VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

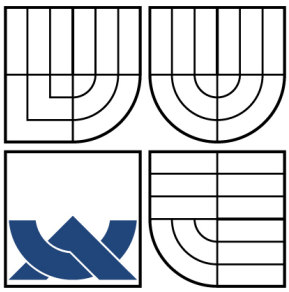
FACULTY OF CHEMISTRY
INSTITUTE OF PHYSICAL AND APPLIED CHEMISTRY

CHEMICAL AND PHYSICAL TRANSFORMATIONS OF HUMIC ACIDS

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

AUTOR PRÁCE — Ing. ZOJA VLČKOVÁ
AUTHOR

BRNO 2009



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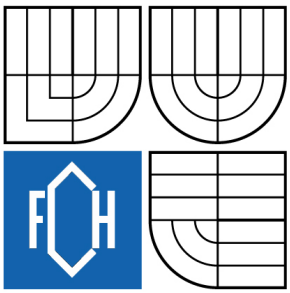

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AUTHOR

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BRNO 2009

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Doctoral thesis Assignment

Number of doctoral thesis:	**FCH-DIZ0020/2008**	Academic year:	**2008/2009**
Institute:	Institute of Physical and Applied Chemistry		
Student:	**Ing. Zoja Vlčková**		
Study programme:	Physical Chemistry (P1404)		
Study branch:	Physical Chemistry (1404V001)		
Head of doctoral thesis:	**doc. Ing. Miloslav Pekař, CSc.**		
Consultants of doctoral thesis:			

Title of doctoral thesis:

Chemical and physical transformations of humic acids

Doctoral thesis assignment:

The aim of the work was to pre-treat South Moravian lignite (a) by oxidation, (b) with short-chained organic acids, to extract respective humic acids, to characterize produced humic acids, to test their biological properties and to research the possible relationships between the structure and the biological properties.

Deadline for doctoral thesis delivery: 15.9.2009

Doctoral thesis is necessary to deliver to a secreatry of institute in three copies and in an electronic way to a head of doctoral thesis. This assignment is enclosure of doctoral thesis.

Ing. Zoja Vlčková Student	doc. Ing. Miloslav Pekař, CSc. Head of thesis	doc. Ing. Miloslav Pekař, CSc. Head of institute

In Brno, 1.9.2006

doc. Ing. Jaromír Havlica, DrSc.
Dean

SOUHRN

Tato práce představuje pilotní studii testující souvislosti mezi biologickými vlastnostmi a strukturou huminových kyselin extrahovaných z původního a modifikovaného jihomoravského lignitu, důl Mír, Mikulčice. V první části práce byly testovány metody vhodné ke zvýšení výtěžku huminových kyselin extrahovaných z lignitu. Oxidace lignitu v plynné fázi nepřinesla uspokojivé zvýšení výtěžku a byla instrumentálně poměrně náročná. Dále proto byla zkoumána jen oxidace v kapalné fázi a modifikace nízkomolekulárními organickými kyselinami. Modifikace organickými kyselinami byla inspirována procesy podporujícími biologické funkce v rizosféře, t.j. kořenový systém vylučuje exudáty způsobující změny v supramolekulové struktuře okolní organické hmoty čímž zlepšuje její mobilitu a prostupnost buněčnými stěnami. Primární struktura huminových kyselin připravených v této práci byla zkoumána prostřednictvím elementární analýzy a spektrálních metod (^{13}C CPMAS NMR, EPR a UV-VIS spektroskopie). Navzdory tomu, že primární struktura vykazovala jen malé rozdíly, měření biologické aktivity a genotoxického potenciálu prokázalo, že huminové kyseliny a jejich humáty získané z lignitu s rozdílnou předúpravou vykazují odlišnou bioaktivitu. Proto byla dále zkoumána supramolekulární struktura vzorků ve zředěných roztocích, a to prostřednictvím vysokoúčinné vylučovací chromatografie, měření ultrazvukové rychlosti a hustoty. Testovány byly dva různé protionty – draselný a amonný. Získané výsledky potvrdily předpoklad, že pozorované změny v kvalitě humátů jsou závislé na protiiontu, koncentraci humátu v roztoku a také na metodě předúpravy původního lignitu. Obě zvolené metody předúpravy lignitu prokázaly svůj potenciál produkovat huminové kyseliny s rozmanitými biologickými vlastnostmi, aplikovatelné v zemědělství, životním prostředí a potenciálně i ve farmakologii.

SUMMARY

This work is a pilot study testing the relationships between biological properties and structure of humic acids extracted from original and modified South-Moravian lignite, mine Mír, Mikulčice. In first part of the work, methods suitable for increase of the humic acid yield were explored. Lignite oxidation in gas phase turned out to be relatively instrumentally demanding and insufficiently effective. Therefore, only oxidation in liquid phase was explored further along with modification with short-chained organic acids. Modification with short-chained organic acids was inspired by processes enhancing biological functions in rhizosphere, i.e. the root system produces exudates causing changes in the supramolecular structure of the surrounding organic matter, improving its mobility and cell-walls penetration. Primary structure of humic acids produced in this work was investigated by elemental analysis, solid state NMR, EPR and UV-VIS spectroscopy. Despite the fact that only minor differences were found, conducted assessment of biological activity and genotoxic potential showed that humic acids and their respective humates isolated from lignite with different pre-treatment show different bioactivity. Therefore, supramolecular structure of samples in diluted solutions was investigated by means of HPSEC, HRUS and densitometry measurements. Two counterions – potassium and ammonium – were tested. Obtained results confirm the assumption that observed quality of humates depends on counterion, concentration of humate and also on the method of lignite pre-treatment. Both selected pre-treatment methods showed potential to produce humic acids with variable biological properties, applicable in agriculture, environmental chemistry and potencially also in pharmacology.

KEYWORDS

lignite humic acids, biological activity, genotoxicity, antimutagenicity, hydration

KLÍČOVÁ SLOVA

lignitické huminové kyseliny, biologická aktivita, genotoxicita, antimutagenicita, hydratace

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PROHLÁŠENÍ

Prohlašuji, že jsem disertační práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Disertační práce je z hlediska obsahu majetkem Fakulty Chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího disertační práce a děkana FCH VUT.

…….
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DECLARATION

I hereby declare that this doctoral thesis was done by myself. All used references were correctly and completely cited. This doctoral thesis (from a content point of view) is the property of Faculty of Chemistry, Brno University of Technology. It can be used for commercial purposes only under permission of the thesis supervisor and the dean of FCH BUT.

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signature of the author

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CONTENTS

1. INTRODUCTION

In the atmosphere of financial, economical and food crisis, whether on regional or global level, the need for maximal exploitation of existing natural resources becomes more and more prominent. This process ought not to be ill-considered, but well-judged, globally planned and controlled in the final outcome.

A precipitous kind of action is for instance the production of biofuels with good intensions to lower the emission rate. In the final aftermath, there is no massive movement in the existing carbon cycle. This may sound cynically and this problem certainly has to be addressed, but it is also necessary to put together all the facts and rethink the results which could be brought about by such an action. It is not exactly clear if or to which extent anthropogenic activity is yet capable to reverse the problem of global warming in current state. Thing is, there are some major natural phenomena, e.g. volcanic activity; and their share to this issue is rather fair-sized.

Needless to say, action of men on this planet has to be temperate and regardful to the natural environment, but the effects of some beneficial operations have to be balanced and their negative impacts on other life spheres ought to be minimized. In global scale, biofuels utilized only regionally and to small extent do not extremely improve on the climate issues. On the contrary, their production interferes very insensitively with the availability of crops, oil extracts of which are used for biodiesel enrichment. Consecutively, lack of these crops on the market is remarkably accountable for recent food crisis.

In the present situation, it is necessary to distinguish which – also natural – sources are a store of easily discharged CO_2 and give added attention to their stabilization. For it is clear, that until the supplies of fossil fuels are completely exhausted, their consumption, virtual prices and negative effects of their usage to the environment will be impossible to maintain on reasonable levels. At least under current political-geographical arrangement.

One of the biggest pools of relatively stable carbon is present in soils. The major problems of lasting soil organic matter stabilization are demanding agricultural exploitation, extensive deforestation and consecutive desertification in certain regions. One of possible solutions to the question of carbon loss in soils could be extensive application of other existing, naturally occurring materials – for example humic substances (HS). They exhibit positive influence on the carbon sequestration in soils and also on the overall biological activity and fertility of soil. By supporting transport processes in soil and possibly enhancing the photosynthesis, humic substances aid the formation of biomass and thus humus. In order to approach this systematically, it is useful to extend some areas of basic research and dedicate a study to the solutions suitable particularly for soil maintenance.

2. STATE OF THE ART

2.1 Humic substances

The term "humus" originates from the Romans, when it was familiarly used to signify the entire soil. Later the term was used to denominate soil organic matter, compost and different parts of organic matter, as well as complexes created by chemical agent treatments to a wide palette of organic substances. It was Wallerius in 1761 who defined humus in terms of decomposed organic matter.

The first relevant study of the origin and chemical nature of HS was worked out by Sprengel in 1839 [1]. His comprehensive study on the acidic nature of humic acids (HAs) is thought to be his most important benefit to humus chemistry. Research on the chemical properties of HS was extended by the Swedish researcher Berzelius, whose main contribution was the isolation of two light-yellow coloured HS from mineral water and slimy mud rich in iron oxides [2].

Despite humic substances undisputable role in sustainability of life, the basic chemical nature and the reactivity awareness of HS is still poor [3]. From the chemical point of view, humic molecules are composed of aromatic and/or aliphatic chains with specific content of functional groups. Their number and position depend on the conditions of formation. Elemental analyses data of humic samples originated from miscellaneous sources show that these HS differ in their elementary composition and reactivity. Although, there exist undisputable differences in way of genesis, humic substances from different sources should be considered members of the same class of chemical compounds [4].

Stevenson [5] stated that humus includes a broad spectrum of organic constituents, many of which have their counterparts in biological tissues. He distinguished between non-HS and HS, the former of which consists of compounds belonging to the well known classes of organic chemistry such as amino acids, carbohydrates, lipids, lignins and nucleic acids, whereas the latter are materials unspecified, transformed, dark coloured, heterogeneous, amorphous and of high molecular weight. From a geological point of view, humic substances are chemical intermediates between plants and fossils. The chemical nature of soils, sludge and sediments can subsequently continually and selectively vary via the conversion and degradation of organic matter [6].

Most frequently, HS are classified according to their solubility in different media. While fulvic acids (FAs) are soluble at any pH, humic acids represent alkali-soluble humus fragments. HAs are commonly extracted using diluted alkali. Subsequently, they are precipitated with an acid and separated from the soluble FAs. Humin represents the insoluble residue [7].

2.1.1 Formation of HS

Although the formation process of HS has been studied hard and for a long time, their formation is still the subject of long-standing and continued research. Some theories have lasted for years; for example, the “sugar-amine” condensation theory, the “lignin” theory or the “polyphenol” theory. A review of such theories can be found for example in a monograph of Davies and Ghabbour [8]. Nowadays, most investigators suppose that humic substances

originate in lignin [9], [10], [11]. Polyphenols come mostly from lignin during its biodegradation, and probably play a key role in the formation process. Polyphenols are also regarded as the main agents in the formation of humic substances from some plants that do not contain much lignin and/or from non-lignin plants. Polyphenols can be considered humic acid precursors. They themselves possess enough reactive sites to permit further transformations, for example some condensation reactions [12].

According to a recently introduced concept, humification in soil can be considered a two-step process consisting of biodegradation of dead-cells components and aggregation of the degradation products [3]. In light of the supramolecular model, one needs not to invoke the formation of new covalent bonds in the humification process that leads to the production of humus. Humification is a progressive self-association mainly of hydrophobic molecules which resist biodegradation. These suprastructures are thermodynamically separated by the water medium and adsorbed on the surfaces of soil minerals and other pre-existing humic aggregates. The exclusion from water means exclusion from microbial degradation and the long-term persistence of humic matter in soil.

2.1.2 Functional groups and reactivity

Physical and chemical properties, as well as the reactivity of humic substances reflect the abundance of individual functional groups in HS. Traditional dividing of HS according to their solubility is a logical consequence of the distribution of polar functionalities in their structure. It is a matter of fact that humins represent the less understood and studied humic material. Accordingly, the information on chemical composition of humin is rare.

Chemical structure of HAs is very complicated. Elemental analysis of different FAs and HAs shows that the major elements in their composition are C, H, O, N, and S. A variety of functional groups, including COOH, phenolic OH, enolic OH, quinone, hydroxyquinone, lactone, ether, and alcoholic OH, have been reported [5]. Except those, nitrogen, sulphur and phosphorus functional groups or bonds can be found in small amount, as well.

Major difference between the functional group content of humic and fulvic acids is that a smaller fraction of the oxygen in the former can be accounted for in COOH, OH, and C=O groups. On the other hand, the quinone C=O content of humic acids is universally higher than for fulvic acids whereas the latter seem to be richer in ketonic C=O content. Another difference is that practically all the oxygen in fulvic acids can be accounted for known functional groups (COOH, OH, C=O) whereas a high proportion of the oxygen in humic acids occurs as a structural component of the “nucleus” (e.g., in ether or ester linkages) [12]. The absolute values in literature reported in $cmole.kg^{-1}$ vary in dependence on origin of humic matter.

2.1.3 Chemical structures identified in HS

It is known that the chemical composition of humic matter includes aromatic rings that interact with each other and with aliphatic chains, giving rise to macromolecular-like aggregates with different masses. Considering that the genesis of HS can involve combinations of several reaction pathways and wide variety of chemical binding systems, it is very difficult to define a clear concept of their composition. Many of the original classical

methods to understand the nature of HS were based on elemental composition, but the results obtained represent averages for agglomerations of molecules and it is impossible to derive precise empirical formulae from these data.

Later, valuable information was gained from chemical degradation techniques (acid/base catalyzed hydrolysis, oxidative and reductive processes and thermal procedures), involving possible chemical constituents and building blocks of HS. However, because the major linkages in the "core" of HS were not hydrolysable, the energy inputs needed to cleave the links between the component molecules give rise to products that could be vastly different from the molecules that compose the macromolecules [13].

Advanced spectroscopic, pyrolytic, and soft ionization techniques were used to reveal the makeup of many of the individual fragments of humic material produced through degradation of biomolecules, and to provide information concerning their relative mobilities. There is substantial evidence that lipid-derived molecules or moieties, including branched and linear alkanes, alkenes, fatty acids, dicarboxylic acids, and long chain alcohols/ethers, ketones/aldehydes, and esters, make up a significant portion of HS. These compounds have been identified as major components of humic fractions in numerous pyrolysis studies, especially when methylation via tetramethylammonium hydroxide (TMAH) is used to preserve carboxyl groups. The distribution of alkanes and fatty acids, which favours an even number of C atoms, indicated microbial or plant origin. While fatty acids were dominant components of the lipid-like portion of the humic matter, smaller amounts of alkyl esters and alcohols/ethers were also observed.

Aromatic fragments observed within humic material may be derived from lignin or other sources. Alkylbenzenes and alkylphenols are detected regularly in the pyrolysates of HS, and can feature alkyl chain lengths of over 25 carbon atoms. However, many of these alkylaromatic molecules may be artefacts created by secondary reactions. Pyrolysis preceded by methylation indicates benzene tricarboxylic acids are common humic constituents, as does ESI-MS. Simple sugars ribose and glucose have been identified using ESI-MS spectra. These spectra also provided evidence for the existence of polypeptides of up to 10 amino acids in length.

Obviously, organic O is the primary source of reactivity in HS, as functional groups containing O provide polarity. Molar O/C ratios of HAs and FAs are typically around 0.5 and 0.7, respectively. These different ratios reflect the pH-dependent properties of many O functional groups, given the operational basis for separation of HAs and FAs [14].

Leenheer et al. [15] noted that a small but significant fraction of humic carboxyl groups display dissociation constants (pK_{aS}) as low as 0.5. Using NMR spectroscopy and methylation, chemical degradation, and pH gradient fractionation, they demonstrated that these strongly acidic structures are probably polycarboxylic acids located near ether or ester functional groups and surrounded by additional electronegative substituents.

Comparison of HS examined with thermochemolysis – MS, or thermochemical degradation and volatilization using TMAH or tetraethylammonium acetate, as well as those processed using multistep chemical degradations and examined with pyrolysis and NMR spectroscopy, indicated that alkyl molecules containing carboxyl groups exist as free acids loosely associated with other moieties, as tightly trapped molecules within the organic matrix, and as acids linked to the matrix via ester bonds.

However, processing by oxidation or acid boiling can produce structures indicating the interaction of unpaired quinone electrons with neighboring H nuclei, and resembling the

spectrum of 2,6-dimethoxy-p-benzosemiquinone. Several 2D NMR studies identify signals from unsaturated ketone groups, consistent with the presence of quinones. Low-temperature fluorescence and EPR studies of a forest soil FAs suggested that quinone rings featuring four protons were common, but that structures featuring substitutions were also present. The EPR studies of quinone moieties in HS indicate that the population of this functional group is small, in the range of $10^{16–18}$ spin g^{-1}, and may be divided into stable and transient organic radicals [14].

Kingery et al. [16] presented 2D NMR evidence of ortho- (1,2) and para- (1,4) substituted phenol groups within a soil HA sample. FAs tend to contain slightly more phenol groups than HAs, and with decreasing apparent molecular size, the proportion of phenols in humic fractions tends to increase.

In summary, modern spectroscopic studies confirm earlier data regarding the variety of oxygen functional groups contributing to the chemical reactivity of HS. Carboxyls, including unusual alkyl polycarboxyl moieties near ester or ether groups, determine the deprotonation and charge behaviour of HS under low pH conditions, while phenols and alcohols play an important buffering role within higher pH solutions. Quinones are not sufficiently numerous to account for the redox behaviour of HS. Ester and ether bonds link different portions of humic molecules. Carbohydrate-based functional groups make up a large portion of humic O groups [14].

Early chemical degradation analysis of humic materials suggested that the carbonaceous structural framework or “skeleton,” of the component molecules was primarily aromatic. The high proportion of alkyl substituents remaining in HA and humin samples after a multi-step chemical degradation process (ultrasonic disruption in methylene chloride, followed by BF_3 methanol transesterification and HI treatment) suggests that these structures are an integral part of the humic carbon skeleton [14].

2.1.4 Molecular characteristics of HS

Several hypothetic supramolecular structures, including different diagrammatic and schematic models without chemical structural formulas, have been proposed during the long history of humus chemistry to account for the chemical composition and behaviour of HS. Yet the primary structure, which refers to covalent bonding within a molecule, is still unresolved. The most frequently adopted view was that humic-like constituents in solution were polymers which will coil at high concentrations, low acidity, and high ionic strength but become linear at neutral acidity, low ionic strength, and low concentration [17]. This random coil model had been a leading concept in humus chemistry for decades. Attempts had been made to find different kinds of structural building blocks and cores for humic matter by means of various techniques. As a matter of fact, the terms polymer, polymeric and polymerization do not have their original meaning in the context of humic matter-like constituents, because, e.g., the term polymer is accurate only when the structural units are linked to each other in a regular manner and by the same kind of linkages.

This random-coiled model for humic matter-like macromolecules had been strongly criticized, e.g. by Wershaw [18], because mathematical equations used to define this model were originally derived for high-molecular-mass linear polymers. On the contrary, Wershaw et al. [18], [19] had presented an alternative schematic membrane model, much like a protein, for the supramolecular structure of humic matter. In this membrane model, humic materials

were pictured as composed of partially degraded molecular components from natural organisms (mainly from plants), which were held together in ordered, membrane-like or micelle-like, aggregated structures by weak interactions, such as hydrogen bonding and π-bonding and van der Waals and hydrophobic forces. These aggregated structures with hydrophobic interiors and hydrophilic exteriors constitute a separate phase in soil-water and sediment-water systems, thus better fitting the theory presented [20] for small organic molecules in aqueous solvents.

It has been explained that many of the most important physical-chemical properties of this humic membrane-like phase are more a function of the nature and structure of the membrane itself than of the properties of the individual molecular species of the aggregate, and alkaline solvents can partially disperse larger structures of these membrane-like aggregates into smaller micelle-like structures, because of an increased charge on membrane surfaces, thus explaining, e.g., why alkaline solvents extract humic matter from soils.

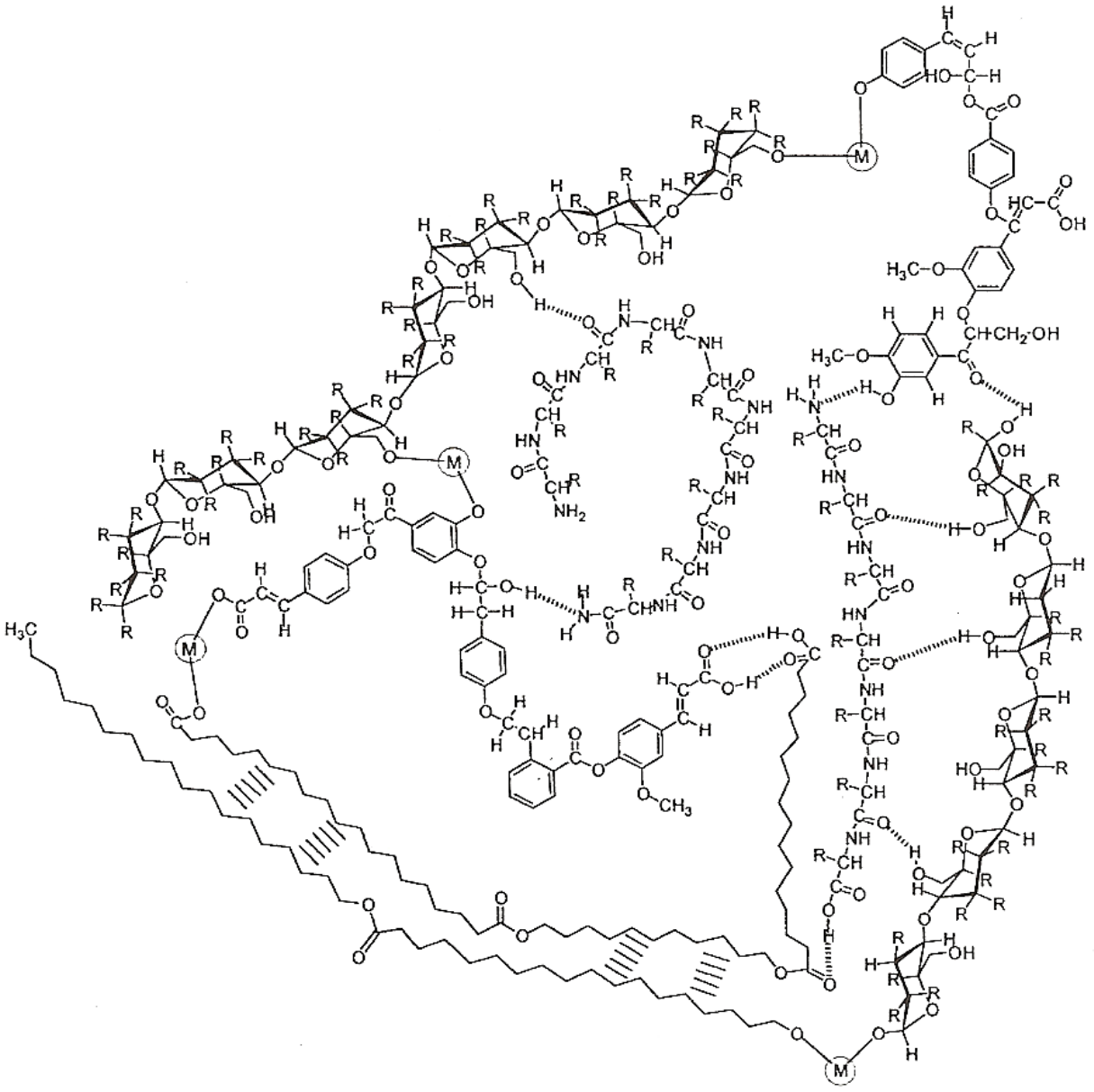

Fig. 1 Recent model structure of humic acid according to Simpson et al. in 2002 [23].

Piccolo et al. [21] have recently presented an extended theory that, instead of being stable polymers, humic constituents at neutral and alkaline acidities are supramolecular associations of relatively small heterogeneous molecules held together by weak dispersive forces such as van der Waals, π–π, CH- π interactions. This conclusion was based on the large-scale experimental data that after addition of modifiers such as natural organic acids, e.g., acetic acid, to the original humic-solute mixture, the macroscopic dimension of this supramolecular association was disrupted in smaller sized associations with reduced chemical complexity. This disruption by organic acid additions was attribute to the formation of new inter- and intramolecular hydrogen bonds which are thermodynamically more stable than the hydrophobic interactions stabilizing humic conformations at neutral acidities [22].

Results obtained later with fluorescence spectroscopy, NMR [24], thermal analysis [25] or mass spectrometry [26] supported such conclusions. Further, other researchers adopted this view [22] although they do not refuse the presence of high molecular moieties which are the rest of parental plant tissues which are protected by humic molecules from biological degradation [14].

2.1.5 Micelle-like arrangement

The mechanism of interaction between dissolved HAs and nonionic organic compounds (especially nonpolar ones) continues to be subject to some controversy. It is, however, clear that these interactions are largely predicated on the detergent character of HAs. It is generally recognized that these materials are surface active and can solubilize a wide variety of hydrophobic species. A view that is widely accepted in present time holds that this is due to a micelle-like organization of HAs in aqueous solution. This concept was introduced in fundamental form by Rochus and Sipos in 1978 [27], and was subsequently elaborated and refined by Wershaw [18], [28], who established the present mode of thinking on the subject. The essence of the theory is that the HA amphiphile consists of an elongated hydrophobic portion with one or more anionic (carboxylate) groups attached at the end. These entities aggregate in the manner of synthetic surfactants, forming micellar or membrane-like structures. It has been recognized for some time that the presence of humate in water will result in a solution which has a significantly lower surface tension than water alone. This observation has prompted speculation that HAs will form micelles in alkaline, aqueous solutions [27].

Interestingly enough, Engebretson et al. found evidence for micelle-like organization in dilute HA solutions which does not feature a critical micelle concentration [27]. In this model, the amphiphilic HA molecules are considered to aggregate both intra- and intermolecularly. The former is made possible by the chain length and flexibility of the humic molecules, which allow them to fold and coil in a manner that directs hydrophilic (e.g., carboxy and hydroxy) groups outward and keeps more hydrophobic (e.g., hydrocarbon) moieties isolated in the centre. This process, which could in principle occur with a single polymer strand, produces an entity that is operationally similar to a conventional micelle, albeit more structurally constrained. Like a micelle, it has a hydrophobic interior and a more hydrophilic surface, giving it distinct solubilizing powers for nonpolar solutes. To indicate both similarities and differences with normal surfactant micelles, these HA structures have been referred to as pseudomicelles.

Spectroscopic evidence for the existence of humic pseudomicelles has been reviewed by von Wandruszka [30]. The structures are considered to exist at low HA concentrations in aqueous solution, although certain variations in composition must be anticipated. It is, for instance, likely that intermolecular aggregation supplements intramolecular coiling in pseudomicelle formation, and that this depends on both the concentration and polydispersity of the solute. The proposed assembly thus consists of coiled humic polymer chains, interspersed with smaller HA fragments. More on colloidal and conformational properties of humic substances can be found in the work of Kučerík et al. [31].

