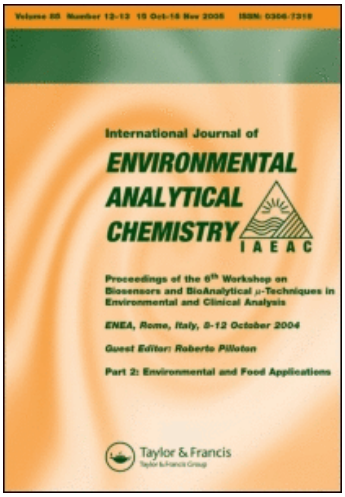

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Step-Wise Fractionation and Recovery of Aquatic Fulvic Acid by Modified Supercritical Fluid CO_2 - Methanol Extraction at Near Critical Temperature

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STEP-WISE FRACTIONATION AND RECOVERY OF AQUATIC FULVIC ACID BY MODIFIED SUPERCRITICAL FLUID CO_2- METHANOL EXTRACTION AT NEAR CRITICAL TEMPERATURE

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Modified supercritical fluid "SF" CO_2 -methanol mixture was used, under step-wise gradient conditions at near supercritical temperature, to fractionate Suwannee River reference fulvic acids (FA) into three fractions. The method was developed after a systematic study of the solubility of FA in SF CO_2-methanol solvent system. Optimum supercritical fluid extraction (SFE) conditions were established at constant temperature of 70 °C and constant pressure of 2,500 psi ; using modified CO_2 fluid mixed with methanol at 18, 24 and 100 percent, respectively. The dynamic extraction conditions were designed to achieve marcketable differences in the fluid solvent power. Fractions were characterized by total uv absorption and fluorescence emission. Under optimum conditions, fractions were collected and characterized by uv absorbance ratio at λ 400/λ 254 nm using non-column HPLC with uv PDA detector. The average total mass recovery of all three fractions was 102 % and coefficient of variation of 6.8%. The first fraction represented 21.5 % of total FA and exhibited absorbance ratio of 0.11. The second fraction represented 55 % of total FA and exhibited almost twice the absorbance ratio. The third fraction represented 25 % of the total FA and exhibited absorbance ratio of 0.30. Total sample and fractions were analyzed using C-18 RP-HPLC with uv-vis PDA and fluorescence detection. RP-HPLC chromatograms of the SFE fractions showed the resolution of the same number of peaks as the total sample, but with different uv absorption and fluorescence emission intensities. The overall results provide some insight on the structural features of FA.

Keywords: Supercritical fluid extraction; aquatic fulvic acids; fractionation of natural organic matter; reversed phase liquid chromatography; UV photodiode array & fluorescence detection

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INTRODUCTION

Supercritical fluid extraction (SFE) is a method applied to both process and analytical applications, in which a supercritical fluid such as CO_2 is used as extraction solvent. Dissolution of the solute into the fluid is the basis of SFE. The unique feature of supercritical fluid as a solvent is that the solvent power is directly related to density , which can be varied as a function of pressure and temperature. In the vicinity of the critical point, large density changes can be produced with either relatively small pressure or temperature changes[1–4]. Dissolution of the solute into the fluid is the basis of SFE. The solubility parameter theory provides the theoretical approach to obtain information on the solubility of a solute in a solvent[5–7]. An expanded approach was developed to calculate the conditions needed to achieve maximum solubility of solutes in SF's[8,9]. The solubility parameter of a fluid (δ_f) is defined as the square root of the cohesive energy density which is the energy content (E, cal/mol) of the fluid per unit molar volume (v, cm^3/mol) relative to its ideal gas state. The solubility parameter of a a pure liquid solute or solvent is a measure of the intermolecular interactions between the compound's molecules. As in the case of liquids when the solubility parameter of a fluid and a solute are about the same, sufficient solubility of the solute in the fluid can be expected. The solvating power of SF's can vary dramatically from that of gas-like to that of liquid-like by changing the pressure and /or the temperature. This character makes selective SF extraction and fractionation possible.

Table I lists typical characteristics of some SF's and liquid solvents, as well as the estimated δ_1 for dissolved HA. For extracting polar solutes modifiers are added to the primary SF to increase the dissolving power of the solvent. The mixture critical constants can be approximated as the arithmetic mean of the critical temperature and pressure of the two components as follows:

$$T_{c(mixture)} = X_{CO2} T_{c(CO2)} + X_m T_{c(m)} \quad (1)$$

$$P_{c(mixture)} = X_{CO2} P_{c(CO2)} + X_m P_{c(m)} \quad (2)$$

Where X_{co2} and X_m are the mole fractions of CO_2 and the modifier, respectively.

