Soil Sci. Plant Nutr., 35 (3), 393–403, 1989

DETERMINATION OF MOLECULAR WEIGHTS OF HUMIC ACIDS BY OSMOTIC PRESSURE MEASUREMENT AND BY PERMEATION CHROMATOGRAPHY ON CONTROLLED PORE GLASS

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Received August 4, 1988

Number-average molecular weights ($\bar{M}_n$) of humic acids (HAs) were determined by osmometry and by permeation chromatography on controlled pore glass with a pore size of 500 Å. $\bar{M}_n$ value of dialyzed HAs tended to decrease in the order of Rp(2)>Rp(1)>P_0 type, and increase in the order of P_0<B<A type. $\bar{M}_n$ value of non-dialyzed HAs did not show any remarkable difference among the HA types, which was considered to be due to the predominance in the number of species with lower molecular weight (<10,000) in HAs. $\bar{M}_n$ value calculated from the permeation chromatograms showed a similar trend to that of $\bar{M}_n$ obtained by osmometry, but the values differed depending on the detection methods. Ultraviolet absorption gave a smaller value for $\bar{M}_n$ while the differential refractive index gave a larger value for $\bar{M}_n$ than that by osmometry. It was shown that both the decrease and increase in the molecular weight take place during the humification process of HAs, where the decrease was considered to be associated with the decomposition of partially decayed bio-polymers and the increase with the formation of a dark colored high polymer intrinsic to soils.

Key Words: gel permeation chromatography, humic acid, molecular weight, osmometry.

As reported by WERSHAW and AIKEN (1985) and STEVENSON (1982), molecular weights of humic substances have been determined by various methods. However, due to the lack of universal molecular weight markers for humic substances as well as the polydispersity and heterogeneity of humic substances, there is a marked discrepancy in the values of molecular weights reported for humic substances. There are few reports on the values of the molecular weights based on the colligative property (thermodynamic property that depends on the number of particles in solution, and not on the nature of these particles). In this study, therefore, the molecular weights of humic acids with different degrees of humification were determined by osmometry which is one of a few colligative methods, as well as by the more convenient permeation chromatography method. The molecular weights of humic acids determined by both methods were compared and their change with the process of humification was investigated.

MATERIALS AND METHODS

1. Humic acids used. Humic acid (HA) samples from five soil types were used. Main characteristics of these samples are listed in Table 1. Elementary composition, functional group composition, degradation products, as well as several spectroscopic characteristics of the HA samples were investigated along with those of other 40 HA samples collected by the authors (KUWATSUKA *et al.* 1978; TSUTSUKI and KUWATSUKA 1978a,b, 1979a,b, 1984).

2. Dialysis of HA samples. Humic acid samples were dialyzed prior to the osmotic pressure measurement with the same membrane which was to be used for the osmotic pressure measurement afterwards. This procedure was adopted, as accurate osmotic pressure can not be determined if any constituent of HA passes through the membrane, and because the Donnan equilibrium should be maintained between the HA solution and the outer solution of dialysis.

Each HA sample (340–390 mg) was dissolved in 10 ml of 1 N NaOH and precipitated again with 12 ml of 1 N HCl and centrifuged. The supernatant was discarded and 1 ml of 1 N NaOH and *ca.* 30 ml of Tris-buffer (0.05 M trishydroxymethylaminomethane, 0.04 M HCl, 0.16 M NaCl, ionic strength 0.2, pH 7.5) were added to the precipitate, in this order. Ultrasonic treatment was applied to dissolve completely the HA pellet, and the HA sample solution was filled up to 50 ml with the Tris-buffer. This solution was transferred into the dialyzing cell connected with gel cellophane membranes (Fuji cellophane No. 600) on both sides and dialyzed against *ca.* 500 ml of Tris-buffer. The outer solution of the dialysis was changed at 1/2–3 days intervals. The dialysis was continued until the outer solution became almost colorless. The absorption at 220 nm of the outer solution, the volume of the outer solution, and the time of the buffer change were recorded every time to calculate the rate of HA seepage through the membrane (Fig. 1). The dialysis was continued for 3 months and the buffers were changed 51 times in total.

