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Research Article
Gastrointestinal Evaluation of Tocte Protein Concentrate (*Juglans neotropica* Diels) and Their Chemical Composition

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Abstract

Background and Objective: Genus *Juglans* also known as walnut has about 23 species distributed around the world. The objective of this study was to obtain tocte protein concentrate (TPC) from *Juglans neotropica* Diels and subject them to a process of *in vitro* hydrolysis simulating gastric digestion and duodenal human digestion using a standardized method. **Materials and Methods:** The TPC was obtained of defatted tocte flour (*Juglans neotropica* Diels) at pH 5.0 of precipitation. The TPC was characterized by SDS-PAGE and RP-UHPLC techniques. The TPC profile amino acids were determined by HPLC. The TPC was subject at *in vitro* simulated gastrointestinal digestion. **Results:** *In vitro* digestibility was determined using pepsin and pancreatin. At pH 5.0 precipitation, yield is 21.6% of TPC with a high protein content of 74.8%. The content of fractions of protein globulin, albumin, glutelin and prolamin were 45.83, 26.67, 18.65 and 8.86% of the total extractable protein, respectively. Gastric digestion at pH 1.2, 2.0 and 3.0 showed a band not hydrolyzed with a molecular weight of 6.5 kDa corresponding to 2S albumin. In the duodenal digestion, all proteins were completely hydrolyzed at the conditions assayed. **Conclusion:** The TPC have high digestibility with pepsin and pancreatin enzymes. The TPC profile of amino acids confirmed that tocte can be an excellent source of plant protein for their amino acid composition. The TPC can be used as functional ingredients and high digestibility.

Key words: Tocte protein concentrate, *in vitro* hydrolysis, *Juglans neotropica* Diels, gastric digestion, duodenal digestion

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Proteins vegetables play an important role in human nutrition, especially in developing countries where protein intake is less than the recommended dose[1]. Now-a-days, there is high interest in the development of products based on vegetable protein, which allows an increase of vegetable protein isolates for the food industry. Currently, products sold are mainly based on soy, quinoa, amaranth, lupine seeds as a source of protein for human dietary supplements[2-4].

Genus Juglans also known as walnut has about 23 species distributed in North, Central and South America, Eastern Europe and Asia. The two most important species are economically the Persian walnut (*Juglans regia*) appreciated for the quality of its nuts and the black walnut (*Juglans nigra*) highly prized for the quality of its timber. In South America, there are the following species: Argentinian walnut (*Juglans australis*), Bolivian walnut (*Juglans boliviana*) and black cedar (*Juglans neotropica*) found in Colombia, Venezuela, Peru and Ecuador and known as tocte[5].

China has a high production of walnut (*Juglans regia*) with 1655 t in 2011, thus being the most dispersed walnut tree in the world[1]. Walnuts have a high nutritional value due to their high composition of oils (65%) and proteins (18-24%). They are used in the manufacturing of chocolates and as ingredients in the production of many food and bakery products[6-8]. Composition of oils has been widely studied. However, the composition and characterization of the proteins is present in very few studies. The Food and Drug Administration (FDA) has approved health claims indicating that diets rich in walnuts can reduce the risk of heart disease[9]. It is known that plant proteins from legume and non-legume plants have two of the main classes of storage proteins. These proteins are named 7S and 11S depending of their sedimentation coefficients. The 11S globulins are hexamers with molecular weights between 300-400 kDa, consisting of two opposed hexagonal rings, each containing three hydrophobically associated pairs of disulfide-linked acidic (29-35 kDa) and basic (18-28 kDa) subunits. 7S globulins are glycoproteins with molecular weights between 150-200 kDa[10]. The occurrence of 11S and 7S type storage globulins in angiosperm seeds has been recognized and accepted[11,12].

