Chapter 2

Chemistry of Oxihumate

1 Introduction

Many attempts have been made to analyse humic acid structure over the years but few have met with success. Oxihumate is a formulation containing oxidatively released humic acid salts derived from a homogenous mix of coal that is reputed to be non-varying and produced by a closely controlled reaction system, which implies that the product is exactly the same in each batch. However to avoid any complications that could possibly arise from variations, all the assays performed in this study were on a single retention batch of oxihumate.

The sub-fractionation of oxihumate relied the solubility differences in different solvent mixtures and were then analysed chemically. Seven major sub-fractions were isolated. The chemical analyses included TLC, HPLC, ash content, UV/visible spectra, infrared spectra, fluorescence spectra, and two fractions that were subjected to Raman spectroscopy. The isolated sub-fractions were further subjected to bioassays to determine anti-inflammatory activity.

2 Materials and Methods

2.1 Materials

Oxihumate, a semi-synthetic humic acid formulation manufactured from a bituminous coal by a mild wet oxidation process (Cronjé, 1988) was supplied by Enerkom (Pty) Ltd. as a dry black powder of 200 to 400-mesh size. This is a formulated potassium salt of the humic acid fraction of the oxidation product.

All reagents used were of the best available grade or analytical reagent grade. Ammonium hydroxide solution (25%) and 32% HCl was from Merck, (Darmstadt, Germany). All solvents used were of analytical reagent or HPLC grade and supplied by Merck (Darmstadt, Germany) or Sigma Aldrich, (St Louis, MO, USA).

Silica gel 60 pre-coated glass or plastic TLC plates were purchased from Merck Chemicals, (Darmstadt, Germany). Initially both fluorescent (F-254) and non-fluorescent TLC plates were used but the fluorescent plates appeared to reveal more compounds.

Water was 18MΩ water produced from the municipal water supply after processing by an Elga Option 4 system fitted with a carbon polishing- filter.

2.2 Methods

2.2.1 Isolation of the humic acid fraction from Oxihumate

The procedure described here follows the standard acid precipitation method first introduced by Achard (1786) and was found to be reproducible and applicable to humic acids from various sources.

The oxihumate was dried in a drying oven at 110°C for 24 hours and allowed to cool to ambient temperature in a desiccator. Samples of this dried material were used to determine the ash content.

A 200 g aliquot of the dried oxihumate was suspended in 2000 ml distilled water and stirred at ambient temperature with a paddle stirrer for 4 hours at 500 rpm. After stirring was complete the suspension was allowed to stand overnight before transferring the upper layer into 500ml plastic centrifuge bottles and centrifuging at 3500*g* for 30 minutes. The supernatant was decanted and the sediment layer from the original suspension transferred to the centrifuge bottles and the centrifugation repeated. The supernatants of both centrifugation steps were combined and the sediments were washed by resuspending in distilled water and again centrifuging to remove the insoluble fraction that is referred to as oxicoal.

The combined supernatants were slowly acidified with 6M HCl to pH 1.0 while stirring continuously to avoid the formation of a thick slurry, allowed to stand for 2 hours and centrifuged at 3500*g* for 30 minutes to isolate the humic acid fraction. The precipitate was referred to as the humic acid fraction while the supernatant represents the fulvic acid fraction (present in the original oxihumate), which is by definition the acid soluble fraction.

2.2.2 Gravimetric determination of the concentration of humic acid

Determination of the concentration of the oxihumate and humic acids were done gravimetrically as follows;

- three aliquots of exactly 300.0ml were pipetted into large flat stainless steel pans that had been dried to constant weight at 110°C and cooled in desiccators.
- the solutions were then dried by heating in a forced air oven at 110°C for 24 hours followed by cooling in a desiccator. The pans with the residues were weighed and the original pan weight subtracted to give the dry mass of the 300.0ml aliquots. These weights in grams were divided by three to give a mass percentage solid.

The concentration of the various sub-fractions of the humic acids were done in the same way except that preweighed 50mm diameter Petri dishes were used and only 2 – 5ml of each sub-fraction used depending on the available volumes.

2.2.3 Ash content of humic acid sub-fractions

One of two methods was used to determine the ash content of the various isolated sub-fractions of oxihumate. In the case of the sub-fractions where there was more than 10g of material available, percentage ash was determined using 50mm flat open fused silica crucibles in a muffle furnace. The second method used was thermogravimetric analysis for the sub-fractions where there was not sufficient sample to perform the analysis in a muffle furnace.

2.2.3.1 Muffle furnace method

Marked silica ashing crucibles were dried at 110°C and cooled in a desiccator to constant weight. The material of which the ash content was to be determined was dried in a forced air-drying oven at 110°C for 24 hours and cooled in a desiccator. This gave a water free sample. Triplicate aliquots of 3,0g of the dried material (oxihumate or isolated sub-fractions) were accurately weighed into individual crucibles. These were loaded into a muffle furnace and the temperature increased from ambient to 500°C over 1.5 hours. The samples were maintained at 500°C for a further 1.5 hours before increasing the temperature to 850°C over 1.5 hours and maintained at this temperature for 3 hours.

The furnace switched off automatically and was allowed to cool without opening the door. When the temperature had dropped to approximately 200°C the samples were removed and placed in a desiccator overnight. The samples were removed from the desiccator individually and weighed as quickly as possible to avoid any absorption of humidity from the atmosphere.

The percentage ash was calculated by using the formula

$$Percent\_Ash = \frac{\textbf{(Weight of crucible with ash)} - \textbf{(weight of empty crucible)} \times \textbf{100}}{\textbf{Original weight of dried sample used}}$$

2.2.3.2 Thermogravimetric analysis

Due to the limited sample sizes available for the tetrahydrofuran precipitated and tetrahydrofuran soluble fractions these isolated sub-fractions were analysed for ash content using a Mettler TGA 851e thermogravimetric analyser with a gas flow controller. Approximately 10mg of each sample was accurately weighed into 70µl alumina sample pans with lids and heated from 30°C to 1000°C at 40°C/minute under a flow of 50ml/minute air. These samples were held at 1000°C for 15 minutes and the mass of the remaining residue used to calculate the percentage ash. These analyses were done in duplicate.

2.2.4 Sub-fractionation of the humic acid fraction of Oxihumate

The acid precipitated humic acid fraction was air dried at ambient temperature in flat pans in a stream of air. An aliquot of the dried humic acid fraction was then vacuum dried over potassium hydroxide and phosphorus pentoxide pellets before being further analysed. A 10g aliquot of the vacuum dried humic acid was suspended in a minimum amount of 1M NH_4OH and stirred until totally solubilized. This resulted in a fairly viscous dark brown solution that on standing formed a small dark precipitate that was removed by centrifugation at 3500*g*.

The first sub-fraction of humic acid was the 60% methanol (MeOH) precipitate. The volume of supernatant was measured and the required volume of methanol to adjust the concentration to 60% MeOH added slowly in 10 – 20ml portions while stirring continuously. If the methanol addition was not done slowly the mixture would thicken and the total fraction would precipitate, leaving a slightly coloured methanolic solution. After all the methanol had been added, the mixture was stirred for a further 2 hours and then left overnight before isolating the precipitated fraction by centrifugation at 3500*g* for 30 minutes. The 60% MeOH soluble fraction was decanted from the sediment. The sediment was washed once with 10 volumes of 60% MeOH and centrifuged again at 3500*g* for 30 minutes.

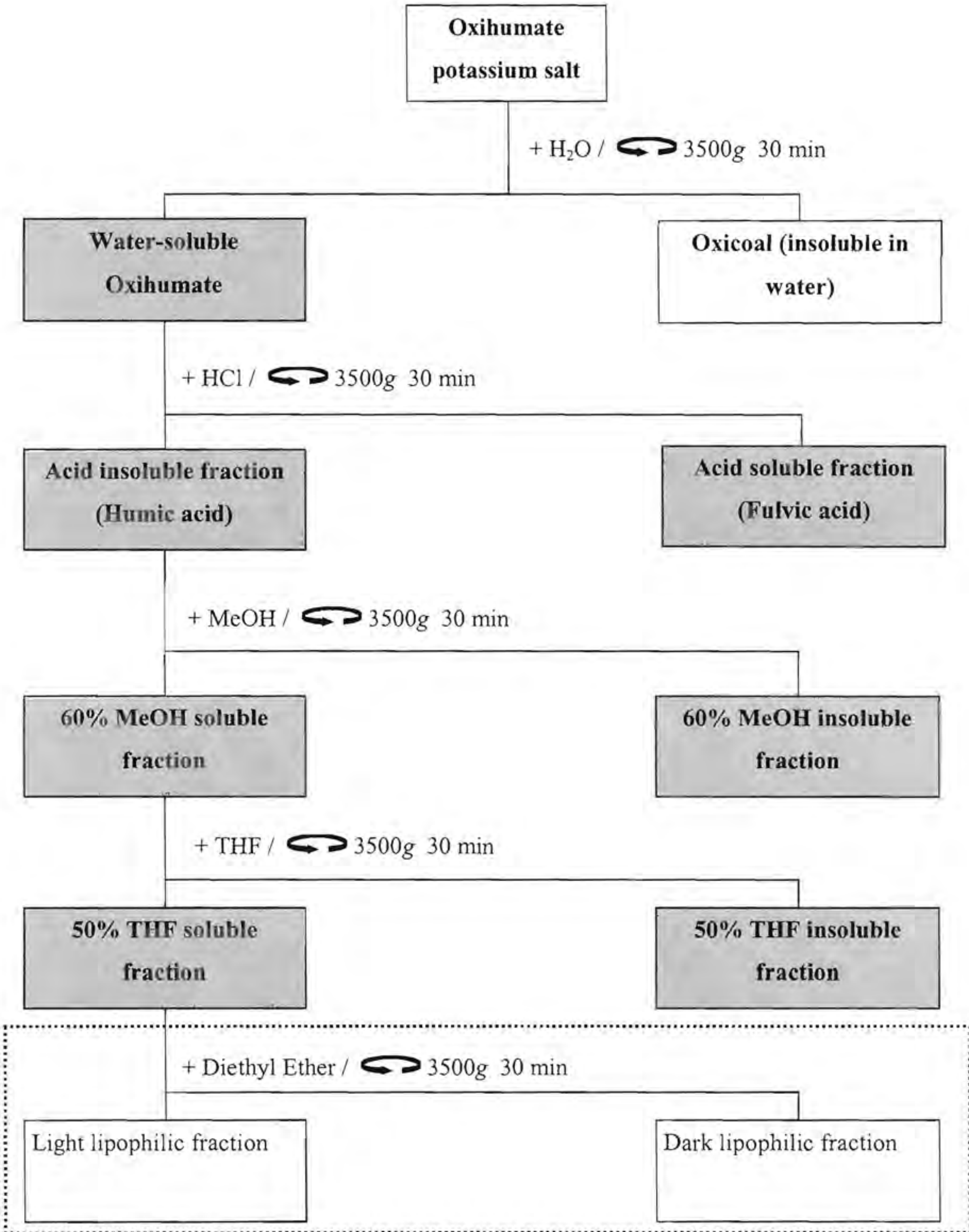

Figure 2-1: Scheme of the sub-fractionation of oxihumate to give 7 fractions. The seven main sub-fractions are shown in grey boxes. The oxicoal and diethyl ether treated lipophilic fractions were not further analysed during this study.

The precipitate was dried at 110°C overnight and weighed to determine the percent dry weight of the fraction. The 60% soluble fraction was adjusted to 50% tetrahydrofuran (THF) by slow addition of THF to the 60% MeOH soluble fraction while stirring continuously. The mixture was allowed to stand overnight before centrifuging at 3500*g* for 30 minutes to remove the precipitated material that was a brown to black amorphous sediment. The supernatant was a reddish-brown solution with a slight green fluorescence. This was air dried at ambient temperature to give a thick dark oil that is referred to as the tetrahydrofuran soluble fraction. The THF soluble fraction was washed with ether to give an orange brown oil that was referred to as the lipophilic fraction. The sub-fractionation sequence is summarized in Figure 2-1. The grey blocks are the sub-fractions that were further analysed.

2.2.5 Thin Layer Chromatography

Thin layer chromatography was performed on each of the isolated sub-fractions of oxihumate using pre-coated silica gel 60 F-254 plates of 20 x 20cm or 5 x 20cm. Initially fluorophore free plates were used but it was found that more compounds could be detected using the fluorescent plates. The glass plates were generally found to give better separations and could be loaded with up to 100μg per spot. Plastic plates could only be loaded with a maximum of 50μg per spot. Generally 2 – 5μl per spot was applied at least 1 cm apart at 1 cm from the lower edge of the plates which were then run in the ascending direction.

Initially several different solvent systems were attempted including those reported in the literature but it was found that the best system depended on the polarity of the fraction being analysed. The plates were run in standard glass TLC chromatography tanks lined with filter paper to ensure atmosphere saturation, in the ascending direction and were generally between 2 and 3 hours per run depending on the polarity of the mobile phase.

The following solvent systems were found to be good for separating the polar sub-fractions of oxihumate from each other:

Ammonium hydroxide solution (25%)-n propanol in the ratio of **7 : 3.**
The tank was pre-saturated for at least 30 minutes prior to developing the TLC.

Water-methanol-acetone-triethyl amine in the ratio of **6 : 6 : 6 : 0.1.**

Acetonitrile-water-ammonium hydroxide (25%) in the ratio **6 : 3 : 1.**

The following solvent systems were found to be good for separating the less polar sub-fractions of oxihumate;

Methanol:chloroform:n-butanol:water:aceticacid in the ratio of **9 : 9 : 3 : 3 : 0.5.**
Filter paper was used to line the tank that was pre-saturated for at least 30 minutes prior to developing the plates (about 2½ hours).

Acetone-n butanol-water-acetic acid in the ratio of **13 : 4 : 2 : 1.**

2.2.6 High Pressure Liquid Chromatography

High-pressure liquid chromatography was carried out on the complete oxihumate as well as the sub-fractions of the humic acid isolated from oxihumate. Although there are some methods published in the literature (Frimmel *et al* 1992; Preuse *et al*, 2000; Woelki *et al.* 1997) none of these were found to give acceptable resolution and reproducibility.

The separations were performed on a Hewlett Packard 1050 HPLC system consisting of a solvent degasser, automated fixed loop injector, column thermostat, quaternary pump, multi-wavelength UV/visible detector and fluorescence detector all of which were controlled from a data station running Chemstation® chromatography software.

All solvents were of HPLC grade and purchased from Sigma Aldrich, (St Louis, MO, USA) or Merck, (Darmstadt, Germany). Different manufacturers columns were used but it was found to have little effect on the chromatograms after about the first 20 injections through the columns. Generally a Phenominex SecurityGuard™ guard column fitted with a C_{18} cartridge was used together with a 150mm x 4.6mm column packed with 5µm C_{18} particles. The columns used were Phenominex "Luna" C_{18} (2), Cosmosil 5C_{18} MS-II (Nacalai Tesque Inc, Japan) and Supelco C_{18} (Sigma Aldrich). Column temperature was maintained at 45°C for all methods.

Detection by the variable multi-wavelength UV detector was simultaneously performed at two different wavelengths each with their own bandwidth and were 230nm with a bandwidth of 20nm for channel A and 270 nm with a bandwidth of 36nm for channel B. The fluorescence detection used 360nm for excitation wavelength and 440nm for the emission wavelength.

Several binary gradient programs were used to elute the injected samples (20µl or 50µl) at a flow rate of 1.0 ml minute^{-1}. The programs were as follows;

Program 1(only the percentage of eluent A is given, the balance was eluent B):

Eluent A: 0.1% trifluoroacetic acid in water

Eluent B: 0.1% trifluoroacetic acid in acetonitrile.

0 – 3.5 minutes	isocratic at 98% A
3.5 – 18.5 minutes	linear gradient to 75% A
18.5 – 24.5 minutes	linear gradient to 75% A
24.5 – 28.5 minutes	linear gradient to 3% A
28.5 – 30 minutes	isocratic at 3% A
30 - 32 minutes	linear gradient to 98% A
30 – 35 minutes	isocratic at 98% A

Program 2 (only percentage eluent A given, the balance is eluent B):

Eluent A: 33 mM Ammonium phosphate in water pH 8.0

Eluent B: acetonitrile.