3. Determination of the HA concentration after dialysis. Three milliliter portions of the dialyzed HA solution and the outer solution were infiltrated into 4 g of cobalt oxide, dried in an oven and subjected to the analysis of C and N by a CN analyser (Yanaco MT500). The concentration of HA was calculated by subtracting the data for the outer solutions from those for the HA solution. The percentage of HA retained was calculated on a carbon basis.

4. Measurement of the osmotic pressure. Four to 5 sample solutions with different concentrations were prepared by diluting the dialyzed HA solutions (master solutions) with the outer solutions of dialysis. The osmotic pressure of each HA solution was measured against that of the outer solution with a Mechrolab 501 high speed membrane osmometer at 30.0°C with the same gel-cellophane membrane as that used for the dialysis. Osmotic pressure measurements of the HA solution and outer solution were carried out alternately.

Table 1. Properties of humic acid samples used.

Humic acid	Origin	Type	RF	$\Delta \log K$	Carboxyl group (meq/g)	Phenolic OH group (meq/g)	Carbonyl group (meq/g)	Amino acid carbon (%)[a]	Hexose carbon (%)[a]
Temmondai	Humic Andosol	A	144	0.527	4.94	2.50	6.38	7.49	1.45
Higashiyama (A)	Dystric Cambisol	B	47	0.664	4.39	2.73	5.40	11.1	1.51
Ohnobaru	Humic Andosol	P_0	39	0.678	3.55	2.42	3.87	12.0	3.30
Anjo	Anthraquic Fluvisol	Rp(1)	21	0.875	2.43	2.03	2.91	16.2	2.51
Kisokoma(F)	A_0 layer of Orthic Podsol	Rp(2)	20	0.806	2.52	3.27	2.64	12.1	2.25

[a]Detected in acid-hydrolysates of HAs and expressed in % of the total carbon content of HA.

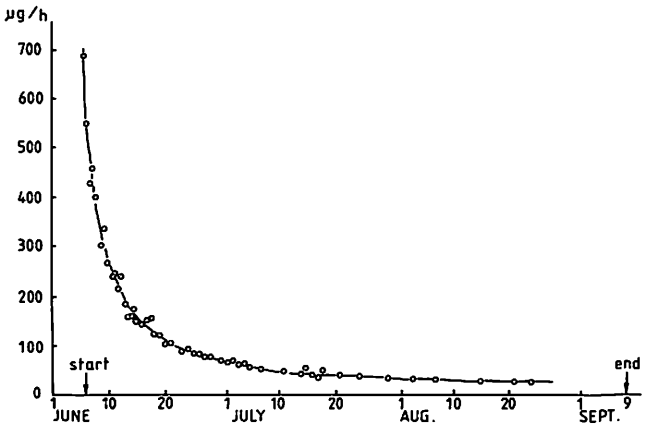

Fig. 1. Rate of seepage of humic acids (Higashiyama(A), B type) during the dialysis through a gel-cellophane membrane.

5. Permeation chromatography of HAs on CPG 10-500. Humic acid solutions before and after the dialysis (50–125 μl) were eluted on a CPG 10-500 column (controlled pore glass 200–400 mesh with an average pore size of 500 Å packed in a glass column of *i.d.* 8.2 mm × length 100 cm) with Tris-buffer (0.05 M trishydroxymethylaminomethane, 0.04 M HCl, 0.16 M NaCl, ionic strength 0.2, pH 7.5). CPG 10-500 was used because the void volume fraction was very small when HAs were eluted. The ionic strength of the eluent was increased to suppress the intramolecular repulsion of negatively charged groups (TSUTSUKI and KUWATSUKA 1984). The flow rate of the eluent was 1.0 ml/min. Elution curves were analyzed by the absorbance at 250 nm with a Hitachi 034 UV-VIS effluent monitor as well as by the differential refractive index obtained with the Waters R401 differential refractometer. The column was calibrated with dextran standard samples with known $\bar{M}_n$ (purchased from Aldrich Chemical Co.).

