Cooked Cassava Leaves: Cyanogenic Compounds Contents, Quality Evaluation, and Processing Conditions

Folhas de Mandioca Cozidas: Teores de Compostos Cianogênicos, Avaliação da Qualidade e das Condições de Processamento

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Abstract

The aim of this study was to evaluate the cyanogenic compounds contents and the microbiological and physicochemical quality of cooked cassava leaves, commercialized in the city of Belém-PA, as well as to verify the processing steps performed in agro-industries and fairs and to perform a diagnosis of the Good Manufacturing Practice (GMP) in two agro-industries. Ten samples were collected, five from fairs and five from supermarkets and cyanogenic compounds, *Salmonella spp*, *Bacillus cereus*, coagulase-positive staphylococci, coliforms at 45 °C, mesophilic aerobes, molds and yeasts, centesimal composition, pH, and acidity were analyzed. Processes for producing cooked cassava leaves at fairs and agro-industries were verified. The GMP was evaluated by applying a checklist. The results of cyanogenic compounds ranged from 1.25 to 8.22 mg HCN/kg, being 100% above the free cyanide legislation. All the samples were in compliance with the legislation for *Salmonella spp*, coagulase-positive staphylococci, and *Bacillus cereus*. For coliforms at 45 °C, 60% of the fair and 20% of the supermarket samples were in accordance with the legislation. The results of mesophilic aerobes and molds and yeasts ranged from <2.40 to 5.38 and <2.88 to 7.30 log CFU/g, respectively. The fair samples were below the minimum recommended for humidity, lipids, and pH and the supermarket samples, below the established for ashes, lipids, and pH. Agro-industries were considered high risk in relation to GMP. Changes in processing influence the cyanogenic compounds content, as well as the GMP scarcity precede contamination.

Keywords: Cassava Crop By-Product. Hydrocyanic Acid (HCN). Physicochemical. Microbiological.

Resumo

O objetivo deste estudo foi avaliar os teores de compostos cianogênicos e a qualidade microbiológica e físico-química de folhas de mandiocas cozidas, comercializadas na cidade de Belém-PA, assim como, verificar as etapas de processamento executadas em agroindústrias e feiras e realizar um diagnóstico das Boas Práticas de Fabricação (BPF) em duas agroindústrias. Foram coletadas dez amostras, sendo cinco de feiras e cinco de supermercado e realizadas as análises de compostos cianogênicos, Salmonella spp, Bacillus cereus, estafilococos coagulase positiva, coliformes a 45°C, aeróbios mesófilos, bolores e leveduras, composição centesimal, pH e acidez. Verificou-se os processos para produção de folhas de mandioca cozidas em feiras e agroindústrias. As BPF foram avaliadas através da aplicação de check-list. Os resultados dos compostos cianogênicos variara de 1,25 a 8,22 mg HCN/kg, estando 100% acima do preconizado pela legislação para cianeto livre. Todas as amostras estiveram em conformidade com a legislação para Salmonella spp, estafilococos coagulase positiva e Bacillus cereus. Para coliformes a 45 °C, 60% das amostras de feiras e 20% do supermercado estiveram em concordância com a legislação. Os resultados de aeróbios mesófilos e bolores e leveduras variaram de <2,40 a 5,38 e <2,88 a 7,30 log UFC/g, respectivamente. As amostras de feiras, estavam abaixo do mínimo preconizado para umidade, lipídios e pH e as do supermercado, abaixo do estabelecido para cinzas, lipídios e pH. As agroindústrias foram consideradas de risco alto em relação às BPF. Alterações no processamento influenciam nos teores de compostos cianogênicos, bem como a escassez das BPF são precedentes a contaminações.

Palavras-chave: *Subproduto da Mandiocultura. Ácido Cianídrico (HCN). Físico-Química, Microbiológica.*

1 Introduction

Cassava (*Manihot esculenta* Crantz) is an angiosperm of the dicotyledonous class from the Euphorbiaceae family, composed of about 7200 species, with the presence of cyanogenic glycosides and latex as characteristic. In its native area, South America, where it was domesticated thousands of years ago, and in the African continent, where it was introduced during the 16th century, is one of the most important sources of staple food, mainly due to its starchy roots (CEBALLOS; LA CRUZ, 2012; FAOSTAT, 2013; GABRIEL *et al.,* 2014;).

The aerial part of cassava (or branch) comprises the above-ground plant portion and is considered to be a by-product generated from the root crop. Depending on the variety, cassava leaves are rich in protein, minerals, carotenes, and B1, B2, and C vitamins. In addition to the lower content of methionine, lysine, and perhaps isoleucine, the protein amino acid profile of cassava leaf compares favorably to milk, cheese, soy, fish, and eggs (SOUZA *et al.*, 2011; SOARES *et al.,* 2016).

Cassava leaves are widely commercialized and used for human consumption in northern Brazil, as a major component in the preparation of a typical dish called maniçoba (LATIF; MÜLLER, 2015; MODESTO JUNIOR; CHISTÉ; PENA, 2019). As a result,

the need for food safety management for cassava derivatives is necessary, given that these products have increasingly been exported to other regions, reaching a larger public.

Although they are a source of valuable nutrients cassava leaves also have toxicity due to the presence of cyanogenic glycosides, and antinutritional factors such as high fiber content, tannins, polyphenols, and phytic acid, which can have toxic effects depending on the processing method and the amount consumed (PESTANA; CASTRO, 2015).

The traditional method for obtaining cassava by-products follows artisanal or semi-mechanized processes that may vary according to each locality characteristics and, due to this, there is the absence of established parameters that guarantee the product identity and quality standard, and therefore there are some critical factors in processing, such as: residual presence of hydrocyanic acid (HCN) at levels above those recommended by current legislation, presence of pathogenic and spoilage microorganisms, hygienic-sanitary problems in processing units, and equipment and utensils made of difficult hygienization materials (ABREU; MATTIETTO, 2016).

Thus, this study aimed to evaluate the cyanogenic compounds content and the microbiological and physicochemical qualities of cooked cassava leaves commercialized in the city of Belém-PA, as well as verify the processing steps performed in agro-industries and main fairs of the capital of Pará and perform a Good Manufacturing Practices (GMP) diagnosis in two agro-industries in northeastern Pará.

