MICHELE HARUMI OMURA

ESTUDO FÍSICO-QUÍMICO E TÉCNICO-FUNCIONAL DE PROTEÍNAS VEGETAIS SONICADAS INDIVIDUALMENTE E EM COMBINAÇÕES BINÁRIAS OU TERNÁRIAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

Orientador: Eduardo Basílio de Oliveira

Coorientadores:
Frederico Augusto Ribeiro de Barros
Hércia Stampini Duarte Martino

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APROVADA: 29 de outubro de 2021.

Assentimento:

Michele Harumi Omura
Autora

Eduardo Basílio de Oliveira
Orientador

Aos meus pais Luiz Omura e Matiko Nishikito Omura,
meus irmãos Rodrigo e Juliana,
com muito amor e carinho, dedico!

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“Cada sonho que você deixa para trás
É um pedaço do seu futuro que deixa de existir.”
(Steve Jobs)

BIOGRAFIA

MICHELE HARUMI OMURA, filha do caminhoneiro aposentado Luiz Omura e da secretária de escola aposentada Matiko Nishikito Omura, nasceu em Marília, interior de São Paulo, em 02 de dezembro de 1988. É membro da Igreja Messiânica Mundial do Brasil, é tricolor paulista, ama a pintura à óleo, cozinhar, reunir os amigos, sair para beber e curtir as lindas paisagens e cachoeiras dos arredores de Viçosa.

Em Agosto de 2007, iniciou a graduação em Tecnologia de Alimentos pela Faculdade de Tecnologia de Marília, concluído em 30 de Junho de 2010, onde recebeu o diploma e medalha de honra ao mérito por ter sido considerada a melhor aluna do curso no período de 2007 a 2010.

Em março de 2012, ingressou no curso de Mestrado do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos da Universidade Federal de Viçosa, defendendo a dissertação no dia 06 de Novembro de 2014. Em 2015, com a pesquisa desenvolvida no mestrado, recebeu da Rede de Banco de Leite Humano o 1° lugar do Prêmio Jovem Pesquisador da Rede de Bancos de Leite Humano na área temática “Processamento, Controle de Qualidade e Utilização do Leite Humano”.

Em 2016, atuou como professora no curso técnico de Alimentos do Senai Londrina – PR. Lecionou as disciplinas de tecnologia de alimentos, tecnologia de massas e farinhas e tecnologia de leite e derivados.

Em Março de 2017, ingressou no Curso de Doutorado no Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos da Universidade Federal de Viçosa, submetendo-se a defesa de sua tese no dia 29 de Outubro de 2021.

RESUMO

OMURA, Michele Harumi, D.Sc., Universidade Federal de Viçosa, outubro de 2021. **Estudo físico-químico e técnico-funcional de proteínas vegetais sonicadas individualmente e em combinações binária ou ternária**. Orientador: Eduardo Basílio de Oliveira. Coorientadores: Frederico Augusto Ribeiro de Barros e Hércia Stampini Duarte Martino.

Proteínas de origem vegetal representam uma alternativa mais sustentável do que aquelas de origem animal, mas não contemplam quantidades suficientes dos aminoácidos essenciais para satisfazer as necessidades nutricionais humana. A área de ciências e tecnologia de alimentos e setores de pesquisa e desenvolvimento industrial, têm buscado maneiras para isolar e otimizar o uso dessas proteínas oriundas de fontes vegetais. Atualmente, o mercado tem disponível para comércio, isolados e concentrados proteicos em pó, mas esses materiais apresentam baixa dispersibilidade, prejudicando e até impossibilitando sua utilização. A tecnologia do ultrassom vem sendo cada vez mais explorada com o intuito de contornar essa problemática. Assim, neste trabalho, isolado proteico da soja (IPS), concentrado proteico de ervilha (CPE) e isolado proteico do arroz (IPA), foram tratados com ultrassom com o intuito de otimizar suas propriedades técnico-funcionais, analisando suas características físico-químicas isoladamente ou em combinações. Por meio do delineamento Box-Behnken, diferentes combinações do binômino potência (256,5; 637,5 ou 712,5W) e tempo (120, 360 ou 600s) do ultrassom, foram aplicados sobre três concentrações das proteínas em meio aquoso (1,0; 3,0 ou 5,0% m/v). O tratamento ultrassônico (USt) promoveu alterações conformacionais no IPS, IPA e CPE, em virtude do aumento na exposição de regiões hidrofóbicas (índice H_0), desencadeando um aumento na capacidade de retenção de óleo (OHC); alterações nos valores do potencial zeta e diâmetro hidrodinâmico (d_h) das partículas dispersas, sem alterar o seu perfil de massa molecular. Pela análise da dispersibilidade, o tratamento de 712,5W|600s, em dispersões a 1,0% (m/v), foi a condição que promoveu um aumento mais pronunciado desta propriedade no IPS, enquanto para o CPE, foi o USt de 712,5W|360s, sobre dispersões 3,0% (m/v). Em contrapartida, nenhuma condição do USt foi capaz de modificar significativamente as propriedades físico-químicas e técnico-funcionais do IPA. Além disso, foi verificado que o USt foi fortemente impactado pela concentração das dispersões. Após o reconhecimento da

efetividade do USt sobre IPS e CPE, o desenho experimental de mistura centroide simples foi utilizado para estudar o desempenho técnico-funcional de misturas binárias e ternária entre os três materiais. Essas misturas foram submetidas ao USt com as condições selecionadas previamente, que revelaram menor capacidade de formação de espuma e gel e maior dispersibilidade, ou seja, 256,5kJ (712,5W|360s). Apesar da baixa efetividade do USt em modificar o IPA de forma isolada, quando este material foi combinado com SPI, essa mistura com USt de 256,5 kJ|1.0% m/v, revelou efeitos sinergísticos, pois promoveu as maiores contribuições na dispersibilidade, no índice da atividade emulsificante e na OHC. Já a mistura entre CPE+IPA, o USt de 256,5 kJ|3,0% m/v revelaram desempenho funcional similares ao do CPE, porém superior ao do IPA. Outros resultados revelaram que a misturas dos materiais combinados ao USt promoveram melhorias na uniformidade e digestibilidade destes sistemas complexos. Assim, este trabalho mostrou que é possível otimizar o uso do IPS, IPA e PPC, por meio do USt ou pelas misturas entre esses materiais proteicos combinados ao USt, resultando em ingredientes constituídos de alto valor biológico, boa digestibilidade, e com boas propriedades técnico-funcionais.

Palavras-chave: Proteínas a base de vegetais. Ultrassom. Otimização. Desenho experimental Box-Behnken. Desenho de mistura centroide simples.

ABSTRACT

OMURA, Michele Harumi, D.Sc., Universidade Federal de Viçosa, October, 2021. **Physicochemical and techno-functional study of sonicated plant proteins individually and in binary or ternary combinations**. Adviser: Eduardo Basílio de Oliveira. Co-advisers: Frederico Augusto Ribeiro de Barros and Hércia Stampini Duarte Martino.

Plant-derived proteins represent a more sustainable alternative than those of animal origin, but they do not contain sufficient amounts of essential amino acids to satisfy human nutritional needs. The área of food science and technology and industrial research and development sectors have been looking for ways to isolate and optimize the use of these proteins from plant sources. Currently, the market has available for commerce, powdered protein isolates and concentrates, but these materials have low dispersibility, hindering and even making their use impossible. Ultrasound technology has been increasingly explored in order to overcome this problem. Thus, in this work, soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) were treated with ultrasound in order to optimize their techno-functional properties, analyzing their physicochemical characteristics, alone or in combinations. Using the Box-Behnken design, different combinations of the binomial power (256.5; 637.5 or 712.5W) and time (120, 360 or 600s) ultrasound were applied on three concentrations of proteins in aqueous medium (1.0; 3.0 or 5.0% w/v). The ultrasonic treatment (USt) promoted conformational changes in the SPI, RPI and PPC, due to the increase in the exposure of hydrophobic regions (H_0 index), which triggering an increase in the oil holding capacity (OHC); changes in the zeta potential and hydrodynamic diameter (d_h) values of the dispersed particles, without changing their molecular mass profile. By the dispersibility analysis, the treatment of 712.5W|600s, in dispersions at 1.0% (w/v), was the condition that promoted a more pronounced increase of this property in the SPI, while for the PPC, it was the USt of 712.5W|360s, over 3.0% dispersions (w/v). On the other hand, no USt condition was able to significantly modify the physico-chemical and techno-functional properties of the RPI. Furthermore, it was verified that the USt is strongly impacted by the concentration of dispersions. After recognizing the effectiveness of USt on SPI and PPC, the simplex centroid mixture design was used to study the techno-functional performance of binary and ternary mixtures between the three materials. These mixtures were submitted to USt under the conditions previously

selected, which allowed for lower foam and gel formation capacity and higher dispersibility, ie, 256.5 kJ (712.5W|360s). Despite the low effectiveness of USt in modifying the RPI alone, when this material was combined with SPI, this mixture with USt of 256.5 kJ|1.0% w/v, revealed synergistic effects, as it promoted the greatest contributions to dispersibility, in the emulsifying activity index and in the OHC. As for the mixture between PPC+RPI, the USt of 256.5 kJ|3.0% w/v revealed functional performance similar to that of PPC, but superior to that of RPI. Other results revealed that the mixtures of materials combined with USt promoted improvements in the uniformity and digestibility of these complex systems. Thus, this work showed that it is possible to optimize the use of SPI, RPI and PPC, through USt or by mixing these protein materials combined with USt, resulting in ingredients with high biological value, good digestibility, and good techno-functional properties.

Keywords: Plant-based protein. Ultrasound. Optimization. Box-Behnken experimental design. Simplex centroid mixture design.

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LISTA DE ABREVIAÇÕES E SÍMBOLOS

Aas	Aminoácidos
Abs	Absorbance
ANS	8-anilino-1-naphthalenesulfonic acid
DLS	*Dynamic Light Scattering*
d_h	Hydrodynamic average diameter
EAI	Emulsifying Activity Index
ESI	Emulsifying Stability Index
FC	Foam Capaciity
H_0	Hydrophobic surface
LGC	Least Gelling Concentration
OHC	Oil Holding Capacity
PDI	Polydispersity Index
pH	Hydrogenionic potential
pI	Isoelectric point
PPC/CPE	Pea Protein Concentrate/ Concentrado Proteico de Ervilha
RPI/IPA	Rice Protein Isolate/ Isolado Proteico de Arroz
R^2	Coefficient of determination
rpm	Rotation per minute
SCD	Simplex Centroid Design
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SH	Sulfhydryls
SM	Supplementary material
SPI/IPS	Soy Protein Isolate/ Isolado Proteico do Arroz
TGI	Trato gastro-intestinal
USt	Ultrasonic treatment
WHO	Water Holding Capacity
ζ-potential	Zeta potential

SUMÁRIO

1. Introdução:

Proteínas são macromoléculas constituídas por combinações de 21 diferentes aminoácidos (Aas) (Damodaran et al., 1996), biossintetizadas por todos os organismos vivos, mas as de origem animal e vegetal são, até o presente, as mais exploradas para alimentação humana. Os termos “essencial” e “não essencial”, quando se referem aos aminoácidos, são de grande importância em nutrição. No primeiro, considera-se aqueles Aas em que o organismo humano não consegue biossintetizar (ou não podem biossintetizar em quantidades suficientes para um metabolismo saudável), precisando ser adquiridos por meio do consumo direto de fontes produtoras. Já no segundo, o próprio metabolismo humano encontra rotas de biossíntese (L.Kathleen Mahan, 2015). Proteínas de origem animal, tais como do ovo, leite, carne e peixe, são consideradas completas neste sentido, ou seja, contemplam todos Aas essenciais em quantidades suficientes. As proteínas vegetais apresentam boa qualidade nutricional, baixa alergenicidade e custos comparáveis às fontes tradicionais. Além disso, apelos sociais relacionados ao consumo de proteínas animais – como a alta demanda de terra, consumo de água e emissões de CO_2 característicos da pecuária tradicional e mesmo questionamentos ideológicos (legítimos) sobre bem-estar animal – desencadearam o aumento (em número) de grupos como vegetarianos, veganos e flexitarianos, o que promove, hoje, maiores demandas por materiais sustentáveis, em particular de origem vegetal.

Os alimentos de origem vegetal podem conter algumas substâncias ditas fitoquímicas (ex: inibidores de proteases, ácido fítico e compostos fenólicos) e fibras que reduzem a absorção e biodisponibilidade dos Aas essenciais. No entanto, as indústrias de alimentos têm recorrido à utilização de concentrados ou isolados proteicos vegetais, garantindo assim que os inconvenientes provenientes da presença de outras biomoléculas sejam minimizados em virtude de sua remoção. Proteínas derivadas de plantas que vêm sendo estudadas ao longo das últimas duas décadas com o objetivo de serem aplicadas como ingredientes alimentares, incluem aquelas provenientes de cereais (p. ex.: trigo, milho, aveia, arroz...), sementes oleaginosas (p. ex.: girassol, abóbora, gergelim, algodão...), leguminosas (p. ex.: soja, ervilha, feijão, grão de bico, lentilha...), nozes (p. ex.: amêndoas, pistache, castanha de caju, amendoim), tubérculos (p. ex.: batata e batata-doce), e pseudocereais (p. ex.: quinoa, trigo sarraceno chia e amaranto) (Nikbakht Nasrabadi et al., 2021).

Dentre essas, proteínas de soja (*Glycine max* L.) ainda permanecem como as

mais utilizadas em formulações de alimentos. A soja é considerada de fácil produção agrícola, apresenta boa qualidade nutricional e funcional, além de custo relativamente baixo (Nishinari et al., 2014). Proteínas da ervilha (*Pisum sativum* L.), concentradas ou isoladas, atualmente é uma das principais alternativas às proteínas da soja, por apresentarem características nutricionais comparáveis, custo industrial acessível, baixa alergenicidade e elevado teor do aminoácido essencial lisina (S. Jiang et al., 2017; Lam et al., 2018). As proteínas do arroz (*Oryza sativa* L.) ainda são pouco utilizadas, devido a sua baixa dispersibilidade em água; porém, ganha atenção devido a sua facilidade de cultivo, boa digestibilidade, valor biológico e hipoalergenicidade (Amagliani et al., 2017). Além disso, proteínas do arroz são ricas em metionina, representando uma opção a se utilizar em conjunto com proteínas de leguminosas para satisfazer a demanda de aminoácidos essenciais (Pietrysiak et al., 2018).

Além dos aspectos nutricionais, as proteínas vegetais também são utilizadas em virtude de suas características funcionais. Contudo, essas proteínas têm seu uso limitado em formulações alimentícias em virtude de sua baixa dispersibilidade em meio aquoso, o que impacta diretamente na exploração de diversas técnico-funcionalidades (Amagliani et al., 2017; Oliete et al., 2018a). Assim, para contornar este problema e otimizar o uso das proteínas vegetais, métodos não térmicos como ultrassom (Wen et al., 2019), alta pressão hidrostática (Primozic et al., 2018), campo elétrico pulsado, micro-ondas e irradiação gama (Han et al., 2018), vêm sendo cada vez mais estudados. No entanto, a aplicação dessas técnicas no setor industrial ainda depende de mais avanços referentes ao *scale-up* de operações unitárias.

Dentre essas abordagens de processamento, a tecnologia de ultrassom de alta frequência pode ser considerada como promissora e prática, que induz perdas nutricionais e sensoriais inferiores àquelas observadas pelo emprego de processos térmicos convencionais (Fan et al., 2020; Ojha et al., 2018). As frequências de onda que variam de 20 a 100 kHz referem-se ao ultrassom de alta potência (Rahman & Lamsal, 2021), e têm sido objeto de diversos estudos para modificar a conformação de proteínas vegetais e avaliar os consequentes resultados de seu desempenho técnico-funcional.

Os principais parâmetros usados na configuração do dispositivo ultrassônico (sonda ou banho) incluem tempo (2-100 min), temperatura (2-50 °C), frequência (20-25 kHz), potência (200-600 W) ou intensidade acústica (22-138 W / cm²) (Gharibzahedi & Smith, 2020). Vários trabalhos reportaram que proteínas vegetais ao

serem modificadas pelo tratamento ultrassônico, não só apresentaram uma melhora significativa em sua dispersibilidade em meio aquoso, mas também tiveram sua capacidade de retenção de água e ou óleo, propriedades emulsificantes, espumantes e gelificantes, aprimoradas (Biswas & Sit, 2020; de Oliveira et al., 2020; Hu et al., 2013; L. Jiang et al., 2014; Malik et al., 2017; O'Sullivan et al., 2016; Omura et al., 2021; Resendiz-Vazquez et al., 2017).

Vale ressaltar que os trabalhos disponíveis na literatura focam em como o ultrassom afeta a conformação molecular e as propriedades tecnológicas de um único isolado/ concentrado proteico. Como relatado anteriormente, o uso de uma única fonte de proteína vegetal não pode suprir a demanda de Aas essenciais para humanos. Assim, conhecendo-se a composição de Aas da proteína vegetal de interesse, é possível identificar seu Aa limitante e combina-la com outra proteína de outro vegetal, cujo Aa esteja em abundância, como acontece com o feijão e arroz, arroz com ervilha, entre outras combinações, satisfazendo assim, a estratégia da complementação.

Logo, existe uma demanda por estudos detalhados de misturas de proteínas derivadas de plantas, sob condições apropriadas do tratamento ultrassônico, para avaliar se há formação de novos compostos proteicos constituídos de alto valor biológico e desempenho técnico-funcional, no mínimo, similar à de seus precursores. Para que essa demanda seja atendida e concretizada, tais estudos devem buscar, primeiramente, compreender as características físico-química das proteínas em meio aquoso, bem como de sua dispersibilidade, de forma isolada e em mistura, além de avaliar características de digestibilidade e interações dentro da mistura de diferentes fontes proteicas a nível molecular ou supramolecular (Jiménez-Munoz et al., 2021).

Do ponto de vista científico, a partir do conhecimento das características físico-químicas das diferentes proteínas vegetais, em sua forma isolada, será possível compreender de que forma as diferentes configurações do dispositivo ultrassônico ou outros fatores externos (por ex: concentração da dispersão) afetarão a conformação das proteínas e, consequentemente, seu desempenho técnico-funcional. Além disso, diante do conhecimento aprofundado dessas características das proteínas de forma isolada, será possível reconhecer e entender as diferenças conformacionais adquiridas pela mistura de diferentes materiais proteicos, bem como encontrar proporções de diferentes isolados proteicos que levam a maiores ou menores respostas quanto a formação de espuma, gel, capacidade em reter água e óleo, formar emulsões, etc.

Do ponto de vista tecnológico, baseado nas respostas funcionais adquiridas como resultado do tratamento ultrassônico e das misturas de proteínas, será possível direcionar melhor as mesmas proteínas para aplicações específicas em termos de desenvolvimento de formulações alimentícias.

Assim, esta tese comtempla informações referentes à otimização do uso de isolados e concentrado proteicos da soja, ervilha e arroz, tanto em sua forma isolada, quanto por meio da mistura entre esses materiais. Para isso, o primeiro capítulo trás uma revisão de literatura referente às proteínas vegetais e tratamento ultrassônico. O segundo capítulo refere-se a resultados experimentais de otimização do tratamento ultrassônico sobre IPS, IPA e CPE, envolvendo três fatores (tempo, potência e concentração), baseados em suas respostas técnico-funcionais. Por fim, o terceiro e último capítulo, traz informações experimentais de misturas entre IPS, IPA e CPE, envolvendo ou não tratamento ultrassônico.

2. Objetivos

2.1 Objetivo Geral

Caracterizar o isolado proteico da soja (IPS), isolado proteico do arroz (IPA) e concentrado proteico de ervilha (CPE), individualmente e em combinações binária ou ternárias, de modo a caracterizar suas propriedades físico-químicas e otimizar uma condição do tratamento ultrassônico baseado em suas respostas técnico-funcionais e de sua digestibilidade *in vitro*.

2.2 Objetivos Específicos

- Caracterizar o IPS, IPA e CPE, em relação a:
    a. Composição centesimal;
    b. Determinação do ponto isoelétrico (potencial ζ, T = 25,0 ± 1,0 °C; pH de 2,0 – 9,0);
- Otimizar uma condição de ultrassom (frequência da onda de 20 kHz, potência de 562,5 à 712,5 W e tempo de 120 a 600 s), por meio do desenho experimental tipo Box-Behnken, em dispersão proteica do IPS, IPA ou CPE variando de 1,0 à 5,0 %, de acordo com suas propriedades técnico-funcional, quanto à:
    a. Dispersibilidade proteica (pH 7,0);
    b. Capacidade de retenção à água e óleo;
    c. Propriedades emulsificantes e espumantes (atividade e estabilidade, pH 7,0);
    d. Propriedades gelificantes (firmeza)
- Avaliar as características físico-química dos 13 distintos sistemas do desenho experimental Box-Behnken, do IPS, IPA ou CPE quanto à:
    a. Distribuição da massa molecular;
    b. Perfil da dispersibilidade proteica à 25 ± 1,0 °C, na faixa de pH de 2,0 – 9,0;
    c. Potencial ζ e diâmetro médio hidrodinâmico (pH 7,0);
    d. Hidrofobicidade superficial (pH 7,0).
- Preparar misturas de IPS, IPA e CPE por meio do desenho experimental de mistura centroide simples, dispersá-las em água na concentração de 1,0 ou 3,0% (w/v), submetê-las à um tratamento ultrassônico de 256,5 kJ (20 kHz, 712,5 W por 360 segundos) e avaliar suas caraterísticas físico-química e propriedades técnico-funcionais em relação à:

a. Perfil da dispersibilidade proteica à 25 ± 1,0 °C, na faixa de pH de 2,0 – 9,0;
b. Distribuição da massa molecular;
c. Potencial ζ e diâmetro médio hidrodinâmico (pH 7,0) ;
d. Hidrofobicidade superficial (pH 7,0) ;
e. Grupos sulfidrilas livres (pH 7,0);
f. Capacidade de retenção à água e óleo;
g. Propriedades espumantes (atividade e estabilidade, pH 7,0);
h. Propriedades emulsificantes (atividade, estabilidade cinética, índice de cremeação, ascpectos macro e microscópicos, pH 7,0);
i. Concentração minima de gelificação.

- Avaliar a digestibilidade *in vitro* das misturas proteicas tratadas ou não com ultrassom.

Capítulo 1

3. Revisão de literatura

3.1 Mercado brasileiro plant-based

Alimentos plant-based, nada mais são que aqueles à base de plantas. Estes alimentos são desenvolvidos a partir de matérias-primas vegetais, atendendo à demanda de consumidores vegetarianos, veganos, flexitarianos, e aqueles que apresentam problemas relacionados a ingestão de proteínas de origem animal (alergia, intolerância). Os flexitarianos correspondem aos consumidores que optaram por reduzir o consumo de produtos de origem animal, sem extingui-los de sua dieta (GFI, 2020).

Em uma pesquisa realizada pelo IBOPE, coordenada pelo GFI e apoiada por 11 empresas do setor de alimentos (GFI, 2020), revelam que dentre os reflexos oriundos da pandemia COVID-19, está a preocupação dos brasileiros em relação à sua saúde e escolhas alimentares. Assim, esta pesquisa mostrou que 50% das pessoas entrevistadas diminuíram o consumo de proteínas animais, 56% ainda consomem frango pelo menos três vezes por semana e 43% de carne bovina. Além disso, 47% das substituições dos alimentos de origem animal é representada pelo consumo direto de legumes, verduras e grãos, ilustrando que a proteína animal ainda ocupa expressivo destaque no prato do brasileiro e que o consumidor ainda não foi completamente atraído pelas opções vegetais disponíveis no mercado. Logo, uma demanda de mercado de produtos plant-based, com características sensoriais pelo menos similares aquelas de origem animal, devem se consolidar nos próximos anos. O Brasil é beneficiado com muitas opções de matérias-primas vegetais e, a Figura 1 ilustra o ranking do valor de produção das principais culturas vegetais que movimentam o mercado brasileiro (IBGE, 2021).

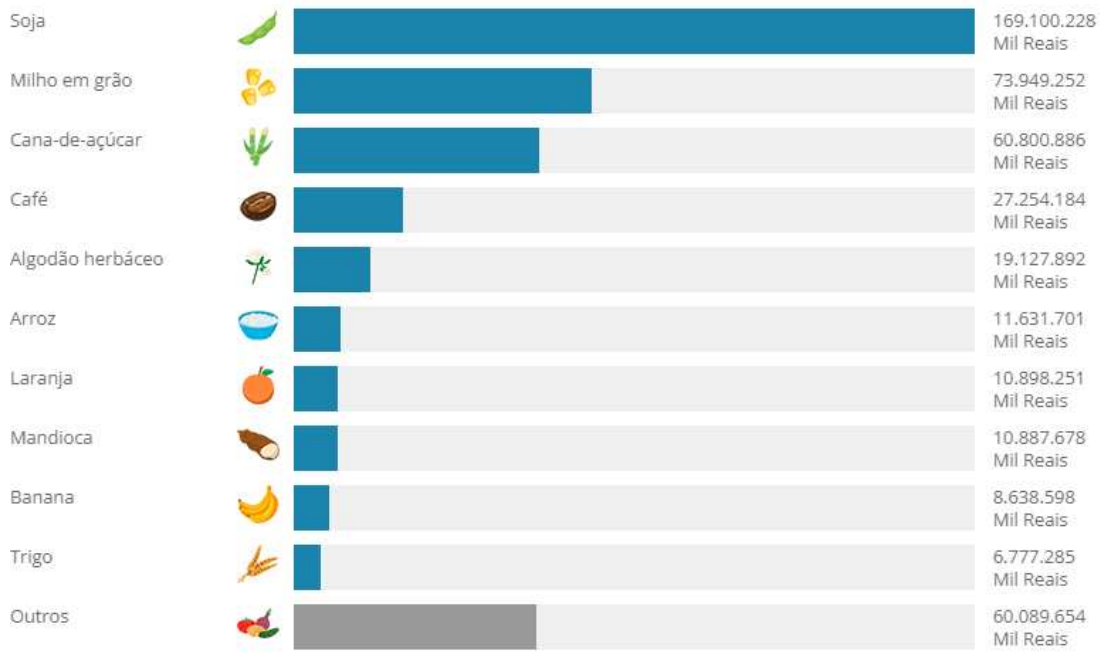


Figura 1. Ranking: Agricultura – Valor da produção (2020).
Fonte: IBGE (2021)

A soja representa a cultura com maior participação do valor de produção, seguido do milho e da cana-de-açúcar (Figura 1). Além destas, culturas minoritárias também atraem interesse de pesquisadores e indústria de alimentos, em virtude de sua composição nutricional. Dentre os constituintes destas culturas vegetais, as proteínas ganham destaque, tanto pelo seu aspecto nutricional, quanto pelo seu valor técnico-funcional, motivo pelo qual o tópico seguinte aborda mais detalhes sobre alguns aspectos das proteínas plant-based.

3.2 Proteínas plant-based

As proteínas são biomacromoléculas constituídas de uma ou mais cadeias longas de resíduos de Aas, com várias funções biológicas (Damodaran et al., 1996). A ingestão regular de proteínas é crucial para o metabolismo adequado e a manutenção da saúde em seres humanos. As proteínas, sejam elas de origem animal e/ou vegetal, devem atingir quantidades de Aas essenciais por grama (g) da proteína de referência, de acordo com às recomendações da FAO/WHO/ONU (ANVISA, 2015; FAO, 2011) (Tabela 1).

Tabela 1. Quantidades recomendadas de aminoácidos essenciais em mg de aminoácido/ g de proteína.

Aminoácidos	Recomendação[1]	Recomendação[2]
Histidina	27	15
Isoleucina	35	30
Leucina	75	59
Lisina	73	45
Metionina + cistina	35	22
Fenilalanina + tirosina	73	38
Treonina	42	23
Triptofano	12	6
Valina	49	39

[1] Padrão de aminoácido tecidual;
[2] Padrão de aminoácido de manutenção.
Fonte: FAO/ WHO/ ONU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition. WHO Technical Report Series N° 935. World Health Organization, Geneva, Switzerland. (2007).

Além da relevância nutricional, as proteínas também desempenham aplicações tecnológicas na formulação de alimentos em virtude de suas propriedades funcionais, como caráter anfifílico e capacidade de formar filmes interfaciais, criando sistemas estabilizadores, como emulsões e espumas, ou interagindo entre si, formando redes como géis e filmes comestíveis. Normalmente, proteínas de origem animal são as mais utilizadas, no entanto, pesquisas recentes tem mostrado bastante interesse em novas alternativas, fazendo com que muitas proteínas vegetais ganhem espaço tanto no meio acadêmico, quanto de aplicação nas indústrias de alimentos.

Muitos vegetais e seus subprodutos são fontes importantes de proteínas. A Figura 2 mostra alguns exemplos de fontes baseadas em três categorias: leguminosas, sementes e cereais que tradicionalmente são utilizadas como suplemento nutricional (Wen et al., 2019).

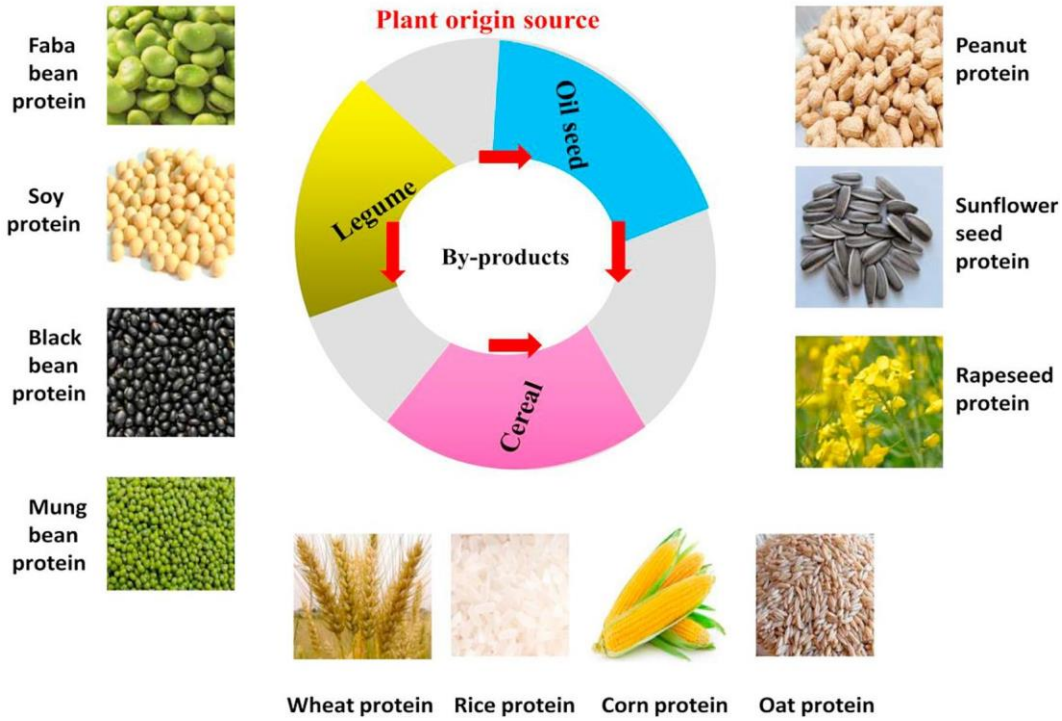


Figura 2. Classificação das principais proteínas derivadas de plantas utilizadas na indústria de alimentos (Fonte: Wen et al., 2019).

De acordo com a ANVISA (Resolução CNNPA n° 12, de 1978), “legume é o fruto ou a semente de diferentes espécies de plantas, principalmente das leguminosas, utilizados como alimentos”. Leguminosas secas, também denominadas por *pulse,* correspondem à sementes comestíveis de leguminosas e representam fonte barata de proteínas, Aas essenciais, peptídeos bioativos, amido, fibras dietéticas e vitaminas (Boye et al., 2010; Ladjal-ettoumi et al., 2015).

Os cereais são as sementes ou grãos comestíveis das gramíneas, tais como trigo, arroz, centeio e aveia (ANVISA, 1978), e são constituídos principalmente por amido, mas suas proteínas perfazem de 10 a 15 % do peso do grão (Sgarbieri, 1996). Estas proteínas são classificadas em albuminas, globulinas, prolaminas e glutelinas que são solúveis em água, solução salina, etanol 70% e álcali diluído, respectivamente (Osborne, 1924). As proporções destas proteínas são diferentes no arroz, milho e trigo, o que lhes conferem propriedades químicas, físicas, funcionais e nutricionais distintas (Sgarbieri, 1996).

Por fim, as sementes são fontes interessantes de proteínas, e muitos estudos de caracterização das propriedades físicas, químicas e funcionais, bem como aplicações alimentícias ou não-alimentícias (plásticos, filmes, materiais de embalagem, etc) já são relatados na literatura (Bandara et al., 2018; Dabbour et al.,

2018; Guo et al., 2018; Kaur et al., 2018; Sánchez-Reséndiz et al., 2018), contudo, em termos econômicos, sua exploração é mais voltada para os óleos.

Apesar destes vegetais representarem fontes de proteínas alimentares, a escolha da localização da proteína dentro do vegetal, bem como o método empregado na extração desta macromolécula, é de suma importância, uma vez que até mesmo pequenas variações nos procedimentos, afetam tanto o rendimento da extração, quanto as características estruturais e funcionais das proteínas de interesse.

Muitos estudos encontrados na literatura reportam sobre processos de obtenção de concentrados e isolados proteicos de origem vegetal, bem como técnicas que otimizam as propriedades funcionais destes produtos obtidos. Os concentrados proteicos são obtidos após o vegetal passar por vários processos mecânicos e operações unitárias que permitem a remoção da maior parte dos demais constituintes do alimento, como a água, minerais e outros materiais orgânicos, restando as proteínas junto a quantidades substanciais de carboidratos e gorduras. Quando o percentual proteico supera 90 %, pode-se denomina-lo como isolado proteico (Kalman, 2014). A seguir, informações da soja, ervilha e arroz são trazidas com enfoque sobre suas proteínas.

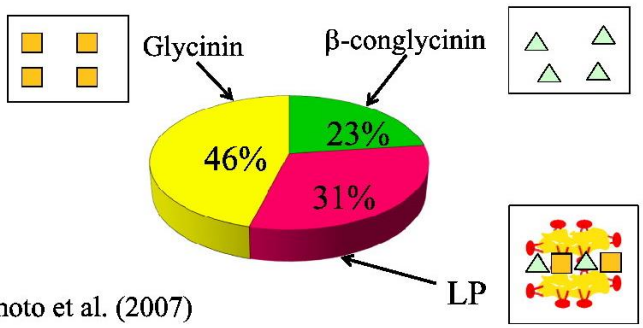
3.2.1 Proteínas da soja

Dentre as proteínas vegetais, as proteínas de soja (*Glycine max* L.) ainda permanece como a mais utilizadas em formulações de alimentos. A soja, corresponde a uma das plantas de maior importância da família das leguminosas em virtude de composição química dos grãos (~40 % de proteína, ~22 % de lipídeos, ~30 % de carboidratos, ~10 % umidade e ~6 % de cinzas), mas estes constituintes são influenciados por fatores genéticos, como tipo de cultivar, e ambientais (localização geográfica e época de semeadura) (De Toledo Benassi et al., 2011). Além disso, são de fácil produção agrícola, geram uma variedade de produtos à indústria de alimentos de qualidade nutricional, funcional e de baixo custo, como óleo vegetal, farinha desengordurada, concentrado proteico, lecitina e isolado proteico de soja (IPS).

As proteínas do grão de soja podem ser extraídas em água após acidificação em pH 4,5 – 4,8 em globulinas de armazenamento (fração precipitada e de maior proporção) e a fração do soro, representada pelas globulinas minoritárias (γ-conglicininas), lipoxigenase (LOX, 102 kDa), β-amylase (61,7 kDa), lectina (33 kDa) e inibidores de tripsina Kunitz (KTI, 20 kDa) (Iwabuchi & Yamauchi, 1987).

As proteínas de armazenamento predominam no IPS, e este tem sido amplamente utilizado na indústria de alimentos e foco de estudos acadêmicos, em virtude das suas características nutritivas e das suas propriedades técnico-funcionais, tais como, formação de espuma, formação de gel e retenção de água e de gordura (Brito-Oliveira et al., 2018; Mattil, 1974; Nishinari et al., 2014). Estas proteínas apresentam tamanho, peso molecular, densidade, carga e estruturas diversas, e são classificadas, de acordo com a velocidade de sedimentação por meio da técnica de ultracentrifugação, em quatro frações: 2, 7, 11 e 15S (Sgarbieri, 1996).

As frações 7S (150 - 190 kDa) e 11S (300 - 360 kDa) constituem 80% da proteína total soja, com proporções de 7S/11S entre 0,5 – 1,3, dependendo da variedade (Saio et al., 1969), e valores equilibrados de Aas polares e não polares (Teng et al., 2009). Além destas, Samoto et al. (2007) relataram que ao se utilizar uma metodologia envolvendo três etapas de acidificação de um extrato aquoso de farinha de soja desengordurada, obtêm-se uma quantidade significativa de uma proteína lipofílica (LP) em IPS (Figura 3), apresentando um conteúdo proteico de 76% (contra 87% do 7S e 93% do 11S) e percentual lipídico de 11,7% (contra 0,8% do 7S e 3,3% do 11S).



LP is consisted of protein (oleosin)–phospholipids complex (OL-PL), glycinin and β-conglycinin. Glycinin and β-conglycinin are "accidentally" involved in OL-PL particles to form LP fraction.

Figura 3. Composição do IPS de acordo com Samoto et a., 2007. Fonte: Matsumura et al., 2017.

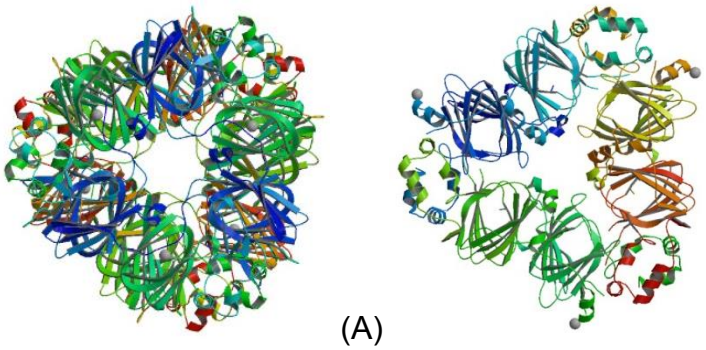
Matsumura et al. (2017) relatam que a LP não foi identificada anteriormente porque esta lipoproteína dificilmente é detectada pela técnica convencional de separação de proteínas (SDS-PAGE), em virtude da baixa sensibilidade do LP à coloração de Coomassie Brillant Blue (CBB).

A família da globulina 11S é representada principalmente pela glicinina, uma molécula hexamétrica (492 resíduos) composta por uma cadeia polipeptídica ácida

(A) (35 - 40 kDa) e uma básica (B) (20 kDa), unidas por uma única ligação dissulfeto (Mujoo et al., 2003; Nishinari et al., 2014). A estrutura secundária se caracteriza como 10 % hélice (53 resíduos) e 29 % como folhas beta (146 resíduos) (Adachi et al., 2003).

Já as globulinas 7S são principalmente representadas pela β-conglicinina (pI: 4,8-4,9), uma glicoproteína trimérica constituída pelas subunidades α' (57 - 71 kDa), α (57 – 67 kDa) e β (42 – 50 kDa) (Nishinari et al., 2014), que contemplam 418 resíduos de aminoácidos e se estruturam como 16 % de hélice e 36 % de folhas beta (Maruyama et al., 1998). As subunidades α e α' são localizadas nas regiões de extensão (α, 125 resíduos; α', 141 resíduos) e do núcleo (418 resíduos), já a subunidade β consiste apenas da região do núcleo (416 resíduos), e esta diferença parece afetar o ponto isoelétrico, hidrofobicidade e estabilidade térmica entre as subunidades individuais, sugerindo assim, diferentes funções físico-químicas (Maruyama et al., 1998).

A proposta atual e aceita em relação às estruturas cristalinas das globulinas 11S e 7S, obtidas por difração de raios X, são apresentadas na Figura 4 e foram obtidas do Banco de Dados de Proteínas (PDB) (http://www.pdb.org/).



(A) (B)

Figura 4. Estrutura quaternária cristalina das principais proteínas (globulina) da soja. A) glicinina (11S) – homohexâmero PDB ID: 1OD5; B) β-conglicinina (7S) – homotrímero PDB ID: 1UIK.

3.2.2 Proteínas da ervilha

Proteínas da ervilha (*Pisum sativum* L.), concentradas ou isoladas, atualmente é uma das principais alternativas às proteínas da soja, por apresentarem características nutricionais comparáveis, custo industrial acessível e baixa alergenicidade (Lam et al., 2018). As ervilhas podem ser cultivadas extensivamente em todo o mundo, sendo seu casco facilmente removível, e as sementes desta leguminosa é comumente industrializada e consumida. Dependendo da variedade,

maturidade na colheita e condições de crescimento, a ervilha contém 60-65% de carboidratos (distribuídos como amido, fibra alimentar, sucrose, oligossacarídeos e celulose), 23,1–30,9% de proteína, 1,5–2,0% de gordura e constituintes menores, como vitaminas, minerais, ácido fítico, polifenóis, saponinas e oxalatos (Lam et al., 2018).

As frações proteicas são constituintes valiosas da semente de ervilha e podem ser extraídas juntas, constituindo um produto heterogêneo designado isolado proteico de ervilha (IPE). Além disso, cada proteína pode ser isolada com base nas características de solubilidade em soluções aquosas, apesar desta realidade ser pouco explorada e empregada pela indústria de alimentos em virtude das precárias informações a respeito da estrutura-função e seu desempenho (Adebiyi & Aluko, 2011; Oliete et al., 2018a).

Considerando a classificação de Osborne (1924), as proteínas de ervilha são representadas pelas albuminas (2S; 5 – 80 kDA), um grupo heterogêneo de proteínas pequenas (enzimas, inibidores de protease, inibidores de amilase e lectina); pequenas quantidades de prolaminas e gluteninas (Saharan & Khetarpaul, 1994), e predominância das globulinas, que são proteínas de armazenamento (65 – 80 % das proteínas totais dentro das sementes), que compreendem três grupos principais: i) legumina (11S), com uma estrutura quaternária hexamérica (6 × ~ 60 kDa); ii) vicilina (7S) e; iii) convicilina (7-8S), tendo estas duas últimas uma estrutura trimérica (3 × ~ 50 kDa e 3 × ~ 70 kDa, respectivamente) (Casey & Domoney, 1999).

As leguminas apresentam uma subunidade ácida (40 kDa) e uma básica (20 kDa) (α-β) ligadas entre si covalentemente por uma ligação dissulfeto, constituindo um monômero dentro da estrutura quaternária não ligada de forma covalente. A cadeia α é dominada pelo ácido glutâmico, tendo a leucina como grupo amino N-terminal, já a cadeia β são constituídas principalmente por alanina, valina e leucina, com a glicina como grupo N-terminal (Lam et al., 2018). As vicilinas possuem 3 subunidades: α, β e γ, unidas por interações hidrofóbicas, cujos Aas constituintes são deficientes em enxofre (metionina e cisteína) e triptofano, mas são ricos em Aas básicos (arginina e lisina) e ácidos (ácido aspártico e ácido glutâmico). Já as convicilinas contém Aas sulfurosos e uma extensão N-terminal altamente carregada (Lam et al., 2018).

Estruturas cristalinas das proteínas da ervilha são precárias na literatura, mas sabe-se que, em sua maioria, são predominadas por estruturas secundárias do tipo folhas-β. Na plataforma PDB, a única representante encontrada para ervilha foi a

estrutura da prolegumina (Figura 5), uma globulina da fração 11S, constituída por 496 resíduos de Aas que se estruturam como 9 % de hélices e 28 % de folhas beta.

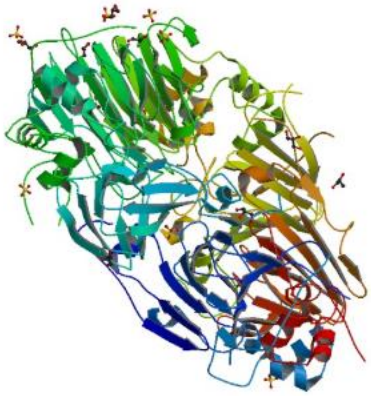


Figura 5. Estrutura cristalina da globulina (11S) prolegumina da ervilha. PDB ID: 3KSC.

3.2.3 Proteínas do arroz

O arroz (*Oryza sativa* L.) é uma planta monocotiledônea que pertence à família *Poaceae* (Champagne et al., 2004). Este cereal é muito consumido em vários países, tendo o amido e a proteína como seus principais constituintes. Assim, o arroz tem sido um alvo recente tanto em pesquisa, quanto para uso em formulações alimentícias, e isto se deve à vários fatores, como facilidade de cultivo, boa digestibilidade proteica e valor biológico, hipoalergenia, entre outras evidências funcionais à saúde humana (Amagliani & Schmitt, 2017).

As proteínas do arroz são encontradas em todas as partes do grão (pericarpo, camadas de aleurona, endosperma e embrião), principalmente no endosperma, localizado em corpos proteicos, entre os grânulos de amido (Sgarbieri, 1996). A composição proteica é variável em se tratando do cereal em sua forma integral, polido ou farelo (albumina 4 – 43 %, globulinas 6 -36 %, prolamina 1 – 7 % e glutelina 22 – 83 %), bem como dependente da variedade e metodologia aplicada para extração (Amagliani et al., 2017).

