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Development of Bio-guided Strategies to Simultaneously Extract Lipids, Proteins, and
Carbohydrates from Full-fat Chickpea Flour

By

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THESIS

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Abstract

Interest in plant-based food products has risen significantly due to strong health, environmental, and social arguments. To supply this demand, cost-effective and efficient extraction processes need to be developed for plant-based substrates. Being a high source of proteins, starch, and fiber, chickpeas can be subjected to sustainable processing strategies to generate added value compounds for food, feed, and fuel applications. To date, there is limited information about the impact of key extraction parameters on extraction efficiency and functionality of chickpea proteins. In addition, current chickpea research has been conducted primarily with the use of flammable/neurotoxic hexane to produce defatted chickpea flour for subsequent extraction of proteins, which has raised health, environmental, and consumer concerns. The goal of this study was to develop aqueous (AEP) and enzyme-assisted extraction processes (EAEP) without flammable solvents that rely on the use of mechanical treatments, water, and specific enzymes to improve the extractability and functionality of oil, proteins, and carbohydrates from chickpea flour. Enzyme-based processes were developed to efficiently extract lipids, proteins, and carbohydrates from chickpea flour, enhance the solubility and digestibility of the extracted protein, reduce flatulence-related oligosaccharides, and release a more diverse pool of oligosaccharides in the extracts. Because high water usage is a major drawback, a two-stage countercurrent extraction process was developed, resulting in the reduction of nearly 60% of the water used in the extraction process and increase in the overall process extractability. The results presented herein signifies a starting point for the development of a mechanistic understanding of the impact of key extraction parameters on structural protein and carbohydrate modifications that can impact key functional and biological properties of chickpea extracts.

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Chapter 1

Current Extraction Strategies of Chickpeas: A Literature Review

1. Introduction

Chickpeas are a source of proteins and carbohydrates used worldwide (Venkidasamy et al. 2019). They are a member of the pulse family, which are legumes that are high in proteins and carbohydrates but low in oil (Hall, Hillen, and Robinson 2017). Pulses can also include lentils, peas, and beans, unlike peanuts and soy which are high in fat content. Chickpeas and other pulses are targets of the plant-based food industry because their proteins are easily extractable and possess desired functional properties (Boye, Zare, and Pletch 2010). Pulse proteins can be used as supplements on their own or as a basis for plant-based substitutes (Shaabani et al. 2018). Recently, a considerable movement has gained traction to increase the development and commercialization of meat and dairy substitutes. To supply these needs, there is a drive to develop extraction methods and processes for plant materials and agricultural byproducts that can efficiently and economically feed the increasing demand. Existing chickpea research has led to new findings for protein quantification and functionality of full fat and defatted chickpea flour (Ladjal-Ettoumi et al. 2016). To date, most processes developed to extract chickpea proteins have made use of upstream removal of lipids by hexane (Ghribi, Maklouf Gafsi, et al. 2015; Sánchez-Vioque et al. 1999; Clemente et al. 1999; Boye, Zare, and Pletch 2010).

However, there is limited information on the impact of extraction conditions on processing extractability. Importantly, there is a critical knowledge gap about the impact of extraction conditions on the production of proteins with desired functional and biological properties. As a matter of fact, the establishment of a connection between extraction conditions and extraction yields and functionality has yet to be elucidated. To bridge this gap, the use of aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) to fractionate chickpeas into its ingredients (i.e., proteins, lipids, carbohydrates, fiber) is attractive as it enables the simultaneous extraction of

its constituents without upstream lipid removal by solvent extraction. The goal of this review is to highlight the existing knowledge of the impact of several extraction methods on the extractability and functionality of chickpea proteins. In addition, special emphasis will be given to the fundamental concepts involved in aqueous and enzyme-assisted aqueous extraction processes to provide significant background and guide future research. Although the focus of this review is on chickpeas, due to limited chickpea research availability, other pulses will be used as a case study when applicable.

2. Chickpea Macronutrient Composition

Chickpeas are composed of proteins, carbohydrates, lipids, and various micronutrients. These macronutrients can be extracted from whole chickpeas and their nutritional benefits could possibly be attained through the consumption of the extracted compounds. Chickpea protein accounts for 23-25% of the chickpea flour (Ghribi, Sila, et al. 2015). As a pulse, chickpea protein is highly coveted and its composition has been thoroughly researched (Wallace et al. 2016). Chickpea proteins are comprised of high amounts of globulins and lower amounts of glutenins, albumins, and prolamin (Chang et al. 2012). Identifying the protein classes in chickpeas can lead to a better understanding of the impact of extractions conditions on protein composition and characteristics such as, thermal and biological properties and functionality. Globulins are the main storage proteins of chickpea, being composed of two major groups: the 11S legumin (320– 400 kDa) and the 7S vicilin (145–190 kDa) proteins (Yust et al. 2003). Further investigation of globulins through SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) under reducing conditions and RP-HPLC (Reversed-phase High-performance Liquid Chromatography) revealed the presence of convicilins with molecular weight from 68 to 70 kDa, vicilins at 43 to 53

kDa, and acidic and basic legumins at 40 and 20 kDa, respectively (Tzitzikas et al. 2006; Romero et al. 1975; Boulter and Croy 1997). In addition, lectins, also known as phytohemagglutinins (PHA), appear in both globulin and albumin fractions and can be found at 27 to 37 kDa (Sathe 2002). Through the extraction process, soluble proteins can be drawn out of the matrix by water solubilization and proteolysis for improved extractability.

Carbohydrates are another component of chickpeas and represent 50-60% of chickpea flour (Ghribi, Sila, et al. 2015). Chickpea carbohydrates are composed of soluble and insoluble fractions. Soluble carbohydrates include simple mono- and disaccharides while starch accounts for 43.8% of chickpea flour (Dalgetty and Baik 2003). Moreover, the insoluble fraction includes fiber composed of cellulose and hemicellulose, oligosaccharides, and insoluble or resistant starch. The insoluble fraction has a reduced rate of digestion, which can lead to desirable metabolic changes such as lower blood glucose and insulin response after consumption (Fredriksson et al. 2000). Dalgetty and Baik 2003 also found that whole chickpeas have 10% insoluble dietary fiber and 8.4% soluble dietary fiber, while chickpea flour with hulls removed has 6.5 and 9.1%, respectively. A negative aspect of chickpea carbohydrates is that they contain flatulence causing oligosaccharides such as stachyose, raffinose, verbascose, and ciceritol (Sánchez-Mata et al. 1998). While these oligosaccharides can be considered prebiotics, the short-chain structure (maximum 4 monosaccharides linked together by alpha linkages) and the simplicity of the monosaccharide composition (only galactose, glucose, fructose, and sometimes xylose) render them easily fermentable by a variety of intestinal bacteria in a non-selective way that results in the production of undesirable gases, causing abdominal bloating and discomfort (Tosh and Yada 2010). Larger carbohydrates can be extracted with the use of specific carbohydrases, which can release possible

prebiotic oligosaccharides and improve protein and oil extractability by creating a more porous structure.

Crude fat or lipids in chickpeas can represent 5-10% of chickpea composition (Ghribi, Sila, et al. 2015). Compared to other pulses, chickpeas, like lupin, are higher in lipid content than a majority of other pulses, like black beans, kidney beans, lentils, and mung beans (Hall, Hillen, and Robinson 2017). Unlike other macronutrients such as proteins and carbohydrates, there is a significant lack of research in lipid extraction and recovery. Chickpea oil analysis has revealed a composition consisting of tocopherols, tocotrienols, and sterols (Zia-Ul-Haq et al. 2007). Tocopherols and tocotrienols found in chickpeas can have important antioxidant and biological properties (Gopala Krishna, Prabhakar, and Aitzetmüller 1997). The selection of the extraction method (i.e., solvent extraction, aqueous or enzyme-assisted extraction) might have a significant impact not only on the extractability of chickpea lipids but also on its composition and stability, which warrants further investigation.

3. State-of-the-art of Chickpea Extraction Strategies

Many methods have been used to extract and recover chickpea proteins. The most common method is to defat chickpea flour with hexane and then vigorously mix the defatted flour with alkaline water or buffer, which will be further described as the extracting medium. Subsequently, the slurry is centrifuged to fractionate the protein-rich extract from the insoluble fiber (Arcan and Yemenicioğlu 2010). During the extraction step, enzymes can be applied to break down large proteins and improve protein extraction yields (De Moura et al. 2008). When analyzing processing conditions for chickpeas, it can be observed that many studies involved the utilization of a solvent defatting step (Table 1). In addition, it is observed that most studies involved further recovery of

the extracted protein via isoelectric precipitation or ultrafiltration to conduct functional analysis

and to characterize and identify the amino acid composition of the extracted protein (Boye et al.

2010).

Table 1: Common extraction conditions for chickpea proteins

Purpose	pH	SLR	Time	Temperature	Defatting	Reference
Protein Functional properties	9	1:10	2 hours	25 °C	Yes	(Ghribi, Sila, et al. 2015)
Protein Characterization	8	1:10	45 minutes	20 °C	No	(Ladjal-Ettoumi et al. 2016)
Functional properties	9	1:15	N/A	35 °C	Yes	(Boye et al. 2010)
Characterization	11.5	1:10	1 hour	N/A	No	(Chang et al. 2012)
Identification	N/A	1:10	N/A	N/A	Yes	(Kou et al. 2013)
Protein Quality	7	1:10	1 hour	37 °C	Yes	(Clemente et al. 1998)
Functional Properties	9	1:10	45 minutes	N/A	Yes	(Papalamprou, Doxastakis, and Kiosseoglou 2010)
Characterization	9	1:20	1 hour	N/A	Yes	(Kaur and Singh 2007)
Functional properties and characterization	12	1:10	1 hour	N/A	Yes	(Sánchez-Vioque et al. 1999)
Rheology study	9.5	1:10	45 minutes	N/A	Yes	(Shaabani et al. 2018)
Amino acid composition	8.3	1:20	1 hour	N/A	Yes	(Singh et al. 1988)
Functional properties	10.5	1:10	1 hour	N/A	Yes	(Yust et al. 2010)

* SLR: Solids-to-Liquid Ratio

Defatting is a unit operation that is almost unanimous for all chickpea extractions described in Table 1. Complete removal of oil is preferred when conducting protein studies as it can affect their functional properties, especially solubility (Carbonaro et al. 1997). Defatting can be difficult and costly due to the necessity to dispose of flammable solvents. These solvents are harmful to people and the environment and are costly to purchase and use (De Moura et al. 2011). Because chickpea lipids have been primarily extracted by the use of flammable solvents, there is a complete lack of information about the impact of aqueous and enzyme-assisted aqueous extraction processes on lipid extractability, composition, and functionality. While chickpea oil may be uncommon in the market, increasing demand for extracting chickpea proteins will likely prompt the development of more environmentally friendly strategies to extract chickpea lipids. Doing an initial search for commercial chickpea oil products, there is currently only one commercial product available for purchasing online, highlighting the limited supply of chickpea lipids. The production of chickpea oil by sustainable processing strategies such as AEP and EAEP may be useful to provide a valuable revenue stream to benefit the economic evaluation of these processes, which would be desirable from an economic and environmental perspective.

3.1. Underlying Chickpea Protein Extraction Mechanisms and Impact of Extraction Parameters on Extraction Yields

Chickpea protein extraction is targeted specifically by the literature listed in Table 1. There is limited information about the impact of key extraction parameters (i.e., pH, SLR, time, temperature, use of enzymes) on the extractability of chickpea proteins and lipids. In addition to gaining a better understanding of the impact of processing conditions on extraction efficiency, their impact on the structure and functionality of the extracted protein and lipids has yet to be

elucidated to enable the development of bio-guided extraction processes to produce high added value chickpea ingredients. The effects of extraction parameters (pH, SLR, extraction time, temperature) on chickpea protein and oil extractability are described below.

3.1.1 pH

As described in Table 1, most chickpea extractions were performed at alkaline pH. It is worth mentioning that the isoelectric point (i.e., pH where protein solubility is reduced) of chickpea proteins is around 4.3 (Sánchez-Vioque et al. 1999). Therefore, the selection of pH values away from the isoelectric point is a common approach to maximize the extractability of chickpea proteins. At higher pH (i.e., more alkaline), cell wall degradation is favored, which often leads to improved extractability due to higher exposure of the intracellular components (Rosenthal, Pyle, and Niranjan 1998). In addition, protein solubility is favored at alkaline pH due to deprotonation of protein side chains, which leads to more opportunities for hydrogen bonding in a polar solvent and, consequently, to improved extraction of protein bodies (K. A. Campbell et al. 2011). If enzymes were to be used in the extraction process, the pH would have to be selected based on each enzyme requirement. Currently, there is no information regarding the effects of different pH values on proteins and lipid extractability from full fat-chickpea flour.

3.1.2 Solids-to-liquid ratio

When analyzing the solids-to-liquid ratio (SLR) used in the extraction of chickpeas, there is a definitive favoring of low SLR conditions (high amounts of water) to maximize extraction yields, with 1:10 being the most commonly used SLR. At low SLR (i.e., more diluted slurry), the solute gradient is higher, which favors the dissolution and diffusion of soluble proteins into the

extraction medium (Rosenthal, Pyle, and Niranjan 1998). In addition, convective mass transfer can increase in low SLR hypotonic conditions. While the underlying extraction mechanism of proteins relies on its solubilization into the extraction medium, lipid extractability is based on its insolubility into the extraction medium. Despite these differences, conditions that favor protein extractability, often favor oil extractability, but at different extents (De Moura, De Almeida, and Johnson 2009; Almeida et al. 2019; Souza et al. 2019; Rosenthal, Pyle, and Niranjan 1998). In addition to improving the extractability of proteins, the use of low SLR can also improve the extractability of lipids. The removal of proteins creates a more porous structure that favors the washing of lipids (Souza et al. 2019; de Queirós et al. 2021; Dias et al. 2020). Increasing SLR (reducing water use) from 1:10 to 1:5 revealed reduced extractability of lipids and proteins from soybeans (De Moura and Johnson 2009). Therefore, for achieving high extractability, extractions should be conducted at conditions that increase the extraction driving force (i.e., protein concentration gradient in the flour and extraction medium), as observed with the use of low SLR. However, the high water usage leads to the production of higher amounts of effluent that needs to be handled, centrifuged, and many times evaporated by spray-drying, thus increasing the use of energy-intense unit operations. Alternately, the use of countercurrent extraction strategies has been shown to increase extraction yields while decreasing water usage during the extraction of lipids and proteins from soybeans and coffee beans can (De Moura and Johnson 2009). None of the studies reported in Table 1 reported extraction yields of chickpea protein and lipids at the extraction conditions employed nor described the impact of SLR on extraction yields.

3.1.3 Extraction Time

Extraction time is another key extraction parameter as it determines the residence time of the substrate exposure to the extraction medium. Extraction time is a parameter that is different than others because it does not have a direct mechanism affecting protein and lipid extractability, whereas other parameters have a defined operational effect. However, sufficient extraction time allows for other extraction parameters to increase their impacts on extraction yields. With more exposure time, for example, enzymes that may be added to the extraction slurry can have more time breaking down structural components which in turn would favor the release of desirable proteins and lipids. Similarly, increased extraction time might favor protein solubilization into the extraction medium thus increasing its extractability. Table 1 reveals most chickpea flour extractions have been performed at 60 min. Shorter extraction times may cut down on energy usage and costs, however, protein extraction is usually favored by a longer exposure time of the solid matrix (i.e., chickpea flour) to the aqueous media during the extraction (Jiang et al. 2010). Reaction time determines exposure time, and a higher exposure, to a certain extent, should increase the release of soluble materials. However, increased extractability at the expense of a higher extraction time could diminish process returns and it should be carefully optimized with other extraction parameters. Reaction times can range between 15 minutes to 3 hours, the selection of which depending on the type of matrix used (type of flour, particle size, heat treatments, among other factors) (Neiva M De Almeida et al. 2019; Souza et al. 2019; K. A. Campbell et al. 2011). Ideal extraction times for protein and lipids are different, with proteins being extracted at faster rates than lipids due to their higher solubility into water. In that view, reaction time should be selected to maximize the extractability of both lipids and proteins. Oil and protein extraction yields from almond flour were best at intermediate reaction times (45 to 60 min), with no further increment in extractability being observed at longer reaction times. (Souza et al. 2019). For soybeans, increased

extractability of lipids and proteins was observed when reaction time increased from 0 to 20 min. Protein and oil extraction yields leveled off at 15 and 20 minutes of extraction time, respectively (Rosenthal, Pyle, and Niranjan 1998). The general trend is that extraction time can have a significant impact within a specific range. However, extraction times above the optimum value will have little to no effect on extraction yields. The optimum extraction time range for chickpea protein and lipid extractability has yet to be determined.

3.1.4 Temperature

Extraction temperature can also affect extraction yields in virtue of its effect on the viscosity of the extraction slurry, which can affect mass transfer extraction mechanisms. At higher temperatures, the reduced slurry viscosity and increased solvent capacity to solubilize the substrate favor both sample wettability and solvent penetration (Mandal, Mandal, and Das 2015) which in turn enhance protein and lipid extractability. Temperature ranging from 25 °C to 65 °C have been used to extract plant matrices, although the use of higher temperatures has led to mixed results including possible protein denaturation and lipid oxidation throughout the extraction (Boye, Zare, and Pletch 2010). Similar to pH, when performing enzyme-assisted extractions, temperature selection must consider the susceptibility of the enzyme selected. Compared to other extraction conditions, extraction temperature was the least common condition reported in the extraction methods used to produce chickpea proteins in Table 1. None of the references revealed protein or oil extraction yields at a specific reaction temperature. As described above, the extraction temperature is impactful as the sample viscosity decreases at higher temperatures, resulting in improved interaction of the substrate with the solute. Higher extraction temperatures are also beneficial when applying enzymatic treatments, but temperature selection needs to be optimized

to avoid enzyme or protein denaturation (Campbell et al. 2011). Of course, with the use of enzymes to assist the extraction, extraction temperature must be selected to fit the best activity range for that specific enzyme. The impact of reaction temperature on extractions yields has not been reported for chickpeas. However, a temperature of 50 °C has been commonly used for other plant-based materials resulting in high protein extraction yields from almond flour (70%), extruded soybean flakes (87%), and almond cake (78%) (Almeida et al. 2019; De Moura et al. 2008; Souza et al. 2019).

3.2 Chickpea Protein and Lipid Extraction Yields

While the scope of research has expanded significantly on chickpeas considering protein functionality and characterization, little has been done to determine the impact of processing conditions (upstream heat treatments before the extraction and extraction parameters) on protein and lipid extraction yields. All of the extraction parameters listed in Table 1 were described with respect to the functionality and profiling of proteins or carbohydrates. One study that uncovered protein recovery was Boye et al. 2010, which found 69.1 and 50.3% protein recovery from Kabuli chickpea by the use of isoelectric precipitation and ultrafiltration, respectively. However, initial protein extraction yields were not reported. Although the extraction parameters described in Table 1 have been used to maximize protein recovery to produce adequate amounts of proteins for functional and characterization studies, a good understanding of the impact of these key extraction parameters is needed to determine their impact on extraction yields, functional and biological properties of the extracted compounds.

Additionally, most of the extraction conditions reported in Table 1 were conducted at a small lab scale. It is of paramount importance to demonstrate the proof of concept of extractions

and separations of the fractions at pilot-scale to understand the potential translation of lab-scale to industrial-scale production. Although extraction processes must maximize the overall processing extractability, understanding the impact of extraction conditions on the functional and biological properties of the extracted compounds is imperative. The development of structure/function-based methods, where extractability and functionality are simultaneously considered and optimized, are needed to guide the development of efficient processes to produce compounds with desired properties.

4. Applications of AEP and EAEP for Chickpea Protein and Oil Extraction

The aqueous extraction process (AEP) is a technique that can eliminate the use of flammable solvents for lipid removal while allowing the simultaneous extraction of lipids and proteins from several matrices (Campbell and Glatz 2009). Similar to previously described methods, in the AEP, extraction is conducted by dispersing full-fat flour, instead of solvent defatted flour, into the aqueous medium at specific pH, extraction time, and agitation (De Moura et al. 2011). After the extraction, the resulting slurry is centrifuged into liquid and solid phases, then the liquid phase is then separated into the protein-rich skim, oil-rich cream, and free oil phases. This separation step takes the place of defatting while allowing for the calculation of yields of oil and protein in the different fractions through mass balancing when each fraction is analyzed for its protein and lipid content. Aqueous extraction methods have been conducted on numerous substrates including soy, almonds, and sunflower seeds (Almeida et al. 2019; Campbell et al. 2016). Overall, AEP has been proven to be effective to simultaneously extract lipids and proteins from several matrices, especially with the addition of a demulsification stage of the cream to free the extracted oil (Wu, Johnson, and Jung 2009). Because most of the AEP extracted oil is entrapped

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in the cream emulsion, instead of as free oil, chemical and enzymatic demulsification strategies have been successfully developed to recover the extracted oil (Dias et al. 2020; Souza et al. 2019; J. De Moura et al. 2008). While the underlying extraction and demulsification mechanisms involved in the AEP have been thoroughly investigated for other matrices (i.e., soy, almonds, coffee), there is limited information about the impact of the AEP with chickpea flour. The overall mechanisms to extract lipids and proteins from chickpeas are similar to those described in Sections 3.1.1 – 3.1.4. The aqueous media can solubilize and transport proteins to the exterior of a food matrix, creating a more porous structure that can facilitate the wash out of the oil droplets (M. H. Cheng et al. 2018; Dias et al. 2020).

