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EFFECT OF ULTRASOUND ON PROTEIN EXTRACTION, FUNCTIONALITY, AND ANTINUTRIENTS OF SOLVENT-DEFATTED SACHA INCHI (*Plukenetia volubilis L.*) FLOUR

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Dedication

“Man finds God behind every door that science manages to open” - Albert Einstein.

To God and my family, fundamental pillars that move my life.

I want to dedicate this research, the time and efforts involved, to every man, woman and child whose lifestyle and health status could be improved through the results described in this work.

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Abstract

Chronic-degenerative diseases, such as obesity, diabetes, hypercholesterolemia, and cancer represented more than 75% of global deaths of year 2020 before the COVID-19 pandemic. In this regard, malnutrition is one of the major driving factors. Proteins are key macronutrients involved in several biological process, nevertheless there are still several low- and middle-income countries where populations do not have access to high quality proteins. On the other hand, the highly consumed animal proteins also contribute to the previously mentioned health problems due to its contents of cholesterol and saturated fatty acids. Furthermore, Sacha Inchi is an underutilized ancient Inca crop, whose edible seeds are high in oil (45-51%) especially polyunsaturated fatty acids, protein (25-28%), and an array of important phytochemicals. After oil extraction or removal, normally by mechanical pressing of seeds, the residue is a protein-rich flour. Interestingly, the Sacha Inchi pressed cake has been previously reported to be rich in essential amino acids, predominantly tryptophan a fundamental precursor of neurotransmitters. Nevertheless, there are several protease inhibitors previously characterized from vegetable protein matrices. Thus, the objective of this work was to evaluate the efficiency of ultrasound to extract Sacha Inchi protein, while reducing its anti-nutrients content without affecting its protein quality. In this work, a 55% extraction yield of protein from solvent-defatted Sacha Inchi flour was achieved using ultrasound (15 minutes, at 1 cycle with an amplitude of 100%) under alkaline conditions (pH 11). Moreover, all the treatments assayed resulted in significative reductions of trypsin inhibitors (less than 20% of their original content); meaning that all the ultrasound-assisted protein extracts obtained herein were safe for human consumption. Likewise, alkaline extracts showed important increases on their essential amino acids, especially Tryptophan (3.4-3.8 grams/ 100 grams of protein). Regarding the *in vitro* protein digestibility, all the ultrasound-assisted under alkaline conditions protein extracts had significantly better protein digestibility (>82%) compared to other pulses and plant-based proteins. Finally, the techno-functional properties suggested that protein extracted from Sacha Inchi using ultrasound could be used as an adequate ingredient in formulation of functional foods and nutraceuticals, especially beverages.

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Chapter 1
Introduction

1.1 Introduction

According to FAO (2022), plant-based protein is a niche sector that has been growing rapidly because of two main reasons, first the constant research for sustainable solutions to feed the projected 9.7 billion people in the world by 2050, and to decrease environmental footprint of traditional animal protein production practices, in terms of water and other non-renewable resources consumption.

Regarding the global feeding solutions, with the accelerated growing world population there is a considerable increasing demand of food supply, which not only consists in fulfilling the calorie intake, but rather is focused on an appropriate supply of macronutrients. In this sense, proteins are one of the most important macronutrients because of its pivotal role in several biological activities, especially the cognitive and immune system development in kids, and achievement of adequate height and weight (FAO, 2013). For many years, proteins from animal origin were considered superior because of their essential amino acid composition and its high biological value, which is a measure of how much the human body uses the consumed protein.

Nevertheless, in the last years the scientific evidence is pointing that there are several health risks of prolonged or excessive animal protein consumption, among the most worrying are: heart diseases, hypercholesterolemia, diabetes, obesity, metabolic syndrome, and premature aging (Huang *et al*., 2020; Micha *et al*., 2014). Shockingly, WHO (2022) reported that at the year 2020 (before COVID-19 pandemic) nearly a 71% of the worldwide deaths were attributed to the non-communicable chronic diseases afore mentioned, especially in low- and middle-income countries. For many years, health systems, governments, and scientists have tried to mitigate these health treats with drug and clinical therapies, rather than focusing on the fundamental role of the diet and lifestyle on the populations health state.

Interestingly, recently food scientists and technologists have reported that several plant-based proteins (soybeans, peanuts, lupinus) nowadays can be comparable to their animal counterparts in terms of essential amino acids contents and biological values (Kumar *et al*., 2021; Loveday, 2020; Pojić *et al*., 2018). Moreover, proteins from vegetable matrices are also very attractive because of its low or non-content of cholesterol, saturated fatty acids, and simple sugars, all are chemical molecules that have been previously reported to be involved in the development and progress of the main chronic diseases with the highest mortality rates in the world (Saxton & Sabatini, 2017).

In this regard, plant-based proteins are actual promisingly alternatives to both, satisfy the rapidly increasing global food needs, and to start mitigating or slowing chronic degenerative diseases rates. In this sense, the panorama is encouraging, because of the recent global consumption tendencies, like vegan and flexitarian diet, which mostly place a large emphasis on eating plant-based proteins. The afore mentioned diets are attracting constantly more assiduous who are conscient of the role of their diet in their health status. Further, the new consumption tendencies are inspiring industries to innovate in dairy commodities products using plant-based ingredients, like cultured meat for hamburgers, sausages, milk alternatives, biopharmaceuticals, vaccines, among others (Amigo & Hernández-Ledesma, 2020; Mistry *et al*., 2022; Sethi *et al*., 2016).

Nevertheless, several plant-based proteins contain anti-nutrimental factors (ANFs) that, if consumed periodically, can compromise the health status of the individual, via affecting some endogenous enzymatic processes, diminishing the nutrient supply, over stressing digestive organs, and so on (Loveday, 2020). In this regard, the ANFs of a food matrix must be reduced to human innocuous levels prior to its commercialization and consumption. For this porpoise, the food industry has implemented several techniques that will be further explained in the next section of literature review. Among the most used processes for the anti-nutrient reductions are the alkali-based and ultrasound application. Individually, both have previously demonstrated to be efficient to reduce ANFs in some plant-based matrices (Amagliani *et al*., 2017; Görgüç *et al*., 2020; O'Flynn *et al*., 2021). Interestingly, Avilés-Gaxiola *et al*. (2018) previously reported that the application of

ultrasound is effective in the inactivation of different enzymes via the phenomena of cavitation, however it has been more studied as a synergistic tool or to enhance the effectiveness of other treatments.

Furthermore, apart from the human's health treats, animal protein consumption must be reduced because animal agriculture contributes to the intensification of the environmental footprint, with higher rates of greenhouse gas emissions, destruction of forests and flora for land uses, also extremely large water consumption rates (Leroy *et al*., 2022). These factors are imperatively modulating some negative climate changes worldwide, in this regard, the flexibility or reduction in animal protein consumption are key actions for mitigating the adverse climate effects. Additionally, concerning animal welfare, each year billions of animals suffer, are killed, and lost their habitat in relation to human food systems.

With all these being said, it seems vital to explore and rapidly characterize new plant-based proteins that contribute to the world's health and environmental concerns afore mentioned. Still, according to Rubio *et al*. (2020), replacing animal protein with plant protein puts many challenges, in the sense that protein content and quality should remain intact while undergoing extraction processes, their cost should be affordable, they should have tasteful flavors, and minimized effects of anti-nutrients and allergens.

1.2 Hypothesis

The hypothesis of this work was that through the combination of different protein extraction techniques, such as ultrasound and pH adjustment, it is possible to recover Sacha Inchi (*Plukenetia volubilis L*.) protein while reducing its anti-nutrient content without affecting its protein quality and functional properties.

1.3 General objective

To evaluate the efficiency of ultrasound for the protein extraction from solvent-defatted Sacha Inchi flour, and to characterize the anti-nutrient content, the protein quality, and functional properties of the extracts.

1.4 Specific objectives

1.4.1 To obtain protein-rich solvent-defatted flours from Ecuadorian Sacha Inchi seeds.

1.4.2 To evaluate the effects of ultrasound and pH adjustment in the protein extraction yield from Sacha Inchi solvent-defatted flour.

1.4.3 To reduce the anti-nutrient contents in the Sacha Inchi protein extracts through the application of ultrasound.

1.4.4 To characterize the protein quality and techno-functional properties of the ultrasound-assisted Sacha Inchi protein extracts.

1.5 Thesis structure

For divulgation purposes, the work described herein was structured in 5 chapters. In **Chapter 1**, a general introduction to the investigation reported here is presented. **Chapter 2** consists of a literature review of the state of the art of plant-based proteins and their global market perspectives. Also, this chapter contains a brief introduction to the cutting-edge bioactive peptides reported in vegetable proteins and their nutraceutical effects on human health. Actual extraction techniques, anti-nutrients, and future considerations are also discussed in this part. **Chapters 3 and 4** present the results of this research study, where the main goal was to extract, assisted with ultrasound, a high-quality protein from Sacha Inchi (*Plukenetia volubilis L.*). Also, all the analytical methods and techniques used in the study are explained and described. **Chapter 5** includes a series of conclusions and personal recommendations for future studies related to the extraction, functionality, and protein quality of Sacha Inchi protein.

Chapter 2

Literature review

2. Theoretical framework

2.1 Global market perspective of plant-based foods

In recent years, the global market has undergone a notable increase in the consumption of plant-based foods to replace traditional animal products such as meat and milk. Animal products are commonly more expensive and require more inputs in terms of land, water and energy usage. Sethi *et al*. (2016) state that these preferences are marked by several factors that may vary depending on the region, among them are cultural and religious reasons, socioeconomic levels, trends or consumption preferences, nutritional education, and health-related reasons.

By the year 2022, the plant-based staple foods market in Mexico is worth 6 629 MXN million with a 12% growth rate. More interesting, it is expected to double its value to 12 790 MXN million by 2027 (Euromonitor, 2022). In the international arena, according to the Canadian National Research Council (2022), the global revenue of plant-based dairy products is expected to reach USD 34 billion in 2025.

According to previous reports, experts and producers in the food sector have identified key venues to take advantage of this growing food market, including launching and diversifying plant-based product lines, varying ingredients, reducing sugar, synthetical substances, and lowering costs (Onwezen *et al*., 2021; Pojić *et al*., 2018; Rizzello *et al*., 2016). Furthermore, it is worth mentioning that all markets around the world are dynamic, so it is crucial to be aware of the constantly varying consumption trends, like flexitarians, a growing niche market of better-educated consumers who balance their diet with animal, vegetarian, plant substitutes, and animal analogs.

Environmental changes and novel health-conscious customers are the two major drivers that favor worldwide markets of plant-based foods. Among the last, the

uncontrolled increase rates in diseases such as lactose intolerance, allergies, hypercholesterolemia, diabetes, and many more, have caused significant shifts of consumers demands and the migration to a healthy eating approach with the minimum consumption of animal origin products. In this regard, nutritional expectations on novel plant-based foods have raised in terms of contents of macronutrients, especially protein.

2.2 Health benefits of plant-based proteins

Table 1. Compilation of some previously reported bioactive peptides associated to different grains.

