I. THE FLUOROMETRIC DETERMINATION OF MALIC ACID, β-NAPHTHOL AND CITRIC ACID.

II. THE SPECTROPHOTOMETRIC DETERMINATION OF TARTARIC ACID.

By

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I

THE FLUOROMETRIC DETERMINATION OF MALIC ACID β-NAPHTHOL AND CITRIC ACID

HISTORICAL

The construction and widespread use of the modern photofluorometer has signalled a new era in fluorescence analysis. The number of papers published in "Analytical Chemistry" on this subject has been, except for the war years, increasing rapidly.

Year	Papers on fluorescence methods published in "Analytical Chemistry"
1929-1934	0
1935	1
1936	0
1937	2
1938	0
1939	3
1940	4
1941	8
1942	0
1943	4
1944	4
1945	4
1946	2
1947	3

These papers dealt with the determination of the following:

Subject	Number of papers*
Riboflavin	8
Thiamine	7
Beryllium	5
Aluminum	4
Zinc	1
Thorium	1
Uranium	1
Gallium	1
Oxygen	1
Methylnicotinamide	1
Nicotinic acid	1
O-nitrophenol	1
P-aminoacetophenone	1
Chlorophyll a	1

* Some papers treated several determinations.

Concurrently Feigl and his coworkers (11) have developed qualitative fluorescence tests for organic acids many of which are based upon condensation with resorcinol to form fluoroscein or umbelliforone like derivatives. Eegriwe (9) has likewise developed several qualitative fluorescence tests for organic acids based upon condensation reactions.

Photofluorometric determinations of organic acids based upon qualitative tests of Feigl's and Eegriwe's or similar reactions have been of very recent origin. Feigl's spot test for citric acid (10) based upon its conversion into citrazinic acid has been investigated as an analytical method (14). Barr (5) has developed a fluorescence method for determining malic acid based upon its condensation reaction with resorcinol.

STATEMENT OF PROBLEM

The requirement for rapid methods for determination of common organic acids in research and industry is apparent. The gravimetric and volumetric methods presently employed are lengthy and tedious, require in most cases empirical corrections, and in the end are subject to error. Interfering substances are often difficult or impossible to remove.

The development of fluorescence methods of analysis for additional organic acids appears feasible. Those fluorescence methods for organic acids which have been developed are rapid and rather specific.

It was therefore proposed to examine the fluorescence test, proposed by Eegriwe (9), for malic acid using β-naphthol in concentrated sulfuric acid as the reagent. The problem was extended to include the fluorometric determination of β-naphthol after preliminary investigation indicated that the same reaction could be used toward that end.

It was proposed to extend the previous study of the fluorometric determination of citric acid (14) with special regard to mechanism of reaction and application of the method.

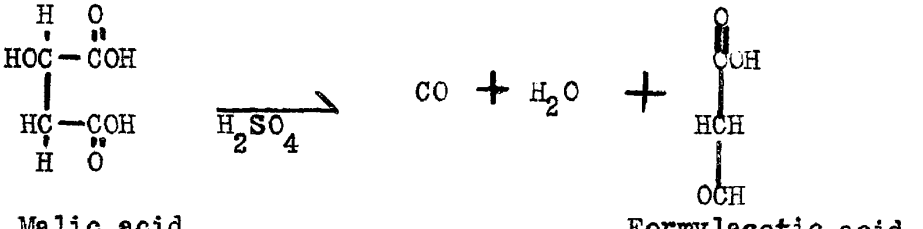
EXPERIMENTAL

A. THE FLUOROMETRIC DETERMINATION OF MALIC ACID AND β-NAPHTHOL.

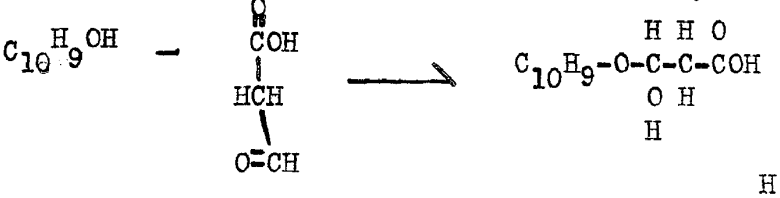
1. Reaction Mechanism.

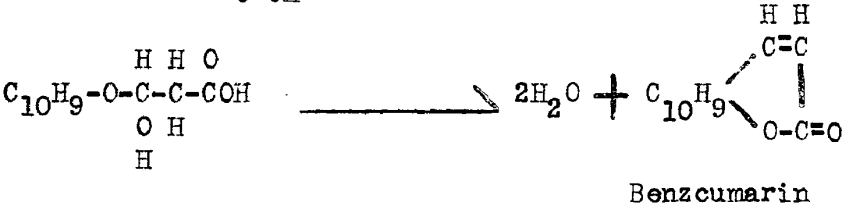
The reaction of malic acid with β-naphthol in concentrated sulfuric acid to give a blue fluorescence was reported by Eegriwe (9) in 1932 as a qualitative test for malic acid. Peckman and Welsh (18) in 1884 had prepared a compound, yielding a blue fluorescence, by the reaction of malic acid and β-naphthol in sulfuric acid. Their compound had a melting point of 146° C. and the empirical formula $C_{13}H_8O_2$.

Peckman and Welsh suggested the mechanism of the reaction to be:

I. 

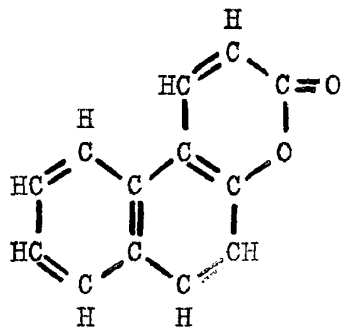
Malic acid — Formylacetic acid

II. 

III. 

Benzcumarin

Boehm and Profft (7) in 1915 demonstrated that two structural forms existed for the isomeric derivatives of malic acid and β-naphthol.



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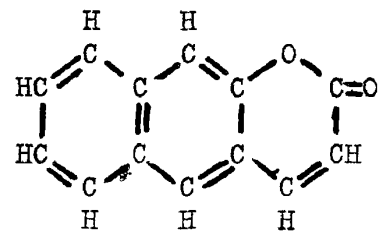
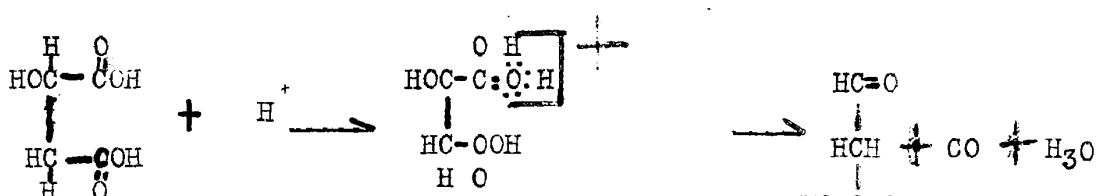


II

They prepared form I, which melts at 118° C. and yields a blue fluorescence, and form II which melts at 163° C. and is not reported as fluorescent, but could not duplicate the work of Peckman and Welsh in regard to the isomer melting at 141° C.

Dey, Rao and Sankaranarayana (8) in 1932 prepared form I, and agreed with Boehm and Profft about its properties. They also reported no success at reproducing the work of Peckman and Welsh.

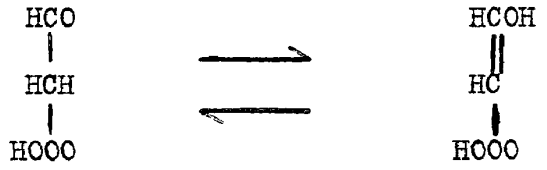
Hammett (12) has demonstrated the decomposition of malic acid by acid catalysis to follow the same general mechanism as outlined by Peckman and Welsh:



Malic acid

Formyl acetate

Formyl acetate has been identified but not isolated (17) and is thought to exist in equilibrium between the following forms:



The existance of formyl acetate as an intermediate in the procedure for the determination of malic acid and β-naphthol is supported by the following experimental evidence.

1. An intermediate is necessarily formed. Fluorescence does not result when malic acid and β-naphthol are added to sulfuric acid in the cold. However, if malic acid is added to sulfuric acid the mixture is allowed to stand several hours, and then β-naphthol is added fluorescence will result.

2. The intermediate formed has characteristics similar to those reported for formyl acetate. It is somewhat stable in cold solutions but unstable in hot. Fluorescence will not result if malic acid is added to sulfuric acid and the mixture heated at a temperature of 100° C. for five minutes before adding β-naphthol. However, the intermediate, after storage for several weeks, will react with -naphthol in the cold to give a blue fluorescence.

2. Apparatus.

The fluorescence intensities were measured with a Lumetron fluorescence meter model 402 EF with 25 ml. cells. The primary filter permitted maximum transmission in the spectral region of 365 millimicrons. The secondary filters consisted of a combination of a yellow filter furnished by the Photovolt Corporation for use in vitamin B_1 determinations which does not transmit emission below 400 millimicrons and a Corning lantern blue filter #5543. This combination of secondary filters permits maximum transmission corresponding to the region of greatest fluorescence of the final fluorescent solution as determined visually. The primary and secondary filters are the same as used in the citric acid determination (14) and are described in greater detail in that connection. The fluorescence standard, a solution of 2.000 g.. of sodium salicylate diluted to 1000 ml., and preserved from mold growth by a few drops of toluene, is the same as described for the citric acid determination and its characteristics are given in greater detail in that connection.

The zero standard for the β-naphthol determination is distilled water. For the malic acid determination it is necessary to prepare a blank of the reagent for this purpose.

3. Variation of the fluorescence intensity of the end-product with temperature and acidity.

Solutions of the end-product were prepared according to the method of Eegriwe (9). Samples of malic acid were evaporated to dryness, treated with a 1 ml. portion of concentrated sulfuric acid containing 25 micrograms of β -naphthol, heated on the steam-bath for 20 minutes, cooled, and diluted to 100 ml. with water.

The fluorescence intensities of a weakly and a strongly fluorescent solution were measured in the fluorescence meter at different temperatures. The data, presented in Table I, indicates that the variation of fluorescence with temperature is small and may be neglected if the temperature is kept within a range of 3 degrees.

TABLE I. Variation in fluorescence intensities with temperature for a weakly and a strongly fluorescent solution.

Temp.	Fluorescence reading of weak solution	Temp.	Fluorescence reading of strong solution
20.0° C.	35.2	22.5° C.	88.2
22.8	35.7	23.5	87.2
23.6	35.3	27.0	86.2
26.0	35.5	30.0	85.3
28.8	35.9		
31.5	34.8		

Aliquots of a strongly fluorescent solution of the end-product were diluted to a definite volume with solutions of sulfuric acid, sodium carbonate or sodium hydroxide to give solutions of varying pH. The fluorescence intensities as presented in Table II

indicate that the fluorescence is rather insensitive to variations of pH on the acid side. If one ml. of 92% sulfuric acid reagent is diluted to 100 ml., as in the standardized procedure the pH of the solution is approximately 0.5.

TABLE II. Variation in fluorescence intensities with pH.

pH	Fluorescence reading
0.2	71.8
0.5	71.3
3	74.0
5	73.5
8	85
12	over 100*

*Fluorescence changes to whitish blue.

4. Variation of fluorescence intensity with different ratios of β-naphthol to malic acid.

Fluorescent solutions of the end-product were prepared by treating dry samples of malic acid with reagent consisting of 1 ml. of concentrated sulfuric acid and varying concentrations of β-naphthol. The samples were heated for 15 minutes on the water bath, cooled, and diluted to 100 ml.

The data is presented graphically in Figure I with fluorescence intensity as a function of the malic acid sample. The curves connect points where equal concentrations of β-naphthol were used.

