VITAE, REVISTA DE LA FACULTAD DE QUÍMICA FARMACÉUTICA
ISSN 0121-4004 / ISSNe 2145-2660. Volumen 18 número 3, año 2011.
Universidad de Antioquia, Medellín, Colombia. págs. 319-324

VALIDATION OF AN ANALYTICAL METHODOLOGY TO QUANTIFY CYANOGENIC GLUCOSIDES IN COMMERCIAL CASSAVA PRODUCTS

VALIDACIÓN DE UNA METODOLOGÍA ANALÍTICA PARA LA CUANTIFICACIÓN DE GLUCÓSIDOS CIANOGÉNICOS EN PRODUCTOS COMERCIALES DE YUCA

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Received: 28 April 2010 Accepted: 27 September 2011

ABSTRACT

A spectrophotometric method for determining cyanogenic glucosides (CGs) was validated in accordance with IUPAC and ICH requirements for new methods. The method demonstrates good linearity in the range of 0.1-0.88 μgg^{-1} hydrocyanic acid (HCN). The accuracy of the method ranges between 95.2% and 96.4%. The precision of the method, reflected as the relative standard deviation of the replicates, is lower than 12%. The method was compared with the most used method to detect CGs in cassava roots, the one established by Essers *et al.* Both methods were applied to quantify CGs in commercial cassava products. The new method is a useful and reliable tool for monitoring levels of CGs in cassava commercial products.

Keywords: Cyanogenic glucosides, cassava, hydrogen cyanide, validation methods, spectrophotometry.

RESUMEN

En este trabajo se valida un método espectrofotométrico para la determinación de glucósidos cianogénicos (CGs) de acuerdo a los requerimientos de IUPAC y ICH para nuevos métodos. El método demuestra una buena linealidad en el rango de 0,1-0,88 μgg^{-1} de ácido cianhídrico (HCN). La exactitud del método está entre 95,2 y 96,4%. La precisión del método reflejada como la desviación estándar relativa de los replicados es menor al 12%. El método se compara con el método más usado para la detección de CGs en raíces de yuca, el método de Essers *et al.* Ambos métodos se usan en la cuantificación de CGs en productos comerciales de yuca. El nuevo método es una herramienta útil y confiable para monitorear los niveles de CGs en productos comerciales de yuca.

Palabras clave: glucósidos cianogénicos, yuca, cianuro de hidrógeno, validación de métodos, espectrofotometría.

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INTRODUCTION

Food products prepared from cassava (*Manihot esculenta* Crantz) are the staple diet for more than 400 million people and they are prepared for consumption in a wide range of presentations, according to the region of the world where they are consumed (1). Products such as garri, fufu and tapioca are traditional in countries such as Nigeria and Mozambique (2). Cassava is a significant source of cyanogenic glucosides (CGs) such as linamarin and lotaustralin. These CGs are widely distributed in the plant, with the highest concentration in the leaves and root peel, and in a lower concentration in the root parenchyma (inside the root) (3). It has been reported that the average levels of cyanide in cassava flour in the Northwest of Mozambique, in a year with normal rainfall, is 45 $mgkg^{-1}$ (4); but in products that involve cooking, levels may be quite lower (0-39 $mgkg^{-1}$) (5, 6). This fact represents a health risk because cassava consumption has been associated with several types of pathological disorders (7). The ingestion of cyanide from bitter cassava varieties with high concentrations of CGs causes two important illnesses: tropical ataxic polyneuropathy in individuals over 40 years old, mainly women (8); and konso (i.e. "tired legs"), the irreversible paralysis of the legs, which occurs mostly in children and women of childbearing age with a low protein intake (8, 9).

