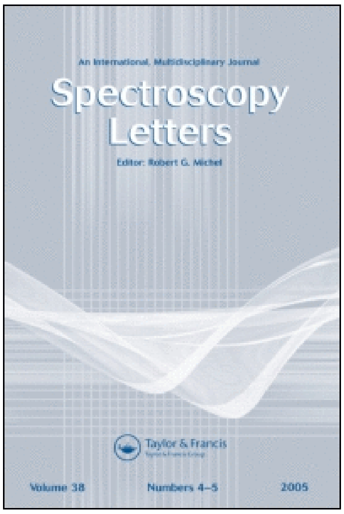

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

Applications of Raman and Surface-Enhanced Raman Scattering Techniques to Humic Substances

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To cite this Article Yang, Yuhui and Chase, Howard A.(1998) 'Applications of Raman and Surface-Enhanced Raman Scattering Techniques to Humic Substances', Spectroscopy Letters, 31: 4, 821 — 848
To link to this Article: DOI: 10.1080/00387019808007402
URL: http://dx.doi.org/10.1080/00387019808007402

SPECTROSCOPY LETTERS, 31(4), 821-848 (1998)

APPLICATIONS OF RAMAN AND SURFACE-ENHANCED RAMAN SCATTERING TECHNIQUES TO HUMIC SUBSTANCES

Key words: humic acid, fulvic acid, backbone, methylation, Raman, FT-Raman, surface-enhanced Raman scattering (SERS)

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ABSTRACT

This paper highlights the use of Raman, FT-Raman and surface-enhanced Raman scattering (SERS) techniques for the study of humic substances. In contrast to other technologies which reveal information only about the average compositions and the kinds of functional groups present in humic substances, Raman and especially FT-Raman spectroscopies characterize the building blocks of humic substances and their changes in derivation and separation processes. Furthermore, surface-enhanced Raman scattering (SERS) techniques are able to readily detect humic substances and co-existing organic species at low concentrations typically found in natural environments and reveal definitive information about the specific groups in humic substances that bind on metal electrodes. Further applications of both Raman and SERS techniques can be extended to complicated systems as well as real environmental samples. Experiments have demonstrated: (1). the backbones of humic substances are structurally disordered carbon networks in most cases; (2). The backbones of humic substances from different sources and types are similar to each other; (3). Normal Raman spectroscopic study of humic

substances should concentrate on the use of near-IR laser(s) resulting from strong fluorescence background and self-adsorption under the excitation with visible laser irradiation; (4). FT-Raman spectroscopy is the required analytical method to assess the effectivity of fractionation methods; (5). SERS spectra of humic substances on metal colloids and films are in most aces very similar to the corresponding Raman spectra of neutralized samples; (6). SERS techniques are very sensitive and highly selective, also both visible lasers and near-IR lasers can be used for SERS study; (7). SERS spectra on metal electrodes may provide additional information about the binding sites and adsorption mechanisms of humic substances on metal surfaces.

ABBREVATIONS: humic acid, HA; Fulvic Acid, FA; Surface-enhanced Raman Scattering, SERS; NMR, nuclear magnetic resonace; EPR, electronic paramagnetic resonance; coherent anti-Stokes Raman, CARS; Super-critical fluid chromatography, SFC; reversed phased high-performance liquid chromatography, RP-HPLC; Liquid chromatography, LC; Super-critical fluid chromatographic - mass spectrometry, SFC-MS; GC, gas chromatography; high-performance liquid chromatography, HPLC; Gel permeation chromatography, GPC.

1. INTRODUCTION

Humic substances affect soil weathering and fertility, pH and alkalinity of natural waters, trace metal chemistry and bioavailability, degradation and transport of hydrophobic organic chemicals, formation of disinfection by-products during water treatment, and heterotrophic production in blackwater ecosystems (1-5). Accordingly, humic substances have attracted extensive attention.

In past decades, extensive efforts have been made to elucidate the structures of humic substances and characterize their behaviour and multiple functions in terrestrial and aquatic environments (1-5). Almost all classical and modern techniques and methodological approaches have been used in this field. In particular, non-separative, non-destructive, spectroscopic techniques have been playing a vital role in the development of this field at each stage. Our current knowledge about the composition, structures and physical-chemical properties of humic substances has greatly benefited from applications of various spectroscopic techniques such as IR (6), NMR (7-8), fluorescence (9) and EPR (10).

This paper summarizes the results and information obtained from a set of novel spectroscopic line, Raman and surface-enhanced Raman scattering techniques, on the structure and chemistry of humic substances.

2. THE USE OF RAMAN AND SURFACE-ENHANCED RAMAN SCATTERING TECHNIQUES FOR CHARACTERIZATION OF HUMIC SUBSTANCES

While current spectroscopic techniques have provided some valuable information about the average composition, as well as the chemistry, of humic substances (1-10), their possible impact on future progress in this field is very limited. For instance, IR spectroscopy, the most powerful tool in the 60 and 70's, is now a routine technique and hundreds of papers in the literature have presented very similar IR spectra of humic substances. Following the pioneering works of MacCarthy et al. (11-12), a few groups have tried to measure the IR spectra of humic substances in wet states, expecting to obtain additional information (11-14). Although measurements can readily be made, this line is not promising as humic substances bind strongly with water and it is difficult to obtain additional information (14). ^{1}H, ^{13}C and ^{15}N nuclear magenetic resonce spectroscopy in 1D and 2D modes have yielded much more information (7-8,15-16). However, the information obtained characterizes the average composition of humic substances rather than their structures (17). Furthermore, both IR and NMR approaches are too insensitive to detect humic substances at the low concentrations typically found in natural environment and can be used only for purified samples in simplified systems. Although EPR (electronic paramagnetic resonance) and fluoroscence methods are sufficiently sensitive (9,10), they cannot provide direct information because both free radicals and fluorophores are only very little parts of the overall composition and structure of humic substances. As a result of the paucity of methods available, little is known about the backbones of humic substances and how they are assembled. In addition little is known about the changes of the backbones in the separation procedures and the effectivity of these separative methods. Much also remains unknown about the specific sites in humic substances responsible for the binding of metal ions. There is therefore an impetus to apply Raman and surface-enhanced Raman scattering (SERS) techniques to the characterization of humic substances. As a complementary method to IR spectroscopy, Raman scattering should be very valuable for characterization of humic substances in the wet state as well as the native state because water is a very poor Raman scatterer and hydrogen bonding causes very little broadening of Raman bands (18). Furthermore, since humic substances are coloured, Raman spectroscopy should in theory be of value not only for structural studies, but also as a probe of the electronic properties (18). Although conventional Raman techniques have low sensitivity, some advanced approaches such as resonance Raman spectroscopy, coherent anti-Stokes Raman (CARS), and surface-enhanced Raman scattering techniques can enhance the Raman signals by factors of up to 10^3-10^6 folds and therefore samples at concentrations as low as ppm level can in theory be investigated. An additional advantage, which will be discussed later, is the high selectivity of Raman and SERS techniques.

However, some obstacles have prevented the success of previous attempts and they are outlined as follows.

