ABSTRACT

Submitted thesis deals with the humic substances, namely the humic acids and their salts, i.e. potassium humates. A literature review about humic substances is presented, the brief history of their research, their structure issues, supramolecular arrangement and applications. A special attempt is paid to review methods of extractions of humic acids from various sources. Further, the papers concerning biological, hormone-like properties are listed and discussed. From the environmental chemistry point of view mainly sorption properties are overviewed. Industrial applications of humics are covered by a wide range of patents and published works such as for instance dyes, polymer additives, etc. In experimental part, the regeneration of lignite and preparation of humic acids and their salts is described. The regeneration principle lies in the oxidation of parental lignite with a range of concentrations of nitric acid and hydrogen peroxide. Novel approach is presented in the characterization humic substances, where the chemical characteristics like Elemental Analysis, Fourier Transform Infra-red Spectroscopy and Thermogravimetry are followed by physico-chemical assessments (Dynamic Light Scattering, Fast Field Cycling Nuclear Magnetic Resonance Relaxometry, High Performace Size Exclusion Chromatography and Fluorescence Spectroscopy in combination with research of humates hydration by means of High Resolution Ultrasonic Spectrometry and Densitometry). These chemical and physico-chemical characteristics are put into the correlation with the biological characteristics, i.e. biological activity of humates, which is assessed by a slightly modified method based on the determination of maize root weight, length and number of lateral roots. Finally, the statistical approach is applied via Pearson's correlation coefficient and as a pilot studies, two environmental applications of regenerated humic materials are designed, in the field of sorption of tetracycline antimicrobial and researching of regenerated lignited as a potential source of fermentable sugars.

KEYWORDS

Humic substances, humates, applications, biological activity, supramolecular structure, physical aspects, sorption.

ABSTRAKT

Předkládaná disertační práce se zabývá huminovými látkami (HL), zejména huminovými kyselinami a jejich solemi, tj. humáty. V práci je prezentována literární rešerše o huminových látkách, stručně je zmíněna historie výzkumu huminových látek, obsáhle jsou pak prezentovány práce o jejich struktuře a supramolekulovém uspořádání. Jsou též zmíněny metody extrakce huminových látek z různých zdrojů. Dále jsou v této práci zařazeny a diskutovány práce zabývající se biologickými „hormonálními“ vlastnostmi HL. Z hlediska chemie životního prostředí jsou zmíněny zejména sorpční vlastnosti HL. Popsané průmyslové aplikace HL pak zahrnují celou řadu patentů a publikovaných prací, zabývajících se využitím huminových látek jakožto barviv či přísad do polymerů, atd. V experimentální části je popsána příprava regenerovaných lignitů, z nichž jsou dále extrahovány huminové kyseliny a připraveny huminové soli, tzv. humáty. Princip regenerace spočívá v oxidaci původního lignitu širokou koncentrační řadou kyseliny dusičné a peroxidu vodíku. K charakterizaci získaných huminových materiálů je výhodně použit nový přístup, kdy jsou kombinovány výsledky z analýz chemických (elementární analýza, infračervená spektroskopie, termogravimetrie) a fyzikálně-chemických (dynamický rozptyl světla, nukleární magnetická rezonanční relaxometrie, vysoceúčelná velikostně-vylučovací chromatografie a fluorescenční spektrometrie se zhodnocením hydratace humátů pomocí vysocerozlišovací ultrazvukové spektrometrie a hustoměru). Tyto metody jsou navíc položeny do kontextu s charakterizací biologické aktivity humátů, která je provedena pomocí modifikované metody založené na měření délky a hmotnosti kořenů kukuřice a laterálních kořenů. V závěru je použit statistický přístup s využitím Pearsonova korelačního koeficientu a jsou též (na základě výsledků pilotních studií) navrženy dvě potenciální environmentální aplikace regenerovaných huminových materiálů – sorpce tetracyklinu a využití regenerovaného lignitu jako zdroje zkvasitelných cukrů.

KLÍČOVÁ SLOVA

Huminové látky, humáty, aplikace, biologická aktivita, supramolekulová struktura, fyzikální aspekty, sorpce.

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STATEMENT

I declare that the dissertation thesis has been elaborated by me and that all the quotations from the used literary sources are accurate and complete. The content of dissertation thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor of thesis and the dean of the Faculty of Chemistry, Brno University of Technology.

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student's signature

PROHLÁŠENÍ

Prohlašuji, že jsem disertační práci vypracoval samostatně a že všechny použité literární zdroje jsem správně a úplně citoval. Disertační práce je z hlediska obsahu majetkem Fakulty chemické Vysokého učení technického v Brně (FCH VUT) a může být využita ke komerčním účelům jen se souhlasem vedoucího disertační práce a děkana FCH VUT v Brně.

.....
podpis studenta

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CONTENT

1. INTRODUCTION

Humic substances may not be beautiful, but they do beautiful things.

[Fritz H. Frimmel, environmental chemist]

Searching for truth may be more valuable than finding it.

[Albert Einstein, theoretical physicist]

Well, I was searching for truth and I tried to do science with the aim to elaborate something useful from the lignite derived humic substances. As it has shown, they indeed do the beautiful things. Therefore, this work has the ambition to be not an ordinary fundamental research dissertation. The biggest part of it is dedicated to the deeper physical-chemical characterization of humic substances in order to extend the overall knowledge about humics, especially those extracted from so called regenerated resources. But there is the second part, where the reader will find not only the literature review about the most of the possible usages for the humic substances, but also an experimental way with aim to "pre-design" a humic agricultural product – the regenerated potassium humate and regenerated lignite. The first it is presented a deeper introduction about our world's resources and agricultural and environmental problems, where the applications of humic substances may do the biggest benefit.

Realizing the brief and basic economic fact that all of the resources are limited, the need for the maximal exploitation of existing natural resources is very prominent and this effort have to be very well judged. Our world is in the beginning of the 21st century and standing before a set of challenges. Quick population growth and local lacks of food and water are mainly the problem of the underdeveloped countries. On the other side, accelerating industrialization and fast growing demand for resources are the dominant issues of the developing countries, like China, India, Pakistan, Malaysia or Brazil. The developed countries are standing before the very question, if they will be allowed to keep their environmental and social standards facing the developing countries in the everyday competition. In the case of vicious circle of the developed countries' agriculture, the subsidies are still motivating the farmers to produce the food using chemical fertilizers and pesticides, however currently the state budget is burdened with expenses on the remediation of soils and treatment of water.

The environmental point of view sadly shows that deterioration of the state of the environment and degradation of the soil quality is omnipresent all around the globe. All of these pessimistic trends are very probably pertinent to each other and not limited only to the geographic place of the primary problem. In every, even in the most rich countries in the world, one can find serious environmental and soil quality problems. If one converge more on the soil issues, all around the planet, serious problems can be seen with soil erosion, desertification and lack of water, which may also be, in particular in developed countries, caused by the extensive agriculture. Since the soil is the essential and precious resource to produce food and grow natural renewable resources, the biggest pool of relatively stable carbon and also a crucial reservoir of water, the soil issues are the terrific danger for the mankind.

Exempli gratia, citizens of the Czech Republic, are nowadays the witnesses of the political and economical struggle over the (probably the greatest Czech) environmental problem – the sanitations and remediation of the long-lived environmental endurances from the time of the socialist dictatorship and Soviet military occupation. The financial cost for dealing with these endurances will be tremendous and will be probably paid by several generations of taxpayers. This should serve as an example, that not only the present population is worsening the state of the environment now, but also some old issues are to be solved. Again, this is a global problem; every country in the world is struggling with it. Every year, more than a billion of U.S. dollars are invested in the United States Comprehensive Environmental Response, Compensation and Liability Act, commonly known as "Superfund", which is the agenda dealing with the old environmental endurances in the U.S.A.

The history teaches us, that even in this situation there is no need for panic. It is already known, that the living standard probably of all the people on the planet is constantly rising – anyway non-uniformly. Local or temporary lack of one of the resources always brought up the invention of an alternative or of a much more efficient exploitation of the particular resource. Inadvertent flings to partial solutions, such as introducing the energetically inefficient biofuels made from plants grown on the soil, which could have been used for growing food, have supposedly even worsen the situation. Therefore, because "the fortune smiles on the prepared" and "preparation is an attitude", the studying of these problems and designing of the correct particular solutions is tremendously important.

Humic substances are such an example of the maximum exploitation of the natural resources. They are easy to obtain by means of extraction from lignite or leonardite (simply from brown coal) or compost and even some waste materials. Traditionally, they are extractable from soils which process is used merely for the scientific research, however it is quite clear, that for the applications, it is more helpful to extract the humics from other raw materials and, if needed, add them to soils. The applicability of humic substances in the agricultural, environmental and applied chemical industry or even medicine is a great topic for research and a business plan for many companies. The author believes that the best what we can do, is to continue in this research and intensify it with the application aim, because it can lead not only to the more sustainable and cheaper agriculture or easier remediation and sorption technologies, but even to some very special and precious applications in the future.

Czech Republic, with its relatively large deposits of lignites, the best resources for the humic acid extraction, is an ideal country for the research and production of humic substances. Yet we have much to catch up. Countries like Italy, Turkey, Pakistan, China, Brazil, Russia or U.S.A. are nowadays much more successful in the humic research than us.

2. STATE OF THE ART

2.1. WHAT A HUMIC ACID IS

2.1.1. The Story of Humic Substances

2.1.1.1. C. 1800-1900

„What is humic acid?“ This very question was posed two hundred years ago by *Jöns Jacob Berzelius*, a Swedish baron and notable chemist, who stands as a father of modern chemistry together with John Dalton, Antoine-Laurent de Lavoisier and Robert Boyle [1]. Of course, Berzelius was not the first who was concerning with the humic substances (HS or humics). The word humus is known since the ancient Rome, where that Latin word meant the entire soil. In the end of 18^{th} century, French-German chemist *Franz Carl Achard* was the first one, who tried to extract HS (obtaining a dark precipitate when applying alkali on peat) [2]. Berzelius succeeded in the isolation of two kinds of HS from mineral water and slimy mud [3]. Swiss chemist *Nicolas-Théodore de Saussure* started to use the term humus only for the dark colored organic material in soil and German chemist *Johann Wolfgang Döbereiner* introduced the term “humussäure” or “humus acid” [4],[5]. German botanist and agricultural scientist *Carl Philipp Sprengel* made a comprehensive study on the acidic nature of humic acids (HA) [6],[7].

2.1.1.2. C. 1900-1950

In the first half of the 20^{th} century, the research of HS and of their origin was greatly extended, even if the answer for the original question is not yet clear. *Oden* classified the HS into humus coal, humic acid, hymatomelanic acid and fulvic acid [8]. *Schreiner and Shorey* identified about 40 organic compounds in soil, including organic acids, hydrocarbons, fats, carbohydrates and nitrogen containing substances [9]. Detailed study was carried out by *Shmook*, who reviewed important aspects of humus chemistry and its formation by soil microorganisms [10]. *Maillard* considered the HS as a result of purely chemical reactions of C=O groups from sugars with NH_2 and COOH groups from amino acids in which the microorganisms do not play a direct role [11]. *Waksman* agreed with the concept, that lignin is a precursor of HS and regarded humus as a mixture of plant-derived materials (including waxes, fats, resins, hemicellulose and cellulose) [7],[12].

2.1.2. Humic Substances State-of-the-art Since 1950 Till Today

In today’s humic science, still the answer about the genesis and structure of HS is unclear, but a great progress in the HS’s physical and analytical chemistry has been done, so as in the research of the HS’ role in the environment. Moreover, many suggestions of applications, (mainly but not only in the agriculture) have been designed [1]. Shortly, many views were taken into account, some scientists have viewed HS as a monomolecular species with a specific structure, some scientist agreed with the HS being described like supramolecules with tendency to aggregate, and the structures have been modeled and remodeled with predicted shape and chelation sites. A few theories have been proposed about the origin of humic substances (*vide infra*) [7].

2.1.2.1. Humic Substances Definitions

There is no distinct definition of humic substances. Humus or soil organic matter is known to include a broad spectrum of organic constituents, many of which have their counterparts in biological tissues. However, according to *Stevenson*, two types of compounds can be distinguished:

- NONHUMIC SUBSTANCES – consisting of compounds belonging to the well-known classes of organic chemistry, such as amino acids, carbohydrates and lipids.

- HUMIC SUBSTANCES – a series of high molecular weight compounds formed by secondary synthesis reactions; they may be generally characterized as being rich in oxygen containing functional groups (COOH, phenolic/enolic OH, alcoholic OH and C=O of quinones) [7].

It is not easy to separate the humic and nonhumic substances, because some nonhumic substances (usually lipids or carbohydrates) may be covalently bonded to the humic matter. Humus probably contains most, if not all, of the compounds of biological origin. Definitions of various substances occurring in natural organic matter are compiled in **Table 1** [7].

Table 1: Various humic substances definitions according to Stevenson [7].

Term	**Definition**
Litter	Macroorganic matter (e.g. plant residues) that lies on the soil surface.
"Light" fraction	Undecayed plant and animal tissues and their partial decomposition products that occur within the soil proper and can be recovered via flotation with a liquid of high density.
Soil biomass	Organic matter present as live microbial tissue.
Humus	Total of the organic compounds in soil exclusive of undecayed plant and animal tissues, their "partial decomposition" products and the soil biomass.
Soil organic matter (SOM)	Vide "Humus".
Humic substances	*Vide supra*.
Nonhumic substances	*Vide supra*.
Humin*	The alkali insoluble fraction of SOM/humus (vide infra).
Humic acid (HA)	The dark-colored organic material that can be extracted from soil by dilute alkali and other reagents and that is insoluble in dilute acid (*vide infra*).
Hymatomelanic acid	Alcohol soluble portion of humic acid.
Fulvic acid (FA)	Fraction of SOM that is soluble in both alkali and acid (*vide infra*).
Generic fulvic acid	Pigmented material in the fulvic acid fraction.

* According to the newest results of extraction experiments (even when using such agents such as urea, dimethyl sulfoxide and sulfuric acid), the humin is mostly constituted of non-polar biological molecules (of probably plant origin) some of them partially degraded in close associations with soil mineral colloids. Therefore, the classification of humin as a humic substance is now a point of scientific discussion [13].

Steelink suggested in his review four definitions of HAs according to the point of view of the involved branch of science, concept and origin and extraction of the HS. He also contributed to the humic acid modeling (vide infra). Steelink's definitions are summarized in the **Table 2** [1].

Table 2: Various humic substances definitions according to Steelink [1].

Question: What a humic acid is?	
Point of view	**Answer**
Molecular & Structural	HA is a discrete molecular structure (at least for humic acids extracted from soil).
Conceptual	HA is a supramolecular species derived from terrestrial plants.
Origin	HAs is an end product of a specific biosynthetic sequence.
Isolation*	HA is the final fraction of a specific extraction procedure.

*The operational "isolation" definitions of HS according to their solubility are very common and probably the same today in 2011 as they were in 1859.

It can be assumed, that humic substances are refractory, dark-colored heterogeneous organic compounds produced as byproducts of microbial metabolism and also, that they are among the most widely distributed organic materials on our planet [7], [14].

2.1.2.2. Humic Substances Genesis

Also in the studying of the HS genesis, there are a few theories and all of them may be partially correct and the real pathway leading to the HS may be their combination.

The oldest **"Lignin-Protein" theory** has been favorized for many years, and consists in that lignin is a primary source of HS in soil. According to this, lignin is incompletely utilized by soil microorganisms and can undergo a series of modifications (loss of methoxyl OCH_3 groups, generation of *o*-hydroxyphenols and oxidations of terminal aliphatic side chains to form COOH groups). Successively, the *o*-dihydroxybenzene units resulting from demethylation of lignin would further oxidize to quinones capable of undergoing condensation reactions with amino compounds and NH_3 produced by microorganisms during the decay of N-containing organic substances. This process would yield first humin, then HA and apparently FA. This theory is supported the fact, that lignin can be demethylated (without further degradation) by a large number of bacteria species and the demethylated and oxidized lignins may be further enriched in COOH groups arising from aromatic ring cleavage. There also exists the possibility, that not only lignins, but other natural macromolecules, like cutin or suberin, are the precursors of HS [7], [12], [15–18].

The more recent, **"Sugar-Amine" theory** lies in the non-enzymatic purely chemical condensation of reducing sugars and amino-compounds formed as byproducts of microbial metabolism, and further polymerization reactions. These polymerizations, which are known to form typical brown nitrogenous polymers, have been postulated to play an important role in

the formation of HS in soil. The initial reaction involves the condensation of an amino group (from e.g. amino acid) with the aldehyde group (from sugar) to form a Schiff base and N-substituted glycosylamine, which are susceptible to various rearrangements and reactions. The rising compounds, like glyceraldehyde, dihydroxyacetone or hydroxymethylfurfural are highly reactive and able to polymerize. These reactions are running in abundance by soil microorganisms, but the condensation reaction proceeds rather slowly at the common soil temperatures and a strong competition exists between the soil microorganisms for the use of these compounds. However, especially in the soils low on lignins or with frequent and drastic temperature changes, this pathway is the realistic possibility how the HS are formed [7],[19],[20].

The most recent **"Polyphenol" theory** states, that the HS originate not only from lignin, but as well from the other plant biopolymers. These biopolymers, when freed from linkage with cellulose during decomposition of plant residues, the side chains of their building units are oxidized and demethylated yielding polyphenols that are converted to quinones by polyphenoloxidase enzymes. Quinones then react with N-containing compounds and polymerize to longer humic chains. Thus, the order of formation of HS would be *vice versa* than in the previous theories, so FA → HA → humin [7],[18],[20].

In conclusion, HS in soil may be formed by all the mechanisms mentioned above, although most researchers favor the polyphenol theory. The number of precursor molecules and the types of reactions is very large, so the count of possible combinations is very high. Soil properties and conditions may determine the relative importance of single precursors, pathways and microorganisms.

2.1.2.3. Humic Acids Composition and Structure

Since this thesis deals mainly with humic acids and their salts and with other humic substances only marginally or not at all, the structure and modeling will be reviewed only for humic acids.

The key for predicting HAs' structures is the **elemental composition**. HS consist of carbon, oxygen, hydrogen and nitrogen (in about 50–60, 30–40, 3–6, 1–5 atomic percent respectively), occasionally small amounts of sulfur and phosphorus can be found too [18], [20].

The next essential knowledge in order to predict structure is the information about **functional groups**. With the progress in chemical analysis instrumentation (UV-VIS, ^{13}C, ^{15}N, ^{31}P and ^{1}H NMR and especially ^{13}C CP-MAS NMR, FTIR, EPR /ESR/) and by means of many degradation and cleavage experiments (acid or alkaline hydrolysis, many various oxidations and reductions and thermogravimetry) interesting functional groups have been found in various samples of humic acids [7],[18]. The meaning of the abbreviations can be found in the Abbreviations list. The main acidic groups in HAs are the carboxyl and phenolic OH groups, but alcoholic OH and carbonyl (quinoid and ketonic C=O) are also well represented, whereas methoxyl OCH_3 groups are found in smaller amounts [7],[18],[21].

The first one, who tried to describe the humic acid **structure**, was Berzelius' student *Mulder* in 1840, who published an empirical formula of $C_{40}H_{30}O_{15}$ for soil HA. This formula prevailed very long time, it was still in account even about 10 years ago [1], [22]. Important models (see **Figure 1**) [1],[23] were published by *Steelink* and *Stevenson* respectively.

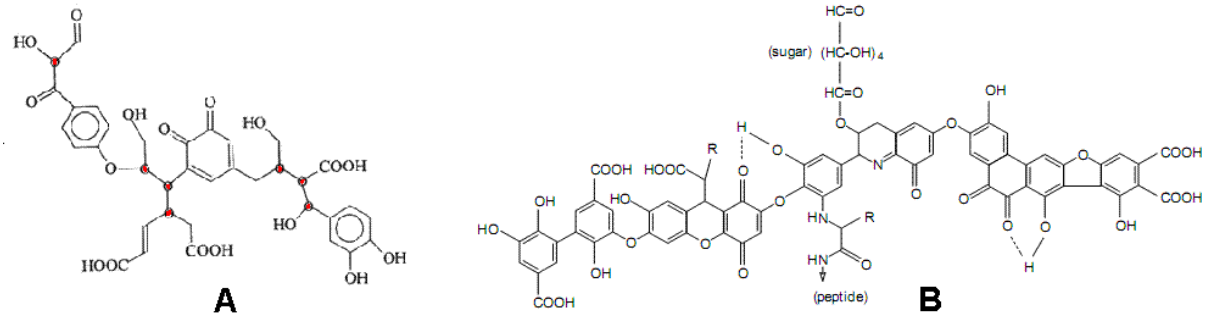


Figure 1: *Steelinks (A) (with red dots as supposed centers of chirality) and Stevensons (B) model of humic acid [1],[23].*

Jansen and coworkers used the Steelinks structure for computer modeling and modified it slightly according to the newer knowledge about elemental composition and chemical structure obtained from circular dichroism and liquid state nuclear magnetic resonance measurements of sample of the humic acid extracted from *Pilayella litoralis* living plant (see **Figure 2**). They also supposed that presented structure is the building block of humic acid.

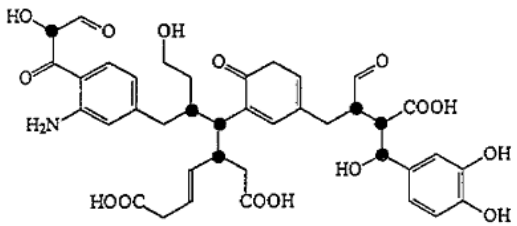


Figure 2: *Jansens modification of Steelinks humic acid model (again with dots as supposed centers of chirality) [1],[24].*

Probably the most recent and accurate model of humic acid structure which is also showing the system complexity was presented by *Simpson et al.* (see **Figure 3**) [25].

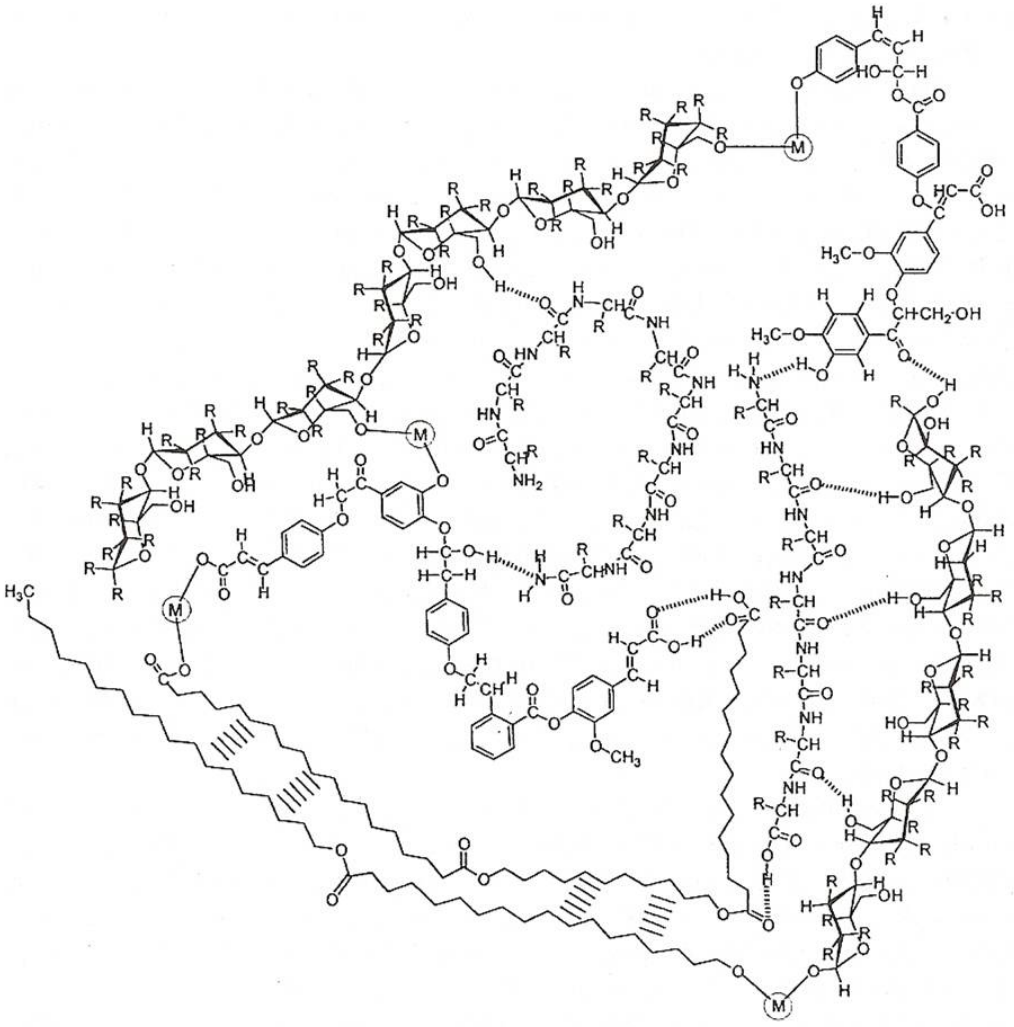


Figure 3: *Recent model structure of humic acid according to Simpson et al. (2002); M stands for metal ion or surface [25].*

2.1.2.4. Humic Acids: Polymers or Supramolecules?

In the past 50 years, the question about humic acid molecular weight appeared and became one of the central problems of the HAs research [26]. The earlier studies favored the **polymer theory**, where humic substances (mainly humic and fulvic acids) were described as randomly coiled macromolecules having elongated shapes in basic or low ionic strength solutions, but becoming coils in acidic or high-ionic strength solutions. These thoughts were mostly based on the results given by ultracentrifugation, sedimentation velocity and diffusion methods [7],[13],[26],[27]. More recently, *Piccolo* carried out an exhausting study of the molecular aspects of humic substances, pointing at the great differences in the predicted molecular weight values of the HS, which were in various studies ranged from 2.6 to 1 360 kg mol^{-1} and even after fractionation, the results were always polydisperse and dependent on the technique used and on the ionic strength of the liquid sample [26 and references therein].

According to the recent information, gained by chromatographic, spectroscopic and also microscopic, pyrolytic and soft ionization techniques, the new concept of humic substances has emerged, since the recent results **were not contingent with the polymer theory**. [14],[26],[28],[29].

Wershaw was the first, who proposed that HS consist of ordered **aggregates of amphiphiles** composed mainly or relatively unaltered plant polymer segments possessing acidic functionality. HS are considered aggregates held together by hydrophobic (π–π and charge transfer bonds) and hydrogen-bonding interactions. The hydrophobic parts of the molecule are in the interiors whereas the hydrophilic part makes up the exterior surfaces [26],[30].

Even though the discussion about the concept of humic substances is still alive [31],[32], the results obtained from an array of many independent analytical techniques are supporting the concept of HS as **collections of diverse low-molecular-mass molecules**.

2.1.3. Humic Acids Extraction and Regeneration

2.1.3.1. Extraction

There exist many various extraction procedures for gaining the main humic substances (humic and fulvic acids) from soil and also from other sources. The agents vary from strong inorganic bases across the neutral salts or organic acid salts to the organic solvents or organic acids. The extraction is necessary because there is no other way how to get the humic substances and they cannot be studied as a part of soil or other source, because there they are the part of complex system, interacting with metal ions, mineral colloids and non-humified organic material. Some of the agents and their use for the specific humic material extraction and assumed yields are summarized in the **Table 3** [7],[18],[33].

Table 3: List of extractants of soil organic materials [7],[18],[33].

Type of Material	Extractant	Yield [%]
Humic substances	*Strong bases*	
	NaOH	To 80
	Na_2CO_3	To 30
	Neutral salts	
	$Na_4P_2O_7$, NaF, organic acid salts	To 30
	Organic chelates	
	Acetylacetone	To 30
	Cupferron	?
	8-hydroxyquinoline	?
	Formic acid	To 55
	Acetone–H_2O–HCl solvent	To 20
	Organic solvents	
	Pyridine	36
	Dimethyl formamide	18
	Dimethyl sulfoxide	23
	Sulfolane	22
Hydrolyzable compounds		
Amino acids, amino sugars	Hot 6*N* HCl	25–45
Sugars	Hot 1*N* H_2SO_4	5–25
Polysaccharides	NaOH, Formic acid, hot water	< 5
Clay-bound biochemicals	HF	5-50
"Free" biochemicals	H_2O, 80% alcohol, ammonium acetate	1
Fats, waxes, resins	Usual "fat" solvents	2–6

Even though there is no universally applicable and accepted method for the humic substances extraction, however, tremendous efforts have been made to develop one. The best candidate method, which can be already used for comparisons within and between laboratories, is described in detail by *Swift*. It gives relatively high yields and it has been found satisfactory for various types of soils [18],[34]. Very briefly and in general, the most common extraction method is symbolized in the **Figure 4** [7].

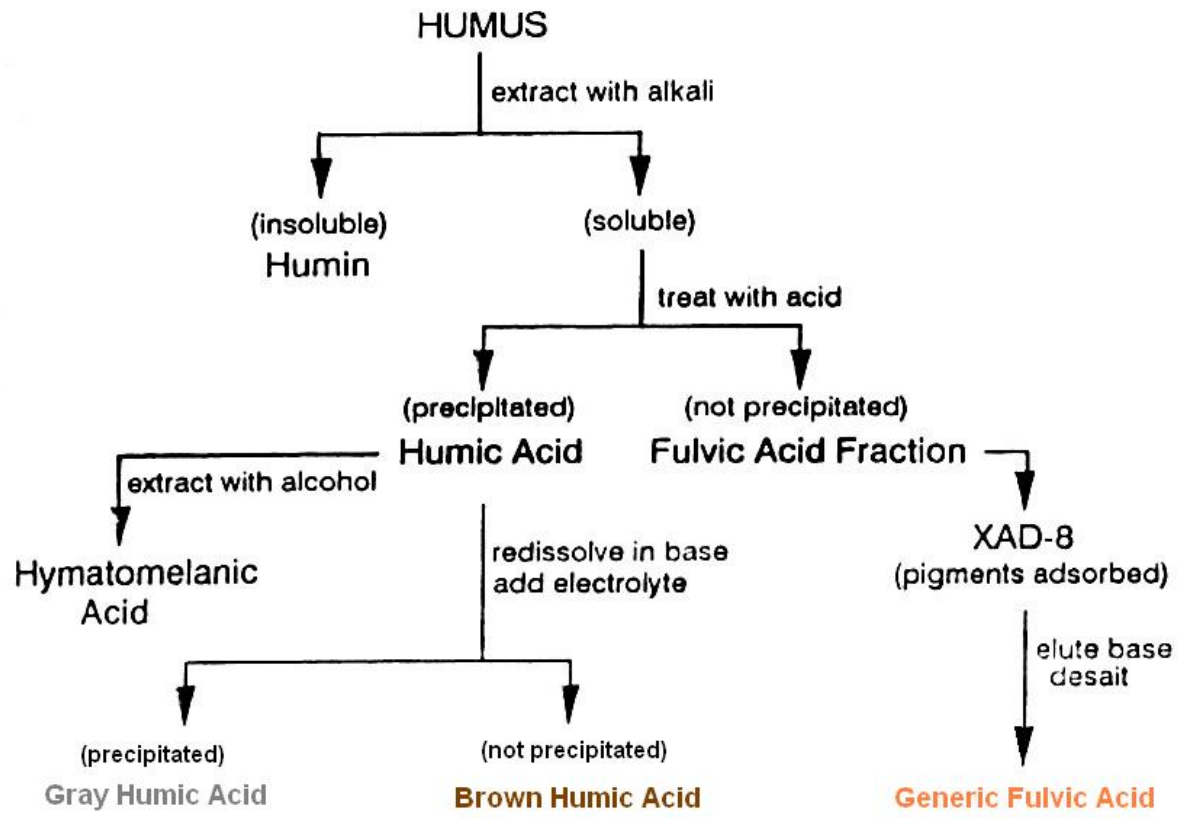


Figure 4: *Scheme for the extraction of humic substances [7].*

2.1.3.2. Other Sources than Soil and Lignite

Humic substances may be extracted from various sources. Humic substances from freshwater, seawater and sediments are not the subject of author's thesis, so they will not be mentioned further in this treatise. Even though the most common extractions are made from various types of soils, the main interest of this thesis is the extraction from lignite (which will be mentioned *infra*). Other sources for extraction of humic substances are peat, shales, and various composts. The extraction procedures are similar to that described by *Swift*. [7],[18],[35],[36].

2.1.3.3. Lignite and Leonardite

Lignite is the youngest coal, usually called as brown coal. Lignite is mostly originating in the Tertiary period, even if some amount originated from Permian and Jurassic, being formed from peat and plant remains in shallow depths at temperatures lower than 100°C. It is the first product of coalification and an intermediate between peat and subbituminous coal. In comparison with black coals (subbituminous, bituminous and anthracite), lignite is less hard and contains more water and oxygen, but less carbon. Simplified scheme of lignite genesis is typified in the **Figure 5** [37]. In conclusion, lignite is morphologically and molecularly polydisperse system consisting of cyclo and aromatic compounds, water (both incorporated in the free space /pores, microcracks/ and physically bonded), special mineral structures based on metal compounds (silicon, iron, aluminum etc.) and macroscopic components. Lignite is usually used as a low quality fuel, mainly in power plants. [38],[39],[40] More efficient utilization of lignite consists in its environmental, agriculture

and “green chemistry” applications. Owing to its special physical and chemical and also surface properties, lignite can be used as a sorbent, remediating and defluoridating agent. The main non-energetic exploitation of lignite stands in its use as a raw material for humic substances [41],[42],[43].

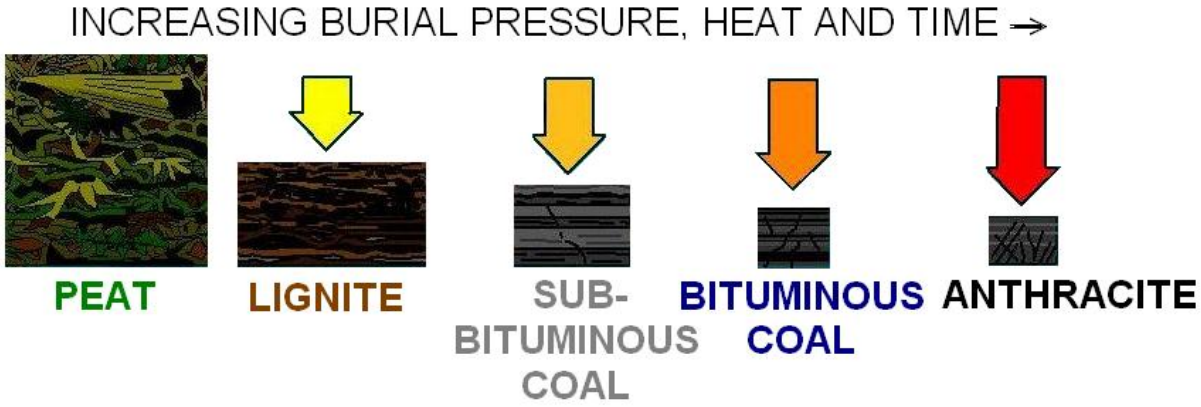


Figure 5: *Scheme of lignite and coal genesis and differences according to Kentucky Geological Survey [37].*

Leonardite is an oxidation product of lignite, associated with lignite near-surface mining. Leonardite is soft and waxy, black or brown, easily soluble in alkalis and rich in humic substances [44]. Lignite is mainly found and mined in the United States of America (ND, ID, WA, WY and in NM), Canada (AB and SK), in Australia, Germany, Poland, Czech Republic, Greece and Turkey, in European and Asian part of Russia and in China. Even though (mainly the shallow near-surface mining) presents negative environmental issues, often the re-naturalized landscape after the mining looks “more natural” than before. The output of lignite is slightly decreasing in many European countries and Russia, yet increasing in U.S.A., Australia, China and Greece [40],[44]. The point of this treatise and thesis is to emphasize the idea, that lignite is very precious chemical raw material and its (non-energetic) exploitation represents a new economical and environmental motivation in the very near future.

2.1.3.4. Humic Substances Regeneration

Since coal is considered as the final product of diagenesis (which runs from low molecular weight substances across the loss of functional groups and condensation reactions to the end of tridimensional poorly soluble network of anthracite), oxidation products obtained from coals by pre-treatment with strong oxidizers like nitric acid, potassium manganate(VII), sulphuric acid or hydrogen peroxide and also by air oxidation have been reported as regenerated humic acids [43],[45],[46]. Ergo the regeneration of coal works, the regeneration of lignite may be an interesting idea too, supposing that it will lead to the change in the resulting HAs structure, enriching them in aromatic structures and semichinoidal structures, furthermore, it may extend the application potential of lignite. The regeneration process is easily symbolized in the **Figure 6** [46]. The optimization of the lignite regeneration process, like choose of agents and their concentration is still subjected to the research and discussion and therefore partial goal of this treatise and future thesis [41],[43],[47]. Interesting products may also be obtained when applying the ammonooxidation process on ligneous materials [48].

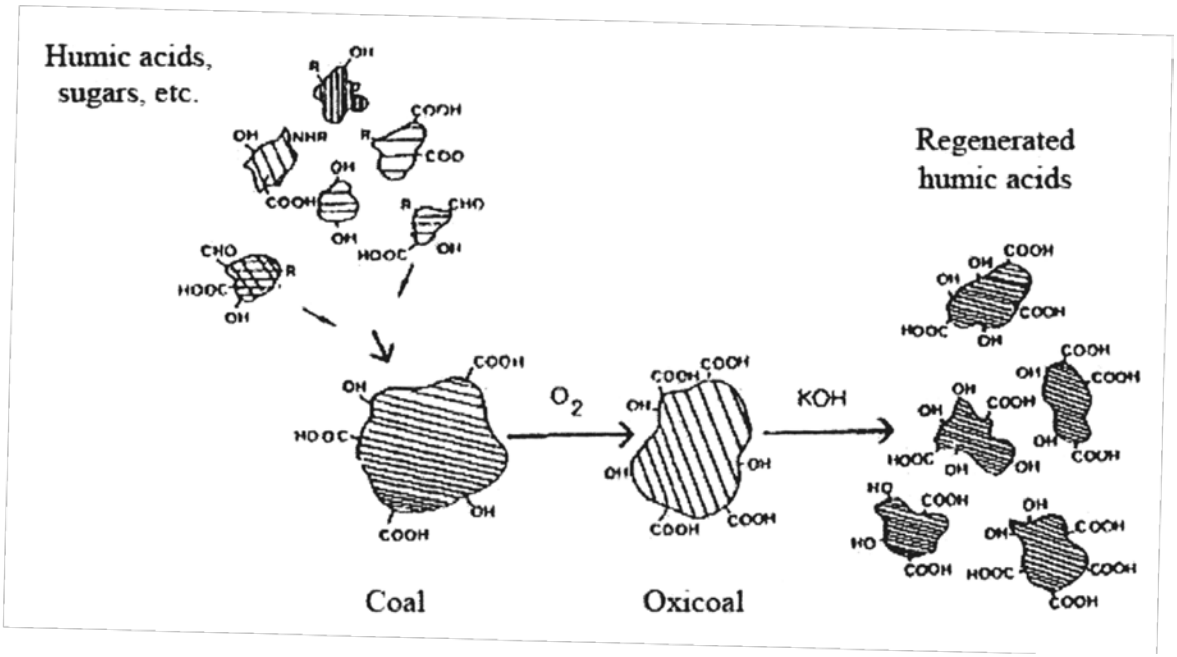


Figure 6: *Coal oxidation as an ideal inverse diagenetic process according to Rausa et al [46].*

2.1.4. Humic substances in the nature

2.1.4.1. The Role of HS in Soils and Environment

HS contribute to plant growth through their effect on the physical, chemical, and biological properties of the soil. They have nutritional function in that they serve as a source of N, P, and S in bioavailable forms, a biological function in that it profoundly affects the activities of microfloral and microfaunal organisms, and a physical function in that it promotes good soil structure, thereby improving tilth, aeration, and retention of moisture, and they reduce thermal conductivity of soil, thereby they protect the biological processes in soils against severe thermal fluctuations. Many of the benefits attributed to HS have been well documented, but it should be noted that the soil is a multi-component system of interacting materials. Accordingly, soil properties represent the net effect of the various interactions and not all benefits can be ascribed solely to the organic component. The biological activity of humic substances will be mentioned separately further [7],[18],[26].

