

## ABSTRACT

### A Structural Characterization of an Aquatic Fulvic Acid (Under the direction of Dr. Russell F. Christman)

The structural characterization of an aquatic fulvic acid was attempted. Several approaches toward obtaining information concerning the structure of a fulvic acid were taken. These included analysis of the products from a mild oxidative degradation, elemental analysis, and spectroscopic analysis of the undegraded fulvic acid.

The oxidative degradation method employed for this research was an alkaline cupric oxide oxidation. Validation of this method with model compounds was accomplished prior to the oxidation of the fulvic acid by this technique. The products obtained from this procedure were analyzed by gas chromatography and combined gas chromatography/mass spectrometry.

Spectroscopic techniques employed for the purposes of analyzing the structure of the undegraded fulvic acid include infrared spectroscopy and proton and solid state  $^{13}\text{C}$  nuclear magnetic resonance spectroscopies.

Additionally, the effects of the oxidative conditions employed for the alkaline cupric oxide oxidation on selected compounds was investigated. Analysis of the resulting products was performed in the same manner as that for the oxidatively degraded fulvic acid.

## Key Words

alkaline cupric oxide oxidation

fulvic acid

mass spectrometry (MS)

gas chromatography (GC)

nuclear magnetic resonance (NMR)

structure

oxidation infrared spectroscopy

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## List of Tables

	<u>Description and Title</u>
Table 4-1	Percent Recovery of Index Phenols from Cupric Oxide Oxidations
Table 4-2	Index Phenol Extraction Efficiency Data
Table 4-3	Percent Recovery of Model Compounds from Cupric Oxide Oxidations
Table 4-4	CuO Oxidation Products from Lake Drummond Fulvic Acid
Table 4-5	Elemental Composition of Lake Drummond Fulvic Acid
Table 4-6A	$^{13}\text{C}$ NMR Chemical Shift Regions and Associated Percent Contributions
Table 4-6B	Proton NMR Chemical Shift Regions and Associated Percent Contributions
Figure 2-1	CuO Mechanism
Figure 4-1	Structures of Index Phenols
Figure 4-2	Chromatogram of Index Phenols
Figure 4-3	Structures of Model Compounds
Figure 4-4	Chromatograms of Group IV Model Compounds: before and after CuO oxidation
Figure 4-5	Mass spectrum of 3,4-Dihydroxybenzaldehyde
Figure 4-6	Diagram of CuO Mechanism
Figure 4-7	Chromatograms for LDFA products from CuO oxidation

Figure 4-8 Infrared spectrum of Lake Drummond Fulvic Acid

Figure 4-9  $^{13}\text{C}$  NMR CP/MAS Spectrum of Lake Drummond Fulvic Acid

Figure 4-10  $^{13}\text{C}$  NMR Dipolar Dephased spectrum of Lake Drummond Fulvic Acid

Figure 4-11  $^1\text{H}$  NMR spectrum of Lake Drummond Fulvic Acid

## INTRODUCTION

Humic materials have been of interest to the investigative scientist for some time (Green and Steelink, 1962, Christman and Ghassemi, 1966). Humic substances are found in both aquatic and terrestrial environments and although humics from these two sources share similarities, differences between the two groups do exist. Humic substances may be grouped into various categories or component matrices depending upon certain characteristics expressed in isolation schemes employed for the separation of the various components comprising the humic material (Thurmond and Malcolm, 1981, Leenheer, 1981). A multitude of techniques have been employed to gather data on the origin, structure, metal chelating ability, and reactions of humics under various conditions (Christman et al., 1984, Schnitzer and Ortiz de Serra, 1973, Hatcher, 1981, Weber, 1983). The accumulation of data pertaining to humic material has generated different schools of thought in areas such as the genesis of humic materials and structural models (Hatcher, 1981, Schnitzer and Ortiz de Serra, 1973, Christman et al., 1984).

Several techniques have been of use in humic research as in studies of structural components of other naturally occurring materials such as lignin and coal (Hedges and Ertel, 1982, Hatcher et al., 1981, Pearl, 1954). The severity of techniques employed varies from nondestructive, as in the case of NMR and IR spectroscopic analysis to destructive as with harsh oxidative conditions coupled with combined gas chromatography/mass spectrometry for the analysis of the oxidation matrix components (Hatcher, 1981, Wilson, 1981, Liao et al., 1982, Norwood, 1986).

Christman and coworkers have noted that the lack of standard isolation procedures and subsequent set of analytical methods for the analysis of organic carbon from aquatic media have hampered the ability of



investigators to easily compare the results of existing databases and propose coherent structural models for humic or fulvic acid (Norwood, 1986).

Norwood has noted the lack of model compound work performed employing the various oxidation techniques which so many investigators have used (Norwood, 1986).

One of the objectives of this research was to standardize the oxidative degradation conditions for the analysis of Lake Drummond fulvic acid. A useful approach to this was to employ the alkaline cupric oxide oxidation procedure as outlined by Ertel and Hedges (1982). This method had to be verified in our own laboratory in the same manner as that of Ertel and Hedges. This was accomplished by checking the stability of compounds previously reported in the literature as stable to the oxidative conditions and procedures outlined by Ertel and Hedges. The series of compounds employed for this purpose are known as the index phenols.

A second objective of the research was to structurally characterize the Lake Drummond fulvic acid oxidation products. This goal was met by employing the previously validated standard oxidative method of alkaline cupric oxide oxidation. Analysis of the resulting product mixture was accomplished via gas chromatography and combined gas chromatography/mass spectrometry. In addition, other methods such as elemental analysis, infrared spectroscopy and proton and  $^{13}\text{C}$  nuclear magnetic resonance spectroscopy were employed to provide additional information concerning the structure of the fulvic acid.

Finally, a third objective of the research was to investigate the relative stability during the standardized oxidation of selected compounds which had been reported in the literature as products of the alkaline cupric oxide oxidation of humic material. This was accomplished by subjecting the compounds to identical alkaline cupric oxide oxidative

conditions as had been previously employed for the oxidation of the index phenols and the Lake Drummond fulvic acid. Analysis of the resulting products was accomplished by employing gas chromatography and combine gas chromatography/mass spectrometry.



## LITERATURE REVIEW

Humic substances are generally complex, base soluble, naturally occurring materials found in both terrestrial and aquatic environments, and are thought to be the products of vegetative decomposition (Thurman and Malcolm, 1981). They may be subdivided into different categories based upon their behavior in various isolation schemes. It is generally accepted that humic acid is the acid insoluble fraction, that which precipitates from solution when the pH of the aqueous medium is less than 2. Fulvic acid is soluble in both basic and acidic media. Hymatomelanic acid is soluble in both basic media and low molecular weight alcohols (eg. butanol). however it is not soluble in dilute hydrochloric acid (Black and Christman, 1963). Humin is a terrestrial humic material component which is not base soluble. This property makes humin difficult to obtain for analysis. Various methodologies have been worked out for the isolation and seperation of the components of humic substances. These vary in approach from distillation under reduced pressure (Black and Christman, 1963) to employing exchange resins of various types (Christman and Ghassemi, 1966, Liao et al., 1982, Thurman and Malcolm, 1981, Leenheer, 1981).

Recent trends in isolation schemes show a marked perference for the macroreticular type resins such as XAD-7 and XAD-8. These resins have a great affinity for high molecular weight polyelectrolytic substances which make them ideal support materials to be employed for the isoation

of humic substances. Leenheer provides a very practice tretise on the various resins employed for isolation of the many components comprising dissolved organic carbon (DOC) in both natural waters and wastewaters (1981). Thurman and Malcolm presented one of the earlier uses of macroreticular resins in combination with cation exchange and lyophilization for the isolation of aquatic humic and fulvic acids (1981). These particular publications have laid much of the groundwork for the methodologies presently employed in the isolation and fractionation of aquatic humic substances. Much of the earlier literature focuses on terrestrial humic materials, however some work on aquatic humic material did parallel some of the earlier findings on terrestrial substances (Christman and Black, 1963, Christman and Ghassemi, 1966).

The literature describes numerous techniques that have been employed by various researchers in their quest for information relating to humic materials. Oxidative degradation techniques comprise much of the earlier literature. By the late 1970's advancements in the application of spectroscopic techniques allowed for a new and different approach towards studying humic substances. Of particular significance is the application of some nuclear magnetic resonance (NMR) techniques such as cross polarization- magic-angle spinning (CP/MAS) and dipolar dephasing. These techniques have provided important information on the intact gross structure of humic substances. The advent of CP/MAS provides a technique to obtain relatively well defined spectra of humic materials in the solid state, an important achievement.

Prior to approaches employing NMR as a structural study tool, the approach towards studying the structure of humic substances was to utilize the degradative techniques available at the time, thus allowing one to examine the fragments of the humic material. However, as is evident in the literature, piecing the subunits back together proves to be quite a

difficult, if not controversial task (Christman et al., 1984).

It is important not to rely too heavily on the information obtained from one particular technique, rather, it is more advantageous to employ the resources of information obtained from as many approaches as possible. However, it is equally important to know the limitations of the techniques that are employed.

One of the earlier oxidative degradation methods employed with humic material was alkaline permanganate oxidation. Schnitzer and DesJardins (1970) found that the major products from alkaline permanganate oxidation on a terrestrial fulvic acid were benzene carboxylic acids. Both 1, 2, 4, 5 - and 1, 2, 3, 5 -benzene tetracarboxylic acids along with penta- and hexabenzene carboxylic acids were found to predominate the oxidation products of reacted fulvic acid. This was found to be the case both when employing premethylated or unmethylated fulvic acid. With respect to smaller, volatile aliphatic acids found, unmethylated fulvic acid produced only acetic acid whereas methylated fulvic acid produced oxalic and acetic acids along with propionic, butyric and isocaproic acids. Acetic acid was also the major volatile aliphatic acid produced in the reaction of methylated fulvic acid with alkaline potassium permanganate in this study. The benzene carboxylic acid portion, although the major degradation product, accounts for only a small portion of the original starting material (0.4 to 1.4 %) (Schnitzer and DesJardins, 1970)

Neyroud and Schnitzer (1974), employing alkaline potassium permanganate oxidation on premethylated terrestrial fulvic acids found that the major degradation product consisted of benzene carboxylic acids. This group was comprised primarily of 1, 2, 4, 5-, and 1, 2, 3, 4-benzene tetracarboxylic acids, benzenepentacarboxylic acid pentamethyl ester and benzene hexacarboxylic acid hexamethylester. The authors indicate that 1,2,3,5-benzene tetracarboxylic acid tetramethyl ester is not detected.

Liao, et al. (1982) utilized alkaline potassium permanganate oxidative conditions on a set of aquatic fulvic and humic acids. Following potassium permanganate oxidation, the product mixture was methylated with diazomethane. From this study, six major categories of compounds were identified. These included benzene carboxylic acid methyl esters, furan carboxylic acid methyl esters, glyoxylic acid methyl esters, aliphatic monobasic acid methyl esters, aliphatic dibasic acid methyl esters and aliphatic tribasic acid methyl esters. Mono-, di- and tribenzene carboxylic acid methyl esters were identified amongst the benzene carboxylic acid methyl esters, however, the tri-, tetra-, penta- and hexabenzene carboxylic acid methyl esters predominate amongst this category and account for approximately 65% of all identified chromatographable products for humic acid and approximately 60% for identified fulvic acid products resulting from oxidation with potassium permanganate. Other individual major products from the humic and fulvic acid degradation mixtures included oxalic, succinic and malonic acid dimethyl esters.

Alkaline nitrobenzene oxidation was a technique used mainly by wood chemists but found application for use by those exploring the composition of soil organic matter. Compared to alkaline potassium permanganate oxidations, alkaline nitrobenzene appears to be less harsh. With alkaline nitrobenzene, less highly oxidized products are obtained. Aldehydes and ketones are readily detected amongst the isolated oxidation products. Morrison (1963) describes a method for the determination of p-hydroxybenzaldehyde, vanillin and syringaldehyde in the product mixture when employing alkaline nitrobenzene as the oxidant. From his findings, Morrison states that phenolic aldehydes account for approximately 1% to 4% of the T.O.C. in peat and 0.5% to 1% of the T.O.C. in soil, whereas in plant material, that parameter measures approximately 7% when employing the same methodology. Other products identified in the product



mixture include p-hydroxybenzoic, vanillic, syringic, coumaric and ferrulic acids along with p-hydroxyacetophenone and acetovanillone.

Acetosyringone is detected but not to the extent of other ketones.

Morrison notes that the composition of the product mixture is altered with respect to choice of reaction temperature. Employing a reaction temperature below 170°C tended to favor the presence of substituted cinnamic acids whereas choosing reaction temperatures above 180 C favored the formation of aldehydes.

In a later study, Gardner and Menzel (1974) examine the aldehyde content of marine sediments after exposing the sediments to alkaline nitrobenzene oxidations. Gardner and Mentzel were attempting to employ the aldehydes as tracers of terrestrial derived organic matter. They chose p-hydroxybenzaldehyde, vanillin and syringaldehyde as their target aldehydes. Syringaldehyde and vanillin were well known lignin degradation products which are derived from higher vascular plants only available from terrestrial sources. They found a range of 0.1% to 1.0% of total organic matter analyzed as being comprised of syringaldehyde and vanillin. The percentage decreased with increasing distance from shore. However, laboratory blanks showed evidence of contaminating amounts of vanillin and p-hydroxybenzaldehyde.

Hedges and Parker (1976) indicate that there are inherent problems associated with nitrobenzene oxidations of organic matter with respect to production fo organic compounds that coelute with p-hydroxybenzaldehyde and vanillin. This was verified by analysis of system blanks for the oxidation conditions employed.

Alkaline hydrolysis is considered mild amongst the spectrum of the oxidative degradation techniques employed by researchers throughout the years. Rupture of ether and ester linkages appears to be the predominant reactions occuring in alkaline hydrolysis (Neyroud and Schnitzer, 1974).

Neyroud, et al. (1974), having first methylated and then fractionated a terrestrial fulvic acid over alumina with solvents of increasing polarity, subjected the two most polar fractions collected to alkaline hydrolysis. The major reaction products isolated and identified by GC/MS consisted of aliphatic acids, phenolic acids and benzene carboxylic acids. Overall yield of products from these two fractions was 9.4% and 7.4%.

Liao, et al. (1982) using aquatic humic substances isolated on XAD-8 macroreticular resin examined the alkaline hydrolysis products by GC/MS. Both humic and fulvic acid samples from two sources were examined under this set of conditions. Products isolated from the reaction mixture were methylated with diazomethane prior to GC/MS analysis. Although in excess of 30 compounds for both humic and fulvic acids from each source were identified, the major alkaline hydrolysis products consisted of the methyl esters of benzene carboxylic acids and of aliphatic acids. Dominant amongst the aliphatic acids were the dibasic acids. Overall yields for products identified varied from 1.2% to 1.7%. The humic acid fractions boasted a yield of approximately two times as much aliphatic dibasic acid methyl ester content compared to the fulvic acid isolated from the same source.

Another degradative technique that has been utilized for studying humic materials is alkaline cupric oxide. With regard to oxidative strength, it is a mild technique, comparable in mildness to alkaline hydrolysis, however it appears to be somewhat stronger than alkaline hydrolysis. Although widely used by wood and lignin chemists, alkaline copper oxide has gained considerable popularity as a degradative technique amongst those studying the structure of humic material (Hedges and Ertel, 1982).

Some of the earlier studies on aquatic humic material employing alkaline copper oxide conditions were performed by Christman and

Ghassemi (1966). They investigated aquatic humic material isolated from several sources in the northwestern United States. The authors reported that the dark color imparted to the humic material was due to the presence of aromatic polyhydroxymethoxycarboxylic acids. Employing thin layer chromatography and various indicator reagent sprays, the authors identified seven degradation products which fell into the above mentioned category. It was also noted that when activated by an appropriate hydroxyl substitution pattern, alkyl side chains of certain phenolic structures were converted to phenolic acids. The alkyl side chain was oxidized to the position where only a one carbon carboxylic acid function remains. This occurs when the alkyl side chain is influenced by the presence of an ortho-para director; i.e., the activator is in a position ortho or para to the alkyl side chain. In this particular case, the ortho-para director is the hydroxyl moiety.

An earlier study by Greene and Steelink (1961) employing aqueous alkaline cupric oxide was performed on soil humic acid. Separation and identification of degradation matrix products was accomplished by paper chromatography. Phenolic structures were also the major components identified in this study and this was also the first time that resorcinol and guaiacyl derivatives were reported as humic acid degradation products. The authors postulated that the presence of these compounds indicate that lignin may not be the sole precursor of terrestrial humic acid.

In a later study performed by Ertel et al. (1984), the potential contribution of lignin as a precursor to aquatic humic substances is assessed by employing alkaline cupric oxide as the oxidative degradation technique. Hedges et al. had utilized this technique extensively in earlier research on the distribution of oxidation products of lignin derived from various sources (1979, 1982). Previous work by Hedges et al. led to the development of the term **index phenols** (1979, 1982). This term applies



to phenols that are commonly and consistently observed in cupric oxide degradation of plant tissues of various origins. The phenols consist of the aldehyde, ketone and carboxylic acid derivative of p-hydroxy, vanillyl and syringyl structures. Cinnamyl phenols, which have trans-propenoic acid functions, are also encountered.

Ertel and Hedges have found that certain types of the phenols are associated with particular types of vegetation. Vanillyl phenols are found amongst the cupric oxide degradation products from both the woody and non woody tissues of angiosperms and gymnosperms, whereas syringyl phenols are only found from angiosperm digests, and cinnamyl phenols from gymnosperm sources. The simplest class of phenols, the p-hydroxy substituted phenols are found amongst the cupric oxide products from vascular and non vascular plant tissues (1979, 1982). Ertel and Hedges (1982) state that a major portion of aquatic humic material comes from vascular plants since these are the only source of lignin.

Ether extractable phenols were not observed when the isolated humic materials were extracted, however, the distribution of the various forms of index phenols were observed amongst the copper oxide oxidation products. The authors felt that this implied that lignin-like structures were present in the humic substances.

In comparing the distribution of the various phenols and their respective form (i.e., aldehyde, ketone, carboxylic acid), Ertel et al. (1984) found that the humic acid fraction had a greater proportion of syringyl and lesser proportion of p-hydroxy phenols relative to the fulvic acid fraction. It was also noted that the fulvic acid fraction yielded higher acid/aldehyde ratios than the corresponding humic acid fraction. The higher degree of demethoxylated and more oxidized moieties observed in the fulvic acid fraction may be the result of a greater degree of biological action upon the fulvic acid fraction compared to the humic acid fraction

With regard to the mechanism of action by alkaline cupric oxide upon complex matrices such as lignin or humic substances, there is a paucity of literature or reported investigations. In a review by Hayes and Swift (1978) the authors mention a plausible mechanism proposed by Chang and Allan. They believe that the oxidation occurs by a one electron transfer process. The authors further elaborate that the abstraction of an electron to produce a phenoxy radical followed by a fast second electron transfer back to the cupric oxide molecule which results in the production of a quinonemethide. A reaction sequence proposed in their review follows;

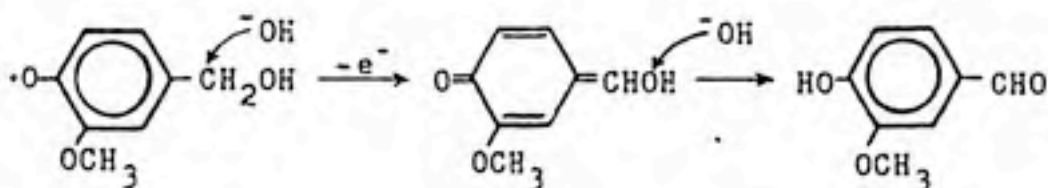


Figure 2-1

The second electron transfer back to the cupric oxide prevents coupling of phenoxy radicals. Employing a high reaction temperature along with excess cupric oxide increases the rate of electron transfer, hence, driving the oxidation more quickly towards completion (Hayes and Swift, 1978).

Whereas oxidative degradation of humic substances followed by chromatographic separation and possible mass spectrometric identification of the matrix components provides one with information concerning the subunit structural entities that comprise the humic

substances, it offers little information with regards to the original overall structure of the intact undegraded humic substance. Recent advances in the area of nuclear magnetic resonance (NMR) allow one to obtain useful information concerning gross overall structural composition of the humic substances. With the advent of cross polarization/magic angle spinning and fourier transform techniques, the high degree of resolution once only available through analysis of samples in the liquid state can now be obtained for intact solids (Wilson, 1981, Hatcher et al., 1981).

Prior to the introduction of CP/MAS, some investigators felt that proton NMR ( $^1\text{H}$  NMR) was a more reliable technique for quantitative structural analysis of humic substances (Ruggerio et al., 1979). However, the introduction of CP/MAS and fourier transform techniques to solid state  $^{13}\text{C}$  NMR analysis has changed that view (Hatcher et al., 1981, Wilson et al., 1981).

In recent years, refinements in the use of the new  $^{13}\text{C}$  NMR techniques have contributed to their applications for quantitative purposes. To employ solid state  $^{13}\text{C}$  NMR, certain parameters such as optimum cross polarization contact time and appropriate pulse delays must be determined experimentally beforehand. In dealing with  $^{13}\text{C}$  nuclei from different chemical environments, different rates of relaxation to the ground state are to be expected once Rf pulses have been applied to the samples (Wilson, 1981). Signals from the various chemical environments within the sample may be seen throughout the spectrum and signals seen in particular spectral regions of arise from distinct classes of structural entities present within the sample.

The positions of the various signals seen in the spectrum may be defined by their chemical shift, which is their resonance frequency difference position relative to a standard such as tetramethyl silane (TMS)

(Wilson, 1981). Hatcher (1983) divides the  $^{13}\text{C}$  spectra of humic substances into five regions. The first region (0 to 50 ppm) is comprised mainly of aliphatic moieties, carbons singly bonded to other carbons. An important entity contained within this region is terminal methyl groups. In region II, (50 to 110 ppm) carbons singly bonded to heteroatoms such as oxygen and nitrogen are observed. Resonances from structural entities such as ethers, carbohydrates, amines, alcohols, methoxyl and acetals are observed within this frequency range. Region III (110 to 160 ppm) mainly reflects the aromatic character of solids, however carbon-carbon double bonds of non aromatic origin also resonate within this region. Carboxyl carbons bonded to heteroatoms such as oxygen and nitrogen resonate within region IV (160 to 190 ppm). Resonances from functional groups such as carboxylic acids, esters and amides are observed in this range. Region V (190 to 220 ppm) reflects the resonances of carbonyl carbons bonded to other carbons or hydrogens, and the resonances arising from ketones and aldehydes are seen in this region.

Another important set of experiments that the nuclear magnetic resonance spectroscopist must perform in order to obtain useful quantitative data is CP/MAS relaxation time experiments. The spin-lattice relaxation time ( $T_1$ ) can be obtained from these experiments. By obtaining this value, the correct parameters for cross polarization contact time and pulse delay time may be established.

Knowledge of relaxation rate parameters may enable one to obtain spectra devoid of signals from CH or  $\text{CH}_2$  moieties (Wilson et al., 1983). This is accomplished through a series of experiments involving dipolar dephasing. Dipolar dephasing involves an instrumental experiment whereby a series of set time delays are inserted into the pulse delay program without decoupling of  $^1\text{H}$  and  $^{13}\text{C}$  nuclei prior to acquisition of data. The dipolar dephasing time ( $T_{dd}$ ) is acquired through this set of



experiments. As Tdd is increased the signal from protonated carbon decreases. Wilson et al., (1983) employing a Maungatua soil sample shows that as Tdd approaches 40  $\mu$  sec the signal from protonated carbon, excluding methyl carbons, is almost completely diminished.

Utilizing this technique, the protonated carbon spectrum can be obtained through a difference spectrum. Subtracting the  $^{13}\text{C}$  spectrum obtained at a long Tdd from that obtained at Tdd=0, will provide the spectrum of protonated carbon minus the methyl carbons. The spectrum obtained will indicate if the aromatic content of the humic material is heavily protonated.

Wilson adds a note of caution concerning interpretation of dipolar dephased spectra with respect to the methyl region. Methyl groups are allowed to rotate freely in the solid state and even at long Tdd's, the methyl carbons do not relax and are present in spectra taken at long Tdds.

It is apparent that the knowledge of relaxation rates and dipolar dephasing techniques provides another approach for functional group analysis. Solid state  $^{13}\text{C}$  NMR with ancillary techniques such as CP/MAS, fourier transform, and dipolar dephasing provides the investigative scientist with additional ways to study the structure of humic substances.

## Experimental Methods

Fulvic acid obtained from the waters of Lake Drummond was the humic substance of choice, employed for the purposes of copper oxide degradation reactions. The reader is referred to the work of Liao for exact details concerning the location and characteristics of Lake Drummond (1981). The fulvic acid was isolated according to the procedure outlined by Christman et al. (1981). The exact isolation procedure is described in detail by Norwood (1985).

Alkaline cupric oxide oxidation reactions on the Lake Drummond fulvic acid (LDFA), the model compounds, and the index phenols were carried out in Monel stainless steel reaction cylinders as diagrammed by Hedges and Ertel (1982) and according to their procedure with minor modifications. Exceptions to the protocol outlined by the authors included the use of a replica of the commercially available 200ml Parr bomb (model 4753). Additionally, an alternative surrogate standard was chosen. Ertel and Hedges employed uniformly ring labeled [ $^{14}\text{C}$ ]-p-hydroxyacetophenone as the surrogate standard for recovery studies. Instead, following the cupric oxide oxidations, known amounts of ethyl vanillin dissolved in pyridine were introduced into the 10 ml Monel minibombs upon opening and prior to any transfer steps (Hedges, personal communication).

Identical gas chromatographic operating conditions were employed with the exception of the instrument, recorder, column and split ratio. A Carlo Erba temperature programmable gas chromatograph (Model HRGC 5160 mega series) interfaced with a Shimadzu C-R3A integrator was employed. A 30m by 0.25mm i.d. fused silica DB-1 capillary column (J & W Scientific) at a split ratio of 10:1 was utilized.

A temperature controller (Cole Palmer Model C-2155-20) equipped with a platinum temperature probe was used to heat the reaction cylinder and maintain the reaction temperature of 170°C. The Monel reaction cylinder was fitted into an insulated heating sleeve mounted on top of a shaker top table. The Monel reaction cylinder, minibombs, insulated heating sleeve, and glove box were manufactured in the UNC School of Public Health instrument shop.

Model compounds, index phenols, ethyl vanillin, trans-cinnamic acid, homovanillic acid, and copper (II) oxide (gold label) were purchased from Aldrich Chemical Company (Milwaukee, WI) and were the best grade commercially available. Structures for the model compounds and index phenols appear in figures 4-3 and 4-1, respectively. Sodium sulfate and ferrous ammonium sulfate were purchased from both Fisher Scientific (Raleigh, NC) and EM Science (Cherry Hill, NJ). Sodium Hydroxide pellets and Whatman extraction thimbles (33mm X 94mm) were purchased from Fisher Scientific (Raleigh, N.C.). Pyrex brand glass wool was purchased from Corning Glass Works (Corning, PA). Distilled in glass pyridine and diethyl ether were purchased from Burdick & Jackson and were the best quality grade commercially available and were used without further purification. For the preparation of trimethylsilylated derivatives, bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% TMCS was employed and was purchased from Pierce Chemical Company (Rockford, IL).

Sodium sulfate, ferrous ammonium sulfate, copper (II) oxide and glass wool were all soxlet extracted with diethylether for 24 hours before being employed for any purposes in any of the reaction steps. Prior to use for extractions, diethyl ether was treated with soxlet extracted ferrous ammonium sulfate in order to reduce any peroxides present.



### GC and GC/MS

As a quality assurance check, stock solutions of the index phenols and various classes of model compounds were prepared, derivatized and analyzed, as their TMS analog, via gas chromatography. The results of the chromatography indicated that all the index phenols and model compounds were free from any interfering substances.

Verification of the identity of the components comprising the various stocks of index phenols and class of model compounds was accomplished via combined gas chromatography/mass spectrometry analysis. Standard solutions of the index phenols and the various classes of model compounds were analyzed, as their TMS derivatives, on a Hewlett Packard gas chromatographic interfaced quadrapole mass spectrometer (Model 5985 B). A 30m X 0.25mm i.d. SE-30 fused silica capillary column was employed for chromatographic separation of the components comprising the various mixtures. A temperature program of 100°C to 270°C at 4°C/min with no initial delay was employed. Samples were analyzed under electron impact conditions at 70eV. A scan rate of 1 second was employed. The identity of the components comprising the mixture isolated after Lake Drummond fulvic acid was subjected to alkaline cupric oxide oxidation, was accomplished on two different combined gas chromatography/quadrapole mass spectrometry instruments. One of the instruments is previously described.

The second instrument employed was a Finnigan 4021 combined gas chromatography/quadrapole mass spectrometer equipped with a 30m X 0.25mm i.d. DB-1 capillary column. A scan rate of 1 scan/second was employed. Samples were analyzed at 70eV. A total of 2600 scans was obtained. The data system employed consisted of a Data General Nova 3

computer equipped with Perkin-Elmer disk drives.

Calibration curves for the index phenols, the four classes of model compounds, and ethyl vanillin were constructed for the purposes of quantifying components in extracted samples. Stock solutions of the index phenols with ethyl vanillin and the 4 classes of model compounds with ethyl vanillin were prepared with known amounts of each component present. Serial dilutions of the stock solutions were made over a 10 fold range. To each solution, precise amounts of the appropriate GC internal standard was spiked into each solution such that there was the same amount of the GC internal standard at each level of dilution. For the purposes of the index phenols, trans-cinnamic acid was employed as a GC internal standard, whereas with respect to the model compounds, homovanillic acid was utilized as the internal standard. On the DB-1 column, under the conditions previously described, homovanillic acid coeluted with vanillic acid, one of the index phenols. It was suggested to use trans-cinnamic acid as a substitute (Ertel, personal communication). Trans-cinnamic acid did not interfere with any of the chromatographed index phenols. Ethyl vanillin was employed as a surrogate standard in all samples for the purposes of estimating overall recovery. The concentration of components chosen for preparing the calibration curves covered a range in the area of the initial starting materials employed for alkaline copper oxide degradations.

#### **Extraction Efficiency**

The recovery of the index phenols was accomplished by measuring the extraction efficiency of known amounts of the index phenols through the complete set of post oxidations steps. A solution of index phenols in a 2 N sodium hydroxide was split into equal volume aliquots and titrated with 6 N HCl until a pH of 2.0 was attained. The solution was then extracted with several portions of anhydrous ethyl ether previously treated with

ferrous ammonium sulfate. The ether extracts were dried by being passed through glass columns containing sodium sulfate. The columns were washed with several aliquots of ferrous ammonium sulfate treated ether and the washings were combined with the previously collected extracts. The extracts were concentrated to a volume of approximately two milliliters via rotary evaporation. The concentrate was transferred to preweighed vials and blown to dryness under a gentle stream of nitrogen. The vials were reweighed, capped with teflon line caps and stored at 0°C until analysis by gas chromatography.

### Functional Group Analysis

Functional group analysis for the Lake Drummond fulvic acid was accomplished by scanning infrared spectroscopy, proton nuclear magnetic resonance spectroscopy and carbon-13 nuclear magnetic resonance spectroscopy.

Scanning infrared spectroscopy was performed using a Perkin-Elmer 710B spectrophotometer, a dual beam instrument (Perkin-Elmer, Norwalk, CT). A 3 mg sample of LDFA was combined with 300 mg of KBr (Harshaw Chemical Company, Solon, OH), thoroughly mixed with a mortar and pestle and pressed into a transparent pellet at approximated 2000 psi for 1 minute. A reference spectra of KBr was also obtained. The reference KBr pellet was prepared in the same manner as the LDFA sample, minus the inclusion of fulvic acid. The KBr had been dried overnight in a drying oven at approximately 120°

Proton nuclear magnetic resonance spectroscopy was performed with a 200 MHz Bruker Fourier Transform spectrometer (Bruker Instruments, Inc., Manning Park, Billerica, MA) The sample was prepared by placing 13.1 mg of LDFA in a 5mm (Aldrich premium grade NMR tube and adding 0.6 ml of D<sub>2</sub>O (Sigma Chemical Co., St. Louis, MO) Enough sodium deuteroxide (NaOD) was added to dissolve the sample (Aldrich, Gold Label). A small amount of

tetramethylsilane was added as a reference standard in order to obtain a lock on 0 ppm. A total of 300 scans were acquired and averaged. A 2 second cycle time was employed.

Solid state C-13 nuclear magnetic resonance spectra for Lake Drummond fulvic acid were obtained under identical conditions as described by Norwood (1985).

