EVALUATION OF PROTEIN EXTRACTION METHODS TO OBTAIN PROTEIN CONCENTRATE FROM CASSAVA LEAF

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ABSTRACT: The cassava leaf, waste generated in the harvest of the roots, is characterized by high content of protein, vitamins and minerals; however, its use is limited due to the high fiber content and antinutritional substances, which can be removed by obtaining protein concentrates. In this context, the objective of this study was to evaluate protein extraction processes, aiming the use of cassava leaves (*Manihot esculenta* Crantz) as an alternative protein. Four methods were tested: 1) Coagulation of Proteins by Lowering the Temperature, 2) Extraction by Isoelectric Precipitation, 3) Solubilization of Proteins and 4) Fermentation of Filter Leaf Juice. To obtain the concentrates, the use of fresh or dried leaves and extraction in one or two steps were also evaluated. The solubilization of proteins (method 3) showed a higher extraction yield; however, with concentrate of low quality. The fermentation of the juice (method 4) produced concentrates with higher quality and lower costs and the isoelectric precipitation (method 2) promoted the obtention of concentrates in less time, both with good prospects for use. The use of two extraction steps was not advantageous to the process and there was no difference between the use of fresh or dried leaf, and the use of fresh leaves is presented as a good option for the simplicity of the method.

KEYWORDS: exploitation, agricultural waste, protein extraction.

AVALIAÇÃO DE MÉTODOS DE EXTRAÇÃO DE PROTEÍNAS PARA OBTENÇÃO DE CONCENTRADO PROTÉICO DE FOLHAS DE MANDIOCA

RESUMO: A folha de mandioca, resíduo gerado na colheita das raízes, caracteriza-se pelo alto teor de proteínas, vitaminas e minerais, no entanto sua utilização é reduzida devido ao alto teor de fibras e substâncias antinutricionais que podem ser removidas durante obtenção de concentrados proteicos. Neste contexto, o objetivo do presente trabalho foi avaliar processos de extração de proteínas, visando ao aproveitamento das folhas de mandioca (*Manihot esculenta* Crantz) como alternativa proteica. Quatro métodos foram testados: 1) Coagulação de Proteínas por Abaixamento da Temperatura; 2) Extração por Precipitação Isoelétrica; 3) Solubilização das Proteínas; 4) Fermentação do Suco de Folhas Filtrado. Para a obtenção dos concentrados, avaliaram-se também a utilização de folhas frescas ou secas e a extração em uma ou duas etapas. A solubilização das proteínas (método 3) apresentou maior rendimento de extração, no entanto com concentrado de baixa qualidade. A fermentação do suco (método 4) produziu concentrados com mais qualidade e menores custos, e a precipitação isoelétrica (método 2) promoveu a obtenção de concentrados em menor tempo, sendo ambos com boas perspectivas de utilização. A utilização de duas etapas de extração não foi vantajosa ao processo e não houve diferença entre a utilização de folha seca ou fresca, sendo que a utilização de folhas frescas se apresenta como boa opção pela simplicidade do método.

PALAVRAS-CHAVE: aproveitamento, resíduo agrícola, extração de proteínas.

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INTRODUCTION

Brazil accounts for almost 13% of world production of cassava, which is grown in all regions of the country, in different production systems that range from subsistence farming to the adoption of new technologies of mechanized planting and harvesting (SCALON FILHO et al., 2005). The leaves of cassava are, most often, wasted in all Brazilian regions, and according to SAGRILO et al. (2008), the production of leaves varies with the harvesting period and can reach approximately two tons of dry matter per hectare, which can be considered agroindustrial waste. The low utilization of cassava leaves (*Manihot spp*.) in alimentation is due to the lack of technical and scientific information to enable detailed knowledge of this food.

The content of crude protein present in cassava leaves can vary between 15 and 40% of dry mass, depending on the age of the plant, climatic conditions and cultural practices (MODESTI et al., 2007; PEQUENO et al., 2007; SILVA et al., 2009; ALETOR, 2010).

