IMPROVEMENT OF FUNCTIONAL AND BIOACTIVE PROPERTIES OF CHIA SEED (*SALVIA HISPANICA*) PROTEIN HYDROLYSATES AND DEVELOPMENT OF BIODEGRADABLE FILMS USING CHIA SEED MUCILAGE

by

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ACKNOWLEDGMENT OF PREVIOUS PUBLICATIONS

Some parts of this thesis contain reprint materials of "Physicochemical characteristics of chia seed (*Salvia hispanica*) protein hydrolysates produced using ultrasonication followed by microwave-assisted hydrolysis" including figures, tables and some discussions.

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ABSTRACT

Chia seed (*Salvia hispanica*) has shown potential as an alternative source of nutrients with a high content of fiber (36 %), protein (25%), and fat (25%). Unfortunately, the presence of a viscous biopolymer (mucilage), surrounding the chia seed (CS), limits the accessibility of the protein and other nutrients. Nevertheless, this biopolymer's chemical composition makes it suitable for the development of biodegradable films. Regarding CS protein, disulfide bonding, and nonprotein-protein interactions often frequent in plant protein, have limited its technological application in food matrices. Therefore, scientists have pointed at processing methods involving enzymatic proteolysis to improve the functionality of plant protein ingredients. The objective of this study was to establish processing techniques to exploit the functionality, extraction, and health benefits of chia seed components. First, ultrasonication followed by vacuum-filtration was used to separate mucilage from CS prior to fat extraction by oil press. Mucilage-free and defatted CS were treated using conventional (enzymatic hydrolysis with alcalase) or sequential (enzymatic hydrolysis with alcalase+flavourzyme), and under water bath or microwave-assisted hydrolysis. Chia seed protein hydrolysates (CSPH) derived from the sequential hydrolysis with microwave treatment showed superior ($p<0.05$) in vitro antioxidant activity. The highest ($p<0.05$) cellular antioxidant activity was achieved by the sequential (94.76%) and conventional (93.13%) hydrolysis with microwave. Dipeptidyl peptidase-V inhibition was higher ($p<0.05$) for sequential hydrolysis with water bath, while Angiotensin-Converting Enzyme (ACE) inhibition activity increased ($p<0.05$) with hydrolysis for all treatments compared to the control. Regarding functionality, sequential hydrolysis with microwave showed higher ($p<0.05$) solubility at lower pH (3 and 5), while conventional hydrolysis with microwave was better at pH 7 and 9. Emulsification properties and foaming capacity were also higher in conventional hydrolysis with microwave, but conventional

hydrolysis with water bath was more stable for foaming properties only. In terms of mucilage applicability, biodegradable films were developed by casting technique where CS mucilage was plasticized with different polyol mixtures (sorbitol and glycerol). CS mucilage films with higher sorbitol content showed superior tensile strength (3.23 N/mm^2), and lower water vapor permeability (1.3*109 g/ m*s*Pa) but had poor flexibility compared to other treatments. Conversely, films with high glycerol content showed high elongation at break (67.55%) and solubility (22.75%), but reduced water vapor permeability and tensile strength. The hydrophobicity, measured as water contact angle, was higher ($p<0.05$) for mixtures containing equal amounts of polyols. Lastly, Raman Spectroscopy analysis showed shifts from 854 to 872 cm^{-1} and 1061 to 1076 cm^{-1}, which corresponded to β(CCO) modes. These shifts represent an increase in hydrogen bonding, responsible for the high tensile strength and decreased water vapor permeability. This study demonstrated that ultrasonication followed by vacuum filtration can successfully separate mucilage from chia seeds; microwave-assisted and enzymatic hydrolysis generated protein hydrolysates with improved bioactivity and functionality. Finally, chia seed mucilage was able to form films with potential to be used in drug delivery and edible food coating applications.

CHAPTER 1. INTRODUCTION

The global population is currently around 7.4 billion, twice the population number that can sustainably inhabit the earth (Nadathur, Wanasundara, & Scanlin, 2017). To ensure food security, food scientists must explore new alternatives to provide nutrients according to availability around the world. Proteins are one of the most important macronutrients required for proper development. Proteins are essential macromolecules composed of individual structures called amino acids, which are indispensable for development and maintenance of our body (Ustunol, 2014). The challenge for obtaining protein through conventional agriculture is enormous; it is estimated that for the year 2050 the world will experience a population growth to 10 billion people (Nadathur et al., 2017). For this reason, the use of emerging and sustainable protein sources has become a trending alternative to help alleviate the environmental impact caused by the production of conventional protein sources such as beef, poultry and dairy products. The need to change eating patterns is necessary to provide adequate amount of protein and nutrients to the world's population. Meat proteins have historically been the primary protein source. Nevertheless, the amount of resources required to produce meat from beef, poultry and pork are unsustainable, causing extreme contamination, and increasing the emission of greenhouse gases (Müller, Wolf, & Gutzeit, 2017). Some plants have shown to be high protein sources, with lower environmental impact. Moreover, the current animal-derived dietary patterns have led to an increased development of chronic diseases. Chronic disorders such as coronary heart disease, type-2 diabetes, neurodegenerative diseases, and renal failures were responsible of 39.5 out of 54.7 million deaths in 2016 (Harris, 2019). There are different ways to address these diseases which include, diet education, smoke prevention and excessive alcohol consumption (Harris, 2019). As food scientists, the main approach we can contribute to alleviate the food demand and the increasing rates of chronic

diseases, is to develop, healthy and sustainable nutrient sources that address these issues. It is well known that proteins have bioactive (antihypertensive, antioxidant, antidiabetic or antithrombotic) and functional (foaming, solubility, emulsifying or gelling) properties, and that these properties might be enhanced by protein modification using enzymatic hydrolysis or new technologies (Mine, Li-Chan, & Jiang, 2010; Pacheco-Aguilar, Mazorra-Manzano, & Ramírez-Suárez, 2008).

Chia seed (*salvia hispanica*) is an emerging protein source, originated from Mesoamerica. The use of this seed as a source of protein, is an alternative that has been explored in the past few years. Chia seeds contain a high content of protein (15-25%) fat (30-33%) and fiber (18-30%) (Mohd Ali et al., 2012). An issue has been encountered in the application of these seeds by the presence of a highly viscous polymer called mucilage that limits the functionality and availability of the protein. For example, researchers have investigated the chia seed protein and determined that its digestibility was low compared to other plant sources, and they attributed this to the barrier effect caused by the mucilage, which protects the seed and its components from gastric acids and enzymes, decreasing the application and digestibility of the protein (Monroy-Torres, Mancilla-Escobar, Gallaga-Solórzano, & Santiago-García, 2008). Multiple methods such as lyophilization (Capitani, Ixtaina, Nolasco, & Tomás, 2013), salt extraction (Muñoz, Cobos, Diaz, & Aguilera, 2012), and oven dying (Campos et al., 2016) have been explored for mucilage extraction, but most of them required laborious work and exhibit low yields. New technologies such as ultrasonication have allowed us to extract highly bounded compounds from plant materials. Ultrasonication has been previously used in the extraction of different compounds including proteins, polyphenols, tannins and polysaccharides; however, exploration using different food matrices is still required to address other possibilities (Vilkhu, Mawson, Simons, & Bates, 2008).

In this thesis, we will identify different protein extraction parameters in order to apply ultrasound as an extraction method of mucilage from chia seeds and enzymatic proteolysis to develop functional and bioactive peptides. Finally, we will develop a biodegradable film using chia seed mucilage.

Research Objectives

Objective 1: Develop an improved mucilage extraction procedure by implementing ultrasound techniques.

Hypothesis: The use of ultrasound technology will improve the extraction yield of mucilage, oil and protein from chia seeds.

Tasks:

- Identify the conditions of separation methods (centrifugation and vacuum-assisted filtration) and temperatures (25, 40, and 55 °C) that increase the mucilage separation yields.

Objective 2: Compare functional and bioactive properties of chia seed protein hydrolysates (CSPH) derived from water bath and microwave-assisted hydrolysis.

Hypothesis: Enzymatic hydrolysis will create chia seed protein hydrolysates (CSPH) with improved functional and bioactive properties.

Tasks:

- Extract protein from chia seeds using microwave energy and enzymatic treatments.
- Develop functional: EAI, FC, SOL, and bioactive assays: DPP-IV, ACE, DPPH, FRAP, MIC, and ABTS.
- Determine structural and amino acid composition of CSPH.

Objective 3: Develop a biodegradable film using chia seed mucilage.

Hypothesis: Mucilage will show to have a good mechanical and barrier characteristics for film development.

Tasks:

- Measure fundamental mechanical and barrier properties of chia seed mucilage films such as tensile strength and elongation at break (TS, EB) and rate of water vapor permeability (RWVP).

Chapter 3 and 4 were developed following a systematic approach **(Fig 1)**, where the products from chia seeds were used in the application of different products.

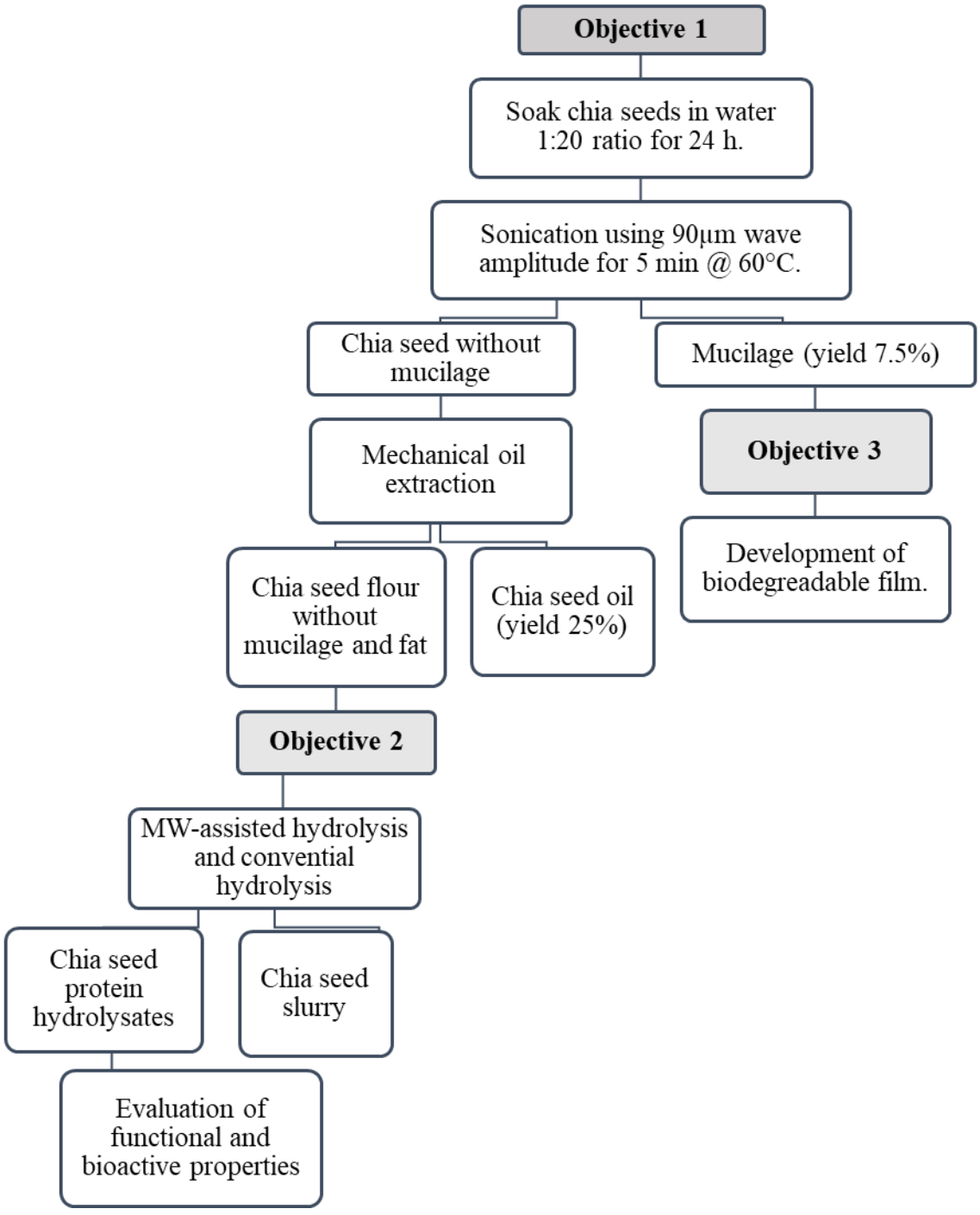

Figure 1. Flowchart for the development of chia seed protein hydrolysates.

CHAPTER 2. LITERATURE REVIEW

2.1 Plant protein composition, structure, and classification

Proteins are molecules essential for life, responsible of structural, functional and biological roles in the body of all living things (Loveday, 2019). Plant proteins contain a broad variety of the 20 amino acids regarding their chemical structure and the fractions present in them. Protein is the most indispensable macromolecule for human and animal nutrition. The amino acids composed of unique hydrocarbon skeletons and elements (nitrogen and sulfur) can't be replaced by any other nutrients, mainly because sulfur and nitrogen are available nowhere else (Wu, 2016). These components are key players in the synthesis of small molecules (glutathione, creatine, nitric oxide, dopamine, serotonin, RNA and DNA) which are required for physiological processes (Wu, 2016). The importance of protein is greatly appreciated in biological processes such as DNA synthesis and replication. In general, the characteristics that define an organism's conformation are stored in their DNA. These molecules mainly contain information for the synthesis of proteins, and proteins are the ones responsible of decoding or copying this information (Damodaran & Parkin, 2017).

2.3.1 Protein structure

The structure of proteins is generally classified in 4 sublevels. First, the amino acids are covalently bonded to one another by the action of the ribosomes via amide bonding and in accordance to the genetic code. The second level is better described as the spatial arrangement of the polypeptide chains due to H-bonding, giving place to an arrangement in form of α- helices, β-turns and sheets, and random coils (Nadathur, Wanasundara, & Scanlin, 2016; Nadathur et al., 2017). The tertiary structure is often described as hydrophobic interactions between non-polar side

chains of amino acids and the disulfide bonds between the cysteine residues that form the secondary structure of the protein itself, then scaffold structures arise as a 3D structure of the polypeptide chain (Nadathur et al., 2017). Is important to mention that the tertiary structure is crucial since it will arise to a vast number of biological roles (e.g., lock and key function in enzymes or receptor sites on cell membranes) (Aldred & Phenols, 2009). Finally, other arrangements exist such as quaternary protein structures, which refer to the spatial arrangement of polypeptides or subunits within the whole structure (e.g., hemoglobulin and ion channels) (Aldred & Phenols, 2009).

2.3.2 Protein classification

Proteins have been classified in different ways throughout the years, and due to the complexity of these macromolecules, a single classification is not possible. Some of the common classifications of proteins are based on solubility, conjugation, shape, function and nutritional value as shown in **Table 1**. One classification that is still used today was established by Thomas B. Osborne in 1908 (Loveday, 2019). This classification is based on solubility and is currently known as the most common plant-protein classification, even though complex mixtures of protein are commonly present in each group and some plant-derived protein groups overlap, it is still a good starting point for protein fractionation (Nadathur et al., 2017).

Table 1. Commonly used protein categories.

Classification criteria	**Categories**
Solubility	Albumins: soluble in water and salt solutions Globulins: not soluble in water, but readily soluble in salt solutions Histones: soluble in acid solutions Prolamins: soluble in ethanol-water solutions Scleroproteins: insoluble in water and salt solutions
Conjugation	Simple: liberates only amino acids when hydrolyzed. Conjugated: liberates other compounds that are usually attached to its structure (e.g., phosphate groups, carbohydrates, lipids, metal groups and nucleic acids)
Shape	Fibrous (rod, fibrous and thread-like) Globular (spherical or ellipsoidal)
Function	Catalytic, structural, regulatory/hormonal, transport, genetic, immune, contractile and storage
Nutritional value	Complete: contains all the essential amino acids Incomplete proteins: missing one or more essential amino acids

(Nadathur et al., 2017).

Plants contain a high amount of proteins that meet biological and structural roles. For plant seeds, the protein content is even higher varying according to the types of seeds (e.g., 10% in cereals, 40% in oilseeds) (Shewry, Napier, & Tatham, 1995). Proteins play metabolic or structural roles to keep the seed integrity and protect it from abiotic factors (e.g., low temperatures, bacterial digestion, cellular respiration), nonetheless a vast portion of the storage proteins serve to store amino acids for seedling growth and germination. Storage proteins are of importance because they will not only determine the protein content in seeds, but also their nutritional quality (Shewry et al., 1995). The main categories found in plants are: albumins, globulins, prolamins, and glutelins. In the case of globulins and albumins, subclassifications are derived from ultracentrifugation-derived Svedberg sedimentation coefficients, which are generally express as Svedberg units (S) (Loveday, 2019). Häkkinen, Nuutila, and Ritala, (2018), identified that albumins are commonly found as 2S whereas globulins as 7-8S and 11-12S. The most common sources of plant protein are from grains (e.g., wheat, rice, millets and sorghum), seeds (e.g., chia, hemp), pulses (e.g., beans,

lentil, peas, lupins), and leaves (e.g., moringa, duckweed) (Nadathur et al., 2017). The distribution of proteins varies according to seed varieties. Studies have shown that cereals such as wheat are especially rich in glutelins, a subgroup of prolamins proteins. In the case of protein from rice, chia seeds, and soy the most abundant type of proteins are the globulins 11-12S (Sandoval-Oliveros & Paredes-López, 2013; Taylor, Taylor, Campanella, & Hamaker, 2016). Legumes are especially abundant in albumins and globulins. Additionally, other type of proteins such as histones are present in much lower concentration in plants but plays a crucial role in the process of post-translational modifications modulating process like floral transition, seed germination organogenesis and morphogenesis (Liu & Min, 2016).

The poor solubility of globulin, prolamins and glutelins is often associated to the condense polymeric state of these fractions caused by high intermolecular disulfide bonding, which results in low hydrophilic residue exposure (Kawakatsu & Takaiwa, 2019). This intrinsic characteristic remains as one of the main challenges limiting the food application of these plant macromolecules in some food matrices (e.g., non-dairy milk substitute, shakes or cereal-protein rich drinks and beverages) (Carbonaro, Maselli, & Nucara, 2012; Clemente et al., 2000; Deshpande & Damodaran, 1989; Ghumman, Kaur, & Singh, 2016; Loveday, 2019).

Other factors such as exogenous interaction of proteins with non-protein components (e.g., starches, dietary fiber, lipids, and tannins) and the high resistance of plant cell walls to digestion, have also limited the digestibility and functionality of these proteins (Pushparaj & Urooj, 2011). Protein structure can greatly influence the capacity of proteolytic enzymes to access the inner structure of proteins, studies have shown that the high presence of β-sheets influenced negatively the protein digestibility of proteins whereas the protein higher ratios of α-helixes, random coils to β-sheets showed a positive correlation to the digestibility values (Bai, Qin, Sun, & Long, 2016).

2.2 Chia seeds (structure, origin, and composition)

Throughout the years, plants have been domesticated and cultivated to obtain novel ingredients and alternative sources of functional foods. One remarkable plant that has shown high potential is chia *(Salvia hispanica)*. Chia is a biannually cultivated plant; it is considered a pseudocereal that produces purple and white flowers that eventually result in small oval shape seeds with sizes varying from 1 to 2 mm (Mohd Ali et al., 2012). Chia seeds were first cultivated in pre-Columbian Mesoamerica. This annual crop was used as a high-value commodity due to its versatility, its medicinal and food applications (Cahill, 2002). The seeds are mainly divided into two semi hemispheric structures that contain the endosperm called cotyledons. The cotyledons are covered by the seed coat, which is composed of three layers endocarp, sclereid and columella **(Fig 2)** (Muñoz et al., 2012).

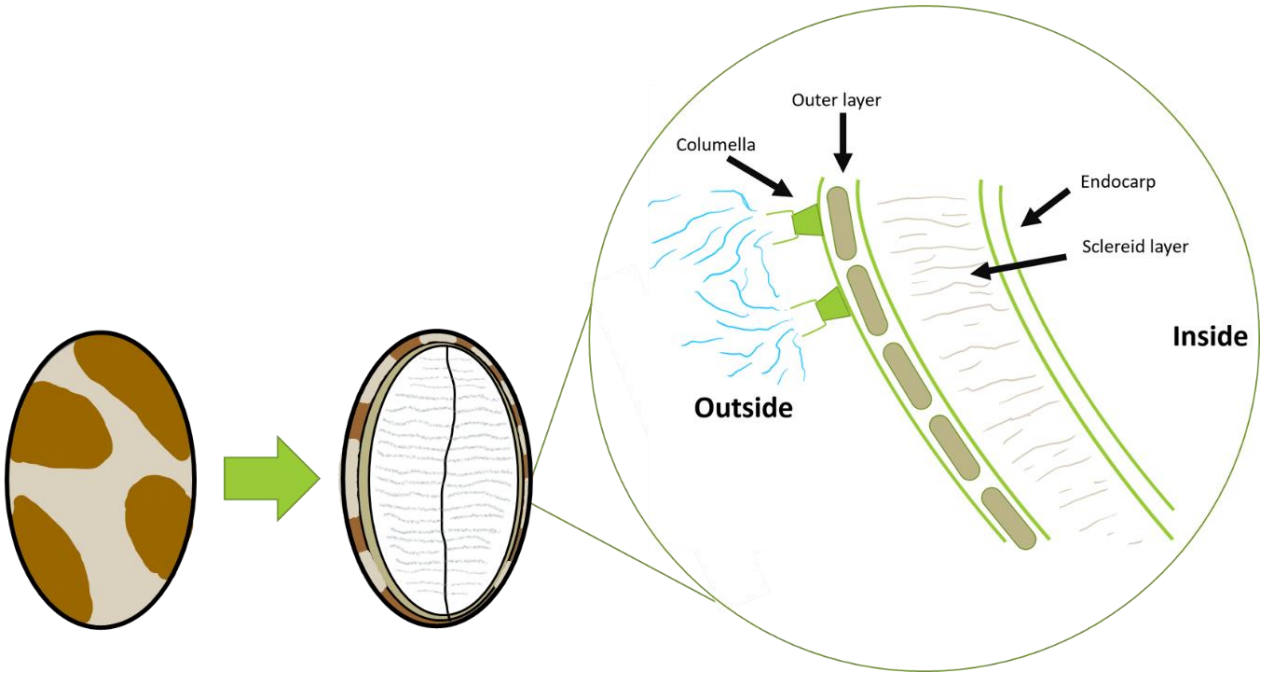

Figure 2. Microscopic structure of chia seed with mucilage exudate.

Chia seeds are predominantly consumed in Mexico and the southern part of Guatemala; nowadays its production has increased expanding to South America, being produced by Bolivia, Argentina, and Ecuador. Chia seeds' nutritional composition consists of protein (15 - 25%), lipids (30 - 33%), carbohydrates (26 - 41%), dietary fiber (18 - 30%) and minerals (4 - 5%) (Segura-Campos, Ciau-Solís, N., Rosado-Rubio, G., Chel-Guerrero, L., & Betancur-Ancona, D. , 2014). Some of the remarkable attributes are the high protein content, being one of the highest sources of protein among edible plants, also containing the highest natural source of alpha-linolenic acid (ω-3), while maintaining remarkable levels of dietary fiber and vitamins (Segura-Campos, Ciau-Solís, N., Rosado-Rubio, G., Chel-Guerrero, L., & Betancur-Ancona, D. , 2014). The high fiber content in chia seeds can be easily seen when the seeds are soaked in water and a copious mucilaginous polysaccharide film forms around the seed (Weber, Gentry, Kohlhepp, & McCrohan, 1991). Chia seed mucilage is a hydrophilic polysaccharide present in microstructures that surround the chia seeds called "seed coats". Mucilage content varies from cultivar to cultivar, researchers have found these numbers varying somewhere between 5-6% (Reyes-Caudillo, Tecante, & Valdivia-López, 2008). These seeds are also a high source of phenolic compounds, including chlorogenic, caffeic acids, quercetin and kaempferol (Sandoval-Oliveros & Paredes-López, 2013).

2.3 Chia seed protein and health benefits

The food industry is constantly aiming for the development of new food products, focusing on superfoods that can satisfy the new tendencies regarding the nutritional needs of consumers. A variety of studies have utilized chia seeds to enhance the healthy characteristics of food matrices. Ayerza Jr and Coates, (2007) fed whole chia seeds and chia seed oil to rats and found that blood serum triglycerides (TG) and low-density lipoprotein (LDL) decreased, while high-density lipoprotein (HDL) and (ω-3) polyunsaturated fatty acids (PUFA) increased. Nieman et al., (2012)

analyzed the effects of chia seed supplementation (25 g/day) in overweight women, showing how plasma α-linolenic acid and EPA increased by 58% and 39%, respectively. da Silva Marineli, (2015) induced rats to overweight and oxidative stress to evaluate the effect of a chia seed diet on these conditions, showing how plasma and hepatic antioxidant capacity values increased.

Newly discovered superfoods contain not only a high nutritional value, but also functional properties. Chia seeds are not the exception, some of its components including polysaccharides, fiber, and protein can be useful for the industry due to their functionality. Chia seeds can be used as thickeners or to develop edible films due to its mucilage content. Chia product development has not been vastly explored, with just a small number of products reported in literature. For example, Sandoval-Oliveros and Paredes-López, (2013) successfully incorporated CS flour into drinks to fortify its protein fraction. Steffolani, Martinez, León, snd Gómez, (2015) studied the effect of the incorporation of chia seed flour to bread, finding that the incorporation of this ingredient in bread had a sensory global score of 6 out of 9 with no difference from the control. More research using this seed needs to be developed to fully explore its potential and applicability in a wide range of food matrices.

Due to their rich oil content, high-quality protein, dietary fiber, vitamin, minerals and phenolic compounds, chia seeds have been highly acknowledged and widely commercialized (Ullah et al., 2016). All these nutrients in conjunction create a super food, which is rich in beneficial characteristics for human health. Amino acid analysis done to chia seed protein, show it contains the nine essential amino acids in abundant amount (Sandoval-Oliveros & Paredes-López, 2013). A 52% of the protein fraction of chia seeds protein correspond to globulins. Globulin structure makes it difficult to break down the protein into smaller peptides and also affects the way the protein is digested (Sandoval-Oliveros & Paredes-López, 2013). This protein is gluten free,

which remarks its applicability in different products including those that cover the celiac market (Ullah et al., 2016). The total amino acid content and the total number of peptide bonds (h_{tot}) of chia seed protein is shown in **Table 2** (USDA, 2011).

