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A Simple Procedure for the Quantification of Intact Linamarin in Cassava and its Products

Benjamin O. Izokun-Etiobhio, Alex C. I. Oraedu and Emmanuel N. Ugochukwu

Department of Biochemistry, University of Benin, Benin City, Nigeria

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ABSTRACT

A thin-layer chromatographic method coupled with a colorimetric technique for the quantitative determination of linamarin is described. The procedure is based on the measurement of hydrogen cyanide released by linamarase hydrolysis of linamarin isolated on the chromatogram. The released HCN is determined by a modified alkaline picrate method employing Conway vessels. The procedure is free from interferences associated with previously described methods and its sensitivity permits as little as 0·8 μg linamarin (≡0·09 μg HCN) to be determined.

Key words: Linamarin, cassava, cyanide, alkaline picrate, thin-layer chromatography.

1 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important source of energy in tropical developing countries.[1] Its use in the livestock-feed, starch, textile and alcohol industries is becoming increasingly important.[2] Cassava has the major disadvantage that it contains the cyanogenic glycosides, linamarin (2-(β-D-glucopyranosyloxy) isobutyronitrile) and lotaustralin (2-(β-D-glucopyranosyloxy) methlybutyronitrile) capable of releasing poisonous cyanide.[3] From 92 to 98% of the total cyanogenic glycosides present in cassava plants[4,5] is linamarin.

Various traditional processing methods are employed to obtain cassava

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seemingly free of harmful amounts of cyanogenic glycosides.[2,3] However, studies[6–9] have shown that cassava food products prepared by these traditional methods contain varying amounts of residual cyanide. The cyanide in these processed cassava products may exist as cyanogenic glycosides (linamarin and lotaustralin) and/or as non-glycosidic cyanide in the form of cyanohydrins and metal cyanides.[8]

Cyanogenic glycosides are generally estimated by measuring the amount of hydrogen cyanide liberated by hydrolysis. Efforts continue to be made to develop suitable methods for the determination of the intact individual glycosides. Use of gas chromatography and high-performance liquid chromatography has been reported,[10,11] but these methods lack specificity[12,13] and require expensive sophisticated equipment not always readily available where the need exists.

Zitnak *et al.*[14] reported a paper-chromatographic procedure for the direct determination of linamarin in biological fluids. The method is based on the reaction of linamarin with *p*-anisaldehyde at 85°C which produces a pigment pink in colour and brightly fluorescent under u.v. light. These workers claimed that linamarin concentration could be estimated by visually comparing the fluorescence of samples with that of standard solutions. Attempts in the present work to use this method have been unsuccessful. Moreover, the reaction is unspecific since many substances, such as carbohydrates, amygdalin, sinigrin, phloridzin, mandelic acid and chlorogenic acid, have been found to produce the fluorescence under the test conditions.

Butler and Butler[15] described a method using picric acid reaction for the detection of cyanogenic glycosides on paper and t.l.c. This method involves a sandwich technique in which the chromatograph is sprayed with an enzyme solution, a perforated sheet is placed on the moist chromatogram and a paper impregnated with a basic picrate solution is placed on top of this. The HCN released by cleavage of the cyanogenic component reacts with the picrate to give orange-brown spots. More recently, Brimer *et al.*[13] modified the sandwich method for quantitative measurement by the use of transparent and hydrophobic picric acid reagent sheets. The sheets are placed in direct contact with the enzyme-wetted silica gel chromatogram. Thus the diffusion of HCN inherent in the original method is minimised. The intensities of the spots on the transparent and hydrophobic reagent strips are then determined by a densitometer, e.g. a Vitratron TLD 100.

The present paper describes a procedure that avoids many of the disadvantages associated with the previously reported methods. The procedure is based on a modification of the t.l.c. method of Bisset *et al.*,[10] which enables selective identification of linamarin. The linamarin spot was scraped off and treated with linamarase in a Conway vessel followed by an alkaline picrate estimate procedure for cyanide. Linamarin concentration is then calculated from the cyanide levels.

The use of the Conway vessels obviates some problems[1,12] associated with the alkaline picrate reaction for the determination of cyanide in cassava extracts. These are due to the unspecific reactions of the alkaline picrate with reducing substances, such as aldehydes and ketones, arising from the enzymic hydrolysis of the cyanogenic glycosides.

