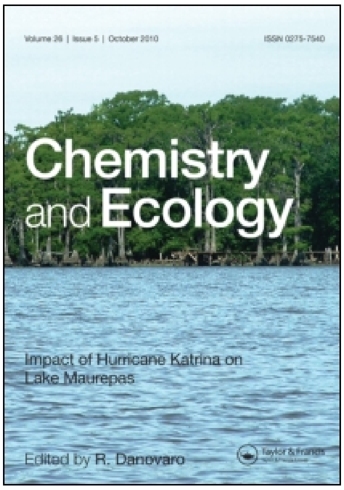

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

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To cite this Article Debska, B. , Banach-Szott, M. , Dziamski, A. and Gonet, S. S.(2010) 'Chromatographic characteristics (HPLC, HPSEC) of humic acids of soil fertilised with various organic fertilisers', Chemistry and Ecology, 26: 4, 49 — 57
To link to this Article: DOI: 10.1080/02757540.2010.501027
URL: http://dx.doi.org/10.1080/02757540.2010.501027

Chemistry and Ecology
Vol. 26, Supplement, October 2010, 49–57

Taylor & Francis
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Chromatographic characteristics (HPLC, HPSEC) of humic acids of soil fertilised with various organic fertilisers

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(*Received 6 June 2009; final version received 9 June 2010*)

The aim of this study was to determine the effect of the size of the soil particle containing organic matter, the type of organic fertiliser (cattle manure, vermicompost, straw) and the decomposition time of these fertilisers in soil on the hydrophilic–hydrophobic properties and polydispersity of humic acid molecules. In the model incubation experiment, organic fertilisers (cattle manure, vermicompost and wheat straw) were mixed with soil material (sandy texture). The soil was sampled for testing after one and three years of incubation. Each sample was separated into two fractions according to graining, grain diameter >0.1 mm (sand fraction) and <0.1 mm (silt and clay fraction), and humic acids (HAs) were isolated. For the HAs isolated, hydrophilic–hydrophobic properties were defined using RP-HPLC, and the division was made into low- and high-molecular-mass fractions (HPSEC). It was demonstrated that introducing organic fertilisers into soil resulted in an increase in the proportion of hydrophobic and high-molecular mass fractions and a decrease in the proportion of hydrophilic and low-molecular mass fractions in the HA molecules. Highest values for the ratio HIL/ΣHOB and lowest values for S1/S2 were noted for HAs of soil mixed with straw. HAs isolated from the fine-grained soil fraction demonstrated a higher degree of 'maturity' than HAs from the coarse-grained fraction, which was seen as higher values for HIL/ΣHOB and lower values for S1/S2.

Keywords: organic fertilisers; humic acids; polydispersity; hydrophilic and hydrophobic fraction

1. Introduction

The organic matter content in arable soils is a derivative of the processes of decomposition of humus compounds and a continuous supply of fresh organic weight [1,2]. The amount and quality of humus is a major indicator of soil fertility and productivity, and depends on many habitat (climate, soil) and anthropogenic factors (tillage and plant growing, fertilisation, crop rotation). Reports to date [3–5] show that the most essential method of maintaining a no-deficit state for the organic matter content in soil is regular organic fertilisation (manure, straw, vermicompost, slurry, green fertilisers, composts). Processes of mineralisation and humification of the organic

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ISSN 0275-7540 print/ISSN 1029-0370 online

DOI: 10.1080/02757540.2010.501027
http://www.informaworld.com

bonds of carbon introduced into the soil with organic fertilisers determine not only the content, but also the properties of the humus substances produced [3,4,6,7].

A large number of factors affecting the course of humification mean that creating compounds of identical composition is practically impossible. For this reason, humic substances show changing elemental composition and a differentiated and very complex structure. Humic substances are characterised by, among other methods, analysing the properties of isolated groups of substances, mainly humic and fulvic acids [6–9].

Humus acids (fulvic and humic acids) are composed of an aromatic core linked with amino acids, sugars, peptides, aliphatic acids and other components of aliphatic structure. The core comprises aromatic rings, mainly of the phenol type. Heterocyclic and condensed rings are sometimes also present. Aromatic rings are connected by bridges of various types: $-O-$, $-CH_2-$, $-NH-$ and $-S-$. Functional groups, such as carboxylic, phenolic, metoxylic, chinoic, carbonyl and alcoholic groups, are an important element of the structure of humic acids (HA) [10].

