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## Method validation of ninhydrin-based spectrophotometry for trace analysis of cyanide residue in an antiseptic alcohol

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### ABSTRACT

In this study, ultratrace level of cyanide was detected based on the catalytic reaction of ninhydrin in the presence of cyanide ion under the optimum conditions. The absorbance of the blue solution of 2-cyano-1,2,3-trihydroxy-2H indene was measured at both maximum wavelengths of 352 and 598 nm. The developed method gave linear range of 0.02-0.1  $\mu\text{g/mL}$  for cyanide ion. Effect of various kinds of alcohols on the absorbance was investigated. Generally, cyanide is a toxic substance with  $LC_{50}$  at  $\mu\text{g/mL}$ , which is rather difficult to determine. Thus, this method was adopted for higher accuracy and precision. At present, alcohols produced from cyanide-rich cassava are commercially available which could be contaminated with the cyanide residue. In this case, an antiseptic alcohol, isopropanol 70% (v/v), was subject as a model solution for cyanide testing with satisfied recovery and without any interference from the diluted isopropanol solution.

**Key words:** cyanide, ninhydrin, isopropanol, catalytic reaction, spectrophotometry

### INTRODUCTION

In principle, the catalytic reaction of 2,2-dihydroxy-1,3-indanedione (ninhydrin) in the presence of cyanide in an alkaline medium proceeds when an aqueous solution of ninhydrin reacts with cyanide ion ( $\text{CN}^-$ ), the hydrindantin as an intermediate product is obtained. This intermediate in sodium carbonate solution pH 8-12 dissociates to be 2-hydroxy-1,3-indanedione of which its proton of hydroxyl group is withdrawn, resulted in monovalent anion giving red solution. Consequently adjusting with sodium hydroxide to increase pH of the solution up to 12.8 the other proton is neutralized to get two forms of divalent anion with blue solution. This method was applied to detect trace cyanide in environment samples. The color solution obeyed Beer's law between 0.04-0.24  $\mu\text{g/mL}$  (at 590 nm,  $\epsilon = 2.20 \times 10^5 \text{ L/mol.cm}$ ) [1]. Later, mechanism of the catalytic reaction of cyanide with ninhydrin was clarified by structural identification [2]. This ninhydrin-based spectrophotometry for trace cyanide determination (LOD 1.5 ng/mL) could be run with short analysis time at 485 and 590 nm. Their parameters affecting the absorbance were studied including pH of solution and UV-Visible light [3]. The absorbance of the catalytic reaction in sodium carbonate solution and in the absence of oxygen was further developed at 485 nm better than that at 590 nm, since at higher pH values the color product was less stable. This method was rapid, cheap and environmentally friendly [4].

Generally, trace determination of cyanide residue in samples can be done with various either chemical reactions or instrumental methods. Both of  $\text{CN}^-$  and thiocyanate ( $\text{SCN}^-$ ) in water samples were determined by GC-FTD followed by reacting with dimethyl sulfate to produce acetonitrile and methyl thiocyanate, respectively. Then both products were extracted with ethyl acetate prior to analysis, giving linearity of 0.05-1  $\mu\text{g/mL}$  ( $\text{CN}^-$ ) and 0.25-10  $\mu\text{g/mL}$  ( $\text{SCN}^-$ ) [5]. No interference was found with inorganic salts of cyanide ion, except ferri/ferrocyanide, with over 90% recovery in urine samples. An indirect method for cyanide determination by using the cyanide as a catalyze in the dissociation of benzil molecule in methanol to get benzaldehyde and methyl benzoate prior to determination by GC.

Linear curve was 0.05-3  $\mu\text{g/mL}$  and LOD was 1  $\text{ng/mL}$  without any interference of anions [6]. An automated microvolume of the reacting agent in flow injection chemiluminescence (CL) system was used for cyanide detection in blood sample following microdistillation (>98% efficiency) compared with an official method. Its linear range was between  $5.0 \times 10^{-7}$  and  $5.0 \times 10^{-5}$  M with LOD of  $2.3 \times 10^{-7}$  M, and RSD for peak height of CL was 1.9% [7].

