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THE PICRIC ACID METHOD FOR DETERMINING MINUTE AMOUNTS OF HYDROCYANIC ACID IN FUMIGATED INSECTS[1, 2]

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INTRODUCTION

IN STUDIES ON the toxic effect of different concentrations of HCN (hydrocyanic acid) on fumigated insects, determinations of the amounts of the gas absorbed and retained are important. To secure these facts, an accurate method of determining amounts of HCN in the range of 0.005 to 0.200 mg is essential. Extensive preliminary investigations showed that the picric acid method had the greatest promise.

Picric acid in alkaline solution has been widely used as a reagent in colorimetry. It has been commonly used in determining soluble sugars in plant extracts and creatinine in animal fluids. In the procedures for determining reducing sugars, the reaction involves the reduction of the nitrophenol to aminophenol. In the presence of reducing sugars, picric acid (trinitrophenol) is reduced to picramic acid when heated in alkaline solution. The same basic reaction is involved in the determination of creatinine. According to the literature, a different reaction occurs between HCN and picric acid in alkaline solution.

Hlasiwetz (1859)[5] was the first to note the formation of isopurpuric acid according to the following equation:

$$\underset{\text{Picric acid}}{C_6H_3N_3O_7} + 3KCN + 3H_2O \rightarrow \underset{\substack{\text{Potassium}\\ \text{isopurpurate}}}{C_8H_4KN_5O_6} + CO_2 + NH_3 + 2KOH$$

Rosenthaler (1923), also, noted that blood-red isopurpuric acid is formed when picric acid and KCN are heated in alkaline solution. Some disagreement exists about the formation of isopurpuric acid. According to Chapman (1911), the reaction is identical with the reducing reaction between picric acid and reducing reagents, resulting in the formation of 2-amino-4,6-dinitrophenol. In the present experiments it was not determined which of the two reactions

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[5] See "Literature Cited" at the end of the paper for complete data on citations, which are referred to in the text by author and date of publication.

occurs. Since either one results in a blood-red coloration, this determination is relatively unimportant.

For determining approximate amounts of HCN in cyanophoric plants, picric acid paper is often used: the crushed plant tissue is incubated in a closed container, from the top of which is suspended a strip of moist picric acid paper (Foy and Hyde, 1937). The HCN given off by the material reacts with the paper, changing the color to a reddish orange. This paper can be compared with a standard, or the color can be extracted from the strips and the resulting solution then compared with a standard solution. It is evident that such procedures are unsuited for quantitative determinations.

Many investigators have used alkaline picrate in HCN determinations (Adriano and Ynalvez, 1932; Nowosad and MacVicar, 1940; Hogg and Ahlgren, 1942). Waller (1910) estimated HCN in animal and plant tissues by distilling it from the sample, which had been acidified with tartaric acid, into a receiving tube containing a measured volume of alkaline picrate. After incubation for 24 hours at 30° C, the color was compared with standards containing known amounts of cyanide. Sullivan (1939), determining the HCN in white-clover plants, distilled the HCN into solutions of NaOH, from which aliquots were taken for the color reaction. He improved the method by measuring the color change with a photometer.

Even with Sullivan's refinement, however, the procedure was not sensitive enough for quantitative determination of such minute amounts as are encountered in fumigated insects. Detailed study of the entire procedure was therefore initiated to eliminate as many as possible of the variables affecting accuracy and to standardize the procedure so that concordant results could be obtained. The procedure given in the following section is based on this study. Some of the factors found to affect accuracy are discussed in a later section.

STANDARDIZED PROCEDURE

Apparatus and Reagents.—The colorimeter used in this work was an A. C. model, Fisher Electrophotometer. The absorption cells consisted of 23-ml cylindrical tubes. A green filter (5,250 angstrom units) proved to be most satisfactory and was used for obtaining the experimental data reported in this paper. A blue filter was first tried; but the instrument could not be balanced at the zero point against the blank solution of the reagents. When the blue filter was used, the colored solutions had to be compared with a distilled-water standard; that is, the most accurate part of the dial scale was used in measuring the difference between the distilled-water zero point and the reading for the blank solution. On the dial there is a calibration scale from which direct logarithmic readings can be obtained. This, designated as "scale A" on the graphs, simplifies the construction of a calibration curve, since the readings vary directly with the intensity of the color. The makers of the instrument advise that results are more accurate if readings fall between the zero point and a scale-A reading of 50.

