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A Qualitative Investigation of the Polymerization Process in the IHSS Humic Substances Extraction Procedure using Model Phenolic Compounds

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A QUALITATIVE INVESTIGATION OF THE POLYMERIZATION PROCESS IN THE IHSS HUMIC SUBSTANCES EXTRACTION PROCEDURE USING MODEL PHENOLIC COMPOUNDS

by

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Roger J. Germay

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Western Michigan University, 2001

Achard (1786) extracted peat with alkali and reported obtaining dark compounds now called humic substances. These are complex, high molecular weight, polyphenolic compounds naturally occurring in soil, in fresh-water and in marine environments. They are biodegradation products of plant materials.

The International Humic Substances Society (IHSS) adapted Achard's method to separate humic substances into three fractions: (1) humic acids (HA), soluble only at high pH; (2) fulvic acids (FA), water-soluble at any pH; and (3) humin, insoluble at any pH. The IHSS method involves contact of soil with 0.1N.NaOH solution, followed by acidification with HCl.

In this study, 0.01 N. solutions of benzenediols in 0.1 N.NaOH were prepared and mixed for 24 hours. The resulting mixtures were then adjusted to pH 7.0 with HCl. Fractions were isolated from these mixtures using Reverse-Phase Preparatory HPLC and freeze-drying. Insights on the structure of the products formed during the 24 hours contact were gained from FTIR, HPLC-MS and Proton-Nmr.

These studies prove that the alkali extraction step of the IHSS humic acid extraction process from soils has a definite potential for producing artifacts because of its polymerization effect on the model compounds studied.

TABLE OF CONTENTS

CHAPTER

CHAPTER

APPENDICES

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Organics in Natural Environments

Nature abounds with a great variety of organic and inorganic compounds. How they interact and how they originated is the focus of a growing interest of research. Phenolic compounds and polyphenolic compounds are very common in natural environments. They are degradation products from plants and can be present in industrial effluents (Jiang, 1996). They are subject to polymerization under natural conditions. Interest in these compounds goes as far back as the beginnings of the science of chemistry itself (Achard, 1786).

Phenolic compounds, it has been suggested by many researchers, play an active role in the formation of humic substances. They have been proposed as structural building blocks of humic substances. These are high molecular weight polyphenolic compounds found in soil and aquatic dissolved organic matter. Many efforts have been made to understand their complex nature and to isolate them. The purpose of this thesis is to investigate experimentally, using model phenolic compounds, the role of polymerization in the humic substances isolation methods.

Humic Substances

Humic substance chemistry has its roots in the work of Achard

(1786). He treated peat with alkali and obtained a dark precipitate that we now know as "humic substances". Later, Baron Jöns Jakob Berzelius (1779-1848), a Swedish chemist considered one of the founders of modern chemistry, introduced the present system of chemical notation in which each element is represented by one or two letters of the alphabet. His student G. J. Mulder published an empirical formula

$$C_{40}H_{30}O_{15}$$

for soil humic acid in 1840 (Mulder, G.J.). Fuchs was one of the first to attempt to assign a structure to humic acid. His structure (Fuchs, 1931), which reflects the polyphenolic character of humic acid, has often been quoted in the chemical literature.

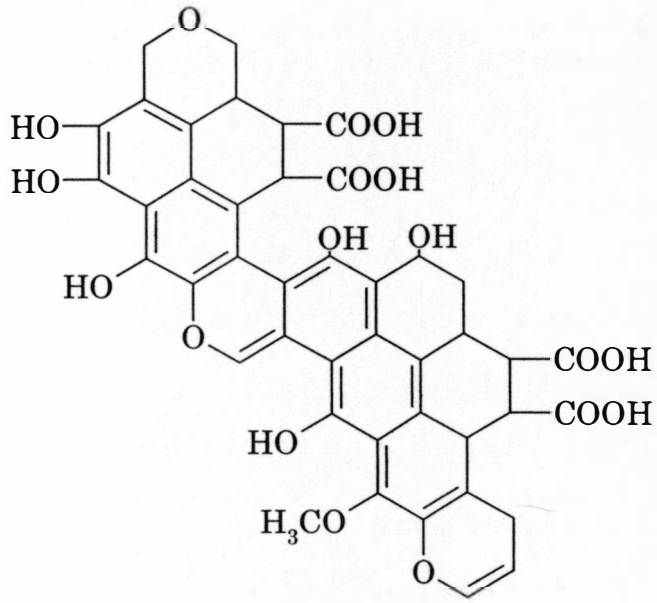


Figure 1. Fuchs' Polyphenolic Structure for Humic Acid (Fuchs, 1931).

Some scientists still believe that soil humic acid has a discrete molecular structure. Paciolla and co-workers (Paciolla et al., 1998-1999)

revised Steelink's (1963) proposed formula by substituting NH_2 for H. The numbered carbon atoms in the structural backbone of the humic acid monomer in Figure 2 below are the chiral centers.

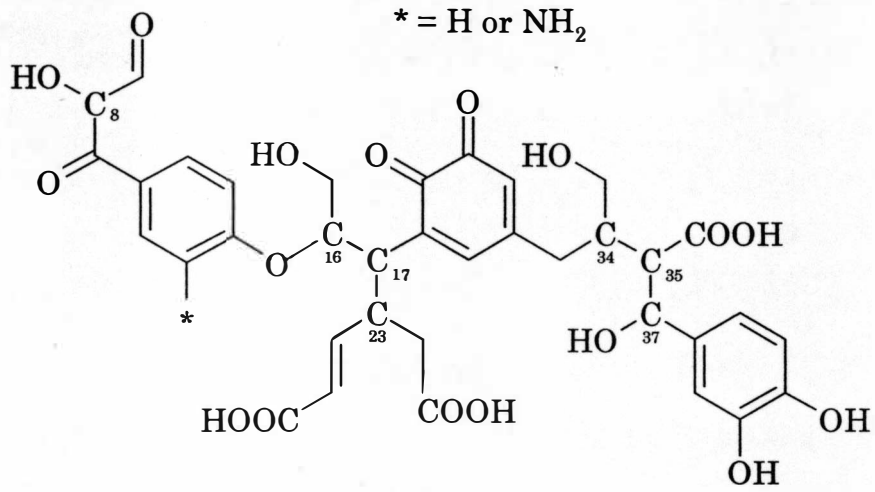


Figure 2. Proposed Structures of Soil Humic Acid Building Block (*= H: Steelink, 1963; *= NH_2: Paciolla et al, 1998, 1999).

Another hypothesis for the origin of humic acids is that they could be the end product of a specific biosynthetic sequence. The Maillard reaction (Labuza et al., 1994), a reaction that starts with the formation of a Schiff base between the carbonyl of a sugar and the nitrogen of an amino compound (amino acids, peptides and proteins). The product then dehydrates, rearranges and condenses many times to yield both simple compounds and very complex brown polymeric nitrogenous products (Stevenson, 1982). The bioavailability of the Maillard reactants in plant cells and the newer methods of monitoring the formation of the Maillard products (^{15}N and ^{13}C NMR) have revived interest in researching this

hypothesis. (Steelink, 1999)

For many years, humic acid has been "operationally defined". This means defined as per the method used for its removal from its original environment. There have been many different methods employed to isolate humic acids (HA's) and humic substances (HS's) in general from the environment. On September 11, 1981, a group of humic substances scientists (Steelink, 1999) held a meeting to organize the International Humic Substances Society (IHSS). They established a set of HS reference standards for soils and waters and agreed upon procedures to isolate these compounds. These reference standard HS's are now available for purchase to anyone doing HS research.

Method of Isolation of Humic Substances

R.S. Swift (1996) described in detail the method agreed upon by the IHSS in 1981 for isolating humic substances. This method was chosen because it had been found to be satisfactory for most soil types and because it could be performed in most laboratories, it was not proposed as the "recommended or approved" method.

The dry soil was passed through a 2.0 mm sieve then equilibrated at a pH of 1 to 2 in room temperature 1 M HCl . The suspension was then shaken for 1 hour. After low speed centrifugation, the supernatant was saved for isolation of fulvic acid using XAD-8 resin. The soil residue was then pH neutralized to 7.0 with 1 M NaOH and enough 0.1 M NaOH, under an atmosphere of N_2, was added to give a final extractant to soil

ratio of 10:1. The suspension, under N_2, was shaken intermittently for a minimum of 4 hours, and then allowed to settle overnight. The supernatant from this suspension was acidified to pH 1 with 6M HCl while stirring, then allowed to separate for 12 to 16 hours into a humic acid precipitate and a fulvic acid supernatant.

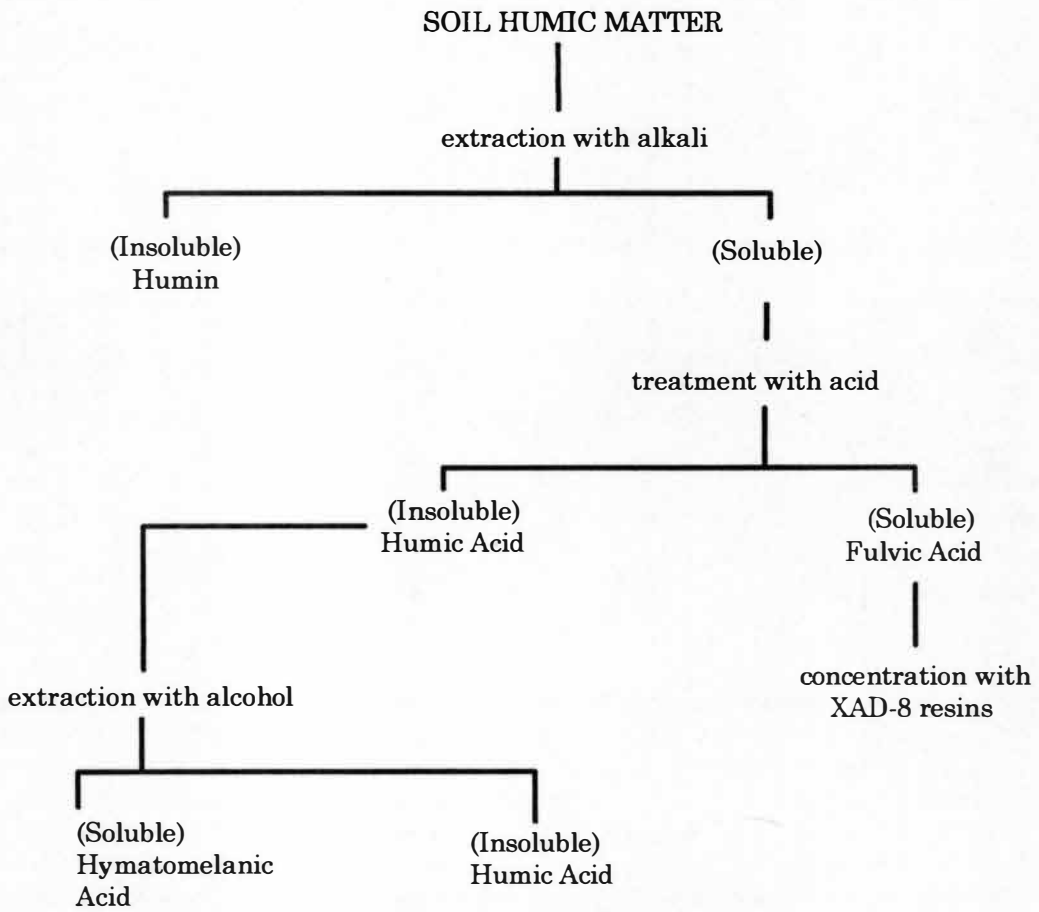


Figure 3. Fractionation Scheme for Humic Substances.

The HA was then purified by redissolving it in 0.1 M KOH, salting it out in 0.3 M KCl, and reprecipitating it in 6 M HCl at pH 1.0 for 12 to 16

hours. Further treatments with 0.1 M HCl / 0.3 M HF reduced the ash content in the purified HA. Mixing the HA with silver nitrate removed the chloride ions and the HA was then freeze-dried. The saved FA fractions were concentrated and purified by a several step procedure using XAD-8 resin filled columns.

Stevenson (1965), (Figure 3), reports the classical scheme for fractionating humus based on extraction of organic matter from soil using caustic alkali, then further subdividing the material according to solubility to pH relationships. He also cautions that the hymatomelanic acid fraction may be an artifact of the extraction method and mentions that some scientists also subdivide the insoluble humic acid fraction into "brown humic acids" (soluble in 5% NaOH) and "gray humic acids" (insoluble in 5% NaOH).

CHAPTER II

LITERATURE REVIEW

Biopolymer Degradation Pathway to Humification

The Lignin-Protein Model

Decomposition of plant material plays an important role in humus formation. Many researchers have recognized that since the mid- to late nineteenth century. One hypothesis emerged by which microorganisms were considered essential to the degradation process, and another that suggested an abiogenic decomposition of plant material, though microbes were necessary to provide the starting materials for that abiogenic process. Fischer and Schrader (1921) proposed lignin as the basic precursor for humic substances. They showed that cellulose and carbohydrates were removed during plant decomposition while lignin was not.

The low nitrogen content of lignin failed to explain the presence of nitrogen in humus. Waksman (1938) proposed his own hypothesis by suggesting that as microorganisms were modifying lignin, they also incorporated protein rich materials from their own composition into it.

Flaig (1966) studied the degradation of ^{14}C-labeled wheat straw and proposed that lignin was degraded oxidatively to produce simple phenols that could then be oxidatively polymerized to form humic substances. The subsequent work of Martin and Haider (1971) with phenols produced by

fungi and other microorganisms showed that these compounds could be polymerized to give humic-like structures.

Plants and microorganisms contain a large variety of biochemicals, some of which are still unknown and are still being discovered. Such complex paraffin-like macromolecules were described for the first time in the 1980's in algae (Largeau et al., 1984) and in plant cuticles. (Nip et al., 1986).

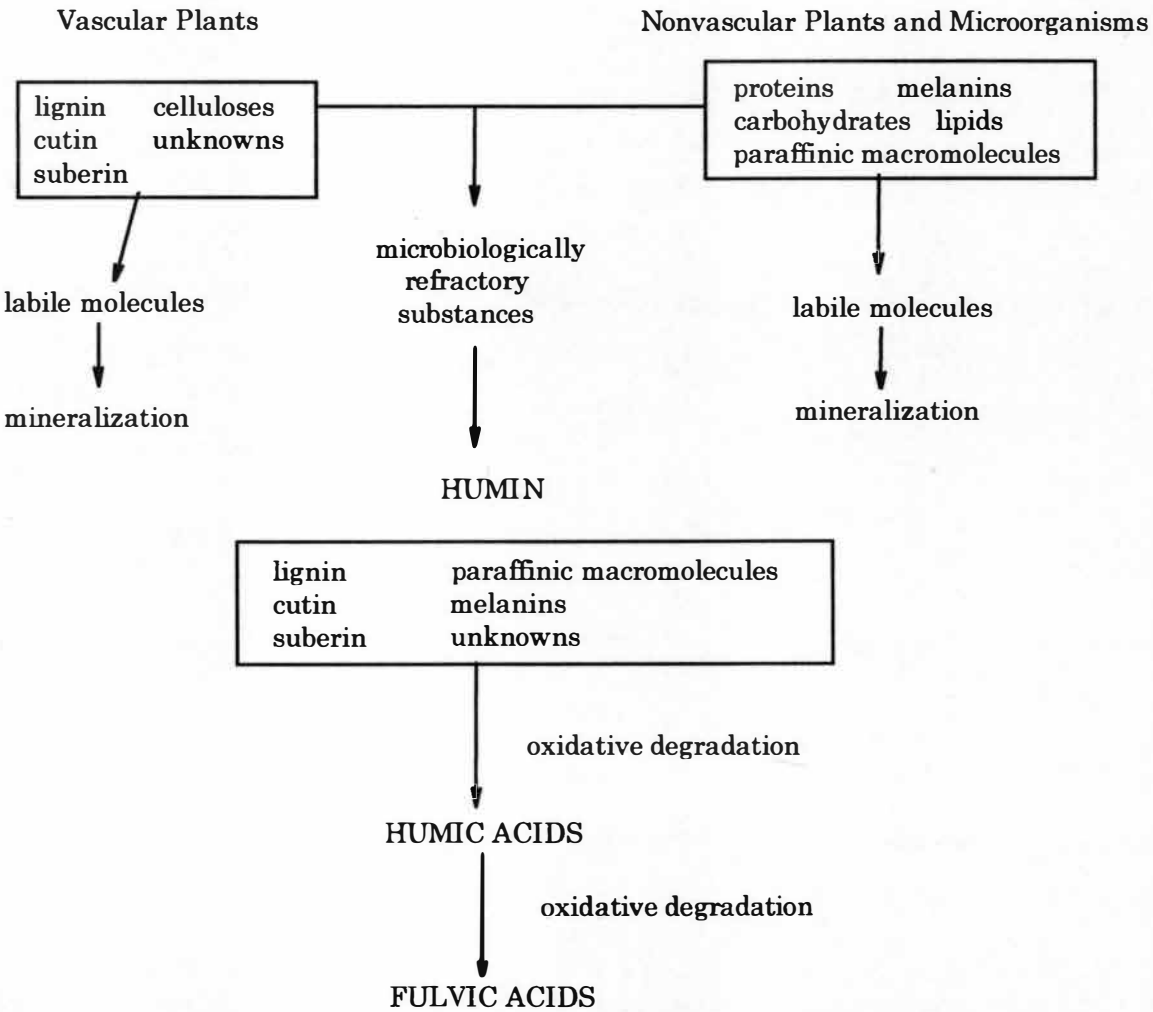


Figure 4. Degradative Pathway to Humic Substances Formation.

Figure 4 outlines the degradative pathway that some Humic Substances researchers, in light of the discovery of these very large biomolecules, believe takes place. During bacterial degradation, some macromolecules are degraded and released, while refractory compounds (lignin, cutins, suberins, melanins, nitrogenated paraffinic macromolecules) are left behind and become humin. Further oxidative degradation produces increased functionality and evolves slowly to humic then to fulvic acids.

Abiotic Condensation Pathways to Humification

The Polyphenol Model

Flaig (1964), Kononova (1966) and Martin and Haider (1971) popularized the polyphenol model (Figure 5). According to this model, plant biopolymers degrade to form simple small phenolic molecules. These molecules then repolymerize to form humic substances as outlined below (Stevenson, 1982).

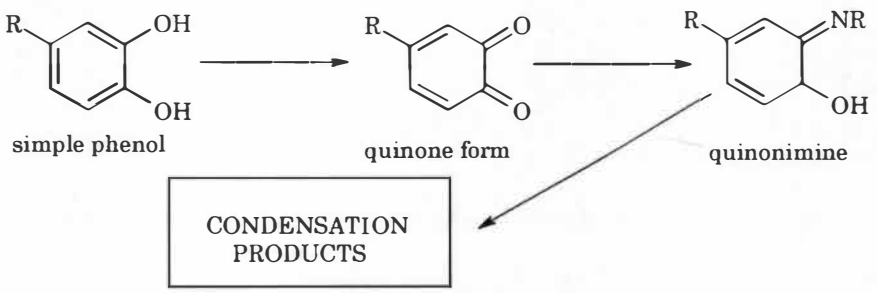


Figure 5. The Polyphenol Model: Schiff Base Formation Between Quinone Intermediates and Amines or Ammonia(Stevenson, 1982).

Quinones easily condense with each other or with amino acids under environmental conditions, forming synthetic polymers (Ladd and Butler 1966, Ertel and Hedges 1983). In addition, polyphenol oxidation to quinones can be spontaneous in aerobic conditions and it can also occur enzymatically (Stevenson, 1982). Since phenols are not easily degraded by microorganisms, they can exist in sufficient quantities in soils to serve as quinone precursors. In marine environments, however, this probability is reduced due to low phenol concentrations in phytoplankton.

The Melanoidin or Browning Reaction Model

The melanoidin model involves the previously discussed Maillard reaction between sugars and amino compounds (page 3 in this thesis). The nitrogen-rich polymers produced under this scheme are similar in composition to marine humic substances (Ishiwatari et al., 1986). The greatest problem of this hypothesis is that the reaction is kinetically of second order and is not rapid at room temperature (Hedges, 1978).

The Polyunsaturated Lipid Model

Polyunsaturated fats are common in phytoplankton on the ocean's surface where they are exposed to ultraviolet solar radiation. According to this model, photolysis occurs, resulting first in formation of marine fulvic acids. Further cross-linking introduces more oxygen and aromatic character into the structure forming finally marine humic acids (Harvey et al., 1983; Harvey and Boran, 1985).

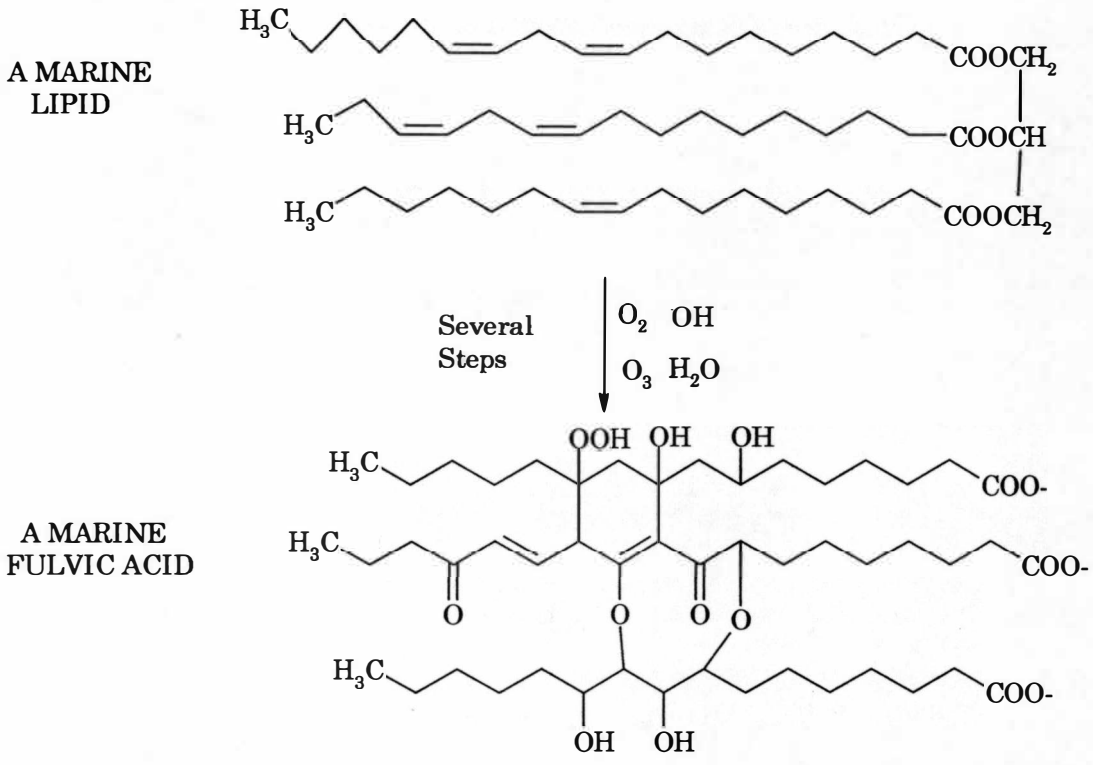


Figure 6. The Polyunsaturated Lipid Model: Formation of a Marine Fulvic Acid (Harvey and Boran, 1985).

