Article

COLORIMETRIC METHOD FOR FREE AND POTENTIAL CYANIDE ANALYSIS OF CASSAVA TISSUE.

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Abstract

Cassava plant accumulates a glycoside that may potentially generate cyanide whose residues remain in cassava roots and their derivatives. This article presents a simple colorimetric method that was tested with root extracts of cassava and leaves of the cultivar "Cascuda" for culinary use. The method presented results that agree with those found in the literature on similar materials. The mean values (mgHCN equivalent kg^{-1} fresh basis) for free cyanide were 40.34 for cortex, 35.02 for parenchyma and 49.40 for roots (cortex + parenchyma). The potential cyanide was higher 49.40 for cortex, 39.43 for parenchyma and 45.10 entire roots. Leaves presented 49.08 as free cyanide and 63.85 as potential cyanide. The method is useful for qualitative and quantitative determination on fresh cassava tissues.

Key words: Cyanide; Cassava; Linamarin; Enzyme; Cyanogenesis, Colorimetric assay

1. Introduction

One of the cassava plant's characteristics (*Manihot esculenta* Crantz) is the general presence of linamarin, a glycoside that is hydrolysable under certain circumstances and releases cyanide (HCN) in a process called cyanogenesis. The hydrolysis of this glycosidase only takes place spontaneously and naturally when a beta-glycosidase enzyme is present. This beta-glycosidase may be the linamarase, an enzyme found on cassava plant. Only free cyanide (CN^-) is toxic, and if hydrolysis does not occur the glycoside remains stable and the food using this product becomes safe. Cyanide or hydrogen cyanide generation takes place when the cassava tissues are broken down and placed together the substrate and enzymes (CAGNON, CEREDA and PANTAROTTO, 2002).

The use of cassava products for food or feed consumption is strongly influenced by the presence of potentially toxic cyanogenic glycosides, and the international literature is concerned about the residual levels

remaining in cassava food (ROSLING, 1987).

Because of this concern about residual levels of cyanide remaining in the cassava plants after processed, the cassava roots were classified according to their potential toxicity to humans and animals. Initially cassava plants were classified into three categories based on their cyanogenic content: a) non toxic (less than 50 mg $HCN.kg^{-1}$ in fresh roots), b) moderately toxic (50-100 mg $HCN.kg^{-1}$ of fresh pulp) and c) dangerously toxic (better or wild) cassava (above 100 mg HCN.kg kg^{-1} of fresh pulp) (BOURDOUX, 1982).

Based on this classification, the maximum cyanide level recommended by the World Health Organization (WHO) for fresh cassava intake is 10 mg of HCN/kg body weight (WHO, 1991 cited by CUMBANA, MIRIONEE and BRADBURY, 2007).

This limit has been questioned because it was established with HCN as gas through inhalation instead of ingestion. Cereda, Ramalho and Lopes (2007) reported an experiment in which it was used linamarin extracted from cassava and given orally to rats. In such experiment the lethal dose (LD50) was 324.86 mg linamarin kg^{-1} for body weight, a value three times higher than that recommend by WHO.

Despite the importance given to the presence of cyanide in food products, the literature has presented the results in a confusing way. The cyanide is sometimes expressed as HCN, free cyanide, total cyanide, and in others as linamarin. The Working Group on Cassava Safety (WOCAS) was formed to determine the correct way to express the results of the analysis of cyanide. According to Bokanga et al. (1994), by using analytical procedure it is possible to determine three compounds in cassava: **Fraction A** as cyanogenic glycosides, **Fraction B** as cyanohydrins and hydrogen cyanide, and **Fraction C**, the amount of hydrogen cyanide. Fraction A should be referred as "total cyanogens content", Fraction B as "non-glucosidic cyanogens content", and Fraction C as "hydrogen cyanide content". The cyanogenic glycoside content is obtained by subtracting Fraction B from Fraction A, whereas the cyanohydrins content is obtained by subtracting Fraction C from fraction B. The recommended unit to be used is "mg HCN equivalent kg^{-1}". Authors should indicate whether their data are calculated on fresh or dry matter basis. The potential for a sample to produce HCN, expressed as total amount of HCN equivalent per weight of sample, has been preferably called **cyanogenic potential**. This way of expressing the cassava cyanide will be used throughout this article.

