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# MOLECULAR SIZE DISTRIBUTION OF HUMIC ACIDS AS AFFECTED BY THE IONIC STRENGTH AND THE DEGREE OF HUMIFICATION

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Molecular size distribution of soil humic acids was investigated by permeation chromatography on porous silica (μBondagel) and porous glass (Controlled Pore Glass, CPG) which enabled rapid analysis. Humic acid molecule was fully expanded at low ionic strength at pH 7.5, but it shrank considerably with increasing ionic strength. This behavior of humic acid molecule was analogous to that of linear polyelectrolyte molecule. Molecular size of humic acids decreased with increasing *RF* value and carboxyl group content and with decreasing $\Delta \log K$ value (namely with the increasing degree of humification). This phenomenon was attributed to oxidative depolymerization and intramolecular condensation during the process of humification. Based on the fractionation experiments on CPG, the fraction with the highest *RF* value and the lowest $\Delta \log K$ value had an intermediate molecular size among the fractions. The excluded large molecular size fraction contained both highly humified components and a large amount of non-colored components. After the fraction with the highest *RF* value was eluted, *RF* value decreased and $\Delta \log K$ value increased steeply with increasing elution volume.

*Key Words:* molecular size, humic acids, permeation chromatography, CPG.

Although numerous studies have been conducted to determine the molecular weight and molecular size of humic substances (*9–11*), several restrictive properties of humic substances (especially humic acids) such as dissociation of functional groups, polydispersiveness, limited solubility, strong light absorption in the visible range, interaction with gel materials, heterogeneous composition, lack of standard material, *etc.* are still hampering the precise determination of molecular weights. Furthermore, the values of the average molecular weight of humic acids seem to be of less practical importance if no information on the molecular weight distribution is supplied with the data. The relationships between the molecular size distribution of humic acids and the degree of humification, their origin, as well as their chemical composition also have not been fully documented. To tackle these problems, various kinds of humic acids with different degrees of humification should be analysed, and a rapid and reproducible chromatographic technique should be developed. The authors applied the permeation chromatography method of humic acids on two types of packing materials: porous silica (μBondagel) and porous glass (Controlled Pore Glass, CPG), and obtained some

preliminary results on the molecular size distribution of humic acids at different ionic strengths and its relationship with the degree of humification and other properties of humic acids.

## MATERIALS AND METHODS

*1. Soil humic acids.* Six humic acid samples were extracted from different kinds of soils. Methods of extraction and purification of humic acids were reported in the previous paper (*7*). Origin and several fundamental properties of humic acids are presented in Table 1. Elementary composition, functional groups, decomposition products by acid and alkali, and spectrophotometric characteristics of these humic acids were investigated extensively along with those of other 36 humic acid samples by the authors (*7, 13–19*).

*2. Preparation of humic acid solution.* Twenty-five mg of humic acid sample was weighed in a centrifuge tube, dissolved in 25 ml of 0.1 N NaOH, precipitated again by adding about 3 ml of 1 N HCl, and centrifuged. The precipitate was dissolved in each eluent buffer and filled up to a volume of 10 ml. In case distilled water was used as an eluent, a small amount of 0.1 N NaOH was added to solubilize humic acid. Before loading to the column, humic acid solutions were passed through a 0.45 $\mu$m Millipore filter.

*3. Standards for molecular weight calibration.* Glucose and dextran were used for making a calibration curve of molecular weight for each column. Dextrans (Sigma) with known average molecular weights were D-2960 (Mw 9,400), D-4133 (Mw 40,000),

Table 1. Properties of humic acid samples.

| Humic acid | Origin | Type | *RF* | $\Delta \log K$ | Carboxyl group (me/g) | Phenolic hydroxyl group (me/g) | Carbonyl group (me/g) | Amino acid carbon (%)[a] | Hexose carbon (%)[a] |
|---|---|---|---|---|---|---|---|---|---|
| Temmondai | Volcanic ash soil | A | 144 | 0.527 | 4.94 | 2.50 | 6.38 | 7.49 | 1.45 |
| Nagara P | Calcareous soil | A | 140 | 0.555 | 6.33 | 1.92 | 8.84 | 4.12 | 0.40 |
| Kuragari | Brown forest soil | B | 61 | 0.634 | 3.69 | 1.94 | 5.74 | 9.79 | 2.28 |
| Higashiyama (A) | Brown forest soil | B | 47 | 0.664 | 4.39 | 2.73 | 5.40 | 11.1 | 1.51 |
| Anjo | Paddy soil | Rp(1) | 21 | 0.835 | 2.43 | 2.03 | 2.91 | 16.2 | 2.51 |
| Kisokoma (F) | $A_0$ layer of forest soil | Rp(2) | 20 | 0.806 | 2.52 | 3.27 | 2.64 | 12.1 | 2.25 |

[a] Amino acid carbon and hexose carbon % were expressed as the ratio of each carbon content to the total carbon content of humic acid.