Table 2. Total amino acid composition for chia seed (USDA, 2011).

Amino Acid	N g/100 g	f_N*N	mM
Aspartic acid	1.69	10.56	79.36
Threonine	0.71	4.44	37.26
Serine	1.05	6.56	62.44
Glutamic acid	3.50	21.88	149.62
Glycine	0.95	5.94	79.06
Alanine	1.05	6.56	73.65
Valine	0.95	5.94	50.70
Cysteine	0.41	2.56	21.14
Methionine	0.59	3.69	24.72
Isoleucine	0.80	5.00	38.11
Leucine	1.37	8.56	65.26
Tryptophan	0.44	2.75	13.47
Tyrosine	0.56	3.50	19.32
Phenylalanine	1.01	6.31	38.21
Lysine	0.97	6.06	41.47
Histidine	0.53	3.31	21.34
Arginine	2.14	13.38	76.78
Proline	0.77	4.81	41.81
Total	19.49	121.81	933.72
h_{tot}/ g			9.33

The h_{tot} (meqv/g) was calculated according to (Adler-Nissen, 1986) by multiplying nitrogen concentration (N) by Kjeldahl conversion factor (f_N). The f_N value used was 6.25 as suggested by (Adler-Nissen, 1986).

2.4 Plant protein hydrolysates

One of the alternatives to increase bioactivity, bioavailability, and functionality of plant protein is through proteolysis. In this process, the molecules are cleaved into small peptides that might have a variety of beneficial effects in the human body and food matrices (Adler-Nissen, 1986). Protein hydrolysis can be produced using acids, bases or enzymes called proteases/peptidases; in some cases, protein catalysis can be developed by extrinsic factors such

as temperature. da Costa, Antonio da Rocha Gontijo, & Netto, (2007) showed the effect of heat and enzymatic hydrolysis on whey protein and determined that an increase in heat indeed increased the separation of peptide bonds causing protein denaturation. Considering the constant applications of protein hydrolysates in the food industry, the best way to break down the peptides is using enzymatic hydrolysis, controlling the extension and type of peptides produced while avoiding hazardous chemical methods, which will lead to risk-free hydrolysates for the consumers.

There are two type of proteolytic enzymes, these include endopeptidases, which cleave peptide bonds within the peptide chain and exopeptidases that focus on breaking down peptide bonds that are at the end of the peptide chain. Chemical methods are most of the time used to determine total amino acid content in food, but this process can destroy some amino acids due to the exposure to such extreme conditions. For example, acid hydrolysis is usually done using 6M HCl at 110°C for 16-24 h, studies have determined that these extreme conditions destroy tryptophan (Tsugita, 1982). The enzymes used to produce peptide breakdown have substrate specificity, and this allows them to break down only the proteins, without decreasing the nutritional value of other compounds such as carbohydrates lipids and vitamins (Tavano, 2013). The proteolytic reaction occurs when the enzyme breaks down the bond of the carboxyl group with the amino group of other amino acid or peptide chain **(Fig 3)**. Depending on the type of enzyme used, the peptide chain will be cleaved in different parts varying in the peptide chain size.

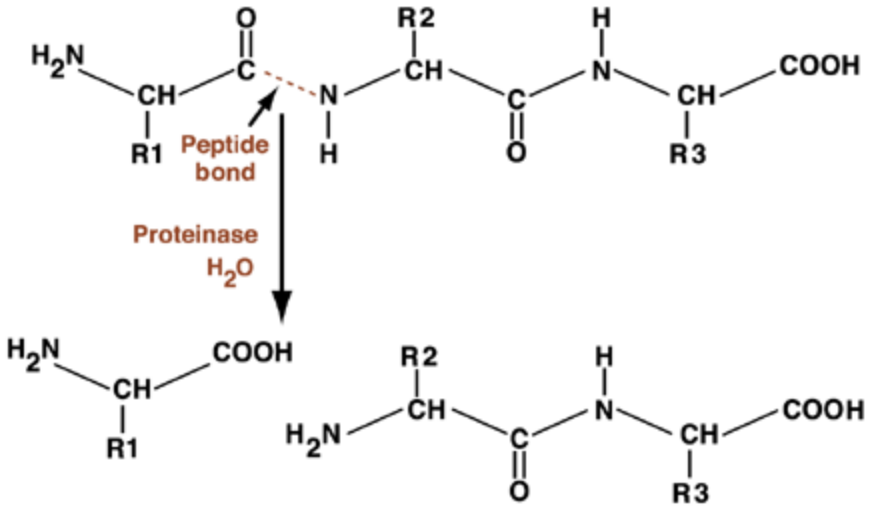

Figure 3. Proteolytic hydrolysis reaction (Donohue & Osna, 2003).

The type of enzyme its specificity will determine at which position the peptide bond will be catabolized, providing a specific arrangement of amino acids which explains the enzyme-substrate interaction (Tavano, 2013). The resulting peptides of one particular type of protease will differ in properties such as bioactivity, antimicrobial, and functionality.

2.5 Bioactive peptides from plant sources

Bioactive peptides (BPs) from plant sources are showing great potential. Some attributes are antioxidant activity, metal chelating capacity, antihypertensive and antidiabetic characteristics. The intrinsic characteristics of plant proteins (e.g., disulfide bonding, packed structures) and the presences of nonprotein-protein interactions have made the extraction of BPs from plants particularly difficult (Carbonaro et al., 2012; Clemente et al., 2000; Deshpande & Damodaran, 1989; Ghumman et al., 2016; Pushparaj & Urooj, 2011). For this reason, BPs of plant proteins are generated using simultaneous hydrolysis that can break down the protein to produce low molecular weight peptides smaller than 3 kDa (Rotimi E. Aluko, 2015). A study conducted by Segura-

Campos, Salazar-Vega, I. M., Chel-Guerrero, L. A., & Betancur-Ancona, D. A (2013) showed that using simultaneous hydrolysis by alcalase and flavourzyme resulted in an increase in the degree of hydrolysates of chia seeds, which will lead to a higher protein cleavage and better peptide bioactivity (Segura-Campos, Salazar-Vega, I. M., Chel-Guerrero, L. A., & Betancur-Ancona, D. A, 2013). These BPs will interact and decrease the pathological intensity of different chronic and metabolic diseases (Aluko, 2015). Hydrolysis produced using *in vitro* digestion of soybean seeds, and soy milk showed a high number of BPs, and had antimicrobial effects in food matrices (Sánchez & Vázquez, 2017). To generate a vast extraction of BPs, the enzyme must cleave peptides located in the primary structure of proteins; these are called encrypted peptides (Sánchez & Vázquez, 2017). The application of the extracted peptides has motivated the food industry to use them in beverage formulations. The antihypertensive and antioxidant capacity of a high protein beverage from walnut, sesame seeds, oat, and soybean showed good *in vitro* activities, indicating the possible application of this type of peptides (Mares-Mares et al., 2017).

2.4.1 Antioxidant activity

Free Radicals are molecular compounds that have a structure with an unpaired electron in their last orbital **(Fig 4)**; this causes them to be extremely reactive. The unpaired electron can stabilize itself by donating or accepting electrons from other molecules. The electron interchange gives origin to oxidative or reducing reactions resulting in diseases, cell disruption and diseases that are reflected depending on environmental factors (Lobo, Patil, Phatak, & Chandra, 2010). All the living organisms produce free radicals through metabolic process, enzymatic reactions, or obtain them from the exposure of critical environmental conditions. Unhealthy diets, smoking, consumption of high saturated fats, drugs, pesticides exposure and inflammation are just some of the factors that contribute to the development of free radicals in the human body.

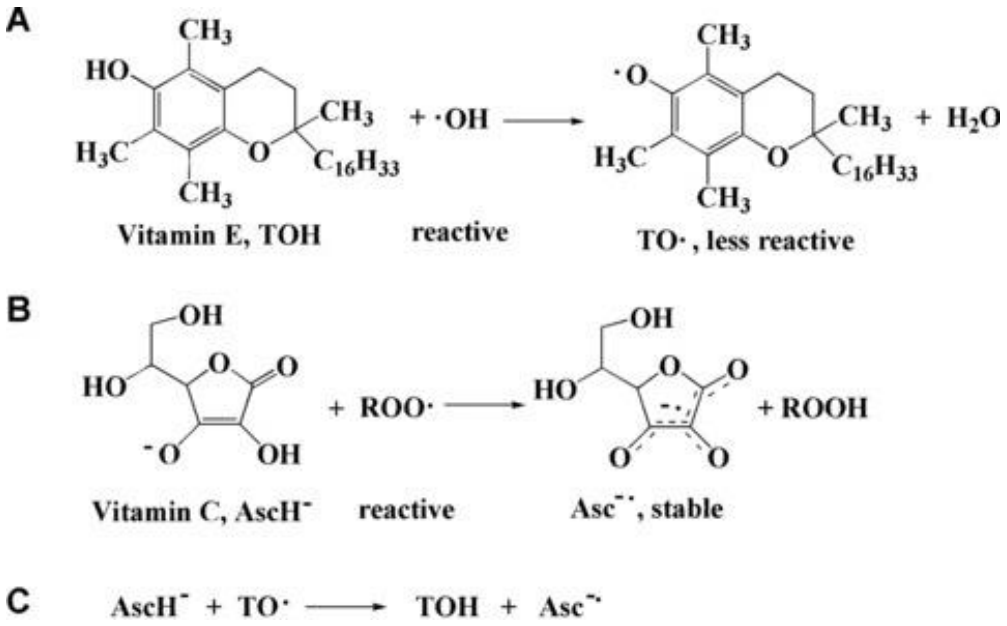

Figure 4. Antioxidant reaction of molecules by electron donation (Lü, Lin, Yao, & Chen, 2010).

The exposure to these factors leads to the development of oxidative stress; this is the general condition in which there is an unbalance amount of free radical production, compares to the capacity of the organism to fight back against free radicals. This results in molecular damage and inefficient metabolic activities, which triggers a second side effect that leads to sickness and aging. Studies correlated high death rates to the consumption of fats and oils showing how these macronutrients can affect the development of leukemia and breast neoplasia; these conditions are generated from the presence of free radicals, which cause cell disruption and lead to the development of diseases of this type (Lea, 1966). Antioxidant components can counter-attack oxidation of food components; antioxidant studies suggest that proteins can inhibit lipid oxidation in different ways such as chelation, scavenging or metal chelation (Elias, Kellerby, & Decker, 2008). Lipids and proteins can be negatively affected by free radicals; the interaction of the protein with free radicals is a multiplex reaction in which a constant electron interchange take place, this suggests the versatility of proteins and the novelty of their antioxidant attributes. Wu, Chen, and

Shiau, (2003) determined that the antioxidant capacity of proteins has a high correlation with the number of peptides present in food. Sustainable sources of protein such as plant proteins have shown to have a high potential in the application as antioxidant compounds. The study of plants like soybean, determined that the peptides delivered from enzymatic hydrolysis increase the inhibitory effect on lipid oxidation compared to the native protein (Penta-Ramos & Xiong, 2002). The way peptides offer antioxidant properties is very vast; some are the indirect effect of other reactions like metal chelation or alteration of the physical and molecular structure of food. The antioxidant capability of an amino acid relies in a significant proportion on the functional R-group (Elias et al., 2008). Depending on the R-group exposed on a peptide chain this one might have a higher antioxidant capacity. If the amino acids that have sulfur or an aromatic ring in their R-group are free to react on the ends of the peptide chain, they will present a higher antioxidant capacity due to the high availability for these hydrogens to be separated from the structure. If the protein structure is complex, it will cause the best radical scavenging amino acids to be inaccessible, hiding the high reactive group within the structures of the native protein. Malaypally, Liceaga, Kim, M., & Goforth, (2015) evaluated the influence of molecular weight on intracellular antioxidant activity of silver carp determining how lower molecular fractions have higher intracellular antioxidant activity due to the high amino acid interaction.

2.4.2 Antihypertension activity

Cardiovascular diseases have increased exponentially through the past years. Around 610,000 people die of heart disease in the United States every year (Centers for Disease Control and Prevention, 2017). Globally the high blood pressure has affected around 30% of adults (Norris, 2013). Most of the heart diseases originate from a series of factors such as the constant buildup of

plaque in the arteries, diabetes, poor diet, physical inactivity and excessive consumption of alcohol (Centers for Disease Control and Prevention, 2017).

Some of the most common diseases derived from these factors are atherosclerosis, arrhythmia and myocardial infarction which might result in strokes and heart attacks if they are not treated in the correct manner and time. Strokes are derived problems which involve the interruption of the blood flow that is directed to the brain, while heart attacks happen when the blood flow to the heart is blocked (American Heart Association, 2017). The American Heart Association states that the most common strokes that patients suffer are called hemorrhagic strokes (American Heart Association, 2017). This type of stroke is caused by uncontrolled hypertension or high blood pressure. Hypertension is a high-risk factor that can be gradually improved by regular exercise, healthy eating, nonsmoking, reduction in sodium intake and other environmental factors such as stress reduction (Norris, 2013).

The incorporation of BPs has shown to reduce hypertension. Knowing the importance of the BPs scientist have focused their efforts on finding sources, procedures or alternatives to obtain or generate these specific peptides. Studies done in casein, and whey protein of foods such as milk, meat, eggs, and seafood which show to release relevant BPs after hydrolysis presenting antihypertensive benefits (Norris, 2013). The way BPs interact with the high blood pressure is by the inhibition of angiotensin-converting enzyme (ACE), which results, a dilatation of the arteries resulting in a reduction of hypertension. The anti-hypertension activity of a peptide will vary depending on the peptide structure; researchers found that peptide containing proline and aromatic amino acid structure has strong inhibitory activities against ACE (Aluko, 2015). Ketnawa, Suwal, Huang, and Liceaga (2018) evaluated the effect of fish protein hydrolysates at 5 mg/mL, the study

showed a 93.48 % of ACE inhibition, suggested how bioactivity of the peptides might be improved using enzymatic treatment.

2.4.3 Antidiabetic

Diabetes is defined as the development of a metabolic disease called hyperglycemia, which is most of the time correlated with high levels of sugar or glucose in the blood. A hormone called insulin regulates the absorption of glucose. The pancreas secretes this hormone, and it helps cells to absorb glucose and use it as an energy source. The National Institute of Diabetes, Digestive and Kidney diseases (NIDDK) explains that diabetes is developed when our body does not synthesize enough insulin or it can't be correctly used, this causes the glucose to stay in our bloodstream and cause health problems (National Institute of Diabetes & Kidney Diseases, 2016). Some of the complications that a person with diabetes might suffer are skin, eye and nerve damage. A study of the World Health Organization (WHO) showed that the population suffering from diabetes has quadrupled in the last four decades (WHO, 2018). An estimation showed that currently, 422 million adults have diabetes and that 1.6 million deaths are attributed to diabetes each year (WHO, 2018). Diabetes is highly correlated with obesity, and the world index showed that 1 out of 3 persons over 18 years old is in an overweight condition, and 1 out of 10 are obese (WHO, 2018). The incorporation of specific compounds to our diet might reduce these conditions. Drugs, synthetic and natural compounds are just some common alternatives used to treat diabetes in traditional and modern medicine. In both cases, the use of plant molecules or compounds has shown to be an exceptional source of medicinal ingredients, extracting active compounds that might be used directly or indirectly to produce hypoglycemic effects (Pamunuwa, Karunaratne, & Waisundara, 2016). Antidiabetic properties have been evaluated by the inhibition of an enzyme called dipeptidyl peptidase-IV (DPP-IV). DPP-IV is a prolyl peptidase responsible of cleaving

proteins or peptides after a proline amino acid residue (Green, Flatt, & Bailey, 2006). The relevance of DPP-IV relies on the ability of this enzyme to inactivate a wide range of peptide hormones such as incretins (Green et al., 2006). Incretins include glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). These hormones contain a high insulin-secretory activity, therefore are responsible for decreasing glucose content in the bloodstream. Studies showed that polyphenols and proteins are the main responsible blocking or regulating symptoms associated with diabetes. Plant proteins have a regulatory effect in numerous types of chronic diseases, studies have been done measuring different hypoglycemic pathways by which the proteins can fight against diabetes (Marya, Khan, Nabavi, & Habtemariam, 2018). Some plant proteins such as bean and oat showed antidiabetic properties; these peptides interfere with the glucose transporters GLUT1 and GLUT2 and the activity of DPP-IV (Marya et al., 2018). One of the most remarkable studies of chia seed protein hydrolysates was done in 2013 using sequential hydrolysis by Flavourzyme® and Alcalase® reaching DH (degree of hydrolysis) of 43.8%, showing that ACE inhibitory activity and antioxidant effects increased as the degree of hydrolysis increases and taught not to have an antioxidant effect when they were incorporated into foods (Segura-Campos, Ciau-Solís, N., Rosado-Rubio, G., Chel-Guerrero, L., & Betancur-Ancona, D. , 2014).

2.5 Extracting bioactive peptides from chia seeds

The rising level of chronic diseases throughout the years due to unhealthy diets, lack of exercise or hereditary conditions have developed the interest of food scientists, which in response to those conditions are focusing in the expansion and development of dietary supplements, compounds or even foods that can improve these medical conditions. These ingredients and supplements have been defined as bioactive compounds (BC), which are constituents present in

plants and high lipid sources (Kris-Etherton et al., 2002). Some of the examples of BC delivered from plants are phenolic compounds, organosulfur compounds, plant sterols and dietary fibers (Kris-Etherton et al., 2002). The scientific community has also revealed the bioactivity of food proteins and peptides. BPs are easily found in dairy products such as yogurts or cheeses, products that had to suffer proteolytic digestion (Park & Nam, 2015). It is well known that BPs may help reduce chronic diseases that are currently causing more than 58 million premature deaths annually (Mine et al., 2010). Some of the biological effects produced by these peptides are antioxidant, anti-inflammatory, anti-thrombotic, antihypertensive and antidiabetic activity.

Chia seed has a high potential of being one of the top protein sources among plants. However, to create bioactive peptides from chia seeds some considerations must be considered. Due to some of the components, raw chia seeds must be preconditioned before using its flour to extract BPs. As mentioned before chia seeds contain a component called mucilage which is a hydrophilic polysaccharide. Due to this component, the chia flour gels in the presence of water forming a barrier between the enzymes and its protein, preventing the hydrolysis from taking place. Mucilage is separated using a method proposed by (Muñoz et al., 2012), where the extraction is optimized considering the variables of temperature, pH, and seed water ratio and then the chia seeds are freeze-dried and rubbed into a mesh. Although efficient, this method is costly, time-consuming, produces low extraction yields and the quality of the mucilage extracted is low due to the extreme manipulation.

2.5.1 Chia seed bioactive peptides

Chia seed protein was studied to determine the inhibitory activity of protein fractions against ACE, showing that peptides from albumin and globulin fractions exhibit the highest ACE inhibitory capacity (Orona-Tamayo, Valverde, Nieto-Rendón, & Paredes-López, 2015). Other

studies focused on protein isolate, which showed high antioxidant power for β-carotene discoloration and iron reducing antioxidant power (Chim-Chi, Gallegos-Tintoré, Jiménez-Martínez, Dávila-Ortiz, & Chel-Guerrero, 2018). Hypertensive effects of chia protein have not been intensively studied, and they may vary depending on the proximate composition of the seed. To our current knowledge no study has measured the antidiabetic activity as the inhibition of DPP-IV enzyme. In terms of chia seed protein antioxidant activity, Orona-Tamayo et al. (2015) evaluated the antioxidant activity of chia seed protein fractions and determined that the highest antioxidant capacity was achieved by prolamin and globulin fractions. The chia seed protein hydrolysates delivered by enzymatic proteolysis showed high antioxidant activity in white bread (0.53–0.55 mmol/L-mg protein) and carrot cream (17.52–18.88 mmol/L-mg) (Segura-Campos, Salazar-Vega, I. M., Chel-Guerrero, L. A., & Betancur-Ancona, D. A, 2013). Despite these two studies, not much information is known of antioxidant mechanism of action and the effect chia seed protein in cellular models.

2.5.2 Microwave-assisted proteolytic hydrolysis

Microwaves are a type of electromagnetic non-ionizing radiation which means it cannot remove electrons from the atom's orbit being harmless to human beings. Other types of energy such as X-ray energy might transfer these electrons causing molecular damage (FDA, 2017). The use of microwaves as an alternative way for delivering energy in a fast and efficient way, although this type of thermal energy has mostly been used for electro domestic purposes, it has become an alternative to generate accelerated chemical and biological reactions. A vast amount of reactions have been assessed using microwave energy. Pan, Niu, and Liu, (2003) used microwaves to aid the extraction of polyphenols and caffeine from green tea leaves and Hua, Low, & Sze, (2006) used microwave energy to assist the chemical digestion of proteins in order to reduce the time of

identification. The acceleration of the reactions is a desired attribute in the industry, increasing productivity while cutting processing times and costs. Some bioactivities might be attributed to the peptide size and distribution. An indirect way to measure the size of a peptide chain is using degree of hydrolysis (DH), which gives an estimation of the portion of cleaved peptide bonds in a protein hydrolysate (Rutherfurd, 2010). The development of high proteolytic hydrolysates (DH>40%) of certain types of foods has been an issue and almost impossible to achieve. Multiple attempts have been used to generate low molecular weight peptides. Pre-digestion, protein isolation and sequential hydrolysis are only some of the common practices done to decrease hydrolysis time. Alternative technologies have been applied to food proteins to increase the hydrolysis. For Example, Mikhaylin, Boussetta, Vorobiev, & Bazinet, (2017) successfully improved the protein susceptibility of β-lactoglobulin to enzymatic hydrolysis using high voltage electrical treatments. Nguyen, Jones, Kim, San Martin-Gonzalez, & Liceaga, (2017) used microwave to assist enzymatic hydrolysis to increase functional and antioxidant properties of rainbow trout by products showing how this process will improve functional and antioxidant activity compared to a conventional method. Knowing this, the application of alternative ways of energy might help the development of enzymatic hydrolysates by generating new active sites for the enzymes to bind and break down the covalent bonds.

2.5.3 Mucilage extraction using ultrasound

The mucilage is natural occurring polysaccharide, and it is composed of high weight polyuronides, which consist of sugar and uronic acid units that form a viscous colloidal dispersion in water. Due to its viscosity it can be used to encapsulate drugs, develop gels and emulsions (Singh, Kumar, Langyan, & Ahuja, 2009). Ultrasonication has been used to assist the removal of different molecules; one example is metabolites from plant and animal tissues, this technique also

increases the extraction yield and decreases the costs and gas emission (Chemat et al., 2017). Ultrasound is defined as a mechanical wave that requires a medium to travel and spread over; they differ from audible sounds waves by their frequency (Rostagno & Prado, 2013). The acoustic frequencies to humans range from 16Hz and 20 kHz, while the ultrasound frequency range from 20kHz to 10MHz (Rostagno & Prado, 2013). The main characteristics to control ultrasound attributes are power (W) the frequency (Hz) and the wavelength (cm). Other attributes such as wave amplitude (μm) are implemented as indirect indicators of the ultrasound wave intensity. Ultrasound waves travel through a medium and its structure, this force causes rapid and periodic longitudinal displacement **(Fig 5)**, resulting in compression and rarefaction phases (Rostagno & Prado, 2013).

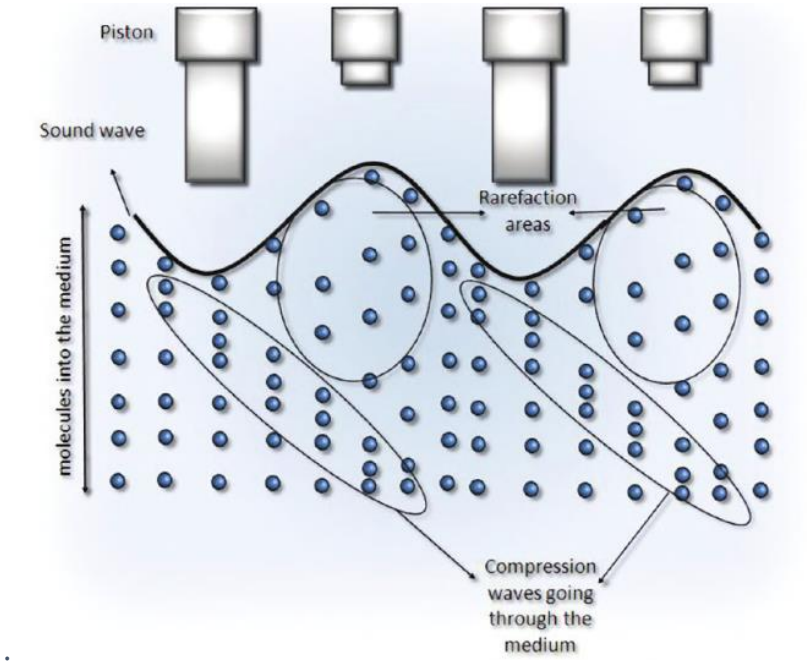

Figure 5. Ultrasound compression and rarefaction cycles (Rostagno & Prado, 2013) – Reproduced by permission of The Royal Society of Chemistry.

Rarefaction exerts a negative force that pulls or expands the molecules of the medium rapidly, and this results in the development of bubbles. The cavitation bubbles surround the solid material submerged in the medium, at that moment they are compressed, they collapse and form micro jets the help liberate specific compounds in plants as shown in **(Fig 6)**.