Due to the imminent danger and high toxicity of several cassava varieties, the Codex Alimentarius Commission has set the limit of 10 $mgkg^{-1}$ hydrocyanic acid (HCN) for cassava flour and cassava starch (10). The expansion of food markets and food imports has forced some countries to introduce a sanitary control to regulate the entry of food products. This is the case of CGs from cassava, the vast majority of countries use the regulation established by the Codex; even though, countries such as Indonesia have set their own limits (11). Colombia has a regulation for CG levels in animal feed (12), but there is no regulation for CG levels in food products for humans. In order to enforce these regulations, it is important to standardize CG detection methods.

This work has three main objectives: the first one consists in validating a methodology for detecting CGs, which is used by the Japanese Ministry of Health, Labor and Welfare (MHLW-J) (13). The second objective consists in analyzing commercial food products prepared from cassava, and evaluating if such products would comply with the Codex CG limits using two methods: the MHLW-J and the method modified by Essers *et al.*, 1993 (14), which is the most used method for the detection of CGs in cassava roots. The third objective consists in comparing the results obtained after analyzing the same samples with methodologies that were mentioned.

MATERIALS AND METHODS

Chemicals

Partially purified linamarase cassava enzyme (EC 3.2.1.21) was donated by the Cassava Quality Laboratory at the Centro Internacional de Agricultura Tropical (CIAT) in Palmira, Colombia. Chloramine T was obtained from Carlo Erba Reagents (Rodano, Milan, Italy). l-phenyl-3-methyl-5-pyrazolone was obtained from Eastman Fine Chemicals (Rochester, New York, USA). Linamarin was obtained from Toronto Research Chemicals (North York, Canada). All the other reagents used were purchased from Merck® Chemical Co. (Darmstadt, Germany).

Preparation of solutions

Citric acid buffer (CAB) was prepared by mixing 128.1 g of citric acid and 64.4 g NaOH in 1 L of distilled water. CAB was diluted (1/10, v/v) and its pH was adjusted to 5.9. Phosphate buffers (PB) (pH 3.0 and 6.0) were prepared using 0.1 M H_3PO_4 with 5 M NaOH. Phosphoric acid buffer (PAB) was prepared by mixing 17 g KH_2PO_4 and 36 g $N_2HPO_412H_2O$ in 1 L of distilled water.

The barbituric acid/isonicotinic (BAI) mixture was prepared as described by Essers *et al.*, 1993 (14): 1,3-dimethylbarbituric (7.0 g) and isonicotinic acid (5.7 g) were dissolved in a solution of 0.46 M NaOH (200 mL) and the pH was adjusted to 7.5.

The solution of pyridinecarboxilic acid-pyrazolone (PAP) was prepared by mixing 2 solutions. Solution A: 0.3 g 1-phenyl-3-methyl-5-pyrazolone in 20 mL of dimethylformamide. Solution B: 1.5 g of 4-pyridinecarboxilic acid in 20 mL of 1 M NaOH. The final volume of 100 mL was reached by adding distilled water.

The chloramine T reagent was prepared daily: 2 g of chloramine T was dissolved in 100 mL of distilled water. The linamarase enzyme (8-9 $EUmL^{-1}$ activity) was diluted (1/1000, v/v) before each test in a phosphate buffer (pH 6.0).

Stock solution and standard cyanide solutions

The stock solution of 100 mgmL^{-1} HCN was prepared by dissolving 25 mg of KCN (previously dried for 12 h in concentrated H_2SO_4) in 100 mL of a 0.33% sodium hydroxide solution. Standard solutions were prepared from the HCN stock solution; dilutions were made using a 0.033% KOH solution until the corresponding levels of concentration required were obtained for the calibration curve.

Measurement of linamarase enzyme activity

0.1 mL of a dilute linamarase solution (1/1000, v/v) were mixed with 0.5 mL of linamarin (5 mM in PB pH 6.0) in a test tube and the mixture was incubated for 30 min at 30°C. Then, 0.6 mL of 0.2 M NaOH were added. After 5 min, the final volume of 4.0 mL was completed with 2.8 mL of PB. 0.1 mL of chloramine-T solution were added to the test tube; which was then placed in an ice bath for 5 min. Later, 0.6 mL of BAI were added and the test tube was left for another 10 min until the color developed (14). The enzymatic activity of the solution was calculated from the measured absorbance at 605 nm, according to the procedure described by Cooke *et al.*, 1979 (15).

