Universidade Federal do Estado do Rio de Janeiro
Departamento de Ciência de Alimentos
Programa de Pós-graduação em Alimentos e Nutrição - PPGAN

EXTRAÇÃO VERDE DE TORTA DE AMÊNDOA: OTIMIZAÇÃO, ESTUDO EM ESCALA PILOTO E CARACTERIZAÇÃO DA FRAÇÃO PROTEICA

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Rio de Janeiro, RJ – Brasil
Março de 2020

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição da Universidade Federal do Estado do Rio de Janeiro como requisito parcial para obtenção do título de Doutor em Alimentos e Nutrição.

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RESUMO

Amêndoas são produzidas principalmente no estado da Califórnia (EUA), que conta com cerca de 77% da produção mundial. A amêndoa é um fruto seco e é considerada um alimento nutritivo, sendo fonte de lipídeos e proteínas. Neste estudo foi utilizado como material inicial a torta de amêndoa, que foi gerada após a extração de óleo por prensagem mecânica a frio. A torta de amêndoa é um material rico em proteína e que ainda contém um residual de óleo. O objetivo deste trabalho foi extrair simultaneamente óleo e proteína da torta de amêndoa, utilizando as técnicas de extração verde: aquosa (AEP) e aquosa-enzimática (EAEP), evitando o uso solventes. Foi realizada uma otimização empregando a ferramenta estatística CCDR (C*entral Composite Rotatable Design*), avaliando efeitos individuais e combinados. Para AEP as condições otimizadas foram 1:12.8 RSL e o tempo de 2 h, enquanto para EAEP foi também 1:12.8 RSL, o tempo de 1 h e o uso de 0.85% da enzima *Alkaline Protease*. Com as condições otimizadas, foi então realizado um estudo em escala piloto, partindo da escala laboratorial de 1 L para 10 L. Além da AEP e EAEP, foi feita uma extração considerada como controle, HEX-AEP (material inicial desengordurado em Soxhlet) em que foram usados os mesmos parâmetros da AEP. Foram geradas três frações após o escalonamento: proteica (*skim*), lipídica (*cream*) e a insolúvel. A fração lipídica foi submetido ao processo de de-emulsificação (química e enzimática) para a liberação de óleo e o melhor resultado foi na EAEP com a de-emulsificação química gerando um rendimento de óleo de aproximadamente 99%. A fração proteica foi avaliada quanto as propriedades tecnológicas, físico-químicas e biológicas além da digestibilidade *in vitro*. A fração proteica da EAEP apresentou maior solubilidade e capacidade de emulsificação, no entanto, esta mesma fração apresentou a menor atividade de emulsificação e menor capacidade e estabilidade de formação de espuma em comparação com a fração proteica de AEP e hexano-AEP. Estes resultados estão correlacionados ao alto grau de hidrolise (23%), mais altos valores absolutos de potencial zeta e aos menores valores de hidrofobicidade de superfície da EAEP. Além disso EAEP apresentou a melhor capacidade antioxidante entre as frações proteicas testadas e um alto potencial de inibição da α-glucosidase (98%) enquanto AEP apresentou a maior inibição de lipase (70%). Na digestibilidade *in vitro* todas as frações proteicas foram eficientemente digeridas pelas proteases digestivas. Este estudo mostrou que diferentes processos de extração alcançaram diferentes propriedades tecnológicas e bioativas da proteína extraída.

Palavras-chave: subproduto, extrações sustentáveis, fração lipídica, fração proteica, propriedades tecnológicas, propriedades físico-químicas e propriedades biológicas

SOUZA, Thaiza Serrano Pinheiro de Souza. **Green extraction of almond cake: optimization, pilot-scale study and protein fraction characterization**. UNIRIO, 2020. 190 pages. Doctoral thesis. Department of Food Science and Technology, UNIRIO.

ABSTRCT

Almonds are produced mainly in the state of California (USA), which accounts for about 77% of world production. Almond is a nut and is considered a nutritious food, being a source of lipids and proteins. In this study, almond cake was used as the starting material, which was generated after oil extraction by cold mechanical pressing. Almond cake is a protein-rich material that still contains a residual oil. The objective of this work was to simultaneously extract oil and protein from the almond cake, using the green extraction techniques: aqueous (AEP) and aqueous-enzymatic (EAEP), avoiding the use of solvents. An optimization was performed using the statistical tool CCDR (Central Composite Rotatable Design), evaluating individual and combined effects. For AEP the optimized conditions were 1: 12.8 RSL and the time of 2 h, while for EAEP it was also 1: 12.8 RSL, the time of 1 h and the use of 0.85% of the enzyme Alkaline Protease. With the conditions optimized, a pilot scale study was then carried out, starting from the laboratory scale from 1 L to 10 L. In addition to AEP and EAEP, an extraction considered as control, HEX-AEP (initial material defatted in Soxhlet) and the same parameters were used as in AEP. After scaling up three fractions were generated: protein (skim), lipid (cream) and insoluble. The lipid fraction was subjected to the de-emulsification process (chemical and enzymatic) for the release of oil and the best result was at EAEP with the chemical de-emulsification generating an oil yield of approximately 99%. The protein fraction was evaluated regarding functional, physical-chemical and biological properties and the *in vitro* digestibility. The protein fraction of EAEP showed greater solubility and emulsification capacity, however, this same fraction showed the least emulsification activity and less foaming capacity and stability compared to the protein fraction of AEP and hexane-AEP. These results are correlated to the high degree of hydrolysis (23%), the highest absolute values of zeta potential and the lowest surface hydrophobicity values of EAEP. In addition, EAEP showed the best antioxidant capacity among the protein fractions tested and a high potential for inhibition of α-glucosidase (98%) while AEP showed the highest inhibition of lipase (70%). In the *in vitro* digestibility, all protein fractions were efficiently digested by digestive proteases. This study showed that different extraction processes achieved different functional and bioactive properties of the extracted protein.

Keywords: by-product, sustainable extractions, lipid fraction, protein fraction, functional properties, physical-chemical properties and biological properties

ÍNDICE

1. **Introdução**

A amêndoa cultivada é designada como *Prunus dulcis*, pertence ao gênero *Prunus* e à família *Rosaceae*, que inclui também maçãs, peras, pêssegos, ameixas e framboesas (MANDALARI et al., 2014; YADA; LAPSLEY; HUANG, 2011). Amêndoas são cultivadas em países de clima temperado e subtropical, sendo frutos adaptados para toda a região do Mediterrâneo, caracterizado por invernos amenos e verões quentes e secos (YADA; HUANG; LAPSLEY, 2013; KODAD; SOCIAS; ALONSO, 2018). São cultivadas comercialmente em locais com verões longos e quentes, sendo os maiores produtores os Estados Unidos (ESFAHLAN; JAMEI; ESFAHLAN, 2010). O estado dos EUA com maior produção é a Califórnia, que é responsável por cerca de 77% da produção mundial deste fruto. A produção mundial prevista para 2020 é de aproximadamente 1.3 milhões de toneladas e esta produção inclui os EUA, a União Europeia, Austrália, China, Turquia e outros. Quanto à exportação e importação, os valores estimados são de 807 mil e 748 mil toneladas respectivamente (USDA, 2019).

Amêndoas são consideradas altamente nutritivas por apresentarem um perfil de compostos saudáveis: são fontes de proteína, ácidos graxos insaturados, fibra dietética, vitamina E e minerais essenciais (MARTÍNEZ et al., 2013; YADA; HUANG; LAPSLEY, 2013). Além disso, são alimentos que fornecem energia, devido à alta porcentagem de lipídios totais, ricos em ácidos graxos monoinsaturados, como o ácido oleico, e poli-insaturados, como o ácido linoleico, além de apresentar baixo teor de ácidos graxos saturados (GRIEL; KRIS-ETHERTON, 2006; SATHE et al., 2008). Quanto a sua composição proteica, amêndoas apresentam proteínas solúveis em água e em soluções salinas devido a amandina ser uma globulina e se apresentar como a proteína de armazenamento mais predominante na composição destas *nuts* (AHRENS et al., 2005).

Com a crescente demanda pelo consumo e produção sustentável de produtos ricos em proteína, aumenta também o interesse por proteínas vegetais alternativas e de menor custo (RODSAMRAN; SOTHORNVIT, 2018; ROMMI et al., 2015). Muitos subprodutos da indústria de alimentos, como por exemplo os gerados após a extração de óleo, são opções interessantes para este fim (ROMMI et al., 2015). A torta de amêndoa

é um subproduto gerado após prensagem a frio da semente de amêndoa: após a extração do óleo, o resíduo formado ainda contém um teor residual de óleo (9%) e alto teor de proteína (49%) (KARAMAN et al., 2015). Diferentes métodos são utilizados na extração de óleo, entre os mais aplicados estão a prensagem mecânica e a extração por solventes, como o hexano (BALVARDI et al., 2015). No caso de materiais com alto teor de óleo (acima de 35%), o processo de extração é realizado em duas etapas, que consistem em uma pré-prensagem mecânica seguida pela extração com solvente (ROSENTHAL; PYLE; NIRANJAN, 1996). Com a extração por prensa mecânica, o óleo extraído apresenta qualidade superior ao extraído por solvente, porém esse método apresenta baixa eficiência de extração. Na extração com hexano é observado um alto rendimento de óleo, mas por ser um solvente inflamável e neurotóxico, é necessário encontrar formas alternativas de extração (LI; SMITH; STEVENS, 2016). As extrações aquosa ou aquosa-enzimática apresentam grande potencial de aplicação, uma vez que são consideradas extrações verdes.

O processo de extração aquosa (*Aqueous Extraction Process* - AEP) e o processo de extração aquosa assistida por enzimas (*Enzyme Assisted Aqueous Extraction Process* - EAEP) são métodos que permitem a extração simultânea de óleo e proteína de sementes oleaginosas (JUNG, 2009). O produto destas extrações é de alta qualidade e adequado para o consumo humano. O interesse nesta abordagem tecnológica tem aumentado recentemente devido ao interesse de realizar métodos de extração mais seguros e de menor impacto ambiental em relação ao uso de solventes (YUSOFF; GORDON; NIRANJAN, 2015). AEP normalmente apresenta baixa eficiência na extração quando comparada a extração por solvente, mas com a aplicação de enzimas proteolíticas, a liberação de óleo é favorecida, o que aumenta o rendimento (ROSENTHAL; PYLE; NIRANJAN, 1996). Estas técnicas (AEP e EAEP) já foram aplicadas em diferentes oleaginosas, entre elas: azeitona (NAJAFIAN et al., 2009), soja (DE ALMEIDA; DE MOURA BELL; JOHNSON, 2014), amendoim (LI et al., 2017) e amêndoa (MOGHADAS; REZAEI, 2017), mas na torta de amêndoa, os processos AEP e EAEP ainda não foram avaliados.

A aplicação de enzimas proteolíticas pode levar à geração de peptídeos biologicamente ativos (NGOH; GAN, 2016). Peptídeos bioativos são definidos como

sequências de aminoácidos, intrínsecas à proteínas ou não, que apresentam atividade biológica, incluindo atividade antioxidante, anti-hipertensiva, antiobesidade, antimicrobiana, entre outras (DE CASTRO; SATO, 2015). As sequências de peptídeos que podem apresentar bioatividade encontram-se inativas dentro da sequência de proteína e podem ser liberados por hidrólise enzimática, geralmente por ação de proteases (NGOH; GAN, 2016). Dependendo do grau de hidrólise, as propriedades tecnológicas das proteínas são afetadas positiva ou negativamente. Entre as principais propriedades tecnológicas afetadas estão a solubilidade e as propriedades de superfície que determinam o comportamento da proteína/peptídeo nas interfaces ar-água e óleo-água (POLANCO-LUGO et al., 2014). As propriedades tecnológicas e os peptídeos bioativos de hidrolisados de proteínas têm sido correlacionadas com a especificidade enzimática, o grau de hidrólise, a composição de aminoácidos e com a sequência dos diferentes peptídeos liberados (ZHAO et al., 2012).

Os métodos, resultados e discussão do presente estudo estão apresentados em capítulos, divididos como segue:

O capítulo 1 é uma revisão bibliográfica clássica sobre assuntos pertinentes à tese em questão.

O capítulo 2 é um artigo de revisão bibliográfica, intitulado *Amêndoas – uma Revisão.* O artigo será submetido para avaliação e possível publicação pelo periódico *California Agriculture*, publicado pela Universidade da Califórnia - divisão de agricultura e recursos naturais. Com esse artigo, pretende-se apresentar aos leitores da principal região produtora de amêndoas, informações atualizadas sobre o cultivo, a produção, as características, composição, potencial alergênico, efeitos benéficos relacionados ao consumo e potenciais aplicações dos frutos de amêndoa.

No capítulo 3, está apresentado o artigo intitulado: *Aqueous and Enzymatic Extraction of Oil and Protein from Almond Cake: A Comparative Study*, que foi publicado pela revista *Processes* (MDPI). Neste estudo foram avaliados os efeitos individuais e combinados da relação sólido-líquido (RSL), tempo de reação e uso de enzimas nos rendimentos de extração de óleo e proteína da torta de amêndoa. Foi realizada uma otimização empregando a ferramenta estatística CCDR (C*entral*

Composite Rotatable Design) para maximizar a extração e distribuição geral dos componentes extraídos entre as frações geradas pelo processo de extração aquosa (AEP) e aquosa assistida por enzima (EAEP). Com este estudo foram obtidos os valores ótimos de RSL, tempo de reação e teor de enzima para então as frações geradas a partir das extrações serem avaliadas em grande escala (capítulo 3).

O capítulo 4 dá continuidade ao processo de otimização e apresenta o artigo intitulado *Scaling-up the Simultaneous Extraction of Oil and Protein from Almond Cake: A Sustainable Approach to Conventional Hexane Extraction*, que será submetido para avaliação e possível publicação pelo periódico *Journal of Cleaner Production* (Elsevier). Neste artigo estão apresentados os resultados de rendimento de óleo e proteína dos parâmetros otimizados realizados em escala piloto em comparação com os resultados em escala laboratorial. Foi avaliado também o método de de-emulsificação da fração lipídica formada em AEP e EAEP para obtenção do óleo livre. Com este estudo mostrou-se a importância do uso de métodos alternativos de extração de óleo e proteína, que puderam ser recuperados de um subproduto, evitando a aplicação de solventes orgânicos, como o hexano.

O capítulo 5 refere-se ao artigo intitulado: *Effect of aqueous, enzyme-assisted and solvent aqueous extractions on functional properties of cold pressed almond cake protein*, que será submetido ao periódico *Food Chemistry* (Elsevier). Neste estudo foi avaliada a fração proteica gerada em escala piloto pelos métodos AEP e EAEP otimizados e HEX-AEP (controle), onde o material inicial foi desengordurado por hexano e utilizou-se os mesmos parâmetros otimizados da extração AEP. As frações proteicas foram avaliadas com relação as propriedades de superfície - potencial zeta e hidrofobicidade - e as propriedades tecnológicas - solubilidade, emulsificação e formação de espuma. Os diferentes processos de extração levaram a diferentes propriedades tecnológicas da proteína extraída, indicando diversos usos potenciais em sistemas alimentares, de acordo com a funcionalidade tecnológica desejada.

No capítulo 6 consta o artigo intitulado: *Impact of enzyme-assisted extraction process on skim biological properties from the almond cake*, que foi submetido ao periódico *Scientific Reports* (Nature Research). Neste estudo foi avaliada a fração

proteica gerada em escala piloto por AEP, EAEP e HEX-AEP (controle) com relação as propriedades biológicas das proteínas extraídas. Foram avaliados o teor total de compostos fenólicos, a capacidade antioxidante (ABTS e ORAC), antimicrobiana, antidiabética (inibição de α-glicosidade) e antiobesidade (inibição da lipase). Além disso, foi medida a digestibilidade *in vitro* nas diferentes extrações. Este estudo mostrou que as diferentes frações proteicas extraídas podem ser utilizadas como ingredientes nutracêuticos, sendo indicados para prevenir diabetes e obesidade, além de todas as frações apresentarem poder antioxidante e fácil digestão pelas enzimas digestivas.

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2. Objetivos

2.1 Objetivo geral:

Extrair simultaneamente óleo e proteína da torta de amêndoa utilizando extrações verdes: AEP (extração aquosa) e EAEP (extração aquosa-enzimática), sem utilizar solventes orgânicos.

2.2 Objetivos específicos:

1) Otimizar as extrações AEP e EAEP na torta de amêndoa;

2) A partir das condições otimizadas, escalonar a produção;

3) Após o escalonamento avaliar métodos de de-emulsificação na fração lipídica;

4) Após o escalonamento avaliar propriedades físico-químicas, tecnológicas e bioativas na fração proteica.

CAPÍTULO 1

Extração e propriedades das principais frações das sementes oleaginosas - uma breve revisão

1. Torta de amêndoa

Na indústria de óleo, cerca de 10 a 30% da produção se torna subproduto após o processamento industrial. Por isso, é crucial que a indústria de alimentos desenvolva sistemas de descarte de resíduos e gerenciamento destes subprodutos e, assim, reduzir os riscos ambientais (KARAMAN et al., 2015).

No processamento de amêndoas, os principais subprodutos gerados são a polpa fibrosa (mesocarpo), a casca dura (endocarpo), a casca fina marrom (exocarpo) e a água de lavagem (quando as amêndoas são branqueadas). Após a extração do óleo desta semente oleaginosas, dependendo do método utilizado, podem ser gerados diferentes tipos de resíduos, onde o produto obtido por prensagem é denominado torta, e quando a extração é por solvente, é chamado de farelo (SMERIGLIO et al., 2016; SUNIL et al., 2015). Neste estudo, o material inicial utilizado foi a torta de amêndoa, após prensagem mecânica a frio do óleo. A torta de amêndoa é um subproduto rico em proteínas e que ainda contém um teor residual de óleo. Este resíduo é normalmente utilizado na alimentação de animais sendo um produto com baixo valor comercial.

A torta de sementes oleaginosas é normalmente rica em proteínas, antioxidantes, fibras, vitaminas e minerais, sendo considerados ideais para suplementação alimentar. Estes subprodutos apresentam então um potencial de recuperação, que é baseado em suas propriedades químicas e em suas propriedades tecnológicas. Eles podem então ser utilizados para enriquecer diferentes produtos alimentares ou como fonte para a produção de diferentes ingredientes alimentares (KARAMAN et al., 2015; SUNIL et al., 2015).

2. Tipos de extração em oleaginosas

2.1 Prensagem mecânica

O método da prensa mecânica é definido como um sistema de separação de fase sólido-líquido para extrair óleo de oleaginosas que contém um teor de óleo acima de

20% (ÇAKALOĞLU; ÖZYURT; ÖTLEŞ, 2018). Neste método, a extração de óleo tem como principal etapa a prensagem, que pode ser definida como a realização da compressão de uma matriz porosa para exsudar um líquido (SAVOIRE; LANOISELLÉ; VOROBIEV, 2013). Este método consiste então em aplicar pressão na oleaginosa para forçar a saída do óleo (YUSUF, 2016).

Há dois tipos de prensa: a hidráulica (processo descontínuo) ou de parafuso (prensas contínuas, chamadas de "*expeller*"). No processo industrial a mais utilizada é a prensa de parafuso contínua, que é alimentada com o material a ser extraído - bruto ou pré-tratado. Os tipos de pré-tratamentos - térmico, redução de tamanho, peneiramento mecânico - variam de acordo com as características do material a ser extraído (SAVOIRE; LANOISELLÉ; VOROBIEV, 2013). A prensagem pode ser feita a quente, onde é possível ocorrer perda de componentes sensíveis à temperatura além de reações de oxidação, ou a frio, que é realizada por meios mecânicos e sem tratamento térmico, gerando um óleo de melhor qualidade e mais adequado para consumo direto (sem refino) (ÇAKALOĞLU; ÖZYURT; ÖTLEŞ, 2018).

Como vantagens, a extração mecânica apresenta simplicidade, rapidez, baixo custo e versatilidade: pode ser aplicada em diferentes oleaginosas e usar pequenas quantidades da matéria-prima. Em contrapartida, este método apresenta baixo rendimento em relação à extração por solvente (ÇAKALOĞLU; ÖZYURT; ÖTLEŞ, 2018). O rendimento de óleo irá depender de diversos fatores: variedade e teor de umidade da oleaginosa, pressão e temperatura aplicadas, tempo de prensagem, além das condições ambientais em torno da planta portadora de óleo, bem como dos procedimentos de pré-tratamento (KHAN; HANNA, 1983; YUSUF, 2016).

2.2 Extração por solvente

A extração por solvente se baseia na diferença de solubilidade de um soluto em duas fases líquidas imiscíveis (LI; SMITH; STEVENS, 2016). Este processo consiste na extração, por lavagens sucessivas em contracorrente com o solvente, do material oleaginoso previamente quebrado, moído ou prensado. O óleo, ao ser extraído por solvente, deve necessariamente passar pelo processo de refino, onde impurezas solúveis e insolúveis são removidas do óleo. Para a remoção do solvente presente no óleo,

utilizam-se evaporadores de filme ascendente ou destilação a vácuo (ROSENTHAL; PYLE; NIRANJAN, 1996).

Sementes oleaginosas ricas em lipídeos normalmente passam por uma pré-prensagem da semente para a obtenção de um óleo de qualidade superior e depois são submetidas à extração por solvente, para a recuperar o óleo residual da torta gerada (GONG; PEGG, 2015), como pode ser observado na Figura 1. A extração por solvente é convencionalmente usada na extração de óleo em sementes oleaginosas que apresentam baixo teor de óleo (< 20%), sendo considerado um dos métodos mais eficientes na extração de óleo vegetal (YUSUF, 2016).

O solvente mais usado na extração de óleo é o n-hexano. A extração com n-hexano apresenta como vantagem alto rendimento de óleo, podendo chegar acima de 96% (DE MOURA; DE ALMEIDA; JOHNSON, 2009). Apesar do alto rendimento, a exposição aguda de humanos a altos níveis de hexano causa efeitos leves do sistema nervoso central (SNC), incluindo tontura, vertigem, náusea leve e dor de cabeça, além de ser altamente inflamável e explosivo (LI et al., 2016a). O n-hexano é então classificado como uma neurotoxina e um composto orgânico volátil (CHANDRASEKARAN, 2016). Entre as desvantagens da extração por solventes tem-se: o alto investimento, alto consumo de energia, problemas de segurança da planta de extração, emissão de compostos orgânicos voláteis na atmosfera e baixa qualidade do produto final (YUSUF, 2016).

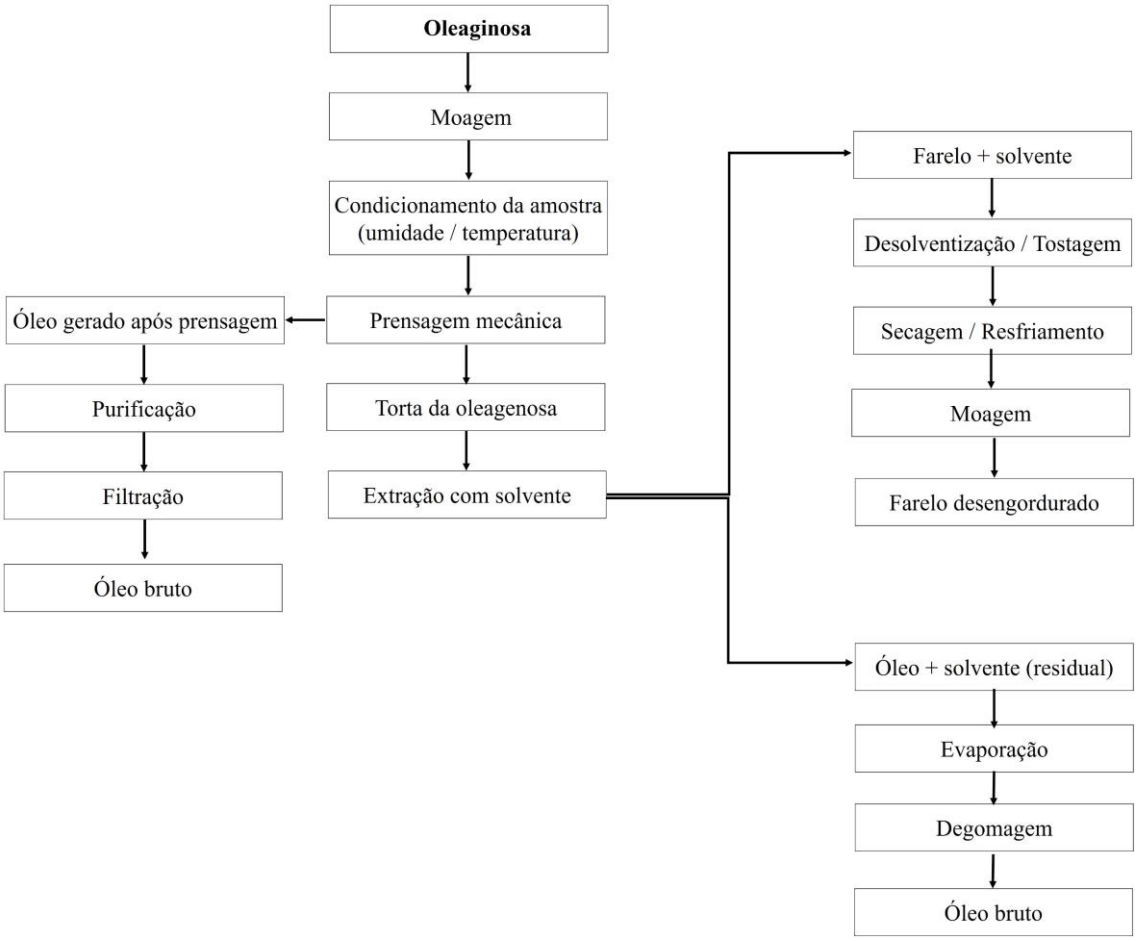

Figura 1: Extração convencional de óleo de oleaginosas, combinando a extração com por prensagem mecânica e solvente (Fluxograma adaptado das fontes: MANDARINO; HIRAKURI; ROESSING, 2015; ROSENTHAL; PYLE; NIRANJAN, 1996)

2.3 AEP e EAEP

Os processos de extração aquosa (AEP) e extração aquosa assistida por enzimas (EAEP) são processos alternativos à extração de óleo por solventes, por serem considerados mais seguros. Além disso, AEP e EAEP recuperam simultaneamente óleo e proteína (concentrada ou isolada) de matérias-primas oleaginosas, enquanto na extração com solvente, o óleo se difunde e é extraído através do solvente e a proteína fica retida no farelo junto com as fibras e os carboidratos (ROSENTHAL; PYLE; NIRANJAN, 1996).

A extração aquosa apresenta menor rendimento de óleo quando comparada à extração por solventes, porém a qualidade do produto final é superior, devido às

condições mais brandas empregadas, que asseguram a retenção de componentes importantes (CASAS; GONZÁLEZ, 2017). A extração por solventes se baseia na capacidade do solvente em dissolver e extrair o óleo, enquanto a extração aquosa usa o princípio da insolubilidade do óleo em água. Na AEP, o óleo não possui afinidade química pelo meio de extração, portanto, não há potencial químico para a dissolução do óleo (ROSENTHAL; PYLE; NIRANJAN, 1996).

As etapas da extração aquosa incluem a mistura das oleaginosas - previamente trituradas - com água; agitação da dispersão - para melhorar a extração de óleo e proteína; centrifugação para a separação das fases (sólida - fração insolúvel e líquida - frações proteica + oleosa); processo de de-emulsificação - para liberar óleo livre da fração lipídica e secagem (Figura 2) (CASAS; GONZÁLEZ, 2017; ROSENTHAL; PYLE; NIRANJAN, 1996). Os parâmetros que influenciam o rendimento da extração aquosa e aquosa assistida por enzima incluem a quebra do material inicial (tamanho de partícula e extensão da ruptura celular), proporção de sólido e líquido, pH, tempo, temperatura, grau de agitação e concentração da enzima (JUNG, 2009; ROSENTHAL; PYLE; NIRANJAN, 1996). A diferença na composição química e estrutura física de cada oleaginosa requer a alteração de condições específicas, como pH e temperatura de extração (ROSENTHAL; PYLE; NIRANJAN, 1996).

O uso de enzimas durante a extração aquosa favorece a recuperação de óleo a partir da hidrólise parcial das paredes celulares. A ruptura do sistema coloidal e a hidrólise de lipoproteínas e lipo-polissacarídeos complexos em moléculas mais simples facilitam a liberação e separação de óleo (CASAS; GONZÁLEZ, 2017). Enzimas como celulase, hemicelulase, pectinase, protease, amilase, glicanase e poligalacturonase foram usadas em estudos anteriores de EAEP (LIU et al., 2016). No caso de oleaginosas com alto teor de proteínas, geralmente é necessário o uso de proteases. As proteases catalisam a hidrólise das ligações peptídicas no interior das cadeias das proteínas, o que resulta na obtenção de fragmentos menores de proteínas ou peptídeos que ajudam a emulsionar e agregar as gotículas de óleo, liberando simultaneamente óleo e proteína. Contudo o uso excessivo de enzima pode levar a formação de um *cream* (emulsão óleo e água) mais resistente, o que dificulta a liberação posterior de óleo livre (CASAS; GONZÁLEZ, 2017).

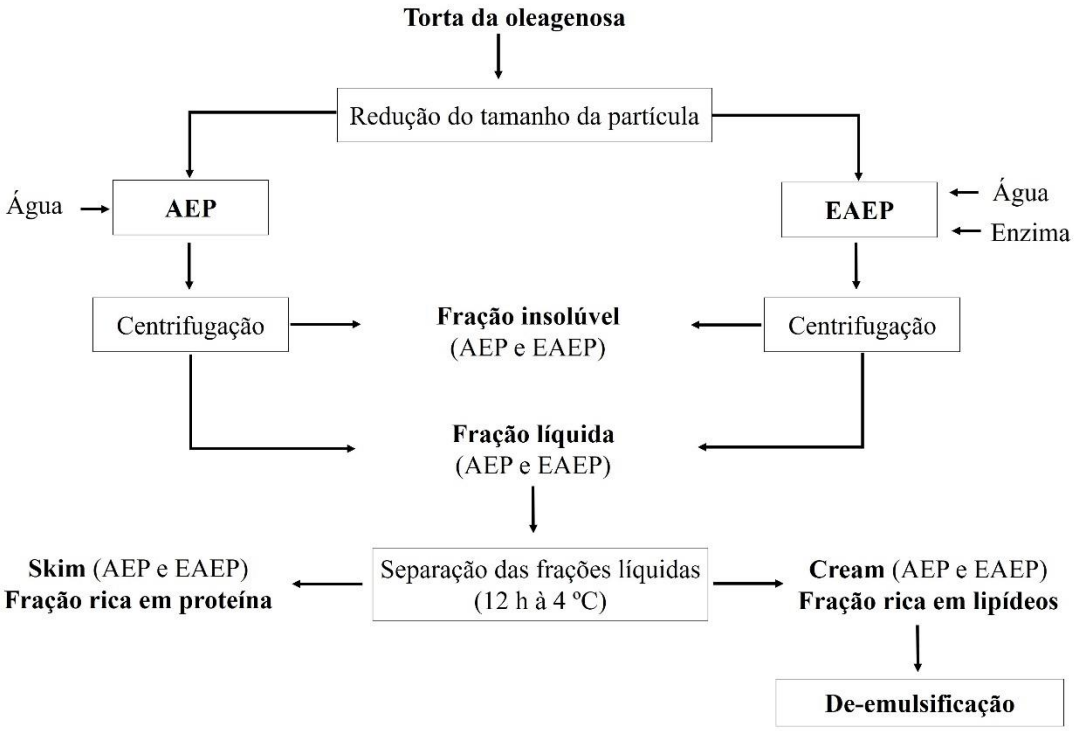

Figura 2: Esquema dos processos e extração aquosa (AEP) e aquosa-enzimática (EAEP)

O processo de extração aquosa agrega valor aos subprodutos, como proteínas, polissacarídeos, óleo e compostos fenólicos (CASAS; GONZÁLEZ, 2017). Além disso, evita-se a diminuição do valor nutritivo de suas proteínas e a presença de substâncias tóxicas a partir da inativação ou remoção de fatores antinutricionais, o que permite a produção de produtos alimentícios em vez de produtos de proteína para alimentação animal (ROSENTHAL; PYLE; NIRANJAN, 1996). AEP e EAEP apresentam como vantagens: menor risco de incêndio, menor perigo operacional e menor impacto ambiental, quando comparado ao uso de solventes; operação mais segura e flexível, que gera menos resíduos e menor consumo de energia; além do óleo extraído ser de melhor qualidade que o óleo extraído por métodos tradicionais, já que durante o processamento aquoso, os fosfolipídios são separados do óleo, assim não é necessária a etapa de degomagem (CASAS; GONZÁLEZ, 2017; LIU et al., 2016; ROSENTHAL; PYLE; NIRANJAN, 1996). Como desvantagens, tem-se: uso de mais tempo de reação e menor rendimento de óleo extraído em relação a extração com solvente e a etapa de de-

emulsificação da fração lipídica é necessária para recuperar e aumentar o rendimento do óleo, pois não é possível evitar a emulsificação do óleo extraído (LIU et al., 2016).

3. **Frações geradas em AEP e EAEP e recuperação**

As frações recuperadas por AEP e EAEP são: a fração insolúvel, rica em fibras; fração aquosa solúvel - *skim*, rica em proteínas e açúcares; fração *cream* que é rica em óleo (emulsão óleo-em-água) e a fração de óleo livre (Figura 3) (LAMSAL; JOHNSON, 2007). A centrifugação é a forma mais utilizada para a separação destas frações, pois desta forma o valor nutricional das proteínas alimentares não é alterado (ROSENTHAL; PYLE; NIRANJAN, 1996). Logo após a centrifugação, a fração insolúvel é recuperada, restando ainda uma fração líquida, que contém as frações *skim* e *cream* mais óleo livre. A separação das fases líquidas é realizada em funil de separação, onde os componentes solúveis se difundem na água e o óleo libertado forma uma fase líquida separada que é parcialmente emulsionado com água (ROSENTHAL; PYLE; NIRANJAN, 1996). Neste processo de separação, o funil de separação é mantido a baixa temperatura (4 ºC) por um longo período (~12 h). A fração liquida então é separada em *skim*, que é uma emulsão liquida rica em proteína e *cream*, uma emulsão cremosa rica em lipídeos.

Figura 3: Frações geradas a partir das extrações AEP e EAEP

A fração proteica (*skim*) pode ser recuperada como concentrado na fase sólida ou como isolado na fase aquosa. O procedimento para recuperar a proteína concentrada consiste na quebra da oleaginosa, seguida de uma extração ácida e por fim é feita a centrifugação para a separação da fração líquida e sólida. A partir da fração sólida a proteína concentrada é então extraída. No caso da proteína isolada, deve haver também

a quebra da oleaginosa, porém a extração é realizada em meio alcalino e então a mistura é centrifugada. Neste caso a proteína é extraída da fração líquida, após passar pelo decantador de 3 fases (centrífuga que separa dois líquidos de diferentes densidades) e então tem-se a fração *cream*, a insolúvel e a *skim*. Esta última fração é então centrifugada após precipitação ácida e por fim obtém-se a proteína isolada (ROSENTHAL; PYLE; NIRANJAN, 1996).

A fração lipídica (*cream*) é formada por uma emulsão estabilizada por diversos componentes celulares, especialmente proteínas (CASAS; GONZÁLEZ, 2017). Portanto, a quebra desta emulsão, por um método de de-emulsificação, é necessária para a liberação do óleo livre. O processo de de-emulsificação por ser realizado com uso de enzimas (fosfolipases ou proteases) ou de forma não enzimática (congelamento-descongelamento ou precipitação das proteínas emulsificantes em seu ponto isoelétrico) (DE MOURA et al., 2008).

4. Fatores que afetam o rendimento da extração

4.1 Proporção de sólidos: líquido

Uma alta proporção de sólido para líquido (mais sólido e menos liquido) é desejável para a obtenção de emulsões menos estáveis e para gerar menos efluentes; contudo, geralmente é necessário usar grandes quantidades de água para obter maiores rendimentos de extração (ROSENTHAL; PYLE; NIRANJAN, 1996). Os maiores problemas do uso de uma grande quantidade água são o aumento do resíduo líquido e a dificuldade no processo de de-emulsificação, o que leva a custos crescentes de tratamento de águas residuais e de de-emulsificação (LIU et al., 2016).

A proporção de sólido para líquido ideal varia de acordo com a oleaginosa a ser usada. Na literatura, já foram reportadas proporções de 1:5 em amendoim (LI et al., 2017); 1:10 e 1:20 em girassol (CAMPBELL et al., 2016); 1:6 (DE MOURA BELL et al., 2013) e 1:10 em soja (DE MOURA; JOHNSON, 2009) e 1:5 em colza (ZHANG; WANG; XU, 2007).

4.2 Tamanho de partícula

O tamanho das partículas da matéria-prima oleaginosa afeta o rendimento de óleo (LIU et al., 2016). Quanto menor o tamanho de partícula, maior o rendimento de óleo, devido à maior rompimento da parede celular durante a redução de tamanho, o que facilita a difusão das enzimas e de componentes celulares (YUSOFF; GORDON; NIRANJAN, 2015). É importante observar que a moagem de forma excessiva favorece a ruptura celular e o aumento da eficiência da extração de óleo e proteína, mas também produz glóbulos de óleo menores, o que torna a de-emulsificação mais difícil. A moagem feita de forma insuficiente, por outro lado, resulta em grandes perdas na extração de óleo (ROSENTHAL; PYLE; NIRANJAN, 1996). Os métodos mecânicos mais utilizados para romper a parede celular são: moagem, corte em lascas, extrusão e tratamento em ultrassom ou micro-ondas (CHANDRASEKARAN, 2016).

4.3 pH

De forma geral, para maximizar os rendimentos de óleo e proteína em matérias-primas oleaginosas, a extração aquosa deve ser realizada em pH longe do ponto isoelétrico (pI). Se houver uso de enzima na extração, o valor de pH deve estar na faixa de pH ótimo para atividade enzimática (CASAS; GONZÁLEZ, 2017). Segundo Rosenthal; Pyle; Niranjan (1996), maiores rendimentos de extração de óleo geralmente coincidem com maiores rendimentos de proteína; assim, a extração aquosa de óleo em oleaginosas pode ser considerada como um processo destinado principalmente à solubilização de proteínas, que resulta na liberação do óleo.

A extração em pH alcalino é mais utilizada, tendo sido testada em várias oleaginosas, confirmando que gera maior rendimento (FETZERA et al., 2018; SARI; BRUINS; SANDERS, 2013). Este alto rendimento pode ser explicado pela maior solubilidade das proteínas em pH básico (CAMPBELL et al., 2011). Embora as condições alcalinas possam melhorar a capacidade de extração das proteínas de oleaginosas, valores de pH muito alcalinos podem gerar efeitos adversos, como redução da digestibilidade das proteínas e dano a alguns aminoácidos (lisina e cisteína) (SARI; BRUINS; SANDERS, 2013). Na literatura, já foram reportados valores de pH em extração aquosa/ aquosa enzimática de 9,0 em soja (DE MOURA et al., 2010; JUNG,

2009) e em amendoim (LI et al., 2016b) e de pH 12,0 em torta de canola (GERZHOVA et al., 2016).

4.4 Tempo

O tempo de reação varia entre as diferentes oleaginosas e as demais variáveis utilizadas nos processo (ROSENTHAL; PYLE; NIRANJAN, 1996). Um longo tempo de reação normalmente aumenta o rendimento da extração, porém é também maior a dificuldade no processo de de-emulsificação. Um curto tempo de reação reduz a eficiência da hidrólise enzimática, o que leva a baixos rendimentos de óleo (LIU et al., 2016). Prolongar o tempo de incubação pode melhorar a degradação dos componentes da parede celular, no entanto, tempos muito longos não são práticos (acima de 9 h), além de ocasionar a diminuição da qualidade do óleo e exigir alto uso de energia (CASAS; GONZÁLEZ, 2017; LIU et al., 2016). Na literatura já foram avaliados tempos de 15 min a 1 h em soja e foi observado que o aumento do tempo de reação gerou rendimento total de óleo semelhante, mas a distribuição de óleo entre as frações foi alterada (DE MOURA; DE ALMEIDA; JOHNSON, 2009); 30 min a 1,5 h em feijão carioca, sendo 1 h o tempo ótimo encontrado (NGOH; GAN, 2016); 30 min a 1,5 h em abobrinha, onde 69 min foi observado como tempo ótimo (LI et al., 2016c) e 3 a 6 h em amêndoa, observando tempo ótimo de 4,6 h (MOGHADAS; REZAEI, 2017).

