitumoral, vasodilatador
Tanino	Antitumoral, inibidor da lipoxigenase
Teobromina	Diurético, estimulante, miorrelaxante
Teofilina	Diurético, colerético, estimulante, vasodilatador, miorrelaxante
Ácido Ursólico	Analgésico, antioxidante, inibidor de protease, antiarrítmico, anticancerígeno

Fonte: Adaptado de (HECK; DE MEJIA, 2007).

Estudos realizados para avaliar o potencial termogênico da erva mate aliado à prática de exercícios físicos apresentaram resultados promissores. Os compostos antioxidantes da erva mate foram recentemente atribuídos à melhora da recuperação da força muscular após a prática de atividade física. Nesse sentido, os compostos ativos presentes na erva mate podem ter desempenhado um papel sinérgico nos efeitos metabólicos durante o exercício. No entanto, mais pesquisas são necessárias para avaliar se os ingredientes ativos apresentam biodisponibilidade após a ingestão (ALKHATIB; ATCHESON, 2017).

Gelificação iônica foi usada para a obtenção de hidrogel de alginato encapsulando extrato de erva mate, obtendo-se bons resultados, pois não houve perda da atividade antioxidante durante a obtenção das esferas de alginato. Durante a digestão, os compostos ativos foram liberados por difusão e erosão das esferas, mostrando-se como uma forma promissora para incorporação de compostos ativos (LÓPEZ CÓRDOBA; DELADINO; MARTINO, 2013).

2.8 Géis

Os hidrocolóides comestíveis, principalmente proteínas e carboidratos são utilizados no processamento de produtos alimentícios principalmente pela sua capacidade

estruturante e estabilizante. O principal mecanismo utilizado é a gelificação de soluções aquosas (hidrogéis), proporcionando a liberação controlada de compostos e estabilização de dispersões e emulsões (GAO et al., 2017). Hidrogéis são estruturas tridimensionais formadas a partir de redes de polímeros hidrossolúveis, que tem grande capacidade de retenção de água, boa adesividade, biocompatibilidade, maciez e extensibilidade. Essas características possibilitam sua aplicação na área biomédica, farmacêutica e alimentícia (XU et al., 2024). As redes de formação dos géis podem ocorrer através de sua reticulação principalmente por via química e física (XU et al., 2024).

A formação de redes estruturadas ocorre a partir da reticulação de hidrocolóides, por três vias: enzimática, química e física. A reticulação enzimática ocorre através da aplicação de enzimas como, por exemplo, a transglutaminase. As reticulações químicas ocorrem através da formação de ligações covalentes, geralmente irreversíveis entre os hidrocolóides utilizados. Para isso são necessárias reações químicas, geralmente utilizando um agente reticulante. Esse tipo de gel é muito estável e são considerados géis verdadeiros (HILAL; FLOROWSKA; WRONIAK, 2023).

A reticulação física, por sua vez, ocorre através de interações não covalentes, nesse tipo de formação de rede as forças precursoras para a estruturação são provenientes principalmente das interações eletrostáticas, pontes de hidrogênio, interações hidrofóbicas que ocorrem entre os polímeros presentes. Hidrogéis por reticulação física podem ser obtidos por diferentes métodos, como: congelamento-descongelamento, formação de complexos estéreos, interação iônica e ligação de hidrogênio (FLETES-VARGAS; ESPINOSA-ANDREWS; PITA-L, 2021; HILAL; FLOROWSKA; WRONIAK, 2023).

A forma como esses géis são obtidos influencia diretamente em suas propriedades de reversibilidade e estabilidade. Em muitos casos, vários mecanismos de reticulação combinados são usados para compor o gel, dependendo da complexidade da matriz de hidrocoloides utilizadas (HILAL; FLOROWSKA; WRONIAK, 2023). A gelificação pode ser um mecanismo utilizado para adicionar estabilidade aos sistemas. Emulsões tradicionais podem ser gelificadas para adquirir maior estabilidade durante o período de estocagem, melhorar a textura causada pelas partículas lipídicas presentes e melhorar a capacidade de retenção de água em produtos cárneos (LIN; KELLY; MIAO, 2020). As emulsões A1/A2, também podem ser gelificadas, para regredir processos de separação de fases além de inibir o processo de floculação e coalescência das gotas (BU et al., 2025).

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CAPÍTULO III

Incompatibility between sodium caseinate - locust bean gum induced by NaCl and yerba mate extract

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Incompatibility between sodium caseinate - locust bean gum induced by NaCl and yerba mate extract

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ABSTRACT: Aqueous two-phase system (ATPS) is a technique used for the separation of biopolymers in two aqueous phases. Some combinations of biopolymers can form a water-in-water (W/W) emulsion due to steric exclusion and thermodynamic incompatibility between these biopolymers under some specific conditions. In this work, the formation of W/W emulsions composed of sodium caseinate (SCN) and locust bean gum (LBG) was evaluated, using NaCl or yerba mate extract as the driving force for the phase separation, which was described by phase's diagrams. Phase diagrams are like fingerprints of ATPS systems, which demonstrate the specific conditions to develop separate phases. Phase diagrams of the two systems show that at the same concentrations of protein and carbohydrate, the addition of NaCl or extract induced the separation of the compounds differently. Salt promotes phase separation by steric exclusion, each phase being rich in one of the polymers. Since extract may also induce other effects, such as the formation of a SCN-extract-LBG complex, migration of LBG to the SCN-rich phase was promoted, modifying the characteristics of the tie lines in the phase diagrams. However, it was feasible to separate the protein in systems containing concentrated phenolic extract, whose incorporation is relevant considering its antioxidant activity.

Keyword: phase diagram; W/W emulsion; yerba mate extract

3.1. INTRODUCTION

Proteins and polysaccharides are biopolymers that impart technological properties to food products such as thickening agent, texture modifier and emulsifier. The combination of proteins and polysaccharides in aqueous systems is generally compatible, forming solutions with a single phase. However, some specific mixtures of proteins and polysaccharides promote the formation of biphasic systems (or aqueous two phase systems) and this immiscibility phenomenon can induce the formation of water-in-water (W/W) emulsions

[1]. Water-in-water emulsions can be used as carrier systems for hydrophilic compounds, with the advantage of being biocompatible and their production does not depend on the addition of synthetic surfactants [2].

Phase behavior of aqueous systems can be described by the Gibbs free energy of the mixture. Miscibility is favored mainly in diluted systems if the entropic variation is greater than enthalpy [3]. However, at higher biopolymers concentration and a given temperature, phase separation can occur if the variation in enthalpy of the system caused by the endothermic molecular interactions is greater than the entropy variation, resulting in positive Gibbs free energy. Therefore, depending on the polymers concentration and temperature, aqueous systems composed of protein and polysaccharides can present the formation of two distinct aqueous phases [4,5]. Different phenomena are responsible for phase separation in aqueous systems through non-covalent interactions categorized as associative or segregative. Associative systems are formed by polymers with opposite charges, promoting the formation of a phase composed predominantly by water and a residual amount of polymers and another aqueous phase containing complexes formed by the combination of the two polymers [1,2,6]. In segregative systems, one polymer is charged and the other polymer is neutral. Thus, the two phases are separated by steric exclusion effects, each enriched with a polymer. The incompatibility of aqueous systems formed by proteins and polysaccharides can be induced with the direct addition of salts, which in high concentrations, promote a salting out effect that favors hydrophobic interactions between proteins and, consequently, biphasic formation. In addition to hydrophobic interactions, pH can change the surface properties of the protein which also affects the separation between molecules [7].

There are several known protein-polysaccharide pairs that form aqueous two phase systems (ATPS), such as starch - gelatin [1], maltodextrin - gelatin [8] and locust bean gum - sodium caseinate [9]. It has been shown that the addition of sodium chloride (NaCl) facilitated the formation of W/W emulsion in mixtures of sodium caseinate (SCN) and locust bean gum (LBG) [10]. Also, ingredients such as sucrose, widely used in food systems, can modify the interaction behavior between milk protein-LBG, changing the symmetry of the tie-lines in the phase diagrams [11]. Other compounds present in food matrices can also influence the phase behavior in protein-polysaccharide systems. For example, phenolic compounds widely found in plant extracts can interact with proteins and carbohydrates through hydrogen bonds, hydrophobic interactions and electrostatic forces [12,13].

Phenolic compounds, such as tannins, flavonoids and anthocyanins can be added to food matrices to bring numerous health benefits with the prevention of various diseases due to their antioxidant properties. Yerba mate (*Ilex paraguariensis*) is a plant native to South

America [14], whose extract is rich in polyphenols as xanthines, saponin and caffeoyl derivatives [15]. In addition to antioxidant activity, it has hypocholesterolemic, hepatoprotective and diuretic properties, as well as to stimulating the central nervous system. It also has the potential to regulate the glycemic index and can be an important natural agent in the treatment of diabetes [16].

The preservation of the antioxidant activity of phenolic compounds is a challenge for their application, as they are susceptible to degradation during processing (pH and high temperatures) and storage. Thus, many studies aim to produce systems to preserve them, such as W/O and W/O/W emulsions [17,18]. However, systems with an oil phase restrict their application or their removal is necessary, which is not always feasible and efficient, making aqueous systems as W/W emulsions more interesting for this purpose.

Considering the relevance of vehiculating phenolic compounds in biopolymeric matrices and the possible influence that these compounds may have on phase behavior of proteins and polysaccharides, the objective of this work was to carry out a comparative study between ATPS (formed by sodium caseinate and LBG) produced with the addition of NaCl or yerba mate extract. The properties of the systems were evaluated from the capacity to produce W/W emulsions through phase diagrams, rheological behavior and microstructure.

3.2. MATERIAL AND METHODS

3.2.1. Material

Sodium caseinate (approximately 82 % (w / w) of protein) was provided by Alibra Ingredients Ltda. (Brazil), locust bean gum (LBG) with approximately 1 % (w/w) of protein donated by CP Kelco (USA) and mate tea extract was kindly granted by Triunfo (Brazil). Sodium chloride (analytical grade) was purchased from Labsynth LTDA. (Brazil). The yerba mate extract was a donation from the company Triunfo (Brazil). According to the manufacturer, it is an aqueous extract, which was dried in a spray dryer and contained 13.6 % (w/w) of protein. Sodium azide (Sigma Chemical Co., USA) was added to protein solutions in order to prevent microbial growth, while Rhodamine B was used to dye protein for fluorescence microscopy (Sigma Chemical Co., USA). All solutions were prepared with deionized water.

3.2.2. Preparation of W/W emulsions

Stock solution of sodium caseinate (SCN) 12 % (w/w) was prepared at 25 °C using magnetic stirring for 1h and then stored for 24h to complete hydration. Stock solution of locust bean gum (LBG) 1 % (w/w) was prepared at 85 °C for 1h with stirring and, after cooling, it was

stored for 24h to complete hydration. In both solutions, sodium azide was added to prevent microbial growth and the pH was adjusted to 7.0. Mixtures of biopolymers were prepared in order to present different concentrations of SCN (2-6 % w/w) and LBG (0.05-0.5 % w/w). In addition, mixtures of biopolymers with the same composition also had the addition of NaCl (0.3 % and 4 % w/w) or extract of yerba mate (2 % w/w). All the mixtures were stirred using magnetic agitation for 1h at 25 ˚C for complete homogenization of the components and then stored at 25 ˚C for 24 h in transparent glass flasks with a lid, in order to have a good visibility of the phase behavior and avoid evaporation.

3.2.3. Building phase diagrams

After 24 hours of storage, it was possible to identify a visual separation of phases of the mixtures, with an opaque bottom phase and a translucent top phase. The bottom phase was identified as rich in protein because it was the densest, while the top phase was defined as rich in polysaccharides. Based on the results found, a visual phase diagram was built to outline the concentration values that occur W/W emulsion formation.

After verifying the visual separation of the phases, each phase was carefully collected with a pipette and transferred to separate flasks. The phases composition was analyzed to build phases diagram with the concentration data evaluated experimentally.

The concentration of SCN and LBG in the initial mixtures (freshly prepared), as well as in the top and bottom phases of the mixtures (after 24h) were analyzed. The protein content was determined by the micro-Kjeldahl method (AOAC, 1996), while LBG concentration was obtained by the colorimetric method of sugar determination using phenol/sulfuric acid reaction [20]. These methods are consolidated methodologies for the determination of proteins and carbohydrates, which have already been used in other studies [9,10,21,22].

A mass balance was performed from the concentrations (% w/w) of carbohydrate and protein to verify the consistency of the data obtained by the tie-lines. For this purpose, the experimental volume of the initial mixture and respective concentrations (% w/w) of protein and carbohydrate were used, comparing with the fractions of carbohydrate and protein found in each experimental volume after phase separation, using the equations (1), (2), (3), (4) and (5).

$$VM_{EXP} = \frac{V_{Mixture}}{V_{Total}} \quad (1)$$

$$VB_{EXP} = \frac{V_{Bottom\ phase}}{V_{Total}} \quad (2)$$

$$VT_{EXP} = \frac{V_{Top\ phase}}{V_{Total}} \quad (3)$$

$$[MB = CM.VM_{EXP} = CB.VB_{EXP} + CT.VT_{EXP}] * 100 \quad (4)$$

$$Error\ (\%) = |100 - MB| \quad (5)$$

Where: VM,B,T_{EXP} is the experimental volume ratio,$V_{Bottom\ phase}$ is the corresponding volume of the formed in the bottom phase, $V_{Top\ phase,}$ is volume formed in top phase. V_{Total} is total volume of the mixture. MB is the mass balance. CM, CT and CB are the concentrations of each biopolymer in the initial mixture (% w/w), the top phase (% w/w) and bottom phase (% w/w), respectively. VM_{EXP}, VT_{EXP} and VB_{EXP} are the volume ratios of the initial mixture, the top phase and the bottom phase, respectively. Error (%) is the percentage error of the mass balance.

3.2.4. Rheological measurements

Rheological measurements of the stock solutions, the initial mixtures (freshly prepared) and the separated phases were performed using a stress-controlled rheometer (TA Instruments, England). Double-wall concentric cylinder geometry was used, in which the internal acrylic cylinder (internal radius 20.38 mm, external radius 21.96 mm) is rotatable and the external cylinder made of stainless steel (internal radius 20 mm, external radius 22.38 mm) is fixed. The flow curves were obtained within the range between 0 and 300 s^{-1}. Three shear stress sweeps (up-down-up steps) were performed to verify the presence of shear time effects (thixotropy). Data from the third flow curve (steady state conditions) were used to fit the power-law model according to Equation 6. Viscosity at 10 s^{-1} was also presented in order to compare the different biopolymers solutions.

$$\sigma = k.\dot{\gamma}^n \quad (6)$$

Where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), k is the consistency index ($Pa.s^n$) and n (dimensionless) is the behavior index.

3.2.5. Fluorescence microscopy

The initial mixtures (freshly prepared) and the separated phases were observed under fluorescence optical microscopy using Carl Zeiss Model AxioScope A1 microscope (Zeiss, Germany), with 100x objective lenses and excitation filter at 515 and 560 nm. Rhodamine B was used as a protein marker, with application of 0.1 μL over 1 μL of emulsion.

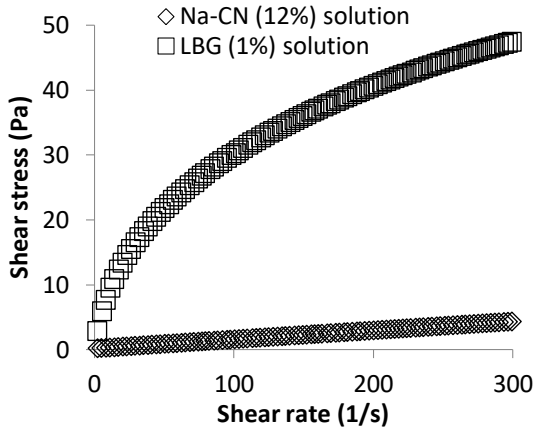
3.2.6. Statistical analysis

Analysis of variance (ANOVA) was performed using a free version of the ASSISTAT 7.7 software. Differences between means were performed using the Tukey test with significance level ($p<0.05$).

3.3. RESULTS AND DISCUSSION

3.3.1 Phase behavior of SCN and LBG systems: definition of composition

Stock solutions of SCN and LBG were characterized in terms of rheological properties, protein and carbohydrate concentration (Figure 3.1).



Solution	SCN 12% (w/w)	LBG 1% (w/w)
$\eta_{10s^{-1}}$ (Pa.s)	0.03	0.82
n	1	0.45
k (Pa.s^n)	0.01	3.77
R^2	0.99	0.99
% Protein (w/w)	10.12 ± 0.04	0.06 ± 0.01
% Carbohydrate (w/w)	0.183 ± 0.01	0.69 ± 0.02

Figure 3.1. Characterization of the stock solutions used to produce W/W emulsions.

