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# A study of the acetic anhydride method for the determination of citric acid

Russell Kent Odland

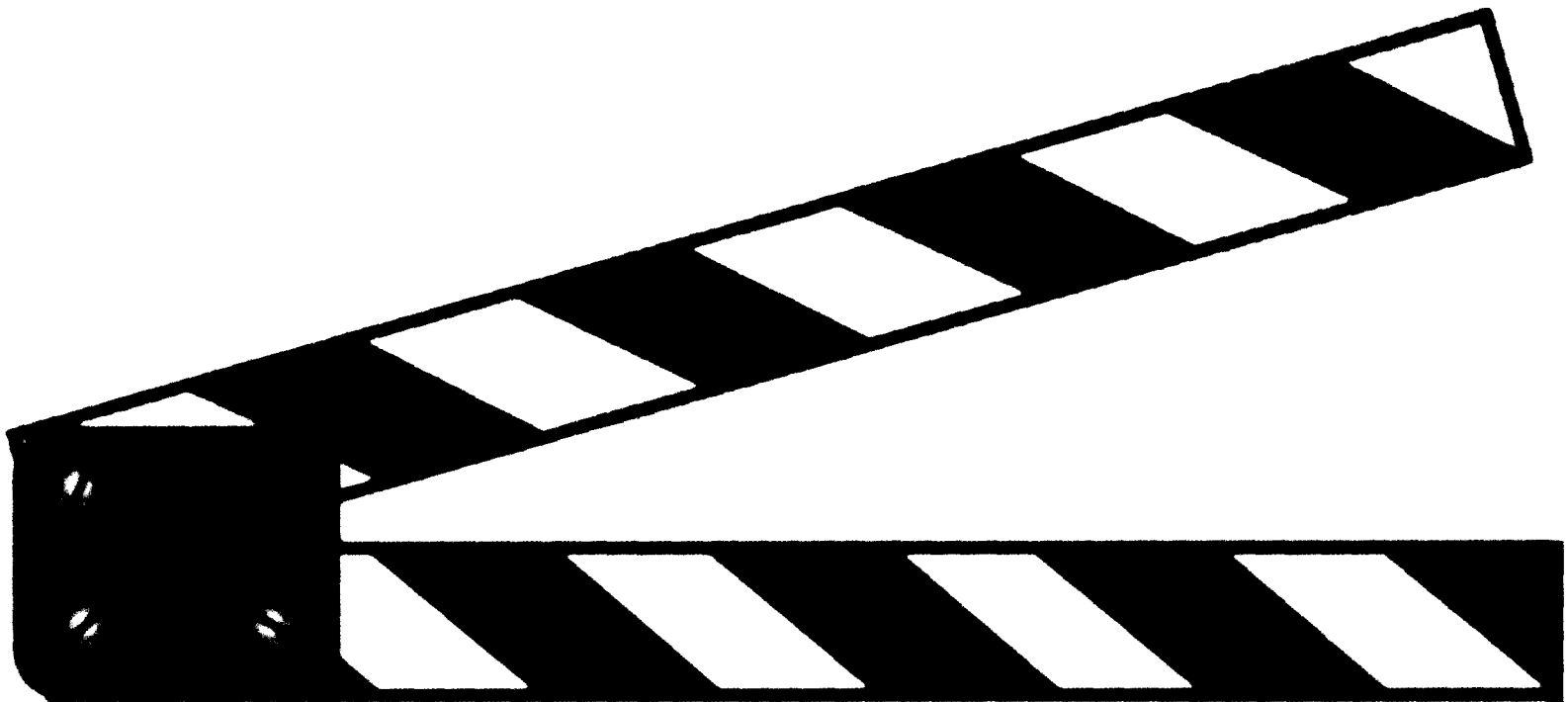
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A STUDY OF THE ACETIC ANHYDRIDE METHOD  
FOR THE DETERMINATION OF CITRIC ACID

BY

RUSSELL KENT ODLAND

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

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#### DEDICATION

This thesis is dedicated to my loving wife, Barbara, and son, Patrick Kent, whose understanding and long patience have made the completion of this work possible.

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## INTRODUCTION

Citric acid is an important compound of many systems. Numerous methods, both qualitative and quantitative, have been used for the determination of the compound. Some of the biological systems investigated include fermentation studies (25), action on yeasts (44), antioxidant in fruits (55), additive in bakery goods (26) and salad dressing preparations (23), amounts in vegetables, fruits (24), milk (54), and grain foods (5). Citric acid in human bone (18), blood and urine (9), muscles (3), teeth enamel (16), liver (21), and brain (48) have been studied. Aside from the biological systems, citric acid has been studied in soap (30), polymer catalysis (28) and reactions (47), tanning (6), cosmetic uses (7), coatings on glass (40), cleaning metals electrolytically (14), metal complexes (49), effect of gamma rays upon (27), and buffer solutions (29). The above examples are not complete; they only show the varied applications of citric acid.

The purpose of this project was to investigate a quantitative spectrophotometric method using anhydrous conditions based on the citric acid-acetic anhydride-pyridine reaction



of Furth-Herrmann (15). Conditions affecting the reaction were investigated and optimized for maximum sensitivity and minimum reaction time. The precision and accuracy of the method were determined, along with interfering substances. A recovery study was made using a tobacco sample. Finally, the pigment formed was characterized by NMR, IR, MS, empirical formula, and molecular weight determinations.

## HISTORICAL

An attempt has been made to show the spectrophotometric methods used for the determination of citric acid. Some advantages and disadvantages of the methods incorporating acetic anhydride and/or pyridine are discussed in detail.

Methods, other than spectrophotometric, for the detection of citric acid include column (42), paper (33), thin-layer (22), and gas chromatography (19). Polarographic (13), enzymatic (34), and potentiometric (37) methods have also been used for the analysis of citric acid. These methods show the wide range of analytical techniques available.

Pucher, Vickery, et. al. (53,39,38) developed one of the first quantitative spectrophotometric methods which is based upon the oxidation of citric acid with potassium permanganate with bromine added, to form pentabromoacetone. The pentabromoacetone was extracted and treated with sodium sulfide. The color produced was measured colorimetrically. This method of producing the pentabromoacetone, with modifications (13) after making the pentabromoacetone, is still used today.

Draganic (12) developed a method using copper benzidine to detect citric, oxalic, tartaric, malonic, lactic, glycolic,

formic, and succinic acids. Crisan and Krausz (11) detected citric, oxalic, and tartaric with lead and ethylenediamine-tetraacetic acid.

Furth and Herrmann (15) were the first to observe the reaction between citric acid, pyridine and acetic anhydride in 1935. Their method was a color test and not quantitative. They also noted that tartaric and aconitic acids gave distinct colors. Casares-Lopez (8) noted that agaric acid produces violet color with these reagents.

Roeder (43) found that instead of pyridine, if one used aliphatic amines, alkali salts of acetic acid, nicotine, quinoline or strychnine, they produced the color complex. Arreguine (1) found that when citric acid was heated in acetic anhydride without any base, a red color was produced. None of the above methods involving the Furth-Herrmann reaction were quantitative.

Saffran and Denstedt (45) developed the first quantitative method for determining citric acid using a modified Furth-Herrmann reaction. In this method water was present, whereas the original Furth-Herrmann test was an anhydrous test. The method was used on kidney and liver homogenates and blood plasma. Trichloroacetic acid was used to precipitate the protein which was found to interfere with the color development.

In the Saffran and Denstedt method, a one ml aqueous

solution that contained 5% trichloroacetic acid and 15-400 micrograms of citric acid were used for the sample. Acetic anhydride (8 ml) and pyridine (1 ml) were added and the sample placed in a water bath at 60°C for 10 minutes. The sample was cooled for 5 minutes in an icewater bath and the absorbance was measured with a Filter 420 or 400. Compounds found to interfere were: tartaric, itaconic, and isocitric acids. Noninterfering compounds were: glucose, urea, glutathione, and succinic, ascorbic, oxalic and malonic, fumaric, pyruvic, and l-malic acids. Beer's Law was not followed and it was suggested that known citric acid samples be run simultaneously with unknown samples. Because of the exothermic nature of the reaction, a water bath was necessary to maintain the temperature at 60°C. The reaction time was low(30 minutes) but the sensitivity of the method was not adequate.

Babad and Shtrikman (2) applied the above method to milk and skim milk powder. The methodology was the same as Saffran and Denstedt (45) and no modification of the procedure was given. Nekhorocheff and Wajzer (36) expanded the Furth-Herrmann reaction to make it applicable to cis-acnitic acid. Instead of pyridine for the base, ammonium hydroxide was used. To 0.5 ml of aqueous cis-acnitic solution to be tested, ammonium hydroxide and 2 ml of ethanol were added and the solution was evaporated to dryness. This procedure was repeated 3 more times, then the dry sample was cooled to 0°C; 3 ml 95% ethanol and 0.5 ml of a mixture of pyridine and acetic anhydride

(3.5:1.5 v/v) were added. The absorbance was measured at 370 millimicrons. The same procedure was followed for citric, except the reagents were added at ambient temperature instead of 0°C. The difference of the two absorbance measurements, at the two temperatures, gave the citric acid concentration. No interfering substances were investigated and the procedure required 50 minutes to complete. Because of the citric acid not reacting at 0°C., the method differentiated between citric and cis-aconitic acids.

Godin (17) used a pyridine-acetic anhydride mixture (7:3 v/v) as a spray reagent for the detection of several organic acids on paper chromatograms. Those acids included: cis and trans-aconitic, itaconic, and fumaric. Reinart and Nesbitt (41) investigated the recovery of citric acid using the Babad and Shtrikman (2) method for milk.

Marier and Boulet (31) did an extensive study on citric acid in milk and related milk products. To a one ml aqueous sample that contained 25-200 micrograms of citric acid, 1.30 ml pyridine and 5.70 ml acetic anhydride were added. The sample was immediately placed in a constant temperature bath at 32°C. After 30 minutes the sample absorbance was measured at 420 millimicrons. The effects of varying amounts of water, pyridine, and acetic anhydride were shown with respect to the sensitivity and stability of the reaction. Various reaction temperatures were investigated using the optimum reagent

concentration. A recovery study using milk samples was performed and possible interfering substances such as trichloroacetic acid, hydrochloric acid, sodium hydroxide, phosphate, and calcium chloride were studied. It was suggested that trisodium citrate dihydrate be used for the standard instead of free citric acid because of the high purity of the salt. The Marier and Boulet (31) method represents the fastest, most sensitive, and accurate procedure for the determination of citric acid using the Furth-Herrmann reagents in an aqueous system. Marier and Boulet (32) modified the method for milk in the presence of casein.

Hartford (20) expanded the Marier and Boulet (31) method to include itaconic, aconitic and fumaric acids. Beer's Law was not followed for the citric acid curve. The method does not distinguish quantitatively between citric and aconitic acids (measured at 435 millimicrons), nor between itaconic and fumaric acids (measured at 385 millimicrons). All conditions were the same as Marier and Boulet (31) with the exception of measuring the acids at different wavelengths.

White and Davies (54) compared the Saffran and Denstedt (45) method with the Marier and Boulet (31) method for analysing citric acid in milk and found the latter method superior in terms of speed and accuracy.

Choy, Quattrone, and Elefant (10) were the first to develop a quantitative method that used the anhydrous Furth-Herrmann reaction. A 2 ml aqueous standard had 4 ml of pyridine added and the solution was evaporated to near dryness on a steam bath.

Sodium sulfate and pyridine were added and the solution was filtered through a glass wool plug into a test tube marked at 8 ml. The solution was diluted to 8 ml mark with pyridine, and 4 ml of acetic anhydride was added. The reaction took place at room temperature for 30 minutes. The absorbance was measured at 385 millimicrons. For protein-containing samples, such as milk and dairy products, the sample was extracted with methanol and passed through a Florisil packed chromatographic column. An aliquot of the eluate was heated on a steam bath until dry and the above procedure was followed. Studies showed that acetic, propanoic, butanoic, valeric, caproic, and lactic acids did not interfere. No data were given to justify their selection of reaction time and the optimum amounts of each reagent. And apparently no studies were made on the stability of the reaction, effects of water, and the nature of the product formed. The amount of citric acid required for the reaction was 10-100 micrograms. This method is superior to the Marier and Boulet (31) procedure because of the increase in sensitivity and simplification of the sample preparation steps.

Valentinis (52) used a Furth-Herrmann (15) reaction with a unique condition for the quantitative determination of citric acid. Instead of pyridine-acetic anhydride reagent, Valentinis only used the acetic anhydride--without any base. Anhydrous conditions prevailed in the system. The aqueous citric acid sample was dried on a steam bath, 10 ml acetic anhydride was added, and the mixture heated at 65°C for 45 minutes. The

purple color produced was measured at 520 millimicrons. It was stated that malic, tartaric, ascorbic, succinic, lactic, gallic, and malonic acids do not interfere. Metals such as molybdenum, aluminum, and tin which interfere with the reaction should be removed from the system before analysis. The sensitivity claimed was 10 micrograms. All of the work was done using pure citric acid and no recovery study was reported or optimum condition studies given.

There was no literature reference found that adapted the Furth-Herrmann reaction to the determination of citric acid in tobacco. Sigmon (50) used the Hartford (20) method (previously discussed) for the analyses for citrates in cigarette paper. The Hartford method was employed without modification and none of the variables of the reaction were investigated.

