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Cyanogenic Glucoside Content of Manioc
I. An Enzymic Method of Determination Applied to Processed Manioc

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Abstract : A method for the determination of the total cyanide content of manioc products is described. Exogenous linamarase has been used to hydrolyse the linamarin in the plant material. Enzyme activity, incubation time and volume of distillate have been varied such that maximum recovery of total cyanide was obtained. Recovery of added HCN and linamarin from manioc substrate was found to be satisfactory. The method was applied to determine the total cyanide content of boiled manioc, manioc flour and manioc starch. Raw manioc on boiling was found to lose ⅓ to ½ of its total cyanide. Steeping with leaves prior to boiling did not alter these values significantly. It was found that while manioc starch contained only small quantities of total cyanide, manioc flour unless specially processed to reduce cyanide content, contained 50 to 250 p.p.m. total cyanide.

1. Introduction

Manioc, *Manihot esculenta* Crantz, contains compounds from which cyanide may be liberated, namely the cyanogenic glucosides linamarin and lotaustralin. In addition, it also has small amounts of free cyanide (HCN) and probably contains cyanohydrins. Although most methods of processing to a great extent eliminate free cyanide, it is clear that unless special precautions are taken, bound cyanide (mainly the cyanogenic glucosides) persists.[4,10,13] The extent of cyanogenic glucoside content remaining in processed manioc has been subject to controversy.[7] This has been mainly due to the accumulation of misleading data in the literature due to : (1) poor sampling[14] and (2) use of sub-optimum conditions for hydrolysis of linamarin.[7] Much of the literature on the topic is therefore of dubious value and it is generally recognized,[3,14] that a fresh set of data, using reliable methods, is an urgent necessity.

This paper presents such a method (previously outlined[10]) where additional linamarase is used to hydrolyse linamarin completely. Although methods with additional linamarase have been used before,[4,5,13] these methods have not been completely satisfactory. Wood[12,13] did not consider all the important parameters concerned with the extent of enzyme hydrolysis and also used unsuitable conditions for recovery of HCN (aspiration in acid conditions). De Bruijn[4,5] used a crude leaf extract for his studies (which necessarily resulted

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in high HCN blanks) and also used the non-specific $AgNO_3$ titration to determine cyanide. Furthermore, he did not pay attention to some variables that have been considered in this study.

2. Experimental

2.1. Materials

Tubers of different varieties of manioc available in the local market were used. Manioc flour was prepared by cutting the edible part into chips followed by drying in a forced-draft oven at 55 to 60°C. The dried chips were then finely powdered and sieved (180 mesh). Manioc starch was prepared by a standard wet process[6] using three resuspensions of starch. Boiled manioc used was prepared (unless otherwise specified) by cutting the edible part into approximately 1 in cubes and boiling for about 45 min in an open vessel.

2.2. Sampling

Flour and starch which lend themselves to complete mixing presented no problems. However, as the cyanogenic glucoside content of the tubers varies lengthwise[5,9] and also radially,[4] special precautions had to be adopted in comparative studies of whole manioc. The tuber was quartered and the opposite quarters pooled; this resulted in two samples of equal cyanide content (3. Results). The samples were then blended in a Waring blendor until completely homogeneous.

2.3. Assay for total cyanide in samples

Samples of starch, flour, homogenised fresh manioc or homogenised boiled manioc (approximately 10 g dry weight) were incubated in 200 ml of water containing 10 ml of citrate buffer (0.2 M. pH 5.0) and about 150 units (μg/ min) of linamarase for 4 h at room temperature (28 to 30°C). The samples were then distilled; approximately 200 ml of distillate was collected in 50 ml of an aqueous solution of 6.25% Na_2CO_3 or of 1.0% NaOH. The distillate (usually 4 ml) was tested for cyanide by the method of Wood[12] (Picrate method) using a spectrophotometer. The cyanide was estimated quantitatively with the aid of standard curves, taking into account the presence of the appropriate amount of Na_2CO_3 or NaOH used. Gradients obtained were .0122 and .0105 (optical density/μg) respectively. Collection in Na_2CO_3 solution was superior to collection in NaOH as use of the latter resulted in a diminution of the colour obtained. Hence Na_2CO_3 was used in nearly all the experiments presented in this paper.