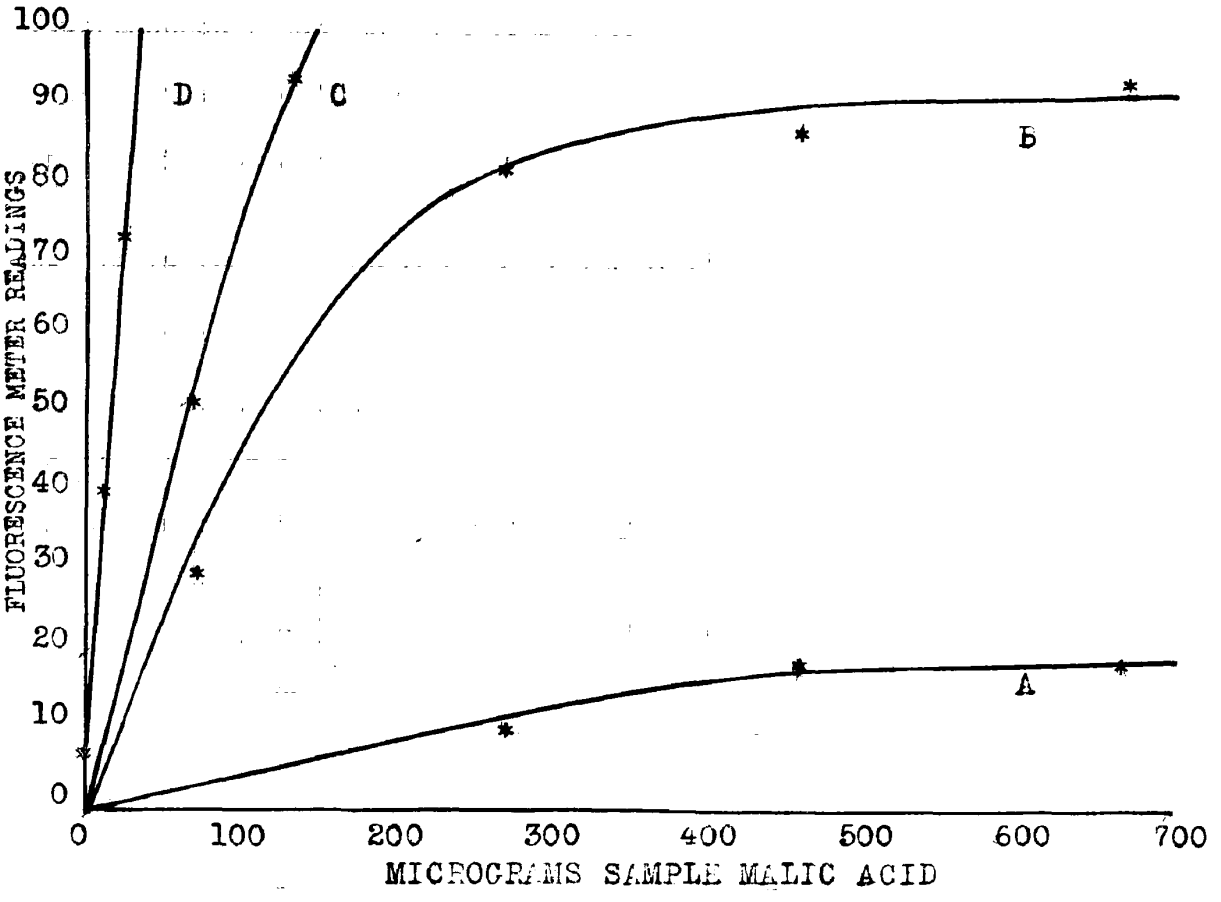


Figure I

Fluorescence intensity readings are given as a function of concentration of β-naphthol and malic acid. The β-naphthol sample in micrograms for each curve is as follows:

A = 25
B = 75
C = 125
D = 250

The instrument is adjusted to zero with the blank solution for each curve except in curve D where greatest possible instrument correction is zero.

From curves A and B it is apparent that for limited amounts of β-naphthol and an excess of malic acid a limiting value of fluorescence intensity is approached. From curves C and D it appears that the fluorescence intensity is a function of the malic acid content when the β-naphthol is in excess.

5. Variation of fluorescence intensity with concentration of sulfuric acid.

TABLE III. Relation of fluorescence intensity to concentration of sulfuric acid. Percentages are by weight.

Sulfuric acid concentration	Fluorescence intensity readings. (parallel samples)
85.2	34.8 33.8
90.5	65.6 63.8
91.7	88.8 88.7
92.5	90.7 92.7
93.9	79.2 78.9
95.1	66.7 65.2

Fluorescent solutions of the end-product were prepared by treating dry samples of β-naphthol for ten minutes at 25° C. with 1 ml. of reagent consisting of an excess of malic acid in

various concentrations of sulfuric acid. The samples were diluted with water to 100 ml.

The fluorescence intensities for the solutions prepared from the sulfuric acid reagent of various concentrations are presented in Table III. The optimum concentration of sulfuric acid for the reaction is approximately 92% by weight.

6. Determination of malic acid.

a. Procedure for analytical method.

In order to facilitate the presentation of the examination of the conditions for the determination of malic acid, the standardized procedure selected for the analytical method is presented first. This procedure is based in part on data which follows.

The standardized procedure is as follows:

A sample containing 1 to 30 micrograms of malic acid is placed in a 10 ml. Erlenmeyer flask and evaporated to dryness in a 105° C. oven. One ml. of reagent consisting of 12 mg. β-naphthol per 100 ml. of 92% sulfuric acid is added, the flask is tipped slightly to insure complete wetting of the bottom surface and the flask is placed in a 90° C. oven for 30 minutes. The flask is removed and cooled. The solution is transferred with water to a 100 ml. flask and brought to volume. The temperature should be 25° $\pm$ 1.5° C. when the fluorescence reading is taken.

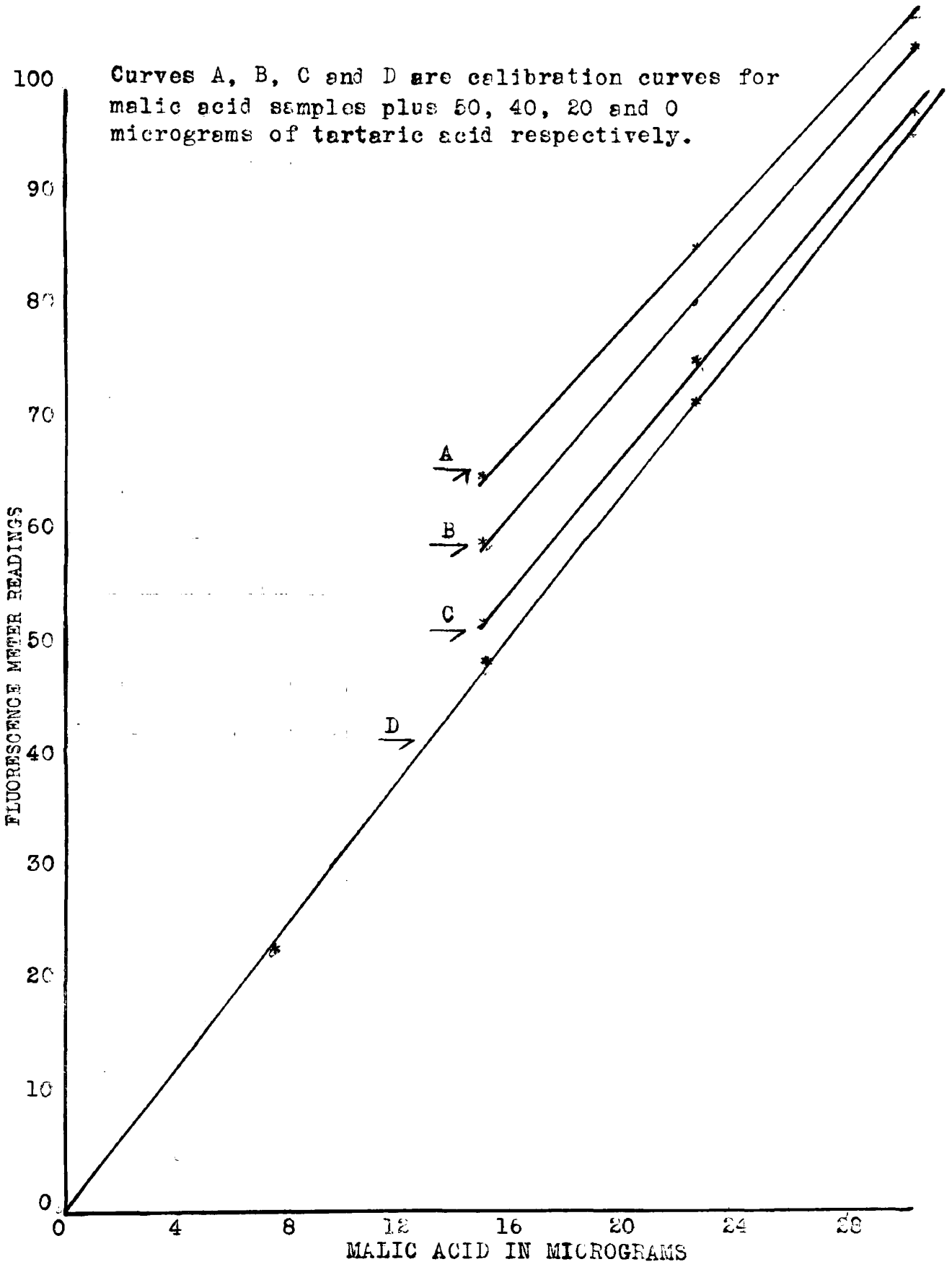


Figure II

Calibration curve

Instrument setting: 0.2% sodium salicylate solution equals 100.

A calibration curve similar to Figure II is prepared from samples of malic acid which are treated in the manner of the procedure. A blank is also run to be used as zero on the fluorescence meter.

b. Reagent.

The concentration of β-naphthol in 92% sulfuric acid to be used for the reagent was selected on the basis of the slopes in Figure I, and the desirability of minimizing the blank reading. It will be noted that curve D has a steep slope but that the blank correction is so large that it cannot be corrected for by the largest correction possible on the fluorescence meter. Curve C has a moderately steep slope and the blank correction can be made by the instrument. Curves A and B have smaller blank corrections but the slopes are not satisfactory.

From these considerations, reagent in the range of 125 micrograms of β-naphthol per ml., appears to display the optimum characteristics. The reagent was usable in every case for at least two weeks when stored in an amber bottle in the refrigerator. One batch showed no change in the calibration curve after storage for ten weeks. Inasmuch as the concentration of β-naphthol is quite critical it is advisable to prepare a new calibration curve with each new batch of reagent.

The β-naphthol used was technical grade which was purified by distillation. The malic acid was technical grade l-malic acid recrystallized from ethyl ether.

c. Time and temperature of reaction.

The fluorescent end-product after being prepared is stable if heated as high as 160° C. However, if the reaction itself takes place at temperatures higher than 110° C. the fluorescence intensity resulting is low. The resulting fluorescence intensity is low if the reaction takes place at less than 80° C. except for very long reaction periods.

The time necessary to reach the maximum reading is shown by Table IV to be 20 minutes at 100° C. and 28 minutes at 80° C.

TABLE IV. Variation of fluorescence intensity readings with time for 80° and 110° C.

Time	37.6 micrograms malic acid at 100° C.	30.1 micrograms malic acid at 80° C.
4 minutes	22.2	0
8	90.2	10.2
12	108*	57.1
16	115*	74.2
20	118*	76.4
24	117*	89.4
28		95.6
32		94.2

*estimated

A comparison of the fluorescence intensity readings obtained at 90° and 100° C. for 30 minute periods, Table V, compare well with those obtained at 80° C. for 28 and 32 minutes.

TABLE V. Comparison of fluorescence intensity readings for reaction at 90° and 100° C. for 30 minutes. 30.1 microgram malic acid samples.

	90° C.	100° C.
Fluorescence intensity readings. Average of 8 samples	95.8	92.4

d. Precision of method.

Table VI lists the fluorometer readings obtained with 31 samples of 30.08 micrograms malic acid each.

TABLE VI. Fluorescence readings from 31 (N) samples of 30.08 micrograms malic acid each.

