Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products

Meredith G Bradbury, Sylvia V Egan and J Howard Bradbury*

Division of Botany and Zoology, Australian National University, Canberra ACT 0200, Australia

Abstract: The simple semiquantitative picrate method for the determination of total cyanogens in cassava flour has been modified by increasing the concentration of the picrate solution used to make up the picrate papers, such that a linear Beer's Law relation between absorbance and cyanogen content is obtained over the range 0–800 mg HCN equivalents kg^{-1} cassava. The method has been adapted to determine the total cyanogen content of cassava roots and the results compared using the picrate method and the acid hydrolysis method for six different roots from five cultivars. The agreement between the results is satisfactory. The simple method for determination of total cyanogens in cassava roots in the field is available in kit form. The methodology has been modified to allow determination of the three different forms of cyanogens present in cassava flour, viz HCN/CN^-, acetone cyanohydrin and linamarin. HCN/CN^- is determined by the picrate method in which cassava flour is reacted with 0.1 M sulphuric acid for 3 h at room temperature. HCN/CN^- plus acetone cyanohydrin is also determined by the picrate method after treating cassava flour with 4.2 M guanidine hydrochloride at pH 8 for 3 h at room temperature. A comparison has been made of the amounts of the three cyanogens present in six cassava flour samples using the semiquantitative picrate and the acid hydrolysis methods. The agreement between the two methods is satisfactory, which shows that the new methodology works well. The picrate method for determination of the three cyanogens in cassava flour is also available as a kit.

Keywords: cassava roots; picrate paper; simple kit; cyanogens; linamarin; acetone cyanohydrin; cyanide

INTRODUCTION

Cassava (*Manihot esculenta*) is the third most important food source in the tropics after rice and maize.[1] The cassava plant produces a cyanogenic glucoside, linamarin, and a small amount of lotaustralin (methyl linamarin) as well as an enzyme linamarase. Catalysed by linamarase, linamarin is rapidly hydrolysed to glucose and acetone cyanohydrin and lotaustralin is hydrolysed to a related cyanohydrin and glucose. Under neutral or alkaline conditions, acetone cyanohydrin decomposes to acetone and HCN/CN^-.

There are three forms of cyanogens present in cassava roots and processed cassava products such as cassava flour, (1) linamarin (+methyl linamarin), (2) acetone cyanohydrin, which is fairly stable under acid conditions, and (3) HCN/CN^-. The total cyanogen content or cyanogenic potential of a sample is the total amount of these three cyanogens and is expressed in mg HCN equivalents kg^{-1} fresh weight. On consumption of a mixture of the cyanogens, acetone cyanohydrin would break down rapidly to CN^- under the alkaline conditions of the gut, whereas it is probable that about one half of the linamarin may pass through the body unchanged.[2–4] Thus, HCN/CN^- and acetone cyanohydrin are essentially more dangerous to humans than linamarin when present in comparable amounts.[4]

We have developed a simple semiquantitative picrate paper kit method for the determination of total cyanogens in cassava flour.[5] The method involves the immobilisation of linamarase in a small filter paper disc also loaded with phosphate buffer at pH 8. The disc is placed in a small vial, cassava flour (100 mg) is added and 0.5 ml water. A strip of yellow picrate paper, previously prepared, is inserted and the vial capped. After 16–24 h at 25–35°C the colour of the picrate paper is matched against a colour chart with 10 shades from yellow to brown corresponding to 0–800 mg HCN equivalents kg^{-1} fresh weight. The method has been used effectively in the field in Mozambique.[6] Increased accuracy is achieved in a laboratory situation by elution of the colour from the picrate paper and measurement of the absorbance at 510 nm. There is a linear relationship between

* Correspondence to: J Howard Bradbury, Division on Botany and Zoology, Australian National University, Canberra ACT 0200, Australia.

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absorbance and cyanogen content which follows the Beer–Lambert law up to almost 400 mg HCN equivalents kg^{-1} fresh weight but, above this, there is a curvilinear relation due to the partial saturation of the picrate paper by HCN gas.[5] This results in decreased accuracy in the range 400–800 mg HCN equivalents kg^{-1} fresh weight. In this paper we have modified the method to overcome this problem.

The adaptation of this simple field method developed for cassava flour[5] to cassava roots would be very useful for agronomists and plant breeders worldwide working in the field on cassava. We have modified the simple method so that it can be used with cassava roots.

Various accurate methods of analysis allow the separate determination of linamarin, acetone cyanohydrin and HCN/CN^- [7–10] but these methods are usually too complex to be used in developing countries. Except in the case of the acid hydrolysis method that does not use linamarase,[9] the high cost of commercial linamarase and its difficulty of preparation has also been a deterrent to use of the accurate methods, but we have developed a new simple kit method of preparation of linamarase.[11] We have developed new methods to allow the determination of the three forms of cyanogens present in cassava products.

