

EXTRACTION AND CHARACTERIZATION OF HUMUS WITH REFERENCE TO CLAY-ASSOCIATED HUMUS

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An extraction-fractionation method was developed with which it is possible to isolate 60-67% of the humus of Chernozemic and Luvisolic soils. Two humic acid fractions were obtained: (1) a conventional alkali-pyrophosphate extractable HA-A; (2) a clay-associated HA-B fraction isolated after ultrasonic dispersion, in water, of the residue of the alkali-pyrophosphate extraction. As compared to the HA-A, the HA-B fractions had lower C contents but greater N contents, narrower C:H ratios, higher molecular weights, and less resistance to hydrolysis in 6 N HCl. It was concluded that the HA-B is a weakly humified, potentially labile humic constituent stabilized by adsorption to clay. Spectral measurements on low-ash (< 2%) humic acids showed a positive correlation between extinction coefficient at 280 nm (E_{280}) and C:H ratio, and negative correlations between E_{280} and percent of hydrolyzable C and N. The $E_4:E_6$ ratio was related to molecular weight, as determined by agar gel filtration, increasing with decrease in molecular weight.

Les auteurs ont mis au point une méthode d'extraction-fractionnement qui permet d'isoler 60-67% de l'humus des sols chernozémiques et luvisoliques. On a obtenu deux fractions d'acides humiques: 1) une fraction HA-A, classique, extractible par un pyrophosphate alcalin et 2) une fraction HA-B associée à l'argile, isolée, après dispersion ultrasonique dans l'eau, du résidu de l'extraction par le pyrophosphate alcalin. En comparaison de la fraction HA-A, la fraction HA-B avait des teneurs en C plus faibles, mais des teneurs en N plus élevées, des rapports C:H plus faibles, des poids moléculaires plus élevés, et une plus faible résistance à l'hydrolyse dans HCl 6N. On en conclut que la fraction HA-B est un composant humique faiblement humifié, potentiellement labile, stabilisé par adsorption sur argile. Les mesures spectrales faites sur les acides humiques à faible teneur en cendres (< 2%) ont révélé une corrélation positive entre le coefficient d'extinction à 280 nm (E_{280}) et le rapport C:H, et des corrélations négatives entre E_{280} et le pourcentage de C et de N hydrolysables. La filtration sur gélose a permis d'associer le rapport $E_4:E_6$ au poids moléculaire, le rapport augmentant en proportion inverse du poids moléculaire.

Contemporary theory describes soil humic constituents as a multicomponent system embracing a wide range of organic heteropolymers varying in size, structure, composition, and degree of association with mineral components. It is not surprising that there is no perfect extractant for such material. The organic matter of base-saturated, humus-rich soils is difficult to extract, because of stable humus complexes with clay, calcium and sesquioxides. Commonly, Tyurin's method, which involves exhaustive ex-

tractions with NaOH after a preliminary decalcification with HCl, has been used (Kononova 1966). Equivalent amounts of humic matter, of similar fractional composition (Baranovskaya 1966) are obtained in a single extraction with the NaOH- $\text{Na}_4\text{P}_2\text{O}_7$ method developed by Kononova and Bel'chikova (1961).

In attempting to extract additional amounts of humic matter following initial pyrophosphate or alkali extractions, most researchers have increased the molar strength of the extractants, thereby promoting a

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strongly flocculated soil system. This increased the possibility of hydrolysis and autoxidation of humic constituents during extraction. Significant changes in extraction and fractionation were effected by Ponomareva and Plotnikova (1968) when, subsequent to alkali extraction, more dilute (0.02 N) NaOH was added to the soil residue and the suspension heated.

We observed that dispersing the soil residue following alkali-pyrophosphate extraction allowed the isolation of additional (up to 25%) amounts of the soil's humus as a dark-colored, humus-very fine clay suspension. This paper reports on the development of an extraction-fractionation method which incorporates this second humus fraction, and discusses its relevance in studies of the nature of soil organic matter.

