REMOVAL OF CYANOGENS FROM CASSAVA ROOTS

Studies on domestic sun-drying and solid-substrate fermentation
in rural Africa

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Cover photo: Basket with dried pieces of cassava root

ABSTRACT

Essers, A.J.A. (1995) *Removal of cyanogens from cassava roots: studies on domestic sun-drying and solid-substrate fermentation in rural Africa.* Ph.D.-thesis at Wageningen Agricultural University, The Netherlands, together with the Unit for International Child Health, Uppsala University, Sweden (131 p, English, French, Portuguese and Dutch summaries)

Cassava is an important staple crop, but its potential toxicity has led to some health problems in Africa. The potential toxicity comes from endogenous cyanogenic glucosides, mainly linamarin, which may degrade by linamarase to cyanohydrins and subsequently to hydrocyanic acid (HCN). A study into a small outbreak of paralysis and poisoning in a cassava-dominated rural area of Mozambique revealed that the walking disability was konzo, a recently identified disease, and suggested that insufficient processing of the bitter cassava roots was a factor in its causation. The usual processing stages to turn roots into flour, sun-drying and heap-fermentation, were studied in Uganda and The Netherlands. For evaluation of initial and resulting levels of the cyanogenic compounds, an analytical assay was tested and improved. Mechanisms of cyanogen removal from cassava by sun-drying and heap-fermentation were elucidated, to allow for its optimization.

Sun-drying removed cyanogens insufficiently from roots with high initial levels. Dynamics of cyanogen levels are described. Continuing drying below moisture levels of 15% did not diminish linamarin levels further, but it was useful for further removal of the cyanohydrins formed. The dehydration rate influenced linamarin breakdown negatively. Reducing the size of the pieces to speed up drying, as done during the konzo outbreak, therefore resulted in higher residual linamarin levels. Linamarin breakdown can be enhanced by reducing the initial dehydration rate. Microbial contamination may need to be controlled to prevent the formation of microbial toxins.

In Uganda and Mozambique certain communities promote fungal growth by heaping and covering the peeled roots. Their aim is to improve the palatability and reduce the toxicity. Cyanogen removal by this solid-substrate fermentation appeared more effective than by sun-drying alone, but several samples of this flour from rural households still had undesirably high levels of cyanogens. Screening of 30 flour samples for mycotoxins was negative, but the formation of mycotoxins cannot be excluded. The humid incubation of cassava extends the time of physiological cell-wall degradation, which allows for linamarase-linamarin interaction. The microflora had an additional positive effect on cyanogen removal by enhancing the cell-wall degradation. The linamarase activity shown by several microorganisms was of lesser importance. The food grade fungi *Neurospora sitophila* and *Rhizopus oryzae* were the most effective in cyanogen removal. Optimization of processing conditions, including the use of starter cultures, is recommended for ensuring safe products.

Key words: cassava processing / cyanogenic glucosides / toxicity

Preface

The research presented in this thesis was initiated during my work as nutritionist in the Provincial Health Service of Nampula, Mozambique, from 1980 to 1985. During this multi task assignment, I became involved in studies of outbreaks of paralysis, associated with high consumption of poorly processed cassava. Nampula province is one of the most cassava dominated areas of Africa. During drought periods, roots of bitter cassava became the sole food crop due to food shortage. This led to the traditional cassava processing being shortened, which was linked to toxic effects. The farming population tried to avoid the acute poisonings they experienced, by altering their processing techniques. It became my task to assist them in their experiments to turn bitter roots into a flour safe for consumption, in a short period of time. When rural Mozambique became too insecure, the investigations continued in other areas of Africa. The Department of Food Science of Wageningen Agricultural University provided an excellent environment for studying the mechanisms of cyanogen removal from cassava tissue. The Netherlands Ministry for Development Cooperation (DGIS) funded the research project 'Detoxification of cassava at household level in rural Africa', from 1990 - 1994. Its aim was to provide knowledge for the improvement of the domestic processing of cassava. Part of the project was carried out in Uganda and Malawi, where it also aimed at increasing the national research capacity. The results have been given in a report which serves as a basis for extension activities. Field studies in Mozambique and Uganda, as well as laboratory studies in Wageningen are presented in this thesis.

Together with colleagues from the Nutrition Department of the Ministry of Health, I recently had the opportunity to feed back results to the target population in Nampula province, Mozambique. Alternative processing methods were tested and adapted in the field with groups of apparently motivated female farmers. We formulated a message and trained local extensionists as part of a campaign to promote effective processing.[1]
It is my hope that the results of these projects and this thesis may benefit the cassava farmers who are occasionally at risk of intoxication by the crop which forms the basis of their existence. I dedicate this thesis to the cassava cultivating peasant population in Africa.

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[1]The poem at page 129/130 was a result of the training course.

Part of a hugh market for heap-fermented cassava in Paída, West-Nile, Uganda (note the high stuffed sacks for export out of the county)

CONTENTS

GENERAL INTRODUCTION

The crop cassava

Cassava is a tropical shrub of which the starchy storage roots, and in some areas also the leaves, are important as food. It was domesticated by Indians living in northeastern South America several thousands years ago. Cassava was brought by the Portuguese to West Africa during the last half of the sixteenth century. It was introduced to East Africa and Asia during the eighteenth century (Jones, 1959: 28-32). Cassava continues to be an important crop in South America, it has become so in Asia, and in Africa it has become extremely important, with an estimated root production in 1986 was 30, 41, and 57 x 10^6 Mt/annum, respectively. Cassava roots provide 30% of Africa's staple food (De Bruijn and Fresco, 1989). The crop's agronomic advantages, such as high productivity, ease and flexibility of cultivation, tolerance to drought, and its ability to grow well on relatively poor soils probably made its adoption in Africa so rapid and extensive.

Drawbacks of cassava roots in terms of food are their bulkiness, their perishability, their low protein content and their potential toxicity. The roots contain about 65% water and they deteriorate within 4 days after harvest. This makes them difficult to transport and market. As the protein level is lower than that of other staple foods, cassava roots have to be supplemented with protein-rich foods to form a balanced diet. Cassava leaves contain a high amount of protein with a moderate net utilization. Considering this, the crop cassava is not low in proteins. The potential toxicity is related to the capacity of all parts of the plant to release hydrogen cyanide from stored cyanogenic glucosides. This ability is known as cyanogenesis.

The character of cassava cyanogenesis

The cyanogenic potential of cassava is due to the presence of two cyanogenic glucosides, linamarin and lotaustralin in a ratio of about twenty to one (Nartey; Butler; Bisset *et al.,* quoted in De Bruijn, 1971). Other compartments of the same tissue contain the enzyme linamarase, which can hydrolyse these glucosides to yield the respective cyanohydrins and glucose. Disruption of the cell structure initiates this hydrolysis. The unstable cyanohydrins yield the toxic compound hydrogen cyanide (Conn, 1973; Cooke, 1978; Fig 1). Due to their capacity to splitt off the

cyanide ion, these three compounds are known as cyanogens.

The term "bitter" cassava, as oposed to "sweet" cassava, refers to the taste of the root parenchyma. Bitterness is associated with higher levels of cyanogenic glucosides (Cock, 1985; Miya *et al.*, 1975). Cultivars that usually give bitter roots are called bitter cultivars. However, certain ecological stress factors, such as pest attacks, prolonged drought and low phosphorus and potassium levels in the soil may cause roots to acquire bitterness, and this coincides with an increase in the levels of cyanogenic glucosides (Ayanru & Sharma, 1984/5; De Bruijn, 1971). Reported levels of cyanogenic glucosides in fresh root parenchyma have been from 10 up to 2000 mg HCN equivalent per kg dry weight (Coursey, 1973).

Linamarin and linamarase levels vary widely between cassava cultivars, between plants of the same cultivar, between different tissues of the same plant, between roots of the same plant and even within the root parenchyma (De Bruijn, 1971; Bourdoux *et al.*, 1980). This heterogenity in the distribution of the cyanogenic glucosides makes research on cassava cyanogen levels demanding in terms of number and size of samples in both experimental and observational studies.
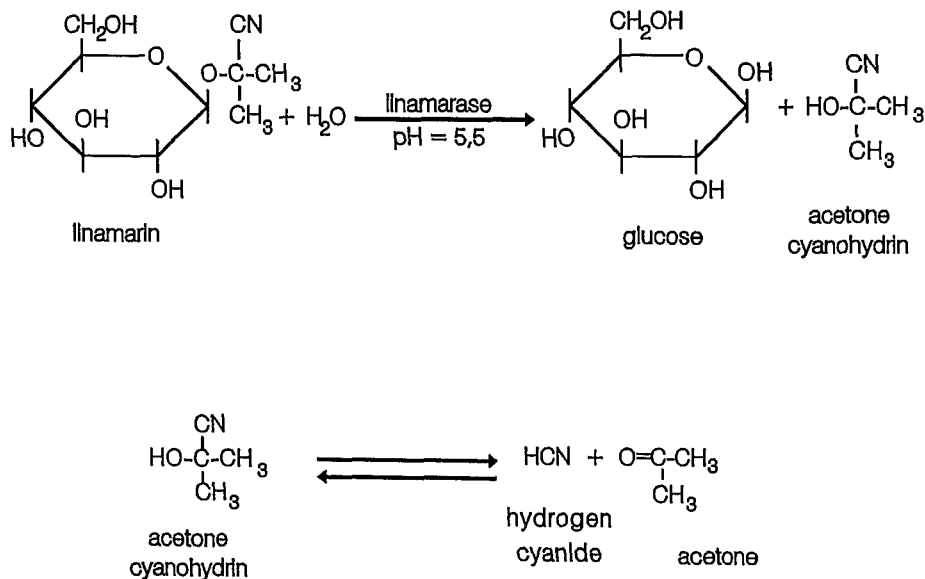

Figure 1 Degradation of Linamarin

Analysis of cyanogenic compounds

To study the degradation and removal of cyanogens during cassava processing, and to monitor the residual levels in obtained products, separate quantification of cyanogenic glucosides, cyanohydrins and HCN is a prerequisite. The analytical methods known showed inconveniences, inaccuracies or systematic errors. The most promising method, an enzymic assay developed by Cooke (1978, 1979), enabled quantification of cyanogenic glucosides, as well as cyanohydrins plus HCN, but it had not been compared with the long-accepted laborious method based on autolysis, steam-distillation and titration (AOAC, 1984).

Processing and cyanogen removal

Cassava starchy roots are processed to make them storable, to reduce their bulk, to increase their palatability, and to reduce their potential toxicity. The large range of processing methods and products has been reviewed by Gietema-Groenendijk (1970) and Lancaster *et al.* (1982). Sweet roots can be eaten raw or boiled, but a more elaborate processing is applied when roots are considered bitter or toxic. Staple foods in Africa are often eaten as a paste with a sauce. A cassava paste can be obtained by mashing boiled cassava roots, steaming the dough of soaked roots, mixing roasted granules with hot or cold water, or by mixing flour obtained from dried root with boiling water. The easiest way to obtain flour is sun-drying of peeled roots. Drying can be preceded by soaking in water, by crushing or keeping roots covered, or by various combinations of these procedures. The organoleptic properties of the products obtained show a great diversity, and the different processing methods are not equally effective in achieving cyanogen removal. The elaborate grating, squeezing and roasting of cassava, leading to the convenience food *"gari"* is applied in several West African countries and appears to be very effective in cyanogen removal (Meuser and Smolnik, 1980). The later introduction of the crop into East Africa without processing methods being communicated can be hypothesized to be a factor responsible for the less elaborate and less effective processing methods applied there. Another explanation may be that, as cultivars with high cyanogen levels had been introduced to East Africa more recently, the necessity for elaborate processing was not there earlier.

Applying only sun-drying to peeled roots, as practised in several East African countries, was found to diminish the total cyanogen level insufficiently from cassava roots with high initial cyanogenic potential (Pieris *et al.*, 1974). This was also observed during the extreme conditions of drought and food shortage in 1981 in Memba district in Mozambique, when roots were also consumed after only short drying periods (Ministry of Health, 1984b). Direct sun-drying remains the main processing method in vast areas of Africa, especially where there is certainty of sunshine for a long period, like in Nampula province in Mozambique. Studies on

the effects of drying on cyanogen levels in cassava roots were reviewed by Coursey (1973) and showed many inconsistencies. These studies did not show if and how, within this process, cyanogen removal may be enhanced.

In some areas of Mozambique and Uganda cassava roots are processed in such a way that they get covered with moulds, resulting in a product which looks dirty to outsiders. The lack of purity and cleanness (Douglas, 1966) has raised concern among the health and agricultural authorities, but no study had so far been done into the reasons for this process, its effectiveness in reducing cyanogen levels, and other toxicological and nutritional aspects of the products.

Toxic effects from cassava cyanogens
Several health problems have been attributed to cyanide exposure from insufficiently processed cassava. Occasional acute poisonings following meals of cassava are known for centuries. In 1887 the crew of Stanley's remarkable expedition through Congo, present-day Zaire, suffered from sometimes fatal acute poisonings when bitter cassava roots were consumed without the extensive soaking as applied by the local inhabitants (Manning, 1985). Acute toxic effects after cassava meals were also reported from the coastal area of Nampula province in Mozambique (Ministry of Health, 1984a). Thiocyanate, the main metabolite of cyanide in humans, had been identified as aggravating iodine deficiency and increasing the prevalence of goitre in a Zairian population consuming insufficiently processed cassava (Ermans *et al.*, 1980). In Nigeria, Osuntokun (1981) found an association between a neurologic degenerative disease, tropical ataxic neuropathy, and long-term moderate cyanide exposure from cassava consumption. An epidemic of spastic paraparesis took place in the coastal area of Nampula province in 1981, and dietary cyanogen uptake from insufficiently processed cassava was suggested as the main etiological factor (Ministry of Health, 1984a,b). The paralytic disease and its possible relation with cassava consumption had previously only been reported from present Zaire, where it was named konzo (Trolli, 1938).

In October 1982, the health authorities in an inland district in Nampula province reported that 11 persons had suddenly been afflicted with paralysis of the legs. It was unclear whether the disease was identical to the one that had been observed in coastal districts in the previous year, and whether the same link to cassava toxicity existed in the inland district. The investigation of the paralytic disease and the simultaneous acute poisonings formed the start of this research and led to the question of how it can be prevented. Answers were sought along the line that the cassava farmers in Nampula province tried to tackle the problem of the acute poisonings from cassava meals: by improving the processing of the roots.

Research objectives

The objective of this research is to elucidate the mechanisms of cyanogen removal during sun-drying and heap-fermentation of bitter and potentially toxic cassava roots, in order to identify key messages for the promotion of processing techniques with effective cyanogen removal.

The more specific aims are:

To study whether an outbreak of paralysis and acute poisoning in a rural population of Mozambique was associated with cassava toxicity. (Chapter 2)

To evaluate and improve a promising method for the determination of cyanogens in cassava products. (Chapter 3)

To study the influence of the dehydration rate on the dynamics of cyanogen removal during sun-drying of cassava roots. (Chapter 4)

To study cyanogen and aflatoxin levels of flour from moulded, domestically sun-dried and stored cassava roots in northern Mozambique. (Chapter 5)

To describe the traditional solid-substrate fermentation applied to cassava roots in Uganda, and evaluate aspects of cyanogen removal and mycotoxin formation. (Chapter 6)

To study if and how the microflora effects the cyanogen removal during solid-substrate fermentation of cassava. (Chapters 7 and 8)

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INSUFFICIENT PROCESSING OF CASSAVA INDUCED ACUTE INTOXICATIONS AND THE PARALYTIC DISEASE KONZO IN A RURAL AREA OF MOZAMBIQUE

AJ Alexander Essers, Per Alsén & Hans Rosling

A small outbreak of paralysis was studied in a rural population in Mozambique. The seven subjects examined had an isolated spastic paraparesis with abrupt onset during a food shortage period following a drought. The diet at onset consisted almost exclusively of leaves and insufficiently processed roots from a recently introduced high-yielding bitter cassava cultivar. The affected population reported frequent symptoms of acute poisonings some hours after meals, and related that to the bitter cassava and the processing applied to it. A high dietary cyanide exposure from cassava in the affected subjects was indicated by high urinary and serum levels of thiocyanate, the main metabolite. We conclude that the observed walking disability was konzo, a recently identified disease entity which has been reported from four widely separated rural areas of Africa. Our dietary and laboratory findings are consistent with those from other konzo affected areas and they support a causal role of cyanide exposure. Effective processing is crucial to avoid toxic effects from cassava.

INTRODUCTION

Due to high yield and drought tolerance cassava is an important staple crop in many parts of Africa. The roots are rich in energy due to high carbohydrate contents, but low in protein and contain varying amounts of cyanogenic glucosides. Bitter cultivars often give higher yields but contain more cyanogenic glucosides (Cock, 1982). Prior to consumption these glucosides must be removed by processing to avoid dietary cyanide exposure (Cooke, 1983). Effective removal of cyanogenic compounds from cassava by household means is possible (Dufour, 1989). Consumption of insufficiently processed cassava may cause acute cyanide intoxication (Cheok, 1978). Moderate cyanide exposure from cassava over many years has been associated with tropical ataxic neuropathy, a paralytic disease with slow onset (Osuntokun, 1981). Continuous high dietary cyanide exposure from cassava for several weeks has been linked to the paralytic disease konzo which is characterized by abrupt onset of permanent spastic paralysis of both legs (Howlett *et al.*, 1990). Konzo is named after the local designation in the Zairian population first affected (Trolli, 1938) and has also been reported as epidemic spastic paraparesis. In addition to Zaire (Tylleskar *et al.*, 1991), konzo has been reported as epidemics in drought affected rural populations in the north east part of Nampula province, Mozambique (Ministry of Health, 1984a) and in two parts of Tanzania (Howlett *et al.*, 1990, Mlingi *et al.*, 1991). The epidemics in East Africa coincided with food shortage and several weeks of exclusive consumption of insufficiently processed bitter cassava roots resulting in high dietary cyanide exposure, as verified by high levels of thiocyanate in serum and urine (Ministry of Health, 1984b). In the human organism cyanide is detoxified by enzymic conversion to thiocyanate using sulphur originating from dietary sulphur amino acids. A high intake of cyanide in combination with a low intake of sulphur amino acids has been advanced as the main aetiology, but other dietary deficiencies and predisposing factors may also contribute to it (Cliff, 1985). A dietary cause has been refuted and an infectious aetiology suggested by Carton *et al.* (1986).

In October 1982 the District Health Officer reported a small outbreak of paralysis affecting 11 women and children in a rural area of Murrupula District in northern Mozambique (Fig 1). This paper reports the clinical, laboratory and dietary investigations of seven of these subjects. They had histories, symptoms and signs typical of konzo. The results from interviews on the dietary and food situation support a toxico-nutritional aetiology of the disease and characterize the agro-ecological problems associated with acute intoxications from cassava and the outbreak of konzo.

STUDY AREA

Murrupula district constitutes the southwest part of Nampula Province in northern Mozambique (Fig 1). A 75 kilometer tarmac road connects the district to the provincial capital. The landscape is slightly hilly at an altitude of 200 - 500 m. Mean temperature is 25°C and rainfall 1000 to 1400 mm per year with a pronounced dry period from May to November (Fig 2). The soils are red brown fersalytic and vegetation is bushy savannah with cultivated fields. Main crops were cassava, rice, sorghum, maize, peanuts and pulses, which were produced in a shifting cultivation, and cashew. Population density was 20 to 40 inhabitants per square km. (Ministério da Educação e Cultura, 1979).
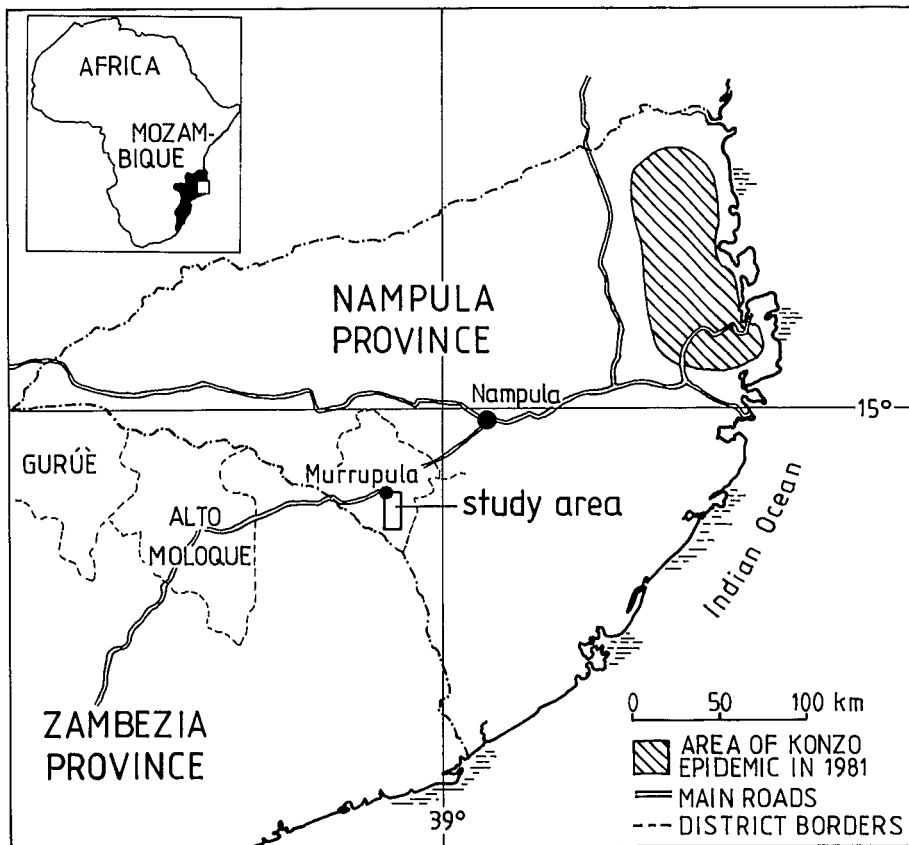
FIGURE 1 Map of study area

SUBJECTS AND METHODS

Seven of the persons suffering from paralysis in both legs were examined on December 7, 1982: five were admitted to the health centre in the small district centre of Murrupula due to this illness, and two persons were examined in their homes in the village of Nahá. All were eating food prepared and provided by their families. The seven paralysed persons were interviewed by a physician (P.A.) regarding symptoms and time as well as type of onset. A general and neurological examination was performed and blood as well as urine specimens were collected. After separation of serum the specimens were kept frozen until analysis. Serum and urine thiocyanate (Lundquist *et al.*, 1979, 1983), urinary sulphate (Lundquist *et al.*, 1980) and urinary creatinine were determined at Linköping University Hospital, Sweden, in order to be used as indicators for the intake of cyanide and sulphur amino acids. Serum was also tested for antibodies to HTLV-1 (Abbott-ELISA).

One of the seven persons affected was living on the outskirts of the district centre, two in the village of Naha 16 km south of Murrupula and 4 were living distant from each other 4-7 km to the south of Nahá. The affected area of about 150 square kilometers was estimated to have 3000 to 6000 inhabitants, suggesting a konzo prevalence of at least one per thousand. In the last week of December each of these seven persons was re-interviewed in their homes with regard to food and dietary situation by a nutritionist from the provincial health service (AJA.E.). Local health staff translated to and from the local language macua. A 24-hour recall food consumption and dietary history study was performed (Den Hartog & Van Staveren, 1986). The affected persons were asked what food items had been consumed in the past 24 hours on a meal by meal basis and how they had been prepared. Subsequently, they were asked what staple foods had been consumed in the weeks preceding the onset, how those had been processed/prepared, what side dishes and snacks were used and if uncommon items had entered the diet. Information was collected in each household and observations were made regarding production of food crops with special reference to cassava cultivars and processing methods used. Information on agriculture and food situation was also collected from key informants in the affected communities, the district health centre and the district state marketing enterprise.
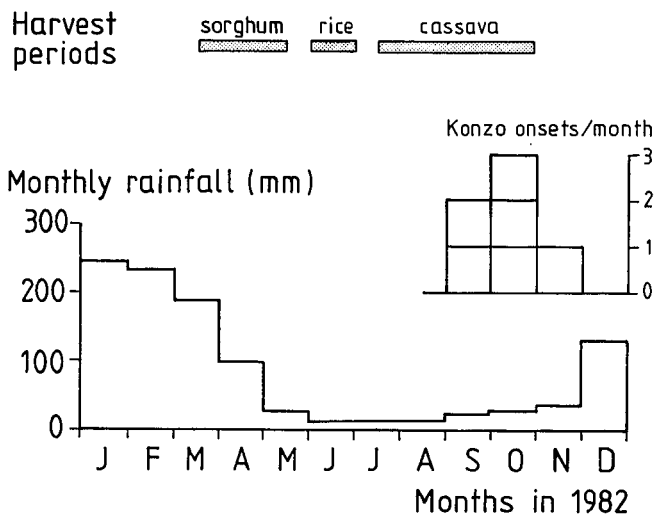
RESULTS

Clinical findings
Six onsets of konzo occurred in September - November 1982 and one onset had occurred in the same season in September 1981 (Table 1, Fig 2). In all seven subjects the onset of paralysis occurred abruptly within one day with some

progression during the following days (range 1 to 7 days). Some minor sensory symptoms in the legs passed after the first week. The symptoms had remained unchanged thereafter in all cases.

All seven had a symmetric spastic paraparesis with bilaterally exaggerated knee reflexes and ankle clonus, but the paraparesis varied in severity. Five cases were unable to walk without support and four of these also had exaggerated arm reflexes. Speech difficulties occurred in two cases. Sensation was normal in all and no other neurological abnormalities were found.

At the time of the interviews, two subjects were in a session with a traditional healer, who was trying to influence the bad spell that was believed to have caused the crippling.

FIGURE 2 Monthly rainfall, harvest periods and onsets of konzo per month

Laboratory findings
The results of the serum and urine analyses, as well as reference data, are given in Figure 3. Urine SCN and SO_4 creatinine ratios have been plotted for six subjects only, as the urine quantity was insufficient for SO_4 and creatinine analysis in the last subject. However, her urine SCN level was in the same range as for the other six. The mean ($\pm$SEM) for serum SCN was 195 ($\pm$39) μmol/l, for urinary SCN/creatinine 51 ($\pm$16) mmol/l and urinary SO_4/creatinine 0.81 ($\pm$0.34) mol/mol, respectively. The highest levels of serum and urinary SCN, as well as the lowest urine SO_4 level were found in subject 3, that also had the most recent onset. The opposite was found in case 5 who had the lowest serum and urine SCN levels and highest urine SO_4 level. All 7 subjects were seronegative to HTLV-1.
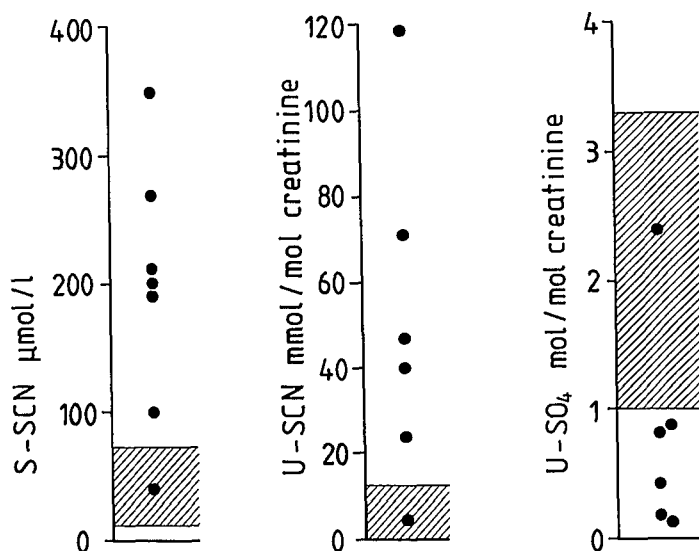
FIGURE 3 Urinary creatinine ratios for thiocyanate and inorganic sulphate and serum thiocyanate levels compared to reference values ($\pm$ 2SEM) for Swedisch children (shaded areas)

General Food Situation

The main staple food was cassava, and sometimes - especially from April until August - rice and sorghum also served as a staple. The rice produced was usually sold. The annual amount of cassava roots needed is harvested during some weeks in September and October and then, after peeling and sun-drying, it is stored for the rest of the year (Fig 2). The staple food, made from cassava flour, is a stiff porridge, which used to be accompanied by a sauce, mainly made from - cassava - leaves, often enriched with some peanuts or cashew nuts (Essers, 1989). The sauce may also contain river or sea fish, crabs, beans, meat, mushrooms or insects.

The overall food production in the area had usually been sufficient. According to the district officer of the state marketing enterprise, the production of rice, sorghum, peanut and cashew was low in 1982 in comparison with earlier years, due to low rainfall. Other local informants confirmed that in 1982 crops apart from cassava had relatively low yields.

Cassava Cultivars

The eight cassava cultivars produced were named: *Makela, Nepathagonha, Nakwale, Ntsiricano, Fernando, Puane, Kampinche* and *Mukorodje. Makela* was the only cultivar with bitter roots and the population unequivocally associated the acute intoxications, described below, with consumption of these roots. The skin of

the root is red and coarse. The leaves are medium broad shaped and dark green. The petioles are red. The plant has a cycle of 18-24 months and reaches a height of about 4 m. All informants stated that its yield is higher than that of other cultivars, especially on poor, dry soils. To quote an illustrative phrase from a local informant: "Those people are poor, they only have fields on the hillside which has poor soil, so only *Makela* can produce there. That is why they only have bitter cassava and get headaches."

Two local informants independently stated that this cultivar was brought to Murrupula from the district of Alto Moloque in the neighbouring province Zambezia by the colonial administrator following a drought in 1967. Production was restricted to his own fields and to those of the colonial supported chiefs. When prisoners were suffering acute intoxication after a meal of sun-dried *Makela*, the administrator ordered the application of heap-fermentation, a processing method used in Alto Moloque. Processed this way, the *Makela* roots did not provoke dramatic reactions anymore. After the independence in 1975 the population attacked the administrator's fields to obtain stem cuttings of *Makela*, and the cultivar was successively propagated in the District.

Cassava Processing
The informants reported that the following four processing methods are applied on peeled *Makela* roots before eventually pounding them into flour, which serves to make a stiff porridge.
Sun-drying of large pieces is practiced by cutting roots into 20 cm long pieces which, if regarded as being too thick, are split longitudinally. Subsequently, the pieces are sun-dried on elevated racks for one to three weeks. The product is called "makaka".
Sun-drying of small pieces is applied on the root extremities, thin roots or on bigger roots that are purposely sliced into pieces of about 1 cm thick. These pieces need sun-drying for only 5 to 7 days and these "ekonho" are the first to be consumed after the cassava harvest.
Fresh-pounding of roots results in crushed particles of millimeter to centimeter size that are sun-dried in one or two days.
Heap-fermentation is a method where a heap of peeled roots is covered with leaves or hay for 2 - 4 days to provoke mould growth. The mould-covered roots are thereafter spread out for sun-drying.

According to the population, the heap-fermentation method is the most effective in preventing acute toxic effects, whereas consumption of fresh-pounded roots implied the highest risk of intoxication. The latter has the advantage of being the fastest, though. We found the heap-fermentation practices to vary between households. Some people cover the cassava well and use for this method the name

"ompopothela", which means "covering". Others just heap the roots uncovered, before sun-drying. "Makaka" stores better and has a higher market value than "ekonho", which has the advantage of being available faster. One elderly peasant woman stated that in the Alto Moloque district, from where this bitter cultivar was introduced, soaking in water was also used to remove the toxic constituents, "But it would be too heavy to bring water up here to soak the roots, so we just cover them", she said.

Diet of Affected Families

All victims stated, retrospectively, that in the weeks preceding the onset of the paralysis, their staple food consisted only of cassava porridge either made solely from *Makela*, or mixed with sweet cassava. Cassava flour used at onset was made from sun-dried small pieces, "ekonho" in most households. One household made flour from freshly pounded roots and another consumed raw roots besides heap-fermented ones (Table 1). The sauce was made up of plain pounded cassava leaves, rarely enriched with other foods. At the time of the interview, some months after the period of the onset of paralysis, cassava was still the only staple food consumed, but the roots had undergone one of the lengthier processing methods and the side dishes were somewhat more varied.

Six of the seven households investigated reported one or more episodes of acute intoxication one or more hours after eating products from bitter cassava over the last two years. The symptoms mentioned were vomiting, diarrhoea, palpitations, dizziness, drunkenness, muscle weakness, apathy and loss of appetite. Those affected were well aware of a link between the consumption of poorly processed bitter cassava and these acute intoxications, but no relationship between this diet and the abrupt onsets of spastic paraparesis was recognised by them.

Three households stated that the 1981 cassava harvest was poorer than normal. This and the poor harvest of other staples in 1982 caused the affected households to start uprooting cassava in 1982 bit by bit before the normal harvest time.

Members of the affected households complained of a lack of side dishes in 1982. The consumption of food items not ordinarily used, due to the relative scarcity of foods, was inquired after, but the answers did not yield any specific item. No cassava peels had been consumed or stored. None of the households had suffered famine. The household of subject 5 seemed slightly better off (considering their housing, tools and literacy) compared to the other affected households and it had a high production of rice, sorghum and cassava in 1982. Still, at the time of onset, this subject also had a diet dominated by recently sun-dried small pieces of bitter cassava. The cassava stock from 1981 had been low due to illnesses in this household, while the other food products had been sold or used for other purposes.