The AEP can be further improved with the addition of enzymes (including proteases and carbohydrases) to assist the extraction (EAEP), which can increase the extractability of proteins, lipids, and carbohydrates. Other reaction parameters (i.e., pH, temperature) have a synergistic impact on extractability with the use of enzymes. The use of enzymes to assist the extraction of plant matrices has been shown to play an important role in improving the process extractability and the functional and biological properties of the extracted compounds (de Souza et al. 2020; Dias et al. 2020). Therefore, it is of critical importance to select enzymes based on their specificity (i.e., proteases, lipases, carbohydrases), which depends on the objective of the study, and to optimize processing conditions (i.e., reaction pH, temperature, and amount of enzyme) to favor its action. Proteases and carbohydrases can be used individually, or in combination for targeted extraction goals in mind. Higher extractability in the EAEP can be attributed to the different modes of action of the enzymes used. When proteases are used in the extraction, they can break down the surface proteins on the lipid membrane releasing lipids into the extraction media. In addition, enzymes catalyze the proteolysis of large protein bodies into smaller and more soluble peptides.

When carbohydrases are used in the EAEP, the breakdown of the cell wall structures can further enhance the release of the intracellular compounds into the extraction medium (Nadar, Pawar, and Rathod 2017). Enzyme concentration can vary widely based on the type of material used and upstream treatments to which the material was subjected to (i.e., flaking, grinding, extruding, heat treatments). Increased enzyme concentration, to a certain extent, generally increases the extractability of proteins and lipids from several food matrices (Souza et al. 2019; Wu, Johnson, and Jung 2009b; De Moura et al. 2008). For chickpeas, the use of Alcalase to produce chickpea protein isolates has been conducted, resulting in chickpea hydrolysates with higher protein purity, decreased molecular weights, and improved solubility and foaming capacity (Ghribi, Gafsi, et al. 2015). However, the enzyme was not applied during the initial extraction stage, but to hydrolyze the protein extracted by the aqueous extraction process. The use of enzymes during the extraction process could have a desired impact on concurrently improving the extractability and functionality of chickpea proteins and lipids. Because enzymes haven't been used to assist the extraction of full-fat chickpea flour, the comparison of extraction yields of AEP and EAEP of chickpea flour is hindered.

AEP and EAEP have not been performed on full-fat chickpea flour and there is limited information about the impact of the aforementioned parameters on extractability. While a similar process to the AEP has been conducted for protein extraction and recovery, the same has made use of a solvent defatting step that the AEP specifically avoids conducting. A better understanding of the impact of extraction parameters on the extractability, composition, and functionality of chickpea constituents in AEP and EAEP is needed to develop efficient structure-function based methods to produce chickpea ingredients with desired properties. Current research for other food matrices (i.e., soybean, almonds, almond cake, peanuts, corn) has shown that careful optimization

of these parameters is imperative to maximize overall process feasibility (i.e., extraction yields, functionality, and biological properties) (De Moura et al. 2008; Almeida et al. 2019; Souza et al. 2019; Campbell et al. 2011).

5. Impact of Extraction Methods on Chickpea Ingredients

5.1 Functional Properties of Extracted Chickpea Protein

The use of proteins in food and industrial applications relies heavily on the functionality of the extracted protein. Functional studies have been performed on various chickpea substrates: raw, cooked, enzyme-treated, and recovered proteins through IEP and ultrafiltration. Protein functionality is significantly affected by the extraction parameters previously described. Sánchez-Vioque et al. 1999 reported that whole chickpea flour (without extraction of the protein from the flour) had 94.7% emulsion capacity, which was higher than the values reported for the protein isolates described in Table 1. However, the protein isolates, recovered from the protein extract by isoelectric precipitation at pH 4.3, had higher water absorption and oil absorption capacity compared to that of the chickpea flour. The change in the functional properties of whole chickpea flour and chickpea protein isolates reflect the impact of extraction methods on changing the composition and the structure of the extracted compounds, which has a direct impact on their functional properties. Boye et al. 2010 found that Kabuli chickpea proteins isolates had higher foaming and emulsifying properties compared to other pulse proteins, and comparable water and oil absorption, and solubility between pH values from 1 to 10. In this study, Kabuli chickpeas were extracted by defatting then mixing with water adjusted in pH 9 and were recovered by two different strategies (IEP and ultrafiltration) before being spray dried. The recovery methods employed had

no significant impact on the protein solubility at pH values from 1 to 10 nor on their emulsifying and foaming properties. However, water holding capacity was higher for proteins recovered by IEP while the fat holding capacity was higher for proteins recovered by ultrafiltration. Ladjal-Ettoumi et al. 2016 also found that chickpea protein, which had been extracted by the AEP, precipitated by IEP, then freeze-dried, had higher emulsification properties and solubility at alkaline pH than that of pea and lentil proteins that were recovered similarly. The strong emulsifying and foaming capabilities of chickpea proteins create potential opportunities for using chickpea proteins as a targeted plant-based egg substitute (i.e., mayonnaise, meringue, etc.). The use of enzymes during the extraction or post-extraction of chickpea proteins can impact the functionality of chickpea proteins as well. Yust et al. 2010 reported that different degrees of proteolysis improved the foaming and emulsifying properties, and water and oil absorption capacities of chickpea hydrolysates compared to unhydrolyzed chickpea proteins. Extractions were performed at conditions listed in Table 1, precipitated, then spray dried. Post-extraction enzyme application has been shown to improve the already robust functional properties of AEP chickpeas proteins (extracted without enzyme). Ghribi, Maklouf Gafsi, et al. 2015 reported that additional hydrolysis of chickpea proteins with Alcalase after the extraction step (defatted flour mixed at 1:3 ratio for 4 hours) produced hydrolysates with higher solubility at all pH values compared to that of the non-hydrolyzed chickpea protein isolates. Chickpea protein hydrolysates with intermediate degrees of hydrolysis also had higher emulsifying capacity and decreased surface tension, showing improvement of functional properties with the enzyme treatment. Chickpea protein extractions can be tailored to achieve high extraction yields and desirable functional properties. As mentioned previously, food applications depend heavily on the functional properties of extracted proteins

which can be altered by tailoring extraction and recovery methods to identify the optimum trade-off between extraction yields and desired functional properties for targeted applications.

5.2 Potential Production of Other Beneficial Chickpea Products

While chickpea extractions have focused primarily on the production of proteins, the production of chickpea oil for antioxidant or skincare treatments is limited and rare. Processing whole chickpeas through AEP/EAEP could produce an oil byproduct, which may be more valuable or as valuable as the chickpea protein stream. AEP or EAEP oil chickpea characteristics should be studied to understand the possible benefits and challenges arising from the use of AEP and EAEP. Extraction conditions could also be tailored to maximize the conversion of fibers into potential prebiotic oligosaccharides, which could help to maintain or modify the gut microbiota. The presence of valuable specific prebiotic oligosaccharides can assist in fulfilling consumer needs for health-promoting foods.

6. Conclusions and Future Trends

Much has been done to evaluate the composition and functionality of chickpea protein extracted by the aqueous extraction process and sometimes modified by post-extraction proteolysis. However, the existing knowledge gap of the impact of extraction conditions on extraction yields and functionality provides an opportunity to elucidate and widen the research scope on chickpeas. Chickpea processing would benefit from the development of processing techniques that could maximize the extractability of chickpea constituents with desired functional and biological properties.

AEP and EAEP have the potential to simultaneously extract lipids and proteins from chickpea flour without the use of flammable solvents. In addition, the use of enzymes in the EAEP can lead to the production of proteins with unique functional and biological properties. To maximize the recovery of the extracted compounds and thus improve the overall process feasibility, the development of demulsification strategies is needed to recover the extracted oil. Importantly, functional and biological analysis of the extracted components are needed to guide the selection of extraction conditions to produce protein extracts and lipids with desired properties. Additionally, alternative modes of extraction, such as countercurrent extraction, are warranted to further improve extraction yields and reduce water usage in the process. These efforts will help to improve the process economics and its environmental impact. In that view, to determine the feasibility of the processes developed, scale-up, proof of concept, and techno-economic analysis are needed. After all, most decisions in the industry depend on the rate of return and payback time, which should be less than 2 years to even be considered as a business opportunity. An economic evaluation of AEP and EAEP of chickpeas has yet to be evaluated and could reveal the process feasibility. These objectives can widen the scope for chickpea processing research and impact industrial standards for the production of beneficial chickpea products.

Chapter 2

A Bio-guided Processing Strategy to Simultaneously Extract Lipids, Proteins, and

Oligosaccharides from Full-fat Chickpea Flour

1. Introduction

Due to the increasing world population and the popularity of alternative protein sources, plant-based proteins are becoming the forefront of sustainable food production. Plant-based protein sources provide many benefits including decreased risk of degenerative diseases and reduced environmental impact from its production (González et al. 2011; WHO 2003). Such benefits have promoted increased production and processing of plant-based products, which in turn requires the development of a critical understanding of the impact of key processing conditions (i.e., extraction and recovery) on the extractability and functionality of many plant-based compounds (i.e., proteins, lipids, and carbohydrates).

An area of growing interest is the processing of pulses, which are part of the legume family. Pulses are generally low in fat and high in protein and fiber (Shevkani et al. 2019). Chickpeas, a member of the pulse family, are an example of a good source of carbohydrates (~60 g/100 g), proteins (19 g/100 g), lipids (6 g/100 g), dietary fiber (~17 g/100 g), among other minor constituents (U.S. Department of Agriculture 2019). The increasing popularity and use of chickpeas in the food industry can be explained by its nutritional value and health benefits associated with its consumption (i.e., low glycemic index, prevention of cardiovascular disease, type-2 diabetes (Wallace, Murray, and Zelman 2016b). Used in food products worldwide, most notably for hummus, chickpeas can be the main source of protein in vegan and vegetarian diets (Duranti and Gius 1997). Chickpea protein isolates can be used not only to improve the nutritional value but the physical and rheological properties of gluten-free food products (Shaabani et al. 2018).

Chickpea proteins, lipids, and carbohydrates can be extracted using numerous methods. The presence of lipids in a food matrix entails the upstream removal of lipids to release the protein

bodies and carbohydrates. Traditionally, upstream lipid removal has been accomplished either by solvent extraction or by the use of mechanical pressing, the selection of which depending on the composition of the material used (De Moura, De Almeida, and Johnson 2009). Despite the environmental and safety issues associated with flammable solvent extraction and low extraction yields associated with mechanical pressing, a protein-rich by-product with varying amounts of residual oil (cake) or compromised functionality (defatted flour) might be produced (L'hocine, Boye, and Arcand 2006; Kim et al. 2021). This sequential approach means that the cake or the defatted flour must be subjected to another processing step to extract proteins and carbohydrates, in addition to removing the remaining lipids.

Alternatively, aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) can be used to simultaneously extract lipids, proteins, and carbohydrates from a food matrix without upstream removal of lipids by solvent extraction or by mechanical pressing (Campbell and Glatz 2009; De Moura, Maurer, et al. 2011). This green processing strategy can reduce the environmental impact of using flammable and hazardous solvents for defatting, by the solubilization and transport of proteins to the exterior of the solid matrix, which creates a more porous structure that favors the washing of the oil droplets by the extraction medium (M. H. Cheng et al. 2018; Dias et al. 2020). Further improving upon the AEP process is the enzyme-assisted extraction process (EAEP), which utilizes enzymes such as proteases and carbohydrases to maximize processing extractability. Increased oil and protein extractability in the EAEP has been attributed to enzymatic hydrolysis of the lipid body membrane, proteins, and cell walls (De Moura et al. 2008; Nadar, Pawar, and Rathod 2017).

AEP and EAEP are accomplished by dispersing the food matrix (i.e., flour, flakes) into water under controlled conditions (pH, temperature, enzyme use) to maximize the overall process

extractability. The slurry is subsequently centrifuged to fractionate the insoluble fraction (the fiber-rich fraction containing unextracted proteins and lipids) from the liquid phase containing the extracted compounds. The liquid phase is further fractionated by density difference into free oil, cream (an emulsion that contains most of the extracted oil), and skim (protein-rich phase) (Wu, Johnson, and Jung 2009; De Moura, Maurer, et al. 2011).

The successful development of extraction methods for new protein sources depends on the development of fundamental knowledge of the impact of the processing conditions employed (i.e., temperature, pH, enzyme use) (De Moura et al. 2011) on the extractability, composition, and functional properties of the extracted compounds. Controlled hydrolysis of chickpea protein isolates by immobilized Alcalase has been shown to produce hydrolysates with higher solubility, oil absorption, foaming capacity, and stability (Yust et al. 2010). However, limited emphasis has been given to the development of a holistic understanding of the effects of key extraction parameters (i.e., solids-to-liquid ratio, pH, temperature, incubation time, amount and type of enzyme) on the overall extractability of both lipids and proteins from full-fat chickpea flour and their impact on the functional properties of the extracted proteins.

Because extraction conditions affect extraction yields and the functionality of the extracted compounds, they have a key impact on the processing feasibility and potential applications of the extracted compounds. This work was undertaken to gain a better understanding of the effects of different enzymatic extraction strategies on the simultaneous extraction of lipids, proteins, and carbohydrates from full-fat chickpea flour and on the functionality of the extracted proteins. Specifically, we evaluated the effectiveness of an upstream enzymatic treatment with carbohydrases (cellulase, hemicellulase, and xylanase) before the use of protease with respect to lipids and protein extractability, solubility and *in vitro* digestibility of the extracted proteins, and

carbohydrate profiling of the extracts. Our working hypothesis was that the use of carbohydrases before the addition of proteases could hydrolyze the cell wall with the potential release of new oligosaccharides and formation of a more porous structure that could favor protein solubilization by the aqueous medium, as well as hydrolysis of the protein bodies and oleosin membrane surrounding the lipid bodies by the protease. That could in turn improve the overall process extractability and concurrently produce more soluble and digestible peptides and release a more diverse pool of oligosaccharides with potential health-promoting effects. High-performance anion-exchange chromatography with pulsed amperometric detection and LC-MS/MS were used to determine the carbohydrate profile of the chickpea extracts.

2. Materials and Methods

2.1 Chickpea Flour and Enzymes Used in the EAEP

Commercial Steamed Chickpea flour of the Kabuli variety was kindly provided by Natural Products, Inc (Grinnell, Iowa, USA). Partially dehulled chickpeas (to increase the fiber content of the final product) were steamed to inactivate enzymes and achieve microbial stability before milling (as described by the manufacturer). The chickpea flour contained $7.37 \pm 0.1\%$ oil, $25.87 \pm 0.07\%$ protein, and $4.69 \pm 0.09\%$ moisture, which were determined as described in Section 2.3.

The following commercial enzymes were used to assist the extraction process (EAEP): (i) FoodPro Alkaline Protease, also known as Protex 6L, a 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 was provided by the Genencor Division of Danisco (Rochester), (ii) Cellulase from *Trichoderma reesei,* with multiple cellulolytic activities (endo and exo-cellulase, β-glucosidase, β-glucanase, hemicellulose, pectinase, and xylanase) and enzyme activity

of 200,000 CU/g at optimal pH from 4.0-6.5 and 45-70 °C, (iii) Hemicellulase from *Aspergillus Niger* with enzyme activity of 600,000 HCU/g which is optimal at pH 2.0-8.0 and 25-90 °C, and (iv) Xylanase from *Trichoderma Longibrachiatum* with enzyme activity of 200,000 XU/g which is optimal at pH 3.5-6.5 and 40-70 °C were provided by Bio-Cat, Troy, Virginia, USA.

2.2. Tailoring Enzymes Use to Maximize the Simultaneous Extraction of Lipids, Proteins, and Carbohydrates from Full-fat Chickpea Flour

The effect of using protease (EAEP), alone or in combination with different carbohydrase pretreatments, was evaluated on the extractability of proteins, lipids, and carbohydrates from chickpea flour (Fig 1). A non-enzymatic aqueous treatment (AEP) was used as the control.

The AEP (control, no enzyme use) was carried out by dispersing 50 g of chickpea flour into 500 mL of water to achieve a 1:10 solids-to-liquid ratio (SLR). The slurry pH was adjusted to pH 9.0 to favor protein solubility and extractability (Neiva M De Almeida et al. 2019) and kept at 50°C under constant stirring. For the EAEP, the potential benefits of using an upstream treatment with carbohydrases, prior to the alkaline protease, were evaluated. The following enzymatic strategies were evaluated: EAEP 1: 0.5% (w/w) of alkaline protease at pH 9.0 for 60 min; EAEP 2: 0.5% (w/w) of carbohydrases (0.25% of cellulase + 0.25% of hemicellulose) at pH 6.0 for 30 min followed by the addition of 0.5% of alkaline protease (w/w) at pH 9.0 for 60 min; and EAEP 3: 0.5% (w/w) of carbohydrases (0.17% of cellulase + 0.17% of hemicellulose + 0.17% of xylanase) at pH 6.0 for 30 min followed by the addition of 0.5% of alkaline protease (w/w) at pH 9.0 for 60 min. For the EAEP, extractions were performed at the same SLR and temperature as the AEP, and pH conditions were selected based on the enzyme manufacturer's recommendations.

After extracting, the resulting slurry was centrifuged at 3000 $x\ g$ for 30 min at 4 °C to separate the insoluble fraction (containing the unextracted compounds) from the liquid phase (containing the extracted compounds). The liquid fraction was placed in a separatory funnel and allowed to settle overnight at 4 °C to separate the oil-rich fraction (cream and free oil) from the protein- and carbohydrate-rich fraction (skim). Each extraction condition was carried out in triplicate.
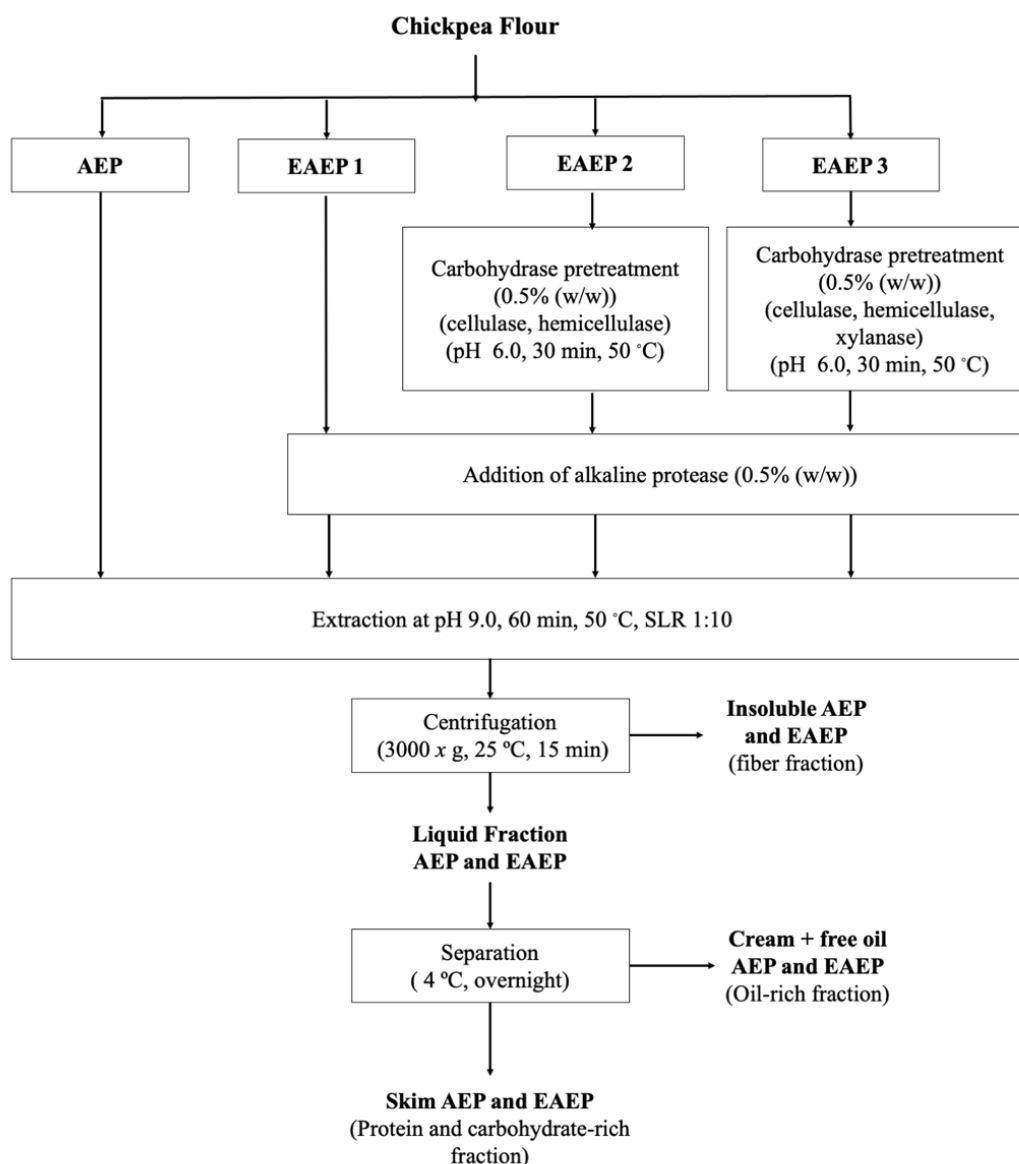
Figure 1. *Process flow diagram for each extraction treatment. AEP: no enzyme; EAEP 1: 0.5% of protease; EAEP2: 0.25% of cellulase and 0.25% of hemicellulase (total of 0.5% of carbohydrases); EAEP3: 0.17% of cellulase, 0.17% of hemicellulose and 0.17% of xylanase (total of 0.5% of carbohydrases). w/w: weight of enzyme/weight of flour.*

Chickpea full-fat flour (starting material for the extraction) and all fractions generated by the AEP and EAEP were characterized for oil, protein, and carbohydrate contents (as described in sections 2.3 and 2.7). Total oil extraction yield (TOE), oil distribution in the fractions (free oil yield, oil yield in the cream, skim, and insoluble), total protein extraction yield (TPE), and protein distribution in the fractions (protein yield in the cream, skim, and insoluble) were determined according to Equations (1)–(4), respectively (Souza et al., 2019):

Total Oil Extraction:

$$TOE\ (\%) = \left[100 - \left(\frac{Oil\ (g)\ in\ Insoluble}{Oil\ (g)\ in\ the\ chickpea\ flour}\right)\right] \times 100 \qquad (1)$$

$$Oil\ distribution\ in\ fractions\ (\%) = \left(\frac{Oil\ (g)\ in\ fraction}{Oil\ (g)\ in\ the\ chickpea\ flour}\right) \times 100 \qquad (2)$$

Total Protein Extraction:

$$TPE\ (\%) = \left[100 - \left(\frac{Protein\ (g)\ in\ Insoluble}{Protein\ (g)\ in\ the\ chickpea\ flour}\right)\right] \times 100 \qquad (3)$$

$$Protein\ distribution\ in\ fractions * (\%) = \left(\frac{Protein\ (g)\ in\ fraction}{Protein\ (g)\ in\ the\ chickpea\ flour}\right) \times 100 \qquad (4)$$

*The fractions relate to free oil, cream, skim, and insoluble.

