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Nutritional assay *Pereskia* spp.: unconventional vegetable

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Abstract: Pereskia *grandifolia* Haworth (PGH) and Pereskia *aculeata* Miller (PAM) are recognized sources of proteins; dietary fiber; vitamins and minerals make this plant leaves, raw, cooked, and braised, an important ally against protein and micronutrient deficiencies. One of the main problems is the presence of antinutritional factors that may interfere in the digestibility and bioavailability of some nutrients. The objective was to evaluate the amino acid profile and the chemical score of the raw leaves and the effects of heating media and time on the total dietary fiber, minerals, trypsin inhibition, oxalic acid and tannins of leaves of PGH and PAM. The samples had similar amino acid profiles and total dietary fiber. With regard to antinutritional compounds, heating the leaves of PGH led to a decrease in trypsin inhibition, primarily after the first minutes of wet cooking. Oxalic acid and tannins predominated in both species. Considering the interaction with time, the variables related to iron and zinc minimized the tannin responses in PGH and PAM, respectively. Heating media and times interfered with the chemical components present in the leaves of Pereskia species and led to high antinutrient retention after heat treatment.

Key words: *Pereskia grandifolia* Haworth, *Pereskia aculeata* Miller, oxalate, tannins, trypsin inhibitor, minerals.

INTRODUCTION

Two edible species of the genus *Pereskia*, *P. grandifolia* Haworth (PGH) and *P. aculeata* Miller (PAM), popularly known as ora-pro-nobis (OPN), are recognized sources of proteins; dietary fiber; vitamins A, B, and C; and minerals such as calcium, zinc, and iron. These compounds make this plant an important ally against protein and micronutrient deficiencies (Hęś et al. 2014, Almeida et al. 2014).

Raw, cooked, and braised OPN leaves are consumed by population. When dried, the leaves can add nutritional value to bread dough and pasta or be used to create other acceptable and low-cost formulations with elevated protein and fiber levels (Moran & Zimmermann 1991, Rocha et al. 2008, Martinevski et al. 2013, Sobrinho et al. 2015).

Leaves are rich in compounds with antioxidant activity (Sim et al. 2010, Agostini-Costa et al. 2014, Almeida et al. 2014) dietary fiber (Rocha et al. 2008, Takeiti et al. 2009, Almeida et al. 2014), minerals, such as calcium, iron, zinc, manganese and magnesium (Albuquerque et al. 1991, Girão et al. 2003, Takeiti et al. 2009, Oliveira et al. 2013, Souza et al. 2016) and monosaccharides such as arabinofuranose, arabinopyranose, galactopyranose, galactopyranosyluronic acid, rhamnopyranose and glucopyranose (Sierakowski et al. 1990).

One of the main problems in exploiting vegetable leaves as a source of nutrients is the presence of antinutritional factors, such as trypsin inhibitors, oxalates, and tannins, among others. These compounds may be toxic and/or interfere in the digestibility and bioavailability of some nutrients, such as proteins and minerals.

The antinutritional factors should be reduced or eliminated when possible. On one hand, heating food may improve some nutritional and sensory aspects; on the other hand, it results in the losses of some nutrients and antinutrients due to heating, leaching, and complexation between substances, hindering their extraction and availability in the body (Correia et al. 2008, Pellegrini et al. 2010).

Trypsin inhibitors can block the action of proteolytic enzymes and cause endogenous losses of essential amino acids, especially those containing sulfur (Pisulewska & Pisulewski 2000). Many researchers have reported that the raw leaves of OPN can inhibit of trypsin (Almeida et al. 2014) and the heating can eliminate the trypsin inhibitors (Pompeu et al. 2014)

Oxalic acid, present in higher amounts in green leafy vegetables (Oliveira et al. 2008, Squena et al. 2009, Almeida et al. 2014) can bind to calcium and iron ions and interferes with the bioavailability of these minerals due to the formation of insoluble salts (Ferreira & Arêas 2010). Ogbadoyi et al. (2006) observed a 65% decrease in oxalate content in ingested vegetable leaves after heating (100°C/5 min) and discarding the water, and others (Benevides et al. 2013, Lisiewska et al. 2011) .

Tannins are able to complex with proteins and minerals, among other polymers, such as carbohydrate after the vegetables that contain them are damaged, making these polymers unavailable to the human body (Delfino & Canniatti-Brazaca 2010, Benevides et al. 2013).

When associated with oxalates and tannins, the fibers may the bioavailability of minerals, such as calcium, zinc, magnesium and iron, among others (Benevides et al. 2011).

Considering that OPN leaves may be used as low-cost sources of proteins, minerals and fibers, and are relevant in preventing and/or treating malnutrition, the present study had two objectives: to determine the amino acid profile of proteins in the leaves of *Pereskia grandifolia* Haworth and *Pereskia aculeata* Miller, and to investigate the behavior of the antinutritional factors and the minerals under different heat treatments.

