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IDENTIFICATION OF PHENOL OXIDATION PRODUCTS

A Thesis

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by

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DEDICATION

To Michael

ACKNOWLEDGMENTS

My thanks go to Monsanto Co. for the use of their laboratories and facilities. I also thank Dr. Zlatkis for his help on my research and thesis.

ABSTRACT

When phenol is treated with sodium hydroxide and oxygen (in the form of air), the salts of organic acids are formed. These acids cannot be analysed by gas chromatography, since the acids are unstable and decompose. Liquid chromatography is a practicable alternative for analysis. Paired ion chromatography has been found to be the best method for the analysis of this mixture of acids, using reversed phase partition chromatography. The acids identified were oxalic, acetic, propionic, croconic, and rhodizonic acid.

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I. INTRODUCTION

We have all probably experienced having phenol, stored in glass bottles, discolor to a reddish brown, after the bottle had been opened once or twice. This discoloration is caused by the reaction of phenol, sodium (from the glass), and oxygen from the air. The phenol is oxidized to form a variety of organic acids[11]. It is these acids that were analyzed in this study.

A variety of analytical methods were attempted before liquid chromatography was considered. Of course, gas-liquid chromatography was first attempted, unsuccessfully. Separation could not be achieved, or most of the sample decomposed on the column. Infrared spectroscopy could identify the fact that organic acids were present, but the mixture could not be conclusively identified. A series of extractions were of little value in separating the mixture. Mass spectroscopy also gave supporting data to the fact that the sample consisted of organic acids, but once again, the fact that the sample was a mixture prevented positive identification of individual components.

Other methods, such as identifying reagents and polarography were also attempted. Precipitation and titration methods also proved unsuccessful. When all else failed, liquid chromatography was tried.

Within the past few years, rapid advances have been made in liquid column chromatography. A major step forward was made with the technology to pack columns efficiently with small particle diameter porous supports (particle diameter <10μ)[7].

The separation and analysis of acid mixtures has historically been done by ion-exchange resins. Ion exchange chromatography is generally applicable to ionic compounds, ionizable compounds (e.g. acids and bases), and compounds which can interact with ionic groups (e.g. chelates, liquids, etc.). Ion exchange materials consist of a porous solid phase, usually a resin, onto which ionic groups are bonded or attached.

The ion exchange process may be carried out in aqueous or nonaqueous media. The mobile phase usually contains a counter ion, in the form of a buffer, opposite in charge to the surface ionic group, which is in equilibrium with the resin in the form of an ion pair. The presence of a solute ion of the same ionic charge sets up an equilibrium, as shown in Figure 1. The counter ion is Na^+ in the case of the cation exchange process and Cl^- in the anion exchange. The equilibrium established can be represented by Equation 1.

$$K = \frac{[\text{--}NR_4^+X^-][Cl^-]}{[\text{--}NR_4^+Cl^-][X^-]} \qquad (1)$$

The higher the value of K, the distribution coefficient, the more strongly the ionic solute interacts with the ion

exchanger. The distribution coefficient is a function of many experimental parameters, such as pH, ionic charge, ionic radius, resin porosity, ionic strength and solvent, temperature, etc.[6]

Recently, however, a different method has been used to separate acids in liquid column chromatography. This method is paired ion chromatography.

Paired ion chromatography has been proven a valuable tool in many uses. Of particular importance are the high separation factors that can be obtained and the high sample capacity.

The basic equilibrium process for the extraction of A^- by counter ion B^+ is

$$A^-_{aq} + B^+_{aq} \rightleftharpoons AB_{org} \qquad (2)$$

where the subscripts "aq" and "org" refer to the aqueous and the organic phases, respectively. It is assumed in Equation 2 that no significant ion pair concentration exists in the aqueous phase, or that any dissociation occurs in the organic phase. The extraction constant E_{AB} is defined as

$$E_{AB} = \frac{[AB]_{org}}{[A^-]_{ag}[B^+]_{aq}} \qquad (3)$$

The addition of secondary chemical equilibrium to the physical distribution process requires the use of the distribution ratio D_A to determine retention in chromatography.[10]

$$D_A = \frac{[AB]_{org}}{[A^-]_{aq}}$$

or

$$D_A = E_{AB}[B^+]_{aq} \qquad (4)$$

The capacity factor k'_A is then

$$k'_A = (E_{AB}[B^-]_{aq})^{-1} V_s/V_m \qquad (5)$$

where V_s = volume of stationary phase and V_m = volume of mobile phase. The relative retention, α, is then

$$\alpha = \frac{k'_{A2}}{k'_{A1}} = \frac{E_{A1B}}{E_{A2B}} \qquad (6)$$

where subscripts A1 and A2 = earlier and later eluting species, respectively.

The retention, and consequently, the separation of the sample is dependent on several variables. As indicated in Equation 6, the retention is dependent on the volume ratio of the two phases. It is also dependent on the type of stationary organic phase and the ionic strength of the aqueous phase (e.g. salting-out effects). The pH is important for acid-base substances. The type of counter ion is important, yet the concentration of the counter ion is of disputed importance. Persson states that it is of primary importance[15], however Eksborg maintains that a system containing a high

concentration of counter ion will allow the partition to be independent of the counter ion concentration as long as the counter ion is present in sufficient excess.[5] This study assumed the latter.

The versatility allowed by controlling retention and the selectivity characteristic of the high performance characteristics of modern liquid chromatography allows rapid separation. Also, since the sample elutes as an ion pair, sensitivity and detection limits can be controlled. Non-u.v. samples can be detected with a u.v. detector if a u.v. active counter ion is selected.

Under certain circumstances, side reactions, such as dissociation or dimerization of the ion pair may occur. This will usually lead to asymmetric peaks. Chromatographic conditions have to be maintained to avoid this effect.

The choice of chromatographic conditions can often be based on the equation for chromatographic resolution (Rs).

$$Rs = N^{\frac{1}{2}} x(1 - D_A/DB) x[4xD_A xV_m/V_s + 2x(1 + D_A/D_B)]^{-1} \quad (7)$$

Unnecessarily long separation times are avoided if $D_A \geq 0.1$. With $N \simeq 300$ and $D_A = 0.1$, a resolution of 2 components in about the same amount ($Rs > 1.5$) is obtained with a separation factor $\alpha < 0.6$.[14]

Ion-pair partition can be applied to all kinds of ionizable organic compounds, aprotic ions such as quaternary ammonium ions, sulphonates and organic sulphates as well as weak protolytes, such as carboxylic acids, phenols, amines and amino acids.[3]

Ion pair extraction has been shown to be very selective and sensitive in the separation and analysis of drugs and biogenic substances in biological fluids. Methods are described for reversed-phase partition chromatography of organic ammonium compounds as ion pairs with inorganic anions. A silicon treated cellulose was used as support, lipophilic alcohols as the stationary phase, and aqueous solutions of inorganic ions as the mobile phase.[20]

Predisolone sodium phosphate has been separated using trihexylamine hydrochloride as counter ion.[8]

Carboxylic acids have been separated using quaternary ammonium ions as counter ions in the station phase. Amines and quaternary ammonium ions have been separated with picrate or β-naphthalene sulphonate as counter ions. Aminophenols have been separated by reversed-phase systems containing bis-(2-ethylhexyl)phosphoric acid in chloroform as the stationary phase.[3]

The separation of codeine, lidocaine, promazine, and strychnine as ion pairs with Cl^-, Br^-, I^-, ClO_4^-, NO_3^-, and SCN^- has been demonstrated.[16]

Choline has been extracted as an ion pair with hexanitrodiphenylamine to dichloromethane. It was then isolated from the extract by partition chromatography as a picrate ion pair and determined photometrically.[4]

Ion pair partition chromatography was used for separation and the quantitative determination of alprenolol and alprenolol metabolites in plasma. The separation was based on the partition of ion pairs with perchlorate using cyclohexane and 1-pentanol as mobile phase.[2]

Phenylacetic, mandelic, and pyridinecarboxylic acids have been separated by ion-pair partition chromatography with 1-pentanol as stationary phase and tetrabutylammonium as counter ion in the aqueous mobile phase.[21]

Thyroid hormones and metabolites and a group of related sulfa drugs have separated and analysed by using ion pairs.[9]

It has been shown that a tri-n-octylamine--aqueous perchloric acid system is suitable for separation of acidic compounds like sulphonic and carboxylic acids by liquid chromatography.[12]

Small quantities of quaternary and tertiary amines used with reversed phase liquid chromatography have been used to separate tartrazine, a sulfonic acid dye from its synthetic intermediates, 3 carboxyl-1-(4-sulfophenyl)-5-pyrazolone and sulfanilic acid.[23]

Paired ion chromatography also proved successful in the separation of the oxidation product of phenol. After the peaks were separated, each peak was recovered in the eluant and the individual acids were analysed by mass spectroscopy.

II. EXPERIMENTAL

A variety of established analytical methods were used before liquid chromatography was used to analyse the oxidation product.

Infrared - A Perkin-Elmer Model 221 double beam spectrometer was used for analysis. The sample was treated as per Figure 2. The samples were analysed as KBr discs.

Identifying Reagents - A variety of reagent grade chemicals were mixed in aqueous solutions of the sample. The tests are described in Table I.

Polarography - A Princeton Applied Research Polarographic Analyzer, Model 174, was used. The tests are described in Table II.

Mass Spectroscopy - A Finnigan Corp. Model 3200 mass spectrometer was used. The sample was acidified with 0.1N HCl, and the sample was analysed at ~300^{o} by chemical ionization. Figure 3 shows a diagram of the mass spectrometer.

Atomic Absorption - A Perkin-Elmer Atomic Absorption Spectrophotometer, Model #306, was used to analyse calcium oxalate precipitate.

Sample Preparation - The oxidation sample was prepared by the following method:

Sodium hydroxide was added to product phenol (which had been stored in metal containers to avoid

contamination from the sodium in glass), to 0.4 weight % sodium hydroxide. The sodium hydroxide immediately reacted with the phenol to form sodium phenate. Air was then bubbled through the melted (100°F) phenol solution for one hour. The phenol solution discolored to a reddish brown liquid.

