ANA PAULA HANKE DE OLIVEIRA

EFEITOS DE PROCESSAMENTO POR ULTRASSOM SOBRE TÉCNICO-FUNCIONALIDADES DE CONCENTRADO PROTEICO DE ERVILHA

Dissertação 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 *Magister Scientiae*.

Orientador: Eduardo Basílio de Oliveira

Coorientadoras: Jane Sélia dos Reis Coimbra

Érica Nascif Rufino Vieira

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RESUMO

OLIVEIRA, Ana Paula Hanke, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Efeitos de processamento por ultrassom sobre técnico-funcionalidades de concentrado proteico de ervilha.** Orientador: Eduardo Basílio de Oliveira. Coorientadoras: Jane Sélia dos Reis Coimbra e Érica Nascif Rufino Vieira.

As novas tendências de consumo voltadas para a saúde e preocupações ambientais tornam as proteínas de ervilha uma fonte proteica promissora, uma vez que apresentam características nutricionais e técnico-funcionais comparáveis a outras proteínas de boa qualidade. Neste trabalho, foi avaliada a influência do ultrassom de alta intensidade (HUS) e diferentes valores de pHs sobre as propriedades físico-químicas, digestibilidade *in vitro* e propriedades emulsificantes do concentrado de proteína de ervilha (CPE). As dispersões proteicas foram tratadas com ultrassom, sob diferentes condições, de modo que o *input* de energia aplicado ao sistema fosse o mesmo (240 kJ). Além disso, foi avaliada a influência dos pHs: 2,8, 4,3, 6,8 e sem ajuste de pH (~ 8,5), em cada tratamento com ultrassom. Nos tratamentos com ultrassom de 412.5 W/581.82 s; 487,5 W/492,31 s; 562,5 W/426,66 s ocorreu aumento da dispersibilidade proteica (no pH 2,8 e sem ajuste de pH) e exposição das superfícies hidrofóbicas (pH 4,3; 6,8 e sem ajuste de pH) e grupos sulfidrilas (pH 4,3 e 6,8), além de redução do diâmetro médio dos agregados proteicos (pH 2,8; 6,8 e sem ajuste de pH). Entretanto, os binômios potência/tempo de 637,5 W/376,47 s; 712,5W/336,84 s podem promover a formação de rearranjos estruturais e, consequentemente, a geração de agregados proteicos. A digestibilidade *in vitro* dos CPEs também foi melhorada pelo tratamento com HUS. Nas propriedades emulsificantes, o pH exerceu a maior influência nos resultados, sendo que os sistemas com valor de pH 2,8 e sem ajuste de pH produziram emulsões mais estáveis. Além disso, o tratamento com ultrassom melhorou a capacidade emulsificante e diminuiu o índice de cremeação das emulsões no pH 6,8. Assim, a aplicação do ultrassom em combinações de potência/tempo até 562,5 W/4266 s é uma tecnologia promissora a ser utilizada pela indústria alimentícia, pois promove alterações físico-químicas e de digestibilidade nas proteínas das ervilhas, além de melhorar as propriedades emulsificantes.

Palavras-chave: Proteína de ervilha. Ultrassom. Emulsão. Digestibilidade *in vitro*.

ABSTRACT

OLIVEIRA, Ana Paula Hanke, M.Sc., Universidade Federal de Viçosa, February, 2020. **Effects of ultrasound processing on technical features of pea protein concentrate.** Adviser: Eduardo Basílio de Oliveira. Co-advisers: Jane Sélia dos Reis Coimbra and Érica Nascif Rufino Vieira.

New consumption trends focused on health and environmental concerns make pea proteins a promising protein source, since they have nutritional and technical-functional characteristics comparable to other good quality proteins. In this work, the influence of high intensity ultrasound (HUS) and different pH values on the physicochemical properties, in vitro digestibility and emulsifying properties of pea protein concentrate (PPC) were evaluated. The protein dispersions were treated with ultrasound, under different conditions, so that the energy input applied to the system was the same (240 kJ). In addition, the influence of pHs: 2.8, 4.3, 6.8 and without pH adjustment (~ 8.5) was evaluated in each treatment with ultrasound. In the 412.5 W/581.82 s ultrasound treatments; 487.5 W/492.31 s; 562.5 W/426.66 s there was an increase in protein dispersibility (at pH 2.8 and without pH adjustment) and exposure to hydrophobic surfaces (pH 4.3; 6.8 and without pH adjustment) and sulfhydryl groups (pH 4.3 and 6.8), in addition to a reduction in the average diameter of protein aggregates (pH 2.8; 6.8 and without pH adjustment). However, the power/time binomial of 637.5 W/376.47 s; 712.5W/336.84 s can promote the formation of structural rearrangements and, consequently, the generation of protein aggregates. The in vitro digestibility of PPCs was also improved by treatment with HUS. In the emulsifying properties, the pH exerted the greatest influence on the results, and the systems with pH value of 2.8 and without pH adjustment produced more stable emulsions. In addition, ultrasound treatment improved the emulsifying capacity and decreased the emulsion creams index at pH 6.8. Thus, the application of ultrasound in power/time combinations up to 562.5 W/426.6 s is a promising technology to be used by the food industry, as it promotes physico-chemical and digestibility changes in pea proteins, in addition to improving the properties emulsifiers.

Keywords: Pea protein, Ultrasound, Emulsions, In vitro digestibility.

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SUMÁRIO

1. INTRODUÇÃO – JUSTIFICATIVA E RELEVÂNCIA

As proteínas são macro nutrientes vitais na dieta humana por fornecerem energia e aminoácidos essenciais que contribuem para crescimento do corpo, e seu funcionamento. Além disso**,** são ingredientes funcionais amplamente utilizados em vários segmentos industriais (alimentícia, farmacêutica, cosméticos) devido à sua aplicabilidade em sistemas como espumas, géis e emulsões (O'SULLIVAN et al., 2015; SHARIF et al., 2018).

Estima-se que o mercado global de ingredientes proteicos será de aproximadamente 46 bilhões de reais até 2022, com uma taxa de crescimento anual de 6,5% ao ano, sendo que o maior crescimento ocorrerá no setor de origem vegetal. Espera-se que em 2054, cerca de 1/3 de todas as proteínas consumidas sejam obtidas de fontes vegetais. Os fatores que conduzem ao aumento da demanda por tais proteínas referem-se ao crescimento populacional, sustentabilidade ambiental com redução do uso da água, mudanças demográficas, posturas pessoais contra o abate e restrições religiosas (HENCHION *et al*., 2017; HOSAFCI, 2017).

Dentre as proteínas vegetais, as de ervilha podem ser consideradas uma alternativa às da soja, que constituem as proteínas de origem vegetal mais tradicionalmente utilizadas para fins alimentícios (CHAO et al., 2016; GEERTS et al., 2017; O'SULLIVAN et al., 2015). De fato, comparada às da soja as proteínas de ervilha possuem valor nutricional similar, sendo ricas em lisina (71 mg/g de proteína) e fibras alimentares (212 mg/g de ervilha). Além disso, apresentam custos industrialmente abordáveis e baixa incidência de alergenicidade (DAHL; FOSTER; TYLER, 2012; DAMODARAN, 2010).

Além dessas características nutricionais, as propriedades técnico-funcionais (gelificantes, emulsionantes, e espumantes; principalmente) contribuem para aumentar o interesse no uso de proteínas de ervilha como um ingrediente alimentar promissor (GRAÇA *et al.*, 2016, XIONG *et al*., 2018; KLOST; DRUSCH, 2018; QIN et al., 2016; ZHOU et al., 2016). No entanto, a utilização/estudo dessas propriedades funcionais das proteínas de ervilha ainda é limitado, o que pode ser atribuído a sua baixa solubilidade na faixa de pH em que os alimentos geralmente se encontram (entre 4 e 7), uma vez que a solubilidade impacta diretamente a manifestação/exploração das demais funcionalidades tecnológicas (LAM *et al*., 2018, JIANG et al., 2017).

Para minimizar essa limitação das proteínas vegetais, várias estratégias envolvendo métodos físicos, químicos e bioquímicos têm sido estudados (OLIETE *et al*., 2018; MCCARTHY *et al*., 2016;. KLOST & DRUSCH, 2018; O'SULLIVAN *et al*., 2016;

O'SULLIVAN *et al*., 2015). Em relação aos métodos físicos, o uso de calor, alta pressão e ultrassom, por exemplo, vêm sendo utilizados a fim de otimizar o uso de proteínas de ervilha, uma vez que promovem alterações na estrutura molecular e diminuição do tamanho de agregados proteicos (OLIETE *et al*., 2018; XIONG *et al*., 2018 ; PENG et *al*., 2016 ; SHEN & TANG , 2012).

O ultrassom é uma tecnologia considerada segura, não tóxica, não prejudicial ao ambiente e induz a menores perdas de caráter nutricional e sensorial dos produtos, quando comparado aos processos térmicos convencionais. Além disso, existe uma ampla oferta de equipamentos disponíveis no mercado incluindo banhos ultrassônicos e diferentes sondas, que podem ser adaptados a diferentes operações apresentando-se como uma opção a ser explorada pela indústria alimentícia (KENTISH & ASHOKKUMAR, 2011; CÁRCEL et al., 2012; OJHA e*t al*., 2018).

O ultrassom de alta intensidade (frequência de 20 a 100 kHz e potência entre 10 e 1000 W/cm^2) é capaz de modificar a estrutura nativa de proteínas em meio aquoso por meio da cavitação acústica. Tal fenômeno refere-se à formação de ciclos diferenciais de pressão, que são originados das ondas sonoras dissipadas no meio. Estes ciclos criam zonas de vácuo onde ocorre a formação de bolhas que colapsam ao atingir zonas de pressões mais elevadas, provocando alta turbulência local, forças de cisalhamento, aumento de pressão e temperatura (MCCLEMENTS, 1995; O' SULLIVAN *et al.*, 2016; ZHOU *et al*., 2016; TAHA *et al*., 2018). Por conseguinte, propicia maior exposição na superfície de grupos hidrofóbicos e de grupos sulfidrilas que na estrutura nativa encontram-se no interior das moléculas proteicas. Isso se traduz em alterações nas propriedades de agregação molecular e atividade interfacial, podendo assim modificar suas técnico-funcionalidades e sua digestibilidade.

A modificação das propriedades físico-químicas de proteínas vegetais, em especial das proteínas de ervilha, pelo uso da tecnologia de ultrassom vem sendo estudada por diversos pesquisadores (O' SULLIVAN *et al.*, 2016; MCCARTHY *et al*., 2016; KLOST & DRUSCH, 2018; ZHOU *et al*., 2016; TAHA *et al*., 2018) no entanto, os mesmos não apresentam estudos que analisam a influência da taxa com que a energia é aplicada ao sistema. Isso pode ser realizado mantendo o *input* de energia fixo e aplicando diferentes combinações de tempo e potência que produzam tal energia.

Deste modo, neste estudo objetivou-se avaliar a influência do ultrassom de alta intensidade, aplicado em diferentes combinações de potência/tempo que forneçam o mesmo

input de energia aos sistemas ajustados a diferentes valores de pH, sobre características físico-quimicas, de digestibilidade *in vitro* e propriedades emulsificantes das proteínas de ervilha.

Desta forma, o presente documento foi estruturado como segue:

i) Um capítulo intitulado "Estudo Bibliográfico" em que é feita uma apresentação sobre as proteínas vegetais, como foco nas proteínas de ervilha. Além disso, foi apresentado definições e estudos relacionados a aplicação do ultrassom de alta intensidade sobre a estrutura proteica. Em sequência foram descritas definições acerca da digestibilidade *in vitro* e propriedades emulsificantes. Com este capítulo, objetiva-se fornecer bases teóricas para a compreensão do caráter inovador da proposta apresentada, bem como, entendimento a respeito de como o trabalho experimental visa responder a problemática apresentada.

ii) Em seguida é apresentado um manuscrito de artigo científico intitulado *Combined adjustment of pH and ultrasound treatments modify techno-functionalities of pea protein concentrates.* Neste estudo será avaliado o efeito de pré-tratamentos com ultrassom de alta intensidade (20 kHz), em diferente combinação de tempo e potência mantendo o aporte de energia fixo, bem como a influência dos pHs 2,8; 4,3;6,8 e sistemas sem ajuste de pH sobre características físico-químicas, de digestibilidade *in vitro* e propriedades emulsificantes do concentrado proteico de ervilha.

2. OBJETIVOS

2.1. Objetivo Geral

Estudar efeitos de pré-tratamentos ultrassônicos, com o mesmo aporte de energia, em diferentes valores de pH sobre características físico-químicas, digestibilidade *in vitro*, e propriedades emulsificantes do concentrado proteico de ervilha (CPE).

2.2. Objetivos Específicos

- Determinar o teor de proteínas do CPE, bem como o ponto isoelétrico (pI) médio das proteínas que constituem tal concentrado.
- Aplicar o pré-tratamento ultrassônico às dispersões de CPE (5% m/v) ajustados a diferentes valores de pH, mantendo o aporte de energia em 240 kJ nas seguintes combinações: 412,5 W por 581,82 segundos, 487,5 W por 492,31 min, 652,5 W por 426,66 min, 637,5 W por 376,47 min, 712,5 W por 336,84 min e sistema controle -sem pré-tratamento de ultrassom.
- Avaliar, para os CPE pré-tratados com ultrassom como descrito acima, as seguintes características físico-químicas:
    a) Dispersibilidade, a 25 °C, em valores de pH compreendidos entre 2 e 9;
    b) Exposição dos grupos hidrofóbicos, a 25 °C, em pH ~ pI previamente determinado, nos pHs 2,5 unidades acima e 1,5 unidades abaixo do pI.
    c) Grupos –SH livres a 25 °C, no pH próximo ao pI, bem como nos pHs abaixo e acima do mesmo.
    d) Distribuição de tamanho hidrodinâmico das partículas dispersas a 25 °C, no pHs mencionados no item c.
    e) Potencial zeta das partículas dispersas a 25 °C, nos pHs citados no item c.
- Avaliar a digestibilidade *in vitro* dos CPE que foram submetidos ao pré-tratamento com ultrassom nos sistemas sem ajuste de pH.
- Avaliar, para os concentrados proteicos de ervilha pré-tratados com ultrassom como descrito acima, as propriedades emulsificantes, a 25 °C, em pH ~ pI previamente determinado, nos pHs 1,5 unidades acima e 2,5 unidades abaixo do pI.

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Capítulo 1
Estudo Bibliográfico

1 REVISÃO DE LITERATURA

1.1 Proteínas

As proteínas são biomacromoléculas constituídas de uma ou mais cadeias longas poliméricas formadas por resíduos de aminoácidos unidos por ligações peptídicas e que desempenham várias funções biológicas (DAMODARAN *et al*., 2010). São essenciais para a vida desde o início da gestação até a velhice, atuando na manutenção da massa muscular, reparo de células danificadas, resposta imune, sinalização celular, entre outros (HENLEY, TAYLOR, & OBUKOSIA, 2010).

As recomendações em relação ao consumo de proteínas na dieta são baseadas nas necessidades de aminoácidos indispensáveis, condicionalmente indispensáveis e nitrogênio necessário para síntese de aminoácidos dispensáveis e outras moléculas não proteicas necessárias para apoiar o crescimento, a manutenção e a reparação tecidual (INSTITUTE OF MEDICINE OF THE NATIONAL ACADEMIES, 2005) como mostrado na Tabela 1.

Tabela 1: Aminoácidos indispensáveis, dispensáveis e condicionalmente indispensáveis na dieta humana.

Indispensáveis	**Dispensáveis**	**Condicionalmente indispensáveis**
Histidina	Alanina	Arginina
Isoleucina	Ácido aspártico	Cisteína
Leucina	Ácido glutâmico	Glutamina
Lisina	Serina	Glicina
Metionina		Prolina
Fenilalanina		Tirosina
Tirosina		
Triptofano		
Valina		

Fonte: LAIDLAW AND KOPPLE (1987).

Os aminoácidos classificados como indispensáveis são aqueles cujo esqueleto de carbono não pode ser sintetizado pelo organismo, necessitando ser obtido pela dieta. Os dispensáveis podem ser sintetizados a partir de outros aminoácidos ou de outros metabólitos de complexos nitrogenados, enquanto os condicionalmente dispensáveis também podem ser sintetizados pelo organismo a partir de outros aminoácidos, no entanto sua síntese é limitada

sob condições fisiológicas especiais tornando a ingestão de tais aminoácidos seja necessária (LAIDLAW AND KOPPLE, 1987).

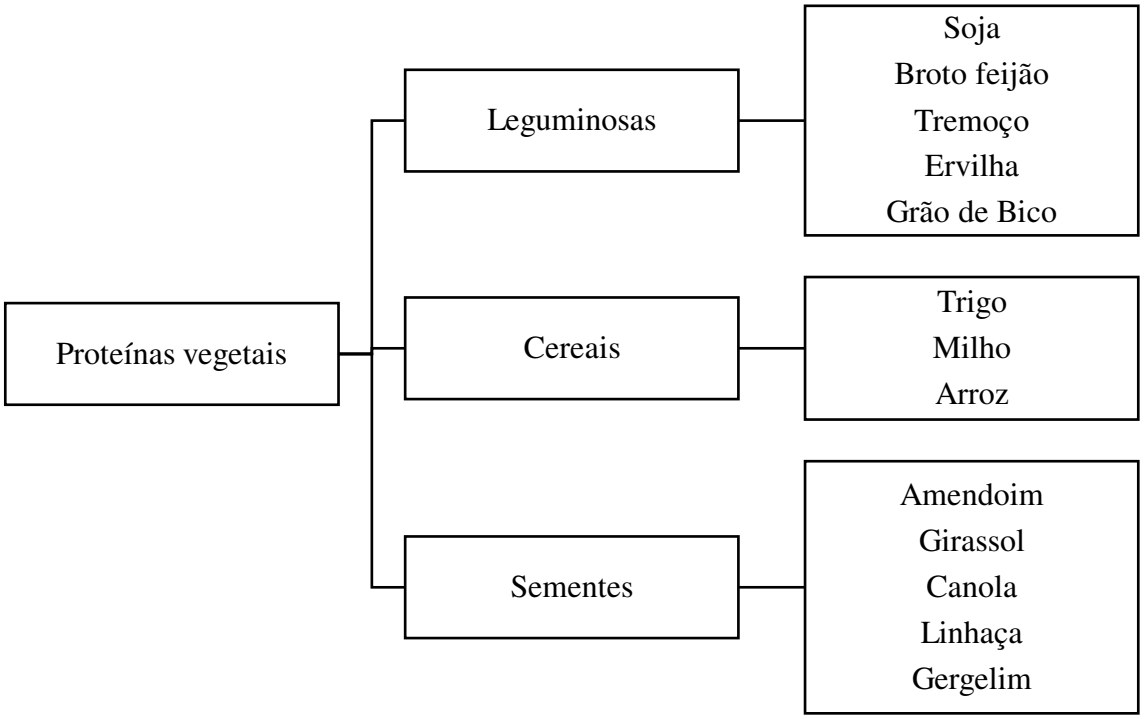
Além dos benefícios nutricionais citados, as proteínas desempenham importantes funções tecnológicas, devido as suas propriedades emulsificantes (ZHAO *et al*., 2018; MCCARTHY *et al*., 2016), gelificantes (KHARLAMOVA; CHASSENIEUX; NICOLAI, 2018; QIN *et al*., 2016) e espumantes (CAO *et al.*, 2018; XIONG et al., 2018).