Studies on HAs must concentrate on obtaining information on the size and properties of specific fractions [6,11–14]. The shape of humic substances, the range of their molecular masses and their hydrophilic–hydrophobic properties often determine the accessibility of a specific group of compounds for the soil microflora and microfauna. They are also of importance for the rate of migration of humic substances down the soil profile.

Application of RP-HPLC allows for the analysis of samples in water solutions, which in the case of humic substances is of considerable importance, offering the possibility to separate soil into fractions of varying hydrophobic character. In their native form, HAs expose their hydrophilic regions to the aqueous solvent, whereas the hydrophobic regions are accumulated in internal complexes. If HAs traverse the stationary phase, the hydrophobic solid matrix enhances the unfolding process. Hydrophobic regions previously hidden within the core become more and more exposed [14].

High-performance size-exclusion chromatography (HPSEC) can be used to analyse the molecular size distribution (MSD) of organic macromolecules. In HPSEC, molecules larger than the gel pores move rapidly through the column with the eluent, whereas smaller molecules penetrate the pores of the gel in relation to both their size and shape and the pore size distribution of the gel [13,15–18].

Numerous authors [19–21] have stressed that organic matter content decreases with an increase in grain diameter, thus fractions of sand are poorest in carbon. Carriers of organic carbon compounds may be made up of larger fragments of dead plant residue, frequently observable with a microscope, and by the forms deposited on the surface of mineral grains. The accumulation of carbon in fractions containing grains smaller and smaller in diameter is due to an increasingly active surface, which enhances the formation of organic–mineral bonds. As reported in the literature [7,22], humus substances connected with the fine-grained soil fraction demonstrate a greater degree of humification ‘maturity’ compared with humus substances that are part of the coarse-grained fraction; hence their higher resistance to decomposition.

Taking the above into consideration, it is clear that evaluation of the effect of the size of the particles to which organic matter is bound on the hydrophilic–hydrophobic properties and polydispersity of HAs is a very important in the study of humic substances. Other interesting information is how organic fertilisers change the properties of HAs occurring in fractions containing particles >0.1 mm and <0.1 mm.

The problem was studied as a three-factor experiment in which the factors were: the size of the soil particles from which HAs were isolated (coarse fraction, particle size >0.1 mm; fine fraction, particle size <0.1 mm); the type of fertiliser (farmyard manure, vermicompost, straw and combinations of manure mixed with straw, and vermicompost mixed with straw); and the decomposition time for fertilisers introduced into the soil (one and three years).

Table 1. Basic chemical properties of soil and organic fertilisers.

	TOC	Nt	P				
Incubated material	$g \cdot kg^{-1}$ d.m.			TOC:Nt	TOC:P	pH in H_2O	pH in KCl
Soil	5.8	0.53	0.10	10.9	71.3	6.2	5.7
Cattle manure	312	31	12.5	10.1	24.8	7.8	7.4
Wheat straw	412	5.1	2.2	80.8	187.3	n.d.	n.d.
Vermicompost	212	14.9	9.9	14.2	22.4	6.6	6.4

Notes: TOC, total organic carbon; Nt, nitrogen; d.m., dry matter; n.d., not determined.

2. Materials and methods

In a model incubation experiment, cattle manure, vermicompost and wheat straw were mixed with soil material (sandy texture). The soil was sampled from the upper layer (0–20 cm) of a brown podzolic soil with the fractional composition of light leamy sand. Vermicompost was produced from bovine manure by Californian worms (*Eisenia fedita* Sav.). The product was subsequently mixed with peat. Basic chemical properties of soil and organic fertilisers are given in Table 1. The experiment was carried out for three years in plastic containers dug in at ground level. The containers (three replicates for each sampling date) were filled with the material as follows:

- 0, soil without additives (control variant);
- 1O, soil + manure (100 kg + 10 kg);
- 2S, soil + straw + NH_4NO_3 (100 kg + 2 kg + 0.014 kg N);
- 3E, soil + vermicompost (100 kg + 8.3 kg);
- 4OS, soil + manure + straw + NH_4NO_3 (100 kg + 5 kg + 1 kg + 0.007 kg N);
- 5ES, soil + vermicompost + straw + NH_4NO_3 (100 kg + 4.15 kg + 1 kg + 0.007 kg N).