2.1.6 Biological activity of HS

Humic substances are resistant to microbial degradation [32] and thus are not generally considered to be dynamically involved in microbial metabolism, especially in anoxic habitats. However, Lovley et al. [33] showed that some microorganisms found in soils and sediments are able to use humic substances as electron acceptors for the anaerobic oxidation of organic compounds and hydrogen. This electron transport is supposed to yield energy to support growth. Later, Scott et al. [34] demonstrated that the electron-accepting capacity (the redox potential) of HS relates directly to their free radical content. As it is known, organic radicals in HS are primarily quinone-like groups, thus their amount could be found important for the biological activity of HS. A series of studies [33], [35], [36], [37] has suggested that HS might support the carbon and electron flow under unaerobic conditions and serve as an electron shuttle.

In more recent work, Canellas et al. [38], [70] investigated the effects of HAs isolated from cattle manure earthworm compost on the earliest stages of lateral root development and on the plasma membrane H^+-ATPase activity. They successfully demonstrated that these HAs enhance the root growth of maize seedlings in conjunction with a marked proliferation of sites of lateral root emergence. According to Canellas et al., they also stimulate the plasma membrane H^+-ATPase activity, apparently associated with the ability to promote expression of this enzyme. In addition, structural analysis revealed the presence of exchangeable auxin groups in the macrostructure of the earthworm compost HA, which could lead to some explanatory conclusions about the assumed hormonal activity of HS, if researched further.

It has already been reported that humic substances can stimulate plant growth by the release of bioactive molecules with auxin-like activities. A multitude of publications by Nardi et al. [39], [40], [41], [42], [69] supported by Piccolo et al. [43] or Pizzeghelo et al. [44] have been devoted to the clarification of mechanisms resulting in bioactivity of HS. Some of their findings showed, that the effects on ion uptake in barley seedlings appear to be selective and their magnitude is related to the concentration of humic substances and to the pH of the medium. In this study, it was also shown that humic matter stimulates carrier-protein synthesis at a posttranscriptional level [40]. Nardi et al. [39], [41] also tried to determine auxin-like activity of HAs according to their size distribution. Especially the low molecular size fraction obtained by disaggregating the humic material with acetic acid, confirmed the effectiveness of the combination of high acidity and low molecular size in influencing the biological activity of the plant system tested [40].

Further results [41] showed that low molecular size fraction of tested HAs (LMS) increased nitrate uptake and strongly inhibited K^+, stimulated ATPase of maize microsomes and H^+ extrusion in a manner similar to gibberellic acid. The stimulation of nitrate uptake by

LMS can not be explained by an effect on the primary transport of solutes but it was suggested that it may act by decreasing the pH at the surface of roots, this facilitating the H^+/NO_3^- symport. The regulatory properties of the LMS appeared to depend on the combination of low molecular size, auxin-like and gibberellin-like activity and to a relevant content of phenolic and carboxyl carbon.

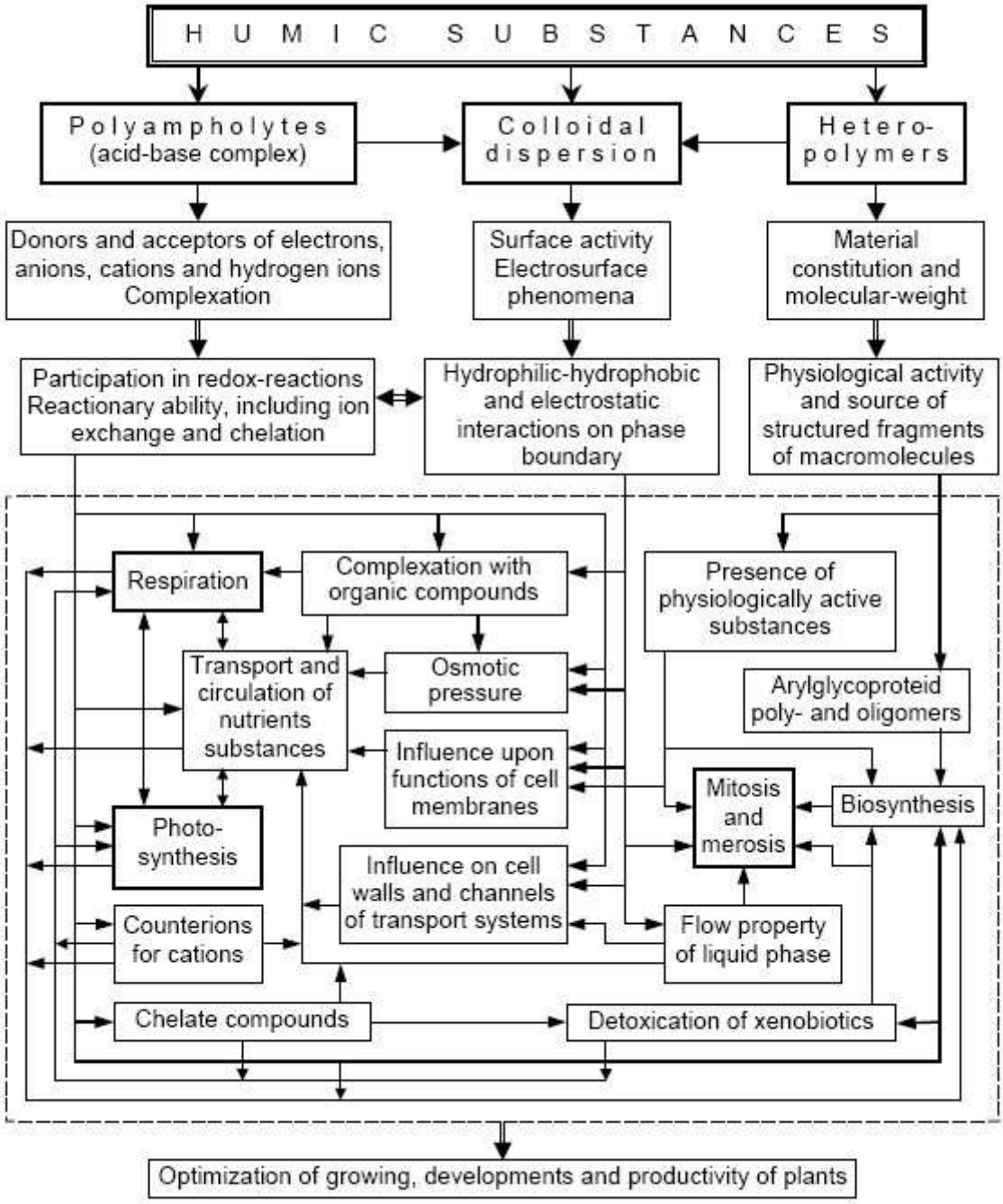

Fig. 2 Participation of humic substances in plant metabolism [45].

As stated before, according to Nardi et al. [42] the overall effect of HS on plant growth depends on the source, concentration and molecular size of humic fraction. While a LMS fraction (LMS < 3500 Da) easily reaches the plasmalemma of higher plant cells and, in part, is taken up into them, a high molecular size fraction (HMS > 3500 Da) is not absorbed and can interact only with the cell wall. Therefore, LMS fraction is considered the major candidate for determining the positive effects of HS on plant growth. Apart from displaying a hormone-like activity, HS may influence both respiration and photosynthesis, although these mechanisms are yet to be explored.

Interesting approach to the question of HS biological activity was described in the work of Popov [45]. He presented a very complex conceptual model reflecting the biological effect of HS on biochemical and biophysical processes occurring in vascular plants (Fig. 2) composed on the basis of scientific literature review and his own experimental data. In accordance with this proposed model, biological activity of humic substances is determined by tree factors; i.e. (1) the presence of varied functional groups, (2) the colloidal characteristics of HS and (3) the material constitution. He terms HS as polyfunctional polyampholites, since they hold various positively (amides, amines, imines, azogroups etc.) and negatively (carbonylic, hydroxylic, carboxylic groups) charged functional groups. Total incidence of these groups determines the reactivity resulting in quickened biosynthesis. Consecutively, as stated by Popov, the processes of respiration and photosynthesis can be optimized by addition of HS into the green plant system.

To evaluate the stimulative effect of HS, it is necessary to quantify their biological activity, i.e. the stimulatory or inhibitory impact on the growth and evolution of green plants. In order to do this, a few methods were designed, as for instance the one by Antošová et al. [65], [66]. This method evaluates biological activity of humic substances on higher plants by measuring the relative increment of a maize seedling experimentee at set conditions.

2.1.7 Practical application of HS

Although humus represents one of the biggest carbon reservoirs on earth, so far, industrial applications of humus and humus-derived products are quite rare. On the contrary, the usage of coal was more abundant and essentially, it constituted the basis of the chemical industry in the second half of the 19th century and the first half of the 20th century. Petroleum was also extensively exploited and it was regarded as the main raw material for the chemical industry of the 20th century. Nowadays, applications of HS can be divided into four main categories: agriculture, industry, environment and biomedicine [12], [46].

Agricultural applications

Humic substances influence soil properties to a degree out of proportion to their small percentage in soil. They strongly bind metals and serve to hold micronutrient metal ions in soil [47], [48]. Because of their acid-base character, HS serve as buffers in soil. The water-holding capacity of soil is significantly increased by HS. These materials increase the soil sorption of organic compounds to which they have a strong affinity [49], [50], [51]. Humic materials in soil also strongly sorb many solutes in soil water and have a particular affinity for heavy polyvalent cations. Thus, water in passing through humic-rich soils is “purified” of its cations.

All in all, HS help break up clay and compacted soils, assist in transferring micronutrients from the soil to the plant, enhance water retention, increase seed germination rates and penetration, and stimulate the development of microflora populations in soils [52], [67]. In addition to the improvement of the soil's physical properties and moisture conditions, HS also show a high cation exchange capacity, which is important for soil fertility [53]. Currently, humic materials are used as additives in fertilizers [54]. The fertilizing effect of sodium humate on plant leaves has been described. Ammonium humate was also found to have a significant growth-stimulating effect [55]. The beneficial effects of HS on plant growth may be related to their indirect (increase of fertilizer efficiency or reducing soil compaction), or direct (improvement of the overall plant biomass) effects. The stimulatory effects of HS have been correlated to the maintenance of Fe and Zn in solution at effective concentrations. In this context, HS have been widely regarded as playing a beneficial role in Fe acquisition by plants [56]. This effect has been mainly attributed to the complexing properties of HS, which increase the availability of micronutrients from sparingly soluble hydroxides [57]. Their effects appear to be mainly exerted on cell membrane functions, promoting nutrient uptake, or plant growth and development, by acting as hormone-like substances [40].

Industrial applications

Humus and humus-containing materials have been used in large-scale building, for instance, as additives to control the setting rate of concrete. Humic materials found use also in the preparation of leather. Initially, they were used as a leather dye, later on as an agent for leather tanning and, finally, as an ingredient of a solution to finish leather. The woodworking industry is another field where HS have been applied. They were used to prepare a natural indigo to dye wood veneer. In addition to this use, humic materials appeared to be suitable agents as a component of water-soluble stains for wood furniture. In the ceramic industry, humic substances were employed mainly as additives to enhance the mechanical strength of unprocessed ceramics, to improve the casting properties of ceramics, to colour clay tiles and among many other applications they were also used in the preparation of earthenware. Furthermore, humic materials have found application in the production of plastics, especially as dyes for coloring Nylon 6 or PVC plastics, hardeners of polyurethane foams or as plasticizer ingredients for PVC. Other industrial applications can be mentioned – ion exchangers, source of synthetic hydrocarbons and fuel oils, foodprocessing and extraction of uranium [58], [12].

Environmental applications

HS have a large capacity to retain transition metals, forming metalorganic complexes, which cause these metals to be more or less available for plants which then include them into the food chain. It has been demonstrated that the presence of HS in natural waters can influence the uptake of radionuclides by natural solids and thus their migration to surface and ground waters. The main task of HS in environmental chemistry is to remove toxic metals, anthropogenic organic chemicals and other pollutants from water. Ion-exchange materials based on calcium humate were found suitable for the removal of such heavy metals as iron, nickel, mercury, cadmium and copper from water and also to remove radioactive elements from water discharges from nuclear power plants. Humus-based filters have been developed for sewage purification, with many applications. The filters are useful to clean chromate

smelter wastewater, to remove oil and dyes from wastewaters and aquatic systems, to filter urban and industrial wastewaters, to remove pesticides from sewage and to remove phenol from water. Humus-containing materials have been also utilized for sorbing gases, e.g. the removal of waste gases from an animal-carcass rendering plant. Slightly modified humates can be applied to remove hydrogen sulfide and mercaptans from municipal gas supplies, and sulfur dioxide from stack gases [59].

Different groups of compounds such as herbicides, fungicides, insecticides, dioxins and also some pharmaceutical products like estrogenic compounds were determined as possible environmental endocrine disruptors. Thanks to their ability to adsorb organic pollutants from the environment, humic substances were found to be useful to remove those contaminants from water, soil and sewage sludges [12], [60], [68].

Biomedical applications

HS produced on a commercial scale are used in veterinary and human medicine. Several studies of the medicinal properties of humic materials have been reported. It was found that humic acids administered prophylactically to rats decreased significantly the extension of gastric damage induced by ethanol. Tolpa peat preparation administered to rats with experimental gastric and duodenal ulcers significantly accelerated the healing process [61]. Pflug and Ziechman [62] reported that HAs are able to interact with the bacterium Micrococcus luteus. In this case humic materials protected the organism against cell-wall disruption by the enzyme lysozyme.

In the last decade there has been an increasing interest in the employment of humic materials in medicine and biology. The possibility of soil humus extract with amino acid complexes and vitamin B analogues being a candidate as a basis for cosmetic and pharmaceutical products has been studied. The main reason for the increasing attention devoted to humic acids can be explain by their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities [63].

Humic materials in aquatic systems and water sediments have been observed to be closely connected with efficacy of hydrotherapy and balneotherapy. Antibacterial and antiviral [64] properties of HS represent new possibilities for their medical application [12].

2.2 Lignite as a source of humic substances

As humic substances origin from plant residues, they can be found everywhere on Earth. They are widely distributed in soils, natural waters, marine and lake sediments, peats, carbonaceous shales, brown coals and other deposits. Nevertheless, the main sources of HS are soils, waters, peats and low-rank coals (lignites). In the area of South Moravia, lignite is extracted from its existing deposits for the purpose of energy generation and heating. Simultaneously, new methods of lignite utilization are explored. In this work, lignite is used as the only source of investigated HAs, therefore, this chapter is dedicated to lignite genesis, composition, to the properties of Czech lignites and HAs production from lignite.

2.2.1 Lignite genesis

Low rank coals and coals generally are formed from peat and the vascular plant remains that accumulate in peat bogs. Anaerobic conditions are considered mandatory for the accumulation and preservation of peat and the formation of coal. Two major types of coals are known: humic coal and sapropelic coal [71], [72]. The former are formed from peat accumulation rich in humic substances derived predominantly from vascular plant remains. The latter represents coal formed from algal (boghead coal) or spore (cannel coal) accumulations. In many respects, sapropelic coal can be considered to have an aquatic origin similar to that of humin of aquatic sediment, which forms from the accumulation of aquatic nonvascular plant debris in clastic sediments.

The general view put forth by van Krevelen [73] and Stach [74] on the formation of coal from HS are as follows:

- Plant debris is first converted to humic acids at the peat stage by the action of aerobic microorganisms that break down the lignin and cellulose to simple monomers which condense to form humic acids.
- Humic acids, when buried to greater depths, condense to form humin and result in chemical changes that involve loss of oxygen functional groups with concomitant coalification.
- As further condensation occurs, the humic acids are all converted to insoluble macromolecules typical of lignite or subbituminous coal.
- Finally, humic substances no longer play a role in coalification as the insoluble coal undergoes further diagenesis leading to bituminous coal, anthracite, and eventually graphite.

Thus, fewer HAs can be extracted from coal of subbituminous or bituminous rank than can be extracted from lignite or brown coal while peat has much greater concentration of HAs. On the other hand, procedures have been described in literature converse to diagenetic process occurring in nature environment and which lead to production of so-called regenerated humic acids (RHAs). Those substances show the same properties as lignite ones, except slightly higher degree of aromaticity.

Apparently, the industrial exploitation of soil or aquatic humic substances is not financially realizable. For instance, the price of humic standards is reported 5-175$ per 100 mg [75]. On the other hand, the availability of humic substances produced in large amount from an inexpensive source (e.g. peats and low-rank coals) through a reliable process appears to be particularly desirable and attractive. In Japan, some thousands of tons per year of nitrohumic acids are produced via nitric oxidation of low-rank coals and commercialized in local market [76]. To sum up, utilization of such carbon-rich sources can bring not only attractive products but also trade revival in locality.

2.2.2 Lignite deposits in Czech Republic

While the Czech Republic has moderate coal and lignite reserves, most are not suited for mining expansion due to environmental and economic factors. Czech Republic has total recoverable reserves of approximately 5.7 billion tons. Of this, approximately 1.8 billion tons

is bituminous and approximately 3.9 billion tons is subbituminous or lignite (brown coal). High volatile lignite is a variety of brown coal, which exhibits the lowest degree of coalification, is of xylitic character with preserved tree trunks and with large or small fragments of wood. From the geochemical and petrological viewpoints, it is a brown coal hemitype. Its dry (ash-free) caloric value is less than 17 MJ/kg.

No boundary between brown coal and high volatile lignite has been established and high volatile lignite is generally included in regular brown coal. In the Czech Republic, however, it is treated separately. High volatile lignite is used in energy generation and for heating. It represents the lowest quality mineral fuel the consumption of which gradually decreases.

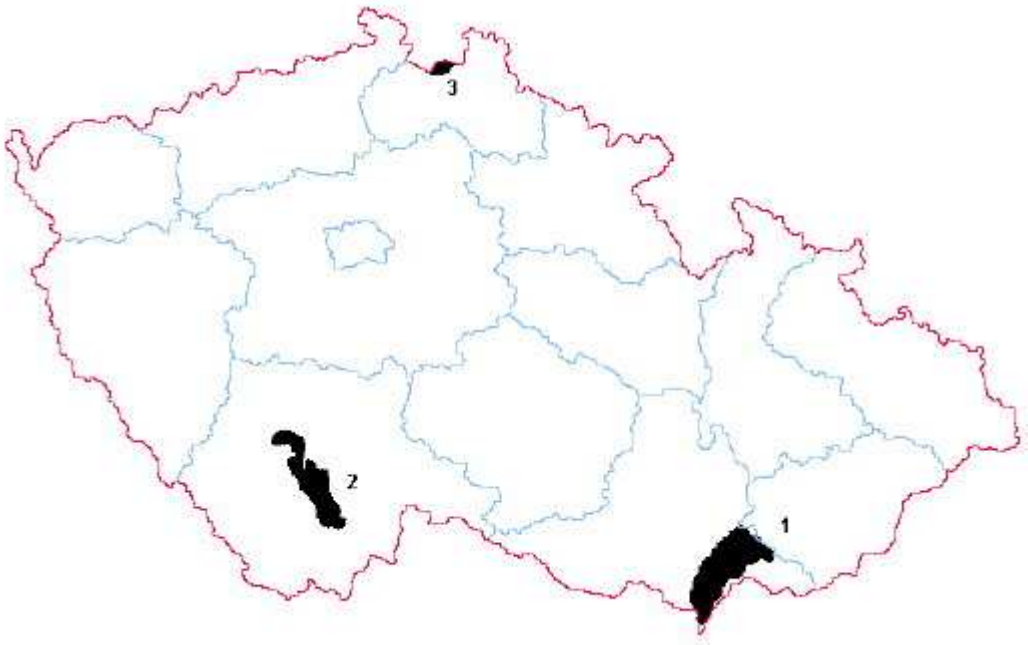

Fig. 3 Registered deposits and their location in Czech Republic. 1 Vienna Basin, 2 South Bohemian Basin, 3 Žitava Basin.

Largest deposits of high volatile lignite occur along the northern margin of the Vienna Basin, which extends from Austria into southern Moravia. There are two lignite seams in the youngest sediments of the Pannonian and Pliocene age. Reserves of the northern Kyjov seam are not sufficient for mining (the last mine Šardice was closed in the end of 1992). Those of the southern Dubňany seam have been mined by only one underground mine Hodonín-Mikulčice. Currently, mine Hodonín-Mikulčice is open and the contract with ČEZ has been prolonged until 2010. Practically all production is burned in the Hodonín power station. Economic reserves are registered at six other deposits, but their exploitation is not anticipated. South Moravian high volatile lignite is of xylodetrital character with numerous tree trunks. It is rich in water (45 – 49%), average content of S is 1.5 – 2.2% and its caloric value is 10 – 12 MJ/kg.

Other deposits of low-quality lignite occur in narrow lobe-shaped bulges of the České Budějovice Basin. Major part of the reserves has been mined out and the remainig ones are not of any economical importance. Smaller isolated occurrences of high volatile lignite (Pleistocene xylite) in the Žitava Basin were mined out to a large extent, as well, and the remaining reserves are not of any importance.

Under current circumstances, there is also the inevitable need to produce chemicals without negative influence on the environment. In this way, lignite should not be viewed as

some kind of low quality fuel only, but also as a valuable raw material for different chemical applications due to the naturally high content of functional groups of its alkali-soluble extracts (i.e.humic acids). South-Moravian lignite contains a relatively high proportion of extractable humic acids (about 25%) [77] which could provide practical applications due to their binding capacity. The oxygenated functional groups, especially carboxylic acids and phenols, are responsible for partial solubility of humic acids and their ability to interact with cationic species, too. As a consequence, the determination of their chemical composition and structure at molecular level (primary humic structure) as well as role of individual humic constituents in the humic supramolecular structure is of importance. With this aim, the stability and degradation behaviour of HS was intensely studied by Válková et al. [78], [79], [80].

2.2.3 Regenerated HAs obtained from lignite

Coal is considered the final result of a diagenetic process which starts with low molecular weight substances (like HAs and FAs) and continues with the loss of functional groups and condensation reactions to form an insoluble tridimensional network [81].

When a hypothetical structure of a medium rank coal as that proposed by Wiser [82] is considered, a few weak points can be identified. These can desintegrate and lead to "depolymerization" of the original structure and possible production of a substrate easily convertible into smaller, soluble fragments. This transformation can be achieved through the oxidation reaction, inverse to the diagenetic process in ideal case. This means the oxidation reaction is able to regenerate the molecules which originally led to the insoluble structure of coal. Humic acids produced this way are generally known [83] as regenerated humic acids (RHAs), although there exist opinions which disagree with this term and state that the occurring processes can not be considered regeneration. For the purpose of this work, samples prepared this way are reported to as samples isolated from pre-treated lignite.

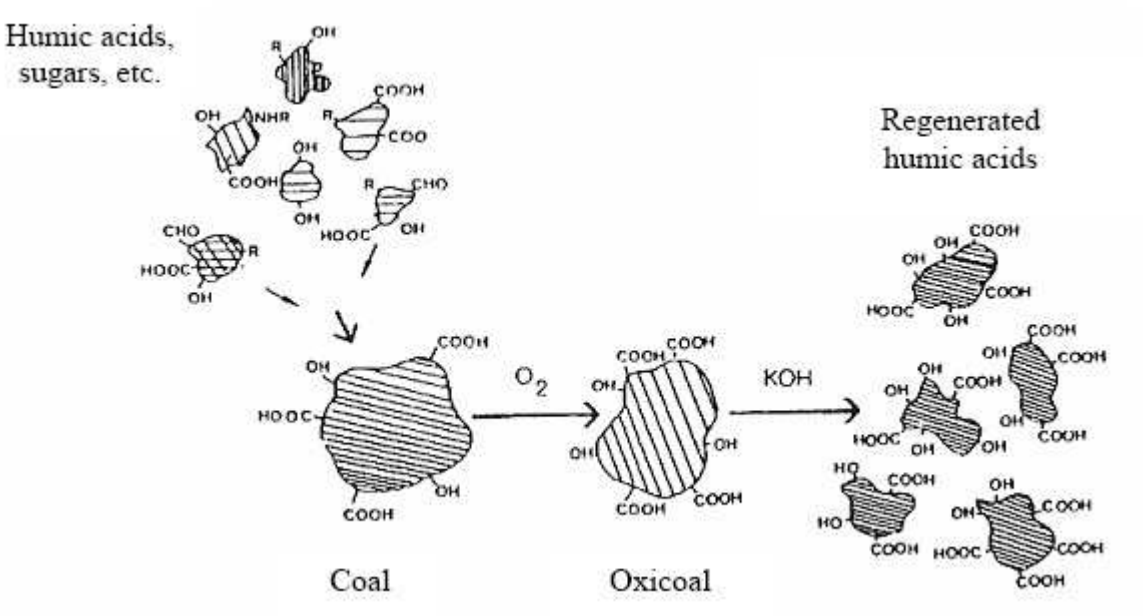

Fig. 4 Coal oxidation as an ideal inverse diagenetic process [84].

The oxidation process itself can be performed either in gas phase or in liquid phase. In gas phase, the lignite is exposed to the oxidative effects of air (or directly oxygen). In liquid phase, various oxidative agents, mineral or organic, can be utilized, e.g. HNO_3, $KMnO_4$. Naturally, the occurring reaction ought to be precisely set and controlled for potential carbon loss or side reactions, so the outcome serves its goal satisfyingly.

RHAs have similar characteristics and chemical behaviour like the original HAs, conversion of which had led to coal formation. Their elemental composition, functional groups content and molecular weights depend on the conditions of preparation procedure, and the original composition of certain lignite type is likely to play an important role, too.

3. WORK OBJECTIVES

The objective of this work is to (1) prepare a variety of humic acids from chemically and physically pre-treated lignite from South-Moravian lignite source; chemical pre-treatment includes several ways of oxidation of parental lignite and physical pre-treatment means rearrangement of supramolecular structure by small organic acids as previously reported [85] (2) identify the individual molecules in HS extracted from respectively pre-treated lignite (3) clarify the mechanisms involved in the influence of investigated humic acids on cell-proliferation in maize seedlings and in their antimutagenic properties determined in *Saccharomyces Cerevisiae* D7 tests.

Principal purpose of this work is to produce humic acids with different properties from South Moravian lignite. The oxidation of parental matter – lignite – was elected as a primary approach. This was performed in gas phase and in liquid phase. Furthermore, physical modifications with some amphiphilic-like agents are tested. Part of this work is focused on the identification of potential causes, physical or chemical, which are likely to be accountable for biological activity of humic acids in terms of cell-proliferation of maize seedlings. As regularly demonstrated in literature [86], [87], [88], [89], [90], [91], [92] humic acids are known to exhibit positive bioactivity under certain favourable conditions. This means that they are able to accelerate, inhibit or otherwise influence the growth of higher plants to some extent. In this study, that influence is quantified in a useful manner and in an attempt to better understand its sources methods of GS-MS and HPSEC are employed. The mass-spectroscopic study is performed to investigate the composition of sugar content, which represents a simple source of energy to be potentially used for executing biological functions. On the other hand, size distribution of examined humic acids ought to discover possible linkages between their molecular weight and bioactivity. Next, the EPR and genotoxicity measurements are involved in order to asses the radical content and genetic hazard and ensure a safe and harmless utilization of such stimulators.

A rather extensive part of this work concentrates on supramolecular structure of prepared humic acids. This is to be considered consequential, as it influences directly the HAs properties. Particularly, the aggregation behaviour differences between HAs produced from individual oxidatively pre-treated and physically modified lignite are monitored by means of HR-US. Also the concentration influence on their stability and structural rearrangement are observed. Finally, the effect of hydration and character of water structures surrounding the humic acid is investigated.

4. OVERVIEW OF RESULTS AND DISCUSSION

One of the ultimate research goals at our institute is to employ a new non-energy exploitation of lignite deposits in South Moravia. This work is part of this research and represents the pilot study on biological exploitation of South Moravian lignite HAs. HAs are one of many environmentally interesting products that can be easily extracted from lignite. In this work, methods of lignite pre-treatment which could presumably bring about a significant increase in yield of respective HAs were employed. These would also have to maintain physico-chemical properties suitable for use in agriculture as additives with biostimulative effect, as a source of stable carbon in soils or in other environmental applications.