D-4751 (Mw 80,700), D-5001 (Mw 173,000) and D-5251 (Mw 500,000). Blue dextran (Pharmacia) was used to determine the exclusion volume. Each standard solution (0.5% in each eluent) was filtered through a 0.45 $\mu$m Millipore filter before being loaded to the column.

*4. Columns.*

1) $\mu$Bondagel: Stainless steel columns (30 cm × 3.9 mm i.d.) prepacked with $\mu$Bondagel E-linear and E-125 (Waters Associates) were used. This packed material is a fully porous silica with controlled porosity and is coated by permanently bonded ether groups. The E-linear column and the E-125 column were linked in series. The E-linear column covered molecular weights between 2,000 and 2,000,000 and the E-125 between 2,000 and 50,000 as dextran. Ten to 20 $\mu$l of sample solutions were loaded on this column, and the flow rate of eluents was 0.5 ml/min.

2) Controlled Pore Glass: Three glass columns (100 cm × 8.2 mm i.d.) were packed separately with 3 types of Controlled Pore Glass (Electro-Nucleonics), CPG-170, CPG-500 and CPG-1000, the pore diameters of which were 167, 493 and 1,038 Å, respectively. Loaded amounts were 75–200 $\mu$l, and the flow rate was 1.0 ml/min. For fractionation, a larger plastic column (85 cm × 25 mm i.d.) was packed with CPG-170. The loaded amount was 1.4 ml and the flow rate was 1.5 ml/min on this column.

*5. Instruments.* A Waters Associate Model 6000A pump and a U6K injector were used for the $\mu$Bondagel columns and the CPG packed in glass columns. For supplying an eluent to the large size fractionation column, a Hitachi 063 Liquid-Chromatograph pump was used. A UV-VIS effluent monitor (Hitachi) and a refractometer (Waters Associate R-400) were used as detectors.

*6. Eluents.* Distilled water, tris-hydroxymethylaminomethane-phosphate buffers (pH 7.5) with different ionic strengths and a sodium borate buffer (pH 9.2) were used as eluents. Tris-buffers (0.025 and 0.05 M), adjusted to pH 7.5 with phosphoric acid, had ionic strengths of 0.0293 and 0.0585, respectively. To adjust the ionic strength to 0.1, 0.25 and 0.5, NaCl was added to 0.05 M Tris-phosphate buffer to the level of 0.0415, 0.1915 and 0.4415 M, respectively. Sodium borate buffer (pH 9.2), 0.02 M in $Na_2B_4O_7$ and 0.05 M in NaCl, was used for the fractionation experiment. Each eluent was passed through a 0.45 $\mu$m Millipore filter and degassed under reduced pressure.

## RESULTS AND DISCUSSION

*Gel-permeation-chromatography on $\mu$Bondagel column*

Three humic acids, Temmondai, Higashiyama(A) and Anjo were eluted with Tris-buffer (pH 7.5) at 5 different ionic strengths on the $\mu$Bondagel column (Fig. 1). Elution curves of humic acids showed only one peak between $V_o$ and $V_o+V_i$ regardless of the ionic strength. In the case of Temmondai humic acid, a slight shoulder was observed in front of the peak at the ionic strengths of 0.0585 and 0.100. The elution of humic acids was not completed within $V_o+V_i$, which suggests that there was some interaction between the $\mu$Bondagel and humic acids. Peaking of the elution curve was