Bioactivity	Peptide Matrix	Release of bioactive peptides technique	Reference
Antioxidant	Whole Wheat	Fermented by BALs (*L. alimentarius* 15M)	Coda *et al*., 2012
	Sacha Inchi seed	Simulated Gastro-intestinal digestion	Zhan *et al*., 2021
Anticancer	Rice bran	Enzymatic hydrolysis (alcalase)	Kannan *et al*., 2010
Anti-inflammatory	Soybean	Enzymatic hydrolysis	Kovacs-Nolan *et al*., 2012
Antihypertensive	Pea	Fermented by BALs (*L. plantarum* 299v)	Jakubczyk *et al*., 2013
	Hemp seed	Enzymatic hydrolysis (pepsin and pancreatin)	Girgih *et al*., 2014
	Sacha Inchi pressed cake	Enzymatic hydrolysis (alcalase-neutrase)	Chirinos *et al*., 2020
Immunomodulatory	Soybean	Enzymatic hydrolysis (trypsin)	Tsuruki *et al*., 2003

Globally there is a constant demand for high-quality proteins in populations diet, which has encouraged food researchers worldwide to a persistent look for novel protein matrices with good bioavailability and high biological value. Vegetable proteins, when consumed in adequate amounts, have been reported to satisfy the nowadays nutritional requirements (Kumar *et al*., 2021), plus they have been well recognized because of their

potential to prevent allergies such as lactose intolerance, and chronic diseases such as hypercholesterolemia and diabetes (McClements *et al*., 2019).

Furthermore, many plant-based proteins yield bioactive peptides composed of sequences of 3-20 amino acid units. These bioactive peptides are considered constituents of functional foods and nutraceuticals because of their beneficial regulatory effects on the human organism (Rizzello *et al*., 2016). In table 1 a brief summary of recently characterized bioactive peptides from grains is presented. The main bioactivities of these peptides assessed both, *in vitro* and *in vivo,* are antimicrobial, antioxidative, antihypertensive and antitumoral.

2.3 Methods used for protein extraction in several food matrices

Regarding plant-based protein technology, developing and standardizing of appropriate cost-effective protein extraction techniques is critical and of paramount importance. Nowadays, conventional protein extraction techniques include biochemical methods (enzymes, alkali-based and organic solvents), physical extractions (ultrasound, microwave, electrical pulses, high pressures, among others), and a few advanced green technologies (supercritical fluids) (Pojić *et al*., 2018).

Despite their wide range of applications, many conventional extraction techniques are barely not eco-friendly, time and energy-consuming, and degrade or affect protein structures. Nevertheless, many industries still depend on these conventional methods due to their economic sustainability. The following section is focused on briefly describing the action mechanism of the most utilized extraction techniques.

2.3.1 Alkali-based

They are the most conventionally used method. NaOH and KOH are frequently used for alkalization of the pH of the medium, thus improving the extraction yield due to several reasons. First, basic pH produces the breakdown of the disulfide bonds which allows an unfolding of the protein structure and a subsequent increase in the percentage of the protein recovery and yield (Contreras *et al*., 2019). On the other hand, it is well known

that in most instances the protein's maximum solubility points occur at high pH values due to the ionization of its amino acids. It is worth mentioning that temperature plays a critical role in alkaline extractions because, if not properly controlled, it can promote denaturation in proteins structure (Kumar *et al*., 2021).

2.3.2 Ultrasound application

Ultrasound extraction has been gaining attention, mostly because its application allows the use of non-toxic and green solvents, thus, it is environment friendly (Rahman & Lamsal, 2021). Ultrasound refers to sound waves applied to produce cavitations that disrupt the cell membranes, providing the liquid phase a spot to extract the organic matter (amino acids) from the plant cells. Several factors can influence the efficiency of ultrasound processes, among them are intensity, energy, solvent ratio, and an adequate combination of temperature, pressure, and time of action. Compared to other technologies, ultrasound requires low time and economic investment, also shorter extraction times.

2.3.3 Enzymatic extraction

Enzymatic procedures are other approaches for recovering high-quality plant-based proteins. According to Görgüç *et al*. (2020), enzymatic extractions are focused on disrupting the cell wall integrity via degradation of its components (cadherins, pectin, cellulose). The specific activity of some enzymes makes this process an organized option for the release of proteins from the seeds. Interestingly, after protein extraction from the matrix, enzymes like proteases can divide high molecular peptides into even smaller and more soluble fractions. It is worth mentioning that each enzyme works under its specific optimum pH, this factor needs to be considered in order to prevent protein degradation (Zhan et al., 2019).

2.3.4 Microwaves application

Microwaves are electromagnetic radiations that can disrupt the hydrogen bonds networks present in the cell membrane of plant matrices, thus increasing the porosity of the wall which allows the infiltration of the solvent that assists the efficient release of

amino acids contained in the intracellular compartment (Phongthai *et al*., 2016). According to Kumar *et al*. (2021), microwave extractions have shown several advantages compared to other thermal methods like uniform heating, increased extraction rate, and lower solvent consumption.

2.3.5 Supercritical fluid extraction (SFC)

SFC is another novel green extraction technology used for the recovery of several molecules like fatty acids, polyphenols, other hydrophilic and lipophilic phytochemicals, antioxidants, and proteins (Triana-Maldonado *et al*., 2017). In terms of protein extraction, Di Domenico Ziero *et al*. (2020) listed some scenarios where water was used as a nontoxic solvent in two thermodynamic conditions, subcritical and supercritical. Each of the mentioned stages allows water to extract polar and non-polar amino acids respectively. However, this type of extraction requires delicate control of the process parameters such as temperature, pressure, reaction time, and pH, all of them need to be aligned in a cost-benefit effective way.

2.4 Sacha Inchi (*Plukenetia volubilis L.)*

Sacha Inchi is also known as maní del Inca, maní del monte, and suwaa. It is an underutilized crop original from the rainforest of the Amazon basin (Sathe *et al*., 2012). Its association with humans has been reported from prehispanic times (3000-5000 years ago), mainly due to its several uses in Inca traditional cooking (Brack, 1999).

The plant cultivation spreads along countries like Ecuador, Peru, Colombia, Bolivia, Venezuela, and Brazil. It is usually found to grow in wet lowland forests below 1000 m and optimal temperatures between 15-37 °C (Cai *et al*., 2012). This crop belongs to the *Euphorbiaceae* family, and it is a monecious perennial vine that contains several staminate flowers. Anthesis typically occurs 5 months after planting and fruiting happens on average at 9 months. Its fruit is a four-carpellate carinate capsule containing 4 to 6 lenticular and albuminous seeds protected by a robust and obscure outer shell of < 2 cm long (Kodahl, 2020).

Sacha inchi (SI) has an interesting nutritional composition as its edible seeds contain high amounts of oil (45-60%), protein (27-30%), and presence of microelements in beneficial amounts for human health (Štěrbová *et al*., 2017). Moreover, the SI lipid fraction is mainly composed of polyunsaturated fatty acids, α-linolenic and linoleic acids (ω-3 and ω-6; 47-51% and 33-36%, respectively of the total lipid content) (Triana-Maldonado *et al*., 2017). More interesting, its protein content is rich in essential amino acids like tyrosine, threonine, tryptophan, phenylalanine, and sulfur amino acids (methionine + cysteine) (Sathe *et al*., 2012). Ruiz *et al*. (2013) reported lysine and leucine as the limiting amino acids in SI protein. Information about variability in the proteomic profiles between different Sacha Inchi cultivars is limited, Rawdkuen *et al*. (2022) compared the *in vitro* digestibility of Thai and Peruvian SI, been the latter slightly better. On the other hand, Čepková *et al*. (2019) previously reported no significant differences.

Sacha Inchi oil and several of its by-products have been evaluated as possible sources of chemical compounds (poly-unsaturated fatty acids, protein, phenolics, and antioxidants) with potential health benefits and disease prevention in previous works (Gonzales *et al*., 2018; Kittibunchakul *et al*., 2022; Valenzuela *et al*., 2014). Further, according to Kodahl (2020), future possible applications of SI for medical purposes include prevention of cardiovascular disease, treatment of arthritis, anti-diabetic, and anti-Alzheimer.

In terms of large-scale production, countries like Peru and Colombia are leading the “Sacha Inchi revolution”, which is the result of some government programs focused on encouraging and supporting local farmers to take advantage of all the Sacha Inchi constituents for the design and large-scale production of various commodity products. First, the seed shells are destined for woodworking and paper making processes, the edible seeds are being commercialized roasted as salty snacks or covered with chocolate. Further, the oil is widely used in the food, medicinal and cosmetic industry, to produce edible virgin oils and cosmetics for skin and hair care, respectively. Moreover, the pressed cake is included in the formulation of some farinaceous products, such as cookies and bread. In addition, the Sacha Inchi leaves are often used to prepare infusions

because of their phytochemicals content (Wang *et al*., 2018). Both, the SI oil and the high protein flour, are increasingly being produced with the clear purpose of exporting these products to global markets, mainly to United States, Korea, and Netherlands, countries where the populations commitment to nutritional health is high (Núñez *et al*., 2021). However, this is a long-term project with such an extensive market that it can still allow the participation of new countries and companies interested in the production of this ancestral crop, like Mexico. In this regard, Kodahl (2020) reports that it is essential to implement sustainable and environmentally friendly production models, also it is important to reinforce the understanding of antinutritional factors associated with the production of Sacha Inchi.

2.5 Vegetable proteins anti-nutrient factors (ANFs)

Despite their previously mentioned nutritional and biological value, the use of several legume seeds like soybean, peanuts, Sacha Inchi, among others are limited by the content of some anti-nutritional factors which are produced endogenously by the plant to protect seeds from insects, viruses, and pests (Avilés-Gaxiola *et al*., 2018). There are particularly aggressive antinutrients in seeds that need extra attention because of their ability to form strong complexes with some digestive enzymes (trypsin and chymotrypsin), resulting in a reduction of the human digestive system effectiveness (Savage & Morrison, 2003). The last mentioned ANFs have received the denomination of protease inhibitors.

Protease inhibitors have been classified in two, Kunitz trypsin inhibitors (18-24 KDa) and the Bowman-Birk inhibitors (7-9 KDa), differing in their molecular weights, the number of disulfide bonds, and their amino acid sequences at the reactive inhibitory sites (Price *et al*., 2016). Regarding their principal treats for human health, Kårlund *et al*. (2021) reported that some protease inhibitors from soybean and wheat may cause gut inflammatory responses in sensitive individuals, and in some cases leading to hypertrophy and hyperplasia of the pancreas. It is worth mentioning that the last could cause poor growth and decreased holistic performance.

Regarding Sacha Inchi, it must be taken into account that this plant belongs to Euphorbiaceas, a family of plants known to produce toxic metabolites for human health (Bueno-Borges *et al*., 2018). There is little data about SI anti-nutrients reported previously, but among them the most relevant are trypsin inhibitors (Bueno-Borges *et al*., 2018; Rawdkuen *et al*., 2016). In this regard, Rawdkuen *et al*. (2022) mentioned that Sacha Inchi seeds contain major proteins, like 11S globulins, resistant to digestive enzymes hydrolysis. Moreover, the 11S protein fraction has previously been reported to affect *in vivo* digestibility of soybean, lupin, and nuts (Quinteros *et al*., 2016a). All the above mentioned reinforces the objectives of this work, focused to improve our understanding of trypsin inhibitors present in Sacha Inchi products, and the need to find new methods for protein extraction that simultaneously allow the reduction or elimination of ANF's.

From the protein quality and digestibility point of view, it has been established to be indispensable to eliminate inhibitors by food processing (Price *et al*., 2016). Fur this purpose, several of the commonly used methods for the reduction or destruction of trypsin inhibitors are based on using heat, including boiling, roasting, heat-soaking, and autoclaving (Vagadia *et al*., 2017). However, most of the mentioned techniques inevitably result in the loss of nutritive and functional components of the food matrix.