In the environment, up to 70 % of the soil organic carbon and up to 90 % of dissolved organic carbon may occur in the form of HS. They influence groundwater properties and the process of formation of fossil fuels; however, they play a major role in the global carbon geochemical cycle. Further environmental importance of humic substances lies in their interactions with organic and inorganic (mainly metal) pollutants. The **organic pollutants** may be adsorbed through physical-chemical binding and forces, including ionic, hydrogen and covalent binding, charge-transfer or electron-donor-acceptor mechanisms, dipole-dipole and van der Waals forces, ligand exchange, cation and water bridging and non-specific, hydrophobic or partitioning processes. The various properties of the specific organic pollutant result in several possible mechanisms that may operate in combination. For a few examples, adsorption via ionic binding or cation exchange involves the carboxylic and phenolic OH groups of HS and applies only to pesticides in their cationic forms, such as diquat and

paraquat. Since the HS possess numerous N and O containing functional groups, the occurrence of H-bonds between HS and acidic pesticides and nonionic pesticides like malathion is suggested. The presence of both electron-donor and electron-acceptor allow HS to form charge-transfer complexes with organic pesticides electron-donor and electron-acceptor features like DDT, dioxins and PCBs or arachlor. Van der Waals forces may have importance into the adsorption onto HS of nonionic and nonpolar pesticides such as carbaryl, alachlor, benzonitrile, DDT, picloram, etc., those pesticides may also interact with HS through hydrophobic adsorption. Other interaction mechanisms may be solubilization, hydrolysis catalysis or photosensitization. One of the most important environmental properties of HS is the ability to interact with **metal ions** to form both water-soluble and water-insoluble complexes possessing various chemical and biological stability and properties. The most processes in which metals are involved in soils are affected by HS, which behave as natural "multiligand", which means that they have many complexing sites per molecule. The principal molecular characteristics of HS involved are polyfunctionality, polyelectrolyte character, hydrophilicity and the ability to change molecular conformation. Most of these metal-HS interactions are measurable by means of ion-selective electrode potentiometry, anodic stripping voltammetry and visible and fluorescence spectrometries. The environmental chemistry of HS is not only highly complex, but it is also a function of the different general properties of the ecosystem in which it is formed, such as vegetation, climate, topography, etc. and all the possible interaction are not yet fully elucidated. Some of the most important environmental properties and applications are mentioned further in more detail [7], [18 and references therein].

2.1.4.2. Physico-Chemical Properties - HS as Colloids

The micellar model of HS was first introduced by *Wershaw* [49] and now is widely accepted [50],[51]. This has been found by employing the experiments studying the same interactions that promoted supramolecular associations. Today's view on the humic acid associations in water is similar to the on micelles formed by surfactants in aqueous solutions, where intra- or intermolecular organization produces interior hydrophobic regions separated from aqueous surroundings by exterior hydrophilic layers. HS organize spontaneously in aqueous solutions, forming aggregates in the colloidal range that have relatively polar exteriors and nonpolar interiors. While in surfactants, these aggregates are called micelles, and consist of discrete monomer units arranged in usually spherical arrays, in HAs the structure is not so exactly defined, since humic molecules have a broad size distribution and a variable allocation of many various functional groups. More probable, the humic molecules are both intra- and intermolecularly organized and the longer chains are coiling up to form domains of different particles. This arrangement is more constrained than the one found in detergent micelles and the term **pseudomicelle** is the best describing one for the HA aggregates.

It has been proved in several works, that the formation of humic pseudomicelles is promoted by the presence of metal ions, especially the multivalent ones, such as Mg^{2+}. This happens probably due to metal ion bridges forming between functional groups on different parts of HA chain. These bridges are drawing the creating of more pseudomicellar domains. These interactions evolve over periods of days or weeks. The value of pH also contributes to the process. Employed techniques were usually the fluorescence; fluorescence anisotropy and electron spin resonance. In a simple way, the head of foam formed when aqueous solutions of

HAs are shaken or stirred, are adverting to the detergent properties of such solutions. Moreover, when the ionic strength and temperature requirements are met, aqueous solutions of HAs exhibit visible clouding point, which can be thermally reinduced. These properties of aqueous HAs solutions may be very crucial and interesting in terms of transport of both organic and inorganic pollutants through soil or the possibilities of removing them from there by the HAs, whose are in the environment naturally ubiquitous. Some of these application presumptions are mentioned further [14],[26],[49–57]. The scheme of the humic acid assembly by *Wershaw* and *von Wandruszka* is shown on the **Figure 7**. The Finnish scientists *Peuravuori* and *Pihlaja* also focused on this behavior of humics, when applying combined techniques such as FTIR, HPSEC and NMR onto humic acid extracted from freshwater, in result they attached to the supramolecular view on humic substances [58],[59]. Recently, the aggregation of HAs into pseudomicelles was supported by studies using dynamic light scattering [60]. When employing the high resolution ultrasonic spectroscopy (HRUS), it has been demonstrated for for a wide range of temperatures and concentrations, that the aggregation of supramolecular domains in a diluted humic substance is dependent on the concentration, temperature and counterion as well as on the humic substance origin [61–63].

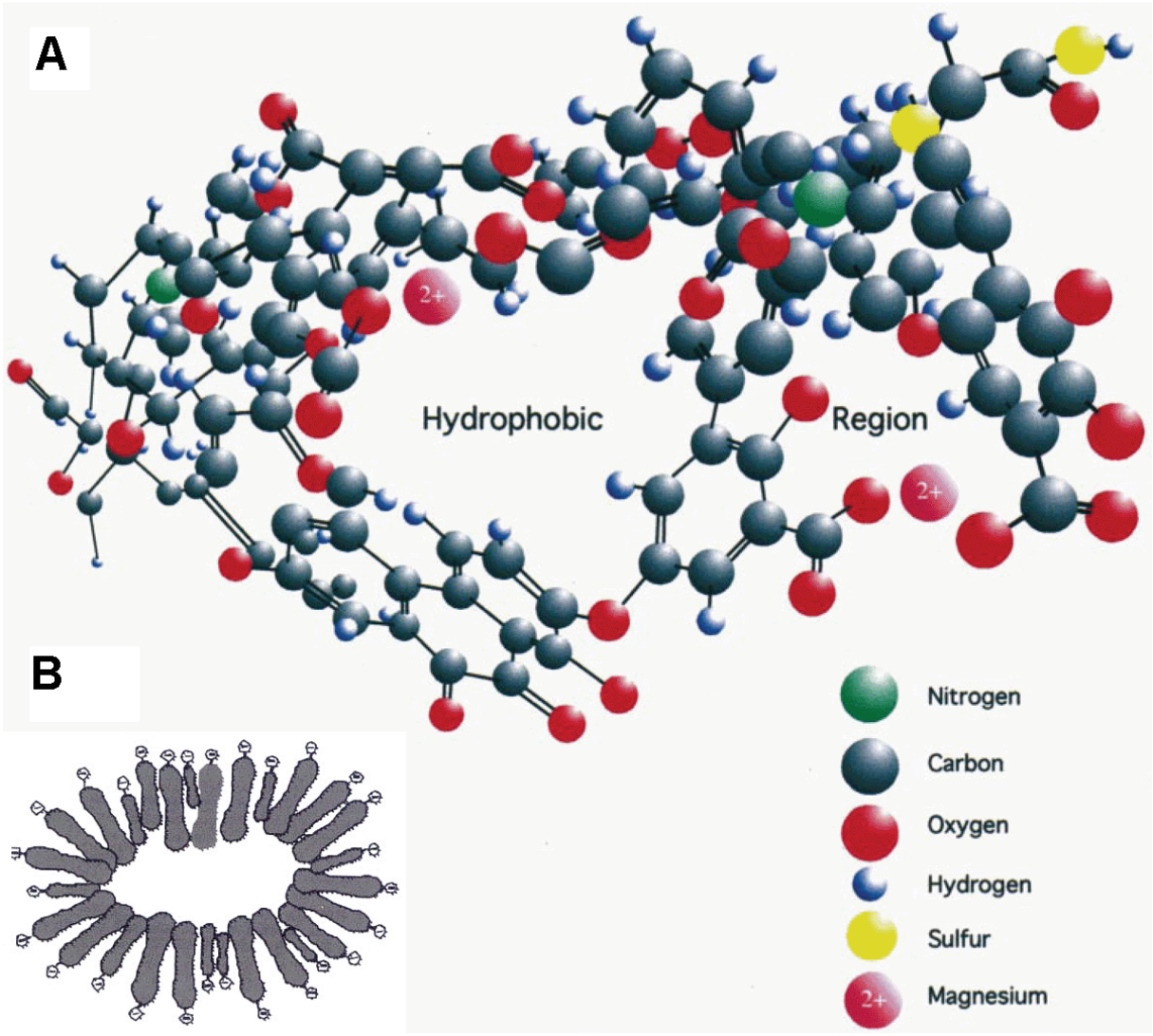


Figure 7: *Humic supramolecular assembly (portion of „type structure“) as designed by von Wandruszka (A) and Wershaw's conception of a humic micelle (B) [49],[54].*

2.1.4.3. Humic Substances Hydration

Both water and humic substances are the essentials of the life on Earth. The specific properties of water are being given mainly by the hydrogen-bonded environment particularly evident in liquid water [64]. Since hydrogen bonds (four from every molecule of water) optimally arrange themselves tetrahedrally around each water molecule, water is supposed to build tetrahedral clusters of four molecules of water around the one (see **Figure 8**), which is the main reason for unusual but so benefitious physical and chemical properties of water [65].

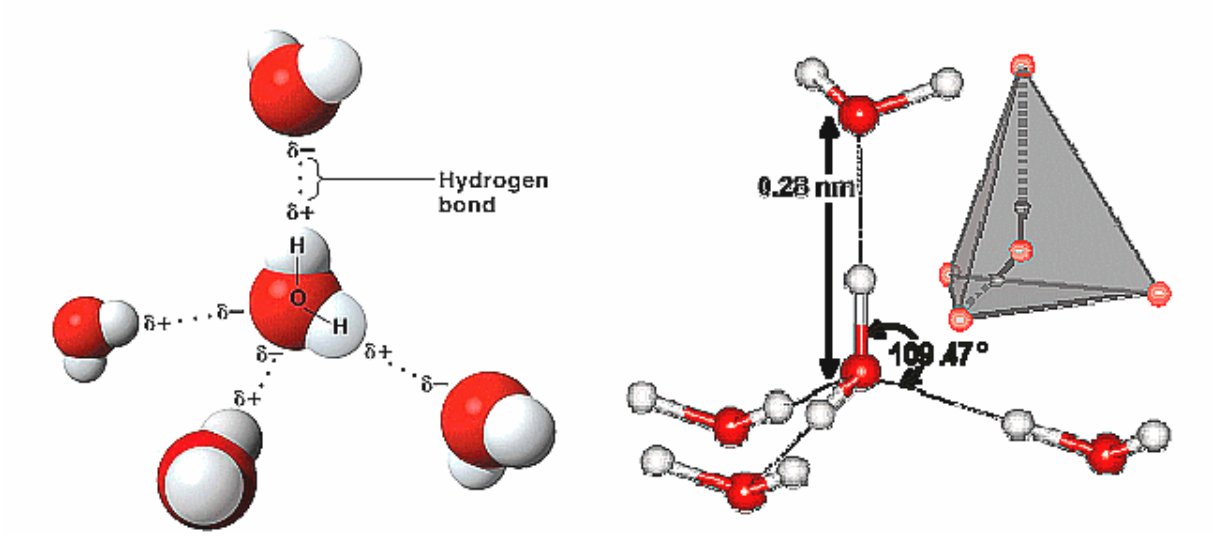


Figure 8: *The cluster of five water molecules in tetrahedral arrangement [65].*

Hydration is generally a term concerning the amount of bound water. This phenomenon is one of the most important factors in playing the role in the biological function of molecules in both living and natural systems. Water is not only the medium for nutrient transport, cell membrane processes and the inducer of biologically active conformation of biomolecules, since water can form various structures with variable physical-chemical features (e.g. clusters), which can drive the biomolecules including humic substances into various complicated organizations. For example, stronger water hydrogen bonding leads water molecules to cluster together and be not so available for biomolecular hydration. In such processes, water is involved in many ways, ranging from direct bridging to collective effects [65].

Due to the vicinity of solids, all the water does not have similar properties in terms of vapor pressure enthalpy, entropy, viscosity and density. Most commonly, two types of water are considered: the free water, the one not affected by the near solid particles and the bound water whose properties are affected due to the present solids [66].

Hydration of humic substances (including the hydration of their protein and carbohydrate moieties) may be a complicated phenomenon. In terms of this work the hydration is studied in a simplified point of view as a comparative method to the other physico-chemical techniques and with an application angle (mainly for agriculture) as well.

2.2. APPLICATION POTENTIAL OF HUMIC ACIDS

In the first engineered uses of humic substances (usually lignite derived or in the form of crude lignite) belong the applications in construction chemistry, where they have been added (together with bitumen and montan wax) into the cement concretes, where they worked as liquidizers and aerating and pore-building additives [67]. The next application was in agriculture, where lignite was tested and applied as organic additive to soils, mostly in order to maintain better water retention and increase porosity of the soil. All of these exploitations of lignite derived HS started in the 1960's and 1970's, associated with the increasing mining of lignite and the first motivation to find a non-energetic use for it [68],[69]. The balneotherapic use of peat (which represents the most significant medical application of HS) is known to mankind since the ancient Babylonia and Rome. The balneotherapy specialized itself in the early 19th century and is broadly used till today [70],[71].

2.2.1. More Sustainable Agriculture

Continua messe senescit ager. (A field becomes exhausted by constant tillage.)

[Publius Ovidius Naso, poet]

The goal of agriculture is not to reach the temporary maximum yields, but to keep them up forever.

[Justus von Liebig, agricultural chemist]

2.2.1.1. Biological Activity of HS

Biological activity of humic substances is a natural phenomenon, studied by mankind almost for one hundred years. The auxin-like activity (auxins are plant hormones, i.e. signal molecules, which regulate the plant growth and many other aspects) was first presupposed by *Bottomley* in 1917 [72]. In the 1970's, as it was already written, the interest in non-energetic lignite applications aroused. In 1980', together with the progress of instrumentation techniques (mainly the electrophoresis, chromatography and nuclear magnetic resonance), the scientific interest in HS biological activity partially shifted from the point of quantifying the effects of HS on plants to elucidating the mechanisms [73].

Even if humic acids are resistant to microbial degradation and not generally supposed to be involved in microbial metabolism, *Lovley et al.* found, that some microorganisms living in soils and sediments are able to use HS as electron acceptors for anaerobic oxidation of organic compounds and hydrogen [74]. Nowadays is also known, that the electron-accepting capacity (the redox potential) of HS is related to their free radical content. Since the radicals in HS are mainly quinone-like groups, their amount is important for the biological activity of HS [75].

It is already well known, that HS stimulate the plant growth and moreover, that HS are taken up into the plant tissues [73]. This effect has been attributed to the formation of complexes between HS and nutrients, which can increase the solubility of certain micronutrients (iron and zinc in particular) [76]. Several works were based on describing the phyto-hormonal-like properties of HS with low molecular mass [77],[78]. However, it has later been suggested,

that the biological activity of HS is related more to their chemical structure, than to molecular mass [79]. The biological activity of the HS with higher molecular mass has been previously proposed by the theory of releasing the HS' low-molecular mass fraction from the supramolecular complex, which is pH dependent [25]. While *Nardi et al.* focused on the auxin-like activity of humic substances [77],[80],[81], *Canellas et al.* studied the effects of HAs (isolated from vermicompost of bovine manure prepared using the *Eisenia foetida* earthworms) on the earliest stage of lateral root development of maize (*Zea mays*) [36],[82].

Nardi's work presented, that especially the low-molecular mass fraction of HS is responsible for the positive influencing of biological activity. Further results showed increased nitrate uptake and inhibited K^+, stimulation of the enzyme ATPase of maize microsomes and the extrusion of H^+ in the form similar to gibberelic acid (which is also a plant hormone regulating growth and other processes). The overall effect of HS on plant growth depends on HS' source, concentration and molecular size of the humic fraction. While the lower molecular size fractions easily reach the plasmolemma of higher plant cells and are partially taken up into them, the higher molecular size fractions (> 3.5 kg mol^{-1}) can interact only with the cell walls. ATPases are class of enzymes, usually anchored in the biological membranes, that catalyze the decomposition of adenosine triphosphate to adenosine diphosphate and phosphate ion, which releases energy used in further reactions. Transmembrane ATPases are also responsible for transport of solutes and maintaining ion gradients across the membrane so they are necessary for cell metabolism. The super-simplified scheme is cartooned on the **Figure 9** [77],[80],[81],[83].

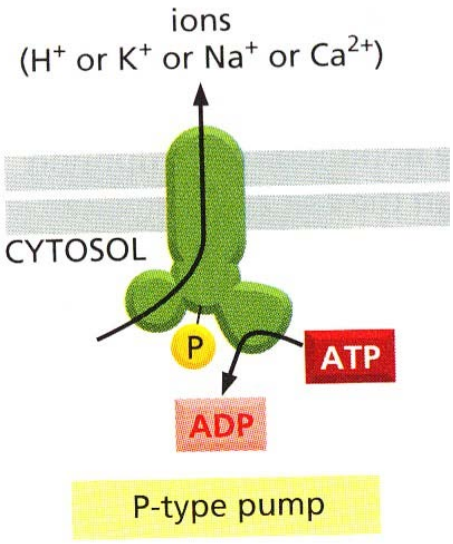


Figure 9: *ATPase mechanism – a P-type pump (which means, that the molecule phosphorylates itself during the cycle; moreover, the pump enzymes can also work in reverse) [83].*

Canellas et al. focused the research on the elucidation of the hormonal-like activity of humic substances. The authors based on the already confirmed information about the auxin-like activity of HS and on the proposal that one of the mechanisms by which auxins stimulate

plant growth is by inducing an increase in the amount of plasma membrane H^+-ATPase, which acidifies the apoplast and thereby loosens the cell wall, allowing the cell elongation. Activation of the H^+-ATPase can also improve plant nutrition by enhancing the electrochemical proton gradient that drives ion transport across the cell membrane via secondary transport systems and on that knowledge, that low molecular weight HA can stimulate the H^+-ATPase of plasma membrane vesicles isolated from roots of several plants (which is probably due to dissipation of electrical potential or enzyme modulation by yet undefined posttranslational mechanism) [84–89]. *Canellas et al*. confirmed the presence of auxin-like structures in the tested HAs (by means of GC-MS), revealed the proliferation effects on the of lateral root emergence in maize roots and supposed the likeness of HA ability to express the plasma membrane H^+-ATPase gene. Furthermore they found out, that addition of HA to the medium promotes inhibition of both ATP hydrolysis and H^+ transport, suggesting that if HA could gain access to the cytoplasm, this enzyme would be inhibited. Recently the team of Canellas discovered, that organic acids (such as malic acid, citric acid or succinic acid) when applied on their humic acid extracted from the vermicompost induce changes in the HA supramolecular conformation and also modify the ability to stimulate maize root growth. Example summary of the Canellas' results of the HA effects on maize roots are shown in the **Figure 10** [36],[82],[84].

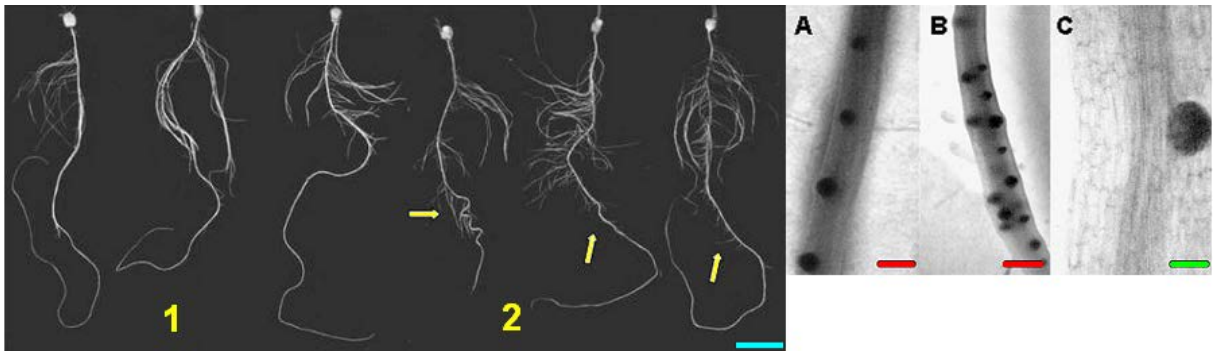


Figure 10.: *Examples of Canellas' results:* ***1*** *– three maize seedling growth for 168 hours in nutrient solution alone and* ***2*** *– in nutrient solution and in 50 mg.L^{-1} HA solution, the arrows are pointing at the additional regions of primary roots with lateral root growth; cyan bar = 20 mm [36].*
A *– control maize root showing some mitotic sites;* ***B*** *– maize root after 40 mg.L^{-1} HA incubation – the hyperinduction of mitotic sites; red bars = 1 mm;* ***C*** *– single mitotic site; green bar = 200 μm [82].*

The most recent results of *Canellas et al.* suggest, that the stimulation of lateral root growth is not so dependent on the molecular size of humic material used [90] (which was previously found by *Nardi et al* [77],[80]). This important new information was achieved by the experiment, where humic acid salt isolated from vermicompost was subjected to a preparative HPSEC fractionation onto fractions with various molecular size and chemical composition (as showed on the NMR). All of these size fractions were principially similarly biologically active – they increased the H^+ATPase activity and lateral root development (showed on Scanning Electron Microscopy (SEM)). The tests were performed on the model organism of thale cress (*Arabidopsis thaliana* ecotype Columbia 4) and again on common maize (corn; *Zea mays*). For summary graphs of results, see **Figure 11** [90].

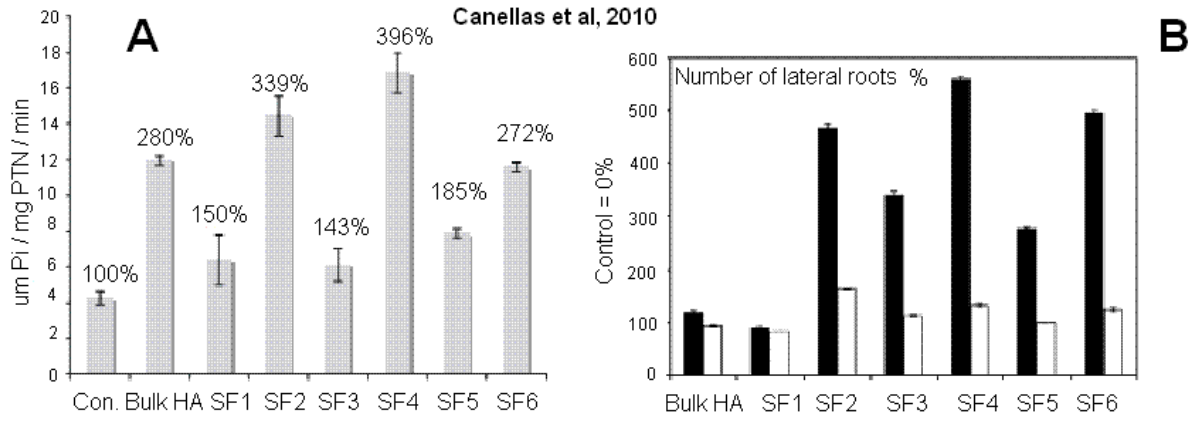


Figure 11: *Canelas' results: A – Effect of size fractions (SF) of humic acid from vermicompost onto plasma membrane H+ATPase activity; B – Effect of ditto onto number of lateral roots [90].*

Interesting approach to the HS biological activity was published by *Popov*, who proposed, that biological activity of HS is determined by the presence of various functional groups, the colloidal characteristics and the material constitution. Popov's model is presented in the **Figure 12** [91].

Some other biological activity research was published by *Valdrighi et al.* in 1996, who favored the compost-derived humic acid and used it in the experiment with common chicory (*Cichorium intybus*) plant growing in sandy soil, resulting that the adding of HA to the soil had positive effects on the plant biomass productivity and on the response of various soil microorganisms. Moreover the authors favored the possibility to extract HAs from compost made up from agricultural, industrial and household organic waste [92].

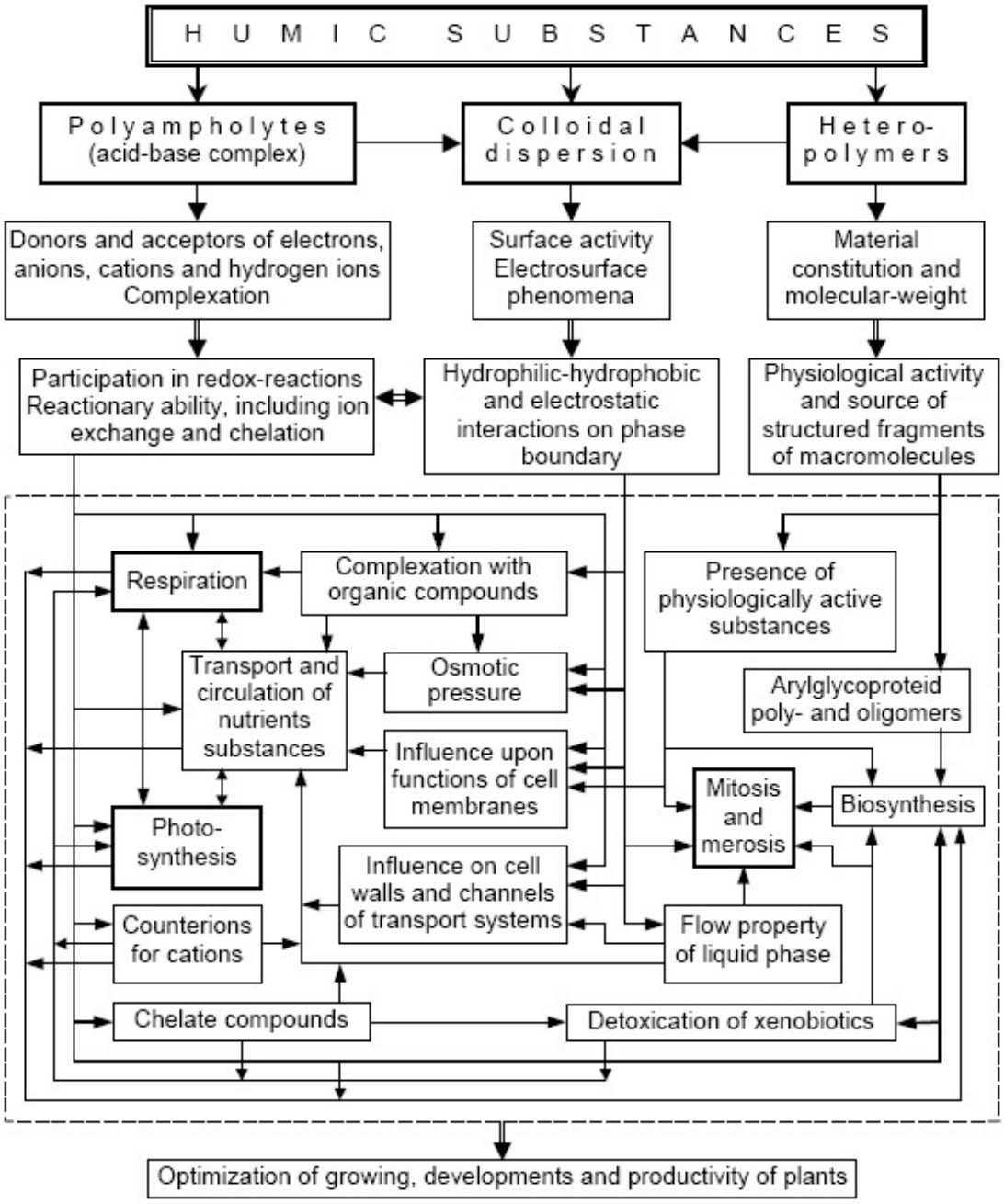


Figure 12: *Participation of humic substances in plant metabolism according to Popov [91].*

Other scientists utilizing maize in the HAs biological activity were *Eyheraigubel et al.*, who focused on the extraction of HS from lignocellulose waste material – poplar sawdust and the performing of the test in hydroponic conditions. This research resulted in the information, that lignocellulose derived mixture of HAs and fulvic acids have positive effects on the biometric factors of the plant (fresh and dry weight of roots, shoot, leaves and flowers), length of roots and leaves and number of leaves and flower. Moreover, the positive effect on lateral root emergence and changes in the nutrient (both macro- and microelements) uptake have been observed. The effects are visible in the **Figure 13** [93].

Figure 13: *Effects of lignocellulosic humic-like substances (left root) on the lateral root growth of maize compared to control (right root) by Eyheraigubel et al. [93].*

Recently, the Czech scientists (*Antošová, et al.*) developed one of the simple methods for quantifying biological activity of HS, using hydroponic system with potassium humate extracted from North Bohemian leonardite. This method evaluates biological activity of HS on several higher plants (common wheat *Triticum aestivum*, common maize *Zea mays*, and cucumber *Cucumis sativus*, etc.) by measuring the relative increment of seedlings at set conditions and by comparing the humate results with those obtained by means of commercial growth stimulant. *Antošová et al.* proved that humates have very good effects on the growth of higher plants; the optimal concentration of humates varied from 60 to 200 mg kg^{-1} [94].

Zancani et al. researched the influence of HAs onto the phosphate level and energetic metabolism of *tobacco BY-2* suspension cell cultures, with that result, that HAs stimulate the excretion of enzymes phosphatases of extracellular origin. The authors also provided rich discussion and recent review on the topic [95].

Important new research was done by *Aguirre et al.* who found, that leonardite humic acids significantly affect the main physiological plant (cucumber, *Cucumis sativus L.* Ashley) responses to Fe deficiency, while increasing the expression of genes (CsFRO1, CsHa1, CsHa2 and CsIRT1) encoding the enzymes like Fe(III) chelate reductase, plasma membrane H^+ATPase and Fe(II) high-affinity transporter. In fact, due to the stimulated expression of these genes, more of the mentioned enzymes were synthesized in the plant resulting in the higher Fe uptake. These results were gained (after harvest of cucumbers treated with humic acid solution and isolation of mRNA transcript from the roots) by means of real time reverse transcription polymerase chain reaction (RT-PCR) [96].

The newest conclusions from *Mora* and *Aguirre et al.* are supposing that the phytoregulators, like cytokinins, gibberellins, or indolacetic acid are not responsible for the biological activity of humic substances, even in spite of the whole generation of scientists who favored this explanation. Since during this research almost none of these phytoregulators were found in the humic acid sample (by means of HPLC-MS/MS), *Mora* and *Aguirre* are preferring the explanation using the phenomenon of activation of root plasma membrane H^+-ATPase activity together with the postulate, that this phenomenon may cause significant changes on root-to-shoot distribution of NO_3^- and therefore of cytokinins and polyamines [97].

As the conclusion, it can be written, that the phenomenon of biological activity of humic substances is not yet fully elucidated, it is a complex biochemical process and a great inspiration for further research, mainly in the field of structure-activity relationships and life science. Recent finds of *Aguirre et al.* [96],[97] are leading the humus research probably to the meeting point of humic science with genetics, proteomics and molecular biology. The humic research probably not only in the biological activity field is reaching the molecular level.

Even writing a full review on this topic will greatly exceed the span of this doctoral thesis and also its primary physical and applied chemistry topic. It can be concluded, that humic substances, mainly HAs and FAs present positive effects on various plant growth probably due to two mechanisms. The first mechanism is the direct one, affecting the transcriptional and post-transcriptional regulation of enzymes and molecular transporters and the indirect one, expressed as the improvement of plant nutrition by increasing the soil nutrient availability [98]. For further reading, the review written by the most recent works by *Canellas et al.* [36],[82],[84],[90], *Zancani et al.* [95] and *Aguirre et al.* [96],[97] are strongly recommendable.

2.2.1.2. Fertilizer Additives

Some scientists also tried to test the effects of humic substances onto plant growth without the pursuit for decoding the process of HS' biological activity. There exist many results and now even applications of using humic substances simply as fertilizer or fertilizer additive, mainly in the form of salts of humic acids (ammonium, calcium or potassium humates) [23],[99–102] *Clapp et al.* studied the possibility to replace the chemical commercial fertilizers with humic substances when enhancing the turfgrass growth. The authors performed the experiments according to *Nelson and Craft* [103], growing the plants (creeping bentgrass – *Agrostis palustris Huds.* and ryegrass – *Lolium perenne L.*) in pouches with nutrient solutions and various humic substances. The experiments resulted in knowledge, that humic and fulvic acids stimulate the root and shoot growth of turf grass [104]. Various agricultural applications of HS are subjected to patents, e.g. the use of the humates (found in association with rutile sands) as additives to the potting media for growing foliage plants or the invention of mixture of water-soluble alkali metal salt of humic acid and other plant nutrient component (containing nitrogen and phosphorus) [105],[106]. Recently, *Chassapis et al.* succeeded in the sorption of humate on perlite (glassy aluminosilicate rock) and proved the product's microbial growth upgradability. Field experiments with this bioinorganic fertilizer are currently processed [107]. Mixing of humic substances with commercial fertilizers is now a great topic for research and commercial applicability of humics all around the world.

2.2.1.3. Crop Tests

Not many scientists have performed the real crop testing. They are very expensive and time consuming, not mentioning the necessity of the possession of suitable crop, which is probably only possible on the land-grant or agriculture specialized universities or other research institutions. Crop test may be connected with foliar fertilizing even if for today, still little is known about the foliar applications of humic substances. *Brownell et al.* proved, that foliar applications of HAs promote growth in a number of plant species such as tomato, cotton and grape [108]. *Xudan* revealed that spraying of wheat growing under dry conditions with fulvic

acids increased the yield, therefore it is suggested, that HS possess the capability to reduce water stress [109]. *Chen and Aviad* indicate the low cost of the foliar application of HS [110]. In 1996, *Fernández-Escobar et al.* made a series of greenhouse and field experiments when applying commercial mixture of nutrients and HAs and FAs extracted from leonardite on the olive trees. The authors proved that foliar application of HAs clearly stimulated the vegetative growth of olive trees, the promotion of growth was independent of the culture system (irrigated or non-irrigated). However, no effect was observed on yield [111].

On the other hand, manufacturers of humic products have conducted extensive field evaluations of their products; however their findings have not been widely disseminated. This can be for two reasons, the first is, that the results may be protected company data, the second one, even if the companies tend to publish such results, they have not so good contacts with scientific sector. For example, LignoTech USA found an increase in harvestable yield in 33 of 38 cases for 12 crops in 8 U.S. states and in five other countries. Helena Chemical together with Horizon Ag found positive responses across the United States on many fields and for many plants too. Moreover, LignoTech USA noted greater diversity of soil microbial species with application of their product. Yet, many of these results were not statistically evaluated, many of these experiments were conducted only once or only few times and it was of course observed, that the results of humic product applications vary dependent on the soil type and quality where they were used [112].

2.2.1.4. Soil Conditioners

It is widely known, that in many agricultural soils in semi-arid and arid Mediterranean climates the exposure to cyclic wetting and drying will reduce the aggregation stability. The following consequence may be enormous and can include the slaking and crusting, accelerated runoff erosion and poor crop productivity. Nowadays, great effort is therefore devoted to apply soil management techniques that can counteract these effects. *Piccolo et al.* observed on the experiment with three Italian soils, that soil treatment with coal derived humic acids (in low rates, ca. 200 kg ha^{-1}) improve the aggregate stability of all the soils tested, moreover, it moderates the magnitude of reduction in structural stability due to successive wetting and drying cycles [113].

By means of experiments with ^{13}C labeled decan-2-ol (as an relatively labile aliphatic compound), *Spaccini et al.* revealed that addition of humic acids to soil significantly increased the sequestration of organic carbon by reducing its mineralization [114].

Egyptian scientists *Selim et al.* performed experiments with Egyptian sandy soils and humic substances, where humic substances applications (the best concentration was 120 kg ha^{-1}) had highly significant effect on improving potato tubers yield and quality as well as macro- and micronutrients concentration remained in soil after the harvest [115].

Some methods of soil conditioning by use of humic and fulvic acids, gels prepared from them or their salts are also subject of patents [116],[117].

2.2.2. Environmental Protection

As it was already written, HS qualify as privileged natural compounds in the interaction with both organic and inorganic pollutants. All the *supra* mentioned processes extents the biodegradation and detoxification, bioavailability and ecotoxicity, accumulation, mobilization and transport, residue persistence and monitoring of organic pollutants. Interactions of HS with organic pollutants have been thoroughly studied mainly from the point of view of sorptive processes involving the partition of organic solutes between the aqueous and soil organic phases; results were generally correlated with octanol – water partition coefficients (K_{OW}) and have been described with reasonable success by the original and modified Flory-Huggins theories. In the case of inorganic pollutants, mainly the trace metals, the most processes in which metals are involved in soils, including the mobility and transport, fixation and accumulation, chemical reactivity and bioavailability are affected by their interaction with HS. Interactions of HS with metals varies with regard to the type of cation considered (group I cations – "hard" ions are likely to undergo electrostatic interactions with HA functional groups while the bonds formed by the group III cations – the "soft" ones – are more covalent in character and the group II cations, such as Cu^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} and Ni^{2+} behave in an intermediate fashion) [18],[118–120].

2.2.2.1. Sorbent and Chelating Abilities

The colloid and sorptive properties of humic substances are used in many environmental applications; however, the main task still lies in the removing of toxic metals, organic chemicals and radioactive elements 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 for removal of radioactive elements from water discharges from nuclear power plants. Humus-based filters have been developed for sewage treatment, with many various applications. Those filters are useful in cleaning chromate smelter wastewater, to remove oil and dyes from wastewaters and aquatic systems, to filter urban and industrial wastewaters, and to remove pesticides from sewage and to remove phenol from water. Thanks to their ability to sequestrate the organic pollutants, HS may be used to remove them from water, soil and sewage sludge. Slightly modified humates can be applied to remove hydrogen sulfide and thiols (mercaptans) from municipal gas supplies and sulfur dioxide from stack gases. Selective binding capacities of HS are also exploited for the destruction of explosives and chemical warfare agents [118],[121–123], [124 and references therein].

Here a few particular examples of HS environmental applications are introduced. *Sanjay et al.* from Arctech, Inc. developed a novel coal humic acid based adsorbent named HUMASORB-CS^{TM}, which works as remediator for contaminated waters. HUMASORB-CS^{TM} is cross-linked (for method see reference [125], an U. S. Patent) and therefore non-soluble in water over a wide pH range. HUMASORB-CS^{TM} was thoroughly evaluated as a very effective removing agent of various toxic metals including lead, mercury or uranium, even in the presence of calcium, and also as very effective in removing non-ionic organic pollutants, such as tetra- and trichloroethylene. All the experiments were performed under simulated barrier conditions (e.g. in a column) at various pressure values in a single treatment step [126].

Zanin and Boetti from HydroGeo North America LLC patented a method and product for purification of waste water (especially of that polluted with phosphorus, nitrogen, heavy metals and chlorinated solvents). The process is based in addition of a metallic salt ($Al_2(SO_4)_2.12H_2O$ or $KAl(SO_4)_2.12H_2O$) and a particular liquid mixture rich in humic and fulvic acids to the waste water and mixing it. The pollutants react with the mixture, which results in stable combination of a flaky precipitate forming sludge, removable by common techniques. The process is hinted in the **Figure 14** [127].