Head-space single drop microextraction in association with fiber optic-based cuvetteless microspectrophotometry was developed for cyanide analysis. Hydrocyanide extracted from 1  $\mu\text{L}$  sample was simultaneously reacted with ninhydrin in sodium carbonate solution (2  $\mu\text{L}$ ). Linearity was found to be 0.025-0.05  $\mu\text{g/mL}$ . No interference was found, since some masking agents were used. LOD was 4.3  $\text{ng/mL}$  [8]. Determination of cyanide in wastewater and natural water including biological samples using the catalytic reaction of ninhydrin with cyanide ion was also reported, but heavy metals like Hg, Cu and Ag might obstruct the reaction. *N*-acetyl cysteine was used as a selective agent for the metal interferences [9]. The method could be applied for cyanide in environmental and biological fluid samples, giving higher selectivity and sensitivity [10].

Colorimetric solid-phase consisting of two channels: extraction and detection of  $\text{CN}^-$  and  $\text{SCN}^-$  was developed. Immobilized aquacyano-corrinoids and immobilized vitamin B12 were used as a cyanide sensor at 583 nm of dicyano complex associated with diffuse reflectance spectroscopy giving LOD of 1  $\text{ng/mL}$  [11]. Fluorescent nanosensors (bovine serum albumin and fluorescein isothiocyanate) to cap with Au-NPs as FITC-BSA-AuNPs probe was used to detect cyanide and iodide in high-salinity and table salts. Trace cyanide could then be detected with LOD of 1  $\mu\text{M}$  [12]. Study of weak acid dissociation in metal ions complexed with cyanide was carried out at 485 nm ( $\epsilon = 1.2 \times 10^5$  L/mol.cm). The kinetic reaction of the red solution at pH 10.8 was also investigated using the cyanide complexes of  $\text{Hg}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  by ligand-exchange with cyanide ion [13].

From these literatures, various chemical reactions associated with spectrophotometry including chromatographic techniques have long been used for cyanide analysis. However, UV-Visible spectrophotometry is still a versatile one for cyanide determination. Thus, this study was aimed to adopt high sensitivity of the catalytic reaction of ninhydrin in the presence of cyanide in an alkaline medium for ultratrace determination of cyanide in an antiseptic alcohol sample as the test model without any interference from the diluted solution of an isopropanol sample.

## MATERIALS AND METHODS

### 2.1 Chemicals and reagents

Potassium cyanide (Merck, Germany), ninhydrin ( $\text{C}_9\text{H}_6\text{O}_4$ ) (Ajax Finechem, Australia), sodium hydroxide (RCL Labscan, Thailand), sodium carbonate (QReC, New Zealand), isopropanol (Riedel Haen, Germany), propanol (M&B, England), methanol (QReC, New Zealand), ethanol (RCL Labscan, Thailand), butanol (RCL Labscan, Thailand), all of analytical grade, were used in this study.

Stock solution (1000  $\text{mg/L}$ ) of cyanide standard was prepared. A series dilution of the cyanide solution was then made with ultrapure water for the trace calibration curve using 5  $\text{mg/L}$   $\text{CN}^-$ . Ninhydrin solution (0.08  $\text{mol/L}$ ) was prepared. Sodium carbonate (0.40  $\text{mol/L}$ ) and sodium hydroxide (2.50  $\text{mol/L}$ ) solutions were also prepared.

Blank solution was also prepared including 0.5 mL ultrapure water, 0.8 mL of 0.08  $\text{mol/L}$  ninhydrin, 0.8 mL of 0.40  $\text{mol/L}$  sodium carbonate and 2.9 mL of 2.50  $\text{mol/L}$  sodium hydroxide, and the blank solution was vortexed and left standing for 3 min.

### 2.2 Apparatus and instruments

Automatic micropipet 100-1000  $\mu\text{L}$  (Rainin, USA) was used. Clean volumetric flask and small glass vials were rinsed with appropriate solvents before use. UV-Visible spectrophotometer (Agilent model 8453, Germany) and analytical balance (model BSA224S-CW) were also used.

