Journal of Chromatography, 209 (1981) 421–431
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,649

HUMIC ACID CHARACTERIZATION OF COLOMBIAN SOIL BY DISC ELECTROPHORESIS AND INFRARED SPECTROSCOPY FOLLOWING GEL FILTRATION

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(Received January 13th, 1981)

SUMMARY

Humic acid extracted from Columbian soil was separated into fractions by means of gel filtration on Sephadex G-25 and G-75. A high proportion of acid has a molecular weight greater than 50,000 daltons.

Analysis of these fractions by disc electrophoresis on polyacrylamide gel and by infrared spectroscopy shows a highly aromatic character for humic and inorganic materials bound to the small fractions.

INTRODUCTION

The aim of this study was to characterize the humic acid of a Colombian soil by non-degradative methods. This particular soil was chosen as it is found in a large area in Colombia, which, even though having a high organic and nitrogen content, shows a low fertility.

The Colombian soil shows a slow mineralization of organic matter[1] and a developed humic system[2]. The high humidification grade is due principally to moisture and clay alofanic materials; however, little is known about the composition and structure of the humic system. The soil under examination contains a high percentage of organic matter and also a high percentage of humic acid[3]. It has been classified as Inceptisoil Cumulic Humitropept[4] and has been studied with respect to its productivity with the addition of nitrogenated compounds[4], its phosphate fixation[5] and its content and distribution of minor components[6]. Its humic acid has already been studied by the usual chemical methods[3].

For the characterization of humic acid we applied the non-degradative techniques described previously[7], owing to its particular properties. The techniques used

0021-9673/81/0000–0000/$02.50

were gel filtration followed by disc electrophoresis under the usual conditions or with urea.

Humic acid must not be treated as a statistically uniformly distributed system, but as a system divisible into homogeneous fractions with different chemical properties as shown by electrophoretic methods. Gel filtration was used to separate humic acid and obtain more homogeneous fractions than total humic acid. These fractions were then submitted to infrared spectroscopy and disc electrophoresis. The resolving power of disc electrophoresis on polyacrylamide gel allows the direct characterization of some properties[8]. Urea (6.0 *M*) was used in the electrophoresis to reduce the weak interactions among the humic acid subfractions. The characterization of the gel was performed with three different staining techniques, one general and two more specific.

EXPERIMENTAL

The soil was Serie Bermeo, collected in the Municipio de Facatativa, Departamento de Cundinamarca, Colombia, at 2640 m above sea-level and an average temperature of 13°C. It had the following general properties: structure, granular; consistency, firm; macro-organisms, rare; roots, copious; internal drainage, quick; external drainage, moderately quick; declivity, 3–7%; erosion, absent; class, Inceptisoil; subclass, Tropept; high group, Humitropept; and subgroup, Humitropept Cumulic[4].

The physico-chemical properties were as follows[3]: pH, 5.4; organic C, 11.2%; organic matter, 19.31%; N, 0.81%; C/N, 13.8; exchange power, 61.0 mequiv. per 100 g; total basicity, 3.47; base saturation, 5.68%; exchange bases (mequiv. per 100 g), Na^+ 0.69, K^+ 0.65, Ca^{2+} 1.22, Mg^{2+} 0.91; and phosphorus content, 30.19 kg P_2O_5/ha.

The humic substance had the following chemical properties: humic acid (HA), 4.5%; fulvic acid (FA), 1.75%; C of HA, 53.85%; C of FA, 31.95%; and C (HA)/C (FA), 1.69. The humic acid had the following chemical properties: moisture, 7.5%; ash, 8.7%; C, 56.64%; N, 2.55% and C/N, 22.21.

Extraction and separation

Dry soil was sieved with a 2-mm mesh. The extraction was achieved with 0.1 *M* sodium hydroxide and 0.1 *M* $Na_4P_2O_7$ solution (150 g of soil per 3 l) with stirring under nitrogen for 1 day[9,10]. After decantation the supernatant was acidified with concentrated sulphuric acid to pH 2–3. The precipitated humic acid was recovered by centrifugation at 4200 *g* for 15 min.