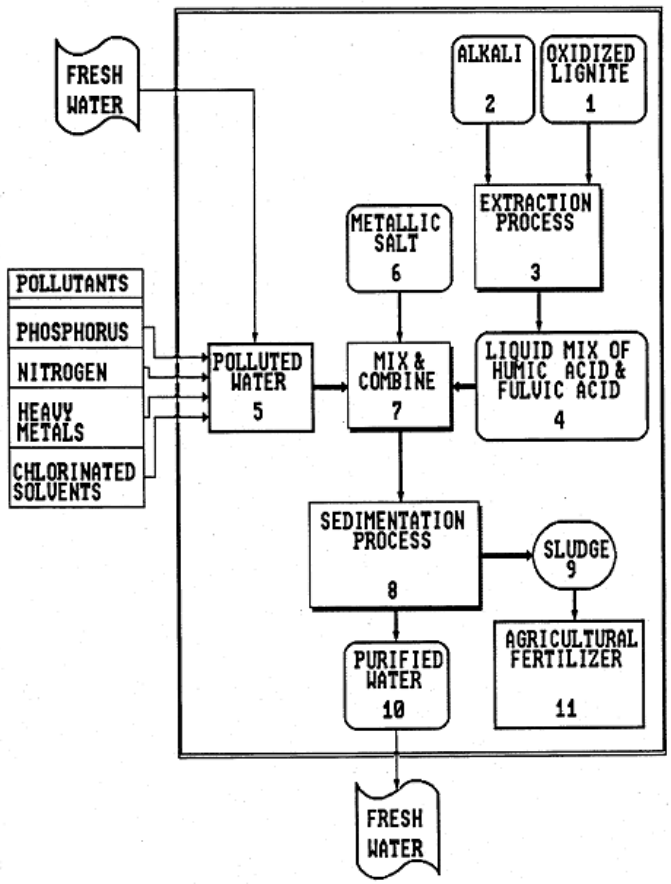
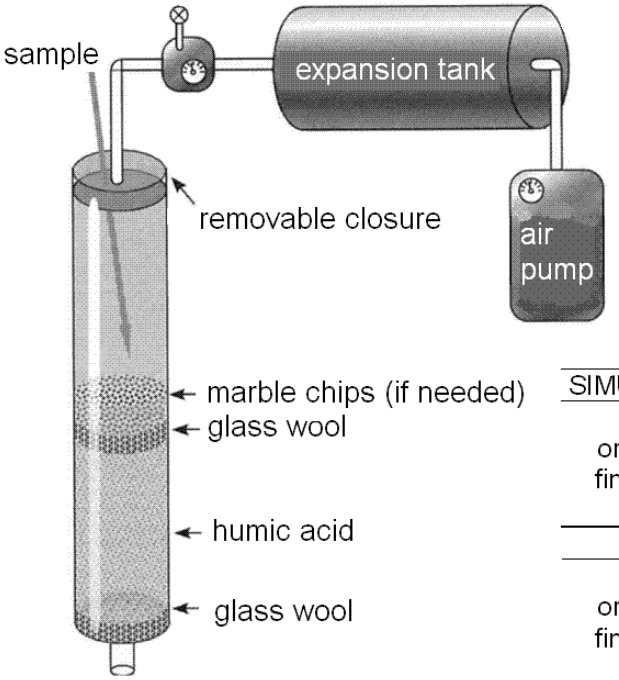


Figure 14: *The process flowchart of the Zanin's and Boetti's invention [127].*

Ray von Wandruszka supposes the difficulty in using HAs and FAs for the decontamination, sorption and other environmental purposes, because the extractions of HAs and FAs are laborious, time consuming and costly (which depends on material from the humics are extracted and on the desired quality). Von Wandruszka states that the Leonardite Humic Acid (LHA) is an exception from these issues, since it is a material, which is found in association with leonardite, is itself mined, therefore cheaply available in bulk, and contains about 80% of humic matter, the remainder being mostly mineral matter, e. g., no further treatment is needed. The unrefined LHA can be used for treatment of polluted water, either by a batch process or (more conveniently) by continuous column extraction. Heavy metals, including Pb^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+} were successfully removed as were the organic compounds like pyrene, difenzoquat, and rhodamine B. Column capacities were about 0.15–2.2 mg g^{-1} LHA for the metals and 5.4 – 29.0 mmol g^{-1} LHA for the organic compounds. Except the low price, the further advantages of LHA are its combustibility and resistance to acids (since metal polluted waters are frequently acidic). Moreover, excellent results were achieved on the

sorption of Zn^{2+} and trichloroethylene (a widely used metal degreaser) when sorbed in the packed column with LHA and marble chips. The experiments results were obtained by means of atomic absorption, UV-VIS and fluorescence spectrometries as well as the gas chromatography [118],[128],[129]. The instrument setup and results are shown in the **Figure 15** [129].



SIMULTANEOUS REMOVAL OF Zn, Mg and Ca			
	Zn	Mg	Ca
orig. conc. [mg.L^{-1}]	100	50	100
final. conc. [mg.L^{-1}]	5.3	40	40
% removed	94.7	20	60
TRICHLOROETHYLENE REMOVAL			
	A	B	C
orig. conc. [mg.L^{-1}]	293	75	21
final. conc. [μg.L^{-1}]	8.3	0.8	4.6
% removed	99.99	99.99	99.99

Figure 15: *Instrument set-up and results of packed column extraction with LHA by von Wandruszka and Newell [129].*

More recently, *Pehlivan and Arslan* performed adsorption and desorption experiments with two variants of local Turkish lignite (Beysehir and Ilgin lignite, from Konya province in Middle Antalya). The sorption of Cu, Pb and Ni metals was studied by batch technique and shaker, the concentration of metals were determined by atomic absorption spectroscopy and the data were assessed according to well known Langmuir and Freundlich equations. The results proved, that the Beysehir and Ilgin lignites are about 60–70% effective in adsorbing the Cu, Pb and Ni cations from water solutions with pH between 3.0 and 5.3 [130].

Most recently, *Havelcová et al.* reported a study about sorption (via batch process) of metal ions (Pb^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}) onto the South Moravian lignite and humic substances (of various quality) extracted from it, and also on humin, the insoluble residue. Moreover, the authors studied the influence of the initial pH of the solutions on the sorption. The authors proved that lignite is both economically and chemically efficient sorbent for toxic metal ions, and suggested, that humin may be used for such sorptions (both from waste waters and polluted natural waters) with very good results too [131].

Of course, more scientists employed themselves with the studying of metal sorption onto lignite and humic acids, for further reading see references by *Janoš et al.* [132], *Klučáková and Omelka* [133], *Klučáková and Pekař* [134] or *Coles and Yong* [135]. *Alvarez-Puebla et al.* performed a study about the sorption of Cu^{2+} ions onto humin [136].

2.2.2.2. Further Environmental Applications of HS

As it was already mentioned, *Ray von Wandruszka* presented a few works about the detergent qualities and the clouding effect of humic substances, which are based on their pseudomicellar character. This may in the future result in the development of natural humic-based surfactant for soil remediation On the other hand, humic acids are able to sequestrate nonpolar organic contaminants and promote their retention in relatively polar environments [47],[51],[53],[118],[137].

Further, a method of removal of oils from solid surfaces and water by means of various (often waste) substances having a high humate level is patented by *Harman et al.* [138].

It is known, that humic substances contain quinoid moieties, which are able to serve as an electron shuttle to metal (e. g. Fe^{3+}) oxides in microbial redox processes and in the biodegradation of priority pollutants [139]. *Kappler and Haderlein* researched, whether the soil (extracted), aquatic (IHSS Suwanee River) and synthesized (via iron catalyzed polymerization of a mixture of different phenols) humic acids can be electrochemically reduced and whether they then can transform pollutants. Humic acids were reduced in aqueous solution at platinated titanium cathode. The reduction capacities of reduced HAs were determined by a redox titration using potassium ferricyanide and they were found higher for the previously reduced HAs than for the untreated HA. Reduced HAs were found to be able to reduce the hexachloroethylene to tetrachloroethylene as a single product. Since reduced HAs are naturally occuring as products of a variety of microbiological processes [140],[141], results obtained by Kappler and Haderlein may be useful for the understanding and developing more sophisticated electroremediation technologies or for remediation only by means of delivering quinone-rich natural organic matter to contaminated soils [142].

Piccolo and Fava with coworkers reported, that humic substances are able to enhance the solubility of polycyclic aromatic hydrocarbons in aqueous environment which is leading to the increase of their bioavailability to microbial metabolism [143],[144].

Very recently, *Stehlíčková and Kozler with coworkers* published a study in which they proved the intensification of phenol biodegradation by potassium humate extracted from oxyhumolite (central European leonardite). The experiments were carried out as a batch cultivation of *Cupriavidus metallidurans* bacteria isolated from industrial wastewater contaminated by various pollutants (predominantly phenol, naphtalene and aniline). The cultivation was performed in liquid basal salt medium with or without potassium humate and with phenol. The biodegradation experiments runned in batch system and a column system packed with ceramic clay. The results showed, that it is possible to accelerate the phenol removal from waters by the addition of potassium humate, moreover, the humate adsorption on the microbial biomass was observed. The reasons may be the interaction between the humate and the cell surface of *C. metallidurans* or the reversible bond establishment between humate and

phenol, which may result in decreasing of phenol toxicity [145]. Some more authors already published the fact, that humates are adsorbed on the cell surface of various bacteria. [146],[147].

Very recently, *Hu et al.* invented a process, in which the waste gases SO_2 and NO_2 are simultaneously absorbed to the solution of sodium humate, which is resulting in Na_2SO_4 and compound humic fertilizer. The authors state, that this process is cheaper and more effective than the usual lime-gypsum process [148].

2.2.2.3. Interactions of HS with a Tetracycline Antimicrobial

As the progress of technologies and medicine goes on, new contaminants are introduced in the environment. For an example of contaminant being introduced in the past 20 years antimicrobial agent tetracycline was chosen.

Tetracyclines (TC) are a class of antimicrobial drugs, usually isolated from microorganisms and modified via chemical synthesis, which are very widely used both in human and veterinary medicine due to their broad antibacterial spectrum of effects and reasonable prices. The structure of tetracycline is based on the napthacene ring (depicted on the **Figure 16**). The side chains may vary as well as the form – the most used one is the water soluble tetracycline hydrochloride [149–151]. The threats of tetracyclines to environment consist in their wide use for both human and veterinary medicine purposes. When dosing tetracyclines to livestock, up to 90 % of the dose may be excreted unmetabolized [152–155]. The concentration of TC in manure may be as high as 20 mg L^{-1} [156] and due to frequent applying of the contaminated manure onto soils, the soil TC concentrations are ranging from 12 to 100 µg kg^{-1} [157],[158]. In surface waters, the general concentrations of TC are in the µg L^{-1} range [159]. The fate of tetracyclines in soil environment is not elucidated yet, but two competing effects are considered. The high water solubility of tetracycline hydrochlorides causes the high environmental mobility [153],[160], yet several investigations showed high sorption of tetracyclines to soil [161].

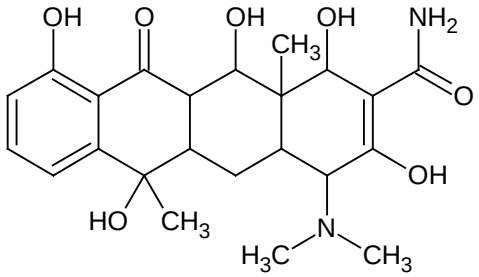


Figure 16: *Structure of tetracycline.*

Tetracyclines in environment may be toxic to water organisms. Even if the concentrations in ground waters are relatively low (ng to µg L^{-1}), it is not known yet, what the antimicrobials may affect the organisms, for which they have not been intended. Moreover, the tetracyclines may degrade to unknown metabolites with unknown effects. The presence of all the antimicrobials in the environment (especially in the low concentrations) may increase the toxic effects of microorganisms and intensify their resistance against the antimicrobial drugs [162],[163]. Also the human medicine tetracyclines are excreted unmetabolized to the

wastewaters. This may cause complications in the biological stage of treatment in the wastewater treatment plants. Moreover, the polar antimicrobials such as tetracycline hydrochloride may not be treated completely and usually are dissipated in the environment via the water coming out of the plant or sorbed in the activated sludge [164],[165].

The sorption mechanisms of TC are not fully elucidated yet either. Some authors have observed the divalent cation binding to clay minerals [166], other studies are describing the non-covalent interactions (intercalation) into clay, sodium-montmorillonite or humic acid-montmorillonite complexes [167–169]. However, no information is known about sorption of TC onto pure humic acid or onto lignite. A different antimicrobial, sulfathiazole, was studied in the terms of cation binding onto the IHSS Leonardite humic acid standard [170]. The most important inspiration for this part of thesis were the works of *Pils and Laird* [153] and *Avisar et al.* [169], who emphasized to gain the information about adsorption of tetracyclines onto montmorillonite clay and humic acid complexes.

2.2.3. "Green" Humic Chemistry Potential

The use of humic substances in industrial processes is widely known. HS, and in particular, HAs were and are in leather industry as a dye, later as a tanning agent and finally as one of more ingredients of a leather-finishing solution [12],[124]. In wood industry, HS have been applied as dyes for the wood veneer [124]. In ceramic industry, humics were used as additives to improve mechanical properties of unprocessed ceramics, to improve the casting properties and to dye tiles and to help to prepare the earthenware. In paper industry the humics serve as agents for production of conducting paper and high tensile strength paper. Humic substances are also used as food and beverage additives [12],[124]. In the uranium mining, HS help to extract the uranium from its ore [171].

2.2.3.1. Polymer Additives

Humic materials have found applications in the production of synthetic polymers, especially as dyes for poly(caprolactam) (Nylon 6) or poly(vinylchloride) plastics, where they work also as the plasticizer [172]. Partial polyvalent metal salts of coal derived humic acids were or are used in rubbers as reinforcing fillers [173] or as dispersants in latex [174]. Preparation of a rubber masterbatch containing a humic acid salt with water soluble volatile base such as ammonia or amine have been patented [175]. Elastomers such as vulcanizable synthetic rubbers blended with various other rubbers (e. g. *cis*-poly(isoprene) or ethylene propylene diene monomer rubber) were prepared with an significant addition of high molecular weight water insoluble humate salt. The humate in these rubbers works as filler, partial or complete substitute for conventional blowing agents and as an "anti-blooming" agent. An example of such a rubber formulation is given in the **Table 4** [176], [177].

Table 4: *Example of a formulation for sponge rubber containing humate [176].*

Ingredient	Parts by Weight
Ethylene Propylene diene rubber (Polysar EPDM 6463)	150
Humic Acid humate (from titanium sand, C content 33 %)	80
Polyterpene resin (Goodyear Wingtac)	10
Zinc Oxide	5
Stearic Acid	1
Zinc–O,–O–di–N–butylphosphorodithioate on inert carrier	3.2 (2 parts active)
Tetramethylthiuram Disulfide	1
Butyl Benzothiazole Sulfenamide	2
Sulfur	1

Antioxidant abilities of humic acids have been demonstrated in composites with poly(vinylchloride) and poly(vinyl alcohol), the antioxidant additives in general and the antioxidant properties of HAs is the subject of collateral intensive research [178–182]. Recently, partially *in our laboratory*, South Moravian lignite derived humic acids have been proposed as an effective, cheap and environmentally friendly antioxidant for poly(vinyl alcohol) [179].

2.2.3.2. Dyes

Except for the already mentioned history of ceramics and leather dyes, humic substances are on the focus of collateral dyes industry, mainly in the purpose as a precursor of cheaper and environmentally friendlier azo-dyes, which they may be thanks to their possession of chromophoric groups. *Kyoungsuk and Wontaik* were probably the first, who studied the possibility of coupling the humic acid with aromatic diazonium salts, resulting in knowledge, that humic and “nitrohumic” acids coupled with aromatic diazonium salts such as aniline, aminonapthalene and aminoantraquinone derivatives are giving various azo-dyes, probably thanks to the many vacant *o*- and *p*- positions on the phenol rings on the humics [183].

Nasir et al. succeeded in preparation of direct azo and acid dyes from Pakistani lignite derived humic acid sodium salt via diazotation/coupling pathway with different arylamines. The structures of products were characterized by FTIR and pyrolysis-gas chromatography-mass spectrometry coupled technique. Applicability and fastness were tested on cotton fabric (for the direct azo dyes) and on the silk and wood fabric for the acid dye. The dyes showed moderate to good light fastness and very good to excellent washing, rubbing, perspiration and sea water fastness properties. The example is shown in the **Figure 17** [184].

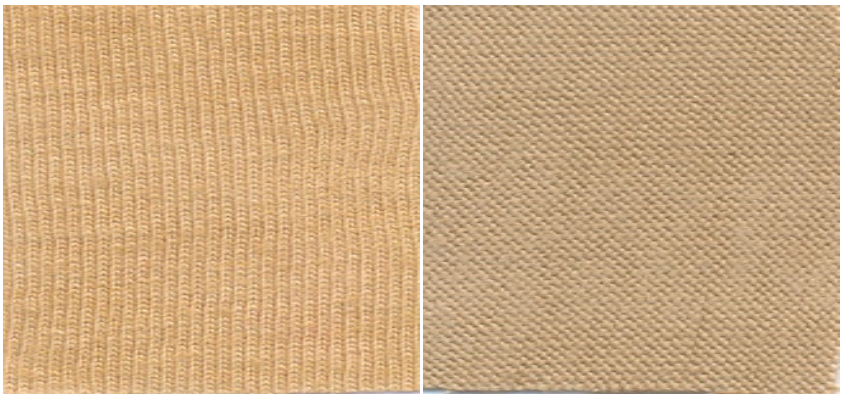


Figure 17: *Example of excellent brown color hues of D3 (left) and D9 (right) lignite humic acid direct azo dye on cotton fabric at 80°C by Nasir et al. [184].*

West and Firt from the Union Camp Corporation are the possessors of the U.S. Patent related to a method of preparation of printing ink formulations containing stabilized aqueous dispersions of multivalent (calcium, chromium, iron, zinc and aluminum) humates, even in today's view on the environmentally friendly printing techniques this method may be disputable [185].

Very recently, from the environmental point of view, the interactions between commercial humic acid (RCNC Co., China) and cationic dye (Toluidine Blue; tolonium chloride; (7-amino-8-methyl-phenothiazin-3-ylidene)-dimethyl-ammonium) were studied by *Sheng et al.* utilizing mainly the UV-VIS and FTIR spectrometries. The main purpose for this research was the modeling of interactions between cationic dye pollutants and aqueous humic acids in ground waters (rivers etc.). Toluidine blue forms complexes with humic acids in aqueous environment, probably due to the existence of many negatively charged groups on the HA [186].

2.2.3.3. HS as Synthesis Precursors

For using humic acid as a synthesis raw material, obviously a different point of view should be applied. Instead of researching the differences between various humic acids from various sources, in this case the scientists are focusing on the similarities in all the humics. Detailed characterization of the used HA is necessary and usually, some of the "standardized" or even "synthetic" HAs are used for the synthesis purposes.

There are not many information about the humics as synthesis precursors. *Duncan et al.* reported in 1981 the possibility to use humic materials as a source of synthetic hydrocarbons and fuel oils [187].

The option to couple humic acids with aromatic diazonium salts was already mentioned in a chapter supra as well as the cross-linking of humic acids to HUMASORB-CS^{TM} sorbent (where the cross-linking serves for reducing solubility but not for any other purpose).

Some studies about alkylation of humic acids have been performed, but mainly with the characterization purpose – as a tool to quantify the hydroxyl sites [188],[189].

Kolla et al. succeeded in application of humic acid as a substrate for alkylation. The authors suggested that all of the humic acids, no matter of their source, possess centers suspected to be active towards various chemistries including substitution, addition, alkylation, reducing and oxidation. Kolla et al. used the simplest model alkylating agent – methyl iodide in two methods: the first one was a simple base catalyzed and thermally induced alkylation utilizing the carboxylic acid functional groups of HA; the anionic nature of HA enables the formation of methyl esters (or methylphenylesters at the phenolic sites as well). The second method lied in the photochemical activation and further C–I bond cleavage, which activates the quinoid radicals for *o*- methylation. Verification of both reactions was easily achieved by using $^{13}CH_3I$ and ^{13}C NMR analysis. The products were mainly the methyl esters of humic acids, insoluble in alcohol and aqueous media from pH 1–10. They still show appreciable (even if a little compromised) metal binding capacity and they are supposed to react with nitrogen and sulfur mustards to eliminate the hazards of these warfare agents. Simplified synthesis reaction pathway is shown in the **Figure 18** [190].

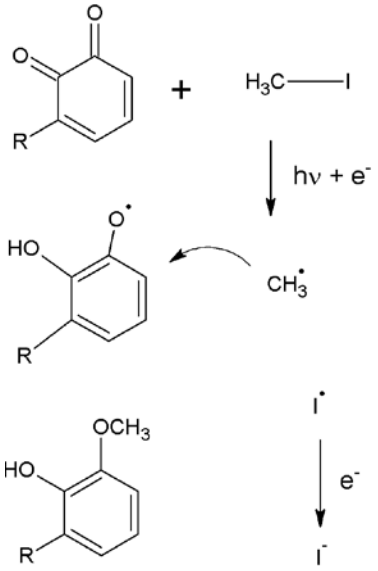


Figure 18: *Reaction pathway of CH_3I reaction with a catechol moiety of HA according to Kolla et al. [190].*

The research group of *Perminova* recently succeeded in the synthesis of quinoid-enriched humic materials, so called humic copolymers from leonardite humic acids. The mechanism consisted in oxidation of phenolic moieties of HAs with various (Fremy's salt, chromium trioxide, potassium ferricyanide, Elbs' reagent and Fenton's reagent) oxidating agents and further reduction with sodium sulfite and consequential formaldehyde polycondensation with catechol and hydroquinone. In addition, radical copolymerization with *p*-benzoquinone was used to obtain hydroquinone-enriched derivatives. Resulting products were purified and characterized by means of FTIR and ^{13}C NMR spectrometries, elemental analysis and capillary zone electrophoresis. All humic copolymers obtained exhibited much higher redox capacity values than the parental HA materials, the values varied from 1.1 to 4.0 mmol g^{-1}. This research may have great potential in ensuring control over redox properties of humic based redox-mediators and reductants needed for their successful application in remediation

strategies and other (mostly ion-exchange based) technologies. The scheme of the synthesis is to see in the **Figure 19** [191].

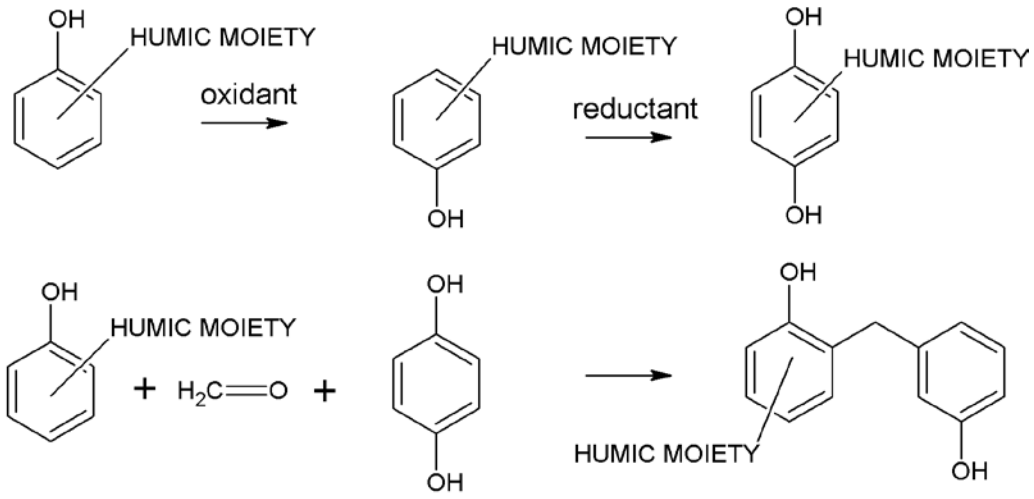


Figure 19: *Schematic pathway for synthesis or quinoid-enriched humic materials by Perminova et al. The **first** reaction is subsequent oxidation and reduction of available phenolic fragments of HA, the **latter** then symbolizes the formaldehyde condensation between phenolic fragments and hydroquinone yielding humic copolymer with pendant hydroquinone units [191].*

Very recently, *Klavins and Purmalis* accomplished a modification of commercial (Sigma-Aldrich) HA with sulphopropyl groups (via 1,3-propanesulfone), trimethylammonio groups (via 1-chloro-2,3-epoxypropane and trimethylammonium chloride), sulphoalkyl groups (via formaldehyde and $NaHSO_3$) and with hydroxyl groups (via 2,3-epoxypropane) in order to increase the surfactant properties. This method was already used for the development of biopolymers with surfactant properties [192]. Resulting products were analyzed by means of FT-IR, ^1H NMR, elemental analysis and surface tension analysis. As conclusion can be written, that the modification occurs at the first benzene rings of humic phenolic structures and at hydroxyl groups and that this modification can allow significant increases in the surfactant properties of HAs [193].

2.2.3.4. HS Extraction from Various Wastes

Recently, as the demand both for humic acids and also for recycling techniques and more efficient waste management increased, several methods appeared, how to extract the HAs not only from coal, lignite, leonardite or soil or compost, but from waste materials too.

Extracting of humics from lignocellulose waste was already mentioned here. *Senesi et al.* published a method, how to gain humic acids from olive-mill wastewaters (which generally contain high amount [about 16 %] of organic matter in suitable form of organic acids, phenols, lipids or amides). Catalytic treatment with a polyenzymatic mixture [194] has been attempted to induce humification processes of this organic matter in wastewater, after 3–7 weeks the HAs were isolated by standard extraction method. Products were characterized by means of UV-VIS, FTIR and fluorescence spectrometries as well as the elemental analysis with conclusion, that the process of gaining HAs from olive-oil mill wastewaters is an

efficient way, particularly with using Mn^{IV} oxide as the humification catalyst. Obtained HAs were similar to those extracted from Italian soil [194].

Unsal and Ok extracted humic substances from a broad variety of waste materials (sewage sludge, brewery sludge, composted grape marc, spent mushroom compost, composted bark, and tobacco dust; all of them obtained in particular plants in Turkey). The most successful extraction was the one from composted bark; the least yielding was the extraction from tobacco dust [195].

Recently, *Quagliotto, Montoneri, et al.* tried to compost the food residues and municipal park green wastes and extract humic acids from the obtained compost. Obtained HAs performed good results as surfactants in soil remediation and textile dyeing [196],[197].

2.2.3.5. Sugar Content in Lignite and its Potential

The research on the origin and constitution of humic acids brought up to light the fact that humic acids contain carbohydrate (sugar) chains. Sugars were found in humics first by *Ogner* [198] and were determined by *Allard*, who studied also the lignitic humic acids. Allard found glucose and galactose as the predominant monosaccharides and xylose, rhamnose and hexoses as the minor monosaccharides in the sample of IHSS lignite humic acid [199]. The presence of sugars (however they might be readily biodegradable) in humics is caused plausibly by some physical stabilization of them or exhausting of the sugar-degrading microbial population during the lignite burial period [199]. *Vlčková et al.* applied chemical clevage on the humic acid samples to find the content of polyols such as threitol, 2-deoxy-arabino-lactone and 2-deoxy-ribonic acid, which are believed as saccharidic compounds and the products of saccharide diagenesis [200]. The Vlčková's humic acid from lignite regenerated with 5 wt% H_2O_2 showed very high content of these polyols [200].

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[201].

2.2.4. Some Companies in Humic Business

In this paragraph, an example of few from many companies carrying in the humic substances business is shown, as an exemplification, that HS can be the driver of successful business plan.

Lignit Hodonín, s.r.o. (now in the portfolio of UVR a.s.) is a Czech company which mines the lignite in southern Moravia since 1982 and the main source of lignite for our laboratory, more information at www.lignit.cz. The company is also the distributor and inventor of the lignite product Terra Clean [202]. *Amagro, s.r.o.* is a Czech trading company selling and distributing lignohumate salts for various purposes, which are made in Russia, in the company called *NPO RET* from cellulose waste, more information at www.amagro.cz and at www.humate.spb.ru .

Humatex, s.r.o. is another Czech company, producing humic acids, humates and fulvates from oxyhumolite (European leonardite), more information at www.humatex.cz. *Humintech GmbH* is a German company producing humic acid salts for agriculture, environmental engineering, industrial applications and veterinary medicine, check www.humintech.com . In the United States of America, many companies carry the humic business, for example the already mentioned *Arctech Inc., HumaTech Inc.* as well as the *and Mesa Verde Resources Ltd., Horizon Ag Products LP,* or *Leonardite Products LLP* (seek information at www.arctech.com, www.humatech.com, www.humates.com, www.horizonag.com and www.leonarditeproducts.com respectively. In Canada, there is the *Black Earth Humates Ltd.* company active on the market (see www.blackearth.com). One of the most known Chinese humic companies is the Bowei Agrochem Co. Ltd., offering humic and fulvic acids, various humates and humate gel (check www.bowei-agrochem.com). From the big multinational corporations, the AMCOL International Inc. (American Colloid Company) is interested in humic business (see www.amcol.com). Many more humics based companies are emerging in Russia, China, Pakistan or Brazil.

In 2010, the Humic Products Trade Association has been established by many of the Northern-American humic companies, in order to promote the commercial applications of humic products and scientific cooperation as well as the addressing of regulatory issues (more information at www.humictrade.org).

2.3. BRIEF REVIEW OF ANALYTICAL TECHNIQUES

These few of brief paragraphs have the purpose to enlighten the basics of function principles of instrumental techniques used during the work on this thesis as well as very shortly discuss the former employment of these methods in humic substances research. Since the purpose of this thesis lies not mainly in the analytical chemistry, this review is very short and do not attempt to explain the analytical techniques completely since each one of the instruments used may present enough inspiration for separate thesis.

For this very reason, only the not-so-common techniques for the humic research are reviewed. Today's state-of-the-art of humic (or organic, inorganic or (bio)polymer chemical research) seems to be so far, that every possible reader of this thesis may be familiar with the basics of the work principles of such an instruments like elemental analysis (EA, CHNS(O) analysis), Fourier transform infra-red spectroscopy (FTIR), thermogravimetry (TGA) or high performance size exclusion chromatography (HPSEC). Also the modern chemist's knowledge of analytical chemistry seems to be including the basics of both gas and (high performance) liquid chromatography (GC, HPLC), with many various sample treatment and introducements (such as solid phase extraction in HPLC (SPE) and also with many analytical endpoints namely the diode array detector (DAD) for HPLC. All of these techniques are described in detail in every textbook of analytical or even organic chemistry, so they are not reviewed here, even if their potential is utterly used during this whole thesis.

2.3.1. Fast Field Cycling Nuclear Magnetic Resonance Relaxometry Principle and Use

Fast field cycling (FFC) relaxometry is a nuclear magnetic resonance (NMR) technique for obtaining the frequency (or magnetic field) dependence of relaxation times (or equivalently relaxation rates) over a six decade range span of magnetic fields (from about 10^{-6} to 1 T). In principle, the sample undergoes the infliction of a Zeeman magnetic field (B_0) which cycles through three different values (polarization, relaxation and acquisition; B_{POL}, B_{RLX} and B_{ACQ} respectively). B_{POL} is applied for a defined period of time T_{POL} to achieve magnetization saturation and sensitivity enhancement. After that, the magnetic field is switched to the new one, B_{RLX}, which is applied for the other period of time τ during which magnetization intensity changes to reach a new equilibrium condition. The final acquisition of the free induction decay (FIDE) is achieved by means of application of the magnetic field B_{ACQ} concomitantly with a 90° pulse on the investigated nucleus. The T_1 relaxation time and consequentially measured longitudinal relaxation rates $R_1 = 1/T_1$ of the observed nuclei are measured at each fixed B_{RLX} intensity by progressive variation of *t* values. The results are represented as nuclear magnetic resonance dispersion (NMRD) profile which lies in the plotting of the longitudinal relaxation rate versus the applied magnetic field strengths. This so called dispersion curves are bearing many various information of the chemical and physical properties of the sample, such as interactions between solvents and solutes, conformations and molecular dynamics. FFC NMR allows the measurement of T_2 as well, but in this case the value or its distribution is expressed only at one frequency (because T_2 is frequency independent) and provides information on character of the interactions between sample and solvent and about the solid samples' interior. Therefore, the FFC NMR technique is effectively employed in the research of tissues, food and seeds, environmental matrices, biopolymers and in the constantly growing interest in the "nano-world" [203–206]. The basic

principles of FFC NMR are depicted on the **Figure 20**. To author's best knowledge, employing the FFC NMR relaxometry onto humic substances research is a complete novelty.

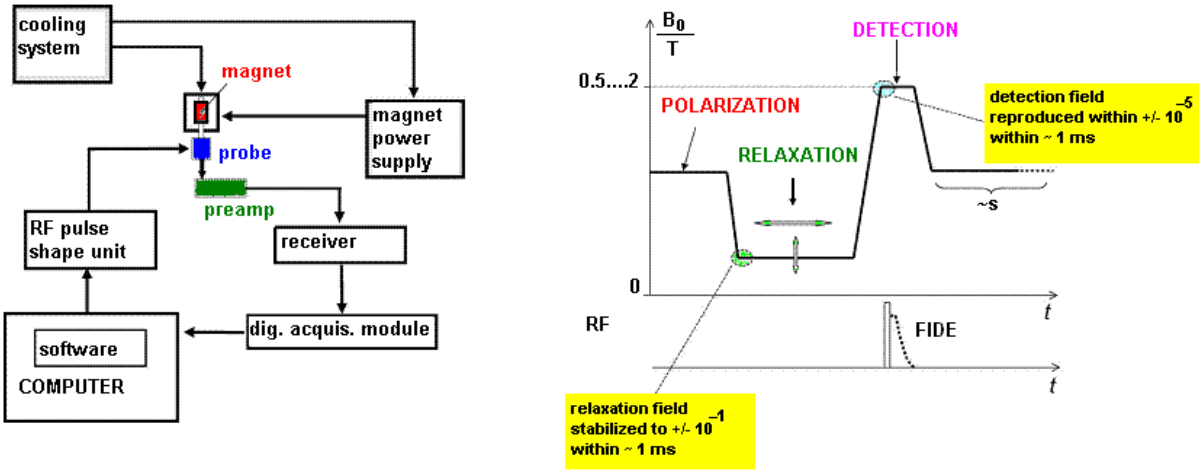

Figure 20: *Scheme of a common FFC NMR spectrometer (left) and a common analysis cycle (right) [203].*

2.3.2. Dynamic Light Scattering Principle and Use

Dynamic light scattering (DLS) method (sometimes called also photon correlation spectroscopy) utilizes the laser light (usually the red light emitted by the He-Ne laser) to measure the diffusion coefficient *D*, which is related to the hydrodynamic diameter of the scattering particle according to the Stokes–Einstein equation **1)**:

$$D = \frac{k_B T}{3\pi\eta d} \qquad 1.$$

where k_B means the Boltzmann constant, T the measuring cell temperature, the diluent viscosity an d the equivalent spherical diameter.

The measurement of diffusion coefficient by DLS involves the correlations of fluctuations in scattering intensity arising from the Brownian motion of the scatterers, obtained at different delay times. The technique enables rapid measurement of particle sizes in solutions, from the low nanometer range to the low micrometer range [60],[207]. The illustration of DLS principles is to see infra on the **Figure 21** [207],[208]. DLS has already been to some utilization in the humic particles research before [60],[209],[210].

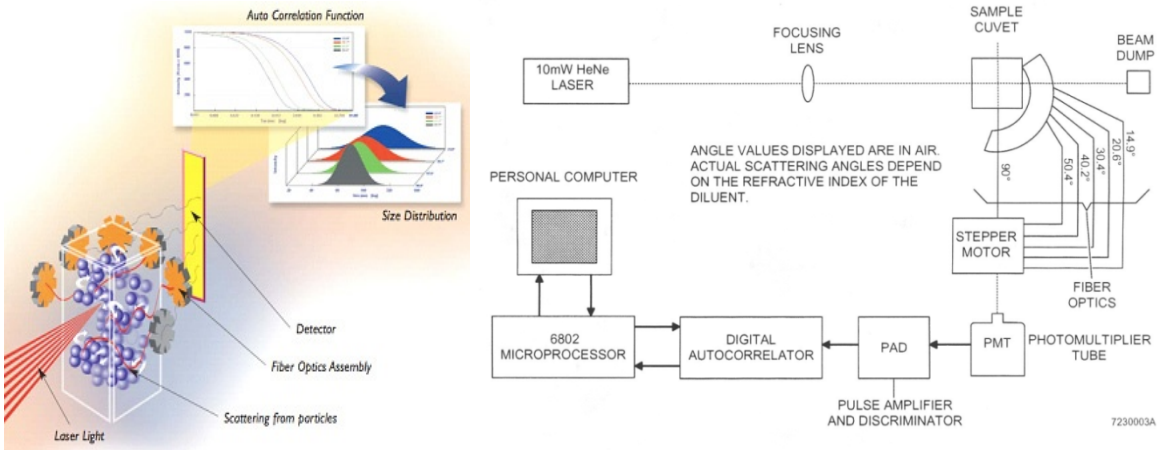

Figure 21: *DLS principle and instrumentation [207],[208].*

2.3.3. High Resolution Ultrasonic Spectrometry Principle and Use

The function of high resolution ultrasonic spectrometer is based on the mechanical nature of the ultrasonic waves. They probe the samples by propagating through them causing oscillating compressions and decompressions. The compressions decrease the distances between neighboring molecules and force them to respond to these compressions by intermolecular repulsion. When under decompression, the attractive forces play the role [211]. The amplitude of deformations caused by analytical ultrasound waves is extremely small and therefore making the ultrasonic spectroscopy a non-destructive technique [212].

Parameters that are traditionally measured in ultrasonic spectroscopy are ultrasonic attenuation and ultrasonic velocity. Attenuation is determined by the energy losses of the ultrasonic waves due to absorption and scattering contributions. In homogenous samples, the periodical compressions and decompressions of the molecules shift the equilibrium of the chemical reactions. A delay in the relaxation of the molecules to the equilibrium state causes absorption of energy. In non-homogenous samples, the presence of particles results in scattering of ultrasonic wave. The ultrasonic attenuation is observed as the decrease of the amplitude of the output wave [213].

The second parameter measured is ultrasonic velocity. It is determined by the density and the elasticity of the medium. The speed of ultrasound is proportional to rigidity of the sample and since the solids are more rigid (which also means having higher elasticity of molecules) then liquids and gases, respectively, the sound propagates through them faster than through liquids and gases. The rigidity of the material is determined by both density and compressibility but the contribution of the compressibility is in the majority of standard samples the leading factor [214]. Ultrasonic velocity is extremely sensitive to the molecular organization and intermolecular interactions in the medium. The (ultra)sound velocity U is simple function of the pressure derivative of density [213], see Equation **2**.

$$U^2 = \left(\frac{\partial \rho}{\partial P}\right)_S^{-1} \qquad 2.$$

where ρ stands for density, P for pressure and S is entropy.

Usually, the main reason why to measure the ultrasound velocity in various media is to determine the elastic properties of the sample. For homogenous media, such as aqueous solutions, the main characteristics describing these properties are adiabatic and isothermal compressibility coefficients (β_S and β_T) defined in the Equations **3**–**5**

$$\beta_S = V^{-1}\left(\frac{\partial V}{\partial P}\right)_S \qquad 3.$$

and

$$\beta_T = -V^{-1}\left(\frac{\partial V}{\partial P}\right)_T = \beta_S + \frac{\kappa^2 T}{\rho C_P} \qquad 4.$$

where κ

$$\kappa = V^{-1}\left(\frac{\partial V}{\partial T}\right)_P \qquad 5.$$

is the volume coefficient of thermal expansion and V is volume. The difference between isothermal and adiabatic compressibility for aqueous solutions is small and usually does not exceed a few percent because for water the values of C_P are large and of κ are small, on the contrary. The adiabatic compressibility K_S of a substance is defined according to Equation **6**

$$K_S = -\left(\frac{\partial V}{\partial P}\right)_S = \beta_S V \qquad 6.$$

A similar expression can be written also for the isothermal compressibility K_T. As follows from **1**, adiabatic compressibility is related to sound velocity by a simple relationship **7**

$$\beta_S = \frac{1}{\rho U^2} \qquad 7.$$

Measurements of ultrasound velocity and density are the only direct ways to evaluate the adiabatic compressibility coefficient of a liquid [214].

