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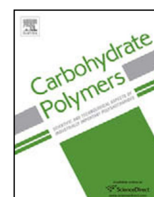

Starch extraction process coupled to protein recovery from leguminous tuberous roots (*Pachyrhizus ahipa*)

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ABSTRACT

The objective of this work was to fit together the starch extraction from *Pachyrhizus ahipa* roots and the recovery of the proteins present in these storage organs, making an improved use of this novel raw material. The replacement of water by buffer PO_4^{-3}/NaCl as solvent in the first extraction steps improved protein extraction without lowering the starch yield. The starches obtained from the traditional and the proposed methods exhibited some differences in appearance and technological and thermal properties, which were endorsed to the adjustment in the methodology of extraction rather than to the use of buffer as solvent. Thus, *P. ahipa* starch obtaining procedure could be coupled to protein extraction with a minimum change in the methodology. This innovation did not significantly shift the characteristics of the starch obtained and allowed to obtain a protein yield of 135.7 mg BSA equivalent protein/100 g of fresh roots.

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1. Introduction

Starch is a ubiquitous semi-crystalline polysaccharide used in food, textile, paper, chemical, pharmaceutical, and biotechnological industries (Falade & Okafor, 2013; Pascoal, Di-Medeiros, Batista, Leles, Lião & Fernandes, 2013; Schirmer, Höchstötter, Jekle, Arendt & Becker, 2013). Likewise, the general concern about greenhouse gas emissions and oil price rise have prompted the bioethanol industry, which has merged as another important application area which constantly requests new starch sources (Pascoal, Di-Medeiros, Batista, Leles, Lião & Fernandes, 2013). In this sense, root and tuber (R&T) crops are now receiving increasing attention as alternative starch sources, oriented to satisfy different commercial demands and associated to the increasing pressure on cereal starches (Falade & Okafor, 2013).

The industrial process of starch isolation from commercial sources consists mainly of the separation of starch from protein and fiber. Important considerations in starch extraction include avoidance of amylolytic or mechanical damage to the starch granules during the initial separation steps, effective deproteinization of the starch, and minimizing the loss of small granules (Lindeboom, Chang & Tyler, 2004).

In the cassava starch industrial production, the consumption of water and energy is decisive, given that production costs and starch quality rely on it. Throughout the extraction process, starch is purified by several stages of fiber screens and starch washers to remove soluble and finely divided fiber. However, the use of a decanter for water separation, as applied in potato starch processing, is optional since the protein and other impurities of cassava are very low (Breuninger, Piyachomkwan & Sriroth, 2009).

Conversely, in the potato starch manufacturing process, although starch itself represents >90% of the commercial value, the co-products (fiber, protein and concentrated deproteinized potato juice) represent almost 10% of the sale value. Likewise, protein quality is becoming increasingly important to the financial side of the entire process (Grommers & van der Krogt, 2009).

Some starch extraction methods use alkali to solubilize protein, allowing to the obtaining of pure starch from flours. Different alkaline agents, such as detergents and sodium hydroxide can be used as extraction solvents (Lee, Htoon & Paterson, 2007). Concerns about the disposal of effluents arise from the use of this kind of extraction media. Likewise, the use of alkaline extraction conditions could induce deterioration in the quality of starch isolates.

The genus *Pachyrhizus* (yam beans), belonging to the *Fabaceae* botanical family is native to Central and Southern America. The

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species *P. ahipa* thrives at the Andes of Peru, Bolivia and Northern Argentina (Forsyth & Shewry, 2002; Sørensen, Døygaard, Estrella, Kvist & Nielsen, 1997; Zanklan, Ahouangonou, Becker, Pawelzik & Grüneberg, 2007). The plant is highly suited to the needs of family farming since it has an efficient nitrogen capturing root system and can be grown with reduced nitrogen fertilizer inputs. Some studies reported the high symbiotic effectiveness of the association between *P. ahipa* roots and bacteria from de genus *Bradyrhizobium* (Kjaer, 1992; Rodríguez-Navarro et al., 2000), equivalent to yields obtained from plants fertilized with nitrate (Rodríguez-Navarro, Camacho, Leidi, Rivas & Velázquez, 2004).

P. ahipa produces enlarged roots weighing between 0.5 and 0.8 kg, which are the only part of the plant suitable for eating. These roots accumulate starch and a relatively considerable content of protein. According to Dini, Doporto, García, &Viña (2013), chemical composition of ahipa roots from different accessions showed the following values (on a dry basis): moisture 78.4–83.5%; total ash 3.32–4.20%; crude protein 7.9–11.5%; total starch 43.7–65.0%; total dietary fiber 20.8–25.9%; and crude fat 0.43–0.63%. As it can be observed, the latter values show that all ahipa accessions had relative low lipid content.

Ahipa roots are analogous to the roots of its close relative *P. erosus* (jicama) and are almost exclusively consumed raw or even cooked. The root skin lifts off quite easily from the internal portion, fleshy, white (Doporto, Dini, Viña & García, 2014; Milanez & Moraes-Dallaqua, 2003) or having purple dots.

Although *Pachyrhizus* tuberous roots have been used habitually for their carbohydrate content, they show between 3 and 5 times the protein content (on a dry weight basis) of other R&T crops, such as potato (Morales-Arellano, Chagolla-Lopez, Paredes-Lopez & Barba de la Rosa, 2001). For example, Gomes, Sirju-Charran & Barnes (1997) reported that the roots of the Mexican yam bean or jicama contained large quantities of two acidic glycoproteins which accounted for more than 70% of the total soluble proteins (about 3% of the root, on a dry weight basis). For *P. ahipa*, root protein functions have been related to metabolism, development and/or protection against pathogens and pests, suggesting that true storage proteins were absent. These observations were consistent with the biological role of ahipa roots as carbohydrate storage organs rather than as propagules (Forsyth & Shewry, 2002).

The objective of the present work was to develop a strategy, coupled to the starch extraction process, for the recovery of the proteins present in *P. ahipa* roots and to determine if the introduced modifications affected the yield and technological characteristics of the starch.

2. Materials and methods

2.1. Plant material

Pachyrhizus ahipa (Wedd.) Parodi plants were cultivated at the Agricultural Experimental Station EEA-INTA Montecarlo, Misiones, Argentina (26° 33′ 40.15″, South latitude and 54° 40′ 20.06″, West longitude). Roots were harvested when aerial plant parts began their senescence. Two batches of roots, harvested in April 2013 and April 2014, were used in this work.

