CHARACTERIZATION OF AQUATIC FULVIC ACIDS BY CHROMATOGRAPHIC METHODS

DISSERTATION

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By

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Several chromatographic and spectroscopic techniques were applied to Suwannee River reference fulvic acids (FA) and their permethylated derivatives. Retention mechanisms, structural characteristics, and thermal stabilities of FA and its derivatives and fractions were evaluated.

Preparative reversed phase high performance liquid chromatography (RP-HPLC) separated the hydrophilic and hydrophobic constituents of FA with a total recovery of about 70%. The analytical separation of FA showed the resolution of seven peaks of comparable featureless ultraviolet-visible (UV-vis) scans in the hydrophilic fraction. The hydrophobic fraction was separated into at least twelve peaks. Their UV-vis scans were characteristic of phenols and conjugated ketones. Several of the peaks exhibited similar scans indicating repeated structural units. The solid state ^{13}C CP/MAS and ^{1}H CRAMPS NMR and FT-IR spectra of the fractions indicated that the hydrophilic fraction contains aliphatic structures and COOH functional groups. Spectra of the hydrophobic fraction indicated the presence of aliphatic and phenolic structures.

Separation of permethylated FA by gas chromatography-flame ionization detection (GC-FID) showed the regular retention pattern characteristic of homologous series compounds. The same pattern was also detected in the GC-FID of the methylated sample after supercritical fluid extraction. Methylated FA was subjected to selected solvent elution on a silica column. Different solvent fractions were characterized by capillary GC-FID. Results indicated that column chromatography can be used to separate methylated FA based on the solvent's strength. Each fraction contained several constituents as indicated by the capillary GC-FID. Efforts to further resolve the fractions into molecular constituents by RP and NP-HPLC were unsuccessful.

Pyrolysis GC-FID of FA and fractions gave characteristic chromatograms at 500°C and 800°C. Pyrograms of the hydrophobic fraction showed regular retention pattern characteristic of polymeric material.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF ILLUSTRATIONS

Figure Page

CHAPTER I

INTRODUCTION

Humic substances (HS) are generally recognized as an ubiquitous component of the organic matter in soils, sediments, and fresh and marine waters. Three major fractions of humic substances are operationally defined in terms of their solubilities.

Fulvic acids(FA). The fraction of humic substances that is soluble under all pH conditions.

Humic acids(HA). The fraction of humic substances that is not soluble in water under acid conditions (pH<2), but becomes soluble at greater pH.

Humin. The fraction of humic substances that is not soluble in water at any pH.

All biopolymers or metabolites of algae, microorganisms, or vascular plants have the potential of being or becoming humic substances through constructive or destructive pathways (1). The detailed mechanism of biochemical and chemical pathways is not well established.

The organic matter in all soils, sediments, and waters exerts significant influence on many agricultural, geochemical, environmental, and treatment processes. Humic substances are vital for maintaining the agricultural

importance of soils, for sustaining the water regime of soils, for holding micronutrients for plant growth in the soil, and for acting as an acid-base buffer in the soil. In environmental contexts, humic substances (HS) play an important role in many processes. For example, HS interacts with metal ions by complexation and ion exchange mechanism depending upon the circumstances; this interaction may lead to solubilization or mobilization of the metal. The association of slightly soluble compounds, pesticides, and organic pollutants, with humic substances in the aquatic environment enhances the solubility of these compounds. Such enhanced solubility can result in an increased mobility of these compounds in rivers and other aquatic environments. Besides the increasing mobility, with the association of organic and inorganic species, humic substances may contribute to greater persistence of the contaminants but with a diminished toxicity (2). The chlorination of indigenous humic substances during municipal water disinfection can produce carcinogenic compounds such as trihalomethane and chlorinated nonvolatiles.

About 50% of the dissolved organic carbon (DOC) in uncolored surface water in the USA consists of humic substances. In colored waters, the fraction of the total DOC in the form of humic substances may be as large as 80%. Up to 90% of the dissolved humic substances in natural waters consists of FA, and the remaining consists of HA and

trace synthetic compounds. This composition is in contrast to humic substances from soils, where the humic acids are a very large excess over the fulvic acids (3).

I-1. General Chemical Properties of HA and FA

For decades, researchers exhausted techniques and instruments to obtain the physical and chemical properties of humic substances. With time, it became apparent that humic substances consist of a heterogeneous mixture of compounds for which no single structural formula could be given. Also, each fraction (HAs, FAs, etc.) came to be regarded as being made up of a series of molecules of different sizes, few having precisely the same structural configuration or array of reactive functional groups. In constrast to HAs, the low-molecular-weight FAs contain higher oxygen but lower carbon contents, and they contain considerably more acidic functional groups, particularly carboxylic groups. Another important difference is that practically all the oxygen in FAs can be accounted for in oxygen containing functional groups (COOH, OH, C=O); a high proportion of the oxygen in HAs occurs as structural component of the nucleus (e.g., in ether or ester linkage).

The characterization data of typical aquatic HAs and FAs are given in Table I.1. The total acidities of the FAs

Table I-1. Characterization of Ogeechee stream fulvic acid (Collected December 1981) (4).

1. Elemental analysis (in percent on a moisture-free and ash-free basis).
   C = 54.56 N = 0.87 % ash = 0.86
   H = 4.97 S = 0.74
   O = 38.20 P = 0.62
2. Carbon distribution by solid-state CPMAS ^{13}C NMR (in percent).
   0– 55 ppm (C—C) = 36 110–145 ppm (C=C) = 12
   55– 65 ppm (C—O) = 8 145–160 ppm (ϕ—O) = 5
   65– 95 ppm (C'—O) = 16 160–195 ppm (COOH) = 16
   95–110 ppm (anomeric) = 3 195–225 ppm (C=O) = 4
3. Aliphatic carbon percentage = (0–110 ppm) = 63%;
   Aromatic carbon percentage = (110–160 ppm) = 17%.
4. Functional groups:
   COOH (titration) = 6.4 Alcoholic OH (^{13}C NMR) = 5.1
   COOH (^{13}C NMR) = 6.8 Phenolic OH (titration) = 1.6
   Carboxyl (^{13}C NMR) = 1.7 Phenolic OH (^{13}C NMR) = 2.1
   Methoxyl (^{13}C NMR) = 3.4
5. Molecular weight = 650–950; radius of gyration = 6 Å.
6. Percent carbohydrate ~5%.
7. Percent of total nitrogen as amino acids ~20%.

Characterization of Ogeechee stream humic acid (Collected December 1981) (4).

1. Elemental analysis (in percent on a moisture-free and ash-free basis).
   C = 55.94 N = 1.27 % ash = 1.13
   H = 4.13 S = 0.93
   O = 36.52 P = 0.25
2. Carbon distribution by solid-state CPMAS ^{13}C NMR (in percent).
   0– 55 ppm (C—C) = 23 110–145 ppm (C=C) = 21
   55– 65 ppm (C—O) = 8 145–160 ppm (ϕ—O) = 9
   65– 95 ppm (C'—O) = 12 160–195 ppm (COOH) = 16
   95–110 ppm (anomeric) = 4 195–225 ppm (C=O) = 7
3. Aliphatic carbon percentage = (0–110 ppm) = 47%;
   Aromatic carbon percentage = (110–160 ppm) = 30%.
4. Functional groups:
   COOH (titration) = 4.7 Alcoholic OH (^{13}C NMR) = 4.3
   COOH (^{13}C NMR) = 6.8 Phenolic OH (titration) = 1.9
   Carboxyl (^{13}C NMR) = 3.0 Phenolic OH (^{13}C NMR) = 3.9
   Methoxyl (^{13}C NMR) = 3.4
5. Molecular weight = 2000–3000; radius of gyration = ~10 Å.
6. Percent carbohydrate ~10%.
7. Percent of total nitrogen as amino acids ~25%.

are higher than those of HAs. Both COOH and acidic OH groups contribute to the acidic nature of these substances, with COOH being the most important. The concentration of acidic functional groups in FAs would appear to be substantially higher than in any other naturally occurring organic polyelectrolyte.

I-2. Environmental Reaction of Humic Substances

The role of humic substances in natural waters, such as interaction with organic contaminants and with metals, and their influence on water treatment, coagulation process, adsorption onto activated carbon, ozonation and chlorination processes, ion exchange and membrane process, etc., are discussed in the 1989 American Chemical Society (ACS) series monograph(5). This section is only confined to the topic of the interaction of humic subtance with metals. This aspect is very important for prediction of the reactivity, toxcity, and tranport of metal ions in the aquatic environment.

The association of positively charged metal ions with negatively charged organic anions goes beyond the concept of cation exchange to specific exchange, complexation, and possible chelation. The fact that protons are released into solution is an evidence of metal complexation with acidic functional groups in humic substances. Other functional groups on aquatic humic substances containing nitrogen,

phosphorus, and sulfur may also be potential complexing sites, but they are believed to be minor compared to the abundance of carboxylic and phenolic functional groups. The relatively high stability of humic-metal complexes and the ability of humic substances to complex polyvalent metal ions such as iron, aluminum, and copper have led scientists to postulate that chelate may be formed.

The factors that influence metal-humic complexation include concentration of humic substances and metal ions, competing ligands and metal ions, source of humic substances, type and speciation (charge) of the metal ion, pH, ionic strength, and temperature. An example of how complexation is affected by type of metal ion, concentration of fulvic acid, and concentration of metal ion is shown in Fig. I-1(6). The effect of pH on complexation can be explained in two ways: (1) The concentration of H^+ ion determines which form of fulvic acid and metal ions are prevalent; (2) H^+ ions compete with metal ions for anionic binding site on FA. As pH is raised, the OH^- ions compete with FA for cationic metal ion. FA becomes more available for complexation, and metal ion becomes less available. Conditional stability constants tend to increase with pH up to a certain point and then decrease.

Humic-metal associations in water occur both in true and colloidal forms. The products of humic-metal association, at moderate to low pH values and low

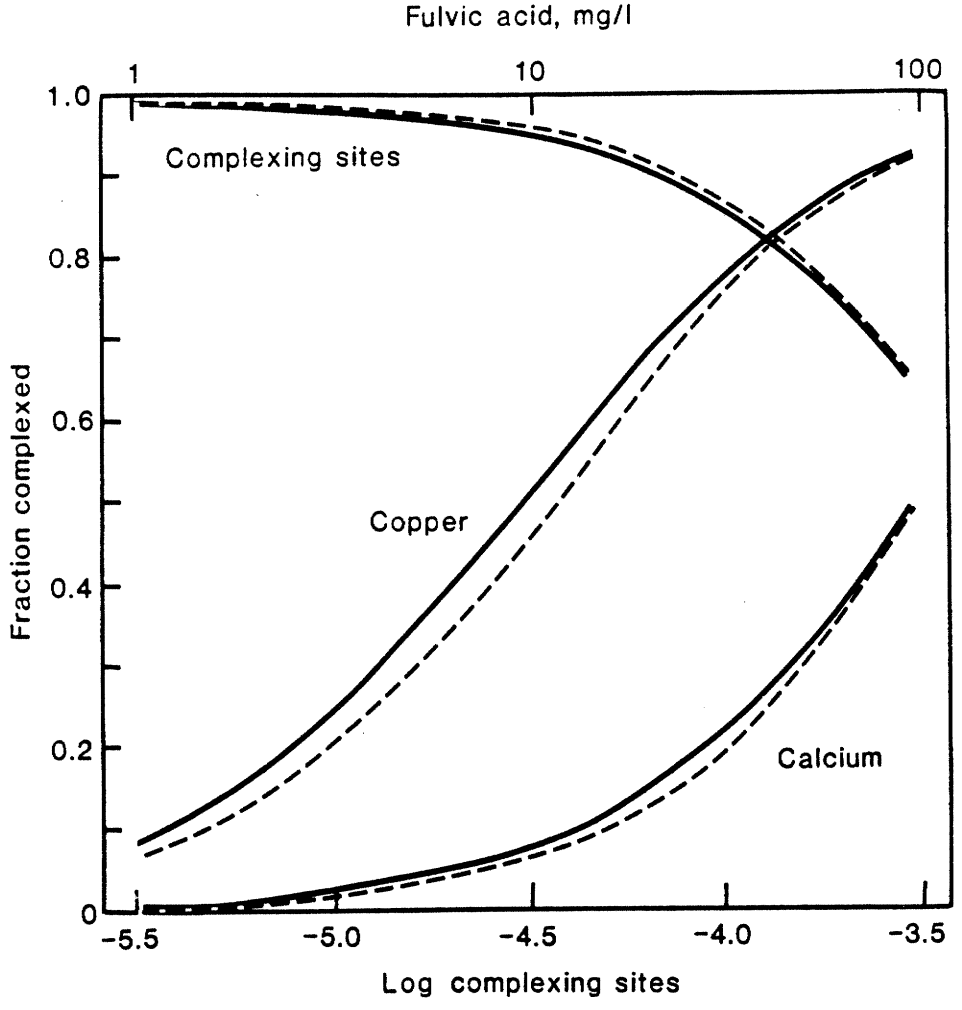


Figure I-1. Degree of complexation of Cu(II) and Ca^{2+} as a function of FA concentration. Total Cu(II) concentrations 1 x 10^{-8} M (solid line) and 1 x 10^{-5} M (dashed lines). From reference 6.

concentration of the metal ion, tend to be dissolved complexes (7). As either the pH approaches neutrality, or the metal to humic ratio increases, the adducts may be either in solution or in a fine colloidal state. If the concentration of humic substances is sufficiently high and the concentration of the metal ion is low, the humic ligand competes effectively with OH^- to keep trace metal complexed in solution or as a finely divided stable colloid. At high metal ion concentrations slightly above or below neutrality, the metal ion is hydrated, resulting in the formation of excess metal-humic flocs (8).

I-3. Characterization of Humic Substances

From the structural studies, we can not only reveal the genesis of humic substances, but also understand the reactivity of these materials in the environment. Recent data accumulated from interlab investigations may lead to major advances in the understanding of the nature and chemical structure of humic substances. Early approaches to the structural elucidation of humic substances commonly utilized "degradative" and "nondegradative" methods.

Degradative Approach

In the degradative approach, macromolecular humic substances are chemically or physically broken down into

various simple compounds representative of main structural subunits. The products are then separated, identified, and quantified. Structural inferences are drawn based on the identities and fields of degradation products and the reaction processes responsible for their generation. The particular process chosen can utilize any type of reaction including:

(1) oxidation with $KMnO_4$, H_2O_2 , CuO-NaOH, nitrobenzene

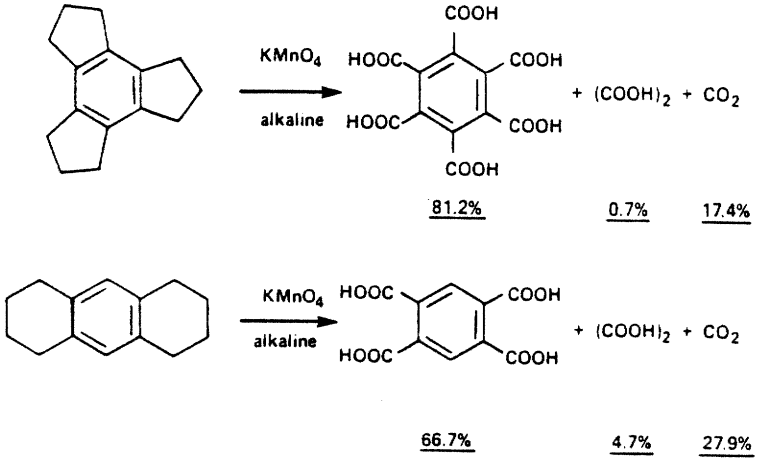
(2) reduction with Zn dust, Na amalgam

(3) pyrolysis

(4) hydrolysis with acids and bases

(5) microbiological degradation

The first three reactions are the most widely used. Potassium permanganate would oxidatively cleave the aliphatic side chain from the aromatic nuclei. Numerous experiments generate significant quantities of benzenecarboxylic acids in various substitution patterns.



Though these patterns could have arisen from aliphatic side chain oxidation or fused ring degradation, they were also obtained from alkaline cupric oxidation treatment, which does not degrade fused aromatic rings. Thus, the oxidative degradation studies with alkaline CuO indicate aliphatic side chains are dominant structures in HS.

In reductive methods, zinc dust distillation gave aromatic fused ring compounds, leading initially to the idea of a stable aromatic core. Sodium amalgam reduction gave mixtures of di and trihydroxy benzenes, also with -COOH or $-CH_2$ substitutents, indicative of possible lignin origin(9). Degradation with phenol cleaves the aliphatic linkages, joining aromatic groups, suggesting structures such as $Ar-(CH_2)_n-Ar$.

The selective rupture of the weaker bonds in a polymer structure can readily be accomplished by analytical pyrolysis. Pyrolysis products included a variety of aromatic, heterocyclic, and straight-chain organic compounds (10). Pattern recognition was used to evaluate FA pyrolysis fragments produced by pyrolysis-MS without separation (11). Carbohydrate and phenolic compounds were more pronounced in FA pyrolysates.

The Structural Inferences from Degradative Studies

Two important conditions are emphasized from the degradative studies (12):

(1) The majority of the studies indicate that these

aromatic nuclei are highly substituted with either crosslinking aliphatic side chains or functional groups such as carboxyl, hydroxyl, and methoxyl. however, the nuclei are not representative of intact lignin/phenolic structures. The sources may come from all kinds of plant biopolymers.

(2) Aliphatic structures have greater importance in the macromolecular structure of many humic substances than previously believed. This is especially true when one considers freshwater aquatic and marine humic substances.

The Pitfalls of the Degradative Approach

These most often applied methods inherit some experimental and inferential pitfalls. The former includes selection of unoptimized reaction conditions, improper use of reaction blanks to detect contamination, selection of an extraction procedure which fails to recover degradation products, and improper identification of degradation products. The latter includes the use of degradation products formed in low yield for major structural inference.

The Nondegradative Approach

This approach seeks to analyze isolated humic substances without chemical or physical alteration utilizing

techniques which allow structural inference to be drawn. The techniques utilized include UV-vis and IR spectrometry, ESR spectrometry and NMR, X-ray analysis, electron microscopy and electron diffraction, chromatography, viscosity and surface tension measurements, and various titration methods. Undoubtedly, the most promising technique in structural identification of pure compounds is NMR. In recent years, the application of ^{13}C in HS research were centered around the aromaticity and aromatic/aliphatic carbon ratio determination of FA and HA. The techniques applied include ^{13}C-^{1}H dipolar dephasing (13); spin-echo and broad band decoupled ^{13}C (14); solid state ^{13}C spectroscopy (15). Hydroxyl functional group were examined with ^{13}C NMR for chemically derived samples (16). The ratio of carboxylic hydroxyl to total hydroxyl content would be determined after samples were permethylated with CH_3I/NaOH. The ^{13}C-^{1}H dipolar interaction and dipolar dephasing experiments indicate qualitative evidence of aromatic ketones exist in humic substances. There is about one ketone group per monocyclic aromatic ring in both FA and HA (17).

There are two structural inferences from nondegradative studies (12):

(1) Humic substances, isolated from different environments and different matrices, differ greatly in chemical structure. The differences usually involve the

relative amounts of aliphatic versus aromatic carbons.

(2) Aliphatic structures have greater importance in the macromolecular structure of many humic substances than previously believed. This conclusion is worth restating since most studies, both degradative and nondegradative, have tended to concentrate on aromatic features of humic structures. The historical oversight has led many investigators to potentially false and dangerous assumptions.

The Pitfalls of the Nondegradative Approach--

The experimental pitfalls for this technique include: (i) the needs of the HS chemist to know enough NMR theory to appreciate relaxation mechanism and to optimize acquisition parameters; (ii) another limitation is sensitivity. Obtaining spectra from larger samples is limited by rotor design and coil size, and frequently it is necessary to increase time of analysis at the expense of quantitation (18); (iii) the interpretation of NMR spectra for a macromolecular system based on chemical shift will not correlate with that of simple compounds, and care must be taken to avoid over-interpretation. In complex macromolecular systems such as humic substances, nuclei can experience a wide variety of chemical shifts.

Solid State NMR of Polymeric Compounds

In solid state NMR, small high resolution features (e.g., the chemical shifts and complex line splittings associated with electronic environments in the molecules) are often masked by static magnetic dipole-dipole interactions which are manifested in spectral line broadening. With the development of multiple pulse and double resonance NMR for dipolar decoupling, it has become possible in many cases, to suppress broadening of solid state NMR. The combined rotation and multiple pulse NMR spectroscopy (CRAMPS) (19) has recently been applied for investigation of ^{1}H NMR of solid polymers and molecular complexes.

The ^{1}H NMR characteristics depend on the spatial distribution of the protons and the nature of the molecular motion. Thus, the spectrum consists of superposition of lines having different widths and also possibly different shapes. In the simplest approximation, we expect a broad line from more rigidly ordered or crystalline regions and a narrower line from more mobile, amorphous regions (20). For randomly oriented solids, the ^{1}H CRAMPS spectrum may still be sufficiently broad because of overlapping chemical shift anisotropies to make chemical shifts associated with structural features unobservable. However, application of CRAMPS in the mapping of the shielding environments of common functional groups seems to be promising. Recently,

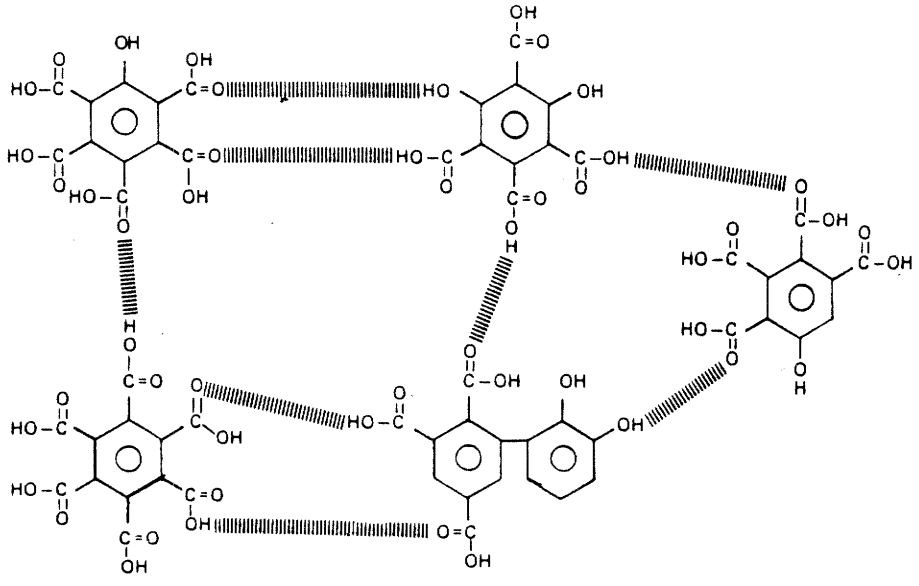
Bronnimann et al. (21) reviewed the technique and its performance and application to solid powder, crystalline compounds, polymers, and organic geochemical samples. Both quantitative and qualitative aspects of the technique were evaluated. Under conditions of thermal equilibrium, the intensities from ^{1}H CRAMPS spectra were proportional to the spin concentration relevant to the corresponding species. In this sense, ^{1}H CRAMPS can provide reliable analytical quantitation and can avoid some of the uncertainties that often plague MAS NMR data obtained via cross polarization. The ^{1}H CRAMPS method is very sensitive, requiring as little as 100 ug of sample. The resolution is often inadequate, and interpretation is still imprecise.

Evolution and Comparision of Structural Models

Over the past two decades, the exact nature of the molecular subunits and interaction/intermolecular bonds present in humic substances derived from various environmental matrices have attracted the interest of scientists from a number of disciplines. Several models were proposed in order to understand the relations between the structure and quantity and the chemical properties and activities of humic substances.

Schnitzer introduced a molecular structural model which is composed of distinct benzene rings substituted by phenolic and carboxylic acids groups as shown in Fig. I-2a

(a) Schnitzer's model (22).



(b) Christman's model (23).

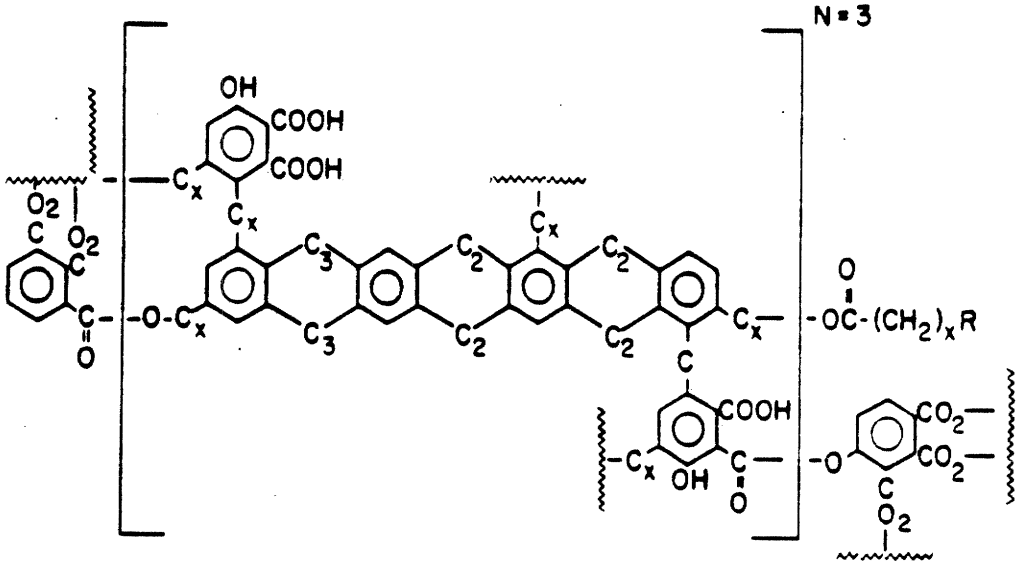


Figure I-2. Proposed structual model of FA and HA.

(22). The interconnection of the so-called "building blocks" was based merely on hydrogen bonds. One of the characteristics of this model is that it is punctured by voids of different dimensions which would trap or fix organic molecules as well as inorganic compounds. Schnitzer's proposed structure was also quite loose or open, which was in agreement with the results of X-ray analysis. Schnitzer's model was strongly criticized for the following reasons: 1) the chemical degradation of humic substances is energy demanding, and the weak intermolecular forces (H-bonding, van der Waal's forces) of this model cannot explain this fact; and 2) the structure does not display polymer properties when dissolved in neutral or basic solution.

Later, Christman et al. proposed a molecular model termed "aromatic matrix" model (Fig. I-2b) (23). The model incorporates structural inference based on identities and relative yields of benzene carboxylic acids after chemical degradation. This model was also criticized for the following reasons:

(1) The model is based on oxidative products identified by GC/MS after CH_2Cl_2 or ether extraction. These products represent only 25% of the initial weight of the starting FA.

(2) The aromatic contents of the model is much higher than could be detected by solid state ^{13}C or ^{1}H NMR. Published data on the aromatic content of FA by solid

state NMR range from 15 to 45%.

(3) The model did not explain the chemical properties of FA, such as color, high O/C ratio, or the percentages of aliphatic carbons.

More recently the membrane model has been proposed by Wershaw and Pinckney (24). The model describes HA as aggregates of small molecules that are held by hydrogen bonding and hydrophobic interactions. The interior of this bilayer is hydrophobic, and the exterior is hydrophilic. It was concluded that humic subtances are composed of mixtures of chemical components derived from partial degradation of plant material. The evidence of this model was derived from 1) measurement of the aggregation of HS by small angle X-ray scattering and ultracentrifugation; 2) studies of the surface -active properties of HS; 3) studies of the sorption of hydrophobic materials in soil-water systems; and 4) the NMR spectra of the various HS and their fractions, which have spectral features.

I-4. Chromatographic Studies of Humic Substances

Chromatographic fractionation has been applied to humic substances for three reasons:

(1) To determine the variation found for properties such as molecular weight, functional group content, etc.

(2) Fractionation procedures have been used as preliminary

steps before spectral measurements or functional group analyses.

(3) To monitor the effect of some other chemical or biological treatment.

In recent years, almost every mode of HPLC has been used in humic substances research. Size exclusion chromatography (SEC) methods include the following inherent limitations: 1) irreversible adsorption of neutral eluites and 2) charge interaction between residual charged groups on the gel and those on the FA. The technique was applied at analytical and semipreparative scales (25,26). A recent study of SCE was applied on methylated FA (27). The molecular mass of the methylated FA ranged between 300-5000 dalton. This result is lower than reported in earlier studies on underivatized FA, possibly due to reduction in hydrogen bonding upon methylation. Up-to-date size exclusion studies did not reveal individual repeated units but indicated the heterogenic nature of FA macromolecules.

Ion exchange HPLC, using weak anion exchange resins such as diethylaminoethyl (DEAE) and pellionex (WAX) have been used to fractionate non-volatile organics from surface and chlorinated water (28,29). Variable resolutions were obtained depending on the column dimensions and type of mobile phase and gradient conditions. Andres et al. (30) fractionated FA on an anion column packed with Amerlite IRI-93 resin with two gradient conditions. The ionic

strength gradient gave more fractions than did the methanol gradient. Fractions were characterized by IR spectroscopy which indicate the heterogeneity of FA fractions. Using linear pH gradient with a high capacity buffer on XAD column, Richardson et al. (31) eluted FA at pH 4. The separation was still inefficient.

Separation of HA, FA, and polymaleic acid (PMA) on an octylsepharose CL-413 column, using sodium chloride and sodium hydroxide as mobile phases, were compared (32). All the samples were fractionated into two distinct peaks. The hydrophobic interaction of FA and PMA with the stationary phase were more similar than that of HA and PMA.

Few publications have appeared in the literature on the application of reversed phase HPLC (RP-HPLC) to humic substances research. Recently, two high efficiency Hypersil ODS and WP-300 microcolumns were applied to separate FA (33,34). Chromatograms showed several poorly resolved peaks (Fig. I-3). The authors implied that better resolution could be obtained with high efficiency columns.

The chromatographic techniques reached a prosperous stage in the 1980s. The rapid development of this technique is supported by two progressive fields: computers with massive data handling ability and detectors with high sensitivity. Those reasons along with the newly synthesized packing materials make HPLC a powerful tool in separation and identification of complex compounds.