The flow curves of the aqueous solutions of SCN and LBG showed different rheological behavior, since the sodium caseinate solution presented almost Newtonian behavior, while the LBG solution presented a nonlinear and shear thinning behavior. Flow curves of both LBG and SCN solutions did not show thixotropy. In addition, the viscosity of the LBG solution presented values approximately 27 times higher, even with a concentration 12 times less than the sodium caseinate solution. Although a concentration of 12% (w / w) of protein has been used for sample preparation, protein analysis indicates that the mixtures have approximately 20% less protein than expected. The same occurred for the evaluated carbohydrate concentrations that have about 30 % less carbohydrates than added. This difference between calculated and actual concentrations may be related to losses during the preparation of solutions and mixtures, as well as the purity of the raw material. Although a difference was observed between the content of biopolymers added and the content quantified in the stock solutions, all the results presented were obtained after the

physicochemical characterization of the concentration of proteins and polysaccharides and not based on the amount initially added. Thus, these differences do not interfere with the reliability of the data presented.

Some process variables are decisive for the formation of W/W emulsions, such as molecular weight and concentration of the biopolymers, in addition to pH and temperature [7,11]. To evaluate the effect of NaCl or yerba mate extract to obtain biphasic systems, preliminary tests were performed keeping the pH, biopolymers concentration and temperature fixed. Concentrations of sodium caseinate (SCN) systems and locust bean gum (LBG) were 4 % (w/w) and 0.4 % (w/w), respectively, as these concentrations presented phase separation without adding salt at pH 5.5 [9]. However, mixtures prepared with the same concentrations (4 % w/w SCN and 0.4 % w/w LBG) did not show visual phase separation after 24 hours without adding salt, although we maintained the fixed pH at 7.

In order to induce phase separation, 0.3 % (w/w) NaCl was added because a similar concentration was used in other systems with SCN-LBG [10]. In addition, the ability to form separate phases with the addition of 2 % (w/w) of yerba mate extract was evaluated. This concentration was chosen because the range of 1-2.5% of extract is generally used for systems for carrying plant extracts rich in phenolic compounds [23,24]. Finally, a mixture of NaCl and yerba mate extract (0.3 % w/w NaCl + 2 % w/w yerba mate extract) was tested and added to the solution containing SCN and LBG. As a control, single solutions containing only protein or polysaccharide were also added with NaCl, extract of yerba mate and the mixture NaCl + and extract of yerba mate. None of the mixtures showed phase separation (results not shown). The protein concentration (% w/w) of the initial mixture and separate phases of the different systems is shown in Figure 3.2.

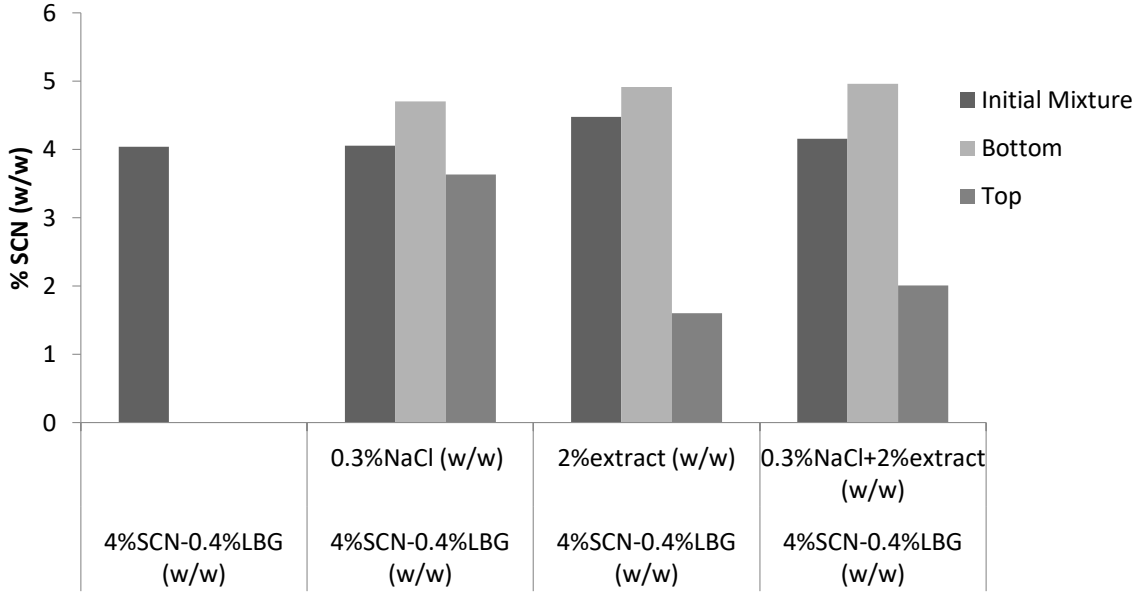


Figure 3.2. Protein concentration (% w/w) of the initial mixture, bottom and top separated phases of systems composed by 4 % (w/w) SCN, 0.4 % (w/w) LBG and NaCl (0.3 % w/w), yerba mate extract (2 % w/w) and NaCl + yerba mate extract (0.3 % w/w + 2 % w/w).

The system containing only SCN and LBG did not show phase separation, as previously mentioned, but it was possible to observe separation after adding NaCl or yerba mate extract. The addition of 0.3% (w/w) NaCl promoted a low protein partition, since the protein fractions of the top and bottom phases were very similar to the initial concentration of the mixture. Studies have shown that phase separation was more intense with increas salt concentration (50mM/300mM) in SCN-LBG systems, while in low NaCl concentration (50mM or approximately 0.3 % w/w) phase separation occurred only in high concentrations of SCN [10]. Thus, in the next experiments, a concentration of 4 % (w/w) NaCl was used to guarantee phase separation.

With the addition of 2% (w/w) extract to the system, there was a better separation of protein between phases, since the bottom phase was more concentrated in protein. However, the separation was more pronounced when both extract and NaCl were added in the system. Yerba mate extract is rich in polyphenols, which can show covalent and non-covalent interactions with carbohydrate hydroxyls and with amino groups of proteins, changing the solubility of these compounds (ZHOU et al., 2020).

Yerba mate extract has approximately 9.6% of caffeine derivatives (caffeic acid, chlorogenic acid, 3,4-Dicaffeoylquinic acid, 3,5-Dicaffeoylquinic acid and 4,5-Dicaffeoylquinic acid), which are the main phenolic compounds with antioxidant activity in mate tea [15]. In this study, the total extract concentration used was 2% (w/w) and, therefore, the total percentage

of phenolic compound was approximately 0.2% (w/w). That is, although the concentration of phenolics is close to the concentration of salt (NaCl 0.3% w/w), there seems to be a greater interaction between the extract and the polymers (SCN-LBG), than with the presence of NaCl.

Although the extract of yerba mate has in its composition important mineral fractions such as manganese and aluminum, the release of these compounds in water is inversely proportional to the presence of phenolics. Therefore, the higher the concentration of phenolic compounds, the lower the release of these minerals in water [15], showing a smaller effect of cations in comparison to phenolics. It should be considered that, in addition to the antioxidant and mineral compounds, the yerba mate extract presented 0.26% (w/w) protein and 0.012% (w/w) carbohydrates, which may also have influenced the effect of the extract on the phase behavior. Due to the complex nature of the extract (phenolics, proteins, carbohydrates and minerals), the concentration of NaCl (4 % w/w) used was twice that of the extract (2% w/w), in order to obtain similar effects on phase behavior.

3.3.2 Visual phase diagram and tie-line determination

The visual phase diagrams were obtained from the identification of separated phases in varying concentrations of SCN and LBG. Figure 3.3 shows the diagrams for aqueous solutions containing SCN + LBG at pH 7 that have added NaCl (4 % w/w).

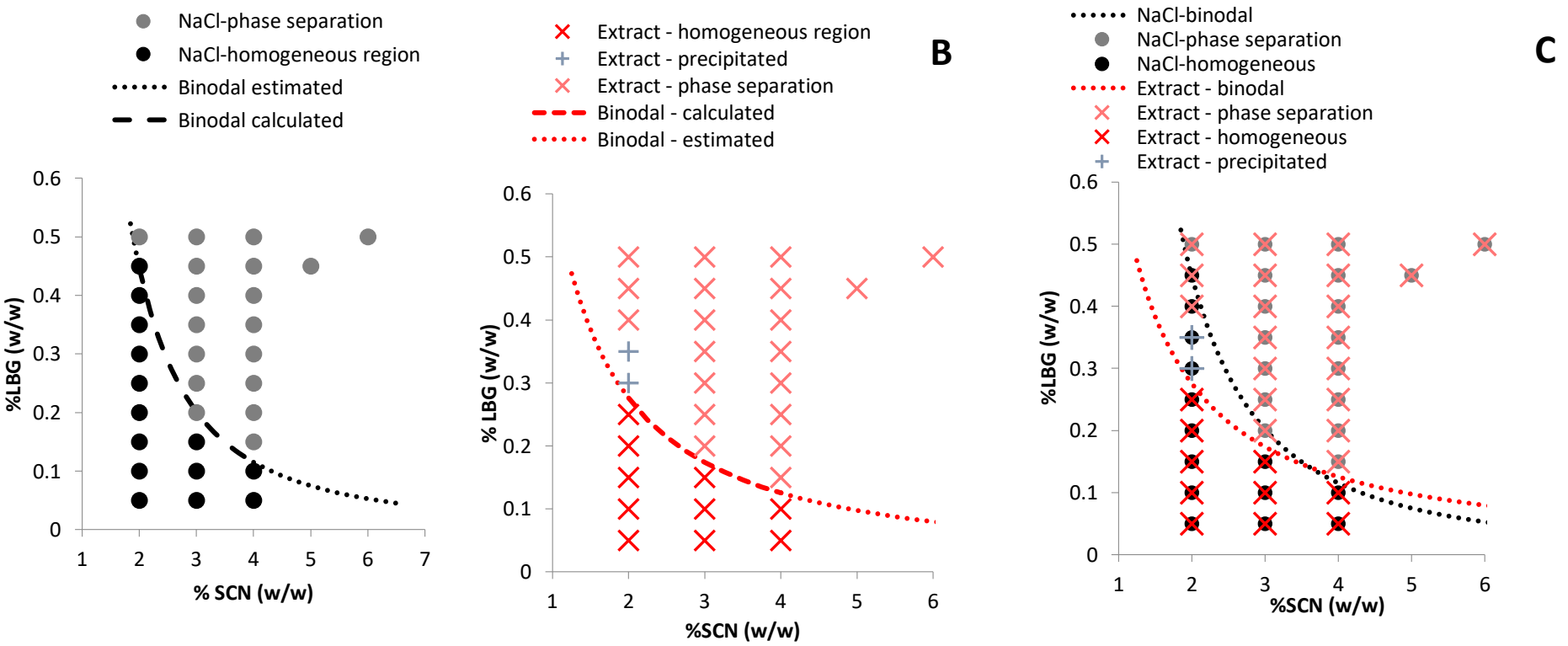


Figure 3.3. Visual phase diagrams of mixtures of SCN and LBG: A- with 4 % (w/w) NaCl, B- with 2 % (w/w) extract and C - overlapping diagrams A and B. Binodal – calculated: obtained from the equation fitted to the experimental data. Binodal – estimated: extrapolation from the empirical equation.

The phase separation was more pronounced with the increase of salt content (0.7M or 4% w/w) compared to the concentration initially used (0.05M or 0.3% w/w). In general, concentrations above 0.1 M are used [25] to observe phase separation. For the diagram containing extract, the same concentration was used for all experiments (2% w/w).

Only the mixture containing 6% (w/w) of protein showed phase separation without the addition of NaCl or extract, considering the concentrations of SCN evaluated in this study (2-6% w/w). However, the addition of NaCl or yerba mate extract allowed the identification of macroscopic phase separation at lower SCN concentrations, forming a whitish opaque phase at the bottom of the flask (rich in protein) and a more translucent top phase, rich in LBG [10]. It is possible to identify the binodal curve that delimits the phase separation regions for the two systems (A and B) in the diagrams (Figure 3.3). The small displacement of the binodal between the two conditions (addition of NaCl or extract) is due to the appearance of samples with precipitate in the diagram with extract. This indicates that the addition of twice the NaCl concentration (% w/w) in relation to the extract exerted a similar effect on visual phase separation.

The volume ratio of the separated phases (%) and the concentration of SCN and LBG (% w/w) of the phases after 24h of preparation were evaluated (Fig. 3.4) for building of tie-lines (Fig. 3.5), to understand the phase behavior.

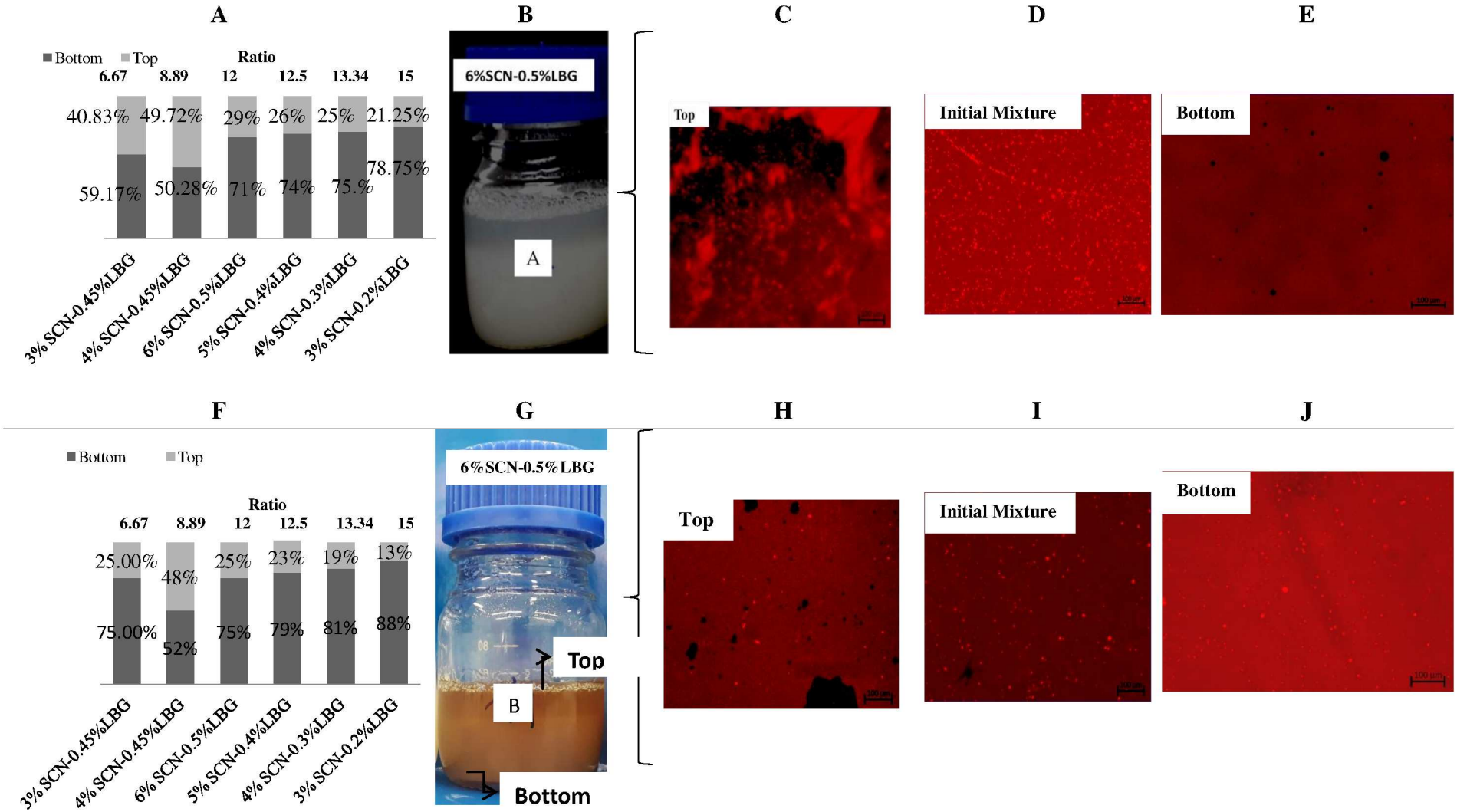


Figure 3.4. Volume phase separation: A, F- Systems with SCN and LBG with phase volumes showed in different SCN:LBG ratios. B, G- 6 % (w/w) SCN-0.5 % (w/w) LBG. C, D, E, H, I, J- Fluorescence microscopy images of 6 % (w/w) SCN- 0.5 % (w/w) of the top phase, initial mixture and bottom phase. A, B, C, D and E- 4% (w / w) NaCl and F, G, H, I, J - 2% (w / w) extract. Protein (SCN) is in red and carbohydrate (LBG) is in black in the microscopies.