In South Moravian lignite the content of HAs (extractable part) is around 20%. Literature data describe the possibility to increase the content by oxidation either in liquid or in gas suspension [77]. The first lignite pre-treatment to be studied for this purpose was the oxidation by air [94]. Details on the lignite air pre-treatment, humic acid extraction and sample characterization can be viewed in the experimental section of **Appendix A**. To broaden the possibilities, both fluid reactor (in combination with mild temperatures of 25 and 50°C) and static reactor (with relatively high temperature of 85 °C) were used. According to our results, both methods eventually lead to increase in the yield of the HAs, although only after a fair amount of time in the case of stationary phase. The more successful experiment in fluid reactor also showcased the simple fact that higher temperature supports the production of HAs. The idea of air oxidation was set up on the assumption that relatively mild conditions could lead to a significant increase of the HAs yield and, simultaneously, have a non-destructive effect on the chemical properties of resulting HAs, e.g. not cause carbon loss due the to intensive oxidation effect. This aim was seemingly achieved, when the elementary analysis and FTIR data confirmed that chemical characteristics of our HAs did not differ significantly, neither among each other nor in comparison to the HA extracted from parental lignite. On the other hand, the HPSEC analysis showed differences among samples indicating difference in supramolecular rather than in primary chemical structure. The highest yield in HAs production was achieved at elevated temperature which can bring about generation of free organic radicals in HS. That represents potential barrier in environmental or agricultural applications.

Thus, concurrently, another study on HAs extracted from both parental and pre-treated lignite was conducted – a study of their antimutagenic and/or genotoxic effect, described in detail in [107]. HA sample processed at high temperature (250°C) was also included in the assay and was the only one to show clear contribution to the genotoxic effect of standard mutagen in all tested concentrations. This is an important finding, despite the fact that in this work, it was the HA itself that was subjected to high temperature, not the lignite. Still, indication exist that although air oxidized lignite samples appear to be chemically of the same value as parental lignite ones, they might be unsuitable for utilization in green chemistry. These conclusions also suggest that the primary structure merely determines the content, but not the behaviour in tested HAs. However, in combination with the amount of time necessary to produce increased yield and demanding experimental conditions, in this work it was decided to dismiss the idea of oxidation in gas phase and to move on to the oxidation in liquid phase with lower temperature.

For the experiments in liquid phase, a well considered line of 8 HAs was prepared in order to carry out an extensive study on their properties, behaviour and agricultural functions in relation to their primary and, as it turned out, more frequently supramolecular structure. The primary aim is to find out if the pre-treatment of lignite leads to the production of HAs with different biological properties, the long-term intention is to find the optimal lignite pre-treatment process, enabling a production of HAs with versatile utilization in all aspects of green chemistry, e.g. as environmentally harmless biostimulants, antioxidants for biocolloids or natural surfactants. The first logical step to characterize produced HAs was the analysis and enumeration of individual chemical elements and groups. Research on distinct chemical classes in plant residues which persevered in parental lignite throughout its diagenesis, such as lignin monomers or stable and labile polyol units was the second step. Next, the assessment of free organic radical content, aggregation behaviour and hydration was necessary in the attempt to uncover the extent of modifications which occurred in the HAs structure due to various lignite pre-treatments. Final step was to examine biological activity and genotoxic (eventually antimutagenic) potential of produced HAs and compare the results to their primary and supramolecular structure.

Production of individual HAs from lignite pre-treated in liquid phase is described in both **Appendix B** and **Appendix C**, which represent the principal part of this work. To explain better the choice of pre-treatment agents, it is appropriate to state that these were selected based on previous experimental experience. Oxidative agents such as nitric acid and hydrogen peroxide have been utilized to increase yield before, approximately 20 to 60% enrichment was achieved [77]. Optimization process was necessary to establish final conditions of described pre-treatment procedure with regard to both sufficient yield and desirable properties of produced HAs. Low temperature (45°C), short period of pre-treatment time (30 min), big volume of liquid phase and relatively low agent concentrations were applied in order to open up functional lignite parts and activate the existing structures for reactions. The two amphiphilic agents, acetic acid and citric acid, were selected for their similarity to short-chained organic acids released into soil by root systems of plants active in the transportation processes in the rhizosphere. Here, they break up the weak interactions holding together the HAs supramolecular structure (aggregates) and thus decrease the aggregation degree of present organic matter. As a result, extracted products should have enhanced ability to penetrate cell-walls, transport the nutrients and sequester water. Acetic acid and citric acid were included into the study with the aim to determine whether they are able to influence – via lignite pre-treatment – aggregation stability of HAs, which is a major factor in various biological properties.

From practical point of view, HAs in agriculture are most likely to be applied by watering and spraying. Therefore, their water-soluble form – humates – was prepared. Properties of complex substances such as HAs in aqueous solutions can be very specific and sensitive even to minor changes in conditions [95]. They can accumulate at interfaces, form self-assemblages, solubilize organic compounds and exist in different colloidal states depending on the solution conditions. Because of the randomness of formation and polydispersity, no uniform behaviour can be expected. Trends, however, can be predicted for properties as surface tension, aggregation and colloidal stability in humate solutions [96]. Next important issue when dealing with supramolecular structure and reactivity of HAs is hydration effect. Investigation of its influence is one of the methods for identifying diversities in HAs of different lignite pre-treatment. To examine also the influence of counterion in humate

solution, it was decided to prepare two sets of humates, first with potassium and second with ammonium counterion. These two ions differ enough to demonstrate how important is it to consider carefully the choice of counterion when preparing humates for a particular purpose.

To comment on the results, similarly to the experiment in gas phase, primary structure of HAs isolated from lignite pre-treated in liquid phase did not significantly vary among each other, as characterized by elemental analysis and solid state ^{13}C CPMAS NMR. This is an important finding for it suggests that primary structure is not the key to the control of HAs behaviour in natural environment. Nevertheless, detailed comments on the elemental analysis, important elemental ratios and the content of functional groups are provided in **Appendix B**. The findings which are of interest are the following: (a) all samples except those with amphiphilic pre-treatment showed slight loss in carboxyl content, (b) all samples except those with amphiphilic pre-treatment and those processed with nitric acid in lowest concentration (5%) showed loss of aromatic structures higher than 5%, (c) samples processed with nitric acid of higher concentrations (10% and 20%) almost doubled content of ethers and alcohols.

Appendix B also offers a list of three most important compounds identified as TMS esthers after HCl hydrolysis of processed HAs. These are threitol, 2-deoxy-arabino-lactone and 2-deoxy-ribonic-acid, polyols originating from carbohydrate precursors reduced during the lignite diagenesis. Other unidentified saccharidic-like compounds were mainly bearing no more than three hydroxyl groups. Polyols content was detected in two successive steps. In the first step, hydrolysis with diluted HCl was used to release mainly labile (unbound) compounds. The second step was conducted on solid residue of the first step. Here, hydrolysis with concentrated HCl aimed to release compounds with more resilient bonds, linked to the organic matrix. It was presumed that higher portion would be released during the second step, as saccharides are strongly associated with the humic matrix, i.e. the aromatic and aliphatic structure composing the major part of humic acids [97].

Generally, the detection of rather low quantities of saccharidic-like compounds in HAs isolated from overaged lignite source was expected, although work of Allard [98] proves that residual carbohydrate structures can be found in lignite HAs, which indicates long-term persistence of these readily degradable products. Allard [98] also states that the nature of such compounds is rather independent of the degree of maturity of the HAs and of their origin as the differences between soil and lignite HAs appear to be quantitative rather than qualitative. Relatively high carbohydrate amounts were reported by Disnar et al. [99] in a peat quality assessment when applying the two-step acid hydrolysis on dry sediments (not HAs). Up to 90 mg g^{-1} TOC were recorded in four of the peat samples analysed, and more than 160 mg g^{-1} TOC in six of them. These amounts were much lower than those found in most terrestrial plants in which carbohydrates are the dominant constituents. Nevertheless, the presence of such amounts of neutral sugars released by peat hydrolysis contradicts the widely accepted idea that carbohydrates are labile compounds which undergo rapid consumption in the sedimentary environment, lignite deposits included [99].

Our results show that the content of saccharidic-like compounds differs among variously pre-treated samples. The values for 5% H_2O_2 processed sample are extremely outstanding (160 mg/g of HA) which means that (a) this sample contains remarkably high amount of polyols, or (b) that the rest of the samples contains remarkably low amount of polyols. Either way, majority of the samples provided very low polyol content (below 20 mg/g of HA) in the first step of hydrolysis and higher amount (20 to 40 mg/g of HA) in the second step.

Interestingly enough, polyol content in the HA processed with acetic acid was almost zero whereas HA process with citric acid came out second best (90 mg/g of HA).

The main reason for this investigation on saccharidic-like content was the possibility that these might serve as storage of energy to be released under certain favourable conditions and enhance the overall bioactivity potential of HAs (will be discussed further below). Although no relation whatsoever was found between these two parameters, our observations provide further evidence that carbohydrates can be preserved in sediments for thousands of years. Also the fact that a simple process of lignite pre-treatment can induce changes in polyol unit numbers of respective HAs is not negligible for there are other properties that can be influenced by their content. The results of Mecozzi and Pietrantonio [100] suggest a different role played by this chemical class during the aggregation process of organic matter and on the formation of aggregate dimension higher than HAs. According to their study carbohydrates along with proteins play a primary role in starting the aggregation process in soil, from dissolved organic matter to the formation of particles and macromolecules by means of their specific interactions with cations and self-aggregation properties. Therefore, the potential application of HAs with increased content of saccharidic-like compounds into soil can bring about stabilization of the soil via connecting of present microaggregates. Recently, it was shown [101] that the shift of balance between micro- and macro- aggregates content presents a significant contribution to the stability of soil organic matter towards microbiological activity represented by soil respiration. In principle, there exist two possibilities of macroaggregates formation. The first is via formation of covalent bonds [101], the second is possible via formation of weak interactions, i.e. so-called sticking by saccharides.

In course of this work, a method suitable for identification of residual lignin monomers in HAs was searched. The so-called DFRC (derivatization followed by reductive cleavage) method was originally developed by Lu and Ralph [102], [103] as a pathway for lignin characterization. The DFRC degradation method includes two key steps – bromination and acetylation with AcBr followed by reductive cleavage with zinc dust. The method was tested on HAs in order to learn whether it is also fitting for characterization of lignite HAs. Optimal conditions (time, temperature) for all reaction steps were explored and the resulting procedure was applied on HAs isolated from parental lignite with a positive outcome reported in detail in **Appendix D**. Results, as detected by GS-MS, show that lignite HAs contain intact lignin monomers. The dominance of conferyl units is in accordance with the gymnosperm origin of the studied lignite. The DFRC method seems to be a convenient option for characterization of lignite HAs by means of detection and identification of its residual lignin monomers. It is also reasonable to expect that DFRC will be suitable for tracing lignin input in several other organic sediments.

To extend the characterization of chemical structure of investigated samples, a quantitative EPR study was conducted with the aim to determine the amount of organic free radicals in all forms of samples – HAs, potassium humates and ammonium humates. Acquired results are commented on in **Appendix C**. Briefly, organic free radical counts in samples of HAs ranged from 1.53×10^{17} spins g^{-1} to 1.12×10^{18} spins g^{-1} which is in agreement with radical counts usually reported for HAs [104]. On the other hand, organic free radical counts in samples of humates were distinctly lower. This observation can be explained by the role of counterion in stability of free radicals in lignite HAs, as demonstrated recently [105]. In this work, it was shown that HAs and ammonium humates exhibit better antioxidant effect towards polyvinyl

alcohol than the sodium humates, due to the disruption of H-bonds stabilizing humic substances supramolecular structure.

When comparing individual pre-treatments, the same observation was made in case of both oxidative agents. While the agent in diluted concentration increases radical content in comparison to non-treated sample, higher concentration gradually decreases radical content. This was attributed to the decreasing amount of aromatic C and carboxylic groups with increasing concentration of the agent. A fairly good correlation (r = 0.79) was found between the free radical concentrations and the carboxyl groups contents as determined by NMR, which suggests that the carboxylic groups contributed to the free radical stabilization in investigated humic samples. Good correlation (r = 0.70) was also found between the free radical concentration and aromatic C, although there are some literature sources which report that aromaticity did not largely influence the free radical content of investigated HAs [106]. Yabuta et al. [106] suggested that there could be more diversity in chemical structure of HAs in the early stage of humification, i.e., free radical content might not be uniformly related to aromaticity but reflected in combinations of various structural properties. Namely, the free radicals are stabilized by combinations of multiple polar components such as carboxyl and nitrogen-containing carbons within HA structures during the early stages of humification. This might especially apply to HAs with lower degree of humification where aromaticity is less than 40%. In our samples, aromaticity ranged from 37% to 46%. Negative correlation (r = -0.62) was found between radical count and content of ethers/alcohols and between radical count and amount of alkyl C (r = -0.68). This suggests that accumulation of alkyl C leads to decrease in free radical content. Line widths also varied (0.50 – 0.57 mT) in HAs samples, implying again structural diversity associated with free radicals.

In humate samples situation is different. Ammonium humates kept the trend set by HAs and their radical content correlated very well with both amount of carboxyl groups (r = 0.71) and aromatic C (r = 0.87). On the contrary, potassium humates showed no relations here. The same pattern occurs for correlations of radical concentration and content of ethers/alcohols and alkyl C. While in ammonium humates Pearson coefficients are (r = -0.50) and (r = -0.84) respectively, potassium humates again show no correlation whatsoever. This might again be related to the issues discussed in ref. [105] and in above paragraphs.

To complete the characterization of investigated samples two more methods were employed, i.e. UV-VIS spectroscopy and HPSEC. Discovered spectroscopic properties are described in detail in **Appendix B**. The obtained absorptivity profiles as well as the provided summary of E4:E6 ratios are in accordance with NMR data and support all previously discussed findings. HPSEC measurements were carried out to address the questions concerning supramolecular structure and presumed aggregation behaviour of HAs in forms of their salts, both potassium and ammonium. Conformational arrangement of their molecules in solution is given by many factors and subsequently determines their properties in solution, as mentioned in above paragraphs. **Appendix B** gives a detailed description of experimental conditions and procedure as well as obtained results summed up in well-arranged overview figures. Essential conclusions are that (a) the humate aggregate size distribution is a function of their counterion, (b) aggregate stability is supported by H_2O_2 generally and by HNO_3 in concentration of 10%, (c) aggregate stability is reduced by HNO_3 in concentration of 20%, (d) size distributions of aggregates in humates processed by short-chained organic acids are analogous to those of humates isolated from parental lignite except for the lowest size fraction

which increased its amount in accordance with our expectations discussed in above paragraphs.

Appendix B further reports detailed information about biological activity assessment and its results in the context of lignite pre-treatment. In essence, the stimulating effect was determined by growth rate of maize seedlings. Again, humates of both counterions were tested with interesting outcome, that (with a few exceptions) the effect was higher in potassium salts than in ammonium ones. Ammonium humates maintained approximately the same level in all tested periods of time, while potassium humates either increased or decreased their stimulating effect with time, in dependence on the used lignite pre-treatment agent. Bioactivity results were statistically compared to molecular size, its distribution, C/H ratio, polyol content and carboxylic content to verify possible relationships between physico-chemical structure and bio-stimulating behaviour. Individual correlations are discussed in **Appendix B**.

In association with HAs, genotoxic behaviour is often reported. Therefore a complex genotoxicity/antimutagenicity assay was performed on investigated samples as these show satisfactory biological activity and might be therefore considered for agricultural applications. The tests were carried out with all samples in form of pottasium and ammonium humates with the aim to evaluate and compare the suitability of individual pre-treatment as well as the influence of counterion from point of view of genotoxicity/antimutagenicity. Description of respective experimental procedure and results summarized in synoptic graphs are available in **Appendix C**, where all findings are commented on. Briefly, the effect of counterion was found to be crucial again, along with the concentration of respective humate. Four different concentrations were tested with the interesting outcome that high dilution of humate sample (0.03%) is the optimal condition for achieving antimutagenic effect of the humate. In course of the testing, certain concentration anomalies were found different for each counterion. This indicates high influence of the character of supramolecular structure in the final genotoxic potential of HAs. What triggers genotoxic/antimutagenic effect in HAs is the blocking or unblocking of molecules contained in humic aggregates during the period of lignite pre-treatment. The explanation would be the same for the effect of biological activity only the agent would be different.

An important fact is that although some humates supported the effect of mutagen, a proof had been obtained that no humate was genotoxic itself. Thus it can be assumed that lignite modification does not induce genotoxic effect in extracted HAs in comparison to the original sample, which is one of the essential conclusions of this assay.

Appendix E deals with densitometry and ultrasonic velocity measurement. Both methods are used to evaluate the process of hydration and its background in terms of concentration and counterion influence. Propagation of ultrasonic wave through humate solution of measured density is investigated and acquired velocity data are used to calculate the adiabatic compressibility. The values of experimentally determined volume fraction of non-interacting solvent for individual lignite pre-treatment and the two different counterions are compared. Obtained profiles give reason to believe that both lignite pre-treatment and specific counterion add up to diversities in supramolecular structure of resulting humates.

From results reported in **Appendix E** the hydration numbers calculated in grams of hydration water (bound water) per grams of humate gave value approximately 0.8 g/g for concentration 1 g/L and increased with dilution confirming the statement about progressive aggregation. That hydration number roughly corresponds to hydration of biomolecules such

as polysaccharides or proteins found in literature. Accordingly, taking into account the content of K^+ (from C content and ^{13}C distribution) the hydration was also recalculated for organic part of humate. Using Pearson correlation coefficient, total and organic part hydration were correlated with results obtained from maize seedling cell proliferation, but no relationship was found.

CONCLUSIONS

Consequential results of this work are summarized in following articles:

- Elemental analysis of HAs isolated from lignite with different pre-treatment shows very similar results. This fact can be considered a proof to the statement that elemental composition is not the only determinant for HAs quality.
- Our observations provide evidence that carbohydrates can be preserved in lignite deposits throughout thousands of years. Their quantity can be both reduced and increased by appropriate pre-treatment of parental lignite, although the mechanism of the formation is unknown.
- The DFRC method designed for lignin monomers detection and identification is suitable for characterization of HAs isolated from lignite and possibly other organic sediments.
- Amount of organic free radicals in HAs is mainly influenced by aromaticity and carboxyl groups. This is probably due to the fact that this work deals with HAs obtained from lignite, i.e. HAs are considerably humified.
- Relationships between amount of organic free radicals and content of individual functional groups in humates indicate that while NH_4^+ counterion secures behaviour exactly alike to that of HAs, K^+ counterion triggers contributions from functional groups other than aromatic carbon.
- Differences in radical concentration as well as in line width values among samples with individual lignite pre-treatments indicate clear structural diversity associated with free radicals.
- The comparison of weight-averaged molecular weights (M_w) of humates provides a proof of that humate properties in solution are counterion dependent. M_w values of ammonium humates are distinctly larger than those of potassium humates, since hard K^+ ion offers stronger self-association to humate than soft NH_4^+ ion.
- Size distribution development is identical for both potassium and ammonium humates and supports the hypothesis that larger portion of polar (oxidized) components with larger hydrophilicity in HAs results in highly hydrated conformations with lower potential to mutually associate.
- The ability of HAs to positively influence cell-proliferation of maize seedlings was proven. However, it is not contingent upon its elemental composition or functional groups.
- The ability of HAs to enhance biological processes in maize seedlings is not associated with the content of saccharidic-like units.
- Unlike the M_w, size distribution influence on biological activity is extensive. The middle molecular size intervals (30-175 kDa) correlate highly positive and high molecular size intervals (175-350 kDa) correlate highly negative in both potassium and ammonium humates. The hypothesis that biological activity is

stimulated by low size fraction was not confirmed, as no relationship was found there.

- Pre-treatment of parental lignite with suitable agent can bring about changes in HAs biological activity in higher plants. The most efficient modifiers are 20% acetic acid and 5% hydrogen peroxide.
- It seems that processes occurring in rhizosphere, i.e. root exudates release and its consequences can be imitated by lignite pre-treatment and used for increase of bio-stimulative efficiency of extracted HAs. These findings are also supported by the size distribution of aggregates in these HAs.
- A proof has been obtained that lignite modification does not induce direct genotoxic effect (i.e. without the presence of other mutagen) of extracted HAs in comparison to the original sample.
- Genotoxic/antimutagenic effect can be influenced by lignite pre-treatment, humate concentration and counterion. It is not contingent upon radical content, since other indicators such as supramolecular structure and aggregate stability seem to play a major role in genotoxic potential of HAs.
- Particularly high antimutagenic effect can be achieved by combination of (a) 5% HNO_3 pre-treatment and K^+ counterion, (b) 5% H_2O_2 pre-treatment and NH_4^+ counterion. Universal antimutagenic effect is also achieved at high dilution of 0.03% for potassium humate of any pre-treatment.
- Lignite pre-treatment agents which produce HAs that are particularly interesting from point of view of biological activity (20% acetic acid, 5% H_2O_2) were found to be either harmless or provide positive effect from point of view of genotoxicity/antimutagenicity, especially in combination with NH_4^+ counterion.
- The increase of density with concentration in investigated samples is not strictly linear and higher density is frequently observed in ammonium humates which suggests that they are mainly the diversities in the structure of dissociated humate which influence this dependency.
- The dependence of ultrasonic velocity on concentration in investigated samples shows higher values mostly for potassium humates which indicates that the total hydration in these cases is higher in potassium humates and the conuterion hydration prevails. The exceptions indicate that humates differ in their supramolecular structure.
- The decrease of volume fraction of non-interacting solvent with concentration is not perfectly linear. Since the contribution from hydration shell of counterion should be linear, the observed non-linearities can only be attributed to the influence of hydration shell of organic part of the dissolved humate.
- Lignite pre-treatment has potential as a method of production of HAs with variable properties. If the correct combination of samples and conditions will be researched in further detail, optimal procedures can be established for successful production of lignite HAs applicable in various fields.

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LIST OF ABBREVIATIONS

^{13}C CPMAS NMR – cross polarization magic angle spinning ^{13}C nuclear magnetic resonance

ČEZ – České energetické závody

DFRC – derivatization followed by reductive cleavage

EPR – electron paramagnetic resonance

ESI-MS – electrospray ionization-mass spectrometry

FAs – fulvic acids

FTIR – Fourier transform infrared spectroscopy

GS-MS – gas chromatography-mass spectroscopy

HAs – humic acids

HMS – high molecular size fraction

HPSEC – high performance size exclusion chromatography

HR-US – high resolution-ultrasound spectroscopy

HS – humic substances

LMS – low molecular size fraction

PVC – polyvinylchloride

RHA – regenerated humic acid

TMAH – tetramethylammonium hydroxide

TMS – trimethylsilyl

TOC – total organic carbon

UV-VIS – ultraviolet-visible spectroscopy

LIST OF APPENDIXES

- Appendix A

  Kučerík, J., Cihlář, Z., Vlčková, Z., Drastík, M.: *Regenerated humic acids obtained by the air oxidation os South Moravian lignite. Part. 1. Production and characterization*. Petroleum & Coal, 2008, 50 (3), 48-54.

- Appendix B

  Vlčková, Z., Grasset, L., Antošová, B., Pekař, M., Kučerík, J.: *Lignite pre-treatment and its effect on biostimulative properties of respective lignite humic acids*. Soil Biology & Biochmistry, 2009, doi:10.1016/j.soilbio.2009.06.013.

- Appendix C

  Vlčková, Z., Duroňová, K., Márová, I., Kučerík, J.: *Lignite pre-treatment and its effect on genotoxicity/antimutagenicity of respectivelignite humic acids and their salts. Influence of concentration and counterion*. Manuscript submitted to Environmental Science & Technology.

- Appendix D

  Grasset, L., Vlčková, Z., Amblès, A.: *Characterozation of lignin monomers in low rank coal humic acids sing the DFRC method*. Accepted for presentation at the 24th International Meeting on Organic Geochemistry in Bremen, Germany in September 2009.

- Appendix E

  Ultrasonic and densitometry measurements. Supplemental information.

APPENDIX A

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REGENERATED HUMIC ACIDS OBTAINED BY THE AIR OXIDATION OF SOUTH MORAVIAN LIGNITE. PART. 1. PRODUCTION AND CHARACTERIZATION.

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Abstract
The influence of lignite air oxidation on the yield and chemical/physical character of regenerated humic acids (RHA) has been studied. RHA were produced under different experimental conditions, i.e. both in static and fluid reactor. Oxidation in the fluid reactor brought first a decrease and after a certain period increase in the yield of RHA. The same observation was done in case of static reactor. It has been verified that higher temperature during the oxidation supports the production of RHA. Despite the difference in the yield of RHA (increase up to 75%) their chemical character was in all cases fairly similar. Some tiny differences were revealed using FTIR which showed the increase in number of ester bonds in RHA produced mainly at temperatures above 80°C causing a significant increase in apparent molecular weight as evaluated by HPSEC.

Key words: lignite, regenerated humic acids, air oxidation

1. Introduction

Humic substances are ubiquitous natural products of microbial degradation of dead plant tissues and animal bodies and represent the major biosphere pool of natural organic matter [1]. In the course of their genesis they underwent processes of humification and in case of lignites also partly coalification. Major components of humic substances are considered to be recalcitrance of plants and algae, including materials derived from lignins, tannins, sporopollenins, and large aliphatic molecules such as algaenans, cutans and suberans. From the chemical point of view, they represent a complicated mixture of distinct chemical compound classes such as lipids, waxes, carbohydrates, polyphenols, proteins and nucleic acids derivates [2]. Their role in nature is vital for sustainable development of life on the Earth. In soils they help to prevent drying and shrinking, improve moisture retaining properties of soils, permit exchange of gases, stabilize structure, enhance availability of micronutrients to higher plants, increase cation exchange capacity, modify application rate of pesticides for effective control, have a direct effect on plant growth [3]. In this respect, they influence the micro algae growth, seed germination, plant growth and development affecting some metabolic processes as respiration, nutrient uptake and inducing morphofunctional changes in the root architecture. In many cases these effects are similar to those induced by plant growth regulators as auxins, gibberellins and cytokinines [4,5].

Operationally humic substances are divided into three groups according to their solubility, i.e. humic acids (HA) soluble in alkali media, insoluble humin (HU) and fulvic acids (FA) soluble at all values of pH [3].

In the past, HA were posited to consist solely of a system of coiled macromolecules having molecular weights in the range of tens to hundreds of thousands of Da [3]. In basic or low-ionic strength solution they had elongated shapes, but became coils in acidic or high-ionic strength solutions. Later, Wershaw [6] proposed that HA consist of ordered aggregates of amphiphilic molecules, composed mainly of relatively unaltered plant polymer segments possessing acidic functionality and held together by hydrophobic (π-π, charge transfer) bonds and H bonding interactions and the hydrophobic parts of the molecules are in the interior, with the hydrophilic part making up the exterior surfaces. Ordered aggregates of humus in soils were depicted as existing as bilayer membranes coating mineral grains and as micelles in solution.

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Recent research brought a new insight into molecular structure of humic substances. As demonstrated, despite the polymer theory which has never been unambiguously proved, the humic mixture consists of various molecules assembled together via weak interactions (π-π, CH-π, van der Waals and H-bonds) forming aggregates of apparently large molecular weight [7]. Low bonding energy implies the possibility of easy separation of distinctive classes of molecules allowing better characterization followed by a potential technological utilization. Based on the polarity of solvents (among others), the sequential extraction techniques allow the separation of free lipids, bound lipids, saccharidic and aromatic fraction (polyphenols, heterocycles) [8].

