Hydrogen cyanide determination in cassava leaves using the picrate paper method

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ABSTRACT

This protocol describes how to prepare and analyse cassava leaf samples for their total hydrogen cyanide content by the picrate paper method. Cyanide in leaves of crops other than cassava can also be prepared and analysed using this protocol. The picrate paper method for leaf cyanide determination relies on endogenous enzymes to breakdown the cyanogenic glucosides (linamarine) in fresh cassava leaf samples. In this protocol cassava leaves are crushed to bring together the cell contents of cassava leaves, including the enzyme linamarase and the cyanogenic glucosides. Hydrogen cyanide is liberated in the process and it reacts with picrate papers that have a colour pigment which darkens with released cyanide. The variations in the darkening of picrate papers is then used to measure the amount of hydrogen cyanide released from fresh cassava leaf samples.

This protocol has been already well outlined by Dr J.H. Bradbury and his team, so please refer to their document (see the attached document below). A kit for cyanide analysis in cassava roots called PROTOCOL E: DETERMINATION OF TOTAL CYANIDE IN LEAVES, has also been designed by them. This protocol describes how the Protocol E kit can be used to determine cyanide in cassava leaves. This protocol is not a replacement of the original protocol. Some of the contents of this protocol have however been directly copied from the original protocol; they are indicated in **bold text**. This protocol however contains additional insights from things encountered during the use of the kit; this information is in **normal text**. In addition to this, this protocol includes insights that Dr Bradbury shared to help troubleshoot a few unforeseen issues; these insights are in ***italics***.

Protocol E.pdf

GUIDELINES

Due to the volatile nature of hydrogen cyanide, errors should be avoided as much as possible when carrying out this analysis. For the results to be accurate the quality of leaves should also be as close as possible to the state they were in right after harvest. The key to using this method is **focus, precision and timeliness**. If these three are maintained throughout the analysis then chances of introducing errors can be reduced making the obtained results comparable to other cyanide determination methods.

MATERIALS TEXT

- Cool box
- Ice packs
- Labels
- Marker pen
- Thick ceramic mortar (inner diameter of about 8 – 10 mm) and pestle
- Small pairs of scissors
- Paper napkins
- A small disposable plastic knife
- 1 or 2 basins (for washing and drying plastic bottles for their reuse)
- 1 L bottled water
- Distilled water
- Thermometer
- Gladwrap or tiny sized plastic bags (2 cm x 10 cm)
- Test tubes or 25 - 50 ml beakers

Protocol E kit

1. **Protocol E; which gives full instructions for total cyanide analysis in leaves.**

2. **A plastic balance with a 100 mg weight glued into one spoon, for weighing 100 mg of cassava root.**
3. **30 flat-bottomed plastic bottles with screw lids.**
4. **Two graduated 1 ml, plastic pipettes.**
5. **Bottle containing 100 buffer papers at pH 6.**
6. **100 yellow picrate papers glued to strips of clear plastic with hobby glue.**
7. **Colour chart with 10 shades of colour which correspond to 0 – 800 ppm total cyanide.**
8. **Ten pink standard papers containing linamarin equal to 50 ppm cyanide.**
9. **Ten paper discs which contain buffer at pH 6 and the enzyme linamarase. These papers have a small black spot, so that they will not be confused with buffer papers.**

BEFORE STARTING

The protocol involves the use of picrate papers and these papers need special care. **When not in use, picrate papers need to be kept in the deep freezer where they remain stable indefinitely**. Place the picrate papers still in their original packaging into a Ziploc plastic bag. The Ziploc plastic bag with its contents should then be placed into a well-sealed firm opaque plastic storage container (a lunch box would be fine). This protects the picrate papers and prevents them from being crushed by external forces.

Picrate papers must be kept dry! Make sure that the deep freezer works well and not as a freezer. Freezers have a tendency of wetting picrate papers, thus destroying them. If the refrigerator you are using has a freezing compartment and not a deep freezing compartment, then do not place the picrate papers in the freezer compartment. Instead, place the picrate papers in the non-freezing compartment (refrigerator). Use the non-freezing compartment when you are not sure if it has a deep freezing function. The non-freezing compartment will not keep the picrate papers below $0^{o}C$, but will at least keep the picrate papers dry. The picrate papers will also be kept cool thus delaying their darkening. Picrate papers become dark when stored at room temperatures or when exposed to sunlight.