As proteínas podem ser originárias de fontes animal ou vegetal sendo que aquelas de origem animal são consideradas completas nutricionalmente, uma vez que contém a maioria dos aminoácidos essenciais (BOBBIO & BOBBIO, 1992). Todavia o consumo excessivo das mesmas pode levar a problemas como obesidade (BUJNOWSKI *et al*., 2011), doença cardíaca (CLIFTON, 2011), aumento da pressão sanguínea (ELLIOTT *et al*., 2006) e aumento de ácido úrico (TRACY *et al.,* 2014). Deste modo, as proteínas de origem vegetal tornam-se uma opção, uma vez que não provocam os malefícios descritos acima, além de apresentarem benefícios à saúde (BIANCHI *et al*., 1993; WANEZAKI *et al*., 2015; LAUERMAN, 1998). Além disso, novas tendências de consumo baseados em questões culturais, religiosas, de sustentabilidade ambiental, alimentação vegana e vegetariana, posturas pessoais contra o abate e custos industrialmente abordáveis justificam o uso de tais proteínas (LAM *et al*., 2018; SHARIF *et al.*, 2018).

1.2 Proteínas vegetais

As proteínas de origem vegetal podem ser divididas em três principais grupos, sendo eles o grupo de leguminosas, sementes e cereais como mostra a Figura 1.

Figura 1- Classificação das proteínas vegetais comumente usadas na indústria de alimentos



Fonte: LIN *et al*. (2017).

As proteínas vegetais classificadas como leguminosas correspondem a sementes comestíveis de leguminosas e representam uma fonte proteica de baixo custo, que contém aminoácidos essenciais, peptídeos bioativos, amido, fibras dietéticas e vitaminas (LADJAL-ETTOUMI *et al.,* 2016; BOYE, *et al*., 2010).

A classe dos cerais são sementes ou grãos comestíveis das gramíneas, constituídos principalmente por amido, no entanto suas proteínas perfazem de 10 a 15 % do peso do grão. As diferenças físicas, químicas, nutricionais e funcionais são decorrentes das proporções distintas das frações de proteína em cada cereal (SGARBIERI, 1996).

As sementes são principalmente utilizadas para a produção de óleo, porém apresentam elevadas porcentagens de proteínas. O farelo obtido na extração de óleo de girassol e no óleo de amendoim, por exemplo, contém cerca de 40-50% e 50-60% de proteína, respectivamente (GONZÁLEZ-PÉREZ; ARELLANO, 2009).

Essas proteínas consistem de várias frações sendo elas: a glutenina, a qual é solúvel em soluções alcalinas aquosas; a fração globulina, solúvel em soluções salinas, seguida pela albumina e prolamina, frações solúveis em água e etanol, respectivamente (OSBORNE, 1909).

Entre as proteínas vegetais as proteínas de ervilha vêm sendo frequentemente estudadas, uma vez que as mesmas apresentam características nutricionais e técnico-funcionais comparáveis as proteínas da soja, as quais são as mais utilizadas pela indústria de alimentos (GEERTS *et al.,* 2017; KLOST; DRUSCH, 2018; MCCARTHY *et al*., 2016; WANG *et al.*, 2019).

1.3 Proteínas de ervilha

A ervilha é uma leguminosa rica em proteínas e carboidratos, com baixo teor de gordura. Dependendo da variedade, grau de maturação e condições de cultivo, a ervilha apresenta cerca de 23,1 á 30,9% de proteínas, 1,5 a 2% de gordura e constituintes secundários. O conteúdo de carboidratos representa em média 60-65% sendo: 35-40% de amido, 10-15% de fibra alimentar e 10-15% de demais carboidratos, tais como sacarose, oligossacarídeos e celulose (LAM et al. 2016). Além disso, contêm altos níveis de lisina, mas tendem a ser limitadas em metionina e triptofano. Deste modo, a ervilha é frequentemente consumida juntamente com grãos de cereais, uma vez que eles apresentam um perfil complementar de aminoácidos essenciais já que proteínas de cereais são geralmente deficientes em lisina, mas contêm níveis mais elevados de aminoácidos sulfurados ácidos (metionina, cisteína) (LAM *et al*., 2018; DAHL et al., 2012).

Dependendo da variedade, maturidade na colheita e condições de cultivo, as sementes de ervilha seca contêm cerca de 25% de proteínas, nas quais predominam duas classes, as albuminas e globulinas, representando 10–20% e 70–80%, respectivamente (CAN KARACA *et al*., 2011).

As albuminas (2S, 5-80 kDa) são consideradas proteínas metabólicas hidrossolúveis, que no caso da ervilha, contêm altas concentrações dos aminoácidos essenciais triptofano, lisina, treonina e metionina quando comparado com as globulinas (BOYE *et al*., 2010). As globulinas são consideradas proteínas de armazenamento solúveis em soluções salinas, as quais compreendem três principais grupos: leguminas, vicilinas e convicilinas, sendo a última em menor quantidade (LAM *et al*., 2018).

As leguminas são divididas em três famílias de acordo com a similaridade de composição aminoacídica. Estas famílias são denominadas como, legumina A, legumina J e legumina S. São proteínas hexaméricas com massa molecular entre 300-400 kDa e coeficiente de sedimentação 11 S. Cada subunidade é clivada proteoliticamente em polipeptídios básicos (β) e ácidos (α) os quais são unidos por ligação dissulfeto. Esses polipeptídios exibem carga e heterogeneidade de tamanho, sendo que quatro/cinco polipeptídios ácidos e cinco/seis básicos

foram identificados. Os tamanhos destes variam de 38 a 40 kDa para os ácidos e de 19 a 22 kDa para os polipeptídios básicos (XIONG et al., 2018a). A cadeia α é dominada pelo ácido glutâmico e possui leucina como aminoácido N-terminal, enquanto a cadeia β contém maiores quantidades de alanina, valina e leucina e possui glicina como o grupo amino N-terminal (SIKORSKI, 2001).

As vicilinas são proteínas triméricas com massa molecular entre 150-190 kDa e coeficiente de sedimentação 7 S. Os monômeros que as compõe possuem massa molecular entre 47-50 kDa e consistem de três subunidades (α, β e γ). São unidas por interações hidrofóbicas uma vez que, não possuem resíduos de cisteína e consequentemente, não podem formar ligações dissulfeto. Em relação a composição aminoacídica, a vicilina contém baixos níveis de metionina e triptofano, e altos níveis de aminoácidos básicos (arginina e lisina) e ácidos (ácido aspártico e ácido glutâmico). Tanto as vicilinas quanto as leguminas são predominantemente compostas por estruturas secundárias do tipo folha β (SIKORSKI, 2001).

A convicilina é a terceira proteína de armazenamento encontrada em ervilhas. Possui uma extensa homologia com a vicilina em seu núcleo, mas possui uma carga adicional e uma sequência de 120-166 resíduos hidrofóbicos próximo a extremidade N-terminal. É uma proteína trimérica que possui polipeptídios com uma massa molecular de cerca de 70 kDa. O perfil de aminoácidos da convicilina difere tanto das leguminas quanto das vicilinas apresentando um resíduo de aminoácido sulfurado (cisteína), além de ser altamente carregada próximo a extremidade N-terminal (BOYE *et al*., 2010; GONZÁLEZ-PÉREZ; ARELLANO, 2009).

Embora as proteínas vegetais apresentem uma alternativa relevante, devido a sua alta disponibilidade, custos abordáveis, baixa alergenicidade e bom valor nutricional, existem desafios a serem vencidos para que seja possível a otimização de sua utilização como ingrediente alimentar. Certas limitações quanto à solubilidade e conseguintes funcionalidades tecnológicas devem ser contornadas.

A dispersibilidade se refere à facilidade com que os agregados em pó são capazes de se separar e dispersar uniformemente na água. Alguns dos principais fatores que influenciam a solubilidade da proteína são o pH do solvente, força iônica, temperatura e propriedades de superfície das proteínas, em particular a quantidade e distribuição de porções de aminoácidos hidrofílicos e hidrofóbicos na superfície (DAMODARAM *et al.*, 2008; LAM *et al*., 2018).

Na água, resíduos de aminoácidos hidrofílicos tendem a se orientar em direção à interface do solvente, enquanto a maioria dos resíduos hidrofóbicos está enterrada no interior da proteína para minimizar a energia livre. Os resíduos hidrofóbicos que permanecem na

superfície, tendem a interagir preferencialmente entre si, diminuindo este modo a dispersibilidade (ALZAGTAT; ALLI, 2002; LAM et al., 2018).

Alebiyi e Aluko (2011) verificaram que a o isolado proteico de ervilha comercial demonstrou uma baixa solubilidade, onde valores máximos de 30% foram alcançados em pH 8.

Métodos físicos e químicos podem ser utilizados com o objetivo de modificar as propriedades físico-químicas e conseguintes propriedades técnico-funcionais das proteínas. Dentre estes métodos o tratamento físico com ultrassom de alta frequência, é uma tecnologia emergente que vem sendo empregada para realizar tais modificações.

1.4 Tratamento de ultrassom

Ultrassom de alta intensidade é um método físico utilizado para modificar a estrutura da proteína. A modificação proteica induzida por ultrassom é frequentemente atribuída à cavitação acústica, que por sua vez auxilia a reduzir o tamanho dos agregados de proteínas e alteram a estrutura molecular da mesma (WEISS *et al*., 2011).

A aplicação do ultrassom promove o aparecimento de ondas sonoras, as quais dissipam através de meios líquidos, resultando em ciclos diferenciais de pressão, com taxas dependendo da frequência. Estes ciclos de pressão criam zonas de vácuo no líquido que eventualmente colapsam durante o ciclo de alta pressão provocando alta turbulência local, forças de cisalhamento, pressões e temperaturas (RAVIYAN, ZHANG, & FENG, 2005, ASHOKKUMAR *et al*., 2009).

As principais vantagens da utilização do ultrassom estão relacionadas a ser uma tecnologia considerada segura, não toxica e não prejudicial ao ambiente (KENTISH & ASHOKKUMAR, 2011). O ultrassom pode ser classificado em duas categorias, as quais são baseadas na frequência e na potência como mostra a Tabela 2.

Tabela 2: Classificação da aplicação de ultrassom em potência e frequência.

Categoria	Classificação	Faixa abrangida
Frequência	Baixa	20 a100 kHz
	Alta	100 kHz a 1 MHz
Potência	Baixa	<1 W cm^{-2}
	Alta	10 a 1000 W cm^{-2}

Fonte: O'Sullivan *et al*. (2016).

A combinação de baixa potência com alta frequência é comumente utilizada para a avaliação analítica de propriedades físico-químicas de alimentos, enquanto que a utilização de baixa frequência com alta potência é empregada para alteração de alimentos, seja física ou quimicamente (O'SULLIVAN *et al*., 2016; CHEMAT *et al.*, 2011; MCCLEMENTS, 1995).

A aplicação de ultrassom em soluções aquosas de proteínas tem sido relatada em vários seguimentos do setor alimentício, como laticínios (caseinato de sódio e isolado proteico de soro) e outras proteínas de origem animal (gelatina e proteínas da clara do ovo) (O'SULLIVAN *et al.*, 2016; O'SULLIVAN *et al.*, 2014; JIANG et al., 2017). Além disso, estudos também mostraram a aplicabilidade do ultrassom em proteínas de origem vegetal (isolado proteico de soja, ervilha e arroz) como apresentado na Tabela 3.

Pode-se observar nos estudos citados, que em geral o ultrassom atua sobre as proteínas diminuindo o tamanho das partículas, melhorando a solubilidade das mesmas, além de expor grupos hidrofóbicos e sulfidrilas que se encontravam no interior da cadeia polipeptídica. Estes eventos contribuem para que ocorra uma alteração das propriedades técnico-funcionais de tais proteínas, otimizando sua utilização pela indústria de alimentos.

Tabela 3: Estudos realizados com a aplicação de ultrassom em proteínas de origem vegetal.

Fonte proteica	Objetivo	Condições de aplicação do ultrassom	Análises realizadas	Principais resultados	Referência
Isolado proteico de soja	Verificar os efeitos da cavitação hidrodinâmica e cavitação ultrassônica sobre as características físico-químicas e técnico-funcionais do isolado proteico de soja.	550 W nos tempos de 0,10,20 ou 30 min.	SDS-PAGE, tamanho hidrodinâmico, viscosidade, H_0 , grupos sulfidrilas livre, solubilidade proteica. Propriedades emulsificantes (IAE e IEE), e espumantes (capacidade de formação e estabilidade da espuma).	Não houve alterações no perfil eletroforético das proteínas, mas foi verificado uma redução da viscosidade e do tamanho hidrodinâmico. Maior exposição de superfícies hidrofóbicas e sulfidrilas. Melhora significativa na solubilidade, propriedades emulsificantes e capacidade de formação de espuma, porém verificou-se uma diminuição da estabilidade da espuma.	(REN et al., 2020)
Isolado proteico de soja	Verificar o efeito do ultrassom de alta frequência sobre as propriedade físico-químicas do isolado proteico de soja produzidos por diferentes métodos de desnaturação.	$80W \cdot cm^{-2}$; frequência de 20 kHz, por 0,10 ou 25 min.	Tamanho de partícula, solubilidade proteica, turbidez, dicroísmo circular, espectroscopia Raman, superfície hidrofóbica, grupos sulfidrilas livres, força do gel, capacidade de retenção de água e microscopia eletrônica de varredura.	A aplicação de ultrassom nos maiores tempos promoveu alterações na estrutura secundária e terciária das proteínas, diminuiu o diâmetro das partículas, aumentou a solubilidade, melhorou a hidrofobicidade da superfície e a propriedade de gelificação de diferentes agregados.	(ZHENG et al., 2019)
Isolado proteico de soja	Avaliar as diferenças físico-químicas de emulsões estabilizadas por IPS usando óleos de MCT, palma, soja e sementes de canola sob diversas condições de aplicação de ultrassom para produção de emulsões.	20 kHz, 50-55W/cm^2, 40% de amplitude Tempos: 2,6,12 ou 18min	Tamanho da partícula, proteína adsorvida, microscopia ótica e fluorescente e estabilidade ao tratamento térmico (50 e 90ºC) e ao sal (50,150 e 300 mM). As análises foram realizadas com óleo MCT, palma, soja e semente de canola.	As emulsões de óleo MCT (18 min) apresentaram menor tamanho de gotícula (d4,3) e tiveram a maior estabilidade, proteína adsorvida e menor tamanho de partícula. Emulsões à base de óleo de palma mostraram alta estabilidade térmica após tratamento térmico a 90 ° C. Tempos mais longos de ultrassom melhoraram a estabilidade da emulsão de diferentes emulsões de óleo em água.	(TAHA *et al*., 2018)
Gelatina bovina, gelatina de peixe, clara de ovo, ervilha, soja, arroz.	Verificar o efeito da aplicação de ultrassom em três proteínas de origem animal e em três proteínas de origem vegetal em relação às alterações nas propriedades físico-químicas e emulsificantes.	20 kHz, com amplitude de 95%, potência ~34 W/cm2. Tempo: Até 2 min.	Solução de proteínas: Espalhamento de luz dinâmico e estático, microscopia eletrônica de varredura criogênica, SDS PAGE e viscosidade intrínseca. Emulsão: Espalhamento estático de luz, tensão interfacial, microscopia eletrônica de varredura criogênica.	Redução do tamanho das partículas e do pH (no primeiro minuto) com o aumento do tempo de sonicação, a exceção do isolado proteico de arroz (IPA). Não houve diferença no perfil de peso molecular após a sonicação. Redução significativa da viscosidade intrínseca á exceção do IPA, sendo que as proteínas de origem animal apresentam maior valor de viscosidade intrínseca. Emulsão: Diminuição do tamanho da partícula de GB, PCO, IPE	(O'SULLIVAN et al., 2016).