The apparatus used in these experiments for distilling the HCN is shown in figure 1. It consisted of a small distilling flask (fig. 1, *B*), with a glass side arm that had an opening 0.6 mm in diameter. The mouth of the flask was fitted

with a ground-glass standard-taper stopper (fig. 1, *A*), through which passed a sealed-in aeration tube extending to within 5 mm of the bottom of the flask. This tube had a lower opening 0.3 mm in diameter. An opening of this size permitted sufficient air to pass through, kept the sample stirred better, and gave more accurate results than did a very fine capillary. Minute quantities of HCN (0.005 to 0.200 mg) could be distilled with this simple apparatus without loss due to rubber stoppers (Morris and Lilly, 1933).

The receiving tube (fig. 1, *C*) was a small test tube, 10 × 1 cm, with a short side arm to connect with the vacuum pump. The distillation flask and receiving tube were of the same size, except that the former had a small bulge blown into it. That small enlargement gave much smoother distillation and prevented the sample from jamming up around the aeration tube.

The reaction containers found most suitable for this determination were Pyrex test tubes graduated at 10 ml and 25 ml. Such tubes eliminate the necessity of transferring solutions to volumetric flasks to bring them all to constant volume. If desired, sugar-analysis tubes graduated at 12.5 ml and 25 ml can be substituted, provided they are used both in constructing the standard curve and in analyzing the sample. The importance of the reaction volume is explained later in this paper (p. 296).

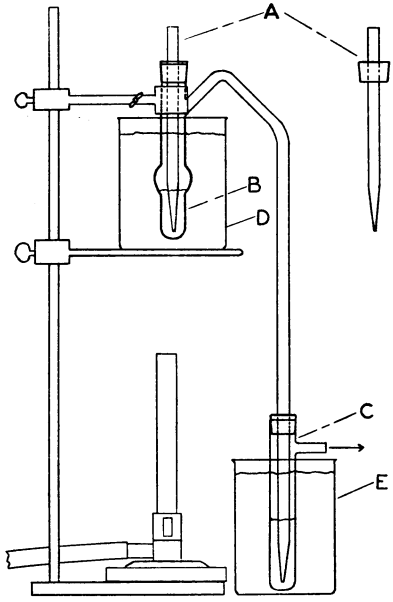

Fig. 1.—Diagram of apparatus used in distilling HCN from insect samples: *A*, aeration tube with standard-taper ground-glass stopper; *B*, distilling flask with connected glass side arm; *C*, receiving tube with outlet to suction; *D*, water bath; and *E*, ice bath.

The reagents used were as follows:

Picric acid solution, prepared by dissolving 10 grams of C.P. picric acid in distilled water and diluting to a final volume of 1 liter.

Sodium carbonate solution, prepared by dissolving 50 grams of the anhydrous salt in distilled water and diluting to a final volume of 1 liter.

Sulfuric acid solution, approximately 10 *N*, prepared by diluting 27.8 ml of concentrated H_2SO_4 to a 100-ml volume.

Sodium cyanide solution, prepared from the moisture-free salt. The solutions used in this work were equivalent to 0.1 mg of HCN per milliliter of solution. The exact amount of NaCN necessary for that concentration depends on the purity of the salt. If 100 per cent pure NaCN were available, 0.1814 gram of the salt, made up to a liter with distilled water, would give the desired concentration. Since most brands of NaCN are only 95 to 96 per cent pure, however, a correction must be made. The HCN content was checked accurately by titration with silver nitrate (Liebig method). A fresh NaCN solution had to be prepared at least once a week, because dilute solutions of the salt decom-

pose in the presence of air. In most instances one preparation will be enough, since the solution is necessary only in constructing the calibration curve.