The liquid (150 ml) was extracted with 100 ml of deionized water. The water layer was recovered and extracted twice with 100 ml dichloromethane, discarding the organic layer each time. The aqueous layer was then concentrated by evaporating ~75% of the water under a nitrogen steam at room temperature. This material was then analysed by the above methods and liquid chromatography.

Liquid Chromatography - All the described liquid chromatograph analyses were made at ambient temperature. The liquid chromatograph is illustrated in Figure 4.

Detector - SF 770 Spectroflow by Schoeffel Instrument Corp., a u.v. detector, set at 254 nm.

Solvent Delivery System - A dual pump system with a solvent programmer was used for gradient elution. The pumps were a Waters M-6000 and a M-6000A. Each pump had a pair of positive-displacement pumping chambers. The solvent programmer was a Waters M-660. The M-660 had eleven programs for gradient elution.

Columns - Four columns were used. They were purchased from the noted companies.

1. Bondapak AX/Corasil (Waters Associates) - 1 meter x 1/8". The Bondapak AX/Corasil column is a permanently bonded, hydrolytically stable, pellicular column-packing material for ion exchange chromatography. It consists of a Corasil substrate with a quaternized alkyl amine functional group chemically bonded to the surface.

2. Zipax-SAX (DuPont) - 22" x 1/8". The Zipax-SAX packing is a spherical particle, normally 30 μM in diameter, with a surface layer 1 μM thick. This layer is composed of micro-beads (500 A) of silica, bonded to the solid core. A strong anion functional group is bonded to the surface.

3. μ Bondapak C_{18} (Water Associates) - ¼" x 22". The packing has a monomolecular layer of octadecyltrichlorosilane chemically bonded to Porasil beads having an average particle size of 10 μ. The H.E.T.P. calculated for this column, based on hydroxybenzoic acid is 0.241 cm. The number of theoretical plates is, therefore, 231.

4. μ Bondapak C_{18} (Waters Associates) - ¼" x 30 cm.

Injector - A stop-flow injector was used.

Reagents - The methanol used was commercial grade methanol prepared by Monsanto. The tetrabutylammonium hydroxide was prepared by Matheson, Coleman, and Bell, 25% in methanol. It was analytical grade. The tetrabutylammonium bromide was prepared by Eastman Chemicals, analytical

grade. The phenol was Monsanto, commercial grade. All other substances were of analytical or reagent grade and were used without further purification.

Two chromatographic approaches were tried. The first was ion exchange chromatography.

Two types of ion exchange resins were tested. The first tested was Bondapax AX/Corasil. The mobile phase was 0.01N $NaNO_3$ buffer pH adjusted to 5.0. 0.01N sodium acetate buffer was used as mobile phase at pH 3.0 and 4.0. The pump rate was 1 ml per minute.

A Zipax-SAX column was evaluated next. The mobile phase was a 0.01N $NaNO_3$ buffer, pH adjusted to 5.0 and 9.0. The pump rate was 1 ml per minute.

The columns used for paired-ion chromatography (PIC) were μ Bondapak C_{18} columns. The mobile phase consisted of a gradient elution of two solutions. Solution A was 0.01M solution of tetrabutyl ammonium hydroxide (TBAH) or tetrabutyl ammonium bromide (TBAB) in water. Solution B was 0.01M solution of TBAH or TBAB in methanol. Various elution rates were used (Figure 5). The pump rate was varied from 1.0 to 2.5 ml/min. The runs were made at ambient temperature. The solvents were degassed under vacuum before use to prevent degassing under pressure in the liquid chromatograph.

Cuts were taken from the preparative column (Column 3) and the solvent evaporated. The tetrabutyl ammonium salt was left in the residue with the acid salt. This mixture was redissolved in water, and the quaternary amine was then complexed with dichloromethane and extracted from the aqueous solution with 2-methyl pentane.[23] This left the salts of the acids which were then reacidified and identified by mass spectroscopy.

III. RESULTS

The infrared spectroscopy scan, Scan 1, is a sample of the oxidation product which was water soluble (see Figure 2, Extration Procedure for I.R.). A broad band at 3420 cm^{-1} is probably due to residual moisture in the sample. The 1630 and 1565 cm^{-1} bands are due to an asymmetric carboxylate stretch. The 1470 and 770 bands are due to $-CH_3$ symmetrical vibration.

Scan 2 is that of recrystallized sodium oxalate. It is easily noted that the major peaks in this scan are also present in Scan 1.

Scan 3 is a scan of the oxidation product (shown in Scan 1) after calcium oxalate has been removed by precipitation. Bands at 3115, 1880, 1630, and 770 cm^{-1} have either been significantly reduced or removed. These are the major peaks on the sodium oxalate scan.

The identifying reagents and the polarography results are given in Tables I and II.

Mass spectrometry, prior to separation by liquid chromatography yielded a wide variety of possible compounds (Scan 4). These possibilities are listed in Table III.

Indirect measurement, based on the precipitation of calcium oxalate and the measurement of excess calcium by atomic absorption gave erratic results, i.e., 118, 358, 1418 ppm, and was abandoned.

The method that was partially successful in separating the oxidation products is shown in Scan 5. It utilized paired ion chromatography. The use of ion exchange columns, Scans 6 and 7, did not separate the acids.

Two general approaches have been taken in using ion pairs in high performance liquid chromatography. One has been to use liquid/liquid partition systems where aqueous solutions of counter-ion were used as the stationary phase.[5] The other approach was gravity-feed systems where the counter ion is added to the mobile phase. The latter approach was used in this study.

The primary advantage of ion pair chromatography is the good possibility of retention volume variations by changes in the nature and concentration of the counter ion. Reversed phase technique has a particularly high versatility since the counter ions are dissolved in easily changeable (or programmable) mobile phase.[20]

In extraction, one desires relatively large extraction constants and separation factors, α. As seen from Equation 5, large E_{AB} values lead to very large capacity factor values in ion pair partition chromatography. It is, therefore, necessary to moderate the conditions required for quantitative extraction in transferring to liquid chromatography (e.g. lower solvating mobile phase, etc.). Moreover, as a result of high column efficiencies, the

selectivity demands are much less severe in liquid chromatography. With these considerations, tetrabutylammonium ion pairs were chosen to separate the oxidation product.

Cuts were taken of each peak eluted in Scan 5 and analysed by mass spectroscopy (Scans 8-13). Chemical ionization was used for analysis. Unlike electron ionization, which is a physical process involving electron-molecule collision to produce ions, chemical ionization is a phenomenon resulting from ion-molecule chemical interactions. Chemical ionization requires gas mixtures at pressures on the order of one torr in the mass spectrometer ion source. The composition of the gas is such that one component of the mixture, the reagent gas, is present as a several thousand-fold excess over the other component, the sample.

$$CH_4 + e^- \rightarrow CH_4^+,\ CH_3^+,\ CH_2^+ \qquad (8)$$

$$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3 \qquad (9)$$

Primary ionization occurs in the reagent gas because of its great excess. These reagent ions then react further with reagent gas in second order processes to produce stable ions characteristic of each gas.

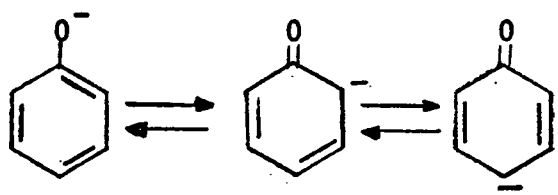
The advantages of chemical ionization over electron ionization are very little fragmentation, since the ions are more stable, and greater sensitivity.

Due to residual tetrabutyl ammonium bromide, even after extraction, it was rather difficult to interpret the mass spectra. From the mass spectroscopy data, it appeared that the low molecular weight acids were not separated. Testing pure compounds on the liquid chromatograph proved this to be true. The results are shown in Table IV.

However, liquid chromatographic analysis of an aqueous sodium hydroxide solution of hydroxyquinone yielded a scan very similar to that of the oxidation product of phenol (Scan 14). The hydroquinone, in the presence of air and sodium hydroxide also discolored in a very similar manner to that of phenol.

IV. DISCUSSION

Phenol, under basic conditions, is known to exist in the state:[22]



Autooxidation is followed with chain reactions of radicals, and the initial stage of the oxidation of phenol in alkaline solution is thought to proceed in the manner shown in Figure 6.

It is not surprising that the oxidation products of hydroquinone would be similar to those of phenol. A proposed mechanism for the oxidation of phenol, Figure 6, involves the formation of quinone. The results of the above analyses would indicate that this proposed mechanism is correct.

Supporting data from the above described analyses would lead to the following conclusions:

(1) Phenol is oxidized through a mechanism which involves the formation of quinone. The oxidation products of phenol and quinone are therefore the same.

(2) A large portion of the oxidation product consists of acids. These acids, as identified with I.R. analysis and mass spectroscopy (after liquid chromatographic

separation), are acetic acid, oxalic acid, propionic acid, and rhodizonic acid. Proposed other acids are croconic and squaric acids.

(3) From the similarities in the liquid chromatograph scans, it is proposed that the unidentified peaks in the phenol oxidation product scan and the hydroxyquinone scan are the di-, tri-, and tetrahydroxyquinones and their various isomers.