2.3 Proximate Analysis

Cream, skim, insoluble, and starting material (chickpea flour) 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). Oil content was determined by using the

Mojonnier acid hydrolysis (AOCS method 989.05), and protein content by using the Dumas method and a conversion factor of 6.25 (Vario MAX cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). Extraction yields of oil, proteins, and solids were expressed as percentages relative to their initial amounts in the chickpea flour as described in Sections 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 (Nielsen et al. 2001). 400 μL of skim was added to 3 mL of OPA reagent which 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 the standard and a blank solution was prepared with distilled water instead of a sample and used as the reaction control. Protein quantification was determined by the Dumas method (conversion factor of 6.25) and the DH was determined as described in Equation (5):

$$DH\ (\%) = \left(\frac{h}{h_{total}}\right) \times 100 \quad (5)$$

where h is the number of hydrolyzed bonds and h_{tot} is the total number of peptide bonds per protein equivalent (7.22 for chickpeas (Kou et al. 2013)). The number of hydrolyzed bonds (h) was determined as described in Equation (6):

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

where α and β values were 1.0 and 0.4, respectively (Adler-Nissen 1986; Nielsen 2001).

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 profile of AEP and EAEP skim proteins produced under optimum extraction conditions as described by Laemmli 1970. Skim fractions were mixed with (1:1, v/v) a Laemmli solution (2x Laemmli Sample Buffer, BioRad, Hercules, CA, USA) composed of 4% SDS, 20% glycerol, 0.004% commassie blue, and 0.125 M Tris HCl, pH approx. 6.8) with 10% of β-mercaptoethanol, 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 for the electrophoretic separation of proteins. 30 µg of protein was pipetted into each well on a precast 12% acrylamide gel (CriterionTM TGX Precast Gels, Bio-Rad, Hercules, CA, USA) and electrophoretic separation was carried out at room temperature for 1 h at 200 V. A low MW range SDS-PAGE standard (14.4–97.4 kDa) (Bio-Rad, Hercules, CA, USA) was used. 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. Solubility Analysis of Skim Proteins

Approximately 15 mL of AEP and EAEP skims from each extraction replicate (n=3) were freeze-dried on a FreeZone 4.5 Liter Benchtop Freeze Dry System (Labconco, Kansas City, MO, USA) and stored at −20 °C for subsequent solubility tests. Protein solubility of freeze-dried AEP and EAEP skim proteins was evaluated by preparing a 10 mL of a 1% (w/v) skim solution in a 30

mL beaker and adjusting the pH of the protein solution to 4.0 and 9.0 by the addition of 1 M HCl or 1 M NaOH solution. Solutions were vigorously mixed at 150 rpm for 1 hour at room temperature and then centrifuged at 10,000 x g at 20 °C for 10 min. The protein content of the supernatant was measured by using the Dumas method and a protein conversion factor of 6.25 (Vario MAX cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). The total protein content was measured by measuring the freeze-dried sample with the same method as the supernatant. All samples were analyzed in duplicate. The solubility (%) was determined as described in Equation (7):

$$Solubility~(\%) = \left(\frac{Protein~in~supernatant~(\%)}{Total~protein~(\%)}\right) \times 100 \qquad (7)$$

2.7. Carbohydrate Profile, Quantification, and α-Galactosidase Treatment of Skim Fractions

2.7.1 Quantification and Profiling of Major Carbohydrates in AEP and EAEP skims

The use of carbohydrases throughout the extraction process and as a post-extraction strategy can result in the production of chickpea extracts containing a diverse oligosaccharide profile while reducing flatulence promoting sugars such as raffinose and stachyose, which are present in high amounts in pulses, including chickpeas. The effects of the use of different carbohydrases during the extraction and post-extraction on the content and profile of carbohydrates of the skim fractions were evaluated by using different analytic techniques.

The total carbohydrate content of the skim fractions was determined using the Phenol-sulfuric method (Masuko et al. 2005). 15 µL of the sample along with 15 µL of nanopure water were added to the well. The well plate was mixed at 300 rpm for 1 min then 150 µL of 98% sulfuric

acid was added to each well. The microplate was then incubated at 85 °C for 15 minutes in an incubating thermal shaker (Thermalshake, VWR, Radnor, Pennsylvania, USA), followed by the addition of 30 µL of 5% (w/v) phenol/water solution. A calibration curve was made using glucose as a standard (from 4 to 20 µg, R^2= 0.9937) was used to quantify the total carbohydrates. After vigorous mixing, the samples were measured using a microplate reader (SpectraMax iD5, Molecular Devices, San Jose, CA) at 490 nm.

2.7.2 Quantification of Oligosaccharides and Simple Sugars in AEP and EAEP Skims

Soluble carbohydrate profiles of skims were quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex ICS-5000+, Thermo Fisher Scientific, Sunnyvale, CA). Aliquots of 200 µL of samples were mixed with 400 µL of ethanol in 1.5 mL tubes, vortexed, and incubated at -30 °C for 1 h. After being centrifuged at 4 °C for 30 min (13,000 × g), the supernatant was dried under vacuum (MiVac Quattro concentrator, Genevac Ltd., Ipswitch, UK). Samples were diluted as appropriate and filtered through a 0.2 µm syringe filter into 1.5 mL vials with septa. Glucose, galactose, and fructose were separated on a CarboPac PA10 column (4 × 250 mm) with a CarboPac PA10 guard column (4 × 50 mm) at a flow rate of 1.0 mL/min. The mobile phase was maintained at an isocratic condition of 10 mM NaOH for 12 min and was increased to 100 mM NaOH in 13 min. Sucrose, raffinose, and stachyose were separated on a CarboPac PA200 column (3 × 250 mm) with a CarboPac PA200 guard column (3 × 50 mm) by isocratic elution using a mobile phase of 50 mM NaOH at a flow rate of 0.5 mL/min. Both columns were washed with 200 mM NaOH for 5 min after each run and equilibrated with the respective initial mobile phases for 10 min before next injection. Calibration curves were built by using 1–60 µg/mL of glucose, galactose, and fructose,

and 0.1–10 μg/mL of sucrose, raffinose, and stachyose. An analytical replicate was conducted for each replicate of extraction and α-galactosidase treatment (n=3).

2.7.3 α-Galactosidase Treatment of Raffinose and Stachyose in the Skim Fractions

Because of the presence of flatulence promoting oligosaccharides in the skim fractions, an α-galactosidase treatment was evaluated to reduce the concentration of stachyose and raffinose in the skim fractions. Because the amount of stachyose and raffinose was not statistically different within the enzymatic treatments, the EAEP 1 skim was selected to demonstrate the effectiveness of the α-galactosidase treatment. 1000 μL of EAEP 1 skim was adjusted to pH 6 with 1 M HCl and α-galactosidase (Bio-Cat, Inc., Troy, VA, USA) was added to achieve 0.25 % (w/v) concentration. The EAEP 1 skim was incubated at 40 °C for 0, 15, 30, and 60 min at 90 rpm in a water bath. Skim samples were placed in an ice bath to stop the reaction and stored at 4 °C until analyzed. Quantification of simple sugar (glucose, galactose, fructose, and sucrose) and oligosaccharide (raffinose and stachyose) were carried out on an HPAEC-PAD as described in section 2.7.1.

2.7.4 Mass Spectrometry Characterization of Oligosaccharides in AEP and EAEP Skims

The oligosaccharide profile of the skims was characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The reconstituted supernatant fractions obtained from ethanol precipitation (described in section 2.7.1) were further purified by solid-phase extraction (SPE). The reconstituted samples (150 μL, equivalent to 30 μL of skims) were premixed with 150 μL 0.2% formic acid and then loaded to Strata-X-C SPE cartridges (30 mg/1 mL, Phenomenex, Torrance, CA, USA) preconditioned with acetonitrile and 0.1% formic acid.

Oligosaccharides were eluted with 3 mL 0.1% formic acid and further loaded to porous graphitic carbon SPE microplate (Glygen, Columbia, MD, USA) preconditioned with 80% acetonitrile with 0.1% trifluoroacetic acid and water. The microplate wells were washed with water for eliminating salts and flushed sequentially with 40% acetonitrile (fraction 1) and 40% acetonitrile with 0.1% trifluoroacetic acid (fraction 2) for eluting oligosaccharides. The collected oligosaccharide fractions were dried in a centrifugal evaporator. Fractions 1 and 2 were combined after dissolving the dried samples in water. For oligosaccharide characterization, the combined samples of the three replicates of extraction were pooled and injected into the LC-MS/MS (one injection for each treatment). For relative quantification, one injection was made for the combined samples of each replicate of extraction.

LC-MS/MS analysis was performed on an Agilent 6520 Accurate-Mass Q-TOF LC-MS with a Chip Cube interface (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent PGC-Chip II (porous graphitized carbon chip with a 40 nL enrichment column and a 75 μm × 43 mm analytical column). The capillary pump delivered 3% acetonitrile with 0.1% formic acid (v/v/v) at a flow rate of 4 μL min^{-1} and loaded samples into the enrichment column. The injection volume was 2 μL for each sample. The nano pump delivered mobile phase composed of 3% acetonitrile with 0.1% formic acid (v/v/v) (solvent A) and 89.9% acetonitrile with 0.1% formic acid (v/v/v) (solvent B). The analytes were separated at a flow rate of 0.3 μL min^{-1} with 0% B from 0.0–2.5 min; 0–16% B from 2.5–20.0 min; 16–44% B from 20.0–30.0 min; 44–100% B from 30.0–35.0 min; 100% B 35.0–45.0 min. The mobile phase was switched to 100% A and equilibrated for 15 min before the next injection. The capillary voltage was set at 1850 V to maintain a stable spray. The drying gas was set at 350°C at a flow rate of 5 L min^{-1}. The scanning mass range was *m/z* 150–2500 for MS and 50–2500 for MS/MS. Collision energy for tandem MS was set by a formula

of $[0.02 \times (m/z) - 3.5]$. Data analysis was conducted in MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). Oligosaccharides were identified by inspecting fragmentation patterns in tandem MS spectra. For relative quantification, peak areas of the identified oligosaccharides were integrated from merged extracted ion chromatograms, including the precursor ions and corresponding in-source fragment ions.

2.8. *In vitro* Skim Protein Digestibility

Protein digestibility of AEP and EAEP skim proteins was measured as described by (Bornhorst and Singh 2013; de Souza et al. 2020). Five mL of liquid skim fractions were mixed with 3.33 mL of SSF (Simulated Saliva Fluid) and vortexed. Subsequently, 6.66 mL of SGF (Simulated Gastric Fluid) 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 (Simulated Intestinal Fluid) 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. Trichloroacetic acid (TCA) was added in a 1:1 (v/v) proportion to the samples to achieve a final 12% (w/w) TCA concentration. The samples were centrifuged at 4,000 rpm for 30 min at 4 °C. The precipitate, protein nitrogen fraction (PN), was analyzed for the protein content. A before digestion control with the sample and water instead of the simulated liquids was performed and an enzyme blank with water instead of samples was also performed. The digestibility was calculated as described by Zhong et al. (2012).

$$Digestibility\ (\%) = \frac{PN_{before} - (PN_{after} - PN_{enzyme\ blank})}{PN_{before}} \tag{8}$$

Where PN_{before} = protein before digestion, PN_{after} = protein after digestion, $PN_{enzyme\ blank}$ = enzyme blank. The PN (protein nitrogen fraction) was measured in the samples by the Dumas method using a conversion factor of 6.25 (Vario MAX cube, HE, DE) before and after the digestion.

2.9. Statistical Analysis

Extractions were performed in triplicate and the functional analysis were performed in duplicate. The results were expressed as the mean ± standard deviation (SD) of the replicates. Replicates of each measurement were analyzed by ANOVA with generalized linear models from the Statistica software (version 13.5.0.17 1984-2018, TIBCO Software Inc, Palo Alto, CA, 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 Effects of Extraction Conditions on Oil and Protein Extraction Yields

The use of selected enzymes to assist the extraction of plant-based matrices has been successfully used as an effective strategy to increase not only the extractability of target compounds (i.e., lipids, proteins, carbohydrates, phenolics) but to impart structural modifications in the food matrix that can lead to the production of compounds with desired functional and biological properties (i.e., higher protein digestibility and solubility, release of prebiotic oligosaccharides and antioxidants, among others (Souza et al. 2020)).

The effectiveness of using alkaline protease, preceded or not by the use of selected carbohydrases, on the extractability of lipids and proteins from full-fat chickpea flour is shown in Figure 2A and 2B. The use of enzymes to assist the extraction significantly increased the overall extractability of lipids from chickpea flour (Figure 2A) compared with the control (AEP, with no enzyme use). When not using enzymes (AEP), 49.78 ± 2.08% of the available oil in the chickpea flour was extracted. However, oil extraction yields increased to 77.15 ± 5.87% for the EAEP 1 (using only protease), followed by 73.45 ± 1.54% for the EAEP 2 (cellulase + hemicellulose pre-treatment followed by protease) and 72.02 ± 1.19% for the EAEP 3 (cellulase + hemicellulase + xylanase pretreatment followed by protease). The higher oil extraction yields observed for EAEP treatments can be attributed to the different modes of action of proteases. Proteases can hydrolyze the oleosin membrane of the lipid bodies, releasing free oil into the aqueous medium (K. A. Campbell et al. 2011). In addition, protein removal from the matrix by solubilization or proteolysis leaves behind a more porous structure that facilitates the release of the oil. On the other hand, the AEP relies primarily on the solubilization of the proteins into the aqueous medium, without the benefit of proteolysis. Therefore, lipids are solely extracted through washing out of the matrix. Despite the higher oil extraction yields observed for the EAEP, compared with the AEP, oil extraction yields were not statistically different among the enzymatic treatments. The additional use of carbohydrase pretreatments did not significantly increase lipid extractability, not justifying the additional use of enzyme, energy, and time. When looking at the oil distribution for the EAEP 1, although 77.15% of the chickpea flour oil was extracted, only 0.43% of the total extracted oil was present as free oil, while 42 and 57% of the extracted oil were present in the cream (oil-rich emulsion) and skim fractions, respectively. Comparatively, for the AEP, only 0.15% of the extracted oil was present as free oil with 15 and 32% being present in the cream and skim,

respectively. While the amount of free oil extracted by the AEP and EAEP was not statically different (0.14 vs. 0.24–0.29% yield), the use of enzyme in EAEP 1 and 2 significantly increased the oil yield in the cream (16.06 vs. 32.23–32.56% yield). Since there are no methods available to recover the diluted oil from the skim fraction, maximizing the amount of lipids in the cream fraction is of key importance to favor the overall recovery of the extracted oil, which entails the development of additional demulsification studies to breakdown the cream emulsion (De Moura and Johnson 2009), which is beyond the scope of this work. Our results are in agreement with the literature (Dias et al. 2020; Souza et al. 2019), which demonstrates that nearly all the oil extracted through the AEP and EAEP is entrapped in the cream fraction.

Similar oil yields were observed for the AEP and EAEP skims. Although the lipid content in the AEP and EAEP skim fractions is low (~0.3 and 0.4%, respectively), the high volume of skim produced accounts for a significant portion of the oil in the chickpea flour (40.58–47.47% yield). At dry basis, the oil content of the AEP and EAEP skims are 7.77 and 7.91%, respectively. When comparing oil distribution with previous studies for other food matrices, lower oil yields have been reported for skim fractions produced by enzymatic hydrolysis (Protex 6L) of extruded soybean flakes (14% yield, De Moura et al. 2008) and almond cake (14% yield, and Souza et al. 2019). This could be attributed to differences in the composition (i.e., lipids, protein) of the starting materials and processing conditions used (i.e., milling, flaking, extruding). As an example, the initial oil content in the chickpea flour is very low (7%) compared to that of soybeans (21%) and almonds cake (16.25%). Because there are no methods available to recover the skim oil, and lipids can reduce the skim protein solubility (Neiva M De Almeida et al. 2019), it is important to identify processing conditions leading to reduced oil in the skim fraction, which should in turn increase the lipid content in the cream fraction for subsequent recover as free oil.

Overall, the addition of a carbohydrase pretreatment in EAEP 2 and 3 did not significantly increase total oil extraction yields when compared to the use of protease alone (EAEP 1), nor altered the distribution of the extracted oil among the fractions. However, the use of protease significantly increased oil extractability and oil yield in the cream when compared with the AEP.

Figure 2B shows the significant increase in protein extractability when enzymes were used to assist the extraction (EAEP) compared with the control (AEP). The use of enzymes in the EAEP significantly increased protein extractability from $62.81 \pm 1.68\%$ (AEP) to $83.49–86.13\%$ (EAEP). However, extraction yields within the enzymatic strategies evaluated were very similar ($83.49 \pm 0.19\%$ for EAEP 1, $84.04 \pm 0.49\%$ for EAEP 2, and $86.13 \pm 1.51\%$ for EAEP 3). The small increase in protein extractability observed for EAEP 3 compared with EAEP 1 could be attributed to the carbohydrase pretreatment applied prior to the addition of the protease, indicating that the breakdown of the cell walls by the carbohydrases and the additional extraction time (30 min) helped with the release of proteins from the chickpea flour. However, considering the additional use of 0.5% of enzyme and the additional reaction time (30 min) when performing the pretreatment with carbohydrases, the modest increment in protein extractability observed compared with the use of the protease alone (83.5 vs. 84.0–86.0%) does not justify the inclusion of the additional pretreatment. As expected, the higher protein extractability observed for the EAEP treatments led to the production of skim fractions with higher yields (77.49–82.62%) compared with the AEP (62.14%). From the 83.5–86% protein extracted, 77–83% and 61% of the extracted protein was present in the EAEP and AEP skims, respectively.

