

CHEMICAL STUDIES ON SOIL HUMIC ACIDS

II. Composition of Oxygen-Containing Functional Groups of Humic Acids

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The levels of alcoholic and phenolic hydroxyl, methoxyl, carboxyl, and carbonyl groups of 33 to 38 humic acids obtained from various types of soils were determined to analyze the relationships between the amounts of these functional groups and the degree of humification or the types of soils. The amounts of various oxygen-containing functional groups examined were all proven to be significantly different among the various types of humic acids by analyses of variance. During humification, generally, the carboxyl and carbonyl groups increased while alcoholic and phenolic hydroxyl and methoxyl groups decreased. Linear and logarithmic regression analyses of carboxyl group contents on *RF* values (optical density of the alkaline solution of humic acids at 600 nm) gave very significant positive correlations. The carboxyl group contents of Rp type humic acids and humic acids from calcareous soils were largely distributed in the upper side of the regression curve. The carbonyl group contents showed a very significant linear positive correlation with carboxyl group contents, and both of them showed high linear positive correlation with *RF* values. Phenolic hydroxyl group contents decreased with humification in Rp(1), B, and A type humic acids. Alcoholic hydroxyl group contents showed a significant negative linear association with *RF* values. Methoxyl group contents decreased rapidly with increasing humification in low humified humic acids, and their negative correlation with *RF* values were proven to be very significant by logarithmic regression analysis. The complicated relationship between oxygen content and *RF* value which was reported previously (7) has been accounted for by the results obtained in the experiments conducted here.

Additional Index Words: elementary composition, calcareous soils, humification, regression analysis.

Humic acids are complicated mixtures of amorphous and high molecular organic compounds. Little is known about their chemical structures although many studies have been conducted to elucidate them (3-5, 11). The purpose of this study is to obtain more detailed information on the chemical properties of soil humic acids and their relationship to the degree of humification or soil type, and also to shed light on their chemical structures and compositions.

In our previous paper (7), elementary compositions of various humic acids were reported. Statistically significant or comprehensible relationships were observed between the hydrogen or nitrogen content and the degree of humification, but such relationships were not observed regarding the carbon or oxygen content.

In this study, the amounts of various oxygen-containing functional groups of various humic acids were determined in an effort to elucidate the chemical structures of humic acids. The relationships between the amounts of the functional groups and the degree of humification or soil types were also investigated.

MATERIALS AND METHODS

1. *Humic acids.* Thirty-three to thirty-eight humic acid samples were used for the determination of oxygen-containing functional groups such as carboxyl, phenolic hydroxyl, alcoholic hydroxyl, methoxyl, and carbonyl groups. The method of extraction and purification, the classification of the soils from which the samples were isolated, and the elementary compositions of these humic acids were reported in the previous paper (7).

2. *Quantitative determination of oxygen-containing functional groups.* (1) Total acidity: The method of MARTIN *et al.* (9) was modified as follows: An accurately weighed humic acid sample (about 50 mg) was placed in a glass centrifuge tube with a stopper and dissolved in 10 ml of CO₂-free 0.1 N NaOH. Twenty-five milliliters of 0.1 N BaCl₂ was added into the solution. The system was stoppered tightly, shaken for 16 hr, and centrifuged. Twenty-five milliliters of the supernatant was transferred into a titration cell and titrated with 0.1 N HCl amperometrically to pH 8.4 using an autotitrator (Hiranuma, Model RAT 11). A blank test was run simultaneously in the same manner.

(2) Carboxyl groups: Carboxyl group content was determined by the calcium acetate method of BLOM *et al.* (1).

(3) Total hydroxyl groups: Total hydroxyl group content was determined according to the acetylation method of DEWALT and GLENN (2), except that the reaction time was extended to 2 hr. By lengthening the reaction time from 5 min to 2 hr, the ratio of the experimental value to the theoretical value increased from 75% to 100% for glucose, from 6.5% to 14% for cellulose powder (200 mesh), and from 100% to 105% for vanillin.

(4) Phenolic hydroxyl groups: 1. The difference between the total acidity and the carboxyl group content was regarded as the phenolic hydroxyl group content (11).

2. Phenolic hydroxyl group content was also determined colorimetrically with Folin-Ciocalteu's reagent. An accurately weighed humic acid sample (0.3–1.5 mg) dissolved in 10 ml of less of 0.1 N NaOH was taken into a 25 ml volumetric flask. Ten milliliters of 20% Na₂CO₃ and 2 ml of Folin-Ciocalteu's reagent were added, and the flask was filled to volume with water. After one hr, the blue color was determined at 720 nm. Phenol was used for making a calibration curve.

(5) Alcoholic hydroxyl groups: The difference between the total hydroxyl group content and the phenolic hydroxyl group content was regarded as the alcoholic hydroxyl group content (11).

