Simple Method for Determination of Thiocyanate in Urine

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Background: **It would be useful to develop a simple kit method for determination of thiocyanate in urine, which could be used to monitor cyanide overload in cassava-consuming populations.**

Methods: **The method was based on the quantitative oxidation of thiocyanate in acid permanganate at room temperature in a closed vial with liberation of HCN, which reacted with a picrate paper. For semiquantitative analysis in the field, the colored picrate paper was matched with a color chart prepared using known amounts of KSCN. In the laboratory, a more accurate result was obtained by elution of the colored complex in water and measurement of the absorbance at 510 nm. Over the range 0–100 mg/L, there was a linear relationship given by the equation: thiocyanate content (mg/L) = 78 × absorbance.**

Results: **The picrate thiocyanate method gave no interference with urine samples containing protein at less than 7 g/L, 21 amino acids, histamine, glucose, NaCl, urea, blood, and linamarin. For 53 urine samples analyzed by an accurate column method and the thiocyanate picrate method, a regression line gave very good agreement (r^2 = 1.000). Quantitative recoveries of thiocyanate added to urine samples were obtained with the picrate method.**

Conclusions: **A simple picrate kit for determination of thiocyanate in urine was developed and is available free of charge for workers in developing countries.**

Cassava is the most important food source in the tropics after rice and maize *(1)*. However, it contains a cyanogenic glucoside, linamarin, that is hydrolyzed in two steps: the linamarin is first hydrolyzed to acetone cyanohydrin and glucose (catalyzed by the enzyme linamarase), and then the acetone cyanohydrin is hydrolyzed to acetone and hydrogen cyanide (HCN). Cassava products such as cassava flour, which is important in East Africa and Indonesia, and gari, which is important in West Africa, and cassava roots have a total cyanogen content of 0–200 mg HCN equivalents/kg cassava (0–200 ppm) [Ernesto et al., submitted for publication, and Refs. *(2, 3)*]. The WHO safe concentration for cassava flour is 10 mg HCN equivalents/kg cassava (10 ppm) *(4)*.

Until recently, in developing countries it was difficult to measure the cyanogen content of cassava roots or products because of various problems associated with the use of accurate methods of analysis. However, a simple, semiquantitative method has now been produced in a readily available kit form to determine cyanogens in cassava roots and products *(5, 6)*. This allows the routine monitoring of cyanogen content of cassava roots and products in developing countries, which can show the likely extent of any cyanogen overload in the food sources of the people.

Because cyanide is converted in the body to thiocyanate, determination of the thiocyanate content of the urine *(7–9)* can be used to check cyanogen overload of the population related to the intake of cassava roots and products. The conversion of cyanide to thiocyanate in the body is catalyzed by the enzyme rhodanese and uses sulfur that originates from the essential amino acids methionine and cystine, which further reduces the essential amino acid content of a diet that may already be marginal in protein intake *(7, 10)*. An accurate method is available for the determination of thiocyanate in urine *(11)*; however, as with the determination of cyanogens in cassava roots and products (see above), this method is not readily used in developing countries because it is difficult and requires somewhat expensive chemicals, resin, and equipment.

For the above reasons, it would be useful to develop a simple semiquantitative kit method for the determination of thiocyanate in urine, which could be used to help monitor cyanogen overload in people and perhaps predict the possible onset of a condition called konzo, which is an irreversible paralysis of the legs *(8, 12)*. Here we report the development of a simple kit method for the measure-

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ment of thiocyanate in urine, based on its quantitative oxidation with permanganate to sulfate and cyanide *(13)*, which is then determined by the picrate method *(5)*.

Materials and Methods

SAMPLES

Urine samples were obtained from volunteer donors in Canberra, including adult males and females, smokers and nonsmokers, and children. These samples were either used the same day or frozen at $-20\ °C$ until required for analysis *(14)*. Fresh blood was obtained from the authors and mixed as required with urine.

