VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
Fakulta chemická
Ústav fyzikální a spotřební chemie

Ing. Zoja Vlčková

CHEMICAL AND PHYSICAL TRANSFORMATIONS OF HUMIC ACIDS

CHEMICKÉ A FYZIKÁLNÍ TRANSFORMACE HUMINOVÝCH KYSELIN

ZKRÁCENÁ VERZE PH.D. THESIS

Obor: Fyzikální chemie

Školitel: doc. Ing. Miloslav Pekař, CSc.

KEYWORDS

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

KLÍČOVÁ SLOVA

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

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 real movement in the existing carbon cycle. It is not exactly clear to which extent anthropogenic activity is yet capable to reverse global warming in current state. There are major natural phenomena, e.g. volcanic activity, whose 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 insensitively with the availability of some crops. 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. 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 desertification in certain regions. One of possible solutions to the question of carbon loss in soils could be extensive application of other existing, natural materials – for example humic substances (HS) which exhibit positive influence on the carbon sequestration in soils and on the overall biological activity and fertility of soil. By supporting transport processes in soil and possibly enhancing the photosynthesis, HS 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 FORMATION OF HS

Although the formation process of HS has been studied hard and for a long time, it is still the subject of long-standing and continued research. Some theories have lasted for years, e.g. the “sugar-amine” condensation theory, the “lignin” theory or the “polyphenol” theory. A review of such theories can be found for example in Davies and Ghabbour [1]. Nowadays, most investigators suppose that HS originate in lignin [2], [3], [4]. 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 HS 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 [5]. According to a recently introduced concept, humification in soil can be seen as a two-step process consisting of biodegradation of dead-cells components and aggregation of the degradation products [6]. 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. Exclusion from water means exclusion from microbial degradation and persistence of humic matter in soil.

2.2 FUNCTIONAL GROUPS AND MOLECULAR CHARACTERISTICS

Physical and chemical properties, as well as the reactivity of HS 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. Elemental analysis of different fulvic acids (FAs) and humic acids (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 are present. Nitrogen, sulphur and phosphorus functional groups or bonds can be found in small amount, as well. Major difference between the functional group content of HAs and FAs is that a smaller fraction of the oxygen in the former can be accounted for in COOH, OH, and C=O groups. The quinone C=O content of HAs is universally higher than for FAs whereas the latter are richer in ketonic C=O content. Another difference is that practically all the oxygen in FAs can be accounted to known functional groups (COOH, OH, C=O) whereas a high proportion of the oxygen in HAs occurs as a structural component of the “nucleus” (ether or ester linkages) [5].

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. 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 [7]. This model had been strongly criticized by Wershaw [8], because mathematical equations used to define it were originally derived for high-molecular-mass linear polymers. Wershaw et al. [8], [9] had presented an alternative schematic membrane model, much like a protein, for the supramolecular structure of humic matter. In this membrane model, humic materials are composed of partially degraded molecular components from natural organisms which were held together in ordered aggregated structures by weak interactions, such as hydrogen bonding and π-bonding, van der Waals and hydrophobic forces.

Piccolo et al. [10] have recently presented a theory that humic constituents at neutral and alkaline acidities are supramolecular associations of relatively small heterogeneous molecules held together by weak dispersive forces. This conclusion was based on large-scale experiments confirming that after addition of modifiers such as natural small organic acids to the original humic-solute mixture, the macroscopic dimension of this supramolecular association is disrupted in smaller sized associations with reduced chemical complexity. This disruption by organic acid additions was attributed 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 [11].

2.3 BIOLOGICAL ACTIVITY OF HS

HS are resistant to microbial degradation [12] and are not considered to be involved in microbial metabolism, especially in anoxic habitats. However, Lovley et al. [13] showed that some microorganisms found in soils and sediments are able to use HS as electron acceptors for the anaerobic oxidation of organic compounds and hydrogen. This electron transport is supposed to yield energy to support growth. Scott et al. [14] demonstrated that the electron-accepting capacity of HS relates directly to their free radical content. Organic radicals in HS are primarily quinone-like groups, thus their amount could be important for the biological activity of HS. Some studies [15], [16] suggest that HS might support the carbon and electron flow under unaerobic conditions and serve as an electron shuttle. Canellas et al. [17] 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 demonstrated that these HAs enhance the root growth of maize seedlings in conjunction with 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.

It has been reported that HS can stimulate plant growth by the release of bioactive molecules with auxin-like activities. A series of studies by Nardi et al. [18], [19], [20] 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 HS and to the pH of the medium. It was also shown that humic matter stimulates carrier-protein synthesis at a posttranscriptional level. Nardi et al. [18], [20] also tried to determine auxin-like activity of HAs according to their size distribution. 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 (LMS) in influencing the bioactivity of a plant system. Further results [20] showed that LMS fraction of tested HAs increased nitrate uptake and strongly inhibited K^+, stimulated ATPase of maize microsomes and H^+ extrusion in a manner similar to gibberellic acid. Nardi et al. [21] attribute the overall effect of HS on plant growth depends on the source, concentration and molecular size of humic fraction.