(6) Methoxyl groups: Methoxyl group content was determined by the method

of VIEBÖCK and BRECHER (15) as modified by PREGL and ROTH (10).

(7) Carbonyl groups: Carbonyl group content was determined by the oximation method of SCHNITZER and RIFFALDI (12) on a reduced scale.

RESULTS AND DISCUSSION

1. Relationships between the amounts of various oxygen-containing functional groups and humic acid types

The levels of major oxygen-containing functional groups in the humic acids used are listed in Table 1, and their mean values for various types of humic acids as well as the indices obtained from the analysis of variance are listed in Table 2. Humic acids are classified into A, B, Rp, and P type according to the definition by KUMADA *et al.* (6). In addition to this, Rp type humic acids are further divided into two groups, one of which consists of humic acids obtained from the A layer of forest soils and the plowed layer of field soils, while the other consists of humic acids from A₀ layer humus and rotted wood. These are designated Rp(1) and Rp(2), respectively, in this report. P type humic acids are also divided into P₀ type humic acids showing no Pg absorption, and P₊₊₊₊ type humic acids showing the distinct Pg absorption, according to KUMADA *et al.* (6). P₊₊₊₊ type humic acids were excluded from the statistical comparison among types, because they are differentiated from other types of humic acids on a different basis such as the presence of Pg, while the other types are classified according to the degree of humification. The sequences of mean values and levels of significance of these relationships obtained by the analysis of variance are given below.

Total acidity : A > Rp(2) ≅ P ₀ ≅ B > Rp(1)	(P < 0.001)
Carboxyl group : A > B > P ₀ > Rp(1) ≅ Rp(2)	(P < 0.001)
Carbonyl group : A > B > P ₀ > Rp(2) > Rp(1)	(P < 0.01)
Total hydroxyl group (A) : Rp(2) > P ₀ > Rp(1) ≅ B > A	(P < 0.001)
Phenolic hydroxyl group (subtracted) (D) : Rp(2) > P ₀ > A > B > Rp(1)	(P < 0.05)
Phenolic hydroxyl group (colorimetric) (C) : Rp(2) > A ≅ P ₀ ≅ B > Rp(1)	(P < 0.01)
Alcoholic hydroxyl group (A-D) : Rp(1) > B > Rp(2) ≅ P ₀ > A	(P < 0.001)
Alcoholic hydroxyl group (A-C) : Rp(2) ≅ P ₀ ≅ Rp(1) > B > A	(P < 0.001)
Methoxyl group : Rp(2) > Rp(1) > B ≅ P ₀ > A	(P < 0.001)

These results show that the levels of oxygen-containing functional groups are significantly different among the various humic acid types.

2. Relationship between the level of each functional group and RF value and relationships of the amounts of functional groups among each other

(1) Carboxyl group. The calcium acetate method for the determination of carboxyl group content is pK dependent, so it does not determine undissociative groups such as esters. Also it may not determine carboxyl groups with high pK values which may occur in polyelectrolytes. This method, however, gives reproducible values and

Table 1. Oxygen-containing func-

Type	No.	Samples	Symbols ^c	$\Delta \log K$	RF	me/g on moisture-			
						Total acidity	Carboxyl	Total A	
A	2	Temmondai	●	0.527	144	7.44	4.94	3.84	
	3	Nagara P	■	0.555	140	8.25	6.33	1.88	
	4	Inogashira-1	●	0.496	138	6.51	5.68	4.10	
	5	Jaana P	■	0.478	132	7.17	4.72	—	
	6	Inogashira-2	●	0.485	115	6.52	4.71	—	
	7	Kinshozan P-1	■	0.543	115				
	8	Shitara Black	●	0.547	114	7.37	4.80	3.56	
	9	Nagara F	■	0.620	102	7.91	5.61	3.09	
	10	Fujinomiya-3	●	0.511	97	6.98	4.50	2.32	
	12	Kinshozan P-3	■	0.565	92	8.24	5.50	3.30	
	13	Komagahara	●	0.542	89	7.00	4.41	2.80	
	14	Nagara OH	■	0.667	89	7.91	5.64	3.10	
	15	Fujinomiya-2	●	0.541	82	7.26	3.92	4.87	
	B	16	Shitara Brown	⊙	0.605	76	5.50	3.35	5.04
		17	Jaana F	▣	0.619	65	5.01		
18		Kuragari-1	⊙	0.634	61	5.63	3.69	4.66	
19		Kinshozan F	▣	0.731	60	6.48	4.84	4.78	
20		Anjo Black	⊕	0.642	57	5.53	3.32	5.01	
21		Higashiyama (A)	⊙	0.664	47	7.12	4.39	6.08	
Rp (1)	28	Ishimakisan F	□	0.729	37	6.67	3.31	5.51	
	29	Sanage-1	⊕	0.711	32	3.94	2.69	4.47	
	31	Jaana OH	□	0.714	30	5.24	2.58	—	
	32	Kinshozan OH-1	□	0.851	29	4.44	3.44	5.40	
	34	Ishimakisan OH	□	0.804	22	5.44	2.81	5.13	
	35	Gifu	⊕	0.853	22	3.62	2.35	5.04	
	36	Anjo	⊕	0.835	21	4.46	2.43	5.30	
	39	Kinshozan OH-3	□	0.897	16	4.02	2.66	5.17	
	Rp (2)	30	Higashiyama (F-H)	○	0.776	31	5.77	2.95	6.18
33		Higashiyama (L)	○	0.809	23	5.80	2.49	6.32	
37		Yamamomo	○	0.968	21	6.58	2.97	4.35	
38		Kisokoma (F)	○	0.806	20	5.79	2.52	6.26	
P ₀	23	Ishimakisan P	▣	0.656	43	6.19	2.95	—	
	24	Kuragari-2	●	0.678	43	6.49	2.83	5.90	
	26	Onobaru	⊙	0.678	39	5.97	3.55	4.89	
	27	Sanage-2	⊕	0.657	38	5.00	2.38	5.35	
P ₊₊	1	Tsubame P _G	⊙	0.272	202	8.47	4.78	3.89	
	11	Tsubame-4	⊙	0.469	94	7.56	4.12	4.03	
	22	Tsubame-1	⊙	0.513	69	5.99	3.33	4.80	
	25	Shishi	⊙	0.506	42	4.55	2.63	—	