Sample preparation

In March, 2006, 1 kg bags of "croquetas de yuca" (cassava croquettes) were bought from different grocery stores (Cali, Colombia). The products were crumbled into pieces by hand, trying to leave it as homogeneous as possible. The resulting sample was re-packaged in 30 g portions in plastic bags and stored at -20°C until the moment of analysis.

MHLW-J Method

After the samples were thawed at room temperature, 15 g of crumpled sample were placed in a glass ball for steam distillation (13). 200 mL of CAB and 2 mL of diluted (1/1000, v/v) linamarase enzyme solution were added. The distillation equipment was hermetically sealed and kept in a water bath at 40°C for 40 min. Then, 100 mL of distilled water was added to the glass ball for steam distillation, and the ball was connected to the condensation column. The lowest part of the condensation column was immersed in 5 mL of 1% potassium hydroxide solution. Distillation was completed when 150 mL of distillate were obtained.

10 mL of distillate, 5 mL of PAB and 1 mL of chloramine T were added to a test tube, which was then covered. After 5 min, 5 mL of PAP were added, and the test tube was placed in a water bath at 40°C for 40 min. The absorbance was measured at 640 nm.

Modified Essers Method

10 g of crumbled sample, previously thawed at room temperature, were homogenized in 150 mL of 0.1 M (25%, v/v, ethanol) phosphoric acid and centrifuged at 3000 *g* for 15 min. The supernatant was separated by filtration with a Watman GF/A filter paper (14).

An aliquot of 0.1 mL of the supernatant was mixed with 0.4 mL of 0.1 M pH 7 PB and 0.1 mL of linamarase enzyme solution in a test tube. After 15 min of incubation at 30°C, 2.8 mL of 0.1 M pH 6.0 PB and 0.1 mL of chloramine T were added and allowed to react for 5 min. Then, 0.6 mL of BAI were added. After 10 min, the absorbance was measured at 605 nm.

Validation of MHLW-J method

The analytical-method validation was performed according to the specifications of Thompson *et al.*, 2010 (16) and ICH, 2010 (17) for the parameters linearity, accuracy, precision, robustness, limit of detection and limit of quantification (13).

Statistical analysis

The Stata v9 software (StataCorp, Texas, USA) was used for statistical analysis of the data. Data were graphed to determine if they were normally distributed; if not, the data were transformed for approximating better this distribution. Paired t tests were run on the HCN results obtained with the MHLW-J and the modified Essers methods. Results were considered statistically and significantly different if $p<0.05$.

RESULTS AND DISCUSSION

According to our own knowledge, this is the first comparison of two methods to quantify HCN in commercial cassava products.

Validation of the method used by MHLW-J

Linearity and linear range

The linearity and linear range responses of the method were evaluated with 16 standard cyanide solutions in triplicate at concentrations ranging from 0.1-3.0 μgg^{-1}. HCN concentration data and

absorbance were related by the least squares linear regression method. Two linear responses were found: one between 0.1-0.8 μgg^{-1}, and the other between 1.2-2.4 μgg^{-1}. The following was the calibration equation of the first linear response (0.1-0.8 μgg^{1}): Absorbance = 1295[HCN] - 0.011 and coefficient of determination (r^2) was 0.9932. The relative standard deviation (RSD) of each point (n=5) was less than 10%. The first interval was selected because the samples were located appropriately within this range.