Purification

The humic acid was dissolved in 0.1 *M* sodium hydroxide solution and reprecipitated with concentrated sulphuric acid three times. Finally, humic acid was dialysed against distilled water until all sulphate had been removed (barium sulphate test) and then lyophilized.

Gel filtration

Humic acid (50 mg in 2 ml of 0.05 *M* Tris chloride buffer, pH 9.0) was loaded on to a Sephadex G-25 column (60 × 2 cm), with the same buffer as eluent, to avoid interactions between the gel and humic acid[11,12]. Fractions of 5 ml were collected and monitored at 578 nm (Beckman ACTA CIII). The fractions were collected in six pools, and each was dialysed against distilled water and lyophilized.

An aliquot of the first batch (25 mg) was re-suspended in the starting buffer (1 ml) and loaded on to Sephadex G-75. Three pools were collected. It should be noted that the two Sephadex gels, especially Sephadex G-25, strongly retain a humic acid fraction (*ca.* 5% of total) that cannot be washed from the gel.

Electrophoresis

The method used has been reported previously[7]; 16% acrylamide was used. The apparatus was a Minivolt vertical model. The concentration of urea, when used was 6.0 *M*.

For characterization of the bands we used a reported staining method[8], with Blue Alcian (BA) as a general stain for acid mucopolysaccharides, Fuchsin (F), decoloured (Zacharius method[13,14]), as a stain for polysaccharides and all other molecules with neighbouring hydroxyl groups and Prussian Blue (PB) as a stain for fractions with reducing properties. All the gels were uniformly distributed despite the slightly different gel length obtained after electrophoresis; this was allowed for by calculating electrophoretic mobilities (EM), defined as[15]

$$\mathrm{EM} = \frac{\text{band migration (after staining) (mm)}}{\text{electrophoretic front migration (at the end of the run) (mm)}} \cdot \frac{\text{gel length (at the end of the run) (mm)}}{\text{gel length (at the end of destaining) (mm)}} \cdot 100$$

Infrared spectroscopy

The IR spectra of total humic acid and of each pool collected were obtained using lyophilized samples; a sample of 0.50 mg per 70 mg of potassium bromide was obtained for dilution. The IR spectra were recorded in the range 4000–600 cm^{-1} with a Perkin-Elmer 247 spectrophotometer.

RESULTS AND DISCUSSION

Gel filtration on Sephadex G-25 of starting humic acid and electrophoretic examination of pools collected

The gel filtration separation is shown at the top of Fig. 1, and below is shown the electrophoretic pattern obtained under normal conditions or with 6.0 *M* urea with the three different stains: Blue Alcian, Basic Fuchsin and Prussian Blue. The initial humic acid and only three pools ($P1_{25}$, $P4_{25}$ and $P6_{25}$) are shown because the differences between one pool and its immediate neighbours are not marked, whereas comparisons are more significant when distant pools are considered. The differences between the gels are only qualitative because quantification of an electrophoretic band (with a scanning photometer) is very difficult: first, the humic acid retains its