^a Phenolic hydroxyl group D, difference between total acidity and carboxyl content. ^b Phenolic hydroxyl group C, determined colorimetrically with Folin's reagent. ^c Symbols: kuroboku soil A ● P₀ ⊙,

tional groups in humic acids.

and ash-free basis					Carbonyl	Methoxyl	Functional	Total O	(E)
Hydroxyl		Alcoholic		A-C			group-O	Total C	Total C
Phenolic		A-D	A-C	A			(E)	(F)	%
D ^a	C ^b			A					
2.50	1.22	1.34	2.62	68	6.38	0.21	0.411	0.438	82.5
1.92	0.94	-0.04	0.94	50	8.84	0.098	0.542	0.523	103.6
0.83	1.12	3.27	2.98	73	6.05	0.25	0.443	0.433	102.3
2.45	1.19	—			4.41	0.14		0.416	
1.81	1.22				11.6	0.18		0.430	
2.57	1.04	0.99	2.57	72	6.83	0.22	0.413	0.441	93.7
2.30	0.83	0.79	2.26	73	18.15	0.25	0.694	0.477	146
2.48	0.97	-0.16	1.35	58	5.02	0.30	0.354	0.487	72.7
2.74	1.10	0.56	2.20	67	6.26	0.30	0.454	0.519	87.5
2.59	1.05	0.21	1.75	63	5.51	0.35	0.383	0.517	74.1
2.27	1.07	0.83	2.03	65	13.65	0.32	0.606	0.473	128
3.34	1.25	1.53	3.62	74	4.80	0.34	0.375	0.449	83.5
2.15	0.85	2.89	4.19	83	11.20	0.51	0.503	0.462	108.9
	1.13							0.450	
1.94	1.06	2.72	3.60	77	5.74	0.44	0.378	0.428	88.3
1.64	0.96	3.14	3.82	80	7.04	0.69	0.450	0.455	98.9
2.21	0.90	2.80	4.11	82	3.75	1.03	0.349	0.459	76.0
2.73	1.32	3.35	4.76	78	5.40	0.73	0.446	0.471	94.7
3.36	1.18	2.15	4.33	79	3.56	0.45	0.373	0.485	76.9
1.25	0.67	3.22	3.80	85	2.39	0.90	0.282	0.435	64.8
2.66	0.72	—			3.79	0.44		0.483	
1.00	0.90	4.40	4.50	83	4.37	1.19	0.388	0.490	79.2
2.63	0.82	2.50	4.31	84	3.07	0.81	0.312	0.443	70.4
1.27	0.83	3.77	4.21	84	2.26	1.18	0.290	0.465	62.4
2.03	0.76	3.27	4.54	86	2.91	1.67	0.321	0.470	68.3
1.36	0.72	3.81	4.45	86	2.22	1.13	0.309	0.474	65.2
2.82	1.30	3.36	4.88	79	4.63	0.80	0.374	0.468	79.9
3.31	—	3.01			2.61	0.89		0.407	
3.61	0.98	0.74	3.37	77	3.15	2.62	0.329	0.454	72.5
3.27	1.25	2.99	5.01	80	2.64	0.86	0.302	0.369	81.8
3.24	1.00	—			3.24	0.43		0.405	
3.66	1.37	2.24	4.53	77	4.46	0.56	0.340	0.391	87.0
2.42	0.98	2.47	3.91	80	3.87	0.74	0.366	0.484	75.6
2.62	0.92	2.73	4.43	83	2.61	0.76	0.280	0.406	69.0
3.69	1.92	0.20	1.97	51	6.37	0.27	0.421	0.510	82.5
3.44	1.80	0.59	2.23	55	4.89	0.33	0.373	0.490	76.1
2.66	1.00	2.14	3.80	79	3.24	0.46	0.322	0.450	71.6
1.92	0.56				2.50	—			

calcareous soil A ■ B ▣ Rp(1) □ P₀ ▤, forest soil B ● P₀ ○, A₀ layer of forest soil ○, paddy soil ⊕, P₊~++++ ⊙.