In the industrial arena, alkaline adjustment of the medium has been previously used for the trypsin inhibitors inactivation (Osman *et al*., 2002). The fundament of this technique is that alkaline conditions lead to cleavage of cysteine which can no further react with lysine (the combination of both amino acids conform the inhibitor reactive site), thus the inhibitor stays permanently unable to perform its action. On the other hand, US has been tested for several applications as previously mentioned, with results that are less time-consuming when compared with its conventional counterparts. Regarding the US effects on protease inhibitors, Vagadia *et al*. (2017) previously reported that ultrasound application resulted in more than a 55 % decrease in the activity of trypsin inhibitors. The reductions of inhibitors were attributed to the ultrasound cavitation effect, which is in function of the waves amplitude applied and the sonication duration. These phenomena

result in perturbations of the disulfide bonds and various conformational changes in the inhibitors structure, leading to its denaturation and loss of activity. Nevertheless, ultrasound is a technology in its first steps in the food industry, whose possible effects on protein extraction and anti-nutrient levels on several matrices should be further explored.

This last statement highlights the actual necessity and the aim of this work to develop new techniques with their optimal conditions that allow us to recover high protein extraction yields and significantly reductions on anti-nutrient levels, all without affecting the nutritional value.

2.6 Vegetable protein extracts future applications

Vegetable proteins are being utilized for the manufacturing of multiple foods, pharmaceutical and cosmetics due to their functional properties, and in the specific case of foods to their nutritional benefits (Jaski *et al*., 2022). Although the plant-based market is controlled by soybean products, the demand for new sources is growing as industries are currently developing new commodities and supplements from cereals and other grains. In this sense, the most important plant-based protein products are beverages like milk substitutes, meat analogous, and supplements (Euromonitor, 2022). These kinds of products are constantly gaining popularity mainly because they are cruelty-free and for the fact that they provide similar nutritional results compared to their animal counterparts.

On the other hand, some important necessary areas of plant-based research that provide opportunities for efficient industry innovation include sourcing, isolation and functionalization, formulation, and processing (Onwezen *et al*., 2021). For example, Fiorentini *et al*. (2020) enlist several cases where texturized and hydrolyzed vegetable proteins are beginning to replace animal or yeast proteins as flavor, texture, and mouthfeel enhancers.

With all this being said, the need for non-thermal green technologies that do not pose any harmful effects on the consumer and that do not affect the biological value of the

proteins is crucial. In this sense, several combinations of the conventional methods previously mentioned have been explored in previous works (Avilés-Gaxiola *et al*., 2018; Ochoa-Rivas *et al*., 2017), with no reports on Sacha Inchi. Thus, the aim of this work was to enhance the extraction yield, and to reduce the anti-nutrients of a novel plant-based protein like Sacha Inchi, without affecting its protein quality.

Chapter 3
Materials and Methods

3.1 Sample preparation

10 kg of Sacha Inchi seeds were purchased in the city of Arenillas, Ecuador (Latitude: 33° 33' 00" S Longitude: 80° 04' 00"W). Similarly, 2 kg of soybeans were purchased from a local supermarket in Monterrey, N.L. with the aim to be used as a control treatment in all essays reported herein. Seeds were transported to the facilities of the Centro de Investigación y Desarrollo de Proteínas (CIDPRO) of Tecnológico de Monterrey where they were stored in double-seal aluminized bags at - 20°C until use.

3.2 Proximate composition

All the proximal essays reported herein were performed according to the official methods of the Association of Official Analytical Chemists (AOAC) as follows: moisture, fat, protein (micro-Kjeldahl; Nitrogen*6.25), crude fiber and ash with AOAC 925.10, 920.85, 978.02, 962.09, 923.03, respectively (AOAC, 1990). Total carbohydrates were calculated by difference using the following equation: 100 - [moisture + fat + protein + crude fiber + ash]. The proximate composition of SI seeds and solvent-defatted concentrate were characterized in triplicate analysis.

3.3 Production of solvent-defatted protein concentrates

The protein concentrates were obtained according to (Cordero-Clavijo *et al*., 2021) with some modifications. Briefly, SI seeds were ground using a mill until a soft consistency paste was obtained. Then, the paste was mixed with n-hexane (1:2 SI paste: hexane, w:v) in 4000 mL amber glass bottles and stirred in an incubator for 1 hour, at 100 rpm and 30 °C. Thereafter, the supernatant or miscella was removed and stored in amber glass bottles. The process aforementioned was carried out five times for each sample or until the fat content was less than 1.5%. The protein concentrates were recovered inside an air extraction fume hood using porcelain Büchner funnels to filter out the hexane. The

concentrates remained in the hood for at least 12 hours for their complete desolventization. Finally, the solvent-defatted Sacha Inchi protein concentrates (sdSIP) were placed in double-sealed aluminized bags and stored at - 20°C until further use.

3.4 Evaluation of the Sacha Inchi protein solubility

sdSIP protein solubility was determined following the protocols previously reported by (Mercado *et al*., 2015; Sathe *et al*., 2012) with slight modifications. Briefly, several suspensions of defatted protein concentrates were prepared using distilled water at a ratio of 1:10 (w/v), subsequently, the pH of the suspensions was adjusted in a wide range (pH values from 2 to 12). All the suspensions were stirred at 300 rpm for 30 min at 30°C. At the end of the mentioned period, the mixtures were centrifuged at 4000 rpm, for 15 min at 4°C, and the supernatants were collected and pooled for protein determination by the Biuret method. Soluble protein percentage was expressed as a relation of protein recovered in the supernatant against the total amount of protein obtained in the protein concentrate by micro-Kjeldahl as previously mentioned. The solubility of SI at each point of pH was evaluated by triplicate.

3.5 Osborne protein fractionation

Protein fractions existing in SI protein concentrates were determined by the Osborne method, according to (Kumar *et al*., 2021; Sathe *et al*., 2012) with brief modifications. First, 10 g of protein concentrate samples were weighed and placed in a 250 mL beaker for a sequential protein extraction with 4 different types of solvents. Distilled water (albumins), NaCl (globulins), 70% EtOH (prolamins), and 0.1 M NaOH (glutelins) were used at a flour-solvent ratio of 1:10 (w/v) at room temperature for 1 h (each solvent extraction) with constant magnetic stirring. At the end of each extraction the suspension was centrifuged (15000 g, 15 min, 4°C), the pellet used for the next solvent extraction and the supernatant collected for protein quantification by micro-Kjeldahl as previously mentioned.

3.6 Ultrasound-assisted protein extraction

Ultrasound protein extractions were performed following the procedure established by (Ochoa-Rivas *et al*., 2017) with some modifications. First, suspensions of SI and soy protein concentrate: distilled water (1:10) were prepared, followed by an adjustment of the pH of the medium. Then, each sample was ultrasonicated for 15 minutes, at 1 cycle with an amplitude of 100%. The equipment employed was a UP400S model (Hielscher Inc., USA) with a probe of 22 mm diameter. Subsequently, to recover the supernatant with the extracted protein, samples were centrifugated at 4500 rpm and 9 °C for 15 min. Finally, the weight of both fractions (supernatants and pellets) was recorded, and samples were stored at - 20°C until further analysis.

Table 2. Treatments used for Sacha Inchi and Soy protein extraction using ultrasound.

Sample matrix	Sample Code[1]	pH adjustment	Ultrasound treatment	Final temperature[2]
SI protein concentrate	usSI7	7	1 cycle, 100% amplitude, 15 min	89 °C
	usSI9	9	1 cycle, 100% amplitude, 15 min	89 °C
	usSI11	11	1 cycle, 100% amplitude, 15 min	92 °C
	usSI11c	11	1 cycle, 100% amplitude, 15 min	42 °C
	SI11h	11	Not-applied	90 °C
	SI11	11	Not-applied	Not-applied
Soy protein concentrate	usSOY11	11	1 cycle, 100% amplitude, 15 min	89 °C

[1]usSI7= ultra-sound SI at pH7; usSI9= ultra-sound SI at pH9; usSI11= ultra-sound SI at pH11; usSI11c= cold ultra-sound SI at pH 11; SI11h= SI at pH11 and heated; S11= SI with just pH 11 adjustment; usSOY11= ultra-sound Soy at pH11.

[2]Maximum temperature (°C) reached inside the ultrasound equipment at each treatment.

[3]All extractions were performed in triplicate.

For the results to be comparable, few sample controls were assayed modulating the extraction factors. Samples performed in this work are listed in table 2. The final temperatures reached at each treatment are included in table 2.

3.7 Protein extraction yield and purity

Protein purity and extraction yields of the ultrasound protein extracts were expressed according to (Espinosa-Ramírez & Serna-Saldívar, 2016; Ochoa-Rivas *et al*., 2017), respectively. Briefly, protein purity was expressed as the protein concentration in the final extract and pellet. On the other hand, protein extraction yield was calculated by relating the weight of total recovered protein against the total amount of protein in the solvent-defatted concentrate, both expressed on a dry matter basis.

3.8 Trypsin inhibitors activity

The presence of trypsin inhibitors in all samples was tested following the Ba 12a-2020 official method of the AOCS (AOCS, 2021), using DL-BAPA as substrate. Briefly, 1 g of sample was mixed with 50 mL of 10 mM NaOH in a 100 mL beaker, the solution was covered with parafilm and mixed for 3 hours at room temperature at 500 rpm with a magnetic stirrer to obtain a sample suspension. Thereafter, 1 mL of the sample extract was placed in a 15 mL glass tube, followed by the addition of 2.5 mL of DL-BAPA working solution and set to incubation in a water bath at 37°C. Then, 1 mL of trypsin working solution was added to the tube to start a 10-minute reaction. Exactly after the incubation period, 0.5 mL of 30% (v/v) acetic acid was added to conclude the reaction, subsequently, the suspension was transferred to a 50 mL corning tube for centrifugation at 6000 rpm, 10°C for 10 min. After centrifugation, the supernatant was filtered using Whatman paper number 1, and the activity of trypsin was evaluated by measuring the absorbance of the filtered solution at 410 nm, mainly influenced by the p-nitroaniline released, a by-product of the activity of trypsin (Rawdkuen *et al*., 2016). The trypsin activity of the samples was always measured by duplicate and compared to a control (reaction using 1 mL of distilled water instead of the sample extract) to ensure the stability of all the reagents.

Trypsin inhibitory activity was expressed as trypsin units inhibited (TUI) per mg of sample, and it was calculated using the equation suggested in the Ba 12a-2020 official method (AOCS, 2021) as follows:

TUI/ mg sample= {(A_{410R}-A_{410RB}) - (A_{410S}-A_{410SB}) x 50}/ (mg of sample used for the assay)

Where,
A_{410R}= control reading
A_{410RB}= control blank reading
A_{410S}= sample reading
A_{410SB}= sample blank
mg sample= (1 ml used for the assay X concentration of the dilute sample extract in mg/ml)

3.9 Protein quality of ultrasound-assisted protein extracts

The amino acid composition of the ultrasound protein extracts was assayed following the AOAC 982.30 official method (AOAC,1990), according to previously reported works (Cordero-Clavijo *et al*., 2021; Espinosa-Ramírez *et al*., 2018). This method involves an HPLC system (1525 binary pump, waters, Milford MA), equipped with a 3.9 x 150 mm AccQ-Taq C18 column and a fluorescence detector (Waters 2475), and an autosampler (Waters 717 plus).