2. Material and Methods

2.1 Sample collection

Ten commercial samples of ground and cooked cassava leaves (cooked manioc) were collected in the city of Belém -PA, namely : five samples purchased in two free markets from artisanal units (codified from I to V) and five samples purchased in a supermarket from agro-industries units (codified from VI to X). All the samples (fairs and supermarkets) were purchased under the same conditions as they are commercialized, at a temperature of approximately 25 ºC in 1 kg polyethylene bags. After collection, all the samples were packaged in isothermal boxes and sent to laboratories for microbiological and physicochemical analyzes as well as cyanogenic compounds contents.

2.2 Cyanogenic compounds contents

Total cyanide (TC), non-glycosidic cyanide (NGC), and free cyanide (FC) contents were determined according to the methodology proposed by Essers *et al.* (1993), with adaptations, when , after cyanogenic compounds extraction, a colorimetric reaction was performed using chloramine T, isonicotinate, and 1,3-dimethylbarbiturate, followed by spectrophotometric reading at 605 nm. The linamarase enzyme (enzymatic activity of >3.0 U/ mL) was obtained and purified according to the methodology proposed by Cooke

(1979). The linamarase enzyme exhibited enzymatic activity >3.0 U/ mL and optimum temperature and pH conditions of 30±1 ºC and pH > 4.0, respectively.

For cyanogenic compounds extraction, 20 g of cooked cassava leaves were homogenized in a processor (Philips Walita Viva Collection RI7762), with 200 mL of ortho phosphoric acid alcoholic solution 0.1M (added of sodium chloride). Subsequently, the solution was centrifuged (centrifuge Excelsa baby Fanem, model I 206-BL, USA) at 5000 rpm for 10 minutes and the supernatant stored in plastic flasks at -18 ºC. For analysis, the sample extracts were placed in test tubes, in triplicate, and prepared differently before the colorimetric reaction, as described below.

For TC analysis, 0.1 mL of extract was added to a test tube containing 0.4 mL of pH 7.0 buffer, followed by the addition of 0.1 mL pre-purified enzyme. After incubation at 30±1 ºC in water bath for 15 minutes, 0.6 mL of 0.2 M NaOH solution was added, and after 5 minutes, 2.8 mL of pH 6 buffer was added, following to the colorimetric reaction. For NGC, 0.1 mL of extract was added into a test tube containing 0.6 mL of pH 6.0 buffer. Then, 0.6 mL of 0.2 M NaOH solution was added, and after 5 minutes 2.9 mL of pH 6.0 buffer was added, following to the colorimetric reaction. While for FC analysis, 0.1 mL of extract was diluted in 3.9 mL of pH 6 buffer prior to colorimetric reaction.

For the colorimetric reaction, in each of the prepared tubes, 0.1 mL of 1 % chloramine T solution was added, and after five minutes, 0.6 mL of isonicotinate/dimethylbarbiturate solution was added. After 10 minutes of reaction, the colored complex formed was read in a spectrophotometer (Thermo Scientific, Evolution 60, Massachusetts) at 605 nm.

TC, NGC, and FC contents were quantified using a seven-point analytical curve (0.5-20.00 µg HCN/mL), with $R^2 > 0.99$, in triplicate and the results expressed in mg HCN/kg. The FC results were analyzed in relation to the cyanogenic compounds parameters established by law (PARÁ, 2016).

2.3 Microbiological analyses

All the cooked cassava leaf samples were analyzed for microbiological parameters required by current legislation (PARÁ, 2016) namely: *Salmonella spp* screening, coagulase-positive staphylococci, *Bacillus cereus*, and coliforms at 45 ºC counts. The count was also made for mesophilic aerobic bacteria and molds and yeasts in order to evaluate the samples hygienic conditions. The analytical methodology used is described in *Compendium of Methods for the Microbiological Examination of Foods* (SALFINGER; TORTORELLO, 2015) and all the analyzes were performed in triplicate.

2.4 Physicochemical analyses

The following analyzes were performed: moisture, ashes, lipids, total proteins with 5.75 factor (vegetable protein), fiber, and carbohydrates by difference. All the analyzes were performed

according to methodologies described in AOAC (2007).

The pH and total titratable acidity (TTA) were also determined according to the methodologies described in AOAC (2007). The pH was obtained by direct reading potentiometer (Tecnal, TEC-51, Brazil). The same system used for pH determination was also used for TTA determination, when 0.1 M NaOH solution was added and the pH was verified simultaneously until it was between 8.1-8.4, checking the NaOH volume spent. The results were analyzed in relation to the physicochemical parameters established by the current legislation (PARÁ, 2016).

2.5 Verification of the processing steps of cassava leaves cooked at agro-industries and fairs

The processing steps of cooked cassava leaves currently used in two large agro-industries, located in the Northeastern region of the state of Pará (Brazil), and in two of the main fairs of the city of Belém (PA-Brazil) were verified through field visits. Visits to places were carried out in the morning, with descriptions of the steps composed by owners reports on each of the steps performed along with photographic material. In agro-industries and fairs, the steps were verified from the fresh cassava leaves reception to the final product.

2.6 Verification of Good Manufacturing Practices (GMP)

To verify the adequacy level of two cooked cassava leaf processing agro-industries in relation to Good Manufacturing Practices (GMP), a Good Manufacturing Practices Checklist (GMPC) was prepared, adapted from RDC nº275 of October 21st, 2002 (Brazil, 2002). GMPC consisted of 165 verification items, distributed in five blocks: Block 1 - Building and facilities; Block 2 - Equipment, furniture, and utensils; Block 3 - Clothing; Block 4 – Food production and transportation; Block 5 - Documentation, the items being evaluated in: as Conformities (C), when in accordance with the law; Nonconformities (NC), when not in accordance with the law and Not Applicable (NA) for items that do not have relevance/application for those industries.

For each block in the list, a percentage value was expressed based on the conformities or nonconformities. All the items attended were summed to obtain the total percentage of the establishment, and the agro-industries classification was performed according to the RDC n° 275 (Brazil, 2002) at Low Risk (76-100 % of conformity), Medium risk (51-75 % of conformity), and High Risk (0-50% of conformity). GMPC was applied in two surveys in each agribusiness. After the first survey, a Corrective Action Plan was prepared and delivered to the owners. Results were expressed as the mean between the two evaluations performed.