Brazil and other South American countries use cassava widely for culinary purposes. The morphology of the cassava plant cannot be used to establish the level of cyanide, which can vary even with fertilization and climate. Laboratory analysis is the only secure way to establish the level of linamarin (innocuous) and free cyanide (toxic).

There are many methods for cyanide analysis described in the literature. The methodology for linamarin and their metabolic analysis is summarized by Cagnon, Cereda and Pantarotto (2003). The authors point out that most of them require a three-step analysis: a) extraction of cyanogenic compounds from plants, b) hydrolysis of glycosides into free cyanide, and c) determination of cyanide. All methods available in the literature are based on some general principles: the extraction of cyanogenic compounds from the plant is performed by using an acid diluted solution, since linamarase is inactive at low pH. Linamarin is then removed and submitted to a series of hydrolyses until cyanide becomes free cyanide. Among the methods cited in the literature, one can cite titration of cyanide with $AgNO_3$ and reaction with alkaline picrate. The most widely used coloring method is based on the König reaction, in which free cyanide (CN^-) is oxidized into cyanogen halide by chloramines T or N-chlorosuccinimide. Another method uses specific electrode for cyanide and voltmeter to measure the potential difference.

Autolysis is a method described by Bradbury (1994) in which reagents pyridine and pyrazolone or pyridine and barbiturates are used. The method has good sensitivity, very good specificity to cyanide, and reasonable accuracy. Another method described by Cooke (1978) was one of the first enzymatic ones, requiring addition of linamarase and use of pyridine and pyrazolone as indicators. This method also has good sensitivity, very good specificity to cyanide and good accuracy. The development of the enzymatic method occurred when Esser (1994) adapted the Cooke´s method by keeping the addition of linamarase and replacing the indicators for isonicotinate and dimethyl barbiturate, which rendered good sensitivity, very good specificity to cyanide and good accuracy. Yeoh and Woon 1992

(1992) describes a method requiring immobilization of linamarase within a polymer disc to release free cyanide, which is then determined by selective electrode. Despite its low-sensitivity, the method has good specificity to cyanide and reasonable accuracy.

Among the methods described in the literature the most internationally recognized is the Esser's one in which the linamarase extracted from cassava tissues is used for linamarin hydrolysis and the released free cyanide is used to establish the standard curve. The linamarase is also used in excess to linamarin hydrolysis in extracts of cassava releasing free cyanide.

Although time-consuming and laborious, the major difficulty of this method is that it requires expensive reagents that depend on authorization of the Brazilian Federal Police for being used. To overcome these drawbacks, Baltha and Cereda, (2006) have used a simple method for determining the free and potential cyanide based on autolysis of cassava roots (BRADBURY, 1994) in which endogenous linamarase enzyme was used to hydrolyze linamarin and release CN^-. Free cyanide was determinate by adapting the method of Smith described by Winton and Winton (1985), which is based on colorimetric reaction of picrate solution. The authors have established that a 15-minute reaction provided the highest values of cyanide, thus indicating an asymptotic curve of color development. Despite its very good sensitivity, this method has the disadvantage that the color reading has great variability due to the non-inhibited action of linamarase.

In order to improve this method, a research was developed to have more stability in lecture and tested on a cassava cultivar recommended for culinary use.

Material & Methods

The experiment was conducted in the Technology Center for Agribusiness Analysis at the Catholic University (CeTeAgro / UCDB) in Campo Grande, State of Mato Grosso do Sul, Brazil. The cassava used was a cultivar popularly known as "Cascuda", grown in the institution's cassava germplasm bank (20 ° 26 ' south, 54 ° 38' west, 532 meters). The cassava roots were randomly harvested at 8 months and analyzed separately cortex, parenchyma, root (cortex + Parenchyma) and leaves.

The method was adapted from Baltha and Cereda (2006) as follows:

2.1. Standard potassium cyanide curve

The standard curve was established with KCN (purchased from Vetec, 96.0% purity) and the reagents were prepared according to item 2.4 (preparation of reagents and solutions).

From the stock solution (0.027 mg HCN/ml) appropriate volumes were taken (**Table 1**) and transferred to a 100 ml flask.

Table 1: Stock solution of HCN in acid conditions (H_2SO_4 0.01M).