A glutelina, como principal fração proteica do arroz, é sub-classificada como i) subunidade α ou ácida (R_a, 7 - 12 bandas, pI < 7, PM 30 a 40 kDa) e ii) subunidade básica (R_b, 5 - 9 bandas, pI > 7, PM 19 – 23 kDa), com base nas diferenças de seus pI (Van Der Borght et al., 2006). Sua caracterização é dificultada em virtude da sua insolubilidade em solventes neutros (Sgarbieri, 1996). As subunidades α e β tendem

a se polimerizar por meio de ligações dissulfeto, formando complexos moleculares de alto peso molecular, insolúveis em água (Amagliani et al., 2017). As demais frações proteicas do arroz ainda apresentam informações inconsistentes, pois a caracterização é muito afetada pela técnica aplicada para extração das frações. Além disso, nenhuma estrutura cristalina de proteína do arroz foi encontrada na plataforma PDB.

Assim, diante do conhecimento das diferentes estruturas das proteínas da soja, ervilha e arroz, é possível compreender melhor os prováveis mecanismos que regem suas propriedades técnico-funcional e biofuncionais.

3.3 Propriedades bio-funcionais

Em termos nutricionais, a longa data emprega-se os conceitos Aas essenciais e não essenciais. No primeiro, considera-se aqueles Aas em que o organismo humano não consegue sintetizar (ou não podem sintetizar o suficiente), precisando ser adquiridos por meio do consumo direto de fontes produtoras. Já no segundo, o próprio metabolismo humano encontra rotas de síntese (Kathleen Mahan, 2015).

Atualmente, admite-se que haja os Aas condicionalmente essenciais, onde sob determinadas condições metabólicas, não são sintetizadas pelo organismo em quantidade suficiente para suprir suas necessidades. Desta forma, a classificação compreende três categorias: i) Aas dispensáveis (alanina, asparagina, glutamato e aspartato), ii) Aas indispensáveis (isoleucina, leucina, lisina, metionina, fenilalanina, treonina, triptofano e valina) e, iii) Aas condicionalmente indispensáveis (histidina, arginina, prolina, cisteína, glicina, glutamina, serina e tirosina), baseados em parâmetros como dieta, estado nutricional e condição fisiopatológica (Waitzberg & Logullo, 2006).

Também deve-se considerar que não é apenas a presença dos Aas essenciais que ditam sua qualidade, mas também a forma como estas serão utilizadas metabolicamente. Desta forma, é necessário que as proteínas possuam (Costa *et al*., 2014):

i) abundância: quantidade e qualidade para atender às necessidades nutricionais;
ii) boa digestibilidade: que a quantidade ingerida seja digerida e absorvida pelo organismo;

iii) disponibilidade: após digestão e absorção, seja utilizada pelo organismo para realização de funções biológicas; e

iv) biodisponibilidade: prontidão do nutriente para ser armazenado e utilizado pelo organismo.

Neste sentido, tipicamente, as proteínas de origem animal (leite, carne, ovos, etc.) são consideras completas (Bobbio & Bobbio, 1992), por apresentarem valores superiores em métodos de avaliação da qualidade proteica, tanto *in vitro* (escore químico), quanto *in vivo* (PER) (Tabela 2). No entanto, vale ressaltar que fatores econômicos, ambientais, religiosos, culturais (Liener, 1962) e alergênicos das proteínas animais, têm levado ao aumento do interesse pelas proteínas vegetais (Lin *et al*., 2017).

Tabela 2. Conteúdo de aminoácidos essenciais e valor nutricional das proteínas oriundas de várias fontes.

Propriedade (mg/ g de proteína)	Fonte de proteína (mg/ g de proteína)											
	Origem animal				**Origem vegetal**							
	Ovo	Leite	Carne	Peixe	Trigo	Arroz	Milho	Cevada	Soja	Ervilha	Amendoim	Vagem
Histidina	22	27	34	35	21	21	27	20	30	26	27	30
Isoleucina	54	47	48	48	34	40	34	35	51	41	40	45
Leucina	86	95	81	77	69	77	127	67	82	70	74	78
Lisina	70	78	89	91	23	34	25	32	68	71	39	65
Metionina + Cisteína	57	33	40	40	36	49	41	37	33	24	32	26
Fenilalanina + Tirosina	93	102	80	76	77	94	85	79	95	76	100	83
Treonina	47	44	46	46	28	34	32	29	41	36	29	40
Triptofano	17	14	12	11	10	11	6	11	14	9	11	11
Valina	66	64	50	61	38	54	45	46	52	41	48	52
Total de Aas essenciais	512	504	480	485	336	414	422	356	466	394	400	430
Conteúdo proteico	12	3,5	18	19	12	7,5	-	-	40	28	30	30
Escore químico (padrão FAO/OMS, 1985)	100	100	100	100	40	59	43	55	100	82	67	-
Quociente de eficiência proteica (PER)	3,9	3,1	3	3,5	1,5	2	-	-	2,3	2,65	-	-

Fonte: Damodaran *et al.*, 2010 (adaptado) apud FAO/WHO/UNU, 1985.

O escore químico (EQ) relaciona o valor de cada Aa essencial de uma proteína-teste e compara-a com o do conteúdo do mesmo Aa de uma proteína padrão, determinando-se assim, o percentual do Aa essencial limitante da proteína-teste. Desta forma, quando o valor de EQ < 1 (ou 100), pode-se afirmar que a proteína é limitante em pelo menos um Aa essencial, mas se o valor for = 1, indica que, em termos químicos, a proteína é de boa qualidade. Já a razão da eficiência proteica (PER) refere-se a uma das principais técnicas de avaliação da qualidade proteica *in vivo*, que se baseia no crescimento do animal, representado pela razão entre o ganho de peso do grupo-teste, pela proteína consumida por este mesmo grupo. Quando o PER < 1,5, é um indicativo de que a proteína teste apresenta baixa qualidade, valores entre 1,5 – 2,0 correspondem a proteínas de qualidade intermediária e > 2, de alta qualidade (Costa *et al*., 2014).

De acordo com o valor do conteúdo proteico informado na Tabela 2, verifica-se que as informações relativas à qualidade proteica (EQ e PER) são referentes aos vegetais *in natura*. Deve-se considerar que concentrados e isolados proteicos apresentam índices de qualidade superiores, fornecendo respostas melhores na utilização das proteínas.

Fernández-Quintela et al. (1998) estudaram o efeito nutricional em ratos de diferentes leguminosas (ervilha, fava e soja), na forma de sementes ou isolados proteicos. Apesar do crescimento e índices nutricionais das leguminosas mostrarem-se inferiores à da caseína (controle), os resultados obtidos com os isolados foram superiores aos da semente, em termos de digestibilidade. Assim, isolados e concentrados proteicos de diferentes fontes vegetais, apresentam-se cada vez mais como potenciais ingredientes proteicos inovadores.

3.4 Propriedades técnico-funcionais

As propriedades técnico-funcionais das proteínas e hidrocolóides em alimentos estão diretamente relacionadas às suas características estruturais, físicas e químicas. Estes fatores ditam o comportamento e desempenho durante a preparação, processamento, armazenamento e consumo, em virtude das interações que ocorrem entre elas mesmas, os demais componentes e água do sistema alimentar. Deste modo, diante da compreensão destas caraterísticas em

conjunto aos seus aspectos nutricionais, é possível otimizar a utilização destas biomacromoléculas no desenvolvimento de produtos. As principais propriedades tecnológico-funcionais estão relacionadas às suas propriedades interfaciais, devido ao seu caráter anfifílico, bem como aquelas relacionadas a capacidade em formar filmes e géis.

As emulsões são sistemas coloidais formados por dois líquidos imiscíveis, com um deles disperso como pequenas gotículas (0,01-10 μm) dentro do outro. As emulsões simples são classificadas de acordo com a natureza de sua fase dispersa; logo, se esta for óleo, ter-se-á uma emulsão do tipo óleo em água (O/A), mas se for água, será água em óleo (A/O) (McClements, 2004). Tais sistemas são a base para inúmeras formulações de alimentos, seja parcial ou totalmente (estado emulsionado) durante sua produção, o que inclui leite, creme, bebidas, sopas, molhos de salada, maionese, sorvete, manteiga, entre outros.

A relação interfacial área/volume das emulsões é enorme, por isso, esses sistemas são termodinamicamente instáveis, tendendo a separação de fases (McClements, 2004). No entanto, é possível minimizar tal instabilidade pelo uso de emulsificantes, que são moléculas ativas de superfície com propriedades anfifílicas, ou seja, possuem regiões hidrofílicas e hidrofóbicas, tais como proteínas.

Para que uma proteína seja um emulsificante eficaz, ela deve ser solúvel em água e estar prontamente adsorvida na interface óleo-água. A habilidade em conseguir desdobrar-se na interface, promove a formação de um filme viscoelástico em torno de gotículas de óleo por meio de interações intermoleculares (Damodaran et al., 1996). Este filme viscoelástico favorece a resistência frente a tensões mecânicas e tende a proporcionar estabilização eletrostática e esterica (Lam & Nickerson, 2013). No entanto, na literatura são encontrados muitos estudos que comprovam que a estabilidade de emulsões pelo uso de proteínas é dependente da composição da solução (pH, força iônica, surfactantes e biopolímeros) e das tensões ambientais (aquecimento, congelamento e secagem) (McClements, 2004). Logo, a escolha do tipo de proteína e sua concentração, para direcioná-lo a algum produto, tem que ser bem embasada para prevenir gastos desnecessários.

As espumas consistem de uma fase contínua aquosa e uma fase dispersa gasosa (ar). Espumas a base de proteínas são formadas pelo

revestimento da bolha de ar por um filme fino e resistente da proteína na interface gás-líquido, garantindo que grandes quantidades de bolhas sejam incorporadas e estabilizadas. Exemplos de alimentos que são espumas: sorvete, bolos, pães, mousse, *marshmallows* (Damodaran et al., 1996). A propriedade espumante, assim como as emulsões, por se tratarem de sistemas dispersos, são altamente instáveis, motivo pelo qual a escolha da substância anfifílica deve ser bem planejada. Fatores como solubilidade, concentração, composição e estado de desnaturação da proteína, bem como presença de sais e outros aditivos, pH e temperatura, tendem a influenciar diretamente o desempenho da proteína neste tipo de sistema.

Gel representa uma fase intermediária entre o sólido e o líquido, e é definido como “sistema substancialmente diluído que exibe um estado constante de fluxo” (Ferry, 1961). O processo de gelificação envolve o emaranhamento das proteínas, por meio de ligações covalentes ou não covalentes, de modo a formarem uma rede tridimensional, aprisionando o solvente (água) em seus interstícios. O uso de calor, enzimas ou cátions divalentes, sob condições apropriadas, tendem a otimizar este processo (Damodaran et al., 1996).

A processo inicial de gelificação envolve o enfraquecimento e consequente rompimento das ligações de hidrogênio e dissulfídricas (geralmente induzida por calor), abalando a conformação tridimensional da proteína. Em seguida, ocorre a polimerização das moléculas, formando uma rede tridimensional, imobilizando fisicamente o solvente por meio de ligações dissulfídricas, interações hidrofóbicas e iônicas, designadas como zonas de junção (Brito-Oliveira et al., 2018).

De acordo com Opazo-Navarrete et al. (2018), existem duas classes diferentes de géis de proteínas: redes de proteínas reticuladas e gel proteico globular. Na primeira, as proteínas são flexíveis e parcialmente desnaturadas. Já as proteínas globulares, envolve o desdobramento e exposição das partes hidrofóbicas, que estavam interagindo entre si no interior da proteína antes do desdobramento, formando aglomerados. Este último é o que representa os géis desenvolvidos por proteínas vegetais.

O grau de desenovelamento da proteína ao longo da desnaturação induzida, ou desdobramento, está diretamente relacionada com o tempo e temperatura do sistema, tipo e concentração da proteína-teste, presença de sais,

valor do pH e interações com outros componentes. Assim, testes e consulta da literatura são primordiais no desenvolvimento dos géis proteicos.

Logo, os isolados e concentrados proteicos de fontes vegetais representam potenciais ingredientes para indústria de alimentos, por apresentarem qualidade em suas propriedades técnico-funcionais e biofuncionais, superiores à sua contra-parte *in natura*. No entanto, o uso destes materiais proteicos a base de vegetais é limitada, principalmente por conta de sua baixa solubilidade em meio aquoso. Assim, atualmente, tem-se recorrido à várias estratégias emergentes para otimizar ainda mais o seu uso, como será relatado a seguir.

3.5 Estratégias emergentes para otimização do uso de proteínas vegetais.

Nos últimos anos, modificação de proteínas por métodos físicos como ultrassom, alta pressão e tratamento térmico vem sendo estudada, aprimorada e aplicada no ramo de alimentos. A aplicação destas estratégias sobre proteínas vegetais constitui uma importante ferramenta para ampliar seu uso, pois parecem trazer melhorias tanto no aspecto nutricional, quanto tecnológico.

O estudo de Wang & Mcintosh (1996) mostrou que o processamento por extrusão ou cozimento (ebulição) de ervilha aumentaram significativamente o ganho de peso corporal (BWG) de ratos, mas não o PER, quando comparado à sua contraparte crua. A ervilha extrusada mostrou melhor eficácia na redução do colesterol plasmático do que a dieta a base de caseína e também foi observado que a combinação de ervilha com trigo em extrusão, melhoraram BWG e PER, em virtude do trigo ter fornecido Aas sulfurosos, que são limitantes nas leguminosas. Alonso et al. (2001) também verificaram melhora da qualidade nutricional da ervilha extrusada, mas foi necessário a suplementação com aminoácidos, pois esta estratégia pareceu abolir os efeitos negativos das ervilhas na deposição de músculos esqueléticos.

Estudos *in vivo*, com foco em qualidade proteica ou valor nutricional de proteínas com tratamento ultrassônico, são escassos na literatura. Em termos de proteínas vegetais, um estudo realizado com microalgas (*Chlorella vulgaris*) mostrou que o ultrassom (pressão de 2,0 – 5,6 bar, entrada de energia entre 961 e 1660 W) melhorou o coeficiente de digestibilidade aparente da proteína bruta do TGI e seu valor biológico, concluindo que a ultrasonicação representa um

processo tecnológico útil no processamento de algas verdes na indústria de alimentos (Janczyk et al., 2007).

Por definição, o ultrassom nada mais é do que uma onda acústica, cuja frequência encontra-se acima do limiar da audição humana, ou seja, superior a 20 kHz (Chandrapala et al., 2012). Este método pode ser classificado em duas categorias, baseado na faixa de frequência como: i) ultrassom de alta frequência (100 kHz a 1 MHz) de baixa potência (<1 W cm^{-2}), mais comumente utilizado para avaliação analítica das propriedades físico-químicas dos alimentos (Chemat et al., 2011) e ii) ultrassom de baixa frequência (20–100 kHz) e alta potência (10–1000 $W \cdot cm^{-2}$), recentemente empregado para a alteração de alimentos, tanto física quanto quimicamente (McClements, 1995).

Apesar do uso ultrassônico ser considerado uma ferramenta útil para melhorar as propriedades de solubilidade e funcionalidade das proteínas, deve-se salientar que as condições a serem empregadas são muito dependentes das características inerentes das proteínas, por isso para conseguir estabelecer condições ótimas, deve-se ter muito cuidado em termos de frequência, amplitude, tempo, temperatura, força iônica, pH e concentração (Higuera-Barraza et al., 2016). Malik et al. (2017) ao avaliarem o efeito do USt de ponta ou banho de 500 W em isolado proteico de girassol, verificaram melhorias na solubilidade e nas propriedades emulsificantes e espumantes até 20 min, mas quando o tratamento se estendia até 30 min, estas propriedades começaram a ser reduzidas. Comportamento similar foi reportado no trabalho de Zhu et al., (2018), que trabalharam com isolado proteico de noz aplicando USt variando potência (200, 400 ou 600W) e tempo (15 ou 30 min). Logo, o USt representa uma estratégia interessante para promover alterações conformacionais nas proteínas plant-based para melhorar sua técnico-funcionalidade, mas, deve-se atentar para não prolongar o tempo de exposição à cavitação acústica, para não dar início à prejuízos em seu desempenho funcional.

A seguir, a Tabela 3 resume alguns artigos encontrados na literatura que avaliaram o efeito do ultrassom sobre proteínas da soja, arroz e ervilha.

Tabela 3. Efeito do processamento com ultrassom sobre as propriedades técnico-funcionais de proteínas da soja, ervilha e arroz.

Proteínas utilizadas	Objetivos	Condição do USt	Análises	Resultados "chave"	Referência
IPS (86%), IPE (86%), IPA (84,5%), gelatina bovina (GB = 86%), gelatina de peixe (GP = 86%) e proteína da clara de ovo (PCO = 85%)	• Discernir os efeitos do tratamento com US em proteínas animais e vegetais; • Avaliar as diferenças no desempenho das proteínas como emulsionantes após o tratamento com US	• Concentração proteica: 0,1-10% em peso; • Volume tratado = 50 mL; • f = 20 kHz; • Amplitude 95%, • Tempo: 2 min • Potência acústica = ~34 W·cm-².	• Tamanho, volume hidrodinâmico e estrutura molecular (DLS), viscosidade intrínseca, SDS-PAGE. • Desempenho emulsionante	• Redução do tamanho, exceto IPA; • Emulsões [> 5%] de PCO, IPS e IPA = gotículas de tamanho mícron (> 10 µm); • Emulsões com GP, IPS e IPA sonicados = tamanhos de gotículas semelhantes às proteínas não tratadas nas mesmas concentrações; • Emulsões GB, PCO e IPE sonicadas com ≤ 1% = tamanho de gotículas menor < contrapartes não tratadas.	O'Sullivan et al., 2016. Food Hydrocolloids.
IPS (86 %) e proteína do trigo (IPT = 90% peso)	• Discernir os efeitos do tratamento com US sobre IPT e IPS. • Eficácia emulsionante de IPT e IPS antes e depois do US	• Concentração proteíca: 0,1-3,0% em peso (água); • Volume tratado = 50 mL • Tempo: 2 min • f: 20 kHz • Amplitude: 95 %. • Potência acústica: ~ 34 $W \cdot cm^{-2}$.	• Microestrutura, SDS-PAGE, volume hidrodinâmico (viscosidade intrínseca) • Emulsão O/A (10% óleo) – Análises: tamanho das gotículas e tensão interfacial.	• US = reduziu tamanho, não afetou o peso molecular, energia insuficiente para hidrolisar a ligação peptídica); • USt melhorou a performance emulsificante e estabilidade das emulsões de IPS e IPT, devido a presença de menores gotículas e viscosidade intrínseca.	O'Sullivan, Park, et al., 2016. Journal of Cereal Science
Glicinina da soja (11S)	• Estudar alterações FQ e estruturais da glicinina de soja devido ao HIU em	• Concentração proteica: 1,0%; • Volume tratado: 20 mL; • f: 20 kHz;	• SDS-PAGE, tamanho de partícula, solubilidade proteica, dicroismo circular, fluorescência, H_0;	• HIU reduziu o tamanho, mudando sua distribuição dos agregados de glicinina, mas, reduziu a turbidez, e aumentou a H_0;	Zhou et al., 2016. Innovative Food Science

	diferentes forças iônicas; • Fornecer características básicas de como o HIU influenciou a estrutura de glicinina da soja; • Otimizar a aplicação da HIU na indústria da soja.	• Tempo: 0, 5, 20 ou 40 min; • Potência: 60%; • Potência acústica: 80 $W \cdot cm^{-2}$); • Forças iônicas (baixa = 0,06, média = 0,2 e alta = 0,6 • pH 7,0;	• Emulsão: 1 mL óleo de milho + 3 mL disp. Glicinina 1,0% - atividade e estabilidade.	• As estruturas secundárias e terciárias quase não foram alteradas; • As três forças iônicas + USt aumentaram a estabilidade da emulsão, alteraram o tamanho e distribuição de partículas, solubilidade, índice de atividade emulsificante e H_0, sendo que as alterações em 0,2 foram mais pronunciadas.	& Emerging Technologies
IPS, proteína = 90,5 %	• Explorar o impacto do HIUS na funcionalidade de um IPS desnaturado em termos de propriedades espumantes e interfaciais.	• Concentração proteica: 6%; • Volume tratado: 10 mL • Tempo: 5, 10, 15 ou 20 min; • T: 75, 80 e 85 °C; • Frequência: 20 kHz; • Potência: 4,27 ± 0,71 W • Amplitude: 20%; • pH 6,9.	• Espuma: 30 mL disp. Proteica 2% a 200 rpm; • DSC, solubilidade proteica, formação, drenagem e colapso da espuma, DLS, balanço de filme superficial.	• HIUS melhorou a capacidade de formação de espuma com resultados sinérgicos com a temperatura, mas a estabilidade não foi modificada significativamente. • Elasticidade invariável dos filmes interfaciais ao longo do tempo.	Morales et al., 2015. Ultrasonics Sonochemistry
Frações 7S e 11S isoladas da farinha de soja	• Investigar as alterações estruturais e F-Q das frações 7S e 11S em função do HIUS.	• Concentração proteica: 3,0 %; • f: 20 kHz; • Potência: 400 W • Tempo: 0, 5, 20 ou 40 min; • Potência acústica: 105-110 $W \cdot cm^2$.	• SDS-PAGE, tamanho de partícula, solubilidade proteica, turbidez, DC, FT-IR, H_0, teor de sulfonil. • Emulsão: 1 mL óleo de milho + 3 mL disp. Proteica (0,5 %) - IAE, IEE	• HIUS diminuiu turbidez e tamanho de partícula de 7S (pH 7,0), e aumentou H_0, solubilidade, IAE e IEE. • HIUS diminuiu a turbidez de 11S, aumentou o IAE, e efeitos mínimos no tamanho de partícula e no IEE. • Os grupos SH das frações 7S e 11S diminuíram, não	Hu et al., 2015. Food Hydrocolloids

				alterou a estrutura secundária mas aumentou ligeiramente a porcentagem de agregados de alto peso molecular	
IPE, 94,3 %	• Investigar os efeitos do tratamento HIUS nas propriedades espumantes e estruturais do IPE.	• Concentração Proteica: 5 %; • f: 20 KHz; • Tempo: 30 min; • Amplitude: 0, 30, 60 e 90%; • Potência acústica: 22–25, 34–36 ou 45–48 W·cm^2;	• MEV, SDS-PAGE, DC, determinação de grupos SH livre, H_0, tamanho da partícula e ζ; • Espuma: disp. Prot. 5%, 15 mL - capacidade de formação de espuma, estabilidade da espuma, microestrutura, MEV e tensão superficial.	• O HIUS não alterou o perfil eletroforético e a estrutura secundária, mas modificou a estrutura terciária = diminuição do tamanho da partícula, aumento da exposição da H_0 e dos grupos SH livre. • A capacidade de formação de espuma aumentou com o aumento da amplitude do US, assim como a estabilidade de espuma após 10 min da formação da mesma. • US = aumentou a taxa de adsorção e diminuiu a tensão superficial.	Xiong et al., 2018. Food Research International.
IPE, 85%	• Verificar o efeito da variação de pH e aplicação de US nas propriedades F-Q de IPE.	• Concentração proteica: 25,5 mg (bs), • pH: 2,4,10 ou 12 • f: 20 KHz (68 W/ 100 mL), • Tempo: 5 min.	• Solubilidade proteína, tamanho da partícula, turbidez, H_0, SH livres e SDS PAGE.	• US aumentou da solubilidade, H_0 e SH livre, e diminuição do tamanho da partícula; • Melhores resultados = combinação de US e pH 12.	Jiang et al., 2017. Ultrasonics Sonochemistry
IPA, 90 %	• Avaliar o potencial do tratamento US assistida por álcali (UAA) para melhorar a aplicação de IPA	• Concentração proteica: 50 g/L • Sol. NaOH: 0,02, 0,04, 0,06, 0,08 e 0,1M; • f: 20 kHz, 50 °C/	• Solubilidade proteica; SDS-PAGE, SH livre, FTIR, microestrutura (PDI, DLS), ζ. • Atividade e estabilidade da emulsão (6 mL sol	• UAA aumentou a solubilidade à medida que aumentou a [NaOH]; • Redução no tamanho e potencial-ζ das partículas e	Zhang et al., 2018. Journal of Cereal Science.

	na indústria de alimentos.	• Tempo: 60 min, • Potência: 600 W • Volume tratado: 100 mL; • Potência acústica: 19,3 W·cm^2;	proteica 1,0 %, pH 7,5 + 2 mL óleo de oliva) • Atividade e estabilidade espuma (40 mL de sol prot 1,0 %),	degradação da subunidade proteica; • UAA alterou a conformação estrutural interna, expondo grupos funcionais, melhorando as propriedades emulsificantes e espumantes.	

DC = dicroismo circular; DSC = calorimetria diferencial de varredura; FTIR = espectroscopia no infravermelho; HIU = ultrassom de alta intensidade; IAE = índice da atividade emulsificante; IEE = índice da estabilidade emulsificante; MEV = microscopia eletrônica de varredura; UAA = ultrassom assistida por álcali; US = ultrassom; USt = tratamento ultrassônico; O/A = óleo em água; SDS-PAGE = dodecil sultato de sódio - eletroforese em gel de poliacrilamida; H_0 = superfície hidrofóbica, SH = sulfidrilas; f = frequência; T = temperatura.

Esses estudos mostraram como a sonicação pode afetar a conformação de um único isolado / concentrado de proteínas. No entanto, o uso de uma única fonte de proteína vegetal não pode suprir a demanda de aminoácidos essenciais para os humanos. Nesse sentido, a utilização de misturas de proteínas de diferentes origens pode ser uma estratégia para a composição de um material contendo aminoácidos em quantidades e proporções adequadas. Além disso, até onde sabemos, não foram realizados estudos que proponham tanto o uso de mistura de proteínas vegetais quanto sua homogeneização por ultrassom de alta potência.

Pietrysiak et al. (2018) produziu uma mistura com proteína isolada de ervilha-arroz (2: 1) por meio de injeção direta de vapor e verificou melhorias em sua funcionalidade sem afetar a composição de aminoácidos. T. Wang, Xu, Chen, & Wang, (2018) e T. Wang, Xu, Chen, Zhou, et al., (2018) dispersaram proteínas de arroz junto com isolado de proteína de soja em diferentes proporções (1:01; 1: 2) sob condições alcalinas e encontrou melhorias em sua funcionalidade usando o método de ciclo de pH, alegando ter promovido o desenvolvimento de um novo hidrocolóide de proteína ou proteína composto. Portanto, misturas de diferentes proteínas vegetais combinadas com diferentes tratamentos podem gerar novos compostos proteicos ou hidrocolóides inovadores, com tecno-funcionalidade aprimorada.

Diante das informações contidas nessa revisão de literatura, esta tese buscou otimizar a utilização dos isolados proteicos da soja e do arroz e concentrado proteico de ervilha, por meio da tecnologia do ultrassom, avaliando suas propriedades físico-química, técnico-funcionais e biofuncionais, com a finalidade futura de utilização destes materiais, de forma isolada ou em mistura, como ingredientes proteicos inovadores.

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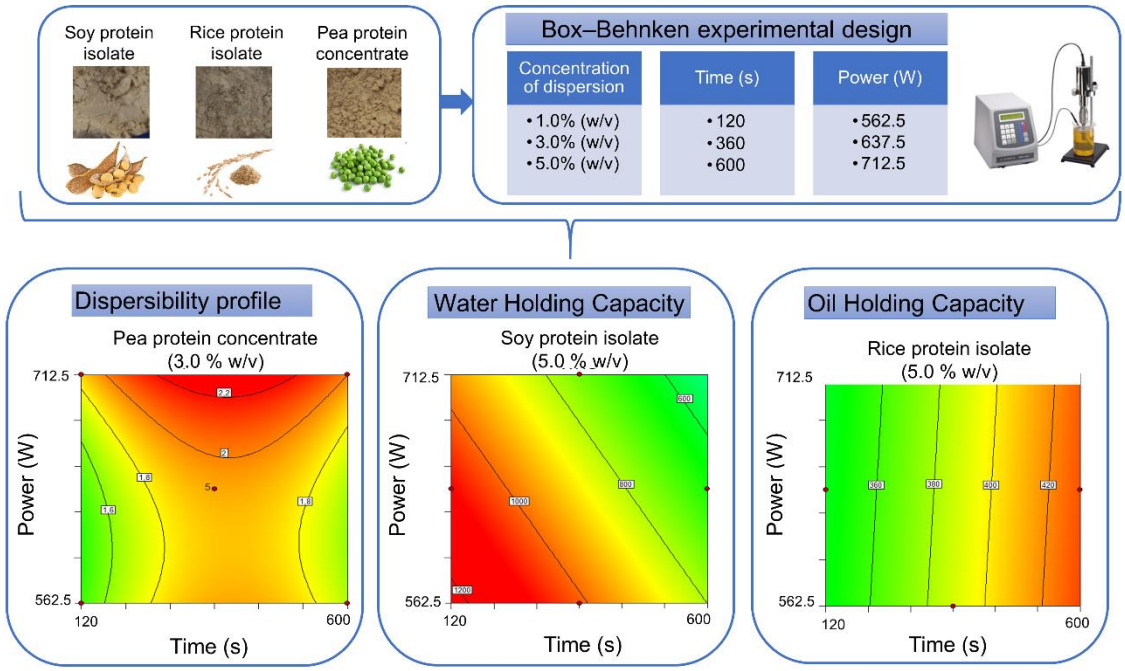
Capítulo 2

4. Effects of protein concentration during ultrasonic processing on physicochemical properties and techno-functionality of plant food proteins[1]

Highlights:

- Ultrasound may change proteins' properties in dispersions at different concentrations.
- Effect of ultrasound was sharply less pronounced in most of protein dispersions at 5%.
- Pea protein concentrate' dispersibility was increased at 3% w/v | 712 W | 360 s.
- Soy protein isolate' water holding capacity was increased at 5% w/v | 562 W | 120 s
- Rice protein isolate was not affected by ultrasound and remains challenging.

Graphical abstract:



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Effects of protein concentration during ultrasonic processing on physicochemical properties and techno-functionality of plant food proteins

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A B S T R A C T

Plant proteins have been increasingly recognized as very relevant food ingredients, due to concerns related to ecology, health and even religion. However, compared to food proteins from animal sources, the former ones have considerably lower dispersibility. Because of this, exploring techno-functional properties of plant proteins in formulations is much more difficult. Ultrasound has been explored in food processing for different purposes and represents a strategic non-thermal operation to enhance the dispersibility (and, consequently, several techno-functionalities) of food proteins. During ultrasonic processing in aqueous medium, the concentration of protein material may favour or impair the action of ultrasound waves, but this fact has been only marginally addressed in literature. Hence, this study investigated the effects of different protein concentrations during ultrasonic processing on techno-functional properties of soy protein isolate (SPI), rice protein isolate (RPI), and pea protein concentrate (PPC). Ultrasonic treatments (USt) at 20 kHz were designed using a Box–Behnken experimental planning, varying ultrasound power (562.5, 637.5 or 712.5 W), application time (120, 360 or 600 s), and concentration of the proteins in aqueous medium [1.0%, 3.0% or 5.0% (w/v)]. Hydrophobic surface index (H0), molecular mass profile, hydrodynamic diameter (dh), and ζ-potential of these three protein materials after USt were evaluated and discussed. Moreover, Response surface methodology was applied to evaluate and to optimize the following techno-functionalities of SPI, RPI, and PPC: water and oil holding capacity (WHC and OHC), and foaming, emulsifying, and gelling properties. After USt, OHC increased for the three protein materials. For SPI, dispersibility and FC at 712,5 W | 600 s | 1.0 % w/v were improved. The WHC (562.5 W | 120 s | 5.0 % w/v), OHC (562,5 W | 120 s | 1.0 % w/v) of SPI were also increased. For PPC, USt led to enhanced dispersibility (712.5 W | 360 s | 3.0 % w/v), WHC (712.5 W | 600 s | 5.0 % w/v), OHC (637,5 W | 120 s | 3.0 % w/v). On the other hand, none of the USt was efficient to significantly modify neither physicochemical nor techno-functional properties of RPI, which remains a challenge. In summary, the present study showed that USt may effectively improve the technological functionality of SPI and PPC. However, concentrations of these protein materials should be kept < 5% (w/v) during ultrasonic processing, otherwise the ultrasound waves are attenuated, and their desirable effects too.

Abbreviations and symbols

Abs	Absorbance
ANS	8-anilino-1-naphthalenesulfonic acid
DLS	*Dynamic Light Scattering*
d_h	Hydrodynamic average diameter
EAI	Emulsifying Activity Index
ESI	Emulsifying Stability Index
FA	Foam Activity
Ho	Hydrophobic surface
LGC	Least Gelling Concentration
OHC	Oil Holding Capacity
pH	Hydrogenionic potential
pI	Isoelectric point
PPC	Pea Protein Concentrate
RPI	Rice Protein Isolate
R^2	Coefficient of determination
rpm	Rotation per minute
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SM	Supplementary material
SPI	Soy Protein Isolate
USt	Ultrasonic treatment
WHO	Water Holding Capacity
ζ-potential	Zeta potential

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1. Introduction

In recent years, the global market for plant-derived proteins has been on the rise (Alves & Tavares, 2019; Sá et al., 2020). This is related to many factors, mostly to the easy digital access to information that has been shaping food consumption trends. Among the several reasons why people are adapting their food consumption, issues related to environmental, social and health aspects - especially those associated with the consumption of animal proteins - triggered the increase (in number) of groups such as vegetarians and flexitarians, that promote greater global demands for sustainable materials. (Sarkar & Dickinson, 2020; Sarkar & Kaul, 2014). Plant proteins present high nutritional quality, low allergenicity and industrially affordable costs. In addition, studies have demonstrated potential techno-functionalities of different plant protein sources for food applications, such as foaming (Amagliani & Schmitt, 2017; Dachmann et al., 2020), emulsifying (Ladjal-ettoumi et al., 2015; Sarkar et al., 2016; Sarkar & Dickinson, 2020; Sharif et al., 2018; S.; Zhang et al., 2020) and gelling properties (Lin et al., 2019; Opazo-Navarrete et al., 2018).

Among plant proteins, soy (*Glycine max* L.) proteins still remain the most often used ones in food formulations, for being of vast agricultural production and generating a variety of products with desirable nutritional and functional qualities, and of low cost (Nishinari et al., 2014). Soy protein isolates (SPI) are constituted mainly by protein fractions 7S and 11S (approximately 80%). Glycinin (11S) is composed of an acid chain polypeptide (A) and a basic one (B) united by a single disulfide bond, whereas β-conglycinin (7S) is a trimeric glycoprotein, consisted of subunits α′, α and β associated mainly via hydrophobic interactions (Nishinari et al., 2014). However, proteins from other plant sources with differentiated amino acid profiles should be investigated in order to analyze if they can present, under different processing conditions, both biofunctional and techno-functional characteristics comparable to soy' proteins. Pea (*Pisum sativum*) and rice (*Oryza sativa*) proteins represent some of these alternatives (Frias et al., 2011; Sá et al., 2020).

Rice proteins are represented mainly by glutelin (22–83%), being this subclassified by its α or acidic (Ra, 7–12 bands, pI < 7, PM 30–40 kDa) or basic subunits (Rb, 5–9 bands, pI > 7, PM 19–23 kDa), based on the differences in their pI (Van Der Borght et al., 2006). These subunits tend to polymerize through disulfide bonds, forming macromolecular complexes of high molecular weight and insoluble in water (Amagliani et al., 2017).

Regarding pea proteins, there are three main groups (60–80% of total proteins): i) legumins (11S), ii) vicilins (7S), and iii) convicillins (7S and 8S) (Casey & Domoney, 1999). Legumins are composed of an acidic subunit (~40 kDa), and a basic subunit (~20 kDa) linked to each other covalently by a disulfide bond (Mertens et al., 2012). Vicillins contain three subunits (α, β and γ) linked by hydrophobic interactions, and convicillins presenting sulfur amino acids, and a highly charged N-terminal extension (Adal et al., 2017; Laguna et al., 2017; Lam et al., 2018; Shand et al., 2007).

The use of plant proteins in formulations is still limited by their low solubility in aqueous media, which impacts directly the exploration of several techno-functionalities (Amagliani et al., 2017; Oliete et al., 2018a). In order to optimize the applicability of plant proteins, the improvement of their functional properties through the use of innovative non-thermal methods, such as ultrasound (Biswas & Sit, 2020), high pressure (Primozic et al., 2018), pulsed electric field and irradiation (Han et al., 2018), have been investigated. Amongst such processing approaches, high-frequency ultrasound technology represents a safe, non-toxic, environmentally friendly alternative, which induces only minor nutritional and sensorial losses of products compared to conventional thermal processes (Fan et al., 2020; Ojha et al., 2018).

Indeed, application of high-intensity ultrasound waves (20–100 kHz; 10–1000 W cm^2) for such purpose has been reported in literature (Gharibzahedi & Smith, 2020; Hu et al., 2013; O'Sullivan et al., 2016). Results generally point out effects related to modifications of native protein structure dispersed in aqueous media, triggering changes in their molecular aggregation properties, as well as in their interfacial activity, thus leading to changes in both their techno-functionalities and digestibility. The effects of ultrasound on food proteins' structures is attributed to cavitation, *i.e.*, the fast formation/collapse of gas bubbles which occurs due to local pressure differentials occurring over short periods of times (a few microseconds). This ultrasonic cavitation causes massive shear, turbulence and a rise in temperature at the neighbouring of bubble collapses. The main effects of acoustic cavitation on proteins include the decrease of proteins aggregate sizes, the partial unfolding of their three-dimensional molecular structures, with the consequent exposure of hydrophobic and/or sulfhydryl groups (Biswas & Sit, 2020; de Oliveira et al., 2020; Gharibzahedi & Smith, 2020; Hu et al., 2013; Jiang et al., 2017; O'Sullivan et al., 2016; Xiong et al., 2018). However, to the best of our knowledge, only few studies have addressed how ultrasound variables (or parameters) alter plant proteins properties (in particular rice and pea proteins) when they undergo such treatments, are available in most common scientific databases. For instnance, O'Sullivan et al. (2016) applied USt at 20 kHz | 712.5 W up to 2 min on SPI (86% protein), RPI (84.5% protein), pea protein isolate (86% protein), bovine gelatin (86% protein), fish gelatin (86% protein) and egg white protein (85% protein); Biswas and Sit (2020) worked with tamarind seed protein isolates (68.3% protein), applying USt at 25 kHz, 100–200 W and 15–30 min; Xiong et al. (2018) studied pea protein isolate (94.3% protein) using 20 kHz, 30–90% amplitude and 30 min; Hu et al. (2013) worked with SPI (>90% protein) at 20 kHz, 200–600 W and 15–30 min — all of them focusing on the effects arising from variations in ultrasound frequency, operating time, power or intensity. (de Oliveira et al., 2020), on the other hand, evaluated the same energy input (240 kJ) but resulting from different combinations of time x power, on physicochemical and emulsifying properties of pea protein concentrates, and showed that different time x power combinations led, indeed, to various differences in such properties.

The novelty proposed in the present study was an assessment of the effects of ultrasonic treatment (USt), not only in terms of ultrasound time and power, but also considering the concentration of soy and rice protein isolate and pea protein concentrate on their techno-functional properties, using a Box-Behnken design (McGrath & Lin, 2008). Though soy proteins have well-known techno-functionalities, they were included in the present research work to serve as a comparison for the two other protein materials. Properties evaluated were: water and oil holding capacity, and emulsifying, foaming and gelling abilities. Moreover, in each case, optimal values of the above cited techno-functional properties were searched by applying the response surface methodology (RSM) analyses on experimental data. The knowledge here reported should be of strategic helpfulness to both academia and industry, as they give indications of how ultrasonic processing may enable the replacement of animal proteins (at least partially) by some common food plant proteins, taking into account existing market demand (Fernandes, 2019; Foods et al., 2020).

2. Material and methods

2.1. Materials

Soy protein isolate (SPI) Pure Pro TM 90B (lot: E330030361, Shandong Xinlai Soybean Biotech Co., Ltd), rice protein isolate (RPI)

Table 1
Coded and uncoded variables of Box-Behnken experimental planning used for ultrasonic processing of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC).

System[a]	Coded variables			Uncoded variables			power x time (kJ)
	Time (X_1)	Power (X_2)	Dispersion concentration (X_3)	Time (s)	Power (W)	Dispersion concentration (%w/v)	
5	−1	−1	0	120	562.5	3	67.5
8	1	−1	0	600	562.5	3	337.5
6	−1	1	0	120	712.5	3	85.5
9	1	1	0	600	712.5	3	427.5
1	−1	0	−1	120	637.5	1	76.5
4	1	0	−1	600	637.5	1	382.5
10	−1	0	1	120	637.5	5	76.5
13	1	0	1	600	637.5	5	382.5
2	0	−1	−1	360	562.5	1	202.5
3	0	1	−1	360	712.5	1	256.5
11	0	−1	1	360	562.5	5	202.5
12	0	1	1	360	712.5	5	256.5
7	0	0	0	360	637.5	3	229.5

[a] The systems' numbering was generated randomly by the software (Design Expert software Version 10, Stat-Ease Inc, Minneapolis, MN).

Oryzatein Silk 90 (lot: JNOP0120180606, Axiom Foods - Los Angeles, CA, USA), and pea protein concentrate (PPC) (lot: 20180415, Shandong Jianyuan Foods Co., Ltd - China) were kindly donated by Nutricium Indústria e Comércio Ltda (Belo Horizonte, MG, Brazil). HCl, NaOH, SDS, boric acid, sulfuric acid, and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and were all used without additional purification. Soy oil (lot: 05 U, Bunge Alimentos, Brazil) was food grade, bought from a local market. Deionized water (electrical resistivity next to 18.2 MΩ·cm^{-2}, at 25.0 °C; Reference A+, Millipore, USA) was used in all experiments.

2.2. Characterization of SPI, RPI and PPC

2.2.1. – Proximate analyses

The proximate composition of SPI, RPI or PPC was determined according to analytical standards of the Association of Official Analytical Chemists (AOAC) (Bodily, 1956): moisture content (AOAC 925.09, 2005), ashes (AOAC 923.03, 2005), lipids (AOAC 920.85, 2005), proteins (AOAC 930.29, 2005), and carbohydrates (by simple difference). Protein content was estimated through Kjeldahl method, by using the following conversion factors: 6.25 for SPI and PPC (Oliete et al., 2018a), and 5.95 for RPI (Agboola et al., 2005).

2.2.2. – Average isoelectric point

Protein powder was dispersed in 20 mL of deionized water (0.1 mg·mL-1), kept under magnetic stirring (BS–2H, Lab Science, China) at 25.0 ± 0.5 °C, during 4 h. Then, the pH was measured, and the dispersion was separated into two parts. In one of part, the pH was increased from 8.0 to 12.0 (using NaOH 1.0 mol L^{-1} or 0.1 mol L^{-1}). In the other one, the pH was reduced from 7.0 to 2.0 (using HCl 1.0 mol L^{-1} or 0.1 mol L^{-1}). As pH reached the values between 2.0 and 12.0, with increments of 1.0 unit (pH/conductometer 914, Metrohm, Switzerland), the dispersions were sampled to ζ-potential analysis. Dynamic light scattering analyses were carried out to estimate the average isoelectric point of the three systems. Furthermore, additional measurements were performed for every 0.1-unit pH increment in a range from 3.5 to 4.5 for RPI, and from 4.0 to 5.0 for SPI or PPC dispersions. Samples were placed in a folded capillary cell (DTS1070; Malvern Instruments, United Kingdom), in order to evaluate ζ-potential values of dispersed protein particles (Zetasizer Nano-ZS, Malvern Instruments, United Kingdom), at 25.0 ± 0.1 °C (Soares, Perim, et al., 2019). ζ-potential values were estimated by applying an electric field and by calculating the electrophoretic mobility, from speed and direction of dispersed protein particles. The Smoluchowski model was used for the double electric layer (Bhattacharjee, 2016).

2.3. Experimental design

Box-Behnken experimental design was used, as described in the work by Khatkar et al. (2018) with modifications, to study the effects of processing time, namely X_1 (120, 360 and 600 s), ultrasonic power, namely X_2 (562.5, 637.5 and 712.5 W) and protein concentration, namely X_3 (1.0%; 3.0% and 5.0%, w/v) and/or the interaction among these independent variables on hydrophobicity surface (H_0), ζ-potential, dispersibility in aqueous medium, water and oil holding capacity, emulsifying activity and stability index (10, 30, 60 or 1440 min), foam activity and stability (30, 60 or 1440 min), and firmness of gels (20.0% (w/v) protein). All 13 experimental series for each protein isolate or concentrate are shown in Table 1, as well as the calculated value of the amount of energy inserted in the system (power x time). Treatment 13 represent the central point, which was performed with five repetitions, and control systems (without ultrasonic treatment) were also studied. RSM was applied on experimental data in order to optimize the processing parameters of ultrasound treatment (USt), and to examine the effect and/or correlation for independent variables on dependent responses. The Design Expert software (Version 10, Stat-Ease Inc, Minneapolis, MN) was used to carry out these experimental design procedures.

2.4. Preparation of protein dispersions and their ultrasound processing

Firstly, soy and rice isolates (SPI and RPI) or pea protein concentrate (PPC) were suspended in deionized water, in order to obtain concentrations of 1.0%, 3.0% or 5.0% (w/v), and kept under magnetic stirring (BS–2H, Lab Science, China) at 7.0 ± 0. 5 °C, during 2 h. Then, an ultrasound device (Viber Cell 750, Sonics, USA), with a tapped step horn with threaded body ½" (13 mm) in diameter, 5 3/8" (136 mm) in length, ¾ (340 g) in weight, 10 mL–250 ml process capacity, and titanium alloy replaceable tip 1/2" (630-0219, Sonics, USA) was used to homogenize 45 mL of protein dispersions placed into 50 mL Falcon tubes, which were sunk in ice baths to avoid overheating. Constant frequency of 20 kHz was used in all experiments (Table 1), excepting the controls without US treatment. After that, all systems were frozen at – 40.0 °C and then lyophilized (LP510, Liotop-Liobras, Brazil), aiming to guarantee microbial stability and ensure the non-variability of each protein material in the subsequent analyses, following recommendations found in reports by Qin et al. (2016) and Hu et al. (2013). Lyophilized materials were packaged in screw-capped plastic pots and stored under refrigeration (±7 °C) until use in subsequent experiments (sections 2.5 to 2.7.4).

