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Chemistry and Ecology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713455114>

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Online publication date: 30 September 2010

To cite this Article Maryganova, Victoria , Szajdak, Lech Wojciech and Tychinskaya, Ludmila(2010) 'Hydrophobic and hydrophilic properties of humic acids from soils under shelterbelts of different ages', *Chemistry and Ecology*, 26: 4, 25 – 33

To link to this Article: DOI: 10.1080/02757540.2010.501138

URL: <http://dx.doi.org/10.1080/02757540.2010.501138>

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Hydrophobic and hydrophilic properties of humic acids from soils under shelterbelts of different ages

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(Received 12 October 2009; final version received 3 June 2010)

A comparative investigation of the hydrophobic–hydrophilic properties of humic acids (HA) extracted with 0.1 M Na₄P₂O₇ at pH 7 from soils under shelterbelts of different ages [14 years old (young) and 200 years old (old)] and adjoining cultivated fields was carried out with the application of ¹³C NMR spectroscopy and hydrophobic interaction chromatography (HIC). The results determined using both methods were in line with the chemical composition of HA. Shelterbelt age was shown to be the principal factor affecting the composition and hydrophobic–hydrophilic properties of HA. HA from soil under the young shelterbelt was found to be less chemically mature and more hydrophilic than HA from the soil of the adjoining cultivated field. By contrast, HA from soil under the old shelterbelt was considerably more chemically mature and hydrophobic than that of the corresponding cultivated field and HA from the soil under the young shelterbelt.

Keywords: shelterbelt; humic acid; hydrophobic–hydrophilic properties; ¹³C NMR; hydrophobic interaction chromatography

1. Introduction

Shelterbelts are rows of trees and groundcover shrubs planted on agricultural land primarily for agronomic and environmental reasons. Shelterbelts restrain soil erosion, improve microclimate for agricultural production, regulate the water regime in soils, participate in the sequestration of carbon and nitrogen to mitigate the greenhouse effect, and can be regarded as biogeochemical barriers decreasing the concentration of many chemical compounds migrating with ground water outflow from adjoining cultivated fields [1–6]. The conversion from arable cropping to forested soil (shelterbelt) has been shown to cause the accumulation of soil organic matter (SOM), organic C and N, with the age of shelterbelts being the principal factor involved. Soil under a shelterbelt of a greater age contains higher amounts of SOM, organic C and N [5].

A major and the most important part of SOM consists of humic substances (HS) which are macromolecular polydisperse biphyllic systems including both hydrophobic domains (saturated

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hydrocarbon chains, aromatic structural units) and hydrophilic functional groups, i.e. having hydrophobic–hydrophilic (amphiphilic) properties [7,8]. The amphiphilic properties of HS and the proportions of hydrophobic and hydrophilic humic fractions are responsible for their solubility, surfactant-like character, susceptibility to biodegradation and interaction with a wide variety of organic and inorganic compounds. Because of that, HS play important roles in environmental processes [8–13].

Hydrophobic interaction chromatography (HIC) is a perspective method for the study of amphiphilic properties of HS [9,14,15]. This method includes the use of hydrophobic interactions between hydrophobic centers of the investigating substance and hydrophobic ligands attached to the uncharged gel matrix. The most hydrophilic components of the sample do not adsorb on the gel and are eluted with a starting buffer. The adsorbed components are fractionated on the basis of successive (or consecutive) reduction of the strength of their hydrophobic interactions with hydrophobic matrix [16]. Humic and fulvic acids (HA and FA) from soils, peats, sapropels and brown coals of different genesis with the application of HIC were shown to be characterised by different proportions of amphiphilic components [9,15,17–19].

One of the most powerful techniques for studying the chemical structure of HS is ^{13}C NMR spectroscopy [20–22]. Using ^{13}C NMR spectroscopy it is possible to obtain direct information on the carbon skeleton of HS, determine the content of hydrophobic and hydrophilic carbon atoms, and calculate the degrees of their hydrophobicity and hydrophilicity [23,24].

The objective of this study was to comparatively investigate hydrophobic–hydrophilic properties of HA from soils under shelterbelts of different age and adjoining cultivated fields using two different methods: ^{13}C NMR-spectroscopy and HIC, and to compare amphiphilic properties of HA with their chemical composition.