In addition, the distribution of extracted proteins was influenced by enzyme specificity within the EAEP treatments. The use of cellulase + hemicellulase + xylanase prior to the addition of the protease (EAEP 3) led to higher protein yield in the skim (82.62%) compared with the use

of cellulase + hemicellulase prior to the addition of the protease (EAEP 2) (77.49%) or protease alone (EAEP1) (77.61%). A similar trend was observed for the cream fraction, where EAEP 1 and 2 led to the production of a cream fraction with a higher protein yield (5.88 and 6.56%, respectively) compared with the AEP (0.67%).
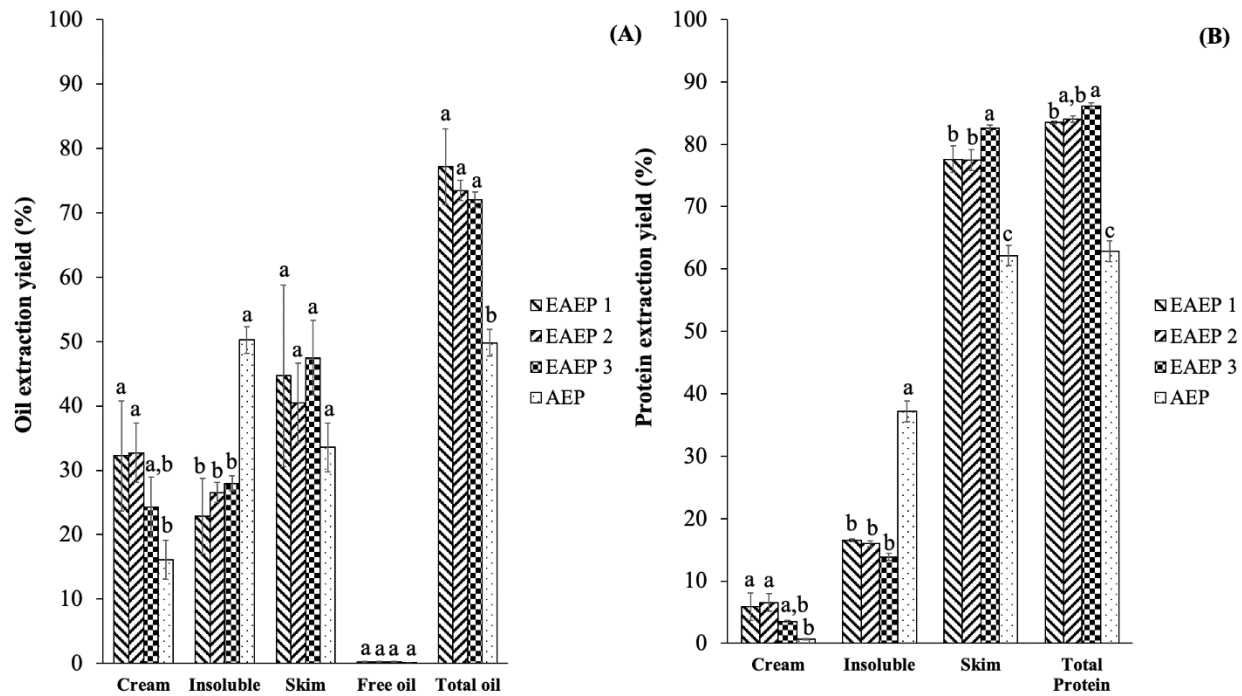
Figure 2. *Oil (A) and protein (B) extraction yields and distribution in the fractions. Different letters indicate statistically significant difference by one-way ANOVA followed by Tukey's test at p<0.05.*

To the best of our knowledge, there are no reports describing the effectiveness of the AEP and EAEP to simultaneously extract lipids and proteins from full-fat chickpea flour, which hinders the comparison of our data with the literature. However, our findings are consistent with the ones presented for the AEP and EAEP of other food matrices. De Moura et al. 2008 reported protein

extraction yields of 85% when using Protex 6L to assist the extraction of extruded soybean flakes. Souza et al. 2019 reported an increase in protein extractability from 69.6% (AEP) to up to 75% when Protex 6L was used to assist the extraction from almond cake.

3.2 Effects of Extraction Conditions on the Degree of Hydrolysis and MW Polypeptide Profile of AEP and EAEP Skim Proteins

During protein hydrolysis, the breakdown of peptide bonds results in increased concentration of primary amines, corresponding to an increase in the degree of hydrolysis (DH). Because the DH often has a significant impact on the functional properties of the extracted proteins (Ghribi, Maklouf Gafsi, et al. 2015), understanding the effects of extraction conditions on the DH and protein functionality becomes necessary to further identify possible industrial applications for the extracted proteins.

The use of enzymes to assist the extraction significantly increased the DH from 10% (AEP) to 23.3, 25.0, and 25.5% for the EAEP 2, EAEP 1, and EAEP 3 respectively (Figure 3). No significant difference was observed for the DH amongst the enzymatic treatments, due to the use of the same amount of protease. Our results are in agreement with the literature where the use of enzymes to assist the extraction leads to a higher DH (Ghribi, Sila, et al. 2015).
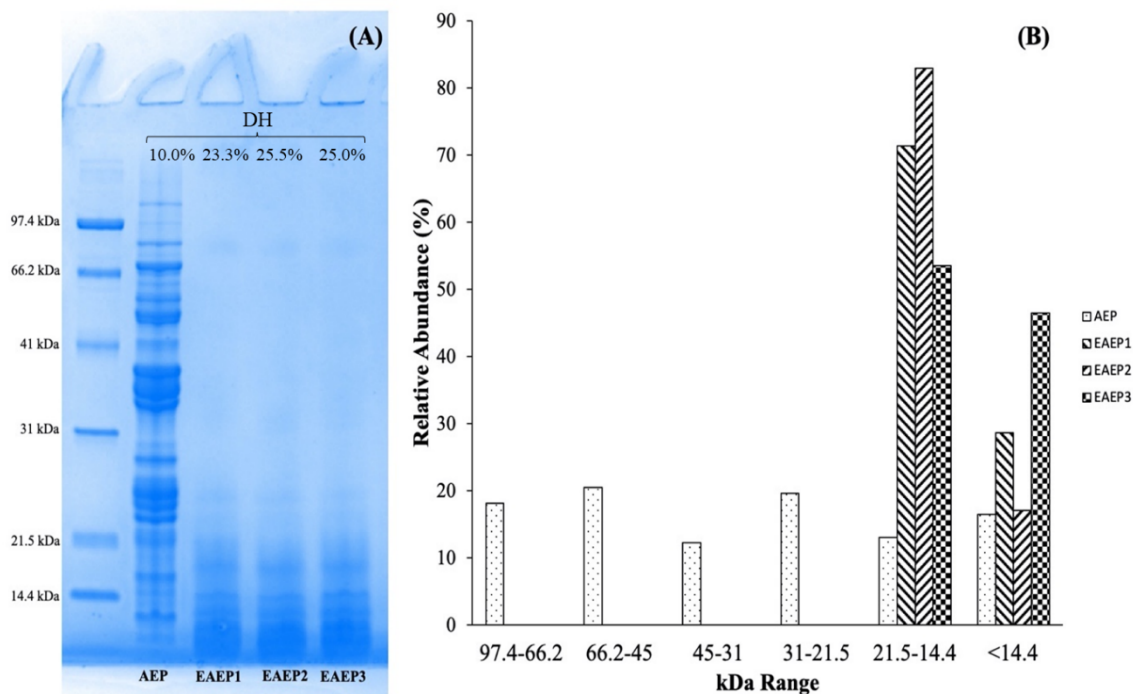
Figure 3. Sodium dodecyl sulfate–polyacrylamide 12% gel electrophoresis profiles (A) of AEP and EAEP skim proteins/peptides and degree of hydrolysis (DH) and (B) molecular weight distribution of AEP and EAEP skim protein bodies.

The protein profile of chickpea skim proteins is shown in Figure 3. The AEP skim proteins (unhydrolyzed proteins) presented a band at ~66 kDa that could be attributed to convicilin, a protein with molecular weight between 68 to 70 kDa (Tzitzikas et al. 2006) corresponding to 18.1% of the protein in the lane. Another intense band can be seen at 45-47 kDa, which might correspond to the vicilin protein, which has three different polypeptide subunits with molecular weights of 53, 47, and 43 kDa (Romero et al. 1975). The major bands seen at ~40 and 20 kDa can be attributed to the acidic (α) subunit of legumins and the basic (β) subunit of legumins, respectively (Boulter and Croy 1997). Moreover, the bands at 37 kDa and 27 kDa could indicate the presence of lectins (Sathe 2002). The legumin alpha-subunit and the lectins correspond to

12.3% of the protein in the lane. Our results are in accordance with the ones reported by Chang et al. 2012, which reported globulin protein 11S legumins and 7S vicilins as the major protein fractions and 2S albumin as the minor protein fraction in chickpea flour.

The use of enzymes to assist the extraction (EAEP 1–3) promoted the complete hydrolysis of proteins with MW > 21 kDa, indicating total hydrolysis of convicilin, vicilin and legumin alpha-subunit, and partial hydrolysis of the legumin beta-subunit, which is in accordance with the significantly higher degree of hydrolysis of those samples. Moreover, a significant increase in the relative abundance of protein hydrolysis products and peptides with MW < 14 kDa can be observed for the EAEP samples. Ghribi, Maklouf Gafsi, et al. 2015 showed a significant decrease in the ~45–66 kDa and ~34–45 kDa molecular weight bands, which occurs due to increasing enzymatic hydrolysis.

3.3. Effects of Extraction Conditions on Protein Solubility

Solubility is an important functional property of proteins because of its impact on food applications. Soluble proteins can be integrated into food products whose pH can vary widely, while insoluble proteins may be limited in their application and desirability. Chickpea protein peptides are needed to be functional, and specifically soluble, to further increase their application in the food industry. Boye et al. (2010) reported that unhydrolyzed extracted chickpea proteins had higher water absorption capacity, higher oil absorption capacity and emulsifying capabilities than other pulse proteins, while having similar solubility and gelation capabilities. With protein hydrolysis catalyzed by enzymes, smaller peptides are released which can be significantly more soluble than larger protein bodies (Carbonaro et al. 1997).

Because enzymatic hydrolysis can significantly affect the functionality of the extracted proteins, we evaluated the impact of the AEP and EAEP on the solubility of extracted proteins at pH 4.0 (which is close to the isoelectric point of chickpea proteins (4.3, (Sánchez-Vioque et al. 1999) and 9.0 (Fig 4 A and B).
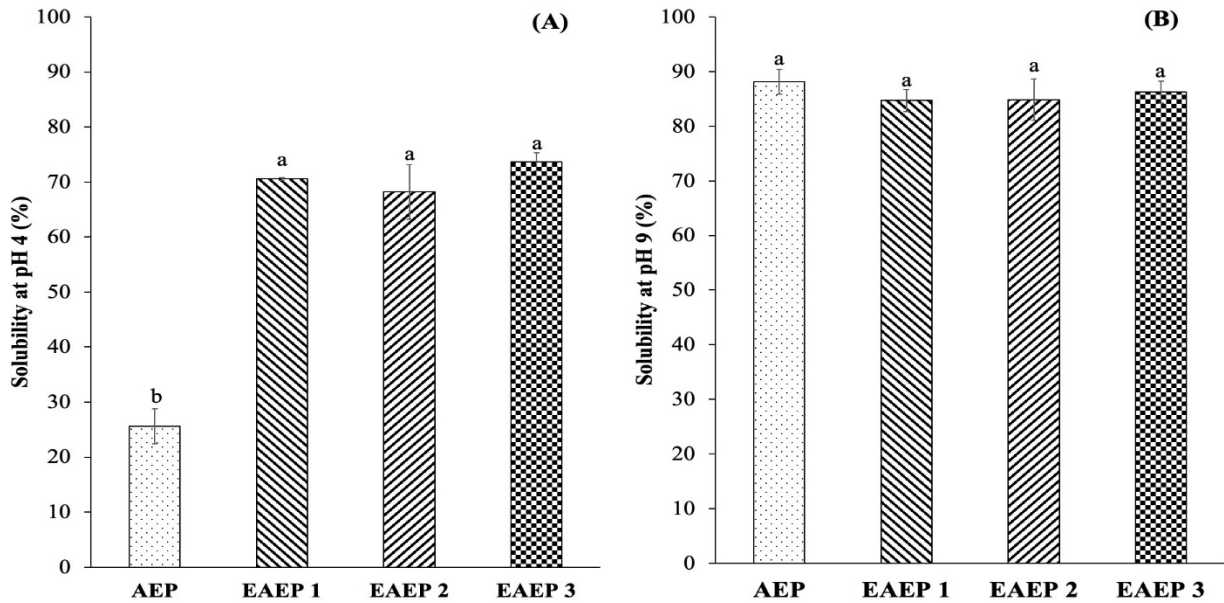
Figure 4. *AEP and EAEP skim protein solubility at pH 4 (A) and pH 9 (B). Different letters indicate statistically significant difference by one-way ANOVA followed by Tukey's test at p<0.05.*

At pH 4, where chickpea protein solubility is unfavored by the proximity to its isoelectric point (pI), the use of enzymes to assist the extraction significantly improved protein solubility (25.6% AEP vs. 68.2–73.6% EAEP) (Figure 4A). However, no statistically significant differences were observed amongst the enzymatic treatments. These results demonstrate that the use of enzymes can indeed generate smaller and more soluble peptides, which was in agreement with previous studies reporting the beneficial effects of proteolysis on the solubility of almond proteins (Souza et al. 2019; Neiva M De Almeida et al. 2019). Increased solubility of EAEP skim proteins

at pH 4.0, compared with the AEP skim proteins at the same pH, is in agreement with the higher degree of hydrolysis of the EAEP skim proteins. However, at pH 9.0, AEP and EAEP skim proteins exhibited similar high solubility, with values ranging from 85 to 88% (Figure 4B). Higher solubility of AEP and EAEP skim proteins at pH 9.0 is attributed to a higher negative net charge of the proteins, which enhances electrostatic repulsion between protein molecules thus favoring its solubility. Conversely, at the isoelectric point, the net-zero charge of the proteins enhances the attractive forces within the protein molecules, which in turn reduces its solubility in the aqueous medium (Zayas 1997). It is not surprising that chickpea protein solubility at acidic pH, which is near the isoelectric point, is lower than that at alkaline pH. In that view, all enzymatic treatments significantly increased protein solubility at acidic pH, which can open up the potential use of the hydrolysates in specific industrial food formulations involving acidic pH.

3.4. Effects of Extraction Conditions on Carbohydrate Content and Profile and α-Galactosidase Treatment

3.4.1 Quantification and Profiling of Major carbohydrates in the AEP and EAEP Skims

In addition to being a source of raffinose, stachyose, and verbascose, chickpeas are a source of dietary fiber (18–22 g per 100 g of flour), from which 10–18 g is comprised of insoluble fiber and 4–8 g is comprised of soluble flour (Tosh and Yada 2010). The use of carbohydrase pretreatments, prior to proteolysis, was evaluated as a strategy to improve the bio-functionality of the protein extracts through the release of potential prebiotic oligosaccharides into the skims through the breakdown of the cell wall polysaccharides.

The effects of the extraction methods evaluated on the total carbohydrate content of the skim fractions are shown in Table 1. Indeed, the enzymatic treatments significantly increased the total carbohydrate content of the skim fractions from 7.68mg/mL (AEP) to 9.17-9.33–8.32 mg/mL (EAEP1–3) (Table 1). The increment in the total carbohydrate for EAEP 2 and 3, content can be attributed to the use of cellulase and hemicellulase in the EAEP 2 and cellulase, hemicellulose, and xylanase in EAEP 3, which likely promoted the breakdown of the cell wall cellulose and hemicellulose (Reese, Siu, and Levinson 1950) into smaller carbohydrate structures. While the EAEP increased the overall extractability of chickpea carbohydrates, the amount of sucrose (4.28–4.70 mg/mL) and major oligosaccharides raffinose (0.83–0.97 mg/mL) and stachyose (2.53–2.80 mg/mL) in the skim fractions was not statistically different within the extraction processes evaluated (AEP vs. EAEP 1–3). This is not surprising because the enzymes used in the EAEP 1–3, including alkaline protease, cellulase, hemicellulose, and xylanase, do not target glycosidic linkages in sucrose, stachyose and raffinose to cause their degradation. Sucrose, stachyose, and raffinose were also not expected to be generated, under the action of the carbohydrases, since they are not part of cell wall polysaccharides' structures. Besides, possibly due to the small size of sucrose, stachyose, and raffinose, their extractability was already high in the AEP and did not further increase when the alkaline protease and carbohydrases were used (EAEP 1–3). Because the increment in the total carbohydrate content in the EAEP skims was not associated with the release of monosaccharides (all in trace concentration) nor with an increase in sucrose and major oligosaccharides such as raffinose or stachyose, LC-MS/MS was used to study the potential of oligosaccharide generation by the enzymatic treatments and characterize the composition of the newly generated oligosaccharides.

Table 1. *Total carbohydrates, raffinose, stachyose, sucrose, and monosaccharide concentrations (mg/mL) of AEP and EAEP skims measured by HPAEC-PAD. Monosaccharides include glucose, galactose, and fructose.*

	Total	*Raffinose*	*Stachyose*	*Sucrose*	*Monosaccharide*
AEP	7.68±0.60[c]	0.88±0.03[a]	2.70±0.15[a]	4.70±0.23[a]	trace
EAEP 1	8.37±0.51[b,c]	0.88±0.03[a]	2.75±0.05[a]	4.70±0.15[a]	trace
EAEP 2	9.17±0.52[a,b]	0.83±0.16[a]	2.53±0.43[a]	4.28±0.64[a]	trace
EAEP 3	9.33±0.29[a]	0.97±0.03[a]	2.80±0.00[a]	4.68±0.03[a]	trace

[1] Different letters in the same column indicate statistically significant difference by one-way ANOVA followed by Tukey's test at $p < 0.05$.

3.4.2. α-Galactosidase Treatment of Raffinose and Stachyose in the Skim Fractions

Chickpeas are rich in raffinose, stachyose, and verbascose, whose simplicity of monosaccharide composition (galactose, glucose, and fructose) render them easily fermentable by a variety of intestinal bacteria in a non-selective way that results in the production of undesirable gases that can cause abdominal bloating and discomfort (Sánchez-Mata et al. 1998). To reduce the concentration of flatulence-causing oligosaccharides stachyose and raffinose in the skims, an α-galactosidase was applied to hydrolyze the glycosidic bonds within raffinose and stachyose (Fig 5).

From Figure 5, we can observe that the α-galactosidase treatment completely hydrolyzed raffinose and stachyose in just 15 minutes, which was corroborated by the concurrent increase in the concentration of galactose released from the cleavage of the α-glycosidic bonds. Although

sucrose concentration should increase with the α-galactosidase treatment, the observed decrease in sucrose reflects the hydrolysis of the glycosidic bonds between glucose and fructose by the α-galactosidase (De Moura et al. 2008), indicating that the enzyme used also possesses invertase activity. This can be observed by the simultaneous reduction in the sucrose concentration and increase in the glucose and fructose concentration. Our results are in agreement with the ones reported by De Moura Bell et al. 2013, who reported the complete reduction of stachyose in the skim fractions generated from AEP/EAEP of soybeans by the α-galactosidase treatment. Our results demonstrate that the α-galactosidase treatment can certainly be an effective and fast treatment to reduce the presence of flatulence-causing oligosaccharides in the chickpea extracts.
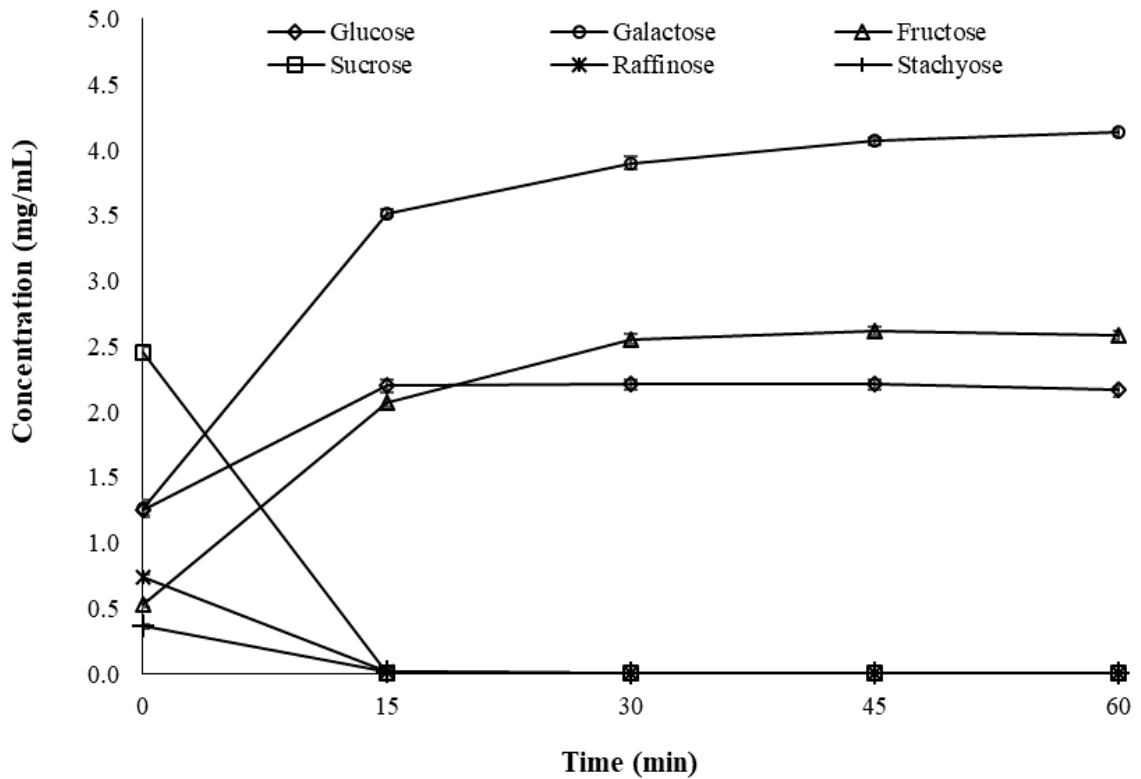

Figure 5. *Sugar concentration kinetics of EAEP 1 skim after α-galactosidase treatment*

3.4.3 Oligosaccharide characterization of AEP and EAEP skims with LC-MS/MS

A total of 60 oligosaccharides were identified from the AEP and the three EAEP skim fractions by inspecting the fragmentation patterns tandem MS spectra (Figure 6 and 7). With the masses of the precursor ions and fragment ions, the monosaccharide compositions of the oligosaccharides were determined. Among the 60 oligosaccharides, 46 contained only hexoses with a degree of polymerization range of 3 to 16 (Figure 6B–F). Stachyose, raffinose, and verbascose peaks, which were identified by comparing the retention times with the authentic standards, were the first three tallest peaks among the 46 hexose oligosaccharides (Figure 6B). As some oligosaccharides could originate from the enzyme formulations used in the EAEPs, the presence of oligosaccharides in the four enzymes used during the extraction was also examined. The results showed that no oligosaccharides were found in the alkaline protease, whereas 31 hexose oligosaccharides found in the skim fractions were also present in at least one of the three carbohydrases used in the EAEP 2 and EAEP 3 (Figure 6D–F). It could therefore be confirmed that the remaining 12 oligosaccharides composed of 3–5 hexose residues and present in similar abundances in the AEP and the three EAEP skim fractions (Figure C–E) are endogenous oligosaccharides in chickpeas.