The modern high resolution ultrasonic spectrometers are commercially available since 2004 when a novel arrangement of ultrasound device based on the resonator method was patented by *Buckin* [215]. Employing this device, which is nowadays produced by Sonas Technologies Ltd., Ireland, it is possible to achieve the resolution of the spectrometer down to 10^{-5} % for ultrasonic velocity and 0.2 % for attenuation measurement. The machine itself consists of two independent measuring cells tempered by common water bath and stirred by electromagnetic stirrers. Ultrasonic velocity is temperature-dependent. Observed changes in measured values are therefore caused both by internal physical-chemical processes and by external fluctuations of temperature. If the second cell as a reference cell is used and is loaded only by the solvent no reactions can occur there. All the changes of measured parameters are therefore caused only by external temperature fluctuations in this cell. Because common water bath secures same temperature in both cells, it will be possible to subtract recorded values (i.e. measuring cell U_1 minus reference cell U_2, further in text denoted as U_{12}) to obtain the values of

ultrasonic velocity and attenuation free of external temperature fluctuations. The scheme of a Sonas HR-US is shown on the **Figure 22**.

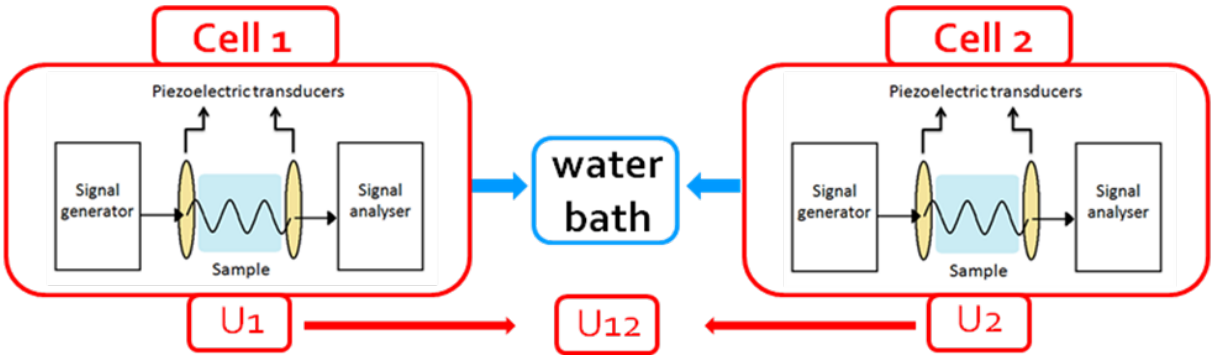

Figure 22: *Scheme of a high resolution ultrasonic spectrometer.*

This kind of arrangement was already utilized on many different fields, especially in food industry, biotechnology and pharmacy. High resolution ultrasonic spectroscopy was employed for characterization of emulsions and dispersions [216], monitoring of heat-induced transitions in biopolymers such as protein denaturation, aggregation and gelation [217], monitoring of crystal formation, kinetics of this reaction, size and amount of crystals [217], studying enzyme activities, reaction mechanisms and kinetic parameters, including inhibition mechanisms and inhibition constants [218], direct real time monitoring of hydrolysis of cellulose [219] and many others.

To author's best knowledge HRUS was employed just several times in the study of humic substances. In the study using lignite humic acids, *Kučerík et al.* [61] stated and confirmed earlier observation about progressive aggregation of humic acids in diluted solutions. Aggregation was noticed also in environment generally considered as unfavorable to aggregation, i.e. at high ionic strength (1 mol L^{-1} NaCl) and at pH as high as 12. Several modifications of humates solutions supported supramolecular theory. In another study, a change in ultrasonic velocity in solutions exposed to a temperature program revealed significant differences in character of hydration at different concentrations [62]. In 2009, *Kučerík et al.* [63] employed HRUS to study progressive aggregation and structural changes in both sodium salts and protonized forms of fulvic acids and sodium salts of humic acids. The standards of the International Humic Substances Society (IHSS) were used to cover wide range of possible sources of HS.

2.3.4. Densitometry Principle and Use

State-of-the-art densitometers are working on the principle of oscillation U-tube. The degassed sample is injected and then flowing in the quartz U-tube and through the reference oscillator and temperature sensor, the information about the resonation frequency of the tube and the measurement temperature are determined, then the sample density is calculated and displayed [220]. The densitometer sketch is to see on **Figure 23**. According to author's best information, employing densitometry in the research of humate solutions is a complete

novelty, although the densitometry is of common use in determining quality of all the branches of chemical industry including food and pharmaceutical production. Densitometry has been used in the research of biopolymers solutions, such as sodium hyaluronate also in combination with ultrasonic spectrometry in order to gain information about the biopolymer hydration [221].

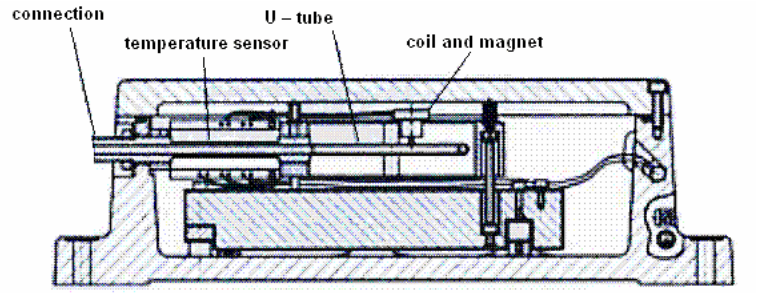

Figure 23: *Oscillation densitometer sketch.*

2.3.5. Hydroponics Root Growth Estimation Principle and Use

The word hydroponics is originating from the Greek words *hydro* (for water) and *ponos* (for labor) and means the method of growing plants using nutrient solutions in water, without soil. Hydroponics is today a well established branch of agriculture industry, utilizing many advantages as high yields, no soil necessity, and enhanced possibility of the plant's nutrition control together with no nutrition pollution runoff to the environment and water recycling and conservation [222]. Hydroponics has got its place not only in industry, but therefore as well in science and several researchers have employed it to research the biological activity of humic substances; either using minimal media or complete nutrition solutions [84],[93],[94]. In contradiction to field tests, the hydroponic experiments are quick, cheap and feasible almost in every laboratory; moreover, their results are not influenced by weather, soil type or other environmental conditions. The plant samples may me suscepted after the harvest to many different analyses, such as measuring, weighing, image analysis, microscopy or determining the hormone or even genetic activity [84],[96].

2.3.6. Brief Image Science Fundamentals

Today's image science is often based on fractals. Fractals (first discovered by *Mandelbrot* [223]) are various scale-invariant objects that can be subdivided in parts, each of which is (at least approximately) a reduced copy of the whole; so the fractals display self-similarity [224]. Fractals are described by means of fractal measure *K* and a noninteger fractal dimension *D*. Fractal measure defines the magnitude of the coverage of space using the elementary cell (e.g. coverage percentage) while the fractal dimension gives us the information about the trend of change of coverage as a function of size of measuring cell, ergo, the *D* measures the degree of fractal boundary fragmentation over multiple scales, so, it determines how the fractal object differs from the Euclidean objects [224],[225]. The main advantage of fractals lies in the ability to describe very complex natural phenomena (e.g. branching of trees, roots or capillaries, structure of cells and microorganisms, clouds, cerebral cortex etc.) by a small set of parameters [224],[226] and so can determine their shape, growth factor, cell aging or colony growth [227].

In conjunction with the recent development in digital imaging technologies, these are finding more and more utilization in the biological and chemical experiments, where the proper quality images for later analysis can be obtained easily using common up-to-date digital cameras and scanners [227],[228]. With advantage, the software HarFa should be used for the sequential analysis of the image. It allows both static and dynamic (video streams) analysis and is usually used to determine the fractal parameters of the analyzed object performing the box counting, wavelet of Fourier transform based methods. HarFa does have an ability to reduce size and threshold images and of a wavelet (Haar) transformation, which is in detail described elsewhere [224],[229]. In a simplified principle, is by this transformation possible to determine the basic structure parameters: the D and K and partially black and white areas D_{BW} and K_{BW} [227],[228].

The HarFa software has been used in many various state-of-the-art research purposes, including biotechnology [227], polymer morphology [230] and neuroscience imaging [231],[232]. Employing HarFa in humics' biological activity research seems to be a novelty.

2.4. STATE-OF-THE-ART SUMMARY

It can be summarized that humic substances are more and more known supramolecular aggregates of organic molecules, ubiquitous in nature as part of salt and fresh waters, soils and coals. Humic acids are the most researched and used part of humic substances; they are extractable by various methods, not only from waters, soils or coals, but recently from composts and various organic wastes too. Humic substances in general and humic acids (or their water-soluble metal salts) in particular have a broad variety of applications, from the promoting of plant growth (affecting the transcriptional and post-transcriptional regulation of enzymes and transporters as well as improving the plant nutrition) and microbial life, soil compacting and carbon sequestration, across ion exchange possibilities, organic contaminants sequestrating and inorganic contaminants sorption, to concrete compacting, polymer improving and textile dyeing. They may be also used as organic synthesis precursors. Humic substances have very many applications in human and animal medicine, however, that lies far beyond the purpose of this treatise. Humics, all around the world, are subjects of business of many companies, of continuous and collateral research and also of thousands of publications and patents. For recently introduced contaminants, humic substances are interesting materials to study the interactions and adsorption (therefore removal) possibilities.

This review does not attempt to be the complete summary of all works and patents about humic acids, but only the treatise made from the most interesting works from the author's point of view and for the purpose of this thesis and the collateral research in the author's laboratory and several cooperating institutions.

3. GOAL OF WORK

The first goal of this doctoral thesis is production of a set of humic acids originating from parental and modified South Moravian lignite. The modification is aimed 1) to increase the yield of humic acids in lignite [43], 2) to influence the chemical and physical character of produced humic matter to obtain the materials with the wide range of properties and 3) to confirm and extend recent results reported in a pilot study dealing with similar subject in the work of *Vlčková* [200],[233].

At first, as a modification mode, the oxidation of parental lignite will be carried out. Oxidation agents of choice will be hydrogen peroxide and nitric acid in a wide range of concentrations. Further, parts of extracted humic acids will be converted into potassium and salts in order to obtain water-soluble samples. Obtained products will be analyzed from several points of view. The stability of these new materials will be assessed by thermal analysis (TA). From the chemical point of view, methods of FTIR will be combined with results from elemental analysis (EA). Structural aspects of humic acid samples in solid state will be investigated by means of FFC NMR Relaxometry.

In case of liquid state analyses, i.e. of potassium humates, the combination of HRUS, DLS, and densitometry is planned. The information about physical structure of humic acids and its relation to their properties published so far is still limited and author of this thesis hopes that extension of traditional approaches and introduction of novelty techniques can bring additional and useful observations regarding this issue. Obtained characteristics, such as state of aggregation, hydration conditions and nature of supramolecular conformation will be correlated with the biological activity and various applicabilities of humates. Author's assumption about those connections is supported by findings of several authors, e.g. *Piccolo* [26], *Canellas et al.* [90] and *Vlčková et al.* [200]. Specifically, the number of water molecules determined at chosen concentration will be correlated with aggregate dimensions obtained from DLS. Considerations will be supported by DAD and RID detected HPSEC, while the DAD detector serves for the high resolution of UV–VIS detection of light absorbing moieties in the humic samples. Final conclusions will be done with respect to the chemical characteristics gained from FTIR and EA, i.e. the efficiency of the pre-treatment of parental lignite with respect to the concentration and character of oxidant and physico-chemical properties of obtained humic acids.

The biological activity of samples will be tested with respect to the root growth and division. Therefore a simple hydroponic method will be derived for the easy use in almost every laboratory. For a proper evaluation of root characteristics image science will be employed. Since the correlation of biological activity and physico-chemical properties of humates (such as hydration, aggregate size or molar weight) is still a discussed novelty, the experimental data will be combined and following scheme will be solved. This represents the added value of humates application on natural systems and it is a first step to design a future regenerated humic crop stimulant / soil conditioner based on lignite humic acids.

Enclosing this thesis, some pilot studies will be indicated about new environmental potential of humic materials. The first one is aimed to check the possible application of humics as a

sorbent for tetracycline antimicrobial (which is a collateral contaminant of growing importance) **********.

For easier understanding of the aim of this thesis, two figures are attached. **Figure 24** [234] with the overall synergistic view on the humic substances research where the combination of physical characterization, chemical analyses and biological activity assessment is applied in order to shed light on the newly prepared regenerated humic materials (lignite, acids and salts) properties and potentials. It stresses out the importance of combination of different approaches, i.e. analytical chemistry brings the information about primary structure which has an influence (still unknown) on both physical structure and on biological activity (partially known) and other properties. Humic substances chemistry is a chemistry of mixtures and non-additive principles are expected. The flowchart (**Figure 25)** then designs the scheme of this thesis work plan, from oxidation of lignite and pilot studies of applicability of the regenerated lignite product, to the humic acid extraction and titration with the deep characterization of the acids and salts together with correlation of the physico-chemical results with the biological activity of humates.

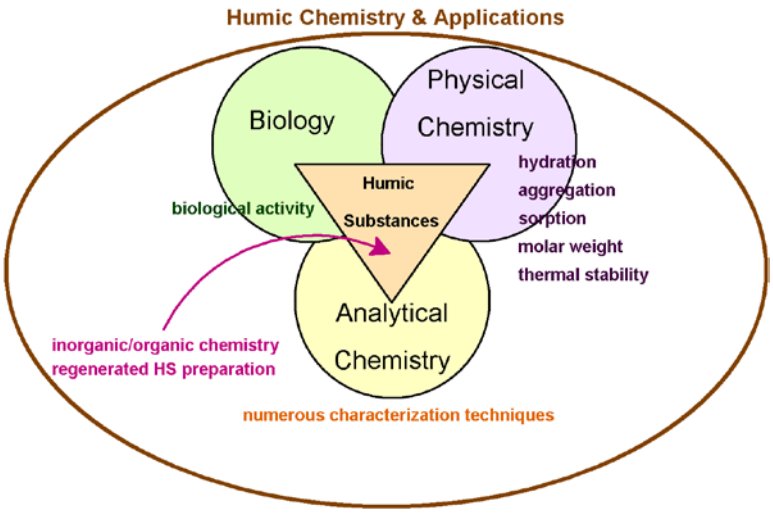

Figure 24: *Synergistic view of this thesis on humic substances chemistry as proposed by Kučerík [234].*

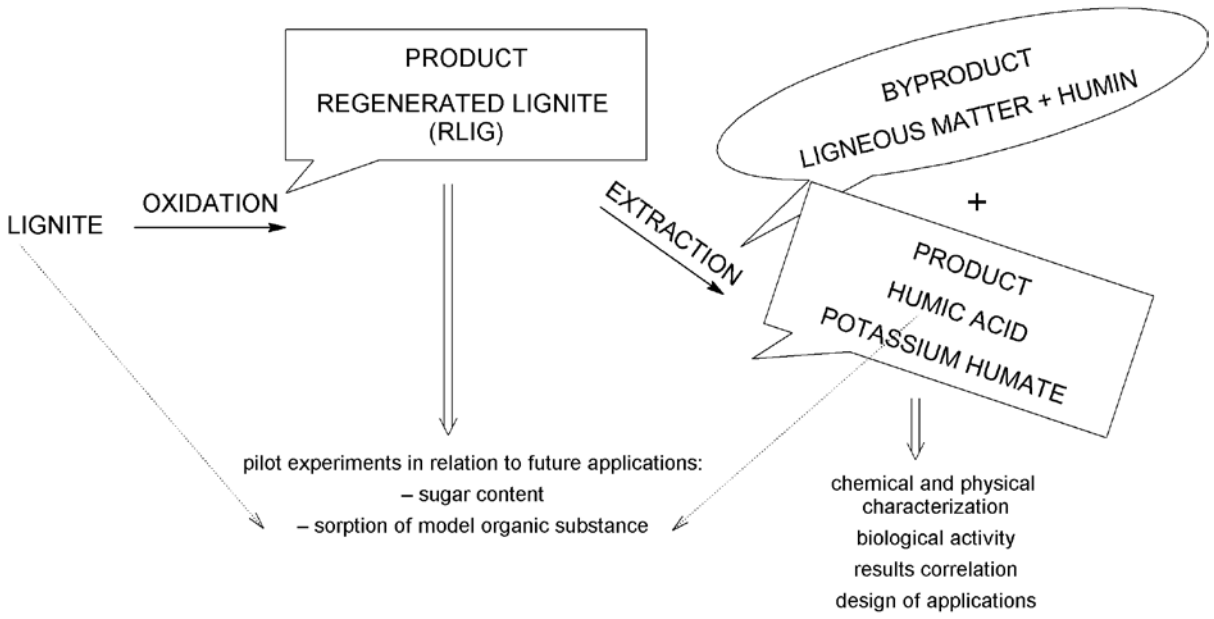

Figure 25: *Flowchart of this thesis.*

In this work, two oxidant agents were used such as nitric acid and hydrogen peroxide. Generally speaking, the oxidation can be carried out by a number of oxidation agents, but in this study, the author would like to continue and to extend the work of *Vlčková* [233] in which *Vlčková* used two different approaches in order to modify parental lignite and extract humic acids of variable "quality" and composition. One of those approaches was aimed to influence mainly the physical structure (organic acids), second one to change the chemical structure. It was demonstrated that chemical composition of extracted humic substances did not differ significantly but their physical structure, as well as their biological activity was successfully altered. Therefore, in this work, we would like to continue one of those approaches, i.e. the oxidative transformation of lignite and focus more in detail on the investigation of physical structure of obtained products with respect to humics applicability.

4. EXPERIMENTAL PART

4.1. SAMPLES PREPARATION

4.1.1. Lignite Regeneration

South Moravian lignite (mine Mír, Lignit Hodonín Ltd., Mikulčice, Czech Republic) was regenerated by means of two oxidizing agents – nitric acid (LachNer Ltd., Neratovice, Czech Republic) and hydrogen peroxide (same producer) in the concentrations of 10, 20, 30, 40, 50, 65 vol% and 5, 10, 20, 30 vol% respectively. Lignite to agent ratio was 1:10 w/w (50 g of parental lignite in 500 mL of regeneration agent). Lignite was regenerated for 30 minutes at the temperature around 30°C in the glass beaker. For the 50 and 65 vol% nitric acid the round bottom flask with reflux and bottom cooling bath was used, since these regeneration reactions were strongly exothermic. The mixture was suction filtered and the filtrate (RFILT) was treated similarly as a fulvic acid fraction, see section 5.1.3. Small amount of wet regenerated lignite (RLIG) (ca. 10 – 12 g) was left to dry on atmosphere and stored in poly(propylene) containers. The rest was used for humic acid extraction. For schematic representation, see **Figure 26**, where the procedure is cartooned then see the actual photo of the lignite regeneration and humic acid dialysis (**Figure 27**).

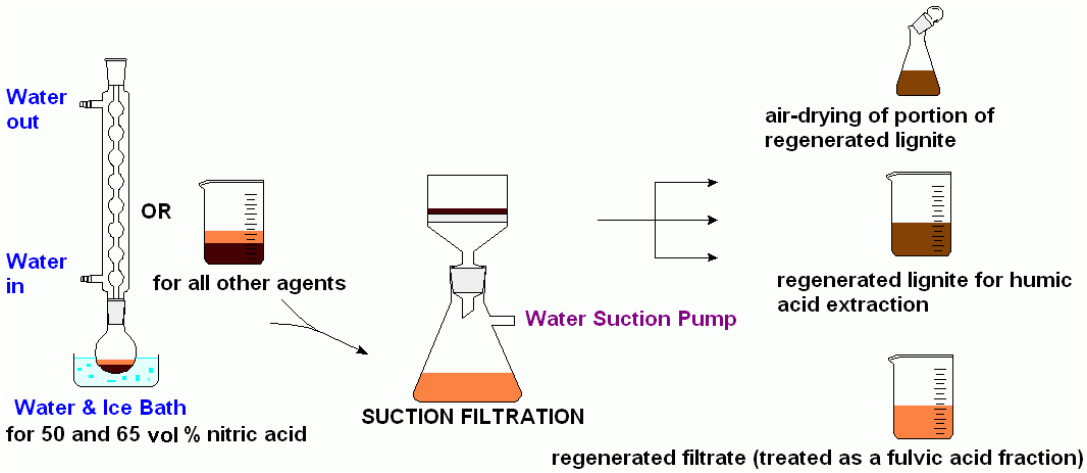

Figure 26: *Lignite regeneration cartoon.*

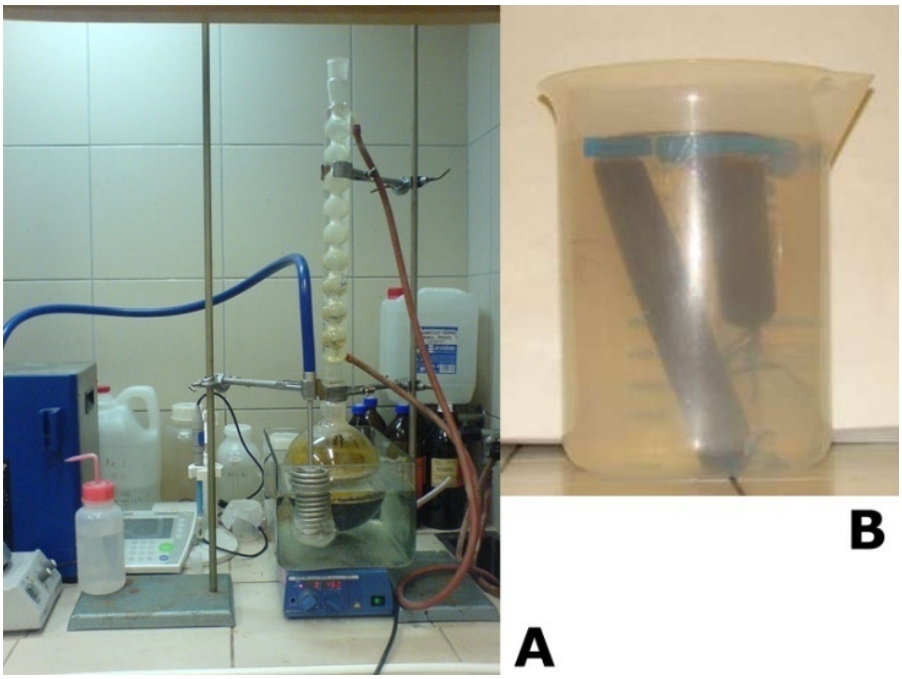

Figure 27: *Lignite regeneration (by 65 vol% HNO_3)(A) and HA dialysis (B) photos.*

4.1.2. Humic Acid Extraction

Humic acids were isolated from parental or regenerated lignite employing the slightly modified procedure described by *Swift* [34]. Shortly, the humic matter was extracted by mixing lignite with 0.5 mol L^{-1} sodium hydroxide (LachNer Ltd., Neratovice, Czech Republic) and 0.1 mol L^{-1} sodium pyrophosphate (SigmaAldrich Co., Steinheim, Germany) extraction agent for 2 hours at 40°C in the glass beaker. The lignite to agent ratio was 0.8–1 : 10 w/w (40 g of regenerated lignite or 50 g of parental lignite and 500 mL of the extraction agent). The resulting mixture was centrifuged for 15 min at 15°C and 4 000 rpm using the Hettich Rotina 46 R centrifuge (Andreas Hettich Ltd., Tuttingen, Germany) and 250 mL Nalgene poly(propylene-copolymer) bottles (Thermo Fisher, Rochester, NY, U.S.A.). The solid part (humin and rests of wooden non-extractable matrix) was separated as waste while the liquid part was considered as the mixture of humic and fulvic acids and acidified via concentrated hydrochloric acid (Penta Ltd., Chrudim, Czech Republic) solution to pH ca. 1, then centrifuged again, the liquid part (fulvic acids) was removed. The inorganic residues in the blend were dissolved by adding the 5 vol% hydrofluoric acid (LachNer Ltd., Neratovice, Czech Republic) in the mixture with concentrated HCl. The whole system was shaken overnight in the poly(ethylene) container employing the Kavalier LT 2 shaker (Kavalier Inc., Sázava, Czech Republic) at mid speed. The blend was centrifuged again, the liquid part was separated and the solid part was remit to dialysis against deionized water in the SpectraPor 1 000 g mol^{-1} cutoff dialysis membranes made from regenerated cellulose (Spectrum Labs Inc., Rancho Dominguez, CA, U.S.A.) until no chloride anions were present in the dialysis water according to the simple drop test using 0.1 mol L^{-1} solution of $AgNO_3$. After dialysis, the humic acid was portioned onto 2 parts, the humic acid (HA and RHA samples) and solution for titration, which was titrated overnight with solution of 0.5 mol L^{-1} potassium hydroxide (LachNer Ltd., Neratovice, Czech Republic) to static pH 7.2, exploiting the Schott TitroLine alpha Plus automated titrator (Schott Inc., Mainz, Germany) (KHA and KRHA

samples). All the solutions were slowly and deeply frozen and freeze dried by means of the Labconco Freezone 4.5 freeze dryer at –50°C and 120–140 mPa (Labconco Corp., Kansas City, MO, U.S.A.) equipped with the Vacuubrand RZ 6 rotary vacuum pump (Vacuubrand Ltd., Wertheim, Germany). Obtained products were crushed in the agate mortar, weighted and stored in sealed vials in dry and dark place. The extraction of humic acids is cartooned on the **Figure 28** together with the regenerated filtrate and fulvic acid treatment scheme. The HA dialysis is to be seen on the **Figure 27**.

4.1.3. Regenerated Filtrate and Fulvic Acid Treatment

This experiment was mainly done with the aim to evaluate the possibility of leaching some portion of humic material into the regeneration agent itself (the RFILT experiments) and the possibility of extraction of fulvic acids from regenerated lignites (which is being discussed to be generally higher, than from the parental lignite, where almost none fulvic acids had been found).

Regenerated filtrate and fulvic acid fractions were purified by means of Amberlite XAD-8 adsorbent resin (Sigma Aldrich Co., Steinheim, Germany) pre-treated according to user's manual (wetted by methanol and washed by deionized water) in a glass column of 3.1 cm diameter. This purification was again performed employing the slightly modified procedure described by Swift [34]. The column packing was made of silane-treated glass wool (Sigma Aldrich Co., Steinheim, Germany) and the Amberlite XAD-8 resin. The bed volume of resin was 0.1 L, so about of 13.5 cm of column height (when the total column volume was 0.5 L). After filling, the column was backwashed with tap water and carefully rinsed by deionized water again.

The column was loaded according to the XAD-8 user's manual and the published Swift's procedure [34] – i.e. 15 bed volumes h^{-1}. The first complete run-through was loaded into the column again while the liquid from the second run was finally discarded. The adsorbed fulvic material was elued using 0.5 L of 0.5 mol L^{-1} solution of sodium hydroxide, carefully, with the speed of 1 – 2 bed volumes h^{-1} and then submitted to 7 days dialysis against deionized water, freezed and freeze-dried (same conditions). For better understanding of the process, see the **Figure 28**.

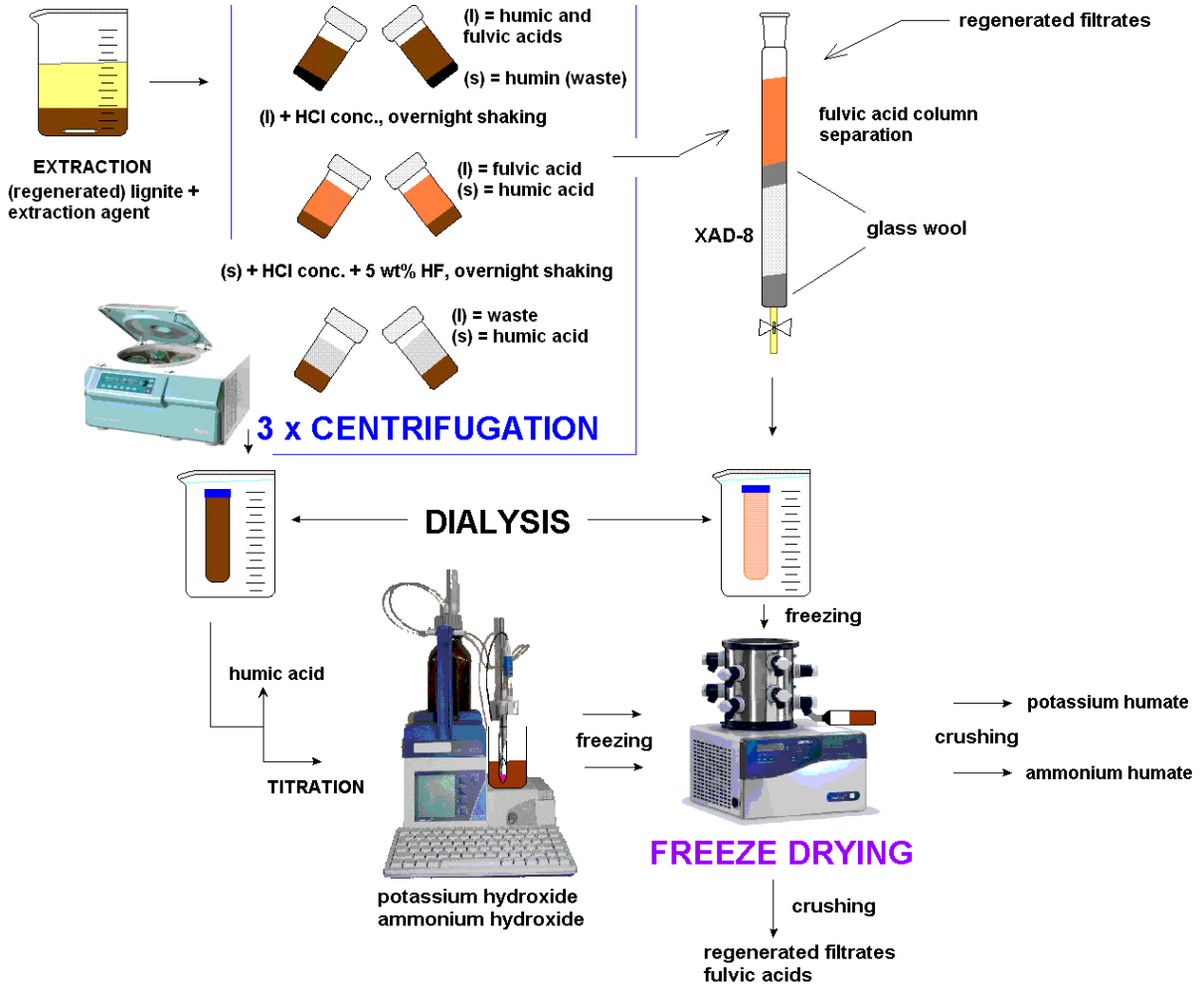

Figure 28: *Humic acid extraction, humate preparation and fulvic acid and regenerated filtrate preparation cartoon.*

4.2. PHYSICO-CHEMICAL CHARACTERIZATION

4.2.1. Elemental Analysis

Elemental analysis was conducted for the HA and RHA samples at the Engineering Test Institute Brno employing the Perkin Elmer 2400 CHNS/O Elemental Analyzer. The oxygen amount was calculated after the humidity subtraction. The purpose of this experiment was to assess the influence of regeneration onto the elemental composition and aliphatic/aromatic carbon ratio of the prepared humic acids.

4.2.2. Thermogravimetric Analysis

HA, RHA and RFILT samples were let to dry in desiccator (sodium hydroxide used as a desiccant) for two weeks, then remitted to thermo-oxidative degradation using TA Instruments Q5000 IR High Resolution Thermal Analyzer (TA Instruments Inc., New Castle, DE, U.S.A.) with 100 µL platinum reusable pans. The analysis was set up as a 10°C min^{-1} temperature ramp from room temperature (RT) to 650°C under 50 mL min^{-1} dried air flux. The pans with dosed samples on the autosampler carousel of the Q5000 are photographed on the **Figure 29**.

Figure 29: *Humic acids (HA)(1–5) and filtrates (RFILT)(6–8) samples on the Q5000 autosampler carousel in Pt pans.*

4.2.3. Fourier Transform Infra-Red Spectrometry

The infrared spectra were collected by the common potassium bromide pellet method, where 1 mg of oven dried (105°C, 3 hours) sample of humic acids (HA and RHA) was mixed with 200 mg of dried KBr (FTIR grade, SigmaAldrich Co., Steinheim, Germany). The pellets were pressed in the Trystom H compactor (Trystom Ltd., Olomouc, Czech Republic). For the spectra collections, the Thermo Nicolet iS10 infrared spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) was employed, the cell was streamed by 7 L min^{-1} of dried air and the machine was set up to resolution of 8 and 64 scans per analysis. The spectra were assessed in the Nicolet Omnic 8 software using the Atmospheric Suppression correction, Automatic Baseline correction and %Transmittance recalculation functions. The important peaks were highlighted by means of the Find peaks function set up to the 85 % of sensitivity. Further, the spectra were elaborated in the Microsoft Excel and OriginLab softwares.

4.2.4. FFC NMR Relaxometry

Proton longitudinal (T_1) relaxation times were evaluated by using a Stelar Spinmaster-FFC-2000 Fast-Field-Cycling (FFC) Relaxometer (Stelar Ltd., Mede, PV – Italy) at a constant temperature of 293 K. Field-switching time was set at 3 ms, while spectrometer dead time was 15 μs. The longitudinal magnetization evolution was recorded at a relaxation magnetic field (B_{RLX}) value of 0.5T, corresponding to a proton Larmor (ω_L) frequency of 20 MHz. The NMR signal was acquired with 8 scans for 32 linearly spaced time sets, each of which was adjusted at every relaxation field to optimize the sampling of the decay/recovery curves. Within the experimental error, all the decay/recovery curves of longitudinal magnetization were exponential. Free induction decays (FIDE) were recorded following a single ^1H 90° pulse applied at an acquisition field (B_{ACQ}) of 0.38T, corresponding to ω_L of 16.2 MHz. A time domain of 100 μs sampled with 512 points was also applied. The decay/recovery curves at each B_{RLX} value (i.e. ^1H signal intensity-vs-τ) were fitted by using 1st order exponential decay/recovery function after exportation of the experimental data to OriginPro 7.5 SR6 (Version 7.5885, OriginLab Corporation, Northampton, MA, USA).

4.2.5. Dynamic Light Scattering

The 250 mg L^{-1} samples of potassium humates (KRHA) were prepared using MilliQ water; according to the counts per second (cps) range ($5\ 10^4$–$1\ 10^6$) determined by the machine,

which was Coulter N4 Plus Submicron Particle Sizer equipped with He-Ne red laser of wavelength of 632.8 nm (Coulter Corp., Miami, FL, U.S.A.). The samples were filtered by means of Amicon 8050 ultrafiltration cell through Millipore GN 0.2 µm filters (both Millipore Corp., Billerica, MA, U.S.A.) and analyzed in 1 cm quartz cuvettes using the Unimodal Analysis Mode, 90° detection angle and 15 min cell temperature equilibration. The measurement was taken for 10 runs of 300 s each. Devious values were excluded according to Dean-Dixon's test [236]. DLS have seen some use in humic acid research before, mainly by *Palmer and von Wandruszka* [60], this experiment is a modified method of their research. Experiments were conducted at 10, 20, 25, 30, 40, 50 and 60°C. Results were evaluated by means of Microsoft Excel software using the standard deviation functions. The machine was calibrated onto the latex beads (Coulter Corp., Miami, FL, U.S.A.). Photo of the machine and Amicon filtration device is shown on the **Figure 30**.

Figure 30: *Amicon filtration device with humic sample (left) and the Coulter N4 Plus Submicron Particle Sizer (right).*

4.2.6. High Performance Size Exclusion Chromatography

The HPSEC experiments were performed according to the work of *Vlčková et al.* [200],[233] in order to distinguish between the humate molecular weight fractions (according to retention time) and their presented absorbance in different wavelengths, which can provide the important information about the molecular/structural composition of our samples. The Dionex Ultimate 3000 Standard Chromatography Station was used (Dionex Inc., Sunnyvale, CA, U.S.A.), embedded with Phenomenex BioSep S2000 600 × 7.8 mm column with Phenomenex BioSep Guard pre-column complemented with 0.2 µm stainless steel inlet filter (Phenomenex Inc., Torrance, CA, U.S.A.). The column was thermostatted at 25°C. The Diode Array Detector was employed in order to gain higher resolution of the UV detection and to shed light on the structure of humic eluates absorbing light at different wavelegths. The whole experiment was set up as follows: 50 mmol L^{-1} solution of NaH_2PO_4 H_2O (Penta Ltd., Chrudim, Czech Republic) in MilliQ water adjusted to pH 7 by means of 1 mol L^{-1} solution of NaOH in order to keep constant ionic strength during the whole analysis to avoid potential ionic exclusion or hydrophobic interactions with the column stationary phase (*Conte and Piccolo* [237]), the mobile phase flow was set at 0.6 mL min^{-1}. Samples were prepared as 0.6 mg mL^{-1} solutions of potassium humates dissolved in mobile phase and 100 µL of the sample was injected.

As standards poly(styrenesulphonates) of 194.2, 145, 32.9, 14.9, 6.53 and 0.91 kg mol^{-1} mass (PSS Polymer Standards Service Ltd., Mainz, Germany) were used as being the most convenient and similar polymers to humic materials. Calibration curve and results were elaborated using the Dionex Chromeleon, Microsoft Excel and OriginLab softwares. As for the results from the Dionex Chromatograph with DAD detector, the M_W and M_N were calculated according the following formulas (**8**,**9**):

$$M_N = \frac{\sum_{i=1}^{N}(h_i M_i)}{\sum_{i=1}^{N}(h_i)} \quad 8.$$

$$M_W = \frac{\sum_{i=1}^{N}(h_i M_i)}{\sum_{i=1}^{N}(h_i M_i^2)} \quad 9.$$

where M_i and h_i are the molecular weight and the height of each i^{th} fraction in the chromatogram respectively [238],[239]. The peak areas were integrated in the OriginLab software. Results obtained from the Agilent Chromatography Station via RID detector were assessed in the attendant software.

The Dionex Chromatography Station is photographed on the **Figure 31**.

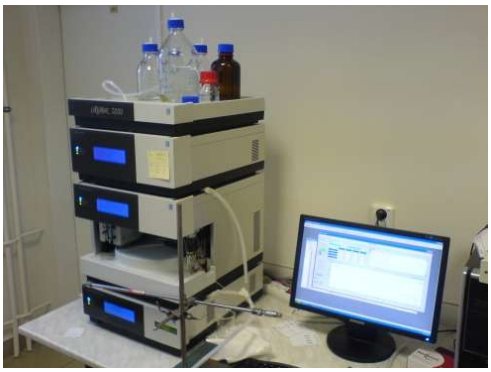

Figure 31: *Dionex Ultimate 3000 Standard Chromatography Station.*

For a comparison, the Agilent 1100 Series Chromatography Station (Agilent Inc., Santa Clara, CA, U.S.A.) equipped with quaternary pump was used, with the very same column and conditions, equipped with the RID detector. Calibration was carried out using polysaccharide standards.

4.2.7. High Resolution Ultrasonic Spectrometry

In this part of the work, a HRUS 102 Ultrasonic Spectrometer (Sonas Technologies Ltd., Dublin, Ireland) was employed. The whole system was carefully thermostatted at 25.00 ± 0.02 °C by means of Thermo Haake C 25 P thermostatic station (Thermo Fisher Scientific Co., Waltham, MA, U.S.A.) The measurements were conducted in two 2 mL independent quartz cells, stirred using bottom (600 rpm) and upper stirrers. Measurements were simultaneously repeated for three different frequencies (5 478, 7 850 and 12 196 kHz) for which the optimal (No. 3, 4 and 7) peaks were found (for a blank sample – the deionized

water). The experiment is based on the determination of the difference of ultrasonic velocities in cell 1 (sample) and cell 2 (water) (the U12 value) and consisted of three steps. At first, 1.00 mL of deionized water was placed in both cells (the A_1 value is found), then 0.25 mL of stock potassium humate solution (1.25 g L^{-1}) is added to the first cell (to obtain a final concentration 250 mg L^{-1} to be able to correlate with the DLS measurements – so the A_2 value is known) and measurement of U12 runs on and on, at last, the humate is added into the second cell (A_3 value is measured). For each step, optimal amount of values (at least 5) are measured and then averaged, see **Figure 32**.

