AN ABSTRACT OF THE THESIS OF

Ae-June Wang for the Master of Science Degree

in Chemistry presented on December 10, 1987

TITLE : HPLC Analysis and Reactions of N-Nitrosamines

ABSTRACT APPROVED : Richard C. Schreiber

N-Nitrosamines are known carcinogens and have already been found in those materials concerned about our daily life. So the object of this project was to develop a simple, rapid and accurate method for N-Nitrosamines and to apply the method to the detection of N-Nitrosamines and to study of their subsequent reactions .

The compounds used here, are N-Nitrosodimethylamine, N-Nitrosodiethylamine, N-Nitrosodipropylamine and N-Nitrosodiphenylamine. The method presented here is to use High-Performance Liquid Chromatography with a reverse-phase column, methanol-water mixtures as the mobile phase and a UV detector. The HPLC chromatograms are well defined and every run can be completed within 10 minutes.

Although some reports stressed that ascorbic acid can block the formation of N-Nitrosamines, from the results of this project, it can be infered that ascorbic acid cannot affect the N-Nitrosamines or reduce them when N-Nitrosamines have already formed.

Finally. the detection of N-Nitrosamines in cigarette smoke was performed and analyzed by HPLC. There were complicate peaks on the chromatogram due to the extra compounds present. In this project, the advanced clean-up procedures were not performed, but two methods for clean-up in references would be recommended.

HPLC ANALYSIS AND REACTIONS OF N-NITROSAMINES

A Thesis

Presented to

the Division of Mathematical & Physical Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Chemistry

by

Ae-June Wang

December, 1987

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ACKNOWLEDGEMENT

I would like to express my gratitude to Dr. David C. Schroeder for his guidance and direction during the investigation and I also would like to express my appreciation to Dr. Charles M. Greenlief for his assistance and advice throughtout my graduate career.

I would like to express my deepest thanks to my father , mother, and also to my sisters - Ai Ping and Ai Yeun, and brother - Yeun Chung. Their loving concern and spirtual support enable me to overcome the difficulties whether it be in my studies or my daily life. This thesis is as much theirs as it is mine.

A special thanks to Kurtis Mitchell for his advice and aid in the correcting and the proofreading of this thesis.

Finally, I would like to thank my close friends - Huang Peifen, Kim Chang Suk, and Yu Cheng Tong. Their friendship and concern have brought me a cheerful and enjoyable life in Emporia.

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INTRODUCTION

N-Nitrosamines are harmful compounds and have already been identified as carcinogens (1). They exist in many materials such as soyabean oil (2), curing meat (3), foodstuffs (4), air (5), and cigarette smoke (6-12). Two ways in which man may encounter nitroso compounds are : [1] by the reaction of nitrite and nitrogen - containing molecules found in the stomach of humans ; and [2] by the ingestion of the compounds as such in food or something else (16). There is concern about there possible widespread occurence in our daily life. How to identify the N-Nitrosamines and to investigate their related reactions will become more important in the future.

A variety of methods have proven useful in the determination of Nitrosamines. Gunatilaka used the Thin-Layer Chromatography (TLC) and staining methods for N-Nitrosamine detection (13), but it is not a convenient or even a precise method. Chamberlain et al. reported a method using Glass Capilary Gas Chromatography (GC) and a nitrogen -phosphorus thermionic detector (NPD) to monitor N-Nitrosamines in cigarette smoke (10). GC-NPD successfully separated the nitrosamines' peaks because NPD would eliminate interferences of non-nitrogen containing compounds the quantitative analysis of the N-Nitrosamines, but the retention time was too long. Another method using Gas-Liquid Chromatography (GLC) with a thermal energy

analyzer (TEA) was reported by Brunnemann & Hoffmann (8-9). They also used GC-TEA to successfully identify the N-Nitrosamine in cigarette smoke (6,11). On the other hand, Fine et al. also mentioned that only a small number of N-Nitrosamines compounds are amenable to Gas-Chromatographic techniques because some N-Nitrosamines compounds will decompose at the high temperature prevelent in the oven of the GC. So they used High-Performance liquid Chromatography (HPLC) and TEA to identify the trace amounts of N-Nitrosamines(15). Although the HPLC-TEA chromatograms are well defined, the HPLC system is not yet as sensitive as the GC-TEA. Because in the HPLC-TEA there is a transition from the liquid phase to the gas phase, the HPLC-TEA interface is considerably more complex than its GC-TEA counterpart.

Although a couple of methods are available, there is a need for a simple, rapid and precise method to identify several N-Nitrosamines. The method proposed here is to use High - Performance Liquid Chromatography and a variable - wavelength UV detector. If the compound can be detected in the UV region, it also can be identified by HPLC. The compounds used here, are N-Nitrosodimethylamine (NDMA) , N-Nitrosodiethylamine (NDEA), N-Nitrosodipropylamine (NDPA), N-Nitrosodiphenylamine (NDPhA). These samples were passed through a reverse-phase HPLC column to separate the various N-Nitrosamines and various methanol-water were utilized as the mobile phase. The reverse-phase HPLC

column was a non-polar one so that the most polar compound would be eluted first. Among these four N-Nitrosamines, NDMA is the most polar and NDPhA is the least polar; therefore the retention time would be incresed as NDMA, NDEA, NDPA, NDPhA.

Though many authors have already reported the HPLC method (12, 15, 16), the method proposed here uses a different column type and detector. By this means, we obtain a rapid, simple way of analyzing the N-Nitrosamines.

Vitamin C (Ascorbic Acid) can block N-Nitrosamines formation as reported as Oppenheimer (1). Some authors also mentioned the effect in curing meat (3,17). Sen et al., indicated that in certain cases, the addition of ascorbic acid or its sodium salt can inhibit the nitrosation reaction and prevent it completely (3). Gough et al., also explained that because ascorbic acid is a reducing agent and can be used in accelerating the reduction of nitrite to nitric oxide and in reducing oxidized pigment so that the formation of N-Nitrosamines in a system containing ascorbic acid is likely to be slowed (17).

Many studies have reported that the formation of N-Nitrosamine can be blocked, but few authors mentioned if the N-Nitrosamines had already formed, whether ascorbic acid can react with the N-Nitrosamines or not. We were interested in making the reaction conditions similar to those in human stomach and put ascorbic acid in N-Nitrosamines solution, then used HPLC to detect N-

Nitrosamines concentration. In addition, we also compared the solution containing ascorbic acid with the solution which did not contain ascorbic acid. The reason for this is that many N-Nitroso compounds are extremely heat- and light-sensitive (15). During the reaction, the temperature is maintained at normal body temperature (37 degree C) for 48 hours. The N-Nitrosamines may decompose and the concentration will be lower than the original value (16). In order to avoid this deviation , we need to compare these two solutions.

Some studies mentioned that the concentration of N-Nitrosamines formed not only depends on temperature, time, and the addition of ascorbic acid, but also on the pH value (3,17). So we also carried out the reaction condition at different pH values to see whether ascorbic acid could reduce the concentration of N-Nitrosamines .

There are many studies concerned with the determination of N-Nitrosamines in cigarette smoke (6-12). These studies have elaborate procedures for the concentration, separation, detection and quantitative analysis of cigarette smoke nitrosamines. Most of them use a smoking machine to collect the smoke condensate and utilize GC, GLC or HPLC with MS, TEA, NPD to analyze the N-Nitrosamines.

The method for the determination of these four Nitrosamines - NDMA, NDEA, NEPA, NDPhA in cigarette smoke, used in this project , was to trap the cigarette smoke

in methanol and water and then analysis of the solution by HPLC. We used the 80% methanol and deionized water as the trapping solution first. There should be more organic compounds in methanol (organic solvent) than in water. The water solution is needed to add methanol to make the solution as the same percentage with the mobile phase then analyze, so the concentration in water solution would be lower than in methanol.

In order to make N-Nitrosamines more soluble in the water solution , sulfuric acid was added to the water. We hoped that the acid solution could trap the nitrosamine but not other organic compounds in the cigarettes.

It was the objective of this study to develop a simple, rapid HPLC method for N-Nitrosamines and to apply the method to the detection of N-Nitrosamines and to study of their subsequent reactions.

EXPERIMENTAL

I. Identify the N-Nitrosamines

A. Apparatus

25 ml syringe was used to add methanol to four stock N-Nitrosamines

B. Reagents

The four stock N-Nitrosamines used here were

N-Nitrosodimethylamine (NDMA),

N-Nitrosodiethylamine (NDEA),

N-Nitrosodipropylamine (NDPA),

N-Nitrosodiphenylamine (NDPhA).

These reagents are CAS-reagent grade and isopac, each one using 1 gram package and were obtained from Sigma Chemical Company. HPLC-grade methanol was used to make nitrosamine solutions.