Organic fertilisers were mixed with soil at doses increasing the organic carbon content by $\sim 10\,g \cdot kg^{-1}$. In the variants with straw, ammonium nitrate was added to lower the TOC:Nt ratio to values close to the other variants.

Soil samples were analysed after one and three years. Prior to analysis, the soil material was air-dried, powdered and sieved through a 1 and 0.1 mm mesh to obtain two fractions, the coarse fraction (0.1–1.0 mm, hereafter the >0.1 mm fraction) and the fine fraction (diameter < 0.1 mm).

HAs were extracted from both fractions with $0.5\,mol \cdot L^{-1}$ NaOH solution after earlier removal of carbonates with $0.01\,mol \cdot L^{-1}$ HCl. HAs were precipitated with HCl solution at pH 2 and purified with a mixture of HCl + HF. The preparations were lyophilised and powdered in an agate mortar. The ash content of the HA preparations was $< 2\%$. The separated HAs were analysed as follows.

1. Hydrophilic and hydrophobic properties were determined with a liquid chromatograph HPLC Series 200 with DAD detector (Perkin–Elmer). Separation involved the use of an X-Terra C_{18} column (5 μm, 250 × 4.6 mm). Solutions of HAs were applied in $0.01\,mol \cdot L^{-1}$ NaOH at a concentration of $2\,mg \cdot mL^{-1}$. A sample of 100 μL was injected into the column. The solvent used was acetonitrile/water and the solvent flow (ratio H_2O/ACN v/v) was as follows: 0–6 min, 99.5:0.5; 7–13 min, 70:30; 13–20 min, 10:90. Detection was carried out at a wavelength of 254 nm. Based on the areas determined under the peaks, the proportion of hydrophilic (HIL) and hydrophobic (ΣHOB = HOB-1 + HOB-2) fractions in HA molecules and the parameter HIL/ΣHOB [6,14,23] were determined.
2. Polydispersity was determined using HPSEC (Chromatograph HPLC Perkin–Elmer Series 200, diode array UV detector operating at 254 nm). HAs were separated using a TSK

G3000SW column (7.5 × 600 mm). Sodium acetate 0.01 mol · L^{-1} at pH 7 was used as the eluent. The guard column was a TSK G3000SW (7.5 × 75 mm). Solutions of HAs were applied in 0.01 M CH_3 COONa at a concentration of 0.05 mg · mL^{-1}. A sample of 100 μL was injected into the column. The first (S1) and second (S2) peak areas were determined, as well as the S1/S2 ratio [11,12].

The qualitative parameters of HAs were analysed for correlations using the statistical software Statistica MS.

3. Results and discussion

Interesting information on the transformation of HAs can be obtained using high-performance liquid chromatography (HPLC). Chromatographic separation of HAs is based mainly on differences in their hydrophobic properties. As a result of chromatographic analysis, and similar to Debska [6], Debska et al. [11] and Woelki et al. [14], our research recorded separation into hydrophilic (HIL) and hydrophobic (HOB-1 and HOB-2) fractions (Figure 1). Hydrophilic fractions correspond to peaks within the range 2.50–4.60 min, whereas hydrophobic fractions are within the range 12.80–17.60 min. Fraction HOB-1 is hydrophobic with a shorter retention time of 12.80–15.60 min, and fraction HOB-2 has a retention time of 15.60–17.60 min. The proportion of hydrophobic fractions considerably exceeded that of hydrophilic fractions (Table 2). The greatest proportion of hydrophilic fractions and the lowest proportion of hydrophobic fractions were recorded for HAs from soil with no organic fertiliser additives. Introducing organic fertilisers into the soil resulted in an increase in the proportion of hydrophobic fractions and a decrease in the proportion of hydrophilic fractions; thus a decrease in the HIL/ ΣHOB ratio.