REAGENTS

Samples of potassium thiocyanate (KSCN, AR grade; Mallinkrodt), potassium permanganate ($KMnO_4$, Analar grade; BDH Chemicals), potassium dichromate ($K_2Cr_2O_7$, Analar grade, BDH), ceric sulfate [$Ce(SO_4)_2$; Ajax Chemicals], linamarin (Sigma Chemical), and bovine serum albumin (Nutritional Biochemical) were used. The amino acids and histamine were from the following suppliers: alanine, glutamine, isoleucine, leucine, methionine, and serine were from BDH; arginine, aspartic acid, cysteine, cystine, glutamic acid, hydroxyproline, lysine, phenylalanine, proline, tyrosine, and valine were from Nutritional Biochemical; histamine and tryptophan were from Fluka; glycine and histidine were Sigma; and asparagine was from Ajax. Multistix 10 SG reagent strips for urinalysis were obtained from Baeyer Australia. Sodium perchlorate (Analar grade; BDH), sodium hypochlorite solution (125 g/L, technical grade; APS Ajax Fine Chemicals), isonicotinic acid (Sigma), 1,3-dimethylbarbituric acid (Fluka), and AG3-X4 anion-exchange resin (100–200 mesh, free base; Bio-Rad Laboratories) were used in the column method *(11)*.

OXIDATION OF THIOCYANATE TO CYANIDE AND DETERMINATION WITH PICRATE PAPER

In preliminary experiments, KSCN solution was reacted with $KMnO_4$, $K_2Cr_2O_7$, or $Ce(SO_4)_2$ in dilute H_2SO_4 in the presence and absence of urine (1 mL) in a 25×50 mm, flat-bottomed polystyrene vial with a polyethylene screw cap (Techno Plas). A 30×10 mm picrate paper, attached to a plastic strip with hobby glue *(6)*, was immediately added to the vial, and the vial was closed with a screw cap. After incubation at 30 °C for 3–16 h, the vial was opened and the plastic strip separated from the picrate paper. The paper was eluted for ~30 min in 5.0 mL of water with occasional stirring, and the absorbance of the solution was measured at 510 nm in a spectrophotometer against a blank solution prepared from an unchanged picrate paper similarly eluted. The HCN liberated from the KSCN was calculated by the equation:

$$\text{HCN content } (\mu g) \text{ from KSCN} = 39.6 \times \text{Absorbance} \quad (1)$$

which was obtained from Eq. 3 of Ref. *(6)*.

In later experiments, 1.0 mL of urine was placed in a plastic vial, 0.1 mL of H_2SO_4 (1 or 2 mol/L), and 0.1 mL of $KMnO_4$ solution (0.1–0.5 mol/L) was added. Various additives, including known amounts of thiocyanate and possible constituents of urine (sodium chloride, glucose, amino acids, bovine serum albumin, linamarin, and blood) *(15)*, were added. A picrate paper was placed in the vial, which was then closed, and the absorbance was measured according to the method described above.

A calibration curve for thiocyanate was prepared using AR-grade KSCN dried to a constant weight at 60 °C. Using an adjustable micropipette, we added accurately known amounts of KSCN solution (1 g SCN^-/L) in duplicate to water to make a total volume of 1 mL, and then added 0.1 mL of 1 mol/L H_2SO_4 and 0.1 mL of 0.1 mol/L $KMnO_4$. A picrate paper was immediately placed in the vial, which was then closed. After incubation at 30 °C for 16 h, the picrate papers were eluted in water and the absorbance of the solutions were determined as described above. The absorbances obtained at each thiocyanate value (2.5, 5, 10, 20, 40, 60, 80, and 100 μg) were averaged. The calibration curve shown in Fig. 1 is linear ($r^2 = 0.999$) over the range of concentrations from 0 to 100 mg/L and is described by the equation:

$$\text{Thiocyanate (mg } SCN^-\text{/L urine)} = 78 \times \text{Absorbance} \quad (2)$$

COLUMN METHOD FOR DETERMINATION OF THIOCYANATE

The general procedure used by Lundquist et al. *(11)* was followed with several changes. Urine (0.5 mL) was diluted with 5 mL of 0.1 mol/L NaOH and applied to a Bio-Rad AG3-X4 anion-exchange Econo-column (2.5×0.7 cm), which retained the thiocyanate. Other soluble constituents of urine were removed by washing with water. The thiocyanate was displaced from the column with 8 mL of 1.0 mol/L sodium perchlorate. Acetic acid (0.3 mL of a 0.5 mol/L solution) was added to a 4-mL aliquot of column
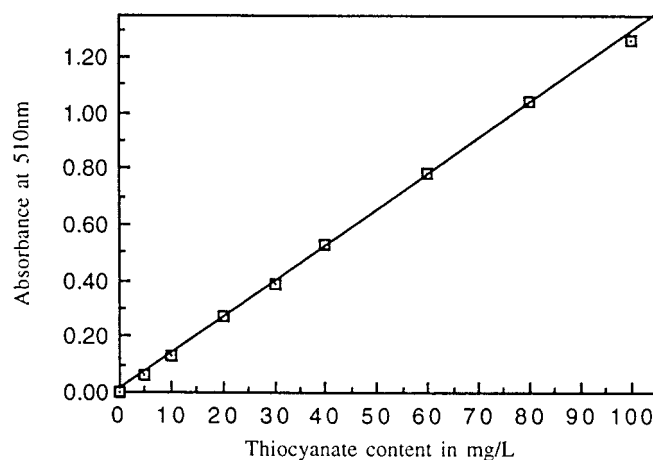