C. Procedure

A stock N-Nitrosamine solution was prepared by taking 1 gram of N-Nitrosamine which is sealed tightly in a glass bottle and using a syringe and needle added methanol in 25 ml increment four times so the total volume was 100 ml. This gave a final concentration of 10 mg of N-Nitrosamine per milliliter at the solution.

(I) Ultraviolet Spectrophotometric Screening Method

A. Apparatus

UV Absorbance was measured with an EU-707 Series GCA/Mcpherson Spectrophotometric Instrument using matched 1-cm path length silica cells.

B. Reagents

A standard N-Nitrosamine solution was prepared by diluting 0.1 ml of stock N-Nitrosamine solution to 25 ml with methanol, giving a final concentration of 40 mg of N-Nitrosamine per liter.

C. Procedure

The four samples were scanned at variable wavelengths from 200 nm to 300 nm. The sample was analyzed against methanol as a blank , set at zero absorbance.

(II) A Method for Determining Four N-Nitrosamines

A. Apparatus

HPLC analysis were performed on a Varian 2010 pump / 2210 system. The UV detector was a variable-wavelength, model 2050, and the wavelength was set at 230 nm. The injector was Rheodyne 7125 with a 10μl loop. All injections were full-loop and an analog strip chart recorder was operated simultaneously, directly from the

detector. The column was an Alltech C18 Cartridge, 25 cm x 4.6 mm i.d., with 5 m particle diameter. 4.25 cm Whatman GF / C Glass Microfibre filters were used to filter the mobile phase.

B. Reagents

Four standard N-Nitrosamine solutions were prepared as decribed in the previous section. A mixed standard was prepared , containing 10 mg/L of each of the four N-Nitrosamines . The mobile phase was prepared with 70% (v/v) HPLC-grade methanol in deionized water. This mobile phase was filtered through a 4.25 cm GF/C glass-fiber filter before use. Other mobile phases containing 65% (v/v) and 60% (v/v) methanol were prepared in a similar manner.

C. Procedure

First, the 70% methanol was used as the mobile phase then power was turned on to the HPLC and pump. The solvent was then pumped through the column to make the baseline smooth. Then each standard N-Nitrosamines solution and the mixture solution were injected to get the chromatograms. All of the HPLC analysis were performed at a flow rate 1.0 ml/min at room temperature. The 1 ml injection syringe was rinsed twice with sample and filled to 0.2 ml for rinsing and filling the injector loop. Each injection was performed in duplicate , except

during the comparison of two reactions then each sample was injected once.

The mobile phase was then changed to 65% methanol and 60% methanol to get each chromatogram.

(III) Calibration Curve for Different Concentration of four N-Nitrosamines

A. Apparatus

HPLC and Filter were used as described the same in the previous section (II).

B. Reagents

N-Nitrosamine standards of 3, 5, 10, 16, 20 mg per liter were prepared by diluting the 40 mg/L standard solution to the required concentration using deionized water.

The mobile phase containing 70% (v/v) methanol was prepared as described in the previous section (II).

C. Procedure

Power was turned on to the HPLC and the solvent was pumped through the column . A wavelength of 230 nm was used and the samples were injected in different concentrations to get each chromatogram. From the peak height , we can calculate the absorbance and then plot the calibration curves. All the HPLC operations were the

same as in section(II).

II. Reaction Rate of N-Nitrosamines and Ascorbic Acid

A. Apparatus

HPLC was used as described in the previous section (II). Two 250 ml Jacketed beakers were used as the reaction vessels. The thermostatic water bath was used to control the temperature at 37 degree C and a stirrer and stir bar were used to push the reaction. Watch glasses were used to cover the reaction vessels.

B. Reagents

Hydrochloric acid solution with concentrations of 0.01 M, 0.05 M and 0.1 M were prepared by diluting 1 M ACS-reagent grade hydrochloric acid solution. Stock N-Nitrosamine solutions were as described in section (II). Ascorbic acid (0.01 g USP-grade) was placed in a 200 ml reaction solution to obtain a final concentration of 50 mg/L. Phosphate buffer solution(pH=7) was prepared as the reaction solution. HPLC-grade methanol was used to make the mobile phase.

C. Procedure

First, prepared 37 degree C water bath in 250 ml Jacketed beakers, two beakers were connected with rubber tubes. Each beaker contained 200 ml 0.01 M HCl solution

on magnetic stirrers.

Added 50 μl Dimethylnitrosamine stock solution (initial concentration 2.5 mg/L). At time intervals of 0, 1 hour, 3 hours, 8 hours, 24 hours, 48 hours , 3 ml of solution were withdrawn by pipet and transfered to 10 ml volumetric flasks, diluted to volume with methanol and immediately analyzed by HPLC. (Actual time is from addition of Dimethynitrosamine until HPLC analysis.)

Then repeated step 2 in the other 250 ml jacketed beaker, this time with 200 ml 0.01 M HCl solution, 50 ul Dimethylnitrosamine stock solution and 0.01 g ascorbic acid (50 mg/L).

After the above procedures, the same steps were repeated the other three N-Nitrosamines. The concentration of each standard nitrosamine solution is 2.5 mg/L. Each solution was then analyzed by HPLC.

Then, Diethylnitrosamine was used as the sample, changing the 0.01 M HCl solution to 0.05 M HCl and 0.1 M HCl solution and repeated step 3-4. Solutions were again analyzed by HPLC.

The Diethylnitrosamine and Dipropylnitrosamine were then used as the samples and changed the HCl solution to 50% buffer solution(pH=7) and 50% water mixture (by volume ratio) and repeated the previous two steps, each standard was analyzed with HPLC.

Repeated the above steps but this time used watch glasses to cover the beaker and used parafilm tighten at

the edge to avoid solution vaporization or letting oxygen in.

III. Identify the N-Nitrosamines in Cigarette Smoke

A. Apparatus

HPLC was used as described in the previous section (II). Water vacuum was used to produce the cigarette smoke. The cigarette smoke collecting equipment was shown in Figure 1. The cigarette smoke solution was filtered through a PTFE membrance filter before HPLC analysis.

B. Reagents

Commercial U.S. cigarettes were without filter tips. HPLC-grade methanol was used to prepare the mobile phase and added to the cigarette smoke solution to make the cigarette smoke solution the same percentage with the mobile phase. Methanol solution (70%, 65% and 80%) were prepared as described in section (II).

C. Procedure

Setting the cigarettes smoke collected equipment, one test tube contain 90 ml water and the other one contain 90 ml 80% methanol solution. First put a cigarette in the left hand side tube then turn on the water vacuum until the solution has a little bubbles, then light

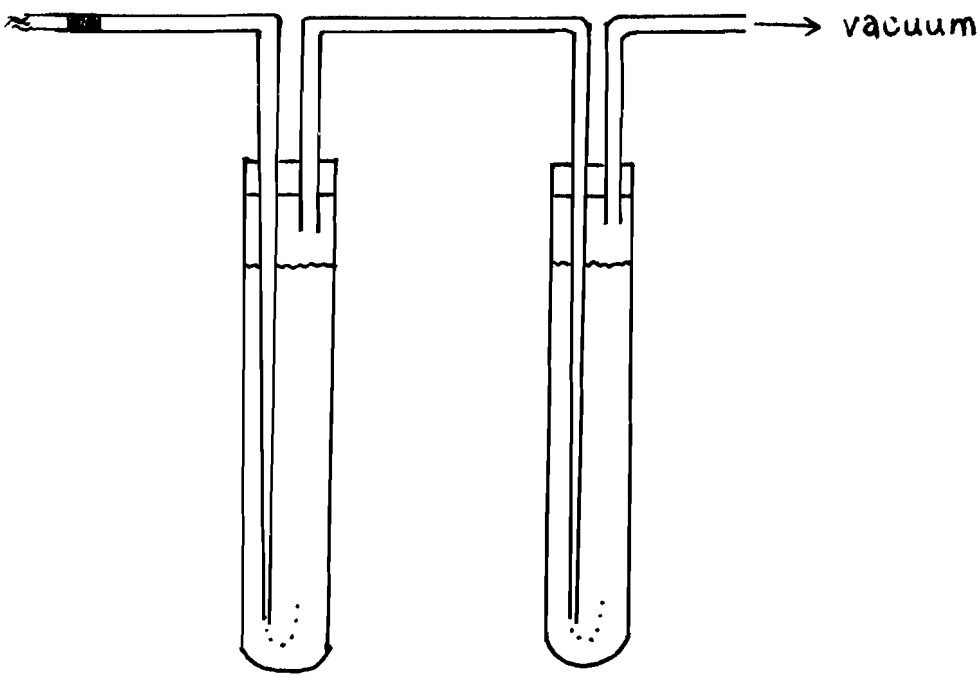
the cigarette. After the cigarette fired out, stopped the vacuum and changed other cigarette. Repeated the collecting steps to gather smoke from 7 cigarettes .

In order to avoid the impurities of the cigarettes in the solution which would obstruct the column, the sample solution was filtered before HPLC analysis. After the filter step, the 80% methanol cigarette smoke solution was directly analyze by HPLC. But the water cigarette smoke solution must have methanol added to be the same ratio with the mobile phase.