3.10 SDS-PAGE

Ultrasound-assisted protein extracts were characterized by SDS-PAGE under reducing (using β-mercaptoethanol) and non-reducing conditions, according to the methodology described by (Espinosa-Ramírez & Serna-Saldívar, 2019). Samples were loaded at a protein concentration of 1 µg/µL on an 18% acrylamide separating gel and 5% stacking gel. Electrophoretic analysis was performed in a Criterion Vertical Electrophoresis Cell (Bio-Rad) at a constant 120 V until the tracking dye migrated to the bottom of the gel (typically 1 hour). The gels were stained with Coomassie Brilliant Blue

R-250 (Bio-Rad). Further, gels were photographed using an isoelectric focusing system Ettan IPGphor (GE Healthcare., Spain).

3.11 *In vitro* protein digestibility

The *in vitro* protein digestibility of the ultrasound protein extracts was measured using a multienzyme technique developed by (Hsu *et al*., 1977). Briefly, for each sample, an aqueous protein suspension (50 mL) containing 6.25 mg of protein/mL was prepared in a 250 mL beaker. The pH of the solution was adjusted to 8 using HCl and NaOH, both 0.1 N. Subsequently, beakers were placed in a water bath for incubation at 37°C. When all samples were at the desired temperature, a multienzyme suspension (containing trypsin, chymotrypsin, and protease from *Streptomyces griseus*) was prepared and adjusted to pH 8. Then, 10 mL of the enzyme solution were added to each sample beaker, followed by a constant stirring at 120 rpm for 10 min inside the water bath at 37°C. The pH drop of each suspension was recorded exactly after the 10 min incubation with the enzymatic solution. Finally, the protein digestibility was calculated using the equation established by Hsu *et al*. (1977) as follows:

$$\mathrm{y} = 210.464 - 18.1\,X$$

Where y is the *in vitro* protein digestibility and X is the suspension pH after the 10 min reaction.

3.12 Assessment of techno-functional properties

Water absorption index (WAI), water solubility index (WSI), and swelling power (SP) were evaluated using the methods previously described by (Cordero-Clavijo *et al*., 2021; Cornejo & Rosell, 2015). Oil absorption capacity (OAC) was determined using the procedure established by (Espinosa-Ramírez & Serna-Saldívar, 2016) using commercial soybean oil. Foaming capacity (FC) and stability (FS) were measured according to

(Espinosa-Ramírez *et al*., 2018) with slight modifications. A water suspension (15 mL) with 0.6 g of protein was prepared and subsequently whipped in an Ultra Turrax (T18 IKA. Baden-Württemberg, Germany) at 14 000 rpm for 1 minute at room temperature. The foam volumes were recorded at 30 s and 20 min and used to estimate foaming properties.

3.13 Statistical analysis

Results are presented as means of three independent replicates ± standard deviations. All data were analyzed using Minitab 17 statistical software. A single factor analysis (ANOVA) was performed followed by the Tukey-Kramer test to evaluate significant differences among treatments ($p<0.05$) at a 95% confidence level.

Chapter 4
Results and discussion

4.1 Chemical characterization of Sacha Inchi raw seeds and solvent-defatted flour

The chemical composition of the SI seeds and solvent-defatted protein concentrate (sdSIP) are summarized in table 3. First, although all samples were stored under identical conditions, the moisture content between seeds and solvent-defatted protein concentrates varied significantly ($p<0.05$). The sdSIP displayed the highest moisture content (11%), nevertheless, Nasir *et al*. (2003) reported that moisture contents below 12% are suitable for functional stability and longer shelf life because meals have a water activity that do not favor growth of microorganisms including molds.

Furthermore, crude protein composition was also significantly higher in the sdSIP due to the oil removed during the n-hexane extraction ($p<0.05$). Compared to previously reported works on similar matrices, the protein content of the sdSIP (67%) was similar to the 66.5% reported by Cordero-Clavijo *et al*. (2021), and higher than values of 57.6% and 61% obtained by Quinteros *et al*. (2016) and Mercado *et al*. (2015), respectively. Due to its comparatively higher protein content, sdSIP can be considered a protein-rich flour, and therefore could be considered as a food ingredient and as a nutritional protein supplement (Kim *et al*., 2021).

Regarding the fat matter, SI seeds content (50.2%) was significantly higher than that reported by Valdiviezo *et al*. (2019) (42%). Variations in oil content between seeds of the same species can be explained mainly due to differences in environment, place and cultivation conditions, landraces, among others (Kiani *et al*., 2020). Moreover, the reduction in oil content observed in sdSIP (1.3%) was similar to the 1.7 % residual fat reported by Cordero-Clavijo *et al*. (2021), reinforcing the efficiency of the solvent defatting process applied to Sacha Inchi.

Additionally, with respect to the fiber and ash contents, the values obtained herein were similar to those reported by Mercado *et al*. (2015) for both, SI seeds and protein

concentrate. On the other hand, the carbohydrate contents of seeds and sdSIP were significantly lower than most values reported by other authors for SI protein concentrates (Benítez *et al*., 2018; Goyal *et al*., 2022). The last is an important fact to take into account because carbohydrates are known to affect the sensory properties and taste of protein concentrates (Bainy *et al*., 2008).

Table 3. Chemical characterization of the Sacha Inchi raw seeds and solvent-defatted protein concentrates.

Parameters	Sacha Inchi	
	Raw seeds	sdSIP[1]
Moisture	6.20 ± 0.08[b]	11.14 ± 0.30[a]
Protein	28.60 ± 0.83[b]	67.00± 0.48[a]
Fat	50.22 ± 2.04[a]	1.29 ± 0.05[b]
Crude fiber	6.82 ± 1.52[b]	8.71 ± 0.50[a]
Ash	5.55 ± 0.06[b]	5.90 ± 0.02[a]
Carbohydrates	6.12 ± 0.42[a]	5.84 ± 0.66[a]

[1]sdSIP= Solvent-defatted Sacha Inchi protein concentrate.

[2]Values are means and standard deviations of three independent replicates, means with different letters within the same row differ significantly ($P< 0.05$).

4.1.1 Protein fractionation by the Osborne method of the Sacha Inchi solvent-defatted flour

The sdSIP soluble protein fractions (figure 1) were characterized using four different types of solvents. The albumin fraction (51%) was observed in significantly higher proportions ($p<0.05$), followed by globulins and glutelins, which did not present significant differences between them. In addition, the presence of a small percentage of prolamins (1.92%) was detected. Protein fractions reported herein for the sdSIP are in concordance with previous investigations on SI seeds (Čepková *et al*., 2019; Zhan *et al*., 2021). Interestingly, the sdSIP albumin fraction was significantly higher than the 44% reported by Sathe *et al*. (2012) for a SI concentrate. Moreover, regarding protein fractions functionality, Amagliani *et al*. (2017) mentioned that hydrolyzed albumins and globulins, both present in sdSIP, from several vegetable matrices are being used in the formulation

of hypoallergenic infant formulas, with the potential to be used in various food systems including the preparation of beverages. Also, the Osborne fractionation results reported herein are attractive because, according to Zhan *et al*. (2021), both the albumins and glutelins showed promising antioxidant activities. Needless to say, both fractions were detected in significative quantities in this work. All the afore mentioned highlights the potential use of SI protein fractions as natural antioxidants that can help to prevent non-communicable diseases (heart disease, cancer, chronic respiratory disease, and diabetes). These results are crucial for future works aimed to evaluate SI protein.

Additionally, all the fractions reported herein for SI were globular proteins. In this regard, globulins have been reported to predominate in several pulses (quinoa, amaranth, lentils), whereas prolamins and glutelins make up 85% of the total protein in cereals (wheat, maize, barley, and rye) (Sim *et al*., 2021). Interestingly, SI herein presented significantly higher contents of albumin (51%), which can be compared to the similar contents of ovalbumin (54%) and lactalbumin (48-58%) reported previously in egg and whey proteins, respectively (Bonnaillie & Tomasula, 2008; Chang *et al*., 2018). It is worth mentioning that albumins have been reported to be a protein fraction highly susceptible to trypsin and chymotrypsin digestion, mainly due to their molecular weight (13-15 KDa), essential amino acids content, and its water solubility (Joehnke *et al*., 2019). Moreover, SI low prolamin contents are encouraging, because of the prolamins previously reported deficiency in essential amino acids, which results in poor protein quality (Holding, 2014).

Furthermore, regarding the techno-functionality of the protein fractions, Sim *et al*. (2021) reported that albumin constitute the highest soluble fraction, mainly due to its higher levels of negatively charged amino acids. In this sense, albumin proteins act as good foaming agents, because their non-polar regions can promote stronger air-water interfaces. Because of all the above mentioned, albumin reported herein for SI should be considered as an indicator of the suitability of the Sacha Inchi protein to be used in the formulation of different types of beverages, such as milk substitutes, supplements, flavored waters, and so on.

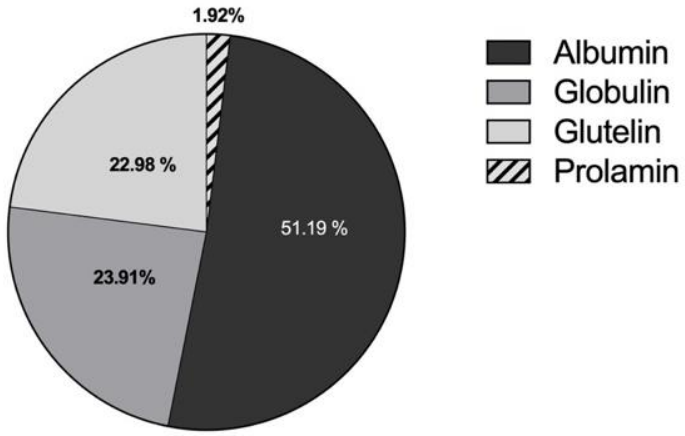

Figure 1. Solvent-defatted SI concentrate (sdSIP) soluble protein fractions (%). Values are means of minimum three independent replicates.

4.1.2 Effect of the pH on the sdSIP protein solubility

The protein solubility profiles of the sdSIP and a soybean protein concentrate (used as a control) on a wide range of pH values (from 2 to 12) are depicted in figure 2. Significative differences were observed among the protein's solubilities ($p<0.05$). The solubility variations can be explained mainly due to their amino acids structural changes, and possible aggregation or denaturation processes of each protein structure at a given pH previously reported (Rahman & Lamsal, 2021). Also, because pH can influence variations in polar amino acids, surface hydrophobicity, particle size, among others (Do *et al*., 2021). Regarding SI, its protein showed a minimum solubility (30.7%) at pH 4, and a maximum solubility (56.1%) at pH 12. These results are in concordance with Sathe *et al*. (2012) who reported pH 4 as the isoelectric point (lower solubility) of a SI protein concentrate. Also, Mercado *et al*. (2015) reported a maximum SI solubility at alkaline conditions (pH 11). On the other hand, solubilities for soybean protein reported herein were similar to the solubility profile of native soy protein reported by Lee *et al*. (2003). Lee and collaborators described that the isoelectric point for the soy protein was at pH 4.3 and that the solubility progressively increased when the pH became alkaline.

Besides, the best protein solubility along the pH scale was achieved by sdSIP ($p<0.05$), especially in the acidic region. These results are in concordance with the

previously mentioned differences in the protein fractions between SI and soybean. In this regard, albumin was the predominant fraction in SI protein (Figure 1), while soybean has been previously reported to be constituted mostly by globulins (Singh *et al*., 2015). It is worth mentioning that previous works described that albumins are the proteins with the best water solubility, while globulins are better solubilized in saline aqueous solutions (Sathe *et al*., 2012; Britannica, 2022). The last statement is also supported by the significantly better solubility of SI against soybean at their respective isoelectric points (both near pH 4) and in some neutral pH values, all these reinforces the idea that Sacha Inchi must be considered as an adequate ingredient for beverages formulation.