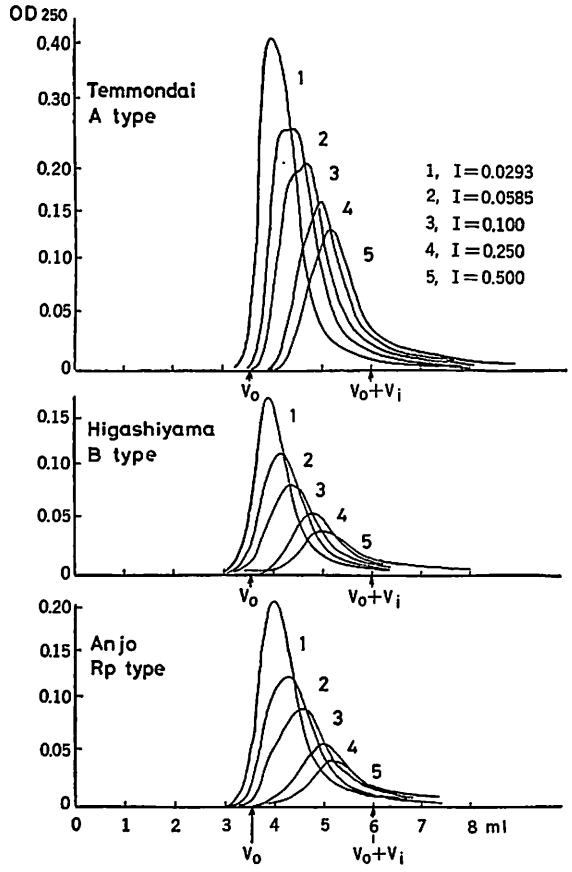


Fig. 1. Elution of humic acids on μBondagel E-linear+E-125 with Tris-buffers (pH 7.5) at different ionic strengths.

delayed with increasing ionic strength in every humic acid. The relationship between the elution volume of the peaks and the logarithm of the ionic strength of eluents is shown in Fig. 2. The elution volume increased linearly with increasing log $I$ in Anjo and Higashiyama(A) humic acids. However, the relationship was not linear in Temmondai humic acid. This difference in the changing pattern of elution volume with ionic strength also suggests the presence of qualitative differences between A type and other types of humic acids. Aggregation of humic acids at a high salt concentration may be one of the possible reasons for the behavior of A type humic acids. Change in elution volume implies a change in molecular size of humic acids. The molecular size of humic acids at an ionic strength of 0.0293 was as large as that of dextran with a molecular weight between 210,000 and 320,000. In contrast, the molecular size of humic acids at an ionic strength of 0.5 was as small as that of dextran with a molecular

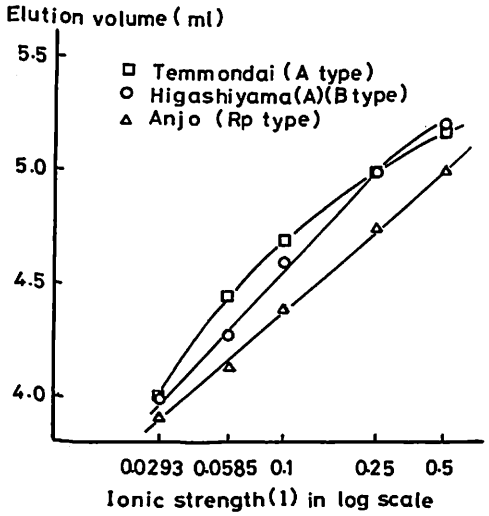


Fig. 2. The relationship between the volumes of humic acids on μBondagel E-linear+E-125 and the ionic strengths of Tris-buffers (pH 7.5).

weight between 2,300 and 4,500. As shown in these changes, the decrease in molecular size of humic acid due to the increase in ionic strength was considerable. The molecule of humic acid may have fully expanded due to the presence of negative charges of carboxylate anion which repulse each other especially at low ionic strength. Such a flexible nature of the humic acid molecule was also suggested by the viscosimetric measurements performed by KUMADA and KAWAMURA (*5*), by data on ultra-centrifugation reported by CAMERON *et al.* (*2*) and by GHOSH and SCHNITZER (*4*).

In this study, no attempt was made to determine the absolute molecular weight of humic acids. Elution curves of humic acids were only used for relative comparison of molecular size distribution. Molecular weight determination of humic acids by permeation chromatography is very difficult, because the molecular size of humic acid depends on the eluent, and any known high polymer can not be used as a standard for the molecular weight determination of humic acid due to its very complex structure and composition. CAMERON *et al.* (*2*) prepared molecular weight standards of humic acids by separating a humic acid into fractions within a narrow range of molecular weights whose average values were determined by applying the ultracentrifuge method. However, it has not been determined whether such a standard obtained from one humic acid sample can also be applied to different humic acids from different origins, because a humic acid molecule with an identical molecular weight can have different molecular sizes and shapes due to the difference in its chemical composition, functional group content, and so on.