Furth and Herrmann (15) indicate that the pigment formed by the reaction of citric acid, acetic anhydride, and pyridine was an acetylated dihydroxycitric anhydride with a composition of  $C_8H_{10}O_{10}$ . This compound was sensitive to oxygen and soluble in pyridine and acetone. This paper was the only one found that attempted to characterize the pigment.



## EXPERIMENTAL

### I. Reagents

All chemicals used were reagent grade including anhydrous citric acid. The pyridine was dried with a molecular sieve, Linde Type 3A-Union Carbide Corporation.

### II. Apparatus

Cary Model 14 Recording Spectrophotometer

Varian A-60 NMR

Perkin-Elmer Model 21 Double Beam Infrared Spectrophotometer

Mass Spectrometer CEC Model 21-103C

Beckman Zeromatic II pH Meter

Perkin-Elmer Model 240 Instrument for Elemental Analysis

Mettler Thermoanalyzer II

Forced Draft Oven

Burrell Wrist Action Shaker

Beckmann Thermometer

Wiley Mill

### III. A Study of the Method

A. Visible spectrum of the citric acid, acetic anhydride, and pyridine mixture.

When dry citric acid was mixed with pyridine and acetic anhydride and heated slightly, a purple pigment was produced. Figure 1 shows the visible spectrum of the product. The acetic anhydride reagent was also used as the solvent for the system. The wavelength maxima were (in millimicrons): 580, 450, 405, and 385.

It should be noted that a color was produced when citric acid and acetic anhydride, without any base, were heated on a steam bath for 30 minutes. The visible spectrum is shown in Figure 2. The maximum wavelengths were (in millimicrons): 550, 470, 392, and 362, which were essentially the same as those reported above, when pyridine was used. When pyridine was added to the citric acid/acetic anhydride mixture and allowed to set for 30 minutes at room temperature, an identical visible spectrum was produced, as in Figure 1.

The main spectral difference with and without pyridine was one of sensitivity. With pyridine, the sensitivity for the lowest wavelength peak was 150 times greater than without the base. Because of this sensitivity difference, a decision was made to use pyridine and to measure the absorbance at 385 millimicrons.

Figure 1

Absorption Spectrum of a Citric Acid

Acetic Anhydride-Pyridine Mixture.

80 micrograms citric acid/10 ml volume

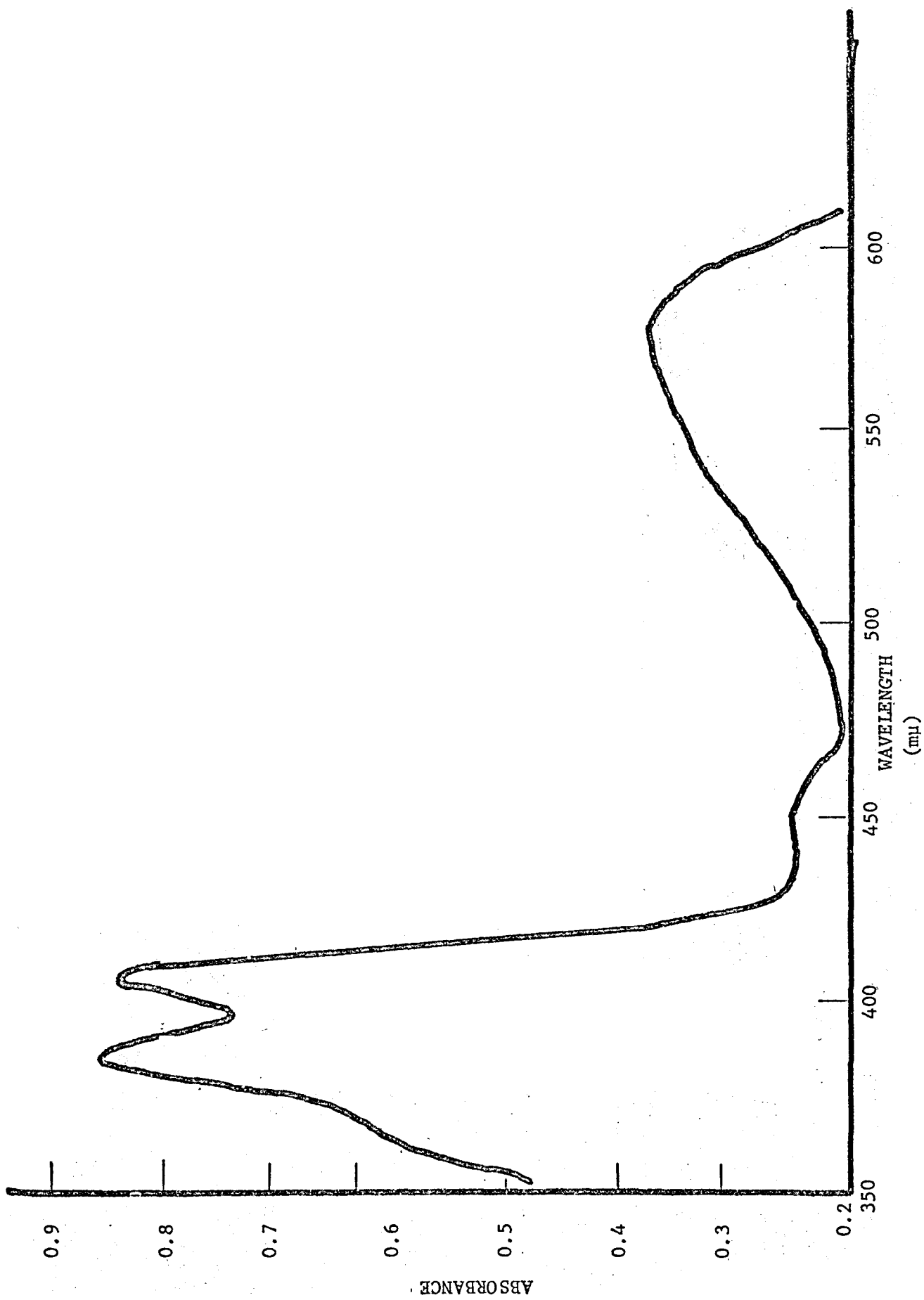
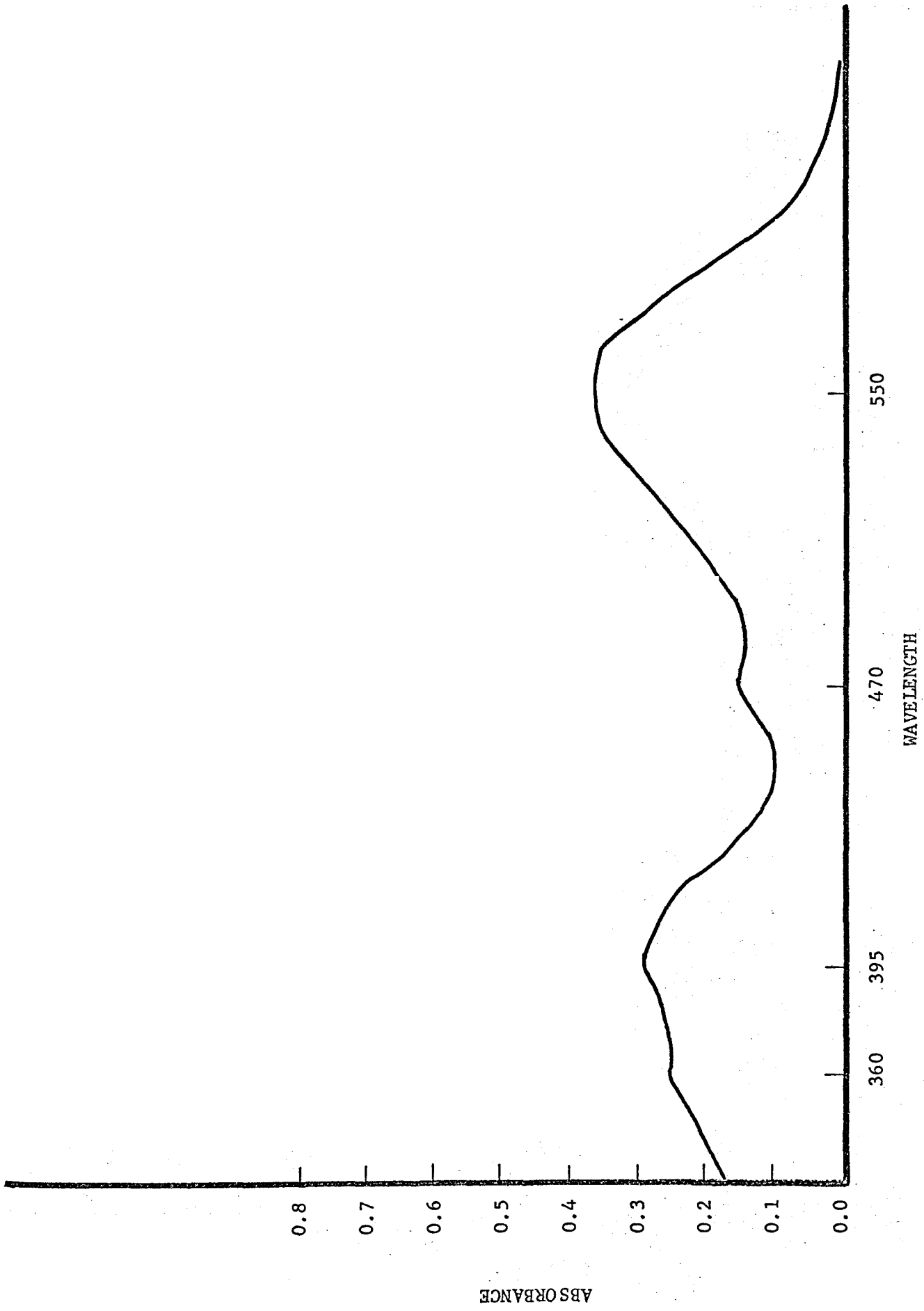


Figure 2

Absorption Spectrum of a Citric Acid-

Acetic Anhydride Mixture

4.4 mg. citric acid/10 ml volume



## B. Optimum Amounts of Reagents

Figure 3 shows the effect of pyridine on the absorbance obtained using 7 ml of acetic anhydride. The absorbance was corrected to a 10 ml total volume for all samples. The reaction time was 30 minutes at room temperature and the absorbance was measured at 385 millimicrons. The reagents should be scrupulously dry. An aqueous solution containing 150 micrograms of citric acid was dried in a forced draft oven at 105° for 60 minutes. The acetic anhydride was used without any modification. The maximum absorbance occurred when 3 ml of pyridine was added.

As with the pyridine, the acetic anhydride was varied from 2 ml to 12 ml. All samples contained 150 micrograms citric acid, 3 ml of pyridine, and the absorbance was corrected to a 10 ml total volume. The maximum absorbance obtained in Figure 4 was at 8 ml of acetic anhydride. The percent difference in absorbance between 7 and 8 ml was less than 2. To simplify the calculations, the colorimetric method for the determination of citric acid was based upon 7 ml of acetic anhydride, and a total volume of 10 ml rather than 11 ml.

The effect of varying the amounts of each reagent on the time required for the reaction and on the stability of the color produced was determined by observing the change of absorbance with increasing time at room temperature. All samples had 150 micrograms of citric acid, and were diluted to a 10 ml total volume using glacial acetic acid. Figure 5 shows that varying

Figure 3

Optimum Amount of Pyridine for Maximum

Absorbance at 385 Millimicrons

Conditions: 7.0 ml acetic anhydride  
all absorbances corrected to  
10 ml total volume  
reaction time 30 minutes



