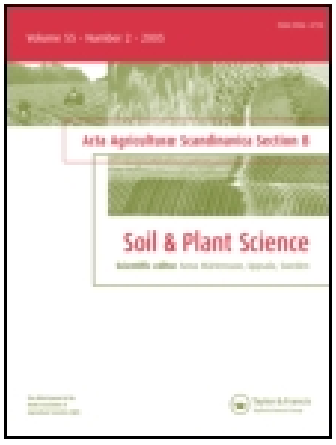

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Fate of humic acids isolated from natural humic substances

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ORIGINAL ARTICLE

Fate of humic acids isolated from natural humic substances

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Composition of humic acids (HA) is a function of plant-derived inputs, degradation processes regulated by microorganisms, organo-mineral interactions and age. Characterization of different origin humic substances is important for evaluation of their contribution to stabile and labile carbon pool in the environment. The relative abundance of chemical components in HA isolated from soils, compost, commercial lignohumates, alginite, acadiane and lignite was studied with aim to quantify content of important biomarkers such as amino acid, lipids and polyphenols. HA were considered as a heterogeneous complex and high concentration of peptides, polyphenols and lipids was determined in acadian-HA to compare with soil-HA. Compost-HA contained much more amino acids to compare with soil-HA samples. Alginite-HA and lignite-HA were similar in biomarkers content to soil-HA. Fourier transform infrared spectroscopy confirmed that chemical composition and functional groups content differs with the origin, humification degree and the age of studied samples. Soil-HA are typically composed of a variety of –OH, COOH–, C–O, C–H_2, (aliphatic and aromatic) groups, quinines, lignin fragments, polysaccharide, monosaccharide and proteins fragments, which are linked together by –O–, –NH–, –H=, >C=O, metal ions and –S– groups. ^{13}C NMR spectroscopy showed that aromatic carbon content was the highest in lignite-HA and soil-HA.

Keywords: natural humic substances; biomarkers; FTIR and ^{13}C NMR spectroscopy

Introduction

Humic substances (HS) are major class of naturally occurring organic compounds. They act as a soil stabilizer, as a nutrient and water reservoir for plant, as a sorbent of toxic metals and organic pollutants and as chemical buffers with catalytic activity. There are many observations suggesting the structure of natural HS as the association of small components to form aggregates in aqueous solution with macromolecular-like properties (Stevenson 1982; Piccolo 2002; Simpson 2002; Tan 2003). Lately it was showed that they exhibit molecular size heterogeneity and have no clearly defined structure. Their precursors were polysaccharides, amino acids, lignin, peptides and lipids.

Recent studies showed that considerable portion of organic carbon in soils is of aliphatic nature. Three main sources of plant-derived aliphatic compounds have been distinguished in soils – free (extractable) lipids, biopolyesters cutin and suberin and non-hydrolysable biopolymers such as cutan and suberan (Tegellar et al. 1995). Monreal et al. (2010) identified following classes of compounds: carbohydrates,

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phenols, lignin monomers, lignin dimmers, lipids (alkanes, alkenes, n-alkyl esters), alkyl-aromatics, N compounds (heterocyclic-N), peptides, sterols and suberin in cultivated Black Chernozem. Mentioned components directly affect recalcitration processes and are supposed to be biologically important biomarkers.

Amino acids directly affect the dynamic of carbon pool. Their origin is proteins and amino acids derived from plants, animals and microorganisms which are transformed and stabilized in the humic substances. In soils, the ratio of nitrogen in hydrolysable protein to the total soil nitrogen (N_t) remains almost constant despite increases or decreases in soil organic matter (SOM) due to different management practices, e.g. manuring and fertilization (Christensen 1996). Most of the nitrogen in soils is organic (>90%) and was found in amino acids, releasable by acid hydrolysis (Stevenson 1982). An increase in the return of organic nitrogen residues (e.g. legumes in the crop rotation) can raise the amount of hydrolysable amino compounds in soils. Conversion of inorganic nitrogen to amino acids, aliphatic amines, polyamines, pyrimidines and purines during photosynthesis is the major source of organic amino compounds. Also the assimilation of inorganic nitrogen by bacteria can be also significant (Kirchman et al. 1991). Amino acids represent about 10 ± 25% of total organic carbon and 30 ± 50% of total nitrogen also in sediments (Cowie & Hedges 1994; Colombo et al. 1998).

Lipid fraction as quoted de Blass et al. (2013) consists of n-alkanes and aromatic hydrocarbons and is supposed to be less efficiently biodegraded than medium chain fatty acids and alcohols. The polarity of aqueous soil solution in which they are contained, leading to self-assemblage phenomena into micelles association reflected into their structure. Wiesenberg et al. (2012) studied recalcitrant effect of lipids, after entering the soil. They came to the conclusion that their composition can be altered mostly by microbial degradation process or by re-synthesis of microbial biomass. Also close relationship between lipids content and soil hydrophobicity and water repellence is known. Lipids association with HS caused their high resilience to biodegradation. In addition lipids, predominantly of bacterial origin are believed to be present as trapped molecules in macromolecular networks (de Blas et al. 2013).

Lignin is the most abundant polymeric aromatic organic substance in environment. Knicker et al. (2008) demonstrated that the lignin backbone can survive charring of plants. Many authors have demonstrated that compositional changes in relation to specific vegetation and soil types, as well as organic carbon preservation/degradation with different organo-mineral associations. Recent studies also suggested, that although, the chemical finger print of natural HS is responsive to its associated vegetation cover, there may be broad commonalities (Grandy & Neff 2008; Buurman et al. 2009; Stewart et al. 2011). Characterization of lignin in fresh, degraded woods and coalified woods was given by Hatcher et al. (1981) and McKinney et al. (1996) the highly aliphatic and resistant biopolymer cutan was determined.

Results of later studies also showed that the transfer of organic carbon may occur between soluble organic carbon (fast carbon pool) and insoluble HS (slow carbon pool). The identification and application of specific biomarkers in soils and sediments is largely based on stability of slow carbon pool in the environment (Brady & Weil, 1999). However, to which extend the biodegradability effects the stability of biomarkers is not well understood yet. Just as well, fate of those constituents formed during humification and assessment of the best analytical method are unknown. The last is given by difficulties with HS chemical extraction, which is tedious and labour intensive and not suitable for large numbers of samples. On the other hand wide range of organic raw materials (coal, oxyhumolite, lignite, brown coal and peat) is potentially suitable for producing humic materials. In spite of their not clear identified composition they are used in soil remediation, as a source of nutrients, and a soil conditioner. They have the advantages of improving soil structure, increasing SOM and enhancing plant growth. Many of them are also available for application in biotechnologies, in human and veterinary medicine and as commercial standards for analytical chemistry.

The aim of our work was to elucidate the chemical composition and character of different origin natural HS. An initial baseline assessment of components across the natural HS helps us to gain more information, which is important for their comparison and evaluation.

Materials and methods

Origin of HS

Studied natural HS were obtained from five soils, compost, alginite and lignite and from artificially produced commercial products – acadian, lignohumates.