Interesting approach to the question of HS biological activity was described by Popov [22]. He presented a very complex conceptual model reflecting the biological effect of HS on biochemical and biophysical processes occurring in vascular plants composed on the basis of scientific literature review and his own experimental data. According to this proposed model, biological activity of HS is determined by tree factors; i.e. (1) presence of varied functional groups, (2) colloidal characteristics of HS and (3) material constitution. Consecutively, the processes of respiration and photosynthesis can be optimized by addition of HS into the green plant system.

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 [23] (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 HAs. As regularly demonstrated in literature [24], [25], [26] HAs 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, a possible energy source for executing biological functions. On the other hand, size distribution of examined HAs 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 HAs. This is to be considered consequential, as it influences directly the HAs properties. Aggregation behaviour differences between HAs prepared from individually pre-treated lignite are monitored by means of HR-US. Concentration influence on their stability and structural rearrangement are observed. Finally, the effect of hydration and character of water structures surrounding the HA is investigated.

4 EXPERIMENTAL

4.1 HUMIC ACIDS

South Moravian lignite collected from the Mír mine in the area of Mikulčice, near Hodonín, the Czech Republic [29] 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. 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. 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. List of samples with respective lignite pre-treatment agent and elemental analysis are reported in Table 1.

Table 1: Elemental composition (atomic %) of HAs samples

Sample	pre-treatment		W[a] (%)	A[b] (%)	C[b] (at. %)	H[b] (at. %)	N[b] (at. %)	S[b] (at. %)	O[b] (at. %)
HA1	–	Mean	3.33	1.84	45.4	36.4	1.17	0.54	16.5
		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
		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
		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
		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
		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
		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
		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
		SD	0.03	0.10	0.3	0.3	0.09	0.16	0.2

4.2 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 an acquisition time of 25 ms, a recycle delay of 2.0 s, and a 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.

4.3 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 and average values were reported.

4.4 DETERMINATION OF POLYOLS BY GC-MS

The HAs hydrolysis was conducted in two successive stages. 10 mg of HAs were introduced in a Pyrex tube with 5 ml of 1.2 M HCl. The tube was closed under vacuum and heated for 3 h at 100°C. After hydrolysis and cooling, the sample was filtered on 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. The second hydrolysis was performed under the same conditions. To each hydrolysate, 1 ml of a 0.2 mg/ml solution of 2-deoxy-D-glucose was added as internal standard. The hydrolysates were then concentrated to 1–2 ml in a rotary evaporator at temperature below 50°C. Subsequently, the residual water and acid were evaporated to dryness at 25–30°C, 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) 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 carrier gas. The GC temperature was programmed from 60 to 300°C at 5°C·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 (with the same GC conditions). The MS was operated in the electron impact mode with a 70eV's ion source energy and the ion separation was operated in a quadripolar mass filter. Products were identified on the basis of their GC retention times, their mass spectra (comparison with standards) and library data (NIST 1.7).

4.5 DETERMINATION OF LIGNIN MONOMERS

The DFRC degradation method includes two key steps: bromination and acetylation with AcBr followed by reductive cleavage with zinc dust. For the derivatization step, 1 g of HA was homogenized and put to a 250 ml round bottom

flask containing 50 ml of the solution of acetyl bromide in acetic acid (8/92). Mixture was stirred for 2 hours in graphite bath at 50°C. After the derivatization, solvent was completely evaporated by rotavator at 45°C. For the reductive cleavage, residue was dissolved in 50 ml of acetic reductive solution consisting of dioxane, acetic acid and water (5v/4v/1v). The mixture was well stirred and 5 g of zinc dust were added as a catalyst. The reaction continued for 30 minutes in graphite bath at 50°C under intensive stirring. Samples were purified, dried, separated according to acidity and dissolved in chloroform. Lignin monomers were detected and identified by GS-MS (the same instrumental configuration as in polyol analysis).

4.6 HPSEC MEASUREMENTS

To assess the molecular size distribution of humic samples, the Biosep S2000 column from Phenomenex with the dimensions of 600×7.8 mm was chosen. The Agilent HPSEC system consisted of a solvent pump equipped with two detectors in series: UV/VIS detector, set to 280 nm, and 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. As the mobile phase $NaH_2PO_4.H_2O$ was prepared in molar concentration of 50 mM and adjusted to pH 7 with 1M NaOH in order to keep constant ionic strenght and minimize potential ionic exclusion or hydrophobic interactions with the column stationary phase. The weight-average molecular weight M_w was calculated according to the following formula: $M_W = \frac{\sum_{i=1}^{N}(h_i \cdot M_i)}{\sum_{i=1}^{N} h_i}$, where M_i and h_i are the molecular weight and the height of each *i*th fraction in the chromatogram. For column calibration standards of known M_w were used. Polysaccharides PSC (404, 212, 112, 47.3, 5.9 and 0.667 kDa) were used to calibrate RI detector and sodium polystyrenesulphonates PSS (194.2, 145, 32.9, 14.9, 6.53 and 0.91 kDa) were used to calibrate UV detector. Calibration curves defined by standards were used to obtain the M_w of humic samples. Each sample was measured twice and 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) and blue dextran (2000 Da) was used to measure the void volume (10.5 mL).