The first time, 70% methanol was used as the mobile phase and the second time 65% methanol was used as the mobile phase. Each sample analysis, was performed twice at two wavelengths - 220 nm and 230 nm in order to compare the results.

The second time , the object will be to change the trapped solution using 80 ml water, 10 ml sulfuric acid (5N) and then repeated the previous steps and analyzed by HPLC.

Figure 1 : Cigarettes Smoke Collecting Equipment



RESULTS AND DISCUSSION

I. Identify the N-Nitrosamines

The first step in analyzing four N-Nitrosamines by HPLC was to find the suitable wavelength in UV detector. From UV screening method, using a standard solution of 40 mg/L, four N-Nitrosamines were analyzed. According to the UV spectrum (Figure 2), it was known that the maximum absorbance in the spectrum was in the wavelength around 230 nm. The wavelength in the maximum absorbance was chosen in order to get the highest, clearest peaks in the HPLC chromatogram.

When the wavelength was set at 230 nm, the different percentage methanol mobile phases could be tested. The results are listed in Table 1. From the results it was found that the 70% methanol is the most suitable mobile phase for N-Nitrosamines analysis because it had a lower retention time than the other two mobile phases and could still separate the four N-Nitrosamines' peak successfully. A typical four N-Nitrosamines chromatogram is shown in Figure 3.

In order to determine the concentration of nitrosamines easily, the calibration curves for each N-Nitrosamine were made. For different concentrations of the standard N-Nitrosamine solutions, the absorbance could be determined from HPLC chromatogram. Plotting absorbance against

Figure 2 : UV Spectrum of four N-Nitrosamines

(40 mg/L)

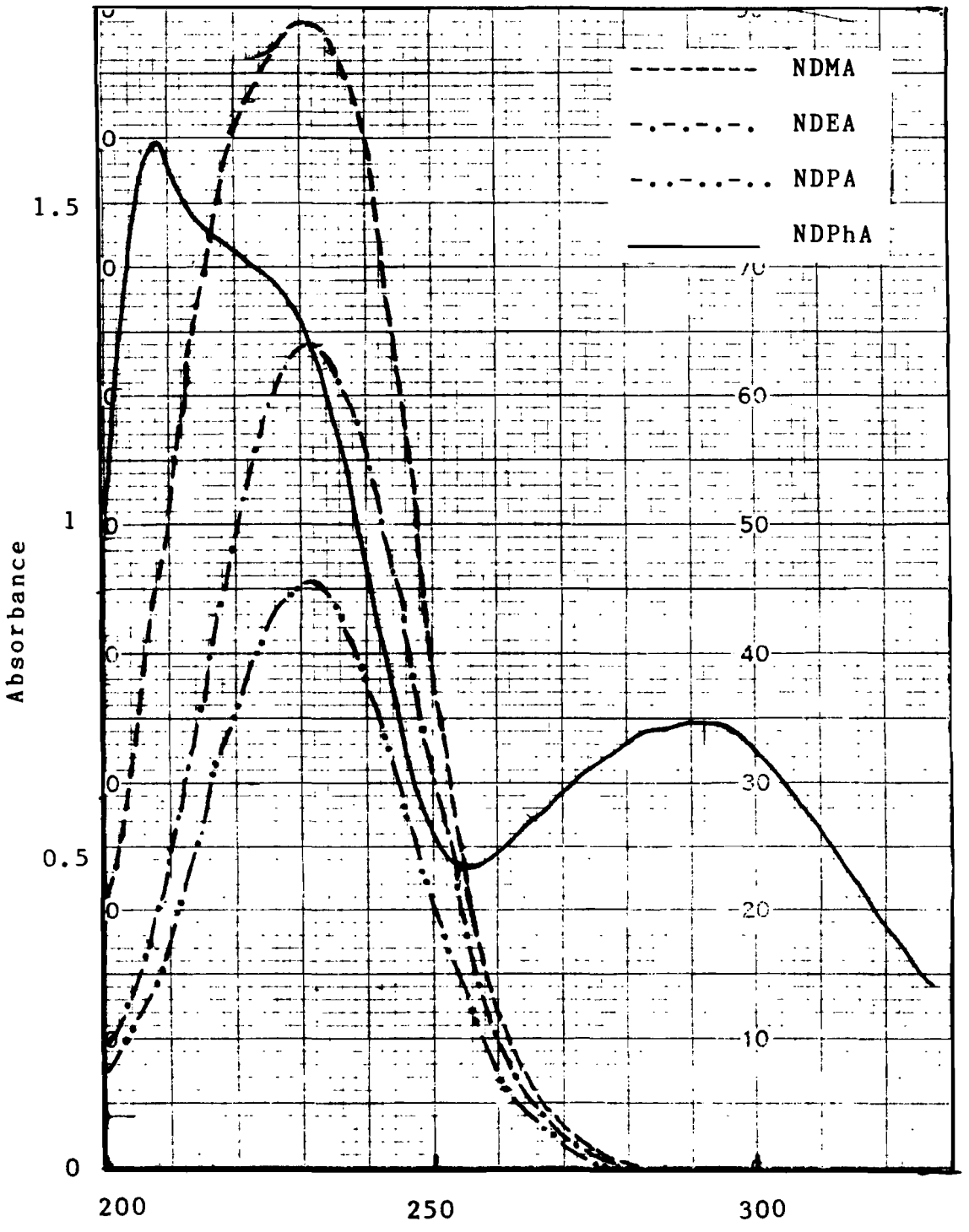
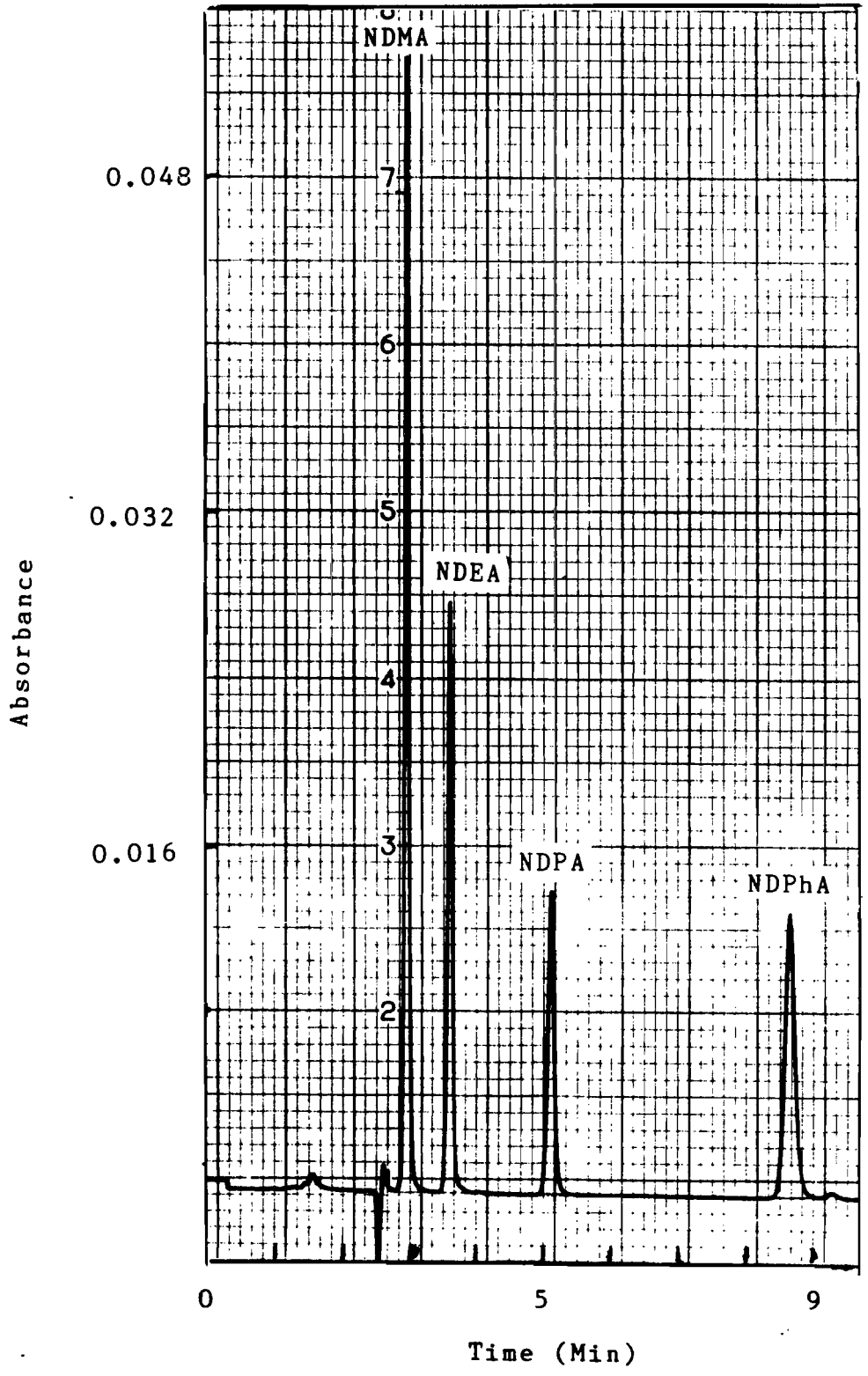


TABLE 1 : The Retention Time of Four N-Nitrosamines in Different Percentage Mobile Phase

Sample	Retention Time (in minutes) in different mobile phase		
	70% methanol	65% methanol	60% methanol
NDMA	2.95	3.00	3.10
NDEA	3.70	3.90	4.30
NDPA	5.00	6.10	7.90
NDPhA	8.55	12.1	19.4

Figure 3 : A Chromatogram for 5 mg/L NDMA, NDEA, NDPA, NDPhA Mixture in 70% Methanol



concentration , the calibration curves are shown in Figures 4, 5, 6, 7 and the data are shown in Table 2. The detector response in absorbance is linearly related to the concentration up to 20 mg/L.

