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"OBTAINING PROTEIN ISOLATES AND POLYPHENOLS FROM QUINOA (*Chenopodium quinoa* Willd.) THROUGH MEMBRANE PROCESSES"

Thesis

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ABSTRACT

The contribution of high quality protein in the daily diet is a global problem especially in developing countries, where a large segment of the population has no access to animal protein. Although the energy supply from these foods might be adequate, insufficient amounts of essential amino acids can cause malnutrition. In this regard, recent studies indicate that quinoa contains elevated concentrations of high-quality proteins, so the aim of this study was to obtain protein isolates and polyphenols from Peruvian quinoa grains (*Chenopodium quinoa* Willd.) by alkaline extraction and ultrafiltration membrane processes. For obtaining quinoa protein isolates, the pH of the extraction and the precipitation processes were evaluated using pH ranges from 7 to 12, and 2 to 6, respectively. Subsequently, a cross-flow ultrafiltration step (UF) was performed with a membrane molecular weight cut-off of 5 kDa. The protein and total polyphenols contents in the quinoa seeds were 12.66% (w/w) and 134.92 ± 0.92 mg eq. of sinapic acid/100 g dry weight, respectively. For the extraction stage, a pH of 12 yielded the highest ratio of protein extraction (64%) with respect to the raw protein content; whereas for the precipitation process, a pH of 4 resulted in the highest yield of quinoa protein isolates. Finally, the use of ultrafiltration membranes helped to increase the recovery of quinoa proteins to 80%. The phenolic compounds were recovered from the protein extraction permeates using acid extraction combined with nanofiltration membranes. The nanofiltration process was suitable for the recovery of 79% of the phenolic compounds present in the quinoa flour. It can be concluded that the use of membrane technologies provides a good alternative for obtaining high quality protein isolates from quinoa and for the efficient recovery of phenolic compounds from natural products.

RESUMEN

La contribución de proteínas de alta calidad en la dieta diaria es un problema global especialmente en los países en desarrollo, donde un gran segmento de la población no tiene acceso a las proteínas animales. Debido a esto, es necesaria la obtención de proteínas vegetales a partir de cereales, leguminosas y otros granos. A pesar de que el suministro de energía de estos alimentos puede ser adecuado, cantidades insuficientes de aminoácidos esenciales pueden causar desnutrición. En este sentido, estudios recientes reportan que la quinoa contiene concentraciones elevadas de proteínas de alta calidad, por lo que el objetivo de este estudio fue obtener aislados proteicos y polifenoles a partir de granos de quinoa peruana (*Chenopodium quinoa* Willd*.)* mediante extracción alcalina y procesos de membrana. Para obtener aislados de proteína de quinoa, el pH de la extracción y los procesos de precipitación se evaluaron utilizando intervalos de pH de 7 a 12 y de 2 a 6, respectivamente. Posteriormente, se realizó una etapa de ultrafiltración de flujo cruzado con un peso molecular de corte de membrana de 5 kDa. Los contenidos de proteínas y polifenoles totales en las semillas de quinoa desengrasada fueron 12.66% y 134.92 ± 0.62 mg eq. de ácido sinápico/100 g de materia seca, respectivamente. Para la etapa de extracción, un pH de 12 produjo la mayor proporción de extracción de proteína (64%) con respecto al contenido de proteína cruda, mientras que para el proceso de precipitación un pH de 4 dio como resultado los mayores rendimientos de aislados de proteína de quinoa. Finalmente, el uso de membranas de ultrafiltración ayudó a incrementar la recuperación de las proteínas de quinoa al 80%. El trabajo posterior fue la recuperación de compuestos fenólicos en donde se utilizó un proceso de nanofiltración; este proceso ayudó a la recuperación total del 79% de compuestos fenólicos presentes en la harina de quinoa.

Se puede concluir que el uso de la tecnología de la membrana proporciona una buena alternativa para obtener aislados de proteína de quinoa de alta calidad; así como la eficiente recuperación de compuestos fenólicos obtenidos como subproductos del proceso.

CONTENT

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List of Abbreviations

Eq. CAT	Catechin equivalents
Eq. Rut	Rutin equivalents
Eq. SA	Sinapic acid equivalents
d.w.	Dry weight
DPPH•	Free radical 2,2-diphenyl-1-picrilhidracilo
EC_{50}	Inhibitory Concentration 50
TP	Total phenols compounds
TF	Total flavonoids compounds
Eq. GA	Gallic acid equivalents
SPI	Soluble protein isolate
PPI	Precipitated protein isolate
MWCO	Molecular weight cut-off
AOAC	Association of Official Analytical Chemists
T_{EC50}	Time required to reach steady state corresponding to the EC_{50}
Trolox	(±) Acid-6-hydroxy-2,5,7,8-tetrametilcroman-2-carboxylic acid

1. INTRODUCTION

Recent studies related to human nutrition have focused on the composition of the diet and the development of chronic degenerative diseases such as obesity, diabetes, cancer, heart disease, among others. One of the determinant factors that favor the development of such diseases is the type and quality of food consumed by the population. For example, it is possible to include in the diet abundant food which not only does not meet the nutritional minimum standards but also are harmful to human health. Recently, the consumption of functional foods containing bioactive compounds or natural products that are able to provide different health benefits is generating new alternatives for the prevention of diseases [1].

Quinoa is a pseudocereal which is currently acquiring great importance in human food due to its high nutritional value. This is mainly due to the fact that it has an adequate balance of essential amino acids, high concentration of lysine in its seeds and leaves, suitable content of vitamins, and high content of calcium and iron [2]. Also, quinoa is considered as the only food of the vegetable kingdom that provides all the essential amino acids to fulfill the human nutrition standards established by the Food and Agriculture Organization (FAO) of the United Nations [3].

The main producer countries of quinoa are Bolivia and Peru. This grain is a prehispanic crop that was used by the ancestral Andean peoples as an important part of their diet (5000-3000 B.C.). Browning, milling and cooking of quinoa are used for its processing, or it can be used as an ingredient in breakfast cereals. In addition, due to malnutrition problems in the developing countries, the FAO declared 2013 as the "International Year of the quinoa", due to its potential to reduce hunger in developing countries and with the aim to achieve food security in those countries, as well as to preserve this millennial crop [4]. The production of this crop had a 3-fold increase from 1992 to 2013 with yields of 1.15 tons per ha, which generates opportunities for the optimal utilization of quinoa and its components.

Considering the increasing demand of proteins for the production of high-quality foods and to the increasing concerns of the consumer by allergies to peanut proteins, lactose in the

milk, as well as the growing number of people who follow vegetarian diets, there is a need for alternative sources of proteins. Oil seeds can be used as a source of high quality proteins, by using membrane technologies for obtaining protein isolates and other compounds of interest.

Quinoa producers in developing countries are looking for new forms of getting the maximum use out the seeds for its commercialization. In this research quinoa seeds are used as a raw material to obtain proteins and bioactive compounds such as polyphenols. To achieve this, it is proposed to use integrated processes that include the alkaline extraction, micro, ultra and nanofiltración for the production of products with high-added value of commercial interest from quinoa grains.

2. LITERATURE REVIEW

2.1. Quinoa generalities

2.1.1. Origin and geographical distribution

Quinoa (*Chenopodium quinoa* Willd) is a pseudo-cereal (Figure 1) which belongs to the Amaranthaceae family, and is native to South America, specifically from the Andean region of Bolivia and Peru [2]. The area of geographic dispersion of this agricultural product is very broad, and at present intensive agricultural and extension techniques are being implemented for the production of this important crop [2].

Figure 1. Quinoa (*Chenopodium quinoa* Willd)

Geographical dispersion of quinoa covers in South America, Colombia, Argentina and Chile; in Mexico a similar species to quinoa, called Huauzontle (*Chenopodium nuttalliae*), is used as an inflorescence vegetable [2]. At present, quinoa production has distributed worldwide since good results for its production and adaptation have been obtained mainly in America, from Canada to Chile; as well as in Europe, Asia and Africa [2].

Historically, there are a few archaeological, linguistic and ethnographic evidences about quinoa cultivation. The archaeological evidence in northern Chile, indicates that quinoa was used before the year 3000 B.C. Findings in the area of Ayacucho, Peru, indicate that the

domestication of the quinoa occurred in the year 5000 B.C. There are also findings of quinoa in the tombs of Tarapacá, Calama, Arica and in different regions of Peru, consisting in seeds and inflorescences. In addition, abundant quantities of quinoa seeds were found in indigenous Chilean graves [5].

2.1.2. Agricultural production of quinoa

Quinoa has an extraordinary ability to adapt to different climatic conditions and agro-ecological zones. It can grow in relative humidity ranging from 40 to 88%. It is cultivated at altitudes from sea level to 4,000 m.a.s.l. and temperature between -4 °C and 35°C. It is possible to produce acceptable yields with low rainfall such as 100 to 200 mm annually. Although quinoa is still not known in many parts of the world, it is becoming increasingly popular in international markets, especially in the developed countries of North America and Europe [6].

The increased production of quinoa comes from the Andean region of Bolivia and Peru. These two countries are the main quinoa suppliers and together represent more than 90% of the world production. Chile and Ecuador are also traditional producers, although on a smaller scale. In the indigenous populations, quinoa is used mainly as a subsistence crop, especially by women, who play a particularly important role in its production and marketing [7].

Bolivia accounted for 45% of the world production of quinoa in 2011. The production in that country has grown steadily since the mid-1990s, with average annual growth rates of 4.5% between 1995 and 2011. Other emerging producers include Australia, Canada, China, Denmark, Italy, India, Kenya, Morocco and Netherlands [6].

Given the nutritional value and great export potential of quinoa, governments of the main producer countries of the Andean region have given priority support for the cultivation of this crop. For example, the Ministry of Agriculture of Peru launched a strategic plan for the production of quinoa during the period 2013-2021, in which quinoa production is projected to grow 64 000 ha of quinoa in 2016 [2]. Also in Bolivia, quinoa yields are expected to

increase from 1.15 tons to 1.50 tons per ha, and the total production will account for 96 000 ton per year. The Ministry of Agriculture of Ecuador also presented an ambitious plan to expand its cultivated area from 1 500 to 10 000 ha in five years, and to increase the annual production from the 712 tons reported in 2012 to 6 818 tons in 2018 [6].

2.1.3. Products derived from quinoa

At present, it is possible to find in the market several products derived from quinoa which include: insufflates, flours, noodles, flakes, granolas, energy bars, etc. In recent years research has conducted for the development of new products to make the production of quinoa more attractive for the consumer [8]. Such is the case of the removal of oils, starch and saponins from the leaves and seeds of quinoa; as well as the use of seeds the bread industry, pastry and drinks. These product diversity can provide quinoa a great economic potential since they can be used in the food, chemical, pharmaceutical and cosmetic industries [8].

2.1.4. Nutritional properties

Quinoa is one of the foodstuffs that can be considered as nutritionally complete; since it provides an adequate balance of proteins (Table 1). This product is considered as the only food that contains all the essential amino acids in comparison with some other basic food commodities. Apart from high quality protein, quinoa seeds also contain carbohydrates, vitamins and minerals such as Ca (94 mg / 100 g), Mg (250 mg / 100 g), P (384 mg / 100 g), S (150-220 mg / 100 g), Fe (13.2 mg / 100 g), and Zn (4.4 mg / 100 g) (Table 2), which are easily absorbed by the human body [9].

Table 1 Contents of macronutrients in the quinoa and in selected foods per 100 g of dry weight [1,8]

Component	Quinoa	Beans	Maize	Rice	Wheat
Energy (kcal/100g)	399	367	408	372	392
Proteins (g/100 g)	16.5	28	10.2	7.6	14.3
Lipids (g/100 g)	6.3	1.1	4.7	2.2	2.3
Total carbohydrates (g/100 g)	69	61.2	81.1	80.4	78.4

The seeds of quinoa contain vitamin E, which has been associated with antioxidant activity of great importance for human health. Also, this food product contains vitamin A, which aids in the improvement of human vision. In addition, quinoa is comprised of greater amounts of thiamine, riboflavin, niacin and ascorbic acid, than those found in cereals such as maize, rice and wheat.

Some researchers as Tang *et al*. [10] recently found that quinoa contains compounds such as polyphenols and phytosterols with possible application in functional food formulations. It also contains fatty acids such as linoleic, stearic, oleic, among others (Table 2) [2]. The composition of these fatty acids is similar to that of corn germ oil.

Table 2. Comparison of the composition of quinoa oil with other vegetable oils [4].

	Fatty acids (%)						
Species and variety of quinoa	**Stearic** C18:0	**Oleic** C18:1	**Linoleic** C18:2	**Linolenic** C18:3	**Eicosanoic** C20	**Dicosanoic** C22	**Tetracosanoic** C24
Quinoa							
Sajama	0.7	23.8	46.2	9.5	2.8	3	0.9
Porotok	0.7	22.2	55.2	4.3	2.6	2.5	0.6
Imbaya	0.7	26.8	50.6	3.9	2.4	2.5	0.6
Cochasquí	1.2	25	48.6	3.9	2.8	2.9	0.6
Average	0.8	24.5	50.2	5.4	2.7	2.7	0.6
Soybeans	4.4	21.6	55.2	9.4			0.7
Peanuts	2	44.7	35.8		4.2	3.4	1.9
Oliva	2.8	79.4	7.6				
Palm	2.9	18.1	2.9				

Carbohydrates are the most abundant molecules in quinoa grains (Table 3). Starch is the major component of carbohydrates of quinoa with concentrations ranging between 57 and 65% dry weight with a 5% of simple sugars. On the other hand, quinoa has a total content of dietary fiber of approximately 4.1% dry weight. Recently beneficial effects to human health have been attributed to dietary fiber such as the reduction of colon cancer and diseases related to the gastrointestinal transit [5,11].

Table 3. Composition of carbohydrates of some varieties of quinoa [12].

	Quinoa (% of dry weight)		
Carbohydrate	**Red**	**Yellow**	**White**
Starch	57.2	58.2	65.2
Reducing sugars (monosaccharides)	2	2.1	1.8
No reducing sugars (disaccharides)	2.6	2.2	2.6
Crude fiber	2.4	3.1	2.1
Pentosanes	2.9	3	3.6

2.1.5. Protein quality of the quinoa

The nutritional quality of the protein is determined by the ratio of essential amino acids present in the protein. These amino acids cannot be synthesized by the human body and therefore must be provided through the diet. Apart from considering the amino acid profile, there are different methods to assess the quality of a protein as the biological value (BV), net use of proteins (NPU) and the coefficient of protein efficiency ratio (PER) [13].

The PER is based on the calculation of the relationship between body weight gain and grams of ingested protein according to the expression:

The net protein utilization (NPU) is given by the ratio:

$$NPU = \frac{N \text{ absorbed and retained by the test subject}}{Nitrogen\ \ ingested}$$

$$PER = \frac{Body\ weight\ (g)}{Consumed\ protein\ (g)}$$

According to the literature reports, quinoa can be used as an economical and excellent

source of high quality protein because its proteins possess a high PER score (2.11 and 3.32 for two varieties of quinoa), in comparison with that of the casein (2.5) that is used as a reference [13]. Similar results comparing the milk proteins of quinoa were reported by White *et al.* [14] and Cardozo and Zea [15].

Ten amino acids are essential only for children: lysine, isoleucine, leucine, phenylalanine, tyrosine, threonine, tryptophan, valine, histidine and methionine. Quinoa proteins can supply around 180% of histidine, 274% of isoleucine, 338% of lysine, 212% of methionine + cysteine, 320% of phenylalanine + tyrosine, 331% of threonine, 228% tryptophan, 323% of valine, according to the values recommended for adult nutrition by the FAO/WHO/ONU [3]. In comparison with wheat, quinoa contains nearly five times more lysine, more than the double of isoleucine, methionine, phenylalanine, threonine, valine, and higher amounts of leucine; all these essential amino acids.

The relative lack of lysine in the diet is an important concern when consumed cereals such as oats, corn, wheat and grain of rice because these contain concentrations of amino acids lower than those recommended by the FAO and the WHO (Table 4). In contrast, the content of amino acids present in quinoa proteins are within the ranges established for nutrition of children from 3 to 10 years.

Table 4. Comparison of the essential amino acid profiles of the quinoa and other crops as recommended by FAO for children's from 3 to 10 years [2,4,16,17].

Amino Acid	FAO*	Quinoa*	Maize*	Rice*	Wheat*
Isoleucine	3	4.9	4	4.1	4.2
Leucine	6.1	6.6	12.5	8.2	6.8
Lysine	4.8	6	2.9	3.8	2.6
Methionine	2.3	5.3	4	3.6	3.7
Phenylalanine	4.1	6.9	8.6	10.5	8.2
Threonine	2.5	3.7	3.8	3	2.8
Tryptophan	0.66	0.9	0.7	1.1	1.2
Valine	4	4.5	5	6.1	4.4

*g/100 g of protein

2.1.6. Structure of the proteins in the quinoa

Under native conditions, proteins of quinoa are mainly composed of two types of proteins: albumins of type 2S represent 35% of the total protein with molecular weights of 20-25 kDa; and globulins 11S represent 65% of the total protein with molecular masses of 30-40 kDa [18]. Brinegar *et al.* [19] isolated and characterized proteins of quinoa by electrophoresis with molecular weights of around 8-9, 22-23, 32-39, and 50 kDa. Additionally, they characterized some protein subunits of 32-39 and 22-23 kDa that were separated by denaturing. The proteins of quinoa are of the albumin type, which constitute the soluble fraction in water and the globulin type which are soluble in saline solutions [20]. The latter contains high quantities of lysine, methionine, threonine, among other essential amino acids.

2.1.7. Antinutrients

Quinoa seeds contain several saponins and phytates in its natural form which interact with some minerals and proteins thus hampering the absorption of the latter. These compounds also affect the sensory characteristics of foods because they confer a bitter taste and dark coloration. In general, the seeds of quinoa contain saponins in their coat, with the exception of the sweet varieties that do not contain saponins, or contain less than 0.11% [3].

Saponins are the most important antinutricional factor of quinoa, mainly present in the seed coat [3]. These compounds contain strings of sugars and one triterpenoid aglycone (sapogenin) in its structure and are classified according to the number of units of sugars in its structure [5]. Commonly these compounds are found in agricultural crops because they have the natural function of defense from the external environment in the soil. Saponins are considered toxic when they are consumed in large quantities, since they form complexes with sterols, which can affect the assimilation of these by the digestive system or break the cell membranes after being absorbed into the bloodstream. Saponins can be partially removed from quinoa by washing with water [8].

Other antinutritional factors present in quinoa are tannins, some protease inhibitors, and phenolic compounds. The latter are considered antinutrients when the production of high quality protein from quinoa is desired, because these compounds generate undesirable flavors and colors in the isolated of protein [21].

2.2. Importance of antioxidants in the diet

Antioxidants are compounds that reduce or prevent the oxidation of an oxidable substrate, by acting as electron donors. In some cases, these compounds can reverse the oxidative damage of the cells. In this way, these compounds can protect the cells from oxidative deterioration, premature aging, and cancer [22].

Free radicals are normally produced during aerobic cellular metabolism, and are used by the cell in different physiological processes as a defense mechanism against infectious agents. However, these molecules are highly reactive and capable of harming other cell

biomolecules [23].

Exogenous factors can cause the formation of free radicals such as environmental pollutants, UV, consumption of certain foods. The increase of the free radical concentration in the cells cause a phenomenon known as oxidative stress. This stress is associated with various chronic degenerative diseases that affect human health and life expectancy. In this regard, a change in the diet that incorporates foods with high antioxidant capacity can be used as a natural therapy to prevent oxidative damage and the consequent health problems [24].

2.2.1. Natural sources of antioxidants

A diet based on foods such as fruits, vegetables, and some meats like fish and birds, will supply adequate amounts of antioxidants, which can reduce the negative effect of oxidative stress on the human body and increase life expectancy and the quality of life. Many fruits contain compounds such as curcumin, resveratrol (red grapes, peanuts), genistein (soy), lycopene (tomato), catechins (green tea), capsaicin (pepper red), beta carotene (carrots) and dietary fiber. These compounds have the ability to interfere with several routes of cell signaling, which helps in the prevention or control of certain types of cancers [22].

2.2.2. Antioxidants present in quinoa seeds

A number of investigations have reported the antioxidant compounds contained in quinoa because of the important role of antioxidants in human health [23]. The compounds with the greater antioxidant activity in quinoa are polyphenols (quercitin and kaemferol), phytic acid, carotenoids, tocopherols and tocotrienols [10,25,26]. These compounds possess high antioxidant activity and are closely related to the functional properties involved in the protection of the membranes from the oxidative damage caused by cellular processes [10].

2.2.3. Importance of polyphenols in the diet

Polyphenols comprise a group of organic compounds widely distributed in the plant kingdom and are the most abundant secondary metabolites present in plants. Through the antioxidant action and/or the modulation of various functional proteins [23]. In terms of

pharmacological activity, these compounds help the body to retain HDL while helping to eliminate the low density lipoproteins (LDL), thus causing a reduction of cholesterol in blood [27].