Elution volume of humic acid at its peak increased in the order of Anjo<Higashiyama(A)<Temmondai when the ionic strength of the eluent was lower than 0.1, sug-

gesting that the molecular size of humic acid decreased in this order. This order corresponded to the increase in *RF* values, the decrease in $\Delta \log K$ (namely, the increase in the degree of humification), and the increase in the carboxyl group content (Table 1). These findings suggest that the molecule of humic acid became compact with increasing degree of humification. The increase in carboxyl group content and the change in the elementary composition of these humic acids with increasing degree of humification (*13*) suggest a progress in the oxidation process. Therefore, the decrease in molecular size of humic acid with increasing degree of humification may be due to oxidative depolymerization or intramolecular condensation.

When humic acids were eluted on μBondagel, a portion of humic acid was eluted beyond $V_o + V_i$, indicating that adsorption on the μBondagel surface took place. With increasing buffer concentration, the relative quantity of humic acid eluted before $V_o + V_i$ decreased (Fig. 1). LOEPPERT and VOLK (*8*), who observed the same phenomena in the chromatography of soil extracts on porous silica (Porasil A and Porasil AX, Waters Associates), attributed this to the decrease in the electrostatic repulsion and the increase in the direct H-bonding interaction between the solute and the Si(OH) groups. Since the Si(OH) groups on μBondagel are blocked by ether groups permanently bonded, H-bonding interaction may be neglected in the case of μBondagel.

*Permeation chromatography on CPG*

Different kinds of humic acids were chromatographed on CPG in order to investigate the patterns of molecular size distribution and the spectrophotometric characteristics of each fraction. No adsorption of humic substances on CPG was observed by DANNEBERG (*3*) as in this study. The effect of ionic strength on the molecular size distribution of humic acids is shown in Fig. 3. A humic acid (Temmondai) extracted

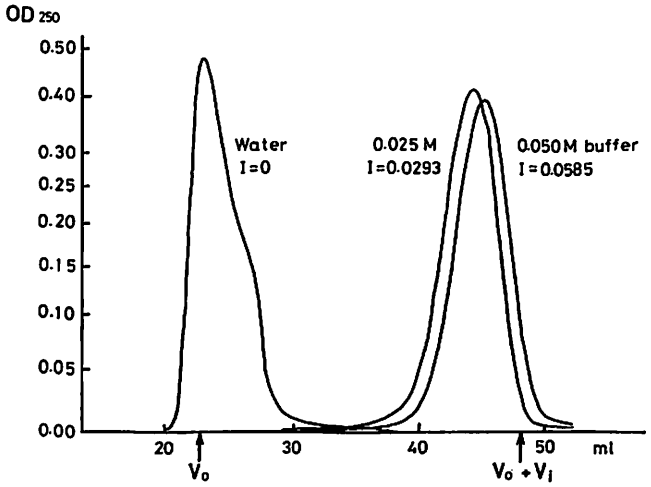


Fig. 3. Elution of Temmondai humic acid on CPG-1000 with water and Tris-buffers (pH 7.5).

from a volcanic ash soil was chromatographed on CPG-1000 using distilled water, 0.025 M Tris-buffer, and 0.05 M Tris-buffer as eluents. When the humic acid was eluted with distilled water, the whole fraction was practically eluted at the exclusion volume, $V_0$. The exclusion may be attributed to the electrostatic repulsion of negatively charged humate molecules by the dissociated Si(OH) groups on the surface of CPG. The structure of the humic acid molecule may be also fully expanded due to the mutual repulsion of dissociated COOH groups on the molecule. On the other hand, when Tris-buffers (pH 7.5, 0.025 and 0.05 M) were used as eluents, the whole humic acid fraction was permeated into the pores of CPG-1000. This phenomenon is attributed to the shrinkage of the humic acid molecules and to the suppression of negative charges on the CPG surface by the large tris-hydroxymethyl ammonium cation. Molecular size of Temmondai humic acid was as large as those of dextrans with a molecular weight of 56,000 and 30,000 in the 0.025 and 0.05 M Tris-buffer, respectively. Molecular weight of the humic acid molecule may be much smaller than that of the dextran which is eluted at the same elution volume, because the expansion of the molecule due to the repulsion of negative charges is assumed to take place in case of humic acid.