95.8	98.1	94.7	94.1
95.7	96.2	97.1	93.8
96.1	97.2	97.8	96.3
97.3	93.4	97.2	97.2
96.2	98.2	96.8	95.2
95.3	97.5	97.7	96.5
96.2	97.3	97.2	97.2
93.8	95.0	95.7	

Average reading($\bar{x}$) = 96.3 units or 30.08 micrograms

Average deviation = 1.12 units or 0.33 micrograms

Standard deviation = 1.28 units or 0.38 micrograms

Where standard deviation = $\left[\frac{\sum (x-\bar{x})^2}{N-1}\right]^{\frac{1}{2}}$

Coefficient of variation = 1.3%

Where the coefficient of variation = $\frac{\text{standard deviation x 100}}{\bar{x}}$

Thus 95% of all samples would be expected to fall within 2.6% of the average value ($\bar{x}$) or the calibration curve. If the average of four aliquots is used 95% of the averages would be expected to fall within 1.3% of the values on the calibration curve.

e. Isomeric forms of malic acid.

L-malic acid was used in the investigations. The effect of dl-malic acid was determined when four samples of dl-malic acid, each of 24.8 micrograms, were determined using the procedure for l-malic acid. The results were as follows:

	24.2 micrograms l-malic acid
	24.9
	25.3
	25.9
Average return =	24.9 micrograms.

It is apparent that the isomeric form of malic acid is immaterial in this procedure. The various forms react in the same manner and therefore the total malic acid is determined by this procedure.

f. The interference of organic acids.

The effect of citric, succinic and tartaric acid upon the determination was examined. Table VII demonstrates how a large excess of each acid, approximately 0.5 mg., affects the reading of the blank and a point high on the calibration curve. Citric or succinic acids in this relatively high concentration is observed to have very little influence upon the fluorescence readings.

The changes which varying amounts of tartaric acid cause on the calibration curve are brought out in Figure II. If the tartaric acid to malic acid ratio is 1:1 the fluorescence intensity readings would be from 3% to 5% high. For a 2:1 ratio it is estimated that the readings would be 10% to 15% high and for a 3:1 ratio they would be 20% to 25% high.

TABLE VII. Effect of adding 0.5 mg. quantities of citric, succinic or tartaric acids to the blank and to 30.08 micrograms malic acid.

Acid added	None	Citric	Succinic	Tartaric
Meter reading blank plus added acid.	0.0	0.2	2.8	20.0
Meter reading malic acid plus added acid.	96.3	93.8 96.3	97.2 95.2	200 approximately
Return in micrograms of malic acid.	30.1	29.3 30.1	30.4 29.8	60 approximately

g. The determination of malic acid in apple juice.

The method of the Association of Official Agricultural Chemists (4) for the separation of malic acid from fruit juices was followed through the first separation as the lead salt. The method was modified in that 0.4 of all quantities was used.

In general the protein matter is precipitated by alcohol and separated along with solids from the juices. The malic acid is precipitated as the lead salt, washed, and put back into solution as malic acid by precipitating the lead as its sulfide.

The procedure for the separation is as follows: A sample containing 20 to 75 mg. of malic acid is selected. The volume is brought to 15 ml. with water or by evaporation. The solution is transferred quantitatively to a 100 ml. volumetric flask and brought to volume with 95% ethyl alcohol. The solution is mixed, again brought to volume, and filtered through a folded filter paper covered with a watch glass. Seventy-five ml. of the filtrate is pipetted into a 100 ml. centrifuge tube, and 10 mg. of citric acid and 1 ml. of lead acetate solution are added. The solution is thoroughly mixed, and centrifuged at 1000 r. p. m. for 15 minutes. The supernatant liquid is tested with lead acetate solution for incomplete precipitation. If additional precipitate appears more lead acetate solution is added and the centrifuging is repeated. The liquid is carefully decanted and discarded leaving the precipitate in the centrifuge tube. The precipitate is washed with 75 ml. of 80% ethyl alcohol added in small portions using a stirring rod to insure a homogeneous mixture. The stirring rod is rinsed with the last portion of 80% alcohol. The solution is again centrifuged and decanted as before. The precipitate is suspended by adding 50 ml. of water

in small portions while using the stirring rod for thorough mixing. The solution is saturated with hydrogen sulfide and transferred quantitatively to a 100 ml. volumetric flask. It is brought to volume with water; the contents are well mixed and filtered through a folded filter paper. Aliquots of the filtrate are taken for the determination of malic acid by the fluorometric method described.

Results obtained by determining the amount of malic acid present in apple juice before and after the addition of known amounts of malic acid is recorded in Table VIII. Results are averages of four aliquots.

TABLE VIII. The determination of malic acid in aliquots of apple juice.

Sample	Malic acid found	Malic acid per 5 ml. aliquot	Added	Found less Added
10 ml.	34.2 mg.	17.1 mg.	none	17.1 mg.
	34.4	17.2		17.2
	36.4	18.2		18.2
5 ml.	24.8	24.8	11.6 mg.	13.2
	25.0	25.0		13.4
5 ml.	54.8	54.8	34.7	20.1
	51.7	51.7		17.0
5 ml.	73.6	73.6	57.8	15.8
	73.4	72.4		14.6

h. Discussion.

The method of the Association of Official Agricultural Chemists for the determination of total malic acid requires the complete elimination of other oxidizable materials after which the malic acid is determined by titration with standard permanganate. The procedure is very long and tedious, and the results require an empirical correction.

The Barr (5) fluorometric method cannot be used if citric acid is present. The techniques required for the Barr method are also somewhat rigorous.

The fluorometric method presented herein may be used in the presence of citric acid. Determinations may be run in several hours which compares well with the Barr method and is much faster than approximately 24 hours for the A. O. A. C. method. For pure solutions the accuracy of the two fluorometric methods are comparable whereas the results for the separation and determination of malic acid in apple juice indicate that the fluorometric method described herein is somewhat less accurate than the Barr method.

7. Determination of β-naphthol.

a. Procedure for analytical method.

In order to facilitate the presentation of the examination of the conditions for the determination of β-naphthol the standardized procedure selected for the analytical method are presented first. This procedure is based in part on data which follows.

The standardized procedure is as follows: A sample consisting of 1 to 15 micrograms of β-naphthol dissolved in benzene is measured volumetrically into a 10 ml. Erlenmeyer flask and evacuated to dryness by means of a vacuum dessicator and a water pump. One ml. of reagent consisting of 1 g. of malic acid dissolved in 100 ml. of 92% sulfuric acid and then aged 24 hours is added. The flask is tipped slightly and rotated to insure complete wetting of the sample. The flask is then placed in a 35° to 40° oven for 10 minutes. The contents are transferred quantitatively to a 100 ml. volumetric flask with water and brought to volume. The temperature should be 25° $\pm$1.5° C. when the fluorescence reading is taken.

A calibration curve similar to Figure III is prepared from samples of β-naphthol which are treated in the manner of the standardized procedure. Zero on the fluorescence meter may be adjusted with distilled water.

b. Reagent.

It was observed that while constant amounts of β-naphthol in concentrated sulfuric acid gave constant fluorescence intensities when heated with an excess of malic acid, (see Figure I), the fluorescence intensities produced from a given amount of β-naphthol could be increased about sixfold by the following procedure:

1. add the excess of malic acid to 92% sulfuric acid and allow the

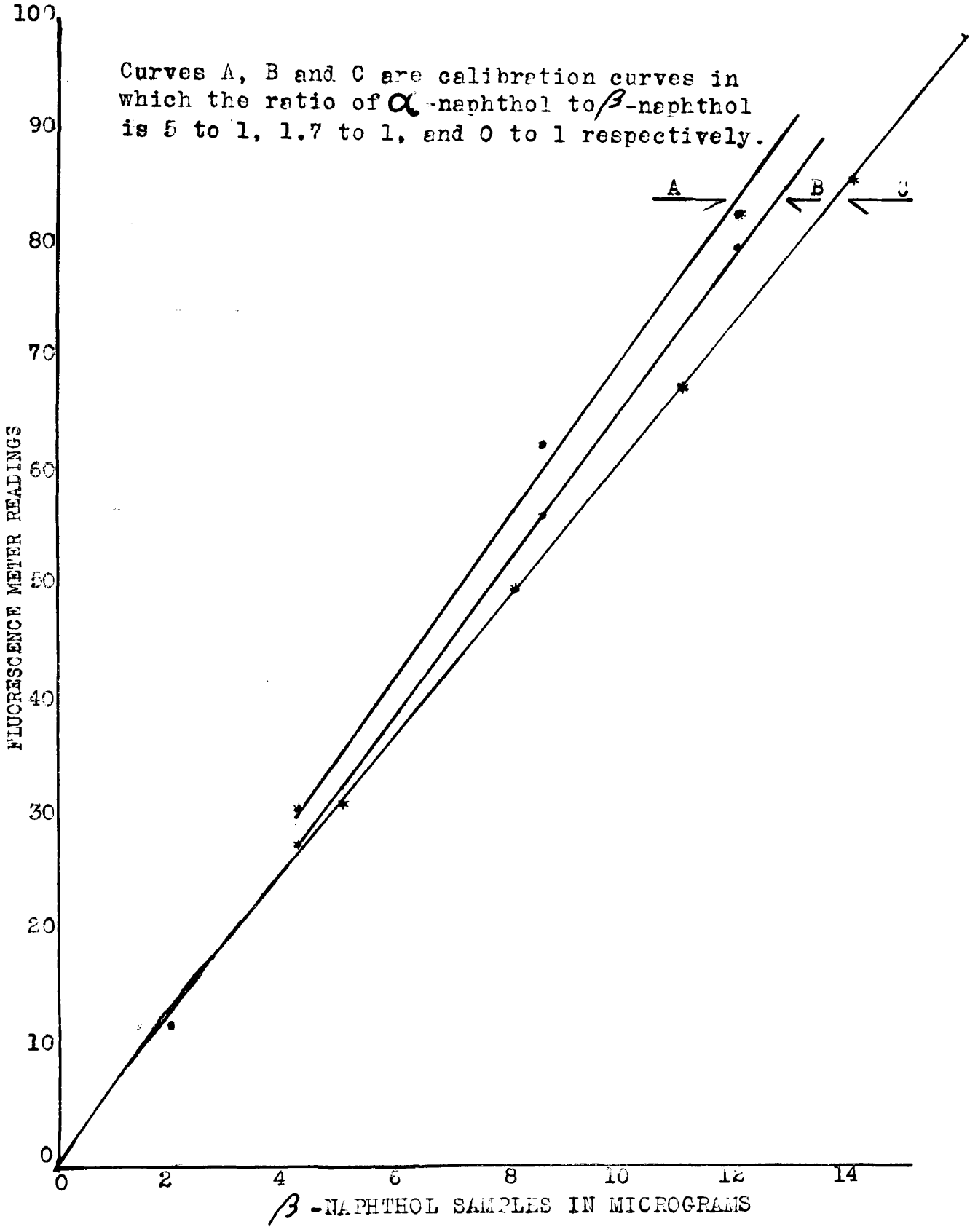


Figure III

Calibration curve

Instrumental setting: 0.2% sodium salicylate solution equals 100.

reagent to age before introducing the sample of β-naphthol.
2. allow the reaction to proceed at slightly higher than room temperature.

The reagent was prepared by dissolving about 1 g. of malic acid in 100 ml. of 92% sulfuric acid. The results obtained by using the reagent after preparation and three hours after preparation are 20% and 65% respectively of the maximum obtained after standing 24 hours. Of three batches prepared on different days from the same dilution of sulfuric acid, there were no deviations in fluorescence intensity readings until ten days after preparation.

The β-naphthol was technical grade purified by distillation. The malic acid was technical grade l-malic acid recrystallized from ethyl ether.

c. Time and temperature of reaction.

From Table IX it is noted that the reaction goes to completion rapidly and a longer reaction period causes no significant variation of the fluorescence intensity.

TABLE IX. Variation of fluorescence intensity with time of heating. 14 microgram samples. Results given are average of 3 samples. Reaction temperature 30° to 40° C.

Time	Fluorescence readings
2 minutes	84.2
3	84.1
10	82.4
20	81.9
60	83.9

The temperature of the reaction is not critical; above 25° C. as illustrated in Table X the results are essentially unchanged. The fluorescence intensity is not increased at reaction temperatures above 40° C. but the possibility of interference due to decomposition of otherwise non-interfering substances is increased.

TABLE X. Variation of fluorescence intensity with temperature. Period is 10 minutes. 14 microgram samples. Results are average of two or three samples.

Temperature	Fluorescence intensity readings
10°	69.3
25	82.4
35-40	84.3
45-50	84.4
55-60	84.0

d. Precision of method.

