FURTHER IMPROVING THE ENZYMIC ASSAY FOR CYANOGENS IN CASSAVA PRODUCTS

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Abstract

A commonly used assay for routine determination of cyanogens in cassava is the enzymic assay developed by Cooke (1979), and improved by O'Brien *et al.* (1991). It compared well with earlier methods and was further improved by more acceptable coloration and more accurate calculation of the cyanogenic glucosides and cyanohydrins (Essers *et al.*, 1993). Isonicotinate/1,3-dimethyl barbiturate, the reagent used in the König reaction, was tested and found to have the following advantages over pyridine/pyrazolone, the color reagent used by Cooke and O'Brien *et al.*: it is less toxic and does not release repulsive vapors; it is quicker, cheaper and easier to handle; it has increased sensitivity and can be stored for longer. The new coloration has been applied successfully in laboratories in Uganda, The Netherlands and Malawi. An assay protocol is summarised. At Wageningen Agricultural University, the new assay was automated using a Gilson automated pipette station. The need for further improvements is discussed.

Key words: Cassava, linamarin, cyanide, analysis, König reaction, isonicotinate

Introduction

Cassava is an important starchy root crop grown in the tropics, but its potential toxicity is a public health concern. The toxicity is due to the presence of cyanogenic glucosides, mainly linamarin, which, following hydrolysis to cyanohydrins, may produce the toxic compound hydrogen cyanide. Ingested cyanohydrins are thought to decompose at the alkaline pH level of the small intestines to yield hydrogen cyanide. The toxicity of ingested glucosides in man is not yet well understood. The potential toxicity of a widely consumed staple food crop requires a reliable, fast and cheap analytical method for measuring the levels of the compound(s) causing toxicity. The separate quantification of the three categories of cyanogens is a prerequisite for the study of the breakdown and removal of cyanogens during processing of cassava, and for monitoring the safety of products. Such a method was developed by Cooke (1979) and subsequently improved by O'Brien *et al* (1991) and Essers *et al* (1993). The method involves homogenization in acid, hydrolysis by exogenous enzyme, pH control, and spectrophotometry. It compared well with the methods previously used which relied on autolysis and separation of HCN by micro-diffusion in a Conway vessel (Cooke and De la Cruz, 1982), and steam distillation followed by titration according to AOAC (Essers *et al.*, 1993). It has proved useful in several studies on cassava products and has the following advantages: it is independent of endogenous linamarase, which also makes it suitable for processed cassava products; it can distinguish between cyanogenic glucosides, cyanohydrins and free cyanide (HCN/CN^-); it is quick; and as toxic repulsive vapors were eradicated with the last improvement, the method is easier to handle.

The complete assay (Figure 1) measures the sum of the cyanogenic compounds (cyanogenic potential) by converting them all to HCN which triggers, quantitatively, the formation of a pigment by the König reaction. Cyanohydrins and free cyanide (non-glucosidic cyanogens), as well as free cyanide alone, are measured by omitting the respective conversion steps in the assay. The concentration of individual compounds is calculated from the difference.

	Diagram	*Rationale*
step 1	extraction in acid cyanohydrins are stabilized ↓	linamarase activity is stopped;
step 2	buffer the extract (pH 6); add linamarase; incubate ↓	glycosides are hydrolyzed to cyanohydrins and glucose
step 3	raise pH to 12 with NaOH ↓	cyanohydrins are converted to CN-
step 4	lower pH to 3 - 7; add Chl-T; add color reagent; incubate 10 min ↓	each CN- ion will trigger the formation of a conjugated molecule with resonance at 605 nm
step 5	measure absorbance at 605 nm ↓	
calibration curve	↓	
cyanogenic potential		
non-glucosidic cyanogens		Same procedure without step 2.
free cyanide		Same procedure without steps 2 and 3; pH has to be kept between 3 and 4.5.

Figure 1. Diagram of the assays of cyanogenic potential, non-glucosidic cyanogens and free cyanide and the rationale of the steps (Source: Essers *et al.*, 1993)

Constraints

The following constraints may be experienced with the enzymic assay described by Cooke (1979) and O'Brien *et al.*, (1991):

- The coloration technique is slow, and the pyridine releases vapors which are toxic, mutagenic, irritating and repulsive.
- The calibration curves obtained for linamarin, acetone cyanohydrin and KCN, with their matching assays, differ. This means that the calculation of glucosides and cyanohydrins by subtraction is not accurate.
- Quantification of existing HCN is not possible, due to its partial vaporization during acid extraction and storage.
- The enzyme, linamarase, used in the assay, is expensive and difficult to obtain.
- Thiocyanate (SCN) is also measured as free cyanide.

