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A modified microdiffusion assay with solid-state detection for the determination of total cyanogens (CNp) in cassava flour. Comparison to the method of O'Brien *et al.* (1991)

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A simplified assay for quantitative determination of the total cyanogen content of cassava flour was developed. It relies on solid state detection after microdiffusion of released HCN from an acid flour extract. The reading of the solid state colour reaction was done by a microplate reader, but may also be done by means of a portable reflectometer. Positive test reactions are stable provided the reacted sheets are stored in the dark. The new method has been compared to the standard method of O'Brien *et al.* (*Journal of the Science of Food and Agriculture*, **56**, 277–289, 1991), and give identical results for levels of total cyanogens higher than approx. 5 mg kg^{-1}. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Products from the roots of cassava (*Manihot esculenta* Crantz) form the staple diet for more than 400 milion people. Cassava roots contain varying amounts of the cyanogenic glucosides linamarin and lotaustralin (Nartey, 1968). If these glucosides and their products of degradation are not reduced to negligible levels by effective processing, consumption may result in dietary cyanide exposure. Such exposure may cause acute poisoning (Mlingi *et al.*, 1992). Consequently, the Codex Alimentarius Commision currently work on a regional standard for edible cassava flour, which will set a limit for the total cyanogenic potential, glucosides + cyanohydrins + HCN = total cyanogens, in flour (CAC, 1988).

In most parts of Africa cassava flour is produced and consumed locally, by pounding or milling. Toxic effects attributed to high amounts of cyanogens in flour made from insufficiently processed cassava roots mainly occur in remote rural areas, i.e. aggravation of iodine deficiency disorders (Delange *et al.*, 1994), tropical ataxic neuropathy (Osuntokun, 1994) and the paralytic disease konzo (Tylleskär *et al.*, 1992). The toxic effects of cassava can be prevented by promotion of effective processing. However, it has been difficult to monitor the effect of such preventions as the available methods for determination of cyanogen levels in cassava flour required transport of speciments to remote laboratories. The aim of the present work was therefore to establish a chemical assay for the total content of cyanogens in cassava flour, that are simpler to handle than the method of O'Brien, and that allows analysis in field surveys; provided extracts can be prepared. To achieve this we modified the microdiffusion and solid state detection method of Brimer and Mølgaard (1986), a method for direct (i.e. without extraction) analysis of cyanogenic glycosides in small samples of green plant material.

The new assay was evaluated by analysing extracts of different flours with both this assay and with the method of O'Brien *et al.* (1991).

MATERIALS AND METHODS

Materials

Chemicals and reagents

Bispyrazolone (GPR grade), 3-methyl-1-phenyl-5-pyrazolone (GPR grade), Chloramine T (GPR grade),

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linamarase (EC 3.2.1.21 from cassava), and potassium cyanide (97%) were obtained from BDH Ltd, Poole UK. Linamarin (L-9131) was from Sigma, St Louis, USA, while all other chemicals were analytical grade from Merck, Darmstadt, Germany. Precoated ion-exchange sheets (Polygram ionex 24-SB-AC, cat. no. 806023) were from Macherey-Nagel, Düren, Germany. Picrate (detection) sheets for the solid state assay were prepared as follows. Precoated ion-exchange sheets (Polygram ionex 25-SB-AC, Macherey-Nagel)) were impregnated by consecutive immersion in two solutions; (1) a saturated solution of picric acid in H_2O, followed by air drying, and (2) a 1 M aq. Na_2CO_3 solution, also followed by air drying.

Cassava flours

Fifty (50) samples of flour were produced by both milling and pounding of 25 batches of sun-dried cassava roots 'makopa' obtained in markets in Dar es Salaam, Tanzania.

Methods

Extraction

First 30 g of flour was homogenized with 170 ml of extraction medium (0.1 M orthophosphoric acid containing 25% v/v of ethanol) for 3×1 min with an interval of 1 min in a Warring blender. Using a further 30 ml of extraction medium the homogenate was washed to a glass-fiber filter (Whatman GFA) and the extract was collected under vacuum.

Moisture determination

Duplicate samples of approx. 10 g were ovendried at 110°C for 16 h and the loss determined by weighing before and after.

Analysis of cyanogens in the extracts

Cyanogens were assayed in duplicate using the method of O'Brien *et al.* (1991) and the new assay. For the method of O'Brien aliquotes of 0.1 ml of extract were assayed for *total cyanogens*, *cyanohydrins* and *HCN*). The content of *cyanogenic glucosides* were calculated from these results.