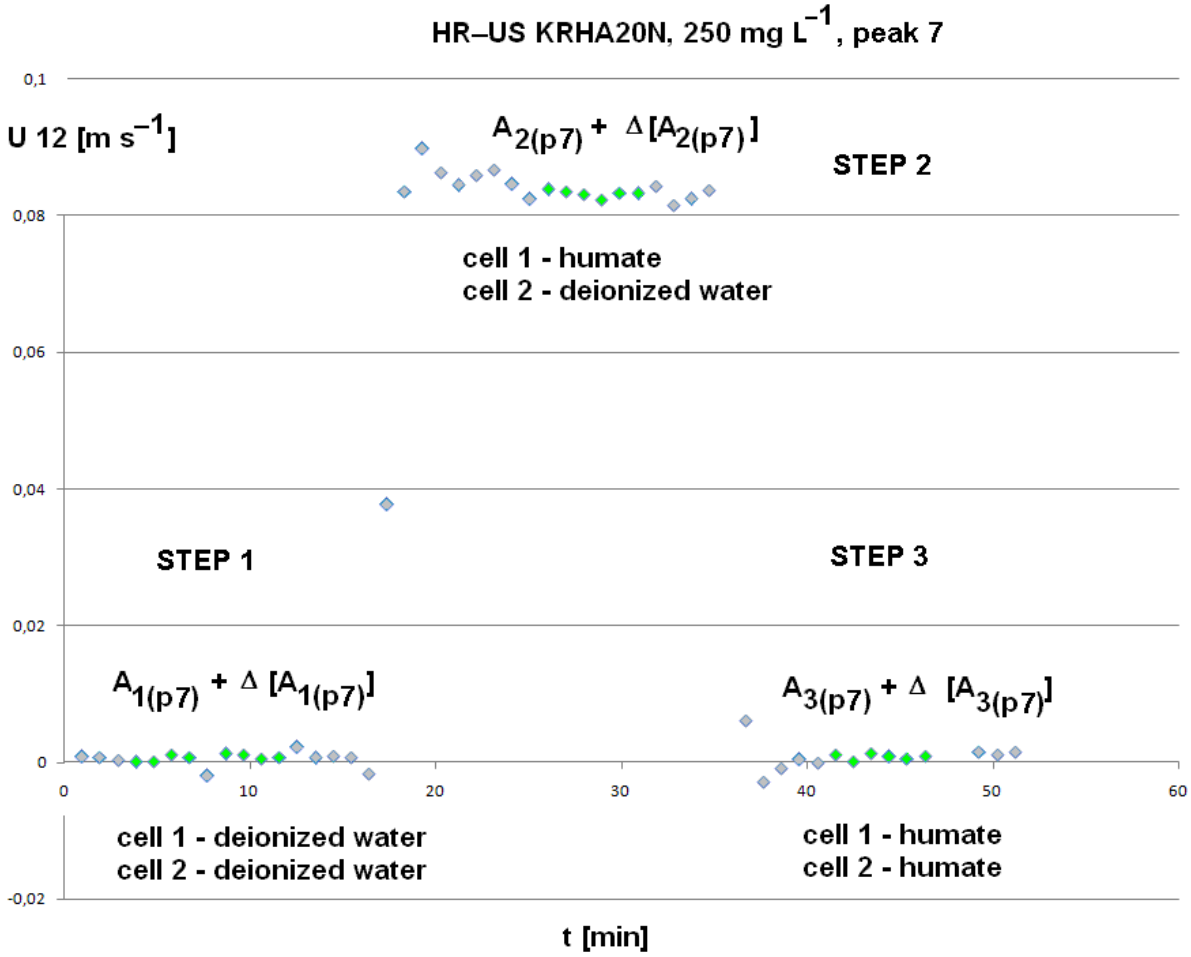

Figure 32: *Principle of HR-US measurement and evaluation – only the green point's values were taken into account (example for 250 mg L^{-1} solution of potassium humate from lignite regenerated with 20 vol% HNO_3, measurement frequency of 12 196 kHz corresponding to peak 7).*

Since from every experiment step, the values are remitted to average, the standard deviation value of the average value is calculated (by means of Microsoft Excel software) This way, three average values of U12 and their standard deviations are obtained (the $A_{step(peak)}$ values), but using the values from all the three frequencies, it makes together nine values. The U12 values from step 1 and step 3 are subtracted from those of step 2 (the higher ones, since in step 2 in cell 1 is a humate solution and in cell 2 the deionized water). The formulas are as following – see **10** and **11**:

$$U_{1A} = A_{2(peak)} - A_{1(peak)} \quad 10.$$

$$U_{2A} = A_{2(peak)} - A_{3(peak)} \quad 11.$$

Resulting two values of ultrasonic velocity (U_{1A} and U_{1B}) are averaged again obtaining the U_{PV} (peak value). All three peak values of U_{PV} are averaged again to become the final average value U_A which is added to a tabeled value of ultrasonic velocity in water (1496.58 m s^{-1}). This way, the statistically correct value of ultrasonic velocity U_1 in 250 mg L^{-1} humate solution is obtained. From the U_1 value, the further magnitudes are calculated according the formulas supra. The errors of these values are calculated like a standard deviation (in the case of average values U12, U_{1A}, U_{1B}, U_{PV} and U_A) in MS Excel software and according to the common formulas for the error calculation [240] in the case of all other calculations. The compressibility is calculated according to the equations **12** – **22**

$$\beta = \frac{1}{\rho U^2} \quad 12.$$

Then the error of compressibility equals

$$\Delta\beta = \frac{1}{\rho U^2}\sqrt{2}\,\frac{\Delta U_1}{4} \quad 13.$$

The error of the compressibilities fraction

$$\left(\beta_F = \frac{\beta_n}{\beta_{n-1}}\right) \quad 14.$$

then equals to

$$\Delta\beta_F = \left(\sqrt{\left[\frac{\Delta\beta_n}{\beta_n}\right]^2 + \left[\frac{\Delta\beta_{n-1}}{\beta_{n-1}}\right]^2}\right)\frac{\beta_n}{\beta_{n-1}} \quad 15.$$

The volume percent of free water and its error are calculated simply

$$V_{FREE} = 100\,\beta_F \quad 16.$$

$$\Delta V_{FREE} = 100\,\Delta\beta_F \quad 17.$$

Volume percent of bound water then

$$V_{BOUND} = 100 - V_{FREE} \quad 18.$$

where the error stays the same.
For the volume of bound water in 1 mL of humate solution stays

$$V_{BOUND1mL} = 0.01\,V_{BOUND} \quad 19.$$

$$\Delta V_{BOUND1mL} = 0.01\,\Delta V_{BOUND} \quad 20.$$

Then hydration of humate in terms of g g^{-1} (grams of bound water per gram of humate) equals to

$$H = \frac{V_{\mathrm{BOUND1mL}}}{0.00025} \quad 21.$$

$$\Delta H = \frac{\Delta V_{\mathrm{BOUND1mL}}}{0.00025} \quad 22.$$

The HR–US 102 Spectrometer is pictured on the **Figure 33**.

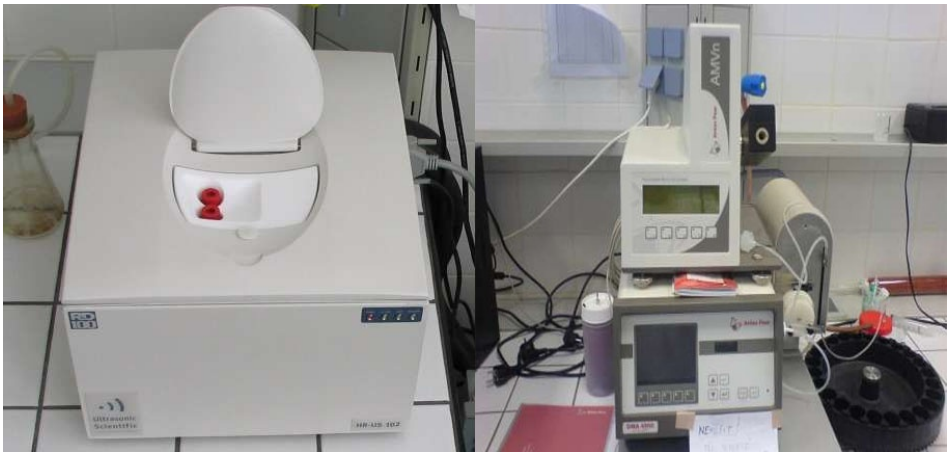

Figure 33: *HR-US 102 Ultrasonic spectrometer (left) and Anton Paar DM A 4500 Density meter (right).*

4.2.8. Densitometry

250 mg L^{-1} solutions of potassium humates were degassed in plastic syringes and injected directly to the clean and dry ultrasonic quartz cell of high sensitive Anton Paar DM A 4500 density meter (Anton Paar Ltd., Graz, Austria). Preliminary to the measurement, the machine was calibrated by means of "density check" function against the blank – deionized water and tempered carefully at 25.00 ± 0.02 °C. The samples in the cell were measured five-times and the results were elaborated in Microsoft Excel software utilizing the standard deviation function again. The used density meter is depicted on the **Figure 33**.

4.2.9. Fluorescence Spectrometry

20 mg L^{-1} solutions of potassium humates were measured in 1 cm quartz cells using Hitachi F-2500 fluorescence spectrometer (Hitachi High Technologies America Ltd., Schaumburg, IL, U.S.A.) in the 240 to 450 nm excitation and 400 to 550 nm emission range at 25°C. Results were assessed as the excitation-emission (EEM) matrices in OriginLab software.

4.3. BIOLOGICAL ACTIVITY OF HUMIC SAMPLES

4.3.1. Plants Growth Experiment

Dressed corns of the common maize (i.e. corn), *Zea mays* CEKLAD 235 species (Oseva Bzenec Ltd., Czech Republic) were selected for their universality and wide spread use for kernel and silage purposes as well as easy availability and top quality (high durability and germinative percent). The experiment is a partial combination of methods previously published by *Zandonadi and Canellas et al.* [84] and *Antošová et al.* [94] and of our modification of these.

4.3.1.1. Seed Germination

The corns were treated for 5 min in the 0.05 mol L^{-1} solution of sodium hypochlorite (NaClO) then carefully washed in deionized water and let to stay in for 4 hours in order to partially wash the dressing and precondition for germination. Germination phase was conducted in wet Fisher Scientific Tork Wiper 430 paper laboratory towels (Thermo Fisher Scientific Ltd., Pardubice, Czech Republic), where the corns were rolled in approx 3 cm gaps. The paper rolls with seeds were put in a glass beaker with certain amount of deionized water at the bottom and let to germinate for 2 days at dark and 28 ± 2 °C employing the BT-120 Biological Thermostat (Laboratorní přístroje Praha Ltd., Prague, Czech Republic).

4.3.1.2. Root Growth

Selected germs (2 to 4 cm) were planted in the poly(styrene) containers onto the foamed poly(styrene) floating beds – 30 germs in every container (they were later observed for mass increment) 5 of them were placed in marked positions on the floating bed (they were later observed for root length and division). In every container was poured 1 L of solution: as both control and sample medium 2 mmol L^{-1} solution of $CaCl_2$ was chosen (for good nutrient properties herewith minimum risk of synergistic activity along with humates on the plant growth [84]). For the comparison of efficacy, the commercial nitrophenolate fertilizer AtonikPro (Arysta LifeScience, Czech Republic) of 0.4 vol% in $CaCl_2$ was tested. The potassium humate were dissolved in $CaCl_2$ in predicted optimum concentration 40 mg L^{-1} [94]. The combinations of AtonikPro+KRHA10N and AtonikPro+KRHA30P were tested as well in the mentioned medium and concentrations.

The growth was conducted in the BT 120 device for 5 days at 25 ± 2 °C every 12 hours at daylight Dennerle NanoLight 9 W lamp, 600 lumen, placed 20–30 cm from plants which was inducting the light intensity about 2500–1500 lux (Dennerle Ltd., Vinningen, Germany) and at every 12 hours at dark. The automated switching was managed by means EMOS 24H switching socket (Emos Ltd., Přerov, Czech Republic).

All the containers were constantly and moderately aerated by means of Sera Precision aquarium pump of 4 W power and 275 L $hour^{-1}$ maximum flow (Sera Ltd., Hainsberg, Germany). All the experiments were performed twice.

The plants growth experiment photos are shown on the **Figure 34**.

Figure 34: *PLANT GROWTH EXPERIMENT PHOTOS: Seed germination (A), corn germs (B), seeding (with 5 marked positions)(C), setting the aerating apparature (D), the biological thermostat BT-120 (E), harvesting the seedlings (F).*

4.3.2. Plants Growth Assessment

4.3.2.1. Manual Assessment

The seeded germs and later the grown 30 plants were carefully weighted and the difference was calculated as a total mass increment. The roots of five marked germs/plants were carefully measured before and after the growing experiment, differences were calculated as a root growth increment.

4.3.2.2. Image Analysis

The five roots of five marked plants were scanned in the plastic foils employing the Epson Perfection 2480 Photo Scanner (Epson Deutschland Ltd., Meersbuch, Germany) against black paper background. Resulting images (300×300 dpi; 2424×3426 pix and 24 bits pix^{-1}) were loaded by means of the HARFA (HARmonic and Fractal image Analyzer) software (http://www.fch.vutbr.cz/lectures/imagesci/), where they were trimmed into desired square images (300×300 dpi; 2048×2048 pix×24 bits pix^{-1}) and saved as bitmaps. Then they were suscepted to 2D Wavelet Analysis function with black and white thresholding set at the value of 160. The *K*[BW] value represents the number of pixels on the black and white border, therefore the value stands for the measure of root division [241].

4.3.2.3. Sugar and Protein Content of the Experimental Maize Seedlings

After the analyses, the whole 5 day *Zea mays* seedlings were dried in the laboratory dryer for three days at 60°C (which is a common method for research of the plant dry matter). The total dry mass was divided for the determination of sugars and protein and both parts were carefully weighted.

Sugar content was determined polarimetrically according to the common *Ewers* polarimetric method. The insoluble starch is transferred by a weak solution of hydrochloric acid onto the soluble and optical active starch solution, which was consequentially analyzed by means of common polarimeter. In short principle, per 5 grams of sample, the 25 mL of 1.124 wt% HCl is used to quantitatively transfer the sample to the 100 mL volumetric flask. The flask was heated for 15 min in boiling water, while it was constantly shaked for 3 min and then shaked occasionally for the rest of the time. After the heating, the content was cooled to 20°C and 30 mL of deionized water was added to the analytical mixture. The mixture was cleared by means of 3 mL of both Carrez I and Carrez II solutions (30 wt% zinc sulfate and 15 wt% potassium hexacyanidoferrate (II) respectively). Finally, the flask is filled with deionized water up to the mark, mixed again and the mixture is filtered through paper filter to a dry beaker, while the first part of filtrate (ca 10 mL) is considered as waste. The clear filtrate was analyzed in the Polartronic E polarimeter (Schmidt+Haensch Co., Berlin, Germany). The sugar content was calculated according to the equation **23** [242]

$$starch\% = 20\frac{100\,\alpha}{\ell\,(\alpha)_{\mathrm{D}}^{20}} \qquad 23.$$

where α is the angle of rotation in degrees, ℓ is the length of polarization tube in dm, and $(\alpha)_{\mathrm{D}}^{20}$ is the specific versatility of starch.

The proteins were determined by a common *Kjeldahl* method [242],[243] for determining of total nitrogen and the result was factorized onto *Zea mays*. An automatic Kjeldahl analyzer FOSS Kjel-Tec^{TM} 2100 (FOSS Inc., Hillerød, Denmark) was used for this determination.

4.4. PILOT STUDIES ON ALTERNATIVE HUMIC APPLICATIONS

4.4.1. Interactions with Tetracycline Antimicrobial

4.4.1.1. Tetracycline Solution Sorption Experiment

As the pilot sorption representatives of humic substances, three samples have been chosen: the dried and milled crude lignite (LIG) (particle size < 0.1 mm), humic acid extracted from parental lignite (HA) and the air dried lignite regenerated with 30 wt% nitric acid (RLIG30N). 0.2 g of sorbents were carefully weighted into the capped 50 mL poly(propylene) centrifugation tubes (Brand Co., Wertheim, Germany) and let to condition for 7 days in 10 mL of deionized water at the Heidolph Reax 2 overhead shaker (Heidolph Co., Schwabach, Germany). Then a 10 mL of tetracycline (>98%, NT, Sigma-Aldrich Co., Steinheim, Germany) solutions in various concentrations were added in order to gain the resulting 20 mL of tetracycline solutions of concentrations of 0.1, 0.5, 1.0, 10.0, 50.0 and 100.0 mg L^{-1}. For each sorbent the blank experiment with deionized water only have been prepared. The tubes were let to shake on the overhead shaker for 48 hours, then the suspension was filtered

manually on the glass funnel with qualitative filter paper cones into plastic capped glass vials and the samples were stored in the refrigerator. The sorption experiment is shown on the **Figure 35**.

A time sorption test was performed using the LIG sorbent and a 100 mg L^{-1} tetracycline concentration, where the samples were filtered and stored after 2, 4, 8, 12, 24 and 48 hours of shaking.

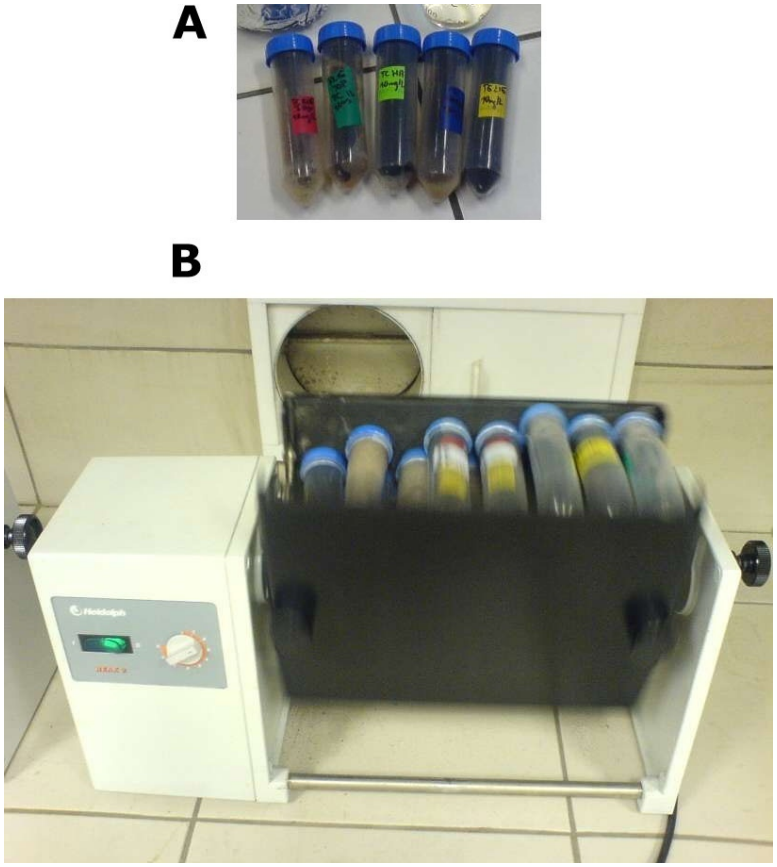

Figure 35: *Tetracycline sorption photos: Particular sorbents with tetracycline solution in tubes (A), Heidolph Reax 2 overhead shaker (B).*

4.4.1.2. Tetracycline Analysis

The analysis has been performed according to *Vítečková* [149] and *Vydrová* [244]. 10 mL of sample was extracted with 10 mL of McIlvaine's buffer (12.9 g of citric acid, 30.2 g of $Na_2HPO_4.12H_2O$, 37.2 g of sodium EDTA together in 1 L of deionized water, mixture pH 3.8). Then the extract was purified and filtered on the SPE (solid phase extraction) columns (Supelco Supelclean ENVI–C18; Sigma Aldrich, Co, Steinheim, Germany) as following: first, every column was conditioned by means of 4 mL of methanol (for HPLC, Sigma Aldrich, Steinheim, Germany) and 3 mL of McIlvaine's buffer. Then, the whole sample (20 mL) was applied onto the column and washed with 3 mL of deionized water, followed by the air drying. Finally, the sample was eluted from the columns into the glass HPLC vials by means of 10 mL of elution agent (20 mmol L^{-1} of oxalic acid in methanol for HPLC).

Concentration of tetracycline in the aqueous solutions after sorption onto the humic substances was determined against external standard using the Agilent 1100 Series Chromatography Station (Agilent Inc., Santa Clara, CA, U.S.A.) equipped with quaternary pump and DAD detector (deuterium and tungsten lamps, 1 024 photodiodes, wavelength range 190–950 nm, slit wide 1–16 mm). The chosen column was the Agilent Zorbax Eclipse XDB–C18, 4.6 x 150 mm.

The gradient analysis has been performed for 23 min and 10 min of washing. Column temperature was set at 20°C, wavelength of detection was chosen at 270 nm and the mobile phase flow was 0.7 mL min^{-1}. The mobile phase gradient is shown in the **Table 5**.

Table 5: Tetracycline HPLC analysis mobile phase gradient

Time [min]	Sulfuric acid (pH = 2.1) [vol%]	Acetonitrile [vol%]
0	90	10
10	80	20
15	70	30
30	60	40

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5. RESULTS AND DISCUSSION

5.1. Sample Yield

In all the cases of lignite regeneration, satisfactory yield was given; the yields are shown in the **Table 6**. The regeneration of parental lignite resulted in higher yields, when performed by means of nitric acid, with the exception of 10 vol% HNO_3. From the hydrogen peroxide regenerated lignites, only the RHA20P sample was richer for a yield, than the humic acid from parental lignite. From the filtrates of the regeneration agent – lignite mixture, only the RFILT65N and RFILT30P yields are of some interest, possibly, the both concentrated regeneration agents are so strong, that they are oxidizing, dissolving or carrying some humic or humic-like matter with itself. The yields of fulvic acids were negligible, so they are not mentioned further.

Table 6: Humic materials yields.

Humics	Yield [g]	Filtrates	Yield [g]
HA	1.783		
RHA10N	1.465	RFILT10N	0.072
RHA20N	3.785	RFILT20N	0.051
RHA30N	3.273	RFILT30N	0.044
RHA40N	2.510	RFILT40N	negligible
RHA50N	4.629	RFILT50N	negligible
RHA65N	4.613	RFILT65N	0.107
RHA5P	1.346	RFILT5P	negligible
RHA10P	1.205	RFILT10P	negligible
RHA20P	1.805	RFILT20P	0.042
RHA30P	0.932	RFILT30P	0.180

5.2. Physical and Chemical Characterization

5.2.1. Elemental Analysis

The results of the elemental analysis of obtained samples are summarized in the **Table 7**.

Table 7: Elemental analysis of HA and RHA samples.

Sample	C [at %]	H [at %]	N [at %]	O [at %]	H/C [1]	N/C [1]	O/C [1]	(N+O)/C [1]
HA	43.53	36.99	0.86	18.62	0.85	0.02	0.43	0.45
RHA10N	40.01	45.81	0.64	13.55	1.15	0.02	0.34	0.35
RHA20N	38.23	42.74	2.12	16.91	1.12	0.06	0.44	0.50
RHA30N	37.89	41.96	2.43	17.73	1.11	0.06	0.47	0.53
RHA40N	38.34	40.92	2.94	17.79	1.07	0.08	0.46	0.54
RHA50N	38.44	41.07	2.66	17.84	1.07	0.07	0.46	0.53
RHA65N	36.66	43.49	2.86	16.98	1.19	0.08	0.46	0.54
RHA5P	41.59	43.43	0.88	14.10	1.04	0.02	0.34	0.36
RHA10P	40.17	44.53	0.78	14.52	1.11	0.02	0.36	0.38
RHA20P	39.24	45.08	0.67	15.02	1.15	0.02	0.38	0.40
RHA30P	38.87	46.60	0.44	14.09	1.20	0.01	0.36	0.37

The most remarkable result of the lignite regeneration is the variation of the nitrogen content of the final humic acids. The N content is rising from the regeneration with 20 vol% to the 40 vol% of nitric acid. Surprisingly, the sample RHA10N performed a lower nitrogen ratio than a humic acid from parental lignite, this could be caused by a fact, that the 10 vol% nitric acid is not enough strong nitrating agent, even if is strong enough to oxidize the lignite. This is a slightly contradictious to the previous results of *Čtvrtníčková et al.* and *Vlčková et al.* [245],[200] where similar result occurred in HA from lignite regenerated by means of 5 vol% HNO_3, but their RHA10N sample was of slightly higher N content than the HA sample. This may support the premise that humics composition can vary even if obtained from the same source and pretreated by the same procedure. Indeed the both previous works' samples were extracted from the lignite of the same source but of different batch. The HA samples made of lignite regenerated with 30, 40, 50 and 65 vol% HNO_3 show a significantly higher amount of nitrogen in their structure, as well as the higher oxygen content and O/C ratio. This, together with increasing H/C ratio, indicates the enhancement of the oxygenous functional groups portion, and potentially also the lowering of the humics aromaticity degree. It may be a consequence of the oxidative attack of the parental lignite followed by the formation of predominantly OH groups; as already stated by *Perminova et al.*, but, these results are in slight contrast with those of Perminova, since in her work the OH groups are supposed to be phenolic [191].

The HAs from H_2O_2 regenerated lignite exhibited lower oxygen content, therefore also the O/C ratio, however the H content and higher H/C ratio suggests the lowering of the aromaticity degree of these samples. In addition, the N content is also lower in this set of HAs.

The N content is rising from the RHA20N to the RHA40N sample; in the RHA50N and RHA65N sample is decreasing again. A possible explanation of this effect is that the increase in concentration of HNO_3 promotes the additional oxidation of other parts of lignite [41]. Another explanation can be done taking into account the relative distribution of N in humic acids vs. their yield during extraction. As reported in **Table 6**, 50 and 65 vol% of HNO_3 showed significantly higher yield of extractable part. It means that in total the extracted part contained larger content of N which was however distributed in a different way as in samples extracted from lignite pretreated by low concentrated nitric acid. While using air as an oxidizing agent for lignite, at elevated temperatures and long time interval, similar observations were reported by *Kučerík et al.* [43]. The H content rises with the regeneration agent concentration from RHA5P to the RHA30P sample.

Regenerating of lignite with HNO_3 may have the beneficial effect on the resulting lignitic humic substances in the nitrogen enrichment, which is usually consistent with a higher degree of humification [246] but simultaneously introducing a higher content of aliphatics, which corresponds to the shorter decomposition time of humic precursors [246]. So, with one agent, it is possible to enhance the quality of lignitic humic substance twice. By a simple comparison with well known IHSS humic standards, the samples from the nitric acid regenerated lignite are at least according to N content reminiscent to more to the HA Elliot Soil than to the Leonardite HA [247].

5.2.2. Thermogravimetry

Since thermogravimetric analysis (TGA) is one of the basic characteristics of every newly prepared material, the regenerated humic acids were throughfully characterized and their TGA results were compared to the humic acid from parental lignite (HA). All the samples presented a standard thermo-oxidative decomposition comparable to lignitic humic substances, performing a little bit indistinct degradation onsets in the interval ranging from 134 to 204°C. The onsets were evaluated from the first derivatives of the thermo-oxidative curves and considered as the measures of the samples' overall thermo-oxidative stability. This was found rather lower for the HAs from regenerated lignites, with the exception of RHA30P (where the value stayed almost the same as for HA) and RHA10N (where the stability is slightly increased, which may correspond to the elemental analysis result, that the 10 vol% nitric acid is an agent not strong agent enough to both nitrate and oxidize the same lignite and therefore to modify the structure of the resulting humic). The samples showed the expected low values of humidity (from about 3 to 6 wt%) both with a low ash content (from about 0.3 to 0.9 wt%). Thus the thermo-oxidative stability of the regenerated humics was found lower, it has no impact or limitation onto their designed future applications in agriculture, environmental protection, and many others fields. All the thermogravimetric results are summarized in the **Table 8** and the thermo-oxidative curves with the clearly visible trend of their first derivatives (DTG curves) are to be seen at the **Figure 36**. It is clearly visible on the DTG curves, that the RHA_N samples perform faster and steeper beginning of thermo-oxidative degradation, than the HA from parental lignite and the RHA_P samples. The RHA_P samples also exhibit slightly steeper beginning of degradation, than the unmodified HA. The first peak of the DTG curve ranging from 150 to 350°C was steeper and of greater area by the RHA samples. This gives the evidence about the enhanced amount aliphatic structures, which are generally less thermo-oxidative stable than aromatics [248],[249]. The

mentioned aromatic systems degradations are represented by the second, high and narrow DTG peak (450 – 550°C). The lower thermo-oxidative stability of the regenerated humics is in agreement with the results of *Gonet and Cieslewitz*, who found significantly negative correlations between the H/C and N/C ratios (both enhanced in the RHA samples) and the thermal stability [250].

Table 8: Thermogravimetry results.

sample	humidity [wt%]	1st onset [°C]	ash [wt%]
HA	5.8	202.8	0.9
RHA10N	5.4	226.6	0.5
RHA20N	5.8	134.8	0.3
RHA30N	5.8	162.4	0.4
RHA40N	4.3	153.1	0.4
RHA50N	4.9	152.6	0.5
RHA65N	5.2	169.7	0.5
RHA5P	6.2	200.1	0.3
RHA10P	6.2	192.2	0.3
RHA20P	4.7	169.8	0.3
RHA30P	3.7	204.0	0.3

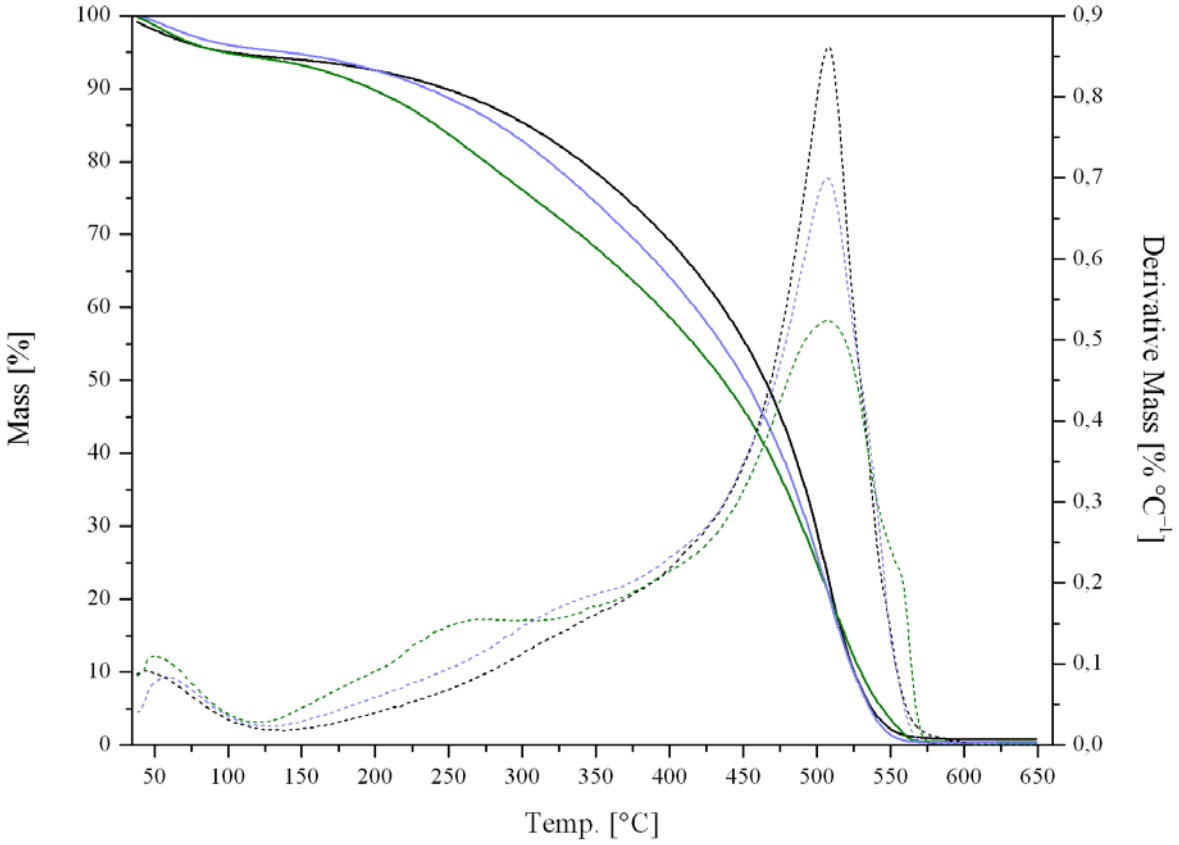


Figure 36: *TGA (solid) and DTG (dashed) curves of HA (black), RHA30N (green) and RHA20P (blue) humic acids – the earlier and quicker degradation of the regenerated samples is clearly visible.*

5.2.3. Fourier Transform Infra-red Spectrometry

FTIR spectroscopy data supported the results from elemental analysis. Obtained vibration spectra of all the samples (HA and RHAs) are comparable to the spectra of humic acids published by many authors before. All the samples performed the ordinary humic substance peaks, like the broad 3400–3200 cm^{-1} (hydrogen bonded OH) and the 2920–2900 cm^{-1} (aliphatic C–H stretching). Sharp peaks have been detected around 1720–1710 $cm^{-}1$ for the C=O of COOH and esters and 1620–1610 cm^{-1} for C=O stretching too, but this was mainly the moiety of COO^{-}, ketonic C=O and aromatic C=C conjugated with COO^{-}. The 1280–1200 cm^{-1} peak signifies the presence of the aromatic rings and aromatic C–stretch. In the RHA samples from lignite regenerated with HNO_3, the aromatic–NH–R moieties may also absorb in this region as well as the aromatic–R–COOR structures in the samples RHA20P and RHA30P; in both the regeneration samples cases are the peaks shifted nearer to the 1280 cm^{-1} value.

As for confirmations, the presence of aliphatic C–H stretching is validated by the 1430–1420 cm^{-1} peak, the OH groups are confirmed by the 1032 cm^{-1} peak and aromatic structures are validated by peaks in the regions of 1510 cm^{-1} and 770–765 cm^{-1}, the COO^{-} groups then by 1376–1369 cm^{-1} peak, however, the 1032 cm^{-1} and 1376–1396 cm^{-1} peaks are not present in the spectra of the regenerated samples, mainly in the RHA30N – RHA65N.

The incorporating of nitrogen atoms into the humic structure by the HNO_3 lignite regeneration is not only reflected by the results of elemental analysis, but it can be also seen in the FTIR spectra, where the 1540–1520 cm^{-1} peaks of Ar–NH/NO_2 are present (RHA20N–RHA65N samples) and the strong oxidative and nitration effect of concentrated HNO_3 is confirmed here by the 1333 cm^{-1} peak of CO–NH and NO_2 (RHA40N–RHA65N samples). In the RHA20P and RHA30P samples, the 1269 cm^{-1} peak of aromatic/R–COOR moieties is to be seen. The infrared spectra of HA, RHA30N and RHA20P are shown on the **Figure 37**.

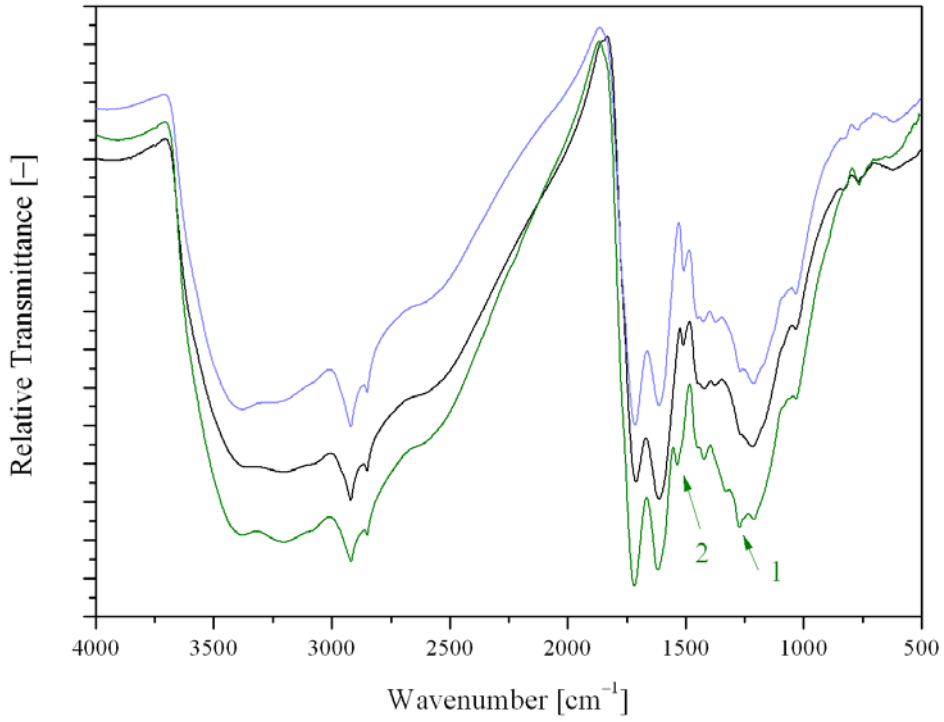


Figure 37: *FTIR spectra of HA (black), RHA30N (green) and RHA20P (blue). The Ar–NH–R peak (1) and Ar, –NH, –NO_2 peak (2) are marked on the RHA30N spectrum.*

To distinguish the differences between the HA and the RHA samples, the ratio of relative intensities of specific absorption bands (2930/1610) cm^{-1}; i.e. the aliphatics vs. the aromatics. Moreover, to assess the lignite modification by means of HNO_3, ratio of relative intensities of the absorption bands (1550–1520 – amidic/1610) cm^{-1} was plotted too. The reason of using of those absorption bands is in the mutual connection and their importance in humic structure as recently demonstrated by *Tandler* [251]. Obtained results are given in the **Figure 38**. All the regenerated samples present slightly higher aliphatic content over aromatic systems when compared to the HA sample from parental lignite. The highest ratio can be seen by the sample RHA40N, followed by the samples RHA65N, RHA50N, RHA20N and RHA5P. The lowest aliphatic content (but still higher than the HA sample) showed the RHA30P sample.

