

Article

Characterization of humin in various soils using ^{13}C NMR with phase-adjusted spinning side bands

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Abstract

Chemical structure of humin, the acid- and alkali-insoluble fraction of humic substances, is much less understood than that of humic and fulvic acids. The objective of this study was to characterize humin in various types of soils using ramp ^{13}C cross polarization/magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy with phase-adjusted spinning side bands (PASS), for which comparisons with humic acids (HAs) and residual humin after lipid extraction in the same soils were also done. Humin samples were obtained in the form of residues after the soluble humus was extracted with 0.1 M NaOH from six soils; a buried Andosol, the surface layer of a grassy Andosol and two forested Cambisols, and the plow layer of a Cambisol and Fluvisol used for an upland field and irrigated rice, respectively. The humin C content in the soil samples ranged from 6.4–84.6 mg C g⁻¹, or 49–64% of total soil C. After the sample was treated with HF-HCl to reduce the ash content, NMR analysis was conducted. Lipids were then extracted with dichloromethane (DCM)-methanol. The % *O*-alkyl C of humins was generally high, while the % alkyl C, % aromatic C, and % carboxy C showed large ranges of 12–42%, 20–49%, and 11–20%, respectively. Humin from the two Andosols and the forested Cambisol was characterized by a high % of aromatic C (35–49%), while that from another forested Cambisol showed a strong alkyl C peak at 29 ppm, of which, the intensity accounted for 42% of the total C. The % alkyl C of humins was commonly higher than that in HAs while the % aromatic C was generally the opposite, in which the DCM-methanol extractable lipids play a significant role. Although the removal of lipids generally resulted in the signal intensities of alkyl C to be reduced, the extent of reduction was limited and alkyl C still accounted for 19–34% of the residual C. There was no significant change in % carboxy C after lipid extraction, suggesting that some of the carboxy C was contained in lipids and was removed with the alkyl C.

Keywords: ^{13}C NMR, Humic acids, Humin, Lipid.

Introduction

Soil humic substances are generally classified into three fractions based on their solubility in acid and alkali, i.e., humic acids (HAs), fulvic acids (FAs), and humin (Stevenson, 1994). Humin accounts for 34–87% of the total soil C in various types of soils (Watanabe and Kuwatsuka, 1991; Grasset et al., 2017; Han et al., 2019; Frazão et al., 2021). However, it is more accurate to consider humin as a fraction of soil organic matter (SOM) rather than humic substances (Song and Hayes, 2023), because humin is obtained as residue after the extraction of HAs and FAs and contains non-humic substances, such as non-polar

biomolecules and other insoluble biomolecules as well as charred plant materials. Lipids, including fats, waxes, resins, and related substances, are representative of such biomolecules (Hays et al., 2017). According to Stevenson (1994), soil lipids account for 1–16% of the total SOM in mineral soils, and these numbers are dependent on the type of vegetation and the pH of the soil. Almendros and González-Vila (1987) proposed that wax in higher plants contributes to the constituents of the humin based on the detection of long-chain alkanes (C15–C31) and fatty acids (C12–C28). Humin may also contain HAs, and possibly FAs, which were not extracted due to their strong adsorption to soil minerals (Rice and

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MacCarthy, 1992; Tsutsuki and Kuwatsuka, 1992). Core components of humin and the quantitative contribution of potential sources to humin have not been clarified.

Humin plays a vital role in the global C balance as a huge terrestrial C pool. It is also recognized that humin has various functions in soil (Guo et al., 2018; Pham et al., 2021), e.g., as an adsorbent for agrochemicals (Murano et al., 2018), organic pollutants (Chefetz et al., 2000; Zhao et al., 2014), and heavy metals (Li et al., 2019), as an electron donor in the chemical reduction of transition metals (Zhang et al., 2019), and as an electron mediator in biochemical processes such as dechlorination (Zhang and Katayama, 2012), denitrification (Xiao et al., 2016), and CO₂-fixing acetogenesis (Ha et al., 2022). Therefore, it is important to clarify their chemical structure. Nevertheless, solution-based analytical methods are not applicable, and even analytical methods for solid samples do not provide satisfactory results due to low organic matter concentration and interference by coexisting substances (e.g., silicates in infrared spectroscopy and paramagnetic elements in nuclear magnetic resonance (NMR) spectroscopy).