2. Materials and methods

2.1. Soil samples

Investigations were carried out on soils under two shelterbelts, the first was 14 years old (young) and the second was ~ 200 years old (old), and adjoining cultivated fields located in the Agroecological Landscape Park in Turew (40 km south of Poznań, West Polish Lowland). The shelterbelts and cultivated fields were introduced on Hapludalfs soils [25]. The old shelterbelt consists mainly of *Robinia pseudoaccacia* with admixture of *Quercus pentrea* and *Quercus robur*. The young shelterbelt is composed of *Quercus pentrea*, *Quercus robur*, *Larix deciduas*, *Pinus silvestris*, *Sorbus aucuparia*, *Sorbus intermedia*, *Tilia cordata* and some others, totalling 24 tree species. Soils of both adjoining fields were under continuous rye cropping. Soil samples were taken from the upper 20 cm of soils (humus horizon) in the middle of the shelterbelts areas (No. 1) and from adjoining cultivated fields 100 m from the shelterbelts (No. 2) during the period of intensive plant growth (i.e. in May 2005).

Total organic carbon (TOC) was analysed on a carbon analyser TOC 5050A with Solid Sample Module (SSM-5000A) produced by Shimadzu, Japan. Dissolved organic carbon (DOC) was determined as described previously [26]. Total nitrogen was estimated by Kjeldahl method. Soil pH was assayed by potentiometric titration in 1 N KCl (1:2.5, v/v).

2.2. Extraction of humic acids

HA from soil samples were extracted with 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ at pH 7 using an extractant/soil ratio of 5:1 under an N_2 atmosphere at room temperature [27]. HA from the young shelterbelt and

adjoining cultivated field were referred to as HA-1 young and HA-2 young, HA from the old shelterbelt and adjoining field were referred as HA-1 old and HA-2 old, respectively.

2.3. ^{13}C NMR spectroscopy of humic acids

Solution ^{13}C NMR spectra were recorded in 0.5 M NaOD on a modified Tesla BS587A spectrometer (Czech Republic) at the frequency of 20.182 MHz in a 'quantitative' regime without nuclear Overhauser enhancement with interpulse delay $> 5T_1$, where T_1 is the time of spin-lattice relaxation. The relative content of different types of C was calculated with an integrator.

2.4. Hydrophobic interaction chromatography of humic acids

HIC of the HA samples was carried out on a column (13×1.6 cm) packed with octyl-Sephacrose 4 Fast Flow (Farmacia, Sweden) in 0.05 M Tris-HCl buffer at pH 8.0 containing 3 M NaCl. Fractionation was performed using step-by-step elution with the following sequence of eluents: (1) 0.05 M Tris-HCl buffer at pH 8.0 containing 3 M NaCl; (2) 0.05 M Tris-HCl buffer at pH 8.0; (3) distilled water; and (4) 0.1 M NaOH solution [17]. The relative contents of the chromatographic fractions of the HA samples were estimated from the areas under corresponding peaks on the chromatograms.

3. Results and discussions

3.1. Characteristics of soils

As shown in Table 1, all the soils investigated are acidic. The pH value of the soil under the young shelterbelt is higher than that of the adjoining cultivated field, whereas the soil under the old shelterbelt is much more acidic than the corresponding cultivated soil.

The accumulation of SOM under shelterbelts is the main mechanism of long-term withdrawal of various elements from recycling in the environment [28]. The TOC content in the soils of cultivated fields adjoining to the shelterbelts was 4.5–4.6 $\text{gC} \cdot \text{kg}^{-1}$ (Table 1). The TOC content in the soil of the young shelterbelt had increased to 8.3 $\text{gC} \cdot \text{kg}^{-1}$ during 14 years. For the old shelterbelt, the accumulation of organic matter over 200 years has led to a substantial amount of TOC, namely 55.2 $\text{gC} \cdot \text{kg}^{-1}$.

The concentration of total nitrogen in the soil under the old shelterbelt, 3.3 $\text{g} \cdot \text{kg}^{-1}$, is the highest value for all the soils under study and is threefold higher than the value for the soil of the adjoining arable field (Table 1). Conversely, for the soil under the young shelterbelt, the total nitrogen content is similar to that of the adjoining arable soil and more than four times lower than the soil under the old shelterbelt. As shown in Szajdak [5], a soil under a shelterbelt of a higher

Table 1. Some characteristics of the tested soils.