Table 2. Significance of the difference in functional group composition among the various types of humic acids.

	Average value within each type (me/g)					Mean square ratio	Level of significance (%)
	A	B	Rp(1)	Rp(2)	P ₀		
Total acidity	7.38	5.88	4.73	5.99	5.91	16	0.1
Carboxyl	5.06	3.92	2.78	2.73	2.93	27	0.1
Carbonyl	8.13	6.63	3.07	3.26	3.55	5.1	1
Total hydroxyl (A)	3.04	5.11	5.15	5.78	5.38	16	0.1
Phenolic hydroxyl (D)	2.32	2.13	1.94	3.25	2.99	4.0	1.1
Phenolic hydroxyl (C)	1.08	1.04	0.83	1.18	1.07	4.4	0.7
Alcoholic hydroxyl (A-D)	0.74	2.99	3.30	2.53	2.48	10	0.1
Alcoholic hydroxyl (A-C)	2.23	4.10	4.31	4.42	4.43	18	0.1
Methoxyl	0.25	0.68	0.97	1.29	0.62	8.0	0.1
O in functional groups/ total C (atomic ratio)	0.468	0.425	0.325	0.335	0.329	4.6	0.7
O in functional groups/ total O (%)	97.4	93.4	69.6	78.1	77.2	3.6	1.9

Notes: phenolic hydroxyl (D), difference between total acidity and carboxyl content; phenolic hydroxyl (C), determined colorimetrically with Folin's reagent.

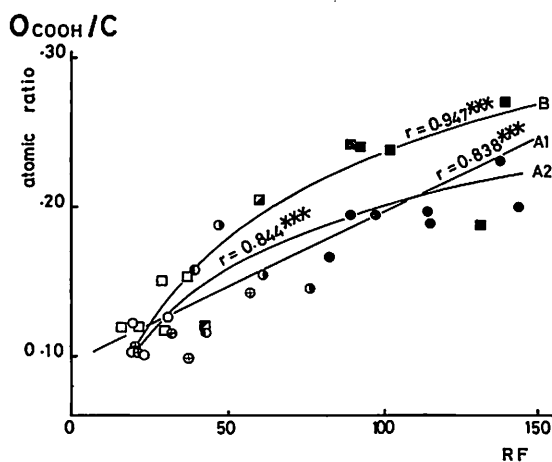


Fig. 1. Relationship between the atomic ratio of carboxyl oxygen to the total carbon and RF value.

A1, a regression line for the all plots; A2, a logarithmic regression curve for the all plots; B, a logarithmic regression curve for Rp type humic acids and calcareous soil humic acids. ■ □ ⊙ denote the A, B, P₀, and Rp(1) type humic acids of calcareous soils.

has been used for humic substances by many researchers, so it was also adopted in this study.

The carboxyl group contents of the humic acids analyzed here ranged between 2.3 and 6.3 me/g, and they accounted for 4.5–13.5% of total carbon content and 18–53% of total oxygen content (Table 1). In Fig. 1, the atomic ratios of carboxyl-O to total-C of humic acids were plotted against *RF* values. The atomic ratios of the oxygen of O-containing functional groups to total-C were adopted in this paper for comparison with the total O/C atomic ratio and for clarification of stoichiometric relationships. The carboxylic group content of humic acids, as a whole, increased with *RF* values (Fig. 1). Linear regression analysis showed a very significant correlation ($r=0.838^{***}$) between them. Logarithmic regression analysis of carboxyl group contents on *RF* values of humic acids gave as highly significant a correlation coefficient ($r=0.844^{***}$) as that by the linear regression analysis. Plots in Fig. 1 were also divided into two groups, one of which consisted of Rp type humic acids and the humic acids from calcareous soils, while the other consisted of humic acids which contained smaller amounts of carboxyl group than the former. Logarithmic regression analysis gave a correlation coefficient (r) of 0.947^{***} for the former group. The logarithmic distribution of carboxyl group contents with regard to *RF* values indicates that the rate of increase in carboxyl content decreases as humification proceeds. The logarithmic distribution may be more possible than the linear distribution because the amounts of precursory functional groups necessary for the formation of carboxyl group decrease during humification. The rapid increase of carboxyl contents of Rp type humic acids may be because Rp type humic acids contain larger amounts of precursory functional groups of carboxyl group such as aliphatic hydroxyl, methoxyl, and methyl group than the humic acids of other types. Humic acid fraction, which has high carboxyl content and able to form stable complexes with metal cations, is presumed to remain in calcareous soils selectively, because organic matter readily undergo oxidation in these soils due to their high pH. On the other hand, humic acid fraction low in carboxyl content and not strongly combined with metal cations is presumed to be more stable in acidic soils than in calcareous soils, because organic matter is oxidized more slowly in acidic soils. Though carboxyl group contents of the two humic acids (Nagara P and Ishimakisan P) obtained from calcareous soils were much lower than those estimated from the logarithmic regression curve B in Fig. 1, these humic acids may be exceptional due to the following reasons. These humic acids represent only part of the whole humic acid fraction in the soils because they were extracted from the soils with 0.1 M Na₄P₂O₇ after the preceding fractions were extracted with 0.1 N NaOH and 0.1 N NaF. In addition, these humic acids may have undergone prolonged humification because they are supposed to be the most stable humic acid fractions in the soils.