TABLE I

IDENTIFYING REAGENT TESTS

	Test Reagents	
Test Material	Lead Acetate	Calcium Carbonate
Oxalic Acid	White Ppt.	White Ppt.
Rhodizonic Acid	Purple Ppt.	Orange Soln.
Tetrahydroxyquinone	Purple Soln.	Gold Soln.
Phenol Oxidation Prod.	No change	No change

TABLE II

POLAROGRAPHY IN A VARIETY
OF SUPPORTING ELECTROLYTES

0.1M KCl	No specific reduction for oxalic acid
pH 7 buffer	No reduction observed
pH 4 buffer	No reduction observed
0.01M HCl - 0.1M KCl	No reduction observed
0.1M HCl	No reduction observed
0.1M KH_2PO_4	No reduction observed
0.01M $HClO_4$ - 0.15 mM In^{+3}	Indium oxalate reduction wave observed, but sample contained interfering compounds

Table III
Possible Compounds
from M.S. Data

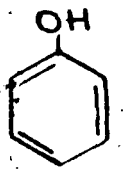
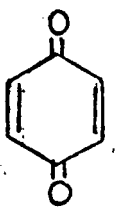
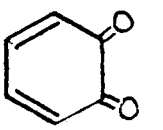
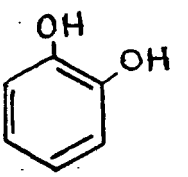
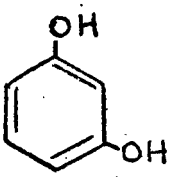
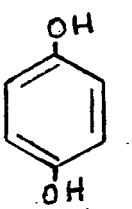
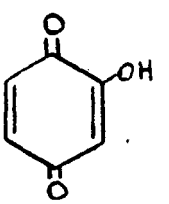
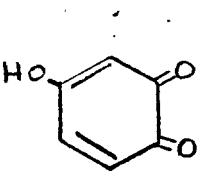
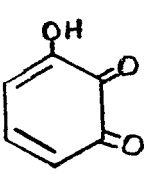
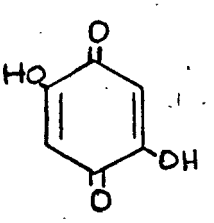
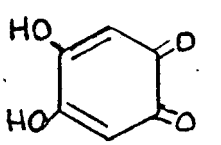
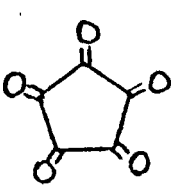
M.W.	Empirical Formula	Structures
94	C_6H_6O	
108	$C_6H_4O_2$	 
110	$C_6H_6O_2$	  
124	$C_6H_4O_3$	  
140	$C_6H_4O_4$	 
140	C_5O_5	

Table III
cont.

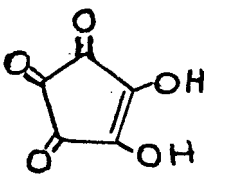
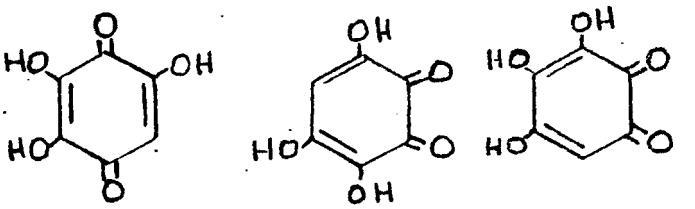
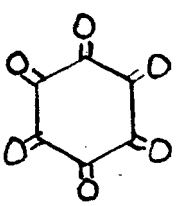
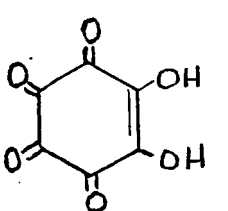
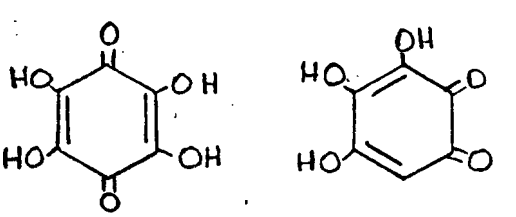
M. W.	Empirical Formula	Structures
142	$C_5H_2O_5$	 Croconic Acid
156	$C_6H_4O_5$	
160	C_6O_6	
170	$C_6H_2O_6$	 Rhodizonic Acid
172	$C_6H_4O_6$	

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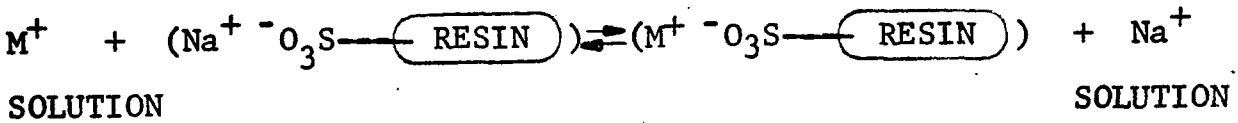
TABLE IV

PURE COMPOUNDS TESTED

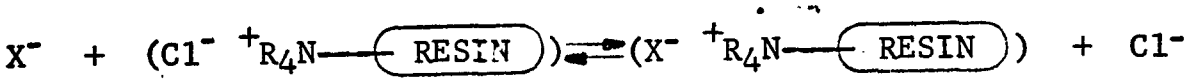
Compound	M.W.	Elution Time L.C. (sec)	M.S. Cut #	Other Analyses
Acetic Acid	60	86	2-7	
Propionic Acid	75	84	2-7	
Oxalic Acid	90	90	4-7	I.R.
Phenol	94	88	2,3	
Hydroxyquinone	110	88	5	
Croconic Acid	142	-	4-7	
Rhodizonic Acid	170	80	6,7	

Figure 1

Ion Exchange Resins



M^+ = Cation



X^- = Anion

Figure 2

Extraction Procedure for I.R.