The greater proximity of the molecular sites available for cross-linking makes this hypothesis a more attractive explanation of the greater aliphatic character found in marine humic substances as compared to soil humic substances. Unfortunately, there exists a discrepancy between the lower $^{13}C/^{12}C$ ratios one would expect in marine humic substances if produced according to this model (Monson and Hayes, 1982) and the ratios actually measured in the environment (Meyers-Schulte and Hedges, 1986). Another drawback is that the model does not provide any mechanism for inclusion of nitrogen into the humic substances.

Diphenol Reactivity in Aqueous Solutions

Polymerization of Diphenols

Catechols and catechol derivatives polymerize into colored products. These reactions are enhanced by the presence of transition metal oxides (such as CuO, MnO_2, ZnO) and cations (such as Fe^{3+} and Mn^{2+}). (Larson, 1980).

Millimolar solutions of phenolic compounds were studied at pH 7.6 in the presence of MnO_2. Absorbance was measured at 360 nm after 25 minutes and after 23 hours. Catechol, and to a lesser extent, hydroquinone, were shown to form colored polymerization products under these conditions, but not resorcinol. (Larson, 1980).

Larson studied the effect of pH on catechol polymerization. He used millimolar solutions in phosphate buffer for 23 hours and he monitored the UV absorbance at 360 nm as a measure of the relative degree of polymerization achieved under the specific conditions. He showed that catechol polymerization increased as pH was increased from 5.00 to 9.40. (Larson, 1980).

CHAPTER III

EXPERIMENTAL METHODS

Materials

Catechol (99.3%), resorcinol (99.2%) and hydroquinone (99.4%) are all position isomers of benzenediol. Starting materials were obtained from Aldrich Chemicals and used without further purification. All aqueous solutions were made using 18 MΩ (milli-Q) water. Sodium hydroxide pellets (97.8%) and 50% sodium hydroxide solution were used to make up approximately 0.1 M NaOH solutions.

Properties of Materials

The structures of catechol, resorcinol and hydroquinone are shown below in Figure 7. The gram-molecular weight is 110.11 g/mole.

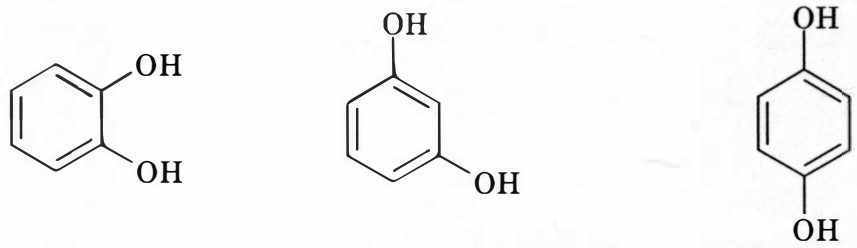


Figure 7. Molecular structures of Catechol, Resorcinol and Hydroquinone. (Left to Right)

Catechol is a tan/off-white crystal. It is soluble in water, in ethyl alcohol, in diethyl ether and very soluble in acetone. It is also soluble in hot benzene, in alkali, in chloroform and in carbon tetrachloride. Its specific gravity is 1.1493. It melts at 105 degrees Celsius and boils at 245 degrees Celsius. It is a weak acid and its pK_{a1} and pK_{a2} values are equal to 9.34 and 12.6 respectively.

Resorcinol is a tan needle-like crystal. It is soluble in water, in ethyl alcohol and in diethyl ether. It is partially soluble in benzene, soluble in any proportion in carbon tetrachloride, very soluble in acetic acid and soluble in hot chloroform. Its specific gravity is 1.2717. It melts at 111 degrees Celsius and boils at 178 degrees Celsius. It is a weak acid and its pK_{a1} and pK_{a2} values are equal to 9.32 and 11.1 respectively.

Hydroquinone is a tan crystal. It is soluble in water and in diethyl ether; it is very soluble in hot water, in ethyl alcohol and in acetone, but insoluble in benzene. It is also soluble in any proportion in carbon tetrachloride. Its specific gravity is 1.328. It melts at 173.4 degrees and boils at 285 degrees. It is a weak acid and its pK_{a1} and pK_{a2} values are equal to 9.85 and 11.4 respectively.

Purity Verification

Melting points were obtained for all the starting compounds as a way to check their purity. The temperature values obtained were in line with reported values in the literature. The three compounds were also tested by High Performance Liquid Chromatography (HPLC). No sign of impurities was detected by this method. In each case, a single sharp peak

was shown to elute. The three compounds were shown to be pure and were therefore used without further purification.

Sample Preparation

Alkali Reaction Solutions

Sample concentrations were used in this study to reflect the concentration of organic compounds naturally in soils (Aiken et al., 1985). In this study, the solutions used were approximately 0.01 M for all three compounds. These were obtained by weighing about 220mg of benzenediol and dissolving it in 200mL of approximately 0.1 M sodium hydroxide. The alkali solution was made by dissolving about 4.0g sodium hydroxide in 1 liter of Milli-Q water or by mixing 1.60mL of 50% (w/w) NaOH (Mallinckrodt) in 200mL water. Solutions were not degassed, covered with a headspace of nitrogen and stirred for 24 hours. At the end of the 24-hour period, the reaction was quenched by lowering the pH to about 7.0 with the addition of hydrochloric acid.

Recovery of Initial Dried Residue Mixture for FTIR

The neutralized residue solutions were initially freeze-dried (to avoid possible sensitivity to heat by these organic compounds). The resulting dried residues were prepared into a Nujol mull and also compressed into a potassium bromide pellet and scanned with a Fourier Transform Infrared Spectrometer.

Recovery of Individual Products and Attempted Characterization

Large quantities of 0.01 M Catechol solution in 0.1 M sodium hydroxide were prepared as described previously. The neutralized reacted sample was then fractionated by preparatory HPLC. The resulting fractions were then combined and freeze-dried. Even with the large amount of initial reaction solution, the individual dried products were in very short supply. In as much as was possible, FTIR, UV-Vis and ^{1}H-NMR spectra were obtained.

Instrumental Technique

Melting Point Apparatus

A Mel-Temp II (Laboratory Devices Inc., Holliston, MA) melting point apparatus was used to determine melting points of starting materials to check their purity. Capillary tubes were filled with sample material and placed in the heated oil compartment. Temperature was increased in a controlled manner and the physical appearance of the material in the tube was monitored as well as the temperature. A temperature range was obtained during which the compound melts.

Gas Chromatography-Mass Spectrometry (GC-MS)

The samples were not volatile enough for direct analysis by Gas Chromatography. To increase their volatility, they were derivatized using a technique first described by Pellizzare (1969). Alltech derivatization reagents HMDS (hexamethyldisilazane), TMCS (trimethylchlorosilane)

and pyridine were used.

A few milligrams of freeze-dried residue were weighed and placed in a sealed vial under a neutral Argon atmosphere. One ml of freshly prepared reagent containing HMDS, TMCS and pyridine (3:1:9) were added and the mixture was vigorously shaken for 30 seconds and all allowed to stand for a minimum of five minutes at room temperature. An aliquot of this mixture was injected directly into the gas chromatograph.

The instrument used was a Hewlett-Packard Model #5890A Gas Chromatograph equipped with a HP-1 capillary column, connected to a Model #5870B Mass Selective Detector. The trimethylsilyl ethers of the study compounds were then detected because of their increased volatility. Figure 8 shows the equation for the formation of the trimethylsilyl ether using resorcinol as a model compound:

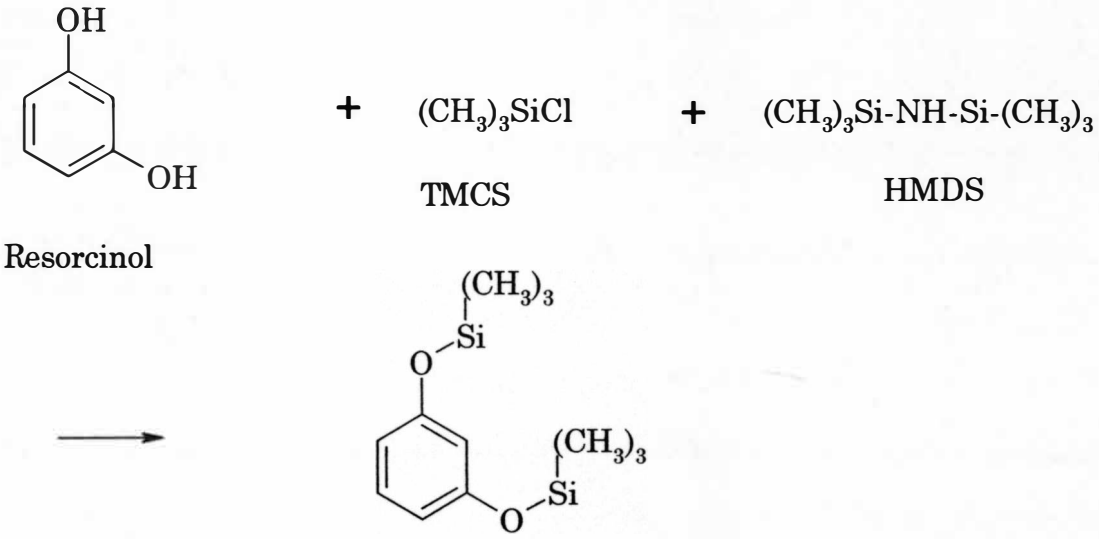


Figure 8. Trimethylsilylation of the Model Phenolic Compound Resorcinol.

Ultraviolet Spectroscopy (UV-Vis)

0.010 molar aqueous solutions of catechol, resorcinol and hydroquinone were prepared in water, mixed for 5 minutes, then diluted 100-fold in water, mixed an additional minute and scanned from 190 nm to 350 nm and from 190 nm to 820 nm on a Hewlett Packard Model 8451A Diode Array Ultraviolet Spectrophotometer. These solutions were measured, against a Milli-Q water blank, in 1cm quartz cuvettes. These spectra provide us with a picture of starting conditions.

Similar 0.010 molar solutions were made in 0.10 molar aqueous sodium hydroxide and mixed for 5 minutes. An aliquot was taken, diluted 100-fold in water, mixed for an additional minute and scanned as above. The solutions were allowed to continue reacting under an argon blanket for 24 hours at which point another aliquot was taken, diluted, mixed, and scanned as above. Millimolar aqueous sodium hydroxide was used as a blank for these tests. These scans provide us, as a function of time, with a picture of the changes, if any, which occur over the 24 hour period.

High Performance Liquid Chromatography with Ultraviolet Diode Array Detection (HPLC-Diode Array)

A RAININ Dynamax HPLC system with UV diode array detector and with an analytical HPLC size flow cell was used with a 25 cm x 4.6 mm Kromasil C-18 analytical HPLC column. The size of the loop was 100 microliters. Neutralized stock catechol residue solution, diluted two-fold with Mobile Phase A was eluted at 1.0 ml/min using gradient elution reverse phase HPLC, as shown in Table 1. This analysis starts out

isocratically at 100% mobile phase A for 12 minutes then ramps a gradient up to 50% A and 50% B by 33.9 min, actually resulting in a 50:50:0.1 H_20:ACN:TFA. The system is then returned to initial conditions and allowed to equilibrate prior to further injections. UV-VIS scans were examined for various peaks in the HPLC spectrum to gain some insight into how different the various fractions were from each other. This feature was also utilized to ascertain the purity of the separated PREP-HPLC fractions later on in our study. See Appendix D for a few examples of these UV-VIS spectra.

Table 1

Gradient Elution HPLC Parameters

	Time	Mobile Phase A	Mobile Phase B
Composition		90:10:0.1	10:90:0.1
		H_2O: ACN: TFA	H_2O: ACN: TFA
	0.0 min	100 %	0 %
	12.0 min	100 %	0 %
	33.9 min	50 %	50 %
	35.0 min	100 %	0 %
	42.9 min	100 %	0 %

Preparatory High Performance Liquid Chromatography (Prep-HPLC)

Two RAININ Dynamax Solvent Delivery Systems, Model SD-1 were

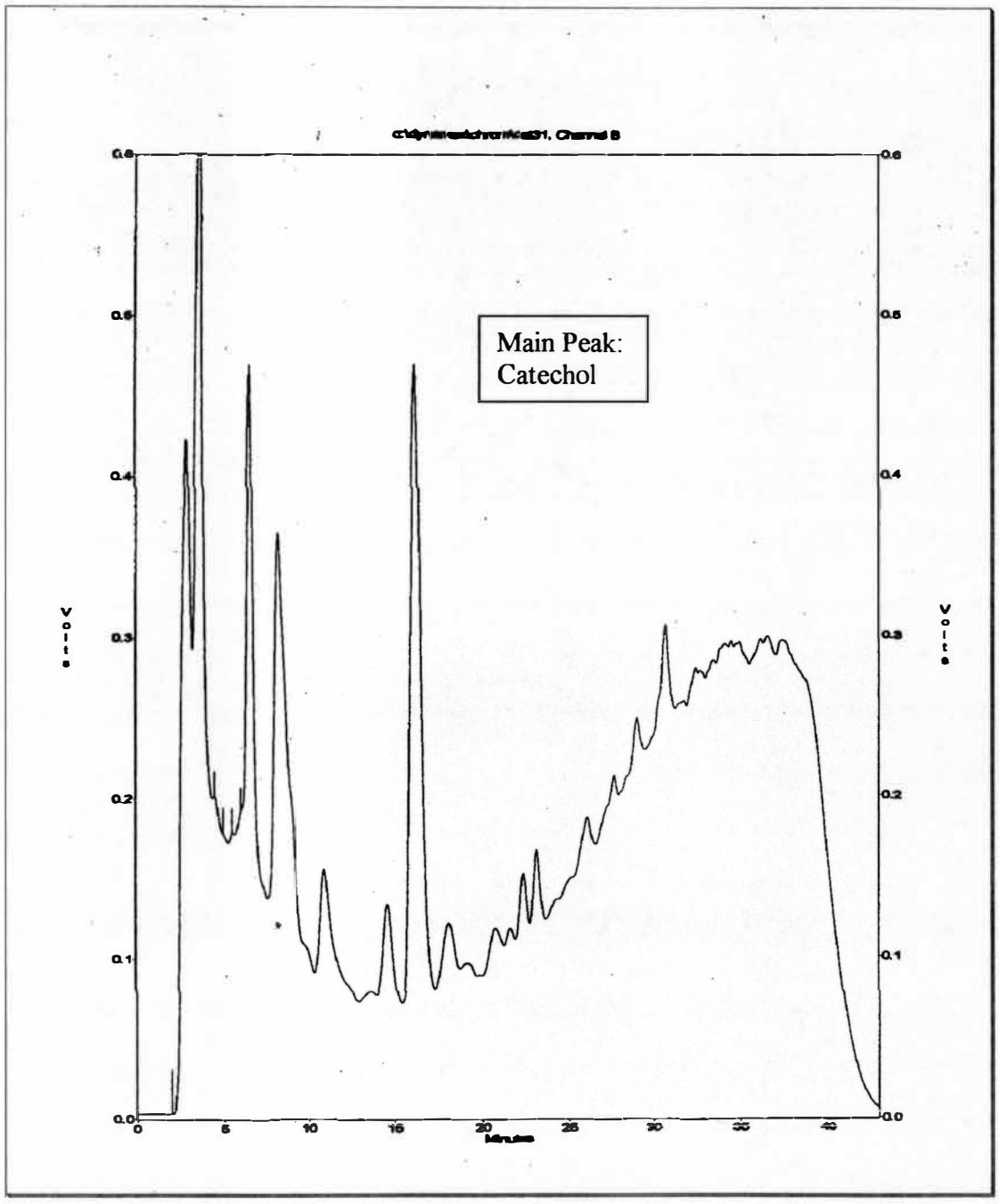


Figure 9. Preparatory HPLC Chromatogram Showing Separation of Numerous Product Peaks.

used to pump, at 18 ml/min, two mobile phases A and B according to a specific gradient over a 25 cm x 21.20 mm PHENOMENEX KROMASIL preparatory HPLC column using a C-18 hydrocarbon monolayer covalently

bonded to 5-micron microparticulate silica as the stationary phase. Peak elution was detected by a RAININ Dynamax Absorbance Detector, Model UV-1, equipped with a 9 mm - 1 mm preparatory flow cell and set at 280 nm. A RAININ Dynamax Fraction Collector, Model FC-1, was used to collect consecutive fractions throughout the run at 30 seconds intervals.

The mobile phase A was 90% water, 10% acetonitrile and 0.5% trifluoroacetic acid; the mobile phase B was 10% water, 90% acetonitrile and 0.5% trifluoroacetic acid (TFA). Mobile phase A was pumped at 100% for 12 min, then a ramp was started to reach 50% A and 50% B at 35 min. This flow was maintained for 1 min then the system was returned to original conditions.

Solid-Phase Extraction (Desalting, Chemical Noise Clean-up)

Pooled fractions from the Prep-HPLC system contain TFA, which interferes in subsequent analysis of the sample by FTIR and ^{1}H-NMR. This impurity was cleaned up using solid-phase extraction. In addition to removing the troublesome TFA, this step concentrates the sample for processing.

The pooled fractions from the Prep-HPLC were diluted in two volumes of water and pumped through a specially chosen HPLC column with higher affinity for the compounds of interest in our study. After

adsorption of the complete sample onto the column, water was pumped over the column to flush out any excess acid from the system and the column was reversed. Acetonitrile was then pumped over the column to elute off the sample. Elution of the adsorbed compound was monitored with a strip chart recorder and the compound was collected by manually turning a valve.

The pump used was a RAININ Dynamax Solvent Delivery System, Model SD300; the detector was an LDC/MILTON ROY Spectromonitor D Variable wavelength detector with Automatic Zero, equipped with a preparatory HPLC size cell. The signal was monitored at a wavelength of 280 nm. The system was operated at a maximum backpressure of 2000 to 2500 pounds per square inch (psi). The collected desalted fractions were then freeze-dried.

Freeze-drying Apparatus

A VirTis Bench Top Freeze Dry system was used in the initial part of this study to produce dried material for preliminary FTIR analysis. It works by subliming the water from the material, that is to say that ice is allowed to evaporate directly without ever forming any liquid. The temperature of the system was maintained at –30 degrees C. The system vacuum pressure was kept in the range of 30 to 60 millitorr. The aqueous solutions were frozen overnight in a freezer then connected to the freeze-dryer by way of a valve. The freeze-drying process was allowed to take place until the samples were completely dry.

During the latter part of the study, an FDS Systems Inc. Flexi-Dry

Microprocessor Control Corrosion Resistant Freeze-Dryer Model FDX3-85A-MP was used for the freeze-drying of the collected fractions from the preparatory HPLC. The collected fractions were pooled, desalted using solid-phase extraction, and then concentrated down to a reasonable volume under stream of nitrogen. The concentrated fractions were then individually transferred into a shatterproof glass vial and frozen in liquid nitrogen. The vials were fitted with caps with an opening and a lint-free cloth covering the hole. They were then placed in another glass chamber fitted through a valve to the drying chamber. The temperature of the system was maintained at about –90 degrees Celsius. The system vacuum pressure was kept at about 96 millitorr.

Fourier Transform Infrared Spectroscopy

Methods of Sample Preparation

Nujol Mull. Two to five milligrams of sample were ground using an agate mortar and pestle. A mull was then prepared by further grinding in one to two drops of Nujol, a high-boiling petroleum oil. The mixture was smoothly squeezed between two salt plates and scanned in the FTIR. (Silverstein, 1974). This method was used to gather preliminary evidence to show that structural changes do occur in some cases.

Potassium Bromide Pellet. 0.1 milligram to 0.8 milligram of sample were ground in an agate mortar, then mixed with about 80 milligrams of KBr (not ground, so as to reduce the possibility of adsorption of atmospheric humidity and likely interference in the spectrum). The mixture was then placed between two anvils and pressed

with a WILMAD No. 107 KBr Quick Press until a clear KBr pellet, 7 mm in diameter, was produced. The collar containing the resulting pellet was scanned in the FTIR. (Wilmad Glass Company Instruction Bulletin). This method was used both for preliminary FTIR evidence and for scanning some Prep-HPLC fractions.

Dried Deposit on Salt Plates. Saturated solutions of the Prep-HPLC fraction material were prepared by mixing minute amounts of sample in a few drops of acetonitrile. The mixtures were transferred to a clean salt plate and dried at room temperature. The resulting sample deposit on the plate was scanned in the FTIR.

Instrumentation

Preliminary FTIR data was obtained by scanning Nujol mulls and KBr pellets from 400 cm^{-1} to 4000 cm^{-1} (KBr pellets also made for two Prep-HPLC fractions). The instrument used was a Nicolet 5DXC Fourier Transform Infrared Spectrometer.

FTIR data from Prep-HPLC fractions was obtained by scanning sample deposits on a salt plate. The instrument used was a Perkin-Elmer Infrared Fourier Transform Spectrometer Model 1710 equipped with Perkin-Elmer "Spectrum for Windows" (Version 1.5) data processing software.

High Performance Liquid Chromatography-Mass Spectroscopy (HPLC-MS)

0.010 molar catechol and hydroquinone solutions were allowed to react in 0.10 molar sodium hydroxide for 24 hours, then neutralized to

pH 7 with hydrochloric acid. These were then injected on an HP 1090 high performance liquid chromatograph, using a 25 cm x 4.6 mm Zorbax Rx C-18 (5 μ particle size) analytical column. Both 20 μL and 25 μL injections were made. The flow was 1.0 mL/min. The gradient was the same as in Table 1 except that the mobile phase compositions were modified to avoid interference by the trifluoroacetic acid (TFA) in the mass spectra. Mobile phase A was replaced by 0.2% formic acid in water while mobile phase B was replaced by 0.2% formic acid in acetonitrile.

A Finnigan LCQ mass spectrometer equipped with ES I Electrospray sample delivery module then analyzed the eluent from the HPLC. The capillary temperature was set at 200°C.

Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR)

Some of the freeze-dried fractions were prepared as saturated solutions of 150 microliters acetonitrile with fraction 21 being dissolved in dimethyl sulfoxide-d_6. The data were acquired on a Varian INOVA 400 NMR spectrometer operating at a proton frequency of 399.80 MHz, and equipped with a Nalorac inverse geometry, triple resonance, micro gradient probe. A standard one-dimensional proton experiment was employed with an interpulse delay of 1.44s and an acquisition time of 2.56s. The spectral width of 6387.7 Hz was digitized with 16384 complex points and was exponentially weighted prior to Fourier transformation. Fractions A and B were acquired with 1024 transients, fraction C with 2048, D with 16384, F with 15168 and 21 with 16384 transients.

CHAPTER IV

RESULTS AND DISCUSSIONS

Preliminary Evidence of Change in Structure

Ultraviolet Spectroscopy Evidence

Ultraviolet Spectroscopy was the technique used to obtain preliminary evidence that shows that changes do occur in some cases upon alkali dissolution of benzenediols. Ultraviolet scans of aqueous solutions of benzenediols were compared to similar scans taken of their alkali solutions as a function of time, after 5 minutes and after 24 hours.

Figure 10 shows the UV-VIS spectrum of a freshly prepared 0.01 M solution of catechol in water. It contains a peak maximum at 200 nm, a shoulder at about 225 nm and another peak maximum at 276 nm. Within 5 minutes of dissolution in 0.1 M NaOH, the three peak maxima move now to 208 nm, 238 nm and 290 nm. By the end of a 24-hour period, the maxima have shifted to 208 nm, 262 nm and 316 nm as shown on Figure 11.

Similar results are obtained in the case of the para-substituted benzenediol, hydroquinone. It is different, however, in the case of resorcinol, the meta-substituted benzenediol. Minimal change is observed in that case as shown below in Table 2.

In Figure 12B, there is a small malformed shoulder that appears on the resorcinol UV-VIS spectrum and stretches from about 315 nm to about

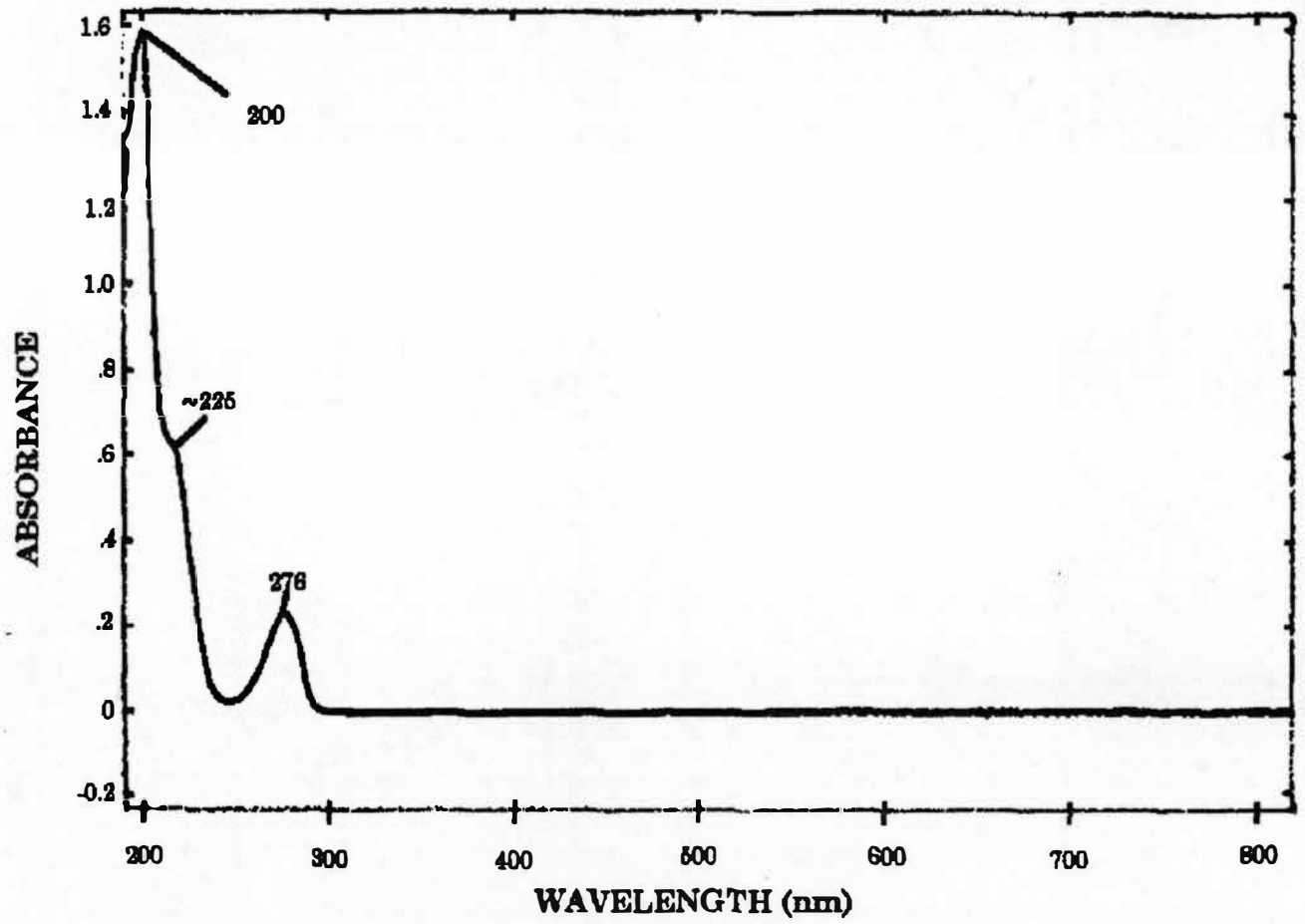


Figure 10. UV-VIS Spectrum of Catechol (0.01 M in Water, Freshly Prepared).

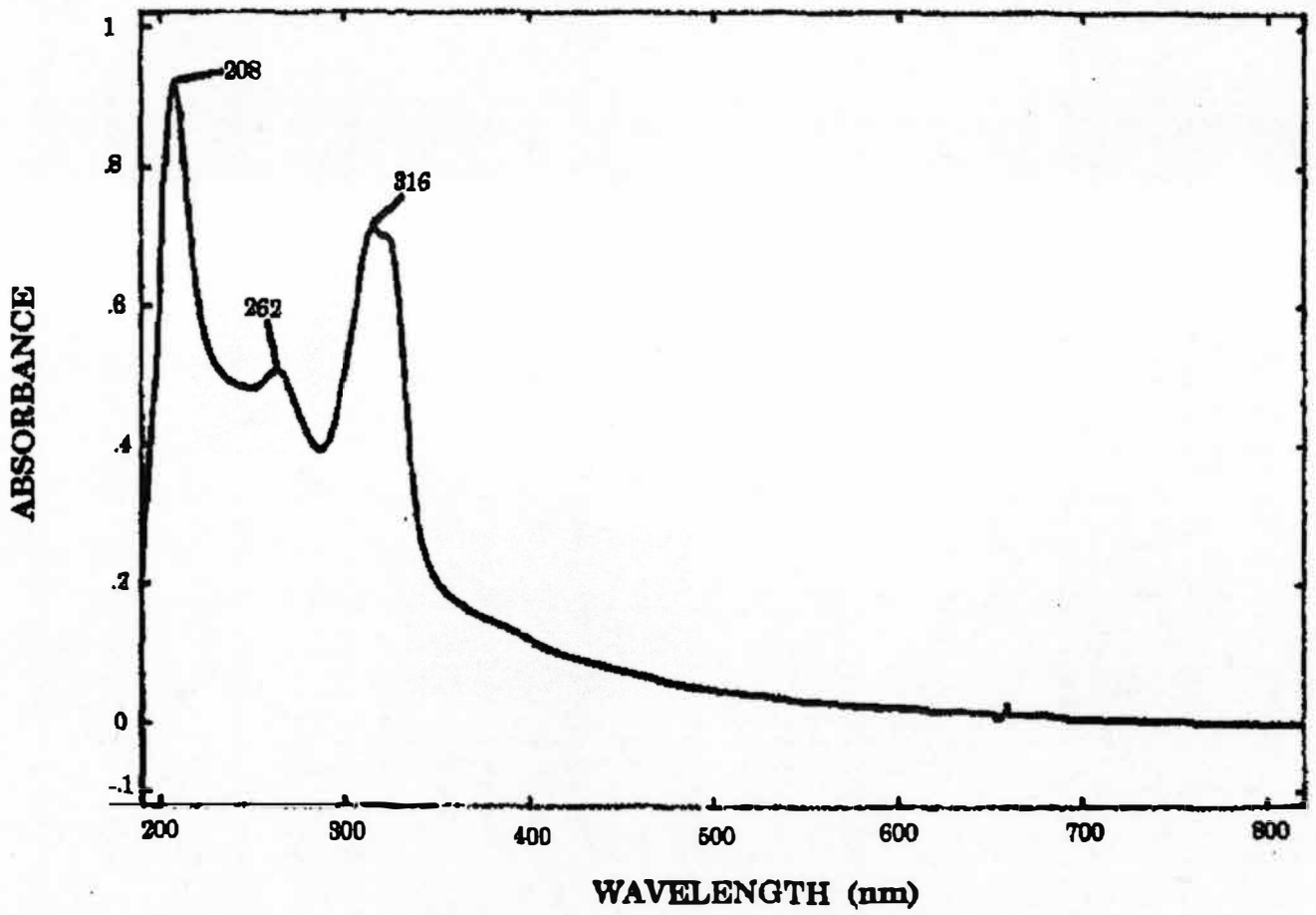


Figure 11. UV-VIS Spectrum of Catechol (0.01M in 0.1 M NaOH, 24 hours contact).

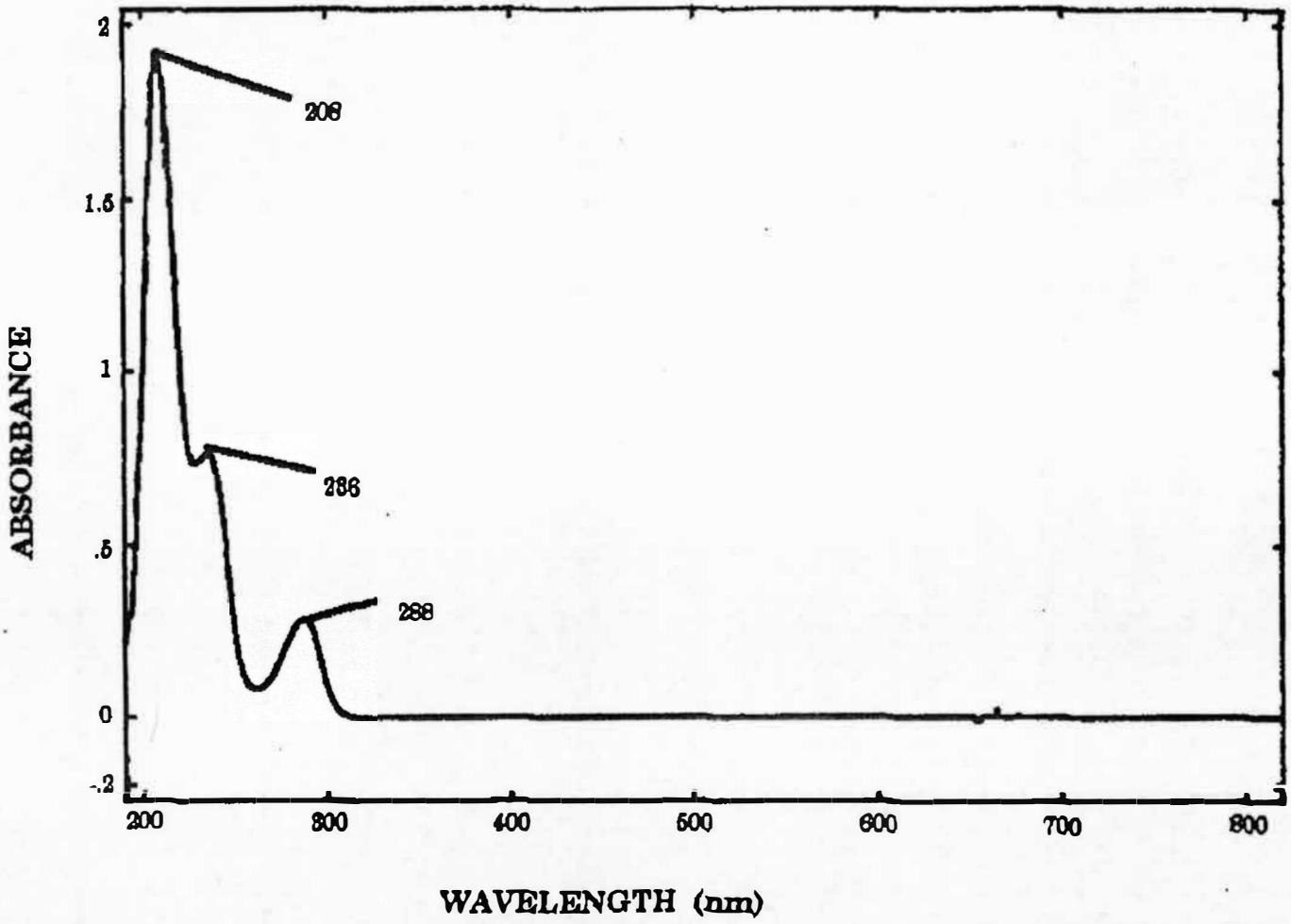


Figure 12A. UV-VIS Spectrum of 0.01 M Resorcinol in 0.1 M Sodium Hydroxide After 5 Minutes.

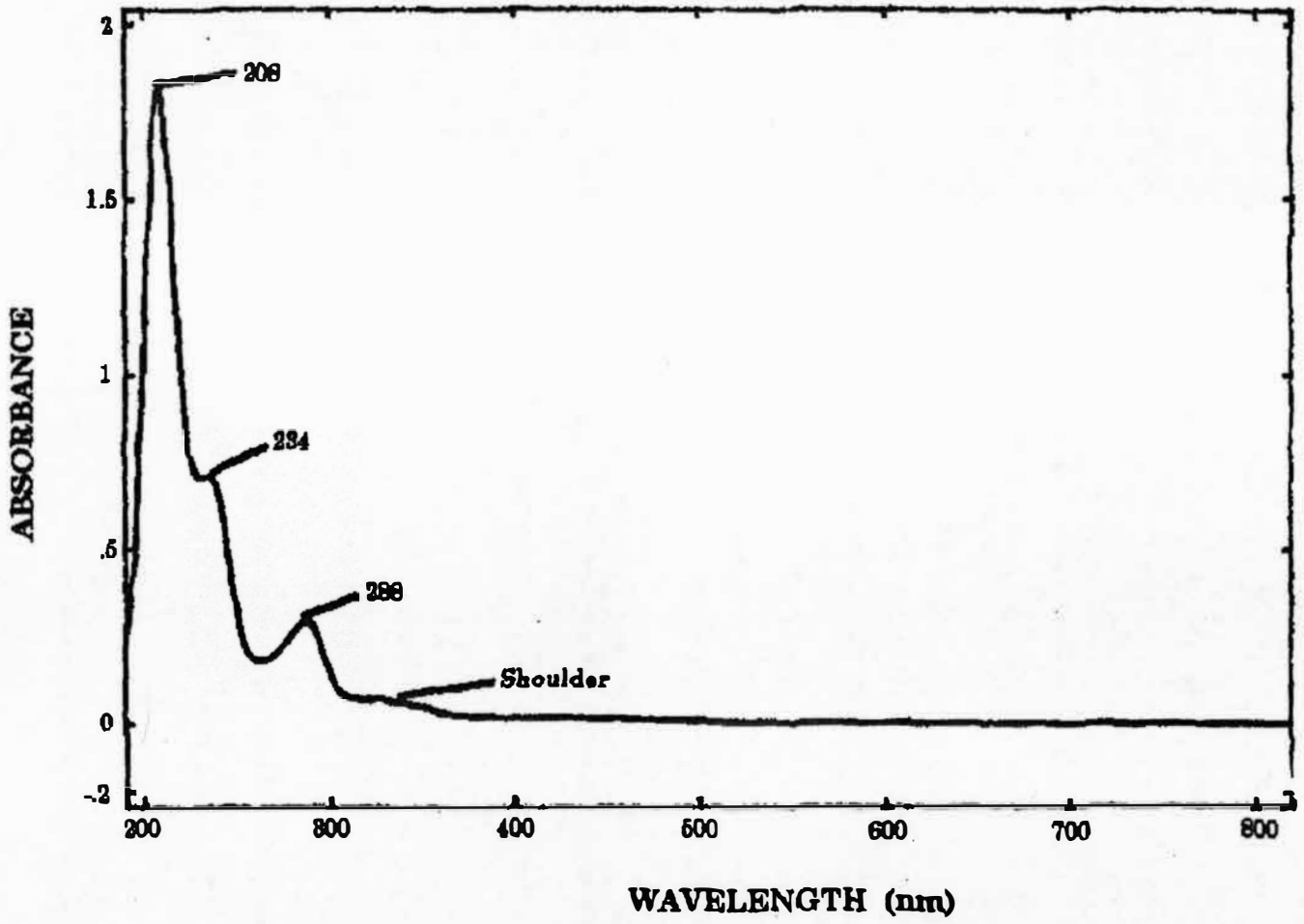


Figure 12B. UV-VIS Spectrum of 0.01 M Resorcinol in 0.1 M Sodium Hydroxide After 24 Hours.

Table 2

UV Absorption Shifts Observed During Alkali Dissolution of Benzenediols

Compound Name	Peak Maxima		
	In Water (5 min)	In NaOH (5min)	In NaOH (24hrs)
Catechol	200, 225, 276	208, 238, 290	208, 262, 316
Hydroquinone	196, 222, 290	212, 309, 402	210, 264, 317
Resorcinol	198-202, 218, 274	208, 236, 288	208, 234, 288

370 nm, this shoulder is not present in Figure 12A. The rest of the spectrum remains essentially unchanged over the course of the 24-hour dissolution of resorcinol in sodium hydroxide. This indicates that we would not expect any change in structure in resorcinol, but we would in catechol and in hydroquinone.

Fourier Transform Infrared Spectroscopy Preliminary Evidence

Benzenediol samples were reacted with alkali for 24 hours, neutralized and freeze-dried. FTIR scans of the resulting residues were obtained (both in cm^{-1} and in μ units) and were compared with scans obtained from the pure benzenediols. The data confirmed what was apparent from the UV-VIS spectra.

Figures 13A and 13B shows that the resorcinol residue is actually essentially still in the form of resorcinol as both the FTIR scans for the pure material and that of the residue exhibit absorption peaks that match.

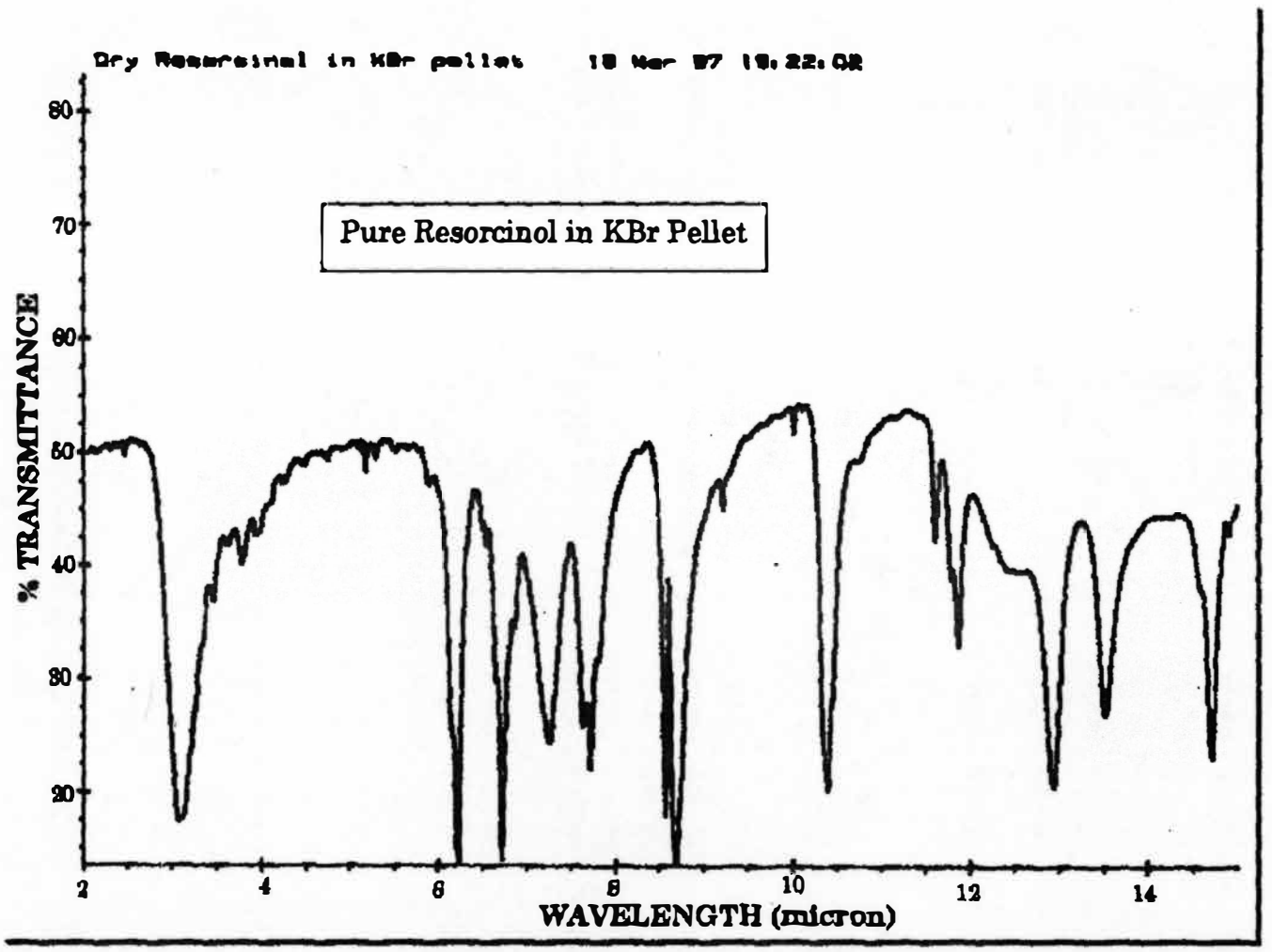


Figure 13A. FTIR Spectrum of Pure Resorcinol .