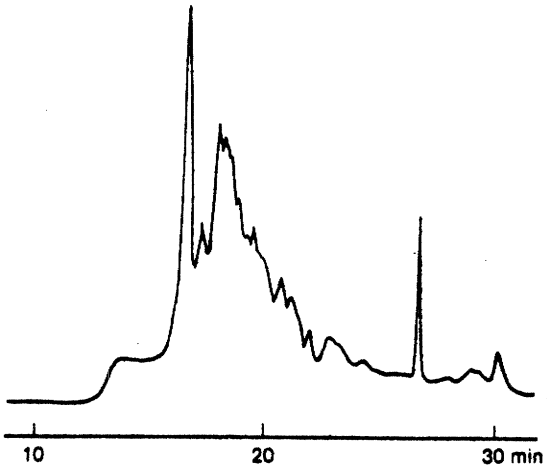


Figure 1

Chromatogram of fulvic acid, obtained with Hypersil ODS column (0.2 mm i.d. × 70 cm).

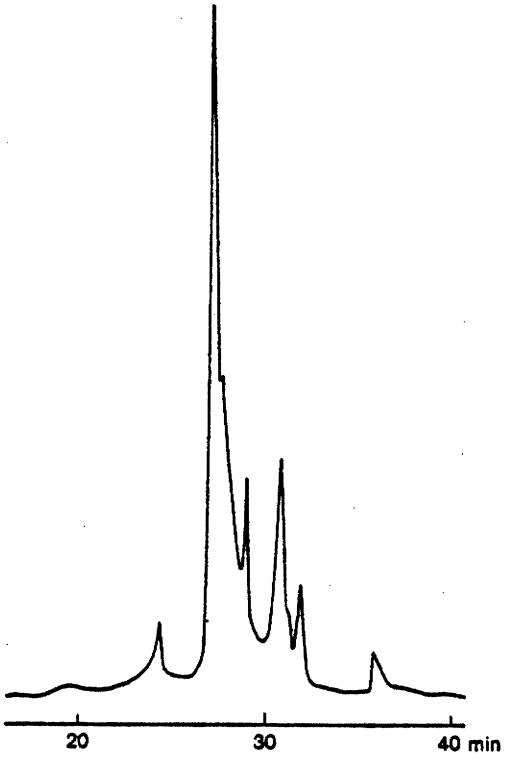


Figure 2

Chromatogram of fulvic acid, obtained with Wp-300 ODS column (0.2 mm i.d. × 1 m).

Figure I-3. Published RP-HPLC chromatogram of FA. The separations were performed by high efficiency microcolumn (34).

A more advanced application of chromatographic techniques includes the study of quantitative structure-chromatographic retention relationships (QSRR) (35). QSRR is statistically derived from a large number of independent structural variables which affect the retention of solutes. Advanced statistical programs used in QSRR are commercially available for personal computers. Once QSSR is established, one can attempt to use them to

(1) explain the molecular mechanism of chromatographic separations,

(2) predict retention behavior of individual substance at special separation conditions, and

(3) determine and select important structural data for the activity of the compounds of interest.

One example of application of QSRR to prediction of retention in HPLC is described in the literature (36). Twelve substituted-benzene derivatives were eluted with different mole fractions of water-methanol binary system and different stationary phases. The correlation of log k (capacity factor) for i th solute in j th stationary phase is shown in Fig. I-4. A general multi-linear QSRR equation was derived describing the RP-HPLC retention data as a function of solute structural parameter E(T) and submolecular polarity parameter δ, the mole fraction of water in binary solvent with methanol X, and the hydrocarbon coverage on the stationary phase C(j); as shown in the

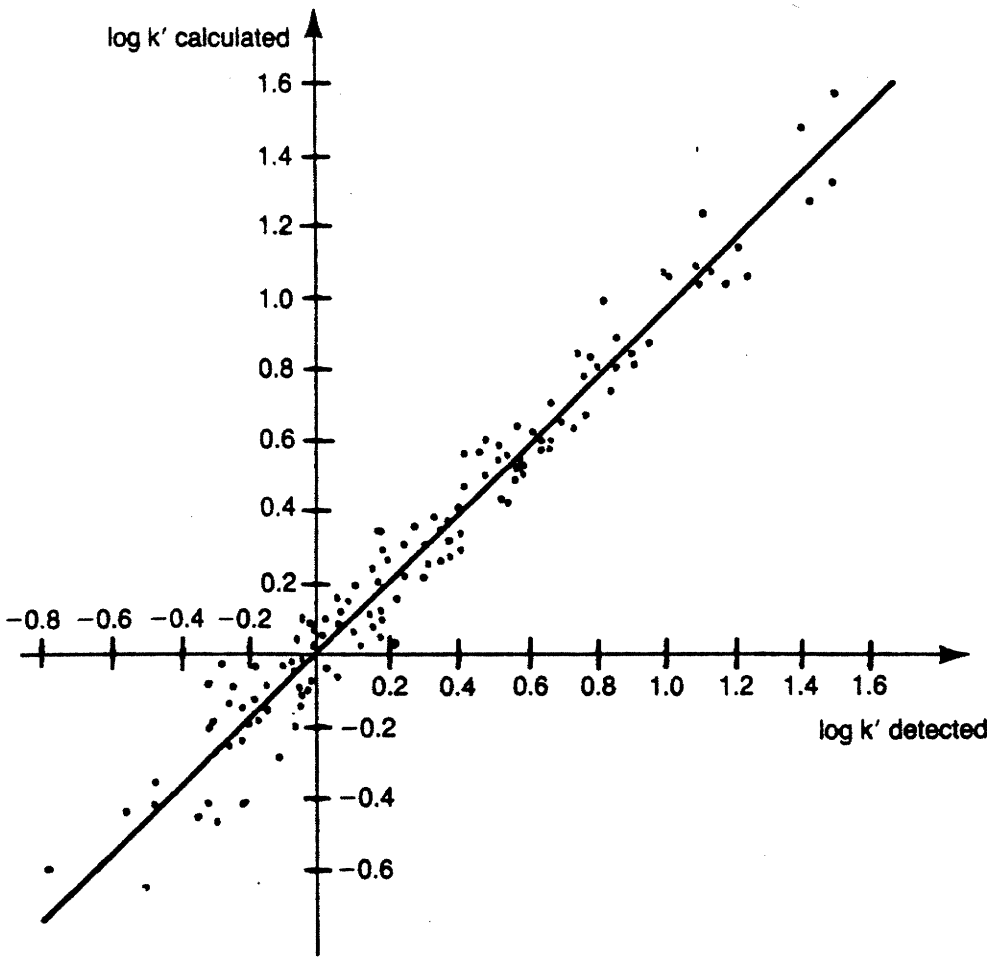


Figure I-4. Correlation of the capacity factors predicted and observed for 12 solutes chromatographed at different mobile- and stationary-phase compositions. (36).

equation:

$$\log k(ijX) = (0.0454(\pm 0.0071)E(T)(i) + 2.6493(\pm 0.9187)\delta(i) - 0.1053(\pm 0.0672)C(j) - 0.4946(\pm 0.5828))X - 0.0381(\pm 0.0039)E(T)(i) + 2.1659(\pm 0.4919)\delta(i) + 0.1696(\pm 0.0359)C(j) + 1.2963(\pm 0.3120) \quad (I\text{-}1)$$

A significant amount of valuable information has already been gathered in the short 10 years since the studies of quantitative relationships between descriptors of the structure of solutes and their chromatographic indices began.

Research Objectives

The purpose of this research project was to develop structural information on purified aquatic FAs through the combined use of RP-HPLC or SFE fractionation followed by spectroscopic characterization. The approach in the research was to 1) optimize the chromatographic separations on purified (reference Suwannee River FA) samples and model compounds, 2) carefully evaluate the retention mechanisms of each mode of separation, 3) apply different spectroscopic techniques to the same original samples and fractions, and 4) evaluate background interferences from solvents, instrument elements, or column materials.

RP-HPLC

In this mode, the hydrocarbon-bonded silica-based packing material is used in conjunction with an aqueous/organic mobile phase to separate wide variety of organic solutes. The selectivity in RP-HPLC is controlled mainly by eluent effects, and it is therefore unnecessary to have an extensive range of packing materials (unlike GC). The separation mechanism is dominated by the solvent effect. Some suggested mechanisms are listed below:

(1) Solvophobic interactions between the solute and the stationary phase (37).

(2) Silanophilic interactions between the solute and the accessible silanol group on the surface (38).

(3) Solvation of the solute and/or the stationary phase by the organic components of the eluent (39).

(4) Secondary equilibria due to interaction with eluent additives (40).

The large number of selectivity parameters makes RP-HPLC very useful in separation science. Sometimes a minor adjustment of parameter affects the interaction solutes between mobile phase and stationary phase dramatically. Thus, RP-HPLC is ideally suited for complex biopolymer mixtures like FA. The important selectivity parameters used in this research included organic modifier, pH, ion-pairing reagents, and added salt. The effect of each of these parameters on RP-HPLC are discussed below.

Organic modifier: Eluents used in RP-HPLC with bonded nonpolar stationary phase are generally polar solvents or mixtures of polar solvent such as methanol with water. The most significant properties of the solvent are surface tension, dielectric constant, viscosity, and eluotropic value. The concentration change of the organic modifier in the eluent will vary these parameters to different extent and affect the solute retention. As a crude approximation, the greater the surface tension of the eluent the greater the retention of a given eluent will be under otherwise identical conditions. On the other hand, high dielectric constant favors interaction between the polar group of the eluent and the mobile phase and therefore will reduce the capacity factor. The viscosity of the hydro-organic mixture affects on the efficiency of the separation by altering longtudinal diffusion. The band spreading of chromatographic peak is reciprocal to eluent viscosity. Thus, column plate height decreases with lower eluent viscosity. Table I-2 gives the properties of the solvent used in this research.

pH: Solute ionization has a pronounced effect on chromatographic behavior, particulary when the pH of the mobile phase is the solute pKa or pKb value (Fig.I-5). The retention and peak shapes of acids, bases, and zwitterions can be controlled by ion suppression through the adjustment of the pH of the mobile phase. The extent of this influence

Table I-2. Properties of solvents used in this research (41).

	Acetonitrile	Methanol	2-Propanol	Water
M.W.	41.0	32.0	60.1	18.0
B.P.(°C)	82	65	82	100
n (ref. ind.)	1.342	1.326	1.375	1.333
UVnm (cut off)	190	205	205	170
Density (g cm^{-3})	0.787	0.792	0.785	0.998
Viscosity (cP)	0.358	0.584	2.39	1.00
Dielectric const.	38.8	32.7	19.9	78.5
Dipole moment (D)	29	22	21	73
Eluotropic value	0.65	0.95	0.82	--

can be predicted by using the secondary equilibria theory and the extended Debye-Hückel theory (43). In predicting the effect of solute ionization on the capacity factors, it is usually assumed that the rate of equilibration of the ionized and nonionized solute molecules in the mobile phase is considerably higher than the rate of the two species with the stationary phase.

Ion-Pairing Reagent: Polar and ionic compounds, which are completely ionized in the pH range of 2 to 8, usually

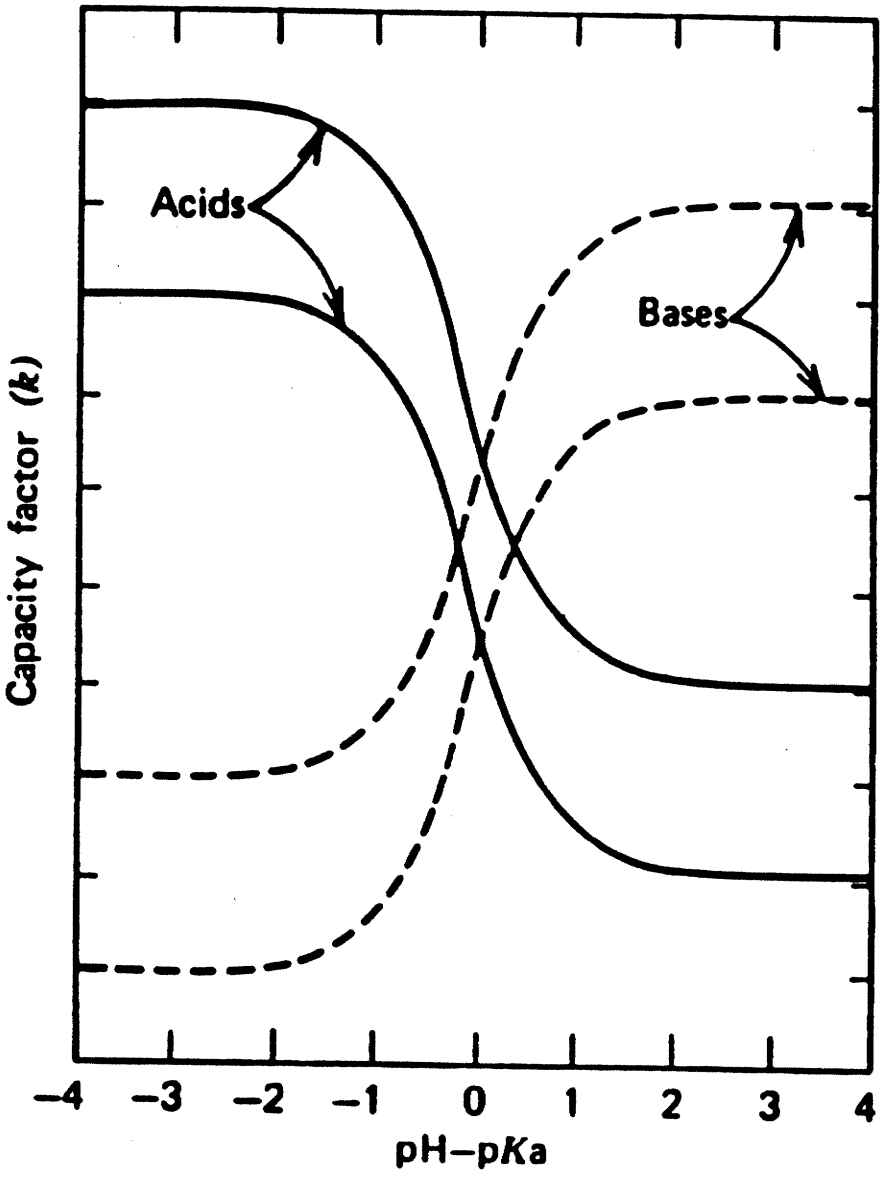


Figure I-5. Effect of the eluent pH on retention of weak acids and bases on nonpolar stationary phases. The scale on the abscissa shows the difference of the solute in the eluent. (42)

give rise to poorly defined and skewed peaks. The peak shape cannot be improved by the adjustment of the composition of mobile phase; however, separation of such compounds can be achieved on RP packing by using the ion-pairing reagent. Four proposed types of equilibria involved in RP ion association are listed in Table I-3.

Table I-3. Equilibria involved in RP ion-pair system as proposed in the literature (40).

	Mobile Phase		Stationary Phase
A	RNH_3^+	$\rightleftharpoons$	RNH_3^+
B	$RNH_3^+ + S^-$	$\rightleftharpoons$	RNH_3S
C	RNH_3^+	+	$S^- \rightleftharpoons RNH_3S$
D	RNH_3^+ $\Downarrow\Uparrow$ Na^+	+	NaS $\Downarrow\Uparrow$ RNH_3S

Although the precise mechanism has not yet been clearly established, intuitively, it is expected that the mechanism of interaction will change with increasing size of the counter ion. Regardless of the nature of retention, it can

be shown that for the reaction

$$R^+_{aq} + I^-_{aq} \rightleftharpoons [RI], \qquad (I\text{-}2)$$

the following relationship holds true:

$$t = \frac{L}{u}(1 + K_{eq}\,\phi[I^-_{aq}]) \qquad (I\text{-}3)$$

where t= retention time, L= column length, u= flow rate, here K_{eq} is the overall equlibrium constant for the ion-pairing process, I^-_{aq} is the concentration of the hydrophobic counter ion, and ϕ is the phase volume ratio. It is evident that retention will be governed by the magnitude of the binding constant and the concentration of the counter ion.

Added Salt: The influence of salts can be explained in terms of reduced electrostastic repulsion between solute molecules and the increase in eluent surface tension. The addition of neutral inorganic salts causes a linear increase in the surface tension of aqueous solutions, according to the following equation:

$$\gamma = \gamma_0 + \sigma m \qquad (I\text{-}4)$$

where γ_0 is the surface tension of pure water, m is the molarity of the solution, and σ is the molal surface tension increment of the salt obtained from the Hofmeister series (44). The effect of changing surface tension on the logarithm of the capacity factor, provided k' and molar

volume of the eluent remain constant, can be expressed by a simplified equation: $\ln k' = A''' + B''$ where A''' contains all the terms in the equation that are influenced by the surface tension, and B'' is:

$$B'' = \frac{N\Delta A + 4.836N^{1/3}(K^{L} - 1)V^{2/3}}{RT} \qquad (I\text{-}5)$$

V: molar volume of solvent

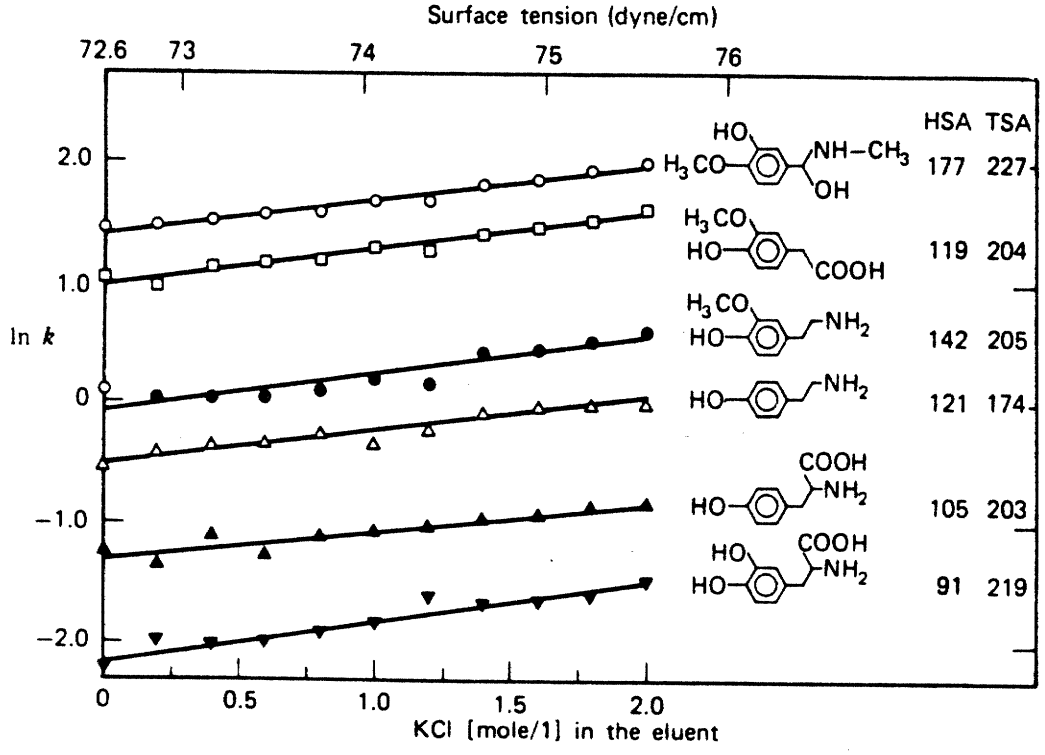
ΔA: reduction in molecular surface are upon of the solute-stationary phase complex

K^{L}: a factor for converting the bulk surface to the molecular dimension.

The change in the logarithm of the capacity factor with the change in salt concentration is illustrated in Fig. I-6.

Gas Chromatography and Pyrolysis GC

Gas chromatography/mass spectrometry has been applied to analyze methylated permanganate-degradation products of aquatic FA (45); amino acids in HCl hydrolysates of FA (46) and pyrolyzates of FA (47,48,49). Methyl esters of benzenecarboxylic acids, furancarboxylic acids, aliphatic mono-, di-, and tribasic acids are major identified products in oxidation fragments. The amino acids account to 4-24 % in fulvic acids collected from seven lakes. Pattern recognition and factor analysis were applied to correlate



HSA: hydrocarbonaceous surface area of solute molecules

TSA: total surface area of solute molecules

Figure I-6. Plot illustrating the effect of salt concentration of the capacity factor. (50).

the source of FA from polysaccharides, lignins, peptides, and lipids. Carbohydrate and phenolic components were more pronounced in the pyrolysis of FA.

Supercritical Fluid Extraction (SFE) and Supercritical Fluid Chromatography (SFC)

A number of potential advantages are possible with SFE compared with conventional extraction methods. These advantages include rapid extraction rate, more efficient extraction, increased selectivity, high recovery and easy purification of extractant, and compatibility with on-line analysis methods such as continuous spectroscopic monitoring or periodic chromatographic analyses.

The potential advantages of SFE are acquired from the properties of a solvent at temperatures and pressures above its critical point. At elevated pressure, this single phase has properties that are intermediate between those of the gas and liquid phases and are dependent on the fluid composition, pressure, and temperature. The compressibility of supercritical fluids is large, just above the critical temperature and small change in pressure result in large change in density change of the fluid (51). The density of a supercritical fluid is typically 100 to 1000 times greater than that of the gas resulting in high solvating chracteristics. However, the diffusion coefficients and

viscosity of the fluid remain intermediate between those of the gas and liquid phases at moderate densities. These properties allow rapid mass transfer of solutes compared with liquid. Many fluids also have comparatively low critical temperatures that allow extractions to be conducted at relatively mild temperatures, e.g., 31°C for carbon dioxide.

In addition to controlling the fluid pressure and/or temperature to regulate the density or solvating power, one can use various fluids or fluid mixtures that exhibit different specific chemical interaction to obtain the desired solvent strength and selectivity. Table I-4 shows the different properties among supercritical fluids, gases and liquids.

Table I-4. Properties of mobile phase used in chromatography (52).

Mobile Phase	Density ($g\ mL^{-1}$)	Viscosity (poise x 10^{-4})	Diffusivity ($cm^2 s^{-1}$)
Gas	$(0.6\text{-}2.0)\times10^{-3}$	0.5-3.5	0.01-1.0
Supercritical fluid	0.2-0.9	2.0-9.9	$(0.5\text{-}3.3)\times10^{-4}$
Liquid	0.8-1.0	30-240	$(0.5\text{-}2.0)\times10^{-5}$

SFE can be used to extract nonvolatile components without degradation which is not possible in GC. In this research, initia efforts on application of SFE to methylated FA were tried.

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CHAPTER II

EXPERIMENTAL

Separation followed by identification is probably one of the most promising approaches for investigating the structure of a complex mixture like FA (1). By using this approach, mixtures are sorted according to physical or chemical properties during the separation process. The subgroup compounds would have more characteristic responses than those of the total mixture. The experimental approach of this research was to seek effective chromatographic method to separate the constituents of fulvic acids, then identify the fractions with different detection instruments. In this research, except in pyrolysis GC (py-GC) all of the analytical methods were nondegradative. Also, the authentic sample analysis was preferred over the chemically treated samples. Figure II-1 shows an outline of the experimental scheme used. Starting with purified SR-FA, samples were subjected to three analytical trains:

Train I: Preparative RP-HPLC of purified SR-FA

Samples were fractionated into two major categories by selective solvent elution. The hydrophilic fraction A-1 and hydrophobic fraction B-1 were subjected to spectrocopic characterization.

Train II: Analytical RP-HPLC

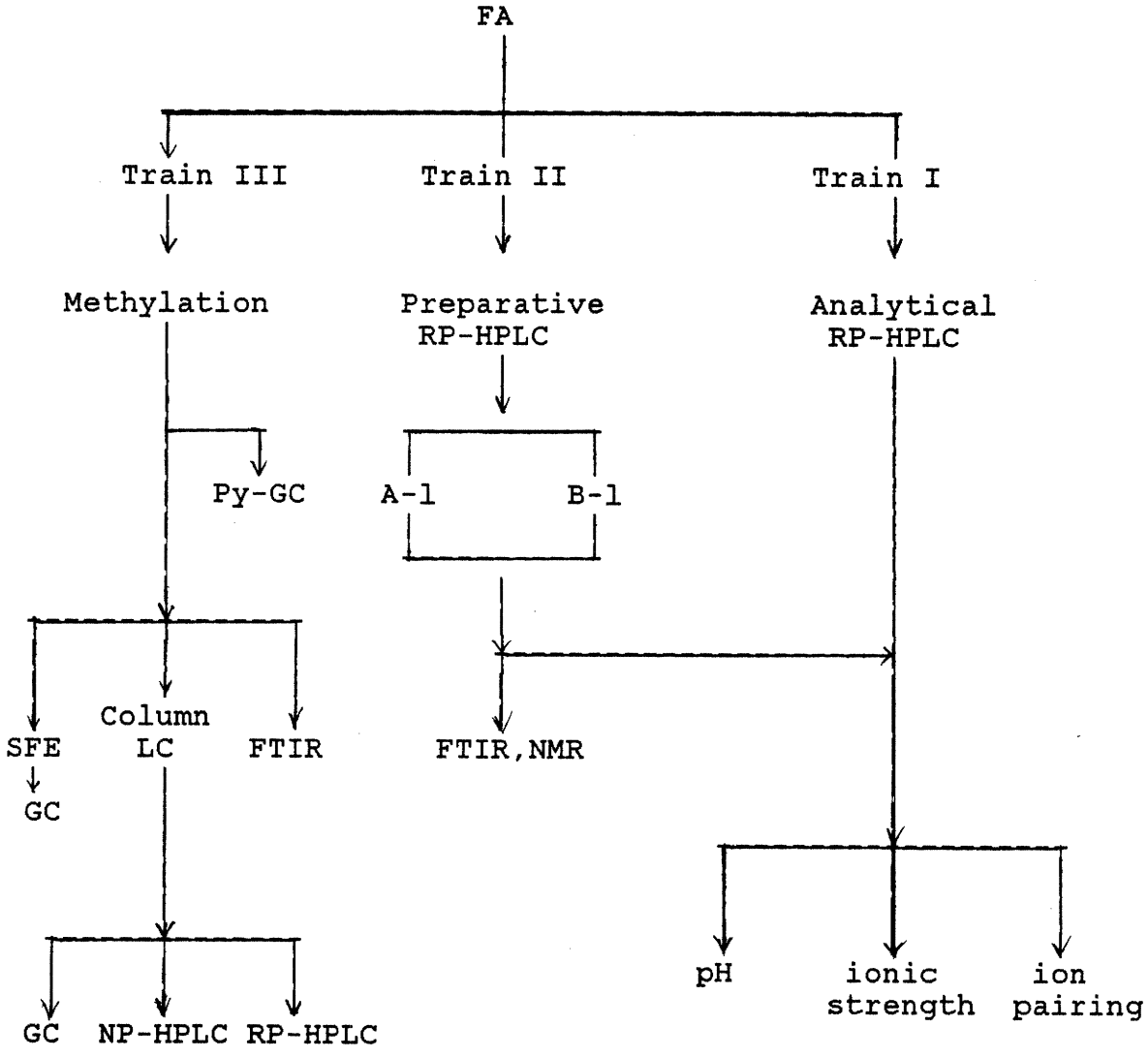

Figure II-1. The outline of research plan.

This train included the analytical RP-HPLC separation of SR-FA and its fractions, buffered or derivatized or Cu^{2+} complexed SR-FA. Both fluorescence and photodiode array detectors were used.

Train III: Pyrolysis GC, GC, SFE

SR-FA and its RP-HPLC fractions were analyzed by Py-GC-FID. Methylated FA was fractionated with column chromatography. The methylene chloride fraction was extracted by CO_2 fluid. All derivatized FA and its fractions were analyzed by GC-FID.

II-1. Material and Reagents

Samples:

The study of the chemistry and environmental significance of aquatic humic substances is greatly dependent on the limitations imposed on the sample by the methods used to isolate this material. Thus, standard and reference Suwannee River fulvic acids were the essential materials used in this research. Fulvic acids from other streams, such as Ogeechee, Ohio, and Beer rivers were used in the HPLC electrolyte effect experiments. Fulvic acids for other experiments were obtained from the International Humic Substances Society (2). The fulvic acids were prepared according to the following procedures (3). Water samples were filtered and acidified with HCl to pH2 in the

pretreatment step. The samples were extracted with alkali solution after they were absorbed on the XAD resins. The extract was acidified to pH < 2 with HCl and centrifuged to separate the HA and FA. In the purification procedure, desalting was used to remove substances that were physically but not covalently bound to FAs. The final products were verified with average elemental analysis and total acidities analysis.

Reagents

All reagents and chemicals in this research are listed below. The sources of manufacture are included for each.

HPLC grade methanol (CAS Reg. 67-56-1), water (7732-18-5), and acetonitrile (75-05-8), were obtained from American Scientific Products. Acetic acid (64-19-7), sodium hydroxide (1310-73-2), adipic acid (124-04-9), resorcinol (108-46-3), phosphoric acid (14335-33-2), potassium phosphate (7778-53-2), monobasic (7778-77-0) and dibasic (7758-11-4), and silica gel (63231-27-4) were supplied from Fisher Scientific Company. Crotonaldehyde (123-73-9), methyl vinyl ketone (78-94-4), maleic acid (110-16-7), 1,2,4-benzenetricarboxlic acid (528-44-9), methylsuccinic acid (1121-07-9), tricarballic acid (99-14-9), vanillic acid (121-34-6), and o-cresol (95-48-7) were purchased from Aldrich Chemical Company. 2,4,6-trihydroxy benzoic acid (83-30-7) was ordered from ICN Pharmaceuticals.

n-Octylammonium chloride(111-86-4) were obtained from Eastman Kodak Company.

GC and spectroscopic-grade methylene chloride (75-09-2) was ordered from Burdic and Jackson. N,N-Dimethylformamide (68-12-2), methyl iodide (74-88-4) and ethyl ether (60-29-7) were obtained from Fisher Scientific Company. N-methyl-N-nitroso-p-toluenesulfonamide (80-11-5), 2-(2-ethoxyethoxy) ethanol (37421-08-2), and sodium hydride (7646-69-7) were purchased from Aldrich Chemical Company.

SFC grade carbon dioxde gas with helium head space was ordered from Scott Specialty Gases.

II-2. Instrumentation

In the first and second trains of experiments HP model 1090 and Waters model ALC-201 systems were used. The third train of experiments applied Varian 6000 GC and Brownlee Microgradient SFC systems.

HPLC systems:

1. Hewlett Packard HP1090 LC with UV-vis photodiode array detector (PAD); DR-5 ternary solvent delivery pump; HP 85B computer and HP 7470 plotter.
2. Waters ALC-201 with a Model 6000 pump. The LC system was connected with Beckman Model 160 UV-vis and Schoeffel Model 970 fluorescence detectors.

GC system:

Varian 6000 capillary GC installed with flame ioniztion detector and interfaced with Chemical Data Model 120 Pyroprobe and Tekmar Model LSC-2 liquid sample concentrator.