Table XI lists the fluorometric readings obtained from 48 consecutive 14.00 microgram samples. From the statistical constants obtained from this data it may be predicted that 95% of all results should fall within 3.1% of the calibration curve. If the average of four aliquots is taken 95% of all averages should fall with 1.55% of the calibration curve.

TABLE XI. Fluorescence intensity readings from 48 samples of 14.00 micrograms β-naphthol each.

88.5	85.8	88.5	85.3	85.6	86.3
88.3	87.8	87.3	86.1	86.1	86.7
87.7	88.4	85.4	85.2	86.2	85.7
88.3	86.8	88.4	87.2	86.7	88.7
89.8	87.2	88.8	88.2	88.4	86.2
89.3	85.8	86.2	84.6	85.8	86.1
85.8	87.2	81.0*	86.2	89.4	86.6

* omitted from calculations.

Average reading = 87.1 units or 14.00 micrograms

Average deviation = 1.2 units or 0.19 micrograms

Standard deviation = 1.35 units or 0.22 micrograms

Coefficient of variation = 1.55%

e. Interference of α-naphthol.

From Figure III it may be noted that the addition of comparatively large amounts of α-naphthol to the sample causes only a small error in the determination of β-naphthol. Where the amount of the two isomers are equal the error is approximately 5% positive. If the ratio of α-naphthol to β-naphthol is 5 to 1 the error is approximately 15%. Wherever the ratio of the isomers may be estimated a correction may be applied for the β-naphthol determination.

f. Discussion.

The method presented herein for the fluorometric determination of β-naphthol may be completed in 40 minutes. It can be used in the presence of small amounts of α-naphthol; in the presence of large amounts of α-naphthol a correction may be applied.

A survey of the literature indicates that no comparable method for determining small amounts of β-naphthol exists. For larger amounts of β-naphthol the bromination and back titration of excess bromine is generally used as a means of determination. This is a general method for many phenolic compounds.

B. THE FLUOROMETRIC DETERMINATION OF CITRIC ACID.

1. Reaction mechanism.

Feigl (10) presents the series of reactions in the test for citric acid as those in Figure IV which proceed via path I from citric acid, aconityl chloride, aconitamide, citrazinic acid to ammonium citrazinate.

The validity of Feigl's mechanism was investigated in the following manner:

1. Overall yield of the reaction from citric acid to citrazinic acid was determined.

2. The reaction yields from citric acid to aconityl chloride and aconitamide to citrazinic acid were determined.

Citrazinic acid was prepared by the method of Behrman and Hofman (6). This synthesis involved the preparation of the methyl ester of citric acid, the ammonolysis of the ester to form citramide, and the closing of the ring to form citrazinic acid with sulfuric acid and heat.

Samples of citrazinic acid were weighed out on the microbalance, dissolved in ammoniacal solution, and the fluorescence intensity compared with the calibration curve for citric acid. Table XII illustrates that the total yield in the procedure of the determination is approximately 37%.

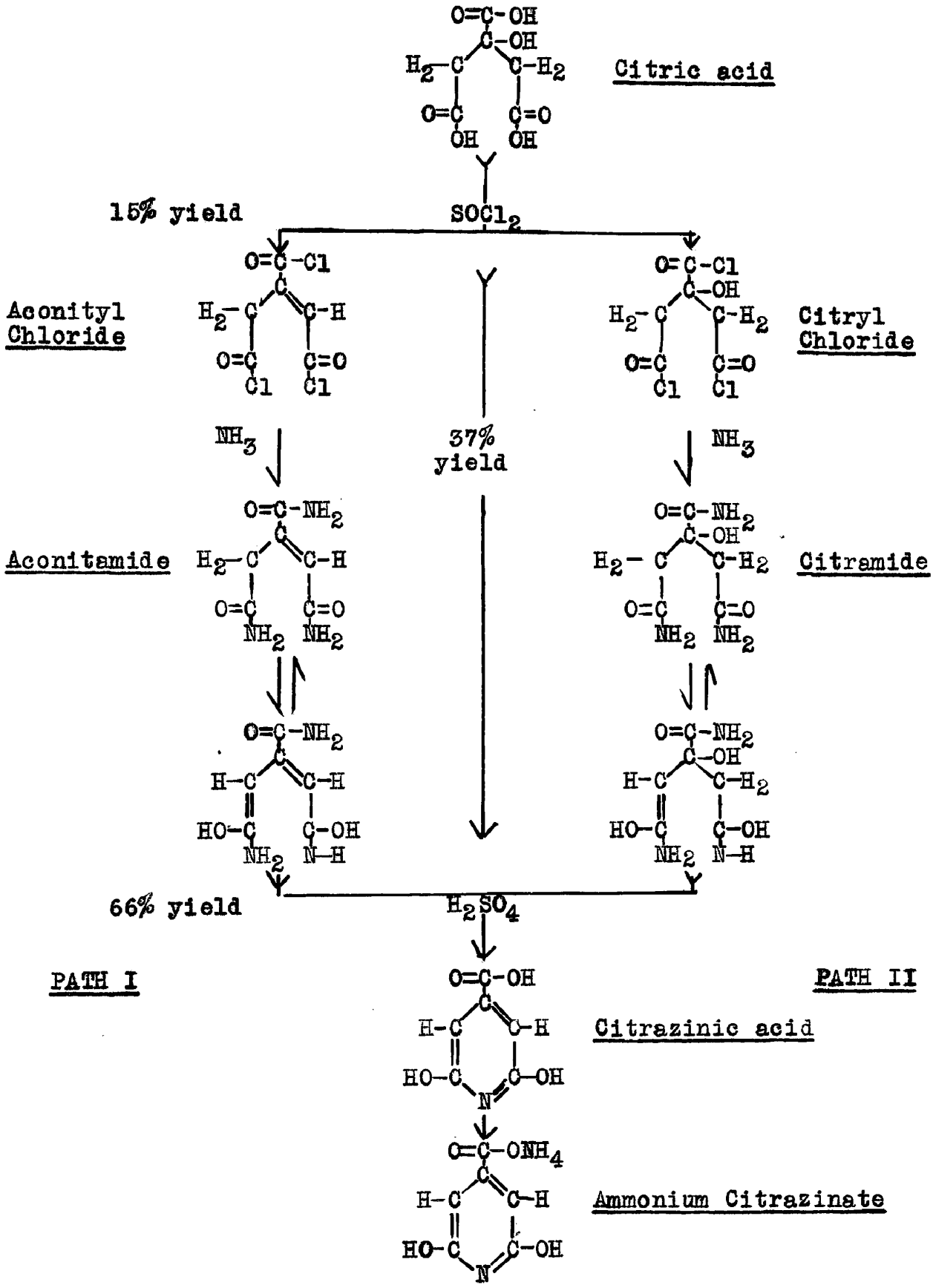

Citric acid
15% yield
$SOCl_2$
Aconityl Chloride
Citryl Chloride
NH_3
37% yield
NH_3
Aconitamide
Citramide
66% yield
H_2SO_4
PATH I
PATH II
Citrazinic acid
Ammonium Citrazinate

Figure IV

TABLE XII. Yield of Citrazinic acid from citric acid.

	I	II	III	IV
Citrazinic acid sample taken	0.960 mg.	0.299 mg.	0.743 mg.	0.854 mg.
Citric acid equivalent to citrazinic acid sample	1.190	0.371	0.921	1.059
Citric acid determined fluorometrically.	3.500	1.010	2.645	2.950
Yield	34.0%	36.7%	38.6%	35.9%

The yield of the reaction from citric acid to aconityl chloride was determined by running 6.33 mg. samples of citric acid through the thionyl chloride reaction of the procedure for determination of citric acid, to produce aconityl chloride. The yield was determined by hydrolyzing the aconityl chloride and determining the aconitic acid polarographically.

TABLE XIII. Polarographic determination of yield of aconityl chloride from citric acid.

	I	II
Citric acid sample taken	6.33 mg.	6.33 mg.
Aconitic acid equivalent to citric acid sample.	5.76	5.76
Aconitic acid found.	0.849	0.870
Yield.	14.7%	15.1%

Acenityl chloride was prepared by the method of Michael and Tissot (16). This synthesis involved the reaction of citric acid and phosphorous pentachloride in a phosphorous trichloride medium. The hydrolysis of the acenityl chloride proceeds at a rapid rate with the evolution of heat. Thus from Table XIV it appears that over 100% of acenityl chloride is present in the samples taken. However, these high results may be accounted for by a partial hydrolysis of the acenityl hydrolysis before the samples were weighed out.

TABLE XIV. Polarographic determination of hydrolysis product of acenityl chloride as aconitic acid.

	I	II
Acenityl chloride sample	2.67 mg.	2.08 mg.
Aconitic acid equivalent to acenityl chloride sample.	1.99	1.60
Aconitic acid found.	2.24	1.98
Yield	112%	123%

The polarographic analysis of aconitic acid was conducted upon a Sargent Model XI polarograph in the manner of Schwaer (20), using 1 N hydrochloric acid as the electrolyte and 0.0024% gelatin as maximum suppressor. The calibration curve is given in Figure V.

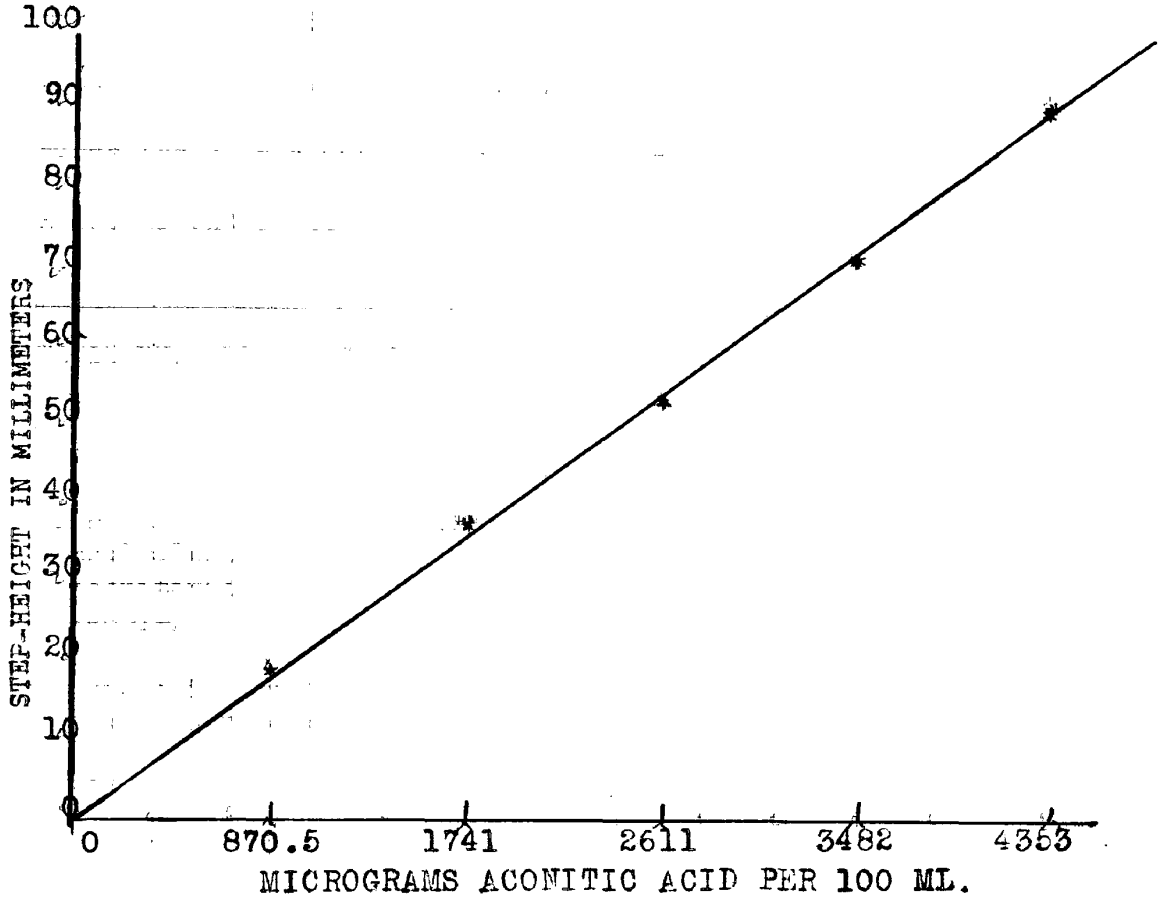


Figure V

Polarographic calibration curve.