1. Humic substances are generally fluorescent (9) and therefore weak Raman scattering will be "buried" beneath the strong fluorescence background. Based on our own experiments, there is a strong fluorescence background from humic substances when the laser lines of wavelength from 250 nm to 700 nm are used as excitation sources.

2. Humic substances are deeply coloured and thereby will adsorb energy from the exciting beam. Thus their Raman specta are usually of poor quality and the procedure can also result in their thermal decomposition. In our experience, under Ar^+ laser laser excitation at 514.5 nm line and Kr^+ laser excitation at 647.1 nm line, the fluorescence of humic substances in concentrated suspensions and in the solid state is quite weak in sometimes and the fluorescence background increases slowly. However, the Raman scattering signals are still too weak to be observed as a result of energy adsorption by

the sample. In fact, fluorescent, coloured and labile samples belong to the "blind" areas of Raman spectroscopic studies (18-19).

3. Several other approaches have also been unsuccessful. The first is the application of chemical quenching methods to eliminate the fluorescence background. Unfortunately, although all the chemical quenching methods in the current literatures, such as the addition of a large excess of KI, Cu^{2+} and Tl^{+} were tried (20-22), the fluorescence of these substances could not be fully quenched and hence the weak Raman scattering could not be observed. The second is the use of the resonance Raman effect. Since humic substances are coloured, they are candidates for resonance Raman studies (18). In principle, Raman signals can be enhanced by factors of up to 10^3 fold when resonance effects occur (18). When the concentrations of humic substances are over 0.1-0.5 g/L, there are several absorption peaks at ca. 450 nm, 480 nm and 530 nm in the UV-visible spectra. The 457.9 nm, 488.0 nm and 514.5 nm lines of Ar^{+} laser are suitable for resonance studies. However, the Raman scattering signals are still beneath the fluorescence background as the quenching methods (20-22) are used at the same time. Another alternative method is the use of coherent anti-Stokes Raman scattering (CARS). Unfortunately, CARS is limited to transparent samples and this approach has also failed (Liang and Kiefer, private communication).

Since humic substances represent the bulk of organic matter in environmental samples (soil, sediment and water) and as various kinds of environmental particles are frequently coated with humic substances (1-5), problems arising from fluorescence and the deep colour of humic substances also prevent the applications of Raman spectroscopy in environmental fields where there are only a few methods available for characterization of samples.

After extensive tests using various Raman modes, strategies to eliminate problems arising from fluorscence and colour have been found, thereby permitting the observation of the Raman scattering signals of humic substances. In practice, Raman spectroscopic measurements of humic substances and related environmental samples are limited to two approaches, FT-Raman and surface-enhanced Raman scattering techniques.

3. EXPERIMENTAL SYSTEMS

3.1 Samples

Well-characterized peat humic acid (HA) from International Humic Substances Society (I.H.S.S.) (23) and fulvic acid (FA) from weathered coal of Tulufan (24) were used. Silver powder (99.99%), copper foil (99.999%, 0.5 mm), gold foil (99.99%, 0.25 mm), MNNG (1-methyl-3-nitro-1-nitrosogunidine, N-methyl-N'-nitro-N-nitroguanidine) and other reagents are all from Aldrich Chemical Company. HPLC grade water was also from Aldrich Chemical Company and used in all the experiments.

3.2 Derivation and Fractionation

3.2.1 Methylation and LC fractionation

The Aldrich MNNG diazomethane generation apparatus (25) was used for the generation of diazomethane. 500 mg FA (fulvic acid) was dissolved in 100 mL methanol, and diethylether saturated with fresh diazomethane was added to the FA solution. The system was stirred overnight and finally, excess diazomethane, ether and methanol were evaporated under reduced pressure. The precedure was repeated twice and 585 mg of the methylated FA was obtained. The methylated FA was applied to a glass column (25 cm x 2.5 cm) containing activated silica (DavisilTM, 60-100 mesh, 150Å, Aldrich). The first fraction eluted with dichloromethane contained 35% of the original sample and the second fraction eluted with methanol contained 62% of the original sample.

3.2.2 RP-HPLC fractionation

The HPLC system for the RP-HPLC separation of the fulvic acid consisted of a Model 110A solvent delivery pump (Beckman Instruments), a Rheodyn Model 7125 syringe injection valve and a Beckman Model 160 ultraviolet detector operated at 254 nm. The stationary phase employed was a 25 cm x 4 mm column of Novapak C-18 (7 um, 300 Å). FA was dissolved in 90% water and 10% methanol at a concentration of 100 mg/L. 20 mL of the FA solution was applied to the column and the hydrophilic fraction was collected. Then a solvent composed of 15% water and 85% methanol was used to elute the hydrophobic fraction. The whole procedure was repeated until sufficient amounts were collected for FT-Raman analysis.

3.2.3 GPC fractionation

Swollen Sephadex G-100, bead diameter 40-120 um, obtained from Aldrich was packed into a glass column (1.6 cm i.d.) and 0.2 M NH_4HCO_3 solution was used as the buffer. Eluent was pumped through the column at 0.5 mL min^{-1} via an LKB 2132 Microperplex peristaltic pump, and the column effluent was monitored at 254 nm by a LKB 2151 Variable wavelength monitor. 0.5 mL of the FA solution (2 g/L) was injected. The first fraction was collected at the void volume of the column (V_0 = 10 mL), determined using Dextran Blue 2000. The second peak with almost the same intensity was collected at 16 mL. The two fractions was dried before FT-Raman analysis.

3.3 Equipment

3.3.1 Raman and SERS measurement using visible lasers

Raman spectra were recorded on two Spex Model 1403 double-grating spectrometers equipped with a gallium arsenide photomultiplier (RCA, Model 31034) used in the single-photon counting mode. A Spectra-Physics 2025-5 Ar ion laser with a 514.5 nm line or a Spectra-Physics 2020 Kr ion laser with a 647.1 nm line was used as the excitation source. Data acqusition conditions: Integration time 1s; scan step length 1 cm^{-1}; slit width 400 um; 3 scans; five-data-point smoothed.

3.3.2 Raman and SERS measurement using Fourier transform mode

FT-Raman spectra were recorded with a Bruker IFS-66 spectrometer connected to a Raman module (Bruker, FRA-106). Radiation of 1064 nm from an Nd:YAG laser was used for excitation with a laser power of 300 mW.

3.4 Normal Raman Measurements

A few mg of HA, FA or their salts and the methylated fractions of the original FA were dispersed on the walls of 5 mm truncated NMR tubes for FT-Raman measurement. In another experiment, 2 mg of the HA was mixed with 100 mg silver powder and then 200 mg KBr (spectroscopic grade). The mixture was homogenized and pressed into a disc. The disc was put into a rotating cell and its Raman signal was measured using 514.5 nm Ar ion laser and/or 647.1 Kr ion laser.

3.5 SER Substrates and Experiments

(1). Copper foil (0.5x0.5 cm) was etched with 3 M nitrate acid for 30 s, then washed with water and ethanol. The activated copper foil (26) was dipped into the samples for 30 s and then taken out. Liquid was blown off with nitrogen gas and then the copper foils containing the samples were used for SERS observation.