SFE system:

Brownlee Microgradient solvent delivery pump connected to HP5809A GC with flame ionization detector.

FTIR: Nicolet Model 60 SAB spectrometer equipped with DRIFTS.

NMR: Nicolet Model NT-150 spectrometer for ^{13}C NMR. Nicolet Model NT-200 for ^{1}H CRAMPS.

Compared with traditional HPLC detectors, the versatility of photodiode array detector (PAD) is generated from the mass data matrix produced from wavelength domain added as a third dimension to time and absorbance. The optical unit directs radiation from a deuterium lamp through a flow cell and then disperses the transmitted radiation onto a linear array of more than 200 photodiodes, monitoring wavelenths in the range 190-600nm. One of the most popular usage of PAD is for peak purity checking, which includes the following methods:

(1) overlay spectra taken at the upslope, apex, downslope of the peak and normalize the absorbance. If the spectra match each with more than 95% confidence, the eluted peak is pure.

(2) plot the absorbance ratio of the peak at two different wavelength. If the ratio is not constant then the peak is coeluted.

(3) plot the isoabsorbance contour. If visible distortion of the plot, it means an impurity exists.

Although mass spectrometry is the best reliable detector for identifying compounds, the LC-MS system is not prevail because of its high cost. GC-MS can not be apllied on nonvolatile compounds. UV-vis photodiode array detector can offer a neat quantitative and qualitative analysis of nonvolatile compounds with curve resolution or deconvolution method (4).

II-3. Methods

Train I: Preparative HPLC

Two semi-preparative HPLC experiments were carried out by two different experimenters on different systems. The sample and the elution conditions are listed in Table II-1.

In Waters system, a column of 50cm x 22.5mm i.d. dry packed with 25 um ST/C18 (Scientific Technology) was used. A water-methanol binary solvent system was used under stepwise gradient conditions. Solvent A-1 consisted of 1% methanol in water, and solvent B-1 consisted of 85 % methanol. An aqueous solution of 1 mg/mL Suwannee River

Table II-1. Experimental conditions for preparative experiments

Conditions	Waters ALC-201	HP 1090
Mode	stepwise	gradient
Mobile phase	methanol:water	methanol:water
	1:99——85:15	1:99——85:15
Elution program	0——14——30min	0——30——35——45min
Back pressure		52 bar 28 bar
Flow	1 mL/min	1.5 mL/min
Equilibrium time	20 min	15 min
Column	50cm L x 2cm i.d.	30cm L x 7.8mm i.d.
Packing	ST/C18 25um	Novapak C18 5um
Sample 1mgFA/5mlH_2O	1 mL	50 uL
Detection	fluorecence	UV-vis PAD
	ex λ= 273nm	254nm
	em λ= 390nm (4).	

reference FA in HPLC water was used for the separation. The sample capacity of the column was experimentally determined as 1 mL/per injection. From each injection, two fractions were collected. The hydrophilic fraction contained solutes eluted with solvent A-1. The hydrophobic fraction contained solutes eluted with solvent B-1. Fractionation was made in batches of 10 mg each. Fractions were freeze-dried to a 10 mL solution, centrifuged to remove particulates, decanted, lypholized and desiccated to constant weight. A procedure blank for HPLC reinjetion involved the collection of the same amount of solvent A-1 and B-1 concentrated to the original 10 mL volume of the sample. A total of 80 mg reference FAs was fractionated in the first experiment. The efficiency of separation in each batch was checked by reinjecting the aqueous solution of the fractions into the analytical RP column. Only batches that showed efficient separation were combined for further spectroscopic characterization (5).

In the second preparative experiment, the HP 1090 system was used with a custom-made preparative column (Novapak C18 30 mm x 7.8 mm i.d.). Gradient elution was used according to the solvent program shown in Table II-1. Method of sample preparation and fraction collection were silimar to the aforementioned methods. The loading capacity of column was examined by increasing the injection volume of sample until the chromatogram showed the tailing peak. If one more

mobile phase blank injection showed no carryover, that was considered the maximum loading of the column. In this experiment, the maximum loading was 50 uL. Forty mg FA was fractionated in two batches. Each batch contained the collected A-1 and B-1 fractions. Fractionation efficiency was checked by reinjection of the concentrated fractions. The useful samples were freeze-dried and set in a desiccator and vacuumed to constant weight.

Train II: Analytical RP-HPLC Separation

Sample preparation: The FA and A-1 fractions were dissolved into HPLC water or phosphate buffer at concentrations of 1 mg/5 mL. The solution must be clear, or filtration through 0.45 um glass fiber filter should be applied before injection into the system. The hydrophobic fractions and methylated FA were dissolved into methanol or methylene chloride. Several analytical columns and mobile phases were evaluated. Table II-2 lists the columns and their characteristics. Table II-3 lists all the mobile phases and the gradient programs used in this research.

Samples were introduced in 5 to 20 uL injections. Each sample was injected at least twice. With each mobile phase after establishing the base line, a procedure blank was injected, followed by the sample. Every few injections, a mobile phase injection was made to check on the stability of the base line and the complete elution of the solutes in the

Table II-2. Specifications of the stationary phases used in this research.

Phase	Description	Particle Size	Carbon No.	%C	Surface Area m^2/g	Column Dimensions[a] cmL x ID
Partisil ODS-10	C_{18} Polymeric, Silica Based (Whatman)	10 μm	C_{18}	10		25 cmL x 0.46 cm ID
Partisil ODS-1	C_{18} Polymeric, Silica Based (Whatman)	10 μm	C_{18}	5	350	25 cmL x 0.46 cm ID
Novapak C_{18}	C_{18} Polymeric, Silica Based (Waters)	5 μm	C_{18}	7		15 cmL x 0.39 cm ID
Novapak C_{18} Prep I		4 μm	C_{18}	7	120	30 cmL x 0.78 cm ID
Scientific Technology	C_{18} Polymeric	25 μm	C_{18}	--	--	50 cmL x 2 cm ID
Versapak	C_{18} Polymeric, Silica Based (Alltech)	10 μm	C_{18}	10	200	25 cmL x 0.46 cm ID
Hypersil ODS	C_{18} Polymeric, Silica Based (Hewlett Packard)	5 μm	C_{18}	10	170	10 cmL x 0.21 cm ID 20 cmL x 0.21 cm ID
Act - II	Copolymerized vinyl pyridine, Resin (Interaction)	10 μm	pH range 3.4 - 10.5	--		15 cmL x 0.46 cm ID
IC-PAK Anion	Polymethylacrylate Containing Quaternary (NH_4^+), Resin Waters	10 μm	---	--		5 cmL x 4.6 cm ID

[a] All Columns are stainless steel

Table II-3. Continuous gradient programs and types of columns and samples used with each.

gradient program	mobile phase	pH at 25°C	gradient t_{min}	%A	%B	column types	samples
I	A: H_2O+HOAc 0.01%	4.0	0	99	1	Novapak C18 5um, 150mm L x 3.9mm i.d. flow: 0.5ml/min	SR-FA,HPLC fractions, model compounds.
	B: CH_3CN		2	70	30		
			16	15	85	Hypersil ODS C18, 200mm L x 2.1mm i.d. flow: 0.3ml/min	SR-FA,HPLC fractions.
			20	15	85		
II	A: H_2O He purged	7.0	0	99	1	Novapak C18 5um, 150mm L x 3.9mm i.d. flow: 0.5ml/min	SR-FA,HPLC fractions, model compounds.
	B: CH_3CN		2	70	30		
			16	15	85	Hypersil ODS C18, 200mm L x 2.1mm i.d. flow: 0.3ml/min	SR-FA,HPLC fractions.
			20	15	85		
III	A: H_2O He purged	7.0	0	99	1	custom made Novapak C18 4um, 300mm L x 7.8 mm i.d., flow: 1.5ml/min	SR-FA second preparativ exp.
	B: CH_3OH		30	99	1		
			35	15	85	Novapak C18 5um, 150mm L x 3.9mm i.d. flow: 1ml/min	SR-FA,HPLC fractions.
			50	15	85		
IV	A: 1mmole $n\text{-}C_8H_{17}NH_2 \cdot HCl$	6.8	0	100	0	Beckman C18 5um, 150mm L x 4.6mm i.d. flow: 0.5ml/min	SR-FA
			1	100	0		
			10	70	30		
			30	0	100		

sample. A typical RP-HPLC method was shown in Fig. II-2.

Train III: Methylated FA by Gas Chromatography and Column LC

FA methylation was performed according to published procedures (2). In the first stage, carboxylic groups in FA were esterfied with diazomethane (CH_2N_2). The hydroxy groups were transformed to etherlinkage by CH_3I in the second step. The methylation device is shown in Fig. II-3. A 20-mg sample of Suwannee River reference FA was dissolved in 2 mL DMF in tube 4. Ten mL of ethyl ether was added to the first tube to saturate the nitrogen carrier gas. Then 1 mL of ether, 1 mL of 2-(2-ethoxyethoxy)ethanol, were added to dissolve 0.1-0.2 g of N-methyl-N-nitroso-p-toluene-sulfonamide in the second tube. One mL freshly prepared 37% aqueous KOH was slowly added into the second tube through a syringe. The base immediately began to release diazomethane from the sulfonamide. The nitrogen flow was adjusted to about 10 mL per minute. Then, the third tube (a safety trap) and the sample tube were positioned to bubble the nitrogen and diazomethane gas mixture through the sample. The reaction was continued about 30 minutes until the slight yellow color of diazomethane persisted in the sample solution. Half of the products were recovered by freeze-drying. The other half of the products proceeded to the next methylation reaction.

One mL CH_3I was added into a three-neck flask, and the

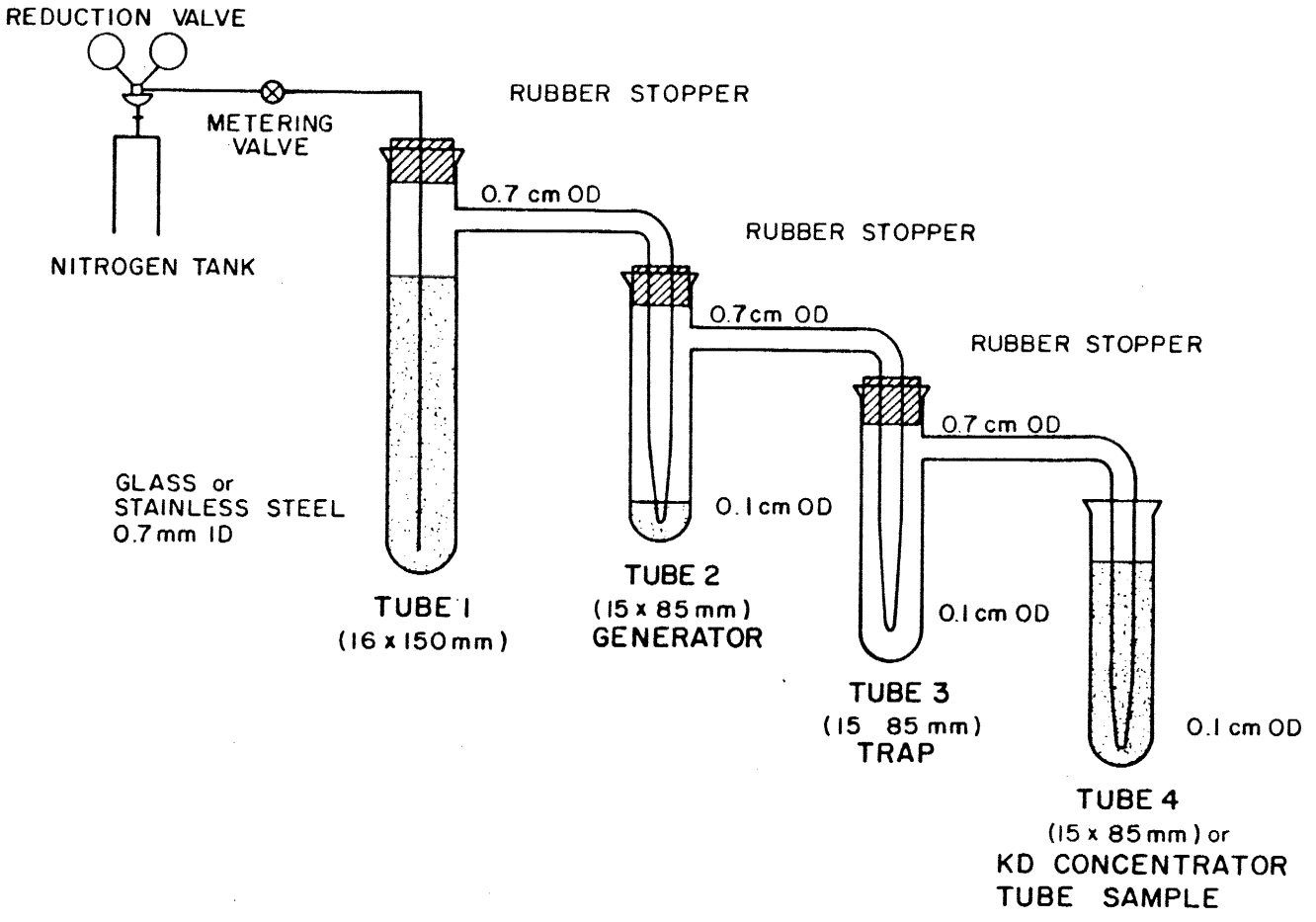

Figure II-3. Diagram of the device used for diazomethane derivatization experiment.

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.* METHOD HP85 *
Comment: None
Hooked External Programs: None
;*END-OF-LIST

;* METHOD SEPARATION
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FLOW      = 0.5
% B= 1    , C1=0
MAXPRESS  = 200
MINPRESS  = 0
OVENTEMP  = 25
STOPTIME  = 20
POSTTIME  = 15
E1=0 E2=0 E3=0 E4=0
AT 0          FLOW    = 0.5
AT 0          % B= 1    , C =0
AT 2          % B= 30   , C =0
AT 10         % B= 85   , C =0
AT 18         % B= 85   , C =0
AT 20         % B= 1    , C =0
;*END-OF-LIST

METHOD1040
DEFAULT
;* DAD Signals
A S=214,4    R=550,100 M=1 I=1
B S=230,4    R=550,100 M=1 I=1 P
C S=254,4    R=550,100 M=1 I=1
D S=280,4    R=550,100 M=1 I=1
E S=0,0      R=0,0     M=0 I=0
F S=0,0      R=0,0     M=0 I=0
G S=0,0      R=0,0     M=0 I=0

H S=0,0      R=0,0     M=0 I=0
;* DAD Intset
A TH = 3       AR = 4
B TH = 3       AR = 4
C TH = 2       AR = 3
D TH = 2       AR = 3
;* DAD Plotset
A RANGE = 120     ZERO% = 5
B RANGE = 100     ZERO% = 5
C RANGE = 80      ZERO% = 5

D RANGE = 60      ZERO% = 5

;* DAD
PEAKWIDTH    = 0.2    ; min
CHARTSPEED   = 0.5    ; cm/min
STOPTIME     = 0      ;
;* DAD Spectra
FROM = 200; nm
TO   = 600; nm
STEP = 2  ; nm
MEM  = 7  ; apex,base,slope

;* DAD Integrator
REPORT TYPE    = 1   ; area%
RATIO SIGNALS =  ,
PLOT MODE      = 2   ; offline
ANNOTATION     = 3   ; markers
;*Signal       [min]       [min]
DD       FROM =0        TO =0
;* DAD Expert
LAMPCURR       = 1       ; low
TIMEBASE       = 0       ; auto

MONITOR        = P
AUTO-OFF       = 1440    ; min
POSTTIME       = 15      ; min
SIGNAL MATCH = 0.125
DEL CALIBTABLE
DEL TIMETABLE
;* DAD Calibration
WINDOW           = 5 %
REF WINDOW       = 5 %
UNITS            =

DEFAULT PEAK# = 0  ;uncal peaks
UPDATE TIME   = 0  ; off
MUL FACTOR    = 1
;* Calibration amounts *
SAMPLE  = 0
ISTD 1  = 0
ISTD 2  = 0
ISTD 3  = 0
CALIB MODE      = 1; area
;*END-OF-LIST