However, SI and soybean proteins presented similar acid isoelectric points (near pH 4), and maximum solubilities at alkaline pH values. Because of this, ultrasound-assisted protein extractions were performed at alkaline conditions for both matrices. Likewise, this information might be considered in further works aimed to study SI protein, especially for those focused on using its protein as an ingredient in the formulation of various food matrices or evaluation of its possible nutraceutical properties.

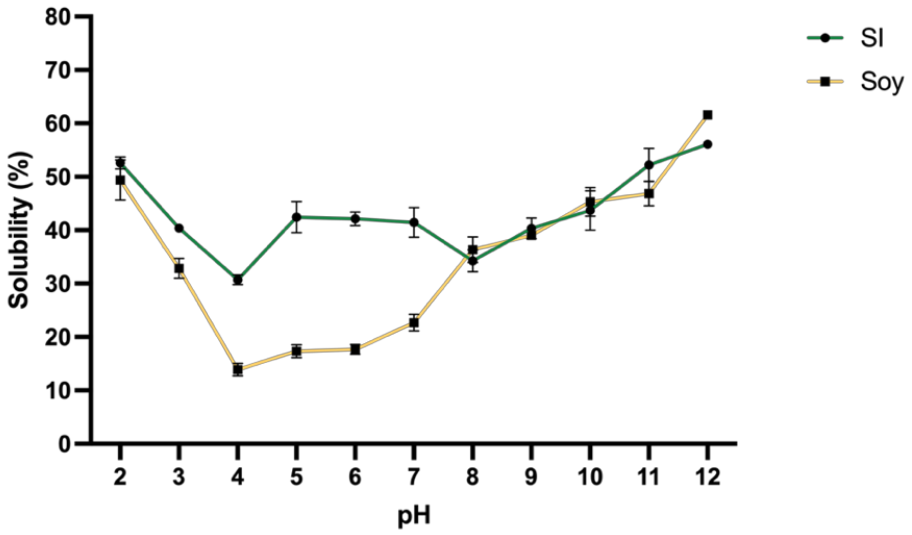

Figure 2. Effect of the pH on the sdSIP and soybean protein solubility. Values are means ± standard deviations (error bars) of minimum three independent replicates ($P< 0.05$).

4.1.3 Evaluation of anti-nutrient of Sacha Inchi and soybean

The results of the protease (trypsin) inhibitors assays were expressed as trypsin units inhibited per mg of sample (TUI) of the sdSIP and soybean protein and are presented in figure 3. Regarding trypsin inhibitors in seeds, results obtained herein indicated that the TUI in soybeans were significantly higher compared to those in SI seeds ($p<0.05$). The difference persisted in solvent-defatted flours mainly due to the increases in the protein concentration of the matrices. These results could be easily explained because trypsin inhibitors (Kunitz and Bowman-Birk) have been previously reported to be peptides corresponding to the globulins, the most abundant protein fraction in soybean (Yang *et al*., 2018). Nevertheless, globulins were also identified in sdSIP (24%), which supports the evidence that protease inhibitors were also present in Sacha Inchi protein.

Furthermore, in the case of soybean, the values of TUI reported herein for beans and solvent-defatted flours were similar to those previously reported by Vagadia *et al*. (2017). Likewise, TUI for both, SI seeds and sdSIP, were higher than the 0.13 reported by Rawdkuen *et al*. (2016) for a partially defatted SI flour. Nevertheless, the results reported herein for SI were lower than the 32 and 46 TUI described by Lázaro (2015) for SI seeds and a protein concentrate, respectively. The variability of protease inhibitors content between matrices of the same species could be attributed to differences between protein fractions contents, growing conditions, and to the seed maturation stage, being the older seeds the ones with the highest contents of trypsin inhibitors (Guillamón *et al*., 2008).

Regarding humans' health concern, the main harmful effects observed from the consumption of trypsin inhibitors is a hypertrophy of the pancreas, caused by the binding of the inhibitor to trypsin, rendering the digestive enzyme inactive (James *et al*., 2005). As a result, an excessive stimulation of digestive enzymes secretion is promoted, which also takes away several essential amino acids from supporting other important body functions. In the case of animals like ruminants, the pancreatic hypertrophy sometimes results in their death. (Savage & Morrison, 2003).

Nevertheless, the main trypsin inhibitors described in soybeans were previously reported to be heat labile due to their low molecular weight and because to the presence of only few disulphide linkages (Kumar *et al*., 2019). However, heat treatments (boiling, autoclaving, microwaving, and so on) are not an optimal solution for anti-nutrients reduction in foods because they can also affect the nutritional value of the final product. Because of the necessity to achieve a balance between destroying the inhibitors, maintaining the nutritional and functional properties of the protein, plus being innocuous for humans' health, according to Liener (1994), most commercially available soybean products should contain 5-20 % of their original trypsin inhibitor activity.

In this regard, soybean protein concentrate presented 12 TUI and sdSIP 9 TUI, meaning that the trypsin inhibitors reported herein for sdSIP were relatively too high for human consume, thus the need of evaluating ultrasound treatment as a tool to inactivate protease inhibitors in Sacha Inchi was pivotal.

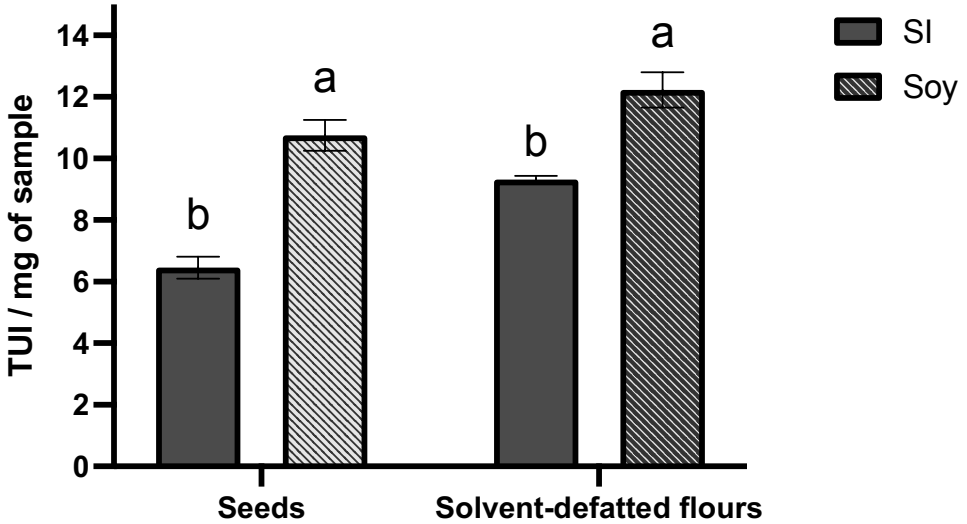

Figure 3. Trypsin units inhibited per mg of sample (TUI) in seeds and solvent-defatted flours from Sacha Inchi and soybean. Values are means ± standard deviations (error bars) of minimum three independent replicates (*P< 0.05*).

4.2 Ultrasound-assisted protein extraction from solvent-defatted Sacha Inchi flour

The ultrasound (US) studies were performed under constant extraction conditions (amplitude 100%, 1 cycle, and 15 min). These conditions were previously reported as the best for peanut protein extraction by Ochoa-Rivas *et al*. (2017). Protein purity depicted in table 4 indicated that the best recovery of organic matter was achieved in SI at alkaline conditions in treatments with and without ultrasound application. The lowest purities were achieved at pH 7 and 9. All these results were in concordance with their respective percentage of solids in each supernatant (extract), which are indicators to estimate the volume of matter extracted. Further, extraction yields show that the highest SI protein extraction was achieved in the SI11 treatment, followed by the usSI11 and usSI11c treatments ($p<0.05$). These results are important, from the point of view that the best protein extraction was achieved applying an alkali-based method alone. When ultrasound was applied in alkaline conditions, extractions resulted on an average of 54% recovery of SI protein. All these results suggest that both, the application of ultrasound and the heat released from the process, are factors that should be strongly considered and controlled in future projects aimed to explore these plant-based protein.

Regarding usSI7 and usSI9, they presented the lowest protein purity and extraction yields, these results could be easily attributed to the previously mentioned low solubility of SI protein at neutral pH values (Mercado *et al*., 2015; Sathe *et al*., 2012). Besides, there is a chance that the low protein extraction yields for ultra-sounded SI at neutral pH could be explained according to Wang *et al*. (2022), who described that sonication and moreover, that the turbulent flow and high-energy produced by ultrasound waves could result in the lowering of the protein solubility.

From these data, it can be noted that the best protein extract, in terms of extraction yields, was obtained from usSOY11. These results are aligned with Preece *et al*. (2017), who previously demonstrated that ultrasound treatments intensify the extraction of soybeans components, leading to increased yields of protein even after just 1 min of treatment. However, all ultrasound-assisted protein extracts under alkaline conditions presented similar protein qualities (table 5). Aminograms show that many of the essential

amino acids were similar between SI and soybean, except for the aromatic amino acid Phenylalanine and Tryptophan, which were significantly higher in usSOY11 and usSI11c, respectively. Attractively, these results suggest that US did not pose any negative affection on the amino acid profile of the alkaline extracts. In this sense, it can be concluded that alkaline adjustment and US treatments were suitable to extract SI protein, however, at some point in the process it was observed that the combination of both techniques and the heat released from the process could result in loses of the protein extraction. Thus, the optimization of the extraction methods applied in this research should be further investigated.

Table 4. Sacha Inchi and soybean protein purity (%) and extraction yield (%) using alkaline method assisted with ultrasound at 100% amplitude for 15 min.

	[2]Protein purity (%)		Protein extraction yield (%)		Solids yield (%)	
Sample[1]	Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet
usSOY11	45.17 ± 2.45^{c}	31.67 ± 5.92^{b}	81.71 ± 1.96^{a}	19.27 ± 3.92^{d}	36.36 ± 7.19^{b}	50.36 ± 6.45^{b}
usSI7	32.71 ± 1.87^{d}	67.10 ± 1.98^{a}	24.28 ± 2.72^{d}	77.50 ± 2.61^{a}	22.97 ± 0.86^{c}	77.23 ± 0.64^{a}
usSI9	33.88 ± 0.95^{d}	66.14 ± 0.83^{a}	20.89 ± 3.86^{d}	77.42 ± 3.07^{a}	19.38 ± 4.63^{c}	78.99 ± 4.63^{a}
usSI11	66.89 ± 2.49^{b}	60.63 ± 2.51^{a}	54.89 ± 4.50^{c}	45.66 ± 3.33^{b}	51.75 ± 6.57^{a}	52.09 ± 6.28^{b}
SI11	77.86 ± 1.55^{a}	57.11 ± 2.73^{c}	66.37 ± 1.78^{b}	34.45 ± 1.64^{c}	55.59 ± 0.36^{a}	42.91 ±1.84^{b}
SI11h	77.49 ± 2.81^{a}	60.38 ± 1.03^{a}	54.30 ± 0.78^{c}	44.47 ± 2.04^{b}	49.28 ± 1.14^{a}	47.90 ± 3.22^{b}
usSI11c	78.69 ± 1.66^{a}	60.70 ± 5.71^{a}	55.47 ± 3.85^{c}	44.53 ± 3.85^{b}	50.83 ± 0.39^{a}	49.17 ± 0.39^{b}

[1]usSI7= ultra-sound SI at pH7; usSI9= ultra-sound SI at pH9; usSI11= ultra-sound SI at pH11; usSI11c= cold ultra-sound SI at pH 11; SI11h= SI at pH11 and heated; S11= SI with just pH 11 adjustment; usSOY11= ultra-sound Soy at pH11.