The 24-hour recall revealed that at the time of the interview the staple food at

TABLE 1
Cassava consumed at onset and at interview by the konzo affected subjects

Case (nr)	Age (yrs)	Sex	Date of onset month	year	Cassava consumed at onset cultivar	processed form	Cassava consumed at interview cultivar	processed form
1	26	F	10	1982	*Makela* +S	heap-fermented +raw	*Makela*	heap-fermented
2	22	F	09	1982	*Makela* +S	fresh-pounded	*Makela* +S	sun-dried large pcs
3	32	F	11	1982	*Makela*	sun-dried small pcs	*Makela*	heap-fermented
4	23	F	09	1981	*Makela*	sun-dried small pcs	*Makela*	sun-dried large pcs
5	6	M	09	1982	*Makela*	sun-dried small pcs	*Makela* +S	sun-dried large pcs
6	8	M	10	1982	*Makela* +S	sun-dried small pcs	S	sun-dried large pcs
7	7	F	10	1982	*Makela* +S	sun-dried small pcs	*Makela* +S	heap-fermented

S = sweet cultivar(s)

all meals of those affected was a porridge made out of cassava, either the bitter *Makela* solely or mixed with sweet cassava, or sweet cassava only. The flour used was from roots that were either heap-fermented or sun-dried in large pieces (Table 1). The sauce was made mainly of cooked pounded cassava leaves, sometimes enriched with cashew nuts, or of flying ants, fish, chicken, or mushrooms.

The use of tobacco was either sparse or absent in the crippled subjects.

DISCUSSION

The findings of a non-progressive isolated spastic paraparesis with acute onset in all examined cases are identical to clinical findings in konzo cases in other affected areas (Ministry of Health, 1984a; Howlett, 1990; Tylleskar *et al.*, 1991; Mlingi *et al.*, 1991). The negative tests for HTLV-1 infection and the abrupt onsets during a certain period of the year in all of the cases exclude HTLV-1 associated tropical spastic paraparesis (Kayembe *et al.*, 1990). The affected population neither grow nor consume lathyrus sativa and the myelopathy called lathyrism caused by exclusive consumption of this pea can also be excluded. We conclude that the studied disease is konzo.

The dietary situation at the time of onset is also very similar to that associated with konzo epidemics in the northeast part of Nampula province in 1981 (Ministry of Health, 1984a), Tanzania (Howlett *et al.*, 1990; Mlingi *et al.*, 1991) and Zaire (Tylleskar *et al.*, 1991). Cassava was the sole staple food, of which insufficiently processed bitter roots formed the main part in all cases, while side dishes consisted almost exclusively of cassava leaves.

When the cell structure is disintegrated during the processing of cassava, the cyanogenic glucosides break down into glucose and cyanohydrins through activity

of endogenous enzymes. Cyanohydrins gradually break down into ketones and hydrogen cyanide, a volatile toxin that escapes rapidly into the air (Bourdoux *et al.*, 1980). If ingested, all three cyanogenic compounds may result in cyanide exposure. Mould growth, which occurs with heaping the cassava, may reduce the levels of cyanogenic compounds considerably (Essers & Nout, 1989). During sun-drying, the levels of cyanogenic compounds drop slowly and when dried, during storage, at an even slower pace (Cooke, 1983). Small pieces dry faster than bigger ones, thereby limiting the time for endogenous enzyme activity. Hence, the breakdown of cyanogenic compounds is likely to be less complete in smaller pieces, leaving them with higher residual cyanogen levels than larger pieces.

The diet at the time of the interviews differed from that at onset with regard to the processing of the cassava roots and the period between harvesting/processing and consumption. The onset of konzo coincided with the consumption of recently processed small cassava pieces, while at the time of the interviews the cassava consumed originated from either sun-dried large pieces or from heap-fermented roots, harvested and processed 1 to 3 months before. These factors will have resulted in a higher level of cyanogenic compounds in cassava flour at the time of onset than at collection of specimen.

The elevated thiocyanate levels (Fig 3) indicate a high dietary cyanide exposure from the almost exclusive consumption of sun-dried cassava revealed by the 24-hour recall. Cassava leaves may also have contributed to the exposure but no other source of cyanide or thiocyanate was traced. The mean urinary SCN/creatinine ratio found in the presently studied konzo affected subjects is more than five times higher than the mean value found in urban Mozambican schoolchildren in the provincial capital of Nampula (Cliff *et al.*, 1985). The thiocyanate levels are somewhat lower than those earlier reported at the onsets of konzo (Ministry of Health, 1984a; Howlett *et al.*, 1990). This can be explained by the collection of specimens 1 to 3 months after onset when stored cassava products had lost more of their level of cyanogenic compounds and briefly processed cassava was not consumed (Table 1). Therefore, SCN levels in the population were already on the decline since right after harvest time. A pronounced seasonal variation in dietary cyanide exposure, with a peak during epidemics has, in fact, been shown in other konzo-affected populations by Casadei *et al.* (1990) and Howlett *et al.* (1990) who, two months after the onset of konzo epidemics, found similar levels of thiocyanate in urine and serum, respectively, as those found in this study.

The low urine levels of inorganic sulphate are indicative of a low intake of sulphur amino acids (Sabry *et al.*, 1965), and are consistent with the low intake of protein-rich foods brought to light in the interviews. Although cassava leaves contain high levels of protein, sulfur amino acid levels are relatively low and their utilisation is further impaired by high tannin content (Ross and Enriquez, 1969).

The mean urinary SO_4/creatinine ratio is nearly a half of the mean value found in urban Mozambican schoolchildren in Nampula (Cliff *et al.*, 1985).

Laboratory findings in the normal range were only found in subject 5, that belonged to the only household with a high food production. However, also in that case the cassava consumed in the period at onset had been exclusively derived from small sun-dried pieces of *Makela*.

The present findings, in a fifth rural area of Africa, of an identical association between konzo and cyanide exposure from insufficiently processed cassava aggravated by low sulphur intake provide strong additional support for an aetiological role of this diet.

The food situation that resulted in the diet inducing konzo and acute intoxications from cassava developed due to several factors. An underlying cause was decreased availability of fertile land which forced families to cultivate on marginal land, where the newly introduced bitter cassava cultivar was the only crop with a good yield. The identical botanical characteristics and common geographical origin indicate that *Makela* is the same as the cultivar named *"Gurue"* in the north-east of Nampula province, which was the one most associated with dietary cyanide exposure inducing the epidemic of konzo (Ministry of Health, 1984a,b). Mota (1970) reported a level of 135 mg of cyanide per kg of fresh root pulp in *"Maquela"*, and considered this cultivar highly toxic. The propagation of *Makela* started in 1975 in Murrupula district and by 1980 there was apparently enough production and subsequent consumption to result in considerable cyanide exposure when inefficient processing methods were used.

Low rainfall deteriorated the food situation in 1981 and 1982, causing the stock of sun-dried cassava from 1981 to be depleted earlier than usual. Although neither the affected households, nor their communities were facing famine, the diet at the end of the dry season consisted almost exclusively of briefly processed roots of bitter cassava, and cassava leaves.

In conclusion, we found a rural community where food security during the lean months of a drought year was provided by a newly introduced high yielding bitter cassava cultivar. The population tried to cope with its toxic properties by changing processing methods, but failed to some extent. This resulted in high dietary cyanide exposure with subsequent acute intoxications and the paralytic disease konzo.

The study shows a situation that several cassava growing areas in Africa are heading for: Sweet cultivars are being replaced by bitter ones, due to higher productivity under adverse agro-ecological conditions. Cassava plants increase their level of cyanogenic glucosides under certain stress conditions such as soils depleted of potassium, prolonged drought (De Bruijn, 1971) and insect attack (Ayanru & Sharma, 1984/5). Under such conditions and time constraints, local processing

methods may not be adequate anymore in reducing the cyanogen contents to innocuous levels. Lack of other foods, especially protein-rich side dishes, which is common in agricultural stress situations, aggravate the toxic effects of the cyanide exposure. Effective processing methods for cassava should be promoted in such areas.

ACKNOWLEDGEMENTS

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STUDIES ON THE QUANTIFICATION OF SPECIFIC CYANOGENS IN CASSAVA PRODUCTS AND INTRODUCTION OF A NEW CHROMOGEN

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The enzymic assay for cyanogens in cassava as developed by Cooke (1978), and improved by O'Brien *et al.* (1991), was compared with a standard method, involving autolysis/steam-distillation/titration, and, after found more useful, further improved with a more acceptable coloration and other minor changes. Cooke's assay, using a linamarin calibration curve, gave similar values for cyanogenic potential in fresh cassava as the standard method. For cassava samples with high non-glycosidic cyanogen levels, Cooke's assay yielded slightly lower values. Isonicotinate / 1,3-dimethyl barbiturate as reagent in the König reaction had the following advantages compared with the so far applied pyridine / pyrazolone colour reagent: It is less toxic and does not release repulsive vapours. It is faster, cheaper and easier to handle, and has increased sensitivity and longer storability. Direct measurement of cyanogenic potential was accurate in extracts containing 35 - 700 μM. Recovery of linamarin supplements was 102 $\pm$ 4%. Separate calibration curves of linamarin, acetone cyanohydrin and KCN were necessary for accurate calculation of cyanogenic glycosides and cyanohydrin levels. Extract storability depended slightly on storage temperature, but was not changed by inclusion of ethanol in the extract medium.

INTRODUCTION

Cassava is an important tropical root crop, ranking fourth on the list of major food crops in developing countries (De Bruijn & Fresco, 1989). An item of concern for public health authorities, plant breeders, producers, processors and principally the consumers, is its toxic potential. The potential toxicity is due to the presence of cyanogenic glycosides, linamarin and lotaustralin in a ratio of approx 20 : 1 (Nartey; Butler; Bisset *et al.*, quoted in De Bruijn, 1971) which, after hydrolysis into cyanohydrins yield the toxic compound hydrogen cyanide. The cyanogenic potential of insufficiently processed cassava has been identified as a factor in health problems like acute toxic effects (Mlingi *et al.*, 1992), iodine deficiency disorders (Ermans *et al.*, 1983), tropical ataxic neuropathy (Osuntokun, 1981) and the paralytic disease konzo (Tylleskär *et al.*, 1992). Ingested cyanohydrins are considered to decompose at the alkaline pH level in the small intestines to yield an equal molar amount of cyanide. The toxicity of ingested glycosides in man is not well understood yet. Toxic effects may occur if suitable glycosidases are present in the gut flora (Bourdoux *et al.*, 1980).

This potential toxicity of a widely consumed staple food crop requires a reliable, yet simple, fast and cheap analytical method to measure the three distinct cyanogenic constituents. The separate quantification of these three cyanogens is also a prerequisite for studies of the breakdown and removal of cyanogens during processing of cassava, and for monitoring safety of obtained products.

Cyanogen analysis

In the analysis of cyanogenic compounds in cassava tissue the following steps can be distinguished: Sampling, homogenisation, extraction, transformation of cyanogenic compounds to HCN and CN⁻, isolation, measurement and calculation from standards. All existing methods involve a combination of several of these steps. Two important methods can be characterized as follows:

1 The "steam-distillation method", which comprises Autolysis of grated cassava, followed by Steam-distillation to isolate the HCN, and subsequent Titration (Williams, 1990; De Bruijn, 1971). It is further referred to as AST method in this article.

2 The "enzymic assay" (Cooke, 1978; O'Brien *et al.*, 1991), which comprises homogenization in acid, filtering, neutralisation, hydrolysis by exogenous enzyme and spectrophotometry after coloration of a pyridine/pyrazolone reagent.

The AST is the since long by the American Organization for Analytical Chemistry (AOAC) officially accepted standard method (Williams, 1990). According to Cooke (1978), the enzymic is superior to the AST method on the basis of minimising assumed side reactions, by avoiding dependency of endogenous linamarase, by an

established high recovery (90-95%) of linamarin supplement in cassava extract, by being faster, and able to distinguish between glycosidic and non-glycosidic cyanide (ngCN). 0.1 mg kg^{-1} was given as the detection limit. However, a direct comparison with the standard AST method was never presented.

Recently, O'Brien *et al.* (1991) improved the enzymic method to the extent that the HCN fraction could be quantified by maintaining the pH low for the free cyanide assay, and inclusion of 25% ethanol in the extraction medium resulted in a larger volume of supernatant in centrifuged gelatinized products. An improved storability of the extracts was suggested. Ikediobi *et al.* (1980) tried to replace the pyridine/pyrazolone colour reagent with picrate but this reagent is not very specific for cyanide in the Guignard reaction (Zitnak, 1973; Arihantana and Buckle, 1986). Bradbury *et al.* (1991) managed to replace the use of the expensive enzyme linamarase by an acid hydrolysis, but this resulted in a more laborious and time-consuming assay and sometimes lower recoveries of linamarin supplement.

We experienced the following constraints with the enzymic assay:

- The coloration technique used is slow, and the involved pyridine releases vapours which are toxic, mutagenic, irritating and repulsive (Sax & Lewis, 1989).
- The calibration curves obtained for linamarin, acetone cyanohydrin and KCN with their matching assays, differ somehow. This means that the calculation of glycosides and cyanohydrins by subtraction is not done accurately.
- Quantification of already present HCN is not well possible, due to its partial vaporization during the acid extraction and storage.
- The prescription of placing the test tubes in iced water after Chloramine T addition was sometimes a problem in developing countries and its practical benefit was also questioned during the development of an automated version of the method.
- The enzyme linamarase, used in the assay, is expensive and difficult to obtain.

The present study reports the comparability of the enzymic assay with the standard AST method, a substitute for the pyridine/pyrazolone colour reagent, accuracy and improvement of the assay, and storability of extracts.

EXPERIMENTAL

Materials

For extraction and recovery experiments fresh peeled roots of cultivars from Costa Rica, Wageningen greenhouses and Uganda were used. Flours were obtained by sun-drying, followed by pounding and sieving.

Linamarin was obtained from BDH Ltd (Poole, UK) and Sigma Chemical Company, St Louis (MO, USA); Linamarase (EC 3.2.1.21) from BDH Ltd; Isonicotinic acid (purum) and 1,3-dimethyl barbituric acid (puriss) from Fluka Chemie AG, Buchs,

Switzerland; acetone cyanohydrin (99%) from Aldridge Chemical Co., Steinheim, Germany; Potassium cyanide (KCN) and chloramine T from Merck, Darmstadt, Germany. Stock solutions and reagents were of analytical grade and prepared as follows: $AgNO_3$ titration solution was prepared by dilution of 1.00 M Titrisol to 0.01 and 0.001 M.

Buffers: pH 4, 4.5, 5, 6 and 7 were prepared from 0.1 M H_3PO_4 and 0.1 M Na_3PO_4.

Extraction media: Acid medium: 0.1 M orthophosphoric acid in water. Ethanol/acid medium: 0.1 M orthophosphoric acid containing 25% v/v ethanol (96%).

Stock solutions: KCN stock (5 mM) was made by dissolving KCN, previously dried over concentrated H_2SO_4, in 0.2 M NaOH. Linamarin stock (5 mM) was made in buffer pH 6. Acetone cyanohydrin (5 mM) stock was made in 0.1 M orthophosphoric acid. Standard solutions were made from the stock solutions by dissolving them in 0.1 M orthophosphoric acid just prior to analysis.

Linamarase solution: Enzyme was dissolved in buffer solution pH 6.0 to give an activity of 5 EU ml^{-1}, stored refrigerated and activity checked periodically, according to Cooke (1979), to be at least 3 EU ml^{-1}.

Chloramine T (0.5 g) was dissolved in 50 ml water and made up fresh every day.

Colour reagent (Y): 3.7 g NaOH was dissolved in 200 ml distilled water. Then, 7.0 g 1,3-dimethyl barbituric acid and 5.7 g isonicotinic acid were dissolved in this alkaline solution by extensive stirring. The pH was adjusted between 7 and 8 with 1 M HCl or NaOH.

Absorbances were measured with Beckman DU-62 and DU-64 spectrophotometers. Homogenization was done with a Braun MX2 kitchen blender.

The Autolysis/Steam-distillation/Titration (AST) cyanogen assay

The AST method according to Williams (1990) and de Bruijn (1971) was executed as follows: Approx. 10 - 20 g of grated, fresh cassava root material was submerged in 200 ml distilled water. After incubation for 18 to 20 hours at 37 °C, the suspension was steam-distilled until 200 ml had passed and collected in 20 ml 0.625 M NaOH solution, which was subsequently titrated potentiometrically with $AgNO_3$.

Revised enzymic cyanogen assay

The cyanogen assays as developed and applied here, based on the assays of Cooke (1979) and O'Brien *et al.* (1991), are schematically presented in Figure 1. They are both outcome and basic method for several tests in this study, and therefore included in this section. The complete assay measures the sum of the cyanogenic compounds by converting them all to HCN which triggers quantitatively the formation of a pigment by König reaction. Cyanohydrins and HCN as well as solely HCN are measured by leaving out the respective conversion steps in the assay. Glycosides and cyanohydrins are calculated from the difference.

	Diagram	*Rationale*
step 1	extraction in acid	linamarase activity is stopped; cyanohydrins are stabilised
	↓	
step 2	buffer the extract (pH 6); add linamarase; incubate	glycosides are hydrolysed to cyanohydrins and glucose
	↓	
step 3	raise pH to 12 with NaOH	cyanohydrins are converted to CN⁻
	↓	
step 4	lower pH to 3 - 7; add Chl-T; add colour reagent; incubate 10 min	each CN⁻ ion will trigger the formation of a big conjugated molecule with a resonance at 605 nm
	↓	
step 5	measure absorbance at 605 nm	

calibration
 curve   →

cyanogenic potential

non-glucosidic cyanogens: Same procedure without step 2.

free cyanide: Same procedure without steps 2 and 3, whilst pH has to be kept between 3 and 5.

FIGURE 1 Diagram of the assays of CNp, ngCN and free cyanide and the rationale of the steps

Assay procedures
Cyanogens were assayed in duplicate using the following formats:
1) Cyanogenic potential (CNp): 0.1 ml extract was added to 0.4 ml buffer pH 7 in a test tube, followed by addition of 0.1 ml linamarase solution. After 15 min incubation at 30°C, 0.6 ml NaOH (0.2 M) was added, followed after 5 min by 2.8 ml buffer pH 6. Coloration as described below.
2) Non-glycosidic cyanogens (ngCN): 0.1 ml extract was added to 0.6 ml of NaOH (0.2 M). After 2 min, 3.3 ml buffer pH 6 was added, followed by coloration.
3) Free cyanide (HCN): 0.1 ml extract was diluted with 3.9 ml buffer pH 4 and assayed colorimetrically.
All assays were carried out in 8 ml glass stoppered test tubes which were opened as short as possible for the necessary additions.

Colorimetric procedure
0.1 ml Chloramine T reagent was added to the 4 ml buffered extract in the stoppered

test tube and mixed. After 5 min, 0.6 ml of colour reagent was added and mixed. The absorbance at 605 nm was measured after 10 min. Reagent blanks were run for each analysis.

Calibration standards
Calibration curves were established, assaying at least 2 quantities in duplicate between 4 and 70 nmol of linamarin, acetone cyanohydrin or KCN per tube with the CNp, ngCN and the free cyanide method, respectively.

Calculation of cyanogen contents
Cyanogen levels were calculated in mg CN equivalent per kg sample on dry weight basis (mg CN eq kg^{-1} dry wt) as follows:

$$[CN] = \frac{x(v+s*\frac{m}{100})}{s(1-\frac{m}{100})*d}*0.026$$

s = sample weight (g), v = weight extraction medium (g), d = volume of extract assayed (ml), m = moisture content (%), and x = quantity of cyanogen in tube (nmol).
x is calculated from the calibration curve: x = $(A_{605}$ - a)/slope. Both slope and intercept (a) are derived from the calibration curve.
For very low values of **a** (<0.005) and higher values of A_{605} (>0.200) the intercept can be left out, as is done by O'Brien *et al.* (1991).

Sample extraction
Fresh root parts were cut into 1 cm^3 cubes and randomised; 50.0 - 70.0 g cassava cubes were homogenized in 250 ml refrigerated extraction medium in a blender for 15 s at low speed, followed by 1 min high speed, 1 min rest and 1 min full speed again. Flour extraction was by swirling gently 4.00 g in 25 ml refrigerated extraction medium in a 50 ml closed container for 5 min.
The homogenates were centrifugated in closed tubes at 10,000 x g for 15 min and the supernatant used as extract.

Adaptation of assay with new chromogen and testing of effect of concentration and ageing of colour reagent, buffer pH, concentration chlorination reagent and assay temperature
We started adapting the colour reagent from the molar ratio Chloramine T / isonicotinic acid / 1,3-dimethyl barbituric acid of 1 : 15 : 15 as applied by Meeussen *et al.* (1989) in an automated analytical set up for soil samples. Cooke (1978) found

that 3.55 μmol Chloramine T, as proposed by Jϕrgensen (1955), was satisfactory for quantitatively chlorinating cyanide in cassava extracts. Preliminary investigations indicated that applying the ratio of 1 : 15 : 15 would require a long incubation time before reaching maximum absorbance. Concentrating the colour reagent 2.5 times (Y) or 5.0 times (Z) gave more satisfying results with respect to incubation time, absorbance maximum and applicable pH range under our conditions. Both concentrations were found useful and have been further tested. The quantity of NaOH was adjusted to neutralize the organic acids. The reagent was maintained slightly alkaline, to avoid the risk of precipitation of the organic acids.

The new colour reagent was tested in concentrations Y and Z in the ngCN assay on standard solutions of 0, 100, 400, 500 and 700 μM of acetone cyanohydrin. Chloramine T concentration was kept at 3.55 μmol per tube. Absorbance readings were recorded during 1 hour, starting immediately after the addition of the colour reagent. Maximum absorbance readings (A_{max}) were recorded together with the time it took to reach that point. Also recorded were the time intervals at which at least 97% and 95% of maximum absorbance took place.

Analyses were carried out on day 0, 3, 7 and 9, using the same reagent prepared on day 0, to check any deviations of maximum absorbance or stability of the developed color. The reagent was stored in a glass bottle at room temperature (22 - 28°C).

The relation between buffer pH and colour development was tested by measuring absorbances obtained for KCN standard solutions of 10 and 70 nmol per tube in buffers of pH 4.0, 4.5, 5.0, 6.0 and 7.0 in the free cyanide assay.

The adequacy of the chlorination step was further tested on fresh and solid-substrate fermented cassava. To measure a maximum of 70 nmol HCN per tube, the originally proposed quantity of chloramine T, 3.55 μmol per tube, results in a 50x excess of reagent. As fermentation may increase the concentration of substances that are subject to chlorination, like reducing sugars, the quantity of chloramine T available for chlorination of the cyanide might become limiting. The effect of Chloramine T concentration on A_{max} and colour stability was evaluated by halving and doubling its concentration in the CNp assays of 7 extracts of fermented and non-fermented cassava with cyanogen levels of up to 75 nmol per tube.

The validity of adding NaOH directly to the extract in the ngCN assay was checked as follows: 0.1 ml of acetone cyanohydrin and linamarin supplemented extracts were incubated with NaOH for 2 and 30 min, both undiluted and after dilution with 0.4 ml buffer in conformity with O'Brien *et al.* (1991).

To check the necessity of immersing the tubes in iced water up to coloration, a mixture of linamarin, acetone cyanohydrin and KCN (5 : 4 : 1), totalling 320 μM, was submitted to the assays for CNp and ngCN in three ways:
A) Keeping the tubes in a 30°C water bath throughout, to imitate tropical conditions;

B) Placing the tubes in iced water immediately after addition of Chloramine T, according to Cooke (1979) and O'Brien *et al.* (1991);
C) Placing the tubes in iced water before NaOH addition, as practised in our earlier assays.

The CNp assay was done in quadruplicate on each of three consecutive days; The ngCN assay in five fold on one single day.

Temperature influence on the colour development and its stability was tested by execution of the free cyanide assay on standard KCN solutions of 8 and 32 nmol per tube at 24 and 34°C, by placing them in a thermostrated water bath between absorbance readings.

Validation and comparisons

Comparison between the enzymic assay (Cooke, 1979) and the AST method
To obtain pieces with similar cyanogen levels, a 5 cm thick cassava root disc was split into halves (De Bruijn, 1971). One segment was subjected to the enzymic assay, starting with homogenization in acid after dicing, and the other to the AST method, starting with incubation in water after grating. Twenty eight root disks were treated this way.

In order to provoke partial hydrolysis of the glycosides, cassava root was grated, hand-mixed and then divided over the two methods and the CNp assayed accordingly. This experiment was done in quadruplicate.

To verify any relation between the ngCN as percentage of CNp, and the CNp yield with each method, ngCN levels were determined according to Cooke (1979) in all acid extracts.

The enzymic assays were as described by Cooke (1979), including the use of the pyridine/pyrazolone colour reagent, but with absorbances related to a linamarin - instead of KCN - calibration curve for CNp, as CNp in fresh roots is mainly in the form of linamarin.

Calibration curves with the pyridine/pyrazolone and the new colour reagent
Standard solutions of 80 and 320 μM of linamarin, acetone cyanohydrin and KCN were analyzed following the assays for cyanogenic potential, non-glycosidic cyanogens and free cyanide as proposed by O'Brien *et al.* (1991) and according to the method presented here with the same standard solutions on the same day. This procedure was repeated three times.

Cyanogen extraction and storability

Recovery of added linamarin, acetone cyanohydrin and KCN from extracts
Fresh cassava was homogenised in acid extraction medium. Parts of the homogenate were spiked with respectively linamarin, acetone cyanohydrin and KCN solutions and

homogenised for another two minutes. The recoveries for each spike (corresponding with about 30 nmol/tube) were determined with all matching assays and calculated as {[cyanogen]$_{spiked\ extract}$ - [cyanogen]$_{extract}$} : {added cyanogen} x 100%. [Cyanogen] was calculated from A_{max} values and the corresponding calibration curves of linamarin, acetone cyanohydrin and KCN from the same analytical run. All tests were done in triplicate. Linamarin recovery was also tested in duplicate with 70 nmol per tube.

Extract storability
Based on experiences and conditions for extract storage in Africa, we tested the changes in cyanogen content of 4 cassava extracts, each under 4 storage conditions. Two random samples from diced cassava roots were extracted with acid and two with ethanol/acid extraction medium. Aliquots of each extract were kept a) at room temperature (21 - 28°C), b) refrigerated (2 - 6°C), c) deep-frozen (-18°C), and d) alternatingly deep-frozen and thawed with an interval of 1 day initially, and later 4 days. The storage condition d) was to imitate power cuts. CNp and ngCN levels were assayed after 4, 28 and 135 days.

RESULTS AND DISCUSSION

Comparison between the enzymic assay of Cooke (1979) and the AST method
Linear regression of CNp levels determined with the two methods in 28 pairs of fresh cassava pieces (Fig 2) revealed a slope of 0.999 with an intercept of 2.4 (mg CN eq. kg^{-1}) at a sample correlation coefficient (r) of 0.99. Cooke's enzymic assay gave on average 3% (SD 4) higher values than AST. This slight difference was significant ($P<0.001$, Student's two-tailed test). Later tests for optimising the AST method, applying chloramphenicol during incubation (Cooke & De la Cruz, 1982), resulted on average in 4% (SD 3.5, n = 4) higher yields than without chloramphenicol.

As CNp levels, using Cooke's method, were related to a linamarin calibration curve, we consider the values so obtained the right ones. However, calculation against a KCN reference as suggested by Cooke (1979), would have resulted in 5 - 10% lower CNp values due to a higher slope of the KCN calibration curve.

The ratio of CNp$_{(enzymic\ assay)}$: CNp$_{(AST)}$ in relation to the ngCN level for each cassava root disc is presented in Figure 3. A statistically significant trend (Student's two-tailed test, $P=0.008$, $r=-0.487$) can be distinguished, showing a drop in the ratio of CNp$_{(enzymic\ assay)}$: CNp$_{(AST)}$ with higher ngCN levels in the cassava sample.

This trend was verified by first grating fresh cassava to allow for formation of more cyanohydrins and HCN. For the grated cassava, AST yielded 418 (SD 3.3%, n=4) and the enzymic assay 371 mg CN eq kg^{-1} (SD 7.4%, n=8), while 57% of it was in the ngCN form. Cooke's method gave statistically significant lower values than AST (Student's one-tailed test, $P<0.0025$). The difference may have been caused by
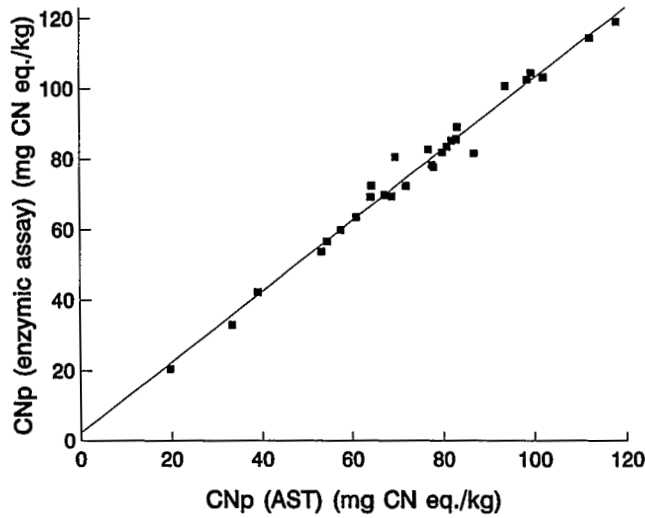
FIGURE 2 CNp values in mg CN eq kg^{-1} of root disc halves, obtained by enzymic assay compared to the halves obtained by AST of the corresponding halves
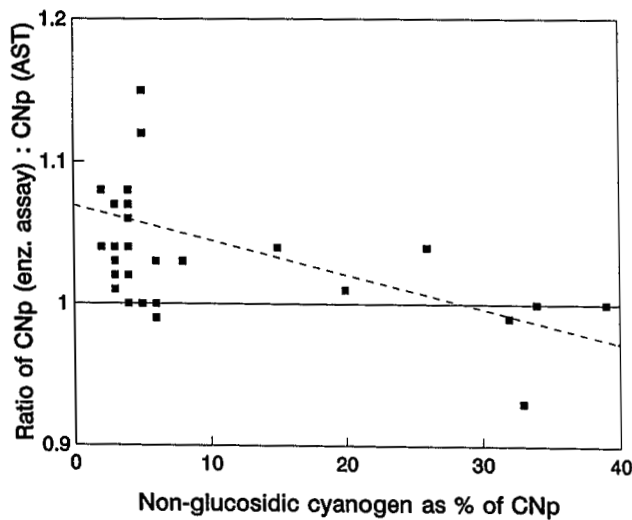

FIGURE 3 The ratio of $CNp_{(enzymic\ assay)}:CNp_{(ASP)}$ in relation to the ngCN level in individual roots and the calculated trend (dotted line)

the combination of a fair part of the high ngCN concentration being in the HCN form and its subsequent escape during homogenisation in the enzymic assay, as the system is not as well closed as in the AST method. Normally, the roots are not to be grated before homogenization with the enzymic assay, making this comparison only useful for samples with already high ngCN levels.

Concluding, we found that the AST and the enzymic method, if related to the appropriate calibration curve, give similar results for CNp in unprocessed cassava roots, unless high levels of HCN are present. Our results also indicate that cyanogen data on fresh cassava, obtained through carefully executed AST, should not be considered inferior as is sometimes suggested. Still, the many advantages of the enzymic assay may shelve the AST method.

Absorbance of the pigment and effects of concentration and ageing of colour reagent, buffer pH, concentration chlorination reagent and temperature influence
In the presence of HCN, the new colour reagent formed a pigment with highest absorbance at a wavelength of 605 nm, giving 6.3 % higher readings than at 600 nm as used by Meeussen *et al.* (1989).

Figure 4 shows that the slope of the calibration curves, as well as the incubation time for reaching maximum absorbance ($t_{A\max}$) and the stability of the formed color, did not change much after 9 days storage of the colour reagent. Later experiments showed equal results even after 12 days of storage. Doubling the concentration of colour reagent resulted in faster reaching the maximum absorbance. This maximum was slightly higher, but the colour was less sTable. We therefore prefer the single concentrated colour reagent (Y) as described in the method section. Absorbance reached maximum within 10 min and could be read with $< 3\%$ loss from 8 up to 40 min for ngCN and up to 25 min for CNp and HCN assays, respectively. With 5% accuracy limits the ranges were 6 to 60 and 6 to 35 min, respectively.

Calibration curves remained linear (correlation coefficient > 0.9995) up to the absorbance of 2.0, corresponding to 70 nmol per tube. This indicates that the excess of colour reagent was sufficient in both concentrations.

Figure 5 shows that with KCN in the free cyanide assay, there was hardly any influence from phosphate buffer pH in the range of 4 to 7 on the colour development.

Sensitivity increase of the free cyanide assay was achieved without problems of cyanohydrin breakdown by adding 0.6 ml acid extract to 3.4 ml buffer pH 6.

Doubling the concentration of chloramine T raised the CNp values in cassava extracts slightly (3%, SD 2, n = 7). No effect on $t_{A\max}$ nor on the colour stability was found. One may therefore opt for a double chloramine T dosage in analyses of fermented cassava.

Adding NaOH directly to extracts in the ngCN assay gave similar values as after dilution with buffer, validating this procedure.