0 – 3.5 minutes	isocratic at 99% A
3.5 – 20.5 minutes	linear gradient to 44% A
20.5 – 22.5 minutes	linear gradient to 4% A
22.5 – 24.5 minutes	isocratic at 4% A
24.5 – 28.5 minutes	linear gradient to 99% A
28.5 – 35 minutes	isocratic at 99% A

In some cases an automated fraction collector was used to collect the column eluent at 30-second intervals starting at injection time. The fractionated eluent from several consecutive separations of oxihumic acid were combined, concentrated rechromatographed on HPLC and by TLC as described in the section above.

2.2.7 Solid Phase Extraction Cartridges

As it appeared that the recovery of the compounds from the C_{18} HPLC column was low, a small study was done to determine the recovery from SPE cartridges packed with a similar stationary phase. The results were compared to controls where the samples were added directly to the eluent and dried in the same way as the SPE eluents.

For the controls 5.0 ml of a 1.0 mg/ml solution of the water-soluble oxihumate sub-fraction was added to 30 ml of a 1:1 mixture of eluent A: eluent B of the acidic solvent system used for the HPLC separations as described above. The mixture was mixed well and quantitatively transferred to preweighed Petri-dishes (that had been dried to constant weight) and dried under a draught of air before transferring to a drying oven at 110°C for 48 hours.

Five clear plastic C_{18} SPE cartridges containing 500mg packing material were activated by washing with 2 x 2ml MeOH and then washed with 2ml distilled water followed by 5ml of eluent A from the acidic mobile phase system described above. One-millilitre samples of the same 1.0mg/ml solution of the water-soluble oxihumate sub-fraction used for the controls were applied to each cartridge and eluted slowly using 2ml of eluent A then 2ml of a 1:1 mixture of eluent A: eluent B and finally 2ml of eluent B from the same acidic solvent system. The eluents from all five SPE cartridges were combined and quantitatively transferred into a preweighed glass petri-dish, dried under a draft of air to evaporate the organic solvent, then for 48 hours at 110°C, cooled in a desiccator and reweighed. The experiment was repeated 3 times with new SPE cartridges each time.

As the cartridges appeared to retain much of the dark coloured compounds, they were then further washed sequentially with 2ml each of MeOH, acetonitrile, THF, ethyl acetate, chloroform and ether in an attempt to elute these coloured compounds. These eluents were combined and treated the same as the acidic eluents. Finally a slow wash with 4ml of a 1:1 mixture of acetonitrile:ammonium hydroxide was done. The total recovered mass from the cartridges was compared to the weight of the controls. These cartridges were washed again about 24 hours later with the last eluent and this displaced a significant amount of the colour from the cartridges.

2.2.8 Ultraviolet and Visible Spectroscopy

UV/visible spectroscopy was performed on a Perkin Elmer "Lambda 2" dual beam UV/visible spectrophotometer using UV WinLab version 2.0 (Perkin Elmer) software to control the spectrophotometer, collect data and graph the data. Distilled water was used as both the reference and blank solutions. Low-UV transparent quartz cuvettes of 10mm path length were used throughout for the analyses.

Generally the sub-fractions or solutions to be analysed were made up as solutions of 1mg/ml and centrifuged at 3500g for 30 minutes to remove any insoluble material. Further dilution to ensure that the absorbance at 200nm was below 2 absorbance units was done to ensure that the spectra were collected in the linear range of the spectrophotometer. The dilution factor and absorbance was then used to calculate the difference in absolute absorbance at 254nm and to normalize the data for each sub-fraction. Absorbance ratios between 465nm and 665nm for each sub-fraction were performed directly after the scans of the samples by the UV Win Lab software.

2.2.9 Fluorescence Spectroscopy

The seven isolated sub-fractions were made up to concentrations of 1mg/ml each in a 10 mM phosphate buffer at pH 7.2. The absorbance at 254 nm was determined for each sample and those with a high absorbance diluted to that of the sample with the lowest A_{254}. This allowed normalization of the luminescence spectra. The samples were scanned in 1cm quartz cuvettes in a Perkin Elmer LS50B Scanning Fluorescence Spectrophotometer between 320 and 700nm with excitation wavelengths repeated with 5nm intervals from 220nm to 400nm. The data was collected to a data station and analysed with FL WinLab version 3.0 software (Perkin Elmer).

2.2.10 Infrared Spectroscopy

Infrared spectra were obtained from KBr windows made by grinding 0.5 – 2 mg of the powdered samples together with 100mg anhydrous KBr until homogenous and pressing 12 mm disks at 320 $kg.cm^{-2}$ in a hydraulic press. The KBr disks were scanned a minimum of 32 scans between 4000 and 400 wavenumbers with a 2 cm^{-1} resolution on a Bruker "Vector 22" spectrophotometer and the data collected and analysed with OPUS version 2.2 software (Bruker).

2.2.11 Raman Spectroscopy

Raman spectra of two of the humic acid fractions were collected on a Diol XY Raman Spectrometer using the 514.5 nm laser line of a 100mW Coherent Innovate 90 Argon ion laser. A backscattering configuration through a microscope fitted with a 50x objective was used at a resolution of $2cm^{-1}$. A liquid nitrogen cooled CCD detector recorded the signal that was then automatically baseline corrected using Dilor Labspec software.

2.2.12 Energy Dispersive Elemental Analysis (EDS)

The ash from each of the major sub-fractions was further analysed by EDS using a Jeol JSM 5800LV scanning electron microscope fitted with a 20KeV X-ray emission source and a Noran Vantage 3rd generation detector. A take-off angle of 35° and a data collection time of 100 seconds were used. At least three different areas of each sample were analysed due to the highly localized nature of the analysis (only $0.01mm^2$ per analysis). Representative samples of ash from major isolated sub-fractions were prepared by sputter-coating a thin layer of gold over a sample of vacuum dried ash mounted on an adhesive strip applied to a sample carrier. At least 3 different areas of ash surfaces were selected and analysed for all elements with atomic number greater than 20 using the emitted k, l and m X-ray lines. The data was collected and analysed with Noran Vantage System 4 Software and the quantitative weight percentage of each of the elements present calculated.

3 Results

3.1 Isolation of the humic acid fraction from Oxihumate

The humic acid fraction of oxihumate was isolated from the formulated potassium oxihumate salt by initial solubilisation in distilled water. Only about 38% of the material was found to dissolve in water although heating the water or increasing the pH of the solution to pH 8 with ammonium hydroxide or triethyl amine could increase this percentage to about 42%. Prolonged stirring did not increase the solubilized amount significantly.

The precipitation of the humic acid fraction by acidification of the aqueous solution resulted in varying percentages of soluble material with the highest concentration remaining in solution when the acidification was carried out slowly while stirring continuously. Between 88% and 92% of the water-soluble fraction precipitated as humic acid. This fraction was then further analysed and sub-fractionated by differential solubility in mixtures of organic solvent. The acid soluble fraction was by definition fulvic acids.

3.2 Gravimetric determination of the concentration of humic acid

Gravimetric determination of the concentration of humic acid appears to be the only reliable method for this determination as all the chromatographic methods can only give qualitative data about the mixture and the recoveries are not reproducible. Triplicate gravimetric analyses generally had a very small variance (less than 0.5%).

3.3 Ash content of humic acid sub-fractions

The ash content of the different isolated sub-fractions is summarized in Table 2-1. Generally it was found that the ash content of the major fractions did not vary much although the colour and texture of the ash from the different fractions were quite different. Glass formation was evident with the acid soluble fraction and it appears as though the major cation present in this fraction is excess potassium. The other fractions tended to form soft loose ash of variable colour. Thermogravimetric analysis of the THF precipitated and THF soluble sub-fractions both gave hard dark grey residues that appeared to be incompletely ashed despite the high temperature and the time at this high temperature. A typical thermogravimetric analysis of the THF soluble fraction is shown in Figure 2-2. The upper section (A) shows a curve of the actual residual percentage mass of the sample verses time in minutes using the left Y-axis for the scale, while the dotted curve represents the temperature of the sample and uses the scale of the right hand Y-axis. Note that the temperature reaches 1000°C at 25 minutes and was held constant for a further 15 minutes. The lower curve, Box (B), shows the rate of percentage mass change per second during the analysis. It can be seen that there are three distinct temperature ranges during which the mass loss was rapid and that even after 10 minutes at 1000°C a slow mass loss was still observed. The residual mass at the end of the analysis was 30%, which is very high for a pure organic compound.

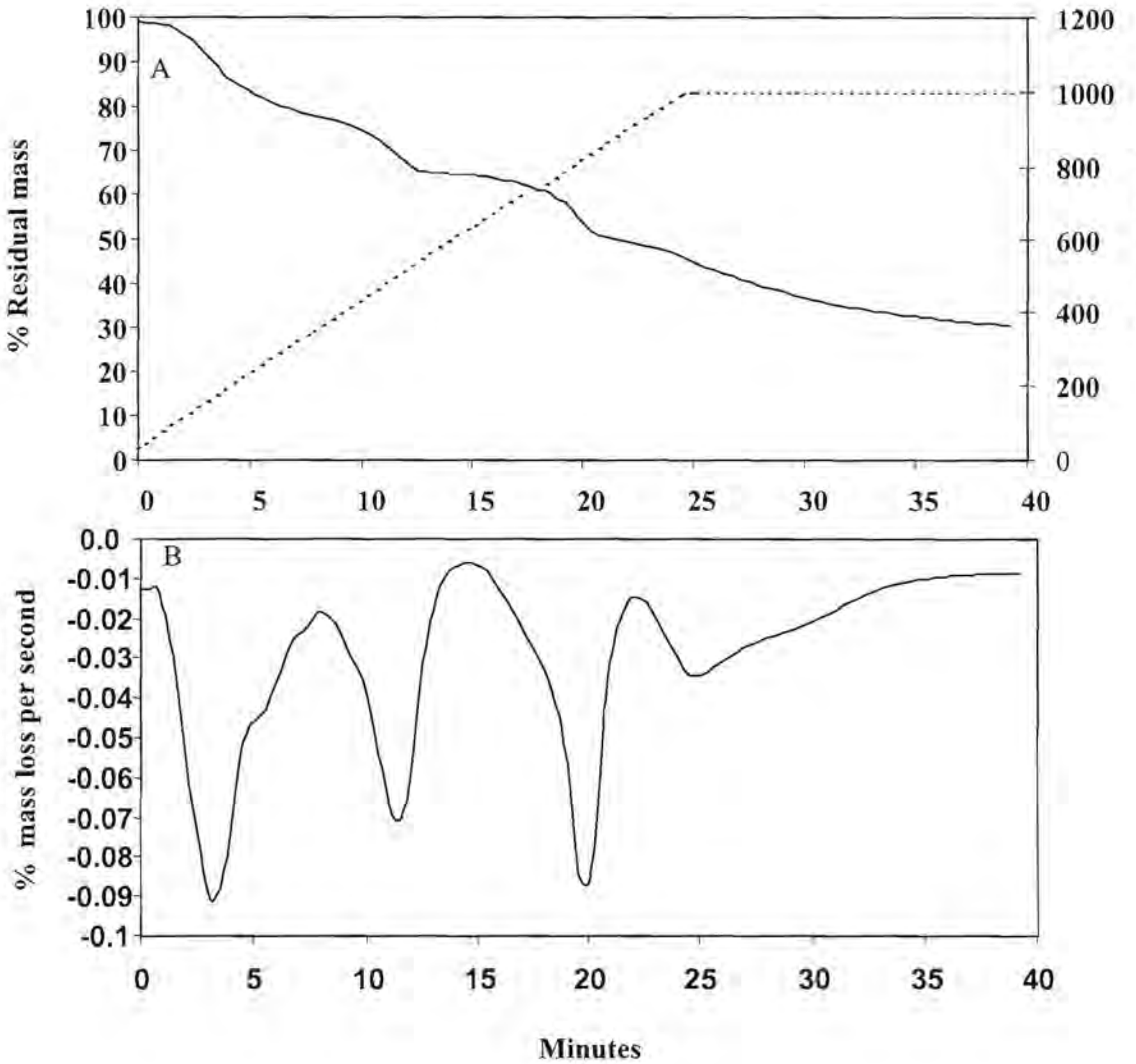

Figure 2-2: A graphic presentation of thermogravimetric analysis parameters for the THF soluble sub-fraction of oxihumate. The solid line in Box A shows the percentage residual mass, using the left ordinate for scale, of the sample while the dotted curve shows the actual temperature of the sample using the right side ordinates scale. The curve in Box B shows the rate of % mass lost per second during heating verses time. Note that there is still significant mass loss for at least 10 minutes after reaching 1000°C at 25 minutes.

Table 2-1: Table summarizing the ash content of different oxihumate sub-fractions. The data is from 5 determinations for each fraction except for the last two sub-fractions where analysis was by thermogravimetric analysis and only two analyses were done (indicated in brackets).

Sub-Fraction	**% Ash**
Potassium Oxihumate (starting material)	26 – 32%
Water-soluble fraction	25 – 34%
Acid precipitate	20 – 22%
Acid soluble fraction	67 – 90%
60% methanol precipitate	17 – 20%
60% methanol soluble fraction	16 – 20%
THF insoluble fraction	13.7% (2)
THF soluble fraction	30.1% (2)

3.4 Sub-fractionation of the humic acid fraction of Oxihumate

The sub-fractionation of oxihumate was found to be fairly reproducible with respect to yields when carried out slowly. Rapid pH changes or organic solvent additions tended to cause a rapid coagulation of the solutions and very little material was then left in solution. Initially triethyl amine salts were made but it was found that later removal of the triethyl amine, which was toxic to the cells used in the bioassay, was very difficult and tedious when compared to ammonia. The most variable fraction was the acid soluble or fulvic acid fraction but this could have been due to large percentages of inorganic material in the fraction. The percentage weight of the seven different fractions and some of the physical characteristics is presented in Table 2-2 below.

Table 2-2: The mass percentages of the different sub-fractions of oxihumate are presented as a percentage of oxihumate and of the fraction from which the sub-fraction was isolated. The physical colour and appearance of each fraction is also indicated

Fraction	Percent of Oxihumate	Percent of parent fraction	Physical appearance
Oxihumate	100	100	Dull black powder
Water soluble fraction	36 - 42	36 - 42	Matt black powder
Acid soluble fraction	6 - 12	25 - 34	Light brown sticky crystals
Acid insoluble fraction	26 - 31	63 – 72	Black powder with a sheen
60% Methanol soluble	3 - 6	15 – 20	Thick dark brown oil
60% Methanol insoluble	20 – 22.5	82 - 90	Lustrous black chunks
Tetrahydrofuran insoluble	1 - 4	20 - 30	Thick dark brown tar
Tetrahydrofuran soluble	2 – 5	72 - 80	Thick red brown grease
Diethyl ether soluble	0.5 – 0.8	12 - 16	Light -orange brown oil

3.5 Thin Layer Chromatography

Thin layer chromatography was the most successful method of quickly gaining information about various fractions or sub-fractions of oxihumate. Several of the separated compounds were visible due to their intense dark colour but many other compounds could not be identified without further visualising techniques. The separated compounds were generally resistant to charring by acids. However, the use of fluorescent TLC plates and UV light inspection permitted detection of several compounds that could not be visualized with general spray reagents. Many compounds in oxihumate showed native fluorescence under long UV light (360nm), varying in colour from bright blue through to a dirty pink. A green and a white fluorescent compound were detected in the THF soluble fraction. The colour of the fluorescence appears to be dependant to some extent on the concentration of the compound in the separated spots with the more concentrated compounds tending to show dark orange to brown fluorescence.