Ciceritol is a digalactosyl-pinitol present in the skim's fractions in a high abundance (with peak areas close to stachyose; Figure 6A) firstly identified in chickpea (Quemener and Brillouet 1983). Besides ciceritol, seven oligosaccharides with relatively lower abundances possess similar structures to ciceritol (Figure 6A). Among them, four are ciceritol isomers containing two hexose residues and one methyl-inositol; two are composed of three hexose residues and one methyl-inositol; one is composed of four hexose residues and one methyl-inositol. Besides ciceritol, di-

galactosyl-pinitol B and tri-galactosyl-pinitol A were identified in chickpeas in previous studies (Laura Ruiz-Aceituno et al. 2013; L. Ruiz-Aceituno et al. 2017). Although the exact structures of the other hexosyl-methyl-inositol derivatives were not determined, this is the first report of their presence in chickpeas to the best of our knowledge,
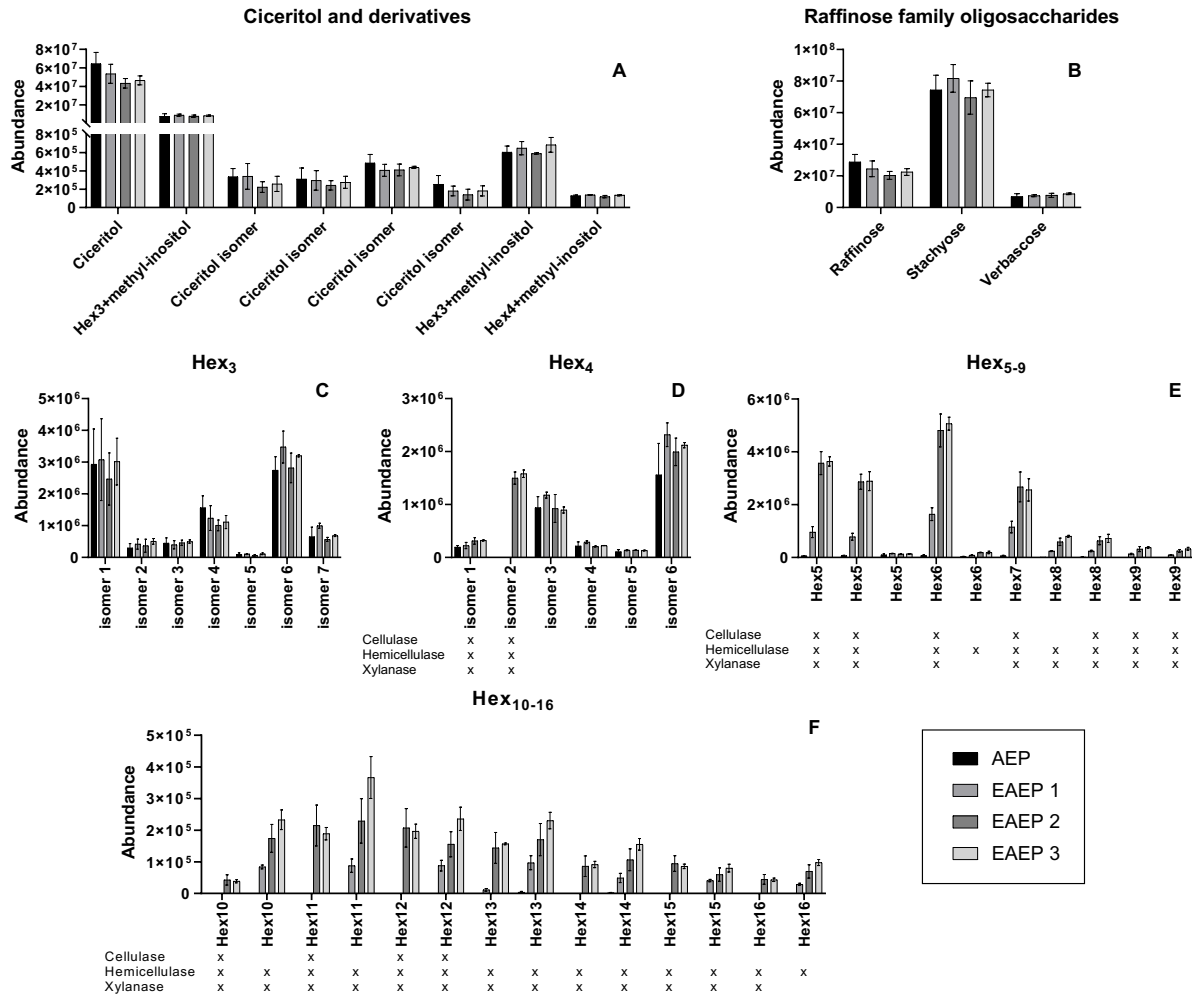
Figure 6. *Relative quantification of oligosaccharides identified in skim fractions generated by AEP and EAEP (excluding the ones exclusively identified in EAEP 2 and EAEP 3).*

Lastly, six oligosaccharides were exclusively found in the skim's fractions generated by EAEP 2 and EAEP 3 (Figure 7). These oligosaccharides were not found in the enzymes used in the EAEP 2 and EAEP 3, indicating that they are likely products from polysaccharide depolymerization under the action of the carbohydrase enzymes used during the extraction. The monosaccharide composition of the six oligosaccharides, with signal intensities from high to low, are Hex_4Pent_3, $Hex_3dHex_3Pent_3HexA_1$, Hex_2Pent_2, $Pent_7HexA_1$, Hex_5Pent_3, and $Hex_2dHex_3Pent_3Hex_1$. Because these oligosaccharides all contain multiple pentose units and other non-hexose monosaccharide units, they were clearly not derived from the depolymerization of cellulose, which consists of linear chains of β-1,4 linked glucose. Based on the monosaccharide compositions, the oligosaccharides were possibly generated from hemicellulose (e.g., xyloglucans) and pectin (e.g., from its component rhamnogalacturonan) (Tosh and Yada 2010; Yoo et al. 2012; Wood et al. 2014). Due to the lack of digestive enzymes in the human gastrointestinal tract able to hydrolyze the glycosidic linkages in plant cell wall polysaccharides, the newly generated oligosaccharides with diverse monosaccharide compositions in the skims of EAEP 2 and EAEP 3 could be potentially novel prebiotics.
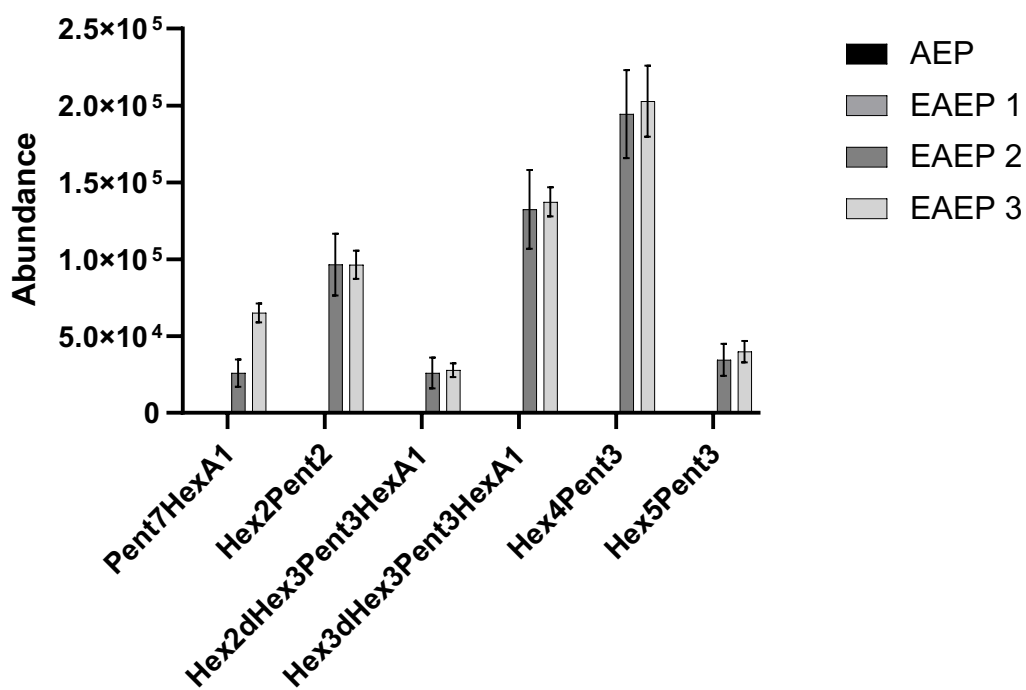
Figure 7. *Relative quantification of oligosaccharides exclusively identified in skim fractions generated by EAEP 2 and EAEP 3.*

It was initially expected that more oligosaccharides could be generated through hydrolyzing polysaccharides by carbohydrases during EAEP 2 and EAEP 3, but in reality, only six oligosaccharides were found in the two skim samples in relatively low abundances. One plausible explanation for this result is that the carbohydrases used in the current study are mainly endo-cleaving enzymes, which can efficiently decrease the size of polysaccharides. Additionally, it is worth to consider that some of the products generated by the enzymatic depolymerization of the polysaccharides might be larger than the size of oligosaccharides (3–20 monosaccharide units) and therefore not measured by LC-MS/MS. The reduction in molecular weight or the increase in solubility of cell wall polysaccharides could lead to the higher total carbohydrate content in the skims of EAEP 2 and EAEP 3 than EAEP 1. However, according to (Brummer, Kaviani, and Tosh

2015), chickpea soluble fiber polysaccharides have a number average molecular weight (M_n) of 419 kDa and a weight average molecular weight (M_w) of 2,103 kDa. The massive size of the soluble polysaccharides may create steric hindrance issues for the l endo-cleaving enzymes decreasing their accessibility and performance. Moreover, the insoluble polysaccharides, which are more abundant than the soluble ones in chickpeas (Tosh and Yada 2010) might have even larger molecular sizes which are not possible to measure with the current analytical tools. Thus, more intense enzymatic treatment might be needed to hydrolyze those and generate oligosaccharides. To further increase the concentration and diversity of novel oligosaccharides from chickpea polysaccharides, additional enzyme screening and process optimization would be needed.

3.5. Effects of Enzymatic Treatment on *in vitro* Digestibility of Skim Proteins

Extraction conditions, especially proteolysis, can significantly alter the *in vitro* digestibility of the extracted protein. In addition to being soluble proteins, increased digestibility of chickpea proteins is another important functional property because it can promote nutritional benefits through higher intestinal absorption. The larger proteins observed in the AEP skim (section 3.2) can hinder digestibility, while the hydrolysis of those larger structures could improve its overall breakdown during human digestion (Sánchez-Vioque et al. 1999).
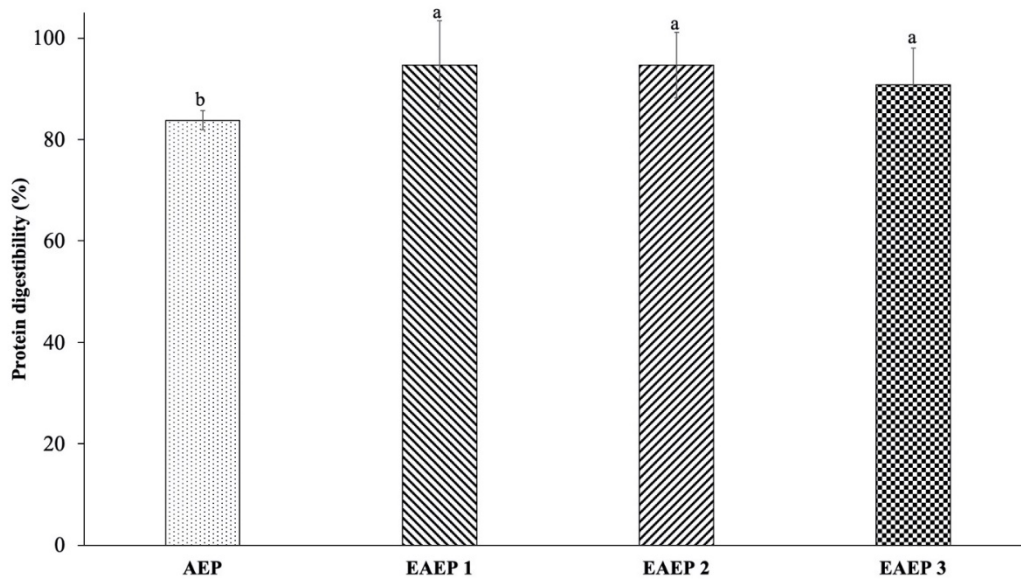
Figure 8. *Effects of extraction processes (AEP and EAEP) on the in vitro digestibility of the skim proteins. Different letters indicate statistically significant difference by one-way ANOVA followed by Tukey's test at p<0.05.*

The *in vitro* digestibility of skim proteins from the non-enzymatic and the three enzymatic treatments are shown in Figure 8. The digestibility of all EAEP skim proteins was significantly higher than that of the AEP skim, highlighting the effectiveness of the use of enzyme during the extraction on protein digestibility. Enzymatic hydrolysis in the EAEP significantly increased protein digestibility from 83.81% ± 1.86 (AEP), to 94.67% ± 8.70, 94.67% ± 6.47, and 90.79% ± 7.21 for the EAEP 1, 2, and 3, respectively, with no significant differences within the enzymatic treatments. As expected, the addition of carbohydrases did not significantly alter the in vitro digestibility of the EAEP skim proteins. Increased protein digestibility of the EAEP skim proteins can be attributed to breakdown of large protein bodies into smaller sizes by the protease (de Souza et al. 2020; He et al. 2015), which is validated by the results from degree of hydrolysis and molecular weight analysis (Section 3.3). Clemente et al. 1998 reported that the digestibility of raw

chickpeas increased from 71.8 ± 1.0 to $83.5 \pm 0.1\%$ after cooking, similar to the findings of Attia et al. 1994. Cooking chickpeas leads to protein denaturation and unfurling of the protein bodies, which improves the access to proteolysis by the saliva, gastric, and intestinal fluids. The digestibility of the AEP skim is similar to that of cooked chickpeas found by Clemente et al. 1998, which could be attributed to the steaming of the chickpeas prior to milling. Goertzen et al. 2020 reported digestibility values of 73.71 and 82.22% for untreated chickpea flour and isolate, respectively. However, no improvements in protein digestibility were observed when pepsin, trypsin, or papain were used to hydrolyze chickpea protein isolate. It is worth mentioning that in their study, enzymes were used to hydrolyze chickpea protein isolate while in our work, enzymes were used to extract proteins from the steamed chickpea flour, which lead to a significant increase in protein digestibility (from 83.8 to 94.6%). Our results highlight that the use of selected enzymes to assist the extraction of full-fat chickpea flour is an effective strategy not only to improve protein extractability but to significantly enhance protein in vitro digestibility and solubility.

4. Conclusions

The extraction methods proposed in this work were effective to improve the overall protein extractability from full-fat chickpea flour and significantly enhance the nutritional quality and functionality of the extracted proteins without the need for upstream lipid removal by solvent extraction or mechanical pressing. Our results highlight that the use of Alkaline Protease in the extraction significantly increased oil (from 49.8 to 77%) and protein extractability (from 62.8 to 84.0%) from full-fat chickpea flour. Enzymatic hydrolysis resulted in the production of smaller (MW <21.5 kDa) and more soluble proteins, which in turn increased protein solubility from 25.6 to 73% at acidic pH, where protein solubility is unfavored. Importantly, enzymatic hydrolysis

significantly increased in vitro protein digestibility from 83.8 to 94.6%, which can therefore enhance the nutritional value of the protein extract. The additional use of an α-galactosidase treatment was successful to completely reduce the presence of flatulence-causing oligosaccharides such as raffinose and stachyose in the protein extracts. While the use of carbohydrases before the addition of the protease did not significantly increase oil and protein extractability, LC-MS/MS revealed the presence of new oligosaccharides structures in the protein extract when the carbohydrase pretreatment was employed, warranting future investigation of the potential prebiotic properties of the new oligosaccharides. The results presented herein highlight that target enzymatic hydrolysis, specifically by protease, can not only maximize the extractability of lipids and proteins but can also produce proteins with high solubility and digestibility, which can be exploited to provide the food industry with plant-based proteins that are highly functional, applicable, and produced by an environmentally friendly process.

Chapter 3

Two-stage Countercurrent Extraction of Lipids and Proteins from Full-fat Chickpea Flour: Maximizing Process Extractability and Economic Feasibility

1. Introduction

Consumers' desire for plant-based diets and the development of new plant-based products by the food industry are driving the surge in pulse crop production such as chickpeas, lentils, dry beans and peas (USDA). The shift towards a plant-based diet is evidenced by the plant protein market growth, which is expected to rise from the $17,222.9 million value in 2019 to $27,965.9 million by 2027 (Joshi 2021). The increasing interest in plant-based products has been primarily attributed to the health benefits associated with the consumption of plant-based diets (i.e., reduction of cardiovascular diseases, gluten-free) and with the sustainability aspect associated with the production of plant-based ingredients (Wild et al. 2014; Naghshi et al. 2020). Environmental impact review of consumption of plant-based ingredients has shown a significant reduction in the production of greenhouse gases and water usage compared to that of traditional food diets and systems (Mekonnen and Hoekstra 2010; Sheldon et al. 2017). This rapid growth in the plant-based market has spurred additional interest in developing and improving processing techniques for the production of highly extractable and functional macronutrients that can supply current plant-based demand.

Chickpeas are an example of a pulse crop whose production in the U.S. increased from 133 million pounds in 2000 to 624 million pounds in 2019 ("USDA Commodity Highlights" n.d.). Like other pulse crops, the consumption of chickpeas is supported by its nutritional value. Chickpeas are a source of proteins (19%), carbohydrates (60%), lipids (17%), and dietary fiber (17%) (U.S. Department of Agriculture 2019). In addition to its high protein content, chickpea proteins have a subtle taste and desired functional properties (Ghribi, Maklouf Gafsi, et al. 2015), making chickpeas a worldwide staple crop that can be used as a matrix to deliver highly sought products and/or ingredients for the manufacture of food products (Duranti and Gius 1997).