It takes about 1 month for picrate papers to darken when they are stored at room temperature. *Once they are darkened in this manner picrate papers cannot be used with the colour chart but are ok with the spectrometer because the colour cancels out.* When they have not been used, picrate papers must always retain their original light yellow colour.

Check the test kit and take note of the standard 50 ppm papers which are pink in colour. **The standard 50 ppm papers must be stored in the refrigerator (non-freezing compartment) as the level decreases gradually over time.**

You will sometimes have to use the picrate paper test kit away from a lab. A number of things must be done if you will be working away from the lab as follows:

- While travelling always place the picrate papers and standard pink linamarin papers in a cool box with ice packs. The picrate papers should still be in the firm storage container they had been placed in, it is convenient to carry them in this way. Also place the small bottle containing the standard pink linamarin in the same storage box as the picrate papers.
- Try to arrange for a deep freezer and fridge at your destination. If this is not possible then you will have to continue using the cool box.
- Try to also arrange for a room where you can conduct your analyses.
- Make sure you have a hard surface to work on
- Arrange for a dark cupboard or place where you can incubate your samples without interference.
- Also carry a basin as you may have to wash the plastic bottles meant for sample incubation, in order to re-use them.

Picrate papers need to be all treated the same way. The time they spend inside and outside the deep freezer or in the cool box must be all the same. In this way the picrate papers will lose colour in a similar manner (not that you want them to lose colour). Thus, if a batch of picrate papers has been differently treated, then it should be treated as one. This means that each batch of picrate papers that has been treated a certain way must have its own blank. Since spectrophotometer readings are only done later on, then a few unused picrate papers must be kept from each batch to serve as blanks for the samples. At least two of these picrate papers should be kept from the batch as extra blanks, this is in addition to the picrate paper used to test the blank during analyses. The quality of the blank is critical for getting reliable results.

Setting up your work area

1 A bench or table will be needed as a working surface. Place the mortar, pestle, scissors, paper napkins and various contents of the Protocol E kit on the work table. The mortar and pestle should have been thoroughly cleaned before the start of the analysis and so should have been the scissors. It would be convenient for you to work in or near a room with a refrigerator. In this way you will be able to keep the leaves and picrate papers cool while you work. Leaf samples should be kept refrigerated until right before preparing them for cyanide analysis. A wash

area is needed for washing up the mortar and pestle after use. The mortar and pestle should be washed to avoid sample contamination with each sample ground. If the room you are working in has a tap and sink this would be ideal for washing up apparatus. A basin and water in a bucket or container could alternatively be used if the room has no washing facility. Place a small brush at the sink for scrubbing the mortar and pestle. The napkins should be used for drying the mortar and pestle and for wiping the scissors clean. Throw them after each use.

As leaf contents will crushed and mixed steps 2 to 6 should be carried out very quickly to minimise losses in hydrogen cyanide from the samples.

2 **Leaves are chopped up with scissors and the small pieces immediately ground up in a mortar and pestle.**

Notes:

- If the leaves still have petioles, remove the petioles from all leaves as it is the cyanide content in the leaf blade that is being measured. However, if you are interested in the cyanide content of petioles, its best you carry out the procedure on petioles alone. It is important that you first check the quantity of leaves in your sample before you begin cutting them up into smaller pieces. This is because, large quantities of cassava leaves need to be sub-sampled to reduce their amount before cyanide analysis. The mortar should never be filled with leaves when grinding, as a large quantity of leaves cannot be effectively and quickly ground together. That is why sub-sampling is important. The sampled leaves could also be large in size and would hence also give more than the needed amount of leaf material for cyanide analysis. Sub-sampling allows you to take a smaller but representative sample of the collected leaves. To carry out sub-sampling, you will have to cut the leaves into three parts i.e. the inner, middle and outer leaf parts as shown in Fig 1.