### 2.3 Real sample

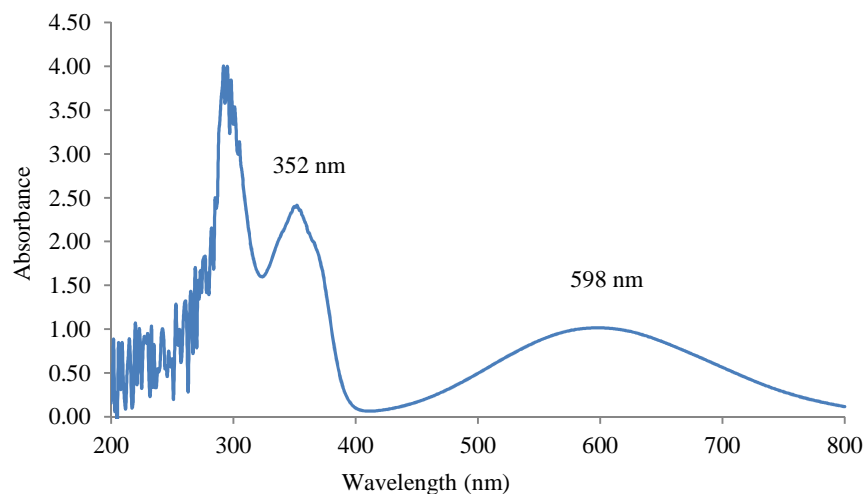
An antiseptic alcohol, 70% (v/v) isopropanol, which is commercially available was purchased from the retail drug store in Khon Kaen University Complex Center and Services. Dilution of the alcohol sample was made with ultrapure water supplied in our chemical laboratory.

## RESULTS AND DISCUSSION

### 3.1 Spectral characteristics of 2-cyano-1,2,3-trihydroxy-2H indene

For optimal maximum wavelength, standard solution of cyanide (1  $\text{mg/L}$ ) 0.20 mL, 0.30 mL deionized water, 0.80 mL of 0.08  $\text{mol/L}$  ninhydrin and 0.80 mL of 0.40  $\text{mol/L}$  sodium carbonate were added in a small glass vial (red

solution). After thoroughly mixing and left for 10 min, 2.90 mL of 2.50 mol/L sodium hydroxide was added, mixed and left for 3 min (blue solution) before recording its UV-Visible spectrum as shown in Figure 1. It was noticed that there are two maximum wavelengths at 352 and 598 nm.



**Figure (1): UV-Visible spectrum of 2-cyano-1,2,3-trihydroxy-2H indene (blue solution)**

However, the effect of the catalytic reaction time of the ninhydrin in the presence of various concentration of cyanide in  $\text{Na}_2\text{CO}_3$  solution was further pointed out in detail. The reaction product with red solution is shown in Figure 2. After addition of sodium carbonate solution, the catalytic reaction of ninhydrin in the presence of cyanide becomes the red solution. The scanning time of the red solution with 1 mg/L  $\text{CN}^-$  as the catalyst was also proceeded until 60 min, giving a constant absorbance after 10 min (Figure 3a). The absorbance increases sharply within 10 min and then keeps constant until about 60 min studied due to completed reaction occurred. So, choice of 10 min is the most suitable time to pursue the reaction system.

Consequently, after addition of sodium hydroxide solution the reaction product changes to blue solution instantly, linearly until 60 min of the measurement. Figure 3b shows the decrease in the absorbance of the blue solution of the final product according to the same scanning time. It was found that the absorbance was linearly decreased upon the measuring time, resulting that a quick measurement of less than 5 min was possible. However, about 5 min constant for the reacting time can be hold for the absorbance measurement of the solution. Since the oxidation of the blue solution can be interfered giving the colorless solution. Then, the absorbance of the solution decreases timely. Thus, the addition of sodium hydroxide solution into the reaction system can be rapidly done within a short period of 3 min as possible. Actually, a few min is preferred for this reaction product.