;&&END-OF-LIST&&
```

Figure II-2. A typical RP-HPLC method in this research.

total volumn was brought up to 20 mL with DMF. The solution was mixed with 0.5 mg NaH powder and set in a ice bath. Esterized fulvic acid from the first methylation step was added into the flask drop by drop. The solution was stirred overnight under nitrogen gas. At the end of reaction, one mL each of CH_3OH and CH_3I was added to the flask, and the solution was stirred for 30 minutes. Ten mL water was added into the final solution, and the products were extracted with 15 mL CH_2Cl_2. The CH_2Cl_2 solution was washed 5 to 6 times with water to remove the DMF and then evaporated to dryness by vaccum.

Column Fractionation of Methylated FA

A 50 cm long 2 cm (inner diameter) glass column was packed with 10 cm long silica gel. The column was rinsed with a 30 mL solution in the sequence CH_3OH, 2-propanol, CH_2Cl_2, hexane. The sample solution was introduced on the top of the packed column. Fifty ml of each solvent was used to elute the sample in the sequence: hexane, CH_2Cl_2, 2-propanol, CH_3OH. The solvents in each eluent were removed by vacuum distillation. Both 0.01% and 1% solution in CH_3OH were prepared for HPLC and GC injection.

Pyrolysis GC

In pyrolysis GC, quartz tube and glass fiber supports were set at clean position until the blank trace showed no impurities. In order to keep loading consistant, the

samples (FA, A-1, and B-1) were made to 1% concentration by dissolving them into acetonitrile. Seventy ul was introduced for each analysis. The quartz tube (15 mm L x 2 mm O.D.) fitted inside the platinum coil and provided good contact with it. During the pyrolysis, the probe was inside an interface chamber which was mounted on the injection port of the Varian 6000 GC. Helium was used as a carrier gas on FID at a flow rate of 3.0 mL/min; the split ratios were 1:11. The pyrolysis parameters for all samples were set as below:

Interface temperature: 220°C.

Ramp rate: 75°C/msec.

Heat interval: 20 sec.

Final temperature: 800°C.

The percentage of pyrolyzates formed was analyzed by using a carbon analyzer (Fig. II-4). The system was standarized to 100 ppm per mL injection. The pyrolyzer then was connected to the system. After cleaning the sample loading device, blank values were detected with the UV lamp in the position off. The total carbon generated was determined by pyrolyzing sample with the UV lamp on; the carbon generated from the functional group was determined with the UV lamp off.

Supercritical Fluid Extraction

A premeasured quantity of C18 support was packed in a

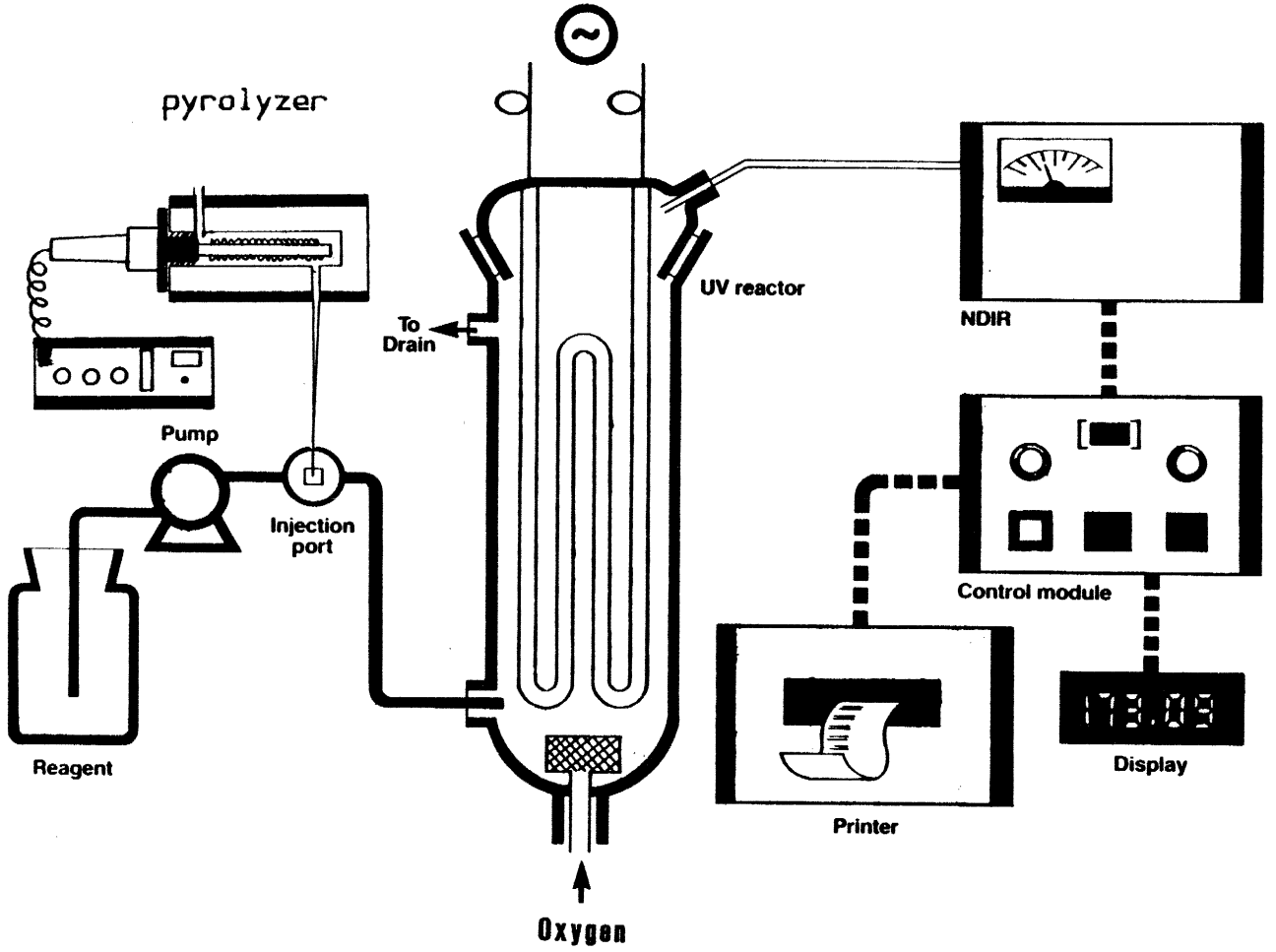

Figure II-4. The flow scheme of carbon analyzer connected to pyrolyzer.

stainless column (50mm L x 4.6mm id). The methylated samples dissolved in the methylene chloride were injected at the top of the column. The column was set in the GC oven at 80°C to evaporate the solvent. In the SFC system, both syringes were filled with CO_2. Pump A was set 50% to the total delivery fluid. The fluid was prepressurized to 2000 to 4000 psi. Oven temperature was set at 120°C to 60°C to vary the fluid density. The extracts were collected at the outlet of the system by dissolving the extracts into 2mL CH_3OH. The collected solution was then concentrated into 0.5 mL CH_3OH and reinjected into GC system for detection. The flow scheme is shown on Fig. II-5.

II-4. Spectroscopic characterization of FA HPLC fractions

Solid State Proton CRAMPS and Carbon 13 CP/MAS NMR

NMR experiments were performed in collaboration with scientists at Regional-NMR center, Colorado State University. Experiments included proton combined rotation and multiple pulse spectroscopy (CRAMPS) on total reference FA, A-1 and B-1. The proton CRAMPS spectra were recorded on modified NT-200 at a proton Lermor frequency of 187 MHz using the BR-24 sequence. The BR-24 cycle time was 108 MS corresponding to τ = 3.0 usec. The RF field strength, ν_H was 210 K Hz. Chemical shifts are reported relative to

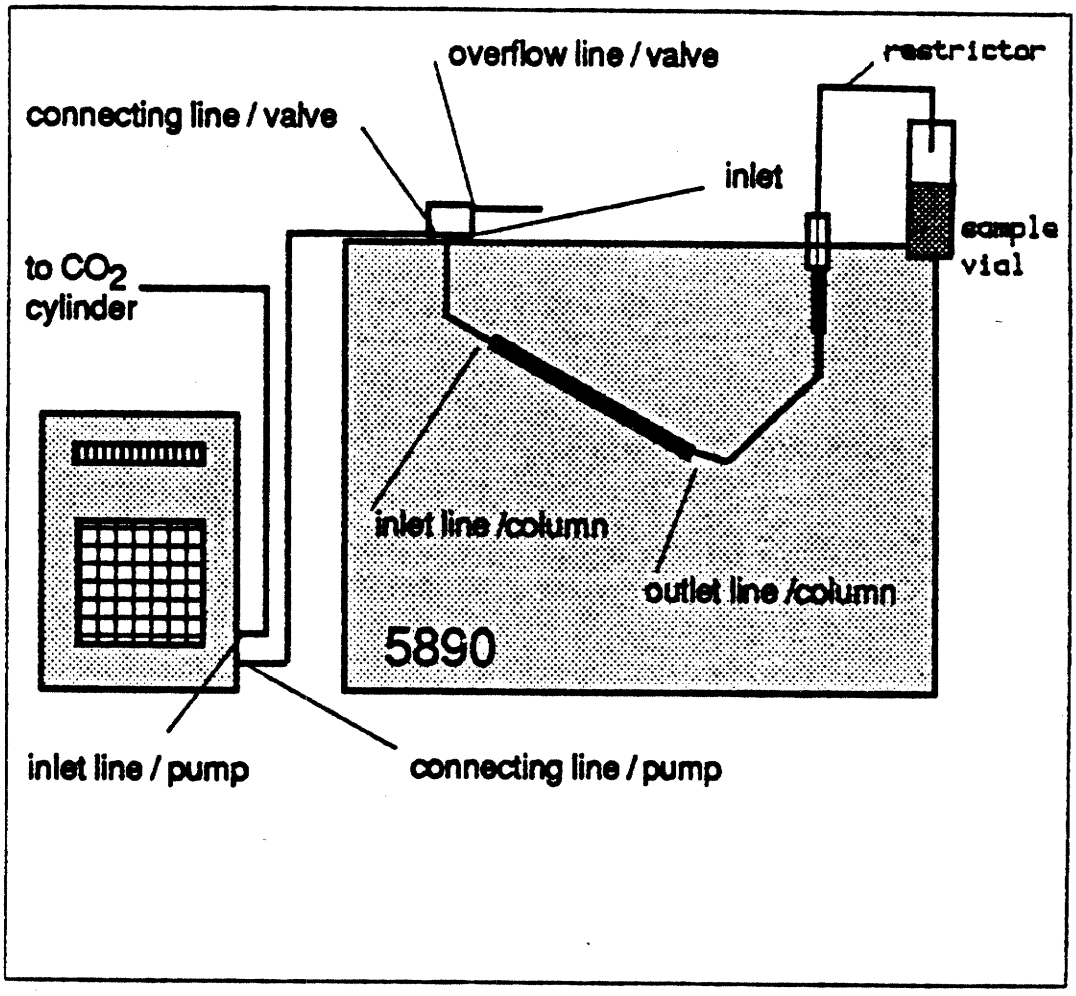

Figure II-5. The flow scheme of SFE.

the proton resonance of TMS and are accurate to ± 0.3 ppm. Carbon-13 NMR spectra were obtained with a Nicolet NT-150 spectrometer at 37.7 MHz using a home-built CP/MAS modification including the probe. The cross polarization of contact time was 1 msec and the pulse repetition time was 1 sec. The ^{1}H irradiation field was 11G and 1K data points were measured with respect to tetramethylsilane via hexamethylbenzene as a secondary substitution reference (aromatic peak at 132.3 ppm).

Fourier Transform Infrared Spectroscopy (FT-IR)

The FTIR analyses were performed by Dr. K. Daugherty's research group in Chemistry Department at University of North Texas. The FA and its methylated samples were analyzed by Nicolet Model 60 SAB equipped with a Spectra-Tech DRIFTS attachment. The operating conditions were as follows: 265 scans, KCl sample background resolution 1 cm^{-1}, and DGTS detector. The methylated FA samples were prepared in KCl pellets using the departmental instrument under the same conditions.

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CHAPTER III

RESULTS AND DISCUSSION

III-1. Preparative HPLC

The preparative separations of complex solute are associated with several inherent problems, which can be summarized as follows:

1) chemical changes to the original material may occur during the separation process;
2) contamination from the mobile phase, solid support, or the instrumental components;
3) loss of resolution due to repeated injection;
4) irreversible adsorption of some of the sample constituents;
5) the usually small amount of purified fractions.

Several trials were made on the preparative sample preparation. The incomplete separation of hydrophilic and hydrophobic regions and peak overlap after reinjection of separated sample was noted in some samples. Other problems included contamination of samples by metal ions during separation or release of the packing material with the sample eluent. Adequate precautions were taken to minimize or completely avoid these problems in subsequent trials. Only the neat samples that showed the distinct boundary in

the reinjection chromatograms were used in the following characterization experiments. The recovery of FA and contribution of each fraction from Waters ALC-201 and HP 1090 are shown in Table III-1. The combined recoveries of both fractions averaged to 64.5% of the total FA used in the first experiment and 74.2% in the second experiment. The average yield of 69% seems excellent compared to the yields by other chemical and thermal degradation methods which do not exceed 25% (1).

The two experiments were run at different periods and using different systems. Not only the total recoveries were close to each other, but A-1 and B-1 fractions were also proportionally close. The experiments are considered to be highly reproducible. The incomplete recoveries are likely due to the irreversible adsorption of FA constituents or due to mechanical loss. Fig. III-1 shows the chromatograms of the total FA and fractions in the first experiment. Fig. III-2 shows the chromatograms from the second experiment. Fractions collected from first experiment were subjected to solid state proton and carbon 13 NMR, FTIR analysis, and analytical separation by LC, GC, and pyrolysis GC.

In the preparative separation, the composition of the mobile phase determines the elution order of the solutes. Two mobile phase compositions were used (1% and 85% methanol in water) as the two limits of the mobile phase elution strength. Continuous gradient programs are listed in Table

Table III-1. Preparative experiments 1 and 2. Percentage recoveries of fractions

	Initial Total wgt in mg	Wgt of A-1 Fraction in mg	Wgt of B-1 Fraction in mg	% Total Recovery A + B	% of A-1 Recovery from Total FA	% of B-1 Recovery from Total FA
Preparative Experiment 1						
Combined Batches (1-5)	50	19.0	11.6	61.2	38.0	23.2
Combined Batches (6-8)	30	11.20	9.8	70	37.3	32.7
Total from Experiment 1	80	30.20	21.4	64.5	37.8	26.8
Preparative Experiment 2						
Combined Batches (1-2)	20	7.91	6.51	72.1	39.6	32.6
Combined Batches (3-4)	20	9.29	5.98	76.4	46	29.9
Total from Experiment 2	40	17.20	12.49	74.2	43.0	31.2
Mean Recoveries Experiment 1and 2	--	--	--	69.4	40.4	29

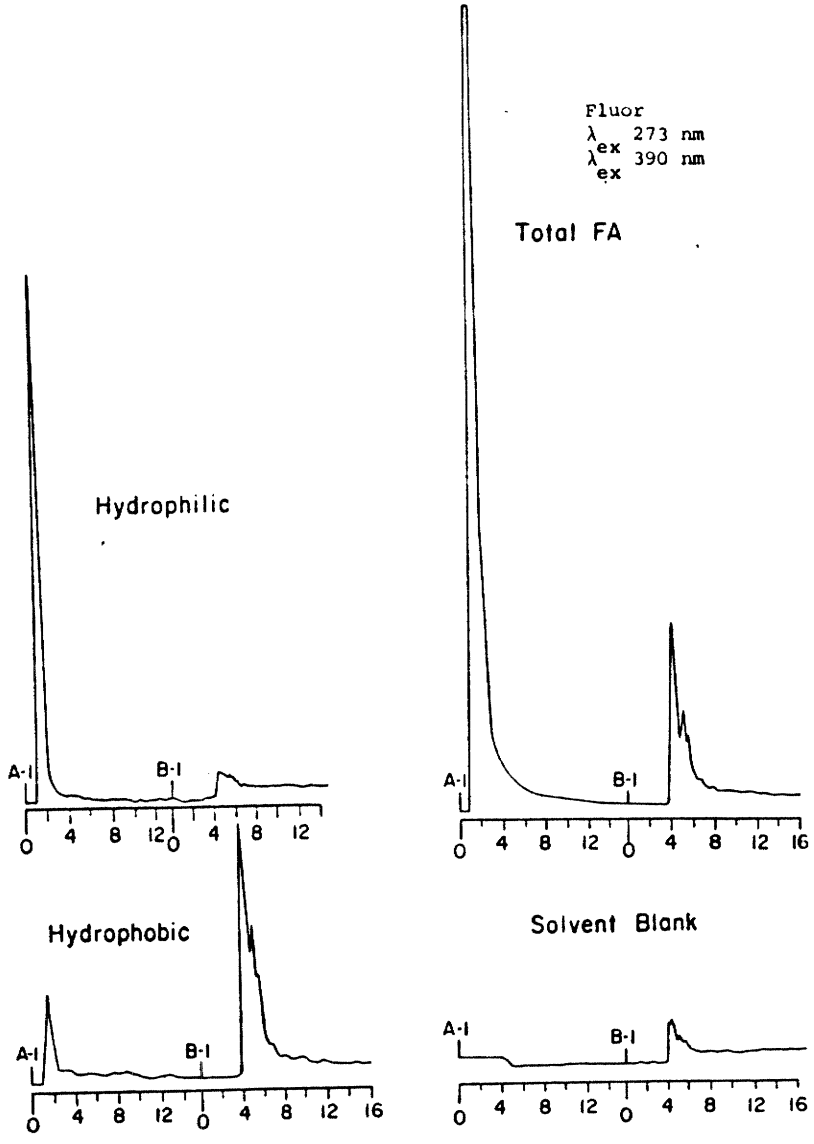


Figure III-1. Preparative experiment 1, analytical chromatogram of total SR-FA and HPLC fractions. Column: Novapak C18 5um, 15 cm L x 4 mm ID, stepwise gradient. Flow rate 1mL/min Detector: fluorescence.

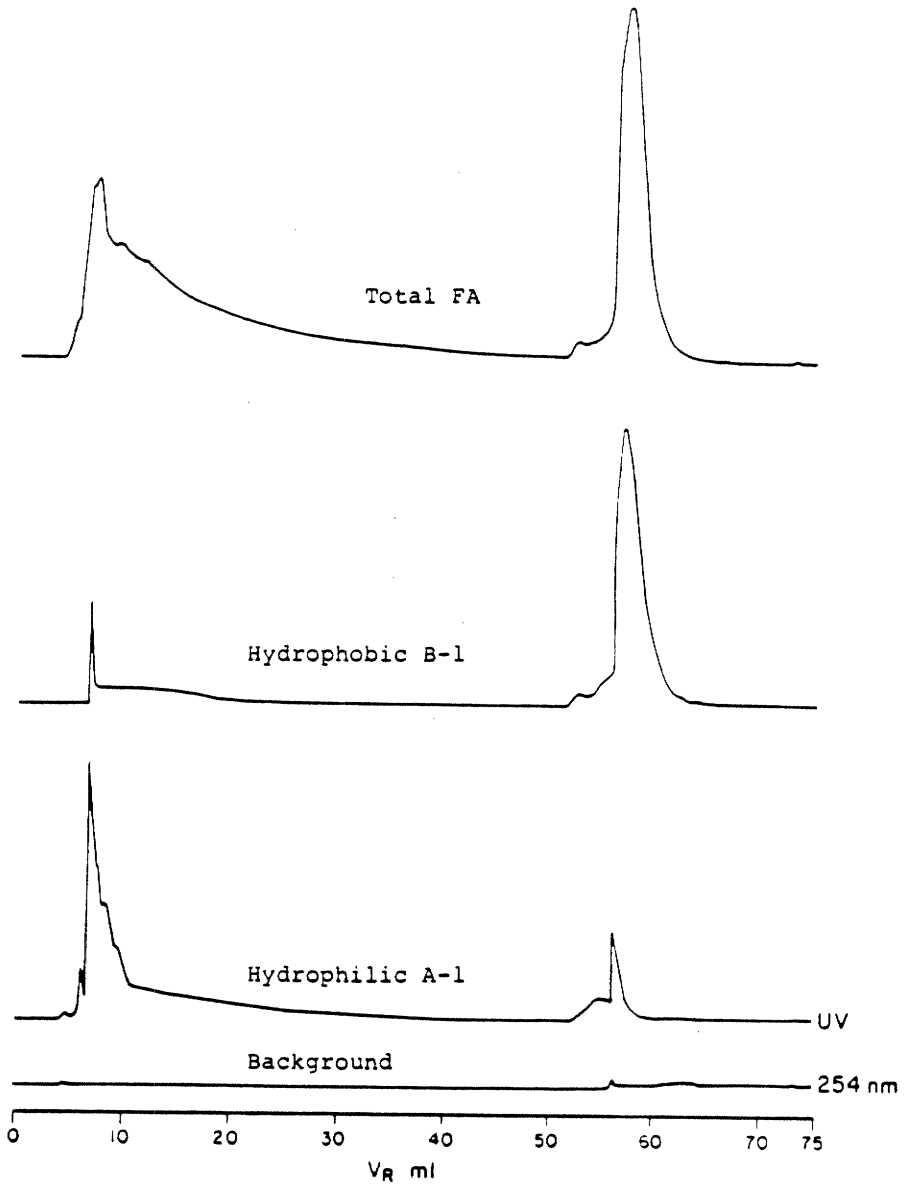


Figure III-2. Preparative experiment 2. UV chromatograms of total FA and fractions on Novapak preparative column experimental conditions in Table II-1.

II-1. The purpose of 1% methanol, which corresponds to 5.86 on polarity scale, is to equilibrate the carbonaceous end of the stationary phase to the aqueous mobile phase at the beginning of elution. At this elution composition, the hydrophilic compounds are likely to be eluted from the column, and the 1% methanol would not desorb any hydrophobic constituent before the organic modifier is increased. In the B-1 part, methanol acts as a solvent for the hydrophobic constituents that are strongly retained on the RP column material. The pH of the mobile phase was maintained at 7 throughout the preparative experiments. At this pH, the dissociated acidic groups are poorly retained on the column.

Some experiments were performed to compare the quality of separation with and without buffer, but the chromatograms were not drastically different. Furthermore, our primary goal was to influence separation of FA constituents using the simple mobile phase to avoid contamination and possible solute-solvent interaction.

Although efforts were made to avoid overloading the column, strong tailing was noted in the second preparative experiment (Fig. III-2). The uncapped silanol groups on the stationary phase strongly interact with the high polar molecules of the eluites and cause the tailing problem. The other problems described in the first paragraph of this chapter could be be alleviated by careful selection of the separation conditions and by examination of the retention

mechanisms and the possible solvent-solute interaction. As far we know, FA has no definite structure, and questions that relate to homogeneity or hetergeneity of FA and HA are not answered yet. The structure of FA is known to change with storage concentration, temperature, light and time (2). For these reasons, the structural changes during separation are intuitive and without dispute. Proper treatment of the mobile phase before use and adequate column rinsing procedures can eliminate the contamination problems. However, polycovalent metal contamination is only avoidable if a nonmetallic instrument is used in HPLC. Since both system and tubings are made of stainless parts, metal contamination was unavoidable in our case. The problem could be minimized by washing the system with 0.1 M nitric acid for two hours before the separation.

Solid State ^{13}C and ^{1}H NMR

Figure III-3 shows the solid state ^{13}C-NMR and ^{1}H CRAMP spectra of SR-FA sample. The ^{13}C spectrum is very similar to SR-FA reported in the literature (3). Resonance from carboxyl carbon centered at about 175 ppm, aromatic carbons centered at about 130 ppm, oxygenated aliphatic carbon centered at about 79 ppm and alkyl carbons centered around 38 ppm. A well-defined resonance line centered at about 200 ppm and can be assigned to carbonyl carbon. Based on the results of dipolar-dephasing experiments, Leenheer et al.(4)