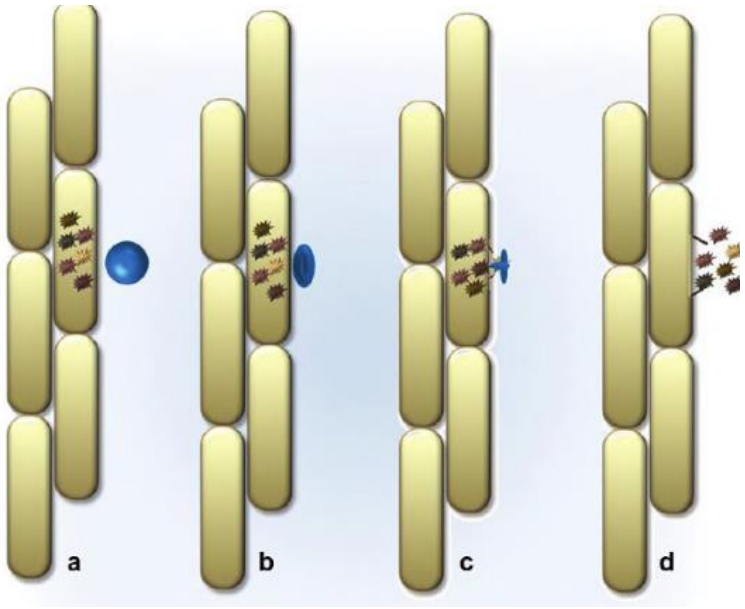

Figure 6. Compounds liberated by microjet in ultrasound (Rostagno & Prado, 2013) – Reproduced by permission of The Royal Society of Chemistry.

The way the cavitation bubbles form depends on the ultrasound parameters such as power, frequency, amplitude or wavelength. Knowing this, the parameters can be manipulated to obtain optimum conditions for different plants and compounds. Ultrasound has been generally used to address the extraction of different plant compounds such as flaxseed oil, wheat bran phenolic compounds and rutin from euonymus alatus (Esclapez, García-Pérez, Mulet, & Cárcel, 2011). Although ultrasound has been mainly used for phenolic compounds other authors have suggest it for polymers from plant sources. Bernardo, Ascheri, Chávez, & Carvalho, (2018) assisted the separation of starch from Yam using a power input of 70% for a period of 15 min.

2.6 Functional Properties of Proteins

The texture, appearance, and flavor are only some of the attributes consumers consider whenever they decide if they like or not food. These characteristics are limited to the food matrix composition. Depending on the food component different attributes might be enhanced. Some components have to be exposed through particular conditions to achieve optimal results (e.g., starch must be heated to undergo gelatinization, high methoxyl pectins require low pH and the presence of sugar to form a gel). Specific ingredients contribute to generate desirable characteristics in a product. Some of the main functional properties are solubility, emulsification, gelatinization, water holding capacity, and foaming capacity.

2.6.1 Solubility

The amount of nitrogen detected determines the solubility of a protein under certain conditions, depending on the solvent, solvent concentration, pH, and charged ions (Sikorski, 1997). Some of the protein classifications rely on the solution in which they are soluble (eg., albumins, globulins, prolamins and glutelins). Denaturation may decrease the solubility of a protein due to the changes in the amino acid arrangement and charged ions exposed in the ends of the peptide chain (Sikorski, 1997). Solubility can be a handy way to determine if an ingredient will maintain a constant suspension in a drink or beverage avoiding precipitation, deterioration or compromising other attributes of the food or fabrication process. Protein solubility depends on the arrangement of the amino acids in the peptide chain, some of the amino acids are very soluble in water (e.g., proline glycine and alanine) while others don't solubilize that efficiently (e.g., cysteine and tyrosine) (Cheung & Mehta, 2014). The individual amino acids are not soluble neither in organic or inorganic solvents the main reason is because the solubility of a peptide chain depends directly on the amount of polar and nonpolar structures distributed throughout the chain or

molecule (Cheung & Mehta, 2014). As the proteins approach the isoelectric point the solubility decreases, this occurs because at that specific pH the electric repulsion between molecules neutralizes and the hydration layers is not enough to prevent protein aggregation (Sikorski, 1997). A study done in baru nuts (*Dipteryx alata*) studying the solubility of baru nuts protein **(Fig 7)** shows how the solubility decreases when the protein gets close to the isoelectric point. As the industry understands the various factors might affect this property, studies have been carried out to determine at which conditions or which protein modification might increase protein solubility the most.

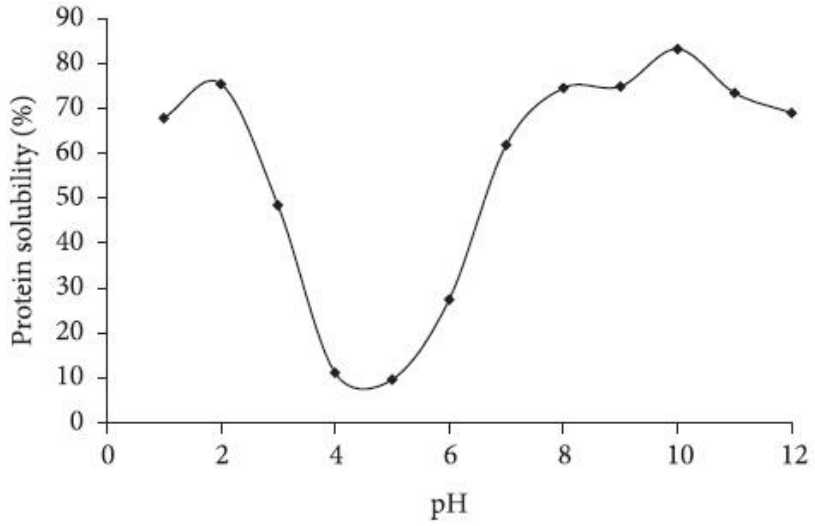

Figure 7. Solubility curve for defatted flour of baru nuts (Guimarães et al., 2012).

Functional properties of pseudo cereals such as chia seeds and quinoa have been studied, but a lack of research in this area has left a small amount of information regarding the capacity or applicability of these grains. Protein solubility of quinoa was evaluated by (Abugoch, Romero, Tapia, Silva, & Rivera, 2008) showing how quinoa protein extracted by alkaline solubilization had its maximum solubility (85%) at pH 7. (Vázquez-Ovando, Betancur-Ancona, & Chel-Guerrero,

2013) studied the functional properties of a chia seed protein rich fraction determining it maximum solubility (60%) was achieved at pH 8.

2.6.2 Emulsion

An emulsion is a way a compound links together two immiscible liquids, having one in a generous amount. In this way, the two liquids hold together in one matrix and don't separate anymore. The emulsion is usually done using water and oil, depending on which substance is in predominant amount, the emulsion can be classified as oil in water (O/W) or water in oil (W/O). To describe each of the interactions correctly, we will refer as a continuous phase to the liquid in significantly higher amount and, dispersed phase as the substance that is present in droplets or small amounts (McClements, 2005). Researchers have determined new possibilities and alternatives such as the development of an emulsion within an emulsion (O/W/O) or (W/O/W). This type of emulsion is possible by developing one emulsion first, for example O/W, and then a second emulsion W/O which allows to obtain a slow release of specific compounds or prevent some compounds from reacting with each other (Oh, Park, Shin, & Oh, 2004). The emulsions might be created using high pressure, for example, milk is homogenized using a high-pressure (10-25 Mpa) process by which the fat globules are entirely dissolved into the aqueous phase and prevent the formation of a fatty layer on the milk. Rather than pressure, some foods need emulsifiers that will interact with both liquid phases helping the emulsion to take place and remain stable for a longer time. There are a variety of emulsifiers (e.g., proteins, polysaccharides, monoglycerides and diglycerides) which work in different ways, compounds that are more soluble in water are great agents to develop O/W emulsions and the ones that are more soluble in a fatty matrix are better for W/O emulsions (McClements, 2005). Proteins usually offer unique emulsifying characteristics by linking the continuous phase (water) and the disperse phase (oil).

They interact with the surface of a fat droplet covering it and allowing this one to be suspended in water (**Fig 8)**, improving the overall solubility and avoiding the separation of the fluids, if the protein is denaturalized or deteriorates an emulsion breakdown might occur.

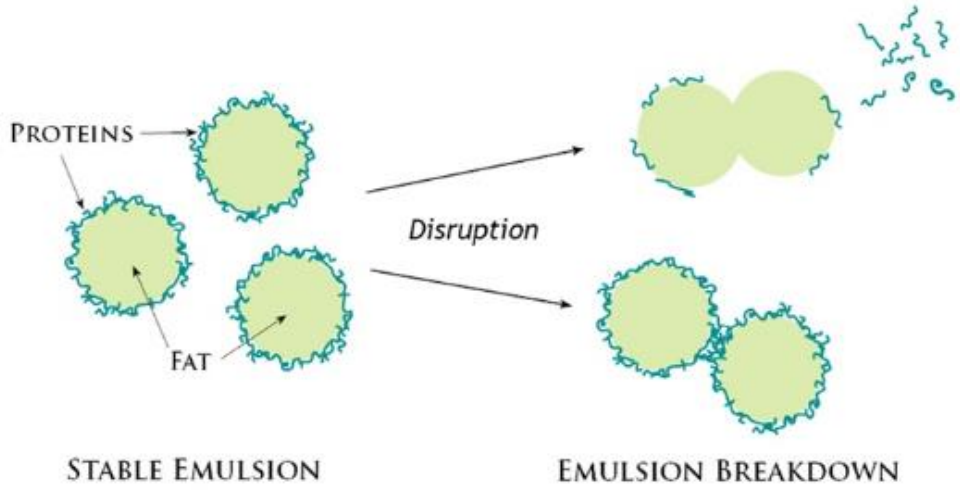

Figure 8. Role of protein in emulsions (Costello, 2017).

An emulsifier will affect the two critical parameters of an emulsion, which are emulsion capacity and emulsion stability (Cano-Medina et al., 2011). Emulsifying capacity refers to the ability of an emulsifier to form an even and well distributed oil and water matrix solution and emulsion stability refers to the time the emulsion is stable and how it disrupts within time. Chia seed mucilage has been well investigated for emulsion capacity and stability but research regarding chia seed protein functionality is still missing. Vázquez-Ovando et al., (2013) investigated the global emulsifying capacity of chia seed flour, which ranged from (50-56%) but not much detail of chia protein has been recorded. Plant source derived proteins such as quinoa, amaranth, buckwheat, barley, wheat, foxtail mille, rice, japanese millet and millets were also evaluated

showing that pseudocereals contain the highest functional and bioactive properties (Asao & Watanabe, 2010).

2.6.3 Foaming

Foaming is a functional property of food that depends on the agent, time and air injection at which a solution is exposed. The foaming agent is evaluated using two criteria, foaming capacity (FC), which refers to the volume of the foam generated and foaming stability (FS), which is the change in volume within time. Generally, foams are done by incorporating air cells or bubbles using a thin continuous liquid layer called the lamellar phase (Zayas, 1997). For the proteins to have a good foaming ability, they must be highly soluble in water; this will ensure a proper formation of a cohesive film at the water and air interface, guarantying an appropriate formation of foam (Cano-Medina et al., 2011). Some studies have evaluated different plant protein sources, and their ability to form foams. Mao and Hua, (2012) assessed the functional properties of walnut showing how high protein concentrations increase the foaming capacity of the solution. Vázquez-Ovando et al., (2013) evaluated chia flour foaming properties showing a maximum foaming capacity of 28%. Foaming capacity of different commercial soy protein isolates had been improved by heat treatments (55°C and 85°C) (Shao, Lin, & Kao, 2016). Mune and Sogi, (2016) compared the functional properties of bambara bean and cowpea protein concentrates derived from two drying method (vacuum-drying and cabinet-drying), determining that cabinet-drying had the best foaming capacity.

2.7 Biodegradable films: Alternative applications of plant materials.

The development of food products is one of the alternatives, but they are some other applications of edible plants. The world is changing an extremely high rate and the use of alternative material to reduce waste had been assessed using plant-derived products due to their compostable and degradable capabilities. As we know plastic pollution is affecting oceans and land fields globally due to the buoyancy and low deterioration rate of this compounds (Eriksen et al., 2014). Ocean tides, winds, and humans spread plastics throughout bays and gulfs leading to polluted coastlines and affected ocean fauna. The extreme use of petroleum-based products such as plastics is leading to a high rate of accumulation of these toxic materials in the ocean. The main affected fauna are marine animals; these can't distinguish plastic waste from their food and ingest plastic material, which blocks in their digestive tracks, avoiding continuing eating, causing to fall in a lack of nutrients and dying (Laist, 1987). The development of petroleum-based food packaging is the primary source of plastic. Due to this problem, alternative ways for food packaging are considering ingredients that increase the biodegradable characteristics. A vast quantity of biodegradable plastics alternatives had considered showing the possibility of gradually replacing plastic with low-cost materials and being less harmful to the environment. Psomiadou, Arvanitoyannis, and Yamamoto, (1996) developed films made from natural resources using microcrystalline cellulose, methylcellulose, and cornstarch showing how compounds can be used together to create certain properties. These types of films are offering an excellent alternative for food packaging and have proved to provide similar characteristics as petroleum-derived plastics. Jiménez, Fabra, Talens, & Chiralt, (2012), used carbohydrates such as starch to make edible films, showing to be an excellent mechanical barrier to hold and protect food products. Some other alternatives for biodegradable films with less permeable characteristics are achieved using

Polylactic acid (PLA). The incorporation of PLA showed a general increase in mechanical properties such as elongation, tensile strength. And also, a decrease in the water absorption, which improved the poor mechanical properties and high permeable characteristic of conventional starch-based films (Hu, Wang, & Tang, 2013).

2.7.1 Biodegradable films using chia seed mucilage

Using natural products to develop biodegradable films is a common practice in the pursuit of a new alternative to petroleum-based plastics. Starch, cellulose, chitin, paper, PLA, plant fiber, epoxidized oils are just some of the natural products used for the fabrication of biodegradable films. Edible films have been produced using chia seed mucilage and glycerol, showing that at higher glycerol concentrations the elongation at break (EB), water solubility and mechanical properties of the film increased (Dick, Costa, Gomaa, Subirade, de Oliveira Rios, et al., 2015). -+

Films developed with glycerol will collapse easily within time and can only be applied to specific products, limiting its applicability. That is why the use of PLA was considered as an alternative plasticizer for chia seed mucilage films. PLA is a component that forms part of the aliphatic polyester derived from lactic acid; this one is generally produced from renewable resources. PLA is a product of starch fermentation, due to its nature it has exceptional biodegradable and compostable characteristics (Garlotta, 2001). A study by Hamad, Kaseem, Yang, Deri, & Ko, (2015) show the novel attributes of PLA, having better mechanical properties (tensile Young's modulus, tensile strength, and flexural strength) compared to common petroleum-delivered polymers such as polypropylene (PP) polystyrene (PS) and polyethylene (PE). One of the unique characteristics of this thermoplastic is its hydrophobic nature, being suitable for the development of biodegradable copolymer films derived from hydrophilic compounds.

2.7.2 Digestion of chia seed mucilage

Chia seed mucilage is a soluble fiber with a highly branched structure, this material contains a high amount of saccharides that include xylose, glucose, arabinose, galactose, glucuronic acid and galacturonic acid (Timilsena, Adhikari, Kasapis, & Adhikari, 2016). These saccharides grant the material a hygroscopic behavior leading to high water holding capacities. Tamargo, A., Cueva, C., Laguna, L., Moreno-Arribas, M. V., & Muñoz, L. A. (2018) showed that chia seed mucilage impacts the bacterial groups, affecting predominantly the aerobes and anaerobes at concentrations of 0.3% of chia seed mucilage. The consumption of chia seed was an associate with an increase of the short chain fatty acids by increasing the butyric, propionic, and acetic acid content, which are known to have beneficial effects on the health including the promotion of gut mucosal health, and increase of apoptosis and cell differentiation of cancer cells (Tamargo, A., Cueva, C., Laguna, L., Moreno-Arribas, M. V., & Muñoz, L. A. 2018). The main impact of this polymer to the gut microbiota was due to an increase in viscosity affecting the different microbial communities. Further research is needed to understand the impact of this polysaccharide in *in vivo* models.

2.7.3 Barrier property

The barrier properties are crucial for the understanding of the quality and resistance of a film, and they will also help to estimate parameters as deterioration and shelf life of the film. The primary objective of a package or film is to keep its content protected from extrinsic factors. All plastics films and materials are relatively permeable to molecules such as gases, water vapor, and liquids, it all depends on the composition of the film which will determine the quality of the film (Siracusa, Rocculi, Romani, & Rosa, 2008). For this reason, the film barrier characteristics are evaluated using three parameters, water vapor transmission rate (WVTR), Carbon dioxide

transmission rate (CO_2 TR) and oxygen transmission rate (OTR). All of these properties evaluate the amount of gas or liquid that permeates per unit area in a determined time, generally expressed as [kg m m^{-2} s^{-1} Pa^{-1}] (Siracusa et al., 2008). For food scientists, these values are of extreme importance to decide which film is feasible for a particular product. A study made by Hamad et al., (2015) analyzed the barrier properties of PLA and other plastics **(Fig 9)**, showing the different permeability abilities to specific components, this will aid food scientist to decide which barrier they are willing to have in their film. Some products require low permeable films to avoid spoilage (e.g., potato chips need a low permeable film to keep the chips crunchy and avoid the loss of gasses that are present in the bag), while others don't require such extreme conditions (e.g., freshly baked bread, will last one or two days in shelf and don't need low permeable conditions).

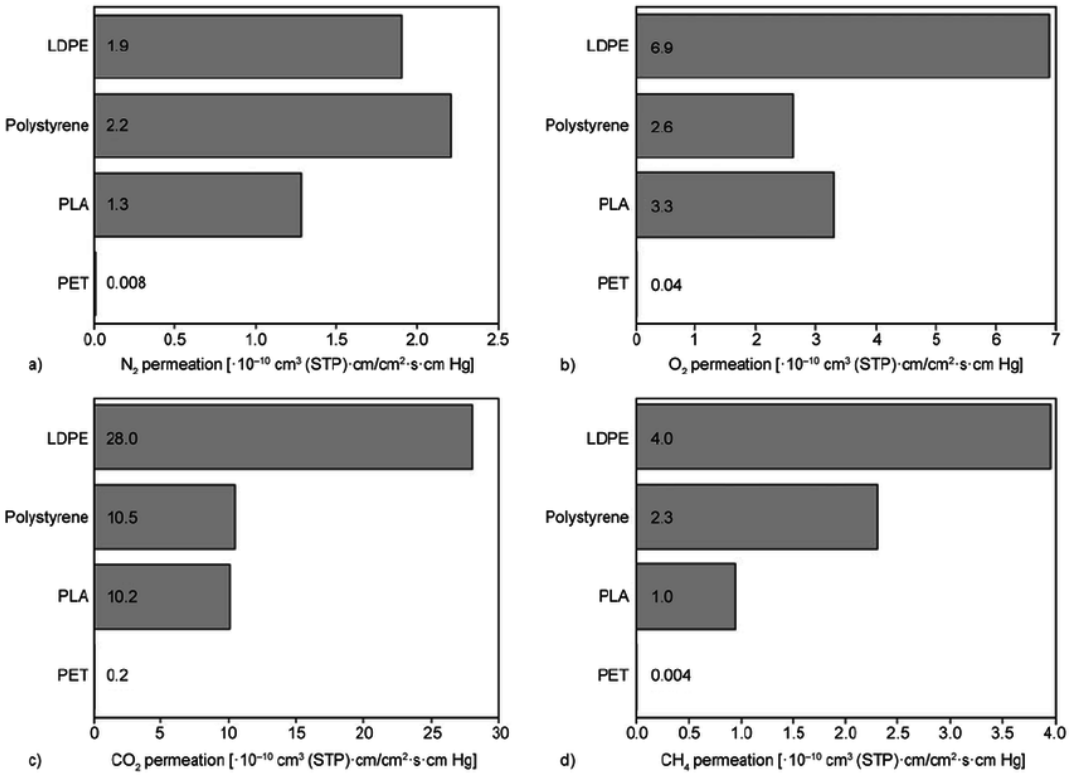

Figure 9. Barrier properties of PLA compared to conventional polymers at 30~C for (nitrogen, oxygen, Carbon dioxide and Methane) (Hamad et al., 2015).

2.7.4 Mechanical properties

The mechanical properties help profoundly in the understanding of the polymer architecture and conditions that might affect its integrity. To evaluate them, the conditions are simulated by exposing the films to standard conditions of stress. Tensile test is a general analysis that recovers information regarding the tensile strength measured in (MPa), the percent of elongation at yield (%) and the percent elongation at break (%) these values will provide essential information of the biodegradable polymer study, understanding it maximum stress and mechanical capacities (Hamad et al., 2015). Other analysis done to films will determine its breaking point and the resistance to pressure exposure which is generally measured as impact and compression properties (Hamad et al., 2015).

CHAPTER 3. PHYSICOCHEMICAL CHARACTERISTICS OF CHIA SEED (SALVIA HISPANICA) PROTEIN HYDROLYSATES PRODUCED USING ULTRASONICATION FOLLOWED BY MICROWAVE-ASSISTED HYDROLYSIS

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Abstract

Chia (*Salvia hispanica*) has gained popularity due to its high nutritional content. Unfortunately, mucilage surrounding the chia seed (CS) limits the technological utilization of the protein. This study evaluated the bioactivity and functionality of CS protein hydrolysates (CSPH) produced by different treatments and a control (unhydrolyzed CS protein). Ultrasonication was used to separate mucilage from CS (7.8% yield). Proteins in defatted-CS flour were enzymatically hydrolyzed using conventional (enzymatic hydrolysis with alcalase) or sequential (enzymatic hydrolysis with alcalase+flavourzyme), and under water bath or microwave-assisted hydrolysis. CSPH from the sequential hydrolysis with microwave treatment showed superior (p<0.05) *in vitro* antioxidant activity. A positive correlation (p<0.05) was established between antioxidant assays and cellular antioxidant activity. The highest ($p<0.05$) cellular antioxidant activity was achieved by the sequential (94.76 ± 1.96) and conventional (93.13 ± 1.07) hydrolysis with microwave. Dipeptidyl peptidase-V inhibition (p<0.05) was higher for sequential hydrolysis with water bath. Inhibition of angiotensin converting enzyme activity increased (p<0.05) with hydrolysis for all

treatments compared to the control. Regarding functionality, sequential hydrolysis with microwave showed higher (p<0.05) solubility at lower pH (3 and 5), while conventional hydrolysis with microwave was better at pH 7 and 9. Emulsification properties and foaming capacity were also higher in conventional hydrolysis with microwave, but conventional hydrolysis with water bath was more stable for foaming properties only. Results show that ultrasonication efficiently separated mucilage from chia seeds. Microwave and enzymatic hydrolysis can generate protein hydrolysates with improved bioactivity and functionality.

Keywords: Chia seed hydrolysates, ultrasonication, microwave-energy, bioactive peptides.

3.1 Introduction

Throughout the years, plants have been domesticated and cultivated to obtain novel potential ingredients and alternative sources of functional foods. One remarkable plant that has shown high potential is chia *(Salvia hispanica)*. Chia is a biannually cultivated plant; it is considered a pseudo-cereal that produces purple and white flowers that eventually result in small oval shape seeds with sizes varying from 1 to 2 mm (Mohd Ali et al., 2012). The seeds are divided into two semi-hemispherical structures, which contain the endosperm, called cotyledons.

Nutritional composition (wet basis) of chia seed consists of protein (15-25%), lipids (30-33%), carbohydrates (26-41%), dietary fiber (18-30%) and minerals (4-5%) (Segura-Campos, Ciau-Solís, N., Rosado-Rubio, G., Chel-Guerrero, L., & Betancur-Ancona, D. , 2014). Its composition allows the seed to have remarkable attributes such as a high content of protein, unsaturated (ω-3) fatty acids, and dietary fiber (Segura-Campos, Ciau-Solís, N., Rosado-Rubio, G., Chel-Guerrero, L., & Betancur-Ancona, D. , 2014). The high dietary fiber content of chia seeds can be observed when the seeds are soaked in water and a copious mucilaginous polysaccharide coating forms around the seed. This polysaccharide is present in microstructures called

collumnellas that surround the chia seed and allow the formation of this gel-like material that limits its digestibility and utilization (Muñoz et al., 2012). The implementation of technologies such as ultrasound processing can aid in the separation of this polysaccharide, while allowing for protein extraction (Vilkhu et al., 2008). Ultrasound methods have shown high extraction yields in a shorter amount of time, for the separation of the polysaccharide from other plant matrices such as lingzhi mushrooms (*Ganoderma lucidum*) and mutamba seeds (*Guazuma ulmifolia* Lam) (Kang et al., 2019; Pereira et al., 2019).

A rising level of chronic diseases throughout the years have led to the development of food-derived bioactive peptides that can help improve these medical conditions. Some of the biological effects produced by these peptides are antioxidant, anti-inflammatory, anti-thrombotic, anti-hypertensive and anti-diabetic. Some proteins, including chia seed proteins, exhibit high resistance to proteolysis, limiting their applicability to generate bioactive peptides. For this reason, different hydrolysis treatments such as high-voltage, electrical (Mikhaylin et al., 2017) and microwave (Nguyen et al., 2017) treatments have been proposed to increase the protein's susceptibility to hydrolysis.

Studies have shown the applicability and attributes of chia seeds. For example, one study showed how plasma α-linolenic acid and eicosapentaenoic acid increased by 58% and 39%, respectively when chia seed was supplemented (25 g/day) in the diet of overweight women (Nieman et al., 2012). Another study by da Silva Marineli, (2015) induced rats to overweight and oxidative stress before evaluating the effect of a diet rich in chia seeds on these conditions, showing how plasma and hepatic antioxidant capacity values increased. Sandoval-Oliveros & Paredes-López, (2013) successfully incorporated chia seed into drinks to enhance the protein content. The aim of this study was to improve the biological and functional properties of chia seed protein

hydrolysates by using ultrasonication to remove the mucilage and microwave-assisted enzymatic hydrolysis to generate bioactive and functional chia seed peptides.

3.2 Materials and methods

3.2.1 Materials

Chia seeds were obtained from Healthworks® (Scottsdale, AZ, USA). Alcalase® (protease from *Bacillus licheniformis*, EC 3.4.21.62) and Flavourzyme® (protease from *Aspergillus oryzae*, EC 232-752-2) were purchased from Sigma Aldrich (St. Luis, MO, USA). Human Dipeptidyl Peptidase IV (DPP-IV, ≥ 4,500 units/ µg protein) and substrate Gly-Pro p-nitroanilide hydrochloride, Angiotensin Converting Enzyme (ACE) from human and substrate Hippuryl-L-Histidyl-L-Leucine (HHL) were all purchased from Sigma Aldrich (St. Louis, MO, USA). All chemicals used were reagent grade and generally obtained by three leading companies VWR International (Radnor, PA, USA), Sigma Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA).