4.7 BIOLOGICAL ACTIVITY ASSESSMENT

Biological activity of the different HAs was tested on *Zea mays* using the methods of [39]. 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 performed in an air-conditioned sterilized container, where the light, temperature and humidity conditions were set according to optimal requirements of used plant species. These were 14 hours of simulated daylight under temperature of 35° and 10 hours of darkness under temperature of 20°. Humidity was fixed to 60 ± 2%. In each experiment 28 non sterilized seeds were used. First, all seeds were placed into a germination box for 2 days where the germinal root of the seed reaches minimal lenght which enables its placement into the experimental container. This consists of 14 tubes, each holding 2 seeds, and is filled with 5 l of experimental solution. Water solution of comercially produced Hydropon fertilizer (0.59 g/l) is used as control solution. In order to prepare experimental solutions, control solution was enriched by tested substance (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 B03H (comparative compound) into the control solution. Root lengths were recorded after 48, 72 and 96 hours. The intensity of stimulating effect of the tested substances is stated in relative units, which express the accrual, eventually the shortening of root length in comparison either to the control solution or to the comparative solution.

4.8 GENOTOXICITY/ANTIMUTAGENICITY ASSAY

The tests were performed with the yeast *Saccharomyces cerevisiae* using the method described in [40]. After 16-18 hours of yeast cultivation 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), the 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. Percentage of inhibition of mutagen in the presence of humate sample was calculated as follows: $\%_{inh} = 100 - [(X_1/X_2)*100]$, where X_1 is the number of yeast colonies in presence of 4-NQO and HA water extract, X_2 is the number of yeast colonies in presence of 4-NQO without humate solution. The results are expressed as means ± SD from 3 measurements. Results were analysed by the Student t-test using Statistica for Windows 5.0. The differences of $p<0.05$ were regarded as statistically significant.

4.9 DENSITOMETRY MEASUREMENTS

The densities of aqueous solutions of 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.

4.10 ULTRASONIC MEASUREMENTS

Ultrasonic velocity of the same samples at the same concentrations was measured with 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. Averaged values from 3 measurements are reported. Values which did not show reproducibility were discarded.

5 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. HAs are one of many environmentally interesting products that can be extracted from lignite as they have various applications in green chemistry. 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.

The first lignite pre-treatment studied for this purpose was oxidation by air [28]. 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. 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 experiment in fluid reactor also showed that higher temperature supports the production of HAs. The air oxidation experimets were based on the assumption that relatively mild conditions could significantly increase HAs yield and, simultaneously, have a non-destructive effect on their chemical properties, i.e. not cause carbon loss due to intensive oxidation effect. This aim was seemingly achieved, when elementary analysis and FTIR data confirmed that chemical characteristics of samples did not differ significantly among each samples or in comparison to the HA extracted from parental lignite.

Concurrently, a study of antimutagenic and/or genotoxic effect of processed HAs was conducted, described in detail in [41]. Sample processed at high temperature

(250°C) was also included in the assay and was the only one to show contribution to the genotoxic effect of standard mutagen in all tested concentrations. Thus, 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, 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 this purpose, a well considered line of 8 HAs (Table 1) was prepared in order to carry out an extensive study on their properties, behaviour and agricultural functions in relation to their primary and supramolecular structure. 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.

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, but with considerable impact on carbon loss in resulting HAs [29]. 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 decrease the aggregation degree of present organic matter and facilitate its penetration of cell-walls, nutrient transference and water sequestration.

From practical point of view, HAs in agriculture are most likely to be applied by watering and spraying. Properties of complex substances such as HAs in aqueous solutions can be very specific and sensitive even to minor changes in conditions. They 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 humates solutions [30]. To examine also the influence of counterion, two sets of humates were prepared, with K^+ and NH_4^- counterion. These ions differ enough to demonstrate the importance of careful consideration of counterion choice when preparing humates for a particular purpose.

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 (Table 2).

This is an important finding for it suggests that primary structure is not the key to the control of HAs behaviour in natural environment. Further interesting findings are: (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.

Table 2: Carbon distribution (%) in different intervals (ppm) of CPMAS ^{13}C-NMR spectra of the lignite HAs samples measured in form of potassium humates.

Sample	261-191 (ketons, aldehyds, quinones)	191-169 (carboxyls)	169-98 (aromaticC)	98-68 (ethers, alcohols)	68-0 (alkylC)
HA1	7.09	7.56	45.80	4.97	34.57
RHA2	5.07	7.26	46.77	5.15	35.74
RHA3	4.57	6.70	40.13	8.34	40.25
RHA4	2.79	5.89	37.58	9.61	44.13
RHA5	5.85	6.91	40.66	5.97	40.59
RHA6	5.96	6.87	40.53	6.71	39.93
RHA7	5.51	7.81	46.34	5.52	34.81
RHA8	4.76	7.48	45.99	6.07	35.69

Three most important compounds identified as TMS esthers after HCl hydrolysis of processed HAs 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 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 [31]. Detection of rather low quantities of polyols in HAs isolated from aged lignite source was expected, although work of Allard [32] proves that residual carbohydrate structures can be found in lignite HAs, which indicates long-term persistence of these readily degradable products.