**Figure (2): The reaction product of ninhydrin in the presence of cyanide concentrations of 0.0, 0.02, 0.04, 0.06, 0.08 and 0.10 mg/L in sodium carbonate solution (red solution)**

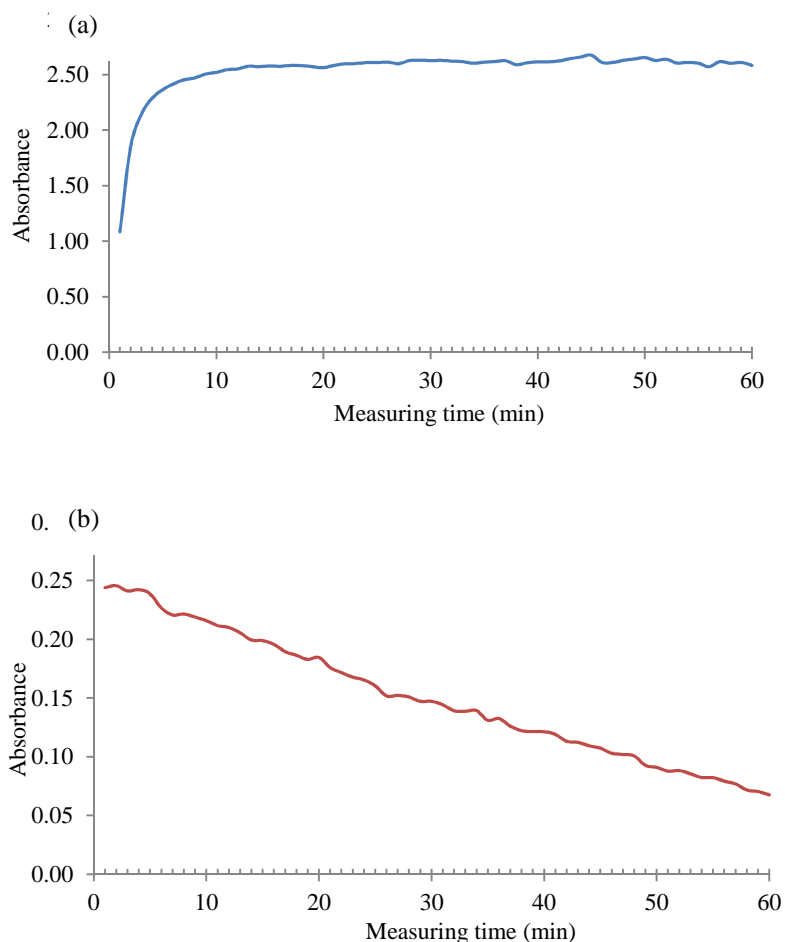
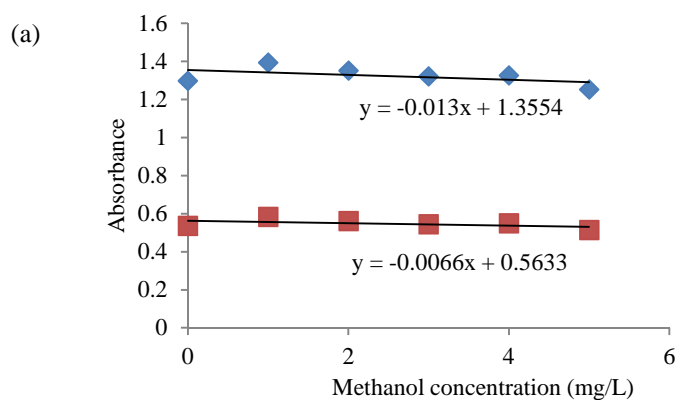


Figure (3): Effect of measuring time on stability of the reaction products: (a) red solution and (b) blue solution

### 3.2 Effect of type of alcohols on the blue solution of the reaction product

To demonstrate the effect of different kinds of alcohols on the absorbance of the reaction product, Figure 4 shows the effect of various concentrations of (a) methanol (1-5 mg/L), (b) ethanol (0.5-2.5 mg/L), (c) propanol (0.02-0.1 mg/L), and (d) butanol (0.05-0.25 mg/L) on the absorbance of the reaction product (blue solution) using 0.02 mg/L cyanide. In particular, Figure 5 shows the color shades of the reaction products using 0.06 mg/L cyanide with red solution (a) and blue solution (b) in the presence of the propanol concentrations of 0, 0.6, 1.2, 1.8, 2.4 and 3.0 % (v/v).