(2) *Carbonyl group.* In this study the oximation method by SCHNITZER and RIFFALDI (12) was adopted for determining carbonyl group contents of humic acids. Hydroxylamine does not react with carboxyl groups in the free form, but it reacts with

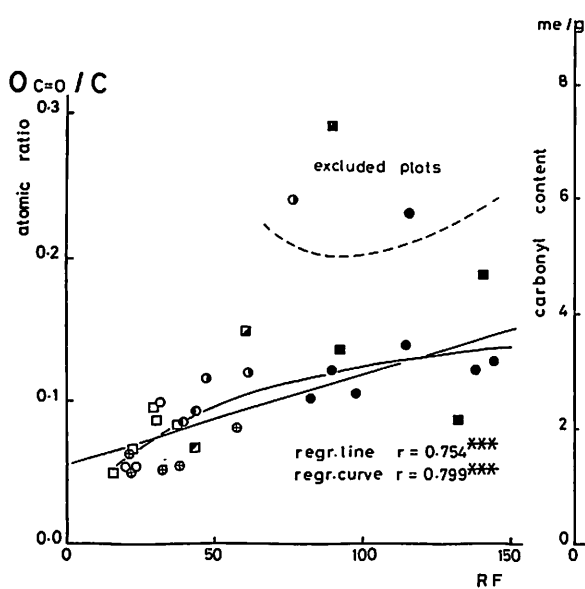


Fig. 2.

Fig. 2. Relationship between the atomic ratio of carbonyl oxygen to the total carbon and *RF* value.

Plots of No. 6, No. 9, No. 14, and No. 16 humic acids were excluded because of their extraordinarily high carbonyl contents.

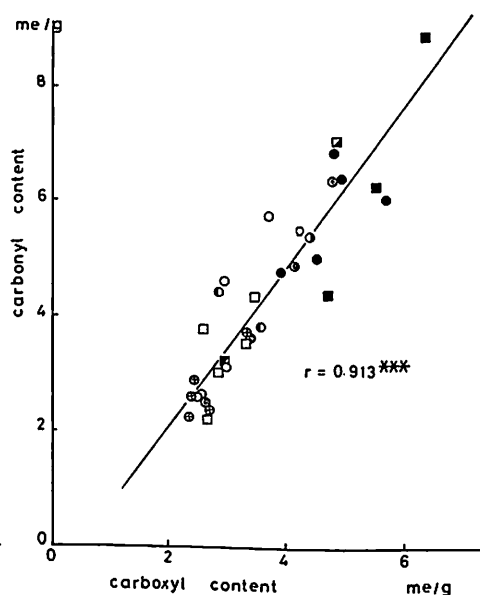


Fig. 3.

Fig. 3. Relationship between carbonyl and carboxyl contents of humic acids.

its esters and amides to form hydroxamic acids. So the reliability of the method itself should be examined further. However, given values were thought to be approximately valid and employed for comparison.

The carbonyl group contents of humic acids varied from 2.2–18 me/g (dry and ash-free basis) (Table 1). Four of these values were extraordinarily high (e.g. No. 6 Inogashira, No. 9 Nagara F, No. 14 Nagara OH, No. 16 Shitara brown). For these humic acids, the values accounted for as much as 50–80% of total oxygen content and 12–19% of total carbon content. The amount of oxygen accounted for by the major oxygen-containing functional groups exceeded 100% of the total oxygen contents of these humic acids. It has not yet been clarified why these extraordinarily high values were observed for these humic acids.

In Fig. 2, the atomic ratios of carbonyl-O to total-C were plotted against *RF* values. Both the logarithmic regression curve and regression line were very significant, but the correlation coefficient of the former was higher than that of the latter.