4.5 Temperatura

A faixa considerada ótima de temperatura para a hidrólise enzimática é entre 40 °C e 55 °C, utilizada na maioria dos estudos de EAEP, onde deve-se observar se a enzima utilizada tem atividade na temperatura escolhida (RUI et al., 2009). O rendimento do óleo é aumentado até uma certa temperatura, ao passar desta temperatura, normalmente observa-se uma taxa constante ou ocorre uma diminuição do rendimento. Além do rendimento de óleo, as características e a qualidade final do óleo também devem ser levadas em consideração ao selecionar a temperatura (YUSOFF; GORDON; NIRANJAN, 2015). Condições de processamento mais brandas, como temperaturas de extração mais baixas, levam ao aumento do rendimento da extração além de melhorar a qualidade do produto final (PURI; SHARMA; BARROW, 2012).

A prensagem convencional e a extração com solventes envolvem altas temperaturas (70 a 90 °C), o que reduz a qualidade e a solubilidade das proteínas, sendo então uma vantagem o uso de temperaturas mais brandas em AEP/EAEP (FETZERA et al., 2018). Em soja foram testadas temperaturas de 25, 50 e 65 °C e a melhor recuperação de óleo livre foi obtida em temperatura média de 50 °C (JUNG; MAURER; JOHNSON, 2009); como exemplos de temperatura que foram fixadas, tem-se: em amendoim, onde a extração aquosa foi realizada a 60 °C (LI et al., 2017) e em girassol, foi aplicada extração aquosa enzimática com temperatura de 50 °C (CAMPBELL et al., 2016).

4.6 Tipo e concentração de enzima

A composição de cada oleaginosa determina a escolha da enzima. Para a obtenção do maior rendimento possível de óleo, pode-se misturar diferentes enzimas (ROSENTHAL; PYLE; NIRANJAN, 1996). O uso de celulase e hemicelulase é necessário para a liberação de óleo quando a matéria-prima oleaginosa contém alto teor dos polissacarídeos celulose e hemicelulose. No caso das oleaginosas com alto teor proteico, geralmente é necessário o uso de proteases (CHANDRASEKARAN, 2016).

Quanto maior a concentração de enzima, maior será a interação entre a enzima e o substrato, o que leva à degradação da parede celular e ao rompimento de mais ligações peptídicas. Segundo a literatura, o rendimento do óleo é normalmente aumentado até uma determinada concentração de enzima, após este limite, a taxa de rendimento fica constante devido à saturação dos substratos (JIANG et al., 2010). Diferentes concentrações de enzima foram testadas para aplicação em EAEP: soja, com 0,5% da protease Protex 6L (DE MOURA et al., 2008; JUNG, 2009); semente de abóbora, com 1,5% de concentração da mistura das enzimas celulase, pectinase e proteinase (1:1:1, v/v/v) (GAI et al., 2013) e colza, adicionando 2,5% da combinação de pectinase, celulase e β-glicanase (4:1:1, v/v/v) (ZHANG; WANG; XU, 2007).

4.7 Agitação

A agitação auxilia a mistura e a ruptura adicional da parede celular e a velocidade de agitação afeta criticamente a distribuição de tamanho das gotas de óleo que, por sua vez, influencia sua separação (ROSENTHAL; PYLE; NIRANJAN, 1998). A

diminuição na velocidade de agitação pode acarretar a diminuição da recuperação de óleo, enquanto o aumento da velocidade pode levar à emulsificação (na presença ou não de enzima) (SHARMA; KHARE; GUPTA, 2002). Sharma e colaboradores (2002) testaram diferentes velocidades de agitação (50, 80, 100 e 200 rpm) e foi observado que de 50 a 80 rpm houve aumento no rendimento de óleo, enquanto de 80 para 100 e 200 rpm o rendimento de óleo foi diminuído. Por isso, é importante selecionar a velocidade de agitação apropriada, que resulte no maior rendimento de óleo possível, considerando tanto o óleo recuperado quanto a estabilidade da emulsão ao final do processo de extração (YUSOFF; GORDON; NIRANJAN, 2015). Na literatura já formam testadas diferentes velocidades de agitação, que foram fixas, a seguir alguns exemplos: semente de gergelim - 80 rpm (HOU et al., 2013), semente de girassol - 120 rpm (LATIF; ANWAR, 2009) e soja - 180 rpm (DE MOURA BELL et al., 2013).

5. Propriedades tecnológicas das proteínas

5.1 Solubilidade

A solubilidade das proteínas está relacionada à sua estrutura, ao pH do solvente, à temperatura e à presença ou não de sais na solução (NICKERSON, 2010). A solubilidade é influenciada pelo equilíbrio de hidrofilicidade e hidrofobicidade na superfície da proteína que, por sua vez, depende da composição de aminoácidos. Quando há um baixo número de resíduos hidrofóbicos em pH acima e abaixo do pH isoelétrico da proteína e há presença de carga elevada, ocorre um aumento da repulsão eletrostática e da hidratação iônica ocorrendo então o aumento da solubilidade (MOURE et al., 2006). No ponto isoelétrico da proteína (pI), a estrutura tem carga superficial líquida igual a zero, o que resulta em solubilidade mínima, pois proteínas vizinhas apresentam uma tendência a se agregar, levando à precipitação dos aglomerados formados (NICKERSON, 2010). A influência da temperatura na solubilidade é variável: em temperaturas abaixo da temperatura de desnaturação da proteína, a solubilidade é tipicamente aumentada com o aumento da temperatura. No entanto, a partir da temperatura de desnaturação, a proteína muda de conformação e expõe aminoácidos hidrofóbicos, antes escondidos, o que resulta na sua aglomeração e perda de solubilidade (NICKERSON, 2010).

A solubilidade da proteína está normalmente relacionada a outras propriedades tecnológicas. Quanto mais solúvel é a proteína, maior a possibilidade de atuar como um bom emulsificante, agente espumante, gelificante ou espessante para uma ampla variedade de aplicações (MOURE et al., 2006).

5.2 Emulsificação

Emulsões são definidas como misturas de dois líquidos imiscíveis (geralmente óleo e água), onde um líquido é a fase dispersa na fase contínua do outro liquido (LAM; NICKERSON, 2013; MCCLEMENTS, 2004). Para formar uma emulsão é preciso a presença de óleo, água, emulsificante e energia (DAMODARAN; PARKIN; FENNEMA, 2008). Devido à imiscibilidade do óleo e da água, as emulsões são inerentemente instáveis e, com o passar do tempo, separam-se em duas fases distintas (NICKERSON, 2010). Dependendo do sistema, a instabilidade em uma emulsão O/A pode assumir diferentes formas: (1) *creaming* é o movimento ascendente das gotículas devido ao fato de elas terem uma densidade menor que o líquido circundante; (2) sedimentação é o movimento descendente das gotículas devido ao fato de elas terem uma densidade maior que o líquido circundante; (3) floculação ocorre quando duas ou mais gotículas se juntam para formar um agregado no qual as gotículas retêm sua integridade individual e (4) coalescência é o processo pelo qual duas ou mais gotículas se fundem para formar uma única gota maior (MCCLEMENTS, 2004).

As emulsões são amplamente encontradas em produtos alimentícios, podendo variar entre emulsões de água-em-óleo (A/O), como margarinas e manteiga, ou emulsões óleo-em-água (O/A), como leite e molhos de salada (DAMODARAN; PARKIN; FENNEMA, 2008; NICKERSON, 2010). Proteínas são preferencialmente escolhidas para aplicação em emulsões alimentícias do tipo O/A pois são comestíveis, ativas na superfície e solúveis em água, além de proporcionarem resistência à coalescência (DAMODARAN; PARKIN; FENNEMA, 2008). Proteínas atuam para estabilizar as emulsões, revestindo a superfície das gotículas individuais de óleo para evitar coalescência ou floculação, assegurando uma boa dispersão das gotículas de óleo dentro da fase contínua da água (NICKERSON, 2010). Devido à sua natureza anfifílica,

proteínas são de grande interesse em termos das suas propriedades emulsionantes e capacidades de formação de película (LAM; NICKERSON, 2013).

5.3 Formação de espuma

Semelhante às emulsões, as espumas são misturas de duas fases imiscíveis com gases e água representando as fases dispersa e contínua, respectivamente e, assim como as emulsões, espumas requerem uma entrada de energia para a sua formação (NICKERSON, 2010). Algumas proteínas atuam como surfactantes, estabilizando a interface de espuma (WOUTERS et al., 2016). As proteínas atuam migrando para a interface ar-água e se reorientam para posicionar os aminoácidos hidrofóbicos em direção à fase gasosa e aminoácidos hidrofílicos em direção à fase aquosa e, em seguida, formam uma película rígida ao redor das bolhas de gás, que confere resistência contra a ruptura (NICKERSON, 2010).

A interface ar-água é termodinamicamente instável, pois as interações entre das moléculas de água entre si são muito mais favoráveis do que as interações entre as moléculas de água e o ar. As espumas colapsam rapidamente, devido a três mecanismos principais de desestabilização (WOUTERS et al., 2016): (1) maturação de Ostwald, que se dá pela difusão de gás em bolhas pequenas em bolhas maiores; (2) drenagem da espuma que leva ao escoamento líquido, que ocorre ao longo da camada de espuma devido à gravidade e (3) coalescência das bolhas, causada pela instabilidade do filme entre elas (DAMODARAN; PARKIN; FENNEMA, 2008).

Espumas à base de proteínas são encontradas na indústria de alimentos, em merengues, mousses e cerveja (NICKERSON, 2010). Proteínas com boa atividade espumante devem adsorver o ar rapidamente durante o batimento; sofrer uma rápida mudança conformacional ao se reorganizar na interface ar-água para reduzir a tensão superficial e formar um filme coeso e visco-elástico através de interações intermoleculares (MOURE et al., 2006).

6. Avaliação das características superficiais das proteínas

6.1 Potencial zeta

O potencial zeta fornece informações sobre o grau de interação entre partículas coloidais, medindo o potencial eletrostático na dupla camada elétrica na superfície das proteínas em solução (GABOR, 2011; GERZHOVA et al., 2016; KORMA et al., 2019). As partículas coloidais acumulam carga em sua superfície, que pode ser expressa como um potencial de superfície (MALHOTRA; COUPLAND, 2004). Quando a carga líquida das partículas é a mesma, as forças repulsivas são predominantes, impedindo a agregação das partículas, que permanecem em suspensão. Por outro lado, quando a carga líquida se torna neutra, não há força repulsiva, o que resulta na agregação e precipitação de partículas em suspensão, fenômeno conhecido como precipitação por ponto isoelétrico (GERZHOVA et al., 2016).

Para medir a carga coloidal é necessária a aplicação de uma tensão elétrica na partícula seguida da medição da velocidade do movimento induzido. Na prática, o potencial determinado não é o da superfície, mas de uma distância curta e indefinida da camada difusa - o potencial z. A carga superficial nas partículas de proteína ocorre devido à ionização parcial de vários resíduos de aminoácidos (MALHOTRA; COUPLAND, 2004). Como as proteínas contêm grupos funcionais ácidos e básicos, sua carga depende do pH e da força iônica do meio (CHALAMAIAH et al., 2017).

6.2 Hidrofobicidade da superfície

Segundo Hayakawa e Nakai (1985), a solubilidade das proteínas está relacionada às características da superfície da proteína, como potencial zeta, e à hidrofobicidade da superfície. Portanto, a avaliação dessas características é essencial avaliar a correlação entre as características superficiais e as propriedades tecnológicas das proteínas.

O número de grupos hidrofóbicos na superfície de uma proteína em contato com um meio aquoso determina a hidrofobicidade da superfície, que desempenha um papel essencial na condução da agregação de proteínas e na atividade interfacial da proteína (DICKINSON, 2003; ZHAO et al., 2012). De acordo com Zhu et al. (2017), a hidrofobicidade da superfície (H_0) é uma característica física importante das proteínas,

que influencia suas propriedades tecnológicas, pois reflete diretamente a distribuição de resíduos hidrofóbicos na superfície da proteína. Segundo Nakai et al., (1980), quanto mais hidrofóbica a proteína, maior será o alinhamento e a integração de proteínas na interface óleo-água, permitindo que a tensão interfacial seja reduzida. Proteínas com maior H_0 tendem a se agregar, via interação hidrofóbica, e formar agregados, que precipitam em solução, então quanto menor a H_0, menor a chance das proteínas se agregarem (NICKERSON, 2010). A H_0 pode ajudar a explicar algumas funcionalidades tecnológicas das proteínas, uma vez que solubilidade, propriedades emulsificantes e espumantes estão correlacionadas às suas propriedades superficiais.

7. **Peptídeos bioativos**

Proteínas são fontes naturais de aminoácidos essenciais e algumas proteínas da dieta alimentar possuem propriedades biológicas específicas, tornando-se ingredientes potenciais para alimentos funcionais (DE CASTRO; SATO, 2015). Para uma proteína ou peptídeo ser considerado um ingrediente funcional, ele deve demonstrar, além do efeito nutricional, efeitos benéficos em uma ou mais funções do corpo e esse efeito deve ser significante para a saúde em geral ou ser capaz de reduzir o risco de doenças (GARCÍA et al., 2013).

Os alimentos funcionais geralmente contêm um ingrediente adicionado com efeitos à saúde confirmados (MENRAD, 2003; NIVA, 2007). No entanto, certos alimentos funcionais contêm constituintes que ocorrem naturalmente com efeitos bioativos conhecidos (ECKERT et al., 2013; LÓPEZ-EXPÓSITO et al., 2007; MCGREGOR; POPPITT, 2013). Durante a digestão ou processamento desses alimentos, as proteínas são degradadas em peptídeos, que podem ter um efeito positivo na saúde humana (MOUGHAN et al., 2014). Esses são chamados peptídeos bioativos, sequências curtas de aminoácidos (AA), geralmente de dois a 20 resíduos (DE CASTRO; SATO, 2015), que são liberados a partir de uma fonte de proteína durante o processamento tecnológico do alimento ou durante a digestão *in vivo* (MAESTRI et al., 2019). Estes peptídeos contêm aminoácidos que apresentam atividade biológica, incluindo atividade antioxidante, anti-hipertensiva, antitrombótica, antimicrobiana, anti-inflamatória e efeitos imunomoduladores (DE CASTRO; SATO, 2015). A bioatividade

é determinada principalmente pela composição, tipo, número e sequência de aminoácidos (AGYEI et al., 2016; DE CASTRO; SATO, 2015).

Entre os métodos mais utilizados para a obtenção de peptídeos bioativos tem-se a fermentação e a hidrólise enzimática. A escolha de uso entre estes métodos depende da fonte de alimento a ser avaliada. Além disso, cada técnica leva a diferentes impactos no tipo e na atividade dos peptídeos formados. A hidrólise enzimática oferece maior especificidade e reprodutibilidade, enquanto a fermentação pode levar a uma variedade de peptídeos com atividades diferentes pois diferentes cepas bacterianas e condições podem ser aplicadas (MAESTRI et al., 2019). Na hidrólise enzimática, a proteína de interesse é submetida a um tratamento enzimático onde a extração ocorre em pH e temperatura específicos. A vantagem deste método é que normalmente há necessidade de menos tempo do que a fermentação microbiana. Na fermentação, a proteína de interesse é hidrolisada por uma cultura de microrganismos, como leveduras, fungos ou bactérias. O grau de hidrólise depende do tempo de fermentação, cepa microbiana e fonte de proteína (CHAKRABARTI; GUHA; MAJUMDER, 2018).

8. **Atividades Biológicas**

8.1 Capacidade antioxidante

A produção de radicais livres nos organismos é regulada por diferentes moléculas antioxidantes, que podem ser endógenas ou podem vir da dieta. Se houver limitação na disponibilidade de compostos antioxidantes, pode haver danos oxidativos na célula (DANTAS et al., 2019). Peptídeos antioxidantes minimizam danos causados por radicais livres aos lipídios, ácidos nucléicos e proteínas podendo então ser utilizados na prevenção da oxidação lipídica (MAESTRI et al., 2019).

Entre as várias classes de antioxidantes que ocorrem naturalmente, os compostos fenólicos - ácidos fenólicos, cumarinas, flavonóides e outros - têm recebido muita atenção (DANTAS et al., 2019). Antioxidantes derivados de proteína oferecem benefícios potenciais, sendo considerados alternativas seguras em relação a antioxidantes sintéticos na preservação de alimentos, além de reduzir o risco de incidência de doenças causadas por estresse oxidativo (LUNA-VITAL et al., 2015).

Atualmente existe grande interesse na busca de antioxidantes naturais, que sejam seguros para evitar a deterioração oxidativa dos alimentos e minimizar os danos oxidativos às células vivas (DANTAS et al., 2019). A oxidação lipídica é o principal fator que causa a deterioração dos alimentos durante o processamento e o armazenamento, produzindo alterações indesejadas na cor, sabor, textura e perfil nutricional, além de gerar produtos de reação potencialmente tóxicos (PIOVESANA et al., 2018).

8.2 Capacidade antimicrobiana

Devido ao desenvolvimento de resistência microbiana aos agentes antimicrobianos existentes, infecções microbianas tornaram-se uma ameaça clínica. Desta forma, descobrir novos agentes antimicrobianos é de grande importância. Atualmente, os produtos naturais têm sido uma das principais fontes de novas moléculas de medicamentos. Produtos de origem vegetal estão entre os mais testados e utilizados como compostos antimicrobianos descobertos até agora. Plantas e outras fontes naturais podem fornecer uma enorme variedade de compostos complexos e estruturalmente diversos (BALOUIRI; SADIKI; IBNSOUDA, 2016). O uso de fontes naturais de compostos antimicrobianos tem um grande potencial, pois possuem características como baixa toxicidade e alta especificidade (DE CASTRO; SATO, 2015).

Entre os produtos mais investigados como potenciais agentes antimicrobianos, estão extratos vegetais e microbianos, óleos essenciais, metabólitos secundários purificados e novas moléculas sintetizadas (BALOUIRI; SADIKI; IBNSOUDA, 2016). Peptídeos com atividade antimicrobiana podem estar presentes naturalmente nos sistemas de defesa de plantas e animais, ou podem ser gerados após a hidrólise de proteínas. A maioria dos peptídeos antimicrobianos é semelhante, apresentando cadeias curtas de aminoácidos, compostas principalmente por aminoácidos catiônicos e hidrofóbicos (DE CASTRO; SATO, 2015).

8.3 Atividade antidiabética e antiobesidade

O diabetes *mellitus* tipo 2 (DM2) e a obesidade são doenças crônicas de importância para a saúde pública como resultado de sua crescente prevalência global (AWOSIKA; ALUKO, 2019). O diabetes *mellitus* (DM) é um distúrbio metabólico

caracterizado por defeitos na secreção e/ou na ação da insulina, que levam à hiperglicemia crônica. O DM é classificado nos tipos 1 (DM1) e 2 (DM2), onde DM2 representa mais de 90% do total de casos de diabetes no mundo (IBRAHIM et al., 2018). O DM1 é uma doença autoimune que leva a um grave defeito na secreção de insulina que não apresenta evidências de ter relação aos hábitos alimentares das pessoas, enquanto o DM2 está relacionado ao estilo de vida e fatores alimentares que levam ao sobrepeso e obesidade com resistência à insulina concomitante no fígado, músculo e tecido adiposo (UUSITUPA; SCHWAB, 2020). A obesidade é considerada como fator de risco para o desenvolvimento e progressão de várias doenças crônicas, incluindo o DM2. Uma abordagem terapêutica eficaz para combater o DM2 e a obesidade pode estar associada à inibição de enzimas digestivas como a α-glucosidase e lipase pancreática (AWOSIKA; ALUKO, 2019).

A α-glicosidase hidrolisa a ligação α- (1 e 4) entre unidades de glicose a partir da extremidade não-redutora liberando moléculas de glicose (DESHAWARE et al., 2017). Inibidores de α-glicosidade reduzem a taxa de absorção dos mono-, di- e tri-sacarídeos pela inibição da degradação de carboidratos complexos no trato gastrointestinal, proporcionando um controle da hiperglicemia pós-prandial, através da modulação da taxa de digestão de carboidratos complexos (IBRAHIM et al., 2019).

A obesidade é definida como uma 'condição caracterizada pelo acúmulo de gordura, que apresenta um risco à saúde'. É resultante da interação complexa entre genética, ingestão alimentar, atividade física, estilo de vida e fatores ambientais. Essas interações resultam em balanço energético positivo a longo prazo e, finalmente, no aumento da massa gorda corporal (AWOSIKA; ALUKO, 2019). A lipase pancreática é uma enzima essencial no processo de absorção de lipídios da dieta, responsável por 50% a 70% da hidrólise dos triacilgliceróis ingeridos em monoacilgliceróis e ácidos graxos livres (WANG et al., 2019). A inibição da lipase pancreática reduz a eficiência da absorção de lipídios no intestino delgado e, assim, inicia uma redução modesta e a longo prazo no peso corporal (JAFAR et al., 2018).

Uma vez que a α-glicosidase e a lipase pancreática são as principais responsáveis pela liberação de substratos energéticos após a ingestão de alimentos, o uso de um

agente que pode inibir as atividades das duas enzimas poderia fornecer um meio mais eficaz de controlar o diabetes e a obesidade (AWOSIKA; ALUKO, 2019). A maioria dos medicamentos antidiabéticos apresenta efeitos colaterais, sendo importante a busca por novos agentes, que sejam seguros e não apresentem efeitos colaterais, como peptídeos bioativos (IBRAHIM et al., 2019). Peptídeos bioativos podem atuar como inibidores de enzimas metabólicas e apresentam potencial uso como agentes terapêuticos contra doenças específicas (AWOSIKA; ALUKO, 2019; IBRAHIM et al., 2019).

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CAPÍTULO 2

Amêndoas: Uma revisão

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RESUMO

Amêndoas são *nuts* adaptadas ao clima temperado e subtropical, produzidas principalmente no estado da Califórnia (77% da produção mundial). Esta semente oleaginosa é considerada um alimento altamente nutritivo, sendo fonte de lipídeos (ácidos graxos insaturados)**,** proteínas, vitamina E, minerais essenciais e fibra dietética, além da presença de metabolitos secundários (em quantidades menores)**.** Há mais de 30 variedades conhecidas de amêndoas cultivadas, que se dividem em dois tipos, podendo ser doce ou amargo, sendo a doce mais comercializada e consumida como *snacks* e como farinha de amêndoa para aplicação em diversos alimentos, como produtos de panificação. Esta revisão traz uma visão geral sobre o cultivo e produção de amêndoas, bem como as características e composição da semente. São também abordadas informações atuais sobre alergenicidade, os benefícios do consumo e potenciais aplicações não apenas na amêndoa como *nut*, mas também dos subprodutos gerados ao longo do processamento.

Palavras chave: amêndoa, produção, composição, benefícios do seu consumo e potenciais aplicações.

1. **Amêndoas: Uma Visão Geral**

A amêndoa cultivada é designada como *Prunus dulcis* (Miller) D.A. Webb, e outros sinônimos utilizados incluem *Prunus amygdalus* Batsch e *Prunus communis* L, bem como sua designação inicial como *Amygdalus communis* L. (YADA; LAPSLEY; HUANG, 2011)**.** Este fruto pertence ao gênero *Prunus* e à família Rosaceae, que inclui também maçãs, peras, pêssegos, ameixas e framboesas (MANDALARI et al., 2014; YADA; LAPSLEY; HUANG, 2011)**.** A classificação em *Prunus* é dividida em cinco subgêneros: *Prunophora, Amygdalus, Cerasus, Padus e Laurocerasus* (KHADIVI-KHUB; ANJAM, 2014). As amêndoas podem ser classificadas como doces (*Amygdalus communis* L. var. *Dulcis*) ou amargas *Amygdalus communis* L. Var. *Amara*) e são consideradas as *tree nuts* mais populares em termos de produção comercial, apresentando uma produção global em torno de 1,3 milhões de toneladas na safra de 2019/20 (USDA, 2019b)**.**

Amêndoas são cultivadas em países de clima temperado e subtropical, sendo frutos adaptados para toda a região do Mediterrâneo, caracterizado por invernos amenos e verões quentes e secos (YADA; HUANG; LAPSLEY, 2013; KODAD; COMPANY; ALONSO, 2018). São cultivadas comercialmente em locais com verões longos e quentes como a Espanha, Marrocos, Armênia, Irã, Itália, EUA e Austrália (ESFAHLAN; JAMEI; ESFAHLAN, 2010; KODAD et al., 2013)**.** Os maiores produtores de amêndoas no mundo são os Estados Unidos, com uma produção em torno de 998,000 toneladas na safra de 2019/20 (USDA, 2019b). O estado dos EUA com maior produção é a Califórnia, sendo responsável por cerca de 77% da produção mundial deste fruto (USDA, 2019).

Amêndoas e outras oleaginosas são alimentos vegetais altamente nutritivos por demonstrar um perfil de nutrientes importantes para a saúde, sendo fontes de proteína, ácidos graxos mono- e poli-insaturados, fibra dietética, vitamina E e minerais essenciais (MARTÍNEZ et al., 2013; YADA; HUANG; LAPSLEY, 2013). Amêndoas fornecem uma grande variedade de nutrientes em quantidades elevadas por porção, já que são minimamente processadas, ou seja, são consumidas diretamente como *snacks,* não necessitando ser processadas antes do consumo além de conter poucos ácidos graxos saturados, açúcares, amido e sódio (DROGOUDI et al., 2013). Além disso, são alimentos que fornecem energia, devido a sua composição nutricional ser caracterizada por uma alta porcentagem de lipídios totais (46 a 76 g por 100 g) - ricos em ácidos graxos insaturados (65% de monoinsaturados e

25% de poli-insaturados), que estão inversamente correlacionados aos níveis séricos de colesterol (GRIEL; KRIS-ETHERTON, 2006; KODAD et al., 2013; SATHE; SZE, 1997; SOUZA et al., 2015).

Estudos tem demonstrado que a ingestão regular em longo prazo de amêndoas, pode trazer benefícios relacionados à saúde como: ajudar a regular o peso; modular a glicose no sangue; reduzir lipídios plasmáticos; melhorar o perfil lipídico em adultos levemente hipercolesterolêmicos; reduzir estresse oxidativo e redução de fatores de risco de doença coronariana (GALLIER; GORDON; SINGH, 2012; JACKSON; HU, 2014; KAMIL; CHEN, 2012; MANDALARI et al., 2014; TAN; MATTES, 2013; WIEN et al., 2003)**.** Desta forma, há um crescente interesse no consumo por ser considerado um alimento saudável (MARTÍNEZ et al., 2013).

2. **Cultivo e Produção**

A plantação de amêndoas começou a ser documentada em locais como Grécia e Chipre há 5000 anos e era comum na Palestina em 1700 A.C. As amêndoas foram introduzidas nos Estados Unidos em 1800, a partir de cultivares específicos importadas do sul da França**.** Há mais de 30 variedades conhecidas de amêndoas cultivadas comercialmente e cerca de dez variedades principais que representam a maior parte da produção (SATHE et al., 2002; YADA; HUANG; LAPSLEY, 2013).

O ciclo de crescimento das amêndoas é adaptado ao clima mediterrânico, que é caracterizado por um inverno úmido e um verão seco. Este fruto é tolerante à seca, mas sua produção, rentabilidade e produtividade são dependentes da irrigação durante cada estação de crescimento (PHOGAT et al., 2013). As condições ideais para o crescimento das amendoeiras são um clima ameno, solos profundos formados por argila, areia, humos e material orgânico e presença abundante de luz solar (ABC, 2015; WIFSS, 2016).

A Califórnia é o local de maior produção de amêndoas dos EUA, em uma área que se estende por mais de 650 Km em que aproximadamente 7.000 produtores individuais cultivam mais de 400.000 hectares de amêndoas (ESFAHLAN; JAMEI; ESFAHLAN, 2010). A maioria das variedades comerciais de amêndoas cultivadas na Califórnia são descendentes de duas variedades não relacionadas - Nonpareil e Mission - originadas de mudas selecionadas a partir de fontes importadas do sul da França. Carmel, Neplus e Peerless são as outras grandes

variedades comerciais, que juntamente com as variedades originais representam mais de 90% da produção total de amêndoa nos Estados Unidos, sendo a variedade Nonpareil responsável por cerca de 35% da produção total de amêndoas (SATHE et al., 2002; YADA; HUANG; LAPSLEY, 2013).

A produção mundial de amêndoa (sem casca), para a safra de 2019/2020 foi estimada em 1,3 milhões de toneladas. Os Estados Unidos são os maiores produtores, com uma produção em torno de 998 mil toneladas. O segundo lugar é ocupado pela União Europeia com uma produção de 121 mil toneladas e o terceiro maior produtor é a Austrália, com 95 mil toneladas. Outros produtores são China, com 45 mil tonadas, Turquia com 15 mil toneladas e outros países com 16 mil toneladas de produção de amêndoas. Os Estados Unidos, além de serem os maiores produtores, são também os maiores exportadores de amêndoa, com uma exportação de 690 mil toneladas, seguidos pela Austrália (75 mil toneladas), União Europeia (25 mil toneladas), Chile (10 mil toneladas) e Turquia (7 mil toneladas) (USDA, 2019b).

3. **Características dos Frutos de Amêndoa**

O fruto da amêndoa é constituído por quatro partes distintas: exocarpo (casca fina coriácea conhecida como a pele marrom), que envolve o mesocarpo (polpa fibrosa), endocarpo (casca da amêndoa) e semente (caroço) (ALONSO; ESPADA; SOCIAS, 2012; DOURADO et al., 2004; ESFAHLAN; JAMEI, 2012; GODINI, 1984), como pode ser observado na Figura 1. A semente é constituída pelo tegumento e pelo embrião. Este embrião contém dois cotilédones volumosos e uma pequena radícula (DOURADO et al., 2004). A importância nutricional do fruto da amêndoa está relacionada a a semente que é a parte comestível, enquanto a casca e a polpa fibrosa (mesocarpo) são utilizadas para alimentação animal e como combustível, sendo consideradas subprodutos. O que difere entre as variedades de amêndoa é a textura da casca que são denominadas como casca dura ou macia (ESFAHLAN; JAMEI, 2012; ESFAHLAN; JAMEI; ESFAHLAN, 2010).

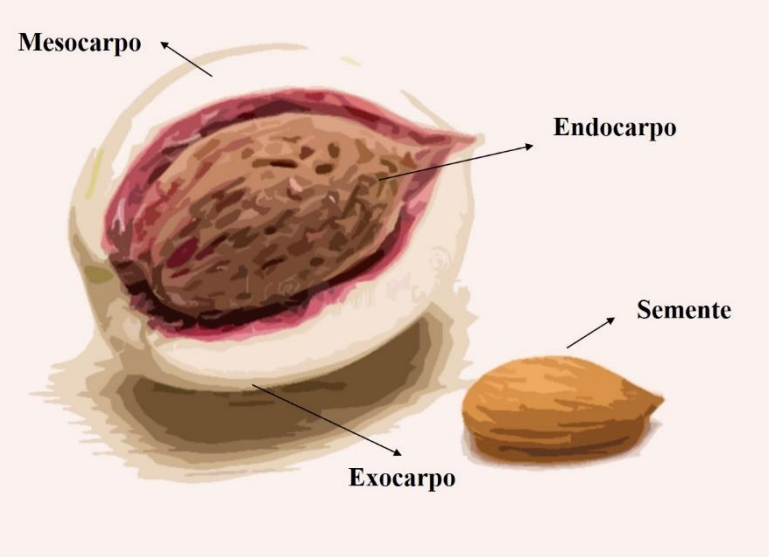

Figura 1: Partes do fruto e semente de amêndoa

Amendoeiras crescem normalmente entre 3 a 4,5 metros de altura. O crescimento da fruta assemelha-se ao do pêssego, até que se aproxime a maturidade e, à medida que o ciclo de maturação continua, a polpa se abre e quando seca pode ser separada da semente com facilidade (ESFAHLAN; JAMEI; ESFAHLAN, 2010). A sua forma é oval e a cor do exocarpo pode variar entre dez tons distintos, indo do verde claro ao avermelhado (KHADIVI-KHUB; ANJAM, 2014). O ciclo de vida das amendoeiras começa no ano zero, que requer o preparo da terra e o estabelecimento do pomar (plantação); os dois anos seguintes são dedicados ao crescimento da árvore onde ainda não há rendimento e neste período há baixo consumo de água e de fertilizantes. Entre os anos 3 a 6 há rendimento crescente de amêndoas e o consumo de água e fertilizantes é aumentado. Por fim, entre os anos 7 a 25 ocorre a maturidade da árvore e os rendimentos são máximos e requerem água em quantidades máximas. Após a colheita no ano 25, o pomar é removido, para ser restabelecido no ano seguinte (KENDALL et al., 2015).

4. Formas de Consumo de Amêndoas

A amêndoa está entre as *nuts* mais populares que são produzidas e consumidas em todo o mundo. A demanda pelo seu consumo vem crescendo nos últimos anos devido às suas características físico-químicas, nutricionais e sensoriais. Amêndoas são consumidas como nozes - *snacks* (produto cru), podendo ser comercializada tanto em fatiadas ou inteiras,

descascadas (sem a pele) ou não (com a pele). Além disso, essas *nuts* são consumidas de forma cozida ou torrada a seco; processadas em pó (ex. farinha); ou como bebidas não alcoólicas (ex. “leite” de amêndoas) e ainda são amplamente utilizadas como ingredientes em produtos de panificação e confeitaria, e na preparação de alimentos em geral (BOLLING, 2017; PRGOMET et al., 2017).

A demanda por alternativas ao leite bovino vem aumentando nas últimas décadas devido a alergia ou intolerância ao leite animal. Entre estas "alternativas não lácteas" tem-se o extrato solúvel de amêndoa – “leite” de amêndoa. O “leite” de amêndoa é uma dispersão coloidal obtida após a mistura de água com amêndoas em pó ou pasta de amêndoa. Desta mistura obtém-se um líquido branco que é então filtrado, homogeneizado (por alta pressão) e por fim é pasteurizado (VANGA; RAGHAVAN, 2018). Outra demanda dos dias atuais é o consumo de produtos *gluten-free*, que está relacionada a patologias associadas à ingestão de glúten como alergia ao glúten, doença celíaca e sensibilidade ao glúten (TORRES et al., 2017). A farinha de amêndoa é então um ingrediente interessante por ser uma farinha alternativa que não contém glúten, além disso, a farinha de amêndoas proporciona mais benefícios nutricionais do que a farinha de trigo e do que as farinhas tradicionalmente usadas para a produção de massas sem glúten (MARTÍNEZ et al., 2017).

A amêndoa fatiada pode ser adicionada a produtos como sorvete e cereais matinais. Para a produção de amêndoa sem pele é realizado um branqueamento, onde a pele é removida pela adição de água quente ou vapor. Os grãos de amêndoa quando moídos em pasta podem ser utilizados em produtos de panificação e na produção de marzipã (doce árabe) (GRADZIEL, 2009). De acordo com um relatório da Innova Market Insights (2018) a introdução de novos produtos de amêndoas cresceu em quatro das principais categorias em todo o mundo, incluindo confeitaria (22%), *snacks* (19%), produtos de panificação (17%) e barras de cereal (16%). Além disso, os principais *claims* usados nas embalagens foram "sem glúten" (24%) e “sem aditivos/conservantes” (15%).

5. **Diferenças entre Amêndoa Doce e Amarga**

Há duas variedades de amêndoa, o tipo doce (*Amygdalus communis* L. var. *Dulcis*) e o tipo amarga (*Amygdalus communis* L. Var. *Amara*) (KARATAY et al., 2014). A principal diferença entre os genótipos de amêndoa é que no tipo doce a prunasina é degradada,

enquanto no tipo amargo, a prunasina é sintetizada no tegumento e transportada para o cotilédone por meio das células de transferência e convertida em amigdalina na semente da amêndoa em desenvolvimento. Esta diferenciação ocorre porque o genótipo doce é rico em atividade citoplasmática e em β-glicosidase no citoplasma e no vacúolo, o que impede a formação de amigdalina ao degradar a prunasina, enquanto no genótipo amargo, a atividade da β-glicosidase nessa camada celular é baixa, não impedindo a formação de amigdalina (SÁNCHEZ-PÉREZ et al., 2008)**.** A amigdalina é um diglicosídeo cianogênico comumente encontrado em frutas como damascos, ameixas, pêssegos, maçãs, mamão e cerejas, além de estar presente em alguns tipos de *nuts* (THODBERG et al., 2018). Amêndoas amargas contêm quantidade significativa de amigdalina (3 a 9%), que está presente apenas em vestígios em amêndoas doces (WIRTHENSOHN et al., 2008; YADA; LAPSLEY; HUANG, 2011).

A toxicidade das amêndoas amargas é causada pelo acúmulo de amigdalina, que libera ácido cianídrico tóxico, após a hidrólise (THODBERG et al., 2018). A amigdalina sofre hidrólise enzimática e é convertida em duas moléculas de glicose, além de mandelonitrila, que devido à sua natureza instável, é espontaneamente convertida em HCN e benzaldeído. A biossíntese da amigdalina em amêndoas começa a partir do aminoácido fenilalanina, que é convertido em prunasina a partir de dois cromossomos (CYP79 e CYP71) e por uma glicosiltransferase (UGT1), então a prunasina é convertida em amigdalina. Na bioativação (Figura 2), a amigdalina é degradada pela amigdalina hidrolase (AH) e são geradas moléculas de prunasina e glicose. A prunasina é então degradada pela prunasina hidrolase (PH) e são geradas uma mandelonitrila mais uma molécula de glicose. A mandelonitrila é hidrolisada pela mandelonitrila liase 1 (MDL1) e por fim são gerados bezaldeído (gosto amargo) e ácido cianídrico (tóxico). Os glicosídeos cianogênicos se encontram em locais diferentes na célula das hidrolases AP e PH (β-glicosidases) então tanto os glicosídeos quanto as hidrolases se encontram inativos. No momento em que o tecido é danificado, os glicosídeos cianogênicos são ativados pelas β-glicosidases e então é liberado o ácido cianídrico (DEL CUETO et al., 2017)**.**

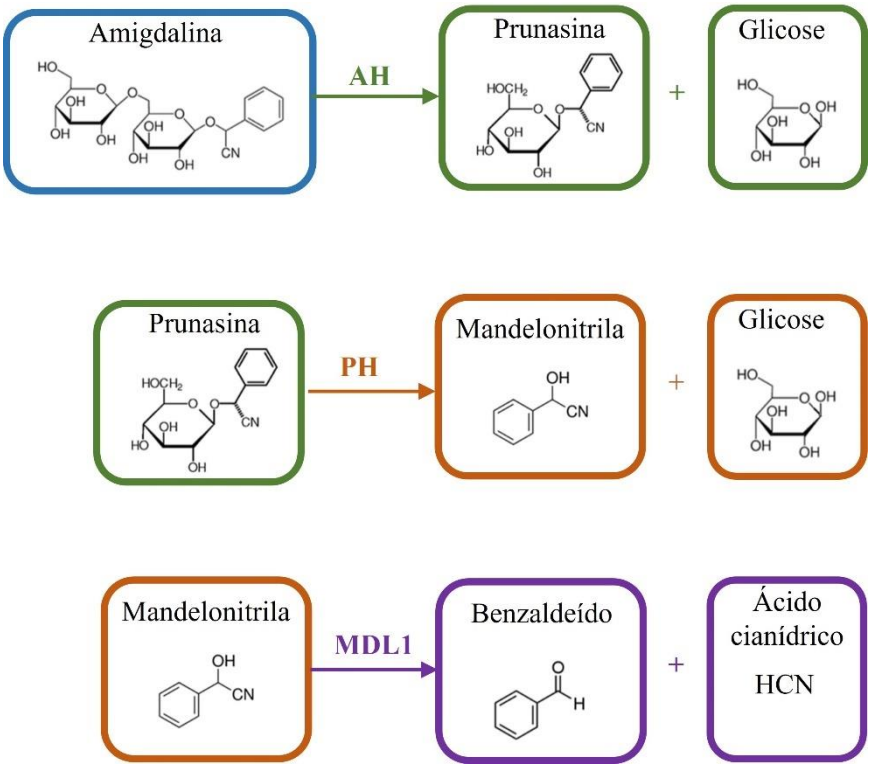

Figura 2. Vias metabólicas da bioativação dos glicosídeos cianogênicos - prunasina e amigdalina em amêndoas. β-glicosidases**:** AH - amigdalina hidrolase, PH - prunasina hidrolase; MDL1 - mandelonitrila liase 1. (Adaptado das fontes: DEL CUETO et al., 2017; JASWAL; PALANIVELU, 2018)

Para a remoção do ácido cianídrico do óleo extraído, a torta de amêndoa amarga é macerada com água por um longo tempo para que então ocorra a hidrólise da amigdalina. Por fim o benzaldeído e o ácido cianídrico são separados por destilação a vapor e o HCN é eliminado, assim o óleo volátil purificado de amêndoas contêm principalmente benzaldeído (EVANS; EVANS; TREASE, 2009)**.** Uma pesquisa mais recente de Zhang e colaboradores (2019) testou diferentes formas de reduzir o teor de HCN em amêndoa amarga e observaram que os tratamentos de cozimento (79%), micro-ondas (87%), ebulição com água (98%) e ultrassom (98%) foram eficientes.