The specie *Juglans neotropica* Diels is the least studied in the composition of both oils and proteins. It is also important to analyze the physiological digestibility of these proteins, as they can be allergens and have resistance to hydrolysis[13]. The process of gastrointestinal digestion of food proteins can release bioactive peptides which are small sequences between 3-21 amino acids, these bioactive peptides can have different activities such as antioxidant, antiviral, anti-inflammatory, antibacterial activities[14]. *In vivo* digestion models are complicate for these reason *in vitro* models have advantage. Another study describe the simulate protein digestion using simulated gastric fluid (SGF) which corresponding at solution (NaCl 0.035 M), this medium is indicated to subject to hydrolysis at food proteins[15].

The objective of this study was to obtain protein concentrate from *Juglans neotropica* Diels determinate their amino acid composition and subject them to a process of *in vitro* hydrolysis simulating gastric digestion and duodenal human digestion using a standardized method.

MATERIALS AND METHODS

Study area: This research study was carried out during the months of January to December of the years 2016 and 2017. It was carried out in the Laboratory of Functional Foods of the Faculty of Science and Engineering of Food and Biotechnology of the Technical University of Ambato (Ambato)-Ecuador) and in the research laboratories of the State University of Bolivar (Guaranda-Ecuador). Tocte nuts (*Juglans neotropica* Diels) were obtained of Andean crop from Otavalo-Ecuador.

Preparation of defatted tocte flour: Defatted tocte flour (DTF) was produced according to the method of Quinteros *et al.*[16]. Tocte (*Juglans neotropica* Diels) nuts were purchased from the supermarket shelves in Ecuador. Walnuts were ground in a windmill. The flour was defatted with hexane [flour/hexane ratio of 1:10 (w/v)] under constant magnetic stirring for 3 h. The slurry was vacuum filtered through a paper filter and the residue was used for subsequent extraction. Hexane extractions were repeated until the filtrate was clear. Residue from the last extraction and filtration step was air dried in a fume hood. DTF was stored at -20°C until further use.

Preparation of tocte protein concentrate (TPC): TPC was prepared according to the process described by Acosta *et al.*[17] with minor modifications. Defatted tocte flour (DTF) was extracted by stirring for 2 h at room temperature (about 25°C) with de-ionized water adjusted to pH 8.0 with 1 M NaOH [flour:water ratio, 1:20 (w/v)]. The slurry was centrifuged at 5,000×g for 30 min at 25°C in an Eppendorf centrifuge (USA). The insoluble tocte protein pellet was re-slurred with pH adjusted de-ionized water as above and centrifuged again. The supernatants mixed were adjusted to pH 3.0, 4.0, 5.0 and 6.0 (isoelectric point), kept for 48 h at 4°C with 1 M HCl and subsequently centrifuged at 5,000×g for 30 min at the same temperature. The precipitate was washed with de-ionized

water, resolubilized in de-ionized water, neutralized to pH 7 with 1 M NaOH at room temperature, then dialyzed against water and lyophilized. All lyophilized protein samples were stored in airtight plastic bottles at -20°C until further use.

Protein fractionation: Protein was extracted sequentially with 1.0 M NaCl (albumin-globulin), then 70% ethanol (prolamin) and finally, 0.1 M NaOH (glutelin) (defatted flour/solvent ratio of 1:10 w/v) for 1 h at 25°C under constant magnetic stirring. The slurry was then centrifuged (5,000×g, 25°C, for 30 min) and the supernatant was vacuum filtered using paper filter to remove insoluble particles. The fractions were then dialyzed against water and lyophilized. All lyophilized protein fractions were stored at -20°C until further use. Extracting rate was calculated as follows: lyophilized protein fractions divided by total protein, according to the Osborne method[18].

Proximate analysis of tocte flour and TPC: Protein, moisture, fat, ash and fiber contents were determined according to the methods of AOAC[19], numbers 920.152, 950.46, 930.09, 920.153 and 934.10, respectively. Determination of soluble solids of the materials was made according to the ISO 2173:2003 method[20]. The protein contents of samples were determined by the Micro-Kjeldahl method[21] using a protein nitrogen coefficient of 6.25. Carbohydrates were determined according to the method of Zhu *et al.*[22]. The contents were expressed on a dry weight basis. Each analysis was done in triplicate and data was reported as means±standard deviation.