3.2.2 Chia seed mucilage extraction

To extract the CS mucilage **(Fig 10)**, seeds were hydrated in distilled water (1:20 ratio by weight) for 24 hours, under refrigerated conditions. Preliminary studies helped develop an ultrasound treatment that offered successful mucilage separation. Hydrated seeds were pre-heated to 55±2°C, followed by sonication at a 75% power input using an ultrasonic cell disruptor (Sonifier® Branson S-150D Danbury, CT, USA). During sonication, the temperature increased to 60±4°C due to molecular friction. This temperature was maintained constant using double walled beaker connected to an immersion circulator control Lauda E100 water bath (Lauda-Königshofen, Germany). Seed were separated using vacuum-assisted filtration. Mucilage-free CS were dried

using a tray dryer (Excalibur Dehydrator 3926TCDB, Sacramento, CA) held at 40°C for 12 h. The weight of the seeds was measured to calculate mucilage extraction yield by weight difference [Eq.1]. Ultrasound mucilage extraction was compared to conventional extraction methods using drying oven and freeze-drying techniques following the methodology proposed by (Campos et al., 2016) and (Capitani et al., 2013), respectively.

$$\% \text{ yield} = \frac{(\text{Weight of dry chia seeds} - \text{Weight of dry chia seeds without mucilage})}{\text{Weight of dry chia seeds}} \times 100 \qquad [\text{Eq. 1}]$$

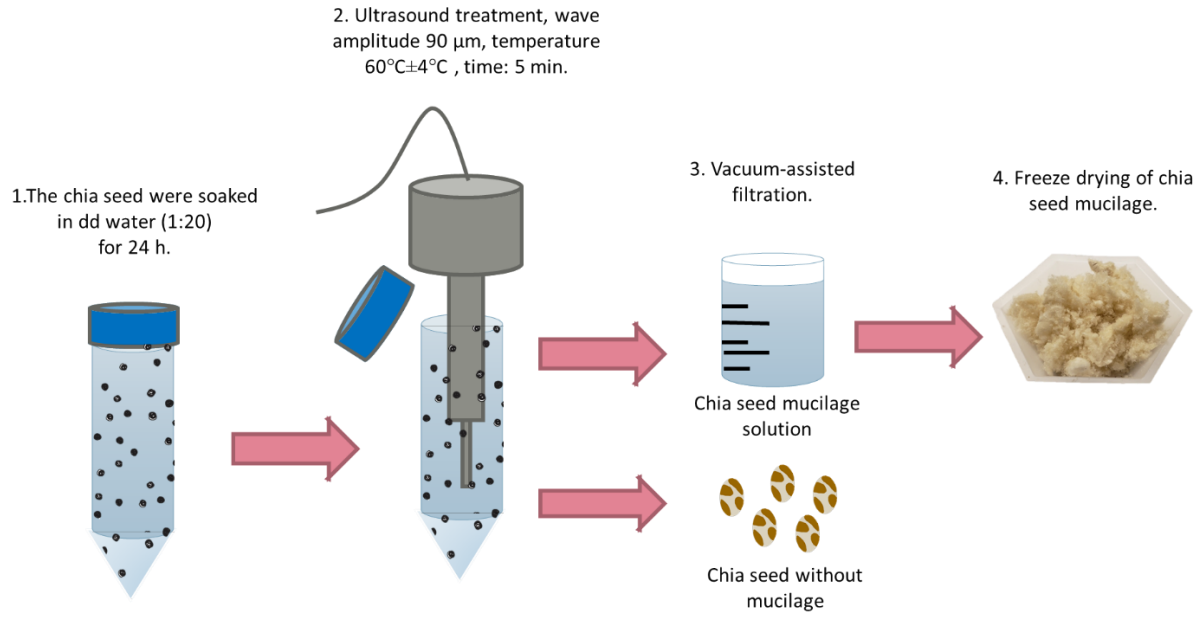

Figure 10. Diagram of mucilage separation (extraction) from chia seeds using ultrasonication and vacuum-assisted filtration.

3.2.3 Chia seed oil extraction

Dried, mucilage-free CS were defatted using a mechanical oil extraction method with a Beamnova Automatic Oil Press Machine (Commercial 304 Stainless Steel Expeller, Guangzhou, China). Seeds were pressed using a stainless-steel endless screw held at 37±2°C. The defatted chia seed was referred to as chia flour. Percentage of oil extraction was calculated by weight difference [Eq.2].

$$\text{yield}\% = 100 \times \frac{(\text{Weight of chia seeds} - \text{Weight of defatted chia seed flour})}{\text{Weight of chia seeds}} \quad \text{[Eq. 2]}$$

In addition, the crude protein content of the chia flour was determined using AOAC methods 984.13 (A-D) by A&L Greatlakes laboratories Facility (Fort Wayne, Indiana, USA).

3.2.4 Chia seed protein hydrolysate (CSPH)

Chia flour was diluted in distilled water to obtain 22.5 mg protein/mL and homogenized using a Sorvall Omni Mixer homogenizer with a macro-attachment assembly (Norwalk, CT, U.S.A). The pH was adjusted to 8.0 using 2 M NaOH, which is the optimal condition for alcalase activity. Proteins were enzymatically hydrolyzed using single enzyme alcalase (A) or a sequential digestion with alcalase+flavourzyme (AF). Proteolysis occurred using conventional (WB) or microwave-assisted (MW) hydrolysis using a microwave accelerated reaction system (MDS, MARS-Xpress/230/60, CEM Corporation, USA). Treatments were denoted as conventional alcalase hydrolysis using a water bath (A-WB) and alcalase microwave-assisted hydrolysis (A-MW). Sequentially (AF) hydrolyzed treatments were coded as AF-WB (water bath hydrolysis) and AF-MW (microwave-assisted hydrolysis). Finally, the control (C) was non-hydrolyzed CS protein. Samples A-WB and A-MW were hydrolyzed for 1 hour with 3% (w/w) Alcalase®. For sequential hydrolysis different times were used, due to the high efficiency of microwave-assisted hydrolysis the time was cut down by half to obtain similar degree of hydrolysis. AF-MW, the reaction was initiated with 2% (w/w) of Alcalase® for 45 min followed by addition of 2% (w/w) of Flavourzyme® for an additional 45 min. For AF-WB, the reaction was developed using 2% (w/w) of Alcalase® for 90 min followed by 2% (w/w) of Flavourzyme® for another 90 min. Hydrolysis was terminated by heating to 95±3°C for 15 min. The hydrolysates were cooled to 4 °C

and centrifuged at 17,636g for 15 min (Avanti J-26S Centrifuge, Beckman-Coulter INC. CA, USA).

3.2.5 Proximal Composition

The moisture determination was developed by the gravitation method proposed by (ASAE, 2002) for rapeseeds using an air convection oven. 10 grams of sample were dried for 4 hours at a temperature of 130 ± 1 ° C. The analysis (moisture, ash, lipid, and protein) of the chia seeds was done following the AOAC methods 920.153, 960.39, and 984.13 (A-D) respectively (AOAC, 2016).

3.2.6 Determination of the degree of hydrolysis

The degree of hydrolysis was calculated following the methodology of Adler-Nissen, (1986) with slight modifications by Liceaga-Gesualdo and Li-Chan, (1999), measuring spectrophotometrically the color formed by free amino groups reacting with Trinitrobenzenesulforonic acid (TNBS). Aliquots (1000 µL) of the sample were added to 1000 µL of 24% TCA solution. The samples were stirred for 1 min and then centrifuged at 21279 g for 5min. Then, Aliquots (1000 µL) of supernatant were added were mixed with 1000 µL of 2.0 M sodium borate buffer (pH 9.2) and 500 µL of 1.0 mM TNBS. The samples were incubated at room temperature for 30 min. The reaction was ended using 500 µL of 2M monosodium phosphate with 18mM sodium sulfide. The absorbance was measured at 420nm using polystyrene cuvette in a Beckmann UV-Visible spectrophotometer (Irvine, CA, USA). The degree of hydrolysis (% DH) was defined as a percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (h_{tot}). The h_{tot} was calculated experimentally using the fully

hydrolyzed chia seed flour, obtaining a value of 9.33 meq/g. The % DH was calculated using equation [Eq.3].

$$\%\, Degree\ of\ hydrolysis\ (DH) = \frac{h}{h_{tot}} \times 100 \qquad \text{[Eq. 3]}$$

3.2.6.1 Amino Acid Analysis

Total amino acid composition of CSPH was analyzed by the method described by Hall, Jones, O'Haire, & Liceaga, (2017) by UPLC Amino Acid Analysis Solution using the AccQ Tag Ultra Derivatization kit with UV detection (Water Corporations, Milford, MA, USA) by the Danforth Center's Proteomics and Mass Spectrometry Facility (St. Louis, Missouri, USA). Samples were pre-oxidized using performic acid and subject to acid hydrolysis with 0.5% phenol/6 M HCl in a vapor-phase hydrolysis vessel under vacuum. The acid hydrolysis will destroy cysteine (Cys), Methionine (Met) and Tryptophan (Trp). The pre-oxidation step using performic acid before the standard acid hydrolysis will yield stable formed of Cysteic acid (Cya) and methionine sulfoxide (MetS), which can, therefore, be measured. The quantity (moles) of amino acids in each peptide fraction was calculated using a series of standard solution runs before the samples. The relative abundance of amino acids (mole %) will be calculated by dividing the quantity (moles) of each amino acid by the sum of the concentration of all amino acids and multiplied by 100 and expressed as mole %.

3.2.6.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

CSPH and control samples were dissolved to 2 mg/mL in zwitterionic-chaotrophic buffer according to Hall, Johnson, and Liceaga, (2018) (2D-gel extraction buffer; 50 mM Tris-HCl, pH 8.8, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 M urea, 2 M thiourea, 67 mM Dithiothreitol (DTT) and stirred for 1 h at room temperature. An aliquot (50 µL) was added to 50

µL Laemmeli sample buffer to obtain a 1 mg/mL final concentration of protein. A sample (20 µL) of this solution was loaded using 4–12% gradient gels (Bis-Tris, NuPAGE, ThermoScientific, Waltham, MA) and ran with MES SDS running buffer (NuPAGE, ThermoScientific, Waltham, MA) at 200 V for 45 min. The gel was stained overnight using Coomassie R-250 and destained overnight using a solution of 40% (v/v) methanol and glacial acetic acid. The molecular weight distribution of hydrolyzed peptides was determined using Precision plus ProteinTM Dual Xtra Prestained Protein Standards (Biorad, Hercules, CA).

3.2.7 Bioactive properties of CSPH

3.2.7.1 2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The scavenging activity of the CSPH was determined according to a method described by Bersuder, Hole, and Smith, (1998) with modifications by Hall et al., (2018). CSPH and control (100 µL) were placed in a 96-well microplate to which 100 µL of (99.5%) ethanol and 25 µL of DPPH solution at a concentration of 0.05% (DPPH/ethanol, w/v) was added. The solution was incubated for 30 min at room temperature in dark conditions, and the absorbance measured at 550 nm using a microplate photometer. Radical reduction was expressed in mM TE/mg sample. Absorbance values were corrected using a sample blank prepared using 25 µL ethanol instead of DPPH solution.

3.2.7.2 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity (ABTS)

The ABTS radical scavenging activity was determined according to Ketnawa and Liceaga, (2017) with some modifications. A solution of 7 mM of ABTS was prepared in 2.45 mM of potassium persulphate and incubated at room temperature for 16 h. After 16 h the ABTS stock

solution was diluted with distilled water to obtain an absorbance at 734 nm of 0.700 ±0.02. A CSPH sample aliquot (20 µL) was mixed with 980 µL ABTS solution and incubated in the darkness at 30°C for 10 min, followed by absorbance reading at 734 nm. The ABTS scavenging activity was calculated by equation [Eq.4] and results expressed as mM Trolox equivalent (TE)/mg sample.

$$ABTS\ scavenging\ activity = \frac{(Abs\ of\ control - Abs\ of\ the\ sample)}{(Absorbance\ of\ control)} \times 100 \qquad [\text{Eq. } 4]$$

3.2.7.3 Metal Ion Chelating (MIC)

The MIC capacity was done following the procedure by Ketnawa and Liceaga, (2017) with modifications. In a 96-well microplate, 200 µL of CSPH samples were mixed with 3.75 µL of 2 mM $FeCl_2$ and 7.5 µL of 5 mM Ferrozine solution. Sample were incubated in the dark for 10 min at room temperature, and the absorbance was read at 522 nm. The MIC capacity was Calculated using the equation [Eq.5].

$$MIC\ ability = \left[\frac{(Abs\ control - Abs\ sample)}{(Abs\ control)}\right] \times 100 \qquad [\text{Eq. } 5]$$

3.2.7.4 Ferric Reducing Antioxidant Power (FRAP)

The FRAP was done according to Girgih, Udenigwe, and Aluko, (2011) methodology with slight modifications by Ketnawa et al., (2018). The 300 µL of protein samples were entirely mixed with 300 µL of 1% potassium ferricyanide (0.2mMolar of phosphate buffer pH 6.6), after this the samples were incubated at 50°C for a period of 20 minutes, 1.2 ml of 10% TCA were added and then mixed homogeneously then centrifuged at 10,000 x g for 5 min. A 100 µL aliquot of the mixture was pipet in a 96-well microplate with 100 µL of dd-water and finally, 20 µL of 0.1%

ferric chloride was added, the mixture was incubated at room temperature for 10 min, and the absorbance was measured at 700nm. Results are expressed in mM TE/mg sample.

3.2.7.5 Oxygen radical absorbance capacity (ORAC)

ORAC was measured according to a modified methodology described by Ou, Hampsch-Woodill, and Prior, (2001). CSPH were diluted to a protein concentration of 0.05 mg/mL in a 75 mM sodium phosphate buffer at a pH of 7.4. The experiment was carried out in a 96-well microplate, each well containing a total volume of 205 µL. 150 µL of Fluorescein (10 nM) was pre-incubated with 25 µL of CSPH sample solution for 15 min at 37°C in dark conditions. Then the reaction was initiated by adding 30 µL of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and the Fluorescence was measured using a spectrophotometer (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Massachusetts, United States) every two minutes for a total time of 90 min using an excitation wavelength of 485 nm and emission of wavelength 535 nm. The results were expressed in µM of Trolox Equivalent by measuring the fluorescein area decay through time (AUC). The values were calculated using the equation [Eq.6].

$$AUC = 1 + \frac{f_2}{f_0} + \frac{f_4}{f_0} + \frac{f_6}{f_0} + \frac{f_8}{f_0} + \cdots f_{90}/f_0 \qquad \text{[Eq.6]}$$

Where f_0 represents the area under the curve at time 0 and f_n the absorbance taken every minute.

3.2.7.6 Cellular antioxidant activity (CAA)

The CAA was evaluated following the methodology proposed by Malaypally et al., (2015) and Wan, Liu, Yu, Sun, and Li, (2015). First, CSPH and control were solubilized in Dulbecco's modified Eagle's medium (DMEM)/high modified phenol red free. Caco-2 cells (100 µL, density of 7.6 x 10^5 cells/mL) were placed in a 96-well black microplate and incubated for a period of 36

h under 5% CO_2 at 37°C. After this, the growth medium was removed using needles, washed using 1x PBS (100 µL), and exposed for 1 h to 100 µL of DMEM (60 µM Dichloro-dihydro-fluorescein diacetate DCFH-DA). CSPH was then added to a final concentration of 5 mg/mL. The solution was removed from each well, followed by a final washed-out with 1x PBS. The cells were exposed to an oxidizing environment by pipetting 100 µL of 500 µM AAPH into each well. Emission by the samples was measured for every 5 min for 1 h using a fluoresce reader Spectra Max Gemini EM spectrofluorometer (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. A sample blank, positive and negative control were required to calculate the cellular antioxidant activity. Sample blanks contained DMEM and (DCFH-DA) without AAPH, negative control wells were prepared incubating cells with DCFH-DA and AAPH and the positive control wells were incubated with cells treated with L-ascorbic acid (50 µM), DCFH-DA and AAPH. Finally, the cellular antioxidant activity was measured with equation [Eq.7], were the blank was subtracted from the sample readings. The fluorescence emission against time data were plotted and used to, calculate the area under the curve in CAA values (%) (Wolfe & Liu, 2007).

$$CAA\ unit = 100 - \left(\frac{\int SA}{\int CA}\right) \times 100 \qquad \text{[Eq.7]}$$

where ∫SA refers to the integral of the sample fluorescence vs. time and ∫CA refers to the integral from the control sample.

3.2.7.7 Dipeptidyl Peptidase IV (DPP-IV) Inhibitory Activity

The DPP-IV inhibitory activity of CSPH was determined following the method by Hall et al., (2018). CSPH samples were dissolved in 100 mM Tris-HCl buffer (pH 8.0) to a final concentration of 1.25 mg/mL. Sample aliquots (25 µL) were pipetted and pre-incubated in a 96-well microplate with 25 µL of substrate Gly-Pro p-nitroanilide hydrochloride (6 mM) at 37°C for

10 min. The colorimetric reaction was initiated by adding 50 µL of human DPP-IV (4.5 unit/mL), followed by incubation at 37°C for 60 min. The reaction was stopped by adding 100 µL of 1 M sodium acetate buffer (pH 4.0). Absorbance of released p-nitroanilide was measured at 405 nm using a Multiskan™ FC Microplate Photometer (Waltham, MA, USA). Sample absorbance was corrected by subtracting blanks in which DPP-IV was replaced with Tris-HCl buffer (100 mM, pH 8.0). The positive control (no inhibitor) used the buffer instead of CSPH sample. For negative control (no DPP-IV activity), the buffer was used instead of DPP-IV solution. Percent DPP-IV inhibition was calculated using equation [Eq.8].

$$DPP\ IV\ inhibition\ \% = 1 - \left[\frac{Abs\ Sample - Abs\ Sample\ blank}{Abs\ positive\ control - Abs\ negative\ control}\right] \qquad [\text{Eq. 8}]$$

3.2.7.8 Angiotensin Converting Enzyme (ACE) Inhibitory Activity

ACE-inhibitory activity was measured according to Hall et al., (2018). CSPH and control samples were dissolved in sodium phosphate buffer (100 mM, pH 8.3) with NaCl (300 mM). Aliquots (25 µL) of CSPH solutions were added to 25 µL of the substrate hippuryl-L-histidyl-L-leucine (HHL) and incubated at 37°C for 4 min. Then, aliquots (80 µl) of human-ACE (50 mU) were added to initiate the reaction, followed by incubation at 37°C in a water bath with constant stirring for 2 h. Reaction was terminated by adding 50 µl of 1 M HCl; then the solution was filtered using a 0.22 µm nylon filter. A control reaction was performed using 25 µl of buffer instead of the inhibitor (CSPH). Hippuric acid (HA) was quantified using high-pressure liquid chromatography (HPLC) (Model 600E, Waters Corporation, Milford MA) with a C18 analytical column (YMC Pack ODS AM 12505-2546WT, YMC America, Inc., Allentown, PA, USA). Percentage inhibition was calculated using equation [Eq.9]

$$ACE\ Inhibition\ \% = \left[1 - \frac{A_{inhibitor}}{A_{control}}\right] \times 100 \qquad [\text{Eq. 9}]$$

where, $A_{inhibitor}$ and $A_{control}$ represent the relative areas (A) with and without inhibitor of the HA peaks. The half maximal inhibitory concentration (IC_{50}) determined the potency of the samples towards ACE inhibitory activity. IC_{50} was expressed in mg of protein per mL. IC_{50} calculations were determined at four different CSPH concentrations (0.05, 0.5, 1, and 5 mg/mL) in triplicate.

3.2.8 Functional properties of CSPH

3.2.8.1 Solubility

Protein solubility was measured following the method described by Chobert, Bertrand-Harb, and Nicolas, (1988) and modified by Hall et al., (2017). CSPH were diluted to 1 mg/mL in 15 mL buffers with pH 3.0 (0.1 M sodium Acetate), 5.0 (0.1 M sodium Acetate), 7.0 (7.4 mM Phosphate) and 9.0 (0.1 M Glycine-sodium hydroxide), respectively. The solutions were stirred for 30 min at room temperature, followed by centrifugation at 12,150 × g (25°C) for 5 min. The protein content in the supernatant was determined using the bicinchoninic acid (BCA) protein assay method with bovine serum albumin as standard. Protein solubility was calculated by the ratio of protein in the supernatant to the protein content in the sample [Eq.10].

$$Solubility\ \% = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content\ in\ sample} \times 100 \qquad [\text{Eq. } 10]$$

3.2.8.2 Emulsion

Emulsifying activity index (EAI) and Emulsion stability index (ESI) were measured spectro turbidimetrically following the procedure of Pearce and Kinsella, (1978) and some modifications by Liceaga-Gesualdo and Li-Chan, (1999). CSPH was diluted to 0.5% (w/v) in 0.1 M phosphate buffer pH 7. A 3 mL aliquot was added to 1 mL of 100% pure canola oil and homogenized at 18,000 rpm for 1 minute, using Sorvall Omni Mixer with micro-attachments assembly (Norwalk,

CT, USA). Aliquots of 50 µL were pipetted immediately and diluted 200-fold into tubes containing 0.3% SDS solution. The tubes were inverted six times gently to ensure homogenization. The turbidity of the mixture was measured at 500 nm using Beckmann UV-Visible spectrophotometer (Irvine, CA, USA). EAI was calculated using equation [Eq. 11].

$$EAI = \frac{(2T) \times (A) \times (df)}{(\emptyset) \times (c) \times (10000)} \times 100 \qquad \text{[Eq. 11]}$$

Where:

T: turbidity at 500 nm wavelength at time zero.

df: dilution factor (200-fold)

L: light path in meters

ø: volume of the oil phase (0.25)

c: concentration of solids (0.5%) in the aqueous phase

Emulsion Stability Index (ESI) was measured by letting samples rest for 30, 60, and 90 min at 500 nm calculating the change in turbidity within time [Eq. 12].

$$ESI\ \% = 100 - \left[\frac{EAI_0 - EAI_T}{EAI_0}\right] \times 100 \qquad \text{[Eq. 12]}$$

Where EAIo is the emulsion activity index at zero min, and EAIt is the emulsion activity index at 30, 60, or 90 min.

3.2.8.3 Foaming

Foam capacity (FC) and foam stability (FS) were determined using the method proposed by Waniska and KIinsella, (1979) with modifications of Pacheco-Aguilar et al., (2008). 0.75 grams of protein were dissolved in 25 mL of dd-water (final pH 6.8) and stirred using a stir bar for 10 min at room temperature. The foam was developed by aerating the mixture exposing to air injection

using Sorvall Omni Mixer with a macro-attachment assembly (Norwalk, CT, USA). FC was expressed as the percentage of increment in volume after aeration [Eq. 13].

$$FC\ \% = 100 \times \frac{Volume\ after\ aeration - volume\ before\ aeration}{Volume\ before\ aeration} \qquad \text{[Eq. 13]}$$

FS was expressed as the percentage of foam remaining after 30, 60 and 90 min after the foam formation.

3.2.9 Statistical Analysis

Results were reported as mean ± standard deviation (SD) of triplicate determinations. A complete randomized design was used as a statistical model with a Duncan separation of means $p<0.05$. The correlation analysis between antioxidant assays and cellular antioxidant activity was calculated using a $p<0.05$. The statistical analysis was carried out using the statistical software SAS 9.4 (Cary, NC, USA). Experiments were done in triplicate.

3.3 Results and discussion

3.3.1 Mucilage ultrasound separation

Ultrasound treatment showed to be a novel approach for the separation of mucilage from chia seeds. In this study the combination of vacuum-assisted filtration and high temperatures increased mucilage separation ($p < 0.05$) the most (**Table 3**), while other tested methods involving other physical separation methods (centrifugation) showed inefficient separation **(Fig 11, B).**

The highest mucilage extraction was achieved at a wave amplitude of 90 µm or a power outage of 75% and a temperature of 55°C, followed by separation using vacuum-assisted filtration. The use of high temperature decreases the viscosity of CS-mucilage mixture. The reduction in viscosity is attributed to an increase in molecular motion and hydrogen bonds in the polysaccharide system

(Timilsena, Adhikari, Kasapis, & Adhikari, 2015). The low viscosity occasioned by high temperatures allows the sound frequency to travel in an easier manner compared to the mixtures where the temperature was lower. The optimized treatment was compared to other conventional methods of mucilage extraction. The mucilage extraction yield was higher ($p < 0.05$) when ultrasound was used (7.65 ± 0.19%), compared to freeze-drying the CS (4.21 ± 0.29%), and oven drying CS (3.65 ± 0.18%). The yield values obtained for conventional extractions methods in this study were lower than the ones reported by Campos et al. (2016) and Capitani et al. (2013). Chemat et al. (2017) showed how ultrasound developed high shear stresses in the proximity between liquid and solid materials, causing oil to separate from basil leaves, addressing the possibility of this application in other food matrices such as chia seed mucilage. Ultrasound frequencies generated microjets in the chia seed surface, targeting structures called columella's that allowed for the physical separation of mucilage in a fast and efficient way. We can conclude that the combination of ultrasound treatment and high temperatures followed by vacuum filtration increased the extraction yield of mucilage from chia seeds.