Isolado proteico de soja (IPS) e glúten de trigo (GT)	Verificar a influência do pré-tratamento de ultrassom nas propriedades físicas e estruturais dos géis formados por misturas de IPS / WG induzidos por MTGase.	40 kHz, potencia 300W. Tempo (0, 10, 20, 30, ou 40 minutos).	Tamanho da partícula, Superfície hidrofóbica, Conteúdo sulfidrila livre, Força do gel, capacidade de retenção de água, microscopia eletrônica de varredura.	Redução do tamanho das partículas, acompanhado pelo aumento da exposição da superfície hidrofóbica e dos grupos sulfidrilas livre. Aumento da capacidade de retenção de água e da força do gel, resultando em redes mais densas e homogêneas.	(QIN *et al*., 2016)
Glicina da soja	Verificar o efeito do ultrassom de alta intensidade nas propriedades físico-químicas e funcionais da glicinina da soja em diferentes concentrações iônicas.	20 kHz a 80 W/cm^2; Tempo: 0 a 40 min; Forças iônicas: (I = 0,06, 0,2 ou 0,6) em pH 7,0.	SDS-PAGE Tamanho da partícula Solubilidade Dicroísmo circular Fluorescência H_0 Emulsão: IAE, IEE.	Não houve alterações significativas nas bandas obtidas no SDS-PAGE, assim como pequenas alterações nas estruturas secundárias e terciárias após o tratamento com ultrassom. Ocorreu uma diminuição do tamanho das partículas e aumento da exposição de grupos hidrofóbicos em todas as forças iônicas, sendo mais pronunciado em I=0,2. Houve um aumento da solubilidade e melhora nos IAE e IEE em todas as forças iônicas, no entanto estes são dependentes do tempo de aplicação do ultrassom.	(ZHOU et al., 2016a)
Isolado proteico de soja e isolado proteico de trigo	Avaliar as propriedades físico-químicas das proteínas de soja e trigo após tratamento de ultrassom, bem como verificar o desempenho emulsificante das proteínas em comparação com Tween 80.	20 kHz, ~34W/cm^2 com uma amplitude de 95%. Tempo: 2 min.	Distribuição de tamanho, SDS-PAG, viscosidade intrínseca. Emulsões: Tamanho das gotículas e tensão interfacial.	O ultrassom reduziu o tamanho dos agregados de ambas às proteínas. SDS-PAGE confirmou que o ultrassom não teve efeito sobre o peso molecular das proteínas (estrutura primária foi observada em ambos os casos, atribuída a energia insuficiente para hidrolisar a ligação peptídica). Emulsões preparadas com as proteínas não tratadas formaram gotículas submicrónicas (~ 150 nm) a concentrações superiores a 0,75%. Emulsões com proteínas sonicadas em [<0,75%] produziram gotículas menores e estabilidade a longo prazo em comparação com suas contrapartes não tratadas.	(O'SULLIVAN *et al*., 2016)
Isolado proteico de soja	Avaliar as modificações das propriedades espumantes e interfaciais de isolados proteicos de soja que passaram pela aplicação de ultrassom.	20 kHz, 4,27 ± 0,71 W e 20% de amplitude Temperatura: 75,80 e 85ºC. Tempo: 5,10,15 ou 20 min	DSC, solubilidade proteica, formação, drenagem e colapso da espuma, DLS, balanço de filme superficial.	O ultrassom melhorou a capacidade de formação de espuma com resultados sinérgicos com a temperatura pela diminuição do tamanho da partícula. No entanto, a estabilidade não foi modificada significativamente. A elasticidade invariável dos filmes interfaciais poderia explicar a estabilidade das espumas ao longo do tempo.	(MORALES *et al*., 2015)
Frações 7S e 11S isoladas de farinha de soja	Investigar as alterações estruturais e físico-quimicas das frações 7S e 11S em função da aplicação de ultrassom.	20 kHz, 400W (105-110 W/cm^2). Tempo: 0,5, 20 e 40 min.	SDS-PAGE, tamanho da partícula, solubilidade proteica, turbidez, dicroísmo circular, FT-IR, hidrofobicidade superficial, sulfidrila livre. Emulsão: IAE, IEE.	A aplicação de ultrassom diminuiu a turbidez e tamanho de partícula de 7S em tampão Tris-HCl 0,05 M a pH 7,0, enquanto aumentou Ho, solubilidade, atividade emulsificante (EAI) e estabilidade da emulsão (ESI). Similarmente, para 11S diminuiu a turbidez, aumentou o EAI, e efeitos mínimos no tamanho de partícula e na ESI. Os grupos SH das frações	(HU *et al*., 2015)

				7S e 11S diminuíram, não houve alteração na estrutura secundária mas aumentou ligeiramente a porcentagem de agregados de alto peso molecular.	
Isolado proteico de ervilha	Verificar o efeito da variação de pH e aplicação de ultrassom nas propriedades físico-químicas de isolado proteico de ervilha.	pH: 2,4,10 ou 12. Ultrassom: 20 kHz (68 W/100 mL), por 5 minutos.	Solubilidade da proteína Tamanho da partícula Superfície hidrofóbica SH livres SDS PAGE.	Aumento da solubilidade, superfície hidrofóbica e teor de sulfidrila livre e diminuição do tamanho da partícula após o tratamento com ultrassom. Os melhores resultados foram obtidos pela combinação de ultrassom e pH 12 a exceção do teor de sulfidrila livre e turbidimetria que apresentaram o melhor resultado a pH 12 sem a aplicação de ultrassom. .	(JIANG *et al*., 2017)
Isolado proteico de ervilha	Investigar os efeitos do tratamento de ultrassom de alta intensidade nas propriedades espumantes e estruturais do IPE	20 KHz, potência (22–25, 34–36 ou 45–48 W/cm2), por 30 minutos.	MEV, SDS-PAGE, dicroísmo circular, determinação de grupos sulfidrilas livre, superfície hidrofóbica, tamanho da partícula e potencial Zeta. Espuma: Capacidade de formação de espuma, estabilidade da espuma, microestrutura e tensão superficial.	Não houve diferença entre a proteína nativa e tratada com o ultrassom em relação ao perfil eletroforético. Não houve alteração na estrutura secundária da proteína, no entanto houve diferença na estrutura terciária uma vez que o tratamento com ultrassom diminui o tamanho da partícula, aumenta a exposição dos grupos hidrofóbicos e dos grupos sulfidrilas livre. A capacidade de formação de espuma aumentou com o aumento da amplitude do ultrassom, assim como a estabilidade de espuma após 10 minutos da formação da mesma. Ocorreu um aumento da taxa de adsorção com a aplicação do tratamento de ultrassom, acompanhando pela diminuição da tensão superficial.	(XIONG et al., 2018b)

Fonte: A autora. **Siglas utilizadas:** SDS-PAGE – Eletroforese em Gel de Poliacrilamida, GB – Gelatina Bovina, PCO – Proteínas da clara do ovo, IPA – Isolado proteico de arroz, MEV- Microscópio Eletrônico de Varredura, IAE – Índice de Atividade Emulsificante, IEE – Índice de Estabilidade Emulsificante, WG- Glúten de Trigo, MCT- Triglicerídeo de cadeia média, MTGase- Transglutaminase microbiana, FTIR- Espectroscopia de Infravermelho, H_0 – Superfície Hidrofóbica, DSC- Calorimetria diferencial de varredura, DLS- Espalhamento dinâmico de luz

1.5 Digestibilidade *in vitro*

A digestibilidade de proteínas é um fator importante para estimar a disponibilidade de proteína para absorção intestinal após a digestão refletindo sobre a eficiência da utilização de proteínas na dieta, e juntamente com a determinação do perfil de aminoácidos, são determinantes para a qualidade das proteínas (FAO 1985; HSU *et al* 1977; FAO / WHO / UNU, 2007).

Os métodos de digestibilidade *in vivo* , usado em animais ou humanos, geralmente fornecem resultados mais precisos, no entanto são demorados e caros, e por esse motivo muito esforço foi dedicado ao desenvolvimento de procedimentos *in vitro* (BOISEN & EGGUM, 1991). Os modelos de digestão *in vitro* fornecem uma alternativa útil aos modelos animal e humano por meio do rastreamento rápido dos ingredientes dos alimentos (COLES, MOUGHAN, & DARRAGH, 2005; (HUR et al., 2011).

O ensaio de digestibilidade proteica *in vitro* simula as condições dos processos digestivos que ocorrem no trato gastrintestinal humano por meio de enzimas proteolíticas e assim é possível medir a percentagem de proteínas que são hidrolisadas por tais enzimas (HUR *et al*., 2011).

As proteínas de origem vegetal apresentam menor digestibilidade quando comparadas como as proteínas de origem animal. A menor digestibilidade pode ser atribuída a fatores como, presença de compostos antinutricionais incluindo inibidores de tripsina, lectinas, fitatos e taninos, os quais promovem a ligação com proteínas ou receptores de proteínas (JAFFÉ, 1968; NIELSEN, 1991; CARBONARO *et al*.,1997). Além disso, fatores estruturais intrínsecos, incluindo estrutura primária e conformação de proteínas leguminosas, também foram propostos como sendo responsável pela baixa digestibilidade (NIELSEN 1991; CARBONARO *et al* 1997; CARBONARO *et al*., 2000).

Deste modo, tratamentos como cozimento, germinação e fermentação, são aplicados com o objetivo de melhorar a digestibilidade de proteínas vegetais. Umas das técnicas emergentes utilizadas para o mesmo fim, refere-se a aplicação de ultrassom, pois o mesmo afeta a estrutura das proteínas levando assim, ao aumento da digestibilidade, uma vez que a abertura da proteína pode potencializar o acesso de enzimas proteolíticas (BURITS E BUCAR, 2000; IN; SWANSON; BAIK, 2007).

1.6 Propriedades técnico-funcionais

As proteínas executam uma vasta gama de funções e por esse motivo a indústria possui grande interesse em sua aplicabilidade. A funcionalidade diversa desta macromolécula surge de sua complexa composição química. Essa diversidade de funções, aliada os aspectos nutricionais tornam tais biomoléculas interessantes para a utilização pela indústria de alimentos (O'CONNELL & FLYNN, 2007; WALSTRA & VAN VLIET, 2003).

Além do papel estruturante, as proteínas contribuem para a consistência e suculência de produto alimentícios devido às suas capacidades de gelificação, texturização, formação de massa, retenção de água e gordura. Além disso, outras propriedades desejadas são a formação e estabilidade de espumas e emulsões (GONZÁLEZ-PÉREZ; ARELLANO, 2009).

- Propriedades emulsificantes

As proteínas são de particular interesse nos sistemas alimentares devido a sua capacidade emulsificante, capacidade de adsorver a interfaces água/óleo e formar filmes interfaciais (FOEGEDING & DAVIS, 2011; LAM & NICKERSON, 2013).

As emulsões são sistemas termodinamicamente instáveis, formadas por dois líquidos imiscíveis, com um deles disperso como pequenas gotículas (0,01-10 μm) dentro do outro. A instabilidade de tal sistema se deve a alta relação interfacial área/volume, o que faz com que o sistema seja induzido a separação de fases (MCCLEMENTS & GUMUS, 2016). No entanto, é possível minimizar tal instabilidade pelo uso de emulsificantes, os quais se posicionam na interface reduzindo a tensão e energia livre global do sistema (MCCLEMENTS, 2004).

Para que uma proteína desempenhe um papel emulsificante eficaz, a mesma deve ser solúvel em água e absorver prontamente na interface óleo-água, desdobrar-se na interface e formar um filme coeso em torno de gotículas de óleo via interações intermoleculares (DAMODARAN, 2006). Estudos que envolveram a utilização de emulsões estabilizadas por proteínas globulares demonstraram que a estabilidade da emulsão depende da composição da solução (pH, força iônica, surfactantes e biopolímeros) e de tensões ambientais (aquecimento, congelamento e secagem) (MCCLEMENTS, 2004).

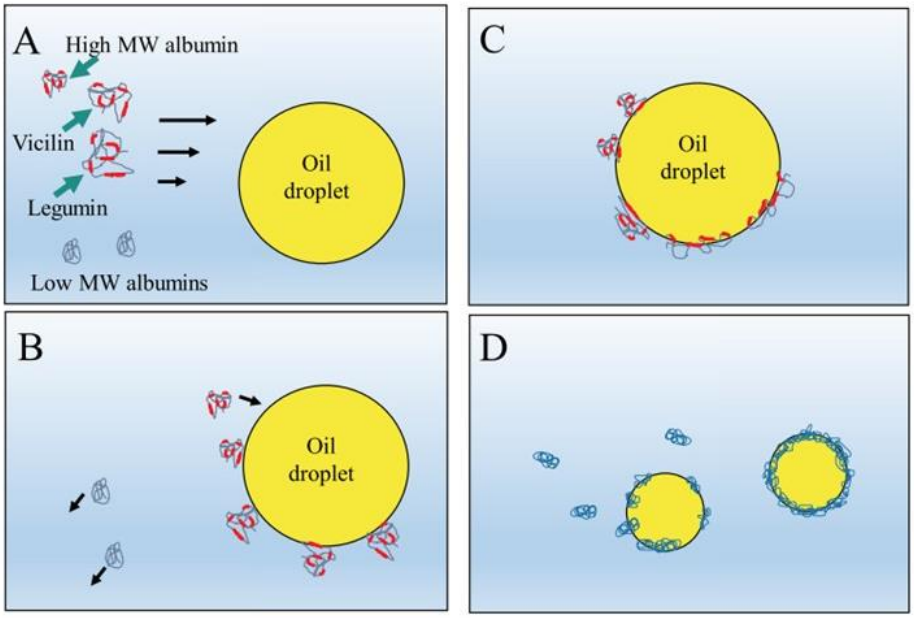
A atividade de superfície das proteínas deve-se à natureza anfifílica que essas moléculas possuem, devido à presença de regiões hidrofóbicas e hidrofílicas em suas cadeias peptídicas (BEVERUNG, RADKE, & BLANCH, 1999; O'CONNELL & FLYNN, 2007).

A adsorção de proteínas para a interface geralmente ocorre em duas etapas. Primeiro, as proteínas (incluindo globulinas e albuminas) migram e se ligam à interface água/óleo (Fig. 2A e B) (McCarthy et al., 2016). Depois que as moléculas de proteína chegam às interfaces e se conectam, as partes hidrofóbicas na superfície da proteína (marcados de vermelhos nas cadeias de proteínas, Fig. 2B) promovem a adsorção. Durante o segundo estágio, ocorre um rearranjo estrutural das proteínas, de modo que as moléculas podem desenovelar-se parcialmente e se realinhar para fazer com que as porções hidrofílicas se voltem para a fase aquosa, enquanto as porções hidrofóbicas interajam com a fase oleosa. Esse rearranjo das moléculas de proteína permite formar um filme viscoelástico na camada interfacial (Fig. 2D) que estabiliza a dispersão das gotículas de óleo por meio de repulsão eletrostática e impedimento estérico (BURGER; ZHANG, 2019).

A capacidade emulsificante das proteínas pode ser melhorada com o desenovelamento parcial das mesmas uma vez que neste processo os aminoácidos hidrofóbicos que se encontram no interior da estrutura proteica são expostos e adsorvidos na superfície de gotículas de óleo, enquanto que os aminoácidos hidrofílicos podem interagir com a fase aquosa atuando como uma barreira estérica contra coalescência e floculação (NISHINARI et al., 2014). Assim, a aplicação do tratamento de ultrassom de alta frequência é uma das maneiras de modificar a estrutura das proteínas bem como provocar o desenovelamento parcial da mesma como exemplificado nas pesquisas listadas na Tabela 3.

A utilização do isolado proteico de ervilha como emulsificante comercial em aplicações alimentares ainda é limitada, o que pode ser atribuído dois fatores: 1) escassez de conhecimento sobre suas propriedades estruturais e funcionais; 2) uma grande lacuna de desempenho entre proteínas de ervilha laboratoriais e aquelas preparadas comercialmente (HAN-NI LIANG & TANG, 2013).

Figura 2 - O comportamento das proteínas de ervilha na interface óleo-água em uma sequência de (A) migração, (B) adesão, (C) desenovelamento e reorientação parcial e (D) formação de um filme viscoelástico na interface.



Fonte: (BURGER; ZHANG, 2019).

Deste modo, este estudo objetivou estudar o efeito de tratamentos ultrassônicos em diferentes combinações de potência/tempo, mantendo a energia final constante, bem como o efeito de diferentes valores de pHs sobre as características físico-químicas do concentrado proteico de ervilha (CPE). Além disso, foram avaliados a digestibilidade *in vitro* e as propriedades emulsificantes do CPEs tratados. Assim espera-se que as modificações produzidas por tais tratamentos, possam contornar as limitações existentes e possibilitar o uso das proteínas de ervilha pela indústria.

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Capítulo II

Manuscrito: Combined adjustment of pH and ultrasound treatments modify techno-functionalities of pea protein concentrates.

ABSTRACT

The effects of ultrasound (HUS) treatments on pea protein concentrates (PPCs), at different pHs, in terms of their physicochemical, emulsifying and *in vitro* digestibility were evaluated. PPCs were studied at pHs 2.8, 4.3, 6.8 and no pH adjustment, each of them submitted to the following HUS power/time binomials: 412.5 W/581.82 s, 487.5 W/492.31 s, 562.5 W/426.66 s, 637.5 W/376.47 s, 712.5 W/336.84 s – total energy input constant equal to 240 kJ. HUS processing led to changes in physicochemical properties of PPC, but such chages depended on both pH and power/time binomials. Whatever the HUS treatment, increased dispersibility (in systems at pH 2.8 and without pH adjustment), increased exposure of hydrophobic surfaces (at pH 4.3, 6.8 and no pH adjustment) and increased free sulfhydryl groups concentration (at pH 4.3 and 6.8). Smallest average hydrodynamic diameters of dispersed PPCs were identified in systems at pH 2.8, 6.8 and no pH adjustment submitted to HUS treatments 412.5 W/581.82 s, 487.5 W/492.31 s, 562.5 W/426.66 s. *In vitro* digestibility of PPCs was also improved by HUS processing. Although all HUS tratments promoted changes in PPCs emulsifying properties, these properties were more clearly affected by pH: emulsions at pH 2.8 presented the highest emulsion stability and the lowest emulsifying activity index, suggesting technological applicabilities in acidic formulations. Although the total energy input was constant, the rate at which it occurred had a great impact in all studied variables. Indeed, power/time combinations 637.5 W/376.47 s and 712.5 W/336.84 s seemed to favor protein aggregation andexposure of hydrophobic amino acid residues, whereas the HUS treatments 412.5 W/581.82 s, 487.5 W/492.31 s, and 562.5 W/426.66 s tended to present an opposite behavior. In summary, this study showed that both pH pre-adjustement of PPCs and power/time binomials of HUS applied to them have to be taken into account, according to the techno-functionality intended to these protein materials.

KEYWORDS

Pea protein, Ultrasound, Emulsions, In vitro digestibility.