II. Reaction Rate of N-Nitrosamines and Ascorbic Acid

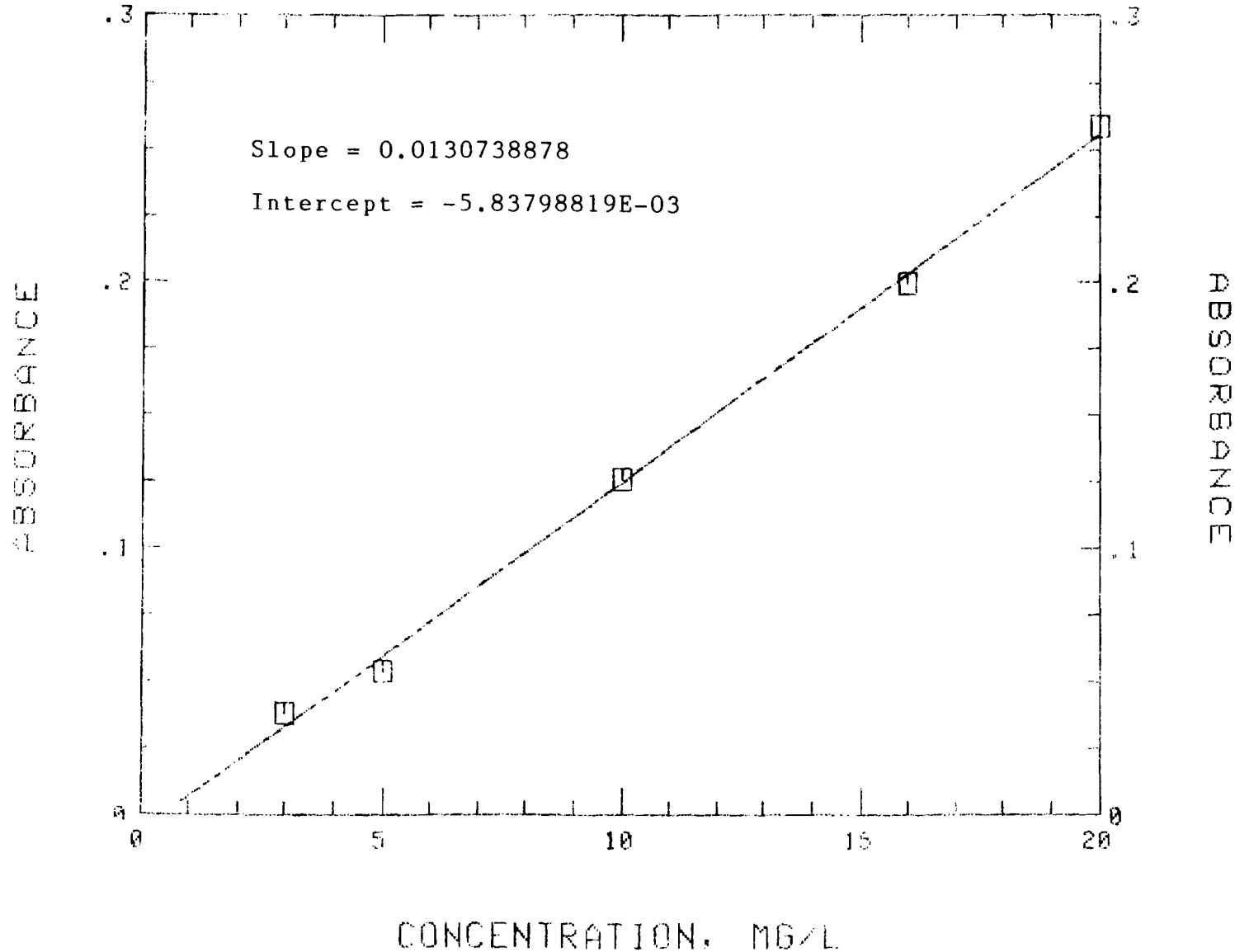
Tables 3-6 list the results for N-Nitrosamines reacting with ascorbic acid in 0.01 M HCl solution compared with an absence of ascorbic acid in the reaction mixture. The concentration of N-Nitrosamines seemed to be decreasing but the concentration of the solution which did not contain ascorbic acid was also decreasing and the ascorbic acid did not decrease. In view of these results, we can conclude that ascorbic acid seems to have no function in reducing the N-Nitrosamines.

On the other hand, Diethylnitrosamine and Dipropylnitrosamine were used as examples, changing the HCl solution to phosphate buffer solution (pH=7) and water mixture (v/v 50:50). From the results shown in Table 7, it was found that the concentration of the two nitrosamines still were not changed a lot but ascorbic acid concentration decreased. The reason for this might be that N-Nitrosamines had not reacted with the ascorbic acid but the ascorbic acid decomposed. Ascorbic acid was easily decomposed in this suitation(pH=7). Gough et al. already reported that at pH values above 3, the dissociation of the

Table 2 : The Data of Calibration Curves for N-Nitrosamines Standards

Sample	Absorbance	Concentration(mg/L)
NDMA	0.2586	20
	0.1997	16
	0.1261	10
	0.0544	5
	0.0381	3
NDEA	0.1376	20
	0.1050	16
	0.0666	10
	0.0285	5
	0.0198	3
NDPA	0.0717	20
	0.0550	16
	0.0339	10
	0.0147	5
	0.0102	3
NDPhA	0.0666	20
	0.0512	16
	0.0320	10
	0.0138	5
	0.0096	3

FIGURE 4 : CALIBRATION CURVE FOR N-NITROSODIMETHYLAMINE STANDARDS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Intercept = -5.83798819E-03
ABSORBANCE
ABSORBANCE
.3
.2
.1
0
0
5
10
15
20
CONCENTRATION, MG/L