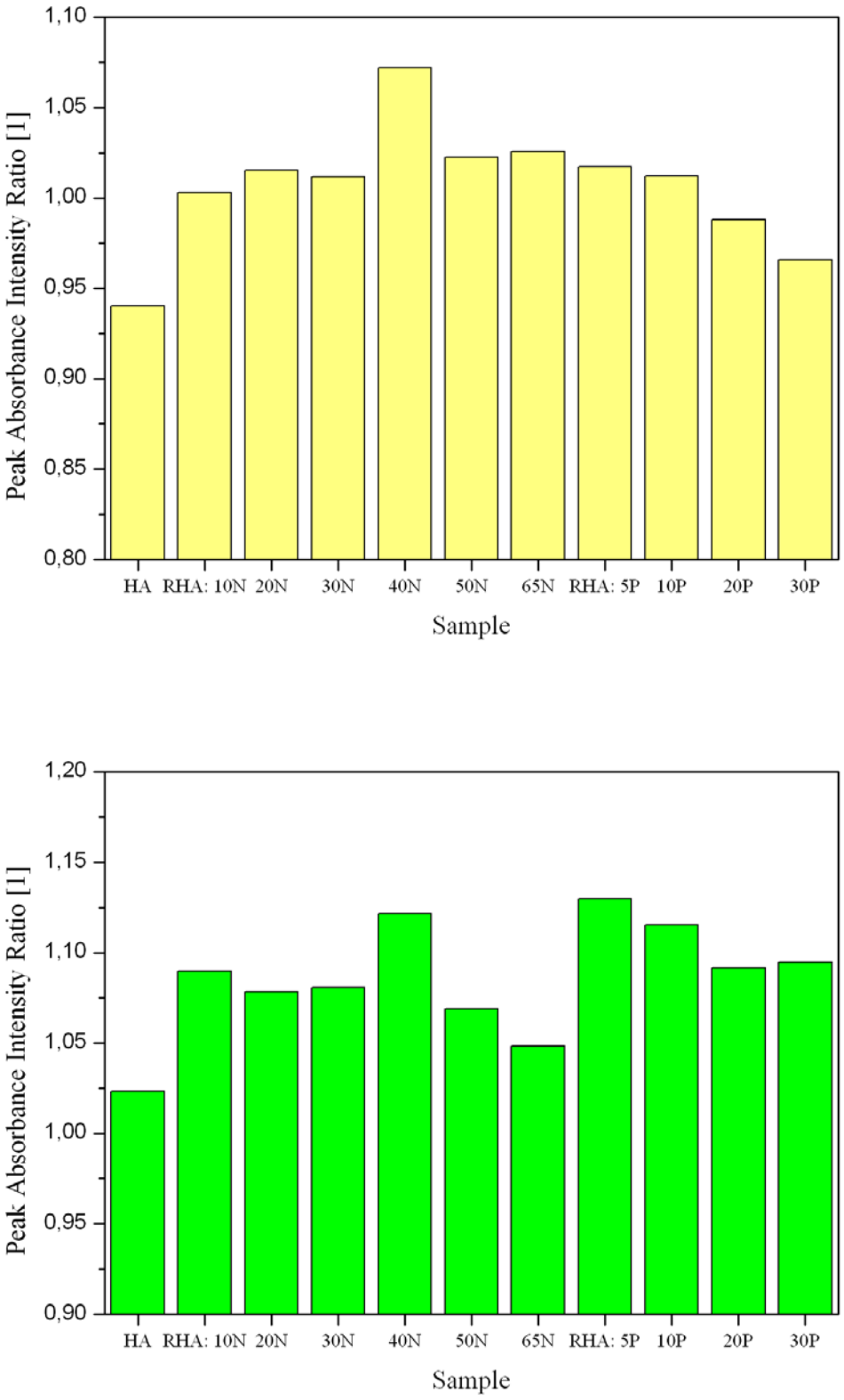


Figure 38: *The charts of the relative intensities of specific absorption bands ratios: 1 – aliphatic vs. aromatic (2930/1610) cm^{-1} (yellow) and 2 – amidic vs. aromatic (1550–1520/1610) cm^{-1} (green).*

5.2.4. Fast Field Cycling NMR Relaxometry

To our knowledge, the application of FFC NMR has never been reported before as a method for characterization of humic substances. In this work, in the first step the T_1 relaxation times were measured in dependency on the Larmor frequency providing the NMRD (Nuclear Magnetic Resonance Dispersion) profiles. Those records are useful for evaluation of water dynamics influencing the physical structure of humic samples. Unfortunately, the results showed that the dynamics of molecular structure of prepared humic samples is very similar and no significant differences were observed. In fact, the obtained NMRD profiles needed the multiparametrical fitting which can provide (mainly in well-defined systems) parameters with physical meaning. It is not a case of humic substances due to their high heterogeneity. Therefore, the author decided to extract data from free induction decay curves obtained at 20 MHz in order to see dynamics of water adsorbed in the inner structure of humic acids. It is necessary to point out that in this case, FFC NMR relaxometry can monitor preferably water protons due to their relatively long relaxation times unlike the protons of humic acids in solid state which are substantially shorter than the dead time of FFC NMR relaxometer used. Therefore, the obtained T_1 distributions reflect the state of water and its distribution in conditioned (equilibrated) samples. Physical structure of humic substances is affected by its heterogeneity and thus a distribution of pores, cavities and inner surfaces of different affinity to water can be found in there. Relaxation times of water protons strongly depend on the environment which they experience. Free water protons show higher relaxation time (seconds) due to “undisturbed” and almost “non-accelerated” free induction decay (FIDE). In contrast, relaxation of water protons present nearby the surface or confined in a cavity is accelerated by the field inhomogeneity caused by other nuclei and electron shielding resulting in faster FIDE and shorter T_1 relaxation times. This effect can be even stronger in the presence of a paramagnetic species such as for example Fe^{III} and Mn^{II} cations (not the case here, see the ash content in **Table 8**). The extracted FIDEs were then transformed by Inversion Laplace Tranformation into distributions reported in **Figure 39** and **Figure 40**. In some case, the obtained data can be deconvoluted, however, since it is still an ill-defined issue the author decided to extract only mean relaxation times (peak maxima) and their widths in the half-height representing the distribution of water protons.

Results demonstrated in **Figure 39** and **Figure 40** show different state of water in produced regenerated humic acids. Taking into account the moisture content presented in **Table 8** it can be expected that all water molecules are present as either adsorbed water or confined water. This was confirmed by the mean relaxation times reaching maximally hundred milliseconds.

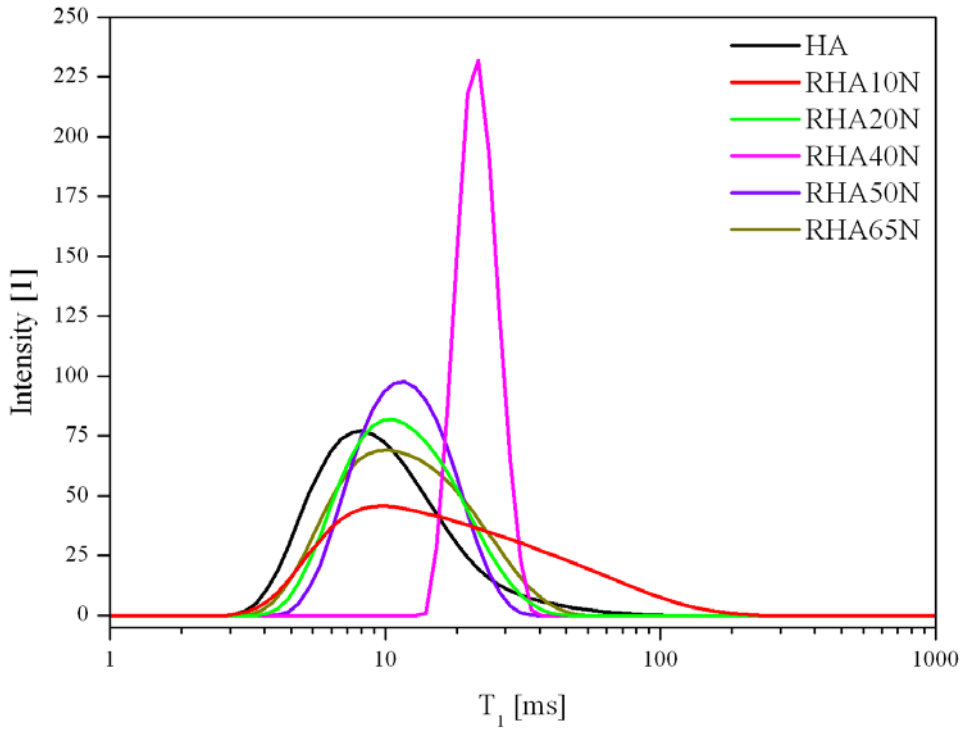


Figure 39: *T_1 distribution of water present in HNO_3 regenerated humic acids and in HA extracted from parental lignite.*

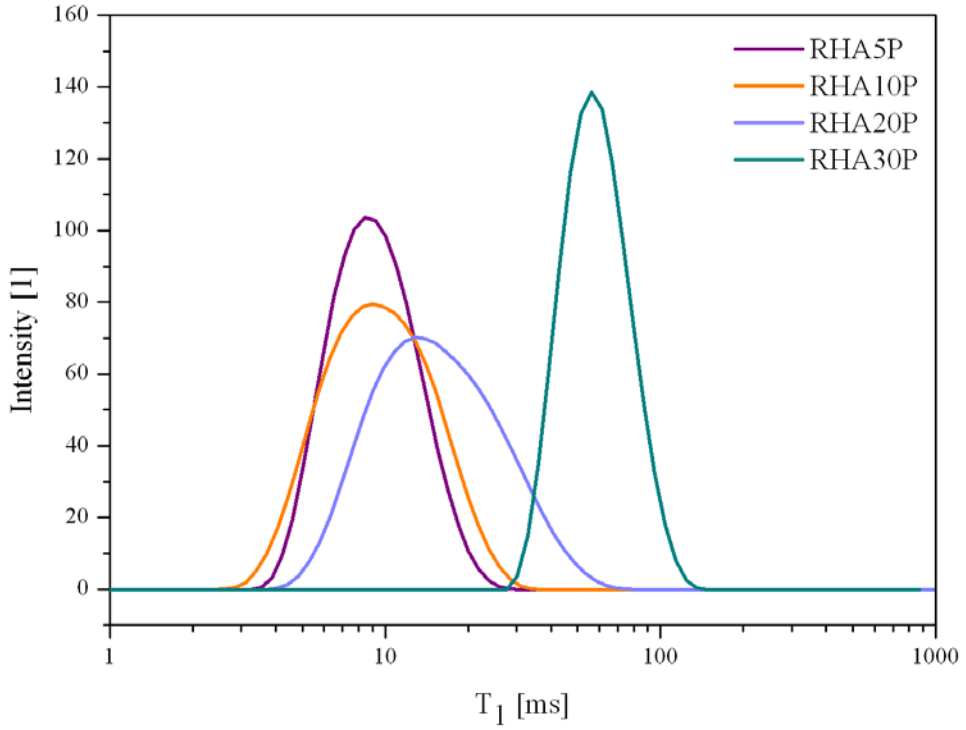


Figure 40: *T_1 distribution of water present in H_2O_2 regenerated humic acids.*

Extracted results are summarized in **Figure 41**. It can be seen that the mean values of relaxation times are increasing with increasing concentration of nitric acid up to 40% and then decreases. In case of hydrogen peroxide, an increase with increasing concentration was determined. **Table 8** shows the comparable water content and therefore, the differences can be attributed to difference in the physical structure. As demonstrated several times (e.g. *Bayer et al.* [252]), the decrease of relaxation time was recorded in progressive penetration of water molecules into the structure of organic matter, i.e. changing of surface to volume (S/V) ratio. That means the higher surface to volume ratio is, the higher the T_1 is. **Figure 41** shows an increase in T_1 at to 40 vol% of nitric acid indicating the increase of the surface of obtained humic acids with respect to water captured inside their structure. Again, this corresponds with or model about the lignite oxidation and production humic acids. Nitric acid was reported as an "opener" of lignite structure [41], i.e. up to 40 vol% is effective to destabilize lignite structure and produce more porosive humic acids. A decreasing tendency after 40 vol% HNO_3 indicates and confirms the theory about the changing oxidation mechanism. To sum the considerations up, 40 vol% nitric acid and 30 vol% hydrogen peroxide allow producing humic acids with higher surface to volume ratio.

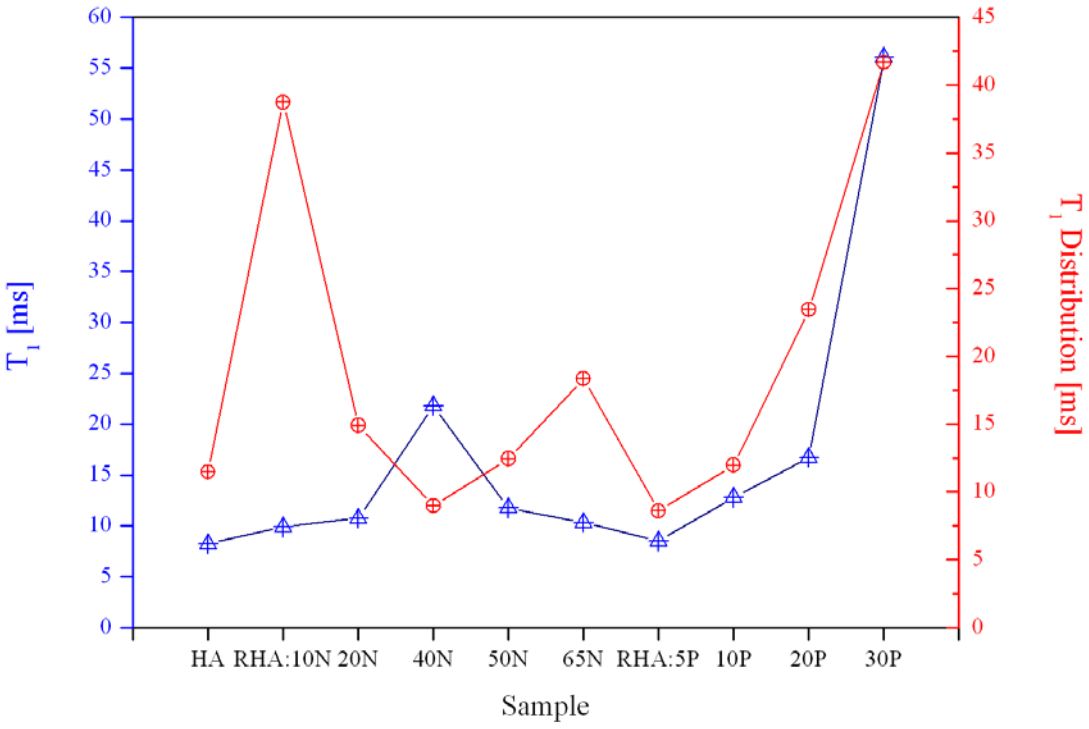


Figure 41: *Relaxation time (T_1) (blue triangles) and its distribution (red dots) in relation to the lignite regeneration agent and its concentration.*

Figure 41 reports also the distribution of water in the pore which is in line with conclusions of *Jaeger et al.* [253], who demonstrated quantitative aspects of obtained peaks and their connection with water content. Monomodal distribution determined in all cases indicates either the continuous distribution of cavity sizes in humic materials or that only one type of cavity is present in humic samples. Anyway, this number reflects the cavity(ies) size, i.e. the broader the peak is, the larger the cavity is. In light of above discussion, sample RHA5P

resembles the original sample HA, i.e. shows both the same S/V and water distribution. It is noteworthy, that sample RHA40N showed low cavity size unlike its S/V indicating a unique physical structure of sample, i.e. low distribution of relatively large-sized cavities (compare **Figure 39**). Unique is also behavior of sample RHA10N, which showed very large distribution of T_1 relaxation times, reaching more than 100 ms. Similar value vas recorded also for sample RHA30P. These T_1 values indicate presence of relatively weak bounded water in both samples. While in sample RHA30P it can be attributed to the surface water, in case of sample RHA10N to relatively low O/C ratio (see **Table 7**) indicating less number of stabilizing H–bonds in the structure. Low O/C ratio can be observed also for sample RHA5N but in this case the mean T_1 value is low, indicating presence of water mainly in the inner structure.

FFC NMR shows its great potential in evaluation of physical structure of produced humic acids even in an "indirect" mode. The information about the water distribution in those materials is of a great importance in estimation their behavior as sorbents of either organic or inorganic compounds. Further discussion on this issue can be seen further.

5.2.5. Dynamic Light Scattering

DLS has seen a limited use in the study of humic materials and most of these investigations considered a single humic or fulvic acid and were focused on instrumental and procedural optimization. For example, *Chin et al.* employed DLS for studying of spontaneous assembling of marine dissolved organic matter into gel-like aggregates [210] while *Palmer and von Wandruszka* came with first light scattering comparative study of the IHSS humic and fulvic samples [60]. The knowledge of humic material aggregate size is not only interesting and novel from the physical-chemical point of view (for example as a comparison with M_N and M_W obtained from HPSEC), but several authors presupposed the aggregate size as one of the factors for biological activity too [77],[254],[255]. Further, knowledge on humic aggregate size and distribution is important in designing of humic-based surfactants [245] and associated phenomenon such as partitioning of humic substances and inorganic contaminants. As demonstrated by *Čtvrtníčková et al.* [245] humic aggregates are playing important role in surface activity, when adsorbed on the available surface (cell, particle, molecular assembly etc.) their physical structure will adopt the appropriate conformation reflecting the conditions and nature of interphase. This is, in our opinion, the reason of the versatility and perhaps also microbiological stability of humic substances.

Investigation of the 250 mg L^{-1} solutions of potassium humates researched showed the presence of large dimension particles, perhaps aggregates, which is in line with the recent observations obtained by HRUS [63] and diffusion ordered NMR spectrometry [256]. Thus, it seems that humates form aggregates even in diluted solutions and do not behave as common monomer surfactants, while they exhibit only partial amphiphilic behavior. Sizes of detected aggregates varied in the interval 100–500 nm (**Figure 42**), which is in agreement with previous results of *Palmer and von Wandruszka*, who analyzed the IHSS standards of humates and fulvates and their own extracted soil humate sample [60].

For the isothermal conditions of the measurements carried out at 25°C value showed that the aggregate size is quite similar for samples KHA, KRHA20N and KRHA30N samples (about

320 nm), KRHA40N, KRHA65N and KRHA20P samples (from about 230 to 260 nm). The highest aggregate size was observed by the samples KRHA10N (471 nm) and KRHA10P (372 nm), in contrary to the lowest aggregate sizes of the KRHA50N, KRHA5P, KRHA30P (all about 135–150 nm) samples

Non-isothermal conditions bought a more complex view on the stability of humate aggregates. KRHA10N sample performs significant fall of the aggregate size at the 50°C, but then the size slightly rises (60°C). Sample KHA showed a decrease of the size of its aggregates at the 50°C too, but without any increase after. Samples KRHA30N and KRHA10P showed the aggregates sizes decrease at 40°C, followed by the rise at 50°C and repeating decrease at 60°C. Aggregates of KRHA65N, KRHA5P and KRHA30P showed any significant decrease of size only at the 60°C. KRHA50N and KRHA20P samples presented no significant changes between the aggregate size and temperature. This aggregate size changes may be ascribed to the complexity of humates composition, their partial amphiphilic nature, their ability to undergo reversible temperature-induced transformations in aqueous solutions [55],[60]. This approach clearly showed a variability of composition of investigated humates, their flexibility to reconform as a response to the change of the external conditions in a unique way.

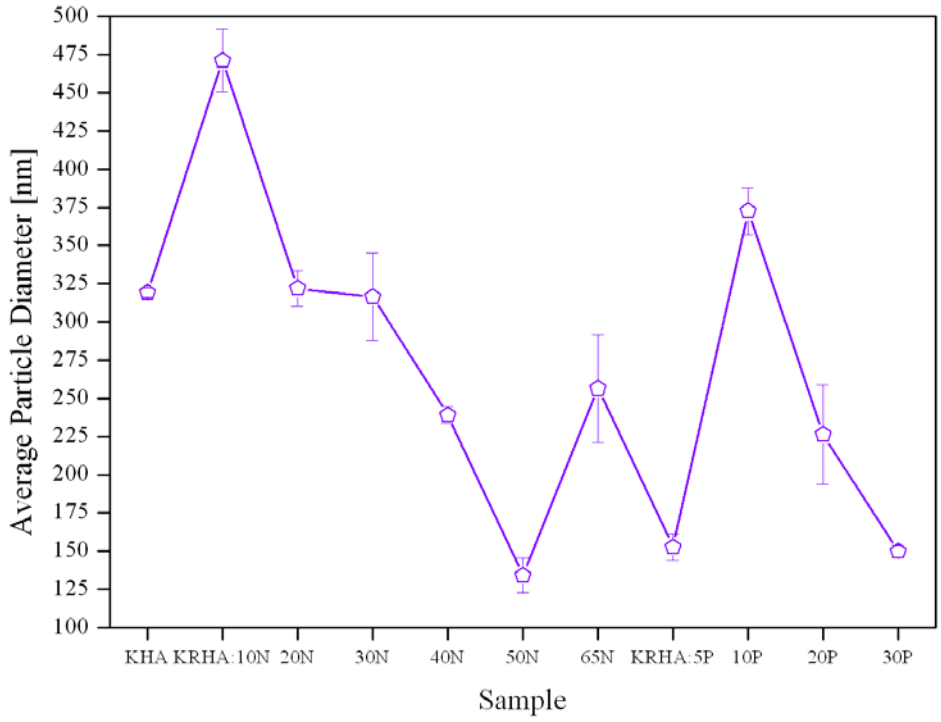


Figure 42: *Average particle diameters of potassium humate aggregates at the 250 mg L^{-1} concentration and 25°C.*

In view of the pseudomicellar model of humic aggregation, the results are slightly distinct from sample to sample, clearly affected by the lignite regeneration. As reported in **Figure 43**, the size of humic aggregates rises from 10 to 20 or 25°C; then is sloping down. According to the previously mentioned results [60], similar behavior was found by *Palmer and von*

Wandruszka by leonardite humic acid IHSS standard (for sizes from 350 to 400 then to 200 nm), as a result of the higher content of condensed aromatic rings system, that impart a certain stiffness to the humic system. The regeneration of lignite brings some bond cleavage and aliphatic content enrichment to the humic structure, therefore the resulting samples behave more like loam humic acid (rise of the aggregate size followed by its fall; from 250 to 450 then to 300 nm) or the soil humic acid (Summit Hill Humic Acid) performing the not-so-significant change of the aggregate size (about from 150 to 200 and then back to 100 nm). This behavior was classified as the result of the possibility of the longer and more flexible chains to fold and bend, i.e. to create both intra- and intermolecular assemblies [60]. In contrast, based on results of HRUS, *Kučerík et al.* hypothesizes that this behavior is a result of both weakening of H–bonds causing transitions in humate physical structure around 25°C and strengthening of hydrophobic interactions and hydration associated with humates dilution (a weakening of surface charge associated with a decrease of hydrodynamic radius above 25°C) [63]. Unfortunately, the size distribution processor mode of analysis (used for aggregate molar weight determination) has been already found unsuitable for highly polydisperse samples like humates [60], so the direct comparison with molar weights is not possible.

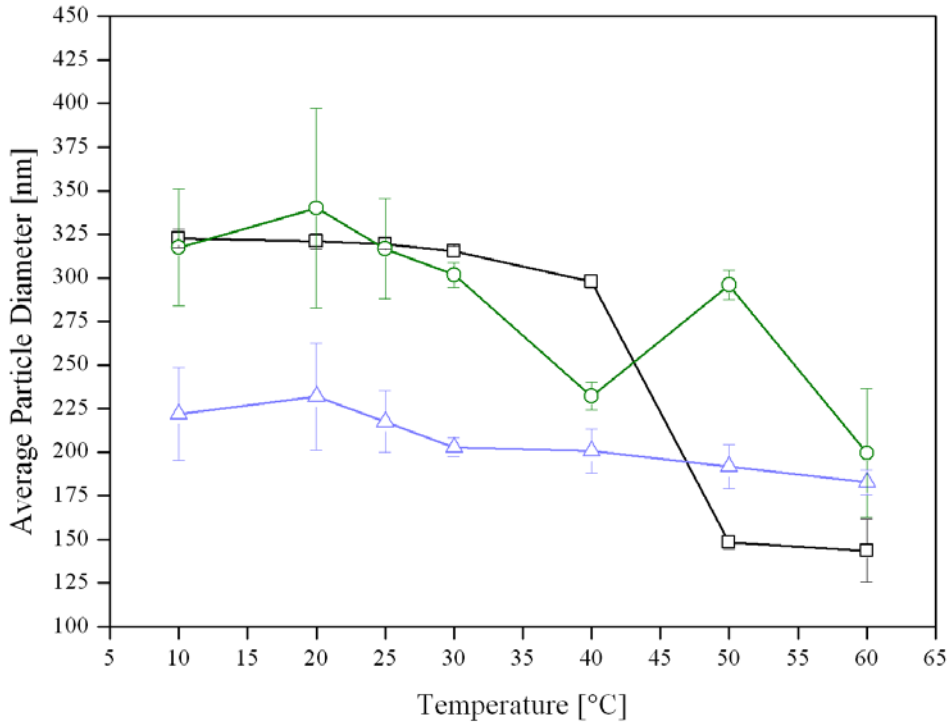


Figure 43: *Average particle diameter of 250 mg L^{-1} KRHA (black squares), KRHA30N (green circles) and KRHA30P (blue triangles) aggregates in dependence on temperature.*

5.2.6. High Performance Size Exclusion Chromatography

In order to further assess the influence of lignite regeneration on the humic supramolecular aggregate (or even humic macromonomer) system heterogeneity, two High Performance Size Exclusion Chromatography (HPSEC) experiments were conducted in two repetitive

measurements on two different chromatography systems and detectors. There have not been observed any significant differences between the results in the duplifications of both DAD and RID HPSEC analyses.

The main motivation of this approach is the evaluation of aggregate size of prepared humic acids, their mutual comparison and by means of DAD detector the extension of the common detection limit of HPSEC experiments. As a rule, only one particular wavelength is employed (either 280 or 256 nm) which is in principle capable to detect only chromophores absorbing in this range. This can lead to the underestimation of some molecular fractions or moieties, mainly those adsorbing in the visible spectrum range. Those can be mainly the highly condensed fractions composed of self-assembled hydrophobic aromatic or unsaturated aliphatic molecules (stabilized by π–π, CH–π and van der Waals interactions). This can be simply understood from a UV–VIS record of humates published in many studies, e.g. *Stevenson* [7]. The highest absorbance can be seen at low wavelengths around 200 nm accompanied by an exponential-like decrease with increasing wavelengths up to 800 nm. In the case of SEC analyses, after injection into the column, the humate sample undergoes a separation depending on the strength of stabilizing interactions among humic molecules, character of the mobile phase and column and pass through the detection chamber. The character of the fraction is determined by the probe, i.e. by the light of the particular wavelength. In the case when there is no interaction between applied beam and humate molecules, detector monitors no mass. In a limit case, in fully saturated aliphatic samples no signal would be recorded. Therefore, it is necessary to employ other wavelengths probing the wider composition of the sample.

The DAD detector used in this study determined the molecular absorptivity in the range of wavelengths from 220 to 415 nm. In all the DAD results, a bimodal distribution can be seen (**Figure 44**). In principle, the sharp and intense peak about the retention time (rt) of 18 min can be attributed to the exclusion of the largest components (aggregates or chain segments), while with increasing rt the smaller aggregates or even molecules are subsequently excluded, resulting in a broader second peak as the detector signal during the 20 to 45 minutes of rt. The extracted results for 4 selected samples detected at 280 nm are reported in the **Figure 45**.

The RI detector gave similar chromatograms for all samples, with only slightly different retention times and overall peak shape (this difference is given by diverse principle of signal generation on the detectors and have been thoroughly discussed in the past, for example see the reference [257]). The sharp on the RI detector peak of largest components is shifted to the 21–23 minutes and the broad peak of smaller moieties is shifted to the 22–25 to 40 minutes of rt (**Figure 46**).

Great differences can be seen between the particular representatives of the potassium humate from the parental lignite and the humates from the regenerated lignites. In general, the nitric acid regeneration brought the enhancing of the both peaks (narrow and broad) ergo enhancing the both detectors' signal response. The KRHA40N and KRHA65N samples showed the suppressed narrow peak and enhanced broad peak. Peroxide regeneration of lignite evoked on both the detectors the enhancement of the narrow peak and suppression of the broad peak.

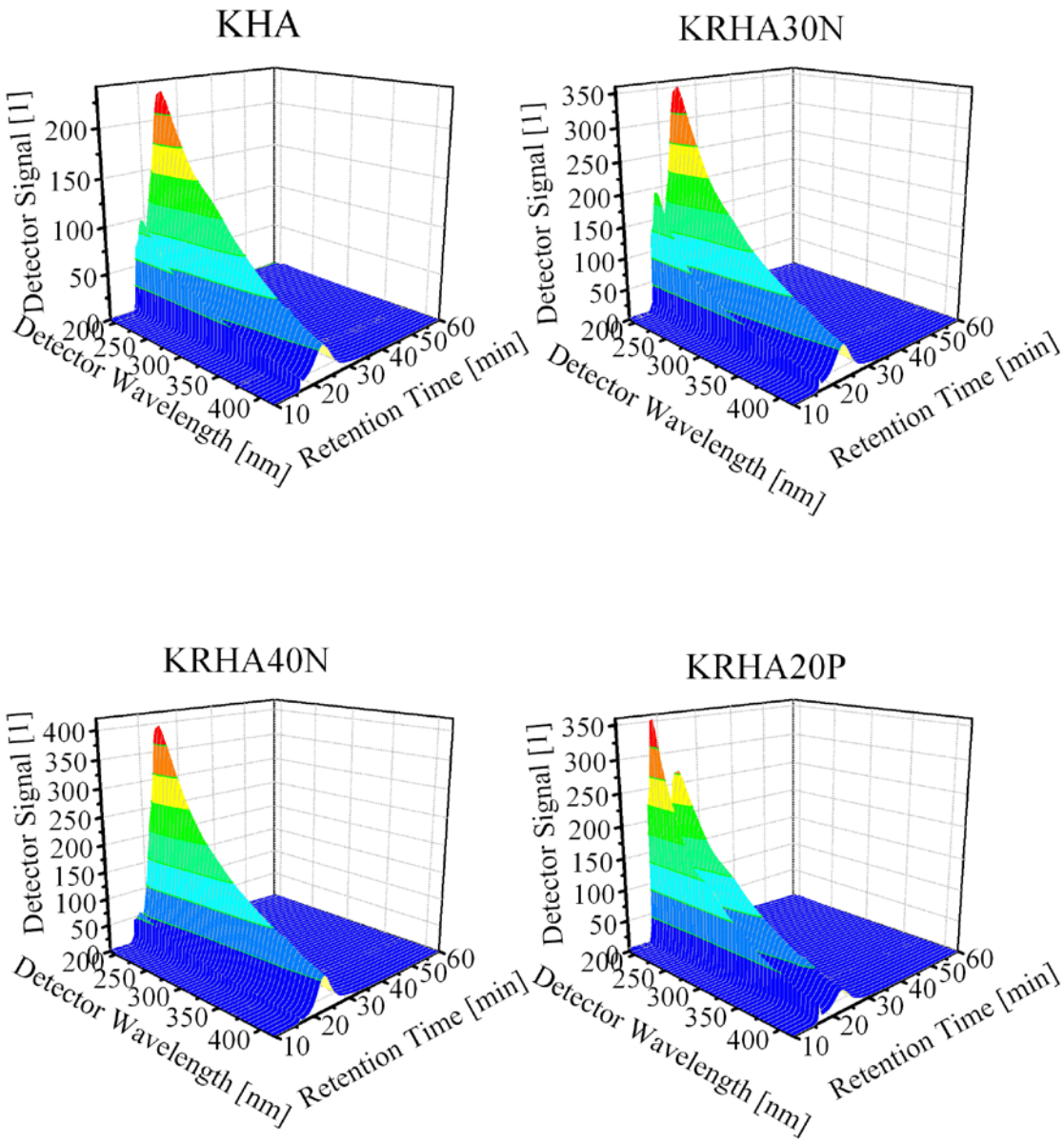


Figure 44: *3D chromatograms obtained by the Diode Array Detector, showing the differences in the representative humic systems composition and absorptivity.*

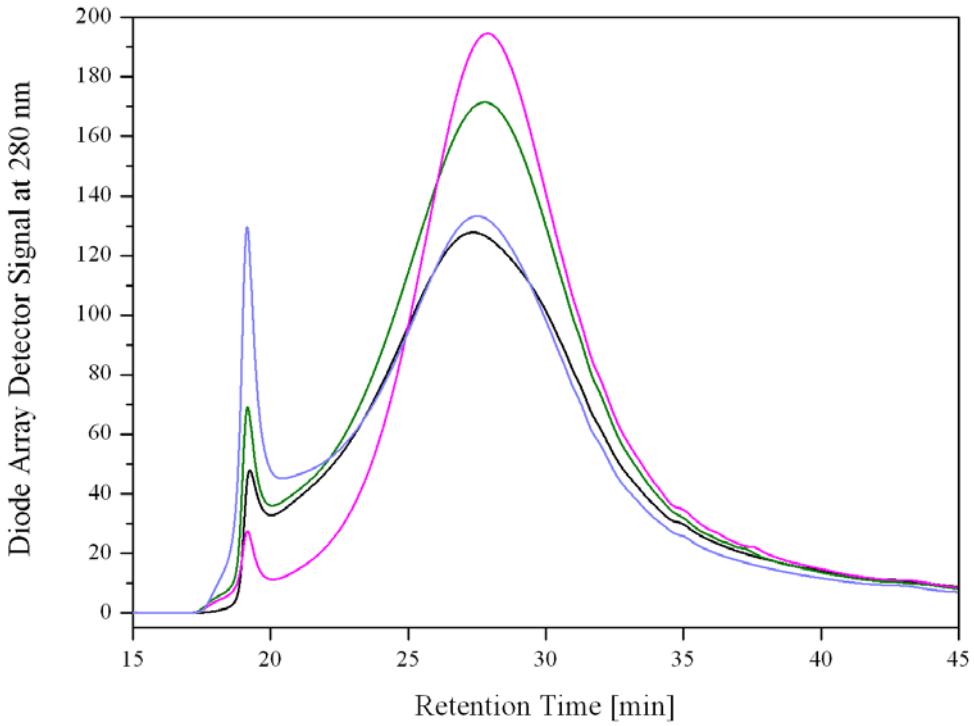


Figure 45: *Diode Array Detector 2D HPSEC chromatogram of KHA (black line), KRHA30N (green line), KRHA40N (magenta line) and KRHA20P (blue line) samples – detector wavelength set up at 280 nm. The differences in magnitudes and areas under respective peaks (narrow at 18 min and broad between 20 and 40 min) are clearly visible.*

According to *Conte et al* [258] the large sized humic aggregates corresponding to the first and narrow peak are composed mainly of the alkyl hydrophobic chains, which are exhibiting only poor fluorescence and UV absorption (e.g. aliphatic saturated lipids). The subsequently eluted smaller size aggregates (broad peak) may be compacted from the shorter but conjugated unsaturated and aromatic carbon chains, showing higher fluorescence and UV absorption. Applying the findings of *Conte et al.* on these HPSEC results, it can be assumed that HNO_3 regeneration of lignite caused an increase in content of unsaturated structures in humic acids. The KRHA40N and KRHA65N samples showed lower content of longer chains, while the samples from peroxidized lignite seem to contain significantly higher amount of longer chains.

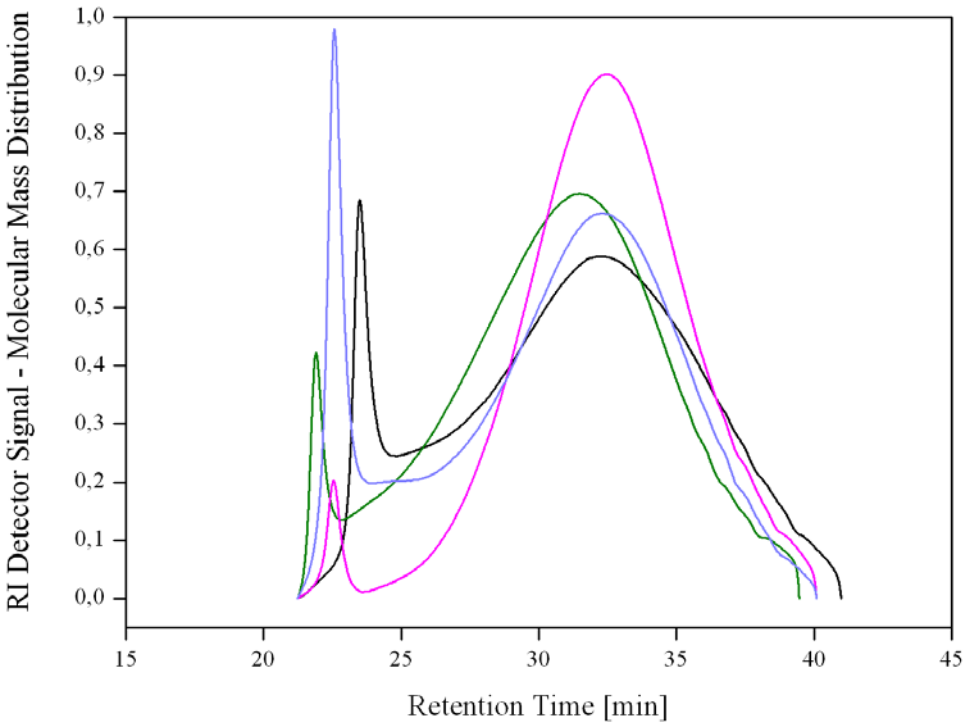


Figure 46: *Refraction Index Detector HPSEC chromatogram of KHA (black line), KRHA30N (green line), KRHA40N (magenta line) and KRHA20P (blue line) samples – detector wavelength set up at 280 nm. The differences in magnitudes and areas under respective peaks (narrow at 22 – 24 min and broad between 23 and 40 min) are clearly visible.*

Results in 3D chromatograms are implicating another feature of the humic systems, the descending of absorptivity (the total DAD peak area) with rising of the wavelength (**Figure 44**). In principle, the peak area at particular retention time and at particular wavelength reflects the content of specific humic components. If the peak areas are plotted against the respective wavelengths the resulting graph representing high resolution UV–VIS detection which is diminishing the effect of interaction among aggregates and thereby emphasizing the differences between investigated samples (**Figure 47** and **Figure 48**). It can be clearly identified, that regeneration, except for KRHA40N sample, increased the content of chromophores in the humic matter. That indicates that the oxidation of lignite has a great impact on the aliphatic moieties while aromatic ones are either untouched or changed only slightly. The behavior of the KRHA40N sample may be explained by higher content of inhomogeneities in the lignite fraction used for the sample preparation.

Additional insight into the differences among humate samples brought the determination of M_W and M_N. Simply, the M_N takes more into account the number or the content of aggregates while the M_W favores the aggregates size. **Table 9** reports the M_W and M_N values based on the detection at 280 nm. It can be seen that M_N was determined lower only for samples KRHA40 and KRHA65, the peroxide line showed M_W values generally higher than nitric acid treated line. Regarding M_W, results were more scattered, except for same samples from N line all the results were lower than for KHA. It is noteworthy, that M_W and M_N values calculated

from RID gave the inverse results when compared with KHA sample. In fact this observation supports the above-statement about the limits of UV detection used at one wavelength. Assuming that RI detector can follow all the eluted humic mass, it can be concluded that nitric acid treatment decreases molecular mass of extracted humic acids while peroxide does *vice versa* (**Table 9**). The ratio between M_W and M_N is used as an indicator of the system polydispersity (PDI). Results reported in **Table 9** indicate that higher concentrations of oxidizing agent cause the narrower mass distribution and while lower concentration causes a slight increase (RID detector). This is in line with recent results indicating that the oxidation of lignite proceeds in several different steps depending on the oxidation time and/or strength of oxidizing agent [43].

In all cases, the molecular mass distribution and the ratio of M_W and M_N confirmed, that the humates are highly polydisperse systems composed of smaller aggregates of shorter conjugated, aromatic and oxidized carbon chains as well as of the longer aliphatic segments.