Slope 0.0280 0.0282 0.0273 0.0281   0.0285 0.0283 0.0281 0.0282
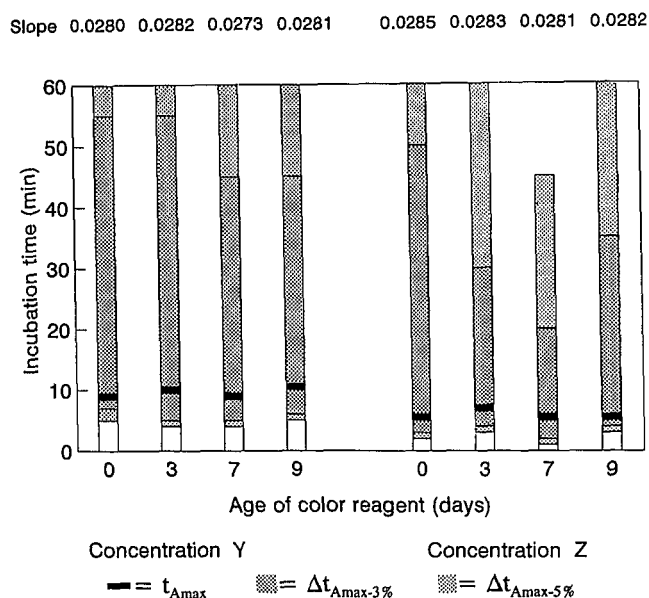
FIGURE 4 The slope and colour development (t_{Amax}, $\Delta t_{Amax-3\%}$ and $\Delta t_{Amax-5\%}$) of acetone cyanohydrin calibration curves after various storage periods of colour reagents with concentrations Y and Z

Slope   0.0305   0.0306   0.0310   0.0296   0.0296
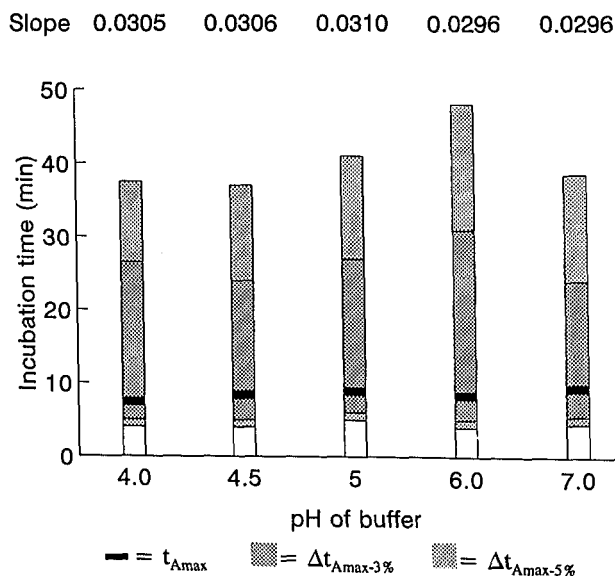
FIGURE 5 Buffer pH in relation to the slope and colour development (t_{Amax}, $\Delta t_{Amax-3\%}$ and $\Delta t_{Amax-5\%}$) of KCN calibration curves determined with the free cyanide assay

TABLE 1

Assay temperatures and related KCN calibration curve slopes, t_{Amax} and colour stability

Temp. (°C)	Calibration curve slope	t_{Amax} (min)	$\Delta t_{Amax-3\%}$ (min)	$\Delta t_{Amax-5\%}$ (min)
24	0.0257	13	9 - 22	8 - 31
34	0.0248	6	4 - 28	3 - 36

TABLE 2

Mean ($\pm$SD) slope values of calibration curves with the assays according to (1) O'Brien *et al.* (1991) and (2) the new assay, measured in one run, repeated on 3 days

Substrate		Assay		
		Cyanogenic potential	Non-glycosidic cyanogens	Free cyanide
(1)	Linamarin	0.0208 ($\pm$0.0015)	-	-
	Ac. cyanohydrin	0.0233 ($\pm$0.0019)	0.0237 ($\pm$0.0008)	-
	KCN	0.0226 ($\pm$0.0025)	0.0237 ($\pm$0.0027)	0.0233 ($\pm$0.0037)
(2)	Linamarin	0.0247 ($\pm$0.0015)	-	-
	Ac. cyanohydrin	0.0276 ($\pm$0.0011)	0.0284 ($\pm$0.0012)	-
	KCN	0.0261 ($\pm$0.0012)	0.0281 ($\pm$0.0007)	0.0271 ($\pm$0.0007)

Placing the test tubes in iced water after Chloramine T addition or before NaOH addition yielded, respectively, 3 - 6% and 7 - 11% higher absorbance values than when omitting this step. However, these steps did not improve the reproducibility. We consider the slight increase in sensitivity alone by this extra step not necessary.

The effect of temperature on colour development is illustrated by Table 1. We see a slight decrease in slope when changing from 24 to 34°C, which may be accounted for by the escape of HCN at the higher temperature, as well as an earlier reaching of A_{max} and a slightly better stability of the colour. A higher temperature seemed to benefit the colour development. It should be noted that the cuvettes in all colour stability experiments remained in the spectrophotometer, which is approx 30°C inside. This implies that with other laboratory conditions, t_{Amax} should be checked before defining the appropriate interval of measurement.

Reproducibility was tested by analyzing CNp in 10 extracts (containing 400 - 900 μM) four times on separate days within 3 weeks. Quadratic mean CV was 2.8%; 90%

of the deviations from median was smaller than 2.6 %.

Comparison between the new and the pyridine/pyrazolone colour reagent
Mean slope values and their standard deviations of calibration curves, made according
to O'Brien *et al.* (1991) and to our revised assay are shown in Table 2. We see a good
correspondence between the patterns of the set of slopes of the curves for CNp, ngCN
and free cyanide, that were obtained with the method of O'Brien *et al.* (1991) and the
new method. This new method gave on average 18 % higher slope values.

Calibration curves appeared to change slightly from day to day, making it
necessary to include some calibration points in each analytical run for appropriate
conversion factors. Ranking of the slope values of different calibration curves
measured on the same day consistently showed highest values for the curves of acetone
cyanohydrin and KCN with the ngCN assay, followed by acetone cyanohydrin in the
CNp assay and KCN in the free cyanide assay, KCN in the CNp assay, and lowest for
linamarin in the CNp assay.

Errors made by subtracting cyanogens measured with different assays
Ideally, equimolar amounts of the substrates linamarin, acetone cyanohydrin and KCN
should give the same absorbance when measured with their matching assays. In
practice, however, it makes a difference if absorbance values are compared with a
linamarin, acetone cyanohydrin or KCN calibration curve. If we consider fresh
cassava, in which nearly 100% of the CNp is in the form of linamarin, a linamarin
calibration curve would be appropriate. Using a KCN curve would then underestimate
the CNp by 6 % according to the Figures from Table 2. However, if a sample is taken
during processing, ngCN levels may be up to 90% of CNp. Then an acetone
cyanohydrin standard would be appropriate for relating the absorbance values. The
proportion is not known beforehand for most products. For proximate assays, KCN
may be used for calibrations in general and linamarin for fresh cassava. For precise
calculations, we should consider the absorbances (A), obtained from the respective
assays, to be constructed as follows:

CNp   : A_1 = 0.0247[linamarin] + 0.0276[cyanohydrin] + 0.0261[HCN]
ngCN   : A_2 = 0.0284[cyanohydrin] + 0.0281[HCN]
free CN : A_3 = 0.0271[HCN]

Solving this set of three equations with three unknowns, yields the concentrations.
Note that the calibration factors (here from Table 2) must be reevaluated each time the
analyses are run.

TABLE 3

Proportional recovery (%, ±SD) of supplemented cyanogens from fresh cassava extracts

Substrate	Assay		
	Cyanogenic potential	Non-glycosidic cyanogens	Free cyanide
Linamarin [a]	102 (± 4)	-	-
ac. cyanohydrin[b]	101 (± 6)	108 (± 4)	-
KCN[b]	67 (±12)	85 (± 7)	80 (±11)

[a] n = 5; [b] n = 3

TABLE 4

Initial cyanogenic potential and non-glycosidic cyanogen values in four cassava samples, extracted two by two with the media phosphoric acid (PA) and ethanol/phosphoric acid (EPA), and the recoveries over time under four different storage conditions

Time (days)			Storage condition [a]						
			a		b		c		d
extraction by PA									
0	CNp	(mg kg⁻¹)	165	221					
0	ngCN	(mg kg⁻¹)	28	34					
4	CNp	(%)	94	94	-	103	100	92	97	95
4	ngCN	(%)	103	103	105	104	108	108	108	108
28	CNp	(%)	99	100	98	102	100	103	98	97
28	ngCN	(%)	89	92	92	101	81	100	93	95
135	CNp	(%)	74	74	84	86	84	83	85	84
135	ngCN	(%)	20	48	29	29	28	28	28	28
extraction by EPA									
0	CNp	(mg kg⁻¹)	231	206					
0	ngCN	(mg kg⁻¹)	29	33					
4	CNp	(%)	97	99	106	100	103	105	99	100
4	ngCN	(%)	100	98	102	100	107	105	110	100
28	CNp	(%)	97	99	104	100	105	105	102	97
28	ngCN	(%)	90	92	87	96	92	103	99	93
135	CNp	(%)	74	79	88	82	87	98	87	85
135	ngCN	(%)	26	23	28	31	26	31	27	25

[a] a = room temperature; b = refrigerated; c = deep-frozen; d = alternately freezing and thawing;

CNp = cyanogenic potential; ngCN = non-glycosidic cyanogens = cyanohydrins plus HCN

Cyanogen extraction and storability

Recovery of linamarin, acetone cyanohydrin and KCN supplements from extracts
From Table 3 we see that the recovery of KCN is incomplete. This is probably due
to the volatility of HCN under acid conditions and a room temperature of 26 °C. These
values reflect the immediate measurements after extraction. Lower values have been
found as well. Incubation at 30°C and the presence of protein (linamarase) may
further have reduced the KCN recovery in the CNp assay. Acetone cyanohydrin
recovery in the ngCN assay was higher than expected, probably indicating the
accuracy errors that occur at both measuring the supplemented and the non-
supplemented extract. The errors are added at subtraction.

Extract storability
The data in Table 4 suggest that storage of extracts, with regard to CNp and ngCN
recovery, does not pose a problem for storage time of one month, but does so for 4
months. We found no significant improvement in storability by the addition of ethanol
in the extract medium as suggested by O'Brien *et al.* (1991). Difference in storage
temperature conditions showed some effect after 4 months, resulting in a slightly
worse CNp recovery after storage at room temperature. This suggests that cooling or
freezing has some advantage if samples are to be stored for more than one month.

General remarks
- The new colour reagent is less toxic and does not release repulsive vapours, thereby
  obviating the need of a well-functioning fume hood.
- The price for the reagent was 0.7 $ cents per assay, compared to 7 $ cents for the
  pyridine/pyrazolone reagent.
- Being in powdered form, transportation of these chemicals to distant laboratories in
  third world countries was easier than transporting pyridine.
- The new coloration method gave less problems with gas development during
  readings.
- We obtained good results, using the new colour reagent as a substitute for the
  pyridine/barbituric acid in the assay of urine thiocyanate according to Lundquist *et
  al.* (1979, 1983). Optimal wavelength in this procedure was 608 nm.

We conclude that the here tested method is a further improved version of the useful
enzymic assay as described by O'Brien *et al.* (1991) and Cooke (1979). Its main
contribution is in the ease of handling by the new coloration step and the increase in
accuracy by using the proper calibration curves.

ACKNOWLEDGEMENTS

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CYANOGEN REMOVAL FROM CASSAVA ROOTS DURING SUN-DRYING

AJ Alexander Essers, Remco M Van der Grift and Alphons GJ Voragen

Linamarin levels in sun-drying cassava root pieces showed an exponential decrease, parallel with the moisture loss, and stabilized when moisture levels reached about 15%. Linamarin degradation in thin root segments was significantly slower and less complete than in thick ones. Disinfected longitudinal root halves, oven-dried at 40°C for 24h, had significantly higher residual linamarin levels than the matched ones submitted to humid incubation at 25°C. Interrupting for one or two days the sun-drying of peeled roots resulted in significantly lower residual levels of linamarin and of cyanohydrins plus HCN. Linamarin degradation was greater when the interruption was earlier or longer. The rate of dehydration influences linamarin degradation negatively. Mechanisms are discussed. Cyanohydrin removal was completed by prolongued drying.

Sun-drying is not very effective in linamarin removal, and speeding up the drying process, e.g. by reducing the size of the pieces, aggravates this. There is a potential for increasing the effectiveness of cyanogen removal by reducing the initial drying rate, followed by thorough final drying.

INTRODUCTION

Two drawbacks of the tropical staple food crop cassava are its potential toxicity and the perishability of the starchy storage roots. Once harvested, the roots deteriorate rapidly and may turn unsuitable for consumption within 4 days (Booth & Coursey, 1974). Although cassava is widely consumed and usually without problems, toxic effects from insufficiently processed cassava have been reported from several areas (Rosling *et al.*, 1993). The potential toxicity comes from cyanogenic glucosides, mainly linamarin, which are present in the cell vacuoles. Disruption of the cells leads to contact with the enzyme linamarase which is located in the cell walls, and to subsequent hydrolysis into glucose and cyanohydrins (White *et al.*, 1994). Cyanohydrins are relatively stable in acid environments but rapidly decompose at a neutral or higher pH into ketones and the toxic hydrogen cyanide (Cooke, 1978). The formed HCN has a boiling point of 26°C and can easily escape into the air.

Effective reduction of cyanogen levels therefore requires two separate treatments. First, a treatment which enhances linamarin-linamarase contact by thorough disintegration of the cell structure. This can be done by crushing, grating or by (microbial) cell wall-degrading enzyme activity. A second treatment is necessary to degrade the cyanohydrins formed and to volatilize the HCN. This can be done by heating or drying at an adequate pH. Traditional processes that fulfil these requirements are applied in several parts of the tropics (Dufour, 1989; Westby & Choo, 1994; Lancaster *et al.*, 1982). A drawback of the most effective and fast methods, like the ones leading to *gari* in West Africa and *farinha* in Brazil, is that they are quite labour demanding (Hahn, 1988; Dufour, 1994), while labour availability is low in sub-Saharan Africa (Jiggins, 1988).

Sun-drying of peeled cassava roots is the easiest way to obtain a storable and transportable product, practised in many parts of the tropics (Cock, 1985; Nweke, 1994). The dried pieces can eventually be processed into flour for human consumption or animal feed. In large parts of Africa cassava roots are mainly processed into flour, which is used for preparing a paste, the generally preferred form for consumption of staple foods (Lancaster *et al.*, 1982). Pieris *et al.* (1974) found high residual cyanogen levels in sun-dried cassava, suggesting ineffective cyanogen removal.

The rate of dehydration of (sliced) cassava roots by sun-drying depends on moisture level, particle size, the material, temperature and loading of the drying floor, the air humidity and temperature, sun-radiation, and wind. If the peeled roots are regarded too thick by the processors, they are split and cut into smaller pieces to standardise or shorten the drying process. Shortening the lengthy drying process is desired to limit microbial spoilage, to reduce the risk of theft, and to quickly have food available and to comply with financial and market demands. Nambisan and Sundaresan (1985) found that thin root discs retained more cyanogenic glucoside than

thicker ones, and attributed this to earlier reaching a low moisture level which would inactivate linamarase. Sun-drying is sometimes interrupted or slowed down, provoking fungal growth, to reduce cassava's potential toxicity (Essers *et al.*, 1995). The role of the dehydration in these processes is not known in detail.

This work was undertaken to study the dynamics of the cyanogen levels and the relation with moisture levels in sun-drying cassava root pieces, to seek practical measures to enhance the cyanogen removal.

MATERIALS AND METHODS

Experimental

A: Monitoring cyanogen and moisture levels during sun-drying
In January 1991, 30 thick straight roots of the bitter variety *Karangwa* were harvested around 8 o'clock (t = 0) in Kiryandongo village, Masindi district, Uganda, and transported to Kampala, where they were measured, peeled rapidly and split lengthwise into 4 segments of similar size, which were dispersed on a cane mat and exposed to sunshine before 14 o'clock. The segments were turned upside down once a day, and sheltered during the night, which is common practice there. Samples of 12 randomly selected segments were taken right after peeling and after 3, 6, 10, 17 and 38 days. Samples were diced into 1 cm^3 cubes, hand-mixed, and 4 sub-samples of approx. 70.00 g were extracted. Cyanogen extraction and pH measurement were conducted within 2 h of sampling, and analysis for cyanogens within 24 h of extraction.

In January 1992, the experiment was repeated in exactly the same way, but to get a more detailed picture, sampling was then after 0, 1, 2, 3, 4, 6, 9, 14 and 30 days.

To measure the reliability of the sampling procedure, 4 samples of 12 fresh roots were taken, sub-sampled and analyzed.

To examine the cyanohydrin and HCN levels in very fresh cassava, two roots of the variety *Karangwa* were peeled, the parenchyma middle disc cut with a sharp knife and cyanogens extracted within 15 min after uprooting. Analysis of cyanogens in the extracts was within one week.

B: Examining the effect of chunk size on cyanogen levels in sun-drying cassava
The effect of chunk size was tested after different extents of sun-drying, by pair-wise comparison of residual cyanogen levels in sun-dried thick and thin segments of the same root.
Twenty thick straight roots of the bitter cultivar *Karangwa* were harvested around 8 o'clock (t=0) in Kiryandongo village and transported to Kampala, where they were peeled and split lengthwise into segments a, b, c, d as shown in Figure 1. Of each root, segments b and d were jointly extracted and analyzed on the same day (0) to
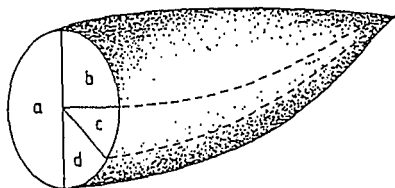
FIGURE 1 Longitudinal division of roots for comparing thick (*a*) and thin (*c*) segments

indicate the initial level of cyanogens, moisture and the pH of that root. Segments a and c were labelled and placed on a mat exposed to sunshine for drying, and sheltered during the night. At each of day 3, 7, 10 and 21, five segment pairs a and c were randomly selected and each piece extracted individually and analyzed for cyanogen levels, moisture and pH.

C: Comparison of cyanogen levels after drying or humid incubation

To test the effect of dehydration on linamarin degradation, residual cyanogen levels of root segments submitted to either drying or humid incubation, were compared pair-wise.

Cassava roots from Costa Rica, obtained commercially in the Netherlands, were peeled and cut into pieces. Ten pieces were disinfected by submerging them for 2 h in 70 % ethanol, to reduce microbial outgrowth. Then, with a disinfected knife, each piece was split lengthwise into two similar segments, which were labelled. This resulted in ten pairs of segments with pair-wise similar levels of cyanogens (De Bruijn, 1973). One segment of each root was placed in a ventilated oven at 40 °C and the other segment in a covered glass jar at 25 °C, avoiding anaerobiosis by leaving a small aperture. After 24 h each segment was extracted and analyzed for cyanogens. Moisture was determined in two combined samples of the dried and two of the humid incubated segments.

D: Interrupted sun-drying

To study the effect of deliberately delaying dehydration of cassava roots on cyanogen levels, residual cassava cyanogen levels after sun-drying and interrupted sun-drying were compared with the initial ones. Equal size roots of the bitter variety *Karangwa* were harvested around 8 o'clock in Kiryandongo village and transported to Kampala, where they were peeled and randomly divided over 6 batches at 14.00 o'clock. One batch was sampled immediately as reference and the others were exposed to sunshine for the 3 remaining hours and subsequently submitted to sun-drying and incubation in a bag following an 8 days schedule as indicated at Table 2. A "sun day" means that the roots were scattered on a cane mat and exposed to sunshine from 09 to 17 o'clock. A "bag day" means that the roots were kept during the same period in a woven polypropylene bag in the shade, to reduce the drying rate. Outside these hours, the roots were kept uncovered in a room to protect them against possible rains and rodents. On day 9, moisture and pH were determined and cyanogens were extracted

from sub-samples of each batch. Analysis for cyanogens was within two weeks.
Experiment D1 was conducted in February 1993. Each batch had 12 roots, which resulted in 4 sub-samples of 3 roots each, as suggested by Bokanga (1994). Each sub-sample resulted in one extract, totalling 24 extracts for the experiment. *Experiments D2 and D3* were conducted in March 1993. Then the six batches consisted of 36 roots each: 6 sub-samples of 6 roots each, resulting in 36 extracts. In *experiment D3*, one initial drying day was added to all treatments to prevent mould growth during the humid weather at that time.

Cyanogen, pH and moisture determination
Moist sub-samples of approx. 70.00 g 1 cm^3 cubes were homogenised in 250 ml 0.1 M H_3PO_4, followed by centrifugation. Samples of dry root pieces were crushed and pounded to flour. Of this, two sub-samples of 4 g were each suspended in 25 ml 0.1 M H_3PO_4, swirled for 5 min, followed by centrifugation. The supernatant was analyzed for cyanogens by enzymic conversion to HCN and colorimetric measurement (Beckman DU-62 in experiment C, otherwise Milton Roy Spectronic 20) after coloration with 1,3-dimethyl barbiturate/ isonicotinate reagent according to Essers *et al.* (1993). The pH was measured 10 min after homogenizing 40 g moist sample in 150 ml or suspending 10 g flour in 100 ml cooled, previously boiled distilled water. Moisture levels were assayed by oven drying at 105 $\pm$ 5° C until constant weight.

Statistical analysis
Random sampling was obtained by lining up all roots or segments and, starting at one end from a randomly determined figure, collecting e.g. every sixth root. The presence of treatment effects was verified by F-test and subsequently judged with Students *t* (Protected Least Significant Difference test, PSD) (Snedecor & Cochran, 1980). Significance of differences are related to two-tailed probability (P) values.

RESULTS

Experiments A: dynamics of cyanogen and moisture decrease
The cyanogen and moisture levels during the sun-drying of root segments are presented in Figure 2a,b. The pH of the roots remained between 6.3 and 7.2 in both experiments. The coefficient of variation (*cv*) of cyanogenic potential of 4 samples at t=0 was 10.4%. The mean ($\pm$SD) root diameter was 6.5 cm ($\pm$1.0) in experiment 1 and 5.5 cm ($\pm$0.8) in experiment 2. Cyanogenic potential in the two roots harvested 15 min before extraction, was 1197 and 866 mg CN eq./kg dry weight, and the cyanohydrin levels 2.0 and 2.4, respectively. HCN was not detectable.
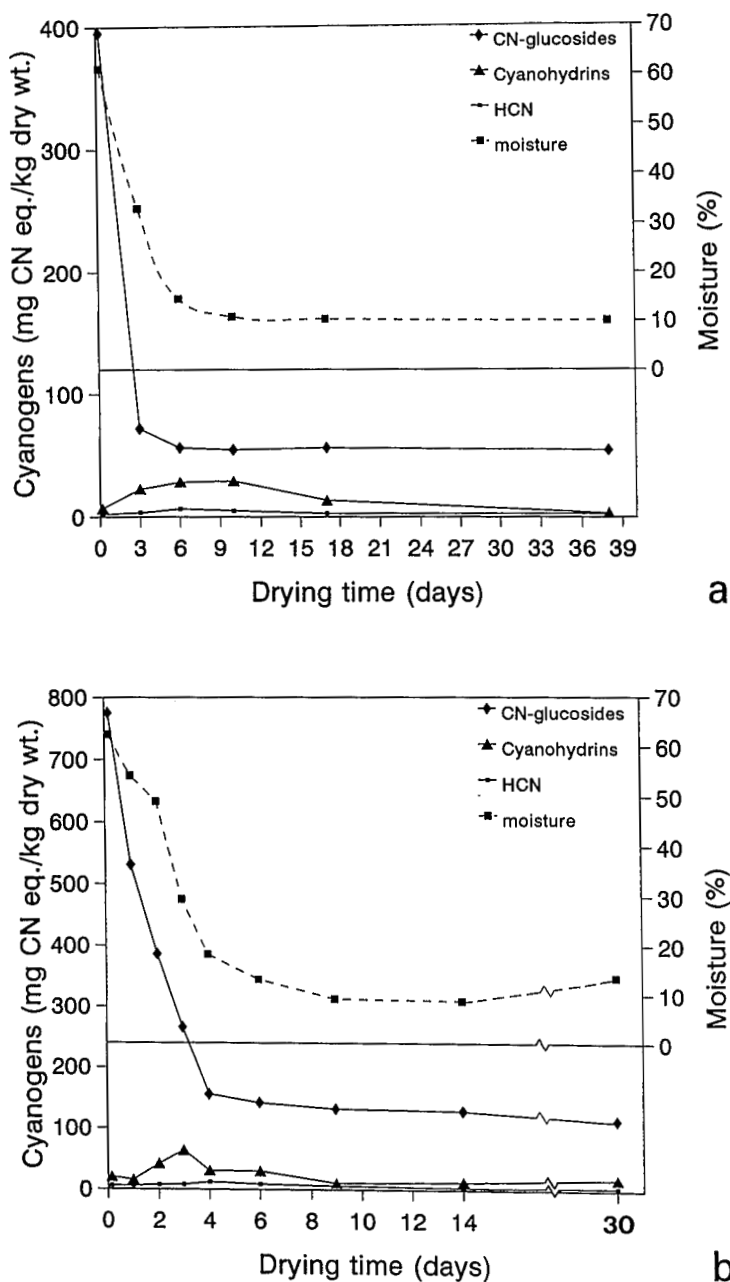
FIGURE 2 Cyanogen and moisture levels in sun-drying cassava root segments (cultivar *karangwa*) over time (batch a in 1991, batch b in 1992)

TABLE 1

Aggregated data on cyanogenic potential of fresh cassava roots and the residual cyanogen and moisture levels in pairs of thick and thin segments after several days of sun-drying

Drying time	Initial levels	Residual levels					
		Thick segments			Thin segments		
(days)	CNp^a	CNg^b	$ngCN^c$	moisture (%)	CNg^b	$ngCN^c$	moisture (%)
3	378 ±94	86.5 ±33.7	34.8 ±23.6	35.7 ±6.3	116.5 ±33.0	25.5 ±21.3	23.1 ±1.8
7	376 ±98	76.3 ±21.5	32.2 ±13.6	22.0 ±3.7	103.6 ±28.3	32.0 ±10.1	15.0 ±1.1
10	369 ±57	64.4 ±26.6	15.0 ± 7.8	15.6 ±2.7	85.7 ±24.6	17.7 ± 5.3	11.8 ±0.3
21	433 ±77	66.8 ±16.4	6.1 ± 1.1	10.0 ±1.6	97.5 ±13.5	5.6 ± 0.9	9.4 ±1.0

[a] cyanogenic potential; [b] cyanogenic glucosides; [c] non-glucosidic cyanogens = cyanohydrins plus HCN; Means ±SD of 5 repetitions are given, as mg CN equivalent/kg dry weight.

Experiment B: thin versus thick segments

The mean (±SD) root diameter was 6.1 cm (±1.0). Cyanogen and moisture levels in drying pairs of thin and thick segments are presented in Table 1. Measured from 3 days of drying onwards, cyanogenic potential and cyanogenic glucoside levels were significantly lower ($P < 0.001$), and moisture levels significantly higher ($P < 0.001$) in the thick segments than in the corresponding thin segments. The cyanogenic glucoside levels in the thick segments were on average (mean ±SD) 72% (±12) of those in the corresponding thin segments. This percentage was not significantly different after 3, 7, 10 or 21 drying days. After 3 and 7 drying days there was still a strong correlation ($r > 0.90$) between initial and residual cyanogenic potential or linamarin levels in the dried pieces. After 10 and 21 drying days, this relationship was weaker, especially in the thick segments.

Experiment C: dried versus non-dried segments

Mean moisture level was 33% in the dried samples and 63% in the non-dried disinfected incubated ones. Mean (±SD) cyanogenic glucoside levels were 67.3 (±13.4) mg CN equivalent/ kg dry weight in the dried segments and 48.8 (±14.6) in the corresponding ones that were prevented from moisture loss. Mean (±SD) non-glucosidic cyanogen levels were 16.2 (±6.1) mg CN eq./kg dry weight in the dried segments and 65.1 (±17.4) in the corresponding non-dried ones. Cyanogenic glucoside levels were significantly higher ($P < 0.005$), and levels of cyanohydrins plus HCN were significantly lower ($P < 0.001$) in the dried segments than in the corresponding ones that were prevented from moisture loss. No apparent fungal or bacterial growth was visible on the dried or incubated pieces.

TABLE 2

Mean levels (±SD) of cyanogenic glucosides, cyanohydrins plus HCN, and moisture in fresh cassava roots and after different sun-drying treatments in experiments *D1*, *D2* and *D3*

Treatment[1]	Cyanogenic glucosides (mg CN eq/kg dry wt)	Cyanohydrins + HCN (mg CN eq/kg dry wt)	Moisture (%)
D1 fresh[2]	490.9 ±20.6	8.4 ± 0.7	57.1
A[2]	31.5 ±23.2	15.8 ± 4.9	18.6
B[2]	7.0 ± 4.1	9.5 ± 1.8	16.9
C[2]	4.3 ± 3.9	7.9 ± 3.5	17.7
D[2]	5.9 ± 3.8	10.9 ± 1.8	18.0
E[2]	1.7 ± 1.6	3.9 ± 0.9	17.8
D2 fresh[3]	779.9 ±80.3	5.4 ± 1.9	59.5
A[3]	83.3 ±10.1	16.5 ± 7.8	16.1 ± 1.7
B[3]	59.7 ±16.3	11.7 ± 4.2	14.1 ± 2.5
C[3]	25.6 ± 9.2	10.4 ± 5.5	15.0 ± 2.0
D[3]	42.3 ±12.0	4.6 ± 2.6	13.0 ± 0.5
E[3]	15.4 ± 8.6	5.3 ± 1.9	14.1 ± 2.2
D3 fresh[3]	783.0 ±63.4	6.4 ± 1.9	58.5
A+[3]	123.8 ±27.7	6.9 ± 2.2	20.0 ± 1.0
B+[3]	78.8 ±23.9	3.1 ± 1.2	18.9 ± 1.7
C+[3]	35.4 ± 7.1	1.5 ± 1.3	21.1 ± 2.6
D+[3]	45.2 ± 6.6	8.1 ± 3.4	17.5 ± 2.3
E+[3]	19.2 ± 3.6	0.5 ± 0.7	13.3 ± 1.2

[1]   number of days in the   [2] n = 4   [3] n = 6

	sun	bag	sun
A:	8	0	-
B:	2	1	5
C:	2	2	4
D:	1	1	6
E:	1	2	5

(+ means one additional initial sun-day)

Experiments D: interrupted sun-drying

Results from the experiments with interrupted sun-drying are presented in Table 2. The pH range in the fresh and treated roots was between 6.0 and 7.1 with a mean (±SD) of 6.5 (±0.3). Coefficient of variation (*cv*) of cyanogenic potential in fresh root samples was 4.1% for 4 sub-samples of 3 roots, and 10.1 and 7.9, respectively, in two experiments with 6 sub-samples of 6 roots. The batches which were processed by interrupted sun-drying had significantly lower ($P < 0.01$) residual cyanogenic potential and linamarin levels than the ones submitted to straight forward sun-drying. Mean (±SD) linamarin levels in interrupted-sun-dried batches were 15% (±7.3), 43%

($\pm$23) and 36% ($\pm$20) of straight sun-dried batches in experiments 1, 2 and 3, respectively. The batches which were incubated after only one day of initial sun-drying had significantly ($P<0.01$) lower residual linamarin levels than when incubated after two days of initial sun-drying. The batches that were incubated for two days had significantly ($P<0.05$) lower cyanogenic potential and linamarin levels than when incubated for one day. The residual levels of cyanohydrins plus HCN together were significantly lower ($P<0.05$) in the cassava pieces that had been submitted to interrupted sun-drying than in the straight forward sun-dried ones.

In several batches a slight fungal growth on the root surface was observed, but not comparable to the degree observed during heap-fermentation (Essers *et al.*, 1995), and neither had the roots become soft. This growth was most pronounced on the roots that had been incubated for two days after only one initial day of sun-shine.

DISCUSSION

Due to the heterogenic distribution of cyanogenic glucosides within and between roots, a good sampling procedure is crucial in such comparative experiments. The reproducibility of the sampling procedures, indicated by the *cv* of the cyanogenic potential of the duplicate samples at t=0, was good. Higher *cv* has been reported as common by Bokanga (1994). The absence of detectable quantities of HCN and the extremely low cyanohydrin levels (below 0.3% of cyanogenic potential) in very fresh roots, are consistent with the theory that these compounds are nearly absent in intact living cassava tissue (White *et al.*, 1994). The small amount of cyanohydrin present must have been formed in the disrupted cells at the cutting edge. We can therefore consider all initial values of cyanogenic potential in our experiments to represent exclusively cyanogenic glucosides at harvest.

In the drying experiments A and B the glucoside degradation was highest at the start, slightly decreasing over the following days, and practically stopped, rather abruptly, before being completed. This decrease coincided with the loss in moisture; the glucoside degradation practically stopped at a moisture level somewhere between 33 and 15% in the first batch, and between 18 and 13% in the second batch. This is slightly higher than the 13% estimated by Mlingi and Bainbridge (1994). We consider the estimate from the second batch more accurate, because of the more detailed information by the shorter measuring-time intervals. Experiment B shows that reducing chip size accelerated the dehydration and reduced the breakdown of linamarin. Contrary to our expectation that the difference in linamarin levels between thick and thin segments would arise or increase after thin segments having reached moisture levels below about 15% while thick segments *still having well above 15%*, i.e. after about 7 days, there was no such trend. The main difference in residual linamarin levels was determined in the first part of the drying stage. Experiment C

shows that drying at 40°C caused less linamarin breakdown than humid incubation at 25°C over a 24 h period. Experiment D shows that extending the period with a higher moisture level enhanced the linamarin breakdown, and that the breakdown was more pronounced in those batches where moisture was retained longer or at a higher level.