Higher values of the SCN/LBG ratio (higher protein concentration) led to lower volumes of the top phase (rich in polysaccharide) (Figure 3.4 A, G). The highest SCN/LBG ratio was 15 for the 3 % (w/w) SCN-0.2 % (w/w) LBG system, in which the lowest volume of the top phase was observed. On the other hand, the lowest ratio was 6.67 for the 3 % (w/w) SCN-0.45 % (w/w) LBG system, although the highest volume of the top phase was observed in the 8.88 ratio (4 % (w/w) SCN-0.45 % (w/w) LBG), both with the addition of NaCl and with extract.

For systems containing extract, the top phase rich in polysaccharide showed lower volume compared to systems containing NaCl in the same SCN: LBG ratios. This phenomenon may be an indication that the addition of the extract did not increase the LBG partition to the top phase due to a possible protein/carbohydrate/extract interaction, an effect also identified in the low slope of the tie lines of the phase diagrams (Fig. 3.5).

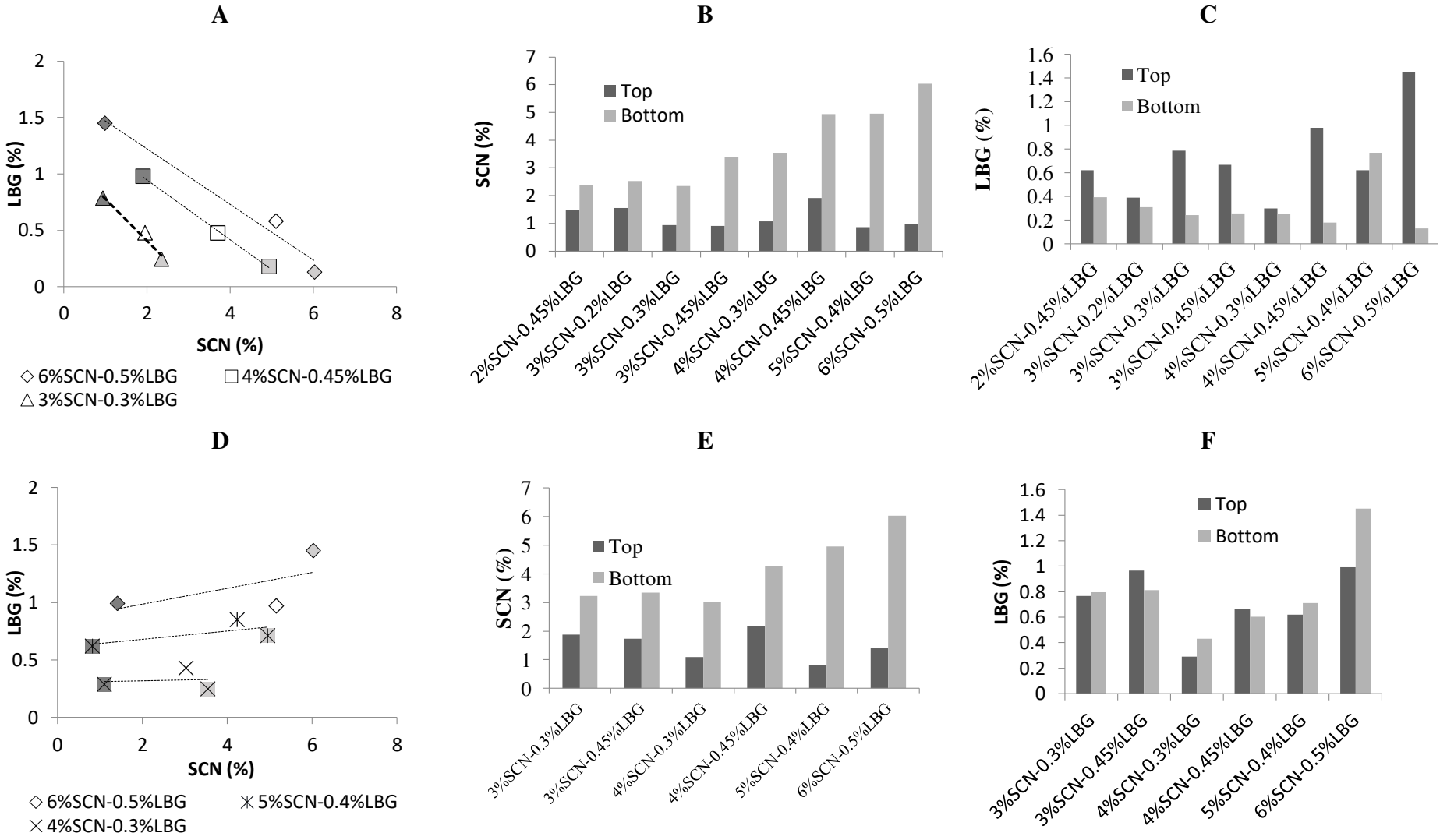


Figure 3.5. Tie-lines and composition of the separated phases. A, D- Tie-lines of the SCN-LBG phase diagrams (initial mixture, top and bottom phases). B, E - % SCN (w/w) of top and bottom phases. C, F- % LBG (w/w) of top and bottom phases . A, B, C- 4% (w / w) NaCl and D, E, F- 2% (w / w) extract.

Protein and carbohydrates concentration of the separate phases were quantified for the diagram of the equilibrium phases with tie lines (Figure 3.5). The equilibrium diagrams obtained with the physicochemical characterization of the initial mixtures and separate phases show that the addition of NaCl (Figure 3.5A) or extract (Figure 3.5D) alters the equilibrium conditions, interfering with the length and slope of the tie lines. Addition of NaCl changes the partition coefficient, to the presence of ions with different hydrophobicities. Another factor that interferes with the phase diagram is the difference in the molar mass of the polymers, because the greater this difference, more asymmetric is the diagram [7,26]. Phase diagrams serve as a fingerprint for ATPS, as they describe the phase behavior for a given temperature and pH. Any system whose composition corresponds to the same tie-line will have the same equilibrium composition after the phase separation. However, the description of the phase behavior of biopolymers mixtures is complex, as it is determined by the electrochemical potential, hydrophobicity, biospecific affinity, molecule size and spatial conformation [2,7]. In addition, it is difficult to accurately predict the effect of molecular interactions in complex systems, such as proteins, carbohydrates and extract [2].

Some studies show that the protein-polysaccharide interaction depends on pH, as in the case of the pair casein - pectin. At pH 6.7, this mixture tends to form two aqueous phases with an increase in the concentration of biopolymers, due to the thermodynamic incompatibility between macromolecules. However, in acidified solutions between pH 4.3 and 5, casein aggregates are formed near isoelectric point (net liquid charge is near zero) and adsorption of pectin on the surface of casein was observed. Therefore, two aqueous phases were also formed, but one of them was predominantly composed by the excess of pectin and the other by casein aggregates with the adsorbed pectin [27]. Similar effects can be seen between caseinate and LBG at pH 7, although LBG is a neutral polysaccharide and pectin is negatively charged. The yerba mate extract is rich in phenolic compounds that can interact with the protein, leading to the formation of complexes [28,29]. With this modification of the characteristics of the protein, LBG may have partly been adsorbed on the surface of protein-phenolic complexes/aggregates, explaining the presence of LBG in the upper and bottom phases.

Despite the limitations faced in the study of these systems, their understanding facilitates the development of formulations in the required working conditions, ensuring the phase separation in a specific region of interest.

The diagram using NaCl (Figure 3.5A) showed a behavior similar to the diagrams found in the literature, in which the top phase is rich in polysaccharide and low in protein, while the

bottom phase is rich in protein and low in polysaccharide [30]. A higher SCN content (% w / w) in the bottom phase was observed (Figure 5B), while the LBG concentration (% w / w) was higher in the top phase (Figure 3.5C), except for 5% SCN-0.4% LBG. Systems with the composition on the tie lines showed that the top phase presented deformed droplets of protein dispersed in a continuous phase with a high concentration of LBG, whose high viscosity may have contributed to the deformation of the droplets. In the bottom phase, LBG droplets were formed in a continuous low viscous protein phase.

The concentration of SCN (% w / w) had the same behavior as mixtures with NaCl (Figure 3.5E) in systems with extract, but the concentration of LBG was similar in both phases (Figure 3.5F). Tie lines in the diagram with extract (Figure 3.5B) show the bottom phase with the highest concentration of SCN and the top phase with the lowest protein concentration. However, LBG concentration of the top and bottom phases showed similar values, which explains the low slope of the tie line. A small slope of the tie lines indicates that, although the mixtures have presented droplets formation and a biphasic appearance after 24 hours, these results cannot be studied only as an ATPS. Another atypical factor was the formation of protein droplets in the bottom phase, which also had a continuous protein-rich phase, making it impossible to identify the presence of carbohydrates by microscopy, although the LBG concentration was not low.

The mass balance of protein and polysaccharide of the phases was performed (Table 3.1) to evaluate errors of the biopolymer concentrations and their effect on the behavior of the tie lines.

Table 3.1. Relative error (%) of the protein and LBG content calculated from mass balance and based on the chemical analyses of the phases and initial composition.

Mixture composition	Biopolymer	Relative error (%)	
		Addition of NaCl	Addition of extract
6%SCN-0.5%LBG	SCN	10.41	5.32
	LBG	11.58	37.61
5%SCN-0.4%LBG	SCN	18.31	3.56
	LBG	66.13	17.23
4%SCN0.45%LBG	SCN	0.92	3.43
	LBG	6.38	3.26
4%SCN0.3%LBG	SCN	11.43	24.99
	LBG	40.59	61.36
3%SCN0.3%LBG	SCN	0.89	3.40
	LBG	14.29	11.17

According to the calculation of the mass balance it is possible to observe that the analysis of proteins and carbohydrates showed a great variation of error without a clear trend. Therefore, it is not possible to state that the errors found from the mass balances are directly related to the difference in the slope of the tie-lines of the systems with NaCl and extract, since the samples with extract showed the same slope trend, both with low as high balance mass error. The biggest error was observed for LBG concentration, which could be attributed to the lowest content of this component and inherent errors associated to the collection of a homogeneous sample.

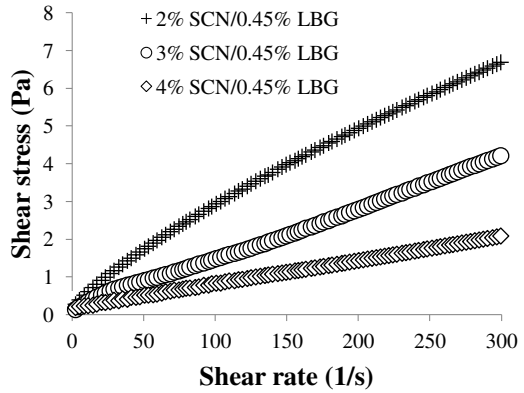
Therefore, the behavior of the tie-lines seems to be related to the physicochemical nature involved during the phase separation process. While samples with NaCl present separation by a segregative phase separation mechanism, samples with yerba mate extract show a more complex separation process. These results indicate that the addition of the extract partitions the biomolecules in a different way than occurs with the addition of NaCl. The tie-lines show a good partition of proteins that are predominantly in the bottom phase in both systems (with addition of NaCl and extract). Nevertheless, the carbohydrate behaves differently when comparing the two systems, because it is predominantly in the top phase in the added salt system and divided into similar fractions both in the top and bottom phases in the extract system. Probably, in addition to the thermodynamic incompatibility, the high complexity of the extract composition may have generated other molecular interactions. Although little

studied, it is possible to form complexes between proteins with a high net charge and neutral polysaccharides under conditions of low ionic strength and at a different pH from the protein isoelectric point [31].

3.3.3 Rheology and Microstructure

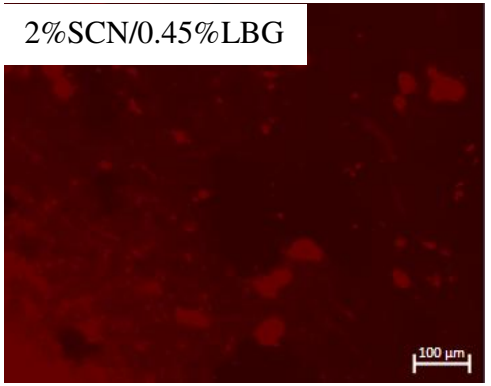
Flow curves of the initial mixture were evaluated with the addition of NaCl (Fig 3.6A) and extract (Fig. 3.6D), where the carbohydrates concentration was fixed at 0.45% (w/w) and protein concentration varied from 2 to 4% (w/w). These systems were chosen to evaluate the effect of the concentration of proteins and polysaccharides on the rheological behavior and microstructure that promotes the formation of W/W emulsions. Rheological flow curves were obtained for the initial mixture and respective phases formed for samples with NaCl and for systems with extract (Fig 3.7), to evaluate the phase separation between SCN and LBG.

A

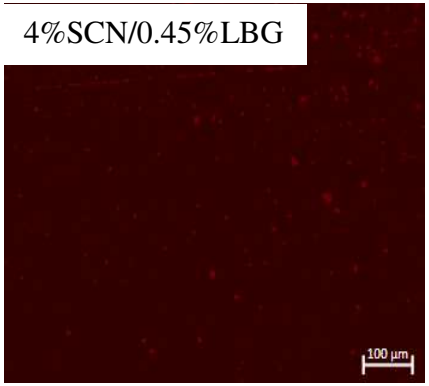


Sample	NaCl $\eta_{(10s)}^{-1}$ Pa.s
2%SCN-0.45%LBG	0.055
3%SCN-0.45%LBG	0.035
4%SCN-0.45%LBG	0.024

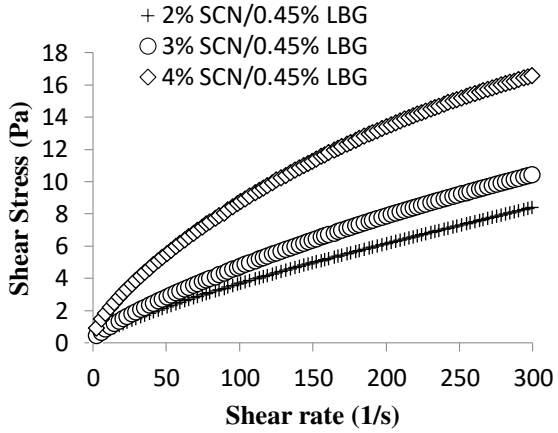
B



C

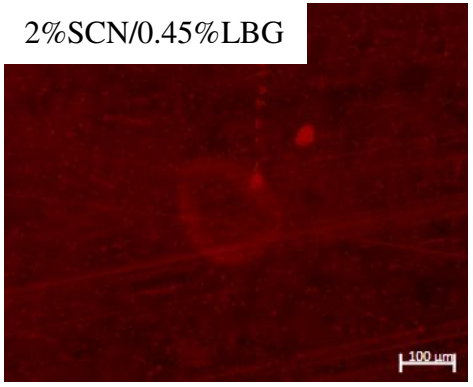


D



Sample	Extract $\eta_{(10s)}^{-1}$ Pa.s
2%SCN-0.45%LBG	0.081
3%SCN-0.45%LBG	0.11
4%SCN-0.45%LBG	0.63

E



F

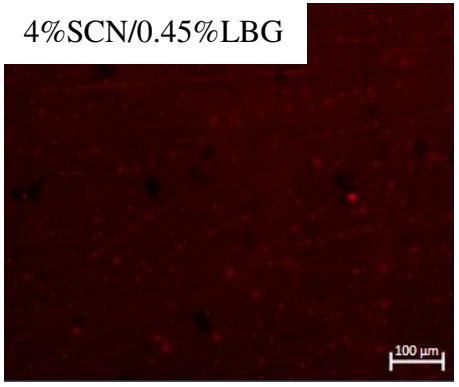
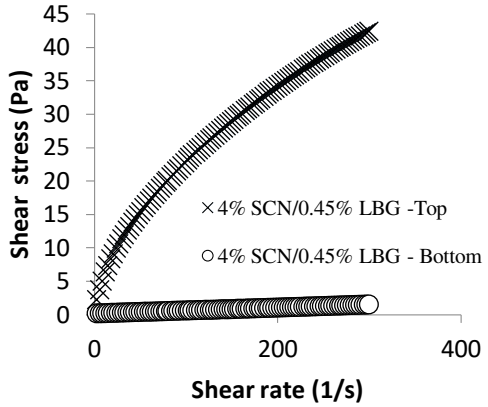


Figure 3.6. Flow curves of the initial mixture of emulsions W/W (A, D) with NaCl (A) and extract (D). Fluorescence microscopy images of the initial mixture of (B, C, E, F) 2% SCN-0.45% LBG with NaCl (B), 4% SCN-0.45% LBG with NaCl (C), 2% SCN-0.45% with LBG extract (E) and 4% SCN-0.45% LBG extract (F). Rhodamine B was used to dye the protein, being SCN represented by light red and LBG by dark regions.