Amino acids composition of TPC: Amino acids analysis was determined according to the method of Zhu *et al.*[22]. Samples of TPC (100 mg) were subjected to acid hydrolysis with 5 mL of 6 M HCl under nitrogen atmosphere for 24 h at 110°C. Each hydrolysate was washed into a 50 mL volumetric flask and made up to the mark with distilled water. The amino acids were subjected to RP-HPLC analysis (Agilent 1100) after precolumn derivatization with O-phthaldialdehyde (OPA) or with 9-fluorenylmethyl chloroformate (FMOC). Methionine and cysteine were determined separately by oxidation products before hydrolysis in 6 M HCl. Amino acid composition was reported as g of amino acid/100 g of protein.

Electrophoresis analysis: The samples were analyzed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE electrophoresis of tocte (*Juglans neotropica Diels*) proteins, proteins fractions and finally products of enzymatic hydrolysis of proteins, were carried out according to Poveda *et al.*[23]. Runs were performed in Miniprotean (Bio-Rad, Hercules, CA, USA). In SDS-PAGE system gels of 12 g acrylamide/100 mL of resolving gel and 4 g acrylamide/100 mL of stacking gels were used. Relative molecular masses of protein were determined by comparison to the molecular weight marker with polypeptide SDS-PAGE standards (MW 6.5-200 kDa and 10-250 KDa) (Bio-Rad) and (MW14-97 kDa GE Healthcare Life Sciences). Gels were fixed and stained with Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St. Louis, MO) for 16 h.

Analysis of TPC using reversed-phase high-performance liquid chromatography (RP-UHPLC): The TPC at pH 5.0 was analyzed using RP-UHPLC technique on Agilent 1200 infinity series UHPLC System (Agilent Technologies, Waldbronn, Germany). The analysis was made using a wavelength of 280 nm. The separation of compounds was made using a column EC C18 (Agilent Poroshell 120, 4.6×50 mm×2.7 µm of particle size). Samples were eluted at 1.0 mL/min with a linear gradient from 0-70% of solvent B (acetonitrile+trifluoroacetic acid) in solvent A (water+trifluoroacetic acid) for 10 min. The injection volume was 100 µL for each duplicated sample[24].

Enzymatic digestion of TPC: The digestive process (10 mg mL^{-1} of TPC at pH 5.0). The environment of the stomach and duodenum was reproduced as closely as possible. This study uses a model which emulates the environment of human gastrointestinal tract with its pH, the presence of enzymes, bile salts, temperature and movement.

Gastric digestion: It used pepsin in a concentration of 2.000 U mL^{-1} of protein at different pHs: pH 1.2, 2.0 and 3.0 dissolved in 0.035 M NaCl, for 2 h at 37°C with agitation. The activity of pepsin was stopped with heat at 90°C, for 10 min.

Duodenal digestion: Pancreatin in a concentration of 100 U mL^{-1} of protein was used. The solution of pancreatin was dissolved in monobasic potassium phosphate solution with bile salt 10 mM and $CaCl_2$ 1.5 M.

One milliliter of gastric digestion at pH 3.0 was mixed with 1 mL of solution of pancreatin in medium for 3 h at 37°C with agitation. The reaction was stopped with heat at 90°C, for 10 min[25,26].

Statistical analysis: Results are presented as means±standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The *post hoc* analysis was performed by the Tukey test. All tests were considered significant at $p<0.05$. Statistical analysis was performed using SPSS (Software version 11.0, Chicago, IL, USA).

RESULTS AND DISCUSSION

Table 1 shows the proximate analysis of defatted tocte flour (DTF) obtained with water. Fat content of DTF and TPC were significantly different ($p<0.05$). Protein content of DTF and TPC also were significantly different ($p<0.05$). The DFT contents 17.7% of protein while the protein content in TPC was 74.8%. The TPC contained a significant higher amount of protein. Protein content reported by Sze-Tao and Sathe[27] had a value of 16.66% and Mao and Hua[1] reported a value of 17.66% for defatted *Juglans regia* L. walnut flour. These results are then in accordance with these previous reports. The carbohydrates content was also low in TPC. The fat content of DTF was high, with a value of 49.1%. The tocte flour was defatted with hexane for 16 h. After being defatted, fat content amounted to 10%.