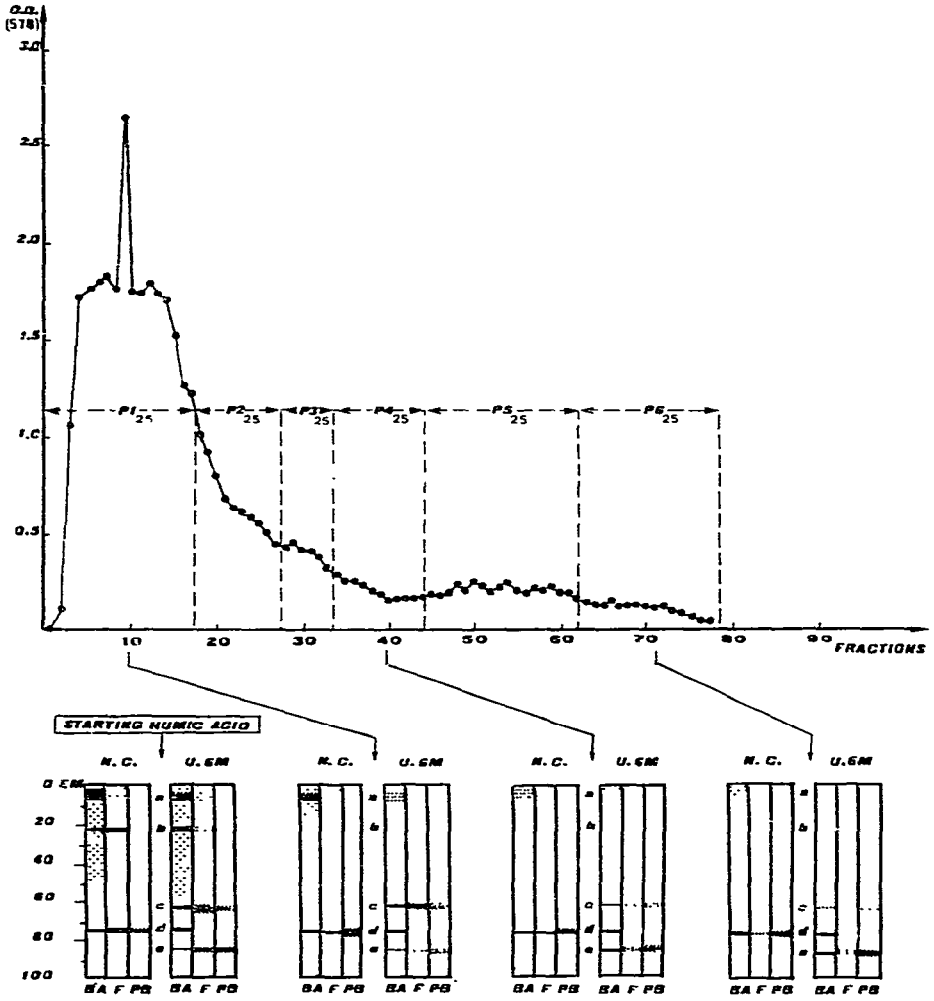


Fig. 1. Gel filtration (Sephadex G-25) of initial humic acid (top) and electrophoretic patterns of the initial acid and of some pools collected from gel filtration (bottom) (see Results and discussion). N.C. = Normal conditions; U. 6M = 6.0 *M* urea; BA = Blue Alcian stained gel; F = Fuchsin stained gel; PB = Prussian Blue stained gel.

own colour, which interferes with the specific stain, even with the use of an appropriate filter (in the Fuchsin staining method there is a slight humic acid decoloration, probably due to the strong oxidation with periodate); secondly, different electrophoretic fractions have different stain binding powers. Gel filtration on Sephadex G-25 shows a very low resolving power and the largest amount of humic acid is eluted without retention on the gel (immediately after one void volume); probably this fraction has a molecular weight of more than 5000 daltons.

Also, if the molecular weight discrimination of Sephadex G-25 is in the range 5000–500 daltons, it is impossible to obtain information on the molecular weight of the pool collected, because appropriate standards do not exist and the last fractions

are eluted after the passage of three void volumes of eluent. This is caused by an abnormal retention by the gel of humic acid, also under the choosen conditions[11,12] (pH of buffer and ionic strength). A proportion of the sample is completely retained by the gel and cannot be recovered. This fraction is retained not because it has a molecular weight smaller than 500 daltons, but probably because Sephadex G-25 strongly binds some part of humic acid. This is supported by a general comparison between the electrophoretic pattern of the initial humic acid and the pools collected: in fact, a loss of fraction b (22 EM units) and generally of a dispersed fraction of low mobility ranging from 0 to 55 EM units can be observed, while the small fractions of high mobility are all present in each pool.

Thus it could be suggested that the apparent low resolving power of gel filtration may be caused by a double effect, *viz.*, normal molecular sieving and abnormal adsorption.

From examination of the electrophoretic patterns with the three stains, the following observations can be made.