MATERIALS AND METHODS

Soils

Samples were obtained from the Ah horizons of virgin pedons. The Orthic Dark Brown Weyburn (W) soil was sampled from a semiarid grassland, the Orthic Black (Ox-1 and Ox-2) from subhumid grassland, the Orthic Dark Gray Whitewood (Wh 1 and Wh 2) from subhumid aspen forest. These soils, developed on moderately calcareous glacial till, are loam-textured, neutral, and 80 to 90% base-saturated. The acidic Gray Wooded (R5) soil has developed on moderately calcareous, clayey lacustrine sediments under aspen-spruce vegetation.

Methods of Analysis

Organic C in the soils and extracts was measured by dichromate oxidation with external heating (Mebius 1960). This technique yielded organic C contents which were $87 \pm 2.8\%$ of those measured by dry combustion. Since all the fractionation data are comparative, a correction factor was not used in the calculations. C and H analyses of the humic materials were made by Mikrolabor, Bonn, West Germany, using dry combustion techniques. N was determined by a semimicro Kjeldahl technique (Bremner 1965). The acid hydrolysis treatments involved boiling humic acids in 6 N HCl under reflux for 16 to 18 h. Ash contents of humic acids were determined after heating to 600 C for 2 h.

The ultraviolet-visible spectra and extinction coefficients were measured on Na-humate solutions containing 17 ppm humate-C, buffered to pH 7 in 0.05 M NaHCO_3 . A Beckman DBG spectrophotometer and a 1-cm cell were used.

The coagulation threshold values were determined by the method described by Kononova (1966). Na-humate solutions containing 34 ppm humate-C were pipetted into a series of CaCl_2 solutions that varied in final Ca^{++} concentration from 5 to 30 meq per liter, shaken, and observed after 2 h. The coagulation threshold was the minimum concentration of Ca^{++} at which humic acid floccules formed.

Relative molecular weights of the humic acid fractions were determined by the agar gel permeation technique (Swift et al. 1970). Twelve percent agar (Oxoid, Ionagar No. 2) ground to less than 120-mesh size was used in 1.6×36 -cm columns. The ammonium salt of the humic acid was eluted through the column with bicarbonate-EDTA buffer at pH 8.5. Humic acid concentration of the eluate was estimated by measuring absorbance at 400 nm.

Extraction-fractionation

The principal extraction (C ext-A, Fig. 1) involved a single treatment with freshly prepared 0.1 N NaOH-0.1 M $\text{Na}_4\text{P}_2\text{O}_7 \cdot 7\text{H}_2\text{O}$, pH 13 (Kononova 1966). The addition of H_2O to the residue of the principal extraction and shaking yielded a disperse Na-soil-water suspension of pH about 8.5. This suspension was insonated to further aid dispersion and the disruption of microaggregates. After 48 h, the peptized suspensions were centrifuged at 10,400 g to yield the dark-colored, humus-very fine clay system

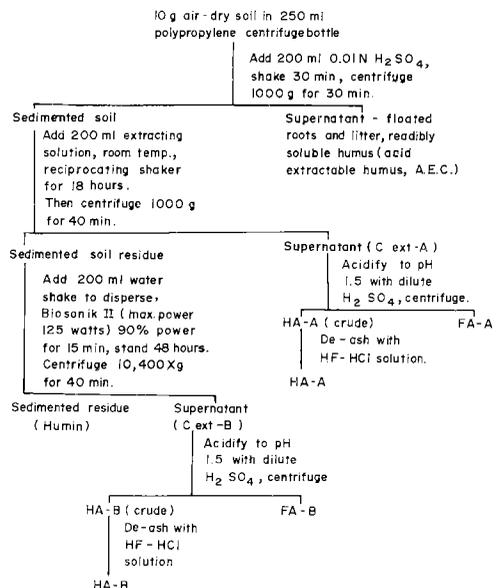


Fig. 1. The extraction-fractionation technique.