Ahipa samples were processed immediately after received. Roots were thoroughly washed with tap water and then sanitized by immersion in NaClO solution (250 ppm of chlorine) for 10 min. Ahipa roots were then hand peeled, cut into 1 cm cubes, mixed and separated into six portions of equal weight for starch and proteins extractions.

For protein recovery, 0.05 M sodium phosphate buffer, pH 7.2 added with 1 M sodium chloride (buffer PO_4^{-3}/NaCl) was used as solvent, as previously described (Forsyth & Shewry, 2002).

2.2. Extraction procedures

Two methodologies were used for starch extraction: the method described by López et al. (2010), referred to as traditional method (T) and the one proposed in this work (P) which included protein recovery from the supernatants obtained after decanting of the starch from the extracted slurries.

2.2.1. Traditional method

The traditional method (T) consists of six starch extractions with water (Fig. 1a): peeled and cut roots were crushed, mixed with water at a ratio of 2 L of solvent per kg of root, and homogenized using a domestic grinder. The mixture was left for 24 h at 4 °C to allow starch separation from the bagasse, then filtered through a muslin cloth, and the filtrate (starch slurry) was left for 24 h at 4 °C for starch decantation. The bagasse retained in the muslin cloth was put again into a bowl; water was added at 2 L per kg root and then homogenized, which corresponded to a second step of starch extraction. The homogenate was left again at 4 °C for 24 h and then filtered. Starch decanted from each slurry obtained was removed (supernatants were discarded), put into a tray and dried at 40 °C in a convection oven.

2.2.2. Proposed method

The proposed method (P) consists of a total of six starch extractions, but buffer PO_4^{-3}/NaCl was used for the first extractions steps, and the lasts were performed with water (Fig. 1b).

The number of buffer extractions was set according to the performance of protein removal from the plant tissue on each extraction step.

Peeled and cut roots were crushed and mixed with buffer (2 L of solvent per kg of root), and homogenized. The mixture was left for 24 h at 4 °C and then filtered through a muslin cloth. The bagasse retained in the muslin cloth was put again into a bowl, buffer was added at 2 L/kg root, homogenized, and the homogenate was left again at 4 °C for 24 h (second step of starch extraction). The filtrate (starch slurry) was left for 24 h at 4 °C for decantation of starch. The supernatant of starch decantation containing soluble proteins was recovered and stored at 0 °C. The filtrate of the second extraction was added to the decanted starch of the first extraction step and left for 24 h at 4 °C (Fig. 1b path A).

Supernatants obtained after decantation of starch from each successive extraction step with buffer were analyzed for their protein content by Bradford assay (Bradford, 1976; Walker, 2002). Bovine serum albumin (BSA) was used as standard and results were expressed as mg BSA/100 g roots.

In the last extraction steps, the scheme was identical but water was used instead of buffer (Fig. 1b path B). The slurries obtained from the aqueous extractions were added to the decanted starch of previous extraction steps; starch cake was resuspended, and left for 24 h at 4 °C for decanting again. These water slurries were used to remove the excess of salts from the decanted starch extracted with buffer. Aqueous supernatants obtained after starch decantation were discarded. Finally, starch cake (containing the total amount of starch extracted) was removed, put into a tray and dried at 40 °C as described above.

2.2.3. Proposed method: number of buffer and water extractions setting

The number of water extractions was determined by the minimum number of washing steps required for the removal of excess salts from the starch extracted with buffer. Starch cake obtained from buffer extractions was resuspended in water at the same ratio used for the starch obtaining procedure (2 L/kg peeled roots). Starch suspension was thoroughly mixed for 10 min and left to decant for 24 h at 4 °C. Supernatants obtained after starch decanting were