The first two constraints have been removed by the improvements in the coloration procedure and the accurate calculation from separate calibrations

1993). Bradbury *et al.* (1991) managed to replace the enzyme by an acid hydrolysis, and although this made the assay less accurate and more complicated, the replacement is relevant when linamarase is not available.

Testing the performance of the new assay

General tests and comparison

The new coloration has been tested extensively and compared with the pyridine/pyrazolone reagent used previously (Essers *et al.*, 1993). The calibration curve slopes of KCN, acetone cyanohydrin and linamarin in the matching assays with the new reagent were comparable and slightly higher. The reproducibility of the new assay was good (CV 2.8%). The pH range of the coloration step was 3 - 6. Changing the assay temperature from 24 to 34°C had no negative effects on color development and stability. Recovery of linamarin added to extracts was 102 ± 4 %, of acetone cyanohydrin, 101 - 108%, and of KCN, 67 - 85%, depending on which of the matching assays was applied. The color reagent was stable for at least 12 days.

Influence of crude enzyme and protein

Since 1992, the method has been successfully applied in laboratories in Wageningen, The Netherlands, in Kampala, Uganda, Zomba, Malawi, and at IITA, Nigeria. However, reduced recoveries have been experienced at CIAT, Colombia (O'Brien, personal communication) when laboratory-made crude linamarase was used instead of the BDH commercial purified enzyme. We therefore compared the use of purified linamarase (BDH, 5.5 EU) and our own laboratory-made crude linamarase (2.2 EU, prepared according to Cooke, 1979) with linamarin (320 μM) in the old and the new assay. As the reduction might have been caused by an excess of non-functional protein in the crude enzyme preparation, we also examined the effect of adding bovine serum albumin (BSA, Sigma A-7906, 2.00 g in 15 mL 0.1 M pH 6 Phosphate buffer). The results in Table 1 show that the high load of bovine serum albumin depressed the linamarin recovery by 20%, in both the old and the new assay.

Table 1. Absorbances from the old and new assay for total cyanogens, after applying different quantities and qualities of linamarase, and addition of bovine serum albumin

Addition to assay				Absorbance			
Linamarase		BSA volume	Linamarin volume	New chromogen		Old chromogen	
vol (mL)	Type	(mL)	(mL)	A_{605}	% of control	A_{630}	% of control
0.1	pure	0.0	0.1	0.814	100	0.547	100
0.1	pure	0.2	0.1	0.586	80	0.441	81
0.3	pure	0.0	0.1	0.858	105	0.577	102
0.3	crude	0.0	0.1	0.847	104	0.625	114

Tripling the amount of pure linamarase gave a slightly higher recovery with the old and the new assay. A tripled quantity of crude enzyme also gave slightly higher recovery with the new assay, similar to the pure enzyme, but it gave a substantially higher recovery with the old assay. The reason for the latter is not known. We were unable to confirm the reduced recoveries of linamarin by crude enzyme in the new assay, as experienced elsewhere. It is recommended that any new crude enzyme preparation be tested at different concentrations.

Artifacts from thiocyanate in cassava

The König reaction is sensitive to both cyanide and thiocyanate (Lundquist *et al.*, 1979). Oke (1969) mentioned that Williams (personal communication) would have found 600 mg SCN per kg of cassava. In order to determine whether thiocyanate from cassava may influence cyanogen levels, the presence of thiocyanate in cassava extracts was measured according to Lundquist *et al.* (1979, 1983), after elimination of the CN^- in 15 extracts. Four extracts were obtained from fresh cassava, four from molded, four from sun-dried, and three from soaked cassava. Thiocyanate was not found in fresh cassava, and only traces (< 2 mg/kg dry weight) were present in 10 cassava products; one sample of molded cassava had 4.5 mg/kg dry weight. It was also found that the enzymic assay of Cooke (1979), which allows up to 5 min chlorination, with the prevailing pH, measured a quarter of an SCN spike. Therefore, thiocyanate in cassava does not normally appear to cause an important artifact in the enzymic analysis of cyanogens, although a slight effect may be found in molded cassava.

Room for further improvements

The enzyme linamarase is, for many laboratories, expensive and difficult to obtain. The replacement of an enzymic degradation of linamarin by an acid degradation is possible, but not always practical for cyanogen measurements in cassava products. Until linamarase is cheaper and more readily available, replacement with a cheap commercial crude enzyme could be beneficial.