Flasks	Stock solution	H_2S0_4 (D)	HCN^- concentration
	ml	ml	mg HCN^-ml^{-1}
0	0	100	0.000
1	1	99	0.026
2	2	98	0.054
3	5	95	0.135
4	10	90	0.270
5	15	85	0.405
6	25	75	0.675
7	50	50	1.350

Equal volumes of picric acid and calcium carbonate (Solution B and Solution C) were mixed for have Alkaline Picrate just in time for the analysis. Concentrations for the standard curve were obtained according to Table 2 by using test tubes with Bakelite screw cap and usable volume near 7 ml. The volume at the test tubes was complete to 4.00 ml with distilled water.

Table 2: Reactive and volume for standard curve of free cyanide concentration (µg HCN).

Test tube	Ml/test tube			
	HCN stock solution	Alkaline Picrate	Distilled water	mg CN
0	0.00	2.0	2.00	0.0000
1	0.04	2.0	1.96	0.00104
2	0.04	2.0	1.96	0.00208
3	0.04	2.0	1.96	0.00520
4	0.04	2.0	1.96	0.01040
5	0.04	2.0	1.96	0.01560
6	0.04	2.0	1.96	0.02600
7	0.04	2.0	1.96	0.05200

The test tubes containing samples and color reagents were incubated for 15 minutes in water bath at 37°C. Before reading were added 15 µL of PA concentrated sulfuric acid (H_2SO_4) to stop the enzymatic reaction and increase the stability of reading. Absorbency reading used a spectrophometer Bel Photonics® Spectrophotometer SP 1105 at 535 nm. The values of absorbance founded were obtained through the standard curve equation. In this experiment the curve pattern was Y = 0.2476X + 0.009 (R^2 = 0.9536) and free cyanide expressed in milligrams (mg) by substitute X for de absorbance. If the reagents changes, a new curve patter should be made. The method sensibility can be calculated in the patter curve by calculate de cyanide concentration as difference between two absorbency values at the best range (0.3 ± 0.01), which in the experimental conditions corresponded to 0.002476 mg of HCN equivalent.

2.2. Analysis of free and potential cyanide in cassava tissues

The cyanide determination was performed using extracts of cassava tissues by separate (parenchyma and cortex) or the cassava root (parenchyma + cortex) and leaves. The previously weighed tissues were disintegrated in 50 ml of distilled water with a mixer Taurus Robot 250 INOX for 60 seconds. The amount of cassava tissues was selected to have the best absorbance values regarding the standard curve. This extract has the endogenous linamarase enzyme to hydrolyze linamarin to acetone cyanidrin according as described by Bradbury, (1994). The test tubes containing samples and color reagents were incubated for 15 minutes in water bath at 37°C. Before reading were added 15 µL of sulfuric acid (H_2SO_4) to stop the reaction and increase the stability of reading. The extract was filtered in a filter paper quality of 185 mm diameter (Whatman ® Sheleicher & Schuell). The lecture obtained for samples readings was used to replaces the X value on equation obtained from the standard curve. The samples were prepared in triplicate and a "white" reading without cassava extract (**Table 3**) was used in order to eliminate possible interference during adjustment of the equipment. In the conditions of the research was not found interfering as glucose or acetone. The result was expressed in mg HCN equivalent/Kg fresh matter.

Table 3: Reactive used for free and potential cyanide determination in cassava extracts.

Tests	Distilled water	Alkaline picrate	Cassava extract
White	2,0	2,0	0,0
Free and potential (HCN^p) cyanide	1,0	2,0	1,0

The difference between free and potential cyanide determinations was possible by stopping the linamarase action either immediately (free cyanide) or 15 minutes after the reaction (potential cyanide).

If it was necessary the 2.0 milliliter of distilled water may be replaced by sample, makes possible a direct reading without dilution. In this case the volume of the sample selected for the best reading of absorbance should be considered for the calculations

Regarding the methodology of Baltha and Cereda (2006) the difference is that phosphate buffer was

eliminated because the pH adjusted to 7.0 by using buffer makes impossible to lower the pH to stop the enzymatic reaction of limarase

2.3. Preparation of the extracts of "Cascuda" cassava cultivar

To compare the methodological changes on Baltha and Cereda (2006) methodology, the tissues from a cassava cultivar were analyzed for free and potential cyanide using both methodologies. The cassava cultivar selected was "Cascuda". The cassava plants were 8 months old, all of them being harvested with roots and leaves. Roots were separated in cortex and parenchyma. The whole roots (cortex + parenchyma), cortex, parenchyma, and leaves were analyzed separately. Extracts were prepared as described in item 2.2 by using 3.0g of each material placed in 50 ml of distilled water.