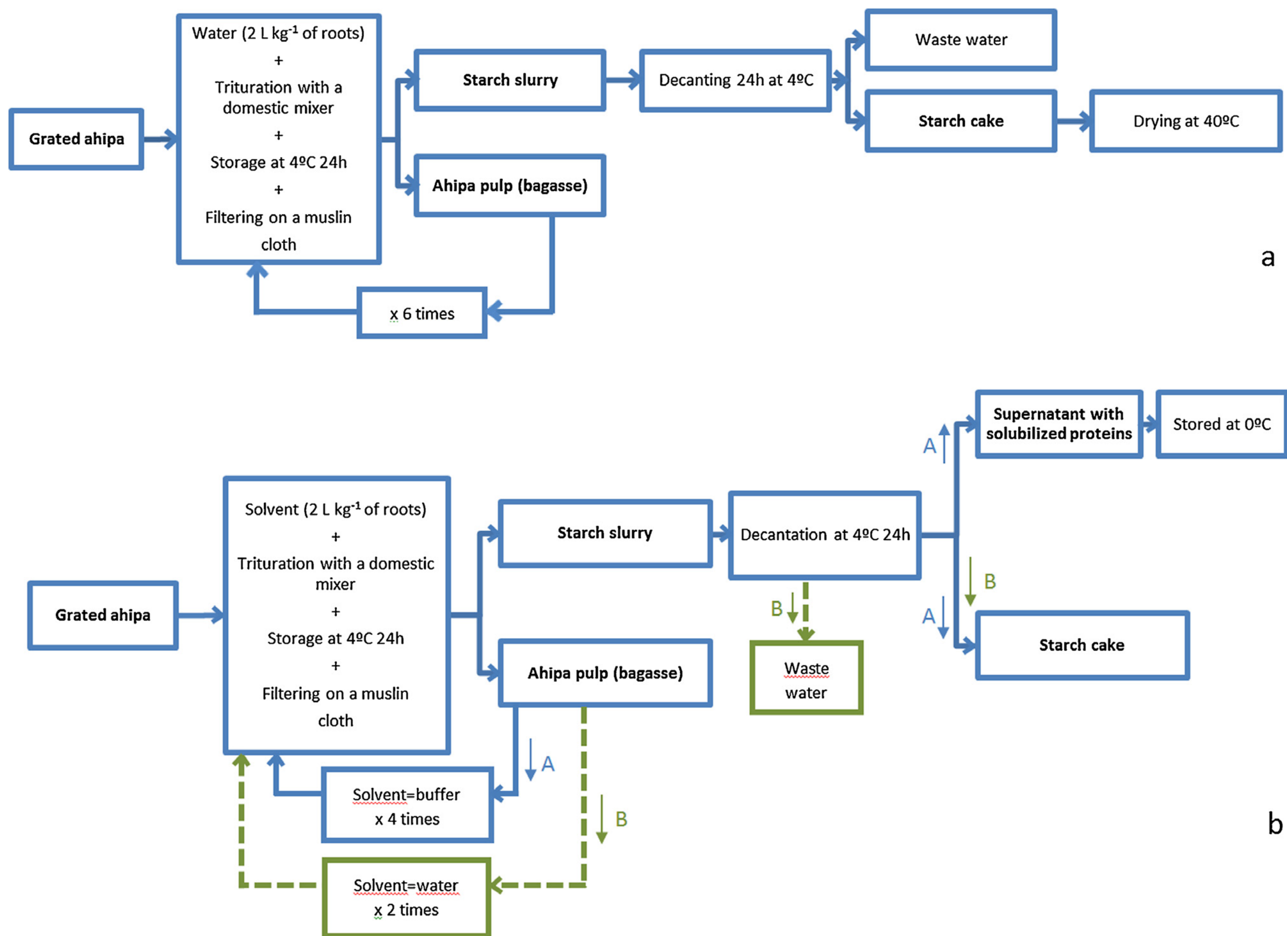


Fig. 1. Fluxogram showing *Pachyrhizus ahipa* starch extraction procedures: a) traditional; b) proposed method: **path A** buffer extraction steps and protein recovery and **path B** water extraction steps and washing of the starch cake after protein recovery.

equilibrated to 10 °C and the conductivity was measured using a PC 510® pH and conductivity bench meter (Oakton Instruments, USA). This procedure was repeated until the value of conductance of the supernatant reached that of the water extracted starch (control).

During starch extraction with the P method, the conductivity of the last decanting supernatant was measured and compared to that of the starch extracted with the traditional method to ensure removal of excess salts from the decanted starch.

2.2.4. Proposed method: effect of the extracting solvent

To ascertain the effect of the solvent used, independently from the extraction procedure, the proposed method was performed as described (with buffer in the first extraction steps, Fig. 1b), or using water in all the extraction steps, referred to as proposed method with water (PW).

2.3. Starch extraction yield and residual protein content

Starch extraction yield was calculated as% w/w through a mass balance, considering the weight of the starch extracted and the initial weight of the roots processed according to Doporto et al. (2014).

For the determination of the amount of residual proteins in ahipa starches, accurately weighed 1.5 g samples were analyzed for total nitrogen content by the Kjeldahl method (AOAC, 1990). Results were expressed as percentage (%) on a dry basis.

2.4. Characterization of the extracted starches

For color measurement, extracted starches were ground in a mortar until a fine powder of uniform particle size was obtained. Samples were placed in petri dishes to cover the bottom in a uniform thickness layer. Luminosity (L^*) and chromaticity parameters (a^* and b^*) of the CIELAB color space were obtained using a CR-400 Konica Minolta colorimeter (Osaka, Japan).

Thermal properties of ahipa starches extracted with the different methods were determined by DSC according to previous works (Dini, Doporto, García & Viña, 2013; López, García & Zaritzky, 2008) using a Q100 differential scanning calorimeter controlled by a TA 5000 module (TA Instruments, New Castle, DE, USA) with a quench-cooling accessory, under a N_2 atmosphere (20 mL/min). Heating range was between 10 and 120 °C at a scanning rate of 10 °C/min. Onset (T_o, °C), peak (T_p, °C), and end temperature (T_c, °C) were determined as well as the enthalpy of the process (area under the peak, ΔH, J/g of the dry sample).

For the analysis of the rheological behavior of starch suspensions, ahipa starch aqueous suspensions (4% w/w) were gelatinized at 90 °C for 20 min. A Rheo Stress 600 ThermoHaake (Haake, Germany) rheometer with a plate-plate system PP35 (gap size 1 mm) at controlled temperature (25 °C) was used. Time-dependent behavior of gelatinized starch suspensions was studied using rotational mode, as described in a previous work (López et al., 2010), and the resulting curves were mathematically modeled according to the Ostwald de Waele equation ($\tau = k\gamma^n$), being k the consistency coefficient and n the flow behavior index.

Viscoelastic behavior of starch pastes were studied by dynamic assays. The linear viscoelasticity range, where sample does not suffer structural damage, was determined in a stress sweep (0–20 Pa) assay at constant frequency (1 Hz). For frequency sweeps 1 Pa was selected as shear stress value, since the linear viscoelastic range was extended up to 2 Pa for all starch pastes.

Frequency sweeps (0.01–100 Hz) were performed at constant stress and the storage (G') and loss (G") moduli, the tangent of the phase angle ($\tan\delta = G''/G'$) and the complex shear stress (G^*) were recorded. Mechanical spectra were obtained from G' and G" versus frequency (f) plots, and they were fitted to the Power law model using the following equations (Steffe, 1996):

$$G' = af^b$$

$$G'' = cf^d$$

where f is the frequency expressed in Hz and a, b, c, and d are the fitting parameters.

For rheological measurements, samples were allowed to rest at the initial temperature for 5 min in order to relax the samples before the dynamic shear. The average of at least three recorded measurements is reported.

2.5. Solvent effectiveness on protein extraction

The capability of each solvent used (buffer PO_4^{-3}/NaCl or water) for ahipa root protein solubilization was evaluated by the quantification of the total amount of proteins extracted with each solvent, and the residual proteins retained in the bagasse after starch extraction. For the quantification of extracted proteins, freeze-dried ahipa roots (harvest year: 2014) were milled into powder and passed through a 16 mesh sieve. 5 g samples were accurately weighed, and 50 mL of the respective solvent (water or buffer) were added. Mixtures were agitated in an orbital shaker (150 rpm) for 1.5 h at room temperature. Aliquots of the supernatants of each extraction were analyzed for protein concentration using the Pierce TM BCA protein assay kit (Thermo Scientific, USA) according to the manufacturer instructions. Calibration curve was performed using BSA standards. Results were expressed as mg BSA/g of lyophilized root.

Residual proteins retained in the insoluble residue (mainly composed of fiber) after starch extraction from ahipa roots with the P and PW extraction procedures were quantified by the Kjeldahl method. For the conversion of total nitrogen to total proteins, a factor of 6.25 was used. Results were expressed as proteins (%) on a dry basis. The total moisture of the fibrous residue was determined gravimetrically after drying at 105 °C until constant weight was achieved.