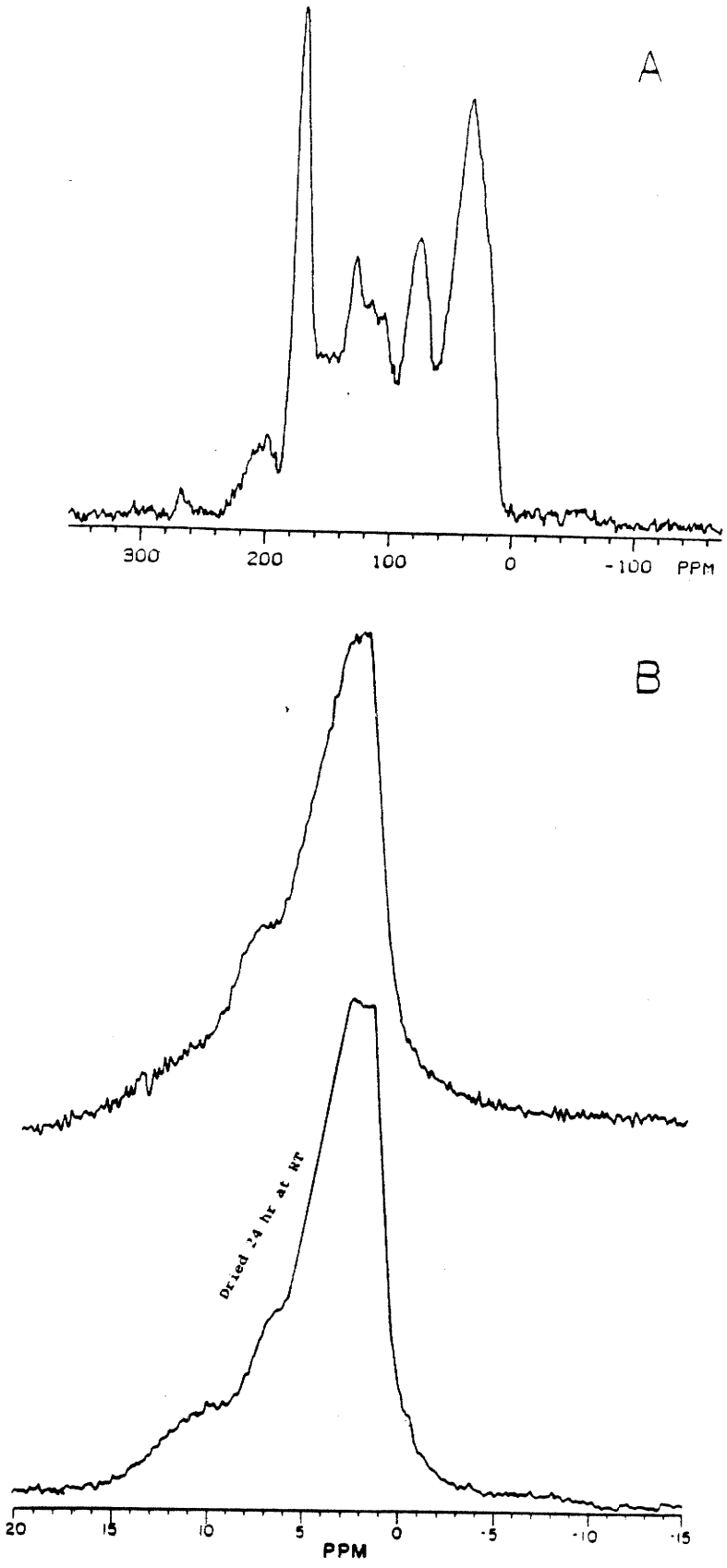


Figure III-3. Solid state ^{13}C and ^{1}H NMR spectra of SR-FA.

assigned ketone rather than aldehyde carbon, to this resonance line. The development of quantitative information from solid state CP/MAS NMR spectra is associated with some uncertainties due to the overlap between signals from different types of carbon. A recent spin-counting study (5) indicated that 97% of the carbons in SR-FA can be detected by ^{13}C CP/MAS. Furthermore, other aquatic FA has been investigated by several groups (6,7), and the results have been rather uniform. In many cases, estimation of aromaticity or acidity of HS from the NMR spectra gave results which were in agreement with other methods. Table III-2 shows the major resonance lines in Figure III-3, carbon assignment and percent composition.

The ^{1}H CRAMPs spectra of total SR-FA is shown in Figure III-3B. Some improvment was noted after drying the sample under vacuum at room temperature for 24 hours. The spectra shows three broad overlapping lines, representing resonance at δ 0.5, 1.8, 3.0, 7.0 and 10.5 ppm. The assignment and composition are also listed in Table III-2. Both ^{13}C and ^{1}H CRAMPS provided comparable quantitative data. No satisfactory ^{13}C NMR spectra were obtained from the A-1 sample. The ^{13}C NMR of the B-1 fraction was very weak and showed a weak broad absorption in the aliphatic region.

Figure III-4 shows the comparison of chemical shifts of ^{1}H CRAMPS among total FA, A-1, and B-1. In the A-1 fraction, the protons on carbon attached to oxygen have high

Table III-2 ^{13}C CP/MAS and ^{1}H CRAMPS chemical shift regions in total FA.

^{13}C NMR

Region	Carbon Type	% Contribution
0 - 50 ppm	methyl, methylene, methine (etc.)	44.47
50 - 110 ppm	alcohol, amine, carbohydrate, ether acetal, methoxyl	17.2
110 - 160 ppm	olefinic, aromatic phenolic	14.10
160 - 200 ppm	carboxylic	18.05
200 - 220 ppm	amide or carbonyl	6.05

^{1}H CRAMPS

Region	Proton Type	% Contribution		
		Rep I	Rep II	Dried
A (0 - 2.0 ppm)	methyl, methylene, methine (etc.)	2.67	4.78	5.66
B (2 - 4.6 ppm)	methyl, methylene (to aromatic ring or carboxyl groups), and groups of indanes and tetralins, protons on carbons to oxygen	40.47	43.31	63.59
C (4.6 - 9 ppm)	aromatic	10.22	6.95	13.48
D (9 - 11 ppm)	acidic	46.62	44.95	13.53

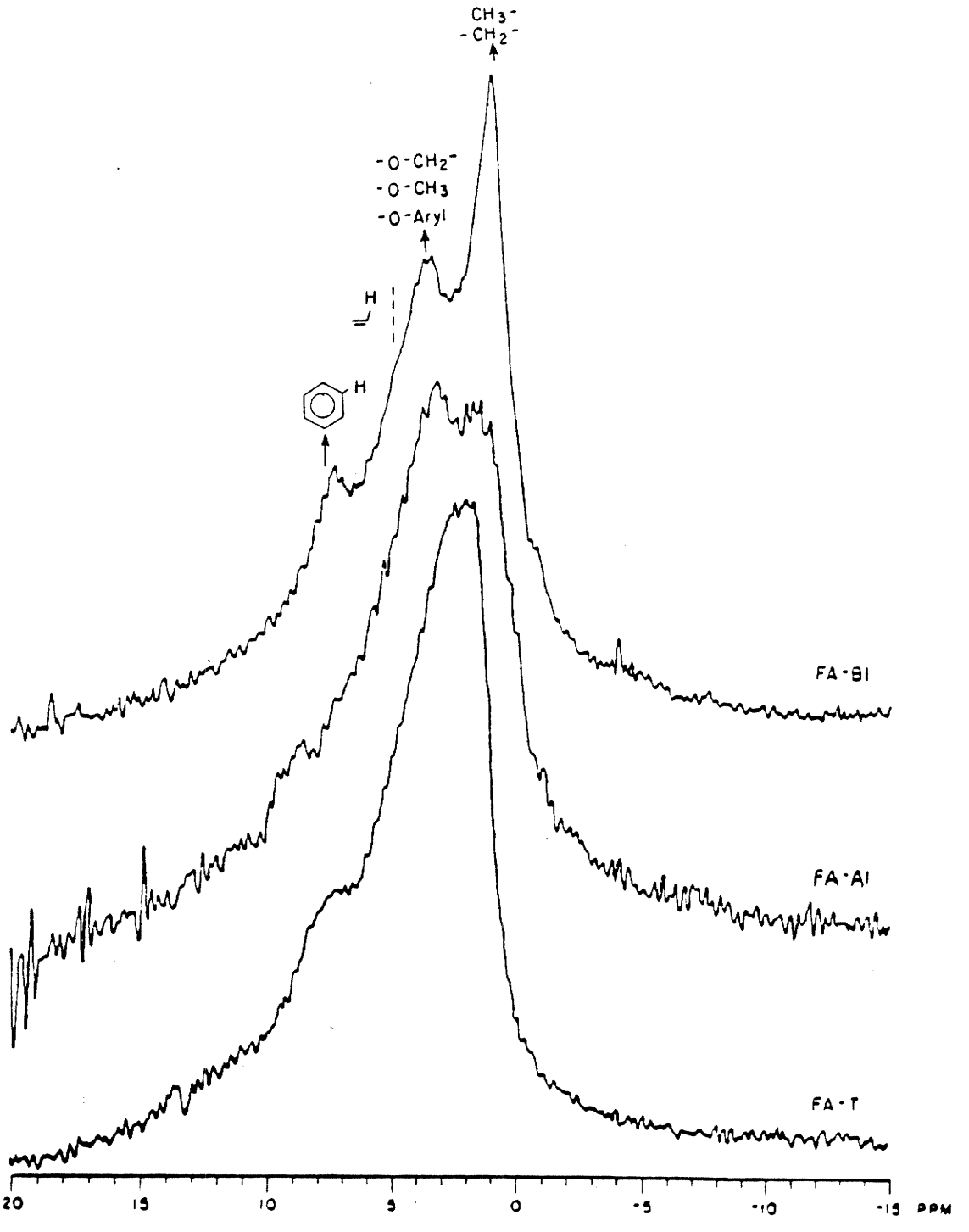


Figure III-4. Comparative ^{1}H CRAMPS spectra of FA and fractions.

absorption. Acidic protons absorption band between δ=9.5 and 12 reveal a clear shoulder indicating more carboxylic groups in A-1 fraction than in B-1 fraction. The intepretation was also apparent in FTIR spectra. p.75.

The B-1 fraction gives three distinct high intensity peaks located around δ 2.2, 4.5 and 8. The strong absorption at δ 2.2 indicates a significant level of benzyl protons or allyl protons in this portion. The absorption around δ 4.5 represents methyl or methylene protons attached to carbon adjacent to oxygen. The δ 8 peak corresponds to aromatic and vinylic protons. From these three absorption bands, the B-1 may contain the molecular structure like alkylphenyl hydrocarbon interlocked with oxygen atoms. The acidic groups between δ 9 to 11 seem to be less than that in the A-1 fraction.

Fourier Transform IR on Total FA and Fractions

The infrared spectra of total FA and fractions are shown in Figure III-5. The main IR absorption bands are listed in Table III-3. The main absorption of total FA is the broad O-H stretch located between 3600 cm^{-1} to 2400 cm^{-1}. The 1730 cm^{-1} band together with the 2600 cm^{-1} band indicate the high COOH content. The 1600 cm^{-1} region represents either aromatic C=C stretching or strong H-bonded C=O group. The aliphatic C-H bending is located at 1440

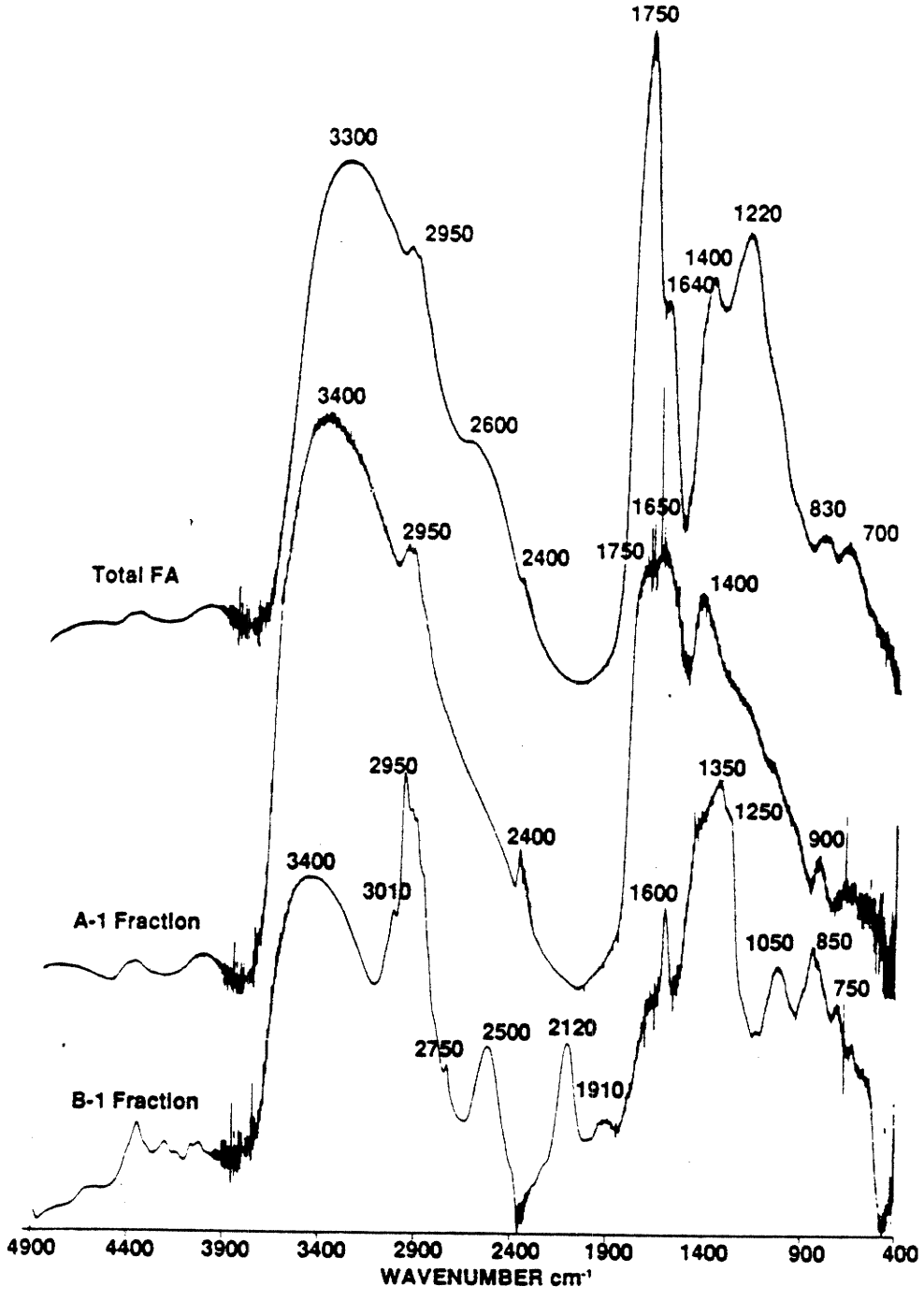


Figure III-5. Solid state FT-IR spectra of FA and fractions.

Table III-3 Main IR Absorption Bands

Frequency (cm^{-1})	Assignment
3400 - 3300	O - H stretching, N - H stretching (trace)
2940 - 2900	Aliphatic C - H stretching
1725 - 1720	C = O stretching of COOH and ketones (trace)
1660 - 1630	C = O stretching of amide groups (amide I bond), quinone C = O and/or C = O of H-bonded conjugated ketone.
1590 - 1517	COO^- symmetric stretching, N - H deformation + C = N stretching (amide II band)
1400 - 1390	O - H deformation and C - O stretching of phenolic OH, C - H deformation of CH_2 and CH_3 groups, COO^- antisymmetric stretching
1280 - 1200	C - O stretching and O - H deformation of COOH, C - O stretching of aryl ethers
1170 - 950	C - O stretching of polysaccharide or polyaccharide - like substance, Si - O of silicate impurities.

cm^{-1} and C-O stretching at 1220 cm^{-1}.

In the A-1 fraction, the absorption of 1720 cm^{-1} and the 2650 cm^{-1} decreases proportionally, but the 1620 cm^{-1} band becomes stronger. The two bands, 1720 cm^{-1} and 1620 cm^{-1}, are assiged to COOH and hydrogen bonded C=O. Thus, the broad absorption at 3400 cm^{-1} is contributed by carboxylic O-H stretching. The C-H bending absorption at 2950 cm^{-1} indicates that the aliphatic structure is still abundant in this fraction. The aromatic C-H bending at 810 cm^{-1} shows that some aromatic moieties are present in the same fraction.

In the B-1 spectrum, a little shoulder at around 1720 cm^{-1} and weak O-H stretching give the hint that COOH is not important in this fraction. Instead, a clear aromatic C=C stretching band at 1610 cm^{-1} together with aromatic C-H bending at 840 cm^{-1} and phenoxy OH stretching at 1020 cm^{-1} support the fact that the phenols are important structures in this fraction. The 3010 cm^{-1} band of C=C-H stretching also supports this explanation. The strong C-H stretching centered at 2950 cm^{-1} and C-H bending centered at 1340 cm^{-1} give a indication of high aliphatic side chain structure in this fraction. Absorption bands at 2520 cm^{-1} and 2110 cm^{-1} are in B-1 only, the reasons are still unknown.

III-2. Analytical RP-HPLC

Electrolyte Effect Experiment

The electrolyte effect experiment was performed at an early stage of the research using the Waters ALC 201 instrument with model 6000 pump. Separations were monitored simultaneously by using a fixed wavelength Beckman UV detector and a Schoeffel fluorescence detector. The purpose of this experiment was to study the effect of adding NaCl as an electrolyte to the mobile phase on the retention behavior of aquatic FA from different streams. Samples used in this experiment were 0.02% aqueous solutions of aquatic FA from Suwannee, Ogeechee, Ohio, and Beer rivers. The electrolyte effect was evaluated at concentration levels of 0.001 M and 1 M, respectively. Aliquots of samples (50 uL) were chromatographed under exactly the same conditions except for the electrolyte concentration in the mobile phase.

Figure III-6 shows the UV and fluorescence chromatograms of each pair of samples. All chromatograms included essentially the same two regions of UV absorption and fluorescence emission. Fluorescence chromatograms of Ohio and Ogeechee rivers contained additional but minor regions of fluorescence. Presence of 0.001 M NaCl in the mobile phase did not cause a detectable difference from the chromatograms of samples run without salt in the mobile

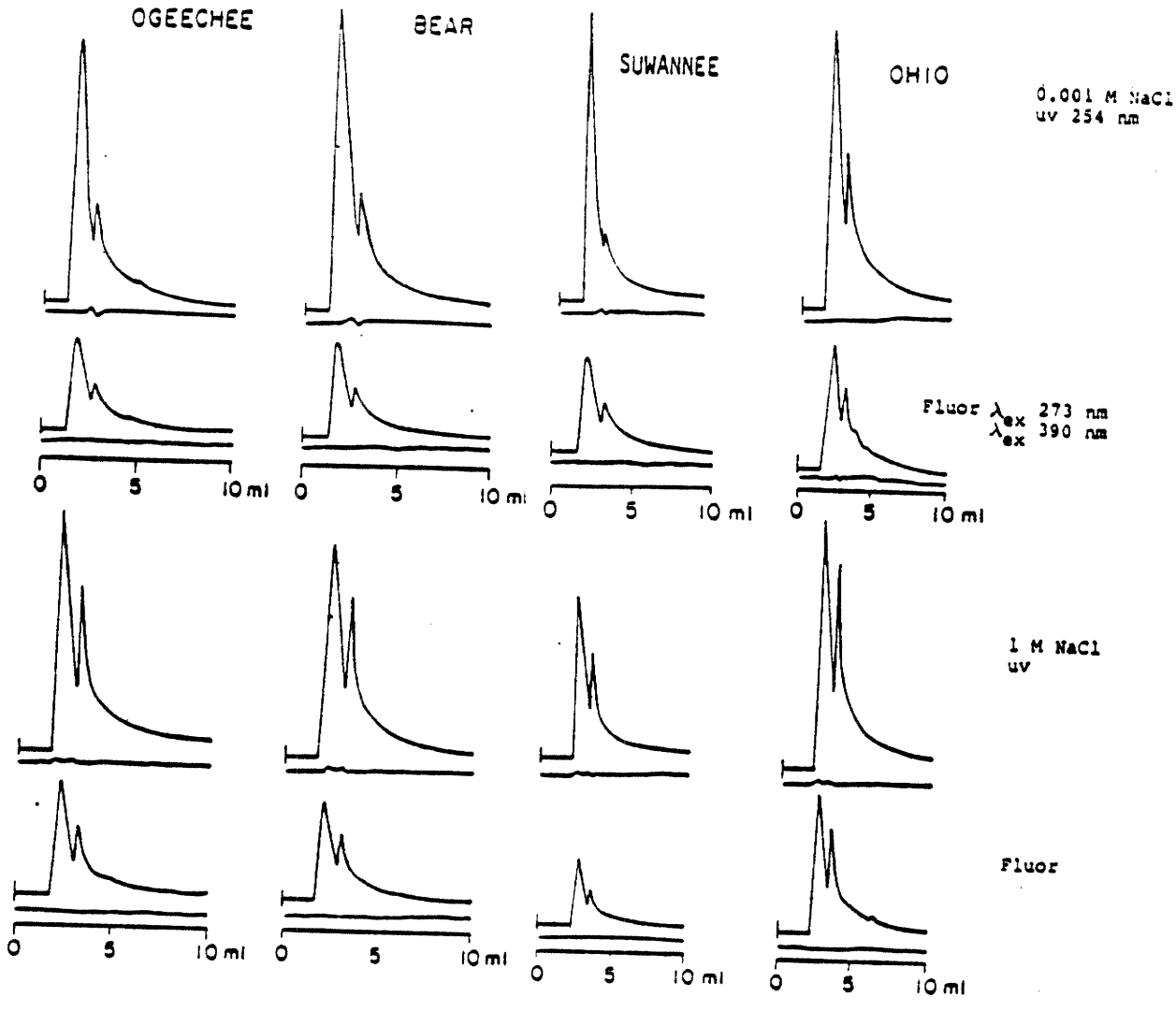


Figure III-6. Effect of added NaCl on retention of FA from different stream.

phase. However, the increase of the electrolyte concentration by a factor of 10^3 induced detectable befects on the retention and signal intensities of FA components. The retention time was increased by almost half a minute in all sample chromatograms that used 1 M NaCl in the mobile phase. Intensities of the UV absorption or fluoresence emission were also influenced. In all the samples, the first peak was reduced by 5-15%, in the presence of 1 M NaCl. The second and third peaks (if present) showed better resolution and signal enchancement in presence of 1M NaCl.

Electrolyte concentration and pH, can have a profound effect on electrostatic interactions and consequently on the retention behavior of ionogenic samples such as FA. A detailed theoretical treatment of these effects has been published (8). Ionization results in a decrease of the retention factor.

The presence of neutral salt in the mobile phase is expected to induce two effects on the ionized species. At low salt concentration where the limiting Debye-Hückel treatment is valid, the predominant effect of added salt is to enchance the ionic atmosphere around each eluite molecule and thus reduce the electrostatic repulsion between the eluites. Thus, the addition of low concentration of electrolyte may cause a decrease in the retention factor. Whereas at higher salt concentrations, further increase in shielding due to salt addition is negligible, the surface

tension of the eluent increases with salt concentration and results in an increase of retention factor. As a consequence, with increasing salt concentration in the eluent, one anticipates an initial decrease followed by monotonic increase in the retention factor of ionized eluites. If the eluite is not ionized, the retention factor should increase with salt concentration in the eluent due to the increasing surface tension.

The simultaneous changes in the UV and fluorescence signal intensities in presence of 1 M NaCl in the mobile phase can be attributed to the shift in the retention time of some of FA constituents due to salting out effect at high ionic strength. The effect would result in increasing the retention time of some solutes. It should be remembered that the two peaks obtained in the FA chromatograms represent simply the split of FA polymeric structure into two units consisting of smaller size.

Continuous Gradient RP-HPLC

Several gradient programs were evaluated using the capabilities of the HP 1090 LC (9). Table II-2 shows a summary of gradient programs and the types of columns used with each program. The mobile phases included binary and ternary solvents. Gradient programs were optimized to achieve the best possible efficiency and reproducibility.

The analytical separations were monitored with UV-vis PAD detector. In successful chromatograms, the UV-vis absorption data points were accumulated and stored in the HP-85B PC. Each separated peak was scanned between λ 200 and 600 nm at three points representing peak maximum, slope upward and slope downward, respectively. Overlap of the spectra collected at these three points is considered to be indicative of peak purity and/or homogeneity. This approach allowed the development of a UV-vis spectral catalogue (10) of FA consituents and several other model compounds, under different gradient programs. Frequently, the fluorescence and amperometric detectors were used, in line with the UV-vis PAD, to monitor the separation.

Gradient Programs

The selected gradient program were developed after initial tests were performed to evaluate the most important parameters. The pH linear gradient(pH 2-12) had attracted several researchers to separate FA (11,12,13). Although the model compounds with different pKa showed good separation at corresponding pH range, in case of FA poor separation was obtained. Even with very precise computer-controlled linear pH gradient, FA separation was unsuccessful (13); the chromatogram appeared with only one major peak and some humps on the slope.