Mixtures containing yerba mate extract showed an increase of shear stress (and, consequently, apparent viscosity) (Fig. 3.6D), with the increase of SCN concentration. This increase in viscosity is expected considering the increase in the total concentration of biopolymers. Fluorescence microscopy (Fig. 3.6E, 3.6F) shows that in both concentrations, in addition to small droplets of SCN, small structures of LBG were identified, although they were more evident at the highest concentration of SCN. The initial mixtures with NaCl showed an unexpected decrease of shear stress (and, consequently, apparent viscosity) (Fig. 3.6A) showed decreasing results with the increase % of SCN concentration (% w/w). As the LBG concentration was constant (0.45% w / w), the phase separation is more intense with the increase of SCN concentration. In this sense, as the samples were always taken at the same point (in the middle of the recipient containing the sample), we assume that more LBG content was collected in samples with 2% (w/w) SCN than the sample with 4% (w/w) SCN, due to the faster phase separation occurring in the presence of NaCl.

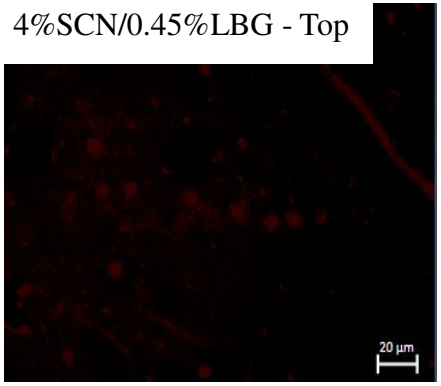
This assumption can be verified by the formation of structures that could be observed in fluorescence microscopy images (Fig. 3.6B and 3.6C), where samples with 2% (w/w) SCN, formed larger protein droplets when compared to the sample containing 4% (w/w) SCN, evidencing the occurrence of phase separation, although the collection and evaluation were carried out right after the preparation of the mixture. In addition, LBG structures were evident in only 2% (w / w) protein. After 24h the top and bottom phases were collected and their rheological properties were measured. Flow curves and fluorescence microscopy are presented in Figure 3.7. The samples containing 4% (w/w) SCN-0.45% (w/w) LBG were chosen, as they presented the most pronounced phase separation.

A



4%SCN-0.45%LBG- NaCl		
	Phase	
	Top	Bottom
$\eta_{10s^{-1}}$ (Pa.s)	0.55	0.019
k (Pa.s^n)	1.66	0.017
n	0.57	0.78

B



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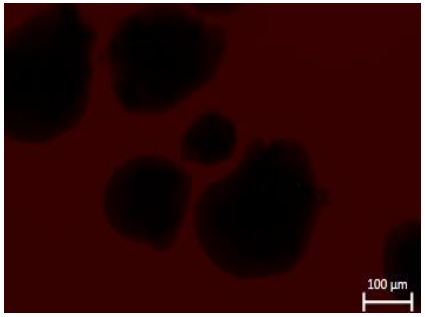

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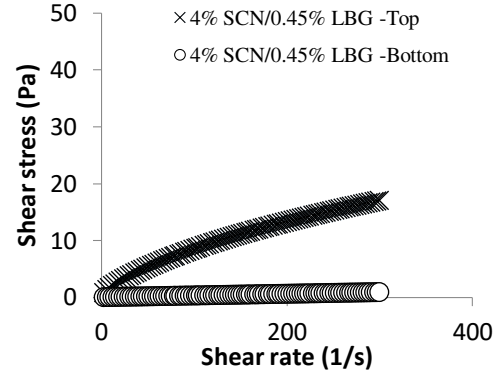
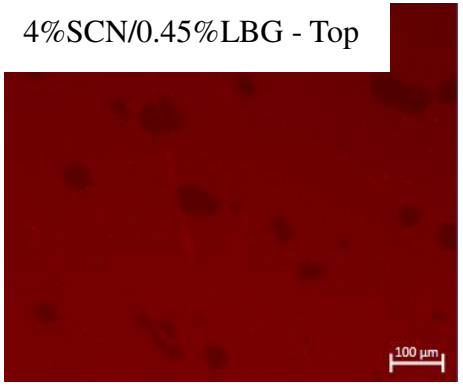


4%SCN-0.45%LBG-Extract		
	Phase	
	Top	Bottom
$\eta_{10s^{-1}}$ (Pa.s)	0.57	0.004
k (Pa.s^n)	4.72	0.004
n	0.17	0.97

E



F

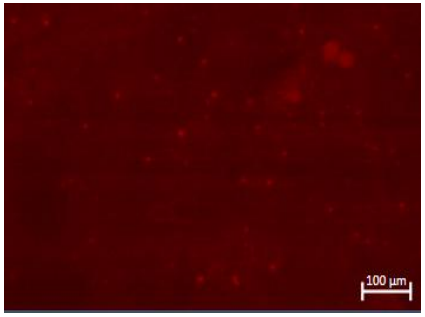
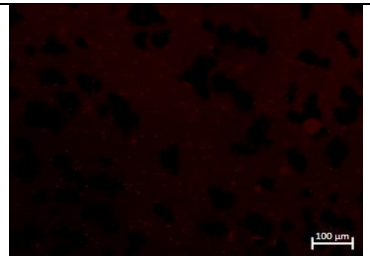
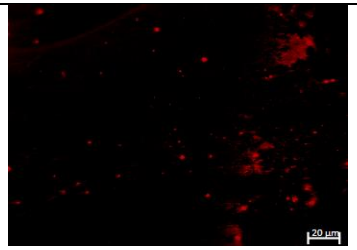
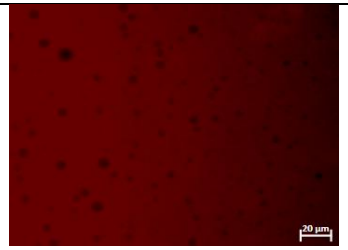
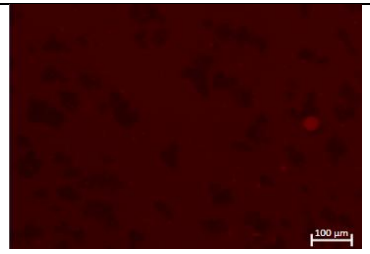
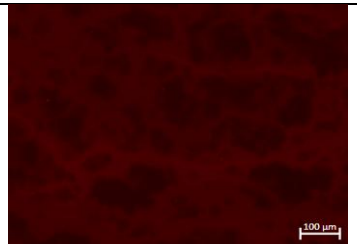
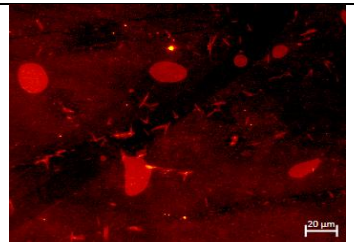
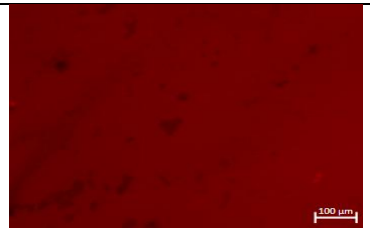
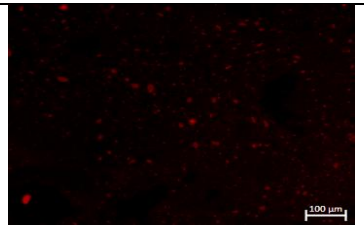
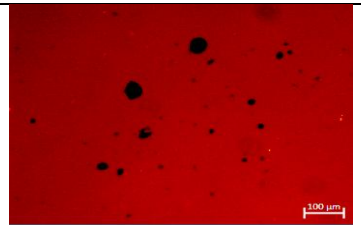
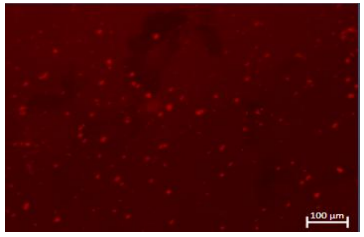
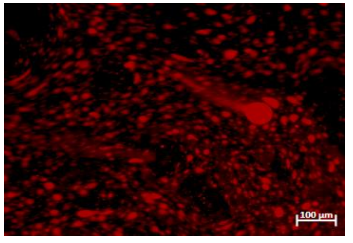
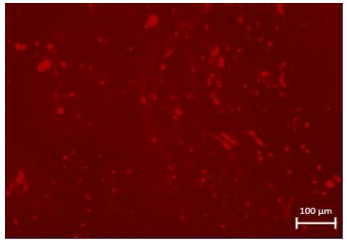


Figure 3.7. Flow curves and fluorescence microscopy images of top and bottom phases for 4%SCN-0.45%LBG. Flow curves of the top and bottom phases (A, D), (A) with NaCl and (D) with yerba mate extract. Fluorescence microscopy images of the top (B, E) and bottom (C, F) phases, being (B, C) with NaCl and (E, F) with yerba mate extract. Rhodamine B was used to dye the protein, SCN is represented by light red and the dark regions representing LBG.

The top phase of systems containing NaCl (Fig. 3.7A) or yerba mate extract (Fig. 3.7D), showed high apparent viscosity and a shear thinning behavior. On the other hand, the bottom phase of these systems showed much lower values of viscosity and Newtonian behavior (Fig. 3.7A and Fig. 3.7D). Rheological behavior of the continuous phase determined the viscosity (and flow curves) of the separated phases. As the LBG concentration increased, the apparent viscosity increased and the shear thinning behavior was more pronounced (data not shown). It is interesting to note that systems with yerba mate extract showed rheology results similar to those of NaCl, although the LBG is more evenly distributed between the phases in the former case. The rheological behavior of an emulsion is influenced by the type of particles, size, shape, distribution and colloidal stability (LUCKHAM; UKEJE, 1999), [32]. However these systems are very complex and the correlations between droplet size and viscosity cannot be studied without considering other factor [33].

Fluorescence microscopy images show that in systems with the addition of NaCl, the top phase was formed by droplets of protein (red) dispersed in the phase formed predominantly by carbohydrate (Fig. 3.7B), while the bottom phase showed the formation of droplets of carbohydrate (black) dispersed in the continuous phase formed by protein (Fig. 3.7C). The addition of yerba mate extract led to the opposite behavior to that found for the NaCl sample, since the top phase (Fig. 3.7E) showed formation of carbohydrate droplets (black) and the bottom phase (Fig. 3.7F) showed formation of protein droplets (red). Table 3.2 presents a comparison between samples with NaCl and yerba mate extract for initial mixture, top and bottom phase, to compare the effect of increasing SCN/LBG ratio on the microstructure.

Table 3.2. Effect of increasing the SCN / LBG ratio on the microstructure of the initial mixture, top and bottom phase, with addition of NaCl and yerba mate extract.

Sample	Ratio SCN/LBG		Initial Mixture	Top phase	Bottom phase
3 % (w/w) SCN-0.45 % (w/w) LBG	6.67	NaCl			
		Yerba Mate Extract			
4 % (w/w) SCN-0.3 % (w/w) LBG	13.34	NaCl			
		Yerba Mate Extract			

Fluorescence microscopy images show that the increase in the SCN/LBG ratio did not change the phase separation behavior in systems with NaCl. There was formation of protein droplets in the top phase (with continuous dark LBG phase) and formation of dispersed LBG droplets in the bottom phase. The increase in protein concentration leads to a higher droplets density in the top phase, while the decrease in LBG concentration (% w/w) promoted a reduction in the number of droplets in the bottom phase. In samples with yerba mate extract, a lower concentration of SCN (% w/w) showed a predominance of carbohydrate structures in the initial mixture and top phase, as well as formation of protein droplets in the bottom phase. With the increase in the concentration of SCN (% w/w) and decrease in the amount of LBG, the droplets formed by proteins are formed in all phases. This inversion may have occurred due to interactions between the extract and the protein, which becomes more intense with the increase in the protein concentration.

3.4. CONCLUSION

The results show that the use of NaCl and yerba mate extract promoted the formation of droplets of both protein and carbohydrate. However, composition of the phases varied according to the components added and their concentration. Phase diagrams show that the mechanisms for obtaining emulsions with addition of NaCl and yerba mate extract can be different. Analyses of volume and composition of the phases indicated these differences, mainly related to the migration of carbohydrates.

There was a predominance of droplets formed by proteins in relation to droplets formed by carbohydrates, both in the NaCl system and in the extract system, although the formation of complexes with extract has modified the behavior of the phases. The addition of plant extracts rich in compounds with antioxidant activity to ATPS systems is important, as it can indicate how they interact in the presence of proteins and carbohydrates, presenting relevant data for the development of new food products.

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CAPÍTULO IV

Production of water-water emulsion gels by yerba mate extract

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Production of water-water emulsion gels by yerba mate extract

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ABSTRACT – Yerba mate extract is a powerful antioxidant compound, but it can also be used as a gel network-forming agent. In this context, this study aimed to investigate the induction of gelation by yerba mate extract in protein-polysaccharide mixtures. W/W emulsions produced with sodium caseinate (SC) and gellan gum were characterized in terms of their physicochemical composition and rheological properties. The mechanical properties of the gels produced from the W/W emulsions in the presence of yerba mate extract were evaluated by uniaxial compression, oscillatory rheology and scanning electron microscopy. Different concentrations of SC-gellan promote distinct protein and carbohydrate compositions of the separated phase, with gellan concentration varying between 0.69 and 1.26% w/w in the top phase. The top phase of these emulsions was used for gel formation by adding varying concentrations of yerba mate extract. The highest firmness (22.41 kPa) was identified with 2% (w/w) extract and the highest gellan concentration, while gels with extreme extract concentrations (0.5% and 8% w/w) and the lowest gellan concentration presented lower hardness (0.6 and 0.92 kPa). Water-holding capacity was related to the quantity and size of the pores formed by the gel network in the different compositions, showing that the protein structures within the pores alter the firmness of the gels formed predominantly of polysaccharides. Our results indicate that W/W emulsions should be more intensively explored for the formation of structures that have different potential applications.

Keywords: Hydrogels; Thermodynamic equilibrium; ATPS; phenolic compounds interaction

4.1 INTRODUCTION

Some mixtures of proteins and polysaccharides can form aqueous two-phase systems (ATPS) due to thermodynamic incompatibility driven by critical factors such as polymer solubility, concentration, pH, temperature, and ionic strength of the aqueous medium (Moschakis et al., 2018). When these critical points are reached, a phenomenon known as steric exclusion occurs, leading to the formation of two distinct phases. To restore thermodynamic equilibrium, a top phase enriched with one polymer is formed, while the bottom phase is enriched with the other polymer (Ye, 2008). In the partitioning of these systems, the formation of dispersed droplets is commonly observed, with the top phase containing protein droplets dispersed in a continuous polysaccharide-rich phase, and the bottom phase comprising carbohydrate droplets dispersed in a continuous protein-rich phase. When this phenomenon occurs, these systems are called water-in-water emulsions (Dumas et al., 2020; Esquena, 2016).

More than a hundred protein-carbohydrate pairs are capable of producing W-W emulsions, such as gelatin-starch, gelatin-maltodextrin, sodium caseinate-LBG, BSA-milk protein and alginate-gellan (Beldengrün et al., 2018; Buldo et al., 2016; Michaux et al., 2021; Perrechil et al., 2009). Mixtures of proteins and carbohydrates are often part of formulations in the pharmaceutical and food industries, increasing the number of studies to understand combinations that promote more interesting structures for different applications. For instance, proteins such as sodium caseinate can be used as emulsifiers (Hanazawa & Murray, 2014; Le, Rioux, & Turgeon, 2017) and gelling agents. Gellan is a multifunctional carbohydrate that has texture-stabilizing, film-forming and gel-producing properties even at low concentrations (Buldo et al., 2016). Due to their high availability, these polymers are used in several applications, although their use in ATPS emulsions is still little explored. The limited application of W-W emulsions is attributed to their low stability due to the small tension

between the two aqueous phases. This condition can be controlled with the addition of stabilizers capable of acting as phase rheology modifiers, making water-water emulsions viable for industrial applications (Beldengrün et al., 2020; Esquena, 2016).

Due to their low stability, W-W emulsions can be gelled to reduce droplet mobility or even be used as a platform to produce microgels with controlled particle size. Although W/O emulsions are the most commonly used to obtain microgels, the continuous phase must be removed by centrifugation, filtration, and washing with organic solvent (Farjami; Madadlou, 2017). However, even with all these steps to remove the oil, residues may remain impregnated in the microgels. In this sense, W-W emulsions offer the advantage of using hydrophilic polymers and, therefore, being fully dispersible in aqueous systems (Esquena, 2016).