#### **Elemental Analysis**

Elemental analysis of the Lake Drummond fulvic acid was performed by M-H-W Laboratories, Phoenix, Arizona. The samples were vacuum dried at 60°C to a constant weight prior to analysis

## Results and Discussion

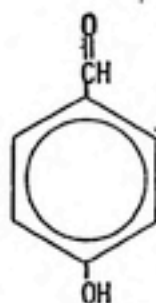
### Index Phenols

A review of the most recent literature on alkaline copper oxide oxidations of aquatic and terrestrial humic substances will reveal the term index phenols, a term coined by Hedges to describe a set of nine substituted phenols that are commonly and consistently found as oxidation products from alkaline copper oxide oxidations of lignin (1975) Figure 4-1). The presence of these phenols amongst the products of humic material subjected to alkaline copper oxide oxidative conditions infers the importance of lignin as a significant or important precursor to humic material formation (Ertel and Hedges, 1981). A major focus of this project was to examine the product mixture obtained from subjecting Lake Drummond fulvic acid to the same alkaline copper oxide oxidative conditions as Ertel and Hedges employed for their studies on lignin (1979). It had previously been demonstrated that the index phenols were relatively stable towards the oxidative conditions employed in the alkaline copper oxide reaction (Hedges, 1975). Since this was the first attempt at alkaline copper oxide oxidations of humic material in our own laboratory facilities, it was appropriate to examine the stability of the index phenols. Approximately 3 milligrams of each compound was employed and when recovery was corrected to that of ethyl vanillin, the surrogate standard, the results indicate nearly quantitative recovery of the p-hydroxyl and vanillyl moieties, however the syringyl compounds survive the oxidation somewhat less than quantitatively (Table 4-1). In his preliminary research on the copper oxide oxidation of the index phenols, Hedges observes the same occurrence (1975). Hedges notes quantitative

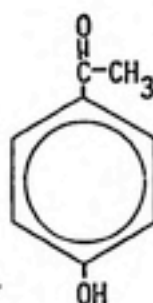


Figure 4-1

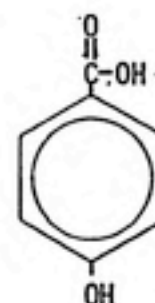
## INDEX PHENOLS



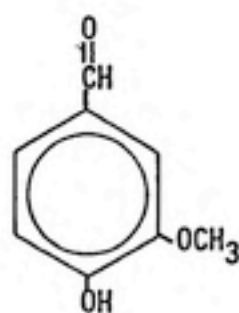
4-Hydroxybenzaldehyde



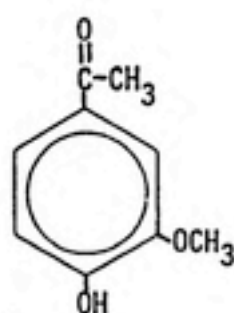
4-Hydroxyacetophenone



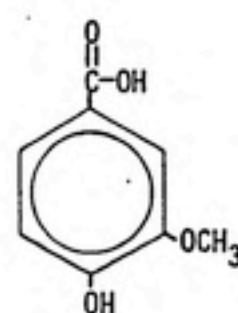
4-Hydroxybenzoic Acid



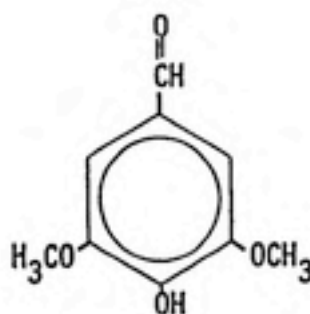
Vanillin



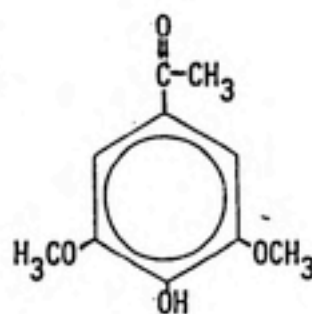
Acetovanillone



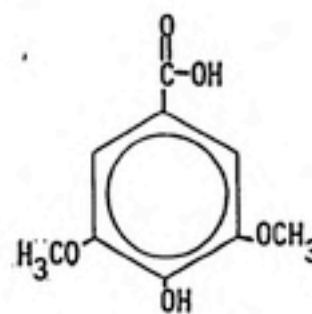
Vanillic Acid



Syringaldehyde

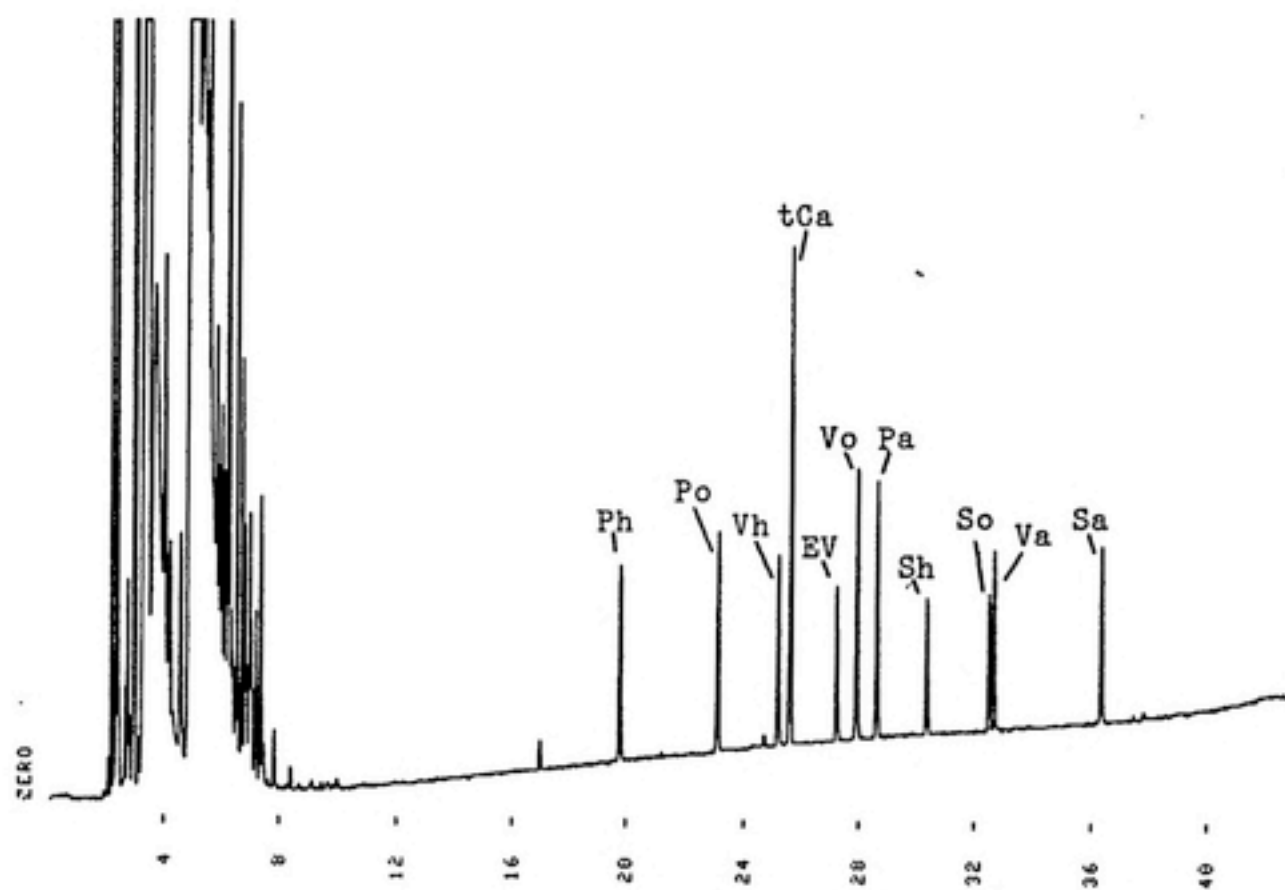


Acetosyringone



Syringic Acid

Figure 4-2  
FID Chromatogram of Index Phenols





recovery for the p-hydroxyl and vanillyl compounds, but only an 84% recovery for the syringyl phenols. The results obtained from this experiment are comparable to those of Hedges, for we see quantitative recovery of the p-hydroxyl and vanillyl moieties and an average recovery of 81% for the syringyl moieties. The losses of syringyl moieties could not be completely ascertained. The possibility of interconversion to vanillyl and p-hydroxyl structures is not precluded, however, it could not be verified by the results from this experiment.

To accompany the copper oxide oxidations of index phenols, the extraction efficiency of these compounds plus the surrogate standard, ethyl vanillin, was investigated. Throughout the course of the post oxidation workup, several steps involving transfers, concentrations and removal of water though the addition of a drying reagent are required. It was of interest to document the recovery of these compounds through the extraction efficiency procedure previously described in the experimental section. From this experiment, one could see how well ethyl vanillin extracts compared to compounds of similar structure and, hence, be able to assess its value as a surrogate standard. Throughout the course of their work involving copper oxide oxidations, Hedges and Ertel employed uniformly ring labeled  $^{14}\text{C}$ -acetophenone for the purposes of calculating recovery. The purchase of uniformly ring labeled  $^{14}\text{C}$ -acetophenone was quite costly, therefore owing to cost restraints, it was decided to employ non-radioactive compound as the surrogate standard. The addition of known amounts of ethyl vanillin to each minibomb following the oxidation was suggested (Hedges, personal communication). The results of the extraction efficiency appear in Table 4-2.

It is obvious that losses occur during the post oxidation work up. The standard deviations associated with the recoveries of the compounds are inflated to a greater degree largely because of the high values associated

Table 4-1Percent Recovery of Index Phenols from Cupric Oxide Oxidation

	<u>Percent Recovery</u>
4-Hydroxybenzaldehyde	99.3%
4-Hydroxyacetophenone	104.6%
4-Hydroxybenzoic Acid	102.9%
Vanillin	98.8%
Acetovanillone	101.3%
Vanillic Acid	106.9%
Syringaldehyde	80.3%
Acetosyringone	93.4%
Syringic Acid	70.0%

**Table 4-2****Index Phenol Extraction Efficiency Data****Percent Recovery for Five Extractions**

	<u>Sample 1</u>	<u>Sample 2</u>	<u>Sample 3</u>	<u>Sample 4</u>	<u>Sample 5</u>	<u>Mean &amp; Std. Dev.</u>
4-Hydroxybenzaldehyde	75.4	68.8	80.4	78.8	114.6	83.6+17.9
4-Hydroxyacetophenone	77.3	69.2	83.4	81.0	117.1	85.6+18.4
4-Hydroxybenzoic Acid	91.9	69.6	87.2	79.2	112.9	88.2+16.1
Vanillin	61.1	50.1	63.8	68.2	96.3	67.9+17.2
Acetovanillone	73.7	62.3	72.2	75.5	108.7	78.5+17.6
Vanillic Acid	90.5	67.2	92.2	80.4	116.3	89.3+18.1
Syringaldehyde	70.0	63.2	70.6	68.6	110.5	76.6+19.2
Acetosyringone	67.4	59.0	75.2	72.4	103.5	75.5+16.8
Syringic Acid	66.5	47.8	72.3	65.4	93.5	69.1+16.4
Ethyl Vanillin	64.0	60.5	65.1	70.5	100.7	72.2+16.4

with sample 5. It is also seen that ethyl vanillin extracts and is recovered in quantities similar to that of the other compounds and its use as a surrogate standard for the purposes of quantitative is justified.

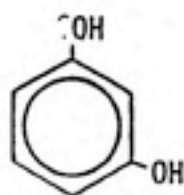
### **Model Compounds**

A series of model compounds were chosen and subjected to the oxidative conditions of the alkaline copper oxide reaction previously described. This set of experiments was performed to investigate the relative stability of some simple compounds which had been reported in the literature as alkaline copper oxide oxidation products of humic material. Additionally, related compounds which had not been cited as products, but which were structurally similar to those reported, were also employed.

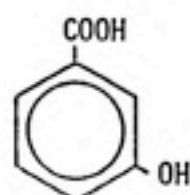
A paucity of data concerning model compound work in relation to alkaline copper oxide oxidations on humic material exists, however, that which has been found deserves mention. Christman and Ghassemi performed alkaline copper oxide oxidations on a series of phenolic and flavenoid compounds. For the substituted phenolic compounds, it was shown that cleavage of the alkyl side chain depends upon the hydroxyl substitution pattern on the ring (1966). The authors stated that the alkyl side chain is more easily cleaved to a one carbon acid function when the appropriate activator resides in a position ortho or para to the alkyl side chain. Results from the flavenoid model compound work indicated that the flavenoid structure is oxidized to more simple phenolic compounds. It must be added that the mode of detection and identification employed consisted of separation of the components by thin layer chromatography followed by spraying with specific indicator reagents.

Hedges subjected the index phenols to the oxidative conditions of the alkaline copper oxide oxidation reaction. He noted that there was approximately a 10% conversion of the aldehydes to their corresponding

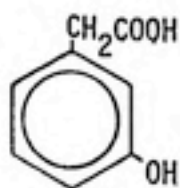
## GROUP III MODEL COMPOUNDS



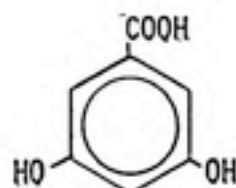
1,3-Dihydroxybenzene



3-Hydroxybenzoic Acid

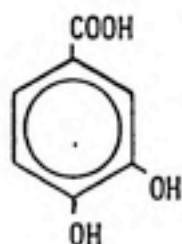


3-Hydroxyphenylacetic Acid

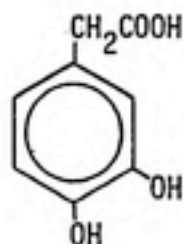
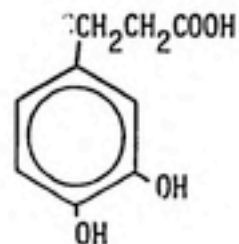
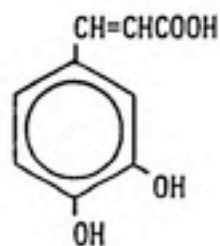


3,5-Dihydroxybenzoic Acid

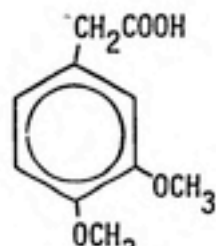
## GROUP IV MODEL COMPOUNDS



3,4-Dihydroxybenzoic Acid

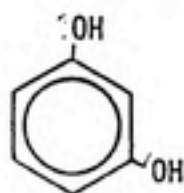
3,4-Dihydroxy-  
phenylacetic Acid3,4-Dihydroxy-  
hydrocinnamic Acid

3,4-Dihydroxycinnamic Acid

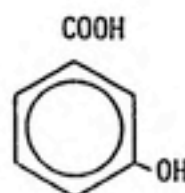


3,4-Dimethoxyphenylacetic Acid

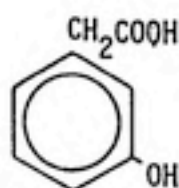
Figure 4-3-(cont.)  
GROUP III MODEL COMPOUNDS



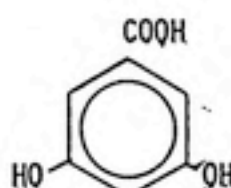
1,3-Dihydroxybenzene



3-Hydroxybenzoic Acid

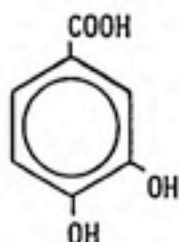


3-Hydroxyphenylacetic Acid

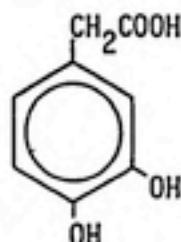


3,5-Dihydroxybenzoic Acid

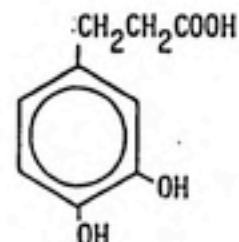
GROUP IV MODEL COMPOUNDS



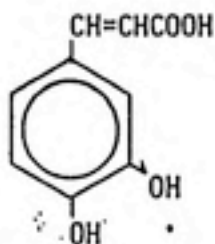
3,4-Dihydroxybenzoic Acid



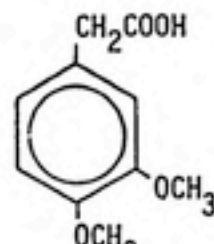
3,4-Dihydroxy-  
phenylacetic Acid



3,4-Dihydroxy-  
hydrocinnamic Acid

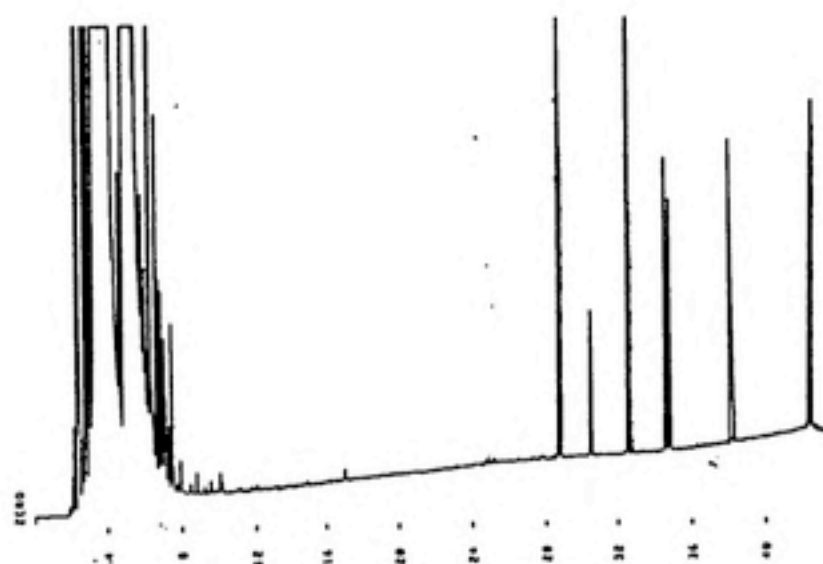


3,4-Dihydroxycinnamic Acid

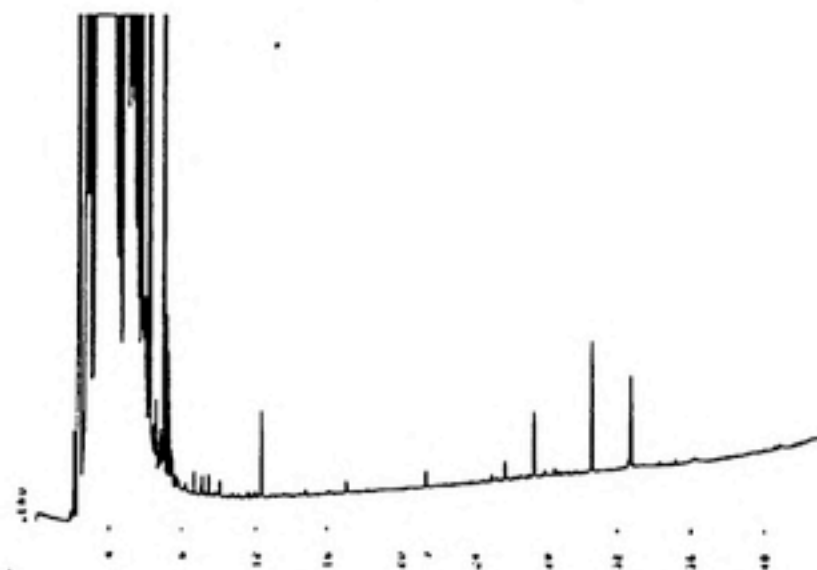


3,4-Dimethoxyphenylacetic Acid

Figure 4-4  
FID Chromatograms of Group IV Model Compounds



Before Oxidation



After Oxidation



acidic form (1975). Under identical conditions, employing syringyl propionic acid as the sole starting material the major oxidation products recovered included syringaldehyde, acetosyringone, and syringic acid. Ferrulic acid was oxidized to vanillin and tyrosine was converted to p-hydroxybenzaldehyde and p-hydroxybenzoic acid. The model compounds chosen for the purposes of this study were all alkyl benzene moieties with various hydroxyl and methoxyl substitution patterns except for one class which did not have any hydroxyl or methoxyl substituents.

The series of model compounds were placed into four categories, Group I through Group IV, according to their structure (Figure 4-3). Percent recovery data for the bombed model compounds appear in Table 4-3. Examination of the results indicates that certain compounds are more resistant to the oxidative conditions than others.

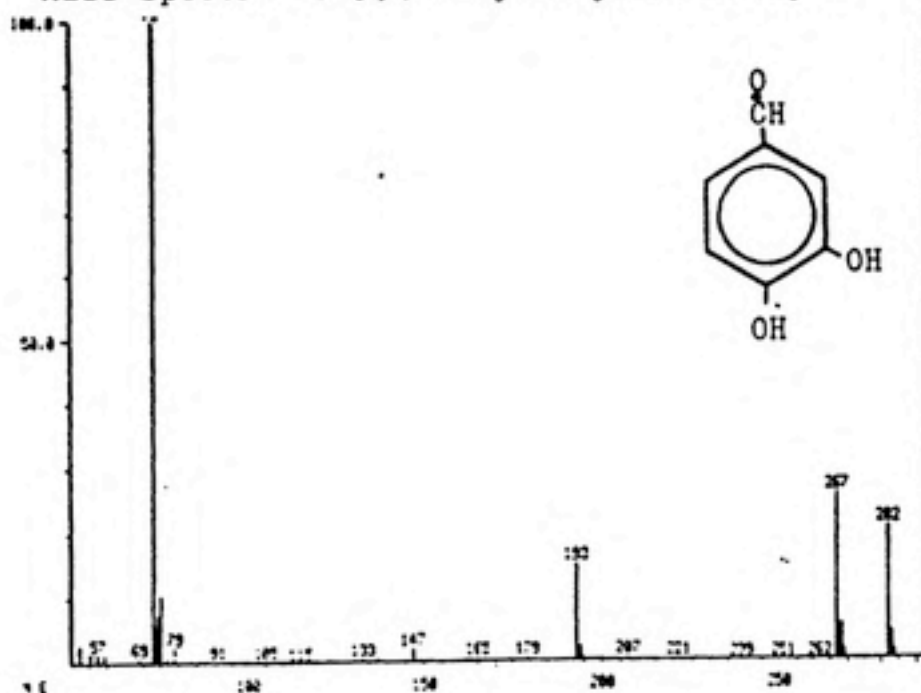
With regard to the data obtained for the Group I model compounds, a note of caution must be made concerning interpretation of the result. One of the model compounds from this class, 3-(4-hydroxyphenyl)propionic acid is not baseline resolved from the internal standard, homovanillic acid, and therefore, these results are somewhat suspect. The choice of another internal standard would clarify this situation.

Examination of the results for the Group II model compounds indicate that these compounds withstand the oxidative condition quite well. Nearly quantitative recovery of the compounds is attained (Table 4-3).

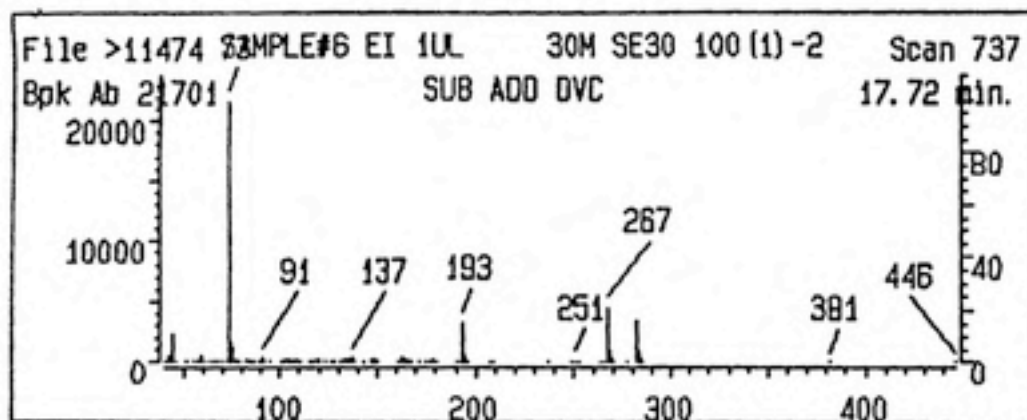
The results for the Group III model compounds readily indicate that 1,3-dihydroxybenzene is greatly affected by the oxidative conditions associated with the reaction. Only 22% of original starting material normalized to the internal standard is recovered, whereas the other Group III model compounds are recovered almost quantitatively. Mechanisms could be proposed with regard to the fate of 1,3-dihydroxybenzene under these conditions, however, based on the data obtained, these mechanisms

Figure 4-5

Mass Spectra of 3,4-Dihydroxybenzaldehyde



Standard



Reaction product obtained from CuO oxidation  
of 3,4-Dihydroxy substituted alkyl benzenes.

would be speculative at best. A further experiment employing 1,3-dihydroxybenzene as the sole starting material would be required to obtain the necessary data from which one could propose plausible mechanisms concerning the fate of this structure. Upon examination of the data for the Group IV model compounds, one sees that the 3,4-dihydroxy moieties are not recovered after the reaction (Figure 4-4). This is readily apparent from comparison of the chromatograms for a stock solution of the compounds and that for the products obtained following the reaction. Aside from the internal and surrogate standards, there are two peaks present. One of them matches the retention index for 3,4-dimethoxyphenyl acetic acid, however, the second peak does not match the retention index for any of the starting material. Analysis by combined gas chromatography mass spectrometry yielded an empirical formula of  $C_{13}H_{22}Si_2O_3$  and a formula weight of 282. Analysis of an authentic sample of 3,4-dihydroxy-benzaldehyde provided a match for both the g.c. retention index and mass spectrum of the secondary oxidation product obtained from the alkaline copper oxide oxidation of the Group IV model compounds (Figure 4-5).

From their review on soil organic colloids, Hayes and Swift suggest a one electron transfer mechanism which proceeds through the formation of a quinonemethide intermediate en route to the formation of an aldehyde as the product.

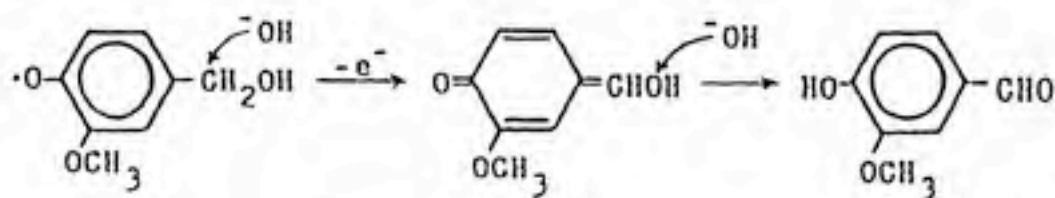


Figure 4-6

Although this mechanism begins with a benzyl alcohol it does emphasize the importance of the hydroxyl group in the para position. Referring to

Table 4-3Percent Recovery of Model Compounds from Cupric Oxide Oxidation

	<u>Percent Recovery</u>
<u>Group I Model Compounds</u>	
2-Hydroxybenzoic Acid	98.0%
4-Hydroxybenzoic Acid	94.4%
4-Hydroxyphenylacetic Acid	78.0%
3-(4-Hydroxyphenyl)propionic Acid	23.7%
<u>Group II Model Compounds</u>	
Phenylacetic Acid	91.6%
Hydrocinnamic Acid	102.7%
Cinnamic Acid	90.7%
<u>Group III Model Compounds</u>	
1,3-Dihydroxybenzene	21.7%
3-Hydroxybenzoic Acid	116.2%
3-Hydroxyphenylacetic Acid	94.6%
3,5-Hydroxybenzoic Acid	102.6%
<u>Group IV Model Compound</u>	
3,4-Dimethoxyphenylacetic Acid	86.0%
3,4-Dihydroxybenzoic Acid	*
3,4-Dihydroxyphenylacetic Acid	*
3,4-Dihydroxyhydrocinnamic Acid	*
3,4-Dihydroxycinnamic Acid	*

\*Not Recovered

Christman and Ghassemi's premise that cleavage of the alkyl side chain will occur when the appropriate activating groups are present on the aromatic ring, it is evident from this study that hydroxyls present in both the meta and para positions exert a greater influence towards cleavage of the alkyl side chain than when present in either the ortho, meta, or para position alone. However, since the data concerning the Group I model compounds should be viewed cautiously, the influence of the ortho and para positions upon cleavage of the alkyl side chain cannot be stated with great certainty. It is also evident that the 3,4-dimethoxy substitution pattern is less activating than the 3,4-dihydroxy substitution pattern.

This is consistent with electrophilic aromatic substituent theory which places the hydroxyl group as a strongly activating ortho-para director and the methoxyl group as only a moderately activating ortho-para director (Solomons, 1976). This previous statement does not suggest that the alkaline copper oxide reaction is classified as an electrophilic aromatic substitution reaction, rather, it merely indicates that the hydroxyl group is believed to be a more potent activator of the aromatic ring than the methoxyl group.

The results from the Group IV model compounds study indicate that 3,4-dihydroxy substituted alkyl benzene may be present in macromolecular compounds such as lignin and humic materials, however, these may not be seen amongst the reaction products from alkaline copper oxide degradations due to secondary reactions occurring during the oxidation. It must be emphasized that the compounds employed are simple structures and are devoid of any near neighbor chemistry such as resonance and/or inductive effects of adjacent moieties which may occur during the oxidation with macromolecular compounds.



### Alkaline Cupric Oxidation of Lake Drummond Fulvic Acid

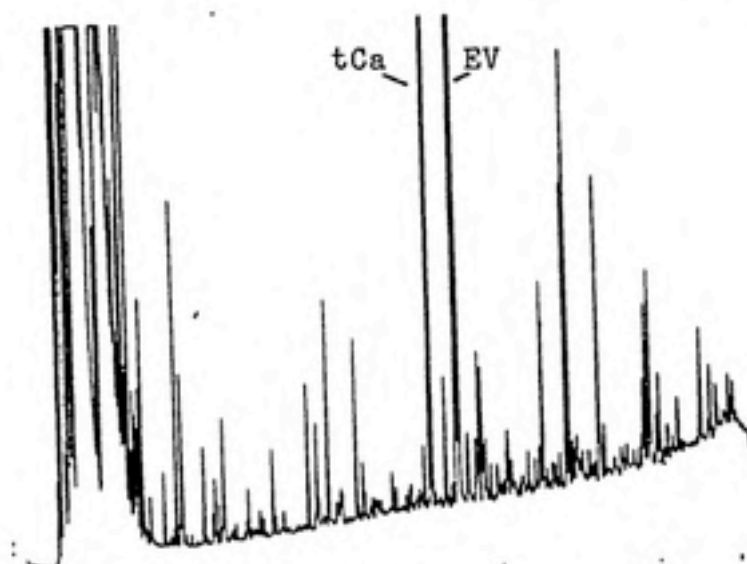
The results of the copper oxide oxidations of Lake Drummond fulvic acid revealed the presence of numerous and diverse structural entities in the product mixture. Both aliphatic and aromatic structures are found and those identified by gas chromatography and combined gas chromatography/mass spectroscopy appear in Table 4-4. This list of compounds comprises approximately 31.4% of the FID chromatogram beyond the large solvent front (Figure 4-7).

Analysis of the data presented in Table 4-4 reveals that the short chain monobasic and dibasic aliphatic acids account for 12.9% of the total peaks present or 41% of the identified products. This indicates that the majority of identified products are aromatic structures. It is interesting to note that the longest aliphatic structures identified, succinic and maleic acids, are only four carbon atoms in length. This is consistent with the premise that the fulvic acid contains shorter chain linkages compared to humic acid, thus making the fulvic acid more soluble and less hydrophobic than humic acid (Christman, 1983, Liao, 1982).

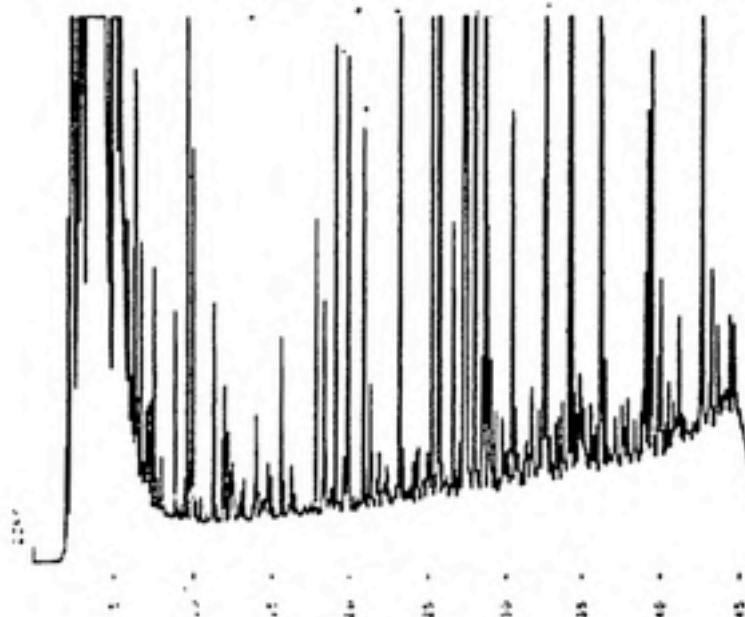
The aromatic moieties comprising the remainder of the product mixture consist primarily of substituted phenols. Many, but not all, of the index phenols described by Hedges were found. Exceptions to this group included syringaldehyde and acetosyringone.