Chickpea protein extraction is traditionally accomplished by the use of an upstream lipid removal step involving defatting of the chickpea flour with organic solvents such as hexane. To address the environmental, safety, and public concerns associated with the use of hexane, flammable solvent-free extraction processes such as the aqueous (AEP) and enzyme-assisted aqueous extraction processes (EAEP) have been used to simultaneously extract proteins, lipids, carbohydrates, among other minor compounds, from several food matrices (K. A. Campbell and Glatz 2009; de Queirós et al. 2021). The immediate advantage it carries is the complete elimination of the upstream lipid removal step by either solvent extraction or mechanical pressing. Instead, AEP and EAEP rely on the solubilization of proteins into the extraction medium (water) which then favors the washing out of oil droplets through the porous structure (De Moura et al. 2011; M. H. Cheng et al. 2018). Enzyme addition to the AEP leads to the EAEP, where proteases and carbohydrases are commonly used to further catalyze the extraction of lipids and proteins (Dias et al. 2020). Oil and protein extractability in the EAEP can significantly increase due to enzymatic hydrolysis of the lipid body membrane, proteins, and cell walls (Nadar, Pawar, and Rathod 2017). Under controlled conditions (i.e., pH, temperature, reaction time, enzyme use), water is mixed with full-fat flour resulting in a slurry with the dispersed food matrix. The slurry is subsequently centrifuged to fractionate the insoluble fraction (the fiber-rich fraction containing unextracted proteins and lipids) from the liquid phase containing the extracted compounds. The liquid phase is further fractionated by density difference into free oil, cream (an emulsion that contains most of the extracted oil), and skim (protein-rich phase) (Wu, Johnson, and Jung 2009; J. De Moura et al. 2011).

We have previously evaluated the effectiveness of different enzymes to assist the extractability and distribution of lipids, proteins, and carbohydrates from full-fat chickpea flour (Chapter 2). The

AEP (pH 9, 1:10, SLR 50 °C, 60 min) achieved oil and protein extraction yields of 48% and 63% while the EAEP (AEP conditions with additional 0.5% (w/w) protease) resulted in oil and protein extraction yields of 77 and 86%, respectively. Besides increasing the overall process extractability, the use of proteases and carbohydrases to assist the extraction generated proteins with higher solubility and digestibility in addition to releasing new oligosaccharides molecules in the protein-rich extract. However, the successful development of extraction methods for new protein sources depends on the development of fundamental knowledge of the impact of key processing conditions employed (i.e., pH, time, type of enzyme and concentration, solids-to-liquid ratio (SLR), temperature) (De Moura et al. 2011) on the extractability, composition, and functional properties of the extracted compounds. Enzyme specificity and concentration play a key role not only in the overall extractability of lipids, proteins, carbohydrates, and other minor soluble compounds from the matrix but also affect the separation of the fractions obtained (cream, skim, and insoluble fractions), the strength of the cream emulsion produced (thus affecting the final recovery of the extracted oil), the functional and biological properties of the extracted proteins, and the recovery of the extracted protein (i.e., by isoelectric precipitation or ultrafiltration) (de Queirós et al. 2021; Dias et al. 2020; Souza et al. 2019; Neiva M De Almeida et al. 2019; De Moura et al. 2011). Common processing challenges in AEP and EAEP include maximizing the overall processing extractability of the desired compounds; to achieve adequate distribution of the extracted compounds (i.e., more oil in the cream and less oil in the skim, and more protein in the skim); to recover the extracted oil, which is mostly entrapped in the cream emulsion, through the development of chemical or enzymatic strategies to breakdown the emulsion to free the cream oil; and to minimize the water usage in the process without reduction in extractability (J. De Moura and Johnson 2009; Dias et al. 2020; Neiva M De Almeida et al. 2019).

Higher extraction yields in AEP and EAEP have been accomplished by the use of single-stage extractions, which require higher water usage. In general, low solids-to-liquid ratio (SLR) (1:10) have been used to maximize process extractability of many food matrices ((K. A. Campbell et al. 2011; De Moura et al. 2008). The use of low SLR (i.e., higher amount of water) has been shown to favor protein solubilization and to a less extent the washing or extractability of lipids. As an example, reducing the amount of water used in the single-stage EAEP of soybeans from 1:10 to 1:5 resulted in reduced extractability of lipids and proteins from soybean flakes as demonstrated by the increase in the lipid (from 5 to 10%) and protein (from 12 to 20%) content of the insoluble fraction (extraction byproduct) (J. De Moura and Johnson 2009).

Countercurrent extraction approaches with the use of flammable solvents such as hexane have been widely used by the oil industry to reduce solvent use without loss in the process extractability (K. A. Campbell et al. 2011). Because of increasingly restrictive environmental, safety, and consumer concerns regarding the use of flammable solvents such as hexane, this strategy has also been applied in the AEP and EAEP of soybeans, defatted sunflower seed meal, and Antarctic Krill (Kerry A. Campbell et al. 2016; Liao et al. 2018; J. De Moura and Johnson 2009). Countercurrent extraction systems rely on contacting the freshest feed (starting material) with a richer extraction medium originating from a previous extraction, where the feed continues to be extracted until near-depleted feed contact the fresh extraction medium. The development of a two-stage countercurrent extraction EAEP for extruded soybean flakes proved to be successful not only to reduce the amount of water used in the process by approximately one-half but also to increase protein (from 87 to 96%) and oil (from 96 to 98%) extraction yields compared with the single-stage extraction process.

While previous studies have unveiled the impact of AEP and EAEP for many other legumes and oilseeds, there is limited information about the effects of key extraction parameters

such as SLR, amount and type of enzyme, extraction pH on the overall extractability of lipids and proteins from chickpea full-fat flour. As a consequence, there is also a lack of economic analysis to compare the processing feasibility of current processing practices (upstream lipid removal by solvent extraction followed by aqueous extraction of chickpea proteins) with the use of AEP and EAEP, which simultaneously extract chickpea lipids, proteins, and carbohydrates without the use of flammable solvents.

The overall goal of this work was to evaluate the impact of the amount of enzyme used to assist the extraction, reaction time, and amount of water used in the process on oil and protein extractability, and processing economics. The specific objectives of this work were to: a) evaluate different concentrations of protease (0 to 0.75%) and extraction time (15 to 75 min) in the single-stage EAEP of chickpea full-fat flour; b) determine the impact of increasing solids-to-liquid ratio (1:15 to 1:6) on the overall extractability of the single-stage EAEP of chickpea full-fat flour; c) evaluate the effectiveness of a two-stage countercurrent extraction to reduce water usage while maintaining or increasing oil and protein extractability, and d) perform an economic analysis of the single-stage and two-stage countercurrent enzyme-assisted extraction processes.

2. Materials and Methods

2.1 Chickpea Flour and Enzyme use in the EAEP

Steamed chickpea flour of Kabuli chickpeas was kindly provided by Natural Products, Inc (Grinnell, Iowa, USA). The starting material was analyzed as described in Section 2.3 and contained $7.37 \pm 0.1\%$ oil, $25.87 \pm 0.07\%$ protein, and $4.69 \pm 0.09\%$ moisture. FoodPro Alkaline

Protease, also known as Protex 6L, a commercial bacterial alkaline endoprotease from *Bacillus licheniformis* with pH activity from 8.0 to 10.5, temperature from 45 °C to 75 °C, and enzyme activity of 580,000–650,000 DU/g (Genencor Division of Danisco, Rochester, NY, USA), was used for enzymatic extractions.

2.2 Effect of Enzyme Concentration and Reaction Time on Extractability of the Single-stage Enzyme-Assisted Extraction Process of Chickpea Flour

Enzyme kinetic studies were conducted to determine the effects of the amount of protease used to assist the extraction and reaction time on total oil (TOE) and protein (TPE) extraction yields. Extractions were performed by dispersing 50 g of chickpea flour into 500 mL of water to achieve a 1:10 solids-to-liquid ratio (SLR). The addition of FoodPro Alkaline Protease at concentrations of 0% (AEP, control), 0.3%, 0.5%, and 0.7% and extraction time of 15, 30, 45, 60, and 75 minutes were evaluated. The slurry pH was adjusted to pH 9.0 to favor protein solubility and extractability (Neiva M De Almeida et al. 2019) and kept at 50 °C under constant stirring of 120 rpm. The insoluble fraction (spent solids) was separated from the liquid (extracted compounds) by centrifugation of the slurry at 4000 x g for 30 min at 4 °C. Subsequent fractionation of the liquid fraction was accomplished by allowing the liquid fraction to settle overnight at 4 °C in a separatory funnel. After overnight settling, the liquid fraction was separated into skim (protein- and carbohydrate-rich fraction), cream (oil-rich fraction), and free oil, which were stored and analyzed for the distribution of the extracted oil and protein (Figure 1 A). Total oil (TOE) and protein extraction yields (TPE) were determined according to Equations (1) and (2), respectively:

Total Oil Extraction Yields

$$TOE\ (\%) = [100 - (\frac{Oil\ (g)\ in\ Insoluble}{Oil\ (g)\ in\ chickpea\ flour})] \times 100 \qquad (1)$$

Total Protein Extraction Yields

$$TPE\ (\%) = [100 - (\frac{Protein\ (g)\ in\ Insoluble}{Protein\ (g)\ in\ chickpea\ flour})] \times 100 \quad (2)$$

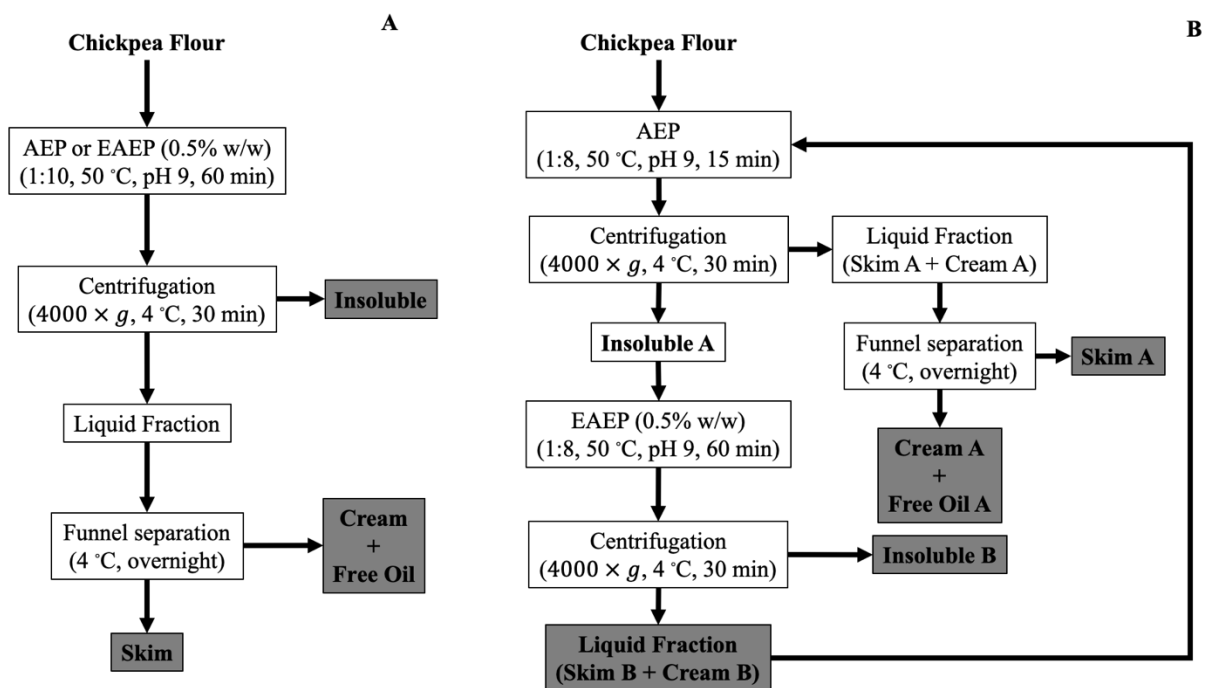Each extraction condition was carried out in triplicate.

Figure 1. *Simplified Single-stage (A) and Two-stage (B) countercurrent EAEP of chickpea flour flow chart*

2.3 Effects of Solids-to-Liquid Ratio (SLR) on the Extractability of the Single-stage Enzyme-assisted Aqueous Extraction Process of Chickpea Flour

After the identification of the amount of enzyme and reaction time leading to higher oil and protein extractability (Section 2.2), the effects of different SLR on extraction kinetics of the single-stage EAEP were evaluated. Extractions were performed using the selected amount of enzyme and reaction time from Section 2.2, except by the SLR, which were 1:6, 1:8, 1:10, and 1:15 (weight of flour/weight of water). Each extraction condition was performed in triplicate and the overall processing efficiency (TOE and TPE) was calculated according to Eq. 1 and 2, as described in Section 2.2.

2.4 Two-stage Countercurrent Enzyme-assisted Aqueous Extraction Process of Full Fat Chickpea Flour

A two-stage countercurrent EAEP was developed to overcome the reduction in the overall process extractability when a higher SLR is used in the single-stage EAEP (Fig 1 B). Two-stage countercurrent EAEP runs were performed in triplicate, with each EAEP run being composed of three samples of chickpea flour that were sequentially extracted. Each sample was subjected to two extractions in batch mode. Extractions were also conducted without enzyme (AEP, control) to compare the enzyme use impact throughout the process. The first extraction of the first run was performed by dispersing 50 g of chickpea flour into water to achieve a 1:8 SLR. The slurry pH was adjusted to 9.0 and the reaction was performed at 50 °C, for 15 minutes under constant stirring. After the first extraction, the slurry was centrifuged to separate the liquid fraction A (skim A + cream A, which left the process) from the first insoluble fraction (insoluble A), which was used as the substrate for the second extraction. The second extraction was performed by dispersing the first insoluble fraction into water to achieve 1:8 SLR and by adjusting the slurry pH to 9.0 prior to

the addition of 0.5% protease (w/w). The second extraction was conducted at 50 °C for 60 minutes under constant stirring. The resulting slurry was centrifuged to separate the second insoluble fraction (insoluble B) from the liquid fraction B (skim B + cream B), which was subsequently used to slurry the first extraction of the second sample, where fresh incoming chickpea flour was used. This was the first time that the enzyme used in the second extraction was recycled into the first extraction of the following sample. The second extraction of the insoluble fraction of the first extraction of the second sample was performed as described for the first sample and this procedure was repeated for the third sample. The sequential extraction of the three samples, constituting one complete run, was repeated three times. Only samples (insoluble, cream, and skim) collected from the third sample of each run were analyzed to ensure that enzyme had been adequately recirculated throughout the process.

Oil and protein yields in the insoluble, skim, and cream fractions of the third sample of each run were calculated according to Equations (3) and (4).

$$Oil\ distribution\ in\ the\ fractions\ (\%) = (\frac{Oil\ (g)\ in\ fraction}{Oil\ (g)\ in\ chickpea\ flour}) \times 100 \qquad (3)$$

$$Protein\ distribution\ in\ the\ fractions\ (\%) = (\frac{Protein\ (g)\ in\ fraction}{Protein\ (g)\ in\ chickpea\ flour}) \times 100 \qquad (4)$$

2.5 Proximate analysis

Cream, skim, insoluble, and starting material (chickpea flour) 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). Oil content was determined by using the Mojonnier acid hydrolysis (AOCS method 989.05), and protein content by using the Dumas

method and a conversion factor of 6.25 (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 chickpea flour. All analyses were conducted in duplicate, and a mass balance was provided for all extracted compounds.

2.6. Viscosity Analysis

The viscosity of slurries with different SLR (1:6, 1:8, 1:10, 1:15) was determined using a viscometer (Ametek Brookfield DV2T Rotational Viscometer, Middleborough, MA, USA). The slurries evaluated in this experiment were produced with 600 g of water basis. 600 g of DI water was placed in a 600 mL beaker and the corresponding flour amounts were added for each SLR by weight; 40 g of flour for 1:15, 60 g of flour for 1:10, 75 g of flour for 1:8, and 100 g of flour for 1:6. Each slurry was raised to 50 °C and pH 9.0. Viscosity analysis of each slurry was performed with the use of the spindle LV-1 and an appropriate rpm was selected such that the % torque would fall within the acceptable range of 10 – 100%. The viscosity of each SLR sample at the appropriate rpm was measured in duplicate.

2.7. Techno-economic Analysis

SuperPro Designer v12.0 (Intelligen, Inc., Scotch Plains, NJ) was used to model the industrial-scale extraction process and equipment used in the single-stage EAEP and two-stage countercurrent EAEP of chickpea flour, as described in Sections 2.2-2.4, for the production of proteins and lipids. The economic impact of both processes, mainly net operating costs, and water usage between the best single-stage EAEP condition and two-stage countercurrent EAEP was compared. The processes were modeled according to processes described in Figure 1, with

the assumption that 100% of the skim protein and cream oil could be recovered. For comparison, the revenue and operating costs were considered to analyze the more profitable process, as well as the return of investment and the payback time.

2.9. Statistical Analysis

Each extraction process was performed in triplicate and each sample was analyzed in duplicate. Replicates of each measurement were analyzed by ANOVA with generalized linear models from the Statistica software (version 13.5.0.17 1984-2018, TIBCO Software Inc, Palo Alto, CA, 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 Effect of Enzyme Concentration and Reaction Time on Extractability of the Single-stage Enzyme-assisted Extraction Process of Chickpea Flour

Previous work demonstrated that the use of enzyme to assist the extraction of full-fat chickpea flour was effective to not only significantly increase oil and protein extractability but also to produce extracts containing more digestible proteins and new oligosaccharides (Chapter 2). The use of 0.5% of FoodPro Alkaline Protease, regardless of the use of a carbohydrase pretreatment or not, increased oil extractability from 49.8 to 77.15% and protein extractability from 62.8 to 83.49%, compared with the AEP, respectively.

To improve upon the extraction processing conditions previously identified, we determined the effects of different FoodPro Alkaline Protease concentrations and reaction time with respect to oil and protein extraction kinetics (Fig 1). Oil and protein extraction yields from the kinetics study are described in Figure 2.
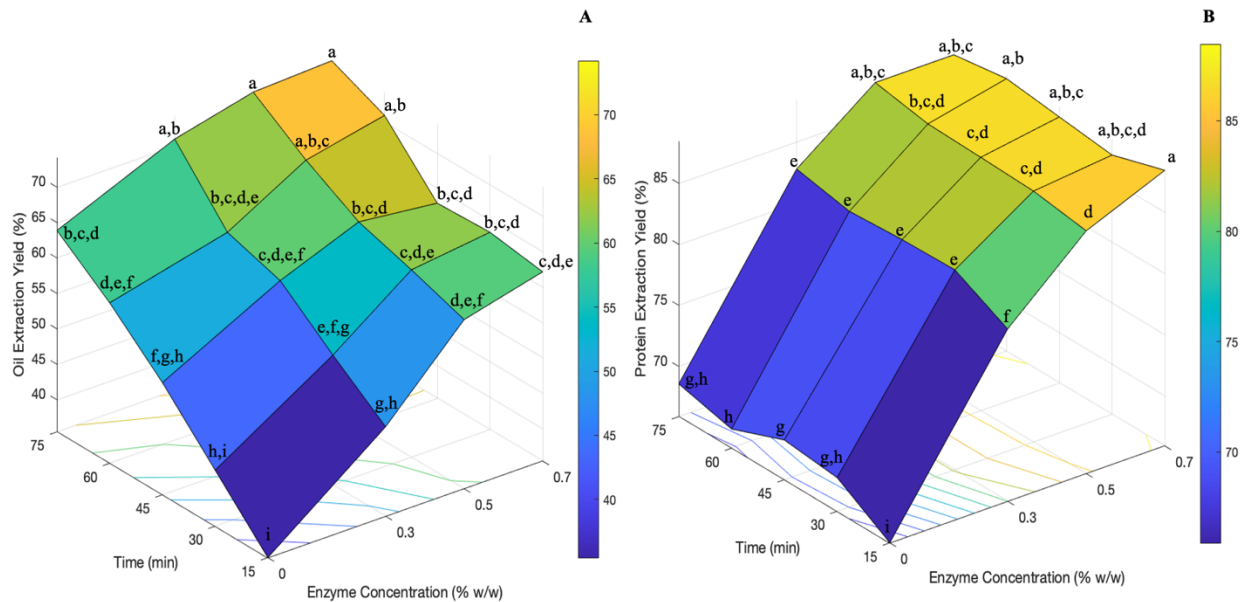
Figure 2. *Total oil and protein extraction yields at each time point and enzyme concentration. Different letters indicate statistically significant differences by a two-way ANOVA followed by Tukey's test at p<0.05.*

For each enzyme concentration evaluated, oil extraction yields increased with higher reaction time; while for each reaction time, the use of higher enzyme concentrations favored oil extraction yields (Fig 2a). However, the statistical significance of the increments observed varied within the experiments. For example, the use of 0.5% enzyme significantly increased TOE from

59.33 to 73.68% when reaction time increased from 15 to 75 minutes, although extraction yields were not statically different ($p < 0.05$) between 60 min (68.53%) and 75 min (73.68%). At reaction time of 60 min, oil extractability significantly ($p < 0.05$) increased from 58.11 to 68.53% when enzyme concentration increased from 0 to 0.5%, with no statistically significant improvement in extractability being observed when enzyme concentration increased from 0.5% (68.53) to 0.7% (70.94%). Overall, the use of a higher extraction time (60-75 min) and higher amount of enzyme (0.5-0.7%) led to increased oil extractability (up to 74.21% for 0.7% of enzyme and reaction time of 75 min). The higher oil extractability observed at longer reaction times and higher enzyme use can be due to increased exposure of the food matrix to the protease, which favors lipid extraction. As described by previous work, proteases can actively hydrolyze oleosin membranes of lipid bodies and release lipids into the extraction medium (K. A. Campbell et al. 2011). In addition, protein removal from the matrix by solubilization or proteolysis leaves behind a more porous structure that facilitates the release of the oil from the matrix (Souza et al. 2019). Increased enzyme concentration allows for higher proteolysis while increased reaction time allows for the protease to fully interact with available substrates. Based on the extraction kinetics results, similar high oil extraction yields (not statistically different at $p < 0.05$), were obtained at the following extraction conditions: 74.21% TOE (0.7% of enzyme and 75 min), 68.53% TOE (0.5% of enzyme and 60 min), 70.95% TOE (0.3% enzyme and 75 min), and 70.94% TOE (0.7% enzyme and 60 min). Based on the lack of statistical difference among the extraction yields above, extraction conditions with reduced enzyme use and reaction time (0.5% of enzyme and 60 min or 0.3% of enzyme and 75 min) could be used as optimum conditions to maximize the oil extractability from full-fat chickpea flour.

