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Chronic Cassava Toxicity

Proceedings of an interdisciplinary workshop
London, England, 29-30 January 1973

Editors: Barry Nestel and Reginald MacIntyre

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Contents

Assay Methods for Hydrocyanic Acid in Plant Tissues and their Application in Studies of Cyanogenic Glycosides in *Manihot esculenta*

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Abstract A survey of the methodology of cyanide assay is presented with particular reference to the determination of linamarin, the cyanogenic glucoside of cassava, *Manihot esculenta* Crantz, and some of the problems in obtaining reliable estimates and reproducible data on the potential cyanide yield.

The measurement of potential cyanide output from plant tissues is a convenient method for medical and toxicological studies as it represents an index of health hazard, and therefore, the actual glucoside content receives little attention even in agronomic studies. Linamarin is unusual in that it is not readily hydrolyzed by acid and therefore endogenous or added linamarase must be employed in the release of cyanide. Since the activity of this enzyme in cassava tissues was only recently elucidated, many of the earlier reports on cyanide yield from these tissues are of dubious value.

The peculiarities of the cyanide assay are reviewed in respect to the principal phases of analytical procedure, namely, the release of cyanide from the glucoside, the isolation or recovery of cyanide, and finally, its analytical determination. Because of the reactivity of cyanide ion, its volatility, and the lengthy incubation for enzymic hydrolysis, the crucial point of a reproducible technique is the total release and isolation of cyanide from the substrate and prevention of losses due to the secondary reactions or to the escape of cyanide from analytical train. It is unfortunate that few research papers have concerned themselves with the reproducibility of given methods and their analytical data, and in particular, with the recovery of cyanide added to plant tissue homogenates. The problems also discussed are the errors arising from sampling bulky plant materials, such as cassava roots, and their preparation for analysis, the two aspects of analytical work which in the past have received little attention or are only superficially covered in published reports.

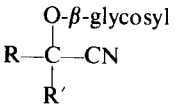
Résumé L'auteur fait la revue des diverses méthodes d'analyse du cyanure, en particulier de la linamarine, le glucoside cyanogène du manioc, *Manihot esculenta* Crantz. Il souligne quelques-unes des difficultés à obtenir des estimés fiables et des données reproductibles sur le rendement potentiel en cyanure.

En médecine et en toxicologie, la détermination de la production potentielle de cyanure par les tissus végétaux est une méthode commode, parce qu'elle fournit un indice du hasard à la santé. Ceci explique le peu d'attention portée à la teneur réelle du glucoside, même dans des études agronomiques. La linamarine est exceptionnelle en ceci qu'elle n'est pas aisément hydrolysée par un acide. On doit donc utiliser de la linamarine endogène ou ajoutée pour libérer le cyanure. Comme l'activité de cet enzyme n'a été élucidée que récemment, plusieurs publications antérieures sur le rendement en cyanure de ces tissus ont une valeur douteuse.

L'auteur examine les aspects particuliers du dosage du cyanure en rapport avec les principales étapes du protocole analytique, à savoir, libération du cyanure du glucoside, séparation et récupération du cyanure et, finalement, son dosage analytique. A cause de la réactivité de l'ion cyanure, de sa volatilité et de la longue période d'incubation nécessaire à l'hydrolyse enzymatique, le point crucial de tout processus reproductible est la libération et la séparation totales du cyanure du substrat, et la prévention des pertes dues aux réactions secondaires ou à l'échappement du cyanure dans le train d'appareils analytiques. Il est regrettable que si peu de publications scientifiques se soient préoccupées de la répétabilité des méthodes utilisées et des données analytiques obtenues à l'aide de ces méthodes, en particulier, de la récupération du cyanure ajouté aux homogénats de tissus végétaux. On discute également des problèmes soulevés par l'échantillonnage de matériel végétal volumineux, tel les racines de manioc, et de sa préparation en vue de l'analyse, deux aspects du protocole analytique qui ont reçu peu d'attention ou ne sont que brièvement couverts dans les publications scientifiques.

CYANOGENIC glycosides occur in a great variety of plant species belonging mainly to the Rosaceae and Leguminoseae families, although there is no discernible pattern in their taxonomic distribution throughout the plant kingdom or within an individual species or plant. High levels of cyanogens may be encountered in one particular tissue of a plant while lacking in the same locus in another plant. The presence of the cyanogens in plant foods and forages is of great concern owing to the release of toxic hydrocyanic acid (HCN) through hydrolysis either by acid within the digestive tract or by endogenous enzymes in damaged or disrupted tissues during crop harvest and food preparation.