Abbreviations and symbols

AOAC – Association of Official Analytical Chemists

PPC – Pea protein concentrate

PPI – Pea protein isolate

DLS – *Dynamic Light Scattering*

FAO - Food and Agriculture Organization of the United Nations

g – gram

EAI – Emulsifying activity index

ICr - Creaming index

SPI - Soy protein isolate

kDa - Kilo-Dalton

kHz - Kilo-Hertz

L - Liter

mg - milligram

mL - milliliter

WHO - World Health Organization

O/W - Oil in water

W/O – water in oil

pH - Hydrogenionic potential

pI - Isoelectric point

ζ - zeta potential

RPM - Rotations per minute

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

H_0 - Hydophobic surface

SH- Free sulphidrillary groups

1. INTRODUCTION

During the last two decades, plant proteins have gained attractiveness and commercial added-value, mainly due to: i) the growing awareness of general health benefits associated with plant-derived foods, ii) the increasing number of vegan and vegetarian people, due to both ethics and religious issues, and iii) environmental concerns for minimizing wastes generated by fruit and vegetable processing industries, making the plant protein ingredients market more and more relevant business [1–3]. In 2017 the global market value for proteins of plant origin was 10.5 billion dollars, and its estimate for 2023 is 16.3 billion dollars. In addition, it is expected that 2054 about 1/3 of all proteins consumed will be obtained from plant sources [4].

Among plant-derived proteins, pea proteins (both protein concentrates and isolates) deserve special attention. Indeed, currently they represent the main alternative to soy proteins, which remain the most consumed vegetable proteins. When compared to soy proteins, pea proteins have comparable nutritional characteristics [5]. In addition, pea proteins present the following advantages: industrially affordable costs [3,4], low incidence of allergenicity (compared to other plant sources, such as soy and peanuts) [6], high antioxidant potential [4,7], elevated contents of essential branched chain amino acids, as well as high lysine content (71 mg / g protein), which is a limiting amino acid in cereals [8]. In addition to the nutritional factor, the potential techno-functionalities of pea proteins, such as emulsifiers, foams and gelling properties, contribute to the make them worthy studying food ingredients. However, restrictions related to their low solubility in pH ranging from 4 to 7 and [5] , mainly, the scarceness of data on their techno-functional properties within this pH range make them still underutilized by the food industry [9,10]. The low solubility of pea proteins is mainly related to their structural features [1,11]. They are mostly globular proteins (approximately 70%), which are soluble in saline solutions and only slightly soluble in water. They are usually divided into the following fractions: legumins (~ 360 kDa) which have a hexameric structure, vicillins (~ 150 kDa) and convicillins (~ 210 kDa) with a trimeric structure [1,12,13].

In order to overcome the techno-functional limitations resulting from reduced solubility of different proteins in aqueous dispersions, chemical (tryptic hydrolysis and pH-shifting) and physical (microfluidization, high hydrostatic pressure, ultrasound) processing approaches have been developed [14–17]. Among these methods, high intensity ultrasound (frequency from 20 to 100 kHz and power between 10 and 1000 $W \cdot cm^{-2}$) has been frequently reported [4,5,18–21]. High-intensity ultrasound is a technology that offers non-negligible advantages compared to other physical approaches. In fact, sound waves are generally considered safe, as they are

environmentally friendly, are not associated with the generation of toxic compounds [22], and promote less nutritional and sensory harming in the processed food products, when compared to other physical methods using heat [23, 24]. Mechanistically, the effect of ultrasound on native structure of proteins is related to acoustic cavitation, which causes differential pressure cycles, shear stresses, turbulence and heating [19,23,25]. The chemical and mechanical effect of these factors promote changes in the structure of protein molecules and supramolecular structures, reducing the size of the aggregates and exposing to the surface hydrophobic and sulfhydryl groups located in the 'core of native proteins' structures. These changes usually exert a great impact on both techno-functional performance and digestibility of proteins [16,23,26–28]

Some studies dealing with high-intensity ultrasound-based approaches to modify physicochemical properties and, therefore, to enhance techno-functionality of plant proteins (including pea proteins) are available [5,15,16,21,29–32]. However, to the best of our knowledge, the most usual scientific databases lack studies addressing specifically the effects of the rates at which ultrasound energy is applied to the pea proteins' dispersions, combined with the effects of pH. Thus, the present study intended to contribute to the research field, by examining the effects of pre-treatments with high intensity ultrasound (20 kHz) on aqueous dispersions of pea protein concentrates, using several combinations of processing time and power (but keeping the total energy input constant), together with variations of pH (near, above and below the average isoelectric point). The pea protein concentrates submitted to each treatment were studied in terms of different physicochemical characteristics, and evaluated for their emulsifying properties as well as their digestibility *in vitro.*

2. Materials and Methods

2.1 Materials

The pea protein concentrate (PPC) was purchased from Shandong Jianyuan Foods Co., Ltd – China (Attachment A), and was a kind gift from Nutricium industry and commerce LTDA. Soy oil (Bunge Alimentos, Brazil, lot: 05 U- may 2019) was bought from a local market. The other reagents were purchased from Sigma Aldrich Corporation (USA), as follows: hydrochloric acid (ID 329753973, purity ≥ 98%), boric acid (ID 329773111, purity ≥ 99.5%), β-mercaptoethanol (ID 57954402 , purity ≥ 99%), 8-anilino-1 naphthalenesulfonic acid (ID 24890494) , sodium hydroxide (ID 329824595, purity ≥ 97%) , 5.5-dithiobis- (2-nitrobenzoic acid)(ID 218200), porcine pancreatin (P7545) and pepsin (P7000). Deionized water was obtained from a Mili-Q system (~18.2 MΩ·cm^{-2}, 25ºC; Reference A^{+}, Millipore, Italy). All chemicals were used without additional purification.

2.2 Centesimal composition of pea protein concentrate

Pea protein concentrate (PPC) chemical composition was estimated according to the Association of Official Analytical Chemists [33]: 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). The conversion factor used for the determination of total proteins was 6.25 [3].

2.3 Average isoelectric point (pI) of pea protein concentrate

Average isoelectric point estimative of PPC was performed by measuring the ζ-potential of PPC aqueous dispersions at different pH values, according to the methodology adopted by [34], with some modifications. Briefly, PPC dispersions [0.1% (w/v)] were prepared in deionized water, and their pH was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 (pH/conductometer 914, Metrohm – Switzerland) by carefully dropping concentrated solutions of HCl or NaOH (1 mol·L^{-1}), under magnetic stirring. ζ-potential measurements were carried out in a Zetasizer analyzer apparatus (Nano Zs, Malvern, England) [35–37].

2.4 Ultrasound treatments of pea protein concentrates

PPC dispersions [5% (w/v)] were obtained by adding appropriate amounts of PPC to deionized water in beakers. Subsequently, the pH of the mixtures was adjusted to three previously defined values (pI, or pI minus 1.5 units, or pI plus 2.5 units). These values were chosen: i) to remain within a pH range technologically logical, ii) to ensure PPC dispersibility enough to proceed to the subsequent steps of the study, and iii) to obtain systems with the

proteins predominantly neutral, positively charged, or negatively charged, respectively. In all cases, the mixtures were kept under magnetic stirring (Magnetic Stirrer BS-2H, Lab Science, China) at 25 ° C for 1h. The beakers were covered with aluminum foil (to avoid effects of light) and sealed with waterproof plastic film (to prevent evaporative water loss). Ultrasound application was performed using an ultrasound device (Sonics, VCX 750, USA) equipped with a 13 mm diameter titanium probe. The dispersions were immersed in an ice bath during the procedure, in order to prevent overheating. The ultrasound was operated at a constant frequency of 20 kHz, with six distinct power and time combinations, providing the same energy input to the system (Table 1). The study contains a total of 24 treatments, which are: three pH values evaluated in five power/time combinations, control of each of the five powers no pH adjustment, three controls with pH values adjusted to the value of pI, or pI minus 1.5 units, or pI plus 2.5 units), without ultrasound treatment and a control no pH adjustment and without ultrasound treatment. Then, the systems were frozen at -40 °C, lyophilized, packed in plastic vials with a lid threads covered laterally with aluminum foil and stored in a refrigerator at 4 ºC ± 1 ºC. These PPC powders were used in the subsequent experiments (sections 2.5 to 2.7).

Table 1: Combinations of pH, time and ultrasound application power.

pH	Power (J/s)	Time (s)	Energy supplied (kJ)
pI minus 1.5 units	0	0	0
	412.5	581.82	240
	487.5	492.31	240
	562.5	426.66	240
	637.5	376.47	240
	712.5	336.84	240
pI	0	0	0
	412.5	581.82	240
	487.5	492.31	240
	562.5	426.66	240
	637.5	376.47	240
	712.5	336.84	240
pI plus 2.5 units	0	0	0
	412.5	581.82	240
	487.5	492.31	240
	562.5	426.66	240

	637.5	376.47	240
	712.5	336.84	240

2.5 Physicochemical characterization of ultrasound treated PPCs

2.5.1 Dispersibility Profile

The dispersibility in aqueous media of ultrasound treated PPCs was determined in the range of pH 2 to 9. Values of pH were adjusted by carefully dropping concentrated solutions of HCl or NaOH (1 mol· L^{-1}) to the dispersions, following the methodology described by Ladjal-Ettoumi et al. (2016) [38], with some modifications. Briefly, PPC dispersions [0.5% (w/v)] were prepared in deionized water, and kept under magnetic stirring (Magnetic Stirrer BS-2H, Lab Science, China) for 4 hours at room temperature (25 °C). Then, the pH (pH/conductometer 914 Metrohm – Switzerland was adjusted to the desired values and the resulting dispersions were centrifuged at 7100 g for 10 min (Centrifuge 5430, Eppendorf, Germany). The supernatant was collected using a 10 to 100 µL micropipette (Eppendorf Research, Germany) and the concentration of the dispersed proteins was determined using the Bradford methodology [39].

2.5.2 ζ potential

ζ potential analysis of dispersed particles was performed as described in section 2.3. However, the dispersions had the pH value adjusted to one of three desired pH values (pI, or pI minus 1.5 units, or pI plus 2.5 units).

2.5.3 Hydrophobic Surface (H_o)

The surface hydrophobicity of ultrasound treated PPCs was determined using a fluorescent marker, 8-anilino-1-naphthalenesulfonic acid (ANS), according to the methodology reported by Kato and Nakai [40], also considering the modifications more recently proposed by Oliete et al. (2018) [3], as follows: PPC dispersions [3.0% (w/v)] were prepared in 10 mmol·L^{-1} phosphate buffer (Na_2HPO_4 - MM 146.96 ; NaH_2PO_4 – MM 119.98,) , which were kept under stirring (Magnetic Stirrer BS-2H, Lab Science, China) for 1h at 25 °C. Then, the dispersions were centrifuged at 10,000 g for 25 min (Centrifuge 5430, Eppendorf, Germany) and the protein content of the supernatant was quantified using the Bradford methodology [39]. Subsequently, the dispersed protein concentration was adjusted to 0.5 mg·mL^{-1} and again quantified by Bradford [39] analysis, with the objective of standardizing the concentration of proteins to be analyzed. Dilutions were performed up to 0.004 mg·mL^{-1}. Then, six microliters of the 1mM ANS solution was added and the mixture was kept in the dark for 15 min. Fluorescence intensity

was measured using an luminescence spectrometer SpectraMax M5 (Molecular devices, California, EUA) at 380 nm (excitation) and 460 nm (emission). The "blank" was composed by the ANS in phosphate buffer only. The initial slope of the fluorescence intensity versus protein concentration ($mg \cdot mL^{-1}$) plot was calculated by linear regression analysis and used as the H_o index.

2.5.4 Free sulfhydryl groups

The content of free sulfhydryl groups was determined by the Ellman reagent method (5,5-dithiobis-(2-nitrobenzoic acid), DTNB), according to the methodology recently described by Xiong et al. (2018) [20], with some modifications. Briefly, PPC samples were dispersed [1.0% (w/v)] in Tris-HCl buffer which contained 86 mM Tris, 90 mM glycine and 4 mM ethylenediamine tetraacetic acid (EDTA), at pH 8.0. The dispersions were stirred (Magnetic Stirrer BS-2H, Lab Science, China) for 1h at 25 ° C and then centrifuged at 7100 g for 20 min (Centrifuge 5430, Eppendorf, Germany). Supernatant protein concentration was estimated using the Bradford methodology [39]. Then, 80 µL DTNB was added to 2 mL of PPC dispersion and the mixture was stirred (Magnetic Stirrer BS-2H, Lab Science, China) for 15 min, in the dark. Subsequently, the absorbance was determined at 412 nm. The "blank" was composed by DTNB in Tris-HCl buffer only. The extinction coefficient used to calculate the free sulfhydryl content was determined to be 13,425 $M^{-1} \cdot cm^{-1}$ [20,41].

2.5.5 Hydrodynamic Size Distribution

Ultrasound treated PPCs were dispersed in deionized [0.1% (w/v)] and kept under stirring (Magnetic Stirrer BS-2H, Lab Science, China) during 1h. The dispersion pH was then adjusted to one of the three desired pH values (pI, or pI minus 1.5 units, or pI plus 2.5 units), and then centrifuged at 7100 g for 25 min (Centrifuge 5430, Eppendorf, Germany). These systems were then placed in a plate with microwells and subjected to analysis at 25 °C, using a Zetasizer ZS Dynamic Light Scattering (DLS) Analyzer (Zetasizer Nano-ZS, Malvern Instruments, United Kingdom) with a scatter angle of 173°, according to the methodology described by Soares et al. and Galvàn et al. [35,36].

2.6 *In vitro* digestibility simulation of ultrasound treated PPCs

The *in vitro* digestibility of ultrasound treated PPCs was assessed according to the methodology reported by Lo and Li-Chan (2005) [42] with modifications more recently proposed by Chen, Zhao, and Sun (2013) [43], using a static gastrointestinal model. Briefly, PPC dispersions [3.0% (w/v)] were prepared in deionized water, which was stirred for 1h. Upon

reaching 37 °C in a controlled environment, the gastric digestion step was started. At this stage, the pH was adjusted to 2 with HCl (2 mol·L^{-1}) and then pepsin (2.0% w/w, protein base) was added. The solution was incubated at 37 °C for 1 h. After that, the material was submitted to the enteric digestion step. The pH was adjusted to 7 with NaOH (2 mol·L^{-1}) and pancreatin (2% w/w, protein base) was added. The dispersion was incubated at 37 °C for 2h and then submerged in a boiling water bath for 5 minutes, to stop the activity of the digestive enzymes. Aliquots were taken for analysis of polyacrylamide gel electrophoresis (SDS-PAGE) before digestion, at the end of the gastric phase and at the end of the enteric phase. The 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.

After each digestibility step, aliquots were analyzed by the SDS-PAGE technique under reducing conditions, according to the methodology developed by Laemmli (1970) [44]. In each case, 10 µL aliquots (taken before digestion and after each digestion step) was added to 5 µL of sample buffer (0.187 M Tris-HCl at pH 6.8; 50% (v/v) glycerol, 6% (m/v) SDS, 1% (w/v) bromophenol blue, 15% (v/v) β-mercaptoethanol) and heated at 90 °C for 5 min. After cooling to room temperature, 15 µL of the solution was inserted into the gel spots, which was composed by 12% (w/v) separating gel and the 4% (w/v) stacking gel of acrylamide. Electrophoresis was performed at 150 V/180 min. Upon the running, the gel was stained using a solution of Coomassie Brilliant Blue R (0.1% (w/v) in 10% acetic acid and 40% (v/v) methanol) for 2 hours, under gentle stirring. The gel was then bleached with a discoloration solution consisting of 10% (v/v) acetic acid and 40% (v/v) methanol and 50% water under gentle stirring until excess dye was removed from the gel. The molecular weight standard used was 10-245 kDa (Sinapse INC2, EUA).

2.7 Emulsifying properties of ultrasound treated PPCs

2.7.1 Preparation of emulsions

Firstly, ultrasound treated PPCs dispersions in water [0.75% (w/v)] were prepared. Next, the pH of these dispersions was adjusted to one of three desired pH values (pI, or pI minus 1.5 units, or pI plus 2.5 units). In each case, O/W emulsions were produced with 95% (w/w) of PPC aqueous dispersion + 5% (w/w) of soybean oil. The two liquids were firstly mixed in a homogenizer (Ultra-Turrax DI 25 Basic, Yellow Line, India) operating at 24,000 rpm for 1 min. Then, the pre-emulsion formed was sonicated (Sonics, VCX 750, USA) at constant frequency

(20 kHz), by applying a power of 525 W for 4.0 min. During the entire emulsification process, the beakers were held within an ice bath, in order to prevent overheating.

2.7.2 Emulsifying activity index (EAI)

The EAIs were empirically assessed by Pearce and Kinsella turbidimetric method (1978) [45], as adapted by Lelas et al. (2009) [46], with some additional modifications, as follows: First, 10 μL of the emulsions were sampled 5 cm from the bottom of the receptors and diluted 1: 200. Sodium dodecyl sulfate (SDS) at a concentration of 0.1% was used as diluent in each case. Then, the absorbances at 500 nm of the diluted emulsions were measured on a UV / Visible spectrophotometer (Cary 50 Probe, Varian, USA) using acrylic cuvettes (path length 1 cm). Finally, the IAE was calculated for each system using Equation 1.

$$\mathbf{EAI}\left(\frac{\boldsymbol{m^2}}{\boldsymbol{g}}\right) = \frac{\mathbf{2} \cdot \mathbf{2,303} \cdot \mathbf{Ao} \cdot \mathbf{DF}}{\boldsymbol{C} \cdot \boldsymbol{\varphi} \cdot \mathbf{10000}} \qquad \mathbf{1}$$

In equation 1: **Ao** (dimensionless) is the absorbance of the diluted emulsion immediately after homogenization; **DF** (dimensionless) is the dilution factor (200); $\boldsymbol{C}$ (0.0075 g·mL^{-1}) is the protein concentration in the aqueous phase prior to emulsion formation and $\boldsymbol{\varphi}$ (dimensionless) is the oil volume fraction of the emulsion (0.05).