Acadian marine plant extract powder (commercial product) marked as SWE Acadian, was obtained from the producer. It derives from select species of marine plants *Ascophyllum nodosum*. Acadian acts as a soil conditioner and is unique when compared to every other seaweed-based product. First of all it

contains a wide range of naturally occurring plant nutrients and trace minerals essential to plant growth, health and productivity. The second it is naturally chelated and therefore is more readily available for plant uptake. The third it stimulates microbial activity and increased HS content in soil. It is an excellent source of organic matter composed primarily of carbohydrates such as alginic acid and mannitol, which contribute to building soil structure, aggregability and quality (Crouch & van Staden 1992; Craigie 2011).

Alginite sample was achieved from Slovakia (locality Lučenec). This is a component of some types of kerogen with a predominance of type II alongside amorphous organic matter. It originates from the biomass of fossilized unicellular algae during several millions of years in volcanic craters (the green algae species *Botryococcus braunii,* order *Chlorococcales*, class *Chlorophyceae*). According to Vass (2003) it contains about 5–50% of organic material, has moderate alkalinity with especially good effects on acidic soils. In addition, the mineral contains up to 22% calcium carbonate and fossil organic matter (up to 19%), mineral substance (at least 81% of this is made up of sand (6%), silt (40%), clay (54%, included 50% smectite, 40% illite and 10% vermiculite/chlorite). Gömöryova et al. (2009) reported also about its positive effect on to soil quality, water retention capacity, nutrient content, microbial activity and colloids content, protect soils against acidification, desiccation and leakage of nutrients.

Lignite sample was obtained from Mikulčice (Czech Republic). It is between coal and peat. According to Madronova (2011) it is the lowest rank of coal, which contains 25–35% of carbon, high inherent moisture (66%) and an ash (from 6% to 19%). Due to its complex composition and inherent heterogeneity lignite possesses non-negligible activity in the natural state. It is used as a sorbent, composite of lignite-biopolymers and polymers, or in nano technologies for cost-effective isolation of humic acids (HA). The sorption properties after certain mechanical and thermal treatment are similar to charcoal. Pekař et al. (2009) and Madronova (2011) showed its high affinity to both organic and inorganic substances. Lignite works as a buffer forcing final pH value to about 5–6 and therefore finds wide application in a variety of soil treatment and environment care technologies.

Lignohumates – commercial product obtained from the producer. It is made of lignin-containing raw material (lignosulfonate, product of wood processing). The final product contains both HA and fulvic acids (FA). Product represents a highly efficient and practically feasible humic (without carrier) fertilizer with microelements in the form of chelates. Besides high-molecular fractions typical of many industrial analogues lignohumates also contain a number of low-molecular humic component salts. Product is not toxic, not cancerogenic, not mutagenic. Residual humates are not traced in plants, as they are quickly included into the metabolism.

Compost was obtained from long-term field experiment (2008–2012) at locality Náměšť n/Oslavou (Czech Republic). It was made from leaves, grass and some portion of soil. Composted products were evaluated every year according to composition degree, maturity and stability of the final products.

Soil-HA were isolated from five different soil (*Haplic Chernozem*, *Luvi-haplic Chernozem*, *Haplic Luvisol and Haplic Cambisol*). All of them are under intensive agriculture and are studied since 2008. Soil HS like others natural HS are not defined in terms of their chemical composition or functional groups. According to Stevenson (1982) they are classified into three major groups according to their solubility. That means: HA, FA and humins. They differ in molecular weight, elemental composition, acidity and cation exchange capacity. Determination and evaluation of HA and FA content and quality was one of the main aim of this study.

Characterization of the HA

The standard International Humic Substances Society (IHSS) extraction method was applied for HA isolation from soils, alginite, acadian and compost as follows: 100 g of air-dried soil sample, sieved at mesh size of 1mm, washed by 10% HCl and stirred for 1–2 hours (decalcination process). After negative reaction for CO_2 (detected by seeing no bubbles) the soil rest washed by 0.05 M HCl. After negative reaction for Ca^{2+} (detected by ammonium oxalate) the soil rest washed by distilled water. After negative reaction for Cl^- (detected by $AgNO_3$), the soil rest was shaken in a 0.1 M NaOH for 7–8 hours. We allowed it to precipitate overnight and then centrifuge 15 minutes at 5000 rpm. Elution with 0.1M NaOH and centrifugation we followed two times and mixed supernatant solutions. Dark-brown solution of HS mixture is precipitated by concentrated HCl to pH = 1. The coagulated HA were decanted, washed several times, extensively purified by 0.5% mixture HCl + HF and dialyzed against distilled water until chloride-free, and freeze-dried (Hayes 1996; Pospíšilová et al. 2011).

Isolation of HA from lignite and lignohumates followed the procedures motivated by the Czech standard CSN 441347 on determination of HS in coal (Hubáček et al. 1962; Stevenson 1982). Commercial lignohumate samples were obtained as a black powder from the producer. Original material

was shaken for 24 hours under nitrogen atmosphere in 0.5 M-NaOH and 0.1 M-$Na_4P_2O_7$ (60 g lignite: 2000 ml of extraction agents) in plastic flasks overnight. HA were precipitated from alkaline extract by adding 6M HCl until pH 2 and treated with a 0.5% (v/v) HCl-HF solution for 24 hours, dialyzed (Spectraphore 3, 3500 Mw cut-off) against distilled water until chloride-free and freeze-dried. Isolation was kindly made at Technical University in Brno.

Fractional composition of HS was determined by short fractionation method (Kononova & Bělčiková 1963; Pospíšilová et al. 2011) as follows: 5 g of air-dried soil sample, sieved at mesh size of 1 mm and extracted by a mixture (1:1, 0.1M NaOH + 0.1M $Na_4P_2O_7$) for 24 h. The sediment was separated by centrifugation at 2800*g* for 10 min, washed with mixture and centrifuge again. Two individual washings were unified with original supernatant, acidified with concentrated H_2SO_4 to pH 1.5. We allowed to precipitate HA overnight. Sum of HS, HA and FA were determined by titrimetric method in aliquot volumes.

Elemental analysis of different origin HA was made by elementary analyser PE2400 CHNS/O. UV-VIS spectroscopy was performed by Varian Cary 50 Probe with optical fibre, within the range 300–700 nm. The FTIR spectra were recorded over the range of 4000–400 cm^{-1} in KBr pellets obtained by pressing under reduced pressure a mixture of 1 mg of samples and 400 mg of dried KBr, spectrometry grade. A Nicolet Impact 400 FTIR spectrophotometer operating with a peak resolution of 4 cm^{-1}, and 128 scans were performed on each acquisition. Nicolet Omnic software was used to obtain the spectra (MacCarthy & Rice 1985; Enev et al. 2014). Infrared spectroscopy was used to elucidate bands of aliphatic and aromatic groups and others functional groups presented in HA molecule. ^{13}C NMR spectra were carried out on spectrometer Varian INOVA 600 (frequency 150,830 MHz). As published Preston (1996), Malcolm (1990), Frùnd and Lùdemann (1991) and Beyer et al. (1993) ^{13}C NMR spectroscopy is able to characterized types of carbon and aromaticity degree of HA sample.