The three most important groups of phenolic compounds are flavonoids, phenolic acids, and other polyphenols. According to Dzialo *et al.*[28], phenols are also antioxidants which can trap free radicals, preventing that they join and damage the cell´s deoxyribonucleic acid (DNA), a critical step in the initiation of carcinogenic processes. In addition, different therapeutic properties have been conferred to polyphenols such as anti-ulcers, anticancer and antimutagenics due to the strong antioxidant nature of these compounds [29].

2.2.3.1. Polyphenolic compounds in the quinoa

Quinoa seeds contain several polyphenols; among which the most abundant are flavonoids, phenolic acids and tannins [5]. Zhu *et al*. [30] isolated six flavonol glycosides of seeds of quinoa; which are kaempferol 3-OR-[β-D-apiofuranosyl(1'-2")]-β-D-galactopyranoside, kaempferol 3-OR-[α-L rhamnopyranosyl (1"-2")]-β-D-galactopyranoside, kaempferol 3-OR-[β-D-apiofuranosyl(1'-2")- α-L-rhamnopyranosyl(1"-6")]-β-D-galactopyranoside, kaempferol 3-OR-(2,6-di-α-L-rhamnopyranosyl)-β-D-galactopyranoside, quercetin 3-OR-[β-D-apiofuranosyl (1'-2")-α-L-rhamnopyranosyl(1"-6")]-β-D-galactopyranoside and quercetin 3-OR-(2,6-di-α-L-ramnopyranosyl)-β-D-galactopyranoside. Since these compounds exhibit antioxidant activity, they may serve as good radicals scavenging agents [30]. Gorinstein *et al.* [31] reported that the content of tannins of quinoa (0.51%) was comparable to that of amaranth; the authors also reported the content of phenolic acids in methanol extracts of quinoa: 251.5 µg/g of ferulic acid, 0.8 µg/g of p-cumáric acid, and 6.31 µg/of caffeic acid.

Alvarez-Jubete *et al.* [32] compared the content of phenolic compounds in quinoa, wheat and amaranth. The content of polyphenols in quinoa (71.7 ± 5.5 mg Eq. GA/100 g of d.w.) was the highest compared to that in wheat (53.1 ± 2.8 mg Eq. GA/100 g of d.w.) and in amaranth (21.2 ± 2.3 mg Eq. GA/100 g of d.w.), which presented the lowest polyphenol content.

2.2.4. Antioxidant activity in quinoa

Quinoa contain compounds such as carotenoids, mostly lutein, and tocopherols (γ - tocopherol), as well as polyphenols, which are regarded as antioxidant compounds, with the phenolic compounds to a lesser extent. Various investigations carried out with quinoa (white, red and black) have demonstrated that the darker color of the quinoa seeds, is related to a higher content of antioxidant compounds [10].

Tang *et al.* [10] evaluated the antioxidant activity of tocopherols, tocotrienols and carotenes in the seeds of three varieties of quinoa (white, red and black) and their contribution to the antioxidant activity. In this study, the concentration of antioxidant compounds was higher in the black seeds ($p > 0.05$), followed by the red and the white quinoa seeds. The total content of tocopherol ranged from 37.49 to 59.82 µg/g and consisted mainly of γ-tocopherol. The black quinoa had higher concentrations of vitamin E followed by the red and the white quinoa. The authors reported the presence of the carotenes, trans-lutein (84.7 to 85.6%) and zeaxanthin, for the first time in the seeds of quinoa; with the black seeds presenting the highest carotene concentration. The antioxidant activities of the lipophilic extracts were positively correlated with polyunsaturated fatty acids, total carotenoids and total tocopherols.

The content of total phenolic compounds in seeds of quinoa determined by Miranda *et al*.[33] in 6 Chilean ecotypes ranged from 14.22 to 65.53 mg Eq. GA/100 g of dry weight. The ecotype Faro presented the highest content of phenols and in turn the best result in antioxidant activity (lower EC_{50} value with 461.89 µg/mL). The variety of quinoa as well as color and location are three of the most important factors by which the content of phenolic compounds can be affected as well as the impact on antioxidant activity.

On the other hand Hirose *et al.* [23] evaluated the antioxidant properties and composition of flavonoids quinoa seed grown in Japan which were compared with various pseudocereals (buckwheat and amarantus). These authors isolated and identified four flavonol glycosides present in quinoa: agliconas, 3- glucosil, 3-galactosil, 2-ramnosil. The quinoa seeds presented greater antioxidant activity than the other pseudocereals studied.

2.3. Processes for separation of the quinoa components

The development of techniques for the separation and isolation of natural products from plants, microorganisms and marine organisms has helped to expand the market of functional foods. In general, modern strategies to achieve this separation and purification are based on chromatography, and spectrophotometry. These techniques have allowed to obtain compounds of interest at the analytical level; however, the current market requires the production of these valuable compounds in greater quantities for use in the food and pharmaceutical supply of the population [34].

Current techniques such as "membrane technologies ", are being used for the separation of different components from natural products such as seeds of canola, mustard, etc. This is because they have a series of advantages such as the high efficiency, simple equipment, low power consumption, etc. With regard to other methods this technique is used as a primary treatment or as a process for product refinement and concentration of the compound or group of compounds of interest [34].

2.4. Membrane separation processes

Techniques such as micro, ultra and nanofiltración have been used to concentrate and purify bioactive compounds and molecules from its biological source. Taking into account that not all natural products can be economically obtained through total chemical synthesis, many of them have to be extracted and separated from their natural sources. These processes are often tedious and consume a lot of time and energy [34].

Advances in the technology and the manufacture of membrane materials have led to the consolidation of membrane filtration techniques as a dominant separation technology since the beginning of 1990. The basic principle is that membrane is a physical barrier between two fluids (one on the side of the feed and the other on the side of the product) that allows, in a selective manner, the passage of certain components of the feed. The feed (F) that passes through the membrane is called permeate (P) and the one that is withheld in the side of the feed is called retentate (R), (Figure 2). The basic equipment needed for the

separation is the membrane ,the module that contains the two fluids [35], and a peristaltic pump which provides the pressure difference to achieve the separation.

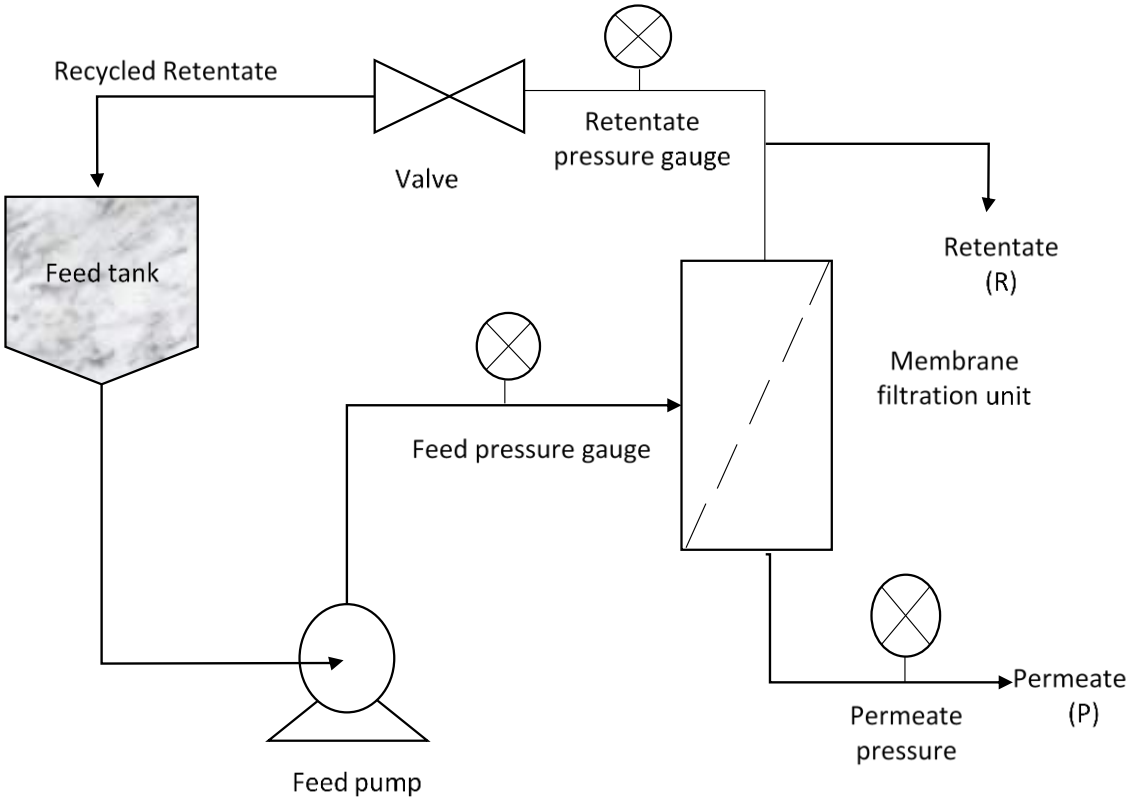
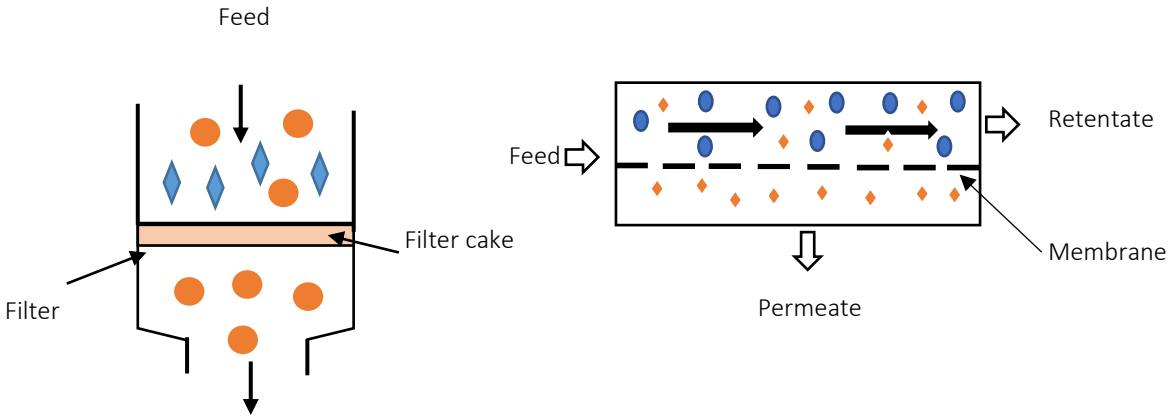


Figure 2. Schematic representation of a membrane system

The membranes have two forms of operation. (a) Front filtration (Dead-end filtration) where the flow occurs perpendicular to the membrane surface (Figure 3a); in this type of process a gel is formed on top of the membrane, which has to be eventually removed, so that the process is not continuous. b) Tangential-flow filtration (Cross-flow filtration) in which the liquid circulates permanently tangentially to the surface of the membrane (Figure 3b). The velocity of circulation and pressure through the membrane are the most important parameters in the operation of a membrane filtration process. In this type of filtration, the continuous tangential flow through the membrane surface, together with some flow turbulence, prevents the accumulation of matter on the membrane surface (cake in the membrane surface), thus allowing this system to be operated in a continuous manner.



a) Front membrane b) Tangential membrane

Figure 3. Membrane separation methods. (a) Front filtration b) Tangential filtration

The equations that describe the physical processes in membrane filtration are based on the assumption that the probability for a particle to pass through the membrane is higher (p = 1) for solutes with 0% rejection. On the contrary, the probability is null (p = 0) for solutes that are fully rejected (100 %) by the membrane. Therefore, the rejection coefficient (R) parameter is defined as:

$$R = \frac{C_R - C_P}{C_R} = 1 - \frac{C_P}{C_R} \qquad (Ec.1);$$

where:

C_R: Retentate concentration

C_p: Permeate concentration

If a solute permeates freely through the membrane, its concentration on each side shall be equal ($C_P = C_R$), then R = 0. As the solute filtration takes place and the solvent passes through the membrane, the volume of the feed is reduced. The volume concentration factor (CF) is given by the following equation:

$$CF = \frac{Vo}{V_R} \qquad (Ec.2);$$

where V_0 is the initial volume of the feed and V_R is the volume retained after filtration.

Assuming that the permeability is constant throughout the process, the concentration of a solute at any time or phase is a function of the concentration factor and the rejection coefficient given as:

$$\frac{C_R}{C_O} = (CF)^R \qquad (Ec. 3),$$

which subsequently becomes:

$$In\left(\frac{C_R}{C_O}\right) = R\ In\ CF$$

with the slope of the resulting straight line representing the average rejection. This equation allows the calculation of the rejection coefficient from the solute concentrations and volume measurements during a filtration process.

There are a wide variety of membrane separation processes which include clarification, concentration, buffer exchange, solvent exchange, purification and sterilization. Membrane processes can be categorized as microfiltration, ultrafiltration, nanofiltración, reverse osmosis, pervaporation and diafiltration. Several methods are used as a driving force for achieving the separation; for example, pressure differences, concentration gradients, and electrical or chemical potential [34].

According to the sizes of particles present in the feed, membranes of micro, ultra and nanofiltration are used.

Three different groups of solutes can be identified according to their molecular size:

- Solutes (size of less than 1 nm), include ions, salts, organic acids and phenolic compounds.

• Colloids (range in size between 1 nm and 1 µm), as polysaccharides, proteins, polymerized phenolic compounds and colloidal aggregates.

• Particles (size greater than 1µm), include microorganism (yeasts and bacteria), cell debris,

colloidal aggregates and crystals.

Table 5 summarizes the spectrum of solute separation related to the solute molecular size for the different membrane processes.

Table 5. Properties of separation system with membranes [36,37].

	Membrane separation process			
Concept	**Microfiltration**	**Ultrafiltration**	**Nanofiltration**	**Reverse Osmosis**
Separate Materials	Separation of particles, bacterium.	Separation of macromolecules	Ions and molecules	Desalination of water
Membrane Material	Organic and inorganic materials (polyacrylamide, ceramics)	Organic and inorganic materials (polyacrylamide, ceramics)	Organic and inorganic materials (polyacrylamide)	Organic and inorganic materials (cellulose triacetate)
Equipment	Plates and frames with spiral	Spiral hollow fiber, plates and frames	Spiral Hollow Fiber	Spiral Hollow Fiber
Principle of separation	Sieving Mechanism	Sieving Mechanism	Solution – Dissemination	Solution - Dissemination
Pressure	< 2 bar	2-10 bar	10-60 bar	10-100 bar
MWCO or pore size	<0.45 µm	5-500 kDa	100-1000 Da	50-100 Da

MWCO: molecular weight of cut-off

2.4.1. Protein isolation

Protein isolates are defined either as products obtained by extraction, purification and recovery of proteins or as products that contain more than 90% of protein. The traditional techniques of alkaline extraction and isoelectric precipitation can be used for protein separation from solid raw materials; however, in these processes antinutritional compounds are formed, thus considerably reducing the purity of protein concentrates. Therefore, membrane processes can be implemented in the traditional extraction method to efficiently obtain isolated proteins with high purity from solid raw materials. This

procedure gives rise to two isolated protein products of canola, both rich in protein and low in phytates [38].

A process for protein isolation using membrane technology has been developed by the Food Engineering group at the Department of chemical engineering and applied chemistry in the University of Toronto. The process was originally developed for canola seeds and consisted of five main stages: alkaline extraction, isoelectric precipitation, diafiltration, ultrafiltration, and drying. The ultrafiltration and diafiltration stages serve to concentrate and purify the soluble protein that is retained after the precipitate is removed.

The process was modified to produce protein isolated from China rapeseed with high purity (90 to 100%) [39]. After that, the process has been applied to the yellow mustard obtaining two isolates with high protein content (> 85%) and non-detectable levels of glucosinolates and phytates [40]. Additional work with canola by this research group include the removal of phenolic compounds from the protein isolates for the purpose of protein purification.

When a protein isolate is applied in a food system, its quality is based not only on the nutritional value, but also on the technological-functional properties and sensory attributes of the isolates. Among the functional properties of proteins are capacity of emulsification, the gelling, the ability of foam formation and stability. The sensory characteristics to take into account in a food system include color, taste, aroma and texture [41].

The market of protein isolates has been very successful in many applications, including processed meats, special foods such as tofu, and other alternatives to animal proteins, with beverages such as soy drinks. Other potential applications of these isolates are in nutritional supplements, infant formulas, protein bars, drinks, confectionery bars, and soups. In addition they can also be used as animal protein substitute in vegetarian products, for example in hamburgers, sausages, etc. [42].

2.4.2. Ultrafiltration

Ultrafiltration (UF) is the procedure most commonly used for the separation of macromolecule components in a mixture. The particle size of species typically rejected by UF membranes include oligomers, biomolecules, polymers and colloidal particles. The driving force for transport across the membrane is the pressure difference. The UF process operates in the range of 2-7 bar, although in some cases pressure differences of 20-30 bar are used. UF membranes can reject molecules within the range of molecular weight cut-offs of 2,000 to 200,000 Da [43] and according to a commercial supplier, the available range is from 5 to 500 kDa. The selection of the MWCO of the membrane depends primarily on the size of the molecule to be separated; for example, hormones, albumins, hemoglobins, enzymes, colloids, etc. [44].

In comparison with mechanical separations, the membrane separation involves high purity, low energy cost, no use of additives, smooth operation conditions, high separation efficiency and easy scaling. On the other hand, a disadvantage of membrane processes is the problem of flux reduction by membrane fouling. Different strategies can be used to prevent membrane fouling and more efficient membranes have been developed to reduce fouling [45].

2.4.3. Nanofiltration

Nanofiltration (NF) is a membrane technology which uses a high-pressure difference with the same principles that reverse osmosis. The main difference is the degree of retention that can be accomplished with these membranes, operating in a range of molecular weights cut-offs of 100 to 1,000 Da [37].

Nanofiltration can be used in applications such as solvent exchange, color removal, organic compounds concentration and purification, desalination. This technology has been used in the dairy industry to recover lactose, and eliminate nitrates and solids in the recovery of whey proteins [46]. Another of the applications of nanofiltración in the food and beverage industry, is the desalination of gelatine to improve its shake and clarification

properties[47]. Additionally, it has used this technology for wastewater recycling and disposal of pesticides [48].

The majority of the NF membranes are made of polymeric materials hydrophilic such as polyethersulfone, polyamides and derivatives of cellulose, which work very well in aqueous system. However, these membranes lose stability and provide a poor performance when organic solvents are used. At present new membranes, have been developed which are made of different polymeric materials with greater stability and resistant to organic solvents. With this NF membranes it is possible to extend the range of applications in the chemical industry, mainly in the area of organic synthesis, and separations of organic compounds [35].

2.4.4. Methods for the separation of proteins and phenols with membranes

The recovery of proteins and phenolic compounds by means of membranes have taken great importance in recent years, because membrane technologies allow the processing of oil seeds to be economically more attractive. The phenolic compounds, glucosinolates and phytates are compounds which can be found together with proteins in some oil seeds. These in turn limits the quality of the protein of the seeds when used as ingredients in food systems, since the protein-associated compounds can be responsible for the astringency and dark color of the isolated proteins.

On the other hand, recent research indicates that phenolic compounds have potential applications as chemoprotectants and antioxidant agents, either as part of a dietary or a pharmacological regime. The recovery of phenolic compounds has been achieved using NF membrane processes to recover polyphenols from the wastewater produced by the process of yellow mustard protein extraction [49].

2.5. Processes reported for the separation of proteins with membranes

To carry out the separation of proteins from mustard seeds, Diosady and collaborators at the University of Toronto [40] conducted primarily an alkaline extraction at pH 11. Subsequently, the supernatant was taken to an ultrafiltration step with a concentration factor of 4, followed by an isoelectric precipitation at pH 5 (Figure 4). After centrifugation of the extract two protein fractions were obtained: precipitated protein isolate (PPI) and soluble protein isolate (SPI) and the meal residue. The SPI isolate is obtained through the use of ultra and diafiltration; whereas the PPI and the meal residue are obtained simply by washing and centrifugation. The final products are obtained by lyophilizing of the protein concentrates. Similar methods have been employed in Diosady´s group for the isolation of proteins from rapeseed and canola seeds [49–51]. To obtain of high quality proteins, the products have to be free of phenols, glucosinolates, phytates and fiber [3,30,52].

Dendukuri and Diosady [53] reported the evaluation of the process of protein extraction from fat-free dehulled mustard using various treatments. Two membrane processes were assessed: microfiltration (Millipore, pore size of 0.1, 0.45 and 0.65 μm) and ultrafiltration (MWCO 10 kDa) with membranes made of polivinilidenefluoride (PVDF) polysulphone and ceramics, respectively. The study focused on the capability of the processes to obtain protein concentrates and remove the antinutritional compounds and peptides. Proteolytic enzymes were used to break-up the aggregates and allow their passage through the membrane. The authors reported that the best process could recover 91% of the mustard protein. The final protein product showed a reduction in the concentration of glucosinolates, phenols and compounds.