2.7.3 Microstructure

The microstructure of the emulsions was studied using an optical microscope (CX40, Olympus, USA), as described by Soares et.al (2019) [46]. Emulsion aliquots (5 μL) were sampled 0.5-1.0 cm below the surface and poured onto microscope slides. The microscope slides were then covered with glass cover slips and observed at a magnification of 100 x. For each emulsion, at least ten images were taken using a digital camera (13 megapixels). The photomicrographies of each emulsion were taken immediately after preparation and at different storage times (1, 5 and 10 days).

2.7.4 Macroscopic kinetic stability

The macroscopic kinetic stability of the emulsions was evaluated in terms of creaming index (ICr) values, measured periodically during 30 days, similarly to the protocol described by Liu and Tang (2013) [42], with slight modifications. Briefly, 10 mL of each fresh emulsion was sampled and transferred by means of Pasteur pipettes to falcon tubes, which were stored vertically at room temperature. Lower serum height (Hs) and total emulsion height (Ht) were recorded during 30 days. In the first seven days, these values were measured every 24 hours,

and then on days 10, 15, 20, 25 and 30 after the production of emulsions. Creaming index was calculated according to equation 2.

$$\boldsymbol{ICr}\ (\%) = \frac{\mathbf{Hs}}{\mathbf{Ht}} \cdot \mathbf{100\%} \qquad \mathbf{2}$$

The curves originating from this analysis were adjusted according to the mathematical model presented in equation 3.

$$\boldsymbol{ICr = ICr_{eq}(1 - e^{-kt})} \qquad \mathbf{3}$$

2.8 Statistical analysis

Statistical analysis were performed using the SAS software (version 9.3, SAS Institute Incorporation, USA; licensed by the Universidade Federal de Viçosa). ANOVA analysis of variance and Tukey's test (with a probability level of 5%, p> 0.05) were used to assess statistical differences between means. Comparisons were made by evaluating all power / time combinations at the same pH value and also the influence of different pH values in each ultrasound treatment. Analyzes of protein dispersibility profile, ζ potential, Ho, SH groups, D_h and EAI were performed in triplicate. Electrophoresis (SDS-PAGE), macroscopic kinetic stability and microstructure were performed in a duplicate. In all cases, three repetitions were performed and the data represented by mean ± standard deviation. In vitro digestibility was performed in triplicate.

3. Results and discussion

3.1 Centesimal composition and average isoelectric point of pea protein concentrate

An analysis of the proximate composition of the pea protein concentrate (PPC) aimed to characterize the material to be used in the study and, mainly, to verify its content of proteins. The composition of the PPC is summarized in Table 1. PPC had a total protein content of approximately 74%, which in fact characterizes it as a concentrate. The studies Jiang et al., McCarthy et al. and Xiong et al. [5,15,20] found a protein content of 85, 82 and 94.3% for pea proteins and Arzeni et al. and Morales et al. [22,47] reported a protein percentage of 94.94 and 90.5% for soy proteins. In all these studies, the protein material was called isolate, showing that protein contents above 80% are being considered as isolates.

Table 2: Proximate composition of pea protein concentrate used in the present study.

Componets	Content (%)
Moisture content	8.9
Ashes	5.3
Lipids	0.4
Proteins	73.9
Carbohydrates and fibers	11.3
Total	**100.0**

The average isoelectric point (pI) of the proteins present in the PPC was estimated. The isoelectric point is defined as the pH value at which the net electrical charge of the proteins is zero. Since the net electrical charge of disperse colloidal particles is directly proportional of their ζ-potential (and the same sign), isoelectric point can be inferred as the pH at which ζ-potential is zero. Thus, as shown in Figure 1, the mean pI of PPC was identified as pH 4.3 (see next section). The pI value found is close 4.5, which is commonly reported as the isoelectric pH of pea proteins. Ezpeleta et al. [48] and Krishina, Croy and Boulter [49] found a pI value for vicillin and legumin of 4.5 and 4.7 respectively, Jiang et al. [5] described that the pI of the pea protein isolate (PPI) (85% of proteins) is between 4 and 5, Chao et al. [1] reported for IPP (82% protein) an average pI of 4.5. Based on the isoelectric point of PPC, the two other pH values were chosen to be investigated in the present study were 2.8 (pI minus 1.5 units) and 6.8 (pI plus 2.5 units). As previously explained, this choice is justified by the following purposes: i) to work within a pH range technologically logical, ii) to ensure PPC dispersibility enough to proceed to the subsequent steps of the study, and iii) to obtain systems with the proteins predominantly neutral, positively charged, or negatively charged (at pH 4.3, 2.8 and 6.8, respectively).

3.2 Physicochemical characterization of ultrasound treated PPCs

3.2.1 Dispersibility profile and ζ-potential

Figure 2 shows the dispersibility profile of PPCs with and without ultrasound treatment, previously submitted to different pHs. Pre-treatment with ultrasound combined with the pH adjustment positively influenced the dispersibility at pH 2.8 and 8.5 (without pH adjustement), although the same was not observed for the other pHs (4.3 and 6.8). In addition, the systems that underwent only pH adjustment, without the ultrasound treatment (No HUS), did not present an increase in dispersibility when compared to the controls (no pH adjustment and without ultrasound treatment).

Treatments without pH adjustment showed the greatest dispersibility, followed by the those whose pH was adjusted to 2.8. One can note a clear the influence of pH on the dispersibility of the PPC (U-shaped dispersibility profile). In particular, in all cases it appeared that the minimum dispersibility occurred in the pH range 4-6, suggesting that the average isoelectric point of PPC is within this pH range. Similar results were reported by Wei et al.; Chavan et al.; Liu et al. [50–52], who found minimal dispersibility of PPI (protein content > 80%), at pH values close to pI (4.5). The knowledge of the pH range in which the system is dispersible is essential for the elaboration of products with the desired technical-functional quality.

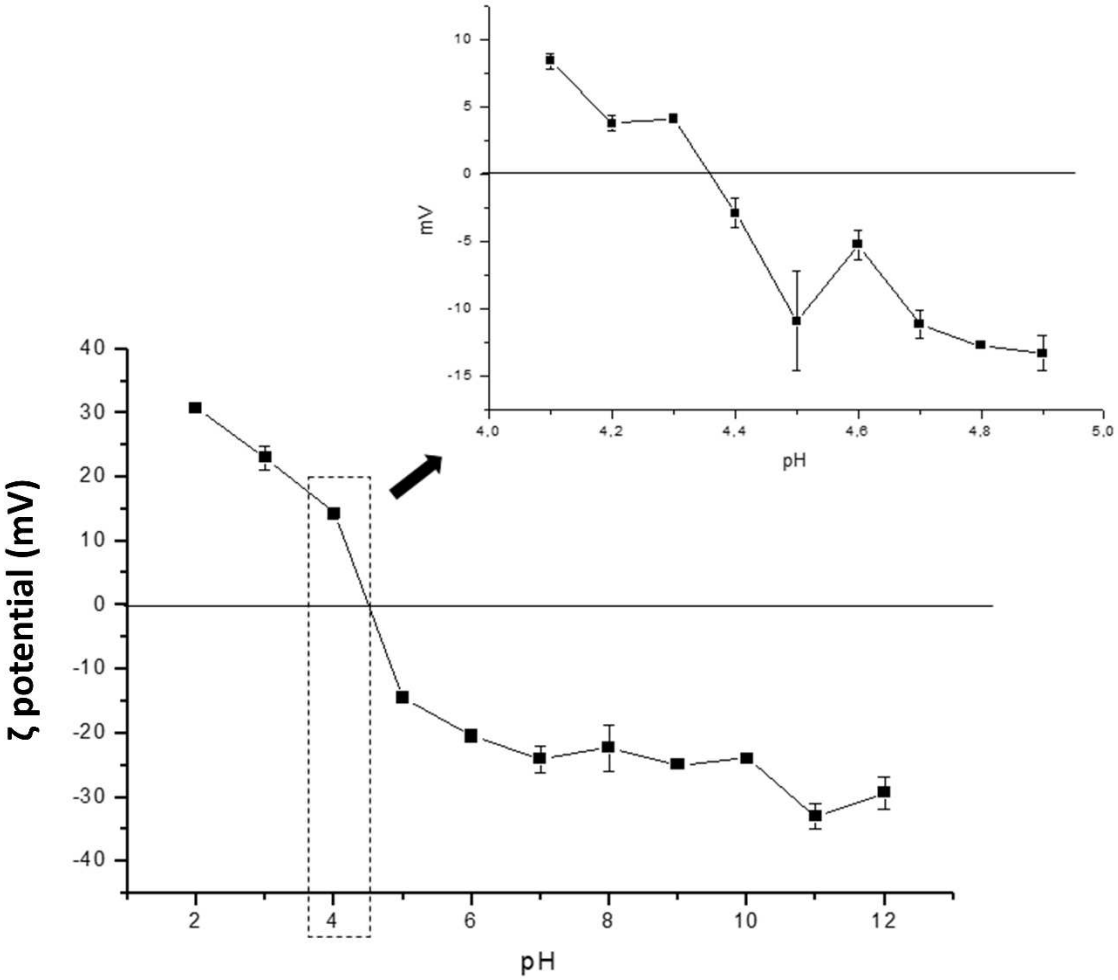

Figure 1: Estimation of the mean isoelectric point (pI) of the PPC proteins in the present study.

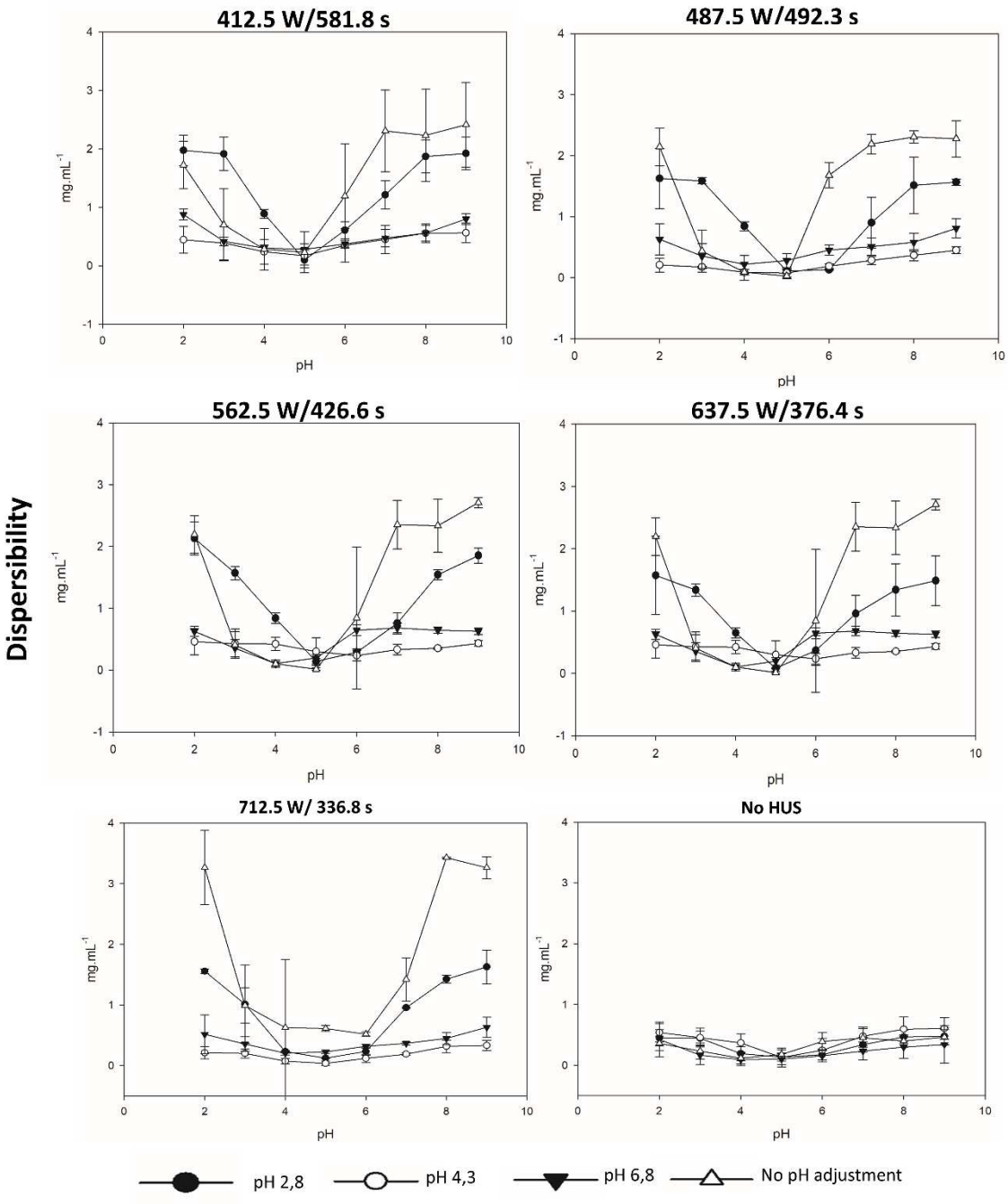

Figure 2: Protein dispersibility profile of samples treated and not treated with ultrasound.

In this study, a minimum dispersibility pH range (4-6) was identified, as the ultrasound pre-treatment may have exposed amino acid residues which, in the native proteins conformations, were buried inside the protein structure. This is further reinforced by the analysis of ζ-potential results (Figure 3A). Indeed, ζ-potential, which reflects the density of electric charges upon dispersed macromolecules and colloidal particles, approached zero at pH ~ 4.3. Therefore, the two other pH values selected for the study were 6.8, at which the proteins constituting the PPC

remained with negative net electric charges, and 2.8, at which these proteins remained with positive net electric charges. The magnitude of the positive charges identified at pH 2.8, as well as the negative charges of PPC without pH adjustment (pH ~8.5), suggests that the greater dispersibility at such pHs values is mainly related to electrostatic repulsion, which seems to be strong enough to prevent proteins from approaching and facilitate hydration (or solvation) of the chains. On the other hand, the lower dispersibility observed in the system with a pH value of 6.8 suggests that the magnitude of negative charges in this pH value was not sufficient to avoid the aggregation process promoted by protein-protein interactions. For systems with pH 4.3 the low dispersibility can be attributed to the weak electrostatic repulsion (close to pI), which could favor the aggregation of proteins.

3.2.2 Hydrophobic surface (H_0)

Through H_0 index of PPCs submitted to different power/time ultrasound and pH treatments (Figure 3B). One can observe that all ultrasound treatments increased the exposure of the hydrophobic domains of the proteins that were previously buried in the protein molecules in their native conformation. Jiang et al. (PPI, with 85% protein) and Hu et al. (SPI, with 90% protein) [5,53], who studied the effect of ultrasound on the properties of pea and soy protein isolates, respectively, also reported a similar effect of ultrasound on the surface hydrophobicity of these proteins. In fact, the increase of the hydrophobic surface is often associated with the enhancement of techno-functional properties of proteins' concentrates and isolates, such as emulsifying, gelling and foaming capacities [54].Significant exposures ($p < 0.05$) of hydrophobic regions occurred in systems at pH 4.3, 6.8 and in those without pH adjustment. When setting the pH at 2.8 and evaluating ultrasonic treatments (lower case comparisons), it appears that the H_0 indices of the systems were not different ($p > 0.05$) from those found for systems without ultrasound treatment.

For systems with pH adjusted to 4.3, the greatest exposure occurred for the ultrasound treatment at 637.5 W/376.47 s, reaching an H_0 index value of 2294.8 whereas the control treatment (No HUS) at the same pH exhibited a value of 305.6 (about 9 times smaller). At pH 6.8, compared with the No HUS system at the same pH value, it was found that there was a significant increase in the exposure of the hydrophobic amino acid residues, and in the highest applied power 712.5 W/336.84 s the increase was about 11 times. For treatments no pH adjustment, the greatest exposure of the hydrophobic regions occurred in the power of 487.5 W/492.31 s, with an increase of approximately 5 times compared to the No HUS system. In

addition, it was found that, at pH 2.8, the ultrasound treatment had no significant influence on the protein unfolding, with consequent exposure of hydrophobic surfaces.

As seen in the dispersibility profiles (Figure 2), dispersions with pHs adjusted to 4.3 and 6.8 were those with the lower dispersibility, which suggests that at this pH range, protein-protein interactions are more expressive than protein-solvent interactions, favoring the formation of protein aggregates. These results are corroborated by H0 indexes results here reported.

The cavitation phenomenon and mechanical tubulence generated by the application of high-frequency ultrasound has the ability to disrupt protein aggregates, thereby exposing hydrophobic regions that previously interacted with each other [55]. For dispersions no pH adjustment, it is reasonable to infer that the expressive exposure of hydrophobic regions was mainly induced by intramolecular electrostatic repulsions that act by unfolding the protein structure. Indeed, such systems presented pH around 8.5, i.e., the proteins were predominantly negatively charged [56].

3.2.3 Free Sulfhydryl Groups (SH)

Figure 3C shows the content of sulfhydryl groups in the PPCs with and without ultrasound treatment at different pH values.

In systems with pH 2.8 and no pH adjustment, there was no significant difference ($p > 0.05$) between samples treated with ultrasound and those not treated (comparisons represented by lowercase letters). The pH value of 4.3 showed a tendency to increase the exposure of sulfhydryl groups with the increase in ultrasound treatment up to 637.5 W/376.47 s. However, there was no significant difference ($p > 0.05$) between the highest applied power 712.5 W/ 336.84 s and the system without ultrasound treatment. At pH 6.8, the greatest exposure of the sulfhydryl groups occurred in power/time combinations of 412.5 W/581.82 s, 637.5 W/376.47 s and 712.5 W/336.84 s.

The content of free SH on the surface is closely related to changes in conformation, unfolding of proteins and disruption of disulfide bonds. The results found corroborate the data obtained in the analysis of H_o, indicating that the ultrasound treatment promoted the rupture of protein aggregates at pH values 4.3 and 6.8 exposing the sulfhydryl groups that were facing the hydrophobic interior. The legumins in peas contains more sulfur-containing amino acids, such as cysteine and methionine, so the significant increase in the free SH content of the pea protein in treatments may be mainly related to changes in the legumins [56,57].