2.6. Electrophoretic profile of ahipa proteins

Protein isolate composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Buffer supernatants obtained after starch decanting in the proposed method were kept frozen at 0 °C until starch extraction was completed (Section 1.2.2). Then, supernatants were thawed at room temperature and put together. Proteins were precipitated by salting out with $(NH_4)_2SO_4$ to a 90% of saturation at 25 °C. The mixture was agitated until complete solubilization of the salt, and then centrifuged for 10 min at 4 °C and 10000 rpm in a Beckman Coulter Avanti J-25 centrifuge. Precipitates were resuspended in phosphate buffer 0.05 M, pH 7.4 (Forsyth & Shewry, 2002), subjected to exhaustive dialysis against distilled water using a 12.4 kDa cut-off cellulose bag, and freeze-dried.

For SDS page electrophoresis, 17 mg of the freeze-dried sample were dissolved in 500 μL of sample buffer [Tris–HCl 0.185 M (pH 8.8), glycerol 12.5% v/v, SDS 2.0% w/v, and bromophenol blue 0.05% w/v]. Mixtures were homogenized and centrifuged for 10 min at 13000g and 25 °C. The volume of the obtained supernatant was divided into two equal parts, and one of them was treated with 12.5 μL of β-mercaptoethanol 5% v/v. Polyacrilamide gel (12% w/v) was loaded with 2, 5 and 10 μL of each preparation, respectively and a MW marker of 20.1, 30, 43, 67 and 94 kDa. Coomassie Brilliant Blue was used as staining agent.

2.7. Statistical analysis

Determinations were carried out at least by triplicate. Results were analyzed by a one way Analysis of variance (ANOVA) and means were compared with the Fisher's least significant difference (LSD) at a significance level of 5% ($p = 0.05$).

3. Results and discussion

3.1. Optimization of the extraction procedure

The proposed extraction procedure was derived from the water extraction method described by López et al. (2010), which has been based on the industrial extraction of cassava starch. In this aqueous extraction method, hereafter named as "traditional method", crushed roots are mixed with water and left in contact for 24 h. The mixture is then filtered, the slurry obtained is left for 24 h at 4 °C, and the bagasse is mixed again with water. After decanting of the obtained slurry, the supernatant is separated from the decanted starch, and the starch cake is removed to be dried out at 40 °C. This extraction scheme is repeated six times in order to be considered exhaustive.

In the extraction method proposed in this work, the total number of steps was maintained but the first extractions were performed with buffer instead of water, in order to favor the solubilization of the proteins present in ahipa roots. The number of buffer extractions was defined according to the performance of protein extraction obtained on each successive step, and the number of water extractions was defined according to the number of rinses needed to eliminate the excess of salts from the extracted starch.

In this proposed method, the slurry obtained from each extraction step with buffer was left to decant, supernatant containing soluble proteins was then removed and stored at 0 °C, and the slurry of the following extraction step was added to the decanted starch. The last extraction steps were performed with water; slurries obtained were added to the decanted starch and mixed before leave them for decanting again. These last water extractions were used to rinse the starch decanted from previous steps to remove the excess of salts. Finally, the last decanting aqueous supernatants were discarded and all the decanted starch was removed in one portion to be dried out at 40 °C (Fig. 1).

The number of washing steps required to remove the excess of salts from the starch extracted with buffer was determined by conductimetric measurements of the supernatants obtained after each water washing step. Washing steps were repeated until the value of conductance of the blank (aqueous supernatant obtained from a water-extracted starch) was reached: 1.2 mS at 10 °C.

Supernatant of the buffer extracted starch exhibited a conductance value of 92.5 mS. Conductance of the first and second water wash supernatants of this starch were of 11.3 and 2.1 mS, respectively.

The conductance of the third washing supernatant matched the value obtained for the washing supernatant of the control, free of excess salts (1.2 mS). Thus, two water washing steps proved to be enough to remove the excess of salts from the starch extracted with buffer.

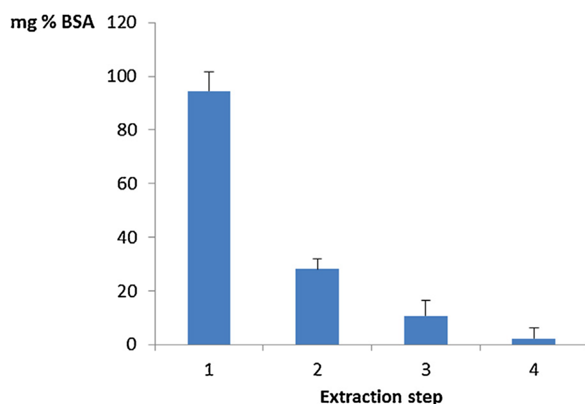


Fig. 2. Protein extracted expressed in mg% BSA (mg BSA/100 g roots) according to the number of extraction steps.

Table 1
Extraction yield and residual protein content of ahipa starches extracted with different methods.

Extraction method	Extraction yield (%)	Residual protein (%)
Traditional	15.70 ± 0.14^a	0.54 ± 0.01^a
Proposed	16.39 ± 0.30^b	0.01 ± 0.02^b

Note: Starch extraction yield is expressed as g/100 g of ahipa root in wet basis. Reported values correspond to the mean ± standard deviation (n = 3). Different letters within the same column indicate significant differences ($p < 0.05$).

Simultaneously, the amount of soluble protein obtained from crushed ahipa roots in the successive steps of extraction using PO_4^{-3}/NaCl buffer was evaluated to set the number of extractions required to obtain an acceptable protein extraction yield. Fig. 2 shows the amount of proteins obtained on each extraction step. The yield of the fourth extraction step dropped off to 2.4% of that obtained in the first one, thus four extractions with buffer were considered enough to obtain the major part of the readily extractable protein.

Based on the results obtained from the conductimetric measurements and the protein extraction yield on each step, the proposed method of protein recovery coupled to the starch extraction method was set to a total of 6 sequential extraction steps, using buffer as solvent for the first 4 extractions and water for the last two steps. Besides the use of buffer, this proposed method also differed from the traditional method in the addition of the starch slurry of each step to the total decanted starch obtained from previous steps, and using the last two consecutive water starch slurries to wash the starch extracted in the preceding ones.

The efficiency and the characteristics of the obtained starches from the traditional and the proposed methods were compared. Table 1 shows the starch extraction yields and the amount of residual protein of starches obtained from both methods. The starch extraction yield obtained with the proposed extraction method (P) with buffer was significantly higher ($p < 0.05$) than that obtained with the traditional one (T), thus the optimized procedure allowed to obtain ahipa proteins as a by- product without entailing starch yield losses.