Blue Alcian. This stain is good for the general characterization of all of the fractions; only fraction a is slightly stained, but retains its own colour. Observing the gel under normal conditions, the above-described loss of fractions after gel filtration and the constant presence of the same fractions in all of the pools eluted must be noted. Fraction a (5 EM units) shows a slight decrease in intensity from $P1_{25}$ to $P6_{25}$.

Comparison with urea-treated gels with the same stain indicates that urea reveals humic acid more clearly in the electrophoretic gel and separates the fraction into a more complete pattern, with five definite fractions.

Band d in the gel under normal conditions is probably the sum of bands d and e in the urea-treated gel; band a with urea treatment should generate band c, as shown by the decrease in intensity of band a compared with the use of normal conditions. These results are in good agreement with the electrophoretic pattern obtained on humic acids from soils of different origin[8]; moreover, a decrease in the intensity of band c (62 EM units), constancy of that of fraction d (75 EM units) and an increase in that of band e (85 EM units) occurs on elution on Sephadex G-25.

Thus, even if gel filtration is unable to separate humic acids into homogeneous fractions, it probably allows the progressive enrichment of small fractions.

Basic Fuchsin. This stain allows the characterization of polysaccharides and of other compounds with neighbouring hydroxyl groups. In contrast with results obtained for humic acids of different origin[8], all of the fractions are stained, both under normal conditions and on urea treatment, except for band d present in urea-treated gel; probably the humic acid is associated with a cellulose matrix in almost all of the fractions; band d obtained on urea treatment shows that only a small amount of this cellulose matrix is released from humic acid; other conclusions are in agreement with those for Blue Alcian.

Prussian Blue. This stain, for fractions with reducing properties, indicates that only bands c and e in the urea-treated gels show reducing properties. These bands, as previously observed, correspond to small molecules of high charge density; moreover, it is impossible to establish if the reducing power of bands c and e is due to oxidizable polysaccharides or other groups, whereas this does not apply to humic acids from other soils[8].

Finally, a progressive decrease of bound stain along the pools must be noted;

this observation, which is not explained by the gel filtration and electrophoretic data, is classified on the basis of the IR results below.

Gel filtration on Sephadex G-75 of $P1_{25}$ and electrophoretic examination of pools collected

Gel filtration on Sephadex G-75 is shown at the top of Fig. 2, and below is shown the electrophoretic pattern of the pools collected. As only three pools were collected, these are all shown. The fraction retained on Sephadex G-75 is negligible, but the large elution volume (three void volumes) indicates that an abnormal interaction also exists between Sephadex G-75 and humic acid.

The gel filtration results show that the largest fraction of $P1_{25}$ has a molecular weight greater than 50,000 daltons and a well defined peak appears after two void