A amêndoa doce apresenta características únicas, pois o fruto inteiro é consumido. Ela pode ser consumida desde o início do seu ciclo de maturação até algumas semanas, enquanto outros frutos em sua família botânica (Ex. pêssego, damasco e ameixa) apenas o mesocarpo (polpa) é consumido, descartando-se a casca e a semente (ESFAHLAN; JAMEI; ESFAHLAN, 2010). O óleo de amêndoas amargas é utilizado na fabricação de aromas

alimentares ou na indústria de cosméticos (KAMIL; CHEN, 2012; WIRTHENSOHN et al., 2008; YADA; LAPSLEY; HUANG, 2011).

6. **Composição**

Os principais componentes encontrados nas sementes de amêndoas são: lipídeos, proteínas, carboidratos (açúcares metabolizáveis), minerais, fibras e metabolitos secundários, que aparecem em pequenas quantidades, mas que influenciam a qualidade das amêndoas (RONCERO et al., 2016). Na Tabela 1 pode ser observada a composição geral das amêndoas. A composição de nutrientes deste fruto é dependente do genótipo, de fatores ambientais, da região de cultivo, dos métodos de cultivo, das condições climáticas que variam entre os anos de colheita, da variação nos fatores de produção (tipo de solo, método de irrigação e variação da temperatura) ou ainda das interações destes fatores (YADA; LAPSLEY; HUANG, 2011).

Tabela 1. Composição dos macronutrientes em amêndoas

Nutriente	Valor por g/100 g
Água	4.4
Energia	579 kcal
Energia	2423 kJ
Proteína	21.2
Lipídio total (gordura)	49.9
Cinza	3.0
Carboidrato, por diferença	21.6
Fibra, dieta total	12.5
Açúcares, total	4.4
Sacarose	4.0
Glicose (dextrose)	0.2
Frutose	0.1
Lactose	0.0
Maltose	0.0
Galactose	0.1
Amido	0.7

Fonte: (USDA, 2019a)

A maior parte da pesquisa sobre composição de amêndoas é concentrada na fração lipídica e na diversidade de ácidos graxos, uma vez que sementes de amêndoas possuem elevado valor nutritivo devido ao alto teor lipídico (KODAD; COMPANY, 2008; RONCERO et al., 2016). Os lipídios presentes em amêndoas são sobretudo lipídios de

armazenamento, que estão presentes como gotículas de óleo intracelular nos tecidos de cotilédones da semente. A fração lipídica neste fruto é constituída predominantemente por ácidos graxos monoinsaturados e poli-insaturados, o que constitui uma importante fonte calórica, mas não contribui para a formação de colesterol em seres humanos, uma vez que a ingestão desses ácidos graxos costuma ser inversamente correlacionada com os níveis séricos de colesterol (KODAD; COMPANY, 2008; YADA; LAPSLEY; HUANG, 2011).

Os ácidos graxos predominantes no óleo de amêndoa constituem 95% do total de lipídios e são: ácido oleico (50 - 80% do teor total de ácidos graxos), ácido linoleico (10 - 26%), ácido palmítico (5,0 - 9,0%) e ácido esteárico (1,5 – 4,0%), enquanto o ácido linolênico é encontrado em concentrações muito baixas (< 0,1%). Na Tabela 2 é apresentada a composição lipídica principal presente em amêndoas. Os componentes secundários no óleo de amêndoas incluem: tocoferóis (450 μg por g de óleo), esteróis (2200 μg por g) e esqualeno (95 μg por g). O principal tocoferol é o α-tocoferol, com 240 - 440 μg por g e os principais esteróis são β-sitosterol, com ~96 mg por 100 g dos esteróis totais e campesterol e estigmasterol com aproximadamente 4,5 e 1,0 mg por 100 g (MAESTRI et al., 2015; WANG et al., 2019). Durante o armazenamento e transporte, a amêndoa pode sofrer rancificação devido à oxidação dos seus ácidos graxos, o que leva à perda de qualidade do produto. Desta forma, a estabilidade do óleo e a composição de ácidos graxos são consideradas importantes critérios para avaliar a qualidade do produto (FORCADA; MARTÍ; COMPANY, 2012).

Tabela 2. Composição das principais frações de ácidos graxos em amêndoas

Lipídios	Valor por g/100 g
Ácidos graxos saturados totais	3.8
16:0	3.1
18:0	0.7
Ácidos graxos, monoinsaturados totais	31.6
16:1	0.2
18:1	31.3
Ácidos graxos polinsaturados	12.3
18:2	12.3
Ácidos graxos, trans total	0.0
Colesterol	0.0

Fonte: (USDA, 2019a)

As amêndoas são também uma boa fonte de proteína, pois seu teor se aproxima ao da carne vermelha, que pode variar entre 14 a 26%. Além disso, a proteína de amêndoa é eficientemente digerida e absorvida (FORCADA; MARTÍ; COMPANY, 2012; YADA; LAPSLEY; HUANG, 2011). O teor de proteína em amêndoas é mínimo durante os primeiros meses da formação do fruto, mas aumenta significantemente nos últimos meses antes da colheita, podendo conter mais de 188 proteínas diferentes (YADA; LAPSLEY; HUANG, 2011; ZHANG et al., 2016b). A proteína de armazenamento predominante em amêndoas é chamada de amandina ou prunina ou simplesmente proteína principal de amêndoa (do inglês: AMP - *Almond Main Protein*). A AMP é uma globulina de tipo 14S, composta por diferentes polipeptídios, de natureza oligomérica (12 polipeptídios por mol) com pesos moleculares estimados entre 42 a 46 e 20 a 22 kDa ligados por ligações de dissulfeto e pertencente à classe das leguminas, contendo em torno de 19% de nitrogênio (MANDALARI et al., 2014; SATHE et al., 2002). As proteínas em amêndoas contêm em torno 30% de aminoácidos essenciais, e estes apresentam um bom balanço, com exceção da metionina, seguida da lisina e treonina, que são os aminoácidos limitantes (KODAD et al., 2013; YADA; LAPSLEY; HUANG, 2011). A proteína presente nas amêndoas é facilmente hidrolisada pelas proteases digestivas, produzindo um hidrolisado proteico de alta qualidade em relação ao balanço de aminoácidos essenciais (KAMIL; CHEN, 2012; KODAD et al., 2013).

Para medir a qualidade de uma proteína, deve-se levar em consideração fatores da proteína, como digestibilidade e disponibilidade de aminoácidos. Uma forma de medir a qualidade da proteína a partir do Índice de aminoácidos corrigido pela digestibilidade proteica (PDCAAS), que é definido como a relação entre o conteúdo do primeiro aminoácido limitante na proteína (mg/g) e o conteúdo deste aminoácido em uma proteína de referência (mg/g), multiplicado pela digestibilidade verdadeira (TPD). O padrão de referência é a necessidade de aminoácidos essenciais para crianças entre 2 e 5 anos de idade (MARINANGELI; HOUSE, 2017). Ahrens e colaboradores (2005) avaliaram a digestibilidade por PDCAAS e concluíram que as proteínas da amêndoa são de baixa qualidade nutricional. Neste estudo, mesmo encontrando um valor alto de digestibilidade verdadeira que varou de 88.5 a 92.2%, dependendo da variedade analisada, ao calcular o PDCAAS para lisina, obteve-se valores entre 0.32 a 0.34 para crianças (2 a 5 anos) e 1.14 a 1.22 para adultos e o PDCAAS para aminoácidos sulfurados (metionina + cisteína) foram encontrados valores de 0.22 a 0.24 e de 0.32 a 0.35 para crianças e adultos respectivamente.

No estudo mais recente de House et al., (2019) foram encontrados valores de PDCAAS para lisina entre 0.43 a 0.48, dependendo da variedade. Portanto, indica-se incorporar quantidades apropriadas de aminoácidos limitante (lisina, aminoácidos sulfurados e tirosina) no desenvolvimento de produtos com a presença de proteínas de amêndoa para crianças. Para adultos, o consumo de amêndoas acompanhado por uma dieta equilibrada e variada, rica em aminoácidos sulfurados pode ser considerada uma fonte de proteína importante na dieta. Segue na Tabela 3 a quantidade dos aminoácidos presente em amêndoas e também a ingestão dietética recomendada e a quantidade de aminoácidos ingeridos ao consumir 100 g de amêndoa. Na Tabla 4 tem-se a digestibilidade medida por PDCAAS em diferentes alimentos.

Tabela 3. Composição de aminoácidos, ingestão dietética recomendada (IDR) e ingestão de aminoácidos na dieta (IAD) de cada aminoácido, considerando o consumo de 100g de amêndoas de acordo com o sexo

Aminoácidos	Valor mg/100g [a]	mg/Kg/dia		%	
		IDR (Homem) [b]	IDR (Mulher) [b]	IAD (Homem) [c]	IAD (Mulher) [c]
Triptofano (W)	200.0	5	5	4000.0	4000.0
Treonina (T)	600.0	20	20	3000.0	3000.0
Isoleucina (I)	800.0	19	19	4210.5	4210.5
Leucina (L)	1500.0	42	42	3571.4	3571.4
Lisina (K)	600.0	38	38	1578.9	1578.9
Metionina (M) + Cistina (C)	200.0 200.0	19	19	1063.2	1063.2
Fenilalanina (F) + Tirosina (Y)	110.0 500.0	33	33	1518.5	1518.5
Valina (V)	900.0	24	24	3750.0	3750.0
Histidina (H)	500.0	14	14	3571.4	3571.4
Arginina (R)	2500.0	-	-	-	-
Alanina (A)	100.0	-	-	-	-
Ácido aspártico (D)	2600.0	-	-	-	-
Ácido glutâmico (E)	620.0	-	-	-	-
Glicina (G)	140.0	-	-	-	-
Prolina (P)	100.0	-	-	-	-
Serina (S)	900.0	-	-	-	-

[a] Valores dos minerais (mg/100 g de amêndoa) derivados da fonte: USDA (2019a) para a referência padrão NDB # 12061, *nuts*, amêndoas; [b] Valores IDR derivados da fonte: INSTITUTE OF MEDICINE (2006), considerando a faixa etária entre 31 a 50 anos para os sexos feminino e masculino; [c] Valores IMR calculados: IMR (%) = C × 100 / IDR, sendo C o nível aminoácido encontrado em amêndoas. Valores calculados para os aminoácidos

essenciais: W, T, I, L, M (+C), F (+Y), Y, V e H. Para os não essências não há IDR: R, A, D, E, G, P e S.

Tabela 4. Digestibilidade da proteína de diferentes alimentos por PDCAAS e DIAAS

Alimentos [a]	Quantidade	PDCAAS [b]
Leite, integral (01077)	852 mL	1.0 (1.10)
Ovo, cozido (01129)	100 g	1.0 (1.05)
Peito de frango (05064)	100 g	1.0 (1.01)
Aveia (08121)	250 mL	0.82
Arroz, branco (20045)	140 g	0.56
Feijão, preto (16006)	250 g	0.6
Amêndoa (12061)	30 g	0.39
Girassol, semente (12036)	30 g	0.66
Grão de bico (16057)	35 g	0.74

[a] Código dos alimentos da fonte: USDA (2019a) para cada referência padrão NDB; [b] PDCAAS (Índice de aminoácidos corrigido pela digestibilidade proteica) da fonte: MARINANGELI e HOUSE (2017)

Os carboidratos estão presentes em baixas quantidades na semente das amêndoas e na forma de açúcares metabolizáveis e como polissacarídeos (fibras alimentares) (COMPANY et al., 2010). O teor de açúcares totais na semente da amêndoa é por volta de 5.5%, onde a composição pode ser afetada pela irrigação, tempo de colheita e condições de armazenamento (KAZANKAYA et al., 2008). No estudo de Nanos et al. (2002) foi observado que a irrigação não afetou o teor de açúcar, mas modificou significativamente a composição de açúcar, após irrigação o maior teor foi de sacarose e rafinose e o menor foi de inositol. Além disso também foi encontrado um maior teor de açúcar na colheita após maturação que foi atribuído ao acúmulo de sacarose e alterações na composição do açúcar, favorecendo a produção de sacarose. Resultado similar ao de Kazantzis e colaboradores (2003), que observaram um teor mais baixo de açucares e muitas alterações na composição de açúcares nas amêndoas colhidas prematuramente. Foram também avaliadas condições de armazenamento, onde ao armazenar as amêndoas tanto descascadas quanto não descascadas ou a 5 ^{o}C ou a 20 ^{o}C (após 6 meses) não foi percebida diferença na quantidade de açucares, mas houve alteração na composição em que a sacarose se manteve constante e os outros açúcares se transformaram em diferentes compostos. A maioria dos açúcares presentes neste fruto é composta por sacarose e rafinose, mas outros açúcares também estão presentes, em menores quantidades, como sorbitol e inositol, e existe traço de xilose, frutose, glicose e galactose (NANOS et al., 2002). Quanto ao teor de fibras, as amêndoas (semente + casca) contém em torno de 12% de

fibra total. Quarenta e cinco por cento da casca é composta por fibra dietética, onde apenas 3 - 4 % são fibras solúveis (substancias pécticas) e a maior fração é de fibras insolúveis (celulose e hemicelulose) (MANDALARI et al., 2010b).

Saura-Calixto; Canellas; Garcia-Raso (1984) analisaram o teor de carboidratos no mesocarpo, endocarpo, tegumento e na semente de amêndoas, por cromatografia gasosa, e observaram que a sacarose é o carboidrato em maior concentração em todas as frações analisadas. Foi observada também a presença de inositol, sorbitol, glicose e frutose em todas frações, com exceção da semente. No estudo sobre a composição de carboidratos na casca de amêndoas realizado por de Sequeira e Lew (1970) a partir de cromatografia gasosa, foram encontrados os seguintes compostos: frutose (8,8%), glicose (10,4%), sacarose (5,2%), inositol (2,5%) e sorbitol (4,6%) e um total de 31.5% de carboidratos presentes na casca. Enquanto em uma pesquisa mais recente de Offeman et al. (2014) a partir da casca, em seis diferentes variedades de amêndoas da Califórnia, formam observadas as seguintes variações de carboidratos: 30,1 a 39,3% de frutose; 33,2 a 43,6% de glicose; 8,0 a 15,1% de sacarose; 2,6 a 6,8% de inositol; 8,3 a 15,8% de sorbitol e 1,7 a 3,4% de xilose.

Os minerais encontrados nas sementes amêndoas são obtidos pela planta a partir do solo em que cresce e da água aplicada na produção (YADA; LAPSLEY; HUANG, 2011). Desta forma, o conteúdo mineral pode ser afetado por muitos fatores ambientais e práticas agronômicas como, por exemplo, a composição do solo, irrigação, recursos hídricos e uso de fertilizantes (YADA; HUANG; LAPSLEY, 2013). Durante o crescimento e amadurecimento do fruto, os minerais se acumulam na semente e na polpa, enquanto na casca ocorre uma diminuição do teor de minerais (SCHIRRA et al., 1994). Os elementos minerais mais abundantes encontrados na semente da amêndoa são potássio, cálcio, magnésio, fósforo, zinco, cobre e manganês (GRUNDY; LAPSLEY; ELLIS, 2016). O conteúdo mineral é normalmente expresso como teor em cinzas, que é o resíduo inorgânico remanescente após a incineração do tecido vegetal. As sementes de amêndoa contêm aproximadamente 3% de cinzas (peso úmido) (YADA; LAPSLEY; HUANG, 2011). Na Tabela 5 pode ser observado o teor dos minerais presentes em amêndoas, a ingestão dietética recomendada e a quantidade de minerais ingeridos ao consumir 100 g de amêndoa.

Tabela 5. Composição, ingestão dietética recomendada (IDR) e ingestão mineral na dieta (IMD) de cada mineral, considerando o consumo de 100g de amêndoas de acordo com o sexo

Minerais	Valor (mg/100g) [a]	mg/dia		%	
		IDR (Homem) [b]	IDR (Mulher) [b]	IMD (Homem) [c]	IMD (Mulher) [c]
Cálcio, Ca	269.0	1000	1000	26.9	26.9
Ferro, Fe	3.7	8	18	46.4	20.6
Magnésio, Mg	270.0	400	310	67.5	87.1
Fósforo, P	481.0	700	700	68.7	68.7
Potássio, K	733.0	4700	4700	15.6	15.6
Sódio, Na	1.0	1500	1500	0.1	0.1
Zinco, Zn	3.1	11	8	28.4	39.0
Cobre, Cu	1.0	0.9	0.9	114.6	114.6
Manganês, Mn	2.2	2.3	1.8	94.7	121.1
Selênio, Se	0.0041	0.055	0.055	7.5	7.5

[a] Valores dos minerais (mg/100 g de amêndoa) derivados da fonte: USDA (2019a) para a referência padrão NDB # 12061, *nuts*, amêndoas; [b] Valores IDR derivados da fonte: INSTITUTE OF MEDICINE (2006), considerando a faixa etária entre 31 a 50 anos para os sexos feminino e masculino; [c] Valores IMR calculados: IMR (%) = C × 100 / IDR, sendo C o nível mineral encontrado em amêndoas.

A vitamina E é a principal vitamina encontrada em amêndoas. Esta vitamina é produzida apenas por vegetais e contem oito diferentes isoformas, e são elas: α-, β-, γ-, σ-, T tocoferol (contém uma cadeia lateral de fitol saturada de 16 átomos de carbono) e α-, β-, γ-, σ, T3 tocotrienol (contém três duplas ligações na cadeia lateral) (LÓPEZ-ORTIZ et al., 2008; YADA; LAPSLEY; HUANG, 2011). As diferentes isoformas são derivadas do 6-cromanol e se diferem pelo número e pela posição dos grupos metila na estrutura (LÓPEZ-ORTIZ et al., 2008). A α-tocoferol é a isoforma biologicamente mais ativa da vitamina E (YADA; LAPSLEY; HUANG, 2011). O conteúdo de tocoferóis é o mais estudado entre as vitaminas, mas além desta há outras vitaminas em amêndoas, como biotina (B7), folato (B9), niacina (B3), ácido pantotênico (B5), piridoxina (B6), riboflavina (B2) e tiamina (B1), que se apresentam em quantidades bem menores. Como as amêndoas apresentam baixa umidade e alto teor de lipídios, é esperado que o teor de vitaminas lipossolúveis, como a vitamina E seja superior ao das hidrossolúveis, como as do complexo B. Na Tabela 6 é apresentada a composição de vitaminas encontrada em amêndoas, além da ingestão dietética recomendada e a quantidade de vitamina ingerida ao consumir 100 g de amêndoa.

Tabela 6. Composição, ingestão dietética recomendada (IDR) e ingestão de vitaminas na dieta (IVD) de cada vitamina, considerando o consumo de 100g de amêndoas de acordo com o sexo

Vitaminas	Valor mg/100g [a]	mg/dia		%	
		IDR (Homem) [b]	IDR (Mulher) [b]	IVD (Homem) [c]	IVD (Mulher) [c]
Tiamina	0.2	1.2	1.1	17.1	18.6
Riboflavina	1.1	1.3	1.1	87.5	103.5
Niacina	3.6	16	14	22.6	25.8
Ácido pantotênico	0.5	5	5	9.4	9.4
Vitamina B-6	0.1	1.3	1.3	10.5	10.5
Folato, total	0.044	0.4	0.4	11.0	11.0
Vitamina E (alfa-tocoferol)	25.6	0.015	0.015	170866.7	170866.7

[a] Valores dos minerais (mg/100 g de amêndoa) derivados da fonte: USDA, (2019a) para a referência padrão NDB # 12061, *nuts*, amêndoas; [b] Valores IDR derivados da fonte: Institute of Medicine (2006), considerando a faixa etária entre 31 a 50 anos para os sexos feminino e masculino; [c] Valores IVR calculados: IVR (%) = C × 100 / IDR, sendo C o nível mineral encontrado em amêndoas.

7. **Alergenicidade**

A literatura internacional indica que cerca de 90% dos casos de alergia alimentar são ocasionados por oito alimentos, que quando utilizados como ingredientes em alimentos embalados devem ser informados como alérgicos, entre eles estão: leite, ovo, peixe, crustáceos, *tree nuts*, amendoim, trigo e soja. A amêndoa é considerada uma *tree nut* e está na lista do FDA (*Food and Drug Administration*), bem como no Regulamento da União Europeia n.º 1169/2011 como um ingrediente alergênico (BUHLER et al., 2015; ZHANG et al., 2016a). Com o aumento do consumo de amêndoas, há maior exposição não intencional de pessoas sensíveis às amêndoas e produtos derivados (SATHE et al., 2002).

A amandina representa aproximadamente 70% do total de proteínas solúveis presentes em amêndoas. Esta proteína ao ser ingerida é reconhecida pela imunoglobulina E (IgE) do soro humano em pacientes alérgicos a produtos à base de amêndoa (SATHE et al., 2002; ZHANG et al., 2016a). Há outros alergênicos presentes em amêndoas além da amandina e estes incluem a profilina, a albumina 2S e a γ-conglutina (MANDALARI et al., 2014). Em diferentes variedades dos frutos pertencentes à família Rosaceae as proteínas de transferência lipídica (PTL) foram associadas a uma variedade de reações adversas com características

alérgicas, incluindo urticária, angioedema facial, edema de glote, edema na língua, vômito e asma. A família PTL apresenta sequências altamente conservadas e estruturas tridimensionais que permitem o reconhecimento da IgE, promovendo a reatividade cruzada entre esses tipos de proteínas. As características moleculares das PTLs, como a presença de oito resíduos de cisteína que formam quatro pontes dissulfureto, conferem uma estrutura compacta peculiar a estas proteínas, diminuindo a probabilidade de degradação em uma variedade de métodos de processamento e tratamentos térmicos em alimentos, aumentando assim a probabilidade de absorção sistêmica e reações alérgicas graves (BUHLER et al., 2015; MANDALARI et al., 2014). A alergenicidade das proteínas alimentares pode ser afetada por tratamento térmico, que destrói certos epítopos estruturais mas, mesmo com este tratamento, a alergenicidade em amêndoas nem sempre é completamente eliminada (MANDALARI et al., 2014).

Ao processar um alimento, o potencial alergênico pode ser afetado. Entre os processamentos mais utilizados, tem-se: aquecimento, fermentação, hidrólise enzimática, tratamento físico (extrusão ou alta pressão), uso de conservantes, alteração no pH, ou combinação de dois ou mais deles (MANDALARI; MACKIE, 2018). Em *nuts* as formas mais utilizadas são o tratamento térmico a quente ou a transformação em manteiga (VERHOECKX et al., 2015). Durante o processamento, as proteínas alimentares podem sofrer modificações, como desdobramento, agregação e modificações químicas. Estas transformações podem alterar a alergenicidade das proteínas alimentares, aumentando ou diminuindo o potencial de reconhecimento pela IgE (ZHANG et al., 2016a).

Venkatachalam et al. (2002) avaliou a principal proteína alergênica em amêndoas inteiras e não processadas da variedade Nonpareil após diferentes métodos de processamento térmico, incluindo torrefação, autoclave, branqueamento e aquecimento por micro-ondas. Neste estudo foi observado que a antigenicidade da amandina foi estável, independentemente do tratamento aplicado. Enquanto no estudo de Zhang e colaboradores (2016a) em amostras de farinha de amêndoa, observou-se que houve mudanças significativas na recuperação de proteínas após tratamentos térmicos ou de alta pressão e também houve modificações na imunorreatividade das proteínas, indicando que as transformações estruturais induzidas por calor e alta pressão contribuem significativamente para as alterações na imunorreatividade das proteínas de amêndoa. Além disso, percebeu-se também que a esterilização em autoclave e o tratamento de alta pressão, que são realizados tipicamente em amostras líquidas ou com

elevados teores de água, podem proporcionar um meio para reduzir a antigenicidade das proteínas de amêndoa.

Segundo uma revisão sistemática feita por McWilliam et al. (2015) a prevalência de alergia à *nuts* em geral varia de 0 a 11,4%. A reação ao ingerir *nuts* pode ser fatal, sendo a anafilaxia a mais grave. Os casos fatais por anafilaxia induzida por alimentos ao ingerir *nuts* representam cerca de 18 a 40% (MCWILLIAM et al., 2015). Nos EUA a taxa de incidência de alergia à *nuts* foi estimada em 0,2 e 0,5% para crianças e adultos, respectivamente. No Canadá, a incidência foi de 0,1% para crianças e 1,1% para adultos (MANDALARI; MACKIE, 2018).

Nos EUA as *nuts* que mais causam alergia são as nozes seguida de castanha de caju e em terceiro lugar as amêndoas. Os dados de incidência de alergia às amêndoas estão disponíveis em sua maioria para crianças (MANDALARI; MACKIE, 2018). Alguns estudos reportaram a quantidade de casos de alergia a diferentes *nuts*, e a amêndoa apresentou: na França 2 casos em dois diferentes estudos (OSTERBALLE et al., 2009; RANCE; GRANDMOTTET; GRANDJEAN, 2005); nos EUA, 7 casos (SICHERER et al., 1999) e em um outro trabalho mais recente 25 casos (SICHERER et al., 2010) e no Reino Unido foram reportados 9 casos (ROBERTS et al., 2005) e 2 casos (VENTER et al., 2007).

8. **Potenciais Efeitos Benéficos Relacionados ao seu Consumo**

As sementes oleaginosas contêm pequenas quantidades de vários compostos bioativos e a presença desses fitoquímicos tais como fibras, fitoesteróis, algumas vitaminas, lipídios e compostos antioxidantes está associado a efeitos benéficos à saúde (KESER; DEMIR; YILMAZ, 2014; LÓPEZ-ORTIZ et al., 2008; SEGURA et al., 2006).

Amêndoas contêm quantidades consideráveis de carboidratos potencialmente prebióticos. A abundância de fibras alimentares (12%) e polifenóis pode estar associada com os efeitos prebióticos na ingestão de amêndoas e de sua casca. A fibra dietética presente nas paredes celulares das amêndoas é resistente à degradação enzimática no trato gastrointestinal superior. Ao ocorrer a fermentação dos polissacarídeos formadores da fibra dietética, é gerado um controle sobre a função colônica, que inclui o equilíbrio da microbiota e da saúde epitelial do intestino grosso. Isto pode levar a mudanças da atividade das bactérias intestinais, induzindo à promoção de fatores benéficos para a saúde e a inibição de fatores prejudiciais.

Foi comprovado que a fibra dietética alterou a composição das bactérias intestinais, levando a melhoria do perfil da microbiota intestinal indicando que a pele de amêndoa pode ser utilizada como prebiótico (LIU et al., 2014; MANDALARI et al., 2010a, 2010b).

Amêndoas, assim como oleaginosas em geral e óleos vegetais, são fontes de fitoesteróis (FORCADA et al., 2015; SEGURA et al., 2006). Os esteróis vegetais incluem uma grande variedade de moléculas, que apresentam estrutura química e função biológica semelhante ao colesterol (FERNÁNDEZ-CUESTA et al., 2012; KESER; DEMIR; YILMAZ, 2014). Nos vegetais, apresentam função de regular a fluidez e a permeabilidade das membranas e são precursores de hormônios envolvidos no crescimento das plantas. Devido à semelhança destes compostos com o colesterol, eles atuam reduzindo a absorção intestinal de colesterol, o que leva à redução dos níveis séricos de colesterol, o que pode vir a reduzir, de forma significativa, o risco de doenças crônicas, como doenças cardiovasculares, câncer ou distúrbios neurológicos, sendo então considerados compostos funcionais (FERNÁNDEZ-CUESTA et al., 2012; FORCADA et al., 2015). O mecanismo de ação tem sido associado à sua hidrofobicidade, que é maior do que o colesterol devido à presença de uma molécula de hidrocarboneto maior (SEGURA et al., 2006). São exemplos de fistoesteróis presentes em vegetais: β-sitosterol, campesterol, estigmasterol e 5-avenasterol, sendo o primeiro o mais abundante de todos (KESER; DEMIR; YILMAZ, 2014; SEGURA et al., 2006). No estudo de Forcada et al. (2015) em óleo de amêndoa foi observado um teor de 73,0% de β-sitosterol, 2,7% de campesterol, 0,73% de estigmasterol e 15,5% de Δ5-avenasterol entre outros fitoesteróis minoritários.

Extratos de semente de amêndoa já apresentaram grande capacidade de eliminar radicais livres. Esta atividade pode estar relacionada com a presença de flavonoides e outros compostos fenólicos, que apresentam-se predominantemente de forma conjugada a açúcares ou outros polióis via ligações glicosídicas ou ligações éster, além da presença de taninos e de tocoferóis (BARREIRA et al., 2008; ESFAHLAN; JAMEI, 2012). Os compostos fenólicos atuam doando e recebendo elétrons, e seu potencial antioxidante pode ser mediado pelos mecanismos de retirada de espécies reativas de oxigênio (ROS), supressão da formação de ROS pela inibição de enzimas ou quelantes de metais envolvidos na produção de radicais livres e regulação ou proteção da defesa antioxidante intrínseca (GOMAA, 2013). Desta forma, extratos de toda a semente, da casca e da pele de amêndoas podem apresentar capacidade antioxidante, com a presença de compostos que impedem ou retardam a oxidação

lipídica ao inibir a iniciação e a propagação de reações oxidativas (BARREIRA et al., 2008; SIVACI; DUMAN, 2014).

O estudo de Valdés et al. (2015) teve como objetivo avaliar o potencial antioxidante da pele de amêndoa (*Prunus amygdalus*). Para isso foi quantificado o teor de flavonoides e compostos fenólicos totais, entre sete cultivares: Marcona, Guara, Planeta, Butte, Colony, Carmel e Padre. O teor de flavonoides totais varou entre 460 a 1162 µg por g de pele, e o teor de compostos fenólicos totais, foi de 54 a 119 mg por g de pele. Além disso, foi realizado o teste de DPPH, em que todos os cultivares apresentaram atividade de eliminação de radicais acima de 90%, indicando que a pele da amêndoa é uma fonte natural de fenólicos e flavonoides, com grande potencial antioxidante. Outro estudo relacionado a extratos fenólicos de dez diferentes sementes de amêndoa da espécie *Prunus amygdalus* L. foi realizado por Esfahlan; Jamei (2012). Nesta pesquisa, o teor de flavonoides variou de 11,3 a 35,6 mg por g de extrato e o teor de compostos fenólicos teve uma variação entre 184,1 a 482,3 mg por g de extrato. E o ensaio de DPPH mostrou porcentagens entre 58,3 a 100% de atividade de eliminação de radicais a uma concentração de 50 ppm de extrato.

O óleo extraído de sementes de frutos em geral tem sido amplamente aplicado em alimentos, além de ser considerado uma boa fonte de compostos antimicrobianos (TIAN et al., 2011). O efeito antimicrobiano destes óleos pode estar associado a presença de compostos fenólicos, que apresentam uma hidrofobicidade parcial. O mecanismo de toxicidade de polifenóis contra microrganismos pode estar relacionado à inibição de enzimas hidrolíticas, como proteases entre outras interações que inativam adesinas microbianas, proteínas de transporte celular e interações não específicas com carboidratos. Enquanto os flavonoides, que são sintetizados pelas plantas em resposta a infecção microbiana, apresentam uma capacidade de formar complexos com proteínas extracelulares solúveis e de formar complexos com as paredes celulares bacterianas (GOMAA, 2013).

Karaman e colaboradores (2015) testaram diferentes concentrações de extratos de óleo de amêndoa (extração em metanol) e estes mostraram atividade antibacteriana. Na menor concentração (4%), o extrato de óleo de amêndoa mostrou atividade antibacteriana apenas contra linhagens de *Listeria monocytogenes* (7 mm de zona de inibição) e na maior concentração (50%), o extrato mostrou atividade sobre todos os quatro microrganismos estudados: *Salmonella Typhimurium* (9,5 mm de zona de inibição), *Escherichia coli* O157:H7

(10 mm de zona de inibição), *L. monocytogenes* (20,5 mm de zona de inibição), *Staphylococcus aureus* (16 mm de zona de inibição), sendo mais eficaz contra as duas últimas espécies, Gram-positivas.

9. **Potenciais Aplicações**

Bebidas à base de amêndoa têm sido utilizadas como alternativa ao leite de vaca por pessoas intolerantes à lactose e mulheres grávidas devido aos altos níveis de cálcio, fosforo e potássio (BERNAT et al., 2015). O "leite" de amêndoa é uma dispersão coloidal, contendo apenas 2% de amêndoas (em pó ou pasta de amêndoa), sendo produzida a partir de uma extração aquosa da amêndoa (*Prunus dulcis*) de substâncias coloidais como proteínas, lipídios, polissacarídeos, taninos, fitatos e a interações destes diferentes compostos. Desta forma, bebidas à base de amêndoa contêm principalmente água filtrada e aditivos (emulsificantes, adoçantes e vitaminas sintéticas) (DHAKAL; GIUSTI; BALASUBRAMANIAM, 2016; CORNUCOPIA INSTITUTE, 2019). Para a obtenção do extrato solúvel de amêndoa em água, as proteínas da amêndoa são essenciais, devido ao seu poder emulsificante, porém para a produção de bebidas de amêndoa fisicamente estáveis, é necessário adicionar um agente emulsificante (como carragena, gomas, óleos, lecitina e sal). E para a reposição de vitaminas perdidas no processamento, as bebidas de amêndoa são geralmente enriquecidas com potássio e vitaminas A e D (SILVA; SILVA; RIBEIRO, 2020; CORNUCOPIA INSTITUTE, 2019). O "leite" de amêndoa contribui para a prevenção de doenças cardíacas, já que apresenta baixo teor de sódio; uma proporção equilibrada de ácidos graxos mono- e poli-insaturados; ajudam na manutenção dos níveis saudáveis de colesterol e apresentam alto teor de compostos antioxidantes (BERNAT et al., 2015)**.**

A goma de amêndoa é um polímero natural que apresenta propriedades interfaciais específicas que permitem a sua aplicação como agente espessante e estabilizante, pois forma filmes interfaciais estruturados (MAHFOUDHI; CHOUAIBI; HAMDI, 2014). Esta goma é exsudada do tronco, galhos e frutos de árvores de *P. dulcis*, após sofrer lesões mecânicas ou infecção por microrganismos (MAHFOUDHI; CHOUAIBI; HAMDI, 2014). Estas frações foram analisadas por Bouaziz e colaboradores (2016) e os resultados revelaram que ambas as frações apresentaram interessante capacidade de retenção de água, capacidade de absorção de gordura, capacidade de emulsificação e estabilização de emulsões. A goma de amêndoa pode

então ser utilizada como aditivo alimentar para melhorar as propriedades tecnológicas de várias formulações alimentares (BOUAZIZ et al., 2016).

A casca de amêndoa (endocarpo) é um subproduto agrícola que representa 4% do peso total do fruto e é geralmente incinerado, já que não apresenta aplicação industrial, devido à sua rigidez (ESFAHLAN; JAMEI, 2012; KACEM et al., 2016). O volume anual de subprodutos do processamento industrial de amêndoas é estimado em aproximadamente 70 a 80%, onde a casca contribui com 40 a 60% deste total (SMERIGLIO et al., 2016). As principais aplicações industriais deste subproduto são a produção de alimentos para animais e de biocombustíveis. Mais recentemente a indústria aumentou seu interesse em aplicar a casca da amêndoa como alimentos nutracêuticos ou suplementos alimentares (indústria de alimentos) e também nas indústrias farmacêutica e cosmética (SMERIGLIO et al., 2016). No estudo de Kacem et al. (2016), foi produzido bioetanol a partir da casca de amêndoa por meio de pré-tratamentos ácidos e alcalinos, o que aumentou teor de celulose (com elevada cristalinidade) e permitiu uma hidrólise enzimática eficiente com as enzimas celulase e xilanase produzidas por *Penicillium occitanis* e a eficiência da fermentação dos hidrolisados foi melhorada com um tratamento com a lacase, aumentando o rendimento do etanol produzido, de 30 para 84%.

Conclusões

Amêndoas são alimentos que fornecem energia, devido a sua composição nutricional. A composição de nutrientes depende do genótipo e de fatores ambientais ou ainda das interações destes fatores, o que pode levar a diferenças em sua composição final. Amêndoas apresentam de 50 a 80% de ácido oleico e de 10 a 26% de ácido linoleico, e não contribui para a formação de colesterol. Além disso, é também fonte de proteína dietética com 14 a 26%, porém ainda não há muita informação genômica e proteômica sobre esta semente oleaginosa. Devido a presença da amandina (principal proteína), amêndoas estão no grupo de alimentos reconhecidos como alergênico. Ainda assim, a semente apresenta vários compostos bioativos, podendo ser utilizada como prebióticos e para a redução dos níveis séricos de colesterol. A casca apresenta potencial capacidade antioxidante e o óleo foi identificado como fonte de compostos antimicrobianos. Além da aplicação clássica de ser consumida como *snacks* e aplicação em produtos de panificação, é uma ótima opção de bebidas. A goma

extrusada do fruto ou dos galhos da amêndoa podem ser utilizadas como agentes espessantes e estabilizantes.

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CAPÍTULO 3

Aqueous and Enzymatic Extraction of Oil and Protein from Almond Cake: A Comparative Study

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Abstract:

The almond cake is a protein- and oil-rich by-product of the mechanical expression of almond oil that has the potential to be used as a source of valuable proteins and lipids for food applications. The objectives of this study were to evaluate the individual and combined effects of solids-to-liquid ratio (SLR), reaction time, and enzyme use on oil and protein extraction yields from almond cake. A central composite rotatable design was employed to maximize the overall extractability and distribution of extracted components among the fractions generated by the aqueous (AEP) and enzyme-assisted aqueous extraction process (EAEP). Simultaneous extraction of oil and protein by the AEP was favored by the use of low SLR (1:12.82) and longer reaction times (2 h), where extraction yields of 48.2% and 70% were achieved, respectively. Increased use of enzyme (0.85%) in the EAEP resulted in higher oil (50%) and protein (75%) extraction yields in a shorter reaction time (1 h), compared with the AEP at the same reaction time (41.6% oil and 70% protein extraction). Overall, extraction conditions that favored oil and protein extraction also favored oil yield in the cream and protein yield in the skim. However, increased oil yield in the skim was observed at conditions where higher oil extraction was achieved. In addition to improving oil and protein

extractability, the use of enzyme during the extraction resulted in the production of skim fractions with smaller and more soluble peptides at low pH (5.0), highlighting possible uses of the EAEP skim in food applications involving acidic pH. The implications of the use of enzyme during the extraction regarding the de-emulsification of the EAEP cream warrant further investigation.