The gelation of protein-polysaccharide mixtures has been extensively studied, and the nature of the formation of these networks confers different properties of structure, aggregation and water holding capacity. In general, the main interactions that promote the formation of protein-polysaccharide hydrogels are electrostatic in nature (pH and ionic strength) between charged biopolymers (Le et al., 2017). An alternative way to obtain hydrogels is the addition of polyphenols into protein-polysaccharide matrices. The addition of compounds such as tannins to these matrices can promote the formation of a ternary protein/polyphenol/carbohydrate complex. Furthermore, polysaccharides (neutral and anionic), such as gellan, xanthan, gum Arabic and carrageenan, act as inhibitors of protein precipitation in the presence of tannins, as they interrupt the binding of proteins to polyphenols, and can also form hydrophobic pockets capable of encapsulating and complexing polyphenols due to hydrogen bonds between the oxygen of the carbohydrate and a hydroxyl group of phenolic compounds (De Freitas, Carvalho, & Mateus, 2003; Ferruzzi, Hamaker, & Bordenave, 2020).

Yerba mate (*Ilex paraguariensis*) is an herb widely used to produce popular beverages, such as mate and tereré, mainly in South American countries such as Brazil, Paraguay, Argentina and Uruguay. This plant has a composition rich in polyphenols and xanthines, purines, alkaloids, flavonoids, amino acids, minerals and vitamins. Due to its rich and complex composition, it has been widely studied as a health-promoting agent in the prevention of cardiovascular diseases, diabetes and obesity, showing promising results (Alkhatib & Atcheson, 2017; Gawron-Gzella, Chanaj-Kaczmarek, & Cielecka-Piontek, 2021; Heck & De Mejia, 2007; Kang et al., 2012; Kungel et al., 2018; Rocha et al., 2018).

Considering these functional and technological characteristics, yerba mate is an excellent candidate for the formation of gelled structures for food, pharmaceutical and cosmetic applications of ATPS emulsions. Therefore, this work aims to produce gels from W-W emulsions of sodium caseinate – gellan gum, using yerba mate extract as a gel-forming agent and evaluating the influence of the composition of the W/W emulsion on the gel-forming capacity to produce tailor made structures.

4.2. MATERIAL AND METHODS

4.2.1 Material

Sodium caseinate was kindly supplied by Alibra Ingredients Ltd. (Brazil), while low acyl gellan gum (Kelcogel®F) and yerba mate extract (50˚ Brix) were donated by CP Kelco (USA) and Triunfo (Brazil), respectively. Sodium azide (Sigma Chemical Co., USA) was applied as an antimicrobial agent and Rhodamine B was used to dye the proteins for fluorescence microscopy (Sigma Chemical Co., USA).

4.2.2. Preparation of W/W emulsion gels

4.2.2.1. Construction of Phase diagrams

First, the two aqueous phases were prepared separately. Stock solution of 12% (w/w) sodium caseinate (SC) was prepared at 25 °C using magnetic stirring for 1 h, and then stored for 24 h. Gellan gum stock solution (1-2% w/w) was prepared at 85 °C for 1 h under stirring and, after cooling, was stored for 24 h. All solutions were prepared with deionized water and the pH was adjusted to 7.0. The two aqueous biopolymer solutions were mixed by keeping the SC concentration at 6% (w/w) and varying the gellan gum concentration (0.3–1% w/w). The mixture was stirred under magnetic stirring for 1 h at 25 °C for complete homogenization of the components. Subsequently, the mixtures were stored in 100 mL transparent glass beakers and covered with PVC film at 25 °C for 24 h prior building phase diagrams.

Phase diagrams were built using a visual analysis of the macroscopic phase separation. The opaque bottom phase was considered rich in proteins due to its higher density, while a translucent top phase was assumed rich in carbohydrates. This observation was performed immediately on the fresh mixture and after 24 h of storage or once phase equilibrium had been achieved. In addition, the physicochemical characterization of fresh W/W emulsions and after phase separation was performed. Protein content was determined by the micro-Kjeldahl method and carbohydrate concentration was obtained by the sugar determination method using phenol/sulfuric acid reaction.

4.2.3 Gelation of W/W emulsions

After achieving phase separation of the W/W emulsions, each phase was collected separately. Each of the phases formed a new W/W emulsion enriched in one of the biopolymers (top polysaccharide-rich and bottom protein-rich) that were tested for gel formation. After the addition of the extract, only the top phase exhibited gel formation and was consequently

selected for further studies. Yerba mate extract (50˚ Brix) was added to the top phase at concentrations ranging from 0.5% to 8% (w/w). The addition of yerba matte extract induced rapid formation of gel that could lead to a heterogeneous structure and the formation of brittle gels. To mitigate this effect and achieve more uniform gelation, a short heat treatment was employed. The extract was added to the top phase of the W/W emulsion in a water bath under agitation for 10 min at 75 °C. The solution was then poured into plastic tubes (approximately 30 mm in internal diameter x 15 mm height), cooled to 25°C and incubated for 48 hours. All gels were prepared in quintuplicate. The pH of both phases was measured before gelation. A control gel containing only 1% (w/w) gellan was prepared to compare with the gels from the W/W emulsions.

4.2.4. W/W properties

4.2.4.1. Rheological measurements

All rheological measurements were performed on a MCR 301 rheometer (Anton Paar, Austria). Flow curves of the W/W emulsions (freshly prepared) and of the separated phases after 24 h were carried out using a parallel plate with a diameter of 40 mm and a gap set at 1.0 mm. A sequential three flow steps (up-down-up) were obtained between 0 and 300 s^{-1} at 25 °C. Thixotropy was qualitatively assessed by the hysteresis between the first and third shear stress versus shear rate curves. The steady-state flow curves (third curve) were adjusted to the power law model (Eq. 1) and the viscosity at 2 s^{-1} was evaluated to compare the different biopolymer blends.

$$\sigma = k \cdot \dot{\gamma}^{n} \qquad \text{(Eq. 1)}$$

Where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), k is the consistency index ($Pa.s^n$), and n is the flow behavior index (−).

Oscillatory tests were evaluated from temperature sweeps between 75 and 25 ˚C, using a stress amplitude of 0.5 Pa (within the linear viscoelastic range) and a frequency of 1 Hz. Cone-plate geometry (angle = 2^o; diameter=60mm and cone truncation of 0.5 mm) was used to perform this measurement. Similar to the gel formation process (section 2.2.2), the top phase of the extract-added W/W emulsions was heat at 75°C for 10 min before being transferred to the rheometer plate at the same temperature. Samples were homogenized in the rheometer using pre-shear at $100s^{-1}$ for 1 min before being subjected to cooling-heating sweeps at 5˚C/min. A thin layer of silicone oil was added around the sample to prevent drying and evaporation. Rheological transitions were evaluated by tan δ (G"/G') and complex viscosity (η*).

4.2.4.2. Fluorescence microscopy

The initial mixtures (freshly prepared) and the separated phases after 24 h were observed under fluorescence optical microscopy using a Carl Zeiss Model AxioScope A1 microscope (Zeiss, Germany) using a 100× objective lenses and excitation filter at 515 and 560 nm. Rhodamine B was used as to stain the protein marker, applying 0.1 µL to 1 µL of emulsion.

4.2.5. Gels properties

4.2.5.1. Water holding capacity (WHC)

Water holding capacity (WHC) was evaluated using a gravimetric method. Gel samples were weighed (2-4g), wrapped in filter paper and placed in 50 mL Falcon tubes. Tubes were closed and left to rest for 48 h. The water holding capacity was calculated according to Eq. 2:

$$WHC = 100 \times \left[1 - \left(\frac{\text{water released}}{\text{water gel}}\right)\right] \quad \text{(Eq. 2)}$$

Where water released is the amount of water released after left to rest (g) and water gel is the initial amount of water in the gel before left to rest (g).

4.2.5.2. Mechanical properties

Mechanical properties were measured under uniaxial compression using a TA-XT-Plus Texture Analyzer (Stable Microsystems Ltd, Surrey, UK) equipped with an acrylic cylindrical plate (30 mm diameter using a crosshead speed of 1.0 mm/s.). Compression was performed up to 80% of the original height of the gels. Hencky or true stress (σ_H) and strain (ε_H) were calculated from the force-deformation data (Steffe, 1996) according to the Eq. 3 and 4, respectively. Rupture properties were obtained from the first maximum peak of the stress-strain curve. Stress at fracture (σ_{rup}) was used as an indicator of gel hardness at rupture, while strain at fracture (ε_{rup}) provides information on the deformability of gels.

$$\sigma_H = \mathrm{F(t)}.\left[\frac{\mathrm{H(t)}}{\mathrm{H_0 \cdot A_0}}\right] \qquad \text{(Eq. 3)}$$

$$\varepsilon_H = -ln\left[\frac{H_t}{H_0}\right] \qquad \text{(Eq. 4)}$$

Where F(t) is the force, Ao and Ho are the initial area and height of the sample, respectively, and H(t) is the height in time t. The rupture or fracture properties were associated with the maximum stress of the stress–strain curve.

4.2.5.3. Scanning electron microscopy (SEM)

Only the samples with the highest gellan concentration (1% and 0.5% w/w - called G1-Top and G2-Top, respectively) were selected for scanning electron microscopy (SEM), since the other samples did not present a suitable structure for this analysis. Samples (approximately 10mm×5mm×5 mm) of gels were fixed for 24 hours in 2.5% (w/w) glutaraldehyde in cacodylate buffer 0.1 M at pH 7.2 and rinsed twice in cacodylate buffer. Fixed gels were

fractured in liquid nitrogen and post fixed in 1% (w/w) buffered osmium tetroxide for 2 h. Gels were dehydrated in a graded ethanolic series (30, 50, 70 and 90%) and finally in 100% ethanol (three changes in 1 h). After dehydration, samples were subjected to critical point drying (Critical Point Dryer CPD03 Balzers, Alzenau, Germany) and then placed on aluminum stubs and coated with gold in a Sputter Coater SCD 050-Balzers. Images were recorded using a VEGA 3 SBU (Tescan, Czech Republic) operated at 15 kV at a magnification of x1000. At least ten images of typical structures were taken for each sample.

4.2.5.4. Statistical analysis

Analysis of variance (ANOVA) was performed using a free version of the ASSISTAT 7.7 software. Significant differences between means were performed using the Tukey test ($p<0.05$).

4.3. RESULTS AND DISCUSSION

4.3.1. Phase diagrams of W/W emulsions

Phase diagrams were obtained by visually identifying the volume and chemical composition of the phases separated from mixtures prepared with varying concentrations of gellan gum. Figure 4.1 represents phase diagrams, both visual and with characterization of the phase composition. Tie-lines of the phase diagrams were obtained from chemical characterization of the initial mixture and after phase separation. Figure 4.1A represents the visual behavior of mixtures prepared with sodium caseinate and gellan gum solutions at different concentrations at pH 7 and evaluated after 24 h to ensure phase equilibrium. Phase diagram shows that at low concentration of biopolymers there is the formation of a single homogeneous phase, but as the concentration of biopolymers increases, there is phase separation and the formation of two-phase aqueous systems.

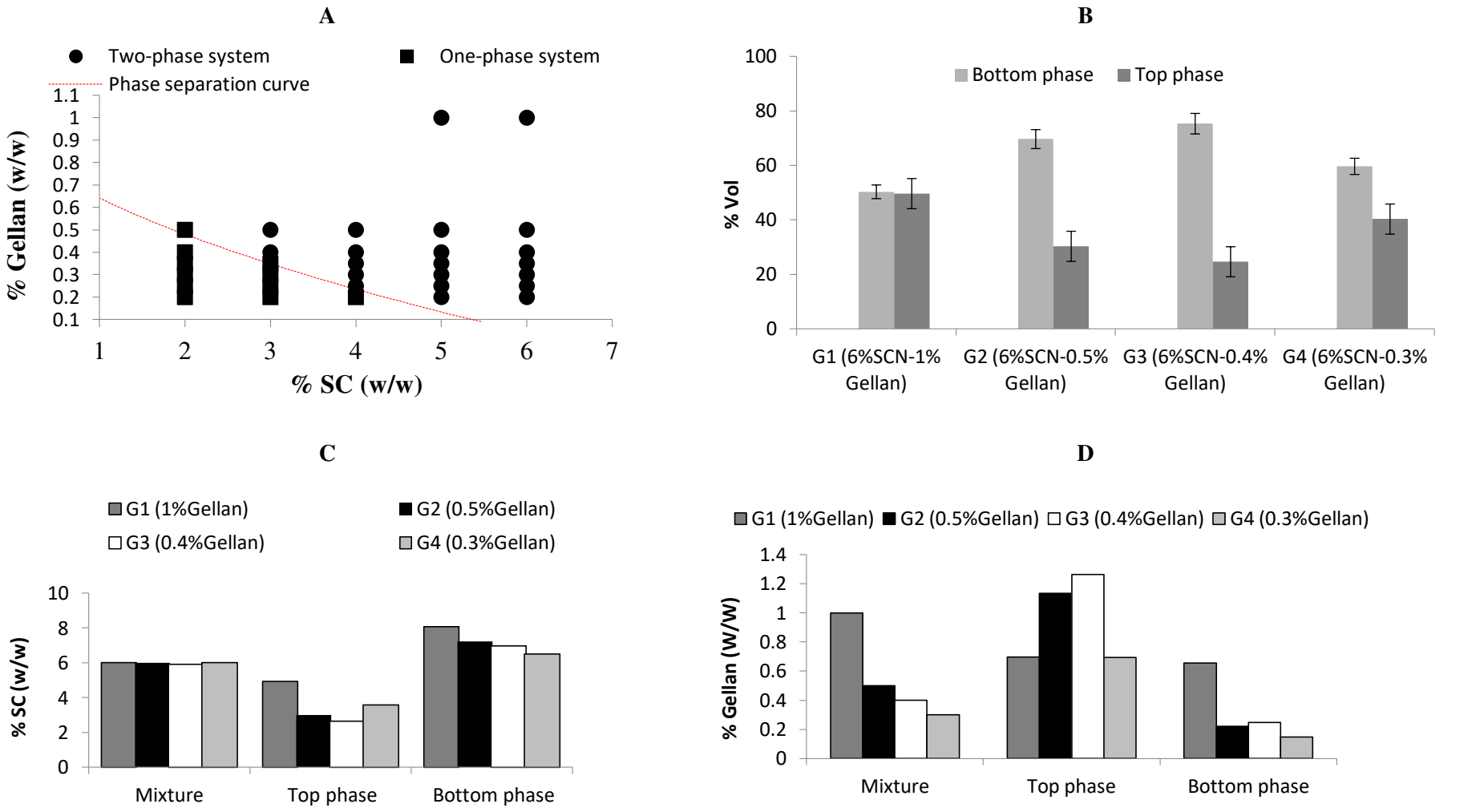

Figure 1. Phase diagrams of SCN- gellan mixtures. A – Visual phase diagram, B – Volume of separated phases, C – Protein composition of the systems, E – D -Carbohydrates composition of the systems. In Figure 1B, C and D the sodium caseinate concentration (SC) was fixed at 6% w/w.

Although gellan gum concentrations varied within a narrow range, Figure 4.1B shows that all mixtures containing 6% (w/w) sodium caseinate (SC) formed two-phase aqueous systems, even with the lowest gellan gum concentration (0.3% w/w). The volume of the top phase showed a decreasing trend as the gellan concentration was reduced (0.5% and 0.4% w/w). However, reducing the gellan concentration to 0.3% (w/w) caused an increase in the volume of the top phase, unlike the other mixtures with 6% (w/w) SC. A higher volume of the top phase indicates a higher protein concentration and, consequently, higher water holding. As the gellan concentration decreases, the repulsive charge interactions between molecules also decrease, facilitating for the protein to remain in the top phase (Rodriguez Patino & Pilosof, 2011). These volume differences depending on the biopolymer concentrations are very interesting, as they generate varied structures and mechanical properties in W/W emulsions (Gao et al., 2017). The behavior regarding the composition of the mixtures and separated phases is shown in Figures 4.1C and 4.1D.

Figure 4.1C shows that protein concentration is directly related to phase volume. It can be seen that the volume of the top phase increases as the protein concentration retained in the phase increases. Such a behavior is confirmed by the gellan concentrations in the top phase, which are inversely proportional to volume. The concentration of gellan in the top phase for sample G1 exhibited the lowest concentration among the samples studied, followed by sample G4 (Figure 4.1D). These results may be related to the steric exclusion effect, which induces phase separation from the initial mixture. The volumes of the separated phases (Figure 4.1B) showed that the top phase/bottom phase volume ratios were 50/50, 30/70, 25/75 and 40/60 for samples G1, G2, G3, and G4, respectively. This phenomenon may have occurred due to the similar volumes of the separated phases (50/50), leading to the formation of bicontinuous systems or even phase inversion (Esquena, 2016).