Distillation.—The sample (100 to 200 mg of insects) was placed in the distillation flask and made to about 2 ml with water. In the receiving tube was placed 1 ml of the Na_2CO_3 reagent, diluted to 3 ml with distilled water to give more volume of alkali for absorption of the distillate.

The distillation unit was then connected and stoppered tightly, with just enough vacuum applied to the receiving tube to maintain a slow but steady flow of bubbles through the Na_2CO_3. One drop of 10 *N* H_2SO_4 was added to the cyanide sample through the aeration tube. The mixture was placed in a

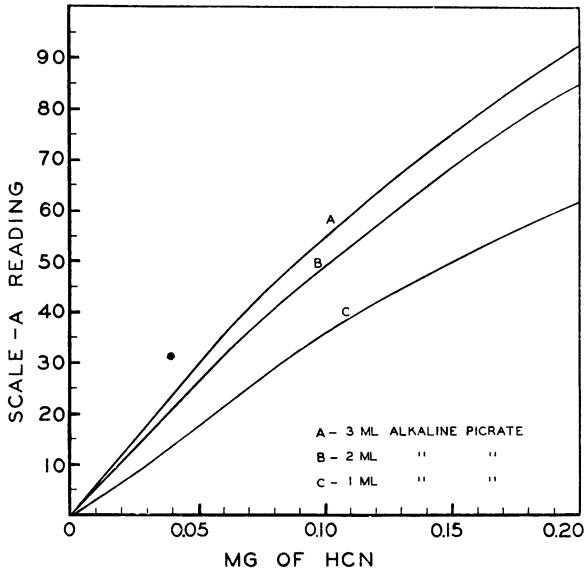

Fig. 2.—Calibration curves of HCN, showing effect of using different amounts of alkaline picrate.

water bath and maintained at the boiling point for 20 minutes. The receiving tube was immersed in an ice bath to keep the carbonate solution cold enough to trap all the HCN in the distillate.

Color Reaction.—After the sample had been distilled for 20 minutes, the distillate was carefully transferred to the reaction tube. The receiving tube and the delivery tube were washed carefully with water to remove any Na_2CO_3 remaining on them. This wash water was added to the distillate, the total volume of the distillate and washings in no case exceeding 9 ml. One milliliter of the picric acid solution was added to the distillate, and the volume was brought to 10 ml with distilled water, if necessary.

After its contents had been thoroughly mixed, the reaction tube was heated exactly 3 minutes in a boiling-water bath and allowed to stand 1½ to 2 hours before its volume was diluted to 25 ml. The tube was then placed in a constant-temperature bath (25° ± 1° C) for 10 minutes, after which its contents were transferred to an absorption cell and the colorimeter reading was taken. The

reference cell in the colorimeter contained a blank solution of the reagents. From this reading, the HCN present was calculated by means of a calibration curve.

Calibration Curve.—For construction of the calibration curve, various amounts of the standard NaCN solution were added to 2 ml of the alkaline picrate reagent, consisting of equal volumes of Na_2CO_3 and picric acid solutions mixed just before using. (The results are more consistent if the carbonate and the picric acid are kept separate until needed.) The cyanide and alkaline picrate mixture was diluted to 10 ml in the reaction tubes. For the color reaction these mixtures were treated exactly the same as the distillates described above. The scale-A readings for the standard mixtures were plotted against the concentration of HCN present (fig. 2).

FACTORS AFFECTING COLOR DEVELOPMENT

In colorimetric analyses the procedure must be exactly the same in each determination. For accurate results, definite rules must be adhered to. Although proposed for visual colorimetry, many of the precautions outlined by Dehn (1917) are even more important in photometry. Preliminary experiments on the alkaline picrate method showed that several important variables must be controlled. For that reason, factors such as reaction volume, length of heating period, time between heating and reading, dilution, and temperature at time of reading were carefully studied to determine how they affect the accuracy of the results.

Reagent Used for Receiving Distillate.—In previous experiments on this method, the HCN was distilled from the samples either into alkaline picrate reagent (Waller, 1910) or into NaOH (Sullivan, 1939). In the present experiments, both these methods proved unsatisfactory for determining minute amounts of HCN.