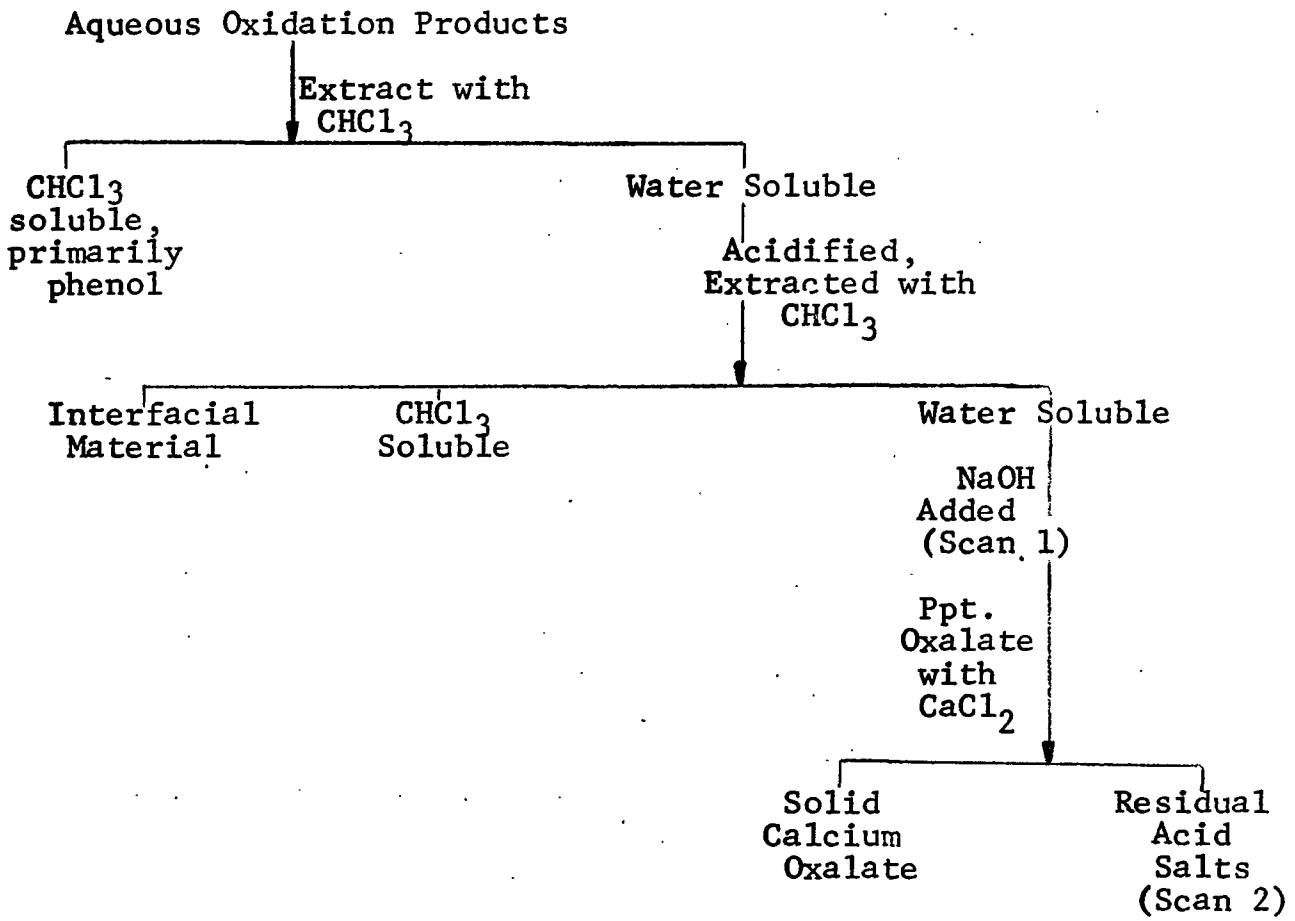


Figure 3

Chemical Ionization
Mass Spectrometer

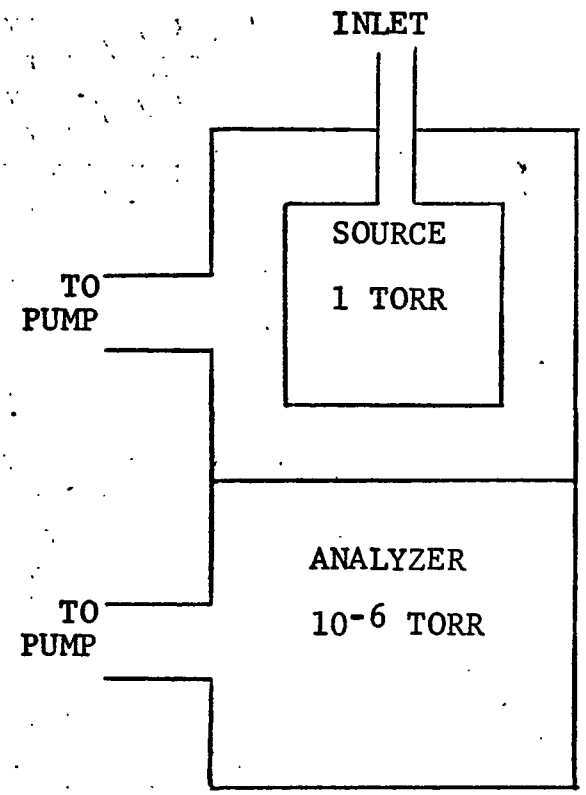


Figure 4
Liquid Chromatograph

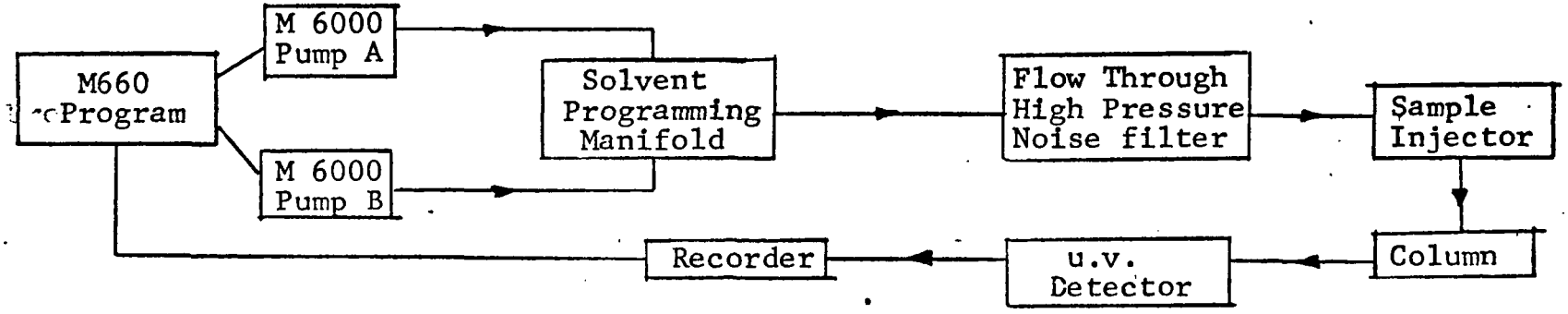


Figure 5
Solvent Programmer
Elution Rates

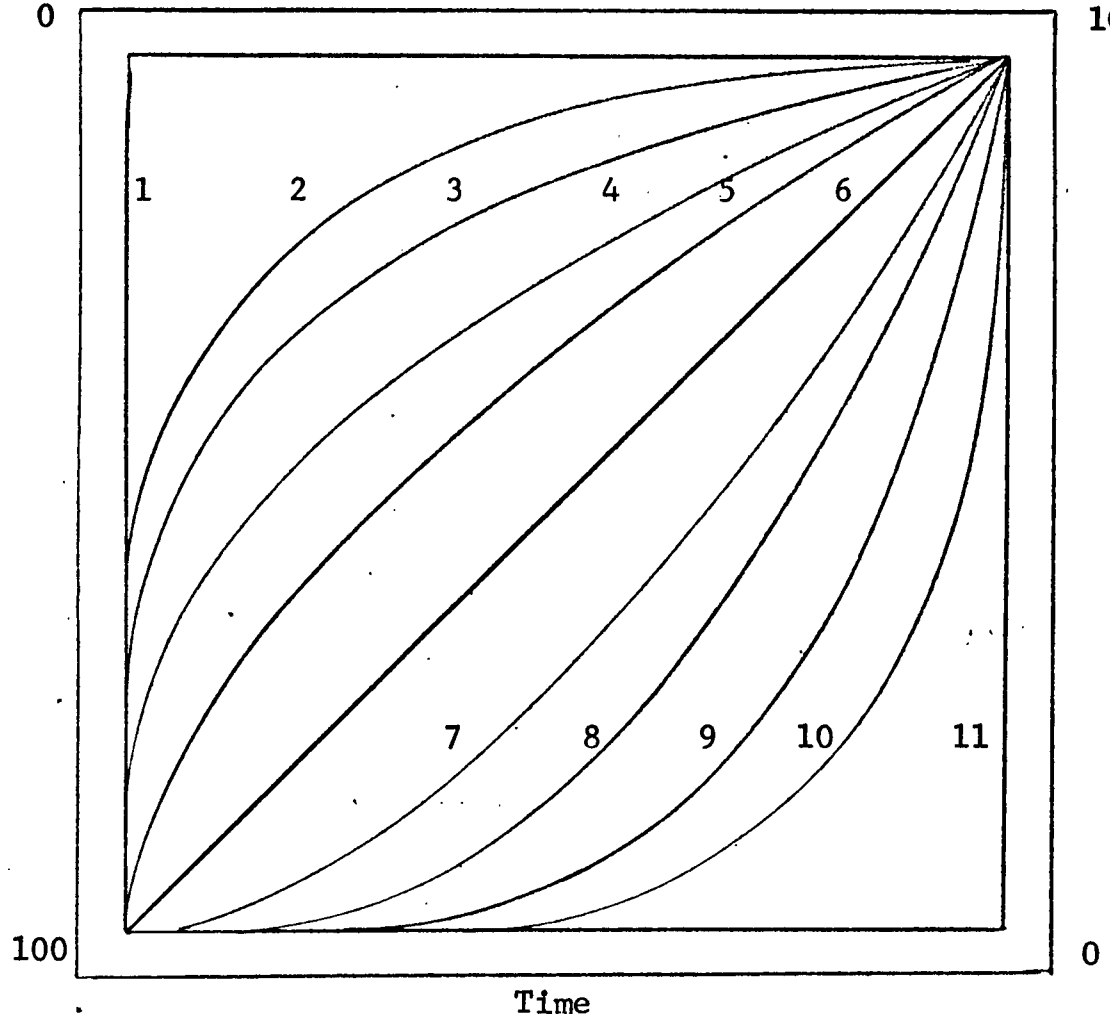
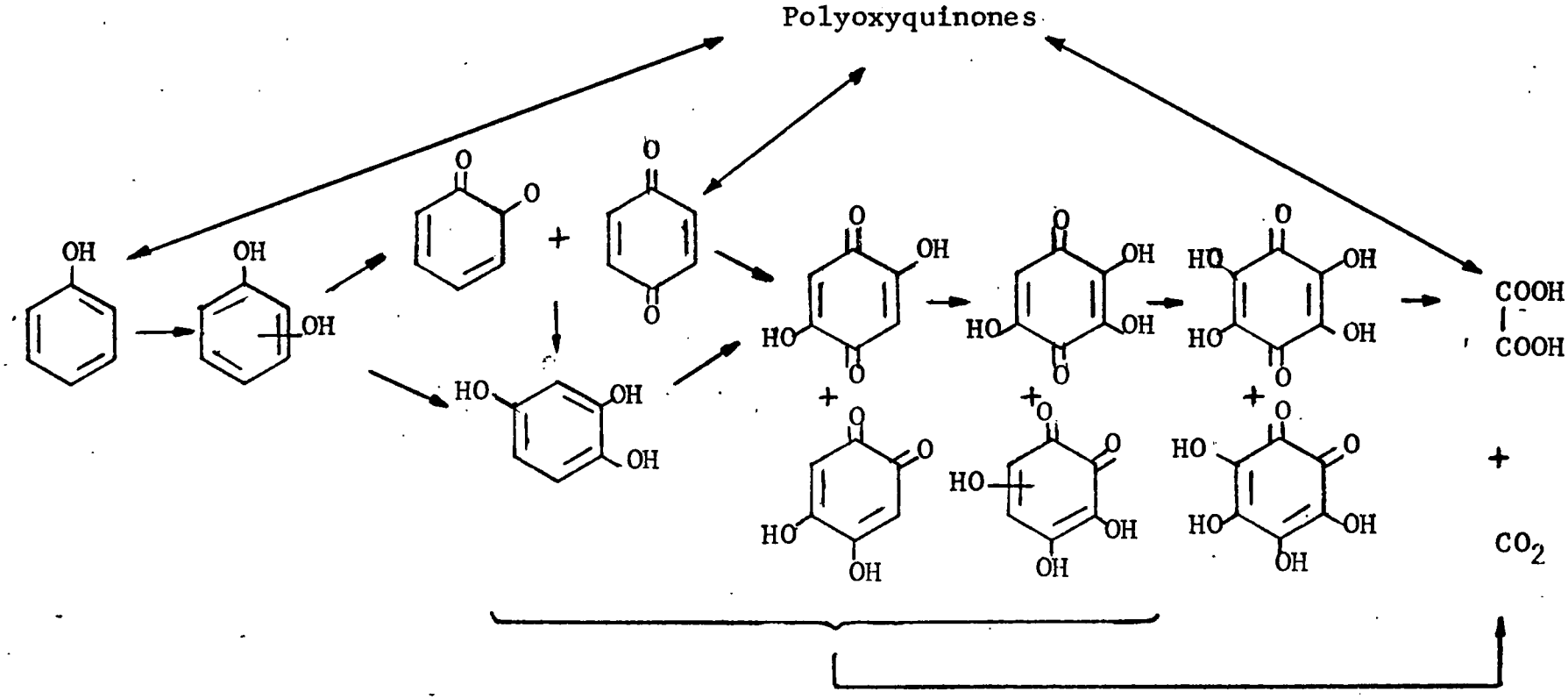
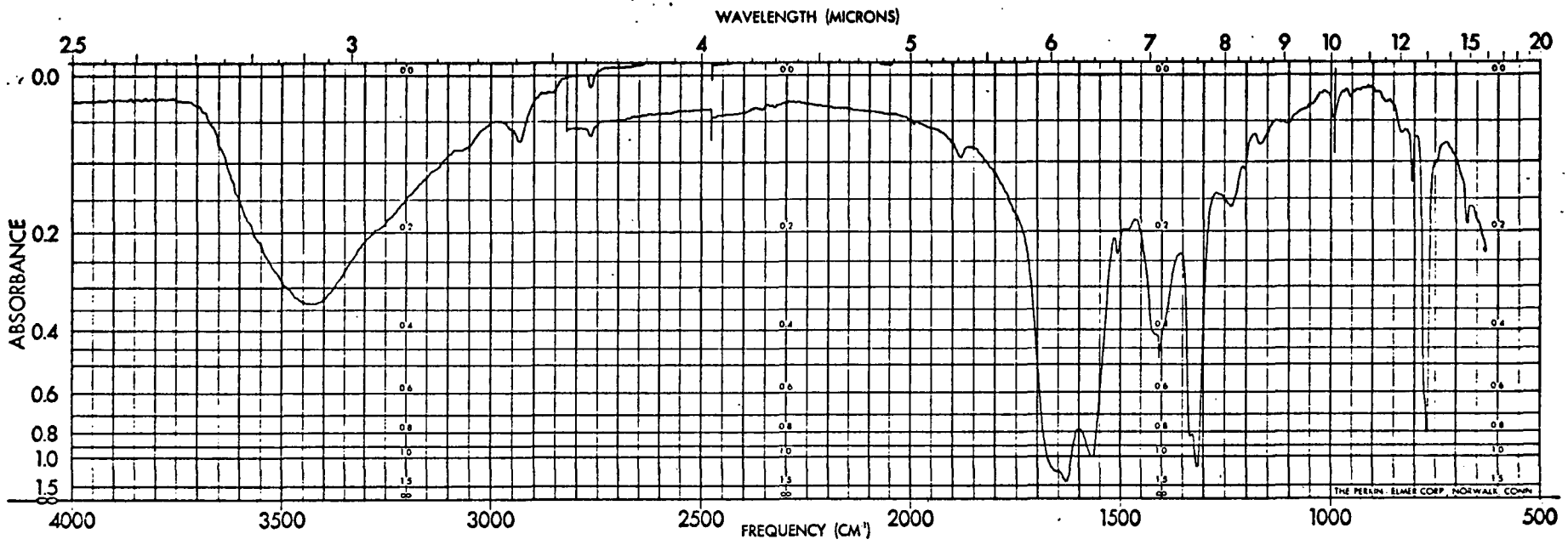