To summarise, in this paper we describe an improvement to the simple kit method for determination of cyanogens in cassava to increase the accuracy in the range 400–800 mg HCN equivalents kg^{-1} fresh weight, new methodology to allow the separate determination of linamarin, acetone cyanohydrin and HCN/CN^- and the adaptation of the method originally developed for cassava flour to determine total cyanogens in cassava root. These methods are available in an improved kit form.

MATERIALS AND METHODS

Materials

Potassium cyanide, AR (98% minimum) was from Prolabo (Paris, France), acetone cyanohydrin (99%) from Aldrich Chemical Co (Milwaukee, WI, USA) and linamarin (95% minimum) from Sigma Chemical Co (St Louis, MO, USA). Picric acid and linamarase were obtained from BDH Ltd (Poole, UK). Linamarase was also prepared as described in Ref 11. Fresh cassava roots and leaves were obtained from the Plant Culture Facility at the Australian National University. Samples of cassava flour were obtained from Dr Julie Cliff (Mozambique), Dr M Djazuli (Indonesia) and Mr Suharno (University of Melbourne).

Simple picrate kit method to determine cyanogens in cassava flour

The method follows Ref 5. A 100 mg sample of flour was placed on top of a 21 mm diameter Whatman 3MM filter paper in a flat-bottomed plastic vial. The filter paper had been previously loaded with 50 µl of 1 M phosphate buffer at pH 8 and, after air drying, 60 µl of a linamarase solution which also contained 1% (w/v) gelatin and 5% (w/v) polyvinylpyrrolidone-10 was added and this was allowed to air dry.[5] Water (0.5 ml) was added and a yellow picrate paper immediately inserted in the vial. The picrate paper was prepared beforehand by dipping a sheet of Whatman 3MM filter paper in a picrate solution, allowing the paper to air dry and cutting it into 3 cm × 1 cm strips. These were glued using a drop of PVA hobby glue to 5 cm × 1 cm clear plastic strips to keep the paper clear of the liquid.[5] The vial was closed immediately with a screw lid and allowed to stand at 30°C for 16–24 h. The colour of the picrate paper was compared with that of a colour chart which contained 10 colours and the cyanogen content read directly from the chart. The picrate paper was separated from the plastic strip and the colour eluted from the filter paper in 5.0 ml water for about 30 min. The absorbance of the solution was measured at 510 nm against a blank which contained a yellow solution produced from a picrate paper not exposed to HCN. The cyanogen content was read off a calibration curve.[5]

Quantitative determination of different cyanogens

The determination of linamarin, acetone cyanohydrin and HCN/CN^- was by the acid hydrolysis method.[8,9]

Improvements in preparation of picrate papers and in the calibration curve

Picrate solutions of various concentrations shown in Table 1 were prepared by weighing out moist picric acid which was dissolved with stirring and warming in a 2.5% (w/v) sodium carbonate solution and made up to volume with the sodium carbonate solution in a standard flask. The absorbance of the alkaline picrate solutions were measured at the absorption maximum (358 nm) after quantitative dilution of the picrate

Table 1. Properties of picrate solutions in 2.5% sodium carbonate

Solution number	*Concentration (%, w/v) of moist picric acid*	*A_{358} of picrate solutions (1 : 500 in water)*
1[a]	0.50[b]	0.582
2	0.50[b]	0.570
3	0.73	0.543
4	1.12	0.831
5	1.28	0.974
6	1.45	1.144
7	1.60	1.241

[a] Solution used in Ref 5.

[b] In these cases the moist picric acid was blotted between filter papers to remove much of the surface water before weighing the picric acid. However, this method is not recommended because it may be a hazard to safety.

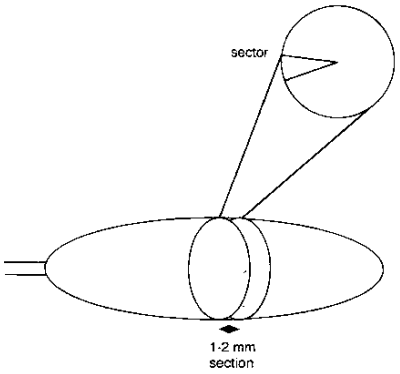

Figure 1. Sketch of a cassava root to show the method by which 1–2 mm thick 100 mg sectors were cut from it.

solutions in water by a factor of 500. Following the procedure given above, Whatman 3MM filter paper was immersed in the various original picrate solutions and the yellow papers allowed to air dry. They were then cut and glued to a plastic strip as described above. In this way picrate papers were prepared which were loaded with different amounts of picrate.