Soil samples	pH (1 N KCl)	TOC $\text{gC} \cdot \text{kg}^{-1}$	N_{tot} $\text{g} \cdot \text{kg}^{-1}$	DOC $\text{gC} \cdot \text{kg}^{-1}$
14-year-old shelterbelt	4.56	8.3	0.8	0.76
Field adjoining 14-year-old shelterbelt	3.97	4.6	0.7	0.29
200-year-old shelterbelt	3.42	55.2	3.3	3.10
Field adjoining 200-year-old shelterbelt	5.84	4.5	1.1	0.30

Notes: TOC, total organic carbon; N_{tot} , total nitrogen; DOC, dissolved organic carbon.

age contains higher amounts of total and organic N. In addition, these differences may be due to the different composition of the shelterbelt plants. The main plant of the old shelterbelt *Robinia pseudoaccacia* is a nitrogen-fixing tree, hence, it significantly increases the total nitrogen content in the soil. This is not the case for plants in the young shelterbelt.

Dissolved organic matter is responsible for microbiological activity in the soil [26]. The investigation revealed the highest DOC content available for microbiological and biochemical activities in the soil under the old shelterbelt (Table 1). This value is 10 times higher than that for the soil of the adjoining cultivated field. The DOC content in the soil under the young shelterbelt is 4 times lower than that in the soil under the old shelterbelt, but 2.5 times higher than the amount of DOC in the soil of the corresponding arable field (Table 1).

3.2. ^{13}C NMR spectra of humic acids

^{13}C NMR spectra of HA under study were divided into five chemical shift regions. In the alkyl C region (0–45 ppm), almost all saturated hydrocarbons, as well as alkyl groups of amino acids and alkyl-substituted aromatics resonate. O,N-alkyl carbons (45–65 ppm) are associated with carbohydrates, methoxyls of lignins and amino acids. Carbons resonating at 65–108 ppm (O-alkyl) are representative mainly of the contribution of carbohydrates and carbohydrate-like components, even though carbons belonging to different structural groups resonate in this region. The region between 108 and 165 ppm is characterised by aromatic C. Signals in the region 165–200 ppm are assigned with carboxyl and other carbonyl-related C (165–200 ppm) [20,29]. Areas of alkyl and aromatic carbons were attributed to hydrophobic carbons, whereas those of O- and N-alkyl carbons, and carbonyl-related carbons represented hydrophilic carbons [23]. The percentages of hydrophobic carbons (HB) and hydrophilic carbons (HI) were used to calculate the degrees of hydrophobicity (HB/HI) for all HA [24].

The relative contents of the different carbon types, as determined from the integration of the chemical shift regions in solution ^{13}C NMR spectra, as well as percentages of hydrophobic and hydrophilic carbon atoms, and the degrees of hydrophobicity HB/HI for the HA under study, are reported in Table 2.

The relative contents of different structural groups in the HA of the both cultivated soils (HA-2 young and HA-2 old) are similar. They contain 17–18% alkyl C, 22% O,N-alkyl C, 25% O-alkyl C, 23% aromatic C and 12–13% carboxyl C. Compared with the HA of the adjoining cultivated soil, the HA from the soil under the young shelterbelt (HA-1 young) is characterised by a lower contents of aromatic C and carboxyl C, as well as a higher percentage of hetero-alkyl C, in particular O,N-alkyl C, associated mainly with methoxyls of lignins and amino acids (Table 2).

Table 2. Carbon distribution over chemical shift regions (ppm) in solution ^{13}C NMR spectra of HA of soils under shelterbelts of different ages and adjoining arable fields (%).

HA samples	0–45 alkyl	45–65 O,N-alkyl	65–108 O-alkyl	108–165 aromatic	165–200 carboxyl	HB ^a	HI ^b	HB/HI ^c
HA-1 young (14-year-old shelterbelt)	18	25	26	21	10	39	61	0.64
HA-2 young (field adjoining 14-year-old shelterbelt)	18	22	25	23	12	40	60	0.67
HA-1 old (200-year-old shelterbelt)	20	18	21	26	15	46	54	0.85
HA-2 old (field adjoining 200-year-old shelterbelt)	17	22	25	23	13	40	60	0.67

Notes: HB^a, per cent of hydrophobic carbon atoms; HI^b, per cent of hydrophilic carbon atoms; HB/HI^c, degree of hydrophobicity.

By contrast, the HA from the soil under the old shelterbelt (HA-1 old) is richer in aromatic C, alkyl C and carboxyl C, but contains lower amounts of hetero-alkyl C compared with those of adjoining cultivated soil (Table 2).