With a few exceptions, cyanogens in plant tissues are nitriles bound in glycosidic form with aromatic or aliphatic aglycones and possessing a general formula:



Some of the typical cyanogenic glycosides are illustrated in Table 1 (Robinson 1963).

In damaged, disorganized plant tissues, HCN is freed from the glycosides by the action of one or more enzymes which are not necessarily present or active in the same cells or tissues. The sweet almond kernels, for instance, contain the enzymes (emulsin) but not the glycoside. This leaves some hope for the plant breeders in their quest for zero-cyanide or zero-glycoside levels in economic plants as a possible means of eliminating the poisonous attributes of these plants in selected clones or cultivars.

Measurement of cyanide output is of such paramount medical and pharmacological significance that few research papers refer to the actual glucoside content but rather are concerned with the potential yield of cyanide as an index of health hazard.

The primary purpose of this paper is to summarize and evaluate available methodology of cyanide assay in plant tissues, and in particular, to focus on their reliability and some of the problems of sampling and sample preparation. Recent developments in enzymology and instrumentation leave some doubts regarding earlier analytical data on potential cyanide yields. This is particularly true for cassava *Manihot esculenta* Crantz, in which the significance of β-linamarase has been only recently clarified (Butler et al. 1965; Wood 1966).

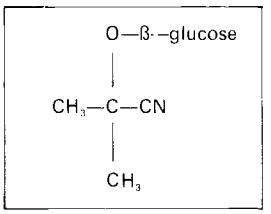
Methods of Cyanide Analysis in Plant Tissues

Direct assay of cyanogenic glycosides, although not unfeasible, imposes physical limitations and would be too tedious and unreliable to be of any practical value. Because of the medical significance of cyanide, and the relative ease of its detection and determination, it is not surprising that most research data are reported in terms of potential cyanide yields rather than the glycoside content itself. Gas chromatography was used by Bissett et al. (1969) to quantitate linamarin and lotaustralin as trimethylsilyl derivatives, while Butler (1965) estimated the same compounds indirectly on the basis of glucose liberated by β-linamarase. Most assays, however, are based on the determination of HCN liberated by acid or enzyme hydrolysis. One of the inconveniences of this assay is the volatility of HCN (bp 26°C) which necessitates working in a closed analytical train.

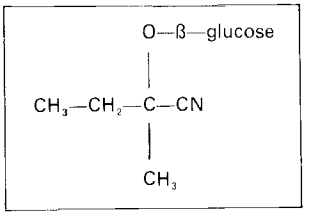
The assays of cyanogens bound with aromatic aglycone components are less difficult to perform

TABLE 1. Common cyanogenic glucosides and their hydrolysis products (McIlroy 1951; Robinson 1963).

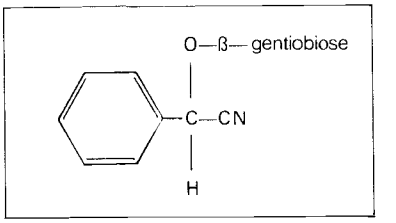
Glucoside	Sugar	Aglycone
Amygdalin	Gentiobiose	Mandelonitrile
Dhurrin	Glucose	*p*-Oxymandelonitrile
Linamarin	Glucose	2-Hydroxy isobutyronitrile
Lotaustralin	Glucose	2-Hydroxy-2-methyl butyronitrile
Prunasin	Glucose	Mandelonitrile



LINAMARIN



LOTAUSTRALIN



AMYGDALIN

TABLE 2. Cyanide detection limits of various methods.

Reaction	Source	Detection limit	λ	Procedure
Silver nitrate	AOAC 1965	0.54–1.08 mg/ml		titration
Picric acid	Snell and Snell 1959	5–50 μg	530 nm	colorimetry
Picric acid	Guignard 1906	30–50 μg/g		qualitative
p-Benzoquinone	Guilbault and Kramer 1965	0.6–150 μg/ml	400, 480 nm	fluorometry
Pyridoxal	Takanashi and Tamura 1970	0.026–1.3 μg/ml	356, 432 nm	fluorometry
p-Chloramine	Pulss 1962	0.01–1.0 μg/ml	582 nm	colorimetry
Picric acid	Gilchrist et al. 1967	5–50 μg/g	515 nm	eluate colorimetry

as they are easily hydrolyzed by acid or exogenous β-glucosidases which are readily available from commercial sources (assay of dhurrin: Gillingham et al. 1969). The assays of linamarins (Table 1) in flax and cassava are rendered more difficult owing to the apparent requirement of a specific enzyme, β-linamarase, which is not readily available and is particularly needed for hydrolysis of prepared or processed food materials (Wood 1965, 1966).