Two standard cyanide solutions were prepared from KCN dissolved in 0.1 M NaOH and made up to the mark in 25 ml standard flasks using 0.1 M NaOH. The cyanide contents were 10 μg and 1 μg HCN equivalents per μl, respectively. Such solutions of KCN in NaOH are stable for at least 6 months at 4°C.[9] Known amounts of the standard cyanide solutions were transferred using a Pipetman micro pipette into small flat bottomed plastic vials followed by 50 μl 1.0 M phosphate buffer at pH 8 and 0.5 ml water. Picrate papers were immediately added to the vials which were then sealed by screw caps. The vials were held at 30°C for 16–24 h. Picrate papers were carefully removed from the plastic strips and immersed in 5.0 ml water for about 30 min. Each absorbance was measured at 510 nm against a control solution prepared from an identical yellow picrate paper which had not been exposed to HCN gas. In this way a set of curves were prepared using picrate papers containing different amounts of picrate.

In the development of a calibration curve to replace that given in Ref 5 a solution of 45.8 mg linamarin in 5.0 ml water was prepared. Amounts of linamarin corresponding to 0, 1, 10, 20, 40, 60 and 80 μg HCN equivalents were delivered using a Pipetman micro-pipette into small plastic vials followed by a 21 mm diameter. Whatman 3MM paper loaded with phosphate buffer at pH 8 and linamarase. Water (0.5 ml) was added, picrate papers were inserted into the vials, which were immediately closed, and kept at 30°C for 16–24 h. The picrate papers were separated from the plastic strips and the former immersed in 5.0 ml water for about 30 min. Absorbances of the solutions were measured at 510 nm. The experiments were carried out in duplicate.

Modification of method to determine separately HCN/CN^-, acetone cyanohydrin plus HCN/CN^- and total cyanogens

In order to determine HCN/CN^- only, it was necessary to inactivate endogenous linamarase to prevent the breakdown of linamarin, which is normally carried out in the accurate methods by extraction of cassava roots or cassava flour with 0.1 M phosphoric acid. The use of phosphoric acid would probably also limit any breakdown of acetone cyanohydrin to cyanide.[9] Accordingly, amounts of KCN, acetone cyanohydrin and linamarin/linamarase corresponding to 40 μg HCN equivalents were dissolved in 0.5 ml of (1) 0.01 to 4 M sulphuric acid or phosphoric acid, or (2) phosphate buffer at pH 8, in a small vial, a picrate paper was added and the vial immediately capped. After different time intervals at 30°C, the picrate paper was separated from the plastic strip, eluted with 5.0 ml water and the absorbance measured at 510 nm.

To determine acetone cyanohydrin plus HCN/CN^-, it was necessary to inactivate any linamarase whilst not reacting with the acetone cyanohydrin or HCN/CN^- present in the sample. Various methods were tried to inactivate the enzyme including inhibitors, change of pH and protein denaturants. Linamarase (0.02–0.05 U), either present in a filter paper disc or in liquid form, was added to 0.5 ml solution in a plastic vial, which contained the inhibitor or denaturant and 40 μg HCN equivalents of linamarin. The pH was usually maintained at 8 with phosphate buffer, but, in one set of experiments, carbonate buffers were used with pH values of 9, 10, 11 and 12. A picrate paper was added to the plastic vial which was sealed with a screw cap and incubated at 30°C for times up to 16–24 h. The picrate paper was separated from the plastic strip, eluted with 5.0 ml water and the absorbance measured at 510 nm against a control solution prepared by eluting in 5.0 ml water a picrate paper which had not been exposed to HCN gas.

Adaptation of method developed for cassava flour to cassava roots

The cyanogen content of the parenchymal tissue of cassava roots is very variable between roots from the one plant, between roots from different plants of the same cultivar, between the same cv. in different environments and between different cultivars.[7,9,12] There is variability within the one root usually with a small longitudinal gradient of cyanogen content from the proximal (stem end) of the root to the distal end. There is usually a larger radial gradient with much more at the periphery of the parenchyma, next to the cortex or peel, than at the centre of the root.[7,9]

Following previous work[7,9,13] a representative sample of the root was obtained by cutting a 1–2 mm thick section across the root about halfway along its length. After removal of the peel, 100 mg sectors were cut from this disc (see Fig 1) and six adjacent 100 mg sectors were analysed using the method described above for analysis of cassava flour which utilises exogenous linamarase. Since cassava roots always contain endogenous linamarase, comparative experiments were carried out with eight different cultivars using five 100 mg sectors with and five adjacent sectors without exogenous linamarase. A thicker transverse section was cut adjacent to the thin disc and the material in the thicker section (20–30 g) was used for the cyanogen content using the acid hydrolysis method. Duplicate analyses were made.