[2]Protein purity (dry base) values are means ± standard deviations of three independent replicates. Means with different letters within a column differ significantly (P< 0.05).

4.2.1 Nutritional protein quality of extracts

The amino acid profiles of all the protein extracts are listed in table 5. After the analysis of all the aminograms it can be concluded that the protein quality of Sacha Inchi is high (Kalkhoran *et al*., 2018). In this regard, all SI treatments met the nutritional FAO recommendations for infants (1-2 years), except for the amino acid histidine and in the case of usSI11 and SI11h they were slightly deficient in lysine. Interestingly, for infants,

all the SI extracts perfectly met the requirements of tryptophan, and in the case of the extracts obtained at alkaline pH values its tryptophan content almost triple the amount observed in the rest of the treatments (FAO, 2013). In this regard, it is important to note that tryptophan was highly susceptible to degradation in the usSI7 and usSI9 treatments. The last is in concordance with Cheng *et al.* (2012), who previously reported that acids can retard and inhibit proteins formation. On the other hand, these results are important in the sense that the mentioned essential amino acid has been previously reported to be a fundamental precursor to various neurotransmitters in the brain, thus regulating several vital functions like appetite, sleep, mood, and pain regulation (Bellmaine *et al*., 2020), especially in children. Consequently, it can be concluded that the alkaline extraction of Sacha Inchi protein was the best option to preserve, and in this case, to enhance its quality.

Further, all the extracts obtained at alkaline conditions presented significantly better IVPD compared to their neutral pH counterparts ($p<0.05$). These differences could be attributed to the good SI solubility at alkaline values, which promotes an adequate susceptibility of the protein to the activity of the digestive enzymes. Moreover, all the alkaline treatments were the best treatment performed in this work to satisfy the nutritional recommendations of the FAO. All the afore mentioned converges in that the best biological values were obtained in all the alkaline treatments.

Table 5. Amino acid profiles of ultrasound-assisted protein extracts

Amino acid (g/100 g protein)	FAO (1-2 years)[1]	Ultrasound-assisted protein extracts[2]						
		usSOY11	usSI7	usSI9	usSI11	usSI11c	SI11	SI11h
Essential AA								
Histidine	1.80	2.72	1.01	1.12	1.67	1.51	1.49	1.64
Isoleucine	3.10	4.74	4.36	4.41	4.89	5.18	5.16	5.07
Leucine	6.30	7.89	7.26	7.23	7.13	7.41	7.30	7.10
Lysine	5.20	6.41	7.66	7.50	5.05	5.71	5.61	5.07
Methionine + Cysteine	2.60	3.18	6.84	6.51	3.45	3.98	4.00	3.52
Phenylalanine + Tyrosine	4.60	9.18	3.14	3.48	7.22	7.30	7.30	7.23
Threonine	2.70	4.13	3.72	3.75	4.63	4.87	4.84	4.54
Tryptophan	0.70	1.32	1.01	1.14	3.46	3.81	3.83	3.65
Valine	4.20	5.05	5.80	5.85	6.28	6.61	6.55	6.43
Non-essential AA								
Arginine		6.94	8.39	8.69	10.31	9.82	9.84	10.34
Alanine		4.46	4.76	4.68	3.78	3.69	3.58	3.65
Aspartic Acid		11.45	7.42	7.71	12.02	12.33	12.48	12.19
Glutamic Acid		17.79	24.20	23.21	14.72	13.89	14.02	14.49
Serine		4.72	3.97	4.15	4.93	4.72	4.77	4.62
Glycine		4.37	5.40	5.69	5.49	4.86	4.77	5.37
Proline		4.96	3.20	3.19	3.77	3.79	3.83	3.74
Hydroxyproline		0.09	0.40	0.37	0.11	0.08	0.08	0.11
Hydroxylysine		0.00	0.43	0.35	0.00	0.14	0.14	0.15
Limiting amino acid score		1	0.56	0.62	0.93	0.84	0.83	0.91
IVPD (%)[3]		83.04 ± 3.76[a]	60.83 ± 1.05[c]	69.22 ± 0.93[b]	83.58 ± 1.55[a]	82.13 ± 1.48[a]	82.25 ± 0.45[a]	84.78 ± 0.69[a]
PDCAAS		0.83	0.34	0.43	0.77	0.69	0.68	0.77

[1]WHO/FAO/ONU suggested requirements for kids (1-2 years).
[2]usSI7= ultra-sound SI at pH7; usSI9= ultra-sound SI at pH9; usSI11= ultra-sound SI at pH11; usSI11c= cold ultra-sound SI at pH 11; SI11h= SI at pH11 and heated; S11= SI with just pH 11 adjustment; usSOY11= ultra-sound Soy at pH11.
[3]The IVPD values are means ± standard deviations of two independent replicates. Means with different letters within the same line differ significantly (P<0.05).

Likewise, the usSOY11 protein amino acid profile perfectly met the nutritional recommendations for both, adults and infants (FAO, 2013). The results obtained herein were in concordance with other studies performed with SI (Mora-Aguirre *et al*., 2020) and soybean (Mohsen *et al*., 2009) proteins. We could conclude that, according to the aminograms obtained, and among all the factors controlled in this investigation, pH is a fundamental variable that modulates the solubility of essential amino acids present in Sacha Inchi protein, especially tyrosine and tryptophan. Similar results were mentioned by Ochoa-Rivas *et al*. (2017) when conducting protein extraction studies of peanuts. Nevertheless, nutritionally speaking, there were not significant differences in the amino acid composition between ultrasound-assisted and sole alkaline extractions. The latter should be considered for future works aimed to extract proteins from Sacha Inchi.

4.2.2 Effect of ultrasound on the anti-nutrients content of Sacha Inchi and soybean protein extracts

As previously mentioned, a food matrix for human feeding porpoises should contain less than 20% of its initial trypsin inhibitors in order to be innocuous for humans health (Kumar *et al*., 2019; Liener, 1994). In this regard, all protein extracts listed in table 2 underwent significant reductions in their TUI compared to their original matrix ($p<0.05$) (figure 4). Therefore, the satisfactory reduction of the trypsin inhibitors for both, Sacha Inchi and soybean protein extracts, could probably be explained via several mechanisms produced mainly by the different extraction factors used in this investigation, which are ultrasound application, alkaline adjustment or the temperature released from the process.

First, interestingly usSI7 and usSI9 showed significative TUI reductions. These results are relevant in the sense that the mentioned extracts were not obtained under alkaline conditions. Thus, their anti-nutrient reduction could be attributed to the application of ultrasound or to the heat involved in the process in concordance with Rahman & Lamsal (2021), who previously reported that the use of US contributed to diminish Kunitz trypsin inhibitors from soybean by disrupting the disulfide bonds to thiol groups, consequently changing the structure of the reactive site of the inhibitor. On the other hand, the SI11 and SI11h treatments were obtained without the application of US and also showed

considerable TUI reductions. These could be perfectly attributed to the well-known modulating effects of the pH and heat on the protein's structures. In this sense, Avilés-Gaxiola *et al.* (2018) previously reported that alkaline pH values can lead to decreases of amino acid residues that conform the active site of the trypsin inhibitors, thus lowering its activity. Moreover, since the trypsin inhibitors are conformed by small peptides (ranging from 7 to 24 KDa), they were susceptible to degradation due to increases in the temperature of the medium.

Furthermore, with respect to the extracts obtained using ultrasound under alkaline conditions (usSI11, usSI11c, and usSOY11), their TUI reductions can be explained via the three mechanisms mentioned above (pH, ultrasound, and temperature). Additionally, the possibility of a synergistic effect between alkaline pH adjustment and the ultrasound application should be further studied in SI and in other novel plant-based alternatives. In this regard, it is worth recommending that the optimization of these factors should be better understanded strongly considering the possible effects of the temperature.

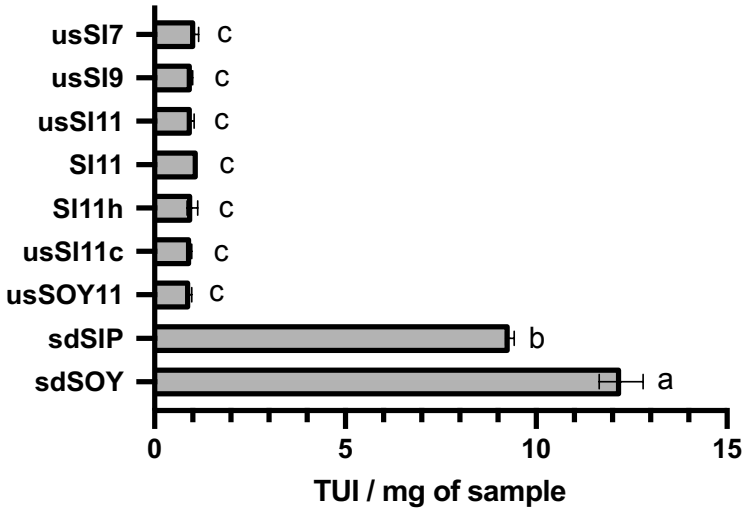

Figure 4. Trypsin units inhibited per mg of sample (TUI) in ultrasound-assisted protein extracts from Sacha Inchi and soybean solvent-defatted flours. Values are means ± standard deviations (error bars) of minimum three independent replicates (*P< 0.05*).

4.2.3 SDS-PAGE electrophoretic patterns of ultrasound protein extracts

All the protein extracts were characterized by SDS-PAGE electrophoresis, resulting in the identification of polypeptides in the 6-65 KDa range. In the case of the SI extracts, all protein electrophoretic profiles were similar, probably suggesting that the impact of the extraction techniques resulted on non-significative differences on the proteins structures. Moreover, the SI electrophoretic profiles in the absence of a reducing agent (Fig. 5a) suggested that protein extracts contained disulfide bond-linked polypeptides, thus the use of mercaptoethanol was necessary to better elucidate reduced fractions or bands. Upon reduction (Fig. 5b), the electrophoresis of SI was composed by peptide dimers of 30-40 KDa and a 6-25 KDa, which means that although a disulfide bond reduction was satisfactory performed, it did not allow their elimination.

The above-mentioned pattern was appreciated in all SI extracts, especially in the usSI7 and usSI9 treatments. In this regard, the wide range of molecular species were in concordance with the different protein fractions identified in Sacha Inchi (figure 1). As previously mentioned, albumins are molecular species with previously reported low molecular weights (5-30 KDa), what makes them highly water soluble and digestible (Joehnke *et al*., 2019; Sathe *et al*., 2012). Regarding globulins, Espinosa-Ramírez & Serna-Saldívar (2019) reported species with molecular weights from 18 to 37 KDa to correspond to the 11S fraction of the globulins, which are normally stabilized by prominent disulfide bridges. The contents of cysteine (table 5) and globulins (Figure 1) in SI support the last statement. Likewise, these results were in concordance with Sathe *et al*. (2012), who earlier indicated that SI proteins are mainly composed of polypeptides with molecular weights from 6 to 70 KDa. Is for all the above mentioned, that we could conclude with high confidence that SI protein is constituted by a complex mixture of storage protein fractions (albumins and globulins) which when they are together can assume different and in some cases complex behavior patterns. (López *et al*., 2016).