When the HCN is distilled directly into alkaline picrate, any volatile reducing substances present in the sample are also distilled into the reagent, and there is no longer any chance of separating the HCN from the impurities.

Experiments were performed to determine whether distillation into NaOH had any effect on color intensity. Alkaline picrate reagent was prepared by mixing equal volumes of the Na_2CO_3 and picric acid solutions described on page 296, and 2 ml of the mixture was put in a reaction tube. Varying amounts of 0.1 *N* NaOH or of 1.0 *N* Na_2CO_3 solution (in addition to the carbonate solution in the alkaline picrate reagent) were added, then 0.05 mg HCN. The volume was brought to 10 ml with distilled water, and the color intensity was determined as described on page 294. According to these data, shown graphically in figure 3, the presence of NaOH decreases the color intensity, whereas excess Na_2CO_3 has no effect at all. The reason, probably, is that the NaOH partially neutralizes the picric acid and causes a greater change in pH than does the carbonate.

According to the results in figure 3, Na_2CO_3 gave the greater promise of being a satisfactory receiving solution. A series of distillates was therefore compared with a series of standard mixtures. In one series, known volumes of the standard NaCN solution were distilled, and the HCN was received in a solution of 1 ml of the carbonate reagent diluted to 3 ml with distilled water.

The standard mixtures were prepared by adding the NaCN directly to alkaline picrate reagent. The amounts of HCN in the distillates and in the standard mixtures were determined by the procedure outlined on page 294. As numerous determinations showed, the variation in the amount of HCN determined in the distillate and in the standard-mixture series was no greater than the experimental variation among duplicate standard-mixture samples of the same concentration of HCN. In all cases, 100 per cent of the HCN was recovered on the distillates (table 1).

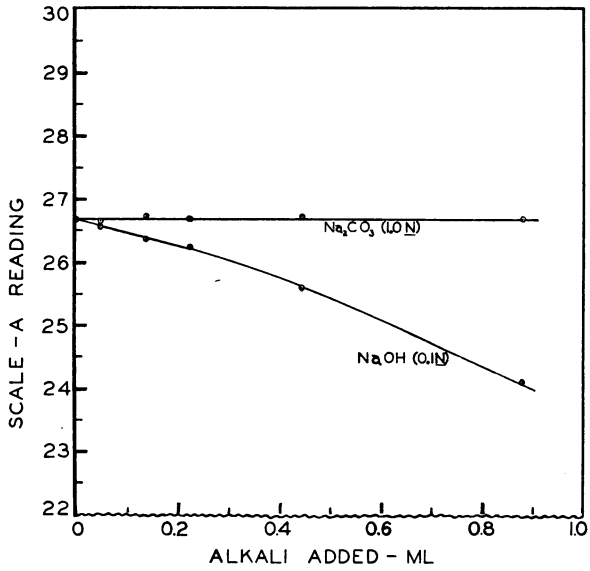

Fig. 3.—Effect of different amounts of Na_2CO_3 and NaOH on the color intensity of alkaline picrate with 0.05 mg HCN added.

Amounts of Na_2CO_3 and Picric Acid.—It is not necessary to use 2 ml of alkaline picrate reagent as described under "Standardized Procedure"; but the amount used in the standard mixtures for the calibration curve must be equivalent to the Na_2CO_3 and picric acid solutions used in analyzing the unknown samples. If a greater volume of alkaline picrate reagent is used, the slope of the curve increases (fig. 2, *A*). For work on fumigated insects a 2-ml volume of the alkaline picrate was very convenient. This amount, made up of equal volumes of the carbonate and picric acid solutions, gives a reagent of the same concentration as that used by Sullivan (1939) and by Hogg and Ahlgren (1942).