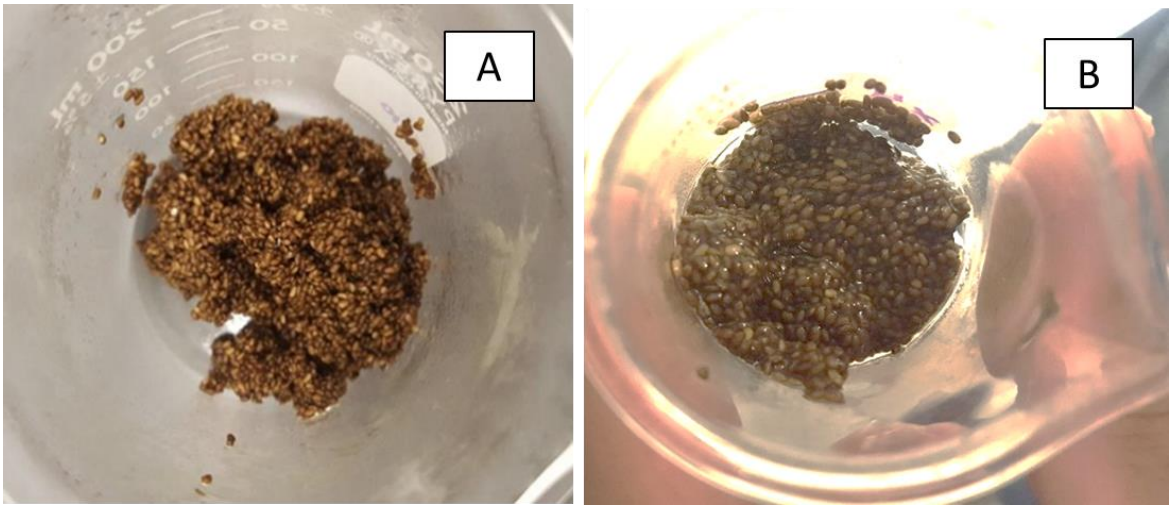

Figure 11. Clean chia seeds after mucilage extraction using ultrasound and vacuum-filtration separation (A); chia seeds with residual mucilage using ultrasound and centrifugation (B).

Table 3. Mucilage extraction from chia seeds.

Temperature ° C	Separation method	Yield of mucilage extraction (%)
55	Vacuum-filtration	7.66 ± 0.23a
55	Centrifugation	6.23 ± 0.56b
40	Vacuum-filtration	4.72 ± 0.17c
40	Centrifugation	3.66 ± 0.17d
25	Vacuum-filtration	3.66 ± 0.17d
25	Centrifugation	3.93 ± 0.17d

3.3.2 Chia seed oil extraction

The oil extraction yield obtained by cold press was 28.24 ± 0.06 g of oil/100 g of chia seeds (without mucilage). The oil content in chia seeds varies between 30-33% (Sandoval-Oliveros & Paredes-López, 2013). Cold pressing of chia seeds without mucilage allows to remove about 95.81 ± 0.12 % of the total fat of the seed. Conversely, CS with mucilage lead to an extraction of only 62.90 ± 0.77 %. This is mainly attributed to the high oil holding capacity of chia seed mucilage (Darwish, Khalifa, & El Sohaimy, 2018). The use of screw-press for oil extraction is a good alternative to reduce the use of hazardous solvents such as isopropanol and hexane in the extraction. Also, removal of oil from seeds (in preliminary experiments) showed to increase the enzyme-protein interaction, facilitating the hydrolysis.

3.3.3 Enzymatic hydrolysis

Table 5 shows the degree of hydrolysis obtained from the different treatments. The highest degree of hydrolysis (46.81 ± 0.19 and 40.68 ± 0.77 %) was obtained by sequential hydrolysis with alcalase and flavourzyme (AF-WB) and (AF-MW), respectively. CS protein is difficult to hydrolyze due to its high content of globulin fractions, which contain several sulfur amino acids directly involved in maintaining the tertiary and quaternary structure of the protein (Sandoval-Oliveros & Paredes-López, 2013). This conformation limits the access of the enzyme to cleavage

sites that are located within the protein fraction. This was confirmed by the amino acid analysis were the content of sulfur-containing amino acids (Cys+Met) **(Table 6)** make up to 7% of the total amino acid composition of chia seed flour (control); a similar value of 6% was reported by (Sandoval-Oliveros & Paredes-López, 2013). The higher hydrolysis in the AF-MW treatment can be attributed to the efficient separation of mucilage and the use of microwave energy. Singh, Orsat, and Raghavan, (2013) evaluated the effect of electromagnetic fields in protein structure and conformational changes, showing how magnetic forces pull dielectric charges in the protein backbone causing protein unfolding or re-orientation. The protein unfolding exposes active sites that allow the proteases to carry out digestion in a fast and efficient way. In addition, separation of mucilage played an important role in the effectiveness of the hydrolysis. The minimum presence of this polysaccharide can block the enzyme-substrate interaction by a process called enzyme immobilization, resulting in low proteolysis. A study conducted by Monroy-Torres et al., (2008) showed that CS flour has a low protein digestibility (79.28%) which is attributed to the presence of mucilage. In general, the utilization of an efficient mucilage separation method and microwave energy decreased the time required to generate higher hydrolysis (DH) and consequently produce lower molecular weight peptides.

3.3.4 Proximal Analysis

The proximal analysis is shown in **(Table 4)**. The content of protein varies from 40-79%. A significant increment in protein content ($p<0.05$) was achieved when protein was enzymatic hydrolyzed compared to the control, but no significant ($p<0.05$) difference was found between enzymatic hydrolysis treatments. These results indicate the ability of enzymatic hydrolysis to liberate protein and remove non protein bound components. Studies conducted in rice bran protein have shown that protein hydrolysis is an alternative way to increase the protein extraction from

complex matrices using enzymes such as flavourzyme and alcalase, increasing the protein extraction to 88 and 81% respectively (Fabian & Ju, 2011). No significant difference was found in ash content for control and hydrolysis treatments. Other studies have shown the ash content of CS to vary between 4-6% (Coates, 2011). Carrillo et al. (2018), study the proximal composition of chia seeds and found the ash to be 4.82%. In this study, the ash content range between 3.74 and 7.44%, minor difference might be attributed to the difference in chia seed sources and removal of other compounds. Ash refers to the inorganic residue that results after a food has been incinerated, this often includes oxides, sulfates, phosphates, chloride, and silicates (Marshall, 2010). The carbohydrate content (calculate by weight difference) ranges from 14.47 to 52.47%. this is mainly attributed to the seed coat still present in the control. Finally, the fat content in this study ranged between 0.37 to 2.61%, studies have assessed the fat content of chia seeds and identified their values to range between 30 to 33% (Mohd Ali et al., 2012). The whole seeds have a fat content of 37.5%, therefore the fat extraction by cold press extracted 95.82% of the fat in the seeds. Additionally, the fat content decreased once enzymatic hydrolyzed, and the fat content increased as the degree of hydrolysis increases. Is known that enzymatic proteolysis helps to release bound non protein components (eg., fats, starches, fibers), the increase in fat content for the sequential treatment AF-MW and AF-WB, is attributed to an increase release of fat bound to protein. Similar observations were observed when tilapia (*Oreochromis niloticus*) was enzymatic hydrolyzed with neutrase and alcalase (Shirahigue et al., 2016).

Table 4. Proximal analysis of chia seed protein hydrolysates. Results are reported on a dry weight basis.

Treatment	Protein (%)	Fat (%)	Ash (%)	carbohydrates (%)
A-WB	79.35 ± 0.64a	0.37 ± 0.00ab	5.73 ± 1.41a	14.54 ± 0.00b
A-MW	77.82 ± 3.29a	0.57 ± 0.01bc	4.80 ± 1.03a	16.54 ± 4.58b
AF-WB	74.69 ± 0.20a	2.61 ± 0.40c	3.89 ± 0.03a	18.53 ± 0.50b
AF-MW	78.16 ± 2.12a	1.22 ± 0.00c	5.76 ± 1.12a	14.47 ± 3.64b
Control	40.54 ± 3.08b	2.53 ± 0.09a	7.73 ± 1.58a	52.47 ± 1.47a

A-WB: chia seed protein hydrolyzed by alcalase enzyme using water bath heating method. A-MW: chia seed protein hydrolyzed by alcalase enzyme using microwave-assisted hydrolysis. AF-WB: chia seed flour hydrolyzed by alcalase and flavourzyme enzymes using water bath heating method. AF-MW: chia seed protein sequentially hydrolyzed by alcalase and flavourzyme enzymes using microwave-assisted hydrolysis. Control: unhydrolyzed chia seed flour.

3.3.5 Antioxidant Activity

The antioxidant properties of the peptides are shown in **(Table 5)**. Overall, the trend for radical scavenging activity of the peptides showed an increase with increasing extent of hydrolysis (DH). AF-MW protein hydrolysates showed overall significant higher ($p < 0.05$) antioxidant properties for MIC, DPPH, ABTS, and CAA. Orona-Tamayo et al., (2015) identified that CS protein between 20-33 kDa corresponds to the globulin protein fraction. Globulin fractions, specifically Globulin 11s, are responsible for the high antioxidant activities in plant-based materials (Delgado et al., 2016). In this study, the same band was observed in the molecular weight distribution corresponding to AF-MW **(Fig 12)**; it is suspected that the combination of sequential hydrolysis and microwave energy caused a partial extraction of globulin fractions. Also, the presence of small peptides in all CSPH treatments displayed strong molecular weight bands below 25 kDa **(Fig 12)**, the presence of low molecular weight peptides can be responsible for the high antioxidant activity of CSPH treatments when compare to the unhydrolyzed chia seed protein (control).

For ABTS, the results for AF-MW are 70-fold higher than those previously reported by Segura-Campos, Salazar-Vega, I. M., Chel-Guerrero, L. A., and Betancur-Ancona, D. A, (2013) where

the protein hydrolysates were produced using sequential hydrolysis and water bath. ABTS and DPPH assays evaluated antioxidant activity by a specific mechanism called scavenging of free radicals. Studies have shown that ability is enhanced depending on factors such as amino acid composition, protein sequences and structural properties (Sarmadi & Ismail, 2010). AF-MW treatment allowed for the release of a specific group of globulin fractions (G3, G4 and G5), which compared to unhydrolyzed CS protein, showed that the globulin fraction has a higher concentration of Phe, Tyr and His (Sandoval-Oliveros & Paredes-López, 2013).

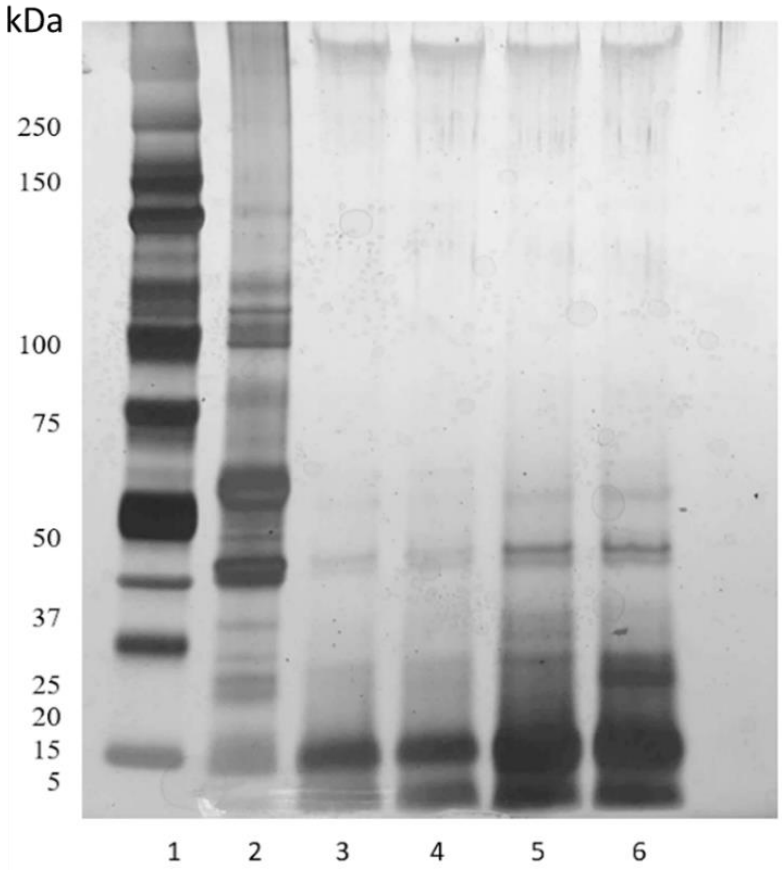

Figure 12. Molecular weight distribution of chia seed protein hydrolysates. Lanes indicate: (1) molecular weight markers, (2) Control: unhydrolyzed chia seed flour, (3) A-WB: chia seed protein hydrolyzed by alcalase enzyme using water bath heating method, (4) A-MW: chia seed protein hydrolyzed by alcalase enzyme using microwave-assisted hydrolysis, (5) AF-WB: chia seed protein hydrolyzed by alcalase and flavourzyme enzymes using water bath heating method, (6) AF-MW: chia seed protein sequentially hydrolyzed by alcalase and flavourzyme enzymes using microwave-assisted hydrolysis.

These results lined up with the ones obtained in this study, were the content of aromatic amino acids (His and Phe) are higher in AF-MW compared to unhydrolyzed CS protein **(Table 6)**. It is hypothesized that protein rotation and unfolding caused by MW and the release of encrypted peptides cause by sequential hydrolysis, enhanced the exposure of these aromatic amino acids responsible of a high donation of protons (Sarmadi & Ismail, 2010). The proton donations from aromatic amino acids to molecules with electron deficiency, improves the scavenging activity stabilizing reactive molecules (Sarmadi & Ismail, 2010). In contrast, the metal ion chelating (MIC) capacity is generally attributed to peptides containing sulfhydryl amino acids such as Cys and Met, which can bind heavy metals and reduce the pro-oxidant activity of some metals. The content of Cys and Met amino acids **(Table 6)** was higher in the control than in other treatments. It is hypothesized that microwave-assisted hydrolysis released encrypted sulfur peptides, making them more bioavailable to interact with free metals, therefore enhancing the MIC capacity.

For FRAP, the not hydrolyzed protein (control) showed the highest antioxidant value, the FRAP mechanism is to quantify the reducing potential of the BPs when it reacts with potassium ferricyanide and ferric chloride creating the emission of light at 700 nm. Bamdad, Wu, and Chen, (2011) showed that vast hydrolyzed proteins suffer a decrease of the reducing activity and that medium size proteins are responsible for ferrous ion chelating activity causing the entrapping of ferrous ions. He attributes this to the decrease of cage structure caused by the high amount of peptide bond cleavage.

Table 5. Bioactive properties of CSPH.

Sample code	Hydrolysis time, (% Enzyme)	Degree of hydrolysis (%)	% MIC Inhibition	FRAP (µmol TE/mg)	DPPH (µmol TE/mg)	ABTS (µmol TE/mg)	ORAC (µmol TE/mg)
A-WB	1 h 3%	33.64 ± 1.44c	66.93 ± 0.57c	83.45 ± 3.87c	131.74 ± 17.33b	465.97 ± 10.46c	1225.49 ± 55.53bc
A-MW	1 h 3%	37.04 ± 2.67bc	72.76 ± 0.42b	171.03 ± 4.92b	166.61 ± 15.34a	435.30 ± 12.96d	1535.81 ± 99.16a
AF-WB	3 h 2%	46.81 ± 0.19a	74.11 ± 0.64b	180.76 ± 4.19b	171.31 ± 8.27a	489.09 ± 3.86b	1482.48 ± 114.63ab
AF-MW	1.5 h 2%	40.68 ± 0.77ab	76.85 ± 0.37a	59.93 ± 3.03c	178.02 ± 15.86a	506.07 ± 4.50a	1122.71 ± 24.74c
Control	0 h 0%	0.00 ± 0.00d	54.43 ± 1.03d	410.26 ± 29.84a	22.99 ± 1.49c	100.57 ± 3.80e	816.33 ± 86.18d

Values are mean ± standard deviation of triplicate determinations. Different letters (a-d) indicate significant differences ($p < 0.05$) between treatments (rows). A-WB: chia seed protein hydrolyzed by alcalase enzyme using water bath heating method. A-MW: chia seed protein hydrolyzed by alcalase enzyme using microwave-assisted hydrolysis. AF-WB: chia seed flour hydrolyzed by alcalase and flavourzyme enzymes using water bath heating method. AF-MW: chia seed protein sequentially hydrolyzed by alcalase and flavourzyme enzymes using microwave-assisted hydrolysis. Control: unhydrolyzed chia seed flour.

ORAC is a common antioxidant assay based on the quantification of the fluoresce emitted by a probe, in this case a protein called fluorescein. Fluorescein is exposed to an oxidative environment by AAPH that causes degradation of the protein, and, consequently, a loss in fluorescence. This assay measures the hydrophilic antioxidant capacity against certain peroxyl radicals (Aruoma & Mutagenesis, 2003). The highest ORAC values ($p<0.05$) were seen in the microwave treatments A-MW and AF-WB at, 1535.81 ± 99.16 and 1482.48 ± 114.63 µmol TE/mg of protein, respectively. Exposure during hydrolysis of encrypted nonpolar residues such as Gly, Ala, Ile, Trp, Tyr and Met are speculated, this in turn can cause an increase in hydrophobic interactions of peptides with oxidizing agents. These results showed a higher ORAC value

compared to other digested materials such as cowpea protein (783.8 µmol/g of protein) and salmon (1541 µmol/g of protein) (Marques et al., 2015).

Table 6. Total Amino acid analysis for chia seed protein and CSPH (g/100 g).

Amino Acid	Control	A-WB	A-MW	AF-WB	AF-MW
Gly	5.128	4.278	4.465	4.231	4.354
Ala	5.405	5.196	4.994	4.972	4.940
Pro	3.573	3.518	3.533	3.477	3.708
Val	4.358	4.462	4.272	4.563	5.021
Ile	2.544	3.096	3.003	3.114	3.474
Leu	5.328	5.685	5.550	5.532	5.985
Met	4.629	3.604	3.986	3.970	3.759
Phe	3.267	3.219	3.408	3.397	3.850
His	1.417	1.358	1.362	1.332	1.358
Thr	3.964	3.608	3.502	3.520	3.567
Cys-Cys	3.046	1.536	1.843	1.797	1.804
Tyr	0.069	0.013	0.017	0.009	0.007
Glu	29.001	30.857	30.675	30.552	29.181
Arg	7.309	7.484	7.985	8.125	8.316
Ser	4.913	5.447	5.545	5.556	5.551
Asp	10.064	12.158	11.710	11.418	10.808
Lys	5.984	4.479	4.151	4.436	4.317
SCAA	7.675	5.140	5.829	5.767	5.564
AAA	4.754	4.591	4.788	4.738	5.215
PCAA	14.710	13.322	13.498	13.893	13.991
BCAA	12.230	13.243	12.825	13.208	14.480

SCAA= sulfur containing amino acids (Met+Cys). AAA= Aromatic amino acids. (Phe, His and Tyr). PCAA= positively charged aminoacids (Arg, His and Lys). BCAA=Branched containing amino acids (Leu, Ile and Val). Sample codes descriptions are provided in Table 5.

The cellular antioxidant activity (CAA) assay indirectly evaluates the permeability of a compound through the cell bilayer while estimating the antioxidant capacity. In this method, a

fluorescent probe DCFH-DA is introduced into the Caco-2 cells, and by the action of esterase is turned into a more polar form of DCFH which emits fluorescence under physiological conditions (Wan et al., 2015). In order to protect DCFH from oxidation caused by AAPH solution, the CSPH peptides must permeate and/or bind to the cell membrane and function a protective barrier against free radicals, thus preventing molecular damage of intracellular organelles and proteins. If the peptides have a good permeability and a high enough antioxidant capacity, they will protect the fluorescent probe from turning into dichloro-dihydro-fluorescein (DCF) and consequently avoiding a decrease in light emission through time (Wan et al., 2015). The highest CAA value **(Table 8)** was observed for the microwave treatments AF-MW and A-MW (94.76 ± 1.96 and 93.13 ± 1.07%, respectively). Wolfe and Liu, (2007) found that the hydrophobicity of the compounds was an important criterion to determine the antioxidant effectiveness in cell culture. This is not the only criteria since structural conformation also plays a crucial role in the quality and effectiveness of the antioxidant activity of these compounds (Wolfe & Liu, 2007). Conradi, Hilgers, Ho, and Burton (1991) found that the permeability of peptide chains was significantly correlated with the total number of hydrogen bonds a peptide can possibly form with water since the major impediment for passive absorption is the energy required to break the peptide-water hydrogen bonds. It is hypothesized that smaller peptides that have a weaker hydrogen bonding capacity and a higher concentration of antioxidant amino acids on the microwave samples (A-MW and AF-MW), are responsible for the increased cellular antioxidant activity observed in this study.

3.3.6 Correlation of antioxidant assays to CAA

A correlation analysis was carried out determining the significance of some antioxidant assays to predict cellular antioxidant activity. The analysis presented a significant correlation ($p<0.05$, $r= 0.888$) of DPPH with respect to CAA followed by ABTS **(Table 7)**. No significant

correlation was found for MIC and CAA. The correlation values between antioxidant assays and CAA was not found in literature for most of the conventional antioxidant methods. Wolfe et al. (2008) established a correlation of ORAC to CAA finding that this assay positively correlates with a Pearson correlation coefficient of ($r= 0.803$) and a significance ($p<0.05$). Their results differ with the results obtained in this study, were ORAC values showed lower correlation ($r= 0.646$, $p<0.05$,). We can conclude that DPPH and ABTS are the most useful screening methods for antioxidant evaluation of compounds since the chemical conditions of these assays allow the effective prediction of *in vitro* antioxidant activity in cellular environments.

Table 7. Correlation analysis between antioxidant assays with cellular antioxidant analysis.

Antioxidant assays	Pearson Correlation Coefficients for CAA (r)	Probability
MIC	0.8626	0.059
FRAP	-0.9156	0.029*
ABTS	0.88192	0.047*
DPPH	0.88887	0.043*
ORAC	0.64662	0.023*

* significant correlation ($p<0.05$) was established.

3.3.7 Antidiabetic properties

The antidiabetic capacity assay measures the peptides' ability to inhibit human DPP-IV. The DPP-IV activity improved in all hydrolyzed treatments compared to the control **(Table 8)**. The highest ($p<0.05$) DPP-IV inhibition was observed in AF-WB ($69.50 \pm 1.05\%$) and the lowest was in the control ($18.18 \pm 1.47\%$). Matsui, Oki, and Osajima (1999), studied the DPP-IV inhibition activity of sardine muscle hydrolysates and attributed the inhibition capacity to di- and

tetra-peptides (Val-Trp and Try -Tyr -Pro -Leu) that match the structure of the substrate of the DPP-IV enzyme. In another study, Nongonierma, Le Maux, Dubrulle, Barre, and FitzGerald (2015) evaluated the DPP-IV inhibitory activity of the protein hydrolysates of quinoa, a similar pseudo-cereal. The IC_{50} value of quinoa protein hydrolysates was of 0.88 ±0.05 mg/mL using a porcine DPP-IV enzyme (0.0025 U/mL). Velarde-Salcedo et al., (2013) evaluated the DPP-IV inhibition of amaranth tryptic digests using porcine DPP-IV (0.0025 U/mL) and found IC_{50} value ranging between 1.2 to 2 mg/mL. To the best of authors' knowledge, no previous report of DPP-IV inhibition activity by CSPH using human DPP-IV (2.25 U/mL) has been reported. In this study AF-WB CSPH had an IC_{50} of 1.28 mg/mL, showing the bioactive potential for CSPH, compared to other pseudo-cereals. Lacroix and Li-Chan, (2015) compared the susceptibility of porcine and human DPP-IV to inhibition and found that porcine DPP-IV is generally inhibited with greater efficacy by protein derived peptides than human DPP-IV. Generally, the DPP-IV inhibition is enhanced with the presence of lower molecular peptides, probably matching the structure of the incretin hormones (GIP and GLP-1). The incorporation of flavourzyme might have influenced the development of tetra- and di- peptides that match the DPP-IV active site. A similar result was obtained for protein hydrolysates derived from Atlantic salmon (*salmon salar*) skin gelatin, were the highest DPP-IV inhibition was achieved using flavourzyme (Li-Chan et al., 2012).

Table 8. Cellular antioxidant activity, Antidiabetic and antihypertensive properties of CSPH.

Sample code	Cellular antioxidant activity %	DPP-IV inhibition capacity		ACE inhibition capacity	
		% inhibition per 2.5 mg of protein	IC_{50} (mg/mL)	% inhibition per mg of protein	IC_{50} (mg/mL)
A-WB	87.54 ± 3.31bc	37.05 ± 0.99c	4.38 ± 0.49a	57.16 ± 1.93a	0.51 ± 0.12a
A-MW	93.13 ± 1.07ab	40.56 ± 0.42c	3.59 ± 0.31b	57.17 ± 3.42a	0.44 ± 0.09a
AF-WB	80.06 ± 2.48c	69.50 ± 1.05a	1.28 ± 0.09d	56.24 ± 0.74a	0.42 ± 0.04a
AF-MW	94.76 ± 1.96a	53.49 ± 0.86b	2.12 ± 0.08c	45.60 ± 2.24b	0.55 ± 0.02a
Control	62.00 ± 9.08d	18.18 ± 1.47d	-	11.18 ± 2.31c	-

Values are mean ± standard deviation of triplicate determinations. Different letters (a-d) indicate significant differences ($p < 0.05$) between treatments (rows). A-WB: chia seed protein hydrolyzed by alcalase enzyme using water bath heating method. A-MW: chia seed protein hydrolyzed by alcalase enzyme using microwave-assisted hydrolysis. AF-WB: chia seed flour hydrolyzed by alcalase and flavourzyme enzymes using water bath heating method. AF-MW: chia seed protein sequentially hydrolyzed by alcalase and flavourzyme enzymes using microwave-assisted hydrolysis. Control: unhydrolyzed chia seed flour.