The presence of the index phenols is generally regarded as evidence of lignin as a precursor to humic material formation (Ertel and Hedges, 1981, 1984). The presence of the p-hydroxyl phenols does not necessarily indicate lignin as a precursor of humic material for these phenols are not exclusively lignin derived, although they are seen as copper oxide oxidation products of non-woody angiosperm tissues and gymnosperm

Figure 4-7  
FID Chromatograms for LDFA Products from CuO Oxidation



With standards Ethyl Vanillin and trans-Cinnamic Acid



Above, spiked with Index Phenols

**TABLE 4-4****CuO Oxidation Products from Lake Drummond Fulvic Acid**

Compound	Retention Index	%Relative Yield
1. Lactic Acid	0.347	3.54
2. alpha-Hydroxyisobutyric Acid	0.359	2.86
3. Levulenic Acid	0.408	1.22
4. alpha-Hydroxybutyric Acid	0.428	0.42
5. Malonic Acid	0.508	0.63
6. Benzoic Acid	0.566	1.19
7. Succinic Acid	0.649	1.82
8. Methyl Succinic Acid	0.670	1.41
9. Maleic Acid	0.684	2.86
10. 4-Hydroxybenzaldehyde	0.720	0.46
11. 1,3-Dihydroxybenzene	0.778	0.81
12. 4-Hydroxyacetophenone	0.848	0.55
13. Vanillin	0.922	0.98
14. 2-Hydroxybenzoic Acid	0.973	0.19
15. Acetovanillone	1.023	1.39
16. 3-Hydroxybenzoic Acid	1.041	0.93
17. 3,4-Dihydroxybenaldehyde	1.045	0.47
18. 4-Hydroxybenzoic Acid	1.049	2.08
19. Phthalic Acid	1.114	1.65
20. Tricarballic Acid	1.178	0.57
21. Isophthalic Acid	1.196	3.36
22. Vanillic Acid	1.206	0.42
23. Terephthalic Acid	1.237	0.65
24. 3,5-Dihydroxybenzoic Acid	1.258	5.79
25. Syringic Acid	1.340	0.88

Retention Index - retention time of analyte relative to that for the surrogate standard, ethyl vanillin. Retention indices reported were obtained from GC data.

% Relative Yield - peak area ratio of analyte versus chromatogram

woods (Ertel and Hedges, 1984). It has been demonstrated that the various vanillyl and syringyl moieties comprising the remainder of the index phenols are lignin derived. The vanillyl structures are copper oxide oxidation products of gymnosperm woody tissues and syringyl moieties are derived from angiosperms. The distributions of these phenols generally reflect the vegetative patterns indigenous to the area from which the humic material is obtained.

Distributions of the different forms of the phenols mentioned within the types of humic material analyzed, i.e., humic acid versus fulvic acid, may also vary. Humic acids generally produce higher aldehyde/acid ratios than the corresponding fulvic acids. Also, it has been shown that fulvic acids produce a greater amount of p-hydroxyl phenols compared to vanillyl and syringyl phenols than the corresponding humic acid (Ertel and Hedges, 1984). This helps to explain the fact that syringaldehyde and acetosyringone are not seen amongst the copper oxide oxidation products of Lake Drummond fulvic acid.

An interesting product which was found amongst many products identified was 3,4-dihydroxybenzaldehyde. Schnitzer and Ortiz de la Serra reported seeing 3,4-dimethoxybenzaldehyde as an alkaline copper oxide oxidation product of a terrestrial humic acid (1973). The products characterized by these authors were methylated following isolation and prior to analysis by GC/MS. Therefore, the possibility exists that 3,4-dihydroxybenzaldehyde was amongst the products isolated, however, since the products were methylated prior to analysis, this position cannot be ascertained. Had the products been analyzed as trimethylsilyl derivatives, the presence of 3,4-dihydroxybenzaldehyde as an oxidation product could be stated more confidently. The significance of 3,4-dihydroxybenzaldehyde as a product of the alkaline copper oxidation of the fulvic acid is emphasized by the results of the alkaline copper oxide

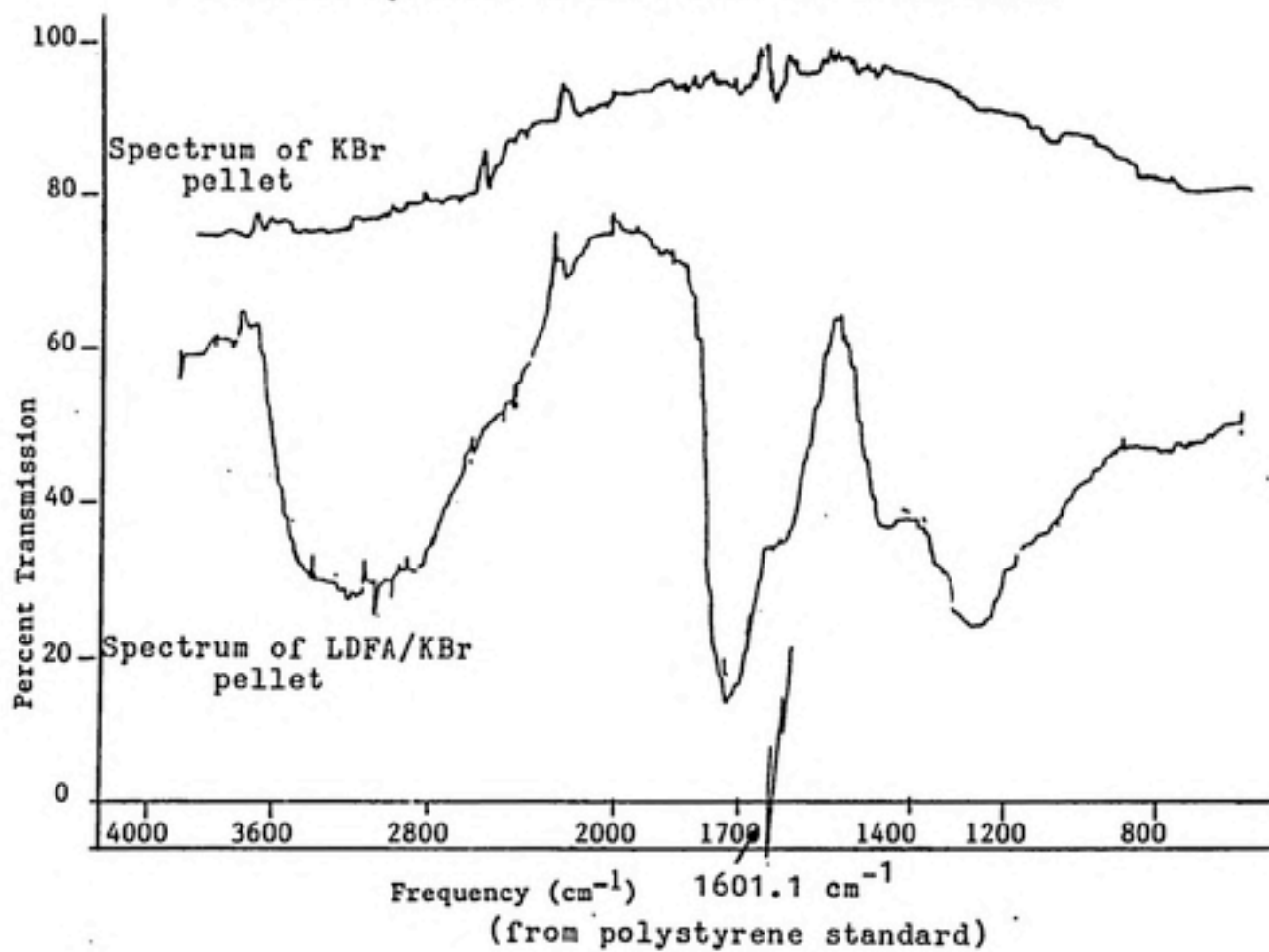
oxidation of the Group IV model compounds. The results of that experiment indicated that 3,4-dihydroxyphenyl alkanoic moieties do not survive the oxidative conditions of the alkaline copper oxide reaction, rather a secondary oxidation product results, namely 3,4-dihydroxybenzaldehyde. It is therefore possible that 3,4-dihydroxyphenylalkanoic moieties may be part of the macrostructure of the humic material, however, upon their release during the alkaline copper oxide oxidation, they are further acted upon producing 3,4-dihydroxybenzaldehyde as one of the products. It must be emphasized that this process is not quantitative and that other competing or subsequent reactions are most likely occurring, further oxidizing these structures.

It was noted during the course of experimentation that some color remained on the sodium sulfate drying column and that this color was not easily removed by diethyl ether, however, a more polar solvent such as methanol would elute the color from the drying columns. The products analyzed in these sets of experiments and those reported by previous investigators are for the most part, the ether extractable organics. It would be of interest to choose an inert polar solvent (more polar than diethyl ether) that would elute the color remaining on the column and see what the product composition of this fraction would be.

### Infrared Spectroscopy

Another approach to functional group analysis of the undegraded Lake Drummond fulvic acid is provided by infrared spectroscopy. The IR spectra for Lake Drummond fulvic acid and potassium bromide appear in Figure 4-8. Several investigators have assigned certain frequencies to various functional groups (Neyroud and Schnitzer, 1974, Thurman and Malcolm, 1985). Hydrogen bonded hydroxyls appear around  $3400\text{ cm}^{-1}$ . As is seen in

Figure 4-8  
Infrared Spectrum of Lake Drummond Fulvic Acid





**Table 4-5****Elemental Composition of Lake Drummond Fulvic Acid**

<u>Sample</u>	<u>%C</u>	<u>%H</u>	<u>%N</u>	<u>%Ash</u>
LDFA 1	50.86	4.09	1.01	0.16
LDFA 2	57.00	5.49	1.07	NIL
LDFA 3	<u>55.15</u>	<u>5.24</u>	<u>0.82</u>	<u>0.40</u>
Mean	54.34	4.94	0.97	0.19

H/C=1.09

C/N=65.36

comparison of the spectrum for the KBr pellet versus that for the fulvic acid, the presence of the hydrogen bonded hydroxyls is a contribution from the fulvic acid. Aromatic C-H stretching appears around  $3000\text{ cm}^{-1}$  and aliphatic C-H stretching occurs near  $2960\text{ cm}^{-1}$  and  $2840\text{ cm}^{-1}$ . An intense signal at  $1720\text{ cm}^{-1}$  is due to carbonyl stretching. A shoulder near  $1620\text{ cm}^{-1}$  may be due to aromatic C=C possibly conjugated to C=O. Aliphatic C-H bending should occur at  $1440\text{ cm}^{-1}$  and C-O-C stretching due to ethers appears at  $1250\text{ cm}^{-1}$ . The infrared spectrum for Lake Drummond fulvic acid is very similar to that for Lake Singletary fulvic acid (Norwood, 1986).

### Elemental Analysis

The results of elemental analysis appear in Table 4-5. The ash content is very low, less than 1%. Also the nitrogen content is low, typical of fulvic acids (Thurman and Malcolm, 1983).

### NMR Spectroscopic Analysis of Lake Drummond Fulvic Acid

Another set of experiments performed to complement the information obtained from the GC/MS analysis of the product mixture obtained from the cupric oxide oxidations of Lake Drummond fulvic acid, was solid state  $^{13}\text{C}$  NMR analysis of the intact fulvic acid. CP/MAS spectra, with and without dipolar dephasing were acquired (Figures 4-9, 4-10). An informative and concise treatise concerning the principles of both C-13 NMR and proton NMR is provided by Wilson (1981). For details concerning the acquisition of the spectra, the reader is referred to Norwood (1985).

Whereas one obtains information concerning the subunit structure of the fulvic acid from analysis of degradation mixtures, information about

the carbon skeleton of the fulvic acid can be obtained from analysis of spectra obtained from various solid state  $^{13}\text{C}$  NMR experiments. The analysis of the spectrum follows the format provided by Hatcher (1981). The spectrum is divided into four regions, each region representative of particular types of carbon moieties. The percent contributions for each region appear in Table 4-6A.

Region I (0 to 50 ppm) consists primarily of paraffinic carbon structures and will contain the signals arising from carbon-carbon single bonds from polymethylene structures and those from terminal methyl groups. Integration of the spectrum reveals a contribution of 21% of the total carbon of the fulvic acid present in this region. Reference to the dipolar dephased spectrum, a prominent peak centered at 20 ppm is indicative of terminal methyl groups present.

Region II (50 to 110 ppm) contains the resonances resulting from carbons bonded to heteroatoms such as oxygen and nitrogen. The bond order of the carbon-heteroatom linkage consists of single bonds and structures representative of the signals one observes in this region, include alcohols, ethers, carbohydrates, acetals and methoxyls. A contribution of 23% of the total carbon contained within the intact fulvic acid was observed for this region. Several resonances which are characteristic of particular structural entities which may or may not be present are worth noting. Wilson et al., indicate the presence of carbohydrates by the occurrence of resonances at 72 ppm and 104 ppm. The signal occurring at 72 ppm is assigned to O-alkyl carbons and that at 104 ppm is due to the presence of dioxygenated carbon structures. The authors further note that these resonances can be more definitively assigned through the use of a dipolar dephase difference spectrum. Also discussed is the signal present at 55 ppm, which the authors attribute to methoxyl carbons ( $\text{O}-\text{CH}_3$ ). With respect to the LDFA CP/MAS spectrum, a shoulder,

rather than a distinct peak is present at 55 ppm. However, in the dipolar dephased spectrum ( $\tau=40$   $\mu\text{sec}$ ), a broad peak centered about 55 ppm is observed. This is probably due to the presence of methoxyl carbons.

Resonances observed from 110 ppm to 160 ppm (Region III) arise primarily from aromatic and olefinic carbon. Results from degradation studies have emphasized the importance of aromatic moieties as major structural constituents of humic material (Liao et al., 1981, Schnitzer and DesJardin, 1970). With the advent of solid state C-13 NMR and the associated techniques of CP/MAS and dipolar dephasing, one can confidently obtain an estimate of aromatic carbon present in the intact undegraded humic material. However, when one views the percent contribution of aromatic/olefinic carbon and compares it to that which is obtained from degradation studies, a disparity between the results from the two approaches is readily evident.

Permanganate oxidations produce mainly benzene carboxylic acids as the major products (Liao et al., 1981) and alkaline copper oxide oxidations show a wide spectrum of phenolic structures (Ertel and Hedges, 1984). The large aliphatic content not detected by chemical degradations is seen via CP/MAS and further emphasized by dipolar dephased spectra. The increase in observable aliphatic content therefore indicates that the aromatic content is lower than previously thought. Region III contributes 27% of the total signal observed in the spectrum. If one assumes that the signal observed in this region is primarily aromatic, then one observes four to five benzene rings per 100 carbons.

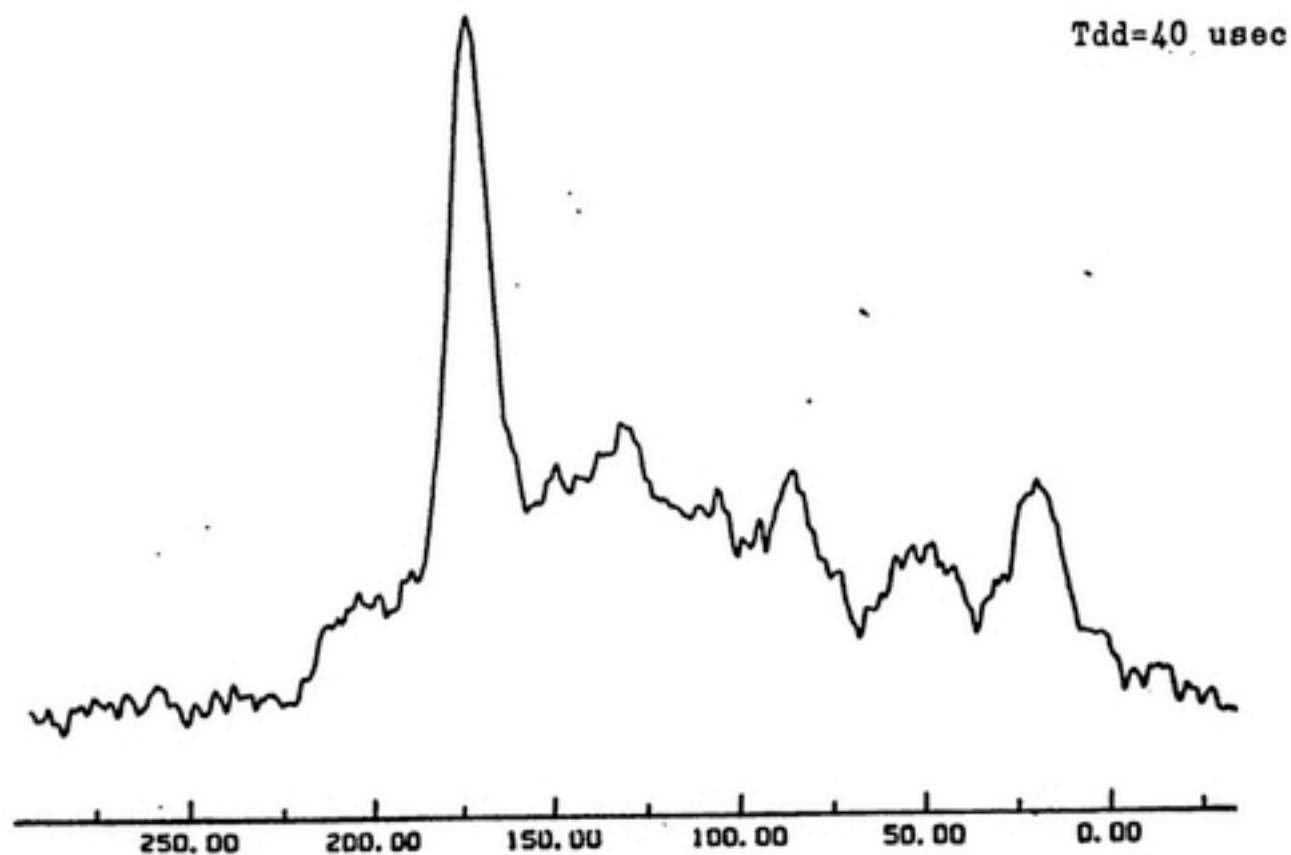
Upfield from the aromatic/olefinic region, one observes a large resonance centered about 173 ppm and a cluster of lesser signals centered approximately at 190 ppm. These signals arise from carbonyl and carboxyl carbons. Contained within this region, Region IV (160 ppm to 220 ppm), are the resonances characteristic of carboxylic acids, esters, amides,

ketones and aldehydes. The large peak centered about 173 ppm is assigned to carboxyl carbons which one may find in structures such as carboxylic acids and esters. The cluster of resonances in the area from 180 to 210 ppm are most likely due to the presence of carbonyl carbons, which are found in structures such as ketones and aldehydes. This region contributes 29% of the total signal observed in the spectrum.

A dipolar dephased spectrum with a  $T_{dd} = 40 \mu$  sec is provided in Figure 4-9. Comparing this spectrum to that of Figure 4-10, one sees a dramatic decrease in area over regions I, II, and III. The dipolar dephased spectrum allows one to view the contribution arising solely from the carbon backbone with any coupling effects due to the presence of attached protons removed. One may note a prominent peak in region I of the dipolar dephased spectrum centered about 42 ppm. This signal arises from rapid spinning terminal methyl groups present in the solid fulvic acid. Due to their rapidly spinning, the coupling of these protons to carbons is not completely disappated.

To complement the information obtained from the C-13 spectra, a proton spectrum was also obtained and appears as Figure 4-11. Percent contributions for various regions of the spectrum appear in Table 4-6B. The format presented and analysis of the spectrum follows that provided by Norwood (1985). In region I, one is primarily observing the signal generated from the presence of protons on terminal methyl, methylene, and methine groups on carbons at least one carbon atom removed from deshielding influences such as ether linkages or carboxyl groups. Region II consists of resonances of mostly methyl and methylene protons on carbons adjacent to carboxyl, aromatic or ether moieties. Methine protons, attached to carbons adjacent to these groups resonate at a lower field and appear in region III. Phenolic - OH protons, some alcohol - OH protons and protons attached to carbons of unsaturated linkages also appear in region

Figure 4-10  
 $^{13}\text{C}$  NMR Dipolar Dephased Spectrum of Lake  
Drummond Fulvic Acid





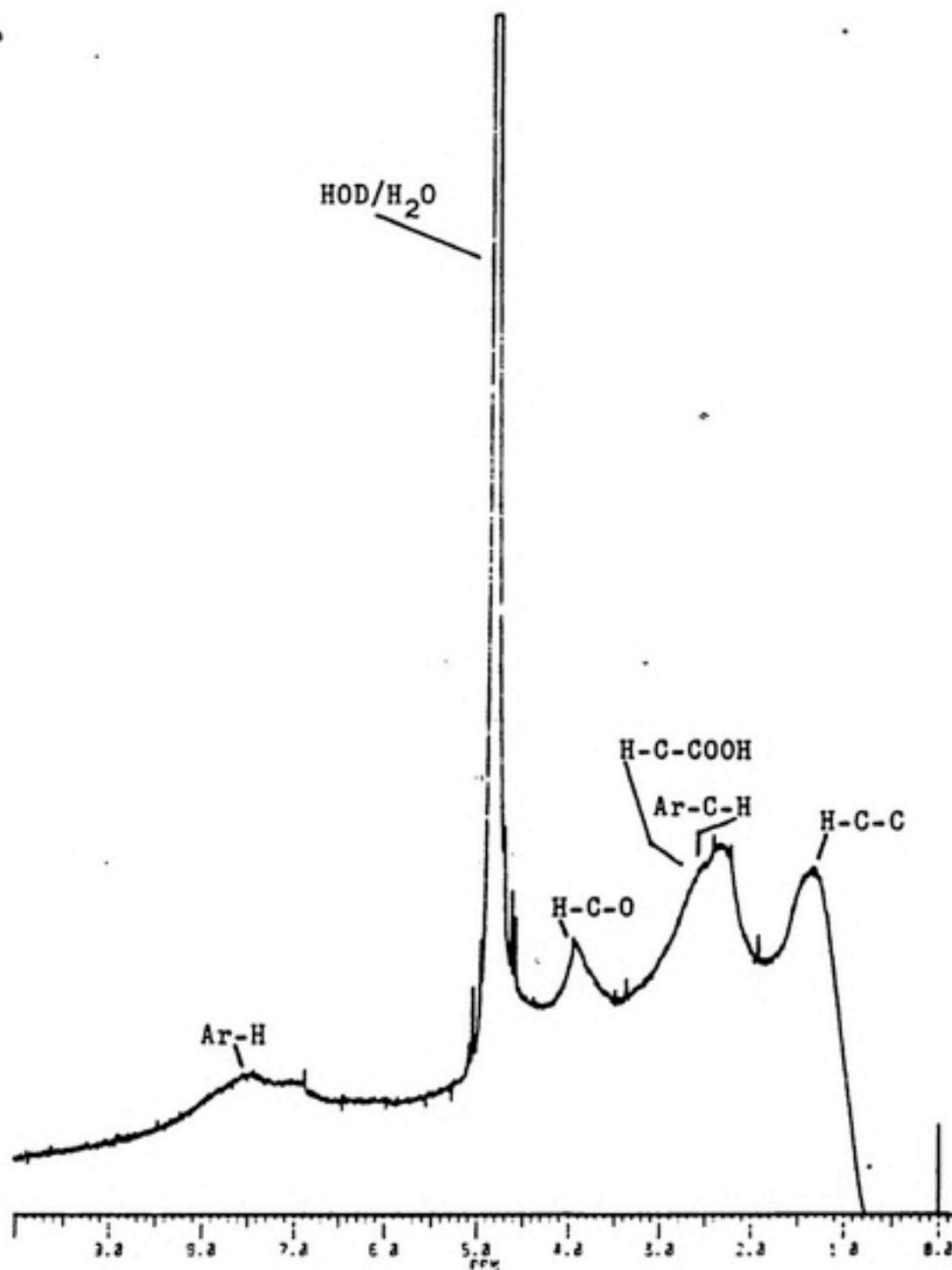
**TABLE 4-6A****C-13 NMR Chemical Shift Regions**

		<u>%Contribution</u>
Region I	0 to 50 ppm	21%
Region II	50 to 110 ppm	23%
Region III	110 to 160 ppm	27%
Region IV	160 to 220 ppm	29%

**TABLE 4-6B****Proton NMR Chemical Shift Regions**

		<u>%Contribution</u>
Region I	0.8 ppm to 1.7 ppm	19%
Region II	1.7 ppm to 3.3 ppm	42%
Region III	3.3 ppm to 4.6 ppm	26%
Region IV	6.5 ppm to 8.1 ppm	13%

Figure 4-11  
 $^1\text{H}$  NMR Spectrum of Lake Drummond Fulvic Acid



III. The large peak centered about 4.8 ppm is due to the presence of  $\text{H}_2\text{O}$  and HOD. Aromatic protons appear in Region IV.

There appears to be very good agreement between the two sets of spectra. Also, with an average of four to five aromatic rings per one hundred carbon atoms and a low percent contribution of signal from the aromatic region of the proton spectrum, it appears as though the aromatic centers are fairly heavily substituted, with approximately three substitutions per ring.

The results presented from the C-13 solid state and proton NMR spectra indicate that the Lake Drummond fulvic acid possesses a great deal of aliphatic structure and the carbonyl and carboxyl content, as seen in the C-13 spectra is significant. The aromatic contribution is significantly less than that postulated from wet chemical methods. The NMR spectra have provided us with important information concerning gross structural features of the intact undegraded fulvic acid and, when coupled with the information from the degradation studies, one may get a clearer picture of the structure of the fulvic acid than that provided from the information of either set of experiments alone.

## Conclusions and Recommendations

Alkaline cupric oxide oxidation of the index phenols revealed that these structures are quite stable under the oxidative conditions employed. Whereas the p-hydroxyl and vanillyl moieties are recovered quantitatively, the syringyl phenols show an average recovery of 81% (the range is 70.0% to 93.4%). To better ascertain the fate of the syringyl moieties, these phenols should be run alone and the product mixture analyzed to see if alkyl side chain cleavage is occurring.

The extraction efficiency data for the index phenols and ethyl vanillin indicates that there is a wide range in the percent recoveries for these compounds and that recovery is generally less than quantitative. It does show that ethyl vanillin is recovered in amounts comparable to the other index phenols and its use as a surrogate standard is justified. This provides one with a more cost effective surrogate standard than that used by Ertel and Hedges, namely uniformly ring labeled  $^{14}\text{C}$ -acetophenone. The necessary precautions to be taken when handling radioisotopes are also eliminated. The numerous transfer, drying and concentration steps employed in the post oxidation workup contribute to the less than quantitative recoveries.

The model compound work has provided us with some insight regarding reactivity of simple phenolic and phenyl alkanoic structures. Although the results of the Group I model compounds suggest that cleavage of the alkyl side chain may be occurring, these results should be viewed cautiously, for one of the compounds, 3-(4-hydroxyphenyl) propionic acid is not completely baseline resolved from the internal standard, homovanillic

acid. The choice of another internal standard would eliminate this uncertainty. The Group II and Group III model compounds are recovered almost quantitatively, with the exception of 1,3-dihydroxybenzene. Another experiment involving 1,3-dihydroxybenzene as the sole starting material would be necessary to fully address the fate of this compound. Mechanisms proposed at this point regarding the fate of 1,3-dihydroxybenzene would be speculative at best. The 3,4-dihydroxy substitution pattern of the Group IV model compounds proved to be very unstable towards the oxidative conditions employed. None of moieties were recovered, rather, a new structure, 3,4-dihydroxybenzaldehyde, appears amongst the products. The 3,4-dimethoxyphenylacetic acid is relatively unaffected by the oxidative conditions, being recovered at a yield of 86.0%. These findings suggest that 3,4-dihydroxyphenyl alkanolic structures may be present in the intact undegraded fulvic acid, however, due to their instability under the oxidative conditions employed, these structures would not be seen amongst the isolated products. However, the results from the model studies cannot account for other influences such as resonance stabilization and inductive effects which may be part of the chemistry of the macromolecular structure.

Analysis of the alkaline cupric oxidation products of the Lake Drummond fulvic acid has provided us with additional information concerning structures associated with aquatic fulvic acids. Norwood performed the same reactions on fulvic acid isolated from Lake Singletary, NC. We now have databases for fulvic acid isolated from two different sources and the results can be compared directly. The list of products and their relative yields are very similar. The product composition for Lake Drummond fulvic acid suggests that the major portion (59%) of the fulvic acid is comprised of aromatic structure and the lesser portion is made of of aliphatic moieties. The largest aliphatic moieties are four carbon and

five carbon atoms in length. It is noted that the products reported are the ether extractable products. Apparently, more polar materials remain on the sodium sulfate drying columns and analysis of these products would require employing an inert, more polar solvent for elution purposes. This would be an interesting endeavor for future alkaline cupric oxide oxidation experimentation on humic material.

The results of the solid state  $^{13}\text{C}$  and the proton NMR experiments provide us with important information concerning the gross overall structure of the intact undegraded fulvic acid. The results indicate that the aliphatic contribution is much greater than that previously thought through the results from wet chemical methods. The aromatic contribution is much less than earlier investigators believed and the aromatic structures have approximately three substitutions per benzene ring.