This all suggests an important role of moisture levels and dehydration rate in glucoside degradation. During drying, water evaporates at the root surface. To restore the water balance in the tissue, water must pass the cell walls. It then depends on the integrity of the membrane system if the glucosides remain within the vacuoles, or are transported along through the linamarase containing cell wall. This forced transport of liquid might be a factor in enabling contact between the glucosides and the linamarase. If this factor would be important, drying pieces should have lower residual linamarin levels than the pieces prevented from dehydration. As the results from experiment C contradicted this hypothesis, we conclude that the dehydration per se does not play an important positive role in establishing glucoside-linamarase interaction, but rather the autolytic processes that occur during the drying period, by affecting the membrane and cell-wall integrity.

The pattern of the decreasing linamarin levels during the sun-drying process may be explained by one or more of the following: The enzymic breakdown of a substrate in solution follows a first or higher order reaction, resulting in a negative exponential curve of linamarin level in grated cassava (Ofuya *et al.*, 1989), and we found this also in homogenized cassava tissue (unpublished findings). The higher initial substrate level therefore logically results in a steeper decline than the lower level which is present after some days. This is irrespective of the drying process or the stage in the incubation or drying process.

Okolie & Oguchukwu (1988) studied several enzyme activities in harvested cassava roots. They found that endogenous cell wall-degrading enzyme activity is highest during the first 24 h after harvest and decreasing during the subsequent days. This cell wall-degradation may reduce the compartmental separation of the linamarase and linamarin and as such imply a higher initial linamarin breakdown. The linamarin levels in our studies showed a similar decrease over time as the pectinase and polygalacturonase activity in their study. The decrease in linamarase activity during the first three days after harvest, shown by Iwatsuki *et al.* (1984), may enhance this effect. The reason for the lowering enzyme activity is not known, but is possibly related to the formation of enzyme-inhibiting compounds such as polyphenoles. This mechanism may be independent from the drying process. It should be noted that, contrary to Okolie & Oguchukwu, Padmaja & Balagopal (1985) found an increase in polygalacturonase activity over the first few days.

Figure 2 suggests that glucoside degradation practically stops when moisture levels are below about 15%. Decreasing moisture levels of 23%, 15% and 13% in cassava starch correspond with a water activity (a_w) of 0.80, 0.42 and 0.33, respectively (Van

den Berg, 1981). In cassava tissue the corresponding a_w must be slightly lower due to the few percent of non-starch cell compounds. In foodstuffs, enzyme interactions are increasingly suppressed from an a_w of 0.8 downward and are absent below an a_w of 0.4 (Belitz & Grosch, 1987).

Upon dehydration, the diffusion coefficient decreases faster for big than for small molecules (Menting, 1969). This would make linamarin diffuse increasingly slower than the small water molecule during the drying, and one can imagine that part of the linamarin molecules finally remain immobilized in the vacuoles due to a lack of bulk water. This would prevent their interaction with linamarase. Menting (1969) found that acetone retention in a drying malto-dextrin solution was higher when the drying was more rapid. Drying rate is higher in thinner than in thicker cassava pieces, as the surface to volume ratio is greater. This different drying rate may explain why in smaller drying pieces linamarin levels remain higher from the start onward, and not only after reaching moisture levels of some 15%.

During drying, the cyanohydrins resulting from the breakdown of the glucosides hardly accumulated during the first 3 days, in spite of the large quantities which must have been formed. Probably, the conversion of cyanohydrins takes place rapidly because of the favourable pH (between 6.3 and 7.1) in an aqueous environment, the presence of α-hydroxynitril-lyase and a temperature of 25 - 45 °C. The low levels of HCN throughout the processing indicate that the formed HCN escaped easily, thereby pulling the equilibrium between cyanohydrin and its metabolites to the latter. The high cyanohydrin levels in the humid incubated root pieces from experiment C show the importance of drying in cyanohydrin removal. It was therefore remarkable that the residual moisture and cyanohydrin levels were lower in the interrupted sun-dried root pieces than in their straight forward sun-dried pairs. The enhanced enzyme-substrate interaction by the moisture retention, together with the final drying step was apparently an effective combination for cyanogen removal.

Although Nambisan & Sundaresan (1985), Mlingi & Bainbridge (1994) and our experiments clearly showed that reducing chip size led to relatively higher linamarin levels after sun-drying, exceptions are possible. As long as the number of cells disrupted by the cutting is small compared to the amount of intact tissue, this will lead to higher residual linamarin levels in thinner particles, as described here. When the number of disrupted cells becomes high compared to that of intact cells, which is achieved by grating, pounding, or dicing with a blunt knife, this relationship may be overruled by the enhanced accessability of linamarin and linamarase. Drying of fresh peeled whole and pounded roots as compared by Mlingi *et al.* (1992) therefore resulted in lower linamarin levels of the latter.

Extending the period with medium moisture levels, as practised with the interrupted sun-drying, led to lower residual levels of cyanogenic glucosides, as well as cyanohydrins plus HCN, over the same period of time. Higher moisture levels during

the incubation led to lower residual glucoside levels but unfortunately also to some mould growth. These fungi may have contributed to the enhanced reduction of the linamarin levels (Essers *et al.*, 1994, 1995), but not much, as they were only superficial and in small amounts, while root softening as in heap-fermentation had not occurred.

Considering the potential toxicity of linamarin (Rosling *et al.*, 1994), and the levels of residual cyanogens in sun-dried roots in these experiments exceeding by far the FAO/WHO (1991) recommended limit of 10 mg hydrocyanic acid equivalents per kg flour, we conclude that sun-drying alone is not appropriate for cassava roots with high cyanogenic potential. For such roots, speeding up the drying process e.g. by reducing chip size is certainly not appropriate. There is a potential for increasing the effectiveness of cyanogen removal in sun-drying cassava by extending the time span with a higher moisture content for linamarin breakdown, followed by a thorough final drying step to reduce the cyanohydrin levels. This may be advisable for roots with medium levels of cyanogenic potential, where water-soaking or more labour intensive methods are inconvenient. It remains to be tested if, by sufficient initial drying, microbial contamination can be controlled and the formation of mycotoxins prevented.

ACKNOWLEDGEMENTS

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THE SAFETY OF DARK, MOULDED CASSAVA FLOUR COMPARED WITH WHITE
a comparison of traditionally dried cassava pieces in north east Mozambique

AJ Alexander Essers & MJ Robert Nout

Fresh cassava roots (9) were split into segments to obtain pieces with similar levels of cyanogenic glucosides. From each root, one segment was deep-frozen immediately and analysed to serve as a reference. Remaining segments were dried and stored for 8 months under traditional household conditions in rural north east Mozambique. In these pieces, a varying extent of fungal growth occurred. They were ground and analyzed individually for moisture, cyanogenic potential and cyanohydrins plus HCN, pH, brightness, aflatoxins and the number and genus of fungal propagules.

Mean ($\pm$SD) initial cyanogenic potential was 399 ($\pm$273) mg CN equivalent per kg on dry weight basis. By the traditional processing and storage a considerable (range 92.3 - 99.5%) loss in cyanogenic potential was achieved. Levels of cyanohydrins plus HCN together ranged from 19 to 89% of the total cyanide. There was no correlation between the initial and residual cyanogenic potential. However, darker flours had significantly lower levels of cyanogenic potential, as well as cyanohydrins plus HCN. Similarly, darker flours showed a higher pH. No aflatoxins could be detected. It is concluded that safety of cassava flour cannot be judged by colour or extent of fungal growth.

INTRODUCTION

Cassava (Manihot esculenta Crantz) is a shrub whose starchy storage roots form the main staple food for an estimated 500 million people in the third world (Lancaster et al. 1982). In north-east Mozambique, cassava is grown mainly on family plots and produces the main staple food for the rural population (Casimiro 1972). In 1981 there was an epidemic of spastic paraparesis in which cyanide from cassava played an etiological role (Ministry of Health of Mozambique, 1984). This aroused alertness on the toxicological aspects of cassava.

The predominant method of processing the roots is as follows: During the main harvest in August/September in the dry season, nearly all mature cassava plants are uprooted. The roots are peeled, cut into pieces of about 20 cm length and split longitudinally if these are regarded as being too thick. These pieces are exposed to the sun by spreading them out neatly on the ground (coastal zone) or on a platform (interior). After about 2 to 3 weeks of sun-drying, the cassava is transferred to a store, which may be a separate building or, in the coastal zone, a loft in the house or kitchen. Eventually, the cassava being consumed from immediately after the drying up to the next year's harvest, the pieces are drawn from the store and pounded into flour for consumption as a stiff porridge on the same or the next day.

Although the harvest takes place during the dry season, occasional showers may cause the drying pieces to become wet, enabling a more or less profuse mould growth to take place. This may also happen to stored dried cassava during the rainy seasons, due to inadequate roof protection.

Infestation with various storage pests is common, but was reported by local peasants to occur later in bitter than in sweet cultivars. In one region, the Murrupula district, part of the rural population encourages mould growth prior to dehydration as follows: The fresh, peeled cassava pieces are kept covered with leaves for three days prior to exposing them directly to the sun. This diminishes the toxic effect of bitter cassava, according to the local peasants. The presence of moulds in the cassava pieces and the products made from them -flour and porridge- usually results in a grey and sometimes greenish grey colour. Sometimes, dark cassava flour is preferred over white by the rural inhabitants of the coastal zone.

In a period when food was rather scarce in the urban areas, the Ministry of Health, to which one of the authors was attached, declared a large quantity of heavily infested and moulded dry cassava as unfit for human consumption. The workers at the mill and other citizens complained about the decision, some of them stating that this was the proper appearance of cassava. Health officials clearly had a different opinion about suitability for consumption from the potential consumers.

Can dried cassava pieces or flour be judged solely by their appearance? In other words, is dark cassava flour less safe than white? There are many aspects to verify

the nutritional value of cassava flour. In this study we limited ourselves to items of toxicological relevance, viz. cyanogens and aflatoxins, and some other parameters in order to understand some of the relationships and processes that lead to higher or lower toxicity.

MATERIALS AND METHODS

Sample preparation

The preparation of the cassava samples was carried out using traditional methods: The handling and treatment of the cassava roots were carried out by local peasants with a minimum of instruction, but under observation by one of the authors.

In a coastal village, nine roots of the bitter cassava cultivar, 'Gurue', were harvested in the morning. They were peeled immediately and cut longitudinally into four segments in order to obtain pieces containing similar levels of cyanogens (De Bruijn 1971). Each segment was labeled. One segment from each root was put immediately into a polyethylene bag and stored in a deep-freeze to serve as a reference. Two segments of each root were spread out on the humid, sandy soil, together with other cassava pieces of the family, to be sun-dried. The pieces were looked after and sometimes turned over by a peasant woman, until they were found to be satisfactorily dried (this was after two weeks) and subsequently stored in her loft, together with the other dried pieces.

The cassava pieces were not affected by rain during the drying period, but during storage some of them became wet during the rainy season because of roof leakage. After 8 months, 14 experimental samples were recovered from the loft, put into polyethylene bags and transferred to a deep-freeze, where they remained until analysis.

Most of the samples showed mould growth; none was infested by insects.

Analyses

Analyses of cyanogen and moisture contents of the fresh reference segments were carried out 2 weeks after sampling, in the National Laboratory of Water and Food Hygiene in Mozambique, as follows: The samples were homogenized in cold water with a blender, after which a sub-sample of the suspension was incubated for 16 - 20 hours at 37 °C for autolysis. The cyanide was then expelled by steam-distillation, until at least 200 ml condensate had been collected in 25 ml of 0.1 M KOH (De Bruijn 1971). Colouration of the cyanide was achieved by forming a complex with pyridine, barbituric acid and hypochlorite (Lundquist et al. 1979) and was measured at 580 nm with a Varian Spectrophotometer. Moisture was measured by incubation of a 5 - 10 g sub-sample at 110±2°C, until constant weight was reached.

Analysis of the experimental segments was carried out after 1 year's storage at -18°C in the laboratories of the Agricultural University, Wageningen, The Netherlands. After having reached room temperature, each segment was unpacked and milled in a mini-hammer mill (Culatti, DFH 48) with a 1 mm mesh sieve, overheating being avoided. The homogenized flour samples were extracted by 8 minutes of gentle shaking of an Erlenmeyer flask containing 1.00 g flour in 20.0 ml 0.1 M orthophosphoric acid.

Total cyanogens, as well as cyanohydrins plus HCN, all expressed as mg of CN equivalent per 100 g on dry weight basis, were determined by the enzymic assay and the pyridine/pyrazolone colourimetric technique as described by Cooke (1979). Determinations were carried out in stoppered test-tubes. Brightness (Y) was determined by light reflection, using a Hunterlab model D25 optical head and served for the ranking of the flours. The pH was established according to Williams (1984) with 2.00 g of flour suspended in 20.0 ml freshly boiled water. Moisture content was determined by oven-drying of 2.0 g sample at 110 ± 2°C until constant weight was reached. The origin of the dark colour was examined microscopically. Fungal counts were determined using oxytetracycline glucose yeast extract agar (Oxoid CM 545), incubated at 22 °C for 5 days. Identification of fungi was based upon their cultural and morphological characteristics. Aflatoxins were analysed semi-quantitatively as follows: A 2 g sample was extracted with 10 ml chloroform for 2 h. The chloroform extract (1 - 10 μl) was spotted on HPTLC plates (Art. 5631 Merck, Darmstadt) together with quantitative references of aflatoxins. After developing with diethyl ether, the plates were developed with chloroform : acetone (90 : 10, v/v) in the same direction. Prior to use, the plates were activated by drying for 2 hours in an oven at 103-105°C, and allowed to cool for at least 1 h in a dessicator. Confirmation of the identity of aflatoxin was carried out according to EEC norm nr. 76-372-EEC. L102 (1976).
All chemical analyses were executed in duplicate. All samples were handled in one analytical run, to allow for comparison between the results.

RESULTS

Most of the dried cassava pieces had dots or larger areas with dark grey and slightly greenish moulds on the surface, as well as dark grey streaks within the pieces. The colour of watery suspensions of the ground pieces, ranged from white to dark grey. Subjective (visual) ranking of the flours corresponded very well to the determinations by brightness measurements. Inspection by microscope revealed mycelium fragments in the flour samples. These were significantly more abundant in the darker flours.

TABLE 1

Means (±SD) of the different parameters in fresh or sun-dried and stored cassava root pieces

Parameters	Fresh root (n=9)	Flour (n=14)
Moisture (%)	65.0 ± 2.4	14.2 ± 0.6
Total cyanogens (mg CN eq./kg dry weight)	399 ± 91	12 ± 14
Cyanohydrins plus HCN (mg CN eq./kg dry weight)		7 ± 8
Cyanohydrins plus HCN as % of total cyanogens		50.5 ± 22.5
pH		8.5 ± 0.8
Fungal counts (cfu/g)		400 ± 530

TABLE 2

Indication of correlations between the different parameters of cassava flours

	ngCN	ngCN%	pH	Brightness	Fungal count
Total cyanogens	+ + +	+	- - -	+ + +	0
Cyanohydrins + HCN (ngCN)		+ + +	- - -	+ + +	0
ngCN as % of total cyanogens			0	0	⊕
pH				- - -	+
Brightness					⊖

Spearman rank correlation: + + +, + + , + : Positive correlation; - - -, - - , - : Negative correlation, statistically significant at, respectively, $P<0.01$, $P<0.05$ and $P<0.1$ (two-tailed); ⊕ and ⊖ : positive or negative tendency ($P<0.1$, one-tailed). 0: no correlation.

Table 1 summarises the analytical results (means ±SD). Table 2 summarises the relationships observed between the parameters of the flour samples, based upon Spearman's rank correlation test (Snedecor & Cochran, 1980). With the same test, no relationship could be detected between (initial) cyanogenic potential of reference samples, and residual cyanogen contents in flour samples of the same root. Since a ranking technique was used, the use of different analytical methods is not likely to influence the probability for correlation, as the same analytical method has been applied within each array of reference or experimental samples. Likewise, no relationship between cyanogen contents of flour samples from the different segments of the same root were observed.

The moisture content of the flours showed marginal differences at the time of analysis and no correlation with other parameters was observed.

The fluorescent spots that developed on the HPTLC plates were initially mistaken for aflatoxin. The size of the spots varied among the flour samples. A positive correlation with the flour brightness was observed: The brighter the flour, the higher the level of the fluorescent compound. However, confirmation of the

identity of aflatoxin with the EEC test was negative.

Various genera of fungi could be identified, including *Rhizopus, Mucor, Penicillium* and *Fusarium* spp. No relationship was observed between the occurrence of any of these fungi and other parameters.

DISCUSSION

Table 1 shows that the darker the flour, the less total cyanogens it contained. Moreover, a lower level of total cyanogens was associated with an even lower level of cyanohydrins plus HCN. In the bioconversion of the experimental samples, the mould growth is the principal factor and independent variable. One of the results of mould growth is the darkening of the flour. This may be caused by fungal spores or by disruption of the integrity of the cells, causing the formation of blue-black streaks (CIAT 1973), or both. Although not all fungi produce dark coloured spores, the fact that the darker flours contained more mycelial fragments than the whiter flours supports this relationship. So does the weak negative correlation between brightness and the count of fungal propagules. The poor relationship, in general, between fungal counts and the extent of mould growth (Nout 1984), may interfere with a statistical analysis of relationships between fungal counts and other quality parameters.

In the tested cassava, a solid-state fermentation of a predominantly fungal nature has taken place. Various fungal enzymes, e.g. proteases, deaminases and decarboxylases, may have contributed to a pH increase. In addition, fungal activity may also have contributed to a more effective decomposition of cyanogenic glucosides, releasing cyanohydrins and HCN, either by the excretion of glucosidases, or by disintegration of the cell wall, thereby facilitating the contact between linamarase from the cell wall and the linamarin, or both. As HCN is assumed to escape easily through evaporation, this is regarded as the main pathway for the loss of cyanogenic potential in cassava during the process of drying and storage.

The "non-glucosidic cyanogens" are thought to contain cyanohydrins, HCN, and other cyanogenic compounds which are measurable without enzymatic or strong acid action. The range of non-glucosidic cyanogens (29 - 89 % of total cyanogens) is quite high. A consequence of this finding, for public health, might be that a larger part of the cyanogens in dried cassava remains in a form that is readily available to the body than has been assumed up until now.

No aflatoxins nor aflatoxin-producing fungi were detected. Care should be taken with aflatoxin determination, as some compound in cassava can apparently be mistaken for aflatoxin in a certain analytical setting. Coker and Tomlins (1986) mention scopoletin as an interfering agent in this respect and make suggestions for a

confirmation test. As high levels of aflatoxin contamination in cassava flour from Mozambique have been reported (Mota 1974), the possible presence of aflatoxin, as well as its determination merit alertness. Although aflatoxins were not detected in the present study, some of the moulds identified are capable of elaborating mycotoxins under some conditions. Consequently, traditional products should be screened for mycotoxins in future investigations.

Although several hypotheses on the processes in the contaminated drying of cassava that led to our findings remain to be tested, this experiment indicates that dark, moulded cassava pieces had much lower cyanogenic potential than white pieces, whilst aflatoxin was absent from all samples. The different residual cyanogen levels could not be accounted for by the initial ones. A causal relationship between the presence of the moulds and diminishing cyanogen levels is likely and merits further research.

In conclusion, we postulate that although dark cassava flour may not always be safer than white, the latter is not necessarily safer than dark flour. Consequently, a judgement of toxicity, based on only colour or mould growth, is certainly not appropriate.

ACKNOWLEDGEMENTS

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REDUCING CASSAVA TOXICITY BY HEAP-FERMENTATION IN UGANDA

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Processing of cassava roots by the Alur tribe in Uganda includes a stage of solid substrate fermentation in heaps. Changes in cyanogen levels during the process, microflora involved, and protein levels, amino acid patterns and mycotoxin contamination of the final products were studied. Processing was monitored at six rural households and repeated at laboratory site, comparing it to sun-drying. Flour samples from rural households were analyzed for residual cyanogens, mutagenicity, cytotoxicity and aflatoxins.

Mean ($\pm$SD) total cyanogen levels in flours collected at rural households were 20.3 ($\pm$16.8) mg CN equivalents kg^{-1} dry weight in 1990 (n = 23) and 65.7 ($\pm$56.7) in 1992 (n = 21). Mean ($\pm$SD) levels of cyanohydrins plus HCN were 9.1 ($\pm$8.7) in the 1992 flours. Total cyanogen levels in the village monitored batches were reduced considerably by heap-fermentation from 436.3 ($\pm$140.7) to 20.4 ($\pm$14.0) mg CN equivalents kg^{-1} dry weight cassava. Residual cyanogen levels were positively correlated with particle size of the resulting crumbs. Heap-fermentation was significantly more effective in reducing cyanogen levels than sun-drying alone, but did not always result in innocuous levels of cyanogens.

Dominant mycelial growth was from *Neurospora sitophila*, *Geotrichum candidum* and *Rhizopus oryzae*. No mutagenicity, cytotoxicity nor aflatoxins could be detected in the flours. Protein quantity and quality were not significantly affected. Cassava gel viscosity pattern was modified to the consumers' preference by this method.

As the removal of cyanogens was more efficient and we found no new obvious health risk, heap-fermentation can be regarded as an improvement compared to sun-drying alone in areas where cassava cultivars with higher cyanogen levels prevail, but we recommend optimization of the process for ensuring still safer products.

INTRODUCTION

Due to several agronomic advantages, the starchy root crop cassava has become the second most important staple food in Uganda, and it presently supplies one fourth of the dietary energy in the country (Romanoff and Lynam, 1992). An aspect of cassava, especially of bitter cultivars, that merits special attention is its cyanogenic potential. The cyanogenic glucosides, mainly linamarin, in the plant cells can be cleaved by the enzyme linamarase, located in the cell walls (Mkpong *et al.*, 1990). The cyanohydrins formed are increasingly unstable at a pH higher than 5, yielding the volatile hydrogen cyanide (HCN) (Cooke, 1978). Dietary cyanide exposure from insufficiently processed cassava is a factor in some public health problems (Rosling, 1993), but the dynamics of cyanogen removal during processing and the mechanisms of cyanide exposure from insufficiently processed cassava are not yet fully understood.

People of the Alur tribe, living mainly in north-west Uganda, traditionally apply a process involving solid substrate fermentation in covered heaps, heap-fermentation, on their -predominantly bitter- cassava roots. The aim is to reduce toxicity and improve palatability of the stiff porridge that they prepare from the resulting flour. For outsiders the process seems an uncontrolled way of rotting, leading to 'a most unappetising looking article' (Anderson, 1944). The method was earlier reported from Kenya by Anderson (1944), from Tanzania by Childs (1961), from Uganda by Goode (1974), and from Mozambique by Essers *et al.* (1992), but it was never studied in detail. The Uganda Ministry of Agriculture recently noted that the use of bitter cassava cultivars and the heap-fermentation practice seem to be spreading from the western part of Uganda to find acceptance in other parts of the country. The Ministry's concern about these developments led to this study.

This paper reports on the processing of cassava roots by heap-fermentation in Uganda. It presents the dynamics of cyanogen removal, and the residual cyanogen levels, associated microflora, absence of mycotoxins, protein quantity and quality of the flours obtained, viscosity of a gel prepared from them, and discusses product safety.

MATERIALS AND METHODS

Study area

Monitoring of the heap-fermentation process, sample collection and interviewing took place in Kiryandongo village, a trading locality in Masindi district, located along a tar road, 200 km north-west of Kampala. In the last decades, much migration has taken place in this area and in the village approx. 50 tribes are represented, according to the District Administration. The village was selected for its situation as one of the rural areas nearest to the capital where cassava is the main staple crop and heap-

fermentation is commonly applied by a substantial number of its inhabitants.

Interviews
After information gathering from several key informants in Kampala, semi-structured interviews were held in 1990 in 11 of the households where cassava samples were collected. The information from observations and these interviews was validated in focus group interviews (Melville, 1993). This was done with one group of about 10 young and elderly women of the Alur tribe and one group of women of the Banyoro tribe in Kiryandongo village. The interviews focused on the common cassava processing and deviations from it, why and when the process is applied, how people learned about it, reasons for choosing bitter cultivars and knowledge of toxic effects from cassava.

Flour sample collection
Dried fermented cassava crumbs of the bitter cultivar *karangwa*, considered ready for conversion to flour and preparation, were collected at consecutive Alur households in Kiryandongo village in April and September 1990 (23 samples) and in October 1992 (21 samples). The number of storage days and the anticipated use were registered. After processing, the products had been stored in this form for 1 to 9 days. In 1990, samples were extracted 2 - 10 days after collection, and the extracts were stored frozen between 1 and 4 months until analysis for total cyanogens. In 1992, samples were extracted on the day of collection. The extracts were stored deep-frozen and analyzed for cyanogens within 14 days.

Monitored processing
All processing steps applied to the cultivar *karangwa* were closely observed at 6 individual households in Kiryandongo village in September 1990. Temperature was measured every morning inside and outside the heaps. One or more samples were taken at every stage (6) of the process for determination of microflora, pH, moisture and cyanogen levels. At 2 households, half (lengthwise) of each fresh root of the initial sample was sliced, sun-dried and pounded into flour, to serve as non-fermented controls. Cyanogen extraction and pH determination were done within 4 h after collection, in the village.

Experimental processing at laboratory site
To verify the results from the village based processing, the same processing method was performed and extended in February 1992 by an experienced Alur farmer from Kiryandongo at a laboratory site in Kampala, as follows: Within 3 h after harvest, 300 roots of cultivar TMS 30572 from Namulonge Research Station were peeled and divided over 5 batches, using a randomized schedule. Roots of the first batch were

split in quarterly segments, to facilitate drying and avoid fermentation during the humid weather, and sun-dried for 6 days on a cane mat. The other 4 batches were submitted to heap-fermentation. On day 6 all remaining roots were pounded batch-wise into flour and cooked into a stiff porridge, *ugali*. Sampling took place at each stage.

Sample preparation at monitored and experimental processing

Twelve whole roots were selected at random and split once lengthwise. Half of each root was discarded to reduce sample size without violating cyanogen levels (De Bruijn, 1971). Remaining halves were diced to approx. 1 cm^3, hand-mixed and 4 subsamples of 70.0 g were each homogenised in a blender (Braun MX2) with 250 ml extraction medium (e.m.), consisting of 0.1 M orthophosphoric acid, supplemented with 25% ethanol in the village-made extracts for improved storability (O'Brien *et al.*, 1991). For moist crumbs, 2 subsamples of approx. 50.0 g were each homogenised in 150 ml e.m. For flours, 2 subsamples of approx. 4.00 g were each suspended in 25 ml e.m. and swirled for 10 min. Dry crumbs were pounded into flour and extracted accordingly. From the cooked flours (*ugali*), 2 subsamples of approx. 50.0 g were each homogenised in 150 ml e.m. Extraction took place by centrifuging the homogenates and suspensions. Samples were extracted within 4 h after collection. Village-made extracts of 1990 were stored below 15 °C for 2 to 16 days and subsequently at -18° C until analysis of cyanogens within 4 months. The extracts from the laboratory-site experiments were stored frozen at -18° C and analyzed within 2 weeks for cyanohydrins plus HCN, and within 2 months for total cyanogens.

Cyanogen, moisture and pH determination

Total cyanogens, as well as cyanohydrins and HCN were assayed according to Essers *et al.* (1993). Moisture levels were assayed by oven drying of 30.00 to 40.00 g at 105 ± 5°C until constant weight. The pH was measured 10 min after homogenizing approx. 40 g cassava dices or crumbs in 150 ml or suspending 10 g flour in 100 ml cooled, previously boiled distilled water.

Consistency characteristics

Viscosity at gelatinization was measured in the village fermented flours and the 2 sun-dried control flours by means of a Brabender Visco-Amylograph, applying 30 g of flour to 450 ml water, a rotation speed of 75 rev min^{-1} and a 350 cm g^{-1} measuring cartridge. Temperature was increased from 50 to 95°C at a rate of 1.5°C min^{-1}, thereafter kept at 95°C for 30 min and then lowered to 50°C also at 1.5°C min^{-1}.

Crude protein and amino acid determination

Crude protein was calculated by multiplying the nitrogen level, assayed with a routine Kjeldahl method, by 6.25. Amino acids were analyzed chromatographically according

to Stein and Moore (1963) with a Merck Biotronic LC-5001 analyzer. The amino acids were released by hydrolysis by boiling for 22 hrs with 6M HCl. The determination of cysteine and methionine was preceeded by oxidation with performic acid, which was later bound to bisulfite. Chemical score per essential amino acid was calculated as mg amino acid per g of crude protein, divided by its value in the reference protein, times 100 (FAO/WHO, 1972).

Mutagenicity and cytotoxicity
The presence of mycotoxins was estimated by assaying mutagenicity and cytotoxicity by Ames test (Maron and Ames, 1983) on extracts of 25 flours and one sample of dried scraped-off moulds, using the tester strains *Salmonella typhimurium* TA100 and TA98, with and without the addition of S9 liver homogenate. Samples of 100 g were extracted according to Filtenborg *et al.* (1983). Calibration curves of mutagenicity were made from commercial aflatoxin B_1 and sterigmatocystine in serial dilutions up to 1.0 and 10.0 μg, respectively, per plate. The recovery of these compounds supplemented to flours was higher than 80%. A positive dose response relation indicates an induced mutagenicity. A negative dose response relation indicates cytotoxicity. The detection limit for mutagenicity was calculated as 2.5 μg of aflatoxin B_1 per kg of flour.

Aflatoxin determination
Aflatoxins were assayed chemically by HPTLC (Soares and Rodriguez-Amaya, 1989) in 10 flour samples after extraction with methanol. The detection limit for aflatoxin was 2.0 μg kg^{-1}

Microbiological analysis
Cassava flour samples were pre-soaked (10 g sample in 90 ml peptone physiological salt solution) for 15 min and then homogenized for 1 min in a *Colworth Stomacher* blender. Serial dilutions were made with peptone physiological salt solution containing 1 g bacteriological peptone and 8.5 g NaCl per litre. Total aerobic mesophiles were counted in pour plates of plate count agar (Oxoid CM 325) incubated at 30° C for 3 days. The same medium was used for counting bacterial endospores in a 10 fold dilution of the sample which was kept at 80° C for 10 min. *Lactobacillus* spp were counted in pour plates of Rogosa agar (LSB agar, BBL 11327) containing 0.2 % Natamycin (Delvocid, Gist Brocades) with overlay and incubated at 30° C for 3 - 5 days. Micrococcaceae were counted in pour plates of Mannitol Salt agar (Oxoid CM 85) incubated at 30° C for 2 days. *Pseudomonas* were counted in surface-inoculated plates of Pseudomonas agar F (Difco 0448-01) incubated at 30° C for 4 days. Enterobacteriaceae were counted in overlaid pour plates of Violet Red Bile Glucose agar (Merck 10275) incubated at 30° C for 24 hrs. Coliform bacteria were counted

in overlaid pour plates of Violet Red Bile agar (Oxoid CM 107) incubated at 30° C for 24 hrs. *Escherichia coli* were enumerated using a 3 tube MPN method with non-selective pre-enrichment in Buffered Peptone water (Oxoid CM 509) at 37° C for 24 hrs, followed by selective enrichment in Brilliant Green Bile (2%) Broth (Oxoid CM 31) at 44° C for 48 hrs. Positive tubes were confirmed using Violet Red Bile agar, incubated at 37° C for 24 hrs. *Bacillus cereus* were enumerated on surface-inoculated plates of Mannitol Egg Yolk Polymyxin agar (Merck 5267) with addition of Polymyxin B Sulphate (Pfizer 90 A02). *Clostridium perfringens* were estimated using a 3 tube MPN method with selective enrichment in Rapid Perfringens medium (Thioglycollate medium Oxoid CM 173 with additions of 60 g gelatin, 5 g peptone, 5 g glucose, 5 g K_2HPO_4, 3 g yeast extract, 1.5 g NaCl, 0.5 g $FeSO_4$, 70 g non-fat milk powder, 0.5 g litmus, 0.075 neomycin sulphate and 0.0125 g polymyxin B-sulphate, per litre) incubated at 46° C for 24 hrs, with confirmation on cycloserine agar (Perfringens Agar Base, Oxoid CM 587 with D-cycloserine, Serra 17685), incubated anaerobically at 37° C for 48 hrs. *Staphylococcus aureus* were counted in pour plates of Baird Parker agar (Gibco 152-00320 M, with RPF supplement Oxoid SR 122), incubated at 37° C for 24 - 48 hrs. *Salmonella* were detected by pre-incubating 25 g sample in Buffered Peptone water at 37° C for 18 hrs, followed by selective enrichment in Muller Kaufmann broth or Rapaport Vasiliadis broth at 42.5° C for 48 hrs and detection on Brilliant Green agar incubated at 37° C for 24 hrs. Yeasts and filamentous fungi were enumerated and isolated on Oxytetracycline Glucose Yeast Extract agar (Oxoid CM 549), Dichloran-Glycerol medium (Oxoid CM 729) and Rose Bengal Chloramphenicol Oxytetracycline agar (Oxoid CM 549, with 150 ppm Rose Bengal). Filamentous fungi were identified on the basis of their macro- and microscopic morphology according to Samson & van Reenen-Hoekstra (1988).

Statistical analysis
Testing for relations between sample variables in the 1992 random collected flours was by F-test, and testing differences between batches was by two-tailed Student's *t* (Snedecor and Cochran, 1980, page 221 and 93, respectively). Logarithmic transformation of the data was applied where necessary to stabilize the variance. Standard deviations (SD) given are with n-1 degrees of freedom.