RESULTS AND DISCUSSION

Improvements to the simple kit method of Ref 5

In earlier work we found more variability in the non-linear part of the calibration graph of absorbance at 510 nm vs cyanogen content in mg HCN equivalents kg^{-1} fresh weight, than in the linear part of the graph (Fig 4 of Ref 5). This variability was particularly evident if different 0.5% (w/v) moist picric acid solutions were used to make up the picrate papers. We believed that this was due to variablities in the actual amount of picric acid because of variable amounts of water present. Since picric acid may explode when dry, it is not safe to handle it otherwise.

Previous work had shown that the linear part of the curve was extended from about 20 μg HCN equivalents with Whatman No 1 paper to about 40 μg HCN equivalents with Whatman 3MM paper (Fig 1 of Ref 5), due to the approximate two-fold increase in capacity of the 3MM compared with the No 1 paper, which is only one half the thickness of 3MM paper. It might therefore be expected that the linear section of the curve would be extended even further by increasing the concentration of the picric acid used.

A number of solutions of various concentrations of moist picric acid ranging from 0.5 to 1.6 g picric acid $100\,ml^{-1}$ solution (% (w/v)) were prepared in 2.5% sodium carbonate and the absorbances at the absorption maximum (358 nm) of 1 in 500 dilutions using distilled water were determined. The results of these measurements are shown in Table 1.

Solutions 1 and 2 were prepared by weighing out picric acid that had been blotted between filter papers to remove most of the surface water before weighing. This may have improved the reproducibility of preparation of picrate solutions, but was discontinued because of the possibility of an explosion. The other solutions, 3–7, were all prepared using moist picric acid straight from the bottle from which excess water had been removed. As expected, there is a linear relationship ($r = 0.999$) between the concentrations of picric acid (%, w/v) obtained for solutions 3–7 from weighing and their absorbances.

Picric acid papers were made from solutions 2, 4, 5, 6 and 7 and these were added to vials containing 0.5 ml 0.1 M phosphate buffer at pH 8 and known amounts of KCN corresponding to 0, 1, 10, 20, 40, 60 and 80 μg HCN equivalents (see above). Experiments were carried out in duplicate and the results are shown in Fig 2. All picrate solutions give the same results up to 20 μg HCN equivalents but, at 40 μg HCN equivalents, solution number 2 gives a slightly lower result than the average of the other solutions. The deviation between solution 2 and solutions 4–7 increases progressively as cyanogen content increases as shown in Fig 2. It should be noted that solution 2 is of the same concentration as solution 1 used in Ref 5, see Table 1, and both give similar graphs (compare Figs 2 and 4 of Ref 5).

Solutions 4–7 give results which are all the same within experimental error and these have been averaged at each level and plotted as one graph in Fig 2. This is a straight line with $r = 0.999$, described by the following equation:

$$\text{cyanogen content (μg HCN equivalents)} = 40.0 \times \text{absorbance} \quad (1)$$

As shown by the results in Table 1 the picric acid solution may be prepared by making a moist picrate solution of concentration 1.12–1.60% (w/v). In practice it would be best to aim for the middle of the range (about 1.4%, w/v) and to check that the absorbance of the resultant diluted picrate solution was around 1.0 (see Table 1).

The straight line graph in Fig 2 was obtained using KCN as a standard. We have also obtained a corresponding line using linamarin as standard following the method described above. The graphs of the two sets of data are shown in Fig 3. The lina-

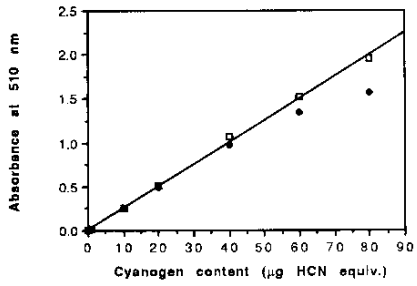

Figure 2. Graphs of absorbance at 510 nm vs cyanogen content (μg HCN equivalents) for picric acid papers made from solution 2, Table 1 (◆) and the mean of results from solutions 4–7, Table 1 (□).