MATERIALS AND METHODS

Plant material

Leaves of the ora-pro-nobis (OPN) species *Pereskia grandifolia* Haworth (PGH) and *Pereskia aculeata* Miller (PAM) were harvested from the Medicinal Plant Garden of the Agriculture Department, UFLA. The leaves were cleaned under water, immersed in sodium hypochlorite (200 ppm), washed with distilled water, and drained.

Sample preparation

One portion of the leaf samples from each species was kept raw for amino acid analysis and another portion was heated using various media and times for mineral, fiber, and antinutritional compound analysis.

The vegetables were cooked according to domestic cooking practices. After optimizing the cooking conditions, wet cooking (boiling) and mixed cooking (braising) techniques were used, according to the heat transfer media (Ornellas 2007). The temperature was kept constant (96 ± 2°C). After the raw leaves were added to enough boiling distilled water (1:4, p/v), they were wet-cooked. The cooking time (1, 2.5, 5, or 10 min)

was measured from the time when the water returned to a boil; then the cooking water was discarded. Mixed cooking was done in 3 mL of soybean oil to 50 g of sample for 1 and 2.5 min, with the same implements and under the same conditions as the wet-cooked leaves.

After cooking, the plant material was cooled in an ice bath and drained. A portion of the samples was placed in an oven at 60° C and dried to less than 10% moisture content. The dried samples were ground to obtain a homogeneous powder (3 times/20 seconds) and stored in amber glass flasks until analysis.

Analyses performed

All chemical analyses were conducted in triplicate and the results expressed in dry matter. The reagents used were obtained from Sigma-Aldrich (USA) or Merck (Germany), and the other chemical products were of analytical grade.

Chromatographic analysis of amino acids and chemical score determination

The amino acid profile of the proteins were determined using the Prates method (Prates 2002) with a weight equivalent to 20 mg of crude protein. 1 mL of sample was filtered through a Millex unit (0.22-mm pore diameter, 13-mm diameter) and placed into an automatic sampler for subsequent injection into an amino acid analyzer under the following analytical conditions: injection volume, 10 mL; oven temperature, 60ºC; fluorescence detector, EX l 350 nm, EM l 450 nm; separation column, Shim-pack Amino-Na; ammonia trap column, Shim-pack ISC-30Na. The amino acid profile was determined using one C18 column with 20 µL injection volume, and an amino acid standard was used to construct the calibration curve.

To test the limiting amino acids, the chemical score of the amino acids (Who 2002) was determined by calculating the quotient of each essential amino acid (mg) contained in the test protein (g) divided by the same amino acid in the reference protein, and then multiplying by 100 (Young & Pellett, 1994). The percentage of protein digestibility was corrected using the protein digestibility percentages reported by Almeida-Filho & Cambraia (1974) (77.7%) and Takeiti et al. (2009) (75.9%) as a base, multiplied by the amino acid content of the protein.

Bioactive analysis

The total dietary fiber (TDF) and fractions were determined using the enzymatic-gravimetric method (Sigma®) according to the AOAC (2005).

The minerals were analyzed according to Malavolta et al. (1997) using an Atomic Absorption Spectrometer.

The extracts, diluted in 1:5 ratios, were prepared according to Souza et al. (2011). A supernatant was used in the enzymatic inhibition assays, and enzyme activity was determined according to Erlanger et al. (1961). Enzymatic inhibition was obtained by determining the line slopes (absorbance × time) of the activity assays of the control enzymes and enzymes with inhibitor, and the absorbance values were converted into µmol of product, using the molar extinction coefficient of BAPNA.

Oxalic acid (OA) was determined using the titration method (AOAC 1990); that is, hot-extracted in HCl and caprylic acid, precipitated, and quantified by titration with potassium permanganate.

The tannins (TAN), extracted with methanol (80%) using the method describe by Swain and Hillis (1959) were measured by the colorimetry method according to AOAC (1990).

Statistical methodology

A univariate statistical technique was used to interpret the trypsin inhibitor levels with

analysis of variance (ANOVA), and the treatment means were compared using Tukey's test at the 5% significance level.

The multivariate analysis techniques were used to evaluate and interact the data related to the TDF, OA, TAN and the minerals levels with which they can complex before and after heating.

After obtaining the results, principal component analysis (PCA) and Biplots (Gower & Hand 1996) were used to identify which heat treatments differed between the species and the variables that most contributed to their differentiation.

A response surface model (RSM) (Anderson-Cook et al. 2009) was fitted for each species to determine the combination of time and value of the most important variable identified in the model that led to minimal response for the TAN variable.

The R Core Team statistical analysis system (R Core Team 2014) was used to perform the analyses and computational routine for PCA and RSM.

RESULTS AND DISCUSSION

Determination of the chemical score and amino acid profile

According to Table I, the amino acid profiles and chemical scores of the protein fraction in raw leaves were quantitatively and qualitatively similar, although the protein levels differed between PGH (27.68 %) and PAM (23.34 %) (preliminary studies). Among the essential amino acids, leucine (6.96%), lysine (5.37%), and phenylalanine (5.02%) prevailed, at values close to those reported in other studies on PAM leaves (Albuquerque et al. 1991, Almeida et al. 2014). Both species also showed a high lysine concentration as compared to other leafy vegetables, exceeding the levels in kale and lettuce by more than 20 times and in spinach by approximately seven times (Mercê et al. 2001).

