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SPECTROPHOTOMETRIC QUANTIFICATION OF GUIGNARD'S SODIUM PICRATE TEST

TAN SWEE LIAN and NOOR AUNI HAMIR*

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RINGKASAN

Ujian Guignard telah diubahkan kepada suatu ujian kuantiti dengan menggunakan larutan cyanide tertentu dan ukuran spektrofotometer. Suatu bandingan di antara cara ujian kuantiti dan pengelasan secara penglihatan telah dibincangkan.

INTRODUCTION

The Guignard's test for HCN released from plant tissues is a rapid qualitative method (AOAC, 1965), and is used in the routine screening of cassava genotypes for low cyanide content (BOLHUIS, 1952; MOH & ALAN, 1972). Essentially, the test involves exposing moist sodium picrate paper strips to the HCN released enzymatically from the ruptured cells of plant tissue being assayed. The paper strips change progressively from an original yellow to orange or to red, depending on the amount of cyanide produced. The colour change is then scored on a scale of 0 to 5. Although this is a useful test, it gives no estimate of the cyanide released in absolute terms, and furthermore, scoring a colour change tends to be subjective.

Attempts have previously been made to quantify the Guignard's test by colorimetric or spectrophotometric analyses, covering a range of cyanide contents from 0.0 to 50.0 (GILCHRIST, *et al.*, 1967; MITCHELL, 1974) or to 100.0 μg HCN (INDIRA & SINHA, 1969). The present article reports the construction of a standard calibration curve by adapting the Guignard's test to spectrophotometric measurements using standard cyanide solutions extending over the range of 0.0 to 400.0 μg HCN. This is to ensure adequate coverage over the cyanide contents usually encountered in fresh root samples of cassava. The quantitative method evolved in this paper is then compared with the classical qualitative one.

MATERIALS AND METHODS

1. Construction of the calibration curve

Sodium picrate paper strips were prepared in the usual manner as when used in the qualitative test for cyanide in fresh plant tissue. Filter paper strips (Whatman No. 1), measuring 1.5 cm x 10.0 cm, were dipped into a 1% w/v solution of picric acid, dried and dipped a second time in a 10% w/v solution of sodium carbonate. The strips were dried again and stored in a dark stoppered bottle until required.

A stock cyanide solution was prepared by dissolving 0.241 g KCN in 1.00 litre of distilled water. (This gave an equivalent of 0.1 mg HCN/ml solution). The stock solution was subsequently diluted to give a range of standard solutions containing 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0, 100.0, 150.0, 200.0, 250.0, 300.0, 350.0 and 400.0 μg CN^-, respectively. Distilled water was used to simulate the standard solution of 0.0 μg CN^-.

The standard solutions were dispensed into test tubes and distilled water added in sufficient quantities to make up a final volume of 8.0 ml in each case. To release the cyanide from the KCN solutions, 3.0 ml of

*Cassava Unit, Field Crops Branch, MARDI, Serdang, Selangor.

3N hydrochloric acid were added to each test tube before inserting a moist sodium picrate paper strip which was kept suspended above the level of the solution by means of a rubber bung serving also to stop up the test tube. After a period of 24 hours (fixed arbitrarily), the paper strip was removed and eluted in 10.0 ml of distilled water.

The absorbance of the resulting coloured solution was measured using an SP6–300 Spectrophotometer (from Pye Unicam) at 550 nm. The blank solution containing no cyanide was used to adjust the absorbance reading to zero. Each set of standard solutions was replicated 12 times, and a calibration curve was constructed with the means.

2. Comparison of the visual and spectrophotometric methods

Fresh root samples from 210 genotypes were used in the comparison. A medium-sized root was chosen in each case, and a slice cut midway along its length. The peel of the root was included in the slice. A finely chopped sample weighing 2 g was taken and placed in a test tube. (Preliminary test have shown that this is an adequate sample weight to produce the range of colour changes required for visual scoring). A few drops of chloroform were added (to facilitate volatilization of the HCN released) and a moist sodium picrate paper strip suspended above the sample as in the previous instance.

After a similar length of time, the colour changes of the paper strips were scored visually into the classes 0 to 5, corresponding to Bolhuis' classification (1964) of the colour reaction (see *Table 1*). The paper strips were then eluted in 10.0 ml of distilled water each, and then diluted by a factor of 2 (to render the subsequent computations equivalent to $\mu g\ CN^-$ per g fresh sample, or mg CN^- per kg fresh sample). A blank solution was included by eluting an unreacted strip in 10.0 ml of distilled water.

Readings of absorbance were recorded at 550 nm as before.