Mixtures of methanol and CO_2 are frequently used. These two solvents are completely miscible at all compositions below the critical point. More elaborate treatment for calculating T_c and P_c of mixtures have been published[10–11]. These data may serve as the guide to select operation temperature.

Application of SFE techniques in environmental analysis is relatively a recent development, but is rapidly gaining interest because of the unique features of the solvent. Strategies for methods development typically considered the solvent

power of the extractant and the properties of the solutes of interest. However several recent studies have shown that environmental matrix components have the potential to aid or hinder the extraction of solute of interest due to the solutes interaction with the matrix components[12,13]. Natural organic matter (NOM) represent most of the organic matrix components in aquatic and terrestrial systems, and is generally referred to as humic acids (HA).

TABLE I Characteristics[a] of Selected Solvents in Supercritical Fluid and Liquid Phases

Supercritical Fluid	$\delta_f (cal^{1/2})$	T_c °C	P_c atm.	*Liquid Solvent*	$\delta_l (cal^{1/2})$
Carbon dioxide	7.5	.31.3	72.9	n-Pentane	7.1
Acetonitrile	6.3	275	47.7	Acetonitrile	11.8
Methanol	8.9	240.5	78.9	Methanol	12.9
2- Propanol	7.4	235.3	47.0	Water	21.0
Water	13.5	374.4	226.8	Dissolved HA	10.3–11.5

(a) From references 1,2 and 19.

Fulvic acid (FA) represents the acid and base soluble fraction of aquatic HA. FA has been subject to extensive characterization in the past decade[14–19]. Its structure includes acidic, basic and uncharged functional groups connected by aromatic and aliphatic carbon linkages. The exact nature and molecular weight of FA have not been unambiguously determined. FA is reported to exhibit extensive molecular weight distribution as idicated by its degree of polydispersity ($M_n/M_w = 2.36$)[18]. Structural models, with several functional groups have been proposed. Fundamental studies on thermodynamic properties or the polarity and/or δ of FA are rare. Data on δ for FA is estimated[20,21] between 10.3 and 11.5 $(cal/cm^3)^{1/2}$. Based on recent studies by differential scanning calorimetry, FA is described as rubbery lightly cross linked polymers which exhibit relative ease of molecular motion[19]. It is also known that FA dissolves in polar solvents such as water methanol and acetonitrile but not in the less polar or non-polar solvents such as methylene chloride and n-hexane. Several fractionation and separation methods have been applied to FA with variable degrees of success[22–24]. The reversed phase chromatograms (RP-HPLC) of aquatic FA are known to be dependant on the mobile phase composition, pH as well as the solvent used to dissolve the sample. Because of variation in the charge and functional group characteristics in different solvents, the polymeric structure of FA exhibit both hydrophilic and hydrphobic components.

The SFE of higher molecular weight oligomers is predominantly determined by their selective solvation in the fluid at the defined densities. Separation is initiated by their differential solvation in the mobile phase[25–26]. Selective solvation of the oligomers is the controlling factor especially for higher molecular weight compounds. Interaction between the mobile phase and the oligomers are of primary importance in polymer extraction.

This paper presents the results of a study conducted to utilize the solvent power of modified SF CO_2 for quantitative fractionation and recovery of reference FA. The approach in this research was to systematically optimize the fractionation based on the extractant solvent power. Total sample and fractions were then subjected to RP-HPLC chromatography.

EXPERIMENTAL

Material and reagents

Suwannee River Fulvic Acid (SR-FA) was used as the sample in this research. The SR-FA was purchased from the IHSS[27].

Reagents

SFC grade CO_2 with He head space was supplied by Scott Specialty gases. Dimethyl dichlorosilane treated glass wool was supplied by Altech Associates. HPLC grade methanol (CAS 67–56–1), acetonitrile (75-05-8), 1,2,4-benzenecarboxylic acid (528-44-9), vanillic acid (121-34-6), and o-cresol (95-48-7) were purchased from Aldrich Chemical Company. The organic free water (Milli-Q) was produced from milli-Q-system.