Calibration curves for KCN, acetone cyanohydrin and linamarin do not coincide in the old and the new assay. This may result in inaccuracies when calculating levels of the three cyanogens which are mainly present in very low quantities. Essers *et al.* (1993) provided a solution to this problem by calculating the levels of cyanogens from the six matching calibration curves. However, in order to avoid this additional complicated activity, changing the assay conditions to obtain identical calibration curves for the three cyanogens would help provide accurate results. We are currently examining the effects of pH control.

HCN levels in extracts of cassava products were always below 10 mg/kg. The low recoveries of KCN spikes measured immediately after extraction, indicate a substantial loss of HCN from cassava samples.

Automation of the assay

In order to facilitate routine measurement of cyanogens, the assay was automated and works as follows:

A pipette station (Gilson, sampler changer Model 222 with diluter Model 401) is used to transfer appropriate quantities of the extracts and reagents to septum-closed, 3 mL reaction containers within the correct time intervals. After incubation, the mixture is transferred to a spectrophotometer. The readings are fed into a computer where the results are shown directly on the computer screen as a bar chart. Both the sampler and the spectrophotometer are controlled by a computer program. The equipment, unlike the continuous flow system of Technicon (Rao and Hahn, 1984) or Skalar (Meeuwsen et

al., 1989), works batch-wise and imitates the manual method exactly. It can handle up to 36 extracts in one batch, gives similar accuracy and works as fast as a well-trained laboratory technician. The equipment requires careful maintenance. Compared to the continuous flow system it has the advantage that far less enzyme is needed and the extracts are stored in closed containers, preventing the escape of HCN. The apparatus can be programmed to measure total cyanogens, non-glucosidic cyanogens and free cyanide. When not in use for cyanogen assays, the apparatus can be programmed for other chemical analyses.

Assay procedures

Sampling

Cyanogen distribution in cassava plants is very uneven, both between and within roots and leaves (De Bruijn, 1971); this should be taken into account when considering the purpose of the assay. To reduce their bulk, roots can be split lengthwise into halves or quarters, and one segment of each root used without seriously affecting the cyanogen levels of the sample (De Bruijn, 1971). To establish cyanogen levels in a field trial, Bokanga (1994) proposed a procedure consisting of sampling 3 roots per plant, 4 plants per plot in field trials with 3 or 4 replications. A total of 12 or 16 extracts from 36 or 48 roots respectively is obtained. Smaller sample sizes are associated with larger standard errors and weaken inferences on the cyanogenic potential of a cassava variety. For describing and comparing the effect of processing, we suggest to take at least four sub-samples of three roots per batch. Such sampling procedure lead to a coefficient of variation (CV) of leass than 10% in our experiments. Leaf sampling can be standardized by collecting the first fully-opened leaf from the top of each plant plus the following two leaves, and omitting the petioles.

Extraction of cyanogenic compounds

Fresh root or moist products are cut into 1 cm cubes and randomized; 50.0-70.0 g cassava cubes are homogenized in 250 mL refrigerated 0.1 M orthophosphoric acid in a blender for 15 seconds at low speed, followed by 60 seconds at high speed, 60 seconds of rest, and 60 seconds at full speed again. For flour, 4.00 g is swirled gently in 25 mL of refrigerated extraction medium in a 50 mL closed container for 5 min. Leaves are cut into 1 cm2 pieces and 4-10 g homogenized in 250 mL refrigerated acid extraction medium. The homogenates are centrifuged in closed tubes at 4,000 g for 10 minutes and the supernatant used as extract.

Extracts are best analyzed immediately, but if not, they should be stored cooled or frozen. Storage data in O'Brien *et al.* 1991, and Essers *et al.* (1993) suggest that average decrease in cyanogenic potential and in non-glucosidic cyanogens in extracts are less than 5% and 10%, respectively, for up to two months, if stored refrigerated or frozen. The poor stability of HCN in these extracts does not allow for storage if free cyanide is to be measured, or if free cyanide is a substantial part of cyanogenic potential or non-glucosidic cyanogens; this is not usually the case.

Chemicals, solutions and reagents

Linamarin can be obtained from BDH Ltd, Poole, UK and Sigma Chemical Company, St. Louis, USA. Linamarase (EC 3.2.1.21) can be purified from cassava peels (Cooke, 1979) or purchased from BDH Ltd. Isonicotinic acid and 1,3-dimethyl barbituric acid are available from several companies e.g. Fluka Chemie AG, Buchs, Switzerland' Acetone cyanohydrin (99%) from Aldridge Chemical Co., Steinheim,

Germany, Potassium cyanide (KCN) and chloramine T from several companies, e Merck, Darmstadt, Germany. Stock solutions and reagents should be of analytical gr

Standard solutions and the color reagent are prepared as follows:

1. KCN stock (5 mM) is made by dissolving 163 mg dry and pure KCN in 500 mL 0 M NaOH. KCN should be dried at least 12 h over concentrated H2SO4.