RESULTS

1. Construction of the calibration curve

A highly significant correlation ($r = 0.9970^{**}$) was obtained between the spectrophotometric readings (absorbance) and the known cyanide concentrations *(Figure 1)*. A regression curve, taking a quadratic form, fitted the data *(Table 2)* to give an equation of $y = 0.005677 + 0.002519\ x - 0.000001409\ x^2$. Standard deviations in the absorbance readings for each cyanide concentration are given in *Table 3*. Variations tended to increase at the higher cyanide concentrations.

For practical purposes, however, where the cyanide content is to be derived from the absorbance reading, the inverse equation of $y = -1.0110 + 371.4679\ x + 167.4901\ x^2$ may be used, where y = cyanide content and x = absorbance reading.

TABLE 1: VISUAL CLASSES OF SODIUM PICRATE – CYANIDE REACTION
(adapted from Bolhuis, 1954)

0	Yellow (no colour change)	Innocuous
1	Light orange	Innocuous
2	Orange	Innocuous
3	Brick red/orangy red	Moderately poisonous
4	Brownish red	Poisonous
5	Dark brownish red	Very poisonous

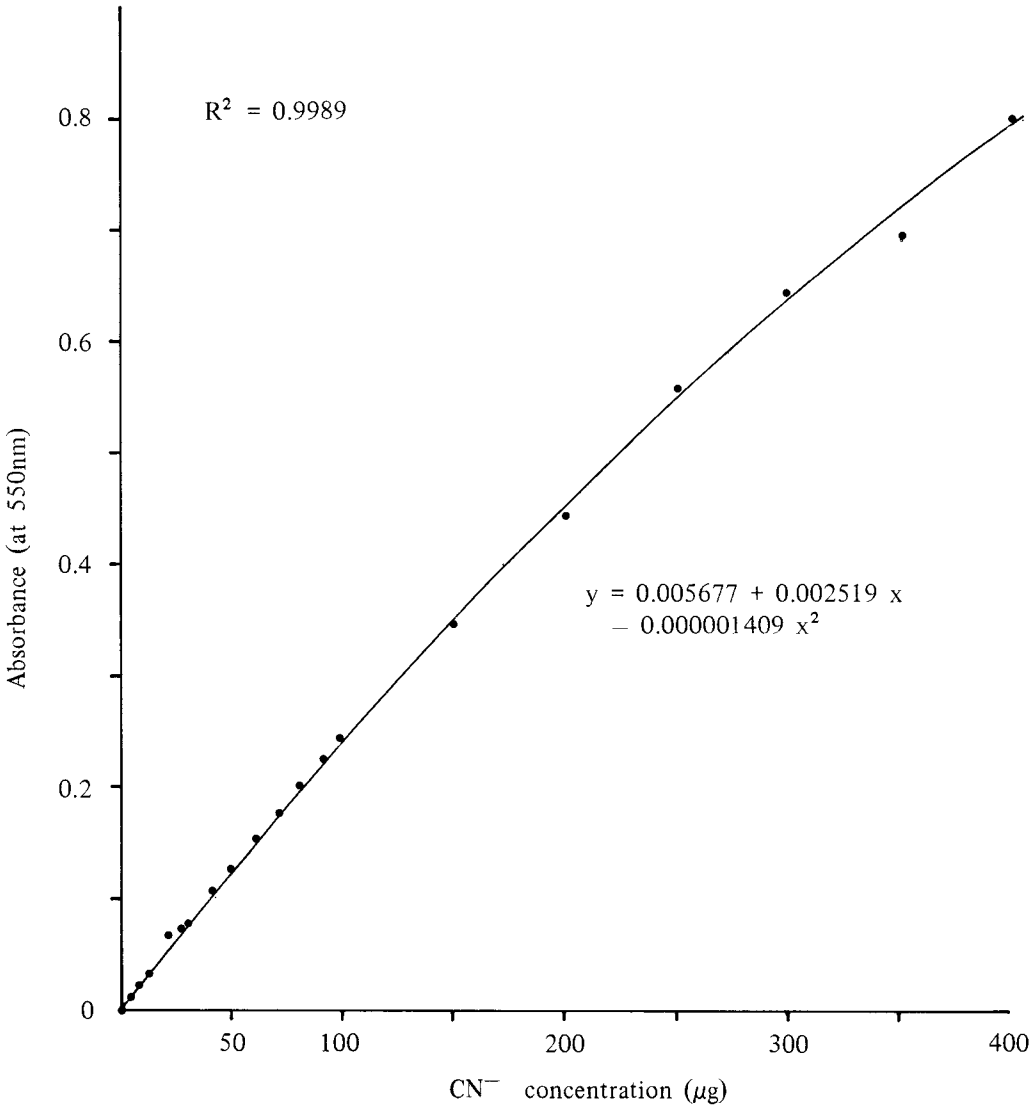

Figure 1. Standard curve for sodium picrate assay of HCN released from potassium cyanide.