Prior to some physicochemical (H_0, ζ and d_h) and techno-functional (emulsifying and foaming properties) characterizations, pHs of dispersions were adjusted to 7.0, using 0.1 mol L^{-1} HCl or NaOH solution. This aimed at standardizing the systems and minimizing influences of ionic

strength, since pH values about 7.8, 7.0 and 8.3 were observed respectively for SPI, RPI and PPC treated by ultrasound, after resuspension in deionized water.

2.5. Physicochemical characterization of protein dispersions

2.5.1. Molar mass distribution

Protein dispersions (5.0% w/w) were kept under agitation overnight, and analyzed by the SDS-PAGE technique under reducing conditions, according the methodology developed by Laemmli (1970, pp. 680–685). Briefly, a 12.0% (w/v) separation gel and 4.0% (w/v) stacking gel were used with 10 μL of each sample added to 5 μL of sample buffer (0.187 mol L^{-1} of Tris-HCl-buffer at pH 6,8; 50% (v/v) glycerol, 6.0% (w/v) SDS, 1.0% (w/v) bromophenol blue, 15.0% (v/v) β-mercaptoethanol); then, the samples were heated at 90 °C for 5 min, cooled to 21–23 °C, and inserted into the stacking gel spots. 5 μL of 10–245 kDa protein molecular weight marker (TrueColor High Range Protein Marker, Sinapse Inc, Brazil) was used. Electrophoresis were performed at 100 V/220 min. Gels were stained with coomassie brilliant blue (0.1% (w/v) in 10.0% (w/v) acetic acid and 40.0% (v/v) methanol) under gentle agitation, during 2 h. Then, gels were immersed in a bleaching solution consisting of 10.0% (v/v) acetic acid, 40.0% (v/v) methanol, and 50.0% water, in order to remove the excess of dye from the gel.

2.5.2. Turbidity and dispersibility profiles of proteins in aqueous media

Turbidity analyses of proteins was studied at pHs ranging from 2.0 to 9.0, with an increment of 1.0 unit, adjusted using 0.1 or 1.0 mol L^{-1} HCl or NaOH solutions. Briefly, dispersions 0.5% (w/v) of SPI, RPI or PPC were prepared in deionized water, kept under magnetic stirring for 4 h at 25 ± 0.5 °C, pH was adjusted and measurements were performed at 600 nm (Abs_{600}) on a UV–Vis spectrophotometer (Cary 50, Varian, Australia), at 25.0 ± 1.0 °C. Deionized water was used as blank solution. Next, absorbance values were expressed as turbidity using Eq. (1). Protein dispersions without USt were used as control.

$$\tau = \frac{2,303 \cdot Abs_{600}}{l} \quad (1)$$

In Eq. (1), $\tau$is the turbidity, and $l$is the optical path corresponding to the width of the cuvette.

Also, in each case, the same systems prepared for the protein turbidity analyses were analyzed in terms of their dispersibility profiles, according to the methodology adapted from Ladjal-Ettoumi et al. (2015) and recently used by Oliveira et al. (2020), were after adjusting the pH, the systems were centrifuged at 7.000 x *g* for 10 min (Centrifuge 5430, Eppendorf, Germany) and the amount of protein (mg·mL^{-1}) in the supernatant was measured by the Bradford methodology. A standard curve was previously built using BSA (0.000, 0.250, 0.500, 1.750, 1.000, 1.250, 1.500, 1.750 and 2.000 mg mL^{-1} to SPI and PPC, or 0.000, 0.010, 0.050, 0.075, 0.100 and 0.500 mg mL^{-1} to RPI), and absorbances were measured at 595 nm (Mæhre et al., 2018).

2.5.3. Zeta-potential, average hydrodynamic diameter (D_h) and polydispersity index (PDI) of dispersed protein

ζ potential measurements of SPI, RPI and PPC dispersed particles, here after USt, were performed as described in section 2.2. Additionally, hydrodynamic average diameter (D_h) and polydispersity index (PDI) for each protein dispersed population present in the dispersions were determined and calculated, respectively, using the equipment Zetasizer Nano-ZS, according to the methodology described by Galván et al. (2018); Soares, Milião, et al. (2019). PDI was estimated from hydrodynamic average diameter (D_h) and standard deviation (SD) relationship for each peak found (Soares, Milião, et al., 2019). The results came from the following apparatus configuration: scattering angle of 173°; refractive index = 1.330; viscosity = 0.8872 cP. Each reported value came from average of three repetitions, which performed 3 runs of 30 s per repetition.

2.5.4. Surface hydrophobicity of proteins (H_0)

Surface hydrophobicity index (H_0) of SPI, RPI or PPC was evaluated using the fluorescent marker 8-anilino-1-naphthalenesulfonic acid (ANS), according to the classical method proposed by Kato and Nakai (1980) with the modifications described by Oliete et al. (2018b). Dispersions of SPI, RPI or PPC [concentration = 1.0% (w/v)] treated with ultrasound, and dispersions for control systems (SPI, RPI or PPC without ultrasound treatment; concentration = 3.0% (w/v)] were prepared using phosphate buffer (10 mmol·L-1), kept under stirring at 25 °C, during 1 h. Next, dispersions were centrifuged at 10.000 x *g* for 25 min, and protein content present in the supernatants was assessed using the Bradford methodology. After that, the protein concentration was adjusted to 0.5 mg mL^{-1} using the same buffer, aiming at standardizing the concentration of proteins during the analyses from 0.05% to 0.001% (w/v). Then, 6 μL ANS solution (1 mmol L^{-1}) were added to 300 μL of each protein dispersion, and the resulting mixtures were kept in the dark for 15 min. To ensure that the fluorescence signal actually came from ANS bonded to the exposed hydrophobic groups exposed of protein molecules, the same analyses were performed using BSA dispersions with the same protein concentrations. In these BSA dispersions, fluorescence signal was proportional to protein concentration, confirming that the fluorescent marker ANS actually bound to proteins. Additionally, two blanks were analyzed: the first one consisted of buffer + ANS, whereas the second control consisted of buffer only. None of them generated fluorescence signal, further confirming that fluorescence was a consequence of ANS binding on hydrophobic surfaces of proteins. The fluorescence intensity was measured using a luminescence spectrometer (SpectraMax M5, Molecular devices, USA) at 380 nm (excitation) and 460 nm (emission). The initial slope of fluorescence intensity as a function of protein concentration (mg·mL^{-1}) plot was taken as the H_0 index.

2.6. Techno-functional characterization of proteins in aqueous media

2.6.1. Water and oil holding capacities (WHC and OHC)

WHC and OHC were determined according to Stone et al. (2015), with few modifications. Briefly, SPI, RPI or PPC were initially weighted using an analytical balance (AUY220, Shimadzu – Japan; accuracy = 10^{-4} g). Then each protein material was mixed with water or with soybean oil. In all cases, 0.05 g of protein material was added to 0.5 g of the respective liquid (oil or water). Subsequently, the mixtures were carefully placed into microtubes and mixed using a vortex stirrer (Phoenix, recnal, Brazil) for 10 s, every 5 min during a total of 30 min. After that, they were centrifuged at 10,000 x g for 15 min. The corresponding supernatants (water or oil) were carefully discarded and the solid pellets were again weighted. WHC and OHC were calculated according to Equation (2).

$$\text{WHC or OHC}\ (\%) = \frac{WG_{PI}}{OW_{PI}} \times 100\% \quad (2)$$

In Eq. (2), $WG_{PI}$is weight gained by each protein material (difference between final and initial weights) and $OW_{PI}$is their initial weights.

2.6.2. Foaming properties

1.0% w/v SPI, RPI or PPC dispersions (pH = 7.0) were placed into graduated cylinder (capacity = 7.5 mL), and then homogenized (Ultra-Turrax T25, IKA-Werke GMBH & CO., Germany) at 10.000 rpm for 1 min during foams preparation (Zhang et al., 2018, with modifications). Foaming capacity (FC) and foaming stability (FS) were calculated by using Equations (3) and (4), respectively:

$$\%\,FC = \frac{V_o}{7.5} \cdot 100\ \% \quad (3)$$

$$\%\,FS = \frac{\Delta V}{V_0} \cdot 100\ \% \quad (4)$$

a

Component % (m/m)	SPI	RPI	PPC
Moisture	5.04 ± 0.18	2.77 ± 0.24	8.91 ± 0.20
Ash	3.6 ± 0.01	1.41 ± 0.02	5.36 0.05
Fat	1.29 ± 0.02	2.55 ± 0.20	0.41 ± 0.25
Protein	83.32 ± 3.23	80.89 ± 1.35	73.98 ± 2.58
Carbohydrate	6.75	12.38	11.34

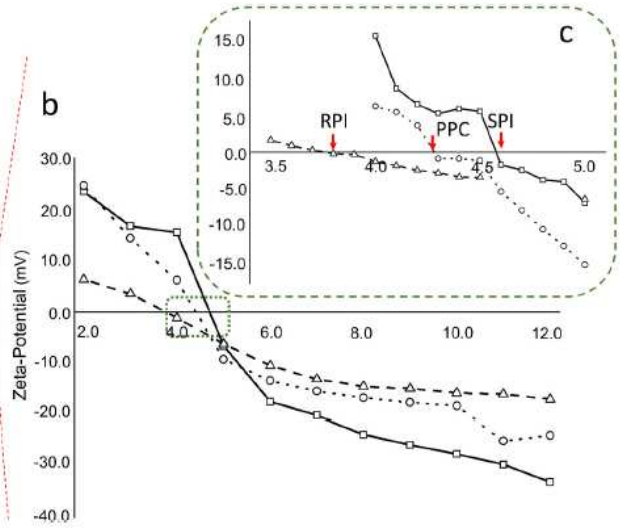

Fig. 1. (a) Proximate composition of SPI, RPI and PPC and (b) average ζ-potential (mV) of dispersed proteins within the pH range 2.0–12.0. In (c), the plot shown in (b) was zoomed between pH 3.5 and 5.0, in order to indicate (red arrows) the pH values at which ζ-potentials were ~0, *i.e.*, the average isoelectric points of the three protein materials [SPI: soy protein isolate (—▫—); RPI: Rice protein isolate (– △ –); PPC: Pea protein concentrate (··○··)]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In Eqs (3) and (4): V_0 is foam volume at time 0 (zero); ΔV is Volume of foam after 30 min, 60 min or 1440 min.

2.6.3. Emulsifying properties

1.0%, 0,75% or 0,5% (w/v) dispersions of RPI, PPC or SPI, respectively, were prepared and their pHs were adjusted to 7.0, as described in previous sections. The different protein concentrations were established according to their dispersibility at this pH, with RPI being that with the lowest dispersibility, followed by PPC and SPI (highest dispersibility). Emulsions consisted of 95% (w/w) protein dispersion (continuous phase) + 5% (w/w) soybean oil (dispersed phase). The emulsification process was carried in two steps, as described by Soares, Milião, et al. (2019). Briefly, a pre-emulsification was performed using a vortex stirrer (Phoenix, Tecnal, Brazil) during 20 s. Subsequently, emulsions were produced using an ultrasonic homogenizer (Viber Cell 750, Sonics, USA), operating at 20 kHz, with a power of 500 W, for 4 min. During emulsification process, the beakers were sunk in ice baths to prevent overheating.

The emulsions were evaluated in terms of Emulsifying Activity Index (EAI) and Emulsifying Stability Index (ESI), according to the methodology firstly described by Pearce and Kinsella (1978), with adaptations recently proposed by Oliveira et al. (2020). Briefly, 50 μL of the emulsions were sampled 3 cm from the bottom of beakers, using a micropipette (volume range = 10–100 μL; Eppendorf Research, Germany) and diluted with 0.1% (w/v) of sodium dodecyl sulfate (SDS) (1:200) at times 0, 10, 30, 60 min, and 24 h after their production. The absorbance of these diluted emulsions was then immediately measured at 500 nm on the UV–vis spectrophotometer (Cary 50, Varian, Australia) using plastic cuvette (path length 1 cm).

EAI and ESI were calculated using Equations (5) and (6).

$$EAI\left(\frac{m^2}{g}\right) = \frac{2{\cdot}2,303{\cdot}\ A_0{\cdot}\ DF}{C \cdot \phi \cdot 10000} \quad (5)$$

$$ESI = \frac{Ao}{\Delta A} \cdot t \quad (6)$$

In Eqs. (5) and (6): A_0 is absorbance of diluted emulsion immediately after homogenization; DF is dilution factor (× 200); c is protein concentration in aqueous phase prior to emulsion formation; ϕ is oil fraction of the emulsion; ΔA is variation in absorbance between 0 and 10, 30, 60 min, or 24h, and; t = time period (10, 30, 60 min, or 24h).

2.6.4. Gelling properties

Least gelling concentration (LGC) of the protein materials was also evaluated according to the approach reported by Boye et al. (2010). SPI, RPI or PPC were weighted in glass vials (capacity = 8.0 mL), with screw caps and adequate amounts to reach protein concentrations of 10.0; 12.5; 15.0; 17.5 and 20.0% (w/v). Then, they were heated up to 95 °C in a thermostatic bath (Phoenix, Thermo Corp., Brazil) for 15 min. Next, the systems were immediately cooled under refrigeration (4 °C) (Pratice, Consul, Brazil) overnight, to induce gelation. After that, the glass vials were inverted in order to determine if the dispersions had formed a gel. In the cases in which gelation occurred, gels firmness (g) was measured using a texture analyser (TA-XT express, Stable Micro Systems Ltd., UK), at room temperature. Then gels were compressed with a 5 mm stainless steel cylinder probe (P/5) into vial using trigger force of 0.0098 N, test speed 0.5 mm s^{-1} and test distance (depth reached by the probe) of 0.5 mm.

2.7. Data treatment and statistical analyses

Physiochemical properties — dispersibility profile of proteins as function of pH, H_0, ζ-potential, d_h and PDI — for SPI, RPI and PPC were evaluated quantitatively in relation to effect promoted by independent variables of ultrasonic processing: time (s), power (W), and dispersion concentration (% w/v). Each response came from at least a duplicate, and the central point was expressed as a mean ± standard deviation of 5 repetitions (Hifney et al., 2016).

For techno-functional properties — WHC, OHC, dispersibility (at pH 7.0), EAI, FC, and gel firmness —, regression models were developed considering the independent variables time cited above. The models' adequacy was evaluated in terms of significance (*p-value* less than 0.05), lack-of-fit, coefficient of determination (R^2) and residual analyses. Dispersion diagrams were observed, and the best fitting regression models were evaluated. We chose to build models with gradual complexity: simple linear, two factor interaction and followed by polynomials of up to sixty order. To choose the most suitable model, the highest score between the two scores composing the Whitcomb Score Equations (7) and (8) (Whitcomb & Anderson, 2016) were considered.

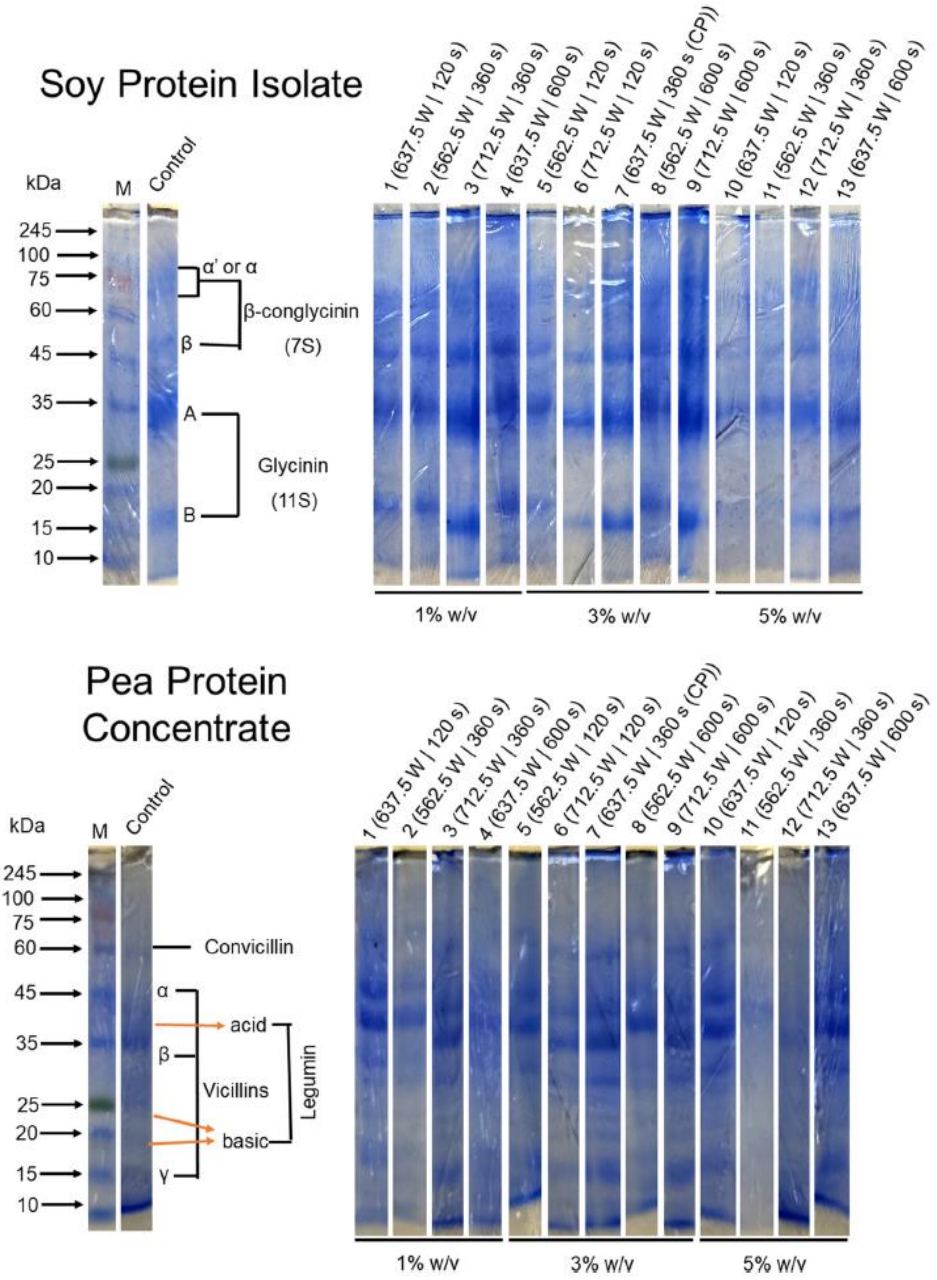

Fig. 2. SDS-PAGE pattern of soluble protein fraction under reducing conditions of (A) soy protein isolate and (B) pea protein concentrate. M is molecular weight marker and control is without treatment ultrasonic.

$$Score\ 1 = (M)(L)\left(Pred\ R^2\right) \tag{7}$$

$$Score\ 2 = (M)(L)\left(Adjusted\ R^2\right) \tag{8}$$

In Eqs. (7) and (8), *M* is the Sequential Model Sum of Squares score: M = 1 if Prob > F value is less than or equal to 0.05; M = 0.05/(Prob > F) if Prob > F value is greater than 0.05 and M = 0 if model is aliased; *L* is the Lack of Fit score: L = 1 if Prob > F value is greater than or equal to 0.10 (or if Lack of Fit not present); L = (Prob > F)/0.10 if Prob > F value is less than 0.10.

Models with highest Whitcomb scores were selected. In the case of a tie in Score 1 and 2, the least complex model was selected. When all model scores where less or equal to zero, a mean based model was chosen. This procedure was adopted for each protein material treated with ultrasound. Analyses of variance (ANOVA) were executed to examine the statistical significance of the terms of regression equation for each response. After establishing the best model, contour graphics were generated, using Design Expert software (Version 10, Stat-Ease Inc, Minneapolis, MN). Other statistical analyses were performed using IBM SPSS Statistics Software (Version 25.0, IBM SPSS Inc., Armonk, NY,

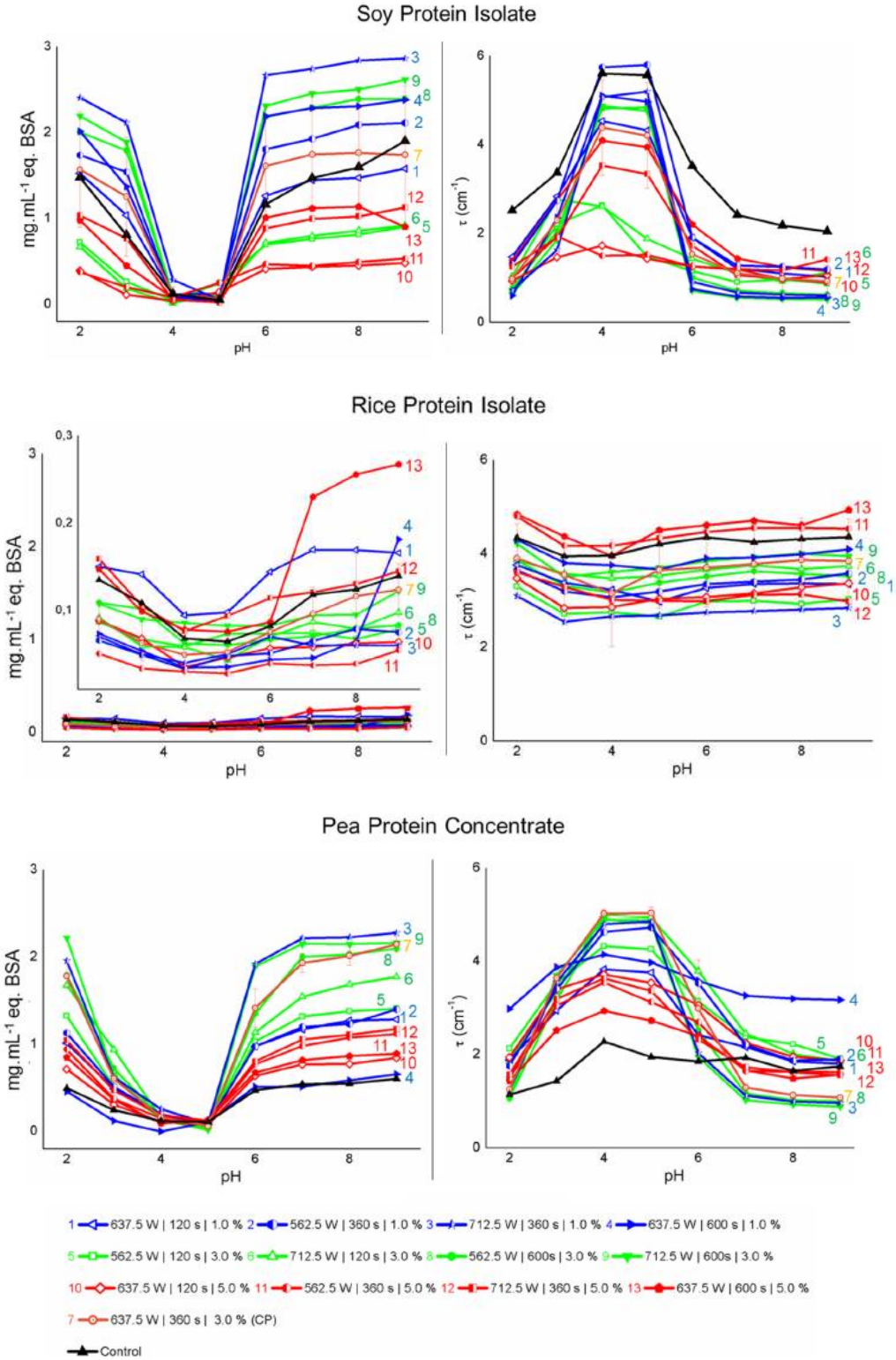

Fig. 3. Dispersibility (first column; mg.mL^{-1} equivalent to BSA in the supernatant of centrifuged dispersions) and turbidity of dispersions with protein concentration = 0,5% w/v (second column) after ultrasonic treatment. Blue lines correspond to dispersions prepared (before ultrasonic treatment) at 1% (w/v), green 3% (w/v), orange is the central point, red 5% (w/v), and black is the control (without ultrasonic treatment). Empty symbols correspond to the lowest amounts of power and time binomials, partially filled with intermediate amounts, and fully filled, highest amounts of power and time binomials. CP is the central point, referring to an average value of 5 repetitions and control is without ultrasonic treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

USA), always considering a significance level of 5% (*p-value* less than 0.05).

3. Results and discussion

3.1. Characterization of soy protein isolate (SPI), rice protein isolate (RPI), and pea protein concentrate (PPC)

Proximate analyses result for SPI, RPI and PPC, – ash, fat, protein, and carbohydrate contents (wet basis) –, as well as ζ-potential values of dispersed protein particles in aqueous media, are represented in Fig. 1. SPI and RPI presented more than 80% of protein content, whereas PPC presented around 74%. Additionally, in all these three materials the second most abundant macrocomponent was carbohydrates, ranging from 6.75% for SPI to 12.38% for RPI. Fat contents were less than 2.55% in all cases, and the ashes contents ranged from 1.41% for RPI to 5.36% for PPC (Fig. 1A). The protein contents ≥80% in SPI and RPI justify their designation as isolates. The highest percentage of carbohydrates found in RPI is due to the fact that this protein material came from a cereal. The highest ash content found in PPC may have influenced the differences in physicochemical and techno-functional properties for this material, as described in sections thereafter. SPI, RPI and PPC presented isoelectric point (pI) values were respectively 4.6, 3.8, and 4.3, respectively (Fig. 1B and C). These results are similar to those reported by the literature: pI = 4.5 for SPI (65% of protein) (Lopes Barbosa et al., 2006), pI = 4.0 for rice residue protein isolates (90% protein) (Hou et al., 2017), pI = 4.5 for rice protein (~80% protein) (Cao et al., 2009), pI = 4.5 for pea protein isolate (Chao & Aluko, 2018), between 4.6 and 4.9 for different pea cultivars (81–89% protein) (Stone et al., 2015). The similar pI values of SPI and PPC may be attributed to the fact that both are from the legume family, being mainly constituted by proteins 7S and 11S.

3.2. Physicochemical characterization of proteins in aqueous media

3.2.1. Molar mass distribution of proteins

In order to assess the molar mass distribution of proteins contained in SPI, RPI and PPC dispersed in water and submitted to different USt (Table 1) were studied by SDS-PAGE under reducing conditions. Results are given in Fig. 2.

Electrophoretic profiles in Fig. 2 show a wide molecular weight variety for SPI and PPC, which ranged from 15 to 75 kDa. Because of the extremely low protein dispersibility of RPI (<0,3 mg mL^{-1} eq BSA, section 3.2.2), this methodology did not yield bands allowing a characterization of molar masses of proteins in this material. Sgarbieri (1996) reported on the difficulty in characterizing rice proteins due to their insolubility in neutral solvents. Cao et al. (2009) reported the low solubility of rice proteins (80%) at pH close to 5.0, and that, since glutellins represent the majority of rice proteins, pH below 3.0 or above 7.0 represent conditions for further exploration of the use of this material. As the aim of the technique was to evaluate the changes promoted by the USt previously defined, we decided not to resort to different, harsher measures for protein extraction only to perform this analysis.

For SPI, four bands were observed, representing the 7S and 11S globulins. The 7S group is composed of subunits α′ or α (~73–76 kDa), and β (~50 kDa) of β-conglycinin. Likewise, the 11S unit is mostly comprised by an acid polypeptide chain (A) (35 kDa) and a basic one (B) (~18 kDa) of glycinin (11S), and these observations were in concordance with different literature reports (Alves & Tavares, 2019; Nishinari et al., 2014; Shand et al., 2007). PPC showed seven bands. Legumin appeared at ~40 kDa (acid subunit) and ~25 and 20 kDa (basic subunit), vicillins at 50 kDa (α); 30–33 kDa (β) and 15 kDa (γ), and convicillins at ~ 71 kDa, in accordance with protein fractions reported by Oliete et al. (2018b); Adal et al. (2017); O'Sullivan et al. (2016); Shand et al. (2007). In the control, a band corresponding to 60 kDa was observed, suggesting the presence of a polypeptide called non-vicillin which was pointed by Mession et al. (2015), also by the SDS-PAGE technique. However, in our study, in the treatments other than the control, the first band (71 kDa) should correspond to convicillin, since this protein is reported in the literature varying its molecular weight between 66 kDa (S. Zhang et al., 2020) up to 77.9 kDa (Adal et al., 2017; Laguna et al., 2017). In fact, the bands exceeding 45 kDa were closer to each other, even if the magnitude of the differences among the molar masses represented by them are larger. This requires more caution when interpretating the electrograms in this molar mass range. These results suggest that the USt may have caused minimal changes on the molecular mass profile of protein structures present in SPI and PPC, since the bands present appear to be practically the same, but their colour intensities were different. O'Sullivan et al. (2016) found that USt at 20 kHz | 712.5 W up to 2 min did not change the molecular weight profile in SPI (86%), RPI (84.5%), pea protein isolate (86%), bovine gelatin (86%), fish gelatin (86%) and egg white protein (85%). Analogous results were also reported in the studies of Biswas and Sit (2020), for tamarind seed protein isolates (68.3% protein, at 25 kHz, 100–200 W and 15–30 min); Xiong et al. (2018) for pea protein isolate (94.3% protein, 20 kHz, 30–90% amplitude and 30 min) and Hu et al. (2013) with SPI (>90% at 20 kHz, 200–600 W and 15–30 min). Therefore, regardless of the concentration of dispersion and the binomial power and time used in the treatment of SPI and PPC, they do not seem to change the molar mass distribution profile of their proteins. In addition, Oliveira et al. (2020) when testing the *in vitro* digestibility of PPC (74% protein) treated with USt corresponding to 240 kJ, suggested that the USt method does not harm its nutritional quality, representing a useful tool for the food industry.

Differences in the colour intensities of the electrophoretic bands suggested differences in protein concentrations applied in each line. Although the dispersions were standardized at 5.0% (w/v) of proteins, the actual amount inserted in each channel was not determined, and varied according to the dispersibility of each protein material (Fig. 3). This observation was evident for SPI, since systems with dispersibility below 0.8 mg mL^{-1}, as shown in the lines 5 (562.5 W | 120 s | 3.0% w/v), 6 (712.5 W | 120 s | 3.0% w/v), 10 (637.5 W | 120 s | 5.0% w/v), and 11 (562.5 W | 360 s | 5.0% w/v) presented the weakest bands in the gel. Moreover, when protein dispersions were prepared in the lowest concentration (1.0% w/v) and received greater amounts of ultrasonic power and time binomials, the corresponding systems yielded more visible and sharp bands. The effects of the lowest dispersibility for PPC was also observed for lines 2 (562.5 W | 360 s | 1.0% w/v), 4 (637.5 W | 600 s | 1.0% w/v), 11 (562.5 W | 360 s | 5.0% w/v) and 12 (712.5 W | 360 s | 5.0% w/v), in addition to the control system. Therefore, it is important to emphasize the importance of quantifying the proteins present in the supernatant before characterizing them by SDS-PAGE, so that at least 1.0 mg mL^{-1} eq. BSA are used.

Structurally, SPI and PPC proteins are more similar among themselves, as they both come from legumes and consist mainly of globulins (Adal et al., 2017; Lam et al., 2018; Nishinari et al., 2014). The dispersibility presented by these protein materials (except for SPI at 5% w/v) may be related to the breakdown of intermolecular interactions (mainly hydrophobic ones) by action of USt (de Oliveira et al., 2020; O'Sullivan et al., 2016; Režek Jambrak, 2017). This should be particularly true for vicillins and convicillins (both from PPC), which may perhaps predominate in the smaller size populations (see section 3.2.4). Differently, for legumin (from PPC) and glycinin (from SPI), which contain more disulphide bonds (Barac et al., 2010; Lam et al., 2018; Nishinari et al., 2014), should result in larger dispersed particles and, hence, it can be hypothesized that they predominate in the larger size populations (see section 3.2.4). The extremely low dispersibility of RPI may be related to the predominance of glutenin, which is soluble in alkaline aqueous media. Such proteins tend to form large oligomers, maintained by disulphide bonds (Van Der Borght et al., 2006). These disulphide bonds should be numerous and strong enough to persist after USt, which is a reasonable explanation for RPI's low dispersibility (see

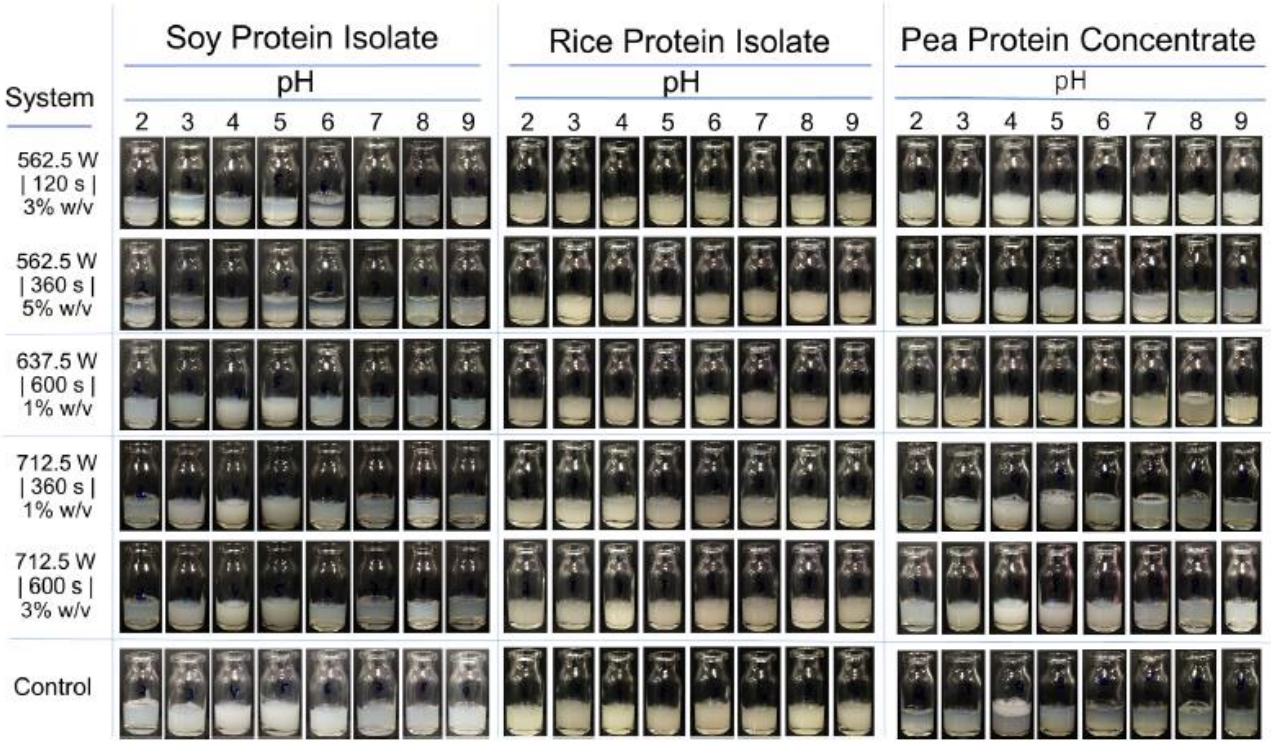

Fig. 4. Visual appearance of soy protein isolate (SPI), rice protein isolate (RPI) or pea protein concentrate (PPC) dispersions at 0.5% (w/v) stirred for 4h (25 ± 0.5 °C).

section 3.2.2), higher hydrodynamic diameters (see section 3.2.4), and impossibility to perform SDS-PAGE analyses, even after all ultrasonic treatments applied in this study.

3.2.2. Turbidity and dispersibility profiles of proteins in aqueous media

Turbidity measurements are useful to suggest about the colloidal state of dispersed particles. Commonly, high values of this index indicate low values of proteins dispersibility. Turbidimetry and dispersibility data for SPI, RPI and PPC are presented in Fig. 3. The macroscopic visual aspect of some SPI, RPI and PPC dispersions mentioned above was showed in Fig. 4. In general, in the pH range 4.0–5.0, lower dispersity and greater turbidity were observed in all cases, as expected, since this correspond to the pH range of the average pI for SPI, RPI or RPI (previously shown in Fig. 1). SPI showed the highest dispersibility (2.86 mg mL^{-1} eq. BSA), as well the most extreme turbidity values (0.51–5.80 cm^{-1}), when compared to PPC or RPI. RPI showed a dispersibility <0.3 mg mL^{-1} eq. BSA, regardless of changes in pH. Similar behaviour found in the work of Pietrysiak et al. (2018) involving a pea-rice protein blend 2:1 w/w, containing 88.7% protein in the pH 3–9 range. In addition, RPI USt turbidimetric values ranged from 2.50 to 4.91 cm^{-1}. The dispersibility profile of PPC varied from 0.01 to 2.29 mg mL^{-1} eq. BSA, whereas the turbidity profile varied from 0.89 to 5.15 cm^{-1}.

The highest protein concentration in SPI supernatant were found for the treatments 3 (712.5 W | 360 s | 1.0% w/v), 4 (637.5 W | 600 s | 1.0% w/v), 8 (562.5 W | 600 s | 3.0% w/v) and 9 (712.5 W | 600 s | 3.0% w/v), showing that higher amounts of applied energies, combined with lower concentrations (1.0%, w/v) tended to increase the SPI dispersibility. On the other hand, dispersions with protein concentration = 5.0% w/v, the protein molecules, after exposure to USt, presented a tendency to agglomerate, which reduced their dispersibility (Fig. 3), and formed larger aggregates that settled. As turbidimetric analyses were performed in samples from the aqueous phase of each system, the turbidimetry values tended to be lower throughout the pH range analyzed (systems: 5 (562.5 W | 120 s | 3.0% w/v), 6 (712.5 W | 120 s | 3.0% w/v), 10 (637.5 W | 120 s | 5.0% w/v), 11 (562.5 W | 360 s | 5.0% w/v), 12 (712.5 W | 360 s | 5.0% w/v) and 13 (637.5 W | 600 s | 5.0% w/v)).

RPI showed the lowest dispersibility regardless the pH of the systems, and the use of USt influenced only slightly on this physicochemical property for this protein. Zhang et al. (2018) also report the low dispersibility of rice proteins (90% protein), even when treated with ultrasound (20 kHz | 600W: protein solubility < 0.5 mg mL eq. BSA); however, in their study, the dispersibility was improved through the combination of alkaline conditions and ultrasound-assisted treatment (improved 230-fold compared to un-treated samples). For RPI, whatever the protein concentration used during USt, proteins presented a high tendency to agglomerate, showing high values of turbidity, that may be associated with the presence of starch, a common component of this protein source (rice), since the percentage of carbohydrates present in the sample was next 12.4% (Fig. 1). The highest values of turbidity found in the entire pH range analyzed were observed in the systems 11 (562.5 W | 360 s | 5.0% m/v) and 13 (637.5 W | 600 s | 5.0% m/v), while the lowest turbidity was verified in the system 3 (712.5 W | 360 s | 1.0% m/v) which corresponds to the USt that combined the highest power with the lowest concentration.

Control system (without ultrasound treatment) of PPC presented both low dispersibility and low turbidity. USt allowed to increase PPC dispersibility, since systems treated with greater powers and lower protein concentrations (system 3 = 712.5 W | 360 s | 1.0% w/v) or accompanied by longer times (system 9 = 712.5 W | 600 s | 3.0% w/v) had higher dispersibility than the control, and showed smaller turbidity values in the pH range between 7.0 and 9.0.

Therefore, USt with combined conditions of lower concentration (1.0% w/v) that received greater power and time binomials tended to improve the dispersibility of SPI and PPC. However, none of the USt conditions tested proved effective to enhance RPI dispersibility. In dispersions at 5.0% (w/v) of SPI, USt even impaired its dispersibility,

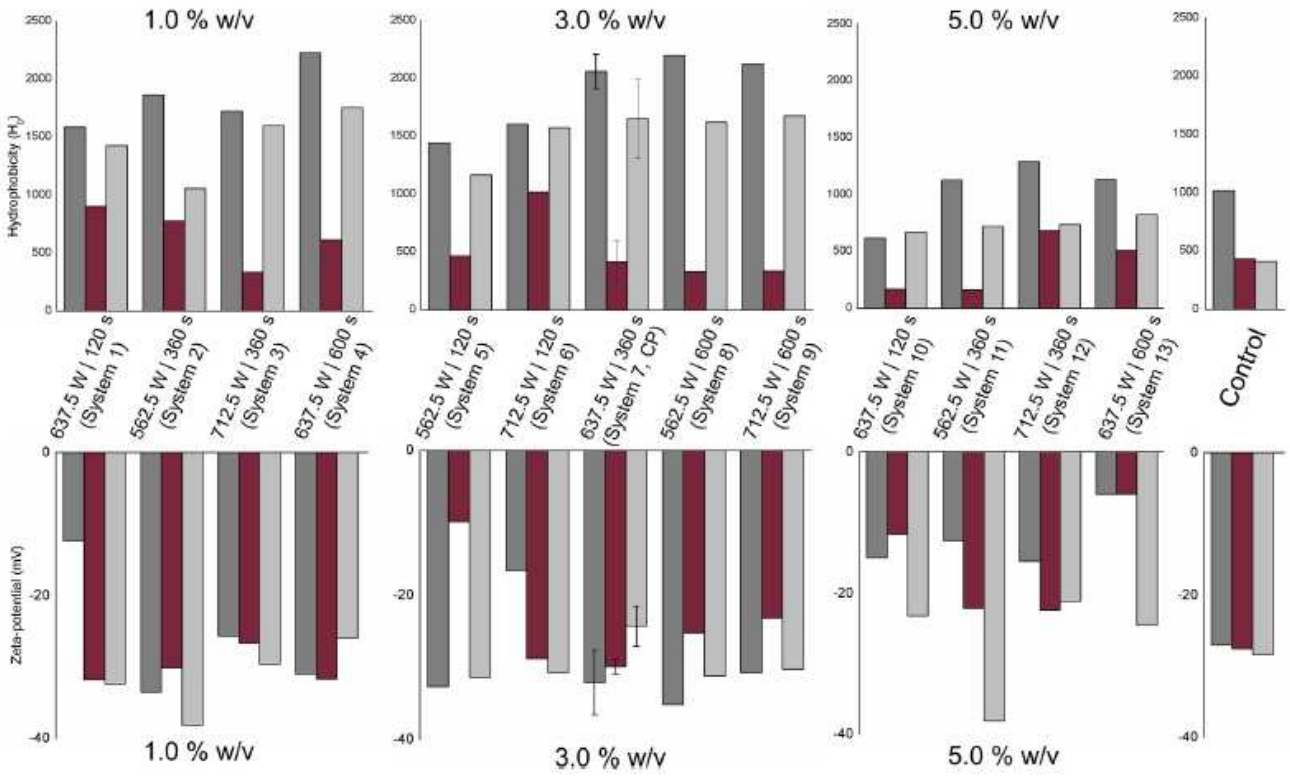

Fig. 5. Hydrophobicity (H_0) and zeta-potentials of soy protein isolate (SPI) , rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasonic treatment. Control system is without ultrasonic treatment and CP is the central point, referring to an average value of 5 repetitions.

Table 2
Effects of ultrasound treatment on the physicochemical and techno-functional properties of soy protein isolate (SPI) when compared to control system (without ultrasound treatment).

System	Treatment			Physicochemical properties			Techno-functional properties							
	DC (%,w/v)	Power (W)	Time (s)	[a]H_0	[a]ζ	[a]TBD	[a]PD	WHC	OHC	[a]EAI	[a]ESI (10, 30, 60 and 1440 min)	[a]FC	[a]FS (30, 60 and 1440 min)	Fm
1	1.0%	637.5	120	↑	↓	↓	↓	↓	↑	↑	↑ - ↓ - ↑ - ↑	↑	↑ - ↑ - ↑	↑
2		562.5	360	↑	↑	↓	↑	↓	↑	↑	↑ - ↑ - ↑ - ↓	↑	↑ - ↑ - ↓	↓
3		712.5	360	↑	↓	↓	↑	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - ↓	↓
4		637.5	600	↑	↑	↓	↑	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - ↓	↓
5	3.0%	562.5	120	↑	↑	↓	↓	↑	↑	↑	↑ - ↑ - ↑ - ↑	↑	↓ - ↓ - ↓	↓
6		712.5	120	↑	↓	↓	↓	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - ↓	↑
7		637.5	360[a]	↑	↑	↓	↑	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - ↓	↑
8		562.5	600	↑	↑	↓	↑	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - =	↓
9		712.5	600	↑	↑	↓	↑	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - ↓	↓
10	5.0%	637.5	120	↑	↓	↓	↓	↑	↑	↓	↓ - ↓ - ↓ - ↑	↑	↓ - ↓ - ↓	↑
11		562.5	360	↑	↓	↓	↓	↑	↑	↑	↑ - ↑ - ↑ - ↑	↑	↓ - ↓ - ↓	↑
12		712.5	360	↑	↓	↓	↓	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - ↑	↓
13		637.5	600	↑	↓	↓	↓	↓	↑	↑	↑ - ↑ - ↑ - ↑	↑	↑ - ↑ - ↓	↑

DC: dispersion concentration, H_0: surface hydrophobicity, ζ: zeta-potential, TBD: turbidity, PD: protein dispersibility, WHC: water holding capacity, OHC: oil holding capacity, EAI: emulsifying activity index, ESI: emulsifying stability index, FC: foaming capacity, FS: foaming stability, Fm: firmness in concentration 20% (w/v).
↑ indicate increase, ↓ indicates decrease, and = means that there was no variation, all in relation to the control (without ultrasound treatment).
[a] pH 7.0, ** central point, referring to an average value of 5 repetitions.

reducing it.