Early work on cyanogens stimulated by acute and sometimes lethal toxicity cases (Montgomery 1969) placed fewer demands on cyanide detection limits since it was sufficient to determine potentially toxic cyanide levels. Recent concern about chronic toxicity through continuous intake of low cyanide foods and forages, and the necessity to study the physiological effects of such intake, stimulated the development of procedures which would allow detection and quantitation of trace amounts of cyanide. Some of the detection limits and ranges for various techniques are summarized in Table 2.

There are three distinct steps in the HCN assay: 1) liberation of cyanide through acid or enzymatic hydrolysis; 2) extraction and isolation of the cyanide from the substrate; and, 3) determination of the isolated cyanide.

Since recent research efforts have provided sufficient, though not fully satisfactory, knowledge about cyanogen hydrolysis (Butler et al. 1965; Wood 1966), the isolation and recovery of cyanide from the digest present the greatest difficulty in obtaining reliable and reproducible data. The assay of isolated cyanide presents the least difficulty since most of the available techniques are reasonably well calibrated and tested for reproducibility (Smith 1929; Asmus and Garschagen 1953; Guilbault and Kramer 1965; Gilchrist et al. 1967).

Also, the manner of selecting a representative sample, sample preparation, and handling creates additional problems in obtaining reproducible data. This is particularly true of bulky cassava roots in which concentration gradients exist in

both horizontal and radial directions (de Bruijn 1971).

Qualitative Tests for Cyanogens

The classical Guignard test (1906) is based on the reaction of alkaline picrate paper with HCN liberated in a closed test tube by spontaneous enzymic hydrolysis of crushed plant material. Cyanide can be detected at 30–50 μg/g concentration. This test has been adapted for quantitation by Boyd et al. (1938), more recently calibrated for colorimetry in the 5–50 μg/extract range by Gilchrist et al. (1967), and proposed for routine cyanogen tests in cassava by Indira and Sinha (1969). Other workable qualitative tests include the ferric ferrocyanide test (Berlin blue) and the benzidine–copper acetate test (Feigl 1960) which according to Wood (1966) detects as little as 0.4 μg of HCN.

Liberation of Cyanide from Cyanogenic Glucosides

Most of the early methods relied on acid hydrolysis or spontaneous autolysis by enzymes contained in ruptured plant tissues, neither of which appears to give a "total potential yield of cyanide" (Winkler 1951). With acid hydrolysis discrepancies occur due to the formation of amides and ammonia while in spontaneous autolysis the activity of enzymes is no doubt influenced by the conditions prevailing in the heterogeneous plant substrate, the accompanying secondary reactions, and the accumulation of products of hydrolysis. Pulss (1962) attributed the incomplete recovery of cyanide from flax and clover to the secondary binding of cyanide in the digest especially when older necrotic tissues were assayed. Wood (1966) claimed a recovery of at least 87% of cyanide present in the glucoside of cassava when extraneous enzyme was used for hydrolysis. On the other hand, Montgomery (1964) found a 3-h acid hydrolysis at 100°C satisfactory for tropical pulses, and de Bruijn (1971) doubted the necessity of using an enzyme preparation and employed a 24-h spontaneous hydrolysis in cassava macerated to liberate the cyanide.

To minimize these interferences and achieve a near ideal field of potential cyanide, it appears desirable to determine individually for each kind of plant tissue the ideal sample size and duration of autolysis which would not interfere with the action of endogenous or exogenous enzymes to approach a first-order enzyme reaction. This could possibly be tested by a two-step hydrolysis: first an endogenous autolysis followed by a second hydrolysis with added enzyme after the products of first action have been removed by distillation. Ideally, the adequacy of cyanide liberation should be tested by the recovery of added linamarin instead of measuring cyanide recovery added to the substrate as carried out by Pulss (1962).