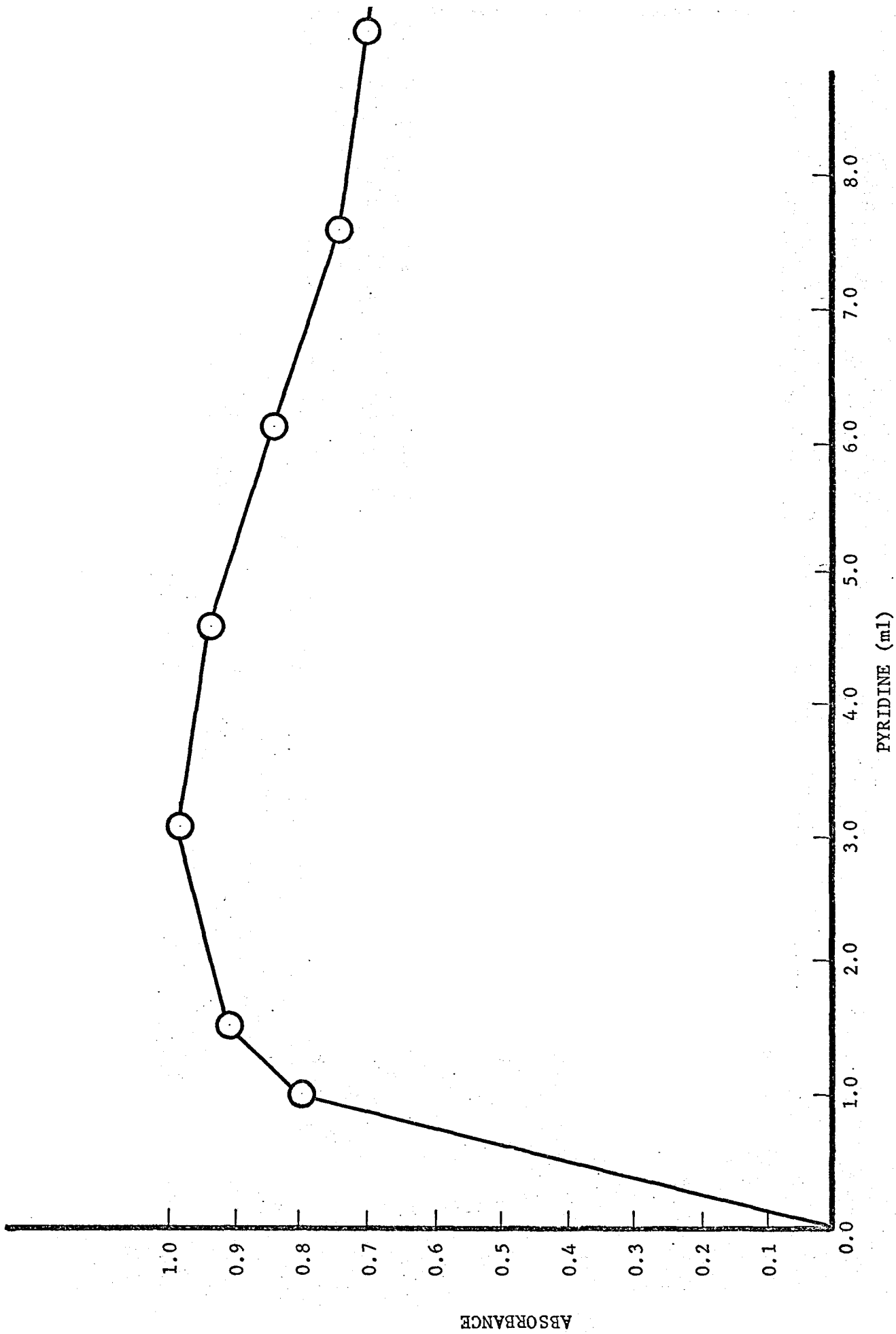


Figure 4

Optimum Amount of Acetic Anhydride  
for Maximum Absorbance at 385 Millimicrons

Conditions: 3.0 ml pyridine  
all absorbances corrected to  
10 ml total volume  
reaction time 30 minutes at  
room temperature

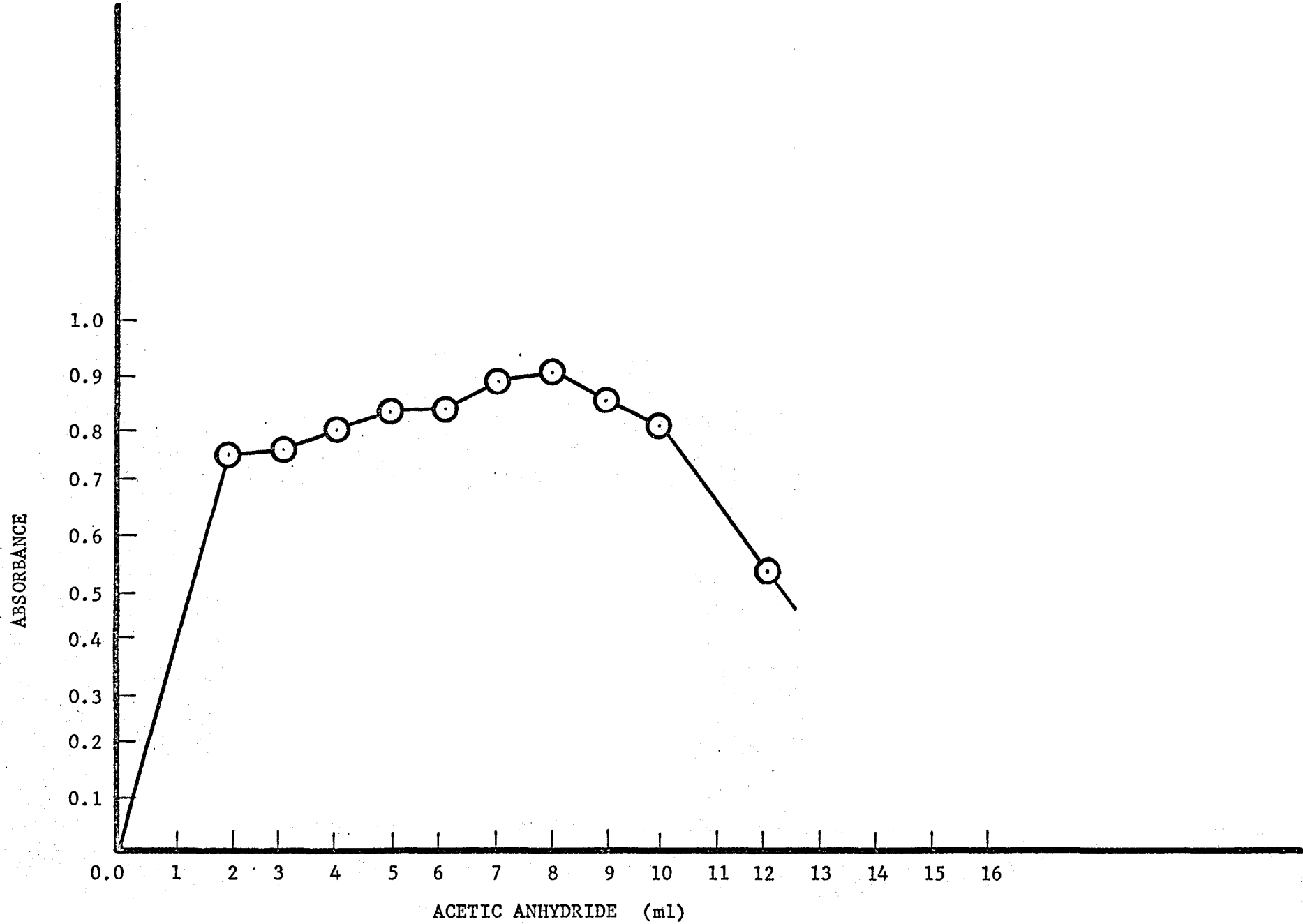
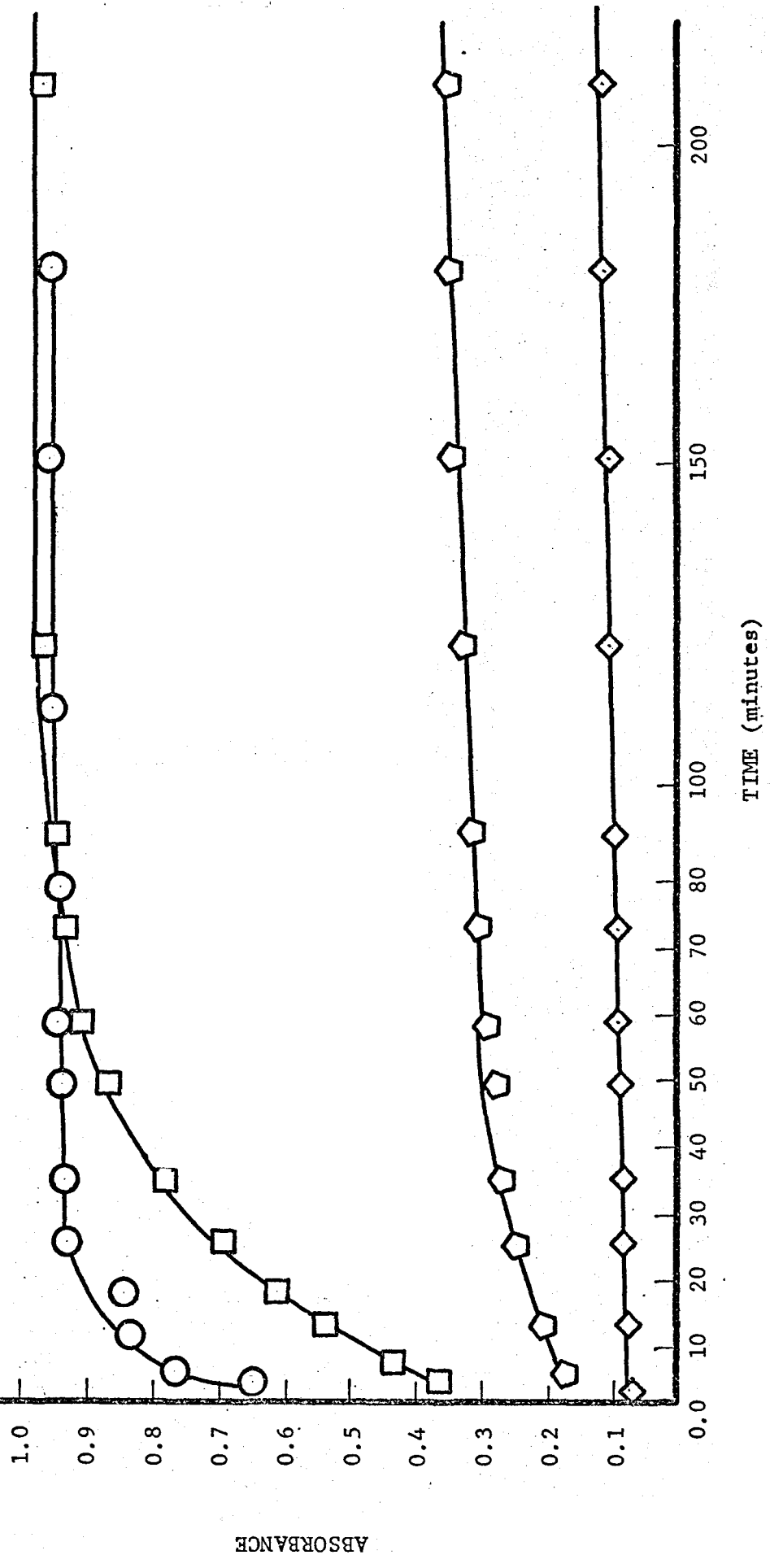


Figure 5

Sensitivity and Stability  
of Various Amounts of Pyridine

Conditions: 150 micrograms of citric acid  
absorbance measured at 385 milli-  
microns  
diluted to 10 ml volume with  
glacial acetic acid (AcOH)

	<u>pyridine(ml)</u>	<u>AcOH(ml)</u>	<u>Ac<sub>2</sub>O(ml)</u>
◇	1	3	6
◻	2	2	6
◻	3	1	6
○	4	0	6



the pyridine between 1 and 4 mls and keeping the acetic anhydride constant at 6 ml, the maximum absorbance was reached within 30 minutes with the maximum amount of pyridine added. The absorbance was stable for at least 3 hours. Figure 6 demonstrates that with the pyridine constant and varying the acetic anhydride between 1 and 7 mls, maximum absorbance was obtained within 30 minutes at 25°C with the maximum amount of acetic anhydride added. Figures 5 and 6 show that the less glacial acetic acid added, the greater the absorbance.

#### C. Order of Reagents

To observe the effect of the order of the reagents (pyridine and acetic anhydride) on the rate of formation of the chromophore, two samples were prepared. Each had 150 micrograms of citric acid. One sample had the 7 ml of acetic anhydride added before the 3 ml of pyridine. The other sample had the same amount of reagents but added in reverse order. Figure 7 shows that the order of reagents does not affect the maximum absorbance. Both rates of formation of the chromophore were the same.

#### D. Effect of Water

The effects of water added before and after the addition of the reagents were investigated. All samples had 150 micrograms of citric acid, 3 ml of pyridine and 7 ml acetic anhydride. A 30 minute development time was allowed. Figure 8 shows the

Figure 6

Sensitivity and Stability of Various

Amounts of Acetic Anhydride

Conditions: 150 micrograms of citric acid  
absorbance measured at 385 milli-  
microns  
diluted to 10 ml volume with glacial  
acetic acid

	<u>Ac<sub>2</sub>O(ml)</u>	<u>AcOH(ml)</u>	<u>pyridine</u>
○	1	6	3
◇	3	4	3
△	6	1	3
○	7	0	3