2.4. Preparation of linamarin and linamarase

Both linamarin and linamarase were prepared by the method of Wood.[13] These preparations when distilled separately produced no colouration with the picric acid test.

2.5. Assay of linamarase

The method of Wood[13] was used except that the reactions were carried out in a syringe (5 ml) such that there was no air space above the liquid. The reaction mixture contained a suitable volume of linamarase extract and excess linamarin (approximately 2.5 mg) in 5 ml which was 0.08 M with respect to citrate buffer (pH 5.0). The reaction was carried out at room temperature. Aliquots (1 ml) were carefully introduced at 3 or 5 min intervals into the detecting mixture and cyanide determined by the method of Wood.[13] Enzyme activity was calculated using the linear part of the reaction curve. A standard curve for equimolar amounts of cyanide and glucose was used in the calculation. The curve is biphasic where r = .014 from 5 μg to 15 μg cyanide and .021 from 15 μg to 80 μg cyanide.

2.6. Assay of linamarin

Two methods were used: (1) the distillation method (after incubation with excess linamarase for 20 h) and (2) direct determination in a syringe using excess linamarase (300 units) and about 1 mg linamarin. Readings were taken at 2 h intervals until no further cyanide was liberated. Other details were the same as in the assay for linamarase.

3. Results

3.1. Preliminary studies

Variation of the quantity of enzyme used and incubation time (Tables 1 and 2) showed that maximum cyanide recovery could be obtained using the conditions described (2. Experimental). Zero time and zero enzyme values in the data are due to free cyanide and possibly residual enzyme activity.

Table 3 shows that the bulk of the cyanide present in the reaction mixture can be collected in the first 125 ml of distillate. However, in practice, 200 ml of distillate was collected. Under these conditions it was found that if KCN is added into the incubation mixture, then HCN could be recovered quantitatively.

TABLE 1. Effect of incubation time on cyanide liberation.

Incubation time (h)	Cyanide liberated (p.p.m.)	
	Sample A	Sample B
—	11	—
0.5	29	26
1.0	36	32
1.5	50	—
2.0	56	48
4.0	55	48

Boiled manioc was incubated with 136 units of linamarase ; for further details see 2. Experimental.

TABLE 2. Effect of enzyme activity on cyanide liberation.

Linamarase activity (μg/min)	Cyanide liberated (p.p.m.)
—	7
68	65
136	67
204	67

In all cases boiled manioc was incubated for 4 h ; for further details see 2. Experimental.

TABLE 3. Effect of volume of distillate on cyanide recovery.

Experiment	Volume of distillate (ml)	Cyanide recovered (p.p.m.)
1	25	69
	50	69
	75	76
2	100	77
	125	84
	150	83
3	175	86
	200	85
	225	88

3.2. Recovery of linamarin

Having separately determined the optimum conditions for liberation of HCN from bound cyanide and its recovery, it was decided to confirm this method by testing the recovery of HCN from linamarin. The results obtained are in Table 4 which shows apparently high values. Further experiments very often gave recoveries about 10% higher than expected. It is believed that this is due to an error in the estimation of linamarin (Table 5).

TABLE 4. Recovery of linamarin added to boiled manioc.

Linamarin added (as μg cyanide)	% recovery
177	104
354	107
531	98
708	119
885	114
1092	117

Linamarin was added into a sample of boiled manioc containing 600 to 700 μg total potential cyanide and the cyanide assay carried out as described in 2. Experimental. Linamarin concentrations were estimated by the syringe method.

TABLE 5. Linamarin estimation by different methods.

Linamarin sample	Cyanide content (mg/ml)	
	Syringe method	Distillation method
1	5.3 ± 0.3	5.9
2	4.3 ± 0.3	4.9
3	1.4 ± 0.1	1.6

3.3. Sampling

Sampling was done as described (2. Experimental). The two samples obtained from each tuber were treated separately and the total cyanide content determined. Results showed that the cyanide content of the two samples were almost identical (Table 6) and as a result this method was used in all studies conducted with whole manioc tubers.