Cassava leaves, despite having good protein content and other nutritional components, have its *in natura* use limited due to the high content of alimentary fiber, which can not be digested in the stomach of humans and monogastric animals, and by factors such as the presence of antinutritive and/or toxic substances, like phenolic compounds, tannins, saponins, lectins or cyanogen (MODESTI, 2006; MELO et al., 2007; WOBETO et al., 2007; SILVA et al., 2010; BLAGBROUGH et al., 2010).

The use of protein from the leaves is possible if the leaf material is subjected to processes that eliminate considerably the toxic and antinutritional agents, aiming also eliminate the fibrous part (BOHNENBERGER, et al., 2010; ALETOR, 2010). An alternative is the extraction of proteins, resulting in cassava leaf protein concentrate (CLPC).

The obtention of leaf protein concentrate (LPC) allows the use of leaf protein as food containing low fiber content and improved nutritional quality (MODESTI et al., 2007; TEO et al., 2010). According to FASUYI & ALETOR (2005), the cassava leaf protein concentrate has reasonable amount of essential amino acids and, due to its high content of lysine, could supplement foods that have this amino acid deficiency, such as cereals.

The process of extracting protein for LPC obtention basically consists of an extraction step, in which you get the green juice and the fibrous residue from the use of extraction solution (water or moderately alkaline solutions) and cell disruption. Subsequently, the green juice, which contains the soluble components of the cells, is subjected to a process that promotes the precipitation of proteins which can be obtained via isoelectric precipitation (pH variation action), thermocoagulation (temperature action), by autocoagulation (fermentation), flocculation, ultrafiltration or extraction with organic solvents such as ethanol, butanol, acetone and ether (MODESTI et al., 2007; DEWAN et al., 2007; TEO et al., 2010).

The protein content of the CLPC may vary from 40 to 70% depending on the extraction technique employed (TEO et al. 2010). The protein concentrate can be applied for various purposes, depending on the type of extraction that was used for obtaining it.

In this context, the aim of this study was to evaluate protein extraction processes, aiming the use of cassava leaves (*Manihot esculenta* Crantz) as an alternative protein.

MATERIAL AND METHODS

Collection and Preparation of the Cassava Leaves:

The cassava leaves (*Manihot esculenta* Crantz) grown in the region of Maripá city, located in the western of the state of Paraná (PR), in Brazil, were randomly collected in plants aged 12 months, in the upper third of the plant and packed in plastic bags for transport to the preparation location. In the laboratory, the leaves were washed with running water and distilled water, the petioles were removed, and the leaves were arranged to shade drying for seven days. Then, the

leaves were placed in a drying oven with forced air circulation of the brand Tecnal, model TE - 394, Piracicaba, Brazil at 40°C for 6 hours to finish the drying. The dried leaves were analyzed for moisture parameters, standard method in oven and crude protein by the Kjeldahl method, using a conversion factor of 6.25 for protein (ADOLFO LUTZ INSTITUTE, 2008).

The dried cassava leaves had moisture content of 11.27 ± 0.51%, average crude protein content of 32.43 ± 1.05% and crude protein on a dry basis of 36.55 ± 1.18%.

Test of Protein Precipitation of Cassava Leaf Juice:

To verify the effect of pH on the precipitation of proteins in the juice of cassava leaves, the test of precipitation of proteins was carried out according to the methodology described by GLÓRIA & REGITANO-D'ARCE (2000), with the purpose of improving the efficiencies of extraction methods. We used for the preparation of leaf juice, 50 grams of leaves (0.42g of total crude protein) and 1000ml of distilled water, ratio 1:20 (w/v). The leaves were mixed and triturated in a blender of the brand Britânia, Bellagio V5 model, Curitiba, Brazil. The leaf juice was filtered in a cotton fabric and the fibrous part was separated. The pH range studied to verify the precipitation of proteins was between 2 and 12, varying in each unit. The equipment used for the determination of pH was of the brand Tecnal model TEC 3-MP, Piracicaba, Brazil. For each pH value, we used two beakers with 50ml of leaf juice each. The initial pH of the juice was 5.51, and we used solutions of NaOH 0.1N and HCl 0.1N for corrections. The pH was adjusted at the start of the test, after 10 minutes, and every 1 hour for 4 hours. At the conclusion of the pH adjustment, the solution was maintained at rest for 1 hour so the precipitate could totally sediment. Then, each solution was centrifuged in a centrifuge of the brand Celm, model LS-3 plus, São Paulo, Brazil, for 5 minutes at 3200 rpm. Aliquots of each phase (precipitate and supernatant) were removed for the determination of crude protein and moisture. The precipitation of proteins was determined by calculating the protein dispersibility index of the liquid phase - PDI (GLÓRIA & REGITANO-D'ARCE, 2000):

$$PDI\% = \frac{\text{supernant tant phase protein } (g)}{\text{sample total protein } (g)} x\,100 \qquad (01)$$

Obtention of the Protein Concentrate:

To obtain the protein concentrates of the cassava leaves, we tested four methods of protein extraction. In the evaluation of the methods, in the step of obtaining the leaf juice, we started with 100g of leaves in 1000ml of distilled water, resulting in the ratio 1:10 (w/v). The assays were performed with four replicates.

METHOD 1 – Coagulation of Proteins by Lowering the Temperature (CEREDA & VILPOUX, 2003): Initially, the leaves were triturated with distilled water for 5 minutes, then the leaf juice was filtered on cotton fabric to remove the fibrous part. The filtered extract was maintained at rest for 24 hours under refrigeration at 4°C. Subsequently, the sample was centrifuged in a centrifuge of the brand Celm, model LS-3 plus, São Paulo, Brazil, for 10 minutes at 3200 rpm, obtaining a supernatant fraction and a precipitate (CLPC). The precipitate passed through the drying process in an oven with air circulating and air renewal and at a temperature of 60°C to constant weight.

METHOD 2 - Extraction by Isoelectric Precipitation (CEREDA & VILPOUX, 2003): The leaf juice prior to filtration on cotton fabric was subjected to adjust the pH value to 8.0 with NaOH 0.1N. After filtration, pH of the extract was again corrected to 4.0 with HCl 0.1N, and then cooled at 4°C. From this point on, it was followed by the same procedures as described in Method 1.

METHOD 3 - Solubilization of Proteins (CEREDA & VILPOUX, 2003): pH adjustment was performed directly on the leaves immersed in water (1:10), with NaOH 0.1N to pH 8.0 before homogenization in a blender without further correction of the pH. After triturating in a blender, the extract was filtered in cotton fabric and the obtained leaf juice was taken to an oven at 100°C for drying the protein concentrate to a constant weight.

METHOD 4 - Fermentation of Filter Leaf Juice (adapted from CHAVES, 1987): The leaves were triturated with distilled water for 5 minutes and then the pH was adjusted to 8.0 with NaOH 0.5N solution. The extract was filtered in cotton fabric. The filtrate was left to ferment naturally in a glass jar for 48 hours at room temperature. With the fermentation, the pH dropped naturally promoting the separation of the fractions. Then, the solution was centrifuged for 10 minutes at 3200 rpm, obtaining a supernatant fraction and a precipitate (CLPC). The precipitate passed through the drying process in an oven with air circulating and air renewal and at a temperature of 60°C to constant weight.

The precipitates (protein concentrates) obtained in the extraction were analyzed for crude protein parameters and moisture. With the results we calculated: a) the crude protein content of the concentrate, b) the mass of crude protein, obtained from 100 grams of dried leaves of cassava (dry basis), c) the yield of protein extraction d) the mass yield of protein concentrate and e) the percentage of mass loss of the extraction process, determined by the mass balance of each method evaluated.

We considered the permanent regime for the calculation of the mass balance.