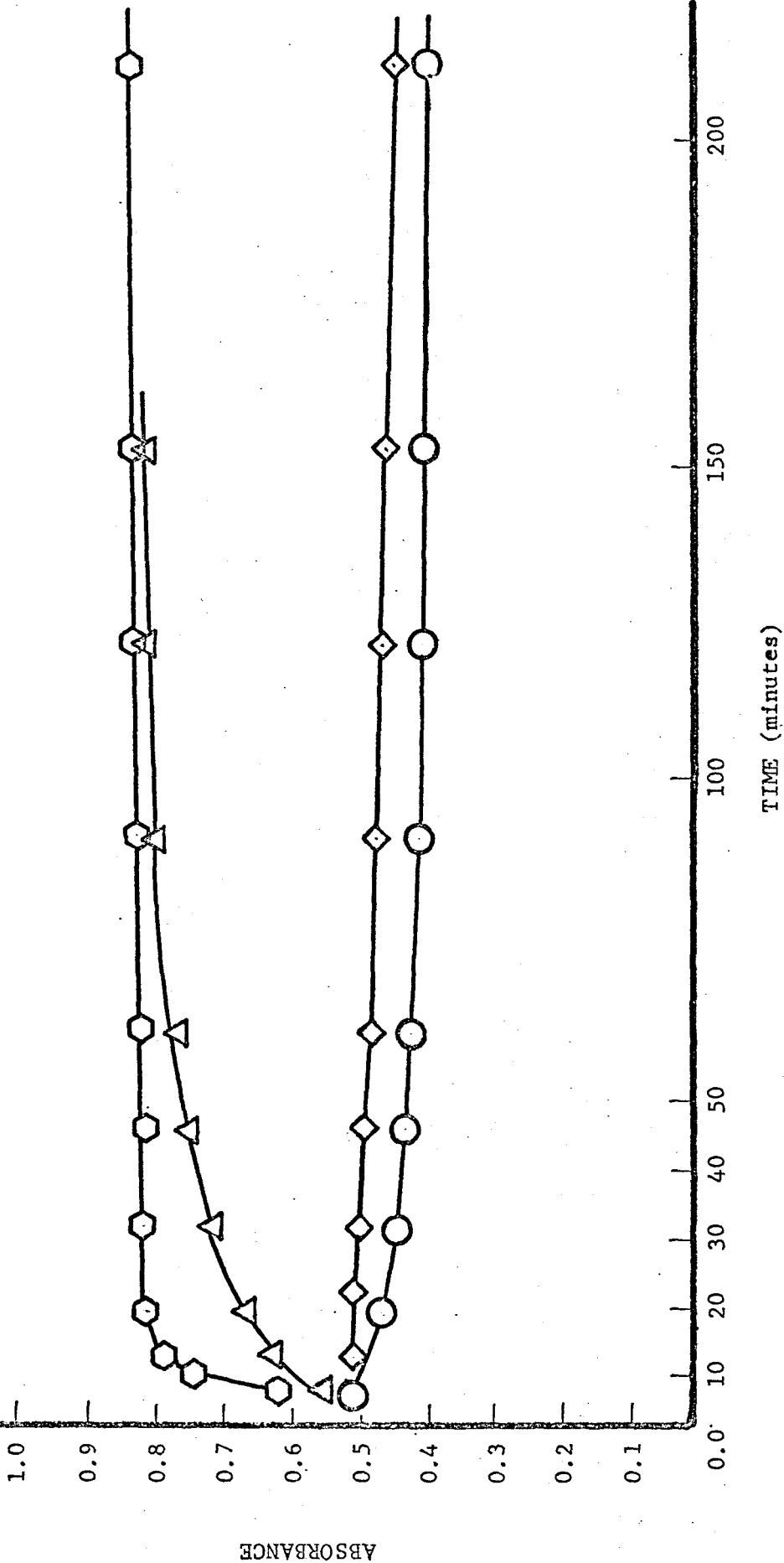




Figure 7

Maximum Color Developed By Reversing

Order of Addition of Reagents

Conditions: 150 micrograms of citric acid  
absorbance measured at 385 milli-  
microns  
reaction temperature 25°C

- Δ 7 ml  $\text{Ac}_2\text{O}$  added; then 3 ml pyridine
- 3 ml pyridine added; then 7 ml  $\text{Ac}_2\text{O}$

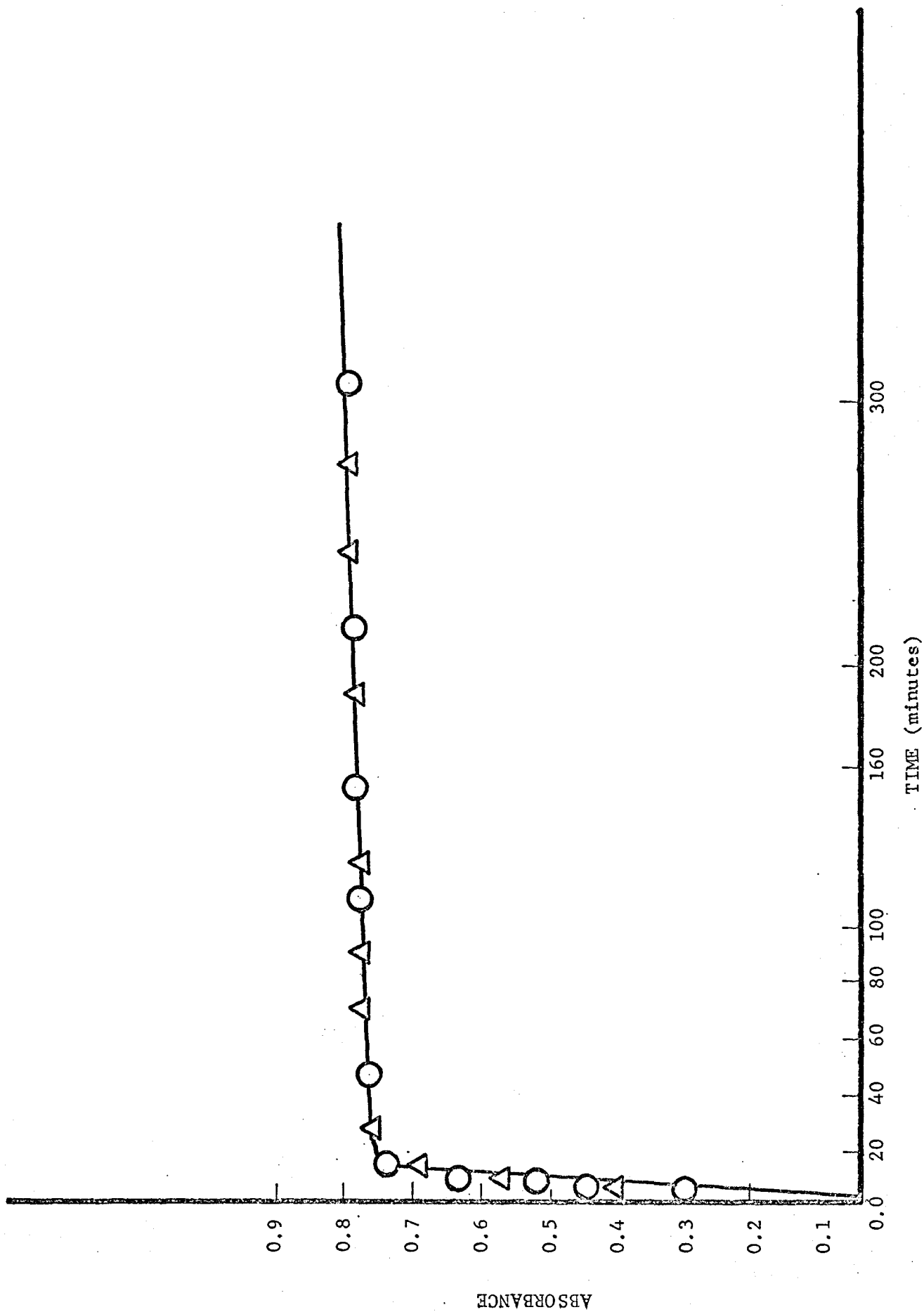
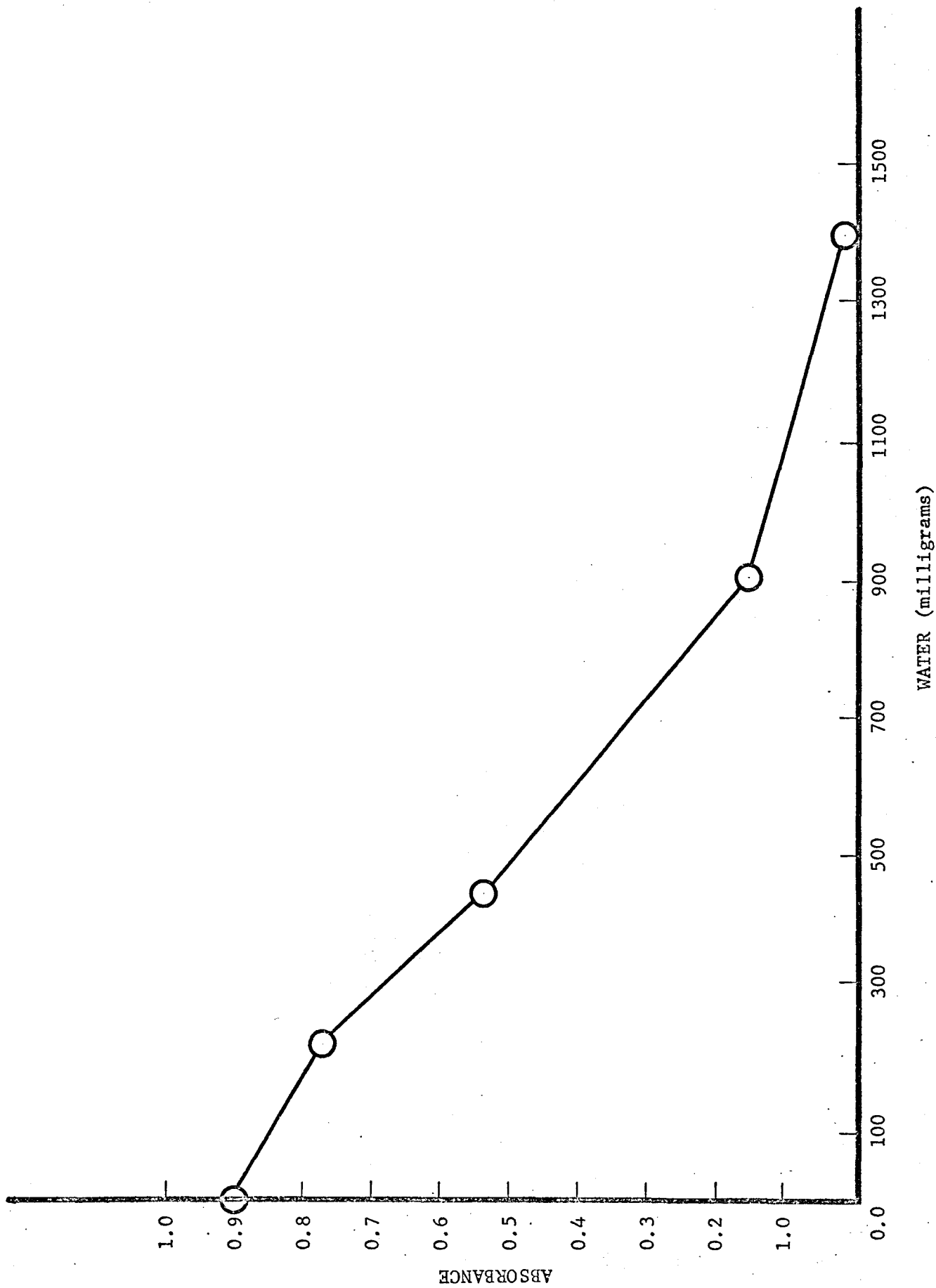


Figure 8

Effect of Water Added to the System

Before the Addition of Reagents

Conditions: 150 micrograms of citric acid  
3 ml pyridine, 7 ml  $\text{Ac}_2\text{O}$  added  
absorbance measured at 385 milli-  
microns and corrected to 10 ml volume  
reaction temperature  $25^\circ$



effect of water added before the reagents. There was approximately a 50% decrease in absorbance when 0.5 ml was added.

When water was added after the reagents, the decrease in absorbance was less dramatic. Figure 9 shows only a 22% decrease in absorbance after adding 0.5 ml of water. The absorbance was not corrected to a 10 ml total volume. The effect of extraneous water in the system prior to the addition of reagents was more detrimental than water entering the system after the reaction was underway.

To ascertain the effect of the absorbance versus increase in reaction time with a known amount of water, two 150 microgram samples of citric acid were prepared. One had 580 mg added before the reagents and the other sample had the same amount of water added after the reagents. Figure 10 again shows that water added before the reagents yields a larger decrease in absorbance.

#### E. Effects of Drying Citric Acid

As shown in the above water addition studies, water added before the reagents causes the maximum decrease in the formation of the chromophore. This is essentially the same as having a wet sample.

Table 1 shows the effect of increasing drying time on absorbance. All samples had 3 ml aqueous solution of citric acid (150 micrograms) in a 25 ml wide mouth Erlenmeyer flask. The forced air draft oven was set at 100°C.