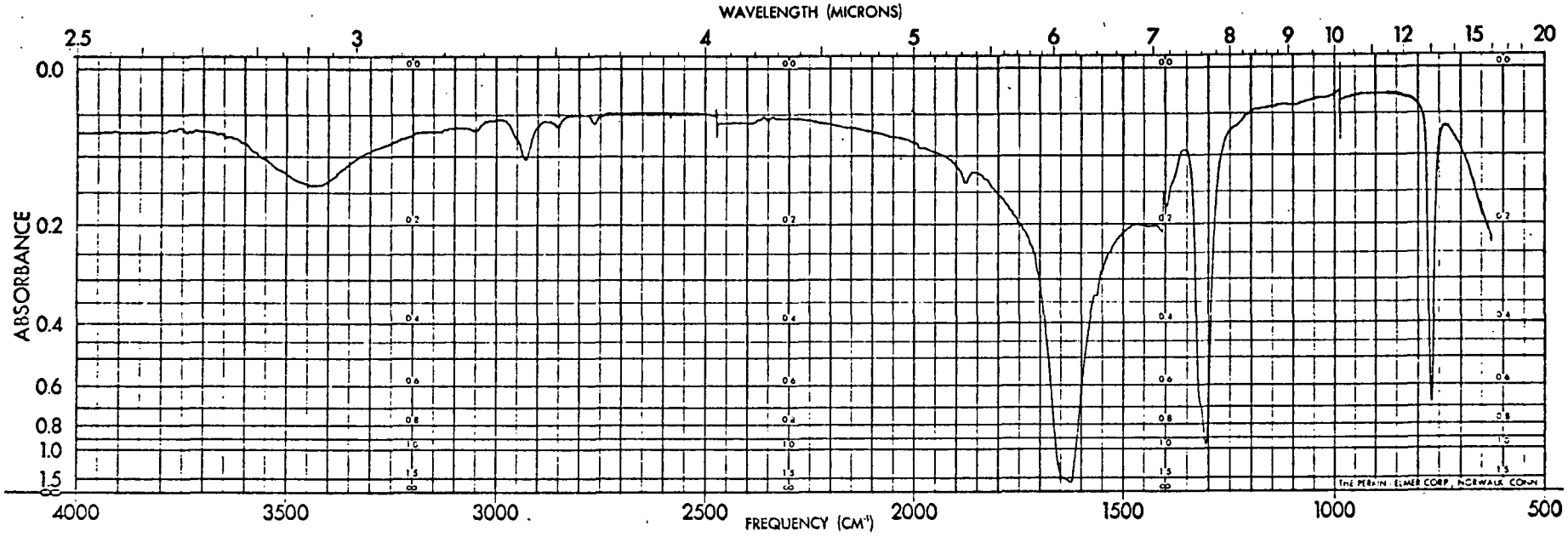
Figure 6
Reaction Mechanism



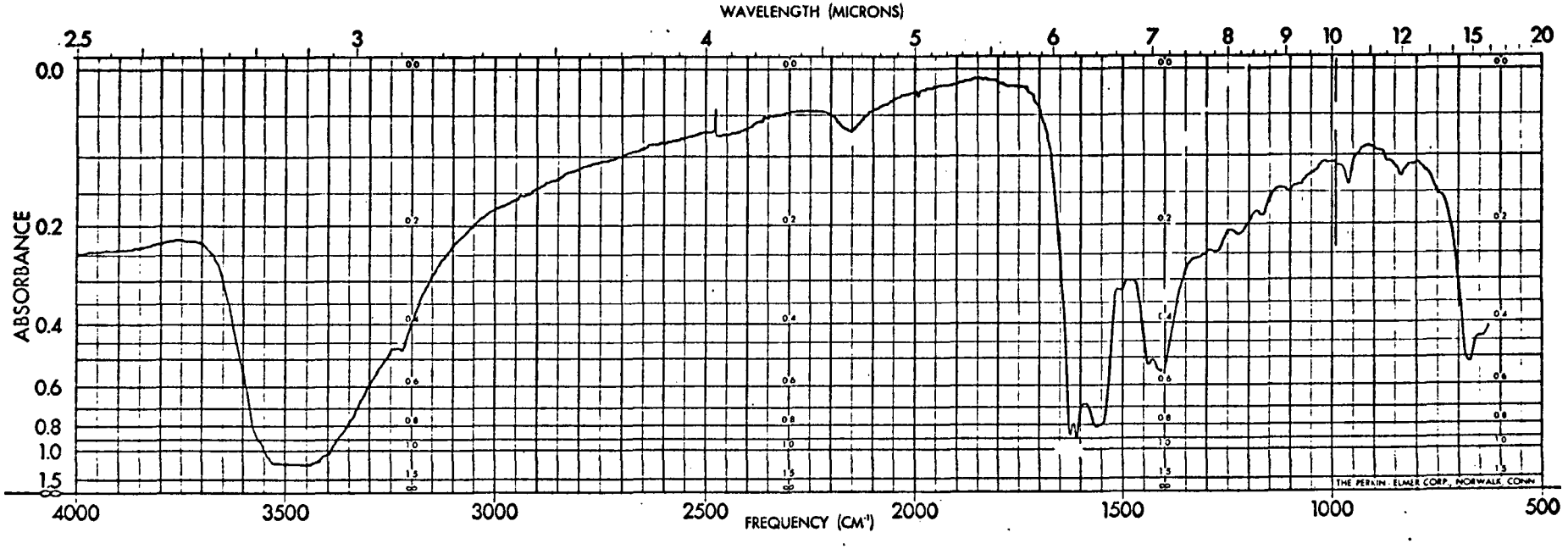
Scan 1
I.R.
Oxidation Product



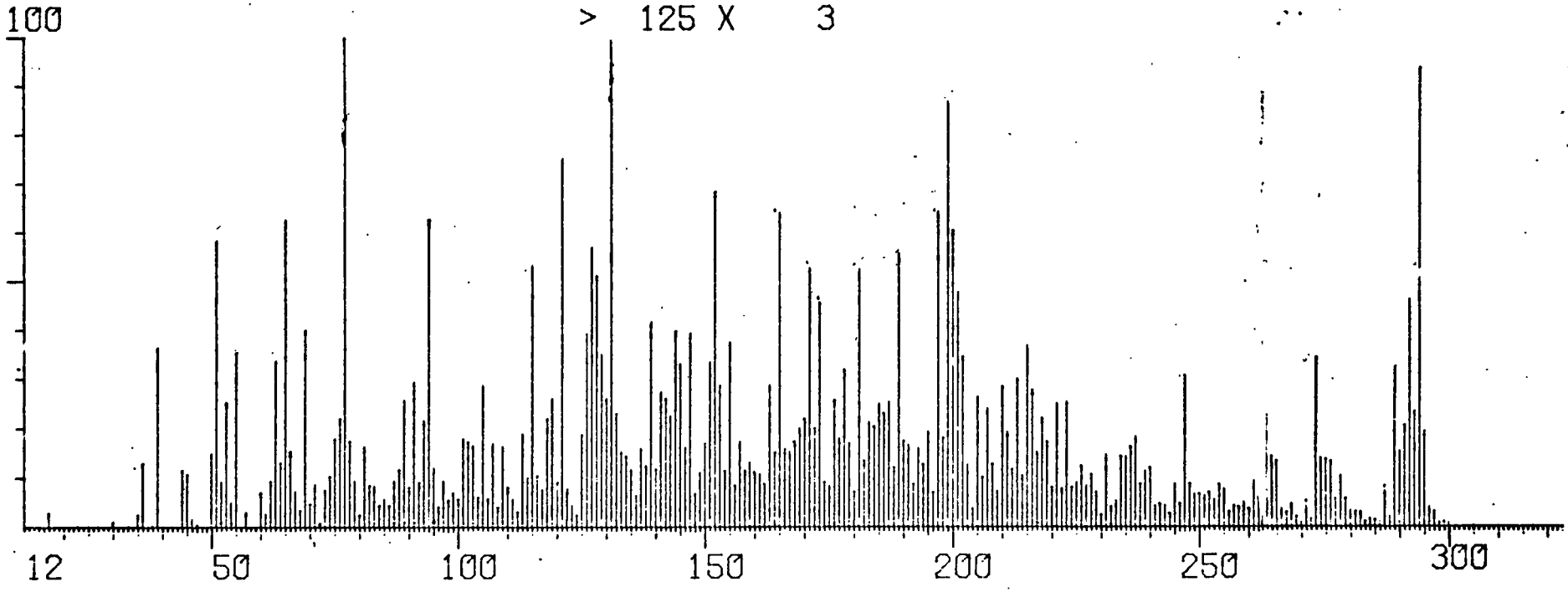
Scan 2
I.R.
Sodium Oxalate

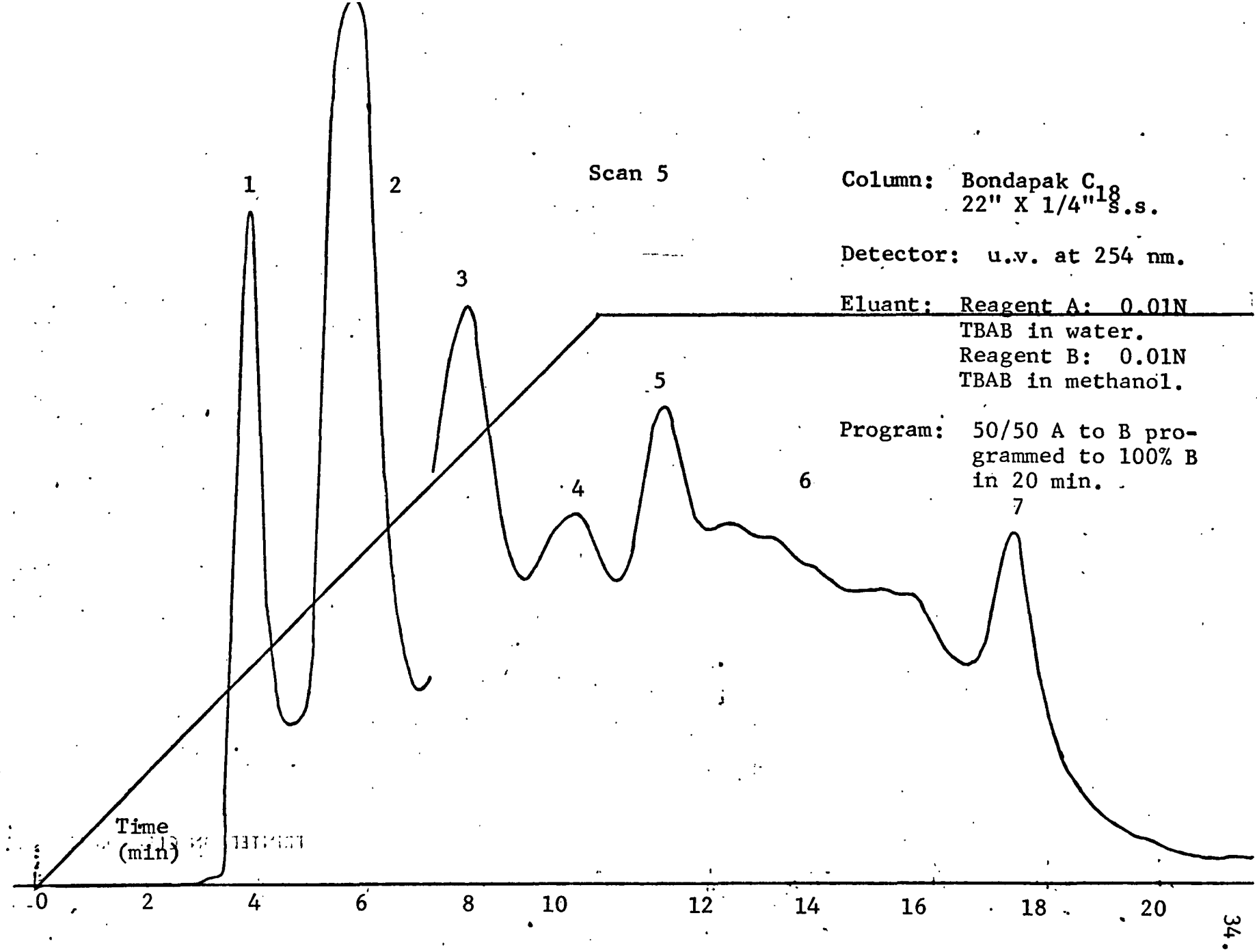


Scan 3
I.R.
Oxidation Products,
Oxalic Acid Removed



Scan 4
M.S.
Unseparated Oxidation Products




Scan 5
Column: Bondapak C_{18}
22" X 1/4" s.s.
Detector: u.v. at 254 nm.
Eluant: Reagent A: 0.01N TBAB in water.