Keywords: solubility; aqueous extraction; oil extraction; protein extraction; almond cake

1. Introduction

Adequately feeding an increasing world population, which is expected to reach 10 billion by 2050, has become a pressing issue for governments, policymakers, and agriculture stakeholders. Providing adequate quantities of sustainable and nutritional protein sources will likely require the combination of animal and plant-based proteins sources [1], which highlights the importance and need of identifying alternative sources of proteins (i.e., agricultural streams and food by-products) to increase the supply and diversity of proteins for human consumption.

Tree nuts are considered a valuable source of lipids and high-quality protein that can be used as food or animal feed [2]. Almonds are a great example of a highly nutritious food which, in addition to being a source of lipids (53–56%) and proteins (16–22%), also contains several health promoting compounds such as tocopherols and antioxidants [3–5]. The lipid fraction in almonds, which is composed predominantly of mono- (65%) and polyunsaturated (25%) fatty acids, has been associated with reduced risk factors for some conditions such as diabetes, obesity, and cardiovascular disease [6]. Almond proteins are mainly composed of a storage protein known as amandin, representing approximately 70% of the total soluble proteins in almonds. Amandin is a 14S globulin composed of different polypeptides [7,8] and has been recognized by human serum immunoglobulin E (IgE) from patients with almond allergy [8,9]. In that view, as required by the Food Allergen Labelling and Consumer Protection Act [10], the addition of any protein from a major food allergen, such as almonds, should be

described in the food packaging label to help allergic consumers to identify and avoid such food products.

Current protein extraction methods are usually preceded by lipid extraction, which can be accomplished by using screw pressing or solvents such as hexane [11,12]. While the use of screw pressing leads to the production of specialty oils, extraction yields are not as high (70–90%) as the yields obtained by the solvent extraction method (usually >95%) [13–15]. Mechanical pressing of almond oil generates a protein-rich cake that might contain a significant amount of residual oil, the quantity of which depends on the treatments to which the sample was previously subjected to (i.e., heat treatments) and mechanical pressing conditions employed (temperature, rotation speed, moisture content). Although protein cakes/meals are primarily used as animal feed [16], the development of efficient and environmentally friendly strategies to extract the residual oil and protein from the almond cake can lead to the production of high-quality protein and lipid fractions that can be used for subsequent food applications.

Aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) are environmentally friendly strategies that enable the simultaneous extraction of oil, protein, and carbohydrate from many oil-bearing materials without the use of flammable and neurotoxic hexane [17,18]. This green extraction strategy relies on the use of upstream mechanical treatments to improve the release of intracellular components (i.e., grinding, flaking, extruding), water, and possible use of enzymes to further improve the release of intracellular compounds [18–20]. The use of proteases alone or in combination with carbohydrases (e.g., cellulases and hemicellulases) has been commonly employed to improve extraction yields [18,21,22]. In the case of oil-bearing materials with high protein content, proteases are generally required to maximize extractability. Proteases catalyze the hydrolysis of polypeptide chain bonds thus releasing more soluble peptides into the aqueous medium. The disruption of the protein network in which the oil bodies are embedded, as well as the hydrolysis of the oil body membrane (oleosin), has shown to improve the simultaneous release of oil and protein [23–25].

The fractions generated by this process (skim - protein-rich, cream - oil-rich, insoluble - fiber-rich, and free oil) can be further converted into food, animal feed, or fuel [18]. The challenges involved in the AEP/EAEP are primarily related to achieving high extraction yields, translating high extractability into high recovery yields, and minimizing the amount of water used in the process [26]. To address these challenges, research has been conducted to evaluate the use of mechanical treatments and enzymes to increase overall extractability [27,28], the use of chemical and enzymatic de-emulsification strategies to release the oil entrapped in the cream fraction [13,28], and the use of counter-current extraction strategies to minimize the amount of water used in the process [29]. However, reducing the oil content in the skim fraction remains a challenge. Till date, there are no methods to further remove the oil from the skim fraction, which might affect the protein functionality [30].

While this environmentally friendly strategy has been evaluated for many food matrices such as soybeans [13,18,31], peanuts [32–34], olive [35,36], apricot [37], and corn [38,39], its application to simultaneously extract oil and protein from almond cake is yet to be evaluated. To the best of our knowledge, there are no reports describing the effects of extraction conditions on the simultaneous extraction of oil and protein from the almond cake produced by mechanical pressing.

Because oil and protein extractability are strongly affected by extraction conditions (i.e., amount and type of enzyme, reaction time, temperature, and solids-to-liquid ratio) [22,40], understanding the extraction mechanisms involved is key to the development of optimized extraction strategies that will enable the utilization of several plant-based protein and oil matrices. The overall goal of this study is to evaluate how processing variables influence the overall extractability of oil and protein from the almond cake as well as their distribution among the fractions generated by the aqueous (AEP) and enzyme-assisted aqueous extraction process (EAEP). The specific objectives of this work were to: (i) Evaluate the effects of solids-to-liquid ratio and reaction time on oil and protein extractability in the AEP of almond cake; (ii) identify the ideal combination of solids-to-liquid ratio, amount of enzyme, and reaction time for increased extractability of oil and protein in the EAEP of almond cake; and (iii)to determine the effects of optimized extraction conditions on the solubility and electrophoretic peptide

profile of the AEP and EAEP skim proteins. In order to identify the best combination of processing variables, a central composite rotatable design (two independent variables, three repetitions in the central point, and four axial points) was used for the AEP and EAEP.

2. Materials and Methods

2.1 Almond Cake

Almond cake was kindly provided by Blue Diamond Growers (Sacramento, CA, USA). Approximately 44 kg of whole natural Butte almonds were mechanically pressed using a M70 Oil Press (Ag Oil Press, Eau Claire, WI, USA). Mechanical expression was performed at 73 – 80 ˚C and 22 – 24 rpm. The press cake was subsequently crushed using a blender (Oster Sunbeam Products, Boca Raton, FL, USA) to produce a more homogeneous starting material for the extraction. The almond cake contained 16.25 ± 0.79% oil, 37.20 ± 0.72% protein, and 9.04 ± 0.18% moisture (see Section 2.3).

2.2 Effects of Processing Variables on Oil and Protein Extractability from Almond Cake and Partitioning of Extracted Compounds

The individual and combined effects of solids-to-liquid ratio and the reaction time were investigated in the aqueous extraction process (AEP) of almond cake. For the enzyme-assisted aqueous extraction process (EAEP), solids-to-liquid ratio and amount of enzyme were evaluated. A central composite rotatable design (CCRD) with two independent variables, three repetitions in the central point and four axial points was used to gain a better understanding of the extraction mechanisms of oil and protein from the almond cake and to identify the optimum conditions for increased extractability and better distribution of extracted compounds (i.e., less oil in the skim fraction) in the AEP and EAEP. Variable levels used in the experimental design were selected based on preliminary tests performed by our group (data not shown) and on enzyme specifications. Central points are the average of levels −1 and +1, and axial points were determined by interpolation ($\alpha = \pm 1.41$). Coded and real values of independent variables and their corresponding dependent variables are shown in Tables 1 and 2.

Table 1. Variables and levels evaluated in the optimization of oil and protein extraction in the aqueous extraction process (AEP) of almond cake.

Experiment #	Solids-to-liquid ratio (X_1)		Reaction time (h) (X_2)	
	Coded value	Real value	Coded value	Real value
1	-1	1:12	-1	0.75
2	+1	1:8	-1	0.75
3	-1	1:12	1	3.00
4	1	1:8	1	3.00
5	-1.41	1:12.82	0	1.88
6	1.41	1:7.18	0	1.88
7	0	1:10	-1.41	0.29
8	0	1:10	+1.41	3.46
9	0	1:10	0	1.88
10	0	1:10	0	1.88
11	0	1:10	0	1.88

Table 2. Variables and levels evaluated in the optimization of oil and protein extraction in the enzyme-assisted aqueous extraction process (EAEP) of almond cake.

Experiment #	Solids-to-liquid ratio (X_1)		Enzyme (%) (wt/wt*) (X_2)	
	Coded value	Real value	Coded value	Real value
1	-1	1:12	-1	0.25
2	+1	1:8	-1	0.25
3	-1	1:12	1	0.75
4	1	1:8	1	0.75
5	-1.41	1:12.82	0	0.50
6	1.41	1:7.18	0	0.50
7	0	1:10	-1.41	0.15
8	0	1:10	+1.41	0.85
9	0	1:10	0	0.50
10	0	1:10	0	0.50
11	0	1:10	0	0.50

* weight of enzyme/weight of almond cake

2.2.1 Aqueous Extraction Process (AEP) of Almond Cake: Processing Optimization and Validation

The effects of reaction time (0.29 to 3.46 h) and solids-to-liquid ratio (1:7.18 to 1:12.82) on oil and protein extraction yields in the AEP were evaluated according to the coded levels (−α,−1, 0, +1, +α) (Table 1). Approximately 50 g of almond cake was

dispersed into water to achieve solids-to-liquid ratios from 1:7.18 to 1:12.82, as described in Table 1. Extractions were performed at pH 9.0, 50 ◦C, at reaction times varying from 0.29 to 3.46 h under constant stirring at 120 rpm. Temperature was selected based on the literature and on the manufacturer's recommendation for the enzyme used in the EAEP experiments. The selection of the reaction pH was based on preliminary data (not shown). After the extraction, the slurry was centrifuged at 3000g for 30 min at 25 ◦C to remove the insoluble fraction, which was immediately frozen at −20 ◦C, from the liquid fraction. The liquid fraction was placed in a separatory funnel and allowed to settle overnight at 4◦C. After overnight settling, the liquid fraction was separated into skim (protein-and carbohydrate-rich fraction), cream (oil-rich fraction), and free oil. Insoluble, skim, cream, and free oil fractions were stored at −20 ◦C for subsequent analysis.

In addition to total oil and protein extraction yields, which represent all oil and protein extracted from the almond cake, the distribution of the extracted oil and protein in the skim, cream, or free oil fraction was determined. Total oil extraction yield (TOE), oil distribution in the fractions (free oil yield, oil yield in the cream, oil yield in the skim, oil yield in the insoluble), total protein extraction yield (TPE), and protein distribution in the fractions (protein yield in the cream, protein yield in the skim, and protein yield in the insoluble) were determined according to Equations (1) – (4), respectively:

$$TOE\ (\%) = \left[100 - \left(\frac{Oil\ (g)\ in\ the\ insoluble\ fraction}{Oil\ (g)\ in\ the\ almond\ cake}\right)\right] x\ 100 \quad (1)$$

$$Oil\ distribution\ in\ the\ fractions\ (\%) = \left(\frac{Oil\ (g)\ in\ each\ fraction\ *}{Oil\ (g)\ in\ the\ almond\ cake}\right) x\ 100 \quad (2)$$

$$TPE\ (\%) = \left[100 - \left(\frac{Protein\ (g)\ in\ the\ insoluble\ fraction}{Protein\ (g)\ in\ the\ almond\ cake}\right)\right] x\ 100 \quad (3)$$

$$Protein\ distribution\ in\ the\ fractions\ (\%) = \left(\frac{Protein\ (g)\ in\ each\ fraction\ *}{Protein\ (g)\ in\ the\ almond\ cake}\right) x\ 100 \quad (4)$$

, where fraction* corresponds to free oil, cream, skim, or insoluble.

All fractions were evaluated regarding oil, protein, and solids content. Optimum extraction conditions identified by the CCRD were validated in triplicate. Approximately 50 g of the skim fraction obtained under optimum extraction conditions (experimental validation) were freeze-dried on a benchtop freeze dryer VirTis-BenchTop™ "K" Series (SP-Scientific, Gardiner, NY, USA) and stored at −20◦C for subsequent solubility tests.

2.2.2 Enzyme-Assisted Aqueous Extraction Process (EAEP) of Almond Cake: Processing Optimization and Validation

Food Pro Alkaline Protease, a commercial bacterial alkaline endoprotease from *Bacillus licheniformis* with pH activity from 8.0 to 10.5, temperature from 45 to 75 ◦C, and enzyme activity of 580,000–650,000 DU/g (Genencor Division of Danisco, Rochester, NY, USA), was used in the EAEP of almond cake. Enzyme selection was based on preliminary data (not shown). The individual and combined effects of solids-to-liquid ratio (1:7.18 to 1:12.82) and amount of enzyme (0.15 to 0.85%) on oil and protein extraction yields in the EAEP were evaluated according to experimental conditions described in Table 2. Extractions were performed by dispersing 50 g of almond cake into water to achieve solids-to-liquid ratio ranging from 1:7.18 to 1:12.82. The slurry was adjusted to pH 9.0 before adding 0.15 to 0.85% of enzyme (weight/weight of almond cake) and extractions were performed at 50 ◦C under constant stirring at 120 rpm. Extraction kinetic evaluations were performed at 1 and 2 h for each experimental condition described in Table 2. After the extraction, the slurry was centrifuged at 3000g for 30 min at 25 ◦C to remove the insoluble fraction from the liquid fraction. The liquid fraction was placed in a separatory funnel and allowed to settle overnight at 4 ◦C. After overnight settling, the liquid fraction was separated into skim (protein- and carbohydrate-rich fraction), cream (oil-rich fraction), and free oil. Insoluble, skim, cream, and free oil fractions were stored at −20 ◦C for subsequent analysis. Total oil and protein extraction yield and the distribution of the extracted oil and protein in the fractions (free oil, cream, skim, and insoluble) were determined according to the equations described in the AEP (Equations (1) - (4)).

All fractions were evaluated regarding oil, protein, and solids content. Optimum extraction conditions identified by the CCRD were validated in triplicate. Approximately 50 g of the skim fraction obtained under optimum extraction conditions (experimental validation) were freeze-dried on a benchtop freeze dryer VirTis-BenchTop™ "K" Series (SP-Scientific, Gardiner, NY, USA) and stored at -20 ˚C for subsequent solubility tests.

2.3 Lipid, Protein, and Solids Recoveries

Cream, skim, insoluble, and starting material (almond cake) were analyzed regarding dry matter, oil, and protein contents. Dry matter content was measured by weighing after drying the samples in a vacuum oven (AOCS method 925.09) [41]. Oil content was determined by using the Monjonnier acid hydrolysis (AOCS method 989.05) [41], and protein content by using the Dumas method and a conversion factor of 5.18 (Vario MAX cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). Extraction yields of oil, protein, and solids were expressed as percentages relative to their initial amounts in the almond cake as described in the AEP and EAEP Sections 2.2.1 and 2.2.2. All analyses were conducted in duplicate and a mass balance was provided for all extracted compounds.

2.4 Degree of Hydrolysis

The degree of hydrolysis (DH) of AEP and EAEP skim fractions was determined by the o-phthaldialdehyde (OPA) method as described by Nielsen et al. [42]. Briefly, 400 µL of skim was added to 3 mL of OPA reagent. The mixture was vortexed and allowed to stand for 2 min at room temperature before measuring the absorbance at 340 nm. A 0.9516 meqv/L L-serine solution was used as standard. A blank solution was prepared with distilled water instead of sample and used as the reaction control. Protein quantification was determined by the Dumas method (conversion factor of 5.18) and the DH was determined as described in Equation (5):

$$DH\ (\%) = \frac{h}{h_{tot}}\ x\ 100 \quad (5)$$

, where h is the number of hydrolyzed bonds and htot is the total number of peptide bonds per protein equivalent (7.58 for almond protein [43]). The number of hydrolyzed bonds (h) was determined as described in Equation (6)

$$h = \frac{SerineNH_2 - \beta}{\alpha} \qquad (6)$$

where α and β values were 1.0 and 0.4, respectively [42,43].

2.5 Low Molecular Weight (MW) Polypeptide Profile Characterization of AEP and EAEP Skim Proteins by SDS-PAGE

SDS-PAGE was used to determine the low MW protein profile of AEP and EAEP skims produced under optimum extraction conditions as described by Laemmli [44]. Skim fractions were mixed with (1:1, v/v) Laemmli solution (2x Laemmli Sample Buffer, BioRad, Hercules, CA, USA, composed by 4% SDS, 20%glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M TrisHCl, pH approx. 6.8), vortexed, and placed in a water bath (95 ◦C, 5 min). A Tris-HCl buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) was used as the running buffer. Electrophoretic separation of proteins was performed by loading 30 µg of protein/well onto a precast 12% acrylamide gel (CriterionTM TGX Precast Gels, BioRad, Hercules, CA, USA). Electrophoretic separation was carried out at 200 V at room temperature for 1 h. A low MW range SDS-PAGE standard (14.4–97.4 kDa) (Bio Rad, Hercules, CA, USA) was used as a standard. Relative quantification and polypeptide distribution were performed using a Gel DocTM EZ Imager system and Image Lab software (Bio-Rad, Hercules, CA, USA).

2.6 Effects of AEP and EAEP Processing Variables on Skim Protein Solubility

Protein solubility of the AEP and EAEP freeze-dried skim fractions obtained under optimum extraction conditions was determined as described by Rickert et al. [45] with few modifications. A total of 10 mL of a 1% (w/v) skim solution was placed in a 30 mL beaker and the pH of the protein solution was adjusted to 5.0 and 9.0 by adding 1 M HCl or 1 M NaOH solution. Protein dispersions were stirred for 1 h at room temperature and then centrifuged at 10,000g at 20 ◦C for 10 min. The protein content of the supernatant was measured using the Biuret method, with bovine serum albumin as a

standard (R^2 = 0.997). The total protein content was measured after solubilizing the samples in a 1 M NaOH solution [46]. All samples were analyzed in triplicate. The solubility (%) was determined as follows (Equation (7)):

$$Solubility\ (\%) = \frac{Protein\ \ in\ the\ supernatant\ (mg/mL)}{Total\ protein\ (mg/mL)} x\ 100 \qquad (7)$$

2.7 Statistical Analysis

Data were analyzed by the Protimiza Experiment Design Software (http://experimentaldesign. protimiza.com.br). The significance of the regression models was assessed by the coefficient of determination R^2 and the F test (analysis of variance or ANOVA) at $p < 0.05$. Optimum extraction conditions suggested by the models for increased oil and protein extraction were further validated in triplicate. Replicates of each measurement were analyzed by ANOVA with generalized linear models from the SAS system (version 9.4, SAS Institute Inc., Cary, NC, USA). Multiple comparisons of least-square means were made by Tukey's adjustment with the level of significance set at $p < 0.05$. Statistical significance differences were denoted by different letters, with the letter "a" being assigned to the highest value.

3. Results and Discussion

3.1 Aqueous Extraction Process of Almond Cake: Process Optimization and Validation

Processing variables such as solids-to-liquid ratio (SLR), particle size, pH, reaction time, and temperature have a remarkable effect on the oil and protein extractability [19,22]. The effects of reaction time (0.29 to 3.47 h) and SLR (1:7.18 to 1:12.82) on the total oil extraction (TOE) and distribution of the extracted oil in the fractions generated by the AEP (free oil, cream, and skim) are shown in Figure1. TOE varied from 34 (experiment #7 - 1:10 SLR, 0.29 h) to 53% (experiment #4 - 1:8 SLR, 3.0 h), with reaction time having a significant impact on TOE. Multiple regression analysis of the data presented in Figure 1 indicates that total oil extraction (TOE) was favored by longer reaction times (X_2) (Y_{TOE} = 44.70 + 5.16 X_2), with the predictive model being statistically significant at $p < 0.05$ (F_{cal} (19.1) > F_{tab} (1,9) (5.12) and R^2 = 68%). Because oil extraction in the AEP is based on the dissolution of soluble

components (i.e., proteins, carbohydrates) in the aqueous medium [47], prolonged incubation times might enhance the dissolution of soluble compounds thus favoring the release of the oil from the original structure [19].

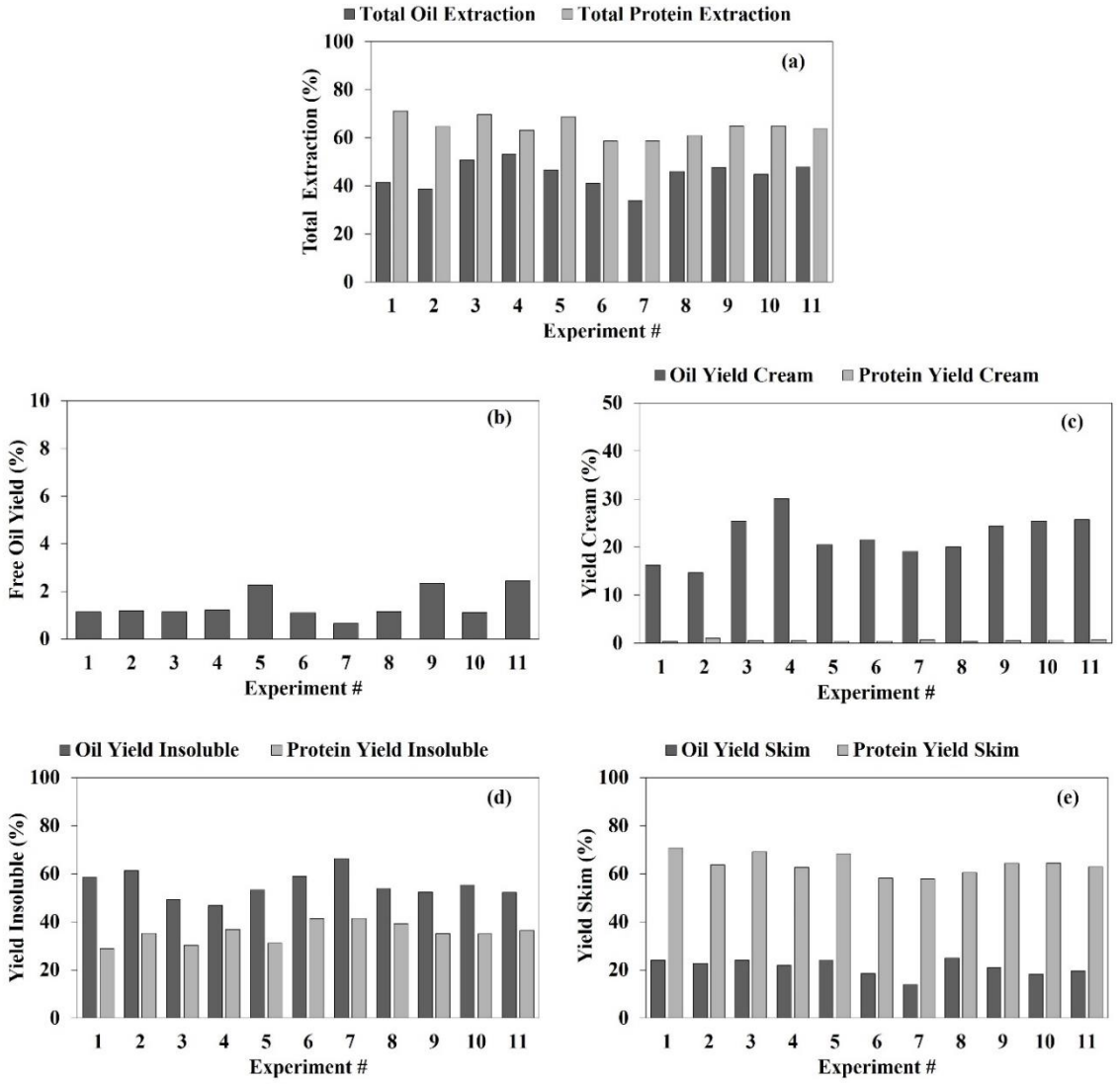

Figure 1. Effects of solids-to-liquid ratio (SLR) and reaction time on extraction yields and distribution of oil and protein among the fractions generated by the AEP: (a) Total oil and protein extraction yields (%); (b) free oil yield (%); (c) oil and protein yield in the cream (%); (d) oil and protein yield in the insoluble (%); (e) oil and protein yield in the skim (%). Experimental conditions: 1 (1:12 SLR, 0.75 h); 2 (1:8 SLR, 0.75 h); 3 (1:12 SLR, 3.0 h); 4 (1:8 SLR, 3.0 h); 5 (1:12.82 SLR, 1.88 h); 6 (1:7.18 SLR, 1.88 h);

7 (1:10 SLR, 0.29 h); 8 (1:10 SLR, 3.46 h); 9, 10, and 11 (1:10 SLR, 1.88 h) (central points).

In addition to having high TOE, the distribution of the extracted oil among the fractions is of great importance to maximize the overall recovery of the extracted oil. Since there are no methods available to recover the oil in the skim fraction, having higher free oil yield, or alternatively, shifting more oil from the skim to the cream fraction is desirable [26]. It is worth mentioning the need for subsequent development of de-emulsification strategies to recover the oil entrapped in the cream emulsion [48,49], which is beyond the scope of this work.

Free oil yield (FOY) varied from 0.65% (experiment #7 - 1:10 SLR, 0.29 h) to 2.4% (experiment #11, 1:10 SLR, 1.88 h), being influenced by reaction time (X_2) but not by SLR (X_1), within the range evaluated. The regression equation of second order ($Y_{FOY} = 1.81 - 0.51\ X_2^2$) (significant at $p < 0.05$, F_{cal} (7.0)>F_{tab} (1,9) (5.12), and R^2 = 44%) indicates that higher free oil yield can be achieved at a reaction time of 1.88 h (central point), with a decrease in free oil yield being observed at shorter or longer extraction time. As for the amount of oil in the cream (OYC), yields from 14.68 (experiment #2 - 1:8 SLR, 0.75 h) to 30.12% (experiment #4) were observed in the experimental runs (Figure1). Higher oil in the cream was favored by longer reaction time (X_2), as described by the regression model $Y_{OYC} = 22.13 + 3.24\ X_2$, which was statistically significant at $p < 0.05$ (F_{cal} (6.0) > F_{tab} (1,9) (5.12) and R^2 = 40.0%).

Although the oil content in the skim was not significantly affected by reaction time and SLR in the range evaluated, the lowest oil yield in the skim (13.9%) was observed at the shortest reaction time (0.29 h), corresponding to the lowest TOE observed (33.7%). Although not statistically significant at $p < 0.05$, undesirable higher oil yields in the skim were observed for experimental conditions where higher TOE was observed, usually at longer reaction times. Overall, longer reaction time (3.46 h) favored TOE and higher oil yield in the cream fraction, which we attribute to higher solubilization and diffusion of proteins into the aqueous medium, which would in turn help with the release of the oil [50].

The effects of reaction time (0.29 to 3.46 h) and SLR (1:7.18 to 1:12.82) on total protein extraction (TPE) and distribution of the extracted protein in the cream and skim are shown in Figure1. TPE varied from 58.6 (experiment #1 - 10 SLR, 0.29 h) to 71.2% (experiment #1 - 1:12 SLR, 0.75 h), being favored by the use of low SLR (X_1) (Y_{TPE} = 64.46 − 3.41 X_1), statistically significant at $p < 0.05$(F_{cal} (10.1) > F_{tab} (1,9) (5.12) and R^2 = 53%). Nearly all extracted protein was present in the skim fraction as evidenced by the protein yield in the skim (PYS). PYS varied from 57.8% (experiment #7) to 71.2% (experiment #1), being also favored by the use of low SLR (X_1) (Y_{PYS} = 63.88−3.50 X_1). The regression model was statistically significant at $p < 0.05$ (F_{cal} (11.0) > F_{tab} (1,9) (5.12) and R^2 = 55%). Overall, low protein yields (0.39 to 1.06%) were observed in the cream fraction, not being significantly affected by the processing variables within the range evaluated. The higher protein extractability observed at low SLR is likely the result of increased protein solubilization and subsequent diffusion into the aqueous medium, which is favored by the higher gradient concentration between solutes and the aqueous medium when low SLR is used. At lower SLR, reduced viscosity of the extraction medium should be expected, thus favoring protein diffusion to the aqueous medium [50]. Our results are in agreement with several reports in the literature [19,29,51], which observed an overall reduction in protein extractability in the AEP and EAEP when a higher SLR was attempted.

The identification of processing conditions that favor the simultaneous extraction of oil and protein and, when possible, the distribution of extracted compounds among the fractions is a key step to improve the overall process feasibility. Our results indicate that TOE and oil yield in the cream are favored by a longer reaction time (3.46 h) at any SLR within the range evaluated, and that TPE and protein yield in the skim are favored by the use of low SLR at any reaction time within the range evaluated. In order to maximize the simultaneous extraction of both oil and protein, validation experiments were performed using the lowest SLR evaluated (1:12.82) at three reaction times (1, 2, and 3 h). This experimental condition was performed in triplicate to test the adequacy of the predictive models. Extractions were performed at pH 9.0 ˚C and 50 ˚C under constant stirring at 120 rpm. Total extraction yields and distribution of extracted oil and protein in all phases generated by the AEP are shown in Figure 2.

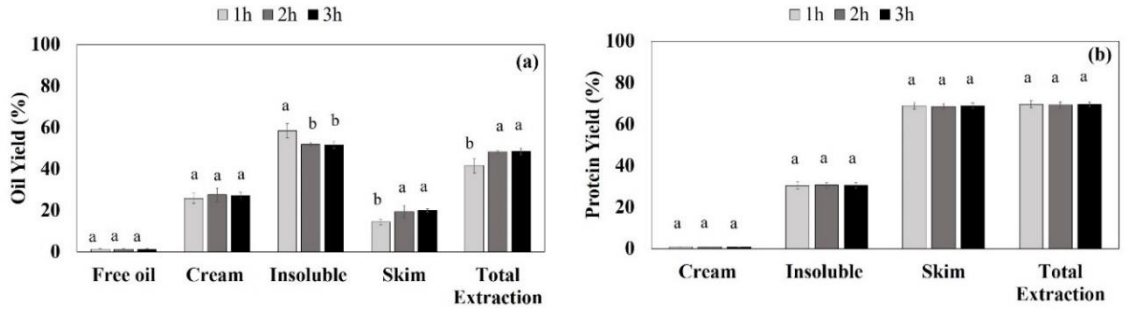

Figure 2. Experimental validation of the AEP conditions (1:12.82 SLR at 1, 2, and 3 h): (a) oil and (b) protein extraction yields for the AEP of almond cake. Means within the same fraction with different letters are statistically different at $p < 0.05$.

Experimental validation of the AEP conditions suggested by the CCRD shows that total oil extraction (TOE) significantly increased from 41.6 to 48.2% (statistically significant at $p < 0.05$) when reaction time increased from 1 to 2 h, with no increment in oil extraction being observed from 2 to 3 h (Figure 2a). TOE of 48.2% is in close agreement with the predicted value of 45.5% at 2 h. Overall, no significant increment was observed in free oil yield (~1.3%) and oil yield in the cream (25.8 - 27.5%) from 1 to 3 h, corroborating with the predicted values of 1.80% for free oil yield and 22% for oil in the cream at 2 h. However, higher oil yield in the skim (14.5% to 19.4 - 20%) was observed when reaction time increased from 1 to 3 h (statistically significant at $p < 0.05$), being in close agreement with the trend observed in the CCRD where higher oil yield in the skim was achieved for conditions where higher TOE was observed.

TPE of 69.6% was achieved at 1 h, being in agreement with the predicted value of 69.3%. No increment in protein extractability was observed when reaction time increased from 1 to 3 h (not statistically different), which is in agreement with the regression model. As observed in the CCRD, nearly all extracted protein (~68.9%) is present in the skim fraction, with the cream having less than 1% of the almond protein. Our results demonstrate that the use of low SLR (1:12.82) and reaction time of 2 h will suffice to maximize the extraction of both oil and protein at pH 9.0 and 50 °C. At these conditions, 70% of protein and 48% of oil can be extracted from the almond cake. The comparison of the results herein with the literature is challenging since there are no reports for the simultaneous extraction of oil and protein from the almond cake resulting

from the mechanical expression of almond oil. For example, oil extraction yields from 79 to 86% have been reported for the AEP of almond flour under optimum extraction conditions [40–43]. However, differences in the starting material used in both studies (theirs being full fat almond flour and ours partially defatted almond cake produced by mechanical pressing) must be carefully considered. Protein extraction yields from 77 to 82% were reported by Esteban et al. [52] when using defatted almond meal produced by solvent extraction and carrying out the extraction under high alkaline conditions (pH 10 - 13.9) and very low SLR (1:1000 wt:vol). It is worth mentioning that, in addition to differences in the starting material used in both studies (theirs being solvent defatted cake and ours being partially defatted almond cake produced by mechanical pressing), the use of strong caustic solutions and high water usage can lead to protein degradation and high volume of effluent, respectively.

3.2 Enzyme-Assisted Aqueous Extraction Process of Almond Cake: Processing Optimization

Enzymatic hydrolysis has shown to improve oil and protein extraction yields from many oil bearing materials, the extent of which depending on the type and amount of enzyme used as well as on the mechanical and thermal treatments to which the sample was previously subject to [19,27,53– 56]. In order to evaluate the effectiveness of using proteases during the extraction, a CCDR was employed to evaluate the individual and simultaneous effects of SLR (1:12.82 to 1:7.18) and amount of enzyme (Alkaline protease, AP) (0.15–0.85%) on oil and protein extractability. Extraction kinetics were evaluated at 1 and 2 h for each experimental condition are described in Table 2.

Oil extraction and partitioning of extracted oil among the fractions are shown in Figure 3. Highest TOE of 55.49% (experiment #5 - 0.50% enzyme (wt/wt) and 1:12.82 SLR) and 55.04% (experiment #1 - 0.25% of enzyme (wt/wt) and 1:12 SLR) were observed at 1 and 2 h, respectively. In general, TOE was favored by the use of low SLR and a low amount of enzyme, the latter being dependent on the reaction time (Figure3). At 1 h, TOE was favored by the use of low SLR (variable X_1), not being affected by the amount of enzyme used (Y_{TOE} 1 h = 39.91 − 6.35 X_1 + 2.71 X_1^2). The regression model was statistically significantat $p < 0.05$ (F_{cal} (72.6) > F_{tab} (2,8) (4.46) and R^2 = 94%). However, at 2 h, TOE was favored by the use of low SLR (X1) and low amount of

enzyme (X_2) (Y_{TOE} 2 h = 46.17 − 3.81 X_1 − 2.63 X_2, statistically significantat $p < 0.05$with F_{cal} (9.5) > F_{tab} (2,8) (4.46) and R^2 = 70.3%). The use of higher amount of enzyme and longer reaction time (2 h vs. 1 h) may have promoted extensive proteolysis and emulsion formation, which can hinder the fractionation of the extracted compounds thus reducing overall oil recovery [19,48]. Although the mechanisms involved in the extraction of oil and protein are rather different [34], in general, conditions that favor oil extraction also favor protein extraction. Our results are in agreement with the ones reported by Ndlela et al. and de Moura et al. [26,40] where oil extraction was favored by the use of low SLR for the EAEP of extruded flaked soybeans. Increased oil extractability at low SLR is likely the result of increased protein solubilization which in turn facilitates the release of the oil from the almond cake into the aqueous medium.

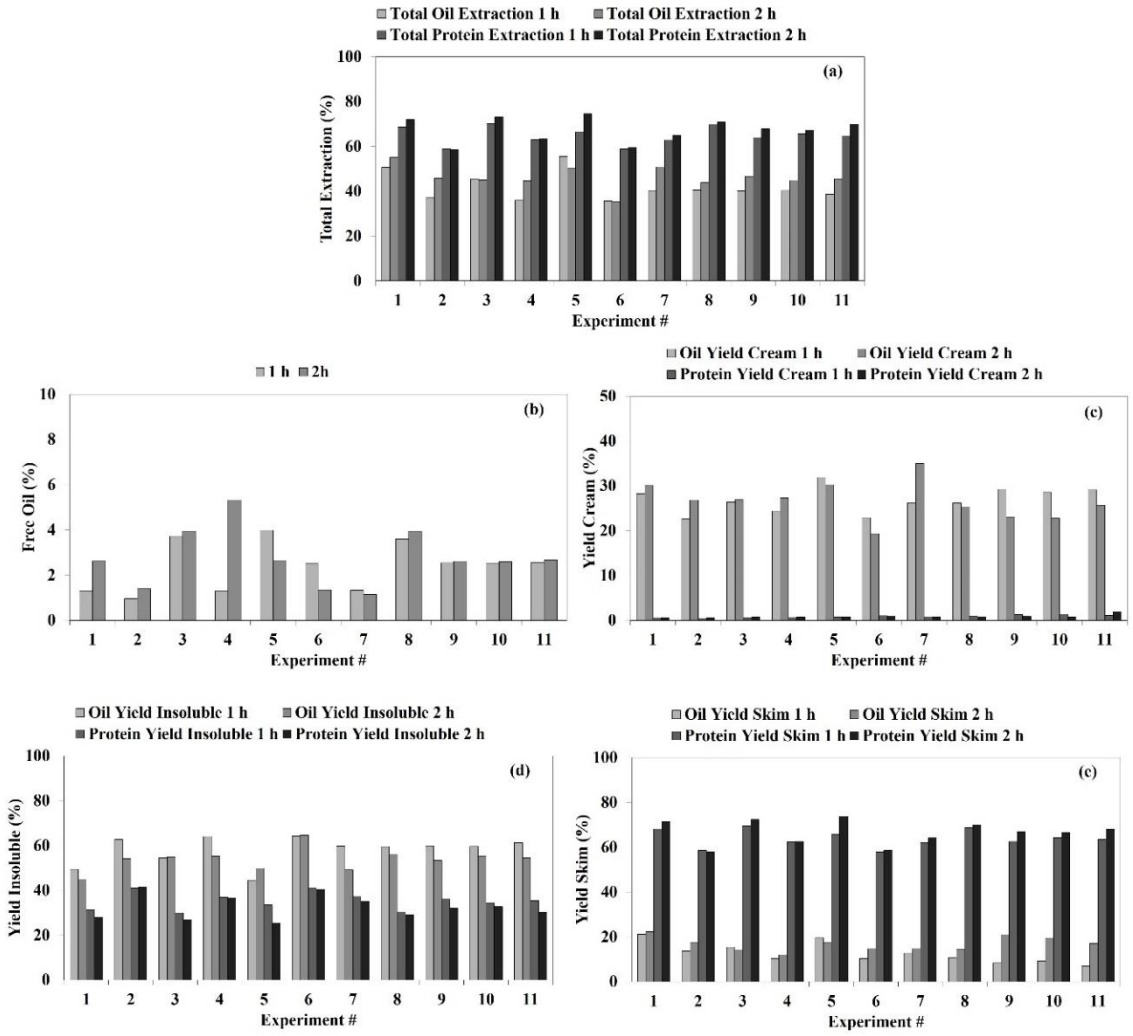

Figure 3. Effects of solids-to-liquid ratio (SLR) and amount of enzyme on extraction yields and distribution of oil and protein among the fractions generated by the EAEP at 1 and 2 h: (a) Total oil Figure 3. Effects of solids-to-liquid ratio (SLR) and amount of enzyme on extraction yields and distribution of oil and protein among the fractions generated by the EAEP at1 and 2 h: (a) Total oil and protein extraction yields (%); (b) free oil yield (%); (c) oil and protein yield in the cream (%); (d) oil and protein yield in the insoluble (%); (e) oil and protein yield in the skim (%). Experimental conditions: 1 (1:12 SLR and 0.25% enzyme (wt/wt*)); 2 (1:8 SLR, 0.25% enzyme (wt/wt)); 3 (1:12 SLR, 0.75% enzyme (wt/wt)); 4 (1:8 SLR, 0.75% enzyme (wt/wt)); 5 (1:12.82 SLR, 0.50% enzyme (wt/wt)); 6 (1:7.18 SLR, 0.50% enzyme (wt/wt)); 7 (1:10 SLR, 0.15% enzyme (wt/wt)); 8 (1:10 SLR, 0.85% enzyme (wt/wt)); 9, 10, and 11 (1:10 SLR, 0.50% enzyme (wt/wt). *(wt/wt, weight of enzyme/weight of almond cake).

Our results indicate that under optimum extraction conditions (1:12.82 SLR and 0.15% of enzyme), high oil extraction yields can be achieved at 1 h (53%) instead of 2 h (55%). At a reaction time of 1 h, free oil yield (FOY) was favored by the use of low SLR (X_1) and higher amount of enzyme (X_2) (Y_{FOY} 1h = 2.39 − 0.61X_1 + 0.75 X_2, statistically significantat $p < 0.05$ with F_{cal} (7.2) > F_{tab} (2,8) (4.46) and R^2 = 64.4%), conditions that will also favor TOE at 1 h. At reaction time of 2 h, free oil yield (FOY) was favored by the use of higher amount of enzyme (X_2) (Y_{FOY} 2h = 2.74 + 1.15 X_2, statistically significant at $p < 0.05$ with F_{cal} (17.8) > F_{tab} (1,9) (5.12) and R^2 = 66.5%). However, those conditions did not favor the TOE at 2 h. In addition, increasing reaction time from 1 to 2 h did not result in higher TOE.