Figure 9

Effect of Water Added to the System

After the Addition of Reagents

Conditions: 150 micrograms of citric acid  
3 ml pyridine, 7 ml Ac<sub>2</sub>O added  
absorbance measured at 385 milli-  
microns  
reaction temperature 25°C

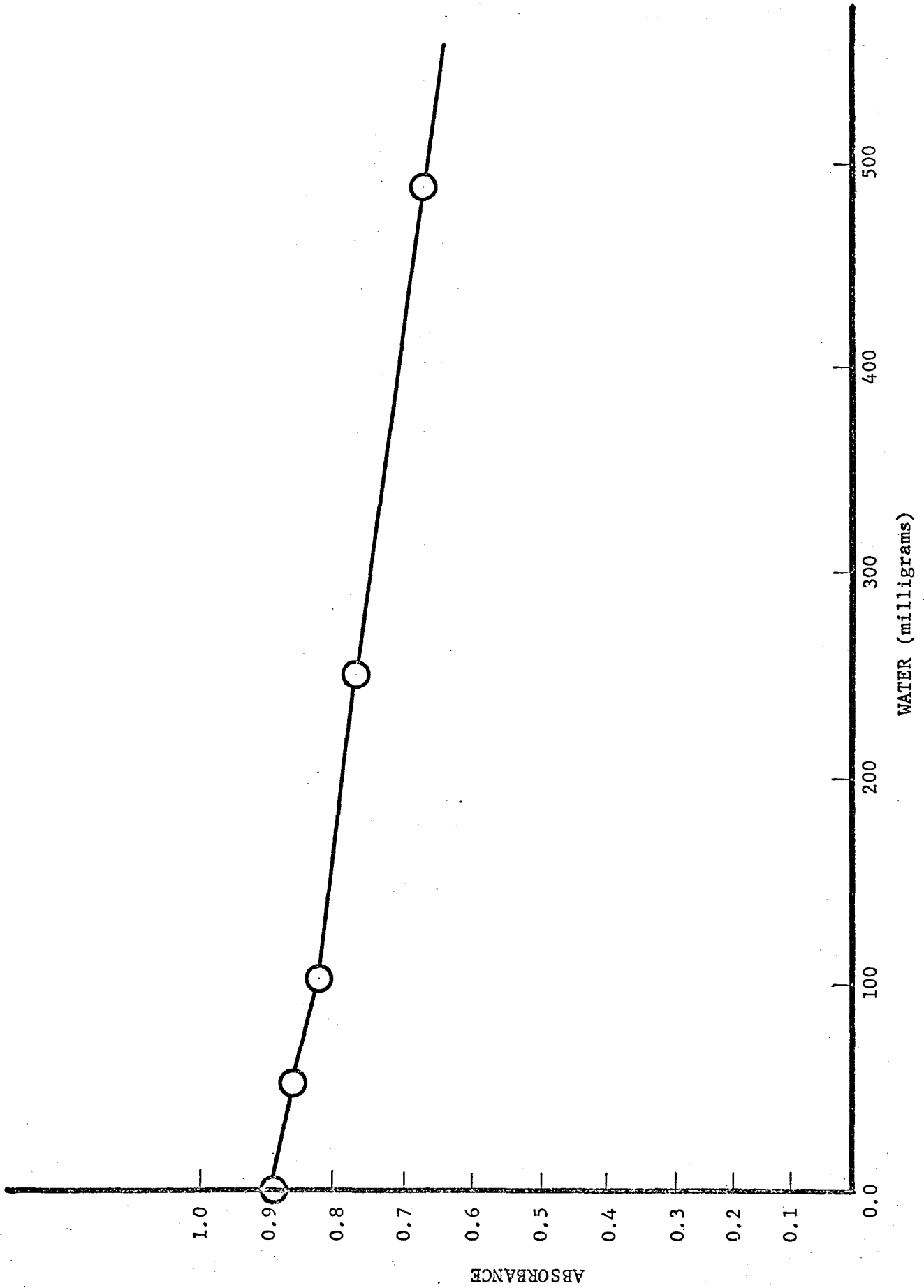


Figure 10

Effect of Water on Absorbance

With Respect to Stability and Sensitivity

Conditions: 150 micrograms of citric acid  
absorbance measured at 385 millimicrons  
reaction temperature 25° C



580 mg H<sub>2</sub>O added; then 3 ml pyridine and  
7 ml Ac<sub>2</sub>O



3 ml pyridine and 7 ml Ac<sub>2</sub>O added; then  
580 mg H<sub>2</sub>O



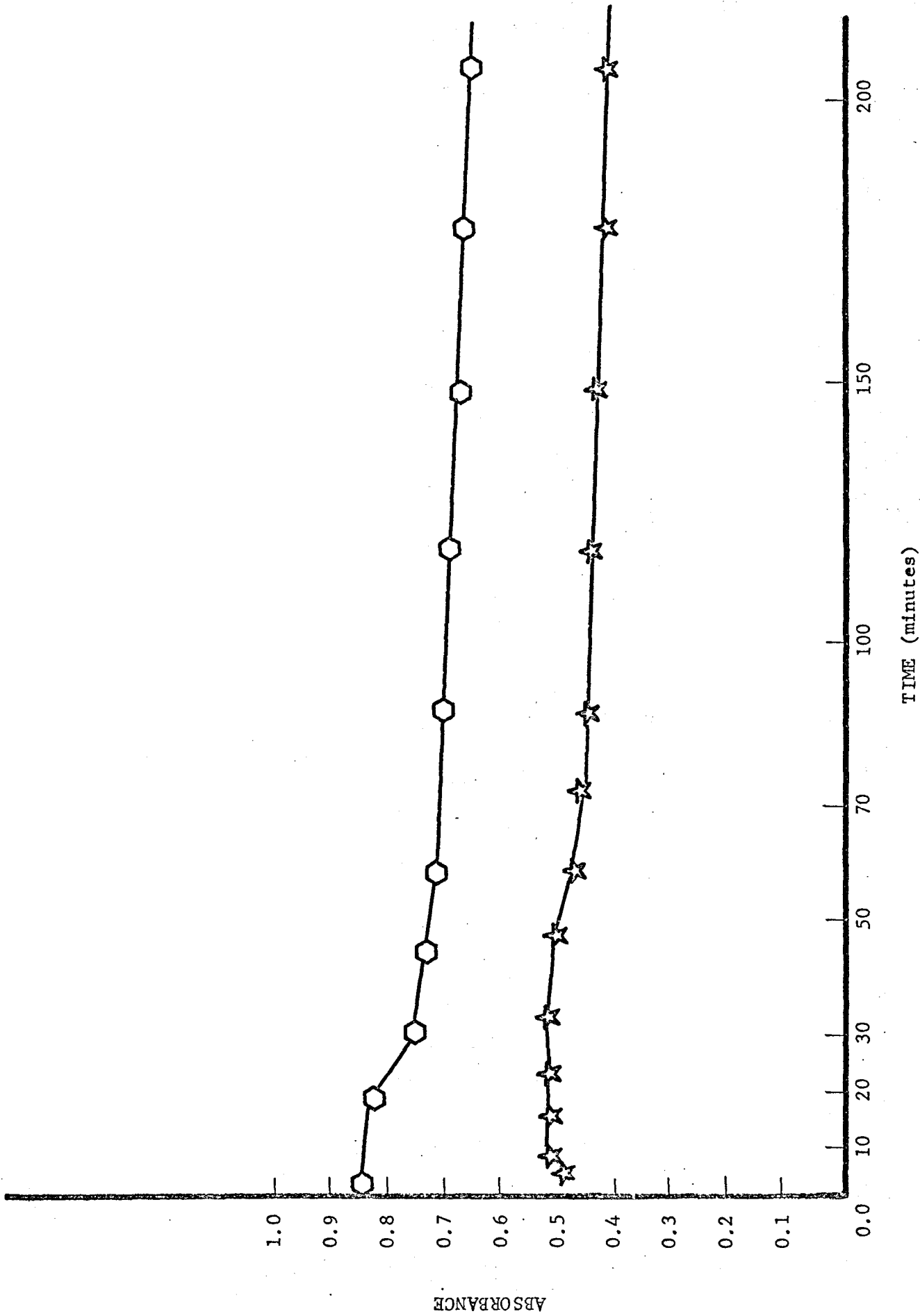


TABLE I

Effect of Drying Time of Citric Acid on Absorbance

<u>Hours in Oven</u>	<u>Absorbance</u>
0.5	.612
1	.905
2	.912
3	.908
4	.921

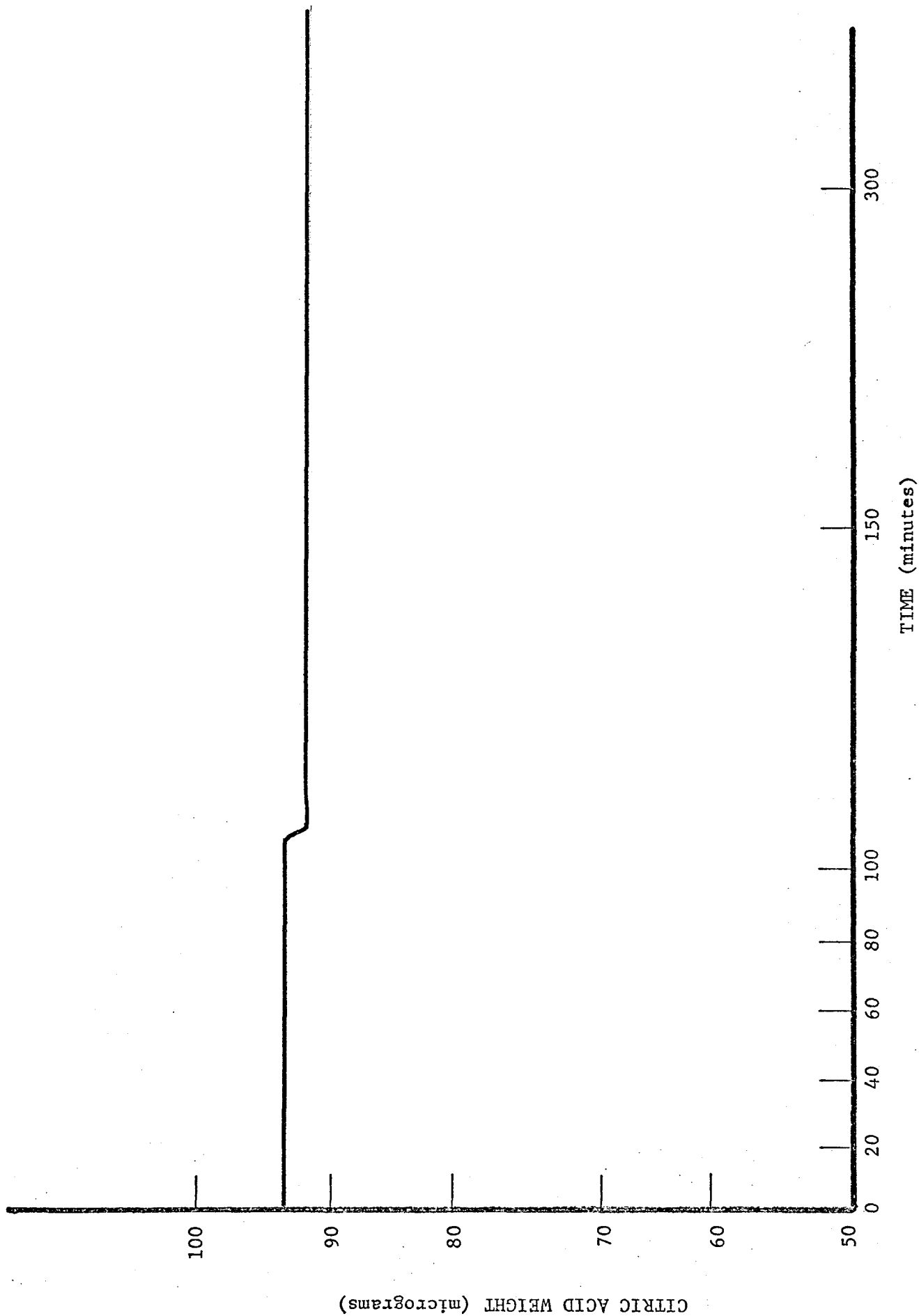
A thermogravimetric analysis (TGA) instrument was used to determine the decomposition, if any, on the citric acid at extended drying times. An expanded scale (0-100 micrograms) was used to amplify the magnitude of weight lost in the sample. The temperature was set isothermally at 123°C and held for five hours. The decrease in weight shown in Figure 12, at 110 minutes corresponds to a net weight loss of less than 0.2%. This was thought to be due to loss of water and not of decomposition.

This study has shown that aqueous samples may be dried in a forced draft oven without danger of decomposition and that 1 hour at 100°C is an adequate drying time. If one uses larger or smaller volumes of solvent, then the drying time would have to be experimentally determined.

Figure 11

Thermogravimetric Analysis of Citric Acid

Conditions: 9.99 mg sample of citric acid  
123°C isothermal temperature, air  
atmosphere



F. Reaction Temperature Study

Table II shows the absorbance of different concentrations and temperatures for the standard curve. The aqueous citric acid samples were dried in an oven at 105°C for 1 hour. Three ml of pyridine and 7 ml of acetic anhydride were added to all samples. The reaction time was 30 minutes for both reaction temperatures.

TABLE II

Absorbance measured at 385 millimicrons

<u>Concentration*</u> <u>(microgms/10 ml)</u>	<u>Absorbance</u> <u>(reaction temp 25°C)</u>	<u>Absorbance</u> <u>(reaction temp 38°C)</u>
20	.020	.208
40	.141	.426
60	.264	.701

\*Each concentration level was the average value of six samples.

Both Marier and Boulet (31) and Hartford (20) noted that citric acid did not follow Beer's Law. If, after the addition of the reagents, the samples were not placed in an oven at 37-40°C, the low concentration levels (e.g. 10-20 micrograms) gave practically no absorbance. When the samples were heated after the addition of the reagents, the low concentration samples reached maximum absorbance and the standard curve was linear.

#### G. Preparation of a Standard Curve

To prepare a standard curve, weigh accurately 100 mg. of anhydrous citric acid and dilute to 100 ml with water (1 mg. citric acid/ml). This was the stock solution. From the stock solution, transfer 2 ml to a 100 ml volumetric flask (20 micrograms/ml) and dilute to volume. It was noted that the standard solutions of citric acid developed a mold in the flask after one month. Fresh standards should be prepared after three weeks.

The pyridine should be previously dried with molecular sieve. A fresh pint size bottle of reagent grade acetic anhydride should be used for the determination of citric acid. After one or two weeks of exposing the opened bottle, while withdrawing the aliquot, the last 25-50 ml of acetic anhydride had become sufficiently contaminated to cause a decrease in absorbance.

Transfer 1, 2, and 3 ml aliquots from the 20 microgram/ml solution to 25 ml glass stoppered wide mouth Erlenmeyer flasks. Place in a forced draft oven at 105°C for 1 hour. The use of some type of tray, to evenly distribute the heat, is recommended because of "hot spots" that developed in the commercial oven that was used.