3.2.3. Zeta-potential, average hydrodynamic diameter (D_h) and polydispersity index (PDI) of dispersed protein

Not surprisingly, all systems presented negative values for ζ-potential (Fig. 5), since all dispersions were prepared at pH 7.0, which is above the average isoelectric points of the three protein materials (Fig. 1). For SPI, all systems that received USt prepared at 5.0% w/v presented smaller densities of negative surface charges (ζ between −5.9 for system 13 = 637.5 W | 600 s and −15.4 mV for system 12 = 712.5 W | 360 s), as well the system 1 = 637.5 W | 120 s | 1.0% w/v (ζ = −12.3 mV), compared to the control without USt (ζ = −27.3 mV) (Table 2). Negative charge densities of sonicated RPI proteins' surfaces were slightly higher than the control (ζ = −27.5 mV), in following systems: 1

Table 3
Effects of ultrasound treatment on the physicochemical and techno-functional properties of rice protein isolate (RPI) when compared to control system (without ultrasound treatment).

System	Treatment			Physicochemical properties			Techno-functional properties						
	DC (%,w/v)	Power (W)	Time (s)	[a]H_o	[a]ζ	[a]TBD	[a]PD	WHC	OHC	[a]EAI	[a]ESI (10, 30, 60 and 1440 min)	[a]FC	[a]FS (30, 60 and 1440 min)
1	1.0%	637.5	120	↑	↑	↓	↑	↑	↑	↑	↑ - ↑ - ↑ - ↑	↓	↓ - ↓ - ↓
2		562.5	360	↑	↑	↓	↓	↑	↑	↓	↓ - ↓ - ↓ - ↓	↑	↑ - ↑ - ↓
3		712.5	360	↓	↓	↓	↓	↑	↑	↓	↑ - ↑ - ↑ - ↑	↓	↓ - ↓ - ↓
4		637.5	600	↑	↓	↓	↓	↑	↑	↑	↑ - ↑ - ↑ - ↑	↑	↓ - ↓ - ↓
5	3.0%	562.5	120	↑	↓	↓	↓	↑	↑	↓	↑ - ↓ - ↓ - ↓	↓	↓ - ↓ - ↓
6		712.5	120	↑	↑	↓	↓	↑	↑	↓	↓ - ↓ - ↓ - ↓	↓	↓ - ↓ - ↓
7		637.5	360[a]	↓	↓	↓	↓	↑	↑	↓	↓ - ↓ - ↓ - ↑	↓	↓ - ↓ - ↓
8		562.5	600	↓	↓	↓	↓	↑	↑	↓	↓ - ↑ - ↓ - ↑	↓	↓ - ↓ - ↓
9		712.5	600	↓	↓	↓	↓	↑	↑	↓	↑ - ↑ - ↑ - ↑	=	↓ - ↓ - ↓
10	5.0%	637.5	120	↓	↓	↓	↓	↑	↑	↓	↓ - ↓ - ↓ - ↓	↓	↓ - ↓ - ↓
11		562.5	360	↓	↓	↑	↓	↑	↑	↓	↑ - ↑ - ↑ - ↑	↓	↓ - ↓ - ↓
12		712.5	360	↑	↓	↓	↑	↑	↑	↓	↑ - ↑ - ↑ - ↑	↑	↓ - ↓ - ↓
13		637.5	600	↑	↓	↑	↑	↑	↑	↓	↓ - ↓ - ↓ - ↑	↓	↑ - ↓ - ↓

DC: dispersion concentration, H_0: surface hydrophobicity, ζ: zeta-potential, TBD: turbidity, PD: protein dispersibility, WHC: water holding capacity, OHC: oil holding capacity, EAI: emulsifying activity index, ESI: emulsifying stability index, FC: foaming capacity, FS: foaming stability, Fm: firmness in concentration 20% (w/v).
↑ indicate increase, ↓ indicates decrease, and = means that there was no variation, all in relation to the control (without ultrasound treatment).
[a] pH 7.0, ** central point, referring to an average value of 5 repetitions.

Table 4
Effects of ultrasound treatment on the physicochemical and techno-functional properties of pea protein concentrate (PPC), when compared to control system (without ultrasound treatment).

System	Treatment			Physicochemical properties			Techno-functional properties							
	DC (%,w/v)	Power (W)	Time (s)	[a]H_o	[a]ζ	[a]TBD	[a]PD	WHC	OHC	[a]EAI	[a]ESI (10, 30, 60 and 1440 min)	[a]FC	[a]FS (30, 60 and 1440 min)	Fm
1	1.0%	637.5	120	↑	↑	↑	↑	↓	↑	↓	↓ - ↓ - ↑ - ↓	↓	↓ - ↑ - ↑	↓
2		562.5	360	↑	↑	↑	↑	↓	↑	↓	↓ - ↓ - ↓ - ↓	↓	↓ - ↑ - ↑	↑
3		712.5	360	↑	↑	↓	↑	↓	↑	↓	↓ - ↓ - ↓ - ↓	↓	↓ - ↑ - ↑	↑
4		637.5	600	↑	↑	↑	↓	↓	↑	↑		↑	↑ - ↑ - =	↑
5	3.0%	562.5	120	↑	↑	↑	↑	↓	↑	↑	↓ - ↑ - ↑ - ↑	↓	↓ - ↓ - =	↑
6		712.5	120	↑	↑	↑	↑	↓	↑	↓	↓ - ↓ - ↓ - ↓	↓	↑ - ↑ - ↑	↑
7		637.5	360[a]	↑	↑	↓	↑	↓	↑	↑	↓ - ↓ - ↓ - ↑	↓	↑ - ↑ - ↑	↓
8		562.5	600	↑	↑	↓	↑	↓	↑	↑	↓ - ↓ - ↑ - ↑	↓	= - ↑ - ↑	↓
9		712.5	600	↑	↑	↓	↑	↓	↑	↑	↓ - ↓ - ↓ - ↓	↑	↑ - ↑ - ↑	↓
10	5.0%	637.5	120	↑	↓	↑	↑	↑	↑	↓	↓ - ↓ - ↓ - ↑	↓	↓ - ↓ - =	↓
11		562.5	360	↑	↑	↓	↑	↓	↑	↓	↓ - ↑ - ↓ - ↑	↑	= - ↑ - =	↑
12		712.5	360	↑	↓	↓	↑	↓	↑	↑	↓ - ↑ - ↑ - ↑	↓	↓ - ↑ - =	↓
13		637.5	600	↑	↓	↓	↑	↑	↑	↓	↓ - ↓ - ↓ - ↓	↓	↓ - ↑ - ↑	↑

DC: dispersion concentration, H_0: surface hydrophobicity, ζ: zeta-potential, TBD: turbidity, PD: protein dispersibility, WHC: water holding capacity, OHC: oil holding capacity, EAI: emulsifying activity index, ESI: emulsifying stability index, FC: foaming capacity, FS: foaming stability, Fm: firmness in concentration 20% (w/v).
↑ indicate increase, ↓ indicates decrease, and = means that there was no variation, all in relation to the control (without ultrasound treatment).
[a] pH 7.0, ** central point, referring to an average value of 5 repetitions.

= 637.5 W | 120 s | 1.0% (ζ = −32.3 mV), 2 = 562.5 W | 360 s | 1.0% w/v (ζ = −30.1 mV) and 6 = 712.5 W | 120 s | 3.0% (ζ = −28.7 mV) (Table 3). For PPC, only the systems prepared at 5.0% that received USt at 637.5 W | 120 s (system 10, ζ = −11.6 mV), 712.5 W | 360 s (system 12, ζ = −21.1 mV) and 637.5 W | 600 s (system 13, ζ = −5.9 mV), showed densities of negative surface charges on proteins' surfaces smaller than the control (ζ = −28.3 mV) (Table 5). At lower protein concentrations (1.0% and 3.0% w/v), conformational changes - promoted by USt - may have exposed electrically charged amino acid residues, previously located in the interior of proteins' structures. On the other hand, in systems with protein concentration = 5.0% w/v, such biomacromolecules seemed to be less structurally impacted by ultrasound effects, which may be attributed to the greater proximity of the molecules (favoured by intra and intermolecular interactions), leading to a decrease of the negative density on the proteins' surface.

The protein isolates and concentrate used in the present study represent a pool of different proteins dispersed in aqueous media. Dispersed proteins can form colloidal agglomerates due to intermolecular interactions among proteins molecules. In this way, the data was represented in terms of two diameter populations, giving a more accurate description about these systems' characteristics. So, results for hydrodynamic diameter (d_h) from intensity distribution showed that all proteins systems were bimodal in terms of dispersed particles diameters (peak 1 + peak 2 = at least 78% of the relative abundance of dispersed particles). Values of d_h, with their respective population percentage are shown in Table 5. PDI for each peak was also calculated, and presented values inferior to 0.7 in most of cases, indicating a small polydispersity within each size population (Table 5). Higher d_h values for SPI, RPI, and PPC before USt (control systems) suggested a highly aggregated state, in accordance with their lower dispersibility in aqueous systems (Fig. 3). The peak 1 was the most representative for all systems. Considering each material, relative abundance of peak 1 varied as follows: from 48.2% (control) to 99.3% (system 9 = 712.5 W | 600 s | 3.0%, w/v) for SPI; from 56.0% (system 3 = 712.5 W | 360 s | 1.0%, w/v) to 95.5% (system 9 = 712.5 W | 600 s | 3.0%, w/v) for RPI; and from 45.1% (system 13 = 637.5 W | 600 s | 5.0%, w/v) to 95.8% (system 3 = 712.5 W | 360 s | 1.0% w/v) for PPC.

For SPI, the highest d_h (5357.7 nm) was observed for peak 2 in the

Table 5
Average dydrodynamic diameter (d_h) with its respective population percentage and polydispersity index (PDI) of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasonic treatment.

System	Treatment			Peak	Soy Protein Isolate			Rice Protein Isolate			Pea Protein Concentrate		
	DC (% w/v)	Power (W)	Time (s)		d_h (nm)	VD (%)	PDI	d_h (nm)	VD (%)	PDI	d_h (nm)	VD (%)	PDI
1	1	637.5	120	1	813.8	53.1	0.50	552.1	89.5	0.39	270.5	75.7	0.31
				2	71.6	25.2	0.26	4905.0	5.8	0.11	26.3	17.0	0.04
2		562.5	360	1	318.7	76.9	0.28	311.3	87.5	0.16	369.5	55.9	0.30
				2	50.1	16.0	0.10	5032.7	12.3	0.07	4073.7	25.1	0.02
3		712.5	360	1	228.4	96.7	0.40	210.0	56.0	0.41	176.8	95.8	0.16
				2	23.3	3.6	0.07	415.9	38.3	0.10	3879.0	4.2	0.12
4		637.5	600	1	346.1	96.9	0.29	323.4	79.0	0.13	341.8	82.6	0.47
				2	4855.3	3.1	0.02	3425.5	20.2	0.05	56.0	12.8	0.11
5	3	562.5	120	1	225.0	51.3	0.06	346.3	72.9	0.82	272.2	87.6	0.42
				2	26.7	45.5	0.06	4265.0	27.1	0.11	3693.7	9.9	0.07
6		712.5	120	1	597.4	48.8	0.25	504.2	80.0	0.50	266.8	67.8	0.35
				2	4243.7	36.0	0.06	4674.3	16.1	0.10	67.3	26.0	0.03
7		637.5	360[a]	1	694.5 ± 443.9	82.0 ± 10.3	0.30 ± 0.11	337.9 ± 80.8	74.6 ± 3.9	0.47 ± 0.13	246.7 ± 35.2	74.7 ± 14.8	0.33 ± 0.15
				2	1318.6 ± 2014	15.4 ± 11.0	0.08 ± 0.04	4557.7 ± 703.1	23.7 ± 6.2	0.10 ± 0.04	1386.9 ± 664.5	21.9 ± 11.0	0.07 ± 0.05
8		562.5	600	1	513.6	90.9	0.41	323.6	83.5	0.77	95.5	65.5	0.50
				2	38.1	4.9	0.07	4306.7	16.5	0.47	1642.5	30.6	0.05
9		712.5	600	1	335.5	99.3	0.24	216.3	95.5	0.77	310.6	73.8	0.47
				2	20.3	2.2	0.07	3699.0	13.6	0.17	2079.5	20.1	0.10
10	5	637.5	120	1	219.3	59.5	0.18	1165.5	88.4	0.45	347.8	66.4	0.59
				2	4829.5	30.8	0.04	117.1	12.7	0.05	4318.0	23.0	0.08
11		562.5	360	1	4401.0	75.0	0.05	433.0	83.5	0.36	422.5	65.1	0.99
				2	20.6	15.9	0.17	3348.0	16.5	0.13	83.1	33.0	0.14
12		712.5	360	1	2981.5	62.3	0.22	220.8	76.7	0.33	321.7	77.9	0.19
				2	102.7	32.9	0.78	284.9	32.0	0.06	4220.0	15.3	0.04
13		637.5	600	1	137.3	63.5	0.10	382.4	83.5	0.43	1231.3	45.1	0.54
				2	5357.7	24.8	0.01	4300.3	16.5	0.29	37.0	30.6	0.06
Control				1	1344.5	48.2	0.31	694.9	94.2	0.41	345.9	71.0	0.31
				2	153.6	34.8	0.24	4870.7	5.8	0.02	4466.7	26.8	0.05

DC: dispersion concentration, VD: volume distribution.
[a] Average of a central point, referring to an average value of 5 repetitions. The other systems are averages from triplicates.

system corresponding to the highest power and time binomials treatment combined with the highest protein concentration (system 13 = 637.5 W | 600 s | 5.0%, w/v), whereas the smallest diameters (20.6 nm and 219.3 nm, for peaks 2 and 1 respectively) were found in system corresponding to the lowest power and time binomials, also at 5.0% w/v (system 10 = 637.5 W | 120 s | and system 11 = 562.5 W | 360 s). The systems prepared at 1.0% w/v, presented lower d_h (in both size peak), compared to the control (except to peak 2, for the system 4 = 637.5 W | 600 s), as well as all peak 1 of the systems at 3.0% w/v. Therefore, regardless of the amount of power and time binomials applied to the systems prepared at 1.0% w/v (before receiving the USt), one can infer that, in these systems, some intermolecular interactions in protein aggregates were more easily broken, once again corroborating the results obtained in the H_0 index analyses. However, at higher concentrations, although such intermolecular interactions been disrupted in some extent, the closer proximity between protein molecules in the systems seemed to lead to new structural rearrangements, forming new aggregates, and this hypothesis is in agreement with the lower ζ-potential (considering their *moduli*), and also with the lower exposure of H_0 groups (Fig. 5).

RPI presented d_h values for peak 1 ranging from 210.0 nm (system 3 = 712.5 W | 360 s | 1.0% m/v) to 1165.5 nm (system 10 = 637.5 W | 120 s | 5.0% m/v). Peak 2 indicated the presence of large clusters (d_h above 2500 nm) for most of systems. There was a tendency to decrease of d_h after USt, except for systems prepared at 1.0% m/v that received less power and time binomials (system 1 = 637.5 W | 120 s and system 2 = 562.5 W | 360 s). Systems 3 and 12, treated at 712.5 W | 360 s, with protein concentrations of 1.0 (m/v) and 5.0% (m/v), respectively, showed different behaviour from the other RPI dispersions, since their d_h were lower. For peak 1, the sizes were 210.0 and 220.8 nm, respectively, and for peak 2415.9 and 284.9 nm, respectively. As also observed for dispersibility and H_0 index analyses, d_h values of RPI was only slightly affected by ultrasound, so protein aggregates naturally occurring in the raw material persisted after USt. This suggests that the power and time binomials tested by the USt, regardless of concentration, was not strong enough to interrupt the intermolecular interactions that maintain the RPI aggregates, indicating that studies with expansion of independent variables may be a perspective of continuation of this study.

Finally, concerning PPC, peak 1 of dispersed particles presented d_h values varying between 95.5 nm (system 8 = 562.5 W | 600 s | 3.0% w/v) and 1231.3 nm (system 13 = 637.5 W | 600 s | 5.0% w/v). Considering peak 2, the control system showed the highest d_h (4466.7 nm), whereas the lowest one was 26.3 nm for the system 1 = 637.5 W | 120 s | 1.0% w/v. Thus, USt reduced the d_h of dispersed particles in peak 2, as well for the majority of peak 1, except in the following systems: 2 = 562.5 W | 360 s | 1.0% w/v, 10 = 637.5 W | 120 s | 5.0% w/v, 11 = 562.5 W | 360 s | 5.0% w/v and 13 = 637.5 W | 600 s | 5.0% w/v.

Results presented and discussed in the above paragraphs can be compared those reported in somewhat similar studies. For instance, Xiong et al. (2018) who worked with pea protein isolate (94.3% protein) reported that dispersions produced at 5.0% (w/v) protein, when receiving USt at 20 kHz, 30–90% amplitude and 30 min, presented Z-average values reduced (from 192.3 to 159.9 nm). O'Sullivan et al. (2016) reported that USt at 20 kHz | 712.5 W during up to 2 min decreased the average dispersed protein size of soy protein isolate (86% protein) from 1799 nm (untreated) to 265 nm, and of isolated from pea protein (86% protein) from 5250 nm (untreated) to 187 nm. However, for RPI (84.5% protein) the same did not occur, as average dispersed protein sizes were 51600 nm for untreated proteins and 52800 nm of USt' proteins. Finally, size reduction for dispersed rice proteins isolates (90% protein) was reported by Zhang et al. (2018), who combined USt

and different alkali concentrations (0.02–0.1 M NaOH). These authors found average diameters of 486.4 nm for the control system (without any treatment) and diameter values ranging from 219.8 to 248.8 nm in systems receiving ultrasound/alkali treatments, reinforcing the lower sensitivity of the RPI to USt.

3.2.4. Surface hydrophobicity of protein (H_0)

Surface hydrophobicity (H_0 index; dimensionless) reflects the density of hydrophobic groups exposed on the surfaces of proteins' molecules. Therefore, higher H_0 values are often correlated to more extensive denaturation. The H_0 index for SPI, RPI and PPC are presented in Fig. 5. SPI without USt (control) presented H_0 = 1019.1. Except for system 10 = 637.5 W | 120 s | 5.0% (w/v), which showed H_0 = 618.2, the USt promoted an increase in H_0 for all systems (Table 2), which presented H_0 values ranging from 1123.9 (system 11 = 562.5 W | 360 s | 5.0%, w/v) to 2223.9 (system 4 = 637.5 W | 600 s | 1.0%, w/v). H_0 of RPI was less influenced by USt (Table 3), since the control presented H_0 = 431.0 and the other systems ranged from 161.9 (system 11 = 562.5 W | 360 s | 5.0%, w/v) to 1017 (system 6 = 712.5 W | 120 s | 3.0%, w/v). USt increased the H_0 value for all PPC systems (Table 4), when compared to the control (H_0 = 410.9), ranging from 669.5 (system 10 = 637.5 W | 120 s | 5.0%, w/v) to 1753.8 (system 4 = 637.5 W | 600 s | 1.0%, w/v). SPI and PPC presented similar behaviours in terms of surface hydrophobicity: those receiving the highest amounts of power and time binomials, combined with the lowest concentrations (1.0 or 3.0%, w/v), presented the highest H_0 index values, highlighting the USt's ability to trigger conformational changes in proteins, exposing groups that were buried within the core of the protein molecules in their native conformation. On the other hand, systems with protein concentration = 5.0% (w/v) showed the lowest H_0 index in all cases. From this result, it can be hypothesized that the ultrasound waves may have exposed hydrophobic residues that were inside the proteins in their native conformations. However, it is known that the exposition of hydrophobic groups to the aqueous medium is thermodynamically unfavourable (since water-water interactions are more prone than water-hydrophobic amino acid residues interactions). Then, in order to reduce the free Gibb's energy of the system, the protein molecules which are closer to each other tend to form hydrophobic interactions among themselves. Therefore, it is perceptible that the concentration (of systems) plays a relevant impact on the performance of USt.

For RPI, the lower H_0 values compared to SPI and PPC (Fig. 5) indicate that the USt had little influence on the agglomerated state of such proteins. Indeed, rice proteins form oligomers which are structured due to numerous disulphide bonds (Van Der Borght et al., 2006), which are strong enough to be kept even after USt. Nisov et al. (2020), when working with rice endosperm protein, reported that if a protein does not have an organized structure, ANS cannot connect simultaneously to the hydrophobic ring of aniline and naphthalene (hydrophobic interaction), and to the polar sulfonate group (electrostatic interaction) leading to an unstable binding to the protein. Consequently, a false value of H_0 could be obtained. Furthermore, since this analysis was performed using the supernatant content (dispersed fraction), the contribution of insoluble fraction on H_0 values was not compiled, which reduces their real value. In addition, it should be noted that as the rice' proteins are multimeric, their hydrophobic faces tend to interact with each other, especially in concentrated systems (dispersions at 5.0% m/v) submitted to mild power and time binomials (Fig. 5), directly contributing to the lowest H_0 indexes.

Our present work emphasized that physicochemical properties of SPI, RPI, and PPC in aqueous dispersion are influenced USt involving different power and time binomials inputs, but, furthermore, it demonstrated that such properties are sharply impacted by the biopolymer concentration present on dispersions to be submitted to ultrasound, as clearly shown by a conjoint analyses of results for H_0 index, D_h, ζ-potential analyses. It is worthy lay emphasis on the fact that concentrated dispersions act as barriers that attenuates ultrasonic cavitation, so the collapse of bubbles affects to a lesser extent the interactions associated to both the three-dimensional structure maintenance and the aggregation state of proteins. These aspects might affect directly their techno-functional properties, as presented in the next section.

3.3. Techno-functional characterization of proteins in aqueous media

The effect of power and time binomials input by USt for systems prepared at 1.0%, 3.0% or 5.0% (w/v) of SPI, RPI or PPC on their capacity to retain water (WHC) or oil (OHC), on their Interfacial (emulsifying and foaming) properties, as well as on the firmness of the gels produced using up to 20% (w/v) protein, were showed in Tables 2–4 Results for these techno-functional properties assessment are presented and discussed in subsections hereafter.

3.3.1. WHC and OHC

To SPI, WHC was reduced as a higher power and time binomial of USt were used, regardless of the concentration (Fig. 1 supplementary material (SM)). This observation corroborates H_0 results, since the increased exposure of hydrophobic groups disfavours protein-water interactions, hence decreasing water retention capacity. Comparisons of different treatments with the control system (WHC = 808.81%) (Table 2), however, reveal that only the following cases presented higher WHC: system 5 = 562.5 W | 120 s | 3.0% (w/v) (894.4%), system 11 = 562.5 W | 360 s | 5.0% (w/v) (1040.5%) and system 10 = 637.5 W | 120 s | 5.0% (w/v) (1093.8%). According to these results, when dispersions at 5.0% (w/v) received less power and time binomials, proteins tend to show increased WHC.

For RPI, all systems showed a slight increase in WHC, where the control showed 253.2%, and the USt system varied from 273.7% to system 2 = 562.5 W | 360 s | 1.0% w/v up to 359.1% for system 11 = 562.5 W | 360 s | 5.0% w/v), suggesting the existence of poorly soluble agglomerates that could form attractive interactions with either water or oil molecules (see also OHC; next paragraph) (Table 3). These results were also in accordance with low values of H_0 and d_h results for RPI, as these physicochemical properties of this protein material were also less affected by USt. Regarding PPC, similarities with SPI were evidenced, since a decrease in WHC was in general triggered by USt (Table 4). However, compared to control (WHC = 482.4%), the system 10 = 637.5 W | 120 s | 5% (w/v) and system 13 = 637.5 W | 600 s | 5% (w/v) showed a negligible increase in WHC (486.9% and 484.4%, respectively), which can be, once again, related to a lesser exposure of hydrophobic groups in systems with protein concentration = 5% (w/v) during the USt.

In terms of OHC, compared to control systems (OHC = 168.4% for SPI; 157.4% for RPI; 224.1% for PPC), USt promoted an increase for this property regardless of protein material evaluated (Tables 2–4). OHC' SPI varied from 264.5% of system 1 = 637.5 W | 120 s | 1.0% (w/v) to 678.5% of system 5 = 562.5 W | 120 s | 3.0,% (w/v) for RPI it was 333.2% (system 6 = 712.5 W | 120 s | 3.0%, w/v) to 944.6% (system 1 = 637.5 W | 120 s | 1.0%, w/v) and PPC from 385.8% (system 12 = 712.5 W | 360 s | 5.0%, w/v) to 840.6% (system 5 = 562.5 W | 120 s | 3.0%, w/v) (Fig. 1 SM). As previously explained, these results agree with those found for H_0 index analyses (Fig. 5), since USt promoted an increase in H_0 in almost all systems, strongly suggesting a partial unfolding/denaturation of proteins and increased surface exposure of hydrophobic groups, which form attractive interaction with triglyceride molecules.

3.3.2. Foaming properties

Protein-based foams are formed by aqueous dispersions of proteins capable of forming a thin and resistant film on the air/water interface, ensuring that large amounts of dispersed phase (air) are incorporated and kinetically stabilized (Damodaran et al., 1996; McClements, 2004). This ability of proteins to be and remain at the interface are the basis of their foaming properties, represented here in terms of capacity (CF) and

Table 6
Descriptive statistics, model, and regression model for techno-functional properties of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasonic treatment.

Test	Protein	Min.	Max.	Mean	Std. Dev.	Model	F-value	P-value	Regression models	R^2	Lack of fit
Dispersibility (pH 7.0)	Soy	0.43	2.74	1.55	0.79	Linear	8.92	0.0018	$Dispersibility_{SPI} = +0.03 + 2.44E\text{-}003\ T + 2.60E\text{-}0,03\ P - 0.34\ C$	0.67	0.97*
	Rice	0.00	0.19	0.07	0.06	2FI	2.02	0.1553*	$Dispersibility_{RPI} = +0.58 + 1.87E\text{-}004\ T - 5.33E\text{-}004\ P - 0.27\ C - 9.72E\text{-}007\ T\,P + 1.41E\text{-}004\ T\,C + 3,33E\text{-}004\ P\cdot C$	0.55	0.35*
	Pea	0.51	2.22	1.49	0.55	Quadratic	4.06	0.0391	$Dispersibility_{PPC} = +8.37 + 4.39E\text{-}003\ T - 0.03\ P + 1.91\ C - 9.72E\text{-}007\ T\,P + 3.70E\text{-}004\ T\,C - 1.5E\text{-}003\ P\cdot C - 6.29E\text{-}006\cdot T^2 + 3.24E\text{-}005\cdot P^2 - 0.19\cdot C^2$	0.84	0.009
WHC	Soy	189.18	1093.76	642.30	277.52	Linear	15.51	0.0001	$WHC_{SPI} = +1759.48 - 0.89\ T - 1.80\ P + 118.24\ C$	0.78	0.99*
	Rice	273.71	365.20	321.43	25.95	Linear	1.41	0.2839*	$WHC_{RPI} = 251.83 + 0.04\ T + 0.05\ P + 6.99\ C$	0.24	0.59*
	Pea	275.14	486.96	412.51	61.63	Quadratic	12.74	0.0015	$WHC_{PPC} = +416.21 - 0.92\ T + 0.62\ P - 44.69\ C + 3,01E\text{-}004\ T\,P + 0.07\ T\,C + 0.16\ P\cdot C + 5.45E\text{-}004\cdot T^2 - 9.33E\text{-}004\cdot P^2 - 8.35\cdot C^2$	0.94	0.88*
OHC	Soy	413.54	944.62	595.48	109.61	2FI	7.8	0.0026	$OHC_{SPI} = +1077.89 - 1,61\ T - 0,05\ P + 98.43\ C + 1.01E\text{-}003\ T\,P + 0,22\ T\,C - 0.33\ P\cdot C$	0.82	0.27*
	Rice	247.63	446.81	380.64	55.45	Linear	2.09	0.1504*	$OHC_{RPI} = +324.62 + 0.18\ T - 0.03\ P + 3.01\ C$	0.32	0.96*
	Pea	385.82	895.58	696.02	146.84	Quadratic	7.1	0.0085	$OHC_{PPC} = -6206.46 - 0.19\ T + 21.32\ P + 359.58\ C - 8.27\ T\,C + 0.16\ T\,C - 0.25\ P\cdot C - 1.06E\text{-}004\cdot T^2 - 0.02\cdot P^2 - 47.80\cdot C^2$	0.90	0.73*
EAI (pH 7.0)	Soy	104.59	178.52	149.32	16.43	Mean				0	–
	Rice	37.51	89.32	51.21	7.31	Linear	2.01	0.1620*	$EAI_{RPI} = +58.05 - 2.03E\text{-}003\ T + 4.05E\text{-}003\ P - 2.9\ C$	0.32	0.005
	Pea	118.63	238.41	153.11	42.07	Mean				0	–
FC (pH 7.0)	Soy	300.00	486.67	405.69	47.22	2FI	2.85	0.069	$FA_{SPI} = -838.47 + 0.68\ T + 1.89\ P + 295\ C - 9.26\ T\,P - 0.01\ T\,C - 0.45\ P\cdot C$	0.63	0.99*
	Rice	10.71	85.71	19.75	17.33	2FI	1.99	0.1605*	$FA_{RPI} = +12.78 + 0.14\ T - 0.05\ P + 1.24\ C + 1.99E\text{-}005\ T\,P - 0.04\ T\,C + 0,01\ P\cdot C$	0.54	0.003
	Pea	250.00	566.67	384.90	91.39	Linear	1.33	0.3072*	$FA_{PPC} = +617,81 + 0.20\ T - 0.42\ P - 12.08\ C$	0.23	0.50*
Firmness gel 20%	Soy	0,0164	0.1492	0,0585	0,0395	Quadratic	1.10	0,46*	$Firmness_{SPI} = -3.27 + 1.07E\text{-}004\ T + 0.010\ P + 0.07\ C - 4.00E\text{-}007\ T\,P - 2.22E\text{-}005\ T\,C - 8.53E\text{-}005\ P\cdot C + 1.72E\text{-}007\ T^2 - 7.63E\text{-}006\cdot P^2 - 2.20E\text{-}004\cdot C^2$	0.58	0.85*
	Pea	0,0123	0,0385	0,0197	0,0069	Quadratic	1.44	0.32*	$Firmness_{PPC} = -0.24 - 1.52E\text{-}004\ T + 9.71E\text{-}004\ P - 0.01\ C + 1.00E\text{-}007\ T\,P + 7.29E\text{-}007\ T\,C + 1.15E\text{-}005\ P\cdot C + 1.06E\text{-}007\cdot T^2 - 8.33E\text{-}007\cdot P^2 + 7.90E\text{-}004\cdot C^2$	0.65	0.0076

WHC: Water Holding Capacity; OHC: Oil Holding Capacity; EAI: Emulsifying Activity Index and FA: Foam capacity. T: Time; P: power and C: concentration. 2FI = two-factor interaction. R^2: R-Square.
*not significant.

stability (FS) (Fig. 2. SM).

SPI's foaming capacity was increased for all USt conditions studied, since control system showed FC = 260%, and the USt systems presented FC ranged from 300% (system 2 = 562.5 W | 360 s | 1.0%, w/v) to 480% (system 3 = 712.5 W | 360 s | 1.0% w/v). These findings are in agreement with the general ensemble of results presented and discussed throughout this report. This time, greater exposure of hydrophobic groups was effective in facilitating diffusion of protein molecules towards air-water interface and their adsorption on it. Foam stability was improved up to 60 min, but after 24 h, only system 1 = 637.5 W | 120 s | 1.0% w/v and system 12 = 712.5 W | 360 s | 5.0% w/v had foam stability greater than the control (Table 2).

For RPI, most of systems showed a reduction for FC, as well as in foam stability, after USt (Table 3). Systems 4 = 637.5 W | 600 s | 1.0% (w/v), 2 = 562.5 W | 360 s | 1.0% (w/v), and 13 = 637.5 W | 600 s | 5.0% (w/v) showed higher percentages of FC (85.7%, 22.9%, and 19.3%, respectively), compared to the control system (18.6%).

Similarly, for PPC' USt decreased FC values found, except for the following systems: 9 = 712.5 W | 600 s | 3.0% (w/v), 11 = 562.5 W | 360 s | 5.0% (w/v), and 4 = 637.5 W | 600 s | 1.0% (w/v). They presented FC values equal to 466.7%, 483.3%, and 566.7% respectively, whereas the control system showed FC = 450%. However, considering that foams were completely destabilized after 24h in the control system, the USt equalled or increased the foam stability after this time (Table 4 and Fig. 2 SM).

3.3.3. Emulsifying properties

Ultrasonic treatments subtly increased the emulsifying capacity of SPI (Fig. 3 SM). systems showed higher values for EAI ranging 141.3 m^2/g (system 2 = 562.5 W | 360 s | 1.0%) to 178.6 m^2/g (system 11 = 562.5 W | 360 s | 5.0%) when compared to control system (EAI = 122.9 m^2/g). The system 10 = 637.5 W | 120 s | 5.0% was the only that presented lower EAI (122.5 m^2/g), and less stability up to 60 min, compared to control (Table 2). After 24 h (1440 min), the system 2 = 562.5 W | 360 s | 1.0% (w/v) was more unstable than control system. In this way, the improved stability of emulsions due to USt can be explained by greater exposure of hydrophobic groups on the surface of proteins (Fig. 5), generating a balanced structure in terms hydrophobic and hydrophilic, which adsorbed maintained well to the interface, as was also observed in FC.

For RPI, only the systems 1 = 637.5 W | 120 s | 1.0% w/v and 4 = 637.5 W | 600 s | 1.0% w/v presented higher EAI (61.2 m^2/g and 69.3 m^2/g, respectively) than the control system (58.7 m^2/g). However, USt increased RPI stability after 24h, where only systems 2 = 562.5 W | 360 s | 1.0% w/v, 5 = 562.5 W | 120 s | 3.0% w/v, 6 = 712.5 W | 120 s | 3.0% w/v and 10 = 637.5 W | 120 s | 5.0% w/v were less unstable than the control system (Table 3), indicating that in some low and medium power and time binomials situations, these systems present greater instability after 24 h. Considering that the USt allowed few structural changes (see results H_0, dispersibility, and D_h), our results were suggesting that the proteins present in the RPI presented greater difficulty in migrate and

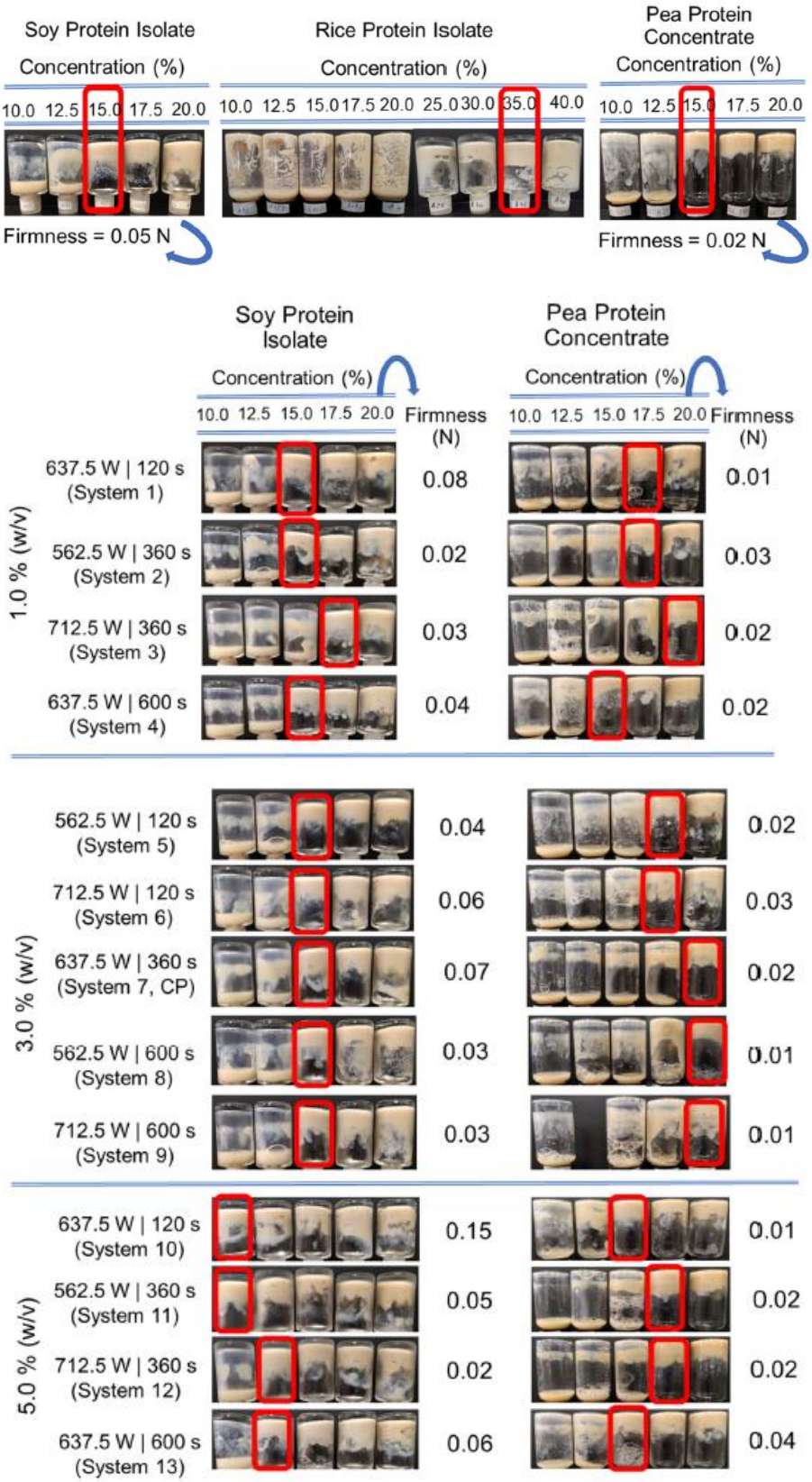

Fig. 6. Least gelling concentration (highlighted in red) of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC)– * Firmness values refer to gels produced at 20% w/v. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

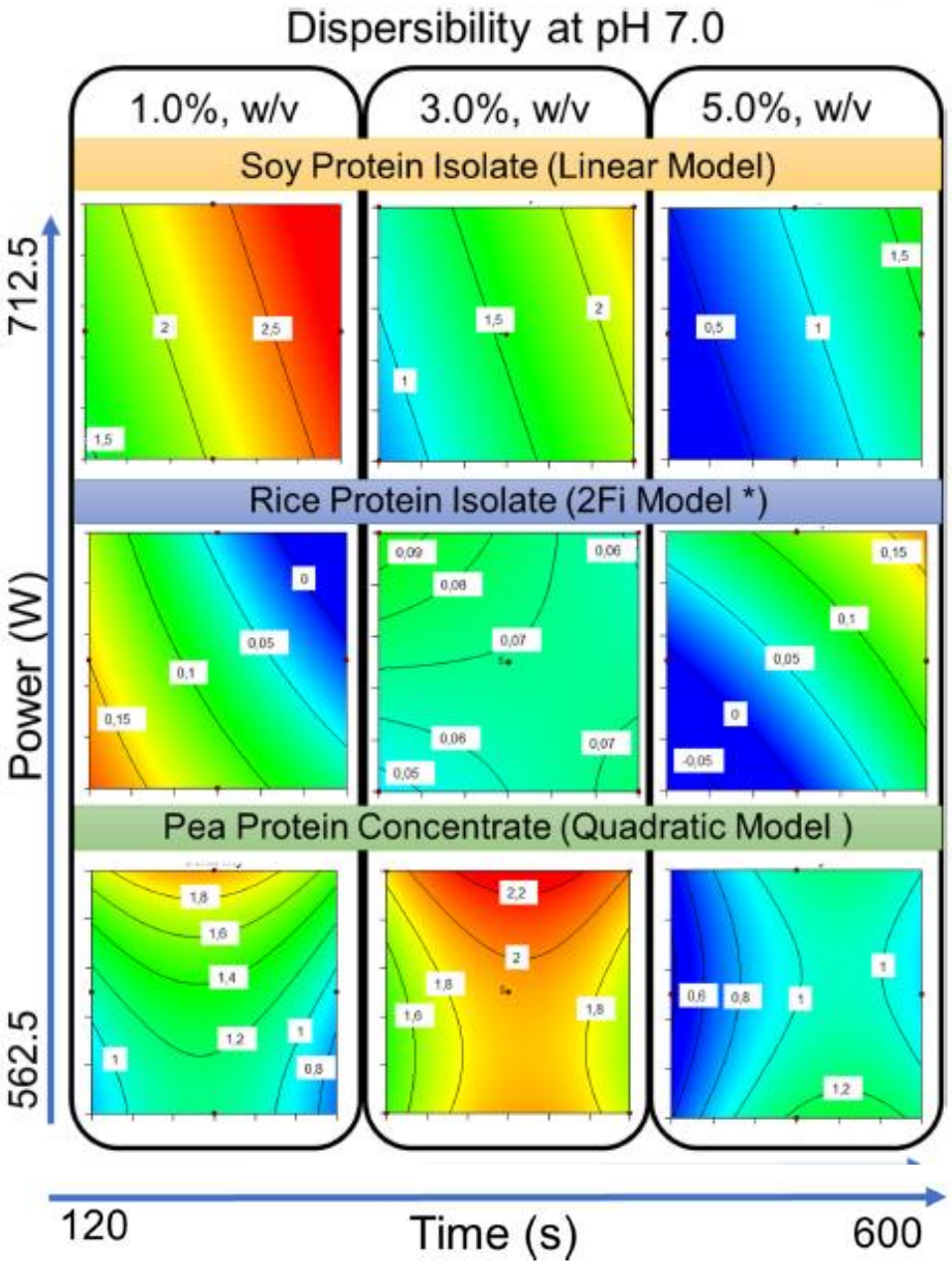

Fig. 7. Contour graphics for dispersibility of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasound treatment – * represent non-significant models (p-value> 0.05) analyzed by ANOVA.

remaining at the interface, due to their large constituent aggregates, resulting in slight changes in their emulsifying properties. Moreover, systems with protein concentration = 1% (w/v) after USt, showed a tendency to improve the stability of emulsions after 24 h.

For PPC, the effect of USt was variable. The control system presented EAI = 135.9 m^2/g, while the systems treated with ultrasound showed values varying from 120.5 m^2/g (system 2 = 562.5 W | 360 s | 1.0%, w/v) to 238.4 m^2/g (system 5 = 562.5 W | 120 s | 3.0%, w/v). There was also an improvement in the kinetic instability of PPC emulsions after 24h in systems 5 (562.5 W | 120 s | 3.0%, w/v), 7 (637.5 W | 360 s | 3.0%, w/v), 8 (562.5 W | 600 s | 3.0%, w/v), 10 (637.5 W | 120 s | 5.0%, w/v), 11 (562.5 W | 360 s | 5.0%, w/v) and 12 (712.5 W | 360 s | 5.0%, w/v) (Table 4), indicating that emulsions prepared with proteinaceous materials that underwent USt at concentrations 5.0% (w/v) or 3.0% (w/v), showed better performance than at 1.0% (w/v). Since USt promoted increase in dispersibility in water, as well an increase in superficial hydrophobicity of PPC, it was expected that the interfacial properties were improved, so that the stability of the emulsion was greater in USt systems, as it happened in SPI, indicating that the structural changes promoted by the treatment have not been balanced in terms of hydrophilicity and hydrophobicity.

3.3.4. Gelling properties

SPI, RPI and PPC dispersions containing different protein

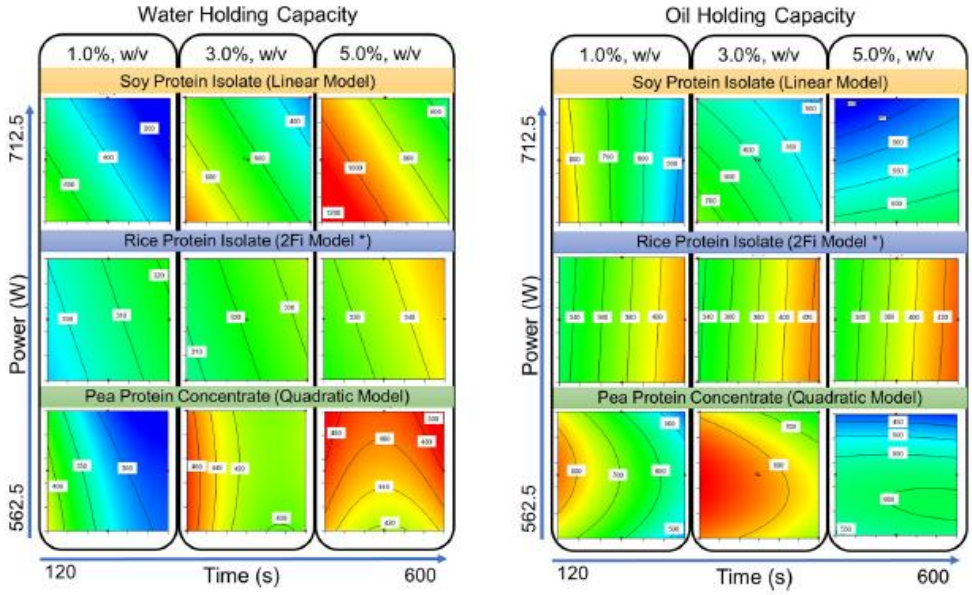

Fig. 8. Contour graphics for water/oil holding capacity (WHC/OHC) of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasound treatment – * represent non-significant models (p-value> 0.05) analyzed by ANOVA.

concentrations were prepared, aiming at to evaluating their least gelling concentration (LGC). Results are depicted in Fig. 5. SPI and PPC required at least 15% (w/v) to form a firm gel, whereas RPI only formed gel with a minimum concentration of 35% (w/v). Considering the technologically impracticable LGC for RPI, this analysis proceeded only with SPI and PPC USt. Heat-induced gelation of proteins is a multi-stage process requiring some unfolding of the native molecules, triggered by temperatures above the protein denaturation temperature, to expose buried interaction sites and thus to form new intermolecular interactions (*i.e.*, hydrogen bond, electrostatic and hydrophobic interactions), allowing the formation of junction zones that lead to a three-dimensional network whose interstices are filled by solvent (generally water) (Ahmed, 2015; Shand et al., 2007).