Instrumentation

The SFE system included; i) Brownlee Microgradient solvent delivery pump connected to HP 5890 A GC oven; ii) The extraction cell was made of a 5 cm L × 2.1 mm ID stainless steel HPLC column; iii) Restrictors were (Dionex), and were made of deactivated fused silica capillaries of 25 μm ID × 40 cm L and 50 μm ID × 80 cm L. The HPLC system included: i) Hewlett Packard HP1090 HPLC with uv/vis photodiode array detector (PDA); a DR-5 ternary solvent delivery pump; a HP 85 B computing system and a HP 7470 plotter; ii) A Schoeffel FS 970 L.C.fluorescence detector, in line with the PDA detector.

Methods of HPLC characterization

Fractions or subfraction samples were collected into methanol and the solutions were brought to predetermined volume and 25 µl was injected into a non-column HPLC system with uv set at λ 254 nm and fluorescence detection set at λ_{ex}273 nm and $\lambda_{em.}$370 nm For analytical HPLC separation of FA and the SFE fractions, two reversed phase C-18 columns were used and the separation was monitored by both uv PDA and fluorescence detectors. Table II shows the experimental conditions for the HPLC characterization of FA and the SFE fractions.

TABLE II RP- HPLC Operating Conditions

Columns :	Nova-pak C_{18} (100 mm 1 × 3.9 mm i.d)	Hypersil- ODS C_{18} (200 mm 1 × 2.1 mm (i.d)
Mobile Phases:	Water +0.01% HAC,pH 2.8	CH_3CN
Gradient Program	%	%
00 min	99	1
02 min	70	30
04 min	40	60
10 min	15	85
14 min	15	85
Flow Rate ml/min:	0.7	0.3
PDA uv Monitor:	λ 254 nm and λ 230 nm	
PDA uv range:	λ 210-nm -λ 470 nm	
Fluorescence:	λ_{exit} = 273 nm ; λ_{em} = 370 nm	
Injection Volume:	25 µl	
Column Temp.:	25 (°C)	

RESULTS AND DISCUSSIONS

SEE method development: rationale and strategy

The optimal SFE method was established with three principal considerations; 1) fluid solvent power; 2) fluid analyte interaction; and 3) extract collection.

Fluid solvent power

In view of the fact that the solvent power of pure supercritical carbon dioxide is close to that of n-heptane, it can be seen that the solvent power of SF CO_2 is not sufficient to extract FA. This judgement was later confirmed in the preliminary experiments when no FA was extracted with pure CO_2 at 4000 psi and 60° C for 50 minutes. Methanol (δ_1 = 12.9 & δ_f = 8.9) posses some superior solvent characteristics and hence was chosen as modifier. For a modified fluid, the solvent power can be controlled by each of the following three variables; a) temperature; b) fluid density (i.e. pressure) and c) fluid composition.

a. Temperature: It is reported that the extraction temperature for non-volatile solutes has a minor influence on the extraction yield and speed[4,28]. This is due to the fact that the extraction temperature would not significantly change the vapor pressure of the non-volatile solutes and thus alter their solubilities in the fluid. Although temperature change do influence fluid diffusivity, it will not make much difference in extraction results, because essentially the extraction of the non-volatile solutes is a solubility controlled process. On the other hand lower extraction temperature will minimize the chance of solute decomposition and derivatization during the extraction. Another reason for lowering the extraction temperature is the fact that the SFE system used for this study was not equipped with a restrictor-heating device and restrictor plugging was encountered during the initial experiments. At lower extraction temperature the temperature differences between the extraction cell and the restrictor, would reduce the possibility or frequency of restrictor-plugging.

b. Pressure: In order to obtain a desired fluid solvent power at fixed extraction temperature , two pressure composition patterns can be utilized; high pressure with less modifier and low pressure with more modifier. The latter pattern was chosen because the more modifier in the fluid, the smoother the required extraction conditions (e.g.. lower extraction pressure and temperature). A lower extraction pressure would also reduce the probability of system's leakage and restrictor plugging since the fluid decompression effect is lessened with lower extraction pressure.

c. Fluid Composition: Three methods are commonly used to mix fluids for SFE; one is premixing the modifier in the reservoir; another is mixing the modifier and the fluid in the pump head, just prior to compression; and thirdly mixing effluent streams of two pumps which is similar to high pressure mixing. Restrictor plugging is a common problem that occurs during SFE extraction. The decompression of the fluid reduces its solvent power within the restrictor and decreases the solute solubilities. On the other hand, fluid decompression and expansion can cause a Joule Thompson cooling effect[29]. These two

effects produce a subcritical condition at the exit end of the restrictor which may lead to solute precipitation and /or moisture freezing and ultimately plugging the inside of the restrictor. Restrictor plugging can be prevented or minimized by improving the restrictor configurations and/or heating the restrictor during extraction.