RESULTS
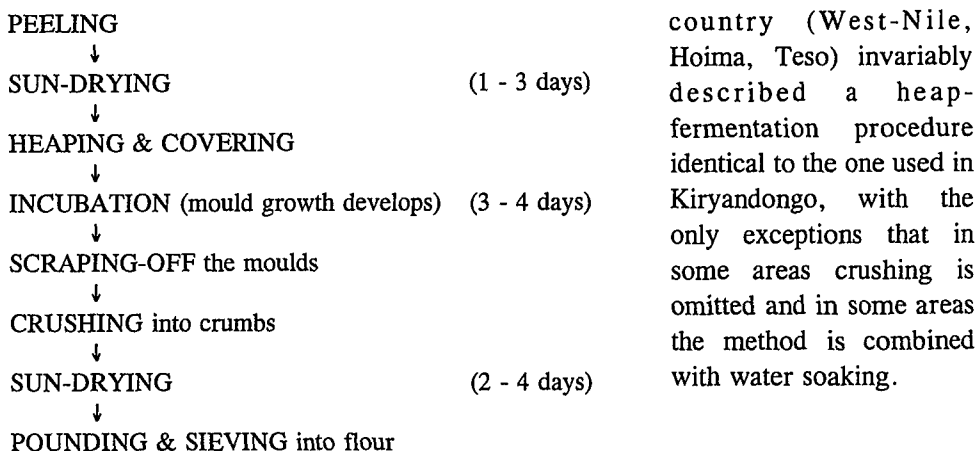
Application of the heap-fermentation process
All 21 Alur interviewees grew both bitter (*makesh*) and non-bitter (*pekesh*) cassava cultivars. The bitter cultivar *karangwa* (also called *tongolo*) was presently grown most, because of its high yields, early maturation, long storability in the soil, and its low susceptibility to theft and herbivore voracy. Bitter cassava roots always undergo heap-

fermentation (*kwogu ungura*), to reduce toxicity, bitterness and rubberiness before consumption in the form of a stiff porridge (*ugali, posho* or *mogo*) made from the resulting flour, and as a preparative step in the processing into *waragi*, a strong distilled alcoholic beverage. Non-bitter cassava roots may be boiled and eaten as a snack, or sun-dried for flour production and subsequent preparation of a *ugali* meal, but they are often also heap-fermented to obtain the preferred palatability. *Ugali* from cassava flour is more rubbery (*dwadwa*) in texture than from cereals, which is not appreciated. This counts especially for *ugali* made of flour from non-fermented roots. Without proper fermentation, bitter cassava is said to remain bitter and induce acute toxic effects like headaches, dizziness, vomiting, diarrhoea, palpitations, muscle-weakness, and even "kill people and animals". The respondents were convinced of this association, and examples of deaths by accidental cassava poisoning were known by all Alurs interviewed. *Waragi* from bitter cassava is said to be stronger than from non-bitter cassava, and thus preferred: "It strikes harder."

The interviewed Alurs considered the processing of cassava by heap-fermentation as part of their tradition which was taught to them by their parents. The process follows in principle a strict schedule (Fig 1) and requires skill for adaptation to weather conditions. Greenish moulds were considered as a sign of spoilage by the processors, caused by an insufficient first drying step. Initially, white moulds appear, but near the end of the heap phase, the roots should be covered with moulds with a black or -less preferred- orange appearance, or both. The crucial criterion for ending the heap phase was not the time spent, but the softness of the roots. It was observed that the particle size after crushing depended upon the softness of the fermented roots, the weather conditions and the anticipated use of the flour. If used for sale, larger pieces were preferred, as less is needed to fill a tin, the marketable volume. The interviews revealed that acute toxic effects have been experienced when people were in a hurry because of food shortage or high market demand. Drying and incubation stages were shortened or steps altered. One short-cut method, considered risky, starts with crushing the peeled fresh roots to small particles, followed by covered incubation for two days and subsequent sun-drying. Acute toxic effects have also occurred when the sun-drying steps were hampered by rainy and humid weather. Toxicity of young roots is believed to be higher than of older roots.

Several people from the Banyoro tribe in Kiryandongo village also cultivated the cultivar *karangwa* for the market and for brewing, as its yields are high. They also processed it by heap-fermentation, which they had learned from the Alur. Some of them, however, recently stopped growing bitter cassava cultivars because of the tiresome and time consuming heap-fermentation, their dislike of the smell and dark colour of the resulting *ugali*, and the recent (1990) lower prices for fermented flour induced by the increased production.

Interviewed students of Makerere University originating from other parts of the

PEELING
↓
SUN-DRYING   (1 - 3 days)
↓
HEAPING & COVERING
↓
INCUBATION (mould growth develops)   (3 - 4 days)
↓
SCRAPING-OFF the moulds
↓
CRUSHING into crumbs
↓
SUN-DRYING   (2 - 4 days)
↓
POUNDING & SIEVING into flour

country (West-Nile, Hoima, Teso) invariably described a heap-fermentation procedure identical to the one used in Kiryandongo, with the only exceptions that in some areas crushing is omitted and in some areas the method is combined with water soaking.

FIGURE 1 Steps and stages in the heap-fermentation processing of cassava roots

Cyanogen levels

Monitoring the processing of cassava by heap-fermentation in 6 rural households showed similar patterns of cyanogen levels over time and the same relation to moisture level and pH, though the levels and the time spans differed between batches. Figure 2 shows the pattern of one of the households as an example. Mean ($\pm$SD) initial total cyanogen level was 436 ($\pm$141) mg CN equivalents kg^{-1} dry weight and residual level in the flours was 20.4 ($\pm$14.0) mg CN eq.kg^{-1} dry weight or 4.5% ($\pm$2.2) of the initial level. Mean residual level of cyanohydrins plus HCN in the flours was 2.8 ($\pm$2.4) mg CN eq.kg^{-1} dry weight. Duration of the processing varied from 5 - 9 days. Mean ($\pm$SD) moisture levels were reduced from 64% ($\pm$3) to 53% ($\pm$5) during the first drying step, and to 13% ($\pm$2) in the resulting flours. Mean ($\pm$SD) initial pH levels were 6.3 ($\pm$0.1), followed at first by a decrease to 5.5 ($\pm$0.4) during the fermentation stage, and gradually increasing to 7.7 ($\pm$0.6) in the flours. Ambient temperature near the heaps fluctuated between 23 and 29°C. Temperature of the incubated roots on the last incubation day was 2 to 12°C higher than this, with a recorded maximum of 35°C. Crushing of the moist fermented roots resulted in crumbs of up to 2½ cm ø. Separate sampling and analysis of smaller (< ½ cm ø) and bigger (> 1 cm ø) crumbs per batch at various moments during drying (Table 1) showed that levels of cyanogenic glucosides, as well as cyanohydrins plus free cyanide, in small crumbs were significantly lower than in big crumbs ($P=0.0016$ and $P=0.015$, respectively, after log transformation). The differences in cyanogen levels between big and small crumbs. could not be attributed to differences in moisture levels. We observed in the village monitored processing, that the bigger crumbs originated mostly from the less affected inner parts of the thicker roots.

TABLE 1

Levels of glucosidic and non-glucosidic cyanogens (mg CN eq.kg^{-1} dry wt) and moisture of small (< ½ cm ø) and big (>2 cm ø) crumbs of crushed heap-fermented cassava from 6 heaps at different moments during the drying stage.

Sample	Big crumbs			Small crumbs		
	CNg[a]	ngCN[b]	moisture	CNg[a]	ngCN[b]	moisture
(nr)	(mg CN eq.kg^{-1} dw)		(%)	(mg CN eq.kg^{-1} dw)		(%)
1	125.3	9.5	49	3.0	7.0	37
2	36.0	3.9	46	3.3	8.4	44
3	65.1	26.9	41	27.5	7.0	45
4	56.2	17.0	39	9.7	3.6	20
5	150.0	6.4	34	23.7	4.0	18
6	16.1	4.5	18	8.9	1.3	14
7	47.0	1.3	16	16.2	0.8	15
8	12.9	8.5	16	7.9	0.8	11
9	15.9	11.6	11	3.2	1.7	12

[a]CNg =cyanogenic glucosides; [b]ngCN =non-glucosidic cyanogens =cyanohydrins plus HCN
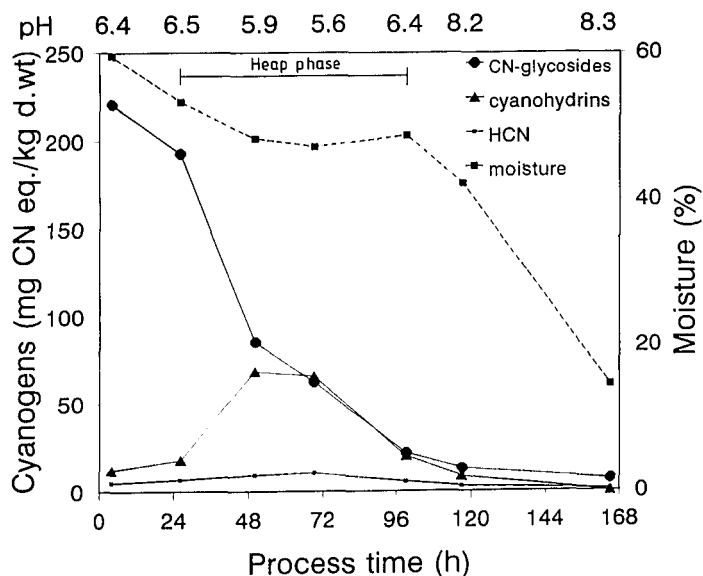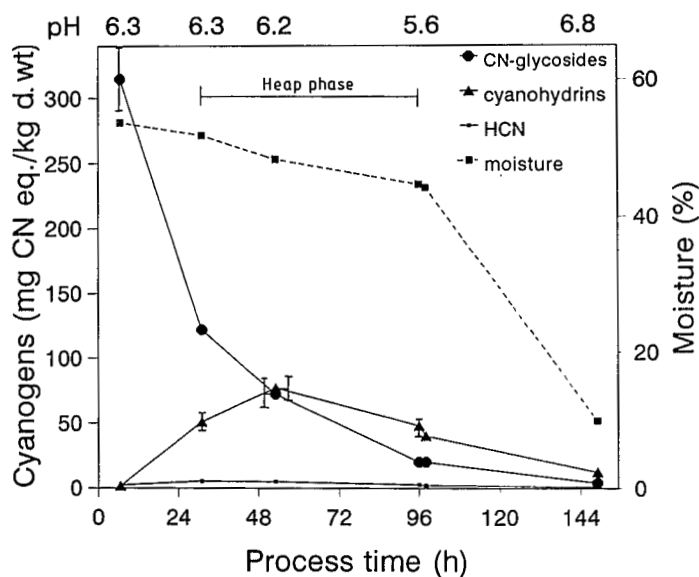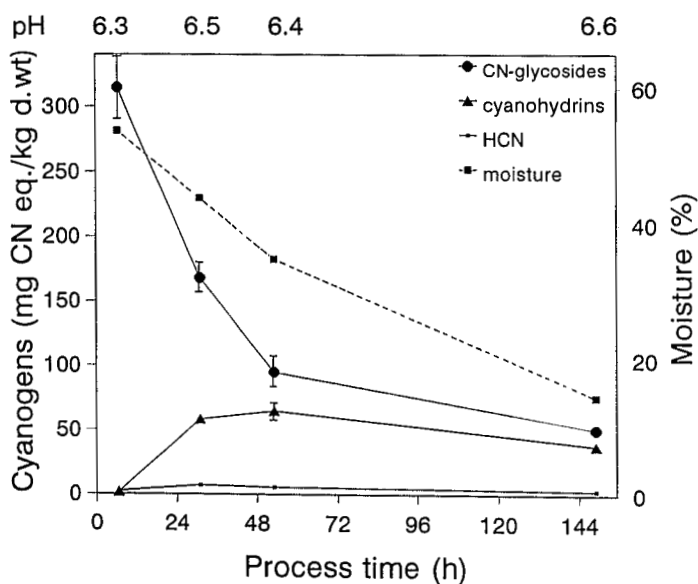

FIGURE 2 Cyanogen levels in a batch of cassava roots (cultivar *karangwa*) during domestic processing by heap-fermentation

FIGURE 3 Mean levels of cyanogens ($\pm$SD), pH and moisture in 4 batches of cassava roots (cultivar TMS30572) during the process of heap-fermentation at laboratory site

FIGURE 4 Mean levels of cyanogens ($\pm$SD), pH and moisture in a batch of cassava root segments (cultivar TMS30572) during the process of sun-drying at laboratory site

Figure 3 shows the mean (±SD) cyanogen levels of the 4 batches during processing by heap-fermentation at laboratory site and Figure 4 shows the mean (±SD) cyanogen levels of 4 subsamples of the batch of sun-drying roots as control. Lab-site experimental processing showed significantly lower levels ($P < 0.001$, $P < 0.02$ after log transformation) of residual glucosides in the heap-fermented batches (3.4 ±1.3) than in the sun-dried cassava (40.3 ±2.7). Also, cyanohydrin levels were significantly lower ($P < 0.025$) in fermented batches (12.3 ±2.8) than in the sun-dried cassava (37.8 ±1.2).

All 10 assayed cooked samples (*ugali*) had similar levels of total cyanogens on dry weight basis to the flours they were derived from.

Residual total cyanogen levels in 23 samples of heap-fermented cassava crumbs, collected in 1990, ranged from 1.4 to 65.8 mg CN eq. kg^{-1} dry weight, with a mean (±SD) of 20.3 (±16.8). Cyanohydrins plus HCN were not assayed separately.

Residual cyanogenic glucoside levels in the 21 samples of crumbs collected in 1992 varied between 7.1 and 202 mg CN eq. kg^{-1} dry weight, with a mean of 56.5 (±53.3). Residual levels of cyanohydrins plus HCN varied between 1.2 and 33.4 mg CN eq. kg^{-1} dry weight, with a mean of 9.1 (±8.7). Moisture levels varied between 13.0 and 19.1% with a mean of 16.2% (±1.4). The data of these samples were examined for relationships between levels of glucoside, cyanohydrin plus HCN and their proportion of the total cyanogen levels on the one hand, and number of storage days, anticipated use and moisture levels, on the other. In the 6 batches that were meant for sale, cyanogenic glucoside levels (110.0 ±65.5 mg CN eq. kg^{-1} dry weight) were significantly ($P=0.0007$) higher than in the 15 batches meant for own consumption (35.2 ±28.4). The relationship was still significant ($P=0.006$) after correction for the other variables. There was a significant correlation between the anticipated use of the flours and the cyanohydrins plus HCN : total cyanide ratio ($P=0.02$, after log transformation), but this correlation was lost after correction for the effect of the other variables. No other correlations were found.

Two batches of fresh roots of the same cultivar, harvested in the same village at the time of flour sample collection in 1992, had total cyanogen levels of 580 and 582 mg CN eq. kg^{-1} dry weight. The cassava roots obtained in 1992 were thinner and derived from younger plants than usual, due to drought and depletion of crops in 1991. In 1992, a severe infestation with cassava mosaic disease was observed.

Proteins
Levels of crude protein and the chemical score of individual essential amino acids in the six fermented and two non-fermented reference flours are presented in Table 2. The levels in fermented and non-fermented samples were not statistically different, although the chemical scores were on average slightly higher in the fermented samples.

TABLE 2

Mean levels (±SD) of crude protein and chemical score of individual essential amino acids in fermented (n = 6) and non-fermented (n = 2) flours

Sample	Crude protein	Chemical score						
	(g/100 g dw)	cys+met (%)	tre (%)	val (%)	ileu (%)	leu (%)	tyr+phe (%)	lys (%)
F[a]	4.5 (0.6)	17.5 (2.5)	32.0 (3.6)	30.3 (3.4)	30.7 (3.9)	26.2 (3.2)	26.0 (2.5)	31.0 (4.7)
non-F	4.7 (0.7)	15.5 (2.1)	28.0 (4.2)	29.1 (3.9)	24.5 (3.5)	21.0 (2.8)	25.5 (9.2)	29.0 (1.4)

[a]F = fermented

Microflora, mutagenicity and aflatoxins

Tables 3 and 4 specify the microflora in the 6 flours obtained from the monitored cassava batches at rural households. On the fermenting cassava, green-blue spots, probably indicating conidia of *Aspergillus* or *Penicillium* spp., were observed only sporadically. From samples taken during the process, microscopic inspection showed that initially several fungi were present which were not recovered in the resulting flour. During the incubation in heaps, the roots started softening from the outside towards the inside, coinsiding with the profuse mould growth, suggesting cellulolytic and pectolytic enzyme activity by the microflora (Okolie & Ugochukwu, 1988).

The Ames test for mutagenicity and cytotoxicity was negative for all 25 tested flours. A very slight mutagenicity was observed only in the sample of dried scraped-off moulds. Aflatoxins were absent in all 10 screened samples.

Consistency

Table 5 shows that the viscosity values of the gelatinizing flour suspensions rose by increasing the temperature to 95°C. Maintaining the temperature at 95°C resulted in a reduction, followed by a set-back at cooling down. Viscosity in the fermented flours was significantly lower ($P=0.01$) than in the non-fermented flours at reaching 95°C, and seemed somewhat higher ($P=0.1$, one-tailed) after reaching a temperature at which consumption takes place.

DISCUSSION

This formerly undocumented heap-fermentation method was capable of reducing cassava cyanogen levels considerably. Starting from the same batch of roots, it resulted in much lower cyanogenic glucoside and cyanohydrin levels than sun-drying alone in the same time span. The high cyanohydrin level and the slightly higher moisture level in the flour from the sun-dried batch indicate insufficient drying. Although the difference in glucoside levels may be explained partly by the splitting of

TABLE 3

Microflora of cassava flour processed by domestic heap-fermentation

	^{10}LogN/g (6 samples)	
	Mean	±SD
Total aerobic mesophiles	8.45	0.23
Lactobacillus spp.	7.50	0.28
Micrococcaceae	7.30	0.20
Pseudomonas spp.	6.60	(1 sample only)
Enterobacteriaceae	6.53	0.50
Bacterial endospores	6.30	0.59
Coliform bacteria	6.28	0.75
Escherichia coli	4.00	(1 sample only)
Bacillus cereus	<2.7	
Clostridium perfringens	<1.7	
Staphylococcus aureus	<1.7	
Salmonella	absent in 25 g	(1 sample only)
Yeasts	6.62	0.44
Filamentous fungi	5.00	0.61

TABLE 4

Filamentous fungi occurring in cassava flour processed by domestic heap-fermentation

	Nr of samples in which detected (n = 6)		
Aspergillus fumigatus	3	*Aspergillus niger*	1
Aspergillus oryzae	4	*Aspergillus parasiticus*	1
Fusarium sporotrichioides	1	*Geotrichum candidum*	6 [a]
Mucor circinelloides	2	*Mucor racemosus*	3
Neurospora sitophila	6 [a]	*Neurospora crassa*	1
Penicillium frequentans	1	*Penicillium waksmanii*	1
Rhizopus oryzae	5 [a]	*Rhizopus stolonifer*	4

[a] Numerous in each positive sample

TABLE 5

Mean (±SD) viscosity of gels from fermented (n=5) and non-fermented (n=2) flours

Samples	Viscosity (Brabender Units)		
	at reaching 95°C	lowest at 95°C	after cooling to 50°C
Fermented	274 ±57	185 ±30	391 ± 114
Non-fermented	450 ± 7	163 ±18	250 ± 14

the roots to be sun-dried, the stage of incubation in heaps seems to have had an additional effect on the cyanogen reduction.

The mean level of total cyanogens in the first 23 crumb-samples, collected in 1990, was similar to the one from the six flours resulting from the domestic processing monitored by us in the same month and village, and very low compared to levels (133 ± 71 mg CN eq. kg^{-1} dry wt) found in sun-dried flours collected in a normal year at households in Masasi, Tanzania (Mlingi *et al.*, 1992). The levels in the products collected in 1992 were higher than the ones from 1990. The levels of cyanohydrins plus HCN in 5 out of 21 samples exceeding 10 mg CN eq. kg^{-1} dry weight were higher than the FAO/WHO (1991) recommended upper limit (10 mg kg^{-1}) for hydrocyanic acid content in cassava flour. The total cyanogen levels in all 21 samples were higher than this limit. However, the relation of the linamarin levels to this recommended limit for hydrocyanic acid, which hardly exists in cassava products, is unclear. If this limit is meant to include linamarin levels, it seems to contain ample safety margins (Rosling *et al.*, 1993).

High residual levels of cyanogens may be caused by a high initial level or an inadequate execution of the process, due to poor judgement, negligence or time constraints on the side of the processor, or on purpose for making "stronger" spirits. Cyanogen levels might have been higher in fresh roots in 1992 due to immaturity. Cyanogen levels in fresh roots were within the same range both years, but the number of two samples from September 1992 was too small to draw conclusions from. Apparently, although useful in principle, heap-fermentation as applied in the studied village does not always lead to sufficient reduction of cyanogens. The 1992 flour samples meant for sale showed significantly higher cyanogen levels than those for home consumption, possibly caused by a shorter fermentation stage. This does not necessarily imply that flour buyers will be more exposed to cyanogens than producers. Marketed fermented flour is often used for brewing, and this purpose was explicitly mentioned for 4 of the 6 products to be sold.

Mechanisms involved in the cyanogen reduction
Levels of cyanogenic glucosides showed an exponential decline during the processes. Cyanohydrin levels rose, due to the breakdown of the glucosides. The conversion rate of glucosides to cyanohydrins was overtaken by that of cyanohydrins to HCN after 1 or 2 days of incubation. HCN disappeared nearly simultaneously and was partly lost during extraction and extract storage. The reduction of cyanogen levels during the short cooking step when preparing *ugali* was negligible.

The first drying step reduced growth conditions for bacteria and enhanced those for fungi. The heap condition, by maintaining adequate moisture levels, extended the time for degradation of cellular structures and linamarin by endogenous enzymes for several days. This process was presumably enhanced by microbial cell wall degrading

enzymes, as the roots, contrary to the non-heaped drying controls, started softening from the outside towards the inside, coinciding with the profuse mould growth. Cassava root softening has been shown to be caused by enzymatic cell wall-degradation (Okolie & Oguchukwu 1988). The barrier of compartmental separation of the linamarin and linamarase was thus further reduced. As several microorganisms can display ß-glucosidase activity (Padmaja & Balagopal, 1985), their direct involvement in linamarin breakdown is also possible. Laboratory experiments that compared the cyanogen reduction of incubated disinfected cassava with inoculated cassava showed a positive effect from microbial activity (Essers *et al.*, 1994). The pH of the fermenting cassava, generally between 5.5 and 6.3, was at the optimum for linamarase activity (Nok and Ikediobi, 1990). Each of these factors may have effectuated a continued breakdown of the cyanogenic glucosides and production of cyanohydrins during the heap phase. The higher pH level towards the end of the process, possibly caused by fungal enzymes like proteases, deaminases and decarboxilases, enhanced the breakdown of cyanohydrins into the volatile hydrocyanic acid. The pH fluctuations during this process thus facilitated the reduction of cyanogen levels. This is quite different from the low pH generally found with anaerobic fermentations as in gari (Vasconcelos *et al.*, 1990) and soaked cassava (O'Brien *et al.*, 1992).

After crushing of the fermented roots, the bigger crumbs showed significantly higher glucosidic and non-glucosidic cyanogen levels than small crumbs from the same batch. This effect was still present after drying. A more thorough crushing of the non-fermented inner parts of the roots would result in faster drying and therefore a shorter period of linamarase activity, which might consequently lead to higher residual glucoside levels. We therefore attribute more value to completion of the fermentation stage than to more intensive crushing for linamarin degradation. Better crushing thereafter may accelerate the drying and thus enhance cyanohydrin removal.

Microflora and mycotoxins
During on-site macroscopic observation of the moulded heaps, the abundant mycelium and black and orange sporulation of *Rhizopus* and *Neurospora* spp. and the white powdered-sugar like spots of *Geotrichum* were remarkable. This corresponds with the finding that these fungi were isolated from all of the flours. Except *Neurospora sitophila* and *Geotrichum candidum*, all fungi isolated by us are reported to be able to produce mycotoxins under pure culture laboratory conditions (Northolt, 1988; Frisvad, 1988). This does not imply that they would produce mycotoxins on cassava in the presence of competing fast-growing microflora. For instance, it was found that *Neurospora* and *Rhizopus* spp. are able to suppress the formation of aflatoxins by *Aspergillus parasiticus* in groundnut (Nout, 1989).
The high number of *Escherichia coli* found in one of the flour samples is indicative

for an unhygienic process. The sporadically found sporeformers *Clostridium perfringens* and *Bacillus cereus* might produce toxins after cooking if the *ugali* would be kept for a long time before consumption.

The absence of aflatoxins, mutagenicity and cytotoxicity in the screened flours does not guarantee that toxic substances were absent. However, it suggests that mycotoxins were not formed in quantities that are detrimental to public health. The occurrence of some suspect fungi as well as a slight mutagenicity in the scraped-off moulds indicate that some mutagenicity might develop during this process. Consequently, it may be useful to maintain the practice of removing the mycelia.

Protein quantity and quality
All flours showed a high crude protein level and low chemical scores, compared to findings by other authors (Leung, 1968; Ekpenyong, 1984; Gomez & Noma, 1986; Ezeala, 1984). The protein content and quality, as expressed by chemical score, varied widely and were of the same order after heap-fermentation as after only sun-drying. As most of the moulds were scraped off, most of the formed mycelial protein was lost. The number of samples was too small to confirm or deny an effect on protein quality in general.

Consistency
The lower viscosity of gels from flours from fermented than from non-fermented roots at 95°C correspond with the findings of Moorthy *et al.* (1993), and the villagers' observation that fermentation facilitates kneading of the *ugali*, which is considered a heavy task. *Ugali* from cassava flour is more rubbery than from cereal flours, which is not appreciated. Reducing this rubberiness was one of the objectives and claimed effects of the fermentation. Measuring viscosity is apparently not appropriate to verify the claimed improvements in this rheological characteristic.

CONCLUSIONS

The heap-fermentation method reduced the cyanogen levels of cassava roots more effectively than sun-drying alone, but it did not always lead to levels below the FAO/WHO suggested limit. The abundant and complex associated microflora probably played a role in the reduction of cyanogen levels and did not result in detectable mycotoxin contamination of the products. We conclude that the process of heap-fermentation is useful in the reduction of cassava's potential toxicity, but there is room for optimization. Improvements should be sought in better ensuring an effective cyanogen removal, a more hygienic product with less suspected fungi, and in shortening the time necessary for the process. These objectives might be met by optimizing processing conditions including the use of appropriate starter cultures.

ACKNOWLEDGEMENTS

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CONTRIBUTION OF SELECTED FUNGI TO THE REDUCTION OF CYANOGEN LEVELS DURING SOLID-SUBSTRATE FERMENTATION OF CASSAVA

AJ Alexander Essers, Carien MGA Jurgens & MJ Robert Nout

The effect of six individual strains of the dominant microflora in solid substrate fermenting cassava on cyanogen levels was examined. Six out of eight batches of disinfected cassava root pieces were incubated for 72 h after inoculation with either of the fungi *Geotrichum candidum*, *Mucor racemosus*, *Neurospora sitophila*, *Rhizopus oryzae* and *Rhizopus stolonifer*, or a *Bacillus* sp., isolated from on-farm fermented cassava flours from Uganda. One non-inoculated batch was incubated as a reference. Levels of initial and final moisture and cyanogens were assayed. The experiment was done in quadruplicate.

Incubation of disinfected root pieces reduced cyanogenic glucoside levels significantly to a mean ($\pm$SD) of 62.7 % ($\pm$2.8) of the initial value. Microbial growth resulted in significant additional reduction of the cyanogenic glucoside levels to 29.8 % ($\pm$18.9) of the ones which were obtained after non-inoculated incubation. Among the tested strains, *N. sitophila* reduced cyanogenic glucoside levels most effectively, followed by *R. stolonifer* and *R. oryzae*. Of all fermented samples, both *Rhizopus* spp. showed highest proportion of residual cyanogens in the cyanohydrin form, followed by the *Bacillus* sp. Flours showed similar patterns of cyanogens as the batches they were prepared from. Cyanogenic glucoside level reduction was significantly correlated ($r=0.86$) with the extent of root softening.

It is concluded that both incubation and microbial activity are instrumental in reducing the potential toxicity of cassava during the solid substrate fermentation and that effectiveness varies considerably between the species of microorganisms applied.

INTRODUCTION

The tropical root crop cassava contains cyanogenic glucosides, which are potentially toxic. Cleavage of the glucosidic bond by the compartmentally separated enzyme linamarase, located in the cell-wall, renders glucose and cyanohydrin (Mkpong *et al.* 1990). Cyanohydrins can be degraded enzymatically, but also decompose spontaneously at pH > 5 into acetone and the volatile HCN (Cooke, 1978). Toxicity of the latter is indicated by the estimated minimal lethal oral dose of 0.5 - 3.5 mg per kg human body weight (Montgomery, 1969). Cyanohydrins may decompose at the pH prevailing in the gut, yielding equal molar amounts of HCN. The toxicity of the glucosides is not yet well understood. Several health problems related to cyanogen uptake from insufficiently processed cassava have been reported from Africa (Rosling, 1993).

The required reduction of cyanogen levels before consumption is achieved by processing and preparation. A processing method applied in several parts of Africa includes a step of solid substrate fermentation (Essers *et al.*, 1992, Essers *et al.*, 1995, Gidamis, 1993). In Uganda (Essers *et al.*, 1995) a common process is as follows: After superficial drying, the peeled roots are heaped and covered to incubate for 3 days to enable profuse mould growth. After removal of the fungal mycelium, crushing and sun-drying, the resulting crumbs are pounded into flour. Although the fermentation stage appeared functional in cyanogen removal, the contribution of the microflora remained unclear. Analyses of the microflora associated with this process in Uganda showed the frequent occurrence and abundance of several fungal species. The pH of the resulting flours was generally higher than 7, in contrast with other fermented products from cassava (Vasconcelos *et al.*, 1990, O'Brien *et al.*, 1992).

The scope of this work is to study the contribution of several representative fungal and bacterial strains, isolated from Ugandan on-farm fermented cassava, to the changes in cyanogen levels by solid substrate fermentation. We examined whether incubation alone of disinfected cassava leads to reduction of the cyanogen levels; whether microbial growth leads to additional reduction of these levels; and whether effectiveness differs among micro-organisms.

MATERIALS AND METHODS

Sample/microbiological preparation
Disinfection of cassava pieces was required to suppress the outgrowth of microbial contamination, without hindering the intended growth of the inoculated strains, and without affecting the tissue structure. After preliminary comparison of options, the following procedure was selected. Cassava roots from Costa Rica were peeled and cut into 15 g pieces, using sterilised equipment. The pieces destined for fungal

fermentation were submerged in oxytetracycline (0.5 g/l water) for 60 sec., the ones for growth of bacteria in ethanol (70% v/v) for 60 sec., while the control batches were submitted to both treatments for 30 sec. Subsequently, the pieces were forced-air dried at 55°C for 1½ - 2 h, to reach a moisture content of 55 - 60 %.

Inoculation was similar for all microbial cultures and was carried out by pressing thus pre-treated root pieces directly on plates overgrown with pure cultures micro-organisms, isolated by the authors from traditionally heap-fermented cassava in Kiryandongo village, Masindi district, in rural Uganda (Essers *et al.*, 1995). Filamentous fungi, identified on the basis of their macro- and microscopic morphology according to Samson & Van Reenen Hoekstra (1988), and a Gram-positive endospore-forming rod-shaped bacterium classified as *Bacillus* sp., not further identified, were deposited in the culture collection of the Department of Food Science of Wageningen Agricultural University (LU). These included the fungi *Geotrichum candidum* (LU243), *Mucor racemosus* (LU360), *Neurospora sitophila* (LU420), *Rhizopus oryzae* (LU582) and *Rhizopus stolonifer* (LU590) which were cultured on Malt Extract Agar, Oxoid CM59, and a *Bacillus* sp. (LU809) which was cultured on Nutrient Agar, Oxoid CM3. Incubation was in sterilised jars of 720 ml during 72 h at 25°C. Screw-caps were left open to avoid anaerobiosis.

Cyanogen analysis

Extraction and analysis of the cyanogenic compounds was by homogenisation of about 70 g in 250 ml 0.1 M orthophosphoric acid, followed by conversion to HCN, which was measured spectrophotometrically after coloration (Essers *et al.*, 1993).

Experimental

Peeled cassava roots were cut into 1½ cm thick discs, which were split into several segments, having near to equal cyanogen levels (De Bruijn, 1971). The adjacent segments were matched to obtain 8 batches of about 350 g with similar cyanogen levels. After disinfection pre-treatment, from one batch 4 sub-samples were taken and extracted immediately (t=0) to serve as a reference for the cyanogen content; one non-inoculated batch and 6 batches inoculated with either of the microorganisms mentioned above were incubated for 72 h at 25°C. Treatment codes are shown in Table 1. After these treatments, a part of each batch was processed into flour by oven-drying at 55°C for one day and subsequent milling (Fritsch Pulverisette type 14.702, Laborgeschaft, Idar-Oberstein, Germany) through sieves of, subsequently, 4 and 1 mm. Extraction and subsequent analysis for cyanogens took place immediately after incubation and after flour preparation.