Lysine represents 5–6% of the total leaf protein content, and considering the importance of this amino acid in the human diet and its limited presence in rice, which is the predominant food in the Brazilian diet and that of many other countries, this is relevant to the chemical composition of PGH and PAM leaves.

Takeiti et al. (2009) evaluated the amino acid profile in PAM and confirmed the suitability of the amino acid profile in its leaves, with the exception of lysine and the sulfur-containing amino acids (methionine + cysteine), which limited the protein quality.

Glutamic acid (12%), proline (12%), and aspartic acid (10%) are dominant amino acids in the leaves of fresh kale, whereas lysine and leucine limit its protein quality (Lisiewska et al. 2008). Lisiewska et al. (2011) observed approximately 25.9% protein (DM) in leaves of spinach, and 49% of this total was essential amino acids. The authors identified cysteine and methionine as limiting amino acids.

Regarding leaf protein quality in PGH and PAM (Table I), the amino acid score was higher than 100% for all analyzed amino acids. However, as this is a plant protein, this value was corrected for its true digestibility of approximately 76%. The amino acid score only remained high for the histidine, aromatic (phenylalanine and tyrosine), threonine, and tryptophan amino acids, further declining for the sulfur-containing amino acids (methionine and cysteine).

Proteins of the cacti in question are classified as incomplete, as they are deficient in one or more essential amino acids. They have the same protein composition as legumes, which are considered as the most adequate protein, and contain 10 to 30% protein. Therefore, they are similarly deficient in sulfur-containing amino acids.

Table I. Mean values and standard deviation of the amino acid levels (dry basis) and chemical score of the essential amino acids of the proteins in leaves of Pereskia grandifolia Haworth (PGH) and Pereskia aculeata Miller (PAM).

Essential amino acids	OPN species		EAA requirements[*1]	OPN species	
	PGH	PAM		PGH	PAM
	(mg g^{-1} protein)			Chemical score[*2] (%)	
Histidine	24.6 ± 0.10	24.0 ± 0.10	15	164 (127.4)	160 (124.3)
Isoleucine	37.5 ± 0.06	36.9 ± 0.04	30	125 (97.13)	123(95.57)
Leucine	69.6 ± 0.07	69.0 ± 1.00	59	118 (91.69)	117 (90.91)
Lysine	54.0 ± 0.20	53.4 ± 0.08	45	120 (93.24)	119 (92.46)
Sulfur (Met + Cys)	17.84 ± 0.4 (16.9+0.9)	17.18 ± 0.04 (16.3+0.9)	22	81 (62.94)	78 (60.61)
Aromatic (Phe + Tyr)	54.37 ± 0.1 (50.5+3.9)	53.71 ± 0.09 (49.9+3.81)	38	143 (111.1)	141 (109.6)
Threonine	30.6 ± 1.05	30.0 ± 0.09	23	133 (103.3)	130 (101.0)
Valine	47.5 ± 0.09	46.9 ± 0.20	39	122 (94.79)	120 (93.24)
Tryptophan	21.7 ± 0.1	21.1 ± 0.12	6	362 (281.3)	352 (273.5)
Total	**357.71**	**352.19**	**277.0**		
Non-essential amino acids					
Arginine	49.7 ± 0.02	49.1 ± 0.01			
Aspartic ac.	6.92 ± 0.02	6.86 ± 0.07			
Serine	2.76 ± 0.09	2.7 ± 0.06			
Glycine	4.72 ± 0.08	4.66 ± 0.07			
Glutamic ac.	10.03 ± 0.08	9.97 ± 0.09			
Proline	5.94 ± 0.09	5.88 ± 0.09			
Alanine	3.74 ± 0.09	3.68 ± 0.09			

[*1]Essential amino acids required by adults - current estimates by the FAO/WHO[20]. [*2]Chemical score of essential amino acids, corrected for true protein digestibility (77.7%).

Considering the fact that vegetable proteins make a considerable contribution to the total protein ingestion of population, mixing different protein sources with complementary levels of amino acids is a good way to improve the nutritional quality of the proteins in these leafy vegetables. Adequate OPN supplementation into prepared meals consisting of cereals (rice, wheat, or corn), or even as a complement to the typical rice-bean mixture, may compose a high-quality protein meal.

Effect of heating on leaf bioactive composition

The main problem in exploiting leafy vegetables as a nutrient source is their antinutritional factors, which can interfere with the bioavailability and digestibility of some nutrients. Table II shows the levels of some of these compounds and the minerals with which they can complex, as determined in raw leaf samples of PGH and PAM and samples cooked in two different media for varying times.

Table II. Mean values of total dietary fiber, minerals, and antinutritionals (oxalic acid, tannins, and trypsin inhibitors) in leaves of *Pereskia grandifolia* Haworth (PGH) and *Pereskia aculeata* Miller (PAM), raw and subjected to wet- and mixed-cooking processes for various times.