The percentage of hydrophilic fractions in HA molecules isolated after one year of decomposition of organic fertilisers in soil was, on average, 15.3% lower for the coarse and fine fractions, and

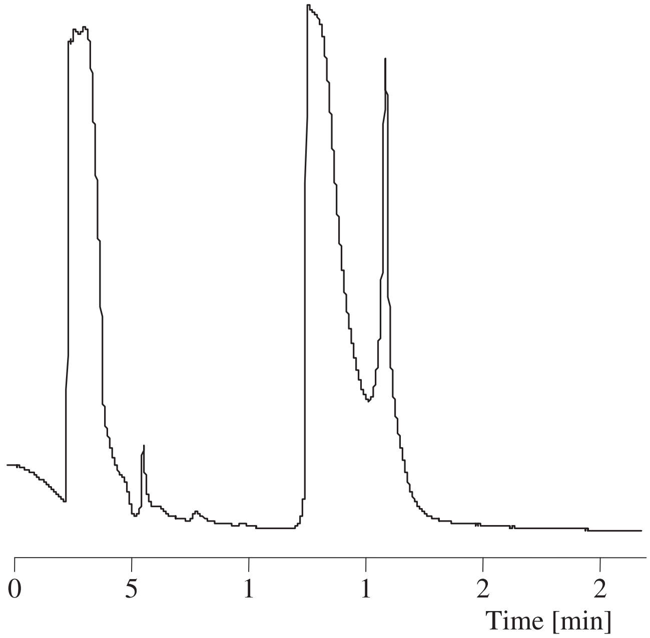

Figure 1. RP-HPLC chromatogram of humic acids variant 4OS, fraction <0.1 mm (after three years).

Table 2. Proportion of hydrophilic and hydrophobic fractions.

Variant	Fraction (mm)	HIL	HOB-1	HOB-2	ΣHOB	HIL/ΣHOB
0	>0.1	35.8	49.2	15.0	64.2	0.557
	<0.1	38.6	40.9	20.5	61.4	0.630
After one year						
1O	>0.1	27.2	53.2	19.5	72.7	0.374
	<0.1	32.8	49.6	17.6	67.2	0.488
2S	>0.1	31.8	50.2	18.0	68.2	0.466
	<0.1	36.1	49.1	14.8	63.9	0.565
3E	>0.1	27.4	49.8	22.8	72.6	0.377
	<0.1	33.0	47.4	19.5	66.9	0.493
4OS	>0.1	27.7	51.4	20.8	72.3	0.383
	<0.1	34.1	45.0	20.9	65.9	0.517
5ES	>0.1	29.7	51.0	19.3	70.3	0.422
	<0.1	35.1	48.2	16.7	64.9	0.541
After three years						
1O	>0.1	29.6	49.1	21.3	70.4	0.420
	<0.1	32.5	47.4	20.1	67.5	0.481
2S	>0.1	30.2	51.4	18.4	69.8	0.433
	<0.1	36.3	49.0	14.8	63.8	0.570
3E	>0.1	31.1	41.5	27.4	68.9	0.451
	<0.1	35.3	47.9	16.8	64.7	0.546
4OS	>0.1	28.9	46.9	24.1	71.0	0.407
	<0.1	36.4	46.6	17.0	63.6	0.572
5ES	>0.1	33.4	48.2	18.4	66.6	0.502
	<0.1	34.1	48.0	17.8	65.9	0.517

9.1% higher than in soil without added fertiliser. After three years, the percentage of hydrophilic fractions increased by 4.2%, whereas the percentage of hydrophobic fractions decreased by 1.9, compared with the proportion in HAs after one year of decomposition of organic fertilisers. These results indicated very clearly that, along with progress in the humification of organic matter, HA molecules demonstrate a higher percentage of hydrophilic fractions than hydrophobic fractions. As a consequence, values of the HIL/ΣHOB ratio are higher.

The highest proportion of hydrophilic, and the lowest proportion of hydrophobic, fractions were found in HAs isolated from samples of soil mixed with straw both after one and three years of decomposition. The lowest values for the HIL/ΣHOB ratio were recorded for variants with manure, which means that of, all the studied samples, HAs from this variant have the lowest proportion of hydrophilic fractions and the highest proportion of hydrophobic fractions.