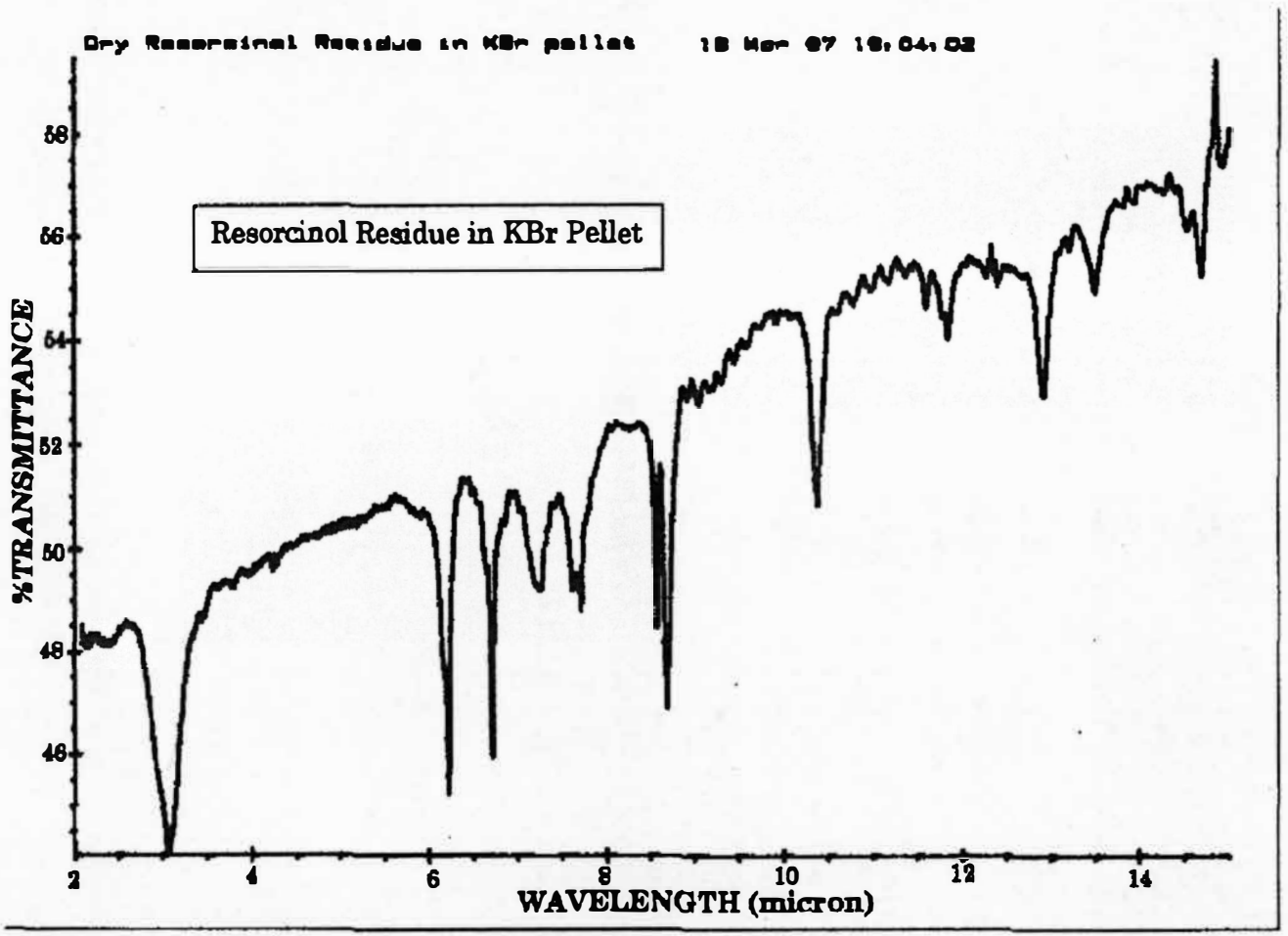


Figure 13B. FTIR Spectrum of Resorcinol Residue Following 24-Hour Treatment with 0.1 M Sodium Hydroxide.

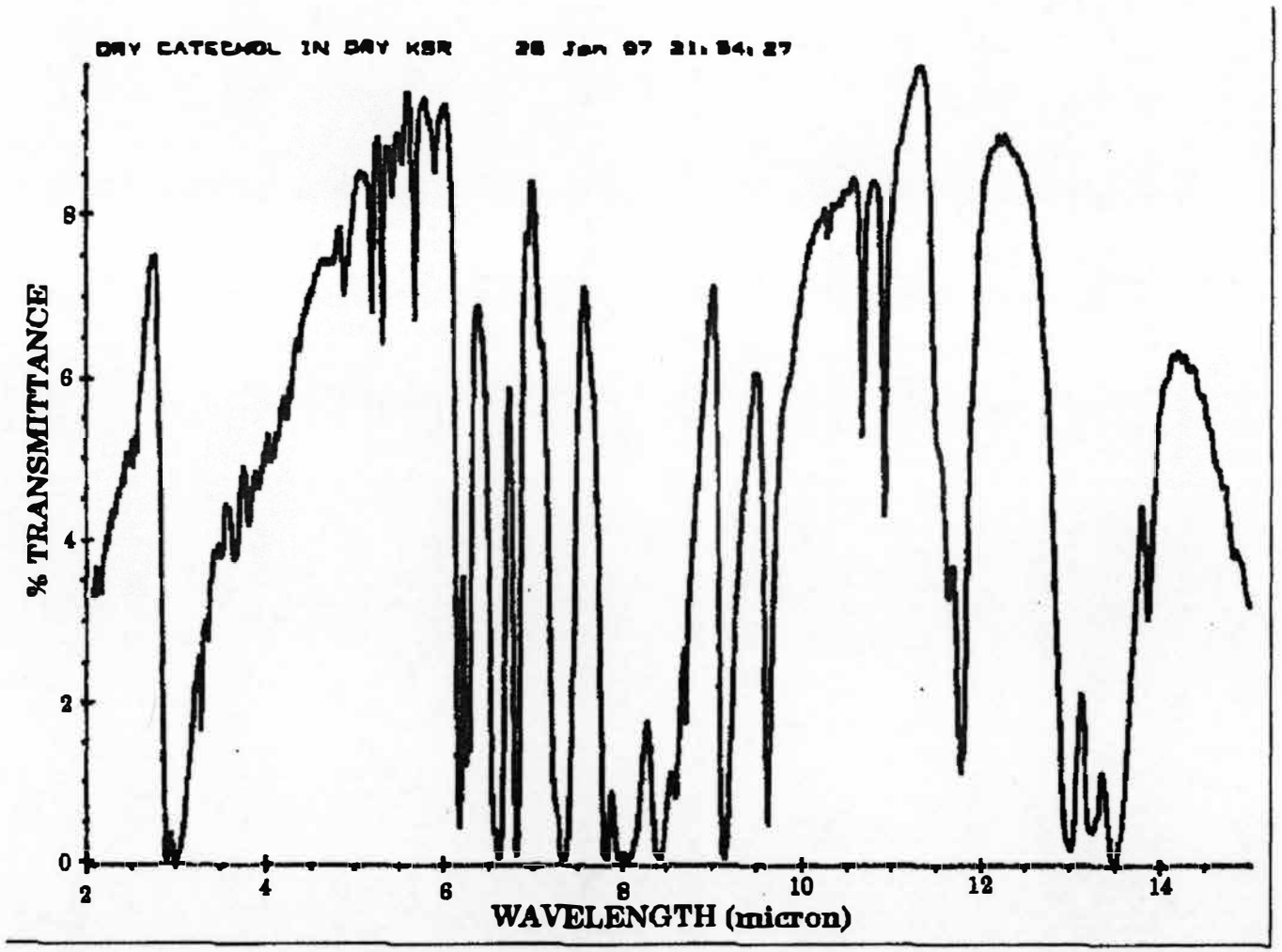


Figure 14A. FTIR Spectrum of Pure Catechol.

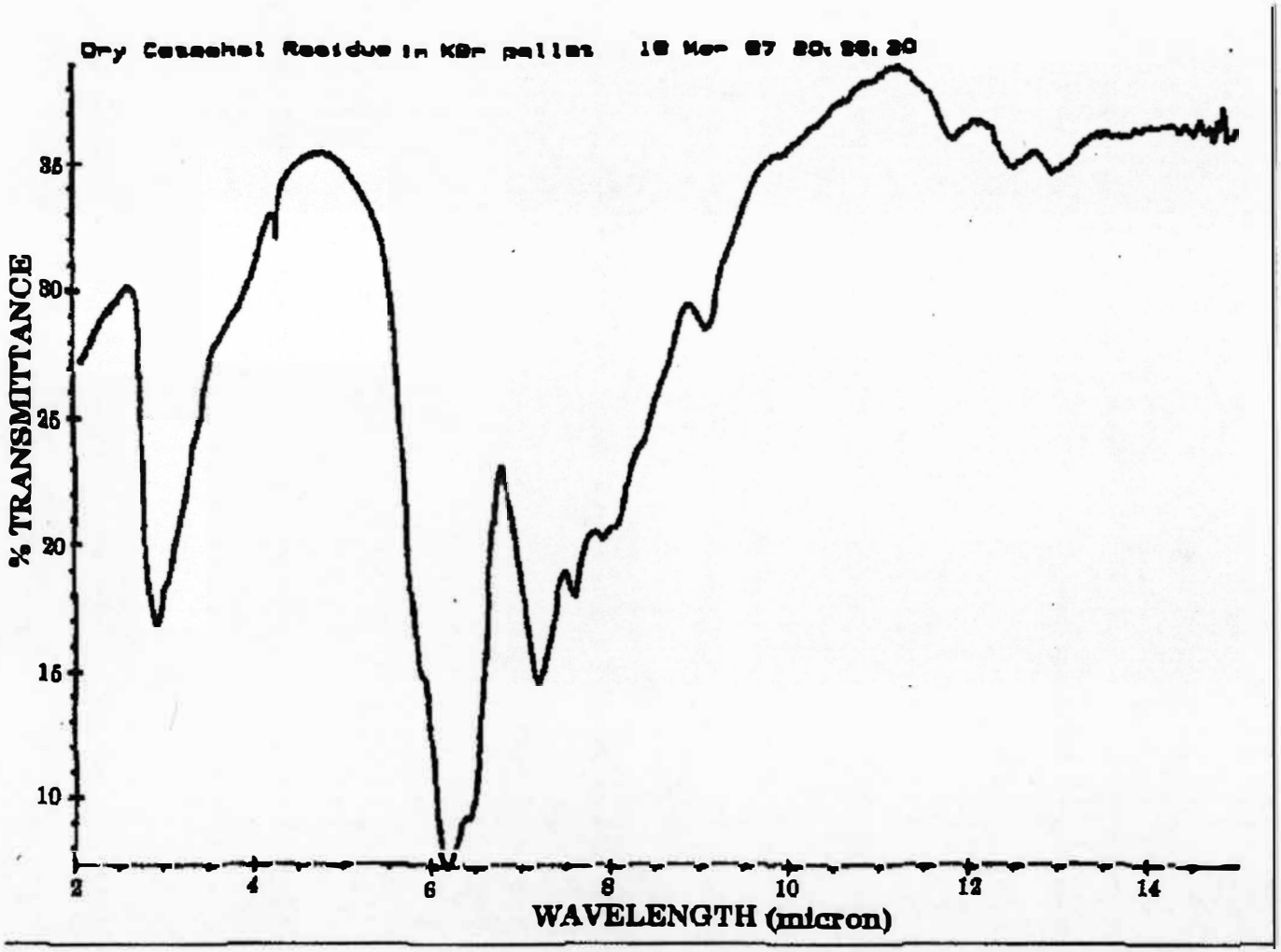


Figure 14B. FTIR Spectrum of Freeze-Dried Catechol Residue After 24-Hour Contact with Sodium Hydroxide.

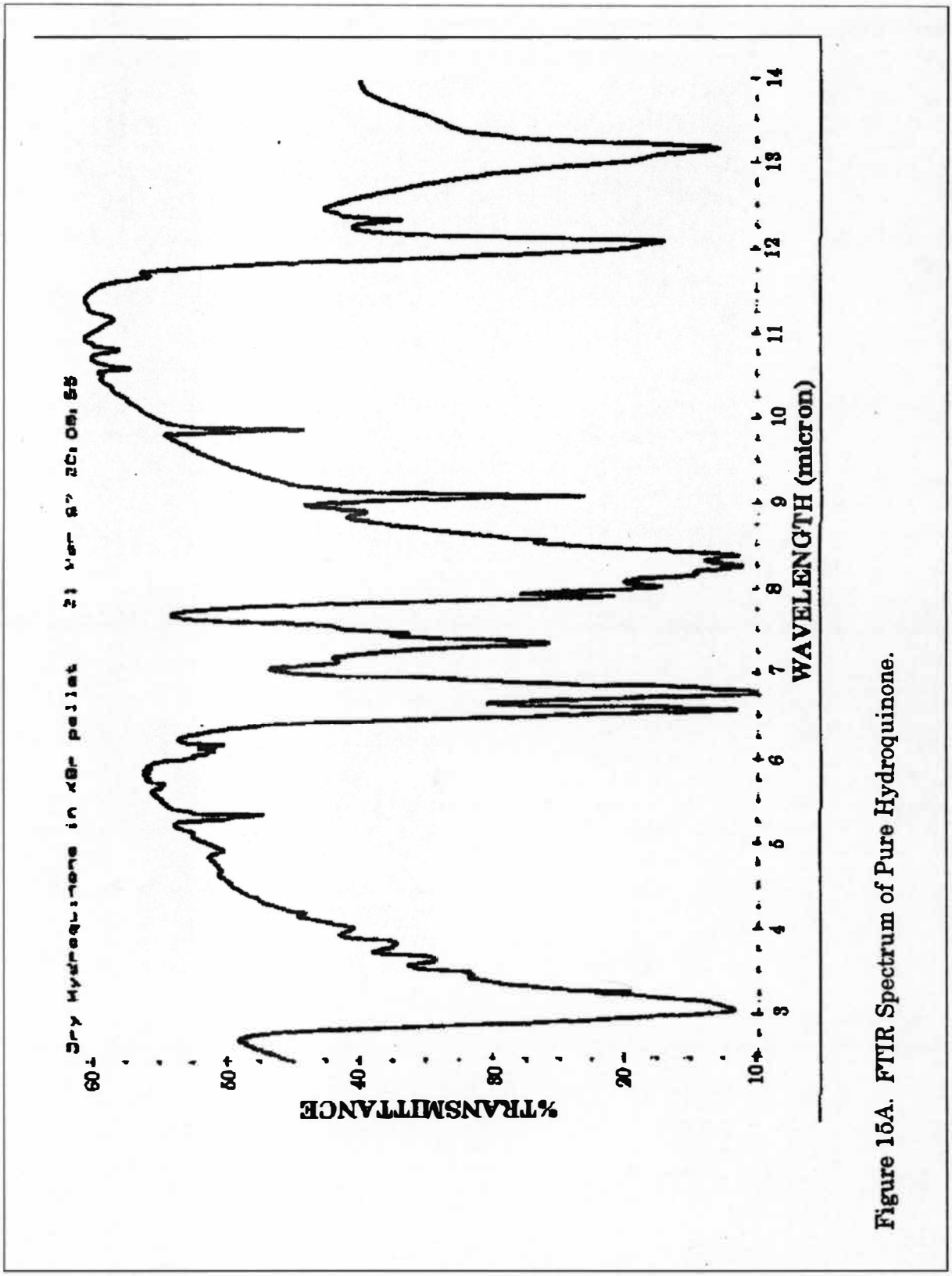


Figure 15A. FTIR Spectrum of Pure Hydroquinone.

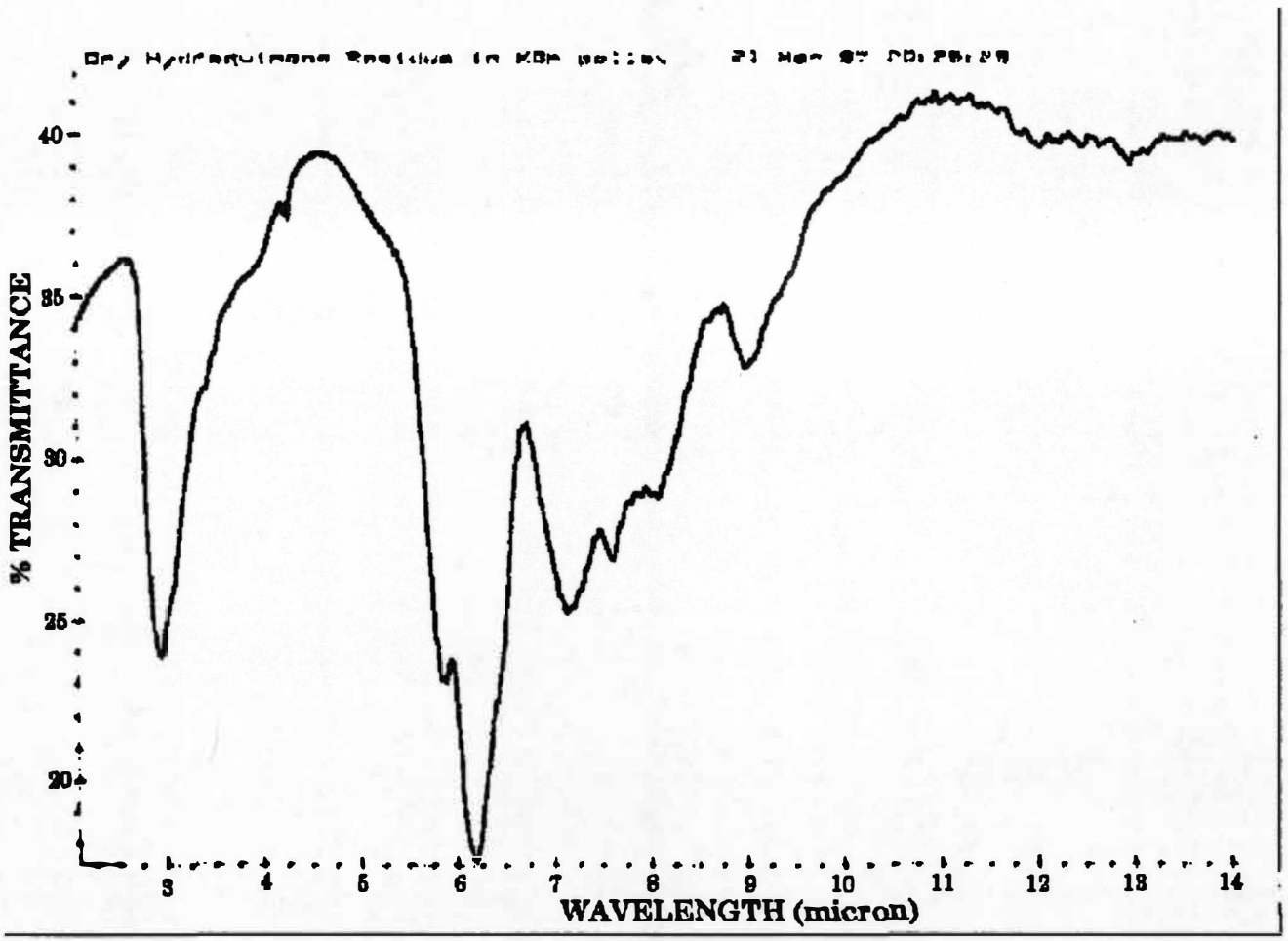


Figure 15B. FTIR Spectrum of Freeze-Dried Hydroquinone Residue After 24-Hour Contact with Sodium Hydroxide.

As expected from the UV-VIS data, this is not the case for catechol and hydroquinone and their respective residues as is well illustrated when comparing sample and residue FTIR scans for catechol in Figures 14A and 14B, and for hydroquinone in Figures 15A and 15B.

Although it is possible to say without a doubt that the FTIR data proves that both the catechol and hydroquinone structures are altered, the scans are not clear enough to determine specific structure changes. This is probably due in part, to the presence of a mixture of various products instead of just one pure compound, but also to the diluting effect of the sodium chloride formed upon neutralization of the sodium hydroxide solution with hydrochloric acid.

Having established that a reaction occurs in the case of the ortho- and the para-dihydroxybenzenes, the research then concentrated in two directions: (1) Attempting to approximate the molecular weight of the products resulting from the reaction and (2) Attempting to separate and gain insight into the possible structure of the above products.

Molecular Mass Considerations

High Performance Liquid Chromatography-Mass Spectroscopy

Catechol and hydroquinone alkali residue solutions were prepared and neutralized to pH 7 for this part of the study. 20 μL of hydroquinone residue and 25 μL of catechol residue respectively were injected into the HPLC-MS system. The mass spectrometer was set to acquire mass data scanning from 80 to 2000 daltons for the catechol injection, and from 100 to 2000 daltons for the hydroquinone injection.

Table 3

Mass Fragments of Greatest Relative Abundance in Catechol Residue

Peak Retention Time	Peak #1	Peak #2
2.77 min	136.5	1221.9
3.34 min	840.8	1424.7
4.67 min	578.7	316.9
6.79 min	735.2	1957.4
7.36 min	655.9	1350.7
8.80 min	249.9	1400.5
9.66 min	1597.5	1573.3
10.87 min	685.5	1500.5
13.57 min	1959.8	1723.6
15.58 min	1036.2	1573.9
15.75 min	1749.1	1722.7
16.38 min	1722.7	1178.5
18.34 min	1736.3	1400.7
18.45 min	1954.7	339.2
20.0 min	1959.5	1573.9

Most HPLC peaks analyzed showed fragments of masses up to and including the high 1900's, some of those including these high mass fragments as their most abundant fragment. Tables 3 and 4 show the masses of the two most abundant fragments for a number of HPLC peaks in the catechol and hydroquinone residue injections, peak 1 representing

the most abundant fragment while peak 2 represents the next most abundant of the two peaks.