In case high free oil yields are not possible, which is commonly the case for most oil-bearing materials, shifting the extracted oil to the cream fraction becomes necessary to reduce the residual oil in the skim [57,58]. Multiple regression analysis of the data in Figure 3 indicates that higher oil yield in the cream (OYC) was favored by the use of low SLR (1:12.82, X_1) and intermediate enzyme concentration (0.5%, X_2) (Y_{OYC} 1 h = 28.99 − 2.55 X_1 − 1.15X_1^2 − 1.74 X_2^2, statistically significant at $p<0.05$ with F_{cal} (15.9) >F_{tab} (3,7) (4.35) and R^2 = 87.2%) at1h. Similar trend was observed at 2h,where the use of low SLR (1:12.82, X_1) and low enzyme concentration (0.15%, X_2) (Y_{OYC} 2 h = 24.34 − 2.27X_1 − 2.05X_2 + 3.08 X_2^2, statistically significant at $p < 0.05$ with F_{cal} (6.9) > F_{tab}

(3,7) (4.35) and R^2 = 74.6%) was shown to favor OYC. Overall, processing conditions favoring higher oil yield in the cream at 1 h (low SLR and intermediate amount of enzyme) can also be exploited to achieve higher TOE at 1 h. It is worth mentioning that strategies to de-emulsify the cream, thus releasing the entrapped oil for subsequent applications, warrant further investigation.

Because any residual oil in the skim fraction (OYS) can significantly affect its functionality [31], reducing the oil content in the skim is desirable to maximize its utilization in subsequent food applications. The use of higher SLR (>1:10, X_1) and higher enzyme concentration (> 0.4%, X_2) (Y_{OYS} 1 h = 8.21 − 3.20 X_1 + 3.82 X_1^2 − 1.51 X_2 + 2.21 X_2^2, statistically significant at $p < 0.05$ with F_{cal} (18.6) > F_{tab} (4,6) (4.53) and R^2 = 92.55%) was shown to reduce overall oil yield in the skim at 1 h but not at 2 h. However, those conditions do not favor TOE, which is favored by low SLR and low amount of enzyme. These results are in agreement with the ones obtained in the AEP experiments, where higher oil extraction was accompanied by the production of a skim fraction with higher oil content. Considering that oil and protein extraction are usually favored by similar processing conditions, the higher oil content in the skim obtained in processing conditions where high oil extraction is observed can be related to increased protein solubilization and/or hydrolysis. Because increased protein solubilization and hydrolysis might favor emulsion formation, the shifting of the skim oil to the cream or to the free oil fraction can be reduced at those conditions. In summary, higher TOE yields higher oil in the cream and intermediate amount of free oil can be obtained by the use of low SLR (1:12.82) and intermediate enzyme concentration (0.50% wt/wt) at 1 h.

Protein extraction and its distribution among the fractions are show in Figure 3. Highest TPE yields were achieved in experiments #3 (70.1% - 1:12 SLR, 0.75% enzyme (wt/wt)) and #8 (69.6% - 1:10 SLR, 0.85% enzyme (wt/wt)) at 1 h and experiments #5 (74.5% - 1:12.82 SLR, 0.50% enzyme (wt/wt)) and #3 (73.1% - 1:12 SLR, 0.75% enzyme (wt/wt)) at 2 h (Figure 3). TPE was favored by the use of low SLR (1:12.82, X_1) and higher amount of enzyme (0.85%, X_2), regardless of the extraction time. As indicated by the predictive models obtained at 1 h (Y_{TPE} 1 h = 64.8 − 3.43X_1 + 1.90 X_2, statistically significantat $p < 0.05$with F_{cal} (21.6) >F_{tab} (2,8) (4.46) and R^2 = 84.4%) and 2 h (Y_{TPE} 2 h = 67.43−5.55 X_1 + 1.80 X_2, statistically significantat $p < 0.05$

with F_{cal} (79.5) > F_{tab} (2,8) (4.46) and R^2 = 95.2%), TPE yields of 72.3% and 77.7% can be achieved under optimum extraction conditions, respectively.

Since most extracted protein is present in the skim fraction, the use of low SLR (1:12.82, X_1) and higher amount of enzyme (0.85%, X_2) resulted in higher protein content in the skim (protein yield in the skim, PYS), regardless of the extraction time. According to the predictive models obtained at 1 h (Y_{PYS} 1 h = 62.93 − 3.49X_1 + 1.85 X_2 + 1.38X_2^2, statistically significant at $p < 0.05$ with F_{cal} (24.6) > F_{tab} (3,7) (4.35) and R^2 = 91.3%) and 2 h (Y_{PYS} 2 h = 66.58 − 5.57X_1 + 1.75X_2, statistically significant at $p < 0.05$ with F_{cal} (123.3) > F_{tab} (2,8) (4.46) R^2 = 98.1%), protein yields in the skim of 73.2% and 75.0 can be achieved under optimum extraction conditions, respectively. Reduced protein content in the cream (protein yield in the cream, PYC), which would result in higher protein content in the skim at same protein extractability, can be achieved at same conditions that will favor TPE (12.82 SLR and 0.85% of enzyme). While this effect was statistically significant at 1 h (Y_{PYC} 1h = 1.25 − 0.26 X_1^2 − 0.28 X_2^2, statistically significant at $p < 0.05$ with F_{cal} (7.3) > F_{tab} (2,8) (4.46) and R^2 = 64.6%), the same was not observed at 2h. Overall, higher TPE and higher protein yield in the skim can be achieved with the use of low SLR (1:12.82) and high amount of enzyme (0.85% wt/wt), regardless of the extraction time. No increase in TPE was observed when reaction time increased from 1 to 2 h. Our results are in agreement with the ones in the literature [24,30,55,59] where increased protein extractability has been attributed to the breakdown of proteins into more soluble peptides by the action of proteases.

Our results indicate that the use of a low SLR (1:12.82) and higher amount of enzyme (0.85% wt/wt) can be used to increase both oil and protein extraction at shorter reaction times (i.e., 1 h). To further confirm the benefits of working with higher enzyme concentration, which was shown to increase protein extractability but can also reduce TOE at longer reaction times, experimental validation was performed using SLR of 1:12.82 and three enzyme concentrations (0.15%, 0.5%, 0.85%, wt/wt). Extractions were performed at pH 9.0, 50 ˚C, under constant agitation at 120 rpm for 1 h. Each experimental condition was validated in triplicate to verify the adequacy of the predictive models. Oil and protein extraction yield as well as their distribution among

the fractions, under extraction conditions suggested by the predictive models, are presented in Figure 4a,b. TOE of 50.6% was achieved during the validation experiment, being in agreement with the predicted value of 52.4% (1 h, 0.5% of enzyme, 1:12.8 SLR) (Figure 4a). As suggested by the predictive model, increasing the amount of enzyme from 0.15 to 0.85 indeed resulted in a very small reduction in TOE (53% vs. 50%), although not statistically significant. Increasing the amount of enzyme from 0.15 to 0.85% significantly increased the protein extraction from 68.3 to 74.9%, which is in agreement with the predicted value of 72.3% (Figure 4b). Based on these results, SLR of 1:12.82 and 0.85% enzyme can be used to maximize the overall extractability of oil (50%) and protein (75%) from the almond cake. As described in the AEP section, the comparison of our results with the literature is challenging due to the lack of studies reporting oil and protein extraction yields from partially defatted almond cake produced by mechanical pressing. For example, while oil extraction yields ranging from75 - 78% have been reported for the EAEP of full-fat almond flour [12,53], no protein extraction yields were reported for both studies. Because of the difference in the starting material used (full fat almond flour vs. partially defatted almond cake), a direct comparison of the results from both studies is difficult.

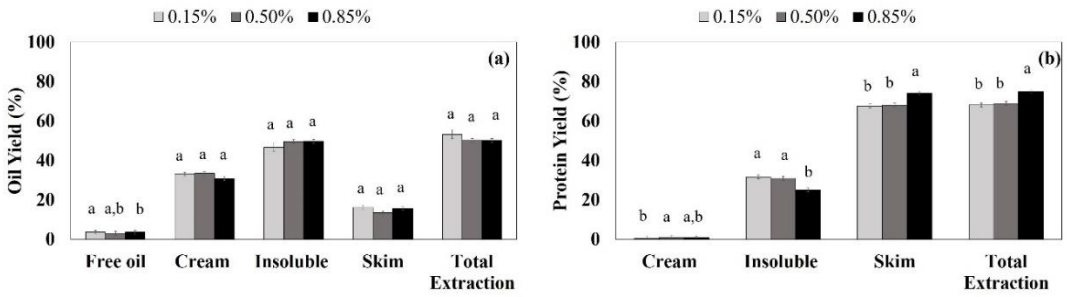

Figure 4. Experimental validation of the EAEP conditions (1:12.82 SLR and 0.15, 0.50, and 0.85% enzyme (wt/wt of almond cake): oil (a) and protein (b) extraction yields. Means within the same fraction with different letters are statistically different at $p < 0.05$.

3.3 Effects of Extraction Conditions on the MW Polypeptide Profile and Solubility of AEP and EAEP Skims

During protein hydrolysis, the breakdown of peptide bonds results in increased concentration of primary amines, which corresponds to an increase in the degree of

hydrolysis (DH) [60]. Because the DH often has a significant impact on the functional properties of the protein [61], understanding the effects of extraction conditions (i.e., AEP vs. EAEP) on the DH and protein functionality becomes necessary to further identify the possible industrial applications for the extracted protein.

The effects of the extraction conditions evaluated during the experimental validation of the AEP and EAEP on the DH and MW profile of AEP and EAEP skim proteins are shown in Figures 5 and 6. As expected, no changes in the DH of AEP skim proteins were observed (~3%) when reaction time increased from 1 to 3 h. However, increasing the amount of enzyme from 0.15 to 0.85% resulted in a statistically significant increase in the DH of EAEP skim proteins (8% vs. 27%) (Figure 5). Our results are in agreement with the literature where the use of a higher amount of enzyme, until a certain extent, resulted in higher DH [48,60].

The DH values described in Figure 5 are in agreement with the SDS-PAGE peptide profile of AEP and EAEP skim proteins (Figure 6). While no statistically significant difference was observed for the DH of the AEP skim at 1h, 2h, and 3h (Figure 5), an increasing DH (statistically significantat $p < 0.05$) was observed when the amount of enzyme used in the EAEP increased from 0.15 to 0.85%. Similar electrophoretic profile was observed for AEP skim proteins, regardless of the extraction time, being in agreement with the constant DH of the samples. AEP skim proteins are mainly composed of proteins and polypeptides bigger than 97 kDa (~14%) and two polypeptide fragments (39kDa (~23%,α-subunit) and 24 kDa (~19%, β-subunit)) which likely correspond to the subunits of amandin, the major protein accounting for 65–70% of extractable almond protein [8] (Figure 6b). Our results are in agreement with the ones reported by Wolf and Sathe [5] and Derbyshire et al. [62], which demonstrated that amandin (62 to 66 kDa subunits) can be converted into acidic (20 kDa) and basic (40 k Da) polypeptides in the presence of mercaptoethanol. Garcia-Mas et al. [63] also reported the presence of two storage polypeptides with 61.0 kDa (prunin-1) and 55.9 kDa (prunin- 2) in almond. These two bands are also present in the AEP skims (5–9%), however, they were completely hydrolyzed in the EAEP skim (0–3%).

The EAEP skim protein profile distribution (Figure 6c) reflected the increase in protein hydrolysis as enzyme concentration increased from 0.15 to 0.85%. The use of 0.85% of enzyme (optimum extraction condition for increased protein extraction) resulted in significant reduction of most polypeptides when compared with the AEP skim and EAEP conditions with reduced amount of enzyme. The use of 0.85% of protease resulted in nearly complete hydrolysis of amandin, with EAEP skim proteins having more than 90% of peptides with MW < 14.4 kD.

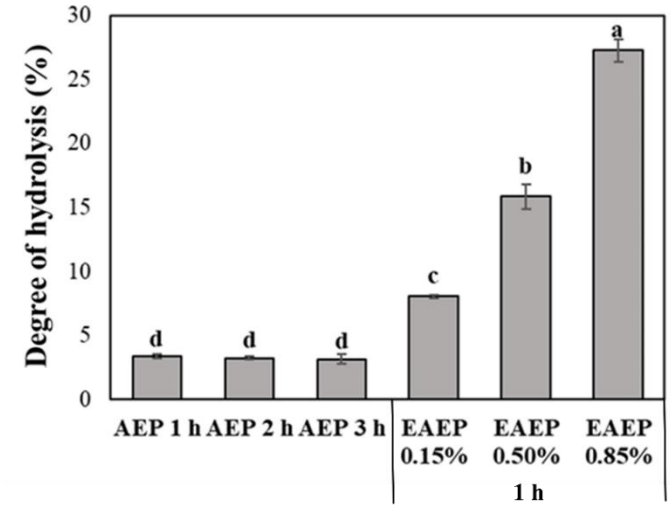

Figure 5. Effects of extraction conditions evaluated during the experimental validation of the AEP and EAEP on the degree of hydrolysis (%) of AEP and EAEP skim proteins. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Different letters indicate significant difference between samples at $p<0.05$.

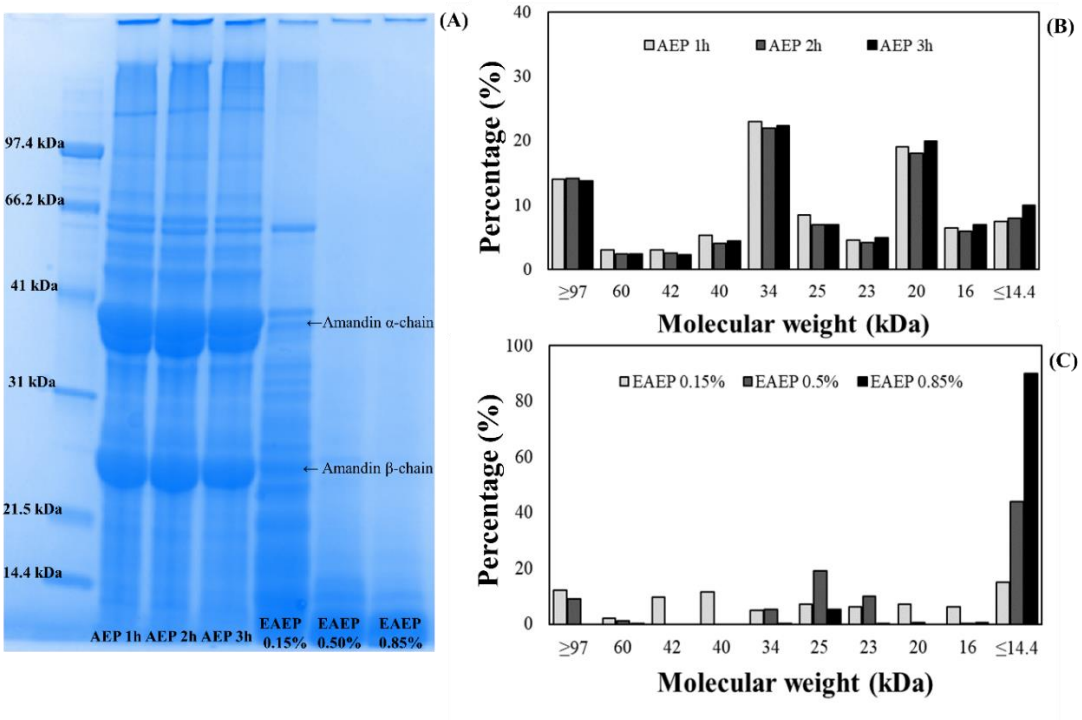

Figure 6. SDS-PAGE protein profile (A) and molecular weight distribution of AEP (B) and EAEP (C) skim peptides.

Because extraction conditions can significantly affect protein functionality [31], we have determined the effects the extraction conditions evaluated during the experimental validation of AEP and EAEP on the solubility of the AEP and EAEP skim proteins. With the goal of identifying possible applications for the extracted protein, skim protein solubility was assessed at an acidic pH (5.0, ~isoelectric point for almond protein, [8]) and alkaline pH (9.0, extraction pH) (Figure 7 At pH 5.0, EAEP skim proteins had a significantly higher solubility than AEP skim proteins). EAEP skim protein solubility at pH 5.0 increased from 25.5 to 48.2% (statistically significant at $p < 0.05$) as enzyme concentration increased from 0.15 to 0.85%, compared with ~19% for the AEP skim protein. Higher solubility for the EAEP skim protein is likely related to the enzymatic release of smaller and more soluble peptides [31,61,64]. As shown in the SDS-PAGE gel, nearly complete hydrolysis of amandin, an insoluble protein under acidic pH [5] was observed in the EAEP skim. The hydrolysis of amandin into smaller peptides can explain the higher solubility of the EAEP skim at acidic pH compared with the AEP skim. Our results are in agreement with the literature where the use of enzyme

resulted in the production of proteins with higher solubility at lower pH [31,60,61]. This behavior, however, was not observed at pH 9.0, where almond solubility is generally favored [65]. AEP and EAEP skim protein solubilities were very similar at pH 9.0, with minimum increments in solubility being observed with increased reaction time for the AEP (87% - 1 h to 92% - 3 h) and increased amount of enzyme for the EAEP (86% using 0.15% of enzyme to 91% using 0.85% of enzyme). AEP and EAEP skim solubilities were higher at pH 9.0 than pH 5.0 (statistically significant at $p < 0.05$).

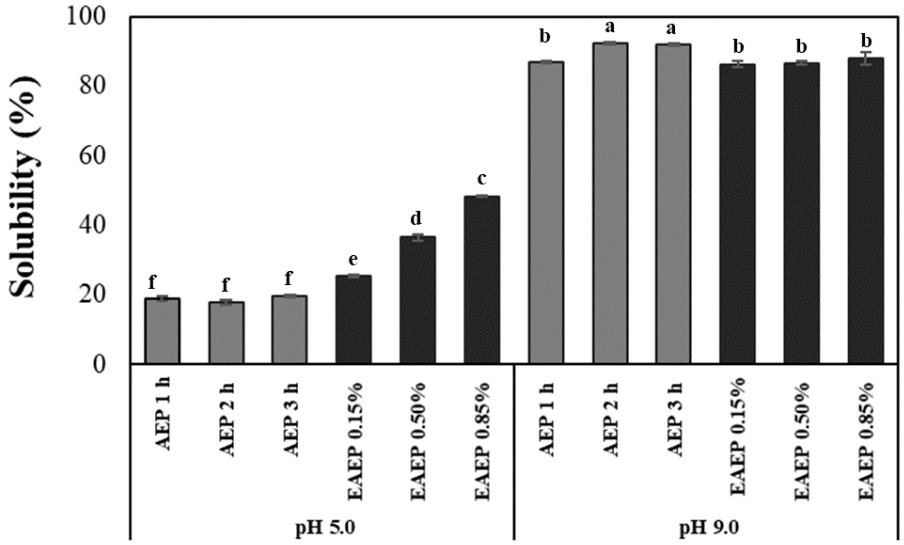

Figure 7. Effects of extraction conditions evaluated during the experimental validation of the AEP and EAEP on the solubility of AEP and EAEP skim proteins at acidic and alkaline pH. Data were analyzed by a two-way ANOVA followed by Tukey's post-hoc test. Different letters indicate significant difference between samples at $p < 0.05$.

Our results are in agreement with the ones reported by Amirshaghaghi et al. [65] which reported reduced solubility of almond proteins at acidic pH (3.2–7.5% at pH 4.0) when compared to alkaline pH (85.3–87.8% at pH 10). Higher protein solubility values observed in our study (AEP–17.9% at pH 5.0 and 92.3% at pH 9.0 at 2 h and EAEP–48.2% at pH 5.0 and 91.2% at pH 9.0 with 0.85% of enzyme) when compared with the ones reported by Amirshaghaghi et al. [65] can be attributed to the differences in the starting material used in both studies (defatted almond flour in their study and partially defatted almond from mechanical pressing in ours) and extraction conditions used. Overall, our results highlight that the use of enzyme during the extraction results in the

production of skim proteins with increased solubility at acidic pH, which can benefit the development of formulations at low pH values.

Conclusions

This study demonstrates that oil and protein extractability from almond cake can be significantly improved by careful optimization of key extraction parameters such as SLR, reaction time, and amount of enzyme. While protein extraction was favored by the use of low SLR in the AEP, oil extraction was dependent on reaction time only. Approximately 48% of oil and 70% of protein were extracted under optimized conditions (1:12.82 SLR, 2 h, pH 9.0, and 50 °C) for the AEP. The use of enzyme during the extraction resulted in increased extraction rate and yields. For the EAEP, the use of low SLR (1:12.8) and 0.85% of enzyme enabled the extraction of 50% of oil and 75% of protein in 1 h, compared with 41.6% of oil and 69.6% of protein for the AEP at same reaction time. While selected processing conditions improved oil and protein extractability, as well as the amount of the oil in the cream, the same benefit was not observed for the oil content in the skim. Regardless of the extraction approach used (AEP or EAEP), increased oil content in the skim was observed for conditions where higher oil extraction was achieved. Nevertheless, in addition to improving overall extractability, the use of enzyme resulted in the production of skim fractions with higher protein solubility at low pH (5.0) when compared with the AEP skim (48 vs. 18%). At alkaline pH (9.0), high solubility was observed for both AEP and EAEP skims (~90%). The higher concentration of soluble hydrolyzed peptides in the EAEP skim could enable its use for specific food or feed applications involving acidic pH.

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CAPÍTULO 4

Scaling-up the Simultaneous Extraction of Oil and Protein from Almond Cake: A Sustainable Approach to Conventional Hexane Extraction

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Abstract

Mechanical oil expression results in the production of a protein-rich cake that still contains large amounts of oil. Subsequent extraction of the residual oil in the cake is commonly performed by the use of hexane, a flammable, and highly polluting solvent. The defatted meal is further desolventized, which results in undesirable changes in the protein quality limiting the use of the cake primarily as feed. In this work, we scaled-up an environmentally friendly approach to simultaneously extract oil and protein from the almond cake by using water (AEP) and enzyme (EAEP) and developed chemical and enzymatic methods to recover the extracted oil. Oil and protein extraction yield of 26.2% (AEP) and 29.1% (EAEP) and 67.7 (AEP) and 70.2% (EAEP) were obtained at pilot-scale (750 g of cake), respectively. Processing scale resulted in reduced oil extractability (26.2% vs. 48.2% at AEP and 29.1% vs. 50.2% at EAEP) but similar protein extraction yields (67.7% vs. 69.3% at AEP and 70.2 vs. 74.9% at EAEP) compared with lab-scale experiments. Regarding de-emulsification results, the highest yield of free oil was found at the EAEP-cream (98.7%), applying the chemical de-emulsification followed by AEP-cream (62.8%) and EAEP-cream (58.9%) using the enzymatic de-emulsification method. At EAEP-cream the use of an enzyme at extraction positively affected the final free oil yield using the chemical de-emulsification analyses, while at AEP-cream the enzymatic de-emulsification showed better results.

Keywords: cold-pressed almond cake; enzyme-assisted extraction, scale-up, de-emulsification.

1. Introduction

There have been increasing environmental, social, and economic concerns related to the production and underutilization of food byproducts generated by the food and agricultural industries (Schanes et al., 2018). As an example, approximately 10-30% of the raw material used to produce vegetable oils becomes byproduct, leading to serious economic and environmental problems (Karaman et al., 2015). Indeed, the recovery of the high organic load present in these byproducts is needed to minimize economic losses associated with the underutilization of these compounds as well as to address environmental issues associated with the disposal of these fractions (Garcia-Garcia et al., 2019; Karaman et al., 2015).

The almond cake is the byproduct from the mechanical expression of almond oil and constitutes a source of valuable proteins and lipids for subsequent utilization (Mandalari et al., 2010). This byproduct contains about 16% oil and 37% of protein (Souza et al., 2019). Different methods are used in oil extraction, among the most used are mechanical pressing-expelling followed by solvent (hexane) extraction, where both present disadvantages, such as low oil yield recovery and safety and environmental issues, respectively (Balvardi et al., 2015; Cheng et al., 2019). The extraction using solvent achieves high oil yields, as this solvent presents a high oil recovery (~95%), a narrow boiling point (63-69 °C), and a great solubilizing ability (De Moura et al., 2009; Oliveira et al., 2019). However this kind of extraction requires high investment, high energy consumption, industry safety issues, emission of volatile organic compounds into the atmosphere, and generates an inferior quality final product (Yusuf, 2018). The removal of the cake oil using hexane is followed by desolventization of the meal, which is commonly used for animal feed, a product with a low added value (Pojić et al., 2018).

Hexane extraction produces a high environmental impact, which generates greenhouse gases (GHG) and pollutant emissions (Cheng et al., 2019). Moreover, hexane causes neurotoxic effects, affecting the central nervous system if inhaled by

humans, as this solvent is soluble in neural lipids (Oliveira et al., 2019). Aqueous extraction processing (AEP) and enzyme-assisted aqueous extraction processing (EAEP), are usually employed in oil-bearing material and simultaneously enables the recovery of oil and protein (Rosenthal, Pyle, 1996). This process is considered an environment-friendly approach that produces higher quality oil and protein more sustainably (De Moura et al., 2008; Yusoff et al., 2015). This method is based on the insolubility of oil in water rather than on the dissolution of oil in organic solvents (Li et al., 2017; Rosenthal, Pyle, 1996). The extraction of the residual oil and protein from the press cake using effective, safe, and eco-friendly strategies such as the aqueous or enzyme-assisted aqueous extraction can lead to the production of high-quality protein and lipid fractions for food, feed, and animal applications (Souza et al., 2019). The replacement of hexane is desirable and relevant due to increasing health and environmental concerns associated with its utilization (Thompson et al., 2006). In general, the AEP and EAEP fractions produced (cream- oil-rich, skim- protein-rich, insoluble- fiber-rich, and free oil) have the potential to be utilized for human consumption (Yusoff et al., 2015).

One of the limitations of this extraction technology is the formation of a stable emulsion (known as cream) during the extraction, which must be broken to release the entrapped oil (Yusoff, Gordon, & Niranjan, 2015; Wu, Johnson, & Jung, 2009). The cream fraction is a lipid-rich emulsion that is formed in the AEP and EAEP. Emulsions formed during these extractions were considered as major obstacles to releasing free oil, and the de-emulsification step is required to recover the oil present in emulsions (Liu, Gasmalla, Li, & Yang, 2016; Rosenthal, Pyle & Niranjan 1996). During the stirring process, the oil present in the starting material is mixed with water, and then the proteins adsorb at the oil/water interface that leads to the formation of an emulsion (Li et al., 2017). The emulsion stability depends on the molecular properties, chemical composition, and physical structure of the oilseeds (Li et al., 2016) as well as on the extraction conditions employed (De Moura et al., 2009). Enzymatic and chemical approaches have been developed to reduce cream stability and free the entrapped oil (Chabrand and Glatz, 2009; De Moura et al., 2009).

To establish the commercial potential of the extractions, it is essential to evaluate the yield recovered testing the process at the pilot-scale. Since our prior research on AEP and EAEP was a lab-scale optimization study (Souza et al., 2019) using 50 g of cold pressed almond cake, that produces less than 1 L of slurry was necessary scale-up both processes. AEP and EAEP were done in pilot-scale using 750 g of cold pressed almond cake using a solid-to-liquid ratio (SLR) of 1:12.8, producing ~10 L of slurry. The aim of the present study was (1) evaluate the differences between lab-scale and pilot-plant scale in all extracted fractions, and (2) determine the effects and the yields of different de-emulsification strategies on the cream fraction generated after the extractions. To date, there are no reports regarding oil and protein extracted by AEP/EAEP at pilot-scale conditions from cold pressed almond cake and further about its free oil yield released by de-emulsification method. Therefore, ultimate goal of this work is to successfully scale-up an alternative environmentally friendly extraction process to replace the use of hexane in the almond oil cake extraction and to produce proteins with target functionalities for food applications. This effort would, in turn, add value to this byproduct in a sustainable way.

2. **Materials and methods**

2.1 Materials

The cold pressed almond cake was kindly supplied by Blue Diamond (Sacramento, CA, USA). The pellets were crushed in a blender (Oster Sunbeam Products, FL, USA) to produce a homogeneous starting material for the extractions. FoodPro Alkaline Protease (Danisco, Genencor Division, NY, USA), an endoprotease derived from a strain of *Bacillus licheniformis* that presents its highest activity at pH values between 8.0 and 10.5 and temperatures from 45 to 75 °C was used during the EAEP.

2.2 Extraction Methods

2.2.1 Aqueous and Enzyme-assisted Extraction Processes

AEP and EAEP extraction conditions were based on previous processing optimization performed at laboratory-scale (50 g of almond cake) (Souza et al. 2019).

For the AEP, 750 g of the almond cake was dispersed in distilled water to achieve a solid-liquid ratio of 1:12.8 (w/v). Extractions were performed in a 10-L jacketed glass reactor with a bottom drain valve (Chemglass, NJ, USA) connected to a thermostat circulating water bath (PolyScience, IL, USA). The temperature of the extraction was maintained at 50 °C, the pH was adjusted to pH 9.0 using NaOH (1 mol/L), and the mixture was maintained under continuous stirring at 120 rpm for 2 h. For EAEP, the reaction time was 1 h after the addition of 0.85% of the alkaline protease at the same conditions of temperature and pH of AEP. A flow chart summarizing the AEP and EAEP conditions, as well as the cream de-emulsification strategies employed to recover the extracted oil are described in Figure 1. Each pilot-scale extraction was performed in duplicate.

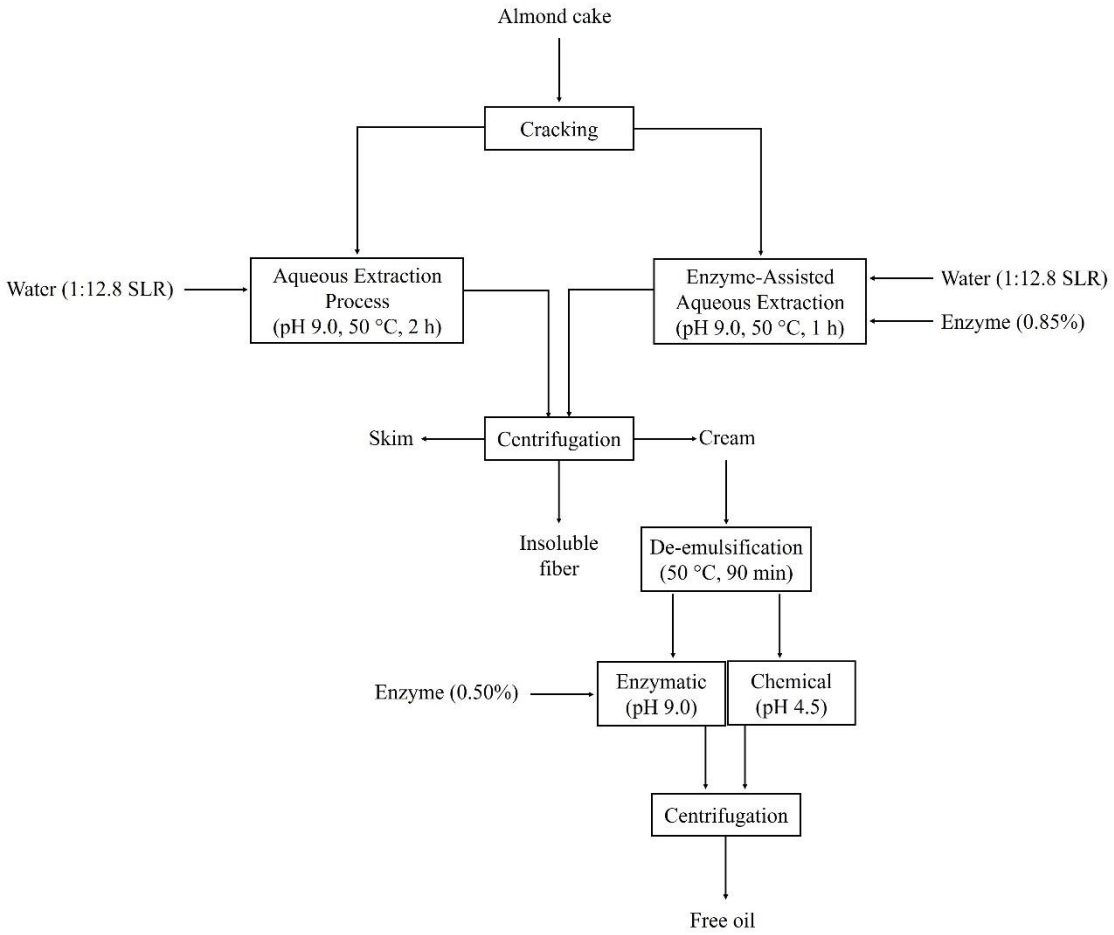

Figure 1. Flow diagram of the aqueous and enzyme-assisted extractions and de-emulsification process

2.2.2 Slurry fractionation

The slurry was centrifuged at 3,000 x g for 30 min at 25 °C to separate the insoluble fraction from the liquid phase. Then, the liquid phase (skim and cream) was transferred to the reactor and allowed to cool down overnight at 4 ºC. On the following day, skim, and cream fractions were separated. The cream phase was immediately characterized in relation to lipids, protein and dry matter contents and then subjected to the de-emulsification process. The insoluble and skim fractions were frozen and stored (-20 °C) for further analyses. The total oil extracted (TOE), total protein extracted (TPE) and the distribution of the extracted oil and protein in all fractions (insoluble, skim and cream) was determined according to equations (1), (2), (3) and (4) as described by Souza et al. (2019).

$$TOE\ (\%) = \left[100 - \left(\frac{Oil\ (g)\ in\ the\ insoluble\ fraction}{Oil\ (g)\ in\ the\ almond\ cake}\right)\right] x\ 100 \quad (1)$$

$$Oil\ distribution\ in\ the\ fractions\ (\%) = \left(\frac{Oil\ (g)\ in\ each\ fraction*}{Oil\ (g)\ in\ the\ almond\ cake}\right) x\ 100 \quad (2)$$

$$TPE\ (\%) = \left[100 - \left(\frac{Protein\ (g)\ in\ the\ insoluble\ fraction}{Protein\ (g)\ in\ the\ almond\ cake}\right)\right] x\ 100 \quad (3)$$

$$Protein\ distribution\ in\ the\ fractions\ (\%) = \left(\frac{Oil\ (g)\ in\ each\ fraction*}{Oil\ (g)\ in\ the\ almond\ cake}\right) x\ 100 \quad (4)$$

, where fraction* corresponds to insoluble, skim, cream, or free oil.

2.2.3 De-emulsification of the cream fraction

The de-emulsification procedure was determined following a method described by Jung, Maurer, & Johnson (2009) with modifications. AEP and EAEP creams were used as starting material for the de-emulsification analyses. Aliquots of 10 g of well-mixed creams (oil-rich fraction) were transferred to a 30 mL beaker and kept submerged in a water bath at 50 °C for 90 min under constant stirring of 150 rpm. Two different de-emulsification processes were evaluated: enzymatic and chemical. For the enzymatic procedure, the pH was maintained at 9.0, and 0.5% of alkaline protease (the same

enzyme used during the extraction) was added. A control experiment, without the addition of enzyme, was performed for the enzymatic de-emulsification. For the chemical de-emulsification process, the slurry pH was adjusted to 5.0 (the isoelectric point of almond protein), with the control experiment being performed at the original pH (9.0) of the slurry. After 90 min of incubation, all samples were transferred to 50 mL centrifuge tubes and cooled down in an ice bath for 15 min. The slurries were then centrifuged at 3,000 x g for 15 min at 25 °C. Right after centrifugation, the free oil layer was removed using hexane, for quantitative purpose. The hexane-lipid fraction was subsequently dried with nitrogen. The free oil yield was calculated using the following equation (Jung, 2009):

$$De - emulsification\ yield\ (\%) \left(\left(\frac{free\ oil(g)}{oil\ in\ cream\ (g)} \right) x\ oil\ content\ (\%) \right) x\ 100 \qquad (5)$$

2.2.4 Proximate analyses

The almond cake and all extracted fractions (insoluble, cream and skim) were evaluated regarding the dry matter, oil and protein contents, according to AOAC (1990), method 925.09, AOAC (1990), method 989.05 and Dumas method, factor of 5.18 (Vario MAX cube, Elementar Analysensysteme GmbH, Langenselbold, Germany), respectively. A mass balance was performed for all components, and all analyses were carried out at least in duplicate.

2.2.5 Statistical analyses

The results were expressed as mean ± standard deviation (SD) of the replicates and the experiments were performed in triplicate. Analyses of variance (ANOVA) were performed, followed by the Tukey test using Graph Pad Prism 5.0 (version 5.04, GraphPad Software, San Diego, CA, USA). Significant differences were evaluated considering the $p < 0.05$.

3. Results and discussion

3.1 Effects of scaling-up on extracted fractions

In order to verify if AEP and EAEP to identify possible pitfalls associated with the overall extractability of oil and protein from the almond cake, the optimized extraction conditions from a previous lab-scale optimization study (Souza et al., 2019) were scaled-up to a pilot-plant, from 50 to 750 g almond cake using 1:12.8 SLR, producing ~10 L of slurry in a jacket glass reactor. The differences between lab-scale and pilot-plant scale in all extracted fractions were evaluated and are presented in Figure 2 (A) and (B), respectively. At lab-scale extraction, higher oil yield at cream fraction for AEP (27.5 vs 8.2%) and EAEP (30.8 vs 13.1%) were obtained as well as at total oil extraction for AEP (48.2 vs 26.2%) and EAEP (50.2 vs 29.1%) when compared to pilot-scale. Regarding protein extraction yield, aqueous extraction did not present a significant difference for any of the fractions observing lab and pilot-scale. EAEP showed slightly lower protein yield at skim fraction (15.7 vs. 16.0%) in the pilot-plant experiments, without significant difference. The solid yield extraction was higher at lab-scale in the cream fraction at AEP (5.3 vs. 1.7%) and EAEP (6.6 vs 3.0%) and in the total solid extraction at EAEP (53.1 vs 45.7%). When the scale has increased, a decrease in oil and solid yields was observed, while protein yield was not affected at AEP and EAEP.