The content of biogenic alpha-L-amino acids (arginine, asparagine, isoleucine, lysine, serine, threonine, valine, leucine, phenylalanine, tryptophan, tyrosine, proline and alanine) was determined using ion exchange liquid chromatography with postcolumn deritatization (AAA-400, Ingos). Peptide content was determined after dialysis on cut-off filter Amicon Ultra 3K (Merk Millipore) using spectrometry detection (SPECORD 210). Profile of polyphenols, measured as a content of 10 polyphenols with significant antioxidant properties (e.g. gallic acid, procatechinic acid, *p*-aminobenzoic acid, chlorogenic acid, caffeic acid, vanillin, *p*-coumaric acid, rutin, ferulic acid and quercetrin) was determined with UV-VIS spectrometry detection (SPECORD 210) using the Folin-Ciocalteu method (Sochor et al. 2011). Content of lipids was determined using Soxhlet extraction. Data were processed using MICROSOFT EXCEL® (USA). Results are expressed as mean ± standard deviation unless noted otherwise (EXCEL®).

Results and discussion

A wide range of natural organic substances is used in agriculture to achieve the best and highest production. Managing the fertility of agricultural soils is a common challenge facing growers throughout the world. In many areas, intensive farming practices have reduced once rich and productive agricultural land into soil with poor physical structure and nutritional deficiencies. Low fertility levels in depleted soils caused limited potential for plant producing. Difficulties with identification of natural HS lead to utilization of not appropriate products and preparations, which could even worse the soil properties and fertility. Results of our investigation showed that natural HS contained the same elements, types of carbon, functional groups and biomarkers but their concentration differs. On the other hand compost-HA contained much more hydrogen, nitrogen and oxygen in their molecule, which indicates young and newly formed HA. We can conclude that elemental composition of HA isolated from natural HS is very similar but some differences could be found between lignohumates-HA, acadian-HA and compost-HA – see Table 1.

Average content of amino acids, peptides, polyphenols and lipids in mineral soils, HA isolated from mineral soils and HA isolated from other sources is listed in Figures 1–3. Mineral soils varied in biomarkers content according to soil, character of organic input and intensity of agriculture. Amino acids and peptides play the most important role in mineral soil. Haplic Cambisol contained amino acids and peptides to compare with Haplic Luvisol and Chernozems. Polyphenols content was similar in all studied mineral soils. Lipids were mostly found in Chernozems – see Figure 1. HA isolated from soils contained less amino acids to compare with compost-HA and lignohumate-HA. Content of peptides, polyphenols and lipids was similar in all soil-HA samples and lignite-HA – see Figure 2. From the Figure 3 it is evident that natural HS varied widely in biomarkers content. HA-lignohumate and HA-compost had the maximum of amino acids. Very

Table 1. Elemental composition of studied samples (atomic%).

Samples	C (%)	H (%)	N (%)	O (%)
Lignohumate	39.00 ± 0.04	42.00 ± 0.10	0.90 ± 0.01	18.50
HA-Haplic Ch.	40.60 ± 0.06	36.80 ± 0.07	2.40 ± 0.02	20.00
HA-Luvi-haplic Ch.	39.00 ± 0.04	38.00 ± 0.11	3.30 ± 0.01	19.50
HA-Haplic Luvisol	35.50 ± 0.02	40.50 ± 0.08	3.40 ± 0.01	19.60
HA-Haplic Cambisol	33.20 ± 0.06	44.00 ± 0.02	2.40 ± 0.02	20.00
HA-acadian	31.85 ± 0.04	42.10 ± 0.03	0.90 ± 0.01	25.10
HA-alginite	35.20 ± 0.02	42.96 ± 0.07	1.90 ± 0.01	19.94
HA-compost	35.50 ± 0.04	41.00 ± 0.08	2.45 ± 0.02	20.00
HA-lignite	39.00 ± 0.02	39.20 ± 0.11	7.00 ± 0.01	15.00
HA-leonardit standard	44.12	33.73	2.70	19.45
HA-Elliott soil standard	44.00	33.70	2.70	19.40

Haplic Ch., Haplic Chernozem; Luvi-haplic Ch., Luvi-haplic Chernozem; nd, not determined.

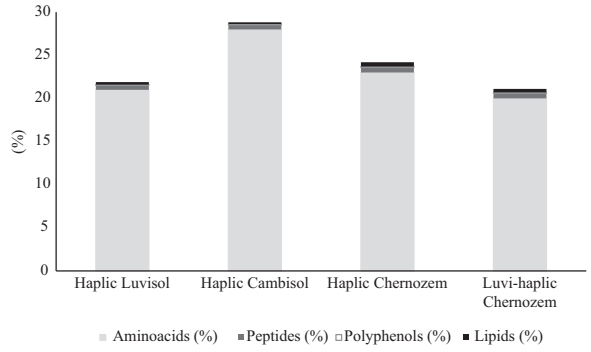

Figure 1. Average concentration of biomarkers in mineral soils.

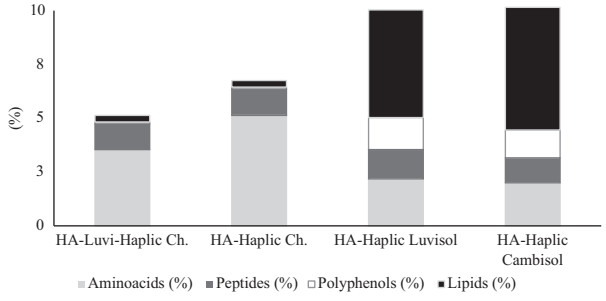

Figure 2. Average concentration of biomarkers in soil-HA.

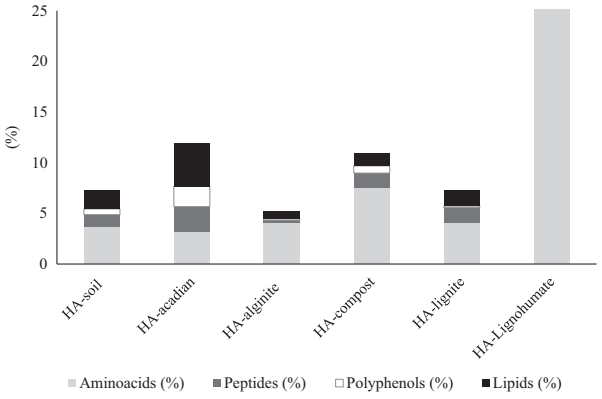

Figure 3. Comparison of biomarkers content in soil-HA and commercial-HA samples.

