Analytical Methods

Rapid wetting method to reduce cyanogen content of cassava flour

J. Howard Bradbury *, Ian C. Denton

Botany and Zoology, Research School of Biology, Australian National University, Canberra ACT 0200, Australia

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In 2005 a simple wetting method was developed that reduced total cyanide content of cassava flour 3–6-fold. The method involved spreading wet flour in a thin layer and standing in the shade for five hours to allow evolution of HCN gas. We found that breakdown of linamarin catalysed by linamarase to acetone cyanohydrin, followed by its spontaneous decomposition to HCN and acetone was greatly increased by standing the wet flour in the sun. Treatment for two hours in the sun gave the same amount of total cyanide remaining as five hours in the shade. This rapid treatment in the sun may be more acceptable to rural women in Democratic Republic of Congo, than five hours in the shade. The two methods are offered as alternatives for use in rural Africa. With adequate linamarase present the residual cyanogen remaining after the wetting treatment was acetone cyanohydrin.

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1. Introduction

Cassava is the third most important food source in the tropics after rice and maize and its production is rapidly increasing, particularly in sub-Saharan Africa, to feed rapidly increasing populations (Nhassico, Muquingue, Cliff, Cumbana, & Bradbury, 2008). Cassava is easy to grow from stem cuttings, yields well even in poor soils in the absence of fertilizer, is drought resistant and the roots can be used as a reserve source of food. Cassava produces two cyanogenic glucosides, linamarin and a small amount of lotaustralin (methyl linamarin), and an enzyme linamarase that catalyses their breakdown to glucose and cyanohydrins. The cyanohydrins are decomposed spontaneously above pH 5 producing hydrogen cyanide (HCN) and a ketone.

Harvested cassava roots deteriorate in air in a few days at ambient temperatures and therefore cassava must be converted into stable products such as flour or gari for storage (Bradbury & Holloway, 1988; Cardoso, Mirione, Ernesto, Massaza, Cliff, et al., 2005). Cassava flour and to a lesser extent gari stored under ambient conditions retain cyanogens over long periods (Bradbury, 2006). However, if flour is mixed with water and the wet flour left in a thin layer for 5 h in the shade at about 30 °C to allow HCN gas to escape, the total cyanide content is reduced 3–6-fold (Bradbury, 2006; Cumbana, Mirione, Cliff, & Bradbury, 2007). The water present swells the flour and allows linamarase to catalyse hydrolysis of linamarin to acetone cyanohydrin which spontaneously hydrolyses at the pH of the flour (ca. 6.5) to give HCN and acetone. This is the basis of the simple wetting method that was successfully field tested by rural women in Mozambique (Muquingue, Nhassico, Cliff, Sitoe, Tonela, et al., 2005; Nhassico et al., 2008). Laminated posters that describe the simple method are available for free in three European and six African languages (<http://online.anu.edu.au/BoZo/CCDN/>).

The wetting method has been introduced into a number of villages around Uvira in South Kivu Province of Democratic Republic of Congo (DRC) where cyanide poisoning and konzo occurs (Karumba, Denton, and Bradbury, 2009). Konzo is an irreversible paralysis of the legs that occurs mainly in children and young women (Howlett, Brubaker, Mlingi, & Rosling, 1990; Ministry of Health, 1984), due to cyanide from bitter cassava. Although the wetting method requires no additional equipment or work, yet it has not proved to be popular with rural women in the villages because of the long period of five hours required for treatment and the need for surveillance of the wet flour over that period, to prevent stealing or spoilage by animals or children. In this paper we show that the time of treatment can be reduced from five hours in the shade to two hours in the sun.