In a similar work, Marnoch and Diosady [40] evaluated a separation process of proteins from mustard seeds using ultrafiltration membranes and diafiltration. A 5 kDa Millipore Pellicon system membrane cartridge made of cellulose was used. With this procedure, the authors reported a protein recovery of 81% comprise into three fractions: 47.3% in the precipitated protein isolate (PPI), 3.8% from soluble protein isolate (SPI), and 29.9% in the meal residue. Xu *et al.* [51] evaluated two membrane processes to purify proteins from

yellow mustard flour. Millipore DIAFLO membranes were used H1P10-20 (Millipore Ltd., Oakville, ON) with a molecular weight cut-off of 10 kDa and an area of 0.05 m^2. The authors reported a protein recovery of more than 85% in the form of two protein isolates. Both isolates contained more than 85% protein and less than 0.5% of phytates and virtually free of glucosinolates.

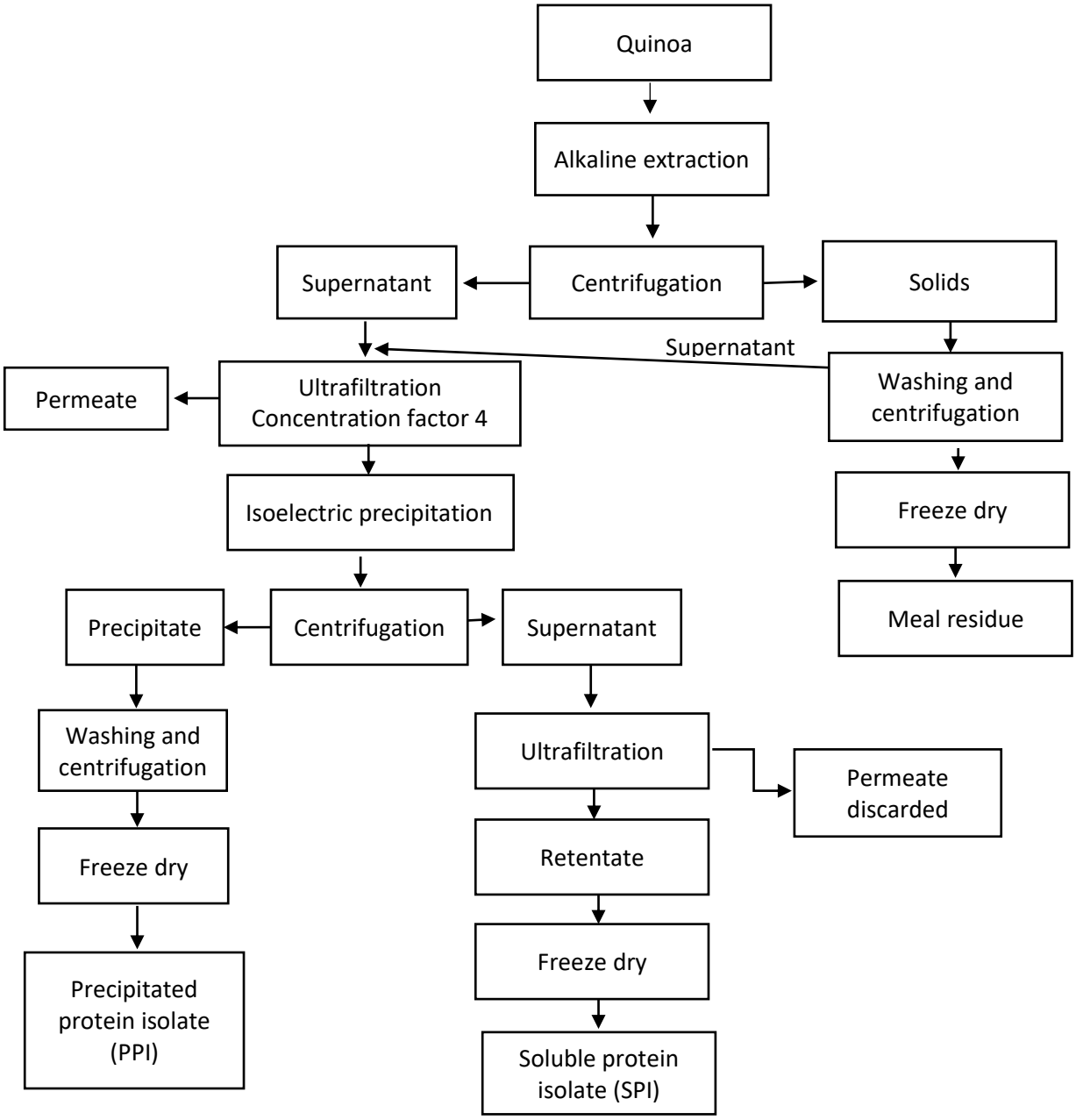


Figure 4. Protein isolation flowchart [40].

2.6. Removal of phenolic compounds

The removal of polyphenols from a solid raw material by using a solid-liquid extraction consists of the transport polyphenolic compounds from the matrix of the plant material to the cell wall, followed by its dissemination to the external solvent medium. Nawas *et al.* [54] reported the extraction of phenolic compounds from grape seeds using ultrafiltration membranes of pore sizes of 0.22 and 0.45 µm. The authors reported a considerably high polyphenols recovery which accounted for 11.4% of the total weight of the seeds; an initial concentration of phenol compounds in the seed of 0.5 mg/mL it was possible to recover 0.057 mg/mL of these compounds with the membrane of pore size of 0.25 µm. Despite the interesting properties of quinoa seeds, to date it has not been reported in the literature a process for the separation of the phenolic compounds from the raw seeds using ultra and nanofiltración membranes.

2.6.1. Use of membranes of nanofiltración for the separation of phenolic compounds

The use of concentration membrane processes (Figure 5) has been used in the literature because nanofiltration membranes provide some process advantages with respect to conventional processes, such as: low temperatures, absence of phases transition and low power consumption.

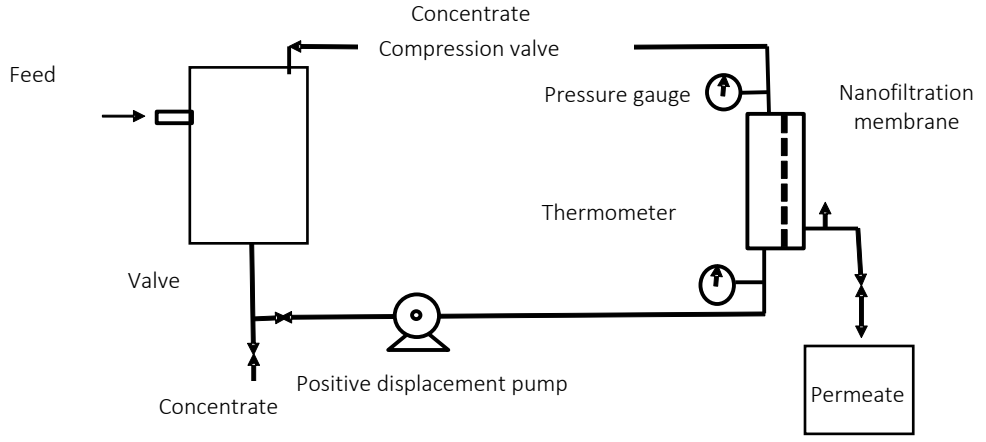


Figure 5. Schematic diagram of the nanofiltration process for solute concentration [55].

Xu and Diosady [56] reported that phenolic compounds provide dark color and undesirable flavors to canola protein isolates in plant tissues, these compounds form complexes with proteins. These authors developed a process to remove from 80 to 90% of the phenols present in canola seeds using an Amicon DIAFLO H1P10 ultrafiltration membrane with a nominal molecular weight cut-off of 10 kDa and a membrane area of 0.06 m^2.

In a further work, Prapakornwiriya and Diosady [49] developed an UF and NF membrane procedure to recover sinapic acid (a phenolic compound) from a wastewater stream of the processing of yellow mustard protein isolation. The UF and NF membranes had MWCOs of 10 kDa and 1000 Da, respectively. The authors reported high percentages of recovery of sinapic acid (95%) from the residual protein-isolation stream.

Similarly, Mello *et al.* [55] used a nanofiltration membrane system to separate flavonoids and phenolic compounds from propolis using NF90 membranes (Osmonics, USA) composed of polyamide and polysulphone with a MWCO 200 Da and a pressure range of 2-6 bar. The authors reported that the membrane retained about 94% of the phenolic compounds and 99% of the flavonoids present in the feed. These concentrations were considerably higher with respect to those obtained with an ethanol extraction that was used as a reference, where recoveries of 53% and 90% were obtained for phenolic compounds and flavonoids, respectively. Considering the above, the nanofiltration process proved to be a highly efficient procedure for the concentration of phenolic compounds.

A relatively new nanofiltration process was reported by Sereewatthanawut *et al.* [57]. The authors reported the fortification and refining of rice bran oil using nanofiltration membranes to separate the glycerides and γ-oryzanol. A cross-flow filtration system was implemented nanofiltration membranes STARMEN ™ 122 and 240, DuraMem ™ 300 and 700 and METD.S. 1000 and 1000X with a MWCO range of 200-1000 Da and nanofiltration pressures varying from 5 to 30 bar. In this study an increase in the amount of γ-oryzanol in the treated oil from 0.95 to 4.1 % (w/w) was reported in addition to a two-fold increase in the antioxidant capacity of the oil. This research demonstrated the potential of nanofiltration to enrich and refine phytochemical compounds in hydrophobic systems.

3. CONCEPTUAL FRAMEWORK OF THE THESIS

3.1. Problem statement

Nowadays there is the global need to include high-quality protein in the daily diets of the world population. However, it results especially difficult to achieve this goal in the case of the population with low income and in poverty, conditions that prevail in the developing countries. People under these socio-economic conditions rarely consume protein of animal origin and must obtain proteins from cereals, legumes and other grains. Even when the energy intake of these foods may be adequate, insufficient concentrations of essential amino acids can cause malnutrition. An alternative to tackle the problem of essential amino acid deficiency is to identify and exploit grains which are rich in proteins of high nutritional value.

Quinoa (*Chenopodium quinoa* Willd) is a pseudocereal that has not been fully exploited yet, despite having a great potential as a food of high nutritional value. Recently, quinoa grains have been recognized as a complete food due to the quality of its proteins, the high quality of quinoa proteins is derived from their superior balance of essential amino acids. An optimal utilization of the proteins quinoa, should imply the absence of substances that interfere with their biological assimilation.

Considering the above and the literature review, in this thesis an ultrafiltration is used to produce quinoa proteins of high quality. In addition, with the use of this technology it is intended to eliminate the antinutritional factors while preserving the quality of the protein.

On the other hand, it has been reported that quinoa contains important concentrations of phenolic compounds that have recently been associated with beneficial effects on the human health. The polyphenolic compounds are obtained as a by-product during the separation process of proteins, in this work, it is also proposed to obtain these compounds using nanofiltration membrane technology.

3.2. Justification for the study

The quinoa products obtained from the membrane processes developed in this work can be beneficial to human nutrition and health, this project will contribute to give an added value to the quinoa seeds; therefore, the use and explotation of the pseudocereal. This in turn, will benefit the producers, processors and people dedicated to the grain commercialization, contributing to improve the economic situation these people and their families.

In addition, considering that quinoa can be cultivated under different climatic conditions, there is the possibility of the implementation of a national agriculture program for the cultivation of this crop. A rational exploitation of this crop can provide important biological products, such as proteins, essential oils and polyphenols compounds. The production of quinoa can help to reduce the serious problem of nutrition in some region of México.

3.3. Originality

Quinoa seeds have an important content of protein and biological compounds which are of vital importance for human nutrition. As mentioned above there are various techniques for the separation of these compounds, which are effective but have some major constraints. For this reason, in this work integrated processes will be used to obtaining protein isolates and concentrates of polyphenols from quinoa grains. The processes include alkaline extraction, isoelectric precipitation, ultra and nanofiltration. According to the literature, there are no reports of protein purification processes from quinoa grains, as well as the recovery of phenolic compounds using membrane technologies. Considering the above, this thesis presents an original work which is scientific and technological relevant worldwide. The results of this work can also provide the milestone for the development of new processes of commercial and industrial importance at national and international level.

3.4. Project Objectives

General Objective

Protein isolates and concentrates of polyphenols from black quinoa seeds using membrane technologies of ultra and nanofiltration.

Specific Objectives

1. Proximal analysis, content of bioactive compounds (polar and non-polar), and their antioxidant activity of black quinoa flour.

2. Obtain of protein isolates from quinoa seeds using alkaline extraction and ultrafiltration processes.

3. Separation and recovery of phenolic compounds present in the grains of black quinoa using nanofiltration processes.

3.5. Goals

The goals of this thesis are related to the specific objectives stated in the previous section.

Objective 1

1.1. Perform the proximate analysis of the quinoa grains.

1.2. Perform the quantification of the bioactive compounds content (polar and non-polar), as well as their antioxidant activity of the quinoa grains.

Objective 2

2.1. Implement an alkaline extraction system for the extraction of protein from quinoa flour.

2.2. Implement an ultrafiltration membrane system for the separation and isolation of proteins from the quinoa flour.

2.3. Analyze the protein content of isolates.

Objective 3

3.1. Implement a nanofiltration system for the separation and concentration of phenolic compounds present in the quinoa flour.

3.2. Analyze the content of phenolic compounds obtained in the nanofiltration process.

4. METHODOLOGY

The methods mentioned below were carried out on the basis of the goals and activities established for the fulfilment of the specific objectives.

4.1. Characterization of the quinoa

4.1.1. Obtaining and sieving of samples of quinoa

Natural black quinoa with the trademark Products de los Andes from Peru, was purchased with a local retailer. Quinoa grains were ground in a Cyclotec ™ 1093 cyclonic mill (FOSS, Spain). Following this, the powder was screened through a stainless-steel mesh No. 20 with a nominal particle diameter of 500 µm and stored in a fresh and dark place for further analyses and processing.

4.1.2. Degreasing of the quinoa flour

Batches of approximately 60 g of de-hulled quinoa flour were defatted with hexane for 24 h using a Soxhlet extractor, and air dried under a fume hood overnight for desolventizing. The combined batches of hexane defatted yellow mustard meal produced a single lot of meal that was used throughout this study.

4.1.3. Proximal analysis

This analysis consisted in the determination of the content of moisture, fat, protein and total ash present in quinoa seeds. All determinations were made in triplicate to obtain the data that are presented below.

a) Moisture

Moisture was determined using the method 925.5 of the AOAC [58]. One g of quinoa flour as weighted in crucibles previously dried and tared. The samples were then placed in an oven at 105 °C for 24 h. After this time, the samples were brought to a desiccator during 20 min for its cooling under anhydrous conditions and were weighed. The calculation of the moisture content of the sample was carried out using the following equation:

$$\%\ humidity\ = \frac{Ww - Wd}{Ww - Wi} \qquad (Ec. 4)$$

Where:

Ww= weight of tray with wet quinoa powder

Wd= weight of tray with dry quinoa powder

Wi= weight of empty tray

b) Determination of the crude fat content by the Soxhlet method

The crude fat content of the quinoa samples was determined by the Soxhlet method according to the NMX-F-089-S 1978 [59]. A round flask with boiling pearls was placed in the oven at 100 °C until constant weight (approximately 2 h). After this, 3 g of quinoa flour was placed in a cartridge of cellulose, and the cartridge was placed in the extractor. Then, the round flask was connected to the extractor, the cartridge was placed in a cylindrical tube which is connected to the refrigerant. Following that, two loads of solvent (ethyl ether) were added through the coolant and the flask was heated to gently boiling, while water was simultaneously circulated by the coolant. After some time, the condensed solvent was obtained with a frequency of about 2 drops per second. The extraction was carried out during 4 h. After all the fat was extracted, the cartridge was removed and the flask continued boiling until the elimination of the solvent. Subsequently, the flask was removed and the extract was dried in the oven at 100 °C for 30 min. After this time, the extract was cooled in a desiccator and its weight was recorded. Calculations were made to calculate the percentage of crude fat according to the following equation:

$$\%\ Crude\ fat = \frac{W_2 - W_1}{M} x\ 100 \qquad (Ec. 5)$$

Where:

W_2 = mass in grams of flask and extracted fat

W_1 = mass in grams of empty flask

M = mass in grams of the sample

c) Determination of protein nitrogen by the Kjeldahl method

For the determination of protein nitrogen was used the method of AACC International Approved Methods of the American Association of Cereal Chemist, AACC Method 46-12, Crude Protein—Kjeldahl Method, Boric Acid Modification

For solid samples weight ~0.2 g of each sample on a nitrogen free paper or for liquid samples weight 5-30 g into each digestion tube; for solid samples place a clean nitrogen free paper in the blank tube or for liquid samples weight 5-30 g of distilled water in the blank tube; later add 4 Kjeldahl Tablets (3.5 g K_2SO_4, 0.175 HgO per Tablet) and 25 ml concentrated H_2SO_4 to each tube. Clamp the suction manifold onto the digestion tubes. Insert the suction tube into the end of the manifold and a tuft of glass wool into the other to allow air passage through the manifold. Turn on the tap water of the aspirator.

Place the connected tubes onto the digestion unit. Heat the tubes at setting 4 during 20 minutes or until the foam subsides. Raise the temperature to setting 6 during 10 minutes or until the foam subsides and the air in the tubes show mist. Then turn the setting up to 10 and digest for 35 minutes ensuring that the walls of the glass are clean and that the solution is color less or very pale yellow for at least 30 minutes before taking off the heat. Later remove the tubes from the digester and place in a rack with the suction continuing until the solution is cool (~ 30 minutes). Then remove the suction tube and place the rack in a fume hood to finish cooling. Remove the glass manifold. Rinse the manifold with water and leave it aside to air dry.

Add 50 ml of distilled water to each tube and stirring until the precipitate is dissolved. Then add 25 ml of sodium thiosulfate solution (8% $Na_2S_2O_3 \cdot 5H_2O$) to each tube and stirring. Cover the tubes and cool before proceeding with the distillation procedure. For the distillation, turn on the Büchi Distillation Unit K-350 and the cooling water line. Wait until the

equipment warms up. Label 4, 500 ml Erlenmeyer flasks (one per sample and one blank). Add 60 ml of 4% (w/v) boric acid and three drops of N-point indicator to each flask, when the machine is ready, rinse for 2 minutes using distilled water in a clean tube; replace the water tube with the blank tube. Place the blank labeled Erlenmeyer flask in the distillate outlet of the unit. Make sure the outlet tube is as far below the surface of boric acid solution as possible.

Add 90 ml of 32% NaOH solution by pressing the reagent button or until the total solution volume is around 180 ml, set the distillation time to 5 minutes and start the distillation, when the instrument is finished replace the current tube with the next sample tube. Replace the current Erlenmeyer flask (rinse off any liquid from the straw into the flask using distilled water) with the corresponding sample flask in the distillate outlet.

Repeat steps 14 to 16 for the remaining samples and titrate the boric acid solutions in the Erlenmeyer flasks with 0.10 N H_2SO_4 from green color to the same pink shade like that in the blank.

Calculate the protein content of the sample using the following equations:

$$\%\ Nitrogen = (0.14)\frac{(m\ sample - m\ blank)}{m}$$, for solid samples

$$\%\ Nitrogen = (0.14)\frac{(Vsample - V\ blank)}{V}$$, for liquid samples

$$\%\ Protein = (\%\ Nitrogen)(6.25) \qquad (Ec.6)$$

Where:

V_{sample}= is the volume of titrant used for the sample (mL),

V_{blank}= is the volume of titrant used for the blank (mL),

m = is the sample weight (g), and

V = volume of liquid sample (mL)

The conversion factor 6.25 was chosen since it was assumed that oilseed proteins have 16% nitrogen.

d) Quantification of total ash

The quantification of total ash was performed using the gravimetric method AOAC 923.03 [60]. A clean porcelain crucible was dried in an oven at 125 °C for two hours to a constant weight. The dried crucible was placed into a desiccator and was left to cool down to ambient temperature. The mass of the crucible was measured in an analytical balance. After this, 2 g of the sample were placed in the crucible and the weight was measured in the analytical balance. Then, the crucible was heated in the oven at 125 ± 5 °C during 24 h and cooled down in the desiccator for 20 min. Following this, the weight of the crucible with the sample was recorded. The sample was then burned using a Bunsen burner and the crucible was subsequently introduced into a muffle furnace to 550 ± 25 °C for approximately one hour. Then, the crucible was removed from the muffle and introduced into an oven at 125 ± 5 °C during 15 min. After this, the crucible was left to cool down to ambient temperature in the desiccator. Finally, the weight of the crucible with the calcined sample was measured in the analytical balance.