Coals of variable degree of coalification possess variable amount of extractable humic acids. Use of oxidation procedures represents an ideal inverse diagenetic process (Fig. 1) resulting in a higher content of extractable humic matter [9]. Oxidation products obtained from coals by pre-treatment with nitric acid, potassium manganate or by air oxidation have been reported as regenerated humic acids (RHA) [9].

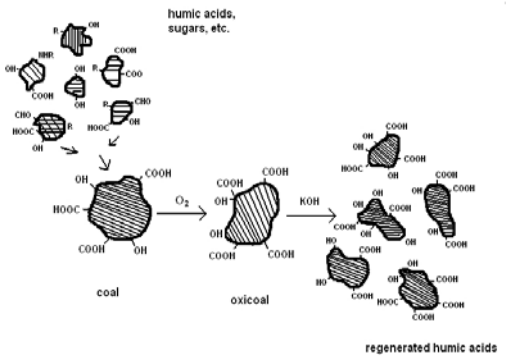

Fig. 1. Coal oxidation as and ideal inverse diagenetic process, adopted form ref. [9]

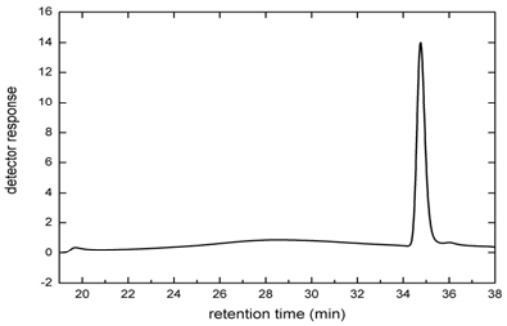

Fig 2. HPSEC analysis of humic acid extracted from parental lignite as detected by RI detector.

Generally, RHA have similar chemical-physical properties as humic substances which were precursors of coal formation. The elemental composition, functional groups content and distribution depends on the conditions of RHA formation and on the chemical properties of parental coal material. In their structure higher content of aromatic structures can be found in comparison with those humic acids extracted from parental coal. RHA also possess more semichinoidal radicals in their structure, i.e. 2×10^{18} spin g^{-1}. That content is minimally twofold higher in comparison with non treated humic samples [3]. Semichinoidal structures are easily oxidizable forming chinones which increases the rate of reduction reactions in soils. Such reactions are responsible for transport of metal ions in soils and support their adsorption by plant roots [9]. Thus technological/agricultural application of RHA bears promising potential.

Air oxidation of coal has already been studied and described in detail [9]. It has been supposed that coal structure contains same “weak” points that can be broken to depolymerize the original structure (Fig. 2). Oxidation at temperatures as high as 180°C was stated the most simplest way of production of RHA [9]. On the other hand, such approach is relatively demanding with respect to the price of the technological performance as well as with respect to the evolution of huge amount of carbon dioxide and other gases.

Since HA as well as RHA represent an interesting raw material useful in technological, agricultural and environmental applications the knowledge on the production is of a great interest [10]. Thus the aim of this work was to investigate the potential of air as a cheap oxidation agent of South Moravian lignite and to assess the chemical-physical character of obtained regenerated humic acids.

2. Experimental

Extraction of HA and production of RHA

Lignite from the Mikulčice locality (Czech Republic) was used in this work. Detailed information on the lignite chemical-physical properties were already reported elsewhere [11,12]. Using several screens, lignite particles were separated according to the particle size into three groups: 1-0.5 mm, 0.5-0,125mm and fraction <125 mm.

RHA were extracted from air pretreated lignite. Lignite was treated by two ways. In the first case, lignite was placed into a container and stream of air made lignite particles “fly” and therefore provided maximal contact with air. Therefore, kind of fluid reactor was used for this purpose. Temperature was

constant either at 25°C or 50°C. The second way of oxidation was carried out in the oven in which the lignite sample was let to be oxidized put on the floor at 85°C for the chosen time period.

Humic substances were extracted from pretreated and parental lignite according to the procedure described earlier[13]. Briefly, a lignite fraction was mixed with the aqueous 0.5 M NaOH and 0.1M $Na_4P_2O_7$ solution (1:10) and stirred for 3 hours. After centrifugation, the supernatant was treated with the concentrated HCl until pH was about 1 in order to precipitate the humic acids (fraction HA). HA were then treated with 0.5% (v/v) HCl-HF solution overnight to remove residual ashes, dialyzed (Spectra/Por® dialysis tubes, 1000 *Mw* cut-off) against distilled water until chloride-free. Obtained products were dried in the oven to constant weight.

All humic samples (50 mg) were suspended in CO_2 free distilled water (60 ml) and titrated to pH 7 with 0.1M NaOH solution employing an automatic titrator as described earlier [14]. The resulting sodium humates were freeze-dried and homogenized in an agate mortar for further HPSEC analyses.

High performance size exclusion chromatography (HPSEC)

The HPSEC system Agilent consisted of a pump equipped with two detectors in series: a UV/VIS detector set to 280 nm for humic analyses, and a refractive index detector (RI). An automatic injector, with a 100 µl sample loop, was used to load HPSEC solutions and a Phenomenex Biosep S2000 (600 × 7.5 mm) column was used for size exclusion separations. The column was preceded by a Biosep Guard column and a 0.2 µm stainless-steel inlet filter. Flow rate was set to 0.6 ml min^{-1} while the HPSEC eluent was a 50 mM $NaH_2PO_4.H_2O$ solution adjusted to pH 7 with 1M NaOH. The buffer concentration was chosen to have a constant ionic strength of 50 mM in order to minimize ionic exclusion or hydrophobic interactions with the column.

All solutions were filtered through quartz filters (Glass Microfibre Filter Whatman International, LTD) before injection. The HPSEC analyses were conducted in duplicate and no significant differences were observed between measurements. For the calculation of weight average molecular weight (*Mw*) following equation was used:

$$M_w = \frac{\sum_{i=1}^{N} (h_i M_i)}{\sum_{i=1}^{N} h_i}$$

where M_i and h_i are the molecular weight and the height of each i-th fraction in the chromatogram, respectively.

Standards of known *Mw* such as polysaccharides (PS) of 186, 100, 23.7 and 12.2 kD, (Polymer Sciences Laboratories, UK) and sodium polystyrenesulphonates (PSS) of 169, 123, 30.9 and 6.78 kD (Polymer Standard Service, Germany) were used for column calibration. Calibration curves were semi-log linear over the range defined by standards and were used to obtain the molecular weights of humic samples.

FTIR spectroscopy

Conventional KBr pellet technique was used. From humic samples previously dried at 105°C for 2 hours and cooled and stored in desiccator were mixed in agate mortar with KBr (1:200 w/w). For this purpose Nicolet Impact 400 was employed.

3. Results and discussion

Production of RHA extracted from South Moravian lignite has been already published [12]. It has been demonstrated that the content of RHA can be increased from 20% to 60% using nitric acid or 45% using hydrogen peroxide. Nevertheless, such approach was associated with a loss of a huge amount of carbon due to the intensive oxidation attack.

3.1 Influence of particle size on the yield of humic acids

In the first step, the attempt was to evaluate the influence of particle size on the yield of extraction of humic acids from lignite. For this purpose 3 particle size fractions mentioned in Experimental were used. Table 1 reports the results of the experiment.

Table 1 Yield of extraction of humic acids from lignite of various particle sizes

Fraction (mm)	1-0.5	0.5-0.125	0.125>
Yield (%)	12.0	23.0	22.0

One can see that the particle size is an important parameter for the production of humic acids. While large particle diameter provided a relatively low yield, particles smaller than 0.5 mm gave yield above 20%. It is logical since lower particle size is connected with larger area and it seems that the extraction agent could not reach to the particles interior and extracted solely the humic material from the particle surface. Additional conclusion can be made considering that humic acids are more abundant in particles with lower diameter. This assumption can be justified by the fact that in the structure of lignite, there can be identified parts of different degree of coalification as well as non-decomposed moieties of parental phytomass having different mechanical properties.

Therefore, in the next experiments, different fractions were used to test the efficiency of suggested oxidation methods.

3. 2 Oxidation in a fluid reactor

This part of the work has been devoted to the evaluation of influence of air oxidation on the yield of RHA extracted from oxidized lignite. For this purpose the fraction 1-0.5 mm was chosen. The obtained data are summarized in Table 2.

Table 2 Oxidation of fraction 0.5-1 mm in the fluid reactor

Oxidation parameters	24 hours at 25°C	72 hours at 25°C	120 hours at 25°C	72 hours at 50°C
Yield* (%)	-15.63	-7.97	75.42	5.46

**Increase (+) or decrease (-) with respect to the value given in Table 1*

As can be seen in Table 2, the oxidation in fluid reactor did not give rise to the yield increase in all cases. We assume that it is caused by the composition of the particles having diameter 0.5-1 mm. In fluid state particles are supposed to be under the mechanical stress (milling) and thus the particle size is decreasing (verified by a parallel experiment). Such processes should lead to the increase in the yield of humic acids. However, it seems that such way of oxidation preferably supports the oxidation of COOH groups of humic acids responsible for their solubility. Kinetics of oxidation of other structures, resulting in formation of other carboxylic groups is evidently slower which is indicated by the yield after 72 hours at 25 and 50 °C and after 120°C.

3.3 Oxidation in stationary phase

In this part of the work we have tested the influence of air oxidation on the yield of humic acids. Since oxidation in fluid reactor was not too sufficient for fraction 0.5-1 mm, we used the fraction 0.5-0.125 to see whether in this case the yield increase will be more intensive. Temperature 85°C was chosen with respect to the results reported recently, which indicated that at temperature above 80°C lipid fraction present in humified matter forming a specific domain is melted [15] and, thus, some parts of lignite are better accessible for oxygen diffusion. Again, similar trend as in case of fluid reactor has been observed (Table 3). Oxidation of lignite brought about a decrease in the yield of RHA with the minimum after 120 hours of oxidation. Further, an increase was observed which resulted in 18% abundance after 456 hours. It seems that lignite is relatively resistant to the oxidation attack under these conditions and the kinetics of oxidation of carbon skeleton is relatively slow. Although the increase of HA content using such simple and chemically mild methodology is low, the next part is devoted to the characterization of selected humic acids obtained during above-mentioned procedures.

Table 3 Oxidation of fraction 0.5-0.125 mm in the static reactor

Oxidation period (hours)	72	120	240	456
Yield* (%)	-22.94	-43.65	-5.51	17.98

**Increase (+) or decrease (-) with respect to the value given in Table 1*

3.4 Characterization of HA and RHA

Selected samples were characterized for their elemental and ash content. Obtained values are reported in Table 4. Values are given in weight %. Chemical properties of humic acids extracted from fraction 1.0-0.5mm did not practically differ from RHA prepared for 24 hours at 25°C, i.e. RHA1, so the latter is used as a reference. Samples produced in fluid reactor were denoted as follows: RHA2 and RHA3 are regenerated humic acids produced for 72 and 120 hours, respectively, and RHA4 stands for regenerated humic acids produced 72 hours at 50°C. Samples RHA5, RHA6, RHA7 and RHA8 were prepared at 85°C in the static reactor for 72, 120, 240, 456 hours, respectively. Sample HA9 represents humic acid extracted from parental lignite.

Table 4 Elemental composition, ash and moisture content of parental and regenerated humic acids, ash content in all samples up to 2.2%, traces of sulphur are included in O content.

	C (%)	H (%)	N (%)	O*(%)	C/H	C/O	Moisture
RHA1	56.07	4.82	1.28	37.22	11.75	1.52	8.34
RHA2	57.37	4.80	1.36	36.47	11.95	1.57	7.63
RHA3	56.62	4.89	1.34	37.15	11.58	1.52	7.85
RHA4	56.81	4.45	1.29	37.45	12.76	1.52	8.16
RHA5	56.91	4.66	1.37	37.06	12.21	1.54	7.61
RHA6	57.21	4.97	1.29	36.53	11.51	1.57	7.98
RHA7	57.72	4.72	1.26	36.30	12.22	1.59	7.03
RHA8	57.42	4.84	1.29	36.45	11.86	1.58	7.48
HA9	57.21	4.62	1.05	37.22	12.38	1.53	n.d.

**calculated by difference*

Decreasing C/H ratio appeared to be related to decreasing aromaticity or degree of unsaturation [13]. Basically, the higher C/H value the higher is the content of aromatic structures in humic material. Ratio C/O indicates the proportion between oxygenated carbons and indirectly also the content of COOH groups.

In fact, the elemental content and its ratios did not show any remarkable changes in composition of RHAs. As mentioned previously, the reason can be seen in the kinetics of oxidation processes as well as in the strength of oxidation agent.

Fig. 2 shows a representative HPSEC of humic acid extracted from parental lignite detected RI detector, Table 5 reports the respective *Mw* of all HA and RHAs based on sodium salt polystyrene sulfonates (PSS) and polysaccharides (PS) calibration. Although the comparison of *Mw* among each other has an importance, the values should be taken as apparental since there is no humic material which can be used for the molecular weight calibration [16]. Further, as mentioned before, humic acids should be considered as a mixture of relatively small molecules and the data reflect more the aggregation properties of humic acids. Aggregates are stabilized by weak interactions (π-π, CH-π and van der Waals and by H-bonds) which depends predominantly on the pH value and ionic strength [17]. In our case, analysis resulted in two distinctive peaks. As demonstrated recently, largest molecular size fractions excluded from column first contain aromatic, alkyl and potentially carbohydrate-like content. The carbohydrate-like and the alkyl chain length seem to decrease with decreasing molecular size. Progressive reduction of aromatic carbon atoms was also observed with decreasing molecular size of the separated fractions. Further, the abundance of hydrophilic molecules have been reported in low molecular fraction [16,18].

Table 5. HPSEC based molecular weight calculated from UV and RI records

	PSS calibration (UV)	PS calibration (RI)
RHA1	9232	9444
RHA2	12646	10760
RHA3	13404	13710
RHA4	7279	8442
RHA5	15213	15030
RHA6	10323	10840
RHA7	12881	13540
RHA8	17460	16940
HA9	9961	nd

In contrast to the elemental analysis the HPSEC analysis revealed better the differences among humic sample (Table 5). Although there is a shift between RI and UV calibrations, the mutual comparison within HA and RHA is the same. Comparison of samples in the first set prepared in the fluid reactor shows the increase in *Mw* with increasing time of oxidation. On the other hand, elevated temperate of oxidation (50°C) brought a significant decrease in *Mw*. It means that the oxidation of lignite progressively liberates the soluble fractions with larger dimensions while temperature can cause their size reduction.

The oxidation in stationary phase at 85°C showed also increasing tendency. The exception is RHA6 which gave a lower value of *Mw* than RHA5, although such value is still higher than that of HA extracted from non-treated lignite. It is noteworthy, that *Mw* of sample RHA8 is almost two-fold larger than the original HA9.

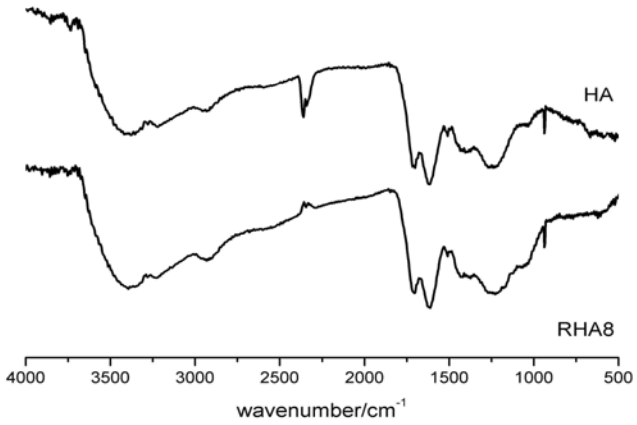

Fig. 3. Comparison of FTIR spectra humic acid extracted from parental lignite (HA) and regenerated humic acid (RHA8)

FTIR spectroscopy measurement of humic acids and RHA showed typical spectra published and described by many authors [3,12]. In this work only the FTIR spectra of HA and RHA8 are reported (Fig. 3). First, some notes to the attribution of peak wave numbers to chemical bonds vibrations. Fig 4 shows an intensive peak within the range 3400–3420 cm^{-1} which can be attributed mainly to the hydrogen bonded –OH; weak peaks in the region 2850–2960 cm^{-1} (aliphatic C–H stretching), strong sharp peaks around 1710 cm^{-1} (C=O of COOH) and 1620 cm^{-1} (C=C stretching, C=O stretching of COO^{-}, ketonic C=O and aromatic C=C conjugated with COO^{-}), a peak around 1510 cm^{-1} (N-H deformation, C-N stretching vibration, C=C aromatic bounds) 1450–1400 cm^{-1} (aliphatic C–H bending, and COO^{-} asymmetric stretching, and possibly C=C and C=N plane vibrations of heterocycles), peaks around 1250 cm^{-1} (aromatic C, C-O stretch), a weak peak around 1050 cm^{-1} (C-O of polysaccharides and Si-O) were observed. The comparison of relative intensities of aliphaic and aromatic bands gave similar results as those reported for elemental analysis. Further, deeper analysis and comparison of peaks in the area 1710 cm^{-1} revealed the slight increase in abundance of esters in samples with higher molecular weight. Therefore, it can be assumed that oxidation of parental lignite led to oxidative disruption of weak points in lignite resulting in progressive increase in yield of extractable humic material. It is likely that higher temperature together with air atmosphere support the reactions between COOH and OH- alcohol groups, i.e. esterification. The increase in molecular weight, i.e. increase in number of ester is significant especially for samples developed at temperatures above 80°C when lipidic fraction is melted and recombination reactions can occur. Such conclusion is in agreement with the ability of regenerated humic acids to form gels having high water holding capacity [9]. However, in this work, gel formation was attributed to the higher ability of regenerated humic acids to form H-bridges due to the increase in content of COOH groups. To shed light on the influence of ester groups on water holding capacity is beyond the scope of this paper and it will be investigated in a special article.

4. Conclusion

The influence of lignite air oxidation on the yield and chemical/physical character of regenerated humic acids (RHA) has been shown. RHA were produced under different experimental conditions, i.e. both in static and fluid reactor. Oxidation in the fluid reactor brought first a decrease and after a certain period increase in the yield of RHA. The same observation was done in case of static reactor. It has been verified that higher temperature during the oxidation supports the production of RHA. Despite the difference in the yield of RHA (increase up to 75%) their chemical character was in all cases fairly similar. Some tiny differences were revealed using FTIR which showed the increase in number of ester bonds in RHA produced mainly at temperatures above 80°C causing a significant increase in apparent molecular weight as evaluated by HPSEC.

Acknowledgements

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APPENDIX B

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Lignite pre-treatment and its effect on bio-stimulative properties of respective lignite humic acids

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ABSTRACT

Humic acids originating from South-Moravian lignite were subjected to a comparative study with the aim to assess the alteration of their physico-chemical properties after various lignite pre-treatments. Physical modification was achieved with two organic acids, such as acetic acid and citric acid and chemical modification by nitric acid and hydrogene peroxide in various concentrations. Elemental analysis, solid-state NMR, GC–MS analysis of polyols and size exclusion chromatography were carried out for chemical–physical characterization of obtained humic acids. Their biological effect, in form of potassium and ammonium humates, was tested on maize (*Zea mays*) seedlings. In these tests, potassium humates achieved far better overall results than ammonium humates. Results were inter-correlated in order to appraise the influence of humic acids physical and chemical properties on biological activity. Surprisingly, fractions with the lowest molecular size (0–35 kDa) showed no correlation with bioactivity results (Pearson coefficient from 0.05 to −0.4). On the contrary, middle-sized fractions (35–175 kDa) showed highly significant positive correlation (Pearson coefficient up to 0.92) and the highest molecular-size-fractions (275–350 kDa) showed negative correlation (Pearson coefficient up to −0.75). These findings were identical for both potassium and ammonium humates. No connection was found between bioactivity of humates and polyols content which was remarkably high; it reached 150 mg per g of humic acids in the most extreme case of 5% hydrogene peroxide pre-treatment. In the final analysis, the preparation mode bore pivotal responsibility for the control of humic acids biological effect and showed the best results for potassium humates obtained from lignite pre-treated by acetic acid and by 2% hydrogen peroxide.

1. Introduction

Long-term deficiency of basic crop products in some regions of the world as well as recent global increase of food prices have aroused a deep discussion concerning current ways of food production, soil productivity and respective soil treatment (Lal, 2007). It is indisputable that soil management system is the crucial determinator for soil organic matter quality and quantity and, as a result, the final soil productivity. Some studies suggest that conventional tillage may be replaced by non-tillage and cropping systems in order to improve the quality of cultivated soils. The study of Bayer et al. (2000) demonstrates how the addition of crop residues under strict non-tillage regime increased total organic carbon (C) and total nitrogen (N) in the subtropical soil to the depth of 0–17.5 cm, and that this increase is directly related to C and N added or recycled by the system. Soil residue concentration at soil surface layer determined great input of metabolisable organic compounds, sometimes higher than the capacity of soil microorganisms to metabolise them. The same experiment brought no significant C and N accumulation under conventional tillage, due to high rates of organic matter decomposition (Bayer and Mielniczuk, 1997). By sinking the CO_2 emissions from soils, non-tillage system benefits both to agriculture and the environment. Similar experiences with a non-tillage approach were reported by Reicosky et al. (1999) for soils in a temperate climate.

Besides the addition of crop residues, added attention to plant growth support is a proper way for increasing the biomass production and maintaining the carbon dynamics in soil. Continuous researches in this field (Malik and Azam, 1985; Lobartini et al., 1992; Govindasmy and Chandrasekaran, 1992; Valdrighi et al., 1996; Khan et al., 2006) show that humic substances from different sources have beneficial effects on plant growth regulation. An overview of these effects was summarized by Popov (2008). Besides the

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influence on osmotic pressure, intercellular diffusion, transport, proteins synthesis, interaction with enzymes and some growth regulators, detoxication, etc., Popov (2008), when describing the biological effect of humic substances attaches a great deal of importance to the surface properties, e.g. influence upon bioelectrical reactions, viscosity of protoplasm, properties of cell walls and transport channel walls, penetrability of intercellular canaliculi and ionophore effect or selectivity of cell membrane.

Despite to some partially successful attempts for foliar applications of leonardite extracts containing humic substances (HS) (Fernández-Escobar et al., 1996), currently, the stimulation of roots is more pronounced than that of overground parts of plant (Antošová et al., 2007). As far as it is known, the interactions between earthworms and microorganisms can produce significant quantities of plant growth hormones and HAs which act as plant regulators (Arancon et al., 2006). Investigation of the earliest stages of root development under the influence of HAs isolated from earthworm compost was conducted by Canellas et al. (2002). Authors observed both enhanced elongation and proliferation of secondary roots. HAs treatment clearly increased the number of lateral root emergence from 7 to 12 times, compared with the control values. It was stated, that this might be associated with H^+-ATPase activity, stimulated indirectly by promoting an increase in the concentration of H^+-ATPase in membrane vesicles of HAs treated samples. They also speculate that this auxin-like effect is due to the presence of intrinsic small bioactive molecules such as indol-3-acetic acid clustered within the HAs supramolecular arrangement. The idea of plant growth hormones adsorbed onto the humates is mentioned in the work of Atiyeh et al. (2002). These might be released due to polarity changes caused by the interactions between soluble HAs and root exudates (Nardi et al., 2000). Similarly, Canellas and Facanha (2004) promote the idea that the activity of the plasma membrane H^+ pumps can be used as a biochemical marker for HS bioactivity. The phyto-hormone-like activity of HS was also a subject of studies by Pizzeghello et al. (2001). They found that acid conditions in soil environment were essential for the auxin-like activity, whereas neutral conditions favoured the gibberellin-like activity. These conclusions were backed up by the follow-up study by Pizzeghello et al. (2006).

The biological effect of HAs was found to be related to their molecular size. Especially low-molecular-size-fractions (LMS) exhibit auxin-like activity and support nitrate uptake by decreasing the pH at the surface of roots and, consequently, facilitating the H^+/NO_3^- transport (Muscolo et al., 1999). These findings were followed by studies by Nardi et al. (2002, 2007) who described the humic LMS effect on enzyme activities and promotion of metabolic pathways. The effect was attributed to a flexible conformational structure of this fraction that allowed a more efficient diffusion of bioactive humic components to plant root cells.

Another easy option for HAs to feed the plant growth would be the utilization of minerals, nutriments and water that are retained by HAs. The clustering capacity of HAs is directly linked to their quantity of organic sites (i.e. carboxyl and hydroxyl). Cations can be released under certain favourable conditions (low pH). The hydration capacity is associated with the quantity of constitutive polymers and low molecular weight compounds, such as carbohydrates (i.e. cellulose and polyols). In HAs extracted from an aged source such as lignite, the content of native carbohydrates is questionable. On the other hand, possibility of a long-term persistence of readily degradable products such as carbohydrates (or their derivatives) was already reported (Allard and Derenne, 2007).

The chief aim of this study is to prepare and evaluate HAs obtained from lignite pre-treated in varied ways. Lignite can be pre-treated either in gas phase or in liquid phase. The air oxidation does not bring satisfactory results and proves to be both instrumentally demanding and time-consuming (Kučerík et al., 2008). Thus, only the liquid phase is used to prepare samples for this study. Chemical oxidation with HNO_3 and H_2O_2 is used in order to produce so-called regenerated HAs (Kučerík et al., 2003). Physical modification is performed with acetic and citric acids. Such short-chained organic acids were previously found to have a significant influence on the quaternary HAs structure (Conte and Piccolo, 1999). The exposure of parental matter to these agents could lead to similar changes in the structure of HAs. These amphiphilic-like agents as well as the oxidative agents applied in lignite pre-treatment could serve for the production of HAs with a certain standard of desirable and well defined properties.

2. Material and methods

2.1. Humic acids

South Moravian lignite collected from the Mír mine in the area of Mikulčice, near Hodonín, the Czech Republic (Kučerík et al., 2003), was used as a source of HAs in this study. Each lignite sample, milled and sieved was pre-treated with various agents, details are shown in Table 1. First, the portions of original lignites were soaked at 45 °C for 30 min under constant stirring in a suspension with the agent in question (50 g of lignite in 500 mL of an agent). Pre-treated lignite was then carefully washed with deionized water on the porous glass until it became agent-free. Next, HAs were extracted by standard alkaline method with 0.5 M NaOH and 0.1 M $Na_4P_2O_7$ as specified before by Conte and Piccolo (1999). For comparison, HAs from parental lignite with no pre-treatment were also extracted. HAs samples (50 mg) were suspended in deionized water and titrated with 0.1 M KOH (0.1 M NH_4OH) solution to pH 7 and freeze-dried to prepare potassium (ammonium) humates to be used for the biological activity assessment and physical–chemical characterization.

2.2. Solid-state NMR measurement

Cross polarization magic angle spinning (CPMAS) ^{13}C-NMR spectra were acquired with a Bruker AVANCE™ 300, equipped with a 4 mm Wide Bore MAS probe, operating at a ^{13}C resonating frequency of 75.475 MHz (CERMANU, University of Naples, Portici, Italy). The humic samples were packed in 4 mm zirconia rotors with Kel-F caps. 1000 scans with 3782 data points were collected over the acquisition time of 25 ms, the recycle delay of 2.0 s, the contact time of 1 ms. The Bruker Topspin 1.3 software was used to collect spectra and the areas of each region of the spectra were attributed to certain groups of carbon atoms, results are reported in Table 3.