Table 1. Organic C isolated after dispersion, or dispersion plus insonation of alkali-pyrophosphate-extracted soils

Soil	Org. C %	C ext-A (NaOH-Na pyr.) %	C ext-B (dispersion) %	C ext-B (dispersion plus insonation) %	C ext-A/C ext-B
Ox-1	3.62	39.4	15.0	24.6	1.6
Ox-2	5.35	38.3	10.5	21.0	1.8
Wh-2	4.77	47.2	13.2	20.2	2.6
Wh-1	4.25	47.6	14.4	18.2	2.1

(C ext-B) as the supernatants. Both the C ext-A and C ext-B were fractionated into humic and fulvic acids by lowering the pH to 1.5. All subsequent analyses were made on low-ash (0-2%) humic acids obtained using a HF-HCl mixture (Lowe 1969).

The non-extractable residue (humin) was fractionated into approximate size fractions by wet-sieving at 63 μ m, by repeated sedimentation and decantation at 5 μ m and by centrifugation and decantation at 2 and 1 μ m (Jackson 1956).

RESULTS

Efficiency of the Extraction Procedures

A single treatment with 0.1 N NaOH-0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ solution (Table 1) extracts substantial amounts of organic matter (C ext-A). Dispersion of the soil residue remaining after the alkali-pyrophosphate extraction and a 48-h "peptization" period results in the isolation of an additional 10-15% of the organic matter. This dark-colored, humus-fine clay suspension has withstood centrifugation at 10,400 g. The addition of an insonation treatment increases the amount of material in this fraction up to 25%. Most of the material isolated by dispersion-insonation is

humic acid; that is, it precipitates at pH 1.5. It is, therefore, designated HA-B.

Following purification by washing in 0.01 N H_2SO_4 and dialysis against distilled H_2O but prior to de-ashing, the HA-B contained about 50% ash, substantially more than the 10-20% ash contents of the HA-A. Preliminary evidence based on the X-ray diffraction analysis of the inorganic colloids associated with the HA-B indicates the presence of some expanding layer silicates.

Chemical Properties of HA-A and HA-B

A summary of the chemical properties of the HA-A and HA-B for nine Ah horizons of grassland and forest soils is shown in Table 2. Although qualitative differences in humic acid composition were noted among the soils, mean values are presented to characterize the HA-A and HA-B fractions. The HA-A fractions contained more C and less H than the HA-B, resulting in substantially higher C:H ratios in the former. As compared to the HA-B, the HA-A was less susceptible to hydrolysis in 6 N HCl.

The stability of humic acids in dilute electrolyte is dependent on the relative pro-

Table 2. Chemical properties of HA-A and HA-B

Chemical property	HA-A		HA-B	
	Mean*	SE†	Mean	SE
C (%)	54.10	0.75	52.30	1.16
H (%)	4.21	0.42	5.82	0.56
C:H ratio	13.00	1.44	9.10	0.80
N (%)	4.31	0.58	5.14	0.46
C:N ratio	12.70	1.46	10.10	0.79
C hydrolyzable in 6 N HCl (%)	33.70	2.58	48.00	4.76
N hydrolyzable in 6 N HCl (%)	61.70	7.83	79.00	9.25

*Values are a mean of nine soil samples, and are statistically different (5% level) using the paired difference test.

†Standard error.

portion of aromatic and aliphatic constituents (Kononova 1966). Lower coagulation threshold values for the HA-A suggest that this fraction has a greater number of hydrophobic, aromatic components which coagulate more readily.

The HA-B contained more N and the C:N ratios were narrower. A large proportion of the N of the HA-B was hydrolyzable in 6 N HCl, particularly the HA-B of grassland soils. McGill (1971) found that a large percentage of the hydrolyzable N derived from similar fractions was α -amino acid N. The other materials removed included carbohydrates and simple phenolic substances leaving the more condensed structures as a residue (Cheshire et al. 1967).