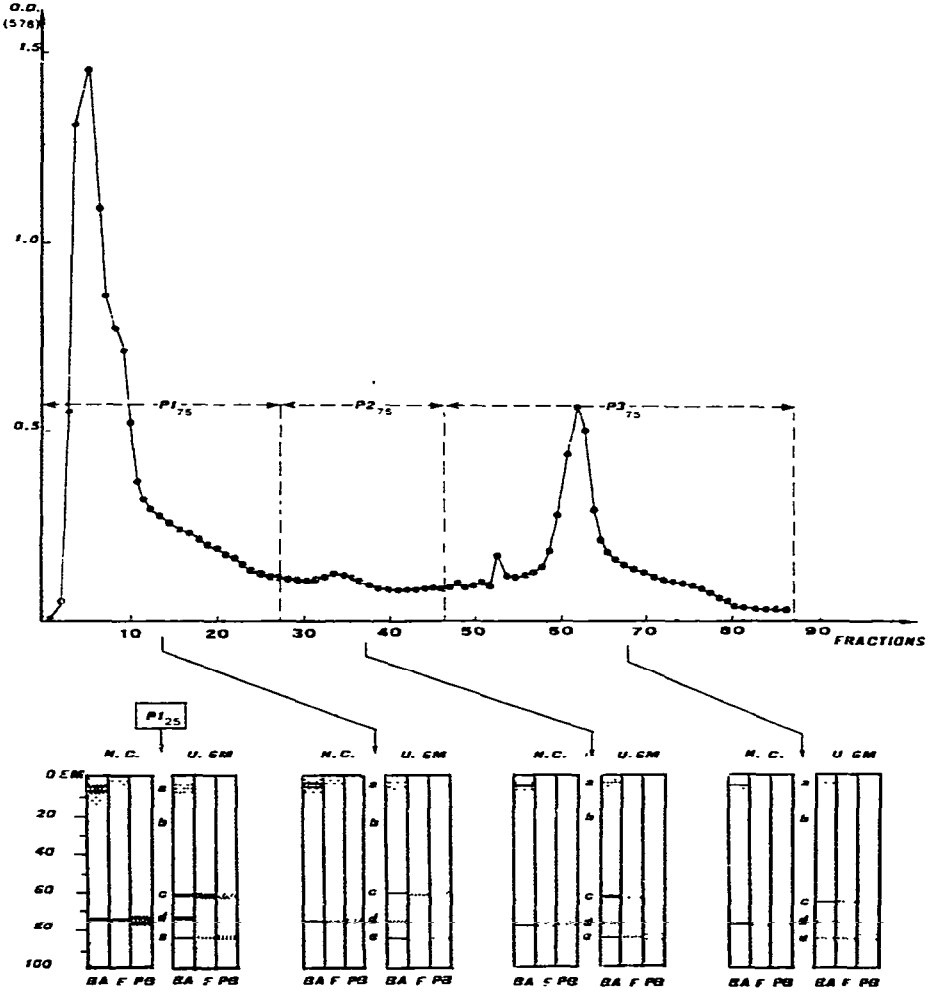


Fig. 2. Gel filtration (Sephadex G-75) of the pool 1 from Sephadex G-25 ($P1_{25}$) (top) and electrophoretic patterns of $P1_{25}$ and of the pools collected from gel filtration (bottom). Abbreviations as in Fig. 1.

volumes of eluent. This should indicate that in the region of 10,000 daltons there is a uniform molecular type; however, the electrophoresis of $P3_{75}$ shows that the uniformity is based only on molecular weight, because this pool has a high heterogeneity.

Examination of the electrophoretic pattern of each pool indicates that, in general, gel filtration is a poor separation method for humic acid. It should be noted that fraction e of $P1_{75}$ in urea-treated gel is stained by Prussian Blue but not by Basic Fuchsin, indicating that the reducing power of this band is probably due to chemical groups other than oxidizable polysaccharides (*i.e.*, *p*- and *o*-diphenols easily oxidizable to quinones). The fact that band c in $P2_{75}$ and $P3_{75}$ is stained by Fuchsin but not by Prussian Blue confirms this hypothesis. Moreover, there is an enrichment of band c in $P2_{75}$ and band a is particularly intense in $P1_{75}$, obviously corresponding to the humic acid fraction of highest molecular weight and lowest negative charge. Other observations are in agreement with results obtained from Sephadex G-25 gel filtration.

Infrared spectroscopy

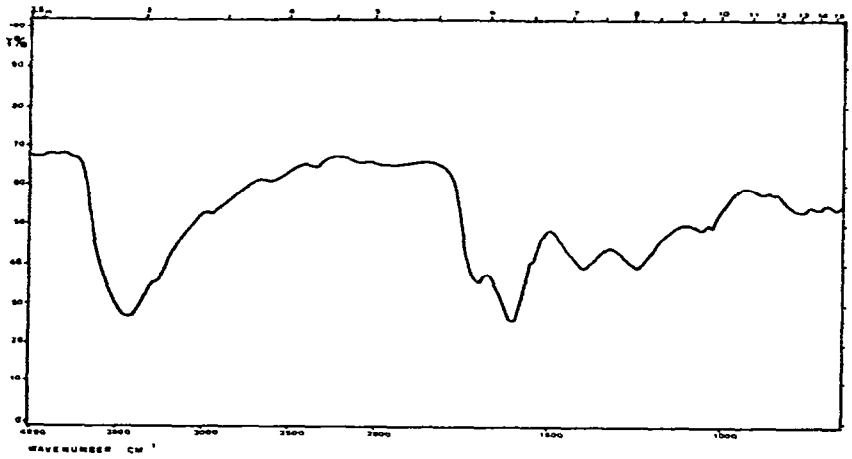
Fig. 3A shows the IR spectrum of the initial humic acid, and Fig. 3B shows the IR spectra of some samples derived from gel filtration on Sephadex G-25, *i.e.*, $P1_{25}$, $P2_{25}$, $P4_{25}$ and $P6_{25}$. The choice was made for the same reason as for electrophoresis.