RESULTS AND DISCUSSION

1. Changes in the characteristics of HAs associated with the dialysis

The dialysis of HAs through the gel-cellophane membrane for a very long time (3 months) was a prerequisite to the osmometry. The ratio of HA retained in the gel-cellophane membrane ranged from 52–67%, and showed no relationship with the types of HAs or the degree of humification (Table 2). The retained fractions had larger E_{600} and smaller $\Delta \log K$ values, in other words, they showed a higher degree of humification, for all the HAs used. The fraction with low molecular weight was lost in the course of the dialysis. Such a fraction is, therefore, not likely to contribute to the dark color of HAs. The gel-cellophane membrane is suitable for use in the

Table 2. Change in spectroscopic characteristics of HAs during dialysis.

Humic acid	Type	Retention % of HA	Change in E_{600}		Change in $\Delta \log K$	
			Before	After	Before	After
Temmondai	A	52.0	77.3	93.1	0.516	0.485
Higashiyama(A)	B	66.5	27.7	36.1	0.679	0.592
Ohnobaru	P_0	56.8	24.8	33.0	0.684	0.600
Anjo	Rp(1)	55.2	12.8	15.3	0.904	0.812
Kisokoma(F)	Rp(2)	57.7	12.4	17.4	0.822	0.712

E_{600}, the absorbance at 600 nm of HA solution at 1 g carbon/100 ml. Before, the value before dialysis; After, the value after dialysis.

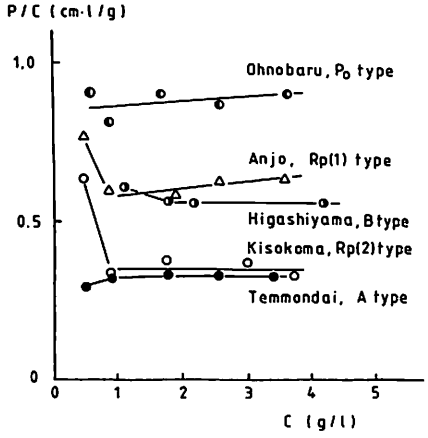

Fig. 2. Relationship between the specific osmotic pressure (P/C) and the concentration of humic acids (C).

measurement of a molecular weight of $>$ *ca.* 10,000 by osmometry. Though the exact limit for the molecular weight of humic substances by the use of this membrane has not been determined under the current conditions, it was tentatively estimated at *ca.* 10,000 for convenience.

2. Number-average molecular weight of HAs determined by osmometry

The osmotic pressures of HAs per unit concentration were almost constant within the concentration range from 1 to 4 g/liter (Fig. 2), which suggested that the molecular weights of HAs were constant in this range. The osmotic pressures of Anjo and Kisokoma HAs, however, tended to increase at a concentration lower than 1 g/liter, suggesting the splitting of the HA molecules at a low concentration.

Like other polyelectrolytes, HA molecules form a micelle at concentrations higher than a certain level (critical micelle concentration; CMC). INOKO and TAMAI (1976)

reported that the CMC of two HA preparation ranged from 4 to 5 g/liter, which was higher than the range of concentrations adopted for the osmometry in this study. Enough data are, however, not available presently to analyze the effect of micelle formation on the molecular weights of HAs.

The number-average molecular weight ($\bar{M}_n$) of HA was calculated by the following equation;

$$P/C = RT(1/\bar{M}_n + AC + BC^2 + \cdots), \quad (1)$$

where P, osmotic pressure expressed as the liquid level(cm) of Tris-buffer. C, concentration of HA solution (g/liter). $R = 8.205 \times 10^{-2}$ liters·atm/(deg·mol) = 84.393 liter·cm/(deg·mol) (1 atm = 1,028.6 cm Tris-buffer). $T = 303$ K. $RT = 255.7$ liters·cm/mol. A and B, virial constants.

In this study, the virial constants were assumed to be zero because the osmotic pressures were almost constant regardless of the concentration. $\bar{M}_n$ values were calculated for all the plots examined and the average was obtained by excluding the three values with large deviations at the low concentrations in the case of the Temmondai, Anjo, and Kisokoma HAs.