2.7 Statistical analyses

The results of cyanogenic compounds, microbiological (expressed in log base) and physicochemical analyzes were expressed as mean ± standard deviation. The means were submitted to analysis of variance (ANOVA) and complementary difference test (Tukey test, $p < 0.05$) for comparison of means, using Statistica® Kernel Release 7.1 software.

3 Results and Discussion

3.1 Cyanogenic compounds contents

The values for total cyanide (TC), non-glycosidic cyanide (NGC), and free cyanide (FC) contents in cooked cassava leaf samples are shown in Table 1. All the samples analyzed (n=10) presented higher values than recommended by legislation (PARÁ, 2016) for FC, which is 0.5 mg HCN/kg, a fact that deserves particular attention, since the cassava products consumption with high cyanogenic concentration can lead to serious illness or even death (GLEADOW; MOLLER, 2014).

Continuous ingestion of unprocessed or improperly processed cassava products containing high cyanogen (linamarin, cyanohydrin, and HCN) levels combined with an amino acid deficient diet containing sulfur (methionine and cysteine) can lead to chronic intoxication, since these amino acids are needed for human organisms cyanide detoxification (JORGENSEN et al., 2011).

Table 1 - Cyanogenic compounds contents (TC, NGC, and FC) of cooked cassava leaf samples from fairs and supermarkets in the city of Belém-PA

Samples	Codification	TC (mg HCN/kg)	NGC (mg HCN/kg)	FC (mg HCN/kg)
Artisanal units (fairs)	I	2.75±0.66[cd]	8.22±0.00[a]	2.33±0.07[cdef]
	II	7.55±0.07[a]	5.20±0.29[c]	2.96±0.07[bc]
	III	7.84±0.88[a]	3.66±0.07[d]	3.29±0.15[ab]
	IV	3.32±0.00[c]	3.95±0.15[d]	3.27±0.22[ab]
	V	2.13±0.07[cd]	1.45±0.44[e]	1.77±0.30[ef]
Agro-industrial units (supermarket)	VI	1.83±0.07[d]	1.62±0.07[e]	1.72±0.07[f]
	VII	2.13±0.07[cd]	2.13±0.07[e]	2.44±0.07[cde]
	VIII	5.67±0.00[b]	6.98±0.52[b]	3.78±0.15[a]
	IX	5.33±0.00[b]	5.01±0.00[c]	2.50±0.30[cd]
	X	1.41±0.07[d]	1.25±0.00[e]	1.94±0.07[def]
PARÁ, 2016		-	-	0.5

Results expressed as mean ± standard deviation (n=3). Mean followed by different letters in the same column are significantly different (Tukey test, $p \leq 0.05$); TC= total cyanide; NGC= non-glycosidic cyanide; FC= free cyanide; -= no parameters established in the standard.
Source: Research data.

The TC values obtained were below the data obtained by Modesto Junior, Chisté, and Pena (2019) in cassava leaves cooked in water for 3 hours at 100 ºC (23 to 82 mg HCN/kg). Cassava leaves in northern Brazil are cooked in water for periods ranging from 48 to 72h (agro-industries) and from five to seven days (fairs), thus, according to Nambisan (2011), the decrease in values may occur due to the linamarin degradation by linamarase, since cassava leaves were milled and added with water, facilitating the linamarin hydrolysis in acetone cyanohydrin, which is hydrolyzed in HCN.

Acetone cyanohydrin are decomposed into ketones and HCN by two routes: spontaneously at pH > 4.0 and temperature > 30 ºC, or due to the hydroxynitrile lyase (HNL) enzyme activity at pH values between 3, 5-6.0 and temperatures ≤ 60 ºC (KALENGA SAKA; NYRENDA, 2012; CÂMARA; SOTO-BLANCO, 2013).

For NGC results, in general, there is little information in the literature about NGC dosages in cassava and its by-products, however, NGC is as important as TC and FC, since it is the cyanide form that is present in larger residual quantities in products in general. Cyanohydrin when ingested decomposes at the alkaline pH level in the small intestine to produce a molar amount equivalent to cyanide. Therefore, ingestion of high cyanohydrin concentrations can be as harmful to human metabolism as cyanide (KALENGA SAKA; NYRENDA, 2012).

FC results are lower than the values found by Modesto Junior, Chisté, and Pena (2019) (3.6 to 25 mg HCN/kg), in their study with cassava leaves cooked for 3h at 100 ºC. An explanation would be that, in general, commercial samples of cassava leaves in northern region of Brazil are boiled with water for periods longer than 48 hours, which leads to greater cyanogen compounds removal. Water boiling treatment is a widely used method in Africa to remove cyanogenic compounds from cassava leaves, usually in periods ranging from 10 to 120 minutes. This process is also very common in Brazil during the maniçoba preparation (MODESTO JUNIOR; CHISTÉ; PENA 2019).

Significant differences in TC, NGC, and FC values found for cooked cassava leaf samples may be related to the amount of cyanogenic compounds in the raw material and the processing conditions (CHISTÉ; COHEN; OLIVEIRA, 2007). It is valid to point out that processing variations may directly influence the cyanide content, such as performing the cooking prior to the grinding step, a common practice in some agro-industries. During the milling process, plant tissue ruptures and the linamarin is exposed to linamarase, initiating the HCN formation, which will subsequently be volatilized (GLEADOW; MOLLER, 2014).

Performing the cooking step prior to grinding, there is enzymatic inactivation (linamarase) before the cyanogenic glycosides hydrolysis to form cyanohydrins and later free cyanide, making the final product still with a high content of cyanogenic glycosides (COHEN; OLIVEIRA; CHISTÉ, 2007).

Another variable that directly impacts cyanogen content would be the cooking time, which varies depending on each processor establishment. Literature suggests periods longer than 72 hours (ABREU; MATTIETTO, 2016), however, this mean cooking time may be shorter or longer depending on the processing location. In fairs it can be from five to seven days while in agro-industries it can be from 48 to 72 h. Cooking time can significantly affect the product cyanide content due to high cyanide volatilization under heating conditions (CAMPOS; CARVALHO; MATTIETTO, 2016).