Calculations:

$$\%\, Total\ ash\ = \frac{C - A}{B - A} X\ 100 \qquad (Ec. 7)$$

Where:

C = weight of the empty crucible

A = weight of the crucible and the dry sample

B = weight of the crucible and the calcined sample

4.1.4. Bioactive Compounds and Antioxidant Activity

a) Quantification of total flavonoids

The determination of total flavonoids in quinoa seeds was performed following the method reported by Lee *et al.* [61].

Preparation of solutions.

- 10% (w/v) $AlCl_3$: In a volumetric flask, 1 g of $AlCl_3 \cdot 6H_2O$ was dissolved in 10 mL of deionized water and the solution was degasified.
- 5% (w/v) $NaNO_2$: In a volumetric flask: 0.5 g of $NaNO_2$ was dissolved in 10 mL of deionized water to a final volume of 10 mL, and the solution was degasified.
- 1 M NaOH: In a volumetric flask, 0.4 g of NaOH was dissolved in 10 mL of deionized water and the solution was degasified.

Rutin standards: 50, 100, 150, 200 and 250 ppm. A stock solution 100 ppm was prepared with the standard rutin for which 0.01 g of rutin was dissolved in degasified methanol to a volume of 10 mL rutin solution standards were prepared using the stock solution and methanol as solvent.

Extraction

An amount of quinoa flour (0.5 g) was suspended in 0.83 mL of distilled and degassed methanol. The mixture was sonicated for 30 min at ambient temperature. Subsequently, the mixture was centrifuged for 4 min at 3000 rpm; and the supernatant was filtered through cotton-wool.

Quantification. The methodology reported by Lee *et al.* [61] was used in this work. The extract obtained previously took 0.25 mL. A volume of the extract (0.25 mL) was mixed with 0.25 mL of 5 % (w/v) $NaNO_2$. The mixture was agitated for 1 min in a vortex. After this, 0.5 mL of 10 % (w/v) $AlCl_3$ was added and the mixture was stirred for 1 min in a vortex. Subsequently, 0.5 mL of 1 M NaOH was added and the solution was mixed in a vortex for 1 min. For the final quantification, 110 µL aliquots were taken of samples and standards, and

read in a ELx808 microplate reader (BioTek, China) cut a 490 nm. From the standard solution measurements, a calibration curve was constructed with rutin concentrations in the range of 50 to 250 ppm. The content of total flavonoids in the extracts of samples of flour was determined using the calibration curve and was expressed in mg equivalent of rutin (Eq. Rutin) per 100 g of dry weight of the sample. The measurements of the standards and samples were carried out in triplicate.

b) Quantification of total phenolic content

Total Phenolic Content (Xu & Diosady) [50]

- **Acetone extraction**

2 g of sample were refluxed with 50 mL of 60% acetone (acidified to pH 3 with $C_2HCl_3O_2$) at 90°C for 30 min. After cooling down, the mixture was centrifuged for 10 min at 1800 x g. The supernatant was collected and the precipitate was refluxed and centrifuged again for a total of 3 times. The supernatants were combined and the acetone was evaporated at 50°C under vacuum.

- **Alkaline hydrolysis**

The acetone-free extract was treated with 20 mL of 4 M NaOH under N_2 for 4 h. The resultant solution after alkaline hydrolysis was acidified to pH 2 with concentrated HCl.

Additionally, the residual meal after acetone extractions was treated with 20 mL of 4 M NaOH under N_2 for 4 h. The mixture was also acidified with concentrated HCl to pH 2 and centrifuged (10 min, 1800 x g).

The supernatant was combined with the acidified extract obtained earlier. The combined extract was diluted to 200 mL.

- **Ethyl acetate/diethyl ether (EA/DE) extraction**

An aliquot of 25 mL of the diluted acidified solution was extracted 6 times with 50 mL of a mixture 1:1 (v/v) of EA/DE. The organic extracts were combined and evaporated to dryness

at room temperature under the fume hood. The extracted phenolic acids were then redissolved in 50 mL of MeOH.

Finally, the phenolic acid content of the MeOH solution was determined spectrophotometrically by Folin method. Five mL of MeOH solution was diluted to 100 mL with reverse osmosis water. Seven mL of the dilute solution where mixed with 0.5 mL Folin reagent, and after 3 min 1 mL of saturated Na_2CO_3 was added. The phenolic content in the sample was expressed as mg of sinapic acid equivalents per 100 g sample based on a calibration curve of sinapic acid.

To carry out the characterization of the quinoa flour was used as the standard gallic acid as it is the one that is mostly reported in the literature, this method is presented in Appendix A2.

c) Quantification of total carotenes

The determination of total carotenes in the samples of quinoa flour was carried out following the methodology reported by Wrolstald *et al.* [62]. An amount of quinoa flour (1 g) was mixed with 2 mL of water. Subsequently, 5 mL of hexane and 5 mL of acetone were added to the quinoa suspension. The mixture was stirred in the vortex during 4 min. The liquid was decanted into a vial and the solids were washed with the same initial volumes of fresh acetone and hexane, and the extraction procedure was repeated. The decanted liquid was placed in a separating funnel and left to stand for 2 min, after which the aqueous phase was discarded. Hexane was added to the organic phase to a final volume of 25 mL, the absorbance of the organic solution was read in a Perkin-Elmer Lambda 35 UV-Vis Spectrophotometer at 450 nm. The sample determinations were carried out in triplicate. To calculate the concentration of total carotenes (Appendix A3), the following equation was employed:

$$carotenes = \left(\frac{A_{450}}{258.84 \frac{mL}{mg}} x\, Vol\ disolution \right) * 100 \quad (Ec. 8)$$

Where:

A_{450} = absorbance of the sample at 450 nm.

Vol $_{dissolution}$= total volume of the hexanic extract.

d) Quantification of the antioxidant activity

The antioxidant activity of the extracts was determined by the method reported by Julián-Loaeza *et al*. [63] for the quantification of the radical scavenging activity using the radical DPPH•.

Preparation of solutions. A 0.1% (p/v) DPPH• stock solution was prepared by dissolving in a volumetric flask 10 mg of DPPH• in MeOH to a volume of 10 mL in the absence of light. From this solution, a 0.004% (w/v) working solution was prepared in the absence of light. The solution was kept in refrigeration and protected from light until its further use.

c: 0.5, 1.0, 1.5, 2.0 and 2.5 ppm. A stock solution of 100 ppm of gallic acid was prepared with 1 mg of gallic acid in a volumetric flask and after adding 90% MeOH until reach a volume of 10 mL. The solution standards were prepared from the stock solution.

Trolox standards: 0.5, 1.0, 1.5, 2.0 and 2.5 ppm. A stock solution of 100 ppm of Trolox was prepared by dissolving in a volumetric flask 0.001 g Trolox in MeOH to a volume of 10 mL. The solution standards were prepared from the stock solution.

Methanolic extracts: Methanolic extracts of quinoa flour samples were prepared with 5 different samples.

Quantification. The quantification was carried out following the method described by Julián-Loaeza *et al.* [63] with some modifications. In a microplate well, 70 µL of the extract or the standard was mixed with 70 µL of 0.004% (w/v) DPPH•. The sample was left at rest for 30 min at ambient temperature in the absence of light and was mixed for 1 min in the

microplate reader. The absorbance of the sample was measured at 515 nm. For the blank of the assay, the sample was replaced by the corresponding volume of MeOH. The antioxidant activity was expressed as the percentage of the radical DPPH• inhibition and was calculated using the following equation:

$$\%\ inhibición = \left(\frac{A_{DPPH\bullet} - A_{EXT}}{A_{DPPH\bullet}}\right) x\ 100 \qquad (Ec. 9)$$

Where:

$A_{DPPH\bullet}$ = absorbance of the blank solution with DPPH•

A_{EXT} = absorbance of the sample

To understand the value of EC_{50} is the source explained the % inhibition *vs* concentration and from the curve obtained is found to concentration corresponded 50% inhibition. The value EC_{50}, corresponds to the amount of sample required to reduce 50% the radical DPPH• and is reported in kg of dry weight of the sample/kg of DPPH•. The EC_{50} value, is obtained from a plot of the % inhibition against quinoa flour concentration.

Kinetics of antiradicalar efficiency. The kinetics was performed with the extract corresponding to the EC_{50} concentration. The absorbance of the sample was measured at 1 min intervals during 120 minutes. The % of DPPH• remaining was calculated from the absorbance values. The T_{EC50} is defined as the time at which the values of % of DPPH• remaining are steady. In this work the T_{EC50}, was obtained by graphing the first derivative of the absorbance values of % DPPH• remaining vs time. The T_{EC50} value was considered when the derivative was constantly close to zero. With the values of EC_{50} and T_{EC50} the antirradicalar efficiency (AE) was calculated using the following equation and was expressed as kg of DPPH•/Kg of dry weight sample x min.

$$\mathrm{AE} = \frac{1}{EC_{50}\ x\ T_{EC50}} \qquad (Ec. 10)$$

4.2. Separation and isolation of quinoa proteins

4.2.1. Optimization of the protein extraction process

With the aim of establishing the optimal conditions for obtaining isolates from quinoa proteins, two critical extraction steps were investigated: the alkaline extraction and the isoelectric precipitation. The parameter that was assessed was the pH. The pH valves that were investigated for these processes are listed in Table 6. The procedures for this assessment are described in the following section.

Table 6. Evaluation of the extraction steps at different pH

Extraction process step	pH tested
Alkaline extraction pH	8, 9, 10, 11, 11.5, 12
Precipitation isoelectric pH	2, 3, 3.5, 4, 5, 6

4.2.1.1. Assessment of the alkaline extraction of quinoa proteins at different pH

The extent of extractability of quinoa proteins was obtained from the alkaline extraction process at different pH following the flow diagram (Figure 6). For each pH condition, 10 g of defatted quinoa flour was used. The quinoa flour was mixed with 180 mL of water, 0.1 g of ascorbic acid in a 250 mL beaker and stirred in a magnetic stirrer. The pH of the solution was adjusted with 25% (w/w) of NaOH solution, at different pH (Table 6) using a pH meter.

The mixture was stirred for 30 min following by centrifugation during 20 min at 9000 x g. The supernatant was kept in a separate flask while the precipitated solids were washed with 60 mL of water and the mixture was centrifuged again. Following this, the supernatant was combined with the supernatant of the first centrifugation cycle. A third washing/centrifugation procedure was repeated as above. The final solid material was dried in a 0V-490A oven (Blue M, United Stated) at 60°C. Protein analyses of the liquid extract and the dried solid material were performed using the method described in section 4.1.3 part C. The extractability assay was repeated twice for each pH tested, thus accounting for

a total of 12 determinations. The percentage of extractability was calculated by dividing the amount of protein in the liquid extract by the amount of starting material and multiplying by 100%.

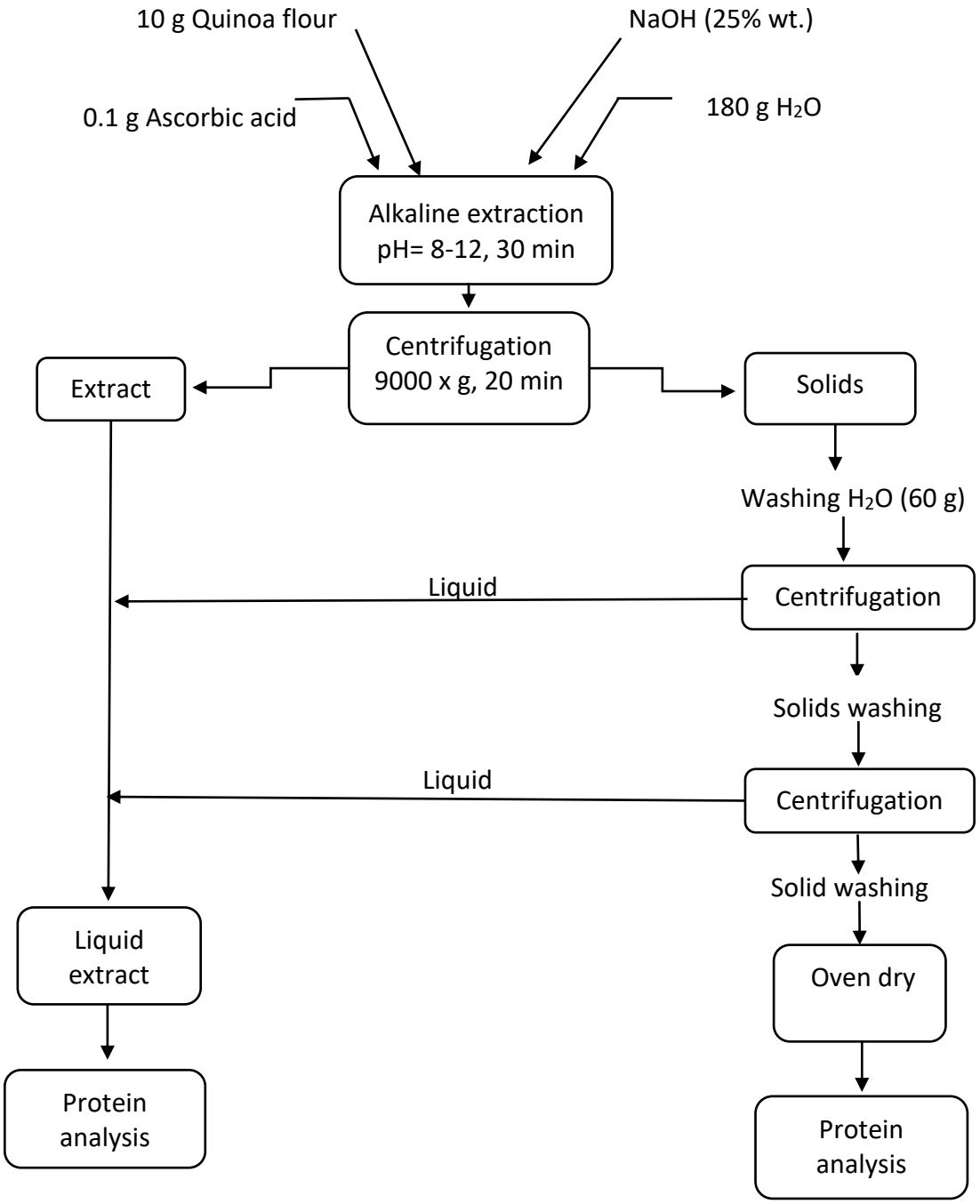


Figure 6. Flowchart of the determination of extractability of quinoa proteins at different pH in the alkaline extraction process.

4.2.1.2. Assessment of the isoelectric precipitation of quinoa proteins at different pH

The isoelectric precipitation of quinoa proteins was assessed to extent of precipitability of the quinoa proteins at different pH (Figure 6). An amount (30 g) of defatted quinoa flour was extracted with NaOH at the pH that was obtained from the alkaline extraction assessment (Section 4.2.1.1,) for 30 min. The extract was separated from the remaining solid through a series of centrifugations and stages of washing (Figure 7). The final extract solution was divided into four portions of 200 g for the precipitability tests and a portion of 60 g for protein analysis of the starting extract material. Each 200 g portion was adjusted to a different pH with a H_3PO_4 solution (6 M) according to Table 6. The suspension was stirred in a magnetic stirrer and the pH was kept constant through the addition of H_3PO_4 during 20 min.

The precipitate solids were separated from the liquid phase through centrifugation followed by a single wash. The liquid streams, which contain the soluble proteins were stored in glass jars in the refrigerator at 4 °C. Before protein analysis, the solutions were left to stand to room temperature with the help of a mixer. The protein tests were carried out in triplicate. On the other hand, the precipitated solids (wet) were stored in aluminum foil bags at refrigeration condition until protein analysis (normally carried out a day after processing); The precipitability tests were performed in duplicate. The percentage of precipitability (%) was calculated as the relation of the amount of precipitate protein the content of protein present in the initial extract.

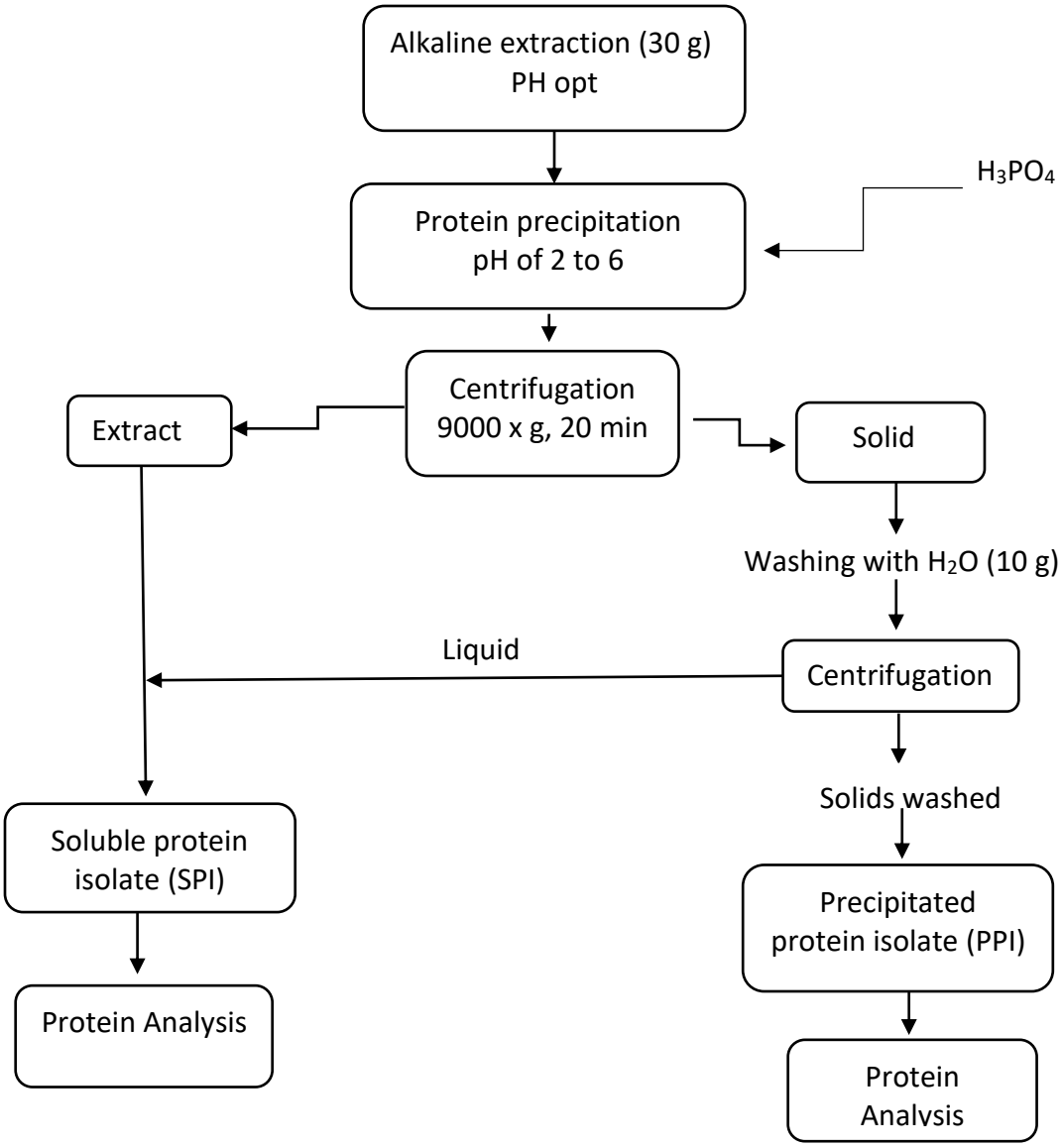


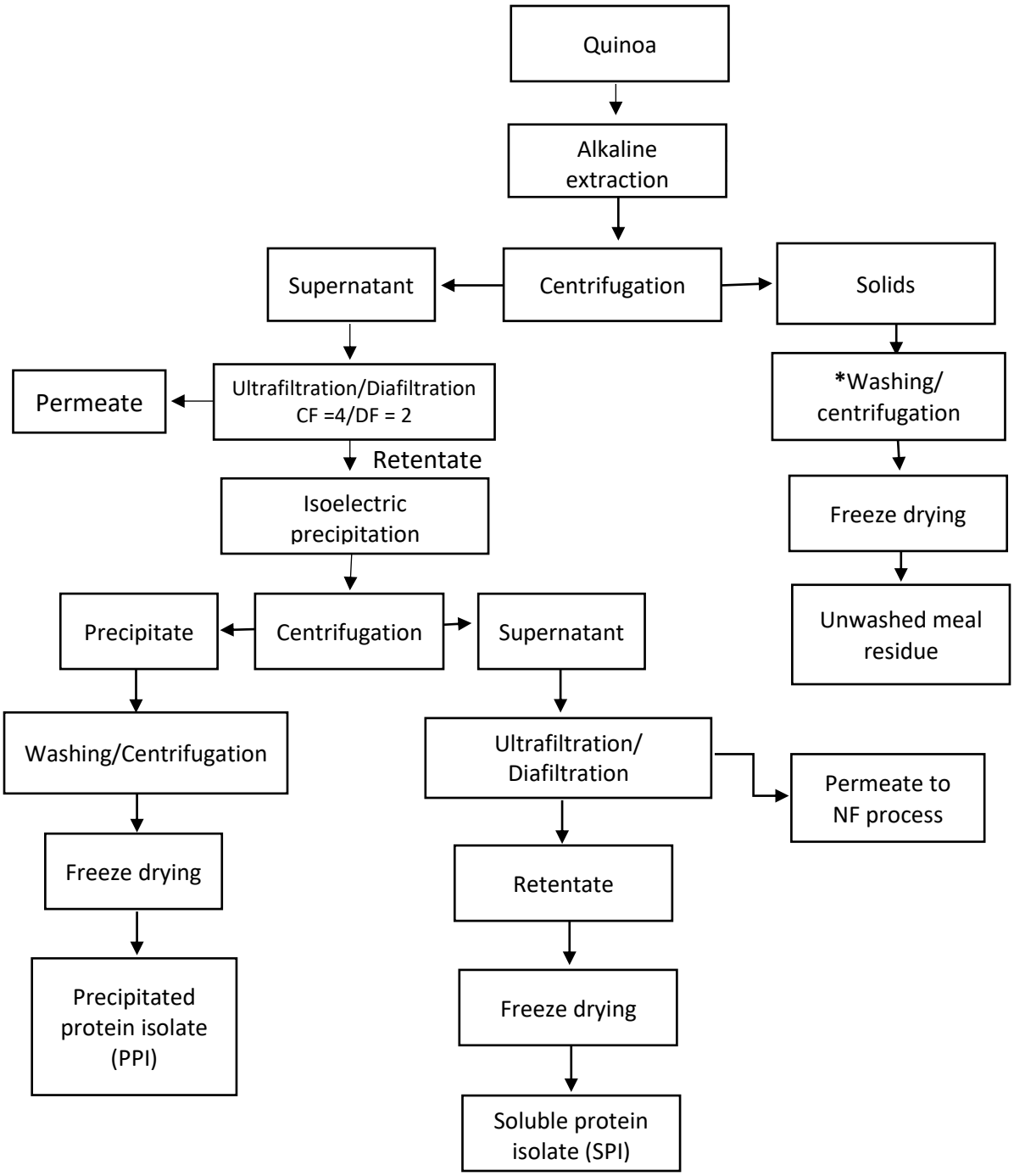
Figure 7. Flowchart of the determination of precipitability of quinoa proteins at different pH in the isoelectric precipitation process.