The extraction yield (EY) of protein was calculated by Equation 02:

$$EY\ (\%) = \frac{CPPC}{CPBE}\ x\,100 \qquad (02)$$

In which:

CPPC = Crude Protein of the Protein Concentrate mass (g);
CPBE = Crude Protein present in the Beginning of the Extraction mass (g).

The mass yield of the protein concentrate (MYPC) was calculated by the equation 03:

$$MYPC\ (\%) = \frac{PCM}{CLM}\ x\,100 \qquad (03)$$

In which:

PCM = Protein Concentrate mass (g) in dry basis;
CLM = Cassava Leaf mass present in the Beginning of the Extraction (g) in dry basis.

The percentage losses of the process were calculated checking each step of the extraction process, the mass differences of input and output.

The protein content of each concentrate was determined by analysis of crude protein, performed at each of the obtained products, in the final step of extraction of the proposed methods. All analysis was performed in triplicate.

We used for comparison of the methods the completely randomized delineation, using the Tukey test with 5% level of significance, by the computer program SISVAR version 4.3.

Use of Extraction in two phases:

To check whether there would be an increase in the yield of the extraction methods under study, we also tested two-phase extraction. The second phase of the extraction was made by reusing the fibrous residue.

In these extraction tests of two phases, for all methods, we used a ratio of water and dried leaf of 1:5 (w/v), for the first extraction. In the second extraction, the fibrous residue was triturated again with water in the ratio of 1:5 (w/v). As a result, the two leaf juices were mixed and followed the procedures described for each evaluated extraction method.

The extraction yield of protein and the yield mass of the protein concentrate were obtained by the equations 2 and 3.

To evaluate the effect of increasing the number of extractions in the performance of the methods, we used a 2x4 factorial experimental delineation, and the factors were the Number of extractions (1 or 2) and the Methods of extraction (four methods), with three replications. We used the Tukey test at 5% level of significance, by the computer program SISVAR version 4.3, to determine whether there was significant difference in the yield of protein extracted, mass yield of protein concentrate and protein content of the concentrates.

Use of cassava fresh leaves in the Protein Extraction:

In this test, we used leaves of a cassava plant aged nine months. The leaves were collected randomly in the upper third of the plant. Then the leaves were placed in plastic bags for transport to the preparation location. In the laboratory, the leaves pass through the process of rinsing with water. First, we washed the leaves with treated water for removal of coarse dirt particles and then with distilled water. The petioles present in the cassava leaves were removed. Shortly thereafter, the leaves were chopped and used in the extraction methods. The cassava leaves showed moisture content of 72 ± 2.0% and of crude protein of 27.7 ± 0.5% (dry basis). At the beginning of the extraction the juice of fresh leaves showed pH equal to 6.4.

To compare these two methods of protein extraction using fresh leaves and dried leaves, we used a 2x4 factorial experimental delineation, and the factors were Types of Leaves (fresh or dried) and Protein Extraction Methods (4 methods), with three replications. We used the Tukey test at 5% level of significance, by the computer program SISVAR version 4.3, to determine whether there was significant difference in the yield of protein extracted and mass yield of concentrate. It was not possible to compare the protein content because it was different leaf ages and different collections.

RESULTS AND DISCUSSION

Test of Protein Precipitation of Cassava Leaf Juice:

The amount of protein (g) present in the supernatant solutions, enabled the calculation of the protein dispersibility index (PDI) (Equation 01), for all the evaluated pH values. Figure 1 shows the values of PDI and the Percentage of Crude Protein in the Precipitate, depending on the pH of the solution.