Our results (Figure 1) show that polyols content differs among variously pre-treated samples. The values for 5% H_2O_2 processed sample are outstanding (160 mg/g of HA) as 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. 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 reason for 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. 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 [33] 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, from dissolved organic matter to the formation of particles and macromolecules by means of their specific interactions with cations and self-aggregation properties.

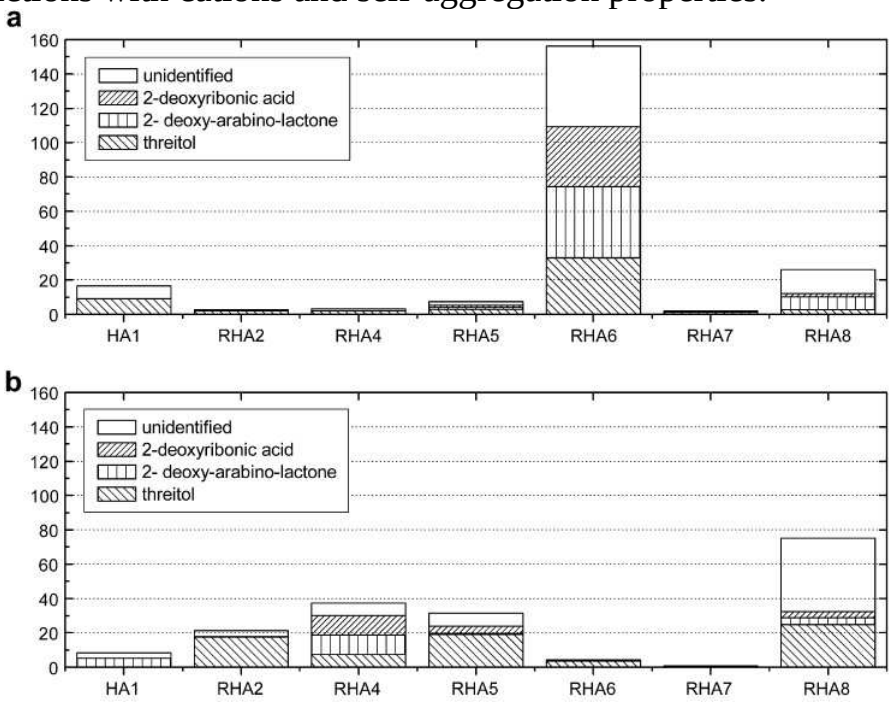


Figure 1: Amounts of saccharidic-like compounds in mg/g of HAs after the first (a) and the second (b) hydrolysis. Data for sample RHA3 were not acquired.

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 [34], [35] 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. Results, as detected by GS-MS (Figure 2), show that lignite HAs contain intact lignin monomers. 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-$CH_2CH{=}CH_2$ 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_2CH_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. Coumaric, coniferic and sinapic acids (P'-CO_2Me, G'-CO_2Me and S'-CO_2Me) are of diagenetically modified lignin. 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.

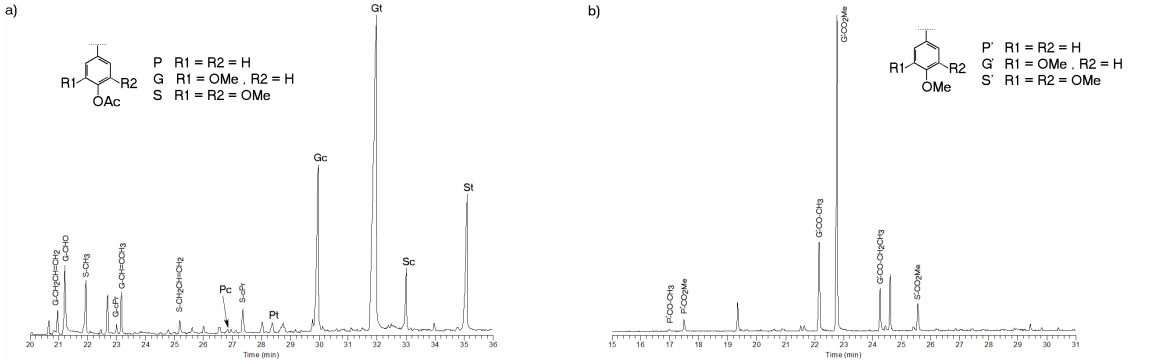


Figure 2: Chromatograms of monomeric DFRC products from lignite HAs bearing (a) alcohol units and (b) keto or carboxyl units.

A quantitative EPR study was carried out. Organic free radical (OFR) 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 [36]. On the other hand, OFR counts in samples of humates were lower. This observation can be explained by the role of counterion in stability of free radicals in lignite HAs, as demonstrated recently [37]. When comparing 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 OFR concentrations and the carboxyl groups contents as determined by NMR, which suggests that the carboxylic groups contributed to OFR stabilization in investigated samples. Good correlation (r = 0.70)

was found between the OFR concentration and aromatic C, although there are some literature sources which report that aromaticity did not largely influence the OFR content of investigated HAs [38]. Yabuta et al. [38] suggested that OFR 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.