Fig. 1. Calibration curve for the simple picrate method.

Thiocyanate content in urine in mg SCN^-/L urine vs the absorbance at 510 nm of the picrate solution ($r^2 = 0.999$).

eluate. The subsequent methodology followed the published procedure *(11)*.

Using the stock solution of KSCN containing 1 g SCN^-/L (see above), we added duplicate amounts of 1, 2, 3, 5, 10, 15, 20, 30, and 40 μg SCN^- to 0.5 mL of water. These solutions were used instead of the 0.5-mL urine sample (see above). A calibration curve was produced, and over the linear range, this curve agreed within experimental error with that shown in Fig. 5 of Lundquist et al. *(11)*. A linear calibration curve ($r^2 = 1.000$) using our data was described by the equation:

$$\text{Thiocyanate } (\mu g) = 4.64 \times \text{Absorbance} \qquad (3)$$

Because a 0.5-mL urine sample was used, the amount of thiocyanate in 1 mL of urine is equal to twice the value given by Eq. 3.

Results

EFFECT OF VARIOUS OXIDIZING AGENTS ON THIOCYANATE

In preliminary experiments, accurately known amounts of KSCN were reacted with $KMnO_4$ *(13)*, $K_2Cr_2O_7$, and $Ce(SO_4)_2$ solutions in the presence or absence of urine. In the absence of urine, all three oxidizing agents, present in excess, gave quantitative liberation of HCN from KSCN; in the presence of urine, there were low recoveries with $K_2Cr_2O_7$ and $Ce(SO_4)_2$ but quantitative recoveries with $KMnO_4$ Presumably, the reaction of thiocyanate with those oxidizing agents that are weaker than permanganate was slow and incomplete in the presence of urine.

The reaction between permanganate and thiocyanate in H_2SO_4, $6\ MnO_4^- + 5\ SCN^- + 13\ H^+ = 6\ Mn^{2+} + 5\ HCN + 5\ SO_4^{2-} + 4\ H_2O$, is very rapid and complete in acidified urine. In the absence of urine, the magenta color from the excess permanganate is retained for the time of the experiment (3–16 h); when urine is present, however, the magenta color disappears in 0.5–3 min because the permanganate reacts with an unknown reducing agent present in the urine. Clearly, the very rapid and quantitative reaction between permanganate and thiocyanate in acidified urine *(13)* supports its use. An attempt to immobilize the permanganate in a 21-mm diameter Whatman 3MM filter paper disc failed because there was a slow loss of the magenta color on drying and deposition of brown manganese dioxide. The brown filter paper gave low HCN evolution in acidified urine solutions to which known amounts of thiocyanate had been added.

CHOICE OF CONDITIONS FOR REACTION OF THIOCYANATE WITH PERMANGANATE

The results from duplicate experiments on six different urine samples in which the concentrations of H_2SO_4 (0.5, 1, or 2 mol/L) and permanganate (0.1–0.5 mol/L) and the reaction time (3–16 h) were varied are shown in Table 1.

Accurate thiocyanate results were obtained by duplicate experiments using the column method. The optimal

Table 1. Choice of reaction conditions for oxidation of thiocyanate by permanganate in acid urine solutions.[a]

Urine sample number	SCN⁻ added, μg	MnO_4^-, mol/L in 0.1 mL added	SCN⁻ content, mg/L, by picrate method, after reaction times of			SCN⁻ content, mg/L, by column method
			3 h	6 h	16 h	
1	0	0.1	0.3	0.8	1.0	1.1
1	0	0.2	0.3	0.6	1.2	
1	0	0.4	1.9	2.2	4.4	
2	0	0.1	2.4	2.8	3.0	3.1
2	0	0.2	2.0	2.3	2.9	
2	0	0.4	1.8	2.5	3.2	
3	0	0.1	0.37	0.67	1.2	1.3
3	0	0.5	0.30	0.44	1.0	
4	0	0.1		0.30	1.0	1.0
4	0	0.5		0.90	1.8	
5	0	0.1		0.37	1.0	1.1
5	0	0.5		1.0	1.9	
6[b]	0	0.1		0.70	1.2	1.3
	5	0.1		5.1	6.3	
	10	0.1		10	11	
	20	0.1		20	21	
	30	0.1		30	31	
	40	0.1		39	41	
	60	0.1		57	59	
	80	0.1		74	77	
	100	0.1		86	96	