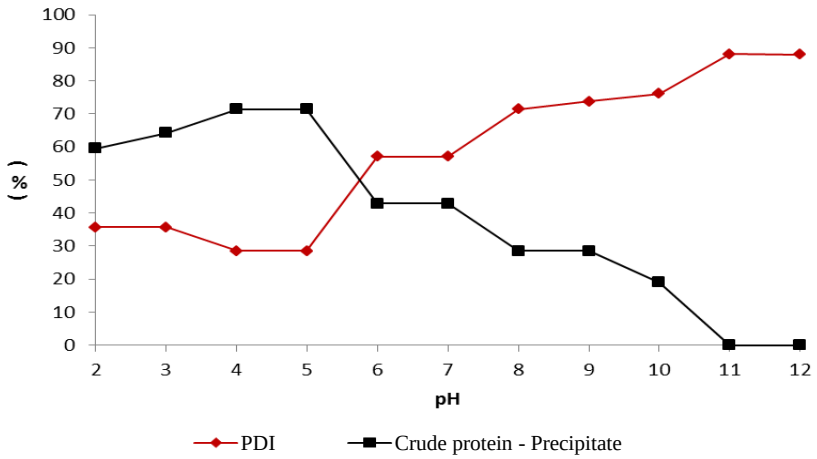

FIGURE 1. Values of PDI and Crude Protein in the Precipitate for each pH condition.

The pH values that showed lower protein dispersibility index were 4 and 5, indicating the lower solubility of the protein in the supernatant phase (liquid) and highest precipitation of it (Figure 1). In this pH range was reached the isoelectric point of the protein present in cassava leaves. The concept of isoelectric point (pI) is derived from the acid-base behavior of amino acids. The isoelectric point is the pH in which positive and negative charges are equal, i.e., zero load and minimum solubility (DERENZO & ALDEIA, 2000). For SGARBIERI (1996), most proteins have isoelectric points or pH between or 4.5 to 6.5, as confirmed in the present study.

The curve of the amount of precipitated protein confirmed that the greatest amount of protein is between pH values of 4 and 5, from these values on the mass of protein present in the precipitate begin to diminish. We can also observe that in pH values between 11 and 12, the protein is almost completely solubilized.

CHAVES (1987), evaluating the influence of pH on the yield of cassava leaf juice proteins, found that at pH above 8 occurred largest solubilization of proteins and at pH below 5, proteins were less solubilized. GLÓRIA & REGITANO-D'ARCE (2000) performed the solubility test of protein concentrate of Brazil nuts and observed that the lower solubility of proteins in pH occurred between 3 and 4 and the greater, at alkaline pH.

The knowledge of the precipitation and solubilization curve of the proteins allows obtaining higher yields of protein concentrates from cassava leaves in order to guide the pH ranges indicated for extraction.

Obtention of the Protein Concentrate:

In the first step of the study, for application of the methods of extraction, we used dried cassava leaves in order to reduce the concentration of toxic compounds present in the leaves. During the drying occurs the contact of the enzyme linamarase with cyanogenic, glycosides, linamarin and lotaustralin, decomposing them to hydrocyanic acid, which being a gas, dissipates into the air (CORRÊA et al., 2002; HELBING & GIGANTE, 2008; BENEVIDES et al., 2011).

In all the methods the extractant was water. With the trituration, occurred the cell disruption of the leaf, delivering the protein to the water. Whereas the dried leaves present protein content of 36.55% and that were used 100g of dried leaves, the process of extracting began with 33.45g of protein mass. The initial pH of the leaf juice was 5.91.

The results obtained with one and two phases of extraction and dried leaves are presented in Table 1.

TABLE 1. Mean values of concentrate mass yield, extraction methods yield and protein content in CLPC using one and two-phase extraction.