In humate samples situation is different. Radical content of ammonium humates correlated 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 [37] and in above paragraphs.

Table 3 Free radical content (in spins g^{-1}) of samples at 25°C.

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}

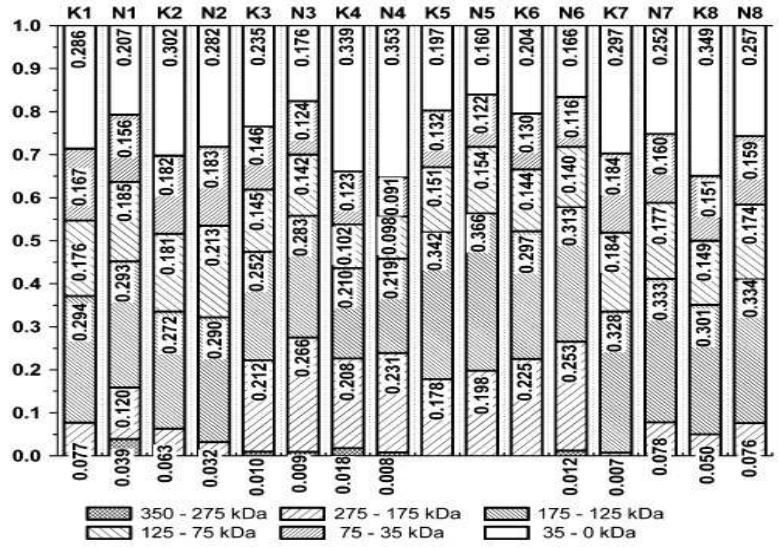


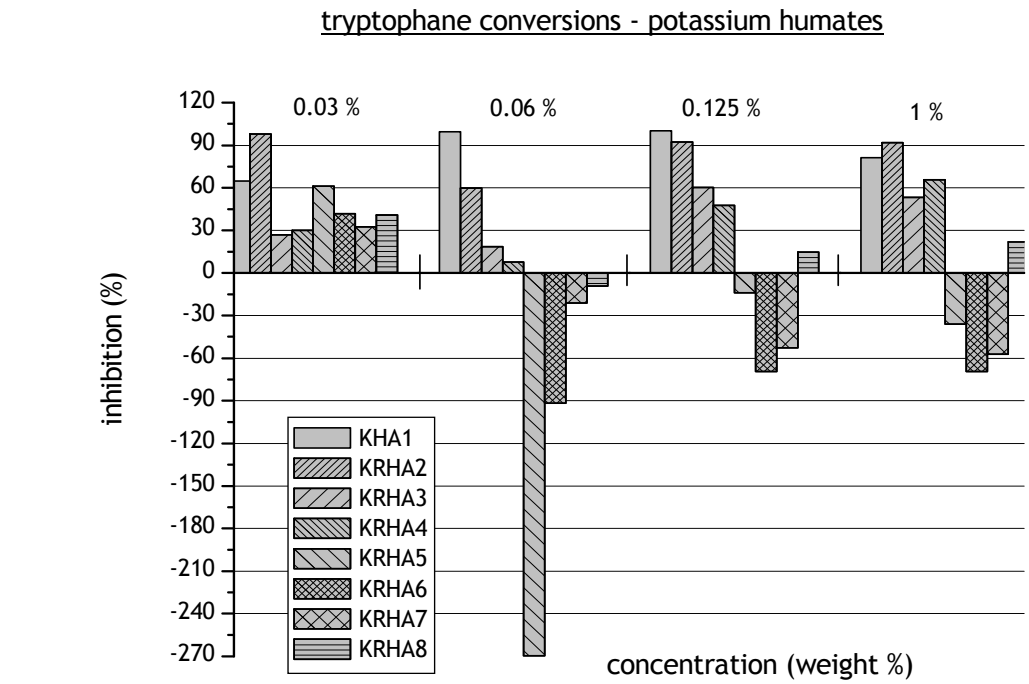
Figure 3 Molar size distribution calculated from data detected by RID. K1-K8 represent respective potassium humates, N1-N8 respective ammonium hmates.

HPSEC measurements were carried out to address the questions concerning supramolecular structure and presumed aggregation behaviour of HAs in forms of their salts. Essential conclusions are that (a) the humate aggregate size distribution (Figure 3) 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.