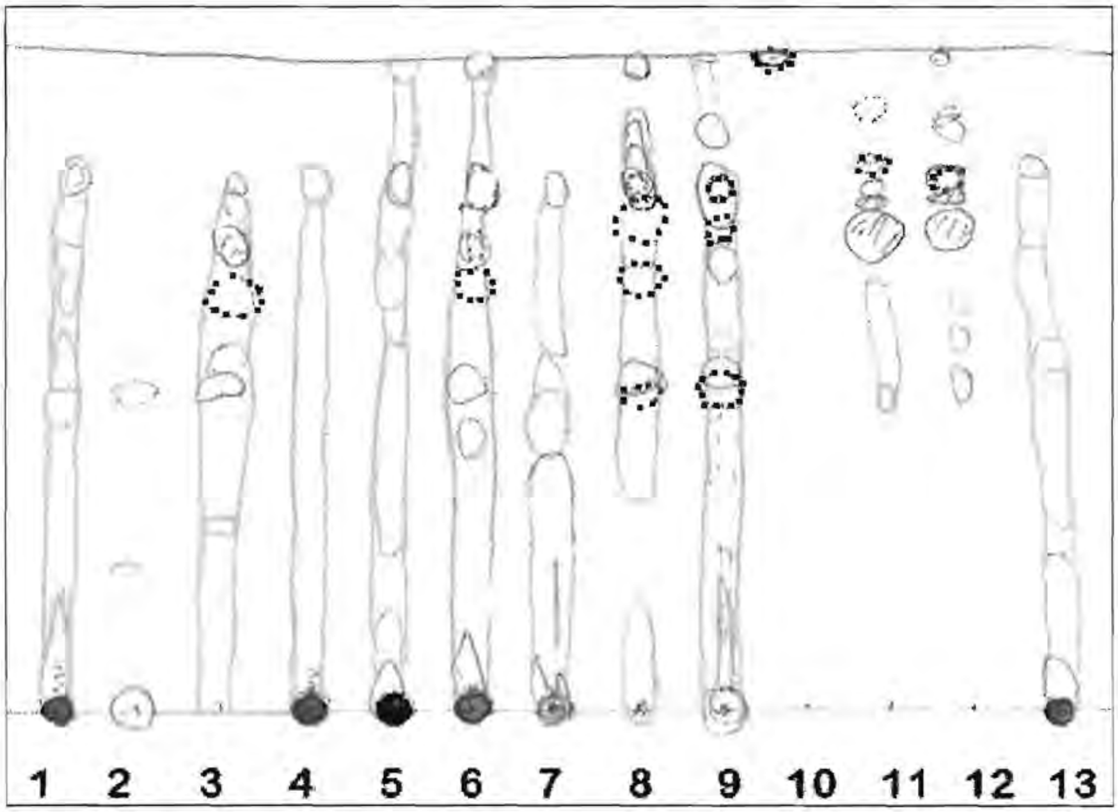

Figure 2-3: TLC separation of the sub-fractions of oxihumate on silica 60 using the methanol: chloroform:butanol:water:acetic acid system. The areas marked by a dotted line stain with I_2 vapour. The stripped areas are strongly blue fluorescent under both 254nm and 360nm light. Lanes are 50μg of: 1, oxihumate; 2, H^+ soluble fraction; 3, methanol soluble from H^+ fraction; 4 H^+ precipitated fraction; 5 MeOH precipitated fraction; 6, MeOH soluble fraction; 7, THF precipitated fraction; 8, THF soluble fraction; 9, Ethyl acetate soluble fraction; 10, petroleum ether soluble fraction; 11, fraction of combined blue fluorescent compounds; 12, chloroform soluble compounds; 13, oxihumate. Lanes 1 & 13 are the same except that lane 1 was not dried prior to developing.

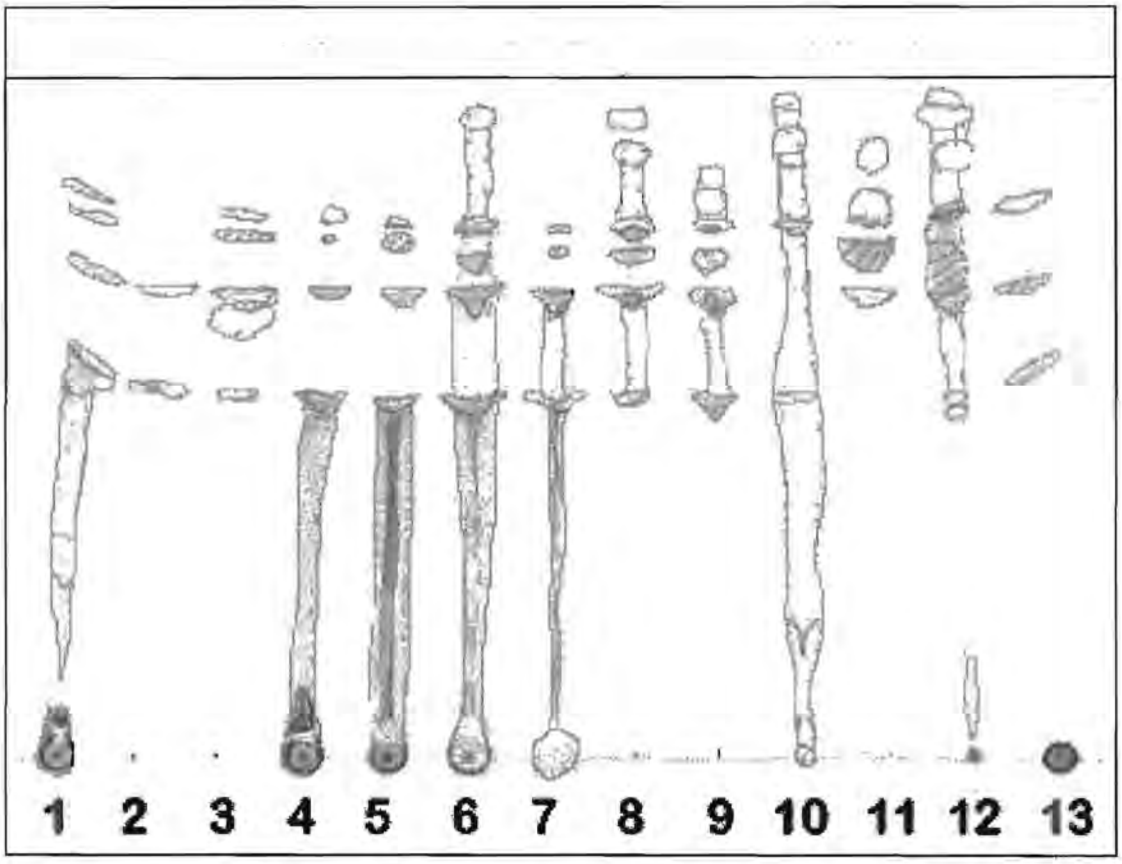

Figure 2-4: TLC separation of the various solvent sub-fractions of oxihumate on silica 60 using the acetonitrile:water:NH4OH system. The areas marked by dotted lines stain with I_2 vapour. Lanes are 50µg of: 1, oxihumate; 2, H^+ soluble fraction; 3, methanol soluble from H^+ fraction; 4 H^+ precipitated fraction; 5 MeOH precipitated fraction; 6, MeOH soluble fraction; 7, THF precipitated fraction; 8, THF soluble fraction; 9, Ethyl acetate soluble fraction; 10, petroleum ether soluble fraction; 11, fraction of combined blue fluorescent compounds; 12, chloroform soluble compounds; 13, oxihumate. Lanes 1 & 13 are the same except that lane 1 was not dried prior to developing.

Typical TLC separations run under different conditions are shown in Figures 2-3 through 2-5, which highlights the differences between the different solvent systems that proved most useful in the separation of the various sub-fractions of oxihumate. The acid solvent system did not move any of the dark compounds from the origin and the non-polar compounds present in the sub-fractions were well resolved with *Rf* values between 0.4 and 0.75. The more polar compounds tended to streak in this development system and were seen as weakly dark-orange fluorescing streaks.

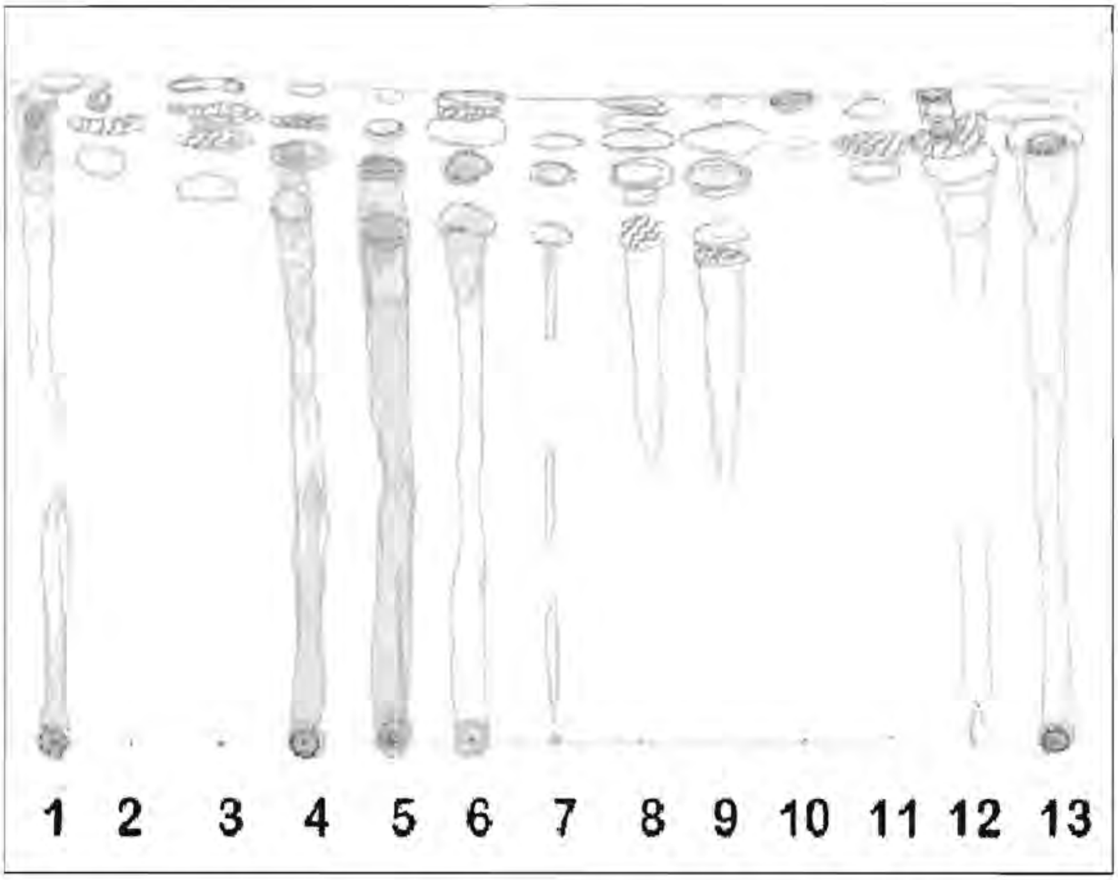

Figure 2-5: TLC separation of the various solvent sub-fractions of oxihumate on silica 60 using the NH4OH propanol system. Note that the majority of the separated compounds run at Rf values of 0.75 and above. Some of the dark coloured compounds have also moved from the origin although they streak severely. Lanes are 50µg of: 1, oxihumate; 2, H^+ soluble fraction; 3, methanol soluble from H^+ fraction; 4 H^+ precipitated fraction; 5 MeOH precipitated fraction; 6, MeOH soluble fraction; 7, THF precipitated fraction; 8, THF soluble fraction; 9, Ethyl acetate soluble fraction; 10, petroleum ether soluble fraction; 11, fraction of combined blue fluorescent compounds; 12, chloroform soluble compounds; 13, oxihumate. Lanes 1 & 13 are the same except that lane 1 was not dried prior to developing.

The alkaline solvent system using acetonitrile:water:ammonium hydroxide appeared to create three solvent fronts on the plates and there were a number of compounds that would move with these apparent fronts. The non-polar compounds tended to run in the zone above the solvent fronts with the fluorescent compounds moving on these pseudo- fronts. There was development of the brown coloured compounds from the origin of the dark coloured sub-fractions but not from the water-soluble oxihumate starting material, even when the applied spot was not permitted to dry on the origin. The dark compounds tended to streak severely and were found mostly at *Rf* values of less than 0.5.

The highly alkaline solvent system using ammonium hydroxide and propanol tended to move more of the dark coloured compounds but at the same time resolution of the less polar compounds was lost with most of these compounds having *Rf* values of 0.75 and above. Many of the dark compounds could be separated into individual spots but there was still a streak of dark colour from the origin to the slowest moving compounds at about *Rf* 0.75. The majority of the dark compounds in unfractionated oxihumate were again not moved from the origin, although there were two dark compounds running at *Rf* 0.89 and 0.94.

Few of the compounds in oxihumate stained with iodine vapour and only a few of the very lipophilic compounds could be visualized with vanillin/H_2SO_4 spray reagent (see Figure 2-6). Charring with sulphuric or chromic acid revealed few compounds, and essentially the same compounds that were visualized by vanillin/sulphuric acid.

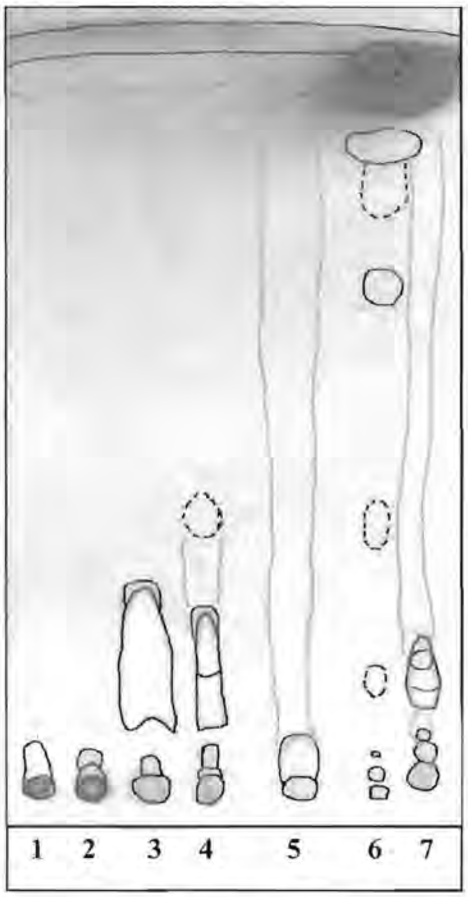

Figure 2-6: A TLC separation of various oxihumate sub-fractions developed using the acetonitrile :water:ammonium hydroxide system and visualized with vanillin/sulphuric acid. Note that only the petroleum ether soluble sub-fraction contains long-chain aliphatic compounds in detectable quantities. Lanes were 50µg of: 1, oxihumate; 2, acid precipitated fraction; 3, MeOH soluble fraction; 4, acetone soluble fraction; 5, chloroform soluble crystalline fraction; 6, petroleum ether soluble fraction; 7, chloroform soluble oil fraction.

3.6 High Pressure Liquid Chromatography

HPLC separations of humic acids on reverse phase columns varied according to the pH of the eluent. A significant fairly sharp pass-through peak (sometimes followed rapidly by a second sharp peak) dominated the first minutes of the chromatogram and was followed later by a large, broad, almost featureless peak eluting over an extended period of several minutes and which was the major feature of the chromatograms of most of the oxihumate sub-fractions when using the alkaline elution system. Several undetected UV transparent compounds were found to elute after this major peak when a fraction collector was used to collect the eluent and the fractions dried under a stream of air. These compounds were found to be waxy lipophilic compounds undetected by monitoring the eluent at a wavelength of as low as 220nm. The dominant peak was a very dark coloured compound or mixture of compounds.

When using the acidic elution system the pass-through peak was very small to insignificant. The main dominant peak that eluted from about 10 minutes and later was better resolved when using the acidic elution than the alkaline conditions, yet still appeared as a broad flat peak with small resolved peaks superimposed on it.

Figures 2-7 and 2-8 illustrate typical chromatograms of the water-soluble fraction of oxihumate separations on the same reverse phase column under the acidic and alkaline elution conditions as described in the materials and methods section above. The chromatograms shown are superimposed traces of a fluorescence detection channel and the two separate UV detection channels at 230±15nm and 270±20nm respectively.