2. Materials and methods

2.1. Materials

Cassava flour samples were obtained from Mozambique and Indonesia and also prepared from cassava grown in the Plant Culture Facility at the Australian National University. Some of the samples have been stored at −20 °C for years with no observable
2.2. Methods

2.2.1. Loss of cyanide from flour on heating at 30 °C and 50 °C

Cassava flour (2 g) was mixed with 2.5 g water in a 100 mL beaker using the same proportion of flour to water (1:1.25) as before (Bradbury, 2006). Duplicate 100 mg samples of wet flour were removed for total cyanide analysis using the semi-micro picrate method, see Section 2.2.2 (Bradbury, 2009), and the beaker weighed. The wet flour was heated for 1, 2 or 5 h at either 30 °C or 50 °C in an oven. After the heating period the beaker was weighed and water added with mixing to replace that lost by evaporation. Duplicate 100 mg samples of wet flour were removed for total cyanide analysis.

2.2.2. Analysis for total cyanide of flour samples

Duplicate 100 mg samples of wet flour were weighed into plastic bottles, a small linamarase/buffer paper was added and 1 mL of 1 M (pH 6.8) phosphate buffer. A 1 cm sensitive picrate paper was added, the bottle closed with a screw lid and left for 16 h at 30 °C. The sensitive picrate paper was removed from its plastic support, the colour eluted with 0.50 mL water for 30 min and the absorbance measured at 510 nm against a blank, using 2 mm wide cuvettes in a Beckman DU 540 recording spectrophotometer. The absorbance was converted to ppm using the equation (Bradbury, 2009).

\[
\text{ppm} = A \times 45.7.
\]

In all cases there was careful water replacement with mixing at the end of the heating period, followed by analysis for total cyanide. The % total cyanide remaining after a treatment was calculated by the equation

\[
\% \text{ total cyanide remaining} = \frac{(\text{ppm cyanide at } t = X) \times 100}{(\text{ppm cyanide at } t = 0)},
\]

where \( t \) = time in h and \( X \) = time of treatment (h) at 30 °C or 50 °C or in the sun.

2.2.3. Loss of cyanide from flour with added linamarase at 30 °C or 50 °C

A 2 g sample of flour from Indonesia (Bogor A) which contained only a very small amount of linamarase (Bradbury, 2006) was mixed with 2.5 mL of water to which different amounts of linamarase had been added from a linamarase stock solution of about 1.6 EU/mL, prepared from BDH lyophilised linamarase. Duplicate 100 mg Bogor A samples were removed for analysis at zero time, and the wet flour heated at 30 °C or 50 °C for the requisite time. The water lost by evaporation was replaced, the flour mixed and duplicate 100 mg samples taken for total cyanide analysis.

In one experiment acetone cyanohydrin was determined by addition of 0.5 mL 0.1 M HCl to duplicate 100 mg samples. A sensitive picrate paper was added to each vial which was closed with a lid and heated at 30 °C for 1 h to denature linamarase. The acid was neutralised by addition of 1 mL of 1 M phosphate buffer at pH 6.8 (Bradbury, 2009). The vials were closed and treated at 30 °C for 16 h after which the sensitive picrate paper was removed and the colour eluted and measured as above. The % total cyanide remaining after the various treatments at different temperatures was calculated, see Section 2.2.2.

2.2.4. Loss of cyanide from flour on exposure to sun

Cassava flour (5 g) was mixed with 6.25 mL water in a weighed, glass Petrie dish containing an iButton to monitor temperature. Duplicate 100 mg samples of wet flour were removed for analysis (Section 2.2.2) at zero time and the wet flour placed in the wintry glass Petrie dish containing an iButton to monitor temperature. The dish was weighed and water added with mixing to replace that lost by evaporation. Duplicate 100 mg samples were removed for total cyanide analysis and the % total cyanide lost was calculated (Section 2.2.2).

3. Results and discussion

3.1. Comparison of loss of cyanide from flour at 30 °C and 50 °C

Fig. 1 shows the % total cyanide remaining in wet cassava flour (MCol 1468(2) fine) vs. time of treatment (h) at 50 °C (■) and 30 °C (●).
initial amount of added water amounts to 55.5%, which drops after 5 h at 30 °C to 45% and to 5.5% at 50 °C. Furthermore at 50 °C the water content at 2.85 h, had reduced to 16% and the small further loss of cyanide from 2.85 to 5 h was because the reaction ceased at low moisture content (Bradbury, 2006). After 5 h, the 50 °C treatment was much more effective in removing cyanide (only 13% left) than the 30 °C treatment (32% left). This probably resulted mainly from the increased rate of breakdown of acetone cyanohydrin to HCN at the higher temperature (White et al., 1994).

