Comparison of cyanide content in arbila beans (*Phaseolus lunatus* L) of East Nusa Tenggara using picrate and acid hydrolysis methods

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**Abstract.** Arbila beans contain cyanogenic compounds in the form of linamarin (cyanoglucosides), acetone cyanohydrin, and free cyanide, all together constitute total cyanide content. The objective of this study was to compare the cyanide content in arbila beans analyzed by picrate and acid hydrolysis methods. Picrate method measures total cyanide only. Cyanide content was identified by using a picrate paper, which turned into yellow. The absorbance was measured by a spectrophotometer at 510 nm. Acid hydrolysis method measures cyanide in arbila beans in the form of linamarin, acetone cyanohydrin, and free cyanide. Linamarin was hydrolyzed in H$_3$PO$_4$ solution. Estimated cyanide levels were measured by using a colorimetric procedure. Data was analyzed using Independent sample t-test (SPSS v.16). The results showed that, there was no difference in the total level of cyanide in both methods. Total cyanide measured by picrate and acid hydrolysis method was 2705.17 ppm and 2693.29 ppm, respectively. In addition, the three forms of cyanide content were as follows: linamarin 926.22 ppm, cyanohydrin 556.01 ppm, and free cyanide 1211.06 ppm. Based on the results, both methods can be used for total cyanide analysis. To determine the form of cyanide other than total cyanide, it is recommended to use the acid hydrolysis method.

**1. Introduction**

Arbila beans (*Phaseolus Lunatus* L) are widely grown in East Nusa Tenggara, Indonesia. It has the potential as a source of protein as most other beans. However, arbila beans also contain a high level of linamarin which releases a toxic compound in the form of hydrogen cyanide (HCN). HCN is formed as a result of linamarin hydrolysis by an endogenous enzyme linamarase. The products of linamarin hydrolysis are glucose and intermediates compound acetone cyanohydrin. Acetone cyanohydrin decomposes easily into hydrogen cyanide and acetone in neutral condition and pH above 4. The overall form of the cyanide can be estimated as total cyanide in the sample [1-4]. Two different quantitative methods namely, the picrate method has been used to measure total cyanide in cassava [1,5,6] whereas the acid hydrolysis method has been used to measure the cyanogenic potential in cassava [7,8].

The picrate method is a general method, however, this method is less accurate because it relies on the hydrolysis reaction facilitated by linamarase. Therefore, the presence of enzyme inhibitors such as tannins will affect the linamarin hydrolysis leading to a negative or extremely low value of total cyanide levels [9]. An attempt has been made to develop the previous general method, namely the acid...
hydrolysis method [7,10]. The acid hydrolysis method is based on cyanogen reaction in acid solution and colorimetric measures with a specific könig reaction. The reaction is to oxidize cyanide into cyanogen chloride with chloramine-T. Then, Cyanogen chloride is reacted with pyridine – barbituric acid mixture to produce purple color and measured by spectrophotometer [11]. This method does not require the presence of enzymes but is able to measure the three different forms of cyanide in the sample (cyanogenic potential) [7]. In the present study, we determined and compared cyanide content in arbila beans by using picrate and acid hydrolysis methods.

2. Material and methods

2.1. Chemical
Potassium cyanide, polyvinylpyrrolidone, picric acid, barbituric acid, deionized water, H₃PO₄, Na₃HPO₄, (NH₄)₂SO₄, 0.2M NaOH, Na₂CO₃ 2.5%, 4 M H₂SO₄, 0.1 MH₂PO₄, Whatman 3 mm was used for picrate paper.

2.2. Plant material
The material used in this study was arbila beans obtained from farmers in East Nusa Tenggara, Indonesia which were harvested in July-August.

2.3. Methods
2.3.1. Preparation of picrate paper. A paper strip of filter paper was previously made by dipping filter paper (Whatman 3mm) in a picric solution. The Picric solution was prepared as follows: picric acid was weighed (1.4 gram) and dissolved in 2.5% sodium carbonate (w/v) made up to 100 ml volume. The paper was dried in the air and then stored in a dark container [5,6,12].

2.3.2 Picrate Method. The picrate method relies on the endogenous enzyme to catalyst hydrolysis of cyanoglucosides into glucose and intermediates acetone cyanohydrin, which is further broken down into hydrogen cyanide. Picrate method procedure was applied by pouring a 100 mg sample of ground Arbila beans placed in a plastic vial. Phosphate buffer (0.5 ml of 0.1 M at pH 8) was added. A picrate paper is attached to the vial and closed with a stopper. The vials were kept in a warm place at 30°C for 16 h. After 16 h, the change in color of the picrate paper was observed and the picrate paper was removed and immersed in water for at least 30 minutes. The absorbance of solutions was measured by spectrophotometer at 510 nm, a blank solution without sample was measured accordingly. The cyanide content was calculated based on the standard KCN curve [5,6,12] prepared previously.