In our laboratory, an Act-II column (150mm x 4.6mm), Interaction Corp., packed with acid-base resistant polymeric

packing material with ionic exchangable sites, was used to separate SR-FA at pH 2-10 range. A flat pH electrode cell was used to monitor the pH at the outlet of the system. Chromatograms did not show satisfactory separation.

Other stationary phases, such as Partisil C8, RSil phenyl were also evaluated but did not show better separation. The C18 stationary phase afforded the best separation of FA. In addition to the long carbonaceous chain effect, another key point is that the higher the carbon loading, the better the performance. Hence, most experiments in this research were finally performed with a Novapak C18 column and a high efficiency Hypersil C18 column. The pH of the mobile phases was chosen at pH 4 and pH 7. There are four reasons for this selection: i) to test the secondary chemical equilibrium effect on separation; ii) the pH of SR-FA sample solution ranged from 3.5 to 3.8, depending on the concentration, and after a few tests, it was found that maintaining the mobile phase at pH 4 produced the best selectivity (14); iii) at a pH lower than 4, the column reequilibration time was longer; and iv) at low pH, the gradient background increased more seriously which made UV-vis detection at wavelength below 220 nm difficult.

In most analytical experiments, acetonitrile was preferred as an organic modifier rather than methanol. The choice was based in the following reasons: i) The elutropic strength of acetonitrile is 3.1 times that of methanol for

C18 stationary phase. Thus, more recovery and shorter retention time is expected by using acetonitrile. ii) The viscosity of acetonitrile is lower, and mass transfer in this phase is faster. The selectivity increases correspondingly. iii) The UV-vis absorption will be stronger in acetonitrile than in methanol.

Gradient Program I: Novapak C18 Column.

Figure III-7 and Figure III-8 show the chromatograms of SR-FA samples dissolved in milliQ water or pH 7 buffer. The UV-vis scans of each peak at three identified point (upslope, apex, and downslope) were plotted on the top of the figures. The experimental conditions in the two chromatograms are exactly the same. Both chromatograms show the resolution of six Uv-vis absorption regions. However, peak intensities and elution order are different. In Figure III-7, the total elution time in the aqueous sample is around 15 minutes, whereas in the buffered sample it is only 10 minutes. This can be attributed to the fast equilibration time in the case of the buffered sample. Also, it is noted that peak no. 6 in the aqueous sample is absent in the buffered sample, while the intensities of peaks 1, 2, and 3 are increased. This effect can be attributed to the electrolyte ionization effect on FA components at pH 7, in contrast to the more protonated form in the case of aqueous mobile phase at pH 4.

The scans of the first two peaks in Figure III-8 are

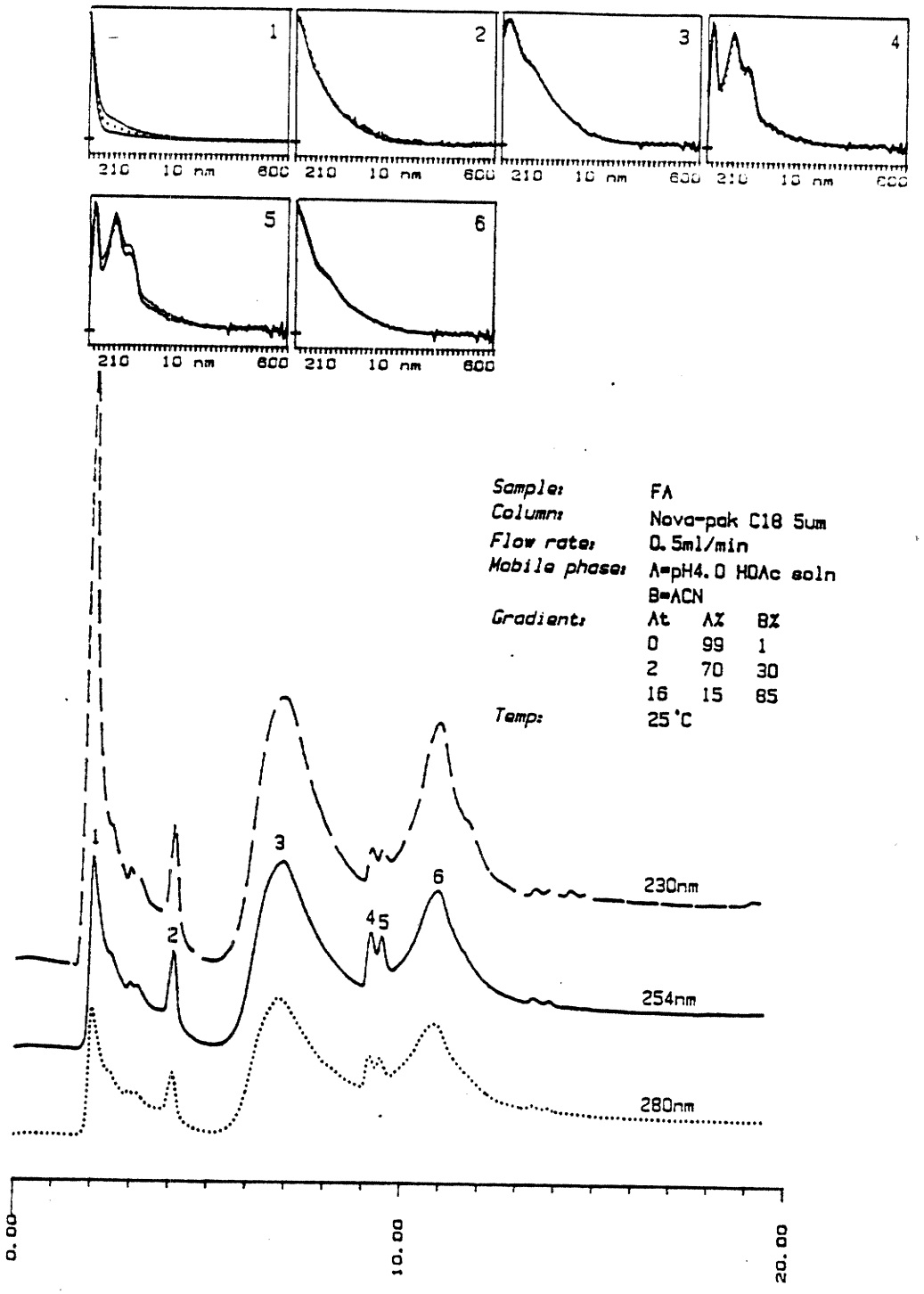


Figure III-7. Chromatogram and UV-vis scans of 50ul 0.02% SR-FA dissolved in milliQ water (stored for one month). Gradient program I.

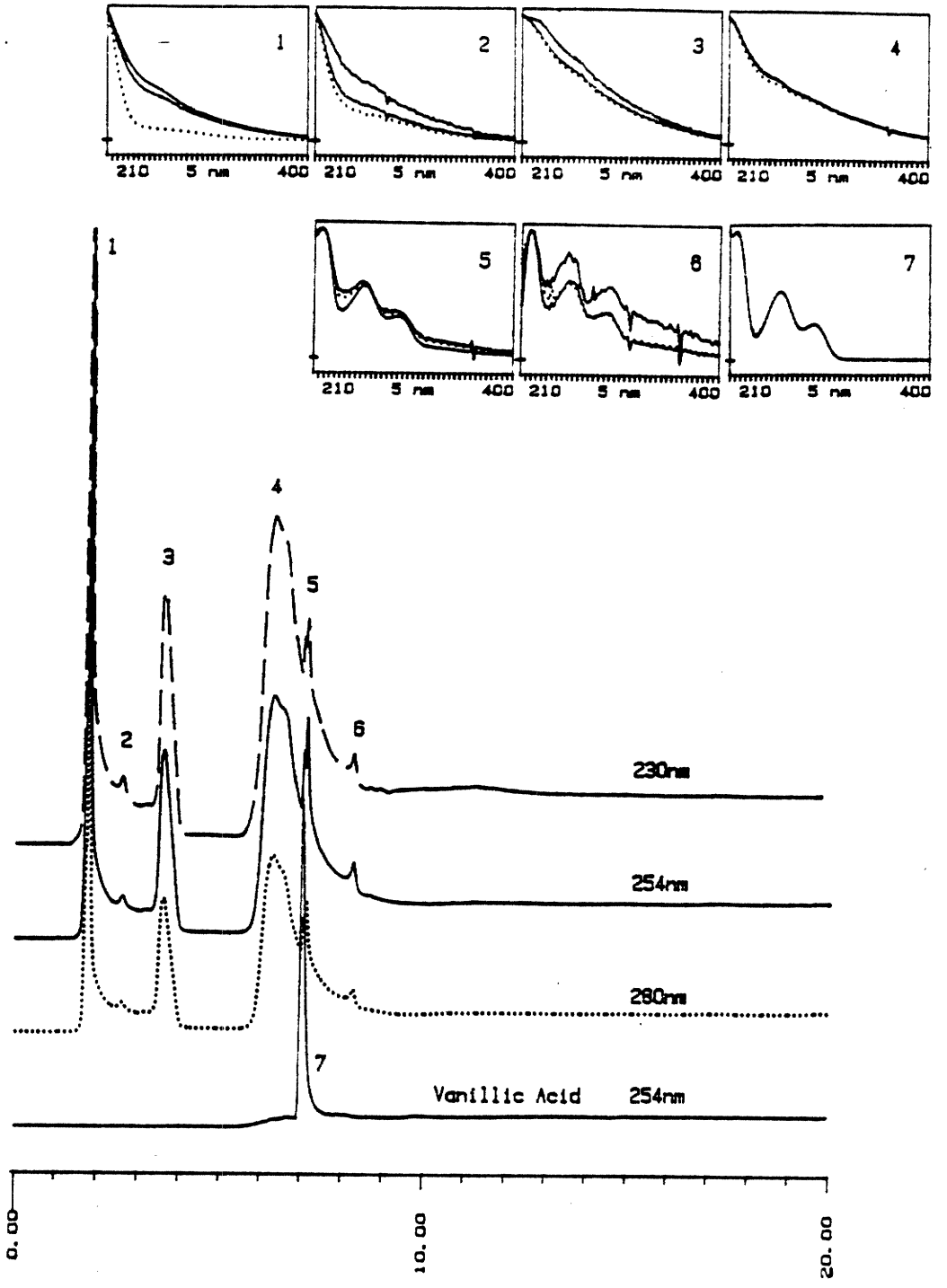


Figure III-8. Chromatogram and UV-vis scans of 50uL 0.02% SR-FA in pH 7 buffer (stored for one month). Gradient program I.

featureless and showed gradual decrease of absorbance with increase of wavelength. Peak 3 in the water-soluble sample is broad and its retention time (t_R) extends from 5.5 to 8.5 minutes. The UV-vis scans of this peak show several minor shoulders at λ 244, 270, 315, 355, 395, 485, 575, and 590 nm. The scans indicate less purity, suggesting that this peak corresponds to a larger fragment of aquatic FA which contains both chromophores and auxochrome structures. The UV-vis scans of the fourth and fifth peaks show well-defined features. Both spectra show two distinct absorption lines at λ 260 and 290 nm. The two lines are characteristic of vanillic acid (VA) structure. Chromatographic peak 6 in Figure III-7 is broad and eluted over a period of two minutes. Its UV-vis scan is similar to peak 3 and is likely due to another large molecular fragment containing chromophores and auxochromes.

In the buffered sample, scans of peaks 1, 2, 3 and 4 are similar to those of peaks 1, 2, 3 and 6 in Figure III-7. The last two peaks (i.e., peak 5 and 6) exhibit the predominant absorption lines of vanillic acid structures noted in peaks 4 and 5 of Figure III-7. The t_R and UV-vis scans of vanillic acid standard solution (peak 7 in Figure III-8) are the same as peak 5 of the sample. This may be the first detection of vanillic acid structural units in FA via HPLC analysis. It is important to note that VA structural units occurred in two chromatographic peaks which

differ in t by 0.4 min in Figure III-7 and by 1 minute in Figure III-8. Detection of additional weak Uv-vis absorption lines in the scans suggests that VA structures are not present in the pure acid form.

The analogy between the UV-vis scans of vanillic acid and those detected in the aqueous or in buffered FA solutions leaves little doubt regarding the presence of vanillic acid in the FA macromolecule. As discussed below, it is interesting to note that the vanillic acid structures were not detected in the chromatograms of either fraction A-1 or B-1. A possible explanation is that vanillic acid is only intermediates formed by the slow degradation from lignin precursors. Once the macromolecule is fractured by the HPLC interactions, vanillic acids may undergo further degradation to simpler carboxylic or phenolic compounds.

Figure III-9 shows the chromatogram and the UV-vis scans of the hydrophilic fraction A-1. Six regions of UV-vis absorption can be identified. The total elution time is only 7.5 minutes. The UV-vis scans of all the six peaks are all similar and all featureless, except for weak shoulders at λ 255 and 365 nm. The scans of the hydrophilic peaks are comparable to the first three peaks in total FA.

Figure III-10 shows the chromatogram and UV-vis scans of the hydrophobic fraction of B-1. As may be expected, the total elution time is extended to 19 minutes. Most of the peaks appear after 6 minutes, indicating strongly

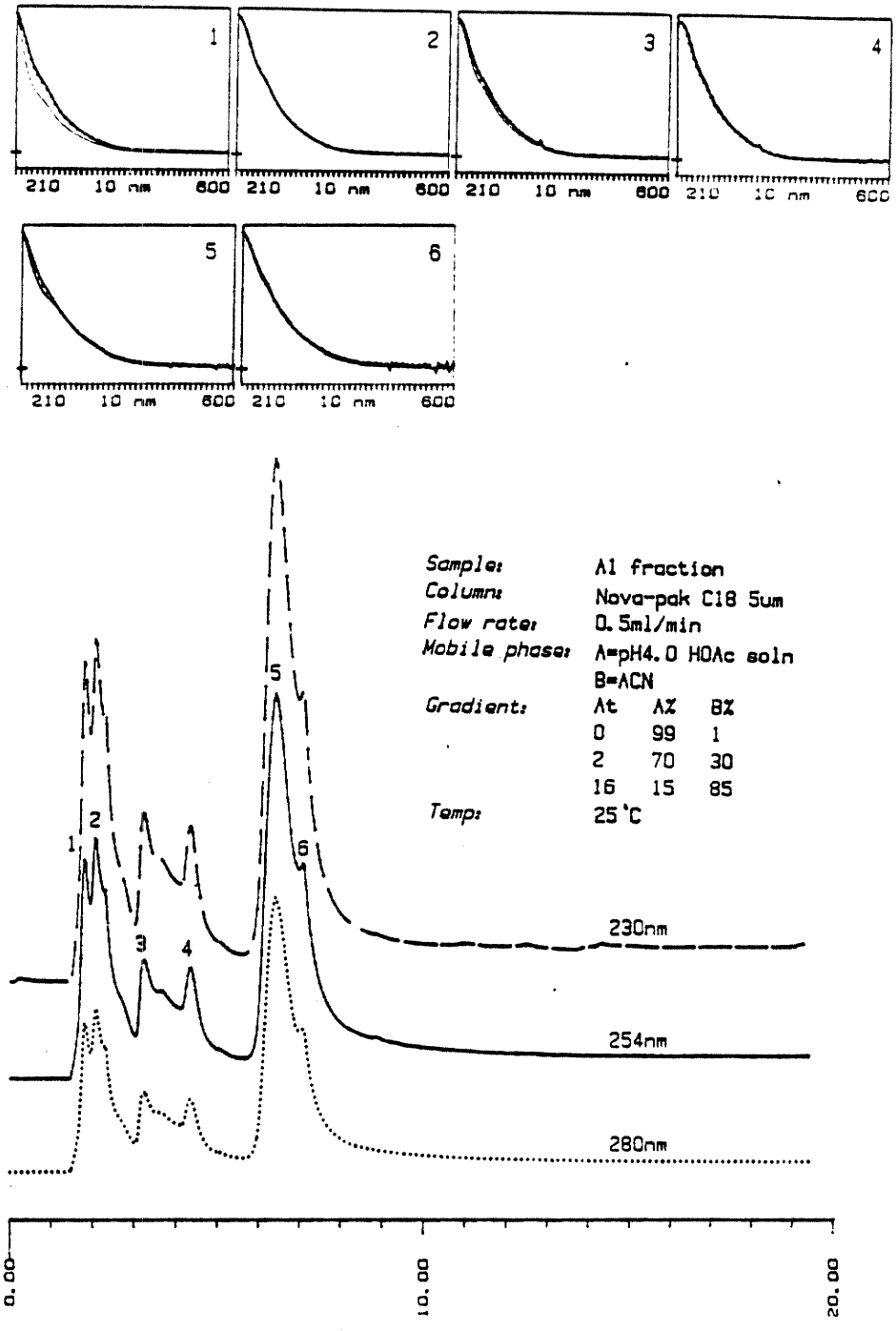


Figure III-9. Chromatogram and UV-vis scans of hydrophilic fraction A-1. Gradient program I.

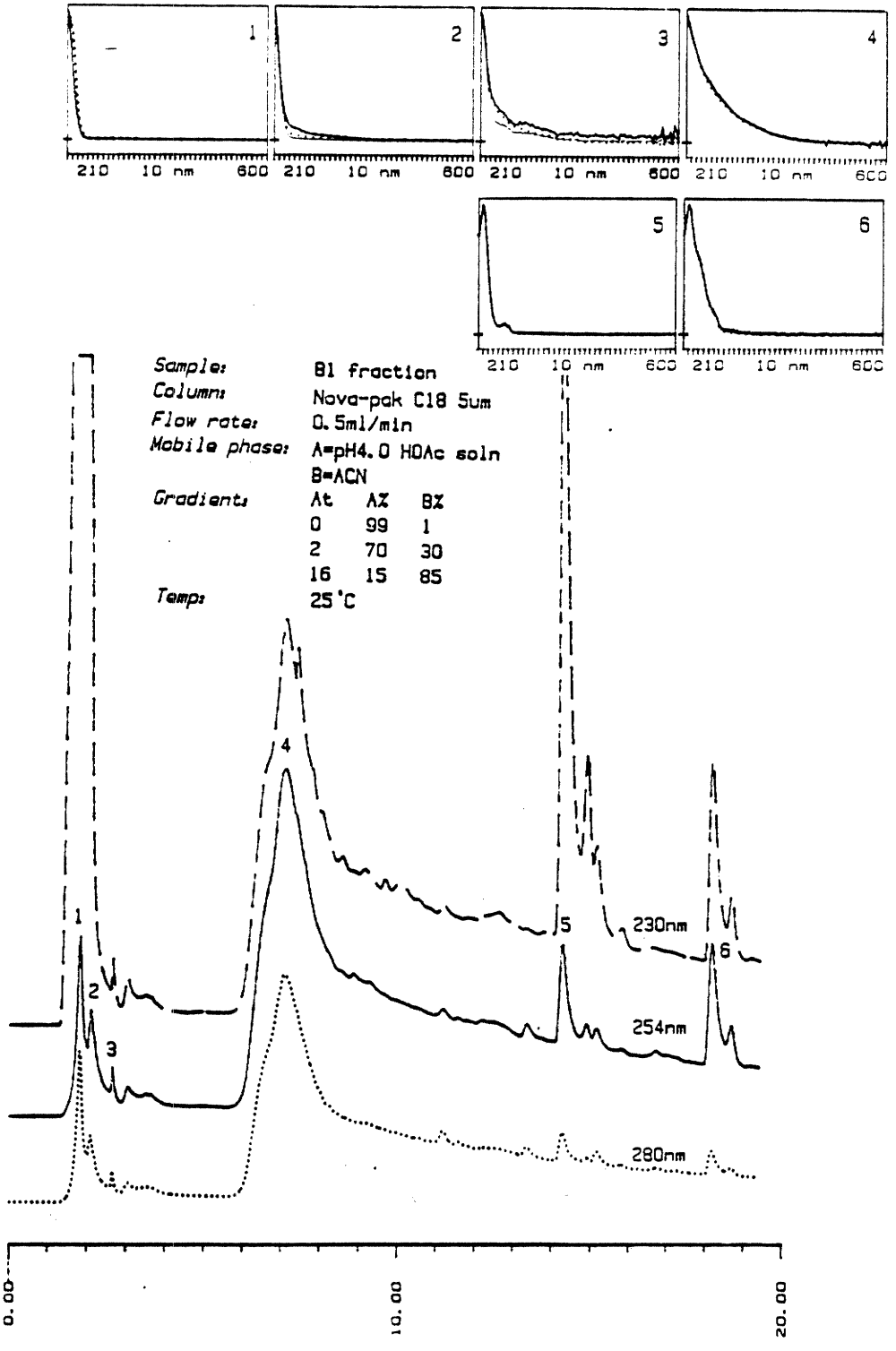


Figure III-10. Chromatogram and UV-vis scans of hydrophobic fraction B-1 of SR-FA. Gradient program I.

hydrophobic interactions. Scans of peaks 1 and 2 are essentially similar except for minor impurities. Both peaks had maximum absorption at λ 212 nm and probably correspond to aliphatic structures. The third peak contains more impurities which may be colored. The fourth chromatographic peak is broad and eluted between 6 and 9.5 minutes. Its scan is featureless and similar to peak 3 and 6 detected in total FA in Figure III-7. The UV-vis scans of peak 5 have a maximum at λ 220 and weak shoulder at λ 265 nm. These absorption features are characteristic of conjugated ketones. Peak 6 shows a charateristic featureless spectrum with minor shoulders at λ 250 and 280 nm.

Gradient Program I: Hypersil C18 Column.

Figure III-11 shows the chromatogram and UV-vis scans of 50 uL of aqueous 0.02% SR-FA and hydrophilic fraction A-1. The chromatograms show modest resolution of three absorption regions. The first two occurred within 3 minutes. The third peak is broad and extends from 7 to 10 min. The UV-vis scans of the first and second peaks were both featureless and similar to the scans of the early eluting peaks with the Novapak column. Peak 3 is also featureless, but it shows some absorption lines in the visible region, indicating the presence of chromophores and auxochromes. This scan represents the predominant polymeric structures in FA. It is likely to consist of two or three structural units containing aliphatic and substituted

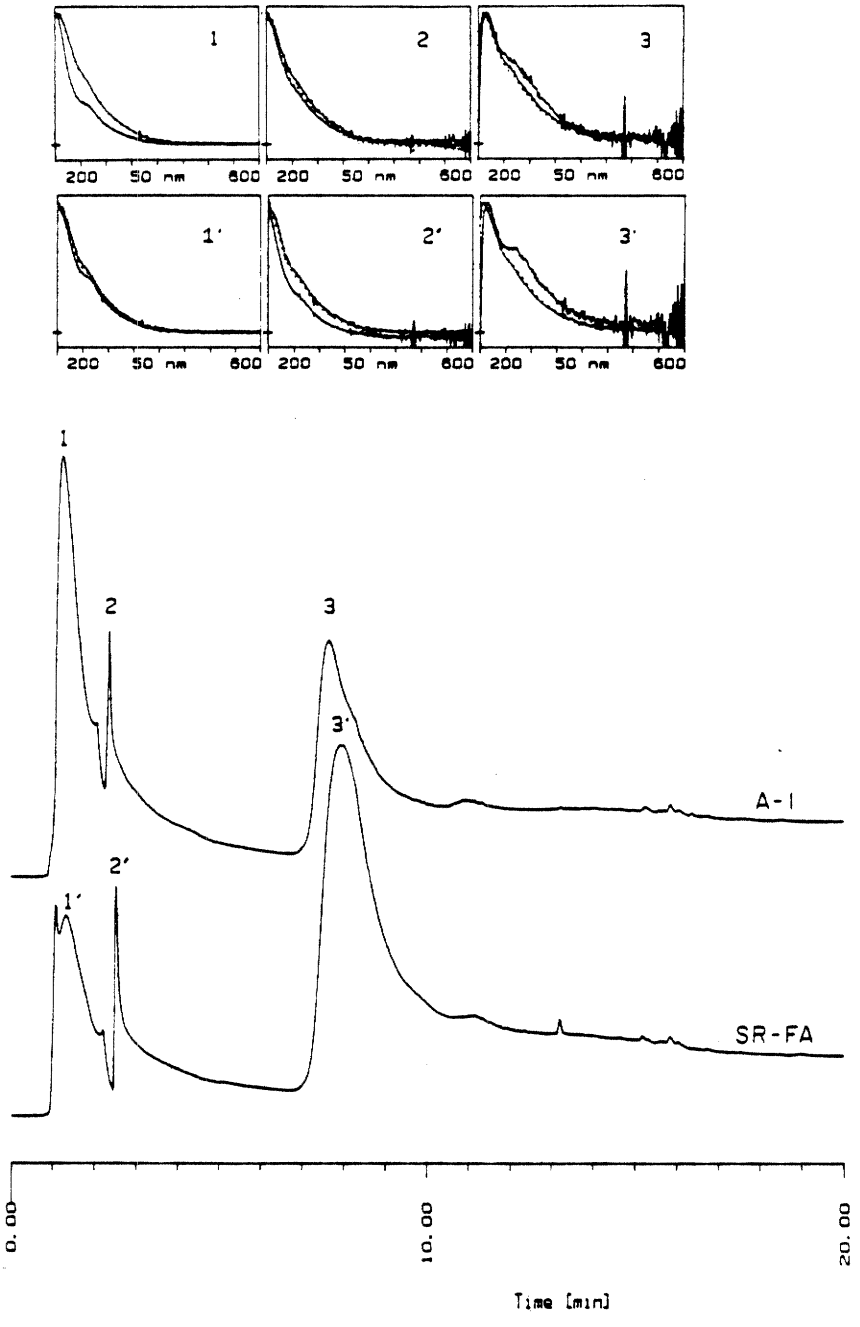


Figure III-11. Chromatogram and UV-vis scans of 20uL 0.02% SR-FA and 20uL 0.02% A-1 fraction dissolved in milliQ Water. Gradient program I. Hypersil ODS column.

aromatic structures.

Figure III-12 shows the chromatogram and scans of the hydrophobic fraction B-1. The chromatogram was monitored at 230 nm. the scans of the early eluting peaks in the first 2 minute are similar to those present in the total sample and hydrophilic fractions. Peak 2-12 occured between 6 and 18 min, with only peak 2 indicating a broad signal with a scan chracteristic of the predominantly colored fragment of FA. Peaks 3, 5, 6, 7, and 8 showed a distinct absorption line at λ 226 nm and a weak line at λ 265 nm. The similarities and intensities of these peaks are the first indication of the presence of repeated structural units in FA macromolecular structure. It should be remembered that these peaks occurred between t_R 8.62 and 11.42 min, i.e., within 2.8 min in the hydrophobic region. It is suspected that these peaks correspond to a conjugated ketone structure. Peaks 4, 9, and 10 are similar and contain a defined additional absorption line at λ 275 nm; such spectra are characteristic of phenolic conpounds. In peak 11, the λ 275 nm line is barely detectable, but a well-defined line at λ 221 nm is noticeable. Peak 12 occurred at t 17.2 min and UV-vis absorption shows several absorption lines at λ 209, 235, 260, 275, and 285 nm. These lines are characteristic of the polynuclear aromatic structures.

The chromatogram of fraction B-1 on a Hypersil column represents one of the most successful chromatographic

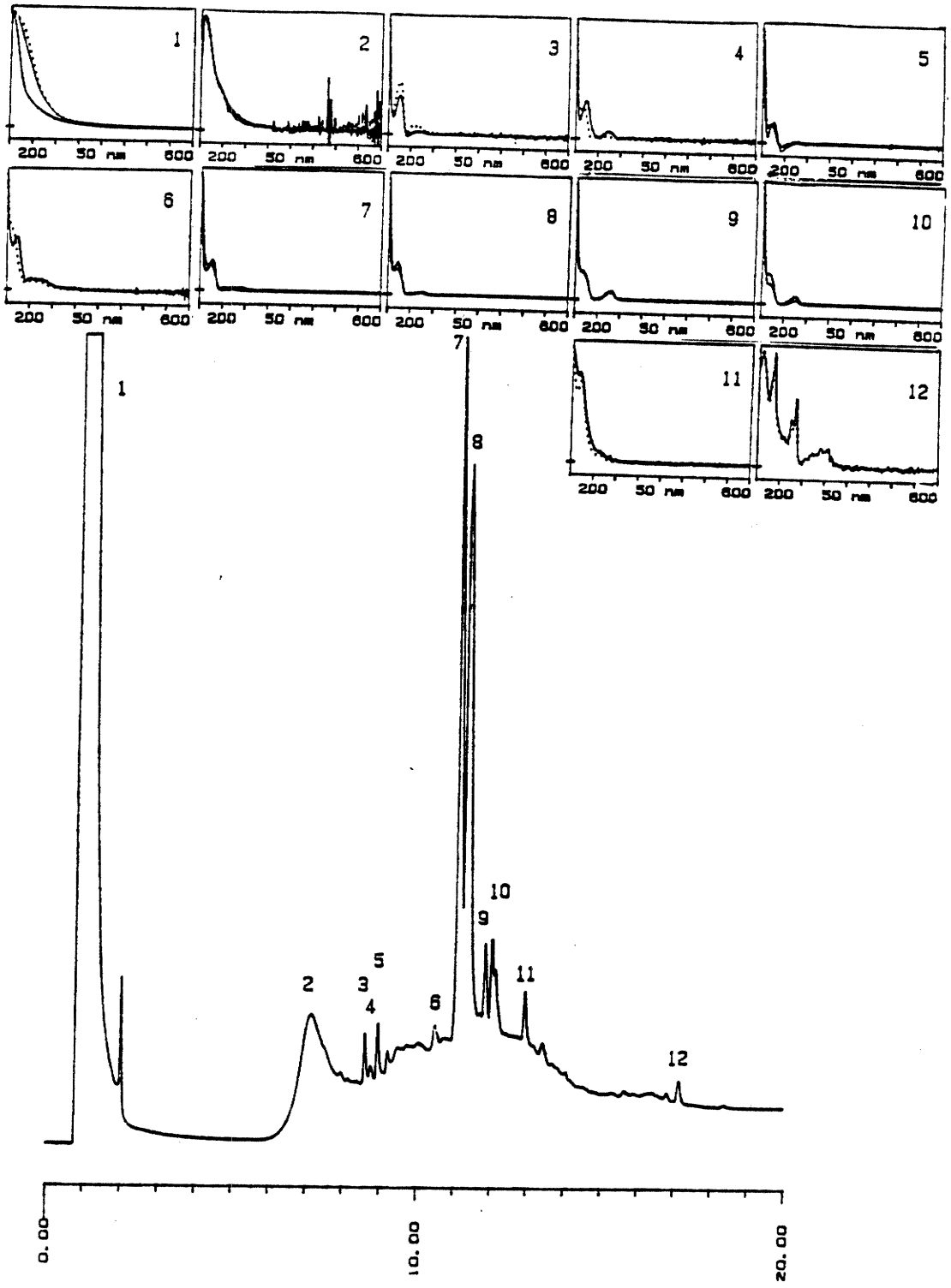


Figure III-12. Chromatogram and UV-vis scans of 50uL of 0.02% aqueous hydrophobic fraction B-1 dissolved in methanol. Gradient program I. Hypersil ODS column.

separations of FA. The UV-vis scans provide the first evidence of the presence of structurally similar units in FA. The effect of storage of the B-1 methanol solution was evaluated by injecting the same sample under the same conditions after storage at 4°C for 5 months and 12 months, respectively. Chromatograms showed essentially the same resolution and the UV-vis scans of each peak are also similar. The only detectable difference was the increase of the magnitude of the phenolic peaks and the decrease in the magnitude of the conjugated ketone peaks (Fig. III-13).

Gradient Program II: Hypersil C18 Column

Because the solvents were purged with He, the pH of the MilliQ water was maintained at 7.00. Figures III-14 and III-15 show chromatograms and UV-vis scans of the total samples and fractions. In Figure III-14, the early eluting peaks (the first 3 min) are stronger than in the case of gradient program I, Figure III-11. This can be attributed to secondary chemical equilibrium influencing the retention behavior of organic acids. This behavior was noted earlier under stepwise gradient conditions (15). It is interesting to note the similarity between the chromatograms of the hydrophobic fraction in Figure III-15 and the one using gradient program I shown in Figure III-12. These results can be taken as evidence of the neutral and hydrophobic nature of the constituents in this fraction. The scans of the peaks

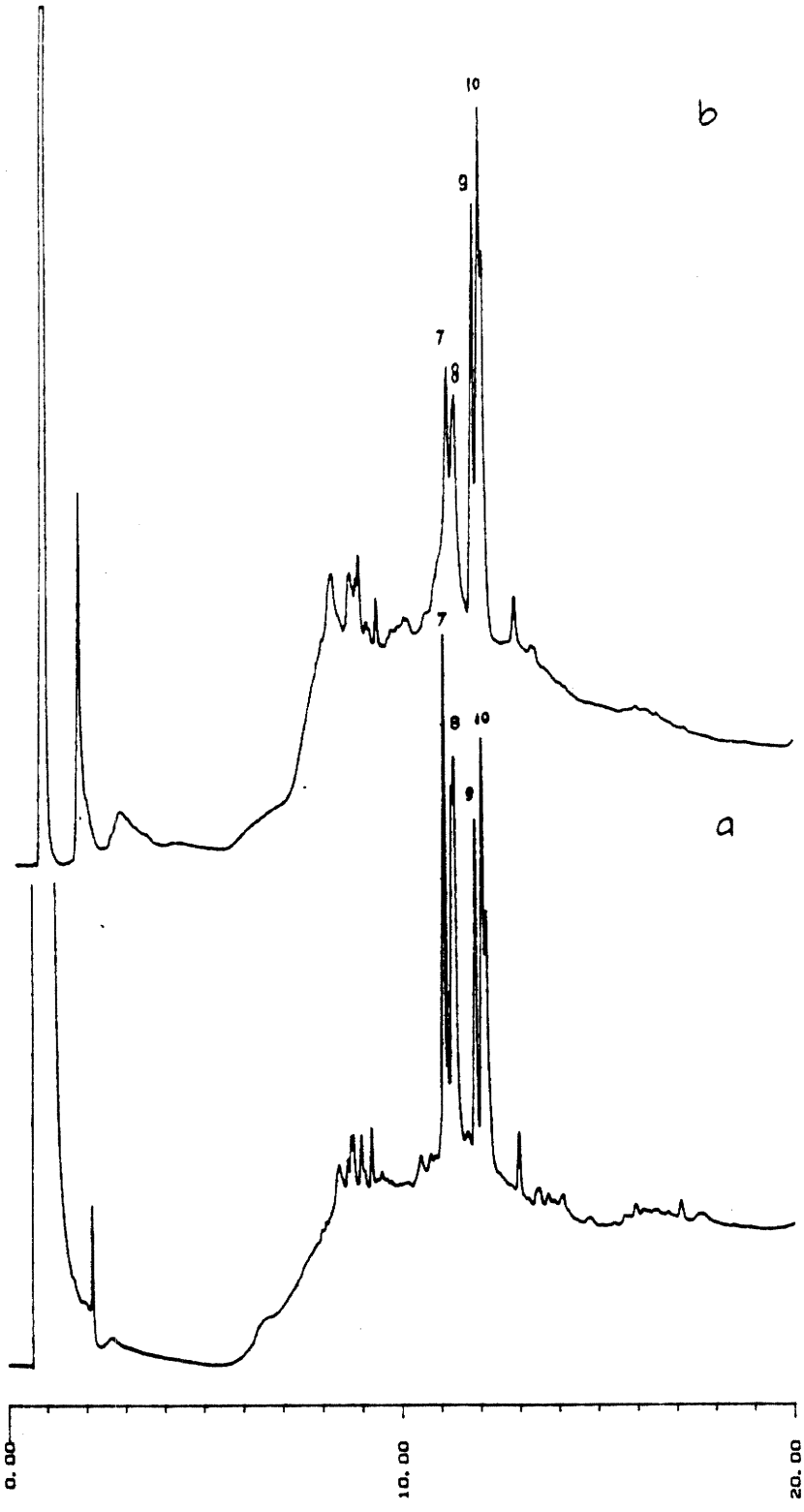


Figure III-13. Chromatogram of B-1 fraction. All conditions are the same as Fig. III-12 except for the sample was stored a) five months b) one year.