Fluid Analyte Interactions

The next consideration was the fluid/solute interaction in terms of flow rate, extraction time and extraction mode. For purified SR-FA, the extraction process is simply solubility controlled and thus the dynamic extraction mode is a more suitable approach. The flow rate and extraction time for this research were adjusted to 10 ml /12 min.,which corresponded to the volume of the pump cylinders, (one for CO_2 and one for methanol).

Because the pump recharge during extraction stops the fluid flow and suspends the extraction operation for about two minutes, the established extraction dynamics would be disturbed which may endanger the fractionation reproducibility. In order to circumvent this problem, the pump recharge was timed to occur between two fractionation steps ie. when the modifier concentration was changed and the dynamic equilibria would naturally shift. The small extraction cell volume would reduce the ineffective fluid consumption and extends the time intervals between pump recharges used. A 25 μm diameter silica capillary proved to be too narrow that it was prone to plug. A silica capillary restrictor of 50 μm in diameter and 80 cm in length, maintained the pressure in the SFE system and controlled the fluid flow rate at about 0.80 ml of pure CO_2 (in liquid) at 2500 psi and 70° C. At this flow rate twelve minutes was the established extraction time.

Extract Collection

The last consideration for SFE method development was the extract collection . Based on the nonvolatile nature of FA, the extracted fractions were easily collected by a solvent in which FA is completely soluble such as methanol. No loss would be expected as long as the solvent was not blown out of the collection vial by the expanded fluid.

Considering the above rationale and strategy, a series of initial experiments were conducted, where solid FA containing 0.60 mg FA were added to the glass wool and packed into the extraction cell. Clogging problems were encountered when a capillary restrictor of 25μm ID and 40 cm in L was used. In order to reduce the restrictor plugging, restrictors of 50μm ID × 80 cm L were used. After initial fractionation experiments, an optimum method was developed and a mass recovery test became feasible. The best fractionation was achieved with metha-

nol percentages of 18 %, 24 % and 100 %, respectively. According to the critical data shown in Table I, and using equation I with an increase of the methanol fraction in CO_2, the T_c of the modified fluid increases. For 0.25 and 0.33 mole methanol in CO_2, at 2500 psi, T_c would be 84 and 100°C, respectively. However exact supercritical conditions were avoided because even minor change in the pressure and or temperature would dramatically change the fluid solvent power and deteriorate the fractionation reproducibility. For this reason a near T_c of 70°C was chosen. Under 100 % methanol the extraction was simply liquid extraction.

Optimum SEE fractionation and recovery experiments

Table III and Figure 1 show the ultimately established fractionation conditions and characteristics of the fractions. Figure 1 shows the SFE fractionation dynamics of 2.00 mg FA where the results indicate almost complete recovery at each stage. The figure clearly demonstrate the fractionation process of FA. In SFE solutes can only be extracted with fluids whose solubility parameters are similar to the solubility parameter of the solutes. The range finding experiments, at pressure of 2,500 psi, supported this fact. In one experiment using 5 % methanol, no FA component was extracted in 50 minutes. In another experiment only 72.2 % of FA was extracted with 20 % methanol in 50 minutes. No further extraction of FA was observed until the percentage of methanol was increased to 100 percent.

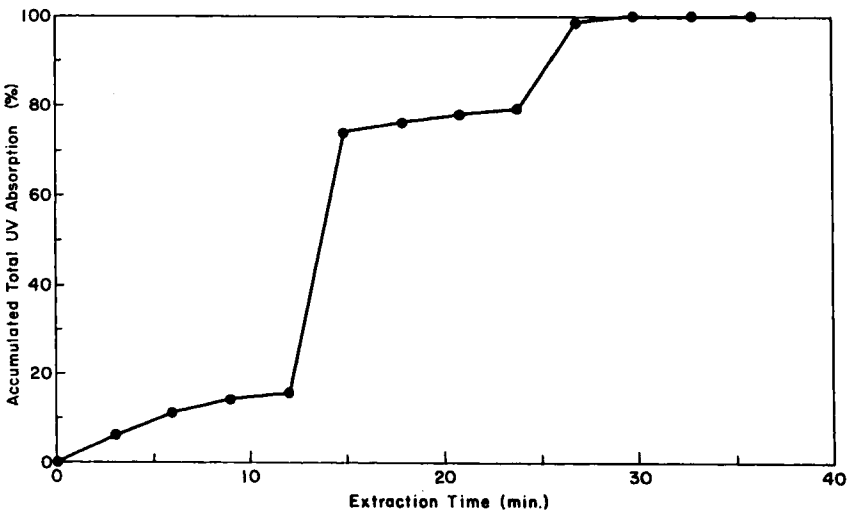

FIGURE 1 Dynamic fractionation of FA under the experimental conditions in Table III