In contrast to previous reports, rechromatography of single peaks isolated from the fractionated HPLC eluent resulted in essentially the collected peaks being eluted, although these peaks appear to be no better resolved than in the original separation of the oxihumate itself. Figure 2-9 clearly illustrates this phenomenon for four of the recombined HPLC fractions collected. TLC analysis of these isolated fractions on normal phase silica plates did however indicate that several components are still present in all these peaks, confirming that they are still not pure compounds.

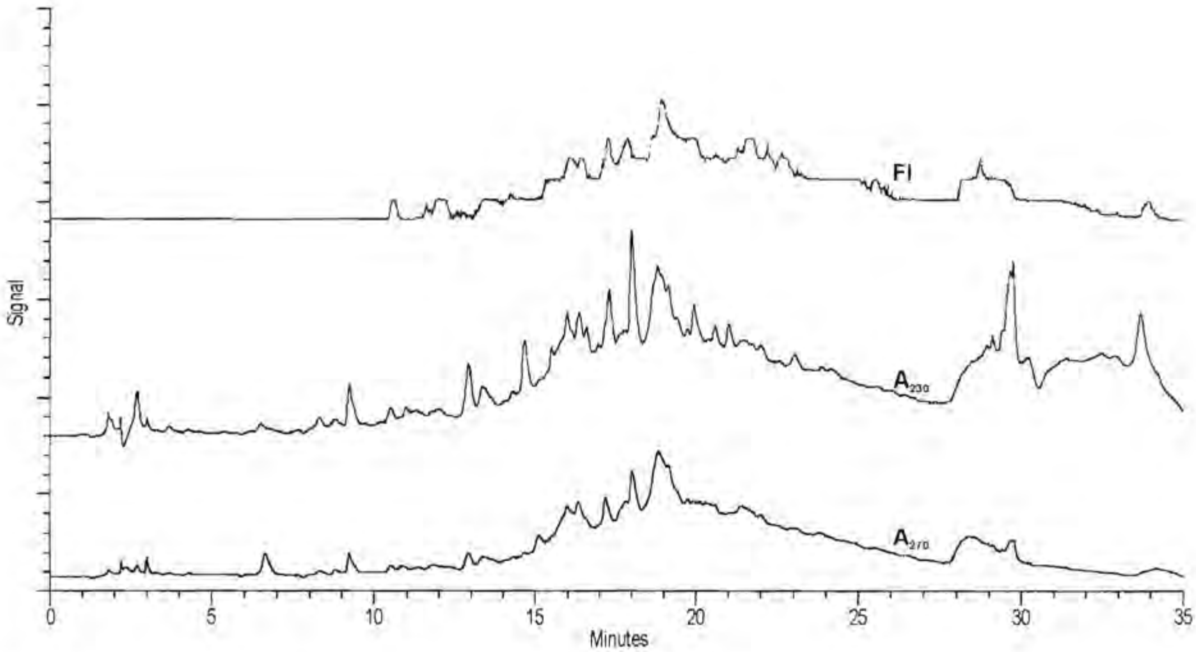

Figure 2-7: HPLC separation of the water-soluble fraction of oxihumate on a C_{18} column using the 0.1% TFA buffer system. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 (**A_{230}**) and UV at 270nm (**A_{270}**). A new C_{18} column was used for this separation.

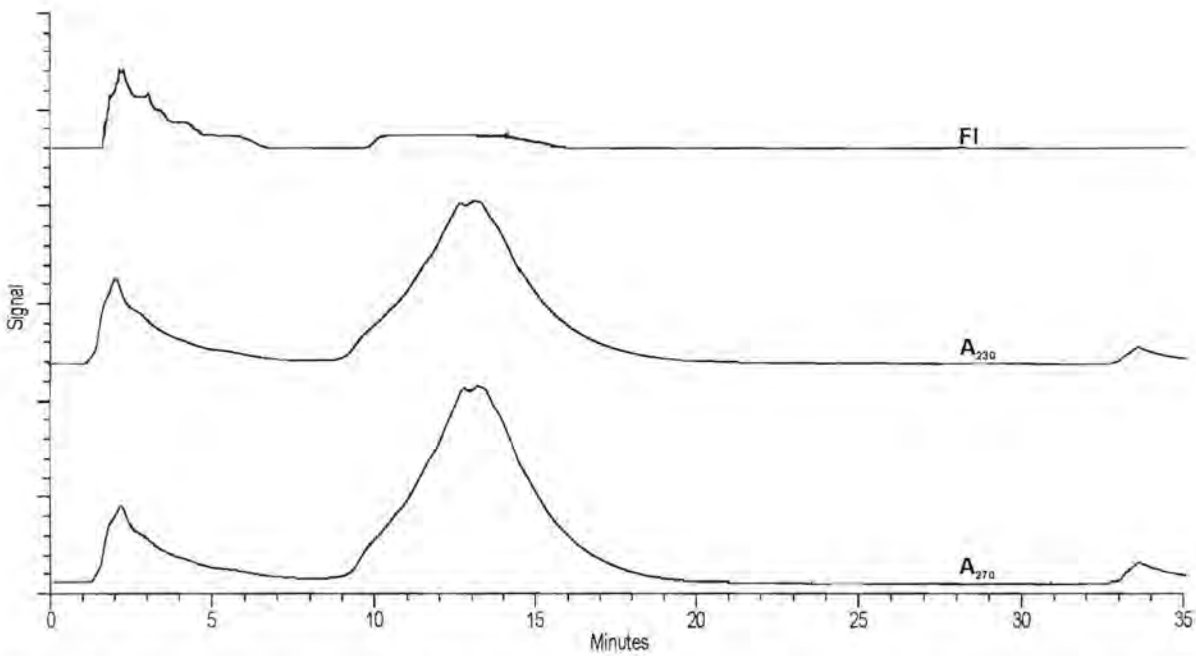

Figure 2-8: HPLC separation of the water-soluble fraction of oxihumate on a C_{18} column using the pH 8 buffer system. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 (**A_{230}**) and UV at 270nm (**A_{270}**). The same column as used for the acidic system in Figure 2-7 was used for this separation.

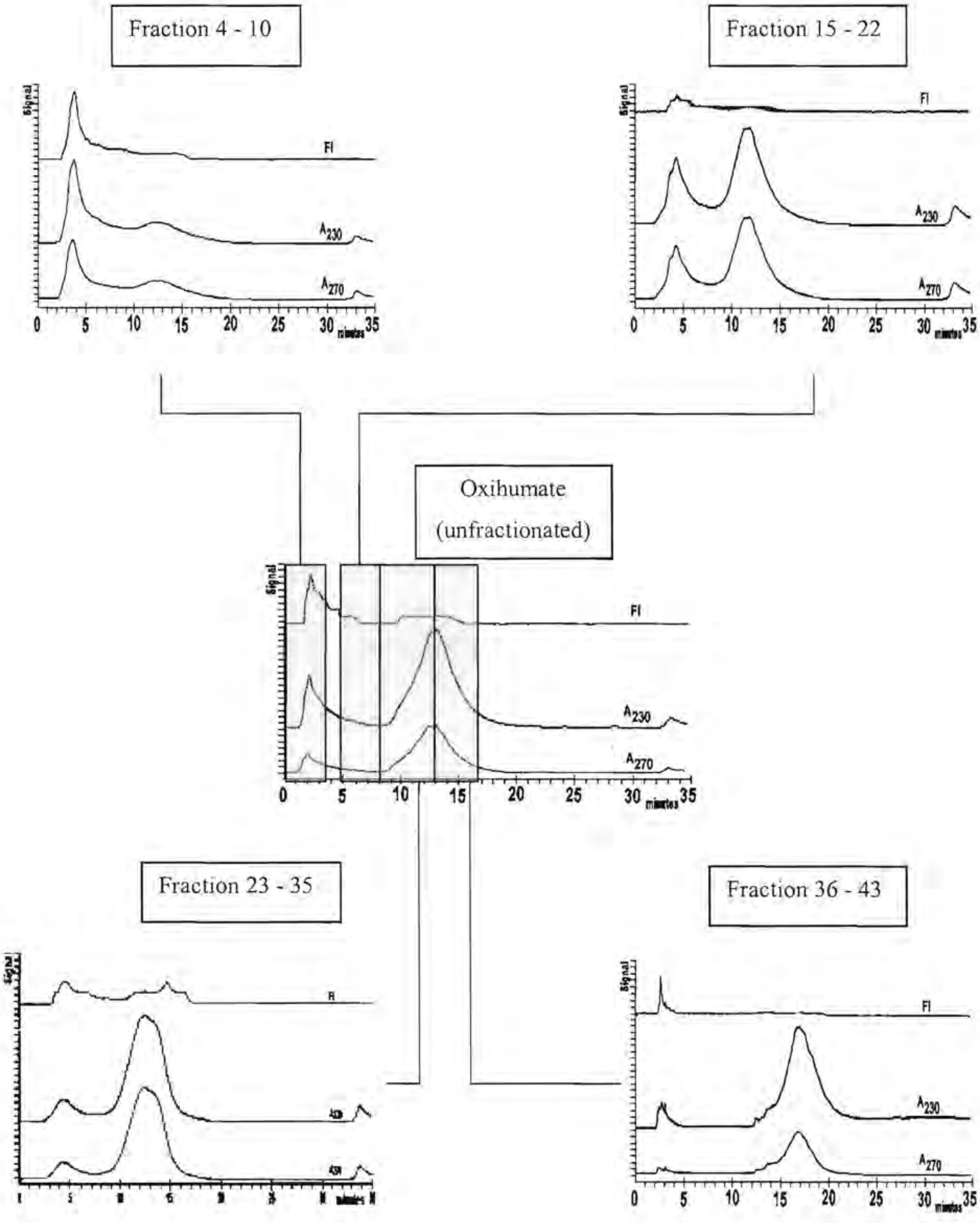

Figure 2-9: Chromatograms demonstrating the separations achieved when re-chromatographing fractions collected during the HPLC separation of oxihumate (centre chromatogram). The chromatograms are labelled with the fraction numbers that were combined before rechromatography.

Figure 2-10 illustrates a TLC separation of the isolated HLPC eluted peaks and it can be seen that there are still several compounds in each of the isolated HPLC peaks.

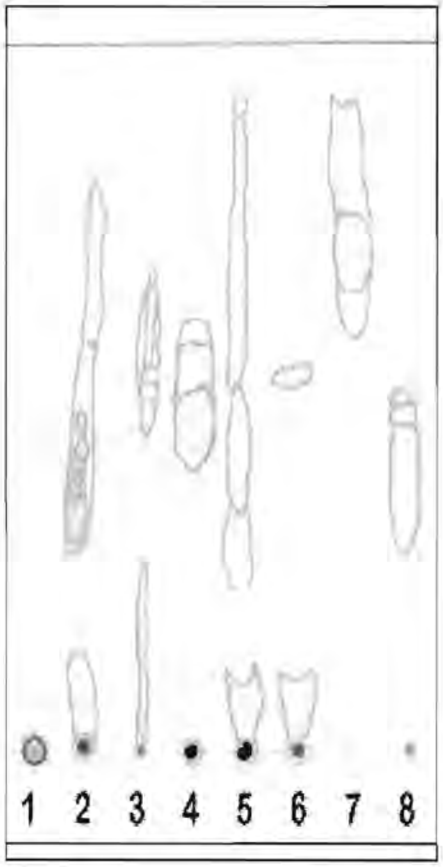

Figure 2-10: A TLC separation of humic acid fractions eluted from a C_{18} HPLC column. Each lane has 50µg of the following sub-fractions 1, oxihumate; 2, fractions 4 – 10; 3, fractions 11 – 15; 4, fractions 15 – 23; 5, fractions 24 – 34; 6, fractions 35 – 44; 7, fractions 45 – 66; 8, column wash.

Sub-fractions isolated from oxihumate by differential solubility were also separated by HPLC using ODS columns and were found to have most of the peaks seen in the chromatograms of the starting material although the ratios of the peak areas varied greatly. The more lipophilic fractions were free of the pass-through peaks but the more polar fractions always exhibited lipophilic peaks. Figures 2-11 through 2-17 illustrate the HPLC reverse phase separations of the seven oxihumate sub-fractions isolated by differential solubility. Differences in peaks that eluted at similar retention times but occurring in chromatograms of different sub-fractions could sometimes be demonstrated by the differences in the fluorescent properties of the peaks.

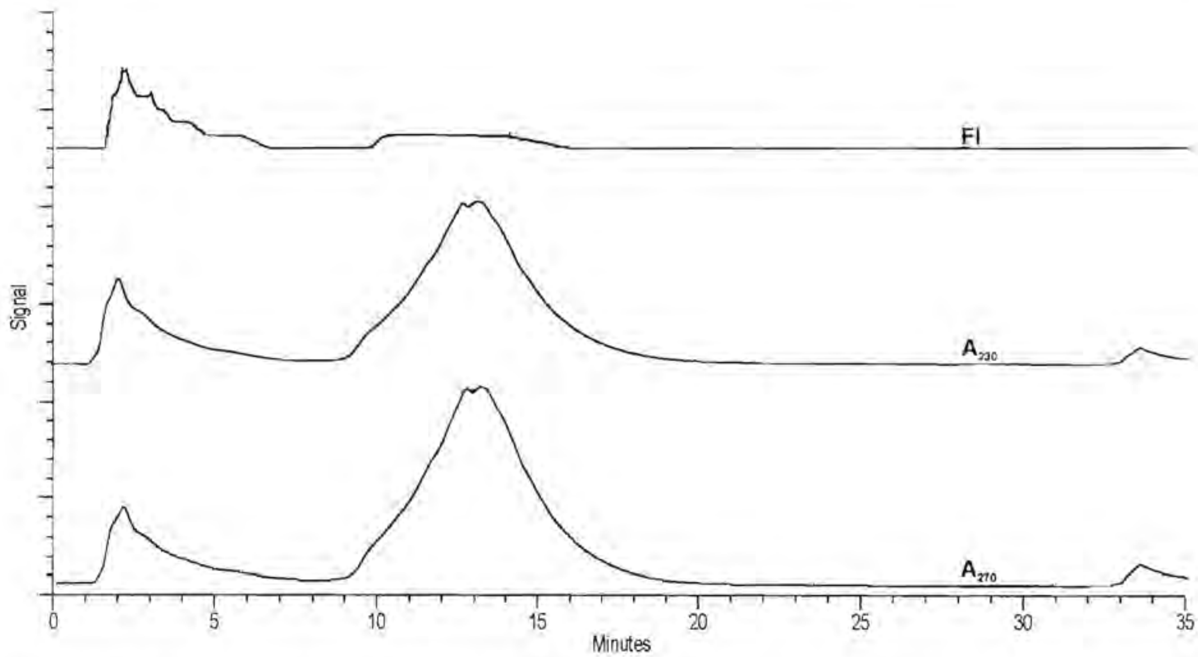

Figure 2-11: HPLC separation of the water-soluble fraction of oxihumate (1) on a C_{18} column using the pH 8 buffer system. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 (**A_{230}**) and UV at 270nm (**A_{270}**).