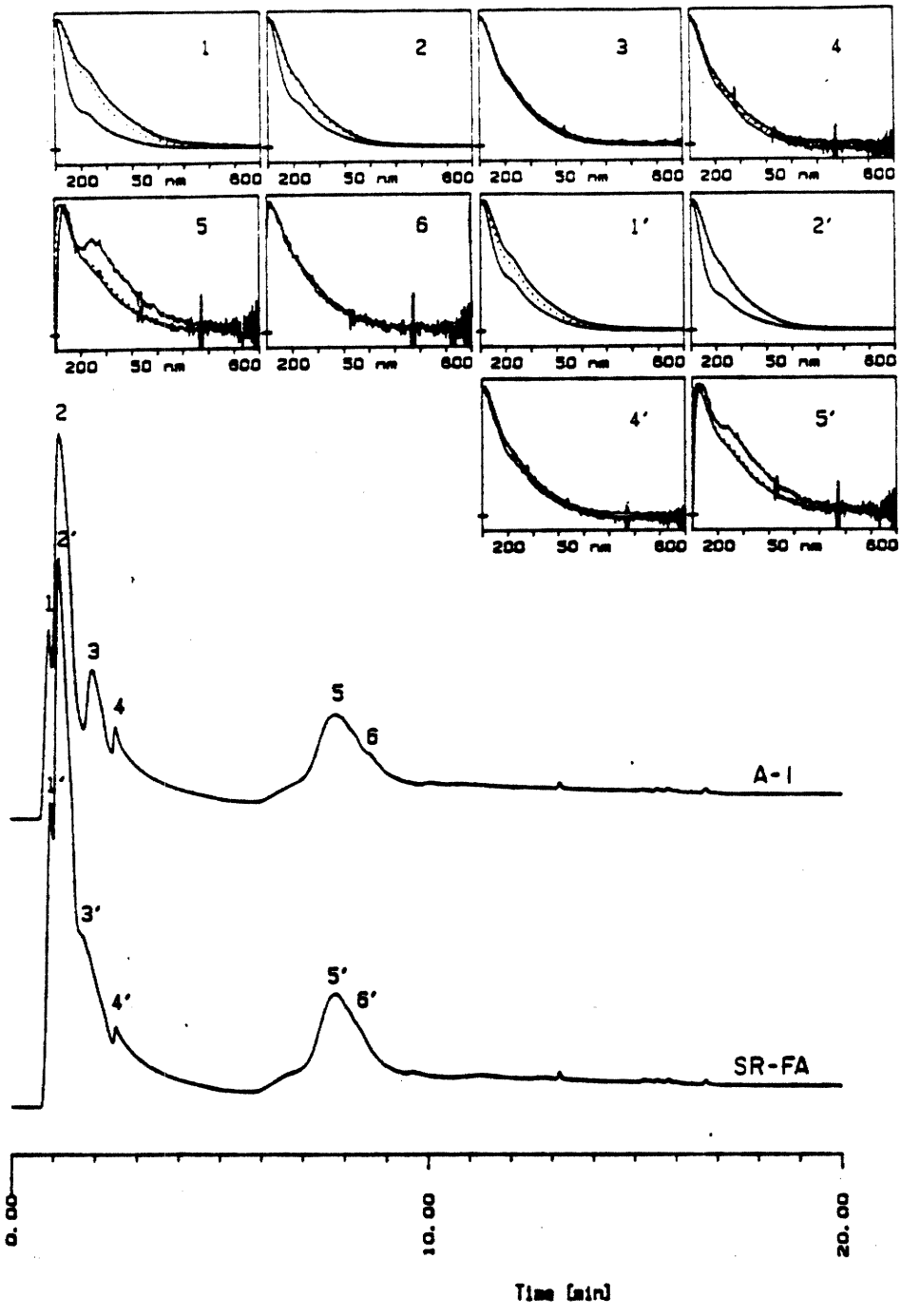


Figure III-14. Chromatogram and UV-vis scans of 50uL 0.02% SR-FA and 50uL 0.02% A-1 fraction dissolved in milliQ water. Gradient program II. Hypersil ODS column.

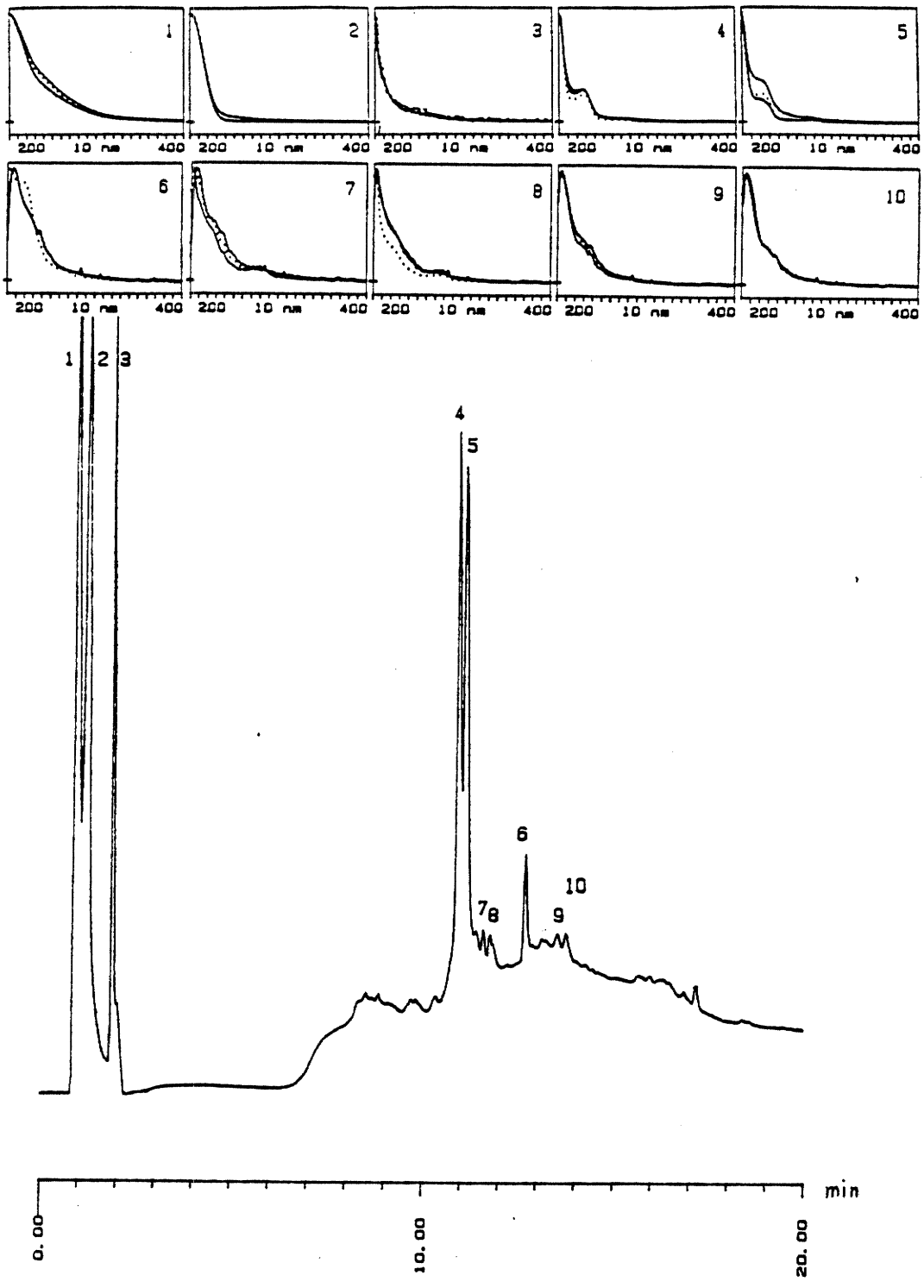


Figure III-15. Chromatogram and UV-vis scans of 20 uL 0.02% hydrophobic fraction B-1 dissolved in methanol: Gradient program II: Hypersil ODS column.

eluted in the hydrophobic fraction are essentially similar to that obtained at pH 4 with gradient program I.

Separation of Twelve Model Compounds

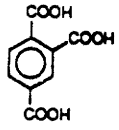
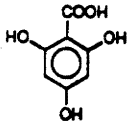
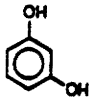
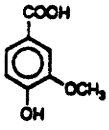
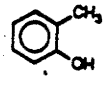
Individual Model Compound Injection

The retention behavior of 12 model compounds using gradient program I and II are shown in Table III-4. The reduced uv-vis spectra of all compounds are shown in Figure III-16. Under both gradient conditions, five acidic compounds were eluted in the hydrophilic region and seven in the hydrophobic one. Compounds eluted in the hydrophobic region included methyl vinyl ketone, resorcinol, vanillic acid, crotonaldehyde, and cresol.

As shown in the table, only the retention times of organic acids were shifted toward shorter retention time at pH 7. The retention times of the phenols were slightly shifted. As expected, the retention times of methyl vinyl ketone and crotonaldehyde did not change with a change of pH from 4 to 7.

The model compounds tricarballylic acid and alanine exhibit featureless spectra. Maleic acid has a maximum absorption at λ 230 nm, the strong aborption extend to 275 nm. 1,2,4-Benzenetricarboxylic acid shows two absorption shoulders at λ 250 and 290 nm. All these compounds were eluted in hydrophilic region. The combination of their absorption spectra is similar to that of FA hydrophilic

Table III-4. List of model compounds structure and retention on Novapak column with gradient program I and II.

compound	structure	t_R min I Novapak	t_R min II Novapak
1. 1,2,4-benzenetricarboxylic acid, pk_1[a] = 2.52, pk_2 = 3.84, pk_3 = 5.20		1.95	1.75
2. alanine, pk_b = 9.87	$CH_3CH(NH_3)COOH$	2.23	2.00
3. maleic acid, pk_1 = 1.91, pk_2 = 6.33	HOOCCH=CHCOOH	2.32	1.70
4. 2,4,6-trihydroxybenzoic acid, pk_1 = 1.68		2.38	1.50
5. tricarballylic acid, pk_1 = 4.42, pk_2 = 5.41	HOCCHCH(COOH)CHCOOH	2.45	2.20
6. methylsuccinic acid, pk_1 = 4.13, pk_2 = 5.62	$HOOCCH_2CH(CH_3)COOH$	6.40	5.71
7. adipic acid, pk_1 = 4.418, pk_2 = 5.412	$HOOC(CH2)_4COOH$	6.68	6.05
8. methyl vinyl ketone	CH_2=CHCOCH$_3$	6.68	6.8
9. resorcinol, pk = 5.2		6.91	6.8
10. vanillic acid, pk = 4.335		7.07	6.2
11. crotonaldehyde	CH_3CH=CHCHO	7.31	7.20
12. o-cresol, pk = 10.26		9.48	9.54

[a] pk in H_2O at 25 °C.

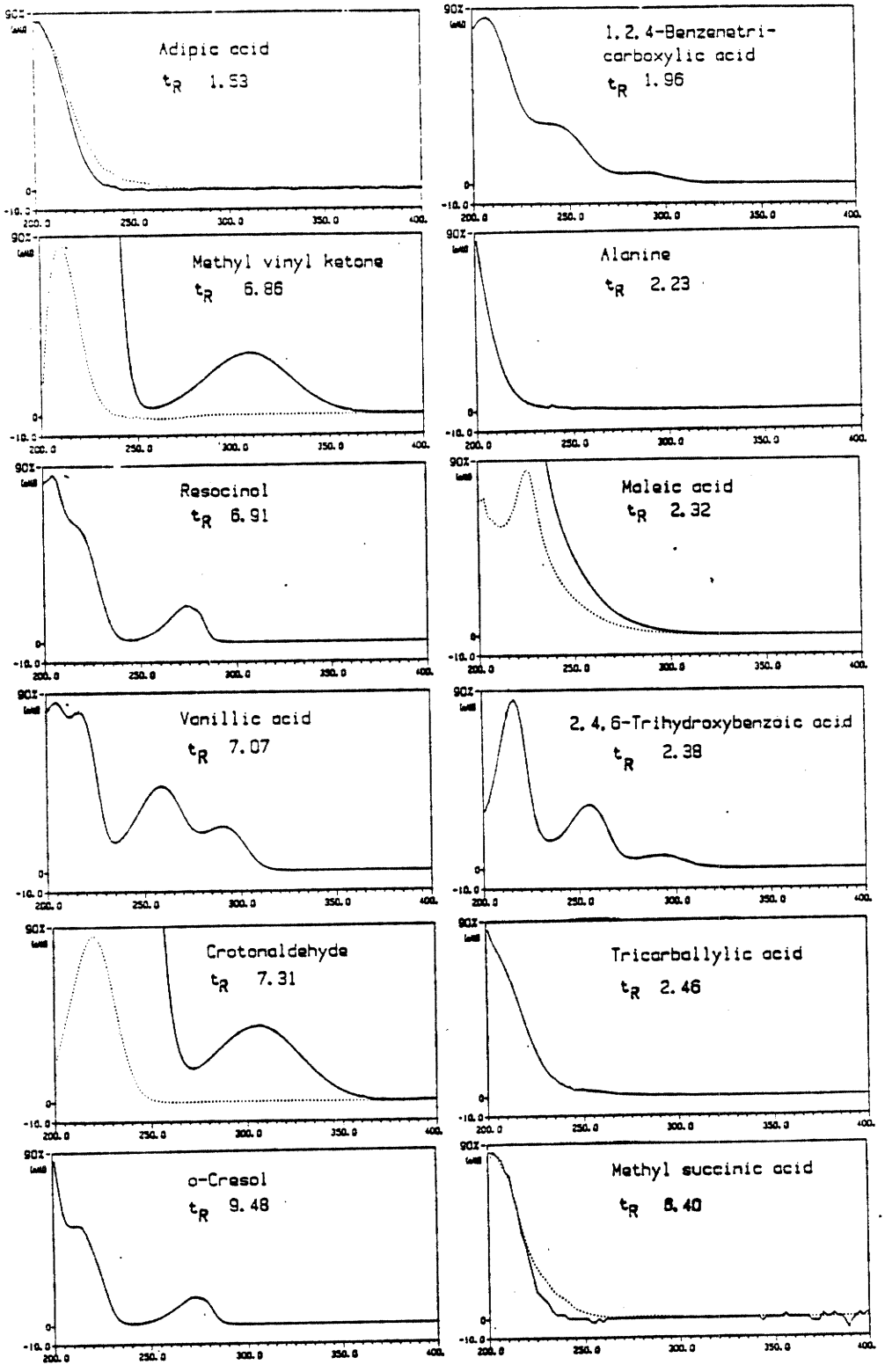


Figure III-16. UV-vis scans of model compounds. Gradient program I. Column Novapak C18.

fraction. Thus, it can be concluded that the hydrophilic fraction of FA contains several aliphatic carboxylic acids and a few aromatic acids. Results from spectroscopic characterization support this conclusion. The retention times and UV-vis spectra of the model compounds crotonaldehyde, resorcinol, and o-cresol were similar to the separated peaks in the hydrophobic fraction.

Mixed Model Compounds Separation

The purpose of this experiment is to demonstrate the separation capability of the system we used. Figure III-17 shows the separation of 12 model compounds in two sets of chromatograms. One set is monitored at 210 nm and the other at 230nm. The compounds in the 210 nm set are free from chromophore structures and are known to have weak photon absorbance even at λ 210 nm. Those in the 230-nm set show strong photon sensitivity even at very low concentration. The retention times of the compounds in the 210 nm set are not consistent with those in Table III-4. This is due to the weak absorption at 210 nm, all the sample concentrations were increased 100-fold compared to those recorded at λ 230 nm. The increased concentration of acidic samples suppressed the pH and increased the ionic strength. Therefore, the retention times of all the samples were increased.

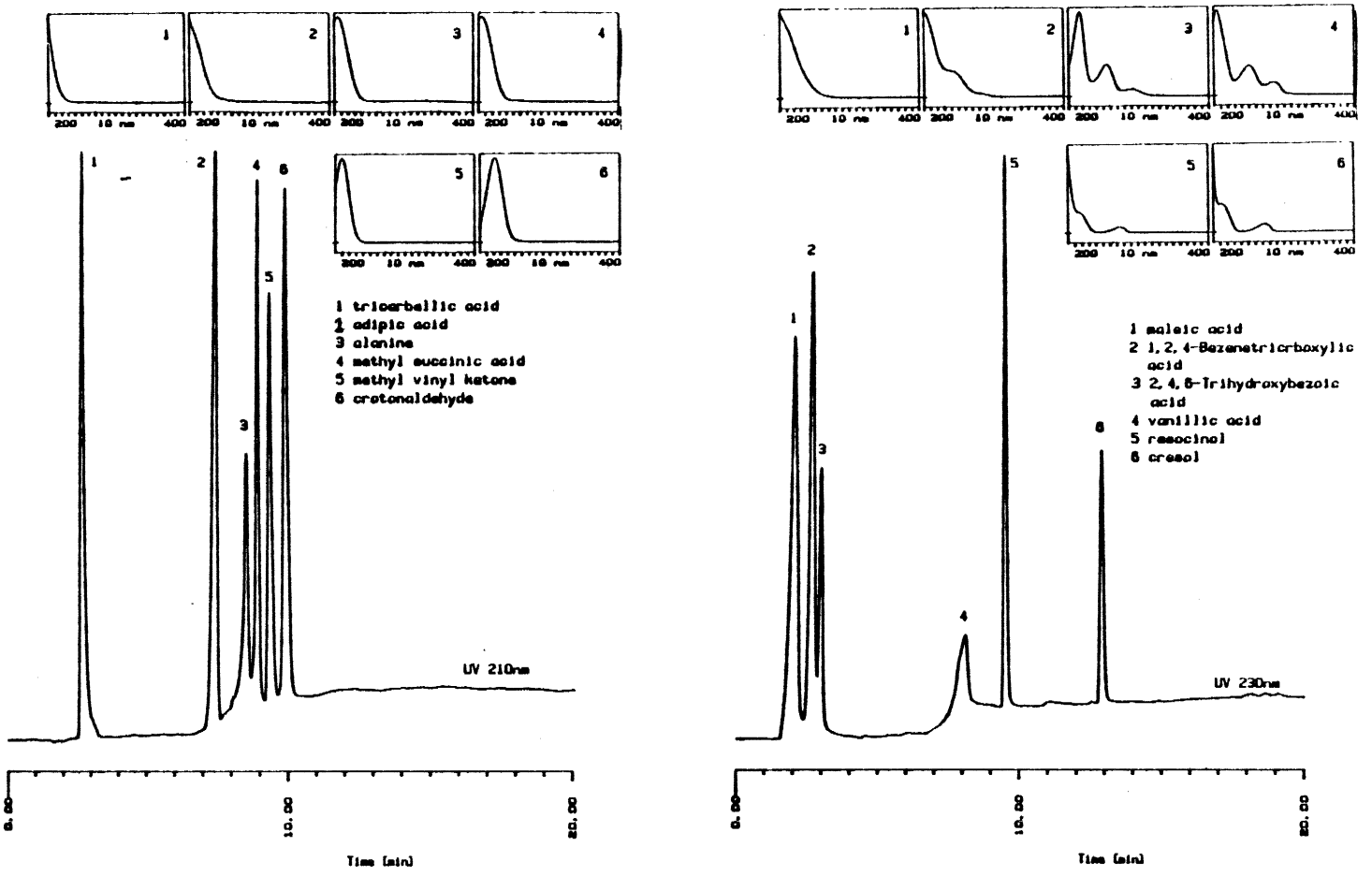


Figure III-17. Chromatograms of 12 model compounds with gradient program I.

Hypersil ODS column 200mm L x 2.1 mm I.D..

III-3. Ion Pairing RP-HPLC of FA

As mentioned before, the retention of strong ionizable eluite will increase under reversed-phase ion-pairing chromatography (RP-IPC). The factors which influence the RPIPC separation include type and size of counterion, pH, concentration of organic modifier and counterion. In this experiment, the solvent program was selected to evaluate the combined effect of pH, and concetration of counterion, and the organic modifiers, on the separation of FA constituents. n-Octylammonium chloride acts as an ion pairing reagent (16). Its effect on ionized solutes are explained as follows. When the mobile phase contains lower organic modifier (A-1 region), the ionic exclusion is the major separation mechanism. The n-octylammonium chloride acts as an electrolyte which may influence the retention of the anions. Large anions are expelled by the eluent more than smaller ones. Then in the mobile phase containing higher organic modifier (B-1 region), the n-octylammonium chloride acts as a surface reactive reagent. At low pH, the retention of the protonated acids are independent of the surface active reagent in the mobile phase. Figure III-18 shows the effect of n-octylammonium chloride concentration on the separation of FA using the Beckman column. According to eq. I-2 (p.30), the retention t_R increases with the concentration of counterion I. Alougth the selectivity is better in 2 mmole mobile phase, the peaks tend to be

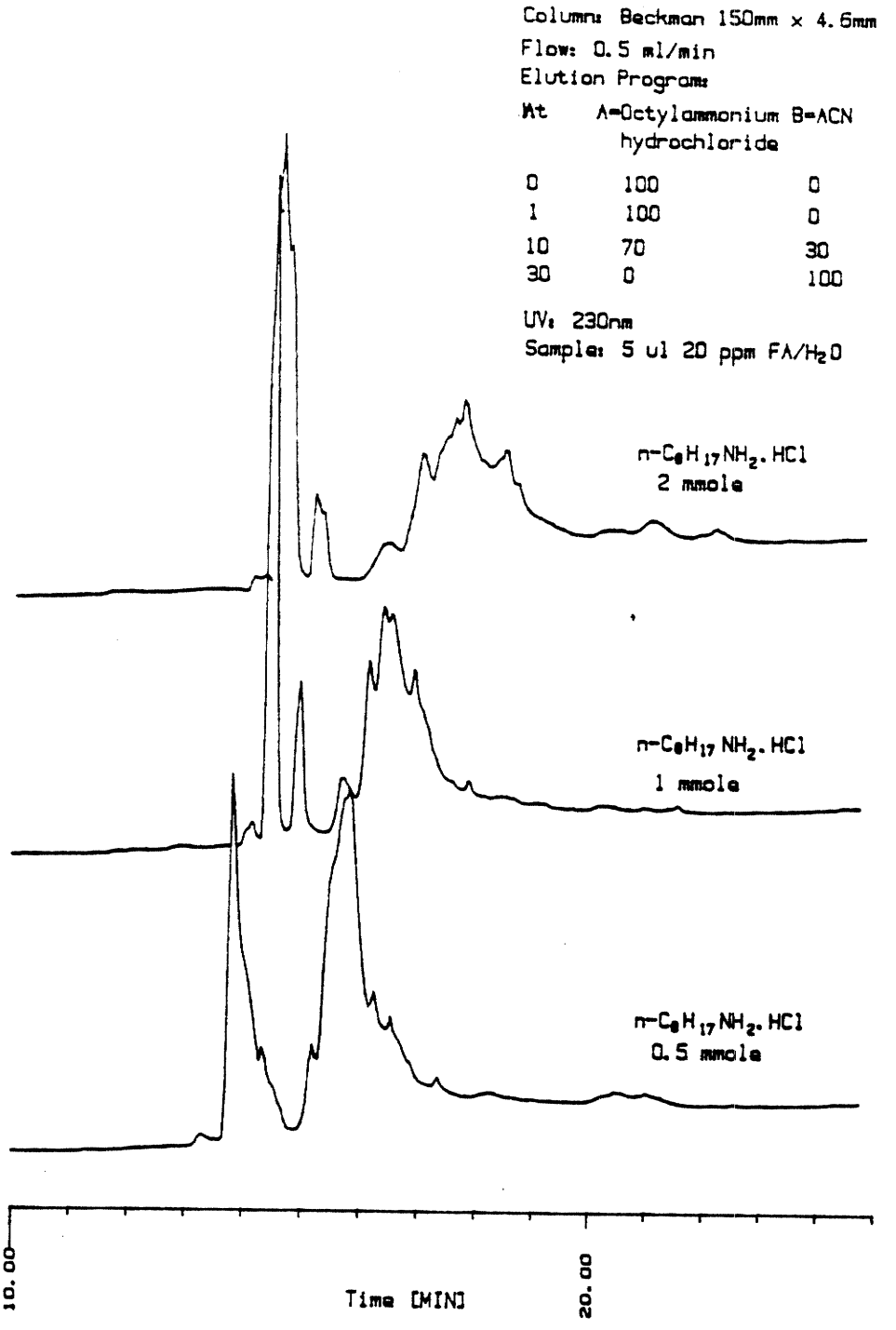


Figure III-18. Chromatograms of FA eluted by different concentration of n-octylammonium chloride mobile phase. Beckman column 150mm L x 4.6 mm I.D. ODS 5um.

flatened. The chromatogram in 0.5 mmole mobile phase does not seem to be well resolved. Since the pH of the mobile phase was 6.8 , most of the solutes were ionized. The insuffient counterions cannot bind the solute anions and thus reduce the selectivity of the eluent. The chromatogram in 1 mmole mobile phase showed moderate separation and well-defined regions with symmetric peaks. The interesting feature of this separation can be seen from either multiple wavelenth chromatogram (Fig. III-19) or three dimensional plot (Fig. III-20). Each chromatogram is divided into two major regions: The two peaks eluting between 14 and 15.5 min show UV absorption similar to that of aliphatic acid model compounds. Below 230 nm, both peaks almost had no absorption. The peaks that eluted between 15 and 17 min showed UV absorption as that of aromatic acid model compounds. This is evident from the continuous absorption in the wavelength region longer than 230 nm. Thus, the peaks in the first region represent the aliphatic constituents in SR-FA, while the second region may contain both aliphatic and aromatic structures. This mobile phase seems suitable for future preparative chromatographic separation of FA. It is recommended that FA first be separated into two major regions, then the collected fractions can be subjected to ion exchange chromatography to isolate the ammonium salts. The spectroscopic properties of both fractions would be distinctly different. Peak 2 which

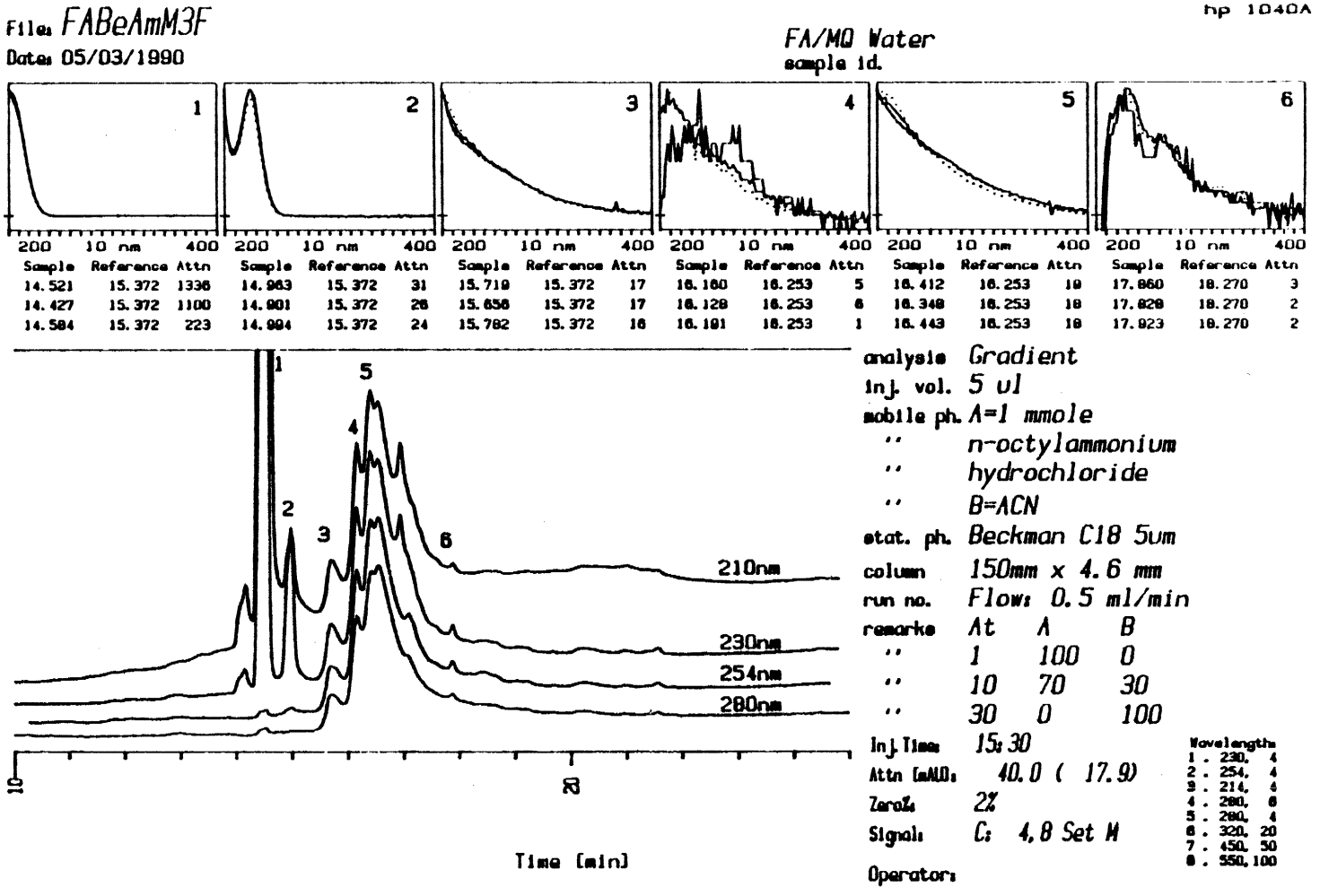


Figure III-19. Chromatograms of FA monitored at different wavelength. Mobil phase: 1 mmole n-octylammonium chloride. Column: Beckman 150 mm x 4.6 mm.

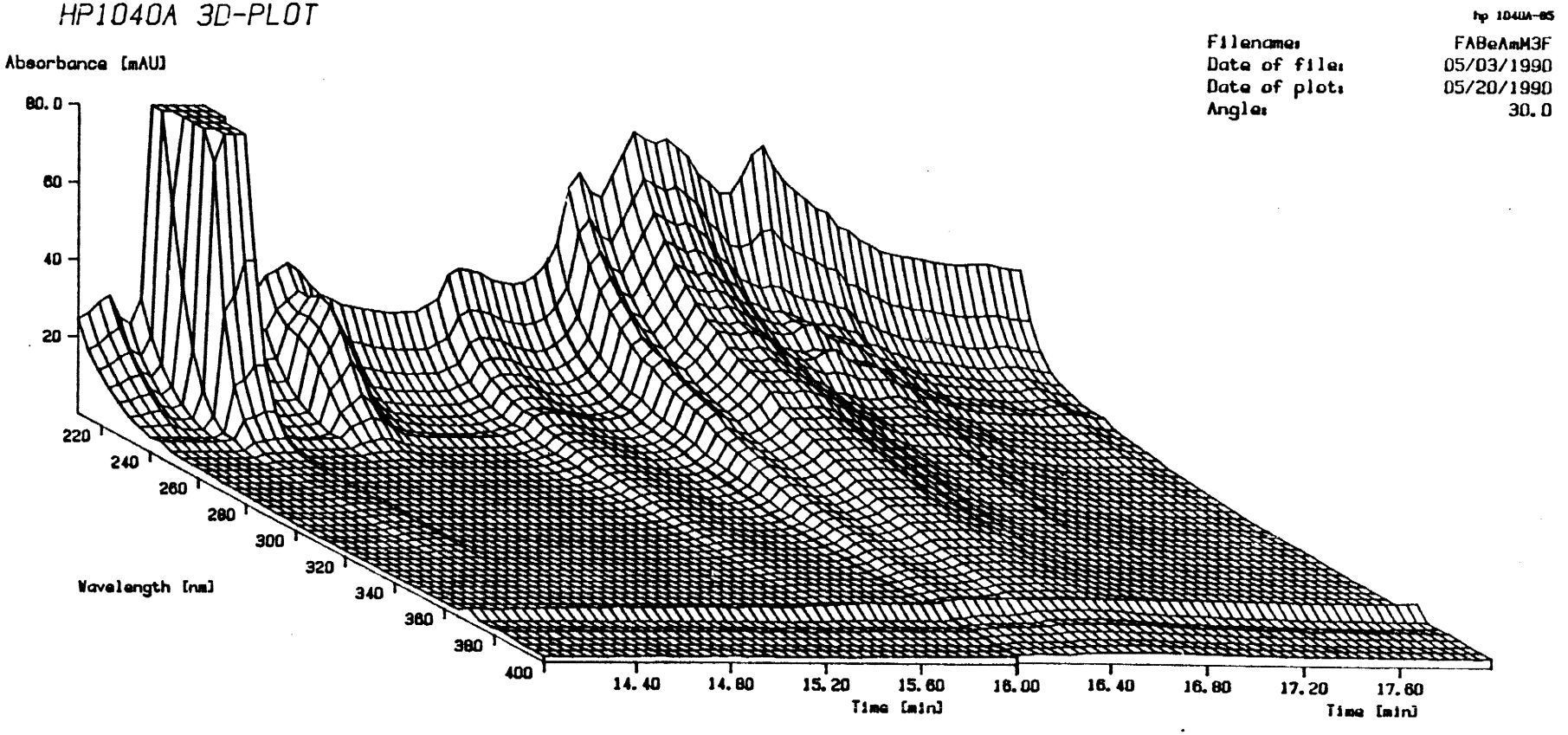


Figure III-20. Three dimensional plot of FA eluted with 1 mmole n-octylammonium chloride. Beckman column 150 mm x 4.6 mm. ODS 5um.

eluted at 15 min showed strong absorption at 225 nm. Its peak purity was checked by overlapping the spectra scanned at different points. The UV spectrum was analogous to conjugated ketone structure detected by solvent program II.

The same sample was separated by Novapak column. The chromatogram (Fig. III-21) is similar to that by Beckman column, but the efficiency of Novapak is lower.

FA-Cu Interaction Experiments

In this experiment, fluorescence quenching (FQ) combined with the chromatographic method were used to study the effect of the interaction between organic ligands of FA and the Cu^{2+} metal ions (17,18) under controlled conditions. A 0.02% buffered solution of SR-FA was divided into four parts. One was used as uncomplexed blank, the other three were added with molar ratios 1:1, 50:1, 100:1 cupric sulfate based on an estimated gfw of FA=1000. Samples were subjected to RP-HPLC on Novapak column using gradient program I. The separation was monitored with the fluorescence detector at λ_{ex} 273 and λ_{em} 390 nm. Only samples that were reacted at molar ratio of 1:1 showed measurable fluorescence quenching. Figure III-22 shows the fluorsecence chromatogram of the uncomplexed and complexed FA. The figure shows that uncomplexed FA is resolved into four major peaks. Complexed FA contained essentially the

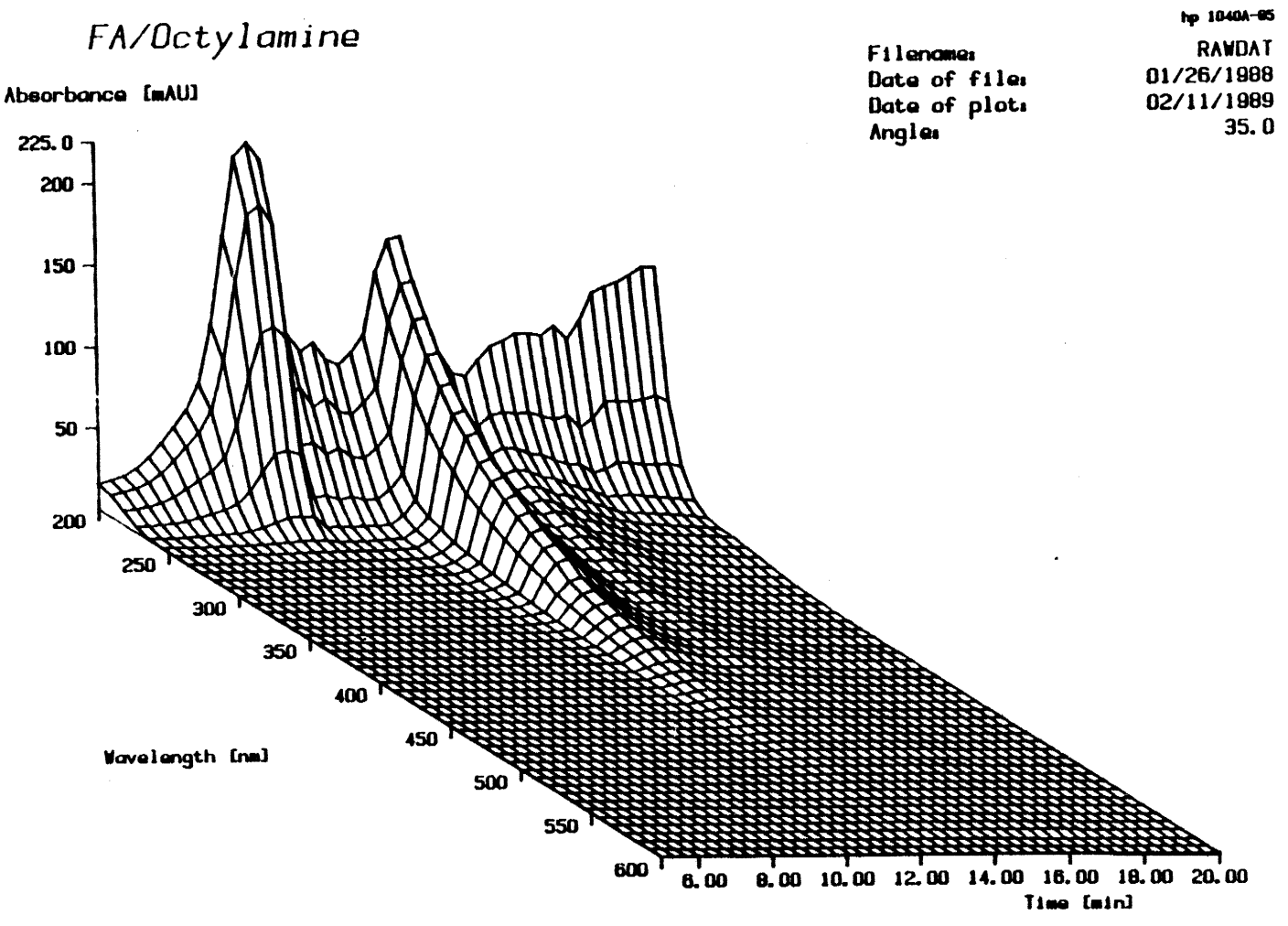


Figure III-21. Three dimensional plot of FA eluted with 1 mmole n-octylammonium chloride. Novapak column: 150 mm x 3.9 mm. ODS 5um.

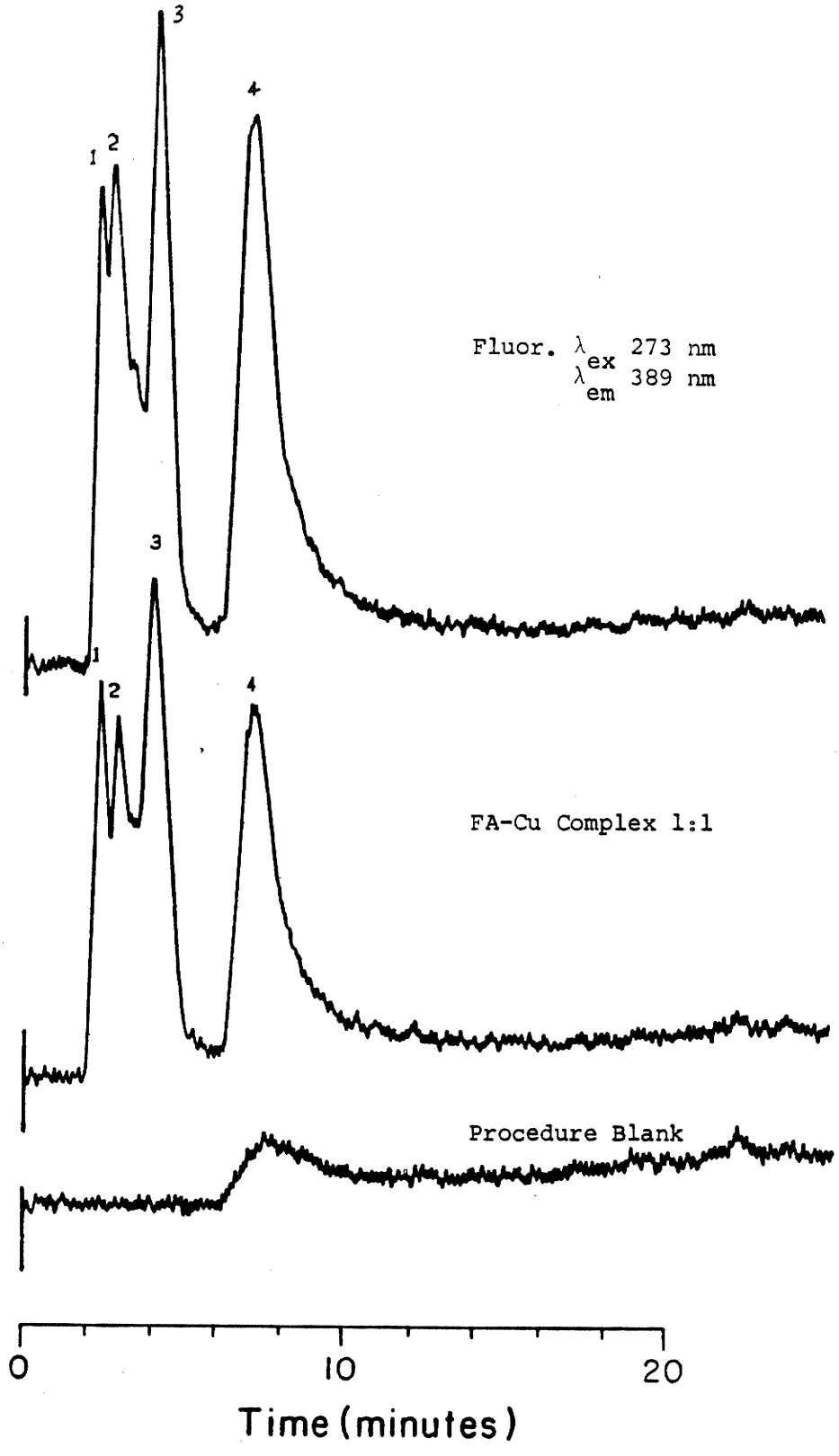


Figure III-22. Fluorescence chromatogram of uncomplexed FA, FA-Cu Complex 1:1 and procedure blank. Column: Novapak. Gradient program I.

same peaks at the same t_R but with variable percent quenching that ranges from 14.46% to 30.43%. Peaks representing the hydrophobic constituents of FA exhibited the highest extent of quenching. If the elution sequence of the peaks are according to the acid dissociation constant of the components, the carboxylic compounds will be eluted earlier than the phenolic compounds. The results suggest that the hydrophobic constituents such as phenolic compounds are better ligands than the hydrophilic ones such as the straight-chain carboxylic acids.

III-4. Chromatography of Derivatized FA

The derivatized FA has been characterized by FT-IR. The dry reference sample and the methylated products were prepared into KBr pellets for FTIR analysis. Figure III-23 shows the spectra of total FA and the CH_2N_2 and CH_3I methylation products. Comparison between the spectra indicated that methylation by CH_2N_2 resulted only in partial decrease of the 3400 cm^{-1} region. The CH_3I treatment has produced an additional decrease in this band; thereby, further methylation of OH groups was accomplished in this step. The minor 3400 cm^{-1} absorption band in the CH_3I spectrum can be attributed to residual H bonding or to the presence of trace amounts of H_2O in the KBr pellets. The 2900 cm^{-1} and 1440 cm^{-1} bands were enhanced by CH_2N_2

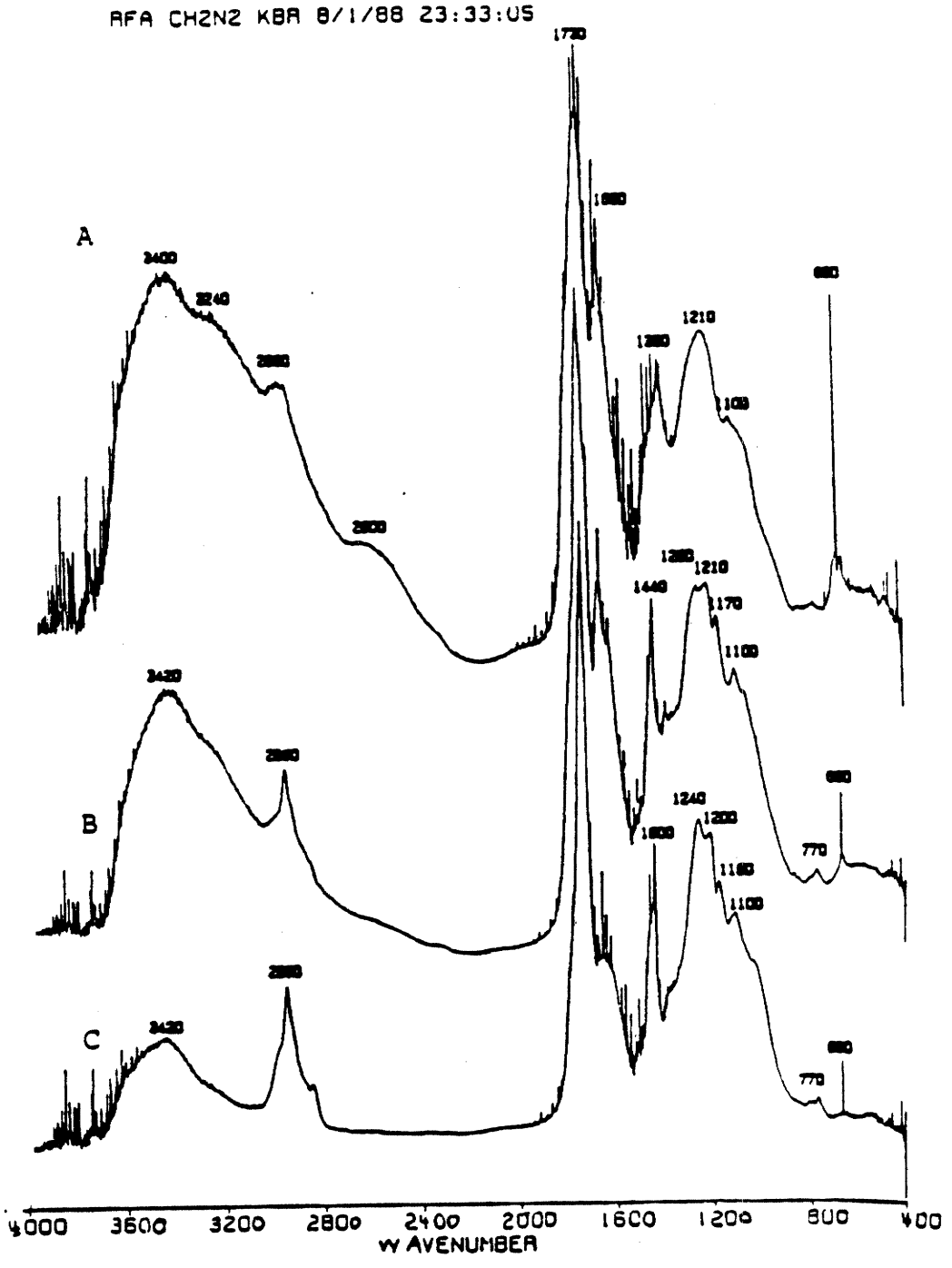
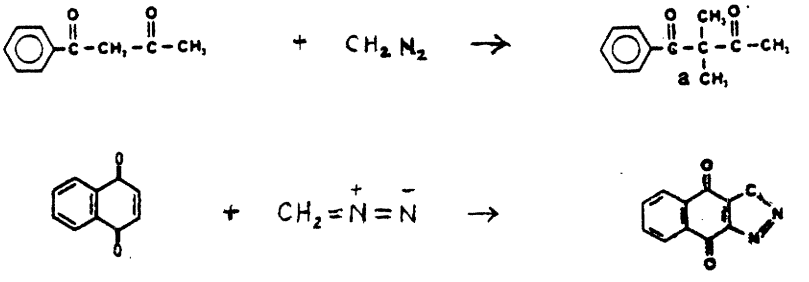


Figure III-23. FT-IR of KBr pellets of A) underivatized FA B) CH_2N_2 methylated product and C) CH_3I methylated product.

methylation and even more enhanced by CH_3I methylation. This is expected due to the increase in C-H stretching and bending vibration. The broad shoulder at 2600cm^{-1} in the FA blank disappeared in the CH_2N_2 spectrum. A shoulder at 2850 cm^{-1} is noted in the CH_3I spectrum due to increase in aliphatic C-H stretching vibrations. The absorption band at 1730 cm^{-1} and 1250 cm^{-1} region are slightly enchanced, The ester structures are more populated. The decrease in the 1620 cm^{-1} band can be attributed to methylation of diketones and 1,3 dipolar addition of quinones (19).