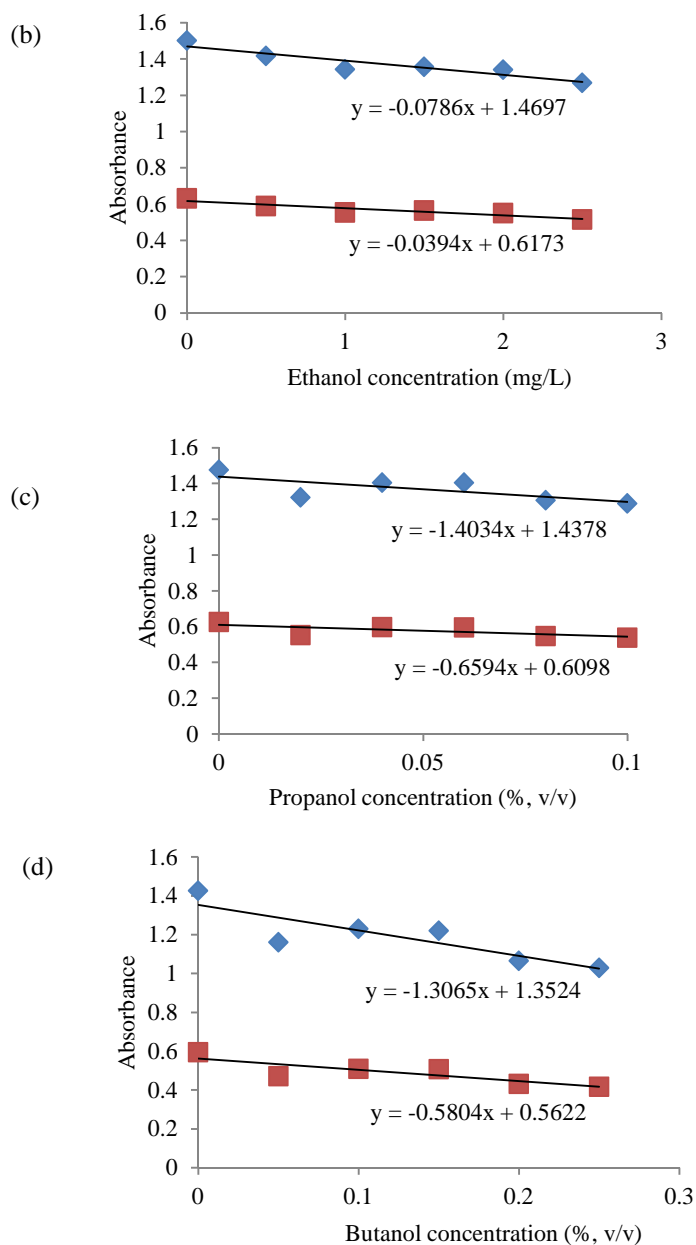


Figure (4): Effects of (a) methanol, (b) ethanol, (c) propanol, and (d) butanol concentrations on absorbance of the reaction product (blue solution) using 0.02 mg/L cyanide



Figure (5): The color shades of the reaction products using 0.06 mg/L cyanide with red solution (a) and blue solution (b) in the presence of propanol concentrations of 0, 0.6, 1.2, 1.8, 2.4 and 3.0 % (v/v)

However, since an antiseptic alcohol sample is mainly composed of 70 % (v/v) isopropanol. Thus, the effect of the isopropanol on absorbance of the catalytic reaction of ninhydrin in the presence of cyanide was also investigated in detail. Figure 6 shows UV-Visible spectra with two maximum wavelengths at 352 and 598 nm of the reaction product using 0.08 mg/L cyanide in the presence of various concentrations (0-8%, v/v) of the isopropanol standard including 7% (v/v) isopropanol in real sample solution.

When using 0.08 mg/L cyanide ion in the catalytic reaction of ninhydrin, the effect of isopropanol concentration on the absorbance of the reaction product decreased linearly between 1% and 8% by volume added (Figure 7). Accordingly, the alcohol sample solution of 7% (v/v) was also quenched the absorbance at the same wavelengths observed. Since dissociation of hydrindantin to 2-hydroxy-1,3-indanedione which is in enol form, its proton is removed by base, and then become red solution. When in the presence of alcohol which is an amphoteric (acid-base property), the alcohol will give back the proton to recover the enol form of 2-hydroxy-1,3-indanedione, resulted in non-absorption. Thus, the absorbance of the reaction product in the presence of the alcohol decreases. This behavior will encounter with the blue product as well. So, dilution of the alcohol sample is needed to be about 0.7% (v/v) in order to determine trace cyanide in the antiseptic alcohol sample with high accuracy and precision.