(2). Metal electrodes were made by embedding the metal foils into Kel-F sheaths (Fluorocarbon Ltd.). The electrochemical roughening required for the surface enhancement mechanisms was achieved by immersion of the electrode in 0.2 M KCl and applying a potential ramp from 0.1 to 1.1 V (all potentials were quoted vs saturated calomel electrode) for the gold electrode, and from -3.0 V to 3.0 V for the copper electrode at a ramp speed of 100 mW S^{-1}. Twenty-five cycles were used, and the electrode surface was kept in the horizontal plane during roughening to ensure that the enhancement was uniform over the electrode surface. The roughened electrodes were dipped into the FA and HA solutions containing 0.2 M KCl for SERS measurement under Kr^+ and/or Nd:YAG laser excitation.

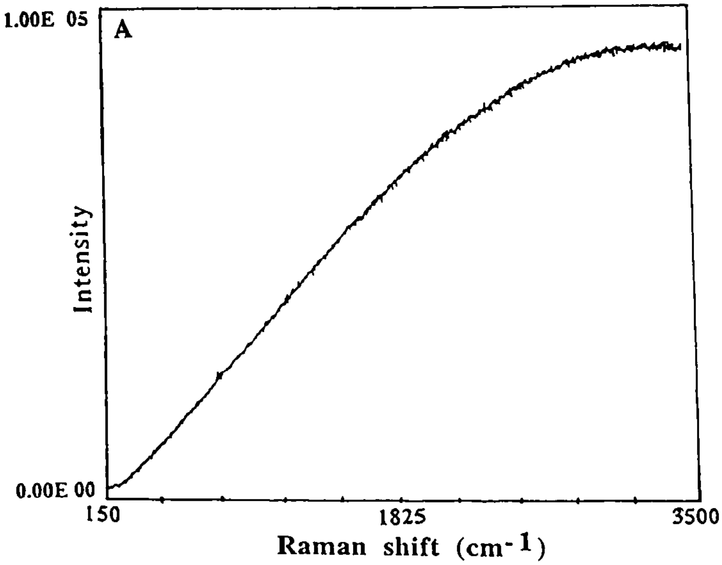
4. RESULTS AND DISCUSSION

4.1 *Measurement of Raman and FT-Raman Spectra*

The Raman spectra of the fulvic acid from weathered coal of *Gongxian* which is different from the fulvic acid used here, the sample with the highest degree of humification, was able to measured using an Ar^+ laser as a result of fluorescence quenching in both wet and solid states (27). The Raman spectra of a few soil humic acids with high degrees of humification in solid state were also obtained (27). It was found that the co-existing inorganic species had little interference on the Raman scattering signals of humic substances, thereby raw fulvic acid without purification could be directly characterized (27). This work established our belief that Raman spectroscopy is attractive for characterization of humic substances. Nevertheless, as indicated above, most of the Raman spectra of humic substances in both visible laser excitation and near-IR laser excitation cannot be measured using conventional methods as a results of their fluorescence and deep colour. In order to solve these problems, silver powder was used as a matrix to prepare Ag-HA(FA)-KBr discs for Raman measurement (28-29). The Raman results of the peat HA in neutralized form are shown in Figure 1.

In Figure 1A, it is clearly observed that the Raman signals of the sample are buried under a strong fluorescence background. Nevertheless, when the sample is diluted in a large excess of silver powder, the fluorescence is quenched as a result of charge transfer and other electronic interactions between the silver powder and the sample (28-29) and thus the Raman signals of the sample are clearly observed (Figure 1B). Besides quenching the fluorescence background, preparation of Ag-HA(FA)-KBr discs weakens the self-adsorption by humic substances of the energy from the excitation irradiation. Three mechanisms, chemical enhancement effects, matrix-dilution effects and metal mirror internal-reflection effects (28-29), enhance Raman signals, thus improving the signal-to-noise ratios (29). Before the advent of the FT-Raman equipment, this method was the main approach to the measurement of the Raman spectra of humic samples, soils, minerals and sediments. Except for a few humic samples with low degrees of humification, the Raman spectra of most humic samples can be readily measured (29).

Nevertheless, humic substances with deep colours show strong adsorption over a wide range of the visible region and thus the Raman signals measured at visible laser excitations may contain resonance effects. In addition, the carboxylic groups of humic substances dissociate when the samples are adsorbed on the surface of the silver powder. Therefore all the spectra thus obtained characterize humic substances in their neutralized form irrespective of the pH of the sample (30). In other words, the information about the building blocks of humic substances in acidic forms cannot be



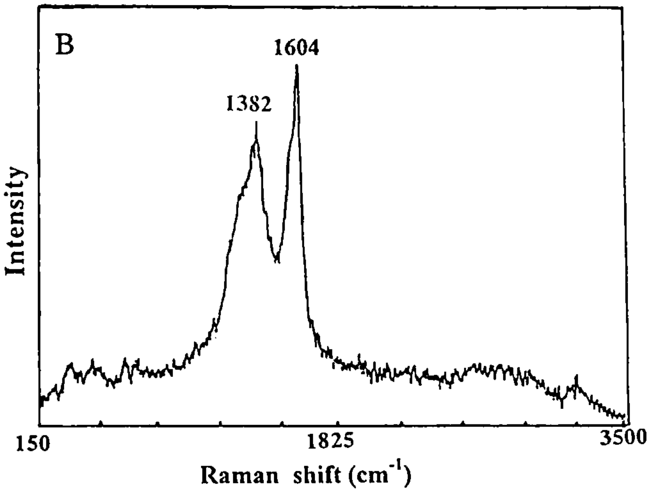


Figure 1. Raman and FT-Raman spectra of peat HA in neutralized form

A. Raman spectrum of the HA sludge. Laser line: 514.5 nm line; Power: 200 mW; scan: 1.

B. HA-Ag-KBr disc (1:100:200). Laser line: 514.5 nm; Power: 200 mW; Scan: 3; rotating speed: 300 rpm/min.

C. FT-Raman spectrum of the HA powder. Laser line: 1064 nm; Power: 300 mW; scan: 500.

D. After subtraction of the thermal emission background.

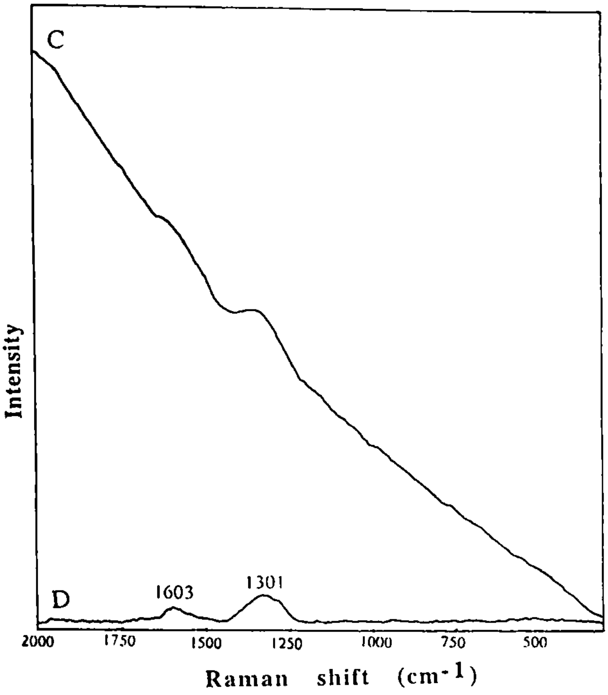


Figure 1. Continued

characterized using this technique. In order to obtain the most comprehensive information, near-IR laser excitation is best since humic substances have little adsorption and fluorescence in this region. Unfortunately, normal sampling techniques, in which samples were placed in capillary tubes were found to be unsuccessful for the Raman measurement as thermal irradation background, resulting from the deep colours of humic substances, predominated in the spectra. This problem can be solved simply by putting a small amount of the sample on the walls of NMR tubes. This is possibly because the energy emitted by the irradiation procedure can easily be transfered into air, the thermal irradation background thus becomes much weaker and thus the Raman scattering signals appear (31). The FT-Raman spectra of the peat humate-Na salt so obtained are shown in Figure 1C and D.