Besides a better extraction performance, a purer starch containing less amount of residual protein was obtained with the proposed method with buffer compared to the traditional one (Table 1).

The proposed method using buffer as extraction solvent increased protein extraction, leading to a purer starch with a content of crude protein below the maximum typically accepted for the production of high-glucose syrups and reducing the risk of the development of Maillard reactions (0.3%) (Hull, 2011).

Table 2
Color attributes of starches extracted with the proposed and traditional methods.

Harvest	Sample	L*	a*	b*
2013	T	95.13 ± 0.36^a	0.41 ± 0.04^a	3.67 ± 0.37^a
	P	95.93 ± 0.12^b	0.36 ± 0.05^a	2.43 ± 0.11^b
	T (G)	35.93 ± 0.54^a	-0.14 ± 0.02^b	-3.59 ± 0.18^a
	P (G)	38.41 ± 0.59^b	0.26 ± 0.05^a	-3.16 ± 0.72^a
2014	PW	95.55 ± 0.61^a	0.18 ± 0.01^a	2.08 ± 0.24^a
	P	95.73 ± 0.62^a	0.49 ± 0.15^a	2.88 ± 0.54^a

Note: Reported values correspond to the mean ± standard deviation (n = 6). Different superscript letters within the same column and harvest year among native or gelatinized (G) starches indicate significant differences ($p < 0.05$).

In order to determine if this difference was due to the type of solvent used (buffer or water) or to the difference in the extraction methodology (sum of slurries removing starch in one final portion or separation of the decanted starch obtained from each extraction step), the proposed extraction method was performed using buffer in the first four steps and water in the other two (proposed method: P) or using water in all the six extraction steps (proposed method with water: PW) on a new batch of ahipa roots (year of harvest: 2014). No significant difference ($p < 0.05$) on the starch extraction yield was observed for the proposed method using buffer or water (data not shown). Despite of the use of buffer and water as solvents, differences detected in the residual protein content of the starches obtained with the proposed method were not significant ($p > 0.05$). Thus, under these experimental conditions, the differences between T and P methods were attributed to the change in the extraction methodology. Probably, the slight increase in the starch extraction yield with the proposed method was due to a lower loss of starch when all the decanted cake was removed in one portion to be dried, instead of taking out the decanted starch of each extraction step separately. The reduction in the amount of residual proteins of the starches obtained with the P method was attributed to the sum of slurries. Each added slurry was used to rinse the decanted starch from previous steps, allowing to the removal of a greater amount of proteins.

3.2. Color attributes of native and gelatinized starches

Color is an important attribute on starches because it influences the acceptability from the consumers as raw material and defines the quality of the final product. Whiteness is associated with a higher purity and quality of the starch. Table 2 shows color parameters of the starches extracted from ahipa roots with different methods. The starches extracted with the proposed method were significantly whiter (higher L* values) than those obtained by the traditional method. The chromaticity parameter b* was also significantly different among starches extracted with different methods. Positive b* values indicated yellowish tones, being those values higher for starches extracted with the traditional method. As for high L* values, low b* values are also associated with higher purity and quality of starch.

To determine the effect of the extraction solvent on the final color of native and gelatinized starches, the proposed starch extraction method was performed with buffer (P) and water (PW). Native starches extracted with the proposed method with different solvents showed no significant differences in any of the parameters analyzed (Table 2).

The color differences observed between the proposed and the traditional methods might be due to the different percentage of residual proteins on the respective starches, which determine the extension of Maillard reactions. In this case, browning by Maillard reactions would be carried out during the drying process of starches at 40 °C. These are mild reaction conditions in terms of temperature and alkalinity, thus browning is also mild, giving a faint yellow

Fig. 3. *Pachyrhizus ahipa* roots and derived products: a) whole and cut roots; b) dried starch cake obtained from the traditional method of extraction; c) milled starches extracted from the traditional (upper row) and the proposed (lower row) methods (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)".

tone to starches, which is sometimes noticeable to the naked eye (Fig. 3c). Besides proteins, ahipa roots contain from 19 to 28% of total sugars, mainly sucrose, and to a lesser extent fructose and glucose, higher level than that found in most edible R&T. Although these saccharides are almost completely eliminated by solubilization during aqueous extraction of starch, the traces remaining in the starch after decantation can favor Maillard reactions.

Starch color is also given by water soluble pigments present in *P. ahipa*, which appear as purple dots within the white flesh of cut ahipa roots (Fig. 3a). The extensiveness of the pigmentation differs from harvest to harvest, to the point that some batch of roots are non-colored (all white) while others have an intense pigmentation. During starch extraction, the first slurries of colored roots are visibly purple, and a purple-gray layer appears on the decanted starch. This dark layer remains after starch cake is dry, but appears as a brownish superficial color on the dried starch, giving a slight homogeneous hue after milling (Figs. 3b and c).

Gelatinized pastes of starches extracted with different methods also showed differences in their superficial color, with significant differences ($p < 0.05$) in the a* and L* parameters (Table 2).

Pastes of starch extracted with the proposed method showed significantly higher L* values, being the difference of L* values greater between starch pastes compared to that among native starches. The increase in the variation of L* values of starch pastes might be due to the increase in browning reactions during cooking.

As for native starches, starch pastes lightness is also an important attribute since pastes clarity could be an indicative of quality for some types of food products. For example, starch used to thicken fruit pie filling should be rather clear, but starch destined to salad dressing should be opaque. Clarity varies noticeably with the source of starch and can be changed by chemical or enzymatic modification of the granules, and by the addition of solutes (Bello-Pérez & Paredes-López, 1996).

3.3. Starch thermal properties

The thermal properties of starches extracted with the P and T methods were analyzed. Thermograms of ahipa starches obtained with different methods showed a single endothermic peak corresponding to the gelatinization process (Fig. 4). Gelatinization temperature ranges were $19.47 \pm 1.74\,^{\circ}$C and $19.31 \pm 3.28\,^{\circ}$C for buffer and water extracted starches, respectively. No significant differences were observed in the onset temperature and temperature range of the endothermic peak, resulting in values similar to data previously reported (Dini, Doporto, García & Viña, 2013; Doporto et al., 2014). Nevertheless, a significant difference was observed in the enthalpy of the process, resulting significantly higher for the starch obtained by the proposed method (Fig. 4). This observation could be attributed to the lower amount of proteins present in this starch.