Reagent B: 0.01N TBAB in methanol.
Program: 50/50 A to B programmed to 100% B in 20 min.
1
2
3
4
5
6
7
Time (min)
0
2
4
6
8
10
12
14
16
18
20

Scan 6
Liquid Chromatography
Ion-Exchange

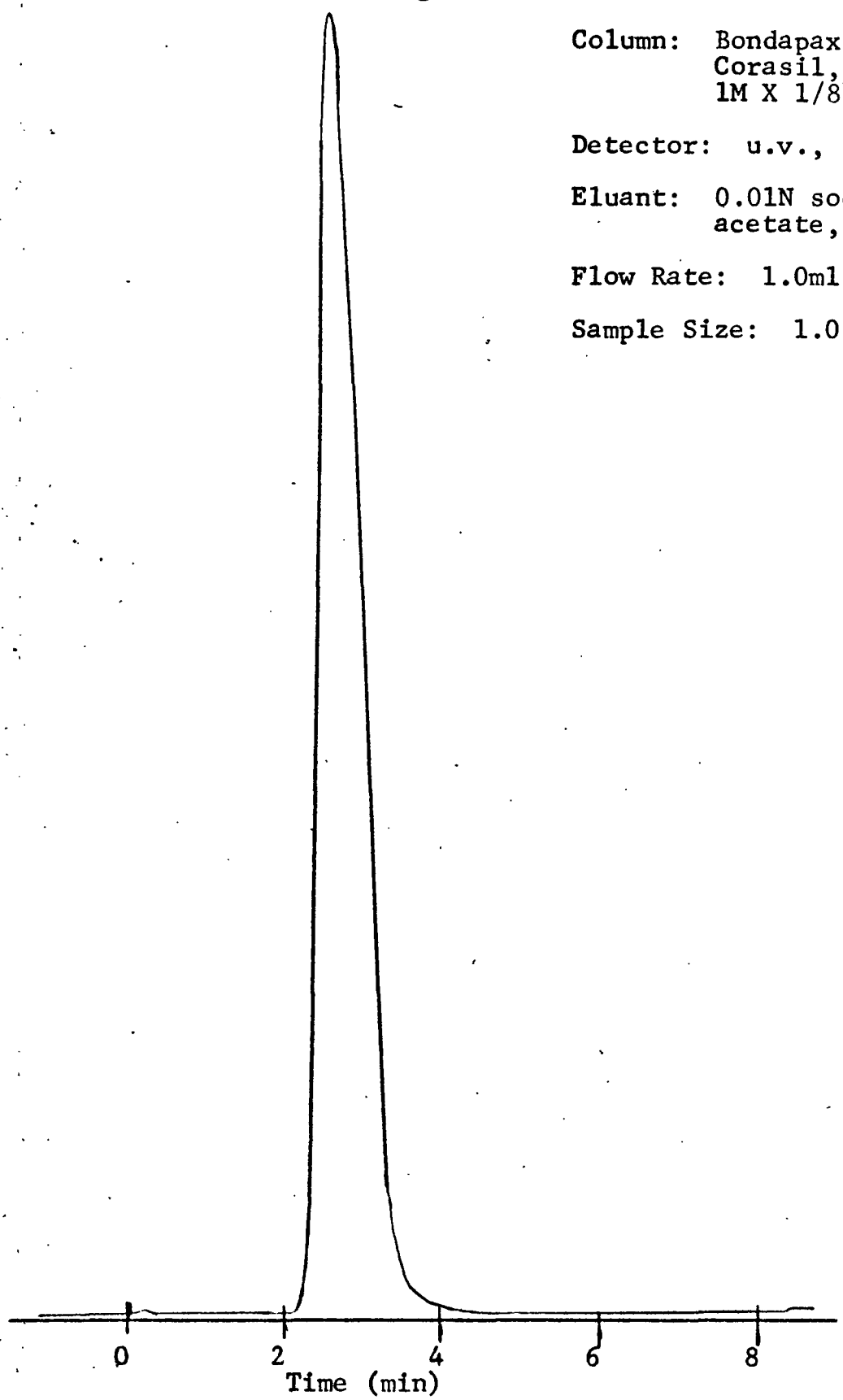
Column: Bondapax AX/ Corasil, 1M X 1/8" s.s.

Detector: u.v., = 220nm

Eluant: 0.01N sodium acetate, pH = 4.0

Flow Rate: 1.0ml/min

Sample Size: 1.0 1



Scan 7
Liquid Chromatography
Ion-Exchange

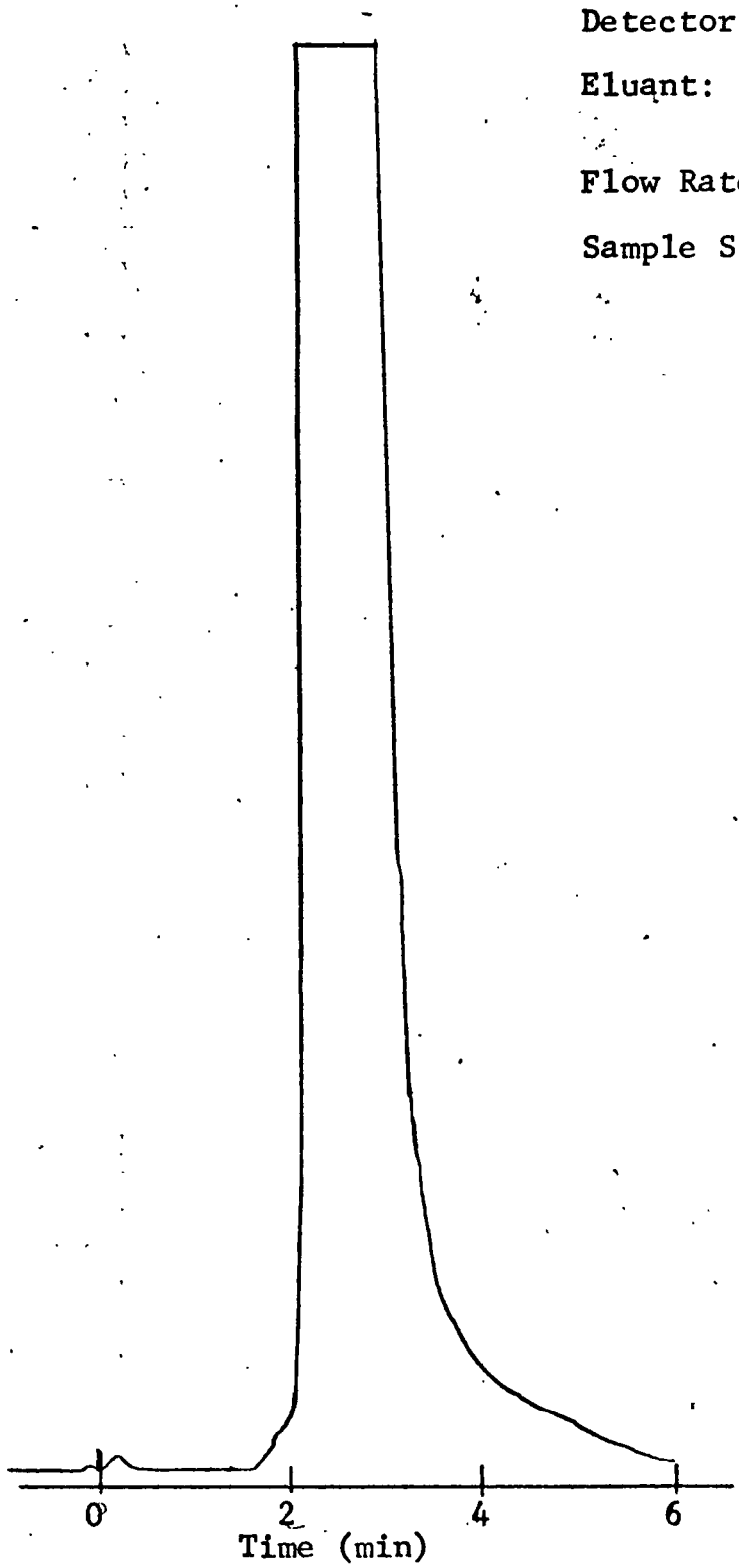
Column: ZIPAX-SAX
22" X 1/8" s.s.