Methods of Extraction[3]	Concentrate Mass Yield (%)[1]		Protein Extraction Yield (%)[1]		Protein Content (%)[1,2]	
	Extraction 1 Phase	Extraction 2 Phases	Extraction 1 Phase	Extraction 2 Phases	Extraction 1 Phase	Extraction 2 Phases
1	20.7 ± 0.7^{a1A1}	16.5 ± 1.5^{a1A1}	34.4 ± 1.3^{a2b2B2}	20.7 ± 1.1^{a2A2}	61.0 ± 2.1^{b3c3B3}	45.9 ± 1.5^{a3A3}
2	35.6 ± 1.8^{b1B1}	28.6 ± 1.0^{b1A1}	48.7 ± 1.0^{b2c2B2}	38.6 ± 0.7^{b2A2}	50.0 ± 1.7^{a3b3A3}	49.4 ± 1.3^{a3A3}
3	49.5 ± 1.6^{c1B1}	41.5 ± 0.7^{c1A1}	59.7 ± 1.4^{c2A2}	56.7 ± 1.5^{c2A2}	44.0 ± 1.4^{a3A3}	50.0 ± 1.3^{a3A3}
4	25.9 ± 1.0^{a1A1}	24.4 ± 0.8^{a1b1A1}	36.2 ± 1.1^{a2b2A2}	35.6 ± 0.9^{b2A2}	51.2 ± 0.7^{a3b3c3A3}	53.4 ± 1.2^{a3A3}

[1] Within the same column, the averages followed by the same small letter do not differ statistically by the Tukey test at 5% level of significance. Within the same line, the averages followed by the same capital letter do not differ statistically by the Tukey test at 5% level of significance. [2] Values calculated in dry basis. [3]Methods of Extraction: 1 –Coagulation of Proteins by Lowering the Temperature; 2 – Extraction by Isoelectric Precipitation; 3 – Solubilization of Proteins; 4 – Fermentation of Filtered Leaf Juice.

We can observe by the results for an extraction, showed in Table 1, that despite the method 3 have resulted in the obtention of a product with a low protein content (44%) compared to the other methods, there was a greater recovery of protein (59,7%), indicating that this method is effective in achieving the objective of the authors. This low amount of protein in the product is a result of the high mass yield value (49.5%), since other compounds were recovered in this method eventually "diluting" the proteins in the final concentrate. The higher protein content was obtained in method 1, in which the action of the temperature was used to precipitate the proteins. However, there was no statistical difference between methods 1 and 4.

The concentrate obtained in method 3 showed brown coloration, differing from the other obtained concentrates, which showed dark green. This may be due to the high drying temperature, which may have caused changes in protein and/or browning of tannins present in the concentrate, which could have been avoided by the presence of reducing agents such as sodium sulfite (TEO et al., 2010). This darkening can also be attributed, in a lesser extent, to the Maillard reaction between proteins and sugars (DAMODARAN et al., 2008). Methods 1 and 2 showed higher contents of protein than those cited in papers that used similar methodology (FASUYI & ALETOR, 2005).

CHAVES (1987) compared four methods of protein extraction from cassava leaves, in terms of dry matter and crude protein. The author found that the yield obtained by fermentation was greater than the yield of precipitation by acids, thermocoagulation and use of an organic solvent. For the method of fermentation, CHAVES (1987) used fresh leaves and 5 days fermentation, obtaining protein concentrates with content of 71.5% protein and dry matter concentration yield of 44%. In the present study, the fermentation was performed in 2 days, obtaining protein content of 51.24% and mass yield of concentrate of 25.86%, indicating the need to increase the length of fermentation.

All methods evaluated in this study proved easy to apply. For the production of protein concentrate in larger scales, the simplest methods to employ would be the method 2 described by CEREDA & VILPOUX (2003) and method 4, adapted from CHAVES (1987). The first method produced a concentrate with a higher protein content compared to the other methods employed, in smaller extraction time, features that are of interest for industrial production. However, the method 4 promoted the formation of clots more consistent and secure, easier to split during the process of filtration with cotton fabric.

The aspects of leaf juice and protein concentrate obtained in tests with two extractions were the same as those observed for tests with an extraction. The pH variation was also the same. Using two-phase extraction (Table 1), the method that had a higher yield of protein concentrate, and yield of extraction was the method 3, which is the method of solubilization cited by CEREDA & VILPOUX (2003), but as already noted previously, the obtained concentrate showed brown coloration, which may impair its use. Methods 2 and 4 do not differ statistically at 5% level of significance, as the mass yield of protein concentrate and extraction yield.