DSC gelatinization temperature is indicative of the degree of order (crystallite perfectness), whilst enthalpy is a measure of the extent of order (amount of crystallinity) (Correia, Nunes & Beirão-da-Costa, 2013). ΔH indicates the required energy for disruption hydrogen bonds within the crystalline zones (Singh, McCarthy, Singh, Moughan & Kaur, 2007).

Protein-starch interactions have been shown to alter the swelling behavior and the rheological and thermal properties of some starches (Considine, Noisuwan, Hemar, Wilkinson, Bronlund & Kasapis, 2011; Debet & Gidley, 2006; Mohamed & Rayas-Duarte, 2003). It has been reported that surface proteins of starch granules can increase or lower the onset temperature and ΔH of the gelatinization peak of starches depending on their botanical origin (Bertolini, Creamer, Eppink & Boland, 2005; Mohamed & Rayas-Duarte, 2003).

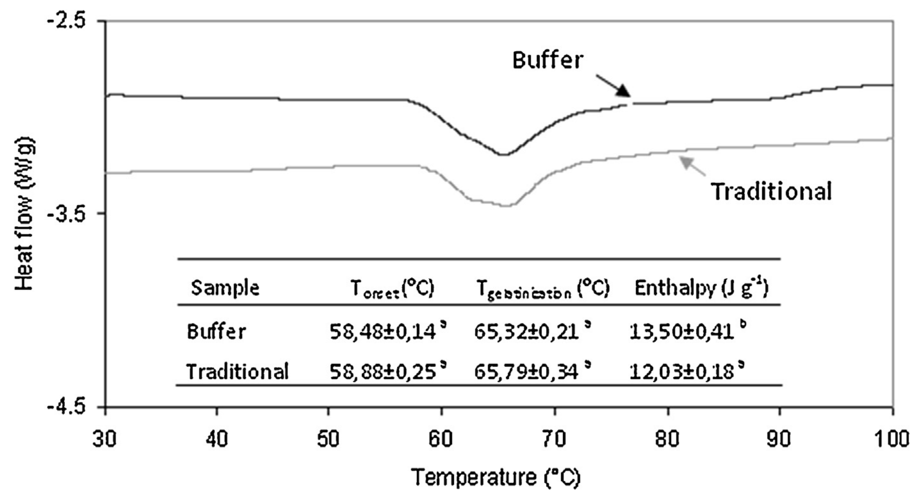
The effect of surface proteins on thermal properties of some starches has also been reported for low amounts of residual proteins (Debet & Gidley, 2006). Although the factors influencing these effects have not been completely elucidated, the higher variations have been mainly associated to low amylose starches (Debet & Gidley, 2006). Such is the case of ahipa starch, with an amylose content ranging from 10 to 14% (Doporto et al., 2014; López et al., 2010), considerably lower than that of other more widely spread starch sources (Debet & Gidley, 2006).

To determine the effect of the extraction solvent in the thermal properties of starch pastes, the proposed extraction scheme (sum of slurries) was performed with buffer and with water respectively in the first four steps. Extracts were performed by adding the respective slurry to the starch decanted in the immediate previous step. No significant differences ($p > 0.05$) were observed in any of the analyzed parameters: peak temperature (PW: $65.5 \pm 0.3\,^{\circ}$C and P: $64.9 \pm 0.6\,^{\circ}$C), temperature range (PW: $16.5 \pm 0.8\,^{\circ}$C and P: $17.7 \pm 1.1\,^{\circ}$C) and enthalpy of the gelatinization process (PW: 10.24 ± 1.8 J/g and P: 11.9 ± 1.9 J/g) of starches obtained from the P and PW methods. Once more, the differences observed between the starches obtained with the P and T methods were attributed to the extraction scheme rather than to the solvent used. Probably, the depletion of surface proteins from starch granules was favored by the washing steps the starch had been subjected to in the proposed method.

3.4. Rheological characterization of starch pastes

The rheological behavior of pastes from starches obtained by different methods was analyzed. Rotational mode assays indicated that pastes presented a pseudoplastic behavior ($n < 1$) satisfactorily adjusted by the Ostwald de Waele model (Table 3). Moreover, starch pastes were characterized as thixotropic ones, indicating that the rheological behavior of these systems was time-dependent. Thixotropic materials exhibit decreasing shear stress and apparent viscosity over time at a fixed shear rate.

The corresponding thixotropic indexes, calculated as the area between the flux curves, were lower for the pastes of starch obtained by the traditional method. No significant differences were



Sample	T_{onset} (°C)	$T_{gelatinization}$ (°C)	Enthalpy (J g^{-1})
Buffer	58,48±0,14[a]	65,32±0,21[a]	13,50±0,41[b]
Traditional	58,88±0,25[a]	65,79±0,34[a]	12,03±0,18[a]

Fig. 4. Thermograms obtained by DSC for *Pachyrhizus ahipa* starches. (—): ahipa starch obtained by the traditional method; (—): ahipa starch obtained by the proposed alternative procedure. Thermal parameters are shown in the inserted Table.

Table 3
Rheological parameters of pastes obtained from ahipa starch extracted with different methods.

Harvest	Sample	Apparent viscosity at 500 s^{-1} (mPa s)	Correlation coefficient r^2	Consistency index, k (Pa s^n)	Flow index, n	Thixotropy index (Pa s^{-1})
2013	P	65.22 ± 2.87[a]	0.9961	1.51 ± 0.17[a]	0,490 ± 0.010[b]	492 ± 47[b]
	T	79.72 ± 2.76[b]	0.9985	2.47 ± 0.15[b]	0,446 ± 0.005[a]	289 ± 31[a]
2014	PW	129.38 ± 7.55[a]	0.9995	2.89 ± 0.86[a]	0,503 ± 0.040[a]	418 ± 141[b]
	P	126.81 ± 2.99[a]	0.9995	2.73 ± 0.07[a]	0,504 ± 0.001[a]	490 ± 40[b]

Note: P: proposed method, T: traditional method, PW: proposed method using water in all stages.
Reported values correspond to the mean ± standard deviation (n = 4). Different letters within the same column and harvest year indicate significant differences ($p < 0.05$).

observed for starches obtained by the proposed method, regardless the extraction solvent used. Consistency index parameter showed a similar trend than apparent viscosity (Table 3).