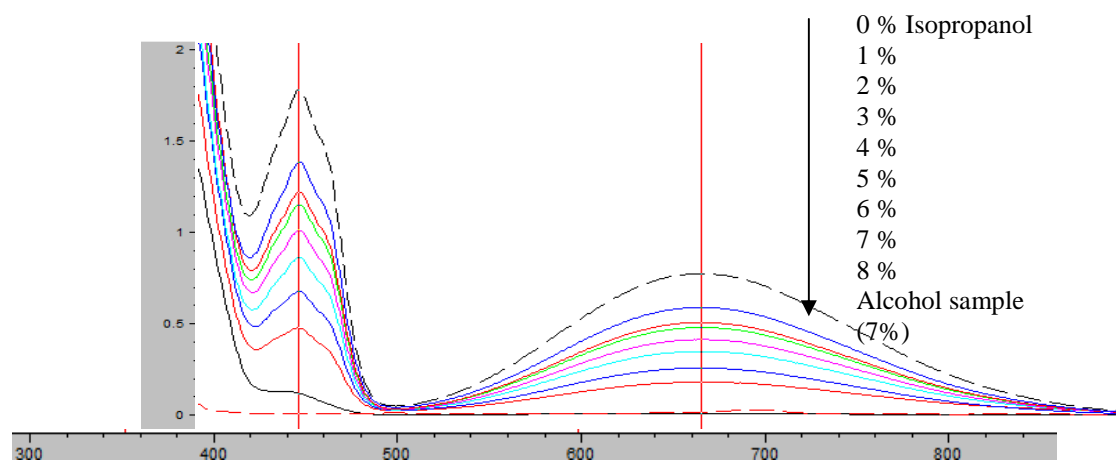


Figure (6): UV-Visible spectra with two maximum wavelengths at 352 and 598 nm of the reaction product using 0.08 mg/L cyanide in the presence of various isopropanol concentrations (0-8%, v/v) including 7% (v/v) isopropanol sample solution

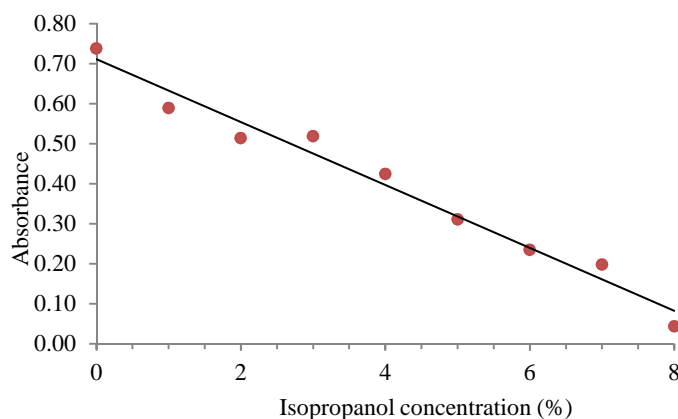


Figure (7): Effect of isopropanol concentration on absorbance of the reaction product

### 3.3 Method validation

**Linearity:** The effect of cyanide concentration ranging from 0.005-0.3 mg/L on the catalytic reaction of ninhydrin was carried out using 0.10 mL of 35% (v/v) isopropanol in the solution mixture of the reaction. Figure 8 shows the effect of cyanide concentration on absorbance of the catalytic reaction of ninhydrin in an alkaline solution at 352 nm (blue triangle) and 598 nm (red circle). However, the linearity of the two curves was effectively found between 0.02 and 0.10 mg/L cyanide ion without any interference of the alcohol solution as shown in Figure 9. Besides, the calibration curves for cyanide ion ranging of 0.06-0.14 mg/L in the presence of 7% (v/v) isopropanol sample solution for both maximum wavelengths of 352 and 598 nm were also investigated (Figure 10).