FIGURE 5 : CALIBRATION CURVE FOR N-NITROSODIETHYLAMINE STANDARDS

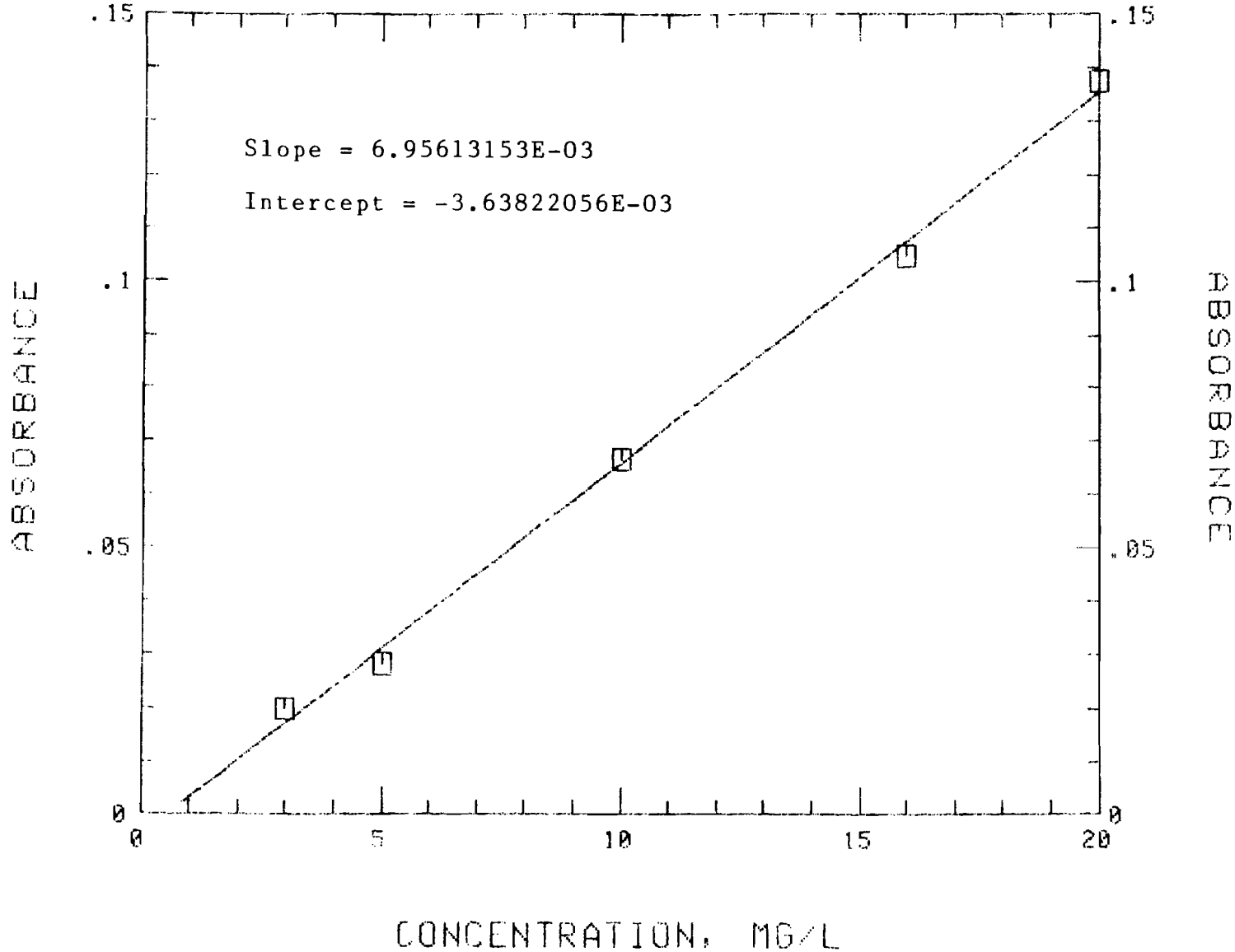
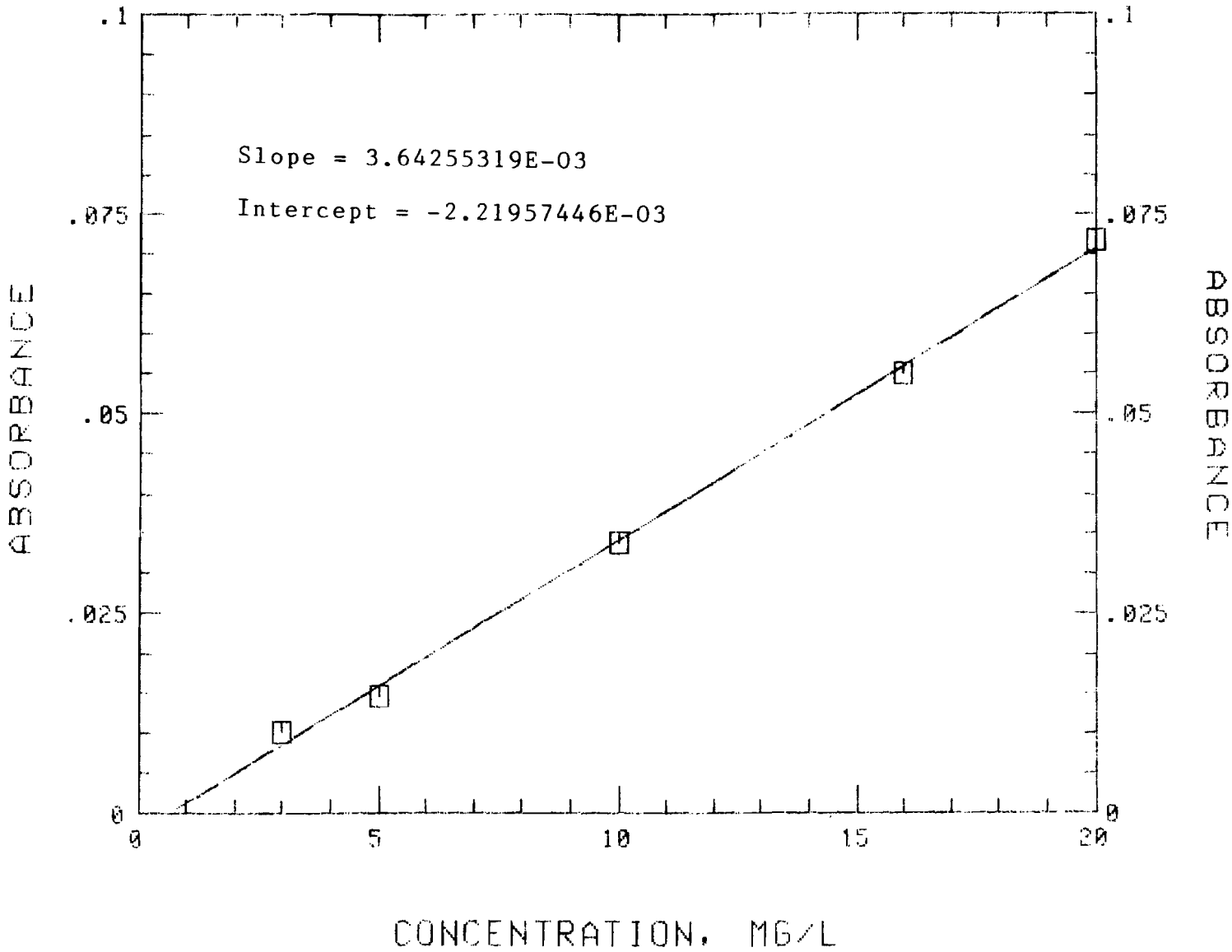

Slope = 6.95613153E-03
Intercept = -3.63822056E-03
ABSORBANCE
ABSORBANCE
.15
.1
.05
0
0
5
10
15
20
CONCENTRATION, MG/L

FIGURE 6 : CALIBRATION CURVE FOR N-NITROSODIPROPYLAMINE STANDARDS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Intercept = -2.21957446E-03
ABSORBANCE
ABSORBANCE
.1
.075
.05
.025
0
0
5
10
15
20
CONCENTRATION, MG/L

FIGURE 7 : CALIBRATION CURVE FOR N-NITROSODIPHENYLAMINE STANDARDS

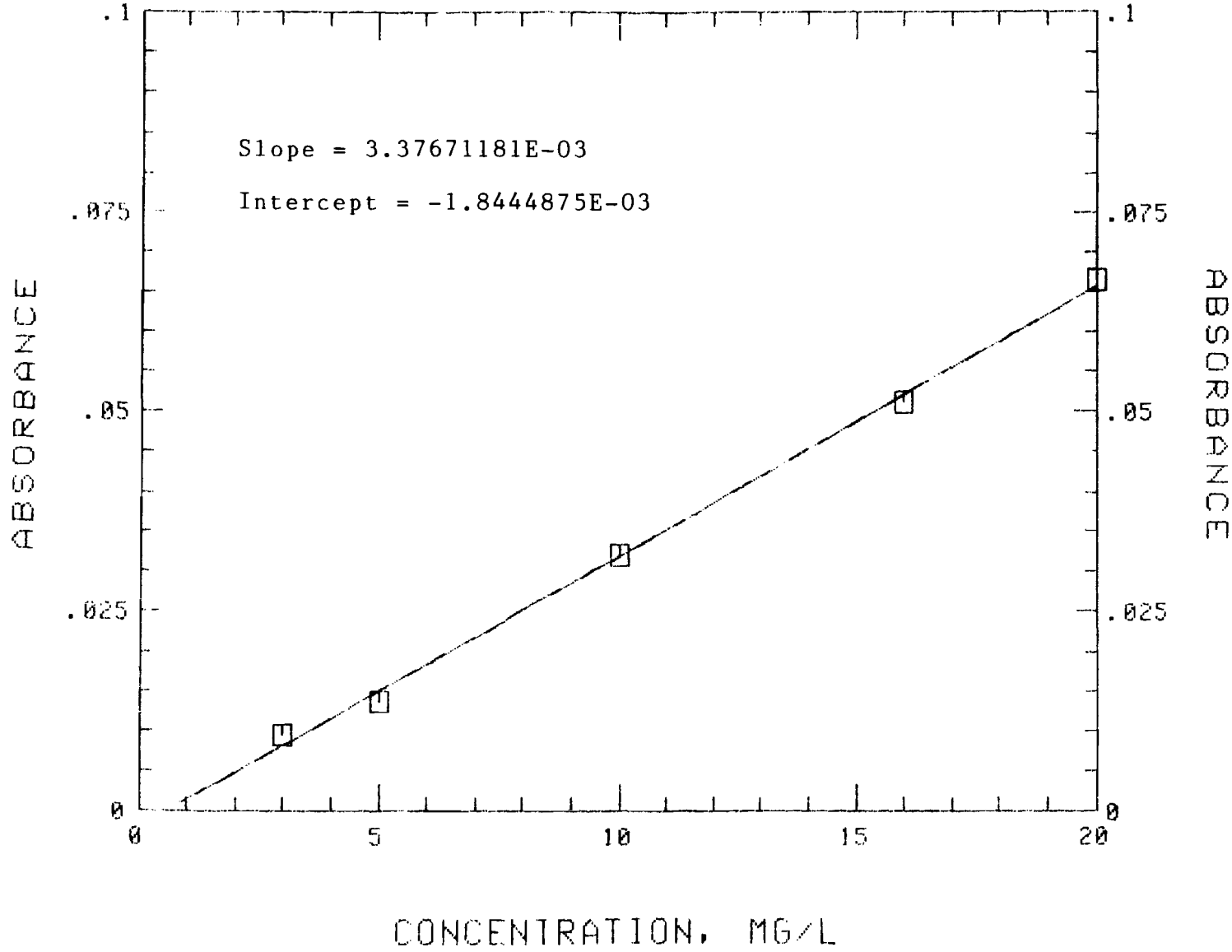

Slope = 3.37671181E-03
Intercept = -1.8444875E-03
ABSORBANCE
ABSORBANCE
.1
.075
.05
.025
0
0
5
10
15
20
CONCENTRATION, MG/L