2.4. Preparation of the reagents and solutions

A) **Stock solution of KCN**: 6.5g of potassium cyanide were diluted in distilled water and completed to 1000 ml with H_2SO_4 0.01M. This stock solution containing 2.6g of CN^- per ml was obtained with the purity of KCN.

B) **Solution of picric acid**: saturated solution of picric acid was obtained by diluting 2.56g in 100 ml of distilled water.

C) **Solution of 5% sodium carbonate** (Na_2CO_3): 5g of sodium carbonate was diluted in 100 ml of distilled water;

D) **Solution of 0.01 M H_2SO_4**: 0.5330 ml of concentrated sulfuric acid was diluted in 1000 ml of distilled water.

2.5 Statistic analysis

The data were submitted to statistical analyses (ANOVA) and the mean, and standard deviation values were obtained and compared by Bonferroni test.

3. Results and Discussion

The proposed method, like several others, only allows the determination of free cyanide. To determine the potential cyanide it is necessary to release free cyanide using an enzyme. To prove that the method is effective in the determination of cyanide in cassava, it was used the plant's own enzyme linamarase in a process called

autolysis, as already established by Bradbury (1994).

To justify the changes in methodology the results for "Cascuda" cultivar regarding whole roots (cortex + parenchyma), cortex, and parenchyma, which were analyzed separately by using both Baltha & Cereda (2006) (**Table 4**) and modified methodologies (**Table 5**). The comparison of methods for cassava leaves is presented in **Table 6**.

The results show variability, even considering that all samples were from the same cultivar, plant and age. According to Nambisan (1994) the linamarin (potential cyanide) content varies in different parts of the plant: leaves, stem, and peel (cortex) contain higher levels of glycoside in relation to the edible part (parenchyma). Different varieties of cassava also have variations in their root's cyanogenic content, ranging from 25 to 450 mgHCN-.kg^{-1} fresh weight.

This variability is also mentioned by Alves (1998) who lists values for cyanide parenchyma, cortex and leaves from the literature. This variability may be result also by different ways to express cyanide for cassava tissues.

Potential cyanide may be higher than or equal to the free cyanide, but never lower. The data obtained by means of the method described by Baltha & Cereda (2006) showed in **Table 4** presented some values for free cyanide higher than potential cyanide. The cortex should present higher potential cyanide that in the parenchyma (edible part) and the combination of both represent the entire cassava root (cortex parenchyma) but the proportion is not the same. Cortex is smaller that parenchyma. Cyanide (free and potential) from parenchyma may influence more the cassava root cyanide content of cyanide that cortex alone.

Table 4: Results (mean of 3 repetitions) for free and potential cyanide regarding cortex, parenchyma, and root (cortex + parenchyma) of "Cascuda" cassava cultivar using the methodology of Baltha & Cereda (2006). Results are expressed as the mean ± SEM (n=3).** p< 0.01,).* p< 0.05 and (s) standard deviation.

	Free Cyanide			**Potencial cyanide**		
	mgHCN equivalent kg^{-1}fresh basis			**mgHCN equivalent kg^{-1}fresh basis**		
	Cortex	Parenchyma	Root	Cortex	Parenchyma	Root
Mean	42.83*	44.77**	43.63*	44.28 *	44.66**	44.77**
s	3.071389	3.960269	5.120609	1.375875	1.862122	3.960269
☑	± 11.77	±2.28	±2.95	±0,80	±1,10	±2.23

The values of free and potential cyanide obtained with the method of Baltha & Cereda (2006) showed values close but statistically different for root and cortex, with significantly higher for cyanide potential, which is expected. Using this method the content of cyanide in the cortex was lower than in the parenchyma and root for both free and potential cyanide. These results founded for cassava tissues agree with literature for cassava for culinary use. However, data showed variation (standard deviation) and consequently the results are not enough precise.precise.