3.2 Microbiological analyses

All the samples analyzed (n=10) showed absence of *Salmonella spp*, coagulase-positive staphylococci, and *Bacillus cereus* counts were < 2 log CFU/g, in accordance with current legislation (PARÁ, 2016).

In a study of a cassava leaves by-product from Madagascar and Tanzania, Abass *et al.* (2019) also found no *Salmonella spp* in any of the analyzed samples (n=90), this is due to the fact that this bacterium is heat sensitive, not surviving at temperatures above 70 ºC. Plants are usually not considered natural hosts of *Salmonella spp*. However, green plant products are considered important vehicles or even additional reservoirs for foodborne pathogens, including *Salmonella spp*, which can contaminate plants during any of the many stages of production, since this pathogen is present in the environment and food chain through manure, insects, water, and soil (PARK *et al.,* 2012; HOU *et al.,* 2013).

The genus *Sthaphylococcus*, especially *Staphylococcus aureus* constitute an important group of pathogens. Contamination may occur directly from animals, infected handlers, or may result from lack of hygiene during food processing or storage (KOTZEKIDOU, 2013).

B. cereus is an aerobic rod-shaped spore-forming bacterium and is commonly present in soil, air, dust, water, dried and processed foods, especially of plant origin (TEWARI; ABDULLAH, 2015). The presence of *B. cereus* in food at concentrations > 4 log CFU/g is known to cause food poisoning (emetic and diarrheal syndrome). The spores survive cooking at high temperatures; therefore, control and prevention of food contamination is necessary (GLASSET *et al.,* 2016).

Table 2 shows the results of coliforms at 45 ºC, mesophilic aerobic bacteria, and molds and yeasts in cooked cassava leaf samples. Values for coliforms at 45 ºC from fair samples ranged from < 0.48 to 2.18 log MPN/g, with 60 % (n=3) in compliance with legislation, that establishes values < 0.48 log MPN/g (PARÁ, 2016). For supermarket samples, the variation ranged from <0.48 to 1.87 log MPN/g, with only 20 % (n=1) being within the legislation scope (PARÁ, 2016). Samples originated from fairs are usually packaged still hot, shortly after cooking, and supermarket samples (from agro-industries) after cooking are also moved to a

cooling stage, which is considered slow, where food is in contact with food handler, surfaces, and utensils, which may lead to product contamination. Thus, it becomes evident the occurrence of food recontamination after cooking, pointing to the poor hygienic-sanitary conditions of the processing establishments.

Table 2 - Coliforms at 45 ºC, aerobic mesophylls, and molds and yeasts counts from cooked cassava leaf samples from fairs and supermarkets in the city of Belém-PA.

Samples	Codification	Coliforms at 45 ºC (log MPN/g)	Mesophilic aerobic bacteria (log CFU/g)	Molds and yeasts (log CFU/g)
Artisanal units (fairs)	I	<0.48[e]	5.05[b]	7.30[a]
	II	2.18[a]	3.24[g]	4.05[g]
	III	1.56[c]	2.73[i]	4.91[d]
	IV	<0.48[e]	4.31[d]	<2.88[h]
	V	<0.48[e]	<2.40[j]	4.76[e]
Agro-industrial units (supermarket)	VI	<0.48[e]	3.39[e]	6.88[b]
	VII	1.56[c]	4.70[c]	5.47[c]
	VIII	1.56[c]	5.38[a]	4.54[f]
	IX	1.87[b]	2.98[h]	<2.88[h]
	X	1.04[d]	3.31[f]	5.47[c]
PARÁ, 2016		<0.48	-	-

Results expressed as means. Means followed by different letters in the same column are significantly different (Tukey test, $p \leq 0.05$). -= no parameters established in the standard.
Source: Research data.

Bacteria of the coliform group are common indicators of the sanitary quality of food and water, and can be found in aquatic environments, in soil and vegetation. They have been included in some cases of life-threatening infections and their presence is used to indicate the presence of other pathogenic fecal origin organisms. High levels of contamination by microorganisms of this group in plants are considered indicative of hygiene problems, pointing to the lack of good practices during cultivation and processing (MACHADO et al., 2018).

There are no parameters established in current legislation for counting of mesophilic aerobic bacteria and molds and yeasts. Mesophilic aerobic monitoring can be successfully used as an indicator of sanitary quality, organoleptic acceptability, application of Good Manufacturing Practices and, to a lesser extent, as an indicator of food safety (KREGIEL, 2015). The molds and yeasts presence can also be considered as an indication of hygienic quality, since according to Kregiel (2015), fungal spores or conidia and mycelia fragments can contaminate food at any processing stage. Just as yeasts, molds can settle in processing industries due to the process poor hygiene or the contaminated packaging spread.

Mesophilic bacterial counts ranged from < 2.40 to 5.05 log CFU/g and 2.98 to 5.38 log CFU/g for fair and supermarket samples, respectively. Agro-industries that process cooked cassava leaves have deficiencies in relation to Good Manufacturing Practices. This fact may be a precedent for contamination, which may occur in many ways, and may justify the high counts of mesophilic aerobic bacteria, being this hypothesis corroborated by Tresseler et al. (2009) who

emphasize that mesophilic bacteria can be introduced into the processing areas by multiple vectors and can settle and multiply, particularly in processing areas that are difficult to clean and sanitize, becoming a contamination source.

In the molds and yeasts count, the results ranged from < 2.88 to 7.30 CFU/g and < 2.88 to 6.68 CFU/g for fairs and supermarket samples, respectively. Abass et al. (2019) found values above 3 log CFU/g in 90 % of cassava by-products samples analyzed in their study. The high fungal load associated with the presence of coliforms suggests poor hygiene during processing, possibly due to the use of water with unsatisfactory characteristics and use of poorly sanitized processing utensils (ABASS et al., 2019).

3.3 Physicochemical analyses

The results regarding the physicochemical characterization of cooked cassava leaves from fairs and supermarkets are presented in Table 3. It was verified that in samples from fairs, 100 % were lower than the minimum recommended by legislation (PARÁ, 2016) for moisture, lipids, and pH. For ashes, 80 % are lower than the minimum required and for proteins, 80 % were higher than the maximum allowed.