Starch pastes obtained from the traditional method showed a significantly higher ($p < 0.05$) apparent viscosity than that obtained with the proposed method, while the use of water (PW) or buffer (P) did not affect this parameter (Table 3). This was probably related to the higher amount of proteins of the starch extracted with the traditional method. This proteins' effect on the apparent viscosity of gelatinized starches has been previously reported for casein on starches from different botanical origins (Bertolini, Creamer, Eppink & Boland, 2005; Doublier, Marzin, Visdeloup & Lefebvre, 1994; Kelly, Van Wagenberg, Latham & Mitchell, 1995). Even low amounts of surface proteins can alter the rheological behavior of starch pastes. Debet and Gidley (2006) reported an increased swelling peak followed by a lower final viscosity for gelatinized wheat and maize starches when native starches were deproteinized with SDS (~0.4% proteins). This change in the final viscosity could be attributed to an increased shear disruption on the more highly swollen granules. Likewise, this effect of the depletion of surface proteins (as well as lipids) enhancing the rate and extent of granule swelling has also been previously reported (Bowler, Towersey, Waight & Galliard, 1985; Debet & Gidley, 2006; Eliasson, Carlson & Larsson, 1981; Tester & Morrison, 1990).

Rheological tests were performed in dynamic mode to characterize the viscoelastic behavior of starch pastes. Frequency sweeps were run at 1 Pa (within the linear viscoelastic range) and mechanical spectra of the samples were then obtained (Fig. 5a). The G' values were in all cases higher than those of G", remaining substantially constant throughout the frequency range used; thus, pastes behaved as weak gels.

Besides, mechanical spectra of pastes stored for 48 h under refrigeration were obtained for studying retrogradation tendency, which determine their potential use as thickener or stabilizer in food formulations. Textural properties of starch gels are important criteria in evaluating the performance of starch in a food system and are mainly related to amylose retrogradation (Sandhu & Singh, 2007). In all cases, stored pastes exhibited higher G' values than freshly prepared ones (Fig. 5b). Thus, increases of 228% and 264% were observed for pastes formulated with starches obtained by the traditional and proposed methods, respectively, being this difference significant ($p < 0.05$).

Upon cooling, the gelatinized starch and water undergo molecular interactions by hydrogen-bonding. The re-association of starch chains results in re-ordering of the system and partial recrystallization with the subsequent displacement of water molecules. Amylose re-association is largely responsible for the initial hardening of the gel, whereas the long-term gelling and retrogradation are mostly determined by amylopectin re-crystallization (Wang, Li, Copeland, Niu & Wang, 2015).

The evolution of the elastic and the viscous moduli as a function of frequency was adjusted to the Power law model (Steffe, 1996). Table 4 shows the parameters corresponding to the model and correlation coefficients r^2, indicating the goodness of fit. Regardless the starch extraction method used, stored pastes exhibited higher values of the *a* parameter than the fresh pastes, in agreement with the results shown in Fig. 5b.

Rheological behavior differences between starches obtained with the proposed method from roots harvested in different years could be attributed to variations in the environmental factors and/or agronomic practices (Asaoka, Blanshard & Rickard, 1992; Asaoka, Blanshard & Rickard, 1993; Zhu, 2015).

3.5. Effect of the solvent on the effectiveness of protein extraction

Differences between the starches obtained with the traditional and the proposed methods were attributed to the methodology of extraction rather than to the solvent used. These differences were

Table 4
Power law fitting parameters of elastic and viscous modulus of pastes obtained from ahipa starch extracted with different methods.

Harvest	Sample	Elastic modulus G'			Viscous modulus G"		
		r^2	*a*	*b*	r^2	*c*	*d*
2013	P	0.984	30.55 ± 9.85[a]	0.144 ± 0.004[b]	0.990	9.47 ± 2.17 [a]	0.34 ± 0.02[b]
	T	0.940	26.73 ± 0.93[a]	0.069 ± 0.005[a]	0.724	3.71 ± 0.13 [a]	0.18 ± 0.02[a]
	SP	0.990	48.71 ± 2.57[a]	0.075 ± 0.005[a]	0.929	6.94 ± 0.53[a]	0.21 ± 0.05[a]
	ST	0.994	47.63 ± 3.44[a]	0.060 ± 0.009[a]	0.995	5.69 ± 0.62[a]	0.26 ± 0.01[a]
2014	PW	0.951	15.83 ± 5.24[a]	0.219 ± 0.004[b]	0.984	6.00 ± 0.38[a]	0.36 ± 0.01[a]
	P	0.978	14.02 ± 0.61[a]	0.148 ± 0.001[a]	0.968	5.17 ± 0.04[a]	0.37 ± 0.01[a]
	SPW	0.995	46.34 ± 0.28[a]	0.082 ± 0.004[a]	0.851	6.53 ± 0.14[a]	0.16 ± 0.04[a]
	SP	0.994	49.91 ± 0.45[b]	0.084 ± 0.001[a]	0.891	7.01 ± 0.04[b]	0.16 ± 0.03[a]

Note: P: proposed method, T: traditional method, PW: proposed method using water in all the stages; S: stored pastes under refrigeration conditions. Power law fitting parameters: *a* and *b* of G' and *c* and *d* of G".
Reported values correspond to the mean ± standard deviation (n = 4). Different superscript letters within the same column, harvest year and storage condition indicate significant differences ($p < 0.05$).