Protein fractionation of TPC: Seed proteins classification according to their solubility was developed with the Osborne method[18], who distinguished 4 fractions: Albumin (water-soluble), globulin (salt-soluble), prolamins (alcohol-soluble) and glutelin (NaOH-soluble). The proportion of walnut proteins fractions is shown in Fig. 1. Globulin was found to be the main protein fraction of *Juglans neotropica* Diels, which content was higher than the one reported[27]. The content of globulin, albumin, glutelin and prolamin were 45.83, 26.67, 18.65 and 8.86% of the total extractable protein, respectively. The yield percentage presents statistical differences with $p<0.05$.

Analysis of TPC by SDS-PAGE electrophoresis and RP-UHPLC: In order to characterize the proteins of *Juglans neotropica* Diels, we studied the presence of proteins, number of bands and the approximate molecular mass using the SDS-PAGE electrophoresis in the presence and absence of reducing agent 2-mercaptoethanol.

The TPC profile was analyzed at reducing conditions (Fig. 2a). The TPC showed eleven bands, out of which 3 bands showed high expression with molecular weights of 20, 35 and 50 kDa (Fig. 2a). Three groups of bands were clear with molecular weights ranging from 6.5-50 kDa. Those 3 groups of bands were clearly stained with Coomassie at reducing conditions.

These results of *Juglans neotropica* Diels were similar to the ones reported by Mao *et al.*[2] to *Juglans regia* L. with masses of 40, 35, 23 and 20 kDa. Both species have then a similar profile of proteins obtained with the alkaline extraction isoelectric precipitation method. The TPC at pH 5.0 presented the highest expression of proteins, those proteins were strongly stained with Coomassie and presented a correlation with the best yield.

The TPC obtained at pH 5.0 was analyzed by RP-UHPLC method. Figure 2b show the profile of different fractions of TPC. Fractions contained in TPC eluted in the eight first minutes of analysis. Four fractions from TPC were identified in the analysis. The first fraction is very soluble in the solvent A because is no retained for the column.

Amino acid composition of TPC: Amino acid composition is an important chemical property of proteins, to determine their nutritional value. The amino acids compositions of walnut proteins were measured and results are shown in Table 2. The analysis of no essential amino acid shows that aspartic acid and glutamic acid are present in low contents. There are high levels of serine and histidine compared to defatted flour and protein concentrate of *Juglans regia*. Content of histidine was very high compared to the FAO and WHO[28] recommended values analysis of essential amino acids shows that methionine is the first limiting amino acid followed with lysine. Both amino acids are limiting amino acid in *Juglans regia*. In the literature, methionine and lysine have been reported

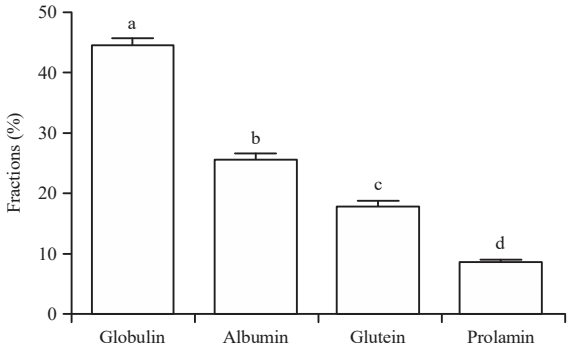

Fig. 1: Fractionation of proteins from *Juglans neotropica* Diels
Different letters indicate statistical differences at $p<0.05$ (ANOVA One-Way)
Defatted tocte flour