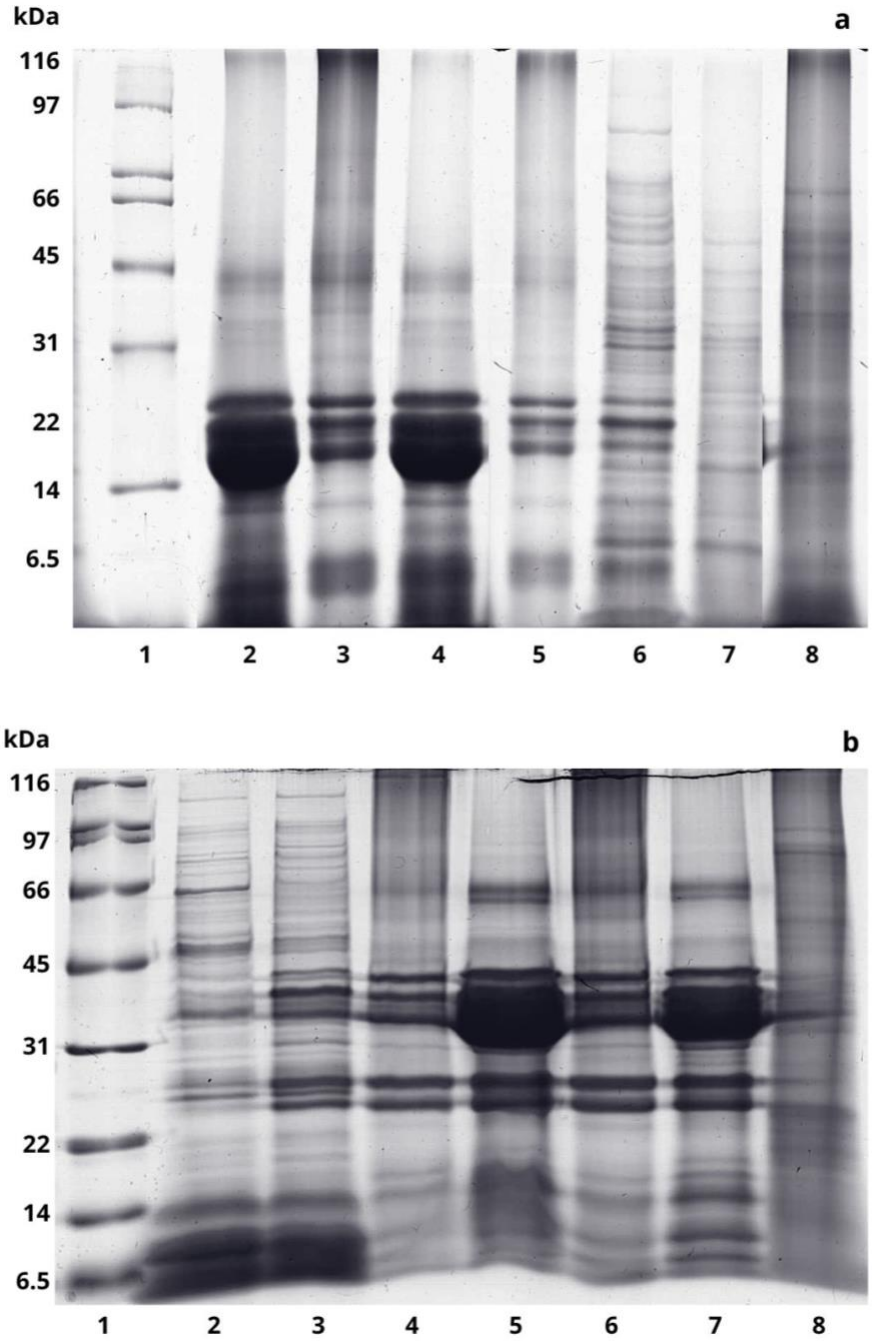

Figure 5. SDS-PAGE (18% acrylamide separating gel) of total ultrasound-assisted protein extracts from Sacha Inchi and Soy in the absence (**a),** and presence (**b)** of a reducing agent (2% v/v of β-mercaptoethanol). **(1)** SDS-PAGE standard broad range (Bio-Rad); **(2)** usSI7= ultra-sound SI at pH7; **(3)** usSI9= ultra-sound SI at pH9; **(4)** usSI11= ultra-sound SI at pH11; **(5)** SI11= SI with just pH 11 adjustment; **(6)** SI11h= SI at pH11 and heated; **(7)** usSI11c= cold ultra-sound SI at pH 11; **(8)** usSOY11= ultra-sound Soy at pH11.

Regarding soybean protein, the electrophoretic profile presented several subunits in the range of 14 to 70 KDa, especially species with molecular weights of 25 and 35 KDa, previously reported to constitute its 11S glycinin fraction (Barać *et al*., 2011). The results presented in this research are in concordance with earlier works who previously stated globulins as the major fraction in soybean protein (Barać *et al*., 2011; Chen *et al*., 2016; Stephan, 2021). Although, these results confirm that protein fractions in soybean are similar between varieties.

With the aim to estimate the industrial applications of the protein extracts obtained herein, the assessments of techno-functional properties were performed (Table 6). The WAI and WSI are useful indexes related to the interaction of the extracts with water, being important factors that can modulate some of the processing steps of the matrix at industrial level, shipping and storage for example. The results for WAI obtained herein demonstrated that among all the SI and soybean treatments obtained using ultrasound there were few significative differences ($p<0.05$). In this sense, almost all the SI extracts presented WAI values similar to those previously reported (Cordero-Clavijo *et al*., 2021; Torres-Sánchez *et al*., 2021). It is worth noting that these results could confirm that no alteration to polar amino acids was displayed after the ultrasound treatment under alkaline conditions. Furthermore, usSI7 and usSI9 showed the lowest WAI values. This could be explained by the previously discussed low solubility of the SI protein at neutral pH values (figure 2).

4.2.4 Techno-functional properties of ultrasound protein extracts.

Table 6. Techno- functional properties of ultrasound-assisted proteins extracted from Sacha Inchi and soybean

	Ultrasound-assisted protein extracts[1]						
Functionality[2]	usSOY11	usSI7	usSI9	usSI11	SI11	SI11h	usSI11c
WAI (g water/ g sample)	3.80 ± 1.62^{abc}	1.98 ± 0.47^{c}	2.29 ± 0.15^{bc}	4.45 ± 1.60^{ab}	3.61 ± 1.12^{abc}	5.53 ± 0.46^{a}	3.68 ± 0.63^{abc}
WSI (g/g) x100	60 ± 0.12^{ab}	62 ± 0.06^{a}	70 ± 0.02^{a}	29 ± 0.05^{c}	53 ± 0.20^{ab}	31 ± 0.01^{c}	37 ± 0.12^{bc}
SP (g/ g sample)	7.93 ± 1.84^{a}	5.29 ± 1.69^{a}	7.68 ± 0.93^{a}	7.59 ± 0.72^{a}	8.04 ± 1.18^{a}	7.99 ± 0.74^{a}	5.93 ± 0.89^{a}
OAC (g/ g sample)	4.67 ± 0.93^{bc}	3.20 ± 0.56^{c}	2.92 ± 0.68^{c}	3.17 ± 1.15^{bc}	7.20 ± 0.93^{a}	6.74 ± 0.69^{ab}	4.79 ± 1.79^{bc}
FC (%)	98.11 ± 2.01^{a}	82.78 ± 2.55^{c}	88.33 ± 1.67^{bc}	91.11 ± 0.96^{abc}	96.07 ± 5.33^{ab}	96.67 ± 5.77^{ab}	91.41 ± 0.44^{abc}
FS (%)	87.78 ± 6.74^{a}	56.67 ± 3.33^{b}	55.56 ± 1.92^{b}	57.78 ± 0.96^{b}	55.48 ± 2.52^{b}	61.33 ± 2.31^{b}	58.28 ± 0.09^{b}

[1]usSI7= ultra-sound SI at pH7; usSI9= ultra-sound SI at pH9; usSI11= ultra-sound SI at pH11; usSI11c= cold ultra-sound SI at pH 11; SI11h= SI at pH11 and heated; S11= SI with just pH 11 adjustment; usSOY11= ultra-sound soybean at pH11.

[2]WAI= water absorption index (weight of sediment/ sample weight); WSI= water solubility index (weight of dissolved solids in supernatant/ sample weight); SP= swelling power (weight of sediment/ sample weight-weight of dissolved solids in supernatant); OAC= oil absorption capacity (grams of oil bound per grams of sample on dry basis); FC= foam capacity (%); FS= foam stability (%).

[3]Values are means ± standard deviations of minimum three independent replicates. Means with different letters within the same line differ significantly (P<0.05).

On the other hand, the usSI7 and usSI9 treatments were significantly higher in terms of WSI values ($p<0.05$), these phenomena could be explained by the predominantly presence of albumins in the SI protein organization. Moreover, as alkaline treatments underwent disulfide bond disrupting for inactivating the inhibitors, structural changes in water binding sites could happen, thus lowering its water affinity (Espinosa-Ramírez & Serna-Saldívar, 2019). However, all the WSI for SI reported herein were higher than those maximum 20% and 29% previously reported by (Cordero-Clavijo *et al*., 2021; Torres-Sánchez *et al*., 2021), respectively, for solvent-defatted SI flours. In the case of the WSI of soybean, it was higher than the maximum 29% reported by (Choi *et al*., 2020) for a defatted soybean flour.

Likewise, there are limited reports on the SP of SI protein, thus all the values obtained here were compared to those previously reported by Kaushik *et al*. (2020) for a lentil protein concentrate. In this regard, SP of all the extracts, for SI and soybean, obtained herein were similar to those reported by Kaushik and coworkers. Nevertheless, the relatively low SP values of SI protein were in concordance with previous reports that state that increases in the protein of a food matrix lead to reductions in their starch elasticity when in contact with water, thus reducing its swelling capacity (Teng *et al*., 2018). The last because the competitive nature of the proteins to bind to water, reducing the water availability to starch. It is worth mentioning, that according to Loveday (2020), the WAI, WSI, and SP values presented herein were in an adequate range to consider the ultrasound-assisted protein extracts under alkaline conditions as good ingredients for novel foods formulations or supplements.

Regarding the OAC, all treatments presented significantly higher values than those reported by Torres-Sánchez and coworkers (1.4), and by Cordero-Clavijo (2.8), both for solvent-defatted Sacha Inchi flours. In the case of usSOY11, its OAC was higher than that reported by *(Brishti et al*., 2017) for a soybean protein isolate. Moreover, SI11 presented the best OAC values, reinforcing the theory that alkaline pH also plays a pivotal role modulating the presence of non-polar amino acids, which are crucial to the protein-oil binding capacity (Cordero-Clavijo *et al*., 2021). Furthermore, according to Brishti *et al*.

(2017), the behavior of proteins when interacting with fat is directly influenced by its conformational and structural features. In this regard, the ultrasound-assisted extracts produced under alkaline conditions presented slightly higher OAC values compared to counterparts obtained at neutral pH (usSI7 and usSI9). Adequate OAC values in plant-based novel proteins are fundamental to improve mouthfeel and flavor retention of certain food products, like supplements for athletes and babies, sausages, salad dressings, and mayonnaise (Amagliani *et al*., 2017).