TABLE III Optimum SFE Conditions, Mass Recoveries and Characteristics of FA Fractions

A-SFE Conditions	*Fraction # 1*	*Fraction # 2*	*Fraction # 3*
Temperature (°C)	70	70	70
Pressure (psi)	2500	2500	2500
Time (min)	12	12	12
Percent CH_3OH	18	24	100
B- Mass Recovery Weight:			
Mean weight (mg)	0.43	1.10	0.52
% CV	13.4	10.6	7.8
% Fraction	21.5	55.0	25.0
C- Characteristics Of Fractions:			
UV λ 400/λ 254 ratio × 10^{-1}	1.13	1.99	3.00
Ratio of FL emission to UV λ 254 nm.	8.57	4.81	0.4

UV measurements of each fraction via the non-column HPLC, at λ 400 nm and λ 254 nm, were used to evaluate the fractions. The ratios of absorbance at these two wave lengths are used as estimates of the ratio between the chromophores and the aromatic structures in FA. The ratio of the fluorescence emission at λ370 nm to the uv absorption at λ254 nm was also used to characterize the fractions. The results in Table III, show that the ratios are different in each fraction. The mass recovery data illustrate sufficient reproducibilities , within the sensitivities of the analytical balance. The measures taken to check the reliability of the SFE method included : i) A procedure blank using the same SFE program without the sample. ii) Reintroduction of individual fractions to the same SFE program. The procedure blanks showed no detectable weight or measurable uv absorbance or fluorescence emissions. The re-extracted fractions showed uv absorption and fluorescence emission only in that specific fraction with the non-column HPLC. The average total mass recovery of all three fractions was 102 % and coefficient of variation of 6.8%. The first fraction represented 21.5 % of total FA and exhibited uv absorbance ratio 400/254 nm of 0.11. The second fraction represented 55 % of total FA and exhibited almost twice the uv absorbance ratio. The third fraction represented 25 % of the total FA and exhibited a uv absorbance ratio of 0.30. This final fraction represents the extraction by pure methanol. These results illustrate the suitability of the developed method for reliable fractionation of FA by SFE. To our knowledge this is the first successful quantitative SFE recovery study of FA.

Characterization of FA fractions by RP-HPLC

The chromatographic separation of FA and the SFE fractions were achieved both on Novapack C-18 and ODS – hypersil column based on published methods[22–23]. Figure 2 shows the three dimensional plots generated by the uv-vis PDA detector of total FA sample. The retention time and the uv scans of the separated peaks are comparable to those published in the literature. The two early eluting peaks in the retention windows of 1.30- 2.00 minutes, and 2.10 – 3.00 minutes are referred to as hydrophilic (1) and hydrophilic (2) components , respectively. The late eluting broad peak within the retention window of 7.00–10.00 minutes is referred to as the hydrophobic (3) components. Figure 3 shows comparison between chromatograms of total FA sample and SFE fractions using both fluorescence and uv PDA detectors. It is noticeable that the chromatographic peak patterns are basically the same in all chromatograms, but the areas under the peaks are different. Table IV shows a comparison between the areas under the peaks for the three retention areas of the fluorescence chromatograms of total FA and the SFE fractions. There is a consistent patten of decrease in the areas under the two hydrophilic peaks in fractions #1, #2 and #3. By contrast area under the hydrophobic peak (peak 3) consistently increased from 63.29% in fraction # 1 to 76.73 % in fraction # 3. Since the HPLC conditions of the total FA and fractions are the same, these results confirm that fractions are different in the relative amounts of components rather than the type of components.

TABLE IV Percent Distribution of Retention Areas in the Fluorescence RP-HPLC Chromatograms of Total FA and SFE Fractions (Figure 3)

Retention Region	*Hydrophilic (1)*	*Hydrophilic (2)*	*Hydrophobic (3)*
Retention time (min.)	1.30–2.00	2.10–3.00	7.00–10.00
Total FA	14.39 %	16.40 %	69.20 %
Fraction # 1	13.29%	18.04 %	63.29%
Fraction # 2	9.59 %	24.93 %	72.36 %
Fraction # 3	8.40 %	14.87 %	76.79 %