Fig. 4 shows similar results for Sephadex G-75. The following conclusions can be drawn: four principal absorption bands occur in all of the spectra, at 3400, 1600, 1400 and 1050 cm^{-1}.

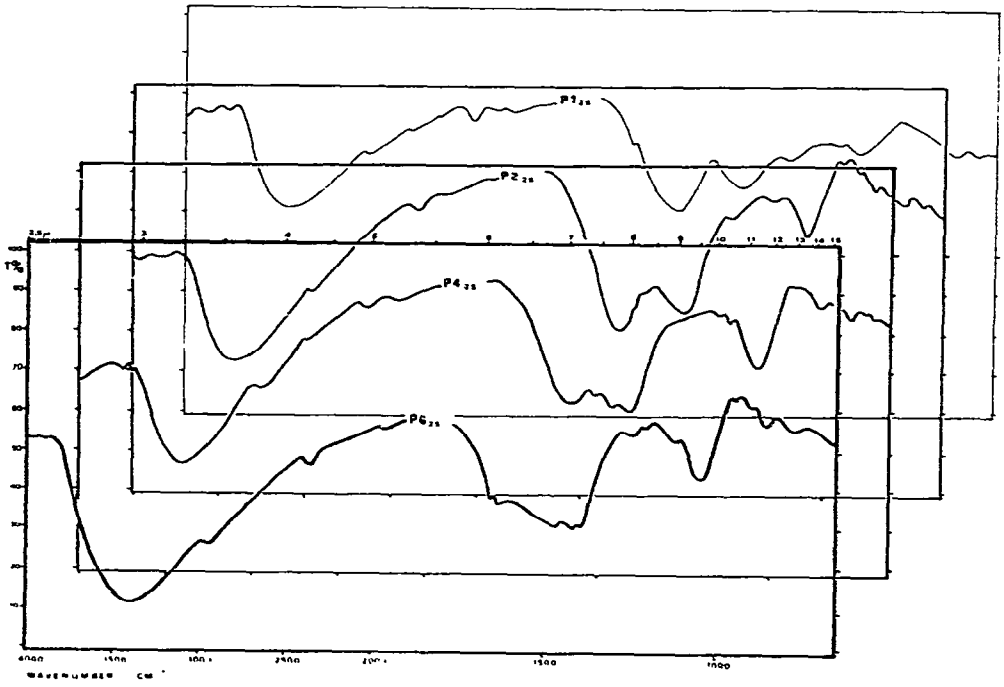
The 3500 cm^{-1} band represents OH from different classes such as phenols, alcohols and organic acids; it is impossible to establish the exact origin of the signal[16,17]. The 1600 cm^{-1} band is characteristic of humic-type molecules and corresponds to double bonds such as C=N, C=O and C=C. The free carboxylic group absorbs at 1725 cm^{-1} and the carboxylate anion at 1600 cm^{-1}. About 1540 cm^{-1} some spectra show a shoulder of various size; from Schnitzer and Khan's interpretation[18] it can be attributed to bound peptides. The 1400 cm^{-1} band corresponds to CH, CH_2 and CH_3 groups; this attribution is confirmed by an absorption band at 2950 cm^{-1}. Carboxylate anion also absorbs at the same frequency (1400 cm^{-1}). The 1050 cm^{-1} absorption band[18] must be attributed to inorganic material, and the Si–OSi band absorbs at 1070 cm^{-1}.