For SPI, no change was noticed in the LCG for systems prepared using SPI that underwent USt at concentration = 3.0% w/v. The system 3 (712.5 W | 360 s | 1.0% w/v) was the only one that presented an increase in LCG (17.5% w/v), compared to the corresponding control (15.0% w/v). In the systems prepared with SPI submitted to USt at concentration 5.0% w/v, the systems 10 (637.5 W | 120 s | 5.0% w/v) and 11 (562.5 W | 360 s | 5.0% w/v) required 10.0% w/v as LCG, while the other ones (systems 12 = 712.5 W | 360 s | 5.0% w/v and 13 = 637.5 W | 600 s | 5.0% w/v), had LCG 12.5%. The gel firmness of the control system was 0.05 N. The system that required 17.5% of LCG showed a firmness of 0.03 N. The system 10 (637.5 W | 120 s | 5.0% w/v) was that with de greatest firmness, which reached 0.15N. The other ones ranged from 0.02 N (systems 2 = 562.5 W | 360 s | 1.0% w/v or 12 = 712.5 W | 360 s | 5.0% w/v) to 0.08 N (system 1 = 637.5 W | 120 s | 1.0% w/v).

For PPC, the USt seems to have minimized the interactions of

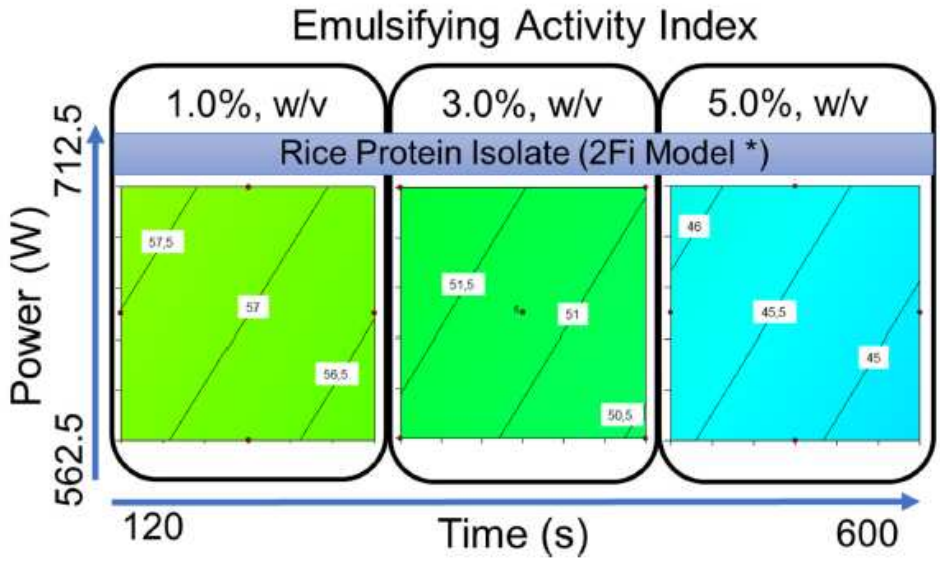

Fig. 9. Contour graphics for foam capacity (FC) of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasound treatment – * represent non-significant models (p-value> 0.05) analyzed by ANOVA.

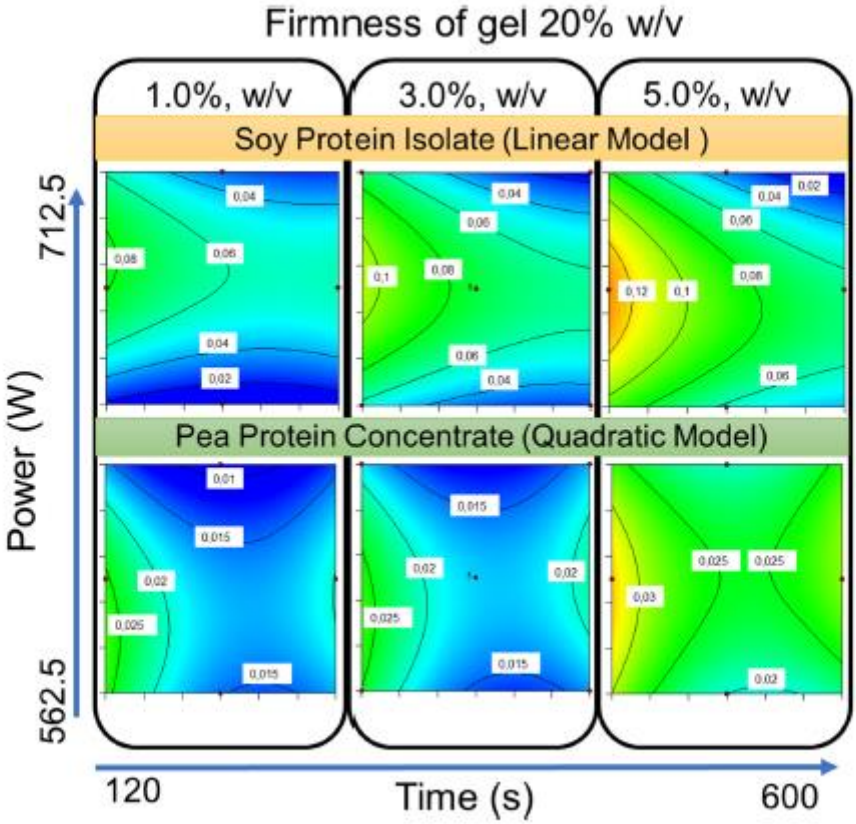

Fig. 10. Contour graphics for emulsifying activity index (EAI) of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasound treatment – * represent non-significant models (p-value> 0.05) analyzed by ANOVA.

junction zone formations that trapped water to cross-link the gel, since only the systems 4 = 637.5 W | 600 s | 1.0% w/v, 10 = 637.5 W | 120 s | 5.0% w/v, and 13 = 637.5 W | 600 s | 5.0% w/v maintained LCG at 15.0% w/v. The system 3 = 712.5 W | 360 s | 1.0% w/v, and those with 3.0% in the highest power and time binomials (systems 7 = 637.5 W | 360 s, 8 = 562.5 W | 600 s and 9 = 712.5 W | 600 s) increased the LCG to 20.0% w/v, and the other systems, gelatinized to 17.5% w/v. Considering that the firmness of the control system was 0.02 N, was demonstrate that USt promotes a little effect on this physical property of gels, whose values varying from 0.01 N (including gels that had LCG at 15.0%, 17.5% and 20.0% in systems that underwent USt at concentrations of 5.0% w/v, 1.0% w/v or 3.0% w/v, respectively), up to 0.04 N (system 13 = 637.5 W | 600 s | 5.0% w/v, whose LCG was 15.0% w/v).

3.3.5. *Optimization of techno-functional properties of soy (SPI) and rice protein isolate (RPI), and pea protein concentrate (PPC) using response surface methodology*

In order to better quantify the effects of the independent variables used in this study on dependent responses, coefficients of adjusted polynomial models and their ANOVA results were calculated and results are shown in Table 6, and contour graphs are shown in Figs. 6–11.

The properties evaluated for all proteins were adjusted for the linear (solubility$_{SPI}$, WHC$_{SPI}$, WHC$_{RPI}$, OHC$_{RPI}$, EAI$_{RPI}$ and FA$_{PPC}$), quadratic (Dispersibilty$_{PPC}$, WHC$_{PPC}$, OHC$_{PPC}$, Firmness$_{SPI}$ and Firmness$_{PPC}$) or 2FI models (Dispersibility$_{RPI}$, OHC$_{SPI}$, FC$_{SPI}$ and FC$_{RPI}$). The responses obtained for EAI$_{SPI}$ and EAI$_{PPC}$ properties did not fit any model, represent dependent responses that did not correlate with the independent variables. In addition, since EAI$_{RPI}$ presented a non-significant model (p = 0.16), we can said that the emulsifying activity analyzed by spectrometry could not be optimized by the experimental conditions used. Not only in terms of EAI, but all properties evaluated for RPI show non-significant models ($p > 0.05$), indicating that higher levels of independent variables should be used to achieve optimized responses to these technical-functional properties.

SPI dispersibility showed a positive effect for power and time binomials input, while the concentration showed a negative effect. Despite the lack of adjustment for Dispersibility$_{PPC}$ ($0.009 < p\text{-value} = 0.05$), intermediate dispersion concentration conditions (3.0%, w/v) and power and time binomials tend to improve its dispersibility.

WHC$_{SPI}$ was increased when dispersions at 5.0% (w/v) received lesser amounts of power and time binomials. WHC from PPC was increased when dispersions at 3.0% (w/v) received less power and time binomials input or when dispersions at 5.0% (w/v) received 712.5 W | 120 s or 712.5 W | 600 s input. Therefore, USt in concentrations of dispersions = 3.0% w/v or 5.0% w/v tend to increase the WHC of proteins. Higher OHC$_{SPI}$ was found in the combination of less concentrated dispersions (1.0%, w/v) with lower power and time binomials input (562.5 W | 120 s). For PPC, the increase in OHC was more pronounced when dispersions at 3.0% (w/v) received USt with 637.5 W | 120 s.

FC$_{SPI}$ was more pronounced in dispersions at 1.0% (w/v) when inputted 712.5 W | 120 s, 712.5 W | 360 s or 712.5 W | 600 s, which correspond to the condition of greater power (712.5 K), regardless of time. High FC$_{SPI}$ was also observed in the 5.0% (w/v) dispersion when applying 562.5 W | 600 s input. The FC$_{PPC}$, whose adjustment of the linear model was non-significant ($p = 0.31$), showed an increase in its FC

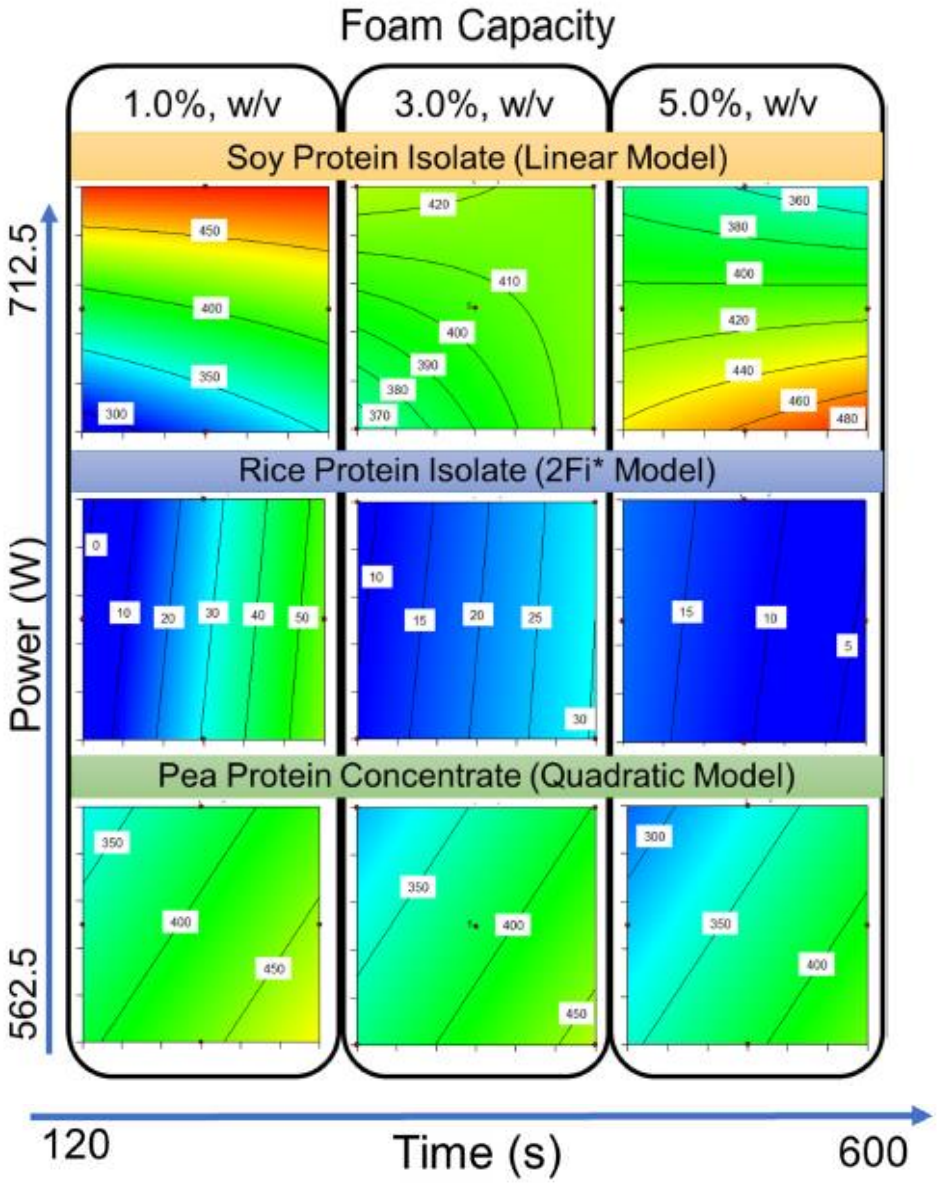

Fig. 11. Contour graphics for firmness of protein gels (20.0%, w/v) of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasound treatment – * represent non-significant models (p-value> 0.05) analyzed by ANOVA.

with power and time binomials input at 562.5 W | 600 s in all concentration evaluated (1.0% w/v, 3.0% w/v and 5.0% w/v).

Finally, the firmness of gels developed with 20% SPI or PPC, despite the suggested quadratic model being shown to be insignificant ($p > 0.05$), showed an increase in this property when the USt was applied dispersions at 5.0%, with power and time binomials input of 637.5 W | 120 s.

In summary of USt conditions that provided minimum and maximum values of the techno-functional properties of the SPI, RPI and PPC are shown in Table 7.

4. Conclusions

Physicochemical properties of soy (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) in aqueous dispersions were influenced by ultrasonic treatment (USt) involving different power and time binomials inputs. Furthermore, it was demonstrated that such properties are sharply impacted by the biopolymer concentration present within aqueous dispersions to be submitted to ultrasound, as clearly shown by a conjoint analyses of results for H_0 index, d_h, ζ-potential analyses. Such effects were much less pronounced for rice protein isolate (RPI), when compared to SPI and PPC. It is worthy to emphasize that in more concentrated dispersions, the proteins themselves seemed to act as

Table 7
Summary of ultrasonic treatment conditions that provided minimum and maximum values of the techno-functional properties of the soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC).

Test	Values	SPI			RPI			PPC		
		T (s)	P (W)	C (% w/v)	T (s)	P (W)	C (% w/v)	T (s)	P (W)	C (% w/v)
Water Holding Capacity	Min	600	712.5	1.0	120	562.5	1.0	600	712.5	1.0
	Max	120	562.5	5.0	600	712.5	5.0	600	712.5	5.0
Oil Holding Capacity	Min	120	712.5	5.0	120	712.5	1.0	600	712.5	5.0
	Max	120	562.5	1.0	600	562.5	5.0	120	637.5	3.0
Emulsifying activity index	Min	–	–	–	600	562.5	5.0	–	–	–
	Max	–	–	–	600	562.5	1.0	–	–	–
Foam capacity	Min	120	562.5	1.0	120	712.5	1.0	120	712.5	5.0
	Max	600	712.5	1.0	600	562.5	1.0	600	562.5	1.0
Dispersibility pH 7.0	Min	120	562.5	5.0	120	562.5	5.0	120	637.5	5.0
	Max	600	712.5	1.0	120	562.5	1.0	360	712.5	3.0
Firmness gel 20%, w/v	Min	360	562.5	1.0	–	–	–	360	712.5	1.0
	Max	120	637.5	5.0	–	–	–	120	637.5	5.0

T: Time; P: power and C: concentration of protein dispersion.

barriers that attenuated ultrasonic cavitation. In these cases, the collapse of bubbles affected to a lesser extent the interactions associated to both the three-dimensional structure and the aggregation state of proteins, which are directly linked to their techno-functional properties.

Among the techno-functional properties studied, oil holding capacity was the only one that increased after USt for SPI, RPI and PPC. Considering that the techno-functional properties are improved as the dispersibility of protein materials increases, a harsher power/time combination (712.5 W | 600 s), in dispersions at 1.0%, can be indicated as the more promising processing condition for SPI. RPI, with or without USt showed a general low techno-functional performance. Moreover, all models of regression were non-significant ($p > 0.05$). As the RPI showed expressively lower foaming and gelling capacities, one strategic applicability to it should be in food for enteral nutrition. In fact, in enteral formulations the formation of foams may impair the flow of the food within the tube or catheter, and semisolid formation may lead to the obstruction of these pipes. In both cases, the arrival of the material within the gastrointestinal tract would be slower, irregular, or even precluded. Finally, PPC showed a more complex behaviour, since experimental data obtained for this material were fitted in most of cases by the quadratic regression model (dispersibility, water/oil holding capacity and firmness). This suggested that the intermediate conditions of dispersion concentration and power/time binomials of USt may be more suitable for this material to be explored as an ingredient in food industry.

CRediT authorship contribution statement

Michele Harumi Omura: Conceptualization, Investigation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Ana Paula Hanke de Oliveira:** Conceptualization, Investigation, Formal analysis, Validation, Writing - original draft. **Lucas de Souza Soares:** Conceptualization. **Jane Sélia dos Reis Coimbra:** Resources, Funding acquisition. **Frederico Augusto Ribeiro de Barros:** Supervision. **Márcia Cristina Teixeira Ribeiro Vidigal:** Formal analysis. **Maria Cristina Baracat-Pereira:** Conceptualization. **Eduardo Basílio de Oliveira:** Conceptualization, Validation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2020.106457.

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Supplementary material.

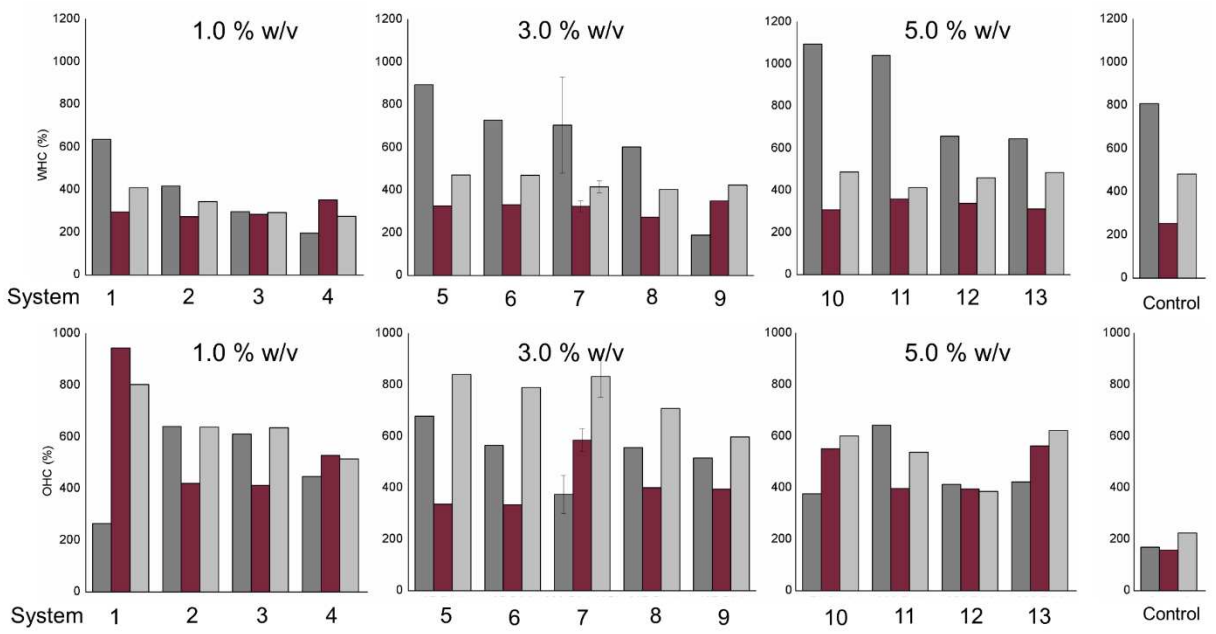

Figure 1 (SM). Water and oil holding capacity of Soy protein isolate , Rice protein isolate and Pea protein concentrate after ultrasonic treatment. System 1 = 637.5 W | 120 s; 2 = 562.5 W | 360 s; 3 = 712.5 W | 360 s; 4 = 637.5 W | 600 s; 5 = 562.5 W | 120 s; 6 =712.5 W | 120 s; 7 = 637.5 W | 360 s (CP); 8 = 562.5 W | 600 s; 9 = 712.5 W | 600 s; 10 = 637.5 W | 120 s; 11 = 562.5 W | 360 s; 12 = 712.5 W | 360 s; 13 = 637.5 W | 600 s. Control system is without ultrasonic treatment and CP is the central point, referring to an average value of 5 repetitions (graphical representation of data summarized in Tables 2-4 within the paper).

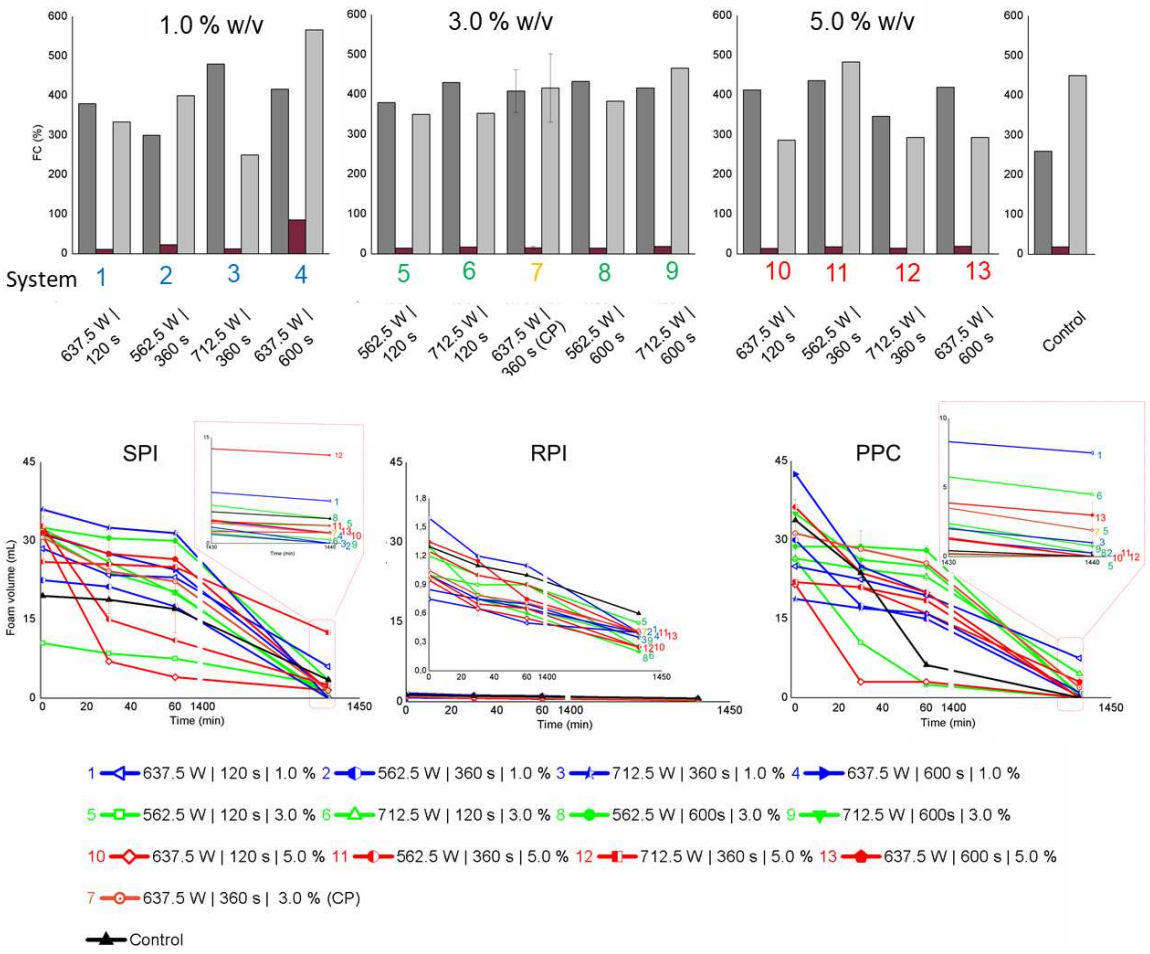

Figure 2 (SM). Foam capacity (FC) and foam stability of soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC) after ultrasonic treatment. Blue lines correspond to dispersions prepared (before ultrasonic treatment) at 1.0 % (w/v), green 3.0 (w/v), red 5.0 % (w/v), and black is the control (without ultrasonic treatment). Empty symbols correspond to the lowest amounts of energy, partially filled with intermediate amounts and fully filled, highest amounts of energy (kJ). CP is the central point, referring to an average value of 5 repetitions (graphical representation of data summarized in Tables 2-4 within the paper).

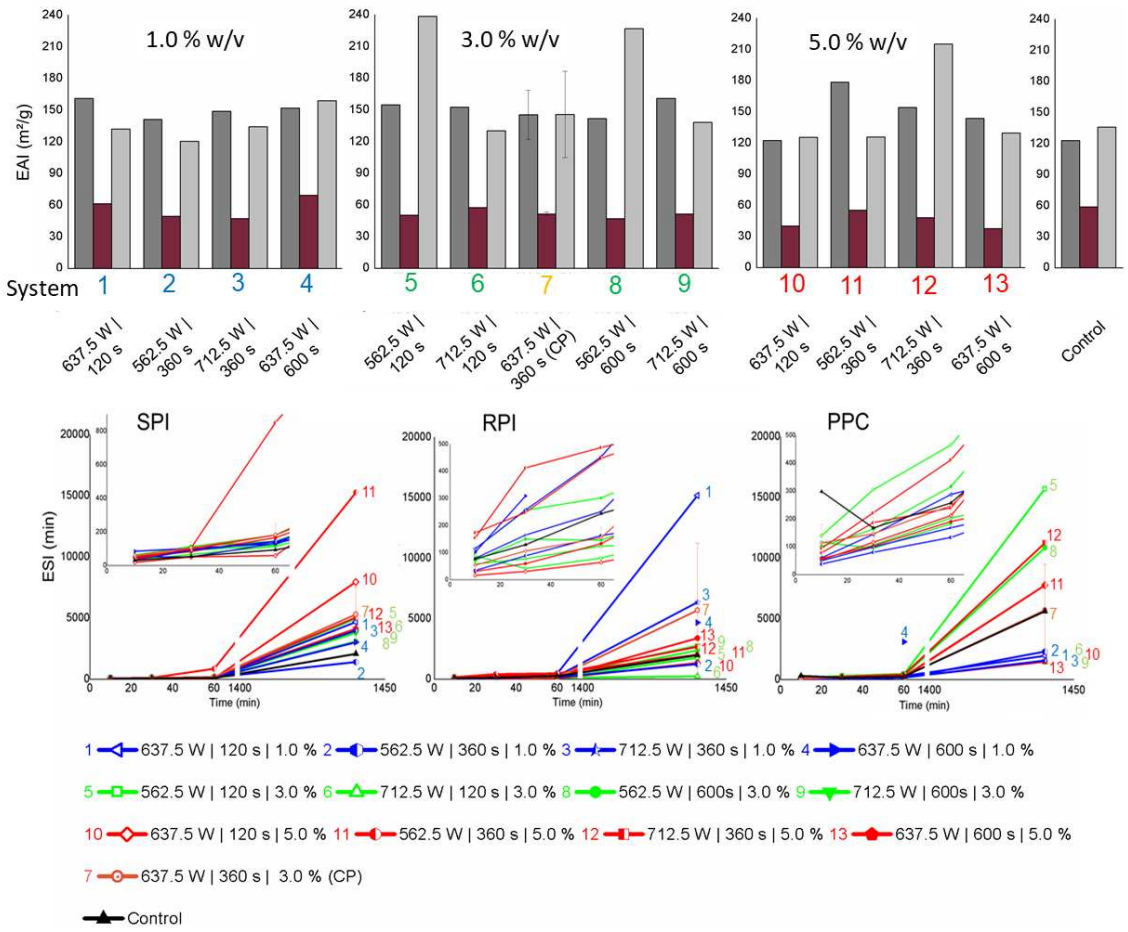

Figure 3 (SM). Emulsifying activity Index (EAI) and emulsifying stability index (ESI) of Soy protein isolate , Rice protein isolate and Pea protein concentrate after ultrasonic treatment. Blue lines correspond to dispersions prepared (before ultrasonic treatment) at 1.0 % (w/v), green 3.0 % (w/v), orange is the central point, red 5.0 % (w/v), and black is the control (without ultrasonic treatment). Empty symbols correspond to the lowest amounts of energy, partially filled with intermediate amounts and fully filled, highest amounts of energy (kJ). CP is the central point, referring to an average value of 5 repetitions (graphical representation of data summarized in Tables 2-4 within the paper).

Capítulo 3

5. Impacts of ultrasound processing on physicochemical and techno-functional properties, and *in vitro* digestibility of plant proteins alone and combined into binary or ternary mixtures[2].

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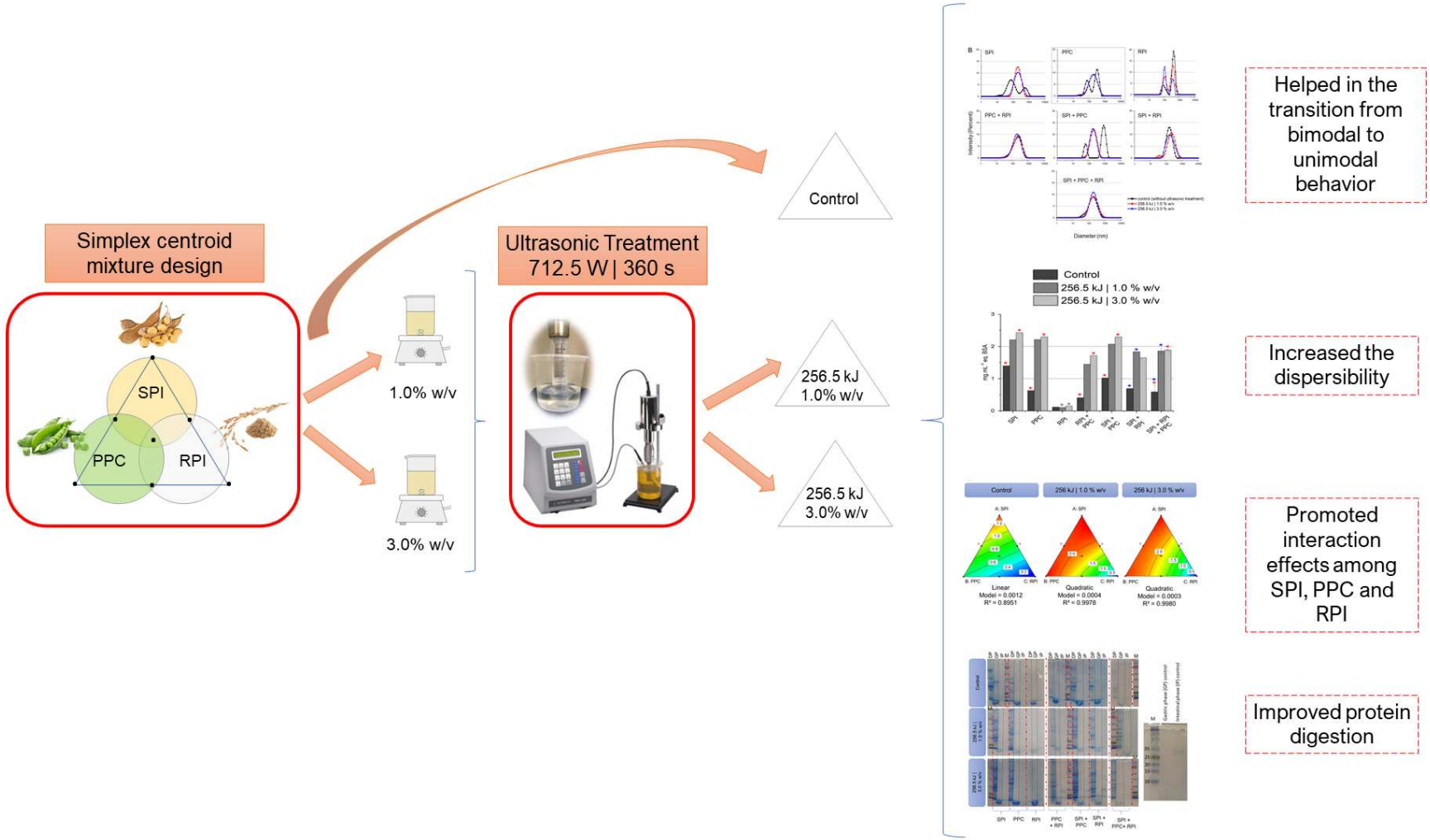
[2]Manuscrito a ser submetido em um periódico internacional.

Graphical abstract:



ABSTRACT:

Plant proteins from different sources can be combined to improve nutritional and techno-funcional properties of mixed products. Thus, the present study investigated the effects of mixtures between soybean protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC), using the experimental design of simplex centroid mixture based on seven mixture-points. In addition, an ultrasonic treatment (USt) at 20 kHz, 256.5 kJ (712.5 W and 360 s) was applied to dispersions of protein mixtures containing 1.0% or 3.0% w/v. The physicochemical properties in relation to dispersibility profile, molecular mass profile, hydrodynamic diameter (d_h), zeta-potential, hydrophobicity (H_0) and free sulfhydryl (SH) groups were evaluated and discussed in relation to the effect of mixtures and USt. The effects of binary and ternary mixtures were evaluated through empirical mathematical modelling, in which the dependent variables were fitted to the quadratic and/ or special cubic regression models. Comparisons between control, USt 256.5 kJ | 1.0% (w/v) and 256.5 kJ | 3.0% (w/v) were performed using the Kruskal-Wallis H test. The USt promoted an increase in the dispersibility of the proteins and changed their surface characteristics, which were showed by the increase in H_0 and reduction in the free SH groups without modifying the molecular mass profile. The effects of USt on the materials caused a reduction in water holding capacity (WHC); an improvement in the foaming capacity, emulsifying activity and oil holding capacity (OHC), as well as an increase the demanded for the last gelling concentration (LGC). The analyses of the results illustrate the potential of plant-based protein mixtures in a wide range of food products, based on the desired techno-functional property. Furthermore, *in vitro* digestibility test showed that the binary and ternary mixtures of SPI, RPI and PPC treated with USt improved protein digestion. Results described the potential of plant-based protein mixtures in a wide range of food products, providing balanced amino acids, improving the techno-functional properties and biological functionality.

1. Introduction:

The current social challenges involve concerns with both the eradication of COVID-19, and reflections on what we have been doing to guarantee the quality of personal and society life. Unsustainable agricultural practices, biodiversity decline, market trends, virtual access to information, and technology development, for example, are among the factors that have shaped our concern with food production. Thus, the development of fairer food systems involves the combination of tools and ingredients, which guarantee a wide range of food production, respecting environmental, social, ethnic and health aspects.

Proteins are inherent compounds of many foods, with important nutritional role, as they provide essential amino acids, in addition to having distinct techno-functional properties. Animal-derived proteins are considered complete when it comes to the aminoacidic profile, but recent research involving alternative sources that minimize environmental impacts and costs, such as those from plant-based, has been gaining ground. Plant-derived proteins that are currently being studied with the aim of being applied as food ingredients include those from cereals (wheat, corn, oats, rice), oil seeds (sunflower, pumpkin, sesame, cotton), legumes (soybeans, peas, beans, chickpeas, lentils), nuts (almonds, pistachios, cashews, peanuts), tubers (potatoes), and pseudocereals (quinoa, buckwheat, chia and amaranth) (Nikbakht Nasrabadi et al., 2021).

Jiménez-Munoz et al. (2021) reported obstacles to the use of plant-based proteins, mainly due to inadequate amino acid balance. They highlighted the demand for detailed studies of plant-derived protein mixtures, not only in terms of the nutritional quality of the composition parameters, but also the digestibility and interactions within the mixture at the molecular or supramolecular level. Furthermore, it is known that plant proteins have limited solubility, and that emerging technologies have been used to improve this property (Avelar et al., 2021). Ultrasound represents a new, green, innovative and sustainable technology, as it is nothing more than an acoustic wave (> 20 kHz). Wave frequencies ranging from 20 to 100 kHz refer to high-power or power ultrasound (Rahman & Lamsal, 2021), and has been used in several studies to modify the conformation of plant-proteins and evaluate the results of their functional performance. For example, Biswas & Sit (2020) found that the solubility, emulsifying and foaming properties, WHC and OHC, of tamarind seed protein isolate were improved after USt, varying the time (15-30 min) and intensity (100-200W). Resendiz-Vazquez et al. (2017) found out that sonication (20 kHz, 200-600 W / 15 min) in jackfruit seed protein isolate improved its OHC, emulsifying and foaming capacity, while affecting the WHC and LGC. Moreover, Zhang et al. (2018) applied an ultrasound-assisted alkali treatment (600 W / 60 min) on rice proteins and managed to improve the solubility of this material 230-fold compared to the control.

The main parameters used in the configuration of the ultrasonic device (probe or bath) include time (2-100 min), temperature (2-50°C), frequency (20-25 kHz), power (200-600 W) or acoustic intensity (22-138 W/cm²) (Gharibzahedi & Smith, 2020). Furthermore, de Oliveira et al. (2020) discovered that even standardizing the energy input (240 kJ) in pea proteins, different combinations of power x time binomials promoted distinct conformational changes. These studies showed how sonication could affect the conformation of a single proteins isolate/concentrate. However, the use of a single plant protein source can-not supply the essential amino acid demand for humans. In this sense, the use of mixtures of proteins of different sources could be a strategy for the composition of a material containing amino acids in appropriate quantities and proportions. Furthermore, to the best of our knowledge, studies proposing both the use of plant proteins mixture and their homogeneization over high-power ultrasound have not been performed. Pietrysiak et al. (2018) produced a blend with pea-rice protein isolate (2:1) through direct steam injection and verified improvements in its functionality without affecting the amino acid composition. Tao Wang, Xu, Chen, & Wang (2018) and Tao Wang, Xu, Chen, Zhou, et al. (2018) dispersed rice proteins together with soy protein isolate in different proportions (1:01; 1:2) under alkaline conditions and found improvements in their functionality using the pH cycle method, claiming to have promoted development of a new protein hydrocolloid or protein composite. Therefore, mixtures of different plant-proteins combined with different treatments can generate new innovative protein composites or hydrocolloids, with improved techno-functionality.

In this study, a mixture design was applied to three plant-proteins: soy protein isolate (SPI), rice protein isolate (RPI) and pea protein concentrate (PPC). Furthermore, based in our recent work (Omura et al., 2021) the produced mixtures were treated with ultrasound (USt) according to the optimized condition (256.5 kJ | 1.0% w/v or 3.0% w/v, for SPI and PPC, respectively), which represent the combination of treatments that promoted higher dispersibility (pH 7.0) with lower values of foam capacity and least gelling concentration (LGC). Considering the impossibility of optimizing the RPI by USt in the previous study, no treatment proposal was applied in this study. The techno-functional characteristics used as a criterion for choose the USt were based on the proposal to obtain a mixture of proteins with potential use as ingredients for powdered protein foods, such as supplements, enteral diet, etc. The protein mixtures USt were evaluated in relation to their techno-functional performance and physicochemical and digestibility characteristics.

2. Material and methods

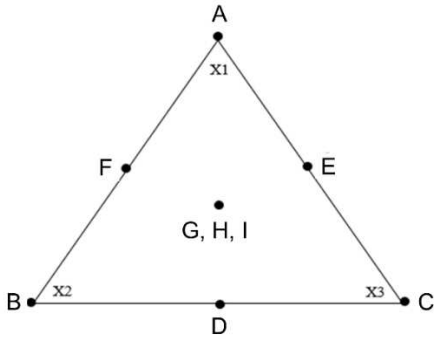
2.1 Materials

Soy protein isolate (SPI) Pure Pro TM 90B (lot: E330030361, Shandong Xinlai Soybean Biotech Co., Ltd), rice protein isolate (RPI) Oryzatein Silk 90 (lot: JNOP0120180606, Axiom Foods - Los Angeles, CA, USA), and pea protein concentrate (PPC) (lot: 20180415, Shandong Jianyuan Foods Co., Ltd - China) were kindly donated by Nutricium Indústria e Comércio Ltda (Belo Horizonte, MG, Brazil). The protein content of these materials was estimated throught Kjeldahl method, by using the following conversion factors: 6.25 for SPI and PPC (Oliete et al., 2018b) and 5.95 for RPI (Agboola et al., 2005). It was found to be 83.32 ± 3.23%, 80.89 ± 1.35% and 73.98 ± 2.58% (wet basis) protein SPI, RPI and PPC, respectively. HCl, NaOH, sodium dodecyl sulfate (SDS), boric acid, sulfuric acid, β-mercaptoethanol, 8-anilino-1-naphthalenesulfonic acid (ANS), 5-5-dithiobis- (2-nitrobenzoic acid, porcine pancreatin (P7545) and pepsin (P7000) were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and used without additional purification. Soy oil (food grade, lot: 05 U, Bunge Alimentos, Brazil) was bought from a local market. Deionized water (18.2 MΩ·cm-2, at 25.0 °C; Reference A+, Millipore, USA) was used in all experiments.

2.2. Experimental design, preparation of protein dispersions and ultrasound processing

The mixture standard design, subtype randomized, no blocks, was selected and Simplex Centroid Design (SCD) was adopted, as described in the work by de Moraes Filho et al. (2018), with modifications. Nine mixtures with plant proteins (X1: SPI, X2: PPC and X3: RPI) were evaluated for their physicochemical properties such as molecular mass profile, surface hydrophobicity (H_0), free sulfhydryl groups, ζ-potential and, hydrodynamic diameter (d_h) as well as its dispersibility profile in aqueous medium, water and oil holding capacity, its emulsifying and foaming properties and last gelling concentration. Figure 1 shows the mapping of the design and the proportions of plant proteins used in the SCD. Points A, B and C (vertices of the triangle) are pure preparations (containing only SPI, PPC or RPI), points D, E and F represent binary mixtures of the plant proteins and point G (center of the triangle) is the ternary mixture, with the same mass ratio (1:1:1 – SPI:PPC:RPI) of the three protein materials. Points H and I are repetitions of the center point. Three complete SCD conditions were adopted: no ultrasonic treatment i) control; and with USt of 256.5 kJ (712.5 W | 360 s) over dispersions ii) 1.0% (w/v) or iii) 3.0% (w/v).

(A)



(B)

Mixture	Mass fraction			
	X_1	X_2	X_3	Total fraction
A	1.00	0.00	0.00	1.00
B	0.00	1.00	0.00	1.00
C	0.00	0.00	1.00	1.00
D	0.00	0.50	0.50	1.00
E	0.50	0.00	0.50	1.00
F	0.50	0.50	0.00	1.00
G	0.33	0.33	0.33	1.00
H	0.33	0.33	0.33	1.00
I	0.33	0.33	0.33	1.00

Figure 1. (A) Coded variables of Simplex Centroid Mixture Design (SCD) of X1: Soy Protein Isolate (SPI), X2: Pea Protein Concentrate (PPC) and X3: Rice Protein Isolate (RPI) and, (B) Mass fraction of each experimental design point.

In order to obtain protein dispersions with 1.0% or 3.0% (w/v), SPI, RPI or PPC were weighed according to the proportion shown in Figure 1B, suspended in deionized water, and kept under magnetic stirring (BS-2H, Lab Science, China) at 7.0 ± 0. 5 °C, during 2 h. Systems were subjected to USt of 256.5 kJ (712.5 W | 360 s), based on the optimized results obtained by Omura et al. (2021). The sonication step (20 kHz) was performed using an ultrasound device (Viber Cell 750, Sonics, USA), with a tapped step horn with threaded body $^{1}/_{2}$" (13 mm) in diameter, 5 $^{3}/_{8}$" (136 mm) in length, $^{3}/_{4}$ (340 g) in weight, 10 mL to 250 mL process capacity, and titanium alloy replaceable tip $^{1}/_{2}$" (630-0219, Sonics, USA). 45 mL of protein dispersions were placed into 50 mL Falcon tubes, which were sunk in ice baths to avoid overheating. The USt conditions were chosen based on combinations of the highest dispersibility profile with the lowest rates of foaming capacity and the minors least gelling concentration (LGC) of SPI and PPC, respectively. As the optimization models suggested for RPI by USt in the previous study showed to be not significant, no USt condition was proposed.

After that, all systems USt were frozen at – 80.0 °C and then lyophilized (LP510, Liotop-Liobras, Brazil), aiming to guarantee microbial stability and ensure the non-variability of each protein material in the subsequent analyses, following recommendations found in reports by Qin et al. (2016) and H. Hu et al. (2013). Lyophilized materials were packaged in screw-capped plastic pots and stored under refrigeration (± 7 °C) until use in subsequent experiments (sections 2.3 to 2.5).

Prior to some physicochemical (ζ, d_h, H_0, and free SH groups) and techno-functional (emulsifying and foaming properties) characterizations, pHs of dispersions were adjusted to 7.0, using 0.1 mol·L^{-1} HCl solution. This aimed at standardizing the systems and minimizing influences of ionic strength, since pH values about 7.5 to 9.6 were observed for SPI, RPI and PPC mixtures after USt and resuspension in deionized water.