3.3.8 Anti-hypertensive activity

In this study, most of the CSPH treatments had similar ACE inhibition capacity except for AF-MW and the control which were significantly ($p<0.05$) lower **(Table 8)**. Other studies have looked at the bioactivity of CSPH incorporated into foods. A study conducted by Segura-Campos, Salazar-Vega, I. M., Chel-Guerrero, L. A., and Betancur-Ancona, D. A, (2013) showed an improvement in IC_{50} value of the ACE-inhibitory capacity of foods by incorporating 5.0 mg/g of CSPH into carrot cream, causing a decrease from 27.67 μg protein/mL to 1.71 μg protein/ml. In our study, the IC_{50} values of CSPH was around 0.40 mg/mL. In the case of the lower inhibitory activity of AF-MW, it might be related to the formation of different structural peptides with a lower binding capacity to the ACE active site. Additional analytical studies are needed to

understand the difference with peptide conformation in CSPH. It is well known that the increase in DH and lower molecular-weight peptides improve the capacity of inhibiting ACE enzymes. Segura-Campos, Salazar-Vega, I. M., Chel-Guerrero, L. A., and Betancur-Ancona, D. A, (2013) evaluated the ACE inhibitory capacity of CSPH (51.64% DH) and obtained 53.8-69.3% inhibition for purified fractions. In this study, the CSPH peptides displayed a 57% inhibition with a 46% DH. Nevertheless, an influence of microwave energy in peptide conformation is not clear for ACE inhibition. Ketnawa et al., (2018) evaluated the ACE inhibition of microwave-assisted hydrolyzed peptides of rainbow trout (*Oncorhynchus mykiss*) and showed that peptides delivered from microwave-assisted hydrolysis had the highest inhibition at 93.5 ± 0.24%.

3.4 Functional Properties

3.4.1 Solubility

The solubility of CSPH showed a dependency of the pH in all treatments, with solubility increasing with increasing pH **(Fig 13)**. This pattern is similar to what is reported for many plant proteins and is related to the low isoelectric point (pI) of CS protein. When the pH of the solution is above or below the pI of a protein the solubility is enhanced because the electrostatic repulsion between molecules is greater than the hydrophobic interactions (Zayas, 2012). CS protein extraction is more efficient at alkaline pH, reaching its maximum solubility at pH 12 (Timilsena, Adhikari, Barrow, & Adhikari, 2016). A previous study by Timilsena et al., (2016) evaluated the solubility of CS protein isolate, reporting 10% solubility at pH 3. A similarly low value was obtained in this study for the solubility of the control (16%) **(Fig 13)**. AF-MW treatment showed the highest solubility at pH 3 (68.32%) and pH 5 (77.17%) when compared to other treatments **(Fig 13)**. This can be attributed to the high degree of hydrolysis of this treatment (40.68 %), where the presence of small peptides increases the exposure of polar and ionizable groups, consequently

increasing their solubility (Nguyen et al., 2017). In addition, enzymatic hydrolysis by alcalase and flavourzyme can increase the number of smaller hydrophilic polypeptides (Zhao et al., 2012). At neutral and alkaline pH, A-MW treatment showed the highest solubility ($p < 0.05$), suggesting that a medium DH (medium size peptides) could improve the solubilization of protein in neutral and alkaline solvents. The application of microwave-assisted hydrolysis can be correlated with an increase production of small peptides and consequently with an increased solubility (Uluko et al., 2013). Overall, hydrolyzing the CS flour improved the solubility of the protein hydrolysates, compared to the unhydrolyzed control at all pH range values.

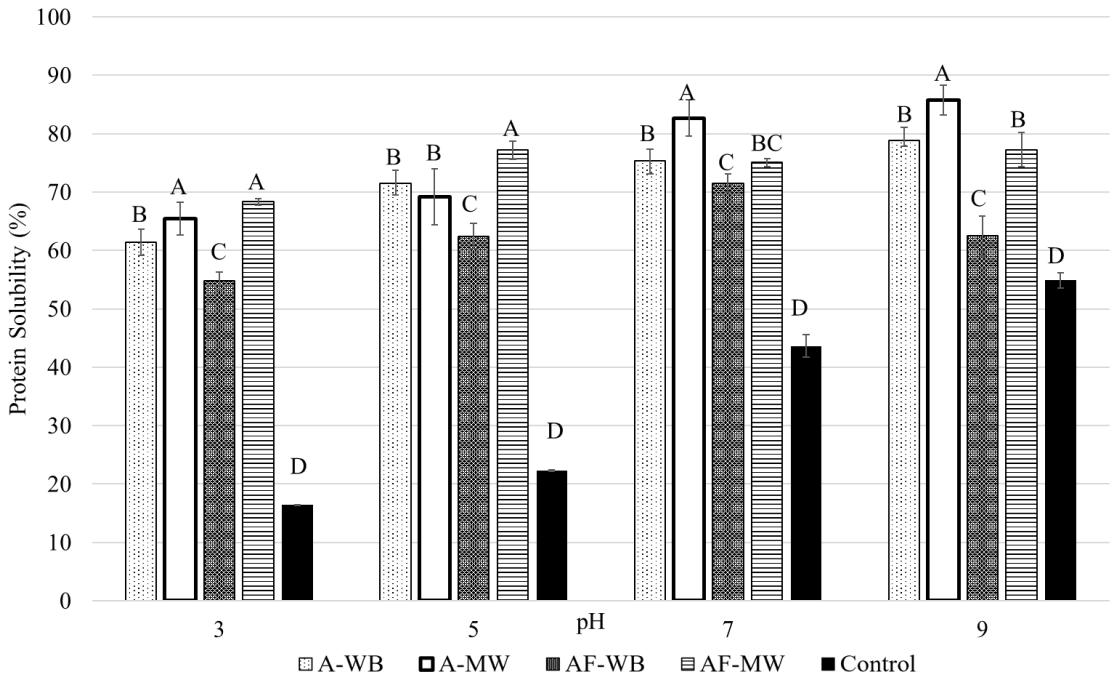

Figure 13. Results for Solubility of chia seed protein hydrolysates. Different letters (a-d) show significant differences ($p < 0.05$) between treatments. Sample codes descriptions are provided in Table 5.

3.4.2 Emulsion

Hydrolysis of CS flour increased its emulsifying capacity (at 0 min) compared to the control **(Fig 14)**. No difference ($p<0.05$) was found for the emulsifying capacities between A-WB, A-MW, and AF-MW. The overall improvement of these treatments in relationship with the control (unhydrolyzed protein) may be due to the enzymatic digestion by alcalase. Similarly, Klost and Drusch, (2019) hydrolyzed pea protein using enzymatic hydrolysis, and found that the oil-droplet-size in hydrolyzed proteins was smaller increasing the emulsion capacity of the unhydrolyzed protein, they attributed this to a vast exposure of hydrophobic residues and the presence of electrostatic repulsion between droplets. Also, a possible effect of microwave irradiation is speculated causing a protein denaturation. Villanueva, Harasym, Muñoz, and Ronda, (2019) evaluated the effect of microwave energy in the viscoelastic characteristics of rice flour, were microwave treatments caused and overall increase in the network formation, they attributed this to the denaturation of the protein. Similarly, Zhang et al., (2019) evaluated the effect of heat treatments in camelia protein cake and found that protein denaturation causes a modification in the secondary structure of the protein, FTIR spectra analysis suggests that temperature cause protein to increase in flexibility, hydrophobicity and exposed charged residues. In contrast, AF-WB had lower emulsifying capacity ($p<0.05$) followed by the control. The reduction in emulsifying capacity in this treatment might be correlated to its high degree of hydrolysis, meaning there is a higher content of low molecular weight peptides. In a study conducted using quinoa seed protein hydrolysates, showed how smaller peptides are unable to form stable films around oil droplets which result in the emulsion collapsing within time (Aluko & Monu, 2003). Differences ($p<0.05$) were observed when evaluating the emulsifying stability over 30, 60 and 90 min, where A-MW showed the best stability at 30 and 60 min, followed by the AF-MW **(Fig 14)**. However, both

alcalase treatments (A-MW and A-WB) had similar emulsion stability at 90 min. A study conducted by van der Ven, Gruppen, de Bont, & Voragen, (2001) showed that whey and casein protein hydrolysates have better emulsifying capacities compared to intact casein and whey protein. Authors attributed this to a more uniform distribution of emulsion droplets size originated from partially hydrolyzed peptides compared to intact protein were the droplets are bigger and collapse faster (van der Ven et al., 2001).

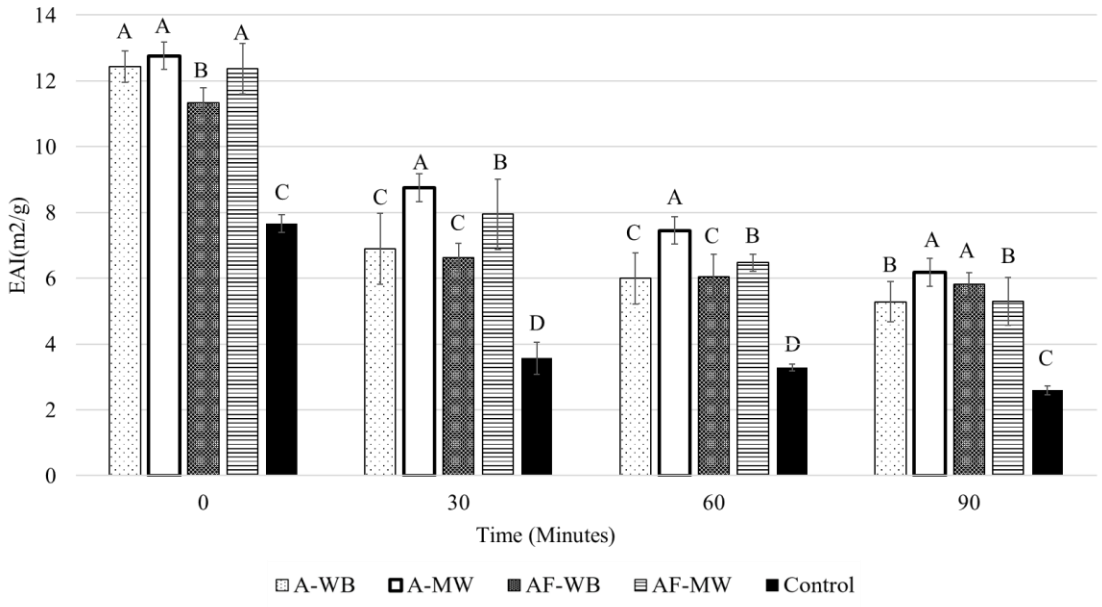

Figure 14. Results for Emulsifying activity of chia seed protein hydrolysates. Different letters (a-d) show significant differences ($p < 0.05$) between treatments. Sample codes descriptions are provided in Table 5.

3.4.3 Foaming

A-MW showed the highest foaming capacity (75%) (at 0 min), followed by A-WB (66.5%) **(Fig 15)**. This result agrees with the highest solubility at pH 7 observed in A-MW, indicating that these peptides are more evenly distributed through the aqueous interface and consequently improve the

way they entrap air molecules. Similarly, Nguyen et al., (2017) developed protein hydrolysates from rainbow trout frames using microwave-assisted hydrolysis, proving that lower molecular weight peptides derived by microwave energy diffuse faster into the air-water interface. CSPH showed a great potential in foaming capacity when compared to other protein sources as whey protein hydrolysates (FC=4%) and rice protein hydrolysates (FC=6%), nevertheless different rice sources have shown foaming capacities as high as 105% (Amagliani, O'Regan, Schmitt, Kelly, & O'Mahony, 2019). Olivos-Lugo, Valdivia-López, & Tecante, (2010) evaluated the foaming capacity of CS protein isolate and reported 70% foaming capacity. The purification and isolation of the protein is suspected to be responsible of the high values reported for the CS protein isolate. Evaluation of foam stability at 30, 60 and 90 min, showed that A-WB resulted in overall higher foaming stability. The decrease in foaming capacity of A-MW compared to A-WB can be attributed to the presence of smaller peptides caused by microwave treatment; smaller peptides are known to have poor stability through time. Nevertheless, A-WB and A-WW have lower DHs amongst all CSPH **(Table 5)** meaning that medium size peptides might be present. Balti et al., (2010) evaluated the influence of DH on foaming properties of cuttlefish and found that foaming capacity decreased slightly as the protein hydrolysis increased. They attributed this to the loss of cohesiveness that is achieved with high molecular-weight peptides and partially hydrolyzed protein.

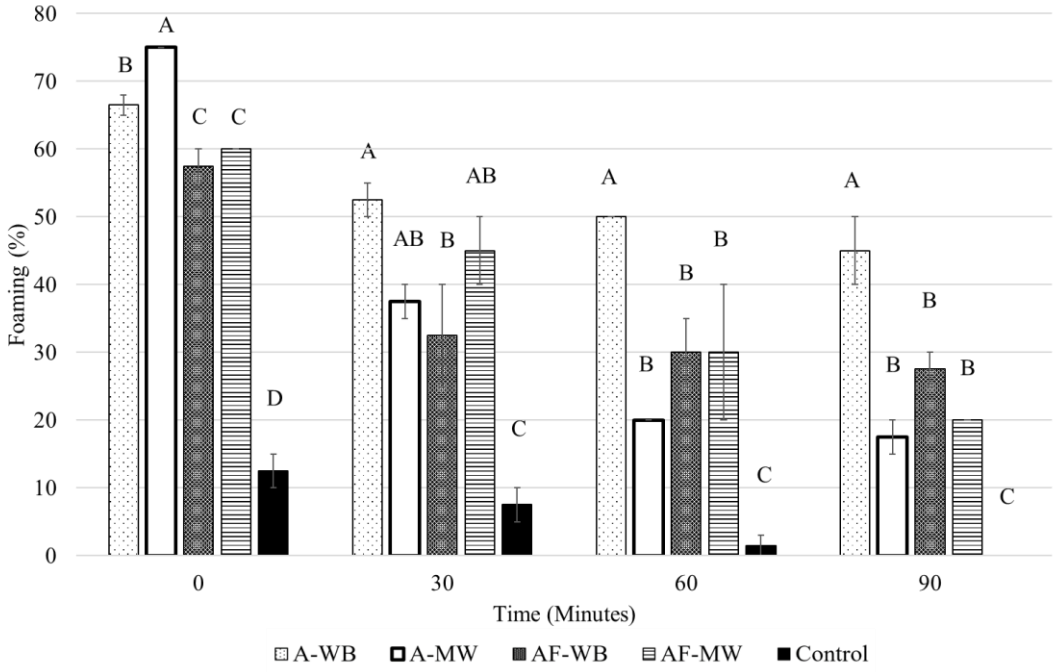

Figure 15. Results for foaming capacity (C) of chia seed protein hydrolysates. Different letters (a-d) show significant differences ($p < 0.05$) between treatments. Sample codes descriptions are provided in Table 5.

3.5 Conclusions

The implementation of ultrasonication and vacuum-filtration, successfully improved the separation of mucilage from chia seeds compared to previous extraction methods using sieves and conventional ovens. The efficient separation of mucilage followed by lipid extraction using a cold screw-press facilitated the separation of protein-rich chia seed flour.

Microwave-assisted hydrolysis with alcalase and flavourzyme can be used to develop CSPH with improved bioactivity and functionality in a shorter amount of time compared to conventional hydrolysis methods. Cellular antioxidant activity (94%) showed the potential antioxidant activity of this peptides in *in vivo* models. A positive correlation between antioxidant assay and cellular antioxidant activity was established, showing that ABTS and DPPH were the most efficient assays in predicting cellular antioxidant activity of CSPH. DPP-IV inhibition

increased as a function of DH, suggesting that higher hydrolysis will result in improved inhibitions. Finally, for ACE inhibition no apparent influence was found regarding type of hydrolysis in their inhibition capacity, suggesting the structural conformation responsible of this bioactivity remains unchanged independently of the treatment applied. Lastly, further studying is required regarding the structural conformation of this peptides to fully understand their inhibition mechanism.

It is known that chia seed shows to be a complex food composed of high polysaccharide, protein, and oil content. Applying different technologies to separate its components is a novel approach to increase yields, expand applications, and investigate bioactivities. Further research is required in ultrasonic field to obtain an industrial scale of separation of this polysaccharide. Chia seed components have a great applicability in food science or in the pharmaceutical sector, as carrier, materials for encapsulation, thickeners or as a source of bioactive compounds.

CHAPTER 4. EFFECT OF POLYOL CONCENTRATION ON PHYSICAL AND BARRIER CHARACTERISTICS OF EDIBLE FILMS FROM CHIA SEED (*SALVIA HISPANICA*) MUCILAGE.

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Abstract

Food packaging is one of the main contributors to the high rates of environmental contamination; researchers have pointed at the use of biopolymers as an alternative material to replace conventional food packaging. Chia seed (*Salvia hispanica*) is recognized by the outstanding nutritional profile, and its surface is especially rich in a polysaccharide called mucilage. The aim of this study was to evaluate the feasibility using of chia seed mucilage (CSM) and a polyol mixture containing glycerol and sorbitol for the development of edible films. Film formulations were optimized implementing a factorial arrangement with two replicates of the central point according to response surface methodology. The independent variables g of glycerol (X_1), and g of sorbitol (X_2) per g of CSM and their interaction (X_1*X_2) showed a significant effect ($p<0.05$) in all mechanical and barrier properties. CSM films with higher sorbitol content showed superior tensile strength (3.23 N/mm^2), and lower water vapor permeability ($1.3*10^{-9}$ g/ m*s*Pa) but had poor flexibility compared to other treatments. Conversely, films with high glycerol content showed high elongation at break (67.55%) and solubility (22.75%), but poor water vapor permeability and tensile strength. The hydrophobicity, measured as water contact angle, was higher ($p<0.05$) for mixtures containing equal amounts of polyols. Raman spectra analysis showed

shifts from 854 to 872 cm^{-1} and 1061 to 1076 cm^{-1}, which corresponded to β(CCO) modes, these shifts represent an increase in hydrogen bonding, responsible for the high tensile strength and reduced water vapor permeability in this study. The optimum conditions of polyol concentration were 1.3 g of glycerol and 2.0 g of sorbitol per g of CSM. CSM films showed potential as a drug delivery, and edible food coating for use in various industry applications.

Keywords: Chia seed mucilage, biofilms, polysaccharide, optimization.

4.1 Introduction

The overuse of petroleum-derived packaging is leading to a high accumulation rate of these toxic materials in the environment. Globally, since 1964, the use of plastics has expanded 20-fold increasing to 322 million tons in 2015 (Wei & Zimmermann, 2017). Packaged food products are the main culprit of single use plastics, due to the utilization of petroleum-based polymers as primary materials in food packaging. Around 72% of the world's plastic production is not recycled, 40% ends in landfills, and about 32% is estimated to end up in natural habitats, such as biospheres and oceans (Andrady, 2015). Buoyancy and slow degradation rate are the reason of the extensive damage and contamination ability of these plastic compounds (Eriksen et al., 2014). Ocean tides, winds, and human activity are mechanisms by which plastics spread through bays and gulfs leading to polluted coastlines and affected ocean fauna. In response to this, the food industry is in search of alternative materials for food packaging consisting of ingredients that increase the biodegradable characteristics of the package. In this regard, plant derived materials have shown a great potential, offering similar structural and barrier functionalities as conventional petroleum-based materials. Ghanbarzadeh, Almasi, and Entezami (2011) developed biodegradable films using corn starch and showed that the mechanical properties are improved by increasing concentrations of citric acid and carboxymethyl cellulose. Oliveira et al. (2019) fabricated films

from plant materials using mucilage extracted from leaves of Barbados gooseberry (*Pereskia aculeate)* combined with glycerol as a cross-linker. Because of their chemical composition, mucilaginous compounds offer a great potential alternative to plastics; very high content of mucilaginous material is present in chia seeds (*Salvia hispanica*). Chia seeds have gained popularity due to their remarkable nutritional composition, including high content of protein (15 to 25 %), fat (30-33%) and fiber (41%) (Ullah et al., 2016). Chia seed protein hydrolysates have also shown bioactive properties such as antioxidant activity, DPP-IV and ACE-inhibition (Urbizo-Reyes, San Martin-González, Garcia-Bravo, López Malo, & Liceaga, 2019). Around 5-7% of the whole chia seed weight corresponds to a complex carbohydrate polymer called mucilage (Tavares, Junqueira, de Oliveira Guimarães, & de Resende, 2018). Chia seed mucilage (CSM) has been used as a thickening agent in a wide range of applications including the development of gluten free pasta (Menga et al., 2017), and breads and cakes (Fernandes & de las Mercedes Salas-Mellado, 2017). A study conducted by Muñoz, Aguilera, Rodriguez-Turienzo, Cobos, and Diaz (2012) showed that CSM and whey proteins can be combined to develop edible or biodegradable films with improved physical properties. CSM has especially a particular high content of xylans (38%) (Timilsena, Adhikari, Kasapis, & Adhikari, 2016), which are the mayor contributors to the crosslinking ability of mucilage films and are known to absorb tens to hundreds of times their weight in water. Because of this, the formation of hydrogels by mucilage-water dispersions is ideal to rearrange the residues biaxially to form films and coatings. Also, mucilage contains a high amount of hemicellulose, which excerpt good barrier properties such as oil resistance and low oxygen permeability (Fu et al., 2019). Scientists have successfully developed films using xylans and hemicelluloses by the incorporation of plasticizers into their matrices. A study showed that incorporating glycerol into cassava starch film increased the elongation at break and water vapor

permeability but decreased the tensile strength of the films, showing the structural influence of plasticizers such as polyols in the physicochemical characteristics of films (Mali, Sakanaka, Yamashita, & Grossmann, 2005). Similarly, studies in gelatin films plasticized with polyols showed reduced water vapor permeability properties when sorbitol was over other common plasticizers (PEG300, EG, DEG, TEG, EA, DEA, and TEA) (Cao, Yang, & Fu, 2009). In this aspect, the incorporation of different polyols might provide unique characteristics to edible films and improve their physical and barrier characteristics. For this reason, the objective of this study was to optimize the physicochemical characteristics of chia seed mucilage films, by manipulation of polyol (glycerol and sorbitol) concentration, and to understand the structural bond conformation using Raman spectroscopy analysis.

4.2 Materials and methods

4.2.1 Materials

Chia seeds (CS) were purchased from Healthworks® (Scottsdale, AZ, USA). Glycerin was purchased from VWR International (Radnor, PA, USA), and sorbitol was purchased from Sigma Aldrich (St. Louis, MO, USA).

4.2.2 Chia seed mucilage extraction

Mucilage extraction was done according to Urbizo-Reyes et al. (2019). Briefly, CS were hydrated in distilled water (1:20 ratio by weight) for 24 hours, under refrigerated conditions. Hydrated seeds were pre-heated to 55±2°C and sonicated at a 75% power input using an ultrasonic cell disruptor (Sonifier® Branson S-150D Danbury, CT, USA). The temperature was maintained constant using a double-walled beaker connected to an immersion circulator control Lauda E100 water bath (Lauda-Königshofen, Germany). Seeds were separated from the mucilage solution

using vacuum-assisted filtration. Finally, the mucilage solution was frozen at -85°C and lyophilized (Labconco FreeZone 2.5 Plus, Kansas City). Dried mucilage powder was stored at 4 ± 2°C until used.

4.2.3 Proximate composition

Proximate analysis (moisture, ash, lipid, and protein) of the CSM was done following the AOAC methods 920.153, 960.39, and 984.13 (A-D) respectively (AOAC, 2016).

4.2.4 Preparation of chia seed mucilage (CSM) films

CSM films were developed by casting methods using polyols (glycerol and sorbitol) as plasticizers. First, the polyol mixtures were prepared according to **(Table 9)**, then solubilized in 15 mL of distillated water and stirred for 40 min. After this, 0.3 g of lyophilized CSM were added and stirred at 25°C until a complete homogenous suspension was achieved. Then, the mixture was held under vacuum for 5 min to remove air that was incorporated in the solution. The films were casted in circular aluminum plates with the following dimensions top 2-1/4" x 3/4" deep (Thomas Scientific, Swedesboro, NJ) and dried in a tray dryer (Excalibur Dehydrator 3926TCDB, Sacramento, CA) at 42 ±2°C for 10 h. Finally, films were peeled and stored in a desiccator for 24 hours prior analyses.

4.2.5 Color measurement

The color of the CSM films was determined using the Hunterlab ColorFlex 45°/0° Spectrophotometer (HunterLab ColorFlex, Hunter Associates Inc., Reston, Virginia, USA). The values were determined using the CIELAB, (L^*, a^* and b^*), where (L^*) measure the lightness and its value range between 0 to 100 (where 0 corresponds to black color and 100 to white). The chromaticity parameter (a^*), the negative values ($-a^*$) corresponds to green color and the positive

values (+a*) corresponds to red color. For the chromaticity parameter (b*), negative values (-b*) corresponds to blue color and positive values (+b*) corresponds to yellow color. The color of the films was measured against a white background taking 3 measurements per treatment. The total difference in color was calculated by comparing the films to a standard white tile where (L*=93.46, a*=-0.79, b*=0.11) according to the [Eq.14].

$$\Delta E^* = (\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \quad \text{[Eq.14]}$$

4.2.6 Tensile strength and elongation at break

Tensile strength and elongation at break was calculated as stated by Garcia et al. (2004) following the official ASTM standard method D882 (ASTM, 2002). First, the CSM films were cut into 25 mm by 63.5 mm rectangles with a razor blade, and then preconditioned in a desiccator for 24 h prior analysis. The maximum tensile strength (g) and elongation at break of CSM films **(Fig 16)** was determined using a TA.XT Plus Texture Analyzer (Texture Technologies Corp., Scarsdale,NY/Stable Micro Systems, Godalming, Surrey, UK) at 25°C according to [Eq.15] and [Eq.16], respectively.

Figure 16. Tensile test specimen under traction.

The thickness of the film was measured four times and the value reported as an average of the readings. The films were deformed at a tensile speed of 1 mm/min.

$$Tensile\ Strength\ \left(\frac{N}{mm^2}\right) = \frac{Breaking\ force(N)}{Cross\ sectional\ area\ of\ the\ sample\ mm^2} \qquad \text{[Eq.15]}$$

$$Elongation\ Break\% = \frac{The\ Increase\ in\ length\ at\ breaking\ point\ (mm)}{Original\ length\ (mm)} \qquad \text{[Eq.16]}$$

4.2.7 Film solubility

The CSM film solubility was determined by a method proposed by Rai and Poonia (2019). The films were cut into 2 cm diameter circles and dried at 105°C to determine moisture content. The precut films were suspended in 50 mL of distilled water and shaken in Erlenmeyer flasks using a Gyratory Shaker (Model G2, New Brunswick Scientific Co. Inc., Edison, NJ) at 25°C and a

rotational speed of 100 RPM for a period of 6 hours. The un-dissolved portions of the films were filtered using a #18 mesh and dried at 105°C. The solubility was calculated according to [Eq.17].

$$Solubility\ (\%) = \frac{Initial\ dry\ mass - final\ dry\ mass}{Initial\ dry\ mass} * 100 \quad \text{[Eq. 17]}$$

4.2.8 Water contact angle

The water contact angle was determined by the methodology proposed by Gezer, Brodsky, Hsiao, Liu, and Kokini (2015), where a VCA Optima contact angle analyzer (AST products, Billerica, MA) was used to photograph 30 s after 2-μL of deionized water was deposited onto the surface of the CSM films. The stable contact angle of the water at the CSM films interface was measured and performed in triplicate for each treatment.