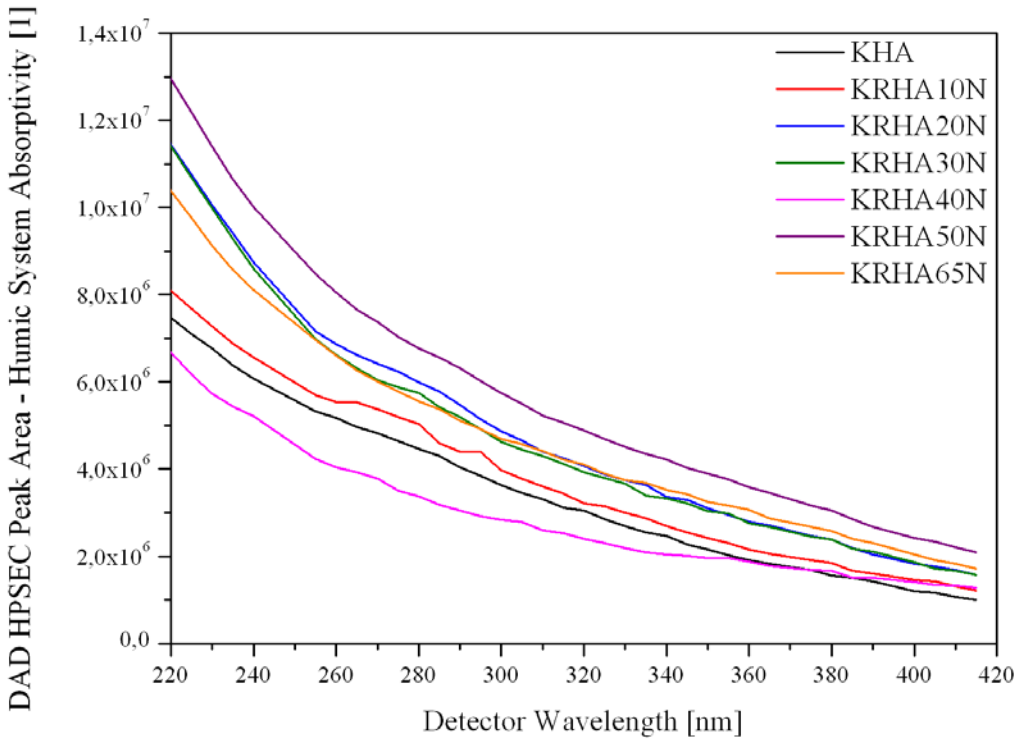


Figure 47: *Absorptivity of humates from lignite regenerated by HNO_3.*

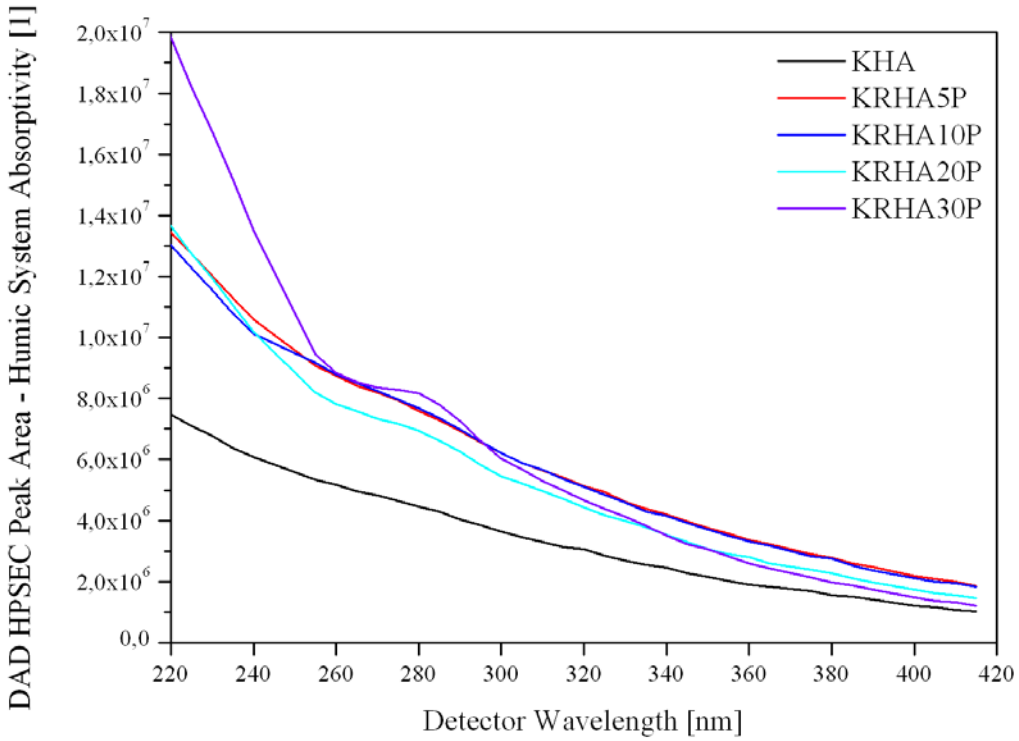


Figure 48: *Absorptivity of humates from lignite regenerated by H_2O_2.*

Table 9: M_N, M_W and PDI of humate samples as detected by DAD and RID HPSEC

Sample	M_N (280 nm DAD) [g mol^{-1}]	M_W (280 nm DAD) [g mol^{-1}]	PDI (280 nm DAD) [1]	M_N (RID) [g mol^{-1}]	M_W (RID) [g mol^{-1}]	PDI (RID) [1]
KHA	7 879	89 733	11.39	6 716	52 915	7.88
KRHA10N	8 954	107 105	11.96	6 474	52 180	8.06
KRHA20N	8 715	91 669	10.52	5 805	36 275	6.25
KRHA30N	8 355	77 605	9.29	5 816	34 420	5.92
KRHA40N	5 797	198 983	34.33	5 240	21 900	4.18
KRHA50N	7 959	73 161	9.19	6 153	36 435	5.92
KRHA65N	6 408	87 198	13.61	6 145	24 755	4.03
KRHA5P	10 782	75 495	7.00	7 361	49 975	6.79
KRHA10P	11 914	86 120	7.23	6 817	55 300	8.11
KRHA20P	11 934	86 066	7.21	6 801	56 390	8.29
KRHA30P	14 643	83 739	5.72	7 766	82 440	10.62

For better recognition of the size distributions and their potential impact on HAs biological activity, the overall area under DAD detected peak (at 280 nm) was integrated. Integrated spectra were virtually separated into 6 intervals (see **Figure 49**). The intervals of distribution showed more clearly the differences between the individual samples. Again, according to the observation of *Conte et al.* [258] 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 slowly while middle-sized content was also favored. The exception can be seen for the sample pre-treated with 40 vol% HNO_3, which eluted profusely both in the range of 0–15 kg mol^{-1} and > 100 kg mol^{-1} suggesting that in this case the oxidation attack resulted in the extensive production of low-molecular-size-fractions, while many of them may have been suscepted to further aggregation onto the large aggregates, ergo, the content of large aggregates is unique by this sample too. For the samples treated with H_2O_2, the low-sized content was reduced in comparison with original humates and the fraction with second highest M_Ws was more prominent again. Generally, these results are in agreement with those of *Vlčková et al.* [200] and the assumptions of *Conte et al.* [258].

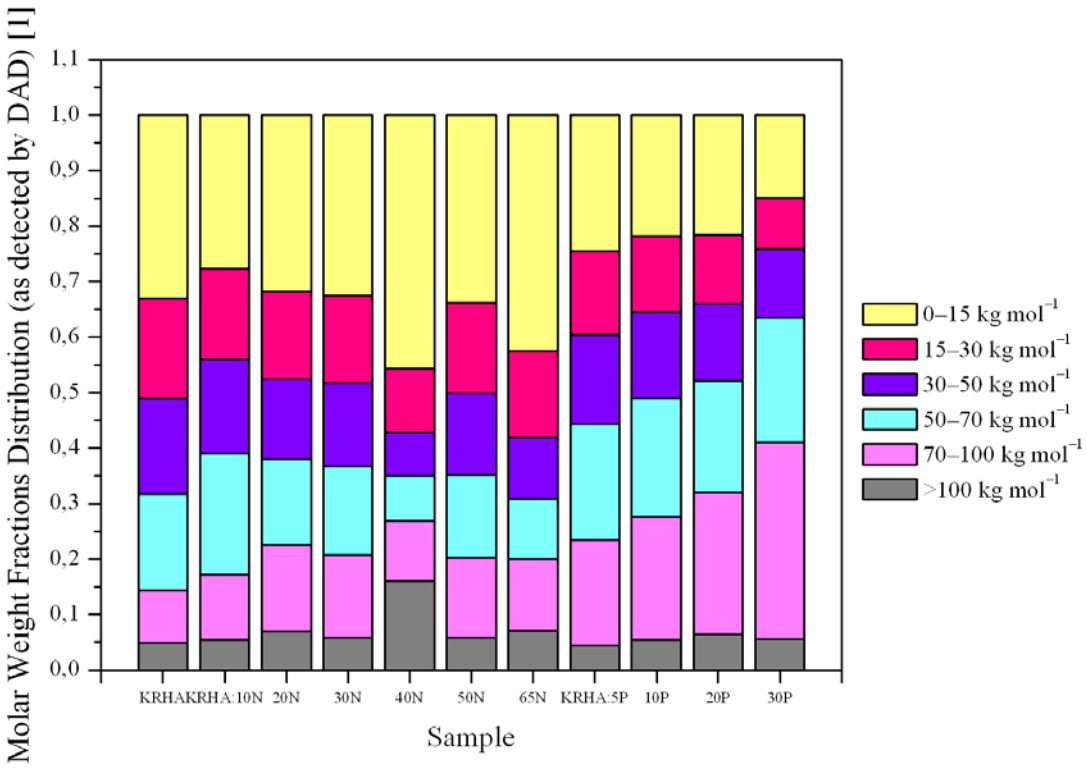


Figure 49: *Molar weights fraction distribution as detected by DAD at 280 nm.*

5.2.7. Humates Hydration

As already mentioned, the physical structure seems to be one of the crucial aspects of humic acids applicability such as for example their biological activity [233]. The author of this thesis hypothesizes that the state of humic acids in solution is determined by their composition and hydration. This hypothesis is based on several observations. First most of biomolecules mainly proteins and polysaccharides functions are closely linked with content and nature of water present their hydration layers. Further, in our recent work we showed that the character of humic substances hydration layer depends on the concentration and both hydrophobic and hydrophilic hydration occurs in the interphase humic aggregate/water [62]. Last, but not least, water is the key factor in aggregate formation and interplay between water polarity and humic aggregate bipolarity influences humic substances reactivity and perhaps also biological activity [245]. Thus the knowledge on the number of water in hydration shell of obtained humates can be vital in understanding of their properties and reactivity.

The hydration of humates was studied by means of high resolution ultrasonic spectrometry and densitometry. The results were calculated by employing the equations **2** to **7** and **10** to **22**. This approach is based on the different compressibility of water in the humate hydration layer and of the bulk water. Subtracting the free water content in % from the value of 100 % was obtained the percent amount of bound water. This amount was recalculated to grams and when related to the sample concentration (250 mg L^{-1}), it gave the value of bound water amount in the sample hydration shell. The graph of ultrasound velocity and density of humate samples is shown in the **Figure 50** as well as the graph of compressibility and humate samples hydration (**Figure 51**).

It can be seen that the ultrasound velocity U12 was generally lower by samples from regenerated lignite than by the sample from parental lignite. Even though, the U12 presented some oscillation character, when the highest values (higher than 1496.665 m s^{-1}) were observed by the samples KRHA20P, KRHA65N and KRHA30N, the lowest values (lower than 1496.655 m s^{-1}) were observed then by samples KRHA50N and KRHA10N. The density presented an oscillation character too, usually with lower values observed in the regenerated samples than the density of KHA sample solution (with the exception of KRHA50N sample). Since the sample compressibility is a calculated value, it had to present an oscillation character too, the highest values were found for samples which presented the lowest values of U12.

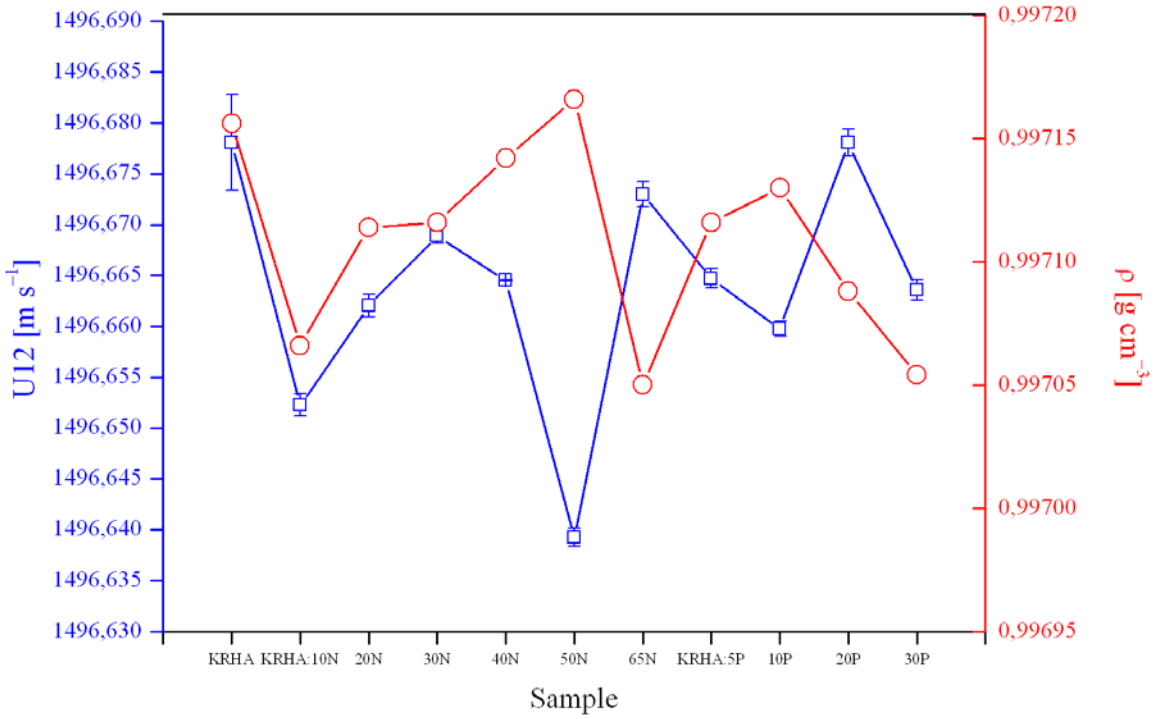


Figure 50: *Ultrasound velocity (U12, blue squares) and density (ρ, red circles) of potassium humate samples.*

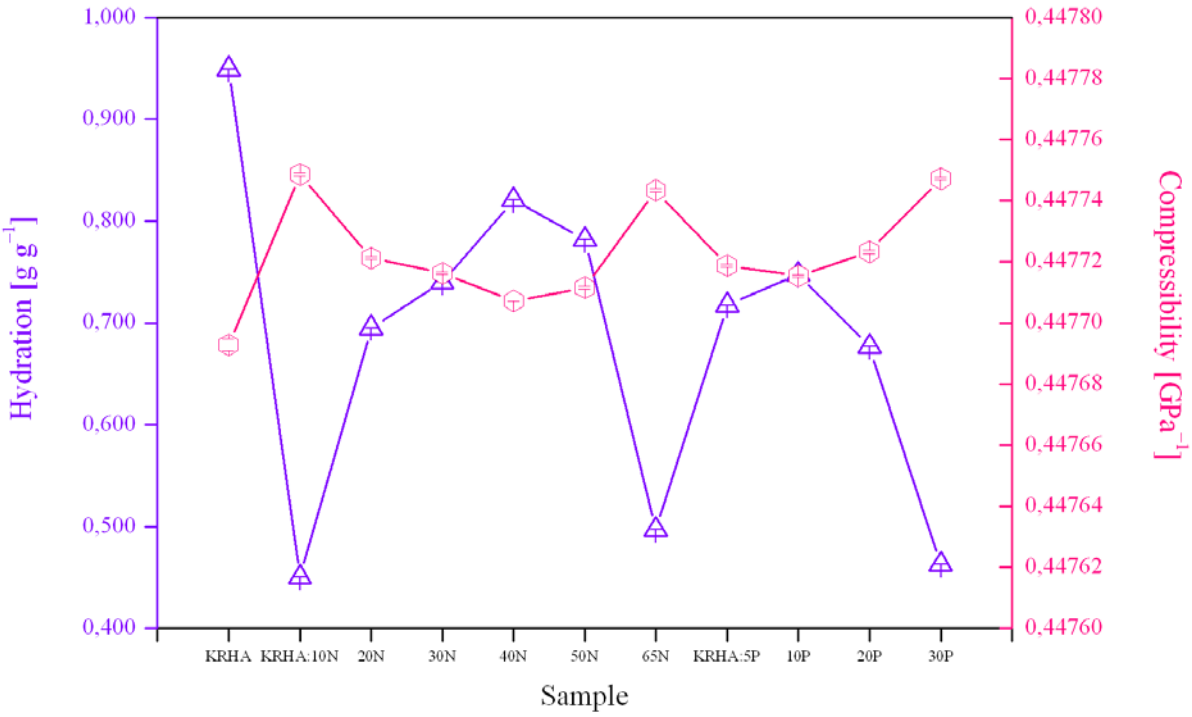


Figure 51: *Hydration (purple triangles) and compressibility (pink hexagons) of potassium humate samples.*

Humate sample hydration values were found between 0.45 and 0.95 grams of water per gram of humate. In such complicated systems like humates, both types of hydration (hydrophobic and hydrophilic) can be contemplated [62]. Since the concentration of samples is still relatively low (250 mg L^{-1}), the hydrophobic hydration is favored as the main contribution to the sample hydration [62]. It is unfortunately not known, how the hydrophobic hydration affects the density and ultrasonic velocity. Supposing the degree of hydrophobic hydration similar in all of the samples, the oscillations in the all observed magnitudes may be the consequence of regeneration of lignite. The values of hydration seems to be quite high, especially in the case of samples KHA, KRHA30N, KRHA40N, KRHA50N and KRHA5P and KRHA10P. They are even similar for example to those discovered for hyaluronan, which is being considered the most hydrated hydrophilic polysaccharide [221]. This can implicate the high porosity level in humic aggregates in liquid state, i.e. determined water in hydration level includes water trapped in the interior of the humic aggregate. When the aggregates is small and compact, determined water layer is mainly on the surface of the aggregate (sample KRHA65N, see M_W and M_N results). Increase in aggregate size increases the hydration layer but at certain dimension, the "cavity" effect has no other effect (sample KRHA30P). This hypothesis reflects the observation about the molecular weight of individual humic molecules which was reported up to 2 kg mol^{-1} [259] and implying capability of humic molecules to form aggregates with limited dimensions due to the low radius of stabilizing weak interactions. This hypothese should be verified in detail, which is beyond the scope of this thesis. In all cases, this may predetermines potassium humates for certain future application, mainly in the arid soil remediation field.

5.2.8. Fluorescence Spectrometry

The analyses of humic acids traditionally include characterization by fluorescence spectrometry. However, as already implied in HPSEC section, application of spectrometries in natural organic matter suffers by the high heterogeneity of the investigated material. On one hand, fluorescence spectrometry is about one order more sensitive than UV analysis but also in this case there are several limitations which are not easy to avoid. First, emission of high concentrated solutions of humic acids is decreased by the inner filter effect. In contrast, low concentrated solutions were demonstrated to be influenced by light scattering [260]. Thus the interval for measurement is relatively narrow and even under convenient concentration conditions the spectral overlapping occurs. In order to recognize the superposition of individual condensed aromatic fluorophores in engine oils, the synchronous fluorescence spectroscopy technique was introduced [261],[262]. The technique is based on the constant difference between excitation and emission wavelength ($\Delta\lambda$) which results in higher resolved records. In case of humic substances it seems that the ideal $\Delta\lambda$ is 20 nm notwithstanding the origin and composition of humic acids [260],[263],[264]. In this case, however, the attribution of peak position is not clear and the informative value is not significantly improved. In all cases, the excitation-emission (EEM) fluorescence is a commonly used method for characterization and distinguishing of respective humic substances. Firstly, according to *Senesi*, the fluorescing characteristics of humics are explained by the polycondensation and conjugation of the unsaturated structures [265]. In 1996, *Miikki and Senesi* used fluorescence synchronous scan spectra for determination of compost maturity and degree of humification, while stating that relative fluorescence intensity decreases with the age of the respective HA, i.e. with the degree of humification [266]. Later, the electron-shuttling ability of humics was

attributed mainly to quinone moieties, which are being considered as a versatile class of biomolecules [74]. Recently, the discoveries on the redox reactivity of humics have illuminated new mechanisms, in which humics can influence the cycling of metals and organic matter in environment [267],[75].

Therefore, in this thesis, based on the fluorescence measurements, the distinguishing between respective regenerated humates in the field of quinone content and redox state is expected. Like the most authors author of this thesis used the EEM approaches, since it produces a fluorescence spectra of a sample at different excitation wavelengths, which makes it advantageous for studying of complex mixtures [268].

The fluorescence spectral data of potassium humates solutions (20 mg L^{-1}) were assessed by means of the OriginLab software. Humate samples presented common humic fluorescence spectra with one or two broad maxima [269–271] at 200–400 nm range of excitation and 400–550 nm range of emission. Interesting changes have been observed in the intensity, which is generally lower by the regenerated samples – particularly by the samples from HNO_3 regenerated lignite. Since the fluorescence in humics is mainly implicated by quinoid and semiquinoid moieties [267], these results may give evidence of the decrease of the quinoid content in humics with regeneration. However not necessarily, because the intensity and locations of EEM peaks depend also on the adjacent moieties, pH, ionic strength, etc. [270],[271],[272], so lower fluorescence intensity may be evoked by oxidation and nitrification by the KRHA_N samples. The chosen fluorescence spectra are depicted on the **Figure 52**. Generally, the oxidized humics show maxima at lower emission wavelengths and higher excitation wavelengths, while the humics at reducing conditions present the maxima shifted to the higher emission wavelength and lower excitation wavelength [271]. The KHA sample has shown the highest intensity and one maximum at $260_{ex}/450_{em}$, KRHA10N and KRHA20P spectra were similar; with lower intensity and KRHA10N with thinner maximum while KRHA30P with broader maximum. KRHA30N and KRHA20P showed two maxima, which may be the result of presence of various functional groups in the vicinity of the fluorescing quinoid groups as well as of the oxidation of the sample [271]. Other samples presented similar spectra, with one or two maxima, but of lower intensities (data not shown). Indeed the optical properties of humic substances are still a “little mystery” but due to their conjugated structure (confirmed by fluorescence spectrometry) and possible ability of charge transport, humic substances may be a good candidate for future pigments of natural origin for industrial applications.

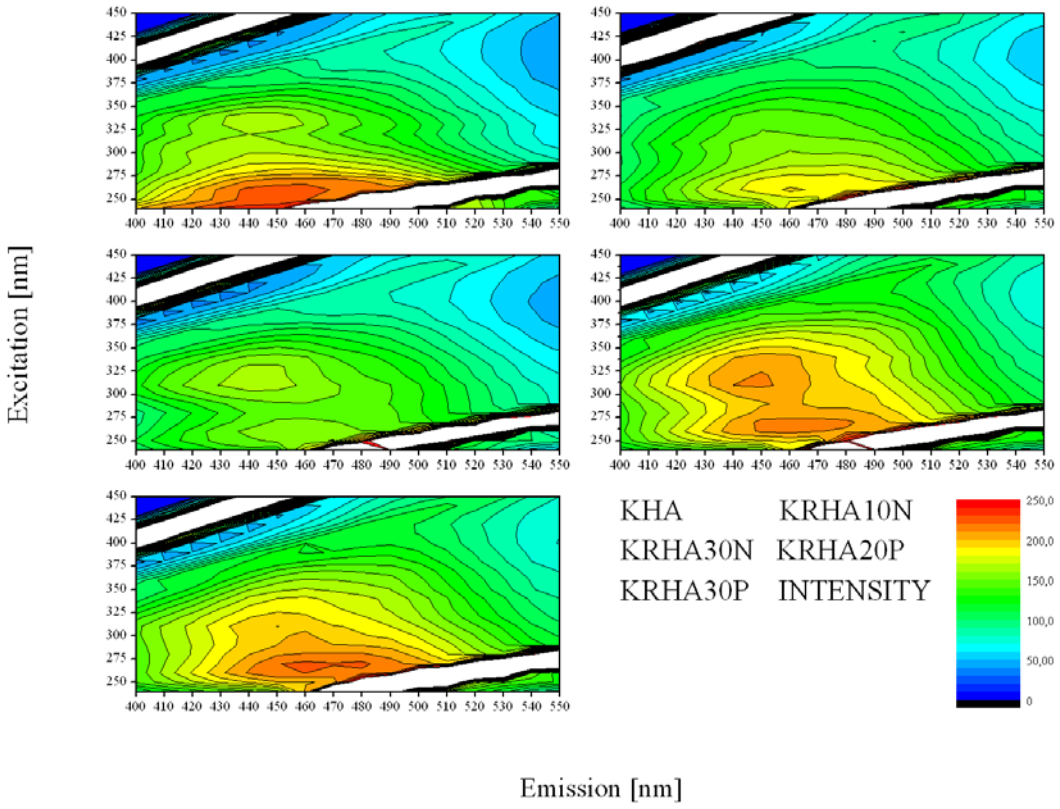


Figure 52: *EEM of selected humates (left to right, top to bottom KHA, KRHA10N, KRHA30N, KRHA20P and KRHA30P).*

5.3. Biological Activity

Together with physico-chemical behavior (e.g. aggregation, sorption properties, etc.), the biological activity is the most interesting feature of the humic substances both from scientific and commercial point of view. In today's humic science it is the most discussed topic and strong efforts are being put in the seeking of assessment methods for this feature. The biological activity evaluation is in today's science present in two forms, **the quantification of the beneficial effect of the humic substance onto the plant**, and **the principle of the beneficial effect**, while the latter is still a subject of scientific discussion [77],[84],[90],[96],[97],[254],[255]. In this work, effort was given to the first form, the quantification of beneficial effects of humates onto the studied plants with the aim to correlate these results with the physico-chemical behavior, which is also a discussed topic (and in this thesis already discussed supra). Usually, the biological activity was compared only to the results of HPSEC and not to other physico-chemical analytical techniques [77],[90],[254]. As a novelty, this work was inspired by *Zandonadi et al.* [84] and *Antošová et al.* [94] to combine their methods of biological activity assessment in order to create a very simple biological activity assessment method suitable for nearly every laboratory, not necessarily a biological or agricultural institution.

The possible influence of the regeneration of lignite on the resulting biological activity was assessed by a simple laboratory method. Five plants from thirty plants were chosen for the

root length and root division measurement, since the experiments were conducted twice, this gave the data for 10 plants, which were averaged and the standard deviations were calculated in MS Excel software. For the mass increment, the whole set of 10 whole plants (with stem and leaves) were carefully weighted. The experiment was also conducted twice, but for all the 30 plants in the container, which is resulting in the higher standard deviation (average was calculated from only two values), but the reliable information lies in the principle, that two times 30 plants have been weighted, so the statistics is hidden in the fact, that the experiment was conducted for 60 plant individuals. The starch and protein content assessments were done similar too, for 30 whole (but dried) plants.

The results and numbers for specific samples are summarized in the **Table 10** and shown on the **Figure 53** to **Figure 56**. Chosen analyzed root scans of *Zea mays* are depicted on the **Figure 57**. The plants grown in the humate solution presented overall higher root growth increment, higher mass increment (with the exception of KRHA30N, KRHA40N and KRHA50N) and higher root division as well as higher nutrition content (by starch content with the exception of KRHA40N). Plants grown in AtonikPro solution showed substantially shorter and less divided roots, but their main roots as well as the lateral roots were significantly thicker and presented the highest mass increments. This may be evocated by the nitrophenolate nature of AtonikPro, since it was mainly designed to increase the yield of crop plants [273]. The highest mass increment was observed by the plants grown in the mixture of 40 mg L^{-1} KRHA10N and 0.04 vol% of AtonikPro (solution 13).

From the humic solution samples, the KRHA50N is considered as the most successful. Although it did not performed higher mass increment, than the control solution and its nutrition content were only slightly higher, it showed the highest root growth increment and root division. Other samples useful for the root growth increment and root division were the KRHA30P and KRHA20P similarly as the KRHA30N, KRHA20N and KRHA10N.

As for the mass increment, most significant results were obtained from solutions with AtonikPro, however the samples KRHA5P, KRHA30P, KRHA20P and KHA showed some significant effect on the plants' mass increase too. Interestingly, the samples prepared by means of HNO_3 regeneration did not present positive effects on the mass increment, while they did on the root length and division.

The root division was assessed by a novel method, where the root scanning was followed by the image science evaluation by means of HarFa software (*vide supra*). Image science is now thoroughly incorporating into the biological and environmental sciences [226],[227]. In this work, the image science will bring better accuracy (over the manual assessment) while keeping to the quick and cheap acquisition (over to the microscopy methods). The images in the same resolution were thresholded to black (background) and white (root) images on the same level of thresholding, therefore the K[BW] – the fractal measure – (by means of HarFa obtained value) shows us the number of pixels on the black and white border, which means the root division or the fractal measure of the root (the more K[BW] pixels, the longer is the border between root and background). The highest root division (which is important for the plant's nurture, anchoring in the soil and survival even at adverse conditions [84]) was

observed on plants grown in KRHA50N, KRHA30N, KRHA30P, KRHA65N and KHA solutions.

Results obtained by this simple *Zea mays* hydroponic test demonstrated that all of the tested humates exhibit positive biological activity on the plants of corn. Both samples with lower molecular weights (ex. KRHA50N) and higher molecular weights (ex. KRHA30P) were found highly positively active, including that fact, that according to the both DAD (at 280 nm) and RID HPSEC chromatograms, the molar weight distribution by the KRHA50N sample was favored to the lower molecular weights moieties while by the KRHA30P sample the higher molecular weight fractions are more dominant. This is in line with the statements of *Canellas* [82], *Zandonadi* [84] or *Vlčková* [200], who determined already, that rather than the molecular weight distribution, the chemical composition and properties may be the driving factor of the humates growth regulation.

Whichever mechanism of the humics biological activity will be in the future stated as the leading one (either the hormone-like activity of the auxin-like moieties, gibberelines or cytokinins and their effects on the amount of plasma membrane H^+ATPase or the principle, that the humics are increasing the expression of genes encoding the H^+ATPase hormone and therefore increasing its activity, together with the NO_3^- root-to-shoot distribution), it is clear that the regenerated humic substances perform biological activity, generally similar or in particular cases (KRHA50N, KRHA30P ...) higher than the KHA sample. This leads to considerations, which have been already predicted by the results of *Vlčková* [200], that oxidation of parental lignite leads to higher biological activity of the resulting humate product, while the mechanism is not yet sure, the oxidation of the humic moiety and/or introducing new acidic, –OH or amino- or nitro- groups may play a role in this effect.

From the simplified point of view of humic substances effect on plant growth, yield or on root division, it can be stated that regenerated potassium humates can achieve effects on maize roots and plants similar to already published results of *Antošová* [94] or *Eyheraguibel* [93], when it is important to note, that root length and division is a key requirement for a plant's ability to survive possible adverse conditions [84].

From the methodology point of view, on the basics of the experiment of *Canellas* [82], *Zandonadi* [84] and *Antošová* [94] it has been successfully developed the most simple, quick, cheap and yet reliable method of basic or pilot testing of the biological activity of humates, which can be performed in almost every laboratory.

Table 10: Biological activity results of humic samples and AtonicPro.

Sample	Concentration	Root growth increment (2×5 plants) [cm]	Mass increment (2×30 plants) [g]	K[BW] – Root division (2×5 plants) [pix]	Starch content (30 plants dry mass) [wt%]	Protein content (30 plants dry mass) [wt%]
Control (0)	–	16.4	26.60	17 513	31.65	10.72
AtonikPro (1)	0.04 vol%	7.3	28.55	13 560	34.81	12.03
KHA (2)	40 mg L^{-1}	18.8	30.20	29 577	33.59	11.23
KRHA10N (3)	40 mg L^{-1}	19.6	29.00	24 807	33.60	11.80
KRHA20N (4)	40 mg L^{-1}	19.9	27.70	21 625	31.97	11.57
KRHA30N (5)	40 mg L^{-1}	20.3	24.75	32 089	31.45	11.86
KRHA40N (6)	40 mg L^{-1}	18.1	26.30	27 648	28.82	11.23
KRHA50N (7)	40 mg L^{-1}	21.3	25.75	33 835	31.45	11.06
KRHA65N (8)	40 mg L^{-1}	18.5	29.25	30 610	33.63	11.86
KRHA5P (9)	40 mg L^{-1}	19.8	31.00	23 723	32.73	12.65
KRHA10P (10)	40 mg L^{-1}	19.4	27.25	25 901	32.94	12.88
KRHA20P (11)	40 mg L^{-1}	20.7	30.15	28 471	31.63	11.51
KRHA30P (12)	40 mg L^{-1}	21.2	31.00	32 089	31.09	10.77
KRHA10N + AtonikPro (13)	40 mg L^{-1} + 0.04 vol%	7.0	38.10	13 580	32.02	11.46
KRHA30P + AtonikPro (14)	40 mg L^{-1} + 0.04 vol%	7.2	32.45	13 144	32.03	11.29

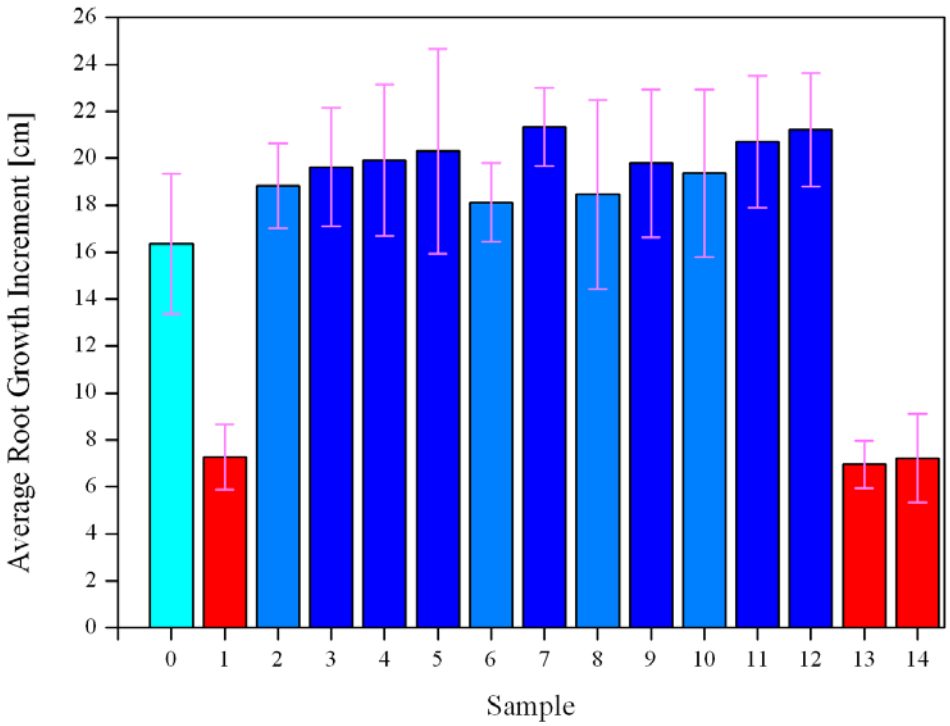


Figure 53: *Average root growth increment in cm for the 2 x 5 selected Zea mays plants and 15 sample solutions (for sample number assignment see **Table 10**).*

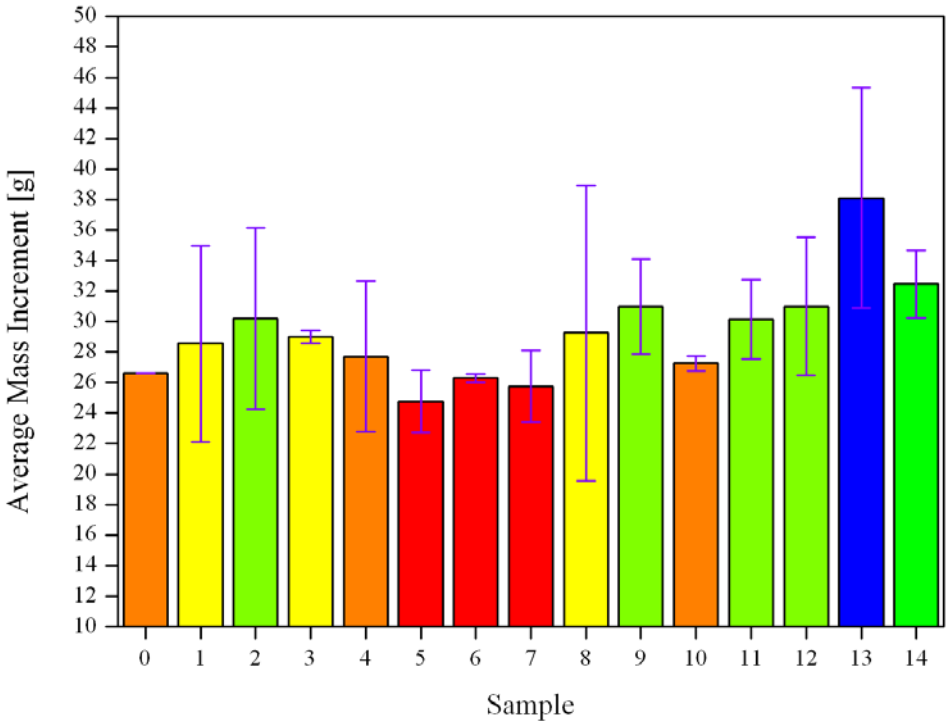


Figure 54: *Average mass increment in g for the 2 x 30 Zea mays plants and 15 sample solutions (for sample number assignment see **Table 10**).*

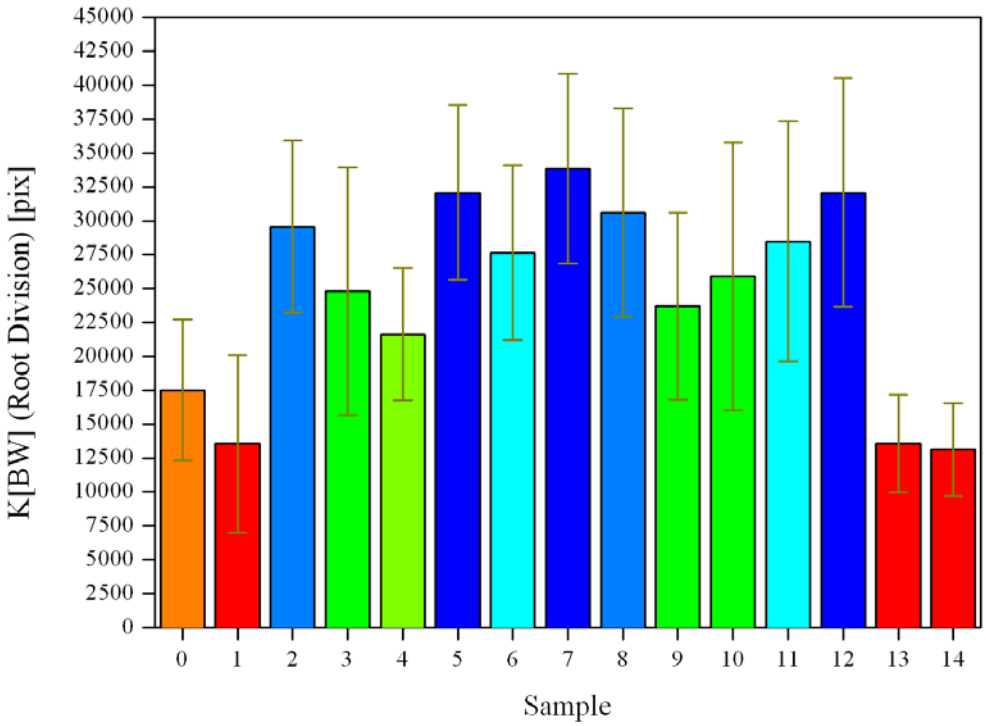


Figure 55: *Average root division (the K[BW] HarFa value in pix) for the 2 x 5 selected Zea mays plants and 15 sample solutions (for sample number assignment see **Table 10**).*