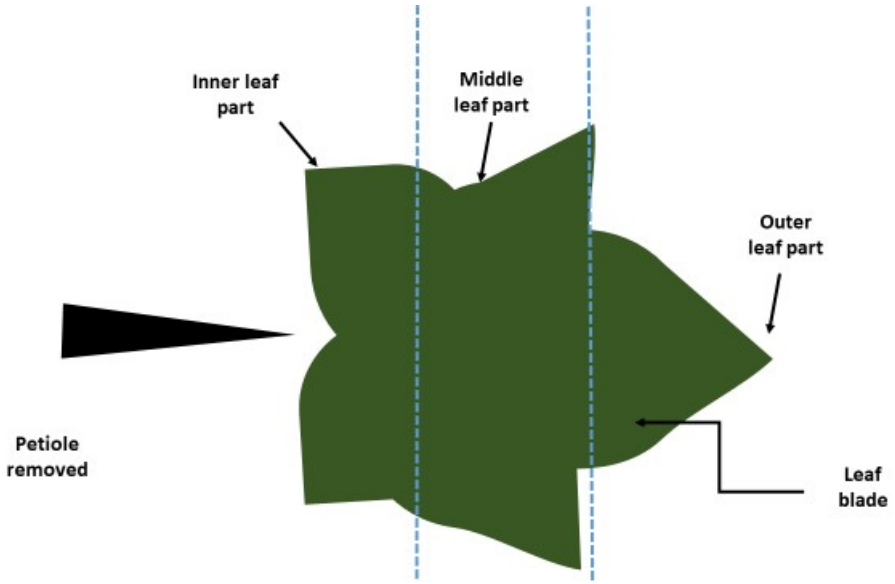

Fig 1. Cassava leaf divided in three sections

- Put the inner leaf parts together and cut them up into smaller pieces (1 cm x 1 cm or smaller) using a scissors. Do the same for the middle and outer leaf parts. Thoroughly mix together each pile of leaf part pieces and pick a small amount of cut leaves from each pile. Place the selected cut leaf part pieces in the mortar. Remember to place an amount that can be quickly and effectively ground to give a small quantity of ground leaf sample. Leaf grinding should be done very quickly to avoid losses in hydrogen cyanide.
- If leaves are few and small in size they can straight away be cut up and ground without sub-sampling.

3 **Immediately weigh out 100 mg of leaves using an analytical balance in the laboratory. If in the field, use a small portable plastic balance which may be supplied in the kit if needed, which has a 100 mg weight glued inside one spoon.**

Notes:

- The small portable plastic balance supplied with the kit is a useful tool for weighing the needed amount of cassava leaf samples, while working in the field. If used correctly it is an accurate tool. Exercise patience when using it to weigh your samples and learn to use it quickly and with high accuracy. The less time ground leaves are left exposed to air the less the introduction of errors through cyanide losses.
- If you are working from a lab you can use a 0.0001 g (0.1 mg) digital analytical balance to weigh your sample. Some labs will however not have balances that can accurately weigh small weights like 100 mg, so the small portable balance supplied with the kit will still have to be used.

4 **Immediately place a round filter paper disc loaded with buffer at pH 6 in a flat bottomed plastic bottle (supplied in the kit), add the ground-up leaves and add 1 mL of water.**

Notes:

- If you do not have access to distilled water, you should use bottled water when performing this step. Tap water may be too variable in composition from one day to the next in some areas. This variability would not be good if you are analysing for cyanide in samples over several days.

5 **Immediately add a yellow picrate paper attached to a plastic strip. The picrate paper must not touch the liquid in the bottle. Immediately close the bottle with a screw capped lid.**

Notes:

- One side of the plastic strip on which the yellow picrate paper is attached is longer than the other. Make sure that this longer side is facing downwards when placing the picrate paper in the bottle. This keeps the water in the bottle further away from the picrate paper. Remember that the picrate papers must always be kept dry. Don't be afraid to place the picrate paper attached to the plastic strip into the bottle as it has been designed to perfectly fit into the bottle.