Precision

The precision was evaluated by repeatability and intermediate precision. The repeatability was evaluated by analyzing 7 replicates of standard working solutions at different HCN concentrations. This procedure was repeated for short periods of time on the same d. The intermediate precision was evaluated similarly but for different d. All samples were prepared and evaluated daily. The RSD in repeatability was lower than 12%. These values were also lower than the ones predicted by the empirical equation of Horwitz, 1982 (18) for the same levels of HCN concentration, which confirms that the method has good precision.

Accuracy

The accuracy of the method was determined using the standard addition method, where the recovery was measured using HCN standards at three concentration levels (12.59, 23.86 and 35.9 μgg^{-1}) in triplicate, which were added to 4 samples of the commercial cassava product. The mean recoveries, expressed in percentage recovery (PR), and their corresponding SD were determined. Table 1 shows the mean PR for the four analyzed samples which ranged between 80.52 and 99.62%.

Table 1. The percentage recovery of HCN in four commercial cassava products, using three known concentration levels.

Sample	Final concentration mgg^{-1}			Standard deviation	Mean percentage recovery (%)
	Mean without doping	Added	Measured		
A	18.45	12.59	12.20	1.55	96.87
		23.86	22.80	1.82	95.54
		35.9	32.19	3.03	89.66
B	17.47	12.59	10.1	0.51	80.52
		23.86	22.1	3.05	92.42
		35.9	33.8	0.34	94.18
C	18.11	12.59	11.58	1.77	91.97
		23.86	23.2	0.27	97.38
		35.9	35.8	1.31	99.62
D	18.17	12.59	10.91	0.40	86.65
		23.86	21.36	0.77	89.51
		35.9	35.48	0.78	98.84

According to AOAC, 1993 (19) and Hubert, 1998 (20), for concentrations between 100 ngg^{-1} and 10 mgg^{-1}, the PR should be between 80 and 110%. The obtained results demonstrate the suitability of the method for measuring CGs in a commercial cassava product.

Robustness

Robustness was evaluated using the 2^3 full-factorial design established by Plackett-Burman, 2002 (21). Three factors considered to be critical in the methodology were selected: incubation temperature, PAP reaction time, and chloramine T reaction time. For every factor, the nominal, maximum and minimum values expected in routine work were encoded as 0, +1 and -1, respectively (table 2).

Table 2. The nominal (0), maximum (+1), and minimum (-1) values established for the experimental factors studied.

Experimental variables	Level "-1"	Level "0"	Level "+1"
IT(°C)	35	40	45
RTPAP (min)	30	40	50
RTCT (min)	3	5	7

IT: Incubation time; RTPAP: reaction time of the pyridinecarboxilic acid-pyrazolone solution; RTCT: reaction time of cloramine T solution.

The results from applying the design matrix are depicted in table 3. From the three mentioned factors, only incubation temperature influenced the results. Therefore, incubation temperature must be carefully controlled.

Table 3. Results obtained for robustness matrix.

	Factors			Interactions				
Experiment	IT	RTPAP	RTCT	(IT)(RTPAP)	(IT)(RTCT)	(RTPAP)(RTCT)	(IT)(RTPAP)(RTCT)	Absorbance
1	-	-	-	+	+	+	-	0.532
2	+	-	-	-	-	+	+	0.760
3	-	+	-	-	+	-	+	0.490
4	+	+	-	+	-	-	-	0.684
5	-	-	+	+	-	-	+	0.578
6	+	-	+	-	+	-	-	0.705
7	-	+	+	-	-	+	-	0.562
8	+	+	+	+	+	+	+	0.589

IT: Incubation time; RTPAP: reaction time of the pyridinecarboxilic acid-pyrazolone solution; RTCT: reaction time of solution of cloramine.

The influence and interaction factors were calculated over each result according to the method established by Capolar-Gautier *et al*., 1992 (22) and their corresponding confidence intervals. It was found that experiment 1 (table 3) has a confidence interval that does not pass through zero, which means that the outcome is influenced by the variation factor. Therefore, the temperature is a critical factor that should be controlled with great accuracy at the time of analysis.