Table 4

Mass Fragments of Greatest Relative Abundance in Hydroquinone Residue

Peak Retention Time	Peak #1	Peak #2
20.02 min	518.5	570.2
22.14 min	398.7	802.6
28.86 min	1319.5	1562.2
30.73 min	1484.2	398.7
31.48 min	1889.7	382.9
32.10 min	1430.5	1490.5
32.85 min	1614.7	1387.3

Careful analysis of the data did not reveal the identity of the M+1 peak in the case of either residue injection. It is possible that the complex compounds we are dealing with may have such large masses that their M+1 peak mass would exceed the range at which the mass spectrometer is scanning. Such large compounds are undoubtedly "polymeric" in nature. It is important to remember that the term "polymer" as applied to humic substances does not necessarily refer to the traditional definition of a repeating unit, or "mere", but refers to very large molecules which may contain some repeating fractions and other fractions with similar though

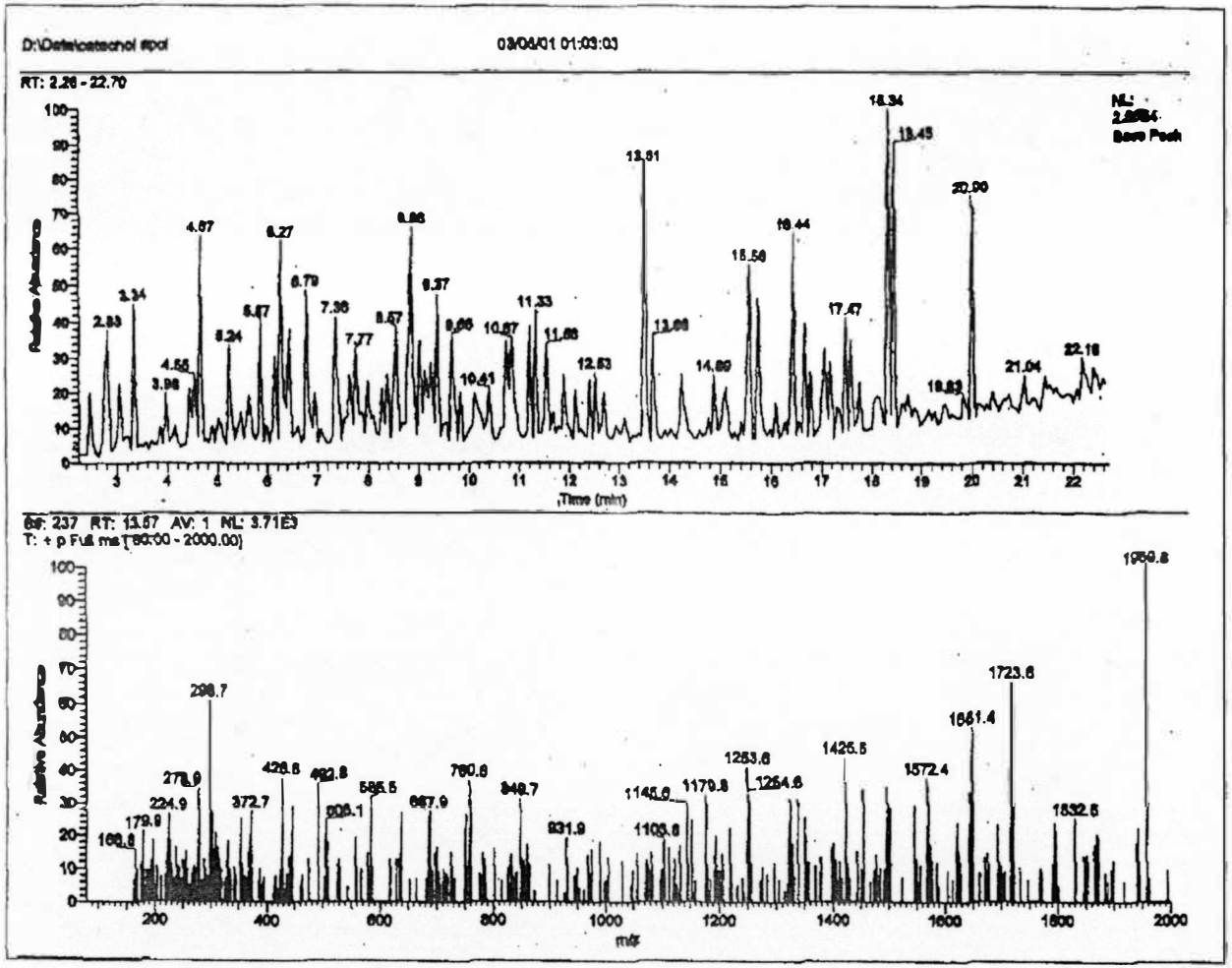


Figure 16A. HPLC-MS Spectrogram of Catechol Residue.

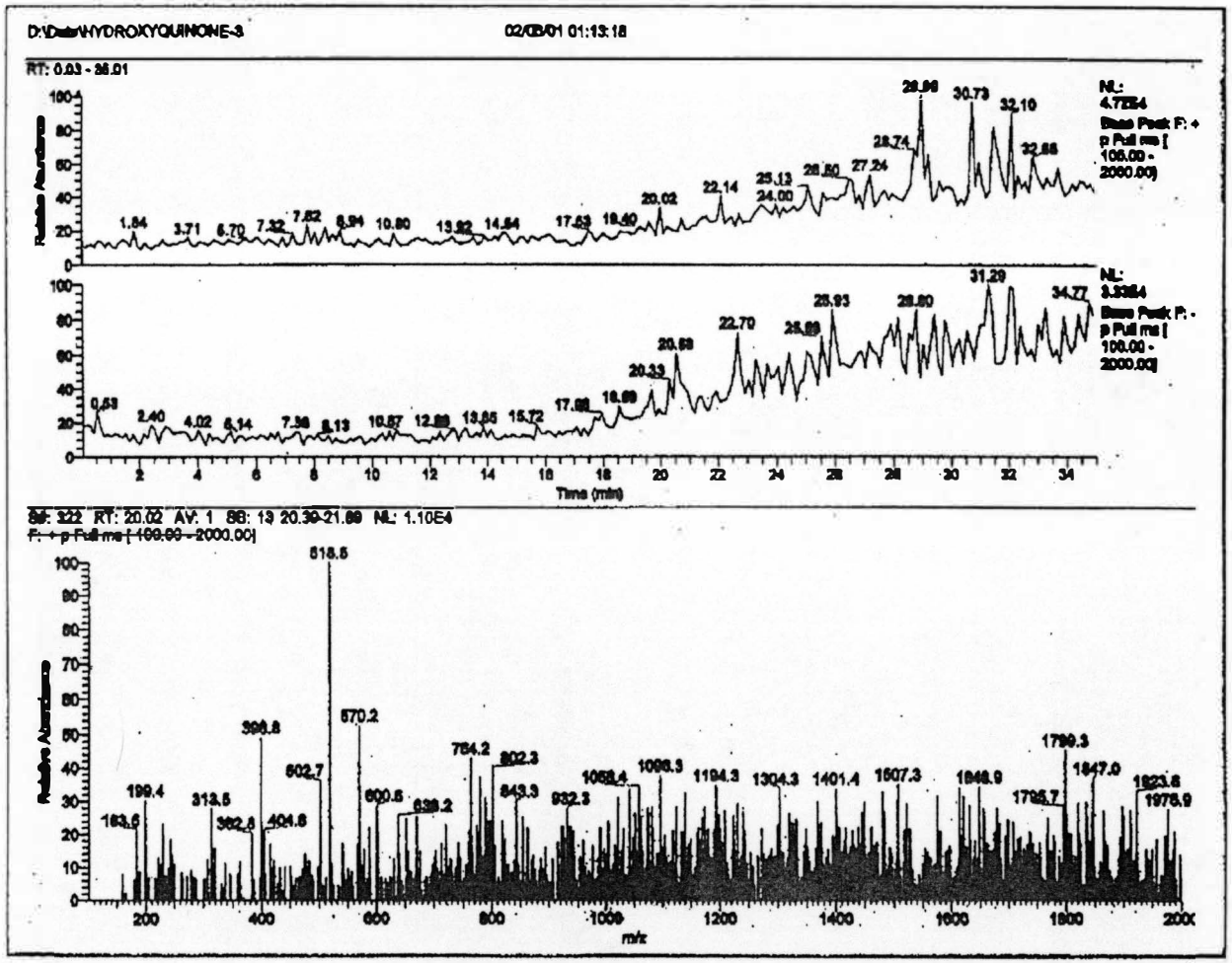


Figure 16B. HPLC-MS Spectrogram of Hydroquinone Residue.

not identical structure. A better term for describing these compounds may be "supramolecule".

Figures 16A and 16B show examples of mass spectrograms from catechol and hydroquinone residues. The catechol example show a very high molecular weight fragment of 1959.8 daltons as the most abundant fragment in the peak eluting at 13.57 minutes. The second most abundant fragment has a mass of 1723.6 daltons. Fragments in this spectrum span the entire mass range. A similar observation can be made regarding the hydroquinone example. Although the most abundant peak is at 518.5 daltons, the spectrum contains peaks of mass as high as 1976.9 daltons.

Gas Chromatography-Mass Spectroscopy

Gas Chromatographic-Mass Spectroscopic data was obtained on the pure benzenediols and also of their respective freeze-dried residues. We attempted to accomplish this by forming and analyzing their more volatile tri-methyl-silyl ether derivatives. Figure 17 illustrates the MS scan obtained from the tri-methyl-silylation of pure catechol. It clearly shows a prominent peak at 73 daltons, indicative of the trimethylsilyl group and also the parent peak at 254 daltons of the trimethylsilyl ether of catechol.

The GC-MS data was expected to confirm the HPLC-MS data and show that high molecular weight compounds are formed during the reaction. A drawback of this method is the extra mass added to each molecule in an effort to achieve volatility. Indeed, each trimethylsilyl group replacing the hydrogen atom in the hydroxyl group adds 73 daltons to the molecule.

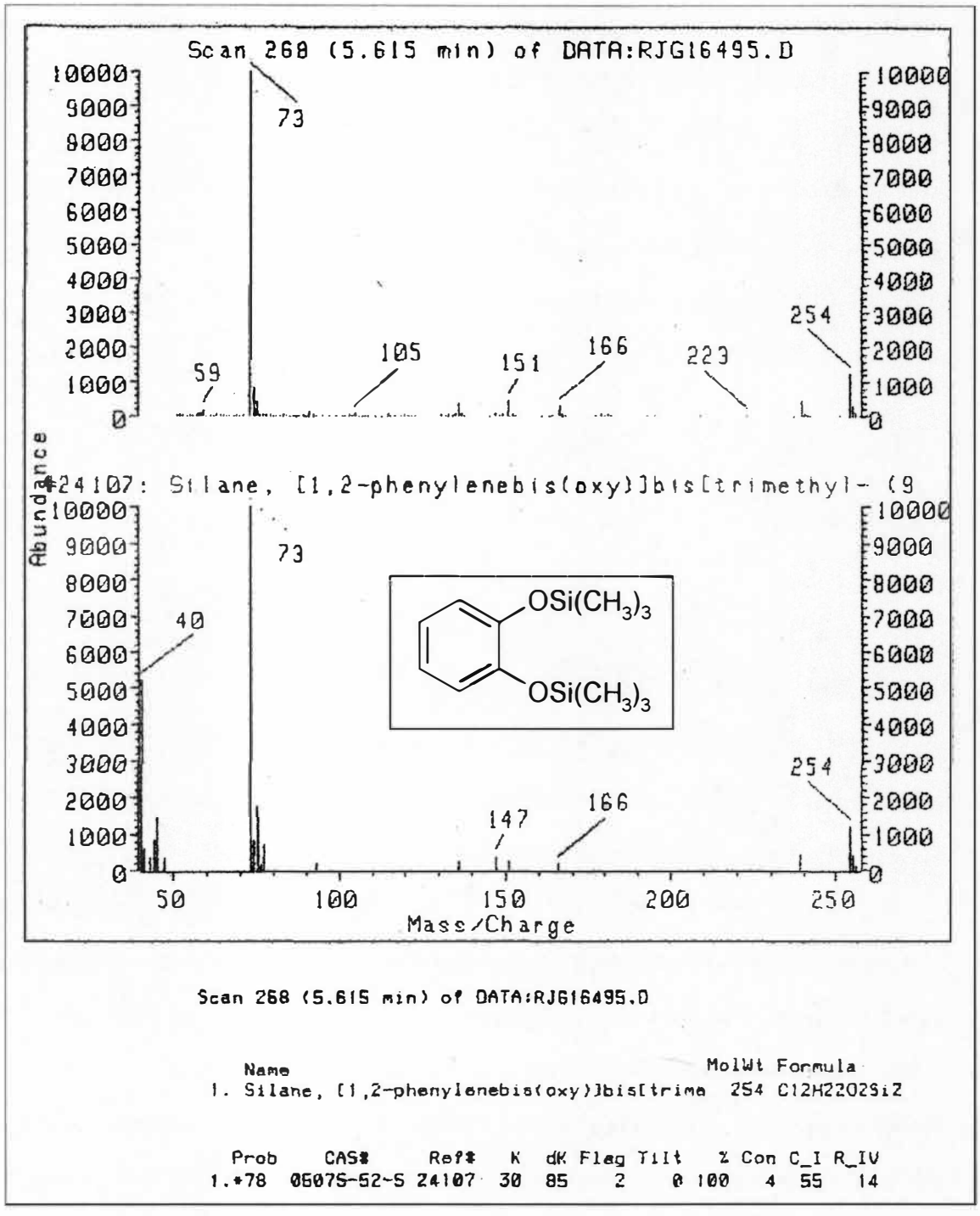


Figure 17. MS Scan of Tri-Methyl-Silyl Ether of Catechol.

The large molecules shown to exist from HPLC-MS data would attain huge masses when derivatized and might actually be too large to be volatile at all. In fact, the Pellizzare method reported successful formations of derivatives with parent ion peaks of only 940 daltons (Pellizzare et al, 1969). With molecules forming fragments in the 1900's in mass, it is entirely possible that they would not be volatile even after trimethylsilylation. This may be why we were not able to detect such peaks.

Structural Considerations

Proton NMR Spectroscopy

Fractions A, B, C and D exhibit a lot of peak activity in the range of 0.8 and 3.0 ppm. See Figures A-2 through A-4 in the appendix. This may provide evidence of the appearance of aliphatic character introduced in the polymeric product, thus indicating that ring cleavage takes place to a certain extent during the reaction.

The region around 6 to 7 ppm also contains a large number of multiplets indicating that the aromatic character is retained as well and that many non-equivalent protons are present. This supports the theory of new bonding creating different environments adjacent to the various C-H units. Good examples of this are found in Figures A-5 through A-7 in the appendix.

Peak multiplets in the region around 3 to 4 ppm are indicative of the presence of hydroxyl groups that may still be intact from the original

alcohol groups of the catechol. Such peaks appear in all the NMR scans in the appendix (See Figures A-2 through A-7).

Due to the complex nature of the polymeric compounds we expect that we are dealing with (See Figures 1 and 2 as examples), it is difficult to make further predictions from the NMR data. As a direct result of the type of reaction we are investigating and the multitude of different products possible, there is a real probability that the PREP-HPLC fractions are not pure, but may contain many compounds, thus complicating further the specific identification of the structures involved.

FTIR Spectra of Prep-HPLC Fractions (Silverstein, 1974; Young, 1995-6)

The infrared spectrum of freeze-dried prep-HPLC fraction "Tubes 36-37" (Figure 17) displays a broad, intermolecularly hydrogen-bonded -OH stretching band in the region of about 3400 cm^{-1}, a sharp C-H stretch at 2849 cm^{-1} attributable to a R-CH_2-OH structure and another sharp C-H stretch at 2913 cm^{-1} attributable to a R-CH_2-OR structure.

Absorptions caused by aromatic C=C stretches occur around 1622 cm^{-1}, thus confirming the retention of aromatic character. However, the appearance of aliphatic character is also indicated by a couple of peaks at 1379 cm^{-1} and 1476 cm^{-1} attributable to C-H bending in CH_2 and CH_3.

Ether linkage is evident in a couple of peaks indicating C-O-C symmetrical stretch at 1076 cm^{-1} and C-O-C asymmetrical stretch at 1258 cm^{-1}, typical of aryl-alkyl ethers.

An ester carbonyl stretching band can be seen at about 1706 cm^{-1}, shifted slightly to lower wavenumber than normally observed, possibly due

Table 5

FTIR Peak Frequencies and Respective Assignments for Prep-HPLC Fraction "Tubes 36-37"

Peak Frequency (cm^{-1})	Vibration Type	Indication
~716	CH_2 Rock	Aliphatic Chain
~790	Not assigned	None
~899	Not assigned	None
~1076	C-O-C Sym. Stretch	Aryl-Alkyl Ether
1185, 1209	Not assigned	None
~1258	C-O-C Asym. Stretch	Aryl-Alkyl Ether
~1379	C-H Bend in CH_2 and CH_3	Aliphatic Chain
~1476	C-H Bend in CH_2 and CH_3	Aliphatic Chain
~1622	Aromatic C=C Stretch	Aromatic Ring
~1706	C=O Stretch	Ester Carbonyl
~2849	C-H Stretch	Alcohol (R-CH_2-OH)
~2913	C-H Stretch	Ether (R-CH_2-OR)
3400-3100	O-H Stretch, H-Bonded	Alcohol –OH

to conjugation involving aromatic rings.

Table 5 lists the frequencies of the various absorption peaks observed in the freeze-dried prep-HPLC fraction labeled "Tubes 36-37" and

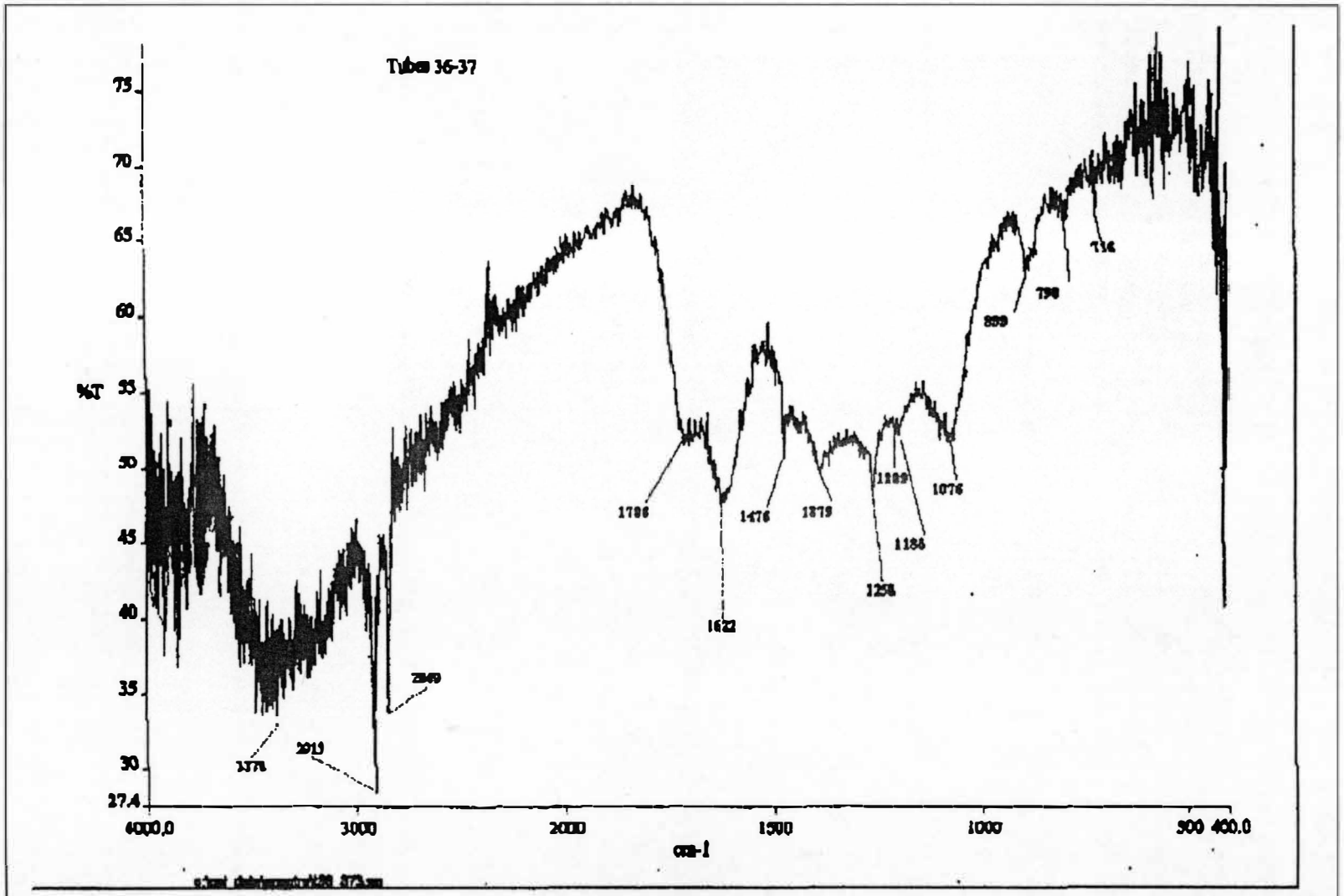


Figure 18. FTIR Spectrum of Prep-HPLC Fraction "Tubes 36-37".

their respective assignments in the spectrogram (Figure 18). Other Preparatory-HPLC fraction FTIR spectrograms are found in Appendix B.

CHAPTER V

CONCLUSIONS

Motivation for the Study

The purpose of this thesis was to investigate the alkali extraction method used by the International Humic Substances Society since 1981 to isolate "standard humic substances" from soil for use in research. We do not question that humic substances exist in the environment as very large molecular weight compounds. In fact, the alkali extraction method may have been one of the best methods available for humic substance isolation from soils at that time (1981). We set out to show that phenolic molecules that are present in the environment do have a real chance of reacting and polymerizing into larger supramolecules when treated with alkali as in the IHSS method.

Experimental Observations and Discussion

Catechol itself is a product of the demethylation of the phenolic methoxyl groups present in lignin (Kirk et al, 1978). It is also formed by the ortho-position hydroxylation of simpler phenols by polyphenol oxidases, enzymes present in plants (Mayer and Harel, 1979).

By subjecting model phenolic compounds (catechol, resorcinol and hydroquinone) to the IHSS alkali extraction method for 24 hours, we were able to show from FTIR data that structural changes occur in the cases of

catechol and hydroquinone, while no change took place in the case of resorcinol.

Mass spectral data obtained with the help of a HPLC-MS system indicated that the smaller phenolic compounds do polymerize into many larger molecules, some of which produced fragments of masses up to more than 1900 daltons. Indeed, Stevenson(1965) had warned of the possibility of artifact production during the alkali extraction of humic substances.

While it was not the goal of this study to propose a mechanism, we believe that the mechanism involved could be similar to previously published mechanisms. It is possible that this polymerization could take place by way of formation of a quinone-type intermediate (Figures 20 and 21)and also quite probably by a radical-involving pathway such as the one proposed by Larson(1980) (Figure 19). This would be consistent with the Polyphenol Model of humic substance formation outlined in Chapter II. Figure 21 shows the formation of a hydroquinone dimer in a polymerization pathway for hydroquinone (Hocking and Intihar, 1985). Our GC-MS data suggestive of such a dimer (See Appendix C) also supports this type of mechanism although we do not know conclusively the exact nature of the radical involved.