Figure 1C indicates that two weak Raman peaks are superimposed on the thermal emission background. Figure 1D is the result after elimination of the thermal emission background using procedures supplied with software of the equipment. In the following spectra, the thermal emission background have been eliminated.

4.2 Assignment of Raman Bands

In most of the Raman and FT-Raman spectra of humic substances, there are only two broad bands (27,29,31). The first band always locates at ca. 1600 cm^{-1}, but the second one shifts from ca. 1380 cm^{-1} to ca. 1300 cm^{-1} as the excitation wavelength changes from the 514.5 nm line to the 1064 nm line, as indicated in Figure 1B and D. In addition, the band at ca. 1600 cm^{-1} becomes weaker, compared with the other band, while the excitation wavelength red-shifts (see Figure 1B and D). This unique phenomenon can be explained only as a result of the unusual excitation-dependent Raman characteristics of carbon-based materials (32-34). The band centred at 1580-1620 cm^{-1} originates from the zone-center E_{2g} mode of graphite and is called the G band (32-34). The other band has been assigned to in-plane c-c photons at the M point of the Brillouin zone and is called the D band (32-34). The D band reveals the degree of disorder of the carbon networks. In previous research, humic substances were hydrolyzed with acid to remove labile constituents, thus leaving only only their building blocks (31). It was found that the FT-Raman bands, characteristic of disordered carbon networks, remained intact and thus the Raman scattering signals must originate from the building blocks (31). Strong, broad D bands and no observations of the second-order peaks in the range between 2000 cm^{-1} and 3000 cm^{-1} in the FT-Raman spectra of humic substances (31) confirm that the backbones of humic substances are low, structurally disordered carbon networks.

The backbones of a few humic samples in protonated form are not graphite-like. As an example, the Raman and FT-Raman spectra of the peat HA in acidic forms are shown in Figure 2. Figure 2A is the FT-Raman spectrum of the sample. The two

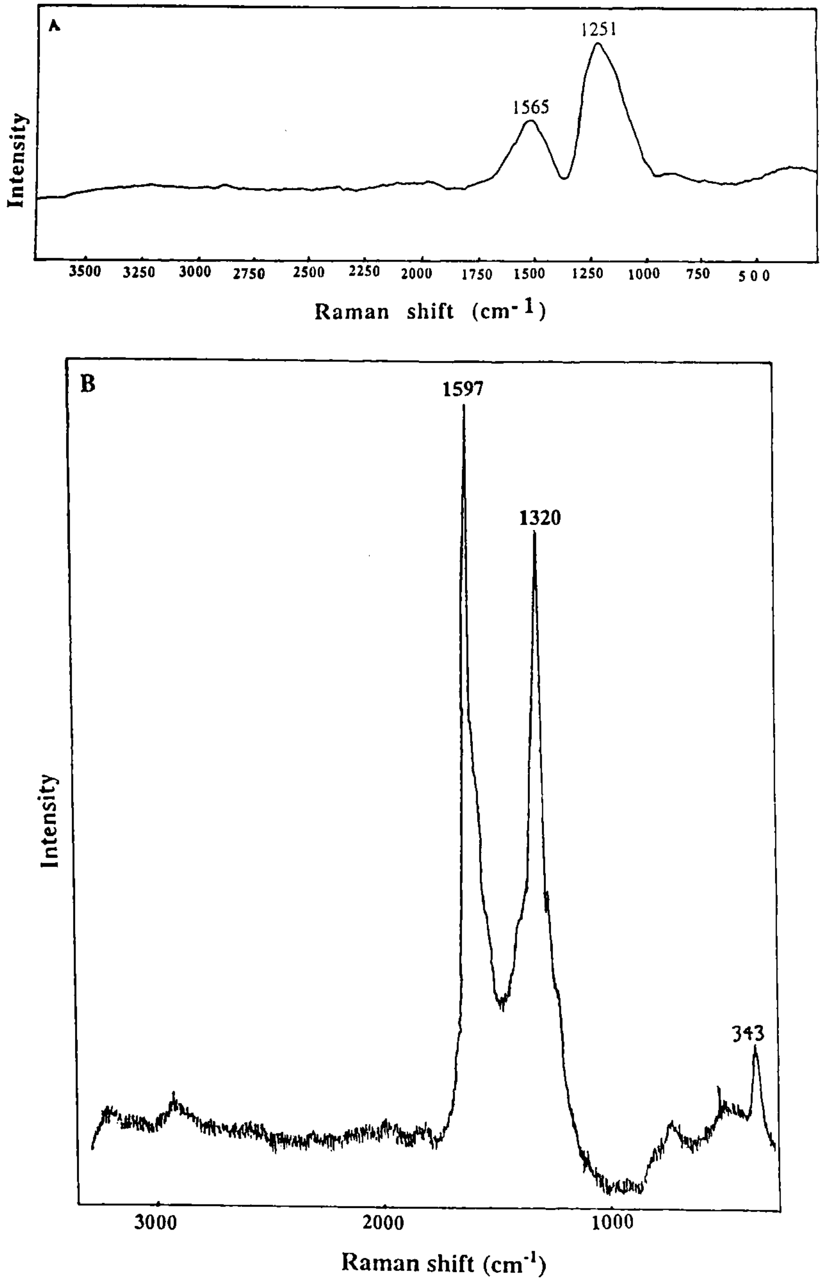


Figure 2. Raman and FT-Raman spectra of peat HA in acidic form
A. FT-Raman spectrum. Laser line: 1064 nm; Power: 300 mW; scan: 500.
B. Raman spectrum. Laser line: 647.1 nm; Power: 200 mW; scan: 3; HA-Ag-KBr disc (1:50:200) with a rotoring speed of 300 rpm/min.

bands at 1550 cm^{-1} and 1250 cm^{-1} all deviate from the characteristic Raman frequencies of the graphite-like materials (32-34) and their assignment cannot be clearly made at the present stage. It has been postulated that it characterizes an amphorous carbon configuration (31). Nevertheless, after neutralization of the sample, the FT-Raman spectrum (Figure 1D) reveals that the backbone of the sample is a disordered carbon network. The silver-aided Raman spectrum of the peat HA in acidic form (Figure 2B) only reveals the graphite-like characteristics since the carboxylic groups are dissociated when the sample is adsorbed on silver surface. When adsorbed on metal surfaces, the dissociation constants of carboxylic groups are enhanced up to ca. 10^8-fold (30). Therefore, Raman spectroscopy in the presence of silver powder cannot reveal the information about humic substances in acidic form. From Figure 2B, it is noted that the D band is located at 1320 cm^{-1} when a Kr ion laser is used, as the result of the unusual excitation-dependent Raman characteristics of carbon-based materials (32-34).