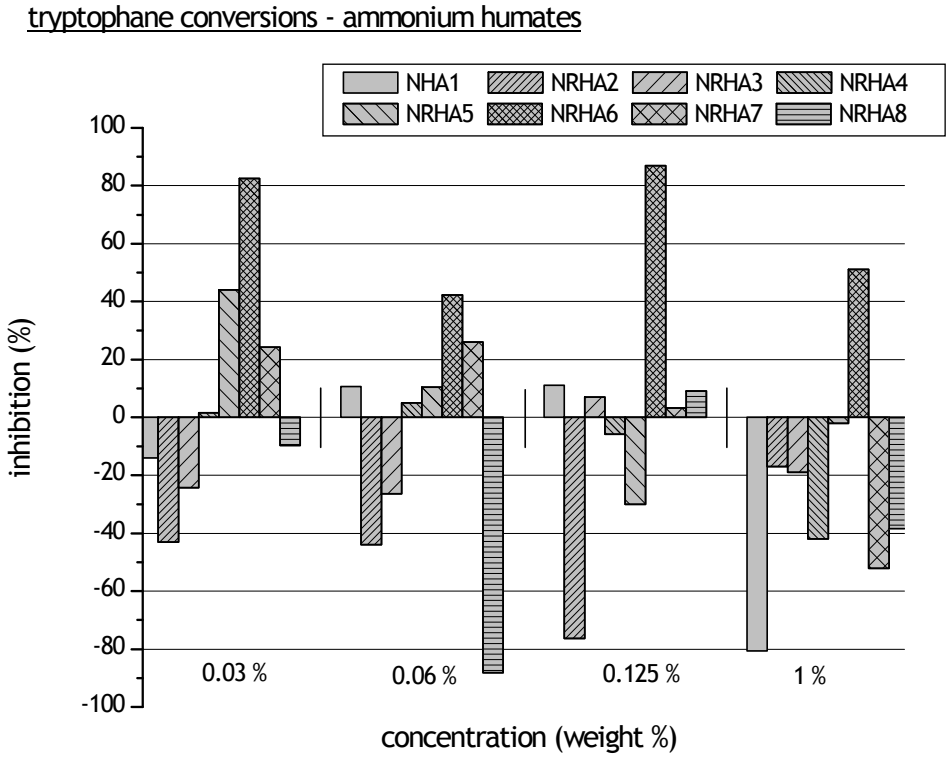
Table 5: Biological activity results in 3 time intervals: (0-48 h), (0-72 h) and (0-96 h). The intensity of stimulating effect of each tested substance is stated in relative units (%), which express the accrual, eventually the shortening of root length in comparison to (a) the control solution of Hydropon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

Sample	Time intervals								
	after 48 hours			after 72 hours			after 96 hours		
	(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

Information about biological activity in the context of lignite pre-treatment can be found in [42]. 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, 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



(a)



(b)

Figure 4: Per cent of inhibition of yeast colony number obtained for tryptophane conversions at presence of mutagen and (a) K^+ humates (b)NH_4^- humates.

coefficient (r) as 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. Correlation between bioactivity and the content of polar carboxylic groups was also

positive, first (after 72 h) increasing then (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 [42] 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 no correlation whatsoever to 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 applies to both humates.

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, results in Figure 4. 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.

Densitometry and ultrasonic velocity measurements are used to evaluate process of hydration in terms of concentration and counterion influence. Density increased with increasing concentration although the trend is not perfectly linear (data not shown). In many cases the density of potassium humate was lower than of 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 number is higher for K^+ ions [43], thus higher density of NH_4^+ humates must be attributable to the influence of dissociated humate itself. That means that either the organic part of NH_4^+ humate is more hydrated, or the organic part of NH_4^+ humate is denser. To access more information on these issues, ultrasonic measurements were carried out. Dependence of ultrasonic velocity on the concentration of humate solution (data not shown) showed contrasting results to density. In most cases, the

ultrasonic velocity in solution of potassium humates showed higher values than in respective ammonium humates. US 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.. Acquired density and velocity data are used to calculate the adiabatic compressibility and volume fraction of non-interacting solvent (example in Figure 5). 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.

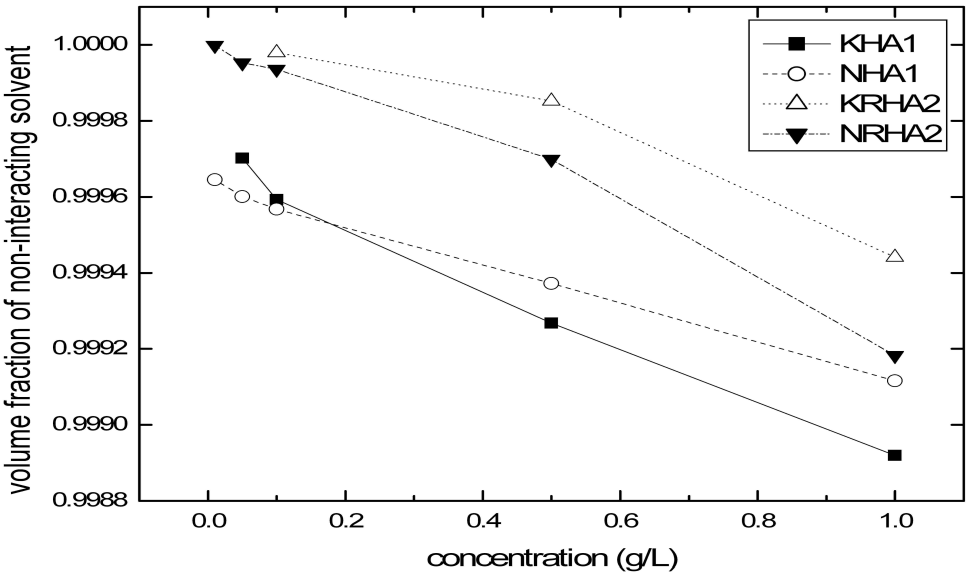


Figure 5: Volume fraction of non-interacting solvent in dependency on concentration of the humate solution for samples KHA1, NHA1, KRHA2, NRHA2.