In Fig. 3, carbonyl group contents were plotted against carboxyl group contents. When the four exceptional humic acids (No. 6, 9, 14, and 16) were excluded, the carbonyl and carboxyl contents showed a highly significant positive linear association

($r=0.913^{***}$). On the other hand, the correlation coefficient decreased to 0.703^{***} when all the data were used in the statistical calculation.

The ratios of oxygen contents accounted for by the sum of carboxyl and carbonyl groups to total oxygen contents of humic acids increased with the humification process, and amounted to about 80% for A type humic acids.

These results suggest that carboxyl and carbonyl groups are important structural components of the humic fraction in contrast to the non-humic fraction of humic acids, and the formation of these functional groups proceeds parallel in the process of humification.

(3) *Phenolic hydroxyl group.* Phenolic hydroxyl contents of humic acids were determined by two methods. In the first method, the value was calculated by subtracting the carboxyl content from the total acidity. The value obtained by this method at times exceeded the total hydroxyl content which is considered to be composed of alcoholic and phenolic hydroxyl contents. This indirect method may have given rather higher values because some carboxyl groups with low acidity are not determined as carboxyl groups but calculated as phenolic hydroxyl groups. By the second method, phenolic hydroxyl contents were determined directly by colorimetry with Folin's reagent using phenol (C_6H_5OH) as the standard material. If humic acids contain polyphenols, phenol contents obtained by this colorimetric method will be rather higher than the real values, because polyphenols are colored more intensely by Folin's reagent than monophenols (8). Nevertheless, the values obtained by this method were about half of those obtained by subtracting carboxyl content from total acidity (Table 1). Consequently, phenol contents obtained by the colorimetric method are thought to be closer to the real values. In Fig. 4, phenolic contents obtained by colorimetry were plotted against those obtained by subtraction. A significant linear association was found ($r=0.587^{***}$). The range of values obtained by colorimetry was narrower than the range of values obtained by subtraction. In Fig. 5, phenolic hydroxyl contents

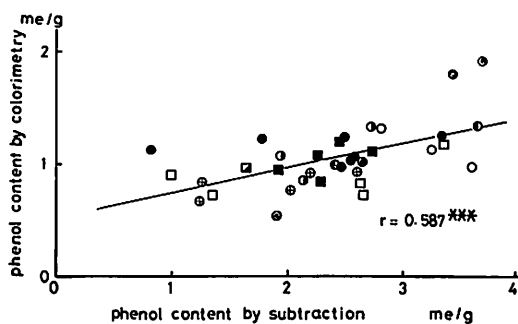


Fig. 4.

Fig. 4. Relationship between the phenol content determined by colorimetry and that by subtracting carboxyl content from total acidity.

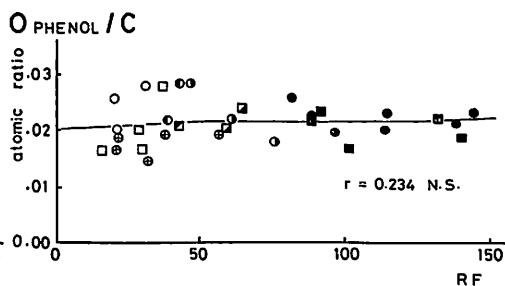


Fig. 5.

Fig. 5. Relationship between the atomic ratio of phenol oxygen to the total carbon and RF value.

determined by the colorimetric method are plotted against RF values. No significant linear association was found between them ($r=0.234$), as was also the case between phenolic hydroxyl contents determined indirectly and RF values ($r=-0.216$).

Nevertheless, from the analysis of variance, the phenolic hydroxyl contents determined by both methods were proven to be significantly different among the various types of humic acids as mentioned in Section 1. In both cases, the phenol contents were highest among Rp(2) type humic acids, which were extracted from the A_0 layer humus or rotted wood of forest soils, and were lowest among Rp(1) type humic acids which were extracted from A and plowed layer soils. The high phenol content among Rp(2) type humic acids may be attributed to the phenolic plant constituents such as lignin, tannin, phenolic acids, and flavonoids remaining in those structures. The low phenol content among Rp(1) type humic acids suggest that such phenols in Rp(2) type humic acids are lost by undergoing humification in soil. The higher phenolic contents of A and B type humic acids compared with Rp(1) type humic acids suggest that some phenols are formed again in humic acid molecules during humification.