Since our reaction vessel was kept in the dark, we cannot invoke a photochemically induced process. Radicals, however, could be produced in other ways. MilliQ water was used in this study and has been shown to contain 8ppm O_2 (Xu, Ling X., 1994). Although efforts were made to preclude any additional oxygen from entering our reaction system (blanket of inert gas such as N_2 or Ar), no attempt was make at removing the oxygen present in the MilliQ water by degassing. Oxygen has been

reported to produce superoxide radical anions as a result of oxidation-reduction reactions with aromatic ions, as in the case of the herbicidal

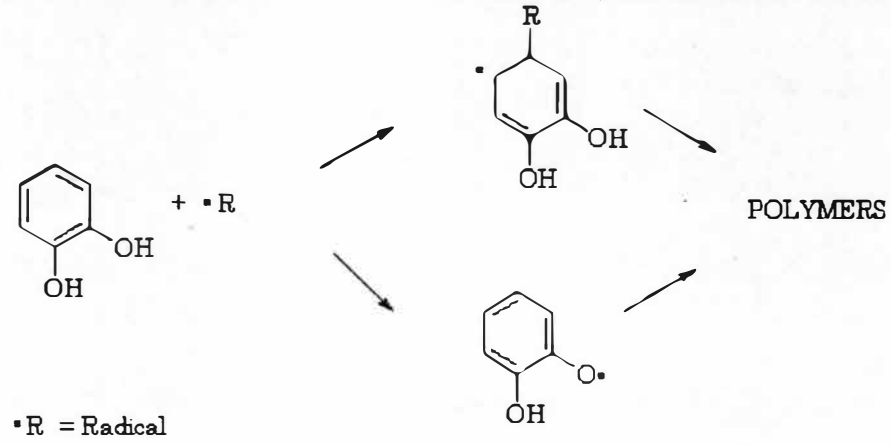


Figure 19. Modes of Attack by a Radical on Catechol. (Larson, 1980).

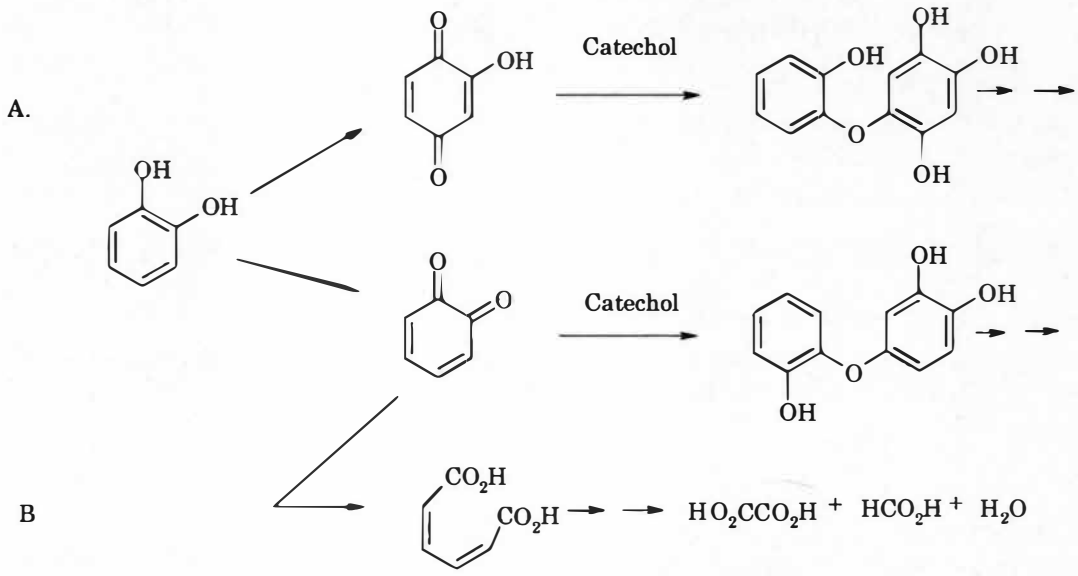


Figure 20. Catechol pathways to (A) Polymerization and (B) Aliphatic Character Formation and Ring Cleavage. (Hocking and Intihar, 1985).

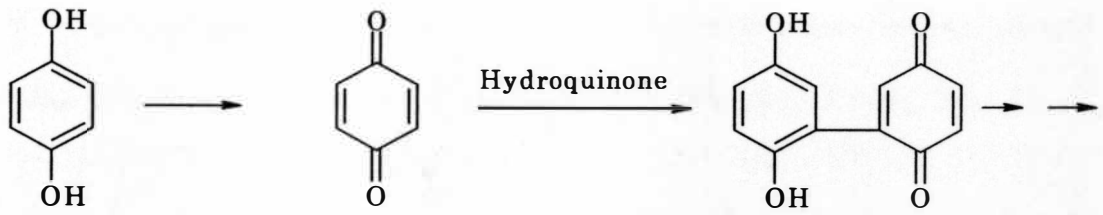


Figure 21. Hydroquinone Pathway to Polymerization. (Hocking and Intihar, 1985).

bipyridinium Paraquat (Parsons, 2000) (Figure 22). As in Figure 22, the dissolved oxygen in our solution could abstract an electron from the reduction reactions with aromatic ions, as in the case of the herbicidal bipyridinium Paraquat (Parsons, 2000) (Figure 22). As in Figure 22, the dissolved oxygen in our solution could abstract an electron from the electron-enriched phenoxide-type anion and produce a superoxide radical anion. This type of radical could react both as a radical and as an anion. Once produced at our pH conditions, it would exist in solution as $O_2\bullet-$, as the pK_a of the peroxyl radical, •O-O-H, is 4.75 (Bielski and Allen, 1977). As such, it can react with catechol or hydroquinone by abstracting a hydrogen atom (proton and electron) or oxidizing the molecule.

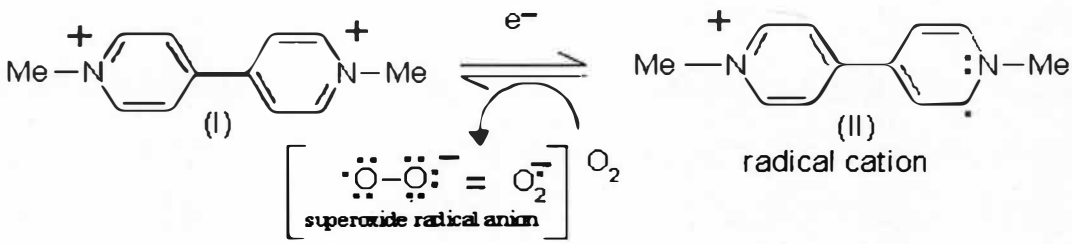


Figure 22. Superoxide Radical Anion Forming Paraquat Mechanism. (Parson, 2000).

Metal cations such as Fe^{3+} are so ubiquitous that they might be present and playing a role contributing in the polymerization process by initiating a radical production process. Indeed, the 50% w/w Sodium Hydroxide solution used in this study contains up to 3 ppm Ag and 3 ppm Fe. Hocking and Intihar (1985) reported that Fe^{3+} cations can form complexes with Catechol in solution. These can undergo an internal oxidation-reduction reaction with an electron transfer from the catechol to the Fe^{3+}, thus yielding Fe^{2+} and a catechol radical.

If these radicals were to be formed, then the resulting quinones could be formed and the reaction proceed by Larson's and Hocking and Intihar's polymerization mechanisms. We do not however have concrete evidence of the presence of these radicals in our solutions.

Final Remarks and Recommended Further Studies

We have shown in this thesis that there is a real possibility of polymerization of phenolic compounds naturally occurring in soils when these soils are subjected to the IHSS humic substances alkali extraction method used to isolate "standard humic substances" from soils. We showed that polymerization does occur in model compounds catechol and hydroquinone, but not in resorcinol. We believe that this is because the meta position inhibits formation of the quinone intermediate.

Our research does not conclusively answer if oxygen and iron are playing a role in radical production or if there is another process at work. This could be investigated by removing oxygen (degassing) or increasing oxygen (bubbling through oxygen). Another approach to understanding the possible role of oxygen in the polymerization process would be to

monitor the dissolved oxygen concentration during the process. Purposefully introducing known concentrations of Fe^{3+} into the system could allow us to monitor what happens and shed some light on the metal cation's role in the polymerization process.

A very unexpected and also very important outcome of our research is that a significant amount of aliphatic character was created during the polymerization process. This is clearly shown in our ^{1}H-Nmr data (great amount of activity in the 0.8 to 3.0ppm region) and also supported by our FTIR data. We would rather have expected highly aromatic polymers to be formed. We cannot explain why this happens nor the mechanism involved in this process.

We also show definite evidence of hydroxyl groups in our products (^{1}H-Nmr and FTIR). Are we forming poly-hydroxyl cyclic aliphatic compounds, are we cleaving the rings or is something else happening? This is an area that definitely necessitates further research to understand the forces at work. A time series of experiments (1, 5 and 10 hours of reaction, for example, instead of the full 24 hours) could be undertaken with Prep-HPLC fractions obtained from neutralized reaction solutions and characterized in the same manner as in this study. ^{13}C-Nmr could be used on the most abundant fractions as well.

No matter which mechanism(s) is/are at work in our reaction system, resorcinol would be the key compound in any further studies to investigate the mechanistic pathways involved. It has been shown to resist great change even after 24-hour sodium hydroxide treatment. This can be due to the meta substitution inhibiting quinone intermediate formation as increased stability brought about by the delocalized electron

is not achieved by resorcinol as in the cases of catechol and hydroquinone.

A small malformed shoulder was noticed after 24 hours in the preliminary UV spectrum. We do not know if this could or could not be the beginning of some change for resorcinol. Could this change perhaps exhibit much slower kinetics than in the cases of the other two positional isomers? This question could perhaps be answered by investigation resorcinol's behavior for periods of, for example, days or even weeks.

In summary, we can state that our research accomplished its goal of showing that there exists, in the IHSS humic substances alkali extraction procedure, a real possibility of forming polymerization artifacts as well as extracting existing compounds. This research also opened up the following three major questions: (1) What are the roles, if any, of dissolved oxygen and possible dissolved metal cations in our solutions? Are they instrumental in radical initiation, if we have radicals at all?; (2) Where does all that aliphatic character come from? Is it the result of ring cleavage, or is it the result of saturation of the aromatic double bonds? What happens to most of the aromatic character we would normally expect in this type of reaction?; and (3) Is resorcinol really completely resistant to this harsh treatment, or are the kinetics of its change much slower than that of the other two positional isomers? Does resorcinol resist change because the quinone intermediate is inhibited, as we suspect, or is there another mechanism at work? We propose that the search for answers to these questions could be the focus of future research in this area.

Appendix A

Proton NMR Spectra of Freeze-Dried Prep-HPLC Fractions of Catechol Residue

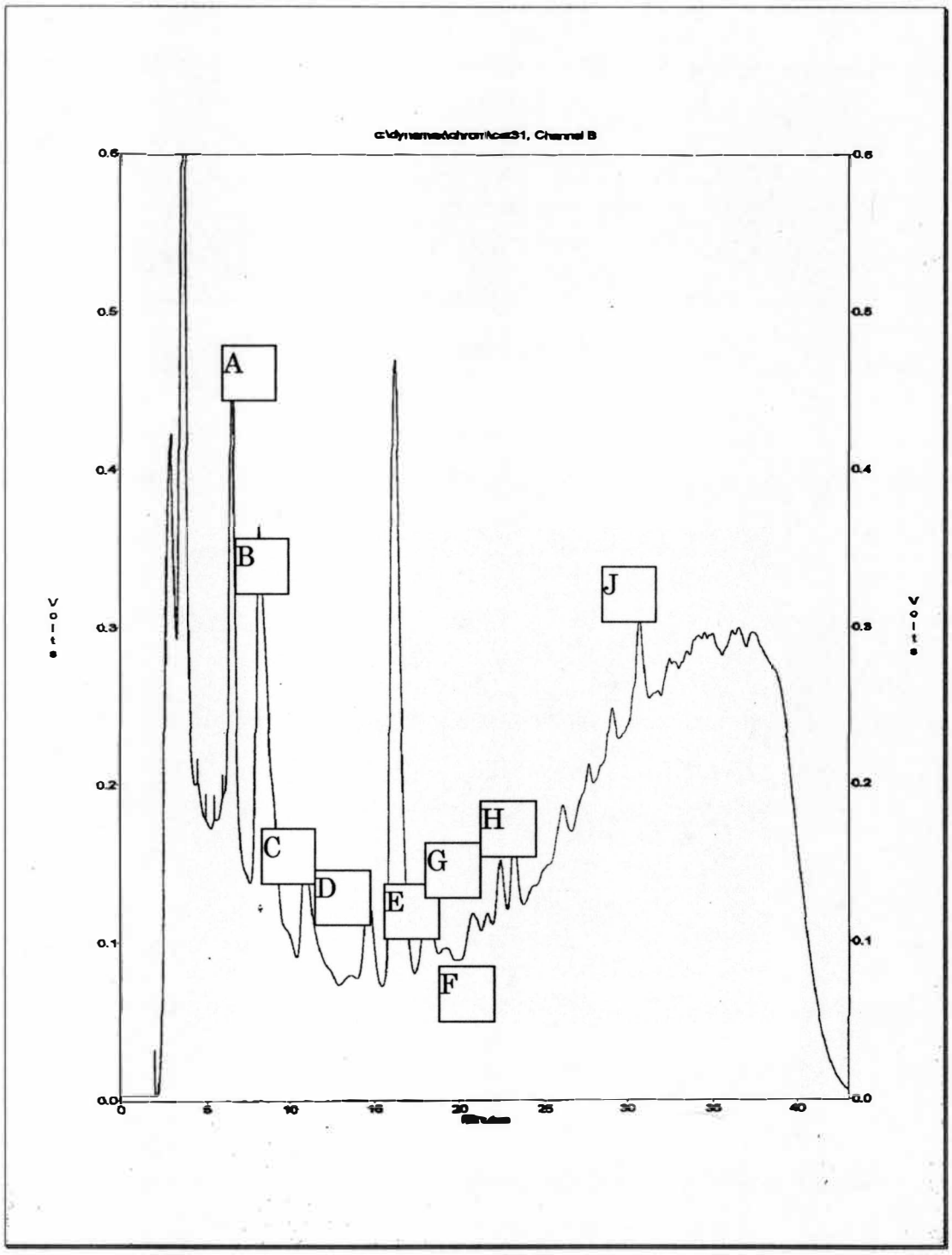


Figure A-1. Prep-HPLC Chromatogram Showing Named Fractions Used in ^{1}H-NMR Spectroscopy.

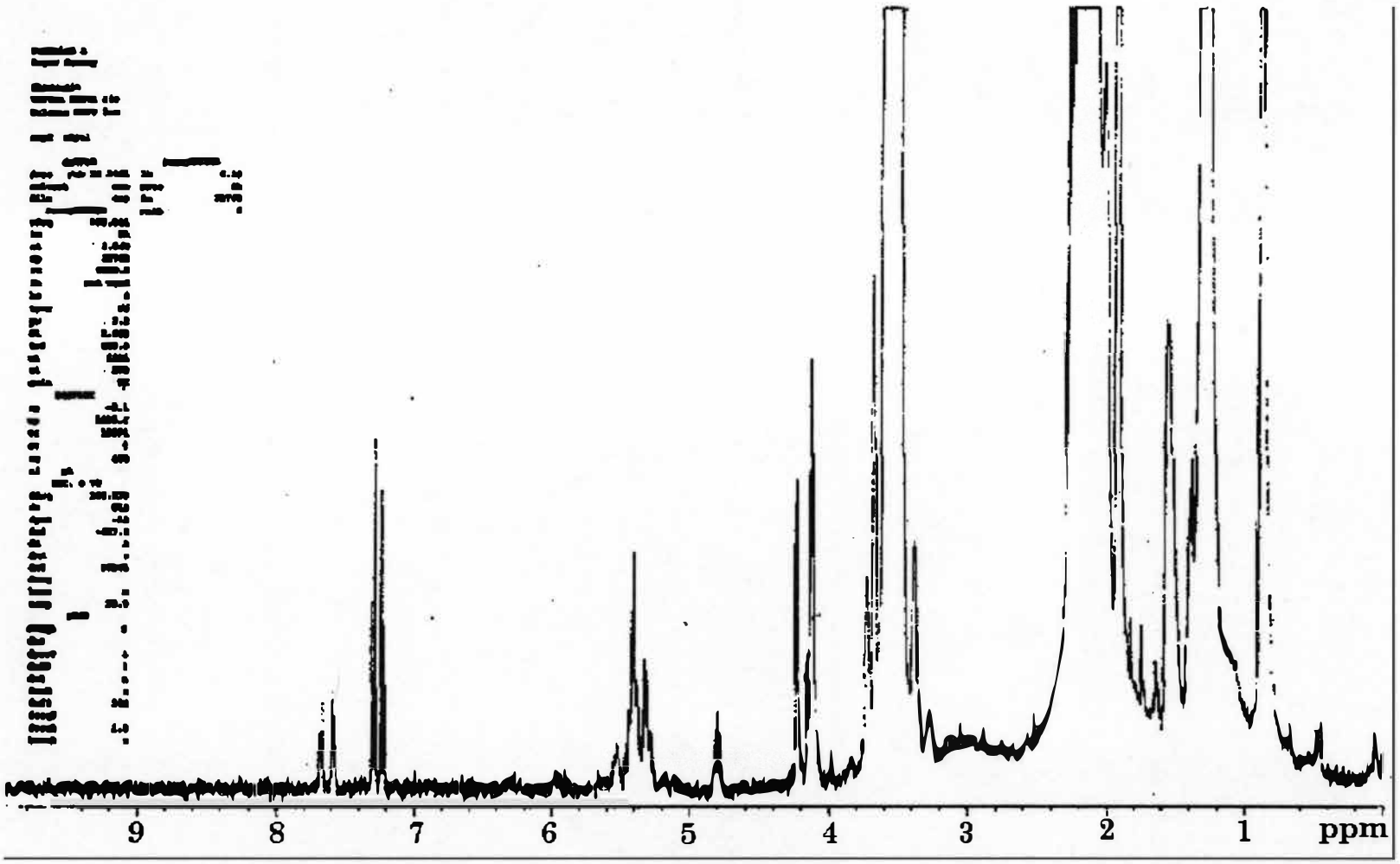


Figure A-2: ^{1}H NMR Spectrum of Fraction A Oxidation/Polymerization Product of Catechol.

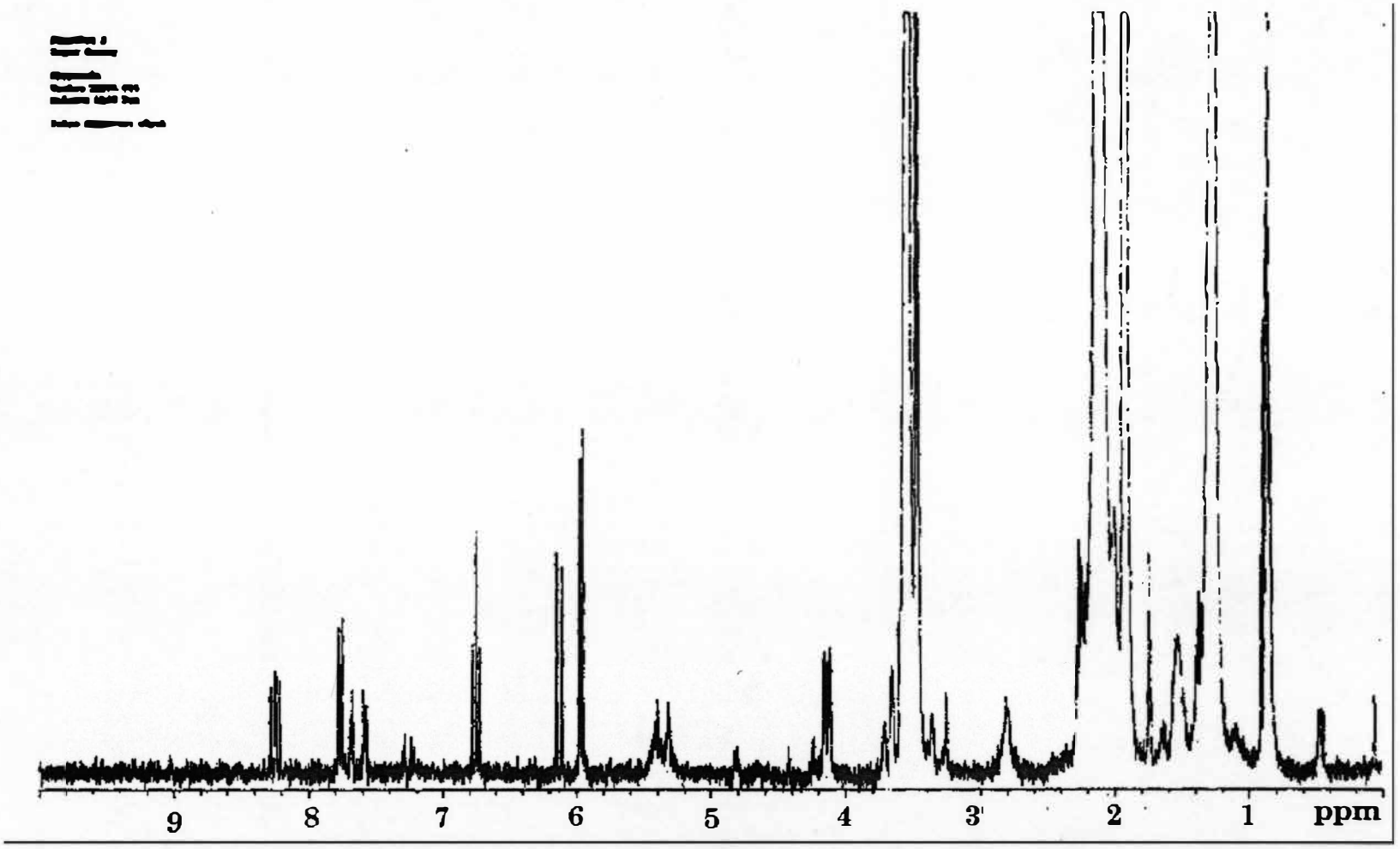


Figure A-3. ^{1}H NMR Spectrum of Fraction B Oxidation/Polymerization Product of Catechol.