Table 1: Proximate analysis of DTF and TPC

Proximate analysis (%)	Protein	Fat	Moisture	Total fiber	Solids	Carbohydrates
DTF	17.70±0.4[a]	49.12±0.5[a]	6.22±0.4[a]	10.60±0.2[a]	2.10±0.2[a]	14.30±0.4[a]
TPC-pH 5	74.80±0.2[b]	10.50±0.2[b]	5.23±0.3[a]	0.13±0.2[b]	2.66±0.2[a]	6.70±0.2[b]

Results are present as mean±deviation standard (n = 3), different letters indicate statistical differences at $p<0.05$ (ANOVA One-Way), TPC: Tocte protein concentrate, DTF: Defatted tocte flour, Source: [a]Mao and Hua[1], [b]Mao *et al.*[2]

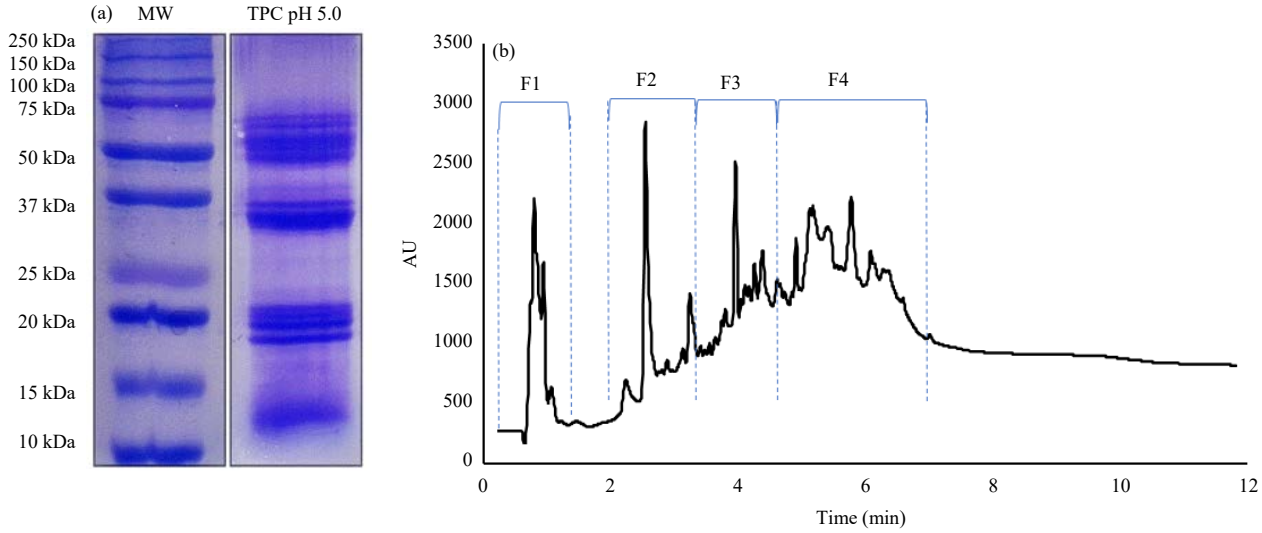

Fig. 2(a-b): Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high-performance liquid chromatography (RP-UHPLC) analysis of tocte protein concentrate (TPC), (a) Profile proteins of TPC by SDS-PAGE and (b) Profile protein of TPC by RP-UHPLC
F: Frequency

Table 2: Amino acid composition of tocte protein concentrate (TPC)

Amino acids	*J. regia*[a] Defatted flour	*J. regia* protein concentrate[b]	TPC pH 5.0 *J. neotropica*	FAO/WHO[c]
No essential amino acids				
Aspartic acid	10.11	10.22±0.43	5.68±0.2	1.9
Glutamic acid	24.06	22.16±0.40	1.87±0.3	
Serine	5.13	5.84±0.12	9.22±0.4	
Glycine	5.20	5.43±0.07	3.15±0.2	
Histidine	2.86	2.38±0.26	7.55±0.3	
Arginine	13.36	14.70±0.42	4.02±0.5	
Alanine	4.93	4.74±0.19	3.47±0.1	
Proline	4.29	4.22±0.29	4.03±0.4	
Essential amino acids				
Threonine	2.23	3.58±0.20	7.41±0.1	3.4
Tyrosine	2.02	2.76±0.11	2.87±0.1	
Valine	4.99	4.18±0.14	3.05±0.2	3.5
Methionine	1.46	1.16±0.12	1.33±0.2	2.5
Cysteine	0.21	0.84±0.08	0.73±0.3	
Phenylalanine	4.44	4.94±0.23	3.59±0.1	6.3
Isoleucine	4.17	3.28±0.15	2.69±0.4	2.8
Leucine	8.70	7.13±0.11	4.80±0.3	6.6
Lysine	3.08	2.58±0.16	2.22±0.2	5.8