Figure 4 shows the RP-HPLC chromatogram of three model compounds 1,2,4-benzenecarboxylic acid , (pk_{a1} = 2.52); vanillic acid (pk_a = 4.34) and o-cresol (pk_a = 10.26) under the same conditions as in Figure 3. Since polarities and acidities of FA components follow a parallel order to the model compounds,

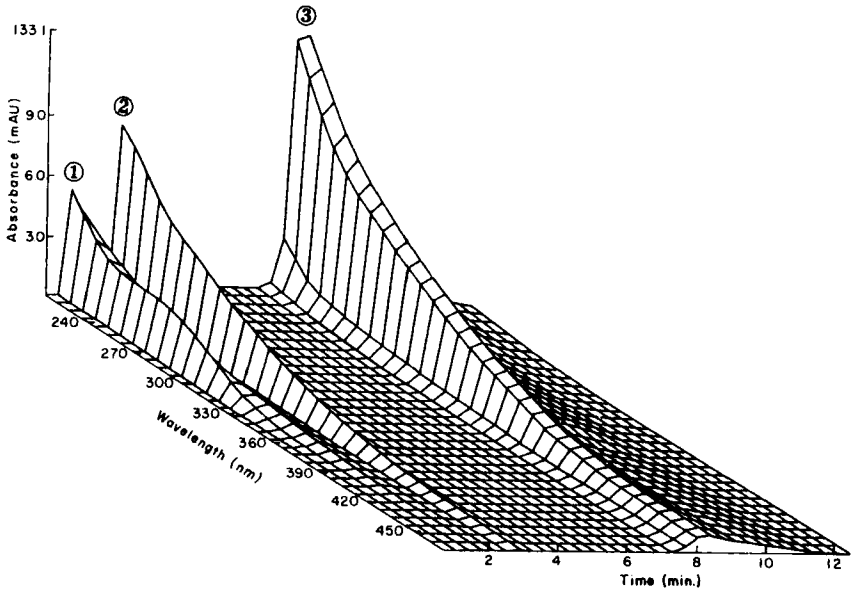

FIGURE 2 Three dimension plot of uv-vis RP-HPLC chromatogram of FA on ODS-Hypersil column. **1** = Hydrophilic 1; **2** = Hydrophilic 2; **3** = Hydrophobic. Experimental conditions are listed in Table II

it can be concluded components of total FA and SFE fractions vary in polarities and acidities as those of the model compounds. A model of FA as a polymeric material consisting of labile (i.e. continually changing) hydrophilic components and rigid (i.e. outer shape maintained by a fixed framework) hydrophobic components is emerging as an acceptable picture of the structural features of FA. It may be argued that polymers would not be separated into 3 retention regions under RP-HPLC conditions but the presence of labile hydrophilic components in FA explains this phenomenon. The retention mechanisms of solutes in RP-HPLC remains unclear, even though RP- HPLC has been widely applied[25–26]. An explanation of solute's retention has been described qualitatively through the hydrophobic effect or by the use of partitioning model. The hydrophobic effect is used particularly to explain the solute- bonded phase interactions. Since the aqueous mobile phase pH is 2.8, solutes are eluted in the order of their overall polarities ie. polar solutes are eluted first (hydrophilic components) and less polar solutes are eluted later (hydrophobic components). Thus while the SFE fractionation yielded three different fractions of different ratios of uv absorbance and fluorescence emissions, the RP-HPLC chromatograms showed only difference in peak intensities. The results indicate that the modified SF CO_2-methanol

extracts more of the labile components of FA in Fractions # 1 and # 2 than of the rigid components of FA. Considering that FA exhibit strong hydrogen bonding and cross linkages, the results are not surprising. In fact the results can be used as a further evidence of the polymer-like structure of aquatic FA.

The overall results of this research indicate the possible use of modified SFE for extraction and recovery of aquatic FA into three distinct fractions. The RP-HPLC chromatograms provide new evidence in support of the polymeric nature of FA which includes labile extractable components and rigid less extractable components. The results of the research also provide useful information on the matrix interference problems encountered in application of SFE to the analysis of pollutants in environmental samples.