Six humic acids were also chromatographed on CPG-170, CPG-500, and CPG-1000. Compared with the elution curves on CPG-500, the relative quantity of the

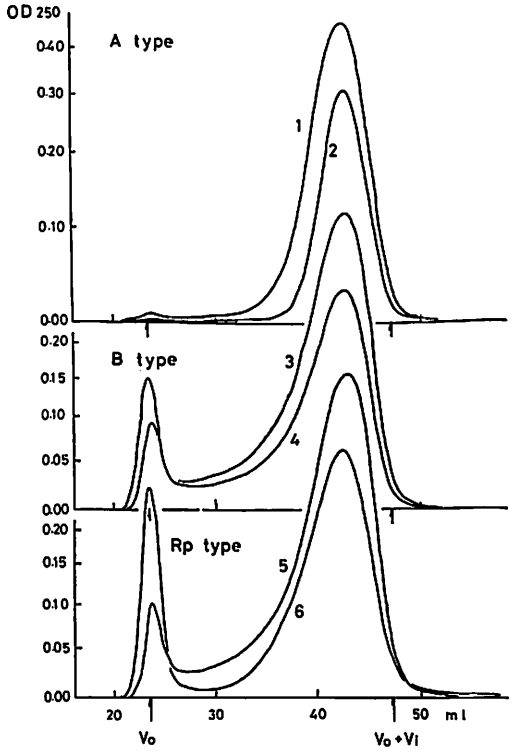


Fig. 4. Elution of 6 humic acids on CPG-500 with a 0.05 M Tris-buffer (pH 7.5).

permeated fraction decreased and that of the excluded fraction increased when CPG-170 was used and the opposite trend was observed when CPG-1000 was used. However, elution patterns of humic acids were similar among different types of CPG, and similar results and discussion may be deduced from the elution curves. Therefore, the elution curves of the 6 humic acids on CPG-500 are shown in Fig. 4. Temmondai and Nagara P humic acids were almost entirely permeated into the pores of CPG-500 and showed a trace amount of excluded fraction. Molecular size distribution of these humic acids was narrower than that of the other humic acids. These two humic acids were characterized by high carboxyl group contents, high *RF* value, and low $\Delta \log K$ value, and therefore by a high degree of humification. They are classified as A type according to the criteria of KUMADA *et al.* (*6*). Elution curves of other humic acids were composed of a relatively small excluded fraction and a large permeated fraction. The relative ratio of the fractions varied with the humic acids. Among the forest soil humic acids, such as Kuragari, Higashiyama(A) and Kisokoma(F), the amount of excluded fraction decreased with increasing *RF* value and with decreasing $\Delta \log K$ value. Paddy soil humic acid, Anjo (Rp type), showed a relatively broad distribution of molecular sizes, but its excluded fraction was smaller than that of Kisokoma(F) humic acid (Rp type) which had a similar degree of humification. The peaks of permeated fractions of the 6 humic acids were all eluted out at almost the same volume (42.5 ml). This elution volume corresponded to the 25,000 molecular weight of dextran.

Former studies (*2*, *4*) described the structure of humic acids as consisting of coiled, long chain molecules, or two or three-dimensional cross-linked molecules whose size and shape in solution were influenced by the pH and the presence of neutral salts. The same description of humic acid structure may also account for the molecular size distribution of humic acids observed in this study. However, the narrower molecular size distribution and the absence of excluded fractions in A type humic acids suggest that the shape of A type humic acids is compact due to oxidative decomposition and intramolecular condensation.

Three humic acids, Temmondai, Higashiyama(A), and Anjo were fractionated on a larger column packed with CPG-170. A broad elution pattern of A type humic acids on CPG-170 was favorable for the purpose of fractionation. A borate buffer (pH 9.2, ionic strength 0.1) was used as eluent. Eluted humic acid was detected by a differential refractometer, and the eluate was fractionated into 5 ml fractions by a fraction collector. For each fraction, UV-VIS absorption spectrum, optical densities (O.D.) at 600 and 400 nm, $\Delta \log K$, *RF* and organic carbon content were determined. Borate buffer was adopted instead of Tris-buffer because Tris-buffer interfered with the determination of organic carbon by the potassium dichromate oxidation method (*12*). The pH 9.2 buffer and the pH 7.5 buffer with an identical ionic strength gave a similar pattern of molecular size distribution. This may be because the dissociated states of carboxyl groups of humic acids were similar at pH 9.2 and at pH 7.5. *RF* value was calculated from the optical density at 600 nm and the organic carbon content of each fraction as follows:

$$RF = \frac{\text{O.D. at 600 nm} \times 1{,}000}{\text{Amount (ml) of 0.1 N } KMnO_4 \text{ consumed by 30 ml of humic acid solution}}$$

where 1 ml of 0.1 N $KMnO_4$ was supposed to be consumed by 0.45 mg humic acid carbon.