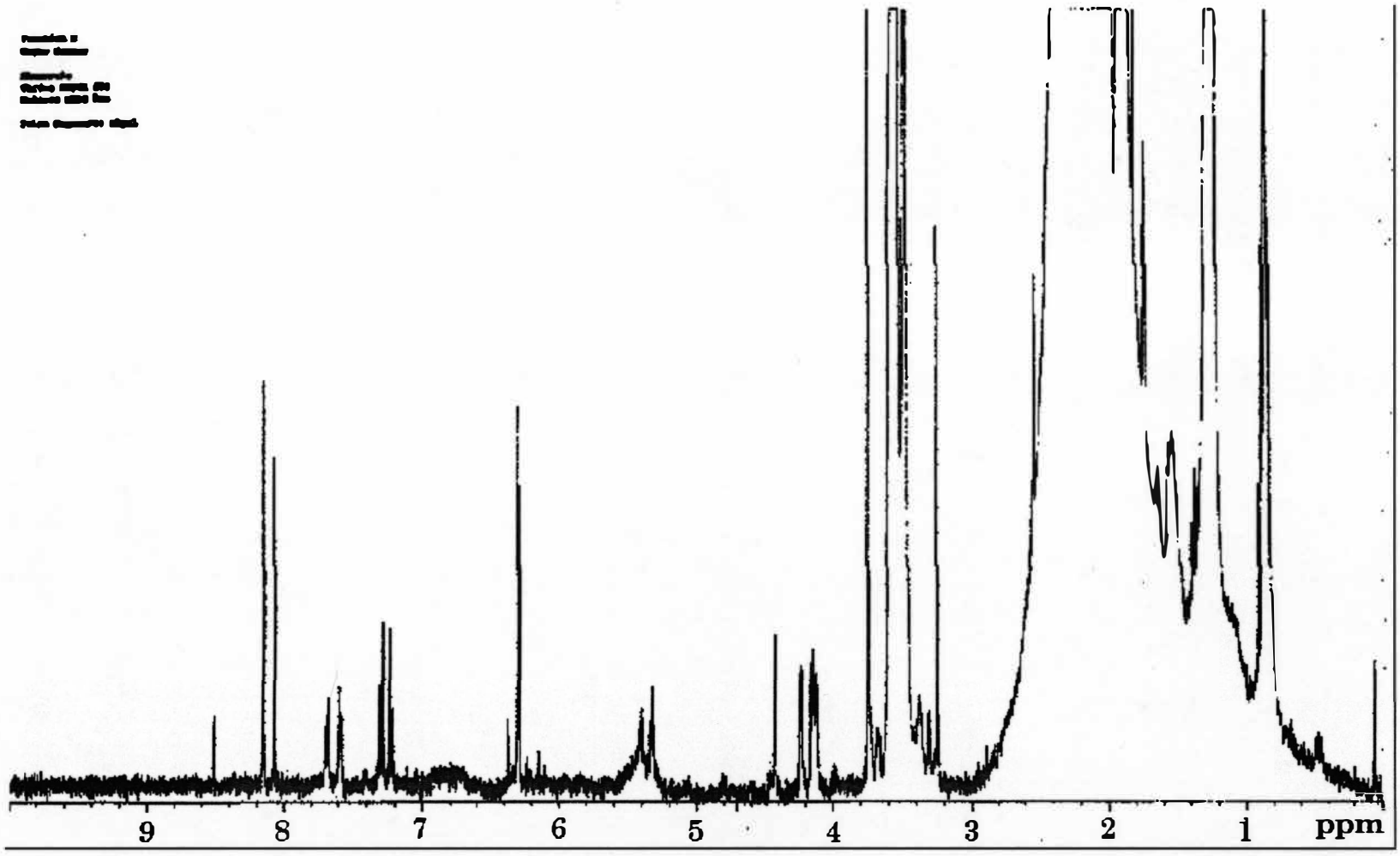


Figure A-4. ^{1}H NMR Spectrum of Fraction C Oxidation/Polymerization Product of Catechol.

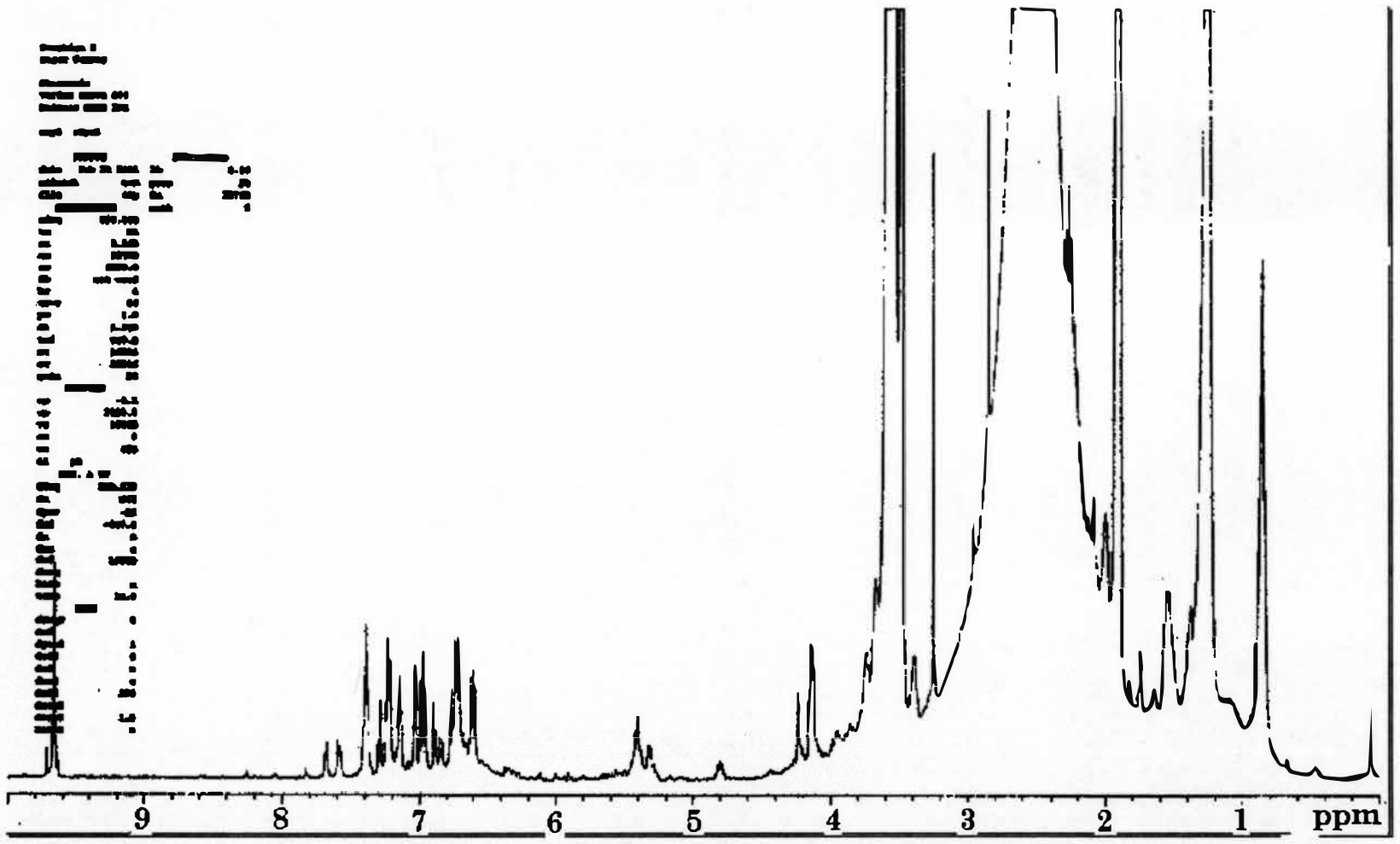


Figure A-5. ^{1}H-NMR Spectrum of Fraction D Oxidation/Polymerization Product of Catechol.

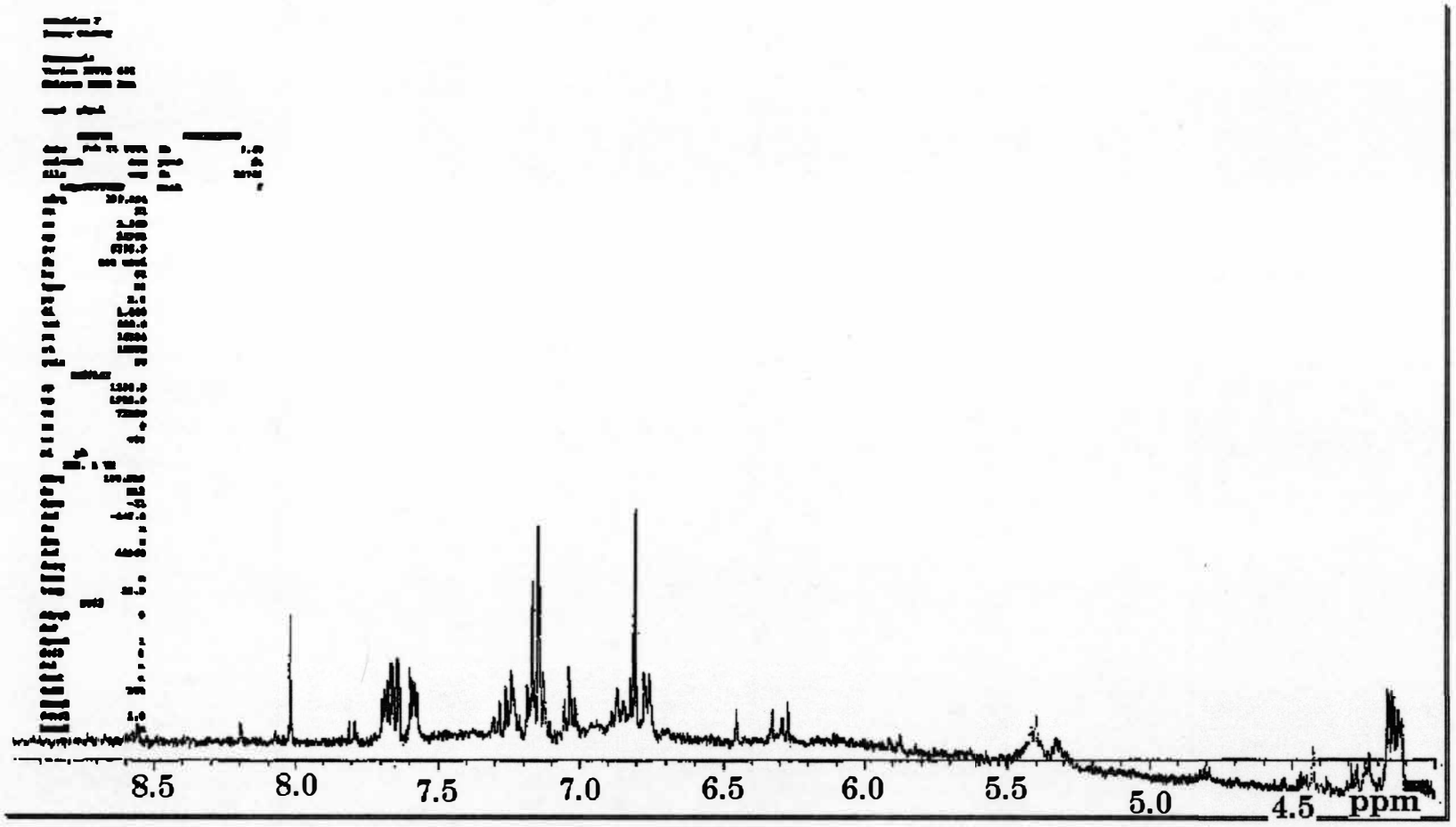


Figure A-6. ^{1}H-NMR Spectrum of Fraction F Oxidation/Polymerization Product of Catechol.

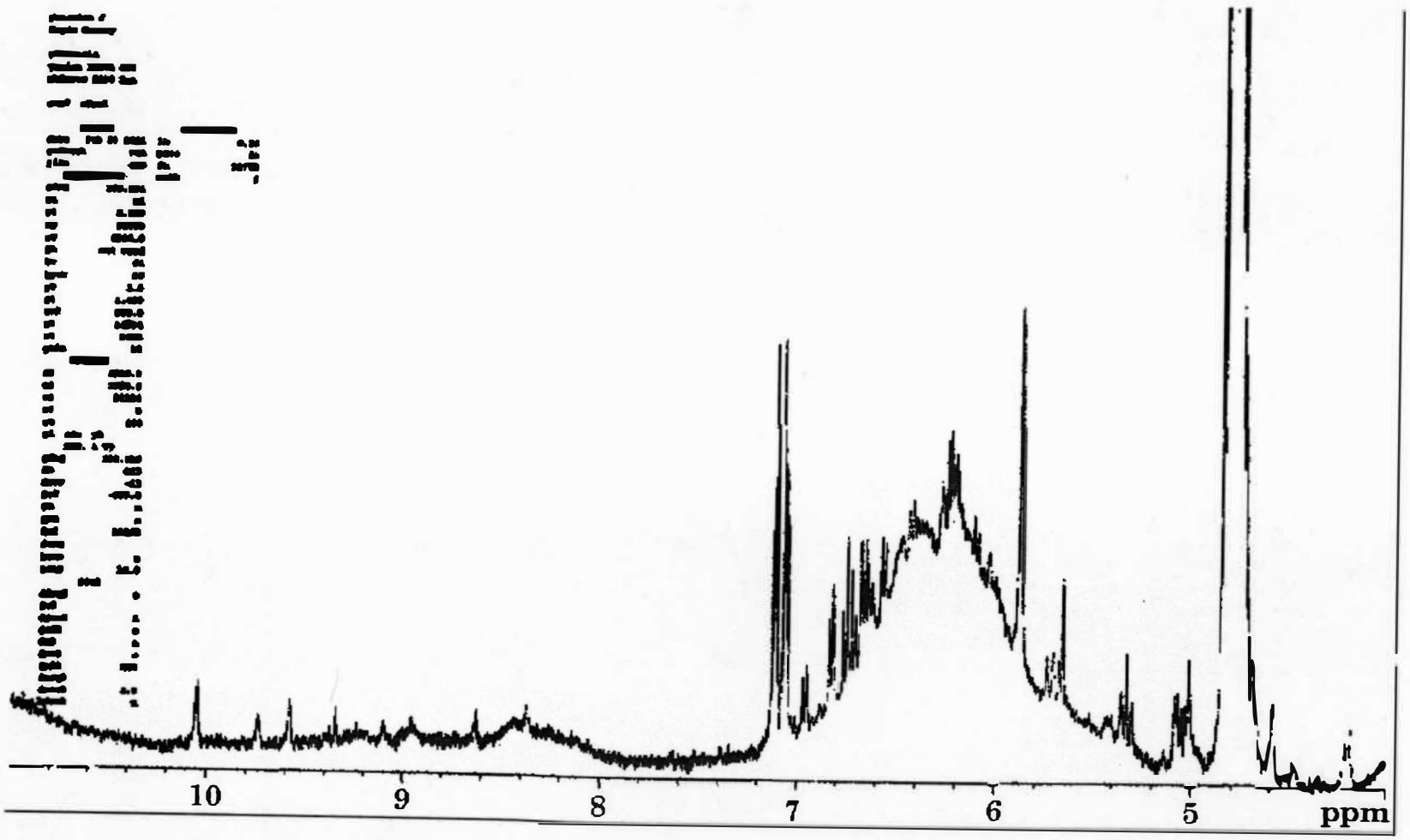


Figure A-7. ^{1}H NMR Spectrum of Fraction J Oxidation/Polymerization Product of Catechol.

Appendix B

Fourier Transform Infrared Spectra of Preparatory HPLC Fractions

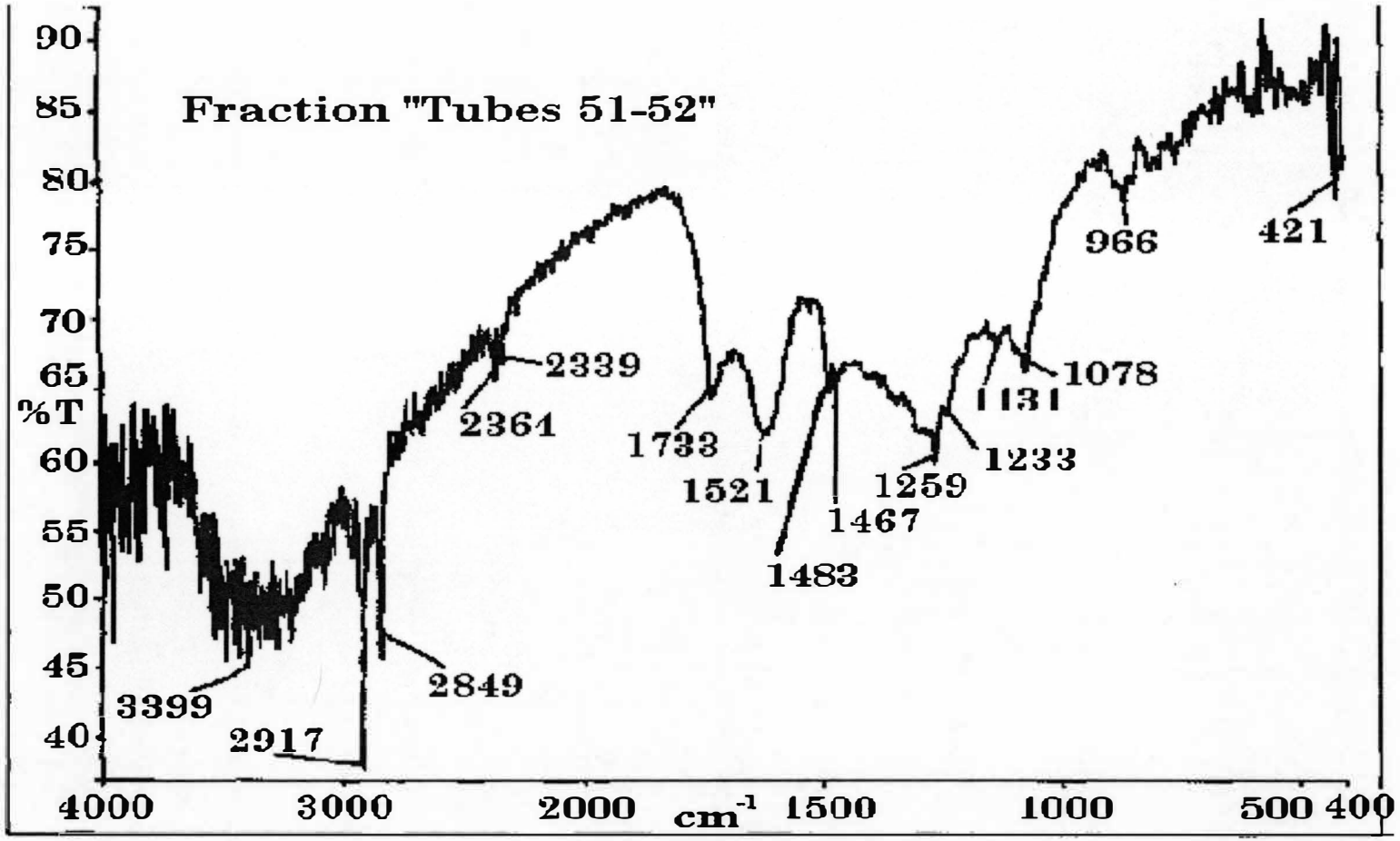


Figure B-1. FTIR Spectrum of Prep. HPLC Fraction "Tubes 51-52".

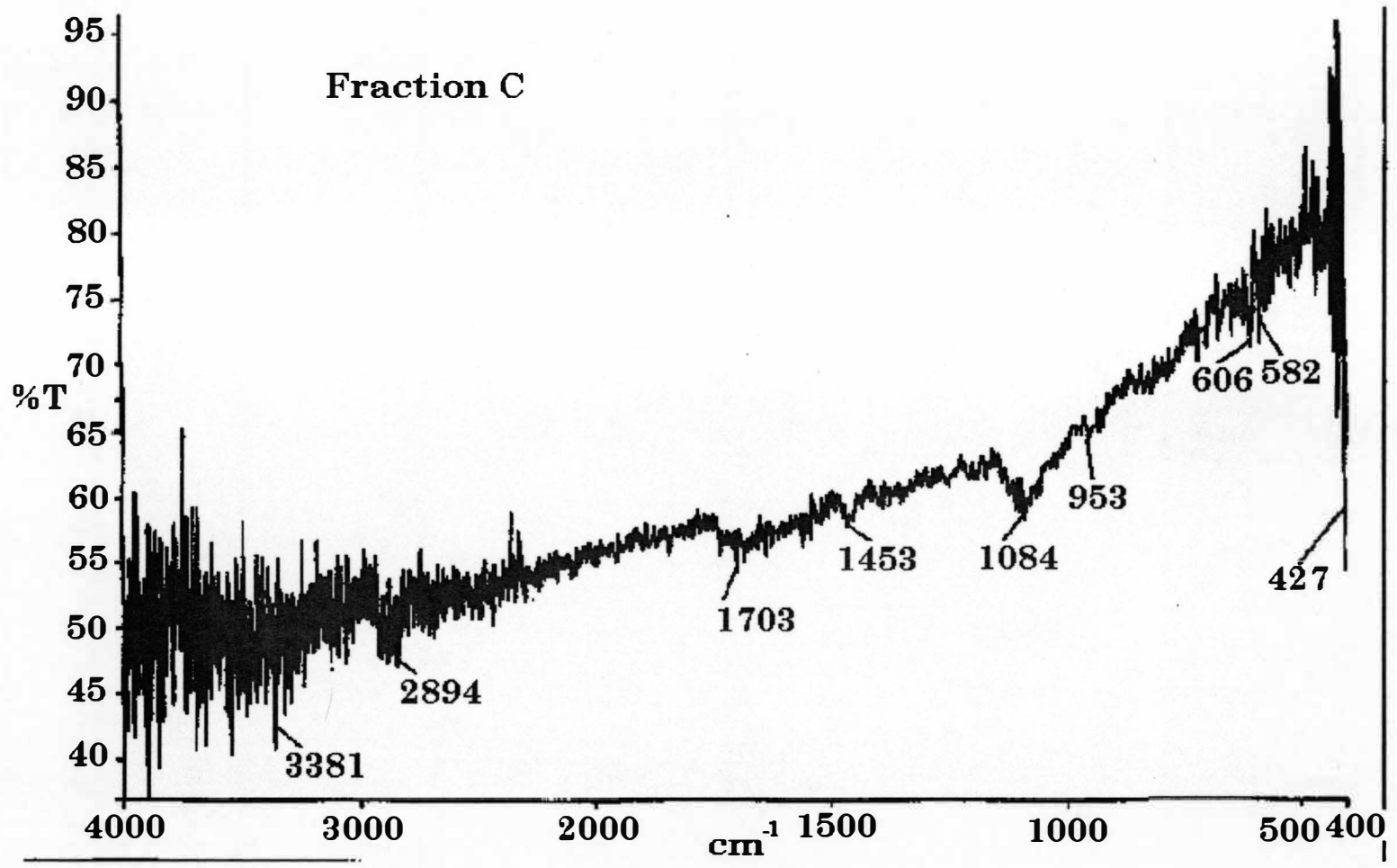


Figure B-2. FTIR Spectrum of Prep. HPLC Fraction "C".