4.3.2. Rheology and structure of the top phase of the W/W emulsions

The top phases of the W/W emulsions were selected for the formation of gels with yerba mate extract, as this phase had the highest concentrations of gellan, in addition to showing varied concentrations of proteins that could influence the mechanical characteristics of the gels. Fluorescence microscopy and rheological properties of the top phases of W/W emulsions are shown in Figure 4.2. Flow curves (in steady state conditions) were well fitted to the power law model. Table 4.1 shows the rheological properties and composition of the top phase at equilibrium conditions.

Similarity in the composition and structure of the mixtures G1-G4 and, mainly, G2-G3 is evident. Emulsions G1 and G4 showed small protein droplets evenly distributed, with a greater quantity in G1 due to the higher concentration of SC (Table 4.1). On the other hand, G2 and G3 presented small protein droplets and aggregates.

The greater complexity of the network of G1 and G4 can be observed by the higher value of the consistency index (k), higher pseudoplasticity (lower n) and viscosity at low shear rate. In addition, flow curves of the top phase of the W/W emulsions show the subtle presence of thixotropy for all formulations. Hysteresis caused by the application of a force over shear time seems to be directly associated with the composition of the phases (Table 4.1). The higher the protein/carbohydrate ratio, the more evident the thixotropy was, which could be related to the Na^+ ions present in caseinate acting as a gel-promoting ion (García, Alfaro, & Muñoz, 2016).

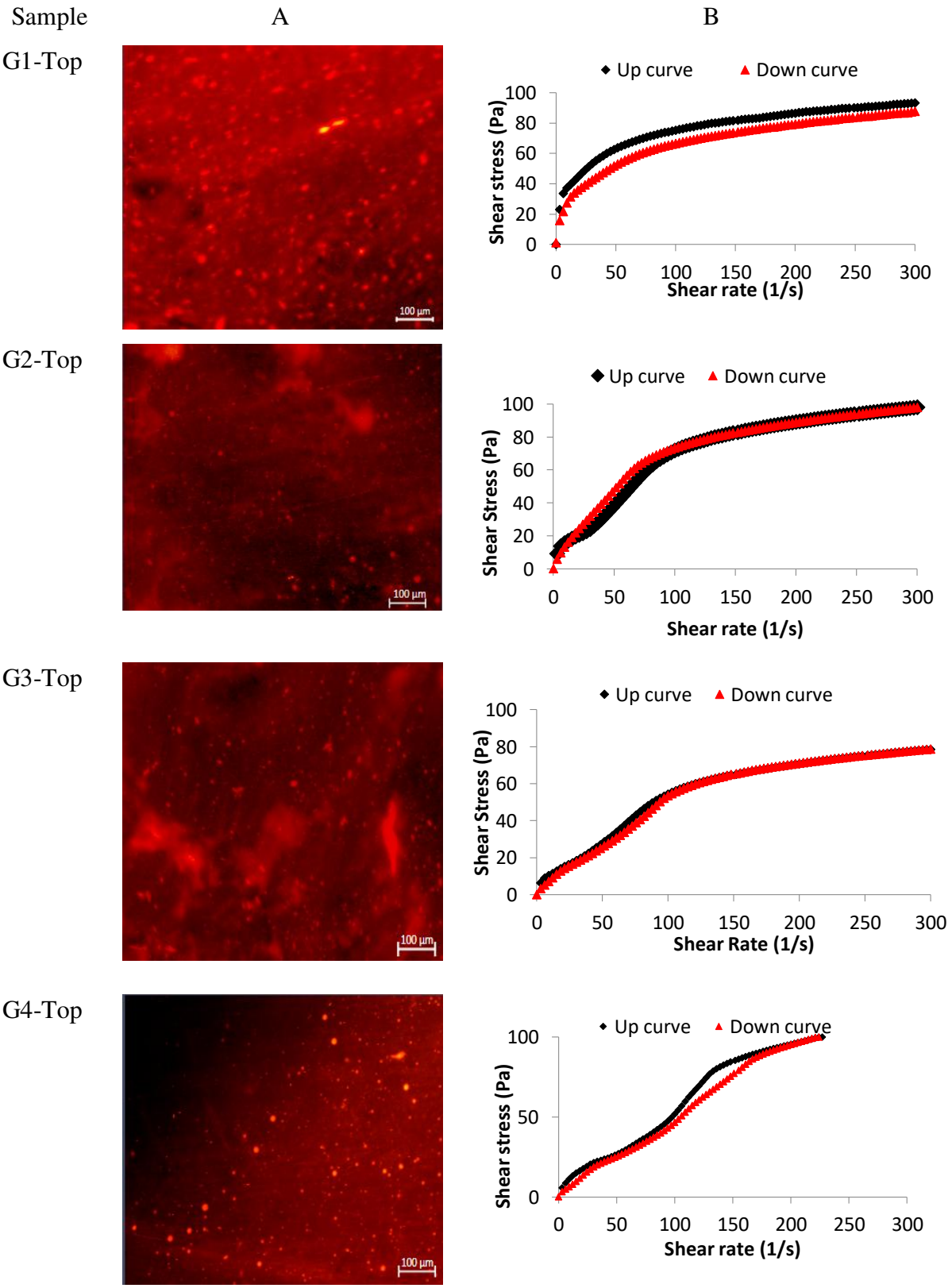

Figure 4.2. A) Fluorescence microscopy and B) flow curves of the top phase in W/W emulsions.

Table 4.1. Rheological properties and composition of the top phase in WW emulsions.

Sample	Top phase						
	SC (% w/w)	Gellan (% w/w)	Ratio SC/gellan	η_{2s}^{-1}(Pa.s)	n	k (Pa.s^n)	Hysteresis (Pa/s)
G1-Top	4.93	0.7	7.07	5.42	0.32	15.18	2376.06
G2-Top	2.99	1.13	2.63	1.44	0.54	4.95	86.57
G3-Top	2.64	1.26	2.08	1.04	0.57	3.38	191.70
G4-Top	3.59	0.69	5.18	1.28	0.44	6.35	831.76

4.3.3 Gelation of top phase

4.3.3.1 Temperature of gel formation

Oscillatory tests were performed to verify the gel network formation temperature. Figure 4.3 shows that, regardless of the phase composition, the addition of extract at concentrations of 0.5, 1, 2 and 4% (w/w) induces similar viscoelastic behavior and gelation temperature between 40 and 50°C. Gelation is associated with a consistent increase in η* with cooling, indicating the formation of a network (Ryu & McClements, 2024). The complex viscosity (η*) increases with the extract concentration, reaching its highest value at 2% (w/w) for all samples. The addition of 8% extract (w/w) induced a different behavior, as structuring occurred even at high temperatures. The significant reduction in pH (Figure 4.4) and the presence of ions derived from the phenolic nature of the extract may explain this behavior. The presence of cations with gelation capacity enhances molecular associations and increases the thermal stability of the gels (Picone & Cunha, 2011; Zhang et al., 2023).

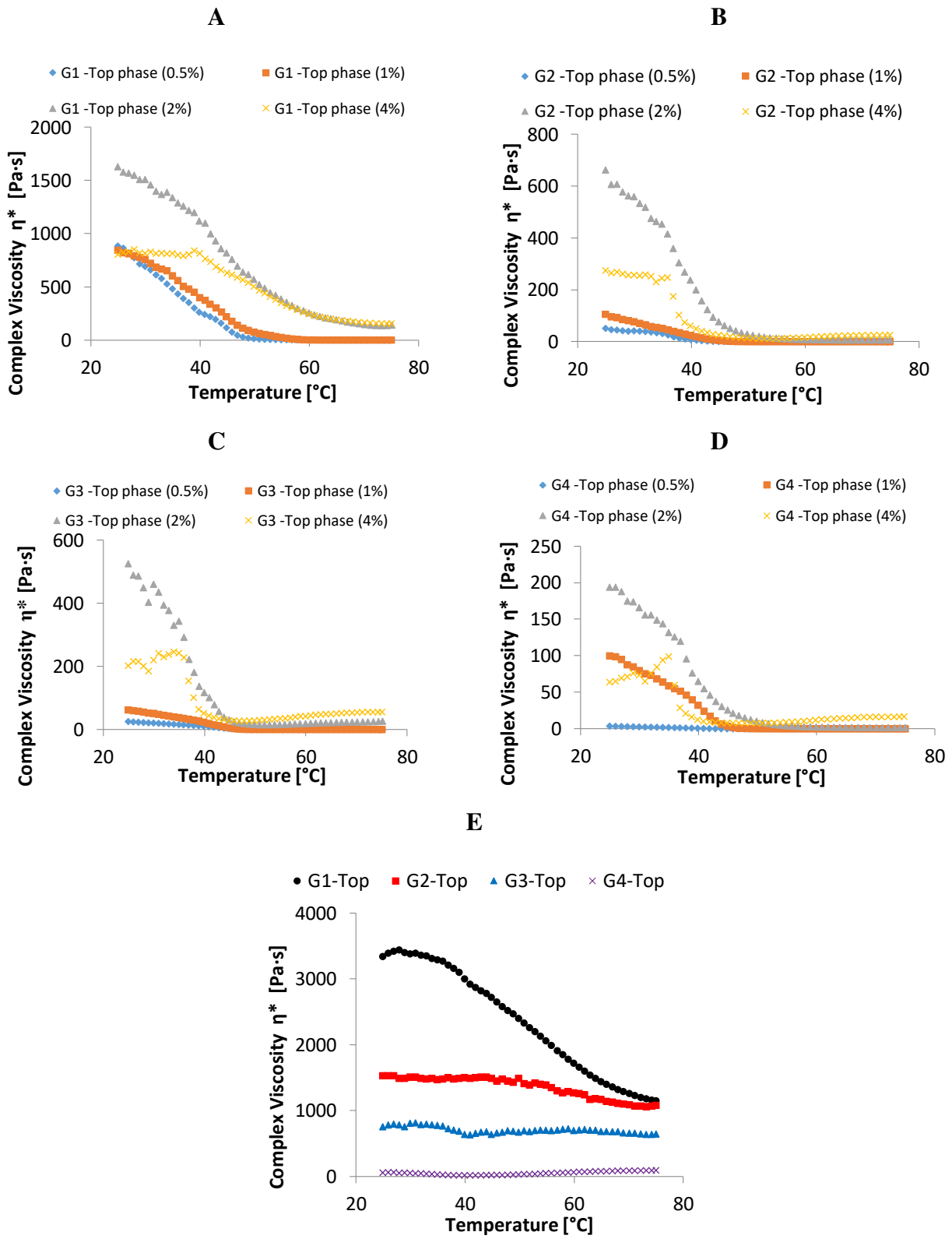

Figure 4.3. Thermal scanning rheology of the W/W emulsion top phase **A)** G1-Top; **B)** G2- Top ; **C)** G3-Top; **D)** G4-Top with addition of different extract concentration (0.5%, 1%, 2% and 4% w/w). **E)** G1-Top, G2-Top, G3-Top and G4-Top with 8 % (w/w) of extract concentration.

Regarding the different phases (but maintaining the same extract concentration), the behavior of η* followed the order: G1>G2>G3>G4. This behavior indicates that at the beginning of the process (cooling), gelation was driven by the interaction between the polysaccharide chains, since η* followed the order of gellan concentration (highest value for G1-Top and lowest for G4-Top). Thus, despite the complexity of the physicochemical composition of the top phases, rheology was driven by gellan concentration and pH (Figure 4.4). Gels produced at different pH values present characteristics that depend on the amount of H^+ ions available to interact with the biopolymers and form gel networks (Picone & Cunha, 2011).

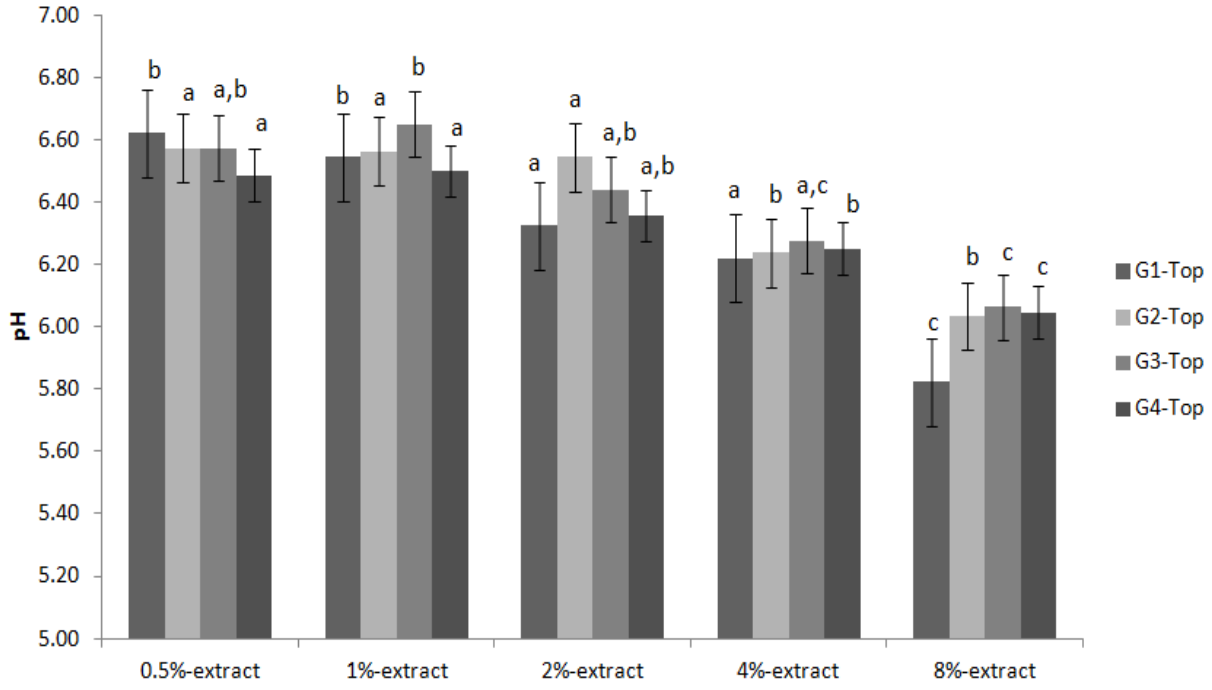

Figure 4.4. pH values of the top phase G1-Top, G2-Top, G3-Top and G3-Top added with different concentrations of yerba mate extract (0.5 %, 1%, 2%, 4%, 8% w/w). Means with different letters show significant differences at $p < 0.05$.

4.3.3.2 Gels properties

Yerba mate extract at varying concentrations (0.5%; 1%; 2%; 4% and 8% w/w) was added to the top phases of the W/W emulsions (G1-Top, G2-Top, G3-Top and G4-Top) and gelation was evaluated over 48 hours. Gels showed a darker coloration as the extract concentration and storage time increased (Figure 4.5). The coloration of the gellan gels obtained with the

addition of the phenolic extract is a function of the pH, since phenolic compounds are more stable at acidic pH and can be degraded with increasing pH (Pascuta et al., 2022). The pH of the yerba mate extract (50 ˚Brix) was 5.28. Thus, after the addition of extract to the emulsions (which were at pH 7.0), there was a reduction in pH (Figure 4.4) that was proportional to the extract concentration.

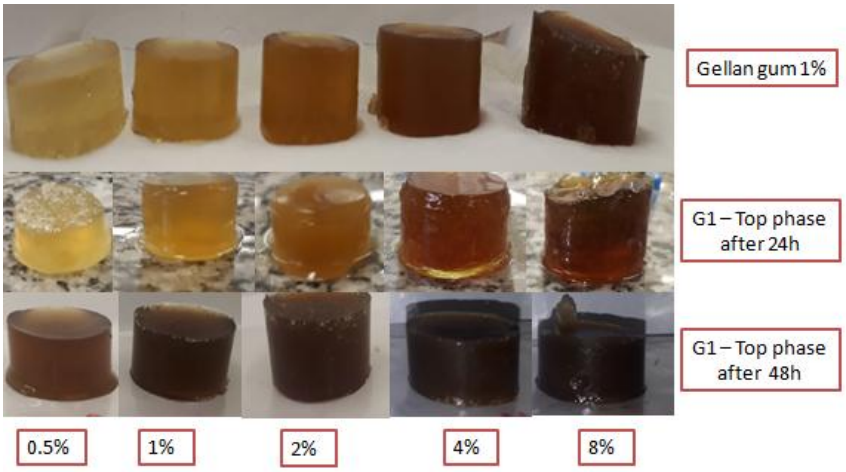

Figure 4.5. Macroscopic appearance of the gels formed with different concentrations of yerba mate extract. Gels: A) control formed with 1% (w/w) gellan, B) top phase after 24 h and C) top phase after 48 h of storage.