Treatments	Dietary fiber (mg 100 g^{-1})	Minerals (mg 100 g^{-1})						Oxalic acid	Tannins (mg 100 g^{-1})	ITU*1 (20 mL·g^{-1})
		P	Mg	K	Ca	Zn	Fe			
PGHRL	**52.8**	**202**	**2333**	**3986**	**2550**	**3.6**	**17.2**	**7891.9**	**2368.7**	**1084.4[a]**
PGHWC1	48.9	191	1968	2435	2800	3.6	15.4	4310.8	2347.8	284.7[b]
PGHWC2.5	51.2	190	1808	2228	2461	3.6	15.5	5135.1	2645.3	155.2[d]
PGHWC5	49.3	195	1795	2258	2655	3.5	17.4	5959.6	2474.9	91.5[e]
PGHWC10	47.5	190	1754	2217	2847	3.5	20.6	6202.7	2606.4	180.5[c]
PGHMC1	48.6	175	1395	3349	1826	2.9	14.9	6060.8	1813.9	181.3[c]
PGHMC2.5	36.8	165	1544	2985	1840	2.8	15.7	6097.7	2496.4	181.0[c]
PAMRL	**51.0**	**184**	**936**	**4425**	**4095**	**6.3**	**21.3**	**5858.8**	**3085.0**	**1324.0[a]**
PAMWC1	41.5	160	1093	3818	4211	6.1	13.9	9729.7	3558.5	221.1[b]
PAMWC2.5	40.2	160	936	3579	4066	5.3	12.3	9993.2	3803.7	185.3[c]
PAMWC5	45.6	160	965	3510	4165	5.2	12.7	9878.4	3616.5	171.8[d]
PAMWC10	36.7	165	763	3276	4275	5.2	11.6	9998.2	3297.9	188.5[c]
PAMMC1	37.2	120	705	2621	3145	3.9	12.3	7168.9	3626.9	224.5[b]
PAMMC2.5	40.6	125	851	2554	3723	4.7	13.8	7493.2	2727.5	224.2[b]

All procedures were conducted in triplicate and the results expressed as dry matter. RL - raw leaves; WC - wet cooking; MC - mixed cooking; Cooking times - 1, 2.5, 5, and 10 min. *[1]Univariate statistics were conducted for ITU analysis (inhibited trypsin units in µmol min-1 g-1 in 20 mL of extract) using Tukey's test. The same letters in the columns do not significantly differ among the treatments, within the same species ($p \leq 0.05$); CV (coefficient of variation) = 0.96%; SE (standard error) = 1.85.

Protease inhibitors are widely distributed throughout the plant (Lisiewska et al. 2011, Pereira et al. 2011) and their presence in OPN leaves, according to Table II, maintains the antinutritional property of the extract from these leafy vegetables. Raw PAM leaves showed the highest inhibition, with 1,324 ITU the inhibition, whereas the inhibition was 1,084 ITU, 20 mL g^{-1} in raw PGH leaf extract. Heating caused a significant decrease in the inhibition of this protease. The inhibition in PGH and PAM leaves decreased by 4 and up to 6 times, respectively, within the first minute of wet cooking, as compared to the raw leaves. There was a trend of decreasing inhibition in all treatments until 5 min of wet cooking. This behavior suggests that most of them are likely protein inhibitors and consequently denatured in OPN leaves.

The significant decrease observed in both species within the first minute of wet cooking may also be related to the denaturation of protein inhibitors that cannot resist the heat and thus more weakly inhibit trypsin; these are most likely *Kunitz*-type inhibitors (Nelson & Cox 2014).

However, the maintenance of inhibitory activity in the leaf during the subsequent minutes of wet cooking suggests the presence of less thermolabile inhibitors and stronger bonds to digestive proteases such as *Bowman-Birk,* due to their compact configuration resulting from a high number of disulfide bonds (Damodaran at al. 2010).

For both mixed-cooking times (1 and 2.5 min), the inhibited trypsin units were still significant in the assay following 10 min of wet cooking, relative to the previous conditions. Although the observed inhibition was lower than that observed in raw leaves, on average, it increased to 16.4% in the extracts of PGH and PAM . The heat generated by boiling the OPN leaves (wet cooking) for 5 min or more or braising them in oil (mixed-cooking) for any length of time may have facilitated the inhibitors, which were still present to interact with other substances in the extract (most likely non-protein in nature), and possibly led to increased enzymatic inhibition. Other antitrypsin factors, such as more heat-resistant tannins, may have also contributed to the increase in inhibitory activity. These compounds have the ability to combine with digestive enzymes and form stable complexes via hydrogen bonds between the phenolic groups and their respective protein sites that confer strong stability to these substances (Hęś et al. 2014, Nelson & Cox 2014).

Despite the lack of consensus about the percentage of tannins necessary to inhibit digestive enzymes, there can be an increase in total free phenols during the cooking process. These can, in turn, penetrate the plant matrix and react with proteins, making them less susceptible to enzymatic hydrolysis (Benevides et al. 2013, Marques et al. 2014).