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Appendix

OLD COUNCIL TREE  
BOND

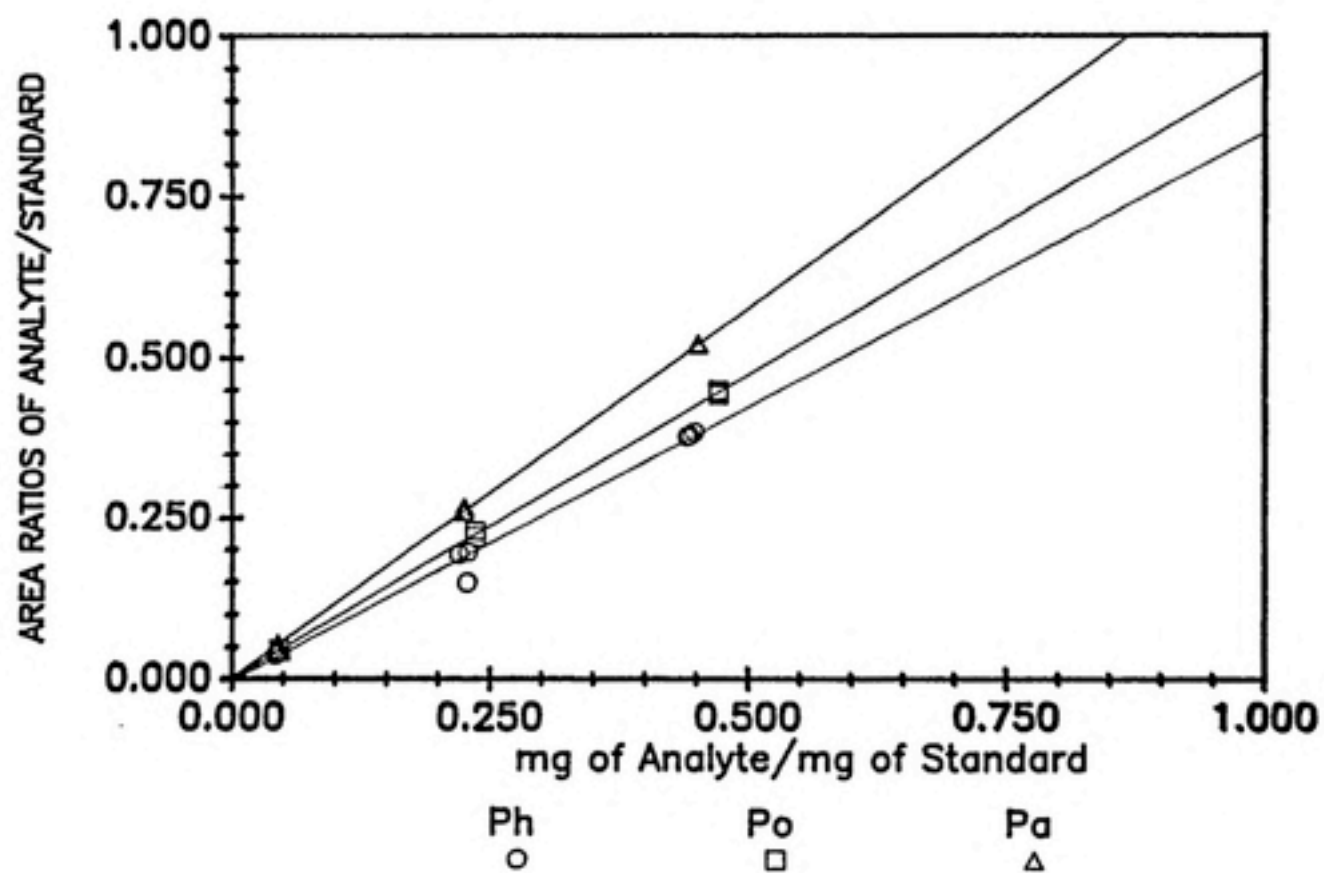
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List of Abbreviations for the Index Phenols and Model Compounds  
Appearing in the Calibration Curves

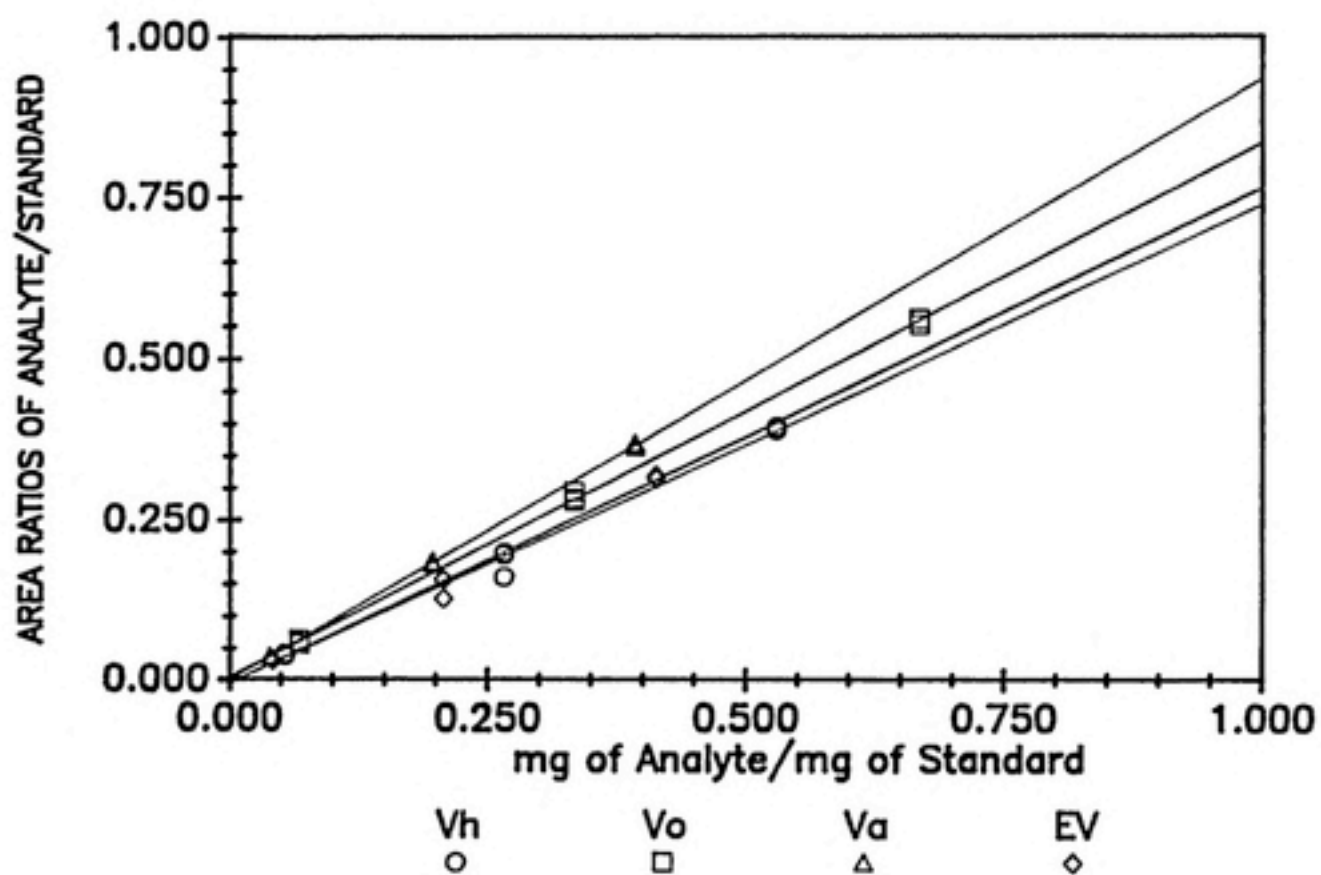
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2.	4-Hydroxyacetophenone	Po
3.	4-Hydroxybenzoic Acid	Pa
4.	Vanillin	Vh
5.	Acetovanillone	Vo
6.	Vanillic Acid	Va
7.	Syringaldehyde	Sh
8.	Acetosyringone	So
9.	Syringic Acid	Sa
10.	2-Hydroxybenzoic Acid	2-HBA
11.	4-Hydroxyphenylacetic Acid	4-HPAA
12.	3-(4-Hydroxyphenyl)propionic Acid	3-4HPPA
13.	Phenylacetic Acid	PAA
14.	Hydrocinnamic Acid	HCA
15.	Cinnamic Acid	tCA
16.	1,3-Dihydroxybenzene	1,3-DHB
17.	3-Hydroxybenzoic Acid	3-HBA
18.	3-Hydroxyphenylacetic Acid	3-HPAA
19.	3,5-Dihydroxybenzene	3,5-DHBA
20.	3,4-Dihydroxybenzoic Acid	3,4-DHBA
21.	3,4-Dihydroxyphenylacetic Acid	3,4-DHPA
22.	3,4-Dihydroxyhydrocinnamic Acid	3,4-DHHC
23.	3,4-Dihydroxycinnamic Acid	3,4-DHCA
24.	3,4-Dimethoxyphenylacetic Acid	3,4-DMPA
25.	Ethyl Vanillin	EV



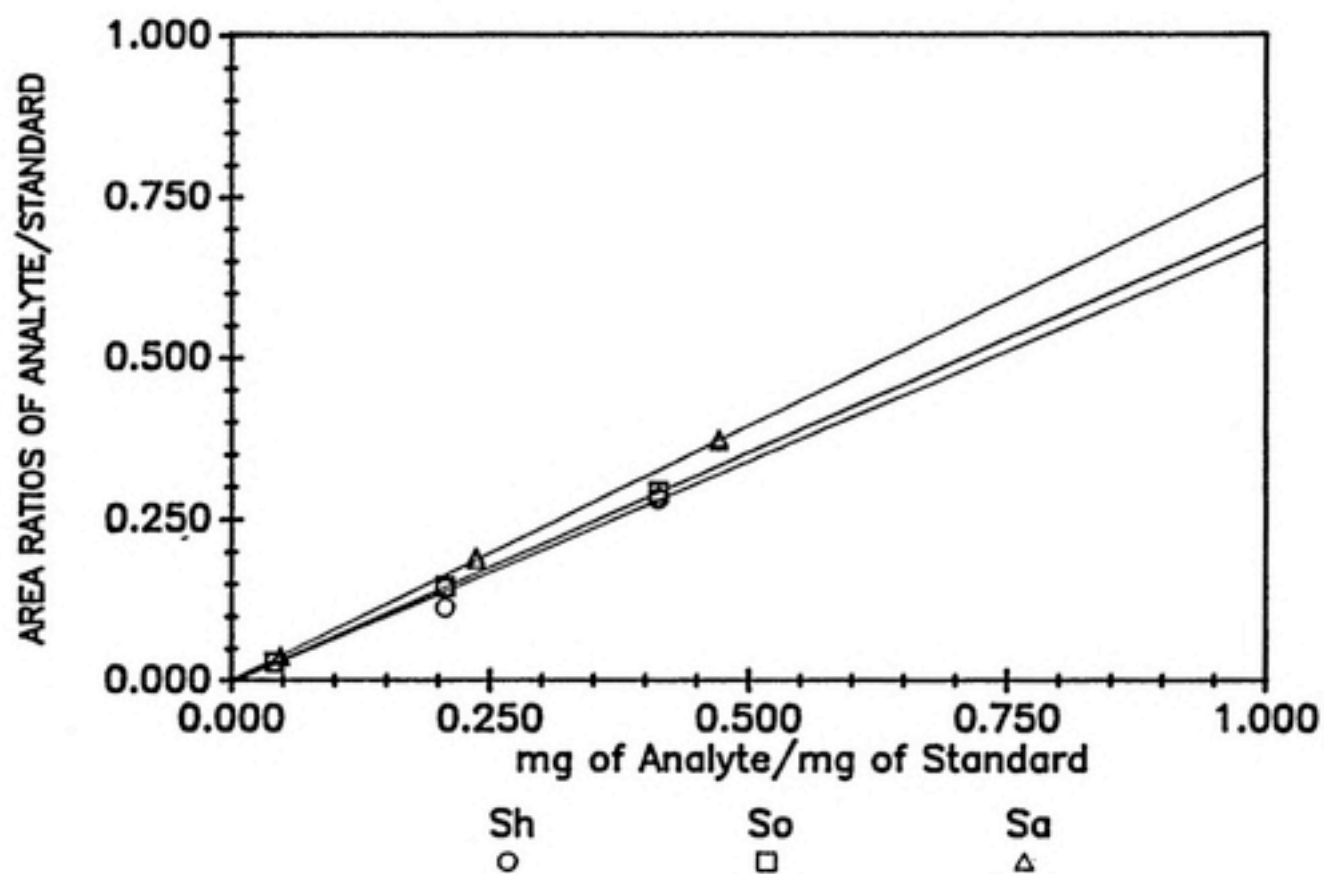
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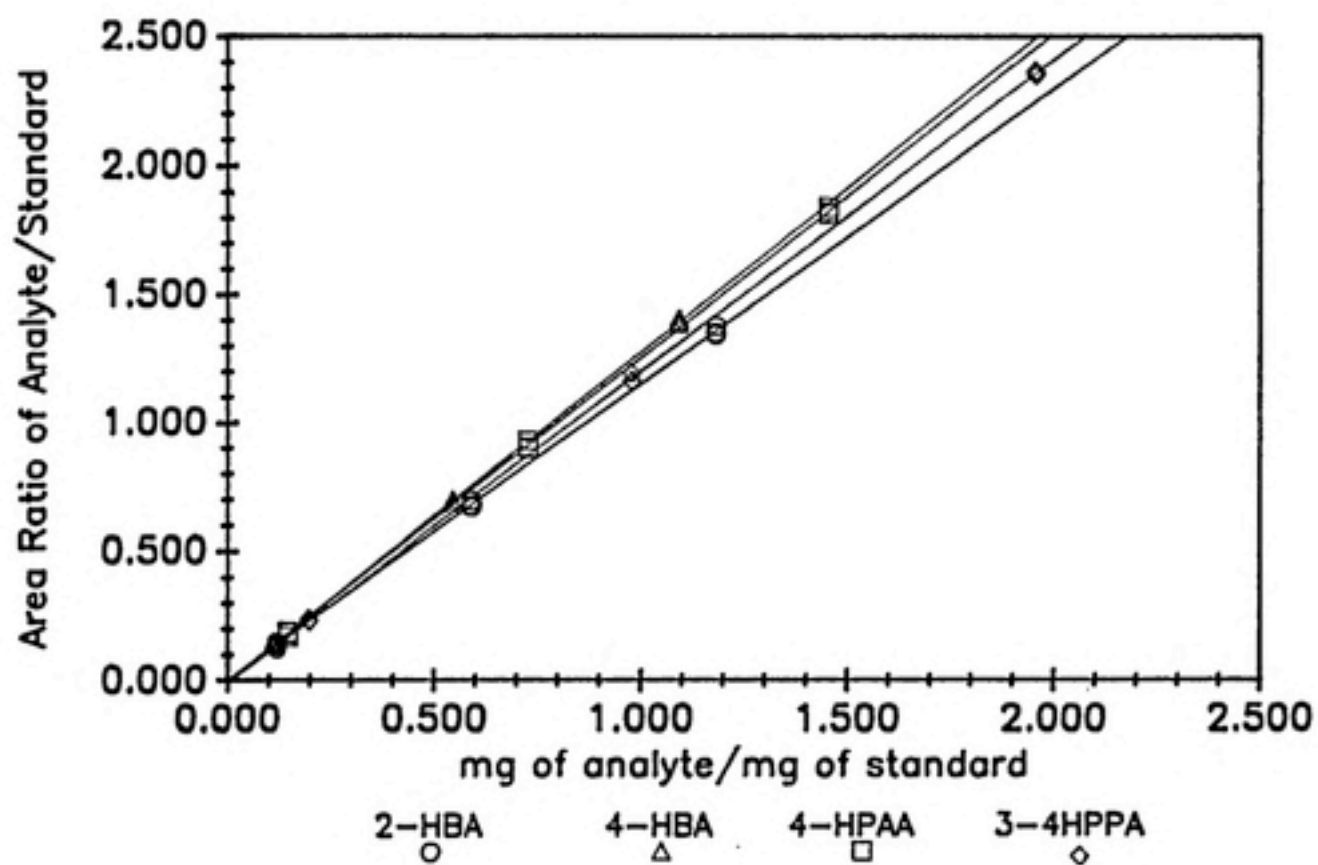
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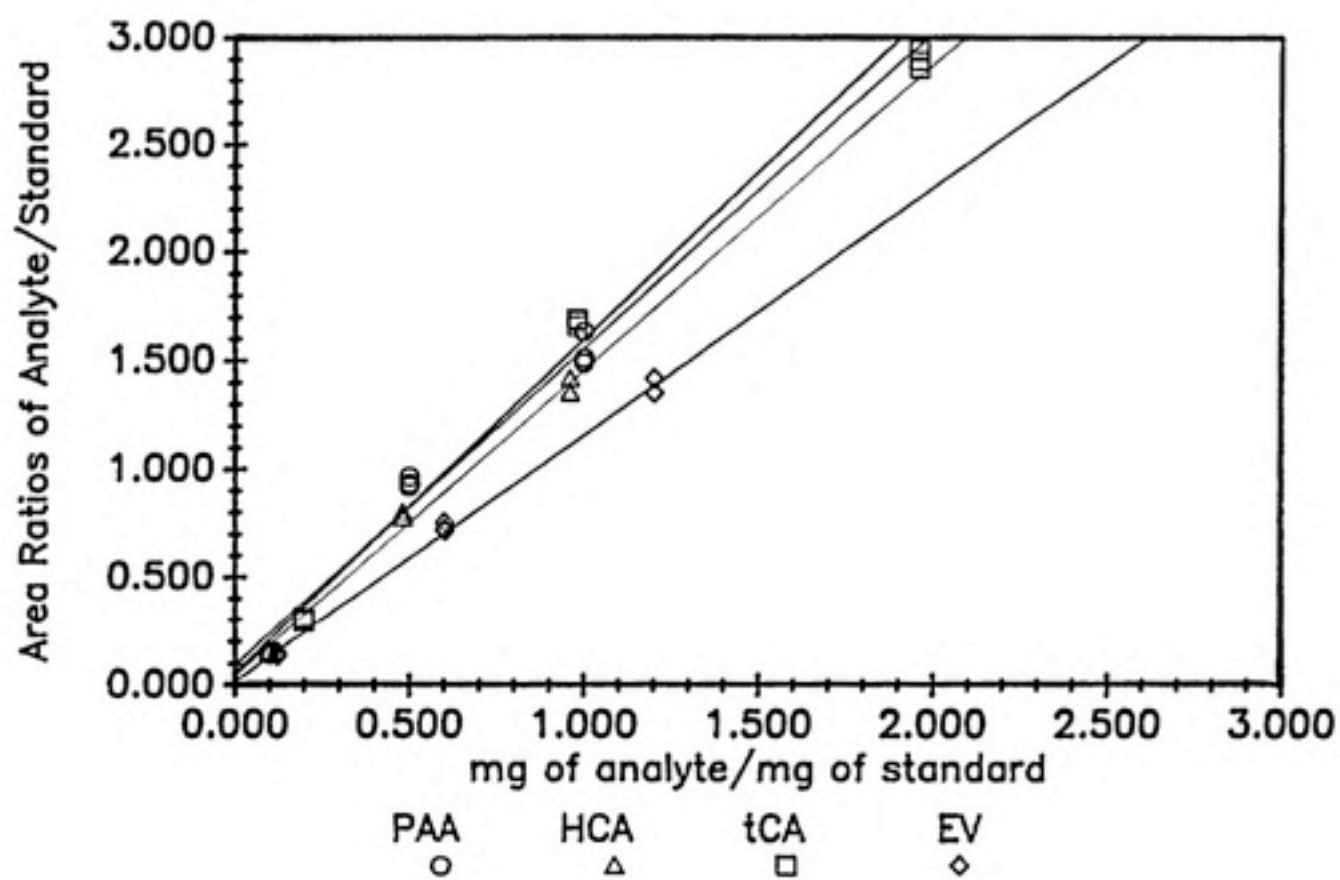
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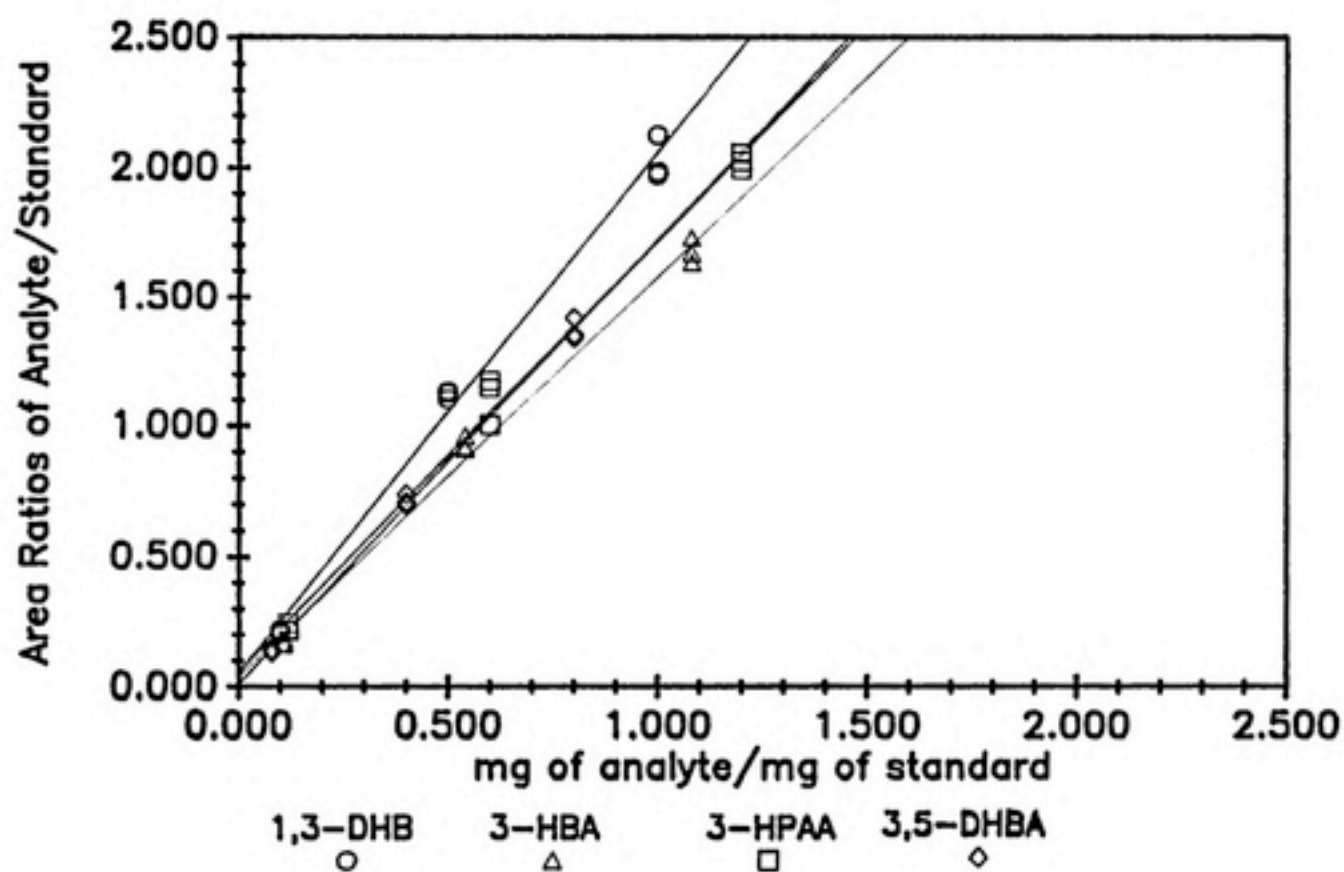
Calibration Curves for Group 1 Model Compounds



Calibration Curves for Group2 Model Compounds



Calibration Curves for Group 3 Model Compounds



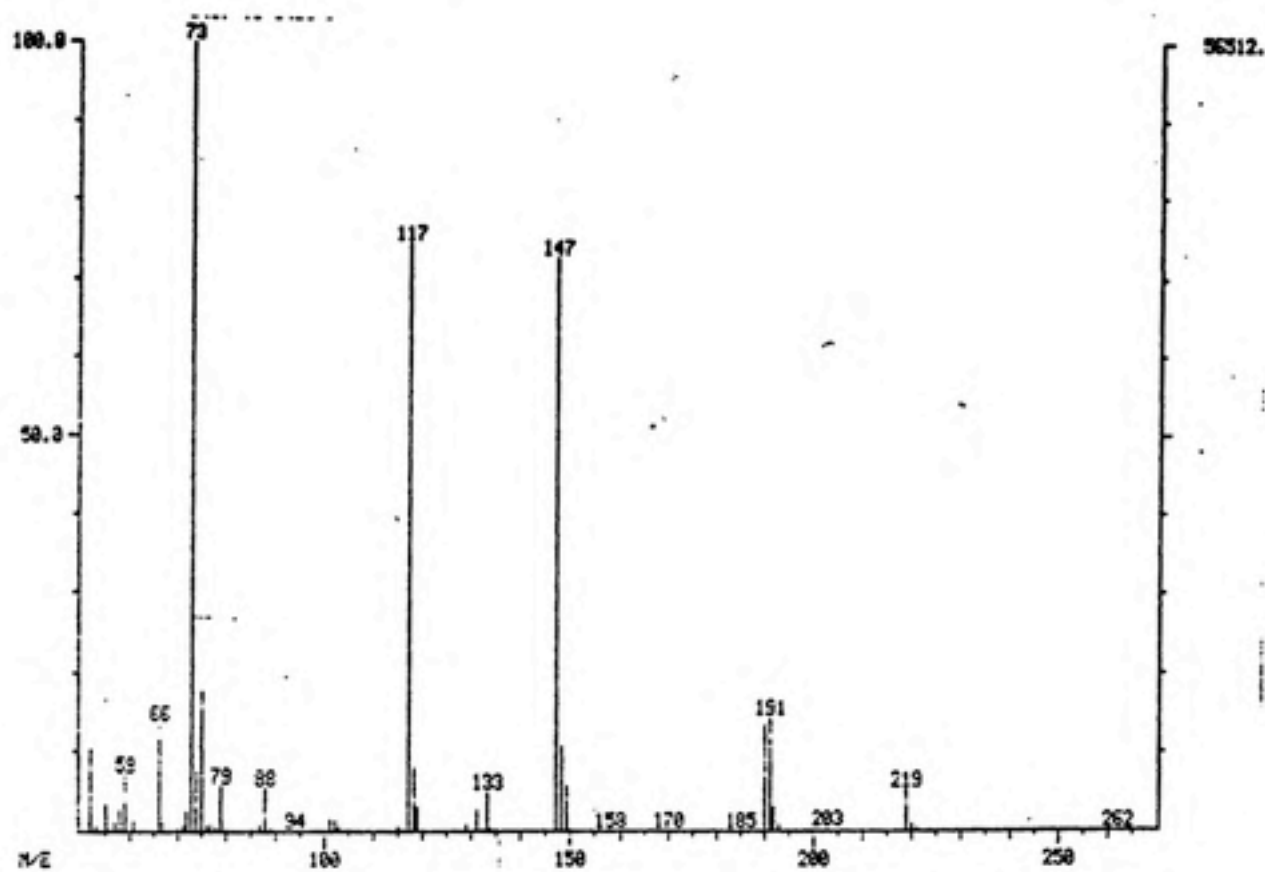
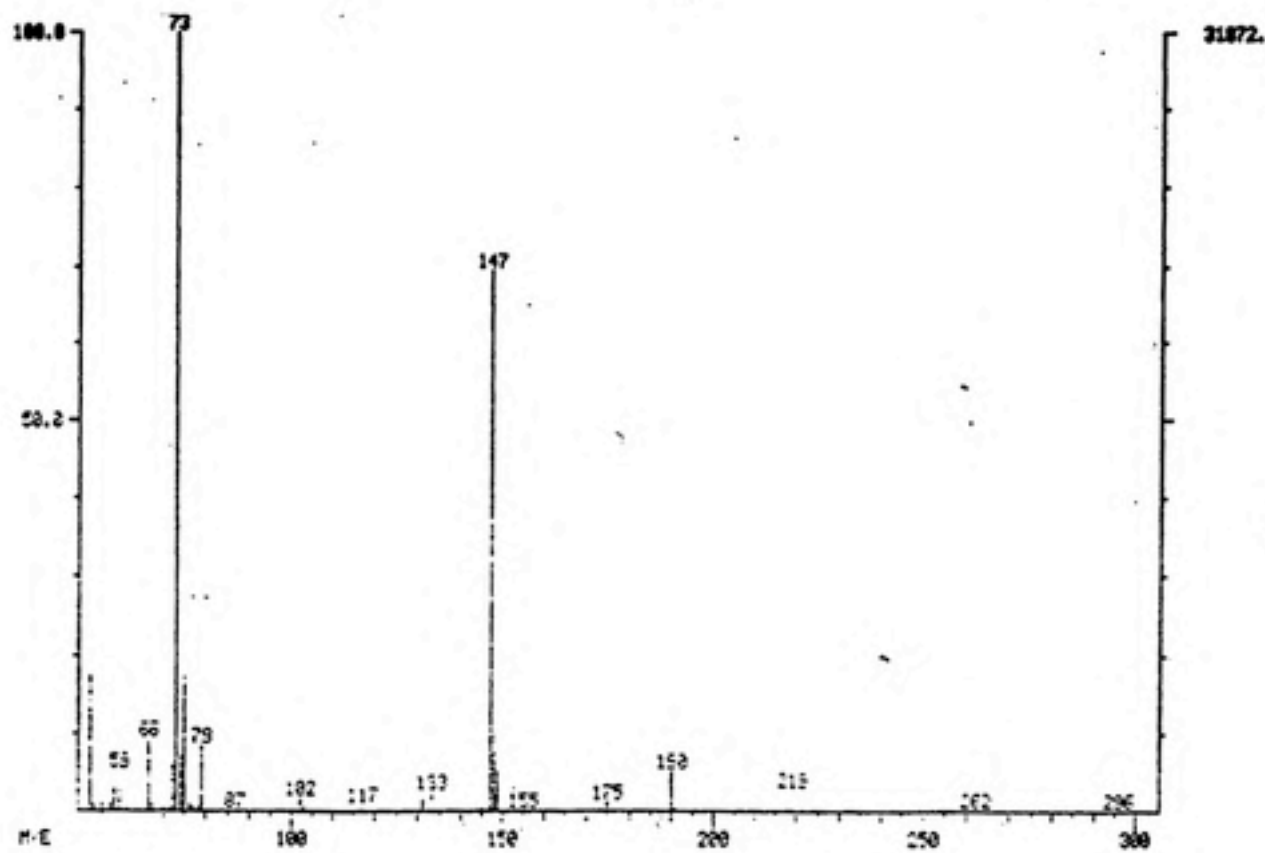


## List of Mass Spectra

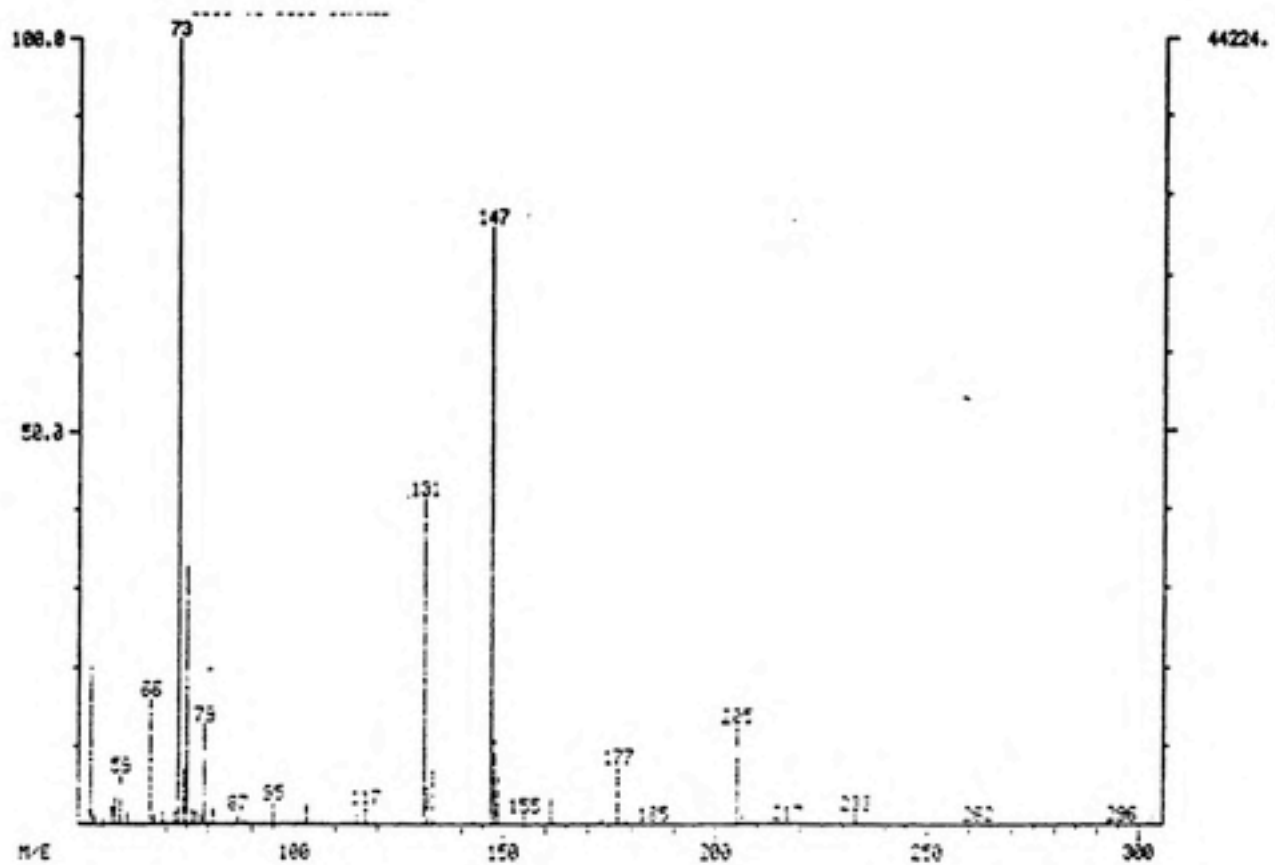
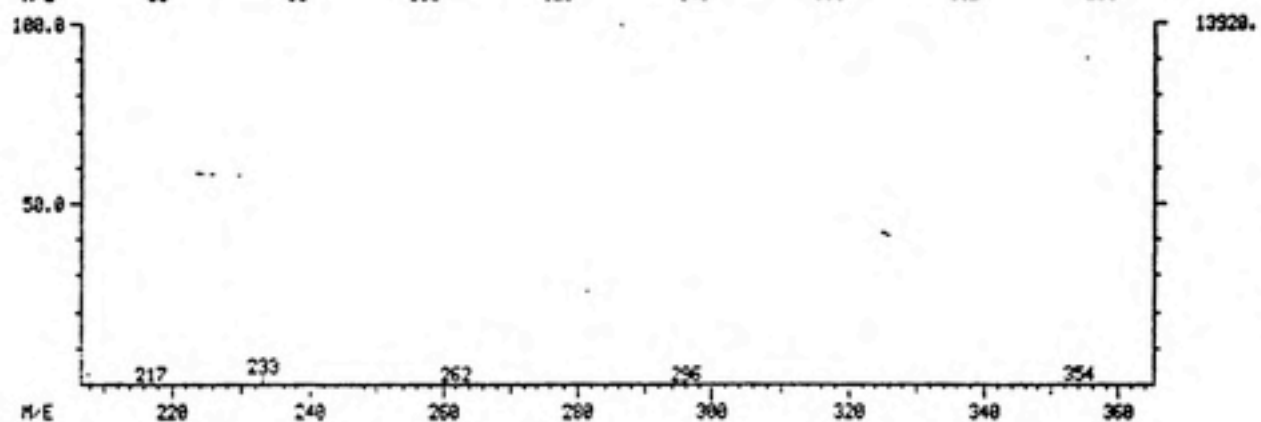
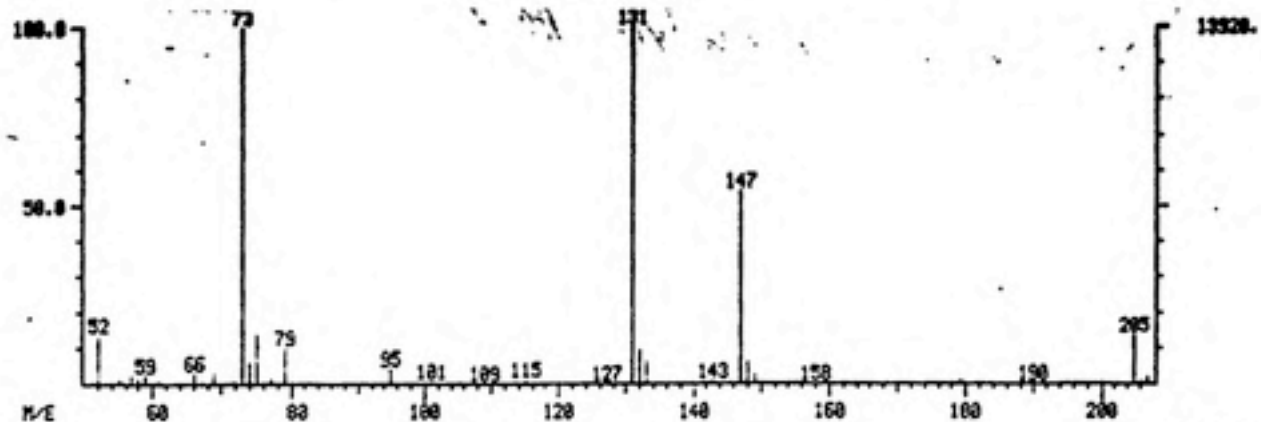
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3. Levulenic Acid
4. alpha-Hydroxybutyric Acid
5. Malonic Acid
6. Benzoic Acid
7. Succinic Acid
8. Methyl Succinic Acid
9. Maleic Acid
10. 4-Hydroxybenzaldehyde
11. 1,3-Dihydroxybenzene
12. 4-Hydroxyacetophenone
13. Vanillin
14. 2-Hydroxybenzoic Acid
15. Acetovanillone
16. 3-Hydroxybenzoic Acid
17. 3,4-Dihydroxybenzaldehyde
18. 4-Hydroxybenzoic Acid
19. Phthalic Acid
20. Tricarballic Acid
21. Isophthalic Acid
22. Vanillic Acid
23. Terephthalic Acid
24. 3,5-Dihydroxybenzoic Acid
25. Syringic Acid

The format for the spectra presented in the appendix shows the standard on the top half of the page and that for the corresponding compound identified from Lake Drummond fulvic acid on the bottom half of the page.