At the pH value of 2.8, it was found that there was no significant difference ($p > 0.05$) in the exposure of sulfhydryl groups in treatments with and without ultrasound. The same behavior was observed in H_o analysis, suggesting that the ultrasound did not promote significant changes in the particle structure at this pH value.

For treatments no pH adjustment, it was observed that there was no significant difference ($p > 0.05$) in the exposure of sulfhydryl groups between systems with and without ultrasound treatment, although the results of the H_O index suggested that the use of ultrasound promoted significant conformational changes between the systems. This can be caused by the oxidation of sulfhydryl groups by hydrogen peroxide produced by acoustic cavitation, resulting in a reduction in the content of the free SH group [58]. Another possibility is that there was the formation of disulfide bonds through SH/SS exchange reactions, favored under alkaline pH conditions [52]. In addition, under alkaline pH conditions, thiol groups tend to be more reactive to form species of mercaptoid ions (S-), which accelerates the oxidation of SH [5].

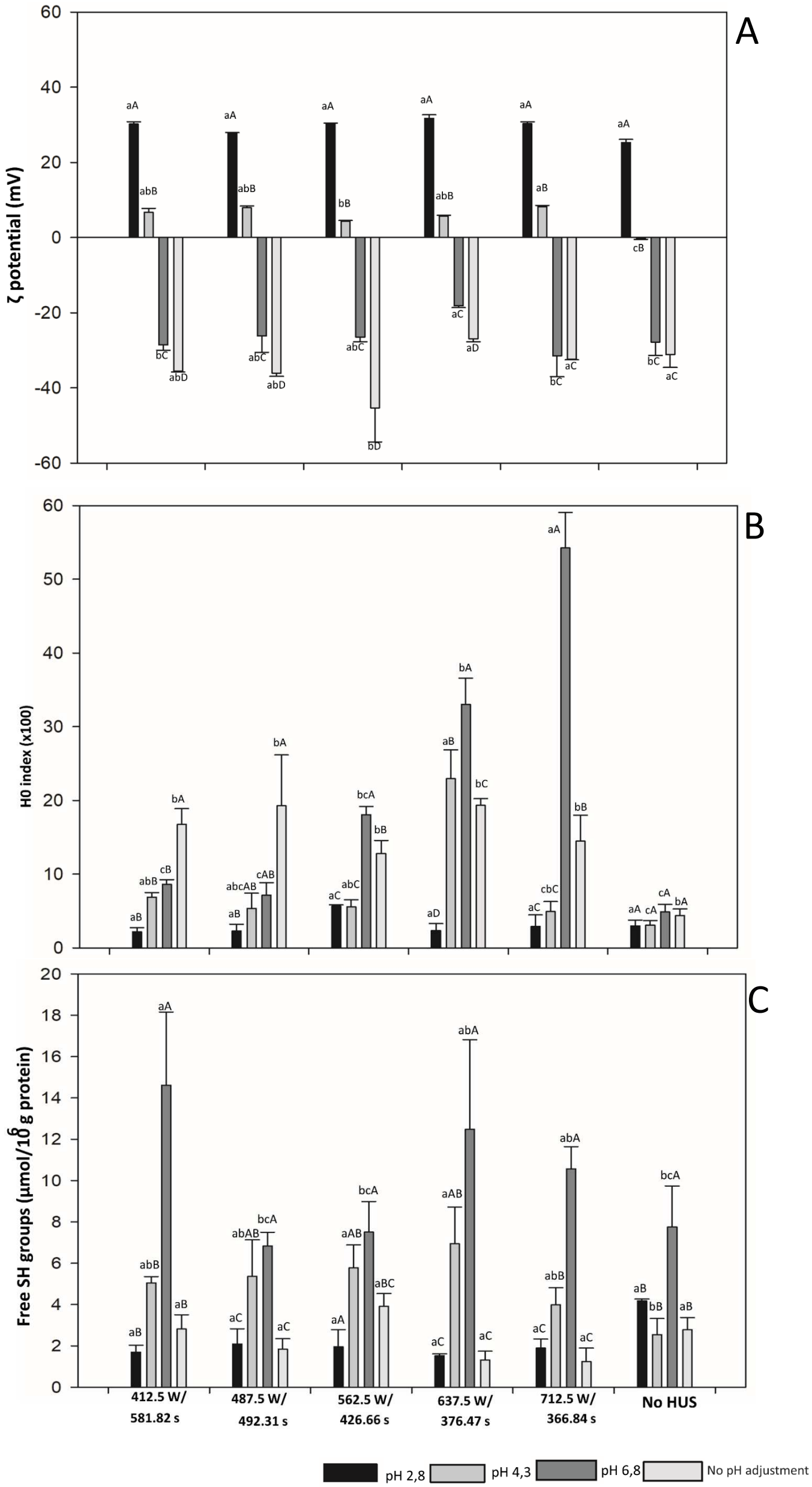

Figura 3: Zeta potential **(A),** Hydrophobic surface **(H_0) (B),** Free sulfhydryl groups **(SH) (C),** of PPC treated and not treated with ultrasound. Lower case letters refer to comparisons between ultrasound treatments at given pH. Capital letters refer to comparisons between pHs for given ultrasound treatment. Tukey means comparison test was used, with a 5% significance level**.**

3.2.4 Average hydrodynamic diameter (D_h)

The average hydrodynamic diameters of the disperse proteins (D_h) are represented in Figure 4. Two populations, both with polydispersity index (PDI) less than 0.1 (Appendix 1), were identified, indicanting that in all cases the populations of disperse particles presented very low polidispersity.

Population 1, in most cases, had the largest mean diameters, which ranged from 301.7 to 2103.68 nm. In systems tretated with power/time combinations of 412.5 W/581.82 and 487.5 W/492.31 s, population 2 has the highest percentage in the data volume, whereas in those tretated with higher powers (637.5 W/376.47 s and 712.5 W/ 336.84 s), peak 1 represented the highest percentage of D_{hs}.

Systems treated with power/time combinations of 412.5 W/581.82 s, 487.5 W/492.31 s and 562.5 W/426.66 s, at pH 2.8 and no pH adjustment, showed lower D_h than those with pH values of 4.3 and 6.8 (comparisons in capital letters). This is in agreement with results previously presented and discussed in sections 3.2.1 to 3.2.3, which point to favored protein-protein interactions over protein-solvent interactions, at such pHs.

The smaller diameters presented by the systems at pH values 2.8 and without pH adjustment can be attributed to the more expressive intra- and intermolecular electrostatic repulsion at these ph values, which are both quite far from the average isoelectric point of PPCs. These findings corroborates those reported by Arzeni et al. [22] which studied the effect of ultrasound on whey and soy protein isolate and egg whites proteins, and found that ultrasound (20 kHz for 15 or 30 min) decreased the size of whey and soy protein aggregates , and caused a slight increase in egg white proteins [59]. Ting et al., Morales et al. also verified a decrease in soy protein aggregates with ultrasound treatments at a frequency of 20kHz [27,46].

Systems treated with 637.5 W/376.47 s and 712.5 W/336.84 s did not show significant differences ($p > 0.05$) when evaluating the different pHs at fixed power (upper case comparisons) and presented a behavior similar to the systems that were not submitted to ultrasound treatment (No HUS). Gülseren et al. [58] studied the effect of ultrasound on the properties of bovine serum albumin and found an increase in mean diameter in longer sonication times. This indicates that more intense treatments can promote the formation of aggregates by structural rearrangements promoted by non-covalent interactions, such as electrostatic and hydrophobic interactions. This result can be correlated with those obtained in the analysis of H_o (section 3.2.2), in which at the highest potencies there was a greater exposure of the

hydrophobic regions, suggesting that the greater exposure of such groups induced the formation of interactions with the objective of minimizing the Gibbs free energy of the system.

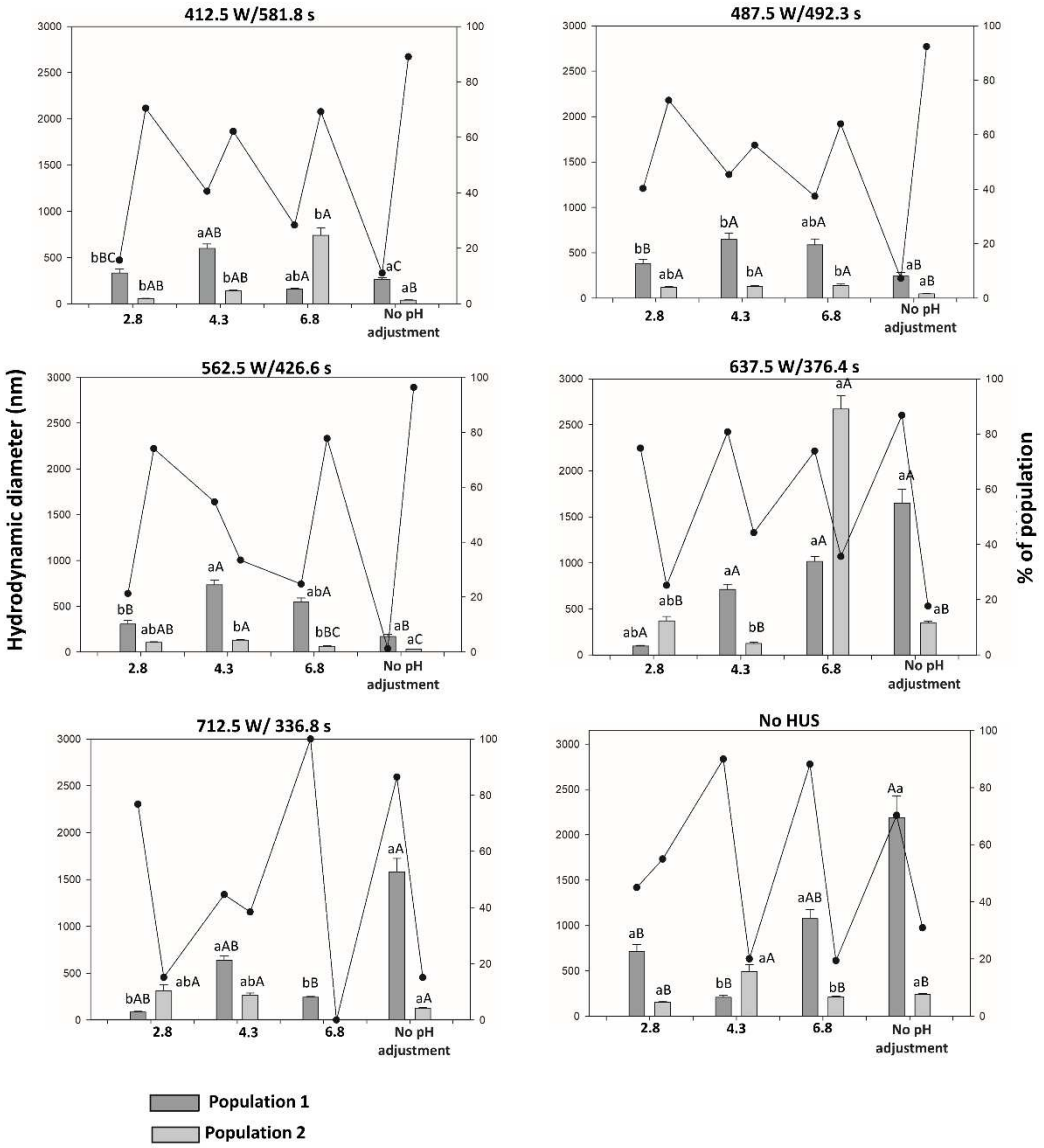

Figure 4: Hydrodynamic diameter of PPC proteins PPC treated and not treated with ultrasound. The lines correspond to the percentage of the population at each peak, while the bars refer to the hydrodynamic diameter. An analysis of comparison of means was performed for each population. Lower case letters refer to comparisons between ultrasound treatments at given pH. Capital letters refer to comparisons between pHs for given ultrasound treatment. The Tukey means comparison test was used, with a significance level of 5%.

It was expected that the size of the clusters at pH 4.3 would be expressively larger than systems at other pH values, however this fact was not observed. In the pH value of 4.3 the proximity to pI possibly caused most proteins to be precipitated at the time of centrifugation and those that remained in the supernatant presumably had a smaller average diameter, since they were in dispersion.

3.3 *In vitro* digestibility simulation of ultrasound treated PPCs

In vitro digestibility analyses were carried out in order to examine if ultrasound treatments are expected to affect the ability of PPCs to be diggested, which is intimately correlated to their nutritional value. By analysing results obtained from the electrophoresis (figure 5), one can note that before digestion the dispersions presented a wide electrophoretic profile, ranging from 15 to 94 kDa, which confirms the variety of proteins in PPC. The bands corresponding to lipoxygenases (LOX), convicillins (CV), vicillins (V), acidic fractions (Lα), and basic (Lβ) of the legumins were identified. Such bands were also identified in the studies performed by Adal et al. [60] that evaluated the electrophoretic profile of PPI (85% of proteins), Oliete et al. [3] that verified the distribution of molecular weights of PPI (96.2% of proteins) submitted to the microfluidization process and Laguna et al.[61] that compared to influences of physical methods (high hydrostatic pressure and ultrasound) on the digestibility of PPI (84% protein).The band corresponding to lipoxygenase was identified at approximately 94 kDa and convicillin appeared close to the molecular weight of 71 kDa. For vicillins three different polypeptides were found, with V1 at around 46 kDa, V2 at around 30 kDa and V3 at 15 kDa. As the electrophoresis was performed under reducing conditions, legumins appeared in their acidic fractions (Lα - 40 kDa) and basic fractions (Lβ1 and Lβ2 at 25 and 20 kDa respectively) [3]. These fractions result from the complete rupture of the disulfide bonds that previously maintained the hexameric structure of the legumins [62]. In addition, the intensity of the identified bands was higher for treatments using higher powers (637.5 W/376.47 s and 712.5 W/336.84 s), and the lowest band intensity was detected for the control system (PPC not treated with ultrasound). This suggests that the application of ultrasound led to a greater dispersibility of proteins, since the systems were centrifuged before electrophoresis, and thus the greater dispersibility caused a greater amount of proteins to be applied to the gel.

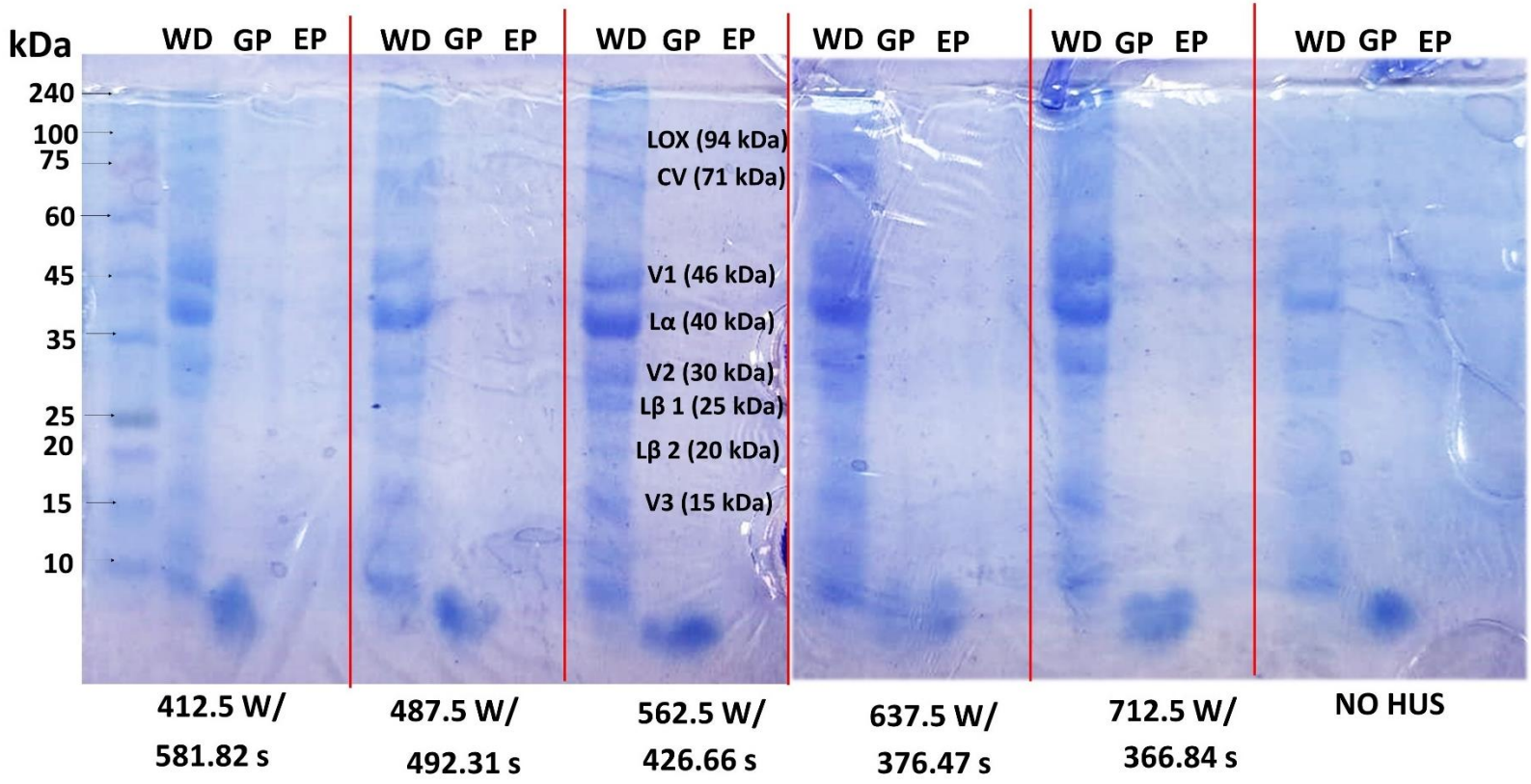

Figure 5: Polyacrylamide gel electrophoresis (SDS-PAGE) of protein dispersions before digestion (WD), after the gastric phase (GP) and after the enteric phase (IP). LOX: Lipoxygenase, CV: convicillin, V: vicillin, Lα: acid fraction of legumin, Lβ: basic fraction of legumin.