[a] Reaction conditions were 1 mL of urine, 0.1 mL of H_2SO_4 (0.5, 1, or 2 mol/L), and 0.1 mL of $KMnO_4$ (0.1–0.5 mol/L) reacted for 3–16 h at 30 °C. The optimal H_2SO_4 concentration was 1 mol/L; hence, only the results for 1 mol/L H_2SO_4 are shown.
[b] Addition experiment.

H_2SO_4 concentration in the 0.1 mL added was 1.0 mol/L. In some samples of urine (e.g., sample 1), $KMnO_4$ solution (0.1 mL) at higher concentrations gave falsely increased results. In all urine samples, 0.1 mL of a 0.1 mol/L $KMnO_4$ solution gave good results. Short reaction times (3 and 6 h) were found to give low results for thiocyanate in some cases, whereas 16 h always gave good results. To check the results at higher thiocyanate concentrations, we added 5–100 mg SCN^-/L urine to sample 6. The method gave a good correlation throughout the range with that expected when the sum of amount of thiocyanate in the urine (1.3 μg) and the added amount was used. Thus, the standard reaction conditions chosen were 1 mL of urine + 0.1 mL of 1 mol/L H_2SO_4 + 0.1 mL of 0.1 mol/L $KMnO_4$ reacted for 16 h at 30 °C in a closed vial using a picrate paper.

EFFECTS OF POTENTIAL INTERFERENTS

In confirmation of earlier work (13), we found that some amino acids reacted slowly with the permanganate, producing cyanide. However, in the presence of 1 mL of urine, the permanganate magenta color disappeared in 0.5–3 min because permanganate reacts with a reducing agent present in the urine (see above). Under these conditions, there was no appreciable reaction of the 21 amino acids and histamine (0.2–2 mg of each used in the tests) with permanganate in acid solution to produce HCN. Three different samples of urine gave zero production of cyanide in every case. The amounts of each amino acid used greatly exceeded the concentrations found in urine (15), hence there is clearly no interference attributable to the presence of amino acids in urine samples.

Bovine serum albumin was added to four urine samples at concentrations of 20, 3, 1, and 0.3 g/L. These solutions matched well the colors obtained using the dipsticks for protein in urine (Multistix 10 SG reagent strips). Duplicate samples of urine (1 mL) containing 0, 0.3, 1, 3, 5, 7, 10, and 20 g/L bovine serum albumin were analyzed for thiocyanate using the standard reaction conditions (see above). With all four urine samples, there was no change in the HCN content with protein concentrations of 0, 0.3, 1, 3, and 5 g/L. At 7 g/L protein, there was a slight decrease (0–10%), which became progressively larger at 10 and 20 g/L protein and was 25–40% at 20 g/L. Protein concentrations in urine usually are very much less than 5 g/L (15); thus it is most unlikely that the picrate method would be affected by protein present in urine.

Blood collected from the index finger of two persons was immediately mixed into two different urine samples, and its concentration was adjusted by dilution with urine to correspond to the values of 200, 80, 25, trace, and negative obtained by color matching using the Multistix 10 SG reagent strips. Duplicate 1-mL samples of urine containing blood concentrations giving reagent strip values of 0, trace, 25, 80, and 200 were then set up and analyzed for thiocyanate using the standard reaction conditions. The HCN liberated was the same in each urine sample at all amounts of blood added for both of the urine samples. Clearly, the picrate method is not affected by the presence of blood in the urine.

Sodium chloride (10 mg), glucose (20 mg), and urea (50 mg) were added separately to 1-mL urine samples, and these were reacted using the standard reaction conditions. Known amounts of thiocyanate were also added to urine samples. In all cases, there were no changes in the thiocyanate content of the urine solutions attributable to the presence of sodium chloride, glucose, or urea.