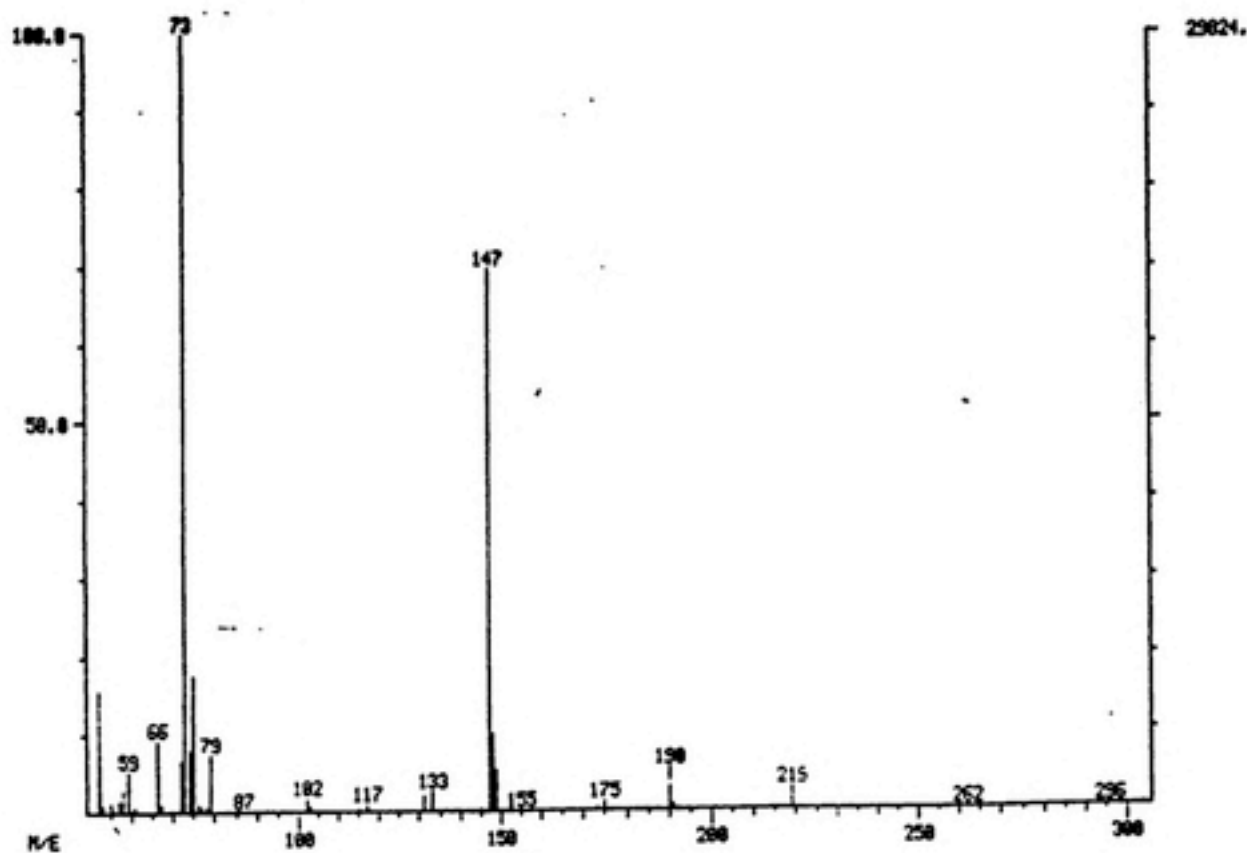
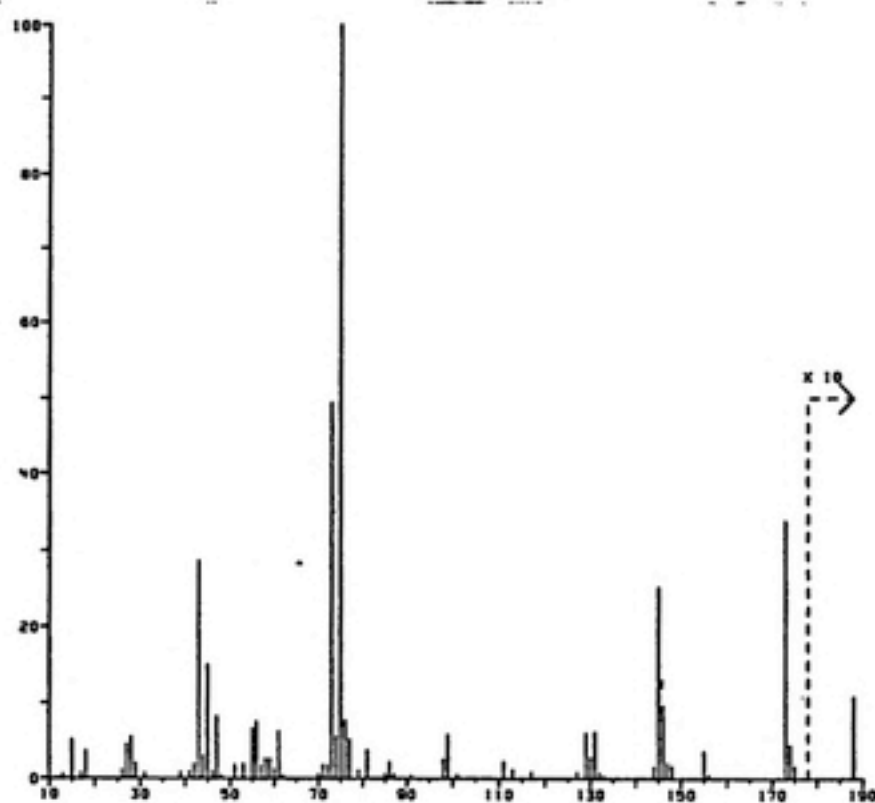
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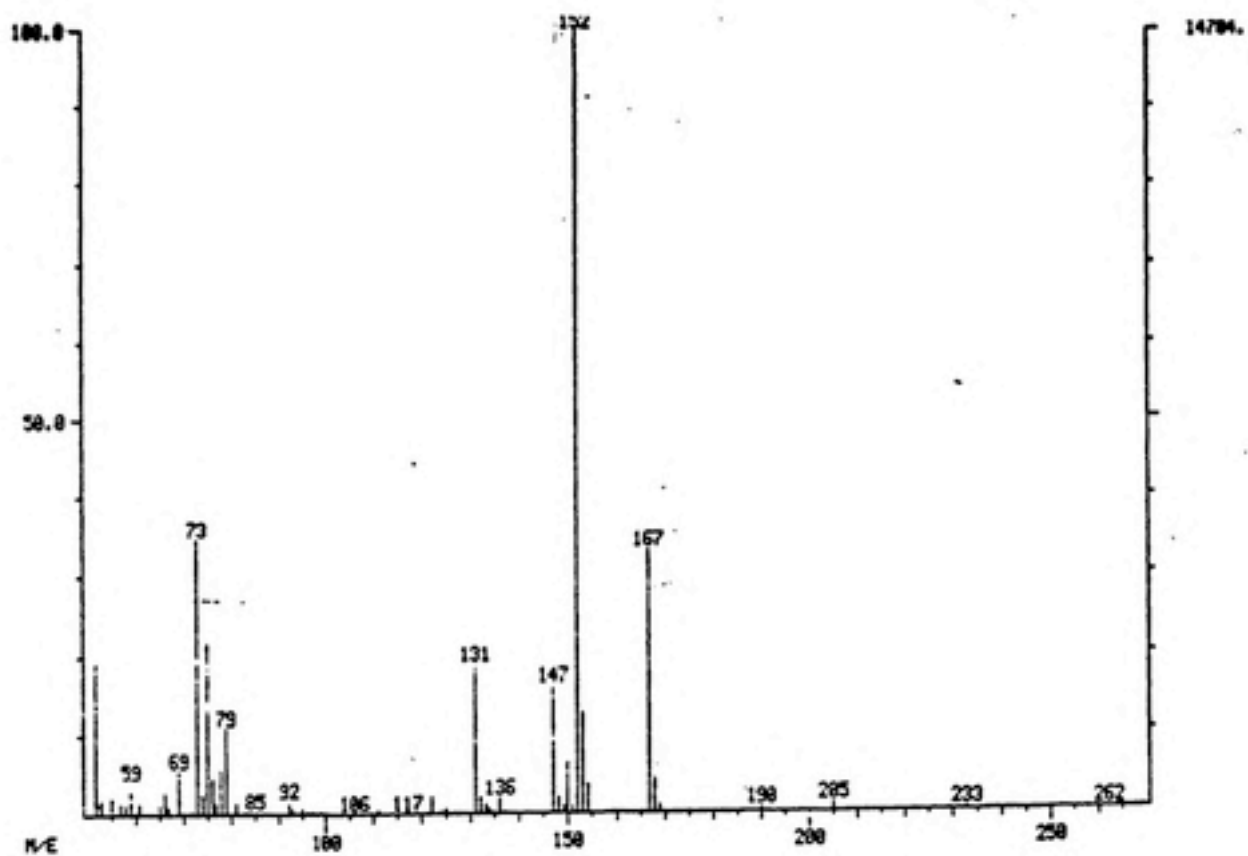
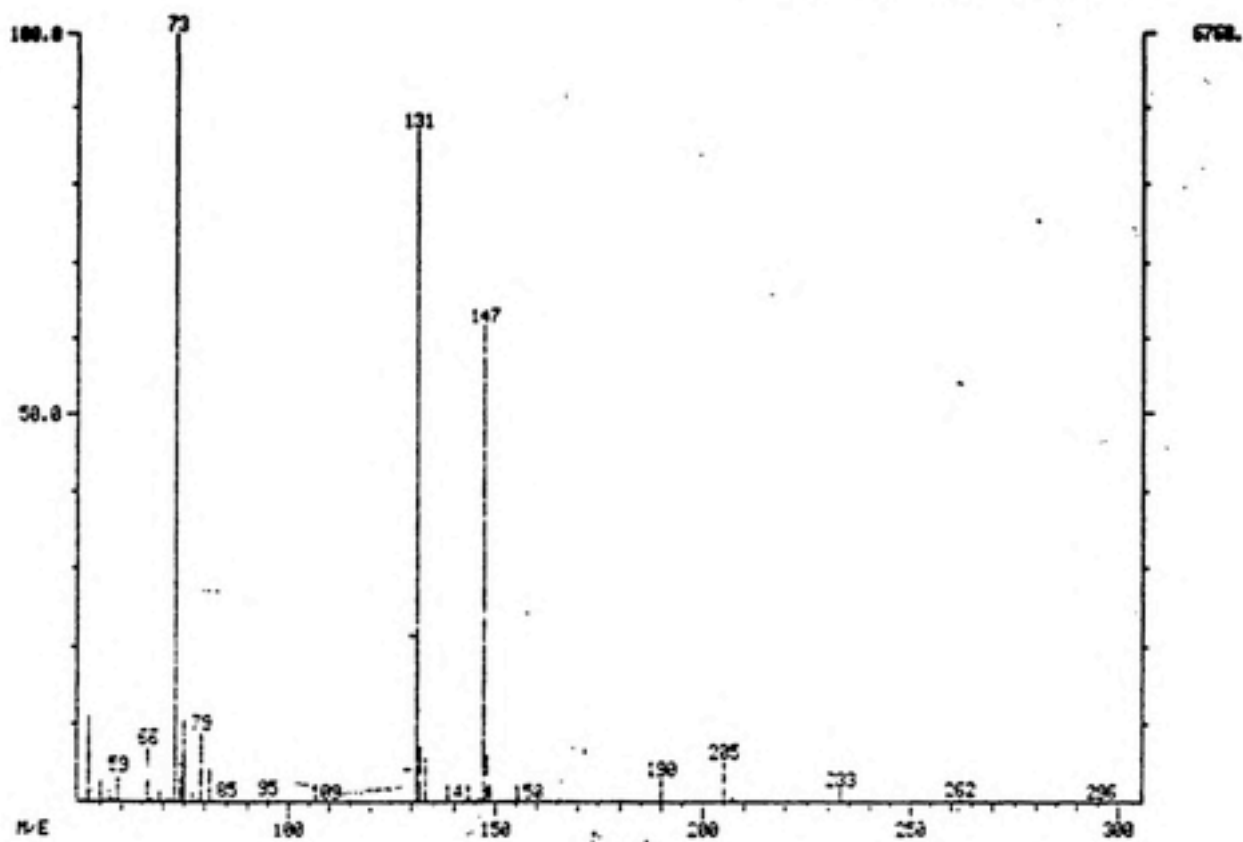
alpha Hydroxyisobutyric Acid



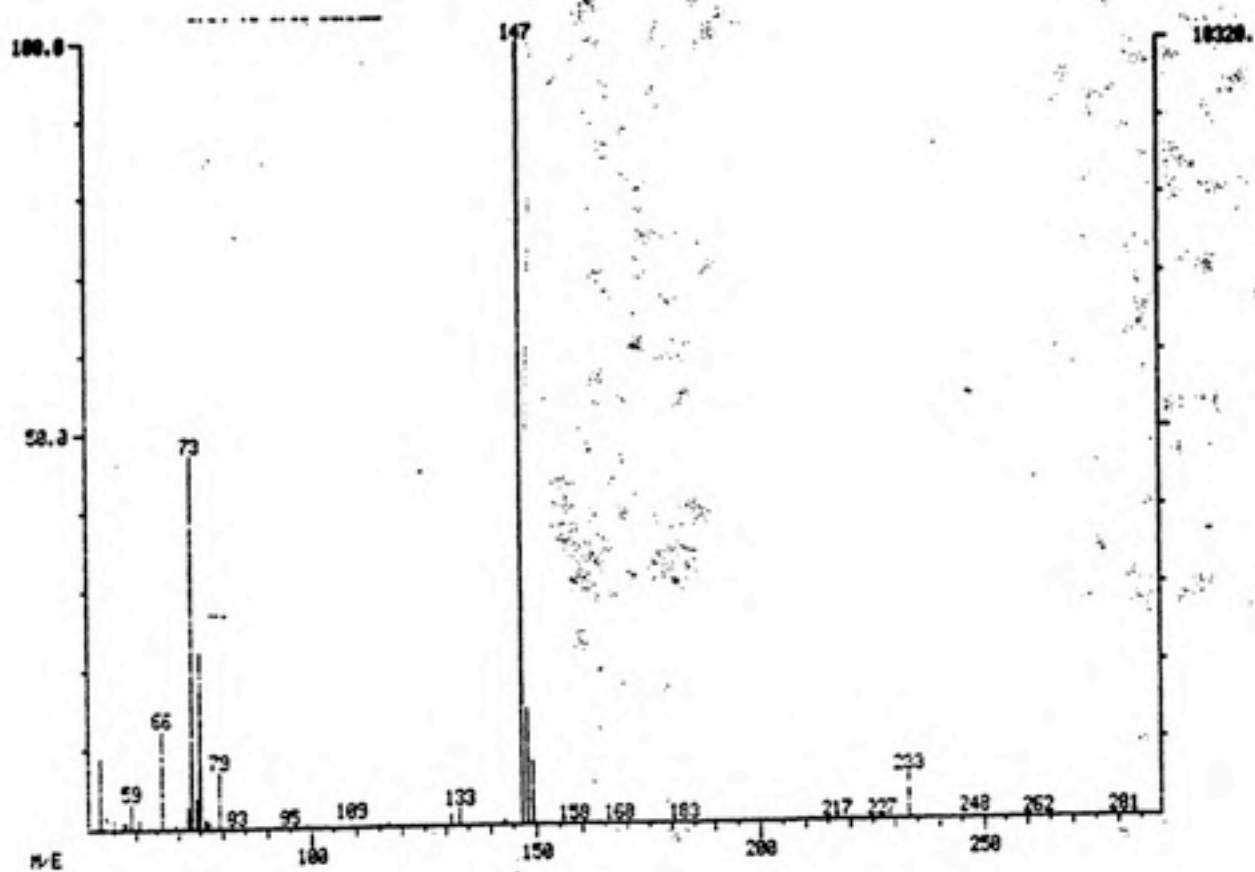
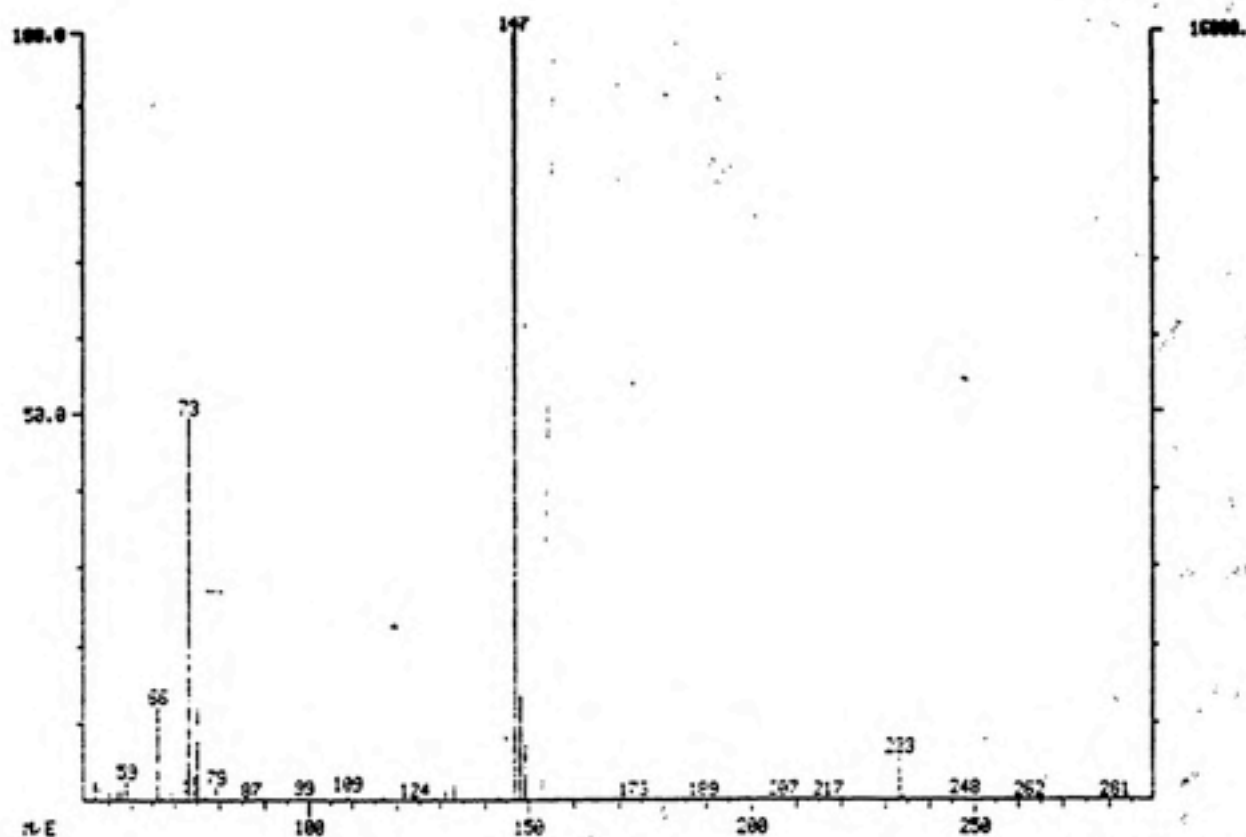
# Levulenic Acid



alpha Hydroxybutyric Acid

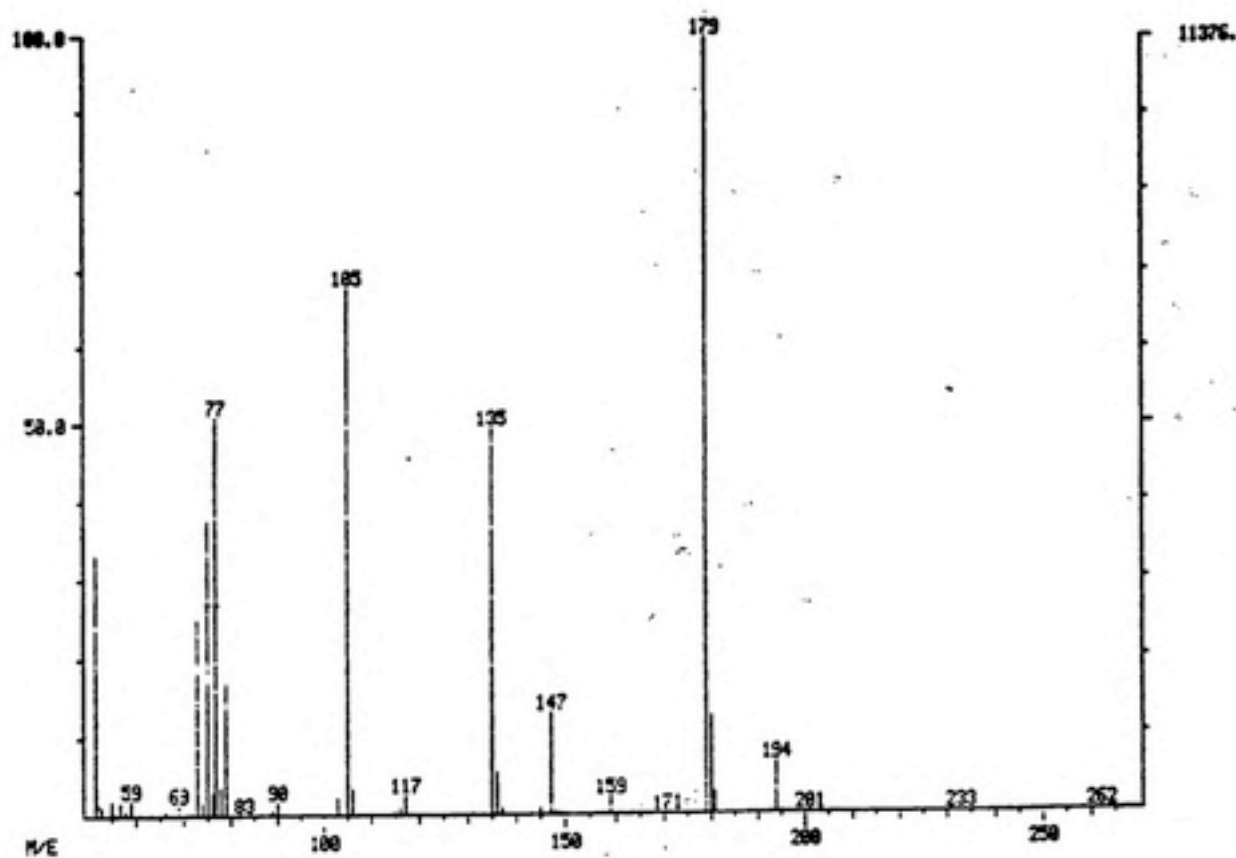
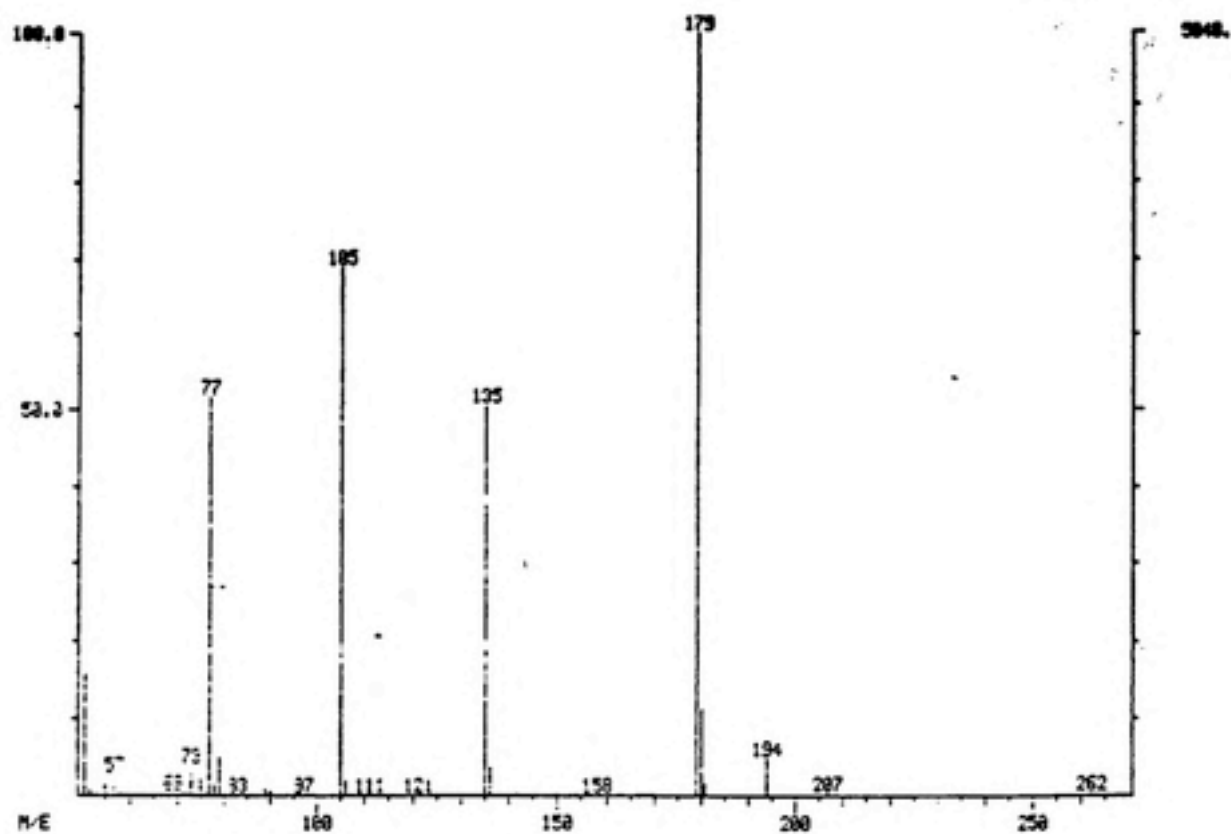


# Malonic Acid

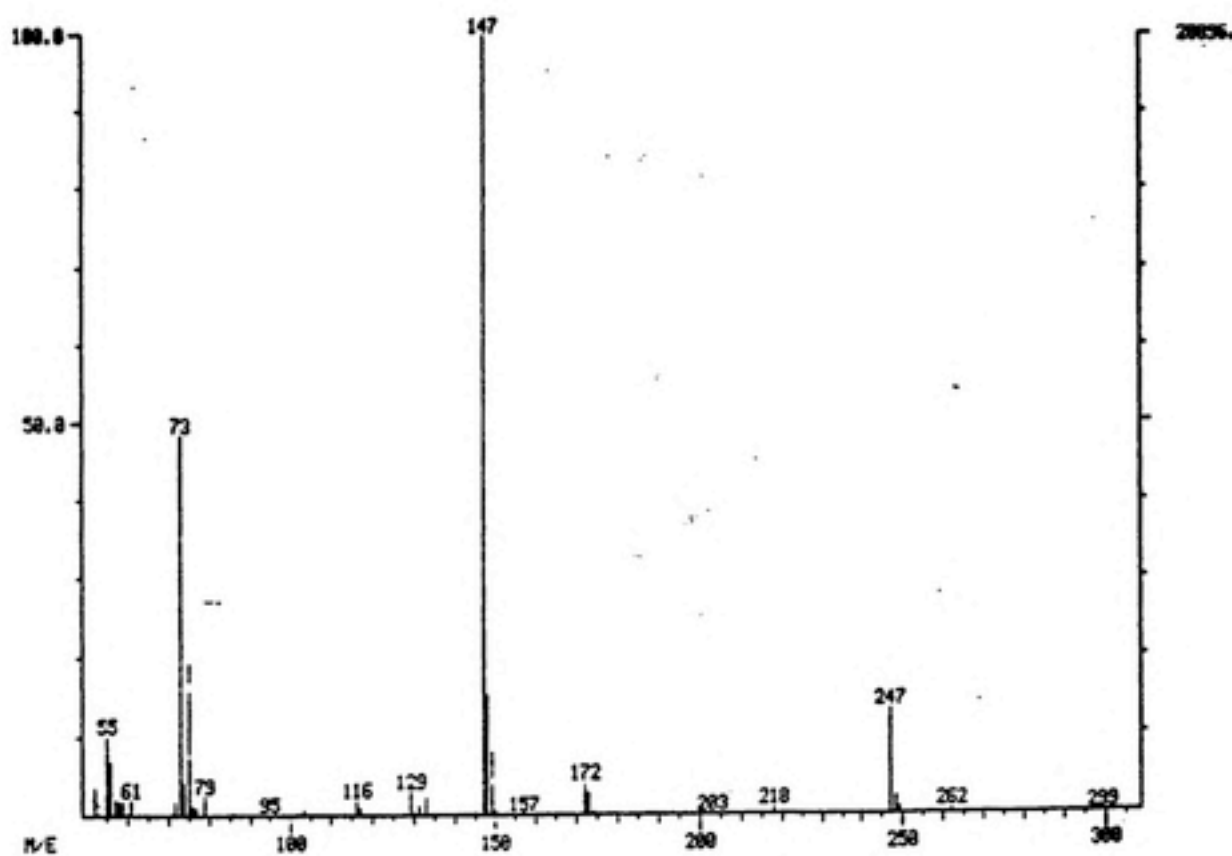
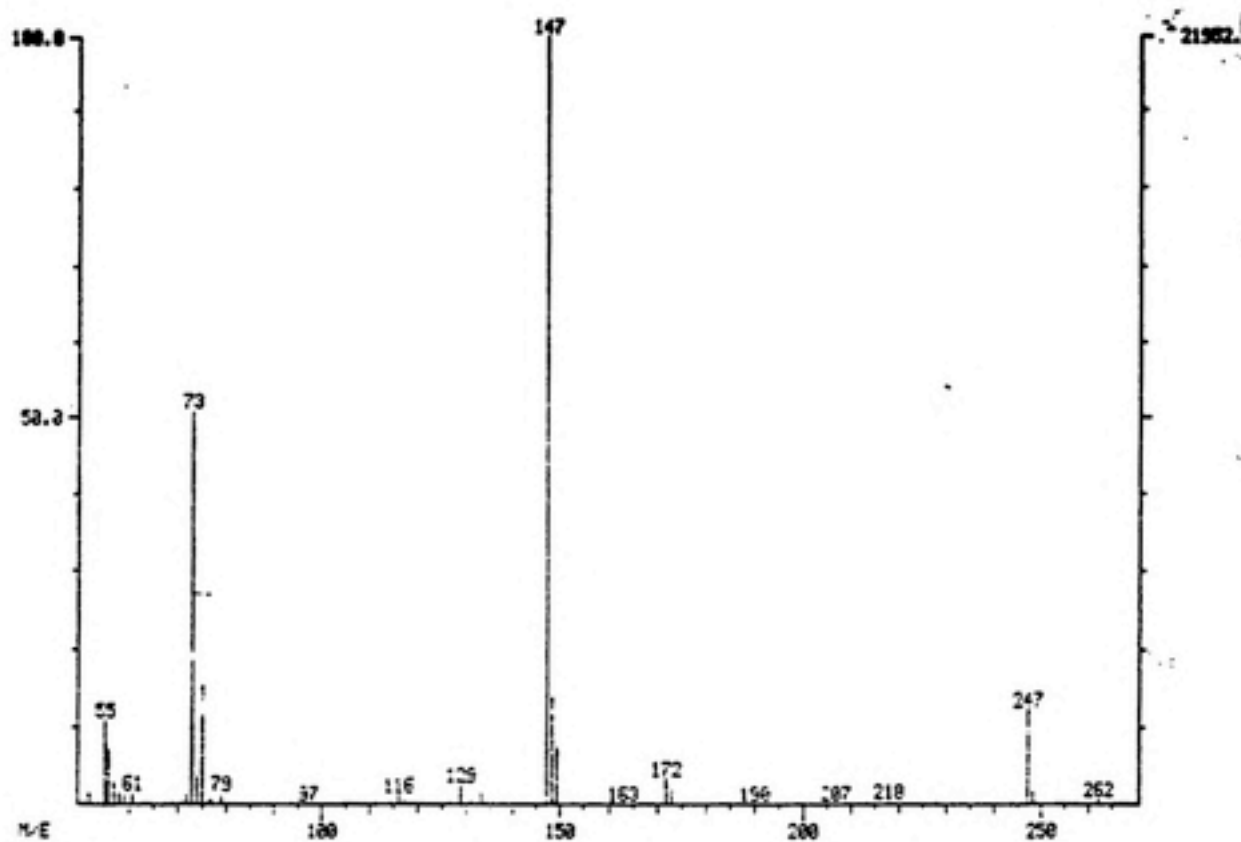