similar content of amino acids was determined in soil-HA, lignite-HA, alginite-HA. Acadian-HA contained more peptides, polyphenols and lipids to compare with soil-HA. Compost-HA contained much more amino acids, peptides, polyphenols and lipids to compare with soil-HA. Lignite-HA had similar biomarkers content to soil-HA – see Figure 3. Compared to others main components, lipids are more abundant in HA than in mineral soil samples. Lipids in soils could be more recalcitrant than other biopolymers. Concentration of amino acids is given in Tables 2 and 3. Hundred times more proline was determined in lignohumates to compare with lignite-HA, acadian-HA and alginite-HA. Soil-HA contained mostly serine, threonine, valine, leucine, arginine, phenylalanine, tryptophan, tyrosine, proline and lysine. Amino acids in soils mainly derived from microbial biomass and they are metabolized by both plant and microorganisms. Tryptophan and glutamic acid directly affected plant root system and high concentration caused inhibition of plant growth. High concentration of amino acids such as alanine, lysine and proline could be also connected with organic fertilizing of agricultural soils (Choi 2013). It is not clear how amino acids composition changes in time but it was found out that they contained the major fraction of labile nitrogen forms in soils (Stevenson 1982; Knicker 2000; Wiesenberg et al. 2012). We supposed that high amino acids content on one hand in lignohumates and low nitrogen content in lignohumates-HA on the other hand could be explained by solubility of hydrolysable nitrogen. So that commercial lignohumates depending on amino acids concentration could directly affect plant growth and soil microbial activity. Jones (1999) attempted to assess life time of soil proteins

Table 2. Average content of amino acids in studied samples (g/kg).

Samples	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
Haplic Ch. (mineral soil)	0.34	0.61	0.39	1.45	136.34	0.67	0.39	0.74	2.06	3.04	3.95	0.02	0.35	0.05	0.36	0.35	42.42
HA-Luvi-haplic Ch.	0.08	0.51	0.00	0.19	143.06	1.18	0.01	1.40	2.37	1.74	6.89	0.18	0.19	1.13	0.13	0.55	72.93
HA-Haplic Luvisol	0.05	1.05	1.01	0.77	157.29	1.03	0.14	0.85	1.86	2.79	5.46	0.12	0.36	0.20	0.44	0.05	37.63
HA-Haplic Cambisol	0.07	1.34	0.63	0.20	226.77	1.04	0.09	1.14	2.14	1.58	1.92	0.14	0.01	0.45	0.06	0.65	40.78
HA-Haplic Ch.	0.82	2.22	2.62	0.13	14.82	1.01	0.13	0.41	1.43	0.46	0.69	3.38	2.62	6.33	2.55	7.162	4.49
HA-acadian	0.42	0.02	1.49	3.47	0.64	0.52	1.94	0.06	1.55	0.24	2.82	0.14	0.29	4.77	1.38	11.26	0.30
HA-alginite	0.50	0.31	1.77	3.80	4.20	0.58	1.93	0.11	1.52	0.10	0.31	2.50	0.54	5.54	3.20	13.32	0.32
HA-compost	2.04	0.28	0.39	0.94	48.71	1.68	2.04	1.68	0.37	2.64	0.27	2.89	9.26	0.28	1.51	0.11	0.37
HA-lignite	0.90	2.48	2.94	0.16	4.31	5.74	0.13	0.77	1.02	0.67	0.75	3.41	2.89	0.36	3.27	8.06	1.06
HA-lignohumate	0.91	0.41	0.96	0.29	213.67	1.46	0.23	0.41	0.78	1.76	6.14	0.18	0.16	0.37	0.06	0.24	52.95
Lignohumate	0.96	1.01	0.17	0.15	36.74	4.08	1.41	0.11	0.30	1.51	0.22	2.85	5.01	0.58	1.07	0.15	0.91

Haplic Ch., Haplic Chernozem; Luvi-haplic Ch., Luvi-haplic Chernozem.

Table 3. LOD and LOQ values for amino acids determination.

Amino acids	LOD (μg/ml)	LOQ (μg/ml)	LOD (uM)	LOQ (uM)
Asp	0.1	0.3	0.7	2.4
Thr	0.1	0.4	1.0	3.2
Ser	0.1	0.3	1.0	3.3
Glu	0.1	0.3	0.6	1.9
Pro	0.8	2.8	7.3	24.3
Gly	0.0	0.1	0.6	1.9
Ala	0.1	0.2	0.7	2.4
Cys	0.2	0.5	1.3	4.2
Val	0.1	0.3	0.7	2.3
Met	0.1	0.3	0.6	2.0
Ile	0.1	0.3	0.7	2.5
Leu	0.1	0.3	0.7	2.2
Tyr	0.1	0.5	0.8	2.6

LOD, limit of detection; LOQ, limit of quantification; nd, not determined.

and peptides and came to the conclusion that radiocarbon dating would include the risk of erratic results when new proteins or peptides formed from old organic matter. Studying of selected biomarkers showed that there is a relationship between the place of origin, character of organic input and the age of natural HS.

Fourier transform infrared (FTIR) spectra of all studied samples suggested stretch vibration and deformation of the following functional groups: -SO_3 H groups at 900–1100 cm^{-1}; polysaccharides at 900–1045 cm^{-1}; various ether and alcoholic groups –O–H and C–O at 1127–1123 cm^{-1}; carbonyl and carboxylic groups at 1225–1223 cm^{-1}; phenolic groups at 1404–1419 cm^{-1}; aromatic C=C groups at 1624–1619 cm^{-1}; carboxylic and amido groups at 1655–54 cm^{-1}; C=O bands at 1690–1716 cm^{-1}; carbonyl and carboxylic groups at 1719–1718 cm^{-1}; –C–H, CH_2 and CH_3 aliphatic groups at 2942–2920 cm^{-1}; amino and amido groups –N–H at 3500–3200 cm^{-1}. Soil-HA isolated from Chernozems contained more aromatic groups to compare with others soil-HA and were similar to HA-lignite (see Figure 4). Soil-HA contained mainly: O–H groups at 3360–3350 cm^{-1}; aliphatic C–H groups at 2924–2922 and 2855 cm^{-1}; aromatic C=C groups at 1624–1619 cm^{-1}; phenolic groups at 1404–1419 cm^{-1}; asymmetric and symmetric vibration of carboxylic groups –CO– at 1583 cm^{-1} and at 1377 cm^{-1}. Our results showed that chemical composition of soil-HA was given by soil type, origin and character of organic input. Similar to us Senesi et al. (1990) and Drozd et al. (1999) showed that chemical composition, functional groups content and elemental composition of soil-HA is given by

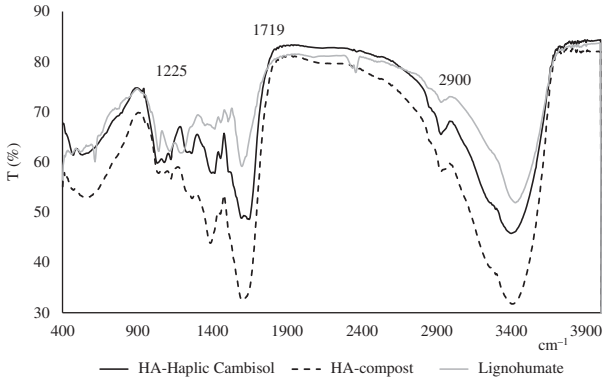

Figure 4. FTIR spectra of HA isolated from soil, compost and lignohumate.