Table 3 : N-Nitrosodimethylamine reacted with ascorbic acid in 0.01 M HCl solution

Time (hour)	NDMA		NDMA + ascorbic acid	
	Abs.	Conc. (mg/L)	Abs.	Conc. (mg/L)
0	2.08E-3	0.82	2.08E-3	0.82
1	2.00E-3	0.81	2.00E-3	0.81
3	1.92E-3	0.80	1.92E-3	0.80
8	1.60E-3	0.75	1.60E-3	0.75
24	9.60E-4	0.66	9.60E-4	0.66
48	8.00E-4	0.64	8.00E-4	0.64

Table 4 : N-Nitrosodiethylamine reacted with ascorbic acid in 0.01 M HCl solution

Time (hour)	NDEA		NDEA + Ascorbic acid	
	Abs.	Conc. (mg/L)	Abs.	Conc. (mg/L)
0	9.12E-3	1.14	9.12E-3	1.14
1	9.12E-3	1.14	9.12E-3	1.14
3	9.28E-3	1.15	9.28E-3	1.15
8	9.12E-3	1.14	9.12E-3	1.14
24	9.12E-3	1.14	9.12E-3	1.14
48	9.11E-3	1.14	9.11E-3	1.14

Table 5 : N-Nitrosodipropylamine reacted with ascorbic acid in 0.01 M HCl solution

Time (hour)	NDPA		NDPA + Ascorbic acid	
	Abs.	Conc. (mg/L)	Abs.	Conc. (mg/L)
0	2.56E-3	1.31	2.56E-3	1.31
1	2.72E-3	1.36	2.72E-3	1.36
3	2.64E-3	1.33	2.64E-3	1.33
8	2.56E-3	1.31	2.56E-3	1.31
24	2.56E-3	1.31	2.56E-3	1.31
48	1.44E-3	1.00	1.44E-3	1.00

Table 6 : N-Nitrosodiphenylamine reacted with ascorbic acid in 0.01 M HCl solution

Time (hour)	NDPhA		NDPhA + Ascorbic acid	
	Abs.	Conc. (mg/L)	Abs.	Conc. (mg/L)
0	2.28E-3	1.22	2.28E-3	1.22
1	2.24E-3	1.21	2.24E-3	1.21
3	2.20E-3	1.20	2.20E-3	1.20
8	1.90E-3	1.11	1.90E-3	1.11
24	1.70E-3	1.05	1.70E-3	1.05
48	8.58E-4	0.80	8.58E-4	0.80

Table 7 : N-Nitrosodiethylamine & N-Nitrosodipropylamine with ascorbic acid reaction in buffer solution (pH=7)and water mixture (50:50 v/v)

Time (hour)	NDEA & ascorbic acid		NDPA & ascorbic acid	
	Abs.	Conc. (mg/L)	Abs.	Conc. (mg/L)
0	5.44E-3	1.31	2.72E-3	1.36
1	5.44E-3	1.31	2.72E-3	1.36
3	5.36E-3	1.29	2.56E-3	1.31
8	5.28E-3	1.28	2.56E-3	1.31
24	5.28E-3	1.28	2.24E-3	1.22
48	5.28E-3	1.28	2.24E-3	1.22

Table 8 : N-Nitrosodiethylamine with ascorbic acid reaction in 0.05 M HCl and in 0.1 M HCl solution

Time (hour)	NDEA & ascorbic acid in 0.05 M HCl		NDEA & ascorbic acid in 0.1 M HCl	
	Abs.	Conc. (mg/L)	Abs.	Conc. (mg/L)
0	5.28E-3	1.28	5.28E-3	1.28
1	5.28E-3	1.28	5.28E-3	1.28
3	5.28E-3	1.28	5.28E-3	1.28
8	5.28E-3	1.28	5.28E-3	1.28
24	5.04E-3	1.25	5.04E-3	1.25
48	5.04E-3	1.25	5.04E-3	1.25

ascorbic acid became significant (17). Our results corresponded with his viewpoint.

Table 8 lists the results for Diethylnitrosamine in 0.05 M and in 0.1 M HCl solutions. Trying to increase the acidity of the solution in order to avoid the ascorbic acid decomposition and to push the reaction between N-Nitrosamines and ascorbic acid. But the concentration of N-Nitrosodiethylamine almost remained the same result as with Table 4.

From Table 3-8, these lists serve to stress one key point, namely that the ascorbic acid did not react with N-Nitrosamines. If the reaction time becomes longer, the solution will appear light yellow in color. The reason is that N-Nitrosamines can not be exposed to light or oxygen or the N-Nitrosamines decomposed. This is also the reason why the concentration of the N-Nitrosamine solution either containing ascorbic acid or not, are all lower.

III. Identify the N-Nitrosamines in Cigarette Smoke

Table 9 represents the result comparing the sensitivity of four nitrosamines at two wavelengths. It could be seen that except for N-Nitrosodiphenylamine, the other three nitrosamines' sensitivities at 230 nm were higher than at 220 nm, but N-Nitrosodiphenylamine had the higher sensitivity at 220 nm. This can be used to confirm the identify of the specific peak in HPLC chromatogram.

In Figures 8-11 are shown the comparison for cigarette smoke solution at two different wavelengths. Figure 8 was the chromatograms for cigarette smoke trapped in 80% methanol and using 70% methanol as the mobile phase. The peaks were complicated because there were also other Nitroso-compounds and tar which could dissolve in metnanol. Each Nitrosamine could be identified by comparing the retention times with the standard reference mixture. NDMA, NDEA, NDPA were all found in the solution. The position in the same retention time with NDPhA has a large peak and it could not verified whether NDPhA existed in the solution or not. The other three nitrosamines all had the higher peak at 230 nm and the lower peak at 220 nm but it was difficult to calculate the ratio compared to Table 9 because of the overlapped peaks.

Figure 9 shows the chromatograms for cigarette smoke trapped in deionized water with 70% methanol as a mobile phase. Comparing Figure 8 with Figure 9, it was found that there were less compounds dissolved in water than in methanol. The addition of the methanol to the water would dilute the samples so the concentration would be lower. The peaks for NDMA, NDEA could be found but NDPA, NDPhA's peaks were not clear. It was also hard to calculate the ratio of two peaks.

In order to make sure of the specific peaks in chromatogram, the mobile phase was changed from 70% methanol to 65% methanol and the distance between each of

of the two peaks would be larger and the overlapped peaks would be more separate to be easily identified. Figure 10 was the chromatogram for cigarette smoke trapped in 80% methanol. From the result, it could be seen that NDPhA had already separated from the big peak and appeared as an independent peak. NDPA and NDMA seemed to be hindered by other peaks. NDEA's peak became independent and clear but the peak at 220 nm was higher than the peak at 230 nm. It was evident that the peak was not NDEA, unless the NDEA's peak was hindered by other peaks. The details were not deeply searched.

Figure 11 is the chromatogram for the smoke trapped in sulfuric acid solution using 65% methanol as the mobile phase. From the result, it was found that NDMA, NDPA, NDPhA either overlapped with other peaks or were not clear so it was very difficult to identify them from the chromatogram. Although NDEA had a very clear peak, after comparing the two peaks at two wavelengths, the same problem could still be seen with Figure 10. The acid trapped solution seemed to be unsuitable.