The visual appearance of the gels obtained from the top phase of the W/W emulsion is similar to the gels obtained with the addition of extract in a 1% (w/w) gellan gum solution, demonstrating the predominance of gellan interactions in the formation of the gel network in 24 hours of storage, as observed at the beginning of gelation in oscillatory rheology (Figure 4.3). Over time (up to 48 h), the gels showed gradual darkening and a stiffer appearance due to the intensification of interactions between components.

The mechanical behavior evaluated from the uniaxial compression of the gels is shown in the Hencky stress-strain relationship (Figure 4.6). The maximum peak represents the first rupture point of the gels, from which the rupture stress and strain were extracted (Table 4.2). Results of strain at rupture showed a tendency of lower deformability of the gels formed by G1-Top

and G2-Top compared to G3-Top and G4-Top. In fact, visually, gels G1-Top and G2-Top were more brittle than samples G3-Top and G4-Top (data not shown). The highest peaks (σ_{rup}) were presented by the gels with the lowest initial gellan concentration (G3-Top and G4-Top), unlike the oscillatory rheology that showed a higher mechanical resistance (complex viscosity) for G1-Top and G2-Top. However, oscillatory rheology was performed to assess the onset of the gelation process (upon cooling) and stress at rupture was obtained after gels storage at rest for 48 h. In addition, Table 4.3 shows that the highest fracture stress values of G3-Top and G4-Top occurred with the addition of 2% (w/w) extract, while G1-Top and G2-Top presented the highest values with the addition of 1-4% and 0.5-1% (w/w) extract, respectively. These results indicate that at the highest gellan content, a lower extract concentration or higher pH may have favored a more ordered (or slower) aggregation of the polysaccharides, resulting in stronger gels.

Interestingly, the gels with lower hardness or stress at rupture were those with addition of 8% (w/w) of extract, unlike oscillatory rheology (highest values of complex viscosity). Our assumption is that the process is initially driven by the rapid interaction between gellan molecules in an acidic medium, but covalent and non-covalent interactions between protein-gellan, gellan-phenolics and protein-phenolics could occur over time. Hydrophilic functional groups of polysaccharides can oxidize in acidic media forming secondary compounds that interact with protein side chains (Pascuta et al., 2022). The interaction between proteins and polysaccharides during the gel formation process at acidic pH has already been observed for sodium caseinate – xanthan systems, where both participate in the formation of the gel network (Braga & Cunha, 2004).

The interaction between carbohydrates and other compounds can influence their technological properties, such as gelation capacity and rheological behavior, as well as their functional applications, including digestibility. Bonds between polysaccharides and

polyphenols are partially reversible and can form rapidly in a non-selective manner. The nature of this interaction is highly influenced by the number of hydroxyl groups capable of establishing strong and intense bonds (Dobson et al., 2019). Protein-phenolic interactions affect the rheological, functional and biological properties of foods, altering protein solubility and thermal stability (Buitimea-Cantúa et al., 2018).

Some proteins have a high affinity for caffeic and chlorogenic acids, as is the case with casein. (Masoumi, Tabibiazar, Golchinfar, Mohammadifar, & Hamishehkar, 2024; Nemli et al., 2024). Therefore, regions with microphase separation may have compromised the structure and, consequently, mechanical properties of the gels.

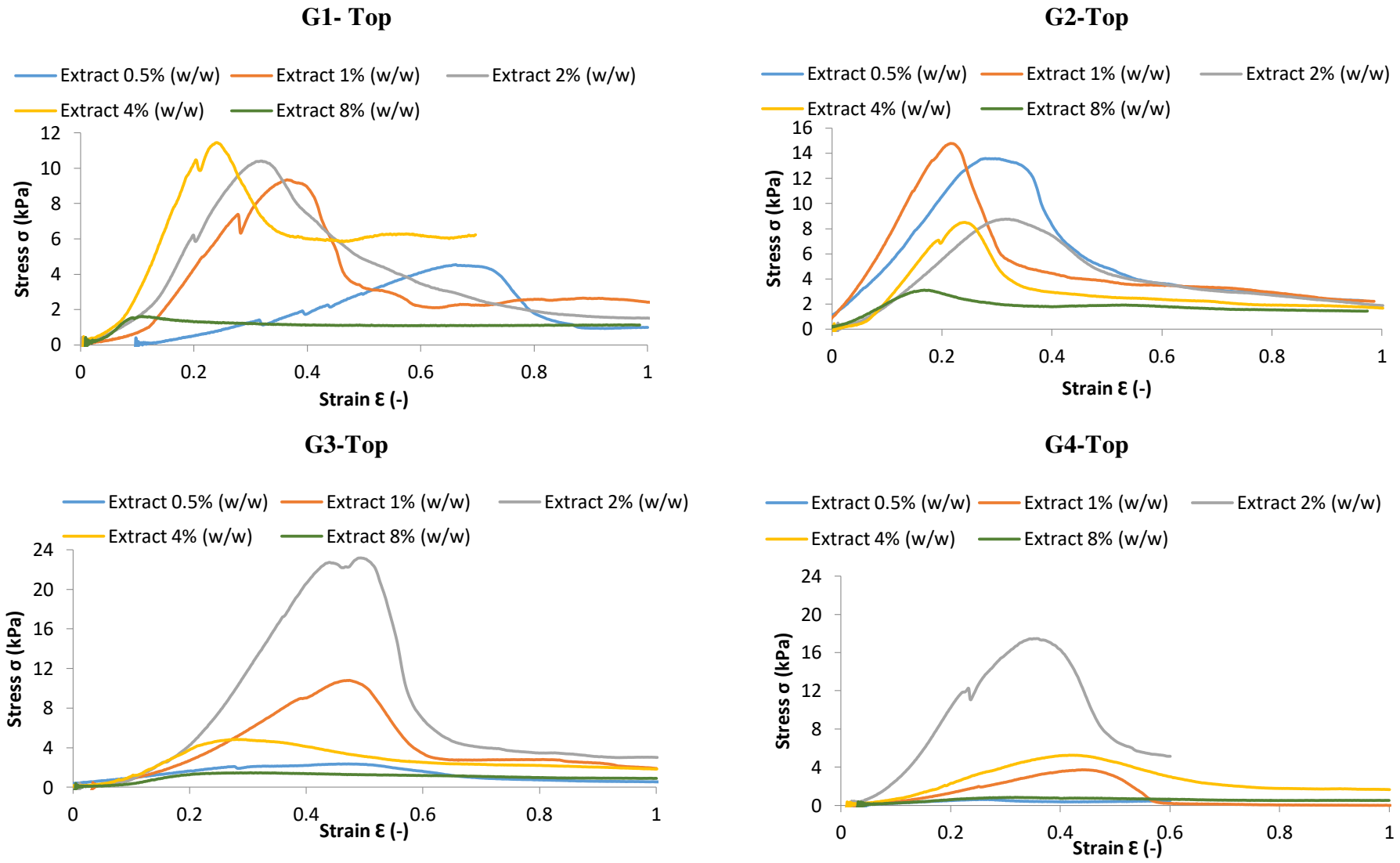

Figure 4.6. Hencky stress vs strain for gels formed from G1-Top; G2-Top; G3-Top; G4- Top with Yerba mate extract addition (1%, 2%, 4% and 8% w/w).

Table 4.2. Stress and strain at fracture for gels formed from G1-Top; G2-Top; G3-Top; G4- Top with Yerba mate extract addition (1%, 2%, 4% and 8% w/w).

Extract	**Stress at fracture- σrup (kPa)**				**Strain at fracture- Ɛrup**			
% (w/w)	G1-Top	G2-Top	G3-Top	G4-Top	G1-Top	G2-Top	G3-Top	G4-Top
0.5	5.21 ± 1.46^{b}	12.24 ± 3.37^{b}	2.00 ± 0.53^{a}	0.60 ± 0.16^{a}	0.58 ± 0.14^{a}	0.38 ± 0.08^{b}	0.53 ± 0.06^{a}	0.25 ± 0.04^{a}
1	8.69 ± 1.64^{a}	13.82 ± 0.81^{b}	10.44 ± 2.56^{b}	4.00 ± 1.13^{b}	0.34 ± 0.03^{a}	$0.29\pm0.05^{a,b}$	0.44 ± 0.03^{a}	$0.40\pm0.06^{a,b}$
2	9.99 ± 1.35^{a}	8.22 ± 1.77^{a}	22.41 ± 4.31^{c}	16.50 ± 2.19^{c}	0.32 ± 0.02^{a}	$0.33\pm0.08^{a,b}$	0.40 ± 0.07^{a}	$0.35\pm0.04^{a,b}$
4	10.39 ± 1.81^{a}	8.04 ± 0.98^{a}	4.20 ± 1.17^{a}	4.84 ± 2.10^{b}	0.25 ± 0.03^{a}	$0.24\pm0.04^{a,b}$	0.33 ± 0.06^{a}	$0.44\pm0.14^{a,b}$
8	1.72 ± 0.88^{b}	1.92 ± 1.30^{c}	1.46 ± 0.77^{a}	0.92 ± 0.41^{a}	0.50 ± 0.50^{a}	0.21 ± 0.14^{a}	0.58 ± 0.34^{a}	0.57 ± 0.45^{b}

Figure 4.7 shows that the gels with the highest water holding capacity contained 0.5%, 4% and 8% (w/w) extract. It is possible to identify a direct linear relationship between hardness (stress at rupture) and water holding capacity, since, for G3-Top and G4-Top, the hardest gels were those with the lowest WHC. This behavior was confirmed by fitting the data with a linear equation showing correlation coefficients of 0.93 and 0.91 for G3-Top and G4-Top.

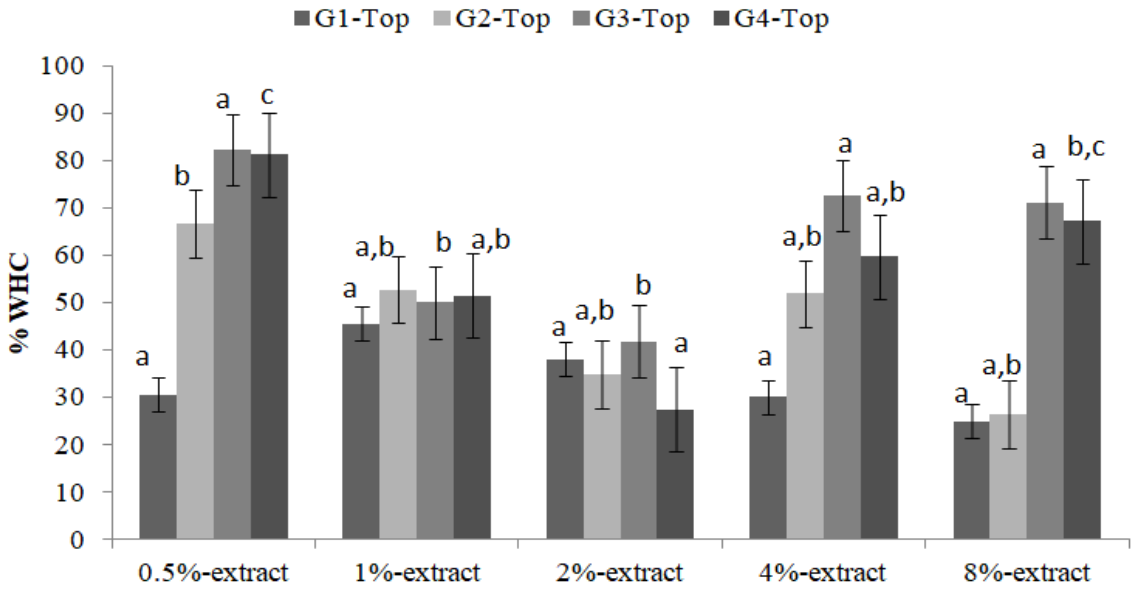

Figure 4.7 Water holding capacity (WHC) of gels obtained from the top phase of W/W emulsions added with Yerba Mate extract. Means with different letters show significant differences at $p < 0.05$.

According to Figures 4.8A and 4.8B, the pore structure and size exhibited different characteristics when the same extract concentration was added to the two samples. The pore sizes were markedly different at 0.5% w/w extract: G1-Top showed larger pores, which contributed to its lower water-holding capacity (Figure 4.7). With the addition of 1% and 2% w/w extract, the pore sizes became more similar, resulting in close water retention. However, coupled structures within the pores were observed due to the distinct composition (G1-Top has a higher protein content). At 4% w/w extract, the pores increased in size again in G1-Top, leading to a larger discrepancy in WHC compared to G2-Top. At 8% w/w extract, the structure became more compact, leading to reduced water retention in the gel.

The structure, quantity, and size of the pores are related to the gel-forming characteristic of gellan in the presence of protein, as well as to the pH reduction caused by the increase in the extract concentration. These factors can modify the typical gel structure, imparting varied properties to the gel (Picone & Cunha, 2011).

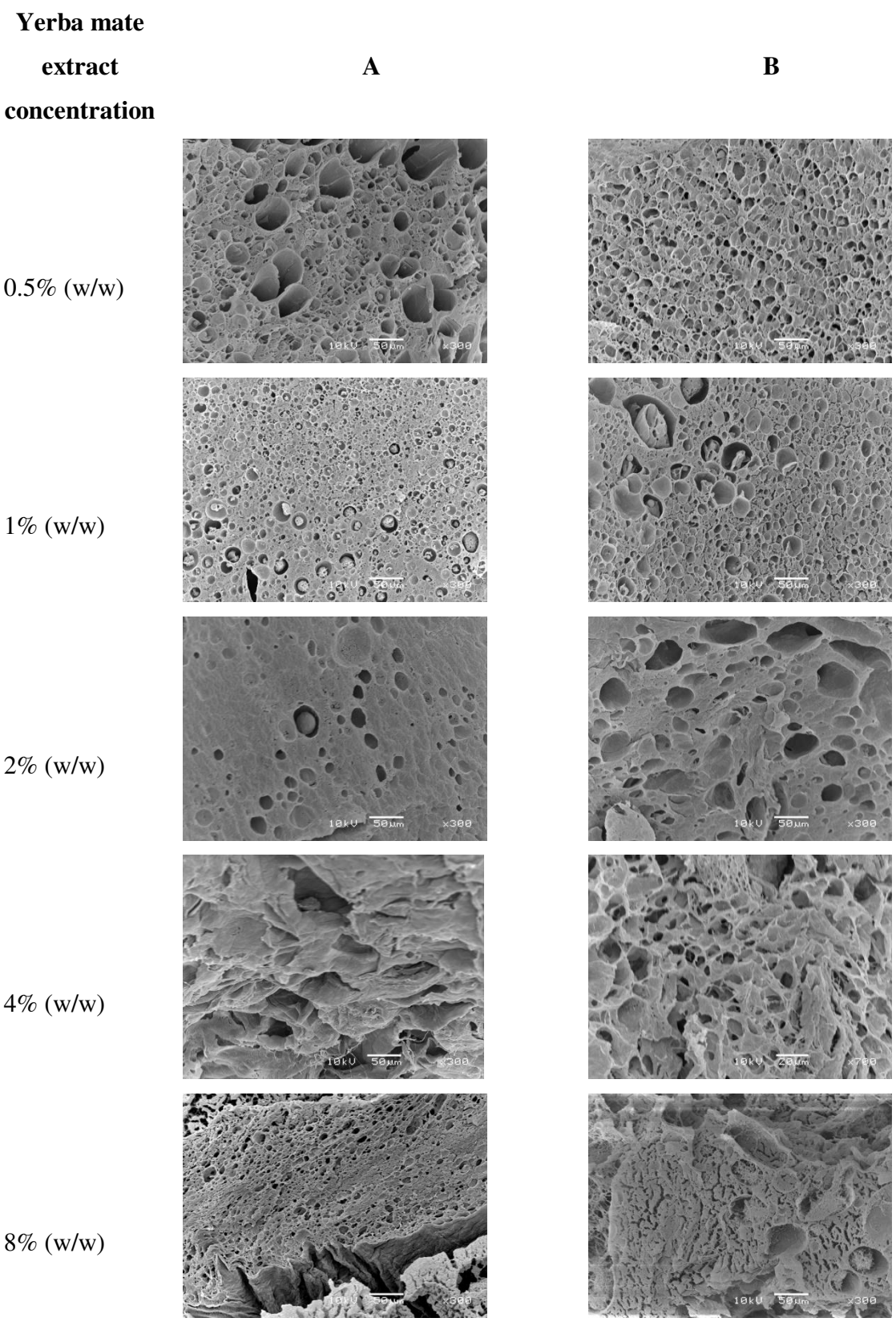

Figure 4.8. Scanning Electron Microscopy (SEM) of gels formed from the top phase of W/W emulsions added with Yerba Mate extract. **A)** G1-Top and **B)**, G2-Top.