A portion of humin can be extracted with a mixture of an inorganic acid solution and an organic solvent, such as dimethyl sulfoxide (64% of total humin; Song et al., 2011) and methyl isobutyl ketone (MIBK) (47–64% of total humin; Rice and MacCarthy, 1988; Almendros et al., 1996). The humin that is not bound to soil minerals can be isolated utilizing heavy liquid separation. Such materials that are occluded in soil aggregates are referred to as inherited humin and accounts for 12–55% of the total humin (Almendros and González-Vila, 1987; Almendros et al., 1996). Both MIBK-humin and inherited humin are thought to be more aliphatic in comparison with HAs or FAs, since the yield of aliphatic compounds such as alkanes and fatty acids upon chemical degradation is higher than that of aromatic compounds (Almendros and González-Vila, 1987). Non-destructive analysis using cross polarization/magic angle spinning (CP/MAS) ¹³C NMR showed that *O*-alkyl C accounted for 43–57% of the C in such samples (Almendros et al., 1996), while a smaller value, 21%, was reported for a DMSO-humin obtained from a Mollisol by Song et al. (2008).

An alternative method for isolating humin, although it is incomplete, is decomposing silicate minerals by

treating the sample with hydrogen fluoride (HF; Preston and Newman, 1992). Samples obtained using this approach appear to have a higher aliphaticity than HAs (Fabbri et al., 1998; Xu et al., 2017, 2019). Xu et al. (2019) reported that the relative abundance of aromatic C in humin in an upland field soil (Calcaric Fluvisol) was higher than that in a paddy soil (Hydragric Anthrosol) and it decreased by the application of organic fertilizer. However, studies on the chemical structure of humin, even including those on some of the fractions mentioned above, are much fewer than those on HAs and FAs (Rice, 2001; Hays et al., 2017). Thus, an accumulation of data from various sources and conditions is needed if we are to understand the common or unique characteristics of humin.

The objective of this study was to characterize the structural properties of humin in various soils using solid-state ¹³C NMR spectroscopy with phase-adjusted spinning side bands (PASS). PASS is a technique that integrates spinning side bands separately and quantitatively recovers them into their inherent center peaks. The results can be used to accurately estimate the C composition of natural organic matter samples (Ikeya and Watanabe, 2016). Prior to analysis, the concentrations of minerals and paramagnetic metals were reduced by treating the sample with a mixture of HF and HCl. To confirm that the chemical structures of humins are different from HAs and the nature of the differences, the HAs in the same soils were also analyzed. The aliphaticity of humin are likely affected by free or loosely bound lipids. To assess their influence on the core structure of humin, a second ¹³C NMR analysis of the humin was performed again after the lipids had been extracted.

Materials and methods

Soil samples used

The two Andosols used were, Yubunbara VIIA-horizon (7.4–8.0 m depth; Oyama, Shizuoka Prefecture, Japan; designated as Yubunbara-7) and Inogashira A-horizon (0–30 cm depth; Fujinomiya, Shizuoka Prefecture, Japan). The dominant plant species at the Inogashira site was *Miscanthus sinensis*. Two Dystric Cambisols, Kuragari (Okazaki, Aichi Prefecture, Japan) and Dando (Shitara, Aichi Prefecture) soils were collected from the A-horizon (0–19 or 0–20 cm depth) in mixed forests (*Quercus*

crispula var. *crispula* *Fagus crenata*, etc.), and another Cambisol, Anjo (Anjo, Aichi Prefecture), was collected from the Ap-horizon (0–15 cm depth) of an upland field. A Eutric Fluvisol, Nagano (Nagano, Nagano Prefecture, Japan), was collected from the Ap-horizon (0–15 cm depth) of a rice paddy. Organic fertilizers had never been applied to both the agricultural soils. These six soil samples were air-dried and sieved (< 2 mm) before use.

Preparation of HAs and humin

Humic acids were extracted with 0.1 M NaOH added at a rate of 300 mL to 1 g total soil C by shaking at 25°C for 24 h under a N_2 atmosphere and in the dark (Ikeya and Watanabe, 2003). The extracted residue was washed twice with the same volume of 0.1 M NaOH and the washings were combined with the extract. The HAs in the extract were then purified according to the NAGOYA method (Kuwatsuka et al., 1992). The residual soil was washed three times with ultrapure water, the pH of the solutions was adjusted to 4–5 with dilute H_2SO_4 , centrifuged to remove liquid phase, freeze-dried, and the resulting solid sample pulverized (humin sample).