Linamarin (0, 1, 5, and 50 mg/L) was added to urine. Duplicate analyses for thiocyanate showed that there was no change in the thiocyanate content for any of the samples to which linamarin had been added. Linamarin added to two other samples of urine gave similar results, which showed that the thiocyanate determination is not affected by the presence of linamarin in the urine, which occurs in cassava-eating populations (9). Because it has been proposed that ingested linamarin may be toxic (9), it is important to check whether linamarin added to urine could be estimated satisfactorily in the presence of thiocyanate. To 0.5 mL of urine, buffered at pH 8, 40 μg of linamarin and different amounts of linamarase were added, followed by a picrate paper. After incubation for 16 h at 30 °C, the picrate paper was removed, the color eluted, and the absorbance measured at 510 nm (5, 6). Using six different samples of urine, we found that the amount of linamarase used must be approximately three-fold higher than the amount of linimarase used with cassava present to obtain complete hydrolysis of linamarin and quantitative recovery of HCN. Presumably, there is some constituent in the urine that partially deactivates the enzyme linamarase, but this is overcome by using at least three times the usual amount of linamarase (5, 6).

COMPARISON OF PICRATE AND COLUMN METHODS

Forty-two different urine samples obtained in Canberra were analyzed for thiocyanate by the picrate method and by the accurate column method. To cover the full range of thiocyanate content (0–100 mg/L) that is obtained in urine samples from cassava-eating populations, 5, 10, 20, 40, 60, 80, and (in one case) 100 mg/L thiocyanate was added to four different urine samples. These urine samples were then analyzed in duplicate by the picrate and in many cases by the accurate column methods. For the four urine samples, the mean (SD) recoveries of thiocyanate from the addition of 5, 10, 20, 40, 60, and 80 mg/L were 104% (4%), 105% (6%), 103% (2%), 105% (2%), 103% (2%), and 103% (2%), respectively. A regression line of the thiocyanate content obtained with the picrate method plotted against the thiocyanate content obtained with the accurate column method for 53 samples (which included the 42 samples above) is shown in Fig. 2. There is a very good correlation between the results from both methods with $r^2 = 1.000$ and a slope of 1.05.
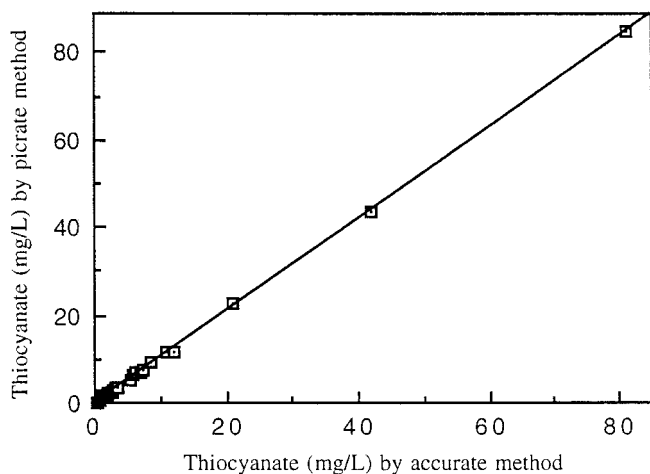
Fig. 2. Comparison of the results of the picrate method and the column method for 53 samples.

The regression line (r^2 = 1.000, $S_{y|x}$ = 0.66) is given by the equation: thiocyanate (picrate method) = −0.039 + 1.05 thiocyanate (column method).

STABILITY OF THIOCYANATE IN URINE

We have confirmed previous work *(11)* that indicated that thiocyanate is stable in urine for at least 6 months at −20 °C. To check the stability of thiocyanate in urine at different temperatures, two urine samples containing 0, 10, and 50 mg/L of added thiocyanate were used. These samples were stored at 4 °C, room temperature (20–25 °C), and 30 °C, and samples were analyzed in duplicate for thiocyanate using the picrate method at periods of 0, 7, and 14 days. No change had occurred after 7 days, but after 14 days at 30 °C, the thiocyanate content of one sample of urine had decreased by ~10% and the other by ~65%. The thiocyanate content of one sample stored at room temperature had decreased by 7% after 14 days, but the other sample was stable. There were no changes in thiocyanate content of any samples stored at 4 °C over 14 days. Clearly, the thiocyanate content of urine samples is constant for at least 7 days at 20–30 °C, at least 14 days at 4 °C, and at least 6 months at −20 °C.