Detector: u.v., = 250nm

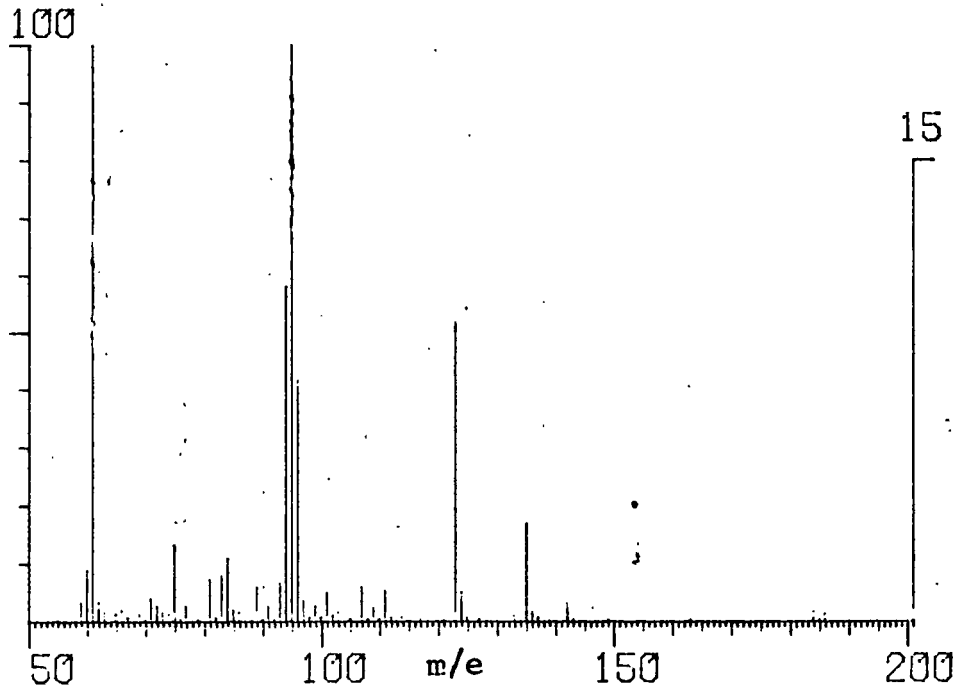
Eluant: 0.01N sodium nitrate, pH = 5.0

Flow Rate: 1.0ml/min

Sample Size: 2.0 1

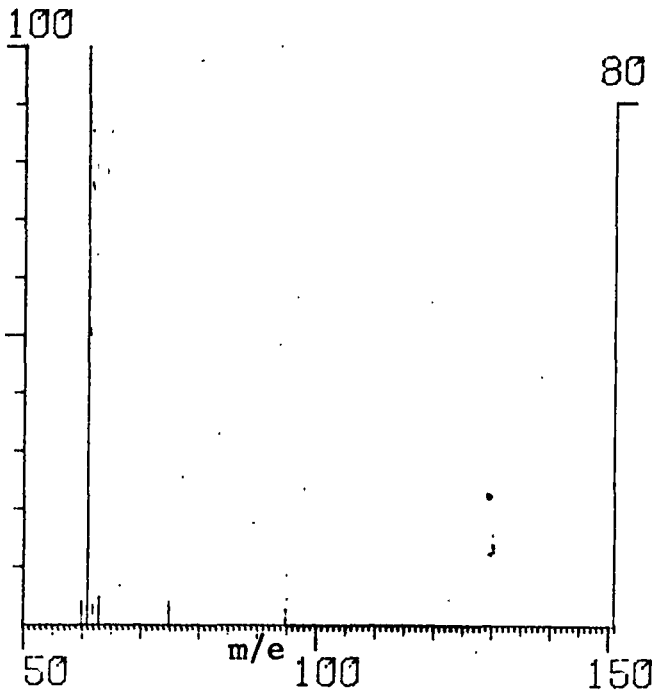


Scan 8
M.S.
Cut #2



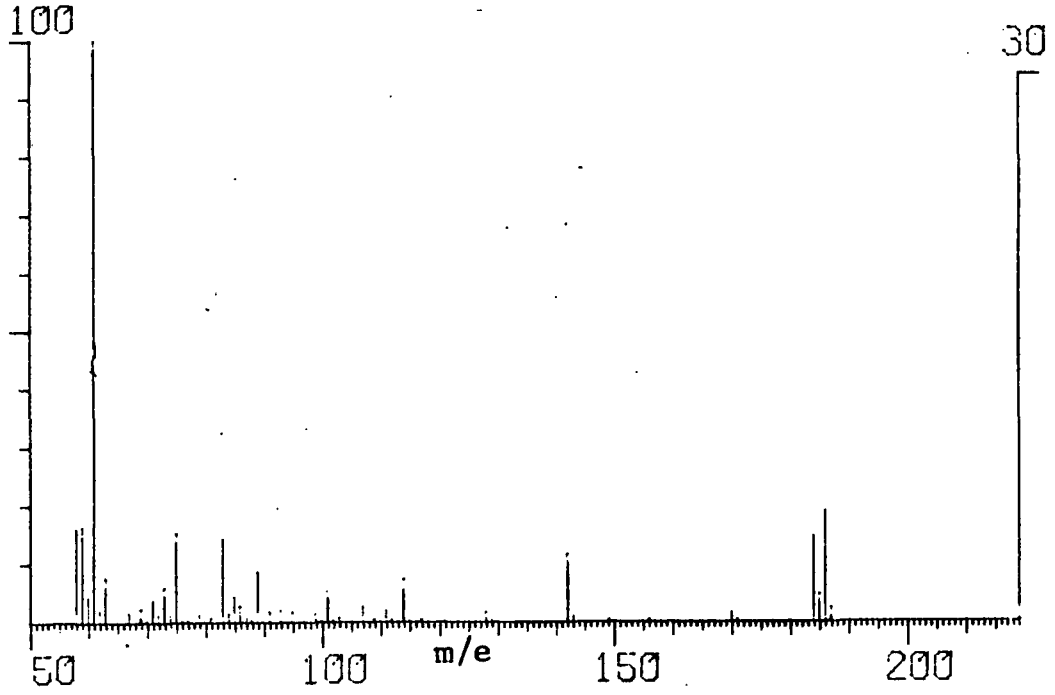
m/e	Compound
61	Acetic Acid
75	Propionic Acid
95	Phenol
123	Hydroxyquinone

Scan 9
M.S.
Cut #3



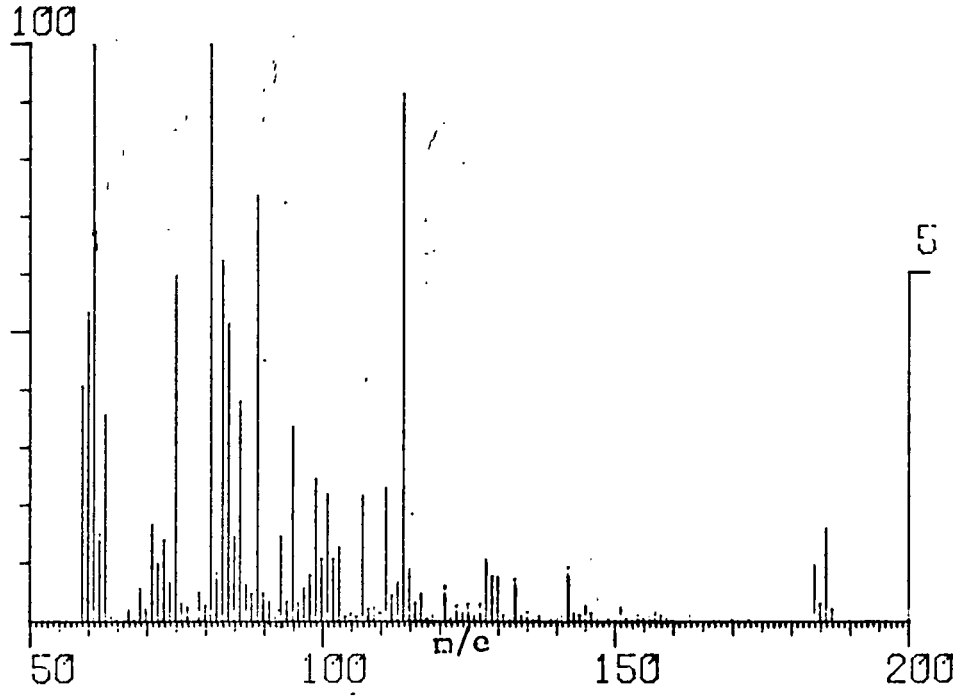
m/e	Compound
61	Acetic Acid
75	Propionic Acid
95	Phenol