As stated in the literature [6,11], the greater the increase in the degree of 'maturity' of HAs molecules, the higher the values for the ratio HIL/ΣHOB. As reported, higher values are found for the HIL/ΣHOB ratio in HAs with a lower hydrogen content, a higher degree of internal oxidation, a higher carboxyl group content and lower values for burning heat, as well as a higher percentage of low-molecular-mass fractions and, as a consequence, a lower molecular mass.

The results given in Table 2 show that the degree of 'maturity' of HAs molecules was determined by not only the type of fertilisers and the decomposition time, but also the size of the soil particles in which the organic matter occurs.

HAs isolated from the finer fraction (< 0.1 mm), compared with HAs from the fraction >0.1 mm, demonstrated a considerably higher proportion of hydrophilic fraction and a lower proportion of hydrophobic fraction and, as a result, higher values for HIL/ΣHOB (Table 2).

The reults confirmed earlier literature reports [7,22,24] that humus substances connected with a fine-grained soil fraction demonstrate a higher degree of humification 'maturity' than those from a coarse-grained fraction.

Compared with HAs from soil without fertilisers, the decrease in the hydrophilic fraction after the introduction of organic fertilisers was more pronounced in HAs occurring in the coarse fraction than in the fraction of particle size <0.1 mm. In HA molecules from the coarse fraction obtained after one year of the experiment, the proportion of the hydrophilic fraction was, on average, 19.6% lower and that of hydrophobic fraction 10.9% higher compared with HAs from soil without fertilisation. The differences in the proportions of the specific fractions in the HA molecules from the fine fraction were 11.4 and 7.1%, respectively. After three years of decomposition of organic matter introduced into soil with organic fertilisers, the percentage of the hydrophilic fraction in HAs from the coarse fraction was 14.4% lower, whereas that of the hydrophobic fraction was 8.1% higher, compared with the preparation from the soil without fertilisers. In the HA molecules from the fine fraction these differences were 9.6 and 6.0%, respectively.

Sample HPSEC chromatograms are given in Figure 2. Application of HPSEC allowed separation of the isolated HAs depending on the size of the molecules [13,15–18]. The chromatogram patterns suggest that the HA molecules include two fractions. The first peak, with a shorter retention time of ∼12.00 to 17.00 min, corresponds to the fraction with larger HA molecules, whereas the other, the maximum of which occurred most frequently at a retention time of ∼19.00 min, is found for smaller HA molecules.

The proportion of the high-molecular-mass fraction in HAs from soil without organic fertilisers accounted for, on average, (fine- and coarse-grained fraction) 25.70% (Table 3). HAs isolated from samples of soil mixed with organic fertilisers showed a higher proportion of the high-molecular-mass fraction and a lower proportion of the low-molecular-mass fraction compared with HAs from soil with no organic fertilisers added. Introducing organic fertilisers into soil, therefore, increases the polydispersity of HAs molecules. As the index of polydispersity of HAs molecules, parameter S1/S2 (the ratio of the proportion of the high-molecular-mass fraction to the proportion of the low-molecular-mass fraction) is assumed. The higher the value of this parameter, the higher the polydispersity of HAs and the higher the resulting mean molecular mass. The lowest values for this parameter were recorded for HAs from soil without additives, −0.346 (mean for coarse- and fine-grained fractions). Values of S1/S2 for HAs from soil one year after the introduction

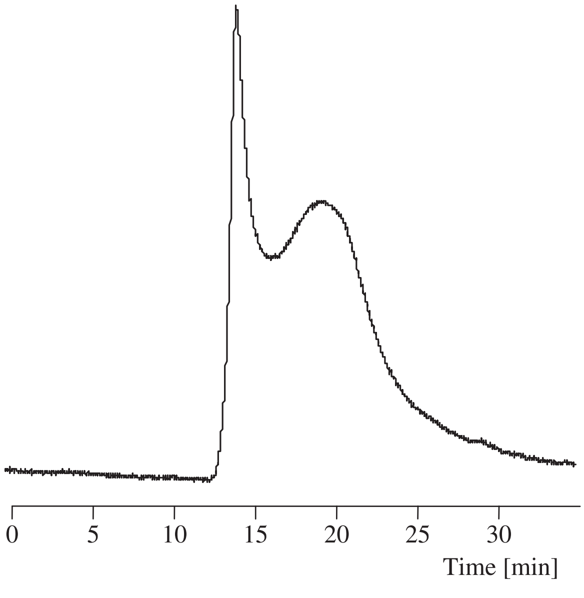

Figure 2. HPSEC chromatogram of humic acids variant 4OS, fraction <0.1 mm (after three years).