Elution curves for Temmondai and Higashiyama(A) humic acids are shown in Figs. 5 and 6. Change in optical densities at 600 and 400 nm, differential refractive index ($\Delta$RI), organic matter content, $\Delta \log K$ and *RF* values with increasing elution volume are also indicated in these figures.

In the case of Temmondai, the curves of O.D. at 600 nm, O.D. at 400 nm, and $\Delta$RI had peaks at 95, 100, and 107 ml of elution volume, respectively. The peak of $\Delta$RI which detects both colored and non-colored materials coincided with that of organic carbon content. The fraction with the highest *RF* value was eluted at 90 ml, which was even earlier than the peak of O.D. at 600 nm. These trends showed that dark colored substances had larger molecular size than non-colored substances in the permeated fraction. *RF* values of eluted humic acid fractions were highest in the middle molecular size fractions, but *RF* values were lower in the excluded fractions

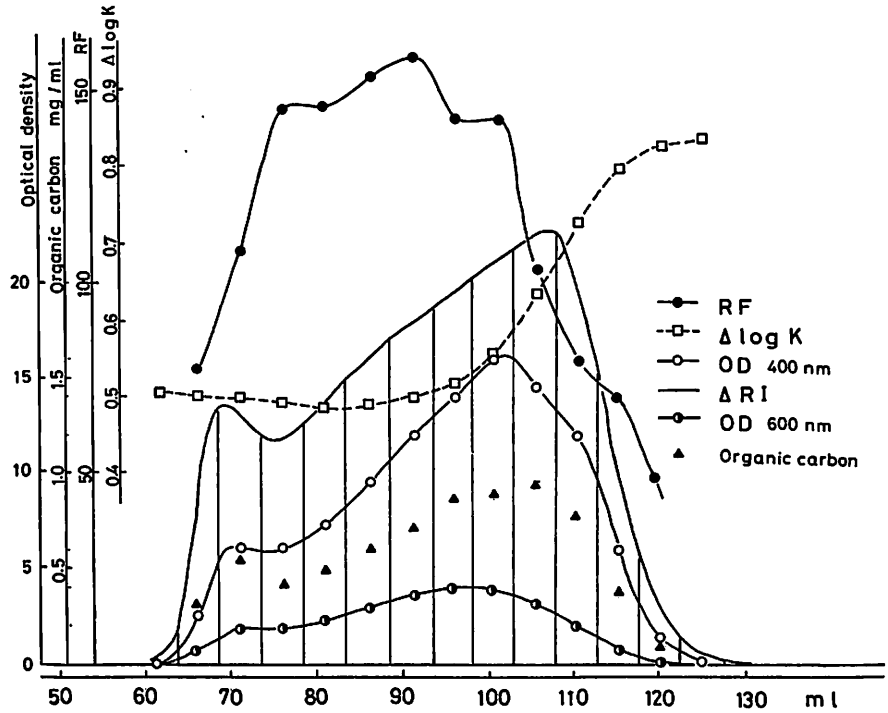


Fig. 5. Elution of Temmondai humic acid (A type) on CPG-170 with a borate buffer (pH 9.1) as monitored by $\Delta$RI, optical densities at 400 and 600 nm, organic carbon, *RF* and $\Delta \log K$.