Specificity of microbial growth was evaluated macroscopically and by characteristic odour of the fermenting products. The extent of growth was monitored semi-quantitatively, using a subjective classification scale from 0 (no visible growth) to 5

(completely covered). Quantitative measurement of fungal propagules was omitted, as this would mainly refer to sporulation of the fungi and not to the more relevant mycelial growth and physiological activity (Nout *et al.*, 1987). Extent of softening of the root pieces was classified on a scale from 0 (no softening) to 5 (completely soft), after manually probing with a round tipped 3 mm ø glass bar.
The experiment was carried out 4 times with intervals of one week.

Statistical analysis
Treatment effects were judged with the Protected Least Significant Difference method (PSD) (Snedecor & Cochran, 1980, p 234). First, the overall treatment effect was examined by an F-test in a 2 way analysis of variance with 7 df for treatments and 21 for residuals. In case of a significant F, the differences between treatment effects were tested by *t* test (Snedecor & Cochran, 1980). In order to stabilize the variance, a logarithmic transformation of the cyanogenic glucoside values was applied, to differentiate between low residual levels.
Significance of differences are related to two-tailed probability (*P*) values.

RESULTS AND DISCUSSION

Microbial growth was good in the inoculated batches and nearly absent in the non-inoculated ones. Although the disinfection treatment does not guarantee exclusive growth of the inoculated micro-organism, macroscopic inspection and characteristic odour release did not indicate significant outgrowth of contaminating microorganisms. Table 1 presents the absolute data on residual cyanogen levels, as well as the softening, in the moist cassava batches and their flours. As the initial total cyanogen levels varied considerably between the 4 experiments, compilation of the results for presentation in Figures 1 and 2 was after calculating the residual cyanogen levels as % of the initial total cyanogen levels. The pH was significantly reduced ($P < 0.0001$) to 4.8 (SD 0.5) after fermentation by *Rhizopus oryzae*, but remained at 6.7 (SD 0.2) after the other treatments (results not shown).

Cyanogenic glucoside levels after incubation only (treatment 2) were significantly lower than those of fresh roots (treatment 1), with $P < 0.0001$ for the moist products and $P = 0.001$ for the flours, indicating that incubation alone led to reduction of cyanogenic glucoside levels. The cause of this decrease is probably associated with the physiological deterioration of the cell-wall and membrane structure (Padmaja & Balagopal, 1985) which, by leaking, allow contact between endogenous linamarase and the cyanogenic glucosides, resulting in breakdown of the latter.
   Cyanogenic glucoside levels in moist products and flours from batches that were incubated after inoculation (treatments 3 to 8) were significantly lower ($P < 0.0001$)
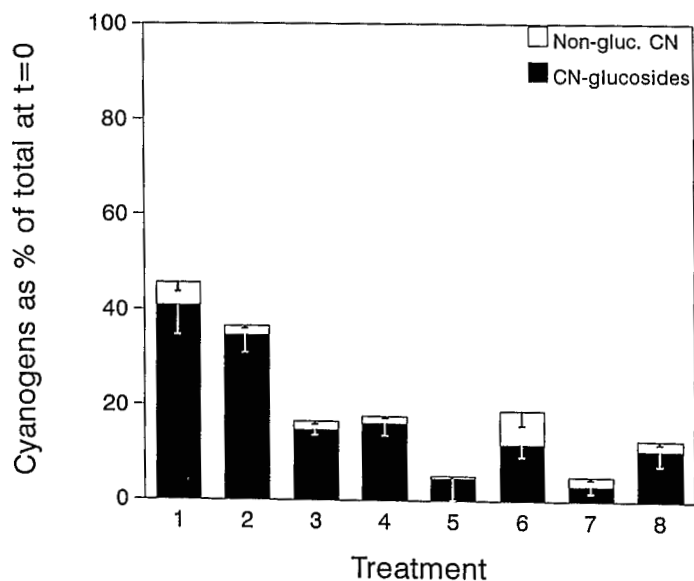
TABLE 1

Levels of cyanogenic glucosides and cyanohydrins in moist cassava and their flours, and softness, after different treatments in the 4 experimental blocks

| Treat-ment[a] | Moist cassava | | Flours | | |
	Cyanogenic glucosides[b]	Cyano-hydrins[b]	Cyanogenic glucosides[b]	Cyano-hydrins[b]	Soft-ness[c]
1	92.7	10.3	46.6	5.4	0
2	60.5	4.0	39.8	2.1	1
3	26.3	15.3	14.7	2.5	2
4	25.1	11.7	15.2	1.6	2
5	3.4	1.5	1.4	0.2	5
6	12.1	43.1	14.7	10.8	5
7	11.7	33.8	2.0	1.7	5
8	14.8	40.8	8.4	0.9	3
1	209.9	16.4	79.5	6.3	0
2	123.3	3.4	68.5	1.9	0
3	62.9	15.7	32.6	3.3	2
4	81.9	2.0	41.0	2.0	1
5	14.9	1.4	22.0	0.9	4
6	24.1	42.2	22.8	17.2	5
7	19.9	44.2	4.2	1.1	5
8	44.0	48.2	29.9	2.5	3
1	219.3	6.1	108.0	15.6	0
2	138.1	49.4	86.7	5.1	1
3	48.2	9.4	37.7	2.6	2
4	53.6	22.8	30.0	3.1	2
5	3.6	0.7	7.2	0.7	4
6	15.0	59.0	31.4	8.5	5
7	11.5	71.6	11.7	4.7	5
8	65.3	77.6	17.9	3.3	2
1	321.5	6.5	120.9	10.9	0
2	204.6	43.3	107.1	4.3	1
3	101.6	8.9	46.6	4.0	2
4	112.1	14.1	63.5	2.6	2
5	27.2	4.6	[d]	1.6	3
6	45.5	64.5	31.2	14.9	5
7	31.6	86.1	12.1	4.3	5
8	127.8	86.5	44.6	9.3	2

[a] Treatments: 1 Control t = 0 h, 2 Control t = 72 h, 3-8 are incubated for 72 h with: 3 *Geotrichum candidum*, 4 *Mucor racemosus*, 5 *Neurospora sitophila*, 6 *Rhizopus oryzae*, 7 *Rhizopus stolonifer*, 8 *Bacillus* sp. [b] values in mg CN⁻ equivalent per kg sample on dry weight base. [c] 0 = no softening, 5 = completely soft. [d] not examined
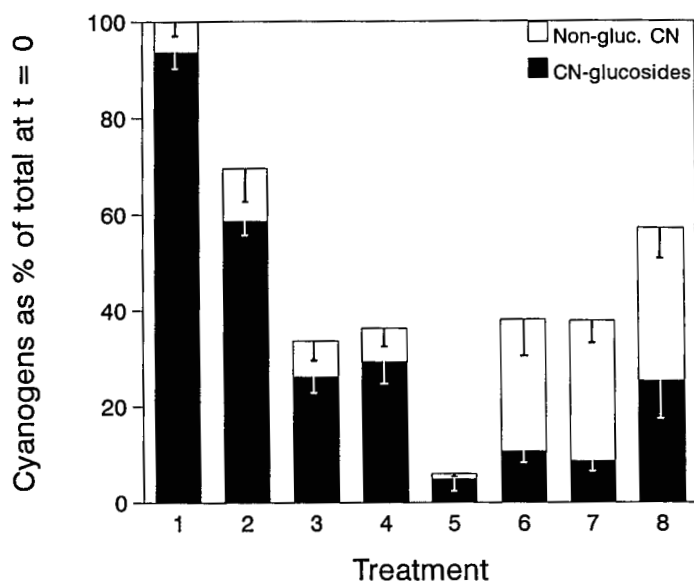
FIGURE 1 Mean (-SD) residual cyanogen levels in **moist cassava** after treatments, as % of total cyanogens at t=0, from 4 experiments (treatment coding as Table 1)

FIGURE 2 Mean (-SD) residual cyanogen levels of **cassava flours** made after treatments, as % of total cyanogens at t=0, from 4 experiments (treatment coding as Table 1)

than samples after incubation only (treatment 2), indicating an additional effect of the microflora. The effect may have been caused by increased endogenous linamarase - linamarin contact through disintegration of cell-wall and membrane structures, or by introducing additional microbial linamarase, or by both.

The effects of the microbial species (treatments 3 to 8) on cyanogenic glucoside levels could be differentiated statistically ($P<0.05$, PSD) as follows: *N. sitophila* fermented batches had lowest levels, followed by the *Rhizopus* spp. fermented ones. Of the flours, *R. stolonifer* and *N. sitophila* fermented batches had significantly lower ($P<0.05$) cyanogenic glucoside levels than the others.

The level of cyanohydrins, as well as their proportion of total cyanogens, was significantly different ($P<0.0001$) after the different treatments, both for moist products and flours. These levels and proportions were significantly higher ($P<0.05$, PSD) in the *R. oryzae*, *R. stolonifer* and *Bacillus* sp. fermented batches than in the others, and after drying remained significantly higher in the flour from the *R. oryzae* fermented cassava. As the loss of the cyanogenic glucosides in this process is through the stage of cyanohydrins, these compounds were apparently better preserved in these last three fermented products. As cyanohydrins are more stable at a lower pH (Cooke, 1978), the higher level of cyanohydrins and their proportion of total cyanogens in the *R. oryzae* fermented products may be explained by the lower pH attained by that fermentation. We cannot explain the high cyanohydrin to total cyanogen ratios in the *R. stolonifer* and *Bacillus* sp. fermented batches.

HCN levels in the moist products ranged from 0.1 - 5.7 mg CN equivalent per kg dry weight with a mean of 2.1 (SD 1.7, n=32). In the flours, these levels did not exceed 2.0 mg CN equivalent per kg dry weight. The sum of cyanohydrins and HCN in each flour sample was below 10 mg CN equivalent per kg dry weight. These low levels are explained by the rigorous final drying step leading to moisture levels of about 5%. It has been observed earlier by Banea *et al.*, (1992) that cyanohydrins are lost at low moisture levels.

The treatments had a significant effect ($P<0.0001$) on the softness of the products. Softening of the pieces was most advanced ($P<0.05$, PSD) after fermentation by the *Rhizopus* spp., followed by *Neurospora. Geotrichum*, *Mucor* and *Bacillus* only caused superficial softening (Table 1). In moist products and flours, residual cyanogenic glucoside levels differed significantly ($P<0.0001$) among the softness classes of the fermented root pieces. The reduction in glucoside levels was significantly correlated ($r=0.86$) with the extent of root softening. Comparing the F values for treatments and softness in moist products after correcting each for the effect of the other, the effect of treatment ($P=0.003$) and softness ($P=0.035$) on the glucoside levels remained significant. For flours, the treatment effect remained significant ($P=0.0003$) after correction for softness of the moist products, but the effect of softness on the residual glucoside levels just lost significance ($P=0.102$) after correcting for treatment effect.

These findings indicate that treatment is more powerful than softness alone for explaining the glucoside level reduction.

The cyanohydrin proportion of total cyanogens in the moist products was significantly related ($P<0.0001$) and correlated ($r=0.73$) to softness after fermentation, but not in the flours. The sequence suggested that the softer the product, the higher this proportion, except for *N. sitophila* fermented cassava. Also here, it appears that although softening has a substantial effect on the cyanohydrin proportion, it does not fully explain the treatment effect.

Softness or structure loss of cassava tissue was induced by the treatments. The fact that correction for the treatment effect reduced the statistical significance of the softness effect on the glucoside levels and cyanohydrins to total cyanogens ratios far more than vice versa, indicates that treatment as such is a stronger determining factor than softness only. It is likely that, in addition to the extent of structure loss, also the type of cell-wall and membrane structure degradation is of importance. The possibility of an additional effect from microbial linamarase activity can not be excluded. Okafor & Ejiofor (1986) found a cyanogen lowering effect from microorganisms that were selected for their linamarase capacity, when seeded on fresh pulp. Maduagwu (1983) and Vasconselos *et al.* (1990) found no such effect in spontaneous fermenting cassava pulp. In the latter cases, the major part may already have been hydrolysed by endogenous enzyme liberated by the cell disruption, before the relevant microorganisms were in sufficient number to cause significant effect. Neither of those studies compare to the present experiment, however, as here no mechanical disruption occurred to liberate the endogenous linamarase. The mechanisms of the microflora bringing about the linamarin level reduction in these experiments is subject of further study.

Although the culture conditions during these experiments were made to resemble average in-situ conditions, the growth and enzyme production of these microorganisms may be favoured differently according to the environmental conditions at household level.

Concluding, we found that both incubation and microbial growth were instrumental in reducing cyanogenic glucoside levels and changing the cyanogen composition in cassava root pieces. The effectiveness differed between the species of microorganisms and was also associated with the root softening obtained. From the perspective of reducing cassava's potential toxicity, *N. sitophila* was most successful of the here tested strains, followed by *R. stolonifer* and *R. oryzae*.

ACKNOWLEDGEMENT

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MECHANISMS OF INCREASED LINAMARIN DEGRADATION DURING SOLID-SUBSTRATE FERMENTATION OF CASSAVA

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Several fungi and bacteria, isolated from Ugandan domestic fermented cassava, released HCN from linamarin in defined growth media. In 72 h, a *Bacillus* sp. decreased the linamarin to 1% of initial concentrations, *Mucor racemosus* to 7%, *Rhizopus oryzae* and *Rhizopus stolonifer* to 30%, but *Neurospora sitophila* and *Geotrichum candidum* hardly degraded the linamarin. Adding pectolytic and cellulolytic enzymes, but not linamarase, to root pieces under aseptic conditions, led to root softening and significantly lower linamarin contents. *N. sitophila* showed no linamarase activity, in contrast to *M. racemosus* and *Bacillus* sp., both of which were less effective in root softening and decreasing the root linamarin content. The most important contribution of microorganisms to linamarin decrease in solid-substrate fermenting cassava is their cell-wall-degrading activity which enhances the contact between endogenous linamarase and linamarin.

The tropical starchy root crop cassava (*Manihot esculenta* Crantz) contains small amounts of cyanogenic glucosides, especially linamarin (Butler, 1965). Disintegration of the tissue structure results in contact of linamarin with linamarase, which is located in the cell walls (Mkpong *et al.*, 1990), and subsequent hydrolysis to glucose and cyanohydrins, which easily break down to ketones and HCN (Cooke, 1978). To prevent toxicity, cassava roots with high cyanogen concentration are usually processed before consumption. Many processing methods have been developed by the cassava-farming population (Lancaster *et al.*, 1982). One of those, which is used in several African countries and has been studied recently (Essers *et al.*, 1995a), involves a 3- to 4-day solid-substrate fermentation in covered heaps after a short initial sun-drying. During incubation, a varied and abundant microflora develops; the roots get covered with a blanket of fungal mycelia and gradually soften from the outside towards the centre. Subsequently, the mycelia are removed, and the roots are crushed and submitted to final sun-drying before being pounded into flour. Incubation alone reduces linamarin contents but the microflora causes an additional effect, associated with root softening (Essers *et al.*, 1995b). Two possible mechanisms contributing to this effect are introduction of microbial linamarase to the cassava, and cell-wall-degrading enzymes that permit contact between the compartmentally separated linamarin and endogenous linamarase.

The aim of the present study was to clarify the mechanism(s) of the solid-substrate fermentation process that enhance breakdown of cyanogenic glucosides. The linamarase activity of several microbial strains was evaluated and the effects of enzymatically induced root softening and exogenous linamarase on the cyanogenic glucosides were compared.

MATERIALS AND METHODS

Roots, enzymes and picrate paper
Cassava roots from Costa Rica, protected by a wax layer, were obtained commercially in the Netherlands. Linamarase (7.6 U/ml) was extracted from fresh Ugandan roots according to Cooke (1979). One unit (U) was defined as the amount hydrolysing 1 μmol linamarin/min at 30°C in 0.1 M phosphate pH 6.0. Picrate impregnated paper was made by immersing strips of filter paper in a solution of equal volumes of saturated picric acid and 5% (w/v) Na_2CO_3. Pectinex Ultra-sp (Novo Nordisk, Denmark) (0.5% w/v solution) was used for cell-wall degradation.

Growth media
The yeasts and moulds, bacilli, and lactic acid bacteria were pre-cultured on yeast nitrogen base (YNB; Difco) plus 1% (w/v) agar (BBL) plus 5% (w/v) glucose, at 25°C for 48h, BHI broth (Gibco) at 30°C for 24 h, and MRS-agar (Merck) plus 1%

(w/v) agar (BBL) at 30°C for 24 h, respectively.

Defined growth media for testing linamarase activity

Linamarin-containing media with a low glucose content were used to screen linamarase activity. For yeasts and moulds, YNB plus 0.5% (w/v) glucose plus 1% (w/v) agar plus 0.01% (w/v) linamarin (Sigma L 9131) in 0.1 M sodium phosphate buffer, pH 6.15, was used. For bacilli, 0.5% (w/v) neutralized bacterial peptone (Oxoid) plus 0.2% (w/v) Lablemco meat extract (Oxoid), 0.1% (w/v) Tween 80 (Merck), 0.02% (w/v) $MgSO_4.H_2O$, 0.0056% (w/v) $MnSO_4.H_2O$, 0.5% (w/v) glucose, 1.0% (w/v) agar and 0.01% (w/v) linamarin (Sigma) in 0.1 M sodium phosphate buffer, pH 6.0, was used. For lactic acid bacteria, the medium for bacilli was used, without agar. For quantification of linamarase activity, the same media were used without agar.

Screening micro-organisms for linamarase capacity

The breakdown of linamarin and subsequent volatilization of HCN were examined with picrate-impregnated paper, fixed in the headspace of test tubes. Microorganisms, isolated from Ugandan domestic solid-substrate fermented cassava, described in Essers *et al.* (1995a), were inoculated on the relevant medium. After 4 days at 25°C (yeasts and moulds) or 30°C (bacilli and lactobacilli), the colour of the picrate-impregnated papers was compared with a standard range.

Linamarase activity of the dominant microorganisms

Five 250-ml Erlenmeyers flasks, each containing 20 ml of the corresponding linamarin-containing medium, were inoculated with *Rhizopus oryzae*, *Mucor racemosus*, *Neurospora sitophila*, *Geotrichum candidum* or a *Bacillus* sp. (one species/flask). A control was left uninoculated. A strip of picrate impregnated paper was fixed in the headspace as a qualitative indicator of HCN. After 72 h at 26°C, residual cyanogens and mycelial weight were assayed. The incubation time and temperature are similar to the conditions observed in domestic processing in rural Uganda. The experiment was performed in quadruplicate.

Enzyme-induced cassava structure loss and linamarin degradation

The effect of tissue structure loss on linamarin breakdown was examined by incubation of submerged cassava root pieces with a cell-wall-degrading enzyme complex, with and without additional linamarase, under aseptic conditions.

Preliminary testing of several commercial enzyme preparations indicated that the cellulolytic and pectolytic activity of Pectinex Ultra-sp (Novo Nordisk, Denmark) was able to soften cassava root tissue. Linamarase activity measured according to the procedure of Cooke (1979), was absent from this preparation. To determine if the proteolytic capacity of Pectinex Ultra-sp (Tableros *et al.*, 1987) would affect

linamarase activity, the activity of succinate buffer (0.05 M, pH 5.5), initially containing 0.26 U linamarase/ml, with and without addition of 0.5% (w/v) Ultra-sp, was measured according to Cooke (1979) after 0, 4 and 23 h. No interference of Pectinex Ultra-sp with linamarase was found. 0.04% (w/v) sodium azide had no interference on the cyanogen assay.

A cassava root was peeled aseptically and cut into two pieces. These pieces were immersed for 30 min in ethanol (70%), then 1 min in sterile water, and, after discarding the 0.5 cm ϕ central column, diced aseptically into cubes of about 1 cm^3. Five batches of disinfected cubes (approx. 11 g) were transferred to 30 ml sterile cups and submerged under 15 ml succinate buffer (0.05 M, pH 5.5), containing 0.02% (w/v) sodium azide to prevent microbial activity. One cup was assayed immediately (control); the remaining four received no addition, 0.5% (w/v) Pectinex Ultra-sp, crude linamarase (0.26 U/ml), or 0.5% (w/v) Pectinex Ultra-sp and crude linamarase (0.26 U/ml), respectively. The amount of linamarase used approximates to the average linamarase activity in 11 g fresh root, according to De Bruijn (1973). After incubation for 16h at 40°C, cyanogens and microbial counts were determined. The experiment was performed in quadruplicate.

Analyses
Semi-quantitative determination of HCN release was by picrate-impregnated paper. This method was derived from the leaf-picrate test (Sadik *et al.*, 1974) and is based on the change in colour of picrate from yellow to brown-red after contact with HCN (Guignard, 1906). Tubes, containing standards of 0 to 100 mg linamarin/l phosphate buffer (0.1 M, pH 6.0), to which 1 U linamarase was added, were fitted with picrate impregnated filter paper in the headspace and incubated for 24 h at 30° C. This resulted in 6 pieces of coloured paper with increasing redness and these were photographed to give a semi-quantitative reference scale.

Cyanogens were extracted and assayed according to Essers *et al.* (1993). In the experiment on enzyme-induced linamarin degradation, each sample of 11 g cassava pieces with 15 ml soak solution was homogenised in 44 ml 0.1 M H$_3$PO$_4$. Moisture in fresh cassava was determined in quadruplicate by oven-drying at 105°C to constant weight. Total counts of mesophilic aerobic microorganisms was carried out by surface plating on plate count agar (Oxoid CM 325), and incubating at 30°C for 3 days. The dry weight of mycelial biomass was assayed by filtering the culture broth through a pre-weighed filter paper (Schleicher & Schuell 595½) and subsequent drying at 100°C overnight. The bacterial biomass was determined in the same way after centrifugation for 10 min at 2000 x *g*. Root softening was considered complete when a 3 mm ϕ glass rod could be pushed through a 1 cm thick root piece without noticeable hindrance.

TABLE 1

HCN release from a linamarin containing defined growth medium after 4 days by several (n) isolates of microorganisms from Ugandan on-farm fermented cassava

| Organism	No. of isolates showing		
	no release	moderate release	strong release
Aspergillus fumigatus	1		
Aspergillus niger	1		
Aspergillus oryzae	1		
Aspergillus parasiticus	5		
Fusarium sporotrichoides	1		
Geotrichum candidum	11		
Mucor circinelloides			2
Mucor racemosus			10
Neurospora crassa	1		
Neurospora sitophila	9		
Penicillium frequentas	1		
Penicillium funicolosum	1		
Penicillium grisofulvum	1		
Penicillium rugulosum	1		
Penicillium waksmanii	1		
Rhizopus oryzae		1	
Rhizopus stolonifer		7	
Scopulariopsis brevicaulis	1		
Scopulariopsis fuxa	1		
Lactobacillus spp.	5	12	3
Bacillus spp.	2	2	
Yeasts	2	10	2

Statistical analyses

Treatment effects were compared with the Protected Least Significant Difference method (PSD) (Snedecor & Cochran 1980). Significance of differences is presented as two-tailed probability (P) values.

RESULTS AND DISCUSSION

Mucor and *Rhizopus* sp. were able to release HCN from linamarin, while *Geotrichum candidum*, *Aspergillus*, *Neurospora*, *Penicillium* and *Scopulariopsis* sp. were unable to do so. Most of the yeasts and lactobacilli released HCN, and two of the four bacilli released a small amount of HCN (Table 1).

Quantification of linamarin breakdown by microorganisms (Fig 1) showed that
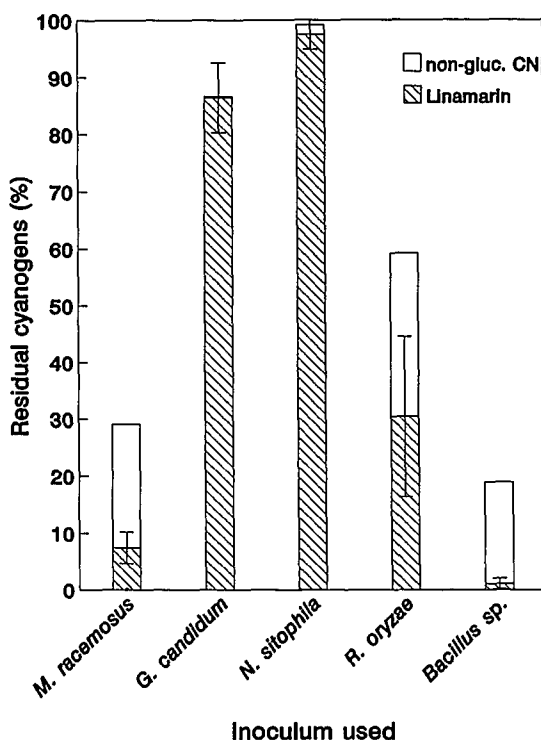
FIGURE 1 Mean (N=4) residual cyanogen levels as % of initial linamarin concentration, in defined growth media after 72 h at 26°C. Error bars (standard deviations) are given for linamarin concentrations.

Neurospora sitophila and *Geotrichum candidum* did not hydrolyse appreciable amounts of linamarin, while *Bacillus* sp., hydrolysed virtually all. *Mucor racemosus* had hydrolysed at least 90% of the linamarin and *Rhizopus oryzae* 45 to 84%. Although *R. stolonifer* was not included in this experiment, earlier experiments showed similar linamarin breakdown by the *R. oryzae* and *R. stolonifer* under the same conditions. The picrate-impregnated paper indicated HCN escape from the media cultured with *Mucor*, *Rhizopus* and *Bacillus*, but not from the ones with *Geotrichum* or *Neurospora*.

The findings of the quantitative experiment were consistent with the findings from the screening, except for *Bacillus* sp. The HCN release by the *Bacillus* sp. was lower than one would expect from the residual linamarin measured. A possible explanation for this is that the *Bacillus* sp. assimilated part of the HCN. It is known that several micro-organisms are capable of metabolizing HCN (Knowles & Bunch, 1986). Padmaja & Balagopal (1985) described linamarase and rhodanese activity, as well as cyanide assimilation by *Rhizopus oryzae* isolated from rotting cassava. Rhodanese can form thiocyanate (SCN⁻) from CN⁻ if suitable sulphur sources are available. The thiocyanate formed may then be used for mycelium formation, but if accumulated, would be assayed as non-glucosidic cyanogen.

Although *Neurospora sitophila* is known to be a producer of β-glucosidase (Oguntimein & Moo-Young, 1991), the strain tested did not break down significant amounts of the linamarin under the prevailing conditions. β-glucosidases are substrate specific and therefore not all can degrade linamarin. The pH of the medium after incubation remained between 5.0 and 6.3, which is adequate for linamarase activity

from various sources (Cooke *et al.*, 1978; Okafor & Ejiofor, 1985).

The mean ($\pm$ standard deviation) fungal biomass developed in 72 h was 171 $\pm$ 21 mg and was the same order for all fungi, while the bacillus biomass was 475 mg $\pm$ 205. Although biomass development and enzyme excretion do not necessarily coincide, the results indicate that the superior linamarase activity exhibited by *Mucor* cannot be attributed to the growth conditions being specifically favourable for this fungus, nor to its higher biomass.

From earlier experiments (Essers *et al.*, 1995b), we know that *Neurospora sitophila* was best at diminishing linamarin in fermenting cassava, followed by *Rhizopus* sp. *Bacillus* sp., *Geotrichum candidum* and *Mucor racemosus* were less effective and also caused less root softening than the *Neurospora* and *Rhizopus* sp. In the present experiments, *N. sitophila* showed no linamarase activity, and there is no indication that this would be different in cassava. This indicates that the structure loss, caused by the enzyme system of *N. sitophila* is of greater importance for linamarin degradation than the combination of low cell-wall-degrading and high linamarase activity of *M. racemosus* and the *Bacillus* sp. Comparing *N. sitophila* and *R. oryzae*, the latter proved at least as effective in softening cassava root tissue (Essers *et al.*, 1995b) and displayed linamarase activity (present study), but it was less effective in diminishing linamarin levels in cassava (Essers *et al.*, 1995b). Therefore, it seems that the type of cell-wall degradation by *N. sitophila* is better suited to enable endogenous linamarase-linamarin contact.

In the experiment on enzyme-induced linamarin degradation, microbial counts in root pieces were $< 10^3$/g in all batches. This excludes interference from microbial contamination. The cassava pieces treated with Pectinex Ultra-sp or Ultra-sp plus linamarase were completely soft after 16 h. Cassava root softening is caused by cell-

TABLE 2

Cyanogens in cassava pieces before and after incubation with enzyme supplements[a]

Enzyme added	Incubation (h)	Total cyanogens[*] (mg CN equiv./ kg dry wt)	Cyanogenic glucosides[*#] (mg CN equiv./ kg dry wt)	Ratio[#] (Cyanogenic glucosides: Total cyanogens)
none	0	118 $\pm$ 12	112 $\pm$14[a]	0.95[a]
none	16	131 $\pm$ 8	64 $\pm$ 3[b]	0.49[b]
Pectinex Ultra-sp (0.68 mg/g)	16	93 $\pm$ 4	12 $\pm$ 5[d]	0.13[d]
Linamarase (0.36 U/g)	16	123 $\pm$ 10	59 $\pm$ 4[b]	0.48[b]
Pectinex Ultra-sp (0.68 mg/g) + linamarase (0.36 U/g)	16	107 $\pm$ 6	28 $\pm$ 3[c]	0.26[c]

[*] Values are means $\pm$ standard deviations for four sets of replicates
[#] Within-columns, values with different superscript letters differ significantly ($P < 0.05$).

wall degradation through combined activity of pectinases and cellulases (Okolie & Oguchukwu 1988). The residual cyanogen concentrations after the treatments are given in Table 2. Treatment with Pectinex Ultra-sp alone resulted in the lowest linamarin concentrations, both in the absolute sense, as well as relative to total cyanogen concentrations. These were significantly lower ($P < 0.05$) than those after other treatments. Second in terms of effectiveness was the combination of Pectinex Ultra-sp and linamarase. This also resulted in absolute and relative linamarin concentrations that were significantly lower ($P < 0.05$) than those in the other treatments. The concentrations after treatment with linamarase alone were not significantly different from the incubated controls.

Facilitating the contact between the substrate and the endogenous linamarase, through cell-wall degradation by Pectinex Ultra-sp, thus proved more effective in reducing the linamarin concentration than supplementing with linamarase. This is due to the inability of the exogenous linamarase to enter intact cassava cells and come in contact with its substrate. Once the cells are disrupted, additional linamarase may accelerate the linamarin breakdown, as was shown by Ikediobi & Onyike (1982). The treatment with both linamarase and Pectinex Ultra-sp was therefore expected to be more effective than Pectinex Ultra-sp alone, but it was not. The reason for this is not clear.

The higher efficacy achieved by adding exogenous cell-wall-degrading enzymes than by exogenous linamarase to intact cassava tissue, was in line with the finding that a root-softening, but linamarase deficient, fungus (*N. sitophila*) could be more effective in diminishing linamarin content than microorganisms displaying linamarase activity.

Overall, the results of the present study indicate that the main contribution of the microflora to the reduction of linamarin concentrations in solid-substrate fermentions of cassava, is their cell-wall-degrading activity which allows contact between endogenous linamarase and the linamarin. Their linamarase capacity is of lesser importance.

ACKNOWLEDGEMENTS

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GENERAL DISCUSSION

Since its introduction four centuries ago, cassava's potential toxicity has not prevented the crop from gaining its position as the single most important staple food crop in Africa. The distribution of cyanogenic glucoside levels in diverse cassava varietal populations suggests that genotypes that plant breeders consider as being low in cyanogens, i.e. with less than 100 mg HCN equivalent per kg fresh weight, are more prevalent than high cyanogenic ones (Bokanga, 1994a). As there is a wide choice of so-called low-cyanogenic genotypes, it is remarkable that farmers still grow cultivars with higher cyanogen levels. In some areas, farmers even intentionally replace low cyanogenic by high cyanogenic cultivars (chapter 6). One can hypothesize that the toxic potential is regarded as unimportant, or that it may be associated with desired other traits, or even that the toxicity itself is desired under some conditions. Farmers who grow cultivars which are toxic and bitter attribute several advantages to them. Lower susceptibility to theft and to herbivore voracity are the characteristics which are most likely to be directly related to the higher toxicity. Because of the association between bitterness and cyanogenic glucoside levels it is difficult to distinguish whether an effect is related to the one or the other trait. An example of this interrelationship is that farmers in Uganda considered the acute toxic effects after cassava meals to be a result of the bitterness which had remained in the flour. Vice versa, they regarded the bitterness as an indicator of toxicity. Purseglove (1981) mentioned that where bitter cassava cultivars are grown, they usually provide the dominant staple. It is likely that in Africa a heavy reliance on cassava does not only relate to the mere benefits of the crop. It may also reflect pressures on the farming system, which forced farmers to embrace this high-yielding and flexible crop (Fresco, 1986; Carter et al., 1992). Such pressures on the food production system may well be linked to food scarcity and might therefore be related to theft of food. Under such conditions, it seems a plausible option to reduce the risk of theft by changing over to a crop which is inedible without time-consuming or labour-intensive processing.

  Whatever hypothesis holds for the farmers, scientists from the agricultural, food and health sectors have shown interest in cassava's cyanogenesis. This is because of the importance of cassava as a high yielding food security crop on the one hand,

and reports on adverse health effects from cassava cyanogens on the other. Food should be safe[1]) at the moment of consumption. Contributing to the safety of cassava consumers, by searching for options for improvements in domestic processing, was the ultimate goal of this study.

When starting this research, several difficulties had to be overcome. One of them was the lack of a generally accepted accurate method for measuring cyanogenic compounds in cassava products, necessary to evaluate processing effects.