Regarding supermarket samples, 100 % was within the range established by the legislation for moisture (PARÁ, 2016). However, all the samples were lower than the minimum required for ashes, lipids, and pH. For proteins, 60 % was higher than the maximum recommended for this parameter.

Table 3 - Physicochemical characterization of cooked cassava leaves samples from fairs and supermarkets in the city of Belém-PA

Samples	Codific.	Moisture*	Ashes**	Protein**	Lipids**	Carbohydrates**	Fibers**	TTA***	pH
Artisanal units (fairs)	I	87.67±0.41[d]	5.21±0.12[c]	28.63±0.52[b]	9.55±0.24[a]	51.06±0.66[de]	16.72±0.18[d]	3.88±0.01[cd]	4.46±0.01[d]
	II	84.72±0.15[f]	4.30±0.12[e]	30.31±0.31[a]	8.64±0.45[b]	51.52±1.24[cde]	15.63±0.25[d]	3.85±0.27[cd]	5.08±0.05[b]
	III	86.09±0.12[e]	5.85±0.11[b]	27.12±0.04[c]	9.21±0.58[ab]	50.74±1.12[de]	15.96±0.44[d]	7.98±1.93[a]	4.21±0.02[g]
	IV	87.67±0.50[d]	3.11±0.04[g]	28.69±0.27[b]	5.90±0.12[e]	56.06±0.44[b]	16.10±0.16[d]	6.48±0.20[ab]	4.25±0.01[fg]
	V	87.09±0.63[de]	7.20±0.15[a]	30.56±0.39[a]	6.69±0.10[d]	49.37±1.04[e]	36.75±1.67[b]	3.07±0.01[e]	4.55±0.01[c]
Agro-industrial units (super-market)	VI	89.62±0.30[bc]	4.59±0.06[d]	23.52±0.77[d]	9.48±0.15[a]	56.62±0.99[b]	19.32±0.49[d]	1.78±0.20[e]	5.24±0.02[a]
	VII	90.36±0.45[b]	3.70±0.02[f]	31.41b±0.34[a]	7.56±0.03[c]	51.17±0.42[de]	39.55±0.79[ab]	3.12±0.27[de]	4.35±0.01[e]
	VIII	90.34±0.9[b]	1.81±0.02[i]	27.95±0.28[bc]	3.38±0.08[f]	60.71±0.25[a]	40.89±2.43[a]	4.81±0.23[bcd]	4.22±0.01[g]
	IX	88.90±0.20[c]	2.72±0.02[h]	30.59±0.31[a]	7.12±0.21[cd]	53.52±0.37[c]	30.98±1.41[c]	7.97±0.45[a]	4.29±0.01[f]
	X	91.49±0.30[a]	5.03±0.09[c]	27.57±0.31[bc]	8.62±0.05[b]	52.66±0.33[cd]	40.55±2.32[a]	5.30±0.28[bc]	4.28±0.01[f]
PARÁ (2016)		88.00 (min)	6.00 (min)	20.47 (min)	18.00(min)	-	-	-	5,5 (min)
		92.00 (max)	8.00 (max)	27.75 (max)	22.00(max)				7,0 (max)

Results expressed as mean±standard deviation (n=3). Means followed by different letters in the same column are significantly different (Tukey test, p ≤ 0.05); * = results expressed in g/100 wb (wet basis); ** = results expressed in g/100 db (dry basis); *** = results expressed in mEq NaOH/g; - = no parameters established in the standard; min= minimum established in the standard; max= maximum established in the standard.
Source: Research data.

Moisture values found are higher than those reported by several authors when analyzing fresh cassava leaves (ONI, 2010; SOUZA *et al.*, 2011; MODESTO JUNIOR; CHISTÉ; PENA, 2019), which can be supported by the fact that in the northern region of Brazil, cassava leaves are cooked with the addition of water and although part of the water is evaporated during the cooking process, much of it is still present in the final product.

The results of ashes, proteins, lipids, carbohydrates, and fibers corroborate the data obtained by Achidi *et al.* (2008) who evaluated cassava leaves cooked in water and found mean values of 5.83; 35.42; 6.57, 32.31 and 18.77 g/100g, respectively.

During the cassava leaves boiling process in water, changes in ash content may occur associated with the leaching of some minerals in water, especially when processing involves water disposal, such as enzymatic *bleaching or food products immersion before further processing*. Enzymatic bleaching with water can increase the protein content in cassava leaves and may also reduce the ash content (ACHIDI *et al.*, 2008). Cassava leaves are quantitatively rich in protein, however, they are deficient in sulfur amino acids (methionine and cysteine) needed for cyanogen detoxification in the human body (BRADBURY; DENTON, 2014).

It is important to emphasize that the lipid results obtained are well below the minimum required by the current legislation, although they agree with the literature. This fact may indicate a possible revision of the limits required by the current legislation.

Fiber content has an inverse correlation with protein content. In the period when the leaves have higher protein concentrations, the fiber content is in the range from 21.86 to 26.66 g/100 (dry matter) on the leaves, thus, the highest amount of protein in relation to the total fibers indicate a high nutritional and technological potential (TROMBINE; LEONEL, 2014).

Regarding TTA, the values obtained were similar to the results found by Modesto Junior, Chisté, and Pena (2019) (2.22 to 4.90 mEq NaOH 0.1 N/g). The importance of decreasing TTA may be related to the microorganism growth control, since this parameter is accompanied by an increase in pH. The decrease in TTA can be explained by the continuous metabolic changes of organic acids present in plants in carbon dioxide and water. In addition, the presence of high levels of reducing substances such as tannins and polyphenols may influence this parameter (AYALA-ZAVALA *et al.*, 2010).

The pH values obtained were lower than those observed by Modesto Junior, Chisté, and Pena (2019) in fresh cassava leaves (4.89 to 5.11), given the fact that, according to the same author, high pH values associated with high moisture values allow the classification of cassava leaves as more susceptible to deterioration if not stored or processed properly.