The obtained number-average molecular weights of HAs ranged from 2.94×10^4 for Ohnobaru HA to 7.82×10^4 for Temmondai HA (Table 3). With reference to the types of HAs, the $\bar{M}_n$ value decreased in the order of Rp(2) > Rp(1) > P_0 type, and then increased in the order of P_0 < B < A type. These sequences suggest that the degradation and depolymerization of high molecular constituents such as lignin and polysaccharides take place in the process of humification from Rp(2) to Rp(1) or to P_0 type, while the formation of the dark colored high polymer intrinsic to soils occurs in the process toward A type.

Opposite sequences among the different HA types have been observed for the changes in the contents of total N, hydrolysable N, amino acid N, and acid-hydrolysable phenolic compounds in HAs (TSUTSUKI and KUWATSUKA 1979a). It is interesting to note that the changes in the $\bar{M}_n$ values of the dialyzed HAs corresponded well to the changes in the chemical characteristics of HAs, because the changes in the chemical characteristics of non-dialyzed HAs did not differ significantly among the HAs of different types as discussed below.

3. Molecular weight determination by permeation chromatography on CPG 10-500

Though osmometry is one of the few methods which give the molecular weight based on the colligative property, this procedure requires a considerably large amount of samples, time and expensive instruments. Moreover the sample should be dialyzed prior to the measurement. The molecular weight obtained by permeation chromatography, on the other hand, should be considered as a relative value, if the column is not calibrated. Permeation chromatography, however, compensates for other shortcomings of the osmometry method. In this study, the samples subjected to osmometry were also chromatographed on the CPG calibrated with dextran to calculate the number-average

Table 3. Number average molecular weights ($\bar{M}_n$) of humic acid samples.

Humic acid	Method	Before dialysis	After dialysis
Temmondai	Osmometry	(1.39×10^4)	7.82×10^4
A type	pc/uv	2.26×10^3	1.49×10^4
	pc/dri		19.1×10^4
Higashiyama(A)	Osmometry	(1.44×10^4)	4.49×10^4
B type	pc/uv	2.34×10^3	1.02×10^4
	pc/dri		9.11×10^4
Ohnobaru	Osmometry	(1.42×10^4)	2.94×10^4
P_0 type	pc/uv	2.30×10^3	5.43×10^3
	pc/dri		7.35×10^4
Anjo	Osmometry	(1.14×10^4)	4.22×10^4
Rp(1) type	pc/uv	1.82×10^3	6.94×10^3
	pc/dri		6.70×10^4
Kisokoma(F)	Osmometry	(1.51×10^4)	7.29×10^4
Rp(2) type	pc/uv	2.47×10^3	9.93×10^3
	pc/dri		6.45×10^4

pc/uv, permeation chromatography with ultraviolet detector; pc/dri, permeation chromatography with differential refractive index. The values in parentheses were estimated by the regression between $\bar{M}_n$ obtained by osmometry and $\bar{M}_n$ by pc/uv (see text for detail).

($\bar{M}_n$) and weight-average molecular weights ($\bar{M}_w$) compared with the $\bar{M}_n$ values obtained by osmometry. The equations were as follows;

$$\bar{M}_n=\sum n_iM_i/\sum n_i=\sum c_i/\sum(c_i/M_i), \tag{2}$$

$$\bar{M}_w=\sum n_iM_i^2/\sum n_iM_i=\sum c_iM_i/\sum c_i, \tag{3}$$

where n_i, number of molecule i; M_i, molecular weight of molecule i; and c_i, concentration (g/liter) of molecule i; which may be substituted by the UV absorbance or by the differential refractive index on the assumption that they are proportional to the concentration. By calibrating the column with $\bar{M}_n$ value of dextran, the following regression equations were obtained between the M_i and the elution volume x_i (ml).

$$M_i=10^{(13.78-0.228x_i)}, \tag{4}$$

for the curve obtained by the UV absorption at 250 nm, and

$$M_i=10^{(13.99-0.228x_i)}, \tag{5}$$

for the curve obtained by the differential refractive index. Small void volume peaks observed in the permeation chromatograms of HAs were omitted in the calculation, because the calibration curve did not cover this molecular weight range. In the elution curves analyzed in this study, excluded fractions were assumed to be small enough.