The lowest yields of concentrate and extraction were obtained in method 1; however, with the action of temperature, a concentrate with protein content of 45.92% and a protein recovery of 20.65% was produced using two consecutive extractions, greater than that found by TEO et al. (2010) who found recovery of about 14% after two extractions.

We can see that, using only one extraction phase, we obtained in all methods highest yields of protein concentrate compared to the two-phase extraction. Statistically, the method 1 for obtaining concentrated yield did not differ by one or two phases of extraction. The same happened to method 4. For these cases, the use of one or two phases did not affect extraction efficiency and is less costly work only with an extraction.

Regarding the protein obtained in each extraction method, method 1 with one phase of extraction has the highest protein content in the leaves concentrate. Using two-phase extraction, the protein content in the concentrate decreased from 61% to 45.94%, with no statistical difference.

For methods 3 and 4, using two-phase extraction, we obtained higher contents of protein, but not statistically different from the extraction of one phase. The same happened to method 2, obtained results using one or two phases of extraction produced approximate protein contents. We can see that the use of one or two phases of extraction did not influence protein contents for methods 2, 3 and 4, since the values were not significantly different.

The realization of two-phase extraction for obtaining protein concentrates, applying extraction methods proposed in this experiment did not cause increases in extraction yield and mass yield. Methods 2 and 3 presented significantly higher yields for the extraction in one phase. This

occurrence can be attributed to the mode of preparation of the leaf juice, since the amount of water used in each phase of the extraction in two phases was half the amount used in the single phase extraction (1:5 and 1:10 dilution for each phase). The highest dilution could promote more effectively the rupture of the cells and release the protein into the extractant (water).

Use of cassava fresh leaves in the Protein Extraction:

Using fresh leaves, the application of the methods of extraction was easier, especially in triturating phase, in which occurs the cell disruption to deliver proteins to the extractant (water). The juice of fresh leaves resulted in a light green color and a softer smell. The appearance of the obtained protein concentrates were similar to those concentrates obtained with dried leaves.

For the mass balance, the amount of fresh leaves in dry mass was 28 grams, being 7.76 grams of crude protein present in the leaves.

When using the method 1, the pH at the end of extraction did not change, for extraction using the methods 2 and 3, the centrifuged liquid and the protein concentrate had the same characteristics of the extraction with dried leaves. The centrifuged liquid resulted in a lighter color and a more viscous concentrate. For method 4, with 48 hours of fermentation, the pH dropped to 3.95, occurring consistent clots, which facilitated the precipitation and separation of the precipitate.

Table 2 shows the results of comparison of extraction depending on the type of cassava leaf used.

TABLE 2. Average values of concentrate protein yield and protein extraction yield using fresh and dried leaves.

Methods of extraction[3]	**Concentrate Yield (%)[1]**		**Protein Extraction Yield (%)[1]**		**Protein Content (%)[1,2]**	
	Dried Leaves	**Fresh Leaves**	**Dried Leaves**	**Fresh Leaves**	**Dried Leaves**	**Fresh Leaves**
1	$20,7\pm0,7^{a1A1}$	$28,6\pm 2,1^{a1A1}$	$34,4\pm1,3^{a2b2B2}$	$58,6\pm 2,8^{a2B2}$	$61,0\pm2,1^{c3}$	$56,7\pm 0, 8^{c3}$
2	$35,6\pm1,8^{b1B1}$	$53,6\pm 1,2^{b1B1}$	$48,7\pm1,0^{b2c2B2}$	$73,7\pm 3,6^{a2B2}$	$50,0\pm1,7^{a3b3}$	$38,1\pm 1,1^{a3b3}$
3	$49,5\pm1,6^{c1A1}$	$46,4\pm 1,5^{b1A1}$	$59,7\pm1,4^{c2A2}$	$67,0\pm 1,0^{a2A2}$	$44,0\pm1,4^{a3}$	$40,0\pm 0,9^{a3}$
4	$25,9\pm1,0^{a1A1}$	$37,7\pm 1,8^{a1b1B1}$	$36,2\pm1,1^{a2b2A2}$	$67,4\pm 1,1^{a2B2}$	$51,2\pm0,7^{a3b3c3}$	$49,6\pm 0,9^{b3c3}$