Electrolyte = 1 N HCl
Suppressor = 0.0024% gelatin
Drop rate = 2.5 drops per sec.
Sensitivity = 5
Half wave potential = 0.5 volts

Sargent model XI polarograph.

Aconitamide was prepared by treating acontityl chloride with ammonium hydroxide. The yield of the reaction from aconitamide to citrazinic acid was determined by treating samples of aconitamide, as in the procedure for the determination of citric acid, with sulfuric acid and heat. The fluorescence intensity of the final solution was determined and compared with the calibration curve for citric acid. The yield from aconitamide to citrazinic acid, as indicated in Table XV is calculated to be about 65%.

TABLE XV. Yield of citrazinic acid from aconitamide.

	I	II	III	IV
Aconitamide sample	0.206 mg.	0.256 mg.	0.117 mg.	0.164 mg.
Citric acid equivalent to aconitamide sample.	0.231	0.286	0.131	0.184
Citric acid found fluorometrically	0.355	0.458	0.189	0.287
Yield	65.0%	62.4%	69.5%	66.3%

Inasmuch as the yield of the reaction, citric acid to acontityl chloride (15%) is less than the total yield of the series of reactions (37%), the reaction mechanism proposed by Feigl can represent only part of the reaction series. If the ammonolysis of acontityl chloride may be assumed to be 100% (the reaction proceeds rapidly in the cold) an estimate may be calculated of the yield by all other mechanisms.

Thus 15% x 66% or 10% represents the yield by path I, (Figure V). The difference between the total yield and the yield by path I represents the yield by all other mechanisms (27%). Therefore, 27/37 or 73% of the total yield is formed by some mechanism other than that proposed by Feigl (path I).

A plausible mechanism by which a part of the 27% may be produced is the series of reactions in path II, (Figure V). In path II the citric acid is converted by means of a different group of intermediates to citrazinic acid. These intermediates are citryl chloride and citramide. The tertiary hydroxyl of the citric acid wholly or in part, may be converted to the corresponding chloride or amine of the intermediate.

2. Dehydrating agents.

Dehydrite, anhydrous magnesium perchlorate, was used in the drying tube (B, Figure VI), during the development of the fluorometric method for the determination of citric acid. Because the refluxing of thionyl chloride through dehydrite might be critiaized as unsafe several other dehydrating agents were examined.

When Drierite, anhydrous calcium sulfate, was substituted for Dehydrite the fluorescence produced, although of the same intensity as that obtained with Dehydrite, was not as reproducible. Cases of lowered fluorescence intensity were accompanied

by darkening of the solution during the thionyl chloride reaction. This result indicates that a small amount of drying agent was getting in the reaction vessel as the same darkening could be obtained with a small amount of sulfate ion. This condition continued even though a layer of fine asbestos was prepared, as in a gooch crucible, over the glass wool plug of the drying tube.

With Hydralo, anhydrous aluminum oxide, in the drying tube the evacuation of the thionyl chloride did not at any time appear complete. Fuming accompanied the admission of dry air to the reaction vessel. In all cases where Hydralo was employed as drying agent the fluorescence intensities were low.

3. Procedure for determining citric acid in solutions free from interferences.

Reagents:

Thionyl chloride; Eastman #246. It must be protected from the moisture of the air at all times. The practical grade causes lower fluorescence intensity and is not satisfactory.

Sodium carbonate; Anhydrous. C. P.

Ammonia; From a compressed gas cylinder.

Sulfuric acid; 76 to 77; Seventy three ml. of concentrated sulfuric acid is added to 35 ml. of water with cooling. The specific gravity ($20°/4°$ C.) of the solution must be within the range of 1.681 to 1.692.

Citric acid solution; A standard stock solution is prepared by dissolving 7.000 g. of C. P. citric acid monohydrate in water and diluting to 1000 ml. The concentration is checked by titration with standard base. The standard stock solution may be preserved with a few drops of toluene. Two ten-fold dilutions of this standard stock solution give a working standard containing 64 micrograms of anhydrous citric acid per ml. The working standard may be conveniently preserved by making it 0.3 N with respect to hydrochloric acid during the dilution.

A sample solution containing 10 to 75 micrograms of citric acid, preferably 1 ml. or less, is accurately measured into the 25 ml. reaction flask (A, Figure VI). The flask is heated for two hours in a vacuum oven at 65 to 70° C. in order to obtain an anhydrous residue.

Approximately 15 mg. of anhydrous sodium carbonate and 2 ml. of thionyl chloride are added to the sample. The combination reflux condenser and drying tube, (B, Figure VI) is connected to the reaction flask and the flask is heated in an oil bath maintained at 95 to 100° C.

After refluxing for 20 minutes, the reaction flask with the tube attached is removed from the oil bath and the excess thionyl chloride is volatilized and evacuated through the drying tube and the three way stopcock (C, Figure VI). During this operation the reaction flask is given a swirling motion to prevent

spattering of the contents. Gentle heat may be applied to hasten the volatilization. The reaction flask is evacuated for four minutes after the residue appears dry and then air is allowed to flow into the flask by means of the capillary, (C-3, Figure VI). The evacuation and flooding with dry air is repeated three times using one minute periods of evacuation. The flask with the condenser attached is introduced into the ammonia chamber (D, Figure VI) under hood. A slow current of ammonia is passed into the ammonia chamber. The tube is disengaged from the reaction flask by means of the wooden block (E, Figure VI) and lifted from the chamber. The cover is then replaced over the chamber opening. After ten minutes the flask is removed from the ammonia chamber, approximately 2 ml. of 76% sulfuric acid is added, and the flask is tipped and rotated to bring the sulfuric acid in contact with the entire residue.

The flask is then heated for 6 $\pm$0.5 minutes at 162° to 168° C. in an oil bath. At this point the solution should be colorless to straw colored. After removal from the oil bath, the solution is diluted with 5 ml. of water and transferred quantitatively to a 100 ml. glass stopped volumetric flask, using 25 ml. of wash water. The solution is made alkaline to litmus with dilute ammonium hydroxide (6 N), made up to 100 ml., mixed thoroughly, brought to 24° $\pm$0.5° C. and the fluorescence intensity determined with the fluorometer.

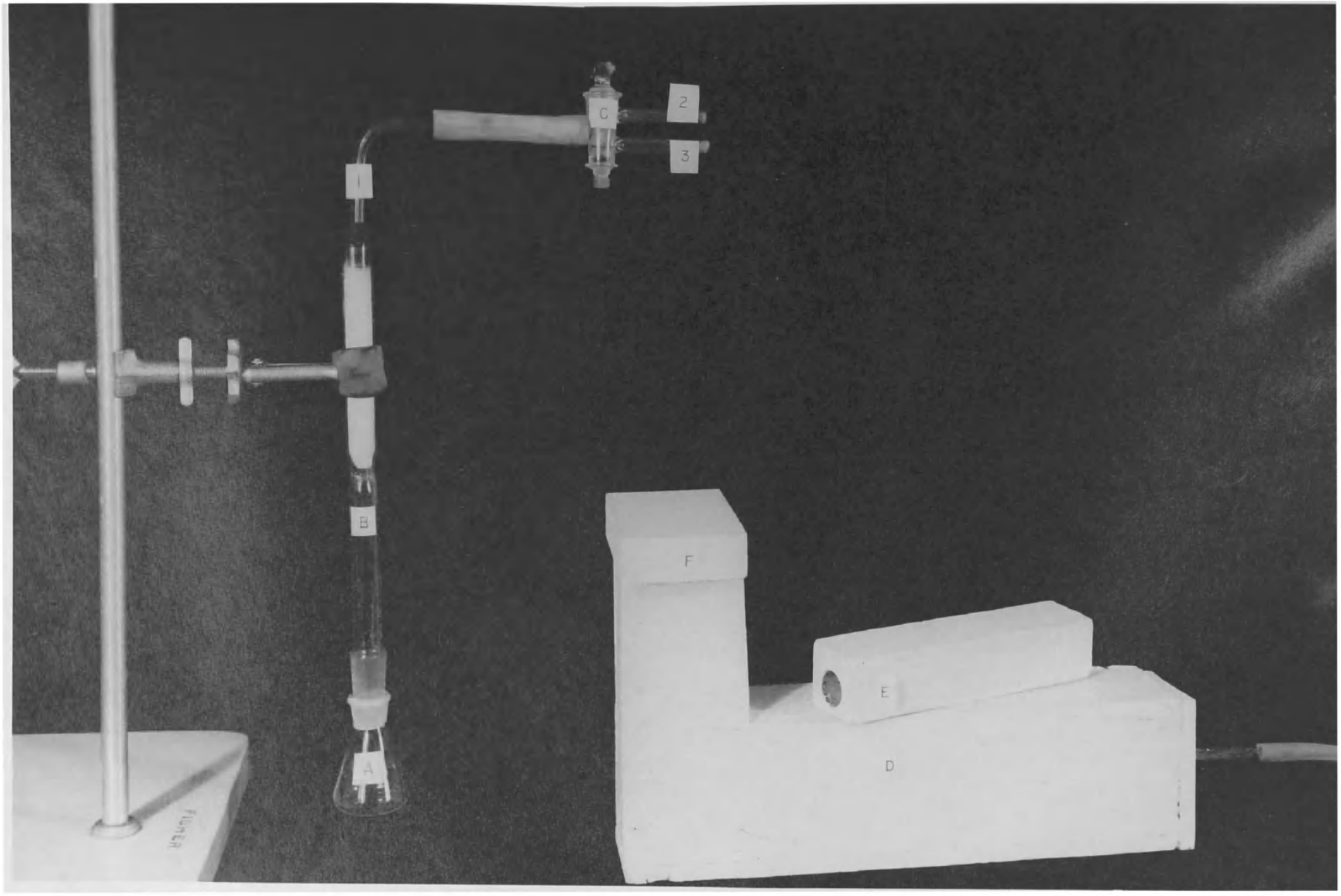


Figure VI

Special apparatus for citric acid determination.

4. Precision of method.

Data obtained in the development of the method (14) has been handled statistically to compute certain constants. The data are reproduced in Table XVI. From these constants the probability of deviations from the average may be predicted. Thus 95% of all determinations should fall within 4.4% of the calibration curve. If the average of four aliquots is taken, 95% of all determinations should fall with 2.2% of the calibration curve.

TABLE XVI. Precision of citric acid determination by fluorometric method. Readings obtained with 31 samples of 60 micrograms citric acid each.

77.8	76.0	78.8	75.2	77.8	80.0	78.0
76.7	76.6	75.6	79.2	74.5	75.6	75.9
78.4	79.8	77.9	79.7	78.7	79.4	77.8
76.1	74.7	76.8	75.6	76.3	70.1*	78.4
78.3	76.9	61.6*				

* omitted from calculations

Average fluorometric reading	=	77.0 units or 60 micrograms
Average deviation	=	1.2 units or 1 microgram
Standard deviation	=	1.6 units or 1.3 micrograms
Coefficient of variation	=	2.2%

5. Interferences.

Compounds which decompose to discolor the solutions resulting from this series of reactions interfere. Tartaric and malic acids fall into this classification. However, as indicated in Figure VII, if tartaric and malic acids are present in amounts less than 100 micrograms per sample taken, the interference is slight; 500 micrograms per sample causes approximately 5% decrease in measured fluorescence intensity. Sulfate ion in very small amounts interferes causing a darkening in the thionyl chloride reaction and a loss of fluorescence. Hydroscopic compounds interfere due to the presence of water of hydration after the drying period.

6. Method for separation of citric acid from citrus juices.

The method of the Association of Official Agricultural Chemists (3) for the separation of citric acid from citrus fruit juices has been modified in that hydrochloric acid is substituted for sulfuric acid and smaller volumes are used. In general, the protein matter is precipitated and filtered along with solids from the juices. The citric acid is precipitated as the lead salt, washed and put back into solution as citric acid by precipitating the lead as the sulfide.

Reagent: Lead acetate solution. Seventy-five g. of normal lead acetate are dissolved in water and 0.5 ml. of glacial acetic acid is added. The solution is made up to 250 ml.