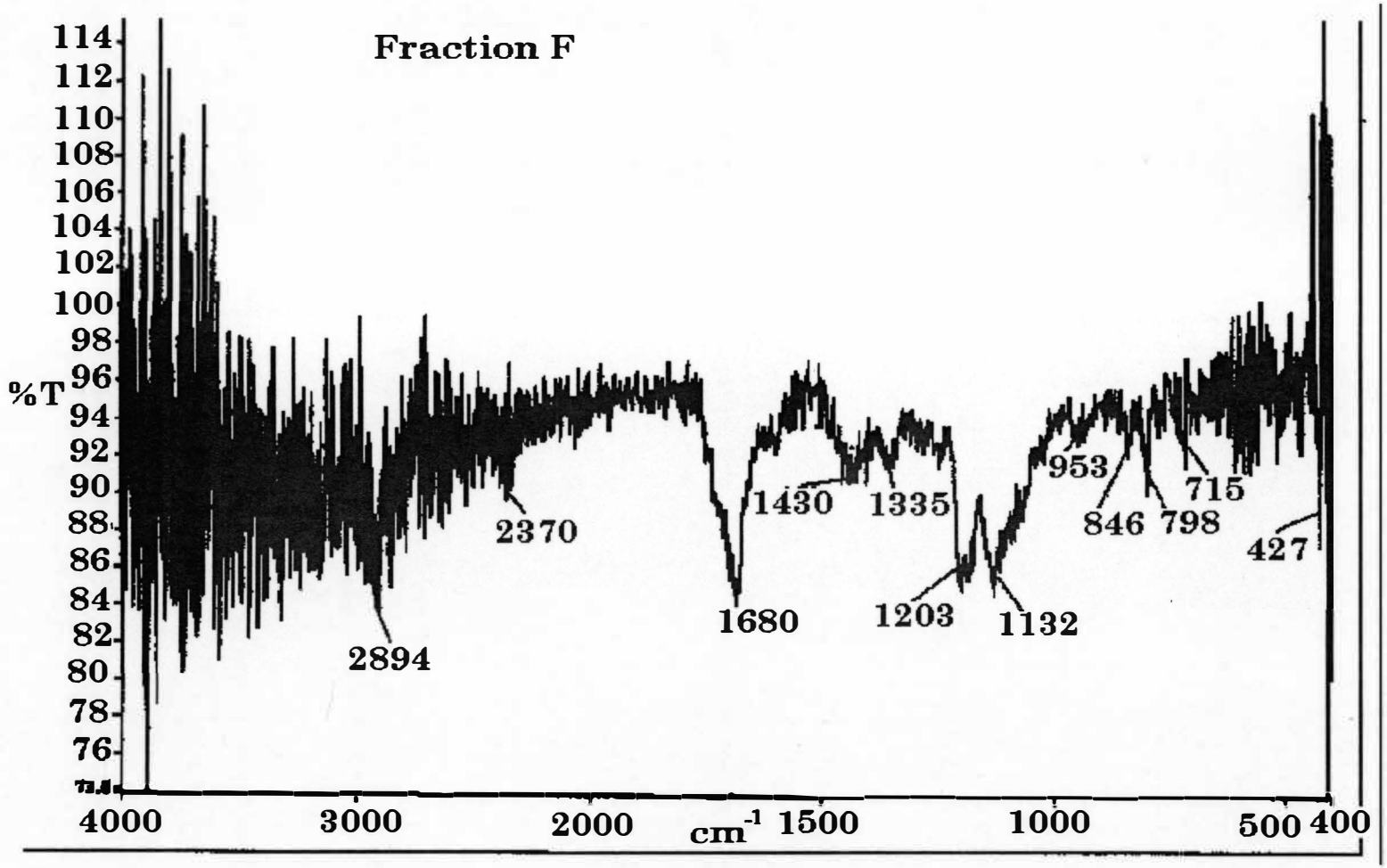


Figure B-3. FTIR Spectrum of Prep. HPLC Fraction "F".

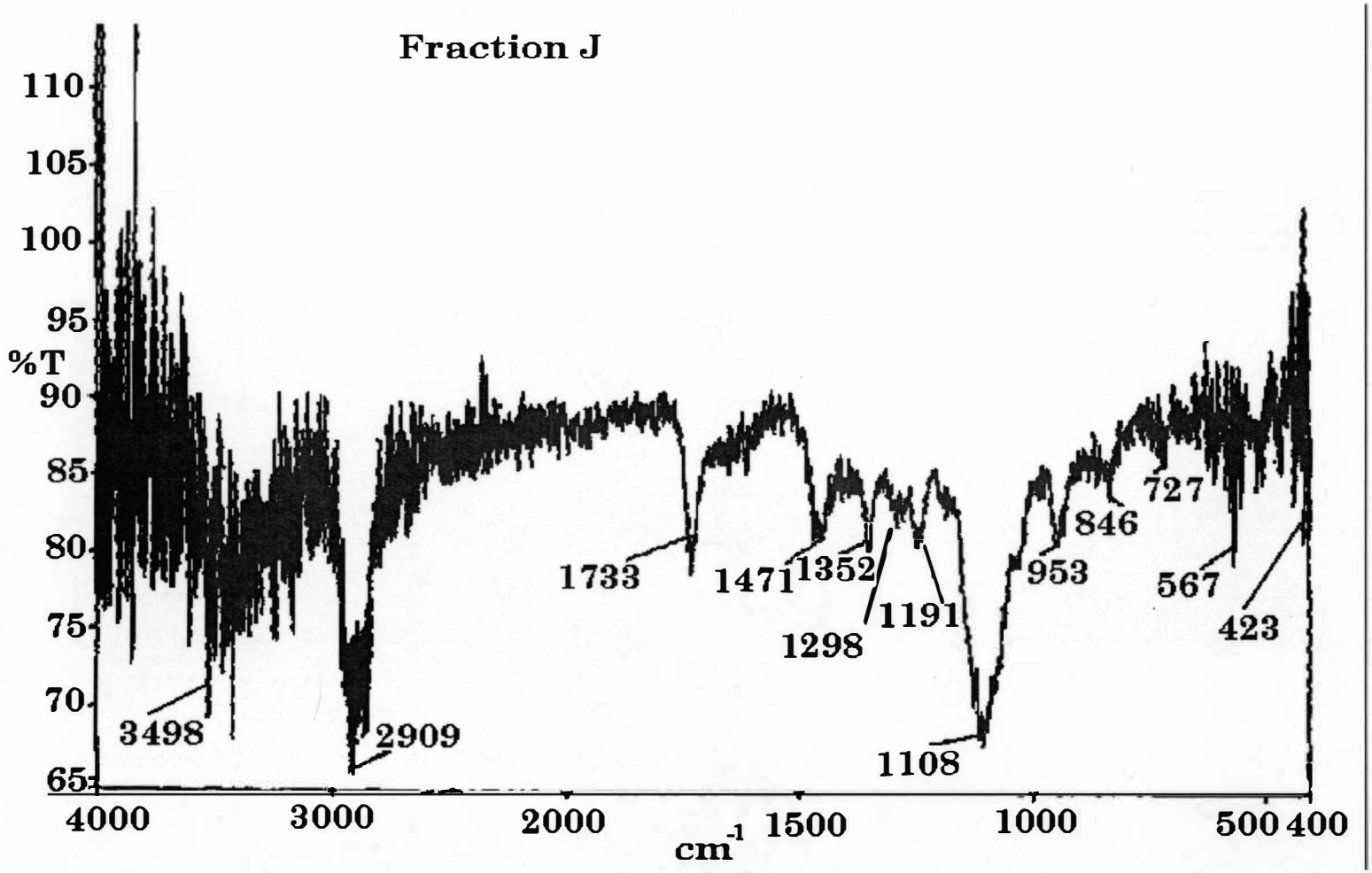
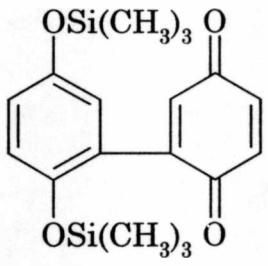


Figure B-4. FTIR Spectrum of Prep. HPLC Fraction "J".

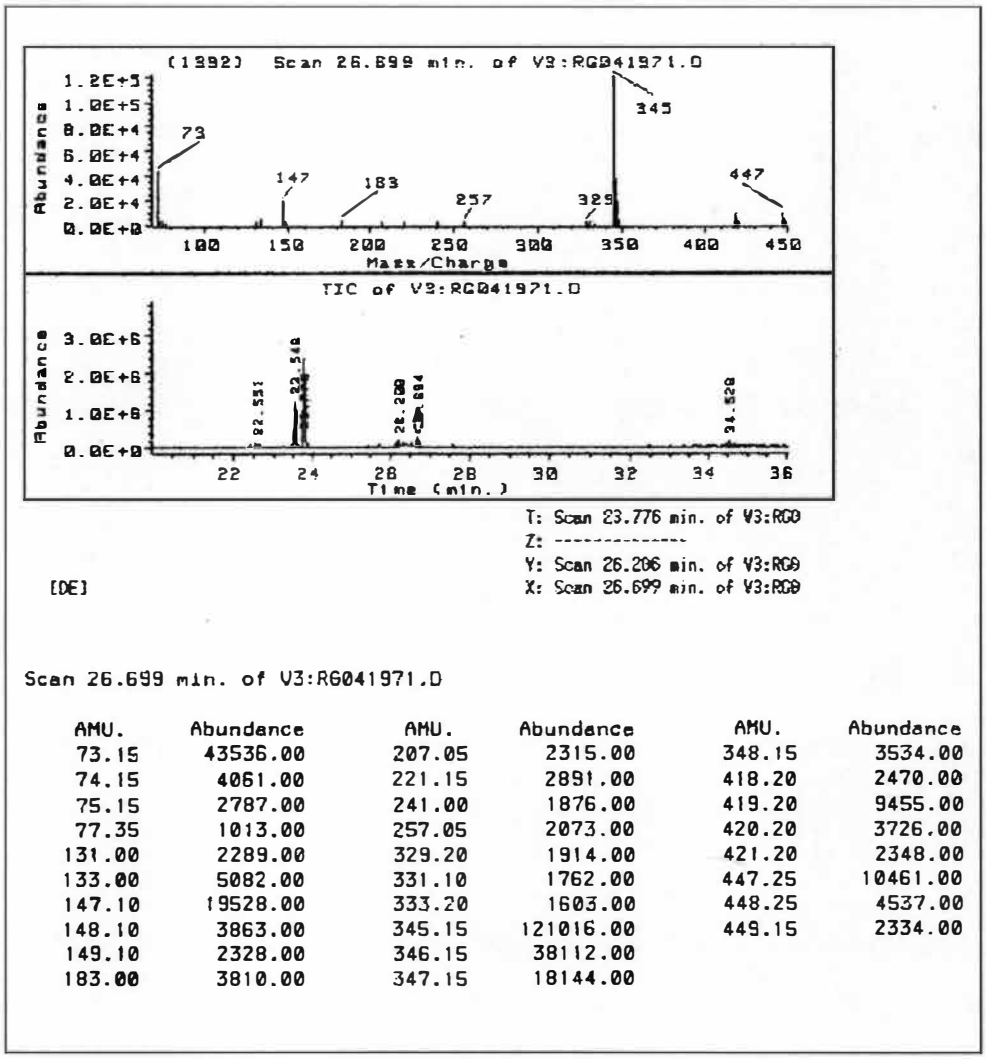
Appendix C

Gas Chromatogram-Mass Spectrogram

Data of Trimethylsilylized Residue of Hydroquinone



(TMS Ether of hydroquinone dimer in figure 24) with loss of methyl group, is consistent with parent peak at 345 amu.



Scan 26.699 min. of V3:RG041971.D

AMU.	Abundance	AMU.	Abundance	AMU.	Abundance
73.15	43536.00	207.05	2315.00	348.15	3534.00
74.15	4061.00	221.15	2891.00	418.20	2470.00
75.15	2787.00	241.00	1876.00	419.20	9455.00
77.35	1013.00	257.05	2073.00	420.20	3726.00
131.00	2289.00	329.20	1914.00	421.20	2348.00
133.00	5082.00	331.10	1762.00	447.25	10461.00
147.10	19528.00	333.20	1603.00	448.25	4537.00
148.10	3863.00	345.15	121016.00	449.15	2334.00
149.10	2328.00	346.15	38112.00		
183.00	3810.00	347.15	18144.00		

Figure C-1: GC-MS Data for Peak Eluting at 26.699 Minutes for TMS Ether of Hydroquinone Residue (Run #RG041971.D).

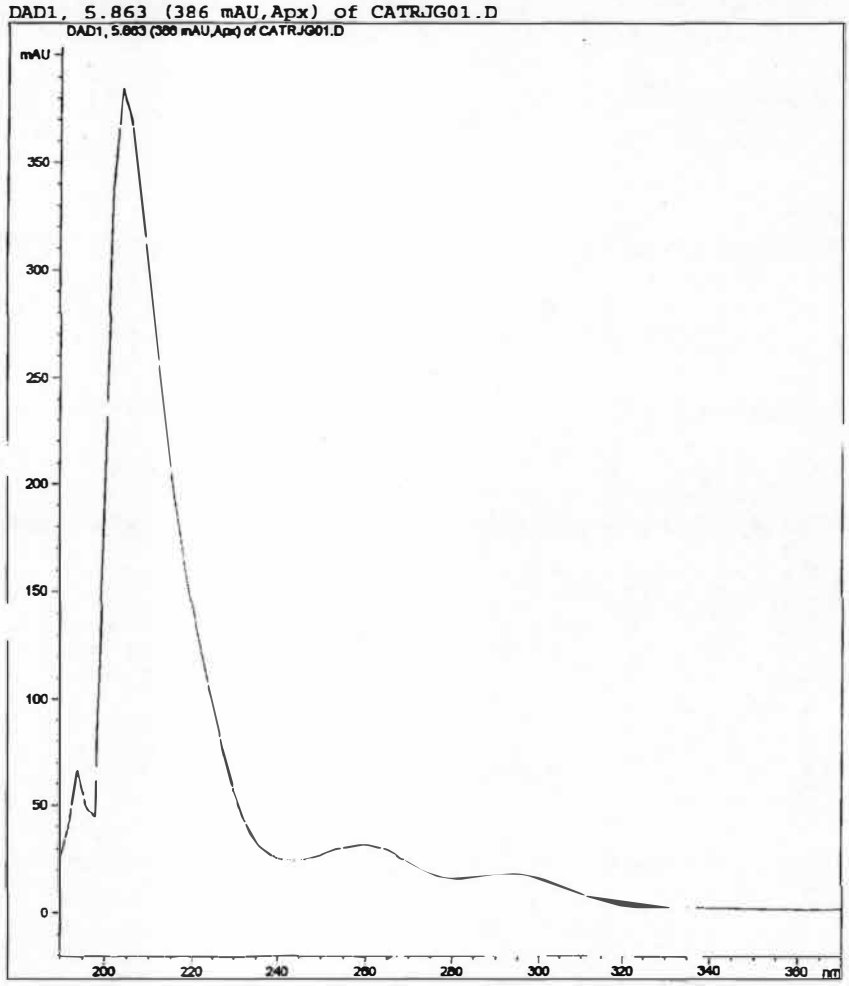
Appendix D

Examples of Ultraviolet Spectra of Individual Preparatory HPLC Fractions

Print of window 39: DAD1, 5.863 (386 mAU,Apx) of CATRJG01.D
KROMASIL C18, MPA = 90-10-0.1

Injection Date : 11/14/00 12:34:14 PM
Sample Name : 50/50 Smp/MobPhA Vial : 100
Acq. Operator : RJG
Inj Volume : 100 ul
Method : C:\HPCHEM\1\METHODS\CATRJG.M
Last changed : 11/14/00 12:20:25 PM by RJG

DAD1, 5.863 (386 mAU,Apx) of CATRJG01.D



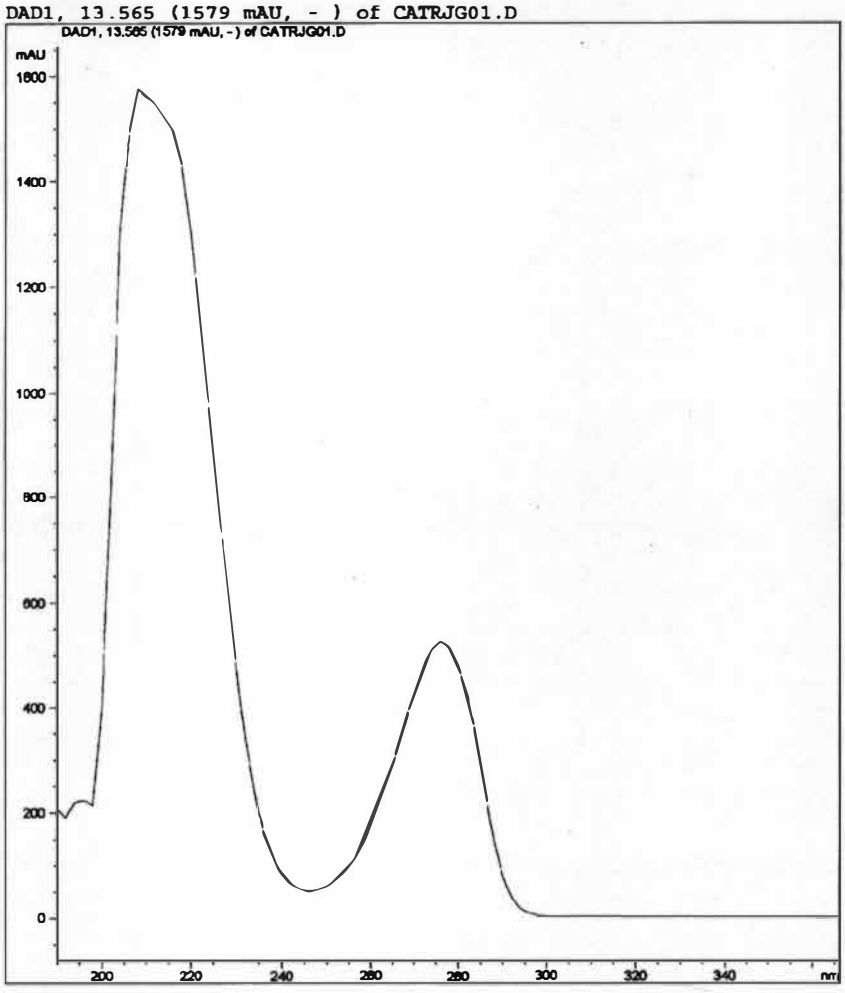
Instrument 1 11/15/00 4:53:15 PM RJG Page 1 of 1

Figure D-1: UV Spectrum of Prep-HPLC Catechol Residue Fraction Eluting at 5.863 min.

Print of window 39: DAD1, 13.565 (1579 mAU, -) of CATRJG01.D
KROMASIL C18, MPA = 90-10-0.1

Injection Date : 11/14/00 12:34:14 PM
Sample Name : 50/50 Smp/MobPhA Vial : 100
Acq. Operator : RJG
Inj Volume : 100 ul
Method : C:\HPCHEM\1\METHODS\CATRJG.M
Last changed : 11/14/00 12:20:25 PM by RJG

DAD1, 13.565 (1579 mAU, -) of CATRJG01.D



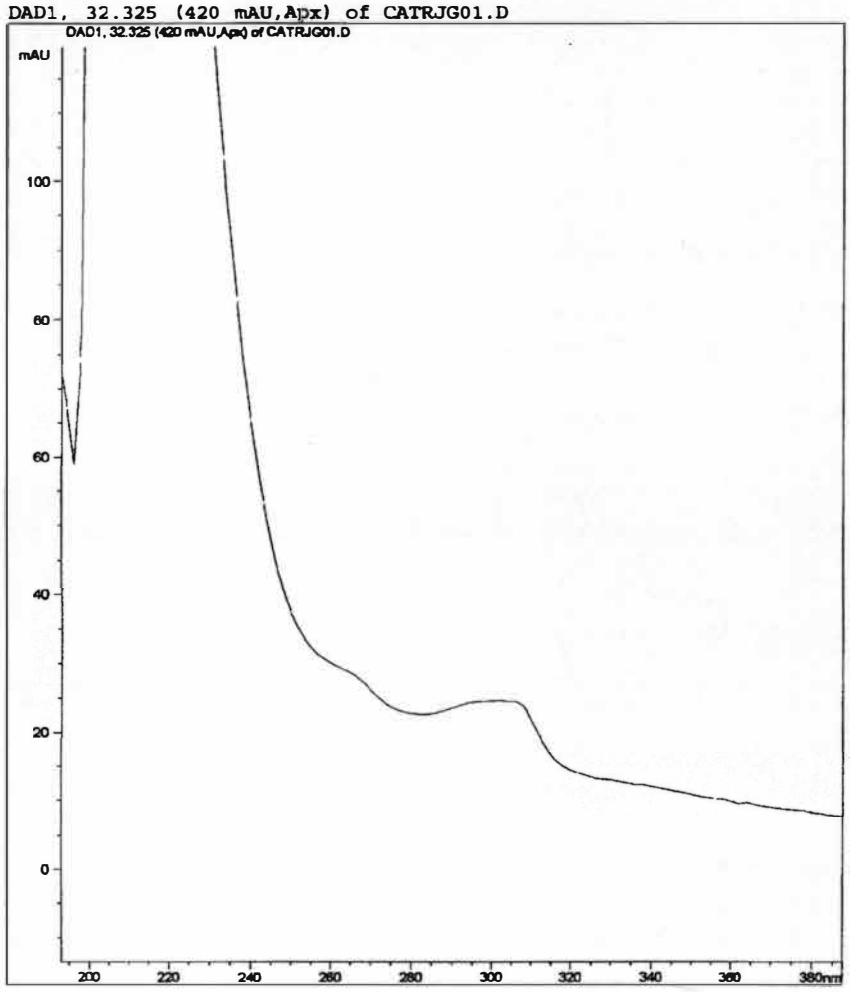
Instrument 1 11/15/00 4:59:06 PM RJG Page 1 of 1

Figure D-2: UV Spectrum of Prep-HPLC Catechol Residue Fraction Eluting at 13.565 min.

Print of window 39: DAD1, 32.325 (420 mAU,Apx) of CATRJG01.D
KROMASIL C18, MPA = 90-10-0.1

Injection Date : 11/14/00 12:34:14 PM
Sample Name : 50/50 Smp/MobPhA Vial : 100
Acq. Operator : RJG
Inj Volume : 100 ul
Method : C:\HPCHEM\1\METHODS\CATRJG.M
Last changed : 11/14/00 12:20:25 PM by RJG

DAD1, 32.325 (420 mAU,Apx) of CATRJG01.D



Instrument 1 11/14/00 2:15:00 PM RJG Page 1 of 1

Figure D-3: UV Spectrum of Prep-HPLC Catechol Residue Fraction Eluting at 32.325 min.

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