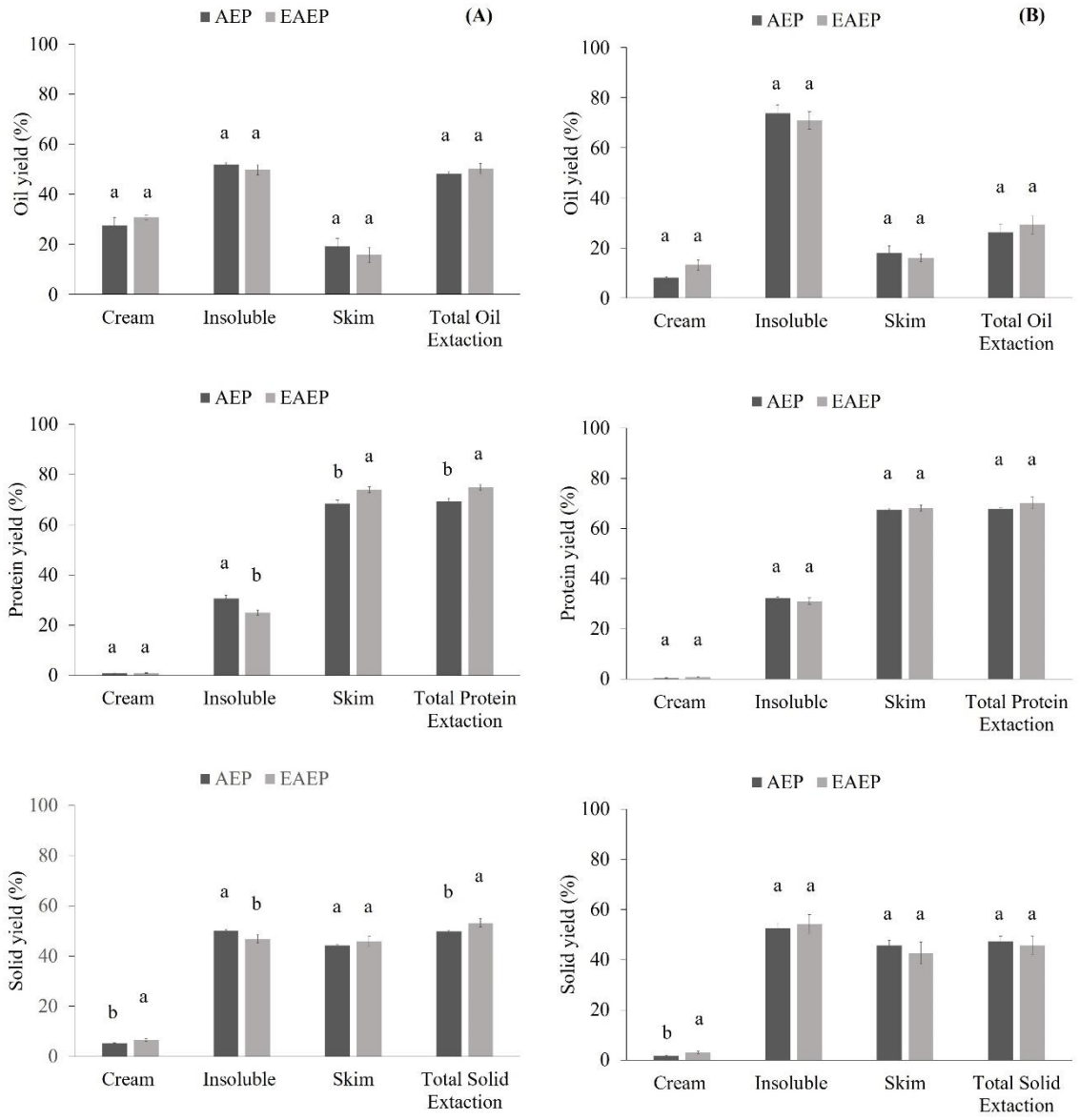

Figure 2. Partitioning of oil, protein and solid at pilot-scale and lab-scale. (A) Lab-scale partitioning of oil, protein and solid; the lab-scale data is from a previous study (Souza et al., 2019); (B) Lab-scale partitioning of oil, protein and solid (% of the total in starting material) produced by AEP and EAEP at pilot-scale and lab-scale from cold pressed almond cake. Different letters indicate statistical difference ($p < 0.05$) between AEP and EAEP. Data represent the mean ± one standard deviation (n = 3)

To establish the commercial potential of the extractions, it is essential to evaluate the yield recovered at the pilot-scale. From the obtained results, a reduction of ~20% in the oil extraction yields, but similar protein extraction yields, at AEP and EAEP, were

observed. The observed yield reduction was significant, which shows the difficulty of scaling up this type of process. A decrease in oil recovery at the pilot-scale was also found by Kapchie et al. (2012), when studying isolated soybean oleosome. The lab-scale experiments produced supernatants with 14 times more residual lipid when compared to the pilot-plant experiments, decreasing from 5.34 to 0.38%, respectively, but the protein content remained stable with no significant difference. The authors attributed the lower oil yield to the lower centrifugation speed for the lab-scale when compared to the higher speed at pilot-scale and the difference of the centrifuge equipment used.

In the present study, the speed and the centrifuge did not change, but the slurry volume generated in the pilot-scale experiments was 10 times higher. The efficiency of the oil recovery is being related to various critical unit operations, such as grinding methods, the conditions used to separate the oil to the other constituents, and the recovery of oil by continuous centrifugation (RHEE et al., 1973). Therefore, centrifugation seems to be a critical point in scaling up the oil extraction process by AEP or EAEP from oilseeds pressed cakes and should be carefully evaluated prior to the implementation of these methods. It is possible that the extracted oil reduced due to the separation of the oil or the creaming at that scale. On a large scale, the formation of a more stable emulsion may have occurred, making it difficult to separate extracted oil trapped in the insoluble.

3.2 Extracted fractions partitioning at AEP and EAEP at pilot-scale

The partitioning of extracted compounds of the generated fractions after scale-up of AEP and EAEP was evaluated and is presented in Figure 2 (B). The oil distribution at AEP indicates that 8.2% of oil is present at the cream fraction, 18.1% is at the skim fraction, and the total oil extracted was 26.2%. At EAEP, 13.1%; 16.0% and 29.1% of oil portioning were at cream, skim, and total oil extracted, respectively. Regarding protein distribution, 0.4% of protein was present at cream at AEP and 0.8% at EAEP; 67.3% of protein went to skim fraction at AEP, and 68.1% at EAEP and the total protein extraction was 67.7% at AEP and 70.2% at EAEP. When comparing the extractions at

the pilot-plant scale, similar oil and protein yields, without significant difference, were observed for AEP and EAEP at the extracted fractions.

As there was no significant difference at the oil and protein fractions (cram, skim, insoluble, and total extractions) between AEP and EAEP, would be unnecessary the use of the enzyme from the point of view of final yield. However, it should be considered that a reduced extraction time was used for the EAEP (1 h instead of 2 h). For the EAEP, the main limiting factor is the cost of the enzyme. However, longer reaction times increase energy use and consequently may also raise the cost of the AEP (Chandrasekaran, 2015; Liu et al., 2016). Considering that half of the time was used for EAEP, it is possible that the reduction in energy and the occupancy cost of the installed capacity may amount to the same as, or even lower than, the cost of the enzyme applied. Besides that, in some cases enzyme does not lead to higher extractability yields but produces a cream that is easier to be demulsified and produces proteins with better functionality. In the present study, the cream + free oil fraction is of greater interest as the cream was used in the de-emulsification step.

3.3 Cream de-emulsification

The present study investigated the chemical and enzymatic de-emulsification of the cream + free oil fractions obtained after AEP and EAEP. The emulsion formed during aqueous or enzyme-assisted extraction of vegetable oil is the major obstacle to recover the free oil, where the extraction conditions play a key role in the resistance of that cream toward the de-emulsification (Chandrasekaran, 2015; Jung et al., 2009). The AEP-cream contained 9.92% ± 1.47 of oil and 1.57% ± 0.01 of protein, whereas the EAEP-cream contained 10.94% ± 0.94 of oil and 1.29% ± 0.03 of protein. The effects of the extraction conditions and de-emulsification conditions used to destabilize both AEP and EAEP cream are described in Table 1. De-emulsification yield of 37.4% and 62.8% were obtained for the chemical and enzymatic approach for the AEP-cream. For EAEP, the de-emulsification yield was 58.9% at enzymatic method and 98.7% at chemical assay. For AEP-cream, the enzymatic de-emulsification presented the highest yield, 25% higher than chemical de-emulsification, and 24% higher than both control samples. For EAEP the pH value (pH 5.0, near to pI of almond proteins) significantly

affected the stability of cream emulsion. There was a 75% increase from the results obtained for the control for the chemical de-emulsification assay. Enzymatic de-emulsification at EAEP presented a similar result as AEP enzymatic de-emulsification, increasing the free oil recovery by 32% compared to the control.

Table 1. Effects of extraction and de-emulsification conditions on AEP and EAEP cream de-emulsification

Cream	Type of de-emulsification	Enzyme content (%)	pH	De-emulsification yield (%)
AEP	Chemical control	no enzyme	initial pH = 9.0	38.8 ± 1.25 ^{b*}
	Enzymatic control	no enzyme	9.0	38.8 ± 1.25 ^{b*}
	Chemical	no enzyme	5.0	37.4 ± 0.89 ^{b}
	Enzymatic	0.50%	9.0	62.8 ± 0.42 ^{a}
EAEP	Chemical control	no enzyme	initial pH = 8.5	23.1 ± 1.44 ^{c}
	Enzymatic control	no enzyme	9.0	26.6 ± 1.31 ^{c}
	Chemical	no enzyme	5.0	98.7 ± 1.90 ^{a}
	Enzymatic	0.50%	9.0	58.9 ± 0.73 ^{b}

Different letters in the same column indicate statistical difference ($p < 0,05$) between the different de-emulsification tests in the same extraction. Data represent the mean ± one standard deviation (n = 3). *As the chemical control was performed at the original pH (9.0) of the slurry and enzymatic control was performed at pH 9.0 without enzyme, both chemical and enzymatic controls were considered as chemical and enzymatic control for AEP extraction.

Recovery of oil at cream fractions during chemical de-emulsification is due to the coalescence of oil droplets. At the isoelectric point, the proteins present their lowest solubility and also favor the coalescence of oil droplets; thus, the emulsion becomes more unstable, reducing electrostatic repulsion between droplets, increasing free oil yield (Chabrand and Glatz, 2009; Wu et al., 2009). This was evidenced by Wu et al. (2009), that studied the size diameter distribution of oil droplet at cream fraction at soybean flakes at different pH values. They observed a droplet size increase close to pI, attributing this to the reduction of electrostatic repulsion, which led to the droplet

coalescence. In contrast, low de-emulsification yields were found for AEP-chemical de-emulsification and AEP-control, characterizing a cream emulsion more stable, that did not favor a high free oil recovery. Possibly the chemical de-emulsification did not affect much the stability of AEP-cream because of the absence of enzymes during extraction. According to Jung et al. (2009), emulsions that are stabilized by hydrolyzed protein are less stable than emulsions that are stabilized by native proteins. In a peanut AEP-cream de-emulsification study, Zhang et al. (2013) obtained similar results, reporting that changing the pH value had little effect on cream destabilization. They attributed this outcome to the lack of enzyme during the extraction and observed a synergistic effect between enzyme treatment and pH adjustment in demulsifying peanut cream.

A significant synergistic effect between pH and enzymatic treatment on de-emulsification was also found by Li et al. (2017) at EAEP-cream chemical de-emulsification. For EAEP-cream, chemical de-emulsification was very efficient in releasing the oil entrapped in the cream. It possibly happened because of the low molecular weight of peptides formed in the EAEP hydrolysates, combined with the pH at isoelectric point, which leads to a high destabilization of the cream layer. Wu et al. (2009) increased the yield of free oil to 100% by totally destabilizing the emulsion on EAEP-cream through the adjustment of the pH to 4.5 (soybean proteins pI). The same was observed by Chabrand & Glatz (2009) at pH 4.5 on EAEP-cream from soybean flour. The authors noticed an increment from 2% to 83% of oil yield recovery.

At enzymatic de-emulsification, the protease hydrolyzes the interfacial proteins that lead to low molecular weight peptides, which helps to break down the cream emulsion, that is stabilized by proteins (Casas & González, 2017). Partial hydrolysis reduces the protein's molecular weight and usually reveals hidden hydrophobic groups that increase the surface hydrophobicity allowing for better adherence to the oil-water interface (Lam and Nickerson, 2013). The increment at de-emulsification yield using the enzyme at AEP-cream extraction showed that the enzymatic de-emulsification made the emulsion less stable, which turned free the entrapped oil for subsequent coalescence, so more free oil could be recovered. Higher de-emulsification efficiency using enzyme was also observed by Li et al. (2017), that studied de-emulsification on the AEP-cream extraction in peanuts. They noticed that the use of proteases (Alcalase 2.4L, Papain,

Protex 6L, Protex 7L, and Protex, 50FP) was more effective on de-emulsification than salts or physical methods. The authors found an increment at de-emulsification yield from 24% at control to 84% using an alkaline protease (Protex 6L), the same enzyme used in the present study.

In the present study, the enzymatic de-emulsification at EAEP-cream was slightly lower than at AEP, and there was no significant difference between the extraction methods applied. This result indicates that at enzyme-assisted extraction, the addition of more enzyme at EAEP-cream did not affect the emulsion stability the same way as for AEP-cream. This possibly occurred because of the high degree of hydrolysis (DH = 23%) at EAEP extraction. The degree of hydrolysis (DH) quantifies the extent of proteolysis and is expressed as the percentage of peptide bonds cleaved (Panyam, Dinakar and Kilara, 1996). The high DH indicates that most of the proteins have already been hydrolyzed at the time of extraction. By adding more enzyme to the medium, there were possibly no more peptide bonds that could be hydrolyzed by the same protease. Extensive hydrolysis in the extraction led therefore to no difference in free oil yield between AEP and EAEP after enzymatic de-emulsification. A different outcome was observed by Jung et al. (2009), where the soybean AEP-cream was significantly more stable than EAEP-cream (74% vs. 100% free oil yield) after enzymatic de-emulsification, leading to a lower free oil yield for AEP-cream. Although these authors applied the same enzyme used in the present study, a significant difference in the concentrations used during extraction and de-emulsification may be pointed out. Jung et al. (2009) used a much lower enzyme concentration during the extraction and a much higher enzyme concentration for the de-emulsification, leading to higher oil yield at EAEP-cream enzymatic de-emulsification than in the present study.

Conclusions

Scaling up both AEP and EAEP was observed that the oil yield was affected, decreasing the oil production by ~20% compared to lab-scale, indicating that centrifugation is a critical step in this process. On the other hand, protein yield was not affected, keeping the yield constant at the pilot-plant scale for both processes. The starting material of the present study (almond cake) contained 16% of oil and 37% of protein. AEP and EAEP

presented similar extraction yield, about 30% of the initial oil, and 70% of the protein. Most of the oil recovered was from the cream fraction (8% in AEP and 13% in EAEP), and as for protein, most were recovered in the skim fraction (67% in AEP and 68% in EAEP). Using a de-emulsification strategy on the cream fractions, the yield obtained ranged from 23.1% (EAEP-cream chemical control) to 98.7% (EAEP-cream chemical de-emulsification). Successful chemical de-emulsification of the cream emulsion recovered during EAEP of the almond cake was also demonstrated, recovering about 99% of the oil extracted in the cream fraction. This study showed that it might be possible to recover oil and protein from the almond cake in a safer way, avoiding hazardous solvents, such as hexane. The implications of the scaling-up and the use of enzymes during the extraction regarding the skim functional protein properties still should be investigated.

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CAPÍTULO 5

Effect of Aqueous, Enzyme-Assisted and Solvent Aqueous Extractions on Functional Properties of Cold Pressed Almond Cake Protein

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Abstract

This study investigated the effects of different protein extraction processes on the almond protein functionality. Proteins were extracted from the almond cake by aqueous extraction process (AEP), enzyme-assisted extraction process (EAEP), and aqueous solvent extraction, using hexane as a solvent to defatted the starting material (hexane-AEP). SDS-page and degree of hydrolysis confirmed that after EAEP extraction, there was extensive hydrolysis (DH = 23%), which interfered in the protein functionalities. Physicochemical properties such as zeta potential (ZP) and surface hydrophobicity (H_0) and protein functional properties, including solubility, emulsification capacity (EC), emulsification activity index (EAI), foaming capacity (FC) and foaming stability (FS) were evaluated. The extraction method significantly influenced the protein properties and the protein composition. EAEP presented higher total oil and protein extraction yield of 29.1% (vs. 26.2 AEP) and 70.2% (67.7 and 63.1% AEP and hexane-AEP), respectively. EAEP presented the highest negative charge at ZP (-43.3 mV) and lowest H_0 (312) at pH 10. Regarding solubility, the highest value was observed for EAEP (95.3%), followed by AEP (87.1%) and hexane-AEP (86.4%) at pH 8, where all extractions showed a U-shape, with the lowest solubility at the isoelectric point (between 4 and 5). EC and EAI showed the same tendency as the solubility curve, but for EC analyses, EAEP presented the highest values, followed by AEP and hexane-

AEP, while EAI presented the inverse result. For foaming properties, hexane-AEP exhibited the highest values among the samples at all pH values.

Keywords: vegetable byproduct, environmental-friendly extraction, physicochemical and functional protein properties

1. Introduction

In order to provide adequate protein in the diet for the global populace, the demand for protein in the world is increasing, so there is a necessity to produce more protein sustainably (Nadathur, Wanasundara, & Scanlin, 2017). The use of byproducts, as from tree nut meals, which presents about 40% of protein, is a suitable choice as an alternative protein resource. The almond cake is a residue generated after the cold-pressed oil extraction from the almond seed, which presents a high protein content (37%) and some residual oil (16%) (Souza, Dias, Koblitz, & Bell, 2019). Although almonds exhibit a high protein content and all essential amino acids (excluding methionine) (Sathe et al., 2002), there are few studies about the protein components of almond and further related to almond cake. The almond proteins are soluble in aqueous media, and the major storage protein is known as AMP or amandin, which constitutes about 70% of total soluble fraction protein (Sathe et al., 2002). Amandin is a legumin type 11 S globulin composed by different polypeptides, with estimated molecular weights between 40 to 42 kDa (acidic subunit) and 20 to 22 kDa (basic subunit) (Zhang, Zhang, Sheng, Wang, & Fu, 2016).

Different extraction methods have been used to extract edible oils, but usually, the industrial process for extraction of oil from oilseeds involves the use of solvents, such as hexane (Rosenthal, Pyle, & Niranjan, 1996). This extraction process reaches high extraction yields (about 95%), but the use of solvents presents several disadvantages, as they are hazardous and polluting (de Moura, Campbell, de Almeida, Glatz, & Johnson, 2011; Rosenthal et al., 1996). Aqueous extraction process (AEP) and enzyme-assisted aqueous extraction (EAEP) has been considered an environmental-friendly alternative approach for oil extraction. An aqueous medium, when compared with solvent use, is considered much safer and cost effective. Another advantage of this extraction is the

simultaneous production of edible oil and protein in the same process (Rosenthal et al., 1996). As processing conditions affect protein composition and functionality, the extraction method should be considered when this protein will be applied as an ingredient with specific functionality (de Almeida, de Moura Bell, & Johnson, 2014; Yu, Ahmedna, & Goktepe, 2007).

To date several authors investigated the extraction effects on protein functionalities in oilseeds, such as peanut (Yu et al., 2007), sunflower (Karayannidou et al., 2007), soybean (de Almeida et al., 2014), canola (Gerzhova, Mondor, Benali, & Aider, 2016), flaxseed (Tirgar, Silcock, Carne, & Birch, 2017), and almond, testing different alkaline solvents (NaOH and buffered saline borate) (Amirshaghaghi, Rezaei, & Habibi Rezaei, 2017). According to the literature, there are no studies on extraction, physicochemical and protein functional properties from the almond cake, produced by aqueous extraction process (AEP) and enzyme-assisted aqueous extraction process (EAEP), and further there is no comparison between these extractions with a defatted starting material using an aqueous solvent extraction (hexane-AEP).

Prior research from our group was a lab-scale optimization study at AEP, and EAEP extractions (Souza et al. 2019) using 50 g of cold pressed almond cake, which produced about 1 L of slurry. To set up the commercial potential of the extractions a pilot-scale using 750 g of cold pressed almond cake and a solid-to-liquid ratio (SLR) of 1:12.8, about 10 L of slurry was produced. The skim fraction from this previous study was then freeze-dried and evaluated. The objective of the present study was 1) extract protein in a different way from the almond cake; 2) evaluate the influence of the extraction process on the physicochemical properties and 3) the protein functionality properties from the skim fractions. Therefore, this study proposed to seek the use of an alternative environmental-friendly process where the hexane is replaced by water/water plus enzyme to extract oil and protein. Besides that, generate a protein fraction with added value that can be applied as ingredient in other foods as those proteins present different functionalities, turning waste into an added value product of economic interest in an environmental way.

2. Material and methods

2.1 Materials

The cold-pressed almond cake was supplied by Blue Diamond (Sacramento, CA, USA). The pellets were processed in a blender (Oster Sunbeam Products, FL, USA) and produced a homogeneous flour to be used on the extractions. An endoprotease – FoodPro Alkaline Protease (Genencor Division of Danisco, NY, USA) was added on EAEP extraction, which is an alkaline enzyme derived from a strain of *Bacillus licheniformis* and has its highest activity at pH 8.0 - 10.5 and 45 - 75 °C. Phthaldialdehyde (OPA), trichloroacetic acid (TCA), serine, 8-anilinonaphthalene-1-sulfonic acid (ANS), and hexane were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biuret reagent and sodium dodecyl sulfate (SDS) were acquired from VWR (Radnor, PA, USA), bovine serum albumin (BSA) and Sudan red 7B from Thermo Fisher Scientific (Waltham, MA, USA) and electrophoreses reagents were purchased from Bio-Rad (Hercules, CA, USA).

2.2 Experimental methods

Three extraction methods were tested to determinate the physicochemical composition and protein functional properties of the freeze-dried skim from the cold-pressed almond cake (AC): AEP, EAEP, and hexane-AEP. The starting material (AC) contained about 16.2% of residual oil (AOCS method 989.05) and 37.2% of protein content (Dumas method). For AEP and EAEP, the AC was used as it was, and for hexane-AEP, the starting material was subjected to the removal of all oil. The sample was defatted exhaustively on a Soxhlet apparatus, using hexane as a solvent, for six hours. After the oil removal, the sample was placed in a fume hood overnight to release any remaining solvent and then was stored in a freezer at -20 °C.

2.2.1 Protein extraction

Proteins were extracted from the almond cake by aqueous extraction process (AEP), enzyme-assisted extraction process (EAEP), and aqueous solvent extraction (hexane-AEP). The scale-up extractions followed the optimized conditions described by Souza et al. (2019). About 750 g of almond cake (AEP and EAEP) and defatted almond

meal (hexane-AEP) were separately dispersed into the water in a solid-liquid ratio of 1:12.8 (w/v) into a 10-L jacketed glass reactor with a bottom drain valve (Chemglass, NJ, USA) connected to a thermostat circulating water bath (PolyScience, IL, USA). For EAEP extraction Alkaline Protease was added. Extractions were performed at pH 9.0, 50 °C for 2 h for AEP and hexane-AEP, and 1 h for EAEP, under constant stirring of 120 rpm. After the extraction, the slurry was centrifuged to remove the insoluble fraction from the liquid fraction, which was allowed to settle overnight, generating the skim fractions that were freeze-dried and stored in a freezer (-20 °C) to be used in future analysis.

2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis from total protein

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was used to evaluate the protein profile concerning the molecular weight of the total protein of the three different extractions as described by Laemmli (1970) with few modifications. The samples were mixed with Leammli solution in a 1:1 (v:v) proportion, thoroughly mixed and heated in a water-bath 95 °C for 5 min. The running buffer used was Tris-HCl buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Thirty micrograms of protein were loaded onto the precast 12% CriterionTM TGX Precast Gels for the electrophoretic separation of proteins. As a molecular weight marker low range SDS-PAGE standard (14.4–97.4 kDa) was used. The electrophoretic separation was carried out at 200 V and room temperature. The gel was imaged using a Gel DocTM EZ Imager system and Image Lab software (Bio-Rad Laboratories) for analysis of the distribution of the protein.

2.4 Degree of hydrolysis (DH)

The degree of hydrolysis was evaluated by the o-phthaldialdehyde (OPA) method, as described by Nielsen, Petersen, & Dambmann (2001). For this assay was used a 0.9516 meqv/L L-serine solution as a standard and was calculated as the following equation:

$$\mathrm{h} = \frac{\text{Serine } NH_2 - \beta}{\alpha} \qquad (1)$$

$$\text{DH}\ (\%) = \frac{h}{h_{tot}}\ x\ 100 \qquad (2)$$

Where: β = 0.4, α = 1 and h_{tot} = 7.58 (Liu et al., 2016).

2.5 Physicochemical analyses

2.5.1 Zeta Potential (ZP)

The electrical charge on the skim surfaces was measured by zeta potential, using the method reported by Gerzhova et al. (2016). The pH of the solutions of the freeze-dried samples (0.01% w/v) in distilled water was adjusted to 4.0, 5.0, 6.0, 8.0, and 10.0, with 0.5 N HCl or 0.5 N NaOH. The solutions with adjusted pH were placed into the measurement chamber of a microelectrophoresis instrument, at 25ºC, and measured using Zetasizer 2000 (Malvern Instruments Ltd., UK). All measurements were made in triplicate for each sample at each pH.

2.5.2 Surface hydrophobicity (H_0)

The surface hydrophobicity was determined using ANS as a fluorescence probe, following the method described by Hou et al. (2017), with modifications. The protein concentration of each sample was determined according to the Lowry method (Fryer, Davis, Manthorpe, & Varon, 1986). H_0 was tested at different pH values (2.0, 4.0, 5.0, 6.0, 8.0, and 10.0). The samples were serially diluted with buffer to obtain protein concentrations of 0.01, 0.05, 0.10, 0.15, and 0.20 mg/mL. Twenty microliters of ANS (8.0 mM) was added to 4 mL of each protein solution and immediately vortexed. Fluorescence intensity of 200 µL (protein solution plus ANS) was recorded at a microplate reader (FlexStation 3; Molecular Devices - San Jose, CA, USA) with an excitation wavelength of 390 nm and an emission wavelength of 470 nm. The H_0 was expressed as the initial slope of relative fluorescence intensity versus protein concentration (mg/mL). Surface hydrophobicity was calculated by linear regression analysis.

2.6 Protein functional properties

2.6.1 Solubility

Protein solubility was determined following a method developed by de Almeida et al., (2014), with modifications. The pH of 10 mL of a 1% (w/w) solution of each freeze-dried skim was adjusted to 2.0, 4.0, 6.0, 8.0, and 10.0 by adding 1 N HCl or 1 N NaOH solution. The dispersions were stirred for 1 h with pH adjusted after 15, 30, and 60 min, if necessary, and centrifuged at 10,000 x g at 20 °C for 10 min. The protein content of the supernatants was measured using the Biuret method, with bovine serum albumin as standard. The total protein content was measured after solubilizing the samples in a 1 N NaOH (Morr et al., 1985). Samples of each extraction and pH were evaluated in triplicate. Solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{protein content in the supernatant}}{\text{total protein content in the sample}} x\ 100 \qquad (3)$$

2.6.2 Emulsification properties: Emulsification capacity (EC) and Emulsification activity index (EAI)

Emulsification capacity (EC) was measured using the method described by Bian et al. (2003), with modifications. The pH of 5 mL of a 2% (w/w) solution of the freeze-dried skim was adjusted to 2.0, 4.0, 6.0, 8.0, and 10.0 by adding 1 N HCl or 1 N NaOH. The protein solutions were blended continuously at 10.000 rpm using a 20 mm shaft on a Polytron PT 2500 homogenizer (Kinematica AG, Lucerne, Switzerland) with vegetable oil, that was dyed with 4 ppm Sudan Red 7B, at 0.25 g/s flow rate, until the inversion point of oil-in-water to water-in-oil. The amount of oil was recorded, and, to confirm the inversion, a conductivity meter (µS/cm) was used, where a significant drop in the conductivity indicates the inversion (Wang & Zayas, 1992). Samples were evaluated in triplicate. Emulsification capacity was calculated as follows:

$$\text{EC (g oil/ g protein)} = \frac{\text{g of oil used to invert the phase}}{\text{g of protein in 5 mL of a 2\% solution}} \qquad (4)$$

Emulsification activity index (EAI) was evaluated using the method described by Jamdar et al. (2010) and Pearce & Kinsella (1978), with modifications. The pH of 15 mL of a 1% (w/w) solution of each freeze-dried skim was adjusted to 2.0, 4.0, 6.0, 8.0,

and 10.0 by adding 1 N HCl or 1 N NaOH. After pH corrections, the dispersions were blended with 5 mL of vegetable oil for 1 min at 20.000 rpm using the homogenizer Polytron PT 2500. The emulsion was analyzed after diluting 40 µL of the sample in 10 mL of 0.1% SDS solution and sampled from the bottom of the beaker just after the homogenization. The absorbance of the diluted emulsion was measured at 500 nm. The emulsion activity index (EAI) was determinate as follows:

$$EAI\ (m^2/g) = \frac{2\ x\ 2.303\ x\ Abs_0\ x\ N}{\varphi\ x\ c\ x\ 10.000} \qquad (5)$$

Where Abs_0 is the absorbance of the diluted emulsion measured immediately after homogenization, N is the dilution factor (x 250), φ is the oil volume fraction (0.25), c is the weight of protein per volume (g/mL). Samples were run in triplicate.

2.6.3 Foaming

Foaming properties were measured according to the method described by Sze-Tao & Sathe (2000) with modifications. The pH of 25 mL of a 1% (w/w) solution of each freeze-dried skim was adjusted to 2.0, 4.0, 6.0, 8.0, and 10.0 by adding 1 N HCl or 1 N NaOH. The protein solutions were whipped continuously at 10.000 rpm in a Polytron PT 2500 homogenizer for 2 min. Immediately after whipping, the suspensions were transferred into a 100 mL graduated cylinder. The foam volume was recorded within the graduate cylinder immediately for foaming capacity (FC) and after 60 min for foaming stability (FS). Samples were evaluated in triplicate. The following equations were used to determinate foaming capacity and stability:

$$FC\ (\%) = \frac{V_A - V_B}{V_B}\ x\ 100 \qquad (6)$$

$$FS\ (\%) = \frac{V_{60\ min} - V_B}{V_B}\ x\ 100 \qquad (7)$$

Where V_A is the volume immediately after whipping, V_B is the volume before the solutions were whipped and $V_{60\ min}$ is the volume after 60 min.

2.7 Statistical analysis

All experiments were performed at least in triplicate, and the values were expressed as mean ± standard deviation (SD) of the replicates. Analyses of variance (ANOVA) were performed, followed by post-tests comparison by Tukey tests to determine the significant differences between the groups using the SAS statistical computer package (version 9.4, SAS Institute Inc., Cary, NC, USA) considering the level of significance $p < 0.05$.

3. Results and discussion

3.1 Extraction yields

EAEP presented similar total oil extraction to AEP (29.1% vs. 26.2% respectively), similar oil yield in skim fraction (16% vs. 18.1% respectively), and at oil yield in the cream fraction (13.1% vs 8.2 respectively), without significant difference at all fractions. As at the hexane-AEP extraction the oil was removed, there was no cream or oil formation. Regarding protein yields, EAEP presented the highest total protein extraction (70.2%) between the extractions, followed by AEP and hexane-AEP (67.3 and 63.1% respectively, with no statistical difference) and also showed the highest protein yield in skim fraction (68.1%) followed by AEP and hexane-AEP (67.7 and 63.1% respectively, with no statistical difference). Slightly lower protein extraction yields were observed for the hexane-AEP (63.1%) ($p < 0.05$). This result might be related to the higher temperature (~68 °C) used during the process of degreasing (Soxhlet extraction), which may have cause protein denaturation and lowered down the protein yield at hexane-AEP.

3.2 SDS-page and degree of hydrolysis (DH)

The SDS-PAGE profiles and the degree of hydrolysis (DH) of skim fractions from the three different extractions are shown in Figure 1. An evident correlation between SDS-PAGE and the degree of hydrolysis in each extraction can be observed. The electrophoretic analyses of AEP and hexane-AEP presented the same profile, showing a higher concentration of polypeptides with molecular weights between 21.5 and 45 kDa, with three very distinct bands in this region (indicated by arrows) and a low

DH (%): 1.82 ± 0.6 and 1.33 ± 0.23%, respectively. In EAEP, a protein/peptide concentrate below 14.4 kDa, as well as a high DH (%) of 22.64 ± 2.80, was found. In addition to the major polypeptides on AEP and hexane-AEP, several minor polypeptides, with low staining intensity and small bandwidth, were noted.

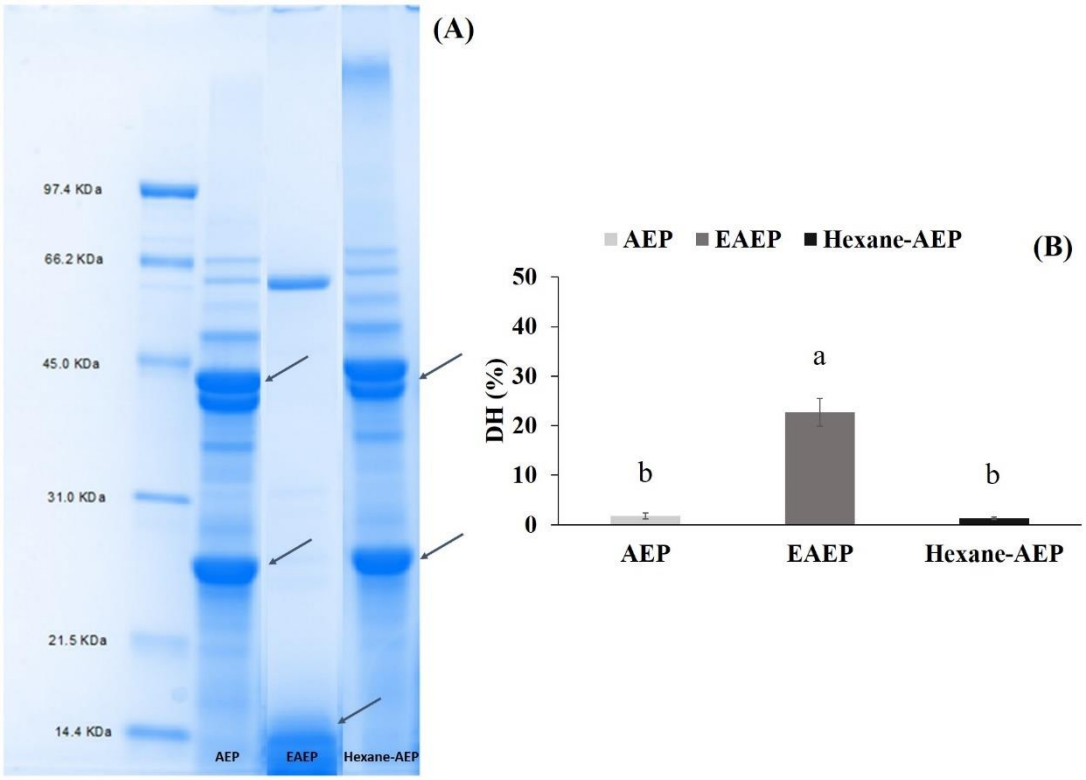

Figure 1. (A) Electrophoretic analysis: SDS-PAGE (12% CriterionTM TGX Precast Gels) analysis of total protein in the extracted skim fractions from the cold-pressed almond cake: lane 1) MW marker standards (14.4-97.4 kDa); lane 2) AEP skim, arrows indicate polypeptides with molecular weights between 21.5 and 31.0 kDa and ~ 45 kDa; lane 3) EAEP skim, arrow indicates polypeptides with molecular weight of 14.4 kDa; lane 4) Hexane-AEP skim, arrows indicate polypeptides with molecular weights between 21.5 and 31.0 kDa and ~ 45 kDa. (B) Degree of hydrolysis of total protein in the different skim fractions. Different letters indicate significant difference ($p < 0,05$) among the extractions. Data represent the mean ± one standard deviation of triplicates.

The results indicate a close relationship between AEP and hexane-AEP and a very distinct behavior for EAEP. AEP and hexane-AEP skim fractions showed very similar polypeptide compositions, as those fractions did not undergo enzymatic hydrolysis,

remaining as whole proteins. EAEP unique profile can be explained by the hydrolysis previously applied in this process. In the presence of the protease, the native almond proteins were hydrolyzed, producing peptides with low molecular weight (>14.4 kDa). The polypeptides composition of AEP and hexane-AEP are in agreement with the results obtained by Sathe et al., (2002), that observed that the structure of amandin, the major almond storage protein, decomposed, in the presence of the reducing agent, into acidic and basic polypeptides with 42 to 46 kDa and 20 to 22 kDa, respectively. This observation suggests the presence of amandin in those extractions. Amandin is constituted by two storage polypeptides of 61.0 kDa (prunin-1) and 55.9 kDa (prunin-2), but in the presence of a reducing agent, such as 2-mercaptoethanol, the polypeptides are converted to 38.6-41.8 kDa and 20.4-22.8 kDa acidic and basic polypeptides of amandin, respectively (Garcia-Mas et al., 1995; Wolf & Sathe, 1998). In an earlier study, tested the behavior of amandin in different pH values and found the same composition of major polypeptides (basic and acid fractions) between pH 6.6 and 11, but it was noted that, by decreasing the pH from 6.0 to 2.0, the most significant polypeptides were observed as 62 kDa and 20 kDa. They attributed the result to the insolubility of amandin at that pH range and the presence of minor proteins in solution.

3.3 Physicochemical analyses

3.3.1 Zeta potential

EAEP presented the highest absolute ZP values, followed by AEP and then hexane-AEP. Hexane-AEP showed the highest positive charge (26.2 ± 0.26) at pH 4 and EAEP, the highest negative charge (- 43.3 ± 3.10) at pH 10 (Figure 2). The ZP charge values closer to zero (0.26, 1.06, and -0.87 mV, respectively) were recorded at pH 4 for AEP and EAEP and pH 5 for hexane-AEP. The solubility of proteins is related to the characteristics of the protein surface, such as zeta potential and surface hydrophobicity. Therefore, it is essential to evaluate the correlation between the superficial features and the functional properties of proteins (Hayakawa & Nakai, 1985). ZP provides information about the degree of interaction between colloidal particles, measuring the electrostatic potential at the electrical double layer on the surface of proteins in solution (Gerzhova et al., 2016). The higher the absolute value of ZP, the weaker the protein-protein interactions. ZP charge indicates if the pH value of the solution is higher

(negative charge) or lower (positive charge) than the isoelectric point of the tested protein.

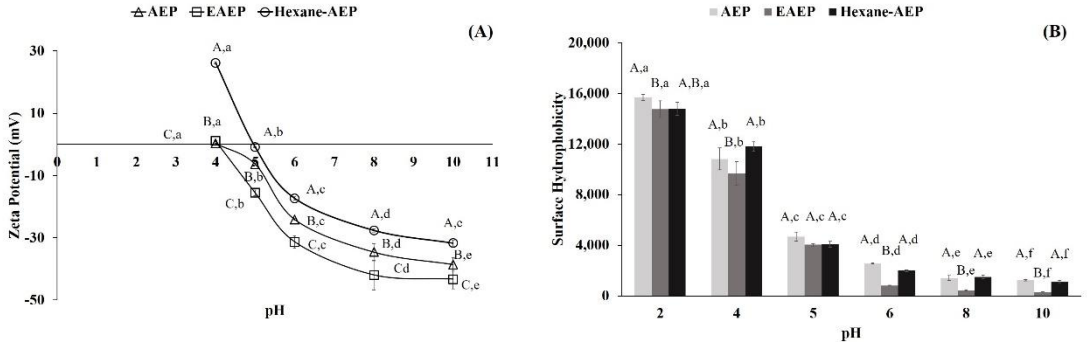

Figure 2. Physicochemical properties for the protein fractions of AEP, EAEP, and hexane-AEP extractions, from cold-pressed almond cake as a function of pH. (A) Zeta potential (mV). (B) Surface hydrophobicity. Different capital letters indicate a significant difference ($p < 0,05$) among the different extractions for the same pH and different lowercase letters indicate a significant difference ($p < 0,05$) among the different pH values for the same extraction. Data represent the mean ± one standard deviation of triplicates.

The results obtained by this study showed EAEP with the highest ZP, which represents a strong repulsive electrostatic force between proteins. These results can be explained by a low ratio of acidic and basic amino acid groups in the peptides of this fraction (Tirgar et al., 2017). The SDS-PAGE (Figure 1) showed that EAEP presented a very low intensity of the acidic and basic polypeptides characteristic to amandin, while AEP and hexane-AEP exhibited high intensity of these bands. This may explain the lower ZP values for the latter extraction fractions. These results are in agreement with Tirgar et al. (2017), who investigated the effect of the extraction on flaxseed proteins and also with Rommi et al. (2015), that studied the physicochemical properties of the protein fractions from defatted rapeseed press cake. Both studies reported higher ZP values for the enzyme-hydrolyzed samples.

Overall, for all extractions, ZP was negative in the tested pH values, and absolute values raised when the pH of the solution was increased. Similar results were found by Gerzhova et al. (2016) in their canola meal study, where the maximum net charge was

found at pH 10, and ZP absolute values decreased as the pH was increased. The minimum net charge was observed between pH 4 (AEP and EAEP) and 5 (hexane-AEP). This shows that for AEP and EAEP, the isoelectric point (pI) was closer to 4 while for hexane-AEP, the pI was displaced to a pH value closer to 5. The pI of a given protein may be shifted by denaturation or chemical modification (Hayakawa & Nakai, 1985). For hexane-AEP, the starting material was exhaustively defatted in a Soxhlet system. N-hexane (the solvent used to remove the lipids from the sample) presents a boiling point of 68-70 °C, so the sample suffered a mild heat treatment for an extended period (6 hours), in contact with a very non-polar solvent, that may have led to protein denaturation.