TABLE 6. Sampling of whole manioc.

Experiment no.	Total cyanide (p.p.m. fresh wt)	
	Sample A	Sample B
1	122	124
2	51	50
3	50	50

Tubers were divided into two parts as described in 2. Experimental and assayed after homogenisation with crushed ice.

3.4. Total cyanide content of raw manioc

After several preliminary experiments it was found that the highest yield of total cyanide was obtained when the material was homogenised with crushed ice. The value obtained was slightly higher than when the tissue was homogenised in 0. 1N H_2SO_4 to deactivate the endogenous linamarase, and considerably higher than when the manioc cubes were warmed in the same strength of acid. Undoubtedly, even homogenisation in ice results in some (very small) loss of cyanide but results obtained by this method were highly reproducible.

3.5. Total cyanide content of boiled manioc

On application of the assay and sampling techniques to test the effect of normal cooking procedures on total cyanide content of manioc, it was found that boiling reduced the concentration of total cyanide to $\frac{1}{3}$ to $\frac{1}{2}$ of the value of raw manioc (Table 7). The percentage decrease varied from sample to sample. The total cyanide content of boiled manioc was found to vary between 11 and 180 p. p. m. (on fresh weight).

It was found that the size to which the material is reduced prior to boiling, affects the final amount of total cyanide slightly; work is proceeding on this aspect and also on the effect of opening and closing the lid of the cooking vessel.

It has been recently reported[2] that when chopped raw manioc is steeped with certain vegetables, the cyanide content of the boiled product is reduced. Studies with leaves, however, showed that the total cyanide content of manioc was not significantly affected by this procedure (Table 8).

TABLE 7. Effect of boiling on total cyanide content.

Sample	Total cyanide content (p.p.m. fresh wt.) Raw	Boiled	% remaining after boiling
1	117	46	39
		46	39
		46	39
2	128	66	52
3*	298	97	33

Manioc tubers were divided into two samples (2.2. Sampling); one was assayed raw and the other after boiling.

*This sample was from the branched tree form of manioc, locally termed "rubber manioc".

TABLE 8. Effect of steeping and boiling with leaves.

Leaf use		Total cyanide content (p.p.m. fresh wt)	
Common name	Botanical name	Control	Experimental
Katurumurunga	*Sesbania grandiflora*	55	45
Katurumurunga	*Sesbania grandiflora*	49	49
Katurumurunga	*Sesbania grandiflora*	33	38
Kankun	*Ipomea aquatica*	61	61
Mukunuwenna	*Alternanthera sessilis*	60	56

Manioc, sampled as described above, was steeped with finely sliced leaves for 2 h and boiled. The second sample was boiled in the normal way and used as Control.

3.6. Total cyanide content of manioc flour

This was previously reported in a short communication.[10] The values reported in that study did not take into account the effect of sodium hydroxide on cyanide-picrate reaction and hence have to be corrected by the factor given in 2. Experimental.

Analysis of manioc flour prepared by the method described here and by more specialized processes[8,11] designed to remove cyanide is shown in Table 9. Clearly, the final total cyanide content of the flour varies considerably (this is mainly dependent on the glucoside content of the raw material). The percentage loss of total cyanide when flour was prepared in this way from manioc has not been calculated in this study as an adequate sampling procedure has still to be worked out. Preliminary studies, however, indicate that losses are small.

The specialized processes[8,11] on the other hand, yield flour of very low total cyanide content.

TABLE 9. Total cyanide content of manioc flour.

Process	Total cyanide content (p.p.m.)
1. As described in 2. Experimental	197
2. As described in 2. Experimental	58†
3. As described in 2. Experimental	240**
4. As described in 2. Experimental	203
5. As described in 2. Experimental	614*
6. Rajaguru method[11]	N.D.
7. Rajaguru method	6
8. JeyaRaj process[8]	8
9. JeyaRaj process	3†
10. JeyaRaj process	10**

* "rubber manioc"
** same raw material
† same raw material
N.D. not detected, less than 2.5 p.p.m.