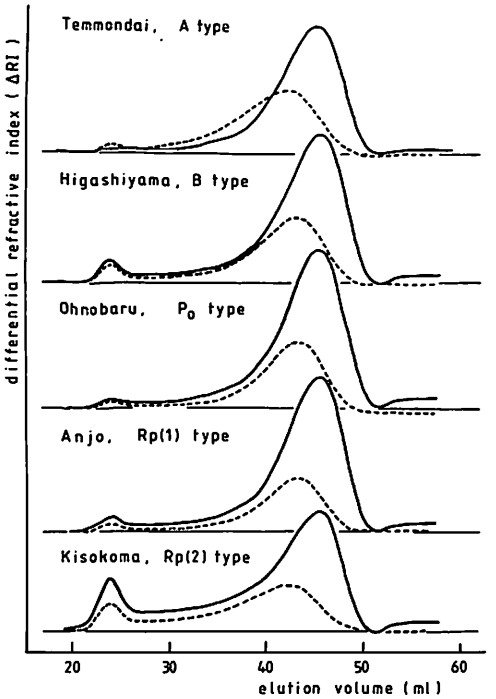

Fig. 3. Elution curves of humic acids on controlled pore glass 10-500 Å before and after dialysis. Solid line, before dialysis; broken line, after dialysis.

Moreover, since the molecular weight of the excluded fractions was *ca.* 100,000 times larger than that near the peak of the elution curve under the conditions employed in this study, the contribution of the excluded fractions to the total number of HA molecules and to the $\bar{M}_n$ value was considered to be negligible.

The $\bar{M}_n$ values for the standard dextrans obtained by the above equations were considerably lower than the values given by the supplying company. This discrepancy between the real $\bar{M}_n$ and the calculated $\bar{M}_n$ values is not surprising, because the peak position of the elution curve usually does not coincide with the $\bar{M}_n$ of high-polymers. This deviation, however, could be corrected by the regression equation between the real $\bar{M}_n$ and the calculated $\bar{M}_n$ for standard dextrans;

$$\text{real } \bar{M}_n = 0.0293\ (\bar{M}_n \text{ by Eq. 2})^{1.497}, \quad r = 0.996. \tag{6}$$

The $\bar{M}_n$ values obtained by osmometry and by permeation chromatography are shown in Table 3. The $\bar{M}_n$ value calculated from the elution curve by UV absorption was always lower than that by osmometry, but it showed almost the same trend as the $\bar{M}_n$ value obtained by osmometry with respect to the HA types. The UV absorption at 250 nm may have put a larger moment on the smaller molecular species, but the

molecular weight obtained was considered to be a relative index of the molecular weight of HAs. On the other hand, the $\bar{M}_n$ value calculated from the elution curve by the differential refractive index was larger than that by osmometry, and increased in the order of Rp(2)<Rp(1)<P_0<B<A type. The differential refractive index may have put a larger moment on larger molecules. The elimination of the void volume fraction in the calculation may also be responsible for the lower molecular weights of the Rp type HAs in this case.

The estimation of the colligative $\bar{M}_n$ value of the non-dialyzed HAs from that obtained by permeation chromatography (detector; UV at 250 nm) was considered to be possible based on the regression between the two types of $\bar{M}_n$ values. Regression equation for the dialyzed HAs was as follows;

$$\bar{M}_n \text{ by osmometry} = 10.35\ (\bar{M}_n \text{ by chromatography})^{0.933}, \quad r = 0.888. \tag{7}$$

On the assumption that the same correlation exists for the non-dialyzed HAs, the colligative $\bar{M}_n$ value of the HA before dialysis was estimated by substituting the $\bar{M}_n$ value obtained by chromatography (UV at 250 nm) of the non-dialyzed HAs into Eq. 7, and shown in parentheses in Table 3. The estimated $\bar{M}_n$ value before dialysis ranged from 1.14×10^4 to 1.51×10^4, and did not vary appreciably among the HAs of different types. This phenomenon may be due to the fact that the constituents of HAs with a molecular weight $<$*ca.* 10,000 contributed significantly to the number of HA molecules, and therefore, also to the $\bar{M}_n$ value of HAs. This observation suggested in turn that the difference in the properties of HAs owes much to the fraction with a molecular weight $>$*ca.* 10,000.