TABLE 2: ANALYSIS OF VARIANCE TO FIT THE REGRESSION CURVE

Source	df	SS	MS	F
Total	19	1.1534452		
Linear regression	1	1.1464884	1.1464884	2966.42**
Deviation	18	0.0069568	3.86489×10^{-4}	
Quadratic regression	2	0.005631	2.8155×10^{-3}	36.10**
Deviation	17	0.0013258	7.7988×10^{-5}	

TABLE 3: MEANS AND STANDARD DEVIATIONS OF ABSORBANCE READINGS AT CORRESPONDING CYANIDE LEVELS OF STANDARD SOLUTIONS

CN^- content	Absorbance		CN^- content	Absorbance	
	Mean	S.D.		Mean	S.D.
0.0 µg	0.000	±0.000	70.0 µg	0.174	±0.042
5.0	0.012	0.004	80.0	0.198	0.050
10.0	0.026	0.011	90.0	0.229	0.049
15.0	0.039	0.014	100.0	0.250	0.065
20.0	0.064	0.027	150.0	0.346	0.072
25.0	0.073	0.021	200.0	0.435	0.095
30.0	0.076	0.016	250.0	0.556	0.099
40.0	0.107	0.028	300.0	0.644	0.106
50.0	0.134	0.040	350.0	0.693	0.136
60.0	0.153	0.035	400.0	0.800	0.122

2. Comparison of the visual and spectrophotometric methods

There was a significant correlation of r = 0.8182** (df = 208) between the visual classes and the spectrophotometric readings. Class means showed a progressive increase in absorbance in relation to the HCN content of the root as estimated by the regression equation obtained previously. *Figure 2* shows the range of cyanide content falling into each of the visual classes. It may be seen that there was some degree of overlap between classes, indicating the relative unreliability of eye-judgements. None of the 210 genotypes tested fell into the visual class 0, although 15 samples in Class 1 gave zero absorbance readings.

DISCUSSION

Selection for low cyanide cassava genotypes is an important objective when the roots are destined for human or animal consumption. A cyanide dose of 50–60 mg HCN is considered lethal for an adult human male weighing 50 kg (BOLHUIS, 1954). The need for a rapid method of screening for these low cyanide genotypes is obvious since in standard selection procedures, especially in the seedling or early clonal generations, hundreds of samples may be handled in a single day. A rapid method such as the Guignard reaction is useful but gives no information on the cyanide content in tangible terms.

Quantitative methods of assay exist, involving titration with silver nitrate (AOAC, 1965) and the exogenous use of the enzyme, linamarase (COOKE, 1979). These methods, however, are tedious and time-consuming, and hence are unsuitable for rapid screening. It appears that a compromise should be sought between rapidity and accuracy. The logical step would, therefore, be to quantify the Guignard reaction, which in itself produces readily discernible colour differences, by carrying it a stage further to measuring the intensity of the colour change spectrophotometrically. Whereas GILCHRIST *et al.*, (1967), INDIRA & SINHA (1969) and MITCHELL (1974) reported such quantifications, the calibration curve they constructed accomodated cyanide contents only up to 50.0 or 100.0 µg. In actuality, cassava root tissues release cyanide often much in excess of 100 µg/g fresh roots