Table 5 shows the data for the same cultivar but analyzed by modified method. The data were similar to the data showed in Table 4, and higher values were ever founded for potential cyanide compared to free cyanide, what make its more consistent. The standard deviation proves that the mean obtained were more stable for variation that the ones obtained with Baltha & Cereda (2006) method. With the adjustment the cyanide content of the cortex was always higher than of the parenchyma and root for both free and potential cyanide, but only the potential

values were statistically differed.

Table 5: Results (mean of 3 repetitions) for free and potential cyanide regarding cortex, parenchyma, root (cortex + parenchyma) "Cascuda" cassava cultivar using the modified methodology; values expressed as mgHCN equivalent kg^{-1}fresh basis. Results are expressed as the mean ± (σ) SEM (*n*=3).* $p< 0.05$; *$p< 0.01$.

	Free Cyanide			Potencial cyanide		
	mgHCN equivalent kg^{-1}fresh basis			mgHCN equivalent kg^{-11}fresh basis		
	Cortex	Parenchyma	Root	Cortex	Parenchyma	Root
Mean	40.34	35.02	36.83	49.40*	39.43*	45.10*
s	0.051962	0.032146	0.262742	0.092376	0.249867	0.234307
σ	±0.03	±0.02	±0.15	± 0.05	±0.14	±0.13

It´s possible that the stability in lectures may be due the reaction between the enzymes linamarase and linamarina was stopped by a strong pH reduction making the reaction more stable.

The values shown in **Table 5** with higher values of cyanide for cortex and smaller for parenchyma agree with the literature, suggesting that the cassava for culinary uses had suitable levels of cyanide in the edible part of the root (parenchyma) ever that cortex is take out. These low values may support the proposal of "Cascuda" cultivar for food, which is also in accordance with the WHO's recommendation (WHO, 1991 cited by CUMBANA, MIRIONEE and BRADBURY, 2007).

The values found for free and potential cyanide are also corroborated by the literature on. Cardoso et al. (2005) described the potential cyanide content found in parenchyma of the fresh cassava roots before and after processing may be considered as safe for consumption by WHO.

According to Gettler and Baine (1938) the colorimetric method using picric acid is not quantitative and is indicated only to detect presence or absence of cyanide. The author also states that if the tissues to be analyzed are rotten, then the method may be invalidated due to substances such as H_2S and SO_2 as they cause a reddish hue. But when fresh cassava is analyzed, this type of interference is absent.

In this experiment the white test was used to detect any interference, so the colorimetric test becomes valid. The risk of human intoxication with low cyanide levels in cassava cultivars is very low. The simple act of peeling the cassava roots before cooking can reduces the level of cyanide in about 50% because the highest levels of cyanide are found in the cortex (HEWE, 1991) similar that the results obtained for the "Cascuda" cultivar cortex and parenchyma.

Table 6 shows the data for leaves of "Cascuda" cassava cultivar. The mean values for free and potential cyanide are similar (de difference was not significantly for both methods), but the standard deviation was smaller than for the data of roots analysis (cortex and parenchyma).

Table 6. Results (mean of 3 repetitions) for free and potential cyanide in "Cascuda" cassava leaves using both Baltha & Cereda (2006) and modified methodologies; values expressed as mgHCN equivalent kg^{-1}fresh basis.

Results are expressed as the mean ± (σ) SEM (*n*=3).* *p*< 0.05; **p*< 0.01 and **(s) standard deviation**.

	Baltha and Cereda (2006)		Modified methodology	
	Cyanide		Cyanide	
	Free	Potencial	Free cyanide	Potencial
Mean	49.27	64.60	49.08	63.85
s	0.342685	0.74054	0.09815	0.092376
	±0.20	±0.42	±0.05	±0.05

Although there is abundant literature on cyanide in cassava and its products is difficult to find comparable results caused by various forms of reporting and different methods of analysis used.

Conclusion

Based on the results obtained it is possible to state that the qualitative and quantitative methods allow the determination of free and potential cyanide contents in different parts of the cassava plant. For cassava leaves, both methods are useful. The method has good sensitivity, good specificity to cyanide, and reasonable accuracy.