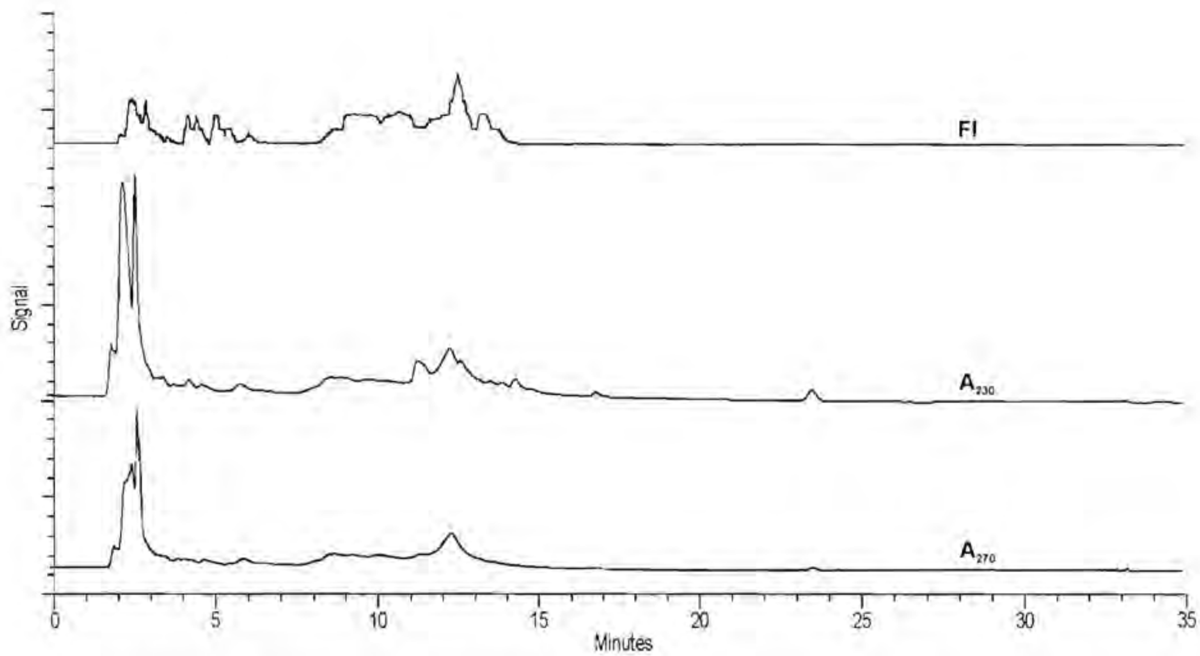

Figure 2-12: HPLC separation of the acid soluble fraction of oxihumate (2) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 (**A_{230}**) and UV at 270nm (**A_{270}**). The dominant humic acid peak does not appear in this chromatogram.

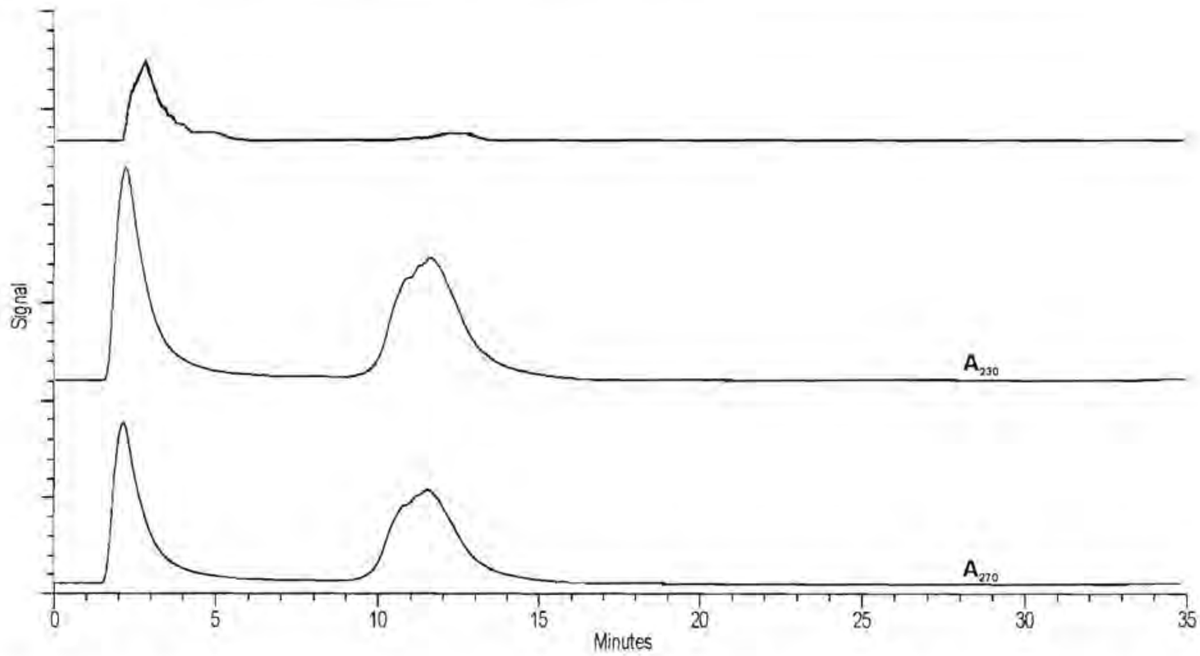

Figure 2-13: HPLC separation of the acid insoluble fraction of oxihumate on a (3) C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (FI); UV at 230 (A_{230}) and UV at 270nm (A_{270}). The UV traces show a narrower main peak than that of the water-soluble fraction and the fluorescent peak on the void appears as the major peak.

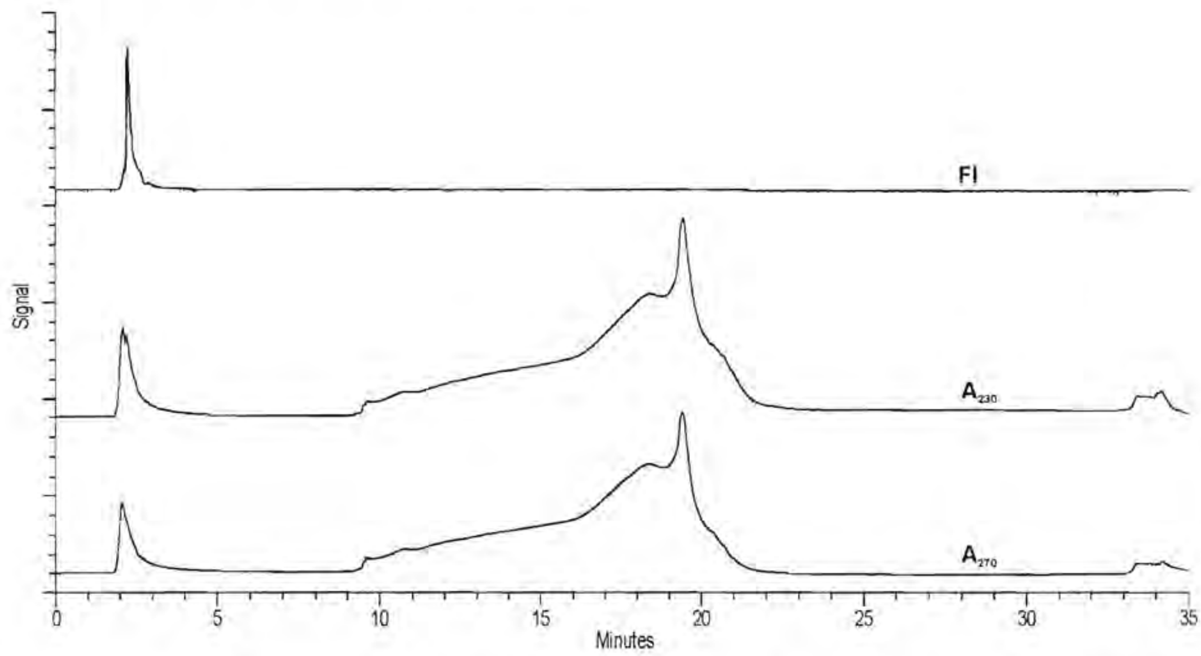

Figure 2-14: HPLC separation of the 60% methanol insoluble fraction of oxihumate (4) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (FI); UV at 230 (A_{230}) and UV at 270nm (A_{270}). The only fluorescent compounds elute on the solvent front and the dominant peak of the humic acids is present with a sharp peak superimposed on this peak.

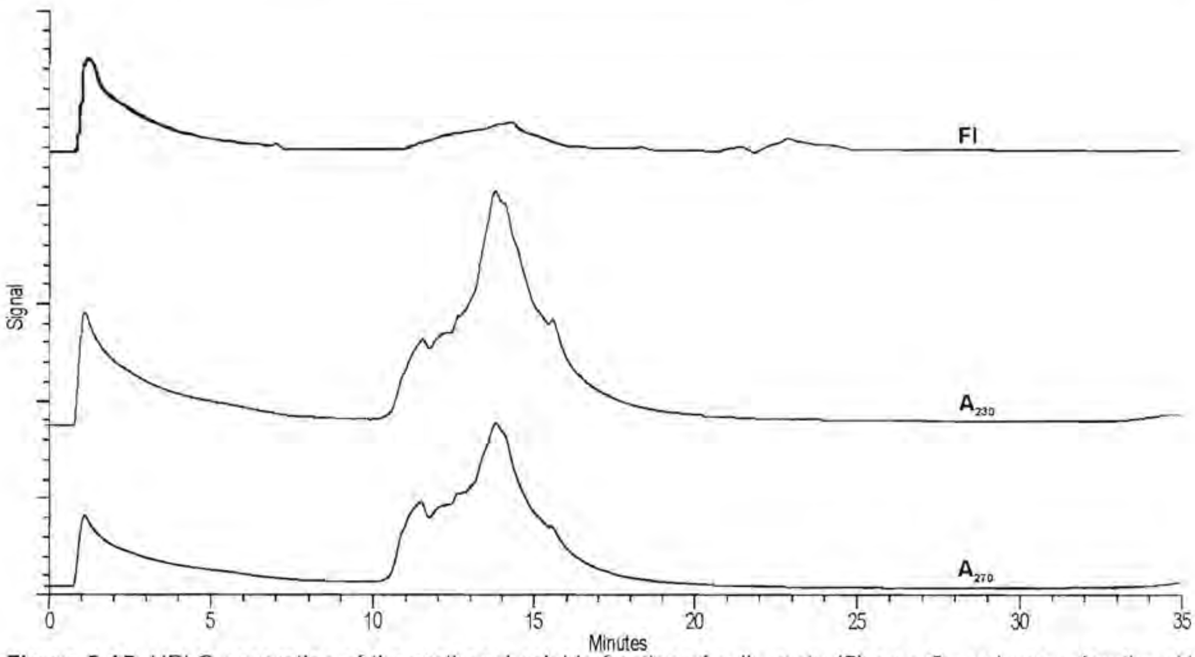

Figure 2-15: HPLC separation of the methanol soluble fraction of oxihumate (5) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (FI); UV at 230 (**A_{230}**) and UV at 270nm (**A_{270}**). The main fluorescent peak elutes on the solvent front with a broad unresolved non-fluorescent peak elutes between 10 and 15 minutes.

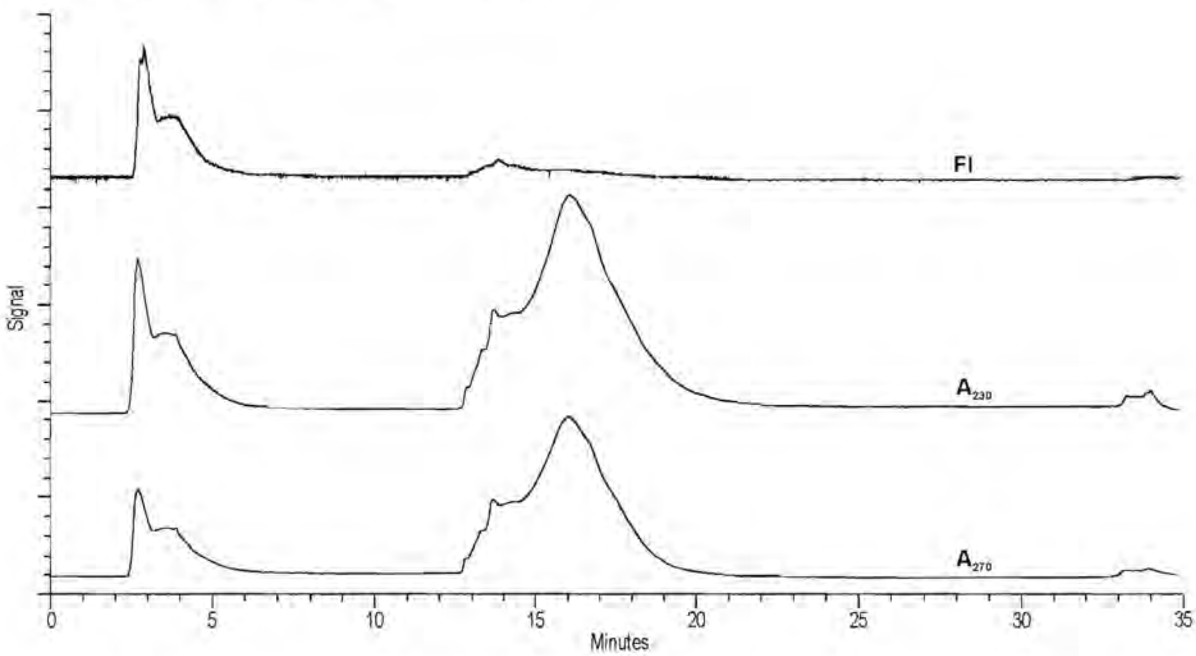

Figure 2-16: HPLC separation of the tetrahydrofuran insoluble fraction of oxihumate (6) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (FI); UV at 230 (**A_{230}**) and UV at 270nm (**A_{270}**). The dominant peak is again the large peak eluting between 12 and 20 minutes – a shift in elution times of the other dominant peak of the fractions. A sharp fluorescent peak elutes on the solvent front implying the presence of polar compounds.

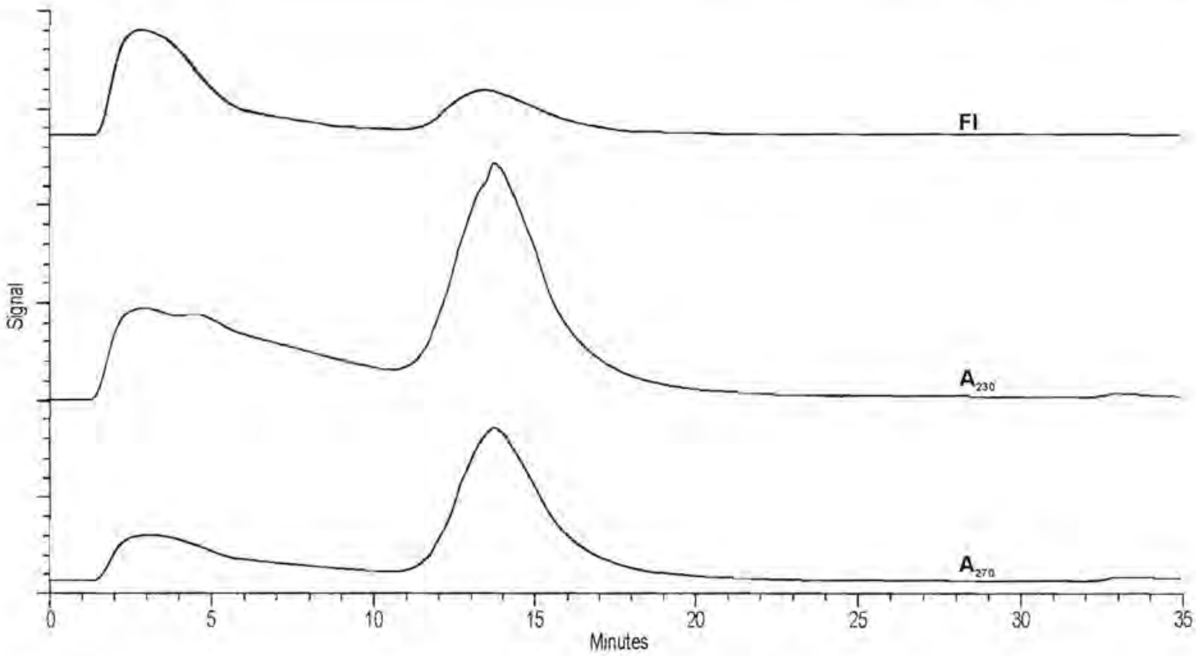

Figure 2-17: HPLC separation of the tetrahydrofuran soluble fraction of oxihumate (7) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 (**A_{230}**) and UV at 270nm (**A_{270}**). The dominant peak appears sharper and elutes slightly earlier than the THF precipitated fraction. An early eluting strongly fluorescent peak is present in addition to the main peak.

3.7 Solid Phase Extraction

The results were a combination of the eluents from five SPE cartridges as initial results indicated that the method could not give accurate results when the recovered material was less than 1mg as the weighing error then became too large.

The recoveries of the controls where samples were added to the mobile phase and dried were all more than 100%. The acid eluent recovery from the SPE cartridges was about 60 – 70% without correcting for any increase in the recovery seen in the control samples. The results are summarized in Table 2-3 below.