It is likely that cyanide is quantitatively released from the glucosides of the mandelonitrile type by acid or a combination of acid–enzyme hydrolysis. This does not seem to be true of the linamarins as Wood (1968) claimed that acid does not hydrolyze but produces artifacts, and its only purpose is to expel the HCN from the substrate. In view of the specificity of β-linamarase (Butler et al. 1965; Coop 1940) and the uneven distribution of linamarin in the cassava root (de Bruijn 1971), there is a need for additional study of factors and conditions determining the complete recovery of cyanide from the glucoside. Winkler (1951) pointed out the variability of hydrolytic power of endogenous enzymes and this can be expected in various cassava tissues subjected to glucoside assay especially when different clones are being studied. Hughes (1969) reported differences in β-glycosidase activity in four different clones of white clover. de Bruijn (1971) observed that in low-glucoside clones a 3-h maceration is sufficient whereas in high cyanide cassava clones a 9–12-h maceration is required for autolysis. One wonders whether this may be due to differences in enzyme activity and/or its impairment by products of hydrolysis. Although the conditions and rates of β-linamarase were thoroughly investigated in model systems, without actual testing of plant homogenates, one can only infer and hope that the action is quantitatively similar in the heterogeneous plant substrate undergoing autolysis.

The endogenous enzyme autolysis may be justified in analyzing the outer peel of cassava roots which was used by Wood (1966) to obtain a crude β-linamarase preparation. With cyanide yield from the peeled tuber often below 10–20-μg/g levels, the use of added enzymes seems more than justified since the enzyme activity in that tissue has not been studied.

The duration of autolysis or hydrolysis by added enzyme also suggests a critical point for recovery as there are variations between and

within plant species. In clover, 4–6 h autolysis, and in flax 8–12 h autolysis, were found satisfactory by Pulss (1962); de Bruijn (1971) employed for cassava a 24-h interval for maximum cyanide yields.

Thus the duration of autolysis, the activity of endogenous enzymes in tested plant tissue, and the efficiency of added enzyme merit closer investigation in order to arrive at an ideal method of liberating quantitatively the glucosidic cyanide.

Extraction and Isolation of Cyanide from Substrate

Cyanide recovery from plant substrate is normally carried out by aspiration with air, nitrogen, or water vapour, and trapping of cyanide in alkaline solution. Critical factors include timing, temperature, volume of substrate, rate of aspiration, sample size, ionic strength, and concentration of solutes in the substrate. The influence of cyanide binding in the substrate has already been mentioned.

According to Wood (1966) the addition of acid merely assists in expelling the cyanide from the substrate. Pulss (1962) found optimum recovery from a mild acid condition (*p*H 5.0) while de Bruijn (1971) and the AOAC method (1965) employ steam distillation without acidification.

Working with flax and white clover, Pulss (1962) carried out a meticulous study on cyanide retrieval from plant substrate including sample size, aspiration, acidity, and interfering substances. Although cyanide is readily and quantitatively recovered from a solution, when added to a clover substrate, as low as 20–30% was recovered with air and 60–80% with nitrogen flushing. A 100% recovery of added cyanide was obtained from clover ash indicating the interference of organic plant substances and oxidative reactions. This clearly underlines the inadequacy of air aspiration and the difficulties in retrieving cyanide from a plant macerate. Reduction of sample size improved the recovery, no doubt due to the dilution of interfering substances. The binding of added nitrogen was particularly pronounced in older, partly necrotic plant material. Recent work at Guelph (Zitnak 1972 unpublished data) indicates a similar binding (10–15%) in acidified cassava substrate with steam distillation. When using a mild acid condition (*p*H 5.0) as recommended by Pulss (1962), these losses are negligible.

With cassava, the most comprehensive study on various conditions influencing analytical data was carried out by de Bruijn (1971). Particular regard was given to sample representation and preservation, time and temperature of autolysis, and sample size for various cassava tissues using steam distillation. In spite of the fact that cyanide recovery was tested with cyanide solution rather than plant macerate, the report provides sound information for future research. It is unfortunate that the cyanide recovery was tested with cyanide solution rather than with plant substrate.