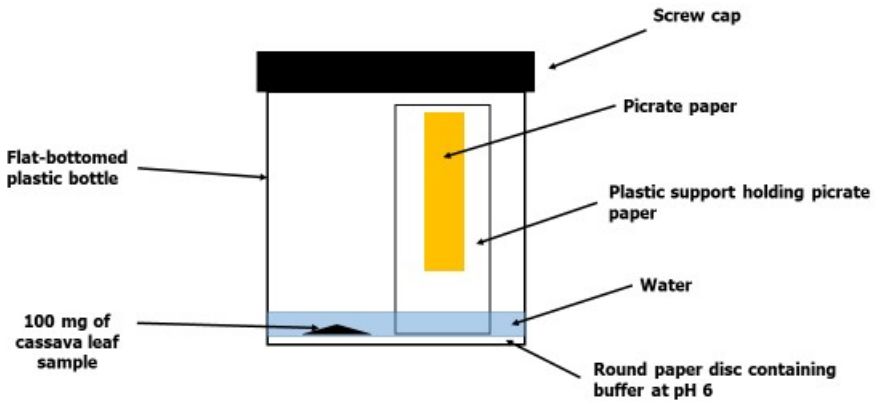

Fig 2. Set up for cyanide capture from cassava leaves using the picrate paper method

6 **Prepare another sample as above but with no leaves, to serve as a blank.**

7 **As a control (or standard) to check on the method, place a filter paper disc loaded with buffer and linamarase in a bottle, add a linamarin paper (see below), 0.5 mL water and a yellow picrate paper. Immediately close the bottle with a screw cap lid.**

8 **Allow the bottles to stand for 16-24 hour at room temperature (20-35^{o}C).**

Notes:

- This is the incubation step. This step should be carried out in the dark. You could use a cupboard for this. Just ensure that you arrange the picrate bottles in manner that will not block you from easily accessing any of them at any time.
- You can take note of the hourly changes in room temperatures during incubation. On cooler days you could decide to leave the picrate papers longer i.e. for the full 24 hours, to allow a fuller reaction time.

Fig 3. Samples left to incubate in the dark

9 **Open the bottles and match the colour of the paper against the colour chart supplied.**

Notes:

- Once you open the bottles, carefully remove the picrate papers, still on the plastic strip, and place them on their plastic caps or on A4 sized paper (Figs. 4 and 5). This is done to allow the picrate papers to first dry-up. Picrate papers become a bit moist during incubation and must not be touched or made to brush against anything before they dry (and avoid directly touching them even when they are dry). Use the plastic strip to hold the picrate paper.
- The plastic caps are used for drying when you do not have to immediately reuse them to incubate other samples. Using the A4 paper for drying however allows you to continue with other samples.
- To easily remember the label identifying each reacted picrate paper that has been left to dry on the A4 paper, write the label on the A4 paper next to the drying picrate paper.
- Once dry you should label the picrate papers, by placing their identity marks on the plastic sheet (Fig 5).