The observed results in our studies corroborate the results of a study by Pompeu et al. (2014) on the leaves of OPN. They evaluated the behavior of trypsin inhibitors using gel electrophoresis and observed that heating at 100°C for 1 min was enough to degrade bands related to protein inhibitors. However, some of the antinutritional factors were not completely degraded.

The trypsin inhibition, mainly in raw OPN leaves, led to a decrease in protein quality after correcting for protein digestibility, especially in the sulfur-containing amino acids, since trypsin and chymotrypsin are particularly rich in sulfur-containing amino acids and divert these amino acids from tissue protein synthesis (Pompeu et al. 2014). Thus, the decrease in inhibition we observed within the first minute of cooking should lead to optimal protein quality in OPN leaves.

Multivariate techniques were used to understand the behavior of the chemical components of PGH and PAM under heat treatments and the antinutritional factors that they can complex with, according to cooking time. Figure 1a shows the plot for the scores of the first two principal components, whereas Figure 1b shows the relationship between the variables.

The first principal components (PC1) responded to the most variance (79.2%). PC1 explained 58.5% of the information associated with the original information, whereas second principal components (PC2) explained 20.7% of the variability in the data matrix.

According to Figure 1a, only PC1 was necessary to differentiate the heat-treated samples according to their chemical behavior, whereas PC2 clustered the treatments according to species.

The treatments of each species clustered together, occupying the same region or close regions in the score plot (Figure 1a), whereas the raw samples remained between the formed clusters. Figure 1a shows the arrangement of the treatments along PC1. The raw and heat-treated samples of PAM occupied more positive regions of PC1, while the other remaining treatments shifted toward more negative values. Figure 1b shows that the variables related to the TAN and OA antinutrients exhibited high weights in PC1, attracting the scores toward more positive values.

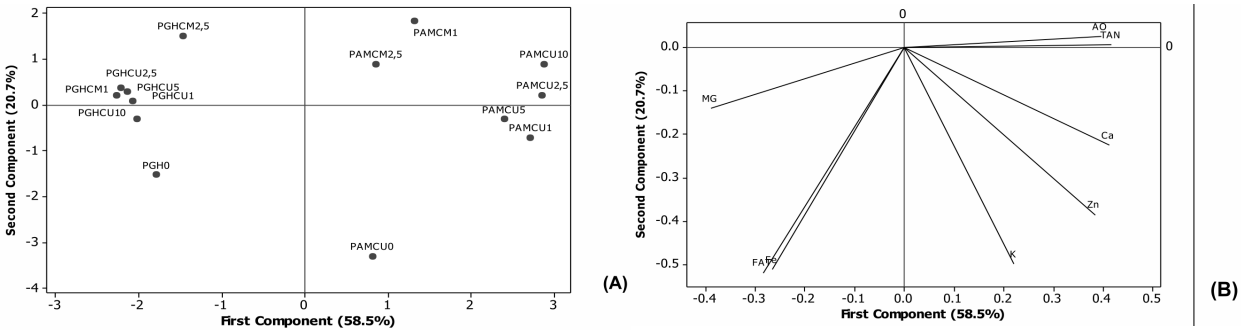

Figure 1. (a) Scores of the first two principal components used to differentiate the heat treatments; (b) Biplots used to indicate the variables that differentiated the treatments in both species.

The proximity of the treatments without the intervention of heat or cooking medium in PGH and the vector related to the TDF variable allowed us to conclude that the highest values of this compound were in the leaves of this species (52.8 mg 100 g^{-1}) and in the raw leaves of PAM (51.0 mg 100 g^{-1}). The TDF levels in the non-heat-treated samples were higher than in the heated ones. A comparison of TDF levels showed that the wet-cooking treatments were most effective at maintaining the levels of these cell wall constituents in OPN leaf tissues.

In the plot of the heated treatments, heat-treated PAM samples exhibited the lowest tissue values of total dietary fiber. However, these were still higher than those of the PAM treatments, which were located in the quadrant opposite the vector representing this compound. Hydrothermal processing for a longer or shorter time affects the macromolecules that form the plant cell wall. The cellulosic fibers swell and partially fragment. The combination of water and heat causes starch to gelatinize, and the consequent cell rupture leads to the release of insoluble fiber, such as lignin, into the medium (Dziedzic et al. 2012). During heat treatment, cellulose can chemically degrade into glucose, and hemicellulose can chemically degrade into arabinose, xylose, and galactose (Rickman et al. 2007).

The proximity of the vectors related to the iron and TDF variables are negative and suggests an association between these two variables and the treatments near them. The highest proportional TDF and iron values in PGH were observed in raw leaves and in those cooked for 10 min. In PAM, raw leaves contained the highest level of this polysaccharide, and a high level was maintained after 5 min of cooking.

The highest amounts of iron in PGH were also observed in raw leaves. After 10 min of cooking, the iron content increased in the leaf tissues of this species and equaled the values observed in raw PAM leaves.