(4) *Alcoholic hydroxyl group.* As we have no direct method for estimating alcoholic hydroxyl groups in humic substances, they were determined indirectly by subtracting phenolic hydroxyl content from total hydroxyl content (11). Since the phenolic hydroxyl content was determined by two methods, two values were also given for the alcoholic hydroxyl content. When phenolic hydroxyl contents determined by subtraction were employed, alcoholic hydroxyl contents ranged between -0.16 and 4.4 me/g. When phenolic hydroxyl contents determined by the direct colorimetric method were employed, alcoholic hydroxyl contents ranged between 0.94 and 5.01 me/g, and their ratios to total hydroxyl contents ranged between 50–74% among A type humic acids, between 77 and 86% among B, Rp(1), Rp(2) and P_0 type humic

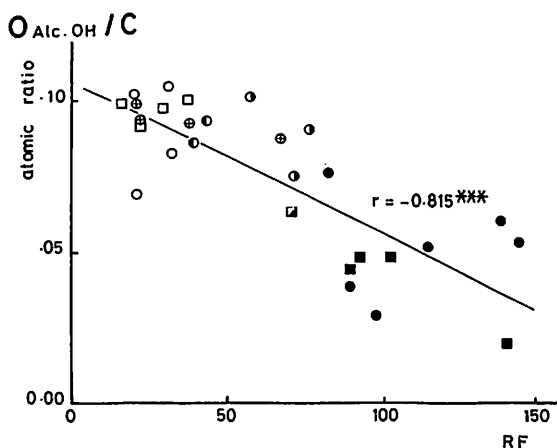


Fig. 6. Relationship between the atomic ratio of alcoholic hydroxyl oxygen to the total carbon and RF value.

acids, and between 51 and 79% among $P_{+...+++}$ type humic acids. That is, the ratios of alcoholic to phenolic hydroxyl contents were about 4 and were similar for most of the humic acids employed. In Fig. 6, atomic ratios of alcoholic-O/total-C were plotted against RF values. A linear association between them was found to be significant ($r = -0.815^{***}$), which indicated that alcoholic hydroxyl content decreased during humification. Though polysaccharide contents of humic acids will be treated in a subsequent paper (14), hydroxyl groups in polysaccharides account for considerably much fraction of the alcoholic hydroxyl contents of humic acids. A significant negative linear association was found between alcoholic hydroxyl content and carbonyl content ($r = -0.679^{***}$), and the decrease in alcoholic hydroxyl content was almost equal to the increase in carbonyl group content.

(6) *Methoxyl groups.* Methoxyl contents ranged between 0.10 and 2.62 me/g and were significantly different among the various types of humic acids. In Fig. 7, atomic ratios of methoxyl-O/total-C were plotted against RF values. A sharp decrease in methoxyl content during humification was quite obvious, and logarithmic regression analysis gave a more critical significance level ($r = -0.768^{***}$) than linear regression analysis ($r = -0.715^{***}$).

High methoxyl levels in low humified humic acids may be attributed to the large amounts of lignin remaining in them (3, 13). A rapid demethylation observed here may be due to microbial degradation (3). Methoxyl groups in humic acids are assumed to be transformed *via* phenolic hydroxyl groups to quinone groups during humification, taking into account the small increase in phenolic hydroxyl contents (Fig 5) and the large increase in carbonyl contents (Fig. 2). In Fig. 8, phenolic hydroxyl

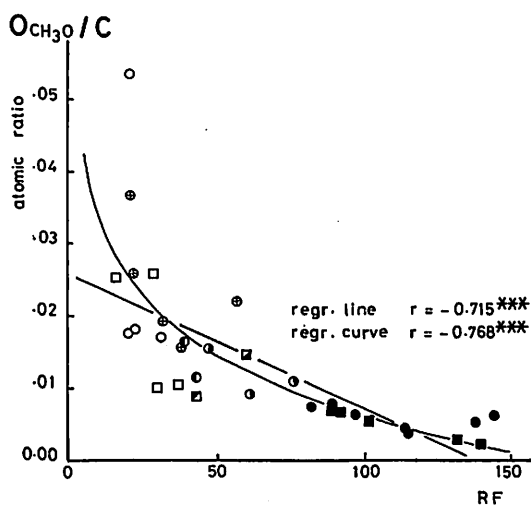


Fig. 7.

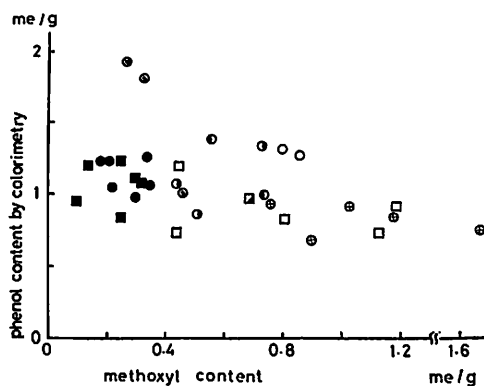


Fig. 8.

Fig. 7. Relationship between the atomic ratio of methoxyl oxygen to the total carbon and RF value.

Fig. 8. Relationship between phenol and methoxyl content of humic acids.

contents were plotted against methoxyl contents of humic acids. Plots tended to group together depending on the humic acid type. Though phenolic hydroxyl contents of A and B type humic acids were higher than those of Rp(1) type humic acids, the increase in phenolic hydroxyl content was too small to account for the decrease in methoxyl content.