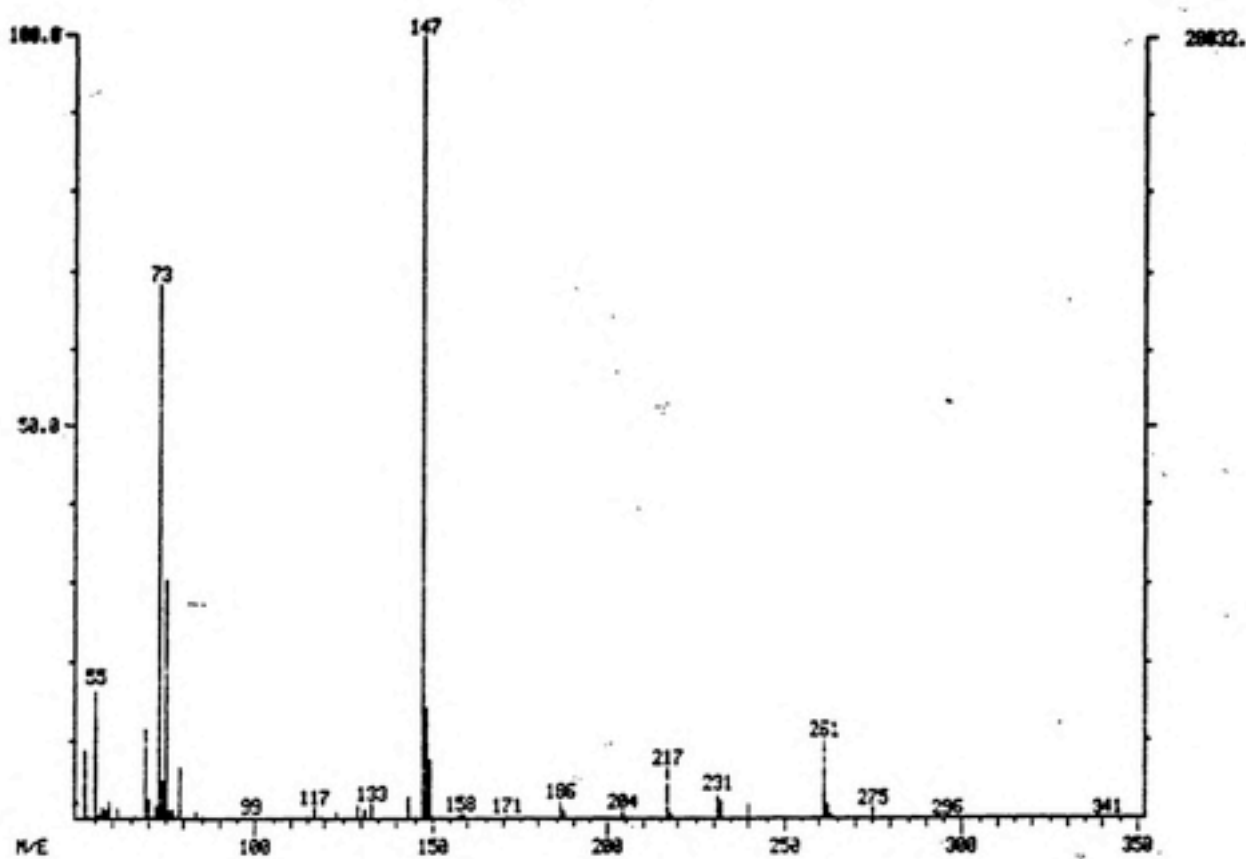
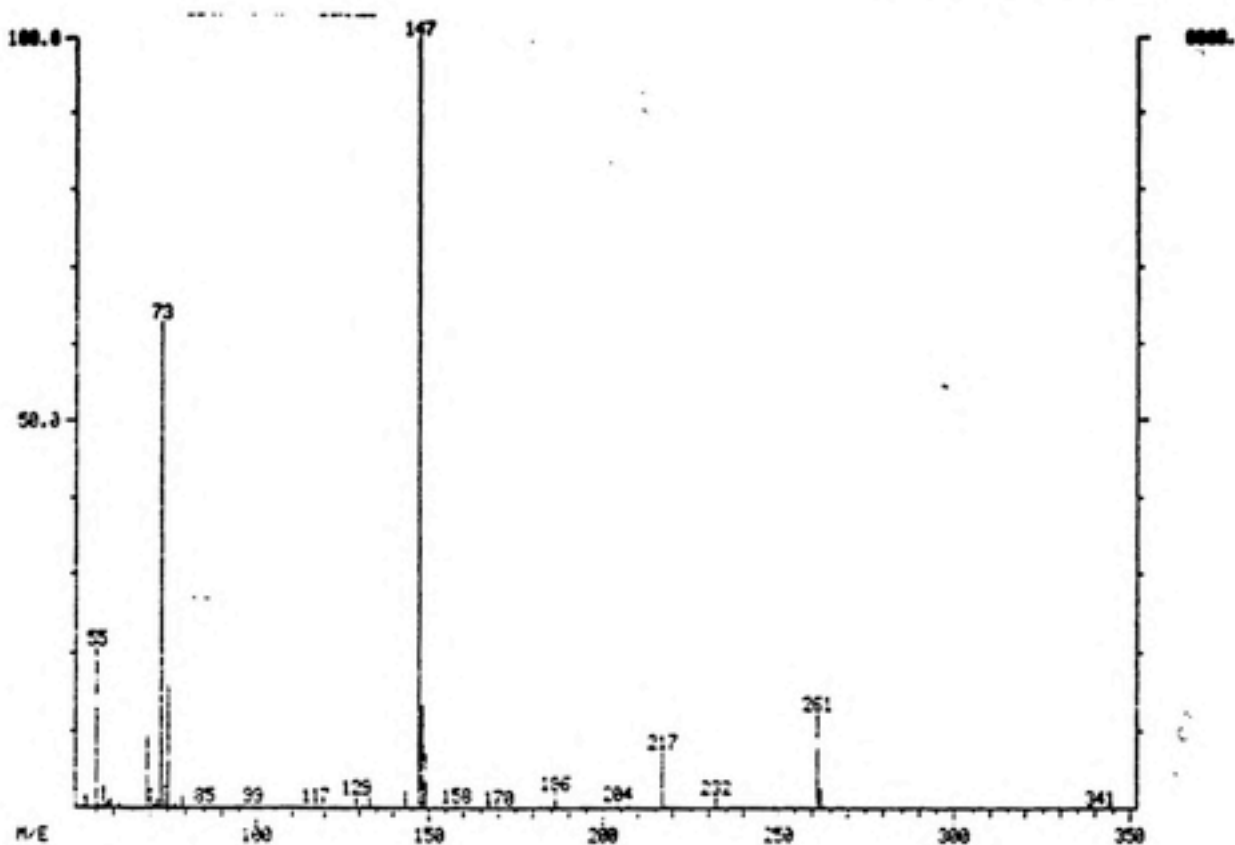
# Benzoic Acid



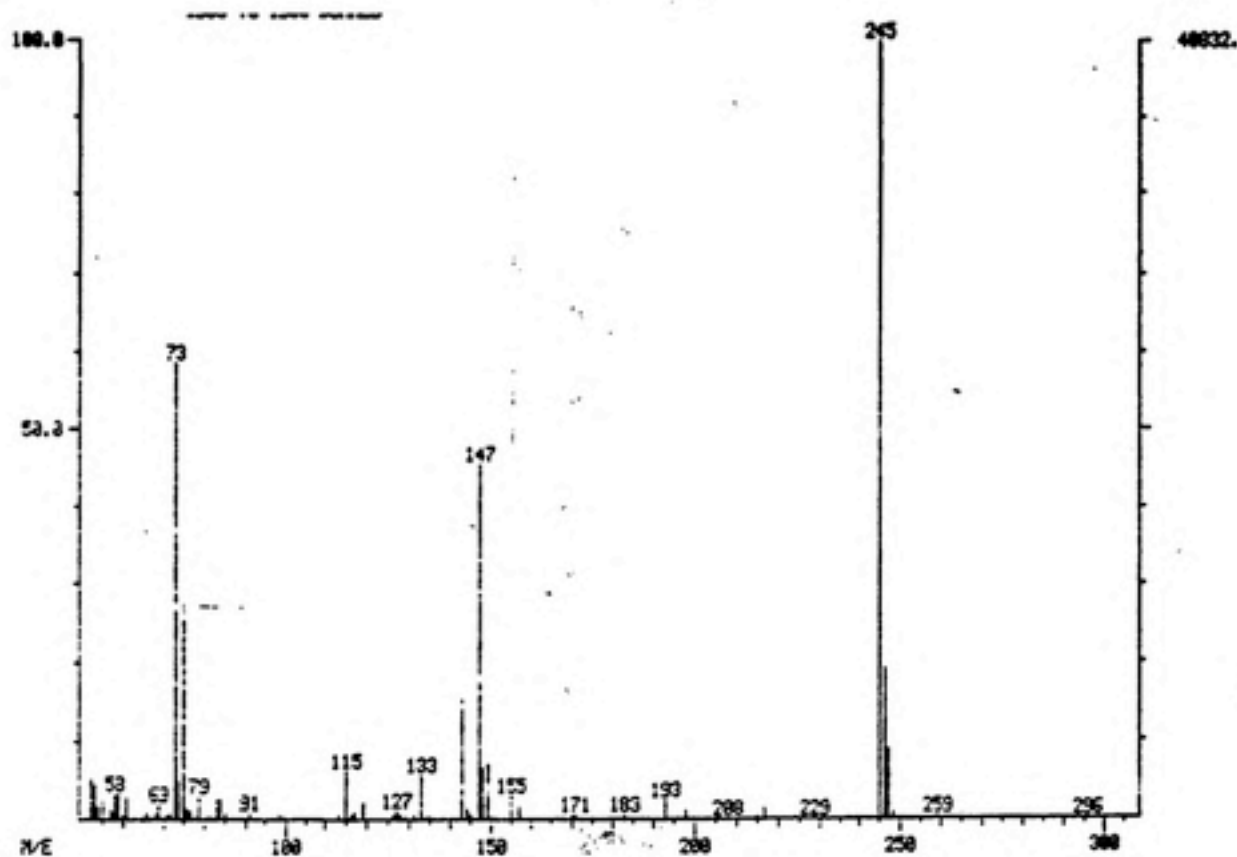
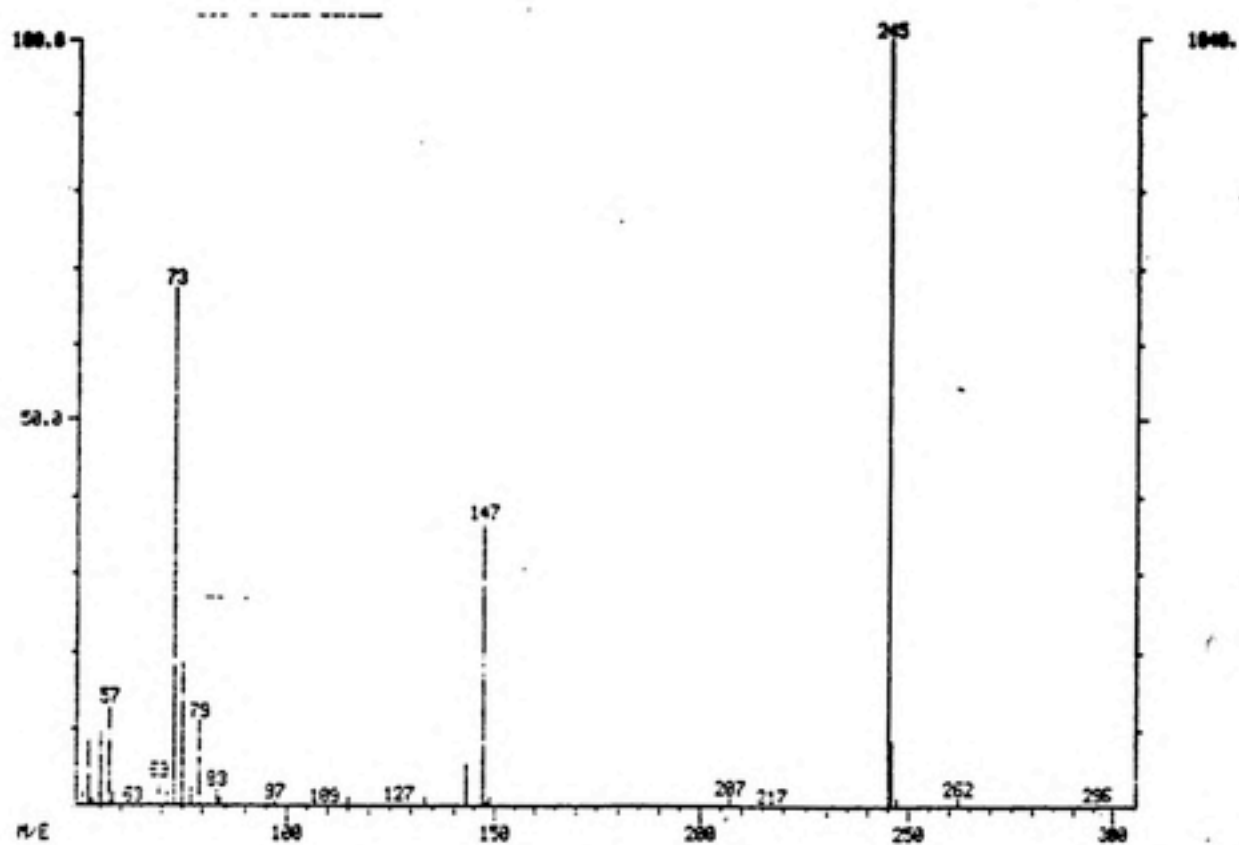
# Succinic Acid



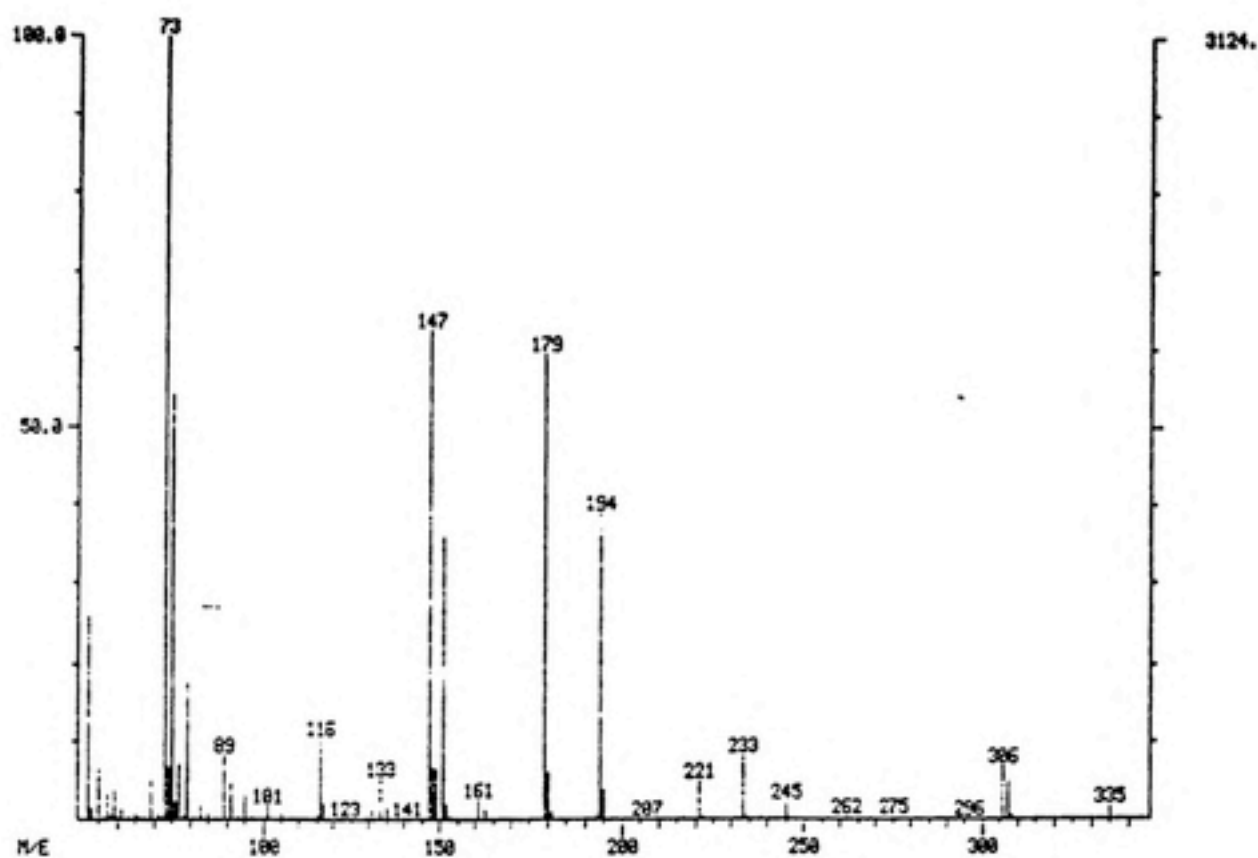
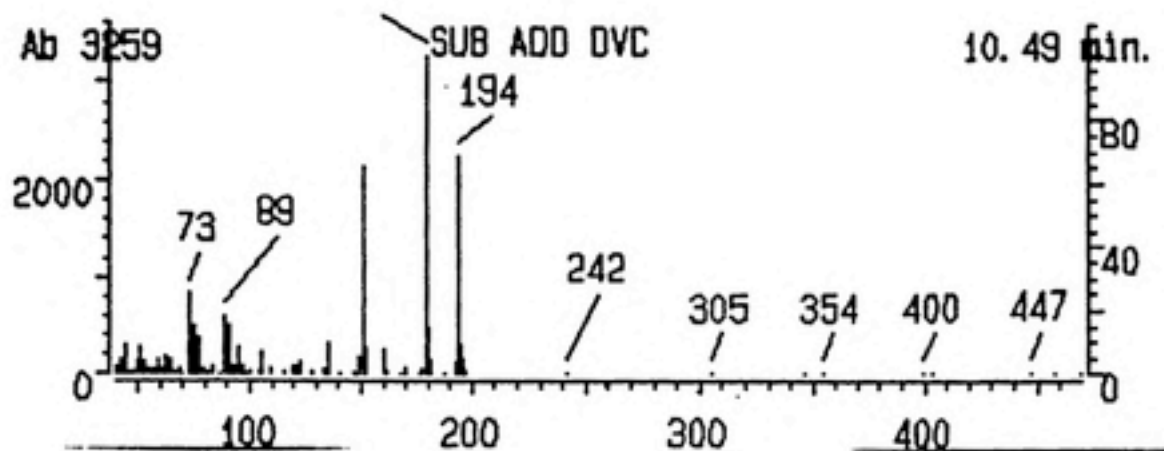
# Methyl Succinic Acid



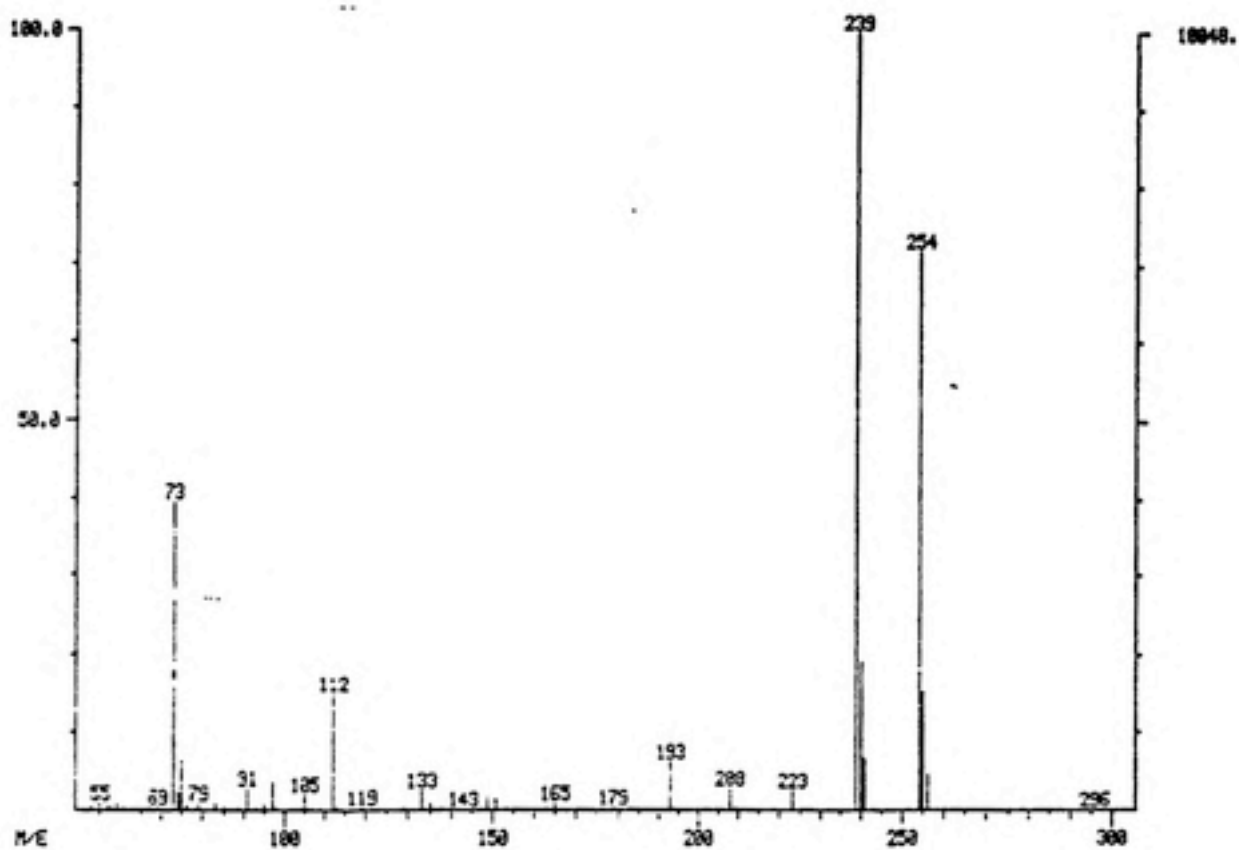
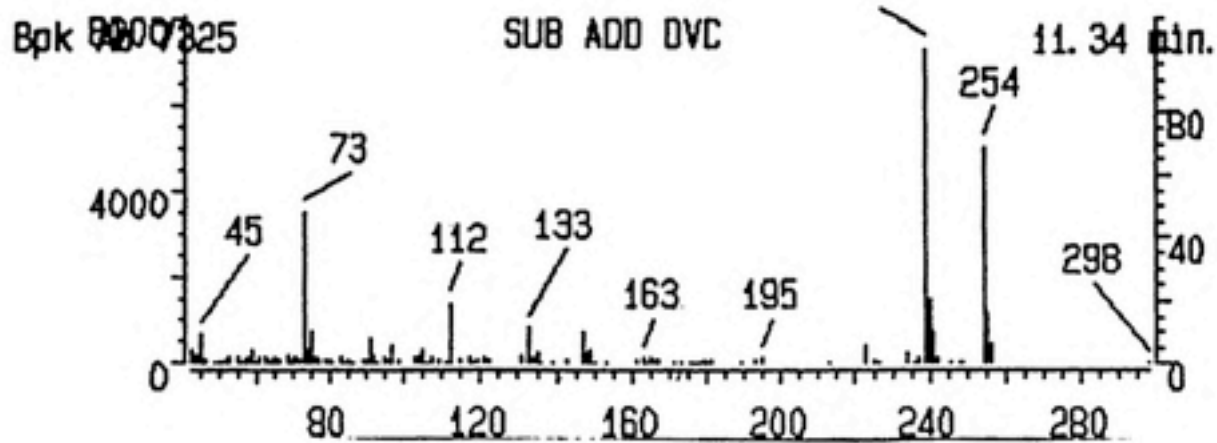
# Maleic Acid



4-Hydroxybenzaldehyde

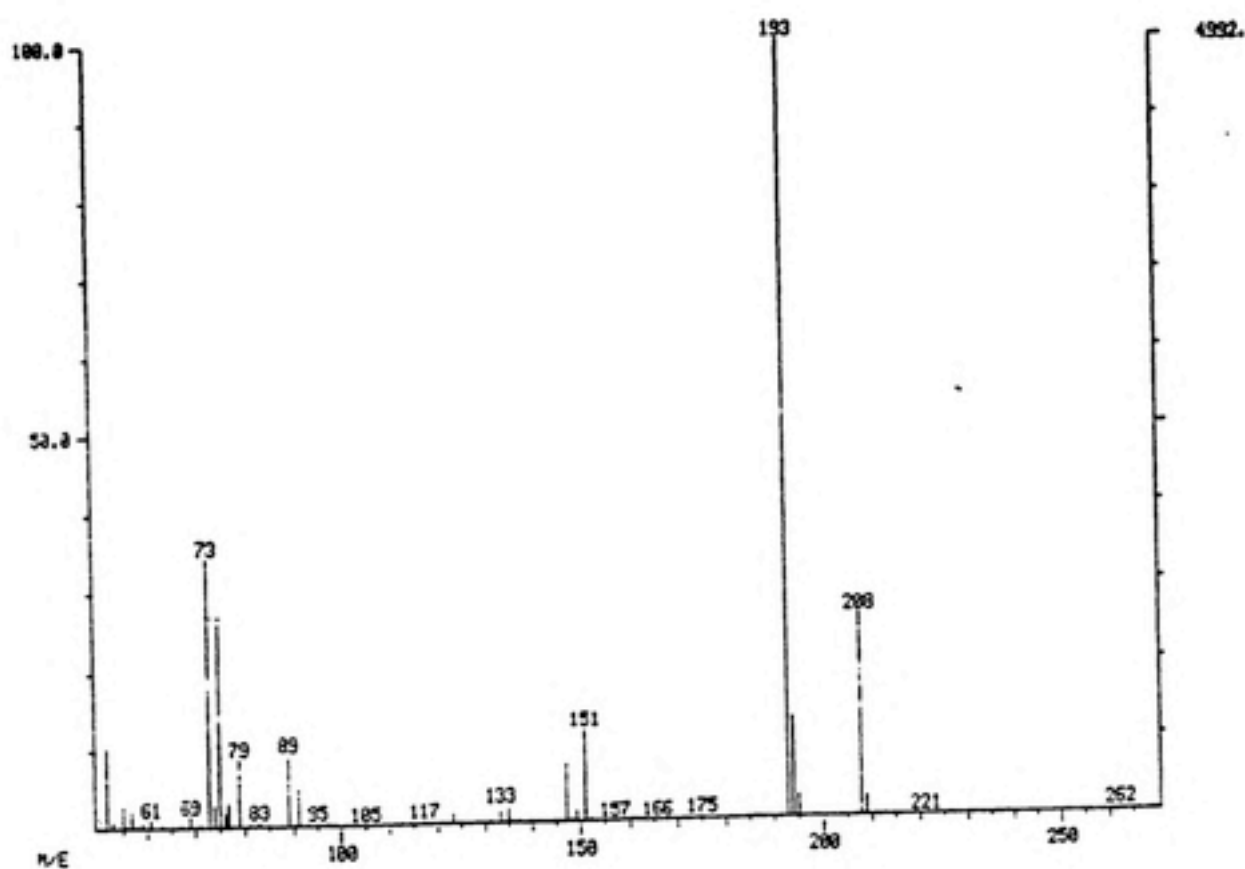
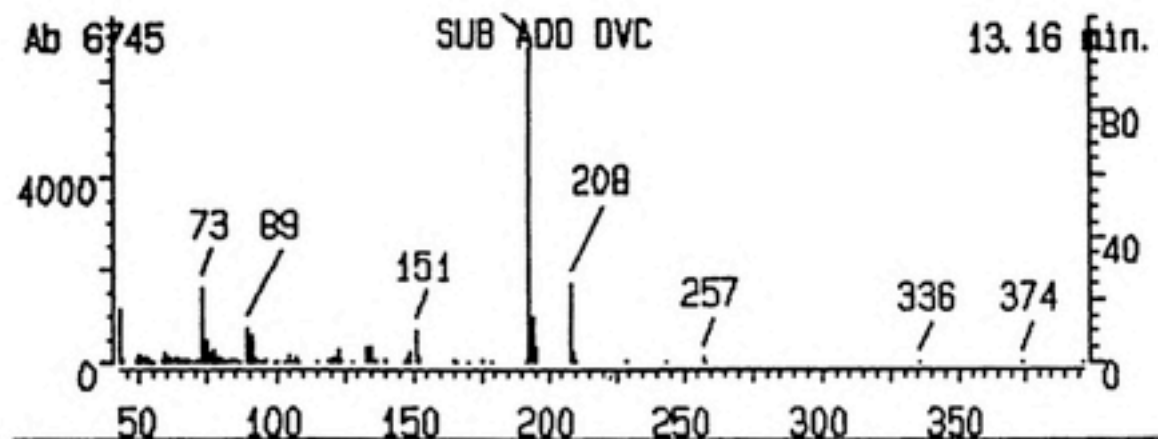


1,3-Dihydroxybenzene

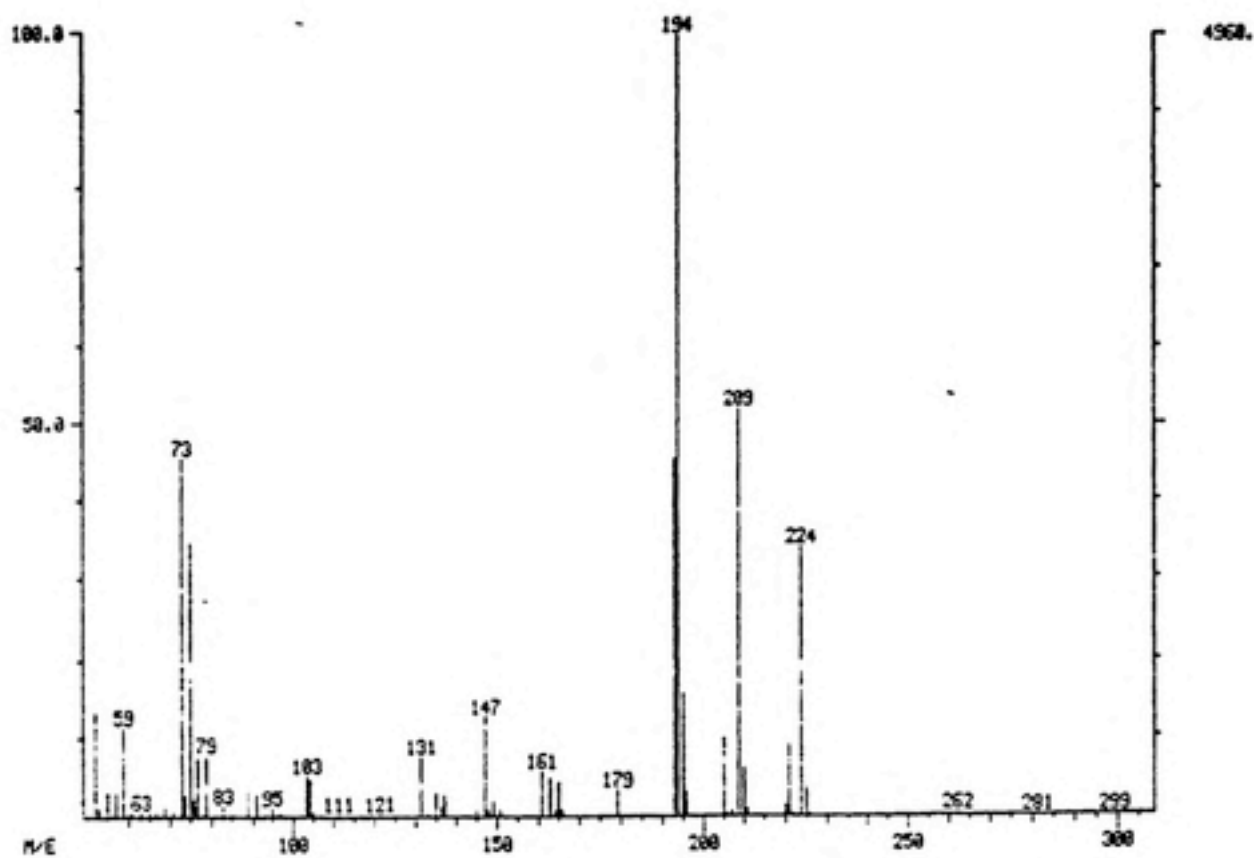
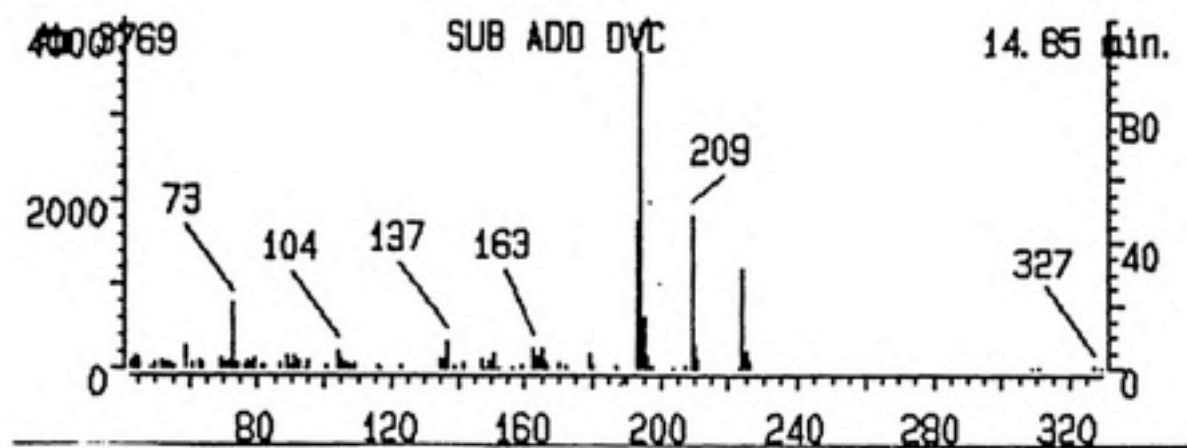