2.3. HPSEC measurement

To assess the molecular size distribution of humic samples the Biosep S2000 column from Phenomenex with the dimensions of 600 × 7.8 mm was used. The Agilent HPSEC system consisted of a solvent pump equipped with two detectors in series: a UV/VIS detector, set to 280 nm, and a refractive index (RI) detector. Humic solutions were loaded by the auto-injector with a 100 μl sample loop and eluted at a constant flow rate of 0.6 mL min^{-1}. The column was preceded by Biosep Guard column with a 0.2 μm stain-less steel inlet filter. The samples were dissolved in the mobile phase to achieve the concentration of 0.6 mg mL^{-1} and they were filtered through a 0.45 μm filter before injection. The mobile phase $NaH_2PO_4.H_2O$ was prepared in molar concentration of 50 mM and adjusted to pH 7 with 1 M NaOH in order to keep constant ionic strength and minimize potential ionic exclusion or hydrophobic interactions with the column stationary phase (Peuravuori and

Table 1
Elemental composition (atomic %) of parental and pre-treated lignite HAs samples and their respective ratios.

Sample	pre-treatment		W[a] (%)	A[b] (%)	C[b] (at. %)	H[b] (at. %)	N[b] (at. %)	S[b] (at. %)	O[b] (at. %)	C/H	C/O
HA1	–	Mean	3.33	1.84	45.4	36.4	1.17	0.54	16.5	1.25	2.75
		SD	0.20	0.22	0.2	0.4	0.07	0.10	0.4		
RHA2	5% HNO_3	Mean	3.14	1.25	45.9	36.2	1.08	0.45	16.4	1.26	2.79
		SD	0.10	0.13	0.4	0.2	0.02	0.13	0.3		
RHA3	10% HNO_3	Mean	2.93	1.84	43	36.6	2.61	0.36	17.4	1.17	2.48
		SD	0.11	0.06	0.2	0.4	0.16	0.11	0.4		
RHA4	20% HNO_3	Mean	2.38	1.29	42.4	36.4	3.08	0.27	17.9	1.16	2.36
		SD	0.08	0.08	0.2	0.4	0.18	0.09	0.2		
RHA5	2% H_2O_2	Mean	2.17	1.74	44	37.8	1.04	0.43	16.7	1.17	2.64
		SD	0.10	0.11	0.3	0.4	0.05	0.25	0.5		
RHA6	5% H_2O_2	Mean	2.14	1.05	43.7	38.4	0.95	0.43	16.6	1.14	2.64
		SD	0.05	0.06	0.2	0.5	0.03	0.23	0.3		
RHA7	20% acetic acid	Mean	2.25	1.44	45.2	36.3	1.16	0.45	16.9	1.25	2.68
		SD	0.12	0.10	0.3	0.2	0.07	0.12	0.3		
RHA8	20% citric acid	Mean	2.1	1.37	45.5	36.2	1.17	0.45	16.7	1.26	2.72
		SD	0.03	0.10	0.3	0.3	0.09	0.16	0.2		

[a] Ash free sample.
[b] Dry sample; SD – standard deviation.

Pihlaja, 1997). The weight-average molecular weight M_w was calculated according to the following formula:

$$M_w = \frac{\sum_{i=1}^{N}(h_i M_i)}{\sum_{i=1}^{N} h_i}$$

where M_i and h_i are the molecular weight and the height of each ith fraction in the chromatogram, respectively (Mori and Barth, 1999).

Standards of known M_w were used for column calibration. Polysaccharides PSC (404, 212, 112, 47.3, 5.9 and 0.667 kDa) from Polymer Laboratories were chosen to calibrate the RI detector and sodium polystyrenesulphonates PSS (194.2, 145, 32.9, 14.9, 6.53 and 0.91 kDa) from Polymer Standards Service were used to calibrate the UV detector. Calibration curves defined by standards were used to obtain the M_w of humic samples. Each sample was measured twice and the standards were put in between each set of 8 samples for reproducibility control.

Water was used to determine the total volume of the column (22.8 mL) whereas blue dextran (2000 Da) was used to measure the void volume (10.5 mL).

2.4. Determination of polyols by GC–MS

The procedure commonly used for the analysis of neutral sugars in soils, water and sediments was adopted (Modzeleski and Laurie, 1971; Oades et al., 1970; Sigleo, 1996; Ogier et al., 2001). The HAs hydrolysis was carried out in two successive stages. The first stage aimed to release rather labile compounds, while the second stage, conducted on the solid residue of the first stage, after soaking with concentrated acid, was performed to release especially compounds linked to the organic matrix with more resilient bonds such as, in soils, the cellulosic glucose present in vascular plant cellulose (Vallentyne, 1963; Cowie and Hedges, 1984). Approximately 10 mg of HAs were introduced in a Pyrex tube together with 5 mL of 1.2 M HCl. The tube was tightly closed under vacuum and heated for 3 h at 100 °C. After hydrolysis and cooling, the sample was filtered through 0.45 μm GF/F filters. The solution was kept for analysis while the solid residue and the filter were introduced in another Pyrex tube and soaked for 12 h at ambient temperature with 2 ml of 12 M HCl. Afterwards, the concentrated acid solution was diluted to 1.2 M HCl and the tube was closed under vacuum. Then, the second hydrolysis was performed under the same conditions as above. In each hydrolysate, 1 mL of a 0.2 mg/mL solution of 2-deoxy-D-glucose was added as an internal standard (Wicks et al., 1991). The hydrolysates were then concentrated to 1–2 mL in a rotary evaporator at a temperature not exceeding 50 °C. Subsequently, the residual water and acid were evaporated 25–30 °C to achieve dry rest, after the addition of 20 mL of propan-2-ol which forms an azeotrope with H_2O and HCl. The two flasks containing the two hydrolysates were dried during 24 h over KOH in a dessicator. Silylation was performed by adding 0.1 mL of pyridine and 0.1 mL of a mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1) (Supelco) and leaving the vials for 1 h at 70 °C. Aliquots of 1 μL of each silylated samples were directly analyzed by capillary GS using a Hewlett–Packard 6890 GC (split injector, 250 °C; flame ionization Detector (FID) at 300 °C) with a fused silica capillary column (SGE BPX 5%, 30 m length, 0.25 mm id., 0.25 mm film thickness) and helium as a carrier gas. The GC temperature was programmed from 60 to 300 °C at $5°C \cdot min^{-1}$ (isothermal for 20 min final time). The GC-MS analyses were performed on a Trace GC Thermo Finnigan coupled to a Thermo Finnigan Automass (under the same GC conditions). The MS was operated in the electron impact mode with a 70 eV's ion source energy and the ion separation was operated in a quadripolar mass filter. The various products were identified on the basis of their GC retention times, their mass spectra (comparison with standards) and literature and library data (NIST 1.7).

2.5. Biological activity assessment

The biological activity of the different HAs was tested on *Z. mays* using the methods suggested by Antošová et al. (2007). In principle, the roots of germinating seeds grow in nutritive solution in the presence of tested substance of defined concentration. The growth rate of roots, which length is measured, determines the stimulative effect of the tested substance.

Experiments were carried out in an air-conditioned sterilized container, where the light, temperature and humidity conditions were set according to the optimal requirements of plant species used. These were for 14 h of simulated daylight at the temperature of 30 °C and for 10 h of darkness under temperature of 20 °C. Humidity was fixed at 60 ± 2%. In each experiment 28 non-sterilized seeds were used (experiments were previously carried out also on microbiologically protected seeds, however, no difference was

Table 2
Composition and physical–chemical properties of the Hydropon control solution.

	% wt.		mg kg^{-1}
N	3	Fe	190
P	1.27	Mn	107
K	4.2	Zn	35.1
Ca	1.92	Cu	<35
Mg	0.5	Mo	<35
pH		5.5–6.5	
Density		1.18 g cm^{-3}	

observed). First, all seeds were placed into a germination box for 2 days. In the germination box, the germinal root of the seed reached the minimal length which enabled its placement into the experimental container. The container consisted of 14 tubes, each holding 2 seeds, and 5 L of experimental solution (Antošová et al., 2007).

Water solution of the commercially produced Hydropon fertilizer (0.59 g/L) is used as control solution. Its composition and physical properties are given in Table 2. In order to prepare experimental solutions, the control solution was enriched by tested substances (potassium and ammonium humates) until the concentration of 100 mg/kg was achieved. A comparative solution of the same concentration was prepared by addition of humate B03H (HAs used as a comparative sample in Antošová et al. (2007) into the control solution.

The root lengths were recorded in given time intervals, i.e. after 48, 72 and 96 h. The intensity of stimulating effect of the tested substances is reported in relative units, which express the accrual, eventually the shortening of root length in comparison either with the control solution or with the comparative solution (Table 5). The work on the biological activity assessment was performed at the Research Institute of Inorganic Chemistry, Inc., Ústí nad Labem, Czech Republic.

3. Results and discussion

3.1. Molecular characteristics

The elemental analysis of samples (Table 1) shows the compositional elements and their ratios distribution throughout the line of eight HAs. Briefly, the carbon content was moderately decreased by all of the applied pre-treatment methods, namely in association with the growing concentration of an agent. Nitrogen content slightly increased in samples pre-treated with HNO_3, decreased with peroxide and did not change with amphiphilic-like treatment. The oxygen content was softly increased in all samples with respect to the original HA1, but the C/O ratio indicated that a really significant difference caused only nitric acid treatment, which was also proved by the C/(N + O) ratio. C/H ratio suggested that in comparison with the original HA1 sample, the aromatic content declined in oxidized samples, in the HNO_3 line in particular and with increasing strength of the acid. Amphiphilic-like agents slightly increased the aromatic content. This finding is also supported by the NMR data. Here, the aromatic content was established within the interval of 169–98 ppm. Further intervals of the NMR spectra were attributed to certain molecular components as listed in Table 3.

Generally, each spectrum of the used HAs was typical of a humic acid extracted from lignite with three major peaks of alkyl carbons at around 30 ppm, aromatic carbons at around 125 ppm and carboxyl groups at around 180 ppm, as depicted in Fig. 1. The differences between original and pre-treated samples as well as between pre-treated samples themselves were slight, but still distinct enough to compare the harms brought about by different oxidizing and amphiphilic-like agents. Hence, the HNO_3 treated samples appeared to be composed of a higher portion of alkyls, ethers and alcohols at the expense of aromatics and C═O components, the alteration getting more distinct with HNO_3 concentration. Surprisingly, the assumption that increased yield of HAs induced by oxidative regeneration (previously published in Kučerík et al., 2003) would be a consequence of increased carboxyl content, was not proven. On the contrary, the carboxyl content decreased clearly with increasing HNO_3 concentration. The reason for such curiosity can be seen in the fact that oxidation was performed with the parental lignite and with on the extracted HAs. Thus, the extractable lignite content (HAs) increased while the average COOH content in HAs slightly decreased. Similar results were already published for the air oxidation of South Moravian lignite (Kučerík et al., 2008). The H_2O_2 samples gave mixed results, the aromatic content decreased, carboxyls and C═O components decreased only slightly and alkyl content was on the highest level in comparison with other samples. Finally, the NMR signal of samples treated with amphiphilic-like agents revealed the interesting fact that those altered neither aromatic nor carboxylic content.

Table 3
Carbon distribution (%) in resonance intervals (ppm) of CPMAS ^{13}C-NMR spectra of the lignite HAs samples (measured in the form of potassium humates).

Sample		261–191 ppm (ketones, aldehydes, quinones)	191–169 ppm (carboxyls)	169–98 ppm (aromatic C)	98–68 ppm (ethers, alcohols)	68–0 ppm (alkyl C)
HA1	Mean	7.09	7.56	45.80	4.97	34.57
	SD	0.21	0.43	0.84	0.71	0.40
RHA2	Mean	5.07	7.26	46.77	5.15	35.74
	SD	0.33	0.52	0.82	0.83	0.36
RHA3	Mean	4.57	6.70	40.13	8.34	40.25
	SD	0.12	0.58	1.07	0.67	0.47
RHA4	Mean	2.79	5.89	37.58	9.61	44.13
	SD	0.23	0.52	0.93	0.82	0.47
RHA5	Mean	5.85	6.91	40.66	5.97	40.59
	SD	0.29	0.49	0.85	0.87	0.41
RHA6	Mean	5.96	6.87	40.53	6.71	39.93
	SD	0.18	0.56	0.77	0.85	0.45
RHA7	Mean	5.51	7.81	46.34	5.52	34.81
	SD	0.09	0.51	0.81	0.73	0.43
RHA8	Mean	4.76	7.48	45.99	6.07	35.69
	SD	0.11	0.46	0.80	0.81	0.45

SD – standard deviation.

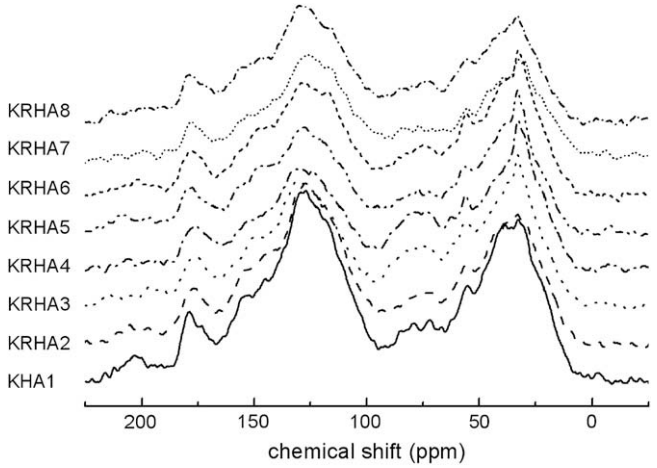

Fig. 1. CPMAS ^{13}C-NMR spectra of lignite HAs samples before (HA1) and after modification (RHA2–RHA8) measured as potassium humates.

3.2. Size distribution of aggregates

Differences in elemental and molecular composition of HAs in solid indicate the differences in conformational arrangement of humic aggregates in solution (Conte and Piccolo, 1999; Piccolo et al., 1999; Cozzolino et al., 2001). Therefore, HPSEC was employed to test further physical–chemical properties of produced HAs, especially the quaternary structure and distribution of aggregate dimensions. Chromatograms of the humates recorded by two detectors produced retention profiles as anticipated, examples of HPSEC are given in Fig. 2. While the UV detector determined the molecular absorptivity of chromophores at the wavelength 280 nm and RI detector evaluated the overall size distribution of humic samples. As it can be seen, there was recorded a bimodal distribution in the UV-detected profile. Generally, the intense peak at the retention time of 18 min represented the largest components (aggregates) of humic sample while with increasing retention time, the smaller ones were excluded. The weight-average molecular weights M_w of all samples based on respective calibrations are reported in Table 4. The comparison of potassium and ammonium humates clearly shows that the humate aggregate size distribution is a function of their counterions. In this case, the M_w values for ammonium humates were distinctly larger than those for

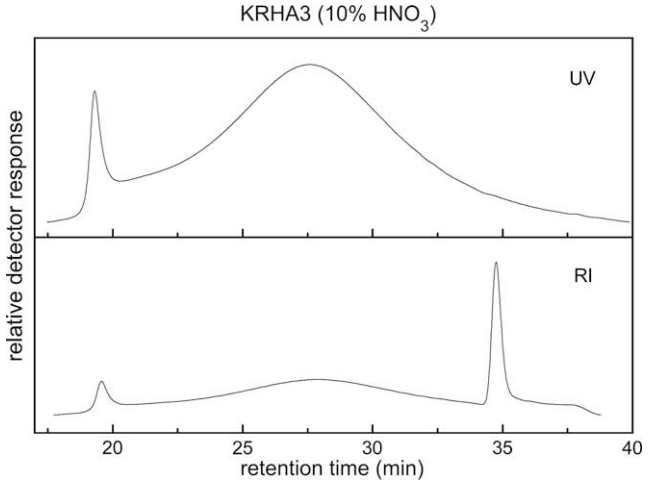

Fig. 2. Exemplary HPSEC chromatograms of the 10% HNO_3 pre-treated sample (KRHA3) detected by UV detection (VWD-UV) at 280 nm and refractivity index (RID).

Table 4
Weight-average molecular weights (M_w) of parental and pre-treated lignite HAs samples (g/mol). VWD-UV stands for data obtained from UV detector at 280 nm and RID means refractivity index.

Sample	VWD-UV	RID	Sample	VWD-UV	RID
KHA1	12,834	15,299	NHA1	15,299	24,427
KRHA2	11,881	15,949	NRHA2	15,949	21,142
KRHA3	15,294	21,223	NRHA3	21,223	26,334
KRHA4	7793	13,083	NRHA4	13,083	14,579
KRHA5	20,594	22,409	NRHA5	22,409	29,602
KRHA6	17,557	21,554	NRHA6	21,554	27,580
KRHA7	12,154	14,679	NRHA7	14,679	20,184
KRHA8	12,161	11,997	NRHA8	11,997	19,166

potassium humates. Plausible reasons for analogous outcome with Na^+ and NH_4^+ have been already discussed by Maia et al. (2008). For the same experimental conditions as used in our study the authors stated that stronger self-association of hard Na^+ counterions to humates was responsible for lower M_w values in sodium humates. Conversely, the softer ammonium ions showed weaker complex-forming abilities towards the hard sites of humate and thus lower capacity of neutralisation of humate negative charges. This would make ammonium humates larger in molecular dimensions than sodium humates, as a result of residual excess of negative charges. These statements are well acceptable from the point of view of the supramolecular theory of humic acid conformation.

The intensity of the peak representing large components varied significantly from sample to sample demonstrating that particularly H_2O_2 and 10% HNO_3 pre-treatment supported the stability of larger aggregates in the sample solution (not shown in the figures). This peak decreased slightly after organic acids treatment and fell vastly in the sample pre-treated with 20% HNO_3, giving the impression that this concentration of pre-treatment agent is a bit destructive and promotes formation of rather unstable humic assemblies. These findings are in good compliance with the chromatograms obtained by RI detector.

For better recognition of the size distributions and their potential impact on HAs biological activity, the overall area under peaks was integrated. Integrated spectra were virtually separated into 5 intervals in the case of UV detection and into 6 relevant intervals in the case of RI detection, the formers declared in Fig. 5. The intervals of distribution showed more clearly the differences between each series of samples. According to the observation of Conte et al. (2007), the alkyl hydrophobic components are mainly distributed in the largest molecular-size-fraction, whereas the amount of oxidized carbons increases with decreasing size of fractions. Thus, for samples prepared by oxidation with HNO_3, with increasing concentration of the agent, larger molecular-size-fractions content increased rapidly, middle-sized content decreased with constant linearity as well as the low-sized content. The exception can be seen for the samples pre-treated with 20% HNO_3, which eluted profusely in the range of 0–35 kDa suggesting that in this case the oxidation attack resulted in the extensive production of low-molecular-size-fractions. For the samples treated with H_2O_2, the low-sized content was reduced in comparison with original humates and the two fractions with highest M_w were more prominent again. Notably, the distribution of aggregates of samples which underwent pre-treatment by either citric or acetic acids seemed to be similar to that extracted from unaltered lignite, except for the 0–35 kDa fraction. In this range, the elution was more pronounced, confirming the expected influence of short-length organic acids on humic matter as described previously by Conte and Piccolo (1999).

The above-described size distribution development was identical for both potassium and ammonium humates and the overall results are in good agreement with the findings of Cozzolino et al.

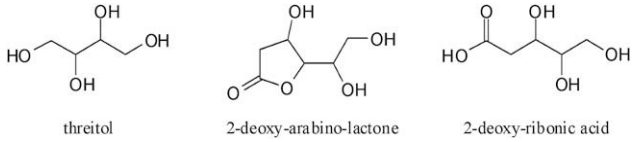

Fig. 3. The three most important compounds identified in prepared HAs as TMS ethers after HCl hydrolysis of humic acids from lignite and modified lignites.

(2001), who manifested that larger content of polar (oxidized) components (larger hydrophilicity) in HAs resulted in highly hydrated conformations loosing the potential to mutually associate. In connection with the fact that the hydration radius of resulting aggregates is generally believed to affect the values of M_w of humic matter (Swift, 1989), it is only natural to find larger molecular dimensions in oxidized samples than elsewhere.

3.3. Polyols identification and quantification

The three most important compounds (as TMS ethers) identified in humic acids were threitol, 2-deoxy-arabino-lactone and 2-deoxy-ribonic acid (Fig. 3). Unidentified compounds were mainly bearing no more than three hydroxyl groups. Carbohydrates reduced during the diagenesis can be considered as the precursors of these polyols. The various pre-treatments influenced the amounts and the nature of saccharidic compounds released after each two steps of hydrolysis. As shown in Fig. 4, the proportion of the rather labile polyols (after the first hydrolysis – HCl 1.2 M) was surprisingly high for the 5% H_2O_2 treated sample. The reason can be seen in the oxidation of short-chained units of HAs by H_2O_2 during the pre-treatment. After the second step (HCl 12 M), required for hydrolysis of more resilient bonds (present in vascular plant cellulose) the highest proportion was obtained for HAs obtained from lignite pre-treated with 20% citric acid.

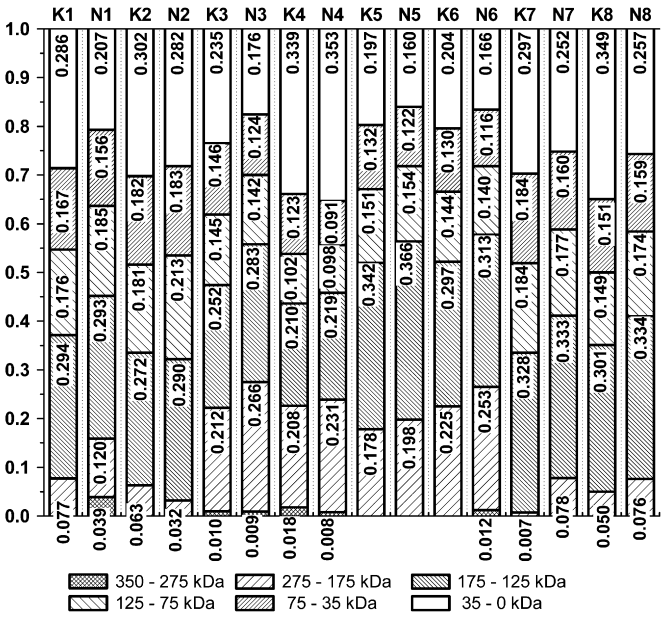

Fig. 5. Molar size distribution calculated from data detected by RID. K1–K8 represent respective potassium humates, N1–N8 represent respective ammonium humates.

3.4. Biological activity in context of lignite pre-treatment

Table 5 reports the relative increments of root lengths with respect to (a) the control solution of commercial Hydropon fertilizer and to (b) the comparative solution of B03H humic acid. The stimulating effect of humic samples as determined by growth rate of roots was found to be in most cases higher with potassium humates in comparison with ammonium humates. Exceptions can be seen in the

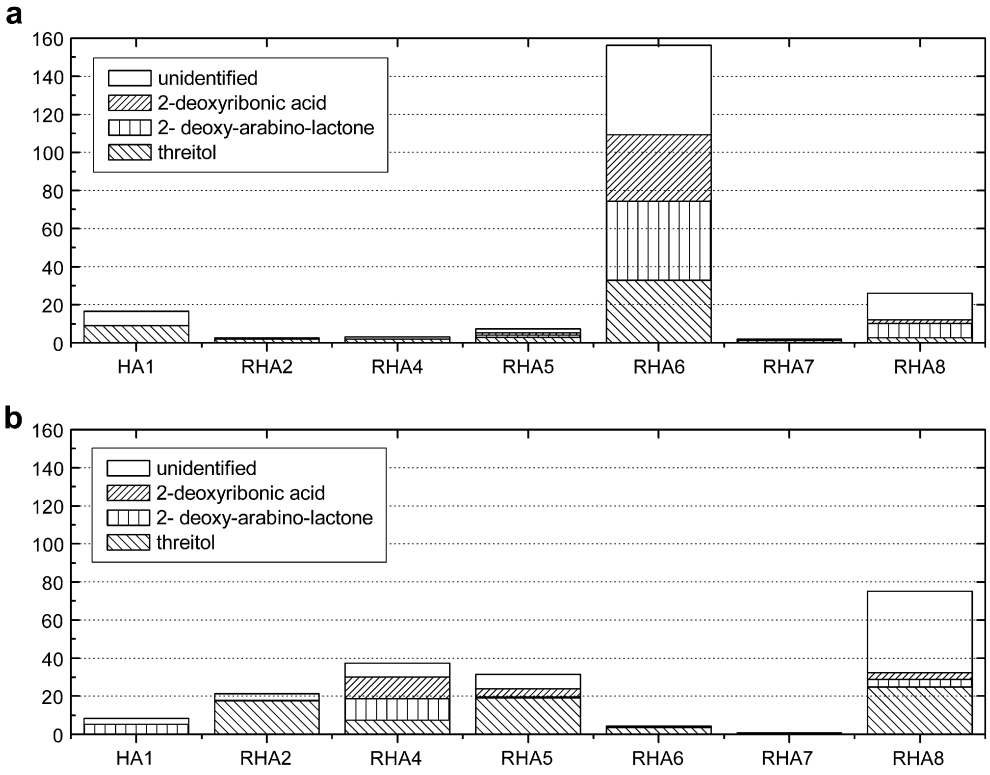

Fig. 4. Amounts of saccharidic-like compounds such as threitol, 2-deoxy-arabino-lactone, 2-deoxy-ribonic acid, and unidentified in mg/g of HAs or RHAs after the first (a) and the second (b) hydrolysis. Data for sample RHA3 were acquired for neither (a) nor (b).

Table 5
Biological activity results in 3 time intervals: (0–48 h), (0–72 h) and (0–96 h). The intensity of stimulating effect of each tested substance is stated in relative units (%), which express the accrual, eventually the shortening of root length in comparison with (a) the control solution of Hydropon fertilizer and with (b) the solution of comparative humic acid B03H.

Sample	Time intervals								
	After 48 h			After 72 h			After 96 h		
	(a) Control solution	(b) B03H solution	pH	(a) Control solution	(b) B03H solution	pH	(a) Control solution	(b) B03H solution	pH
KHA1	163 ± 21	89 ± 11	5.15	142 ± 14	85 ± 8	4.80	130 ± 10	83 ± 6	4.93
KRHA2	168 ± 14	92 ± 8	5.12	149 ± 13	89 ± 8	4.78	136 ± 12	87 ± 7	4.68
KRHA3	155 ± 20	85 ± 11	5.07	138 ± 14	82 ± 8	4.71	125 ± 11	79 ± 7	4.56
KRHA4	138 ± 21	75 ± 11	5.20	131 ± 17	78 ± 11	4.78	128 ± 15	81 ± 9	4.57
KRHA5	134 ± 15	91 ± 10	5.31	132 ± 9	97 ± 7	5.26	128 ± 8	100 ± 6	5.13
KRHA6	115 ± 17	78 ± 11	5.27	116 ± 10	86 ± 8	5.01	117 ± 10	92 ± 8	5.05
KRHA7	137 ± 13	93 ± 8	5.02	131 ± 7	97 ± 5	4.78	128 ± 5	100 ± 4	4.75
KRHA8	116 ± 15	79 ± 10	5.11	122 ± 12	90 ± 9	4.87	126 ± 10	98 ± 8	4.77
NHA1	115 ± 24	81 ± 17	4.56	117 ± 14	83 ± 10	4.41	116 ± 15	86 ± 11	4.29
NRHA2	131 ± 14	91 ± 10	4.57	126 ± 11	89 ± 8	4.25	124 ± 9	91 ± 7	4.15
NRHA3	125 ± 12	87 ± 8	4.49	120 ± 9	84 ± 6	4.09	114 ± 7	84 ± 5	4.00
NRHA4	97 ± 17	68 ± 12	4.64	99 ± 13	70 ± 9	4.33	99 ± 9	73 ± 7	4.16
NRHA5	122 ± 19	81 ± 13	4.41	115 ± 17	82 ± 12	4.20	107 ± 13	79 ± 10	4.04
NRHA6	114 ± 21	74 ± 14	4.51	110 ± 14	78 ± 10	4.20	99 ± 13	73 ± 9	4.00
NRHA7	123 ± 15	82 ± 10	4.42	117 ± 14	83 ± 10	4.18	110 ± 9	82 ± 6	3.98
NRHA8	103 ± 15	69 ± 10	4.58	107 ± 16	76 ± 11	4.32	105 ± 13	77 ± 9	4.02

case of humic acids RHA2 and RHA3. Generally, the application of potassium humates caused either increase or decrease of bioactivity with time while ammonium humates maintained approximately the same level of bioactivity in all periods of time. In potassium humates, the difference in comparison with control solution and B03H standard solution were also notable. In samples KHA1, KRHA2 and KRHA3 had the trends decreasing tendencies in due time. On the contrary samples KRHA4, KRHA5, KRHA6, KRHA7 and KRHA8 had decreasing trend with respect to control solution while an increasing trend to B03H in due time. These samples showed a more positive trend with the increasing time than the comparative substance B03H.