Properties of the Humic Fraction

A considerable proportion of the non-extractable organic constituents (humic) was associated with the silt and sand fractions (Table 3). Visual examinations of this material revealed that much of it was in the form of particulate plant and fungal residues in varying stages of decomposition. Charcoal fragments were common in the humic fractions of forest soils. The particulate organic material had remained in the soil despite the flotation treatment. The flotation treatment was effective in removing 3.2, 3.5, and 16.7% of the organic C from the Ox-1, Ox-2 and W soils, respectively. The particu-

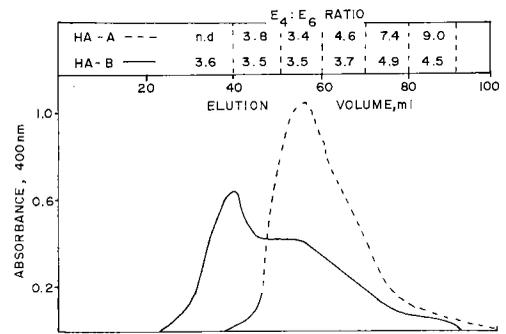


Fig. 2. Molecular weight distribution of HA-A and HA-B, and variation in $E_4:E_0$ ratio with molecular weight.

late material not removed was probably mechanically entangled or entrapped within soil aggregates. A portion of these materials would be soluble in alkali, and make a slight contribution to the HA-A and FA-A fractions. Constituents released by the rupture of fungal cells during the insolation treatment, and subsequently adsorbed to clay, would be included with HA-B fraction (McGill 1971).

Agar Gel Fractionation

The approximate molecular weight distribution of the HA-A and HA-B was determined using an agar gel permeation technique and bicarbonate-EDTA buffer as eluant. The elution curves presented (Fig. 2) are repre-

Table 3. Distribution and nature of non-extractable organic material fractionated as to approximate size

Soil	Approximate size fraction μm	Prop'n of soil C (%)	Org. C (%)	N (%)	C:N	C hyd. (%)	N hyd. (%)
W	>5	5.3	0.4	-	-	-	-
	5-2	5.3	4.8	0.39	12.3	-	-
	2-1	10.0	7.8	0.83	9.4	-	-
	<1	15.4	5.8	0.70	8.2	70	95
Ox-1	>5	5.4	0.3	-	-	-	-
	5-2	5.5	3.9	0.35	11.0	-	-
	2-1	13.3	7.4	0.86	8.6	-	-
	<1	14.2	4.6	0.63	7.3	72	94
R5	>5	12.5	5.7	0.11	16.3	18	57
	5-2	7.8	3.7	0.29	12.6	36	-
	2-1	4.1	2.7	0.40	6.8	42	63
	<1	12.2	2.1	0.44	4.7	44	59

sentative of those observed for all soils investigated. The HA-B possesses an initial maximum, corresponding to a high molecular weight component, and a secondary maximum at a point approximately equal to the peak elution volume of the HA-A.

Visible and UV Spectra

Absorption spectra in the region 260-720 nm of the HA-A and HA-B of the Ah horizon of the Ox-2 soil (Fig. 3) are representative of those observed for the A horizons of all the soils studied. The spectra show a gradual decrease in extinction coefficient with increase in wavelength. Similar characteristics have been reported for the optical properties of clay-associated humus isolated by boiling in 0.02 N NaOH the residue of soils previously extracted with more concentrated NaOH (Ponomareva and Plotnikova 1968). Certain spectral parameters were related to the chemical properties of the humic acid fractions. The strong correlation between C:H ratio and extinction coefficient at 280 nm (E_{280}) is particularly noticeable (Table 4). C:H ratios are commonly employed as indices of aromatic condensation for both soil (Kononova 1966) and coal humic acids (Swain 1970). Values for E_{280} were negatively correlated with the levels of C and N hydrolyzability of the HA-A and HA-B.

There were no significant correlations between $E_4:E_6$ ratios and chemical properties related to the aromatic character of the humic acids, such as the C:H ratios or the levels of C and N hydrolyzability. Low $E_4:E_6$ ratios, thought to be characteristic of a high degree of aromatic condensation, were observed for the high molecular weight, weakly condensed, more aliphatic HA-B.