The samples should be taken out of the oven and set aside for 10 minutes (or until ambient temperature is reached) with the samples closed to the air using glass stoppers. Accurately transfer 3.0 ml of pyridine and 7.0 ml of acetic anhydride

to the Erlenmeyer flask containing the citric acid and a blank. One should use a suction bulb to transfer the reagents because of the toxicity of the pyridine (46) and the reactivity and lachrymatory properties of the acetic anhydride. Stopper the flask and put in an oven at 37-40°C for 30 minutes. Measure the absorbance at 385 millimicrons and subtract the blank.

Figure 12 shows the standard curve for a concentration range of 0-60 micrograms. Each concentration was done in sextuplet. The percent relative deviation was 3.0. The slope of the curve was 0.1147. This value was obtained from an IBM 1130 computer using a standard polynomial regression program.

TABLE III

Deviation From Standard Curve (Figure 13)

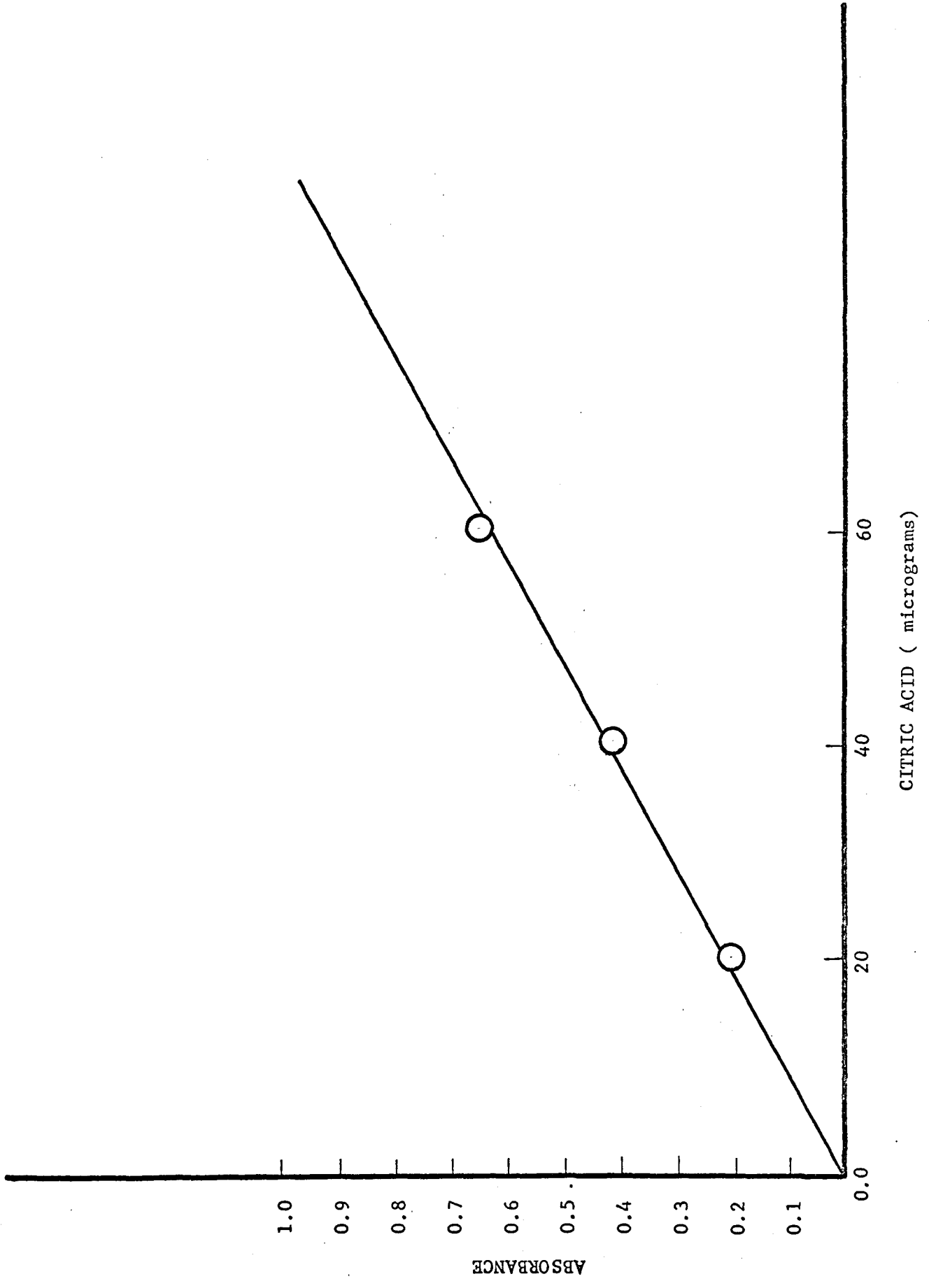
<u>20 ugm CA</u>		<u>40 ugm CA</u>		<u>60 ugm CA</u>	
<u>A</u>	<u>Dev.</u>	<u>A</u>	<u>Dev.</u>	<u>A</u>	<u>Dev.</u>
.203	.002	.451	.010	.713	.002
.192	.013	.448	.007	.720	.009
.217	.012	.429	.012	.703	.008
.205	.000	.434	.007	.710	.001
.218	.013	.441	.000	.717	.006
<u>.195</u>	<u>.010</u>	<u>.443</u>	<u>.002</u>	<u>.705</u>	<u>.006</u>
Ave. .205	.008	.441	.006	.711	.005
Std. Dev.	$10.8 \times 10^{-3}$	Std. Dev.	$8.31 \times 10^{-3}$	Std. Dev.	$6.65 \times 10^{-3}$
% Rel. Std. Dev.	5.3	% Rel. Std. Dev.	1.9	% Rel. Std. Dev.	1.0

Figure 12

Standard Curve for Citric Acid

Conditions: absorbance measured at 385 milli-  
microns  
total volume, 10 ml  
reaction time, 30 min.  
3 ml pyridine and 7 ml Ac<sub>2</sub>O added





#### H. Interferences of Other Acids

Known acids at the 150 microgram level were substituted for citric acid employing the method developed. Several of the acids listed in Table IV did not react at this low concentration level but did give a color when reacted at the 1-2 gram level. These acids will be discussed in the next section of the experimental. All of the acids listed were done in duplicate and the absorbance was measured at 385 millimicrons. After the addition of the reagents, the samples were not placed inside an oven for the required time but were left at room temperature for 30 minutes.

TABLE IV

#### Acids Substituted for Citric Acid in the Method

<u>Acid</u>	<u>Micrograms citric acid*</u>
citric	150
cis-aconitic	144
trans-aconitic	153
itaconic	158
isocitrate**	0
fumaric	7
tartaric	3
succinic	0
d-malic	0
l-malic	0
dl-malic	0
oxalacetic	1
alpha-keto-butyric	1
alpha-keto-glutaric	3
alpha-keto succinic	0
beta-keto adipic	0
l-ascorbic	0

\* All absorbance calculated from citric acid standard curve, 10 ml total volume

\*\* The barium salt was used. No color is produced. When the the salt is acidified, then reacted with the reagents, a color is produced only at high concentration levels (mg/10 ml range).

#### IV. Recommended Procedure for the Determination of Citric Acid

The following is the recommended procedure for the determination of citric acid. In a 25 ml glass stoppered flask, place samples containing 0-60 micrograms of citric acid in a forced draft oven at 105°C for one hour. (If more than 3 ml water in sample, a longer drying time is needed). Remove from the oven, stopper the flasks, and let samples cool to room temperature for 10 minutes. Add 3.0 ml of dry pyridine and 7.0 ml acetic anhydride, stopper immediately and mix thoroughly. Place in an oven at 37-40°C for 30 minutes. Measure the absorbance at 385 millimicrons. Follow the above procedure for a reagent blank. The flask should be dried before adding the reagents. Compare the absorbance with a standard curve and calculate the amount of citric acid in the sample by the equation:

$$\text{Citric Acid (micrograms)} = \frac{(\text{Absorbance-blank}) \times \text{Dilution factor}}{\text{Slope of Standard Curve}}$$

#### V. Determination of Total Citric Acid in a Tobacco Blend

The following determination of citric acid in a tobacco sample was based on an extraction method by Harvey, Hale, and Ikeda (19). After the extraction of the tobacco, they used

esterification and subsequently analyzed the methyl esters by gas chromatography. A modification of their extraction procedure was used for quantitatively removing the citric acid from the tobacco. In place of the absolute methanol-sulfuric acid mixture used for the extraction and esterification, a mixture of methanol, hydrochloric acid, and water was used. Also 20% water was added to the extraction procedure to prevent any esterification that would take place. Harvey, Hale and Ikeda showed that with 10% water, one can only expect to get 18% esterification. An acid is necessary to convert any citrates in the tobacco to free citric acid. The HCl was used in place of  $H_2SO_4$  because of its volatility.

The following procedure was used for analyzing citric acid in a commercial cigarette tobacco. Grind the tobacco in a Wiley mill using a 20 mesh screen and dry it overnight at  $105^{\circ}C$  in a forced draft oven. Accurately weigh 1 gram of tobacco and place in a 125 ml Erlenmeyer flask. Add 10 ml water and 50 ml of a methanol, HCl,  $H_2O$  mixture (89:1:10 v/v) and shake for 30 minutes using a mechanical shaker. Filter with one piece of #40 Whatman filter paper. Transfer 1.0 ml of extract to a 10 ml volumetric flask and dilute to volume with methanol. Transfer 1.0 ml of the diluted extract to a small separatory funnel and add 5 ml  $H_2O$ . Extract with 5 ml portions of diethyl ether four times into a 25 ml ground glass Erlenmeyer flask. Evaporate the ether to near dryness (less than 3 ml) and place in a forced draft oven @  $105^{\circ}$  for 30

minutes. Let the samples cool at room temperature, with the flask stoppered, for 10 minutes. Add 3.0 ml pyridine and 7.0 ml acetic anhydride, stopper, swirl flask, and place in oven at 37-40°C for 30 minutes. Measure the absorbance at 385 millimicrons. The average of 3 determinations gave a value of 2.59% of citric acid on a dry weight basis of tobacco. An independent method of analysis showed the same tobacco to have 2.24%.

Table V shows the recovery study of 40 and 60 micrograms of citric acid added to 1 gram of dry tobacco.

TABLE V

Recovery of Citric Acid Added to Tobacco Sample

<u>micrograms citric acid added</u>	<u>micrograms found</u>	<u>% Recovery</u>
40	38.4	96.0
40	35.2	88.0
40	36.9	92.3
40	36.7	91.7
Average	36.8	92.0
60	57.0	95.1
60	56.7	94.5
60	55.3	92.5
60	55.5	92.5
Average	56.3	93.6

The equation used to find the amount of citric acid was:

$$\text{micrograms citric acid} = \frac{(\text{Abs.}-\text{blank})(\text{dilution factor})}{\text{Slope of standard curve}}$$

## VI. Characterization of the Pigment Formed By the Reaction of Citric Acid, Acetic Anhydride, and Pyridine

### A. Product Formed Using Different Bases and Anhydrides

Several bases were added to citric acid and acetic anhydride to determine if pyridine was a specific base necessary for the formation of the colored product. Sodium hydroxide (1 pellet) was added to 1 gram of citric acid and 10 ml of acetic anhydride. The sample had the same wavelength maximum (550-560 millimicrons) as when pyridine was added. Potassium hydroxide gave the same result. Another base, n-butylamine, was added and an identical absorption was noted. It should be mentioned that n-butylamine reacted violently when added to the citric acid-acetic anhydride mixture. When added drop-wise, the reaction was controllable. It appears that any base added to citric acid and acetic anhydride generated the same visible spectrum.

Four anhydrides were reacted with citric acid and pyridine to determine if any specific anhydride was needed for the reaction. Succinic anhydride (15 grams) was mixed with 10 gm citric acid and 100 ml pyridine. The solution was heated in a beaker on a hot plate. The reaction is exothermic and the heater was used only to initiate the reaction. The mixture went from light green to yellow to orange to a light brown color. The temperature was 88°C after 16 minute reaction time. Phthalic anhydride (15 gms) was added to 10 grams citric acid

and 100 ml pyridine. The solution went from a light yellow to green to light brown. After 20 minutes reaction time, the temperature was 86°C.

Maleic anhydride (20 gms) was added to 10 gms citric acid and 150 ml pyridine. The solution turned dark brown immediately. The solution started to boil and the temperature was 85°C.

Citric acid (10 gms) and 25 ml pyridine were mixed together. Hexafluoroacetic anhydride (less than 1 ml) was added dropwise. The reaction was instantaneous with a white gas evolved. No odor above that of the pyridine could be detected. Extreme care should be taken when mixing this reagent because of the violent reaction that takes place. A pink color was produced and wavelength maxima in acetone were: 572, 540, 455, and 360 millimicrons.

Finally, 8 grams of pyromellitic dianhydride was added to 10 grams of citric acid and 100 ml of pyridine. The solution turned blue and finally a brown color developed. A precipitate formed.

It appeared that succinic, maleic, phthalic, pyromellitic, and hexafluoroacetic anhydrides gave products that were similar, if not the same as the final product obtained with acetic anhydride.