The amount and composition of plant litter were shown to be essential controlling factors for the formation of SOM and humification processes in terrestrial ecosystems [30,31]. The chemical composition of the primary resources control decomposition and humification by changing the turnover rates. Resources rich in phenols, waxes and lignins are known to decompose more slowly and contribute markedly to the stable SOM pool in comparison with proteins and sugars. The residues of young plants decompose more rapidly than those of older plants, the latter often containing more stable cell-wall compounds [30]. In soils with high total N and low C/N ratio aromaticity of HA tended to be higher [32].

The input of organic matter with crop residues for arable soils is usually lower than litter input for forest soils [31,33]. Conversion from arable cropping to a newly forested soil (under a young shelterbelt) leads to an increase in fresh litter input followed by increasing microbiological activity. The organic compounds released in the soil during mineralisation of the fresh residues of young plants are involved in the process of humification with the formation of the young immature humic molecules enriched with carbohydrate and peptide structural units [27]. Because of that, the HA from the soil under the young shelterbelt has a lower percentage of aromatic C and higher proportions of O,N-substituted aliphatic C, i.e. has a lower degree of humification and is younger than the HA of the corresponding cultivated field.

Advanced stages of humification in the soil under the old shelterbelt are characterised by a higher aromatic carbon content [32]. As shown in studies of forest soil profiles and litter bag experiments [34], the relative amount of alkyl C increases during biodegradation and humification, whereas the amount of O-alkyl carbon shows a relative decrease. This is associated with loss of the most easily metabolisable carbohydrates and amino acids, and an accumulation of alkyl C in such recalcitrant biopolymers as cutin and suberin. Strong microbial utilisation of HA from different soils, including a forest soil, has already been established [35,36]. Aliphatic structural units in HA, mainly carbohydrates and peptides, are preferentially utilised by microbes, and the remaining HA contains more condensed aromatic structures [36]. The DOC content, and thereby the microbiological activity in the soil under the old shelterbelt are much higher than in all other soils (Table 1), which may be the reason for the highest aromaticity of the corresponding HA. Old trees of *Robinia pseudoacacia* and a significant total N content in the soil may contribute to the aromaticity of HA-1 old. The increase in carboxyl C may be related to the side-chain oxidation of plant-derived lignin-phenolic compounds and/or incorporation of carbonyl-rich material from fresh vegetal tissues [32,37]. Therefore, the HA from the soil under the old shelterbelt can be regarded as the most humified and mature HA among all the HA under study.

These findings are in line with the results of previous researches [27] which showed that HA isolated from soil under 9-year-old shelterbelt with 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ at pH 7 was less chemically mature and younger than HA from the adjoining arable field, whereas HA from the soil under 160-year-old shelterbelt had a higher degree of humification and was more chemically mature than a corresponding arable field. This indicates that the processes of humification with the formation of young immature humic molecules in the soil under the young shelterbelt are more intensive than in the soil of an adjoining arable field and in the soil under the old shelterbelt.

The percentage of hydrophobic carbons HB and the degree of hydrophobicity HB/HI calculated from the ^{13}C NMR data are highest for the HA from the old shelterbelt, and lowest for the HA from the soil under the young shelterbelt. For the HA from the both cultivated soils, the degrees of hydrophobicity were found to be similar and a little higher than the value for the young shelterbelt (Table 2).

3.3. Hydrophobic interaction chromatography of humic acids

Hydrophobic–hydrophilic properties of HA depend on their chemical composition, structure and conformation [17–19,38–41]. The most chemically mature peat HA fraction was shown to contain the highest proportion of hydrophilic components, whereas a less chemically mature HA fraction contained the largest amount of the most hydrophobic compounds. However, peat FA were almost completely represented by hydrophilic components. The most hydrophilic components of peat HA obtained by the HIC fractionation were characterised by the largest amount of carboxylic groups and the lowest proportion of saturated hydrocarbon chains. By contrast, the most hydrophobic fraction of peat HA contained the largest amount of saturated aliphatic structures and the least percentage of carboxylic groups [17]. The author [40] found that the hydrophobic fraction of HA isolated from a southern chernozem using HIC contained an appreciable amount of benzoid structures, as well as short and ramificated aliphatic chains. The hydrophilic HA fraction was rich in carbohydrate fragments, carbonylic groups and aliphatic chains. The humification processes in forest soils and in incubated plant residues were shown to be connected with an increase in hydrophilic fractions and a decrease in hydrophobic fractions of HA [38,42].