Although raw PAM leaves had the highest iron values (21.3 mg 100 g^{-1}), PAM exhibited the lowest iron retention after heat treatment. The highest retention value was observed in leaves cooked for 1 min, but only slightly more than half of the iron was retained (65.5%). At longer cooking times, there was a trend toward decreasing by more than half (on average, 57.13%).

Raw PGH leaves had lower iron content (17.2 mg 100 g^{-1}) than raw PAM leaves, and this content decreased approximately 10% within the first minutes of heat treatment. However, there was a trend toward maintaining the original content over the succeeding minutes of wet cooking, and this increased in relation to the raw leaves within 10 min of cooking.

The vectors related to iron and TDF exhibit contrasting signals to the antinutrients OA and TAN. This corroborates the variation in these compounds, particularly in PAM, since the levels varied in the exact opposite direction as those of the antinutrients. When combined with oxalates and phenolic substances, fiber can compromise the bioavailability of minerals such as calcium, zinc, magnesium, and iron, among others. These are present at substantial levels in OPN, which can lead to their intake into the human body (Almeida et al. 2014, Takeiti et al. 2009).

According to the Figure 1a, the vector related to TAN, which was closest to the origin, exhibited the highest weight, contributing more to PC1 and reflecting the highest variability in the treatments. Thus, there is evidence to support the assumption that the PC1 modeled the antinutritional behavior of the treatments. Due to its sufficient weight (negative), the TDF/iron variable also aided in differentiating the treatments. Thus, the treatments on the right side of the plot had the highest antinutritional value in their leaves, according to Table I. In Figure 1, the proximity of the axes related to the OA and TAN variables (Figure 1b) in the PAM10 and PAM2.5 treatments (Figure 1A), respectively, demonstrated that the highest antinutritional values were observed in this species after these respective cooking times.

According to principal component analysis (Figure 1), which showed the relationship among the treatments in the score plot, the Ca, Zn, and K variables in supplemental Figure 2b were more strongly associated with PAM, particularly in raw leaves (K and Zn) and leaves cooked for 1 and 5 min (Ca). Calcium is abundant in green leaves, mainly in the form of calcium pectate, and composes the middle lamella of the cell walls, adding structural integrity to the membranes and the cell wall (Kinupp & Barros 2008). The temperature most likely favored the rupture of cell walls, and could be responsible for the increase in observed mineral content in the samples subjected to heat treatment. Santos et al. (2008) observed that the leaves of Brassicas subjected to different boiling times maintained their calcium levels with increased cooking times, similar to the behavior of magnesium, which exhibited a small decrease until 8 min of boiling. Similar behavior was observed in the present experiment. The location of this mineral on the plot suggests that the highest values were in the raw PGH leaves, which exhibited a 75.2% retention index after 10 min of wet cooking.

Regarding zinc, the location of the vector (Figure 1b) indicated that the highest values were in raw PAM leaves (6.3 mg 100 g^{-1}), whereas raw PGH leaves contained slightly more than half of that amount (3.6 mg 100 g^{-1}), according to Figure 1a. Ora-pro-nobis flowers evaluated by Almeida

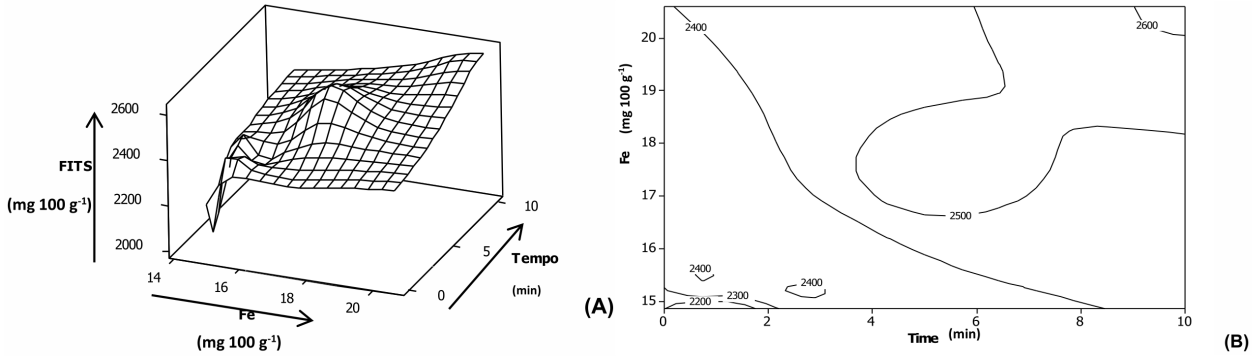

Figure 2. (a) Response surface; (b) Contour plot to study minimization of the response predicted by the model in relation to the iron compound and time in PGH.

et al. (2014) also exhibited more abundant zinc levels in PAM; however, the reported values of 4.93 mg 100 g^{-1} for PGH and 7.30 mg 100 g^{-1} for PAM exceeded the observed levels in this study. The first minute of wet cooking did not affect the zinc content in PGH, as the retention index was approximately 96.2%. The retention index remained the same from 1 to 5 min of wet cooking (83.4%). Regardless of time, wet cooking led to higher zinc losses in PAM; however, the highest decrease in the leaf tissues of both species was observed after mixed-cooking.