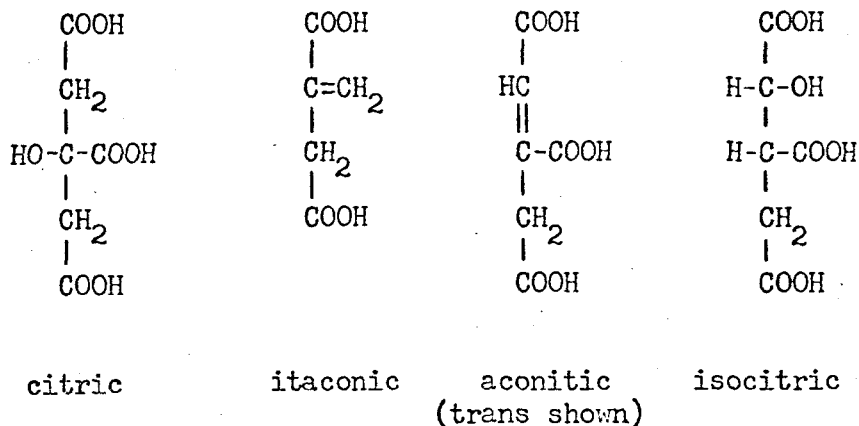
#### B. Reaction With Different Acids

As shown in Table III, cis and trans-aconic, and itaconic

acids produce essentially the same absorbance at 385 millimicrons as citric when present at the same concentration level. At a higher concentration level, isocitric produces an identical wavelength maxima but at much lower intensity. (The isocitric as a barium salt was acidified with 1N HCL, evaporated to dryness and then reacted in the usual manner.) The rates of formation of the acetic anhydride product of the citric, itaconic, isocitric, and aconitic acids were qualitatively determined. Approximately 1 gm of each acid with 10 ml acetic anhydride were placed on a steam bath and the time in which the color first developed (eye observation) was noted. The citric and itaconic took 6 minutes while isocitric took 2 minutes and aconitic, 1 minute, to develop color.

The wavelengths at which absorption occurs for citric, isocitric, and cis and trans-acconitic are identical to Figure 1. Itaconic was identical except the peak at 550 millimicrons was absent.

The structure of the following compounds are listed below:





The compounds shown above are very similar. All are di or tri-carboxylic acids. Citric and isocitric could eliminate one molecule of water readily to form the aconitic acid. Methyl citrate does not react with the reagents to form any chromophore.

### C. Decarboxylation of Citric Acid

The following qualitative experiment was carried out to determine if the citric acid decarboxylated while forming the product when acetic anhydride was added (without any base). A helium tank was connected to a 50 ml flask which was connected to a gas washing bottle with a fritted disk stopper. The bottle was connected to a "U"-tube of LiOH to keep air from entering the system. A  $\text{Ba}(\text{OH})_2$  solution was prepared as follows:  $\text{Ba}(\text{OH})_2$  was added to 20 ml water until saturated. The solution was filtered and the clear filtrate added to the gas washing bottle which had previously been flushed with dry helium. Citric acid (500 mg) and 30 ml acetic anhydride were added to the flask and flushed immediately with helium. A positive test for  $\text{CO}_2$  was obtained. A blank consisting of acetic anhydride alone, was added to the flask and flushed with helium. No precipitate was observed. These facts indicate that citric acid decarboxylates when acetic anhydride was added.

#### D. Isolation of the Pigment

The pigment, formed by the mixture of citric acid, acetic anhydride and pyridine, was isolated for physical tests to determine the structure of the pigment.

An extraction procedure was first contemplated. The separation of the pigment from the reagents did not appear feasible because of the solubility of the acetic anhydride. The anhydride was soluble in benzene, toluene, chloroform, carbon tetrachloride, acetone, ether, ethyl alcohol, and water. Pentane and acetic anhydride were not miscible but the pigment stayed in the anhydride.

By trial and error, it was found that if the pigment solution was added to a large amount of diethyl ether, the pigment precipitated from solution.

When 10 gms citric acid, 30 ml acetic anhydride and 14 ml pyridine were mixed together, the reaction was exothermic and carbon dioxide was evolved. The reaction was allowed to proceed for 30 minutes at room temperature. The covered beaker was cooled in an ice-water bath for 15 minutes and then poured into 200 ml of diethyl ether cooled in an ice-water bath. After 5 minutes, the precipitate was filtered with filter paper using suction. Approximately 200 ml of ether was poured over the precipitate to remove any residual acetic anhydride, pyridine, or citric acid. The wet precipitate was immediately put into a vacuum desiccator and evacuated to dryness. If the

precipitate was exposed to the atmosphere, the reddish brown powder turned black in color and started to bubble. After decomposition, the black residue had a shiny, glossy appearance with some bubble shapes intact. When examined closely, the residue appeared like a very thin piece of black glass that was shattered.

The sodium salt of the pigment was prepared several times. Sodium hydroxide pellets were substituted for pyridine and the precipitate was isolated as stated above. The sodium salt was more sensitive to moisture and/or air and appeared only slightly soluble in acetic anhydride when an attempt was made to regenerate the original color in solution. Further sodium salt isolations were discontinued.

Because of the heat evolved during the reaction when the reagents were mixed to form the pigment, the temperature was observed (See Figure 13) as the reaction time progressed. At 1.5 minutes, the solution appeared to have been boiling. The temperature at this time was 59°C. Because of the relatively high boiling reagents used, the bubbles were apparently carbon dioxide.

#### E. Infrared Spectrum of Pigment

The pigment was isolated (See Section VI, D) and dried before obtaining the IR spectrum. Figure 14 shows the spectrum obtained using a KBr pellet.

Figure 13

Temperature of the Reaction

Conditions: 10 grams citric acid  
30 ml  $\text{Ac}_2\text{O}$   
15 ml pyridine

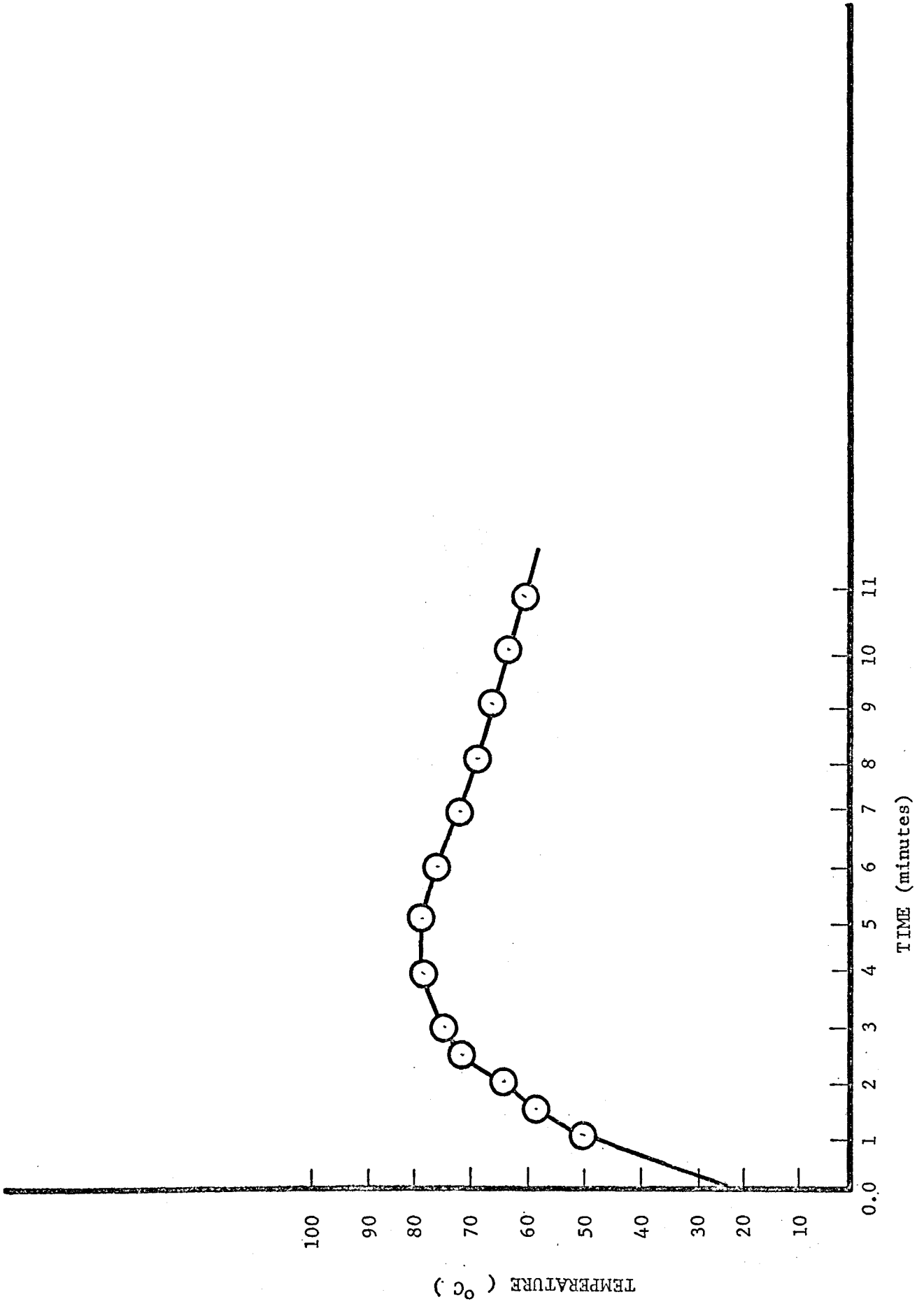


TABLE VI

Wavelength and Frequency Maxima of IR Spectrum

Wavelength (microns)	Frequency (cm <sup>-1</sup> )
2.90	3425
3.25	3077
5.62	1779
5.72	1748
6.20	1613
6.50	1538
6.70	1493
7.10	1408
7.26	1377
7.48	1337
8.00	1250
8.32	1202
8.58	1165
8.90	1124
9.38	1066
9.82	1018
9.92	1008
11.03	907
12.82	780
13.35	749
14.74	678

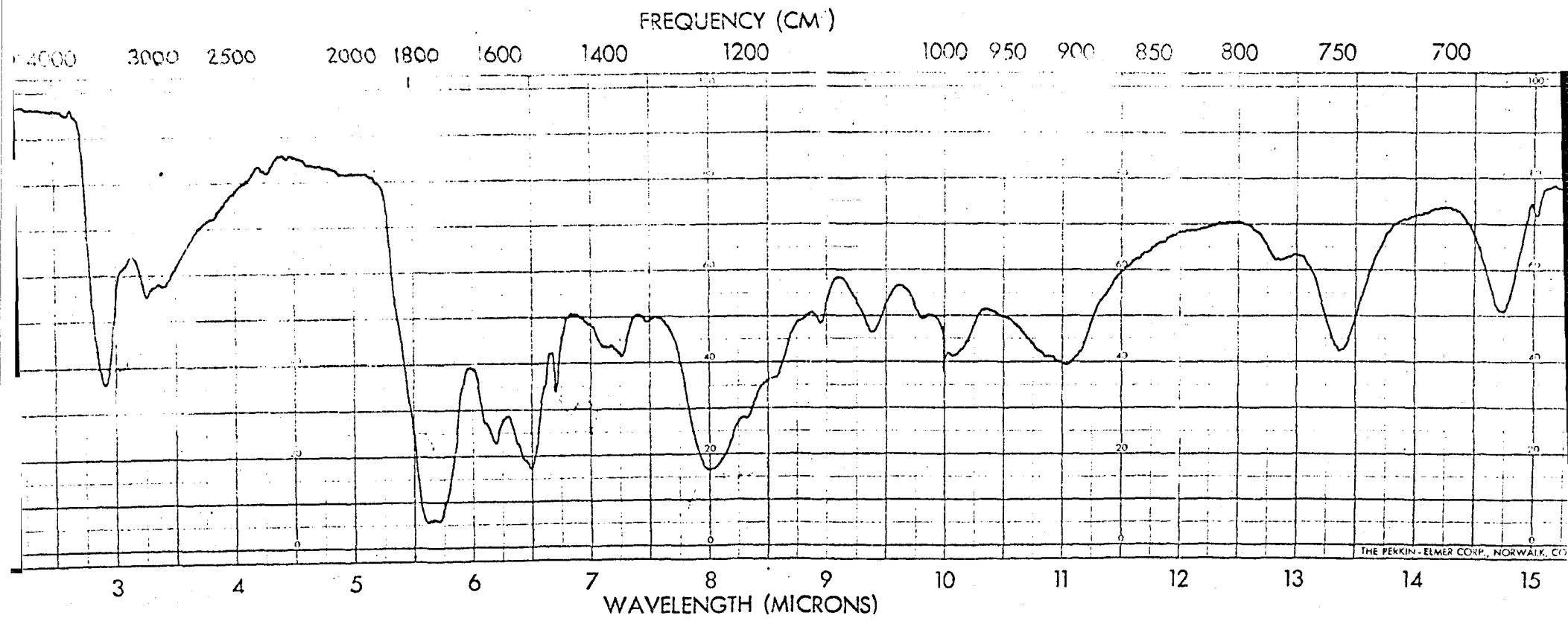
The following assignments of IR absorption bands were taken from Silverstein and Bassler (51) and Bellamy (4).