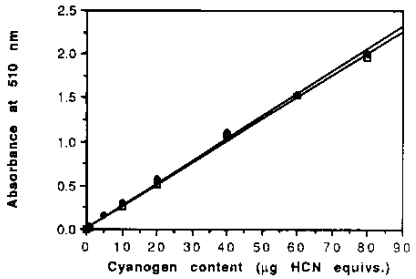

Figure 3. Graphs of absorbance at 510 nm vs cyanogen content (µg HCN equivalents) for the mean of results from picric acid solutions 4–7, Table 1 using KCN as standard (◆) and the results using linamarin as standard (□).

marin line is fitted by the equation

$$\text{cyanogen content (µg HCN equivalents} = 39.1 \times \text{absorbance} \quad (2)$$

with $r = 0.998$. These represent better calibration curves, especially in the range 40–80 µg HCN equivalents, than that used in our earlier work (Fig 4 of Ref 5). The best calibration line is obtained by averaging the two lines represented by eqns (1) and (2) to give eqn (3), namely

$$\text{cyanogen content (µg HCN equivalents)} = 39.6 \times \text{absorbance} \quad (3)$$

Since, in the kit method, only 0.1 g of cassava root or flour is weighed out, the observed cyanogen content is in µg HCN equivalents/0.1 g cassava = 39.6 × absorbance, which on conversion to µg HCN equivalents/g cassava product gives the final equation

$$\text{cyanogen content (µg HCN equivalents/1.0 g cassava)} = 396 \times \text{absorbance} \quad (4)$$

In earlier work we found that yellow picrate papers gradually darkened when stored in the dark at room temperature (see Fig 2 of Ref 5) and we recommended that they should not be stored for longer than one month. We have now found that these papers are stable for four months if kept in the refrigerator at 2–4°C or the deep freeze. The time study is still continuing.

Modifications to determine separately HCN/CN^-, acetone cyanohydrin plus HCN/CN^- and total cyanogens and comparison of results using different methods on cassava flour

In the previous study we developed a simple method to determine total cyanogens in cassava flour,[5] which involves addition of exogenous linamarase to ensure complete hydrolysis of linamarin to acetone cyanohydrin. The reaction is buffered at pH 8 to ensure complete hydrolysis of acetone cyanohydrin to HCN/CN^- and quantitative liberation of HCN gas, which reacts with the picrate paper. As already indicated, it is important to be able to determine the different cyanogens separately, namely, HCN/CN^-, acetone cyanohydrin and linamarin, in cassava products. This is normally carried out by separate determinations for HCN/CN^-, acetone cyanohydrin plus HCN/CN^- and total cyanogens, from which the amounts of acetone cyanohydrin and linamarin are obtained by subtraction from the data. We have also used this approach.

Determination of HCN/CN^-

To determine only HCN/CN^- it was necessary to prevent the breakdown of linamarin by inactivating any linamarase present and also the decomposition of acetone cyanohydrin, whilst allowing the liberation of HCN gas from any cyanide salts present in the substrate. It was found that over 16 h at room temperature there was no liberation of HCN from 40 µg HCN equivalents of linamarin in the presence of linamarase in 0.5 M phosphoric acid. Under these conditions there was considerable decomposition of acetone cyanohydrin to HCN. A time series showed that there was complete liberation of HCN from 40 µg HCN equivalents of KCN in 3 h in acid solution and also in pH 8 buffer. Thus it was possible to reduce the reaction time from 16 to 3 h.

The breakdown of 40 µg HCN equivalents of acetone cyanohydrin was then studied in 0.5 M phosphoric acid at different times up to 5 h. It was found that the liberation of HCN from acetone cyanohydrin followed a linear line with time and amounted to 0.5 µg HCN equivalents (1.3%) after 3 h. In order to optimise the acid conditions, experiments were made of the breakdown in 4 h of 40 µg HCN equivalents of acetone cyanohydrin in 0.01–0.5 M phosphoric acid or sulphuric acid. It was found that sulphuric acid appeared to be slightly better at preventing the breakdown of acetone cyanohydrin than phosphoric acid at the same concentration and the best results were obtained with 0.1 M sulphuric acid. The final conditions chosen for determination of HCN/CN^- were 0.1 M sulphuric acid for 3 h at room temperature.

Determination of HCN/CN^- plus acetone cyanohydrin

An inhibitor of linamarase, D-gluconic acid lactone (Sigma Chemical Co) was initially tried. No HCN was liberated from 40 µg HCN equivalents of linamarin/linamarase at pH 8 in presence of 0.5 M D-gluconic acid lactone, which showed that the enzyme was effectively inhibited. Unfortunately, the D-gluconic acid lactone was found to react with acetone cyanohydrin and hence this approach was abandoned. Reaction of 40 µg HCN equivalents of linamarin in carbonate buffer solutions of pH 9, 10, 11,

12 containing linamarase showed a progressive decrease in the amount of HCN liberated but, even at pH 12, about 4% HCN was produced. Furthermore, control experiments using 40 µg HCN equivalents of acetone cyanohydrin or KCN showed that the liberation of HCN over 16 h was about 18% lower at pH 12 than at pH 8. Experiments at high pH were thus not useful. Linamarase was not inactivated in solutions containing 10% sodium dodecyl sulphate or 13% ethylene diaminetetraacetic acid and 8 M urea reduced the evolution of HCN from linamarin/linamarase to about 10% of the expected amount, but did not eliminate it altogether.