The 5 h treatment at 30 °C gives a higher value for % cyanide remaining than a 2 h treatment at 50 °C, see Fig. 1. Comparisons were then made under these conditions using different samples of flour from Mozambique, flour prepared in Canberra and one sample from Indonesia (Bogor A), that contained only a small amount of linamarase, with different amounts of exogenous linamarase added (Bradbury, 2006). Results in Table 1 show that for seven different flour samples and the three Bogor A experiments with added linamarase, about the same amount of cyanide remained using either 2 h at 50 °C or 5 h at 30 °C. As shown in Fig. 1 there is further loss of cyanide if the 50 °C treatment is continued after 2 h, when the moisture content is 32%, but very little loss of cyanide beyond a heating period of 3 h, because the flour sample is too dry.

The % cyanide remaining for the different flour samples ranges from about 9% for MAus7(3) coarse to 88% for Bogor A, which has very little linamarase (0.005 EU/g). As exogenous linamarase is added to Bogor A the % cyanide remaining decreased from 54% at 0.02 EU/g to 23% at 0.08 EU/g to 12% at 0.24 EU/g. Clearly the range of enzyme concentrations 0.02–0.24 EU/g covers the enzyme concentrations present in the flour samples shown in Table 1. For the wetting method to be effective in removing linamarin from cassava flour there must be a reasonable amount of linamarase remaining in the flour, which is usually the case. However, if the wet flour is artificially dried during preparation at temperatures above about 80 °C then linamarase is denatured (as in Bogor A) and the wetting method becomes ineffective.

### Table 1

Comparison of % cyanide remaining from cassava flour heated at 50 °C and 30 °C in an oven and in the sun.

<table>
<thead>
<tr>
<th>Sample of flour</th>
<th>%Cyanide remaining after</th>
<th>Standing in sun for 2 h</th>
<th>Heating at 50 °C for 2 h</th>
<th>Heating at 30 °C for 5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mozambique</td>
<td></td>
<td>46</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>Nampula 3</td>
<td></td>
<td>48</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>Mozambique JC 4</td>
<td></td>
<td>40</td>
<td>25</td>
<td>56</td>
</tr>
<tr>
<td>M Aus 7(3) fine</td>
<td></td>
<td>15</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>M Aus 7(3) coarse</td>
<td></td>
<td>25</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>M Col 1468(2) fine</td>
<td></td>
<td>17</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>M Col 1468(2) coarse</td>
<td></td>
<td>–</td>
<td>89</td>
<td>86</td>
</tr>
<tr>
<td>Bogor A</td>
<td></td>
<td>–</td>
<td>56</td>
<td>52</td>
</tr>
<tr>
<td>Bogor A + 25 µL linamarase</td>
<td></td>
<td>–</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Bogor A + 100 µL linamarase2</td>
<td></td>
<td>–</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Bogor A + 300 µL linamarase</td>
<td></td>
<td>–</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

1 In all experiments the initial % moisture was 55.6%. Mean % moisture after 2 h at 50 °C was 32%, after 2 h in sun at 39–46 °C was 22% and after 5 h at 30 °C was 45%.

2 25,100 and 300 µL amounts of a 1.6 EU/mL linamarase solution were added to 2 g Bogor A flour, hence the linamarase added amounted to 0.02, 0.08 and 0.24 EU/g flour, respectively.

#### Fig. 2.

% Total cyanide remaining in wet cassava flour (Bogor A) treated at 50 °C for 2 h (●) and 5 h (■) vs. volume of linamarase solution added.