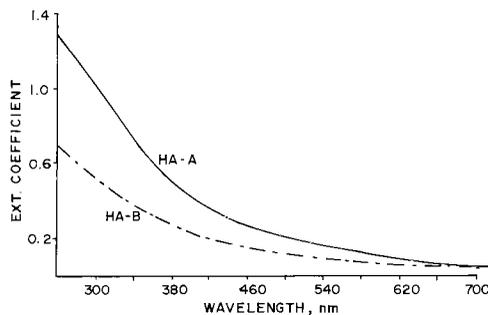


Fig. 3. UV-visible spectra of HA-A and HA-B fractions of Ox-2 Ah horizon.

The $E_4:E_6$ ratios increased with decrease in molecular weight (Fig. 2).

DISCUSSION

The conventional humic fractionation technique followed by dispersion in a dilute alkali system and insonation resulted in the separation of 15-25% of the soil organic matter not normally isolated. This fraction, the C ext-B, from which the HA-B is precipitated, was considered to be clay-associated or clay-adsorbed humus. It may contain some material not in the form of clay-humus complexes; examples are non-complexed material entrapped in microaggregates or constituents released by the sonic rupture of cells and adsorbed to the mineral colloids. A comparison of similar pairs of soils in the same soil zone revealed greater amounts of C ext-B in the more clayey soils (McGill 1971). The C ext-B is also more abundant in grassland than in forest Ah horizons (Table 1).

The lower extinction coefficients and $E_4:E_6$ ratios of the higher molecular weight

Table 4. The correlation of chemical and spectral properties of humic acids

	<i>n</i>	<i>r</i>	Regression equation
C:H ratio and E_{280} (HA-A and HA-B)	18	0.96**	$E_{280} = -0.43 + 0.12$ (C:H ratio)
E_{280} and C hyd. %†(HA-A and HA-B of A horizons)	18	-0.95**	C hyd. % = $0.65 - 0.26$ (E_{280})
E_{280} and N hyd. %† (HA-A and HA-B of A horizons)	18	-0.93**	N hyd. % = $1.0543 - 0.384$ (E_{280})

**Significant 99% level.

†% of material hydrolyzable in 6 N HCl.

HA-B, as compared to the HA-A, agree with earlier reports of decreases in extinction coefficient and ratio of absorbance at 400 and 600 nm with increase in molecular weight (Butler and Ladd 1969; Swift et al. 1970). The higher coagulation threshold values of the HA-B further substantiate those chemical and spectral data that indicated that the HA-B has a more disperse, more aliphatic structure than the HA-A. The HA-B fractions have greater N contents, narrower C:N ratios, and are more susceptible to hydrolysis than the HA-A. High molecular weight constituents that could include a wide spectrum of organic polymers appear to be important components of this fraction.

Humification is often regarded as a process involving both the formation of humic substances and the oxidative degradation and condensation of the original polymers to yield more aromatic humic acids (Kononova 1968). Swift et al. (1970) postulate that the final stages of the humification process involve an oxidative degradation of high molecular weight humic material to more strongly aromatic components of lower molecular weight. Under these terms, the HA-B may be considered weakly humified, the HA-A more strongly humified. It is possible that the characteristics of the HA-B which indicate a low degree of humification are a consequence of its adsorption to fine mineral colloids. It has been suggested that clay is able to adsorb and protect organic matter rich in nitrogen (Swift and Posner 1972), and that flexible, high molecular weight polymers are readily adsorbed to clay (Greenland 1965).

The characteristics of the non-extractable residue (humin) generally agree with descriptions of this fraction as a wide spectrum of alkali-insoluble carbonaceous constituents that includes particulate, partially humified plant residues and humic material intimately associated with mineral colloids (Perraud et al. 1971). The properties of the clay-associated humin (< 1 μm humin, W and Ox-1 soils) such as the narrow C:N ratio and the high susceptibility to acid hydrolysis suggest that this material is relatively similar to the HA-B.