Sources: [a]Mao and Hua[1], [b]Mao *et al.*[2], [c]FAO/WHO[28]

as amino acids limiting to walnut seed amino acid composition. Pea, like other grain legumes, is deficient in the sulfur-containing amino acids methionine and cysteine but has a relatively high content in lysine; hence, pea essential amino acid profile is complementary to the ones of cereal grains[29]. Threonine amino acid (7.41%) presents a high content in TPC compared to the FAO and WHO recommended values (3.4%). Tryptophan was not identified as analysis conditions caused destruction when acid hydrolysis with solution of 6 N HCl is used. The total content of amino acids (TAA) in TPC was 60.13% (without the contribution of Tryptophan which could not be detected). From these results, we concluded that walnut proteins could be a good source of essential amino acids for adults. TPC has a good nutritive value.

Gastrointestinal simulation of TPC: Products introduced into the human body are subject to a complex process of digestion, during which some components are digested, while others remain in an unchanged form. The TPC obtained at

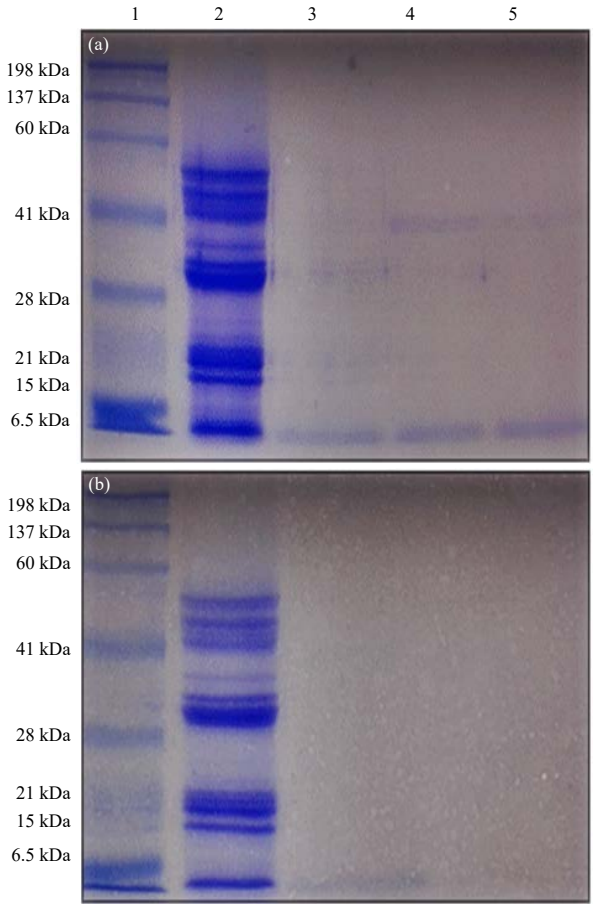

Fig. 3(a-b): Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of gastrointestinal digestion of tocte protein concentrate (TPC), (a) Gastric digestion of TPC, Lane 1: Molecular weight, Lane 2: TPC without hydrolysis, Lane 3: Gastric digestion at pH 1.2, Lane 4: Gastric digestion at pH 2.0, Lane 5: gastric digestion at pH 3.2 and (b) Duodenal digestion of TPC, Lane 1: Molecular weight, Lane 2: TPC without hydrolysis, Lane 3: Gastric hydrolysis of TPC at pH 3.2, Lane 4: Duodenal digestion of TPC, Lane 5: water