It is recommended that the use of FT-Raman spectroscopic techniques with the novel sampling method (31) is a popular and reliable way to characterize humic substances in both acidic and neutralized forms and other samples with deep colour such as coal, carbon powders, soils, sediments and minerals.

4.3 Characterization of The Fractionation of Humic Substances

There are two possibilities concerning the nature of the backbones of humic substances. The first is that humic substances are polymers with networks of chemically linked carbon atoms and thus no methods can separate them effectively. The second possibility is that humic substances are aggregates composed of small and medium-sized molecules and these aggregates form disordered carbon networks. In order to separate humic substances effectively, the aggregates must be disrupted and the success of the fractionation should be able to be observed by FT-Raman spectroscopy. In this work, the fulvic acid is fractionated using RP-HPLC, GPC and LC methods.

The RP-HPLC chromatogram of the fractionation of FA is not shown here because it is very similar to the result in a previous publication (35). FA is separated into hydrophilic and hydrophobic fractions. The FT-Raman spectra of the original sample and the two fractions are demonstrated in Figure 3A, B and C. It is observed that no change occurs to the building blocks of the FA as the two fractions obtained possess the same building blocks as that of the original FA. Two fractions were also collected using a GPC method whose chromatogram was also reported previously (36) and their FT-Raman spectra are shown in Figure 3D and E. These spectra are also very similar to that of the original sample. The experiments suggest that RP-HPLC and GPC methods are ineffective for the separation of humic substances. Yu tried to separate FA from Gongxian by electrophoresis (37). After separating the sample into fractions, further separation of these fractions by another electrophoresis technique showed that the fractions contain the same constituents (37).

Our SFC experiments also support the result from FT-Raman analysis (38). The four fractions obtained from the RP-HPLC and GPC fractionations was methylated for SFC analysis and SFC analysis proved that the four fractions and also the fractions collected after electrophesis had almost the same SFC results as that of the methylated sample without previous fractionation (38). I.e., the SFC chromatograms which contain 60-70 peaks are very similar to each other. Since the compounds in humic substances aggregate together via hydrogen bonding, electrostatic interactions, p-π bonding, van der Waals forces and salt bridges (39), it is possible that humic aggregates cannot be effectively separated in their native state since no ways are available to disrupt this type of aggregation. The fractions obtained by different methods may be aggregates which have different degrees of aggregation, but contain essentially the same backbones.

If humic substances are indeed aggregates rather than polymers, the interactions among molecules would be greatly weakened when the samples are methylated. Methylation end-caps carboxylic groups and phenolic groups in humic substances, thereby eliminating hydrogen bonding and electrostatic interactions to a large extent,

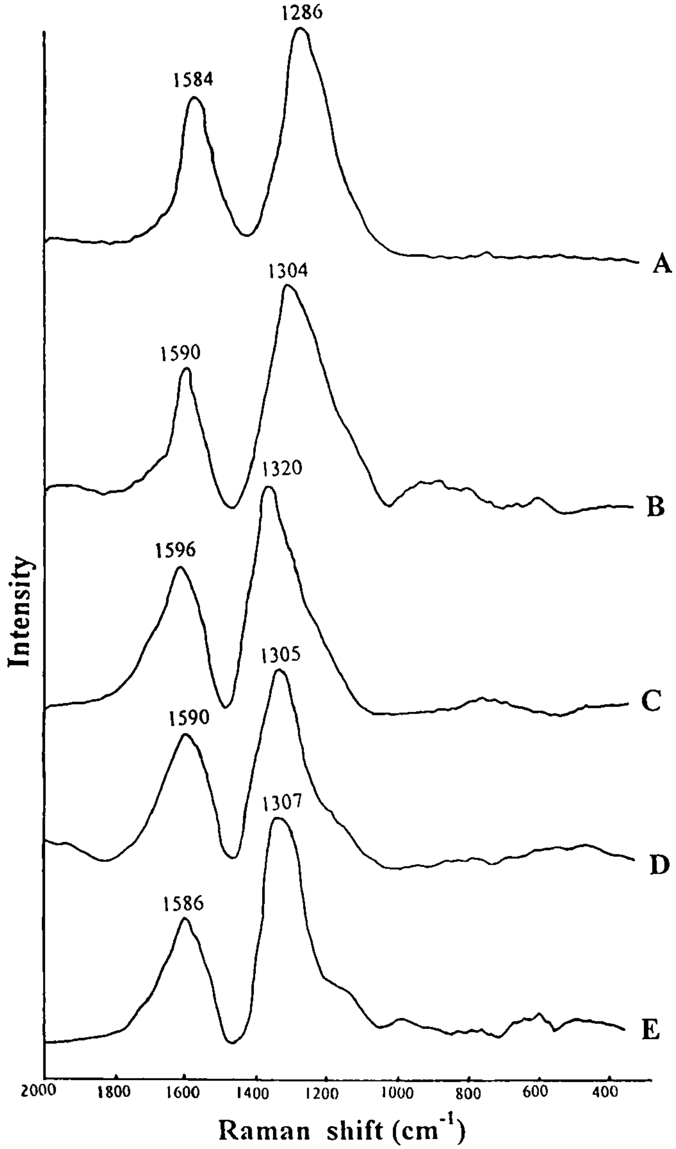


Figure 3. FT-Raman spectra of weathered coal FA powder in neutralized form. Laser line: 1064 nm; Power: 300 mW; scan: 500.

A. the original sample
B. hydrophilic fraction after RP-HPLC fractionation
C. hydrophobic fraction after RP-HPLC fractionation
D. the first fraction in GPC fractionation
E. the second fractio in GPC fractionation

and also weakens other forces such as *van der Waals* attractions. It is our assumption that methylated samples should be able yo be fractionated more effectively.

Methylated FA was separated into two fractions using a normal LC method described in **EXPERIMENTAL** SYSTEMS and the FT-Raman spectra of the fractions are shown in Figure 4. Figure 4A is the spectrum of the original MFA (methylated FA). It can be seen that the backbone of MFA is a disordered carbon network since only two bands characteristic of graphite-like structures are observed. After fractionation, the FT-Raman spectra show great changes, as seen by comparing Figure 4A with Figure 4B and C. In Figure 4B and C, there are very strong bands at 2955 cm^{-1} and 2845 cm^{-1} assigned to symmetric and asymmetric stretch vibrations, respectively, of CH_3 groups as a indicator of methylation. Evidence of methylation is also observed at 1731 cm^{-1} assigned to the C=O stretch vibration of methylated esters. In Figure 4B, the presence of the bands at 1604 cm^{-1} and 1288 cm^{-1} may imply that the backbone is not completely disrupted. However, some extra information such as the bands at 1156cm^{-1} assigned to aliphatic OH, 996 and 956 cm^{-1} characteristic of the conjugated aromatic C-H out-of-plane deformation (39) are observed. In Figure 4C, besides the same bands at 2955 cm^{-1}, 2845 cm^{-1} and 1731 cm^{-1} as are found in Figure 4B, the D band at ca. 1300 cm^{-1} disappears, and this implies that the aggregate has been disrupted. Although further research is necessary to confirm the assignments of some bands, it is noted that the peaks in the range between 1700 cm^{-1} and 700 cm^{-1} are very similar with those observed in the FT-Raman spectra of lignin (40). The results imply that additional valuable information can be observed only after the disruption of the backbones. Further characterization of the methylated fractions using NMR, SFC, SFC-MS and IR is being undertaken and will be reported elsewhere.