4.2.9 Water vapor permeability (WVP)

The WVP was calculated according to García, Pinotti, Martino, and Zaritzky (2004) using the E96 method (ASTM, 2000). The films were sealed with metal clamps in 10 cm^2 circular opening PVC permeation cells containing anhydrous calcium chloride (0% RH), and stored at 25°C in a desiccator prior analysis. A 75% relative humidity (RH) gradient was established using a saturated solution of sodium chloride in a desiccator at 25°C. The water vapor transport rate was determined gravimetrically. Ten weight measurements were made, and the change in weight was plotted as a function of time. A regression analysis of each sample was conducted and the slope of the straight line (g/s) was divided by the area (m^2) to calculate the water vapor transmission rate (WVTR). Finally, the WVP was calculated according to [Eq.18] and expressed as (10^{-9} g/ m*s*Pa)

$$WVP = \left(\frac{WVTR}{S(R1-R2)}\right) * d \quad \text{[Eq.18]}$$

Where *S*: the saturation water vapor pressure at 25°C, *R1*: relative humidity outside the cell, *R2*: relative humidity inside the cell. The driving force (*S(R1-R2)*) was determined to be 1753.55 (Pa).

4.2.10 Raman spectroscopy

The structure characterization of the CSM films was determined using a Bruker FT-Raman spectrometer RFS 100/S (Bruker Optics, Lubeck, Germany) with a germanium detector cooled by liquid nitrogen. CSM films were cut in 2.5 cm by 2.5 cm squares using a razor blade and placed on a steel holder for Raman analysis. The analysis was conducted with an excitation wavelength of 1064-nm from a ND: YAG laser. A total of 300 scans were collected per sample, at a 4 cm^{-1} of resolution and a laser power of 220 mW. The spectra was collected at a range between 3500 and 73 cm^{-1}. The baseline and smoothness of the spectra was first corrected using the software OPUS 6.5 (Bruker, Karlsruhe, Germany). Finally, the main peaks were identified using KnowltAll® (Analytical Edition, ID Expert, Bio-Rad Laboratories, Inc.).

4.2.11 Statistical analysis

The statistical analysis was done using a central composite design, evaluating the influence of the independent variables glycerol (X_1) and sorbitol (X_2) concentration (g) on the dependent variables (Y_n). The response surface methodology (RSM) was conducted using the statistical software Statistica 10.0 ®. The quantities of the independent variables were coded as follows (-1, 0 and 1) whereas the central points were codified as (0, 0). The coded and decoded values are shown in **(Table 9)**.

Table 9. Coded and decoded levels of polyols incorporated to chia seed mucilage films.

Treatment	Coded variables		Decoded variables	
	Glycerol	Sorbitol	Glycerol (g/g of mucilage)	Sorbitol (g/g of mucilage)
1*	0	0	1.67	1.67
2*	0	0	1.67	1.67
3	-1	-1	0.67	0.67
4	1	-1	2.67	0.67
5	-1	1	0.67	2.67
6	1	1	2.67	2.67

*Central point.

The number of treatments was established using [Eq.19]. The levels (g) of the polyols were determined by preliminary data, identifying ideal central point, conditions and limitations in the incorporation of polyols in the mucilage films. The experiments were repeated 3 times before analysis.

$$n = 2^k + m \qquad [\text{Eq. } 19]$$

Where 2^k= factorial points, m=repetitions for the central point, and $n = 2^2 + 2 = 6\,TRT$. Finally, the independent variables were optimized using a desirability simulation of the software Statistica 10.0 ®. A 100-step simulation was run according to the values observed in this study. In the optimization process, the dependent variables (Y_n) elongation at break, tensile strength, contact angle and solubility were maximized, whereas the water vapor permeability was minimized.

4.3 Results and discussion

4.3.1 Proximate analysis of chia seed mucilage

The proximate composition of CSM showed content of 11.44 ±0.37% of crude protein, 0.25 ±0.05% of ash, 11.99 ±0.88% of moisture, 0.09 ±0.01% of fat and 76.35 ±0.60% of carbohydrates (calculated by weight difference). The carbohydrate content includes acid detergent fiber of 10.30 ±1.30%. Proximate composition of CSM, in this study falls within values reported

in other studies. Segura-Campos, Ciau-Solís, Rosado-Rubio, Chel-Guerrero, and Betancur-Ancona (2014), extracted CSM and reported a higher protein (25%) and fat (26%) content, whereas extracted and purified CSM by Timilsena et al., (2016) and reported lower content of protein (2.6%) and higher fat content (0.6%). The compositional difference is attributed to variances involved during mucilage extraction. Nevertheless, ultrasound-assisted mucilage extraction method used in this study, resulted in higher extraction yield (7.8%) (Urbizo-Reyes et al., 2019) than other physical methods (5.6%) (Timilsena et al., 2016). Ultrasound energy and the high shear forces developed under the sonic field could be responsible for increased protein extraction within the mucilage matrix. The molecular composition of the stored mucilage is still unknown, and a possible intramolecular bonding of proteins in the stored mucilage matrix could be the reason why residual proteins are still present in the extracted material. Timilsena et al. (2016), hypothesized that a possible covalent linkage might exist between protein and mucilage. It has been widely demonstrated that the ultrasound technique can help extract other compounds from plant matrices such as proteins, phytochemical compounds, and minerals (Chemat et al., 2017). Recently, some studies reported an increase on protein extraction when ultrasound was applied to spirulina (Vernès et al., 2019). Higher protein extraction was attributed to the erosion, shear effects and fragmentation caused during sonoporation, which generated cellular lysis that released protein from plant cells (Vernès et al., 2019).

4.3.2 Chia seed mucilage films thickness and color

In preliminary trials, CSM begun to combust at temperatures between 180-200°C limiting its capacity to be extruded or mixed with PLA. For this reason, its applicability on conventional 3D printing was restricted; however, the application on a bio-3D printing at lower temperatures might be feasible and further studies are required to understand this possibility. CSM films were

effectively casted in aluminum plates with variating concentrations of polyols (glycerol and sorbitol). Casting in plastic or glass surfaces made film peeling inefficient and laborious. Excessive incorporation of polyols as plasticizers (6.0 g of polyols/g of mucilage, wet basis) resulted in unmanageable films with lack of structure and a syrupy texture. Conversely, low concentration of plasticizers (less than 1.0 g of polyols/g of mucilage, wet basis) resulted in films with poor flexibility and film's peeling from the plate became almost impossible. Film thickness depended on treatment and ranged from 0.381 to 0.392 mm. The incorporation of polyols has shown to cause an increase in thickness; this is attributed to an increase of free-volume when polyols are incorporated intermolecularly (Cerqueira, Souza, Teixeira, & Vicente, 2012). The films had a slight yellow color as indicated by the color parameters **(Table 10)**. The natural color of these films might serve as a barrier for UV light. This could allow the material to serve as a UV protectant of photosensitive foods extending the shelf life of the products by decreasing photo-oxidative degradation. Dick, Costa, Gomaa, Subirade, de Oliveira Rios, et al. (2015) evaluated the UV absorption capacity of chia seed mucilage films and found that light transmittances was very low for the UV regions between 200-280 nm, indicating the potential role of this material as a UV barrier in food packaging. The L^* values were significantly lower (darker) ($p<0.05$) for films with higher content of glycerol. Dick, Costa, Gomaa, Subirade, de Oliveira Rios, et al. (2015) showed similar results, where the incorporation of glycerol resulted in a decrease in lightness (L^*). An increase in glycerol also resulted in increased red (a^*) and yellow (b^*) colors. The low incorporation of polyols caused overall lower (b^*) values (more blueish colors). The total difference in color (ΔE) indicated a significantly higher ($p<0.05$) difference for films with high glycerol (treatment 4) from the standard white plate, and the lowest difference was achieved with low polyol concentrations (treatment 3). This change in color is often attributed to non-enzymatic

browning that occurs between glycerol and residual protein in the mucilage matrix (Barrett, Briggs, Richardson, & Reed, 1998). Nevertheless, the films retained a semi-transparent appearance as the polyol composition changed.

Table 10. Color determination of chia seed mucilage films.

Treatments	*L**	*a**	*b**	ΔE*
1*	50.67 ± 0.08a	1.76 ± 0.04b	15.22 ± 0.07ab	2065.76 ± 0.07b
2*	50.95 ± 0.51a	1.74 ± 0.22b	15.12 ± 0.47b	2038.41 ± 0.47bc
3	51.07 ± 0.33a	1.64 ± 0.09b	14.28 ± 0.24d	2003.95 ± 0.24c
4	49.42 ± 0.02b	2.12 ± 0.08a	15.55 ± 0.22a	2186.26 ± 0.22a
5	50.94 ± 0.66a	1.62 ± 0.19b	14.61 ± 0.12cd	2023.90 ± 0.12bc
6	50.79 ± 0.26a	1.65 ± 0.13b	14.94 ± 0.25bc	2046.18 ± 0.25bc

The treatment assignment is specified in Table 9. *: Central point.

4.3.3 Mechanical properties (tensile strength and elongation at break)

Naturally edible and biodegradable films developed using biopolymers such as proteins or polysaccharides are brittle and collapse with manipulation. The incorporation of plasticizers is necessary to avoid premature fracture, poor flexibility and improve mechanical properties as tensile strength, elongation at break, and stickiness of the films (Sothornvit & Krochta, 2005). Dick, Costa, Gomaa, Subirade, de Oliveira Rios, et al. (2015) showed that incorporating glycerol to CSM increased the elongation and permeability and decreased the tensile strength of the films. In this study, we determined that different polyols had a significant effect ($p<0.05$) in the mechanical properties of the films made from CSM **(Fig 17)**. The incorporation of glycerol (X_1), sorbitol (X_2) and their interaction (X_1*X_2) had a significant ($p<0.05$) effect in the predicting models **(Table 11)** of tensile strength ($R^2= 0.91$) and elongation at break ($R^2= 0.99$). Films with a higher content of sorbitol exhibited a higher tensile strength **(Table 12)**, whereas films with a high content of glycerol or glycerol-sorbitol concentration showed poor strength. Glycerol is a hydrophilic molecule that consists of a 3-carbon backbone with 3 hydroxyl groups. Because of the small molecular nature of this polyol, the number of hydroxyl groups is higher in a given volume

when compared to sorbitol (Santacruz, Rivadeneira, & Castro, 2015). The high content of hydroxyl groups contributes to the incorporation of water molecules, resulting in an increased moisture content, that at the same time can increase the molecular mobility of the films (Mendelovici, Frost, & Kloprogge, 2000). Similarly, Espino-Díaz et al. (2010) developed edible films from prickly pear (*Opuntia ficus-indica)* using glycerol as a plasticizer and showed a maximum tensile strength of 0.95 N/mm^2. Generally edible and biodegradable films have tensile strengths that ranges, between 1-10 N/mm^2 (Han, 2014). The highest tensile strength in this study was achieved with 0.67 g of glycerol and 2.67 g of sorbitol per g of CSM (3.23 N/mm^2). It seems plausible that an increase in interactions between sorbitol-mucilage and mucilage-mucilage is responsible of the high tensile strength of these two films. Another study using pea starch, compared different plasticizers and found that a higher tensile strength was achieved by sorbitol compared to glycerol (Y. Zhang & Han, 2006). For elongation at break, higher values were obtained when a higher content of glycerol was incorporated. The small molecular nature of glycerol allows it to be inserted into adjacent polymeric chains, resulting in decreased molecular attractions that cause an increase in molecular mobility and decreased rigidity (Dick, Costa, Gomaa, Subirade, de Oliveira Rios, et al., 2015). Films with a higher content of glycerol (2.67 g of glycerol and 0.67 g of sorbitol per g of CSM) reached elongation at break values as high as 68%. Whereas in films with a high content of sorbitol (0.67 g of glycerol and 2.67 g of sorbitol per g of CSM), the elongation at break reach a maximum value of 21%. Talja, Helén, Roos, and Jouppila (2007) explored the mechanical properties of edible films made with starch plasticized with different polyols. Their findings showed that films plasticized with sorbitol had the lowest elongation at break compare to xylitol. The difference in elongation at break has been attributed to a possible polyol crystallization, common in sorbitol and

xylitol. This process is also known to enhance the crosslinking within the starch network causing differences in mechanical properties (Talja et al., 2007).

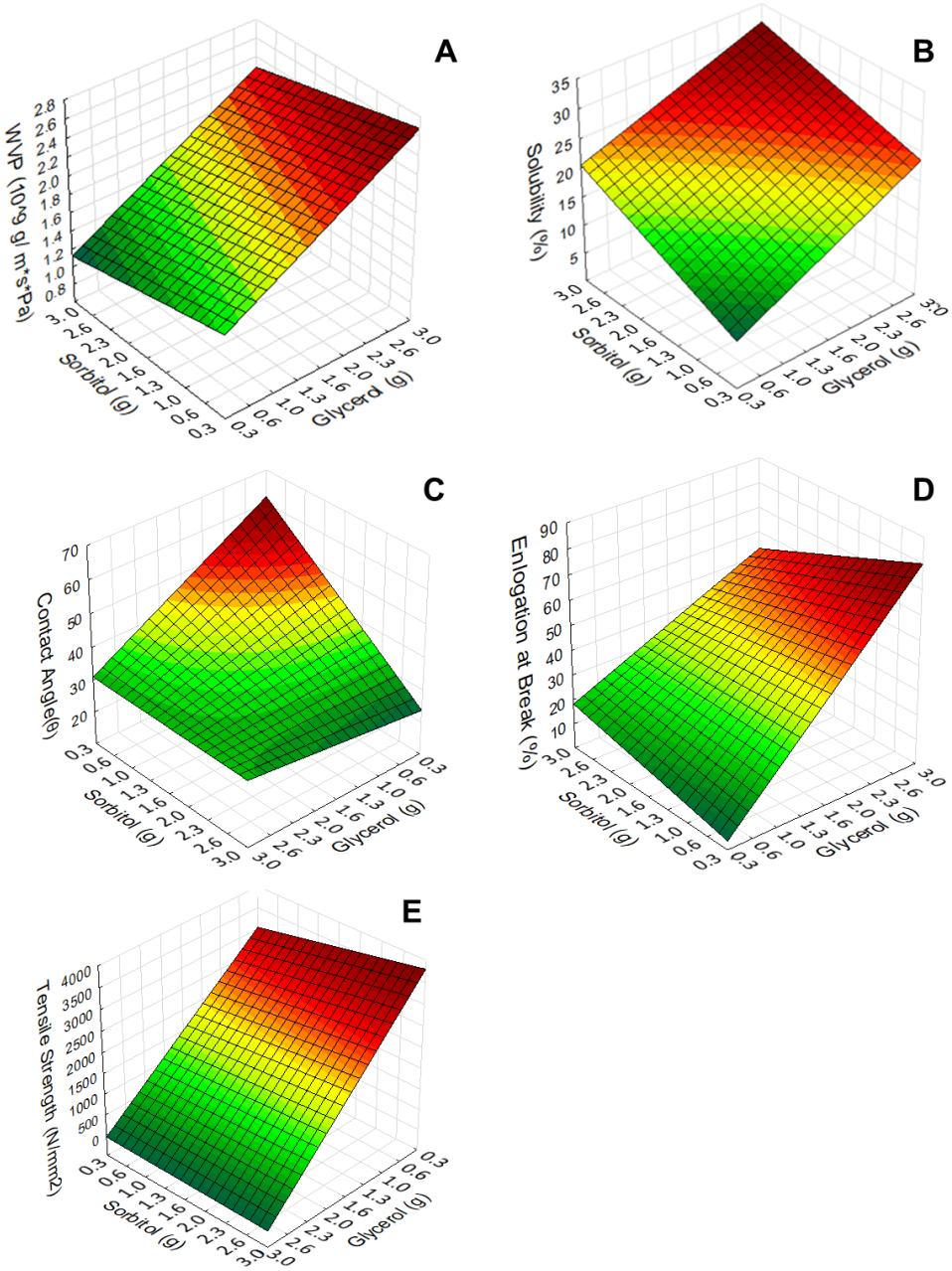

Figure 17. Response surface diagrams; Effect of glycerol (g) and sorbitol (g) per gram of chia seed mucilage (CSM) on the response variables: water vapor permeability (A), solubility (B), contact angle (C), elongation at break (D), tensile strength (E).

4.3.4 Water vapor permeability (WVP)

Glycerol, sorbitol and their interaction showed a significant ($p<0.05$) effect in the predicting model **(Table 11)** for WVP ($R^2= 0.96$). An increased concentration of glycerol, or polyol mixture resulted in higher WVP **(Fig 17)**. Similarly, Dick, Costa, Gomaa, Subirade, Rios, et al. (2015), showed an increase in WVP with an increase in glycerol concentration. They hypothesized that the small molecular size of glycerol, allows the polyol to penetrate the intermolecular matrix, causing a lower amount of polysaccharide-polysaccharide interactions and an overall increase in free volume and molecular movement. On the other hand, films plasticized with sorbitol showed a more compact and rigid structure, limiting the permeation of water vapor molecules through its structure. A higher molecular mobility allows water vapor molecules to permeate more easily through the film. Hence, sorbitol being a larger molecule than glycerol, showed a reducing effect in the WVP of the CSM films. Likewise, Antoniou, Liu, Majeed, Qazi, and Zhong (2014) compared the WVP of edible films from tara gum (*Caesalpinia spinosa*) and found that films made with sorbitol exhibited lower permeability when compared to glycerol films. They attributed this to the lower hydrophilic nature of sorbitol compared to glycerol. In this study the lowest WVP **(Table 12)** achieved was $1.30*(10^{-9}$ g/m*s*Pa), this value is lower than those reported for tapioca starch-microcrystalline cellulose composite films $3.37*(10^{-8}$ g/m*s*Pa), plantain seed gum $12.77*(10^{-9}$ g/m*s*Pa) and blends of cassava starch $5.05*(10^{-7}$ g/m*s*Pa) (Niknam, Ghanbarzadeh, & Hamishehkar, 2019; Othman et al., 2019; Parra, Tadini, Ponce, & Lugão, 2004). Nevertheless, these observations differ from other studies that evaluated the effect of other polyols in the permeability of starch-gelatin edible films, where the highest WVP was achieved when sorbitol was incorporated in the film matrix (Al-Hassan & Norziah, 2012). It is speculated that a difference in polymer structure is responsible of the difference in the film

permeability. The WVP of CSM films is still relatively high, this also can be attributed to the hydrophilic nature of this polysaccharide. A study conducted by Coorey, Tjoe, and Jayasena (2014) assessed the functional properties of CSM and identified a high water-holding capacity (267 g/g of mucilage). They associated this to the hydrophilic nature of xylans and arabinoxylans, which are known to absorb hundreds of times their equivalent weight in water.

4.3.5 Water contact angle

Water contact angle measures directly the angle formed by a water droplet when it is deposited on the surface of a film. This is a way of determining the hydrophobic nature of the surface of a material. This method is based on image processing and a curve fitting technique, analyzing the contact angle form with the baseline and the tangent of the drop boundary when deposited on a surface (Karbowiak, Debeaufort, Champion, & Voilley, 2006). The contact angle (θ), provides information of the wettability of a surface, a film is categorized as a hydrophilic ($\theta < 90$) or a hydrophobic ($\theta > 90$) boundary (Karbowiak et al., 2006). In this study, glycerol, sorbitol and their interaction showed a significant ($p<0.05$) effect in the predicting model **(Table 11)** for contact angle ($R^2=0.99$). An increase in both polyols caused a decreased in the contact angle, whereas a decrease in polyols increased it **(Fig 17)**. The contact angle was maintained between 46-48 as the polyols increased the contact angle to reach maximum values of 48.39 at the central points (1.67 g of glycerol and 1.67 g of sorbitol per g of CSM); nevertheless, the incorporation of more than 3.34 g of a polyol per g of mucilage showed to decrease the water contact angle drastically **(Table 12)**. The low contact angle values are related to the hydrophilic nature of mucilage fibers and polyols. CSM is a biomolecule composed of a branch of saccharides and acids that are hydrophilic in nature, these saccharides include xylans, arabinose, glucose, galactose, glucuronic acid and galacturonic acid (Timilsena et al., 2016). The incorporation of the polyols as

plasticizers may increase the hydrophilicity. Similarly, Jouki, Yazdi, Mortazavi, and Koocheki (2013), evaluated the water contact angle of edible films made with quince seed (*Cydonia oblonga*) mucilage and showed that the contact angle decreases with an increase in glycerol content. Most authors attribute a decrease in contact angle to the hydrophilic nature of polyols, which cause a decrease in the hydrophobicity of polysaccharide films (Jouki et al., 2013). The same behavior has been reported for other edible films made from whey proteins, psyllium gum and tara gum (Ahmadi, Kalbasi-Ashtari, Oromiehie, Yarmand, & Jahandideh, 2012; Antoniou et al., 2014; Kokoszka, Debeaufort, Lenart, & Voilley, 2010).

4.3.6 Film solubility

Glycerol, sorbitol and their interaction showed a significant ($p<0.05$) effect in the predicting model **(Table 11)** for film solubility ($R^2= 0.98$). The increased content of polyols resulted in a higher solubility **(Fig 17)**. No apparent trend was observed in film solubility when plasticized with sorbitol and glycerol. Films plasticized with a low concentration of polyol mixture had the lowest solubility (11.61%). It seems plausible that the low quantity of polyols allowed the films to maintain a rigid structure and higher hydrophobicity, as shown from the contact angle values. Other researchers reported similar trends, where glycerol was relatively more efficient to increase the solubility of the films; this was attributed to the lower molecular weight of glycerol, that increased the exposure of hydroxyl groups and resulted in higher water binding (Ghasemlou, Khodaiyan, & Oromiehie, 2011). However, both polyols are hydrophilic and increasing their content will increase the water solubility of the films, with values as high as 29.34%. In preliminary trials, the films were completely solubilized completely in 12 h. These films have high capacity to absorb moisture due to their hydrophilic nature, thus limiting their functionality as a moisture barrier, However, their molecular attributes make them suitable for other applications such as

oil/grease barriers and oxygen barrier (Mikkonen & Tenkanen, 2012). Other applications of xylan rich films include nanoencapsulation and delivery of drugs such as ibuprofen, in this regard these materials offer ideal release times of drugs due to their soluble nature (Daus & Heinze, 2010). Ultimately, optimum film solubility will depend on the type of product and function wanted to be accomplished. Generally, food needs to be protected from moisture migration to keep the food quality at optimum condition prior to consumption. The solubility, hydrophilic nature of these films might be desirable in some type of products which require a broad range of solubility, such as delivery of bioactive compounds, fruit coatings, soft gel capsules, microcapsules, among others (Han, 2014).

Table 11. Statistical models for response variables, as a function of independent variables grams of glycerol (X_1) and sorbitol (X_2) per gram of mucilage.

Response variables	Statistical model	F value >F critical	R^2
WVP	$y = 1.49 + 1.40x_1 - 0.55x_2 - 0.11x_1x_2$	52.64>4.54	0.96
SOL	$y = 4.69 + 19.82x_1 + 15.97x_2 - 6.25x_1x_2$	115.46>4.54	0.98
CA	$y = 65.78 - 44.90x_1 + 53.90x_2 - 59.21x_1x_2$	5.22>4.54	0.99
EB	$y = -7.36 + 101.70x_1 + 24.64x_2 - 64.53x_1x_2$	26>4.54	0.99
TS	$y = 3079.61 - 3715.17x_1 + 1095.48x_2 - 1308.43x_1x_2$	25>4.54	0.91

WVP: water vapor permeability. SOL: solubility. CA: water contact angle. EB: elongation at break. TS: tensile strength.

Table 12. Effect of independent variables on response variables.

Treatment	Glycerol (g)	Sorbitol (g)	WVP (10^{-9} g/m*s*Pa)	SOL (%)	CA (θ)	EB (%)	TS (N/mm^2)
1*	1.67 (0)	1.67 (0)	2.05 ± 0.25	19.65 ± 0.03	46.95 ± 4.65	41.84 ± 0.49	977.74 ± 11.80
2*	1.67 (0)	1.67 (0)	2.30 ± 0.13	19.60 ± 0.06	48.20 ± 3.02	41.73 ± 0.95	991.32 ± 1.60
3	0.67 (-1)	0.67 (-1)	1.62 ± 0.10	11.61 ± 0.57	48.39 ± 7.02	14.27 ± 0.26	2732.39 ± 27.87
4	2.67 (1)	0.67 (-1)	2.28 ± 0.39	22.75 ± 0.28	28.55 ± 3.66	67.55 ± 2.54	346.28 ± 8.98
5	0.67 (-1)	2.67 (1)	1.30 ± 0.16	20.44 ± 1.15	23.15 ± 3.00	21.31 ± 0.42	3232.67 ± 22.53
6	2.67 (1)	2.67 (1)	2.20 ± 0.45	29.34 ± 1.79	24.63 ± 1.37	51.35 ± 0.89	375.52 ± 3.53

*central points. Coded values are shown as follows (n). WVP: water vapor permeability. SOL: solubility. CA: contact angle. EB: elongation at break. TS: tensile strength.

4.3.7 Raman analysis of chia seed mucilage and chia seed mucilage films

The CSM **(Fig18)** and CSM films **(Fig19)** showed very defined spectra corresponding to the regions of 73-600, 600-1600 and 2900-2930 cm^{-1}. The corresponding assignment of the FT-Raman spectra bands is shown in **(Table 13)**.