Reaction Volume.—The HCN and alkaline picrate mixture, when heated in the water bath, must be rigidly controlled at a constant volume for all determinations. Any change in the concentration of the reacting substances causes a similar change in the concentration of the colored compound produced. In effect, therefore, an increase in volume of solvent is a decrease in concentration of solute. As figure 4 shows, the intensity of color for a given amount of HCN, reacted with a 2-ml sample of alkaline picrate, decreases rapidly with an increase in reaction volume.

If desired, the reaction volume can be made more or less than 10 ml, the volume that was used in this problem in order to obtain color differentiation from small quantities of HCN. If the volume is increased, however, it is advisable to increase the alkaline picrate in each mixture. If, for example, the reaction volume is to be 25 ml, 5 ml of the reagent should be used. When a small reaction volume—10 ml—is used, the final dilution eliminates any error resulting from unequal evaporation during heating.

Length of Heating Period.—In the first experimental work, reaction mixtures were heated in boiling water for 5 minutes, according to the procedure

TABLE 1

RELATIVE AMOUNTS OF HCN DETERMINED BY THE PICRIC ACID METHOD ON STANDARD MIXTURES AND ON DISTILLATES FROM KNOWN QUANTITIES OF HCN

HCN added	Standard mixture,* scale-A reading	Distillate†	
		Scale-A reading	HCN recovered
mg			*mg*
0.005	2.4	2.4	0.005
.010	5.1	5.2	.010
.020	10.7	10.7	.020
.030	16.1	16.0	.030
.040	21.4	21.4	.040
.050	26.8	26.7	.050
.060	31.5	31.6	.060
.080	40.6	40.7	.080
.100	49.3	49.4	.100
.150	69.4	69.6	.150
0.200	86.3	86.3	0.200

* Standard mixtures prepared by direct addition of NaCN to 2 ml of alkaline picrate were diluted to 10 ml, heated 3 minutes, allowed to stand for 2 hours, diluted to a final volume of 25 ml, and then read at 25° C.

† Distillates were prepared by distilling the NaCN into 1 ml Na_2CO_3 (50 grams per liter), according to the procedure described. (Text, p. 294.)

of Sullivan (1939) and of Hogg and Ahlgren (1942). Investigations showed, however, that excess heat diminishes the color development. Maximum color intensity was obtained when the heating time was 2½ to 3 minutes. Heating for a longer period appears to decompose the color products formed in the first 2 or 3 minutes. All values reported in this paper were obtained from solutions heated 3 minutes.

Time between Heating and Reading.—As the heated solution cools to room temperature, the color intensity begins to increase. This development extends over several hours. One must therefore read both the standard and the unknown mixtures in the photometer at about the same length of time after heating. In this way the error from unequal color development is eliminated. Two hours gave more consistent results than a shorter period.

Dilution When HCN Concentration Is High.—The amounts of HCN in the insect samples tested were so minute that the photometer readings came within the recommended range (scale-A reading 0 to 50) with no further dilution than is indicated in the standardized procedure—a final volume of 25 ml, all the distillate being used.

If there is much more HCN in the distillate, dilution will be necessary to bring the readings within the desired range. Such dilution, however, may introduce errors. Willaman and Davison (1924), while investigating the picric acid method for determining sugars, found that dilution gave results 5 to 10 per cent high.

An experiment was therefore conducted to determine the point in the procedure at which the dilution can most safely be made. To test the effect of dilution after the reaction, a series of solutions was prepared, all having a reaction volume of 10 ml with 2 ml of alkaline picrate, but with the NaCN solution varied to give the equivalent of 0.05 to 0.50 mg of HCN. Duplicate

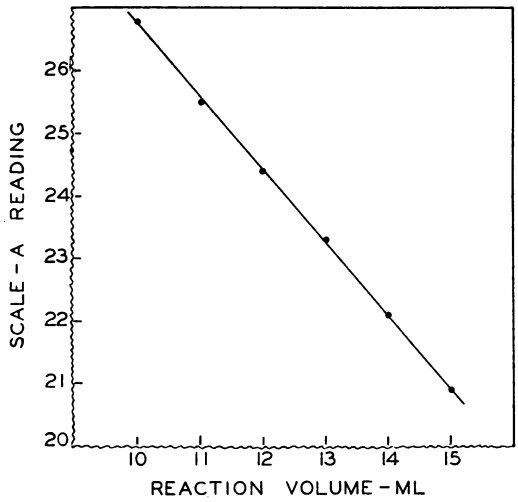

Fig. 4.—Effect of changes in the reaction volume on color development in 2 ml alkaline picrate with 0.05 mg HCN added.