The hydration numbers calculated in grams of hydration water (bound water) per grams of humate gave values 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.

6 CONCLUSIONS

- 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) in 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.

7 REFERENCES

[1] Davies, G., Ghabbour, E. A. (eds.): *Humic substances, structures, properties and uses*. The RSC, Cambridge 1999.

[2] Oglesby R. T., Christman R. F., Driver C. H.: *The biotransformation of lignin to humus facts and postulates*. Adv. Appl. Microbiol., 1967, 9, pp. 171-184.

[3] Burdon J.: A*re the traditional concepts of the structures of humic substances realistic?* Soil Sci., 2001, 166, pp. 752-769.

[4] Davies, G., Ghabbour, E. A., Steelink C: *Humic acids: Marvelous products of soil chemistry*. J. Chem. Educ., 2001, 78, pp. 1609-1614.

[5] Peña-Méndez, E. M. et al.: *Humic substances – compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine*. J. Appl. Biomed., 2005, 3, pp. 13-24. ISSN 1214-0287.

[6] Piccolo, A.: *The supramolecular structure of humic substances*. Soil Science, 2001, 166, 11, pp. 810-832.

[7] Swift R. S. In Humic Substances II, in *Search of Structure*; Hayes M. H. B., MacCarthy P., Malcolm R. L., Swift R. S., Eds. John Wiley & Sons: New York, 1989, pp. 449-495.

[8] Wershaw R. L.: *A new model for humic materials and their interactions with hydrophobic organic chemicals in soil-water or sediment-water systems*. J. Contam. Hydrol., 1986, 1, pp. 29-45.

[9] Wershaw R. L.: *Application of a membrane model to the sorptive interactions of humic substances*. Environ. Health Perspect., 1989, 83, pp. 191-203.

[10] Piccolo A., Conte P., Trivellone E., van Lagen B., Buurman P.: *Reduced heterogeneity of a lignite humic acid by preparative HPSEC following interaction with an organic acid. Characterization of size-separates by Pyr-GC-MS and ^{1}H-NMR spectroscopy*. Environ. Sci. Technol., 2002, 36, pp. 76-84.

[11] Peuravuori, J., Pihlaja, K.: *Preliminary Study of Lake Dissolved Organic Matter in Light of Nanoscale Supramolecular Assembly*. Environ. Sci. Technol., 2004, 38, pp. 5958-5967.

[12] McKnight, D. M.: In Perdue, E. M., Gjessing, E. T. (eds): *Organic acids in aquatic ecosystems*. John Wiley and Sons, New York, 1990, pp. 223-243.

[13] Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P., Woodward, J. C.: *Humic substances as electron acceptors for microbial respiration*. Letters to Nature, 1996, vol. 382.

[14] Scott, T. D., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E., Lovley, D. R.: *Quinone-like moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms*. Environ. Sci. Technol., 1998, 32, 2984-2989.

[15] Lovley, D. R., Woodward, J. C., Blunt-Harris, E. L., Hayes, L., Phillips, E. J. P., Coates, J. D., J. Acta Hydrochim. Hydrobiol., 1998, 26, 152
[16] Coates, J. D., Ellis, D., Blunt-Harris, E. L., Gaw, C., Roden, E., Lovley, D. R., Appl. Environ. Microbiol., 1998, 64, 1504.
[17] Canellas, L. P., Olivares, F. L., Okorokova-Facanha, A. L., Rocha Facanha, A.: *Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+-ATPase activity in maize roots*. Plant Physiology, 2002, 130, pp. 1951-1957.
[18] Nardi, S., Panuccio, M. R., Abenavoli, M. R., Muscolo A.: *Auxin-like effect of humic substances extracted from faeces of Allolobophora Caliginosa and A. Rosea*. Soil Biol. Biochem., 1994, 26, pp. 1341-1346.
[19] Nardi, S., Concheri, G., Dell'Agnola, G.: Biological activity of humus. In Piccolo, A. (ed): *Humic substances in terrestrial ecosystems*. Elsevier, New York, 1996, pp. 361-406.
[20] Nardi, S., Pizzeghello, D., Gessa, C., Ferrarese, L., Trainotti, L., Casadoro, G.: *A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings*. Soil Biol. Biochem., 2000, 32, pp. 415-419.
[21] Nardi, S., Pizzeghello, D., Muscolo, A., Vianello, A.: *Physiological affects of humic substances on higher plants*. Soil Biol. Biochem., 2002, 34, 1527-1536.
[22] Popov, A. I.: *The probable mechanism of biological effect of humic substances*. Proceedings of the 14th international meeting of the IHSS, 2008, vol. 2, pp. 453-456.
[23] Kučerík, J., Conte, P., Pekař, M., Piccolo, A.: *Conformational behavoiur of lignite humic fractions separated by sequential pH-extractions*. Fressenius Environmental Bulletin, 2003, 12, 683-689.
[24] Flaig, W.: *About the effect of humic substances on the plant metabolism*. 1963, Int. Peat Congress, Leningrad, Russia, 48 p.
[25] Kononova, M. M.: *Soil organic matter: its role in soil formation and in soil fertility*. 1966, Pergamon Press, New York, 544 p.
[26] Sladký, Z., Tichý, V.: *Application of humus substances to overground organs of plants*. 1959, Biologia Plantarum, Praha 1, pp. 9-15
[27] Kučerík, J., Čechlovská, H., Bursáková, P., Pekař, M.: *The thermodynamic stability and molecular feature of lignite humic acids aggregates studied by high resolution ultrasonic spectroscopy.* J. Therm. Anal. Cal., 2008, accepted.
[28] Cihlář, Z., 2007. Bachelor thesis, VUT Brno.
[29] Kučerík, J., Pekař, M., Klučáková, M.: *South-Moravian lignite – potential source of humic substances*. Petroleum and Coal, 2003, 45, pp. 58-62.
[30] Tombácz, E.: *Colloidal properties of humic acids and spontaneous changes of their colloidal state under variable solution conditions.* Soil Science, 1999, 164, 814-824.
[31] Ogner, G.: *Analysis of the carbohydrates of fulvic and humic acids as their partially methylated alditol acetates*. Geoderma, 1980, 23, 1-10.