Cyanogen assay

The enzymic assay of Cooke (1978, 1979) was recently improved by O'Brien *et al.* (1991), by allowing for separate measurement of the three categories of cyanogens. It gave similar results to those found with the long-established laborious "autolysis/steam-distillation/titration" method (AOAC, 1991). The former was further improved with respect to accuracy, cost, speed and ease of handling (chapter 3). The replacement of the toxic and repulsive-smelling pyridine in the colouration also made it more useful for less sophisticated laboratories and reduces the health hazard for laboratory technicians. The improved colouration has meanwhile found acceptance in major cassava laboratories, and is now applied to assay thiocyanate in body fluids (Lundquist, 1995). In order to facilitate routine measurements, the assay was automated in our laboratory. The set-up of pipette station, spectrophotometer and computer works batch-wise and mimics the manual method in performance, accuracy and speed. Still, there is room for further optimization of the assay with respect to being less vulnerable to minor external influences and replacing the expensive linamarase.

Cyanogenic glucosides are the only cyanogens in fresh intact cassava root and there is no appreciable amount of cyanohydrins or HCN (chapter 4). The levels of residual cyanogenic glucosides, cyanohydrins and hydrogen cyanide in cassava products depend on the initial cyanogenic glucoside levels and the processing applied. Considering the rapid initial diminishing of cyanogenic glucoside levels and the low accumulation of cyanohydrins and HCN in harvested cassava (chapter 4, Fig 2), the freshness should be questioned of "fresh root" samples in which more than 5% of the cyanogenic potential is in the form of cyanohydrins and HCN. It is doubtful whether such samples represent the cyanogenic potential at harvest.

The levels of HCN found in cassava products are rarely above 7 mg per kg dry weight (chapters 4 and 6; Mlingi *et al.*, 1992; Banea *et al.*, 1992; O'Brien *et al.*, 1992). This is due to the volatility of HCN at ambient temperature, resulting in its rapid disappearance from cassava products. The low levels found may partly be an artifact due to evaporation during the usual acid extraction and the extract storage. This is illustrated by the recovery of only 67 to 85% of the KCN added to a

cassava extract when assayed within 1 h after homogenization (chapter 3). As the levels of HCN measured are always low, and because the toxic effect of cassava cyanohydrins is probably similar to that of HCN with which it can be assayed in one fraction, the separate measurement of HCN in dry products for routine quality control might as well be omitted.

Health effects of cassava cyanogens

HCN levels in cassava products were always lower than cyanohydrin levels. Ingested cyanohydrins are considered to decompose at the alkaline pH level in the gut to yield an equal molar amount of cyanide (Bourdoux *et al.*, 1980). Toxicity of cyanohydrins is therefore probably similar to that of HCN, but the effect may be delayed with some hours and stretched, due to the slow release of the stomach content into the gut. The toxicity of ingested glucosides in man is not yet well understood, but it was recently found that a variable portion is converted to cyanide in the human body, possibly by glucosidases present in the gut (Carlsson *et al.*, 1995; Hernández *et al.*, 1995).

Acute poisonings within hours of consumption of insufficiently processed cassava have been experienced by several cassava growing populations. The symptoms range from drowsiness, headache, dizziness, vomiting, diarrhoea, palpitations, muscle weakness, convulsions, coma and, possibly and ultimately, death within several hours after consumption of a cassava meal (Cheok, 1978; Mlingi *et al.*, 1992; Akintowa & Tunwasche, 1992). These symptoms are in agreement with those reported from the Mozambican community described in chapter 2.

Konzo is the name given to a newly identified paralytic disease. A number of recent epidemics helped to draw renewed attention to cassava's toxic potential. Konzo was formerly described as epidemic spastic paraparesis. Outbreaks of *konzo* have been reported to occur in Mozambique (Ministry of Health, 1984a; Cliff, 1994), Tanzania (Howlett *et al.*, 1990), Zaire (Tylleskär *et al.*, 1992), and the Central African Republic (Tylleskär *et al.*, 1995). These epidemics have disabled several thousands of people, mainly children above the age of three and women in their fertile years. The clinical features are an abrupt onset of a permanent, but not progressive, symmetrical spastic paraparesis (paralysis of both legs) in a previously healthy person. Associated involvement of the arms and, occasionally, optic nerves may be present in cases with severe spastic paraparesis. The range of disabilities varies from a slightly spastic gait in mild cases to an inability to walk in more severely affected cases (Howlett, 1994).

The cases of paralysis described in chapter 2 had exactly the same clinical characteristics of spastic paraparesis with sudden onset, and dietary history at occurrence: nearly exclusive consumption of insufficiently processed cassava, as

those mentioned above. The high urinary thiocyanate levels found indicate a high exposure to cyanide. The low urinary inorganic sulphate levels indicate a low intake of dietary sulphur amino acids. The latter provide sulphur for converting cyanide to thiocyanate. Acute cassava poisonings occurred simultaneously in the affected population, a finding which is also reported from the other epidemics of konzo. The coherent findings, consistent with those reported from other konzo affected populations, support a causative role of a high cyanide intake from insufficiently processed cassava, although no mechanisms for the neurological damage have yet been elucidated (Tylleskär, 1994).

Since 1982 several outbreaks of konzo and acute cassava poisonings have occurred in Nampula province in Mozambique. The last decade was characterized by social insecurity, war, displacement and drought that led to inadequate diets based on insufficiently processed cassava (Davis & Howarth, 1993). In the small locality of Nahá alone (chapter 2), about 50 new konzo cases were reported, resulting in 21 cases per 1000 inhabitants in 1993 (Cliff, 1994). During my recent visit to Nampula province, farmers and villagers from the districts Mogincual, Murrupula, Meconta, Mogovolas and the area surrounding Nampula town repeatedly mentioned that over the last decennium bitter and toxic cassava cultivars have become more important sources of food than the sweet ones. Not only theft-avoidance had created this shift, but also actual theft of sweet rooted plants had decimated their existence.

Avoidance of toxic effects
Epidemics of acute poisonings and konzo have only been reported from very poor rural populations in cassava-growing areas of sub-Saharan Africa, where the diet during the month preceding the outbreak consisted almost exclusively of insufficiently processed bitter cassava roots and leaves. The background information provided in these studies shows that the underlying cause of these health problems is rural poverty and food insecurity (chapter 2), induced by an agro-ecological collapse in a shifting cultivation system. Additional stress, such as war, a drought-provoked food shortage or new market opportunities, has been identified as leading to short-cuts in processing which triggered konzo epidemics. Improving rural economy and the production, diversification and accessability of food would prevent such toxic effects from bitter cassava. However, this is difficult to achieve in the short term.

Toxic effects of cassava can also be avoided by growing cultivars with low cyanogen levels, by adequate processing and, to some extent, by optimizing plant growing conditions and by consumption of a balanced diet. Farmers sometimes prefer to grow toxic cultivars as opposed to less toxic ones for a variety of reasons, some of which are probably directly linked to toxicity. Substitution with low-

cyanogenic cultivars will therefore not always satisfy the farmers' needs. Cultivars that are generally low in cyanogens may become more toxic by certain kinds of ecological stress, such as prolonged drought, lack of phosphorus and potassium in the soil (De Bruijn, 1971), and attacks by certain insects (Ayanru & Sharma, 1984). A diet with an adequate protein supply is necessary to maintain the body's limited cyanide detoxification capacity, but in this context we are dealing with conditions where adequate balanced diets are desired, but not accessible. Amazon Indians have shown that processing, even with ingenious "stone-age" equipment, can reduce the cyanogen content of roots of even the most toxic cultivars to safe levels (Dufour, 1989). Good processing therefore seems essential to avoid toxic effects.

Improving processing?
The health problems originating from cyanogen exposure from cassava in several African communities could be avoided if effective processing techniques were applied. However, the role of technological intervention in preventing toxic effects has to be considered against the background of the combination of socio-economic and environmental factors that affect the food situation of the population at the community and household level. Long-term exposure with low and occasionally medium levels of cyanogens under stable conditions must be distinguished from exposure under conditions like war, natural disaster, extreme poverty and collapse of the commercial or agricultural system, i.e. circumstances which lead to higher exposure levels associated with severe poisonings and konzo epidemics. As these are associated with a breakdown of the socio-economic situation and household food security, whilst effective processing methods based on grating are labour-intensive, it is not yet certain whether alternative processing techniques will be feasible for the affected population to implement.

When promoting effective processing, a distinction must be made between inducing small improvements to prevailing processing methods and introducing fast and effective methods for emergency situations. The latter would be supplementary to, and not a replacement of, the dominant processing methods.

Processing by sun-drying
Sun-drying of peeled roots is a common and simple way to make the bulky and perishable roots storable and transportable, and to reduce the potential toxicity. It was shown that straight-forward sun-drying of roots with medium to high initial levels of cyanogenic glucoside does not reduce the levels of total cyanogens to the FAO/WHO (1991) safety limit of 10 mg HCN equivalent per kg flour (chapter 4). During drying cyanogenic glucoside levels initially showed an exponential decrease, which stopped when moisture levels reached about 15%. Although the conversion

of cyanohydrins to HCN and subsequent escape of the latter occurred largely simultaneously with the linamarin degradation, a thorough final drying stage was necessary to reduce the cyanohydrin levels to below 10 mg HCN equivalent per kg.

Experiments with controlled dehydration rates showed that the drying rate is inversely correlated with the rate of linamarin degradation. Post-harvest traumas and physiological deterioration provoke membranes to leak to some extent, which allows linamarin to escape from the vacuoles (White *et al.*, 1994) and to reach the linamarase, which is located in the cell-walls (Mkpong *et al.*, 1991). Menting (1969), using other compounds, found that with faster evaporation large molecules have increasingly lower diffusion rates than small ones. This mechanism may well be the main cause for a lower linamarin diffusion in faster drying cassava root pieces which leads to less linamarin-linamarase interaction. This explains not only why faster-dried pieces have higher residual linamarin levels when completely dry, as found by Mlingi & Bainbridge (1994) and Nambisan & Sundaresan (1985). The latter attributed this to the moisture levels at which linamarase is inactive being reached earlier. The present explanation goes further by explaining why immediately from the start the decline in linamarin levels is slower in faster evaporating pieces. It also explains why in earlier studies, summarized by Coursey (1973) and later also found by Gomez & Valdivieso (1984, 1985), unexpected differences were found in residual linamarin levels after oven-drying at different temperatures. We therefore hypothesized and confirmed in practice that slowing down or interrupting the initial dehydration improves the effectiveness of cyanogen removal in sun-drying cassava. This shows a potential for optimizing cyanogen removal in the process of sun-drying.

Risks and benefits of moulds

A negative consequence of reducing the drying rate is the increased risk of outgrowth of microbial contamination. This might lead to spoilage or even toxin formation. In domestically dried and stored root pieces in Mozambique unintentional moistening and subsequent fungal growth resulted in significantly reduced cyanogenic potential (chapter 5). However, no aflatoxins were found in 10 of these samples nor in 10 other samples that had intentionally been allowed to grow mould (chapter 6). Cytotoxicity and mutagenicity as indicators of microbial toxins were also absent from all 30 screened samples of intentionally moulded cassava (chapter 6). Contamination of cassava flour or dried pieces with aflatoxins reported earlier (Mota & Lourenço, 1974) must be considered with caution, as a phenolic compound behaving like aflatoxin in a common analytical TLC method is present in dried cassava (Coker & Tomlins, 1986; chapter 5). However, Westby *et al.* (personal communication) found unacceptable contamination of sun-dried cassava root pieces with several other mycotoxins in samples from Ghana and

Uganda. It is well possible that microbial toxin formation constitutes a long-term harmful side-effect of prolonged sun-drying, carried out to prevent the acute toxic effects of cyanogens. The outcome may resemble the choice between Scylla and Charibdis. Either a clean, quickly sun-dried product with relatively high residual cyanogenic glucoside levels with a risk of acute poisoning, or a slowly sun-dried product with low cyanogen content, but with microbial toxin levels which might lead to adverse long-term health effects. A way to overcome this dilemma is to stop or to control the microbial growth.

Traditional solid-substrate fermentation
Solid-substrate fermentation in heaped cassava roots, as applied in Uganda, purposely enhances an abundant mould growth. Some control of the microflora is exercised by applying a short initial drying stage, by using the inoculum of earlier fermented cassava roots, and by discarding the roots on which undesired moulds dominate. This method is applied by the Alur and some other tribes to improve the palatability of the cassava roots and to reduce their potential toxicity. The method is also applied in parts of Mozambique, Tanzania, Kenya, Zaire and Malawi. Recently, Hahn (1989) and Nweke (1994) mentioned the application of air-fermentation to cassava roots in Africa, but they did not differentiate this process further from other processes leading to dried pieces or flour. To my knowledge it has not been the subject of serious study so far. A possible explanation for this is that, to the outsider, it seems an uncontrolled "rotting" of roots, which is ascribed to negligence or to strange taste preference. Sometimes it is not clear whether contamination with moulds was intentional or that it occurred accidentally during sun-drying. For these reasons, solid-substrate fermentation may not have been recognized adequately as being a processing method distinct from sun-drying alone. From a toxicological point of view, as well as according to the processors, it certainly is, although intermediate forms exist. In our experiments the method was seen to be more effective in the removal of cyanogenic glucosides, as well as cyanohydrins, than sun-drying alone. This is in line with the findings of the unintentionally moulded cassava in Mozambique (chapter 5), the data on interrupted sun-drying (chapter 4), and the information on the risks of toxic effects provided verbally by farmers in Mozambique (chapter 2) and Uganda (chapter 6).

We found that the incubation as such of cassava root pieces led to the degradation of cyanogenic glucosides and that this degradation was enhanced by the activity of several microorganisms. The various microorganisms differed in their ability to enhance the glucoside breakdown in cassava roots, the most effective ones being the fungi *Neurospora sitophila*, *Rhizopus oryzae* and *Rhizopus stolonifer*. The main contribution of the microflora to the removal of cyanogens was not their linamarase capacity, although present in some strains, but their membrane- and cell-

wall-degrading enzyme activities. The latter activities break down the compartmental separation between the endogenous linamarase and the linamarin. Cell-wall-degrading activity has also been found to be the main mechanism by which lactic acid fermentation contributes to the removal of cyanogenic glucosides during the soaking of roots. However, during soaking the glucosides are released partly unaffected into the water (Westby & Choo, 1994), while in solid-substrate fermentation the entire degradation route of cyanogenic glucosides via cyanohydrins to HCN and volatilization of HCN has to take place.

The flour from heap-fermented cassava, used in rural households, often had linamarin levels and rarely had cyanohydrin levels which were above the Codex Alimentarius' safety limit (FAO/WHO, 1991). It is not known whether, in those cases, shortcuts had been made in the established process, or if roots with higher glucoside levels had been used. On the other hand, the standard set by the Codex Alimentarius may well be a safety guarantee, but it is not necessarily an optimal instrument for evaluating product safety, nor consumer risks. If this standard would be used, a large part of Africa's cassava flour would probably be deemed unsuitable for human consumption. Realistic criteria for safety evaluation of cyanogen levels in cassava products still have to be developed. In any case, the cyanogen levels in several flour samples exceeding this limit by a factor 4 or more, the occurrence of several suspect fungi and the contamination with *Escherichia coli* in at least one of them indicate a need for process optimization. The request of the processors for a quicker process is another reason. The process has potential for being improved, as the food grade fungi *Neurospora sitophila* and *Rhizopus oryzae* were the most effective ones in a process which can adequately reduce cassava cyanogen levels. Processing conditions could be optimized, including the use of starter cultures of these fungi, to ensure a hygienic product with low levels of cyanogens.

Since the start of this research in 1982, increased attention has been paid to the issue of cassava cyanogens. Much new knowledge in this field has been generated. The advances were recently reviewed in a workshop on cassava safety (Bokanga *et al.*, 1994b). I was fortunate that the research that forms the basis for this thesis became an integral part of these developments. The knowledge generated in this study gives several options to help cassava growers to enhance the removal of cyanogens from cassava roots. In order to better evaluate the adequacy of processing methods, the criteria to assess safety of cassava consumption need revision. To this end accurate data on the intake of the distinct cyanogens, food items and the health status are needed from several cassava consuming populations. Useful analytical methods are now available. Farmers' motives for their choices in processing need elucidation to enable extensionists to formulate correct messages for the introduction of improvements.

[1]) Safe food is defined here as food which does not cause significant adverse effects to its consumers. It can be considered as food in which any potentially toxic compound is in such a low concentration that with an expected use of the food, including processing, preparation and its proportion of the diet, and taking into account possible neutralizing food factors, no relevant adverse health effects in its consumers are to be expected in the short or long run. These health effects should be considered relative to other common health expectations and risks. The limit below which cyanogen levels in fresh cassava roots may be considered safe may be higher in a population which applies extensive processing, than in one which is used to consume unprocessed roots. Cassava products with residual cyanogen levels that in the long run might induce goitre, may be considered relatively safe for a population facing acute famine. Therefore, "low" and "high" levels of cyanogens are relative concepts. Rosling (1994) pointed at the discrepancy between the limit below which plant-breeders consider genotypes low-cyanogenic, and the Codex Alimentarius' limit for cyanogens in cassava flour. The first value is 30 times higher than the second one.

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Background

Cassava is an important staple root crop of the tropics. It contains cyanogenic glucosides, mainly linamarin, which, after enzymatic conversion to cyanohydrins, may release the toxic hydrogen cyanide (HCN). These compounds can be removed by processing. Insufficient removal has led to some health problems in rural Africa. Such problems were studied in Mozambique. Cyanogen removal during the usual processing methods, sun-drying and heap-fermentation, was studied in Uganda and The Netherlands. The aim was to explore possibilities for the optimization of cyanogen removal.

The problem

A study of a small outbreak of paralysis in a rural population in Mozambique revealed that the seven paralyzed subjects examined had an isolated spastic paraparesis with abrupt onset during a period of food shortage. The diet at onset consisted almost exclusively of insufficiently processed roots and leaves of a recently introduced high-yielding bitter cassava cultivar. The affected population also reported frequent symptoms of acute poisonings some hours after meals, and related that to the bitter cassava and the processing applied. A high dietary cyanide exposure from cassava in the affected subjects was indicated by high urinary and serum levels of thiocyanate, the main metabolite. The observed walking disability was diagnosed as konzo. Konzo has been identified as a distinct disease entity in 1990 and it has been reported from five other geographically separate rural areas of Africa. The dietary and laboratory findings were consistent with those from other konzo affected areas and they support a causal role of cyanide exposure.

Cyanogen assay

To be able to evaluate changes in cassava cyanogen levels, a promising enzymic assay, developed by Cooke, was compared with the laborious standard AOAC method. Cooke's assay, if using a linamarin calibration curve, gave similar values for the cyanogenic potential in fresh cassava as the standard method. For samples with a large proportion of HCN, Cooke's assay yielded slightly lower values, but it had the advantage of being independent of endogenous linamarase, and it was less laborious. The method was further improved by using isonicotinate and 1,3-dimethyl barbiturate as colour reagent. Compared with the usually applied pyridine with pyrazolone, the new reagent is less toxic, it does not release repulsive vapours and can be stored longer. The improved method is faster, cheaper, easier to handle and has greater sensitivity. Separate calibration curves of linamarin, acetone cyanohydrin and KCN resulted in more accurate calculation of cyanogenic glucoside and cyanohydrin levels than before. Recovery of linamarin supplemented to extracts was $102 \pm 4\%$. Direct measurement of cyanogens was accurate in cassava extracts containing $35 - 700$ μM.

Sun-drying of roots

The removal of cyanogens from cassava root pieces by sun-drying was studied in field and laboratory experiments. Cyanogenic glucoside levels decreased exponentially during sun-drying and stabilized when moisture levels reached about 15%. The degradation rate of cyanogenic glucoside was inversely correlated with the dehydration rate. This may be explained as faster evaporation lowers the diffusion rate of the cyanogenic glucoside molecules, as they are larger than water molecules, and thereby decreases the possibility for glucoside-linamarase interaction. Application of straight-forward sun-drying leads to insufficient cyanogenic glucoside removal from roots with high initial levels. Speeding up the drying process, e.g. by reducing the size of the pieces, results in higher residual levels. On the other hand, thorough drying was necessary to minimise the cyanohydrin content. Interrupted initial drying, followed by rigorous drying, reduced the levels of cyanogenic glucosides and cyanohydrins significantly more than straight-forward drying in the same time span.

In rural Mozambique cassava root pieces with known initial cyanogen levels were sun-dried and subsequently stored for 8 months under traditional household conditions. On these root pieces, varying extents of fungal growth had occurred. The traditional process resulted in 92.3 - 99.5 % loss of cyanogenic potential and the cyanohydrins plus HCN ranged from 19 to 89 % of the residual total cyanogen levels. There was no correlation between the initial and residual levels of total cyanogens. The samples of flour that had become darker by mould-growth had significantly lower levels of cyanogens and a higher pH. Aflatoxins were absent. Therefore, dark moulded cassava flour is not necessarily less safe than clean white, as was previously assumed.

Traditional solid-substrate fermentation

The Alur tribe in Uganda processes cassava roots by solid-substrate fermentation in heaps, to improve the palatability and diminish the potential toxicity. Mean ($\pm$SD) total cyanogen levels in flours from rural households were 20.3 ($\pm$16.8) mg CN equivalents per kg cassava on dry weight basis in 1990 (n = 23) and 65.7 ($\pm$56.7) in 1992 (n = 21). Mean ($\pm$SD) levels of cyanohydrins plus HCN were 9.1 ($\pm$8.7) in the 1992 flours. Total cyanogen levels in 6 monitored batches of on-farm fermented roots were reduced by heap-fermentation from 436.3 ($\pm$140.7) to 20.4 ($\pm$14.0) mg CN equivalents per kg cassava on dry weight basis. Residual cyanogenic potential was positively correlated with particle size of the resulting crumbs. Crumbs meant for brewing or selling had higher levels than those meant for home consumption as food. Heap-fermentation was significantly more effective in reducing cyanogen levels than sun-drying alone, but it did not always result in the low levels recommended by the Codex Alimentarius Committee of the FAO/WHO. No mutagenicity, cytotoxicity nor aflatoxins could be detected in the 30 flours analyzed, but the formation of mycotoxins cannot be excluded. Protein quantity and quality were not significantly affected. The

cassava gel viscosity pattern was modified to the consumers' preference by this method.

The role of the fermentation in the reduction of cassava cyanogen levels, as well as the mechanisms behind these effects were studied in six individual strains of the dominant microflora. Disinfected cassava root pieces were incubated for 72 h after inoculation with either of the fungi *Geotrichum candidum*, *Mucor racemosus*, *Neurospora sitophila*, *Rhizopus oryzae* and *Rhizopus stolonifer*, or a *Bacillus* sp. Incubation of disinfected root pieces reduced cyanogenic glucoside levels significantly to (mean $\pm$SD) 62.7 $\pm$2.8 % of the initial value. With microbial growth, residual glucoside levels were 18.6 $\pm$11.7 % of the initial ones, which is significantly lower than without inoculation. Among the tested strains, *N. sitophila* reduced cyanogenic glucoside levels most effectively, followed by *R. stolonifer* and *R. oryzae*. Of all fermented samples, both *Rhizopus* spp. showed the highest proportion of residual cyanogens in the cyanohydrin form, followed by the *Bacillus* sp. Flours showed lower absolute levels, but similar patterns of cyanogens to the batches they were prepared from. Cyanogenic glucoside level reduction was correlated ($r=0.86$) with the extent of root softening.

In defined growth media, several of the fungi and bacteria released HCN from linamarin. A *Bacillus* sp. decreased the linamarin to 1% of initial concentrations, *M. racemosus* to 7%, *R. oryzae* and *R. stolonifer* to 30%, while *N. sitophila* and *G. candidum* hardly degraded linamarin within 72 h. Adding pectolytic and cellulolytic enzymes, but not linamarase, to root pieces under aseptic conditions, led to root softening and significantly lower linamarin contents. *N. sitophila* showed no linamarase activity, in contrast to *M. racemosus* and *Bacillus* sp., both of which were less effective in root softening and decreasing the root linamarin content. The most important contribution of microorganisms to linamarin decrease in solid-substrate fermenting cassava therefore is their cell-wall-degrading capacity which enhances the contact between the endogenous linamarase and cyanogenic glucosides.

Conclusions

The effectiveness of cyanogen removal can be enhanced by initially reducing the drying rate, followed by a thorough final drying step. Microbial contamination should thereby be kept under control.

The removal of cyanogens by heap-fermentation was more effective than by sun-drying alone. As we found no new obvious health risk, the process can be regarded as an improvement compared to sun-drying alone in areas where cassava cultivars with higher cyanogen levels prevail. It is recommended to optimize the processing conditions, including the use of starter cultures of the effective food grade fungi *Neurospora sitophila* and *Rhizopus oryzae*, to ensure a more hygienic product without mycotoxins and with innocuous levels of cyanogens.

Contexte

Le manioc est une importante plante alimentaire de base dans les pays tropicaux. Il contient des glucosides cyanogénétiques, essentiellement de la linamarine, d'où peut se libérer de l'acide cyanhydrique toxique (HCN), après la transformation enzymatique en cyanohydrines. Ces substances peuvent être éliminées par un certain traitement. L'élimination insuffisante de ces substances a engendré plusieurs problèmes de santé en Afrique rurale. De tels problèmes ont été étudiés au Mozambique. L'élimination des substances cyanogénétiques lors du traitement habituel des racines de manioc, à savoir le séchage au soleil et la fermentation en milieu solide, a été étudiée en Ouganda et aux Pays-Bas. Le but était d'explorer les possibilités d'optimalisation de l'élimination des substances cyanogénétiques.

Le problème

Une petite épidémie de paralysie a été étudiée auprès d'une population rurale au Mozambique. Les sept personnes examinées, présentant une paralysie aux deux jambes, étaient atteintes d'une paraparésie spastique sporadique, subitement apparue lors d'une période de disette. Pendant cette période, l'alimentation se composait presque exclusivement de racines et de feuilles insuffisamment détoxiquées d'une variété de manioc à haut rendement, plus amère, qui avait été introduite quelques années auparavant. Des symptômes d'intoxication aiguë apparaissaient généralement quelques heures après la consommation de manioc. Les valeurs élevées de thiocyanate, le métabolite essentiel, dans l'urine et le sérum, indiquaient une forte exposition au cyanure, provenant du manioc. Ce syndrome était diagnostiqué comme konzo. Le konzo a été identifié en 1990 comme une maladie particulière, signalée entre-temps dans cinq autres régions rurales d'Afrique, géographiquement dispersées. Les résultats de l'anamnèse alimentaire et les analyses biochimiques correspondaient à ceux des autres régions ravagées par le konzo et indiquaient le rôle étiologique de l'exposition au cyanure.

Analyse des substances cyanogénétiques

Afin de déterminer les modifications des teneurs en substances cyanogénétiques du manioc, une méthode enzymatique prometteuse, établie par Cooke, a d'abord été comparée avec la méthode standard de l'AOAC, qui est laborieuse. La technique de Cooke donnait les mêmes résultats que la méthode standard pour le potentiel cyanogénétique du manioc frais, à condition qu'une courbe d'étalonnage de la linamarine soit utilisée. Dans les échantillons ayant une forte teneur en cyanohydrine et en HCN, la méthode de Cooke donnait certes des résultats quelque peu inférieurs, mais présentait l'avantage d'être indépendante de la linamarase et était moins laborieuse. La méthode a encore été améliorée en remplaçant le réactif coloré, pyridine-pyrazolone, par de l'isonicotinate associé au 1,3 diméthylbarbiturate. Le nouveau réactif est moins toxique et ne répand pas d'odeur nauséabonde ; il est plus

sensible, plus rapide, moins cher, se conserve plus longtemps et est plus commode d'emploi. Des courbes d'étalonnage distinctes de la linamarine, de l'acétone-cyanohydrine et du KCN ont permis d'obtenir un calcul plus précis qu'auparavant des concentrations de glucoside cyanogénétique et de cyanohydrine. Environ 102% (écart-type = 4) de la quantité de linamarine ajoutée à l'extrait a été ainsi redéterminée. Une mesure directe des substances cyanogénétiques était possible dans des extraits de manioc en contenant 35 - 700 μM.

Séchage au soleil des racines

L'élimination de substances cyanogénétiques dans des morceaux de racines de manioc séchés au soleil a été étudiée dans des expériences effectuées sur le terrain et au laboratoire. La concentration de glucoside cyanogénétique diminuait de façon exponentielle lors du séchage et se stabilisait à une teneur en eau d'environ 15%. La vitesse de décomposition des glucosides cyanogénétiques avait une corrélation négative avec la vitesse de déshydratation. Cela pourrait s'expliquer par le fait qu'une évaporation plus rapide réduit la vitesse de diffusion de la molécule du glucoside cyanogénétique - plus grosse que celle de l'eau - d'où la possibilité d'interaction du glucoside avec la linamarase. L'utilisation du séchage au soleil comme unique étape de traitement conduit à une élimination insuffisante des glucosides cyanogénétiques des racines ayant de fortes teneurs initiales. L'accélération du processus de séchage, par exemple en diminuant la taille des morceaux à faire sécher, conduit à des teneurs résiduelles supérieures. Par ailleurs, un séchage approfondi est nécessaire pour diminuer la teneur en cyanohydrine. Un ralentissement du séchage au début, suivi d'un séchage intensif, assurait une baisse plus significative des teneurs en glucoside cyanogénétique et en cyanohydrine qu'un séchage simple sur la même période.

Dans la zone rurale du Mozambique, des morceaux de racines de manioc, ayant une teneur en cyanogène connue, ont été séchés au soleil puis stockés pendant 8 mois dans des conditions domestiques. Ces morceaux ont moisi, à différents degrés. Le processus traditionnel s'est traduit par une diminution de 92,3 - 99,5% du potentiel cyanogénétique. La part de cyanohydrines et d'HCN s'élevait à 19 - 89% du potentiel cyanogénétique résiduel. Il n'y avait aucun rapport entre les valeurs initiales et finales du potentiel cyanogénétique. Les échantillons de farine, devenus plus foncés en raison de la moisissure, présentaient des teneurs très inférieures en substances cyanogénétiques et un pH supérieur. Il n'y avait pas d'aflatoxines. C'est pourquoi la farine de manioc foncée et moisie ne présente pas nécessairement plus de risques que la farine de manioc propre et blanche, comme on le pensait auparavant.

La fermentation solide traditionnelle

Pour traiter les racines de manioc écorcées, la tribu Alur, en Ouganda, les empile et les recouvre, ce qui entraîne la fermentation. Elles sont ensuite séchées. Ce processus

a pour but d'améliorer le goût et de diminuer la toxicité potentielle. Le potentiel cyanogénétique moyen (± écart-type) des échantillons de farine des ménages ruraux en 1990 (n=23) était de 20,3 (±16,8) mg équivalent CN par kg de poids sec. En 1992 (n=21), ce potentiel était de 65,7 (±56,7); en outre, la teneur en cyanohydrine avec HCN était de 9,1 (±8,7) mg équivalent CN par kg de poids sec. Le potentiel cyanogénétique des racines empilées, qui étaient à fermenter, sous observation, dans 6 ménages, passait d'une teneur de 436,3 (±140,7) à 20,4 (±14,0) mg équivalent CN par kg de poids sec. Après traitement, le potentiel cyanogénétique avait une corrélation positive avec la taille des particules. La farine destinée à la vente ou à la production de boissons alcoolisées présentait des teneurs supérieures en substances cyanogénétiques à celles de la farine destinée à l'autoconsommation. La fermentation solide était significativement plus efficace que le seul séchage au soleil, pour la diminution des teneurs en substances cyanogénétiques, mais ne conduisait pas toujours aux faibles teneurs recommandées par la Commission Codex Alimentarius de la FAO/OMS. Aucune mutagénicité, cytotoxicité ou aflatoxine n'a été trouvée dans les 30 échantillons de farine analysés, mais on ne peut pas exclure la formation de mycotoxines. La concentration et la qualité de la protéine du manioc n'étaient pas modifiées de façon significative. La bouillie préparée à partir de cette farine présentait un autre type de viscosité, conforme au goût du consommateur.

L'effet de la fermentation sur les teneurs en substances cyanogénétiques et leurs mécanismes d'action ont été étudiés en laboratoire au moyen de six souches de la microflore dominante. Des morceaux de racines de manioc désinfectés ont été incubés après inoculation d'une des moisissures *Geotrichum candidum*, *Mucor racemosus*, *Neurospora sitophila*, *Rhizopus oryzae* et *Rhizopus stolonifer*, ou une espèce *Bacillus*. L'incubation des morceaux de racines désinfectées diminuait de façon significative les teneurs en glucoside cyanogénétique jusqu'à en moyenne (± écart-type) 62,7% (±2,8) de la valeur initiale. La croissance microbienne a permis de réduire la teneur en glucoside cyanogénétique jusqu'à 18,6% (±11,7) de la valeur initiale, ce qui est significativement inférieur au résultat obtenu sans inoculation. Parmi les souches testées, *N. sitophila* diminuait le plus la teneur en glucoside cyanogénétique, suivie par *R. stolonifer* et *R. oryzae*. Le manioc couvert d'une espèce *Rhizopus* avait la proportion la plus forte de potentiel cyanogénétique subsistant sous la forme cyanohydrine, suivi par l'espèce *Bacillus*. Les farines avaient des teneurs en cyanogène qui étaient proportionnellement inférieures à celles des racines fermentées dont elles provenaient. La baisse de la teneur en glucoside cyanogénétique était corrélée de façon significative (r=0,86) au degré de perte de structure de la racine.