2.3. Physicochemical characterization of protein dispersions

2.3.1 Molar mass distribution of proteins

Protein dispersions (5.0% w/v) were kept under agitation overnight, and analyzed by the SDS-PAGE technique under reducing conditions, according to the methodology developed by Laemmli (1970). Briefly, a 12.0% (w/v) separation gel and 4.0% (w/v) stacking gel were used with 10 µL of each sample added to 5 µL of sample buffer (0.187 mol·L^{-1} of Tris-HCl-buffer at pH 6.8; 50.0% (v/v) glycerol, 6.0% (w/v) SDS, 1.0% (w/v) bromophenol blue, 15.0% (v/v) β-mercaptoethanol); then, the samples were heated up to 90 °C for 5 min, cooled to 22 ± 1.0 °C, and inserted into the stacking gel spots. 5 µL of 10-245 kDa protein molecular weight marker (TrueColor High Range Protein Marker, Sinapse Inc, Brazil) was used. Electrophoresis was performed at 100 V/220 min. Gels were stained with Coomassie brilliant blue (0.1% (w/v) in 10.0% (w/v) acetic acid and 40.0% (v/v) methanol) under gentle agitation, during 2 h. Then, gels were immersed in a bleaching solution consisting of 10.0% (v/v) acetic acid, 40.0% (v/v) methanol, and 50.0% water, in order to remove the excess of dye from the gels.

2.3.2 Turbidity and dispersibility profiles of proteins in aqueous media

Turbidity analyses of proteins were studied in the pH range from 2.0 to 9.0 with an increment of 1.0 unit, adjusted using 0.1 or 1.0 mol·L^{-1} HCl or NaOH solutions. Briefly, SPI, RPI or PPC dispersions (0.5% w/v) were prepared in deionized water and, kept under magnetic stirring for 4 h at 25.0 ± 1.0 °C. Then, pH was adjusted and measurements were performed at 600 nm (Abs_{600}) on a UV-Vis spectrophotometer (Cary 50, Varian, Australia), at 25.0 ± 1.0 °C. Deionized water was used as blank solution. Absorbance values were expressed as turbidity, using the Eq. 1. Protein dispersions without USt were used as control.

$$\tau = \frac{2{,}303 \cdot Abs_{600}}{l} \quad (1)$$

In Eq. (1), τ is the turbidity, and l is the optical path corresponding to the width of the cuvette.

Also, in each case, the same systems prepared for the protein turbidity analyses were analyzed in terms of their dispersibility profiles, according to the methodology adapted from Ladjal-Ettoumi et al. (2015) and recently used by Oliveira et al. (2020). After adjusting the pH, the systems were centrifuged at 7,000 x *g* for 10 min (Centrifuge 5430, Eppendorf, Germany) and the amount of protein (mg·mL^{-1}) in the supernatant was measured by the Bradford methodology. A standard curve was previously built using BSA (0.000, 0.010, 0.025, 0.100, 0.500, 1.000, 1.250, 1.750, 2.000 and 3.000 mg·mL^{-1}), and absorbances were measured at 595 nm (Mæhre et al., 2018).

2.3.3 Zeta-potential, average hydrodynamic diameter (d_h) and polydispersity index (PDI) of

dispersed protein.

Protein powder was dispersed in 20 mL of deionized water (0.1 mg·mL^{-1}), kept under magnetic stirring (BS-2H, Lab Science, China) at 25.0 ± 0.5 °C, during 4 h. The pH was measured (pH/conductometer 914, Metrohm, Switzerland), and adjusted to 7.0 (using HCl 0.1 mol·L^{-1}). Then, a dispersion was centrifuged at 7,000 x g for 10 min (Centrifuge 5430, Eppendorf, Germany) and the supernatant was analyzed. Samples were placed in a folded capillary cell (DTS1070; Malvern Instruments, United Kingdom), in order to evaluate ζ-potential values of dispersed protein particles (Zetasizer Nano-ZS, Malvern Instruments, United Kingdom), at 25.0 ± 0.1 °C (Soares, Perim, et al., 2019). ζ-potential values were estimated by applying an electric field and by calculating the electrophoretic mobility, from speed and direction of dispersed protein particles. The Smoluchowski model was used for the double electric layer (Bhattacharjee, 2016). Additionally, hydrodynamic average diameter (d_h) and polydispersity index (PDI) for each dispersed protein population were determined and calculated, respectively, using the equipment Zetasizer Nano-ZS, according to the methodology described by Galván et al. (2018); Soares et al. (2019). PDI was estimated from hydrodynamic average diameter (d_h) and standard deviation (SD) relationship for each peak found (Soares, Milião, et al., 2019). The results came from the following apparatus configuration: scattering angle of 173 °; refractive index = 1.330; viscosity = 0.8872 cP. Each reported value came from an average of three repetitions, which performed 3 runs of 30 s per repetition.

2.3.4 Surface hydrophobicity of proteins (H_0)

Surface hydrophobicity index (H_0) of SPI, RPI or PPC mixtures was evaluated using the fluorescent marker 8-anilino-1-naphthalenesulfonic acid (ANS), according to the classical method proposed by Kato & Nakai (1980) with the modifications described by Omura et al. (2021). Dispersions of SPI, RPI or PPC (3.0%, w/v) treated with ultrasound, and dispersions (5.0%, w/v) for control systems (SPI, RPI or PPC without USt) were prepared using phosphate buffer (10 mmol·L^{-1}), kept under stirring at 25 °C, during 1 h. Next, the dispersions were centrifuged at 10,000 x g for 25 min, and the supernatants' protein content was assessed using the Bradford methodology. After that, the protein concentration was adjusted to 0.5 mg·mL^{-1} using the same buffer, aiming at standardizing the concentration of proteins during the analyses from 0.05% to 0.001% (w/v). Then, 6 µL ANS solution (1 mmol·L^{-1}) were added to 300 µL of each protein dispersion, and the resulting mixtures were kept in the dark for 15 min. To ensure that the fluorescence signal actually came from ANS bonded to the exposed hydrophobic groups exposed of protein molecules, the same analyses were performed using

BSA dispersions with the same protein concentrations. In the BSA dispersions, fluorescence signal was proportional to protein concentration, confirming that the fluorescent marker ANS actually bound to proteins. In addition, the value found for the buffer containing ANS was discounted from all analyzed samples. The fluorescence intensity was measured using a luminescence spectrometer (SpectraMax M5, Molecular devices, USA) at 380 nm (excitation) and 460 nm (emission). H_0 index was calculated from the slope of fluorescence intensity (a.u.) as a function of protein concentration ($mg \cdot mL^{-1}$) plot.

2.3.5 Free sulfhydryl groups

The content of free SH groups was determined by the Ellman reagent (5,5-dithiobis-(2-nitrobenzoic acid); DTNB) method, according to the methodology described by Xiong et al., (2018), with the adaptations of de Oliveira et al. (2020). Briefly, Ellman's reagent (4 mg/mL) was prepared by dissolving 40 mg of DTNB in 10 mL of Tris-HCl (86 mM Tris, 90 mM glycine and 4 mM ethylenediamine tetraacetic acid (EDTA), pH 7.0). In addition, 1.0% (w/v) protein dispersions were prepared in the same Tris-HCl buffer, kept under stirring (Magnetic Stirrer BS-2H, Lab Science, China) for 1 h at 25 ± 1.0 °C and centrifuged at 7,100 g for 20 min (Centrifuge 5430 Eppendorf, Germany). The protein concentration in the supernatant was estimated using the Bradford methodology. Then, 80 µL of DTNB were added in 2 mL of this supernatant, which was kept under stirring (Magnetic Stirrer BS-2H, Lab Science, China) for 15 min in the dark and the absorbance was determined at 412 nm. The "blank" was composed of DTNB only in Tris-HCl buffer. The content of free SH groups was calculated as reported by Oliete et al., (2018b), with an extinction coefficient of 13,425 M^{-1} cm^{-1} (T. Xiong et al., 2018; W. Xiong et al., 2016).

2.4 Techno-functional characterization of proteins.

2.4.1 Foaming properties

7.5 mL of 1.0% (w/v) dispersions containing mixtures of SPI, RPI or PPC (pH = 7.0) were placed into graduated cylinder (capacity = 50.0 mL), and then homogenized (Ultra-Turrax T25, IKA-Werke GMBH & CO., Germany) at 10,000 rpm for 2 min during foams preparation (Zhang et al., 2018, with modifications). Foaming capacity (FC) and foaming stability (FS) were calculated by using Equations 2 and 3, respectively:

$$\% FC = \frac{V_o}{7.5} \cdot 100\ \% \quad (2)$$

$$\% FS = \frac{\Delta V}{V_0} \cdot 100\ \% \quad (3)$$

In Eq (2) and (3): V_0 is foam volume at time 0 (zero); ΔV is Volume of foam after 60 min or 1440 min.

2.4.2 Emulsifying properties

1.0% (w/v) dispersions, containing mixtures of SPI, RPI or PPC, were prepared and their pHs were adjusted to 7.0, as described in previous sections. Emulsions consisted of 95% (w/w) protein dispersion (continuous phase) and 5% (w/w) soybean oil (dispersed phase). The emulsification process was carried in two steps, as described by Soares et al (2019) with modifications. Briefly, a pre-emulsification step was performed using an ultra-turrax stirrer (Ultra-Turrax T25, IKA-Werke GMBH & CO., Germany) during 1 min at 10,000 rpm. Subsequently, emulsions were produced using an ultrasonic homogenizer (Viber Cell 750, Sonics, USA), operating at 20 kHz, with a power of 500 W, for 4 min. During the emulsification process, the beakers were sunk in ice baths to prevent overheating. After preparing the emulsion, 10 mL were sampled and transferred to 15 mL falcon tubes, using Pasteur pipettes. The tubes were closed and stored vertically, at 25.0 ± 1.0 °C.

2.4.2.1 Emulsifying Activity Index (EAI) and Emulsifying Stability Index (ESI)

EAI and ESI were determined according to the methodology firstly described by Pearce & Kinsella (1978), with adaptations proposed by Oliveira et al. (2020) and Omura et al. (2021). Briefly, 50 µL of the emulsions were sampled 8 cm above the bottom of the tube, using a micropipette (volume range = 10-100 µL; Eppendorf Research, Germany) and diluted with 0.1% (w/v) of sodium docecyl sulfate (SDS) (1:200) at times 0, 60 and 1440 min after their production. The absorbance of these diluted emulsions was immediately measured at 500 nm in the UV-vis spectrophotometer (Cary 50, Varian, Australia) using plastic cuvette (path length 1 cm).

EAI and ESI were calculated using Equations 5 and 6.

$$EAI\ \left(\frac{m^2}{g}\right) = \frac{2 \cdot 2{,}303 \cdot \mathrm{A_0} \cdot \mathrm{DF}}{C \cdot \varphi \cdot 10000} \quad (4)$$

$$\mathrm{ESI} = \frac{\mathrm{Ao}}{\Delta A} \cdot t \quad (5)$$

In Eq. (4) and (5): A_0 (dimensionless) is the absorbance of diluted emulsion immediately after homogenization; DF (dimensionless) is the dilution factor (200); C is the protein concentration in aqueous phase (0.01 $g.mL^{-1}$) prior to emulsion formation; φ is the oil fraction of the emulsion (0.05); ΔA is the variation in absorbance between 0 and 60 or 1440 min, and; t is the time period (60 or 1440 min).

2.4.2.2 Macroscopic kinetic stability of the emulsions

The creaming index (CI) of the emulsions was evaluated according to the methodology adopted by de Oliveira et al. (2020). The lowest serum height (Hs) and total

emulsion height (Ht) were recorded on days 1, 2, 3, 4, 5, 6, 7, 10, 15, 20, 25 and 30. The ICr was calculated according to Equation 6.

$$ICr\ (\%) = \frac{Hs}{Ht}\ x\ 100\% \quad (6)$$

2.4.2.3 Microstructure of the emulsions

The microstructure of the emulsions was studied according to the methodology described by Soares, Milião, et al. (2019). 5 µL aliquots of each emulsion were sampled 1.5 cm below the surface and inserted into microscope slides, which were covered with glass cover slips. The slide was observed under an optical microscope (CX40, Olympus, USA) with 100x magnification. At least 6 fields of each emulsion-containing slide were recorded using photos taken with a digital camera (48 megapixels). Photomicrographs of each emulsion were taken immediately after preparation and at different storage times (1, 5, 15 and 30 days).

2.4.3 Oil and water holding capacities (OHC and WHC)

OHC and WHC were estimated according to Stone et al. (2015), with some modifications. Briefly, SPI, RPI or PPC were initially weighed using an analytical balance (AUY220, Shimadzu – Japan; accuracy = 10^{-4} g). Then each protein material was mixed with water or with soybean oil. In all cases, 0.05 g of protein material was added to 1.0 g of the respective liquid (oil or water). Subsequently, the mixtures were carefully placed into microtubes and mixed using a vortex stirrer (Phoenix, Tecnal, Brazil) for 10 s, every 5 min for 30 min. After that, they were centrifuged at 12,000 x g for 15 min. The corresponding supernatants (water or oil) were carefully discarded and the solid pellets were weighed again. WHC and OHC were calculated according to Equation 7.

$$\text{WHC or OHC}\ (\%) = \frac{WG_{PI}}{OW_{PI}} \times\ 100\% \quad (7)$$

In Eq. (7), WG_{PI} is the weight gained by each protein material (difference between final and initial weights) and OW_{PI} is their initial weights.

2.4.4. Least gelling concentration (LGC)

Least gelling concentration of mixtures of SPI, RPI or PPC, with or without USt, was evaluated according to the approach reported by Boye et al. (2010). Therefore, adequate amounts of protein mixtures were weighed, in glass vials (8.0 mL), to reach protein concentrations with 10.0; 12.5; 15.0; 17.5, 20.0 % (w/v). In some cases, when 20.0% (w/v) were not enough to develop a gel, 22.5%, 25.0, 27.5, 30.0 or 40.0% (w/v) concentrations were also tested. Then, they were heated up to 95 °C in a thermostatic bath (Phoenix, Thermo Corp., Brazil) for 15 min. Next, the systems were immediately cooled under refrigeration (4 °C)

(Pratice, Consul, Brazil) overnight, to induce gelation. After that, the glass vials were inverted, in order to determine if the dispersions had formed a gel.

2.5 In vitro digestibility

The *in vitro* digestibility of the protein materials was assessed according to the methodology reported by de Oliveira et al. (2020), with modifications. Protein dispersions (3.0% w/v) were prepared in deionized water and kept under magnetic stirring (BS-2H, Lab Science, China) at 25.0 ± 0.5 °C, during 1 h. 2 mL of the protein dispersion were taken as control, and 10 mL were placed into graduated tube (capacity = 50.0 mL), taken to dubnoff metabolic bath (TE-053, Tecnal Brazil), under gentle agitation, until reaching a temperature of 37.0 ± 2.0 °C. The gastric digestion step was started by adjusting the pH to 2.0 with HCl (2.0 mol·L^{-1}). Then, 2.0% w/w (protein base) of pepsin was added to the proteins' dispersions, which were incubated at 37.0 ± 2.0 °C, for 1 h. Subsequently, the material was submitted to the enteric digestion step, adjusting the pH to 7.0 with NaOH (2.0 mol·L^{-1}) and adding 2% w/w (protein base) of pancreatin, followed by incubation at 37.0 ± 2.0 °C for 2h. To interrupt the activity of digestive enzymes, aliquots of the gastric and enteric phases were heated up to 95 °C in a thermostatic bath (Phoenix, Thermo Corp., Brazil) for 5 min. Next, these aliquots were centrifuged at 10,000 g for 10 min at 25 °C (Centrifuge 5430, Eppendorf, Germany), and the supernatant was collected and stored at −20 °C until analyses. Aliquots were analyzed by SDS-PAGE under reducing conditions, as described in section 2.3.1. To ensure that the used digestive enzymes did not interfere with the electrophoretic profile of the protein material, the same conditions of *in vitro* digestion were applied to deionized water.

2.6 Data treatment and statistical analyses.

Regression models were developed to analyze physicochemical (dispersibility, d_h, ζ-potential, H_0, free SH groups – at pH 7.0) and techno-functional properties (WHC, OHC - without pH adjustment, EAI and FC - at pH 7.0) of the mixtures containing SPI, RPI and/ or PPC. The models' adequacy was evaluated in terms of significance (*p-value* ≤ 0.05), lack-of-fit, coefficient of determination (R^2) and residual analyses. Dispersion diagrams were observed, and the best regression curve fitting models were evaluated. We chose to build models with gradual complexity: simple linear, quadratic, special cubic, full cubic and fourth order polynomial. The most suitable model was chosen considering the highest order polynomial, in which the additional terms are significant and the model is not aliased. This procedure was adopted for each protein material treated, or not, with ultrasound. Analyses of variance (ANOVA) were performed to examine the statistical significance of the terms in the regression equations for each dependent variable. After establishing the best model, contour graphics were generated,

using Design Expert software (Version 11, Stat-Ease Inc, Minneapolis, MN). Each response came from at least a triplicate, and the central point was expressed as a mean ± standard deviation of 3 repetitions. For each mixture, the distribution curves of each physicochemical and techno-functional properties, in the three treatments, were compared using the Kruskal-Wallis H test, followed by the post hoc test of Dunn and submitted to Bonferroni adjustment. Statistical analyses were performed using IBM SPSS Statistics Software (Version 25.0, IBM SPSS Inc., Armonk, NY, USA), considering a significance level of 5%.

3. Results and discussion:

3.1 Physicochemical characterization of proteins in aqueous media.

3.1.1 Molar mass distribution of proteins

The molar mass distribution of SPI, RPI or PPC, as pure preparations or as binary or ternary mixtures, with or without USt, under reducing conditions (SDS-PAGE) are shown in Fig. 2.

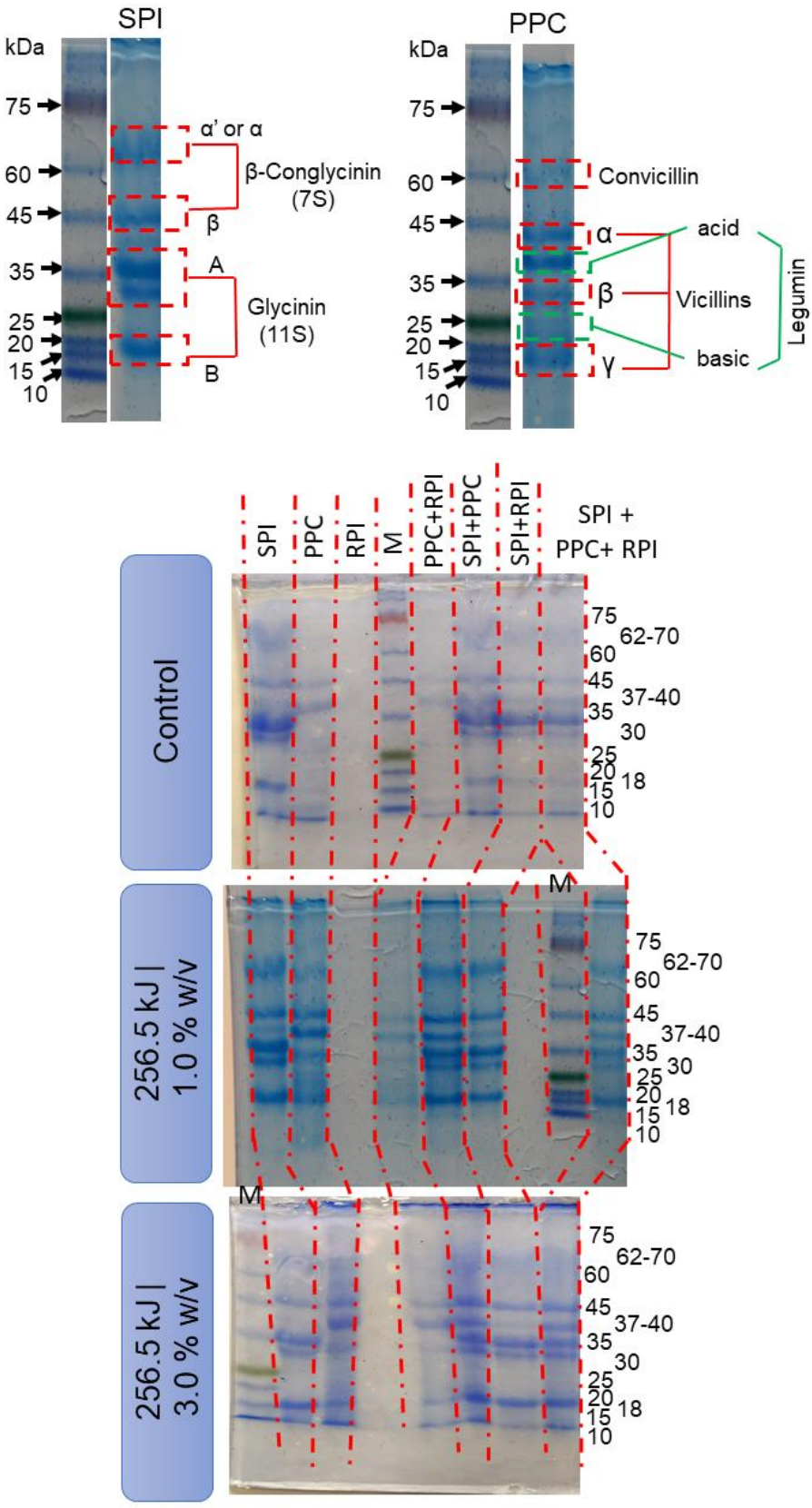


Figure 2. SDS-PAGE pattern of soluble protein fraction under reducing conditions of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture, with or without (control) ultrasonic treatment. M is molecular weight marker and control is without treatment ultrasonic.

In general, the bands present in the gels were similar in the three conditions evaluated (control, 256.5 kJ | 1.0 % or 3.0 % w/v) and they varied between 15 and 70 kDa. The SPI presented four bands, corresponding to the α′ or α (~ 62-70 kDa) and β (~ 45 kDa) subunits of β-conglycinin (7S globulin), along with an acidic (A) (35 kDa) and a basic (B) polypeptide chain (~18 kDa) of glycinin (11S globulin). The identity and molar mass of these bands (glycinin: 20–40 kDa and β-conglycinin: 46 to 80 kDa) are in agreement with the literature (H. Hu et al., 2013; Q. Hu et al., 2021; Liu et al., 2021; Tao Wang, Xu, Chen, Zhou, et al., 2018; Xia et al., 2021; Zhao et al., 2020). On the Other hand, PPC presented 6 bands, which are suggested to correspond to the convicilins (~60 kDa), the acidic (~37 kDa) and the basic (~25 kDa) subunits of legumin, and the α (~40 kDa), β (~30 kDa) and γ (~15 kDa) subunits of vicilins, being also similar to those reported in the literature (Omura et al., 2021; Klost et al., 2020; Tanger et al., 2020). RPI did not present bands in its soluble fraction, neither as a pure preparation, nor as a combined with other proteins (SPI or PPC). RPI as a pure preparation was the only system that did not reach at least 1 $mg \cdot mL^{-1}$ eq. BSA in the supernatant, as suggested in the work by Omura et al., (2021). The impossibility of reaching such concentration is due to its low dispersibility (section 3.1.2). However, the molecular mass profile of the RPI (glutenins) could be observed when this material was subjected to the SDS-PAGE technique using protein dispersion, that is, without centrifugation (Figure 1, SM). Studies involving different USt and plant-based proteins have reported similar results, that is, there was no change in the electrophoretic patterns of proteins due to sonication (Biswas & Sit, 2020; L. Jiang et al., 2014; O'Sullivan, Murray, et al., 2016; Rahman et al., 2020; T. Xiong et al., 2018)

Electrophoresis is commonly used to characterize the primary structures of proteins. Since the pattern of the profiles were similar, under reducing conditions (Figure 2), it is suggested that the proteins in the binary or ternary mixtures, with or without USt, did not interact to form new structures with molar mass between 10 and 245 kDa. Studies of plant protein mixtures with USt are not included in the literature. However, a mixture of pea-rice isolate protein (2:1) through direct steam injection processing (Pietrysiak et al., 2018) and rice proteins with IPS (ratio of 1:01, 1:2 at pH 12) (Tao Wang, Xu, Chen, & Wang, 2018; Tao Wang, Xu, Chen, Zhou, et al., 2018) also found the coexistence of intact protein subunits (similar molecular weight), which reinforce the findings of non-alteration and non-formation of protein structures by protein mixtures. Therefore, other instrumental analyses (upcoming sections) were applied to assess possible structural modifications of the proteins.

3.1.2 Turbidity and dispersibility profiles of proteins in aqueous media

Turbidity and dispersibility data for SPI, RPI, PPC as pure preparations, binary or

ternary mixture with or without USt are presented in Fig. 3.

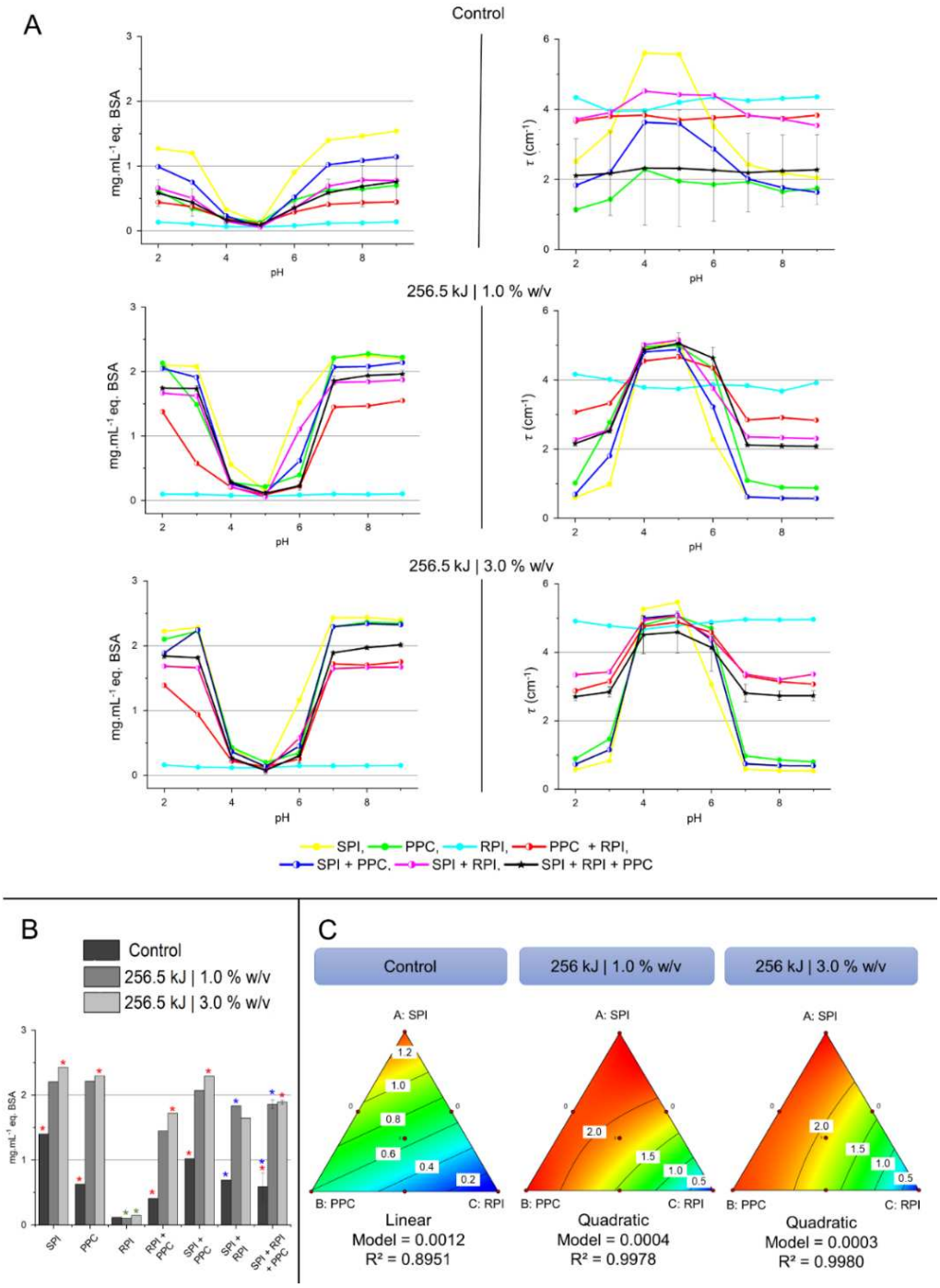


Figure 3. Simplex-centroid mixture design and responses to A: dispersibility (first column; mg·mL^{-1} equivalent to BSA in the supernatant of centrifuged dispersions) and turbidity of dispersions with protein concentration = 0.5 % w/v (second column), as a function of pH (2.0 - 9.0); B: pH 7.0 dispersibility histogram, and C: pH 7.0 dispersibility contour graph of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture, with or without (control) ultrasonic treatment.

* represents statistical differences by the Kruskal-Wallis U test, at 5% significance level, which:
* difference between control and USt of 256.5 kJ | 1.0% w/v;
* difference between control and USt of 256.5 kJ | 3.0% w/v and
* difference between USt of 256.5 kJ | 1.0% w/v and 256.5 kJ | 3.0% w/v

Noticeably, the highest turbidity values agreed with the lowest dispersibility values, within the pH ranged between 3.5 and 5.5, which correspond to the mean values next to the isoelectric point (pI) of the SPI, RPI and PPC (3.8 to 4.9) (de Oliveira et al., 2020; Hou et al., 2017; Omura et al., 2021; Stone et al., 2015; Zhao et al., 2020). This happens because pH values close to the pI of the protein represent a condition in which the net charge of the protein is next to zero; therefore, protein-protein interactions favor aggregation, due to the reduction of intermolecular electrostatic repulsion. Outside this pH range, it was observed that SPI and PPC, alone (USt) or in combination, had the highest dispersibility values, constituting the typical U-shaped curve of plant-proteins (Zhao et al., 2020), indicating that the acidic and alkaline conditions, far from the pI, are more suitable for dispersing such proteins (Avelar et al., 2021) or their mixtures. On the other hand, regardless of pH and USt, RPI had the lowest dispersibility values (<0.2 $mg \cdot mL^{-1}$ eq. BSA). This behavior has already been reported in the literature (Omura et al., 2021; Pietrysiak et al., 2018; Zhang et al., 2018; Zhao et al., 2020), and is related to the high percentage of glutellins in RPI. Glutellins are insoluble in water, due to their extensive aggregation, disulphide bond cross-linking and glycosylation (Day, 2013). For this reason, the presence of this material in a binary or ternary mixture with SPI and/or PPC tended to reduce the dispersing capacity of the mixtures, as it can be seen in Fig. 3.

Different from what was observed in the control, USt systems presented higher dispersibility profiles (≥ 1.5 $mg \cdot mL^{-1}$ eq. BSA) of the proteins at acidic and alkaline pH values. These results indicate the effectiveness of the cavitation phenomenon in affecting the interactions that maintain the native conformation of proteins, increasing the matrix porosity, which induces the formation of micro-fissures and channels that facilitate the interaction with water (Tiwari, 2015). Furthermore, at pH 7.0, the point of maximum dispersibility with little effect of the ionic strength of the materials involved it is noticeable (Fig. 3C) that USt did not only influence the increase in dispersing capacity, but also promoted an interaction effect between the proteins, as the model fit changed from linear to quadratic.

In the control condition (Fig 3C), the observed linear model indicates that the highest dispersibility was achieved when using only the SPI (1.40 $mg \cdot mL^{-1}$ eq. BSA), while the lowest was achieved when there was only the RPI (0.12 $mg \cdot mL^{-1}$ eq. BSA). Upon USt, the RPI remained as the material with the lowest response. Conversely, the suggested quadratic model indicated that, although SPI remains as the mixture that promotes greater dispersibility, the SPI + PPC mixture tends to exert a negative effect on this property.

In terms of statistical differences, with the exception of the RPI, all USt mixtures showed superior protein content in the supernatant (at pH 7.0) when compared to the control condition (Fig. 3B). For the SPI, PPC, RPI + PPC, SPI + PPC and SPI + RPI + PPC mixtures,

such superiority was observed for the USt of 256.5 kJ | 3.0 %, w/v. In the SPI + RPI and SPI + RPI + PPC mixtures, the difference was in relation to USt 256.5 kJ | 1.0 %, w/v. The RPI, even with little influence of pH and USt, revealed a statistical difference between the USt systems (p = 0.027). Thus, the USt in 3.0% w/v dispersions promoted greater dispersibility in protein mixtures. In addition to, in most cases, contemplating greater amounts of soluble proteins, for a proposal for industrial application, it would correspond also to an energy saving.

The impact of USt on the hydration characteristics (solubility/dispersibility) of plant proteins has been studied and the results have been positive, suggesting that the use of US improves this property (Biswas & Sit, 2020; de Oliveira et al., 2020; H. Hu et al., 2013; L. Jiang et al., 2014; Malik et al., 2017; Resendiz-Vazquez et al., 2017) and, consequently, it optimizes its use in food formulations, as a result of the improvements in its functionality. The way that USt improves protein solubility is reported in the literature as an effect of cavitation, in which hydrogen and hydrophobic bonds are broken, reducing the size of protein aggregates, changing the secondary and tertiary structure of proteins, and promoting its partial unfolding in order to increase the surface area that will be in contact with solvent (H. Hu et al., 2013; L. Jiang et al., 2014; Rahman & Lamsal, 2021; Wen et al., 2019). Thus, analyzes of d_h, ζ-potential, H_0 index and free SH groups were performed and are presented and discussed in the following sections.

3.1.3 Average hydrodynamic diameter (d_h), polydispersity index (PDI) and zeta-potential of dispersed protein.

The results from the d_h analysis are shown in Table 1. Since the protein materials are composed by a pool of different proteins fractions, which were dispersed in an aqueous media, Table 1 provides information referring to two populations in diameter (peak 1 + peak 2 = at least 93.63% of the relative abundance of dispersed particles) for more detailed description. Then, a contour graph (Fig. 4A) illustrates the influence that different protein materials bring to the d_h diameter of the most representative population, in this case, peak 1. Fig 4B illustrates the diameter distribution profile (nm) of the dispersed particles in relation to the intensity distribution.

Table 1. Average hydrodynamic diameter (d_h) with its respective population percentage and polydispersity index (PDI) of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment.

Mixture	Protein	Peak	Control			256.5 kJ | 1.0 % w/v			256.5 kJ | 3.0 % w/v		
			d_h (nm)	VD (%)	PDI	d_h (nm)	VD (%)	PDI	d_h (nm)	VD (%)	PDI
1	SPI	1	89.35*	67.97	0.31	220.50	100.00	0.19	232.17*	99.60	0.29
		2	575.80	25.67	0.14	-	-	-	1600.33	0.40	0.01
2	PPC	1	314.10	62.20	0.09	214.73	96.20	0.33	209.33	97.70	0.34
		2	80.58	37.27	0.09	26.68	2.73	0.06	4774.50	1.80	0.02
3	RPI	1	422.20	77.80	0.05	359.70	64.20	0.06	630.47	48.63	0.03
		2	87.42	22.20	0.04	107.05	35.80	0.06	117.60	51.37	0.04
4	PPC + RPI	1	225.00	99.20	0.31	228.90	96.40	0.06	197.57	100.00	0.30
		2	-*	-	-	4871.33**	2.53	0.02	-*	-	-
5	SPI + PPC	1	863.27	75.63	0.08	213.67	100.00	0.22	196.90	100.00	0.19
		2	61.64**	24.37	0.05	-*	-	-	-*	-	-
6	SPI + RPI	1	161.57*	98.87	0.16	235.37*	94.40	0.24	211.20	100.00	0.37
		2	5313.00	1.70	0.01	37.92	5.1	0.06	-	-	-
7	SPI + PPC + RPI □	1	213.50 ± 50.25	92.22 ± 5.88	0.36 ± 0.18	233.67 ± 21.71	98.46 ± 0.48	0.37 ± 0.06	212.52 ± 7.4	99.52 ± 0.84	0.27±0.07
		2	33.47* ± 4.02	6.43 ± 2.22	0.06 ± 0.01	5016.83* ± 154.06	2.18 ± 0.71	0.01 ± 0.00	-**	-	-

256.5 kJ: ultrasound treatment (712.5 W | 360 s). DC: dispersion concentration, VD: volume distribution;
□ average of a central point, referring to an average value of 3 repetitions. The other systems are averages from triplicates.
* represents statistical differences by the Kruskal-Wallis U test at a 5% significance level, which:
* = difference between control and USt of 256.5 kJ | 1.0% w/v;
* = difference between control and USt of 256.5 kJ | 3.0% w/v and;
* = difference between USt of 256.5 kJ | 1.0% w/v and 256.5 kJ | 3.0% w/v.

The largest d_h was verified in the control system of the binary mixture between SPI and RPI (peak 2 = 5313.00 nm) (Table 1). The smallest was peak 2 of PPC in the condition 256.5 kJ | 1.0 % w/v (26.68 nm). In both cases, the contribution in the population percentage was small (< 2.73%). The highest d_h with the greatest contribution in terms of volume distribution was the mixture between SPI and PPC in the control condition (peak 1 = 863.27 nm, VD = 75.63%). However, when the system SPI + PPC was submitted to the USt, the lowest representative d_h of the conditions 256.5 kJ | 1.0 % w/v (213.67 nm) and 256.5 kJ | 3.0 % w/v (196.9 nm) was verified. Considering that the highest PDI value observed in the three experimental conditions was 0.37 (SPI + PPC + RPI at 256.5 kJ | 1.0 % w/v and SPI + RPI 256.5 kJ | 3.0 % w/v), it can be inferred that the populations presented low polydispersity.

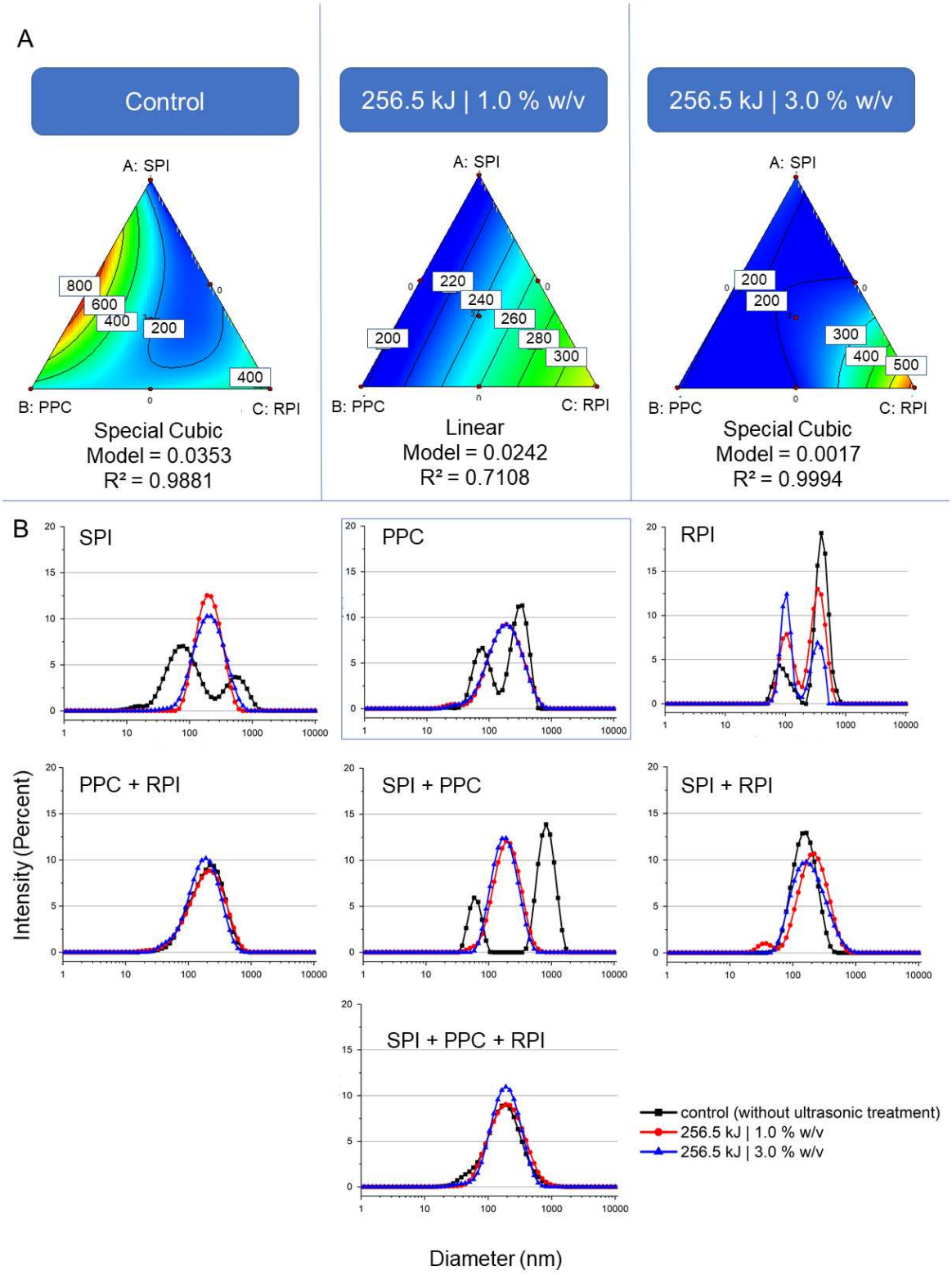


Figure 4. Simplex-centroid mixture design and the responses to A: average hydrodynamic diameter (d_h) of peak 1 and B: diameter distribution by intensity as a function of particle size for soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment.

A significant increase in d_h of peak 1 after USt was observed only in two situations: i) when SPI, as a pure preparation, went from 89.35 nm (control) to 232.17 nm (256.5 kJ | 3.0 % w/v); and ii) the SPI + RPI mixture went from 151.57 nm (control) to 235.37 nm (256.5 kJ | 3.0 % w/v). In these situations, however, when observing the dispersibility profile (Fig. 3) at pH 7.0, after USt, there was an increase in the amount of protein dispersed in the supernatant and a reduction in turbidity. These results show that despite the increase in d_h in these systems, these aggregates are more dispersible than in their initial condition (control). The statistical differences observed in peak 2 were due to the absence of this population in some of the conditions of applied treatment (PPC + RPI, SPI + PPC and SPI + PPC + RPI).

Although not significant, an increase in d_h after USt of 256.5 kJ | 1.0 % w/v was observed in peak 1 of SPI, PPC + RPI and SPI + PPC + RPI, and at peak 2 for RPI and SPI + PPC + RPI. In USt of 256.5 kJ | 3.0 % w/v, the increase in d_h was observed at peak 1 of RPI and SPI + RPI and peak 2 of SPI, PPC and RPI. With exception of RPI, in these systems, an increase in dispersibility and a reduction in turbidity were also observed. H. Hu et al. (2013) verified through microstructure analysis that SPI formed larger aggregates upon applying a total energy input of 180, 360, 540, 720 or 1080 kJ (20 kHz, power: 200, 400 or 600 W and time: 15 or 30 min) over SPI (> 90.0% protein) due to the exposure of free SH groups that reacted with themselves (forming disulphide bonds) or oxidized. L. Jiang et al. (2014) also found particle sizes greater than the control after applying a total energy input of 108, 216, 432, 324 or 648 kJ (20 kHz, power: 150, 300 or 450 W and time: 12 or 24 min) in black bean protein isolate (84.72% protein), and they attributed this result to turbulent ultrasound forces, which increase the speed of collision and aggregation, resulting in the formation of unstable aggregates through non-covalent interactions. Despite the increase in size, in both studies, an increase in protein solubility was observed. In other situations, the USt promoted a reduction in the d_h of protein mixtures.

Fig. 4A shows the most representative responses of d_h (peak 1). As a pure preparation, the RPI represents the material with the highest d_h in the three evaluated treatments. Considering that the USt 256.5 kJ | 1.0 % w/v was fitted to the linear model, it can be concluded that the RPI contributes to the increase in d_h, while the PPC contributes to the reduction, without significant influence of the binary and ternary mixtures. In the control conditions, the adjustment to the special cubic model showed that the ternary mixture promotes the most reduction in d_h, and that the binary mixtures SPI + RPI and PPC + RPI also contribute to this behavior. However, the SPI + PPC mixture

leads to the formation of large agglomerates (~800 nm). The USt 256.5 kJ | 3.0 % w/v showed that the ternary mixture contributes to the increase in d_h, while all binary mixtures contribute to the decrease. Therefore, it can be concluded that the USt, in general, tends to reduce the d_h of protein aggregates. The reduction of plant-protein size by USt were also found for soy protein isolate/ concentrate (Jambrak et al., 2009), pea (de Oliveira et al., 2020; T. Xiong et al., 2018), wheat (O'Sullivan, Murray, et al., 2016), walnut (Zhu et al., 2018), and sunflower (Malik et al., 2017).

In addition to promoting the reduction of d_h, the USt directly contributed to a greater uniformity of proteins dispersed in aqueous media, since, according to the distribution profile (Fig. 4B), the bimodal behavior of the SPI, PPC and SPI + PPC systems, observed in the control condition, became predominantly unimodal. Although the RPI as a pure preparation has presented two populations, when it was combined with SPI or PPC, it tended to have an almost unimodal behavior. It is noteworthy that, considering the low dispersibility of RPI in aqueous media (Fig. 3), the contribution of this material in the distribution profile with other materials tends to be underestimated. Opposite behavior was verified in the work of L. Jiang et al. (2014), because regardless of the USt applied, the black bean protein isolate changed from unimodal to bimodal distribution. However, it is noteworthy that in addition to being a protein material different from the present study, their USt was applied in a dispersion 10.0% w/v, and the abundance of protein molecules along the USt may have promoted protein-protein interactions that developed this bimodal distribution.