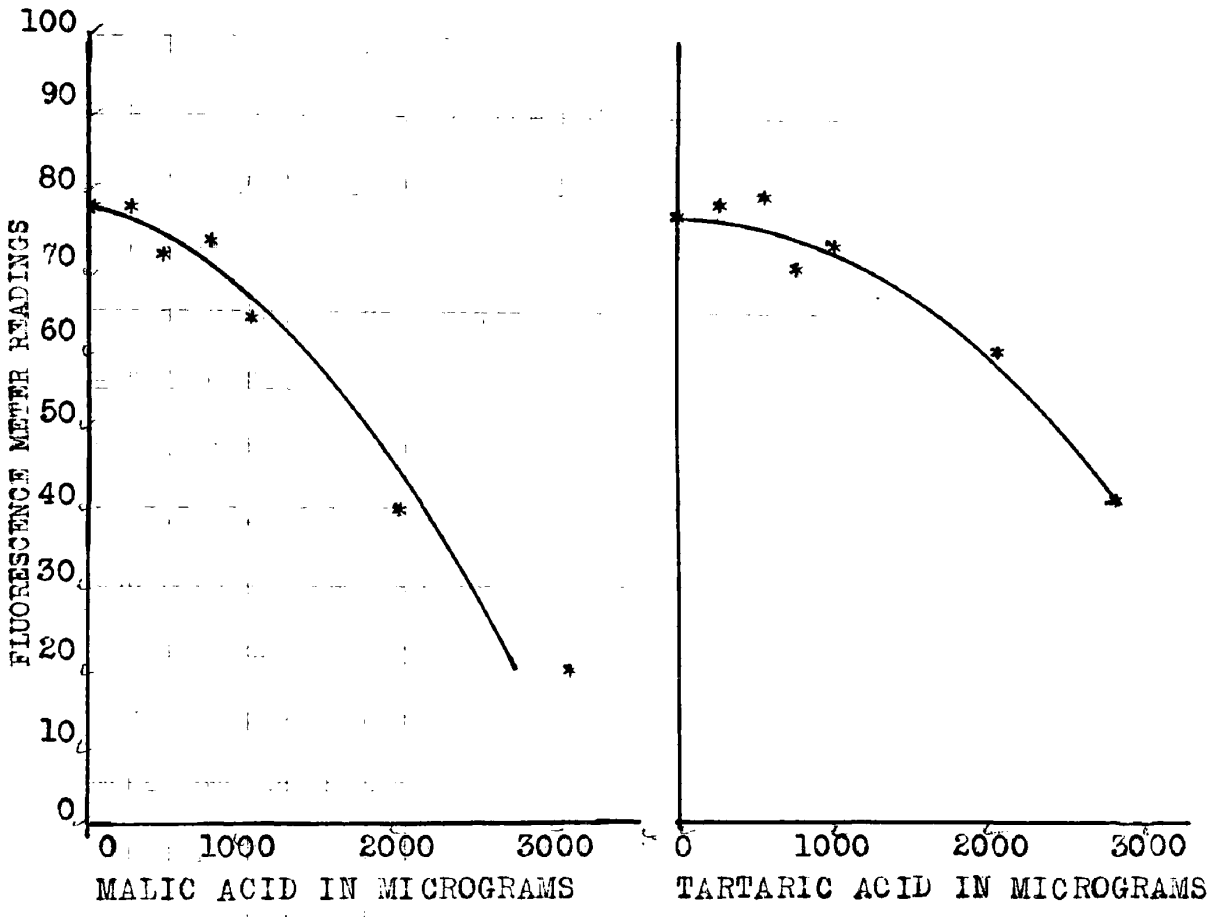


Figure VII

The fluorescence intensity readings for 60 micrograms samples of anhydrous citric acid are plotted as functions of the amount of addition malic or tarteric acid.

METHOD OF SEPARATION: A weight of sample containing 40 to 75 mg. of citric acid is selected. The volume is brought to 15 ml. with water or by evaporation. After adding 1.5 ml. of 1 N. hydrochloric acid, the solution is heated to 50° C., and is transferred quantitatively into a 100 ml. volumetric flask. The flask is cooled, brought to volume with 95% alcohol and the solution is well mixed. The solution is filtered through a folded filter paper covered with a watch glass. Fifty ml. of filtrate is pipetted into a 100 ml. centrifuge tube, 2.5 to 3.0 ml. of lead acetate solution is added, the solution is thoroughly mixed, and centrifuged at 1000 r.p.m. for 15 minutes. The supernatant liquid is tested with lead acetate solution for incomplete precipitation. If additional precipitate appears more lead acetate solution is added and the centrifuging is repeated. The liquid is carefully decanted and discarded leaving the precipitate in the centrifuge tube. The precipitate is washed with 50 ml. of 80% ethyl alcohol added in small portions using a stirring rod to insure a homogeneous mixture. The stirring rod is rinsed with the last portion of the 80% alcohol. The solution is again centrifuged and decanted as before. The precipitate is suspended by adding 50 ml. of water in small portions while using the stirring rod for thorough mixing. The solution is saturated with hydrogen sulfide and transferred quantitatively to a 100 ml. volumetric flask. It is brought to

volume with water; the contents are well mixed and filtered through a folded filter paper. The filtrate is diluted one part to four parts of water. One ml. portions are taken for fluorometric analysis. The citric acid in the original sample is found by multiplying the amount found in one ml. portion by 1000.

The error incurred by passing known amounts of citric acid through this separation and the fluorometric determination is illustrated in Table XVII. Four samples, each containing 63.3 mg. of anhydrous citric acid were used. In each of the four separations four 1 ml. aliquot portions were determined. The errors incurred in these separations and determinations are within the same range as those found in the determination alone.

TABLE XVII. Errors incurred in separation of pure citric acid.

Separation	Citric acid present	Citric acid found	Error
A	63.3 mg.	61.9	
		60.9	
		62.9	
		61.0	
		av. 61.7	2.4%
B	63.3	61.7	
		60.7	
		62.0	
		59.7	
		av. 61.0	3.5%
C	63.3	62.5	
		62.9	
		62.0	
		63.4	
		av. 62.7	0.9%
D	63.3	62.7	
		62.5	
		62.3	
		59.9	
		av. 61.9	2.1%

7. Citric acid determination in citrus juices.

Values obtained by this method for some citrus juices are compared with values obtained by the use of the pentabromoacetone method of the Association of Official Agricultural Chemists (3) in Table XVIII. Each fluorometric result given is an average of four aliquots.

TABLE XVIII. Comparison of citric acid determinations by the fluorometric and A.O.A.C. methods for some citrus juices.

	Citric acid by A.O.A.C. method	Citric acid by Fluorometric method
Canned Grapefruit juice	1.48%	1.54%
	1.48	1.42
	1.47	1.49
	1.43	1.43
Canned Orange juice	0.845	0.858
	0.841	0.820
	0.835	0.863
		0.827
Canned Lemon juice	5.18	5.50
	5.14	5.54
	5.23	5.28
		5.45

8. Discussion.

Three hours are required for a determination by the method described for citric acid free from interferences of which two hours are required for drying the sample. A complete separation and determination of citric acid in citrus juices requires 4.5 hours compared to nearly 24 hours elapsed time for the standard A. O. A. C. pentabromoacetone gravimetric procedure. In the A. O. A. C. method a period of approximately 12 hours is required for complete precipitation of the pentabromoacetone.

SUMMARY

A fluorometric method for the determination of malic acid is presented which requires three hours for the separation and determination of malic acid in apple juice. Samples from one to thirty micrograms may be used and the coefficient of variation for thirty microgram samples of malic acid is 1.3%.

A fluorometric method for the determination of β-naphthol is presented which requires 40 minutes. β-naphthol samples of one to fifteen micrograms are used and the coefficient of variation with fourteen micrograms samples is 1.55%.

A change in the accepted reaction mechanism for the fluorometric analysis of citric acid is proposed. The application of the method to citrus juices is demonstrated.

II

THE SPECTROPHOTOMETRIC DETERMINATION OF TARTARIC ACID

INTRODUCTION

The production of a green color by the reaction of β-naphthol and tartaric acid in concentrated sulfuric acid has been described by Pinerra (19). Similar reactions between tartaric acid and resorcinol (11), and gallic acid (7) in sulfuric acid media have been reported in which colored solutions result.

In connection with the fluorometric determination of malic acid (Part I, A, 6) it was observed that tartaric acid interfered due to the formation of a green-blue color. It was proposed to investigate the color test in which a blue color develops when β-naphthol and tartaric acid are heated in sulfuric acid as a quantitative method.

A rapid method for the determination of tartaric acid would have application inasmuch as the present methods, the bitartrate (1, 2) and the racemate (2), are time consuming. The bitartarate method depends upon a precipitation of tartaric acid as potassium acid tartrate followed by titration with standard base and the racemate method is based upon the precipitation of calcium racemate followed by titration with standard permanganate.

EXPERIMENTAL

1. Apparatus.

The Cenco-Sheard Spectrophotolometer, serial no. 12317, with 1 cm. cells and a 3 millimicron slit opening was used for the preparation of the transmittancy curve, the preliminary investigation of the method and for the analysis of cream of tartar, baking powder and grape juice for tartaric acid. Some comparative measurements were made with the Coleman Universal Spectrophotometer Model 11.

2. Transmittancy curves.

The transmittancy curves for the blue-green colored solutions prepared from measurements on both the Cenco-Sheard and the Coleman Universal Spectrophotometers are given in Figure VIII. From a large number of such curves prepared from colored solutions developed under slightly differing conditions it was observed that the transmittancy between 600 and 660 millimicrons remains essentially unchanged while that from 400 to 600 and from 660 to 700 millimicrons may vary in an unpredictable manner. For this reason, and because the curve is flattest at 620 millimicrons, the wavelength of 620 millimicrons was selected for further measurements.

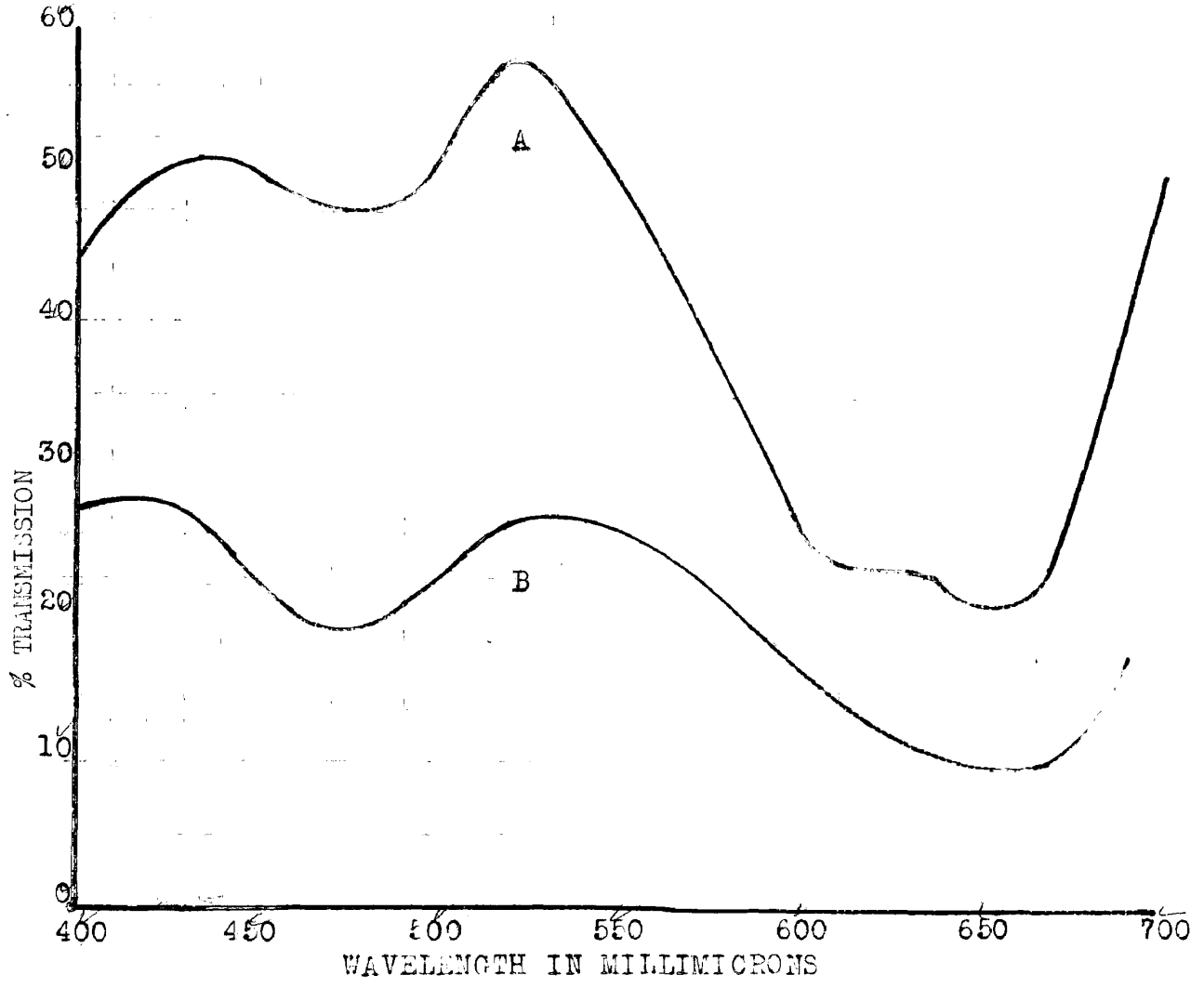


Figure VIII

Transmittancy curves for blue colored solution resulting from treating 250 micrograms of tartaric acid as in the procedure described.