The interferences of organic plant substances occur, according to Pulss (1962), more often with the distillation procedure than with aeration of clover material, however, this is overcome by using the colorimetric method of Asmus and Garschagen (1953) instead of the usual silver nitrate titration. The latter overestimates cyanide yield in clover by 8–14%. Regardless of the accuracy and sensitivity of cyanide determination methods, it is quite clear from the foregoing remarks that the accuracy and reliability of plant assays is primarily dependent on adequate evaluation of the cyanide recovery procedure and its reproducibility, the weight of analytical sample, and the kind of plant tissue to be assayed. It appears quite necessary to perform recovery tests with individual plant species, and even different tissues of the same plant, in relation to their physiological state.

Among recent studies on variability of cyanide yields in cassava, Sinha and Nair (1968) gave no indication concerning sampling, replication, reproducibility of tests, or even the source of assay method that would allow the reader to pinpoint the reliability of the data presented. Similarly, Indira and Sinha (1969) report on a rapid cyanide determination based on the colorimetry of picrate paper reaction (Gilchrist et al. 1967) without an attempt to assess the cyanide recovery from plant substrate. This otherwise simple method appears to have a great potential for routine agronomic studies provided that a method is calibrated with plant substrate tests. There is an obvious need to obtain such data for cassava tissues for this or any other method attempting to determine the potential yield of cyanide as truly representative of the glucoside content. Only in this way can one make a sound comparison of research data from different geographical regions. In cassava, only Wood (1966) attempted to appraise his assay procedure in terms of glucoside content and linamarase

activity. For practical agronomic segregation of high–low cyanide clones, such accuracy may be of lesser concern, however, for the sake of physiological or toxicological studies. It is essential that even in such investigations one should aim for the potential cyanide yield (Winkler 1951) as clearly representative of the glucoside content.

Determination of Cyanide

Numerous quantitative methods, differing in sensitivity and detection limits, have been employed in determining the isolated glucosidic cyanide. Since it is beyond the scope of this paper to encompass them in number and in detail, a few general remarks will be made on their applicability in cassava studies. Some indication of sensitivity limits is given in Table 2.

(*a*) Titration with acid or alkaline *silver nitrate* is a well-known standard procedure (AOAC, 1965), suitable for macro determinations (0.5–1.0 mg/ml). Its main disadvantage is in introducing errors through secondary reactions (Pulss 1962) and difficulties in obtaining clear end-points for some plant tissues.

(*b*) Colorimetry with *picric acid* (Snell and Snell 1959): The reaction detects cyanide in the 5–50-μg range, and although nonspecific due to the interference of other substances, it is applicable to aspiration or distillation extracts of plant tissues. A novel adaptation of this method is the colorimetry of picrate paper eluate as modified and calibrated by Gilchrist et al. (1967). Liberated cyanide reacts with a constant size of saturated alkaline picrate paper and the reaction products are eluted and measured colorimetrically at 515 nm. This test, developed for testing *Sorghum* spp. dhurrin content, was also used by Indira and Sinha (1969) for cassava root and leaf material. Although Indira and Sinha presented only meagre data concerning the variance and reproducibility of measurements, this method can be developed into a useful agronomic routine test. It still needs to be amended to determine cyanide recovery from plant substrate, since Gilchrist et al. (1967) introduced "paper recovery" standards using solely acid-hydrolyzed cyanide solutions for calibration for the assay of dhurrin.

(*c*) Potentiometric measurement with *cyanide-sensing electrode*: A cyanide ion detection electrode with a claim of 15% accuracy (Orion Research Inc. 1967) can detect CN ions in the 10^{-6} to 10^{-2} M range (0.026–260 μg/ml). This electrode, which has a multitude of industrial applications, was evaluated for two plant cyanogens, namely in cigarette smoke and in *Sorghum* forage (dhurrin). The method for *Sorghum* (Gillingham et al., 1969) was reported to have a ± 0.956 correlation with picric acid colorimetry of cyanide retrieved by aspiration, unfortunately with an overestimate of 40%. However, Orion Research Inc. have provided a regression line which allows more accurate calculations of the cyanide content. This method merits further evaluation for cassava plant material primarily because of its wide detection range, provided that the endogenous β-linamarase activity in individual tissues is sufficient for quantitative release of cyanide (the original method employed exogenous β-glucosidase) and that all cyanide can be retrieved from the plant substrate.

(*d*) Two *fluorometric* methods have been developed to date and both should be applicable for measurement of cyanide in biological fluids since both of them are based on specific cyanide reactions.