5. References

ALVES, A.A.C. Physiological and developmental changes in cassava (*Manihot esculenta* Crantz) under water deficit. 1998. PhD Thesis on Plant Physiology, Cornel University, Ithaca, New York. 160p.

BALTHA, A.D.T. de B.; CEREDA, M.P. 2006. Cassava free cyanide analysis using KCN or acetone-cyanidrin as pattern**.** In: INTERNATIONAL MEETING ON CASSAVA BREEDING, BIOTECHNOLOGY AND ECOLOGY. 1. Brasilia: Proceedings... p.132. Cassava improvement to enhance livelihoods in sub saharan África and Northweast Brasil. Brasília. University of Brasília. Ministério do Meio Ambiente.

BOKANGA, M.; ESSERS, S. ; POULER, N. ; ROSLING, H. ; TEWE, O. ; ASIEDU, R. and BRADER, L.1994. Preface. Acta Horticulturae,Wageningen, v. 375, p.1-2.

BOURDOUX, P. et al. 1982. Nutritional factors involved in the goitrogenic action of cassava. Ottawa, qnt, IDRC. 100 p.

CAGNON, J.R.; CEREDA, M.P. and PANTAROTTO, S. 2002. Glycosides of cassava cyanogen: biosynthesis. distribution. detoxification and analytical methods. In: Cereda, M.P. Agriculture: Latin America starchy tuberous and roots. Cargill Foundation, São Paulo, v.2, chap.5, p.83-99 (in Portuguese).

CARDOSO, A. P. ; MIRIONE, E.; ERNESTO, M.; MASSAZA, F.; CLIFF, J.; HAQUE, M.R. and BRADBURY, J. H. 2005. Processing of cassava roots to remove cyanogens. Journal of Food Composition and Analysis, v.18, n.5, p.451-460.

COOKE, R.D. An enzymatic assay for the total cyanide content of cassava. Journal of the Science of Food and Agriculture, London, v.29, p.345-352,1978.

CUMBANA, A.; MIRIONEE, E.; CLIFF, J. and BRADBURY, H. 2007. Reduction of cyanide content of cassava flour in Mozambique by the wetting method. Food Chemistry, v.101, n.3, p.894-897.

ESSER, A.J.A. Removal of cianogens from cassava roots. ESSER, A. A. J.; BOSVELD, M.; GRIFT R. M.; VORAGEM, A.G.J. Studies on the quantification of specific cyanogens in cassava products and introduction of a new chromogen. Wagninger University,1995, p. 21-38.

GETTLER, A.O. and BAINE, J.O. 1938. The toxicology of cyanide. American Journal of Medical Science, v.195, p.182-198.

HEWE, O. O. 1991. Detoxification of cassava products and effects of residual toxins on consuming animals. In: PROCEEDINGS OF FAO EXPERT CONSULATION MEETING ON ROOTS, TUBERS, PLANTAINS AND BANANAS. 81p.

NAMBISAN, B. 1994. Evaluation of the effect of various processing techiniques on cianogen content reduction in cassava. BOKANGA, M.; ESSER, A.J.A.; POUTER, N.; ROSLING, H.; TEWE, O. In: INTERNATION WORKSHOP ON CASSAVA SAFETY. Ibadan, Acta Horticulturae, Wageningen, v. 375, p.203-207.

RAMALHO, R.T.; LOPES, A.M.; CEREDA, M.P. 2007. Oral Letal Dosis evaluation (DL_{50}) of linamarin extracted from cassava in rats. In: Brazilian Journal of Toxicology, ISSN 1415-2983 In: CONGRESSO BRASILEIRO DE TOXICOLOGIA. Búzios, v.20, additional n°3, p. 145, 2007. (in Portuguese)

ROSLING, H. 1987 Cassava toxicity and food security: a review of health effects of cyanide exposure from cassava and of ways to prevent these effects. Uppsala. UNICEF/African Household Food Security Programme. 40p.

YEOH,H.H. AND WOO, H.C. 1992 Cassava leaf b-glucosidase specificity and inhibition. Phytochemestry, p.2263-2225.

WINTON, A.L.; WINTON, K.B. 1958. Análisis de Alimentos. 2.ed. Barcerlona: Hispano Americana , S.A. p. 262-266. (In Spanish).

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