4.2.2. Alkaline extraction of quinoa proteins using membrane ultrafiltration

After extraction of the quinoa protein by means of an alkaline solution, a procedure based on membrane ultrafiltration [49] was used to concentrate and isolate the proteins present in the liquid extract.

The Figure 8 shows the flow diagram for the aqueous extraction combined with membrane processes. For alkaline extraction, the quinoa flour was mixed with a 1:18 relation of water in a beaker until a smooth paste, free of lumps, was obtained. The mixture was homogenized during 3 min using a Silverson L2R mixer (Thermo scientific, Mexico) at maximum speed, followed by the addition of ascorbic acid (1% w/w) as an antioxidant. The natural pH of the mixture of around (4.7) was adjusted to 12, with the addition of NaOH solution (25 % w/w). The extraction was allowed during 30 min, after which is the mixture was poured uniformly in 3 bottles of 1 L and centrifuged for 30 min at 9000 g.

From the centrifugation, two fractions were obtained: a solid precipitate and the liquid supernatant. The solid precipitate was resuspended in water (6:1), homogenized for three minutes and extracted again under the same conditions. After centrifuging, the protein solution was collected and weighed. The alkaline extract was adjusted with 6 M H_3PO_4 to a pH of 9.



***** After the washing and centrifugation process the liquid extract was mixed with the supernatant obtained from the previous process.

Figure 8. Flowchart of the process of proteins isolation from quinoa powder using alkaline extraction and ultrafiltration.

For the isolation UF experiments, approximately 2000 g of the protein alkaline extract at pH 12 was used.

Subsequently, the pH of the solution was lowered to 9 and NaCl was added to the solution, following by heating at 55-58 °C for 30 min. Then, the solution was subjected to ultrafiltration with a concentration factor of 3.9. The average flux for this operation was about 37.2 L /(m^2 x min). In a batch, 2808.63 g of permeate and 526.69 g of retentate (avg.) were produced. After ultrafiltration, the retentate was continuously diafiltered by the continuous addition of a sodium chloride solution (0.05 M) (with a total addition of about 1040.84 g) at pH 9 to obtain a diafiltration volume of 2.8. The polyethersulfone membrane was used in a SEPA CF II Membrane Element Cell (GE Osmonics Inc., MN, USA) equipment. After the membrane processing, the protein solution concentrate had a dark brown color. The isoelectric precipitation was carried out by the addition of about 7.74 g of 6 M H_3PO_4 and treated as described in section 4.2.2. The color of the protein suspension changed to a slightly lighter color and increases in the suspension viscosity were observed with the addition of acid.

The use of food grade NaCl was included in this integrated process as NaCl is recommended for the alkaline extraction of proteins since it increases protein solubility of proteins. The addition of NaCl improved the extractability results in the alkaline extraction process since the protein extractability increased from 60 to 64% with the presence of NaCl.

4.2.3. Statistical analysis

The results of % extractability and % precipitability were statistically analyzed performing an analysis ANOVA using the software Minitab 14 (Minitab, Inc. PA, USA). A p value of 0.05 was used. Data are expressed as the mean ± the standard deviation (SD). Three replicates were used for each experimental determination.

4.2.4. Membrane process of the protein solution and precipitation isoelectric

After alkaline extraction and isoelectric precipitation, the purification and isolation of the proteins present in the extracts were conducted using ultrafiltration membranes (Figure 9). The protein solution obtained from the alkaline extraction process was filtered using a filter paper Whatman No. 1. After filtration, RO water was added to the solution to obtain a concentration factor of 4 in the ultrafiltration process. The solution was heated to a temperature between 55 and 60 °C for a period of 30 min. After warming up, the solution was cooled to 40 °C. Subsequently, a sample was taken for the determination of proteins.

The ultrafiltration equipment consisted of a filtration system SEPA CF II fitted with a diaphragm pump HydraCell, a Baldor electric motor with a variable speed engine. An ultrafiltration polyethersulfone membrane with a MWCO of 5 kDa with an effective area of 0.015 m^2. This equipment has been used in the Food Engineering laboratory at the University of Toronto for high-pressure membrane processes.

The molecular weight profiles of the quinoa protein extracts [64] show that most of the quinoa proteins have a molecular weight greater than 5 kDa; therefore, a membrane with a MWCO of 5 kDa was selected for the ultrafiltration and diafiltration processes to obtain the maximum protein recovery. After ultrafiltration, the retentate was taken to a diafiltration stage with the continued addition of sufficient 0.05 M NaCl solution at pH 9 to obtain a diafiltration volume of 3. At the end of the membrane processes a sample was withdrawn for the determination of proteins[64]; The process was carried out once. At the end of each experiment, the unit was immediately drained and washed with distilled water.

For the isoelectric protein precipitate, 6 M H_3PO_4 was added drop by drop with continuous agitation to reduce the pH to a value of around 4. The solid suspension was centrifuged at 6000 rpm for 30 min. The supernatant was separated for the process of ultrafiltration, while the solid fraction was resuspended in water. Subsequently, the suspension was washed with 5 times its weight of RO water. Finally, the suspension was centrifuged and the precipitate was freeze dried for protein analysis.

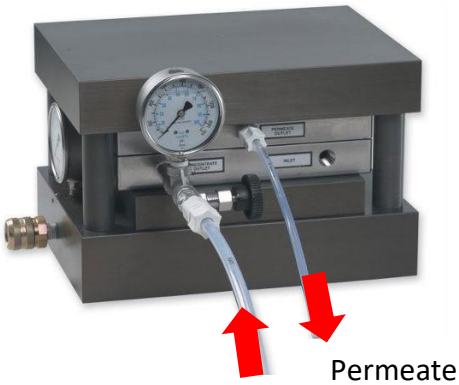


Figure 9. Filtration SEPA CF II Membrane Element Cell (GE Osmonics Inc., MN, USA).

4.3. Separation of polyphenols in the quinoa

4.3.1. Nanofiltration process.

In order to carry out the nanofiltration process it was decided to choose a nanofiltration membrane of 150-300 Da. (Table 7), with an effective area of 0.015 m^2. The nanofiltration process was carried out with the same equipment for the ultrafiltration process (Figure 9), where the following conditions were chosen based on previous tests with reverse osmosis water; the manometer was changed to the equipment to adjust the pressure to 16-17 bar, the feed flow obtained was approximately 1.5 L/min. The equipment allowed the batch work as the configuration of the same does not allow feedback and work on a continuous form.

Table 7. Specifications of the nanofiltration membrane used.

MWCO	150 – 300 kDa
Construction	Composed of thin film
Material	Polysulphone base and top layer of polyamide
pH range tolerated (25°C)	2-11
Rejection	M_2SO_4
Maximum temperature tolerance	80°C
Maximum pressure tolerance	40 bar

To carry out the separation of the polyphenols present in the permeate of the ultrafiltration process the method used at the University of Toronto by Legorreta [65] was used, with slight modifications (Figure 10). This procedure consists in separating the phenolic compounds from the permeated obtained from the ultrafiltration step of the protein isolation process (Figure 8).

As mentioned above, the starting solution for the polyphenol recovery process was the UF permeate of the protein isolation process. A batch of the permeate from UF was unfreezed and then centrifuged at 9000 x g for 20 minutes. Following this, the supernatant was filtered (polishing) through a filter Whatman No. 54 and the resulting filtrate was taken to nanofiltration processing. After nanofiltration the retentate was frozen with liquid nitrogen and freeze dried.

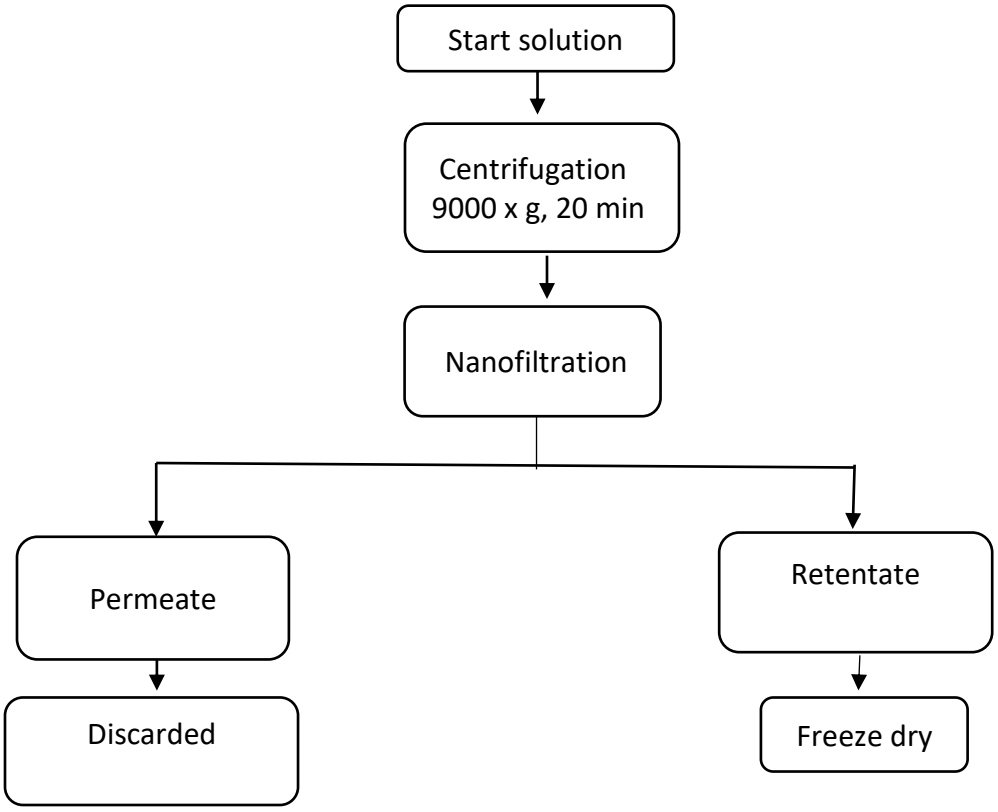


Figure 10. Flowchart of the process of polyphenol recovery from the UF permeate of the protein isolation process by using nanofiltration.

5. RESULTS AND DISCUSSION

This section describes the discussion of the results obtained for the process of protein and polyphenols recovery and isolation from quinoa (*Chenopodium quinoa* Willd.) seeds through membrane processes.

5.1. Characterization of the quinoa

The black quinoa seeds used in this study were characterized by performing a proximate analysis. The Table 8 shows the average values and standard deviations of the proximal determinations of the seeds of quinoa that include moisture, crude fat, total protein, and total ash.

Table 8. Proximal composition of the quinoa grains.

Determination	**% (g/100 g d.w. sample)**
Moisture	14.51 ± 0.78
Crude fat	10.93 ± 0.59
Total proteins	14.83 ± 0.74
Total ashes	1.07 ± 0.03

d.w. Dry weight.

The moisture content (14.51 ± 0.78 g/100 g) of the black quinoa used in this study was greater than that described by Reyes *et al.* [13], who reported an average of 12.07 ± 0.17 g/100 g for different varieties of quinoa (white, sweet pink, bitter). The moisture content quinoa is within of various cereals such as maize (10.37 g/100 g), wheat (10.94 g/100 g) and rice (11.62 g/100 g) [66]. The moisture content of oil seeds can be affected by several factors such as the variety of quinoa, storage time, and agroclimatic cultivation conditions[67].

The content of crude fat in black quinoa (10.93 ± 0.59 g/100 g d.w.) was greater than that reported by Miranda *et al.* [68], who reported values in quinoa of Chile ranging from 5.5 to

8.5 g/100 g of d.w. The variations observed may be due to the composition of different cultivars, genotypes, ecotypes, and cultivation conditions.

On the other hand, the total protein content of the black quinoa was of 14.83 ± 0.74 g/100 d.w. In the literature, there are very few protein determinations for black quinoa; the majority of the reports have been described for white, pink and yellow quinoa. Nevertheless, the content of protein obtained in this study is similar to the range reported in the literature: 7-16 g/ 100 g d.w. [52,64,69,70].

The quinoa studied in this work has a protein content similar to that of cereals such as wheat (13.68 g/100 d.w.). However, wheat grains do not contain all the essential amino acids in the required amounts [4,71]. Finally, the content of total ash was 1.07 ± 0.03. This value is similar to the determinations reported by Ogungbenle *et al.* [72] for quinoa (*Chenopodium quinoa).*

The variation in the concentration of proteins, lipids and other compounds depends of several factor such as the place in where the product is being grown since the environmental and climatic conditions influence either positively or negatively, stress factors to which the plant is exposed, growing conditions such as soil, irrigation, agrochemicals etc. All of these factors can vary the content of the above mentioned compounds [73,74].

5.2. Bioactive compounds and antioxidant activity

This section describe the results of the analysis of the content of total flavonoids, total phenols and total carotenes, as well as the determination of the antioxidant activity of the quinoa flour.

5.2.1. Total flavonoids

In the literature it has been reported that quinoa seeds contain a wide variety of flavonoid compounds such as catechin, rutin, quercetin, among others [23,31,75,76]. These compounds are found both in the form of aglicones or glycosides.

In the present study, the content of total flavonoids of the methanolic extracts from quinoa flour was 99.64 ± 4.63 mg Eq. Rut/100 g d.w. The flavonoid content of the black quinoa was greater than that reported by Pasko *et al.* [77], who reported a 36.00 ± 1.1 mg Eq. Rut/100 g d.w. for quinoa of Bolivia.

In an chemical analysis study of quinoa Andean: quinoa (*Chenopodium quinoa*) red, cream, black and yellow, kañiwa (*Chenopodium pallidicaule*) yellow and brown, Ritva *et al.* [29] identified the flavonoids and other phenolic compounds present in the grains by HPLC. The flavonoid content of quinoa and kañiwa was exceptionally high, varying from 36.2 to 144.3 mg/100 g of d.w.

The results obtained in this work show a greater content of total flavonoids respect to those value reported by by Ritva *et al.* [29] was 69.2 ± 3.6 mg/100 g for a commercial variety of black quinoa, being myricetin, quercetin and kaempferol the most abundant.

5.2.2. Total phenolic compounds

The content of total phenolic compounds (TPC) in the black quinoa samples was 96.29 ± 7.70 mg Eq. GA/100 g d.w. The phenolic content of the quinoa studied in the present work was greater than those reported by Alvarez-Jubete *et al.* [32], who reported 71.7 ± 5.5 mg Eq. GA/100 g d.w. for quinoa of Bolivia.

Dini *et al.* [78] determined the content of total phenolic compounds in seeds of bitter (black) and sweet (white and gold) quinoa seeds, before and after a boiling process. The authors reported that before the boiling process the black quinoa contained a greater amount of phenolic compounds (86.4 ± 1.41 mg Eq. GA/100 g d.w.) as compared with the quinoa seeds (77.2 ± 1.67 mg Eq. GA/100 g d.w.). After the boiling, the total phenolic content was reduced for both quinoa varieties: 59.4 ± 0.23 mg Eq. GA/100 g d.w. and 28.7 ± 0.28 mg Eq. AG/100 g d.w. in the black and sweet quinoa seeds, respectively. The results obtained in this research are similar to those obtained by Dini *et al*. The possible small variation between the results may be due to the place where the seed comes from, in this study comes from the Andes of Peru and those studied by Dini *et al*. from Ecuador.

In the investigations performed by Ritva *et al.* [52] and Pasko *et al.* [79], higher concentrations of phenolic compounds were reported in comparison with the values obtained in this work: 261.04 ± 3.68 mg Eq. GA/100 g d.w. and 375 ± 0.05 mg Eq. GA/100 g d.w., respectively. This is possibly due to the fact that Ritva *et al.* used freshly harvested quinoa that did not receive treatment before its packaging for the elimination of antinutritional factors; while Pasko *et al.* [79] carried out sowing of quinoa in the laboratory and possibly could be subjected to stress conditions. Apart from saponins reduction, these quinoa washing and drying pretreatments can also remove other compounds which are sensitive to temperature and processing time such as the phenolic compounds.

Chlopicka *et al.* [75] made a comparative analysis of the content of total phenols of wheat, amaranth and quinoa, in which it was determined that wheat contained the highest concentration of these compounds; followed by quinoa and finally the amaranth with concentrations of 696 ± 0.11 , 280 ± 0.1 and 271 ± 0.1 mg Eq. AG/100 g d.w., respectively.

With respect to other common cereals, the content of total phenolic compounds obtained in this study was higher than that of wheat (56 mg/100 g d.w.), barley (88 mg/100 g of d.w.; and similar to that of rye (103 mg/100 g of d.w.) and lower than that of millet (139 mg/100 g d.w.). Therefore, it is concluded that quinoa is a food that contributes higher content of phenolic compounds respect to other cereals.

5.2.3. Total carotenes

The content of total carotenes present in black quinoa samples was 0.997 ± 0.373 mg of β-carotene /100 d.w., which was lower that reported by Tang *et al.* [10], who obtained values of 1.12 ± 0.13, 1.5 ± 0.09 and 1.76 ± 0.19 mg of β-carotene /100 g of d.w. in white, red and black quinoa varieties, respectively. Similarly to the flavonoid reports, the results of these authors [10] indicate that the total carotene concentration of quinoa seeds increases with the darkness of the quinoa seeds.

The content of β-carotene determined in black quinoa was lower than that obtained by Tang *et al.* in quinoa (black, red and white) possibly by the time and solvents used for the

extraction of these compounds since in this study a mixture acetone/hexane was used for extraction, while in the study by Tang et al. [10] methyl *tert*-butyl ether/tetrahydrofuran (MTBE/THF).This solvent concomitance may possibly increase the amount of compounds extracted from the sample, in addition to that the extraction time was higher than that evaluated in this work, possibly the prolonged interaction with the solvents helped to increase the extraction.

5.2.4. Antioxidant capacity

The determination of the antioxidant activity of the quinoa methanolic extract was conducted to test the ability of the extract to inhibit of radical DPPH•. It was observed that the quinoa extract had a low capacity to scavenge the radicals DPPH•. For this assessment, we determined the EC_{50}, which is the concentration of the sample required to reduce 50% the initial concentration of DPPH•. The EC_{50} of the methanolic extracts of the quinoa samples (0.0158 g/mL) was approximately a ten-fold higher than those of the controls: gallic acid (0.0017 g/mL) and Trolox (0.0027 g/mL). This indicates that it is needed 10 times more amount of quinoa flour than that of the controls in order to reduce 50% the initial concentration of DPPH•.