Capillary GC-FID

As discussed in the literature review (see chapter I), most previous GC/MS studies on FA involved a prior chemical reaction to oxidize or hydrolyze the macromolecules. Thus, the results of such studies detected only the fragments of FA. In this experiment, the two-step methylated FA was analyzed by GC-FID without chemical degradation. No significant peaks showed in the first-step methylated chromatogram. This shows that the one-step methylated sample still contains strong hydrogen bonding components;

thus, the volatality is low. Hence, all the methylated samples represented here refer to the CH_3I methylated FA. Chromatograms in Figure III-24 show the poor reproducibility of two methylated FA samples. Those two methylated samples were injected under the same GC operation conditions. In the methylation experiment, it is hard to control the reagents quantitatively in a micro-scale system, especially with very reactive reagents such as diazald and NaH. However, the GC chromatograms show an important result, i.e., both chromatograms show the regular retention pattern characteristic of repeated structural units in FA. The evenly separated intervals of peaks eluted between 24 to 35 min appeared in both chromatograms. These pattern indicate the existence of homologous series or polymeric compounds in the derivatized FA. Four hydrocarbon standards, C16, C18, C20, and C22 were injected under the same conditions, and all appeared before 6 min. However, all the significant peaks of derivatized FA samples showed up after 8 min. The peaks with longer t_R and at higher temperature indicate that they are homologous series with high polar functional groups. This assumption excludes the formation of polymeric products from CH_2N_2 polymerization which, is not shown on the blank chromatogram.

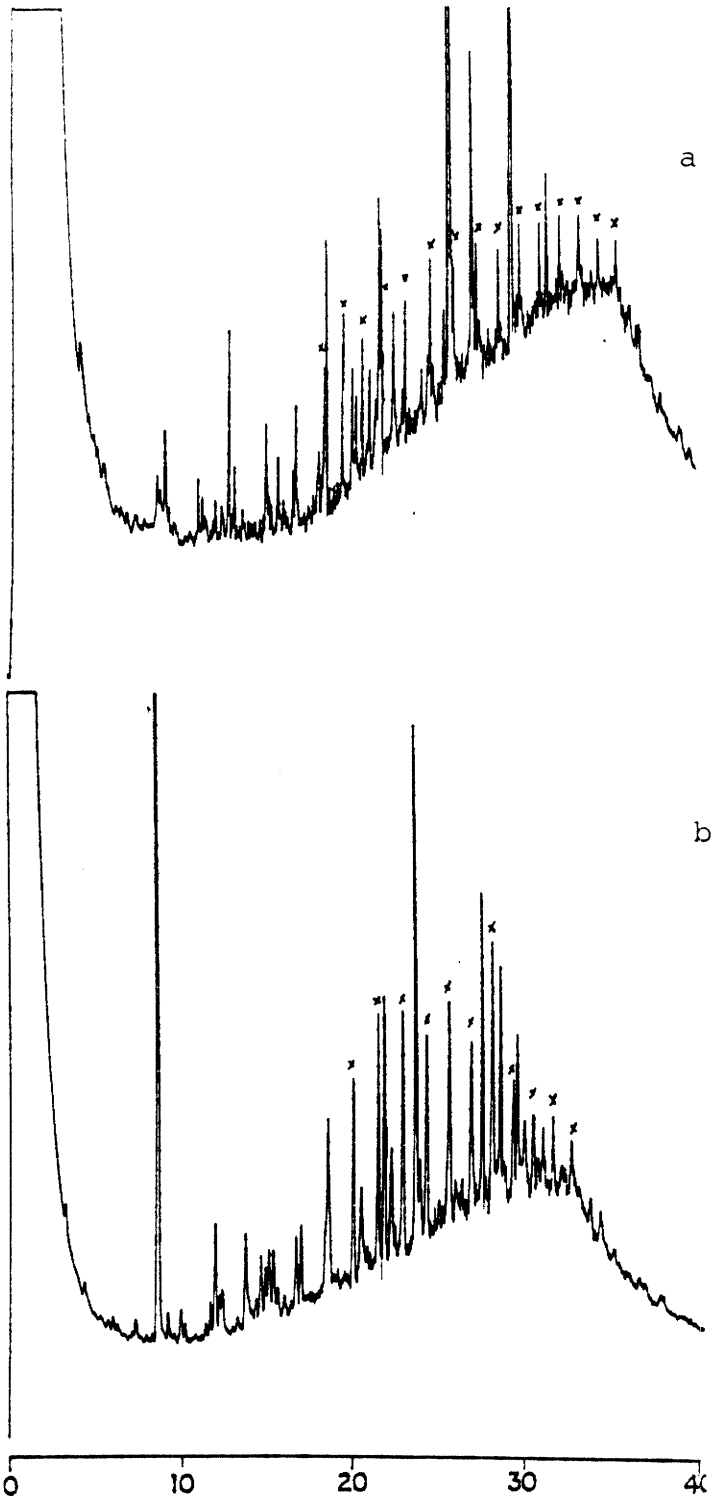


Figure III-24 The GC chromatograms of two methylated samples a and b. Column : SPB-608 Supelco. Temp. Prog.: 40°C 2min, ramp rate 6°C/min to 220°C then hold for 8 min, Flow: He 2 mL/min. Sample: 1 uL 2% solution in CH_2Cl_2.

Column Chromatography

The sample in Figure III-24b was separated by column chromatography into four fractions. The polarity parameter (20) of each eluent are in the order are 0.2 (hexane), 3.1 (methylene chloride), 3.9 (2-propanol), 5.1 (methanol). The recovery of each fraction (in mg) is:

Total FA	Procedure blank	Hexane extr.	CH_2Cl_2 extr.	2-Propanol extr.	Methanol extr.
16.43	0.73 clear	1.05 clear	0.99 light yellow	5.26 dark yellow	6.21 dark yellow
% recovery					
82.22		6.39	6.03	32.01	37.80

The data show that the high polar constituents are eluted with methanol and 2-propanol, and represent 70% of the total. The unrecovered constituents represent 18 % and maybe due to the solute-stationary interaction.

GC-FID of Column Chromatography Fractions

All four fractions were prepared to the same concentration (1%) and were nalyzed with Varian 6000 GC. Figure III-25 shows chromatograms of four extracts chromatographed under the same conditions as Figure III-24. The procedure blank showed no response to FID. Comparing the four chromatograms with the total sample chromatogram, each fraction chromatogram represents a part of that of total sample. The three strong peaks in the total sample appear in all fractions and are marked with circles. According to the "like-dissolves-like" principle, the hexane

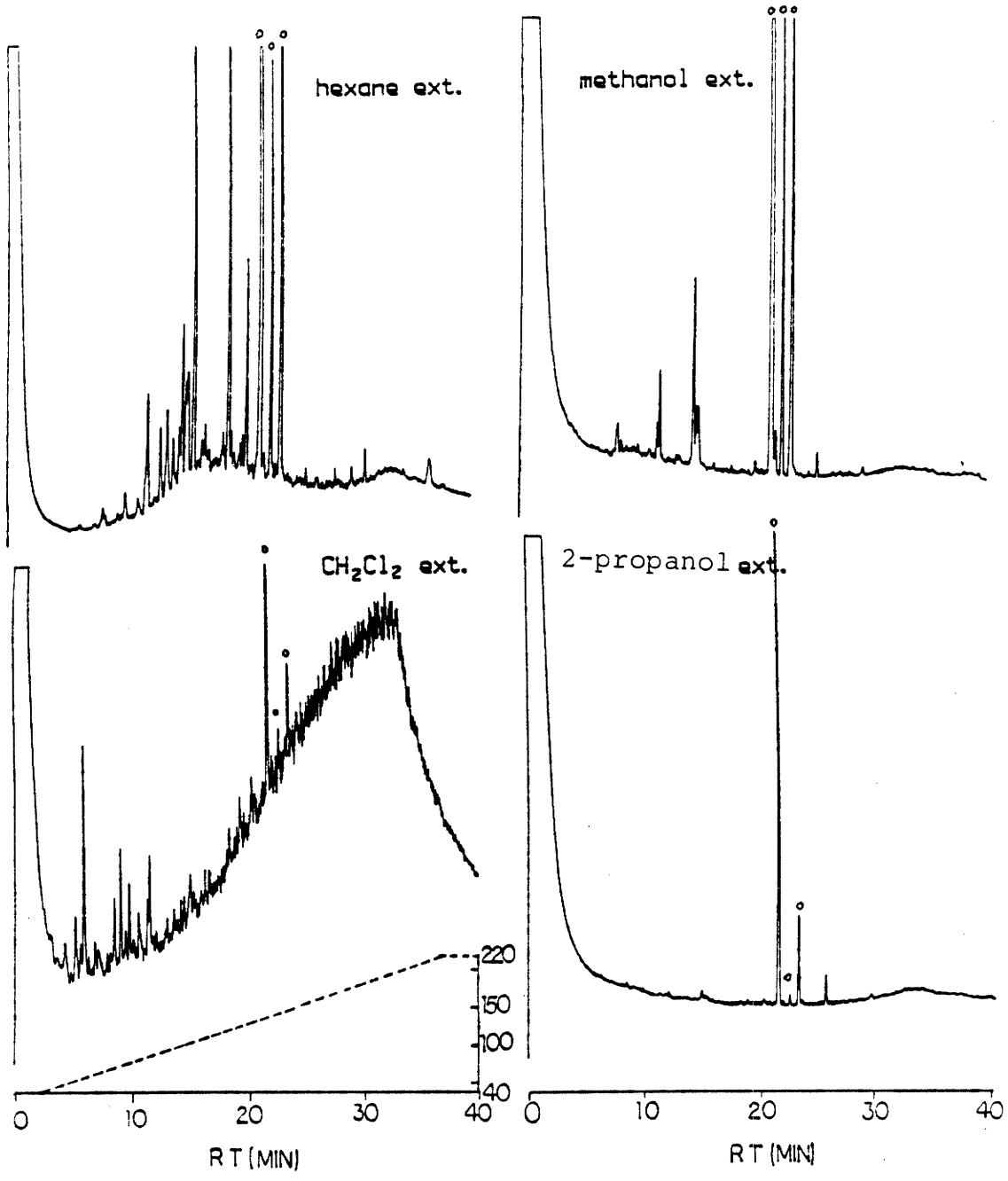


Figure III-25. GC Chromatograms of four extracts. Chromatographic conditions are the same as in Fig. III-24.

extracts represent the higher volatile nonpolar compounds which are easily detected by FID and give better reponse. The chromatogram of CH_2Cl_2 extracts showed the high background and few resolved peaks. No repeated structural units were detected in this chromatogram. The 2-propanol and methanol extracts represent about 70% of the recoveries, and were both dark yellow in color, and the chromatograms consist of several peaks including the circled ones.

Supercritical CO_2 Fluid Extraction

SFE was applied to analysis of methylated FA. Since the SFE mechanism enmploys solvation rather than evaporation, the high molecular weight and polar substances can be easily eluted at high density fluid or high polar mixed fluid. In addition, SFC could be connected to both HPLC and GC detectors, provided that adquate interface is used.

The CH_2Cl_2 fraction was subjected to SF extraction. The GC chromatograms of the SF extracts are shown on Figure III-26. The chromatograms show that components in the CH_2Cl_2 extract were separated at different fluid density. There is only one peak that appears in the low density fluid chromatogram. In the high density state, more components were extracted due to the higher solvational capability of the fluid. The regular retention pattern resurged in the high density chromatogram.

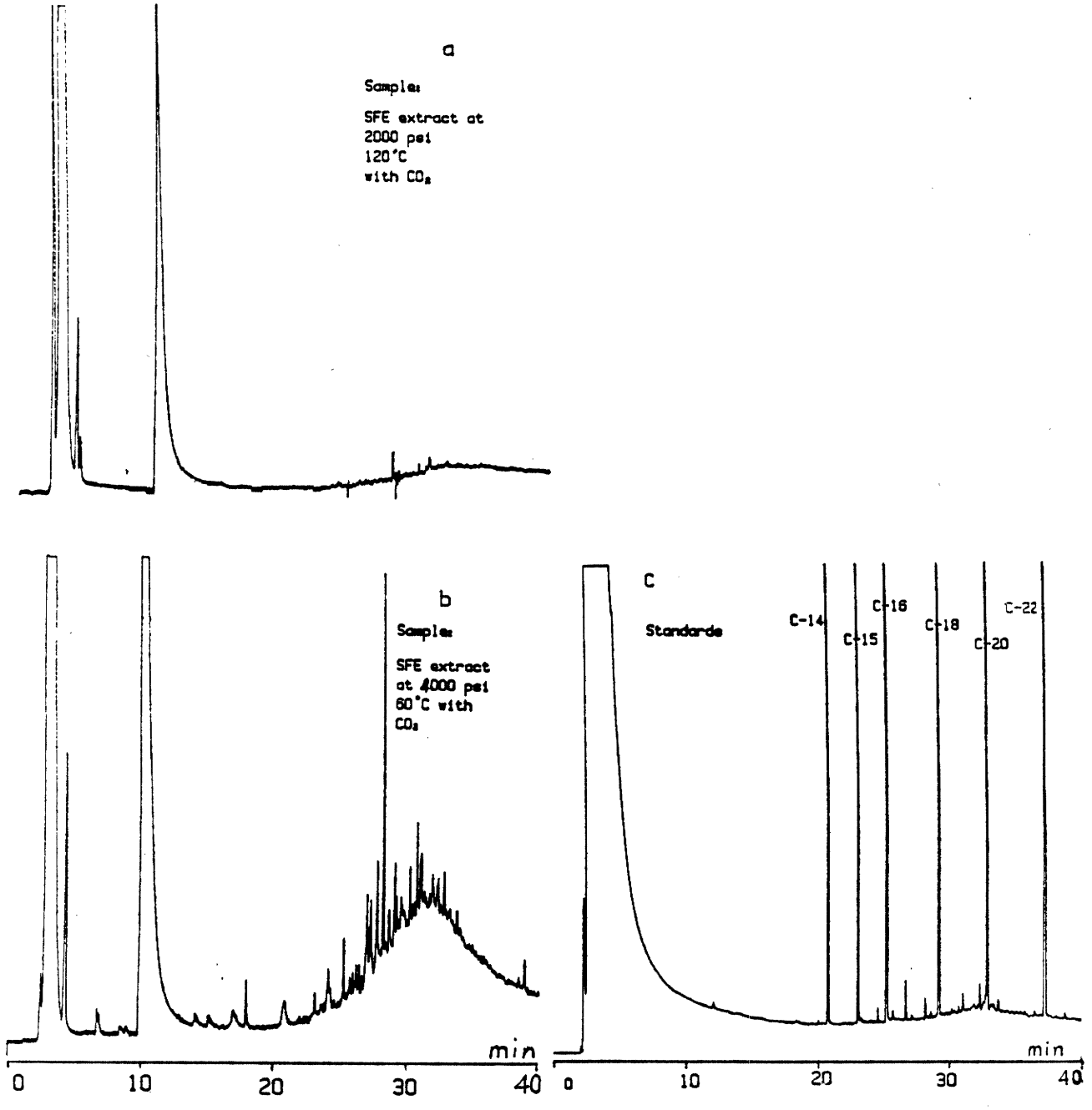


Figure III-26. The GC chromatograms of SF extracts from CH_2Cl_2 column fraction. RSL-300 BP 60m x 0.25mm 0.5um. Flow rate of He: 3 mL/min, Temperature program : 40°C 2min, ramp rate 6°C/min to 220°C then hold for 8 minutes.

NP- and RP-HPLC of Column Chromatography Fractions

Both reversed (RP) and normal phase (NP) HPLC were utilized to analyze the fractions of methylated sample. Figure III-27 shows the chromatograms of fractions in NP-HPLC. All the chromatogram appearances are very simple. The column used is a C18 stationary with only 5% carbon loading, and it does not represent a typical absorption column. The elution mechanism in this type of column is complicated, and the elution order at which the components are eluted cannot be predicted as is the case with adsorption chromatography. All the samples are eluted in 100% CH_2Cl_2 as mobile phase. Methanol and CH_2Cl_2 extracts show the similar profiles. The peaks extend from 16 to 23 min, 2-propanol extract from 18 to 23 min, hexane extract from 20 to 23 min. Generally, the separation of this mode is not useful. Figure III-28 shows the chromatograms of fractions in RP-HPLC mode. Since the column is a typical RP column, the more polar components eluted earlier. This fact can be seen in the elution region between 10 to 20 min. There are two regions in all chromatograms. The t_R of first peak is 13 min, the second one is 17.5. In methanol extract, most components are eluted in the first region, less in second region. For hexane extract, the elution order is reversed.

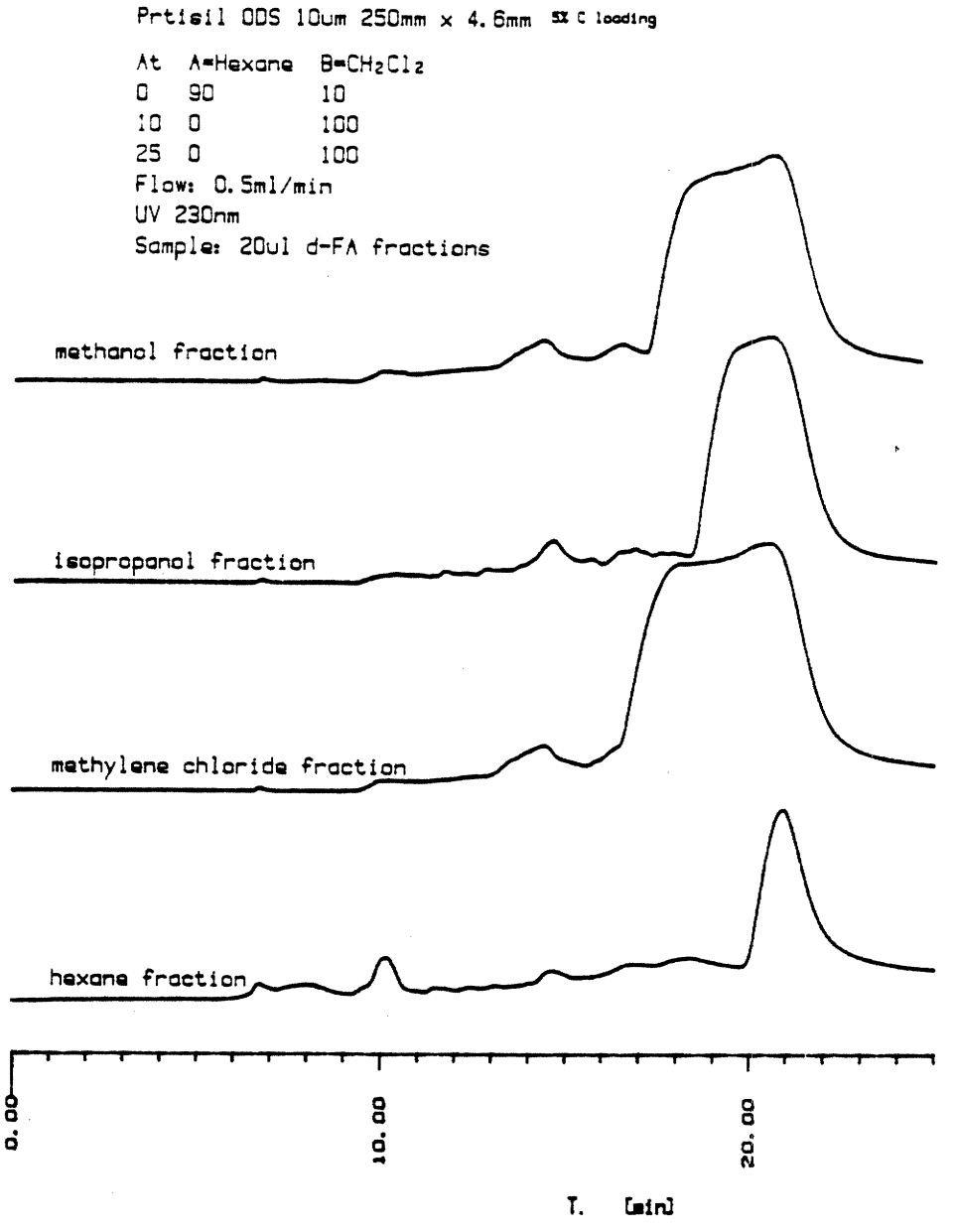


Figure III-27. Chromatograms of four extracts by NP-HPLC.

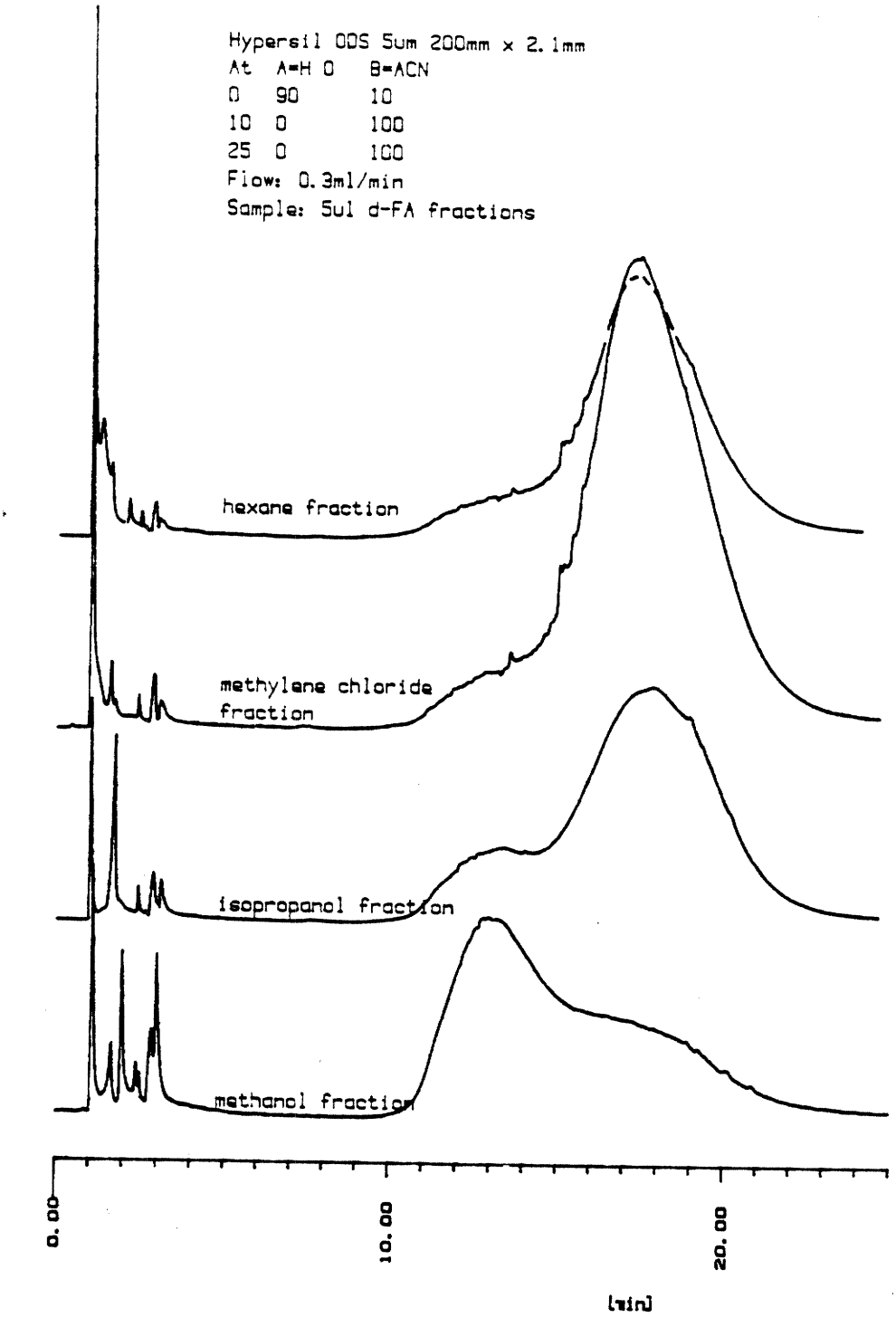


Figure III-28. Chromatograms of four extracts by RP-HPLC.

Pyrolysis Capillary GC of FA and Fractions from Prep-HPLC

The effects of pyrolysis temperature, heating period, heating rate on FA were studied in the same lab previously (21). Four standards were injected in this experiment to compare their production changes at different pyrolysis temperature. Figure III-29 shows the pyrograms of total FA and fractions at 500°C and Figure III-30 at 800°C. The HPLC and spectroscopic study in early sections showed the structural differences among the total FA, A-1, and B-1 fractions, but the pyrograms of the three samples at 800°C are silmilar to each other. It is interesting to note that three chromatograms showed essentially the same fragments. The total carbon combusted in this experiment was checked with the organic carbon analyzer. There was 26±3% carbon in FA pyrolyzed at 800°C. The functional group carbon represented 12±3%.

At 500°C (Fig. III-30), the FID chromatograms of all samples are weaker than those at 800°C. Since the molecular weight of the samples are still high, at this temperature the degradation of high molecular weight compounds could include elimination of functional groups and degradation of aliphatic molecules. The aromatic 'nuclei' is just starting to rupture (22). The benzene peak in the FA pyrogram showed moderate response at 500°C. The other standard peaks, phenol, toluene, o-cresol, are less than those peaks in 800°C pyrograms. All target peaks are present in both

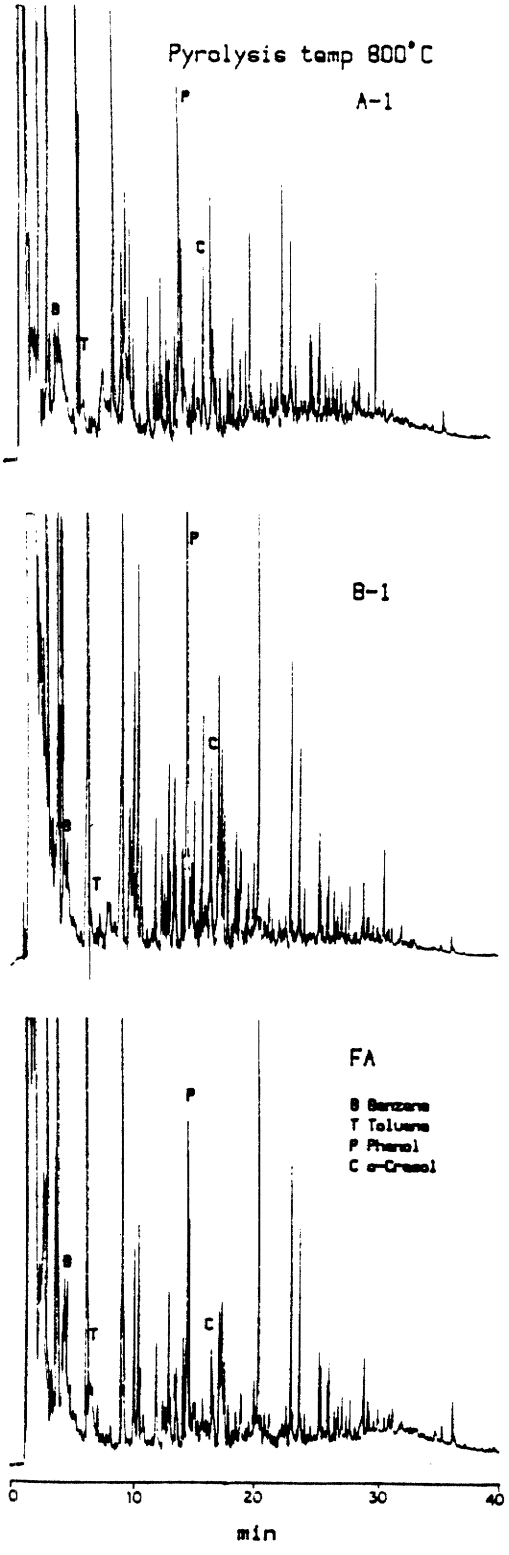


Figure III-29. Pyrograms of FA and fractions pyrolyzed at 800°C and 500°C. GC conditions as Fig. III-26.

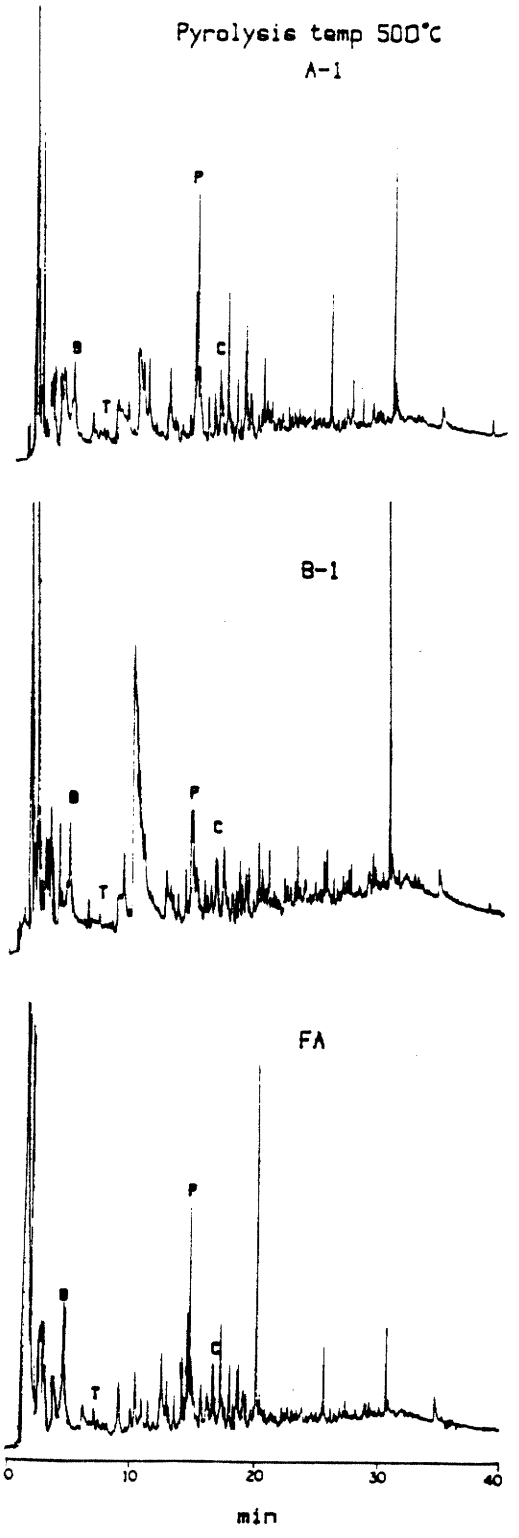


Figure III-30. Pyrograms of FA and fractions at 500°C. Conditions same as Fig. III-29.

fraction A-1 and B-1 pyrograms at 500°C. Pyrogram of B-1 at 500°C shows detectable regular retention pattern characteristic of homologous or polymeric series of compounds. The pyrogram is also distinguishable from that of A-1 at 500°C.

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CHAPTER IV

CONCLUSIONS

The overall purpose of this research project was to develop better understanding of the composition and structural features of aquatic fulvic acids. The approach was i) to utilize an initially purified sample (Suwannee River FA), ii) to carefully evaluate and apply RP-HPLC separation, and iii) to utilize independent spectrometric methods to characterize the total sample and separated fractions.

Through careful selection and evaluation of the separation and characterization methods, two important conclusions can be made regarding aquatic FA. For the first time, the feasibility of separation of constituents of FA at the molecular level by using nondegradative method has been demonstrated. This was illustrated by the use of step-wise preparative and analytical RP-HPLC. Peaks representing vanillic acids and phenols were easily identifiable. Other peaks were less identifiable but represented pure compounds. Second, the presence of repeated structural units in FA was illustrated by more than one technique: it was detected in the B-1 analytical RP-HPLC chromatogram. Later, it was detected in the pyrogram of the B-1 fraction. Finally, it was noted in the GC-FID of the SF extract from methylated

FA.

The following results were achieved:

1. A recovery of 70% was obtained from the preparative RP-HPLC experiments. This is excellent recovery compared with degradative methods which do not exceed 25%.
2. The hydrophilic fraction constitutes 40% of the total FA and can be resolved into five peaks. The UV-vis absorption of these peaks were featureless and similar to those of FA.
3. The hydrophobic fraction of FA represents ca 30% of the total sample and can be resolved into 12 peaks by analytical RP-HPLC. The UV-vis absorption of 4 peaks were similar to those of phenolic compounds.
4. The vanillic structure was detected only in the analytical separation of total FA. This fact indicates that vanillic acid is an intermediate structure in FA.
5. From the solid-state NMR and FTIR spectra, there is a surplus quantity of aliphatic structure and carboxylic groups in the hydrophilic fraction exceeding those in the hydrophobic fraction.
6. Ion-paired HPLC showed partial resolution of aliphatic components of FA. The technique is promising for future structure identification or property testing.
7. Column chromatography of methylated FA allowed the

separation of four fractions based on their solvent extraction polarity. Fractions were characterized by GC-FID, Py-GC-FID, NP-HPLC and RP-HPLC.

CHAPTER V

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