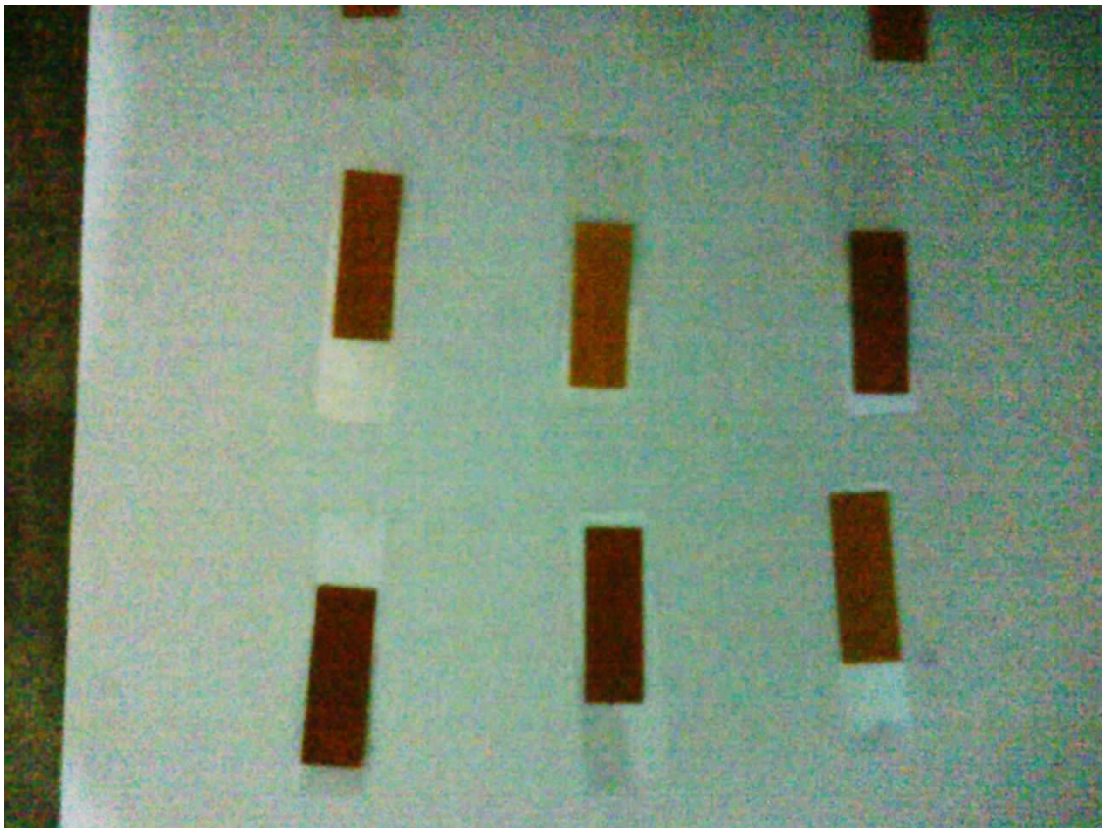

Fig 4. Picrate papers left to dry on A4 size paper and still attached on their plastic strip

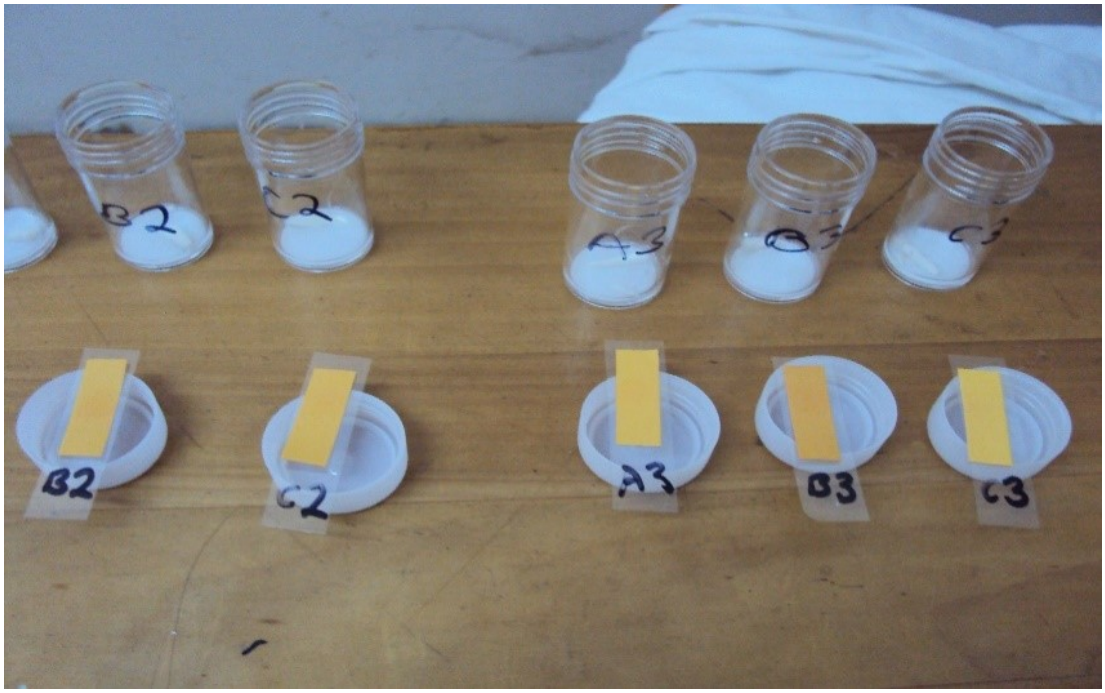

Fig 5: Labelled picrate papers that were left to dry on screw cap lids

10 **From the colour chart, read off the amount of total cyanide in ppm in the leaves. Also check that the blank corresponds to zero and the control gives the expected value.**