The results of the ζ-potential are shown in Fig. 5. All systems presented negative values, since the analysis was performed at pH 7.0, above the pI of the three materials studied (SPI, PPC and RPI). The highest density of negative charges was observed in the control system involving SPI and PPC (ζ = -39.67 mV). The lowest was the RPI (ζ = -20.93 mV) submitted to the USt of 256.5 kJ | 3.0 % w/v.

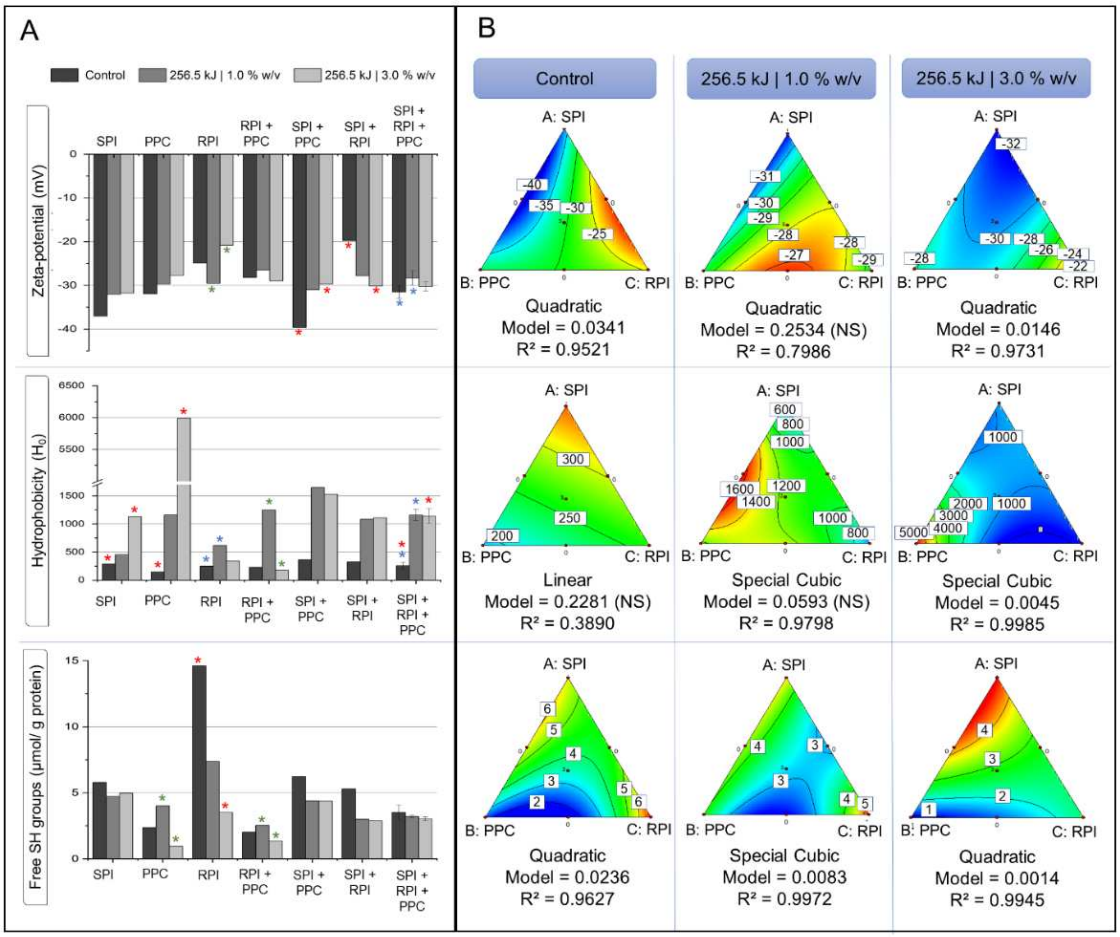


Figure 5. Simplex-centroid mixture design and the responses to zeta-potential, hydrophobicity surface (H_0) and free sulfhydryl groups (SH) of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment, presented as A: histogram, and B: contour graph.
* represents statistical differences by the Kruskal-Wallis test at a 5% significance level, which:
* difference between control and USt of 256.5 kJ | 1.0% w/v;
* difference between control and USt of 256.5 kJ | 3.0% w/v and
* difference between USt of 256.5 kJ | 1.0% w/v and 256.5 kJ | 3.0% w/v

It was found that the systems containing SPI and PPC showed negative charge densities on protein surfaces lower than the control after USt (SPI, PPC, SPI + PPC and SPI + PPC + RPI mixtures). The differences presented by SPI and PPC, as pure mixtures, were not significant as those verified in the SPI + PPC (control and USt of 256.5 kJ | 3.0% w/v) and SPI + PPC + RPI (control and the USt at 256.5 kJ | 1.0% w/v) mixtures. The minimization of negative charges on the surface of SPI, PPC and SPI + PPC mixtures after USt indicates a reduction of electrostatic repulsion, which allowed protein-protein interactions and helped standardize the d_h of the proteins dispersed in an aqueous medium, changing their distribution from bimodal to unimodal (Fig. 4B).

The presence of RPI in the mixtures contributed to changes in the ζ-potential of the systems in a distinct way. Alone, it showed a statistical difference between the two USt. The greater ζ-potential of RPI' treated at 256.5 kJ | 1.0 % w/v suggested a more pronounced effect of ultrasonic waves in the diluted dispersions. This observation is reinforced by the lowest d_h of RPI in their peaks 1 and 2, shown in Table 1. Finally, the statistical difference observed for SPI + RPI mixture between the control and USt 256.5 kJ | 3.0 % w/v suggest that, the conformational changes triggered by the USt, despite promoting an increase in the d_h of protein aggregates (Table 1), exposed groups with negative surface charges, which may have directly contributed to the increase in the dispersibility of the mixture (from 0.69 mg·mL^{-1} eq. BSA of the control, to 1.65 mg·mL^{-1} eq. BSA, Fig. 3).

3.1.4 Surface hydrophobicity of proteins (H_0)

The results for the H_0 index are shown in Fig 5. Except for the mixture between RPI + PPC USt at 256.5 kJ | 3.0 % w/v, the USt promoted an increase in H_0 index. The increase in surface hydrophobicity due to USt has been verified in several plant-based protein materials (de Oliveira et al., 2020; H. Hu et al., 2015; L. Jiang et al., 2014; Malik et al., 2017; Omura et al., 2021; Tong Wang et al., 2021). In this study, a significant increase was observed for SPI, RPI and PPC as pure preparations; as well as, in the ternary mixture (Fig. 5A). In the case of SPI and PPC, the statistical difference was observed between the control and the USt of 256.5 kJ | 3.0% w/v, whereas for the RPI, it happened between the control and USt at 256.5 kJ | 1.0% w/v. In the ternary mixture (SPI + RPI + PPC), both USt were statistically different from the control. In the case of SPI, PPC and SPI + RPI + PPC mixtures, the statistical differences found between the treatments were equal to those verified in the dispersibility at pH 7.0 (Fig. 5). Similar behavior was verified in the work of H. Hu et al., (2013), in which they applied a total energy input of 180, 360, 540, 720 or 1080 kJ (20 kHz, varying power 200, 400 or 600 W and time 15 or 30 min) over SPI (> 90.0% protein) and found that both the hydrophobicity and the solubility of the protein were directly proportional to the applied energy.

The RPI + PPC mixture, in addition to being the only system that presented H_0 index lower than the control after USt at 256.5 kJ | 3.0% w/v, also showed to be the only system that did not present a significant difference between the USt. The higher value observed in this binary mixture after USt at 256.5 kJ | 1.0% w/v may be a result of the more pronounced effect of cavitation on the more dilute dispersion. In the 3.0% dispersion, the presence of more protein molecules along the USt may have promoted

the unfolding of the protein molecules, extended their chains, and facilitated the intermolecular interactions among them. As result, protein aggregates were formed, protecting the hydrophobic region from the surrounding polar environment, possibly within a disulfide network. (L. Jiang et al., 2014; Rahman & Lamsal, 2021). The more pronounced effect of cavitation at the dispersions containing 1.0% w/v or protein may have been the reason for the statistical difference observed for the RPI.

The linear model suggested for the control condition shows that the SPI contributes to the increase of the H_0 index, while the PPC decreases (Fig 5B). The USt promoted conformational changes so that the proposed model fit was suggested as special cubic, that is, the binary and ternary mixtures influenced the response of the H_0 index in a complex way. At USt 256.5 kJ | 1.0% w/v, the SPI + PPC mixture promoted high hydrophobicity, while the ternary mixture tends to decrease it. Now for USt 256.5 kJ | 3.0% w/v, the ternary mixture contributed the most to the increase in the H_0 index, and the SPI + PPC and PPC + RPI mixtures tend to decrease this index.

Despite the low dispersibility observed for the RPI, both in the control condition and after USt (Fig 3A), the little influence against the H_0 index is suggested by the favoring of protein-protein interactions that turn towards the interior of the protein, exposing less hydrophobic groups on its surface. Despite the formation of RPI protein aggregates after USt, it could be observed that the application of USt in 1.0% w/v dispersion, in addition to promoting a statistical difference in the H_0 index compared to the control, also presented d_h (Table 1) lower than those verified in the RPI' USt in dispersion at 3.0% w/v, indicating that the USt in more diluted dispersions in the RPI, can be a useful strategy in improving its applicability in the food industries.

The increase in the H_0 index promoted by the use of USt indicates that cavitation triggered conformational changes in the proteins in order to expose hydrophobic clusters that were previously buried in their molecular core (native conformation). These changes could be fitted to the special cubic model significantly in the USt applied at 3.0% w/v dispersions. The quadratic model was better adjusted for the ζ-potential for this same treatment condition. These results show greater reliability in predicting H_0 index and ζ-potential when USt is applied in 3.0% w/v dispersions than in 1.0% w/v.

3.1.5 Free sulfhydryl groups

The content of free -SH groups are also shown in Fig 5. Among the pure mixtures, RPI represents the material with the highest content of free SH groups (control = 14.6 μmol/ g protein), while PPC contains the lowest content (256.5 kJ | 3.0% w/v = 0.95 μmol/ g protein). Although this analysis refers to the content of free SH groups on

the surface of the protein, this result is directly associated with the content of sulfur amino acids (methionine/cysteine), in which cereals contain more of these amino acids, while for legumes, they are limiting (Jiménez-Munoz et al., 2021).

Different from the behavior observed in H_0 index, the conformational changes promoted by USt tended to decrease the content of free -SH groups, except for PPC and PPC + RPI USt at 256.5 kJ | 1.0 % w/v. For these exceptions, statistical differences (p < 0.05) were observed between the USt applied, in which dispersions prepared at 1.0% w/v when receiving the USt, in addition to having increased free SH content, compared to the control, were statistically superior to at 256.5 kJ | 3.0 % w/v. This increase in the content of free SH groups is a result of the more pronounced effect of cavitational pressure and shear of the USt in the more diluted dispersions, which exposed these groups that were previously found inside the protein, breaking the disulphide bonds and reducing the SS for the formation of the SH groups. Similar behavior was verified in different plant-based protein materials USt, such as soy protein isolate (90% protein), 20 kHz, 0 W, 200 W, 400 W, 600 W for 15 min or 30 min (180-1080 kJ) (H. Hu et al., 2013); pea protein isolate (85% protein, dry basis) USt at 68 W/100 mL for 5 min (20,4 kJ) (S. Jiang et al., 2017); rice proteins (90% protein, wet base) under different alkaline conditions and USt at 19.3 W/cm^2 (20 kHz, 600 W, 60 min = 2160 kJ) (Zhang et al., 2018), isolated protein extracted from bran of dephenolated sunflower, USt with probe (20 kHz, 58-61 W/ cm^2, 150 - 900 kJ), or by bath (40 kHz, < 0.5 W/ cm^2, 150 - 900 kJ) (Malik et al., 2017).

For the other systems, in which a decrease in free -SH content was observed, it is possible that intra and intermolecular structural rearrangements occurred, reducing the presence of sulfhydryl groups on the surface of the proteins. Furthermore, since amino acids can crosslinking within or between protein molecules, during USt, disulphide bond between the sulfur-containing amino acids may have happened, promoting a polymerization phenomenon of the protein (Corredig et al., 2020). In addition to polymerization, it is known that when water is used as a solvent during sonication, it tends to decompose into its hydroxyl radicals and hydrogen atoms, and may react with each other, forming hydrogen peroxide and molecular hydrogen, which is capable of oxidizing SH groups (Rahman et al., 2020; Rahman & Lamsal, 2021). When this oxidation occurs, the content of free SH groups decreases. Between the control and the USt at 256.5 kJ | 3.0% w/v, the only system in which there was a significantly (p < 0.05) decrease in the free SH groups content was in the RPI. This indicates that free SH groups reductions were due to the oxidation, conjointly with the effect of polymerization, which resulted in

higher d_h aggregates (630.47 nm, table 1), leading to structural rearrangement that buried the free SH groups inside the protein. A decrease in the content of free SH groups was also observed for SPI and its subunits (7S and 11S) after USt of 20 kHz at 45 - 180 kJ (Rahman et al., 2020) or 120-960 kJ (H. Hu et al., 2015), and these authors justify these results due to the oxidation of free sulfhydryl groups by free radicals, mainly hydroxyl.

For control systems (Fig. 5B), the RPI represents the protein with the greatest contribution to the content of free SH. After, being mixed with PPC or SPI, some interactions occurred in a way to decrease this index. As with the control, the USt of 256.5 kJ | 3.0% w/v was fitted to the quadratic model. Again, the SPI + RPI and RPI + PPC mixtures reduced the content of free SH, but it was the SPI who contributed the most to the increase in these groups. The USt of 256.5 kJ | 1.0% w/v, adjusted for the special cubic model, showed that the ternary mixture and the SPI expose more free sulfhydryl groups on the surface, while the same binary mixtures that involved the RPI decrease this exposure.

Our results for physicochemical properties (dispersibility, d_h, ζ-potential, H_0 index and free SH) of the systems were evaluated through adequacy of the models (*p-value* ≤ 0.05, lack of adjustment, R^2 and residual analysis). The analyses of these properties suggest that USt improves the uniformity of the proteinaceous materials, either as pure preparations or binary or ternary mixtures. Significant *p-values* with high R^2 were observed more frequently in the USt of 256.5 kJ applied over dispersions prepared at 3.0% w/v. These findings indicate that USt in more dilute dispersions appears to receive a more pronounced effect from cavitation, whose conformational changes more complex. Therefore, in physicochemical terms, the application of USt in 3.0% w/v dispersions is more interesting for the current demand for plant protein blends.

3.2 Techno-functional characterization of proteins.

The effect of mixtures between SPI, RPI and PPC, with or without USt, in relation to its dispersibility, interfacial properties (foam capacity and emulsifying activity), and oil (OHC) or water (WHC) holding capacity, are presented in Table 2. The discussion of dispersibility is presented in section 3.1.2 to introduce and study the effects of aggregation and denaturation over a pH range of 2.0 - 9.0, and contextualizes them with the following topics relating to d_h, ζ-potential, H_0 and free SH groups at pH 7.0. Contour graphs of the adjusted model (Table 2) are presented and discussed throughout the following sections, together with histograms that provide raw data information and

statistical comparisons between treatments (control, 256.5 kJ | 1.0 % w/v and 256.5 kJ | 3.0% w/v). The least gelling concentration was also evaluated, but in a qualitative character (section 3.2.4).

Table 2. Descriptive statistics, model, and regression model for techno-functional properties of SPI, RPI and PPC as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment.

Test	Treatment	Min.	Max.	Mean	Std. Dev.	Model	F-value	p-value	Regression models	R^2	Lack of fit
Dispersibility (pH 7.0)	Control	0.1182	1.3987	0.6554	0.3763	Linear	25.61	0.0012	$Dispersibility_{control} = +1.32 \cdot S + 0.59 \cdot P + 0.05 \cdot R$	0.8951	0.7289*
	256.5kJ | 1.0% w/v	0.1013	2.2138	1.72	0.6509	Quadratic	266.55	0.0004	$Dispersibility_{256.5kJ \mid 1.0\% w/v} = +2.21 \cdot S + 2.21 \cdot P + 0.10 \cdot R - 0.57 \cdot S \cdot P + 2.72 \cdot S \cdot R + 1.16 \cdot P \cdot R$	0.9978	0.9701*
	256.5kJ | 3.0% w/v	0.1481	2.43	1.80	0.6778	Quadratic	294.57	0.0003	$Dispersibility_{256.5kJ \mid 3.0\% w/v} = +2.44 \cdot S + 2.30 \cdot P + 0.16 \cdot R - 0.46 \cdot S \cdot P + 1.25 \cdot S \cdot R + 1.81 \cdot P \cdot R$	0.9980	0.0896*
FC (pH 7.0)	Control	193.33	524.44	432.96	100.42	Quadratic	9.84	0.0444	$FC_{control} = +463.89 \cdot S + 449.44 \cdot P + 187.22 \cdot R - 271.11 \cdot S \cdot P + 706.67 \cdot S \cdot R + 584.44 \cdot P \cdot R$	0.9425	0.2072*
	256.5kJ | 1.0% w/v	323.49	600.00	491.01	85.54	Quadratic	11.01	0.0381	$FC_{256.5kJ \mid 1.0\% w/v} = +471.55 \cdot S + 424.88 \cdot P + 321.71 \cdot R + 13.43 \cdot S \cdot P + 762.00 \cdot S \cdot R + 535.33 \cdot P \cdot R$	0.9483	0.7138*
	256.5kJ | 3.0% w/v	189.84	600.00	450.23	127.32	Quadratic	11.02	0.0380	$FC_{256.5kJ \mid 3.0\% w/v} = +310.95 \cdot S + 470.96 \cdot P + 187.46 \cdot R + 563.09 \cdot S \cdot P + 996.75 \cdot S \cdot R + 401.19 \cdot P \cdot R$	0.9484	0.7435*
EAI (pH 7.0)	Control	55.69	135.32	109.42	30.73	Special Cubic	528.18	0.0019	$EAI_{control} = +103.61 \cdot S + 122.33 \cdot P + 55.69 \cdot R - 11.57 \cdot S \cdot P + 201.61 \cdot S \cdot R - 108.66 \cdot P \cdot R + 831.92 \cdot S \cdot P \cdot R$	0.9994	
	256.5kJ | 1.0% w/v	54.83	149.05	123.31	29.69	Quadratic	33.39	0.0078	$EAI_{256.5kJ \mid 1.0\% w/v} = +103.2 \cdot S + 147.88 \cdot P + 53.66 \cdot R + 13.79 \cdot S \cdot P + 248.22 \cdot S \cdot R + 73.31 \cdot P \cdot R$	0.9923	0.0721*
	256.5kJ | 3.0% w/v	59.04	155.63	114.36	30.06	Special Cubic	159.91	0.0062	$EAI_{256.5kJ \mid 3.0\% w/v} = +116.38 \cdot S + 155.63 \cdot P + 59.04 \cdot R + 51.63 \cdot S \cdot P + 22.25 \cdot S \cdot R - 71.61 \cdot P \cdot R + 313.06 \cdot S \cdot P \cdot R$	0.9979	
OHC	Control	145.96	196.79	161.61	14.93	Quadratic	30.80	0.0088	$OHC_{control} = +196.65 \cdot S + 163.91 \cdot P + 145.82 \cdot R - 68.35 \cdot S \cdot P - 18.71 \cdot S \cdot R - 23.82 \cdot P \cdot R$	0.9809	0.7978*
	256.5kJ | 1.0% w/v	349.77	1264.84	1029.88	278.43	Quadratic	16.38	0.0218	$OHC_{256.5kJ \mid 1.0\% w/v} = +1099.59 \cdot S + 1263.99 \cdot P + 348.92 \cdot R + 134.43 \cdot S \cdot P + 1392.80 \cdot S \cdot P + 412.28 \cdot P \cdot R$	0.9647	0.9493*
	256.5kJ | 3.0% w/v	316.50	1056.18	612.19	217.59	Linear	16.28	0.0038	$OHC_{256.5kJ \mid 3.0\% w/v} = +979.06 \cdot S + 593.01 \cdot P + 264.48 \cdot R$	0.8444	0.5686*
WHC	Control	229.72	835.67	607.93	178.63	Linear	24.74	0.0013	$WHC_{control} = +834.59 \cdot S + 723.80 \cdot P + 265.40 \cdot R$	0.8918	0.6533*
	256.5kJ | 1.0% w/v	47.73	333.76	141.72	84.05	Quadratic	63.82	0.0030	$WHC_{256.5kJ \mid 1.0\% w/v} = +79.83 \cdot S + 115.56 \cdot P + 334.96 \cdot R - 219.06 \cdot S \cdot P - 157.35 \cdot S \cdot R - 164.22 \cdot P \cdot R$	0.9907	0.5375*
	256.5kJ | 3.0% w/v	120.48	508.71	235.17	131.68	Quadratic	35.95	0.0070	$WHC_{256.5kJ \mid 3.0\% w/v} = +143.50 \cdot S + 511.65 \cdot P + 370.82 \cdot R - 760.75 \cdot S \cdot P - 297.17 \cdot S \cdot R - 590.19 \cdot P \cdot R$	0.9836	0.4562*

FC: Foam capacity; EAI: Emulsifying Activity Index; OHC: Oil Holding Capacity; and WHC: Water Holding Capacity. S: Soy Protein Isolate; R: Rice Protein Isolate and P: Pea Protein Concentrate.

* not significant

All the techno-functional properties of the evaluated protein mixtures were adjusted for linear (dispersibility$_{control}$, OHC$_{256.5\ kJ\ |\ 3.0\%\ w/v}$ and WHC$_{256.5\ kJ\ |\ 1.0\%\ w/v}$), quadratic (Dispersibility$_{256.5\ kJ\ |\ 1.0\%\ w/v}$, Dispersibility $_{256.5\ kJ\ |\ 3.0\%\ w/v}$, FC$_{control}$, FC$_{256.5\ kJ\ |\ 1.0\%\ w/v}$, FC$_{256.5\ kJ\ |\ 3.0\%\ w/v}$, EAI$_{256.5\ kJ\ |\ 1.0\%\ w/v}$, OHC$_{control}$, OHC$_{256.5\ kJ\ |\ 1.0\%\ w/v}$, WHC$_{256.5\ kJ\ |\ 1.0\%\ w/v}$, WHC$_{256.5\ kJ\ |\ 1.0\%\ w/v}$) or special-cubic models (EAI$_{control}$, EAI$_{256.5\ kJ\ |\ 1.0\%\ w/v}$), revealing that, when mixed (binary or ternary), the protein interacted at a molecular level, changing their structures, as previously stated, and affecting the dependent variables.

3.2.1 Foaming properties

The foaming capacity (FC) and stability (FS) of the proteins, alone or in association after 1 or 24h, are shown in Figure 6.

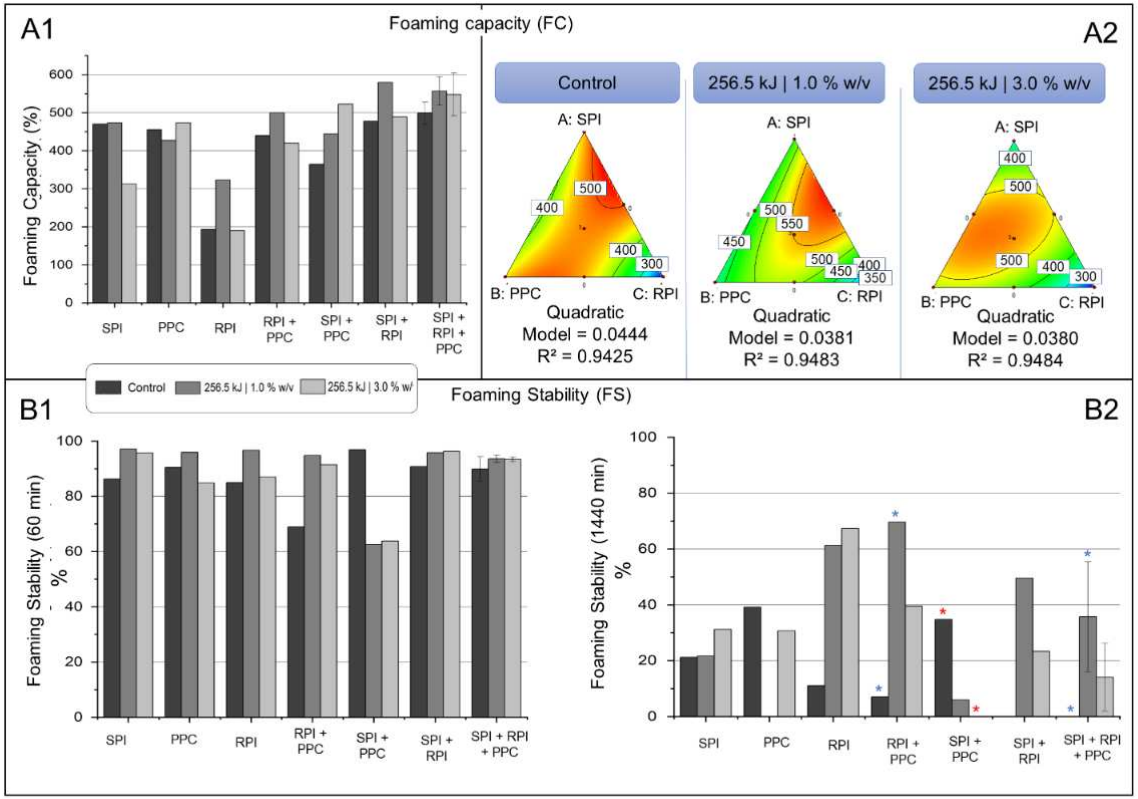


Figure 6. Simplex-centroid mixture design and the responses to foaming capacity (FC), presented as A1: histogram, and A2: contour graph; and foaming stability (FS), presented as B1 after 60 min and B2 after 1440 min, of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment, at pH 7.0.
* represents statistical differences by the Kruskal-Wallis test at a 5% significance level, which:
* difference between control and USt of 256.5 kJ | 1.0% w/v;
* difference between control and USt of 256.5 kJ | 3.0% w/v and
* difference between USt of 256.5 kJ | 1.0% w/v and 256.5 kJ | 3.0% w/v

Noticeably, the USt did not promote statistical differences in the FC of the proteins (Fig. 6 - A1). However, with the exception of PPC as a pure preparation, the USt applied in 1.0 % (w/v) dispersions increased the FC of the mixtures. As for 3.0% w/v dispersions, the increase was only verified in the mixtures of PPC (473.33%), SPI + PPC (522.22%), SPI + RPI (488.89%) and SPI + PPC + RPI (548.15%). In these mixtures, it can be inferred that the USt promoted structural changes in the proteins, which favored their ability to diffuse to the air/water interface and form a resistant film, which incorporated greater amounts of the dispersed phase (air). Among the main events related to the promotion of the diffusion performance to the interface of these mixtures is the increase of the hydrophobic surface (Fig. 5). When exposed to the gas phase, hydrophobic regions reduce the interfacial tension and may develop a viscoelastic film at the air-liquid interface through intermolecular interactions (T. Xiong et al., 2018). The correlation between the increase in hydrophobic surface area with FC, due to USt was reported in pea protein isolate (T. Xiong et al., 2018), tamarind seed protein isolate (Biswas & Sit, 2020), sunflower protein isolate (Malik et al., 2017), and jackfruit seed protein isolate (Resendiz-Vazquez et al., 2017). In this current research, RPI + PPC mixture treated with USt 256.5 kJ | 3.0% w/v was the only one in which there was a decrease in the H_0 index, which justifies the observed decrease in its FC. Conversely, for the SPI and RPI mixtures, USt 256.5 kJ | 3.0 % w/v showed increased H_0 indexes, and reduced FC values. This observation may be attributed to the increase in the d_h of these mixtures (Table 1), since larger protein aggregates tend to presents less flexibility, which makes their adsorption at interfaces more difficult. The PPC USt 256.5 kJ | 1.0% w/v, had its d_h reduced, hydrophobicity and dispersibility increased, but these conformational changes somehow developed protein aggregates whose surface characteristics reduced their ability to adsorb at the air/water interface.

The FC contour graphs (Fig. 6 - A2) showed that the quadratic model was well adjusted for the three conditions evaluated. Noticeably, RPI systems tended to decrease the FC, however, when combined with SPI (SPI + RPI), this binary mixture was the one that most contributed to the foam increase, both in the control and after the USt. The protein composites produced by Tao Wang, Xu, Chen, Zhou, et al. (2018) with a mixture of rice proteins (RP) and SPI, adjusting pH to 12, followed by its neutralization to pH 7.0, showed similar results. The RP showed lower indices of FC and FS, but when it interacted with SPI to form the soluble composites, these indices were greatly increased at pH values from 4.0 to 8.0. These authors reported that RPs and SPIs unfolded, interacted and formed complex structures at pH 12.0, maintained after neutralization, resulting in a

water-stable hydrophilic compound with increased amphiphilic properties. Thus, reinforcing the findings that the three conditions fit the quadratic model, it is suggested that the joint dispersion of binary mixtures leads to the formation of protein composites with increased dispersibility characteristics and interfacial properties.

The low contribution of the RPI in FC may be related its low dispersibility and, higher d_h values, even after USt, revealing greater resistance to structural alteration, which resulted in rigid structures that make it difficult to reduce interfacial tension for the foam formation (Gharibzahedi & Smith, 2020). The need for a strong treatment to break the inter/intramolecular interactions in rice protein aggregates to induce improvements in their functionality are shown in the work by Zhang et al. (2018). They observed that rice proteins (~90.0% protein) promoted an increase in FC after an ultrasound-assisted alkali treatment (0.04, 0.06, 0.08 and 1.0 M NaOH), whose total energy input was 2,160 kJ (20 kHz, 600 W for 60 min), that is, more than 8 times the energy value used in the present work.

FS reports a percentage ratio of the remaining foam volume to the initial foam volume, after a certain period of time. In this work, after 60 min of foam production (Fig. 6 - B1) there were no statistical differences for FS, as well as for FC, between treatments for protein mixtures. Similarly, Malik et al. (2017) did not observe statistical differences in FS of foams produced with sunflower protein isolate dispersions treated with USt, (total energy input ranging from 150 to 900 kJ) after 30 min of foam production. Additionally, it was noted that all systems maintained more than 60.0 % of the foam after 60 min and that the USt increased the FS of the mixtures: SPI, RPI, RPI + PPC, SPI + RPI and SPI + RPI + PPC. The increase in FS, due to USt was also observed in 1.0% dispersion of rice proteins (Zhang et al., 2018) and 2.0% dispersion jackfruit seed protein isolate (Resendiz-Vazquez et al., 2017) after 30 min, or after 60 min in 10.0% dispersion SPI (Jambrak et al., 2009) and 3.0% dispersion of tamarind seed protein isolate (Biswas & Sit, 2020). One day after its production, FS_{1440} min results showed that the foam was totally undone in the SPI + RPI and SPI + RPI + PPC control, 256.5 kJ USt | 1.0 % w/v PPC and 256.5 kJ USt | 3.0 % w/v of SPI + PPC. It was also observed that the RPI, despite having low FC, showed high stability after USt, both in the pure preparation form and in the mixture containing SPI and/or PPC. Such results suggest that despite the lower adsorption effectiveness at the interface, systems containing RPI may have formed a firmer film, which best prevents coalescence.

3.2.2 Emulsifying properties

Results of the activity index (EAI) and stability index (ESI) of SPI, RPI and PPC as pure preparation or in a binary or ternary mixture, with or without USt are shown in Figure 7. Besides, the contour surface of EAI, for each treatment, is highlighted. The microscopic aspect of the emulsions immediately after their production and after 5, 15 and 30 days, are shown in Figure 8.

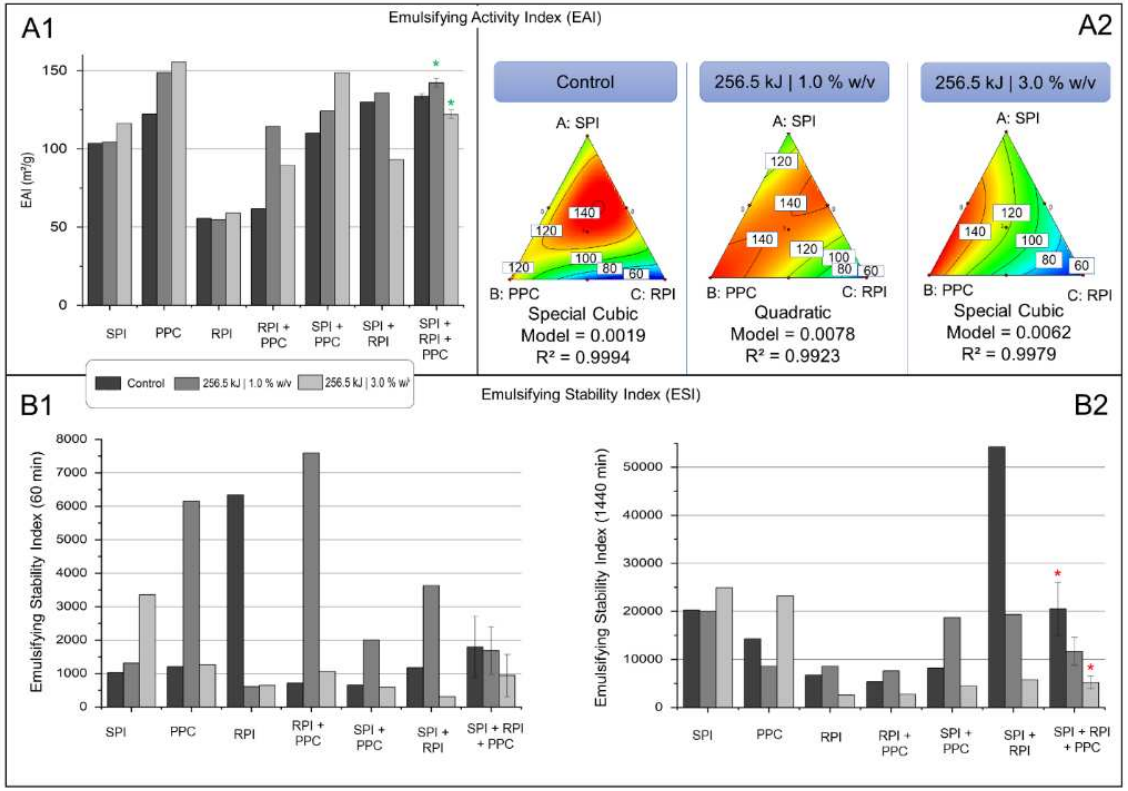


Figure 7. Simplex-centroid mixture design and the responses to emulsifying activity index (EAI), presented as A1: histogram, A2: contour graph; and emulsifying stability index (ESI) after 60 min (B1) or 1440 min (B2) of the emulsions of soy protein isolate (SPI)), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment, at pH 7.0.
* represents statistical differences by the Kruskal-Wallis test at a 5% significance level, which:
* difference between control and USt of 256.5 kJ | 1.0% w/v;
* difference between control and USt of 256.5 kJ | 3.0% w/v and
* difference between USt of 256.5 kJ | 1.0% w/v and 256.5 kJ | 3.0% w/v

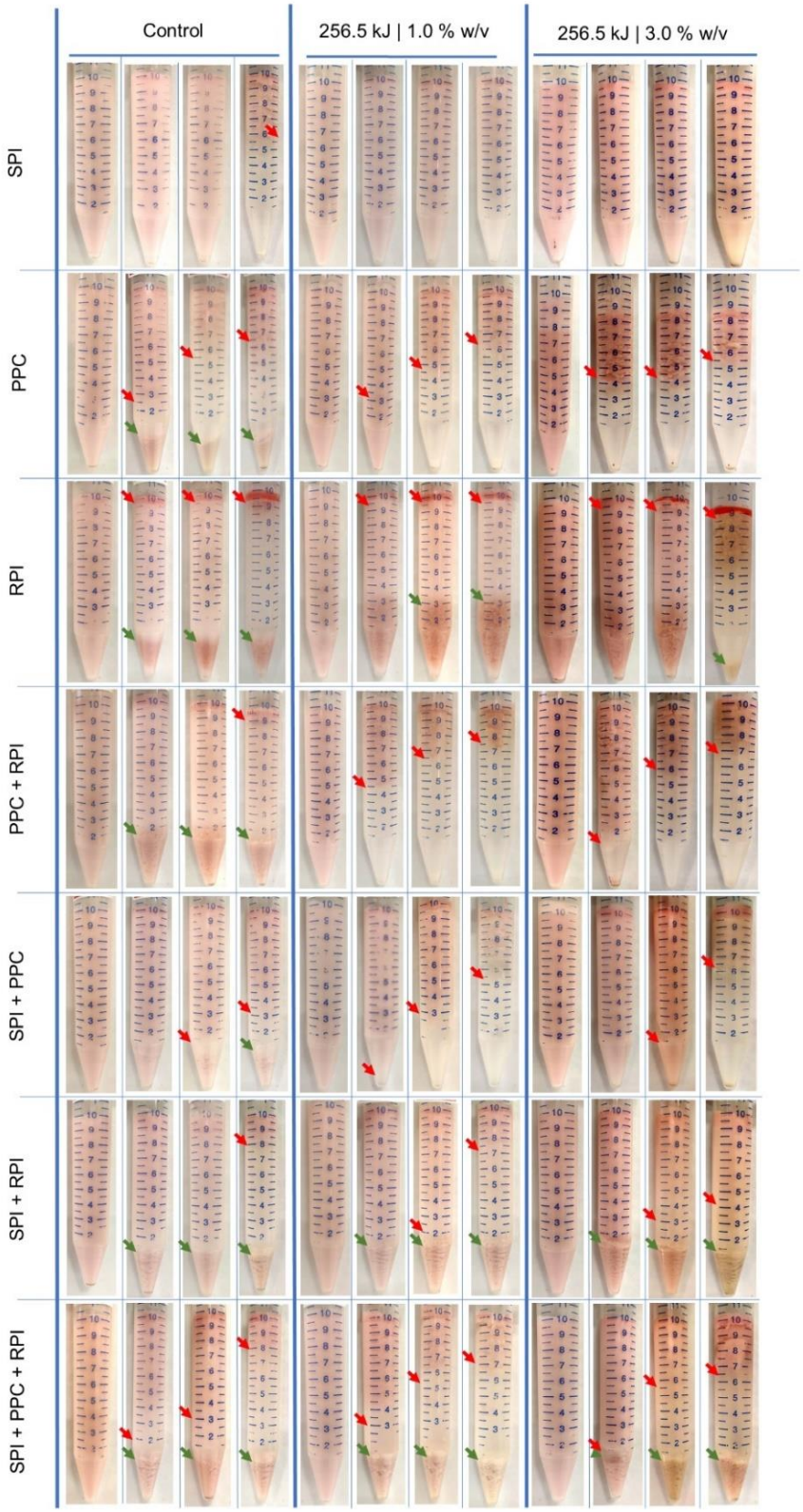


Figure 8. Simplex-centroid mixture design and the responses to visual appearance of emulsions with soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment, at pH 7.0, during 30 days of storage.
Red arrows indicate serum height, and green arrows show the biopolymers precipitation.

The EAI refers to the relationship between the oil/water interface area that can be covered per unit of mass of a surfactant, in the case of this study, the proteins. Thus, high EAI values represent a greater ability of the protein to organize, interact and create a film at the oil/water interface (David Julian McClements, 2004). Similar to the behavior observed in FC, the RPI also presented the lowest EAI values with (256.5 kJ | 1.0 % w/v = 54.83 and 256.5 kJ | 3.0% w/v = 59.04 m²/ g) or without (control = 55.7 m²/ g) USt, proving the low adsorption performance at both the air/water (Fig. 6) and oil/ water interfaces (Fig. 7 – A1). The limited capacity of the RPI to diffused to the oil-water interface and form a rigid film to coat oil droplets, even after the ultrasonic cavitation, is illustrated by photomicrographs (Fig. 8), in which it is observed the presence of larger drop sizes since its preparation (time 0). The control and USt of 256.5 kJ | 3.0 % w/v, revealed a significant influence of the ternary mixture on the EAI (Fig. 7 – A2). Additionally, in the control condition, the ternary mixture showed the highest EAI (133.73 m²/ g), followed by the SPI + RPI (130.06 m²/ g) and PPC (122.33 m²/ g) mixtures. Such results, highlights that the simple combination of SPI, PPC and RPI may be a promising strategy for products that demand high emulsifying capacity.

In relation to SPI, PPC and RPI as pure mixtures, the USt applied to dispersions at 3.0% (w/v) tended to develop higher EAI than that of dispersions prepared at 1.0 % (w/v). In binary mixtures, this behavior was observed only for SPI + PPC. For the other binary (RPI + PPC and SPI + RPI) and ternary (SPI + PPC + RPI) mixtures, the USt of 256.5 kJ | 1.0 % (w/v) showed higher EAI than in those at 256.5 kJ | 3.0% (w/v), and in the ternary mixture, this difference was significant. Both USt condition increased the EAI in the SPI, PPC, RPI + PPC and SPI + PPC mixtures, compared to the control. The increase in EAI in protein mixtures after USt is also related to the increase in dispersing capacity and surface hydrophobicity, along with the decrease in protein d_h. Together, the effect of USt increased the adsorption of proteins (alone or a mixture) at the oil-water interface. Other plant-based proteins researchers report EAI improvements by using USt through turbidimetric analysis. For example, for soy (Jambrak et al., 2009), pea (de Oliveira et al., 2020), rice (Zhang et al., 2018) and walnut (Zhu et al., 2018).

A decrease in EAI after USt of 256.5 kJ | 3.0% w/v was observed in the SPI + RPI and SPI + RPI + PPC mixtures. The RPI USt at 256.5 kJ | 1.0% w/v also had its EAI reduced, but the decrease was subtle. The reduction of EAI in these systems suggests that the complex interaction effect of the special cubic model of these mixtures, promoted a type of aggregation between protein molecules, favoured by the increase of hydrophobic groups on the surface, in order to impair the orientation and adsorption of

proteins on the interface, so that it was possible to visualize the presence of slightly larger droplets in the photomicrographs at time 0 (Fig. 8). Effects of protein aggregation promoted by USt that decrease emulsifying activity were also observed for materials/ proteins under severe conditions of potency or pH of jackfruit protein isolate (69.62% protein) (Resendiz-Vazquez et al., 2017) or time for sunflower protein isolate (Malik et al., 2017).

Knowing that emulsions do not have thermodynamic stability, stable emulsion is the one that manages to avoid the separation of oil and water phases for a longer time. Among the processes that lead to phase separation of emulsions are: cream formation, flocculation and coalescence, which can occur individually or in combination (David Julian McClements, 2004). In this work, we aim to illustrate the immediate destabilization of system by means of turbidimetric analysis (ESI) at times 60 and 1440 min after emulsion production (Fig. 7-B1 and B2, respectively), and to evaluate emulsion destabilization mechanisms through macroscopic aspects (Fig. 8), microscopic (Fig. 9-A) and creaming index (Fig 9-B), immediately after its production and after 1, 5, 15 and 30 days.

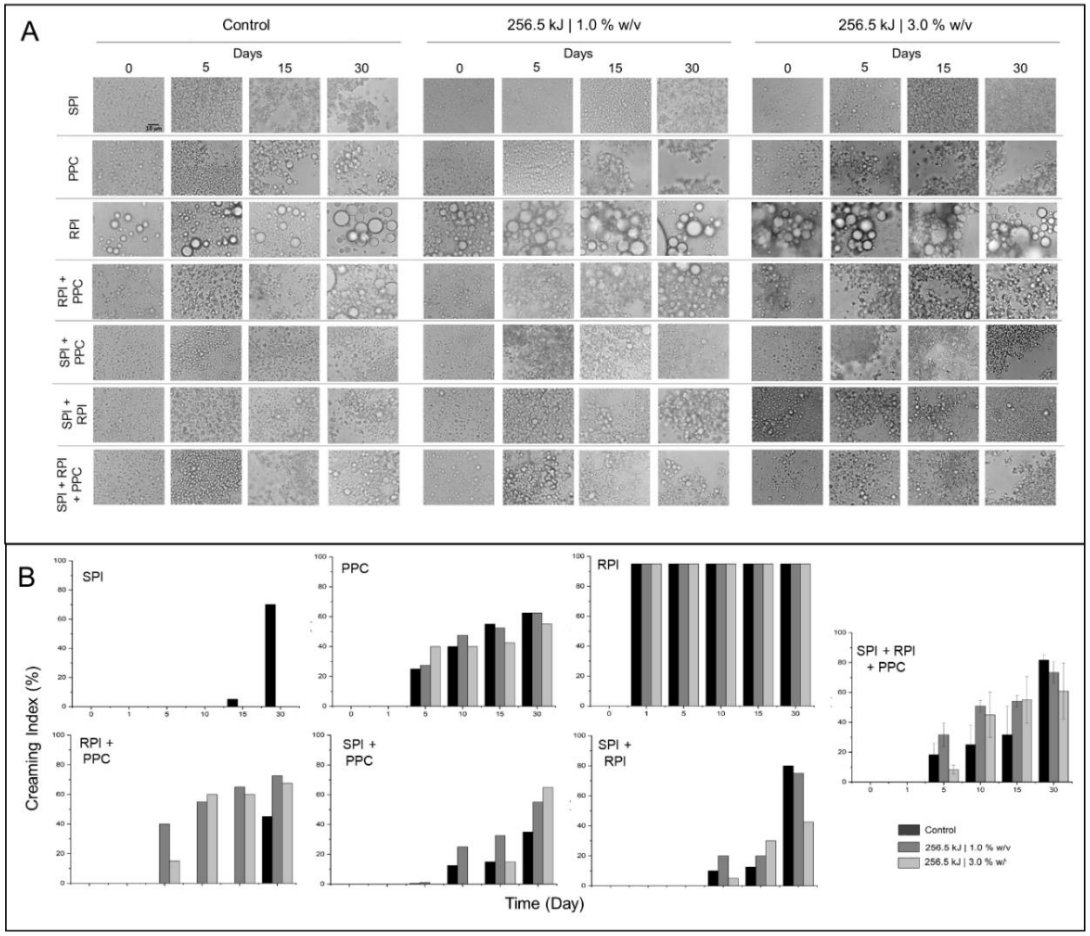


Figure 9. Simplex-centroid mixture design and the responses to optical microscopy (A) and creaming index (B) of the emulsions of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment, at pH 7.0, during 30 days of storage.