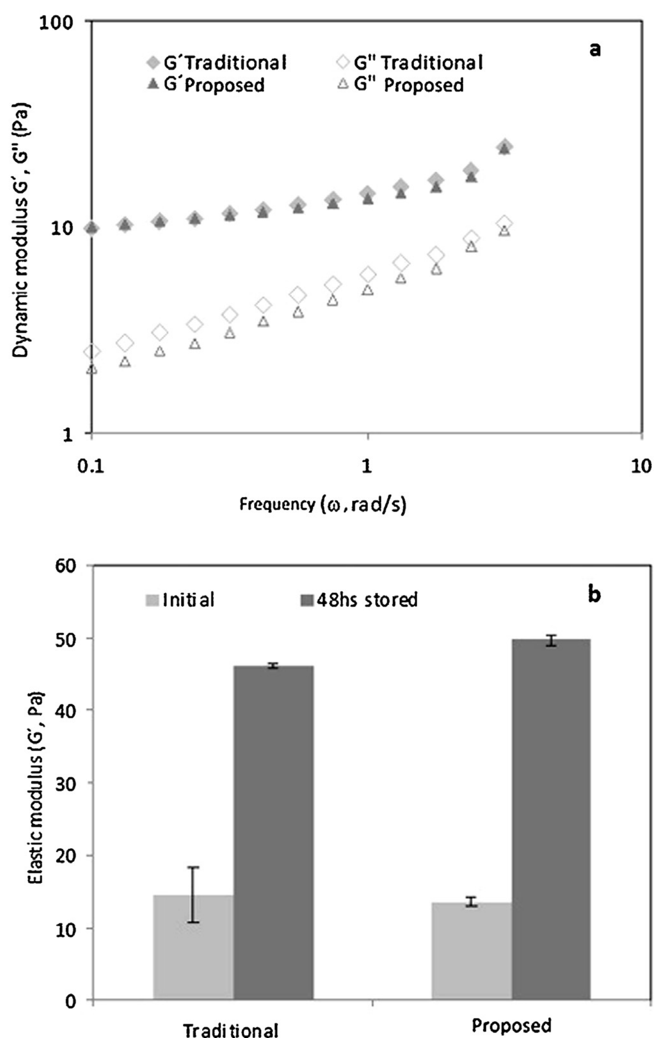


Fig. 5. a) Mechanical spectra at constant amplitude = 1 Pa; b) Storage modulus (Gí) at 1 Hz of freshly prepared (initial) or refrigerated stored (48 h) pastes of ahipa starches obtained by the traditional or the proposed method.

mainly associated to the addition of slurries of each extraction step to decanted starches from previous steps, which serves as rinsing of all the extracted starch before being removed and dried.

To determine if the use of buffer is justified, extraction was performed with the proposed method using water and buffer. The efficiency of each solvent for protein extraction was measured using freeze dried roots since the amount of proteins extracted from fresh roots is relatively small, and differences between solvents performance are dificult to detect. The residual proteins present in the insoluble residue (mainly composed of fiber) obtained after starch extraction from fresh roots was also quantified to determine the effectiveness of protein removal from the plant tissue.

Table 5
Protein extraction yield and baggase residual protein content.

Solvent	Protein extraction yield (mg BSA/g)	Bagasse residual protein (%)
W	62.78 ± 0.14[a]	3.85 ± 0.05[b]
B	69.57 ± 1.51[b]	2.80 ± 0.18[a]

Note: W: water, B: buffer. Results are expressed in% on dry basis of roots or bagasse, respectively. Reported values correspond to the mean ± standard deviation (n = 3). Different superscript letters within the same column indicate significant differences ($p < 0.05$).

Results in Table 5 show that significantly higher amounts of proteins were extracted when buffer was used as solvent, while the residual protein content of the bagasse was significantly reduced. Therefore, the use of buffer as solvent during starch extraction produced no considerable modifications in the properties of the obtained starch but increased the harnessing of ahipa proteins in more than 10%.

3.6. Electrophoretic profile of ahipa proteins

Fig. 6 shows the electrophoretic separation of proteins in polyacrylamide gel, at three different concentrations, with or without the addition of β-mercaptoethanol.

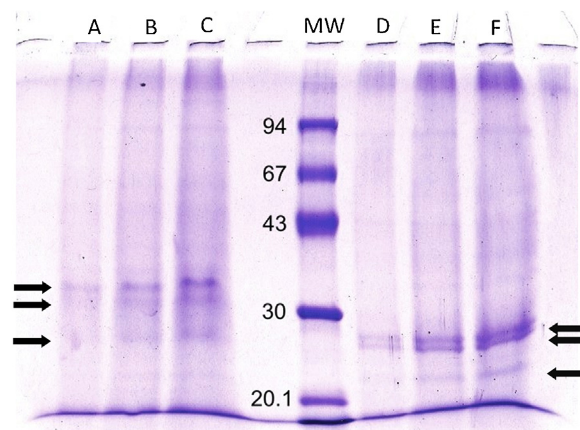


Fig. 6. SDS PAGE of 2, 5 and 10 μL samples of the protein fraction extracted from *P.ahipa* root without (lines A, B and C) and with β-mercaptoethanol (lines D, E, and F). MW: molecular weight marker. The arrows show the distinctive bands observed.

Profiles obtained without β-mercaptoethanol exhibited three main bands, two of them with molecular weights between 30 and 43 kDa and another one between 20.1 and 30 kDa. Thus, at least three different kinds of polypeptides with different molecular mass were present in the extract. When samples were treated with β-mercaptoethanol, the bands above 30 kDa were lost and three new bands of lower molecular mass appeared. Two of these bands were close to 30 kDa and the third one was revealed closer to 20.1 kDa, suggesting that some peptide species were joined by disulfide bonds. These results are in accordance with those reported by Forsyth and Shewry (2002) who found four bands between 17 and 30 kDa when the protein extracts obtained by solubilization in PO_4^{-3}/NaCl buffer were treated with β-mercaptoethanol.

Results were similar to those reported by Gaidamashvili et al. (2004) for the main protein fractions (DB1, DB2, DB3) of sweet potato (*Dioscorea*), which showed low molecular mass and minor subunits joined by disulfide bonds.

The lower molecular mass suggests higher digestibility of the proteins (Guimarães, Favaro, Viana, Braga Neto, Neves & Honer, 2012). From a techno-functional point of view, small proteins and peptides with high solubility are very useful as food ingredients. In this sense, protein hydrolysis is a widely used technique in the food industry to reduce the size and secondary structure of proteins, increasing their solubility and the functional properties directly related to this feature, such as foaming and emulsification (Culbertson, 2006). Additionally, ahipa proteins remain highly soluble in the presence of high amounts of sodium chloride which makes them suitable ingredients for salty foods (Culbertson, 2006).

4. Conclusions

Ahipa roots are valuable raw materials for the food industry mainly because of being rich in starch. The traditional method of cassava starch extraction has been successfully applied for these roots, but *P. ahipa* has a considerably higher amount of proteins than cassava and other edible roots and tubers, which are lost during this starch extraction process. The use of buffer as solvent improved protein extraction from ahipa roots without impairing the extraction yield of starch. The starches obtained from the traditional and the proposed methods exhibited some differences in their appearance and technological and thermal properties, which were attributed to the modification in the methodology of extraction rather than to the use of buffer as solvent. The washing steps of the starch extracted by the proposed method increased surface proteins depletion and yielded a clearer and purer starch.

Protein extraction can be coupled to the starch obtaining procedure with a minimum change in the methodology, without substantially altering the characteristics of the starch obtained, and enabling the exploitation of this valuable by-product.

Conflict of interest

The authors have declared no conflict of interest.

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