Table 3. Proportion of high- and low-molecular-mass fractions.

Variant	Fraction (mm)	S1	S2	S1/S2
0	>0.1	26.3	73.7	0.357
	<0.1	25.1	74.9	0.335
		After one year		
1O	>0.1	37.7	62.3	0.605
	<0.1	32.3	67.7	0.477
2S	>0.1	33.5	66.5	0.504
	<0.1	31.2	68.8	0.453
3E	>0.1	36.8	63.2	0.582
	<0.1	33.1	66.9	0.495
4OS	>0.1	37.6	62.4	0.602
	<0.1	34.2	65.8	0.520
5ES	>0.1	33.9	66.1	0.513
	<0.1	32.6	67.4	0.484
		After three years		
1O	>0.1	31.6	68.4	0.462
	<0.1	33.2	66.8	0.497
2S	>0.1	27.6	72.4	0.381
	<0.1	28.4	71.6	0.397
3E	>0.1	32.7	67.3	0.486
	<0.1	31.8	68.2	0.466
4OS	>0.1	28.2	71.8	0.393
	<0.1	25.1	74.9	0.335
5ES	>0.1	32.3	67.7	0.477
	<0.1	33.4	66.6	0.502

of organic fertilisers ranged from 0.453 to 0.602, whereas after three years values were lower, ranging from 0.335 to 0.502 (Table 3). The lowest values for parameter S1/S2, after both one and three years of decomposition of organic fertilisers, were recorded for HAs of soil mixed with straw.

Our results confirmed earlier reports by Aleksandrova [25] and Debska et al. [11]. These authors [11,25] showed that HAs at a very early stage of plant material humification are characterised by a lower degree of polydispersion and, as a consequence, a higher mean molecular mass, whereas during humification, the molecular mass of the HAs becomes lower.

Taking this into consideration, it can be concluded that, in general, HAs isolated from soil samples with larger particles have a higher molecular mass (a higher percentage of fraction S1) (Table 3) than HAs from the fine fraction. Similar to changes in the proportions of hydrophilic and hydrophobic fractions described earlier, one year after the introduction of organic fertilisers into the soil, changes in the percentage of the low and high molecular mass fractions (S1 and S2) were higher in HA molecules from the coarse fraction than those from the < 0.1 mm fraction, compared with controls. The differences in the proportions of low- and high-molecular-mass fractions in HAs isolated after one and three years of the experiment were, respectively, 15.1 and 8.5% for the coarse fraction, and 7.0 and 3.4% for the fine fraction.

Analysis of the correlation revealed the existence of a significant negative relationship between polydispersion and the hydrophilic–hydrophobic properties of HAs (values of the correlation coefficient between S1/S2 and HIL/ΣHOB at $\alpha < 0.05$ was 0.656). Egeberg and Alberts [26] showed that the hydrophobic fraction has a higher percentage of larger molecules. As a consequence, the proportion of the high-molecular-mass fraction is also higher, as confirmed in this study.

Analysis of the results allowed us to speculate that the transformation of organic matter occurring in the coarse fraction is more intense than in the fine fraction because more fresh organic matter is concentrated in the fraction with larger particles.

4. Conclusions

Introducing organic fertilisers into soil resulted in an increase in the proportion of hydrophobic and high-molecular-mass fractions and a decrease in the hydrophilic and low-molecular-mass fractions in HA molecules.

After the introduction of organic fertilisers into soil, the highest values for the ratio HIL/ΣHOB and the lowest values for S1/S2 were noted for HAs in soil mixed with straw.

HAs isolated from a fine-grained soil fraction demonstrated a higher degree of 'maturity' than those from the coarse-grained fraction, which is seen as higher values for HIL/ΣHOB and lower values for S1/S2.

Fresh organic matter introduced together with fertilisers into soil is concentrated to a greater extent in particles >0.1 mm, compared with the fine fraction, as confirmed by the differences in the properties of HAs from both fractions.

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