Negligible amounts of dark coloured compounds were recovered from the organic solvent washes of the cartridges although a transparent waxy residue accounting for about 20% of the mass was recovered in this eluent. Some dark compounds were eluted by the ammonium hydroxide:acetonitrile solution, which were clearly visible, but the recovered mass was too small to determine. Interestingly, elution of the cartridges with the same eluent after a further 24 hours displaced more of the dark colour but could not displace all the retained coloured material.

Table 2-3: Summary of the recoveries of the water-soluble oxihumate sub-fraction from C_{18} SPE cartridges. The controls were performed by adding 1.0ml sample to the acidic eluent and drying the samples as for the SPE eluents. The experiments are all a combination of the eluents from 5 separate SPE cartridges.

	Acid elution (%)	Organic phase elution (%)	Ammonium hydroxide: acetonitrile (%)
Control 1	122	-	-
Control 2	132	-	-
Control 3	116	-	-
Experiment 1	62 Dark brown	16 White wax	Too small to determine Dark brown/black
Experiment 2	72 Dark brown	20 White wax	Too small to determine Dark brown/black
Experiment 3	66 Dark brown	20 White wax	Too small to determine Dark brown/black

3.8 Ultraviolet and Visible Spectroscopy

Although limited information could be derived from the UV/visible spectra it was found that there were some distinct differences in the spectra of the different sub-fractions derived from the same humic acid starting material. The dilution factors required to ensure that the absorbance at 200nm was below two Absorbance Units was different for each fraction and is summarized in Table 2-3. The $E_{4/6}$ absorbance ratios were calculated for the diluted solutions after normalising the absorbance at 254nm. These ratios are summarized in Table 2-4. Although the general trends of the absorbance spectra were similar for all the oxihumate sub-fractions with the absorbance decreasing logarithmically with increasing wavelength there were distinct differences in the spectra. This is illustrated in Figure 2-18 where the UV spectra are overlaid in a staggered pattern to enable differentiation of the different sub-fractions spectra. It can be seen that the acid soluble and the THF soluble sub-fraction have a faster decrease in absorbance than all the other sub-fractions and that the THF sub-fraction has a distinct broad shoulder between 250 and 300nm. This shoulder can only just be discerned in most of the sub-fractions and does not appear to influence the $E_{4/6}$ ratio that is commonly used to characterize humic acids.

Table 2-4: Dilution factors required to normalize the absorbance at 254nm of 1mg/ml solutions and the absorbance at 465nm, 665nm and the E4/6 ratio.

Sub-fraction	Dilution factor	A_{465}	A_{665}	$E_{4/6}$
Oxihumate	24	0.135	0.054	2.513
Acid solubles	1	0.066	0.044	1.514
Acid precipitate	27	0.108	0.028	3.935
Methanol precipitate	30	0.212	0.107	1.981
Methanol solubles	12	0.117	0.055	2.108
THF precipitate	3	0.140	0.058	2.410
THF solubles	8	0.093	0.052	1.777

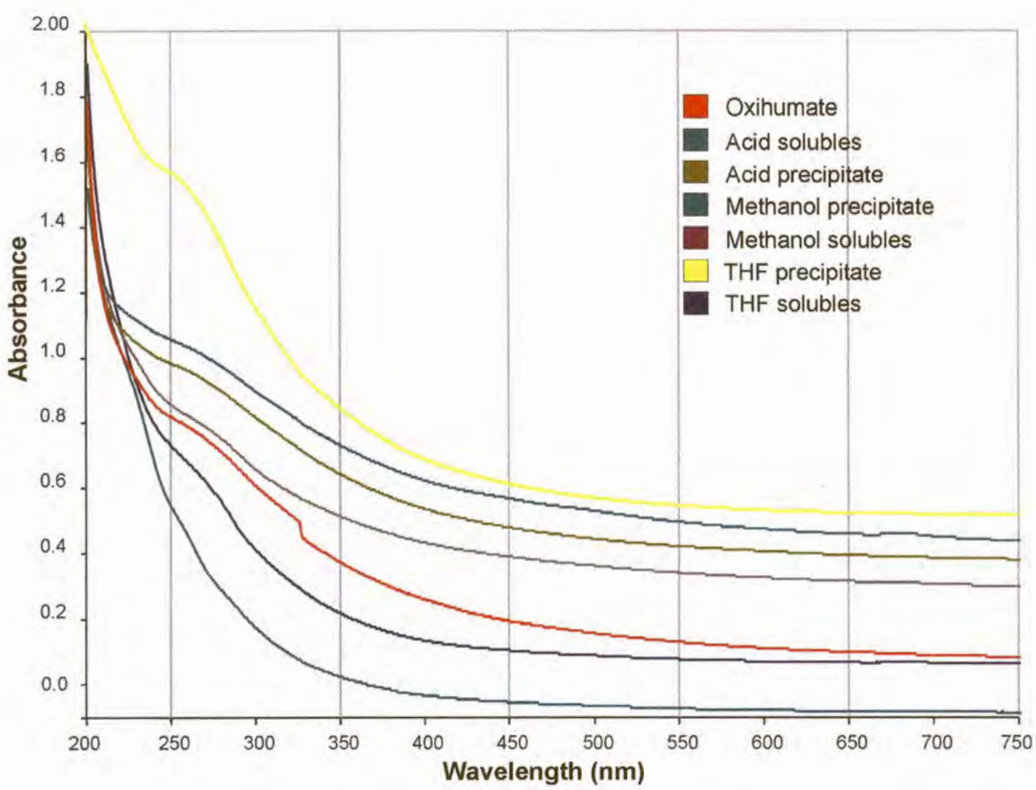

Figure 2-18: Staggered UV/visible spectra of the seven sub-fractions of oxihumate. The absorbencies were normalized at 254nm. Note the similarity and featureless absorbance above 350nm for most of the sub-fractions.

3.9 Fluorescence Spectroscopy

Fluorescence spectroscopy was performed on each sub-fraction at 36 different excitation wavelengths and a 3D emission profile generated from the individual scans using the FL WinLab software version 3.0, the same software that was used to control the instrument and collect the raw data.

It was found that the fluorescent intensity of the various sub-fractions of oxihumate varied greatly from each other although all 7 of the isolated sub-fractions exhibited a maximal emission as a broad almost symmetrical peak centred on 432nm. Figure 2-19 is an overlay of all seven isolated sub-fractions to illustrate the different fluorescent maxima when the samples were excited with light at 280nm.

The acid soluble sub-fraction, that would represent the fulvic acids, had a fairly intense fluorescence centred on 432nm with a broad shoulder appearing around 385nm when the excitation wavelength was at about 280nm.

The THF precipitated sub-fraction showed an intense fluorescence with the main emission centred slightly higher at 440nm This emission wavelength appeared to be independent of the excitation wavelength as the same emission peak was seen over a very wide range of excitation wavelengths.

The THF soluble sub-fraction was the most fluorescent sub-fraction and required a further dilution of at least 1:30 more than the other fractions to remain within the scale limits set for the remaining fractions. The main fluorescent emission wavelength for the THF soluble sub-fraction was centred on 375nm with a second emission peak centred on 432nm and a slight shoulder was seen at around 480nm.

The THF precipitated and THF soluble sub-fractions both revealed the 480nm shoulder when excited at wavelengths below 300nm. The methanol soluble sub-fraction, like the THF soluble sub-fraction, had a fluorescent emission peak centred at 375nm although it was broad and weak and was a secondary peak in the methanol soluble sub-fraction. The fluorescent intensity could be severely affected by the dark colour of the sub-fractions and the fact that this fluorescent emission falls within the region where intense UV absorption occurs. This could account for the low fluorescence displayed by the darker sub-fractions.

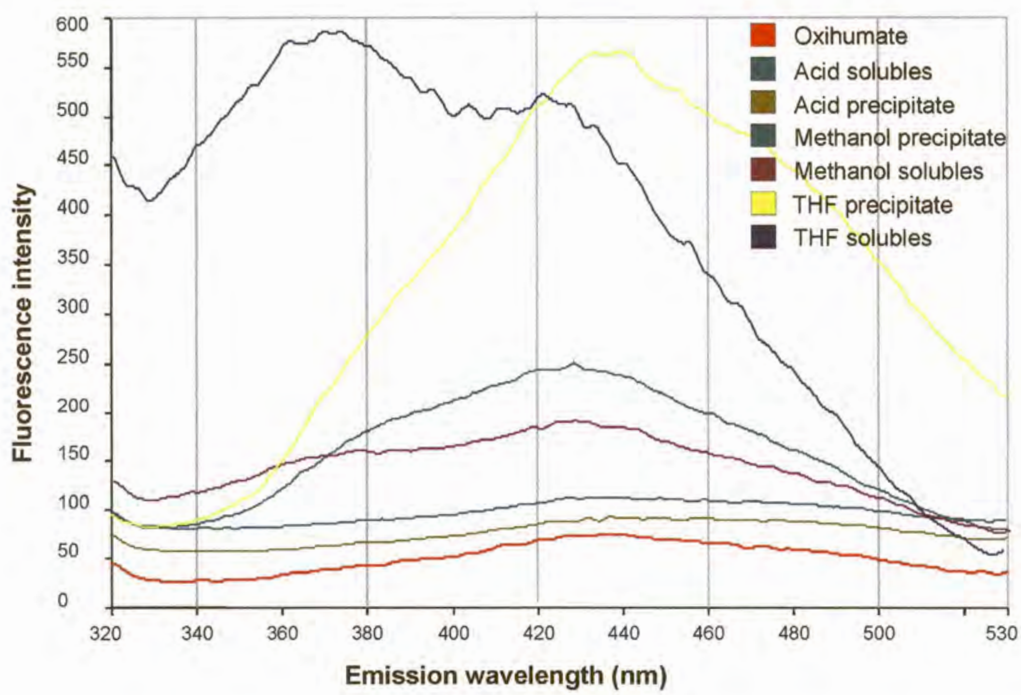

Figure 2-19: Fluorescent emission spectra of the seven sub-fractions of oxihumate, all recorded using an excitation wavelength of 280nm.

3.10 Infrared Spectroscopy

Infrared spectroscopy of the seven different sub-fractions provided the most chemical structural information. Except for the THF soluble sub-fraction, the similarity between the spectra of the different sub-fractions was obvious, with the main absorption bands appearing in all the sub-fractions.

The broad band from 3500 to 3200 cm^{-1} is due to hydrogen bonded υ(O-H) stretch of carboxylic, alcohol and phenol groups. The small bands at 2950 – 2840 cm^{-1} are the asymmetric and symmetric υ(C-H) stretch of aliphatic chains.

The intense bands at 1720 cm^{-1} υ(C=O) seen in the THF soluble sub-fraction can be seen as an insignificant inflection on the oxihumate spectrum but becomes more visible in the sub-fractions isolated from the organic solvent modified solutions. This band is assigned to the carbonyl groups of α, β unsaturated or aromatic esters or for ketones.

The very intense band with a maximum absorption between 1570 to 1685 cm^{-1} is the dominant peak in all the dark coloured sub-fractions and is probably due to υC=O of carboxylic acid salts that overlaps with the υ(C=C) of aromatic and conjugated double bonds. Alkyl chains are associated with bands that occur between 1400 - 1462 cm^{-1} for $\delta(CH_2)$ and CH_3 deformations at 1375 cm^{-1}.

Phenolics are indicated by the bands around 1250 - 1280 cm^{-1} due to υ(C-O) and the small sharp band resulting from free υ(O-H) at 3690 cm^{-1} that is at the high extreme for this stretching absorbance, occurring when there is alkyl substitution of the phenolic ring. The sharp but medium intensity bands between 1030 - 1095 cm^{-1} are due to υ(C-OH) and υ(C-O-C) of ethers and alcohols with the lower wavenumbers associated with aromatic ethers.

The combination of bands from 400 – 800 cm^{-1} would point towards terminal double bonds in alkanes or substituted phenolic compounds. As a complication, Si-C bonds and several covalent sulphur compounds demonstrate absorption in this region. All these bond types could well occur in oxihumate.

Figure 2-20 is a staggered overlay of infrared spectra of all 7 sub-fractions of oxihumate and illustrates the strong similarity of spectra, implying that the different sub-fractions must be composed of very similar structural entities. The only two spectra that have obvious differences are the acid soluble sub-fraction that would be the fulvic acid sub-fraction and the THF soluble sub-fraction. Both these sub-fractions lack the strong absorption band at 1587 cm^{-1} but do show a strong absorption band at 1720 cm^{-1} indicating the presence of carbonyl compounds that are not carboxylic salts.

The broad absorption bands, especially in the fingerprint region, would indicate that there is a mixture of compounds with similar but not identical structures. The fact that all the dark coloured sub-fractions showed an intense absorption band over a broad region from above 1720 cm^{-1} to below 1550 cm^{-1} and that the fulvic acid and THF soluble sub-fractions had less intense absorption at the upper limit of this region (above 1700 cm^{-1}) could indicate that the latter two sub-fractions account for the absorbance at the higher wavenumbers and can be extracted from the complex due to a weaker association with the bulk of the humic acid complex.

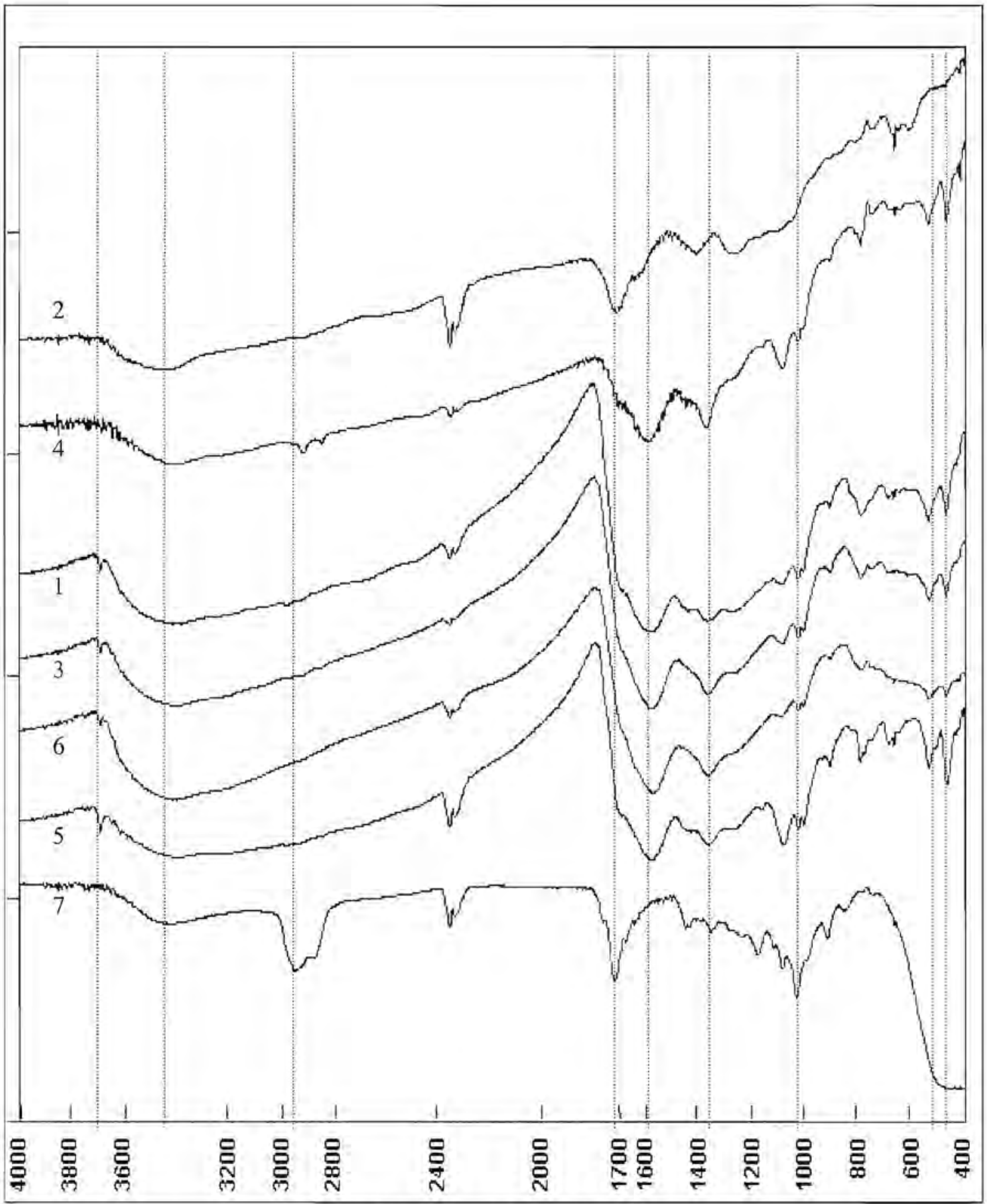

Figure 2-20: Staggered overlays of the infrared spectra of the seven sub-fractions of oxihumate. The spectra are for the following sub-fractions: 1, oxihumate; 2, acid soluble; 3, acid precipitate; 4, MeOH precipitate; 5, MeOH soluble; 6, THF precipitate; 7, THF soluble. The vertical dotted lines indicate peaks that are common to most spectra and correspond to 3695 cm^{-1}, 34 41 cm^{-1}, 2952 cm^{-1}, 1720 cm^{-1}, 1596 cm^{-1}, 1375 cm^{-1}, 1038 cm^{-1}, 537 cm^{-1}, 457 cm^{-1}. The peaks at 2358 and 2343 cm^{-1} are due to CO_2.