2 EXPERIMENTAL

2.1 Cassava and cassava products

Cassava parenchymal tissue, 'gari' and 'akpu', were obtained as previously described.[8]

2.2 Chemicals

Linamarin was a product of Calbiochem. Ltd, San Diego, USA. All the other chemicals used were of analytical grade and were obtained from BDH Chemicals Ltd, Poole, Dorset, UK. Partially purified cassava linamarase was prepared according to the method of Ikediobi *et al.*[16]

2.3 Standard solutions

Preparation of standard solutions of linamarin was according to the method of Zitnak *et al.*[14] A stock solution containing 20 mg of linamarin in 10 ml (2 $\mu g\ \mu l^{-1}$) 80% ethanol was used for a series of dilutions in glass test tubes such that a 10 μl volume of each of the dilutions provided a range of 0·8–20 μg linamarin standards equivalent to 0·09–2·19 μg HCN.

2.4 Apparatus

Merck Kieselgel (5627) pre-coated plates were used for the t.l.c., and a Pye-Unicam Sp-1800 spectrophotometer was used for the colorimetric assay in the alkaline picrate procedure.

2.5 Methods

2.5.1 Preparation of extract of cassava parenchymal tissue

Ten grams of fresh cassava tissue was added to 90 ml 90% v/v ethanol. The mixture was homogenised in a Waring blender for 3 min. After boiling for 10 min to inactivate the endogenous linamarase the clear slurry was filtered. The filter cake was washed with an equal volume of 90% ethanol. The combined extract was centrifuged at 1200×g for 5 min. The filtrate was concentrated in a rotary vacuum evaporator at 40°C followed by drying in a desiccator. The residue was extracted with 10 ml methanol and centrifuged to remove a small quantity of flocculent particles. The resulting supernatant was stored in the refrigerator until analysed.

2.5.2 Preparation of extract of 'gari' and 'akpu'

About 20 g of 'gari' or 'akpu' were extracted with 125 ml of distilled water using a Gallenkamp flask shaker at 4 cycles s^{-1} for 20 min. The mixture was centrifuged for 5 min at 1200×g. A 5-ml aliquot of the supernatant was freeze-dried. The residue was then dissolved in 0·5 ml distilled water for analysis.

2.5.3 Thin-layer chromatography

The technique was essentially that of Bisset *et al.*[10] except that Merck Kieselgel (5627) pre-coated plates were used instead of silica gel G. Aliquots (5–20 μl) of the extracts of cassava, 'gari', 'akpu' and linamarin standard solutions were applied to two identical plates and run simultaneously. Identification of the

linamarin spots was carried out on one chromatogram. The linamarin concentration could be estimated visually by comparison of the brown–black colour of unknowns with a set of calibration standards. The chromatograms could be preserved for up to 6 months by washing off the excess sulphuric acid and drying in an oven at 110°C. The second identical chromatogram was used for quantitative analysis.

2.5.4 Determination of cyanide/linamarin

The area occupied by linamarin on the untreated plate was identified by comparison with spots on the stained chromatogram and marked with a pointed instrument. This small area of the plate was scraped off and the scrapings were placed in the outer well of a Conway vessel whose inner chamber contained 1 ml 0·1 M NaOH. Excess partially purified cassava linamarase (50–100 units) was added to the scrapings. The air-tight Conway vessel was left for 24 h at room temperature (28°C) for complete hydrolysis of the linamarin and diffusion of the HCN to occur. An alkaline picrate estimation of cyanide according to Ikediobi *et al.*[16] was carried out on an aliquot of the alkaline cyanide solution in the centre well of the Conway vessel as well as on the neat extracts. The linamarin equivalent of the estimated cyanide was calculated using the mole ratio (linamarin : HCN, 247 : 27).

3 RESULTS AND DISCUSSION

The efficiency of the proposed method was assessed by carrying standard linamarin solutions through the procedure described. The results in Table 1 show that the quantitative yield of linamarin cyanide obtained in all the cases approximates very closely to the theoretical values obtained by calculations. The recoveries were within 94–101%. There were no significant differences between the linamarin concentration of the standard solutions and the linamarin concentrations calculated from the experimentally obtained hydrogen cyanide.