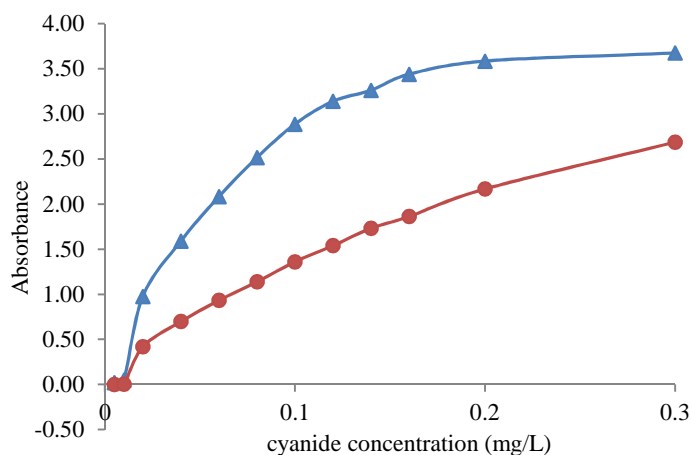


Figure (8): Effect of cyanide concentration on absorbance of the catalytic reaction of ninhydrin in an alkaline solution at 352 nm (blue triangle) and 598 nm (red circle)

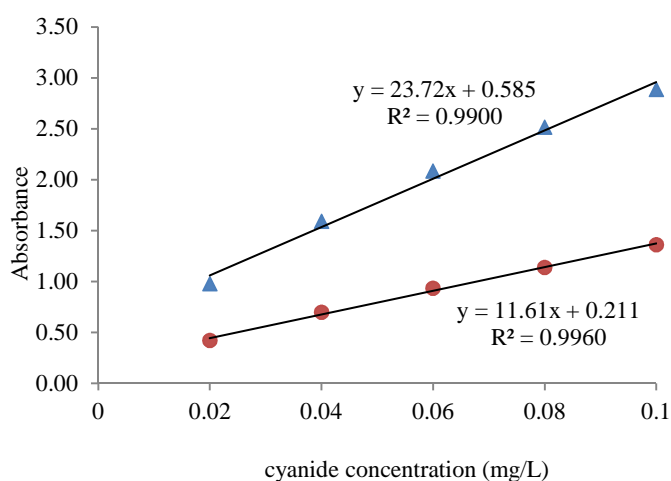


Figure (9): Calibration curves for cyanide determination according to the reaction product (blue solution) for both maximum wavelengths at 352 nm (blue triangle) and 598 nm (red circle)

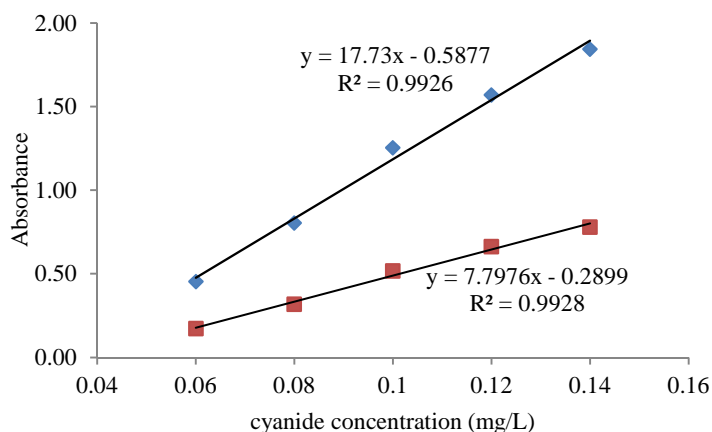


Figure (10): Calibration curves for cyanide ion ranging of 0.06-0.14 mg/L in the presence of 7% (v/v) isopropanol sample solution for both maximum wavelengths of 352 and 598 nm

*Limits of detection (LOD) and quantitation (LOQ):* Using of blank solution of the catalytic reaction of ninhydrin in the absence of cyanide ion, the standard deviation ( $n = 11$ ) of the absorbance of the blank solution at 598 nm was equivalent to be 0.004 mg/L. Based on both  $S/N = 3$  and  $S/N = 10$  ratios, both LOD and LOQ were, therefore, found to be 0.012 and 0.040 mg/L, respectively.

**Accuracy and precision:** The accuracy of the method was expressed as the recoveries of cyanide ion spiked with three levels of 0.02, 0.05 and 0.10 mg/L into the 0.7% (v/v) isopropanol sample solution determined by calibration curve of the blue solution. The results are shown in Table 1, giving between 45.0-104% recoveries. The precision of the method was also evaluated from the slopes of the calibration curves carried out for both an intra-day and an inter-day analysis as shown in Table 2. Their satisfied RSDs(%) were found to be 2.72 and 5.33 for the data obtained from the intra-day and the inter-day analysis, respectively.