Infrared spectra obtained from Sephadex G-25 pools. In the spectrum of initial humic acid it can be seen that the intensity of the 1600 cm^{-1} band (aromatic C=C) is greater than that of the 1390 cm^{-1} band. In the $P1_{25}$ spectrum these intensities are very similar to those of total humic acid but the 2950 cm^{-1} band, corresponding to aliphatic C–H, is absent. In the $P2_{25}$ spectrum the intensities of the 1600 and 1390 cm^{-1} bands are identical and the 2950 cm^{-1} band slowly increases. In the $P4_{25}$ spectrum the relative intensities of the bands change, the 1390 cm^{-1} band becoming greater relative to the 1600 cm^{-1} band and the 2950 cm^{-1} band increasing even more. The $P6_{25}$ spectrum shows the same characteristics as $P4_{25}$. Hence it can be concluded that $P1_{25}$ is principally an aromatic fraction, while an increase of aliphatic character along the pools was detected.

None of the spectra from Sephadex G-25 shows the 1725 and 1200 cm^{-1} bands, confirming that part of humic acid is lost in the separation (fraction b on

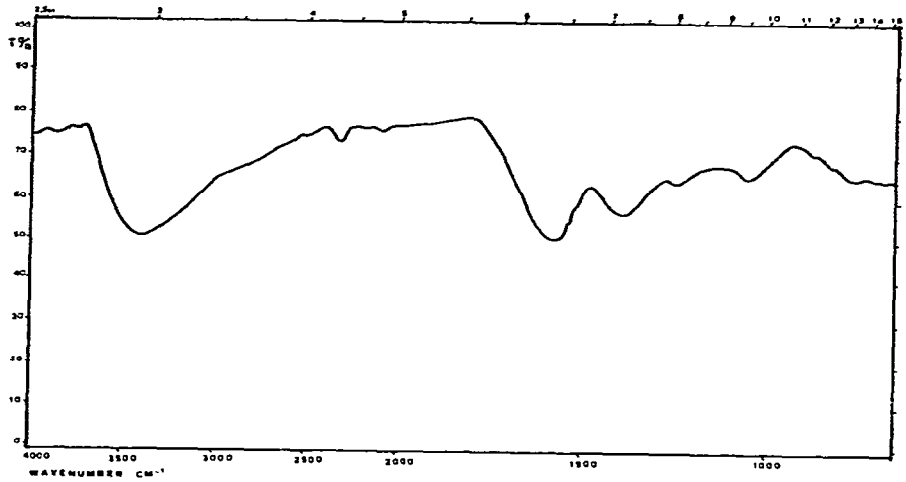


A.