3.7. Total cyanide content of manioc starch

Three samples of manioc starch prepared by a standard wet process were analysed. The total cyanide content of these samples were 11, 4 and 4 p.p.m. respectively.

3.8. Free cyanide content in processed manioc

In nearly all cases the free cyanide content was low, generally between 0 and 10 p.p.m. Little attention was paid to free cyanide in this study as results in this range of concentration are not easily calculated with the detecting reagent used here ; besides, free cyanide generally forms only a small percentage of the total cyanide in most manioc products.

4. Discussion

The first part of this study was devoted to the determination of the optimum conditions of liberation and isolation of cyanide. It was found that 150 units of linamarase used with an incubation time of 2 h was sufficient to liberate bound cyanide. However, in this study, the incubation time was increased to 4 h as an additional precaution. The liberated cyanide was recovered by steam distillation, 200 ml of distillate being collected. Results showed that further increase of (1) the quantity of enzyme used, (2) incubation time and (3) volume of distillate collected produced no further recovery of cyanide.

Next, the efficiency of recovery of total cyanide was tested. Addition of KCN into the reaction mixture showed that quantitative recovery was possible. However, this is not the best test of recovery as the main product of enzyme hydrolysis is keto-cyanohydrins which are in equilibrium with HCN, the position of the equilibrium being very much towards the cyanohydrins. Due to this and because keto-cyanohydrins decomposed to give HCN at 85°C, recoveries using linamarin are of more significance. Difficulties in obtaining a quantitative recovery from linamarin appeared to be mainly due to an approximately 10% underestimation of the linamarin as determined by the syringe method ; the reasons for this are not clearly understood. Furthermore, the mean value obtained by this method has a standard deviation of 5 to 6%. Taking these factors into account, it appears that a quantitative recovery of linamarin has been achieved. In addition, there does not appear to be a way by which either linamarin or one of its products could be converted to a volatile compound that would give a more intense picrate colour reaction than cyanide.

Manioc does not appear to contain any other volatile compound that will produce a colour reaction with picrate. This is deduced by the facts that firstly, those samples with no free cyanide do not produce any colouration in the absence of added linamarase (i.e. all colour produced is enzyme dependent) and secondly, flour processed to be free of total cyanide has given optical densities as low as 0.005 (where the normal optical densities are in the range of 0.10 to 0.60).

It seemed possible that some volatile compound might enhance the colour formed by picric acid and cyanide (especially at high cyanide concentrations) and therefore lead to spurious results. This appears not to be the case as : (1) drastic variation of sample size does not alter the quantity of cyanide liberated /g of sample, (2) the absorption spectrum of the cyanide-picrate colour reaction of the distillate is identical to that formed by the same quantity of pure HCN and (3) the early studies (using aspiration) showed that estimation of cyanide liberated by the picrate method and 'Alridges method' gave the same result (<2% error). These points together with the high reproducibility of the results obtained in this study underline the validity of the method. Furthermore, a simple technique of sampling whole manioc such that any two given treatments may be reliably compared has been devised.

Applications of the technique to boiled manioc showed that $\frac{1}{3}$ to $\frac{1}{2}$ of the glucoside still remains after the standard cooking procedure. In some instances (18 out of 26) the amount remaining is not sufficient to produce a lethal dose of cyanide in a meal (for the average healthy adult) even if all the glucoside is hydrolysed in the alimentary canal. In other cases (8 out of 26), however, the quantity of total cyanide exceeds 40 mg/250 g dry weight and in these instances complete hydrolysis in the alimentary canal could produce a lethal effect. Fortunately, it appears probable that the extent of glucoside hydrolysis after consumption is relatively small ; this may explain why a meal of manioc is rarely fatal.

These studies have also shown that steeping with vegetable leaves, etc. (as recommended recently to the public[2]) does not significantly affect total cyanide concentration. This is to be expected as the manioc glucoside and the leaf enzyme (if present) have no chance of interacting. Similar results have been obtained by another independent study.[11]

Studies on manioc have clearly shown the need for well organised processing of manioc products. Dependable methods[8,11] are now available (this paper presents the results) that reduce cyanide level from an alarming level of 200 p.p.m. to a relatively harmless level of 0-10 p.p.m.

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