At the end of the gastric phase of simulated diggestion, a cluster of polypeptide chains smaller than 10 kDa was identified in all treatments, indicating that the proteins and their respective identified fractions were hydrolyzed into smaller polypeptides. Lower band intensities were also observed for treatments using higher powers (637.5 W/376.47 s and 712.5 W/336.84 s), showing digestibility with greater efficiency and suggesting that in these cases, the proteins were more intensively digested, forming polypeptides smaller than 10 kDa, which could not be detected by this specific electrophoresis technique. In the study carried out by Laguna et al. previously cited [61], it was found that high hydrostatic pressure increased the gastric digestibility of pea proteins when compared to systems that did not undergo physical treatments.

Finaly, after the enteric phase, no polypeptide fragments were identified, indicting that proteins were completely digested during the gastric phase, as expected. In the material obtained after the enteric phase, peptides were smaller than the pores of the electrophoresis gel and had molecular masses smaller than the molecular weight standard used.

3.4 Emulsifying properties of ultrasound treated PPCs

3.3.1 Emulsifying activity index (EAI)

EAI analysis (Figure 6) shows that there is no significant difference ($p > 0.05$) between ultrasound-treated and untreated systems, when evaluated at the same pH value (lower case comparison) in systems with a pH of 2.8, 4.3 and systems no pH adjustment.For the pH value of 6.8, significant differences were found between the systems treated with power/time combinations 412.5 W/581.82 s, 487.5 W/492.31 s, 562.5 W/426.66 s and the system no pH adjustment, with these powers having the highest IAE. However, this difference was not identified ($p > 0.05$) for systems treated with 637.5 W/376.47 s, 712.5 W/ 336.84 s and no pH adjustment. In addition, at the lowest powers (up to 562.5 W/426.66 s), the highest EAIs were found for pH 2.8, followed by pH 6.8, no pH adjustment and, finaly, pH 4.3. In the systems treated with higher powers (637.5 W/376.47 and 712.5 W/336.84 s) and in systems no HUS tratement, a similar behavior occurred; however, the system no pH adjustment showed EAI higher than those with pH adjusted to 6.8. The emulsifying activity index is related to the amount of active surface molecules to cover a given the water/oil interface area, thus reducing the interfacial tension [63]. The ability of proteins to adsorb at the interface of droplets in emulsions may be associated with physical characteristics of proteins, such as hydrophilic-

lipophilic balance, net electric charge, and average D_h (which affects the diffusibility of molecules in the medium) [53,54].

Ultrasound treatments are expected to increase the exposure of hydrophobic residues buried in the core of the native conformation of protein molecules, improving their affinity for interfaces. In fact, this occurred in systems with pH 6.8 treated with the lowest ultrasound powers (412.5 W/581.82 s, 487.5 W/492.31 s and 562.5 W/426.66 s). In treatments at powers of 637.5 W/376.47 s and 712.5 W/ 336.84 s, the exposure of hydrophobic amino acid residues occurred more extensively, as demonstrated by the H_o results (section 3.2.2), leading to higher IAEs. Similar findings were reported by Zhou et al. [64], who found an increase in the IAE of ultrasound-treated soy glycinin (20 kHz to 80 $W \cdot cm^{-2}$ from 0 to 40 min) for shorter periods of exposure, as well as a decrease in this index in longer times of application. Whatever the ulltrasound treatment, systems with pH 2.8 had high EAI values, due to the electrostatic repulsion among protein molecules at this pH (as they arepredomintantly negatively charged), favoring the separation of protein aggregates, thus reducing their hydrodynamic diameters. Smaller protein aggregates can more easily migrate to interfaces, making adsorption more efficient. In addition, electrostatic repulsion works by stabilizing the droplets formed [56]. This behavior was similar to that observed for systems no pH adjustment, in which proteins molecules have a predominantly negative surface charge, since their pH is higher than 8.0.At pH 4.3, the EAI were the lowest ones, whatever the ulltrasound treatment, which may be attributed to the greater protein aggregation at this pH value, since it is close to the isoelectric point (section 3.1) and thus protein-protein interactions predominate over. This is, indeed, corroborated by reported results for protein dispersibility (section 3.2.1) and average hydrodynamic diameter (section 3.2.4).

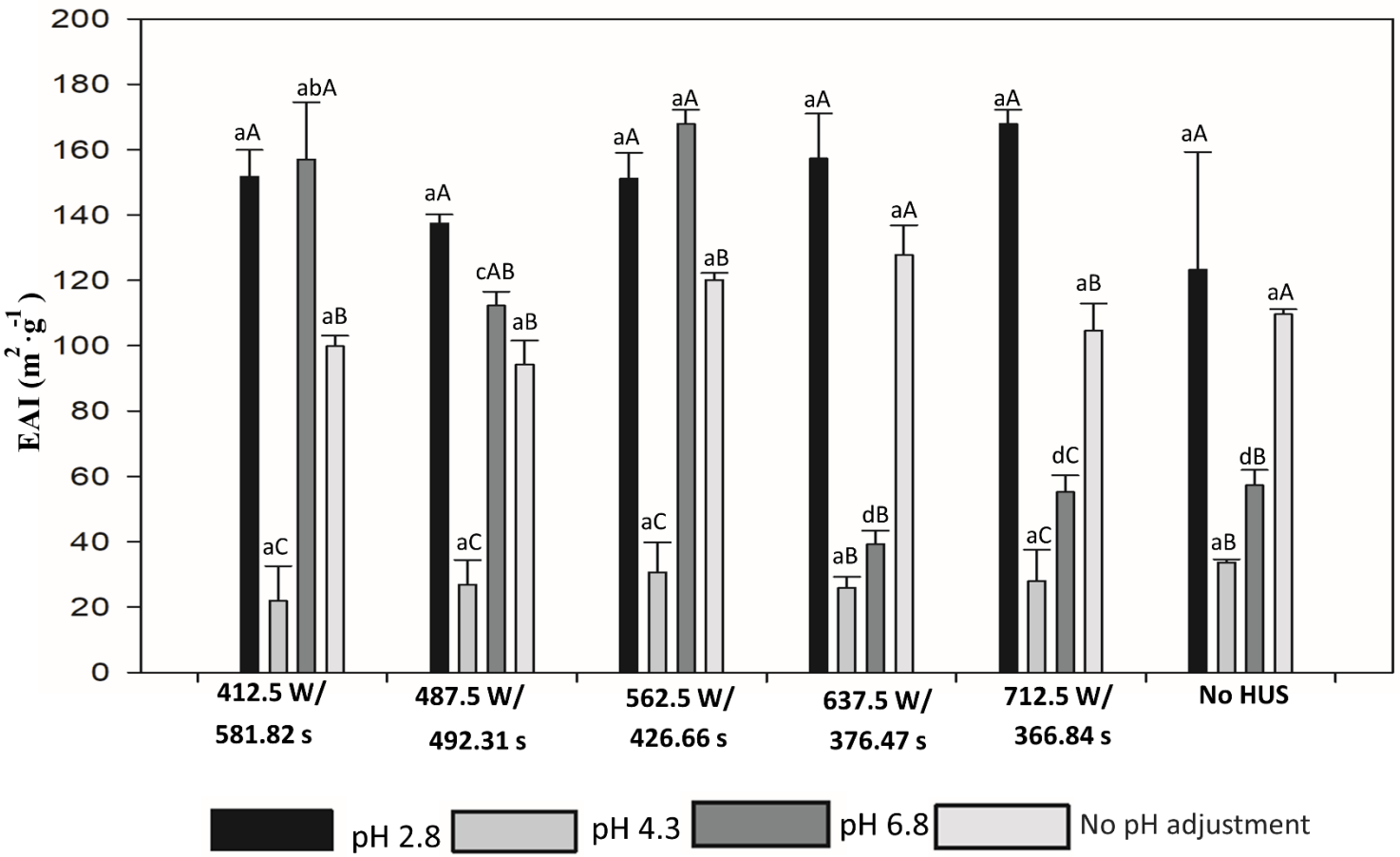

Figure 6: Emulsifying activity index (EAI)of PPC treated and not treated with ultrasound. Lower case letters refer to comparisons between ultrasound treatments at given pH. Capital letters refer to comparisons between pHs for given ultrasound treatment. Tukey means comparison test was used, with a 5% significance level**.**

3.3.2 Microstructure

Optical microscopy of the emulsions was performed as soon as they were produced, five days later, and on the tenth day after their production. As shown by photomicrographs in figure 7, systems at pH 2.8 presented the smallest droplet sizes, either with or without ultrasound treatment.

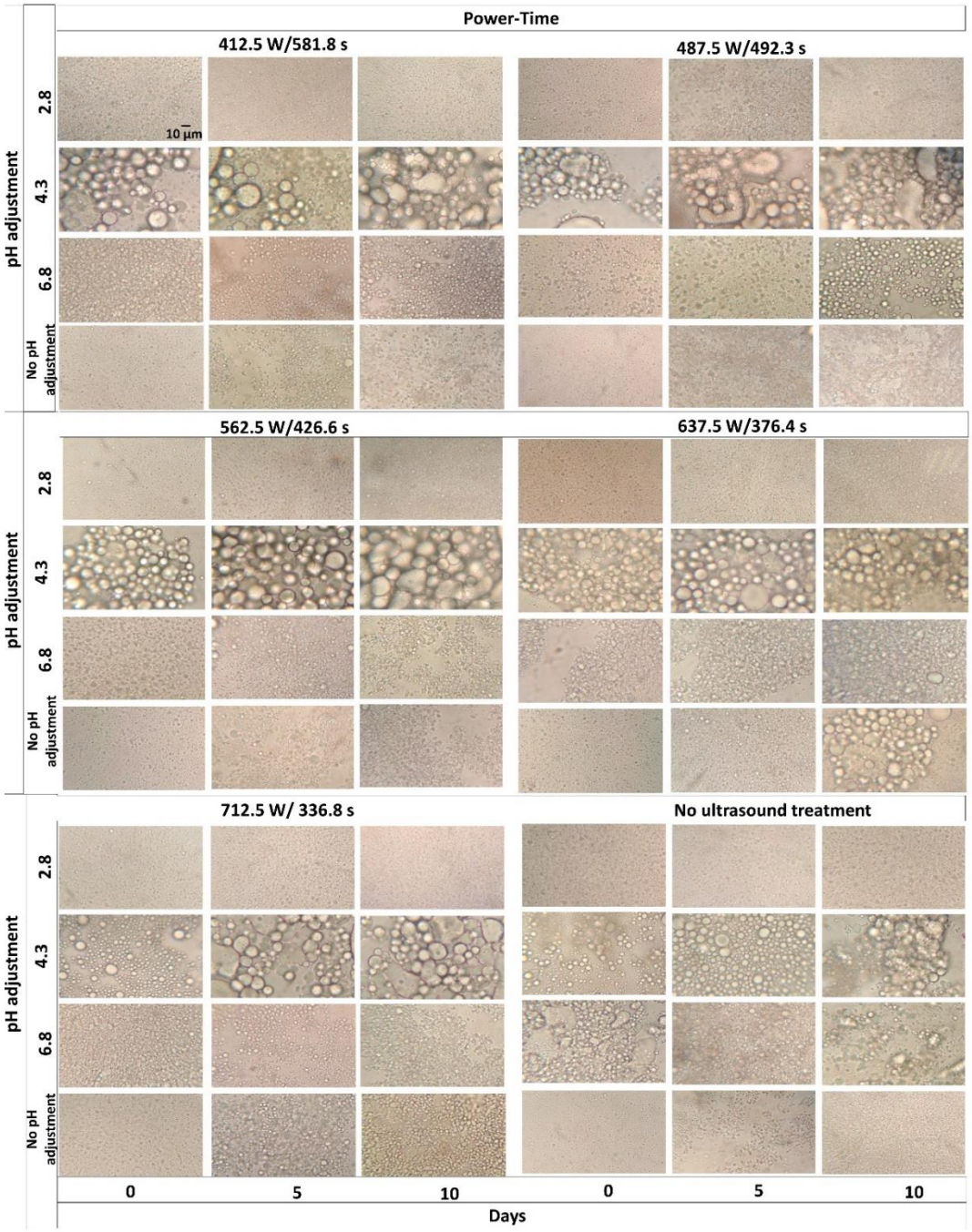

Figure 7: Optical microscopy of emulsions produced at pH values 2.8.4.3, 6.8 and pH adjustement, treated with ultrasound at 412,5 W/581.82 s; 487.5 W/492.31 s; 562.5 W/426.66 s; 637.5 W/376.47 s; 712.5 W/ 336.84 s and no ultrasound treatment.

Systems with pH 4.3 exhibited the largest droplet sizes in all cases, followed by those with pH 6.8. For emulsions fabricated using protein dispersions no pH adjustment, small droplets were formed in all cases; however, the application of power/time combinations of resulted in an increase in the size of the droplets over the days (see next subsection). It is also noteworthy that, with the exception of systems with pH adjusted at 2.8, some droplet aggregation was observed. It is also worth mentioning that, with the exception of systems with pH adjusted to 2.8, some droplet aggregation was observed. These aggregates may have arisen through destabilization mechanisms, such as flocculation by an attractive balance of interactions, which possibly occurred due to the repulsive forces (negative charges) being smaller in module than the attractive forces (mainly hydrophobic interactions).

The analysis of these results suggest that the electrostatic repulsion among droplets in systems with pH 2.8 (high density of positive electric charges) was not only favorable to the formation of smaller droplets but also sufficient to maintain the stability of the emulsions during the evaluated period of time (see next subsection). However, systems with pH 6.8 and no pH adjustment behave differently, even with they carry droplets with high negative charge densities. The greatest droplet aggregation presented by emulsions with pH 4.3 is related to the proximity to the average isoelectric point of proteins, which are adsorbed at droplets' interfaces. At such condition, electrostatic repulsion among droplets is minimized. Corroborating these findings, Keerati-u-rai and Corredig (2009) who studied the effect of heat treatment on the emulsifying stability of soy protein isolate, and Geerts et al. (2017) that evaluated the emulsifying properties of aqueous extracts rich in pea proteins at pH 6.5 and 3.8 [59,60] observed that aggregates can be adsorbed on the emulsion interfaces, however, there is a greater propensity for destabilization by flocculation.

In emulsions with pH 6.8 without ultrasound treatment, the formation of agglomerates is verified, corroborating with studies of Ben-Hanb et al. [65] who evaluated the microstructural properties of gelled emulsions of pea protein isolates at pH 7. It was noticed that in emulsions formed only with PPI they presented larger droplet sizes than emulsions formed by the combination of milk and pea proteins. In addition, evidence of aggregation was observed. Peng et al [66] verified the effect of heat treatment on the emulsifying properties of pea proteins (protein concentrate with 79.9% proteins) and observed that emulsions produced with untreated PPC at pH 7 produced emulsions in which part of the oil droplets were in its individual form, and the rest remained flocculated. The studies presented show that the formation of aggregation occurs in emulsions prepared at pH 7. In this study, this fact is verified at pH 6.8 (~ 7), however

the application of ultrasound promoted a decrease in the size of the droplets, which can delay the aggreation process.

3.3.3 Macroscopic kinetic stability

Figure 8 shows the kinematics of creaming of emulsions formed at different pHs in treatments with and without ultrasound, during storage for 30 days. While in figure 9 the visual appearance of the emulsions is presented immediately after their formation and stored for 5, 10 and 30 days. The empirical mathematical model represented in Equation 3 was adjusted to expermental data. The parameters ICr_{eq} (which is related to the maximal cream index after an indefinitely long time) and K (which is related to the creaming rate at short times) were compiled in Table 3.

Table 3: Parameters calculated from the mathematical adjustment presented in equation 3.

Power/time	pH	ICr_{eq}	K	RAdjust
412.5 W/581.82 S	4,3	82.46	3.58	0.01
	6,8	68.18	0.45	0.92
	No pH adjustment	59.57	0.23	0.70
492.31 W/492,31 S	4,3	65.94	2.22	0.005
	6,8	83.67	1.17	0.92
	No pH adjustment	95.69	0.07	0.89
562.5 W/426,66 S	4,3	85.90	2.44	0.03
	6,8	6.85	0.18	0.92
	No pH adjustment	75.56	0.35	0.74
637.5 W/376.47 S	4,3	89.91	2.21	0.02
	6,8	59.51	0.32	0.08
	No pH adjustment	83.23	0.20	0.75
712..5 W/336.84 S	4,3	79.94	2.07	0.01
	6,8	70.52	0.38	0.80
	No pH adjustment	73.51	0.25	0.91
NO HUS	4,3	75.94	2.13	0.03
	6,8	90.42	2.14	0.38
	No pH adjustment	64.75	0.04	0.88

For systems with pH 2.8 the model could not be adjusted, due to the absence of creaming in the studied period. The pH 4.3, showed no correlation (R = 0) with the proposed adjustment is due to the almost instantaneous creaming of the emulsions.