2.3.3. Acid hydrolysis method. The principle of the acid hydrolysis method is extractions of cyanogens from the material using an acid solution, which inactivates linamarase and prevents the catalytic breakdown of linamarin. This is to ensure that hydrolysis of cyanogens is solely done by acid hydrolysis and to release hydrogen cyanide. Hydrogen cyanide is measured based on the colorimetric determination. The linamarin, acetone cyanohydrin, and free cyanide (HCN and CN-) measured by the acid hydrolysis method have been reported by Bradbury et al. [7] modified by Bradbury et al. [8]. Linamarin was extracted from Arbila beans by diluting it in phosphoric acid (H₃PO₄) (0.1 M with low pH) and mixed for 2 – 3 minutes. The mixture was filtered using Whatman no 1 paper filter then centrifuged at 8000 g for 10 minutes. The filtrate was stored at 4°C overnight. Total cyanide content was determined by adding filtrate with 2 ml of 4 M hydrochloric acid (H₂SO₄) in a test tube with a stopper. The solution was heated up in boiling water of 100°C for 50 minutes and cooled with ice-cold water. The solution was left overnight at 4°C. After that, 5.0 ml 3.6 M sodium chloride (NaOH) was added into the solution and the mixture was incubated for 5 – 10 minutes to complete the reaction, afterwards continued with the colorimetric procedure. In this step, the solutions must be in alkaline conditions to ensure spontaneous decomposition of acetone cyanohydrin into free cyanide. Meanwhile, to determine any HCN in the filtrate solution, 5 ml of extract (filtrate solution) was added
into 5 ml of 0.2 phosphate buffer (pH 6) and continued with the colorimetric procedure. The cyanide and acetone cyanohydrins content were measured by adding filtrate with 2 ml of 4 M H$_2$SO$_4$ in a test tube with a stopper and rapidly cooled with ice and cold water. After that, 5 ml of 3.6 M NaOH was added to the solution and the colorimetric procedure was carried out. The colorimetric procedure was performed by adding 1 ml of the solution with 7 ml of 0.2M phosphate buffer (pH 6) into two test tubes. To one of the test tubes, 2 ml of water was added to serve as a blank solution and the second test tube was added by 0.4 ml 5 g / litre chloramine – T solution. The tubes were cooled in ice for 5 minutes and added with 1.6 ml of pyridine in barbituric acid solution and were incubated for 60 – 90 minutes until purple colour was produced. The absorbance of the purple solution was measured at 583 nm against the blank solution. The cyanide content was calculated based on the standard KCN curve.

2.3.4. Proximate analysis. The proximate analysis was carried out based on AOAC methods [13]. Proximate analysis of arbila beans includes water content, total fat, protein content, ash content, carbohydrate content.

2.3.5. Statistical analysis. The analysis of total cyanide using picrate and acid hydrolysis methods was carried out in three experimental replications. The results were treated by analysis of independent sample t-test using SPPS v.16 for Windows. The results were presented as averages ± standard deviations followed by corresponding letters which indicate the significant differences and analyzed considering a confidence level of 95% (p<0.05).

3. Result and discussion

3.1. Proximate analysis of arbila beans
The arbila beans were analyzed for chemical composition, the results are shown in Table 1. The chemical composition of arbila beans were within range of those reported by others [14,15] slightly variations may be due to the area of cultivation, cultivars and climate. Arbila beans contain a moderate level of protein. However, the levels of cyanide content limit its utilization. Therefore it is necessary to remove or maximize the reduction of cyanide content prior to consumption. To ensure safety, using the appropriate method to determine all forms of cyanide in arbila beans is required.

| Analysis parameters | Arbila beans (%)$^a$ |
|---------------------|----------------------|
| Water content       | 13.4 ± 0.04          |
| Protein             | 18.75 ± 0.25         |
| Fat total           | 1.43 ± 0.03          |
| Carbohydrate        | 62.85 ± 0.29         |
| Ash content         | 3.58 ± 0.03          |