COMPARISON OF COLOR CHART WITH ABSORBANCE MEASUREMENT METHOD USING PICRATE KIT

A color chart was prepared by exposing freshly prepared Whatman 3MM, 30 × 10 mm picrate papers to HCN liberated from the oxidation of known amounts of thiocyanate by excess permanganate, using the treatments described above. The papers that contained 0, 1, 2, 4, 6, 10, 20, 40, 60, and 100 mg/L were aligned on a chart. The colors of the 30 × 10 mm papers were matched using computer technology to give a permanent color chart record with 10 different colors over the whole range. A direct comparison was made between the results obtained with the simple color chart method and the absorbance measurement for a urine sample to which 10 concentrations of thiocyanate from 1 to 80 mg/L had been added. Duplicate analyses were made at each concentration, and

these were scored using the simple color chart by two independent observers. The mean deviation between the two methods was 11%. One urine sample with added thiocyanate was analyzed by measuring the absorbance over 10 identical samples; the mean (SD) result was 11.0 (0.12) mg/L.

DEVELOPMENT OF A THIOCYANATE PICRATE KIT METHOD

To 1.0 mL of urine in a plastic, flat-bottomed vial, we added 0.1 mL of 1 mol/L H_2SO_4 and 0.1 mL of 0.13 mol/L $KMnO_4$ (prepared by dissolving 100 mg of $KMnO_4$, weighed out using a small portable balance, in 5.0 mL of water). A 30 × 10 mm picrate paper, attached to a 50 × 10 mm plastic strip with hobby glue, was added to the vial, which was then closed. Another sample was prepared as above but with no urine to serve as a blank. As a control to check the method, a 21-mm Whatman filter paper previously loaded with 4 or 40 μg of thiocyanate was placed in a vial, 1 mL of water was added, and acid, permanganate, and a picrate paper were added as above. The three closed vials were left at room temperature (20–37 °C) overnight. The vials were opened, and the colors of the papers were matched against the color chart. The color of the blank should correspond to zero on the chart, and the controls should correspond to 4 or 40 mg/L.

To obtain a more accurate result, the plastic strip was removed from the picrate paper, which was immersed in 5.0 mL of water for about 30 min. The absorbance of the solution was measured at 510 nm against the blank yellow solution. The thiocyanate content in milligrams per liter was calculated by Eq. 2. There should be reasonable agreement between the semiquantitative color chart result and the more accurate result for both the urine sample and the control.

COMPONENTS OF KIT FOR THIOCYANATE PICRATE DETERMINATION

A kit containing the following components was prepared:

1. A protocol that gives a stepwise method for analysis of thiocyanate in urine
2. A small portable balance for weighing out 100 mg of $KMnO_4$
3. Clear plastic, flat-bottomed 25 × 50 mm vials with screw lids
4. Graduated plastic 1-mL pipette
5. Filter paper discs, 21-mm diameter, loaded with 4 and 40 μg of thiocyanate (These are stable at room temperature for at least 6 months.)
6. Picric acid papers glued to strips of clear plastic with PVA hobby glue [The picric acid papers were stable for only 1 month at room temperature but were stable in a freezer for at least 8 months *(6)*]
7. A color chart from 0 to 100 mg/L thiocyanate for 30 × 10 mm papers

Discussion

The optimal conditions for the permanganate oxidation of thiocyanate in 1 mL of urine were as follows: addition of 0.1 mL of 1.0 mol/L H_2SO_4 and 0.1 mL of 0.1 mol/L $KMnO_4$. The HCN liberated was reacted with a yellow picrate paper in a small closed vial over a period of 16 h at 30 °C. The brown picrate color was eluted in 5.0 mL of water, and the absorbance was measured at 510 nm. The method was not affected by the presence of potentially interfering compounds present in the urine, such as protein at <7 g/L, 21 amino acids, histamine, glucose, sodium chloride, urea, blood, and linamarin.

The picrate method and a column method were compared using 53 urine samples, and a very good regression line was obtained (see Fig. 2). To evaluate the use of the method in the field, we measured the stability of urine on storage at different temperatures and produced a color chart with 10 shades of color to allow visual comparison of colors. Satisfactory agreement was found between the simple visual comparison and the more accurate measurement of absorbance. This analysis served as the basis for the development of a thiocyanate picrate kit method. The detailed methodology used in the kit is described above, together with the components needed for its operation.

These kits are available free to health workers in developing countries, funded by the Australian Centre for International Agricultural Research (ACIAR), and can be obtained by writing to Dr. J Howard Bradbury.

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