The characteristics of the HA-B and clay-associated humin indicate that most of the

material associated with clay in the grassland soils of Saskatchewan is a largely aliphatic, weakly condensed, high molecular weight, N-rich, potentially labile humic constituent. It is postulated that such materials are stabilized by adsorption to clay and further biochemical alterations are slowed down.

The extraction-fractionation method described is relatively simple and efficient. It differs from conventional techniques in that the secondary extraction yields a disperse system which can be fractionated into various humus-mineral colloid fractions by high-speed centrifugation. It is suited to the study of humus in different genetic soil groups and to the dynamics of C (Shields and Paul 1973) and N (McGill 1971) within the complex soil humus system.

BARANOVSKAYA, V. A. 1966. Comparative study of methods of determining the composition of soil humus and their statistical evaluation. *Sov. Soil Sci.* **11**: 1157-1160.

BREMNER, J. M. 1965. Total nitrogen. Pages 1149-1178 in C. A. Black et al., eds. *Methods of soil analysis, Part 2. Chemical and biological properties*. Amer. Soc. Agron., Madison, Wisconsin.

BUTLER, J. H. A. and LADD, J. N. 1969. Effect of extractant and molecular size on the optical and chemical properties of soil humic acids. *Aust. J. Soil Res.* **7**: 229-239.

CHESHIRE, M. V., CRANWELL, P. A., FALSHAW, C. P., FLOYD, A. J. and HAWORTH, R. D. 1967. Humic acid — II. Structure of humic acids. *Tetrahedron* **23**: 1669-1682.

GREENLAND, D. J. 1965. Interactions between clays and organic compounds in soils. *Soils Fert.* **28**: 415-425 and 521-532.

JACKSON, M. L. 1956. *Soil chemical analysis — advanced course*. 2nd printing. Published by the author, University of Wisconsin, Madison. 991 pp.

KONONOVA, M. M. 1966. *Soil organic matter*, 2nd ed. Pergamon, Oxford. 544 pp.

KONONOVA, M. M. 1968. Transformations of organic matter and their relation to soil fertility. *Sov. Soil Sci.* **8**: 1047-1055.

KONONOVA, M. M. and BEL'CHIKOVA, N. P. 1961. Quick methods of determining the humus composition of mineral soils. *Sov. Soil Sci.* **10**: 1112-1121.

LOWE, L. E. 1969. Distribution and properties of organic fractions in selected Alberta soils. *Can. J. Soil Sci.* **49**: 129-141.

- McGILL, W. B. 1971. Turnover of microbial metabolites during nitrogen mineralization and immobilization in soil. Ph.D. Thesis, University of Saskatchewan, Saskatoon, Sask.
- MEBIUS, L. J. 1960. A rapid method for the determination of organic carbon in soils. *An. Chim. Acta* **22**: 120-125.
- PERRAUD, A., KHA, NGUYEN and JACQUIN, F. J. 1971. Characterizing humin forms in different soil types. *C.R. Acad. Sci., Paris*, **272**: 1594-1597.
- PONOMAREVA, V. V. and PLOTNIKOVA, T. A. 1968. Methods and some results on the fractionation of Chernozem humus. *Sov. Soil Sci.* **11**: 1562-1572.
- SHIELDS, J. A. and PAUL, E. A. 1973. Decomposition of ¹⁴C-labelled plant material under field conditions. *Can. J. Soil Sci.* **53**: 297-306.
- SWAIN, F. 1970. *Non-marine organic geochemistry*. Cambridge University Press.
- SWIFT, R. S. and POSNER, A. M. 1972. The distribution and extraction of soil nitrogen as a function of soil particle size. *Soil Biol. Biochem.* **4**: 181-186.
- SWIFT, R. S., THORNTON, B. K. and POSNER, A. M. 1970. Spectral characteristics of a humic acid fractionated with respect to molecular weight using an agar gel. *Soil Sci.* **110**: 93-99.