All these factors led to the PGH treatments exhibiting more negative PC1 values and the PAM treatments exhibiting more positive values, according to the arrangement of the plots in Figure 1a.

Researchers have tried various methods of decreasing antinutritional factors in vegetables, and heat treatment is one of the most frequently discussed in the literature (Benevides et al. 2013). In addition, the leaching of substances and the action of other compounds to decrease antinutritional factors can be facilitated by the presence of water (Naves et al. 2010).

In the present study, water-soluble antinutritional compounds were expected to transfer into the liquid medium due to heating and leaching, as in other conventional cooking procedures such as those reported by Ogbadoyi et al. (2006) who observed a 65% decrease in oxalate content in ingested vegetable leaves after heating (100°C for 5 min) and discarding the water, and others (Benevides et al. 2013, Lisiewska et al. 2011) .

However, this hypothesis was not confirmed, mainly due to the TAN levels. The variation in the TAN and AO levels may have occurred due to the chemical components of this vegetable and their possible interactions with the antinutrients (Damodaran et al. 2010).

Other exploratory analyses were used to evaluate the reasons for the large retention of chelating antinutritional minerals in PGH and PAM in order to minimize the levels of such compounds in the leaves of these vegetables, primarily after wet cooking.

Consistent with the proposed objective, the aforementioned PCA (Figure 1) was used to identify compounds that led to a minimal response for the variable related to leaf TAN levels. Due to the proximity of the vectors, these conclusions were extended to the variable related to leaf OA content as well.

The percentage of sample variation explained by the first two components, approximately 80%, was considered adequate for this study, and the results obtained using these scores (Figure 1a) showed that the variables (Figure 1b) differentiated the heat treatments in both species. The biplots (Figure 1b) confirmed the effect of the compounds on the clustering of the treatments. The results determined the selected variables to be used for constructing response surfaces to study the minimization of TAN, as shown in the tridimensional plots (Figures 2a and 3a), and for their projection in the plane, using contours (Figures 2b and 3b).

The compounds' interactions with time (T) relative to the response of TAN were modeled based on the PCA, considering the parameters represented by the compound-time interaction. Supplementary Material (Table SI) and Figures 2a and 2b show the estimates of the fitted linear model for analysis of the response surface in order to minimize the TAN response observed in PGH.

According to the results shown in Supplementary Material (Table SII), the fitted model explained 64.5% of the sample variation and is an adequate fit considering the predictive power used in analysis of the compounds that differentiate the heat treatments. Due to the

estimated coefficients, the iron compound showing no interaction with time made a higher contribution to the minimization of the TAN response. This decrease was also observed in the interaction with time, albeit to a lesser extent. The response surface fitted to the times and responses of the iron compound is shown in Figure 3a.

Figure 2a shows that minimization of the TAN response was attributed to one region with lower iron and time values.

These values are shown in Figure 2b. According to the response surface for the analyzed factors in this figure, minimization of the mean TAN responses occurred at a temperature of 96 ± 2°C, for times shorter than 2 min, and with Fe responses lower than 16 mg 100 g^{-1}.

The TAN levels in the PGH leaves reached their maximum value when the heat treatment lasted for a longer time, regardless of cooking medium, and according to the contour plot, the 4- to 6-min interval most strongly contributed to their increase.

For PAM, Supplementary Material (Table SII) and Figures 3a and 3b show the estimates of the fitted linear model for analysis of the response surface related to minimization of the TAN response.

Supplementary Material (Table SII) shows that not all parameters were significant; however, given the quality of the model fit indicated by the determination coefficient (R^2 = 73.96%), the model had good predictive power.

According to the results, the Zn compound, interacting with time, contributed to the minimization of TAN. In determining the other parameters, the T × Zn interaction exhibited a higher decrease in the mean response of the TAN variable. Figures 3a and 3b indicated, respectively, the response surface and contour plot of the predicted values for the fitted model.

Regarding the fitted linear model for TAN, Figure 3a shows that minimization of the predicted response was observed in one region with low Zn and time values. The more precise location of these parameters is reinforced by the contour plot (Figure 3b), in which times shorter than 4 min and Zn compound values produced minimal responses for TAN, with estimates between 3,000 and 3,200 mg 100 g^{-1} of tannins in PAM leaves.