Additionally, the antiradicalar efficiency was calculated with the values of EC_{50} and T_{EC50} for the methanolic extract of the quinoa samples. The extract had T_{EC50} values of 59 min. These values are greater than those obtained for the controls: gallic acid (28 min) and Trolox (17 min). The antiradicalar efficiency of the quinoa extracts was 2.14×10^{-5} Kg of DPPH/g of quinoa. In comparison with this result, the antiradicalar efficiency of gallic acid was 27 times greater (5.7×10^{-4} Kg of DPPH/g of gallic acid) and that of Trolox was 4 times greater (5.35×10^{-1} Kg of DPPH Trolox/g). According to the classification of antioxidant power with respect to their antiradicalar efficiency reported by Gramza *et al.* [71], the methanolic extract of the quinoa samples obtained in this work corresponds to a low antioxidant power (Table 9).

Table 9. Classification of the antioxidant power of an extract according to the antiradicalar efficiency [80].

AE Interval **(Kg of DPPH/g of dry sample*min)**	**Classification**
$AE \leq 1 \times 10^{-3}$	Low
$1 \times 10^{-3} < AE \leq 5 \times 10^{-3}$	Media
$5 \times 10^{-3} < AE \leq 10 \times 10^{-3}$	High
$AE > 10 \times 10^{-3}$	Very High

As part of the traditional diet, quinoa can be implemented as a usual component for its consumption since apart from the contribution in the main nutrients for a good diet, they can also be important source in the prevention and treatment of cardiovascular diseases due to the efficiency low antiradicalar that is related to the prevention and treatment of certain cardiovascular diseases. Flavonoids have been shown to prevent platelet aggregation and induce muscle relaxation, along with proteoglycans, flavonoids exert an inhibitory effect on allergic symptoms [81,82].

5.3. Separation and isolation of quinoa proteins

5.3.1. Optimization of the protein extraction process

5.3.1.1. Assessment of the alkaline extraction of quinoa proteins at different pH

With the aim of optimizing the pH of the protein recovery from the alkaline extraction stage, different pH conditions were assessed for this process. The recovery of proteins from quinoa flour increased with the pH of the alkaline extraction process, from 37.45 ± 0.16% at pH 8 to 60.17 ± 0.10% at pH 12. Since a significant increase in the extractability of quinoa protein was observed as the pH of the extraction stage was increased (Figure 11), it results evident that a high extraction pH should be used (pH of 12) to achieve adequate protein

recoveries. This increase in extractability with pH is the result of interactions of NaOH and the extractable quinoa proteins through the disruption of ionic bonds, hydrogen bonds, etc.

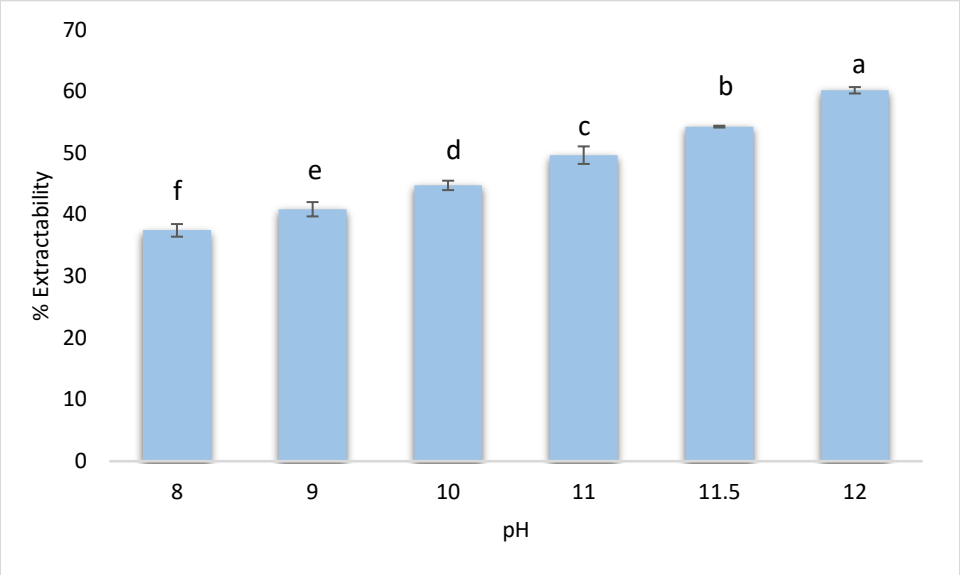


Figure 11. Extractability curve of quinoa proteins as a function of the pH of the alkaline extraction stage. Data are expressed as mean of three measurements ± standard deviation. Means with different letters indicate significant differences ($p<0.05$).

The extractability obtained in this work was low with respect to that of canola flour and defatted yellow mustard, which presented extractabilities of 69.5% and 85% at pH 12, respectively [38,40].

On the other hand, pH above 12 was not investigated because of the concern about the formation of lysinolalanine, which is a toxic compound. In membrane processes similar to those reported here, Deng et al. [83] demonstrated that rapeseed/canola protein preparations contained very low levels of lysinolalanine concentrations (<500 µg/g lysinoalanine) like that found in commercially produced casein and soybean protein isolates. Nevertheless, as a food safety measure, it is recommended that extreme pH values (> 12) are avoided during the production of quinoa protein isolate, in addition, using pH

higher than 12 pose a risk to damage the membrane integrity as the pH range for the 5 kDa UF membrane should not exceed a pH of 12 [84].

Some ways to improve the protein extractability of the process are increasing the temperature and the extraction time, and the addition of NaCl [85–87]. The protein that was not extracted during the alkaline extraction step is recovered as a byproduct of the process (meal residue) and can be used in other applications. Considering the factors above, pH of 12 was chosen as the optimal pH for the alkaline extraction stage of quinoa protein.

5.3.1.2. Assessment of the isoelectric precipitation of quinoa proteins at different pH

The results of protein precipitability for the isoelectric precipitation stage as a function of the precipitation pH are shown in Figure 12. The maximum protein precipitability (74.42%) was achieved at pH 4, as at this ionic state the increasing protein-protein interactions reduce the protein solubility around the isoelectric point (pH ≈ 4); therefore, the net electrostatic charges of proteins are minimal and there is less interaction of the protein molecules with the aqueous medium.

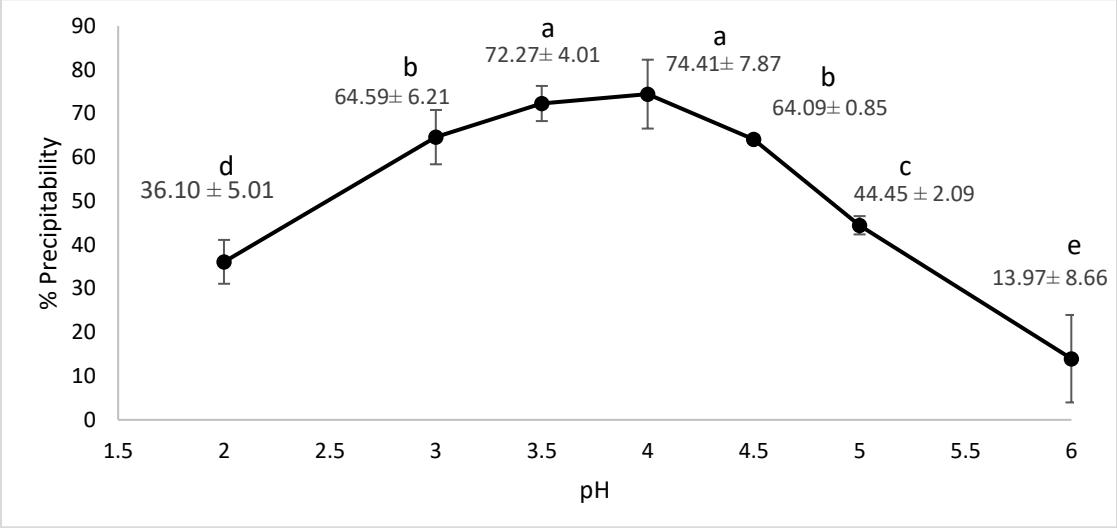


Figure 12. Precipitability curve of quinoa proteins as a function of the pH of the isoelectric precipitation stage. Data are expressed as mean of three measurements ± standard deviation. Means with different letters indicate significant differences ($p<0.05$).

It is observed that from pH 4.5 to 6 protein precipitability decreases as the pH increases. The sharp decrease in protein precipitability at pH above 4 is due to the increase of the hydrophilic interactions between the proteins and water; therefore, the proteins have a low precipitability.

Similar behaviors were observed in the studies reported by Marnoch and Diosady [40] with yellow mustard where the protein precipitation had an optimum value of 71 ± 2% as the pH of the solution was increased to 5.5. Several investigations have determined the isoelectric point for proteins of a number of cereals and pseudo-cereals such as amaranth, wheat, soy, among others, which range between 4 and 5. The isoelectric point obtained in this study for quinoa proteins (pH = 4) is in agreement with that reported in the literature [51].

The standard deviation values of the precipitability curve are higher than those obtained in the extractability curve because in the precipitation process the samples are taken from the wet precipitate, which causes a larger error in the measurements.

The use of $CH_3OH/NH_3/H_2O$-hexane for the quinoa flour degreasing process may be a good alternative to improve yields of precipitated protein isolates since in works by Xu *et al.* [51] It is found that the percentage of PPI is significantly increased with the use of $CH_3OH/NH_3/H_2O$-hexane than with hexane alone. However, the % protein extractability is slightly decreased with the use of $CH_3OH/NH_3/H_2O$-hexane and should therefore be taken into account for the choice of this solvent for the degreasing of the starting material.

5.3.1.3. Alkaline extraction of quinoa proteins using membrane ultrafiltration

The optimized process for the obtaining protein isolates using UF membranes is shown in Figure 13.

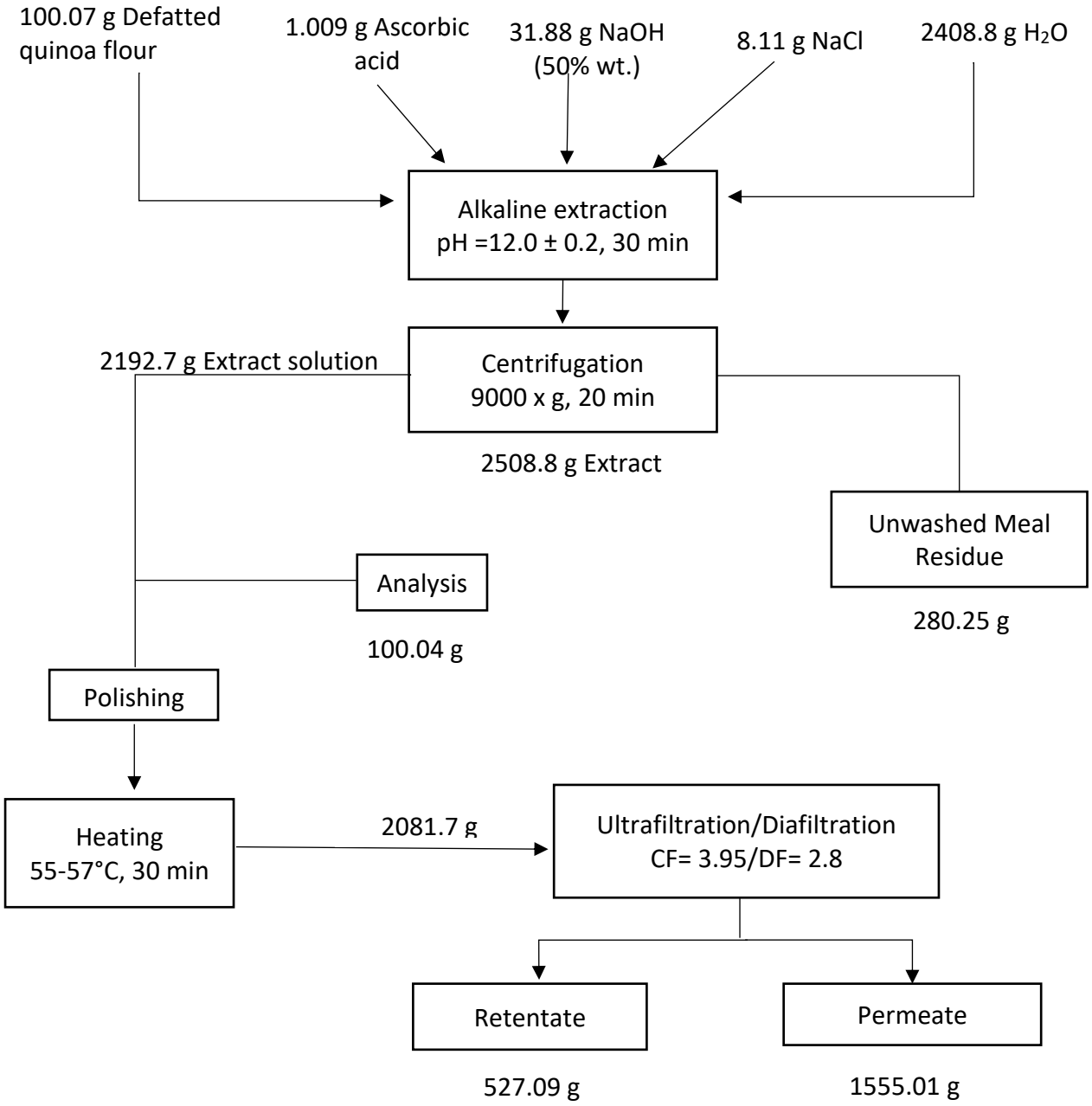
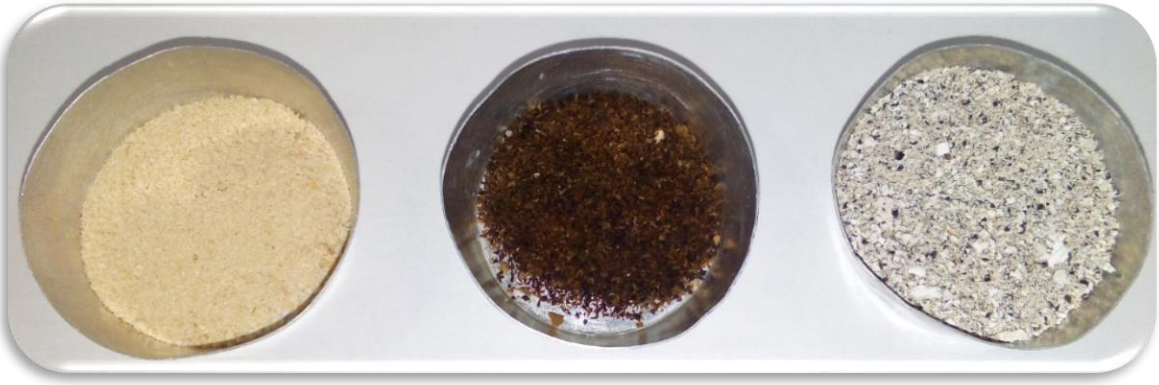


Figure 13. Optimized process for isolation of quinoa proteins using alkaline extraction and ultrafiltration membranes.

The integrated alkaline extraction and ultrafiltration membrane processes for quinoa protein isolation (Figure 8) resulted in three main protein fractions: the precipitated protein isolate (PPI), the soluble protein isolate (SPI), and the meal residue, which pictures are observed in Figure 14.



Soluble protein isolate (SPI) | Precipitated protein isolate (PPI) | Meal residue

Figure 14. Products obtained by ultrafiltration-extraction of quinoa seeds: soluble protein isolate (SPI), precipitated protein isolate (PPI) and meal residue

The overall process was carried out in a single batch, following the conditions of Figure 8; The batch of 100 g quinoa flour as the starting material yields 4.46 g of precipitated protein isolate (PPI), while the recovered soluble protein isolates (SPI) were 2.57 g and 74 g of flour residue. These products represent 81.03% of the raw material.

Comparing with the results obtained by Marnoch and Diosady [40] in terms of the amount of soluble and precipitated protein isolates, it can be observed that the soluble protein isolate content is similar to that obtained in mustard 2.9 g, however the amount of precipitated quinoa protein isolate is much lower when compared to the 21.7 g obtained in the defatted mustard protein isolates. This gives rise to the investigation of new alternatives for the precipitation of quinoa proteins and the obtaining of higher yields of precipitated protein isolates.

The addition of NaCl in the alkaline extraction process has been shown in previous works with canola that can break the ionic bonds between proteins and phenolic compounds [50]. In general, the concentrations of neutral salts applied to these processes are in the range from 0.05 to 1 M; thus, increasing protein solubility can also prevent the formation of protein-phenol complexes. However, at salt concentrations higher than 1 M, the solubility of the proteins decreases, which may lead to protein precipitation since this condition favors stronger protein-protein interactions. Considering the above, in this work NaCl concentrations of 0.05 M was used in the alkaline extraction process.

In the literature, the PPI isolates have been associated to globulin proteins, whereas the SPI isolates are constituted of albumins [86].

5.3.1.4. Membrane selection for the process of ultrafiltration of the quinoa liquid extract

For the ultrafiltration of quinoa protein extracts a 5 kDa polyethersulfone membrane was used. In previous work done with yellow mustard and oriental mustard, it was observed that an important nitrogen fraction contained in the alkaline extract was permeated through a 10 kDa membrane [88]. In these work nitrogen-compound losses between 15 and 30 %. Were quantified indicating a possible loss of protein through the membrane. The analysis performed on the ultrafiltration permeates indicated that there were proteins with MWCO lower than 10 kDa present in the yellow mustard extracts. Therefore, it was decided to use a 5 kDa membrane for the UF protein purification process in this work. With the implementation of this membrane, the nitrogen-compound losses decreased by about 7-12%.

Protein analyses of the UF permeates showed that the 5 kDa membrane was suitable for the retention of most of the proteins present in the alkaline extract. The losses in the permeates accounted for 4% or protein, which was probably due to the permeation through the membrane of small nitrogen-compounds including small peptides and free amino acids.

5.3.1.5. Effect of the concentration and diafiltration factors

In previous works, it has been observed that with the use of a diavolumen of 2 a rejection coefficient of impurities of 86% is attained; with a diavolumen of 3, 95% of impurities are rejected and a diavolumen of 4 rejects 98% of impurities. Therefore, as the diavolume increases, the rejection coefficient of impurities increases [56].

The concentration factor used for the ultrafiltration process was 3.95, whereas a diavolumen of 2.8 was used for the diafiltration stage. The use of this ultrafiltration/diafiltration configuration resulted in the elimination of 88.7% of the impurities from the protein extract. It was not possible to increase the concentration factor and the diavolumes due to the fact that the feeding and holding hoses are very long, so that the amount of retentate is insufficient to continue retentate recirculation,

Xu & Diosady [50] evaluated several processes for the elimination of phenolic compounds from protein extracts. The diafiltration process was carried out with a diavolumen equal to 5; for PPI 1094 mg/100 g sample of phenolic acid was measured in the control, a decrease of 16.2% was observed with the diafiltration a decrease of 16.2% in the amount was reduced to 917 mg/100 g of sample of phenolic acid. In the case of SPI, the control concentration was 1053 mg/100 g sample of phenolic acid, representing a 22% decrease in these compounds at 823 mg/100 g phenolic acid sample.

Also, there is a direct relationship between the diavolumenes used for the diafiltration and the color of the final isolates since the larger the diavolumen used in the process the possibility exists that the color of the protein isolates is clearer than if it were use small diavolumenes, because they are eliminating impurities in greater or lesser percentage [40,50,51,56].

5.3.1.6. Protein mass balance in the protein isolation process

After the freeze drying of the isolated products, an average of 4.46 g of precipitated protein isolate and 2.57 g of soluble protein isolate were recovered in one batch. These amounts represent the 7.03% of the initial mass of the quinoa flour. The meal residue accounted for 74% of the initial quinoa flour.

The protein mass yields of the integrated protein isolation process are distributed as shown in Figure 15. The initial protein in the quinoa flour was distributed in the following fractions: the protein isolates SPI and PPI, had a total yield of 5.28% and 28.19%, respectively; and the meal residue accounted for 46.76% of the protein in the starting material. The yields of isolated proteins obtained in this work were higher than those obtained for canola flour by Xu [89], with yields of 9% and 15% for the SPI and PPI isolates.