[1] Within the same column, the averages followed by the same small letter do not differ statistically by the Tukey test at 5% level of significance. Within the same line, the averages followed by the same capital letter do not differ statistically by the Tukey test at 5% level of significance. [2] Values calculated in dry basis. [3]Methods of Extraction: 1 –Coagulation of Proteins by Lowering the Temperature; 2 – Extraction by Isoelectric Precipitation; 3 – Solubilization of Proteins; 4 – Fermentation of Filtered Leaf Juice.

In general, for fresh leaves, the methods 2, 3 and 4 showed statistically similar concentrate yields. The best yields of concentrate were obtained by methods 2 and 3.

Comparing each method, evaluating fresh leaves and dried leaf, we observed that the methods 1, 2 and 3 are similar. Only for method 4 there was difference using fresh leaves due to better clot formation, which favor the separation of the concentrate. Assessing the extraction yield, we observe higher yields using fresh leaves, however, without presenting significant difference for methods 1, 2 and 3.

It was not possible to compare the contents of protein, using dried leaves and fresh leaves, because they are not the same cassava leaves assessed for the two cases. The ages of the plants used were different. To apply the methods with dried leaves, we used leaves of twelve months old, and for the application of the methods with fresh leaves we used leaves of nine months old. Therefore, in the case of fresh leaves, we obtained protein concentrate with lower protein.

The fresh leaves are easier to be used, however, the durability is lower due to its degradation occur very quickly after harvest and can not be stored. Dehydrating the leaves, it is possible to store them for later use for extraction.

Mass losses in the extraction processes

The mass loss in each extraction method was calculated by the reduction of the initial mass of the leaves, in each phase of separation. The results are shown in Table 3.

TABLE 3. Results of mass losses in the extraction processes of each method evaluated in the experiment.

Methods[2]	Mass Losses of the Process (%)		
	Dried Leaves Extraction 1 Phase	Dried Leaves Extraction 2 Phases	Fresh leaves
1	17.3[a]	2.1[b]	30.0[a]
2	18.6[a]	6.0[a]	18.1[c]
3	10.7[b]	1.9[b]	27.2[ab]
4	10.7[b]	0.6[c]	25.3[ab]

[1] Within the same column, the averages followed by the same small letter do not differ statistically by the Tukey test at 5% level of significance. [2] Methods of Extraction: 1 –Coagulation of Proteins by Lowering the Temperature; 2 – Extraction by Isoelectric Precipitation; 3 – Solubilization of Proteins; 4 – Fermentation of Filtered Leaf Juice.

Observing the extraction in two phases, there is a decrease in mass losses in the studied methods. The purpose of applying more than one extraction phase is to minimize the losses in the process and thus increase yield. However, extraction in two phases did not improve the yield, since the mass loss refers to other constituents, showing that the protein is easily extracted in the initial phase of extraction.

Mass losses in the processes of protein extraction of fresh leaves were greater in percentage terms than using dried leaves. In this case, the larger part of mass loss was in the filtration phase.

CONCLUSIONS

The solubilization of proteins (method 3) showed higher extraction yield, however, the obtained protein concentrate showed low quality.

The fermentation of the juice (method 4) produced concentrates with more protein quality and lower costs and isoelectric precipitation (method 2) promoted the obtention of concentrates in shorter time of extraction, both with good prospects for use.

The use of two extraction phases was not advantageous to the process and there was no difference between the use of fresh or dry leaves, and the use of fresh leaves is presented as a good option for the simplicity of the method.

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