The absorption at 6.70, 13.35, and 14.74 microns are pyridine bands. With known pyridine, the 14.74 band was at 14.25 microns. The carboxylate anion absorbs at 6.50 and 7.10 microns. The carbonyl bands (at least 2 possibly 3 or 4) absorb at 5.62 and 5.72 microns with inflection points at 5.54 and 5.78 microns. Double bond character was evident by observing bands at 6.20, 10.08, and 11.03 microns. The carbonyl bands in conjunction with the bands at 8.00 and

Figure 14

Infrared Spectrum of the Pigment

KBr Pellet





11.03 microns indicate a conjugated cyclic anhydride. The band at 8.00 microns in conjunction with the carbonyl strongly suggested an acetate group. The bands at 2.92 and 6.20 microns were water from the KBr. The compound appeared to be a pyridine salt of an unsaturated, acetylated cyclic anhydride acid.

#### F. Degradation of the Pigment---Analysis by Mass Spectrometer

The vapor pressure of the pyridine salt pigment was not sufficiently high to obtain a spectrum of the compound. A relatively large amount of the pigment was placed in a glass tube connected to the inlet of the mass spectrometer. The purpose of the experiment was to heat the pigment and analyse the gases evolved. The pigment was pumped down and 10 minutes later, a sample was taken. The first run showed a small amount of carbon dioxide (m/e 44) and larger amounts of pyridine (m/e 79) and acetic acid (m/e 60). The pigment was evacuated for 1 hour to eliminate all of the gases. The pigment was then heated with a heat gun and a gas sample removed for analysis. This second run showed an abundance of pyridine.  $\cong$  carbon dioxide  $\cong$  acetic acid. After all the gases were vented from the pigment, the sample was heated again and an aliquot of the gases taken. This third spectrum showed the amount of carbon dioxide  $\cong$  pyridine  $\cong$  acetic acid.

No m/e above 80 was detected. The only three compounds found by degrading the pigment were acetic acid, carbon

dioxide and pyridine.

#### G. NMR Analysis

Figure 15 shows the NMR analysis for the pigment. The solvent used was deuterated dimethyl sulfoxide. The peak at 1.92 ppm was due to an acetyl group. The broad peak between 2.00 and 2.45 was due to methylene groups. All of the peaks past 8.00 ppm were attributed to pyridine. The peaks at 0.00 ppm and 2.54 should be disregarded as they were due to internal standard (tetramethylsilane) and solvent, respectively. Of the nine NMR spectra obtained on different pigments, all were very similar except the ratio of the acetyl group kept changing with respect to the rest of the spectrum. This indicates that there was probably some free acetic acid in the sample as a decomposition product (suggested by MS analysis). There were no olefinic hydrogens shown in the spectrum. Several spectra were made with deuterated water added. The spectra were the same as Figure 15.

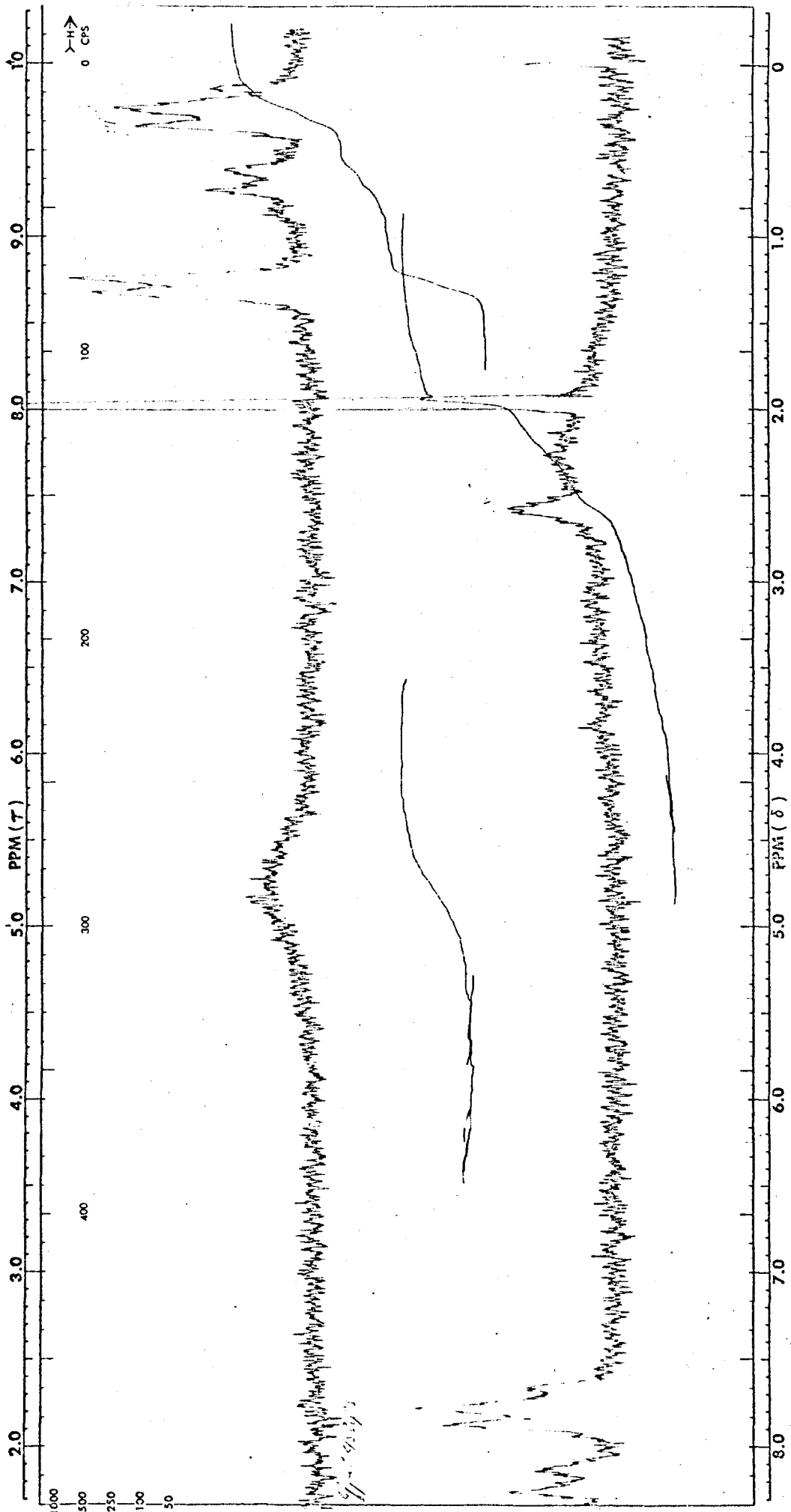
#### H. Melting Point of the Pigment

Samples of the pigment that were placed into melting point tubes and heated all showed irreversible melts. The melting point was very difficult to observe visually because the dark red-brown pigment turned light black color. All of the samples decomposed between 103°-110°C.

Figure 15

NMR Spectrum of the Pigment

Conditions: 60 megacycle instrument  
internal standard-tetramethylsilane  
solvent-deuterated dimethyl sulfoxide  
top scan offset 450 cps



Finally, a sample was submitted for differential thermal analysis (DTA). The pigment was heated from room temperature to 200°C at a rate of 5°/min. No melting point was detected. The sample decomposed on heating in air.

### I. CHN Analysis

CHN analysis was performed on the pigment produced by the addition of citric acid, acetic anhydride, and pyridine. It is believed that the pigment is a pyridine salt of an unsaturated anhydride acid. In Table VII, each empirical formula shown is calculated on the assumption that one molecule of pyridine is present per molecule of pigment.

TABLE VII

C	Found			O (by difference)	Empirical Formula-Pyridine (C <sub>5</sub> H <sub>5</sub> N)
	H	N			
56.41	2.53	2.48	38.58		C <sub>21.5</sub> H <sub>9</sub> O <sub>14</sub>
56.36	2.42	2.21	38.58		C <sub>24.5</sub> H <sub>10</sub> O <sub>15.5</sub>
57.32	2.86	2.32	37.50		C <sub>24</sub> H <sub>12</sub> O <sub>14</sub>
57.42	3.07	2.77	36.74		C <sub>19</sub> H <sub>10</sub> O <sub>11.5</sub>
55.30	3.47	3.40	37.83		C <sub>14</sub> H <sub>9</sub> O <sub>10</sub>
57.16	3.09	2.71	37.04		C <sub>19.5</sub> H <sub>11</sub> O <sub>12</sub>
54.89	3.25	3.07	38.79		C <sub>16</sub> H <sub>10</sub> O <sub>11</sub>
56.42	3.47	3.08	37.03		C <sub>16</sub> H <sub>11</sub> O <sub>10.5</sub>

All of the above data were made on independent pigment preparations. The first four were made using benzene and toluene as solvents for precipitating the pigment. It had been observed later that residual traces of benzene are difficult to remove using a vacuum pump. In the last four analyses, diethyl ether was used to precipitate the pigment. This solvent gave better yields and there was less chance of contamination because of the solubility of pyridine, acetic anhydride, acetic acid, and unreacted citric acid in ether. (The preparation of the pigment used in the last four analyses is found in section VI, D). The last two analyses in the CHN table were determined nine months apart on newly precipitated pigments. There appears to be a fairly good check.

The method used to determine the CHN analysis was checked by submitting a known pyridine oxalate. The results were:

Calcd. for $C_7H_7NO_4$ :	C, 49.70;	H, 4.18;	N, 8.28;	O, 37.83
Found	: C, 48.47;	H, 4.08;	N, 7.98;	O, 39.47

#### J. Other Experimental Observations

Several other techniques were used for analysing the pigment and/or its reaction products.

Column chromatography was used to purify the pigment. Aluminum and silica oxide columns were used with several solvent systems. In all cases the pigment turned black in color and decomposed 1-2 cm. down the column.

Because the NMR, MS, and IR suggest an acid and anhydride, an attempt was made to make the esters of the decomposition products. The pigment was added to water, made basic with sodium hydroxide, and evaporated to dryness on a steam bath. The residue was used to prepare the methyl esters of the acids present, using the procedure of Harvey, Hale and Ikeda (19). Only one ester was evident during a gas chromatographic analysis of the methyl esters. The ester was identified by MS as methyl acetate.

Several attempts were made to determine the molecular weight of the pigment. Because of the solubility of the pigment in acetone and p-dioxane, the boiling point elevation (acetone as solvent) and freezing point depression (p-dioxane as solvent) techniques were used.

For the boiling point elevation experiment, a Beckmann thermometer was placed in a round-bottom, two neck flask, along with a reflux condenser. The top of the condenser was fitted with a drying agent that excluded water from the system. A heating mantle connected to a powerstat regulated the temperature. The acetone (B.P. 56.5°C) was dried with sodium sulfate. The molecular weight of known citric acid (M.W. 192) ranged from 322 to 951. This wide range for different concentrations of citric acid was probably due to the extent of association (dimerization, trimerization, etc.) of the acid (35). To check the procedure of the molecular weight determination, ethyl caproate (M.W. 144) was substituted for citric

acid. The experimental result was 185. The molecular weight of the pigment (4 determinations) ranged from 253 to 340. Some of the pigment may have been decomposed and/or ionized by the solvent (acetone). The freezing point depression method using p-dioxane was unsuccessful because the pigment precipitated out of the p-dioxane as the solution approached the freezing point of 11.7°C.

#### K. Experimental Data Correlation

Several general statements concerning the structure of the pigment are presented. Because of the spectral similarities of the reagents used to form the pigment and the pigment itself, no definite chemical structure may be assigned. The NMR spectra showed an acetyl group(s) with methylenes. No olefinic carbon-hydrogens were present. The IR spectra had carbonyls at higher frequency than an acetate carbonyl. It also shows unsaturation, other than pyridine, as a vinyl or tetrasubstituted olefin. It is assumed, based upon the NMR data, that a tetrasubstituted olefin(s) was present. The visible spectrum (See Figure 1) indicates extensive conjugation because of the 385, 405, and 550 millimicron maxima. The IR high frequency carbonyls and the bands at 8.0 and 11.0 microns suggest an anhydride. Di- or tricarboxylic acids are usually dehydrated to form anhydrides in the presence of acetic anhydride. The CHN analysis showed a very high oxygen content. The minimum amount of carbons



according to CHN analysis was 16.

Taking the above facts into consideration, it is suggested that the pigment is a pyridine salt of a small polymeric cyclic anhydride acid with conjugation. It is possible that one carboxylic acid group forms an anhydride with one carboxylic acid group of a different molecule of citric acid. Another possibility is a highly conjugated ketone type acid salt instead of an anhydride.

## SUMMARY

A method for the determination of citric acid employing anhydrous conditions has been studied. The method was based on the Furth-Herrmann reaction of citric acid, acetic anhydride, and pyridine. The chromophore produced was measured spectrophotometrically at 385 millimicrons. The conditions of the method, such as amount of reagents, order of reagents, effect of water, and temperature were optimized for maximum sensitivity and minimum reaction time. Sixteen different acids were substituted for citric to determine if any interfered with the method.

A satisfactory procedure was developed to determine citric acid and the method was applied to citric acid in cigarette tobacco.

The pigment produced in the method was isolated. It was found to decompose with traces of water and was soluble in acetone, acetic anhydride, and pyridine. Visible, IR, NMR, and MS spectra of the pigment were obtained. Though the exact chemical structure was not identified, general structure types were proposed.

In conclusion, a method for the determination of citric acid in the 0-60 microgram range was developed. A procedure was developed to isolate the pigment formed by the Furth-Herrmann reaction. The pigment was characterized by several spectral techniques and general structures proposed.

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