3.4 Verification of the processing steps of cassava leaves cooked at agro-industries and fairs

Regarding the processing steps verification of cooked cassava leaves currently used in agro-industries and fairs, it was observed that in fairs, the process begins with the leaves reception, which are acquired in the frequency of twice to three times a week, stored in raffia bags with 30 kg capacity. Subsequently, they are washed by immersion in a water tank and dried by manual shaking, followed by the grinding stage

(industrial meat grinder).

In high demand periods, cooking takes place in the licensed houses and in low demand periods in the fairs own premises, although this last option is a practice prohibited by the City Hall of Belém-PA. In the cooking step, the period varies according to each sale place, being reported periods from five to seven days. After this step, the cooked cassava leaves are packed still warm, without temperature control. According to Abreu and Mattietto (2016), after cooking, it is a common practice to pack the product while it is still warm in 1 kg plastic bags, which is valid as long as it is cooled down as quickly as possible.

In agro-industries the processing begins with the fresh leaves reception, which are packed in raffia bags (30 kg) and stored in the raw material reception area for up to five days. In the processing area, the leaves are washed by soaking in tanks with water and then dried by hand shaking, then grinded (industrial meat grinder, similar to those used at the same step in fairs) and placed in industrial aluminum pans.

The practice of the following steps sequence was verified: the leaves, after the excess water removal step, follow to the cooking step and then grinding. This fact had already been reported by Abreu and Mattietto (2016), where two production ways of cooked cassava leaves in the northern region of Brazil are shown: grinding the leaves before or after cooking, with pre-cooking grinding being the most common way used.

In the cooking stage, the leaves are boiled with water, boiling for 48-72 h in industrial aluminum pans. According to Abreu and Mattietto (2016), besides the issue of food safety, eliminating all cyanide from the leaves, cooking time can also influence the sensory product attributes. During cooking, the elimination of the present hydrocyanic acid occurs, due to being highly volatile, and the linamarase enzyme inactivation at high temperatures, which affects the linamarin hydrolysis process, responsible for releasing the cassava toxic principle (COHEN; OLIVEIRA;CHISTÉ; 2007).

After cooking, the material proceeds to the cooling stage, where the food can remain in the cooking vessel until it reaches room temperature, or is fractionated into smaller containers where it can remain for up to 48 h. According to Abreu and Mattietto (2016), there is a tendency to leave cassava leaves cooked cooling at room temperature. Slow cooling allows microorganism spores germination that were not inactivated during cooking and may cause changes in the product.

After reaching room temperature, the product is then packed in plastic material with 1kg capacity, and then packed in larger packages, with 30kg capacity at room temperature until they are transported to the sale places.

3.5 Verification of Good Manufacturing Practices (GMP)

The percentages of Conformities (C) referring to the application of GMPC in the cassava leaf processing agro-industries A and B are presented in Table 4.

Table 4 - Mean percentages of conformities (C) and overall assessment obtained from surveys carried out on cassava leaf processing agro-industries A and B

Block	Verification items	Agro-industry A		Agro-industry B	
		C	%	C	%
Block 1	77	31	40.26	16	20.78
Block 2	21	7	33.33	11	52.38
Block 3	14	6	42.86	4	28.57
Block 4	28	8	28.57	9	32.14
Block 5	25	0	0	0	0
GE	165	52	31.52	40	24.24

Block 1 - Building and facilities; Block 2 - Equipment, furniture, and utensils; Block 3 - Clothing; Block 4 – Food production and transportation; Block 5 – Documentation; C – Compliant items; GE – General evaluation.
Source: Research data.

Both were classified as high risk, with an overall percentage of 31.52% (agro-industry A) and 24.24% (agro-industry B). GMP is scarce for the cassava and derivatives production in the state of Pará-Brazil, a fact found in a joint inspection by the Public Prosecutor's Office and the Agricultural Defense Agency of the State of Pará (ADEPARÁ) in various agro-industries (JUSBRASIL, 2012).

Regarding agroindustry A, for block 1 (building and facilities), among the evaluated compliances the following ones stand out: the presence of paved internal access roads, floors, walls, and ceiling in good condition and easy hygiene material and presence of protected windows against insects and pests and for agroindustry B it was possible to observe the presence of a roof with easy hygiene and in good condition. The NC percentage in block 1 for agro-industry A was due, among other factors, to the lack of bulging corners on the walls and ceiling, and for agro-industry B were largely attributed to unsatisfactory conditions in the sanitary facilities sub-item.

Most of the cassava derivatives are manufactured by family agro-industries and due to this, the facilities infrastructure is lacking, which may compromise the food produced quality, as these conditions make it difficult to implement GMP (SCARPIN, 2011).

For block 2 (equipment, furniture, and utensils) the C percentage in agroindustry A was attributed to factors such as the existence of an appropriate place to store the utensils. For agroindustry B, block 2 was the one with the highest percentage of conformities among the blocks evaluated in this location, highlighting the presence of furniture and utensils in number and in an adequate state that allow easy hygiene. CN percentage of block 2 for agroindustry A was attributed to items related to the utensils used (inappropriate material, damaged surfaces, inadequate condition, and drawings that make proper hygiene difficult). Both industries A and B were deficient concerning standardized control on the cleaning frequency of equipment, furniture, and utensils.

According to statistics from the World Health Organization (WHO), more than 65% of reported cases of foodborne illness are due to hygienic-sanitary carelessness of handlers,

improper processing practices, and unhygienic physical structure, utensils, and equipment (WHO, 2015).

Block 3 (clothing) obtained the highest percentage of C in agro-industry A, where it was verified the use of light-colored uniforms, in an adequate state of conservation and the existence of training in Good Manipulation Practices by the handlers with training records. In agro-industry B, the wearing of light-colored and well-maintained uniforms was also verified. The NC percentage in this block for both A and B agro-industries occurred due to the observation of the use of earrings and rings by the handlers and the absence of posters in the production area guiding them about the correct hand hygiene. Handlers may exhibit inappropriate attitude when handling food, such as talking while performing tasks and not performing careful hand cleaning which can be considered a simple execution measure that can minimize the contamination risk (LEITE *et al.,* 2013).