3.11 Raman Spectroscopy

Raman spectroscopy did not reveal any useful information with regard to chemical structures or functionality. A problem experienced with this technique was the intense fluorescence exhibited by the oxihumic acid under the argon ion laser. The greater the fluorescence the less defined the spectra became. Another observation was that the small area of the samples of the oxihumate sub-fractions exposed to the intense laser beam appeared to bleach to an off-white colour during the analysis.

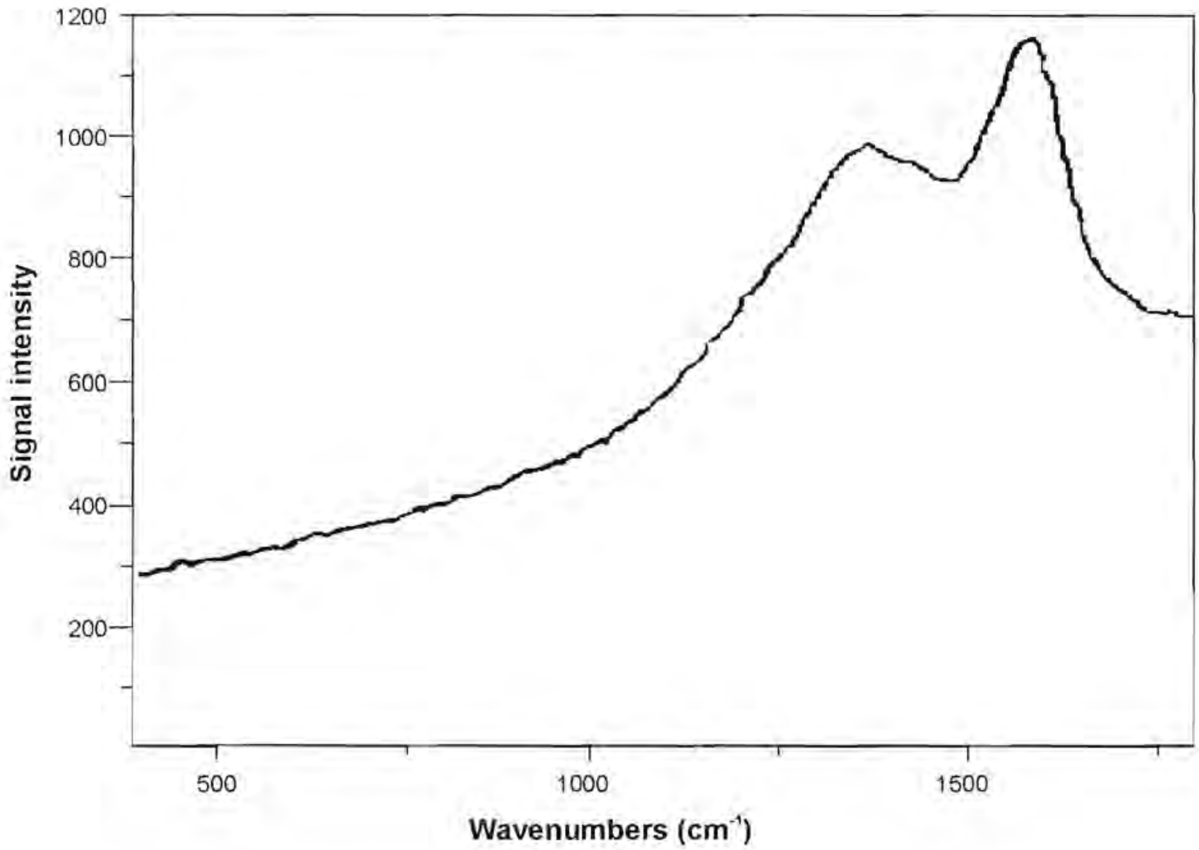

Figure 2-21: A typical Raman spectrum of the water-soluble fraction of oxihumate. Note that the peaks are relatively broad and that there is an apparent baseline drift.

The Raman spectra of all the fractions revealed only two fairly broad bands centred around 1370 and 1591 cm^{-1} and a typical trend of decreasing background absorbance with decreasing wavenumbers. These bands can be assigned to polycondensed aromatic functionality and amorphous carbon structures. The fact that the relative intensities of these absorption bands in the different isolated sub-fractions changed only marginally again implies that the basic chemical structure of all the sub-fractions is very similar. The acid soluble fraction exhibited a slightly different Raman spectrum that could be explained by the very intense fluorescence and high ash content implying a high inorganic salt content. A typical Raman spectrum of water-soluble oxihumate is presented in Figure 2.21.

3.12 Energy Dispersive Spectroscopy (EDS)

EDS analysis of the ash obtained from the various isolated sub-fractions revealed a high content of potassium and silica in most of the fractions. The major salts that were detected in the samples were aluminium, silica, sulphur and potassium. Minor components were chloride, sodium, titanium and iron.

The elements found in the different fractions varied from sub-fraction to sub-fraction but it was also found that there was a fairly large variation within the same sample due to the small area of the ash particle that could be analysed at a time. A typical EDS spectrum of an ash sample is illustrated in Figure 2-22. The results of the elemental composition of the ash from the different isolated sub-fractions is summarized in Table 2-5.

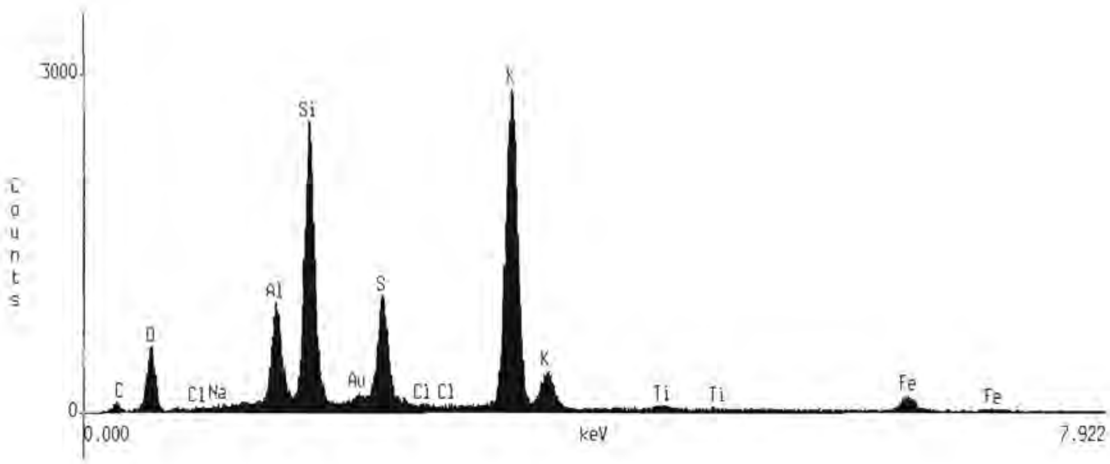

Figure 2-22: A typical EDS spectrum of an ash sample, in this case of the methanol precipitated sub-fraction of oxihumate. The area analysed per observation is about 100 x 100 µm. Note that there are two emission lines for most elements being the k and the l x-ray lines.

Table 2-5: A summary of the percentage inorganic element composition of five of the different isolated sub-fractions as determined from their ash. The ash was obtained by heating the samples in a muffle furnace at 500C and 850°C as described in the ashing method. Samples were analysed by EDS in several different areas to obtain an average element composition. Only the detected elements are reported in the table. ND: not detected.

Sample / Element	237 H2O sols	H+ sols	H+ ppt	MeOH ppt	MeOH sol
Na	0.08	ND	0.04	0.14	0.05
Mg	ND	ND	0.01	0.03	ND
Al	1.10	0.08	0.35	1.98	1.76
Si	6.93	0.03	4.68	6.29	4.21
P	ND	ND	ND	0.02	0.02
S	1.83	0.64	0.66	0.46	1.79
Cl	0.17	39.58	3.29	0.24	0.04
K	20.42	43.54	11.07	6.23	7.65
Ca	0.10	0.35	0.11	0.09	0.08
Cr	ND	ND	0.11	0.01	ND
Fe	1.03	0.01	1.26	2.23	1.19
Ti	0.14	ND	0.12	0.31	0.18
Percent ash	31.8	84.2	21.7	18.0	17.0

4 Discussion

Humic acid structure has been an elusive parameter to characterize despite the introduction of new technologies and instrumentation over the last 50 years. Arguments have been put forward as to the structure, origin and mechanism of formation but consensus has not yet been reached (Hayes, 1998; Novac *et al*, 2001; Wershaw, 1993; Wilson *et al*, 1987). One popular hypothesis at present is that humic acid is in fact a complex mixture of chemical compounds that are strongly associated or weakly bound to each other (Conte & Piccolo, 1999; Piccolo & Conte, 2000). The original material from which the humic acid was formed would determine to a large extent which compounds would most likely be found in the humic acid complex and conditions of formation would determine the physical characteristics (Ziechmann *et al*, 2000). The environment and conditions to which the humic acid mixture has been exposed would also play a role in the final chemical compounds present, the minerals incorporated and other inclusions found in the humic acid complex and therefore play a role in their physical properties. The data presented in this study supports the hypothesis that humic acid is a complex of many different compounds held together in a tight association.

The relatively high ash content and the refractory behaviour of the humic acid to combustion at temperatures (as high as 1200°C) indicate that there is probably some highly stable condensed aromatic compounds or volatile inorganics in the humic acids, especially in oxihumate, which is derived from coal by a relatively mild oxidation process. Thermogravimetric analysis has revealed that there is a fairly large percentage of refractory organic matter that requires extended time at or above 1000°C before total combustion occurred. The bulk of the ash appears to be potassium salts, which would be expected as potassium hydroxide was added during the formulation of the oxihumate. Even the THF soluble fraction that had been through four solubility dependant fractionation steps of increasing concentrations of organic solvents still exhibited a high ash content of 30%. The high percentage of organic solvent in this fraction's mother liquor is unlikely to solubilize any pure inorganic salts, indicating that the inorganic ions involved must be complexed to large lipophilic organic acid compounds. The acid soluble fraction (the fulvic acids) ashed to give almost pure potassium chloride, implying that the organic material is all burned away and that the silica and alumina salts are almost quantitatively precipitated during the acid treatment used to isolate this sub-fraction. In the sub-fractions where a "glass melt" formed during the ashing process, it was found that the predominant element in the glassy residue was silica. Potassium, silicon, aluminium, sodium, sulphur, chloride and iron were the major inorganic ions found in oxihumate ash by EDS micro-assays as summarized in Table 2-5.

The percentage solubility of the various sub-fractions was affected by the rate of the change from the soluble to the insoluble state; rapid changes were characterized by precipitation of almost all the compounds in solution while slow changes left more of the soluble compounds in solution. The fact that up to 5% of the soluble portion of oxihumate was soluble in aqueous tetrahydrofuran but that this sub-fraction cannot be extracted directly from the oxihumate by the same solvent mixture indicates that the changes need to be done subtly and slowly to avoid co-precipitation or re-inclusion into the chemical complex/association.

The use of TLC to gain qualitative information of the sub-fractions of oxihumate was the fastest and most successful analytical technique. Many of the compounds extracted from the oxihumate proved to be highly fluorescent or UV absorbing at 254nm, which presented a convenient method to detect these compounds on TLC plates if impregnated with a fluorescent agent. Not many compounds could be visualized with "universal" visualizing agents like chromic acid nor did many of the separated compounds stain with iodine vapour. The most successful visualizing agent for the more lipophilic sub-fractions (those that were soluble in methanol, THF or ether) was vanillin/sulphuric acid, a reagent that visualizes long chain aliphatic alcohols, steroids, essential oils and phenols. Several visualizing reagents used revealed no compounds whereas others could visualize only one or two compounds that had separated from the bulk of the very dark material. Antioxidant visualizing reagents like reduced Fast Blue or 0.05% $KMnO_4$ or 10% molibdophoshoric acid revealed a limited number of compounds in the various sub-fractions except the acid soluble sub-fraction (fulvic acids) which appeared to be comprised of several strong antioxidants.

Most of the dark material did not move off the origin of silica gel TLC plates. When dark material did move from the origin it streaked severely indicating poor solubility in the solvent systems used. The more alkaline and polar the solvent system, the more coloured material moved from the origin, but the dark compounds always exhibited severe streaking. This is illustrated in Figures 2-4 and 2-5, which show the acetonitrile:water:ammonium hydroxide and the ammonium hydroxide:propanol mobile phase separations respectively. The acetonitrile:water:ammonium hydroxide system runs with a series of four apparent solvent fronts, each pseudo-front carrying with it a mixed band of compounds. This would imply that solubility is limited and that only the compounds that are solubilized move on the pseudo-front. The fastest moving front is the least polar and would carry the non-polar lipophilic compounds whereas the zone behind the slowest moving front is the most polar and alkaline that carries the polar and coloured compounds from the origin. A distinct increase in the apparent amount of coloured material that moves from the origin was observed for the isolated sub-fractions of oxihumate with the least polar sub-fractions leaving almost no coloured compounds on the origin. The ratio of colourless compound to coloured compound increases in the isolated sub-fractions that are soluble in the more non-polar organic solvents. This may result from the coloured compounds being soluble in the mobile phase when there is a high enough concentration of non-polar compounds present. This increase in their solubility results in an increase in the movement of these polar coloured compounds.

When using the ammonium hydroxide:propanol system, more of the coloured compounds move from the origin due to the strong solvating effect and the highly polar nature of this solvent system as well as deactivation of the silica stationary phase. A disadvantage of this solvent system is that all the non-polar compounds move in a relatively tight band between *Rf* 0.75 and *Rf* 1.0 - the solvent front. Although the dark coloured compounds move from the origin more readily than with the less polar acetonitrile:water:ammonium hydroxide solvent system, the same trend is seen that the less polar isolated sub-fractions still show more of the dark coloured compound moving off the origin. Again it could be speculated that the more lipophilic compounds are solvating the polar dark coloured compounds and this is why more of the latter compounds move off the origin. Another observation that supports the solvation effect is that if the oxihumate fraction is applied to the plate and not allowed to dry before developing, more of the coloured compounds move from the origin.

Contrary to the results with alkaline solvent systems, the acidic solvent systems all gave good separations of the non-polar compounds in the more lipophilic sub-fractions but did not move any coloured compounds from the origin. In the case of the water-soluble oxihumate sub-fraction only a trace of these non-coloured compounds could be detected moving from the origin – implying that the complete humic acid complex is very strongly associated and not easily disrupted. Although the lipophilic sub-fraction of oxihumate accounts for about 5% of the soluble mass of oxihumate, the amount of detectable material separating from the bulk of the material left on the origin appears to be far less than 5% of the mass. The coloured compounds in oxihumate do not move off the origin as these compounds are insoluble in acidic solvents and solubility is a prerequisite for chromatographic separation. The non-polar compounds in the isolated sub-fractions did however move from the origin and were well resolved over a wide region of *Rf* values from 0.4 to 1.0 (the solvent front). Some compounds in the isolated sub-fractions appeared to streak over the full length of the separation, indicating poor solubility. The most lipophilic compounds moved with the solvent front, stained with iodine vapour and coloured with the vanillin/sulphuric acid visualizing spray, both indications that they were probably aliphatic type compounds as reactive phenolics would have bleached the iodine vapour due to iodination reactions taking place.