When looking at the impact enzyme concentration and reaction time on protein extractability (Fig 2 b), enzyme concentration was the major processing parameter significantly impacting protein extractability, with minimum to no changes in protein extractability being observed within the different reaction times evaluated. The effect of reaction time was only statistically significant at enzyme concentrations varying from 0 to 0.5%, although the magnitude of the increase in protein extractability observed was small, as demonstrated by the general slope, which is not nearly as pronounced as the one describing the impact of reaction time on oil extractability.

For example, at 75 min of reaction time, total protein extractability (TPE) increased from 68.57 to 87.58% when enzyme concentration increased from 0 to 0.5%, with no significant increase in extractability being observed when enzyme concentration was further increased from 0.5 % (87.58) to 0.7% (87.89). At 0.5% enzyme, TPE increased minimally (although statistically significant at $p<0.05$) from 85.78 to 87.58% when reaction time increased from 15 to 75 min, although extraction yields between 30 min (85.78%) and 75 min (87.58%) were not statistically different. According to Fig 2b, the highest TPE (88.47%) was achieved by the use of 0.7% of enzyme and reaction time of 15 min. However, similar TPE (87.59%) was also achieved by the use of a smaller amount of enzyme (0.5%) and a longer reaction time (75 min).

Because the feasibility of EAEP depends on maximizing the extractability of both lipids and proteins from the chickpea flour, the use of 0.5% of protease and reaction time of 60 min, which favored the simultaneous extractability of lipids and proteins, was selected for subsequent experiments. The results presented herein are in agreement with our previous work where oil and protein extraction yields from full-fat chickpea flour of 77.15 and 83.49%, respectively, were achieved when 0.5% of enzyme and reaction time of 60 min were used.

Besides, the results presented herein are in agreement with the ones presented for other food matrices such as soybeans, where the use of an intermediate enzyme concentration (0.5%) favored oil and protein extraction of the EAEP of the almond cake (Souza et al. 2019). Although the extraction kinetics experiments presented herein were useful to determine the necessary amount of enzyme and reaction time needed to maximize the extractability of lipids and proteins from chickpea flour using the single-stage EAEP, the impact of different SLR, which impact the overall process extractability and economics, had yet to be evaluated for the single-stage EAEP of chickpea flour.

3.2 Effects of Solids-to-Liquid Ratio (SLR) on the Extractability of the Single-stage EAEP of Chickpea Flour

Building on the results of the impact of amount of enzyme and reaction time on extraction kinetics, where 0.5% of enzyme and reaction time of 60 min were selected for subsequent extractions, we evaluated how different SLR impact oil and protein extractability from chickpea flour. It is well known that, to a certain extent, higher water usage (low SLR) leads to enhanced protein solubilization into to extraction medium and, to a lesser extent, also enhances oil extractability (J. De Moura and Johnson 2009). The effects of increasing SLRs from 1:15 to 1:6 on oil and protein extractability of the single-stage EAEP of chickpea flour are shown in Fig 3a and 3b.
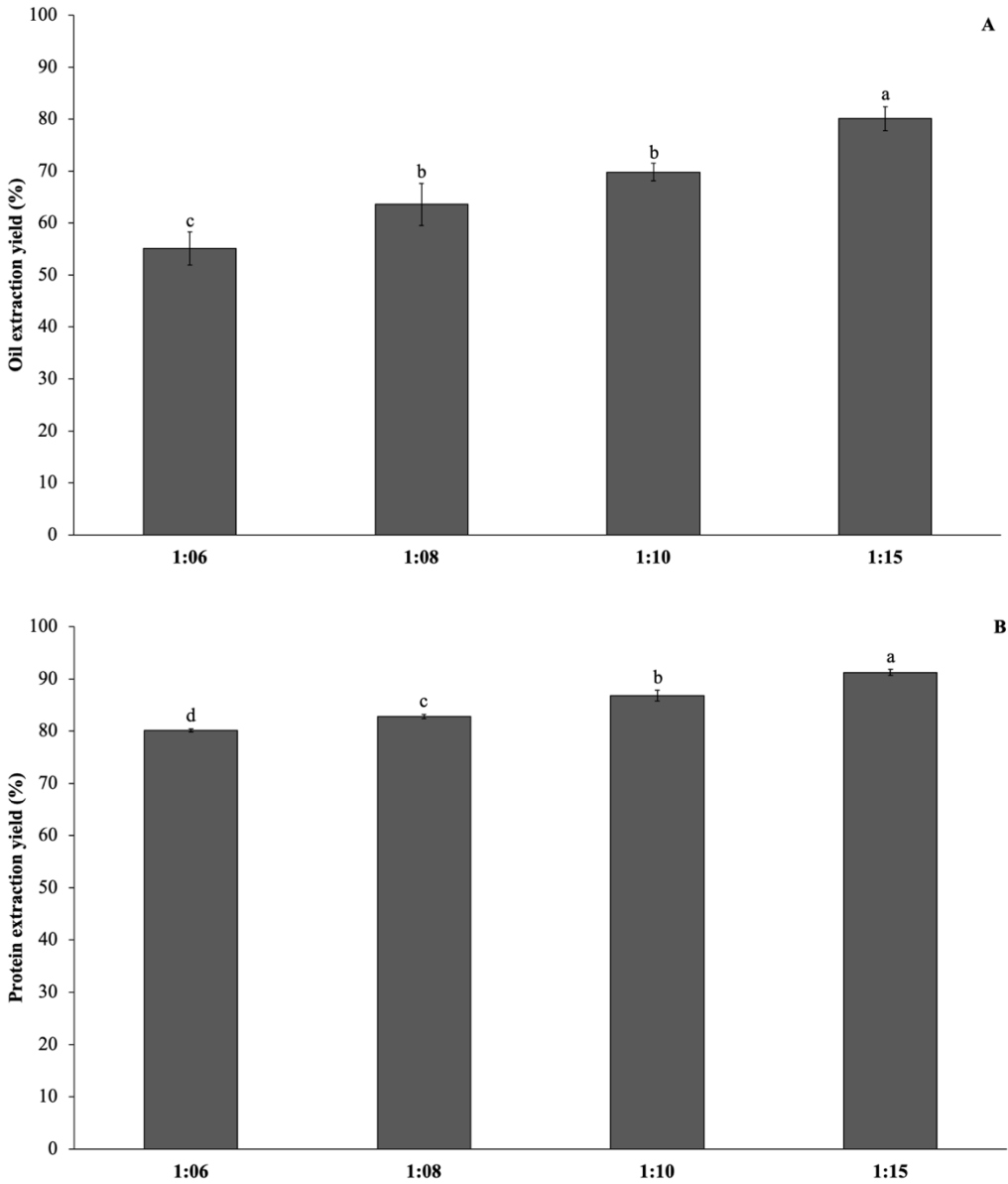
Figure 3. Total oil (A) and protein (B) extraction yields at different solids-to-liquid ratios (SLR). Different letters indicate statistically significant difference by one-way ANOVA followed by Tukey's test at p<0.05. Extractions were performed at pH 9.0, 0.5% of protease (w/w), 50°C, 60 min).

As expected, oil and protein extraction yields significantly increased as SLR decreased from 1:6 to 1:15. Although oil and protein extraction mechanisms are different, often, conditions that favor protein extractability also favor lipid extractability, although at different extents (Souza et al. 2019). Oil extraction yields increased from 55.10 to 80.07% when SLR decreased from 1:6 to 1:15, in agreement with the trend observed for protein extractability. Because of the insolubility of lipids in water, lipids are washed from the porous structure within the chickpea flour matrix into the aqueous solvent (Dias et al. 2020), which is enhanced by reduced viscosity of the extraction medium when SLR decrease. In addition, oil extraction yields can benefit from reduced SLR because of reduced interfacial coverage of lipid bodies by adsorbed proteins (K. A. Campbell and Glatz 2009), which are released into the extraction medium, thus reflecting higher protein extractability.

As depicted in Fig 3B, protein extraction yields significantly increased from 80.14 to 91.20% when SLR decreased from 1:6 to 1:15. At lower SLR (1:15), the higher amount of water increases the gradient between the matrix solute and the aqueous media solute (K. A. Campbell and Glatz 2009), thus favoring the extractability of the solute. In addition, higher water usage also reduced the slurry viscosity, as demonstrated by viscometry analysis (Table 1). The slurry viscosity decreased by ~ 50% when SLR decreased from 1:6 (10.18 cP) to 1:15 (5.15 Cp), in agreement with the higher oil and protein extraction yields achieved when decreasing SLR from 1:6 to 1:15 (Fig 3a and 3b). For soluble materials like proteins, dissolution and diffusion are highly favored under low SLR and reduced viscosity, leading to an increase in extraction yields (Rosenthal, Pyle, and Niranjan 1998).

Our results are in agreement with previous results (J. De Moura and Johnson 2009; Neiva M De Almeida et al. 2019; Souza et al. 2019), which demonstrated that higher extraction yields were achieved at lower SLR for soybeans, almonds, and almond cake, respectively.

Considering the enzyme kinetics and SLR study results, higher oil (80%) and protein (91%) extraction yields can be achieved using 1:15 SLR, 0.5% protease, pH 9.0, 50°C, and reaction time of 60 min, being higher than the 68.5% oil extraction and 86.7% protein extraction achieved under optimum extraction conditions (1:10 SLR, 0.5% protease, pH 9.0, 50°C, and reaction time of 60 min) identified in the enzymatic/reaction time kinetics study. However, the higher extractability observed was achieved at the expense of a lower SLR (1:15 vs. 1:10), which unquestionably has an impact on processing economics. High water usage in the AEP/EAEP leads to higher volumes of effluent that need to be handled, centrifuged, and subsequently spray-dried to produce protein concentrates and isolates, increasing the use of unit operations that are energy-intensive ((J. De Moura et al. 2011; Cater et al. 1974). To address the challenge of maintaining extractability while reducing water usage, a two-stage countercurrent EAEP was developed with the use of 1:8 SLR, which had an intermediate loss in extractability but could bring a significant reduction in water usage compared with 1:15 SLR. Therefore, extraction conditions for the development of the two-stage countercurrent EAEP were: 0.5% enzyme, 1:8 SLR, pH 9 and 50 ºC for 15 (first extraction) and 60 min (second extraction).

Table 1. Effects of SLR on slurry viscosity at different RPM. *Different letters indicate statistically significant difference by one-way ANOVA followed by Tukey's test at p<0.05 within the same RPM row.*

Viscosity (cP)

rpm/SLR	1:6	1:8	1:10	1:15
150	10.18 ± 0.08^a	7.69 ± 0.07^b	6.90 ± 0.17^c	5.16 ± 0.11^d
200	12.20 ± 0.03^a	9.05 ± 0.04^b	7.97 ± 0.02^c	6.12 ± 0.04^d
250	13.96 ± 0.04^a	10.20 ± 0.07^b	8.88 ± 0.01^c	6.80 ± 0.07^d

3.3 Two-stage Countercurrent AEP and EAEP of Chickpea Flour

To reduce the amount of water used in the single-stage EAEP without loss in oil and protein extractability, a two-stage countercurrent extraction process was developed using 0.5% of enzyme, 1:8 SLR, pH 9.0, 50° C and 15 (first extraction) and 60 min (second extraction). A control experiment was performed at the same conditions except for the lack of enzyme use (AEP).
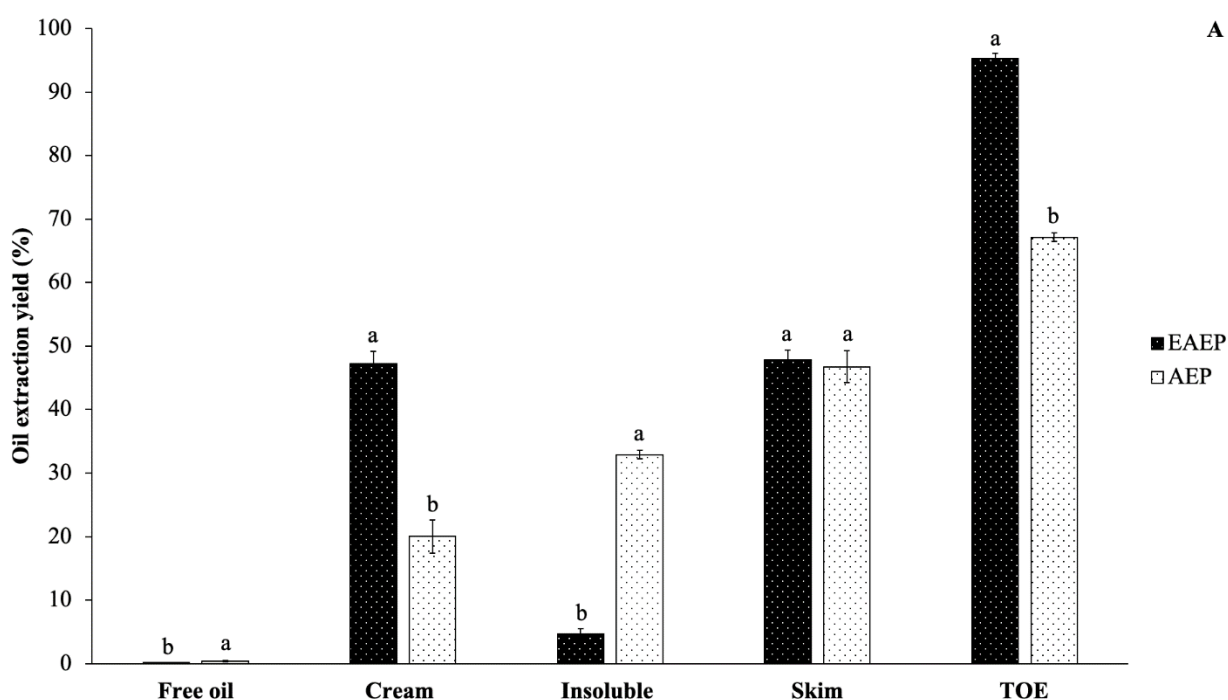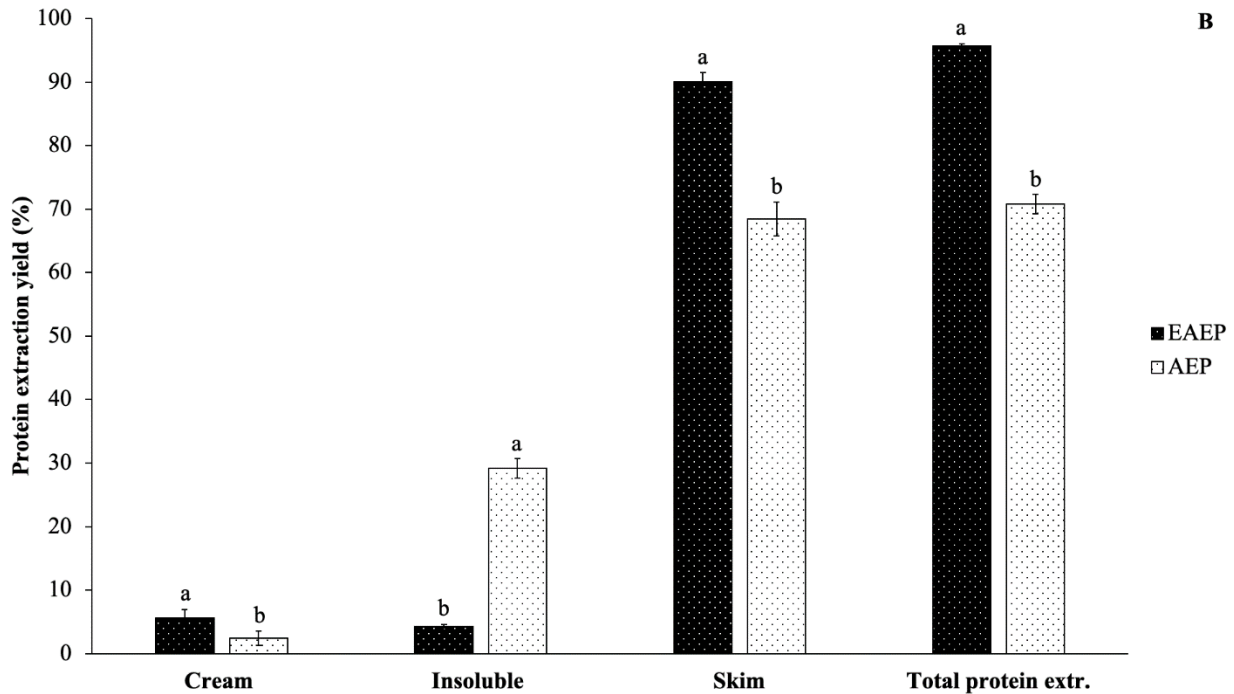
Figure 4. Oil (A) and protein (B) extraction yields and their distribution in the fractions. Different letters indicate statistically significant difference by one-way ANOVA followed by Tukey's test at p<0.05.

Fig 4 a and b describe the effects of the two-stage countercurrent EAEP and AEP on oil and protein extraction yields as well as on their distribution among the fractions generated by the extraction processes of chickpea flour. By contacting fresh incoming chickpea flour with the skim fraction arising from the second-extraction from a previous extraction (where enzyme addition happens), the two-stage countercurrent EAEP (0.5% enzyme) extracted 95.26% of the chickpea flour oil at 1:8 SLR, compared to 61.72% for the two-stage countercurrent AEP (control, no enzyme) (Fig. 4a), highlighting the effectiveness of using a protease to increase TOE yields. As previously discussed, the use of protease during the extraction can benefit oil extractability by

either hydrolyzing the lipid body membrane (i.e., oleosin) or by hydrolyzing the protein matrix, which in turn favor the release of the oil from the matrix (K. A. Campbell et al. 2011; Rosenthal, Pyle, and Niranjan 1996). The extracted oil in the two-stage countercurrent was distributed as 46.35% in the cream and 47.86 % in the skim in the EAEP, in contrast to 20.62% in the cream and 46.74% in the skim for the AEP. Such change in the distribution of the extracted oil demonstrates that the additional oil extracted by the two-stage countercurrent EAEP, compared with the two-stage countercurrent AEP (control), was present in the cream fraction, which could therefore be recovered as free oil for subsequent use if adequate demulsification strategies are developed to breakdown the cream emulsion and release the oil. Since there are no viable methods available to recover the diluted oil present in the skim fraction, shifting more oil from the skim to the cream becomes necessary to maximize the recovery of the extracted oil (N M De Almeida 2014). The TOE yields presented herein were measured for the third sample of each run when the enzyme had been adequately recycled throughout the process. The TOE was also measured for each of the three samples composing each extraction run. Average TOE of 88.12%, 91.93%, and 95.78% and 51.18%, 60.17%, and 73.13% were achieved for samples 1, 2, and 3 of the three runs of the EAEP and AEP, respectively (Fig 5a).   Increased extractability from the extraction of sample 1 to sample 3 might indicate the impact of the adequate recycling of the second skim (saturated solution containing enzyme) into the subsequent extraction (first extraction of incoming flour), favoring overall extractability. TOE in the two-stage countercurrent EAEP (1:8 SLR) of 95.26% was much higher than the optimum 80.07% TOE yield achieved by the single-stage EAEP at 1:15 SLR, indicating the effectiveness of the two-stage countercurrent EAEP in reducing the amount of water used in the process by 60% while increasing TOE by 18.97%.