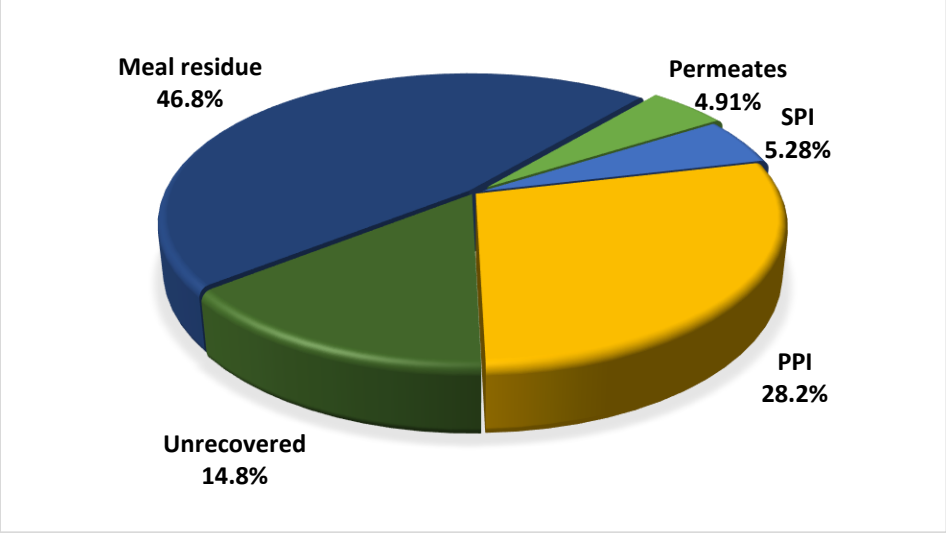


Figure 15. Protein yield obtained from the ultrafiltration process of quinoa.

With the ultrafiltration process, the total recovery of quinoa protein was 80.23%, which was higher than that obtained for canola (55% recovered) by Xu and Diosady [56]. In other studies, the use of ultrafiltration membrane processes for protein isolation from oriental mustard and yellow mustard seeds resulted in protein recoveries of 93% and 91.7%, respectively [49,51,90]. The variations in protein recoveries in the different studies are

mainly due to the type of seed, the extraction efficiency, as well as the type of proteins present in the seeds.

Evaluations performed by Xu *et al.* [89], which determinations of mass and protein distribution were made in the different output streams of the process; starting from 100% of the starting material it was possible to determine that 15.3% of the mass was recovered in PPI, 8.5% recovered in SPI and 58.3% corresponded to the flour residue. For the case of the mass yields obtained in the different outflows of the quinoa process (Table 10) as the canola was based on 100% of the starting material it was possible to determine that 4.46% of the mass was recovered in PPI, 2.57% was recovered in SPI and 74.0% corresponds to the meal residue unwashed, the losses in the process in both cases for quinoa and canola were similar of 19 and 17.9% respectively.

This difference in percentage of mass recovered is mainly due to the protein content present in both seeds since the quinoa has a lower protein content than canola; another factor by which the mass content can be modified is the % extractability protein since less is extracted in quinoa (64%) than in canola (70%); likewise, the % precipitation of quinoa (74.42%) is different than canola (67%), which considerably affects the mass % recovered in PPI, SPI and flour residue.

As for the distribution of the protein in the different products obtained can be observed for the case of the quinoa that the PPI contains percentages of protein similar to those of the canola that is to say 80.21% and 87%, respectively. In the SPI, there is a significant difference between the percentage of protein obtained since in the case of canola SPI this has values of 91.6%, in the case of quinoa the % of protein obtained is much less 26.2%. The quinoa residue has a higher yield (46.7%) compared to canola (33.8%) for the same reason that canola has the highest % extractability of its proteins. What possibly happened with the SPI obtained with quinoa is that there is probably little soluble protein because the content is lower, or that this interacting soluble protein with other compounds, a possible solution is to increase the NaCl content or add treatments combined with SDS (sodium lauryl sulphate) in the extraction process.

Table 10. Mass and protein distribution among major process streams.

Product	Dry Matter		Protein	
	g	%	G	%
Defatted quinoa seeds	100	100	12.66	100
Precipitated Protein Isolate(PPI)	4.46	4.46	3.57	28.2
Soluble Protein Isolate (SPI)	2.57	2.57	0.67	5.28
Meal Residue	74.0	74.0	5.92	46.7
UF and DF permeates	n.d.	n.d.	0.62	4.91
Unrecovered	18.9	19.0	1.87	14.8

n.d.= no determinate

Some studies in Diosady´s group research have shown that the protein isolate has the potential to be applied whether in the protein fortification of beverages and foods or to improve some of the functional properties of foods and chemical products [38,50,56].

5.3.1.7. Color and taste of the PPI and SPI isolates

Variations in the color of the process streams were observed during different stages which affected the final color of the isolates. After alkaline extraction, the color of the extract turned dark brown. In addition, the color of the retentate became darker after the ultrafiltration process as the extract was being concentrated. After isoelectric precipitation, the color of the soluble protein stream changed to a light color. From this stage, the color of both isolates was maintained after the freeze-drying process (Figure 16)

Soluble protein isolate (SPI) Precipitated protein isolate (PPI)

Figure 16. Powders of soluble protein isolate (SPI) and precipitated protein isolate (PPI) obtained in black quinoa by ultrafiltration.

Since protein isolates are intended to be used as functional ingredients in foods, it is desirable that they do not contribute to the flavor, or provide a minimum and complementary flavor to foodstuffs. Therefore, studies in canola by Xu and Diosady [50] evaluated their taste using sensory test methods to determine both taste intensity and acceptability. PPI and SPI have different functional properties [89], with different application in food systems, therefore, they must be evaluated and compared differently.

These results show that the aqueous extraction using membrane ultrafiltration processing of quinoa seeds constitutes a feasible and efficient method to produce high quality quinoa protein. In addition, the products obtained with these processes remarkably increase the value of raw quinoa seeds, and can be used as suitable replacements of animal-derived proteins. Considering the above, the process of protein isolation from quinoa seeds developed in this work, creates a good opportunity for attending the malnutrition problems in our country and in other developing countries, as well as in populations with socioeconomic vulnerability.

5.4. Recovery of polyphenols from the UF permeate of the protein isolation process

5.4.1. Evaluation nanofiltration process

For the recovery of phenolic compounds from the UF permeate of the protein isolation process, a molecular weight cut-off membrane of 150-300 Da was used for the nanofiltration process. This membrane was used to avoid the passage of most phenolic compounds since according to the literature the majority of the phenolic compounds present in quinoa flour are greater than 150 Da [76,91,92]. Considering this, the permeate of the nanofiltration should contain mostly water, salts, and some low molecular weight organic compounds. Figure 17 shows the process performed for the recovery of phenolic compounds from the ultrafiltration permeates with the indication of the mass amounts of the different process streams.

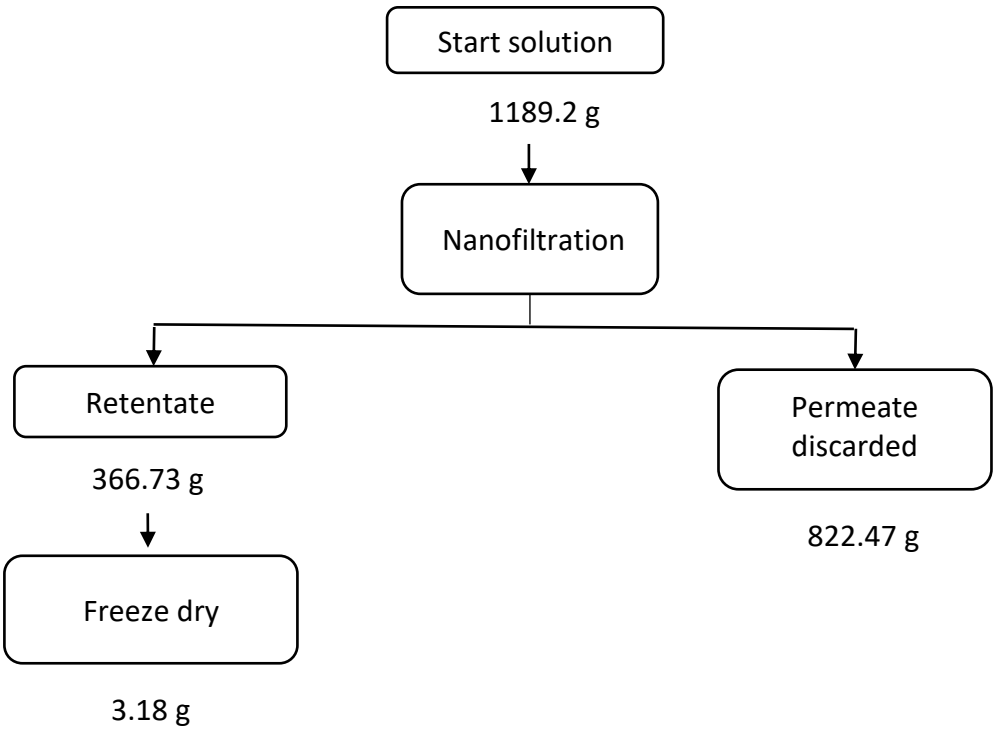


Figure 17. Flow diagram for obtaining phenolic compounds by nanofiltration.

The nanofiltration process was relatively fast as it was completed in only 2 h. A concentration factor of 3.3 was used it was lower than that used by Legorreta [65] since the amount of liquid extract is lower than that used in yellow mustard. After NF completion, the retentate (366.73 g) it was then lyophilized to obtain the final retentate powders rich in phenolic compounds (Figure 18).

Figure 18. Powder rich in phenolic compounds obtained from the freeze drying of the nanofiltration retentate.

The Figure 19 shows pictures of the solutions of the NF process streams. Significant color differences were observed for the different solutions.

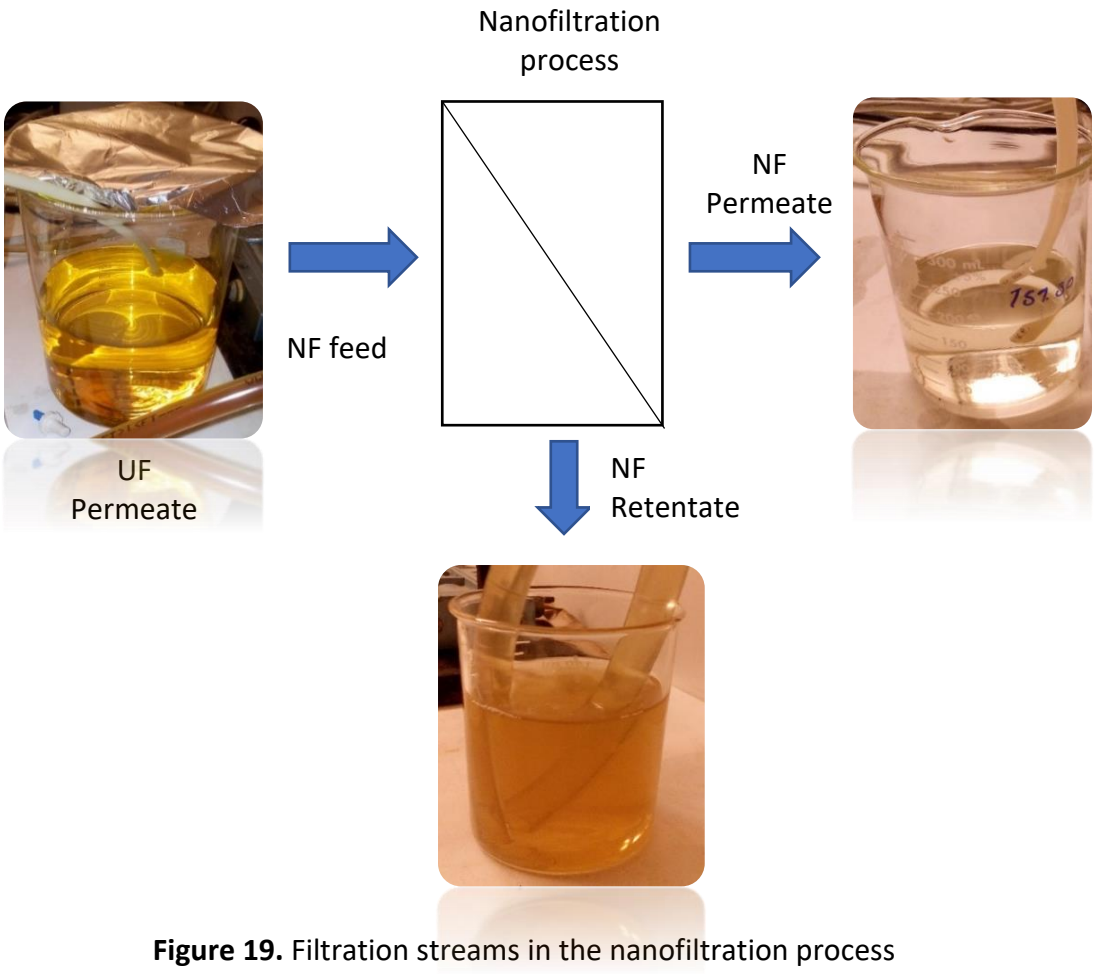


Figure 19. Filtration streams in the nanofiltration process

The starting solution had a light-yellow color which can be associated to the presence of phenolic compounds present in the UF permeate. At the end of the nanofiltration process the NF retentate solution became darker and more viscous because the phenolic compounds present in the feed were concentrated. On the other hand, the NF permeate resulted in a transparent colorless solution. The color change of the permeate with respect to the feed was most probably due to the removal of most of the phenolic compounds by the NF membrane, resulting in a solution mostly comprised by water, salts and some organic compounds smaller than 5 kDa.

5.4.2. Recovery of the phenolic compounds present in the UF permeate using nanofiltration

The complete process for the recovery of phenolic compounds is shown in Figure 20 with the total phenolic compounds contents indicated for the relevant streams of the process. Total phenolic concentrations were expressed as mg eq. of sinapic acid/L of solution.

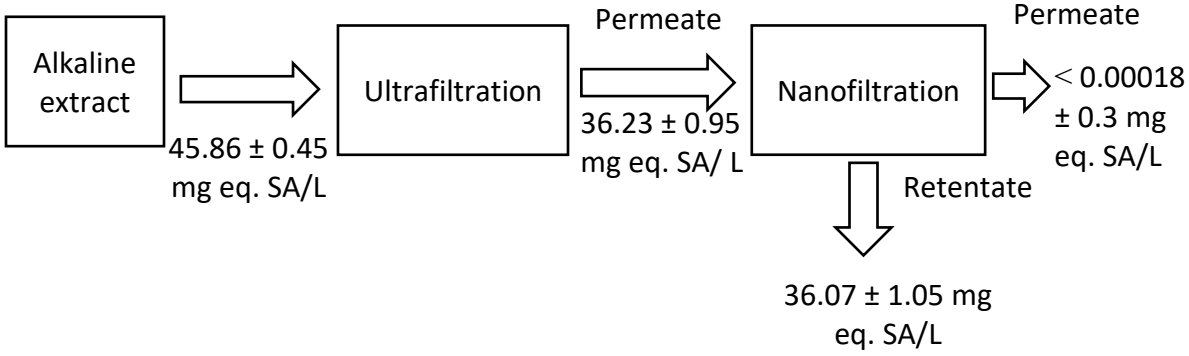


Figure 20. Concentration of total phenols in the different extraction-filtration streams.

Initially the content of phenolic compounds was 45.86 ± 0.45 mg eq. SA/L of solution present in the liquid extract from the ultrafiltration permeates which represented the 100%. In the course of the ultrafiltration process approximately 21.00% of these compounds were lost, leaving only 36.23 ± 0.95 mg eq. SA/L of solution which represented the 79%. 21.0% of non-recovered compounds may have been lost in the centrifugation processes or have interacted with the ultrafiltration membrane or at the same time react with the proteins and be present in the ultrafiltration retained.

The retentate of the nanofiltration process weighted 366.7 g accounting for 30.84% of the NF feed. The lyophilized powder obtained from the retentate of the nanofiltration process contained 36.07 mg eq.SA/L of solution which represented the 78.65%. Considering this, 99.5% of the phenolic compounds present in the UF permeate were recovered in the nanofiltration retentate.

The permeates obtained weighted 822. 47 g, accounting for 69.2% of the NF feed. From the mass balance of the process was estimated that almost 100% of the phenolic compounds are effectively recovered. This was further confirmed as the samples of the NF permeate because the concentrations of the phenolic compounds in this stream can be considered insignificant for the purpose of this study (< 0.00018 ± 0.3 mg eq. SA/L).

The process carried out by Legorreta [65] to obtain phenolic compounds of yellow mustard by nanofiltration processes in acidic and alkaline conditions it was obtained the following results; 1221.08 g of liquid extract from the ultrafiltration process were feed, of which 271.35 g were obtained in the retentate, representing 22.22% of the total feed. In the case of the permeate, 949.73 g of extract were recovered, which represented 77.7%. These proportions are greater and better than those of the nanofiltration process of quinoa extract since in the work done by the same it was possible to be used in concentration factor of 4.5 greater than 3.3 obtained in quinoa. However, the recovery rate of phenolic compounds in the quinoa nanofiltration retentate is higher than those obtained in mustard for both acidic conditions (77%) and alkaline conditions (64%).

6. CONCLUSIONS

- The proximal analysis of black quinoa flour showed that the data obtained are similar to those reported in the literature.
- The use of 5 kDa membranes to perform ultrafiltration and diafiltration resulted in a crude protein recovery of 80.21% of the total protein in the quinoa defatted flour.
- An amount of 4% of the protein was lost in the permeate fraction. Additional protein losses in the overall process may be attributed to the permeation of small polypeptides and amino acids (< 5kDa) through the UF membrane.
- The protein isolation process resulted in three protein products: i) A precipitated protein isolate accounting for 28.19% of the total initial quinoa protein with a protein content of 80.21% d. w. ii) A soluble protein isolate accounting for 5.28% of the total initial quinoa protein, with a protein concentration of 26.2% d.w. iii) A meal unwashed residue accounting for 46.76% of the total initial quinoa protein, with a protein concentration of 7.62% d.w.
- The purity of quinoa isolates was 80.21% for PPI, 26.2% for SPI and 7.62% for residue.
- The overall nanofiltration process to of the phenolic compounds obtained from the ultrafiltration permeates allowed to recover and concentrate a total of 78.65% of the phenolic compounds.
- The nanofiltration process allowed to recover 99.5% of the phenolic compounds in the nanofiltration feed.
- The results of this work show that the aqueous extraction using membrane ultrafiltration processing of quinoa seeds constitutes a feasible and efficient method to produce high quality quinoa protein.

7. PERSPECTIVES

Considering the results obtained in this study, a series of investigations should be conducted to better understand the membrane system and to optimize the process for improves the recovery of isolated quinoa proteins, as well as phenolic compounds. Since the meal residue contains a significant content of proteins, it is suggested to add enzymes before the extraction process to increase the overall yield of protein extraction. Also, considering that two protein isolates are derived from this work, it is recommended to study the functional properties of these isolates, as well as their sensory characteristics in a food system. The amino acid profile of the quinoa protein isolates obtained in this work must be determined to corroborate its nutritional value; as well as to carry out structural elucidation and sequencing the primary structure of proteins. In addition, considering the importance of the phenolic compound concentrate as an antioxidant/functional ingredient, it is suggested to perform the identification of the phenolic compounds present in the powders obtained from the nanofiltration process.

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9. APPENDIX A

Analytical Methods

A1. International Approved Methods of the American Association of Cereal Chemist, AACC Method 44-15A, Moisture -- Air-Oven Methods

2 g of sample were placed in a preweigthed aluminum tray, and the weight was measured; The tray was covered with a punctured piece of aluminum foil, after replicates were prepared, the samples were placed in a hot air oven at 105°C overnight afterwards, the samples were removed from oven, and cooled to room temperature in a desiccator.

The weight of the dried samples was recorded.

The moisture content (M) was calculated using the following equation:

$$M = \left(\frac{W_2 - W_0}{W_1}\right) * 100$$

Where

M= Percent moisture content,

W_2= Weight of the dry sample and the tray (g),

W_0= Weight of the tray (g), and

W_1= Weight of the initial sample (g)

A2. Quantification of total polyphenols

The content of phenolic compounds was measured following the method reported by Julián-Loaeza *et al*. [63].

Preparation of solutions.

- 90% (v/v) EtOH: In a volumetric flask, 90 mL of EtOH was added to 10 mL of water.
- 0.5% (w/v) Na_2CO_3: In a volumetric flask, 50 mg of Na_2CO_3 was dissolved in 10 mL of water.
- 0.1 M Folin-Ciocalteu reagent: 0.5 mL of the 2 M Folin-Ciocalteu reagent was dissolved in water in the absence of light to a final volume of 10 mL in a volumetric flask.
- 100 ppm gallic acid stock solution: In a volumetric flask, 1 mg of gallic acid, was dissolved in 90% MeOH to a volume of 10 mL. The gallic acid solution standards of 10, 20, 30, 40 and 50 ppm were prepared from the gallic acid 100 ppm stock solution.