Spectrophotometer	Curve
Cenco-Sheard	A
Coleman	B

3. Standardized conditions selected for analytical method.

In order to facilitate the presentation of the investigation of the conditions of the color development the standardized conditions selected for the analytical method are given first.

Reagents:

91-92% sulfuric acid: Five hundred ml. of 95% sulfuric acid are added to 35 ml. of distilled water. The specific gravity at 20°/4° C. must lie between 1.8195 and 1.8240.

β-naphthol: Technical grade β-naphthol was purified by distillation.

β-naphthol sulfuric acid solution: One hundred mg. of β-naphthol are dissolved in 100 ml. 91-92% sulfuric acid and stored in an amber tinted bottle. Usable for 48 hours after preparation.

80% sulfuric acid: Four parts by volume of 95% sulfuric acid are added slowly and with cooling to one part of water.

The sample containing 20 to 200 micrograms of tartaric acid is placed in a 15 ml. pyrex Erlenmeyer flask and evaporated to dryness in a 110° C. oven. One ml. of β-naphthol sulfuric acid solution is pipetted into the flask. The reaction vessel is heated at 145° C. for 20 minutes. Ten ml. of 80% sulfuric acid are added and the solution is well mixed.

The calibration curve, Figure VIII, is prepared by treating known amounts of tartaric acid as in the procedure for the determination. A blank solution is run simultaneously with each set of

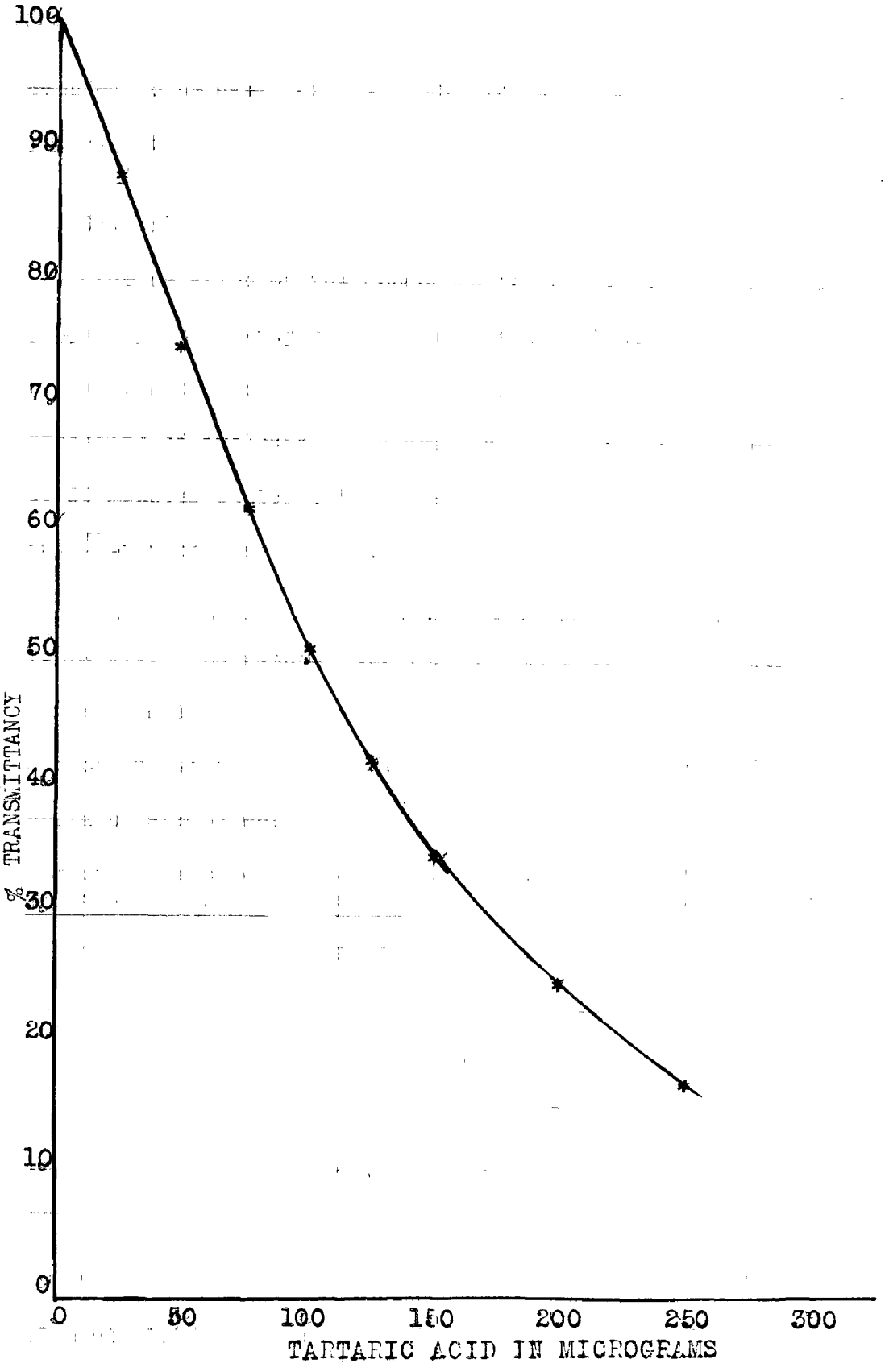


Figure IX

Calibration curve
Cenco-Sheard Spectrophotometer

samples to be used for the "100% transmittancy" setting of the instrument.

4. Reagent.

The relation of the concentration of sulfuric acid used in preparing the β-naphthol sulfuric acid solution was determined by varying the concentration of sulfuric acid in the standardized procedure of analysis. The results in Table XIX for heating period of 20 minutes indicate that the 92% concentration is optimum for the β-naphthol sulfuric acid solution.

TABLE XIX. Instrument readings for 100 microgram samples of tartaric acid as concentration of sulfuric acid and time of reaction are varied. Cenco-Sheard Spectrophotometer. All percentages are by weight.

Period of reaction at 140-145° C.	Concentration of sulfuric acid. 89.0%	90.5%	92.0%	93.5%	95.0%
10 minutes	54.8	48.2	48.8	63.0	69.0
15			51.3		
20	60.0	49.9	52.9	50.2	60.8
25			50.0		
30	67.0	47.8	52.0	50.1	67.0

It was noted that the hue gradually changed from green at 89.0% to blue at 92.0% to blue-black at 95% concentration of sulfuric acid.

In a similar manner, the β-naphthol in the sulfuric acid was varied while the other conditions of the standardized procedure of analysis were observed. The relation of the transmittancy of the final solutions obtained with the α-naphthol concentrations used is illustrated in Table XX. The most intense coloration is produced for the lesser amounts of tartaric acid if less than 1 mg. of α-naphthol per ml. is used. For larger amounts of tartaric acid the most intense coloration is produced with more than 0.75 mg. of α-naphthol. A large excess of β-naphthol, such as 3.00 mg. per ml., produces a change of hue to green.

TABLE XX. Instrument readings for samples of tartaric acid as β-naphthol concentration is varied. Cenco-Sheard spectrophotometer. 1 ml. -naphthol solution used per sample.

Micrograms of tartaric acid	Mg. -naphthol per ml. solution. 0.25	0.50	0.75	1.00	1.25	1.50
50	71.8	71.8	71.8	74.4	75.0	
100	51.5	48.5	49.3	52.5	52.2	54.8
250	28.0	20.0	17.0	16.0	16.9	17.9

The sulfuric acid and β-naphthol may be added separately to the sample. It is inconvenient to measure out 1 mg. portions and therefore the possibility of dissolving the β-naphthol in

benzene was examined. The results obtained were comparable with those obtained later in the investigation by mixing the β-naphthol and sulfuric acid except that occasionally unexplainable variation in color intensity developed.

The prior solution of the β-naphthol in the 92% sulfuric acid was then investigated. The difficulty in the use of such a solution was found to be that sulfonic acid derivatives of β-naphthol are formed which are somewhat colored. This reaction normally causes the reagent to become useless after 24 hours due to the increasingly large blank. It was found that storage in an amber tinted bottle extended the usefulness of the β-naphthol sulfuric acid solution to 48 hours.

5. Reaction time and temperature.

As illustrated in Table XXI, variations in temperature in the range of 120° to 145° C. for the reaction do not cause excessive variations in the instrument readings obtained. Below 115° C. the color development is very slow. Too high a temperature is undesirable for the reaction as it increases the interference due to decomposition of other compounds, such as malic acid, commonly associated with tartaric acid.

Inasmuch as the temperature is not critical an ordinary laboratory constant temperature oven adjusted to 145° C. was found satisfactory, after testing with a number of standard samples.

TABLE XXI. Variation of readings for 100 microgram samples of tartaric acid at various oil bath temperatures. Cenco-Sheard Spectrophotometer. Twenty minute period.

	120-125° C.	130-135° C.	140-145° C.
Instrument readings for 3 samples	50.0	54.2	51.0
	56.0	50.7	54.2
	55.8	53.8	52.3
Average reading	53.9	52.9	52.5

Table XX illustrates that the time for the reaction to reach completion using 91 to 92% sulfuric acid with 1 mg. β-naphthol per ml. sulfuric acid is 10 minutes. Twenty minutes was selected as standard for this step as a longer time does no harm. For the period of 10 minutes, samples if grouped in the oven, may not reach reaction temperature. For the period of 20 minutes, it was found that the oven could be "overloaded"; that is, if over 15 samples were placed in the oven at one time they did not reach reaction temperature and the color intensity was less than expected.

6. Dilution of colored solution.

At the conclusion of the reaction the volume is very close to 1 ml. which was the volume of reagent added to the dry sample. This volume is too small for most cuvettes and therefore the solution must be diluted to a constant volume. Dilution with water causes

instantaneous loss of color. Dilution with sulfuric acid of concentration varying from 60% to 95%, if readings are taken without delay, do not cause a variation in the final color intensity. Dilution with the 60% sulfuric acid is undesirable because there is a gradual change in the diluted solution of the hue from blue to green accompanied by a loss of intensity of 5% per hour. The 95% sulfuric acid presents difficulties in that it is too viscous for ready handling. An 80% solution of sulfuric acid was selected as diluent inasmuch as it lacks the undesirable features of the 60% diluent and is somewhat less viscous than the 95% diluent.

It was found convenient to dilute the 1 ml. of reaction solution by adding with a pipette the desired volume of 80% sulfuric acid. Ten ml. were added for the runs carried out in this investigation.

7. Precision of method.

For the Cenco-Sheard Spectrophotelometer Table XXII gives readings obtained from 30 consecutive 100 microgram samples and the statistical data obtained therefrom. From these data it is estimated that 95% of samples will fall within 8% of the mean. If averages of four aliquots are taken 95% of results would be expected to fall within 4% of the mean.