**Table (1): Recoveries of cyanide spiked into 0.7% (v/v) isopropanol sample solution**

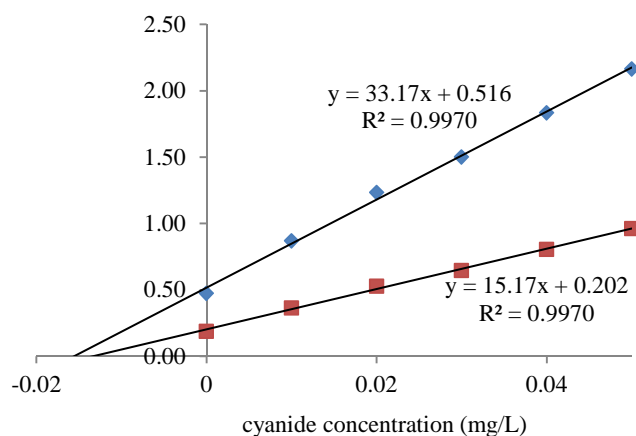
Cyanide added (mg/L)	Recovery (%)
0.02	45.0
0.05	104
0.10	98.0

**Table (2): Regression equations for both an intra-day and an inter-day analysis**

	Regression equation	
	Intra-day (n = 3)	Inter-day (n = 5)
	y = 11.879x - 0.1332	y = 11.610x - 0.2110
	y = 11.438x - 0.0620	y = 11.720x - 0.1129
	y = 11.272x - 0.0920	y = 10.902x - 0.0926
		y = 10.470x - 0.1348
		y = 11.879x - 0.1332
RSD (%)	2.72	5.33

### 3.3 Standard addition method for real sample

Aliquots of the diluted sample solution, 0.7% (v/v) isopropanol alcohol in ultrapure water, were spiked with 0.02, 0.05 and 0.10 mg/L cyanide solutions for their recovery study and determined following the same calibration range used (0.01-0.05 mg/L). The results showed both linear curves for maximum wavelengths (352 nm and 598 nm) as shown in Figure 11 for 0.02 mg/L cyanide spiked level. Those data obtained in Table 3 for the other two spiking levels were not shown.



**Figure (11): Standard addition curves for both two maximum wavelengths (352 nm in blue and 598 nm in red) for 0.02 mg/L of cyanide spiked into 0.7% (v/v) isopropanol sample solution**

**Table (3): Recovery of cyanide spiked into the sample solution determined by standard addition method**

Cyanide added (mg/L)	Cyanide found (mg/L)	Regression eqn. (R <sup>2</sup> ) at 352 nm	Regression eqn. (R <sup>2</sup> ) at 598 nm	Recovery (%)
0.02	0.013	y = 33.17x + 0.516 (0.9970)	y = 15.17x + 0.202 (0.9970)	65.0
0.05	0.065	y = 22.32x + 1.606 (0.9790)	y = 10.71x + 0.694 (0.9800)	130
0.10	0.157	y = 33.67x - 0.153 (0.9730)	y = 14.72x - 0.078 (0.9640)	157

## CONCLUSION

Ultratrace level of cyanide in the alcohol sample solution was detected based on the catalytic reaction of ninhydrin in the presence of cyanide ion under the optimum conditions. The absorbance of the blue solution of the reaction product was measured at both maximum wavelengths of 352 and 598 nm. The ninhydrin-based spectrophotometric method was adopted for higher accuracy and precision. Both LOD and LOQ were found to be 0.012 and 0.040 mg/L, respectively. It gave a calibration range of 0.02-0.1 µg/mL for cyanide ion. The effect of different type of



volatile alcohols on the absorbance of the blue product was also investigated. At present, alcohols produced from the cyanide-rich cassava are commercially available which could be contaminated with the cyanide residue. An antiseptic alcohol, isopropanol 70% (v/v), was therefore subject as a model solution for cyanide testing with satisfied recovery (98.0-104%) and without any interference from the diluted isopropanol solution.

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