For the ^{13}C NMR analysis, 10 or 20 g of a humin sample was treated with 10 M HF-0.2 M HCl (1:1, v/v) added at a ratio of 5:1 (v/w). The suspension was shaken at 25°C for 12 h, centrifuged, and the supernatant was then removed. This treatment was repeated a total of five times. The final residue was repeatedly washed with ultrapure water and freeze-dried.

A portion of the HF-treated humin was subjected to Soxhlet extraction with dichloromethane (DCM)–methanol (2:1) for 8 h (lipid-removed humin), and the residual weight was determined.

The C content in the soil, humin, and lipid-removed humin was determined using an elemental analyzer NC2500 (Thermo Fisher Scientific, Waltham, MA, USA).

Measurement of ^{13}C CP/PASS NMR spectra

Ramp CPMAS ^{13}C NMR spectra of the HAs, humin, and lipid-removed humin with PASS were recorded at 176 MHz on ECA 700 spectrometer (JEOL, Tokyo, Japan). Operating conditions are as follows: contact time, 1.0 ms; delay time, 1.0 s; spinning rate, 9 kHz; and number of data accumulation, 1560–9620. Chemical shifts are quoted relative to tetramethylsilane

(0 ppm) and adjusted with hexamethylbenzene (17.36 ppm). Signals in the spectra were divided into five regions of 0–45 (saturated alkyl C), 45–105 (alkyl C substituted by O or N; represented by *O*-alkyl C), 105–160 (aromatic C), 160–190 (carboxy C), and 190–220 ppm (ketone C) and their cumulative intensities were regarded as the proportion of each C group in total C.

Significance of the difference in the relative abundance of each C group between humin and HAs or between humin and lipid-removed humin was determined by the Holm-Sidak test.

Results

The humin C content of the six soils (Table 1) ranged from 6.4–84.6 mg C g^{-1} , which correlated positively to the total soil C content ($P < 0.005$). The distribution of the proportion of soil C that was present as humin C was narrow, 49–64%, irrespective of the fact that the difference in total C content was more than 10 times among the soils.

Figure 1 shows ^{13}C CP/PASS NMR spectra of humin and HAs. The HF-HCl treatment resulted in the C content in the humin samples to be increased from 67–151 mg C g^{-1} to 100–429 mg C g^{-1} . The humin showed major resonances at 20–22 ppm (methyl C), 27–28 ppm (polymethylene C), 52–55 ppm (methoxy C), 70–71 ppm (secondary alcohol C), 101–103 ppm (acetyl C), 126–128 ppm (aromatic C–C), 149–153 ppm (aromatic C substituted by O or N; represented by *O*-aromatic C), and 170–172 ppm (carboxy C) (Mao et al., 2017). The distribution range of humin C that is present as alkyl C, aromatic C, and carboxy C (Table 2) was substantial: 12–42%, 20–49%, and 11–20%, respectively. The spectral patterns were similar between Yubunebra-7, Inogashira, and Kuragari humin with differences in the signal intensity of alkyl C. In these samples, the relative abundance of aromatic C was the highest among the five C groups, 35–49% (Table 2), while % alkyl C varied, 12–29%. The ^{13}C CP/PASS NMR spectra of the Nagano and Anjo humins were similar, in which the differences between % alkyl C (28–31%), % *O*-alkyl C (29–31%), and % aromatic C (24–26%) were minimal. In contrast, the Dando humin was characterized by strong alkyl C signals, accounting for 42% of the total humin C.

Major resonances of HAs were observed at 20–21, 27–28, 52–54, 70–71, 101–103, 126–127, 151–152, and 170–172 ppm. Two Andosol HAs (Type A; Lu et

al., 2020) showed larger aromatic C signals with a maximum at 126–127 ppm, while the resonances in the alkyl C and *O*-alkyl C regions of the Yubunbara-7 HA from the buried Andosol were very weak. The methoxy C (55 ppm) and *O*-aromatic C (152 ppm) signals were greater in the Nagano and Anjo HAs (Type Rp). In the Nagano and Anjo HAs, strong *O*-aromatic C peaks contributed to the % aromatic C (33–34%; Table 3) which was comparable to the Kuragari and Dando HAs (Type B; both 34%), and the strong methoxy C peak resulted in a higher % *O*-alkyl C (27–29%) than the other HAs (4.5–23%).