Figure 4.8 illustrates the potential modifications in the structure and gel network formation as influenced by the emulsion composition and the concentration of the added extract. For the G1-top (Figure 4.8A) and G2-top (Figure 4.8B) samples, varying pore sizes and numerous arrangements are observed. As the extract concentration increases, the distribution becomes more balanced, leading to a more organized gel network formation. However, with an extract concentration of 4%, the network becomes disorganized, and at 8%, the network's behavior and organization exhibit structural characteristics distinct from the others, likely due to the intensified interaction between the protein and the extract. These results are confirmed by the high standard deviation of the structures formed. The increase in pH could lead to the exposure of the protein's hydrophobic groups, enhancing interaction with certain phenolic compounds, (Masoumi et al., 2024; Nemli et al., 2024; Ozdal, Capanoglu, & Altay, 2013)**.**

4.4. CONCLUSION

Yerba mate extract has been shown to be an efficient gelling/structuring agent for producing hydrogels from immiscible biopolymer mixtures, also called water-in-water emulsions. The separate phases of these emulsions, composed by gellan-sodium caseinate pair, showed droplets of one biopolymer surrounded by a continuous phase composed by the other biopolymer. The addition of yerba mate extract at different concentrations formed gel networks with varied properties, depending on the composition. The number and size of pores in the network directly interfered with the water holding capacity and mechanical properties. It was also observed that despite the prevailing gellan network, yerba mate extract may have interacted with the protein, causing a change in properties that was observed by the darker color and harder structure at long-term. In general, the complex nature of the interactions between phenolic compounds and biopolymer compounds in W/W

emulsions are capable of forming networks with varied properties for different potential applications.

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CRediT

Karine Cristine Kaufmann: Conceptualization, Investigation, Methodology, Writing - Original Draft. **Gabriela Feltre:** Methodology. **Douglas Fernandes Barbin:** Writing - Review & Editing, Supervision. ***Rosiane Lopes Cunha:*** Writing - Review & Editing, Supervision, Project administration.

Conflict of interest

The authors declare that they have no conflicts of interest.

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CAPÍTULO V

Discussão geral

5. DISCUSSÃO GERAL

Emulsões água/água podem ser obtidas através da combinação de pares específicos de proteína-polissacarídeo, em função desse fenômeno, o estudo da interação de algumas dessas combinações com extrato de erva mate, rico em compostos fenólicos, foi abordado nesse trabalho. A incompatibilidade termodinâmica entre os pares caseinato de sódio (SCN) - goma jataí (LBG) e caseinato de sódio (SCN) - goma gelana foi abordada nos capítulos III e IV, respectivamente.

No capítulo III, a capacidade de formar sistemas aquosos bifásicos foi avaliada através da adição de NaCl ou extrato de erva mate. Verificou-se que, em ambos os casos, houve incompatibilidade termodinâmica dos biopolímeros. A separação visual de fases foi observada e o volume de fases separadas foi dependente da concentração dos polímeros, que também influenciou as propriedades das fases separadas. Essas diferenças ficaram evidentes no diagrama de fases, construído com dados de composição de proteínas e carboidratos. O diagrama de fases para os sistemas produzidos com NaCl apresentaram comportamento típico para sistemas ATPS, com maior composição de carboidrato na fase superior como relatado em outros estudos (GAO et al., 2017; MOSCHAKIS et al., 2018). Entretanto, os resultados mostrados pelo diagrama de fases do sistema caseinato-LBG-extrato apresentaram comportamento distinto. A concentração de carboidrato na fase superior foi similar a da fase inferior, com consequente modificação da inclinação das linhas de amarração. A migração da LBG para a fase inferior é um indicativo de que houve uma interação entre as moléculas de caseinato de sódio-LBG- extrato formando complexos de alta massa molecular que migraram para o fundo.

A microscopia permitiu verificar a formação de gotas nos sistemas, porém com diferenças entre os sistemas com NaCl e extrato de erva mate. O sistema contendo NaCl mostrou formação de gotas de LBG na fase inferior, que se comportou como a fase dispersa. Para o sistema contendo extrato de erva mate, esse comportamento típico de formação de gotas de carboidratos na fase inferior não foi verificado. Foi possível observar uma predominância na formação de estruturas de proteínas, este efeito pode ser explicado pela maior interação entre o extrato e a proteína que se torna mais intensa com o aumento da concentração de proteína. O extrato de erva mate é rico em compostos fenólicos como o ácido clorogênico, e as interações entre os ácidos fenólicos e as proteínas podem ser influenciadas por diversos fatores, como pH, concentração e tipo de carga da proteína que podem alterar a solubilidade e, consequentemente, as propriedades tecnológicas das

proteínas (TARAHI et al., 2024).

No capítulo IV foi verificada a incompatibilidade entre o caseinato de sódio/goma gelana. A partir disso, foram obtidas emulsões A1/A2, explorarando a influência de suas características nas propriedades de géis, induzidos pela adição de extrato de erva mate. A obtenção de ATPS foi observada sem a adição de outros componentes (como o extrato de erva mate) e, após 24 horas, todas as misturas formuladas com concentração de proteínas a partir de 5% (m/m) apresentaram separação de fases. Os sistemas apresentaram formação de gotas, com a fase superior rica em gelana e fase inferior rica em caseinato de sódio. Como a fase superior das emulsões apresentou maior concentração de gelana, elas foram utilizadas para a produção de géis, através da adição de extrato de erva mate em diferentes concentrações, como agente indutor da gelificação.

Foi constatada a formação de géis através dos ensaios reológicos oscilatórios, mostrando que a viscosidade complexa aumentou proporcionalmente com a concentração de gelana. No entanto, as propriedades mecânicas dos géis apresentaram comportamento distinto do esperado, pois a concentração de extrato teve grande influência nos resultados. As amostras com maior concentração de gelana e maior concentração de extrato apresentaram géis mais frágeis, que podem ter ocorrido pela rápida formação da rede. As formulações com baixas concentrações de gelana, por sua vez apresentaram géis menos quebradiços. Esses resultados indicam que menor concentração de gelana e menor concentração de extrato, promovem uma formação mais lenta da rede de gel, favorecendo uma agregação mais ordenada, o que levou à obtenção de géis mais resistentes. A concentração de extrato mostra grande impacto na estrutura dos géis formados, uma vez que as microscopias mostraram que a partir de 4% (m/m) de extrato a característica estrutural dos géis apresenta redes mais desordenadas, devido à forte interação entre as proteínas presentes nas emulsões e o extrato. Essa interação também foi observada na coloração dos géis. Após 24h, os géis produzidos a partir das emulsões A1/A2 apresentaram coloração semelhante aos géis de gelana 1% (m/m) mostrando a predominância da gelana na agregação polimérica e formação da rede de gel. Porém após 48h, os géis apresentaram mudança de coloração, indicando que interações de outra natureza continuaram a ocorrer mesmos após a estruturação da gelana, apontando a interação entre as proteínas e o extrato rico em compostos fenólicos.

CAPÍTULO VI

Conclusão Geral

6. CONCLUSÃO GERAL

Os resultados mostram que o uso de NaCl e extrato de erva-mate promoveu a formação de gotículas tanto de proteína quanto de carboidrato em sistemas caseinato de sódio- goma jataí. Entretanto, a composição das fases variou de acordo com os componentes adicionados e sua concentração. Os diagramas de fases mostram que os mecanismos de obtenção de emulsões com adição de NaCl e extrato de erva-mate podem ser diferentes. As análises de volume e composição das fases indicaram essas diferenças, principalmente relacionadas à migração de carboidratos. Houve predominância de gotículas formadas por proteínas em relação às gotículas formadas por carboidratos, tanto no sistema NaCl quanto no sistema extrato, embora a formação de complexos com extrato tenha modificado o comportamento das fases. A adição de extratos vegetais ricos em compostos com atividade antioxidante aos sistemas ATPS é importante, pois pode indicar como eles interagem na presença de proteínas e carboidratos, apresentando dados relevantes para o desenvolvimento de novos produtos alimentícios.

O extrato de erva-mate demonstrou ser um agente gelificante/estruturante eficiente para produzir géis a partir de misturas de biopolímeros imiscíveis, também chamadas de emulsões água/água. As fases separadas dessas emulsões, compostas pelo par caseinato de sódio – gelana apresentaram gotículas de um biopolímero cercadas por uma fase contínua composta pelo outro biopolímero. A adição de extrato de erva-mate em diferentes concentrações formou redes de gel com propriedades variadas, dependendo da composição. O número e o tamanho dos poros na rede interferiram diretamente nas propriedades mecânicas dos géis formados. Também foi observado que, apesar da rede de gelana ser predominante, o extrato de erva-mate pode ter interagido com a proteína, causando uma mudança nas propriedades que foi observada pela coloração e estrutura. Em geral, conclui-se que a complexa natureza das interações entre compostos fenólicos e compostos biopoliméricos em emulsões A/A são capazes de formar sistemas e redes com propriedades variadas para serem exploradas em diferentes aplicações agregando valor tecnológico e funcional.

CAPÍTULO VII

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CAPÍTULO VIII

Apêndice I: Atividades Desenvolvidas e Divulgação dos Resultados

8. APÊNDICE I: ATIVIDADES DESENVOLVIDAS E DIVULGAÇÃO DOS RESULTADOS

8.1 Artigos publicados relacionados aos resultados da tese:

KAUFMANN, KARINE CRISTINE; CZAKOSKI, ALINE ; BARBIN, DOUGLAS FERNANDES ; DA CUNHA, ROSIANE LOPES . Incompatibility between sodium caseinate - locust bean gum induced by NaCl and yerba mate extract. INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES, v. 183, p. 276-284, 2021.

8.2 Artigos publicados em colaboração

CZAIKOSKI, ALINE ; GOMES, ANDRESA ; **KAUFMANN, KARINE CRISTINE** ; LISZBINSKI, RAQUEL BESTER ; DE JESUS, MARCELO BISPO ; CUNHA, ROSIANE LOPES DA . Lignin derivatives stabilizing oil-in-water emulsions: Technological aspects, interfacial rheology and cytotoxicity. INDUSTRIAL CROPS AND PRODUCTS, v. 154, p. 112762, 2020.

8.3 Resumos Publicados em anais de congresso

KAUFMANN, K. C.; BARBIN, D. F. ; CUNHA, R.L. . 'BUILDING PHASE DIAGRAMS USING MULTIVARIATE CALIBRATION MODELS'. In: 14° Simpósio Latino Americano de Ciência de Alimentos - SLACA: Impacto da Ciência de Alimentos na Saúde e na Doença, 2021, Campinas. Anais do 14 SLACA - Simpósio Latino Americano de Ciência de Alimentos, 2021.

KAUFMANN, K. C.; CZAIKOSKI, A. ; BARBIN, DOUGLAS FERNANDES ; CUNHA, R.L. . Rheological properties and phase diagram of water-in-water emulsions. In: VIII International Conference on Food Proteins and Colloids, 2020, Campinas. Book of Abstracts of the VIII CIPCA International Conference on Food Proteins and Colloids. Campinas: Faculdade de Engenharia de Alimentos. Universidade Estadual de Campinas, 2020.

CZAIKOSKI, A. ; Gomes, A. ; **KAUFMANN, K. C.** ; CUNHA, R.L. . Emulsifier capacity of lignin derivatives: a comparison with wey isolate protein. In: VIII International Conference on Food Proteins and Colloids, 2020, Campinas. Book of Abstracts of the VIII CIPCA International Conference on Food Proteins and Colloids. Campinas: Faculdade de Engenharia de Alimentos. Universidade Estadual de Campinas, 2020.

8.4 Participação em eventos científicos

14° Simpósio Latino Americano de Ciência de Alimentos - SLACA: Impacto da Ciência de Alimentos na Saúde e na Doença. BUILDING PHASE DIAGRAMS USING MULTIVARIATE CALIBRATION MODELS. 2021. (Simpósio).

VIII International Conference on Food Proteins and Colloids. Rheological properties and phase diagram of water-in-water emulsions. 2020. (Congresso).

8.5 Estágio Docente

Participação em Programa de Estágio Docente (PED), categoria C - Atividades de Apoio a Docência Parcial, como bolsista. Componente "FT621 – Processos Mecânicos", sob supervisão da Profa. Dra. Rosiane Lopes da Cunha.

CAPÍTULO IX

Anexo I: Permissão pra a utilização de dados publicados

9. Anexo I: Permissão pra a utilização de dados publicados

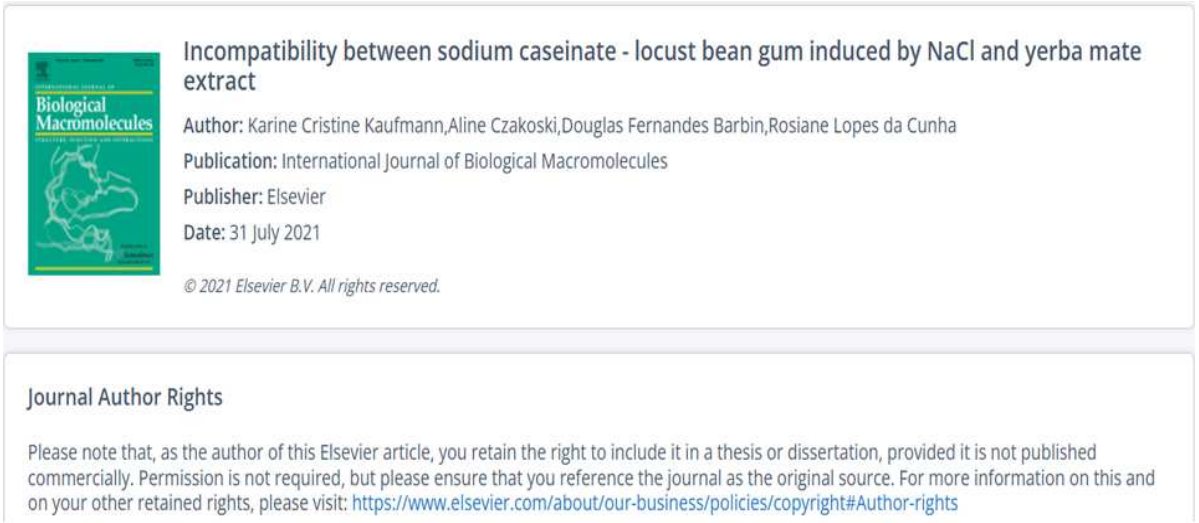

Figura 9.1. Permissão para incluir o artigo: "Impatibility between sodium caseinate - locust bean gum induced by NaCl and yerba mate extract", como capítulo da tese.

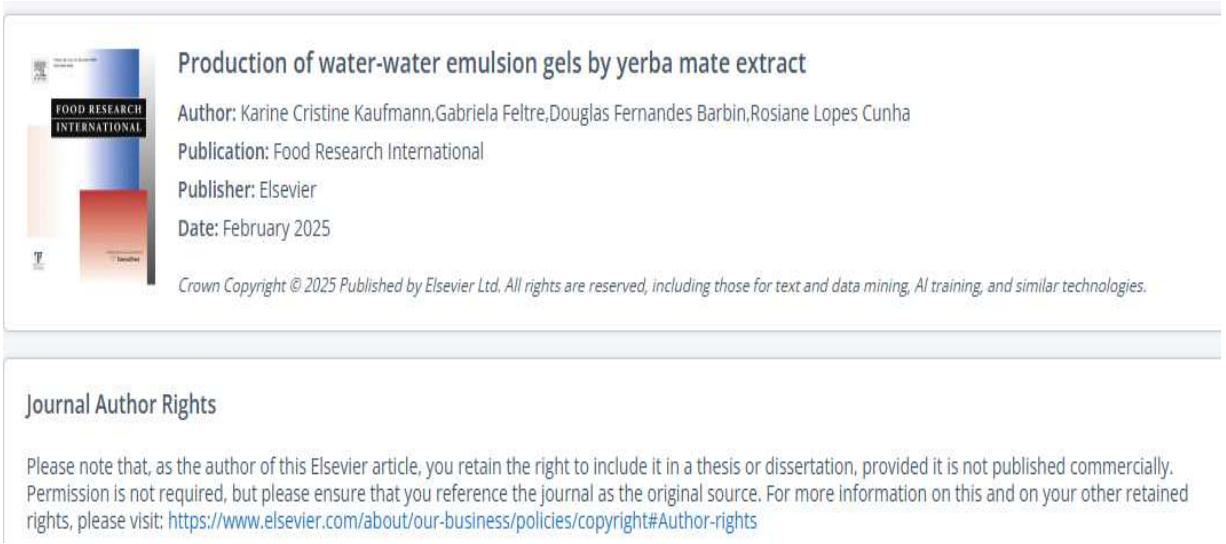

Figura 9.2. Permissão para incluir o artigo: "Production of water-water emulsion gels by yerba mate extract", como capítulo da tese.