For block 4 (Food production and transportation), the C percentage was verified in both the presence of an isolated area from the processing area for the raw material reception and product label according to current legislation. The NC percentage for both A and B agro-industries was largely attributed to the lack of storage control at the raw material reception, absence of temperature control in the area requiring thermal control (cooking area), and absence of "dirty area" insulation in the processing area. This fact had already been reported by Dias *et al.* (2012) when observing that in the raw material control item the diagnosed industries produced beyond their capacity, which causes problems mainly in the raw material storage, in the reception area organization, and production flow sub-items.

Block 5 (documentation) was considered the most unsatisfactory evaluation block in both agro-industries, where they presented 100% NC. This is due to the fact that they do not have a Guide of Good Manufacturing Practices, Standard Operating Procedures (SOPs), or any records of the activities performed. Lack of documentation is one of the most common irregularities reported in GMP audits across food industries (COSTA *et al.,* 2010; GUIDO *et al.,* 2010; DIAS *et al.*, 2012).

4 Conclusion

The samples analyzed presented free cyanide contents above the recommended by the current legislation, however they do not offer risks to consumers regarding the microbiological quality, since the presence of pathogenic microorganisms was not detected. The presence of coliforms, high counts of bacteria, and molds and yeasts show product recontamination. Significant differences were obtained regarding the physicochemical quality that may be related to variations due to factors such as plant varieties and age. Regarding GMP, agro-industries were assessed as high risk according to current legislation. There is currently a joint effort between the state inspection agencies and the cassava by-products processing agro-industries to adapt the processing sites, especially regarding GMP.

References

ABASS, A.B, *et al.* Enumeration of the microbiota and microbial metabolites in processed cassava products from Madagascar and Tanzania. *Food Control.,* v.99, p.164-170, 2019. doi: 10.1016/j.foodcont.2018.12.025.

ABREU, L.F.; MATTIETTO, R.A. Cassava culture: socioeconomic aspects, genetic improvement, cultivation system, pest, disease and agribusiness management: Embrapa Amazônia Oriental Documents. Chapter 13: Cassava derivatives manufacturing procedures: recommendations for obtaining safe and quality products, p. 223-230, 2016.

ACHIDI, A.U. *et al.* The use of cassava leaves as food in Africa. *Ecol Food Nutr.*, v.44, p.423-435, 2006. doi: 10.1080/03670240500348771.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (AOAC). Official methods of analysis of the 18th international. Published by AOAC International edition; 2007.

AYALA-ZAVALA, J.F. *et al.* Antioxidant enrichment and antimicrobial protection of fresh-cut fruits using their own by-products: Looking for integral exploitation. *J. Food Sci.,* v.75, n.8, p.175-181, 2010. doi: 10.1111/j.1750-3841.2010.01792.x.

BRADBURY, J.H.; DENTON, I.C. Mild method for removal of cyanogens from cassava leaves with retention of vitamins and protein. *Food Chem.,* v.158, n.1, p.417-420, 2014. doi: 10.1016/j.foodchem.2014.02.132.

BRAZIL. National Health Surveillance Agency. Resolution RDC n°. 275, of October 21, 2002. Provides for the Technical Regulation of Standardized Operating Procedures applied to Food Producing / Industrializing Establishments and the Checklist for Good Manufacturing Practices in Food Producing / Industrializing Establishments.2002. doi 10/23/2003.

CAMPOS, A.P.R.; CARVALHO, A.V.; MATTIETTO, R.A. Effect of fermentation and cooking on physicochemical characteristics and cyanide content during tucupi processing. Brazilian agricultural research company Embrapa Documents 107. 2016.

CÂMARA, A.C.L. ; SOTO-BLANCO, B. Cyanide: occurrence, characteristics and applications. Hauppauge: New Science Publishers, 2013.

CEBALLOS, H.; LA CRUZ, G. Cassava in the third millennium: modern production, processing, use, and marketing systems Chapter.2: Cassava Taxonomy and Morphology. *CIAT.*, p.574, 2012.

CHISTÉ, R.C.; COHEN, K.O.; OLIVEIRA, S.S. Study of tucupi physicochemical properties. *Food Sci. Technol.*, v.3, n.27, p.437-440, 2007. doi: 10.1590/S0101-20612007000300002.

COHEN, K.O.; OLIVEIRA, S.S.; CHISTÉ, R.C. Quantification of Total Cyanogenic Compound Content in Products Made with Cassava Roots. Brazilian agricultural research company Embrapa Documents 290.2007.

COOKE, R.D. Enzymatic assay for determining the cyanide content of cassava and cassava products. *CIAT.*, p.14, 1979.

COSTA, F.S. *et al.* Hygienic-sanitary evaluation of honey processing industries. *Hig. Aliment.*, v.24, p.184-185, 2010.

DIAS, M.A.C. *et al.* On the implementation of good manufacturing practices in a small processing unity of mozzarella cheese in Brazil. *Food Control.*, v.24, n.1, p.199-205, 2012. doi: 10.1016/j.foodcont.2011.09.028

ESSERS, A.J.A. *et al.* Studies on the quantification of specific cyanogenes in cassava products and introduction of a new chromogen. *J. Sci. Alimentos Agric.*, v.63, n.3, p. 287-296, 1993. doi: 10.1002/jsfa.2740630305.

FAOSTAT. Food and Agriculture Organization of the United Nations -FAO. World cassava production data in 2013. Available at: <http://faostat.fao.org/ site / 339 / default.aspx>. Accessed on: 05/10/2018.

GABRIEL, L.F. *et al.* Bimulating cassava growth and yield under potential conditions in southern Brazil. *Agron J.*, v.106-1119, 2014. doi: 10.2134/agronj2013.0187.

GLASSET, B. *et al.* Bacillus cereus-induced food-borne outbreaks in France, 2007 to 2014: epidemiology and genetic characterisation. *Eurosurveillance.*, v.21, n.48, p.304-313, 2016. doi: 10.2807/1560-7917.ES.2016.21.48.30413.

GLEADOW, R.; MOLLER, B. Cyanogenic Glycosides: Synthesis, Physiology, and Phenotypic Plasticity. *Annu. Rev. Plant Biol.*, v.65, p.155–185, 2014. doi: 10.1146/annurev-arplant-050213-040027.