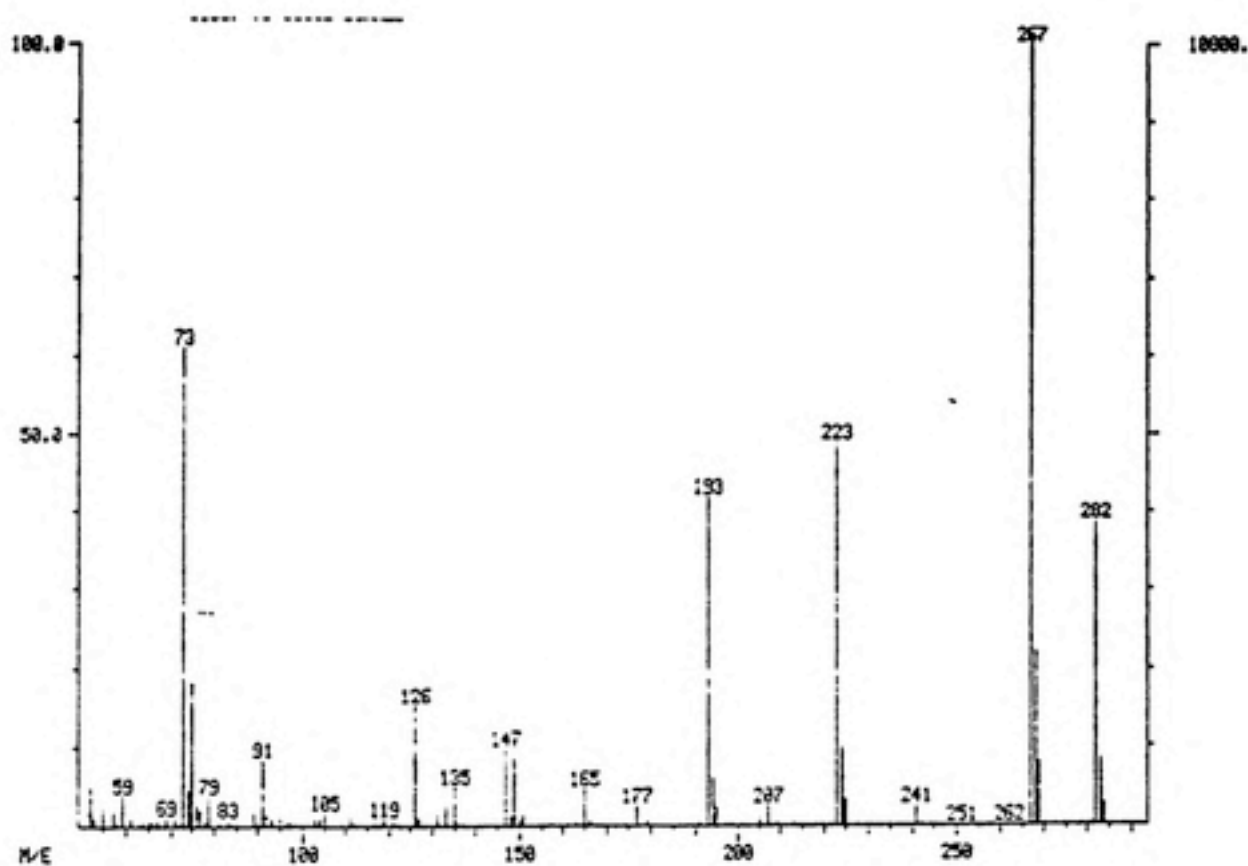
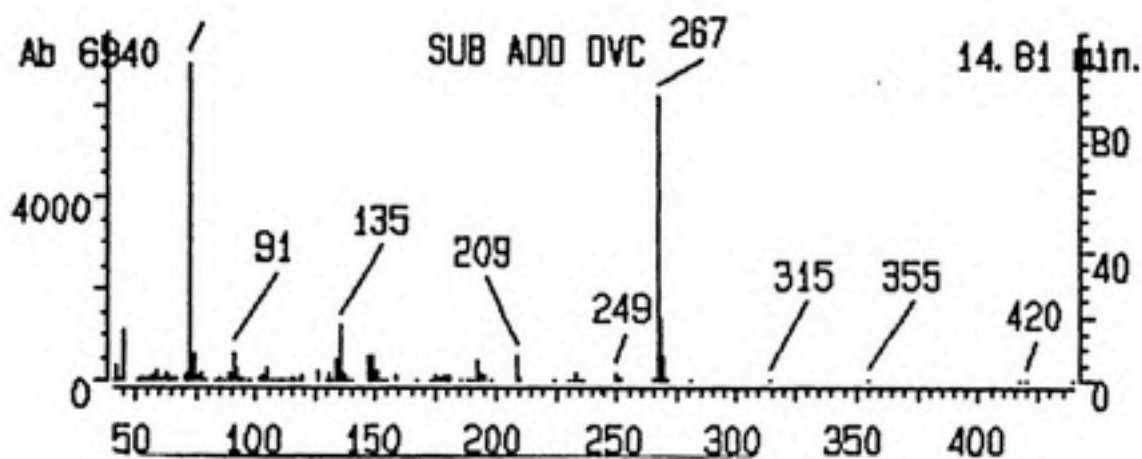
4-Hydroxyacetophenone



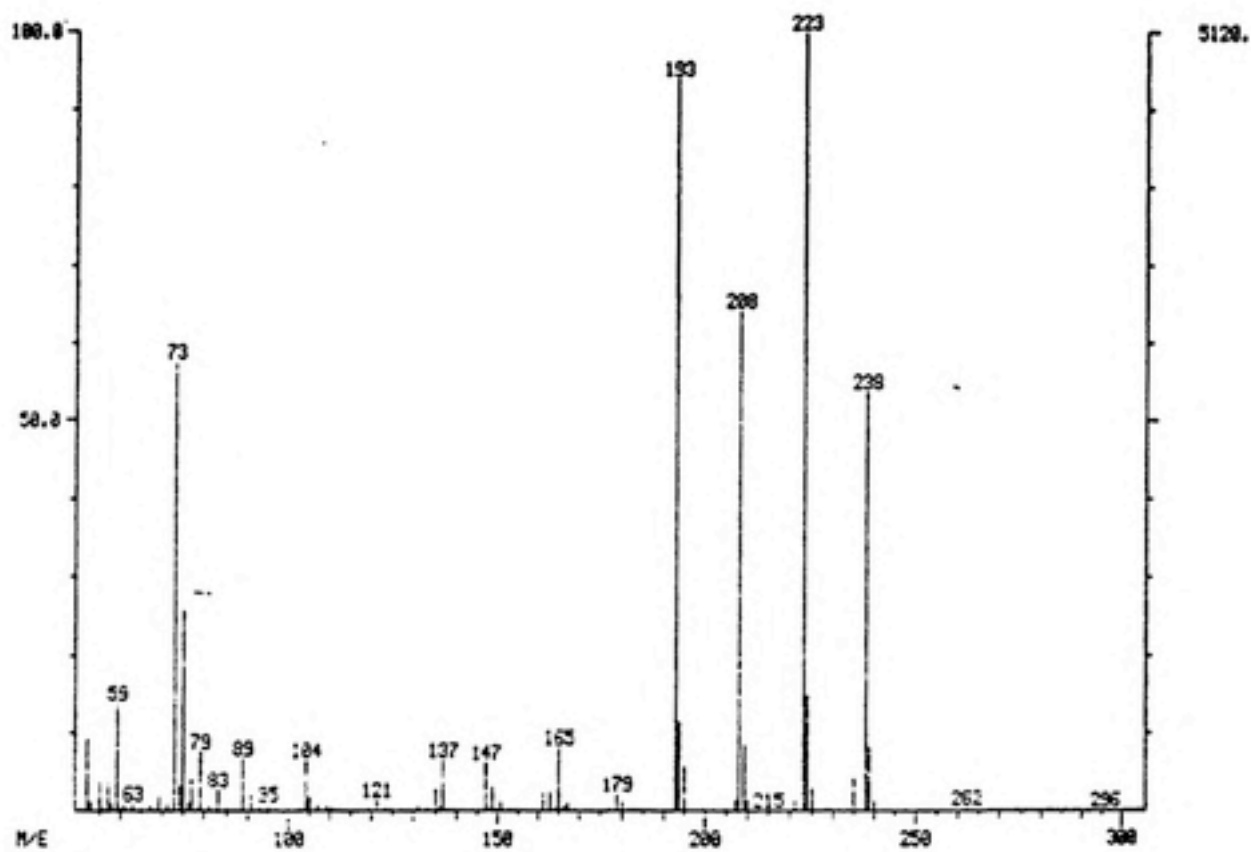
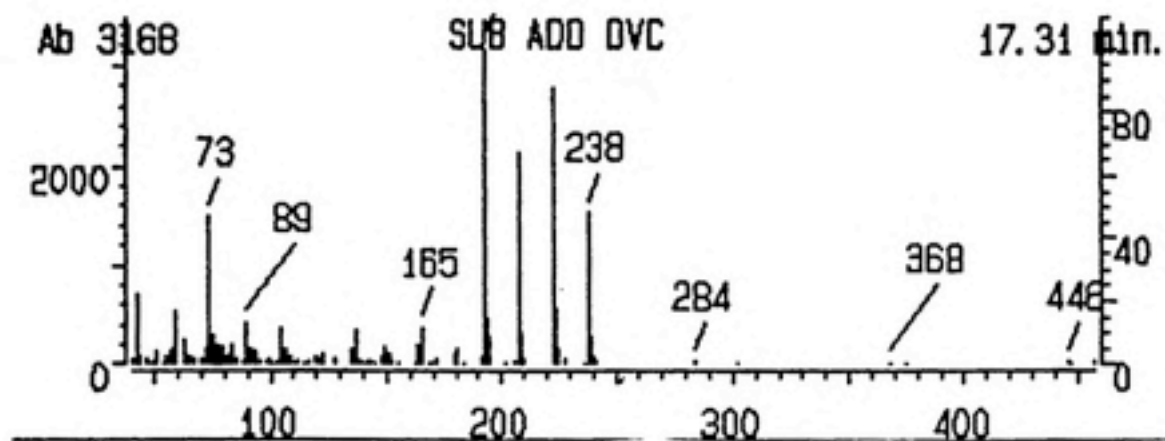
# Vanillin



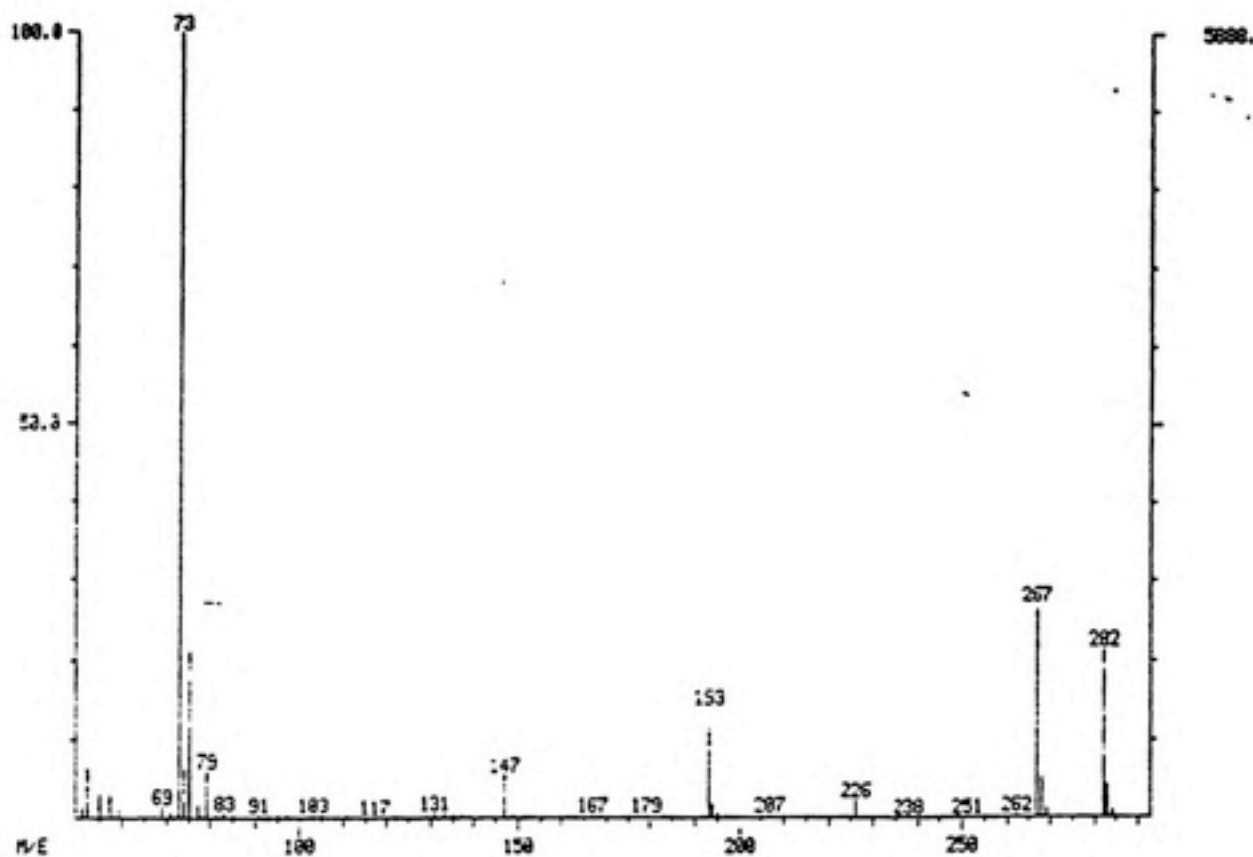
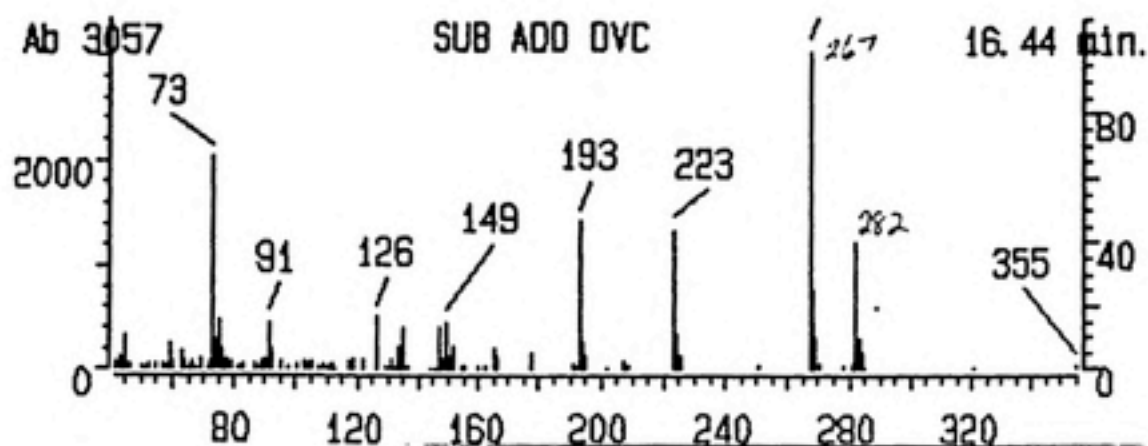
2-Hydroxybenzoic Acid



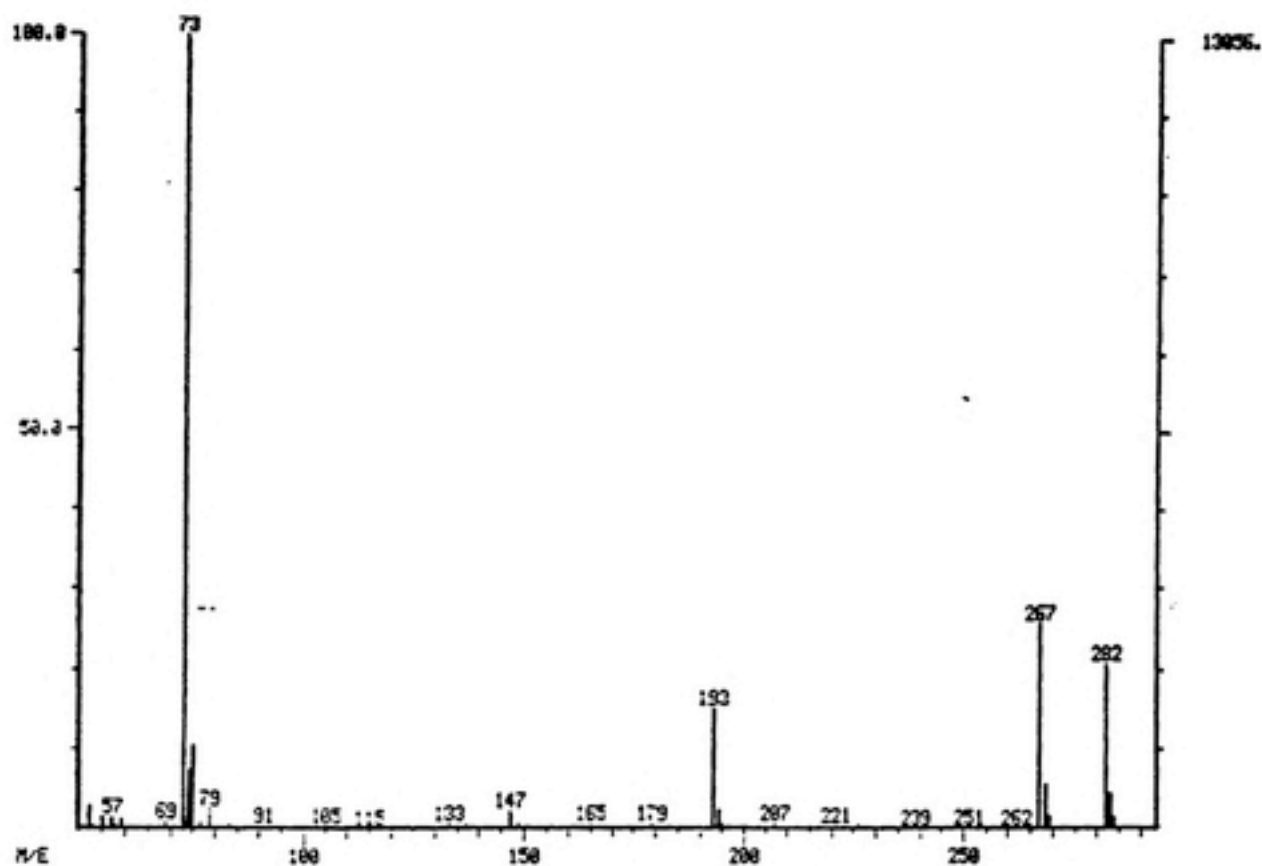
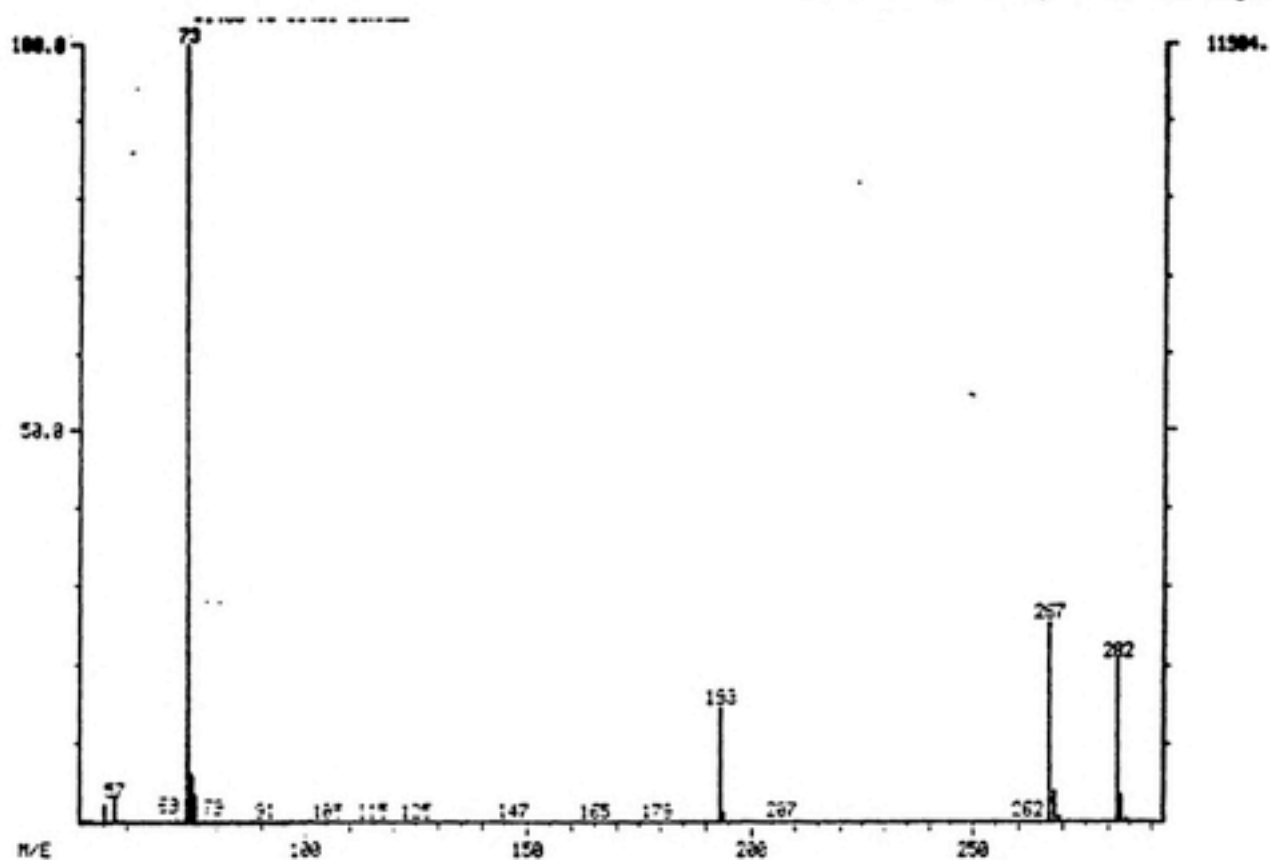
# Acetovanillone



# 3-Hydroxybenzoic Acid

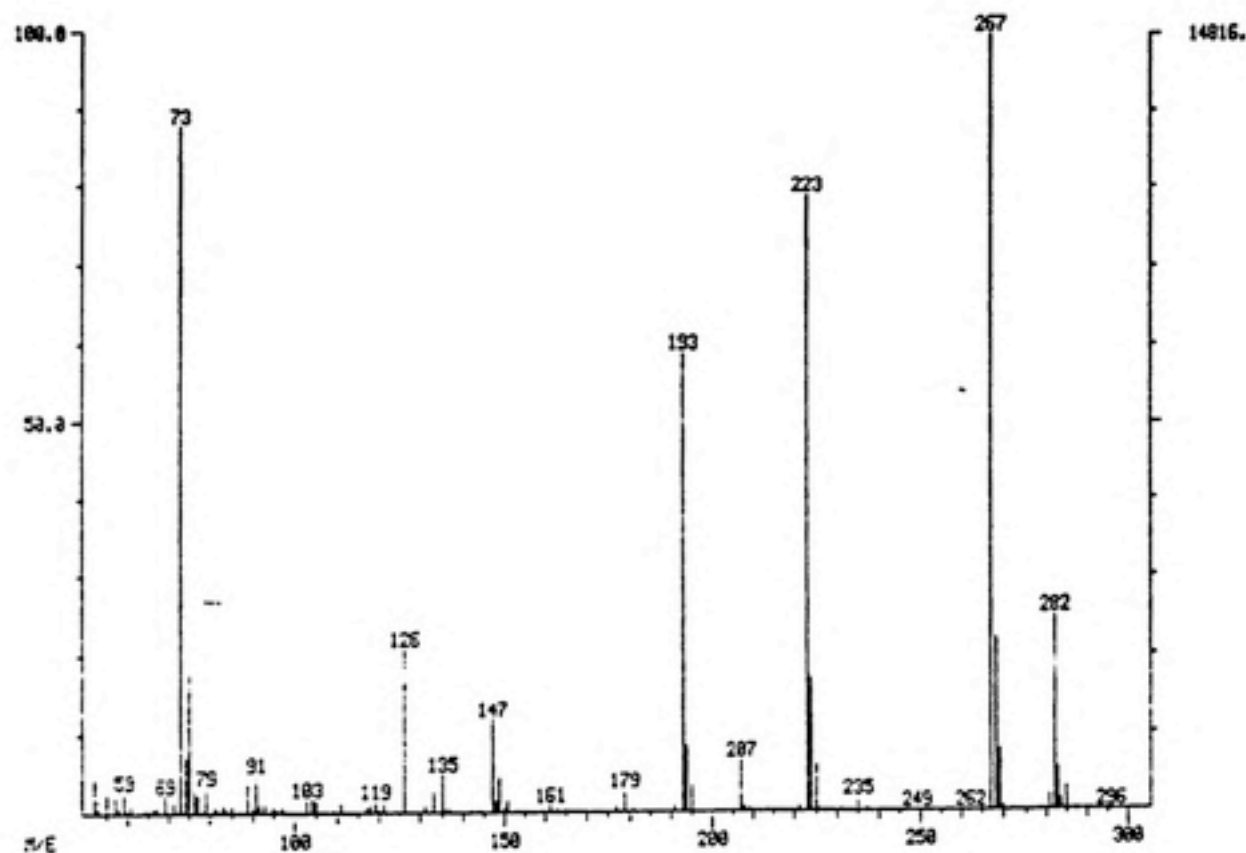
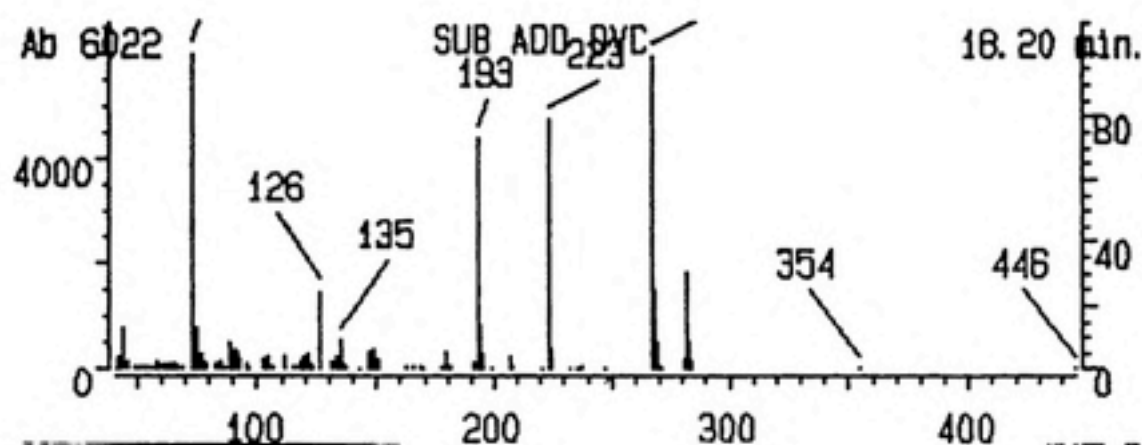


# 3,4-Dihydroxybenzaldehyde

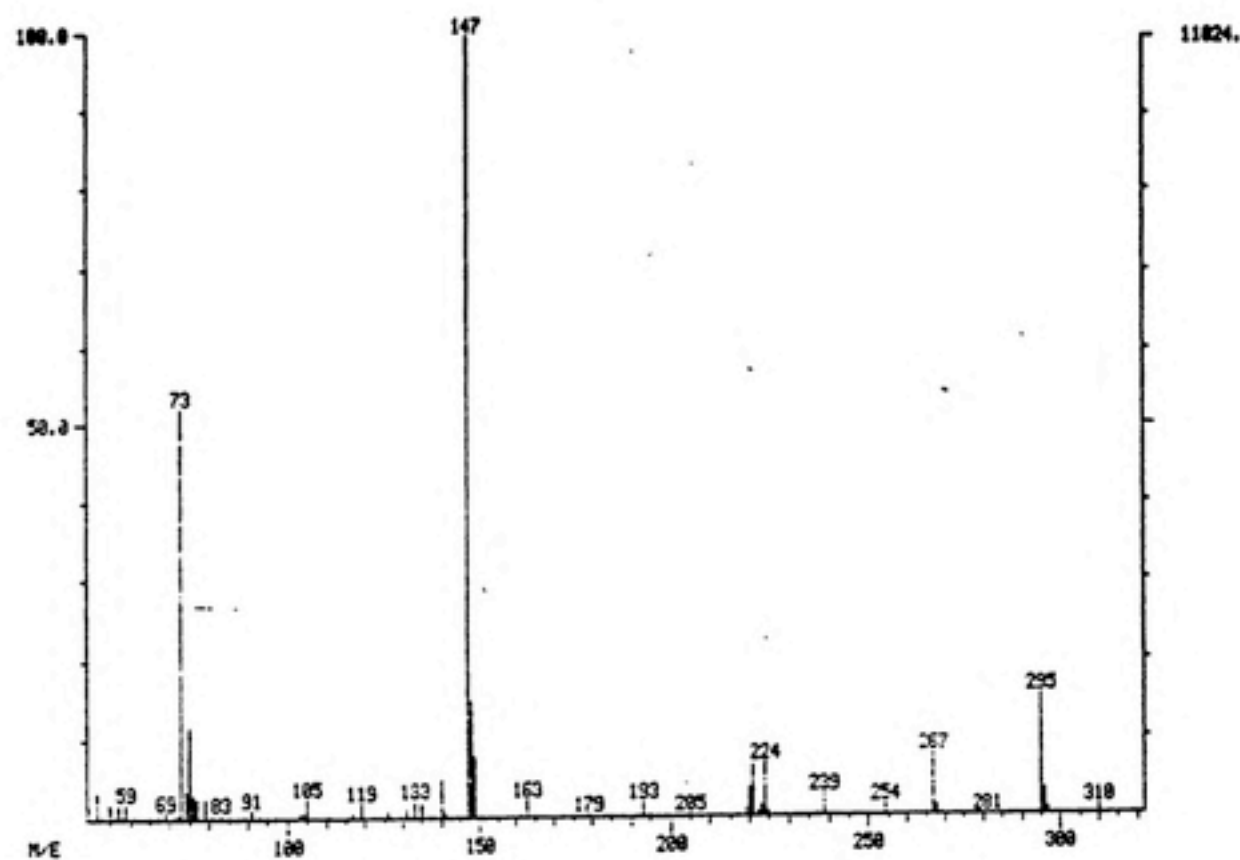
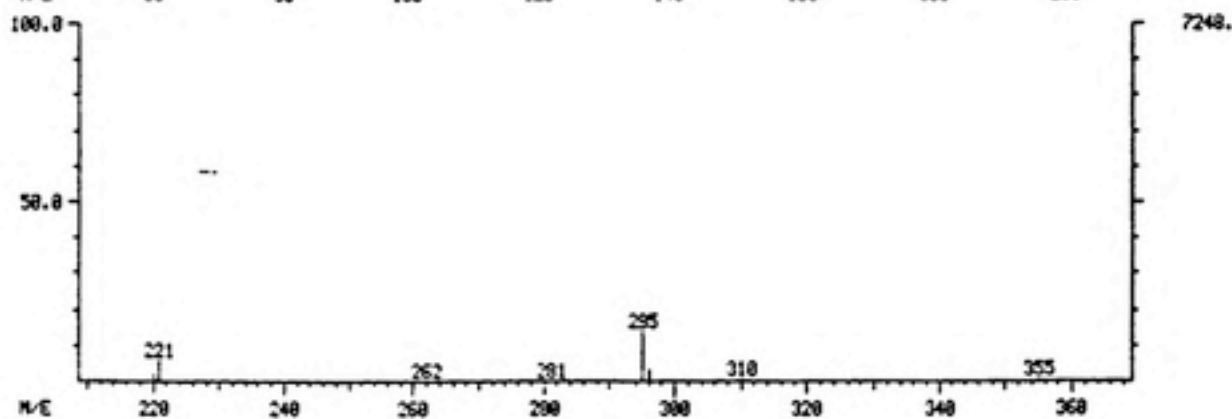
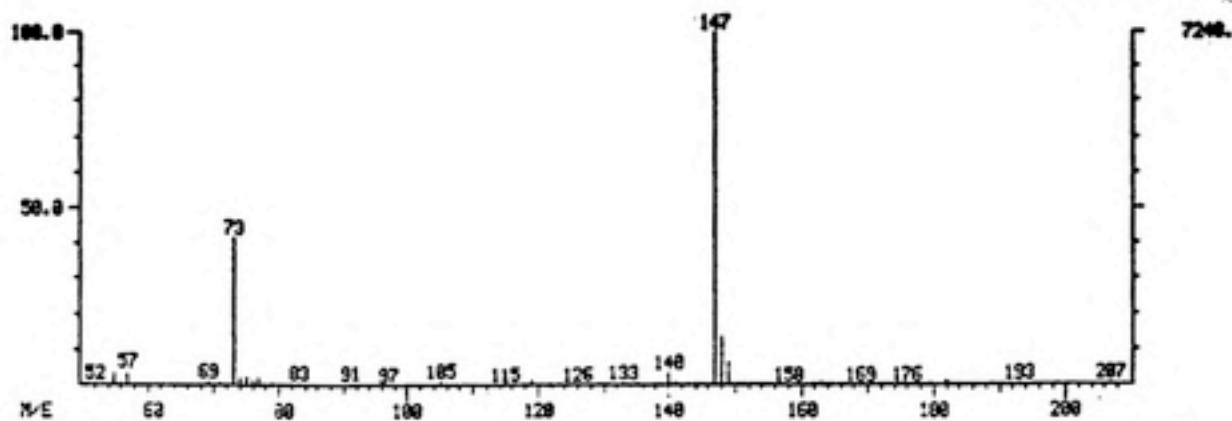