Table 9 : A Comparison of N-Nitrosamines Sensitivity at Two Wavelengths

Sample	Sensitivity (abs./ g)		Ratio
	230 nm	220 nm	230 nm / 220 nm
NDMA	1.210	0.896	1.35
NDEA	0.685	0.452	1.52
NDPA	0.301	0.188	1.60
NDPhA	0.226	0.264	0.86

Figure 8 : A Comparison of Cigarettes Smoke in 80% Methanol at Two Wavelengths (70% Methanol as The Mobile Phase)

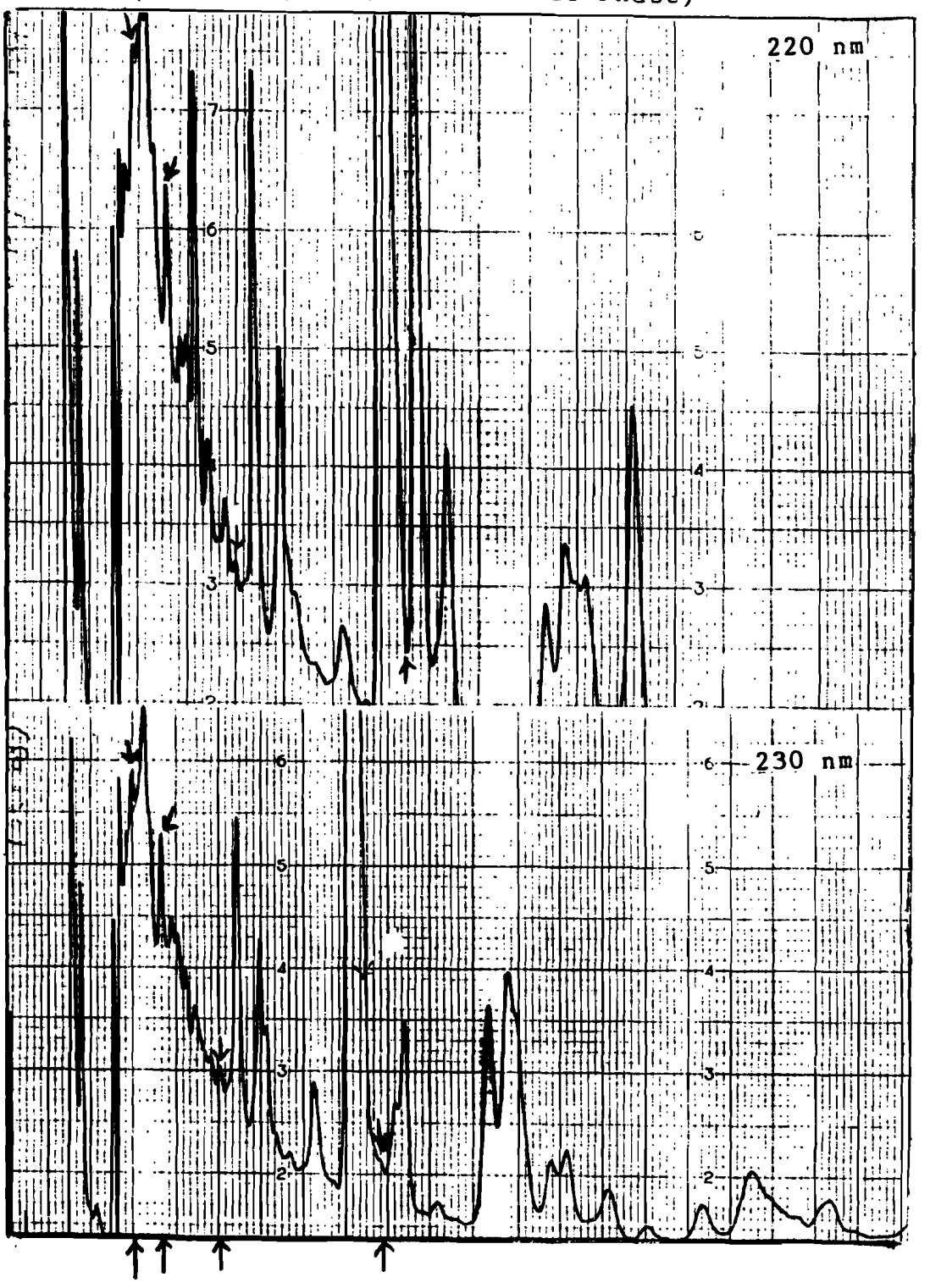


Figure 9 : A Comparison of Cigarettes Smoke in Water at Two Wavelengths (70% Methanol as The Mobile Phase)

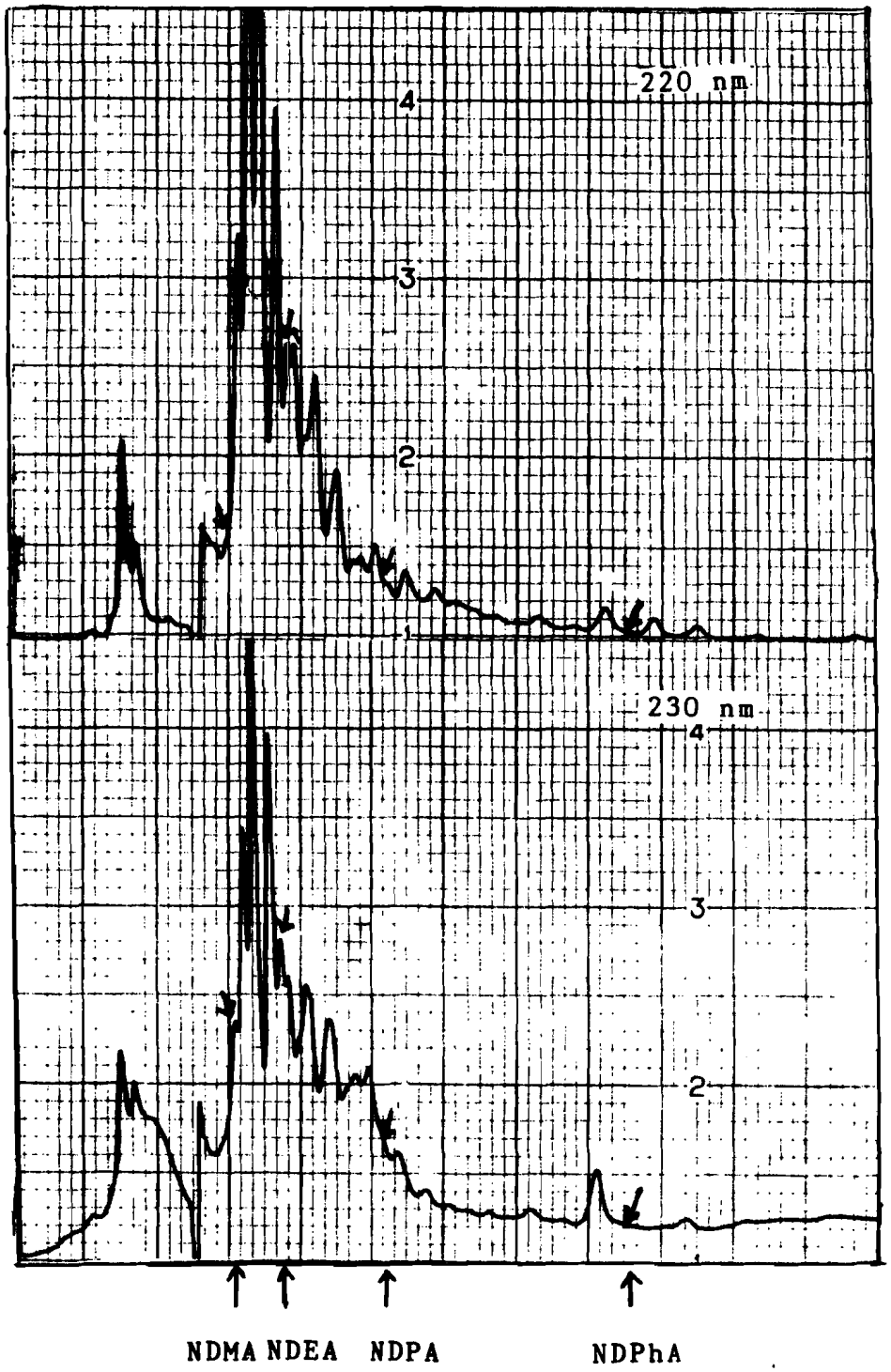


Figure 10 : A Comparison of Cigarettes Smoke in 80% Methanol at Two Wavelengths (65% Methanol as The Mobile Phase)