GUIDO, E.S. *et al.* A university extension approach to improve milk quality in the production chain in the municipality of Barbosa Ferraz (Paraná). *Bol. Centro Pesqui. Process. Aliment.*, v.28, n.2, p.303-312, 2010. doi: 10.5380/cep.v28i2.20446.

HOU, D.B. *et al.* Detection of water-quality contamination events based on multi-sensor fusion based on an extended Dempster–Shafer method. *Meas. Sci. Technol.*, v.24, n.5, p.055801, 2013. doi : 10.1088/0957-0233/24/5/055801.

JORGENSEN, K. *et al.* Biosynthesis of the Cyanogenic Glucosides Linamarin and Lotaustralin in Cassava: Isolation, Biochemical Characterization, and Expression Pattern of CYP71E7, the Oxime-Metabolizing Cytochrome P450 Enzyme. *Plant Physiol.*, v.155, p.282-292, 2011. doi: 10.1104/pp.110.164053.

JUSBRASIL. Public Ministry of the State of Pará-. Belém, Sta. Izabel and Castanhal: MP wants to regularize products derived from cassava. Available at: <https://mp pa.jusbrasil.com.br/noticias/3086514/belem-sta-izabel-e-castanhal-mp-quer-regularizar-produtos-derivados-da-mandioca>. Accessed on: 15. May.2018.

KALENGA SAKA, J.D.; NYRENDA, K.K. Effect of two ethnic processing technologies on reduction and composition of total and non-glucosidic cyanogens in cassava. *Food Chem.*, v.130, n.3, p.605-609, 2012. doi: 10.1016/j.foodchem.2011.07.082.

KOTZEKIDOU, P. Microbiological examination of ready-to-eat foods and ready-to-bake frozen pastries from university canteens. *Food Microbiol.*, v.34, n.2, p. 337-343, 2013. doi: 10.1016/j.fm.2013.01.005.

KREGIEL, D. Health Safety of Soft Drinks: Contents, Containers and Microorganisms. *Biomed Res. Int.*, v.1, p.1-15, 2015. doi: 10.1155/2015/128697.

LATIF, S.; MÜLLER, J. Potential of cassava leaves in human nutrition: a review. *Trends Food Sci Technol.*, v.44, p.147-158, 2015. doi: 10.1016/j.tifs.2015.04.006

LEITE, M.A.G. *et al.* Sanitary hygienic conditions in the handled

sectors in supermarkets in the municipality of Barra do Garças-MT. *Rev Aliment. Nut.*, v.24, n.1, p.37-44, 2013.

MACHADO, E.R. *et al.* Frequency of Enteroparasites and Bacteria in the Leafy Vegetables Sold in Brazilian Public Wholesale Markets. *J. Food Prot.*, v.81, n.4, p. 542–548, 2018. doi: 10.4315/0362-028X.JFP-17-358.

MODESTO JUNIOR, E.N.; CHISTÉ, R.C.; PENA, R.S. Oven drying and hot water cooking processes decrease HCN contents of cassava leaves. *Food Res. Int.*, v.119, p.517-523, 2019. doi: 10.1016/j.foodres.2019.01.029.

NAMBISAN, B. Strategies for elimination of cyanogens from cassava for reducing toxicity and improving food safety. *Food Chem Toxicol., v.*9. n.3, p.690-693, 2011. doi: 10.1016/j.fct.2010.10.035.

ONI, A.O. *et al.* Chemical composition and nutritive value of four varieties of cassava leaves grown in South-Western Nigeria. *J Anim Physiol An N.*, v.95, p.583-590, 2011. doi: 10.1111/j.1439-0396.2010.01086.x.

PARA. Normative Instruction No. 1 of 05/06/2016, provides for the technical regulation of the production of the cooked bean and other measures. Standard of Identity and Quality of Cooked Maniva (cooked cassava leaves) for production and commercialization in the State of Pará - Agricultural Defense Agency of Pará - ADEPARÁ. DOE: 09 May 2016.

PARK, M. *et al.* Characterization and Comparative Genomic Analysis of a Novel Bacteriophage, SFP10, Simultaneously Inhibiting both Salmonella enterica and Escherichia coli O157:H7. *Appl. Environ. Microbiol.*, v.78, p.58-69, 2012. doi: 10.1128/AEM.06231-11

PESTANA, T.C.; CASTRO, G.H.F. Potential of cassava branch for use in feeding ruminants: review. *PubMed Vet e Zoot.*, v. 9, n.10, p. 457–466, 2015.

SALFINGER, Y.; TORTORELLO, M.L. Compendium of methods for the microbiological examinations of foods. 2015 Apha.

SCARPIN, D. Evaluation of the microbiological quality of food before and after the implementation of good manufacturing practices in agribusinesses in the extreme west of Santa Catarina. Santa Catarina: University of the west of Santa Catarina, 2015.

SOARES, I.A. *et al.* Protein concentrate obtained from cassava leaves (Manihot esculenta crantz) of three commercial varieties. *Acta Amb. Cat.*, v.13, n.1, p.1-7, 2016. doi: 10.24021/raac.v13i1.3644.

SOUZA, A.S. *et al.* Nutritional value of fractions of the fresh forage of four cassava varieties. *Rev. Bras. Saúde Prod. Anim.*, v. 12, n.2, p.441-455, 2011.

TEWARI, A.; ABDULLAH, S. Bacillus cereus food poisoning: international and Indian perspective. *J. Food Sci. Technol.*, v.52, p. 2500-2511, 2015. doi: 10.1007/s13197-014-1344-4.

TRESSELER, J.F.M. *et al.* Microbiological quality evaluation minimally processed vegetables. *Ciênc. AGROTEC.*, v.33, p.1722-1727, 2009. doi: 10.1590/S1413-70542009000700004.

TROMBINE FRM, LEONEL M. Physicochemical composition and technological properties of cassava leaves flour. *Energ. Agric.*, v.29, n.1, p.76-81, 2014. doi: 10.17224/EnergAgric.2014v29n1p76-81.

WHO. World Health Organization. Foodborne disease 2015. Available at: <http://www.who.int/topics/foodborne_diseases/en/>. Accessed on: 19. Mar 2020.