In a comparison of the ^{13}C CP/PASS NMR spectra between the humins and HAs in the same soils (Fig. 1), the signals at around 28 ppm assignable to polymethylene C were stronger in the humins than in the HAs while those of methoxy C and *O*-aromatic C were generally less intense in the humins. Regarding C composition (Tables 2 and 3), % alkyl C was typically higher ($P < 0.005$) in the humin than in the HAs. On the contrary the % aromatic C was generally lower ($P < 0.05$) in the humin than in the HAs.

The proportion of lipid C in the humin C (Table 1), as estimated from the residual amount of C after lipid extraction, ranged from 16–31%. There were no relationships between these values with soil type or land use type. The ^{13}C CP/PASS NMR spectra of the lipid-removed humin showed signals with chemical

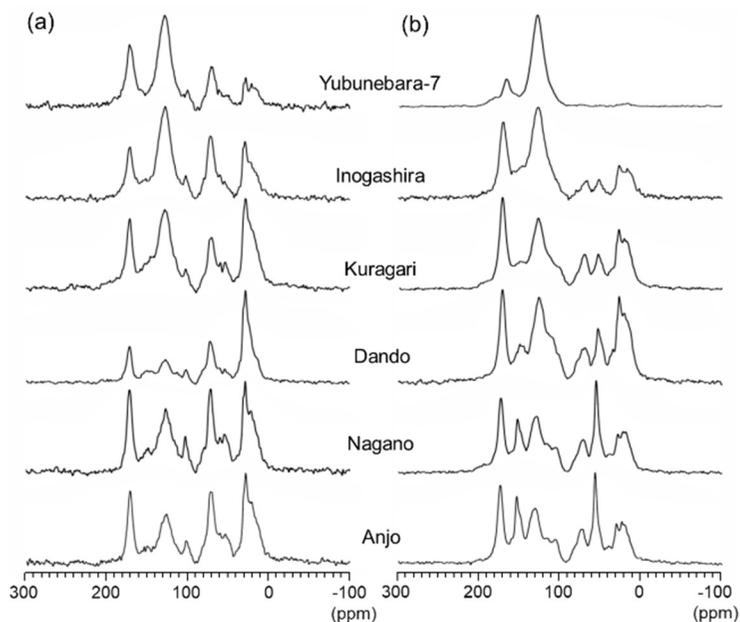


Fig. 1. ^{13}C CP/PASS NMR spectra of humin (a) and humic acids (b) obtained from six soils.

shifts similar to the humin before DCM-methanol extraction (Fig. 2), with lower intensities in the alkyl C region. DCM-methanol extraction resulted in a decreased % alkyl C in all samples except for Yubunbara-7 ($P < 0.005$; $n = 6$), but still accounted for 19–34% of the residual C. The % *O*-alkyl C and % aromatic C were generally higher ($P < 0.005$; Table 4) in the lipid-removed humin than in the humin before DCM-methanol extraction, while the % carboxy C was not significantly different between them.

Table 1 Total C content, humin C content, and proportion of lipid C in humin C

Soil	Total C (mg g ⁻¹)	Humin C (mg g ⁻¹)	Proportion of lipid C in humin C [‡] (%)
Yubunbara-7	43.2	25.7 (59.5) [†]	30.8 (18.3) [‡]
Inogashira	167	84.6 (50.6)	16.1 (8.2)
Kuragari	65.6	33.5 (51.1)	20.5 (10.5)
Dando	61.9	33.0 (48.5)	30.7 (14.9)
Nagano	14.9	9.5 (63.7)	27.8 (17.7)
Anjo	12.3	6.4 (51.7)	24.5 (12.7)

[†]Values in parentheses denote the proportion of humin C in total soil C (%).

[‡]Estimated from the residual C amount after lipid extraction.

[‡]Values in parentheses denote the proportion of the lipid C in humin in total soil C (%).