Notes:

- Colour chart readings are often the same as spectrophotometer readings and are usually just as reliable. If you are not sure of how to

take the chart readings then consider using the spectrophotometer. It is however ultimately better to get readings from a spectrophotometer.

- When taking colour chart readings, do not remove the picrate papers from the plastic strips if you plan to later get spectrophotometer readings from the same picrate papers. However, do not compare the reacted picrate papers by comparing the side covered by the plastic strip with the colour chart. *It is the uncovered reacted picrate paper that must be used to get the colour reading*. This is why it is advised to remove the plastic strip from the picrate paper when taking colour chart readings. You might however risk losing the label on the plastic strip if you remove the picrate paper from the plastic strip.
- If you have to travel to a research lab to use a spectrophotometer, then you will have to prepare the picrate papers for transportation. It is advisable to still take the colour reading in case something accidentally happens to the picrate papers while in transit. To better preserve the picrate papers they will need to be individually wrapped in plastic. *Gladwrap can be used for this purpose*. You can alternatively place the picrate papers in tiny plastic bags (2 cm x 10 cm). Remember to group together individually wrapped reacted picrate papers from the same field.
- Remember to still keep the picrate papers away from light and do not also expose them to room temperatures for long. The firm opaque storage container and cool box will again be handy. Do the transportation as quickly as possible and immediately place the picrate papers in the deep freezer once you have arrived at your destination.
- Storing reacted picrate papers in this manner is also necessary when you have to wait to take the spectrophotometer readings. *Although picrate papers can stay stable indefinitely in the deep freezer try to carry out the spectrophotometer reading within 1 – 2 days*.

USING THE SPECTROPHOTOMETER TO DETERMINE LEAF HCN LEVELS FROM THE PICRATE COLOUR CHANGES

11 **Carefully remove the plastic backing sheet from the picrate paper.**

12 **Place the picrate paper in a test tube and add 5.0 ml of water measured accurately with a pipette.**

Notes:

- 25 - 50 ml beakers can also be used instead of test tubes. Unlike test-tubes they are wider and picrate papers can be easily removed from them. Swirling is also easier when beakers are used. However, getting several beakers of this size may be difficult thus any lab ware that can be used as a beaker can be alternatively used.
- Be careful not to spill out any of the solution while swirling the contents.

13 **Leave the test tube at room temperature for about 30 min with occasional gentle stirring.**

Notes:

- After the 30 minutes have elapsed remove the picrate paper from the test-tube or beaker using a plastic disposable knife. The picrate paper easily attaches to the plastic knife. Do not use something metallic or something with a suction capability to remove the picrate paper. All the colour of the picrate paper should have been lost by the end of 30 minutes.

14 **Measure the absorbance at 510 nm of the picrate solution from 13 against the blank from 6.**

Notes:

- Use the picrate solution from the blank to zero the spectrophotometer, then read off the absorbance of the reacted picrate solutions of the leaf samples.

15 **The total cyanide content in ppm is calculated by the Equation 1**

Total cyanide content (ppm) fresh weight = 396 x absorbance ..... Equation 1

16 **The cyanide content obtained for the same sample of leaf, from both the colour chart and spectrophotometer, should be about the same. Also check that the standard value agrees using both methods.**

17 **Carefully remove the plastic backing sheet (it may be washed and used again) from the picrate paper.**

Bibliography

1. Bradbury MG, Egan SV, Bradbury JH. Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens

18 in cassava products. J Sci Food Agric. 1999;79:593–601.
2. Egan SV, Yeoh HH, Bradbury JH. Simple picrate paper kit for determination of the cyanogenic potential of cassava flour. J Sci Food Agric. 1998;76:39–48.