Scan 10
M.S.
Cut #4



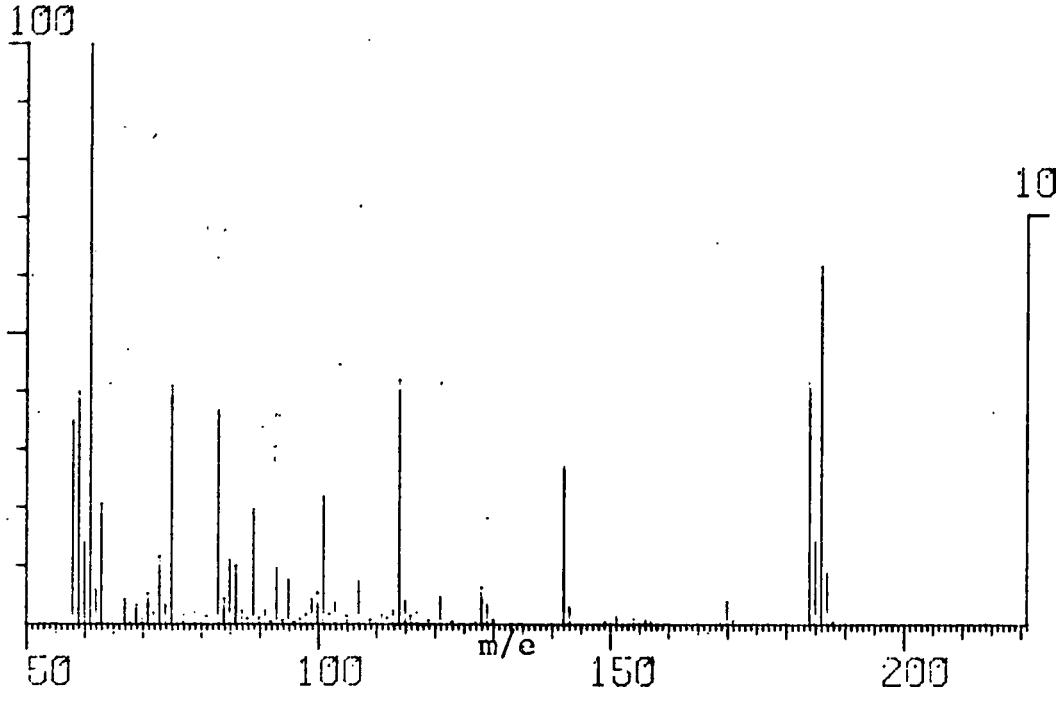
m/e	Compound
61	Acetic Acid
75	Propionic Acid
89	Oxalic Acid
142	Croconic Acid

Scan 11
M.S.
Cut #5



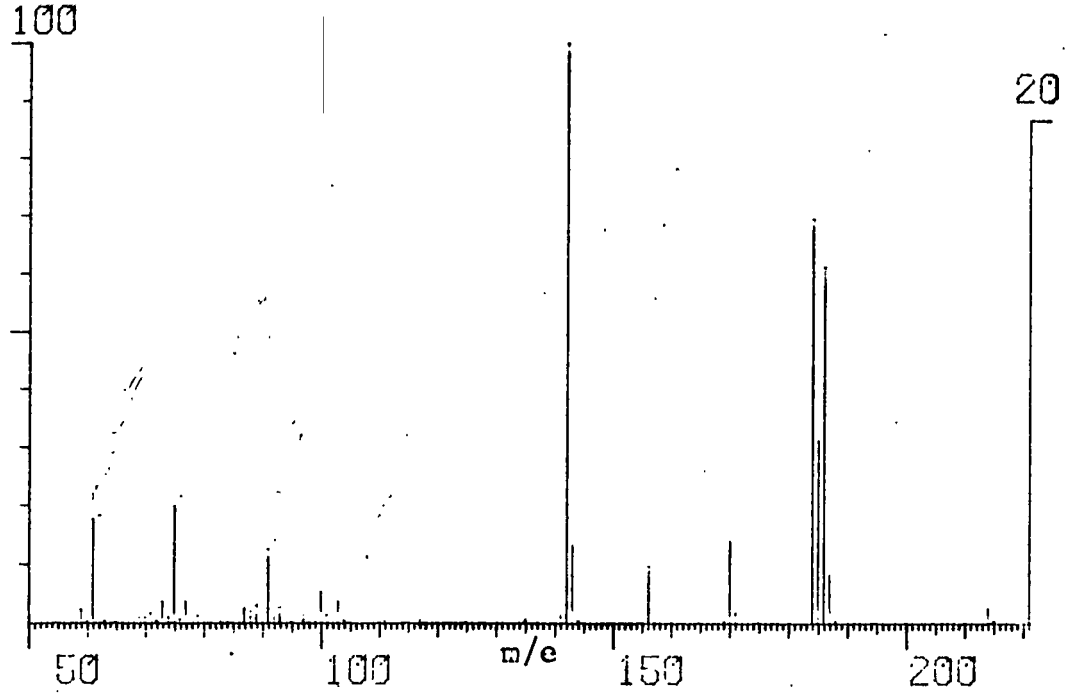
m/e	Compound
61	Acetic Acid
75	Propionic Acid
89	Oxalic Acid
107	Quinone
142	Croconic Acid

Scan 12
M.S.
Cut #6

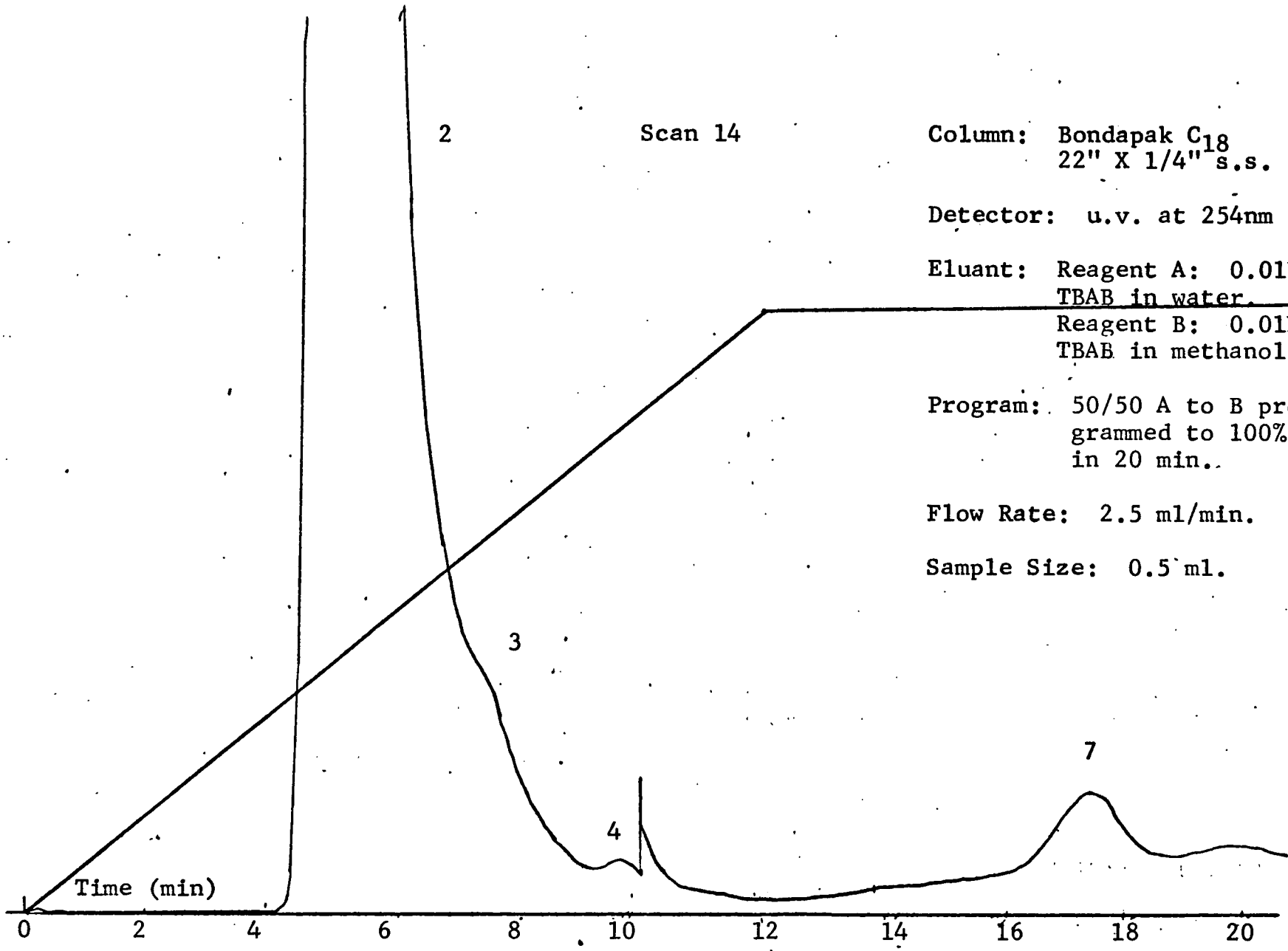


m/e	Compound
61	Acetic Acid
75	Propionic Acid
89	Oxalic Acid
142	Croconic Acid
170	Rhodizonic Acid

Scan 13
M.S.
Cut #7



m/e	Compound
61	Acetic Acid
75	Propionic Acid
89	Oxalic Acid
142	Croconic Acid
170	Rhodizonic Acid


2
Scan 14
Column: Bondapak C_{18}
22" X 1/4" s.s.
Detector: u.v. at 254nm
Eluant: Reagent A: 0.01N TBAB in water.
Reagent B: 0.01N TBAB in methanol
Program: 50/50 A to B programmed to 100% B in 20 min..
Flow Rate: 2.5 ml/min.
Sample Size: 0.5 ml.
3
4
7
Time (min)
0
2
4
6
8
10
12
14
16
18
20

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