Regarding the lower tannin content in the PGH and PAM leaves, Figures 2 and 3 allowed us to draw inferences about the causal effects on the responses of interest in order to optimize the response variable. Simultaneously considering the response surface plots, the oxalic acid

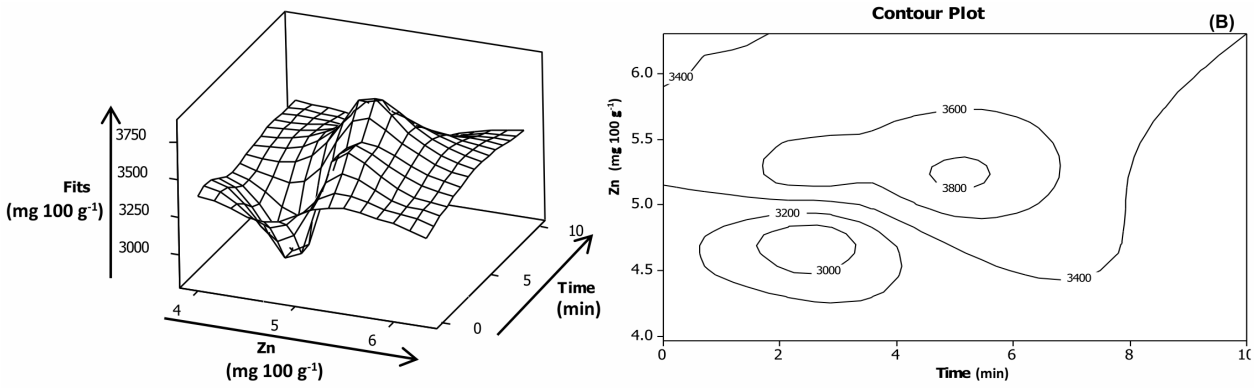

Figure 3. (a) Response surface; (b) Contour plot to study the minimization of the response predicted by the model in relation to the Fe compound and time in PAM.

values increased with increasing cooking time, especially for wet cooking in PAM, and with the tannin values in both species.

Based on the literature, there is strong evidence that hydrothermal processing of the plant tissue physically and chemically altered cell wall properties (Dziedzic et al. 2012), and consequently, interfered in the interrelationship between the variables.

The chemical composition of these vegetables may be largely responsible for the behavior observed in this study. Fiber, present in large amounts in the leaves of these species (Table II), has polar groups with high water stability and confers water retention capacity to these compounds (Lattimer & Haub 2010, Schroeder et al. 2013). PGH and PAM have high biopolymer content, including arabinogalactan, which has properties that allow it to chelate metal ions (Carvalho et al. 2014, Lima-Junior et al. 2013). These factors most likely affected the movement of compounds between the inner leaf and cooking media.

Proteins, oxalates, phytates, lignin, phenolic substances, and minerals, among other inorganic compounds, are also associated with cell wall polysaccharides. Tannins emerge among the antinutritional factors as one of the most studied compounds. They are mainly observed in plant vacuoles, which are released into and allow the action of these antinutrients in plant tissues after plant damage (Hęś et al. 2014).

According to these factor models (Figures 2 and 3), prolonged contact with heat during boiling most likely favored the rupture of cell walls and partial release of tannins that were bound to proteins or other polymers, such as carbohydrates, and increased the availability of free tannins, which leach into the medium once they become soluble (Delfino & Canniatti-Brazaca 2010). However, as tannins, among other phenolic classes, have hydroxyls and aromatic rings, they are able to combine and form stable complexes (Hęś et al. 2014, Marques et al. 2014). Thus, longer contact with the heat certainly favored the complexing of these antinutritionals. Once free, they could interact with multivalent minerals such as iron and zinc and form insoluble complexes that would hinder the migration of these chemical constituents into the medium.

These results can be extended to oxalic acid, which is present in higher amounts in green leafy vegetables. The combination of soluble oxalate, sodium, and potassium salts that forms is easily released from the food when subjected to leaching processes; behavior further favored by increasing temperature (Ferreira & Arêas, 2010). However, due to oxalic acid's strong chelating ability to multivalent cations (Ogbadoyi et al. 2006, Nawirska-Olszánska et al. 2014), insoluble salts such as ferrous oxalate or slightly soluble salts such as calcium oxalate can form.

In the routine preparation and consumption of these vegetables, the interrelationships between the chemical components present in OPN leaves led to the observed behavior of high antinutritional retention.

These effects are particularly important considering the prevalence of high levels of micronutrient deficiency and malnutrition among the poorest individuals in developing countries and vulnerable groups such as elderly, pregnant women, adolescents, children, and individuals with deficient intake of proteins and micronutrients.

CONCLUSIONS

The amino acid profiles and chemical scores of the protein fraction in raw leaves were similar. Among the essential amino acids, leucine, lysine and phenylalanine (5.02%) prevailed. The amino

acid score was higher than 100% for all analyzed amino acids. However, as this is a plant protein, this value was corrected for its true digestibility of approximately 76%.

The thermal treatments decreased the levels of total dietary fiber and minerals in both species. The wet cooking was more effective in maintaining dietary fiber and minerals levels.

The first few minutes of cooking significantly reduced the percent inhibition of the digestive protease for both species of OPN. In relation to the tannin and oxalic acid levels, the treatments were effective in reducing these antinutrients to the PGH, but for PAM there was an increase in these compounds.

In routine situations of preparation and consumption of these vegetables the interrelationships between the chemical components present in leaves of ora-pro-nobis culminated with the behavior of great antinutricional retention verified.

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SUPPLEMENTARY MATERIAL

Table SI. Estimates of the linear model with interactions used to optimize the TAN response observed in PGH.
Table SII. Estimates of the linear model with interactions used to optimize the observed TAN response in PAM.

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