The acidic solvent systems proved particularly useful to determine the free iodide content of radioactively labelled fractions of oxihumate as the bulk of the oxihumate remained on the origin while the free iodide had an *Rf* of approximately 0.73. From the autoradiograms made from TLC plates of ^{123}I labelled oxihumate, several previously minor unseen compounds were revealed that ran between the origin and the iodide as well as a compound that ran very close to the solvent front at an *Rf* (0.92) which is higher than that of iodide. This observation alone points to the fact that the humic acid in oxihumate contains several chemically different compounds, some that carry a functionality that can easily react with iodine and that these compounds can easily be separated from the bulk of the humic acid.

HPLC is a technique that in general has not proved to be very successful for the separation of compounds in humic acids to date. Several attempts in the past have all shown that there is an exclusion peak and a major peak that elutes over a fairly long time with limited resolution (Susic & Armstrong, 1990; Schulze *et al*, 1999; Preuse *et al*, 2000; Frimmel *et al*, 1992). This effect was seen in the HPLC separations performed on C_{18} columns in this study. It was found that the recovery of the injected sample was low and that the column backpressure would increase rapidly after about 50 injections with a simultaneous deterioration in the resolution of the eluted peaks. A washing sequence of the column at this stage could not improve the resolution and had only a minor effect on the backpressure. However if the column washing sequence was performed after 20 injections a waxy residue could be eluted and the increase in backpressure on the columns was delayed. The resolution however still deteriorated progressively. If the columns with high backpressure were disassembled it was found that the stationary phase had almost completely stained dark brown with only a short length at the outlet end showing only slightly stained particles. This dark colour could not be completely washed from the stationary phase even if using concentrated ammonium hydroxide, a treatment that could be used to clean C_{18} SPE cartridges fairly effectively.

The recovery of the material from the HPLC column was therefore not quantitative and the material that was remaining on the column was altering the chemistry of the column, which caused the rapid decrease in column efficiency.

The compounds eluting in the first peak appeared to be polar and contained the highest concentration of fluorescent compounds (except for the acid soluble sub-fraction). The compounds eluting later (between 10 and 16 minutes) and which made up the main unresolved peak became progressively darker. This large unresolved peak represented most of the dark compounds eluted from the column. Ultraviolet transparent compounds eluted at the end of the gradient and these compounds were waxy and very lipophilic. As these compounds were UV transparent they were not detected by either the UV or the fluorescent detector but were found when a fraction collector was used to collect the eluent. These undetected compounds eluted at acetonitrile concentrations of greater than 70% and were found to be waxy white solids with no distinct smell.

It was found that C_{18} HPLC eluted peaks collected by fraction collector were not as soluble as they were prior to chromatographic separation. This could have been due to breaking up a complex or association of compounds or the result of denaturing the compound similar to what can occur to some proteins when chromatographed on C_{18} columns.

Re-chromatography of the HPLC eluted fractions on silica TLC plates using an acidic mobile phase showed that the dark compounds remained at the origin, implying insolubility in acidic medium. When alkaline mobile phases were used severe streaking occurred, implying low solubility or ionic compounds that bind strongly to the silica.

The general pattern of HPLC separation of oxihumate is similar to that reported in the literature for humic acids (Frimmel *et al*, 1992; Gremm *et al*, 1991) using C_{18} columns and similar mobile phases. This elution pattern was found to be reproducible and appeared to have a slightly better resolution than those reported above, especially with respect to the second major "peak" eluting from 10 to 16 minutes. The number of injections through the column however had a major role in the resolution of the different compounds in the oxihumate and it would be difficult to compare the methods due to the different sources of the analysed humic acids because the number of injections through the column was not reported in the above literature.

The isolated sub-fractions (separated according to solubility differences) also gave reproducible elution patterns. Despite the reproducibility, quantitation was not possible as the sample recovery varied greatly from fraction to fraction.

Complications in attempting to quantitate the compounds in oxihumate (and other humic acids) are that the very lipophilic compounds and the C_{18} HPLC column washings were not detectable by the UV or fluorescence detectors. A further complicating factor is that the eluted compounds were not completely resolved. The absolute absorbance for individual compounds could therefore not be determined and calibration curves were unreliable.

The use of a C_{18} HPLC column and an acidic mobile phase (0.1% trifluoroacetic acid in both water and acetonitrile) resulted in good resolution of the compounds of the main peak, eluting between 10 and 16 minutes, but the recovery was very low with almost none of the dark coloured compounds being eluted. This method was later only used when sub-fractions had been previously separated by low-pressure column chromatography and the bulk of the dark compounds had already been removed from the sample. The UV transparent lipophilic compounds were strongly retained when using the acidic mobile phase and, like with the alkaline mobile phase, needed to be washed from the column after 20 injections. This result would imply that these lipophilic compounds are very non-polar and unaffected by the pH of the mobile phase indicating an absence of any ionic functionality in these compounds.

Due to the low recoveries on HPLC, especially using acidic mobile phases, a small study was done in an attempt to determine the percentage recovery using C_{18} SPE cartridges. Although the method used would not give precise results it was a far better method than comparing peak areas on the HPLC system with and without a column as the retention behaviour of any compound distorts the apparent concentration of individual compounds grossly. The UV transparent compounds in the oxihumate were only detected after collecting the eluent and drying these fractions. These compounds, although adding to the mass of the injected sample would not be detected by the HPLC system detectors with or without an analytical column and would therefore have distorted the apparent recoveries in a positive direction.

The fact that the control samples appeared to have gained mass is probably due to the potassium in the oxihumate fraction forming salts with the stronger trifluoroacetic acid in the eluent. Although the eluent is completely volatile, potassium trifluoroacetate would not be volatile and would then add to the mass of the sample. If this increase in mass of the control samples were factored into the percentage recovery of the SPE eluent, then the actual recoveries of the oxihumate from the SPE cartridges would be only 60% at most. The waxy residues that were eluted by the organic solvents accounted for a further 15 – 20% (corrected for the controls) of the mass, which means that there is still approximately 20% of the mass of the sample retained on the cartridges.

It was obvious that the coloured compounds were being strongly retained on the C_{18} cartridges and that they were not easily recovered. This same effect was seen on the HPLC columns, where the dark compounds eventually contaminated the entire length of the stationary phase resulting in poor resolution. The retained compounds however appeared to be in a slow equilibrium that would allow displacement of some of the dark compounds to occur under strongly alkaline conditions, conditions that result in deterioration of silica based stationary phases. This phenomenon was also reported by Susic and Boto (1989) who reported that SPE columns had unpredictable behaviour and appeared to establish a new equilibrium on standing.

It would appear from these results that there are several compounds with very different characteristics present in the oxihumate and that these compounds must have a mutual effect on the other compounds in the oxihumate. The solubility, chromatographic retention, UV absorbance, fluorescence and colour are all affected by the stripping out of some of the compounds from the humic acid complex.

Fluorescence spectroscopy revealed that all the isolated sub-fractions had some fluorescent compounds present but that the THF soluble sub-fraction accounted for most of the fluorescence of oxihumate. Two particularly strongly fluorescing compounds were present in this sub-fraction. The general appearance of the sub-fraction was a brown oil with a slight fluorescent green tinge when seen in sunlight and gave a very strong blue fluorescence under both 254nm and 360nm UV light. On TLC it could be shown that several compounds in the THF sub-fraction had native fluorescence that was only visible under a 360nm light. The emission colour of these compounds varied from almost white through blue and green to orange and "brown". The colour and intensity of the fluorescence appeared, at least to some extent, to be dependant on the concentration of the spots and how well the spots were resolved. When the isolated sub-fractions were scanned in a spectrofluorometer, the compounds were still a complex mixture of compounds and a cumulative fluorescent effect was observed. There is a strong possibility that interference and quenching effects play a major role in the observed fluorescent spectra due to the many fairly similar chemical structures that would be present in the sample (Senesi *et al*, 1991). The excitation and emission maxima reported by Alberts (Alberts *et al*, 2000; Tacacz & Alberts, 1999) for humic acids from aqueous environments matched those found for oxihumate in this study the closest, although there were small differences. Luminescence spectra (3D spectra) (Mobed *et al*, 1996) reveal that a broad emission band between 375 and 500nm existed even when the excitation wavelengths were varied by more than 100nm. The maximal emission wavelength is approximately 430nm for all the sub-fractions despite the fact that they are mixtures of several compounds. This could indicate that there are a limited number of strongly fluorescent compounds or that there is a reproducible quenching or interference mechanism present.

In concentrated samples it was seen that the scattered excitation and second order harmonic wavelength emission were not present in the emission spectra although when the same sample was further diluted, both these emission wavelengths were exhibited as strong peaks. This could indicate that a strong quenching effect is evident. Furthermore the fact that the more concentrated samples show darker and even a "brown" fluorescence on TLC also indicates some form of quenching, the mechanism of which is not known at this time.

Infrared proved to be a very useful technique in determining the presence of chemical functionality in oxihumate although the results are not as well defined as would be expected if a single compound were under scrutiny. The intense absorption in the "carbonyl region" appears to be fairly universal for all humic acids, irrespective of where they are sourced (Khairy, 1989; Theng & Posner, 1967). The spectra of the THF and the fulvic acid sub-fractions were very different from all the other sub-fractions in the carbonyl region. The fairly sharp absorbance by these two sub-fractions at 1720 cm^{-1} would indicate the presence of esters or unsaturated ketones. The THF sub-fraction also has a strong absorbance band at 1038 cm^{-1} which is in the absorbance region of the C-O stretch of esters, alcohols and ethers. The value is low for typical simple esters but could be due to α-unsaturated esters or α-unsaturated free acids. Aromatic ethers show a typical combination of peaks at 1250 cm^{-1} and 1030 cm^{-1} a combination of bands that is also seen in the THF sub-fraction. An alternative structure also absorbing in this region is internal tertiary carbon groups of substituted alkanes.

The fulvic acid sub-fraction also shows the strong ester absorption band at 1720 cm^{-1} but lacks the C-O stretch band of the alcohols, ethers and esters. This sub-fraction shows a shoulder at 1630 cm^{-1} that falls into the alkene, substituted aromatic ring, carboxylic acid salt or amine absorption region. The 1410 cm^{-1} absorption however, reinforces the carboxylic salt structure. The high ash content and high potassium concentration in the ash would again point towards the carboxylic salt structure.

The presence of strong hydrogen bonded hydroxyl absorption could result from acidic or alcohol groups, both of which fit the generally accepted chemical functionality that would be found in humic acids. The old debate that has not been resolved is whether the structure is predominantly aromatic or aliphatic (Farmer & Pisaniello, 1985; Ikan *et al*, 1986; Schnitzer, 1985; Stevenson, 1994). This cannot be seen from the IR spectra of the oxihumate nor from most of its dark coloured sub-fractions. The THF soluble sub-fraction was the only sub-fraction to show a distinct aliphatic CH_3 and CH_2 peaks at 2950 cm^{-1} and 2850 cm^{-1}. The aromatic C-H stretching bands (3000-3100 cm^{-1}) did not stand out in any of the sub-fractions, which raises the question whether this functionality is really involved in the main chemical structure. However, if there were few aromatic C-H bonds present due to extensive substitution of the aromatic rings these classic aromatic absorption bands would be very small to insignificant.

The insignificant aliphatic C-H stretching in all the fractions except the THF sub-fraction (which is rich in aliphatics) cannot be explained by the same argument. Even though the THF soluble sub-fraction accounts for only 5 – 6% of the mass, the aliphatic band should be visible in all the sub-fractions from which this sub-fraction was isolated. The literature generally does not describe the effect that mixtures of compounds would have on the spectra of each other and whether some form of quenching could occur. From the results of the UV/visible, fluorescent and Raman spectra, where distinct interference or quenching effects appear to be evident, it would not be surprising if some form of interaction, which includes quenching or energy transfer, does occur in infrared spectra.

The Raman spectroscopy was relatively disappointing in that the bands were broad and there was a strong baseline shift. These effects were attributed to quenching or interference. A problem experienced with the Raman spectra of oxihumate sub-fractions was the strong fluorescence exhibited by the different isolated sub-fractions that appeared to interfere with the scattered light pattern of the spectra. The spectra obtained for the water-soluble sub-fraction pointed to polycondensed aromatic ring structures and/or amorphous carbon. If this is considered together with the IR spectra it would appear that there are nearly no hydrogens bound to aromatic rings, leaving highly oxygen and carbon substituted ring structures. In this case there would be many C=C bonds which also absorb in the 1500 - 1600 cm^{-1} region, the region of very strong absorbance in the IR spectra of oxihumate. This region of absorbance overlaps the carbonyl absorption of carboxylic acid salts, which makes the peak assignments difficult. The fact that the absorption bands are mostly broad, again implies that the humic acid is probably a complex mixture of slightly different compounds. The fact that the THF soluble sub-fraction can be separated into at least 30 different compounds on TLC, many which are UV absorbing or fluorescent, again supports the concept that humic acid is a tight association of many different compounds.

Although not shown, liquid phase NMR spectra that were performed on oxihumate during this study showed a very complex combination of resonance bands, including aromatic and aliphatic hydrogens and carbons, but the spectra were not of good quality, had very large solvent (H_2O) peaks and could not give quantitative data with respect to the relative proton or carbon numbers. What was obvious was that there were many different resonance bands of compounds present in different apparent concentrations and that there were many "inactive" carbon atoms present in the complex. These NMR results confirmed the presence of both the aliphatic and aromatic functionality in oxihumate.

If all the chemical and physical data collected for oxihumate and its isolated sub-fractions is considered it would appear to support the hypothesis that humic acids are in fact tightly associated or loosely bound chemical complexes. Some compounds can be separated from the structure under certain conditions but the bulk of the humic acids remain in the complex due to similar chemical structure or physical properties. It would appear that the remaining humic acid, after extraction of the least tightly bound compounds, would re-equilibrate and this would allow more compounds to be extracted from the complex. The presence of aromatic functionality could not easily be confirmed by the techniques used to determine chemical structures during this study although there are several characteristics such as the refractive nature of the oxihumate to combustion in air, the strong UV absorption and the fluorescence that would imply that such structures do exist in the complex.

This study has used several analytical techniques that have not been used in combination by any other research group reporting on their humic acid analysis so far. The individual and combination of techniques all point to a complex mixture of compounds and not to a single chemical structure that could be isolated and characterized as "humic acid". Taking into account the fact that these compounds are derived from plant material that would be a mixture of many different chemical compounds to start, and that these compounds have been through a degradation process and chemical alteration due to environmental and physical conditions over a period of several million years it would not be surprising to find that there are still many different compounds in the complex making up humic acid. Despite the fact that there are sub-fractions that can be extracted and that these can be further separated chromatographically into many compounds it is uncertain whether these compounds were chemically bound within the humic acid structure or just associated with a core compound that is not soluble.

Another complicating fact is that there is a relatively high ash content for a compound that is organic. This can be explained by the binding of inorganic salts and metal ions but there appears to be a large percentage of silica and aluminium oxides, both elements associated with soil or clay. This would indicate that the organic compounds are "contaminated" with soil or that degraded soil is complexed by the humic acids. By washing these isolated sub-fractions with hydrofluoric acid it should be possible to remove most of the silica and this would indicate the amount of clay in the humic acid.

Considering the data collected during this study, it would be reasonable to state that humic acid, and especially oxihumate derived from a bituminous coal by an oxidation process, are complex mixtures of many individual chemical compounds held together by either weak chemical bonds or merely in a tight association with each other and that the environment and treatment of this complex mixture would determine the physical and chemical characteristics of the humic acid as a whole.