pedogenetical processes. HA-compost showed the main differences in the finger print region at the wavelength 1700–1000 cm^{-1}; 1035 cm^{-1}; and 900–1045 cm^{-1}. They contained much more C=O carbonyl, −COO−, ketonic groups and polysaccharides in their molecule – see Figure 4. Drozd et al. (1999) quoted that during composting process content of alkyl groups −CH2− is decreasing and gradually increased content of C=O bands in carboxylic bands. So that infrared spectroscopy is a useful tool for evaluation of composting process. HA-lignite had the highest content of carboxylic groups (−COOH) at 1000–1200 cm^{-1}, polysaccharides, C–O and −OH groups at 1000–1220 cm^{-1}; C=C aromatic and C–O groups at 1620–1655 cm^{-1}; C= bands in COOH groups at 1700–1720 cm^{-1}; asymmetric carbon in $-CH_2$ groups at 2930–2850 cm^{-1}; and −OH and H− at 3300–3400 cm^{-1} – see Figure 5. Veselá et al. (2005) determined in lignite HA semichinoidal and chinoidal structures (2-methylnaftochinone) and confirmed their high reduction ability for metallic ions (Cr, Hg, Au, Pb, etc.) and Madronova (2011) published similar results. We supposed that high concentration of chinone in lignite HA come from covalent bounds of the last with metal ions and are not the results of microbial enzymatic activity. Lignohumate-HA differs in lower wavelength (finger print region) and contained more carboxylic groups at 1581 and at 1414 cm^{-1}; $-SO_3-$ groups at 1105 cm^{-1}; and $-SO_4-$ groups at 620 cm^{-1}; −OH groups in the region 2000–3700 cm^{-1}. Intensity of the last vibration in the region from 2000 to 3700 cm^{-1} confirmed that HA-lignohumate did not contain long aliphatic rings. Acadian-HA had some differences from soil-HA in the finger print region at the wavelength 1700–1000 cm^{-1}; 1035 cm^{-1}; and 500–1045 cm^{-1}. That means they are, like compost-HA, rich in C=O carbonyl; −COO−; ketonic groups and polysaccharides. Presence of carboxylic groups (−COOH) was detected at 1000–1200 cm^{-1}; polysaccharides, C–O and −OH groups at 1000–1220 cm^{-1}; C=C aromatic and C–O groups at 1620–1655 cm^{-1}; and C= bands in −COOH groups at 1700–1720 cm^{-1}. Alginite-HA differs from soil-HA mainly at the region 1000–17000 cm^{-1}. Presence of carboxylic groups (−COOH) was detected at 1000–1200 cm^{-1}; polysaccharides, C–O and −OH groups at 1000–1220 cm^{-1}; C=C aromatic and C–O groups at 1620–1655 cm^{-1}; and C= bands in COOH groups at 1700–1720 cm^{-1} – see Figures 4 and 5.

^{13}C NMR technique allows to elucidate in detail chemical structure of heterogenic organic compounds. ^{13}C NMR is significant contribution to deepening the knowledge structure of HA (Enev et al. 2014; Novák & Hrabal 2011). Quantitatively can detect different carbon types (e.g. carbonyl, carboxyl, aromatic, olephinic, anomer, aliphatic carbon) and for this reason is very useful technique to determine chemical structure in HA of different origin. The most important ^{13}C NMR parameters from the point of view of chemical structure of HA

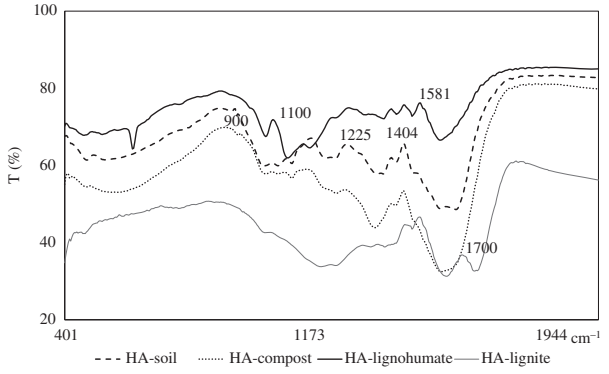

Figure 5. FTIR spectra of HA isolated from soil, compost, lignite and lignohumate.

Table 4. Integral region and types of carbon according to Malcolm (1990).

Region No	Spectral region (ppm)	Type of carbon
1	230–184	Carbonyl in keto- and aldehyde
2	184–157	Carboxyl in acids or esthers
3	157–143	Aromatic C–O
4	143–106	Aromatic and olephinic, C–C, C–H
5	106–87	Anomers
6	87–43	sp^3 carbon, C–O, C–N
7	43–15	sp^3 carbon, C–C

Table 5. The relative integral intensity in studied samples (spinning site peaks).

Sample	Integral regions			
	15–43 ppm	43–106 ppm	106–157 ppm	157–230 ppm
Soil-HA				
HA-Haplic Ch.	20	25.8	37	17.2
HA-Luvi-haplic Ch.	20	25.9	37	17.1
HA-Haplic Luvisol	26.2	18.3	36.4	18.5
HA-Haplic Cambisol	28.5	25	22.3	20.3
HA from different sources				
HA-acadian	18	31	33	15
HA-alginite	18.95	48.54	21.84	10.07
HA-compost	20	24.9	30	25
HA-lignite	22	26	39	18
HA-lignohumate	17	30	34.2	18.8

are percentage of aliphatic (Caliph) and aromatic (Car) carbons. From these parameters it can be calculate aromaticity degree (α). Studied spectral regions according to Malcolm are given in Table 4. Calculation of aromaticity degree suggested by Hatcher et al. (1981) and Schulten and Schnitzer (1995) was used. The content of different carbon types in samples depends on HA origin. Results showed that the content of aromatic carbon was decreasing in order: *lignite-HA* > *soil-HA* > *lignohumate-HA* > *acadian-HA* > *compost-HA* > *alginite-HA* (see Table 5). Maximum of aromatic carbon was identify in lignite-HA followed by soil-HA. The lowest aromatic and the highest of aliphatic carbon content were found in alginite-HA. Precursor of alginite-HA are marine organic materials with some contribution of algae and not higher plants as in soil-HA or lignite HA. It is evident that mainly alginite-HA, but also HA isolated from compost, lignohumates and acadian represent young, newly formed HA, with low aromaticity degree (about 40%). Aromaticity degree (α) was higher in HA from soils and lignite, which is connected with high stability and low tendency to oxidation (Table 6). Lawson and Stewart (1989) and Highasi et al. (1998) also confirmed that content of ^{13}C phenolic carbon (C=O) at 143–157 ppm and sp^3 carbon (C–O) at 43–87 ppm was the highest in HA-lignite. Madronova (2011) found out that the content of C–O and C=O groups increased in HA molecule with their molecular weight. Preston (1996) characterized relative signal intensity at 30 ppm as a presence of long ring –O–alkyl and $-CH_2-$ groups. Very low content of the last groups was found in lignohumates, which was also confirmed by FTIR spectroscopy. Malcolm (1990) identified at 50–60 ppm presence of aliphatic and methoxylic groups, which originated from plant biopolymers (lignin). Rossell et al. (1995), Beyer et al. (1993), Barančíková (2002) and Barančí-ková et al. (2003) showed that there are significant changes in content of functional groups in soil-HA and these changes correlated with intensive agriculture. All changes are mainly in the region 43–15 ppm and 157–143 ppm (according to ^{13}C NMR spectral analysis) and functional groups presented there are responsible for soil aggregability and nutrition

Table 6. Calculated values of Aromaticity degree (α) and carbon distribution in HA according to Hatcher et al. (1981).