3.2. Loss of cyanide from wet flour in the sun

Table 1 shows the results obtained with wet flour spread in a < 1 cm thick layer exposed for 2 h in the sun in a glasshouse to raise the temperature in the winter sun. The maximum temperature recorded by an iButton placed in the direct sun and surrounded by wet flour was 39–46 °C. The results in Table 1 show that the % cyanide remaining on standing in the sun for 2 h is about the same as that obtained by heating in an oven at 30 °C for 5 h or at 50 °C for 2 h. The moisture content after 2 h in the sun (22%) was lower than after 2 h at 50 °C in an oven (32%) because of the greater amount of evaporation from the exposed shallow Petri dish than from a 100 mL beaker in an enclosed oven at 50 °C. The moisture content after 5 h at 30 °C (45%) was much higher, as would be expected from the lower temperature.

Since both wetting treatments, 2 h in the sun and 5 h in the shade, remove the same amount of total cyanide from cassava flour, it is useful to compare their relative merits for use by rural communities in Africa. The major advantage of the former treatment is the shortening of the time from 5 h to 2 h, which is really important if the sample of flour requires constant surveillance to prevent thieving or spoilage by birds or children (Karumba, Denton, & Bradbury, 2009). On the other hand the 5 h treatment in
the shade is more secure if placed inside the house, less subject to spoilage by insects and animals and less water is lost by evaporation, which is important where water is in short supply, as in parts of east Africa. It is clearly advantageous that there are now two alternative methods available that can be used by rural people as they wish to suit their own needs.

3.3. The nature of residual cyanogens in flour after wetting treatment

In using the wetting method it was found that residual cyanogen remained in the wet flour after treatment, see Fig. 1 and Fig. 2 of Bradbury (2006). Residual cyanogen also remains after production of gari, which has been shown to be nearly all acetone cyanohydrin (Bradbury, 2009), and is stabilized by the low pH of gari (about 4.2) and its hard, gritty nature. Neither of these gari problems occurs with cassava flour which is soft and has pH ca 6.5, yet it is difficult to remove the last traces of cyanogen. To ascertain the nature of this residual cyanogen, we added increasing amounts of linamarase to wet Bogor A flour, which contains only a small amount of linamarase (Bradbury, 2006), and measured the total cyanide remaining after 2 h at 50 °C in an oven. The results in Fig. 2 show that the% total cyanide remaining drops rapidly from 89% when there is no added linamarase to 12% at 300 μL linamarase solution and there is no further decrease on doubling the amount of linamarase to 600 μL. The most likely explanation of this result is that at 300 μL linamarase solution added, all the linamarin has been hydrolysed to acetone cyanohydrin catalysed by linamarase and hence additional linamarase has no effect. After 2 h treatment at 50 °C with 300 μL linamarase solution added, analysis of the flour for acetone cyanohydrin gave 10.6% remaining and for total cyanide 10.4% remaining, the same result within experimental error, which showed that all residual cyanogen was present as acetone cyanohydrin. Flour with 300 μL linamarase solution added and treated for 5 h at 50 °C, gave 6% total cyanide remaining (see Fig. 2), due to a further drop in the acetone cyanohydrin content between 2 h and 5 h, as also observed in Fig. 1. Under these conditions acetone cyanohydrin is the sole residual cyanogen present in flour after the wetting treatment of 2 h at 50 °C. Similarly in gari virtually all the residual cyanogen present is acetone cyanohydrin (Bradbury, 2009).

4. Conclusions

The wetting treatment for removal of cyanogens from cassava flour involved leaving the wet flour in the shade for 5 h in the tropics at ambient temperature, (Bradbury, 2006; Cumbana et al., 2007). The long treatment (5 h) was found to be a disincentive to its use in the Uvira area of DRC (Karumba et al., 2009). We have found that an alternative treatment of 2 h in the sun is equally effective in removing cyanogens, and the two treatments 2 h in the sun and 5 h in the shade, are offered as two options for people in rural Africa. In flour samples dried at temperatures above about 80 °C linamarase is denatured and the wetting method becomes ineffective, unless endogenous linamarase is added. The residual cyanogen left after completion of the wetting treatment with adequate linamarase present, was shown to be acetone cyanohydrin.

Acknowledgments

We thank Dr. Julie Cliff for the supply of flour samples from Mozambique.

References