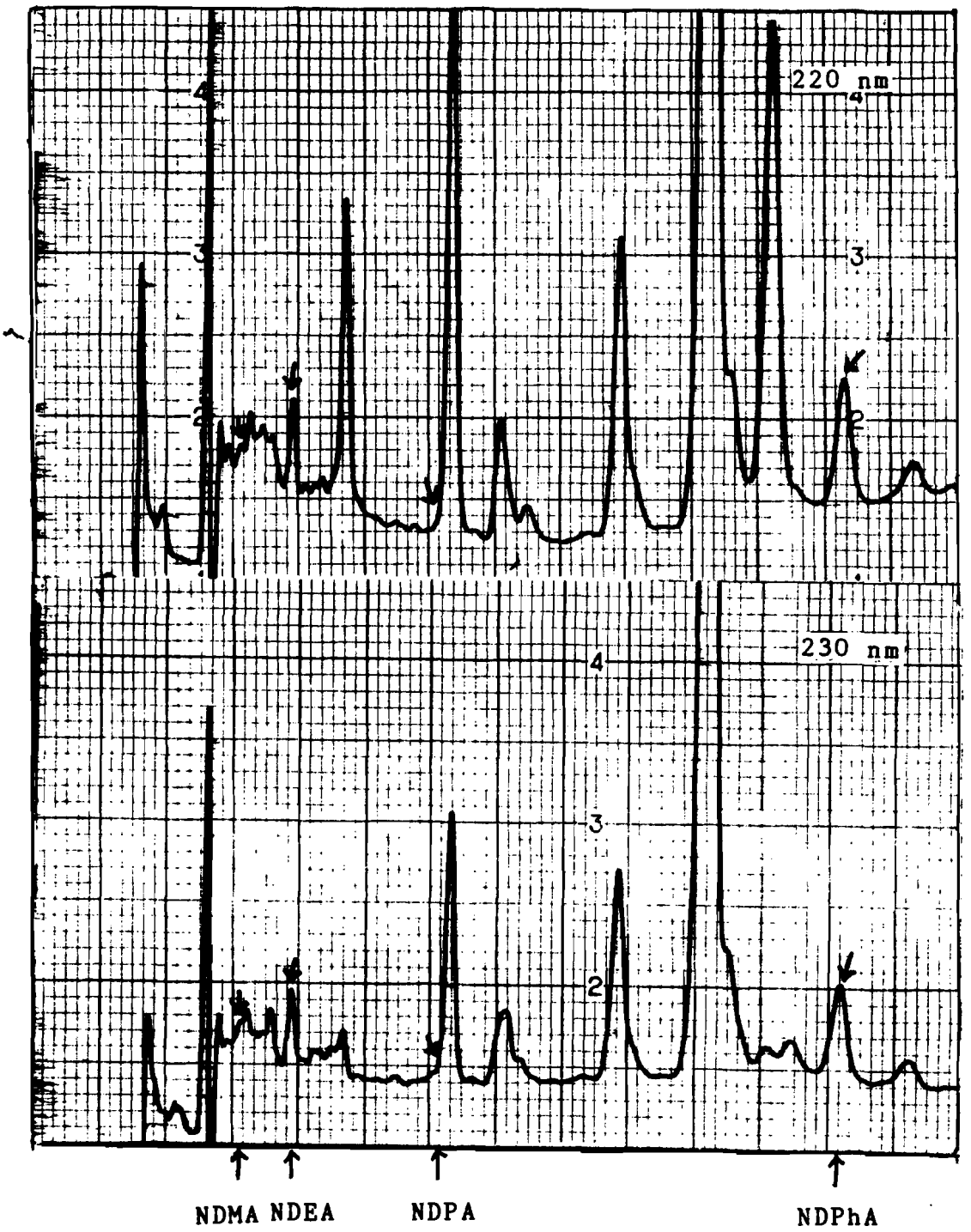
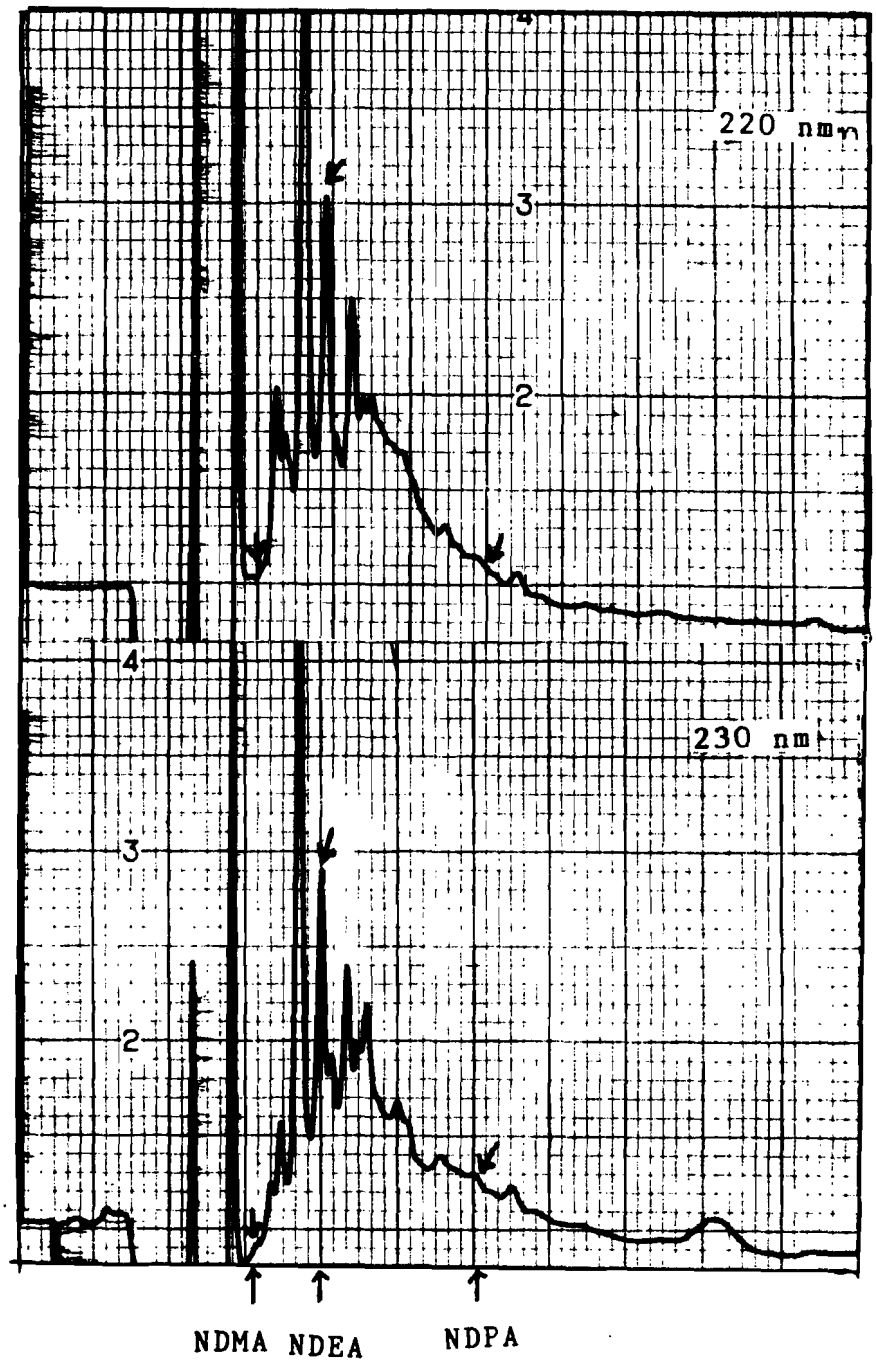


Figure 11 : A Comparison of Cigarettes Smoke in Sulfuric Acid Solution at Two Wavelengths (65% Methanol as The Mobile Phase)



CONCLUSION

Using reverse-phase HPLC in the analysis of N-Nitrosamines - NDMA, NDEA, NDPA, NDPhA proved to be a useful method. Every run can be completed within 10 min and four peaks are separate and obvious. From the calibration curves, the detection limit concentration is up to 20 mg/L. Needless to say, it can applied to qualitative nitrosamines analysis or trace - quantitative nitrosamines analysis. This method is not only simple, rapid and accurate, but also has multiple uses.

In addition, we find that ascorbic acid cannot reduce the N-Nitrosamines neither in acid solution nor in neutral solution. Although many reports stressed that ascorbic acid can inhibit the formation of N-Nitrosamines, from our results it can be infered that ascorbic acid might block the N-Nitrosamines formation in certain suitations but cannot affect the N-Nitrosamines when they have already formed.

Finally, N-Nitrosamines analysis in cigarettes smoke was performed . Though we might guess NDMA, NDEA, NDPA, NDPhA exist in these types of cigarettes , from the chromatogram we cannot make sure due to the extra compounds present.

In this project, the advanced clean-up procedures were not performed, but two methods for cleaning up have been found in references. Some studies reported that the smoke

was trapped in ascorbic acid buffer solution at pH 4.5 and extrated with dichloromethane, washed with 2 N NaOH and the organic phase was then chromatographed on basic alumina and analyzed by GLC-TEA (18). Other groups also reported one method utilizing solvent-partitioning and HPLC for clean-up steps (16). It is recommended that all the steps be followed until the extraction step . It is then recommended to concentrate the solution by removing all of the solvent and then adding the mobile phase. Some compounds might not dissolve in the mobile phase so filtering the sample for HPLC analysis is appropriate. It is believed that this method will also remove other compounds from the cigatette smoke solution.

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