2. Linamarin stock (5 mM) is made by dissolving 31.0 mg in 25 mL buffer pH Weighing should be done quickly as the compound is hygroscopic.

3. Acetone cyanohydrin (5 mM) stock is made by first dissolving 570 μL (o.532 g) 25 mL 0.1 M orthophosphoric acid, and from this solution, making 1.00 mL up 50 mL with 0.1 M orthophosphoric acid. Because of the poor performance of pure compound in a pipette, the added quantity should also be weighed for acc calculation of concentration. The refrigerated stocks can be stored for several week to months.

4. Standard solutions of 80 and 320 μM are made from the stock solutions by mak 1.60 mL of them up to, respectively, 100 and 25 mL with 0.1 M orthophosphor acid. This results in 8 and 32 nmol/tube and absorbances of about 0.220 AU 0.880 AU, depending on the spectrophotometer. KCN standards are made just pri to analysis and applied as 0.1 mL to 0.5 mL orthophosphoric acid and 3.4 ml buffer, pH 6, or as 0.1 mL to 3.9 mL buffer, pH 4.

5. Color reagent : Pure NaOH (3.7 g)is dissolved in 200 mL distilled water. Then, 7 g 1,3-dimethyl barbituric acid and 5.7 g isonicotinic acid are dissolved in this alkali solution by extensive stirring. The pH is adjusted between 7 and 8 with 1 M HCl NaOH. This reagent can be kept for at least 12 days at room temperature.

Analytical assay

Cyanogens are assayed in duplicate using the following formats:

1) Cyanogenic potential: 0.1 mL extract is added to 0.4 mL buffer, pH 7, in a test tube, followed by addition of 0.1 mL linamarase solution. After 15 min incubation at 30°C, 0.6 mL NaOH (0.2 M) is added, followed after 5 min (range 2 - 15) by 2.8 mL buffer, pH 6. Coloration as described below.
2) Non-glucosidic cyanogens: 0.1 mL extract is added to 0.6 mL of NaOH (0.2 M). After 2 min (range 1 - 15), 3.3 mL buffer, pH 6, is added, followed by coloration.
3) Free cyanide (HCN): 0.6 mL extract is diluted with 3.4 mL buffer, pH 6, and assayed by colorimetry.

All assays are carried out in glass stoppered test tubes. The contents of the tubes are mixed after each addition.

Colorimetric procedure

Chloramine T reagent (0.1 mL, 2% w/v) is added to the 4 mL buffered extract in the test tube and mixed. After 5 min (range 2 - 30), 0.6 mL of color reagent is added and mixed. The absorbance at 605 nm is measured after 10 min (range 8 - 30). Reagent blanks are run for each analysis. Pigments already present in leaf extracts should be compensated for by measuring extract blanks as well. In a digital single beam spectrophotometer, the absorbance can be measured between 0.050 and 2.000 Absorbance Units (AU). In less sophisticated analog equipment, absorbance can be measured between 0.100 and 1.200 AU with acceptable accuracy

Calibration standards

Calibration curves are established by assaying at least two replicate quantities, with between 8 and 40 nmol of cyanogen per tube. For proximate assays, KCN may be used for calibrations in general, and linamarin for fresh cassava. For precise calculations, standard solutions of linamarin, acetone cyanohydrin and KCN should be measured with all matching assays as described elsewhere (Essers *et al.*, 1993). Calibration curves change slightly from day to day, so it is necessary to include some calibration points in each analytical run for appropriate conversion factors.

Calculation of cyanogen contents

Cyanogen levels are calculated in mg HCN equivalent per kg sample on dry weight basis (mg HCN equivalent kg^{-1}, DWB) as follows:

$$[HCNequivalent] = \frac{x(v + s\frac{m}{100})}{s(1 - \frac{m}{100}) * d} * 0.027$$

where s = sample weight (g)
v = volume extraction medium (mL)
d = volume of extract assayed (mL)
m = moisture content (%)
x = quantity of cyanogen (nmol) in the tube
x is calculated from the calibration curve

$$x = \frac{(A_{605} - a)}{slope}$$

where A_{605} is the absorbance measured at 605 nm. Both *slope* and intercept, *a* are derived by linear regression of the calibration points by means of a calculator. For very low values of *a* (<0.005) and higher values of A_{605} (>0.200) the intercept can be neglected.

Acknowledgments

I thank my colleagues Margaret Boweld, Remco van der Grift and Fons Voragen who participated in the development of the method and Gerry O'Brien for independently testing it. This study was financed by the Netherlands Ministry for Development Cooperation.

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