Reaction with *pyridoxal* (Takanashi and Tamura 1970) produces a highly fluorescent compound ($\lambda_{ex} = 356$ nm, $\lambda_{em} = 432$ nm) allowing measurements in the 10^{-6} to 10^{-5} M (0.026–1.3 μg/ml) range. This appears to be a suitable method for micro-scale study of linamarin distribution in the cassava tuber.

Reaction with *p-benzoquinone* (Guilbault and Kramer 1965) in dimethylsulphoxide ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 480$ nm) has a wide range, 0.6–150 μg/ml and is suitable for a glass filter fluorometer for routine testing. The disadvantage is the nonaqueous medium, limit of the aqueous sample aliquot to 0.1 ml, and the need to readjust it to a *p*H 7.5 before the reaction. This method is currently being investigated at the University of Guelph with moderate success.

(*e*) A direct cyanide *gas chromatography* is also available but untested on cassava material (Woolmington 1961), although paper and thin-layer and column chromatography were extensively used in the last decade for identification, procedure testing, and plant extract examinations (Bissett et al. 1969; Butler and Conn 1964; Clapp et al. 1966; Wood 1965). Butler (1965) used paper chromatography for indirect assay of linamarin and lotaustralin. These methods require sophisticated laboratory instrumentation and possibilities for routine testing are rather limited. There is, however, a possibility of developing paper or thin-layer chromatography for a densitometric direct assay of the glucosides which is yet to be investigated.

(*f*) Perhaps the *colorimetric* procedure of Asmus and Garschagen (1953) used by Pulss (1962) is worthy of testing on cassava plant material. It is based on cyanide reaction with *p*-chloramine and barbituric acid and is very sensitive, with detection limits of 0.01–1.0 μg/ml at 582 nm. As mentioned earlier, the method eliminates some interferences normally occurring with steam distillation and silver nitrate titration.

In considering the reviewed methods, the accuracy, detection limits, and reproducibility with cyanide solutions are fairly well established and the main factor in considering their use is the ease of manipulation and technical difficulties. The

matters of most serious concern are, as emphasized earlier, the liberation of cyanide from glucosides contained in the plant substrate and the retrieval of the cyanide after its liberation.

Problems in Sampling and Sample Preparation

One can not omit from this review a few pertinent remarks on sampling and preparation procedures which in the end are the determinants of reliable data. Too often the sample methodology is treated superficially and much of the accumulated experiences finds little favour and acceptance on the part of editors of research papers unless the methodology is the prime objective of the research. Sample size or representation and replication are too often sacrificed to speed and quick acquisition of data. Papers by Sinha and Nair (1968) and Indira and Sinha (1969) are greatly lacking in this respect as contrasted with de Bruijn's (1971) thorough investigation of analytical conditions and sample materials.

A representative sample for bulky cassava roots is difficult to obtain without homogenization of kilogram quantities of material as done by de Bruijn (1971) who for final analysis used 27-g homogenate samples. In contrast, Wood (1965) used only 4–12-g samples for homogenization and a 1-g sample for the assay proper. There is indeed an urgency to investigate this matter more thoroughly and perhaps the sensible way would be mapping of linamarin distribution within the peeled root in order to arrive at a reproducible method of obtaining a smaller but representative tissue sample. If a uniform sampling procedure could be developed, and approved by common agreement, including as well a standard procedure for liberation and isolation of the cyanide from cassava material, one would have a greater faith in the data reported by various research workers. de Bruijn's (1971) work provides ample directions for sample preparation and preservation.

Perhaps the only other serious concern is whether endogenous β-linamarase in peeled root tissues is sufficiently active to release quantitatively the cyanide from the glucoside. The use of added β-linamarase appears imperative for dried or processed cassava products such as gari or konkonte flour (Wood 1966). Since the enzyme is not available commercially the preparation of β-linamarase according to Coop (1940) might be too cumbersome for obtaining a continuous supply. Present work at Guelph (Zitnak 1972 unpublished data) concerns developing a simplified procedure using 5-day-old flax seedlings to achieve this goal. This procedure is quite successful since greater quantities can be obtained than with Wood's (1966) method using cassava peel as the raw source of the enzyme.

In conclusion, I have attempted to focus attention on some of the problems to be faced by the analyst in the quest for assembling reliable assay data on the occurrence and fluctuation of cyanogens with particular regard to those of *Manihot esculenta*. Hopefully, it will stimulate action and research for the development of standard and uniform analytical procedures.

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