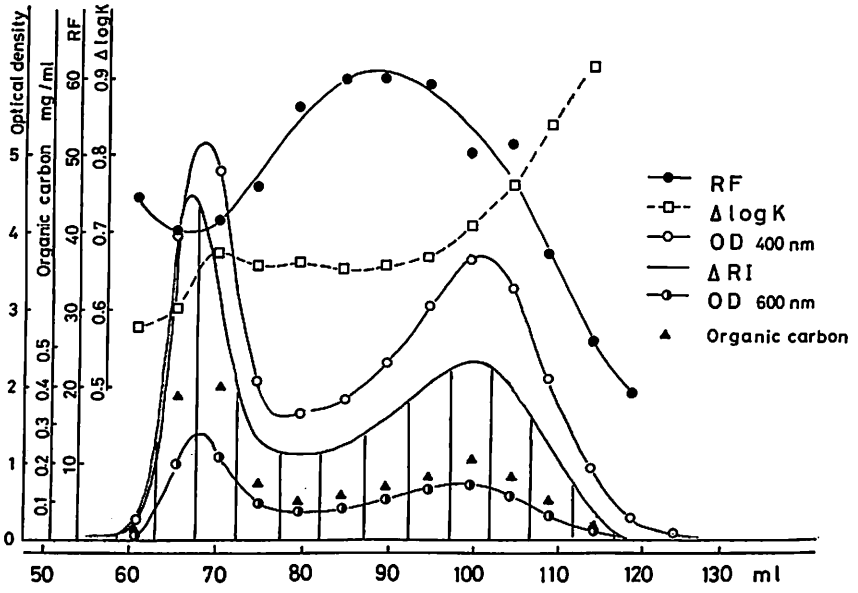


Fig. 6. Elution of Higashiyama(A) humic acid (B type) on CPG-170 with a borate buffer (pH 9.1) as monitored by $\Delta$RI, optical densities at 400 and 600 nm, organic carbon, *RF* and $\Delta \log K$.

which had the largest molecular size. On the other hand, $\Delta \log K$ values were almost identically low among the fractions eluted before the peak of *RF*.

*RF* value is defined as the optical density at 600 nm of humic acid per unit concentration. Therefore, *RF* value reflects not only the degree of humification of humified component, but also the composition of colored and non-colored fractions in humic acids. On the other hand, the value of $\Delta \log K$ represents the inclination of the absorption curve in the visible range, and therefore, it is not affected by the co-existence of non-colored materials. A low $\Delta \log K$ value indicates the occurrence of highly humified components in the fraction.

Behavior of $\Delta \log K$ values of eluted fractions showed that highly humified components also existed in the fractions eluted before the peak of *RF* value appeared.

Relatively low *RF* values of excluded fractions showed on the other hand that non-colored components such as polysaccharides contribute to these large molecular size fractions. BARKER *et al.* (*1*) also showed that the molecular size of soil polysaccharides was much larger than that of colored materials.

After the peak of *RF* value was reached, *RF* values of fractions decreased and $\Delta \log K$ values increased with increasing elution volume.

Elution curve of Higashiyama(A) humic acid (Fig. 6) was characterized by a larger excluded fraction than that of Temmondai humic acid. The peak of $\Delta$RI and O.D. at 400 nm was eluted earlier in Higashiyama(A) humic acid than in Temmondai humic acid, which suggests the greater contribution of large molecular size components in

Higashiyama(A) humic acid. The patterns of changes in the $RF$ and $\Delta \log K$ values were almost the same as those of Temmondai humic acid. The change in these parameters suggests that the middle molecular size fraction had the highest degree of humification, and that highly humified components exist together with the large amount of non-colored components in the excluded fraction. Similar results were obtained with Anjo humic acid.

*Comparison of μBondagel and CPG as packing materials*

Utilization of μBondagel and CPG as packing materials enabled a rapid and reproducible permeation chromatography of humic acids. The most remarkable difference between the results obtained by using μBondagel and CPG was found in the peaking of the elution curves. In the case of μBondagel, the elution curve of each humic acid showed a peak at different elution volumes. On the other hand, when humic acids were chromatographed on CPG, elution curves of humic acids belonging to different types and origins had peaks at the same elution volume.

Because the retention of humic acids by hydrophobic interaction is likely to occur when μBondagel is used, a better eluent system which eliminates undesirable interactions should be sought. In the case of CPG, it was difficult to eliminate the excluded fraction of B and Rp type humic acids even when a large pore size CPG such as CPG-1000 was used.

The results obtained both in the μBondagel and CPG chromatography were as follows.

1) Elution volumes of humic acids depended on the ionic strength of eluents.
2) Average molecular size of A type humic acids was smaller than that of B type and Rp type humic acids. Molecular size distribution of A type humic acids was narrower than that of other types of humic acids.
3) Elution curves of different humic acids were very similar to each other.

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