[32] Allard, B.: *A comparative study on the chemical composition of humic acids from forest soil, agricultural soil and lignite deposit: Bound lipid, carbohydrate and amino acid distribution*. Geoderma, 2006, 130, 77-96.
[33] Mecozzi, M., Pietrantonio, E.: *Carbohydrates, proteins and lipids in fulvic and humic acids of sediments and its relationships with mucilaginous aggregates in the Italian seas*. Marine Chemistry, 2006, 101, 27-39.
[34] Lu, F., Ralph, J.: *Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: Protocol for analysis of DFRC monomers*. Journal of Agriculture and Food Chemistry, 1997, 45, 2590-2592.
[35] Lu, F., Ralph, J.: *The DFRC method for lignin analysis. 2 Monomers from isolated lignins*. Journal of Agriculture and Food Chemistry, 1998, 46, 547-552.
[36] Jezierski, A., Czechowski, F., Jerzykiewicz, M., Chen, Y., Drozd, J.: *Electron paramagnetic resonance (EPR) studies on stable and transient radicals from compost, soil, peat and brown coal*. Spectrochimica Acta Part 1: Molecular and Biomolecular Spectroscopy, 2000, 56, 379-385.
[37] Kučerík, J., Bakajová, B., Pekař, M.: *Antioxidant effect of lignite humic acid and its salts on the thermo-oxidative stability/degradation of polyvinyl alcohol blends*. Environmental Chemistry Letters, 2008, 6, 241-245.
[38] Yabuta, H., Fukushima, M., Kawasaki, M., Tanaka, F., Kobayashi, T., Tatsumi, K.: *Multiple polar components in poorly-humified humic acids stabilizing free radicals: Carboxyl and nitrogen-containing carbons*. Organic Geochemistry, 2008, 39 (9), 1319-1335.
[39] Antošová, B., Novák, J., Kozler, J., Kubíček, J., Kimmerová, I.: Methodic for testing biological activities of humic substances in higher plants. In Barosso, M. I. (ed): *Reactive and functional polymers research advances*. NovaScience Publishers, 2007, pp. 191-203.
[40] Zimmermann, F.K., von Borstel, R.C., von Halle, E.S., Parry, J.S., Siebert, D., Zetterberg G., Barale R, Loprieno, N., 1984. *Testing of chemicals for genetic activity with Saccharomyces cerevisiae*. Mutat Res 133, 199-224.
[41] Márová, I., Kučerík, J., Mikulcová, A., Duroňová, K., Vlčková, Z., 2009. *Study of antimutagenic and/or genotoxic properties of processed humates*. Accepted with revisions to Environmental Chemistry Letters.
[42] Vlčková, Z., Grasset, L., Antošová, B., Pekař, M., Kučerík, J., 2009. *Lignite pre-tretment and its effect on bio-stimulative properties of respective lignite humic acids*. Soil Biology & Biochemistry, doi:10.1016/j.soilbio.2009.06.013.
[43] Zavitsas, A.A.: *Properties of water solutions of electrolytes and nonelectrolytes*. Journal of Physical Chemistry, B 2001, 105, 7805-7817.

8 CURRICULUM VITAE

Name:	Zoja Vlčková
Date of birth:	19.04.1983
Place of birth:	Nitra, SR
Education:	1997 – 2001 Secondary school, Párovská 1, Nitra, SR 2001 – 2006 Faculty of Chemistry, Brno University of Technology, diploma thesis: EPR study of selected compounds decomposition by the method of spin trapping. 2006 – 2009 Faculty of Chemistry, Brno University of Technology, dissertation thesis: Chemical and physical transformations of humic acids.
Languanges:	advanced: English, German; intermediate: French, Portuguese
Scientific training:	2007: EMBRAPA, Colombo, Brazil 2008: University of Poitiers, Poitiers, France
Teaching praxis:	2006 – 2008: Seminar in physical chemistry II, FCH, BUT
Course:	2006: Inerpretation of vibration spectra, Prague

9 ABSTRAKT

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.