Hydrophobic interaction chromatograms of the HA under study are shown in Figure 1, and the relative contents of their chromatographic fractions with different hydrophobic and hydrophilic properties are given in Table 3.

All the HA samples were fractionated on five fractions differing in their capacity to react with hydrophobic gel, as shown for HA from peat, sapropel and brown coal [17–19]. Each eluent eluted

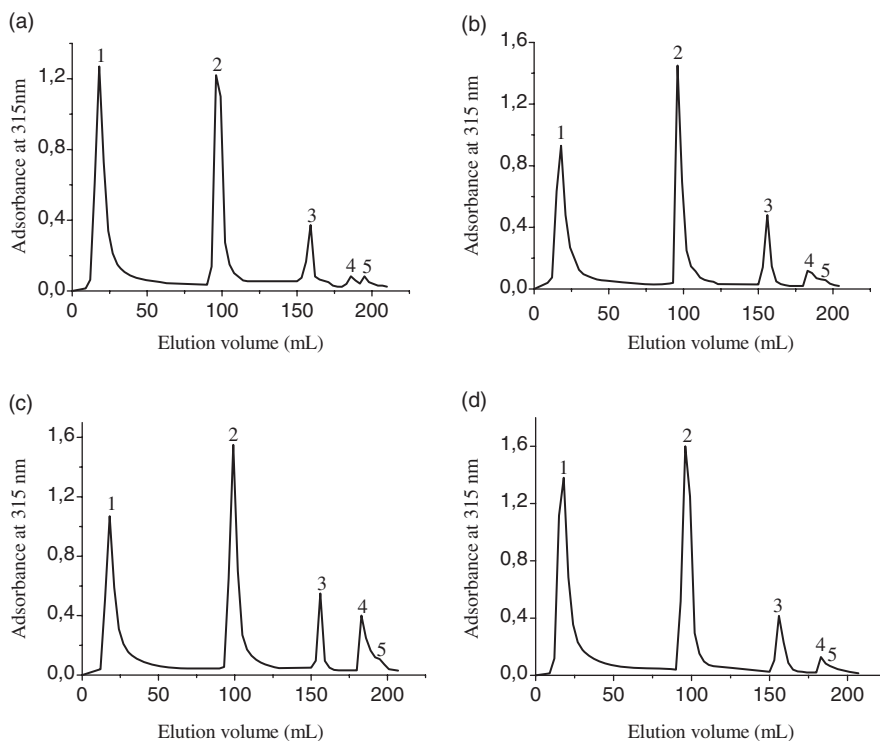


Figure 1. Chromatography of hydrophobic interaction of HA-1 young (a), HA-2 young (b), HA-1 old (c), HA-2 old (d) on an octyl-Sepharose 4 fast flow column (13 × 1.6 cm) using step-by-step elution with: 0.05 M Tris–HCl buffer at pH 8.0 containing 3 M NaCl (peak 1); 0.05 M Tris–HCl buffer at pH 8.0 (peak 2); distilled water (peak 3); and 0.1 M NaOH (peaks 4 and 5).

Table 3. Relative contents of chromatographic fractions obtained by HIC of HA from soils under shelterbelts of different ages and adjoining cultivated fields on an octyl–Sephacryl 4 Fast Flow column (13 × 1.6 cm) using step-by-step elution with: 0.05 M Tris–HCl buffer at pH 8.0 containing 3 M NaCl (fraction 1); 0.05 M Tris–HCl buffer at pH 8.0 (fraction 2); distilled water (fraction 3); and 0.1 M NaOH (fractions 4 and 5) (% of the total area of the HA chromatograms).

HA samples	Fractions				
	1	2	3	4	5
HA-1 young (14-year-old shelterbelt)	48	41	8	2	1
HA-2 young (field adjoining 14-year-old shelterbelt)	44	39	11	3	2
HA-1 old (200-year-old shelterbelt)	34	43	11	9	3
HA-2 old (field adjoining 200-year-old shelterbelt)	44	44	9	2	1

one fraction, except for the 0.1 M NaOH solution which eluted two fractions. The applied sequence of eluents reduces the strength of hydrophobic interactions between the hydrophobic sites of the HA and the hydrophobic ligands on the gel matrix [16]. This leads to a gradual increase in the hydrophobicity of the HA fractions from fraction 1 to fraction 5. The most hydrophilic fraction 1 of the HA samples did not adsorb on the hydrophobic gel and was eluted with a starting buffer. It comprised from 34 to 48% of the total areas of the HA chromatograms. The proportion of more hydrophobic fraction 2 ranged from 39 to 44%. The relative contents of the most hydrophobic components (fractions 3 + 4 + 5) ranged from 11 to 23%.