Methanolic extract. The methanolic extract was prepared by mixing 0.5 g of the flour in 1 mL of MeOH. The mixture was sonicated for 30 min at ambient temperature. Subsequently, the mixture was centrifuged for 4 min at 3000 rpm, and the supernatant was recovered for the quantification.

Quantification. The determination of total phenol content was carried out using the colorimetric method of Folin-Ciocalteau [93] with some modifications. In a 1.5 mL Ependorf tube, 0.5 mL of the sample or the standard was added together with 0.5 mL of the 0.1 M Folin-Ciocalteau reagent. The mixture was left at rest for 3 min at ambient temperature, followed by stirring in a vortex for 15 s at low speed. Following this, 0.5 mL of 0.5% Na_2CO_3 was added to the mix with a multichannel micropipette, and the mixture was left in repose for 30 min at ambient temperature. Sample or standard aliquots of 120 µL were taken to the microplate reader. The sample or standard was shaken for 1 min at medium speed in the reader, after which the absorbance of the sample or the standard was read

using a 750 nm filter. For the blank of the sample or standard water was added instead of the Folin-Ciocalteu reagent. The calibration curve was constructed using the measurements of the gallic acid solution standards and the total phenolic content of the samples was expressed as mg eq. of gallic acid per 100 g of dry weight of the sample (mg Eq. GA/ 100 g d.w.). All the determinations were carried out in triplicate.

A3. Obtaining equation 8, to determine the carotenes (Wrostald et al. [62]).

From the equation of Lambert-Beer (Equation 11)

$$A = \varepsilon * C * l \qquad (Ec. 11)$$

$$\therefore \qquad C = \frac{A*Mw}{\varepsilon*l}$$

Where:

A = absorbance of the sample at 450 nm

ε = molar absorptivity of β-carotene = 139 L/mol x cm

C = Concentration of solute (mol • L^{-1})

l = Bucket length (1 cm)

Mw: molecular weight of β-carotene

$$= \frac{A_{450} * \ 537 \ \frac{mg}{mmol}}{\left[139000 \ \frac{l_{dissolution}}{mol \ _{\beta-\text{carotene}} * cm}\right]\left[\frac{1mol \ _{\beta-\text{carotene}}}{1000 \ mmol}\right]}$$

$$= 0.38 \ mg \ eq. \ \frac{_{\beta-\text{carotene}}}{l \ issolution} \qquad (Ec. 12)$$

Aliquots of quinoa flour samples of 25 mL was prepared; these results were performed in triplicate.

$$C = \frac{m}{v}$$

$$\therefore \quad m = C * v \qquad (Ec. 13)$$

Where:

m: mass

v: volume

$$m = 0.38\ mg\ eq.\ \frac{\beta-\text{carotene}}{l\ issolution} * 0.025\ l = 0.0095\ mg\ \beta - \text{carotene}$$

APPENDIX B

B1. Quinoa flour analysis

Protein content as is

Experiment #	Samples	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P
	Blank	0	0	0	0
1	1	0.5119	7.51	2.05	12.84
	2	0.5170	7.59	2.06	12.85
	3	0.5165	7.60	2.06	12.88
2	1	0.5041	7.29	2.02	12.65
	2	0.5049	7.33	2.03	12.70
	3	0.5031	7.22	2.01	12.56
3	1	0.5042	7.25	2.01	12.58
	2	0.5014	7.10	1.98	12.39
	3	0.5069	7.22	1.99	12.46

Average	12.66
SD	0.174

Moisture content quinoa flour

Sample	Initial weight (g)	Final weight (g)	Moisture (%)
1	.8069	1.9791	10.3
2	.8247	1.9322	10.3
3	.8062	1.9362	9.3

Average	10.1
SD	0.29

B2. Extractability curve

Experiment 1			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa (%)**	**g of protein in the starting material**
10.00	295.2	12.66	1.266

Experiment 2			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.06	284.1	12.66	1.273

pH 8							
Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the solution**	**Extractability (%)**
1	Blank	0	0	0	0	0	0
	1	20.0	3.72	0.026	0.163	0.480	37.9
	2	20.0	3.70	0.026	0.162	0.478	37.7
	3	20.0	3.69	0.026	0.161	0.477	37.6
2	1	20.0	3.80	0.027	0.166	0.472	37.1
	2	20.0	3.79	0.027	0.166	0.471	37.0
	3	20.0	3.82	0.027	0.167	0.475	37.3

Average	37.4
SD	0.38

pH 9			
Experiment 1			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.00	284.1	12.66	1.266

Experiment 2			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.05	278.9	12.66	1.272

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the solution**	**Extractability (%)**
1	Blank	0	0	0	0	0	0
	1	20.0	4.23	0.030	0.185	0.526	41.5
	2	20.0	4.20	0.029	0.184	0.522	41.2
	3	20.0	4.18	0.029	0.183	0.520	41.0
2	1	20.0	4.22	0.030	0.185	0.515	40.5
	2	20.0	4.24	0.030	0.186	0.517	40.7
	3	20.0	4.20	0.029	0.184	0.512	40.3

Average	40.87
SD	0.47

pH 10			
Experiment 1			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.00	282.8	12.66	1.266

Experiment 2			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.03	**279.2**	**12.66**	**1.270**

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the solution**	**Extractability (%)**
1	Blank	0	0	0	0	0	0
	1	20.0	4.60	0.032	0.201	0.569	44.9
	2	20.0	4.62	0.032	0.202	0.572	45.1
	3	20.0	4.60	0.032	0.201	0.569	44.9
2	1	20.0	4.65	0.033	0.203	0.568	44.7
	2	20.0	4.60	0.032	0.201	0.562	44.2
	3	20.0	4.62	0.032	0.202	0.564	44.4

Average	44.74
SD	0.34

pH 11			
Experiment 1			
Quinoa Weight (g)	**Weight extract(g)**	**Protein content quinoa**	**g of protein in the starting material**
10.00	285.2	12.66	1.266

Experiment 2			
Quinoa Weight (g)	**Weight extract(g)**	**Protein content quinoa**	**g of protein in the starting material**
10.10	279.5	12.66	1.279

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the solution**	**Extractability (%)**
1	Blank	0	0	0	0	0	0
	1	20.0	5.00	0.035	0.219	0.624	49.3
	2	20.0	5.00	0.035	0.219	0.624	49.3
	3	20.0	5.10	0.036	0.223	0.636	50.3
2	1	20.0	5.30	0.037	0.232	0.648	50.7
	2	20.0	5.10	0.036	0.223	0.624	48.8
	3	20.0	5.19	0.036	0.227	0.635	49.6

Average	49.65
SD	0.71

pH 11.5			
Experiment 1			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.00	285.5	12.66	1.266

Experiment 2			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.02	291.8	12.66	1.269

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the solution**	**Extractability (%)**
1	Blank	0	0	0	0	0	0
	1	20.0	5.50	0.039	0.241	0.687	54.2
	2	20.0	5.49	0.038	0.240	0.686	54.1
	3	20.0	5.51	0.039	0.241	0.688	54.3
2	1	20.0	5.40	0.038	0.236	0.689	54.3
	2	20.0	5.39	0.038	0.236	0.688	54.2
	3	20.0	5.40	0.038	0.236	0.689	54.3

Average	54.28
SD	0.08

pH 12			
Experiment 1			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.01	282.1	12.66	1.267

Experiment 2			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
10.03	277.0	12.66	1.270

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the solution**	**Extractability (%)**
1	Blank	0	0	0	0	0	0
	1	20.0	6.21	0.043	0.272	0.766	60.5
	2	20.0	6.20	0.043	0.271	0.765	60.4
	3	20.0	6.20	0.043	0.271	0.765	60.4
2	1	20.0	6.28	0.044	0.275	0.761	59.9
	2	20.0	6.29	0.044	0.275	0.762	60.0
	3	20.0	6.26	0.044	0.274	0.759	59.7

Average	60.17
SD	0.31

B3. Precipitability of quinoa proteins as a function of pH

Alkaline extract			
Experiment 1			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
30.05	910.4	12.66	3.804

Experiment 2			
Quinoa Weight (g)	**Weight extract (g)**	**Protein content quinoa**	**g of protein in the starting material**
30.02	904.9	12.66	3.800

Experiment #	**Sample**	**Vol (ml)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**
1	Blank	0	0	0	0
	1	20.0	5.80	0.041	0.254
	2	20.0	5.81	0.041	0.254
	3	20.0	5.82	0.041	0.255
2	1	20.0	5.82	0.041	0.255
	2	20.0	5.83	0.041	0.255
	3	20.0	5.82	0.041	0.255

Average	0.254
SD	0.0005

B4. Soluble protein isolated

pH 2

Experiment 1	Experiment 2
Weight extract (g)	**Weight extract (g)**
202.4	205.8

Experiment #	Sample	Vol (mL)	SO_4H_2 0.1 N (ml)	% N	% P	g of protein in the starting solution
1	Blank	0	0	0	0	0
	1	20.0	1.72	0.012	0.075	0.152
	2	20.0	1.91	0.013	0.084	0.169
	3	20.0	1.92	0.013	0.084	0.170
2	1	20.0	2.10	0.015	0.092	0.189
	2	20.0	2.10	0.015	0.092	0.189
	3	20.0	2.00	0.014	0.088	0.180

pH 3

Experiment 1	Experiment 2
Weight extract (g)	**Weight extract (g)**
202.1	201.8

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the starting solution**
1	Blank	0	0	0	0	0
	1	20.0	1.51	0.011	0.066	0.134
	2	20.0	1.51	0.011	0.066	0.134
	3	20.0	1.50	0.011	0.066	0.133
2	1	20.0	1.50	0.011	0.066	0.132
	2	20.0	1.55	0.011	0.068	0.137
	3	20.0	1.55	0.011	0.068	0.137

pH 3.5

Experiment 1	Experiment 2
Weight extract (g)	**Weight extract (g)**
200.04	200.01

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the starting solution**
1	Blank	0	0	0	0	0
	1	20.0	1.29	0.009	0.056	0.113
	2	20.0	1.30	0.009	0.057	0.114
	3	20.0	1.40	0.010	0.061	0.123
2	1	20.0	1.49	0.010	0.065	0.130
	2	20.0	1.48	0.010	0.065	0.130
	3	20.0	1.41	0.010	0.062	0.123

pH 4

Experiment 1	Experiment 2
Weight extract (g)	**Weight extract (g)**
202.10	199.9

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the starting solution**
1	Blank	0	0	0	0	0
	1	20.0	1.50	0.011	0.066	0.133
	2	20.0	1.51	0.011	0.066	0.134
	3	20.0	1.51	0.011	0.066	0.134
2	1	20.0	1.50	0.011	0.066	0.131
	2	20.0	1.50	0.011	0.066	0.131
	3	20.0	1.49	0.010	0.065	0.130

pH 4.5

Experiment 1	Experiment 2
Weight extract(g)	**Weight extract(g)**
200.02	200.02

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the starting solution**
1	Blank	0	0	0	0	0
	1	20.0	1.40	0.010	0.061	0.123
	2	20.0	1.41	0.010	0.062	0.123
	3	20.0	1.42	0.010	0.062	0.124
2	1	20.0	1.50	0.011	0.066	0.131
	2	20.0	1.52	0.011	0.067	0.133
	3	20.0	1.51	0.011	0.066	0.132

pH 5

Experiment 1	Experiment 2
Weight extract (g)	**Weight extract (g)**
202.1	197.7

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the starting solution**
1	Blank	0	0	0	0	0
	1	20.0	1.8	0.013	0.079	0.159
	2	20.0	1.80	0.013	0.079	0.159
	3	20.0	1.81	0.013	0.079	0.160
2	1	20.0	1.99	0.014	0.087	0.172
	2	20.0	2.10	0.015	0.092	0.182
	3	20.0	2.10	0.015	0.092	0.182

pH 6

Experiment 1	Experiment 2
Weight extract (g)	**Weight extract (g)**
202.1	200.8

Experiment #	**Sample**	**Vol (mL)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**g of protein in the starting solution**
1	Blank	0	0	0	0	0
	1	20.0	2.79	0.020	0.122	0.247
	2	20.0	2.79	0.020	0.122	0.247
	3	20.0	2.70	0.019	0.118	0.239
2	1	20.0	3.30	0.023	0.144	0.290
	2	20.0	3.30	0.023	0.144	0.290
	3	20.0	3.31	0.023	0.145	0.291

B5. Precipitated protein isolated

pH 2

Experiment 1
Weight extract (g)
202.4

Sample	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P	Precipitate DW	g of protein in the precipitated	g of protein in the starting solution (SPI)	% Precipitability
Blank	0	0	0	0	0	0	0	0
1	0.5074	7.81	2.155	13.47	0.446	0.0601	0.1523	39.44
2	0.5041	7.80	2.166	13.54		0.0604	0.1691	35.70
3	0.5009	7.52	2.102	13.14		0.0586	0.1700	34.46
1	0.5051	8.31	2.303	14.40	0.461	0.0664	0.1891	35.10
2	0.5044	8.69	2.412	15.07		0.0695	0.1891	36.75
3	0.5069	7.95	2.196	13.72		0.0633	0.1801	35.13

Average	36.10
SD	1.81

pH 3

Experiment 1
Weight extract (g)
202.10

Experiment #	**Sample**	**Mass (g)**	**SO_4H_2 0.1 N (ml)**	**% N**	**% P**	**Precipitate DW**	**g of protein in the precipitate**	**g of protein in the starting solution**	**% Precipitability**
1	Blank	0	0	0	0	0	0	0	0
	1	0.5018	9.00	2.511	15.69	0.575	0.0902	0.1335	67.59
	2	0.5023	9.30	2.592	16.20		0.0932	0.1335	69.77
	3	0.5060	8.89	2.460	15.37		0.0884	0.1326	66.65
2	1	0.5088	10.98	3.021	18.88	0.442	0.0835	0.1324	63.02
	2	0.5013	10.68	2.983	18.64		0.0824	0.1368	60.21
	3	0.5017	10.70	2.986	18.66		0.0825	0.1368	60.28

Average	64.59
SD	4.01

pH 3.5

Experiment 1
Weight extract (g)
200.04

Experiment #	Sample	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P	Precipitate DW	g of protein in the precipitate	g of protein in the starting solution	% Precipitability
1	Blank	0	0	0	0	0	0	0	0
	1	0.5091	11.48	3.157	19.73	0.433	0.0854	0.1129	75.67
	2	0.5064	11.45	3.165	19.78		0.0857	0.1138	75.30
	3	0.5011	11.42	3.191	19.94		0.0863	0.1225	70.47
2	1	0.5034	11.30	3.143	19.64	0.452	0.0888	0.1304	68.09
	2	0.5009	11.90	3.326	20.79		0.0940	0.1295	72.55
	3	0.5073	11.32	3.124	19.52		0.0883	0.1234	71.53

Average	72.27
SD	2.90

pH 4

Experiment 1
Weight extract (g)
200.60

Experiment #	Sample	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P	Precipitate DW	g of protein in the solution	g of protein in the starting solution	% Precipitability
1	Blank	0	0	0	0	0	0	0	0
	1	0.5004	9.11	2.549	15.93	0.666	0.1061	0.1326	79.99
	2	0.5008	9.10	2.544	15.90		0.1059	0.1335	79.31
	3	0.5079	9.30	2.563	16.02		0.1067	0.1335	79.92
2	1	0.5059	10.49	2.903	18.14	0.504	0.0914	0.1312	69.71
	2	0.5076	10.39	2.866	17.91		0.0903	0.1312	68.81
	3	0.5072	10.30	2.843	17.77		0.0896	0.1303	68.73

Average	74.41
SD	5.85

pH 4.5

Experiment 1
Weight extract (g)
200.02

Experiment #	Sample	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P	Precipitate DW	g of protein in the solution	g of protein in the starting solution	% Precipitability
1	Blank	0	0	0	0	0	0	0	0
	1	0.502	11.30	3.151	19.70	0.401	0.0790	0.1225	64.47
	2	0.5062	11.42	3.158	19.74		0.0792	0.1234	64.15
	3	0.5091	11.42	3.140	19.63		0.0787	0.1243	63.34
2	1	0.5025	11.19	3.118	19.49	0.437	0.0851	0.1313	64.87
	2	0.5057	11.20	3.101	19.38		0.0847	0.1330	63.67
	3	0.5062	11.20	3.098	19.36		0.0846	0.1321	64.03

Average	64.09
SD	0.55

pH 5

Experiment 1
Weight extract (g)
200.3

Experiment #	Sample	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P	Precipitate DW	g of protein in the solution	g of protein in the starting solution	% Precipitability
1	Blank	0	0	0	0	0	0	0	0
	1	0.5056	8.10	2.243	14.02	0.498	0.0698	0.1592	43.86
	2	0.5045	8.23	2.284	14.27		0.0711	0.1592	44.66
	3	0.5061	8.25	2.282	14.26		0.0710	0.1600	44.38
2	1	0.504	10.00	2.778	17.36	0.454	0.0788	0.1721	45.79
	2	0.5026	9.90	2.758	17.24		0.0782	0.1816	43.08
	3	0.5062	10.40	2.876	17.98		0.0816	0.1816	44.93

Average	44.45
SD	0.93

pH 6

Experiment 1
Weight extract(g)
200.2

Experiment #	Sample	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P	Precipitate DW	g of protein in the solution	g of protein in the starting solution	% Precipitability
1	Blank	0	0	0	0	0	0	0	0
	1	0.5093	7.22	1.985	12.40	0.255	0.0316	0.2467	12.82
	2	0.5098	7.52	2.065	12.91		0.0329	0.2471	13.32
	3	0.5068	7.60	2.099	13.12		0.0335	0.2391	13.99
2	1	0.5067	7.60	2.100	13.12	0.252	0.0331	0.2899	11.41
	2	0.5011	7.60	2.123	13.27		0.0334	0.2899	11.54
	3	0.5070	7.80	2.154	13.46		0.0339	0.2908	11.67

Average	12.46
SD	1.08

B6. Protein content in fractions

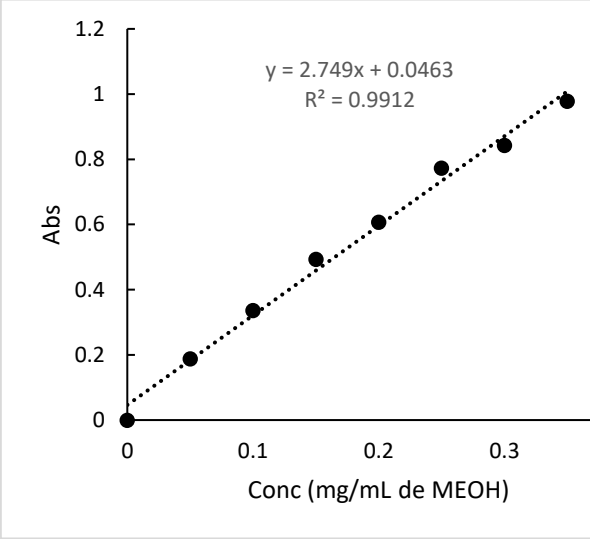
Meal Residue (dry basis)

Experiment #	Sample	Mass (g)	SO_4H_2 0.1 N (ml)	% N	% P
	Blank	0	0	0	0
1	1	0.5119	2.89	0.790	4.94
	2	0.517	2.85	0.772	4.82
	3	0.5165	2.84	0.770	4.81
2	1	0.5089	2.60	0.715	4.47
	2	0.5067	2.59	0.716	4.47
	3	0.5087	2.60	0.715	4.47

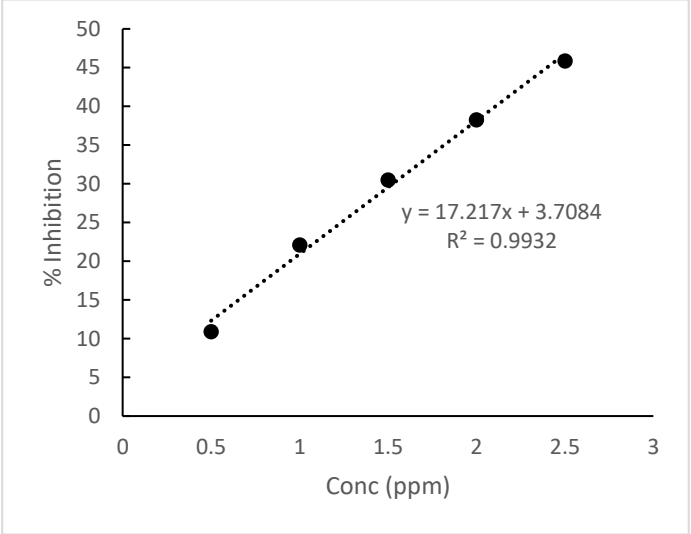
Average	4.66
SD	0.22

APPENDIX C

C1. Standard curve for sinapic acid



C3. Standard curve for trolox.



C.2. Standard curve for gallic acid.