Dans des milieux de culture contrôlés, diverses bactéries et moisissures étaient en mesure de libérer l'HCN de la linamarine. Une espèce *Bacillus* diminuait la linamarine jusqu'à 1% de la concentration initiale, *M. racemosus* jusqu'à 7%, *R. oryzae* et *R. stolonifer* jusqu'à 30%, alors que *N. sitophila* et *G. candidum* ne dégradaient

pratiquement pas la linamarine en 72 heures. L'addition d'enzymes pectolytiques et cellulolytiques, mais pas de linamarase, aux morceaux de racines, dans des conditions aseptiques, conduisait à une perte de la structure de la racine et à une réduction significative des teneurs en glucoside cyanogénétique. *N. sitophila* ne présentait pas d'activité linamarasique, contrairement à *M. racemosus* et à l'espèce *Bacillus,* qui étaient moins efficaces pour ramollir les racines et diminuer la teneur en glucoside cyanogénétique. C'est pourquoi l'apport essentiel des microorganismes à l'élimination des glucosides cyanogénétiques, lors de la fermentation solide, est leur activité destructive de la paroi cellulaire, favorisant ainsi le contact entre la linamarase endogène et le glucoside cyanogénétique.

Conclusions

Dans la production de farine de manioc par séchage au soleil, l'efficacité de l'élimination de substances cyanogénétiques peut être améliorée en ralentissant initialement la vitesse de séchage puis en effectuant un séchage intensif. Le développement microbien doit être maintenu sous contrôle.

L'élimination des substances cyanogénétiques par le processus de fermentation solide est plus efficace que par le seul séchage au soleil. Aucun nouveau risque particulier pour la santé n'a été décelé. C'est pourquoi le processus peut être considéré comme une amélioration par rapport au seul séchage au soleil, pour les racines de manioc ayant de fortes concentrations en glucoside cyanogénétique. Afin d'obtenir un produit plus conforme aux normes sanitaires, sans mycotoxines et présentant des teneurs inoffensives en substances cyanogénétiques, il est recommandé d'optimaliser le processus, ainsi que d'appliquer des cultures de départ de moisissures efficaces, de qualité alimentaire, *Neurospora sitophila* et *Rhizopus oryzae*.

Contexto

Mandioca é um importante alimento de base na zona tropical. As células da planta contêm glucosides cyanogénicos, principalmente linamarina, de que, após uma transformação enzimática em cianohidrina, pode libertar-se o ácido cianídrico (HCN) que é um gás tóxico. Estas substâncias podem ser removidas por processamento. A remoção insuficiente de estas substâncias tem conduzido a problemas de saúde nas zonas rurais de África. Tais problemas têm sido estudados em Moçambique. A remoção de substâncias cianogénicas durante o processamento regular de raízes da mandioca, secagem ao sol e fermentação do substrato sólido, tem sido investigado em Uganda e Holanda. O objectivo foi explorar as possibilidades para optimizar a remoção de substâncias cianogénicas.

O problema

A investigação de um surto de paralisia numa população rural em Moçambique mostrou que as sete pessoas examinadas com ambas pernas paralisadas, tiveram uma paraparesia espástica que iniciou abruptamente num período de carência alimentar. A alimentação naquele período consistiu quase exclusivamente de folhas e raízes não adequadamente processados de uma variedade amarga de mandioca, que foi introduzida uns anos antes.

A população afectada relatou sintomas frequentes de envenenamento agudo umas horas após a ingestão de refeições a base de mandioca e relacionou esses sintomas à mandioca amarga bem como ao processamento aplicado. O consumo da mandioca implicou uma exposição elevada do ácido cianídrico da mandioca, como foi comprovado através dos níveis elevados de thiocianato, o metabólite principal, nas urinas e soros. A incapacidade de andar foi diagnosticada como konzo. Konzo tem sido identificado em 1990 como uma doença distinta que entretanto foi denunciada em cinco outras áreas rurais, geograficamente dispersas em África. Os resultados da pesquisa alimentar e das análises bioquímicas são similares aos encontrados em outras populações afectadas por konzo e suportam um papel causal do cianeto.

Análise de substâncias cianogénicas

Para estabelecer mudanças nos níveis de substâncias cianogénicas da mandioca, primeiro um método enzimático esperançoso, desenvolvido por Cooke, foi comparado com o método laborioso estandarte da AOAC. O análise de Cooke rendeu os mesmos valores da potência cianogénica em mandioca fresca como o do método padrão, com a condição que fôr utilizada linamarina para a sua calibração. Em amostras com um alto nível de cianohidrinas e HCN o método de Cooke resultou em valores ligeiramente inferiores, mas o método tinha a vantagem de ser independente de linamarase e foi menos laborioso. Este método foi melhorado através da substituição do reagente de coloração piridina-pirazolona pela combinação de isonicotinato com 1,3-dimethylbarbiturato. Este reagente novo é

menos tóxico, não difunde um cheiro repugnante; é mais sensível, mais rápido, mais barato, a sua utilização é mais fácil e mantém melhor a sua qualidade depois da armazenagem. Fazer curvas de calibração separadamente de linamarina, acetona cianohidrina e KCN resultou em cálculos de glucosidas cianogénicas e cianohidrina mais precisos que anteriormente. Por média 102% (desvio padrão 4%) da quantidade linamarina acrescentada aos extractos foi recuperado. A análise directa de cianeto foi possível em extractos com uma concentração de 35 - 700 μM.

Secagem ao sol

A remoção de substâncias cianogénicas de pedaços de raízes da mandioca que são secas ao sol, foi estudado em ensaios no campo e no laboratório. A concentração de glucosido-cianogénico diminuiu exponencialmente durante a secagem e estabeleceu-se ao chegar uma humedade de uns 15%. A velocidade de degradação da glucosido-cianogénico foi correlacionada negativamente com a da desidratação. Isto poderia ser explicado pela evaporação acelerada baixando a velocidade de dispersão da molécula glucosido-cianogénico -por ser maior que a da água-, diminuindo assím a possibilidade de interacção da glucosido com a linamarase. Aplicar somente a secagem ao sol como único processamento conduz a uma remoção insuficiente de glucosido-cianogénico de raizes com altos níveis iniciais. Acelerar a secagem, por exemplo através da diminuição do tamanho dos pedaços, conduz a níveis residuais mais altos. Por outro lado é necessário secar bem para diminuir o nível de cianohidrina. Secagem travada no início, seguido por uma secagem intensiva resultou em níveis significativamente menores de glucosido-cianogénico e cianohidrina que secagem simples durante o mesmo tempo.

Numa área rural de Moçambique pedaços de mandioca com níveis conhecidos de glucosido-cianogénico foram secos ao sol e depois armazenados durante 8 meses em condições caseiras. Nestes pedaços diferentes níveis de contaminação por bolores foram encontrados. O processo tradicional resultou numa redução de 92,3 - 99,5% da potência cianogénica. A proporção de cianohidrina com HCN foi 19 - 89% da potência cianogénica residual. Não havia relação entre o nível inicial e residual da potência cianogénica. As farinhas mais escurecidas por bolores tinham níveis significativamente inferiores de substâncias cianogénicas e um pH maior. Aflatoxinas não estavam presentes. Concluiu-se que as farinhas de mandioca escurecidas por bolores não são necessáriamente menos seguras do que as farinhas limpas e mais branca, como foi anteriormente assumido.

Fermentação do substrato sólido tradicional

A tribo Alur em Uganda processa raizes de mandioca guardando-as em montões cobertos, causando a criação de bolores. Depois são expostas ao sol para serem secadas. O fim do processamento é o melhoramento do paladar e a diminuição da

potência tóxica. A média (± desvio padrão) da potência cianogénica nas farinhas de lares rurais em 1990 (n=23) foi 20,3 (±16,8) mg CN equivalente por kg de matéria seca. Em 1992 (n=21) isso foi de 65,7 (±56,7), e o nível de cianohidrinas com HCN foi 9,1 (±8,7) mg CN equivalente por kg de matéria seca. A potência cianogénica em montões de raízes que foram fermentadas sob observação em 6 lares diminuiu de 436,3 (±140,7) a 20,4 (±14,0) mg CN equivalente por kg de matéria seca. A potência cianogénica depois do processamento estava correlacionada positivamente com a grossura dos pedaçinhos. Farinha destinada à venda ou à produção de bebidas alcoolicas tinha teores maiores de substâncias cianogénicas do que aqueles para o consumo próprio. A fermentação do substrato sólido foi significativamente mais efectivo na redução dos níveis de substâncias cianogénicas do que somente a secagem ao sol, mas nem sempre resultou em níveis inferiores aos valores baixas recomendados no Codex Alimentarius da FAO/OMS. As 30 amostras analisadas não mostraram mutagenicidade, citotoxicidade nem tinham aflatoxina, mas a formação de micotoxinas não pode ser absolutamente excluída. A concentração e a qualidade da proteína da mandioca não foram alteradas significativamente por este processo. Papas preparadas de este farinha mostraram um padrão de viscosidade alterada, conforme os desejos dos consumidores.

O papel da fermentação nos níveis de substâncias cianogénicas e os mecanismos do função foram estudados no laboratório a partir de seis linhagens da microflora dominante. Pedaços desinfectados de raízes de mandioca foram incubados depois da inoculação com *Geotrichum candidum*, *Mucor racemosus*, *Neurospora sitophila*, *Rhizopus oryzae* e *Rhizopus stolonifer*, ou um *Bacillus* sp. A incubação dos pedaços desinfectados reduziu os níveis de glucosido-cianogénico até por média (± desvio padrão) 62,7% (±2,8) do nível inicial. Com o crescimento microbiano, o nível de glucosido-cianogénico foi reduzido a 18,6% (±11,7) do nível inicial, que é significativamente menos do que sem inoculação. Das linhagens investigadas a *N. sitophila* reduziu o nível de glucosido-cianogénico o mais, seguido por *R. stolonifer* e *R. oryzae*. Os pedaços cobertos com *Rhizopus* spp. tinham a proporção maior na forma cianohidrina, seguido pelos com *Bacillus* sp. A redução dos níveis de glucosido-cianogénico foi correlacionada significativamente (r=0,86) com a perda de firmeza da raiz. Os padrões de substâncias cianogénicas nas farinhas foram parecidos aos das porções molhadas de que foram feitas, menos que os níveis tenham sido inferiores.

Em meios alimentares definidos, varias bactérias e fungos foram capazes de libertar HCN de linamarina. Um *Bacillus* sp. reduziu a linamarina até 1% da concentração inicial, *M. racemosus* a 7%, *R. oryzae* e *R. stolonifer* a 30%, enquanto *N. sitophila* e *G. candidum* quase não degradaram a linamarina dentro de 72 h. A adição de enzimas pectolíticas e celulolíticas , mas não linamarase, a

pedaços de raiz em condições asepticas, conduziu à perda de firmeza da raiz e níveis de glucosido-cianogénico significativamente inferiores. *N. sitophila* não mostrou actividade de linamarase, em contraste com *M. racemosus* e *Bacillus* sp., que foram menos efectivos em amolecer as raizes e reduzir o nível de glucosidos cianogénicos. Por isso, a contribuição mais importante de micro-organismos na remoção de substâncias cianogénicas durante a fermentação de substrato sólido é a actividade de degradação das estruturas celulares, promovendo o contacto entre a linamarase endogénica e o glucosido-cianogénico.

Conclusões

Na produção de farinha de mandioca por via de secagem ao sol, a efectividade da remoção de substâncias cianogénicas pode ser ampliada por travar a secagem no início, seguida de uma secagem intensiva. Pode ser necessário restringir o crescimento de bolores.

A remoção de substâncias cianogénicas pelo processo de fermentação de substrato sólido é mais efectiva do que somente por secagem solar. Não foi encontrado um risco adicional de saúde. Por isso, o processo pode ser considerado como um método melhorado em comparação à secagem solar de raizes com níveis altos de glucosido-cianogénico. Para assegurar um produto mais higiénico sem micotoxinas e com níveis inócuos de substâncias cianogénicas, é recomendada uma optimização do processo, incluindo a aplicação de culturas de arranque dos fungos de grau nutricional *Neurospora sitophila* e *Rhizopus oryzae*.

Achtergrond

Cassave is een belangrijk basis-voedselgewas in de tropen. Het bevat cyanogene glucosiden, voornamelijk linamarine, waaruit na enzymatische omzetting in cyanohydrines het giftige blauwzuurgas (HCN) kan vrijkomen. Deze stoffen kunnen door verwerking verwijderd worden. Onvoldoende verwijdering van deze stoffen heeft in ruraal Afrika geleid tot een aantal gezondheidsproblemen. Dergelijke problemen werden bestudeerd in Mozambique. De verwijdering van cyanogene stoffen tijdens de gebruikelijke verwerking van cassavewortels, zon-drogen en vaste-stof fermentatie, werd bestudeerd in Uganda en Nederland. Het doel was om mogelijkheden voor optimalisatie van de verwijdering van cyanogene stoffen te exploreren.

Het probleem

Een kleine epidemie van verlammingen in een plattelandsbevolking in Mozambique werd bestudeerd. De zeven onderzochte personen met verlamming aan beide benen hadden een geïsoleerde spastische paraparesis die abrupt inzette tijdens een periode van voedselschaarste. De voeding tijdens die periode bestond bijna uitsluitend uit onvoldoend ontgiftigde wortels en bladeren van een hoog-produktieve bittere cassave cultivar die enkele jaren tevoren geïntroduceerd was. Symptomen van acute vergiftiging enkele uren na cassave maaltijden werden hierbij vaak ervaren. Een grote blootstelling aan cyanide uit cassave bleek uit hoge thiocyanaat waarden, de belangrijkste metaboliet, in urine en serum. De kreupelheid werd gediagnostiseerd als konzo. Konzo is in 1990 geïdentificeerd als een afzonderlijke ziekte waarover inmiddels gerapporteerd is uit vijf andere, geografisch verspreide rurale gebieden in Afrika. De resultaten van de voedingsanamnese en biochemische bepalingen kwamen overeen met die uit andere door konzo geteisterde gebieden en duiden op een oorzakelijke rol van blootstelling aan cyanide.

Analyse van cyanogene stoffen

Om veranderingen van de gehaltes cyanogene stoffen in cassave te bepalen, werd eerst een veelbelovende enzymatische methode, ontwikkeld door Cooke, vergeleken met de bewerkelijke standaard-methode van de AOAC. Cooke's bepaling gaf dezelfde waarden voor het cyanogeen potentieel in verse cassave als de standaard methode, mits een linamarine ijklijn werd gebruikt. In monsters met een hoog gehalte aan cyanohydrines en HCN gaf de methode van Cooke weliswaar iets lagere waarden, maar hij had het voordeel onafhankelijk van linamarase te zijn, en hij was minder bewerkelijk. De methode werd verder verbeterd door het pyridine-pyrazolone kleurreagens te vervangen door isonicotinaat met 1,3-dimethylbarbituraat. Het nieuwe reagens is minder giftig, het verspreidt geen walgingwekkende geur; het is gevoeliger, sneller, goedkoper, langer houdbaar en gemakkelijker te gebruiken. Afzonderlijke ijklijnen van linamarine, aceton

cyanohydrine en KCN resulteerden in nauwkeuriger berekening van de cyanogeen glucoside en cyanohydrine concentraties dan voorheen. Gemiddeld 102% (SD=4) van de aan extract toegevoegde hoeveelheid linamarine werd hiermee terugbepaald. Een directe meting van cyanogenen was mogelijk in cassave extracten die 35 - 700 μM bevatten.

Zon-drogen van wortels

De verwijdering van cyanogene stoffen uit stukken cassave wortel die in de zon gedroogd worden, werd bestudeerd in veld- en laboratoriumexperimenten. De concentratie aan cyanogeen glucoside nam exponentieel af gedurende de droging, en stabiliseerde bij een vochtgehalte van ongeveer 15%. De afbraaksnelheid van cyanogeen glucoside bleek negatief gecorreleerd te zijn met de dehydratatiesnelheid. Dit kan mogelijk verklaard worden doordat snellere verdamping de diffusiesnelheid van het -ten opzichte van water grotere- cyanogeen glucoside molecuul verlaagt, en daarmee de kans op interactie van het glucoside met linamarase. Het toepassen van zon-drogen als enige verwerkingsstap leidt tot onvoldoende verwijdering van cyanogeen glucoside uit wortels met hoge begingehaltes. Het versnellen van het droogproces, bijvoorbeeld door het verkleinen van de te drogen stukken, leidt tot hogere residu-gehaltes. Anderzijds is grondig drogen nodig om het gehalte aan cyanohydrine te verlagen. Vertraging van de droging in het begin, gevolgd door intensief drogen, zorgde voor significant lagere gehaltes aan cyanogeen glucoside en cyanohydrine dan bij rechttoe rechtaan drogen over dezelfde periode.

In ruraal Mozambique werden stukken cassave wortel met bekend gehalte aan cyanogenen in de zon gedroogd en vervolgens gedurende 8 maanden opgeslagen onder huishoudelijke omstandigheden. Deze stukken raakten in verschillende mate beschimmeld. Het traditionele proces resulteerde in 92,3 - 99,5% afname van het cyanogeen potentieel. Het aandeel van cyanohydrinen plus HCN bedroeg 19 - 89% van het restgehalte aan cyanogenen. Er was geen verband tussen begin- en eindwaarden van het cyanogeen potentieel. De monsters van door beschimmeling donkerder geworden meel hadden significant lagere gehaltes aan cyanogene stoffen en een hogere pH. Aflatoxinen waren afwezig. Donker, beschimmeld cassavemeel is daarom niet noodzakelijkerwijs onveiliger dan schoon wit cassavemeel, zoals eerder gedacht werd.

Traditionele vaste-stof fermentatie

De Alur stam in Uganda verwerkt geschilde cassavewortels door ze gestapeld en afgedekt te bewaren, waardoor fermentatie optreedt. Daarna worden ze gedroogd. Met dit proces wordt beoogd de smakelijkheid te verhogen en de potentiële giftigheid te verlagen. Het gemiddelde ($\pm$SD) cyanogeen potentieel in meelmonsters van rurale huishoudens in 1990 (n=23) was 20,3 ($\pm$16,8) mg CN equivalent per kg

droog gewicht. In 1992 (n=21) was dat 65,7 ($\pm$56,7); daarbij was het gehalte aan cyanohydrines plus HCN 9,1 ($\pm$8,7) mg CN equivalent per kg droog gewicht. Het cyanogeen potentieel in stapels wortels die onder observatie in 6 huishoudens gefermenteerd werden, verminderde van 436,3 ($\pm$140,7) tot 20,4 ($\pm$14,0) mg CN equivalent per kg droog gewicht. Het cyanogeen potentieel na verwerking was positief gecorreleerd met de deeltjesgrootte. Meel bestemd voor de verkoop of voor de produktie van alcoholische drank had hogere gehaltes cyanogene stoffen dan dat voor eigen consumptie. Vaste-stof fermentatie was significant effectiever dan uitsluitend zon-drogen in het verlagen van de gehaltes aan cyanogene stoffen, maar resulteerde niet altijd in de lage gehaltes die de Codex Alimentarius Commissie van de FAO/WHO voorschrijft. Er werd geen mutageniteit, cytotoxiciteit noch aflatoxine aangetroffen in de 30 geanalyseerde meelmonsters, maar de vorming van mycotoxinen kan niet worden uitgesloten. De concentratie en kwaliteit van het cassave-eiwit waren niet significant veranderd. Pap bereid van dit meel vertoonde een veranderd viscositeitspatroon overeenkomstig de voorkeur van de consument.

De rol van de fermentatie op de gehaltes aan cyanogene stoffen en de werkingsmechanismen hiervan werden in het laboratorium onderzocht aan de hand van zes stammen van de dominante microflora. Gedesinfecteerde stukken cassavewortel werden geïncubeerd na beënting met een van de schimmels *Geotrichum candidum*, *Mucor racemosus*, *Neurospora sitophila*, *Rhizopus oryzae* en *Rhizopus stolonifer*, of een *Bacillus* sp. Incubatie van gedesinfecteerde stukken wortel verlaagde de gehaltes van cyanogeen glucoside significant tot gemiddeld ($\pm$SD) 62,7% ($\pm$2,8) van de beginwaarde. Door microbiële groei werd het gehalte cyanogeen glucoside teruggebracht tot 18,6% ($\pm$11,7) van de beginwaarde, hetgeen significant lager is dan zonder beënting. Van de geteste stammen verlaagde *N. sitophila* het cyanogeen glucoside gehalte het meest, gevolgd door *R. stolonifer* en *R. oryzae*. De met *Rhizopus* spp. begroeide cassave had de grootste proportie van het overgebleven cyanogeen potentieel in de cyanohydrine vorm, gevolgd door de *Bacillus* sp. De melen hadden gehaltes cyanogenen die evenredig verlaagd waren ten opzichte van de gefermenteerde wortels waarvan ze gemaakt waren. De verlaging van het gehalte aan cyanogene glucosiden was significant gecorreleerd (r=0,86) met de mate van structuurverlies van de wortel.

In gedefinieerde voedingsbodems waren diverse bacteriën en schimmels in staat HCN uit linamarine vrij te maken. Een *Bacillus* sp. verminderde het linamarine tot 1% van de beginconcentratie, *M. racemosus* tot 7%, *R. oryzae* en *R. stolonifer* tot 30%, terwijl *N. sitophila* en *G. candidum* nauwelijks linamarine afbraken binnen 72 uur. Het toevoegen van pectolytische en cellulolytische enzymen, maar niet linamarase, aan stukken wortel onder aseptische omstandigheden, leidde tot struktuurverlies van de wortel en significant lagere cyanogeen glucoside gehaltes. *N. sitophila* vertoonde geen linamarase activiteit, in tegenstelling tot *M. racemosus*

en *Bacillus* sp., die minder effectief waren in het zacht maken van wortels en het verlagen van het cyanogeen glucoside gehalte. De belangrijkste bijdrage van microorganismen aan het verwijderen van cyanogene glucosiden tijdens vaste-stof fermentatie is daarom hun celwand-afbrekende activiteit die het contact verhoogt tussen het endogene linamarase en cyanogeen glucoside.

Conclusies

Bij de produktie van cassavemeel door middel van zon-drogen, kan de effectiviteit van de verwijdering van cyanogene stoffen vergroot worden door aanvankelijk de droog-snelheid te verlagen, gevolgd door een intensieve droging. Microbiëele uitgroei moet daarbij onder controle gehouden worden.

De verwijdering van cyanogene stoffen door het proces van vaste-stof fermentatie is effectiever dan door uitsluitend zon-droging. Hierbij is geen nieuw opvallend gezondheidsrisico gevonden. Daarom kan het proces beschouwd worden als een verbetering ten opzichte van alleen zon-drogen, voor cassavewortels met hoge concentraties cyanogene glucosiden. Om een hygiënischer produkt zonder mycotoxinen en met onschadelijke gehaltes aan cyanogene stoffen te bewerkstelligen, wordt optimalisatie van het proces aanbevolen, inclusief het toepassen van starter culturen van de effectieve 'food grade' schimmels *Neurospora sitophila* en *Rhizopus oryzae*.

ACKNOWLEDGEMENTS

I wish to express my personal thanks to all the many people who and institutions that have contributed to this research. Firstly, I thank my parents, Pieter Essers and Hetty Avink, for having provided me with an education that made such a research possible.

I am grateful to the peasants in Nampula province in Mozambique who participated in the research with enthusiasm and to the Nutrition Section of the Ministry of Health of Mozambique, which allowed me to work on this subject. I appreciate the present Head, Lourdes Fidalgo, who, genuinely engaged with improving people's nutrition, urged me to return with the results to the field. It was a rewarding duty.

Although I had been studying the issue of preventing toxic effects from cassava in Mozambique, it was a challenge to restart the research in a new setting after some years and to build new institutional linkages from scratch. Hans Rosling, Frits van der Haar, Louise Fresco, Bertus Haverkort, Gerard de Bruijn, Louk Box and Sonja Vlaar inspired and encouraged me to re-initiate this research. I thank them for their moral support and good advice.

At Wageningen Agricultural University: Many thanks to Jan Koeman, professor of toxicology, for drawing my attention to cassava toxicity and the value of indigenous processing early on in my studies, and to professor Walter Pilnik for inviting me to the Department of Food Science with this research. I thank the Head of Department, my supervisor Fons Voragen, for giving me free rein, enormous confidence and many-sided support to carry out this research, and, together with other colleagues of the Department of Food Science, for fruitful discussions; Robert Nout for sharing his microbiological expertise and research experience in an amicable way, he was as a a co-supervisor to me; Martin van Montfort for his patience while giving advice on statistics; Eric van Munster who spent many days with me on the bookkeeping of project funds, and the colleagues at the chemical store who always had to deal with someone in a hurry on his way to Africa. I highly appreciate Margaret Bosveld for thorough laboratory work, being a loyal colleague and for giving unceasing positive support to the students and me. The students Marjon Bennik, Jane Caffe, Anneke van Drongelen, Lucy Elburg, Franceska Erdelmann, Remco van der Grift, Nancy Jansen, Jurgen Jansen, Karien Jurgens, Nancy van der Kley, Irene Lobbezoo, Annemieke Schoenmaker, Nico

MOTHER CASSAVA

Cassava, you are great.
Cassava, you are good to us.
Cassava, it's you who feeds us.
In difficult conditions, when maize does not yield,
in poor soils, you, cassava, still yield.
During drought, you, cassava, still feed us.
Your roots give us energy,
your leaves strengthen our body.

Sometimes, cassava, you turn bitter,
as not everyone is always in a good mood.
When you are irritated by drought or poor soils,
which means that you are lacking water and food,
you turn bitter. Who would not do so?
Some types are always bitter,
like the persons with a suspicious mind ...
They are not very inviting.
You must have your reasons.

This bitter cassava affects us with her bitterness,
it seems as if she wants to poison us!
Sometimes, you bitter cassava, you have brought us illness.
When we were hungry and you, only you had remained in the field,
you provoked vomiting and konzo in some of us,
because we did not have the patience to make you gentle.

With your bitter words you warned us,
that you need time before being consumed:
Soaking in water for days until soft,
or becoming soft by moulds, before being dried.
That is how we made you show your friendly face.
It has served us well, when we had time,
and so it will serve us well, if we have time.

But when there is hunger at the door, and you, only you are in the field ...?
Now we know how we will use you then:
We are not going to wait until you are soft,
because we want to eat the same day,
yes, on the same day!
We get rid of your poison by grating your roots,
squeeze out your bitter juice, and roast the crumbs.
This way, cassava, even if you were so bitter,
you have turned delicious.
The poison has left and we can use you with soup or relish.
Now, Mummy cassava, now that we know your secret,
you will serve us even better.

Sander Essers (original in Portuguese, Nampula, Mozambique 29/7/94)

MAMÃ MANDIOCA

Mandioca, és grande.
Mandioca, és boa para nós.
Mandioca, és tu que nos alimenta.
Em condições difícis, quando o milho não rende,
com solos pobres, tu, mandioca, ainda produzes.
Quando temos seca, tu, mandioca, ainda nos alimentas.
As tuas raízes dão-nos energia,
a tua mathapa faz o nosso corpo forte.

Às vezes, mandioca, te tornas amarga,
como nem todas as pessoas sempre andam bem dispostas.
Quando estás irritada por seca ou por solos pobres,
então, quando te falta bebida e comida,
te tornas amarga. Quem não faria isso?
Alguns tipos sempre estão amargos,
como as pessoas que sempre andam desconfiadas ...
Não permitem facilmente a sua aproximação.
Deves ter as tuas razões.

Esta mandioca amarga está a afectar-nos com a sua amargura,
parece que quer-nos envenenar!
Às vezes, tu mandioca amarga, tens nos trazido doenças.
Quando ficamos com fome em casa, e tu, só tu estavas na machamba,
deixaste-nos com vómitos e algúns de nós com konzo,
porque não tivemos a paciência para te tornar manso.

Com as tuas palavras amargas, tu nos avisaste,
que precisas de tempo antes de ser comida:
Posta de molho, até que fiques mole,
ou que fiques mole por bolores, antes de te secar.
Isto foi a maneira para mostrar-nos a tua cara doce.
Isso serviu-nos bem, quando tivemos tempo,
pois, servirá bem, quando temos tempo.

Mas quando há fome no lar, e tu, só tu estás na machamba ...
agora já sabemos como te aproveitar:
Não vamos esperar até que fiques mole,
porque queremos comer no mesmo dia,
sím, no mesmo dia.
Acabamos com teu veneno por ralar as tuas raízes,
espremer o teu suco amargo, e depois torrar.
Assím, mandioca, mesmo se fores tão amarga,
o teu aspecto já torna-se delicioso.
O veneno saiú e podemos acompanhar-te com mathapa ou caril.
Assím, mamã mandioca, como agora sabemos o teu segredo,
assím vais servir-nos ainda melhor.

Sander Essers, Nampula, 29/7/94 (inspirado por Florence Nwapa)

CURRICULUM VITAE

Adrian Jan Alexander ("Sander") Essers was born on September 26th, 1950 in Den Haag in The Netherlands. From 1957 to 1961 he lived in Surinam. In 1969 he graduated at the Rembrandt Lyceum in Leiden, after which he began studying Technical Physics in Delft. In 1971 he went to Wageningen Agricultural University to pursue a study in Human Nutrition. Before obtaining his academic degree in 1980, he did extended research training in the Peruvian highlands and held a post at the International Agricultural Centre in Wageningen on the International Course in Food Science and Nutrition. In 1980 he started to work for the Ministry of Health in Mozambique, mainly in public health and rural development. This multi-task assignment contained participatory research on cassava processing with a rural community suffering from cassava-cyanide intoxications. From 1987 to 1989 he worked for the Information Centre for Sustainable and Low-input Agriculture of the ETC Foundation in Leusden. From 1989 onward, he initiated and managed the research project 'Detoxification of cassava at household level in rural Africa', based at the Department of Food Science of Wageningen Agricultural University with branches in Uganda and Malawi. This thesis is a result of that research.

Presently, Sander Essers works at the Department of Toxicology at the same University on principles of risk assessment of naturally occurring toxins in plant foods.

PROPOSITIONS / STELLINGEN

1   The crop cassava is of great importance; its toxicity problem is relatively small but not to be neglected. (this thesis)

2   Removing cassava **cyanogens** after harvesting may be more practical and beneficial than eliminating the expression of cassava **cyano-genes** before cultivation. (this thesis)

3   Dissemination of grating practices for cassava processing may have a greater effect on public health than increasing the linamarase levels in the cassava plant. (this thesis)

4   The FAO/WHO norm (1991) for cyanogen levels in cassava products is unrealistic for many communities: Strict application of the norm would imply the rejection of most cassava products for human consumption in Africa, thereby creating a famine of unprecedented size. (this thesis)

5   Food safety and food security may be conflicting goals if food processing is ignored. (this thesis)

6   Half a kilogram of the cassava roots which are commercially available in the Netherlands can potentially release a lethal dose of hydrogen cyanide, if eaten raw. With the increasing popularity of tropical foods and the unacquaintance with cassava, a limit should be set on cyanogen levels in marketed roots and information provided that they are not to be eaten raw. (this thesis)

7   The proud statement of going to the field with an open mind, when studying people of another culture, often implies ignorance of one's own biasses.

8   The enormous gap between local and international salaries is a major cause for the lack of sustainability of development projects.

9   If you want to know how things really are, try to change them. (K. Lewin)

10 Slumber is like an ecological wetland of the mind: Copies of words and images are decomposed and the elements rearranged, bringing to life new insights.

11 A researcher should mark the "scientific dead-end streets" he/she encounters during experimental work, not as a mere tribute to the science philosopher Karl Popper, but to prevent other scientists from wasting time on those options.

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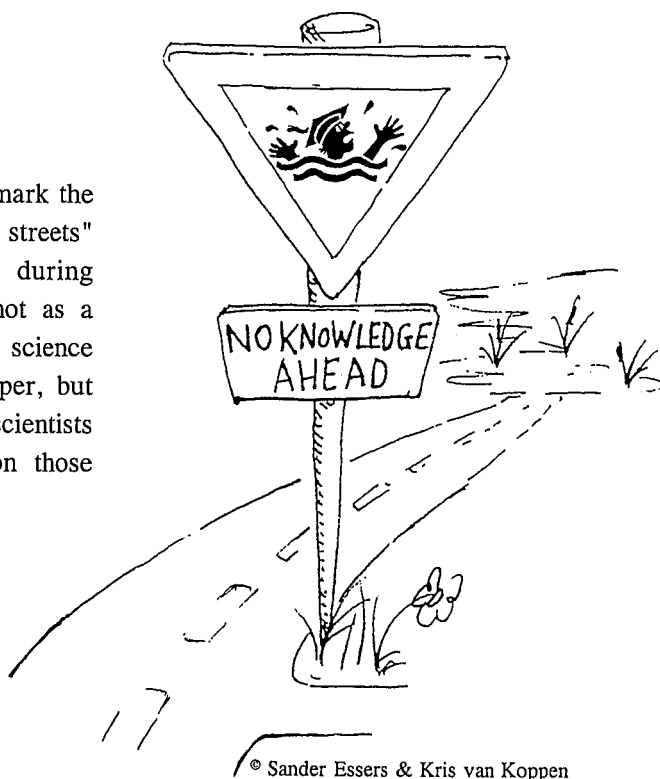
12 Advice from supervisors and co-authors from different disciplines to leave out 'irrelevant' information from a draft publication is more often complementary than overlapping, leaving the obedient Ph.D.-candidate with nothing to publish.

13 The crop cassava is nutritious: I thrived even by studying it.

Propositions belonging to the thesis
REMOVAL OF CYANOGENS FROM CASSAVA ROOTS
Studies on domestic sun-drying and solid-substrate fermentation in rural Africa
by Sander Essers, Wageningen, The Netherlands, 2 June 1995.