Additionally, the foaming behavior of proteins is directly dependent on its concentration, structure in terms of protein fractions that are present in the matrix, and polar amino acids content, which modulate their ability to form foams. The last are defined as air bubbles dispersed in a solid or liquid matrix formed after a mechanical whipping process (Heredia-Leza *et al*., 2022; Uluko *et al*., 2016). In this regard, foaming capacities of all the SI and soybean extracts obtained at alkaline pH were lower than those reported by (Cordero-Clavijo *et al*., 2021) for a solvent-defatted SI flour, nevertheless, all treatments were higher than the 73% reported by Rawdkuen *et al*. (2022) for a SI protein isolate obtained by alkaline extraction and acid precipitation. In the case of usSOY11, its FC was significantly better than the previously reported value of 69% observed for a soybean protein isolate (Brishti *et al*., 2017). Likewise, results indicated that the only significative differences in FC were found between pH adjustment treatments, this is worth mentioning in the sense that it could be concluded that the ultrasound application did not affect the foaming properties of the Sacha Inchi protein. Furthermore, the best FC were obtained at alkaline pH, these results were in concordance with the previously mentioned water affinity of the albumins, the major fractions in the Sacha Inchi protein.

Interestingly, there were no significative differences in the FS among SI extracts, which means that all extracts obtained herein presented a far more stable foam than the maximum 40% previously obtained by Cordero-Clavijo *et al*. (2021). These positive results could be attributed to the conformational changes to the protein structure, provided by US or alkali adjustment, which gives the peptides more flexibility to move towards and to stabilize the air-water interface in bubbles (Heredia-Leza *et al*., 2022). The FC of

soybean was the best of all protein extracts, and the finding is in concordance with previously reported works (Brishti *et al*., 2017).

Finally, the *in vitro* protein digestibility of the protein extracts was evaluated as an indicator to estimate the bioaccesibility and to help to evaluate future possible applications of the Sacha Inchi protein in subsequent works. In this sense, all the SI protein extracts obtained by ultrasound under alkaline conditions presented significantly higher IVPD values compared to the previously reported 60-70% for simulated gastro-intestinal SI protein digestibility (Rawdkuen *et al*., 2018, 2022; Zhan *et al*., 2021). The observed SI IVPD could be attributed to possible improvements in the structure and conformation of the protein due to the extraction treatment, which can lead to higher release rates of soluble nitrogen (Chirinos *et al*., 2020). Moreover, the reduction of anti-nutrients and their peptide bonds has also been previously reported to enhance the ability of digestive enzymes. All the afore mentioned could be perfectly attributed to the alkaline treatment, this assumption could be confirmed by looking at the significatively low IVPD of the usSI7 and usSI9 extracts, which also underwent US and heat treatment but still presented the lowest digestibility values. Nevertheless, the usSI7 and usSI9 extracts showed IVPD similar to other plant-based proteins, such as peanuts and some beans (Rizzello *et al*., 2016). Moreover, usSOY11 presented digestibility values similar to those reported by (Gan *et al*., 2009). It is worth mentioning that the best IVPD was achieved in all treatments at pH11, confirming the hypothesis that alkaline-based techniques are adequate options to improve SI protein solubility, disrupt its peptide bonds, and to reduce its trypsin inhibitors without affecting its protein quality. All the afore mentioned in this research invite us to conclude that in general terms (extraction yields, anti-nutrients reduction, protein quality and techno-functional properties) the best treatment was SI11, meaning that alkaline extraction alone was the best technique for the objectives of this work.

Chapter 5
General conclusions and recommendations for future works

Results obtained herein highlight the suitability of Sacha Inchi flour to provide protein-rich concentrates (>65%) with high protein quality which can be used as novel ingredients for the development of innovative food products. For the last-mentioned purpose, the holistic understanding of the SI protein relation with water was elucidated via studying its complete profile of protein fractions and better understanding of how they influence their water solubility. In this regard, the Sacha Inchi and soybean proteins were significantly different, because they presented different major protein fractions, albumins and globulins, respectively. Nevertheless, both exhibited similar isoelectric points (near pH 4) and increased solubilities as the pH gradually increased or became more alkaline. Moreover, it is worth mentioning that some of the nutraceutical and functional properties that have been attributed to soy albumins and globulins may possibly be present in Sacha Inchi. Further understanding in this area is crucial for future works aimed to evaluate the effects of Sacha Inchi digested or hydrolyzed proteins and peptides in humans' health.

Moreover, results obtained along this thesis demonstrated that it was possible to extract protein from solvent-defatted Sacha Inchi flours using an ultrasound treatment under alkaline conditions. The combination of ultrasound-assisted extractions under alkaline conditions and the heat involved in the process reduced the trypsin inhibitors contents in SI and soybean protein concentrates without affecting their protein qualities. Regarding extraction yields, nevertheless the ultrasound worked on SI protein extraction, it was noted that the conventional alkaline adjustment alone was the ideal technique for extracting its protein. These can be explained mainly due to the predominant effect of the pH on the electronegativity of the amino acids, essential for the protein-water interaction, also to the low solubility of the Sacha Inchi protein under neutral pH conditions confirmed in this work (figure 2), and to the disulfide bonds previously reported in the Sacha Inchi protein.

Attractively, all the protein extracts obtained in this work underwent significant reductions on their protease inhibitors contents. These results were observed in ultrasound extracts under alkaline and neutral conditions; nevertheless, we should suggest that when considering using ultrasound to achieve reductions on the trypsin inhibitors of Sacha Inchi protein without affecting its nutritional quality and techno-functional properties, temperature is a crucial factor to be controlled. Nutritionally speaking, the aminograms of all the Sacha Inchi extracts (table 5) indicated that the essential amino acids increased significantly when ultrasound was applied under alkaline conditions. These results are in concordance with previously reported evidence of the pH ability to modulate the solubility of a protein. Still, this information is helpful and must be considered in future investigations aimed to increase the biological values of food matrixes using Sacha Inchi protein, because it suggests that US did not pose any negative affection to the aminograms of the protein.

Additionally, after SDS-PAGE electrophoresis was performed, we could reinforce the importance of albumins, globulins and disulfide bonds on the Sacha Inchi protein organization. These are crucial factors that influence the presence of anti-nutrients, and also collaborate to regulate the solubility and digestibility of the protein. Moreover, polypeptides observed in Sacha Inchi are in concordance with the protein fractions determined in this research and with other previously reported works.

Furthermore, after the techno-functional evaluation of all the SI extracts, we could confirm that none the ultrasound application or the alkaline pH adjustment posed any reduction on the functionality of the protein. These results are important in the sense that they ensure to the food industrials and researchers that the ultrasound is an extraction technique that will not negatively affect the functionality of their products in any stage (shipping, production, storage, sensorial testing, among others).

Finally, it is worth mentioning some pivotal connotations for future research works focused on Sacha Inchi or in other novel plant-based proteins. Regarding protein extraction, it could be interesting to study the effects of an enzymatic method assisted

with ultrasound. According to the Sacha Inchi protein conformation and its alkaline maximum water solubility, the use of an alcalase could potentiate the protein extraction from Sacha Inchi and the rate of water solubility. Moreover, SI extracts could become an interesting protein-rich ingredients for novel food formulations, especially beverages, for example plant-based milk analogs alternatives, and in other commodities products like bread and other bakery items. Because of its nutritional characteristics and content of bioactive compounds, the Sacha Inchi protein extracts could help to increase the nutraceutical effect of supplemented food matrixes. Therefore, it is pertinent to recommend that further research should be designed with the aim to evaluate the possible nutraceutical effects of Sacha Inchi in humans' health.

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MSc Products

Hereby the main contributions made within the period of the MSc program are detailed. It includes published articles, articles in preparation to be submitted derived from this research project, and participations in international conferences.

Published article in a journal

- Cordero-Clavijo, L., Serna-Saldívar, S., Lazo-Vélez, M., Avilés-González, J., Panata-Saquicilí, D., Briones-Garcia, M. (2021). Characterization, functional and biological value of protein-enriched defatted meals from Sacha Inchi (*Plukenetia volubilis*) and chocho (*Lupinus mutabilis*). *Journal of Food Measurement and Characterization*, *15*(6), 5071–5077. https://doi.org/10.1007/s11694-021-01084-5

Articles to be submitted

- Cordero-Clavijo, L., Serna-Saldívar, S., Chuck-Hernández, C., Espinosa-Ramírez, J., Lazo-Vélez, M. (2022). Sacha Inchi (*Plukenetia volubilis L.*): A highly nutritional and phytochemical emerging superfood.

- Cordero-Clavijo, L., Serna-Saldívar, S., Chuck-Hernández, C., Espinosa-Ramírez, J., Lazo-Vélez, M. (2022). Effect of ultrasound on the protein extraction and trypsin inhibitors from Sacha Inchi (*Plukenetia volubilis L.*).

- Cordero-Clavijo, L., Serna-Saldívar, S., Chuck-Hernández, C., Espinosa-Ramírez, J., Lazo-Vélez, M. (2022). Sacha Inchi protein extracts obtained by different procedures at pH 11: influence in dough rheology and bread quality.

National and international conferences

- Cordero-Clavijo, L., Lazo-Vélez, M., Panata-Saquicilí, D. (2021). Comportamiento térmico de mezclas de harinas desgrasadas de Sacha Inchi y Chocho por calorimetría diferencial de barrido (DSC). II Congreso Internacional de Cereales, Leguminosas y Afines, de la Red de Cereales y Afines, llevado a cabo en la Universidad Técnica de Ambato. October 11th – October 15th, 2021.

- Cordero-Clavijo, L., Serna-Saldívar, S., Chuck-Hernández, C., Espinosa-Ramírez, J., Lazo-Vélez, M. (2022). Effect of ultrasound on protein extraction and trypsin inhibitors of Sacha Inchi (*Plukenetia volubilis L*.). V Congreso Internacional de Alimentos Funcionales y Nutracéuticos. June 8 th- June 10 th, 2022.

Appendix

Artículo publicado: Characterization, functional and biological value of protein-enriched defatted meals from Sacha Inchi (*Plukenetia volubilis*) and chocho (*Lupinus mutabilis*). *Journal of Food Measurement and Characterization*, *15*(6), 5071–5077. https://doi.org/10.1007/s11694-021-01084-5

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Characterization, functional and biological value of protein-enriched defatted meals from sacha inchi (*Plukenetia volubilis*) and chocho (*Lupinus mutabilis*)

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Abstract

A deficit in protein intake can severely halt children growth, and is associated with both structural and functional pathologies of the brain, as well as increase the risk of contracting infectious diseases. Protein-enriched defatted meals from sacha inchi (*Plukenetia volubilis*) (sdSI) and chocho (*Lupinus mutabilis*) (sdCH) were obtained by a solvent defatting procedure. The concentration of protein was higher in sdSI (64%) than in sdCH (60%). Lysine and tryptophan were the limiting amino acid (AA) in each case. However, the AA profile of the protein meals met daily intake FAO requirements for adults, while their blends met the requirements for infants. The sdSI had an in vitro digestibility of 100. Regarding functional properties, sdSI presented higher values for water absorption index (3.5), emulsifying activity (56%), and foaming capacity (167%), while the sdCH had a slightly better oil absorption capacity (2.8). However, when mixing sdSI with sdCH some improvements in functional properties were observed. The nutritional and functional properties of the mixtures of meals can be used to develop protein-enriched foods and beverages.

Curriculum Vitae

Luis Mateo Cordero Clavijo was born in Loja, Ecuador on February 22nd, 1998. He obtained his bachelor's degree in Food Engineering in October 2020 from Universidad del Azuay at Cuenca, Ecuador. He started his MSc. in Biotechnology in the Instituto Tecnológico y de Estudios Superiores de Monterrey at Monterrey, Nuevo León, México in February 2021, under the supervision of PhD. Sergio Serna Saldívar. During his studies, he published a paper of the Sacha Inchi biological value, and this report represents, apart to be the required thesis required to complete his master studies, a potentially published research project focused on plant-based novel proteins.

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