A 6 M solution of guanidine hydrochloride (Fluka, Switzerland, puriss) was effective in preventing the evolution of HCN from 40 µg HCN equivalents of linamarin in the presence of linamarase over 16 h at pH 8. Control experiments with acetone cyanohydrin and KCN showed that they were unaffected in their liberation of HCN by the presence of 6 M guanidine hydrochloride, hence the latter was not involved in any side reactions. The breakdown of 40 µg HCN equivalents of acetone cyanohydrin to HCN in pH 8 phosphate buffer was followed from 0.5 to 4 h and was found to be complete after 2–3 h. Thus the time for this experiment could be reduced from 16 to 3 h.

Preliminary experiments had shown that 3 M guanidine hydrochloride was insufficient to prevent the liberation of some HCN from linamarin/linamarase at pH 8 over 16 h. Accordingly an experiment was set up with cassava flour which contained endogenous linamarase and had an acetone cyanohydrin plus HCN/CN^- content of 4 mg HCN equivalents kg^{-1} and a total cyanogen content of 95 mg HCN equivalents kg^{-1} (sample 1, Table 2). Amounts of guanidine hydrochloride of 200, 250 and 287 mg were added to 0.5 ml of pH 8 buffer which corresponded to 4.2 M, 5.2 M and 6 M guanidine hydrochloride, respectively. The cassava flour (100 mg) was added, a picrate paper, the vials closed and kept at 30°C for 2, 4 and 18 h. The absorbances of the aqueous solutions from the picrate papers were measured as described above. There was no significant difference between any of the duplicate results, which gave a mean value of 4.3 mg HCN equivalents kg^{-1} and standard deviation of 1.7. This result was in good agreement with that obtained by acid hydrolysis (see Table 2). It showed that 200 mg of guanidine hydrochloride (4.2 M) was sufficient to inactivate the linamarase and a time of 2–3 h was sufficient time for the liberation of HCN from acetone cyanohydrin.

Comparison of results for cassava flour by simple picrate and acid hydrolysis methods

The results of a comparison of the picrate method and the acid hydrolysis method for determination of the three different cyanogens in cassava flour samples is shown in Table 2. The agreement between two methods is satisfactory, particularly when one considers the appreciable errors which are inherent in the measurement of small absorbances using both methods at low cyanogen values (< 10 mg HCN equiv kg^{-1}). These results show that the methodology developed for determination of HCN/CN^- and HCN/CN^- plus acetone cyanohydrin described above is satisfactory.

Table 2. Comparison of the amounts (mg HCN equivalents kg^{-1} cassava flour) of the three different cyanogens present in cassava flour samples determined by the picrate method and acid hydrolysis[a]

Sample number[b]	*Cyanogen content in mg HCN equivalents kg^{-1} cassava flour*		
	HCN/CN^-	*Acetone cyanohydrin*[c]	*Linamarin*[c]
1	0.4 (1.6)	2.7 (2.1)	87 (91)
2	0.8 (1.6)	5.3 (7.6)	68 (62)
3	1.8 (3.4)	6.9 (7.9)	19 (28)
4	0.6 (2.2)	0.4 (0)	24 (34)
5	0.4 (0.9)	2.8 (3.5)	12 (8.5)
6	0.4 (0.8)	3.0 (4.2)	4.5 (2.9)

[a] The acid hydrolysis results (from Table 3 of Ref 5) are given in parentheses after the corresponding result, obtained from duplicate analyses by the picrate method.
[b] Samples 1, 2, 3, 4, 5, 6 correspond to samples 19, 23, 24, 20, 30 and 31, respectively, from Table 3 of Ref 5. The picrate analyses were carried out in duplicate.
[c] Total cyanogens in the cassava flour were determined in duplicate using the method of Ref 5, but using a stronger picrate solution for preparation of picrate papers (see above) and eqn (4) to calculate the cyanogen content. The content of linamarin and acetone cyanohydrin was calculated using eqns (5) and (6), respectively.

Adaptation of method developed for cassava flour to cassava roots

The methodology used for cassava flour involved the weighing out (using a small portable plastic balance) of 100 mg of finely divided flour. This was shown to be a representative sample of the flour by absorbance measurements using the semiquantitative picrate method made on repeated samples taken from the same batch of flour. For two different samples of flour, 6 lots of 100 mg were analysed and the results were (1) 91 ppm (SD 10.6), coefficient of variation (CV) = SD × 100/mean = 12% and (2) 190 ppm (SD 9.6), CV = 5%.