Bioactivity results, i.e. relative increment in respective intervals of (0–48 h), (0–72 h) and (0–96 h), were compared with molecular size, its distribution, saccharidic compounds content, C/H ratio and carboxylic content using the Pearson coefficient (r) as a statistic parameter. With potassium humates, values of r comparing bioactivity and C/H ratio decreased with time down to 0.17, suggesting that there is no linear relationship between the variables. On the contrary, with ammonium humates the same parameter increased with time reaching positive values up to 0.67. The correlation between bioactivity and the content of polar carboxylic groups was also positive, first (after 72 h) increasing, later (after 96 h) decreasing with time for both potassium and ammonium humates to reach identical value of $r = 0.50$.

Interestingly enough, M_w calculated on the base both UV and RI detections did not satisfactorily correlate with the bioactivity of our humic samples, as the statistical analysis showed low correlation coefficients here, r values from −0.02 to 0.40. On the contrary, size distribution revealed some linear relationships. Unlike the literature data, our low molecular intervals (0–35 kDa) showed absolutely no correlation with the bioactivity results, r values from −0.32 to −0.05. The middle molecular size intervals (35–175 kDa) correlated highly positive (r up to 0.92) and high molecular size intervals (175–350 kDa) correlated highly negative (r down to −0.75) with bioactivity results. This observation applies both to potassium and ammonium humates.

Finally, the possible relation between amounts of polyols (Fig. 4) and bioactivity was investigated. No correlation was found for potassium humates (r from 0.05 to 0.15) and negative correlation was found for ammonium humates (r values up to −0.63). Therefore, it is clear that the ability of HAs to enhance biological processes is not associated with the content of polyol units. Nevertheless, the amount of polyols itself is surprisingly high and could be exploited in other applications to be researched.

In overall results, concerning biological function in higher plants it is clear that the pre-treatment of parental lignite using appropriate modifiers can bring about the relatively significant differences in biological activity of extracted HAs. In this study, it has been demonstrated on the South Moravian lignite that in this respect, the most efficient modifiers are the 20% acetic acid and 5% hydrogen peroxide. While the potential of lignite oxidation in production of HAs with higher biological activity could be expected, the physical pre-treatment based on the interaction of a modifier molecule with a humic hydrophobic domain was unpredictable. As it follows from the results published here, the crucial point in this pre-treatment is the chemical character of the modifier. As demonstrated in previous papers (e.g. Cozzolino et al., 2001; Conte and Piccolo, 1999), the conformation of humate quaternary structure is significantly affected by the interaction with an organic molecule. It indicates either the potential unblocking of biologically active molecules or re-aggregation of humic aggregates forming smaller units having higher potential to penetrate through the cell walls and play a role in processes of cell proliferation.

Added N in the form of NH_4^+ counter ion did not improve the agricultural quality of humates, which is again associated with the importance of character of mutual inter-molecular interactions as well as a feature of the resulting aggregates.

The application of lignite HAs as additives in soil maintenance is efficient, presumably less expensive in comparison with contemporary commercial fertilizers and environmentally preferable as they are natural products. However, as suggested by our results, mechanisms of lignite-based humic substances in cell proliferation processes differ in comparison with humic substances found in soils (see Introduction). Apparently, the way of humic development, i.e. origin, plays a role in biological processes and such notion is critical in designing of humic-based products applications. Furthermore, it has been proven that the processes occurring in rhizosphere, i.e. root exudates release, can be imitated by the lignite pre-treatment and used for the increase in bio-stimulative efficiency of extracted humic acids. It is our hypothesis, that the strength of citric acid was not as strong as that of acetic acid and therefore, the resulting HAs provided different results. It seems that the future research should be aimed to find the appropriate and most efficient combination of humic substances/pre-treatment

agent to simulate more effectively the molecular re-aggregation in parental lignite. In fact, the exudates composition is specific for all root systems, thus, it is probable that the most effective pre-treatment or "pre-chewing" can be reached using the same compounds combination as released by roots. Therefore the determination of such interrelationship represents a future challenge.

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APPENDIX C

Lignite pre-treatment and its effect on genotoxicity/antimutagenicity of respective lignite humic acids and their salts. Influence of concentration and counterion.

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Abstract

The effect of South Moravian lignite pre-treatment with oxidative agents (HNO_3, H_2O_2) and small organic acids (acetic and citric acid) on genotoxicity/antimutagenicity of respective humic acids was studied. Samples were converted into soluble forms of potassium and ammonium humates to investigate also the influence of counterion (NH_4^+, K^+). All humates were characterized by UV-VIS spectroscopy using the absorptivity curves and humification index (E4/E6 ratio) and their free organic radical content in solid state was investigated using EPR spectroscopy. Obtained characteristics were compared with the results of a complex genotoxicity/antimutagenicity assay performed with the yeast *Saccharomyces cerevisiae* D7. In the tests some humates supported the effect of mutagen but none of them was genotoxic itself, which is the most important observation. It was concluded that modification of lignite did not induce genotoxic effect in extracted HAs in comparison with the original sample. On the contrary, the right combination of pre-treatment agent, humate concentration and counterion can provide considerable antimutagenic effect. Particularly high antimutagenic effect can be achieved by combination of (a) 5% HNO_3 pre-treatment and K^+ counterion, (b) 5% H_2O_2 pre-treatment and NH_4^+ counterion. Universal antimutagenic effect is also achieved at high dilution of 0.03% for potassium humate of any pre-treatment. Correlation between genotoxicity/antimutagenicity with free organic radical content of investigated humates showed no relationship; it is more likely that the genotoxic/antimutagenic effect was caused by changes of humic quaternary structure induced by pre-treatment of parental lignite.

Key words: lignite humic acids, humification, free radicals, genotoxicity, antimutagenicity

Introduction

Humic substances are widely researched for use in environmental, agricultural and medical applications (Pena-Mendez et al., 2005), therefore the studies on their genotoxic potential are well justified. Mutagenicity study of Hemming et al. (1986) showed that 15-30 % of observed genotoxic effect in humic substances is caused by chlorination intermediate products, such as furanones. Although furanones are mutagenic to bacteria and they cause DNA damage in laboratory animals, these compounds are also very effective anti-carcinogenic agents in the diets of animals which are being treated with known cancer-inducing compounds (Klöcking and Helbig, 2005). Evidence for therapeutic antiviral and anti-inflammatory properties of humates was found by van Rensburg et al. (2002) and Jooné and van Rensburg (2004). Desmutagenic activity of humic acids (HAs) was reported by Sato et al. (1987) and Cozzi et al. (1993). According to Sato et al. (1987) the desmutagenic effect was heat-resistant, increased with an increase of the HA molecular weight and was caused by adsorption of mutagen, not by its decomposition. Typical individual components of HAs had no desmutagenic effect.

Ferrara et al. (2006) investigated the capacity of two different environmental HAs (leonardite and soil) to reduce the mutagenicity of mitomycin C in the human lymphoblastoid cell line TK6 in three performed tests (genotoxic, desmutagenic and antimutagenic). Neither of the HAs used alone did produce genotoxic effect and both reduced mutagen activity, especially in the desmutagenic test. Both desmutagenic and antimutagenic activity were more pronounced for leonardite HA than for soil HA, which the authors attributed to the higher carboxylic group content and lower phenolic group content of leonardite HA. The total genotoxicity including DNA damaging effect of anthropogenic genotoxines and of natural components such as humic substances was closer investigated by Erbes et al. (1997) using so-called comet assay, a sensitive technique which detects DNA strand breaks in single cells. They concluded that the total genotoxicity is always influenced by humic substances because they act as a reservoir and a mask for genotoxines.

Genotoxicity in humic systems is often reported to be related to the reactive oxygen species (ROS). Since HAs are polyphenolic compounds, they generate ROS such as superoxide anion which initiate oxidative stress on red blood cells resulting in their dysfunction (Cheng et al., 1999). HAs from artesian well water in Taiwan are considered as a factor that causes the Blackfoot disease (BFD). Moreover, within the BFD endemic areas cancers occur at significantly higher rates than in areas free from the BFD (Chen et al., 1988).

Findings of Hseu et al. (2008) suggest that HA can induce oxidative DNA damage and genotoxicity in human lymphocytes, the predominant pathway being changes in Ca^{2+} homeostasis. HA-induced DNA damage can be decreased by superoxide, hydrogen peroxide and ROS scavengers. Lu et al. (2006) attempted to identify the tumor promoting activity of HA in mouse epidermal JB6 clone 41 cells and observed a significantly increased numbers of ROS which may mediate the process of transformation in epidermal cells.

In our recent study (Vlčková et al., 2009) the attempt was made to prepare various humic acids – lignite pre-treatment with oxidative agents and short-chained organic acids was demonstrated to be a feasible way to do so – and to test their respective potassium and ammonium salts for to their biological activity. However, during the pre-treatment organic carbon in parental lignite is subjected to physical and chemical modification which may bring about some undesirable properties, e.g. increased genotoxic potential. Therefore, in this work, a genotoxicity assay on the eukaryotic organism *Saccharomyces cerevisiae* D7 accompanied by a spectroscopic (UV-VIS and EPR) survey was carried out. The aim was to verify the possible genotoxicity of HAs and, simultaneously, their possible antimutagenic action against 4-nitroquinoline-N-oxide. Finally, the relations between pre-treatment-induced changes in chemical properties of HAs and their genotoxic/antimutagenic effect were investigated.

Material and methods

Humic acids

South Moravian lignite collected from the Mír mine in the area of Mikulčice, near Hodonín, the Czech Republic (Kučerík et al., 2003) was used as a source of HAs for this study. Each lignite sample, milled and sieved was pre-treated with different oxidizing or amphiphilic-like agent, details are shown in Table 1. First, the portions of original lignites were soaked at 45°C for 30 min under constant stirring in a solution of the agent in question (50 g of lignite in 500 mL of an agent). Pre-treated lignite was then washed with deionised water on the porous glass until agent-free. Next, HAs were extracted by standard alkaline method with 0.5 M NaOH and 0.1 M $Na_4P_2O_7$, purified and freeze-dried as specified in detail in Vlčková et al. (2009). For comparison, HAs from parental lignite with no pre-treatment were also extracted. All HA samples (50 mg) were suspended in deionized water and titrated with 0.1 M KOH (0.1 M NH_4OH) solution to pH 7 and freeze-dried to prepare potassium (ammonium) humates.

UV-VIS spectroscopy

Spectrophotometric analysis was performed with Varian Cary50. UV and VIS spectra of humic samples in form of aqueous solution in 1 cm cuvette were recorded separately, UV spectra in the range 200 – 400 nm with sample dilution 0.02 g/l, VIS spectra in the range 400 – 800 nm with sample dilution 0.3 g/l. In order to compensate the possible disproportions in sample preparations the UV and VIS profiles (Fig. 1a and Fig. 1b, respectively) were reported as the absorbance/concentration (absorption coefficient) in dependence on the wavelength. The E4/E6 ratio method by Chen et al. (1977) was used to determine the ratio of absorbances of humate samples at 465 and 665 nm; results are summarized in Table 1.

EPR spectroscopy

EPR spectra of each sample in three forms (humic acid, potassium humate, ammonium humate) were recorded using SpectraNova EPR 70-03 XD/2 device. 20 mg of solid sample were placed into quartz cuvette and measured at 25°C. Relative area of EPR signals was obtained by double integration and compared with EPR measurements of standard sample of known spin per gram content. Line width values recorded for the main peak in spectra were also calculated. Measurements were carried out twice, reported values are average of the measurements.

Genotoxicity/antimutagenicity assay – the Saccharomyces cerevisiae D7 Test

Genotoxicity/antimutagenicity tests were performed with the yeast *Saccharomyces cerevisiae* using the method first described in Zimmermann et al. (1984) and first tested on HAs by Márová et al. (2009). After 16-18 hours of cultivation of the yeast in liquid YPD medium the cell suspension in logarithmic phase of growth was divided into centrifuge tubes (10 ml) and centrifugated at 4500 rev min^{-1} and 20°C for 5 min. The cell sediment was suspended twice in a phosphate buffer (pH 6.98). 10 ml of the cell suspension was influenced with 0.1 ml of 0.06 mg/ml 4-nitroquinoline-N-oxide (4-NQO) which was used as standard mutagen. Simultaneously with the mutagen 0.5 ml of humate sample dissolved in DMSO was added. Four different concentrations of humate solution (0.03%, 0.06%, 0.125% and 1%) were tested. 0.1 ml of the cell suspension (10^6 cells /ml) was inoculated on the selective

medium for tryptophane conversions testing. 0.1 ml of the cell suspension (10^7 cells /ml) was inoculated on the selective medium for isoleucine reverse mutations analysis. Numbers of the yeast colonies grown in Petri dishes were counted after 5-10 days of the incubation at 28°C. All measurements were done triplicate.

The percentage of inhibition of mutagen in the presence of humate sample was calculated according to the equation:

$$\%_{inh} = 100 - [(X_1/X_2)*100]$$

where X_1 is the number of the yeast colonies in the presence of 4-NQO and 0.03 %, 0.06 %, 0.125 % and 1 % humic acid water extract, X_2 is the number of the yeast colonies in the presence of the 4-NQO without humate solution. The results are expressed as means ± SD from three measurements. Results were analysed by the Student t-test using Statistica for Windows 5.0 (Statsoft, USA). The differences of $p<0.05$ were regarded as statistically significant.

The mutagen 4-nitroquinoline-N-oxide was purchased from Sigma-Aldrich.

Results and discussion

Chemical analysis of humates

Lignite humates used in this work were previously tested for their biological activity and some chemical composition such as elemental analysis, ^{13}C carbon distribution, molecular size distribution and content of saccharidic-like components (Vlčková et al., 2009). Generally, potassium humates showed higher effect on the cell proliferation of maize (*Zea mays*) seedlings than ammonium humates; the best results were achieved for humates extracted from lignite pre-treated by acetic acid and by 2% hydrogen peroxide. Comparison of biological activity with results of size exclusion chromatography showed positive correlation with molecular fraction 35-175 kDa and negative correlation with molecular size fraction 275-350 kDa. No relation was found between biological activity and polyols content.

In this study the UV/VIS and EPR spectroscopy were used to complete the overall characterization and find the possible relationship between chemical structure and genotoxic/antimutagenic behaviour of investigated humates.

Both ultraviolet and visible spectra (Fig. 1a, Fig. 1b) are featureless and show continuous decrease in light absorption with increasing wavelength. Highest absorption

coefficient was found in humic samples extracted from lignite pre-treated with organic acids. Also HA extracted from 5% HNO_3 pre-treated lignite shows higher absorptivity than HA extracted from original lignite. Conversely, in comparison to HA extracted from original lignite, HA samples extracted from 10% HNO_3, 2% H_2O_2 and 5% H_2O_2 pre-treated lignite show lower absorptivity values, especially in middle wavelength area. 20% HNO_3 pre-treated lignite produced HA with the lowest absorptivity profile. These data may be related to the higher amount of conjugated aromatic rings (in accordance with NMR results published previously in Vlčková et al., 2009) which are efficient in light absorption, especially in visible range (Kononova, 1966). Table 1 gives a summary of the E4:E6 ratios. Significantly higher values for 10% and 20% HNO_3 pre-treated samples confirm their lower condensation degree (Stevenson, 1982).

EPR spectra of HAs samples showed well resolved singlet peak with no hyperfine structure, typical for semiquinone-type radical detected in humic substances as it is stabilized in aromatic conjugated ring system, condensed to various extent (Bayer et al., 2002). As reported in Table 2, organic free radicals in HAs samples varied from 1.53×10^{17} spins g^{-1} to 1.12×10^{18} spins g^{-1}. The difference of stable free radical content among variously pre-treated samples is not marginal. The 5% HNO_3 pre-treatment increased radical content when compared to non-treated sample, but with higher HNO_3 concentration radical content gradually decreased. The same behaviour was observed when 2% and 5% H_2O_2 pre-treated samples were compared with the sample extracted from original lignite. This can be caused by the decreasing amount of aromatic C and carboxylic groups with increasing concentration of the agent. A fairly good correlation ($r = 0.79$) was found between the free radical concentrations and the carboxyl groups contents (NMR data published in Vlčková et al., 2009), which suggests that the carboxylic groups contributed to the free radical stabilization in investigated humic samples.

Lignite pre-treatment with short-chained organic acids brought about remarkable increase in free radical concentration for both agents but the values were notably higher for acetic acid than citric acid pre-treatment. In overall results, both acetic and citric acid pre-treatments produced HAs with highest amount of stable organic radical concentration (1.12×10^{18} spins g^{-1} and 8.70×10^{17} spins g^{-1}, respectively) followed by 2% H_2O_2 (6.94×10^{17} spins g^{-1}) and 5% HNO_3 (5.39×10^{17} spins g^{-1}) pre-treatment. The lowest radical concentration (1.53×10^{17} spins g^{-1}) was found for 20% HNO_3 pre-treated sample.

In humates, the organic free radical content was lower, ranging from 5.19×10^{16} spins g^{-1} to 4.01×10^{17} spins g^{-1} in potassium humates and from 8.32×10^{16} spins g^{-1} to 3.83×10^{17}

spins g^{-1} in ammonium humates. Such observation is in agreement with recent observation concerning the role of counterion in stability of free radicals in lignite humic acids and respective humates with respect to their antioxidant properties (Kučerík et al., 2008a). It was demonstrated that HAs and ammonium humates show better antioxidant effect towards polyvinyl alcohol than the sodium humates, due to the disruption of H-bonds stabilizing humic substances quaternary structure. The comparison of pre-treatment influence for humates led to similar observation as for HAs (Table 2). Since the semiquinone free radical concentration (major part of free radicals in humic substances) is consistent with the humification index of organic matter (Martin-Neto et al., 1998), HAs samples with higher radical content (lignite pre-treated with organic acids, 2% H_2O_2 and 5% HNO_3) are supposed to be have such index higher than those with lower radical content (lignite pre-treated with 5% H_2O_2, 10% and 20% HNO_3). Therefore, the findings made by EPR spectroscopy are in good compliance with the above discussed UV-VIS spectroscopy results.

The line width, which represents another informative parameter obtained from the EPR signal, varied among the HAs samples with different lignite pre-treatment from 0.50 mT (5% HNO_3, 10% HNO_3 and 5% H_2O_2) to 0.57 mT (acetic acid) and 0.58 mT (2% H_2O_2). For comparison, the HA from original lignite produced a signal of 0.56 mT line width. The two remaining HAs (20% HNO_3 and citric acid) produced a signal of 0.54 mT line width. In humates, both potassium and ammonium, the values of line width were generally higher (from 0.60 mT to 0.79 mT) and again the widest signal was found for acetic acid and 2% H_2O_2 pre-treated samples (Table 3). The line width is a parameter directly proportional to the extent of delocalisation of unpaired electrons onto neighbouring molecules (Senesi and Steelink, 1989) and the variation in its values can arise from factors such as spin-spin interactions caused by close proximity of radical species (Watanabe et al., 2005). The more free radical is interacting with its neighbours, the more its relaxing time decreases and the line width increases.

Genotoxicity/antimutagenicity

This assay was performed on all samples in form of pottasium and ammonium humates with the aim to evaluate and compare the suitability of individual regeneration/modification as well as the influence of counterion from point of view of genotoxicity/antimutagenicity. Per cent of inhibition of yeast colony number obtained for tryptophane conversions (Fig. 2) are considered fundamental, while the results obtained for

isoleucine reverse mutations (Fig. 3) fulfil the controlling function and for that matter support adequately and satisfactorily the former.

As for the influence of counterion, our results show that potassium humates are more suitable for this study as they show better solubility and stable pH values (Vlčková et al., 2009). It is noteworthy that comparison of both lines provided an interesting outcome that the effect of pre-treatment was exactly the opposite in potassium humates than in ammonium humates. Specifically, in pottasium humates the original sample demonstrates high antimutagenicity and with increasing strength of HNO_3 this positive effect slightly declines. Thus, although the whole HNO_3 line is positively harmless, only 5% HNO_3 pre-treatment is profitable in terms of antimutagenicity when compared to the original sample. In ammonium humates, all samples in HNO_3 line including the original one supported mutagen activity, the worst case being 5% HNO_3 regeneration. The simple fact, that added nitrogen in form of HNO_3 lignite pre-treatment works better with K^+ then NH_4^+ counterion, is an interesting observation.

Pre-treatment with H_2O_2 bolstered positive effect in ammonium humates, especially in 5% H_2O_2 pre-treated sample which did extremely well when considering overall results for the ammonium line. On the contrary, H_2O_2 line of potassium humates supported mutagen activity with the only exception of high sample dilution (0.03%). Modification with organic acids produced mostly harmless humates, with positively outstanding results only for highly diluted samples (0.03%). In detail, the acetic acid lignite pre-treatment brought about moderately antimutagenic properties in respective ammonium humate, which diminished with growing concentration reaching negative values for the concentration of 1%. Respective potassium humate demonstrated the same trend with even faster decline, the only concentration securing antimutagenic effect was the 0.03%. Lignite pre-treatment with citric acid is not recommendable when considering ammonium humates as they developed properties supporting the effect of mutagen. Conversely, this pre-treatment produced rather harmless potassium humates.

In overall results, the concentration of 0.03% seems to play a key role in antimutagenic properties of tested humates. Especially with potassium humates, there is no exception to the quite decent antimutagenic effect of investigated samples. With ammonium humates the effect is less pronounced, because of the above mentioned HNO_3 line issue. Across all the tests an anomaly in behaviour of concentration line was discovered for concentration 0.125% in ammonium humates and the same anomaly shifted to concentration 0.06% in potassium humates. This is likely to be related to some special physico-chemical

properties of humate samples at these particular concentrations since the conformation of humates is known to be strongly concentration dependent (Kučerík et al., 2009).

The preparation of samples 7 and 8 was inspired by processes occurring in rhizosphere where humic acid in close proximity of a plant root is reconformed by root exudates, typically organic acids as used in this study. The change of conformation induced in samples 7 (acetic acid) and 8 (citric acid) in comparison to conformation of parental sample 1 can be the reason for above described behaviour of sample 7 with concentration, i.e. fast decline from positive influence of the humate (both K^+ and NH_4^+) to negative values. While concentration dependence is linear for sample 7, in sample 8 the situation is more complicated. Basically, all K^+ humates are slightly antimutagenic with the exception of the concentration with anomaly (0.06 %), whereas all NH_4^+ humates considerably support mutagen activity with the exception of the concentration with anomaly (0.125 %). In both cases, concentration dependence is different, but supports our former statements. Hence, when preparing a material with certain properties it is necessary to contemplate the availability of toxic groups and take into account anomalies occurring at specific concentrations in combination with particular counterion.

An important fact is that although some humates supported the effect of mutagen, according to lethal controls (colony numbers in the presence of humate only, without mutagen), which were less than 10, no humate was genotoxic itself. Thus we conclude that lignite modification does not induce genotoxic effect in extracted HAs in comparison to the original sample.

Genotoxicity/antimutagenicity in context of lignite pre-treatment

Lignite pre-treatment with oxidative agents and small organic acids was examined by means of EPR to assess the organic free radical content in respective HAs which gives an indication of the overall reactivity of the molecules and the linewidth of HAs signal which characterizes the structure of a molecule bearing the radical, i.e. its availability for other reactions such as organic pollutants or metal binding. Both are closely related to the total genotoxic potential provided by HAs.

As discussed above, the only pre-treatment suitable for production of K^+ humate with versatile antimutagenic properties is 5% HNO_3. This humate also happens to contain the highest amount of free radicals. On the other hand, the non-pre-treated sample in the K^+ line of humates – also demonstrating antimutagenic activity – contains the lowest number of free radicals. This observation suggests that free radical content is not the only indicator of

antimutagenic activity. It is our hypothesis that these are structural factors such as existence of humic aggregates with variable size and stability (Kučerík et al., 2009). Different pre-treatment agents may block or unblock molecules or aggregates with genotoxic potential contained in these humic assemblies. This theory is supported by the fact, that potassium humate obtained from lignite processed by acetic acid with average free radical count demonstrates antimutagenic effect only in high dilution (0.03%). High sample dilution has significant influence on the quaternary structure of HAs in solution. The same goes for potassium humates obtained from lignite processed with 2% and 5% H_2O_2 with relatively high free radical numbers. Both supported mutagen activity with the only exception of high sample dilution.

As discussed in previous sections, structural features of humates are strongly dependent on the counterion. The agent giving the best results with K^+ (5% HNO_3) gave the worst results with NH_4^+ producing a humate strongly supporting mutagen in wide range of concentration. Again, this sample scored one of the highest free radical numbers. Similarly high free radical numbers for NH_4^+ humates were also found for citric and acetic acid pre-treatment. While citric acid brought about mutagen supporting properties in respective NH_4^+ humate, acetic acid caused the resulting NH_4^+ humate to support mutagen only when dosed in high concentrations. In NH_4^+ line, 5% H_2O_2 pre-treatment produced the only humate with remarkable antimutagenic properties and simultaneously the lowest free radical amount. But the same lowest free radical amount was also observed for sample NRHA4 (20% HNO_3) which was found to be harmless in low concentrations but supported mutagen in higher concentrations. Thus again, the free radical count does not seem to play a key role here.

Conclusion

Technological application of humic substances is well recognized. Main sources of humic substances represent deposits of low rank coals (lignites, leonardites) and peat. Both of them, however, can provide humic substances only with specific properties, biological activities or in limited quantity. Peat is generally richer in content of humic substances in comparison to low rank coals but humic substances often contain relatively high amount of carboxylic groups which is undesirable in some agricultural applications due to high actual acidity of treated soils. On the contrary, low rank coals provide lower content of humic substances. It can be increased by additional pre-treatment which leads to production of so-called regenerated humic acids (Rausa et al., 1994). There exist two options for production of

regenerated humic acids which are oxidation in suspension with liquid agent or oxidation by air. The latter can be carried out either at ambient (Kučerík et al., 2008b) or elevated temperatures high up to 180°C (Rausa et al., 1994). At lower temperature the oxidation takes very long time providing low yield; in contrast higher temperature promotes the kinetics of oxidation and the yield of humic acids is high. Although, no genotoxicity was reported for humic acids obtained by air oxidation at elevated temperatures in Rausa et al. (1994), recent results reported in Márová et al. (2009) indicate contrasting results. For sodium humate which was heated up shortly the significant increase in genotoxicity was observed. Therefore, it seems that application of non-treated humus-containing material does not bear a risk (Márová et al., 2009) but humic substances produced by additional pre-treatment of parental material should be used with care and tested for their potential harmful effect on the environment.