TABLE XXII. Precision of method using Cenco-Sheard Spectrophotometer. Instrument readings for 30 samples of 100 micrograms tartaric acid each.

52.5	49.2	50.0	51.5	53.2	53.0
51.3	51.0	50.0	51.1	54.8	51.8
53.0	52.6	52.1	50.9	50.0	51.3
53.0	50.8	50.7	50.4	51.2	52.8
49.0	51.4	52.9	51.0	51.8	51.7

Average reading: 51.5 units or 100 micrograms

Average deviation: 1.01 units or 2.5 micrograms

Standard deviation: 1.62 units or 4 micrograms

Coefficient of variation: 4%

For the Coleman Spectrophotometer Table XXIII gives readings obtained from 30 consecutive 75 microgram samples and the statistical data obtained therefrom. The difficultyin the use of the Coleman instrument is that the readings appear to shift from day to day so that for each day a new calibration curve would be required. The calibration curve for the Cenco-Sheard spectrophotometer remained constant for the length of the investigation. Some determinations run on the Coleman spectrophotometer, when run simultaneously with the preparation of the calibration curve, gave substantially the same results as those obtained with the Cenco-Sheard instrument (Table XXII).

TABLE XXIII. Precision of method using Coleman Spectrophotometer. Instrument readings for 30 samples of 75 micrograms tartaric acid each.

41.8	46.8	46.0	39.8	42.0	45.0
43.3	45.2	46.2	44.8	40.9	41.5
43.0	43.1	42.0	41.5	45.8	41.1
42.6	42.6	38.2	42.2	42.2	44.3
42.8	44.5	41.2	40.2	39.8	41.0

Average reading:	42.8 units	or 75 micrograms
Average deviation:	1.72 units	or 3.0 micrograms
Standard deviation:	2.13 units	or 3.53 micrograms
Coefficient of variation:	4.7%	

For the Coleman spectrophotometer 95% of the samples would fall within 9.4% of the mean and if the average of four aliquots is taken the results would fall within 4.7% of the mean.

8. Interferences.

Possible interference by acids sometimes found associated with tartaric acid was investigated. Succinic acid and citric acid do not interfere if present in great excess. Figure X demonstrates that amounts of malic acid, approximately equal to the weight of tartaric acid, must be present before interference is noted. As the amount of malic acid is increased the color intensity is first

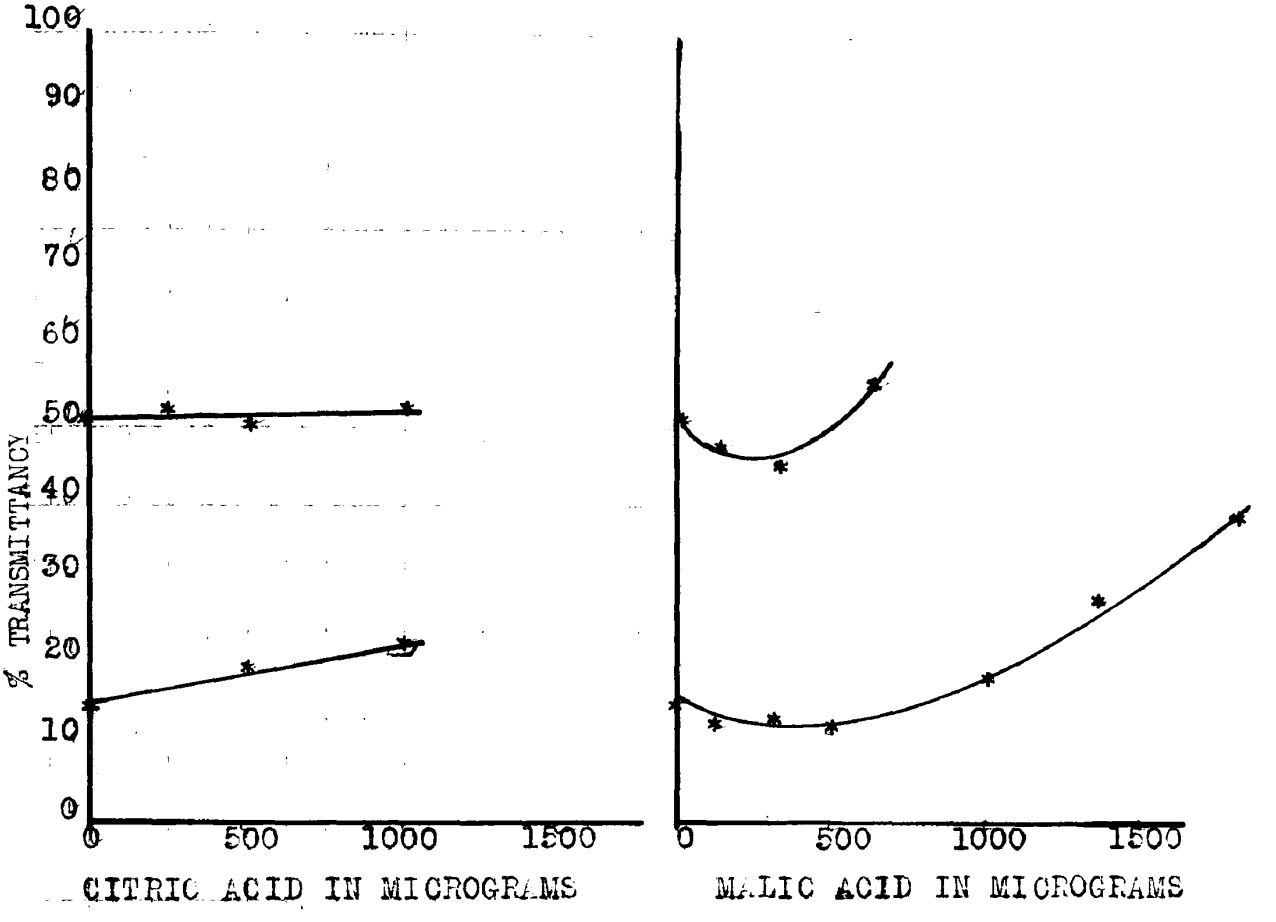


Figure X

Transmittancy readings for 100 and 250 microgram samples of tartaric acid are plotted as functions of the amount of citric or malic acid added. Coleman spectrophotometer.

increased and then as the ratio of malic acid to tartaric acid approaches three the intensity begins to decrease.

9. Application of method to potassium acid tartrate and baking powder.

The method was tested on potassium acid tartrate, (cream of tartar) without prior separations. A solution of pure potassium acid tartrate was prepared by dissolving 253 mg. of potassium acid tartrate in 100 ml. of distilled water. Samples of 0.05 ml. portions or 100.9 micrograms as tartaric acid were used. Table XXIV presents the analysis of the samples for tartaric acid. It is apparent that the calibration curve could be prepared from potassium acid tartrate as well as from tartaric acid.

TABLE XXIV. Determination of tartaric acid content of pure potassium acid tartrate. Theoretical calculated: 100.9 micrograms per sample.

Instrument	Micrograms determined	
Cenco-Sheard		97.4
		96.8
		102.3
		100.3
	Average	99.2
Coleman		106.4
		105.2
		91.2
		100.0
	Average	100.7

The baking powder was analyzed for tartaric acid by the bi-tartrate method of the Association of Official Agricultural Chemists (1), as well as the spectrophotometric method described herein. The samples for the spectrophotometric was prepared for analysis by dissolving 0.250 g. of baking powder in 25 ml. Samples of 0.05 ml. portions were taken for analysis. Table XXV illustrates the results from these parallel analysis.

TABLE XXV. Comparison of analysis of baking powder for tartaric acid by A. O. A. C. and spectrophotometric method. Cenco-Sheard spectrophotometer.

Determination by A.O.A.C. method	Average of 3 aliquots, Spectrophotometric method
28.8%	28.9%
29.2	29.4

10. Application of method to grapejuice.

The method of the Association of Official Agricultural Chemists (2) for the separation of tartaric acid from fruit juices has been modified in that the factor of 0.4 was used in all volumes. Also, the separation was carried through 1, 2 or 3 precipitations as lead tartrate as indicated in Table XXVI. In general, the protein matter is precipitated and filtered along with solids from the juices. The tartaric acid is precipitated as the lead salt, washed and put

back into solution as tartaric acid by precipitating the lead as the sulfide.

Reagent: Lead acetate solution; Seventy-five g. of normal lead acetate are dissolved in water and 0.5 ml. of glacial acetic acid is added. The solution is made up to 250 ml.

METHOD OF SEPARATION: Twenty ml. of grapejuice are selected as sample. The volume is brought to 15 ml. by evaporation. After 1.5 ml. of 1 N sulfuric acid, the solution is heated to 50° C., and is transferred quantitatively into a 100 ml. volumetric flask. The flask is cooled, brought to volume with 95% alcohol and the solution is well mixed. The solution is filtered through a folded filter paper covered with a watch glass. Fifty ml. of filtrate is pipetted into a 100 ml. centrifuge tube, 1.2 ml. of lead acetate solution is added, the solution is thoroughly mixed, and centrifuged at 1000 r.p.m. for 15 minutes. The supernatant liquid is tested with lead acetate solution for incomplete precipitation. If additional precipitate appears more lead acetate solution is added and the centrifuging is repeated. The liquid is carefully decanted and discarded leaving the precipitate in the centrifuge tube. The precipitate is washed by adding 50 ml. of 80% ethyl alcohol in small portions using a stirring rod to insure a homogeneous mixture. The stirring rod is rinsed with the last portion of the 80% alcohol. The solution is again centrifuged and decanted as before. The precipitate is suspended

by adding 50 ml. of water in small portions while using the stirring rod for thorough mixing. The solution is saturated with hydrogen sulfide and transferred quantitatively to a 100 ml. volumetric flask. It is brought to volume with water; the contents are well mixed and filtered through a folded filter paper. Aliquots of this solution are taken for spectrophotometric determination as indicated in Table XXVI after one precipitation.

Twenty ml. of the filtrate from the first precipitation, described above, are heated to boiling to expel hydrogen sulfide. The solution is transferred to a 100 ml. centrifuge tube with alcohol and brought to a volume of 100 ml. with alcohol. One ml. of lead acetate solution is added. The solution is mixed, centrifuged as before, decanted, water added as before and the tartaric acid liberated as before with hydrogen sulfide. The solution is brought to a volume of 100 ml. as in the first precipitation, and filtered. Aliquots of the filtrate were taken for spectrophotometric determination as indicated in Table XXVI after two precipitations.

Fifty ml. of the filtrate from the second precipitation were evaporated to 20 ml. and treated as described for the second precipitation. Aliquots of the filtrate from the third precipitation were taken for Table XXVI after three precipitations.

The spectrophotometric method is compared to the bitartrate method for tartaric acid in Table XXVI for commercial grapejuice.

TABLE XXVI. Comparison of determination of tartaric acid in grapejuice by A. C. A. C. and spectrophotometric methods. Cenco-Sheard spectrophotometer. All spectrophotometric figures are average of 3 aliquots.

% tartaric acid by A.O. A.C. method	% tartaric acid by spectrophotometric method. After 1 precipitation	After 2 precipitations	After 3 precipitations
0.442	0.606	0.469	0.448
0.452	0.613	0.498	0.464
0.441	0.606	0.488	
0.448		0.482	
0.443		0.476	
		0.480	

11. Discussion.

The spectrophotometric method is not as precise as the bitartrate method. However, the spectrophotometric method is more rapid; both the analysis of cream of tartar and baking powder for tartaric acid may be completed within one hour whereas the bitartrate method requires an elapsed time of nearly 24 hours. For the grapejuice analysis the spectrophotometric method may be completed within 3 hours whereas the bitartrate method requires about 24 hours elapsed time. The difference in the results between the A. O. A. C. method and the spectrophotometric method after two precipitations may be due to the A.O.A.C. method being low. Hartman and Hillig (13) average about 2% low when using the bitartrate method for fruit juices. When the same method is used for baking powders an empirical positive correction is called for.

SUMMARY

A spectrophotometric method for the determination of tartaric acid is presented which requires four hours for the separation and determination of tartaric acid in grapejuice. Tartaric acid samples of 20 to 250 micrograms may be used with a coefficient of variation of 4% for 100 micrograms samples.

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