The values of the weight-average molecular weights ($\bar{M}_w$) based on the permeation chromatogram (Table 4) were *ca.* 100 times larger than the $\bar{M}_n$ value of HAs. In the case of the non-dialyzed HAs, the $\bar{M}_w$ value for Rp(2) type HA, which had the lowest

Table 4. Weight average molecular weights ($\bar{M}_w$) of humic acid samples.

Humic acid	Method	Before dialysis	After dialysis
Temmondai	pc/uv	2.07×10^5	2.11×10^6
A type	pc/dri		3.74×10^6
Higashiyama(A)	pc/uv	6.26×10^5	1.22×10^6
B type	pc/dri		2.75×10^6
Ohnobaru	pc/uv	2.64×10^5	5.21×10^5
P_0 type	pc/dri		1.96×10^6
Anjo	pc/uv	4.36×10^5	7.32×10^5
Rp(1) type	pc/dri		2.13×10^6
Kisokoma(F)	pc/uv	2.00×10^6	2.52×10^6
Rp(2) type	pc/dri		5.38×10^6

See the footnote of Table 3 for abbreviations.

degree of humification, was extremely high, while the $\bar{M}_w$ value for A type HA with the highest degree of humification was the lowest. Because the constituents with higher molecular weights contribute more to the value of $\bar{M}_w$ than to that of $\bar{M}_n$, it is considered that Rp(2) type HA contains a larger amount of slightly decayed biopolymers, resulting in the very high $\bar{M}_w$ value for this type of HA. On the other hand, the $\bar{M}_w$ value of dialyzed HAs decreased from Rp(2) type *via* Rp(1) type to P_0 type, but increased from P_0 type *via* B type to A type.

4. Discussion

Average molecular weights of HAs belonging to different types and the order in which they varied differed largely according to the method of determination of the molecular weight. This phenomenon is attributed to the fact that the molecular weights of HAs are spread over a very wide range, and each method of molecular weight determination puts different moments on different molecular weight ranges. The average molecular weight itself, in addition, does not provide information on the polydispersity. Therefore, the molecular size distribution determined by gel permeation chromatography enables to analyze the behavior of the molecular weight of HA. In the previous paper (TSUTSUKI and KUWATSUKA 1984), we reported that the average molecular size of A type HAs was smaller than that of B type and Rp type HAs, and that the molecular size distribution of A type HAs was narrower than that of other types of HAs. However, when elution curves by different detection methods were compared in detail, it was also suggested that the dark-colored substances had a larger molecular size than the non-colored substances in the permeated fraction of HA and that the fraction with a medium molecular size exhibited the highest degree of humification.

The same conclusion can also be derived from the results presented in this study. The number-average molecular weight ($\bar{M}_n$), with a lower dependence on the macromolecular constituents such as the excluded fractions of HAs, could more correctly reflect the change in molecular weight of HAs near the peak area of elution curves. The elimination of the low molecular constituents prior to the osmotic pressure measurement also high-lighted the change in the molecular weight of the HAs in the medium molecular weight range ($>$ *ca.* 10,000). Thus, it was demonstrated in this study that both the decrease and the increase in molecular weights took place during the process of humification of HAs. The decrease in the molecular weight occurred in the early stage of humification especially in the Rp(2), Rp(1), and P_0 types. The random coil solution conformation for HA molecules proposed by CAMERON *et al.* (1972) and HAYES and SWIFT (1978) may be best applied to this stage of HAs, and the splitting of long side chains in the process of humification can be inferred. On the other hand, the increase in the molecular weight occurred in the subsequent stage of humification especially in the P_0, B, and A types. The increase in the medium range molecular weight eluted at the peak position of the permeated fraction was responsible for this increase. The formation of a conjugated core structure with a medium molecular size

by polycondensation of phenolic and quinonoid units was suggested in this stage.

Acknowledgments. The authors extend their gratitude to the late Prof. (emeritus) K. Kumada for his valuable suggestions in this study, and to Prof. (emeritus) M. Nagasawa, Faculty of Engineering of Nagoya University, for the use of the Mechrolab 501 high speed membrane osmometer, and to Dr. H. Higo of the above laboratory for his guidance in osmometry.

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