As observed for TOE, the two-stage countercurrent EAEP achieved significantly higher total protein extractability (TPE) than the two-stage countercurrent AEP (control) as well (Fig 4b). The two-stage countercurrent EAEP (0.5% enzyme) extracted 95.70% of the chickpea protein, compared to 68.39% for the two-stage countercurrent AEP (control, no enzyme) (Fig. 4b). Increased TPE yields for the EAEP can be attributed to the ability of proteases to hydrolyze large protein bodies into smaller peptides which become more soluble in the aqueous media, thus improving protein extractability (de Souza et al. 2020). The extracted protein in the two-stage countercurrent was distributed as 90.07% in the skim and 5.63 % in the cream for the EAEP, in contrast to 68.39% in the skim and 2.43% in the cream for the AEP. Increased protein content in the skim can lead to higher protein purity when producing concentrates or isolates, which would be the main goal of the downstream processing of the skim. The two-stage countercurrent EAEP achieved TPE yields of 95.7% at 1:8 SLR, being higher than the optimum 91.2% TPE yield achieved by the single-stage EAEP at 1:15 SLR. The two-stage countercurrent EAEP was effective in reducing the amount of water used in the process by 60% while increasing TPE yields by ~5% compared with the single-stage EAEP. The higher protein extractability and lower usage of water of the two-stage countercurrent EAEP result in a skim fraction that has a significantly higher protein concentration (3.7%) than that of the single-stage EAEP (1.3% for EAEP) at optimum extraction conditions (0.5% enzyme, 60 minutes, 1:15 SLR). As previously mentioned, this can be beneficial for reducing downstream processing costs of the skim for industrial production of protein concentrates and isolates. The TPE yields presented herein were measured for the third sample of each run, when all fractions had been adequately recycled throughout the process. TPE yields were also measured for each sample composing each of the three runs. TPE increased from 93.67%, 95.62%, and 95.66% from sample 1 to sample 3, reflecting the adequate recycling of the

skim containing the enzyme used in previous second extraction by the extraction of the third sample. The third sample was the first sample to be extracted by recycling the skim fraction that was generated from the second sample extraction, which had enzyme used in both first and second extractions. That was evidenced by the higher protein extractability of sample 3. Although TPE were not statistically different among samples 2 and 3, only samples from the third sample were collected and analyzed to calculate protein extraction yields. TPE yield in the two-stage countercurrent EAEP (1:8 SLR) of 95.70% was much higher than the optimum 91.19% TPE yield achieved by the single-stage EAEP (1:15). Our results demonstrate the effectiveness of countercurrent extraction conditions for improved yields of both oil and protein and reduced water usage.

The total oil and protein extraction yields presented herein are consistent with previous studies which found higher extraction yields with concurrent reduction in the amount of water used in the process (40%) with the two-stage countercurrent EAEP of soybeans, mustard flour, and green coffee flour (De Moura and Johnson 2009; Tabtabaei and Diosady 2013; Souza Almeida et al. 2021)
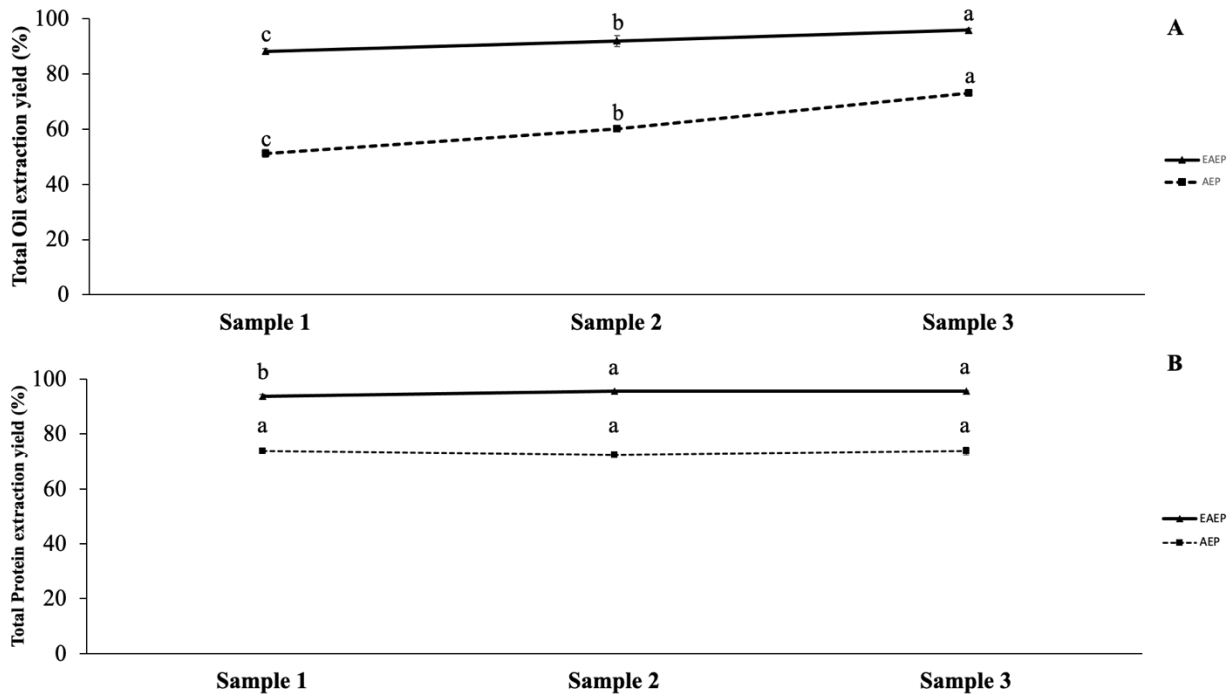
Figure 5. *Total oil and protein extraction yields for the three samples composing each extraction run. Different letters indicate statistically significant difference by one-way ANOVA followed by Tukey's test at p<0.05.*

The two-stage EAEP achieved extraction yields of 95.26% for oil and 95.70% for protein, which is greater than the ones achieved by the single-stage EAEP using 1:10 SLR where oil and protein extraction yields of 77.15 and 83.49% were achieved (23.47 and 14.62% improvement for oil and protein extractability, respectively). The use of a two-stage countercurrent EAEP led to oil and protein extractability increments of 25.71 and 10.14% for green coffee (Souza Almeida et al. 2021) and 2.08 and 5.75% for soybeans (De Moura and Johnson 2009), respectively, when compared with their respective single-stage EAEP. The aforementioned increments in extractability were lower than ones achieved in our study, however, it is important to consider that in addition to the different matrices used in the studies above described, extraction conditions

employed in the single-stage and two-stage countercurrent EAEP were also slightly different (i.e., SLR, reaction time, enzyme use). With the application of the two-stage process, water usage decreases; the amount of water per extraction is significantly less. Additionally, the fact that the two-stage countercurrent EAEP of chickpeas was able to improve extraction yields by such a wide margin over the single-stage EAEP makes the two-stage EAEP a potential alternative strategy to maximize protein and oil extractability.

3.4 Techno-economic Analysis

Comparing the economics of the single-stage EAEP and the two-stage countercurrent EAEP first requires the modeling of the two processes (Figure 6A and B), which was accomplished by the use of SuperPro Designer v12.0 (Intelligen, Inc., Scotch Plains, NJ) and by following the schematic representation of both processes described in Fig 1A and 1B. Continuous models representing the experiments performed at lab scale were developed, and for this scale, the amount of 500 kg of chickpea flour per hour was considered. All materials added to the process were considered to be at room temperature when added to the blending tanks, which would heat the slurry to 50 °C and allow for mixing of the materials with specific residence time. Once the extraction is completed, the centrifugation then separates and cools the slurry down to 25 °C while processing. The selected price points for the revenue streams and material costs were equivalent for both processes (Table 4). In addition, the total amount of chickpea flour used per year was the same to prevent any discrepancies between the two processes. To understand the economic impact of the processes, only the extraction and centrifugation steps were considered and modeled for both processes. Optimum extraction yields (80.07% for oil and 91.20% for protein at 1:15 SLR) were used to model the techno-economic analysis of the single-stage EAEP and for the two-stage

countercurrent EAEP (95.26% for oil and 95.70% for protein at 1:8 SLR). The total net revenue

from each process and the initial equipment investment were used to analyze the feasibility of both

processes. The selling costs of oil and protein listed in Table 4 were gathered from bulk retail

prices and uses the bulk concentration of the desired product in the final outlet revenue stream,
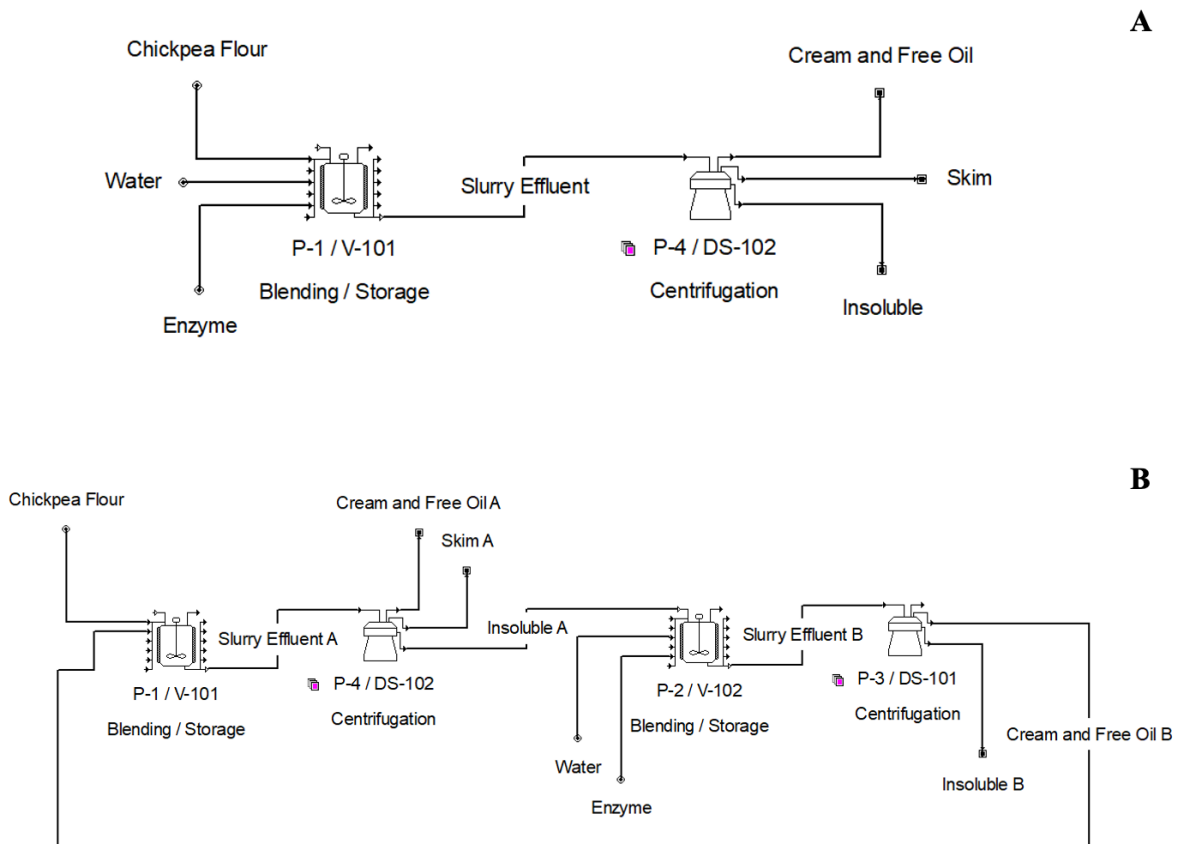
assuming full recovery from them.

Figure 6. *Modeling the techno-economic analysis of single-stage EAEP (A) and two-stage*

countercurrent EAEP (B) of chickpea full-fat flour

Table 4. *Price points, early production and consumption, yearly revenue and operating costs, and total net profit of single-stage EAEP and two-stage countercurrent EAEP of chickpea full-fat flour.*

		Cost per Unit	Single-Stage		Two-Stage	
			Units per Year	Cost per Year	Units per Year	Cost per Year
Revenue	Protein	$20.00	902,021 kg	$18,040,420.00	966,849 kg	$19,336,980.00
	Oil	$15.00	70,091 kg	$1,051,365.00	144,433 kg	$2,166,495.00
Operating Costs	Flour	$2.43	3,960,000 kg	$9,622,800.00	3,960,000 kg	$9,622,800.00
	Enzyme	$23.00	19,800 kg	$455,400.00	19,800 kg	$455,400.00
	Water	$0.0011795	59,400,000 L	$70,062.89	31,680,000 L	$37,366.87
	Labor	$69.00	22,629 h	$1,561,401.00	45,257 h	$3,122,733.00
	Power	$0.10	1,877,163 kWh	$187,716.30	2,012,097 kWh	$201,209.70
	Steam	$12.00	2,996 MT	$35,952.00	3,934 MT	$47,208.00
	Chilled Water	$0.40	429,382 MT	$171,752.80	532,663 MT	$213,065.20
Net Profit				**$6,986,700.01**		**$7,803,692.23**

Overall, the annual net profit is greater for the two-stage process than that of the single-stage process. To determine the net profit, price points for all materials were thoroughly researched. For the revenues, protein and oil costs were determined through retail prices of high-quality chickpea protein and oils. Using resources available, a high-quality chickpea protein concentrate can be sold at $20 per kilogram ("Chickpea Protein Isolate Cost" 2020). Chickpea proteins have a wide range of applications in the food industry due to its renowned mild taste and high solubility and digestibility which can justify the higher price point for chickpeas proteins compared with other plant-based proteins (Chapter 2). Because of the reduced lipid content of chickpeas (~6%), there is a limited supply of chickpea oil that drives its current price to approximately $63 per

kilogram, which was derived from a small-sized product ("Chickpea Oil 30mL" 2020). Chickpea oil is a desirable cosmetic and because of its scarcity, it can command a high price and could possibly be more profitable as a revenue stream per its output compared to the chickpea protein powder. The price point was set to be a quarter of the retail price ($15 per kg), as bulk price points should be lower than the retail price. For the operating costs, the price points for chickpeas are $2.43 per kilogram of flour (NPI Soy FOB, Grinnell, Iowa, USA) and $23 per kilogram of enzyme ("PROTEX 6L Protease 25kg" n.d.), which were the costs of materials used in the lab-scale research. Additionally, the price for water was calculated through local water rates (Davis, CA, USA) to be $0.0011795099 per liter of water ("Water Rates" n.d.). Labor and utilities (power, steam, chilled water) costs were provided for by the SuperPro Designer database.

It is worth mentioning that potential differences in extraction yields and ease of separation into fractions through centrifugation, which might alter the distribution of the extracted compounds within the fractions generated by the process, should be acknowledged when considering process scale-up. While the experimental data used for the technical-economic analysis was based on lab-scale experiments using 500 grams of flour per extraction, the economic analysis was conducted at an industrial scale; using 500 kg of flour per hour. However, based on existing literature (J. De Moura and Johnson 2009; J. De Moura et al. 2011) for soybean processing, oil and protein extraction yields and cream demulsification yields at pilot-scale (75 kg of soybeans) were similar to the ones obtained at lab-scale (2 kg of soybeans) for the integrated two-stage countercurrent extraction, where the enzyme was added during the demulsification was further recycled into the two-extraction stages. While AEP and EAEP generate a protein-rich fraction (skim) that can be subjected to isoelectric precipitation and/or spray-dried or freeze-dried to produce protein concentrates or isolates, the extracted oil is entrapped in an oil-rich fraction (cream) that requires

the development of a demulsification process (i.e., chemical or enzymatic) to breakdown the emulsion and free the oil for subsequent use as food or fuel (Wu, Johnson, and Jung 2009). The use of enzymatic demulsification has been shown to be not only effective to breakdown the emulsion produced by the two-stage EAEP of soybeans but to reduce the overall enzyme use in the process by 35% by recycling the enzyme used during the cream demulsification into the extraction (J. De Moura et al. 2011). Further research is needed to optimize key demulsification parameters (i.e., enzyme use, incubation time, reaction pH) to maximize the release of the cream oil for chickpeas.

When analyzing the revenue stream, the increased protein extractability achieved by the two-stage EAEP led to a slight 7.19% increase in revenue. From analyzing the chickpea oil revenue, a 106.05% revenue increase was observed when moving from the single-stage EAEP to the two-stage countercurrent EAEP due to the significantly higher amount of oil extracted by the two-stage countercurrent EAEP. As discussed previously, the two-stage process improved oil extraction yields to a greater extent (from 80.07% to 95.26%) than did for e protein extraction yields (from 91.20% to 95.70%). When comparing the operating costs of the two processes, the cost of the flour and enzyme is the same, while water costs are higher for the single stage. The use of the two-stage countercurrent EAEP enabled a 47% water cost reduction compared with the single-stage EAEP, reducing yearly water usage from 59,400 MT to 31,680 MT. Finally, labor and utility costs are also different between the single-stage EAEP and two-stage countercurrent EAEP. We observed a large difference in labor costs, where more operational costs are present in the two-stage due to the necessity of managing cyclical extractions and additional reactor and centrifuge usage. The utilities for the two-stage are also higher, but not to the same extent of the labor costs. Because the working volume of the two-stage extraction slurry is reduced, compared with the single-stage

process, the utility demand for the two-stage EAEP is only slightly higher because of the reduction of water usage. The increased revenue and decreased water usage of the two-stage countercurrent EAEP greatly outweighed its higher operating costs. Conservation of water is important to reduce usage demand and lessen the environmental impact of the extraction process and can also decrease the operational and energetic costs associated with further processing separation of the fractions by centrifugation and water removal by spray-drying to produce protein powders. Reducing water usage is a key step to reducing centrifugation costs for the aqueous materials and protein recovery costs for processing the skim, which can satisfy commercial demands (J. De Moura and Johnson 2009).

To understand the feasibility of both processes, the return on investment and the payback time were calculated using the equipment costs provided by the SuperPro database (Table 5).

Table 5. *Equipment costs of the single-stage and two-stage countercurrent EAEP.*

Single-Stage EAEP			Two-Stage Countercurrent EAEP		
Item	**Units**	**Total Cost**	**Item**	**Units**	**Total Cost**
Blending Tank	1	$271,000.00	Blending Tank	1	$206,000.00
Disk-stack centrifuge	4	$2,024,000.00	Blending Tank	1	$261,000.00
			Disk-stack centrifuge	3	$1,533,000.00
			Disk-stack centrifuge	2	$1,080,000.00
Net Equipment Costs		**$2,295,000.00**			**$3,080,000.00**

The payback time was calculated using Equations (5), (6), and (7) (M. Cheng, Rosentrater, and Wang 2016). Scenario 1 represents the investment costs and profit of the single-stage and Scenario 2 represents that of the two-stage process.

$$\Delta Capital\ Investment = Investment\ Scenario\ 2 - Investment\ Scenario\ 1 \quad (5)$$

$$\Delta Net\ Profit = Profit\ of\ Scenario\ 2 - Profit\ of\ Scenario\ 1 \quad (6)$$

$$Payback\ Time = \frac{\Delta Capital\ Investment}{\Delta Net\ Profit}\ years \quad (7)$$

Additional investment is required for scenario 2 (two-stage process) and must be considered for comparison with scenario 1 (single-stage process). Using Equation (7), the payback time for the difference between the two processes was calculated to be 0.96 years. This is due to the difference in net profit being higher than the difference in the capital investment when comparing both scenarios. Overall, the two-stage countercurrent EAEP has higher net profits than that of the single-stage EAEP, which can be attributed primarily to its higher extractability and reduced amount of water used in the process. The two-stage countercurrent EAEP is more advantageous compared with the single-stage EAEP from economic and environmental perspectives.

4. Conclusions

This study revealed the impact of critical extraction parameters (amount of enzyme, reaction time, and solids-to-liquid ratio) and modes of extraction (single-stage and countercurrent extraction) on the simultaneous extraction of lipids and proteins from full-fat chickpea flour and economic process feasibility. While extraction kinetics revealed that protein extractability was primarily favored by higher enzyme use, oil extractability, which benefits from the removal of protein from the matrix, required higher enzyme use and longer reaction times. About 68.5% oil and 86.7% protein were extracted by the use 0.5% protease, pH 9.0, 50°C, reaction time of 60 min,

and 1:10 SLR. Further evaluation of the impact of SLR demonstrated that lower SLR significantly reduced the slurry viscosity, thus increasing oil and protein extractability to 80 and 91%, respectively (1:15 SLR, 0.5% protease, pH 9.0, 50°C, and reaction time of 60 min). The development of a two-stage countercurrent extraction process was successful to reduce the amount of water used in the single-stage extraction while further improving oil and protein extraction yields to 95.78 and 95.66%, respectively. The results of techno-economic analysis of the single-stage and two-stage countercurrent EAEP showed that the two-stage countercurrent EAEP had higher yearly net revenue but also a higher equipment investment cost. The results presented herein further widen the scope of processing standards for full-fat chickpea flour and add to the elucidation of the impact of key processing conditions on the extractability and economic feasibility of the production of chickpea ingredients for subsequent food/nutraceutical applications.

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