The ESI is the ratio between the absorbance observed immediately after its production and the change in absorbance observed after a given time, multiplied by the time in question. Thus, the higher the value, the greater its ability to prevent phase separation. When evaluated the ESI after 60 min (Fig. 7 - B1), it is observed that the RPI without USt, PPC and RPI + PPC USt at 256.5 kJ | 1.0% w/v showed the highest ESI, but their indices do not represent differences significant in relation to the other treatments applied. Furthermore, USt was shown to better minimize the instability of emulsions in SPI, PPC, RPI + PPC, SPI + PPC mixtures, but it harmed RPI and SPI + RPI + PPC. Similarly, Zhu et al. (2018) found that USt (total energy input of 180, 360, 540, 720 or 1080 kJ) improved the stability of emulsions (20.0% oil) prepared with walnut protein isolate (80.32% protein) 10 min after its production, justifying being a joint effect of the reduction of the formed droplets or changes in the surface chemistry of the surface of the lipid droplets that altered the repulsive and attractive interactions between the droplets. Resendiz-Vazquez et al. (2017) also found an improvement in the stability of emulsions (50.0% oil) produced with jackfruit seed protein isolate (69.62% protein) treated with ultrasound after 30 min, alleging improvements in the orientation of proteins at the interface due to the turbulent effect caused by USt. These are some examples found in the literature that report the beneficial effect of USt on ESI of plant-proteins. Nonetheless, a decline in the stability of the emulsion (25.0% oil) produced with rice protein (90.0% protein) after USt was observed in the work by Zhang et al. (2018) after 15 min. In this study, when we observed the presence of more and larger droplets in the RPI photomicrographs (Fig. 9-A), the decrease in the stability of the rice protein emulsion after USt is associated with a weaker interfacial layer, thus allowing a greater degree of coalescence.

After 1440 min (1 day), the SPI + RPI mixture in the control condition showed the highest ESI values (54,271.42 min), followed by SPI (24,947.04 min) and PPC (23,225.73 min) in the USt 256.5 kJ / 3.0% w/v. The only statistical difference was observed in the ternary mixture between control and USt at 256.5 kJ / 3.0% w/v. Although the USt improves the EAI of most mixtures, the ESI1440 min showed that although the USt increases the diffusion and adsorption efficiency at the oil-water interface, the film developed by the smaller and more flexible protein is not always effective in preventing destabilization of the emulsions. Improvements in the kinetic stability of emulsions prepared with proteins treated with USt were also reported by Jambrak et al. (2009) using 3.0% dispersion of soy protein concentrate and isolate after 24 hours, and they attributed this event to the better orientation of protein at the oil-water interface after

these receive the turbulent effect produced by the USt.

It is observed that the studies carried out with the ESI available in the literature comprise different protocols, in terms of oiled phase percentage, homogenization condition, different times used for the evaluation of the stability and others. Furthermore, discussions around this index are very similar to those proposed for EAI, but they do not address the possible mechanisms of emulsion destabilization. Thus, below, we will discuss the evaluation of the creamation index (CI), together with the macro and microscopic aspects up to 30 days of storage at 25 ± 2 °C.

Immediately after the production of the emulsions (time 0), the macroscopic aspect (Fig 8-A) of all mixtures in the three conditions of applied treatments was uniform, without any signs of destabilization, which is why the CI was considered to be zero (Fig. 9-B). However, in microscopic terms, observed that the RPI droplets are much larger than those of the other systems, comprising different sizes and even aggregation in the USt of 256.5 kJ | 3.0% w/v, while the SPI presented with smaller and uniform droplets.

SPI, as a pure preparation, constituted the material with the best emulsifying performance, since, after USt, its macroscopic appearance was uniform throughout the 30 days of storage (without cream formation) and, in the control condition, only after 15 days of storage the emulsion began to show signs of destabilization, presenting in the 30-day photomicrograph aspects of droplet aggregation. The PPC in the control condition had a gradual increase in its CI from the 5th day of storage, as well as a precipitation of proteins at the bottom of the tube. This behavior may be the result of the combined effect of the lower water dispersibility with the low value found in the H_0 index, since after the USt, this precipitation was absent. The RPI one day after its manufacture already showed oil displacement on the tube surface, illustrating the phase separation, confirming its lower emulsifying capacity. Furthermore, in the control condition (day 5), USt 256.5 kJ | 1.0% w/v (day 15) and 256.5 kJ | 3.0% w/v (day 30), protein precipitation at the bottom of the tube was also observed, reinforcing the hypothesis of the combined effect of lower dispersibility with low H_0 indices.

The SPI + PPC mixture started to show signs of creaming after 5 days. For SPI, the mixture effect reduced its stability, but for PPC improvements were observed. This behavior was also observed in the SPI + RPI mixture, with improvements for the RPI and loss for the SPI. In addition, precipitation at the bottom of the tube was observed after 5 days and creaming on day 10. Finally, in the ternary mixture, destabilization of the emulsion by creaming and protein precipitation were observed from day 5, with the CI increasing until day 30. The USt of 256.5 kJ | 3.0% w/v applied to this ternary mixture

proved to be less unstable.

Considering that the aliquot for microscopy analysis was removed 2 cm from the top of the emulsion, the mechanism of destabilization was promoted at least by creaming. The result of destabilization could be observed in photomicrographs by the presence of larger droplets, mainly on day 30 and all times and treatments of the RPI. From day 5 onwards, it was also possible to verify aggregation of the droplets of the mixtures in USt 256.5 kJ | 3.0% w/v and in the control condition (in SPI, on day 15). At USt 256.5 kJ | 1.0% w/v, in most cases, aggregation was more evident from day 15 onwards. The precipitation observed at the bottom of the tubes also indicates the destabilization mechanism of the emulsions by depletion, being mainly present in the emulsions produced in the presence of the RPI. This may be the result of an effect of the gravitational force on the creaming, since the protein structure could neither efficiently adsorb itself at the interface nor interact well with the water.

When observing the positive effect of USt on EAI and ESI, associated with the evidence that the destabilization mechanisms of the all emulsions of binary and ternary mixtures start around day 5, it can be inferred that these mixtures are very promising for use in the powdered formulated food industry, considering that in addition to ensuring a more complete supply of essential amino acids, this type of food prepared at home is for immediate consumption, but if a few days' storage is necessary, it is likely that it will be able to maintain its stability for at least one day.

3.2.3 Oil and water holding capacities (OHC and WHC)

Oil and water holding capacities data for SPI, RPI, PPC as pure preparations, binary or ternary mixture with or without USt are presented in figure 10.

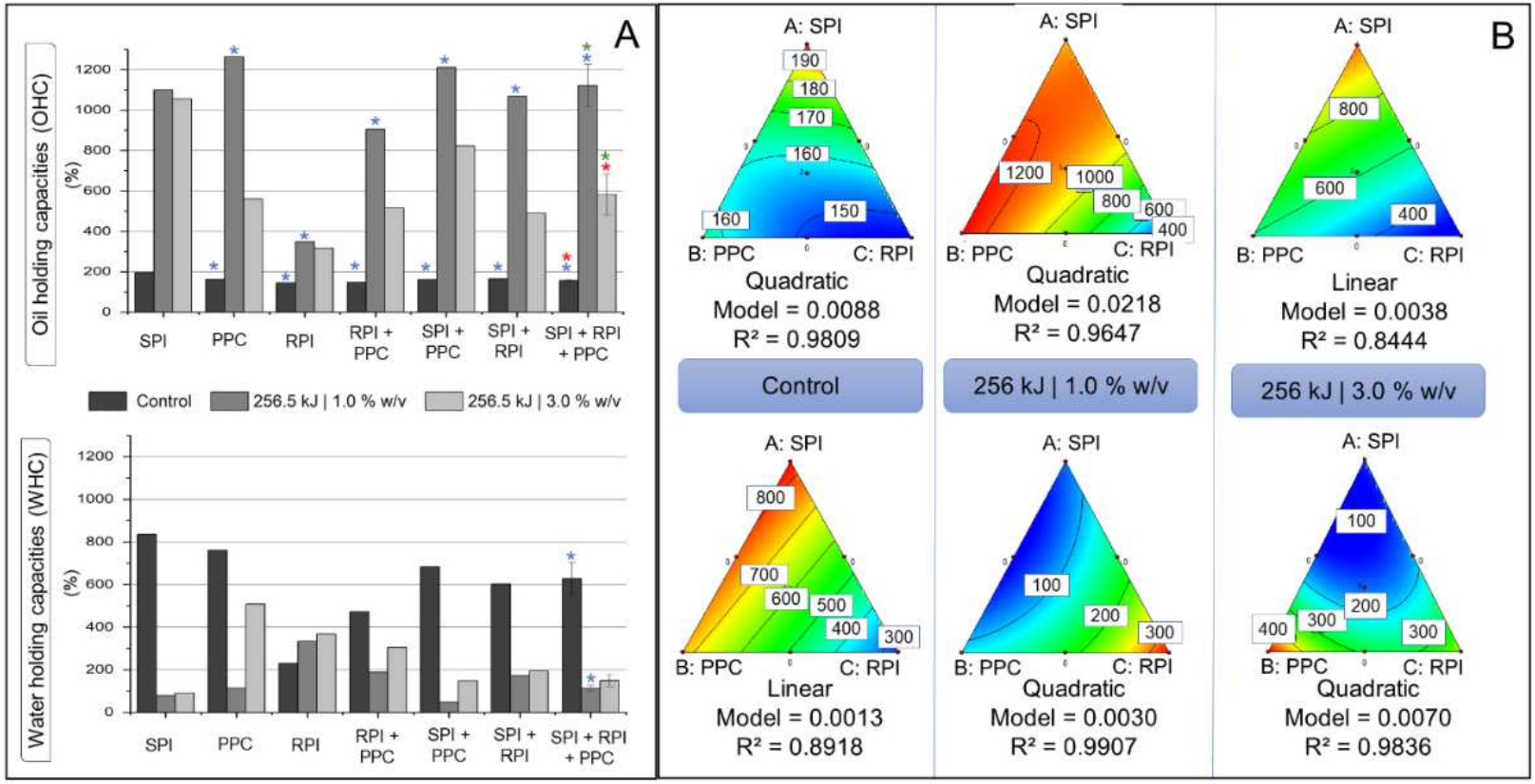


Figure 10 1Simplex-centroid mixture design and the responses to oil holding capacities (OHC) and water holding capacities (WHC) of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment, presented as A: histogram, and B: contour graph.

* represents statistical differences by the Kruskal-Wallis test at a 5% significance level, which:

* difference between control and USt of 256.5 kJ | 1.0% w/v;

* difference between control and USt of 256.5 kJ | 3.0% w/v and

* difference between USt of 256.5 kJ | 1.0% w/v and 256.5 kJ | 3.0% w/v

In the control condition (without USt), the OHC values ranged from 145.96% (RPI) to 196.79% (SPI), revealing little variation between the systems. Binary and ternary mixtures also showed OHC with little variation among them. However, the adjustment to the quadratic model showed that there was an interaction effect between the proteins, with the SPI being the one that contributed the most to this parameter, whereas the binary mixtures, had a negative effect. Zhao et al. (2020), reported similar results in relation to proteins from soy, pea, rice and wheat, as all proteins showed similar OHC values.

It is noteworthy that USt promoted an increase in OHC in all systems, showing a greater magnitude in systems prepared with 1.0% (w/v) than those with 3.0% (w/v) (Fig 10. A). Except for SPI, statistical differences were observed for all mixtures, comparing the control with the USt of 256.5 kJ | 1.0 % w/v. In the ternary mixture, all three treatments showed statistical differences among them, with the USt of 256.5 kJ | 1.0 % w/v presenting the highest OHC. Again, the RPI had the lowest technical-functional performance in the three conditions tested. PPC and SPI as pure preparation were mainly responsible for the increase in OHC under conditions 256.5 kJ | 1.0% (w/v) and 256.5 kJ | 3.0% (w/v), respectively. At USt 256.5 kJ | 1.0% (w/v), mixtures were also fitted to the quadratic model, indicating the positive influence of binary mixtures on the OHC, which was not observed for the 256.5 kJ | 3.0% (w/v) systems, since they were fitted to the linear model. Therefore, it was verified that the USt of 256.5 kJ | 1.0% (w/v) allowed to reach higher OHC values than 256.5 kJ | 3.0% (w/v). However, the prediction in this one is easier to obtain, since higher values are obtained with more SPI, and the smaller ones, having more RPI.

The increase in OHC in all mixtures, after USt, indicates that the collapse of cavitation bubbles near the protein molecules was strong enough to promote conformational changes in the protein structures, expose the non-polar hydrophobic amino acids side, increase their binding capacity to oil molecules. The greater exposure of hydrophobic patches on the proteins, after USt was confirmed by the increase in the H_0 index (Fig. 5).

Regarding the WHC, in the control condition, SPI showed the highest water retention capacity (835.67%), while the RPI showed the lowest (229.72%). Since the WHC variable was adjusted to the linear model, it can be inferred the direct contribution of these proteins in predicting this property.

Although the USt increased the OHC, the consequence of the greater exposure of hydrophobic groups on the surface of the protein mixtures was the reduction in the

WHC. With the exception of the RPI, the USt promoted a reduction in the WHC of the protein mixtures and, in all systems, the USt in the 3.0% (w/v) dispersions decreased less than the WHC than at 1.0 % (w/v). Although the RPI had the lowest techno-functional performance for FC, EAI and OHC, for WHC after USt at 256.5 kJ | 1.0% (w/v), represents the protein that most contributes to this property, while in USt at 256.5 kJ | 3.0% (w/v), was the PPC. Considering the adjustment to the quadratic model of both USt, it was observed that the interaction effect in the binary mixtures somehow minimized the WHC of the protein mixtures.

The effect of USt in promoting an increase in OHC and, consequently, a decrease in WHC, was also reported in canola protein isolate (Flores-Jiménez et al., 2019), sunflower protein isolate (Malik et al., 2017) and jackfruit protein isolate (Resendiz-Vazquez et al., 2017), which attributed this behavior to alterations in protein structure after USt, exposing hydrophobic groups, which interact with hydrocarbon chains in fat molecules, resulting in better oil adsorption.

3.2.4 Least gelling concentration

The amount of SPI, RPI, PPC as pure preparations, binary or ternary mixture, with or without USt, needed to build a firm gel network are shown in Fig. 11.

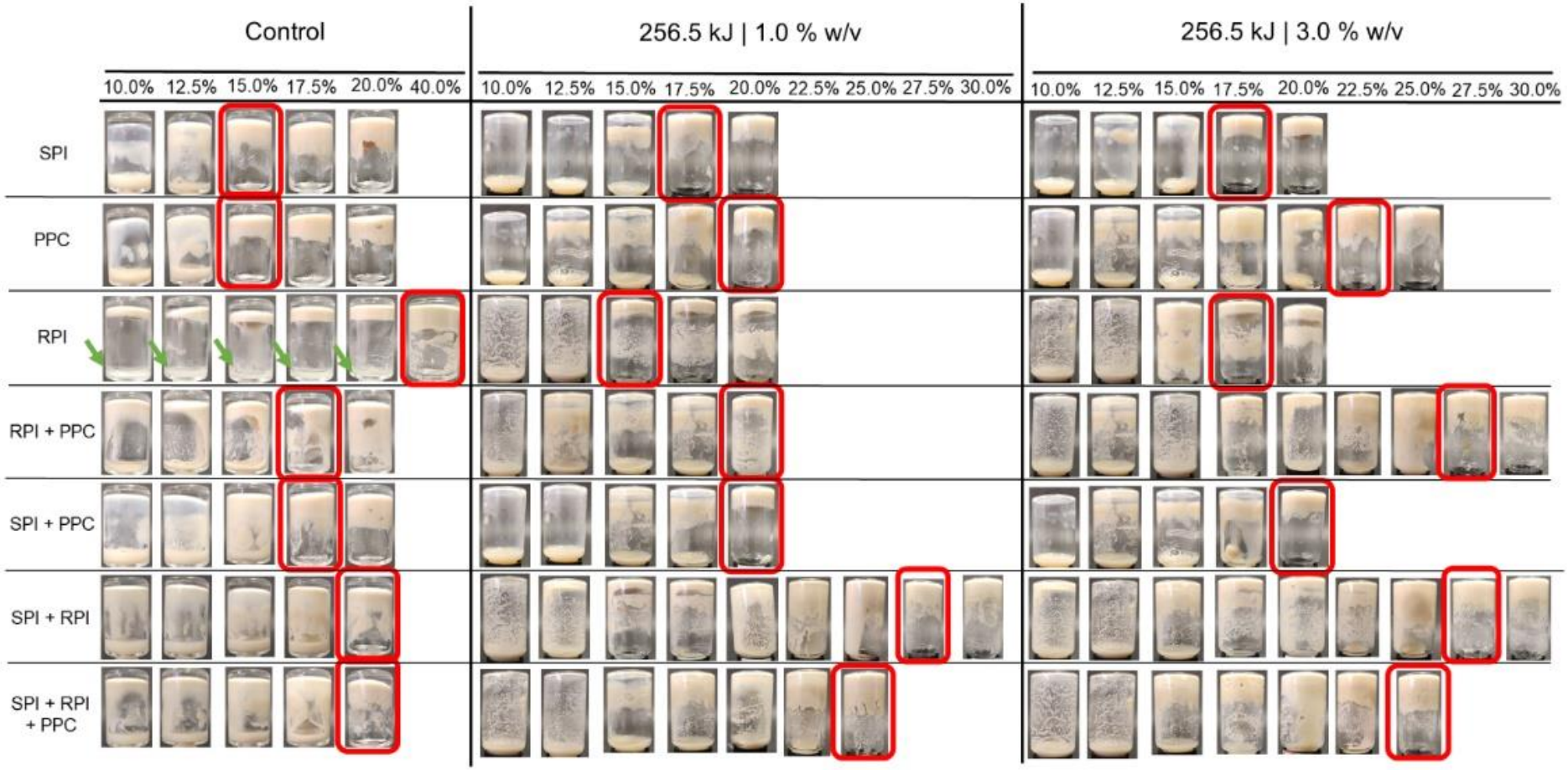


Figure 11. Simplex-centroid mixture design and the responses to least gelling concentration (LGC) of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment.

In the control condition, the LGC for SPI and PPC was 15.0% w/v, whereas for the RPI it was 40.0% w/v. For the later system, up to 20.0% of accumulated water was observed at the bottom of the flask. This lower water affinity of the RPI was also observed in the dispersibility and WHC results. Despite the high amount of RPI needed to form a gel, the simple mixture of this protein with PPC or SPI, promote aggregation between their molecules, allowing the formation of self-sustainable gels with 17.5 and 20.0%, respectively. The SPI + PPC mixture, showing LGC of 17.5% w/v, reveals that the aggregation between its proteins disfavors the inter and intramolecular interactions that promote the gel structuring.

The amount of RPI after USt was 15.0% (256.5 kJ | 1.0% w/v) or 17.5% w/v (256.5 kJ | 3.0% w/v). Such result highlights that, regardless of the condition, USt may be a good strategy to improve the gelling capacity of RPI. This is possibly due to the ability of US waves to modify the structure of the protein, exposing hydrophobic aminoacids residues and favoring intermolecular interactions, and consequently junction zones, to occur. Indeed, it is well known that the formation of gels by proteins is mainly driven by the hydrophobic effect, which consists in the association of hydrophobic residues in order to reduce contact of this non-polar species with water. Thus, the strong correlation between WHC and LGC is noted, since the RPI after receiving the USt increased the WHC and decreased the LGC.

On the other hand, the LGC of binary and ternary mixtures after USt, slightly increased, mostly because of protein molecules' coaggregation, which hindered the formation of the junction zones and water entrapment in the interstices. As observed in more detail in the physicochemical analyses, USt promotes conformational changes in proteins, changing the density of electrical charges (ζ-potential), reducing the d_h of the aggregates, exposing more hydrophobic groups and decreasing the free sulfhydryl groups on the surface of proteins. Therefore, the unfolded protein, by exposing hidden functional groups to the surface, presents other factors besides the increase in hydrophobicity that affect the formation of gels in the mixtures.

Khatkar et al. (2020) applying an energy input ranging from 90 to 360 kJ in soy protein concentrate (80% protein) and Resendiz-Vazquez et al. (2017), who used 180 to 540 kJ in jackfruit protein isolate (69.62% protein) found that the USt decreased the LGC. However, it is noteworthy that these authors applied the USt in 10.0% w/v dispersions, and Omura et al. (2021), when applying a USt ranging from 67.5 kJ to 427.5 kJ in SPI and PPC, found that the USt when applied in dispersions at 5.0 % w/v, LGC is lower than those applied over 1.0% w/v or 3.0 % w/v dispersions, indicating that USt studies with

different energy inputs on plant-based protein dispersions under different concentrations are needed.

3.3 *In vitro digestibility*

The *in vitro* digestibility of SPI, PPC and RPI proteins, alone or in a mixture, with and without USt, were analyzed using the electrophoretic profile (SDS-PAGE), as shown in Figure 12.

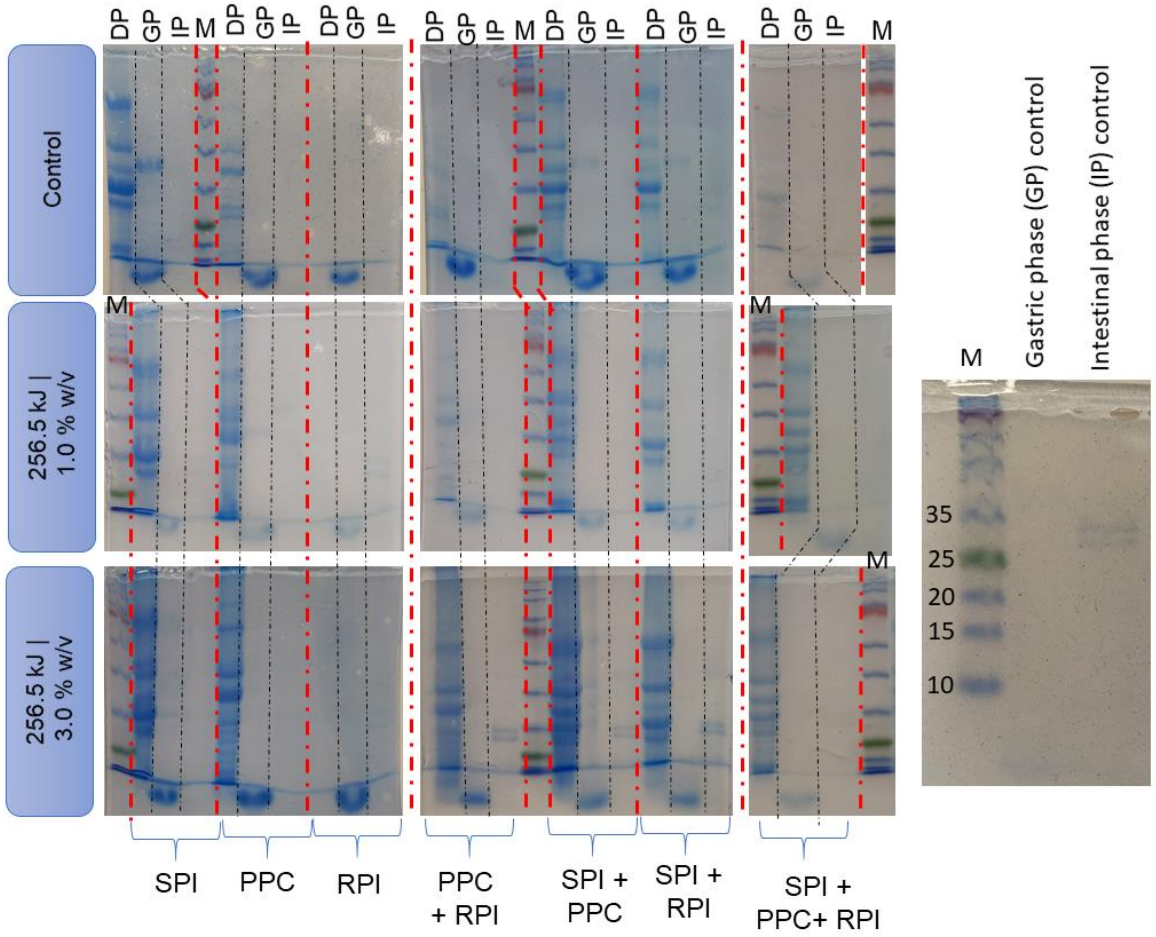


Figure 12. Simplex-centroid mixture design and the responses to in vitro digestibility of soy protein isolate (SPI), pea protein concentrate (PPC) and rice protein isolate (RPI) as pure preparations, binary mixtures or ternary mixture with or without (control) ultrasonic treatment.
DP is protein dispersion, GP is gastric phase, IP is intestinal phase and M is molecular weight market.

The description of the molecular mass profile (characterization) was described in section 3.1.1 and correspond to the first column of the *in vitro* digestibility characterization, in this section identified as protein dispersion (DP). The second column corresponds to the gastric phase (GP). It is observed that, with the exception of the control condition involving the SPI and SPI + PPC mixture, there was the presence of a single band with less than 10 kDa, suggesting the disruption of the interactions that maintained the main SPI, PPC and RPI subunits, with consequent digestion of them. These small fragments, which were obtained through pepsin activity at its optimal pH, are delivered to the intestine (IP). The third column represents the intestinal phase (IP). Considering that the control condition revealed a faint band with ~27kDa, the subtle presence of this band in the samples can be disregarded. Thus, it can be inferred that at the end of the IP, all protein material was digested.

Considering that currently the food industries consider highly relevant aspects based on compositional data, without taking into account the effect of processing on the digestibility and bioavailability of protein sources (Jiménez-Munoz et al., 2021), when we observe the presence of two bands in the control system (SPI and SPI + PPC), as well as the presence of less intense bands (10 kDa) than those systems that have gone through the USt in GP, it is suggested that the ultrasound technology represents an useful tool to increase the digestibility of plant proteins, especially when applied over a dispersion prepared at 1.0% w/v. The presence of the less intense GP band after USt 256.5 kJ | 1.0% w/v than 256.5 kJ | 3.0% w/v indicates more pronounced protein digestion. Furthermore, in the case of RPI, it is noteworthy that, despite the impossibility of characterizing the dispersion supernatant by SDS-PAGE, and the insensitivity to extreme pH conditions (Fig. 3), the presence of the FG band in all three conditions analysed, reinforced the effectiveness of the FG conditions adopted in the digestion of rice proteins.

4. Conclusions:

This work brought, for the first time, an approach to the effect of mixtures between three plant protein: two legumes (SPI and PPC) and one cereal (RPI), that were submitted (separately or together) to ultrasound treatment (256.5 kJ) and two dispersion concentrations (1.0 or 3.0% w/v).

The USt promoted interaction effects among SPI, PPC and RPI, enabling improvement in both the physicochemical and techno-functional aspects. The effects of binary and ternary mixtures were evaluated through mathematical modelling, in which

the dependent variables fitted to quadratic and special cubic regression models, respectively. In addition, the USt promoted an increase in the dispersibility of the seven mixtures of the simplex centroid design, changing their surface characteristics, such as increased hydrophobicity and reduction of free SH groups, as a result of the conformational changes in proteins. Knowing that solubility is a critical parameter to be evaluated to guide the use of an ingredient in food products, it can be concluded that the USt of 256.5 kJ | 1.0% w/v is the most suitable for the SPI + RPI mixture, while the USt of 256.5 kJ | 3.0% w/v was more suitable for the PPC + RPI mixture.

The similarity in the bands of the molecular weight profile gels, under reducing conditions, of SPI, PPC and RPI as pure preparations or binary and ternary mixtures in the three conditions analyzed (control, USt 256.5 kJ | 1.0% or 3.0% w/v) indicated that USt neither altered the primary structure of proteins nor promoted interactions or formation of new structures with molecular mass between 10 and 245 kDa, reinforcing that the different results found in the present work are consequences of the alteration in three-dimensional conformational of the proteins.

The changes observed in d_h and in the distribution profile, showed that the USt represents a useful tool in improving the uniformity of SPI, RPI and PPC both alone and in mixtures. In general, it can be concluded that the USt affected the hydration characteristics, promoting an increase in dispersibility and a decrease in WHC; improved the interfacial properties, as it increased the foaming capacity, emulsifying activity and OHC and altered the texture characteristics, increasing the demand for proteins in the LGC. The conjoint analyses of the results illustrate the potential of using plant-based mixtures in a wide range of food products, based on the desired techno-functional property. Furthermore, in the *in vitro* digestibility test, USt showed to improve protein digestion, indicating that the binary and ternary mixtures of SPI, RPI and PPC treated with ultrasound represent a strategy to obtain products with nutritional functionality, both in terms of providing balanced amino acids, and biological functionality. Therefore, the USt binary and ternary mixtures contemplate a balance between functional performance, cost, sustainability and nutritional functionality, presenting themselves as promising protein ingredients.

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Suplementary material

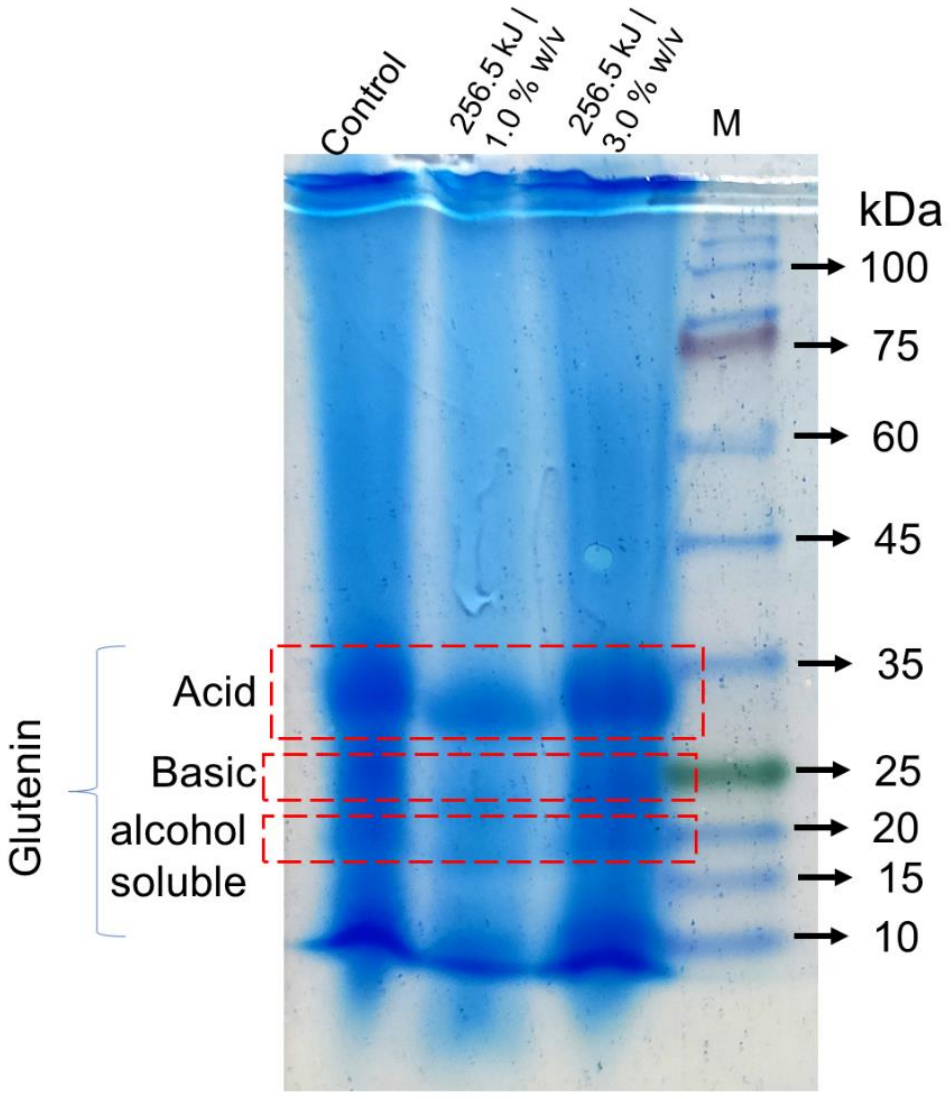

Figure 1 (SM). SDS-PAGE pattern of protein dispersion of rice protein isolate (RPI). M is molecular weight marker and control is without treatment ultrasonic.

6. Conclusões Gerais

Esta tese comtemplou que a tecnologia do ultrassom, é uma ferramenta útil para modificar e/ou desenvolver misturas de proteínas da soja, ervilha e arroz. No entanto, para encontrar ajustes de otimização adequados para esses materiais, individualmente, é necessário que o estado inicial de agregação dos isolados/ concentrados proteicos, sejam pelo menos dispersíveis em meio aquoso.

No primeiro estudo experimental, o tratamento ultrassônico otimizou o uso do isolado proteico de soja (IPS) e concentrado proteico de ervilha (CPE), baseada em suas respostas funcionais. Por meio do delineamento experimental Box-Behnken, foi possível esclarecer os efeitos de diferentes entradas do binômio potência e tempo do processamento por ultrassom, envolvendo concentrações distintas das dispersões dos materiais proteicos. O isolado proteico de arroz (IPA) também foi estudado. No entanto, o efeito do tratamento ultrassônico pouco afetou as respostas deste material. O tratamento ultrassônico é fortemente impactado pela concentração do biopolímero em dispersões aquosas, uma vez que as próprias proteínas atuaram como barreiras que atenuavam a cavitação ultrassônica. Logo, quanto maior a quantidade de proteínas no momento do tratamento ultrassônico, as ondas afetam em menor grau as interações associadas tanto à estrutura tridimensional quanto ao estado de agregação das proteínas.

Em relação as propriedades técnico-funcionais, o tratamento ultrassônico, ao alterar a conformação das proteínas, aumentando a exposição de regiões hidrofóbicas, aumentou de forma unânime a capacidade de retenção de óleo dos materiais proteicos. Além disso, sabendo que a dispersibilidade em água das proteínas é um fator chave para seu uso em formulações alimentícias, o tratamento de 712,5 W por 600 s, em dispersões a 1,0% (m/v), foi a condição que promoveu maior dispersibilidade no IPS, enquanto para o CPE, foi de 712,5 W por 360 s, sobre dispersões 3,0% (m/v).

Nesse trabalho, foi proposto combinações binárias e ternária entre IPS, CPE e IPA, para avaliar o desempenho técnico-funcional dessas combinações

que se complementam em termos de composição nutricional. Essas misturas foram submetidas ao tratamento ultrassônico com as condições selecionadas a partir do primeiro estudo, que, em princípio, possibilitou menor capacidade de formação de espuma e gel e maior dispersibilidade.

Assim, no segundo estudo experimental, efeitos de mistura por meio de modelagem matemática, foram obtidos. Foi observado pela dispersibilidade no pH 7,0, uma transição do ajuste do modelo linear para o quadrático, evidenciando o efeito de interação entre duas proteínas. Além disso, o USt promoveu um aumento na dispersibilidade de todas as misturas. Novamente, destacando o papel da dispersibilidade como um parâmetro crítico a ser avaliado para orientar a utilização de um ingrediente em produtos alimentícios, conclui-se que o USt de 256,5 kJ | 1,0% (m/v) é o mais adequado para a mistura SPI + RPI, enquanto o USt de 256,5 kJ | 3,0% (m/v) foi mais adequado para a mistura PPC + RPI. Interessantemente, apesar da baixa efetividade do USt em modificar o IPA de forma isolada, quando este material foi combinado com SPI, essa mistura com USt de 256,5 kJ | 1.0% m/v, revelou efeitos sinergísticos, pois promoveu as maiores contribuições na dispersibilidade (pH 7,0), no índice da atividade emulsificante (pH 7,0) e na OHC. Já a mistura entre CPE + IPA, ao receberem USt de 256,5 kJ | 3,0% m/v revelaram desempenho funcional similares ao do CPE, porém superior ao do IPA, representando assim, uma maneira de otimizar o uso do IPA e com potencial de disponibilizar um ingrediente de origem totalmente vegetal que contempla todos aminoácidos essenciais.

Com base nos resultados obtidos nos testes da distribuição da massa molecular, distribuição de diâmetro hidrodinâmico e da digestibilidade *in vitro*, pode-se concluir que esta tecnologia, quando conduzida com bastante critério, tende a melhorar a uniformidade de sistemas complexos, como misturas de diferentes proteínas, promove melhorias em sua digestão, e tudo como resultados de alterações na conformação tridimensional das proteínas.

Este trabalho mostrou que é possível otimizar o uso das proteínas da soja, arroz e ervilha, melhorando sua dispersibilidade e, consequentemente, sua funcionalidade, por meio do USt ou por misturas entre esses materiais proteicos

combinados ao USt. Pode-se concluir ainda que IPS + IPA e CPE + IPA tratados com ultrassom, representam misturas constituídas de alto valor biológico em termos de composição e de digestibilidade e, em alguns casos, com propriedades técnico-funcionais superiores a sua forma pura.

ANEXOS

CERTIFICATE OF ANALYSIS FOR ORYZATEIN® SILK 90

ORYZATEIN® SILK 90

Lot #: HZN14026
PO #: 3509
Manufacture: August 19, 2014
Expiration date: August 18, 2016
CofA Date of Report: February 26, 2015

Usage: Rice Protein is an excellent source of vegan, hypo-allergenic protein. It is being used as a replacement for Soy Protein and Whey Protein in food grade products including protein supplements, bars and nutritional drinks. Rice protein concentrate is very low in factors causing flatulence. Oryzatein uses non-GMO sprouted brown rice and has an amino acid profile closest to mother's milk. Oryzatein is Identity Preserved and Kosher.

Description: Rice protein concentrate is the concentrated fraction of rice grain, resulting from the rice wet milling process.

Characteristics: Color is light brown or beige; Taste is bland; Fragrance is clean and bland; Texture is free flowing meal, free of foreign material and not gritty.

	Technical Standard	Testing Method	Test Results	Unit
ID Testing		Organoleptics	Conforms	
pH:	4.5-7.0	USP	6.73	
Protein (dry basis):	90% min	AOAC 920.152	90.8	%
Fat:	5% max	AOAC 948.15, 922.06	1.9	%
Fiber:	6% max	AOAC 991.43	<6	%
Moisture:	5% max	AOAC 930.15	3.78	%
Ash:	3.5% max	AOAC 923.03 (321.05)	<3.5	%
Total Carbohydrate:	15% max	By Calc.	<15	%
Cyanuric Acid	Negative	LC/MS / MS method LIB #4421	Negative	
Melamine	Negative	LC/MS / MS method LIB #4421	Negative	
Ammelide	Negative	LC/MS / MS method LIB #4421	Negative	
TPC (cfu/g):	15,000 max	USP <2021>	<100	cfu/g
Coliform (cfu/g):	30 max	AOAC 991.14	<10	cfu/g
Salmonella (cfu/10g):	Negative	USP <2022>	Negative	cfu/10g
E. Coli (cfu/10g):	Negative	USP <2022>	Negative	cfu/10g
Staph aureus (cfu/10g):	Negative	USP <2022>	Negative	cfu/10g
Yeast and Mold (cfu/g):	100 Max	AOAC 997.02	<10	cfu/g
Heavy Metals (ppm)	<1	ICP-MS	<1	ppm
Arsenic (ppm)	<0.20	ICP-MS/ AOAC 993.14	0.107	ppm
Cadmium (ppm)	<0.30	ICP-MS/ AOAC 993.14	0.192	ppm
Lead (ppm)	<0.25	ICP-MS/ AOAC 993.14	0.146	ppm
Mercury (ppm)	<0.045	DMA	0.040	ppm
Gluten (ppm)	<20	ELISA	<10	ppm
Aflatoxins B1, B2, G1, G2 (µg/kg	<5	AOAC 999.07	<2	µg/kg
Ochratoxin A (µg/kg)	<10	AOAC 999.07	3.3	µg/kg
Vomitoxin (Deoxynivalenol) (µg	<750	LC - MS/MS	<10	µg/kg
Zearalenone (µg/kg)	<200	LC - MS/MS	41	µg/kg
Bacillus Cereus (MPN/g)	<1,000	AOAC 980.31	<3	MPN/g
Particle size (Analyzer):	Min 95% pass through a 600 Mesh			
Packaging	25 kg bag with inner polyethylene liner			

Country of Origin: China
Approval Signature: Heather Szucs, Director of Quality
Doc Update: 7/18/2014

AMIOM FOODS

Where Nature and Technology Meet

Supplying the food, health and nutraceutical industries

ORYZATEIN 90 / ORYZATEIN 90 SILK

AMINO ACID PROFILE - % OF TOTAL AMINO ACIDS

AMINO ACID	% of Total AA
Alanine	4.9
Arginine	6.82
Aspartic Acid	9.1
Cystine	2.4
Glutamic	17.21
Glycine	3.15
Histidine	2.24
Isoleucine*^	5.83
Leucine*^	9.1
Lysine*	5.12
Methionine*	2.3
Phenylalanine*	4.38
Proline	6.1
Serine	4.92
Threonine*	4.75
Tryptophan*	1.56
Tyrosine	4.1
Valine*^	6.02

*Essential Amino Acids
^Branch Chain Amino Acids

PurePro™ 90B

SOY PROTEIN ISOLATE
BEVERAGE GRADE

PRODUCT SPECIFICATION AND DATA SHEET

DESCRIPTION

Soy Protein Isolate Beverage Grade is an isolated soy protein that has excellent dispersing ability and very low viscosity. It is specially designed for powdered beverages like Milk, Dual protein Milk, High protein Milk, and other powdered beverages.

PHYSICAL CHARACTERISTICS		TYPICAL BACTERIOLOGICAL ANALYSIS	
Protein (DRY BASE)	90% min	Standard plate count	10,000/g max
Moisture	7% max	Enterococcus	100/g max
Ash	6% max	Coliforms (in 0.1g)	negative
Crude fiber	4% max	Salmonella (in 25g)	negative
Fat	0.5%max	Yeast and mould	50/g max
PH	7±0.5		
Color	light		
Flavor	neutral		

FATTY ACIDS (G/100G) * (BASED ON FAT BY ACID HYDROLYSIS)

Aturated	1.0	Trans fat	<0.05
Cholesterol	0	Sugar	<1

* average value

PARTICLE DISTRIBUTION(TYPICAL ANALYSIS)

98% through 100# U.S. standard screen

ESSENTIAL AMINO ACID (G/100G) *

Arginine	6.85	Methionine	0.916
Cystine	\	Serine	5.05
Tryptophan	\	Lysine	2.86
Histideine	3.05	Phenylalanine	3.74
Tyrosine	2.9	Threonine	3.64
Leucine	5.32	Valine	2.48
Glutanicacid	22.60	Asparticacid	11.4
Glycine	3.91	Alanine	4.56
Proline	4.39	Isoleucine	4.60

* average value

Packing: 20kg net weight, paper/plastic bag poly-lined.

Storage: below 75°F and 60% relative humidity promotes longer shelf life.

Certificate: HACCP, ISO9000, IP, QS, Kosher & Halal certificated

Shelf life: Two years.

山东健源食品有限公司
SHANDONG JIANYUAN FOODS CO.,LTD.
地址：山东省招远市开发区民营工业园区盛泰路 电话(Tel)：0086-535-8139206-8139211
Add：Shengtai Road，Zhaoyuan City，Shandong，China 传真(Fax)：0086-535-8139200
网址（Http)：//www.jianyuangroup.com 电邮（E-mail)：trade@jianyuanfoods.cn

Pea Protein Isolate

DESCRIPTION: Non-GMO pea protein isolate, especially suitable for health drinks, cereal snack foods, baked foods, meat products, and healthcare products

MANUFACTURER: Shandong Jianyuan Foods Co., Ltd CHINA

TECHINICAL DATA:

Appearance	Light-yellow powder	
Moisture	≤8%	GB 5009.3-2010
Ash	≤8%	GB 5009.4-2010
Crude Fiber	≤5%	GB/T5009.10-2003
Protein	≥80%	GB 5009.5-2010
Fat	≤2%	GB/T5009.6-2003
PH	7.0~8.0	Laboratory method
Particle(Through 80 mesh)	Min 95%	80 mesh Sieve Analysis

TYPICAL AMINO ACIDS (g/100g Product):

ASP	12.7	GB/T 5009.124-2003
THR	4.19	
SER	6.56	
GLU	13.3	
GLY	3.08	
ALA	4.57	
PRO	4.65	
VAL	4.07	
MET	1.20	
ILE	4.51	
LEU	7.53	
TYR	6.38	
PHE	5.10	
LYS	5.46	
HIS	3.45	
ARG	6.65	
Amino Acids	93.4	

HEAVY METALS (mg/kg Product):

Lead	≤1.0	GB 5009.12-2010
Arsenic	≤0.5	GB/T 5009.11-2003
Zinc	69	GB/T 5009.14-2003
Selenium	1.7	GB 5009.93-2010
Cadmium	0.01	GB/T 5009.15-2003
Chromium	015	GB/T 5009.123-2003
Copper	0.81	GB/T 5009.13-2003
Mercury	≤2.0	GB/T 5009.17-2003

PACKAGING:
25kg net bags with inner plastic bags

STORAGE:
The shelf life is 24 months, in cool and dry place. Keep away from strong odor or volatile materials and moisture.

CERTIFICATE: Kosher, Halal, HACCP, ISO9001:2008, BRC