Hayes et al. claimed that humic substances resisted all attempts to separate them into reasonably pure fractions (17). Here it is suggested that a reason is because no analytical methods were available to characterize the changes of the backbones following the separation procedures. FT-Raman spectroscopy is the required analytical tool and this preliminary study suggests that further fractionation should be conducted with methylated samples. It is well-known that normal phase LC and

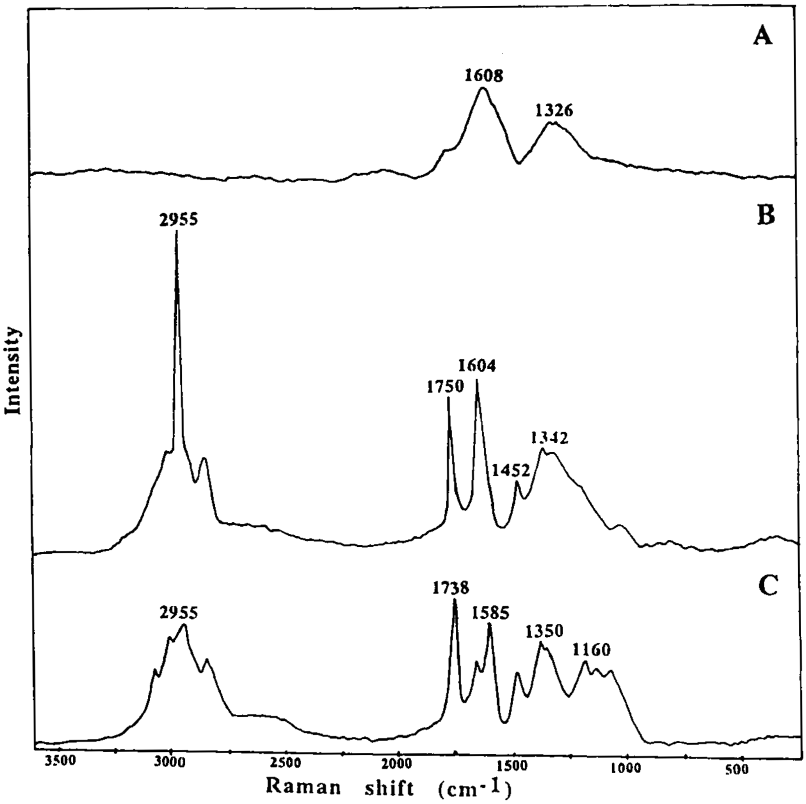


Figure 4. FT-Raman spectra of methylated FA. Laser line: 1064 nm; Power: 300 mW; scan: 500.
A. the methylated FA without fractionation
B. the methylated fraction desorbed from activated silica column using CH_3OH
C. the methylated fraction desorbed from activated silica column using CH_2Cl_2

HPLC methods do not provide sufficient resolution for separation of methylated samples and the use of GC is limited to volatile compounds in humic substances. Nevertheless, super-critical fluid chromatography (SFC) may be a powerful technique yielding high resolution and its potential can now be established (38). It is also noted that the backbones of humic substances are similar to coal. Although there are many common characteristics between humic substances and lignins, the backbones are definitely different to each other.

4.4 Role of Surface-enhanced Raman Scattering (SERS) Techniques in The Study of Humic Substances

The disadvantage of normal Raman and FT-Raman spectroscopy is their low sensitivity. Strong self-adsorption of the laser irradation further lowers the Raman sensitivity and attempts to measure the FT-Raman spectra of humic substances in the soluble state are unsuccessful. However, surface-enhanced Raman scattering techniques are proven to be powerful tools for detection of humic substances and co-existing organic species at trace levels as a result of their high sensitivity, high selectivity and ability to quench fluorescence (41-48). When organic species are adsorbed on roughened Ag, Cu and Au surfaces, their Raman signals are enhanced 10^6-fold. The effect is called surface-enhanced Raman (SER) effect (49-51). In SER experimental systems, three kinds of substrates are frequently used (41-48): metal sols, metal films and metal electrodes. Most of the SER substrates have been tested in previous studies (41-48) and HNO_3-etched copper foil is tried here.

Of all the SER substrates, chemical-reduced yellow and/or grey silver sols are the most simple and sensitive (49-51). However, addition of humic or fulvic acid solutions into the silver sols does not induce aggregation of silver sols and no SER signals of humic substances can be observed (42). This is because at neutral and basic conditions, humic substances act as polyelectrolytes with a number of negative charges and thus there is strong static repulsion between these substances and the silver sols. In addition, the large size of the aggregates also makes their interaction with charged colloidal surface unfavourable, so the samples tend to stay in the aqueous phase (42). A method of solving this problem is to adjust the pH to acidic conditions and this procedure is effective in inducing the adsorption of humic samples on aggregated silver sols, thereby producing SER effects (42). However, under acidic conditions, chemically reduced sols are unstable and it is difficult to get reliable quantative data. The most significant application of chemically reduced silver sols is the direct detection and characterization of the co-existing organic species, since humic substances do not produce any Raman interference at neutral and basic conditions (43). In addition, humic substances induce an additional pre-resonance effect under 514.5 nm laser excitation (43). It is possible to add small quentities of humic substances during the SERS investigation of many organic species and biological samples, thereby enhancing signal-to-noise ratios and preventing them from decomposing (43). It was found that a photo-reduced $AgNO_3$ substrate is the best for the trace detection of humic substances (35). In order to detect the presence of humic substances in natural waters, aquatic samples can directly mixed with $AgNO_3$ (1:1 v/v) and the SER signals of humic substances can be readily observed with little interference from co-existing organic species (35).

Although colloidal silvers are the most sensitive substrates, only trace amount of humic substances can be adsorbed onto them. When the concentrations of humic substances are higher than 10 mg/L, excess humic substances in solution fluoresce and prevent the detection of SERS signals. In addition, co-existing chloride ions scavenge the SERS signals since humic substances cannot compete with chloride ions on the surface active sites (35). In order to obtain SER spectra, many other substrates such as vacuum-deposited metal films (44), chemically etched silver foil (41,45), $AgNO_3$-modified semi-conductors (46), gold electrodes (44,47) and copper electrodes (48) have also been tried. These substrates have relative low sensitivity, when compared with colloidal silvers, and higher concentrations of humic substances (100 mg/L) are necessary for SER measurements. It is found that HNO_3-etched silver and copper foils are the most simple and reproducible substrates amongst the family of metal films. The SER spectrum of peat humic acid adsorbed on a HNO_3-etched copper foil is shown in Figure 5.