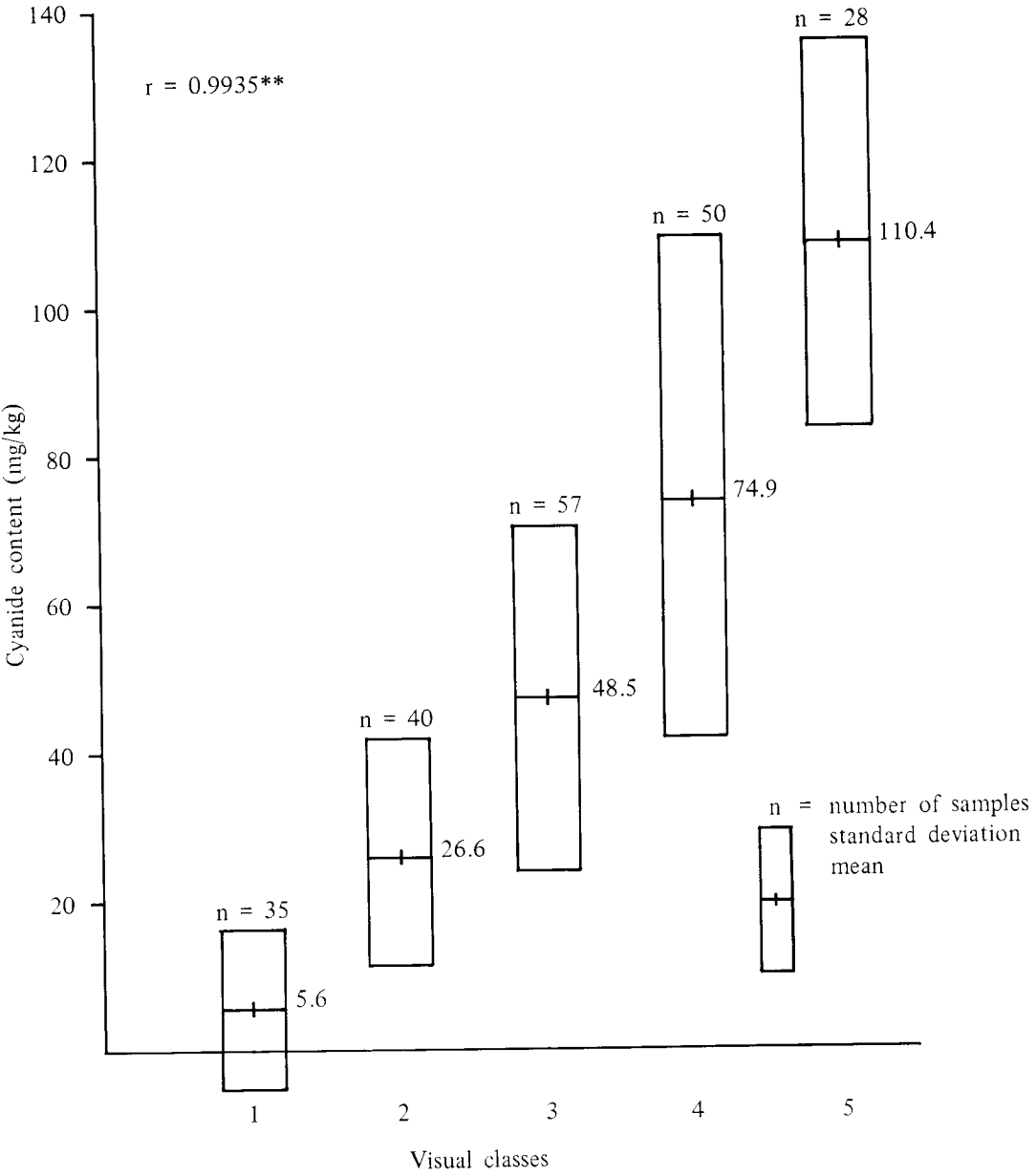

Figure 2. The range of cyanide content (as determined spectrophotometrically) in each visual class.

(ROGERS, 1963; SINHA & NAIR, 1968; SINHA 1970). BOLHUIS (1954) considered the limits for HCN content per g of fresh roots to lie largely between 10 and 370 μg. Furthermore, results from the present studies indicate that when the cyanide concentrations of the standard solutions were increased to 400.0 μg, the relationship with absorbance was no longer a linear-type regression but had become curvilinear. The straight-line relationship held only at the lower range of cyanide contents (as was also reported by the earlier workers), following Beer-Lambert's Law on the penetration of light through a liquid medium. This implied an additional source of error if a linear regression equation

was extrapolated to cover higher cyanide contents. It would be more preferable to work within the linear range by appropriate dilutions of the eluate. The regression equation for the linear range (i.e. from 0.0 to 100.0 $\mu g\ CN^-$) as obtained from the present data is $y = 0.002542\ x$, where the line is forced through the origin ($R^2 = 0.9984$).

When visual scoring was compared with the present method, it became obvious that the classes tended to overlap due to the inaccuracy of visual assessments. Nevertheless, it was still possible to separate with relative ease the innocuous classes, 1 and 2, from the poisonous ones, 4 and 5 (see *Figure 2*). Class 3 representing moderately poisonous levels of cyanide fell in an ambiguous area bridging Classes 2 and 4.

By being able to measure changes in colour intensity by spectrophotometric means, it is possible to estimate the cyanide content of the plant tissue in units of mg/kg material. It should be noted that the mean cyanide contents of the various visual classes correspond very well with the toxicity classification system compiled by KOCH (1933), *viz.*

Cyanide content (mg/kg fresh root)	Toxicity
Less than 50	Innocuous
50 – 100	Moderately poisonous to poisonous
More than 100	Very poisonous

Moreover, absorbance readings better reflect the continuous variation exhibited by this trait of cyanide content in cassava as class groupings are entirely artificial.

The use of the quantitative method of estimating cyanide content may be extended to cover other plant tissues, such as cassava leaves.

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SUMMARY

The Guignard's sodium picrate test was quantified by the use of standard cyanide solutions and spectrophotometric measurements. A comparison between the quantitative method so developed and the usual visual classification is presented.

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