3.3.2 Surface hydrophobicity

At surface hydrophobicity analyses, EAEP presented the lowest values for H_0, while no difference could be observed between AEP and hexane-AEP. The highest H_0 value was found at pH 2 for AEP (15,668 ± 230) and the lowest, at pH 10, for EAEP (312 ± 2.00) (Figure 2).

The number of hydrophobic groups on the surface of a protein in contact with an aqueous medium is indicated by the surface hydrophobicity, which plays an essential role in conducting protein aggregation and interfacial protein activity (Zhao et al., 2012). The surface hydrophobicity of the protein, as measured by the ANS probe, derives from the binding of the probe to the hydrophobic regions on the surface of the proteins. The detection of the fluorescence corresponds to hydrophobicity, and the higher the signal, the higher the surface hydrophobicity (Alizadeh-Pasdar & Li-Chan, 2000).

The lowest surface hydrophobicity was found for EAEP, indicating that the hydrolysis of the protein structure reduced the number of hydrophobic amino acids to react with the ANS probe at the resulting peptide's surface. These results agree with the previous study by Tirgar et al. (2017), that evaluated the effects of the extraction method on the functional properties of flaxseed protein and also with Polanco-Lugo, Dávila-Ortiz, Betancur-Ancona, & Chel-Guerrero (2014), who studied the effects of enzymatic hydrolysis on lima bean protein isolate. Both studies also observed a similar

decrease in surface hydrophobicity after proteolysis. The highest surface hydrophobicity for all extractions was found at pH 2. At low pH values, protein subunits tend to disassociate, which exposes hydrophobic moieties, leading to a high H_0 (Lam & Nickerson, 2013). However, this is not in agreement with the findings of Wolf & Sathe (1998) for the behavior of amandin in acidic pH. An alternate explanation may be a limitation of the method applied, as described by Alizadeh-Pasdar & Li-Chan (2000). The low H_0 and a high ZP provided to EAEP a low propensity for protein-protein interaction, which prevents proteins from aggregating, thereby decreasing surface tension and tending to increase solubility, when compared to the other extractions (AEP and hexane-AEP).

3.4 Protein functionality

3.4.1 Solubility

The effect of pH on the solubility of AEP, EAEP, and hexane-AEP freeze-dried skim fractions is shown in Figure 3. All three samples presented U-shaped curves, which is the typical profile found for the solubility of plant proteins as a function of pH. Usually, higher solubility can be observed at the extreme ends (acidic or basic pH values), and lower solubility is found when values close to the isoelectric point (between pH 4 and 5) are used, as can be observed in Figure 3. The highest solubility was found for EAEP at pH 8 (EAEP 95.3% vs. 87.1 and 86.4 for AEP and hexane-AEP, respectively) followed by pH 10 (EAEP 90.9%, vs. 84.9 and 87.5% AEP and hexane-AEP, respectively). EAEP also showed higher solubility at all pH range tested, except for pH 2 (66.2% vs. 84.0 and 81.4% for AEP and hexane-AEP, respectively). The lowest solubilities were found for AEP at pH 4 and 6 (28.2 and 30.9%, respectively).

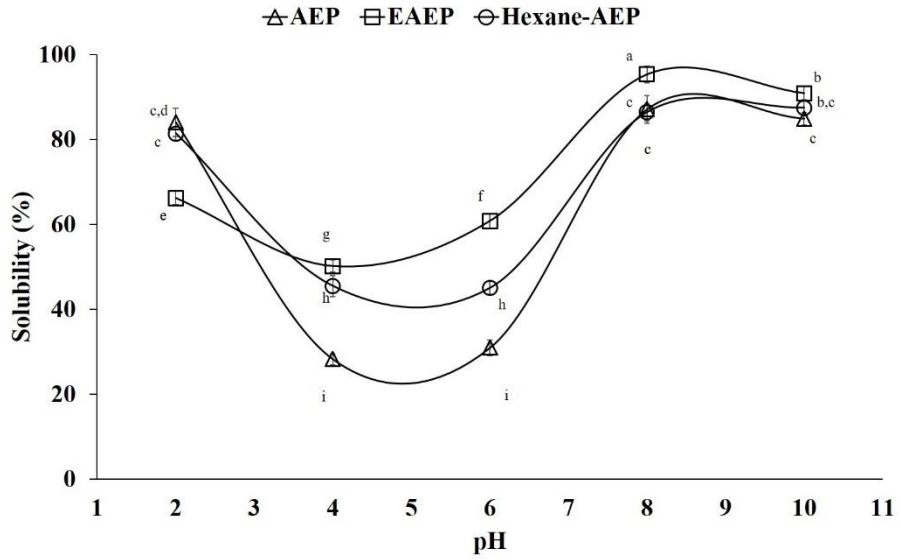

Figure 3. Protein solubility profile as a function of pH for the protein fractions of AEP, EAEP, and hexane-AEP extractions, from cold pressed almond cake. Different letters indicate significant difference ($p < 0,05$) among the different extractions. Data represent the mean ± one standard deviation of triplicates.

An essential factor to be considered regarding the solubility of a protein is the pH of the solution. This parameter affects the electrostatic and hydrophobic interactions among the proteins. EAEP presented the highest solubility at almost all the pH range tested, as was already expected. The enzymatic hydrolysis reduces the molecular weight and liberates ionizable groups from the proteins, which tend to raise their solubility (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). These results are also in accordance with the low H_0 and the high ZP negative charge found for EAEP, which point to greater solubility. The three extractions showed lower solubility at pH 4 and 6, and the explanation for this is the isoelectric point of the almond protein. At an equal net charge of particles (positive or negative), the repulsive forces are induced avoiding protein aggregation, but at neutral net charge (pI = net charge is zero), the repulsive forces are decreased, resulting in the aggregation and precipitation of suspended particles (Gerzhova et al., 2016). Overall, all the samples presented higher solubility at the extreme ends (pH 2, 8, and 10). These results are in agreement with most of the studies on the solubility of plant proteins. Amirshaghaghi et al. (2017) tested protein isolates from wild almonds extracted with NaOH and buffered saline borate and

observed the lowest solubility at pH 4 (7.5% and 3.2%) and the highest at alkaline pH (85.3 and 87.8%). De Almeida et al. (2014) evaluated soy protein after different treatments, in several pH values, and noted solubilities between 0.0 to 24.6% at pH 4 (close to soy proteins pI, 4.5) and 48.5 to 77.0% at alkaline pH (8 to 11).

3.4.2 Emulsification capacity (EC)

The effect of pH on the emulsification capacity (EC) and the emulsification activity index (EAI) of freeze-dried protein fractions of AEP, EAEP, and hexane-AEP extractions are shown in Figure 4. EC is measured by the volume of oil that can be emulsified by one gram of sample before the inversion of oil-in-water to a water-in-oil emulsion occurs (*i.e.*, inversion point) (Damodaran, Parkin, & Fennema, 2008). The profiles for the EC results were very similar to the U-shaped profiles found for solubility, for all three samples. EAEP showed higher ($p < 0.05$) EC at all pH range tested when compared to AEP and hexane-AEP samples, which presented no significant difference between them. The highest EC was found at pH 2, 8, and 10 (419, 430 and 430 g oil/ g protein) for EAEP. AEP and hexane-AEP presented the lowest EC values at pH 6 (225 and 260 g oil/g protein, respectively) followed by pH 4 (304 and 298 g oil/g protein, respectively).

EC is related to the protein's capacity to reduce the interfacial tension between water and oil (Wang & Zayas, 1992). For proteins with low surface charge, the protein-protein interaction dominates, and the proteins tend to associate in large aggregates, which leads to precipitation. Similar behavior is observed when the proteins show high surface hydrophobicity, where they tend to aggregate, via hydrophobic interactions, and also form large aggregates, which precipitate (Nickerson, 2010). At low H_0 and high ZP values, the occurrence of protein-protein interaction decreases, increasing solubility, and the greater the solubility, the greater the water-oil interaction (Tirgar et al., 2017). Overall, the EAEP protein fraction demonstrated significantly higher EC in comparison with AEP and hexane-AEP. EAEP presented the highest surface charge (ZP), highest solubility, and lowest surface hydrophobicity (H_0) among the samples. These results are in agreement with Karaca, Low, & Nickerson (2011), that studied emulsifying properties of chickpea, faba bean, lentil, and pea proteins isolates and observed a

positive correlation for EC and solubility and a negative correlation between EC and surface hydrophobicity. Similar results were also found by Tirgar et al. (2017), who evaluated flaxseed protein concentrates and observed that the enzymatic and alkali extracted proteins showed higher EC when compared to the enzymatic-solvent extraction.

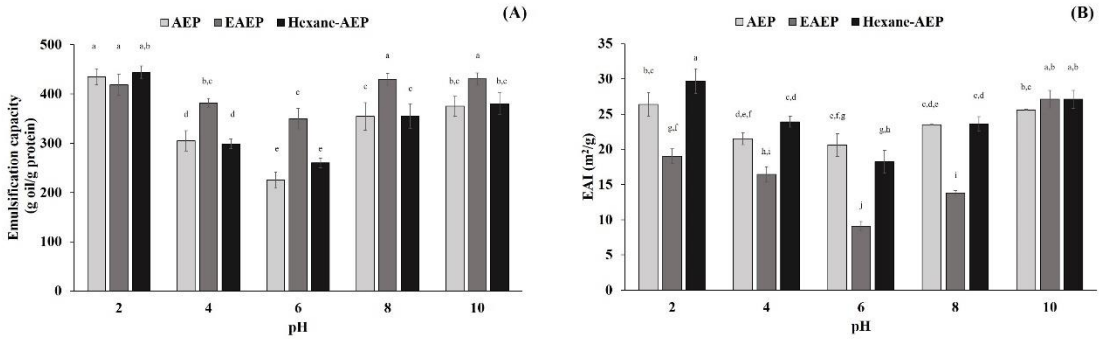

Figure 4. Emulsification properties for the protein fractions of AEP, EAEP, and hexane-AEP extractions, from cold-pressed almond cake as a function of pH. (A) Protein emulsification capacity (g oil/g protein). (B) Protein emulsification activity index (m^2/g). Different letters indicate significant difference ($p < 0,05$) among the different extractions and pH values. Data represent the mean ± one standard deviation of triplicates.

3.4.3 Emulsification activity index (EAI)

EAI represents an estimate, as measured by turbidimetry, of the interfacial area, which is stabilized per weight unit of protein, in a diluted solution, describing the ability of that protein to form an emulsion (Pearce & Kinsella, 1978). The three samples tested showed profiles similar to those for solubility and EC. When samples were compared to each other, however, their behavior was quite the opposite: hexane-AEP showed higher EAI and AEP, and EAEP presented lower EAI at all pH range tested. The highest EAI was found at pH 2 for hexane-AEP (29.7 m^2/g) and the lowest at pH 6 for EAEP (9.1 m^2/g) (Figure 4).

In this study, the samples with higher H_0 showed higher EAI values, which is in accordance with Polanco-Lugo et al. (2014) that also correlated higher EAI results to the increased hydrophobic surface at hydrolyzed lima bean protein isolate. Nickerson

(2010) evaluated cruciferin-rich protein isolates and found that EAI was positively correlated with surface hydrophobicity. These results were attributed to greater alignment and integration of the proteins into the oil-water interface, reducing the interfacial tension. Karaca et al. (2011), on the other hand, evaluated different non-hydrolyzed proteins (chickpea, faba bean, lentil, and pea) and observed an increase in EAI for proteins with higher surface charge, higher solubility, and lower surface hydrophobicity, as opposed to the present study. According to Tirgar et al. (2017), higher surface charges lead to more repulsive electrostatic forces and prevent coalescence in the emulsion. The use of hydrolysis leads to the formation of smaller droplets. Small peptides in an emulsion present a significative coalescence, which is considered an instability factor in the emulsion (Damodaran et al., 2008). Coalescence reduces the interfacial area, leading to a lower turbidimetry and, in consequence, to a lower EAI (Pearce & Kinsella, 1978).

In general, EAI was higher at extreme pH values (2 and 10). These pH values might induce the unfolding of the proteins, exposing some hydrophobic groups previously buried in their native conformation, which improves the adsorption of the protein at the oil/water interface, leading to higher EAI results (Zhu, Lin, Ramaswamy, Yu, & Zhang, 2017). EAI, however, did not positively correlate to solubility. This lack of correlation was also found by Zhao et al. (2012), who studied the functional properties of enzyme-hydrolyzed rice dreg proteins and suggested that smaller peptides (generated after hydrolysis), although more soluble, could not unfold and reorient at the interface due to charge repulsions.

EAEP showed the best results for EC but the worst for EAI. Until now, there is not a clear relationship between peptide size and emulsification properties (Zhao et al., 2012). However, regardless of the size of the peptide, its amphiphilicity plays an essential role in interfacial and emulsifying properties (Klompong, Benjakul, Kantachote, & Shahidi, 2007). According to the results, it can be observed that EC was favored by higher solubility and surface charge and by lower surface hydrophobicity, while EAI was favored mainly by higher H_0 but also by higher surface charge.

3.4.4 Foaming capacity (FC) and stability (FS)

Foaming capacity (FC) and foaming stability (FS) of AEP, EAEP, and hexane-AEP protein fractions as a function of pH are presented in Figure 5. Foaming capacity (FC) is evaluated by the amount of interfacial area that can be created by the protein. Foaming stability (FS) represents the ability of the protein to maintain the foam volume over a defined period of time against gravitational forces (Damodaran et al., 2008; Nickerson, 2010). Hexane-AEP showed the highest FC and FS among all samples. The highest FC was observed for hexane-AEP at pH 2 (~142%) followed by pH 4 (~95%) and the lowest for EAEP at pH 8 and 10 (4% for both pH values). The highest FS was found for hexane-AEP at pH 4 (~95%) followed by pH 2 (~58%) and the lowest for AEP at pH 8 and 10 (2%) and EAEP at the same pH (0%). Regarding the influence of the pH, for hexane-AEP, the FC results were directly related to the solubility results, while the FS results were inversely related. For the other samples, both results (FC and FS) behaved inversely to solubility.

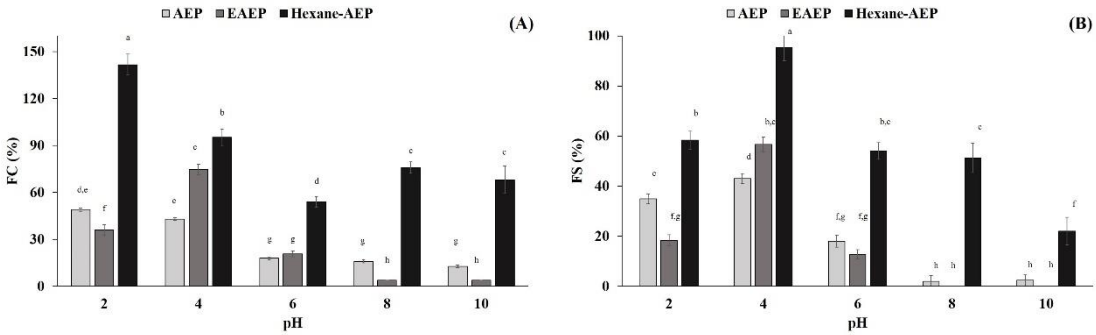

Figure 5. Foaming properties for the protein fraction of AEP, EAEP, and hexane-AEP extractions, from cold-pressed almond cake as a function of pH. (A) Protein foaming capacity (%). (B) Protein foaming stability (%). Different letters indicate significant difference ($p < 0,05$) among the different extractions and pH. Data represent the mean ± one standard deviation of triplicates.

Overall, hexane-AEP exhibited better foaming properties when compared with AEP and EAEP, regardless of the pH of the solution. The starting material for the hexane-AEP sample was exhaustively defatted prior to extraction, while AEP and EAEP still contained some lipid residue. The presence of lipids can significantly decrease protein foaming properties. Lipids are more active than proteins in the surface

and thereby adsorb to the air-water interface, promptly inhibiting protein adsorption (Damodaran et al., 2008). This leads to a decrease in the protein film thickness, which reduces the foam stability (Damodaran & Paraf, 1997). In addition to the absence of lipid, the protein in the hexane-AEP extraction may have undergone a partial denaturation, which may also have contributed to the superior results of this sample.

In general, for AEP and EAEP, FC and FS decreased as the pH increased. These results agree with Sze-Tao & Sathe (2000) that tested almond protein isolate at pH 5.0, 6.5 and 8.2 and obtained higher volumes of foam with lower pH. The results also agree with Klompong et al. (2007), that tested different degrees of hydrolysis in yellow-stripe trevally (*Selaroides leptolepis*) protein and observed that by increasing DH (%) and pH, there was a reduction in FC. EAEP extraction presented the lowest results for FC and FS. These results are related to EAEP high degree of hydrolysis ~23% (Figure 1). Foaming properties can be impaired by excessive hydrolysis, which results in the formation of peptides with low molecular weight and prevents the establishment of a cohesive film on the interface (Damodaran & Paraf, 1997; de Almeida et al., 2014).

According to ZP analysis (Figure 1), the isoelectric point (pI) of the protein from almond varied with the extraction method and was found near pH 4 for AEP and EAEP and near pH 5 for hexane-AEP. At pH values close to the pI, the lack of repulsion between the charges favors protein-protein interaction and facilitates the formation of a viscous film at the interface, which favors foaming properties (Damodaran et al., 2008). This behavior close to pI led to a higher FC (EAEP) and FS (AEP, EAEP, and hexane-AEP). Mita & Matsumoto (1978) studied wheat protein (gluten, gliadin, and glutenin); Kapchie, Towa, Hauck, & Murphy (2012) assessed soybean protein obtained by ultrafiltration and Polanco-Lugo et al. (2014) evaluated enzyme-hydrolyzed lima bean protein isolate. Regarding FS, all these studies found the highest foam stability near the pI of the tested proteins. These results indicate that FC and FS were more affected by the extraction method and its influence on the pI than by solubility or surface hydrophobicity.

Conclusions

The protein extraction yield was quite similar for AEP (68%), EAEP (70%) and hexane-AEP (63%), however, the functional properties of the protein fraction produced by each treatment were different, which might justify the use of either the AEP or EAEP extraction depending on the protein application sought. Overall, the skim fraction produced by the EAEP showed higher solubility and emulsification capacity at alkaline pH compared with the other extracted skim protein, likely due to the use of enzymes during the extraction process. However, EAEP skim proteins had the lowest emulsification activity and lowest foaming capacity and stability in comparison with AEP and hexane-AEP skim protein. This study showed that different extraction processes achieved different functional properties of the extracted protein, which indicates a good potential use in food systems depending on the specific functionality. Thus, based on the results achieved, is recommend when solubility and emulsification capacity are required, EAEP > AEP > hexane-AEP should be used and when foaming and emulsification activity are needed, hexane-AEP > AEP > EAEP. This study evidencing that possible to recover oil and protein from an oilseed byproduct environmentally and safely, avoiding hazardous solvents producing a better-quality final product. The implications of the different extraction processes regarding the skim biological protein properties still should be investigated.

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CAPÍTULO 6

Biological Properties of Almond Proteins Produced by Aqueous and Enzyme-Assisted Aqueous Extraction Processes from Almond Cake

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Abstract

The almond cake is a protein-rich residue generated by the mechanical expression of the almond oil. The effects of the aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) on the biological properties of the almond cake protein were evaluated. Total phenolic content (TPC), antioxidant capacity, inhibitory effects against crucial enzymes related to metabolic syndrome, antimicrobial potential, and *in vitro* protein digestibility profile were assessed. EAEP provided the best results for antioxidant capacity by both ORAC (397.2 μmol TE/g) and ABTS (650.5 μmol TE/g) methods and also showed a very high (98%) potential for α-glucosidase inhibition. AEP provided the highest lipase inhibition (70%) in a dose-dependent way. Enzymatic kinetic analyses revealed that EAEP generated uncompetitive inhibitors against α-glucosidase, while EAEP, AEP, and HEX-AEP (used as control) generated the same kind of inhibitors against lipase. No protein-rich fraction was effective against any of the bacteria strains tested at antimicrobial assays. An *in silico* theoretical hydrolysis of amandin subunits corroborated with the results found for the antioxidant capacity, the enzyme inhibitory experiments, and antimicrobial activity. After digestibility tests, the results indicated that the digestive proteases were efficient in hydrolyzing almond proteins, regardless of the extraction applied and that HEX-AEP presented the highest digestibility (85%). In summary, the skim obtained by EAEP and AEP showed potential

to be used as a nutraceutical ingredient, indicated to prevent the risk of developing metabolic syndrome: EAEP skim associated with diabetes and AEP skim with obesity.

Keywords: byproduct, environment-friendly extraction, bioactivities, α-glucosidase, lipase, digestibility

1. Introduction

The consumption of almonds (*Prunus dulcis*, syn. *Prunus amygdalus*) has been associated with various health benefits. Its antioxidant activity is usually attributed to the presence of α-tocopherols and polyphenols, with the latter being associated with reduced risk of metabolic syndrome - through the regulation of postprandial hyperglycemia and reducing the occurrence of diabetes *mellitus* type II - and potential antimicrobial effect[1]. Almond polyphenols are mainly found at the lipid interface and contribute to improving whole almonds shelf-life due to their antioxidant and antimicrobial activities[2]. In addition, almonds are considered a good source of protein, although methionine, lysine, and threonine are essential limiting amino acids. Almond's major storage protein is called amandin and represents about 70% of the total soluble proteins in the seed. Amandin is formed by two subunits: prunin-1 and prunin-2, which are composed of basic (20 - 22 kDa) and acidic (42 - 46 kDa) polypeptides[3].

Because almonds are a good source of lipids and proteins, prior removal of oil is needed to produce defatted protein fractions. The mechanical expression of almond oil generates a protein-rich cake which is commonly used as animal feed. While the composition of the cake is intrinsically related to the pressing conditions, which in turn dictates the extraction efficiency, oil and protein contents of 16 and 37% have been observed for the almond cake, respectively[4]. The residual oil in the protein-rich cake is commonly extracted by the use of hexane, a practice that has raised environmental, safety (flammability), and health (neurotoxic effects) concerns[5]. These concerns, along with increasingly restrictive regulations, have prompted the search for more environmentally friendly extraction approaches[6]. Aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) are environmentally friendly strategies that can replace the use of hexane and enable the simultaneous extraction of oil, protein, and

carbohydrate from many oil-bearing materials[7]. In addition, the EAEP offers the possibility of generating fractions with improved functionality and biological properties[8]. Research has shown that protein hydrolysis may generate bioactive peptide, improving the functional and biological properties of the original proteins[9]. Bioactive peptides have been associated with various biological properties such as antioxidant, antihypertensive, antimicrobial, antithrombotic, hypocholesterolemic and immunomodulatory functions[10]. Valorization of the almond cake, as many other food byproducts, could be achieved by the tailored extraction of its major constituents (oil, proteins, and bioactive compounds) for subsequent use in food, feed, fuel, and nutraceutical applications. These compounds may be used in the formulation of functional foods or to improve the nutritional characteristics of other food products while contributing to the reduction in food waste[11]. The search for bioactive compounds (i.e., bioactive peptides) in agricultural and industrial byproducts has been increasing, as they bear substances with properties of interest to the food and pharmaceutical industries[12].

The overall goal of this study was to evaluate the effects of the aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) on the biological properties of the skim fraction (protein-rich fraction) produced from the almond cake and from the hexane-defatted cake (HEX-AEP) as commonly performed by the oil industry. The specific objectives of this study were to evaluate the *in vitro* protein digestibility, the total phenolic content, and the bioactive properties (antioxidant capacity, inhibitory effect against α-glucosidase and pancreatic lipase, and antimicrobial activity) of the skim fractions generated from the AEP and EAEP of almond cake. These properties were compared with the skim fraction produced by the AEP of the hexane-defatted almond cake (used as a control). Therefore, the aim of this work was to use an environmentally friendly extraction processes to generate proteins with improved biological functions, ultimately leading to potential health benefits, from an underutilized food byproduct.

2. Materials and methods

2.1 Materials

Cold pressed almond cake was supplied by Blue Diamond (Sacramento, CA, USA). For EAEP, an endoprotease (FoodPro Alkaline Protease from *Bacillus licheniformis*; Danisco - NY, USA) was used. Hexane, Folin-Ciocalteu reagent, gallic acid, 1,1-diphenyl 2-picrylhydrazyl (DPPH), 2,20–Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulfate, Trolox, fluorescein, 2,2'-Azobis (2 methylptopionamidine) dihydrochloride (AAPH), α-glucosidase, p-nitrophenyl-α-D-glucopyranoside (p-NPG), lipase from porcine pancreas type II (EC 3.1.3), 4-nitrophenyl laurate (p-NP-Laurate) were purchased from Sigma-Aldrich (St. Louis, USA). The pathogenic strains tested were supplied by Fiocruz (Rio de Janeiro, Brazil).

2.2 Aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP)

Optimum extraction conditions identified by Souza et al.[4] were scaled up for the AEP (aqueous extraction process), EAEP (enzyme-assisted aqueous extraction process) and HEX-AEP (solvent aqueous extraction process). The almond cake (oil 16.25g.100g^{-1}, protein 37.20 g.100g^{-1}, and moisture 9.04 g.100g^{-1}) was dispersed in distilled water in a ratio of 1:12.8 (w/v) into a 10-L jacketed glass reactor (Chemglass, NJ, USA). For HEX-AEP, the almond cake was previously defatted with hexane in a Soxhlet device for 6 h at 68 ºC. Process conditions were: 50 ºC, pH 9.0, 120 rpm, reaction time - 2 h (AEP and HEX-AEP) or 1 h and addition of alkaline protease 0.85 g.100g^{-1} (EAEP). The slurry was centrifuged at 3,000 x *g* for 30 min at 25 ºC and the supernatant was allowed to cool down overnight at 4 ºC to separate skim from cream fraction when the latter was formed. Approximately 16 Kg of each skim fraction was freeze-dried (Lyophilizer - Virtis, CA, EUA) generating about 650 g of freeze-dried sample, as each extraction was done in duplicate.

2.3 Total phenolic content (TPC)

Samples of 1g of the freeze-dried skim were mixed with 10 mL of distilled water, ethanol (100%) or methanol (100%), stirred for 1 h and centrifuged (5000 x g/ 5 min)[11].

TPC was quantified by the Folin-Ciocalteu reagent method as modified by Singleton et al.[13]. Readings were performed in a microplate reader (FlexStation 3; Molecular Devices, CA, USA) and the absorbance was recorded at 750 nm. TPC was calculated from the equation of a standard curve of gallic acid ranging from 5 to 130 µg. mL^{-1} (R^2 = 0.9982, y = 0.0102x - 0.0215). TPC was expressed as mg of gallic acid equivalents (GAE) per 100 g of dry sample.

2.4 Antioxidant capacity

2.4.1 ABTS assay

ABTS assay was based on Ngoh and Gan[10] with slight modifications. Samples (10 µL), at concentrations ranging from 0.5 to 5 mg. mL^{-1} (in distilled water) were mixed with 190 µL of diluted ABTS reagent. Absorbance was recorded at 720 nm, in a microplate reader, at 37 °C, in triplicate. Antioxidant activity was calculated from the equation of a standard curve of Trolox ranging from 50 to 750 µM (R^2 = 0.979, y = -0.0004x - 0.0117). The antiradical activity was expressed as µMol of Trolox equivalent per g of sample.

2.4.2 ORAC assay

The ORAC method was performed according to Zulueta et al.[14]. Samples (80 µL) at concentrations ranging from 0.025 to 0.200 mg. mL^{-1} (in distilled water), PBS (blank) or Trolox (standard) were mixed with 80 µL of fluorescein (78 nM) followed by 40 µL of APPH (221 mM). The fluorescence was measured every minute for 90 min (excitation - 485 nm; emission - 535 nm). A calibration curve using Trolox solutions (10-120 µM) (R^2 = 0.9891, y = 62.444x + 734.12) was used and the antiradical activity was expressed as µMol of Trolox equivalent (TE) per g of sample using Equation 1.

$$\text{ORAC } (\mu\text{M TE}.\, g^{-1}) = \left(\frac{(AUCs - AUCb) - b/a}{C} \right) \qquad (1),$$

where AUCs and AUCb are the areas under the curves of the sample and blank, respectively; a is the intercept and b is the slope from the standard curve, and C is the sample concentration tested in the experiment.

2.5 Enzymatic inhibition

2.5.1 α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was evaluated according to Ibrahim et al.[15], with slight modifications. Samples diluted in water (50 µL) at final concentrations of 0.5 to 40 mg. mL^{-1} or ultrapure water (control) were incubated with 25 µL of 0.5 $U.mL^{-1}$ α-glucosidase solution in PBS (100 mM, pH 6.8), at 37 °C, for 1 h. After pre-incubation, 25 µL of p-NPG substrate solution (5 mM) in PBS (100 mM, pH 6.8) was added, and the mixture was incubated at 37 °C, for 30 min. One hundred microliters of glycine-NaOH buffer (pH 10.0) were added to stop the reaction. The absorbance was measured at 405 nm in a microplate reader. The inhibitory activity was expressed as a percentage of a control sample (without the inhibitors). The α-glucosidase inhibitory activity was calculated by using Equation 2.

$$\text{α-glucosidase inhibitory activity (\%)} = \left(1 - \frac{As}{Ac}\right) x\ 100 \qquad (2),$$

where As and Ac are absorbances of sample and control, respectively.

2.5.2 Pancreatic lipase inhibition assay

The pancreatic lipase inhibitory activity was evaluated based on the method described by McDougall et al.[16], adapted to a 96-well microplate. Samples (15 µL) at final concentrations of 5 to 10 mg. mL^{-1} or ultrapure water (control) were incubated with 60 µL of pancreatic lipase (10 mg. mL^{-1}) solution in Tris-HCl buffer (100 Mm, pH 8.2), at 37 °C, for 30 min. After pre-incubation, 135 µL of p-NP-laurate (2.5 mM, in 5 mM sodium acetate buffer, pH 5.0, with 1% Triton X-100) was added to start the reaction and was incubated at 37 °C, for 2 h. The absorbance was measured at 405 nm using a microplate reader. The inhibitory activity was expressed as a percentage of a control sample (without the inhibitors). The pancreatic lipase inhibitory activity was calculated by using Equation 3.

$$\text{pancreatic lipase inhibitory activity (\%)} = \left(1 - \frac{As}{Ac}\right) x\ 100 \qquad (3),$$

where As and Ac are absorbances of sample and control, respectively.

2.6 Kinetics of enzyme inhibition

To identify the type of inhibition exerted by the extracted proteins on α-glucosidase and pancreatic lipase, an enzyme inhibition kinetic experiment was performed according to Ibrahim et al.[15]. For the α-glucosidase and pancreatic lipase inhibition assays, a range of concentrations from 0.15 to 5.0 mM of p-NPG and 0.05 to 2.5 mM of p-NP-Laurate was used. Lineweaver-Burk plots were used to determine the kinetic constants, K_m (Michaelis constant) and V_{max} (maximum velocity).

2.7 *In silico* theoretical hydrolysis

In silico digestion of prunin-1 and prunin-2 sequences by the subtilisin enzyme (EC 3.4.21.62) was conducted using the enzymatic action tool incorporated into the BIOPEP platform[17]. Prunin-1 and prunin-2 sequences were taken from the UniProt platform (accession number Q43607 and E3SH29, respectively). The antioxidant peptides were evaluated by the BIOPEP biological activity database limiting the activities evaluated to antioxidants. For α-glucosidase and pancreatic lipase inhibitory peptide profiles, the criteria suggested by Ibrahim et al.[18] and Ngoh and Gan[10] was used.

2.8 Antimicrobial assay

For the antimicrobial assay, the gram-positive bacterial strains *Staphylococcus aureus* (NCQS 00402), *Bacillus cereus* (NCQS 00445) and *Listeria monocytogenes* (NCQS 00673) and the gram-negative bacteria strains *Escherichia coli* (NCQS 00595) and *Salmonella enterica* subsp. *enterica* (NCQS 00236). The bacterial strains were consecutively sub-cultured with 24 h intervals. After the activation, were made a bacterial suspension, which was adjusted to 0.5 McFarland scale.

2.8.1 Agar disk diffusion method

The antimicrobial effects were firstly determined by the agar disk diffusion method according to Balouiri et al.[19] and Kim et al.[20], with modifications. Filter paper discs were placed on the tryptic soy agar surface. On top of discs, 10 μL of the diluted sample in water (20 mg. mL^{-1}), the standard (amoxicillin, 2 mg. mL^{-1}) or the blank (saline solution) was added. Petri dishes were incubated for 24 h and observed. A clear

zone (halo) characterizes a positive result and no halo formed means negative result for bacterial growth inhibition.

2.8.2 Broth dilution method

The broth microdilution test according to Balouiri et al.[19] and Kim et al.[20] was performed with modifications. Serial two-fold micro dilutions were prepared in a 96-well microplate. In each well 50 μL of the sample (diluted in water) or the standard (amoxicillin) and the same volume of overnight bacterial suspension stains, in the range of 0.5 to 70 mg. FmL^{-1} (final concentration in well) was added. The microplates were incubated at 37 °C, for 24 h. Bacterial growth was estimated by absorbance readings at 660 nm.

2.9 Digestibility

2.9.1 *In vitro* protein digestibility

Protein digestibility was measured as described by Roman et al.[21] and Bornhorst and Singh[22]. The composition of the digestive solutions is presented in Table 1. Five grams of freeze-dried skims were mixed with 3.33 mL of SSF and vortexed. Subsequently, 6.66 mL of SGF was added. Afterward, the pH was adjusted to 3.0 and the samples were placed into a water-bath (37 °C, 140 rpm, 2 h). Then, 10 mL of SIF was added, and the pH was adjusted to 7.0. The samples were incubated into a water-bath at 37 °C, 140 rpm, for 2 h. To stop the digestion, samples were heated in a water bath at 85 °C for 3 min. TCA (12 $g.100g^{-1}$) was added in a 1:1 (v/v) proportion and the samples were centrifuged at 4,000 rpm for 30 min at 4 °C. Total nitrogen (NT) and nonprotein nitrogen (NPN) - soluble fraction after TCA (12 $g.100g^{-1}$) precipitation - were measured in the samples, by the Dumas method using a conversion factor of 5.18 (Vario MAX cube, HE, DE) before and after the digestion. In vitro protein digestibility was calculated by using Equation 4[23].

$$D\ (\%) = \frac{NPN_{after} - NPN_{before} - NPN_{enzymes\,(blank)}}{NT_{before} - NPN_{before}}\ x\ 100 \qquad (4),$$

where: NPN_{after} = protein after digestion, NPN_{before}= protein before digestion, NPN_{enzyme} = enzyme blank and NT_{before} = total protein before digestion.

Table 1. Composition of the simulated fluids

Simulated Saliva Fluid (SSF)	Final concentration (mg. mL^{-1})	Final pH
mucin	1.0	7.0
NaCl	0.117	
KCl	0.149	
$NaHCO_3$	2.1	
Simulated Gastric Fluid (SGF)	**Final concentration (mg. mL^{-1})**	**Final pH**
pepsin	0.75 (2000 U/mL)	1.8 - 2.0
gastric mucin	1.5	
NaCl	8.78	
Simulated Intestinal Fluid (SIF)	**Final concentration (mg. mL^{-1})**	**Final pH**
pancreatin	8.0 (800 U/mL)	7.0
bile extract	10.0	
$NaHCO_3$	16.8	

2.9.2 *In vitro* digestibility evaluation by SDS-page

SDS-PAGE was used to evaluate different stages during the digestion of skim samples. The protein profile was assessed as described by Laemmli[24] with few modifications. Thirty micrograms of protein were loaded onto the precast 12% acrylamide Criterion™ TGX Precast gel. A low range SDS-PAGE standard (14.4–97.4 kDa) was used as a molecular mass marker. The gel was imaged using a Gel Doc™ EZ Imager system and Image Lab software (Bio-Rad Laboratories, CA, USA).

2.10 Statistical analyses

The experiments were performed at least in triplicate, and the results were expressed as mean ± standard deviation (SD) of the replicates. Analyses of variance (ANOVA) were performed, followed by the Tukey test using Graph Pad Prism 5.0 (version 5.04, GraphPad Software, CA, USA). Associations between antioxidant

capacity and phenolic compounds were assessed by Pearson correlation. Significant differences were established at $p < 0.05$.

3. Results and discussion

3.1 Total phenolic content (TPC)

The effects of different extraction conditions on the content of phenolic compounds in the skims are shown in Fig 1a. It can be observed that EAEP generated a skim fraction with the highest TPC, regardless of the solvent used to extract the phenolics. Among the solvents evaluated, higher phenolic extraction was achieved when using water as a solvent. TPC in the skims ranged from 7.0 to 308.6 µg GAE/100g dry basis, depending on the solvent used and the sample extracted. Using water as a solvent, TPC of 308.6, 198.3, and 145.7 µg GAE per 100g dry sample were observed for the EAEP, AEP, and HEX-AEP skim fractions, respectively.

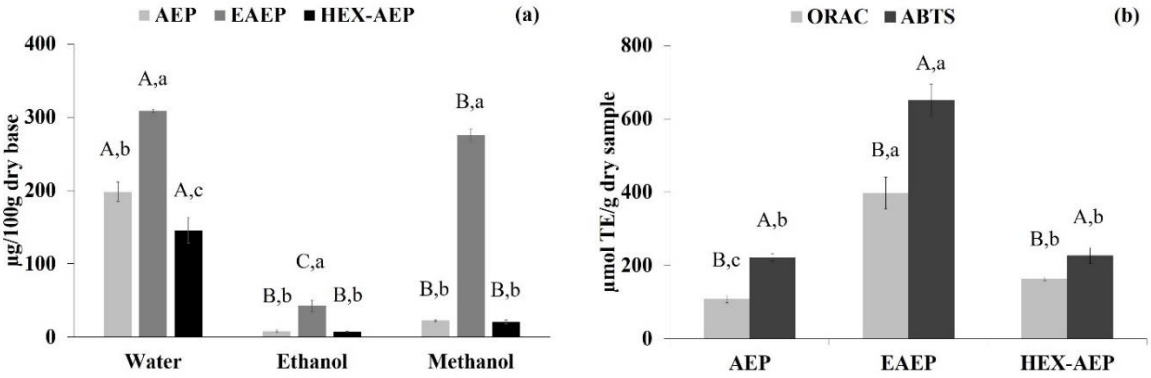

Figure 1. (a) Total phenolic content extracted by water, ethanol, and methanol. Different capital letters indicate significant difference ($p < 0.05$) among the solvents (water, ethanol, and methanol) for the same skim and different lowercase letters indicate significant difference ($p < 0.05$) between the different skims (AEP, EAEP, and HEX-AEP) within the same solvent; (b) Antioxidant capacity evaluated by the ORAC and by the ABTS methods. Different capital letters indicate a significant difference ($p < 0.05$) between the antioxidant activity analyses (ORAC and ABTS) for the same skim and different lowercase letters indicate a significant difference ($p < 0.05$) between the different skims for the same antioxidant activity evaluation method. Data represent the mean ± one standard deviation of triplicates.

The solvent polarity plays an important role in the recovery of polyphenols from different matrices, as it affects the solubility of the phenolic compounds[25] and, according to Abarca-Vargas et al.[26], water, ethanol, methanol, and their mixtures are the most frequently tested solvents for TPC extraction. In this study, TPC yield related to the solvent as follows: water > methanol > ethanol. The results suggested that the higher the polarity of the solvent, the higher the extractability (polarity Index: water = 9.0; methanol = 6.6 and ethanol = 5.2)[26]. A similar trend was also found by Gomaa[27], where higher TPC yields in bitter almonds and sweet apricots were achieved when using water as a solvent, compared to methanolic and ethanolic extracts. In a previous review regarding almond polyphenols, Bolling et al.[28] showed that the most abundant polyphenols were proanthocyanidins (epicatechin and catechin), hydrolyzable tannins (gallotannins, ellagitannins, and phlorotannins), and flavonoids (anthocyanidins, flavan-3-ols, flavonols, flavanones, and biflavone). Proanthocyanidins have also been identified as primary phenolic compounds in whole almonds by other authors[29]. Zam et al.[30] evaluated TPC at pomegranate's peel and observed that water presented the highest extraction yield of polyphenols in general and of proanthocyanins in particular. The high extractability can be related to the weakening of the hydrogen bonds in aqueous solutions, and the low solubility of these polyphenols in absolute organic solvents is strengthened by hydrogen bonds between polyphenols and proteins in these solvents[31]. This observation may explain the higher solubility of the phenolic compounds from the almond cake skim fraction in water than in the other solvents.