Generally, SER spectra of humic substances do not show obvious differences from those of the normal Raman spectra using the same excitation wavelength, as can be seen by comparing Figure 5 and Figure 2B. The main difference is that the carboxylic groups in humic substances dissociate on metal surfaces and thus all SER spectra characterize neutralized humic substances. Nevertheless, SER spectroscopy is still

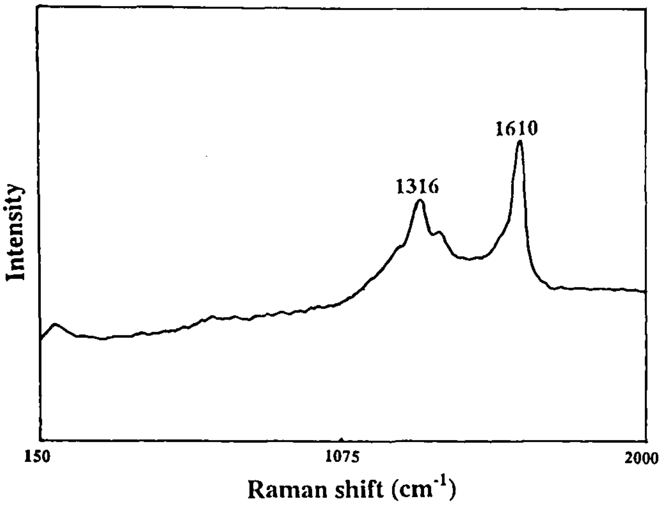


Figure 5. SERS spectrum of peat HA in acidic form. The HA was dissolved in DMF at a concentration of 100 mg/L. A drop of the sample (0.2ml) was spotted on the actived copper foil (0.5x0.5 cm) and the DMF was drown-off using dry nitrogen. Laser line: 647.1 nm; Laser power: 200 mW; scan: 1.

attractive as a result of its unique advantages. First, fluorescence is quenched in SERS investigation and all common lasers can be used for SERS study while normal FT-Raman study is limited to near-IR excitation. Thus, many more laboratories have access to suitable equipment to perform SERS studies of humic substances and other environmental samples. Second, humic substances and co-existing organic species typically exist in the natural environment at trace levels. SERS spectroscopy can be used directly for the analysis and characterization of these environmental samples without the need for pre-concentration and separation. Third, SERS spectroscopy using colloidal silvers has a higher selectivity to that of normal Raman spectroscopy (43) although both of them are supervior in selectivity to most of the alternative methods in this field. Fourth, when metal electrodes are used, the surface potentials can be changed to such a degree that there are some metal ions and/or adatoms on the surface, thereby revealing the binding mechanisms of humic substances onto the surfaces. As two typical examples, the spectra of humic acid and fulvic acid adsorbed on gold and copper electrodes are shown in Figures 6 and 7, respectively.

Figure 6A is the SER spectrum of peat humic acid adsorbed on an Au electrode. Besides the broad bands at 1594 cm^{-1} and 1322 cm^{-1} characteristic of disordered carbon structure, there are several peaks at 1554 cm^{-1},1468 cm^{-1}, 1382 cm^{-1} and 1028 cm^{-1} in the spectrum. The peak at 1382 cm^{-1} is umambiguously assigned to the symmetric stretch vibration of the carboxylate groups (39) and the peak at 1028 cm^{-1} arises from the ring breath vibration of the pyridine-like rings (49-51). The 1468 cm^{-1} peak may be from the bending vibration of CH_2 and CH_3 groups (39). The assignment of the peak at 1554 cm^{-1} is that it probably arises from amide II band and aromatic rings (39). The data shows that humic acid is adsorbed on the gold surface via both physical adsorption and specific chemical binding modes involving carboxylic groups, pyridine groups and so on. Figure 6B is the SER spectrum of fulvic acid adsorbed on Au electrode. In this spectrum, the disappearance of the peak at 1468 cm^{-1} is as a result of very low content of aliphatic chains in the structure of fulvic acid with very high degrees of humification (24). Since the aromatic rings in the structure of the fulvic acid are very highly substituted with carboxylate groups, it is unfavourable for it to bind "end on" to the surface via carboxylate groups and thus the peak assigned to symmetric stretch vibration of carboxylate groups disappears. According to the surface selection rules, only the groups near the surface can be significantly enhanced (49-51). Instead, there is a strong band at 1669 cm^{-1} assigned to C=O strech vibration . It implies that there are three adsorption modes. The first is the physical adsorption revealed by the two characteristic bands at 1604 cm^{-1} and 1305 cm^{-1}. In other words, the fulvic acid aggregate lies flats on the surface. The second mode is that parts of the fulvic acid aggregate or a specific group becomes attached to the substrate surface via carbonyl group. The third mode is attachment to the surface via pyridine rings.

Liang et al found that SER spectra on copper electrodes revealed very specific information totally different from the spectra arising from the backbones of humic substances (48). This method has been repeated here and the main difference in our experiment from the previous research (48) is to use an ex situ oxidation reduction

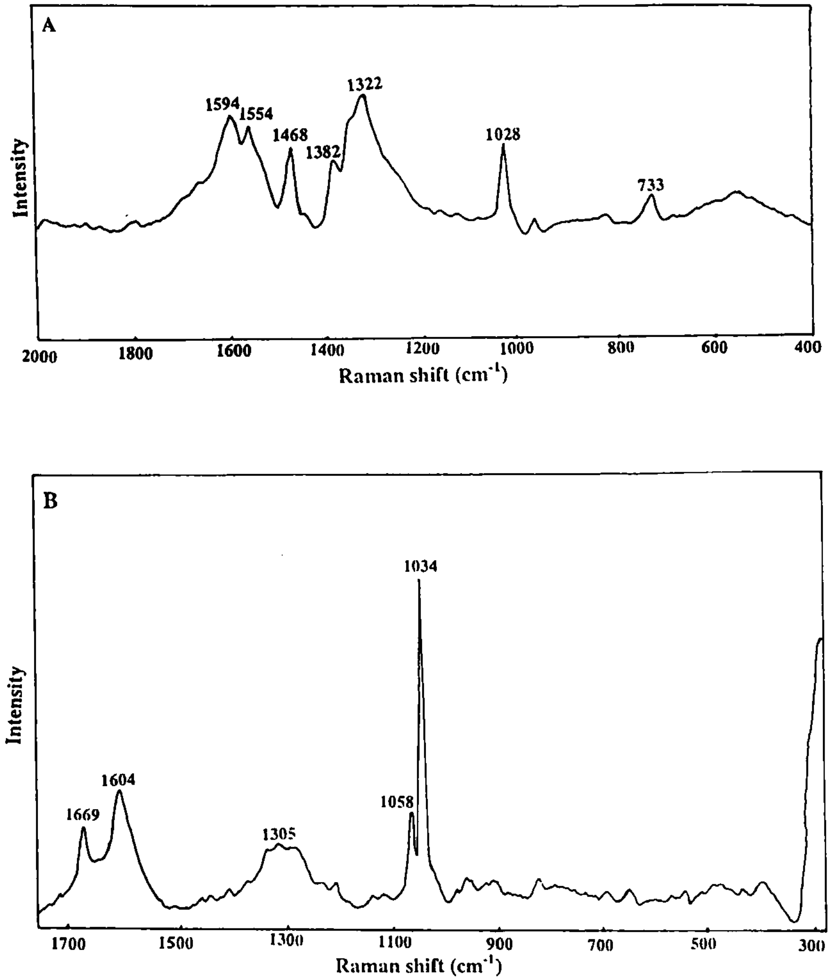


Figure 6. SERS spectra. Concentration: 100 mg/L; pH: 7; Laser line: 1064 nm; Au electrode; Laser power: 300 mV; surface potential: +0.2 V; scan: 100.
A. peat HA; B: weathered coal FA.