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Table and Figure Captions

Table 1

List of HAs samples with respective lignite pre-treatment agent and calculated value of E4/E6 ratio recorded by UV-VIS spectrometer in form of potassium humate.

Table 2

Free radical content (in spins g^{-1}) of samples at 25°C.

Table 3

Linewidth values (in Gauss units) recorded at 25°C for the main peak of the spectra.

Figure 1

(a) UV profile (b) VIS profile of HAs samples recorded in form of potassium humates.

Figure 2

Per cent of inhibition of yeast colony number obtained for tryptophane conversions at the presence of a mutagen and (a) potassium humates (b) ammonium humates.

Figure 3

Per cent of inhibition of yeast colony number obtained for isoleucine reverse mutations at the presence of a mutagen and (a) potassium humates (b) ammonium humates.

Table 1

Sample	Pre-treatment agent	E4/E6
HA1	–	6.2520
RHA2	5% HNO_3	6.5821
RHA3	10% HNO_3	8.8745
RHA4	20% HNO_3	10.0232
RHA5	2% H_2O_2	6.1049
RHA6	5% H_2O_2	6.7581
RHA7	20% acetic acid	6.1046
RHA8	20% citric acid	6.2112

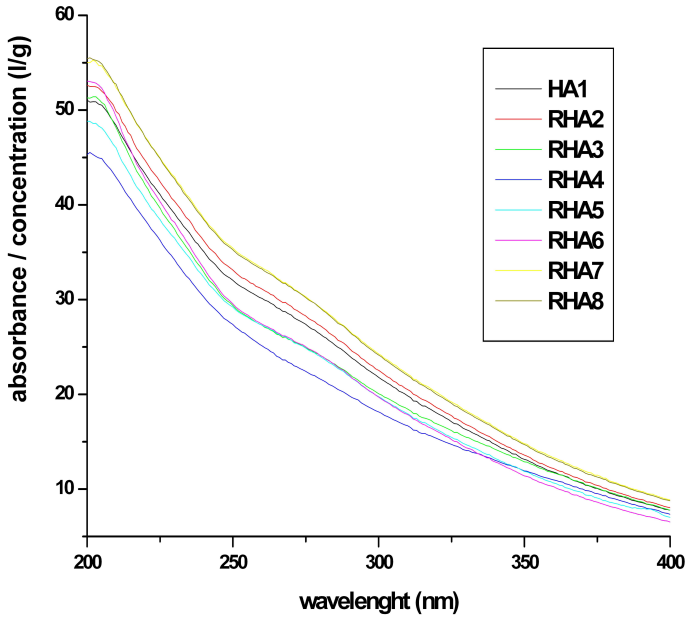
Table 2

Sample	Radicals	Sample	Radicals	Sample	Radicals
KHA1	5.19 e^{16}	NHA1	3.23 e^{17}	HA1	4.76 e^{17}
KRHA2	4.01 e^{17}	NRHA2	3.80 e^{17}	RHA2	5.39 e^{17}
KRHA3	1.96 e^{17}	NRHA3	2.54 e^{17}	RHA3	3.59 e^{17}
KRHA4	6.28 e^{16}	NRHA4	1.61 e^{17}	RHA4	1.53 e^{17}
KRHA5	2.68 e^{17}	NRHA5	8.32 e^{16}	RHA5	6.94 e^{17}
KRHA6	1.37 e^{17}	NRHA6	1.61 e^{17}	RHA6	2.44 e^{17}
KRHA7	1.96 e^{17}	NRHA7	3.83 e^{17}	RHA7	1.12 e^{18}
KRHA8	2.38 e^{17}	NRHA8	3.58 e^{17}	RHA8	8.70 e^{17}

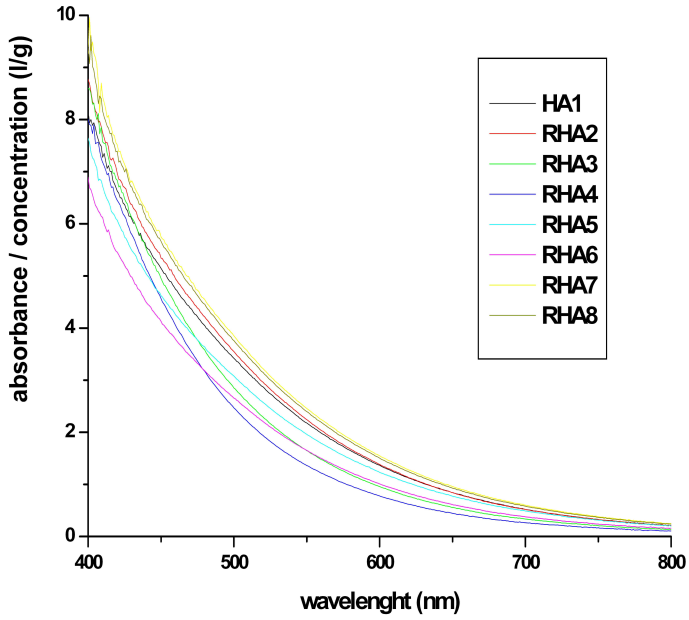
Table 3

Sample	Linewidth	Sample	Linewidth	Sample	Linewidth
KHA1	7.22	NHA1	6.65	HA1	5.60
KRHA2	7.00	NRHA2	6.08	RHA2	5.00
KRHA3	6.43	NRHA3	6.26	RHA3	4.97
KRHA4	6.56	NRHA4	5.95	RHA4	5.37
KRHA5	7.11	NRHA5	7.89	RHA5	5.81
KRHA6	6.54	NRHA6	5.53	RHA6	5.02
KRHA7	6.36	NRHA7	6.69	RHA7	5.68
KRHA8	6.49	NRHA8	6.06	RHA8	5.35

Figure 1

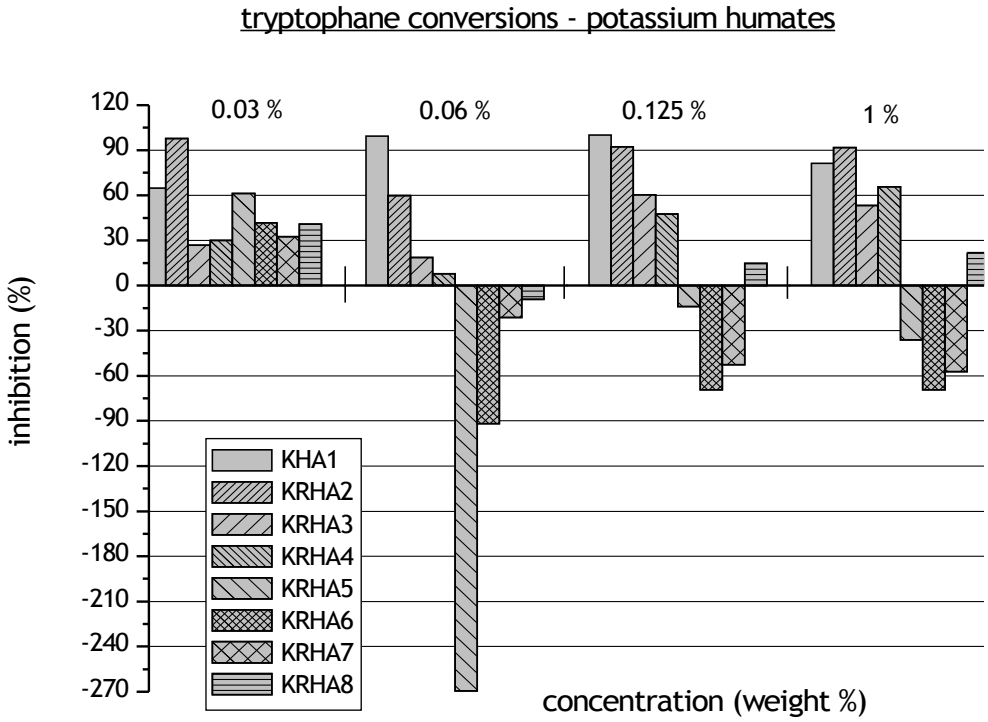

absorbance / concentration (l/g)
wavelenght (nm)
HA1
RHA2
RHA3
RHA4
RHA5
RHA6
RHA7
RHA8

(a)

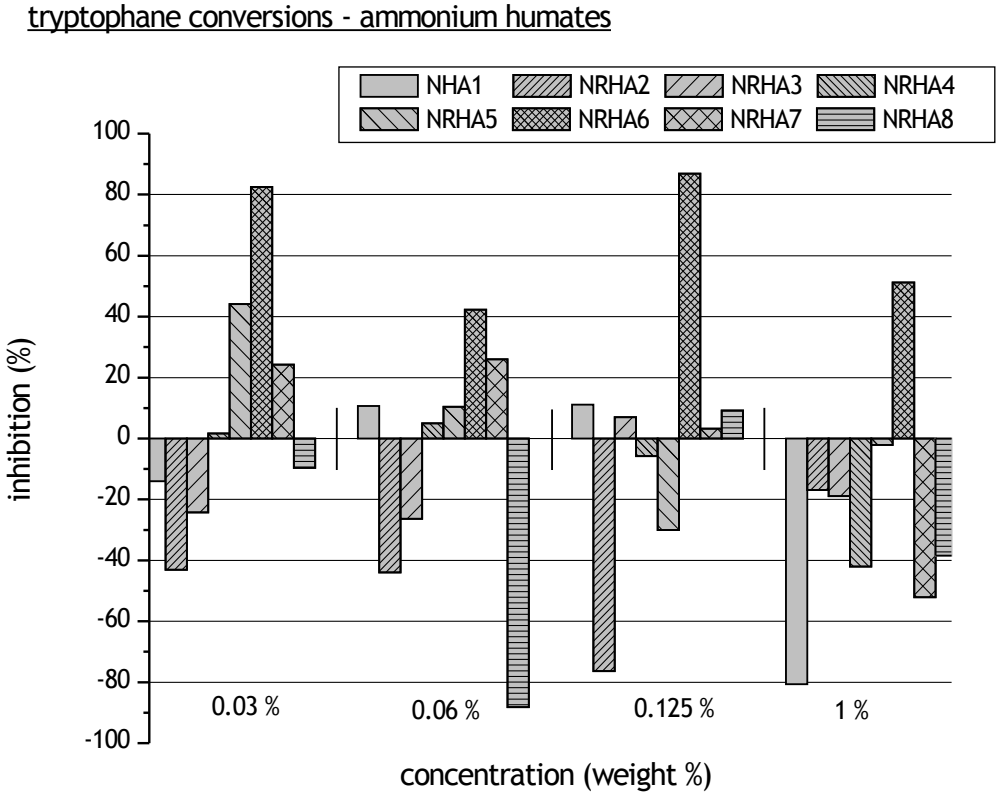

absorbance / concentration (l/g)
wavelenght (nm)
HA1
RHA2
RHA3
RHA4
RHA5
RHA6
RHA7
RHA8

(b)

Figure 2

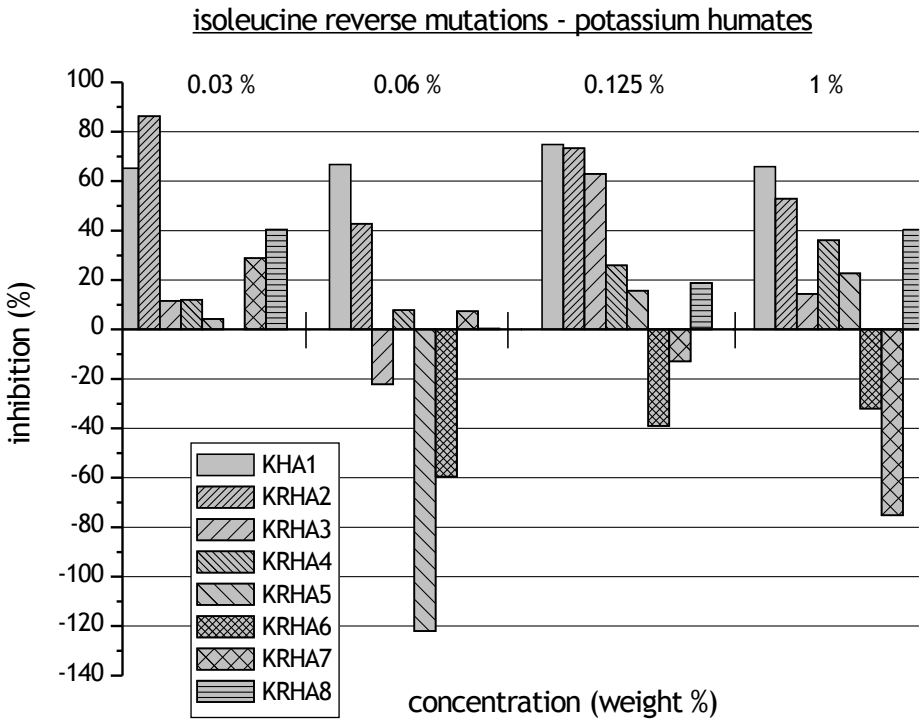

tryptophane conversions - potassium humates
0.03 %
0.06 %
0.125 %
1 %
inhibition (%)
120
90
60
30
0
-30
-60
-90
-120
-150
-180
-210
-240
-270
KHA1
KRHA2
KRHA3
KRHA4
KRHA5
KRHA6
KRHA7
KRHA8
concentration (weight %)

(a)

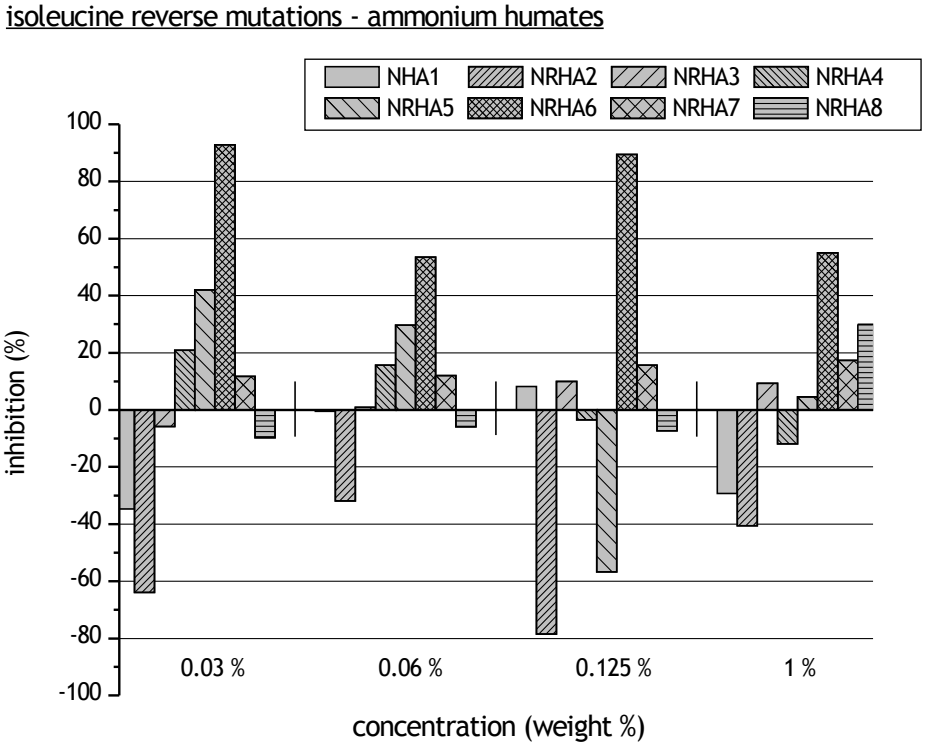

tryptophane conversions - ammonium humates
NHA1
NRHA2
NRHA3
NRHA4
NRHA5
NRHA6
NRHA7
NRHA8
inhibition (%)
100
80
60
40
20
0
-20
-40
-60
-80
-100
0.03 %
0.06 %
0.125 %
1 %
concentration (weight %)

(b)

Figure 3


isoleucine reverse mutations - potassium humates
0.03 %
0.06 %
0.125 %
1 %
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
inhibition (%)
KHA1
KRHA2
KRHA3
KRHA4
KRHA5
KRHA6
KRHA7
KRHA8
concentration (weight %)

(a)


isoleucine reverse mutations - ammonium humates
NHA1
NRHA2
NRHA3
NRHA4
NRHA5
NRHA6
NRHA7
NRHA8
100
80
60
40
20
0
-20
-40
-60
-80
-100
inhibition (%)
0.03 %
0.06 %
0.125 %
1 %
concentration (weight %)

(b)

APPENDIX D

Characterization of Lignin Monomers in Low Rank Coal Humic Acids using the DFRC Method

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Lignin monomers are among the most effective biomarkers of terrestrial material because they provide an unambiguous fingerprint for vascular plants. Using lignin monomers, plant source types can be differentiated as being either woody or non-woody or of angiosperm or gymnosperm origin. Much of the work to examine lignin in natural systems such as soils and sediments has been conducted using the CuO oxidation method [1], or the thermochemolysis method using tetramethylammonium hydroxide (TMAH) [2,3]. Each method results in a different suite of lignin products, because they differ in the mechanism of decomposition (oxidation and hydrolysis with CuO, hydrolysis and methylation with TMAH). Then, a research target is to find efficient and more selective methods for *β*-O-4 ether cleavage because *β*-O-4 ethers are the most frequent interunit linkages in lignin. A method exploiting derivatization followed by reductive cleavage (the DFRC method) is highly efficient for cleaving lignin *α*- and *β*-aryl ethers and suitable for lignocellulosic materials [4].

In this study, the DFRC method was applied for the characterization of lignin monomers of the aklalinosoluble part (i.e. humic acids) of a Czech's lignite (low rank coal).

The DFRC degradation method includes two key steps: bromination and acetylation with AcBr followed by reductive cleavage with zinc dust [4]. The gas chromatograms of monomeric products from lignite humic acids submitted to the DFRC method are shown in Figure 1.

In addition to the major **P** (*p*-coumaryl peracetate), **G** (coniferyl peracetate) and **S** (sinapyl peracetate) monomers that arise from *β*-ether units, many minor components originating from other structures were also found. Vanillin acetate (**G-CHO**) result from acetylation of released vanillin that survived the Zn step, whereas 4-acetoxy,3-methoxytoluene (**S-CH$_3$**) is from syringaldehyde after Zn reduction or from syringyl alcohol brominated with AcBr and produces the toluene form in the .Zn step. Eugenol and isoeugenol acetates (**G-CH$_2$CH=CH$_2$** and **G-CH=CHCH$_3$** respectively) and guaiacylcyclopropane (**G-cPr**) could come from coniferyl alcohol endgroups. In the same way, **S-cPr** is presumably from sinapyl alcohol. **G'-CO-CH$_2$CH$_3$** compounds arise from guaiacyl α-CO *β*-ether units. Whether methoxyphenones (**P'-CO-CH$_3$** and **G'-CO-CH$_3$**) coming from lignin, it is still unclear although they have been found in plants.

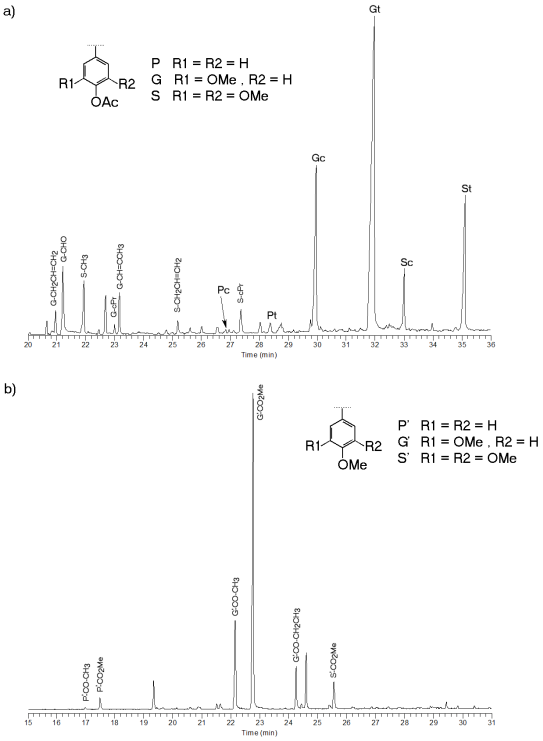

Fig. 1. Chromatograms of monomeric DFRC products from lignite humic acids bearing alcohol units (a) and keto or carboxyl units (b) (for abbreviations see text in bold, c: cis, t: trans).

Coumaric, coniferic and sinapic acids (**P'-CO$_2$Me**, **G'-CO$_2$Me** and **S'-CO$_2$Me**) are of diagenetically modified lignin.

Results show that low rank coal humic acids contain intact lignin monomers. The dominance of conferyl units is in accordance with the gymnosperm origin of the studied lignite. It is reasonable to expect that DFRC will be suitable for tracing lignin input in several other organic sediments.

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APPENDIX E

Ultrasonic and densitometry measurements.

The parameters of density and ultrasonic velocity are often measured to determine total hydration and hydration shells, eventually the conformation of various biomolecules, such as polysaccharides or proteins. Combination of both methods can provide information about adiabatic compressibility and other physical-chemical parameters important for the study of biomolecules in solutions. In previous part of the work, mainly primary structure of produced HAs was investigated. The aim of this part of the work is to use the methods of densitometry and ultrasonic velocity measurement to gain some information about their supramolecular structure, mainly about their hydration and conformation in solution, and to see whether it is possible to obtain such information by means of these methods.

Experimental

Sample preparation

Detailed information on sample origin and preparation can be found in Vlčková et al, 2009.

Table 1 List of pre-treatment angents and respective sample abbreviations. In the following text, KHAx (KRHAx) will stand for potassium humtes and NHAx (NRHAx) for ammonium humates.

Sample abbreviation	Pre-treatment agent
HA1	–
RHA2	5% HNO_3
RHA3	10% HNO_3
RHA4	20% HNO_3
RHA5	2% H_2O_2
RHA6	5% H_2O_2
RHA7	20% acetic acid
RHA8	20% citric acid

Densitometry measurements

The densities of aqueous solutions of ammonium and potassium humates at concentrations of 0.01, 0.05, 0.1, 0.5 and 1 g/L were measured using Anton Paar DMA 4500 densitometer at

25±0.01°C. The stock solution of 1 g/L was prepared in milliQ water at least 24 hours before measurement to avoid the possible additional dissolution of solid humates in water. The solutions with lower concentrations were prepared by dilution of stock solution several hours before measurement. Before injection into the apparatus, sample was well-shaken and degassed. To control the reproducibility, some samples were measured in triplicate. The standard deviation never exceeded ±0.00003 g/cm^3. Results are reported in Figures 1-4.

Ultrasonic measurements

Ultrasonic velocity of the same samples at the same concentrations was measured employing HRUS 102 at 25.00±0.02°C. Cell 1 was loaded up by 1 mL of degassed sample and cell 2 was loaded up by 1 mL of degassed milliQ water. Samples were stirred by rod stirrers at 600 RPM. Measurements of ultrasonic velocity were conducted at frequencies of 5478, 8219 and 12196 kHz. The values given in Figures 5-8 are an average of 3 measurements, the missing values are those which did not show reproducibility.

Data evaluation

Propagation of ultrasonic wave through a liquid phase is described by following equation:

$$u^2 = \frac{1}{\rho\beta},$$

where u is the measured ultrasonic velocity, β is the adiabatic compressibility and ρ is density.

If the hydrated humate and its counterion can be regarded as incompressible in the bulk of water, than the following equation can be used (Davies et al, 1982):

$$V_{0,H_2O} = \frac{\beta_{HUM}}{\beta_{H_2O}},$$

where $V_{0,H2O}$ is experimentally determined volume fraction of non-interacting solvent, β_{HUM} and β_{H2O} are the respective compressibilities of humate solution and water determined from ultrasonic velocity and density data.

Results and Discussion

Figures 1-4 report the obtained dependencies of density on concentration in the range of concentration from 0.01 to 1 g/L. As expected, the density increased with increasing concentration. However, the trend is not perfectly linear, particularly not in the concentration interval from 0.01 to 0.5 g/L. In this range, the increase is steeper than at higher concentrations for non-pre-treated samples and samples processed organic acids and 2% H_2O_2, but less steep or balanced for samples pre-treated with HNO_3 (in all concentrations), 5% H_2O_2. It is not a general observation but in many cases the density of potassium humate was lower than the respective ammonium humate.

Density is a parameter which includes the contributions of (a) density of bulk water, (b) density of dissociated humate, (c) density of hydration shell of the counterion and (d) the counterion itself. Hydration of counterion is an issue of many studies. For example hydration number for NH_4^+ was reported from 8.4 (Dang and Smith, 1993) to 1.2 (Burakowski and Gliński, 2009) depending on the used method, concentration of the solution and composition of the solution. Situation with K^+ is similar, values are reported in the range from 2 (Zavitsas, 2001) to 4-8 (Ohtaki and Radnai, 1993). For our considerations, we used the numbers reported in Zavitsas (2001), i.e. 2.1 ± 0.3 for K^+ and 1.3 ± 0.4 for NH_4^+, since the same technique was used for both ions and concentrations were similar to those reported in this study. The radius of K^+ and NH_4^+ ions were reported 1.38 and 1.6 Å, respectively (Kiriukhin and Collins, 2002). Therefore, if the hydration number is higher for K^+ ions, the (more frequently) higher density of NH_4^+ humates reported in Figures 1-4 must be attributable to the influence of dissociated humate itself. There are two possible explanations for this conclusion. Either the organic part of NH_4^+ humate is more hydrated, or the structure of organic part of NH_4^+ humate is denser. To access more information on these issues, ultrasonic measurements were carried out.

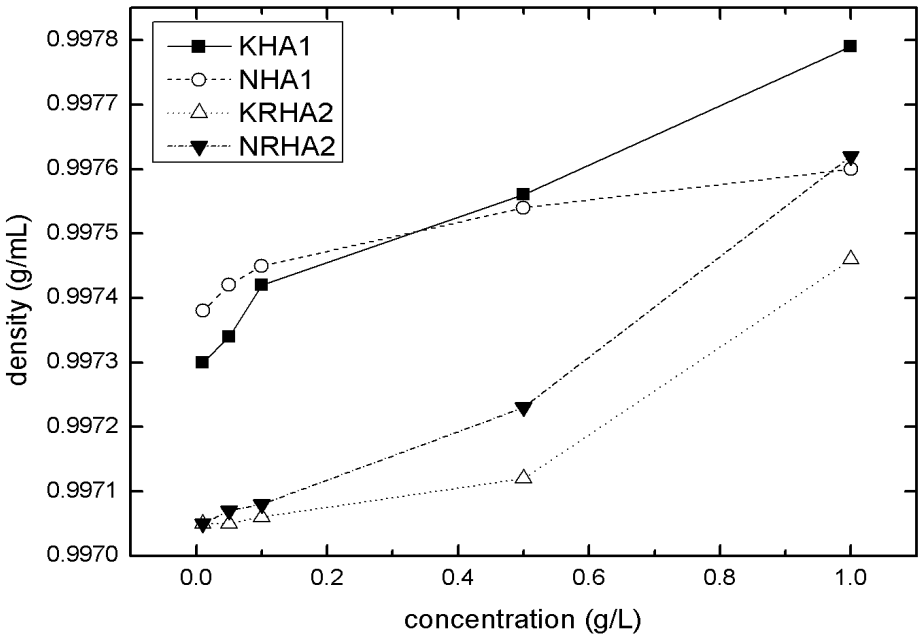

Figure 1 Density of samples KHA1, NHA1, KRHA2 and NRHA2 in dependency on concentration of the humate solution.