3. General discussion on the relationship between oxygen contents and the degree of humification

The ratio of oxygen content, expressed as the sum of carboxyl, carbonyl, phenolic hydroxyl, alcoholic hydroxyl, and methoxyl contents, to total-C increased when the degree of humification increased (Tables 1 and 2). In other words, the amounts of oxygen which can not be accounted for by these functional groups were higher among less humified humic acids. Humic acid-oxygen that is not accounted for by these functional groups may be in the form of ethers in lignin-like substances and hydroxyl groups of polysaccharides which could not be determined because the percentage recovery of the hydroxyl contents of polysaccharides by the quantitative acetylation method was not more than 15%.

In Fig. 9, the atomic ratios of oxygen content, expressed as the sum of carboxyl, carbonyl, phenolic hydroxyl, alcoholic hydroxyl, and methoxyl contents, to the total carbon content were plotted against *RF* value. A linear association ($r=0.723^{***}$) and a logarithmic association ($r=0.733^{***}$) were found to be significant between them. In Fig. 9, regression curves and lines for the levels of these functional groups against *RF* values were graphed together. In Fig. 10, atomic ratios of total-O/total-C were plotted against *RF* values. The trend of total-O/total-C versus *RF* value was different from the trend observed when the ratio of the sum of oxygen contents of these functional groups to the total-C was plotted against *RF* values especially among the less humified humic acids. That is, the total oxygen content tended to decrease during humification among the less humified humic acids while the sum of oxygen contents of functional groups tended to increase. Though methoxyl and alcoholic hydroxyl contents decreased indeed among these humic acids with the increase in *RF* value (Fig. 9), the decrease in total oxygen content could not be explained solely by the decrease in methoxyl and alcoholic hydroxyl contents. Oxygen which was not present in the form of one of the functional groups studied here was assumed to be in the form of ethers in lignin and polysaccharides as well as hydroxyl groups of polysaccharide in less humified humic acids. The amount of such oxygen is presumed to decrease rapidly during humification.

The amount of oxygen present in each functional group and the ratio of each to the total oxygen content of humic acids obtained from calcareous soils were higher when compared with humic acids whose *RF* values were similar (Fig. 9). This may be because the formation of carboxyl and carbonyl groups is enhanced under alkaline conditions in calcareous soils and the dissociative functional groups and carbonyl groups are stabilized by the formation of complexes with calcium. On the contrary,

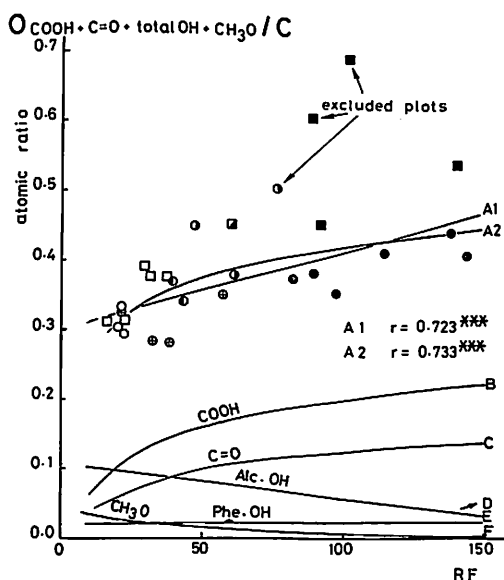


Fig. 9.

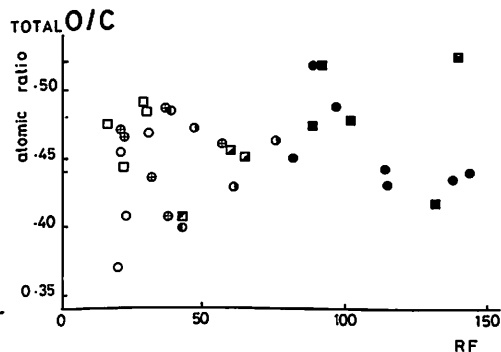


Fig. 10.

Fig. 9. The relationship between the sum of the atomic ratios of carboxyl, carbonyl, alcoholic hydroxyl, phenolic hydroxyl, and methoxyl oxygen to the total carbon and *RF* value.

A1, A2 are the regression line and curve for the plots exhibited here, but plots of No. 9, No. 14, and No. 16 humic acids were excluded because of their extraordinarily high carbonyl contents. B, C, D, E, and F are the regression curves and lines for the relationships between the atomic ratios of each functional group oxygen to the total carbon *versus* *RF* value, where B, C, D, E, and F correspond to carboxyl, carbonyl, alcoholic hydroxyl, phenolic hydroxyl, and methoxyl groups, respectively.

Fig. 10. Relationship between the atomic ratio of the total oxygen to the total carbon and *RF* value.

in the case of humic acids from paddy soils, the amount of each functional group and their sum, except the methoxyl group, were lower than the other humic acids when compared with humic acids whose *RF* values were similar, possibly reflecting the reductive conditions in paddy soils.

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