Sample	C_{arom} (106–157 ppm)	C_{alif} (15–106 ppm)	sp^3 C (87–15 ppm)	α (%)
Soil-HA				
HA-Haplic Ch.	37.00	45.80	34.50	44.69
HA-Luvi-haplic Ch.	37.00	45.90	34.50	44.63
HA-Haplic Luvisol	36.40	45.50	40.30	44.44
HA-Haplic Cambisol	22.30	53.50	50.60	29.40
HA from different sources				
HA-acadian	33.00	46.00	40.00	41.77
HA-alginite	21.80	67.50	58.20	24.41
HA-compost	30.00	44.90	37.00	40.05
HA-lignite	39.00	48.00	36.00	44.82
HA-lignohumate	34.20	47.00	40.40	42.12

regime. Typical ^{13}C NMR spectra of HA isolated from different sources are listed in Figure 6–11. As it was mentioned before more aromatic carbon was determined in HA isolated from lignite and Haplic Chernozem (region 106–157 ppm) – see Figures 6 and 8. Less content of aromatic carbon (region 106–157 ppm) and more aliphatic carbon (region 15–106 ppm) was found in alginite, compost, acadian and lignohumates – see Figures 7, 9–11.

Results of elemental composition, functional groups content, carbon distribution and concentration of biomarkers (peptides, polyphenols, amino acids and lipids) confirmed that studied parameters differ with the origin, humification degree and age. Soil-HA are typically composed of a variety aliphatic and aromatic groups, quinines, lignin fragments, polysaccharide, monosaccharide and proteins fragments, which are much more stable and play an

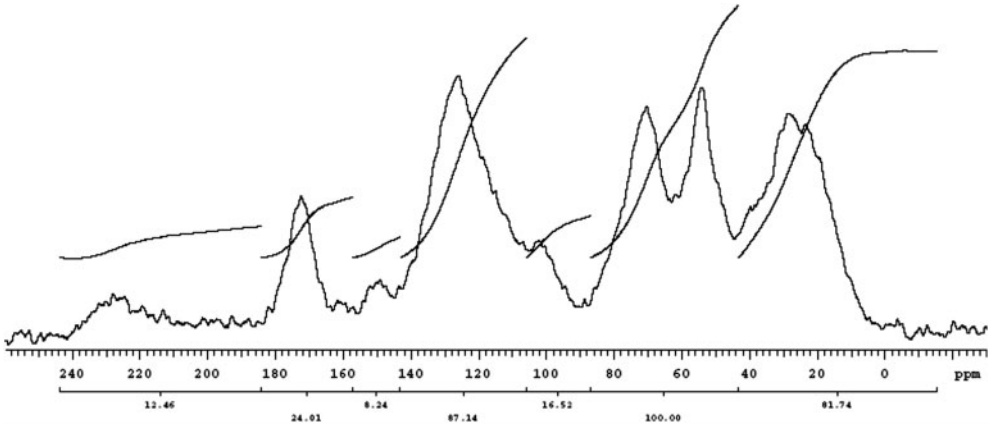

Figure 6. ^{13}C NMR spectra of HA isolated from Haplic Chernozem.

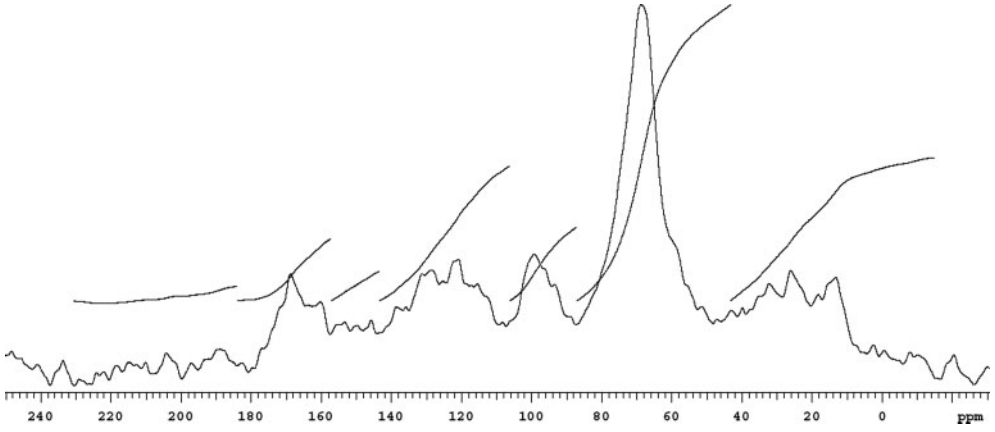

Figure 7. ^{13}C NMR spectra of HA isolated from alginate.

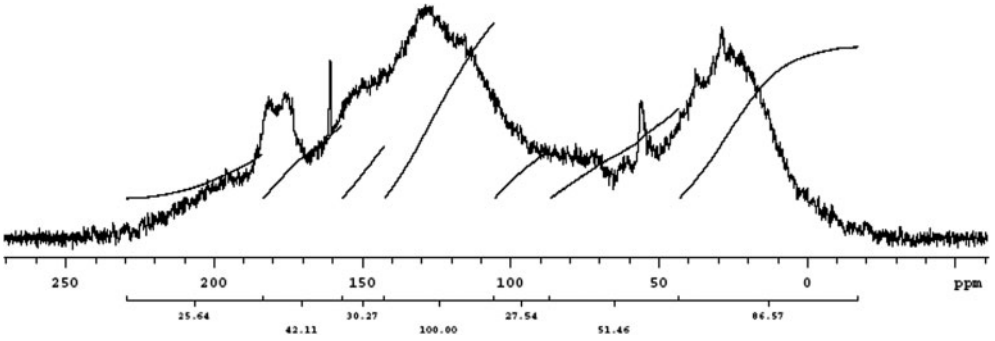

Figure 8. ^{13}C NMR spectra of HA isolated from lignite.