HA-1 young from the soil under the young shelterbelt is characterised by the largest proportion of the most hydrophilic fraction 1 (48%), and the lowest content of the most hydrophobic fractions 3 + 4 + 5 (11%). For HA-2 young from the soil of adjoining arable field, the content of the most hydrophilic fraction is 8.3% lower, and the percentage of the most hydrophobic components 45.5% higher than the former (Table 3). By contrast, HA-1 old from the soil under the old shelterbelt is characterised by the lowest amount of the most hydrophilic fraction 1 (34%), and twice the proportion of the most hydrophobic components, compared with that of the corresponding cultivated soil (Table 3).

Thus, fractionation of HA under study by HIC allowed us to separate polydisperse and heterogeneous systems of HA on humic components with different hydrophobic–hydrophilic properties. Our data showed that HA with different chemical composition and structure had different amphiphilic properties. HA-1 young from the soil under the young shelterbelt showed the lowest amount of hydrophobic aromatic C and the highest percentage of hydrophilic hetero-alkyl C, in particular O,N-alkyl C (Table 2). In accordance with this, the amount of the most hydrophilic fraction was the highest, whereas the proportion of hydrophobic components was the lowest (Table 3). The increased percentage of the hydrophilic fraction in the HA from the soil under the young shelterbelt is accounted for by incorporation of the organic compounds released in this soil during mineralisation of additional fresh plant residues [27].

For HA-1 old from the soil under the old shelterbelt, the opposite was observed: this HA was richest in aromatic C and alkyl C, which can be regarded as hydrophobic carbon atoms, but contained the least amounts of hydrophilic hetero-alkyl C atoms (Table 2). So, despite the highest proportion of hydrophilic carboxyl carbons, this HA was characterised by the lowest amount of the most hydrophilic fraction 1 and the highest percentage of the most hydrophobic components (Table 3). This result is understandable, taking into account the high microbiological activity in this soil (the highest DOC content) which results in strong microbial utilisation of the mainly hydrophilic fractions in HA-1 old and its enrichment with hydrophobic compounds.

Consequently, the hydrophobic and hydrophilic properties of the HA under study, determined using the HIC method, are in line with the ¹³C NMR spectroscopy data. In light of these results, HA extracted from the soil under the young shelterbelt contains the highest percentage of the

most hydrophilic fraction and is more hydrophilic than HA from the soil of adjoining cultivated field. By contrast, HA isolated from the soil under the old shelterbelt is characterised by the lowest proportion of hydrophilic compounds and is considerably more hydrophobic (and therefore, more stable) than HA from the cultivated field and from the soil under the young shelterbelt.

4. Conclusions

The age of shelterbelts was shown to be the principal factor affecting the composition and amphiphilic properties of HA. HA from the soil under the 200-year-old shelterbelt (old) was found to be richer in aromatic, alkyl and carboxyl carbon atoms, but contained lower amounts of hetero-alkyl C compared with that of adjoining cultivated soil, and, hence, can be regarded as a more humified and chemically mature HA. The number of hydrophobic carbon atoms and the degree of hydrophobicity calculated from the ^{13}C NMR data were also higher for the HA from the old shelterbelt. The HA extracted from the soil under the 14-year-old shelterbelt (young) was characterised by a lower amount of aromatic C, and a higher percentage of O,N-alkyl C, predominantly in carbohydrate and polypeptide structures compared with that from adjoining arable field, i.e. had a lower degree of humification and chemical maturity, and was younger. Correspondingly, HA from the soil under the young shelterbelt had a lower proportion of hydrophobic carbon atoms and less hydrophobicity. The hydrophobic–hydrophilic properties of HA, determined using the HIC method, were shown to be in line with results calculated from ^{13}C NMR spectroscopy. HA from the soil under the old shelterbelt was characterised by a significantly lower amount of the most hydrophilic fraction and twice the proportion of the most hydrophobic components, compared with the HA of the adjoining cultivated field, i.e. was more stable. By contrast, for HA from the soil under the young shelterbelt, the amount of the most hydrophilic components was higher, and the percentage of the most hydrophobic fractions lower, compared with HA from the soil of the corresponding cultivated field.

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