In applying this method to cassava roots, a sampling procedure was needed which would give a result which was representative of the cyanogen content of the root. As described above, the methodology involved cutting a wedge-shaped sector as shown in Fig 1. In order to check whether variations in thickness of the sectors caused variability in the results, an experiment was conducted in which a transverse section of thickness 4–5 mm of a root was cut. After removal of the peel, an annular shaped ring about 5 mm thick was cut from the transverse

section. The doughnut-shaped ring was then cut into small cubes, each of which weighed 100 mg. Adjacent cubes were analysed using the picrate method with every alternate cube being cut into slivers of 1–2 mm thickness, before being set up for analysis. There were 19 analyses of cubes of thickness about 5 mm and 19 analyses of adjacent cubes cut into about 1 mm thick slivers before analysis. In general, the 5 mm cubes gave a lower result than the adjacent 1–2 mm thick slivers. A paired t-test showed that the difference was significant ($P = 0.05$). The lower result for the thicker samples is probably due to incomplete breakdown of the linamarin in the sample, presumably because of the inability of the linamarase to penetrate fully the substrate, which results in incomplete hydrolysis of the linamarin. The general agreement between the results of the picrate and acid hydrolysis methods in Table 3 shows that sectors of 1–2 mm thickness allow complete reaction of linamarin.

To check whether there was any loss of cyanogen during the time period needed to cut a 100 mg sector of parenchyma tissue for analysis, an experiment was made in which adjacent 100 mg sectors were cut and left in the air for 0, 1 and 2 h before being set up for analysis. Seven adjacent sets of sectors were prepared, 21 samples altogether. Examination of the results showed no consistent pattern of behaviour but certainly no drop in cyanogen content with time as would have occurred if there had been appreciable breakdown of linamarin to HCN over 2 h. Nevertheless, we would recommend that, once the sectors are cut, they should be set up for analysis without delay.

In order to check whether additional (exogenous) linamarase is necessary over and above what is already present in cassava roots, comparative experiments were carried out using eight different cassava cultivars. Ten adjacent 100 mg sectors were cut from each cultivar and alternate sectors were analysed in the absence and presence of exogenous linamarase. There was no significant difference between the mean values obtained in the presence and absence of added linamarase, which shows that it is not necessary to add additional linamarase in the analysis of cassava roots.

Table 3. Comparison of cyanogen contents of cassava roots determined by semiquantitative picrate and acid hydrolysis methods

Cultivar	*Cyanogen content mg HCN equivalents kg^{-1} fresh wt using*	
	Semiquantitative picrate method[a]	*Acid hydrolysis method*[b]
TMS 4(2)1425,rootA	67 (39)	73
TMS 4(2)1425,rootB	39 (38)	36
TMS 50395	41 (37)	58
TMS 63397	47 (64)	51
TMS 91934	16 (94)	17
TMS 60506	5 (60)	11

[a] Mean and coefficient of variation in brackets of 6 results obtained from 1–2 mm thick, 100 mg adjacent sectors of cassava roots.
[b] Mean of duplicate analyses.

The variability of the results obtained by this method from 6 adjacent sectors taken over 6 different roots are given as CV values in Table 3. These CV values range from 37 to 94% (mean value 55%) and are clearly much higher than the average value of 9% obtained with two samples of cassava flour (see above). The reason for this is the considerable variability in the cyanogen content, particularly across the diameter of the cassava root,[7,9] and it is recommended that three sectors be analysed for each root studied.

There is good agreement between the results of the accurate acid hydrolysis method and the semiquantitative picrate method for 4 of the entries in Table 3, but the result for TMS 50395 is about one standard deviation lower than the corresponding acid hydrolysis result. The root with the lowest amount of cyanogen (11 mg HCN equivalents kg^{-1}) gave poorer agreement between the two methods, because of the larger experimental errors that occur as the limit of sensitivity of both methods is approached.[5]

Preparation of a new simple kit to determine separately linamarin, acetone cyanohydrin and HCN/CN$^-$ in cassava roots and cassava products

Total cyanogens

The method involved either (1) weighing out 100 mg of finely ground cassava flour or (2) cutting a sector of cassava root as shown in Fig 1 and adjusting its weight to 100 mg using the balance. (1) A cassava flour sample was placed in a small plastic vial on top of a 21 mm diam. Whatman 3MM filter paper disc (which had been previously loaded with phosphate buffer at pH 8 and linamarase[5]). (2) A cassava root sector was placed in a small plastic vial on top of a filter paper disc previously loaded with phosphate buffer but not with linamarase. Water (0.5 ml) was added and then a picric acid paper. The picric acid paper was prepared beforehand by dipping Whatman 3MM filter paper in a solution made by dissolving 1.4 g of picric acid in 100 ml of 2.5% (w/v) sodium carbonate. The paper was allowed to air dry and was then cut to size and attached to a plastic strip as described previously. The vial was immediately closed and allowed to stand for 16–24 h at room temperature (25–37°C). The bottle was opened and the yellow to brown colour of the picric acid paper compared with a colour chart with 10 shades of colour corresponding to 0–800 mg HCN equivalents kg^{-1} cassava.