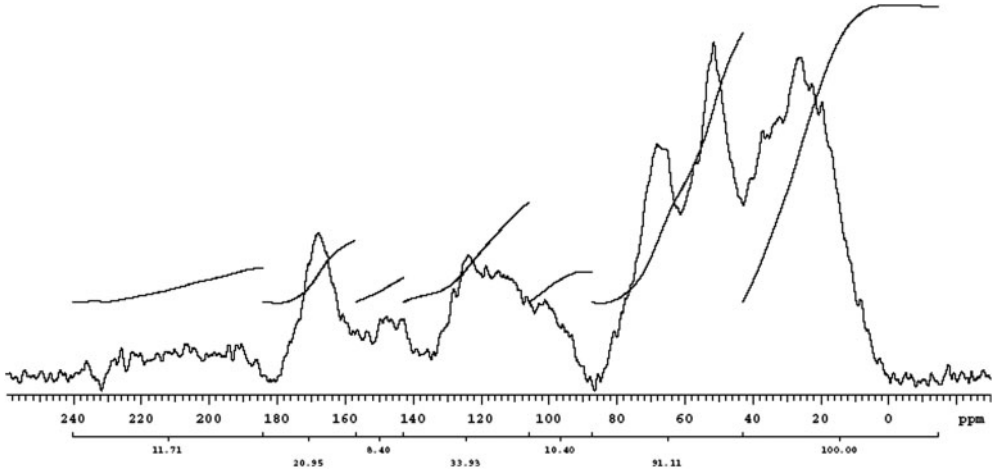

Figure 9. ^{13}C NMR spectra of HA isolated from compost.

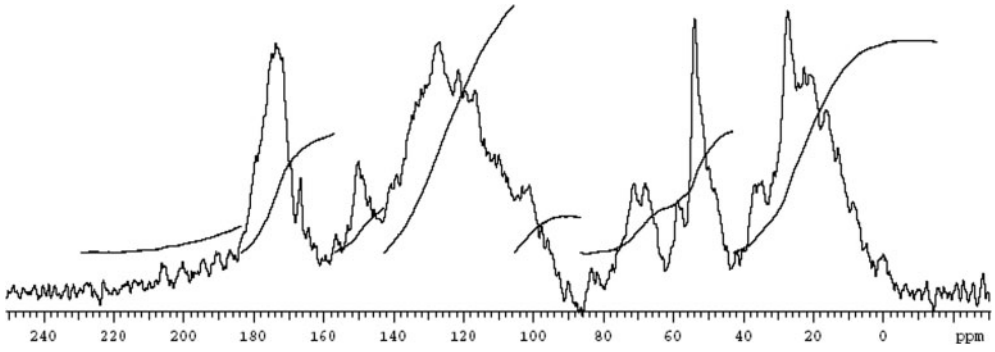

Figure 10. ^{13}C NMR spectra of HA isolated from Acadian.

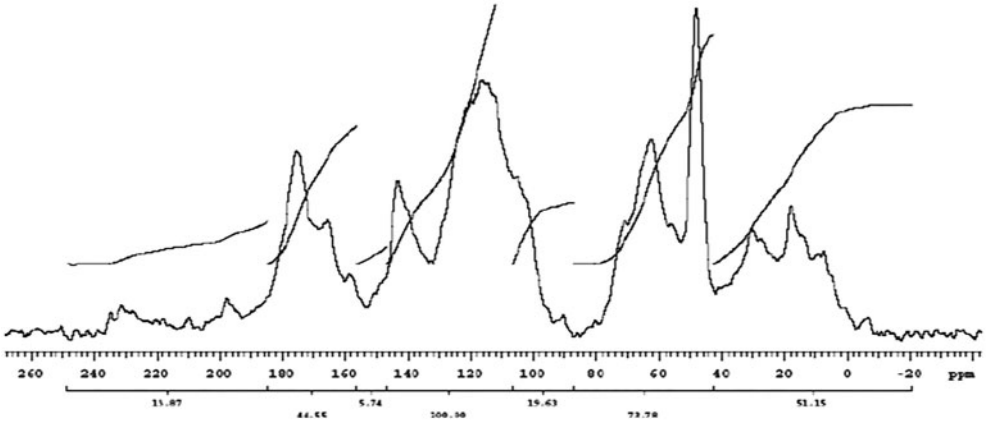

Figure 11. ^{13}C NMR spectra of HA isolated from lignohumate.

important role in stabile carbon pool of soil organic matter. On the other hand, we can easily identify HA isolated from other sources, which are usually less stable and take place in labile carbon pool of SOM.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Barančíková G, Klučáková M, Madaras M, Makovníková J, Pekař M. 2003. Chemical structure of humic acids

isolated from various soil types and lignite. Humic Subst Environ. 3:3–8.

Barančíková G. 2002. Changes of humic acids structure on selected key monitoring localities of arable soils. Soil, Plant Environ. 48:40–42.

Beyer L, Schulten HR, Frund R, Irmler U. 1993. Formation and properties of organic matter in forest soils, as revealed by its biological activity, wet chemical analysis, CP-MAS ^{13}CNMR spectroscopy and pyrolysis – field ionization mass spectrometry. Soil Biol Biochem. 25:587–596.

Brady CN, Weil RR. 1999. The nature and properties of soils. 12. ed. Upper Saddle River (NJ): Prentice Hall; 881 p.

Buurman P, Nierop KGJ, Kaal J, Senesi N. 2009. Analytical pyrolysis and thermal assisted hydrolysis and methylation of eurosoil humic acids samples – a key to their source. Geoderma. 150:10–22.

Choi J-W, Kim J-Y, Nam Y-J, Lee W-S, Han J-S. 2013. Comparison of compositional characteristics of amino acids between livestock wastewater and carcass leachate. Environ Monit Assess. 185:9413–9418.

Christensen BT. 1996. Carbon in primary and secondary organo-mineral complexes. In: Carter MR, Stewart BA, editors. Advances in soil science: structure and organic matter storage in agricultural soils. Boca Raton (FL): CRC Press; p. 99–165.

Colombo JC, Silverberg N, Gearing JN. 1998. Amino acid biogeochemistry in the Laurentian Trough: vertical fluxes and individual reactivity during early diagenesis. Org Geochem. 29:933–945.

Cowie GL, Hedges JI. 1994. Biochemical indicators of diagenetic alteration in natural organic matter mixtures. Nature. 369:304–307.

Craigie JS. 2011. Seaweed extract stimuli in plant science and agriculture. J Appl Phycol. 23:371–393.

Craigie JS, MacKinnon SL, Walter JA. 2007. Liquid seaweed extracts identified using 1H NMR profiles. J Appl Phycol. 20:215–221.

Crouch IJ, Van Staden J. 1992. Effect of seaweed concentrate on the establishment and yield of greenhouse tomato plants. J Appl Phycol. 4:291–296.

de Blas E, Almendros G, Sanz, J. 2013. Molecular characterization of lipids from extremely water-repellent pine and eucalyptus forest soils. Geoderma. 206:75–84.

Drozd J, Jeziersky A, Licznar M, Liecznar SA, Weber J. 1999. IR spectra and elemental analysis of humic acids isolated from municipal compost in different stages of maturity. Humic Subst Ecosyst. 3:11–15.