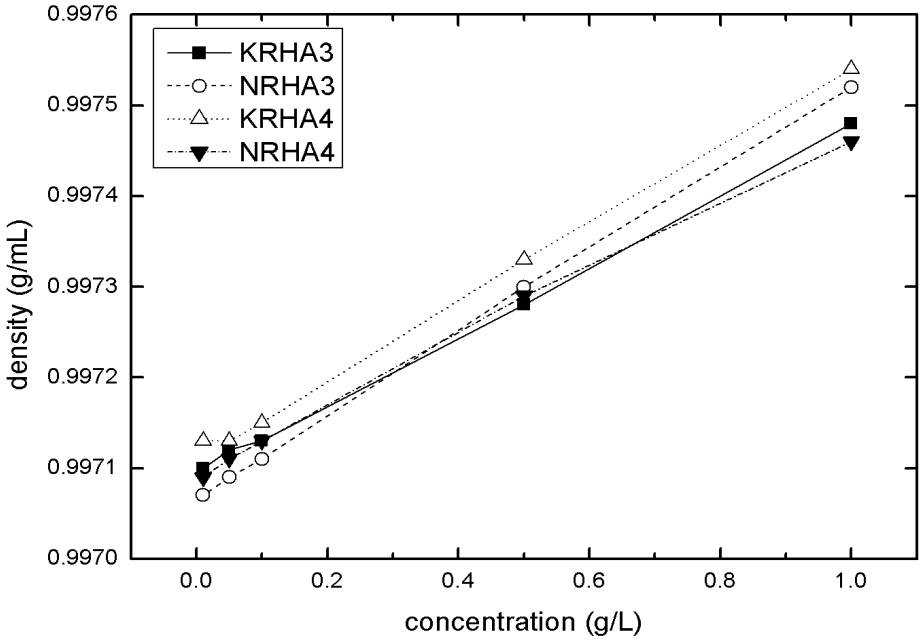

Figure 2 Density of samples KRHA3, NRHA3, KRHA4 and NRHA4 in dependency on concentration of the humate solution.

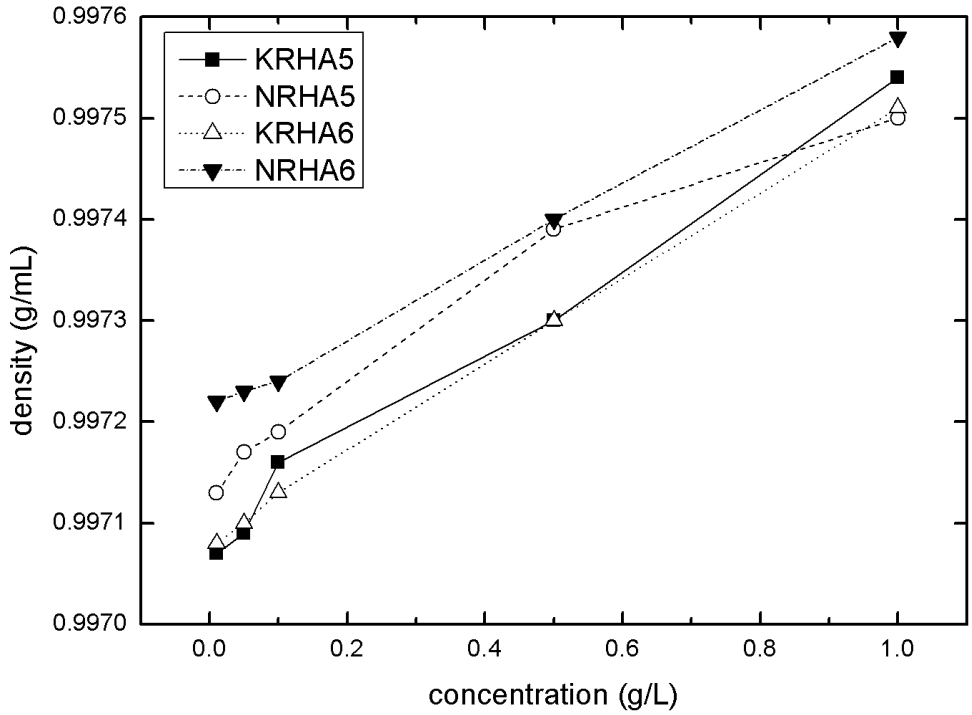

Figure 3 Density of samples KRHA5, NRHA5, KRHA6 and NRHA6 in dependency on concentration of the humate solution.

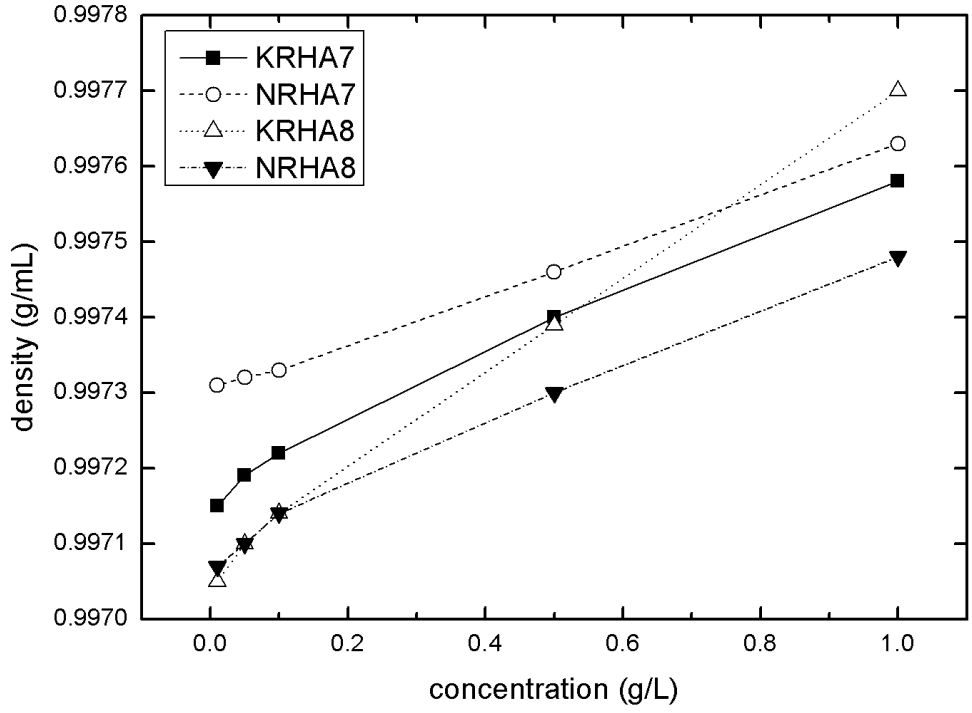

Figure 4 Density of samples KRHA7, NRHA7, KRHA8 and NRHA8 in dependency on concentration of the humate solution.

Dependence of ultrasonic velocity on the concentration of humate solution, as depicted in Figures 5-8, showed contrasting results to density. In most cases, the ultrasonic velocity in solution of potassium humates showed higher values than in respective ammonium humates, the most exemplary cases being samples processed with small organic acids and H_2O_2 (in both concentrations). The ultrasonic velocity in solution depends on three factors, (a) on hydration of molecules and ions which increases the velocity value (hydration shell is less compressible than the bulk of water), (b) on inner compressibility of aggregates (higher compressibility decreases the velocity) and (c) on the compressibility and density of the bulk of solvent (water). Therefore, in most cases the influence of counterion (K^+) hydration prevails. In cases where ultrasonic velocity is higher in ammonium humates, either the hydration of organic part or its compressibility and density play more significant role. This hypothesis is consistent with the conclusions concerning the humate densities. Since no fluctuation of ultrasonic velocity during the measurement at various frequencies (5478, 8219 and 12196 kHz) was observed, one can assume that micelles with hydrophobic interior and hydrophilic surface are not present in the supramolecular structure.

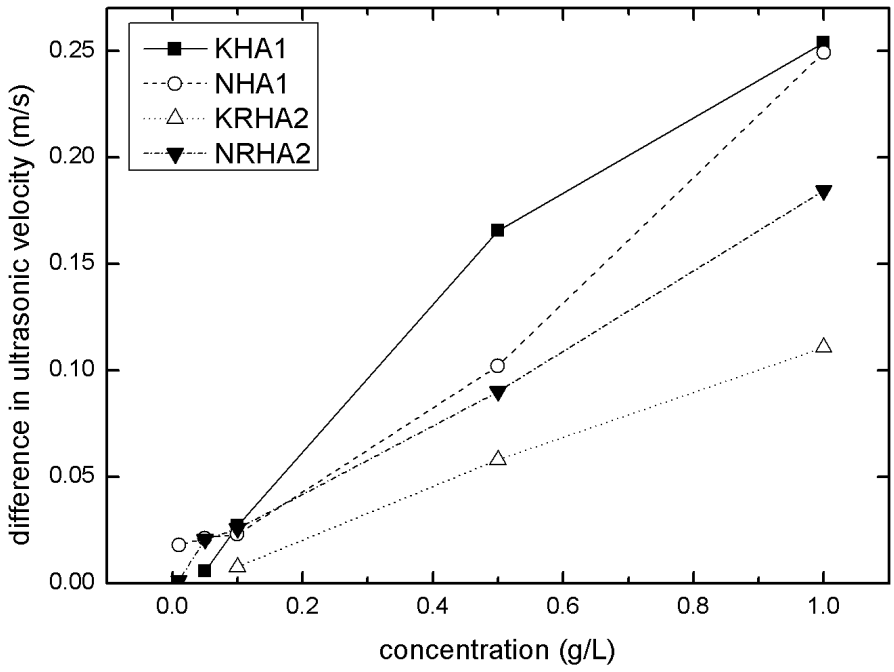

Figure 5 Difference of ultrasonic velocity (sample – water) in dependency on the concentration of the humate solution for samples KHA1, NHA1, KRHA2, NRHA2.

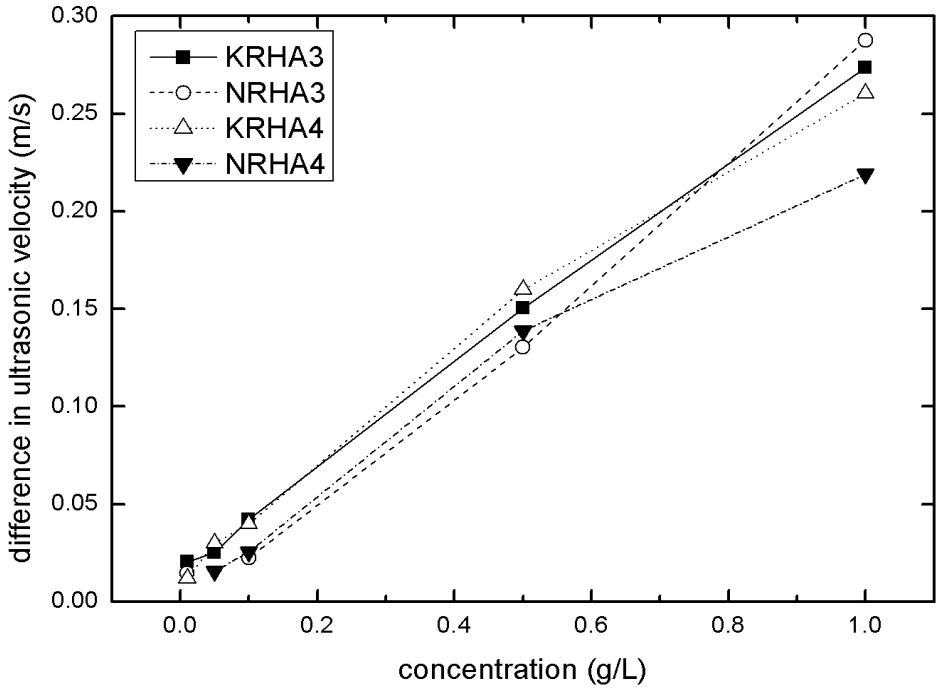

Figure 6 Difference of ultrasonic velocity (sample – water) in dependency on the concentration of the humate solution for samples KRHA3, NRHA3, KRHA4, NRHA4.

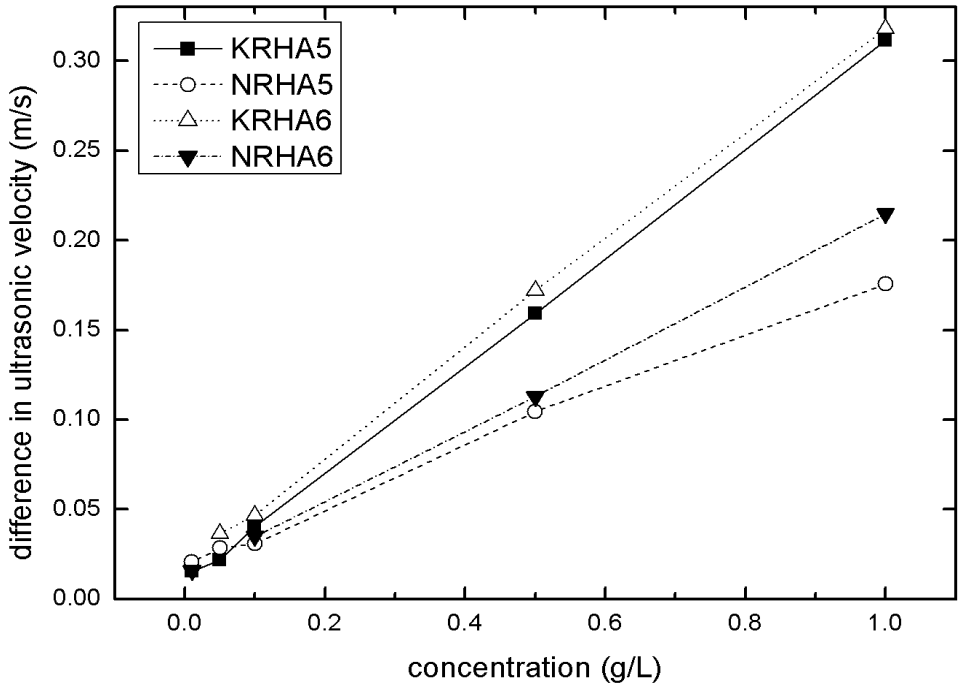

Figure 7 Difference of ultrasonic velocity (sample – water) in dependency on the concentration of the humate solution for samples KRHA5, NRHA5, KRHA6, NRHA6.

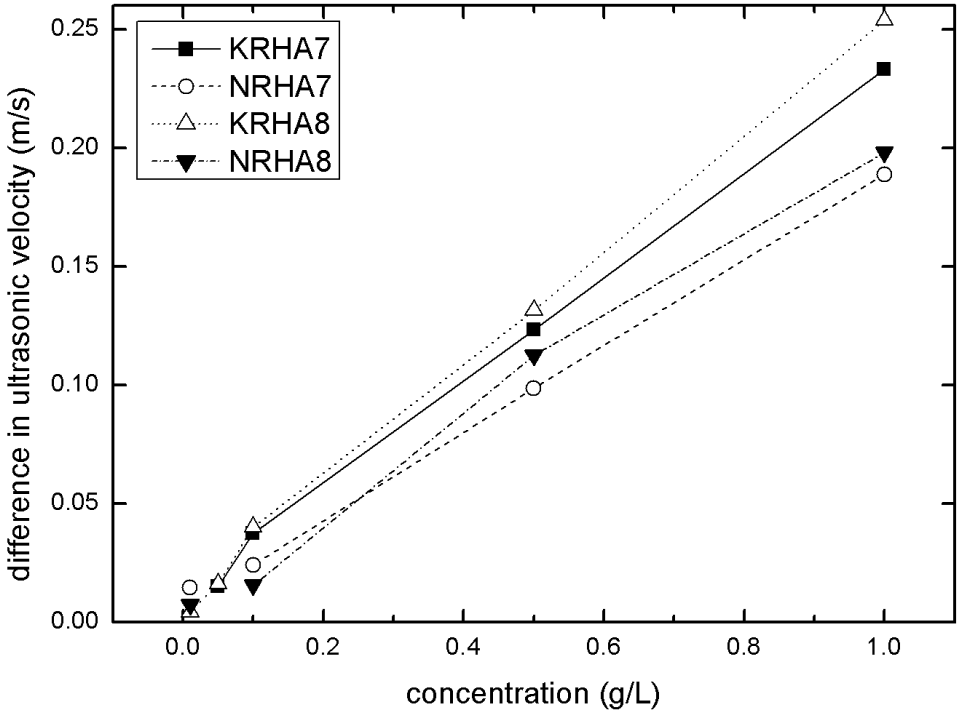

Figure 8 Difference of ultrasonic velocity (sample – water) in dependency on the concentration of the humate solution for samples KRHA7, NRHA7, KRHA8, NRHA8.

In order to better recognize the conformation differences between humates, the adiabatic compressibility was determined. Compressibility is a measure of the relative volume change of the solution in the response to a pressure. It can serve for determination of volume fraction of non-interacting solvent which provides information about the hydration of the whole humate with respect to all parameters discussed above. Results are reported in Figures 9-12.

As expected, with increasing concentration the volume fraction of non-interacting solvent is decreasing. This volume fraction consists of all water molecules except those trapped in hydration shell of the counterion and in hydration shell of the humic part. Fact is that increase of hydration with increasing concentration is linear for counterions, because (a) their concentration is increasing proportionally to the concentration of solution and (b) the experiment operates in a range of low concentrations (Zavitsas, 2001). Therefore, it has to be the humic part which causes the non-linearity seen in Figures 9-12. This observation is similar to the behaviour of other biomolecules, e.g. polysaccharides which demonstrated unfolding of structure and gradual hydration caused by gradual dilution (Davies et al., 1982). Our findings

are also in line with recent statement about the gradual aggregation of humates in aqueous solutions at low concentration without the exhibition of critical micelle concentration (Kučerík et al., 2009).

Determination of hydration of humic molecules is important with respect to the biological activity of humates. For example, Zavitsas (2001) discussed the similarities of K^+ and Na^+ cation in relation to the mechanisms concerned with K^+ channel proteins which are more permeable to K^+ than to Na^+ (by factor 10^3-10^4) despite the chemical similarities of the two ions and despite the smaller size of Na^+. Different potential for creating hydration shells for cation and its partner anion in solution can be a decisive point in diverse properties of seemingly similar ions.

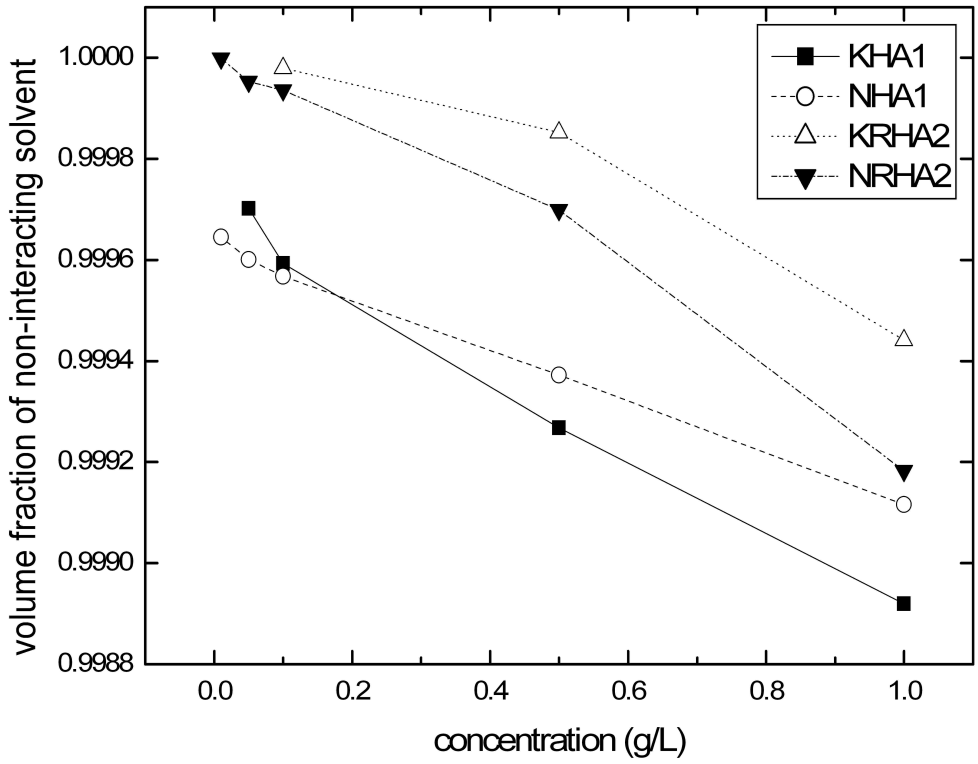

Figure 9 The volume fraction of non-interacting solvent in dependency on concentration of the humate solution for samples KHA1, NHA1, KRHA2, NRHA2.

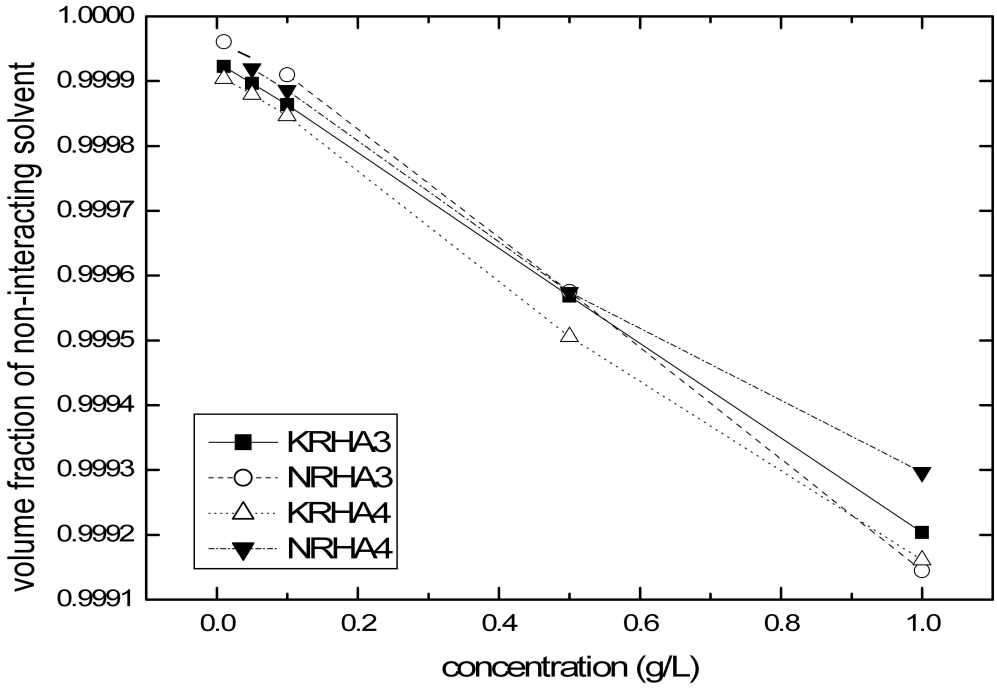

Figure 10 The volume fraction of non-interacting solvent in dependency on concentration of the humate solution for samples KRHA3, NRHA3, KRHA4, NRHA4.

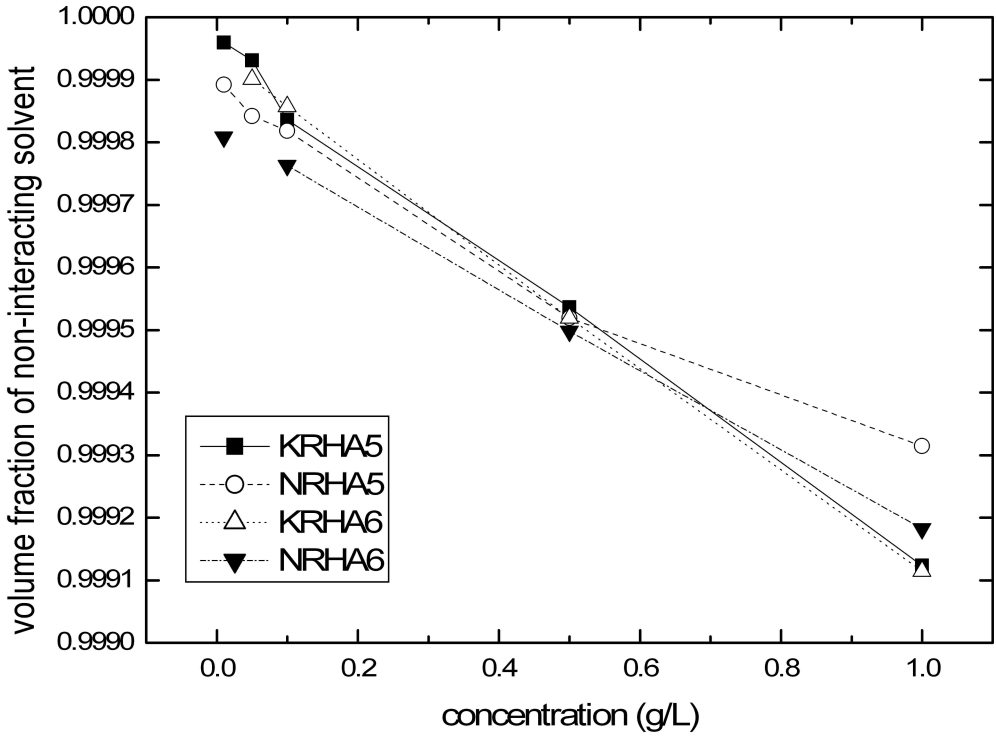

Figure 11 The volume fraction of non-interacting solvent in dependency on concentration of the humate solution for samples KRHA5, NRHA5, KRHA6, NRHA6.

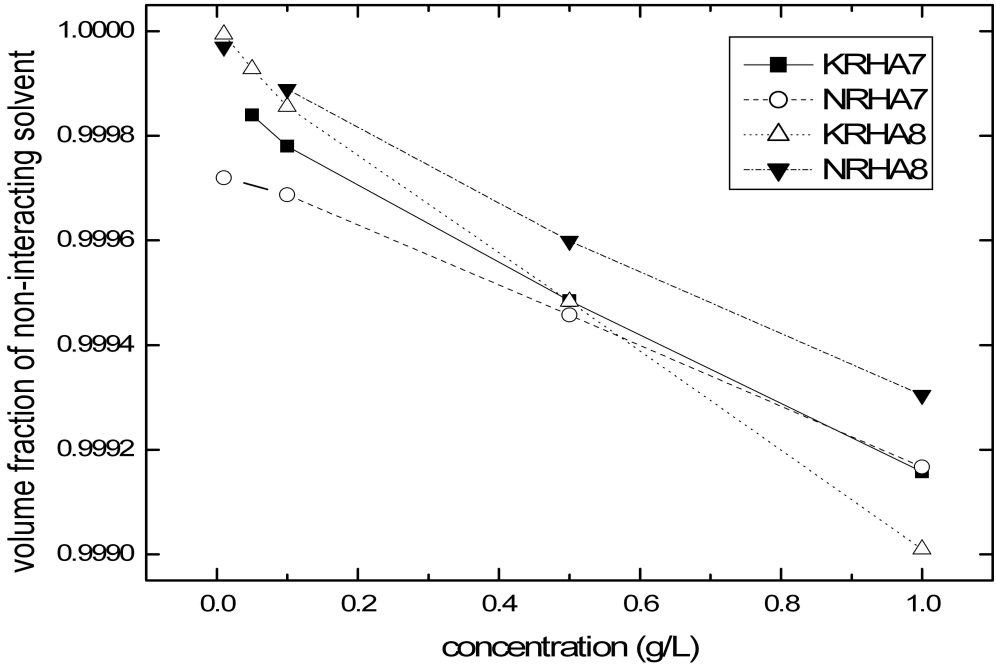

Figure 12 The volume fraction of non-interacting solvent in dependency on concentration of the humate solution for samples KRHA7, NRHA7, KRHA8, NRHA8.

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