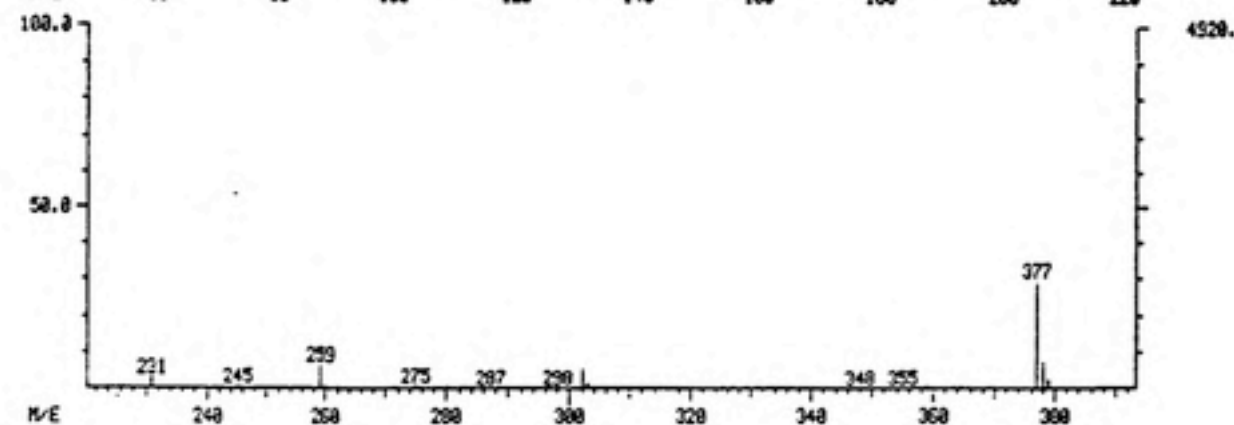
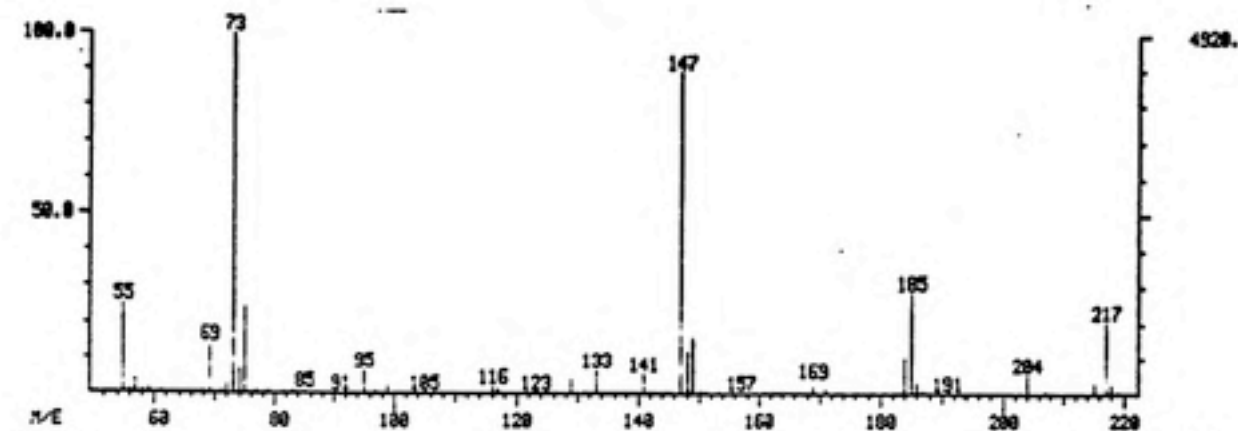
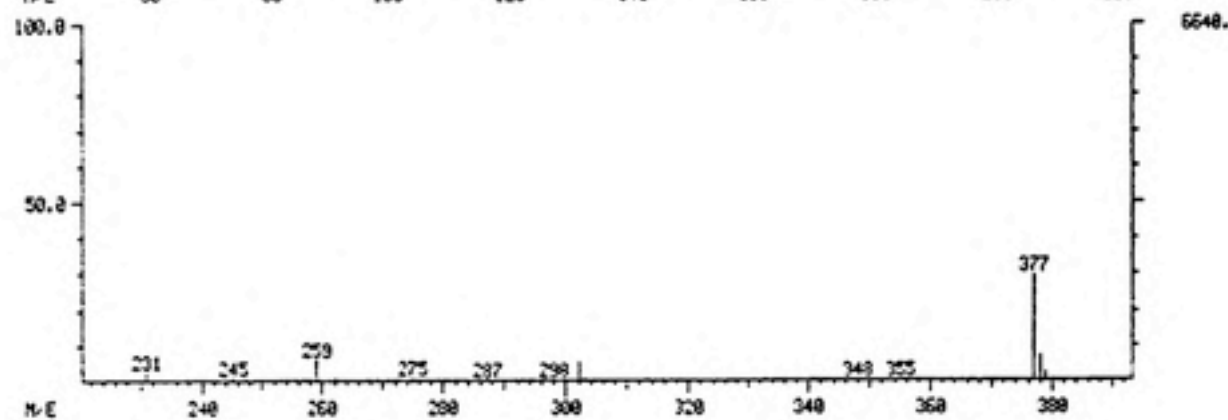
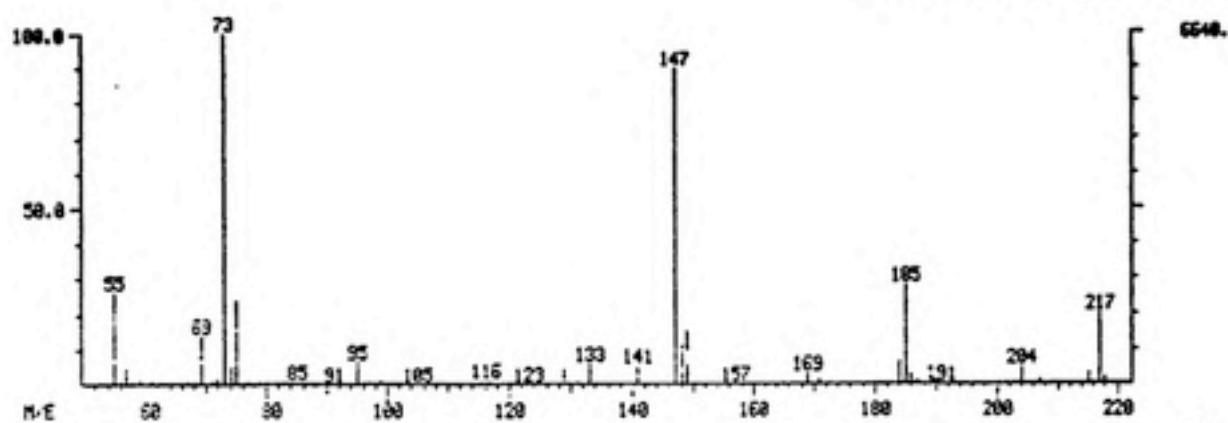
# 4-Hydroxybenzoic Acid



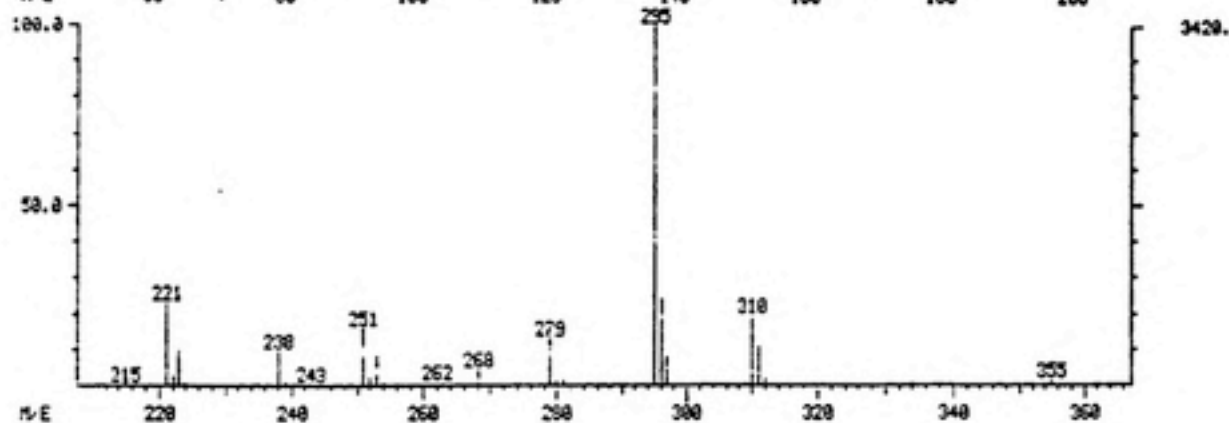
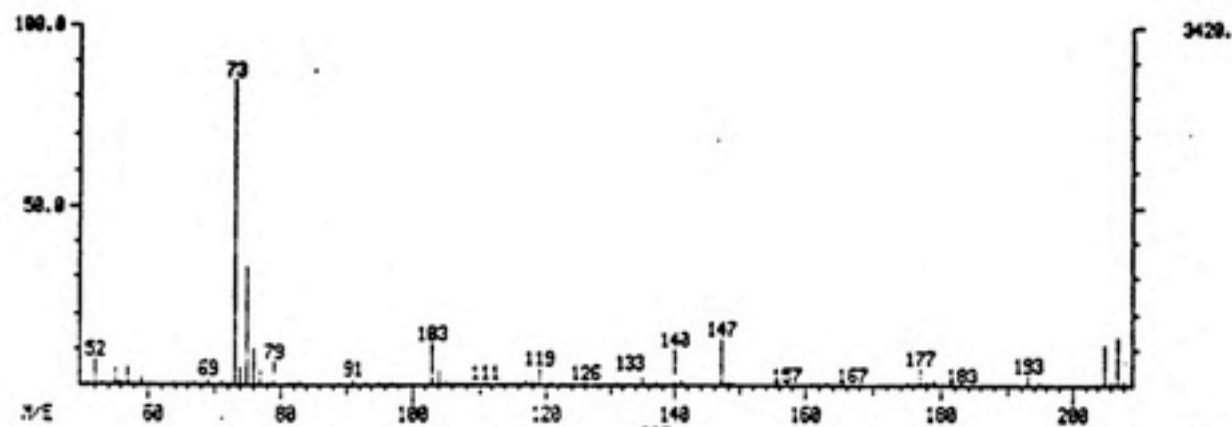
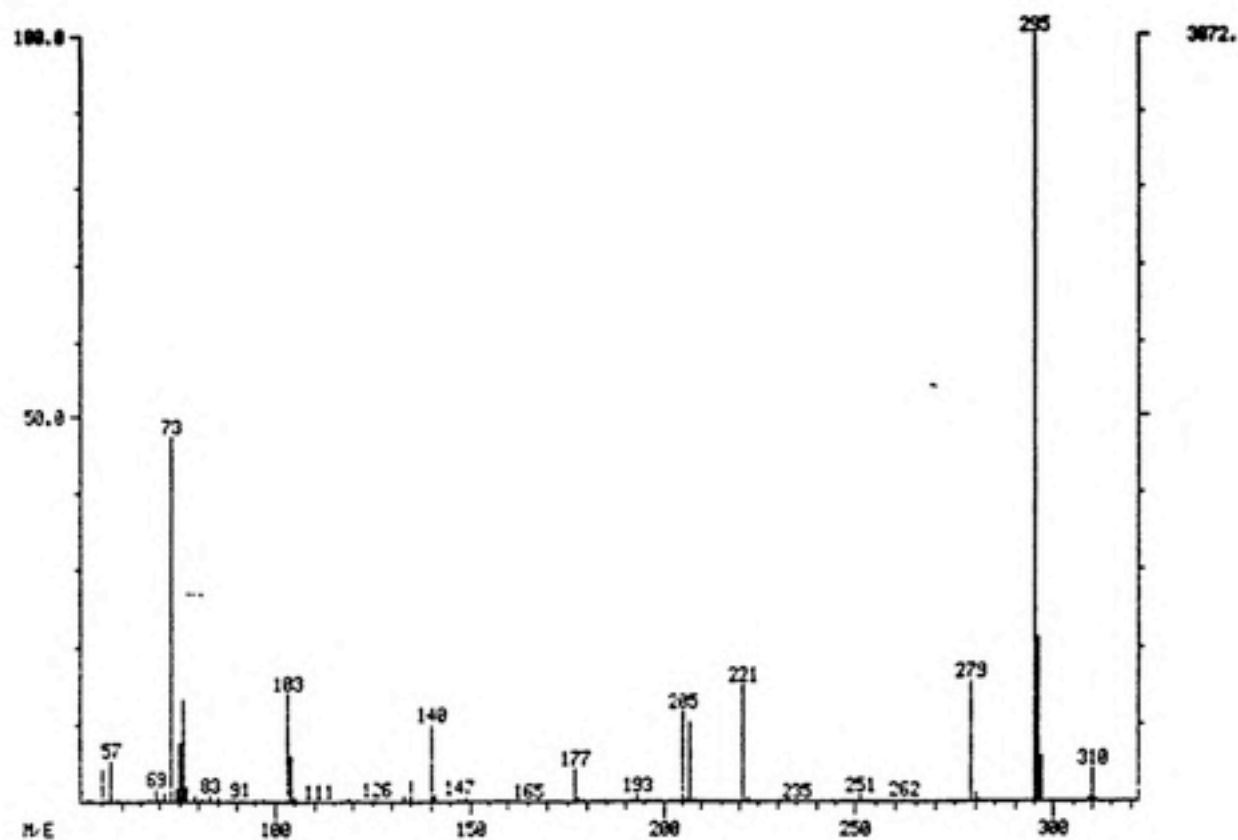
# Phthalic Acid



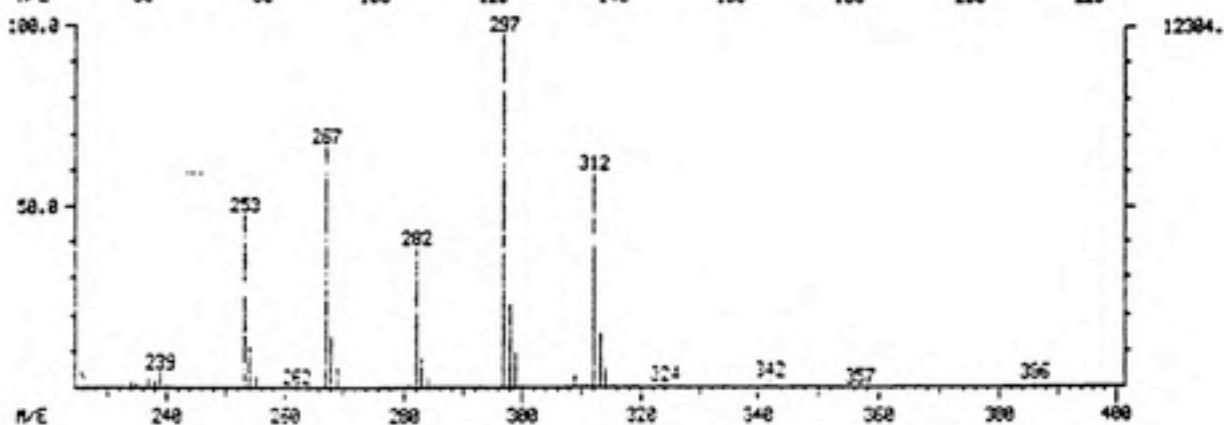
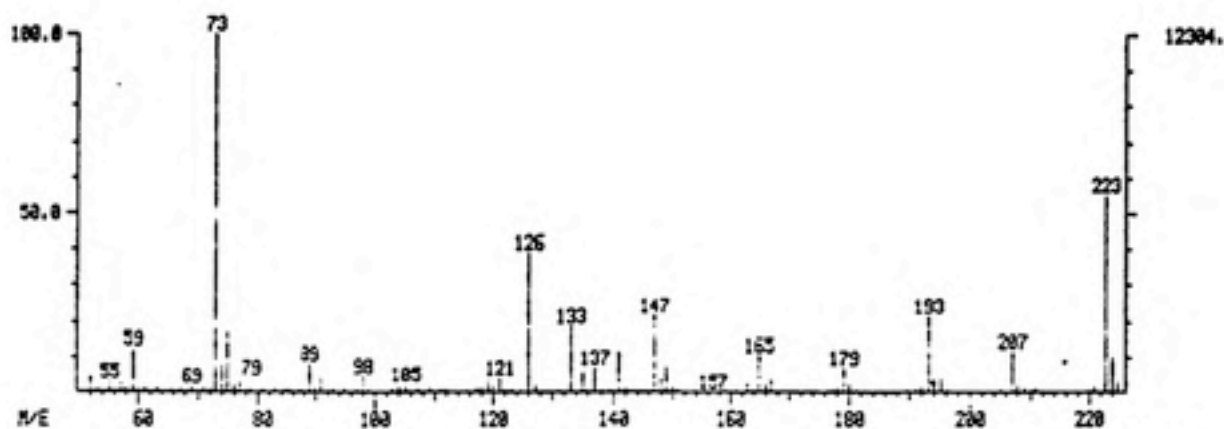
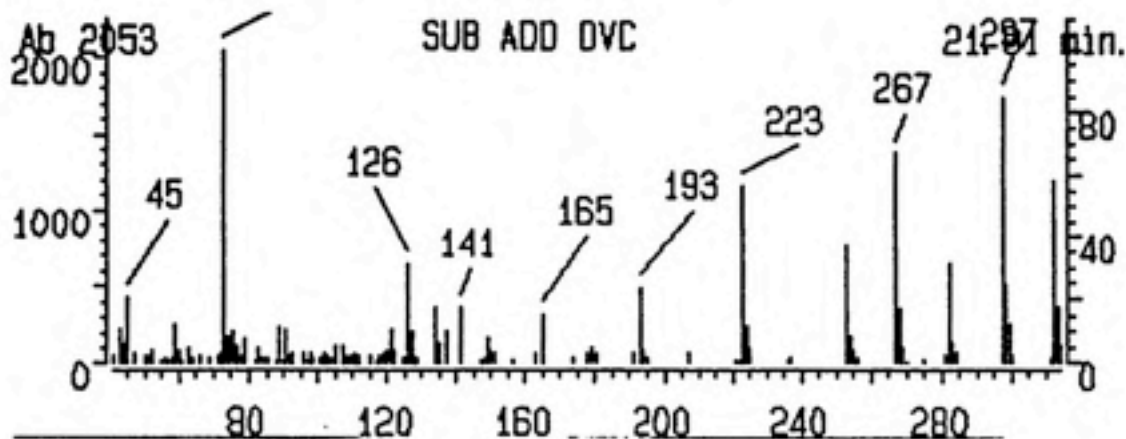
# Tricarballic Acid



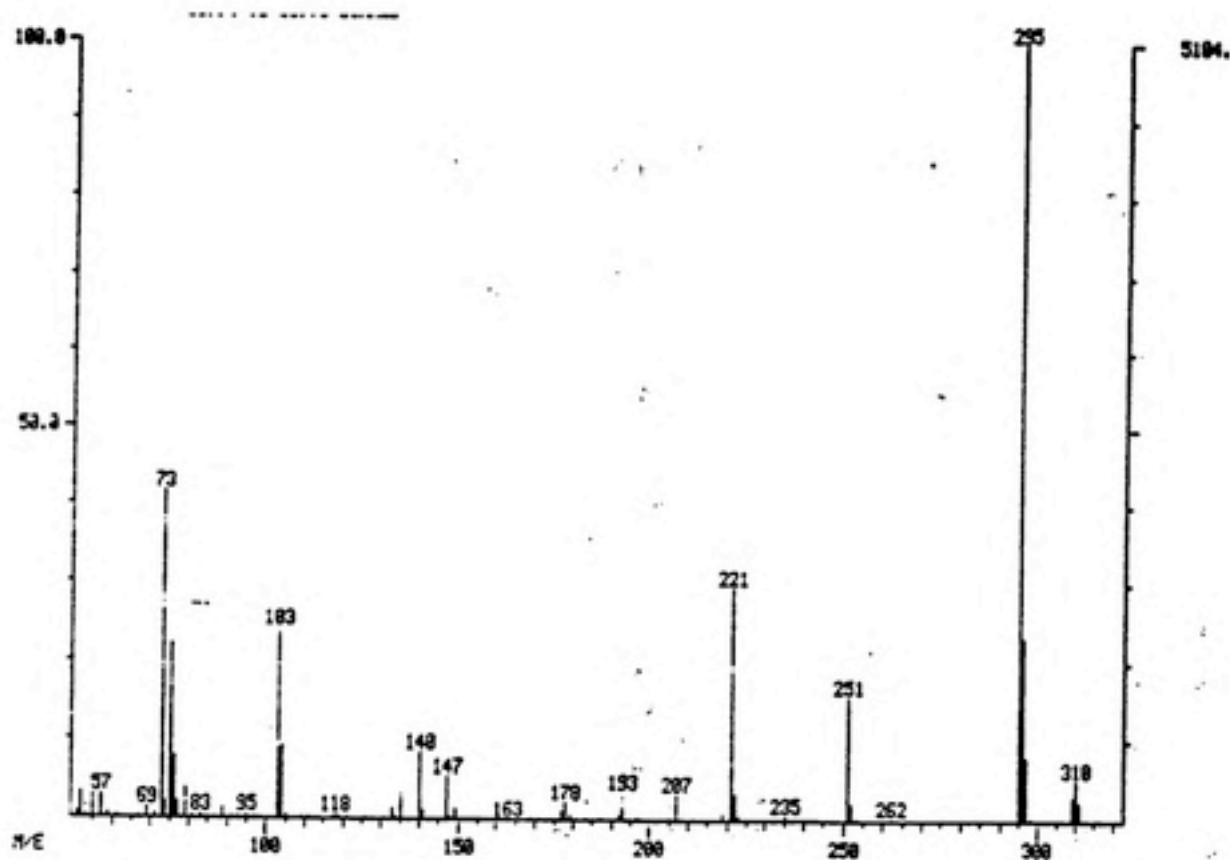
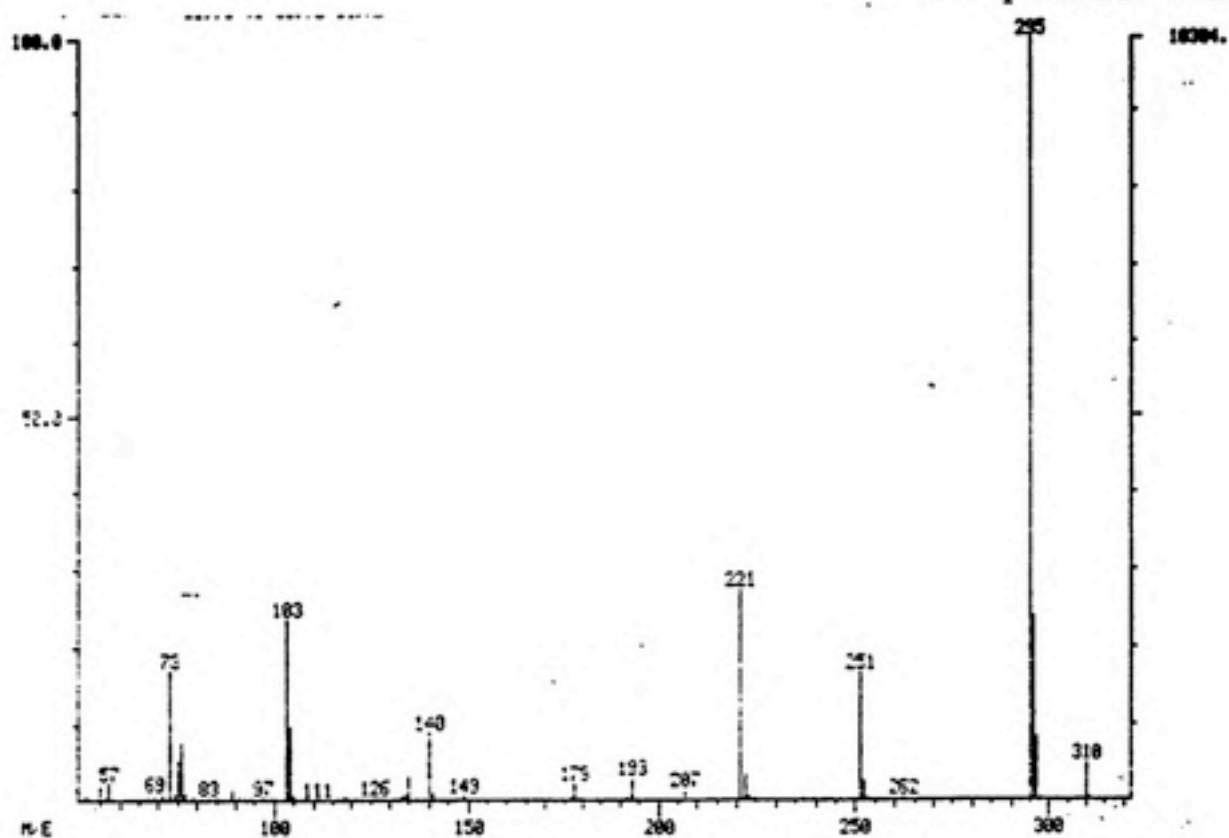
# Isophthalic Acid



Vanillic Acid

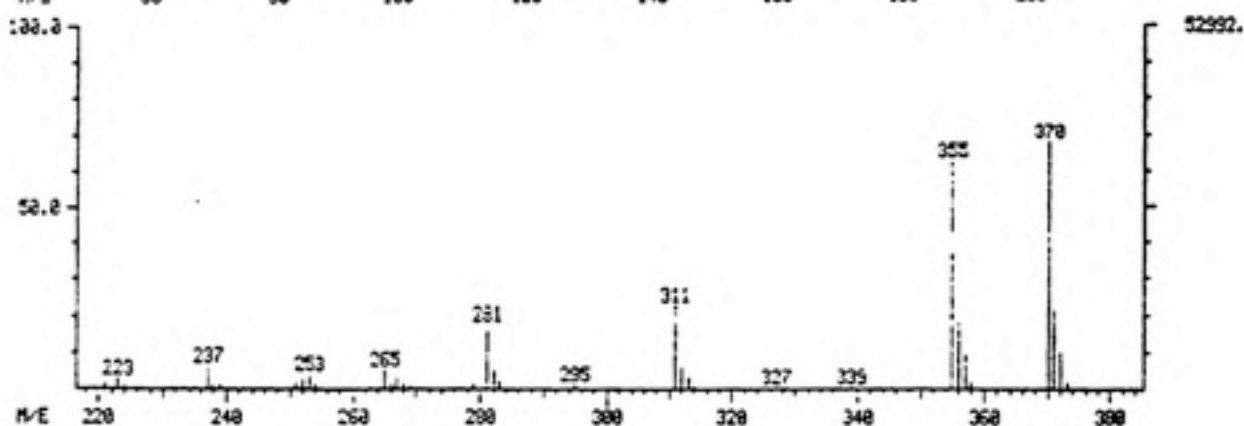
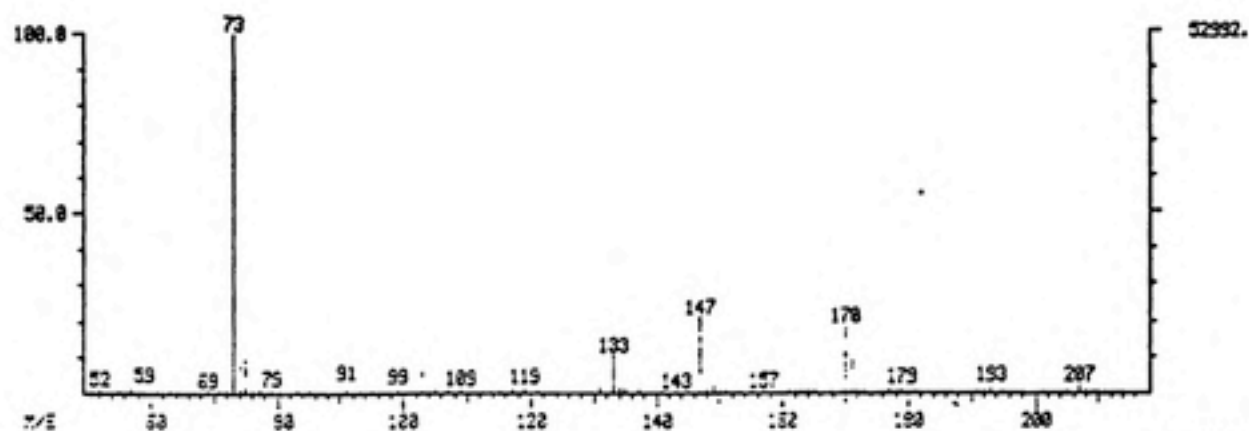
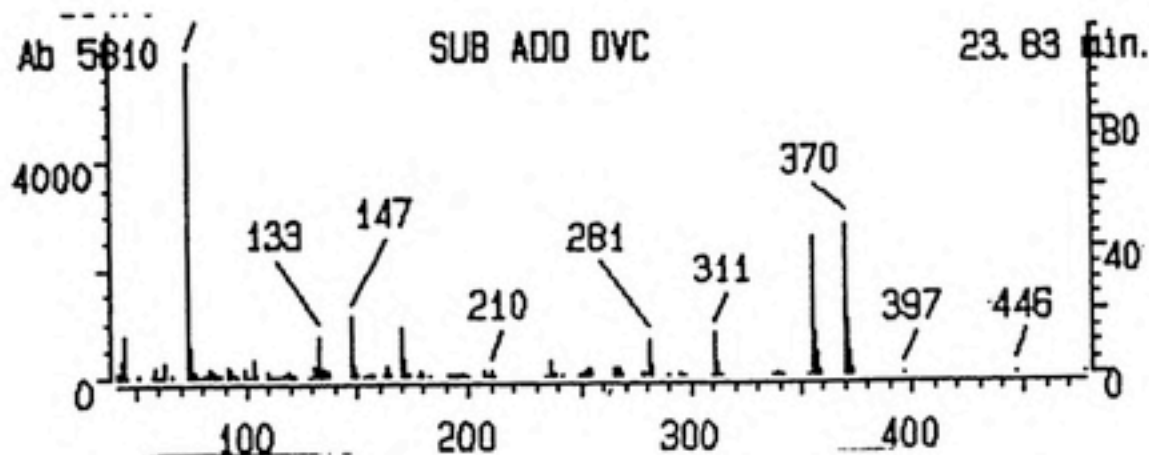


# Terephthalic Acid





# 3,5-Dihydroxybenzoic Acid



# Syringic Acid