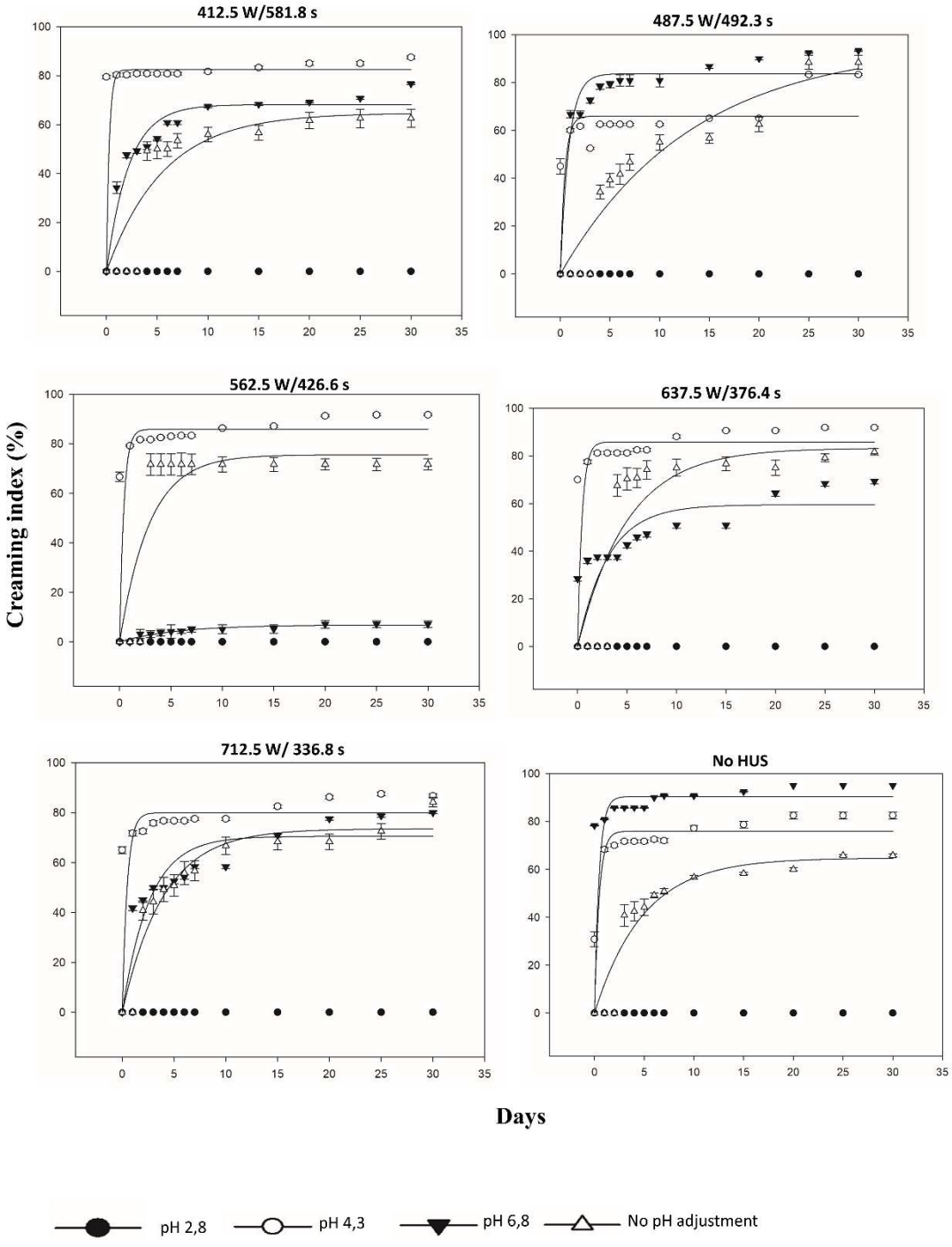

Figure 8: Evolution of the creaming index of emulsions formed at different pH values, during 30 days of storage.

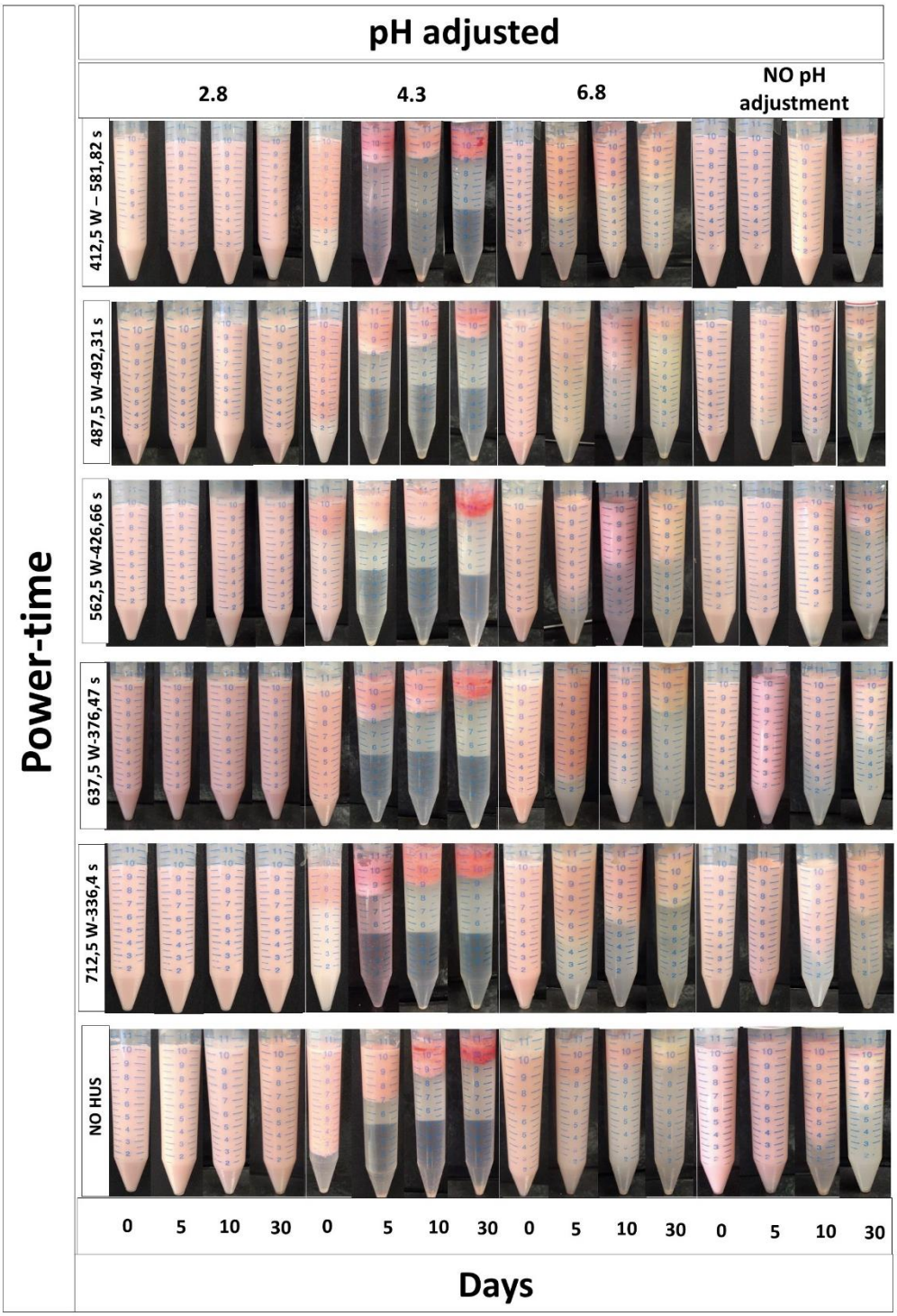

Figure 9: Visual appearance of emulsions on days 0, 5, 10, 30 of storage at 25°C.

Furthermore, for emulsions with this pH value, higher values for both ICreq and K parameters were found, indicating an extensive and fast destabilization of these systems. In most cases, emulsions formed at pH 6.8 and no pH adjustment showed correlation with the

proposed model (R> 0.70), and the values of the parameters ICreq and K were lower, showing that such systems were more kinetically stable than tose with pH 4.3 mentionned above, emulsions prepared with dispersions with pH 2.8 remained stable throughout the evaluated period, with creaming indexes practically equal to zero, either for systems with or without ultrasound treatment. Emulsions with pH 4.3, had instantaneous destabilization, with creaming index values at values as high as 60-80%, immediately after emulsification. At pH 6.8, ultrasound treatment decreased the rate of emulsions creaming, so ICr, reached a plateau of 60-80%, between 10 and 20 days of storage. Emulsions with this pH, prepared with dispersions that received the ultrasound treatment 562.5 W/426.66 s, showed a different behavior from the others, presenting ICr inferior to 10%, after 30 days of storage. Instead, for those without ultrasound treatment, instantaneous phase separation occurred, reaching a ICr plateau of about 95% after 20 days of storage. For emulsions no pH adjustment, those fabricated with PPC dispersions that received the ultrasound treatments 487.5 W/492.31 s, 637.5 W/376.47 s and 712.5 W/336.84 s presented slightly higher ICr values than those produced with PPC dispersions no ultrasound treatment. Such emulsions reached the plateau with ICr superior to 80% after 20 days of storage, whereas emulsions without ultrasound treatment stabilized at ICr values close to 70%. The ultrasound treatments 412.5 W/581.82 and 562.5 W/426.66 s led to results similar to those found for emulsions no HUS.

Creaming is one of the most common destabilization phenomenon in food-grade O/W emulsions. It refers to the upward movement of the oil droplets, due to its lower density of this material compared to the aqueous phase [67]. Normally, ICr (which is a numerical indicator of the fraction of the emulsified oil that “got out” from the emulsion) is affected by many variables. According to Stokes' law [68], not only the difference in density between the dispersed and continuous phases directly impacts creaming, but also the diameter of the droplets. The kinetic stability observed for emulsions with pH 2.8 is related to the smaller droplet size, shown in figure 7 discussed in section 3.3.2, as well as to the expressive electrostatic repulsion among droplets, since the proteins at their interface carry positive net electric charges. The instantaneous creaming occurred in emulsions with pH 4.3, whatever the ultrasound treatment of PPC, can be related to the low EAI presented by the systems at this pH value (section 3.3.1). In fact, at pH close to pI, protein-protein interactions are favored, leading to the formation of larger protein aggregates (section 3.2.4), which diffuses more slowly and promotes a less regular covering of the droplets’ interfaces. Therefore, flocculation and creaming become more prone to occur. At a pH value of 6.8, ultrasound-treated systems resulted in a lower ICr after 30

days of storage, compared to the same pH system no ultrasound treatment. A similar result was obtained by O'Sullivan [28] who studied the effect of ultrasound on bovine gelatine, fish gelatine, egg white protein, with 86, 86 and 85% protein respectively and pea protein isolates (86% protein), soybean (86% protein) and rice (84.5% protein), treated with ultrasound at 20kHz, at an acoustic power of 34 $W \cdot cm^{-2}$ for 2 minutes. And verified that after 28 days of storage the emulsions produced with PPI remained stable and that the diameter of the droplets of the emulsions with PPI treated with ultrasound was smaller than the droplets of the emulsions prepared with untreated PPI.

In systems no ultrasound treatment, the ICr of emulsions no pH adjustment was lower than that of emulsions with pH 6.8. This result corroborates the results of Liang and Tang 2013 [58], in which emulsions produced with PPI at pH 9 had a lower ICr than emulsions with pH 7. This result can be explained by the greater magnitude of electrostatic repulsion and the lower droplet size obtained in emulsions no pH adjustment. As with pH 6.8, phase separation occurred over time, which can be attributed to destabilization mechanisms, such as flocculation, which can lead to droplet coalescence and, consequently, phase separation.

4 CONCLUSIONS

This study showed that high intensity ultrasound processing, at specific power/time combinations, promote changes in physicochemical characteristics of pea protein concentrate (PPCs) previously exposed at different pHs, leading to improvements in their emulsifying properties and *in vitro* digestibility. Although the ultrasound energy injected into the systems was constant in all treatments (240 kJ), the input rate of such energy was shown of great relevance, as at the different power/time combinations led to clearly different results. The treatments 637.5 W/376.47 s and 712.5 W/336.84 s promoted more pronouced changes in proteins, favoring their aggregation. On the other hand, the ultrasound treatments 412.5 W/581.82 s, 487.5 W/492.31 s and 562.5W/ 426.6 s increased protein dispersibility (at pH 2.8 and no without pH adjustment), increased exposure of hydrophobic surfaces (at pH 4.3, 6.8 and without pH adjustment), increased free sulfhydryl groups (at pH 4.3 and 6.8), and decreased the average hydrodynamic diameter of dispersed protein (at pH 2.8, 6.8 and without pH adjustment). The ultrasound also promoted changes in emulsifying properties, although pH changes led to more clar differences in such properties. Whatever the ultrasound treatment, emulsions at pH 4.3 presented the lowest kinetic stability and the lowest emulsifying activity index, while those at pH distant from isoelectric point (2.8 and no pH adjustment) opposite behavior. In addition, the application of ultrasound enhanced the *in vitro* digestibility of PPCs,

indicating that such processing approach may make these proteins more bioavailable. These results show that ultrasound is an alternative technology that can be used to improve the characteristics of pea proteins, and that the combinations of time, power and pH have to be chosen according to the final technological application intented for these protein materials.

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Conclusão geral

As proteínas de ervilha apresentam uma fonte alternativa de proteínas com elevado valor nutricional, no entanto, seu uso pela indústria de alimentos ainda é restrito devido ao conhecimento limitado sobre a relação estrutura/função e baixa dispersibilidade nos valores de pH de interesse (entre 4 e 7). Assim, alternativas devem ser avaliadas para contornar tais barreiras.

O uso do ultrassom de alta intensidade aliado ao ajuste de pH promoveu alterações nas proteínas de ervilha melhorando suas propriedades físico-químicas, emulsificantes e de digestibilidade *in vitro*. Ao fornecer aos sistemas a mesma quantidade de energia (240 kJ) em diferentes combinações de potência/tempo, verificou-se que a aplicação das combinações de potências/tempo de 637.5 W/376.47 s e 712.5 W/336.84 s promoveram modificações mais expressivas nas proteínas favorecendo a formação de agregados. No entanto, sistemas tratados com ultrassom nas combinações de 412.5 W/581.82 s, 487.5 W/492.31 s e 562.5W/ 426.6 s melhoraram as características físico-químicas e emulsificantes do CPE por meio do aumento da dispersibilidade das proteínas e exposição de superfícies hidrofóbicas e sulfidrilas livres, com diminuição do tamanho das partículas. Além disso, foi verificado um aumento do índice de atividade emulsificante.

O pH exerceu grande influência sobre as características dos sistemas, de modo que em valores distantes de pI (2,8 e sem ajuste de pH) exibiram os melhores resultados. Além disso, a análise de digestibilidade *in vitro* mostrou que a aplicação de ultrassom de alta intensidade melhora a dispersibilidade das proteínas, aumentando sua disponibilidade em sistemas aquosos.

Por meio deste estudo, verificou-se que o ultrassom é uma tecnologia promissora a ser utilizada pela indústria, uma vez que melhora o desempenho técnico-funcional das proteínas de ervilha. As combinações de tempo, potência e pH a serem utilizados dependem da aplicação final em que as proteínas de ervilha serão destinadas.

APÊNDICE A - Potência acústica calculada a partir de cada binômio potência/tempo.

Uma maneira alternativa de expressar a energia aplicada ao sistema é por meio da potência acústica, a qual é calculada calorimetricamente como descrito por O'Sullivan *et al.* (2016), pelas equações 1 e 2.

$$I_a = \frac{P_a}{S_a} \qquad 1$$

Onde,

$$P_a = m \cdot c_p \left(\frac{dT}{dt}\right) \qquad 2$$

Nas Equações 1 e 2, $\mathbf{P_a}$ (W) representa a potência acústica, $\mathbf{S_a}$ é a área superficial da superfície emissora de ultrassom, **m** é a massa da solução tratada com ultrassom (g), **cp**: calor específico do meio (4,18 kJ / gK) e **dT/ dt**: taxa de variação de temperatura em relação ao tempo, começando em t = 0

Table 1: Cambinações de potência/tempo de aplicação do ultrassom de alta intensidade.

Potência (J/s)	Tempo (s)	Energia (kJ)	Potência acústica (W·cm^{-2})
0	0	0	0
412,5	581,82	240	10.60
487,5	492,31	240	13.92
562,5	426,66	240	18.86
637,5	376,47	240	22.15
712,5	336,84	240	23.91

APÊNDICE B - Índice de Polidispersidade dos partículas em dispersão.

O índice de polidispersidade das partículas presentes na dispersão proteica é apresentado na Tabela 2.

Tabela 2: Índice de polidispersidade das dispersões de concentrado proteico de ervilha (CPE).

Power/time	pH	PDI - Pico 1	PDI - Pico 2
412.5 W / 581.82 s	2,8	0,016	0,003
	4,3	0,007	0,003
	6,8	0,012	0,004
	Sem ajuste de pH	0,016	0,006
492.31 W/492,31 s	2,8	0,011	0,003
	4,3	0,012	0,012
	6,8	0,018	0,007
	Sem ajuste de pH	0,005	0,001
	2,8	0,007	0,004
562.5 W/426,66 s	4,3	0,017	0,006
	6,8	0,005	0,015
	Sem ajuste de pH	0,003	0,001
	2,8	0,049	0,012
637.5 W/376.47 s	4,3	0,005	0,007
	6,8	0,001	
	Sem ajuste de pH	0,013	0,006
	2,8	0,013	0,027
712.5 W/336.84 s	4,3	0,009	0,002
	6,8	0,019	0,004
	Sem ajuste de pH	0,029	0,019
	2,8	0,023	0,019
Sem tratamento com ultrassom	4,3	0,009	0,003
	6,8	0,008	0,002
	Sem ajuste de pH	0,012	0,002

ANEXO A – Especificações do concentrado proteico de ervilha

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SHANDONG JIANYUAN FOODS CO.,LTD.
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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

MICROBIOLOGICAL DATA:

Salmonella	Not Detected	GB 4789.4-2010
Yeasts	<100cfu/g	GB 4789.15-2010
Coliforms	<30MPN/100g	GB 4789.3-2010
Aerobic plate count	≤10000cfu/g	GB 4789.2-2010
Moulds	<10cfu/g	GB 4789.15-2010
Escherichia coli	Not Detected	GB/T 4789.38-2008
Aflatoxin B1	Not Detected	GB/T 18979-2003
Aflatoxin B2	Not Detected	
Aflatoxin G1	Not Detected	
Aflatoxin G2	Not Detected	
Ochratoxin A	Not Detected	GB/T 23502-2009
Listeria monocytogenes	Not Detected	GB 4789.30-2010
Staphylococcus aureus	Not Detected	GB 4789.10-2010
Shigella	Not Detected	GB/T 4789.5-2003

TYPICAL MINERALS:

Sodium	1071mg/100g	GB/T 5009.91-2003
Calcium	1.4×10^{3}mg/kg	GB/T 5009.92-2003
Iron	2.0×10^{2}mg/kg	GB/T 5009.90-2003

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网址（Http)：//www.jianyuangroup.com 电邮（E-mail)：trade@jianyuanfoods.cn

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