pH 5.0 was digested with pepsin (gastric digestion) and with a double digestion system pepsin and pancreatin (duodenal digestion). Those 2 types of digestions were examined using the SDS-PAGE electrophoresis (Fig. 3). Gastric digestion was evaluated with pepsin at different pHs: pH 1.2, 2.0 and finally pH 3.0 in SGF solution. The TPC profiles were characterized with the presence/absence of proteins with molecular weights between 6.5-60 kDa (Fig. 3a Lane 1). The hydrolysis with pepsin at pH 1.2, 2.0 and 3.0 leads to the disappearance of bands on SDS-PAGE gels. Only the band with a molecular weight of 6.5 kDa was resistant to hydrolysis with pepsin. The 6.5 kDa band corresponds to 2S tocte albumin.

The 2S albumin storage protein is an important type of common allergenic protein in many plant species, including several oilseeds, legumes and nuts[30]. Different authors have reported resistance to hydrolysis with *in vitro* gastrointestinal models of 2S albumins. Moreno and Clemente[31] reported resistance to hydrolysis of 2S albumins of Brazil nut named Ber e 1. The most potent allergens with resistance to hydrolysis come from peanut and are known as Ara h 6 and Ara h 7. Orruno and Morgan[32] have reported resistance to proteolytic digestion of 2S albumins of sesame protein (Ses i 1). Lehmann *et al.*[33] have reported two 2S peanut albumins with high resistance to hydrolysis with duodenal enzymes, as described by other authors. Resistance to hydrolysis of 2S mustard named Sin a 1 and Bra J 1 has also been reported by Pastorello *et al.*[34] and Sen *et al.*[35]. Other food proteins have been studied for their allergenicity such as milk proteins that affect a big number of infants. Milk proteins can be substituted in the infant formulas for different vegetal proteins such soybean, rice and other cereals for their high digestibility and little antigenicity[36-39].

The 45 kDa band found corresponds to residual pepsin (Fig. 3a Lane 2-4). TPC was totally hydrolyzed with a double system (pepsin and pancreatin). All tocte proteins were susceptible to hydrolysis with pepsin plus pancreatin. Bands corresponding to the range of 6.5-60 kDa were not observed (Fig. 3b).

The present study reports the characterization of nuts (*Juglans neotropica* Diels) proteins grown in the Andean region of Ecuador. Report for the first time the composition of amino acids presents in these proteins. It describes the hydrolysis of the nuts touch proteins under an *in vitro* hydrolysis model that simulates physiological conditions in humans with two phases of digestion (gastric digestion and duodenal digestion). The characterization of proteins has been carried out by electrophoresis and HPLC analysis. In the future it would be necessary to perform identification using HPLC-MS-MS. The peptides present in the hydrolysates can also be identified in the future using mass spectrometry and the identified peptides could be synthesized to know their biological properties. Obtaining protein touch concentrates opens the possibility for it to be used for food purposes.

CONCLUSION

In summary, the results of this study have shown that the extractability of tocte protein is pH sensitive and more specifically at pH 5.0. This study showed the characterization of proteins from *Juglans neotropica* Diels and its fractions of albumin, globulins, glutelin and prolamins. The results of this study showed the digestibility of tocte proteins with pepsin and pancreatin. From all tocte proteins studied, the tocte 2S albumin was highly stable to digestion with pepsin at pH 1.2, 2.0 and 3.2. All tocte proteins were susceptible of pancreatin hydrolysis. Composition of amino acids in tocte proteins indicated that proteins have a high nutritive value and can be a good source of plant proteins for human nutrition.

SIGNIFICANCE STATEMENTS

The present study focused on obtaining tocte protein concentrate from nuts of *Juglans neotropica* Diels a species distributed by several countries of South America but with few studies on their proteins. This study describes the chemical composition of nuts. Its proteins were characterized and the amino acid composition of the protein concentrate was determined. *In vitro* digestibility of touch proteins was evaluated.

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