The new method used incubation blocks (Brimer, 1994) allowing a subsequent reading of the solid state reaction with a microplate reader (Brimer, 1994). Aliquots (0.2–0.5 ml) of extract were mixed with 1 ml of phosphate buffer 0.5 M, pH 6.0 and 0.1 ml of linamarase solution (3 EU ml^{-1} in 0.1 M orthophosphatebuffer pH 6) in a 2 ml tube. The tubes were placed in the incubation block and the block was immediately closed by means of a picrate sheet and incubated overnight at room temperature (not less than 20°C). The picrate sheet was air dried and the reaction spots read using a EAR 400 FW, Easy Reader (SLT, Groedig/Salzburg, Austria) microplate reader at 550 nm (Brimer, 1994). Determinations were based on a standard curve obtained from the hydrolysis of 5, 10, 20, 40 and 60 nmol of linamarin respectively (Fig. 1), one curve being produced on each detection sheet containing 18 sample analyses.

Calculation of the total cyanogen content (CNp) in mg HCN equivalents kg^{-1} dry wt was for both assays done as described in O'Brien *et al.* (1991) to include sample moisture. Thus, for the new method a calculation example looks as follows:

$$\text{Total cyanogens (mg kg}^{-1}\text{ as HCN)} = \frac{27 \times N \times V'}{S \times DW}$$

N = nmol of CN as calculated from the calibration curve, S = volume of sample in µl, DW = dry wt of sample; V′ (extraction volume adjusted to include the sample moisture) = V + (M×FW)/100; M = moisture in %, FW = freshweight of sample.

RESULTS AND DISCUSSION

Cooke (1978) developed a method for the extraction of cyanogens from cassava roots, employing standarized homogenization in 0.1 M orthophophoric acid using a Warring Blender. This method was later modified to give maximum yield of cyanogens from processed products (O'Brien *et al.*, 1991). In the present work, extracts were prepared and analysed by using spectrophotometry as in O'Brien *et al.* (1991), and by a modification of a microdiffusion assay with solid-state detection (Brimer and Mølgaard, 1986; Brimer and Rosling, 1993).

The modifications of the solid-state assay were (1) the use of an extract of the commodity to be analysed (the method of Brimer and Mølgaard used small pieces of tissue), (2) the use of 0.1 ml of a (3 EU ml^{-1}) solution of

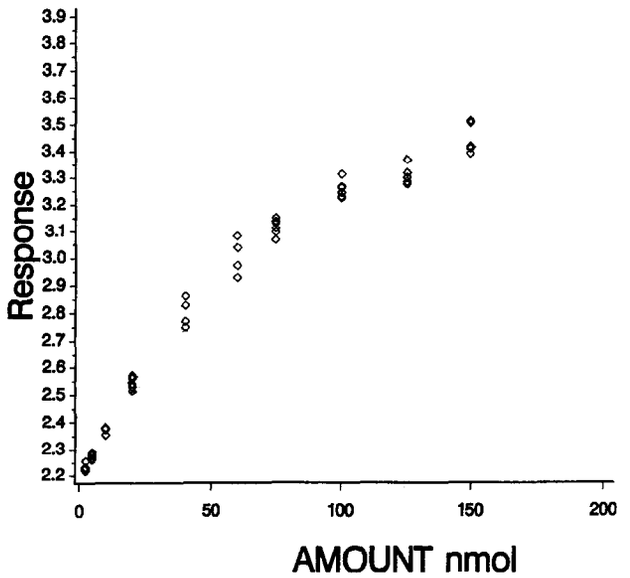

Fig. 1. Typical example of a standard graph (2.5–200 nmol HCN equivalents) as measured by absorption of transmitted light at 550 nm using a microplate reader. Only the interval 5–60 nmol was used in the present work (see Materials and Methods).

linamarase from cassava in 0.1 M of phosphate buffer, pH 6 (same composition and amount as in the O'Brien assay) as a source of enzyme (crude enzymes from *Helix pomatia* = wineyard snail were used earlier), and (3) the use of a stronger (0.5 M) phosphate buffer for the stabilization of the pH in the final incubation mixture.

The results from determinations of total cyanogens in 50 samples of flour are presented in the scattergram in Fig. 2. A linear regression analysis including all points shows a coefficient of regression (r) of 0.915. However, neglecting the point (13.7;45.4) which is obviously due to an error in the determination with the method of O'Brien *et al.*, since the point representing the corresponding pounded flour fits the pattern perfectly, the coefficient of regression (r) was found to be 0.944, the slope (α) 1.06, and the intercepts with the y-axis : −1.26. For x-values of 5 and 80 mg kg^{-1} this formula results in y-values of 4.1 and 83.6 mg kg^{-1}, respectively. Thus, the comparison clearly proves the new assay to be useful for routine analysis of cassava flour.