Enev V, Pospíšilová L, Klučáková M, Liptaj T, Doskočil L. 2014. Spectral characterization of selected humic substances. Soil Water Res. 9:9–17.

Frŭnd R, Lŭdemann HD. 1991. Quantitative characterization of soil organic matter and its fractionation products by solid state high resolution C-13 (CPMASS) NMR spectroscopy. Naturforsch. 46C:982–988.

Gömöryová E, Vass D, Pichler V, Gömöry D. 2009. Effect of alginite amendment on microbial activity and soil water content in forest soils. Biologia. 64:585–588. Section Botany.

Grandy AS, Neff JC. 2008. Molecular c dynamic downstream: the biochemical decomposition sequence and its impact on soil organic matter structure and function. Sci Total Environ. 404:297–307.

Hatcher PG, Schnitzer M, Dennis LW, Maciel GE. 1981. Aromaticity of humic substances in soils. Soil Sci Soc Am J. 45:1089–1094.

Hayes MBH. 1996. Chemistry of the colloids of soils, sediments, waters. Birmingham: UB; 363 p.

Highasi R, Fan MT, Lane WM, Andrew N. 1998. Association of desferrioxamine with humic substances and their interaction with cadmium (II) as studied by pyrolysis-gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. Analyst. 123:911–918.

Hubáček J, Kessler MF, Ludmila J, Tejnický B. 1962. Chemistry of coal. Praha, Bratislava: SNTL, SVTL; 469 p.

Jones DL. 1999. Amino acids biodegradation and its potentional effects on the organic nitrogen capture by plants. Soil Biol Biochem. 31:613–622.

Kirchman DL, Suzuki Y, Garside C, Ducklow HW. 1991. High potential turnover rates of dissolved organic carbon during a spring phytoplankton bloom. Nature. 352:612–614.

Knicker H. 2000. Biogenic nitrogen in soils as revealed by solid state carbon-13 and nitrogen-15 nuclear magnetic resonance spectroscopy. J Environ Qual. 29:715–723.

Knicker H, Hilscher A, González-Vila FJ, Almendros G. 2008. A new conceptual model for the structural properties of char produced during vegetation fires. Org Geochem. 39:935–939.

Kononová MM, Bělčiková NP. 1963. Soil organic matter (Organičeskoje veščestvo počvy.). Moskva. 228–234. Russian.

Lawson GJ, Stewart D. 1989. Coal humic acids. In: Hayes BHM, McCarthy P, Malcolm RL, Swift RS, editors. Humic substances II, in search of structure. Chichester: Wiley; p. 642–686.

MacCarthy P, Rice JA. 1985. Analytical methodology for elemental analysis of humic substances. In: Aiken JR, McKnight DM, Wershaw RL, McCarthy P, editors. Humic substances in soil, sediment and water: geochemistry, isolation and characterization. New York (NY): Wiley-Interscience; p. 433–455.

Madronova L. 2011. Humic acids from raw materials of the Czech Republic. New York (NY): Nova Science Publishers, Inc; 133 p.

Malcolm ML. 1990. The uniqueness of humic substances in each of soil stream and marine environments. Anal Chim Acta. 232:19–30.

McKinney DE, Bortiatinsky JM, Carson DM, Cliffort DJ, Leeuw JW, Hatcher PG. 1996. Tetrathymethylammonium hydroxide (TTAH) termochemolysis of the aliphatic biopolymers cutan: insight into the chemical structure. Org Geochem. 24:641–650.

Monreal CM, Sultan Y, Schnitzer M. 2010. Soil organic matter in nano-scale structure of a cultivated Black Chernozem. Geoderma. 159:237–242.

Novák F, Hrabal R. 2011. Kvantitativní ^{13}C NMR spektroskopie huminových látek. Chem Listy. 105:762–760. Czech.

Pekař M, Sýkorová I, Koutník I. 2009. Progressive and efficient non-energy application of lignite. Acta Res Rep. 18:11–15.

Piccolo A. 2002. The supra-molecular structure of humic substances. A novel understanding of humus chemistry and application in Soil Science. Adv Agron. 75:57–134.

Pospíšilová L, Formánek P, Kučerík J, Liptaj T, Lošák T, Martensson A. 2011. Land use effects on carbon quality and soil biological properties in Eutric

Cambisol. Acta Agric Scand, Sect B – Plant Soil Sci. 61:661–669.
Preston CM. 1996. Application of NMR to soil organic matter analysis. History and prospects. Soil Sci. 161:144–166.
Rossel RA, Zech W, Haumaie RL, Miglierina AM. 1995. Physicochemical properties of humus of a semiarid pampaean soil under two crop rotations. Arid soils Res Rehab. 9:379–387.
Schulten HR, Schnitzer M. 1995. Analysis of organic matter in soil extracts and whole soils by pyrolysis-mass spectrometry. Adv Agron. 55:168–217.
Senesi N. 1990. Molecular and quantitative aspects of the chemistry of fulvic acids and its interactions with metal ions and organic chemicals. Part II. The fluorescence spectroscopy approach. Anal Chim Acta. 232:77–106.
Simpson AJ. 2002. Determining the molecular weight, aggregation, structures and interactions of natural organic matter using diffusion ordered spectroscopy. Magn Reson Chem. 40:S72–S82.
Sochor J, Skutkova H, Babula P, Zitka O, Cernei N, Rop O, Krska B, Adam V, Provazník I, Kizek R. 2011. Mathematical evaluation of the amino acids and polyphenol content and antioxidant activities of fruits from different apricot cultivars. Molecules. 16:7428–7457.
Stevenson FJ. 1982. Humus chemistry – genesis, composition, reactions. New York (NY): Wiley Interscience; 445 p.
Stewart CE, Neff JC, Amatangelo KL Vitousek PM. 2011. Vegetation effect on soil organic matter chemistry of aggregate fractions in a Hawaiian forest. Ecosystems. 14:382–397.
Tan KH. 2003. Humic matter in soil and environment. Principles and controversies. New York (NY): Marcel Decker; p. 386.
Tegellar EW, Hollman G, Van Der Vegt P, De Leeuw JW, Holloway PJ. 1995. Chemical characterization of the periderm tissue of some angiosperm species: recognotion of an insoluble, non-hydrolyzable, aliphatic biomacromolecule (Suberan). Org Geochem. 23:239–250.
Vass D, Bublinec E, Halas L, Belacek B. 2003. Overview of Pincina alginite fertility. Foldtani Kutatas. 15:75–80.
Veselá L, Kubal M, Kozler J, Innemanová P. 2005. Structure and properties of oxihumolite types natural humic substanbces (Struktura a vlastnosti přírodních huminových látek typů oxihumolitů). Chem Listy. 99:711–717. Czech.
Wiesenberg CIB, Schneckenberger K, Schwark L, Kuzyak Y. 2012. Use of molecular rations to identify changes in fatty acid composition of Miscanthus giganteus (Greef et Deu.) plant tissue, rhizosphere and rood free soil during a laboratory experiment. Org Geochem. 46:1–11.