$^a$ Values are means ± SD (Standard deviation of means) of triplicate

3.2. Cyanide measured by picrate method
The method was applied to analyze cyanide with three experimental replications. The results are shown in Table 2, the total cyanide in arbila beans was 2705.17 ± 5.31 ppm (mg/kg). The total cyanide content indicated that there is the hydrolysis of Linamarin by linamarase to produce acetone cyanohydrin. During the test, the pH of the sample was 8 to allow acetone cyanohydrin to decompose into free-HCN [5,16]. Phaseolus lunatus, contains anti-nutritional compounds such as tannin 0.005 mg/g, phytate 5.07 mg/g, total phenol 6.06 mg/g, saponins 265µg/g [17]. The picrate method can be
less accurate because it relies on the hydrolysis reaction of the linamarase enzyme in the sample. If the sample contains enzyme inhibitors such as tannins, flavonoids, phytate, or saponins, it will inhibit linamarin hydrolysis and produce a negative or low value of cyanide.

3.3. Cyanide measured by acid hydrolysis method

Table 2 shows the cyanide content in various forms (linamarin, acetone cyanohydrin, hydrogen cyanide, and cyanide ion) produced by the Acid Hydrolysis method. The results showed that the total cyanide of arbila Beans with the Acid Hydrolysis method was 2693.29 ppm (mg/kg). Cyanide can be measured in other forms, namely free cyanide (HCN) and HCN + acetone cyanohydrin, whereas linamarin and cyanohydridr were calculated using the formula [8]:

Linamarin = total cyanide - (acetone cyanohydridr + free cyanide) \[ (1) \]

Cyanohidrin = \[ (\text{acetone cyanohydridr} + \text{free cyanide}) - \text{free cyanide} \] \[ (2) \]

The acid hydrolysis is a method which is based on cyanogen reaction in acid solution and colorimetric measures with a specific könig reaction, to oxidize cyanide into cyanogen chloride with chloramine-T. Then, Cyanogen chloride is reacted with pyridine – barbituric acid to produce purple color and measured by spectrophotometer [11]. The chemistry of the pyridine-barbituric acid reaction in acid hydrolysis method is shown in Figure 1 [11].

![Figure 1. The chemistry of colorimetric determination of cyanide [11].](image)

3.4. Comparison of picrate methods and acid hydrolysis methods

The mean total cyanide content was 2705.17 ppm (mg/kg) analyzed by picrate method whereas by using acid hydrolysis method was 2693.29 ppm (mg/kg) (Table 2). These results show that the total cyanide content by acid hydrolysis was lower than the picrate methods. These results were similar to those reported by [9] using similar methods. They reported that 7 out of 10 samples (Sorghum leaf, Peach stone, Plum stone, Nectarine stone, Apricot stone And Giant taro leaf) when analyzed its total cyanide content using the acid hydrolysis method produces lower values than those of the picrate method. However, statistically, it showed that there was no difference in total cyanide levels in both methods. It is known that the total cyanide level using the picrate method is higher but not different from the acid hydrolysis method, so it can be concluded that the total cyanide test can be carried out using both methods. The result shows that both methods can be used for total cyanide analysis. To analyze the form of cyanide other than total cyanide, it is recommended to use the acid hydrolysis method. The Acid hydrolysis method can measure the overall forms of cyanide in the sample.
Table 2. Cyanide content arbil beans using picrate and acid hydrolysis methods.

| Methods            | Total of cyanide (ppm) | HCN + Acetone cyanohydrin (ppm) | Free HCN (ppm) | Linamarin (ppm) | Acetone cyanohydrin (ppm) |
|--------------------|------------------------|---------------------------------|----------------|-----------------|--------------------------|
| Picrate            | 2705.17 ± 5.31*        | -                               | -              | -               | -                        |
| Acid hydrolysis    | 2693.29 ± 10.31*       | 1767.07 ± 12.52                 | 1211.06 ± 14.78| 926.22 ± 10.84  | 556.01 ± 13.17           |

* Values are means ± standard deviation followed by corresponding letters which indicate the significant not differences and performed considering a confidence level of 95% (p > 0.05)

4. Conclusions

The picrate and acid hydrolysis methods are generally applicable to determine the total cyanide content of arbil beans. Therefore, both methods can be used to determine total cyanide analysis because no significant difference in the levels was observed for both methods. However, the acid hydrolysis method can measure the overall forms of cyanide in the sample which is the method of choice to determine the total cyanide content of arbil beans to ensure safety.

Acknowledgement

The present study was part of the study on Cyanide Detoxification of Arbil Beans and Its Utilization as Protein Source Alternatives funded by Hibah Peneliti Unggulan – Brawijaya University Research and Community Service Council, Contract No. 1383.164/UN10.C10/PN/2020 to Dr. Siti Narsito Wulan.

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