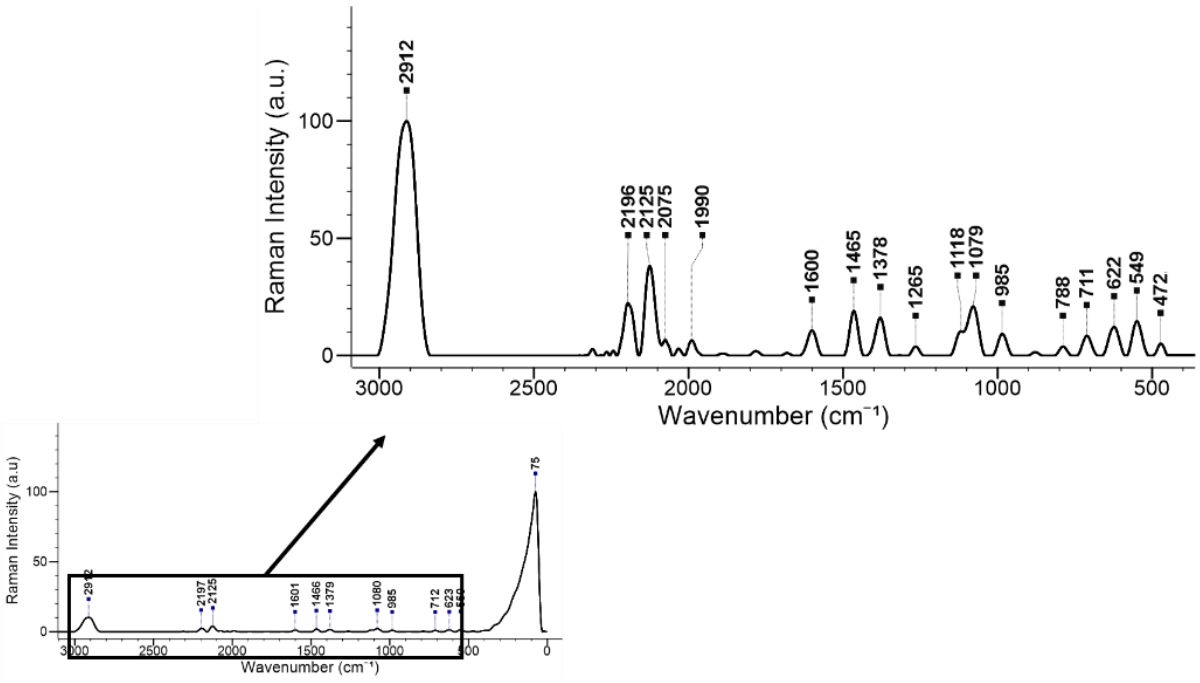

Figure 18 . Raman spectra for chia seed mucilage.

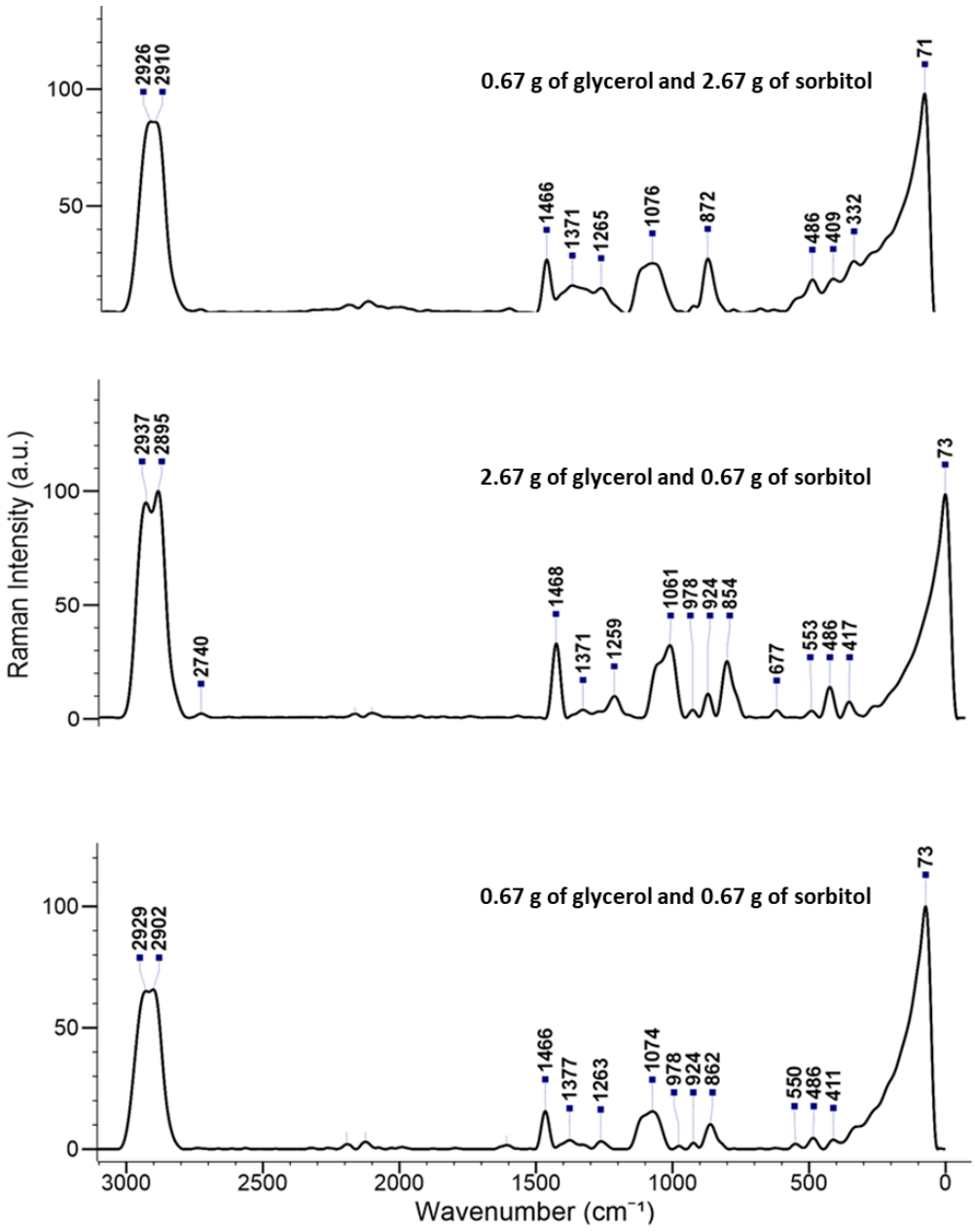

Figure 19. Raman spectra for edible films from chia seed mucilage at different polyol concentrations

In the first region (73-600 cm-1) we see a predominant peak (73 cm-1) for both CSM and CSM films, this area corresponds to the skeletal structure of CSM (Salgado-Cruz et al., 2013). Auzanneau, Combes, and Zwick (1991) identified very low frequencies (< 300 cm-1) as intermolecular hydrogen-bond stretching and bending, generally present in the skeletal structures

of polysaccharides. Authors have also reported this low region as corresponding to dipole-dipole interactions. One of the main differences found between CSM and CSM films was observed at the band located at 486 cm-1. Wiercigroch et al. (2017) associated this band to β(COC) ring deformations usually present in polyols and starches (amylose and amylopectin) as C1OC4 glucosidic bond. The band at 549 cm-1 is principally associated with glyosidic vibration β(OCO) bonds of maltose (Wiercigroch et al., 2017). For the 600-1600 cm-1 region, the peak at 677 cm-1 is observed in the CSM film containing high glycerol concentrations (2.67 g of glycerol and 0.67 g of sorbitol per g of CSM), and it is associated to δ(CC) bending in glycerol (Gryniewicz-Ruzicka et al., 2011). Mendelovici et al. (2000) evaluated the effect of cryogenic treatments on glycerol and determined that this peak remains unchanged when hydrogen bonding is induced. A slightly different Raman shift was observed in every CSM film at the frequencies of 854, 862 and 872 cm-1. Mendelovici et al. (2000) associated the Raman peak of 852 cm-1 to β(CCO) symmetric stretches in glycerol, where these bonds showed not to be involved in hydrogen bonding when glycerol is treated cryogenically. In a study conducted by De Veij, Vandenabeele, De Beer, Remon, and Moens (2009), a shift of the β(CCO) stretching vibration occurred by increasing the carbons and number of OH groups of the polyols, 851 cm-1 for xylitol, and 878 cm-1 for sorbitol. The Raman shift aligns to the results of this study, where the CSM films with a high content of sorbitol presented the higher shift (872 cm-1), conversely the lowest frequency (854 cm-1) was achieved when a low content of sorbitol and a high content of glycerol were incorporated. Bands at 924 and 978 cm-1 were observed in films plasticized with at least 50% of glycerol (g of glycerol/g of polyols), but not in films with high content of sorbitol (Fig 19). Similarly, Mendelovici et al. (2000) found peaks at 928 and 987 cm-1 in Raman spectra of glycerol, they assigned these observations to β(CCH) vibrations, additionally they reported Raman peak shifts in

this frequency when the material was cooled down, suggesting the shift observed in this study might be caused by hydrogen bonding. In a study on starch-based films, peaks between 920-960 cm-1 were assigned to glyosidic linkages developed by hydrogen bonding of the glycerol with films (Nobrega, Olivato, Müller, & Yamashita, 2012). One of the main differences was observed between the frequency range of 1061-1076 cm-1, assigned to ν(CCO). The films plasticized with a minimum of 50% of sorbitol (g of sorbitol/g of polyols) showed peaks at a Raman frequency higher (1074-1076 cm-1) than films with higher glycerol content (1061 cm-1). These films also showed the highest tensile strength (Table 12), we hypothesize that an increase in network bonding between mucilage-mucilage and mucilage-protein is responsible for these observations. Similarly, Mendelovici et al. (2000) observed a similar Raman shift in the symmetric ν(CCO) stretching modes (1054 cm-1), when treating glycerol cryogenically, attributing this to the increasing number of hydrogen bonding and network formation between molecules. This band (1054 cm-1) is commonly present in sorbitol and other polyols such as mannitol (De Veij et al., 2009). Additionally, the presence of protein in the mucilage matrix might play a role in the formation of highly resistant films when low polyol concentrations was present. Nobrega et al. (2012) assessed the Raman spectrum of oat globulins and identified peaks at 1245 and 1450 cm-1 as amide III (disordered structure) and (C-H bending) correspondingly. In this study, similar bands where observed at 1265 and 1465 cm-1 in CSM. A slight shift in this region was observed from 1259 to 1265 cm-1 for films plasticized with at least 50% of sorbitol (g of sorbitol/g of polyols), the Raman shift increase as sorbitol increase in concentration. This shift might suggest a protein-sorbitol interaction, causing an increase in bond formation.

In summary, CSM films containing a low polyol concentration or a high content of sorbitol, showed very marked band shifts at 862-872 cm^{-1} and 1061-1076 cm^{-1}, corresponding to ν(CCO).

We hypothesize that a possible increase in interaction between sorbitol-mucilage and mucilage-mucilage is responsible for the shift in this type of linkages and might explain the high mechanical properties of these two films. The bands at 472, 549, 1118, and 1379 cm^{-1} are typically observed in most polysaccharides such as starch and mucilage, and are attributed especially to CH_2OH (Nobrega et al., 2012). Most of these bands correspond to the glucose finger print observed in both the CSM and its films Wiercigroch et al., 2017).

The region corresponding to 2900-2930 cm^{-1} has been associated with the presence of pentose and hexoses. X. Zhang, Chen, Ramaswamy, Kim, and Xu (2017) and Kačuráková et al. (1999) have identified the region between 2800-2900 cm^{-1} to be associated with high content of D-xylose and glucose, commonly referred as v(CH)-vibrations. It is known that CSM contains around a 38% of xylans (Timilsena et al., 2016). Xylans are hemicelluloses with a wide application in the food industry as sweetening, thickening and emulsifying agents (Kačuráková et al., 1999). This hemicellulose is one of the most abundant biopolymers in the plant kingdom, its structural composition is usually described as multiple side chains bonded to a backbone of linear chains corresponding to (1→4)-linked β-D-xylopyranosye (Ebringerová & Hromádková, 1999). Xylans generally form a net-like cross-linked structure, and since their side chains are mainly composed of hydroxyl groups; this provides them with high water dispersibility and water holding capacity, making them suitable materials for encapsulation and film development applications (Fu et al., 2019).

Table 13. Raman Spectra analysis for chia seed mucilage and its films.

Chia seed mucilage	2.67 g GLY + 0.67 g SOR	0.67 g GLY + 2.67 g SOR	0.67 g GLY + 0.67 g SOR	Assignment
73	73	71	73	skeletal structure (δs(C–C))
		332		β(CCO), t(COHO)
	417	409	411	δ(CCO)
472				β(CCO)
				β
	486	486	486	(COC)
549	553		550	β(OCO)
622				β(COC)
	677			δ(CC)
711				γ(OH)
788				β(CCC), β(CCO), β(OCO)
	854			ν(CCO)
			862	ν(CCO)
		872		ν(CCO)
	924		924	β(CCH) β (COH)
	978		978	ν(CO) ring, β(CCH)
985				ν(CCO)
	1061			ν(CCO)
1079		1076	1074	ν(CCO), β(COH)
1118				νs(COH) from C-2
1265	1259	1265	1263	Amide III
1378	1371	1371	1377	ω(CH2)
1465	1468	1466	1466	δ(CH2) δ(CCH)
1600				ν(CO)ν(CC)
1990				NI
2075				NI
2125				NI
2196				NI
2912	2895	2910	2902	νs(CH2)
	2937	2926	2929	νs(CH2)

NI: not identified, GLY: glycerol, SOR: sorbitol.

4.3.8 Optimization

The polyol content of CSM films was optimized using a desirability simulation, running a 100-step simulation according the values observed in this study. In the optimization process, the dependent variables elongation at break, tensile strength, contact angle and solubility were

maximized, whereas the water vapor permeability was minimized. The simulation **(Fig 20)** estimated the optimum conditions to be at 1.29 g of glycerol and 2.03 g of sorbitol per every g of mucilage. This process might be useful for development of food coatings, and the desired parameters might be adjusted accordingly to required characteristics. Other studies have optimized film composition according to plasticizer and mucilage content (Oliveira et al. 2019). Future studies in these films need to be conducted, evaluating the food applications, and how different polyols interact with food (e.g. fruits and vegetables) or micro- and nano-encapsulation of bioactive components.

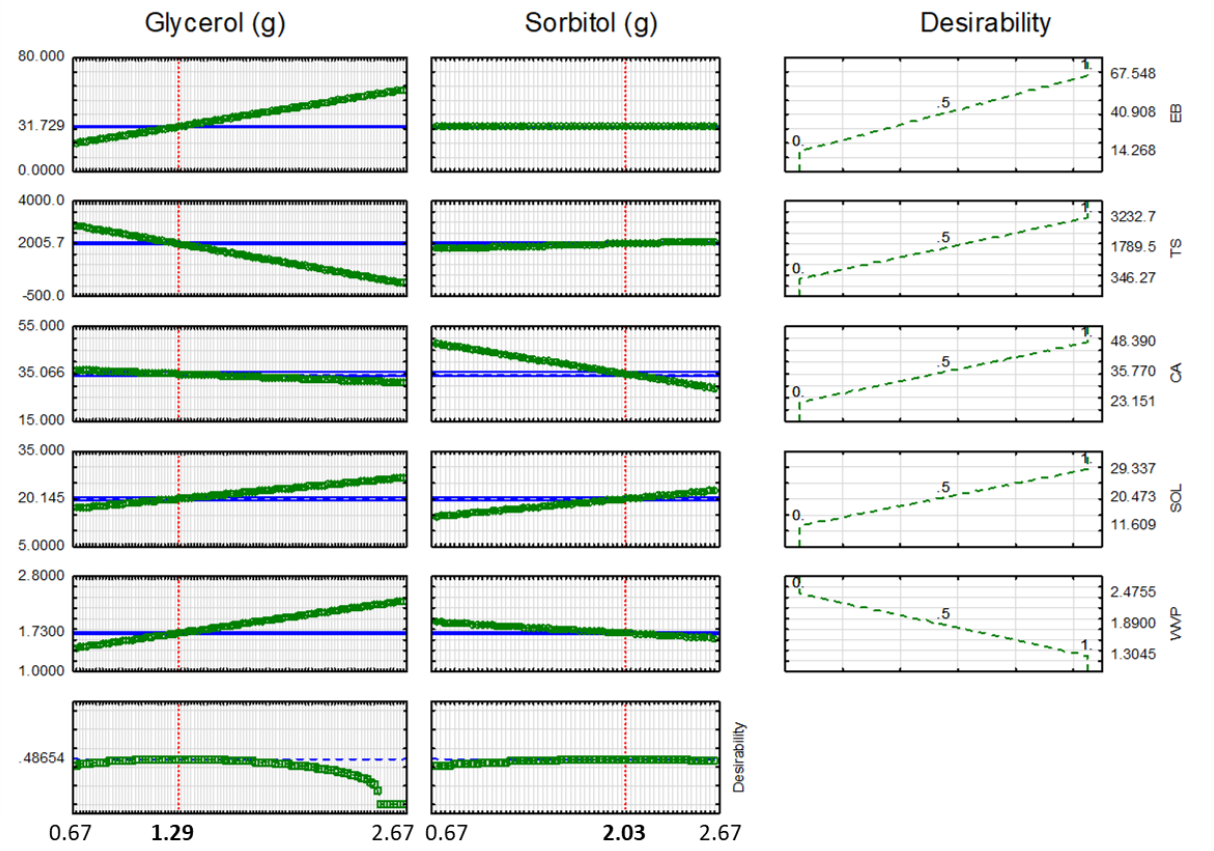

Figure 20. Optimization of edible films from chia seed mucilage.

4.4 Conclusions

CSM, as other biomolecules, has a great potential in the development of biodegradable materials from plants. CSM films were effectively developed by casting methods, using polyols as plasticizers to improve their physical structure. This research showed that each polyol imparts unique properties to the films and that the ideal content or mixture will depend on the intended use of the material. In this regard, the utilization of both polyols, sorbitol and glycerol, in combination showed to significantly ($p<0.05$) impact the physical and barrier properties of CSM films. Sorbitol provided CSM films with higher strength and a certain degree of hydrophobicity, whereas glycerol provided an increase in elongation at break, flexibility and hydrophilicity of the films. Raman spectroscopy revealed marked band shifts at 862-872 cm^{-1} and 1061-1076 cm^{-1}, corresponding to (CCO), and confirming that hydrogen bonding is induced with polyol treatment, which aligns with the mechanical observations in this study. The applications of this material might need to be geared towards products that do not require low water vapor permeability, such as nanoencapsulation and micro delivery carriers. Furthermore, the hygroscopic nature of CSM films might potentially confer good barrier properties against oxygen (O_2) transfer. Future studies should focus in applying this material in such environments to understand its behavior and develop further applications. The material begun to combust at high temperatures limiting its applicability on conventional 3D printing. However, the application on bio-3D printing might be feasible and further studies are required to understand this possibility.

CHAPTER 5. OVERALL CONCLUSION AND FUTURE DIRECTIONS.

5.1 Overall conclusions

- Chia seeds (*Salvia hispanica*) can be used as a source of functional and nutritional ingredients, due to their high content of fiber, high-quality fat, and protein.
- Ultrasound, along with vacuum filtration, is an excellent technology for the extraction of mucilage from chia seed and also had a high impact on lipid separation.
- Microwave-assisted hydrolysis with sequential enzymatic treatments produced protein with improved functionality and health benefits, especially as antioxidant agents.
- Mucilage from chia seeds showed high potential in the development of biodegradable and edible films.

5.2 Future directions

- Evaluate the functionality of chia seed protein hydrolysates in food prototypes and evaluate their sensory characteristics.
- Identify peptide sequences responsible for the inhibitory capacity of DPP-IV and ACE.
- Assess the bioactive properties of chia seed protein hydrolysates *in vivo*, especially for antioxidant activity.
- Implement and evaluate chia seed mucilage as an edible coating for fruits, vegetables, or even as a carrier to nano-deliver drugs.
- Evaluate the oxygen barrier properties of chia seed mucilage.

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APPENDIX A. FORMS

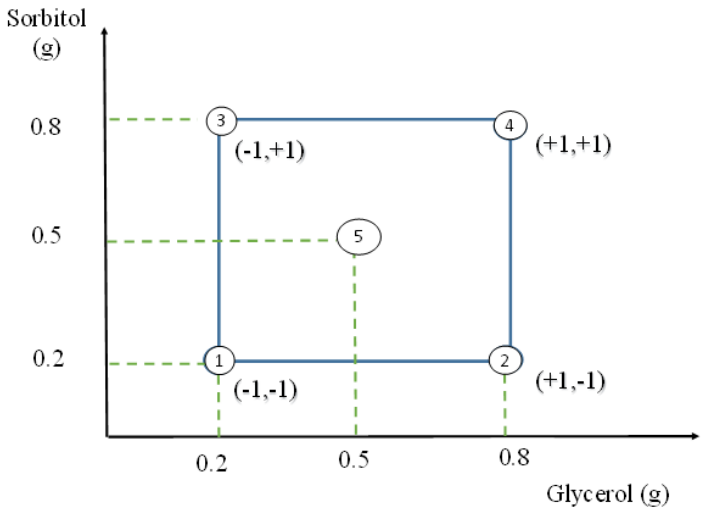

Figure 21.Central composite design assignment for CSM films.

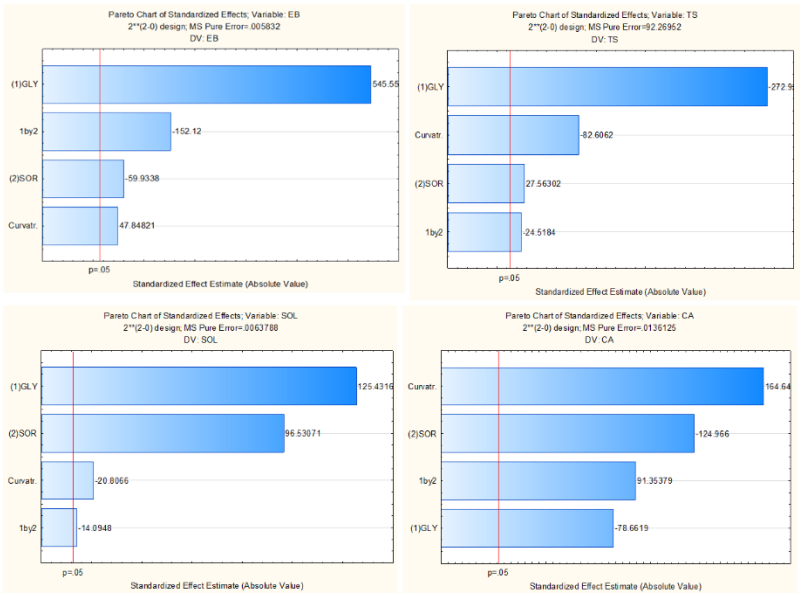

Figure 22. Pareto charts, significance effect of polyol and their interaction on mechanical and barrier properties of CSM films.

Figure 23. Ultrasonic removal of mucilage from chia seeds.

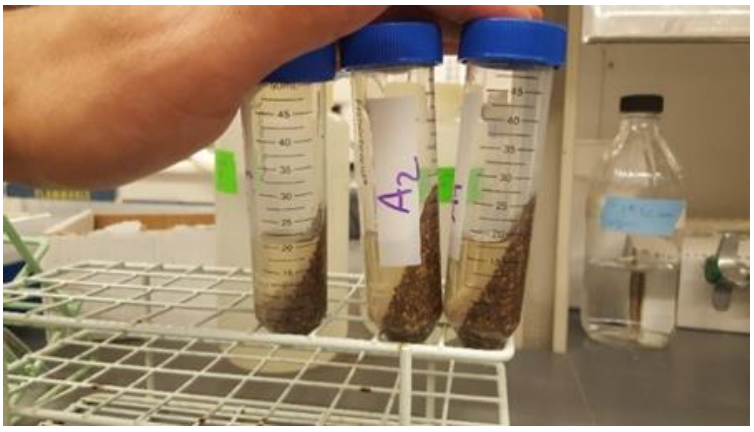

Figure 24. Centrifugation as a separation method for chia seed mucilage.

Figure 25. Chia seed oil extraction by oil press.

Table 14. Regression coefficients and confidence limits.

TS					
Factor	Regression coefficient	Std.Err.	P	Cnf.Limt._ -90%	Cnf.Limt +90%
Mean/Inter.	3079.610	17.931	0.004	2851.770	3307.440
Glycerol	-3715.170	31.117	0.005	-4110.550	-3319.800
Sorbitol	1095.480	31.117	0.018	700.110	1490.860
1L by 2L	-1308.430	53.365	-24.518	0.026	-1986.490
EB					
Factor	Regression coefficient	Std.Err.	P	Cnf.Limt._ -90%	Cnf.Limt +90%
Mean/Inter.	-7.365	0.143	0.012	-9.177	-5.554
Glycerol	101.708	0.247	0.002	98.564	104.851
Sorbitol	24.641	0.247	0.006	21.498	27.784
1L by 2L	-64.539	0.424	0.004	-69.930	-59.148
CA					
Factor	Regression coefficient	Std.Err.	P	Cnf.Limt._ -90%	Cnf.Limt +90%
Mean/Inter.	65.783	0.308	0.003	61.871	69.695
Glycerol	-44.903	0.528	0.007	-51.612	-38.194
Sorbitol	-53.907	0.528	0.006	-60.616	-47.198
1L by 2L	59.214	0.905	0.010	47.708	70.719
SOL					
Factor	Regression coefficient	Std.Err.	P	Cnf.Limt._ -90%	Cnf.Limt +90%
Mean/Inter.	4.699	0.151	0.020	2.783	6.616
Glycerol	19.823	0.259	0.008	16.536	23.111
Sorbitol	15.976	0.259	0.010	12.689	19.264
1L by 2L	-6.254	0.444	0.045	-11.892	-0.616
WVP					
Factor	Regression coefficient	Std.Err.	P	Cnf.Limt._ -90%	Cnf.Limt +90%
Mean/Inter.	1.499	0.007	0.003	1.415	1.583
Glycerol	1.401	0.011	0.005	1.256	1.546
Sorbitol	-0.551	0.011	0.013	-0.696	-0.405
1L by 2L	0.111	0.020	0.112	-0.139	0.360

WVP: water vapor permeability. SOL: solubility. CA: water contact angle. EB: elongation at break. TS: tensile strength.