SUMMARY AND CONCLUSIONS

Modified supercritical fluid "SF" CO_2 -methanol mixture was used, under step-wise gradient conditions at near supercritical temperature, to fractionate Suwannee River reference fulvic acids (FA) into three fractions of different composition. The method was developed after a systematic study of the solubility of FA in SF CO_2- methanol solvent. Optimum supercritical fluid extraction (SFE) conditions were established at constant temperature of 70 °C and constant pressure of 2500 psi; using modified CO_2 fluid mixed with methanol at 18, 24 and 100 percent, respectively. Fractions were characterized by total uv absorption and fluorescence emission. The SFE fractions exhibited different uv absorption and fluorescence emission characteristics . Under optimum conditions, individual fractions were re-extracted only in the corresponding fraction. Fractions were also characterized by RP-HPLC with uv-vis PDA and fluorescence detection. The average total mass recovery of all three fractions was 102 % and coefficient of variation of 2.8%. The first fraction represented 21.5 % of total FA and exhibited uv absorbance ratio 400 nm/254 nm of 0.11, The second fraction represented 55 % of total FA and exhibited almost twice the uv absorbance ratio. The third fraction represented 25 % of the total FA and exhibited a uv absorbance ratio of 0.30. The RP-HPLC chromatographic peak patterns total FA and the SFE fractions are basically the same in all chromatograms, but the areas under the peaks are different. Each chromatogram consisted of two hydrophilic peaks and one broad hydrophobic peak. The SFE fractions showed a consistent patten of decrease in the areas under the two hydrophilic peaks in fractions #1, #2 and #3. By contrast area under the hydrophobic peak (peak 3) consistently increased from 63.29% in fraction # 1 to 76.73 % in fraction # 3. A model of FA as a poly-

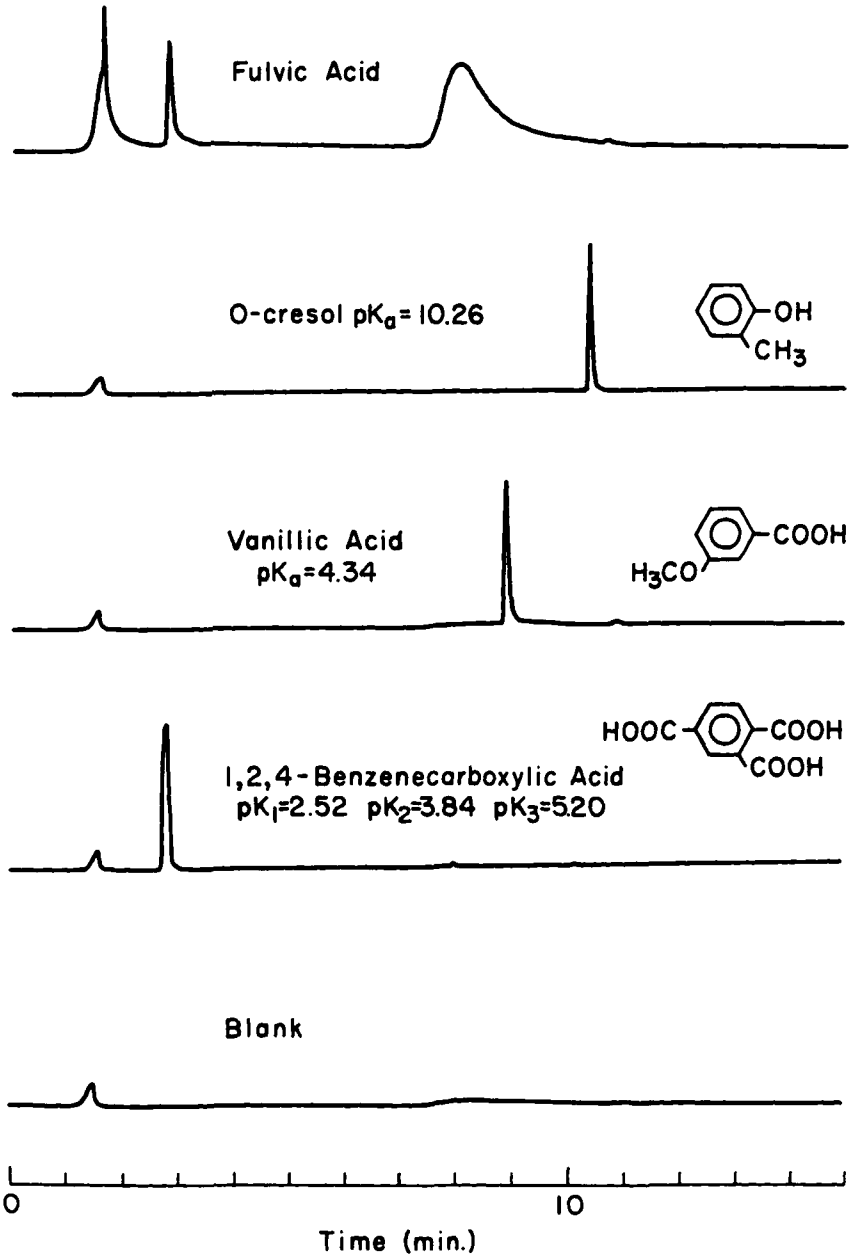

FIGURE 3 UV λ 254 nm and fluorescence RP-HPLC chromatograms of FA and SFE fractions on ODS Hypersil Column. Experimental conditions are listed in Table II