Discussion

^{13}C CP/PASS NMR analysis of the humin indicated that the structures varied widely among various types of soils. Humin from the two Andosols was visually darker and characterized by strong aromatic C signals (Fig. 1a) as was also observed for HAs (Fig. 1b). Similar % aromatic C between humin and HAs was also reported for Hapludolls under upland cultivation in the USA (Pikul Jr. et al., 2009) and a Chinese Mollisol (Zhang et al., 2019). The black C content in the Yubunbara-7 and Inogashira soils was reported to be 48% and 32% of the total soil C (Lu et al., 2020), while the aromatic C in humin was estimated to be 29% and 21% of the total soil C (Table 2). Thus, the aromatic structure of the humin in these soils may be greatly affected by the presence of insoluble black C. The major difference between humin and HA in the Yubunbara-7 soil was the presence of significant signals in the alkyl C and *O*-alkyl C regions for the

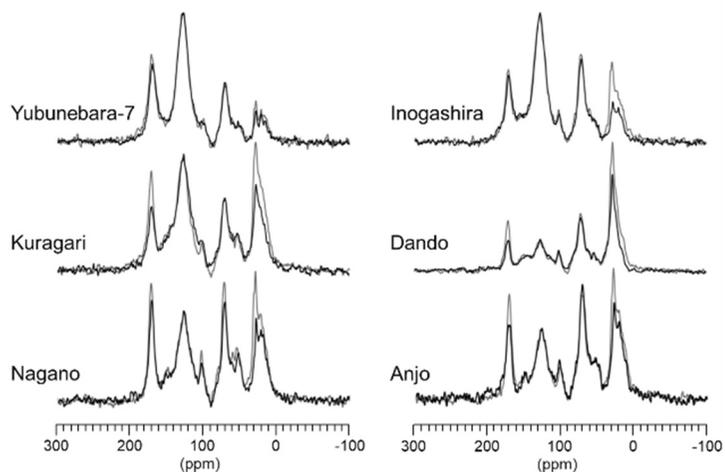


Fig. 2. ^{13}C CP/PASS NMR spectra of lipid-removed humin (black lines) with those of original humin (gray lines) from six soils.

humin. Although their relationship to aromatic structure is unknown, it is likely that the aliphatic components of humin were protected physically or by binding to soil minerals for a long period (ca 10,000 years in case of Yubunbara-7; Watanabe and Takada, 2006).

The peaks or shoulders for methoxy C and *O*-

Table 2 Carbon composition of humin

Soil	% Alkyl C	% <i>O</i> -alkyl C	% Aromatic C	% Carboxy C	% Ketone C
Yubunbara-7	11.6	18.7	48.6 (29) [†]	19.6	1.5
Inogashira	19.1	24.1	41.7 (21)	14.0	1.1
Kuragari	28.5	19.5	35.4 (18)	15.1	1.5
Dando	42.1	26.2	19.7 (9)	10.9	1.1
Nagano	28.1	29.4	26.3 (17)	15.5	0.7
Anjo	31.2	30.6	24.0 (12)	13.5	0.7

[†]Values in parentheses denote the proportion of aromatic C in humin in total soil C (%).

Table 3 Carbon composition of humic acids

Soil	% Alkyl C	% <i>O</i> -alkyl C	% Aromatic C	% Carboxy C	% Ketone C
Yubunbara-7	2.0	4.5	75.6	16.7	1.2
Inogashira	15.1	13.0	48.9	21.3	1.7
Kuragari	22.5	21.8	33.7	20.4	1.6
Dando	26.3	22.9	33.8	15.5	1.5
Nagano	19.1	28.5	33.2	16.9	2.3
Anjo	19.8	26.8	34.3	17.9	1.2

aromatic C were larger in the humin in four soils other than Andosols (Fig. 1a). In the Anjo and Nagano soils, the aromatic C in humin accounted for 12% and 17% of the total soil C (Table 2) while the black C content was reported to be 10% and 7%, respectively (Lu et al., 2020). These findings suggest that sources other than black C, such as lignin, tannins, and microbially synthesized melanins (Chatterjee et al., 2015; Hays et al., 2017) also contribute to the aromatic C in humin in these two soils. The removal of lipids from the Nagano humin and Kuragari humin resulted in an increased % aromatic C (31% and 40%; Table 4) to similar or higher levels compared to the HAs (33% and 34%; Table 3). Nevertheless, the methoxy C and *O*-aromatic C signals in their ¹³C CP/PASS NMR spectra (Fig. 2) were still smaller than those in the HAs (Fig. 1b). We therefore conclude that the contribution of lignin to the aromatic C in humin is generally small, or that the lignin-derived components in the humin have already lost their specific functional groups.