In addition to the solvent used, the use of enzyme or not during the extraction also influenced the total recovery of phenolic compounds from the skims. The EAEP skim showed the highest yields of TPC compared with the AEP, which is likely due to the effect of the protease action during the extraction. The phenolic compounds can bind to proteins and carbohydrates, therefore hydrolysis of these complexes by proteases might help the release of entrapped phenolics[32]. Pinelo et al.[32] studied the release of phenolic compounds from apple skin and observed that proteases favored their release. In the present study, the phenolic compounds that were inaccessible to the AEP and HEX-AEP skims were possibly released after the addition of the protease in the EAEP, enabling their solubilization into the solvent. Among the sample, HEX-AEP presented

the lowest content in water. This might be related to the previous fat removal of HEX-AEP, where the sample was exposed to a mild heat treatment at ~68 °C and extraction temperature above 65 °C may lead to phenolics degradation[33]. Our results are much lower than the values obtained by Bolling et al.[2] where concentrations from 88 to 159 mg GAE per 100 g dry basis (whole almond) were reported. According to Phenol-Explorer (www.phenol-explorer.eu)[28], the whole almond and the dehulled almond contain 287 and 61 mg GAE per 100 g, respectively, which is about 1000 times higher the TPC values herein presented. Reduced phenolic content in our samples might be in part due to the processing steps to which the samples were subjected to. Pasqualone et al.[34] and Smeriglio et al.[35] suggested that blanching the whole almonds before the peeling and crushing may be responsible for the leaching of a significant part of the phenolic compounds.

3.2 Antioxidant activity

The antioxidant capacity was assessed in the extracted skims using the ABTS and ORAC assays (Fig. 1b). EAEP showed the highest antioxidant capacity for both ORAC (397.2 µmol TE/g) and ABTS (650.5 µmol TE/g) methods. HEX-AEP (163.5 µmol TE/g) showed higher antioxidant capacity followed by AEP (108.7 µmol TE/g) by the ORAC method, while HEX-AEP (226.7 µmol TE/g) and AEP (221.7 µmol TE/g) showed no significant difference by the ABTS method. Although both methods followed the same trend and presented an extremely high (R^2 = 0.98) correlation coefficient by Pearson's evaluation, in general, the values found by the ABTS assay were higher than by the ORAC method. EAEP showed about 65% more antioxidant capacity than the other two samples when the ORAC method was applied, whereas this feature was 59% higher than the second-best sample (HEX-AEP) when the ABTS assay is considered.

Higher antioxidant capacity of EAEP skim can be in part explained by the highest TPC in this sample (Fig. 1a), as proanthocyanidins, already identified in whole almonds, are recognized for increasing antioxidant capacity[28,29] and, according to Pearson's correlation coefficient, ABTS (R^2 = 0.94) and ORAC (R^2 = 0.87) assays strongly correlated with the TPC for each sample. However, when comparing the results

obtained by Pasqualone et al.[34] and Smeriglio et al.[35], much higher TPC led to significantly lower antioxidant capacity. For instance, a sample with 703.03 mg GAE per 100 g presented 69.5226 µmol TE per g (ABTS) and 162.5940 µmol TE per g (ORAC)[35]. These results indicate that the presence of other components (i.e, proteins, peptides) can also contribute to their antioxidant capacity. Thus, another explanation for the higher activity in EAEP skim may be the formation of potential antioxidant peptides during the enzymatic extraction (Fig. 4). The protease used for EAEP processing might have hydrolyzed the proteins present in the almond cake and later in the skim fraction, generating peptides that might present antioxidant activities. In fact, the theoretical hydrolysis (Supplementary Table S1) of the most important storage protein of almonds (amandin) revealed the formation of 4 different antioxidant peptides (HL, IY, VY, PHW). Some studies have identified antioxidative peptides from several plant-based proteins, such as peanuts[8] and okara[36]. These studies correlated the antioxidant capacity with the degree of hydrolysis (DH %), whereas the higher DH %, the higher the antioxidant capacity. The DH % of the skims were evaluated in a previous study from our group and 23, 1.8, and 1.3% were achieved for the EAEP, AEP, and HEX-AEP skim fractions. The high DH % found for EAEP skim may have contributed to the higher antioxidant capacity observed, which is in agreement with the above-cited studies. AEP and HEX-AEP skims showed no significant differences for DH %, nor for TCP, when methanol and ethanol were applied, or for the antioxidant capacity measured by the ABTS assay.

To the extent of our knowledge, to date, no study has investigated the effects of extraction conditions on the antioxidant properties of the almond cake protein. Interestingly, the data from this study showed that extracting the protein from the almond cake by the EAEP can improve the antioxidant capacity of the extracted protein, as revealed by the ABTS and ORAC assay results.

3.3 α-glucosidase and pancreatic lipase inhibition

Preliminary tests evaluated potential inhibitory effects of the extracted skims against α-glucosidase (Fig. 2a) or pancreatic lipase (Fig. 2b) as well as the lowest concentration that exerted the highest inhibition. It seems evident that, among the

samples tested, only the EAEP skim presented α-glucosidase inhibitory activity (Fig. 2a). In addition to the concentrations evaluated in Fig. 2a, higher concentrations of AEP and HEX-AEP skims (10, 20, and 40 mg. mL^{-1}) were also evaluated but showed no activity. The EAEP skim, on the other hand, showed very high inhibition, even in the lowest concentration tested: 0.5 $mg.mL^{-1}$ inhibited 84.6% of the α-glucosidase activity while 2.0 mg. mL^{-1} inhibited 97.8% of the α-glucosidase activity, with no significant improvement for higher concentrations (5.0 mg. mL^{-1} - 98.9%). Unlike α-glucosidase, the pancreatic lipase was inhibited by all the samples tested (Fig. 2b). AEP showed the highest dose-dependent inhibitory activity followed by HEX-AEP skim, which showed little over half of AEP's inhibitory activity. EAEP skims showed the lowest inhibition of the pancreatic lipase, which remained unchanged ($p > 0.05$) regardless of the concentration tested. A kinetic study was performed to verify the type of inhibition taking place in the skim fraction, applying the least concentration of each sample that provided the highest inhibition (Fig. 3).

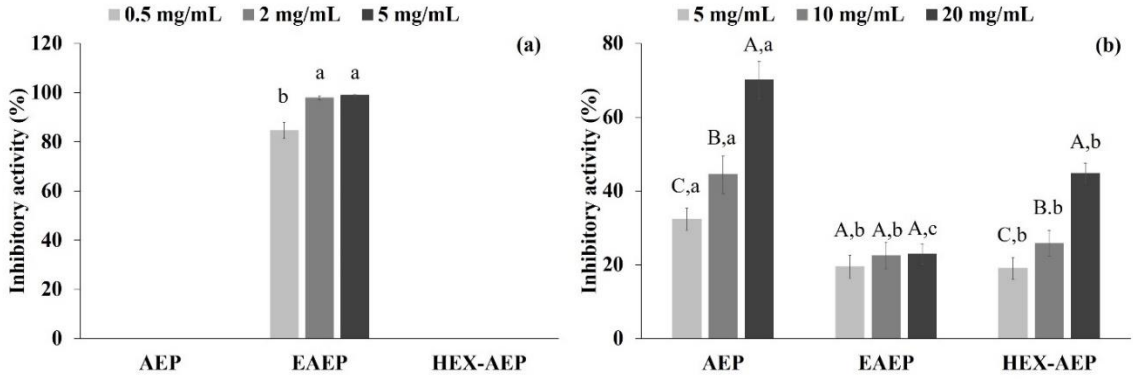

Figure 2. (a) α-glucosidase inhibitory activity of EAEP skim. Different letters indicate a significant difference ($p < 0.05$) among the concentrations evaluated. (b) Lipase inhibitory activity of AEP, EAEP, and HEX-AEP skims. Different capital letters indicate a significant difference ($p < 0.05$) among the concentrations evaluated (5, 10 and 20 mg. mL^{-1}) for the same skim and different lowercase letters indicate a significant difference ($p < 0.05$) among AEP, EAEP, and HEX-AEP skims for the same concentration. Data represent the mean ± one standard deviation of triplicates.

The enzymes tested in this study - α-glucosidase and pancreatic lipase - are a part of the digestion of carbohydrates and triacylglycerols in the digestive tract[37]. Alpha-

glucosidase is involved in the digestion of starch, catalyzing the hydrolysis of oligo- and disaccharides to release free molecules of glucose[9,15]. Therefore inhibitors of α-glucosidase aid to control postprandial hyperglycemia by modulating the digestion rate of complex carbohydrates such as starch and may be employed in the prevention of diabetes and other associated diseases[15].

In this study, 2 mg. mL^{-1} EAEP skim inhibited ~98% of the α-glucosidase activity, while AEP and HEX-AEP skims did not present an inhibitory effect. These results may be explained by the generation of bioactive peptides, through the alkaline protease activity in the enzymatic extraction (EAEP) compared with the aqueous extraction process (AEP nor HEX-AEP) where no enzyme was used during the extraction. Bioactive peptides are encrypted within the primary structure of proteins and may be released by proteolysis. These peptides may act as inhibitors of metabolic enzymes and present potential use as therapeutic agents against specific diseases. Ibrahim et al.[18] identified the structural and physicochemical requirements to design an active α-glucosidase inhibitory peptide. According to their findings, α-glucosidase inhibitory activity is related to short peptides, with 3 to 6 amino acid residues, containing a hydroxyl or basic side-chain amino acid at the N-terminus and a proline residue closer to the C-terminal with methionine or alanine occupying the final C-terminal position. The theoretical hydrolysis of amandin subunits (Supplementary Table S2) showed the formation of 13 different peptides with at least one of the above characteristics. It is therefore possible that, after proteolysis, some bioactive peptides were released from the almond cake proteins, leading to the high inhibitory activity of EAEP against α-glucosidase. Awosika and Aluko[9] reported that 20 $mg.mL^{-1}$ of yellow field pea peptides inhibited up to 53.3% α-glucosidase activity and Oseguera-Toledo et al.[38] evaluated peptide fractions from pinto Durango beans and observed inhibitory activity of 76.4% against α-glucosidase. This study is, however, the first report of such activity for almond protein hydrolysates from the almond cake.

Pancreatic lipase is responsible for the digestion and absorption of dietary fat through the hydrolysis of the triacylglycerols to glycerol and fatty acids[9,37]. The inhibition of pancreatic lipase prevents the breakdown of dietary fat into fatty acids, therefore reducing their absorption in the gut, which may be a viable approach to the

control of hyperlipidemia and obesity[37]. Some *in vitro* studies reported plants with a high concentration of fat or high content of tannins as pancreatic lipase inhibitors[16,37]. Proanthocyanidins, already reported in almonds[29], are among the primary active tannins and their activity was attributed to their ability to bind proteins, leading to the complexation and precipitation of the enzymes[39]. The same authors, however, claim that the mechanism of polyphenolic compounds on pancreatic lipase inhibition remains unclear. In the present study, all skim fractions showed some degree of lipase inhibition: AEP skim exhibited the highest inhibitory percentage, followed by HEX-AEP and EAEP skims. The TPC in this same samples did not follow this trend; on the contrary, EAEP skim showed the highest TPC of all samples (Fig. 1a). Thus, it is unlikely that the inhibition activity will be related to the TPC content.

Information about lipase inhibition by proteins or peptides is scarce in the literature, and there is even less information on pancreatic lipase activity inhibition by food protein-derived peptides[9]. However, a few recent studies reported peptides as pancreatic lipase inhibitors: Ngoh and Gan[10] found bioactive peptides in pinto beans that inhibited the lipase activity in a range between 23 to 87% and Stefanucc et al.[40], who discovered novel tripeptides as lipase inhibitors, observed inhibitions from 50 to 100 mg Orlistat (i.e., standard drug to treat obesity) equivalent per g of sample, depending on the peptide sequences. Ngoh and Gan[10] verified, through docking analysis, that most of the amino acid residues of the peptides involved in pancreatic lipase inhibition were hydrophobic amino acids such as proline (P), leucine (L), glycine (G), phenylalanine (F), alanine (A) and methionine (M). The theoretical hydrolysis of amandin (Supplementary Table S3) showed the formation of 17 highly hydrophobic peptides, due to the presence of these amino acid residues. Awosika and Aluko[9] evaluated yellow field pea protein hydrolysates and observed that trypsin and alcalase generated peptides with higher lipase inhibition capacity than chymotrypsin and pepsin. Alcalase is subtilisin similar to the alkaline protease used in the EAEP. Although EAEP skim showed the highest TPC and peptide content, this sample was not the most efficient for lipase inhibition. This inefficiency may have occurred due to the high degree of hydrolysis (DH = 23%) of the EAEP skim, which possibly generated peptides with a different profile from those naturally present in the AEP and HEX-AEP skims

(Fig. 4). These results indicate that the AEP skim is a good candidate for use as a pancreatic lipase inhibitor, while the EAEP skim could be used as a highly efficient α-glucosidase inhibitor.

3.4 Kinetics of enzyme inhibition

Kinetics experiments were performed to reveal the mechanisms of action of the different skim samples on α-glucosidase and pancreatic lipase inhibition, applying the least concentration of each sample that provided the highest inhibition (Fig. 3): for α-glucosidase, 2 $mg.mL^{-1}$ of the EAEP skim was applied; for lipase 20 $mg.mL^{-1}$ of the AEP or of HEX-AEP skims and 5 $mg.mL^{-1}$ of EAEP skim were tested. Lineweaver-Burk plots revealed that EAEP skim behaved as an uncompetitive inhibitor for both, α-glucosidase and pancreatic lipase (Table 2). Likewise, AEP and HEX-AEP skims also behaved as uncompetitive inhibitors of pancreatic lipase (Table 2). The results showed that the lines (control vs. inhibitors) intersected at both y-axis and x-axis, at different points, in the Lineweaver-Burk plots (Fig. 3). This indicates that, in the presence of the inhibitor, both the Michaelis constant (Km) and the maximum velocity (Vmax) decreased when compared to the control. These results were confirmed by calculating the apparent Km and Vmax (Table 2). Inhibition of pancreatic lipase by AEP and HEX-AEP, however, showed inhibition graphs that cross before reaching the y-axis. This kind of behavior was related by Park et al.[41] to competitive inhibition, although without the corroboration of Km or Vmax values.

Table 2. Apparent Km (Michaelis constant) and Vmax (maximum velocity) and mode of inhibition of α-glucosidase and pancreatic lipase

α-glucosidase			
	Km	Vmax	Mode of inhibition
Control	8.67 ± 6.58 [a]	2.81 ± 1.45 [a]	-
EAEP skim (2 mg. mL^{-1})	0.17 ± 0.02 [b]	0.02 ± 0.004 [b]	Uncompetitive
Lipase			
	Km	Vmax	Mode of inhibition
Control	0.24 ± 0.01 [a]	0.68 ± 0.01 [a]	-

AEP skim (20 mg. mL^{-1})	0.17 ± 0.01 ^{b}	0.62 ± 0.02 ^{b}	Uncompetitive
EAEP skim (5 mg. mL^{-1})	0.19 ± 0.02 ^{b}	0.59 ± 0.02 ^{b}	Uncompetitive
HEX-AEP skim (20 mg. mL^{-1})	0.19 ± 0.01 ^{b}	0.61 ± 0.02 ^{b}	Uncompetitive

Different letters indicate a significant difference ($p < 0.05$) among the different samples and control for Km or Vmax. Data represent the mean ± standard deviation of triplicates.

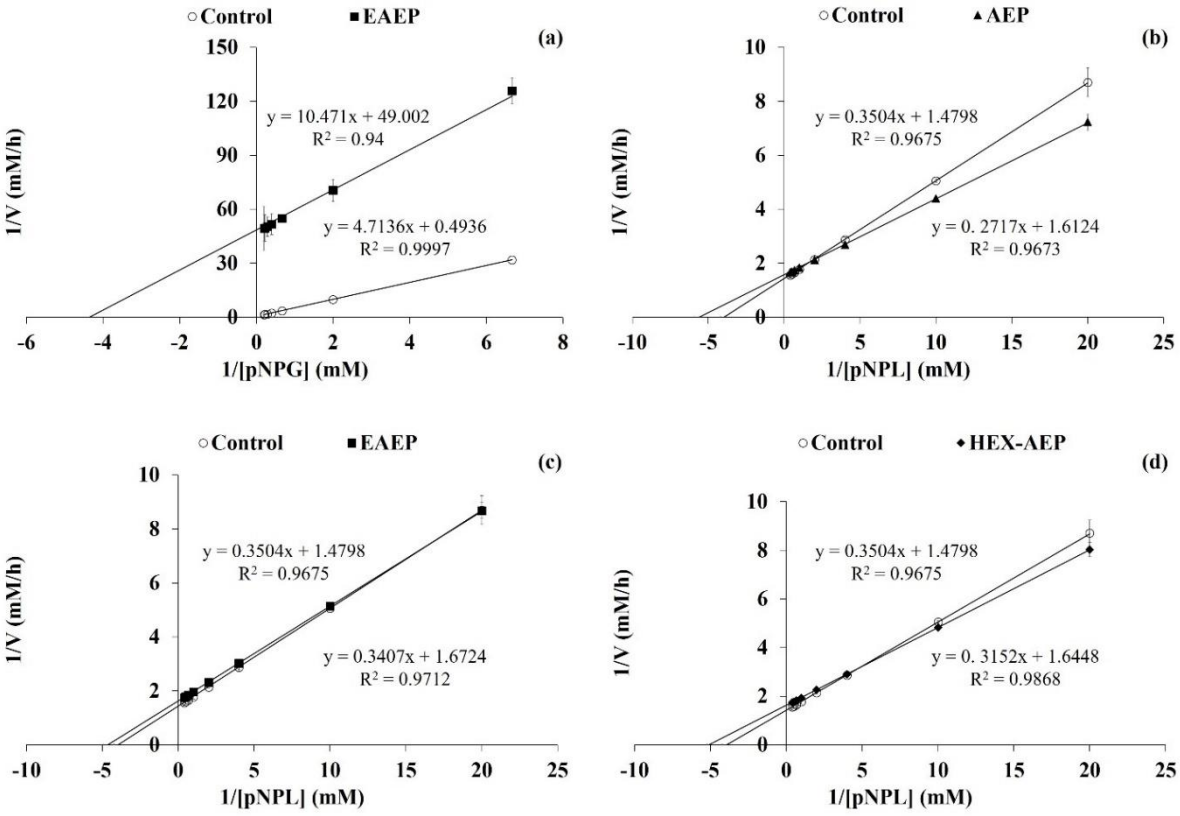

Figure 3. (a) Lineweaver-Burk plots of α-glucosidase inhibition for control (no inhibitor) and EAEP skim (2 mg. mL^{-1}); (b) Lineweaver-Burk plots of lipase inhibition for control (no inhibitor) and AEP (20 mg. mL^{-1}); (c) EAEP (5 mg. mL^{-1}) and HEX-AEP (20 mg. mL^{-1}) skims.

For uncompetitive inhibition, the inhibitor binds the enzyme-substrate complex in an allosteric site and has no substrate-like structure. The uncompetitive inhibitor causes a structural distortion of the active and allosteric sites of the complexed enzyme, which prevents the catalysis to occur and results in a decrease in Km and Vmax values[42].

According to Leskovac[43], uncompetitive inhibition works better in the presence of high concentrations of substrate, a condition likely to occur in the digestive tract during digestion. EAEP skim inhibition of α-glucosidase produced a Km over 50 times lower and a Vmax around 140 times lower than the control. These results indicate that the peptides generated by the proteolysis of almond protein showed a strong ability to bind to the α-glucosidase-substrate complex. Thereby, EAEP skim may be an excellent pool of peptides to delay the breakdown of dietary carbohydrates and consequently reduce the rate of glucose release at the small intestine[15]. Pancreatic lipase inhibition at AEP showed Vmax approximately 10% lower than the control and Km up to 40% lower than the control, showing a weak binding between inhibitors and enzyme-substrate complex.

The uncompetitive inhibition of α-glucosidase by peptides was also observed by Ibrahim et al[15], who identified two active α-glucosidase inhibitory peptides - SVPA and SEPA. SVPA acted as an uncompetitive inhibitor and SEPA was a non-competitive inhibitor against α-glucosidase. In contrast, Awosika and Aluko[9] studied yellow field pea protein-derived peptides and found a non-competitive inhibition of α-glucosidase. The uncompetitive inhibition is considered as a rare type of inhibition that may occur in multimeric enzymes[42]. To the best of our knowledge, the first study on inhibition of pancreatic lipase by food-derived bioactive peptides was published by Ngoh and Gan[10] and dealt with bean protein hydrolysates. Therefore, there is scant information relating peptides and pancreatic lipase inhibition, especially on the enzyme kinetics. Most of the studies at lipase kinetics are related to phenolic compounds, such as the study from, Park et al.[41] who identified a non-competitive or competitive inhibition against lipase at flavonol-3-O-glycosides and flavonol aglycones in *Polygonum aviculare* L., respectively.

Overall, the EAEP skim presented a high inhibitory activity against α-glucosidase and AEP, EAEP, and HEX-AEP skims showed varying degrees of inhibitory activity against lipase. Unlike the α-glucosidase inhibitory effect, AEP skim showed higher inhibitory activity against pancreatic lipase compared with EAEP skim. However, it must be taken into account that the concentration of the EAEP skim (5 mg. mL^{-1}) was 4 times lower than the active concentration of the AEP skim (20 mg. mL^{-1}). The lower concentration needed by the EAEP skim can be addressed to its chemical reaction order.

EAEP skim presented a zero-order behavior, which means a constant rate of inhibition, independent of the concentration tested. The zero-order kinetics occurs at the limit where the enzyme is saturated with substrate and an increase in the initial substrate concentration will have no effect on the rate of reaction, as no free enzyme is available[43]. The result indicates that the protein fraction extracted from the almond cake by the EAEP has the potential to be used as a source of bioactive protein/peptides to control hyperglycemia and obesity. EAEP skim can retard the release of glucose from complex dietary carbohydrates and could thus be subsequently evaluated regarding potential hyperglycemia reductions. All skim samples may partially suppress and delay the triacylglycerol digestion and consequently help to control hyperlipidemia and obesity[15,37].

3.5 Antimicrobial activity

The antimicrobial potency of the extracts against five bacteria strains was evaluated by agar disk-diffusion, which measures the formation of a halo, known as the zone of inhibition, and also by broth microdilution, that indicates the minimum inhibitory concentration (MIC). The samples were tested by agar disk-diffusion at a concentration of 20 mg. mL^{-1}. None of the skim fractions showed inhibition by this method, as they did not cause the formation of inhibition zones (halos). Although the positive control (amoxicillin) did. To eliminate the hypothesis that the concentration tested was too low to exert some antimicrobial activity, all samples were tested against the same bacterial strains at the concentration of 70 mg. mL^{-1}, by the broth dilution method. Nevertheless, the samples did not exhibit antimicrobial activity against the five strains tested. The theoretical *in silico* hydrolysis and BIOPEP search did not reveal any antibacterial, antiviral or antifungal peptides, corroborating the *in vitro* assays.

Different extracts (water, methanol, and ethanol) of bitter almonds were tested against human pathogenic bacteria by Gomaa[27]. The authors observed inhibition activity against only 4 of the 11 microorganisms tested. They attributed the significant inhibitory effect to the phenolic compounds in the extracts. The antimicrobial activity of almond skin[35], almond oil[44], and cold press edible oil byproduct[11] has already been tested. According to the literature, a plant extract depends on which part of the plant

was evaluated, the method and solvent used for extraction and finally the concentration tested to be a potent antimicrobial extract[11].

3.6 In vitro protein digestibility

The profile of each skim protein fraction during oral, gastric, and intestinal digestion was evaluated by SDS-PAGE (Fig. 4). SDS-PAGE showed similar initial protein profiles for AEP and HEX-AEP skims, presenting proteins with a molecular mass between 21.5 and 45 kDa, although some low molecular weight protein bands may be also observed (Fig. 4 - Dig 01). On the other hand, for the EAEP skim, a concentration of low molecular mass proteins and peptides at 14.4 kDa and below became evident. Dig 02 (Fig. 4) shows the profile for samples that received salivary solution (SSF) plus gastric solution (SGF), immediately after addition, and Dig 03 (Fig. 4) shows the same samples after 2 hours of reaction. Overall, there was little difference in the same sample, between Dig 02 and Dig 03, indicating fast digestion of the extracted almond cake proteins by pepsin. In a previous study regarding almond protein digestibility[45], pepsin was able to hydrolyze the proteins in 5 min, indicating rapid hydrolysis capability. Although EAEP showed initially an already digested profile due to the partial hydrolysis caused by the use of protease during the extraction, the addition of pepsin seemed to reduce the peptide cluster at the bottom of the gel - below 14.4 kDa, indicating further digestion and formation of lower mass peptides. Dig 04 (Fig. 4) presents the profile of the samples containing salivary solution (SSF), gastric solution (SGF) and also the intestinal solution (SIF), after another 2 h reaction. At this stage, all samples showed similar profiles, with the higher molecular mass bands probably derived from the different proteins in the pancreatin solution added. The results indicate that both pepsin and pancreatic proteases were efficient in hydrolyzing almond cake proteins independent of the extraction method.

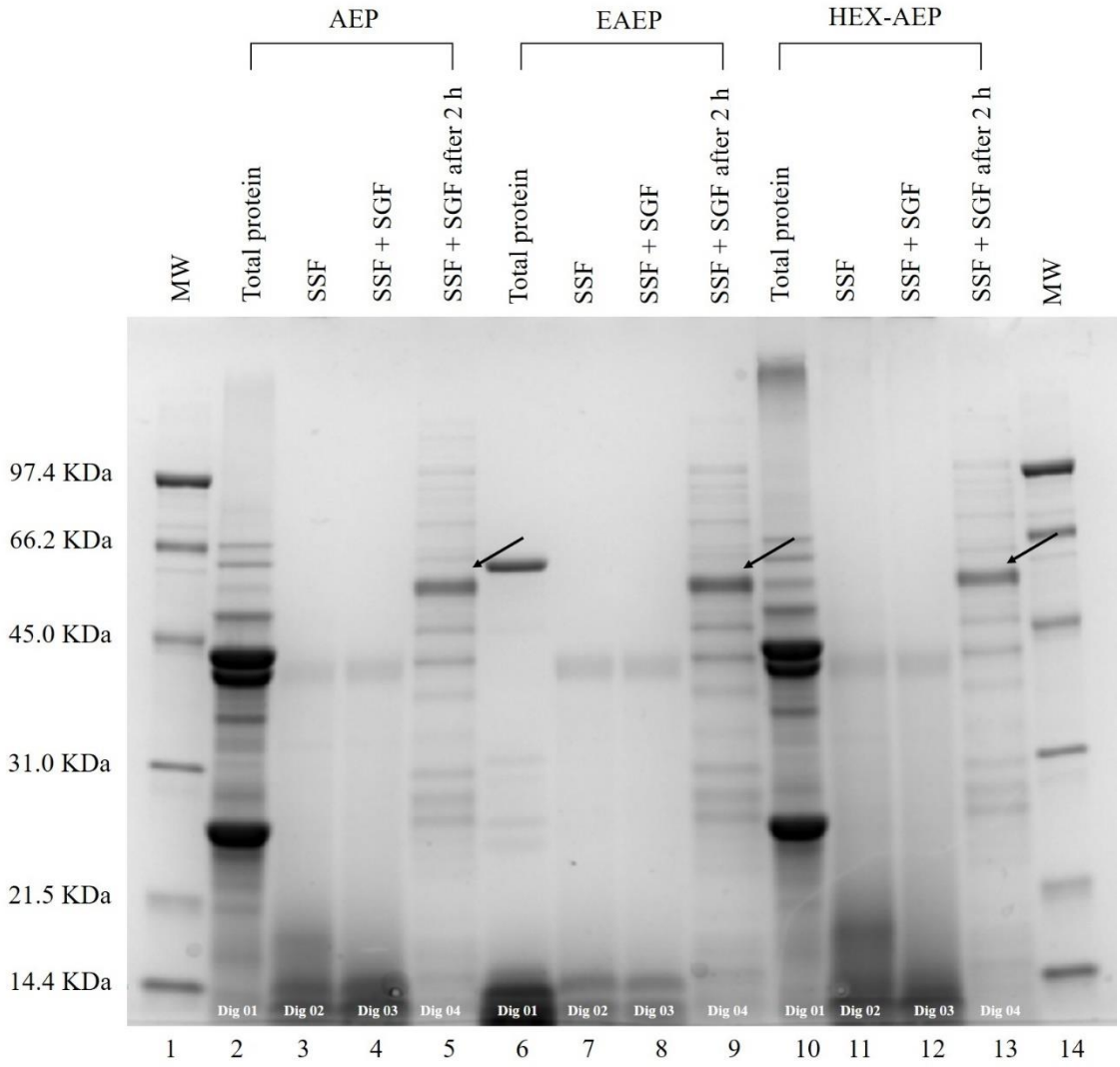

Figure 4. SDS-PAGE (12% Criterion^{TM} TGX Precast Gels) analysis of the different stages of in vitro digestion of EAEP, AEP, and HEX-AEP: lane 1) Molecular mass marker standards (14.4-97.4 kDa); lane 2) AEP total protein; lane 3) AEP plus SSF and SGF; lane 4) AEP plus SSF and SGF after 2 h reaction; lane 5) AEP plus SSF, SGF, and SIF after 2 h reaction; lane 6) EAEP total protein; lane 7) EAEP plus SSF and SGF; lane 8) EAEP plus SSF and SGF after 2 h reaction; lane 9) EAEP plus SSF, SGF, and SIF after 2 h reaction; lane 10) HEX-AEP total protein; lane 11) HEX-AEP plus SSF and SGF; lane 12) HEX-AEP plus SSF and SGF after 2 h reaction; lane 13) HEX-AEP plus SSF, SGF, and SIF after 2 h reaction; lane 14) MW marker standards (14.4-97.4 kDa). Arrows indicate the pancreatin (Dig 04) in each skim fraction.

Simulated digestion includes an oral, a gastric and an intestinal phase. These methods involve the use of digestive enzymes and their concentrations as well as the pH, digestion time, and salt concentrations to simulate physiological conditions *in vivo*[46]. Among the tested samples, the highest digestibility was found for the HEX-AEP (85%) skim, followed by the AEP skim (73%) and the EAEP skim (64%). The high digestibility regardless of the extraction is consistent with a previous True Protein Digestibility (% TPD) *in vivo* test from Ahrens et al.[47], who found digestibility from 82 to 92% depending on the varieties of the whole almonds. Moreover, Sathe[48] and Sze-Tao and Sathe[45] also reported high *in vitro* almond protein digestibility.

The high digestibility of HEX-AEP skim may be explained by the removal of the residual oil by hexane extraction (in a Soxhlet). During the hexane extraction, the almond cake was subjected to mild temperatures (68-70 °C) which might have resulted in protein denaturation. In some situations, some degree of protein denaturation might improve enzyme accessibility to the protein thus improving the digestibility[49]. Conversely, the EAEP skim presented the lowest digestibility despite its higher DH. Typically, digestibility increases with protein hydrolysis[50]. However, the extensive hydrolysis achieved by the EAEP (DH = 23%) entailed fewer attack sites available to the digestion enzymes (pepsin and pancreatin), which led to an underestimation of this parameter[50]. Similar results were found by Betancur-Ancona et al.[50], who reported higher digestibility in the raw materials (*Phaseolus lunatus*) than for the hydrolyzates produced.

Conclusions

The effects of the aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) on the biological properties of the AEP and EAEP skims were evaluated. The use of an enzyme to assist the extraction of the almond cake resulted in the production of a skim fraction with higher TPC and the presence of bioactive peptides associated with increased antioxidant capacity and inhibitory effects against α-glucosidases. The *in silico* theoretical hydrolysis indicated the presence of 4 antioxidant peptides, 13 peptides with structural requirements for α-glucosidase inhibition and 16 highly hydrophobic peptides, likely to inhibit pancreatic lipase. The AEP skim presented a

similar profile as the HEX-AEP skim regarding antioxidant capacity, TPC and digestibility, but provided the highest lipase inhibition potential, and therefore may be used in the prevention of obesity. Although further studies are required to characterize the active compounds and the mechanisms of action associated with the observed bioactivities, the present study demonstrated that the almond cake can be transformed into value-added health-promoting products by the application of environmentally friendly extraction processes.

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Supplementary Information for:

Biological properties of almond proteins produced by aqueous and enzyme-assisted aqueous extraction processes from almond cake

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Table S1. Summary of resultant antioxidant peptides from the major almond protein subunits after subtilisin digestion *in silico* using BIOPEP database

Amandin subunit	ID	Sequence	Location	Name	Function	Activity	MI Mass	Chemical mass
Prunin-1	3317	HL	[81-82]	*	Antioxidative	Antioxidative	268.1420	268.3020
	7873	IY	[92-93]	peptide from soybean protein isolates: beta-conglycinin and glycinin	Antioxidative	Antioxidative	294.1470	294.3300
	8037	PWH	[429-431]	synthetic peptide	*	Antioxidative	438.1890	438.4720
	8224	VY	[439-440]	antioxidative peptide	Free radical scavenging	Antioxidative	280.1310	280.3030
Prunin-2	3317	HL	[72-73]	*	Antioxidative	Antioxidative	268.1420	268.3020
	7873	IY	[83-84]	peptide from soybean protein isolates: beta-conglycinin and glycinin	Antioxidative	Antioxidative	294.1470	294.3300
	8224	VY	[386-387]	antioxidative peptide	Free radical scavenging	Antioxidative	280.1310	280.3030

H – histidine; L – leucin; I – isoleucine; Y – tyrosine; P – proline; W – tryptophan; V – valine. Digestion simulation was conducted with subtilisin in the BIOPEP database [1]

Table S2. Overview of the presence of specific amino-acids suggested as important requirement for the α-glucosidase inhibition activity

Amandin subunit	Sequencie	R, L, S, T or Y at N-terminus	A or M at C-terminus	P close to C-terminus
Prunin-1	QQRR**A**	no match	**A**	no match
	RAL	**R**	no match	no match
	RNGIY	**R**	no match	no match
	RQL	**R**	no match	no match
Prunin-2	IPQNH**A**	no match	**A**	no match
	RADF	**R**	no match	no match
	RAL	**R**	no match	no match
	RIS	**R**	no match	no match
	RPS	**R**	no match	no match
	RQHIF	**T**	no match	no match
	TNTL	**T**	no match	no match
	TPHW	**T**	no match	no match
	N**P**S	no match	no match	**P**
	R**P**S	no match	no match	**P**

A – alanine; M – methionine; P – proline; R – arginine; K – lysine; S – serine; T – threonine; Y – tyrosine. The criteria used was suggested by Ibrahim *et al.* [2], that proposed the structural requirements for α-glucosidase inhibition as tri – to hexapeptides with serine S, T, Y, L or A as the ultimate N-terminal residue and P preferably at the penultimate C- terminal position while A or M at ultimate C-terminal position.

Table S3. Overview of the presence of specific aminoacids suggested as important requirement for the lipase inhibition activity

Amandin subunit	Sequence	Hydrophobicity (%)	Hydrophobic residues
Prunine-1	V**A**I**PAG**	83.33	A, P, G
	M**A**K**AF**	80.00	A, F
	AQ**AL**	75.00	A, L
	R**AL**	66.67	A, L
	MAN**GL**	60.00	M, A, G, L
	N**AP**Q**L**	60.00	A, P, L
	GA	50.00	G, A
Prunine-2	**L**	100.00	L
	W	100.00	W
	AQ**AL**	75.00	A, L
	AR**L**	66.67	A, L
	P**AG**	66.67	A, G
	R**AL**	66.67	A, L
	VN**AP**Q**L**	66.67	A, P, L
	AG	50.00	A, G
	GA	50.00	A, G
	QN**AF**	50.00	A, F
	R**AD****F**	50.00	A, F

A – alanine; L – leucine; P – proline; F – phenylalanine; G – glycine; M – methionine; W – tryptophan. The criteria used was suggested by Ngoh et al. [3], that proposed the presence of hydrophobic residues as characteristic of a lipase inhibitor. To evaluate the hydrophobicity of the sequences, the platform: Peptide Hydrophobicity/Hydrophilicity Analysis Tool, was used.

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Conclusões gerais

Com este trabalho foi avaliado um subproduto da extração de óleo de amêndoa, rico em proteína e um residual de óleo, para adicionar valor ao resíduo, realizando extrações sustentáveis - utilizando as extrações aquosa e aquosa enzimática ao invés do hexano. As extrações AEP e EAEP foram otimizadas e 1:12.8 (p/v) foi a melhor proporção de sólidos e líquidos para as duas formas de extração; o tempo otimizado foi de 2 h e 1 h, respectivamente. O teor de enzima utilizado na EAEP foi de 0,85%. Após aumento de escala o rendimento de óleo diminuiu em cerca de 20% enquanto o rendimento de proteína se manteve constante.

Na fração lipídica (*cream*), EAEP foi melhor pois apresentou em torno de 99% de rendimento de óleo após a de-emulsificação química. A fração proteica (*skim*) sofreu hidrólise, o que gerou maior solubilidade em pH 8.0 (95%), bem como a maior capacidade de emulsificação, no mesmo pH (430 g óleo por g de proteína), mas menor atividade de emuksificação em pH 6.0 (9.1 m^2 por g de proteína) e baixa capacidade e estabilidade de formação de espuma em pH 10.0 (4 e 0%, respectivamente). Este método é então indicado para aumentar a solubilidade e reduzir a tensão interfacial entre água e óleo em produtos com pH básico, porém não é indicada para formação de emulsão ou espuma. Estes resultados podem ser explicados pelos altos valores absolutos de potencial zeta (-43.3 mV em pH 10.0); aos baixos valores de hidrofobicidade na superfície (312 em pH 10) e ao alto grau de hidrolise (~23%) da extração aquosa enzimática.

Na fração *skim* também formam testadas diferentes atividades biológicas, onde a proteína obtida por EAEP apresentou maior capacidade antioxidante tanto por ORAC (397,2 µmol TE por g de amostra seca) quanto por ABTS (650,5 µmol TE por g de amostra seca) em relação à AEP e HEX-AEP e também mostrou um alto potencial de inibição da α-glucosidase (98%), sendo esta fração mais indicada para ser utilizada como antioxidante além de mostrar um potencial para ser utilizada para prevenir a diabetes. Enquanto AEP proporcionou a maior inibição de lipase (70%) de forma dose dependente, podendo ser indicada como ingrediente nutracêutico, para prevenir o risco de desenvolver obesidade. Nenhuma fração proteica foi eficaz contra qualquer uma das

bactérias testadas nos ensaios antimicrobianos. Quanto a digestibilidade *in vitro*, os resultados indicaram que as proteases digestivas foram eficientes na hidrólise das proteínas de amêndoa, independentemente da extração aplicada.

Com isso, pode-se afirmar que além de gerar maior rendimento de óleo, apresentar maior solubilidade e capacidade de emulsificação, a extração assistida por enzima também pode ser utilizada como um ingrediente antidiabético, enquanto a extração aquosa é indicada para o uso como ingrediente antiobesidade.

Além disso, AEP e EAEP são métodos mais sustentáveis e geram produtos finais (óleo e proteína) de qualidade superior em relação à extração com hexano, que é um solvente tóxico. Desta forma o objetivo do estudo foi alcançado mostrando que o uso de processos alternativos, geraram frações proteicas com diferentes propriedades tecnológicas, transformando subprodutos em produtos de valor agregado de interesse econômico de forma mais ecológica.