In order to obtain a more accurate result, particularly at low concentrations (<10 mg HCN equiva-

lents kg^{-1}), the picrate paper was separated from the plastic strip (which may be used again after washing) and the paper was immersed in 5.0 ml of water for about 30 min at room temperature. The absorbance of the solution at 510 mm was measured against a blank obtained by immersing a picrate paper not exposed to HCN in 5.0 ml of water for 30 min. The cyanogen content was calculated using eqn (4), see above. In each series of measurements it was essential to run a check analysis, which consisted of a square Whatman 3MM paper loaded with a known amount of linamarin such as 5 µg or 40 µg HCN equivalents of linamarin which equals 50 and 400 mg HCN equivalents kg^{-1} cassava, respectively.

Acetone cyanohydrin plus HCN/CN⁻

Since the amount of acetone cyanohydrin plus HCN/CN^- is small in cassava roots but may be considerable in cassava flour, it is unlikely that this methodology would need to be used for cassava roots. A 100 mg sample of cassava flour was weighed out and placed on top of a 21 mm diam. Whatman 3MM paper containing phosphate buffer at pH 8 (without linamarase). Guanidine hydrochloride (200 mg) was added followed by 0.5 ml water. A picrate paper was immediately placed in the vial which was closed by a screw cap lid. After 3 h the vial was opened, the colour of the picrate paper compared with that of the colour chart and the cyanogen content recorded in mg HCN equivalents kg^{-1} cassava. Because of the likelihood of the result being in the low range (<10 HCN equivalents kg^{-1}), in which the usefulness of the colour chart is greatly reduced, it is recommended that the colour be eluted from the paper in 5.0 ml of water, the absorbance measured at 510 nm and the cyanogen content calculated by eqn (4).

HCN/CN^-

A 21 mm diameter Whatman 3MM filter paper (previously loaded with 50 µl of 1.0 M sulphuric acid and allowed to air dry), was placed in a vial and 100 mg of cassava flour was added. Water (0.5 ml) was added and a picrate paper was immediately placed in the vial which was closed by the screw lid. After 3 h at room temperature the picrate paper was removed from the vial. Comparison with the colour chart was not useful because these results are usually <5 HCN equivalents kg^{-1}. The plastic strip was separated from the picrate paper, which was immersed in 5.0 ml water. After 30 min the absorbance of the solution was measured at 510 nm against a blank as described above. The amount of cyanogen was determined using eqn (4).

The amount of linamarin and acetone cyanohydrin was calculated by the following equations:

$$\text{linamarin content} = \text{total cyanogens} - (\text{acetone cyanohydrin} + HCN/CN^-) \quad (5)$$

$$\text{acetone cyanohydrin} = (\text{acetone cyanohydrin} + HCN/CN^-) - (HCN/CN^-) \quad (6)$$

CONCLUSION

The simple semiquantitative kit picrate method of Ref 5 has been improved by using a more concentrated picrate solution than formerly to produce a linear Beer's Law dependence between absorbance and total cyanogen content over the full range from 0 to 800 mg HCN equivalents kg^{-1} cassava. The simple methodology, originally developed for cassava flour has been adapted for use with cassava roots and it is hoped that this may be useful for agriculturalists working in the field. The kit is essentially as described previously, but with picrate papers that contain more picrate than formerly. It is simple, compact and requires only 0.5 ml of water for each analysis. The yellow picrate papers darken after 1 month at room temperature but remain stable for at least three months if stored in the refrigerator.

We have also introduced new methodology to determine the three forms of cyanogens present in cassava products by measuring

(1) HCN/CN^- using 0.1 M sulphuric acid, which inactivates linamarase and minimises the breakdown of acetone cyanohydrin,
(2) acetone cyanohydrin plus HCN/CN^-, using 4.2 M guanidine hydrochloride at pH 8 which inactivates linamarase.

These methods should be useful for cassava products to assess the amounts of acetone cyanohydrin and HCN/CN^- present, since these compounds are completely broken down to cyanide in the human gut, whereas only about 50% of linamarin may be absorbed by the body.[2–4] Simple kits are now available for the determination of total cyanogens in cassava roots and also for the determination of the three different forms of cyanogens in cassava flour and other cassava products.

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