samples were prepared. The solutions were heated 3 minutes, allowed to stand 2 hours, and then diluted to final volumes proportionate to the NaCN added, the volume used with 0.05 mg of HCN being 25 ml as in the standardized procedure. If the color formed were proportionate to the HCN added, all volumes should thus give the same reading as the 25-ml volume.

Table 2 indicates that this does not happen. The 50- and 100-ml volumes gave readings slightly higher than the 25-ml, but the differences are probably not significant. The 200- and 250-ml volumes, on the other hand, gave readings considerably lower. A possible explanation is that a comparatively large amount of HCN will not give a full depth of color because of the change in the equilibrium ratio between the amount of HCN and the amount of picrate reagent present. Though the experiments do not indicate the underlying reasons, Beer's law apparently does not hold for these dilutions.

The only accurate method of bringing high HCN concentrations within the scale range, then, is to dilute the distillate before adding the picric acid. Often one will have to run a trial-and-error series of dilutions to determine the approximate range. Dilution of the distillate affords an opportunity to take

aliquots for replicate determinations, a check not possible with the low HCN concentrations in the tested insects.

Temperature at Time of Reading.—Although variations in temperature cause only slight variations in the readings, solutions should be brought to the same temperature before the readings are made. In the HCN and alkaline picrate system, a temperature rise causes an increase in the reading. The latter is small, however, in comparison with the temperature increase. When 0.05 mg HCN was present, an elevation of 15 degrees Centigrade resulted in an increase of 1 unit on the scale-A reading. A water bath of any arbitrary

TABLE 2

EFFECT OF DILUTION ON THE COLOR INTENSITY OF THE HCN-ALKALINE-PICRATE SOLUTION*

Amount of HCN	Final volume of picrate solution	Scale-A reading			HCN recovered, average of two samples	
		First sample	Second sample	Average	Amount	Percentage
mg	*ml*				*mg*	*per cent*
0.05	25	26.8	26.8	26.8	0.050	100.0
.10	50	27.4	27.7	27.6	.103	103.0
.20	100	27.3	27.4	27.4	.204	102.0
.40	200	24.8	24.8	24.8	.368	92.0
0.50	250	23.0	23.1	23.1	0.423	84.6

* All solutions had reaction volume of 10 ml with 2 ml of picrate; they were heated 3 minutes and allowed to stand 2 hours, then diluted to the various volumes, and read at 25° C with green filter.

temperature (preferably 18° to 25° C) may be used to bring the solutions to the same temperature, allowing a deviation of ± 1 degree. All readings in this report were made at 24° to 25°.

DISCUSSION

The chief criticism of this method is the nonspecificity of the reaction. Any volatile reducing substances possessed by the material being distilled are usually carried over with the steam into the receiving flask, and subsequently react with the alkaline picrate. Some insects—for example, the confused flour beetle, *Tribolium confusum* duV.—have been found to give off volatile reducing substances during the distillation. Preliminary distillations on the unfumigated insects are therefore essential, to determine whether substances that might react with the picric acid are present in the distillate. Most of the insects experimented upon were free of such substances. Partial success has been achieved in eliminating these reducing impurities, when present, by redistilling the distillate at a lower temperature.

With this method, the possible range in concentration for the determinations is 0.005 to 0.200 mg HCN; but the most suitable range is 0.01 to 0.10 mg HCN. Use of the smaller range results in readings between 0 and 50 on the A scale of the colorimeter. One can vary the range somewhat, if necessary, by changing the volume of alkaline picrate used for each determination.

This method has been used in determining HCN from fumigated scale insects and walnut-husk-fly pupae. The experimental data on these insects are published in the accompanying paper (Lindgren and Sinclair, 1944).

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