In this study, there was no significant difference in the % *O*-alkyl C between the humins and HAs (Tables 2 and 3). However, this was due to a larger methoxy C content in the HAs, especially in the Nagano and Anjo soils (Fig. 1). The C α of amino acids and C-NH groups in side chains may also contribute to the resonance in the 45–60 ppm region. On the other hand, the carbohydrate C content was higher in the humin than in the HAs, as indicated by the more intense secondary alcohol C-O signals with a maximum at 70–71 ppm in their ¹³C CP/PASS NMR spectra. This was also estimated based on a comparison of the composition of monosaccharides released upon acid hydrolysis among the humus fractions (Itoh et al., 2007). According to them, the monosaccharide composition of humin was characterized by larger proportions of

glucose and xylose, suggesting that cellulose and hemicellulose were a source of humin components, although glucose can be derived both from plants and microbes (Murayama, 1988).

The lipid C in humin corresponded to 8–18% of the total soil C (Table 1). These values were likely higher than the general soil lipid content reported in the literature (Stevenson, 1994). The removal of silicate minerals by the HF-HCl treatment might have increased the extraction efficiency, the possibility that these values were overestimated due to incomplete recovery of residual samples from cylindrical glass fiber filter papers after the DCM-methanol extraction cannot be excluded. The major C that was removed by extraction with DCM-methanol appeared to be alkyl C. The fact that humin contains a higher % alkyl C than HAs is less conclusive in some cases after DCM-methanol extraction. That no enrichment in carboxy C during the extraction was detected suggests the simultaneous removal of some of them along with the alkyl C, for example in the form of fatty acids and their esters.

The proportion of alkyl C that was removed by DCM-methanol extraction was estimated to be up to 39–49% (Table 4), even allowing for the possibility that the amount of lipid extracted was overestimated. In the Inogashira and Kuragari humins, a larger reduction in the peak intensity at 28 ppm suggested that more molecules containing polymethylene chains were extracted. However, the peak intensity reduction was more limited in the Dando humin. In the ¹³C CP/PASS NMR spectra of Dando humin before and after lipid extraction (Fig. 2), the signal intensity at approximately 20 ppm (methyl C) relative to 29 ppm was smaller than that of the other humins. A broad peak (shoulder) at 80–90 ppm, where OC (quaternary)

Table 4 Carbon composition of lipid-removed humin

Soil	% Alkyl C	% <i>O</i> -alkyl C	% Aromatic C	% Carboxy C	% Ketone C
Yubunbara-7	9.3 (45) [†]	21.3	50.1	18.6	0.7
Inogashira	11.7 (49)	26.6	45.2	15.1	1.4
Kuragari	21.3 (41)	25.8	39.8	11.5	1.6
Dando	34.0 (44)	32.1	22.8	9.1	2.0
Nagano	22.7 (42)	29.5	31.0	15.5	1.3
Anjo	25.2 (52)	34.9	24.4	13.2	2.3

[†]Values in parentheses denote the proportion of alkyl C in humin that was removed by DCM-methanol extraction (%).

carbons appear (Mao et al., 2017), relative to the peak of secondary alcohol C-O at 71 ppm was larger. As such, the Dando humin may contain larger amounts of longer polymethylene chains and/or branched polymethylene chains with ether bonding or hydroxyl group. However, the ¹³C CP/PASS NMR spectra did not indicate that there were distinct structural differences between the extracted and unextracted lipids in any of the samples. Therefore, it was considered that the non-extractable alkyl components in humin stably accumulated by forming complexes with polyvalent metals, clay minerals, and/or by binding to *O*-alkyl and aromatic components in humin.

Conclusions

Humin in various types of soils is typically characterized by a higher % carbohydrate C and by a higher alkyl C content than HAs in the same soil. The aromatic C content is generally smaller than that for HAs, with some exceptions possibly due to the contribution of black C. The contribution of lignin, at least for which their specific functional groups were maintained, to the aromatic structures in humin was consistently small. The high alkyl C content can partially be explained by the presence of lipids that are extractable with DCM-methanol.

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