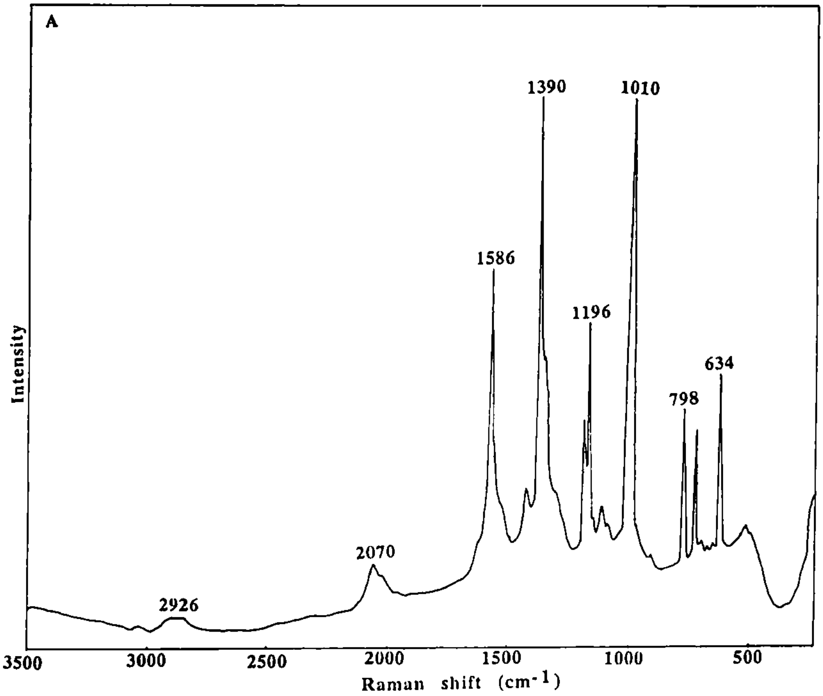


Figure 7.SERS spectra. Copper electrode; surface potential: -2.6 V; Laser line: 1064 nm; Laser power: 300 mW. scan: 100; Concentration: 100 mg/L; pH 7.
A: peat HA; B: weathered coal FA.

cycle (ORC) activation procedure without the presence of humic substances. The procedure avoids denaturation of humic substances. The spectra attained are shown in Figure 7 and are quite similar to those from Liang et al. (48). The most significant characteristic of the spectra is the very high resolution, totally different from most of the poorly resolved molecular spectra of humic substances reported in the literature (1-5). The data imply that the samples are adsorbed on the surfaces mainly via chemical binding. The doublets at 1596 and 1582 cm^{-1} are assigned to the C=C stretch vibration of aromatic rings (39). The peak at 1390 cm^{-1} is assigned to the symmetric -COO^{-} stretch and it is weak in the spectrum of the fulvic acid as a result of the reasons

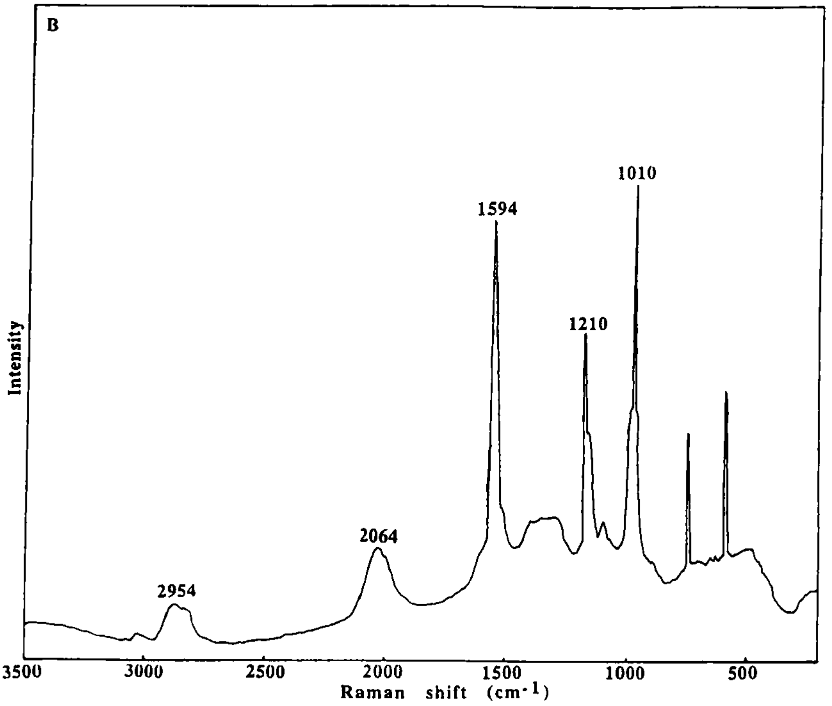


Figure 7. Continued

mentioned above. The band at 1010 cm^{-1} is assigned to the ring breathing mode of pyridine (49-51). Further assignment of the bands has been discussed previously (48).

Copper is much active than gold and silver in chemical activity, thereby chemically binding with humic substances. It is not surprising that a high-resolved SER spectrum, totally different from those arising from the backbones of humic substances, is observed as the result of chemical binding. The SER spectrum reveals only a few kinds of functional groups (carboxylic group and pyridine rings) and constituents in the humic substances specifically adsorbed onto the copper surface. Since the other parts of the humic substances are far from the surface, their SERS signals are too weak to be observed as a result of the surface selection rules (49-51). In this case, SERS spectroscopy screens out the bulk information and only detects the specific binding groups.

5. CONCLUSIONS

1. Raman and FT-Raman scattering techniques mainly reveal information concerning the backbones of humic substances. These methods are highly selective and suitable for characterization of humic substances in wet states as well as native states. FT-Raman technique with the modified sampling technique (31) is the most popular technique and is reliable.
2. Humic substances from different sources and kinds have the same backbones.
3. FT-Raman spectroscopy may be of value in the assessment of the effectivity of the fractionation methods.
4. SERS spectra are very similar to the normal Raman spectra of neutralized samples. As a result of high selectivity and high sensitivity, SERS spectroscopy is of great value for detection of trace amounts of humic substances and co-existing organic species. In addition, humic substances can be used as an additive in further colloidal silver-aided SERS investigations to prevent the decomposition of the target molecules and to further enhance their SERS signals.
5. SERS spectroscopy on metal electrodes may provide very specific information about the binding sites and mechanism, but its role in revealing comprehensive information about the chemical nature of the bulk aggregates is very limited.

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Date Received:December 16, 1997
Date Accepted:January 22, 1998