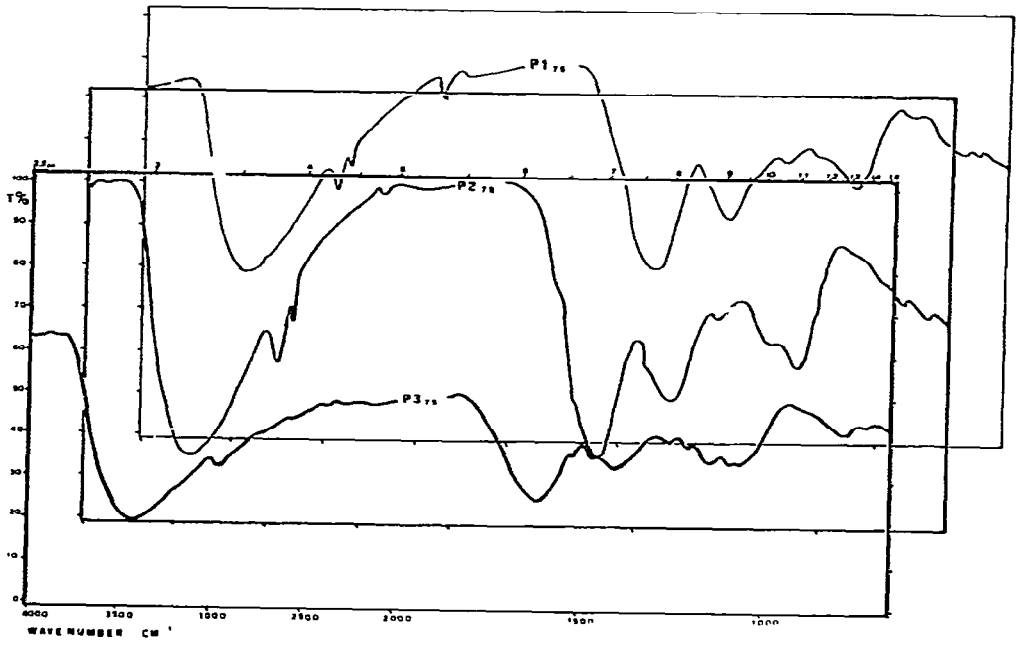


B

Fig. 3. Infrared spectra of (A) initial humic acid and (B) pools collected from gel filtration on Sephadex G-25.



A.



B.

Fig. 4. Infrared spectra of (A) $P1_{25}$ and (B) pools collected from gel filtration on Sephadex G-75.

electrophoresis) and should be rich in carbonyl groups and C–O bonds (the 1200 cm^{-1} band is assigned to C–O stretching).

The 1050 cm^{-1} band (Si–O bond) present in the initial humic acid increasing with increasing elution volume, showing that the small fractions are more strongly bound than higher fractions to the inorganic matrix. This is confirmed by the decreasing staining power of these fractions, as can be seen in the electrophoretic patterns[7].

$P1_{25}$ shows a great difference to the other pools, justifying the choice of Sephadex G-75 for subsequent separation.

Infrared spectra obtained from Sephadex G-75 pools. In the $P1_{75}$ and $P2_{75}$ spectra the 1050 cm^{-1} band is well defined, whereas it is absent from the $P3_{75}$ spectrum. According to gravimetric experiments (data omitted), the small fractions have a greater ash content and so the IR spectra of Sephadex G-75 pools should have a small 1050 cm^{-1} band, as suggested from the Sephadex G-25 results. This discrepancy can be explained by assuming that the band is also due to other groups, such as C–O–C. The interpretation of IR spectra from gel filtration fractions by Russel and Anderson[19] confirms this hypothesis. They suggested that the bonds of silicates and humates could be superimposed in some IR regions. Moreover, in the spectra of these pools, as could be predicted from Sephadex G-25 data, the 1725 and 1200 cm^{-1} bands are absent. It should be noted that the $P3_{75}$ spectrum is very similar to that of $P1_{25}$, the starting pool for the second gel filtration.

CONCLUSIONS

The general application of gel filtration to the preparative separation of humic acids has serious limitations: the fractions obtained show high heterogeneity and information on its molecular weight is uncertain owing to the absence of appropriate standards and to the interaction between humic acid and Sephadex, which causes a loss of some fractions. However, combination with disc electrophoretic and IR spectroscopic data allows the elucidation of properties of the fractions obtained.

These methods are very powerful, providing a large amount of information with little consumption of sample (*ca.* 1 mg). For these reasons it is advisable to find a preparative method with better resolution than gel filtration, whereas electrophoresis could be proposed as general technique. However, some workers[20] have reported that isotachophoresis is more efficient, separating the humic acid into up to eleven distinct fractions, but this method requires complex apparatus.

From gel filtration it could be established that a high proportion of total humic acid has a molecular weight greater than 50,000 daltons. Bendeck[3] reported that 93% of the humic acid of the soil studied had a molecular weight smaller than 100,000 daltons; hence this fraction of molecular weight ranging from 100,000 to 50,000 daltons, of high condensation and aromatic character (as proved by the IR spectra), can account for the low soil fertility, in spite of the large amount of organic matter.

The explanation of the low availability of organic material could be extended even to the small fractions, tightly bound to inorganic material; however, this conclusion requires confirmation by performing the same analysis on humic acids of different origin.

These comparisons with other soils are also desirable for establishing the common properties of humic acids of different origin in order to clarify the general

humification process; conversely, the differences in properties should explain particular origins of the humic acids and account for soil fertility.

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