Limit of detection (LD) and quantification (LQ)

Following the methods of ICH and Miller (17, 21), LD was determined to be 0.003 mgg^{-1} and LQ was 0.011 mgg^{-1}. Hence, all experiments were conducted in a region above the method's LQ.

Analysis of commercial cassava products

The HCN content of three bags of the commercial cassava product is shown in table 4. The HCN values reported by the MHLW-J method are higher than those reported using the modified Essers *et al*., 1993 (14) method for bags 1 and 3 ($p < 0.0001$) but not for bag 2 ($p = 0.37$). A possible cause for such results could be due to the fact that the modified Essers *et al*., 1993 (14) method is exposed to more interference. This interference occurs because the colorimetric reaction takes place in the same solution where the enzymatic hydrolysis reaction takes place.

Table 4. Total content of HCN in the commercial cassava product as determined by two different methodologies.

	Bag 1 (n=65), $mgkg^{-1}$		Bag 2 (n=69), $mgkg^{-1}$		Bag 3 (n=48), $mgkg^{-1}$	
	MHLW-J Method	Modified Essers Method	MHLW-J Method	Modified Essers Method	MHLW-J Method	Modified Essers Method
Mean of HCN, (SD)	25.5 (3.2)	23.3 (1.2)	21.3 (4.5)	21.8 (2.1)	18.5 (1.3)	14.7 (1.1)
p value†	<0.0001		0.37		<0.0001	

† According to paired t tests.

There are two interfering factors associated with CGs that could affect the detection of cyanide: first, by impeding the enzymatic hydrolysis of all CGs in the sample; and second, by hindering the reactions that cause the formation of the colorimetric complex. The MHLW-J method used has the advantage that distillation occurs after the enzymatic reaction; thus, the high temperatures can foster conditions for a complete hydrolysis of CGs. Moreover, in the MHLW-J method, the hydrolyzed CGs are captured by water vapor during distillation, and they are released into the KOH medium (which prevents the evaporation of HCN). The solution is then quantified by the cyanide in it without interference from the cassava matrix. This is an improvement over the modified Essers *et al*., 1993 (14) method, in which quantification is performed on a solution that includes parts of the cassava matrix.

It is noteworthy that for both methods, the commercial samples exceeded the maximum HCN content allowed by the Codex (10 $mgkg^{-1}$) (10). In other words, if the tested bags had been exported to a country with the Codex limits, such bags, and potentially the whole shipment, would have been rejected.

The greater sensitivity of the MHLW-J method could be advantageous for food companies that sell products that have natural high levels of CGs because, if such method is appled, it will reduce the chances of the product being rejected.

CONCLUSIONS

The MHLW-J method for the detection of CGs in a food matrix of commercial cassava products was validated; the linearity (correlation coefficient) was 0.9932 at the range of 0.1-0.88 μgg^{-1}. The accuracy of the method is greater than 80.52%. The precision of the method, reflected as the relative standard deviation of the replicates, is lower that 12%. These facts show that the MHLW-J methodology complies with the basic parameters for a valid analytical method.

The MHLW-J method provided data with a statistical significance higher than the data obtained by the modified Essers *et al.*, 1993 (14) method (table 4); for this reason, it can be concluded that the MHLW-J method is more sensitive than the modified Essers *et al.*, 1993 (14) method. HCN levels in commercial cassava products sold in Colombia were higher than the ones recommended by the Codex for human consumption.

ACKNOWLEDGEMENTS

The authors acknowledge the following departments at CIAT: Cassava Quality Laboratory, Nutrition Quality Laboratory and Cassava Genetic Improvement Program; as well as the funding provided by Congelagro McCain-Colombia, AgroSalud (CIDA7034161), the Monsanto Fund and CIAT. The authors would also like to thank the Chemistry Department of Universidad del Valle, Colombia.

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