A more detailed look into the flour samples analysed was gained by analysing for water content, and for the content of cyanogenic glucosides, cyanohydrins and free HCN (method after O'Brien *et al.* (1991)). The results proved the samples to vary considerably in total cyanogenic potential (Fig. 2), moisture content and in the proportion of the three different classes of cyanogens. Moisture varied from 11.4 to 40.3%. The range of cyanogens expressed as mg CN eq. kg^{-1} dry wt were for glucosides 0.0–54.2, for cyanohydrins 0.0–26.8, and for HCN 0.2–4.3. Thus, the new assay is reliable for total cyanogens in flours with varying levels and proportions of cyanogens, on the basis that the extraction is quantitative. The difference between the results as measured by the two methods being less than 20% at levels higher than 5 mg CN eq. kg^{-1} dry wt, it is concluded, that the new assay can be used with a lower limit for quantification of 5 mg CN eq. kg^{-1} dry wt. This, is well below the limit of 10 mg kg^{-1} dry wt proposed in the preliminary work on a regional standard for safe cassava flour (CAC, 1988). This makes the method well suited for control analysis. The assay developed is based on circular solid state spots of dia. 10 mm as in the standard method of Brimer and Mølgaard (1986). As discussed by Brimer (1994) the lower limit of detection can be enhanced, by restriction of the spot diameter, however, this was not found to be necessary for routine analysis of flours.

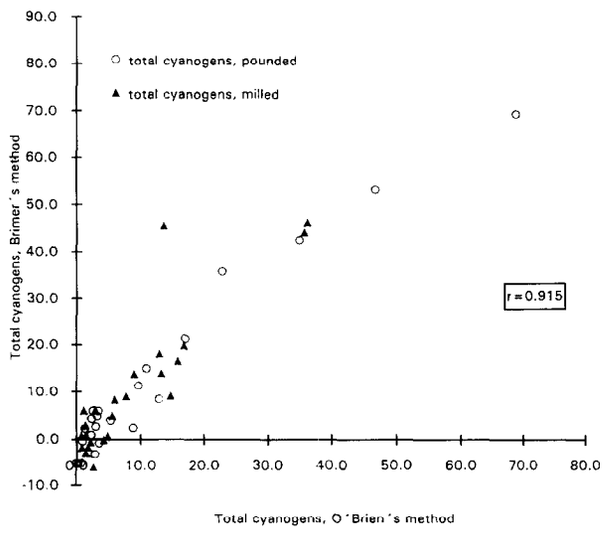

Fig. 2. Comparing total cyanogen determination (mg HCN eq. kg^{-1} dry wt) by the method of O'Brien and the new method.

The new assay uses the same type of ortho-phosphoric acid extraction as the spectrophotometric methods of Cooke (1978) and O'Brien *et al.* (1991), but differs in a number of other points. It includes a considerably smaller number of procedures and reagents than the spectrophotometric methods. It does not need cooling as the Cooke (1978) and the O'Brien *et al.* (1991) methods nor boiling as methods using acid hydrolysis of the glucosides (Bradbury *et al.*, 1991, 1994). The solid state colour reaction obtained in the new assay may with good results be rated visually against a standard strip (Brimer and Mølgaard, 1986), measured instrumentally in the field, or since it is stable over months if protected from light (Brimer *et al.*, 1983), after returning to a stationary laboratory. The instrumental reading may be based on either absorbtion of transmitted light or on reflection (Brimer, 1994). Determination of absorption of transmitted light as measured by means of a stationary microplate reader (used in this study) thus is only one of several possibilities (Brimer, 1994). Portable instruments allowing quantitative rating in the field are available and have been evaluated, based both on absorbtion (e.g. the portable microplate reader 'Dynatech M-250'; Brimer *et al.*, 1994) and on reflection (e.g. the portable reflectometer 'Nycocard Reader'; Brimer, 1994).

Many different products are made from roots of cassava, some fermented some gelatinized (IFS, 1992). More detailed investigations are in progress concerning the applicability of the solid state assay on fresh cassava roots as well as on products other than flour, i.e. for example gari for which Codex Alimentarius has a limit for total cyanogens of 2 mg kg^{-1} (FAO, 1989). In addition, a new blender has been constructed and is being tested for its reliability in field surveys.

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