Sensitivity tests were carried out using progressively decreasing concentrations of linamarin, and it was found that the lower limit of detection and quantification was 0·8 μg linamarin corresponding to 0·09 μg of hydrogen cyanide released by hydrolysis.

TABLE 1
HCN Content of Linamarin Standard Solutions Determined by the T.l.c./Modified Alkaline Picrate Method[a]

Linamarin (standard) μg	*HCN (theoretical)* μg	*HCN (estimated)* μg	*Linamarin (calculated)* μg	*Recovery* %
4	0·44	0·41±0·01	3·77±0·20	94·35±3·31
8	0·87	0·85±0·04	7·77±0·25	97·17±3·20
12	1·31	1·33±0·03	12·15±0·26	101·24±2·13
16	1·75	1·70±0·07	15·55±0·34	97·16±2·63
20	2·19	2·07±0·07	18·92±0·82	94·61±1·99

[a]The results are expressed as the mean ±s.e. based on four replicates.

TABLE 2
HCN/Linamarin Content of Cassava and its Food Products[a]

Material	*Moisture content %*	*T.l.c./alkaline picrate method mg HCN kg^{-1} dry wet*	*Linamarin mg kg^{-1} dry wt*	*T.l.c./modified alkaline picrate method mg HCN kg^{-1} dry wt*
Fresh cassava tuber	63	817·41±4·34	7477·79±39·67	826·06±1·25
'Gari'	13	3·60±0·07	32·89±2·30	3·33±0·05
'Akpu'	54	2·46±0·15	22·53±1·38	2·30±0·06

[a]The results are expressed as the mean ±s.e. of determination on four different extracts.

In order to demonstrate the utility of the method, extracts of fresh cassava parenchymal tissue and cassava food products 'gari' and 'akpu' were separated by t.l.c., and the amount of linamarin corresponding to the linamarin spot was determined (Table 2). When the same extracts were incubated with linamarase to hydrolyse the endogenous linamarin and the thin-layer chromatographic procedure was repeated, the spots corresponding to linamarin were absent. This indicated that the linamarin spots contained no contaminants. Thus in contrast to the alkaline picrate method of Ikediobi *et al.*[16] modified by Izokun-Etiobhio and Ugochukwu[8] for the measurement of intact cyanogenic glycosides (linamarin and lotaustralin) in cassava, this procedure enables separate quantification of linamarin in fresh cassava tubers where it forms 92–98% of the total cyanogenic glycosides.[4,5] The slight apparent increase (<5%) in cyanide content of fresh cassava tuber obtained by the modified alkaline picrate method could, therefore, be due to lotaustralin whose spot on the chromatogram could not be identified during this work because of the lack of an authentic lotaustralin preparation.

There were no significant differences between the cyanide contents of 'gari' and 'akpu' as determined by the proposed t.l.c./alkaline picrate method and those obtained by the modified alkaline picrate method which measures only intact cyanogenic glyosides.[8] It can be assumed that the residual intact cyanogenic glycosides in cassava food products, 'gari' and 'akpu' are mainly linamarin.

Having established the efficiency and reliability of the method when applied to linamarin standard solutions it was applied to 'gari' samples already analysed by

TABLE 3
Recovery of Linamarin from Gari (5·0 g) Fortified with Linamarin[a]

Linamarin content (mg)	*Linamarin added (mg)*	*Total linamarin (mg)*	*T.l.c.-modified alkaline picrate method mg HCN per 5 g 'gari' (dry wt)*	*Linamarin (calculated) (mg)*	*Recovery (%)*
0.16	5.00	5.16	0.55±0.03	5.03±0.05	97.42±1.50
0.16	10.00	10.16	1.05±0.02	9.61±0.08	94.23±2.8

[a]The results are exprerssed as the mean ±s.e. (mean) of four estimations on the same extracts.

the method and then fortified with different concentrations of linamarin. The results of the studies are shown in Table 3 from which the recovery of linamarin and reproducibilty of the method can be seen to be quite good.

The method of assay described gives good recoveries of linamarin from standard solutions, is inexpensive and is suitable for the estimation of linamarin in cassava, its products and derivatives. It is particularly suitable for materials containing interfering substances as these are eliminated chromatographically. It may be possible to use this technique also to measure lotaustralin in fresh cassava tissues given a standard preparation.

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