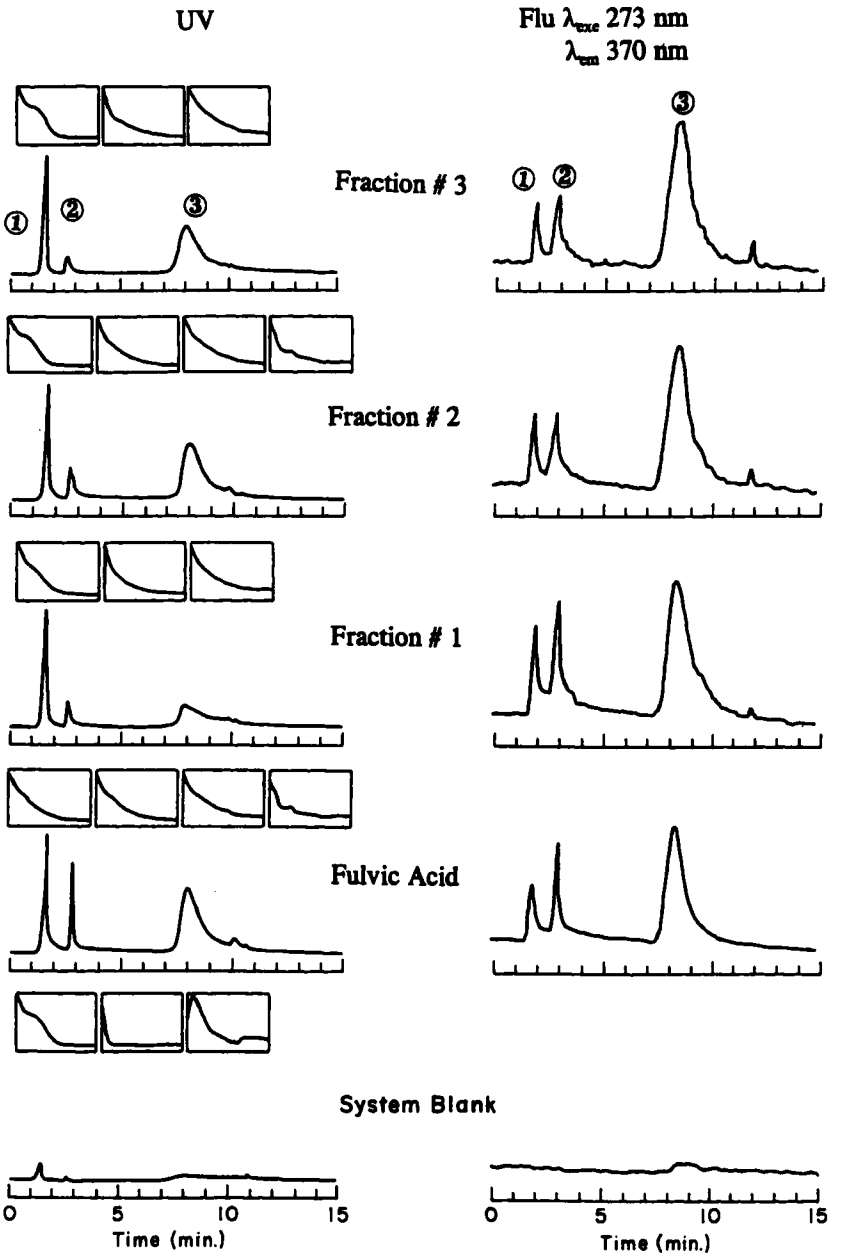

FIGURE 4 UV λ 254 nm RP-HPLC Chromatogram of three model compounds and FA sample. Experimental conditions are listed in Table II

meric material consisting of labile hydrophilic components and rigid hydrophobic components is emerging as an acceptable picture of the structural features of FA. The RP-HPLC chromatograms provide new evidence in support of the polymeric nature of FA which includes labile extractable components and rigid less extractable components. The results of the research also provide useful information on the matrix interference problems encountered in application of SFE to the analysis of pollutants in environmental samples.

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