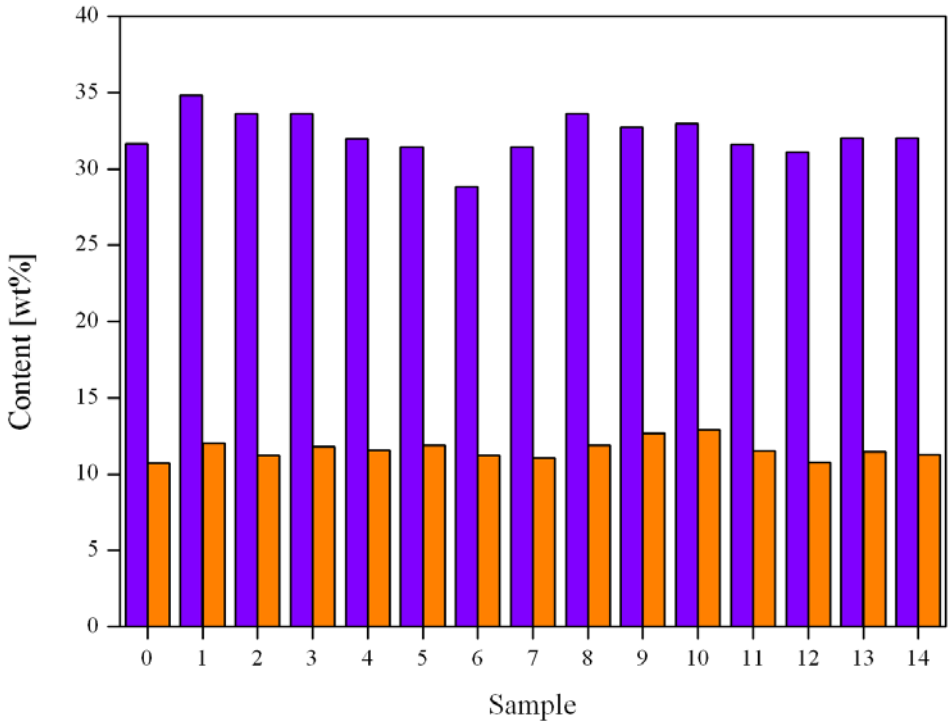


Figure 56: *Starch (violet) and protein (orange) content in wt% in the dry mass for the 30 Zea mays plants and 15 sample solutions (for sample number assignment see **Table 10**).*

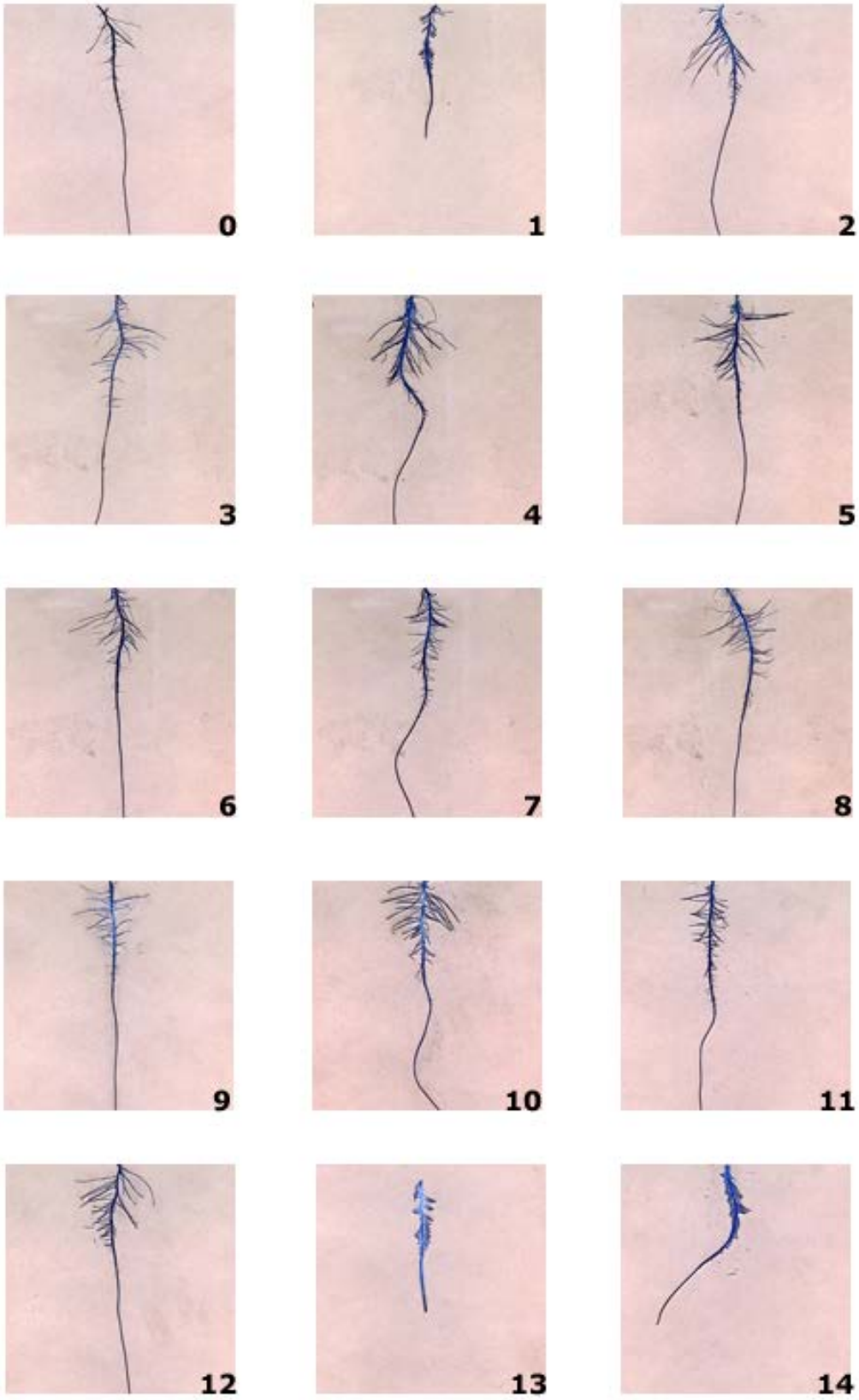


Figure 57: *Selected pictures of root scans of Zea mays grown in 15 sample solutions – differences in length and division may be seen simple by eye. For better readability and printability, the scans are turned negative (for sample number assignment see* ***Table 10****).*

5.4. Pilot Studies on Alternative Humic Applications

5.4.1. Interactions with Tetracycline Antimicrobial

In this pilot testing, the main goal was to distinguish lignites (as available raw materials or byproducts) from the extracted humic acid from the point of the future use as a sorbent of organic pollutants. This application is not completely novel, however in the case of South Moravian lignite materials, mostly the sorption of inorganic compounds was researched. For this purpose, crude milled lignite (LIG), humic acid extracted from parental lignite (HA) and RLIG30N (as a representative of regenerated lignite) were chosen while the sorbate tetracycline antimicrobial was assessed, as an increasingly emerging pollutant [149],[158]. Time test for 8 values from 2 to 48 hours have been performed on the LIG sample and 100 mg L^{-1} tetracycline concentration (**Table 11** and **Figure 58**). Generally, the concentration of tetracycline was decreasing with time.

Table 11: Time test of tetracycline (100 mg L^{-1}) sorption onto LIG (crushed and dried lignite) sample.

Time. [h]	Resulting conc. [mg L^{-1}]	Decrease (i.e. sorbed amount) [mg L^{-1}]	Decrease [%]	Decrease [mg]	Decrease [g_{TC} $g_{sorb.}^{-1}$]
2	64.469	35.531	35.53	0.711	0.00355
4	42.446	57.554	57.55	1.151	0.00576
8	27.834	72.166	72.17	1.443	0.00726
12	31.721	68.279	68.28	1.366	0.00683
24	17.151	82.849	82.85	1.657	0.00828
48	10.144	89.856	89.86	1.797	0.00899

In the concentration tests, the parental lignite (LIG) was assessed as the most successfull sorbent for tetracycline, while RLIG30N was the least successfull. Therefore, probably no effort is needed either in regeneration of lignite or humic acid extraction, when the desired application lies in tetracycline sorption. Generally, all the designed sorbents showed complete sorption of tetracycline in concentration from 0.1 to 1.0 mg L^{-1} which is again in the line with potential applications, since the concentrations of tetracycline in the environment vary from ones to hundreds of μg L^{-1} in waters [159], μg kg^{-1} in soils [157],[158], up to 20 mg L^{-1} in manure [156]. The LIG and HA samples may be considered suitable also for the treatment of higher concentrations of tetracycline, e.g. in manure or in some major spills.

Since this is a pilot study and even despite the time test of sorption has been provided, the equilibrium concentration has not been reached. With respect to the findings of *Borisover and Graber*, who stated, that the sorption of organic compounds onto organic matter (incl. humic substances) is strongly affected by the phenomena of humic material hydration and swelling. From numerous results of sorptions of organic contaminants like *m*–nitrophenol, nitrobenzene, acetophenone and benzyl alcohol onto IHSS Pahokee peat and respective humic acid and humin, from the environments of water and *n*–hexadecane (i.e. from wet and dry environment), it became evidential, that hydration may affect the sorption both by increasing or decreasing, according to the particular sorbate applied. In particular cases, the sorption isotherm could not have been determined [274]. Later, *Borisover et al.* supposed a mechanism, how sorbent–sorbate interactions may be weakened by the water–sorbate

competition over the available places for sorption on organoclays, like montmorillonite, etc. [275]. Recently, *Borisover et al.* presented results of a sorption of carbazepamine (*5H*–dibenz[*b*,*f*]azepine–5–carboxamide) onto parental natural organic matter (NOM) – the IHSS Pahokee peat from water and *n*–hexadecane with the result of possibility of construction of absorption isotherm, however, the equilibration of the system mixture in water took 14 days. For carbazepamine, in particular, the hydration of the NOM extended the interactivity with the sorbate [276].

Independently on the experiments of *Borisover*, *Jaeger et al*., and *Schaumann and LeBoeuf* reached the proof of the humic material hydration phenomena and surface changes evocated by this hydration by means of different methods, namely the Temperature Modulated Differential Scanning Calorimetry (TMDSC) and aforementioned ^{1}HNMR Relaxometry. The slow swelling process in humic materials experiencing a hydrating environment may take from days to weeks or even months and this may affect the sorption and transport processes of pollutants [277],[253],[278] mainly with respect to the progressive and up to now not predictable change in the sorption surface. Unfortunately, many of these results (like [276] and [253]) have been published after the author of this thesis performed these experiments. Nevertheless, results of this work support the observation about the non-equilibrium conditions used in many papers issued in last several decades to determine equilibrium parameters of Freundlich or Langmuir isotherms. *Jaeger et al.* [253] observed the progressive change in the state of water in peat sample as long as seven months and concluded that hydration and swelling of organic matter is governed by three processes with time constants in the range of minutes (fast processes), hours (medium fast processes) and weeks/months (slow processes) with related apparent activation energies of 5–50 kJ mol^{-1} indicating the breaking of hydrogen bonds, water diffusion and reorientation of SOM chains during hydration, respectively.

Thus, in this pilot and novelty study, an effort has been given to test the suitability of obtained and prepared solid humic materials (LIG, HA and RLIG30N) for tetracycline removal from water and not to elucidation of such mechanisms, which would greatly extend the range of this pilot study in terms of this dissertation. As for the results, the best tetracycline removal from water was achieved employing the parental lignite (LIG) as the sorbent, where the lowest resulting concentration of tetracycline and the highest ratio of grams of tetracycline sorbed per grams of humic material sorbent was achieved. Humic acid from the parental lignite presented mediocre results, apparently, some of the moieties attractive for the tetracycline sorbate were either lost during the HA extraction from the lignite or the effective surface of lignite differed with the comparison with resulting material. In the lignite regenerated by means of 30 vol% nitric acid, poor results were achieved. The explanation of this appearance may be in the sorbent material surface change as well as partially enhanced dissolving in the water environment, which may be induced by the regeneration. Supposably, the tetracycline may interact also with the solved moieties of the regenerated lignite, therefore stayed in the supernatant and not in the solid sorbent. For the clear overview of these results, see the **Table 12** and **Figure 58**. This leads to the first conclusion, that for the environmental sorption application, the easiest and cheapest way should be followed, the usage of the raw lignite itself. When compared to the literature, the results are comparable to the findings of *von Wandruszka and Newell* (sorption of trichloroethylene onto raw leonardite humic acid, where about 99.98 to 99.99 wt% of the sorbate in initial concentrations from 20 to 300 mg L^{-1} was removed) [129] and to the revelations of *Avisar et al.* (where about 87–88 % of tetracycline was sorbed from water onto Na–montmorillonite clay in the presence of Sigma-Aldrich humic acid) [169].

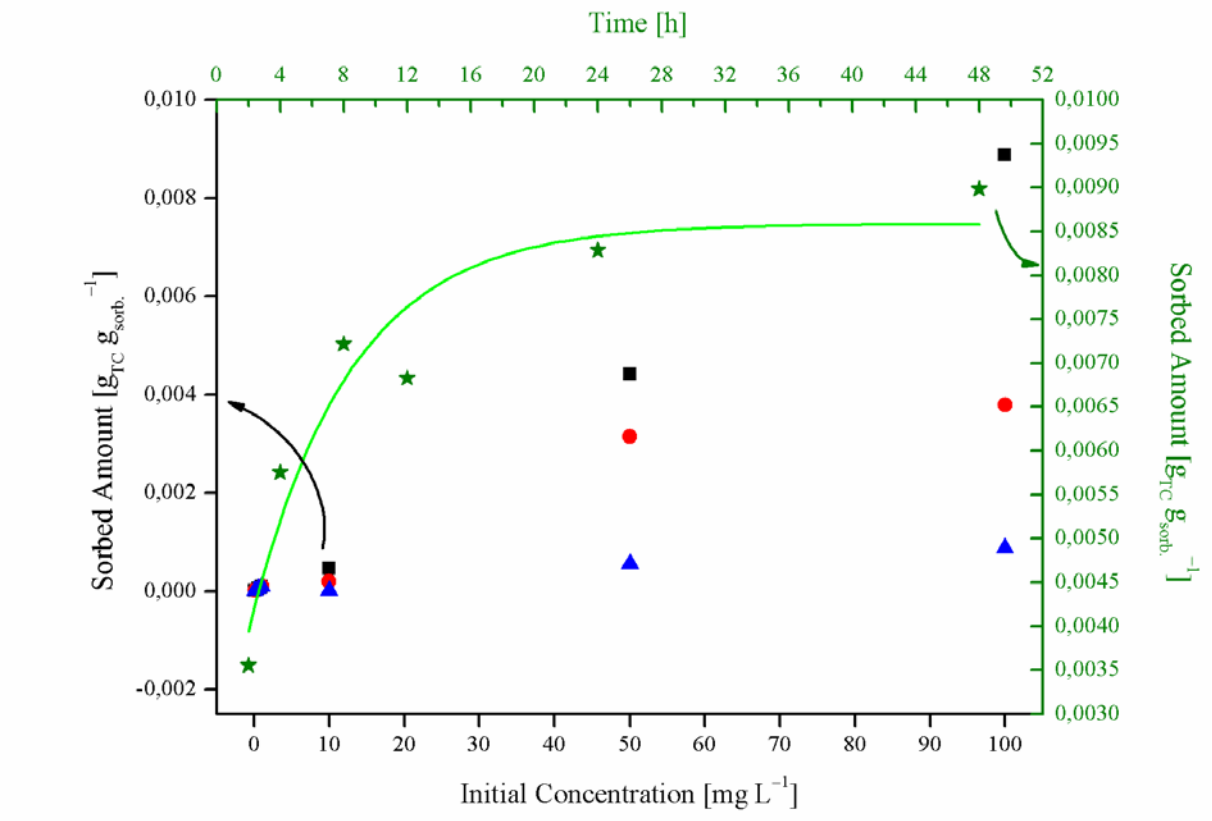


Figure 58: *Sorbed amount of tetracycline (after 48 hours) onto lignite (black squares), humic acid (red circles) and RLIG30N (blue triangles). The time test results are added as green stars and light green exponential decay fitted line (sorption of 100 mg L^{-1} TC onto LIG).*

Table 12: Tetracycline sorption results.

Sorbent	Initial conc. [mg L^{-1}]	Resulting conc. [mg L^{-1}]	Decrease (i.e. sorbed amount) [mg L^{-1}]	Decrease [%]	Initial amount. [mg]	Decrease [mg]	Decrease [g_{TC} g_{sorb}^{-1}.]
LIG	0	0.000	0.000	–	0.000	–	–
	0.1	0.000	0.100	100.00	0.002	0.002	0.00001
	0.5	0.000	0.500	100.00	0.010	0.010	0.00005
	1.0	0.000	1.000	100.00	0.020	0.020	0.00010
	10	5.373	4.627	46.27	0.200	0.092	0.00046
	50	5.853	44.147	88.29	1.000	0.883	0.00441
	100	11.217	88.783	88.78	2.000	1.776	0.00888
HA	0	0.000	0.000	–	0.000	–	–
	0.1	0.000	0.100	100.00	0.002	0.002	0.00001
	0.5	0.000	0.500	100.00	0.010	0.010	0.00005
	1.0	0.000	1.000	100.00	0.020	0.020	0.00010
	10	7.965	2.035	20.35	0.200	0.041	0.00020
	50	18.514	31.486	62.97	1.000	0.630	0.00315
	100	62.020	37.980	37.98	2.000	0.760	0.00380
RLIG30N	0	0.000	0.000	–	0.000	–	–
	0.1	0.000	0.100	100.00	0.002	0.002	0.00001
	0.5	0.000	0.500	100.00	0.010	0.010	0.00005
	1.0	0.000	1.000	100.00	0.020	0.020	0.00010
	10	9.959	0.041	0.41	0.200	0.001	$4.1\ 10^{-6}$
	50	44.372	5.628	11.26	1.000	0.113	0.00056
	100	91.194	8.806	8.81	2.000	0.176	0.00088

5.4.2. **********

********** [279],[200],[233].

**********[201].

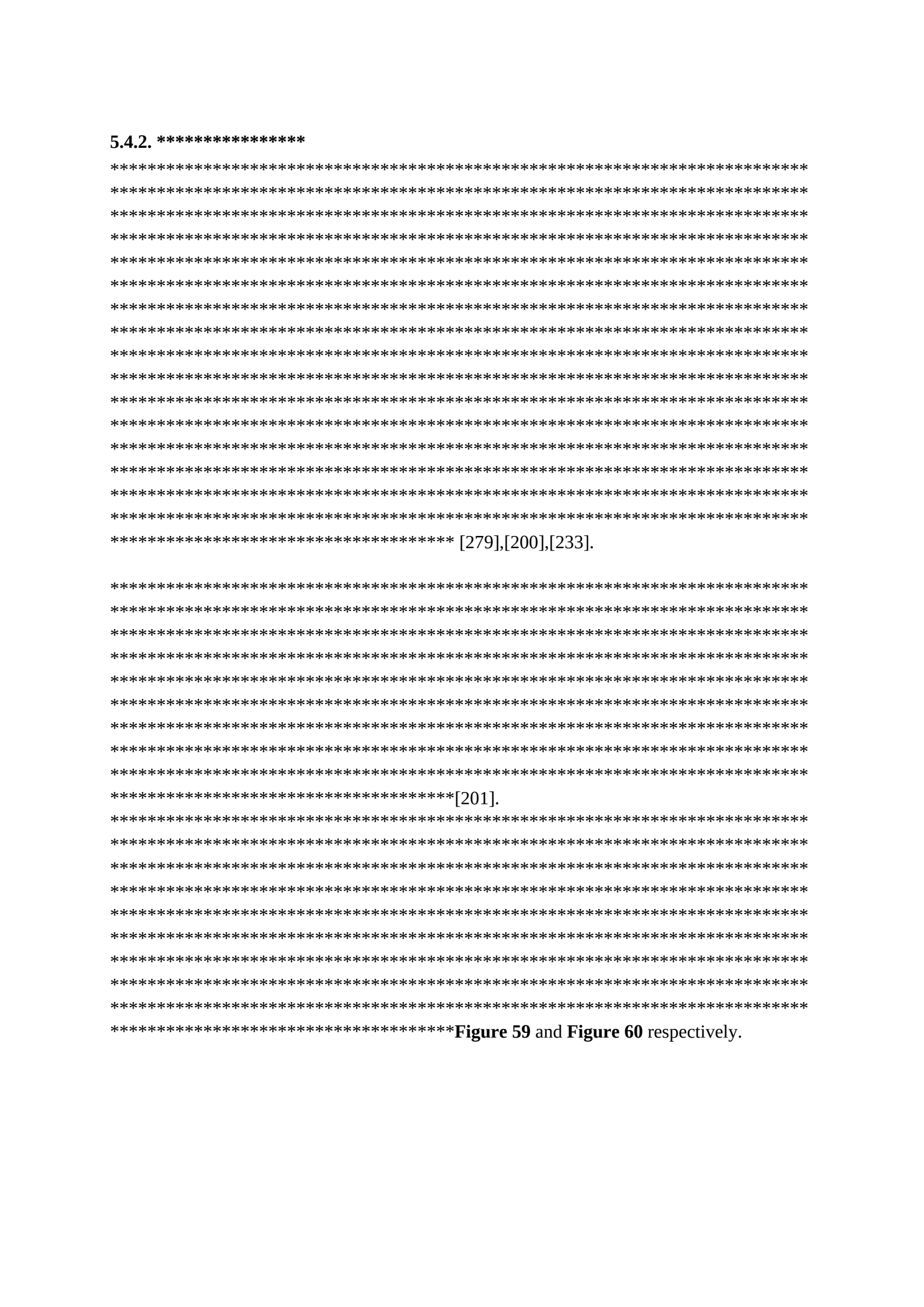
**********  **Figure 59** and **Figure 60** respectively.

Figure 59: **********

Figure 60: **********

5.5. Overall Statistical Approach

Generally, with employment of such a high amount analytical techniques together with biological activity assessment, a need for a statistical method in discussion of the results is emerging. Pearson's correlation coefficient (*r*) is (however supplying information about linear correlations only) an advantageous used statistical analysis for results obtained from multiple techniques. From humic substances aimed works, *exempli gratia*, it has been used in the work of *Vlčková et al.*, where it served for the appreciation of the influence of molecular weight and polyols and carboxylic content or C/H ratio onto the humics' biological activity [200].

Principal Component Analysis (PCA) is a statistical technique used for reduction of symptomatic space dimension and identifying patterns in the data in the way that helps to highlight similarities and differences in the particular data. With advantage, it is applied on data, where the number of results (variables, features ...) exceeds the number of samples. Its matematical apparatus is in detail described in other publications, therefore is no need to

repeat it here (e.g. [280–282]). PCA has experienced a limited use in the humic substances research, e.g. *Thomsen et al.* used it for describing the similarities and distinctnesses of particular set of humic acids, fulvic acids and natural mixtures of both from the point of view of aromaticity/aliphaticity and increasing/decreasing content of ketonic and O–substituted aromatic groups (when analyzed by means of liquid state ^{13}C NMR and UV–VIS) [282]. As for other authors, *Peña-Méndez et al.* used PCA for describing the similarity/difference of fingerprint MS spectra of various humic acids [283]. In this work it is used mainly for the comparison of respective humic acid samples and to their assortment according to similarities and distinctnesses. Since a lot of data from many different techniques are obtained here, PCA seems to be a valuable method for the final statistical assessment.

The Pearson's correlation coefficients are reported in **Table 13**, **Table 14** and **Table 15**. The first two tables show correlations of the samples in solid state and in liquid state separately. The third table presents correlations of the solid state characteristics of humic acids with the liquid state of respective potassium humate salts in terms of the biological activity together. Usually, the solid state samples and liquid state samples should be correlated separately, however, the elemental analysis and presence of functional groups detected by means of FTIR are, from the work of *Vlčková et al.*[200] presumed to perform a significant influence on biological activity, therefore they are (as an exception) correlated here together too. The **Table 13** which stands for the solid samples correlation, tells generally only that information, that the results of FTIR in terms of aliphatic, aromatic and amidic groups content do correlate with results of elemental analysis, which describe the C, H, O and N content. Moreover, the information from thermogravimetry (about the lesser thermogravimetric stability of regenerated humics) is confirming the fact [284], that the regeneration (while introducing oxygen and nitrogen contaning moieties into the humic acid structure) is collaterally reducing the thermal stability of the samples, as it can be deducted from the negative correlation of O and/or N content and 1st TGA onset temperature. Generally, the more H (aliphaticity) in the humic sample, the lower is the amount of ash after the thermogravimetrical analysis, since there is a negative correlation between (r = – 0.778). This negative correlation implies, that in the ash content some residual carbon may be present.

In **Table 14** the correlations between the properties of liquid potassium humate samples are reported. Generally, it can be assumed, that measured parameters perform only limited amount of correlation. The average aggregate particle size (AAPS) assessed by means of DLS interestingly seems to have no correlation with the aggregate sizes (molar weights assessed by means of both DAD and RID detected HPSECs. There is a slightly negative trend indication between sample absorptivity (detected by DAD HPSEC) and AAPS (r = –0.410), which implies that the absorptive moieties may have possessed a lesser propensity to aggregation. No interesting correlations of density and ultrasonic velocity with other magnitudes were observed, while obvious correlations (e.g. density and hydration, since hydration is calculated using density) are not discussed. Only the hydration performed a very slight trend indication with DAD peak absorptivity (r = –0.311), so, the more absorptive samples may tend to be more difficult to hydrate. As for the HPSEC results, the M_N values obtained by means of DAD detector (respective for the 280 nm set wavelength) are in a good correlation with those obtained by means of RID (r = 0.844), however, the correlation of DAD M_W and RID M_W is negative (r = –0.413). As well, negative is the correlation of M_N and M_W obtained by DAD,

while the correlation of both molar weights obtained by RID is positive (r = 0.893). This can be ascribed either to the fact, that the respective detectors are sensitive to different moieties. Unfortunately, it is necessary to remark, that since the results of KRHA40N sample (mainly the DAD HPSEC M_W result) are obviously strongly deviated but still included in and contributing to this statistical approach. On the basis of these findings, the KRHA40N sample may be labeled as artefactual. The author of this thesis regrettably has no other explanation for this behavior of the KRHA40N sample, as something may have influenced either the regeneration of lignite (with consideration of the possibility, that 40 vol% HNO_3 concentration may have cleaved the lignite moieties into some specific other ones, which may be enriched with N containing groups and tend to aggregate back together, therefore may have created a unique sample) or the extraction of the humic acid sample. Also, the inhomogeneities could have been included in the specific portion of the crude lignite, which was used for this sample preparation. The biological activity properties correlations have shown some interesting results: the root division K[BW] presented slight negative trend with AAPS (r = –0.419) while the AAPS presented also slight negative trend with growth increment (r = –0.432). Thus it seems that root growth and root division are more promoted by humates, which do not tend to aggregate into the greater aggregates. The molecular weights, in particular the M_N as obtained from the RID had shown moderate positive correlations with the root growth increment (r = 0.617) and the mass increment of the whole set of 30 plants (r = 0.772) (where also positive correlation with the M_W obtained by the RID (r = 0.627) was found). The DAD results showed positive correlation with the mass increment of the whole set of 30 plants (r = 0.500) in the case of M_N, while negative trend (r = –0.598) was the result in the case of M_W. It is necessary to have a respect to the possibility of influence on these correlations of DAD results from the artefactuality of the KRHA40N sample. Positive correlations of molecular weights with root growth increment and plants mass increment are in line with the previous findings of *Nardi et al.* [77] and *Canellas et al.* [90] that the molecular weight of humates may not be the driving influence onto their biological activity and the higher molecular weight humates may present a biological activity too (however not penetrating to the plant itself, but remaining on the root surface). The more absorptive moieties containing humates seem to induce the root growth of *Zea mays* according to the correlation of r = 0.694. From the point of view of correlations between the biological properties, root division presented the expected slight trend with Root growth (r = 0.328). Plants with higher root division contained slightly lower levels of proteins (r = –0.527).

In the **Table 15**, the correlations between the solid state properties and biological properties are reported. There can be seen positive correlations of O and N content as well as the aliphatic and amidic FTIR Peak intensity ratios with root division and root growth, however also surprising negative nitrogen content in humic material trend onto the biological activity in terms of plants mass increment (r lies ca. between –0.600 and –0.700).

Table 16 shows the correlations between particular molecular weights fractions (as detected by DAD at 280 nm) (see **Figure 49**) and biological characteristics. Habitually, the high molecular weight fractions (> 100 000 g mol^{-1}) as well as the low molecular weight fractions perform no correlations or negative trends with the biological characteristics (e.g. r = –0.571 in case of trend of 30 plants mass increment and > 100 kg mol^{-1} mass fraction or

r = –0.654 in case of root growth and 0–15 kg mol^{-1} fraction). In general, the molecular mass fractions of humates seem not to show any influence on the plants' root division, therefore, the already mentioned crucial factor of plants' survival looks as not to be dependent on the molecular mass fraction of applied humate. The most influential in the biological activity's point of view are the middle weight fractions of humates (usually from 30 to 100 kg mol^{-1}) where generally slightly positive (or at least trendy) correlations have been found, such as r = 0.614 and r = 0.538 between the root growth and 70–100 kg mol^{-1} fraction and 30 plants mass increment and 50–70 kg mol^{-1} fraction respectively. Similar results have been obtained for the plants' nutritional properties (sugar and protein content). This results are both in the line with previous results of experiments with generally milder regenerated humates of *Vlčková et al.* [200] as well as the results of *Canellas et al.* [90], (in the particular case of root division) who's results actually first tested the presumption, that humate molecular weights may have an influence onto root growth and/or division, and concluded this presumption as wrong. As for the work of *Nardi* [77], these results are not in the agreement with the *Nardi's* presumption of greatest biological activity of humates of molar weights to 3.5 kg mol^{-1}, but in line with the later works, which presuppose the biological importance of the higher molar weight humates, either penetrated into the plant's root or aggregated on its surface [80],[82],[84],[90].

Table 13: Pearson's correlation coefficients of solid humic acid samples.

	Elemental Analysis [at%]								TGA [°C]	TGA ash [wt%]	FTIR Transmittance				FTIR Relative peak intensities		
Variables	**C**	**H**	**N**	**O**	**H/C**	**N/C**	**O/C**	**(N+O)/C**	**1st onset**	**at 650°C**	**aliph. 2930 cm^{-1}**	**Ar C=C 1459 cm^{-1}**	**C–O, –OH 1422 cm^{-1}**	**amidic 1520-1550 cm^{-1}**	**2930/1459**	**1459/1422**	**amid/1459**
C	1	–0.362	–0.663	–0.135	–0.769	–0.690	–0.501	–0.579	0.612	0.459	0.417	0.414	0.388	0.546	0.348	–0.359	–0.739
H	–0.362	1	–0.406	–0.864	0.872	–0.375	–0.619	–0.550	0.290	–0.778	0.106	0.152	0.214	0.222	0.326	0.922	–0.303
N	–0.663	–0.406	1	0.723	0.062	0.999	0.895	0.954	–0.776	0.094	–0.513	–0.566	–0.583	–0.736	–0.728	–0.268	0.932
O	–0.135	–0.864	0.723	1	–0.516	0.706	0.925	0.872	–0.625	0.612	–0.301	–0.336	–0.390	–0.479	–0.433	–0.828	0.687
H/C	–0.769	0.872	0.062	–0.516	1	0.098	–0.158	–0.073	–0.109	–0.753	–0.135	–0.102	–0.046	–0.125	0.048	0.815	0.183
N/C	–0.690	–0.375	0.999	0.706	0.098	1	0.890	0.951	–0.773	0.077	–0.508	–0.560	–0.575	–0.733	–0.721	–0.239	0.939
O/C	–0.501	–0.619	0.895	0.925	–0.158	0.890	1	0.987	–0.781	0.357	–0.420	–0.451	–0.488	–0.628	–0.521	–0.582	0.886
(N+O)/C	–0.579	–0.550	0.954	0.872	–0.073	0.951	0.987	1	–0.798	0.269	–0.461	–0.500	–0.531	–0.680	–0.604	–0.478	0.927
TGA 1st onset [°C]	0.612	0.290	–0.776	–0.625	–0.109	–0.773	–0.781	–0.798	1	0.160	0.370	0.391	0.415	0.518	0.377	0.354	–0.663
TGA ash at 650°C	0.459	–0.778	0.094	0.612	–0.753	0.077	0.357	0.269	0.160	1	0.230	0.193	0.145	0.106	–0.048	–0.690	0.154
aliph. 2930 cm^{-1}	0.417	0.106	–0.513	–0.301	–0.135	–0.508	–0.420	–0.461	0.370	0.230	1	0.994	0.990	0.952	0.718	0.041	–0.520
arom. C=C 1459 cm^{-1}	0.414	0.152	–0.566	–0.336	–0.102	–0.560	–0.451	–0.500	0.391	0.193	0.994	1	0.998	0.970	0.785	0.059	–0.556
C–O, –OH 1422 cm^{-1}	0.388	0.214	–0.583	–0.390	–0.046	–0.575	–0.488	–0.531	0.415	0.145	0.990	0.998	1	0.972	0.789	0.126	–0.567
amidic 1520-1550 cm^{-1}	0.546	0.222	–0.736	–0.479	–0.125	–0.733	–0.628	–0.680	0.518	0.106	0.952	0.970	0.972	1	0.834	0.113	–0.737
2930 / 1459	0.348	0.326	–0.728	–0.433	0.048	–0.721	–0.521	–0.604	0.377	–0.048	0.718	0.785	0.789	0.834	1	0.113	–0.687
1459 / 1422	–0.359	0.922	–0.268	–0.828	0.815	–0.239	–0.582	–0.478	0.354	–0.690	0.041	0.059	0.126	0.113	0.113	1	–0.203
amid/1459	–0.739	–0.303	0.932	0.687	0.183	0.939	0.886	0.927	–0.663	0.154	–0.520	–0.556	–0.567	–0.737	–0.687	–0.203	1

Table 14: Pearson's correlation coefficients of liquid potassium humate samples.

Variables	DLS AAPS*	ρ	HRUS U12	Hydration	DAD HPSEC P.A.	DAD HPSEC at 280 nm M_N	M_W	PDI	RID HPSEC M_N	M_W	PDI	K[BW] Root division	Growth increment	30 Plants mass increment	Sugar content	Protein content
DLS AAPS*	1	–0.151	0.070	–0.127	–0.410	–0.225	0.108	0.065	–0.281	–0.111	0.009	–0.419	–0.432	–0.166	0.466	0.346
ρ	–0.151	1	–0.242	0.928	–0.238	–0.347	0.158	0.234	–0.345	–0.314	–0.258	0.043	–0.067	–0.491	–0.257	–0.003
HRUS U12	0.070	–0.242	1	0.137	–0.178	0.036	0.034	0.033	0.110	0.059	0.029	–0.041	–0.391	0.423	0.118	0.051
hydration	–0.127	0.928	0.137	1	–0.311	–0.340	0.174	0.251	–0.310	–0.297	–0.252	0.028	–0.219	–0.338	–0.217	0.017
DAD HPSEC P.A.	–0.410	–0.238	–0.178	–0.311	1	0.840	–0.700	–0.797	0.744	0.637	0.575	0.044	0.694	0.315	0.171	0.275
DAD HPSEC at 280 nm M_N	–0.225	–0.347	0.036	–0.340	0.840	1	–0.457	–0.669	0.844	0.910	0.880	–0.043	0.617	0.500	0.066	0.097
M_W	0.108	0.158	0.034	0.174	–0.700	–0.457	1	0.959	–0.557	–0.413	–0.400	–0.169	–0.598	–0.245	–0.630	–0.226
PDI	0.065	0.234	0.033	0.251	–0.797	–0.669	0.959	1	–0.696	–0.621	–0.618	–0.059	–0.668	–0.351	–0.584	–0.239
RID HPSEC M_N	–0.281	–0.345	0.110	–0.310	0.744	0.844	–0.557	–0.696	1	0.893	0.812	0.021	0.428	0.772	0.381	0.124
M_W	–0.111	–0.314	0.059	–0.297	0.637	0.910	–0.413	–0.621	0.893	1	0.983	0.032	0.525	0.627	0.200	–0.094
PDI	0.009	–0.258	0.029	–0.252	0.575	0.880	–0.400	–0.618	0.812	0.983	1	–0.007	0.543	0.540	0.202	–0.110
K[BW] Root division	–0.419	0.043	–0.041	0.028	0.044	–0.043	–0.169	–0.059	0.021	0.032	–0.007	1	0.328	–0.228	–0.209	–0.527
Growth increment	–0.432	–0.067	–0.391	–0.219	0.694	0.617	–0.598	–0.668	0.428	0.525	0.543	0.328	1	0.010	–0.118	–0.282
30 Plants mass increment	–0.166	–0.491	0.423	–0.338	0.315	0.500	–0.245	–0.351	0.772	0.627	0.540	–0.228	0.010	1	0.420	–0.007
Sugar content	0.466	–0.257	0.118	–0.217	0.171	0.066	–0.630	–0.584	0.381	0.200	0.202	–0.209	–0.118	0.420	1	0.452
Protein content	0.346	–0.003	0.051	0.017	0.275	0.097	–0.226	–0.239	0.124	–0.094	–0.110	–0.527	–0.282	–0.007	0.452	1

Table 15: Pearson's correlation coefficients of chemical composition with humates' biological activity.

	Elemental Analysis[at%]								FTIR Relative peak intensities ratios		
	C	**H**	**N**	**O**	**H/C**	**N/C**	**O/C**	**(N+O)/C**	**2930 / 1459**	**1459 / 1422**	**amid/1459**
K[BW] Root division	–0.267	–0.186	0.270	0.397	0.025	0.276	0.442	0.396	0.050	–0.120	0.421
Growth increment	–0.114	0.381	–0.285	–0.282	0.310	–0.281	–0.212	–0.241	0.527	0.240	–0.197
30 Plants mass increment	0.475	0.290	–0.696	–0.528	–0.024	–0.686	–0.645	–0.676	0.350	0.291	–0.631
Sugar content	0.391	0.044	–0.399	–0.247	–0.150	–0.389	–0.359	–0.379	0.364	0.064	–0.323
Protein content	0.181	0.214	–0.183	–0.398	0.040	–0.180	–0.398	–0.332	0.168	0.248	–0.377

Table 16: Pearson's correlation coefficients of molecular weight distribution fractions (detected by DAD at 280 nm) with humates' biological activity.

	> 100 kg mol^{-1}	**70–100 kg mol^{-1}**	**50–70 kg mol^{-1}**	**30–50 kg mol^{-1}**	**15–30 kg mol^{-1}**	**0–15 kg mol^{-1}**
K[BW] Root division	–0.036	0.137	–0.200	–0.221	–0.118	0.100
Growth increment	–0.514	0.614	0.538	0.311	–0.199	–0.654
30 Plants mass increment	–0.371	0.420	0.502	0.206	–0.195	–0.491
Sugar content	–0.752	–0.173	0.422	0.713	0.628	–0.203
Protein content	–0.279	–0.082	0.271	0.353	0.230	–0.145

Principle correlation analysis results are reported in **Figure 61** and in **Figure 62**. The set of humic samples properties was assessed separately for the solid state humic acid samples and dissolved potassium humates. The measure of similarity here is the distance of the sample marks (blue dots), the lower distance the higher similarity (as it was already interpreted before in the work of *Thomsen et al.* [282]. The overall variability in the two components (as reported in the graphs is 80% by the solid samples but only 60% by the liquid samples. Here, the reset may be affected by the artefactuality of the KRHA40N sample. The most significant variabilities (the sample properties which discriminate the sample from the others the most) are depicted as red dots and described in italic font. They belong to the nearest sample. In the graph of solid samples (**Figure 61**) it can be seen, that the samples can be divided into 5 groups (HA, then RHA30P, then RHA40N, then the first (RHA65N + RHA20N + RHA30N + RHA50N) and second (RHA10N + RHA10P + RHA5P + RHA20P) groups of similar samples. According to this PCA, the regeneration of lignite showed significant influence onto the resulting humic acid properties. The HA sample from parental lignite has no similar samples around, as well as the RHA30P and RHA40N. The HA sample presented differences in the thermogravimetric as well as in the constitution (EA and FTIR) results. RHA30P sample differs by its H/C ratio, i.e. aliphaticity. Distance of the RHA40N sample may be ascribed to its already mentioned artefactuality but also to its FTIR relative C–O, –OH and amidic peak intensities.

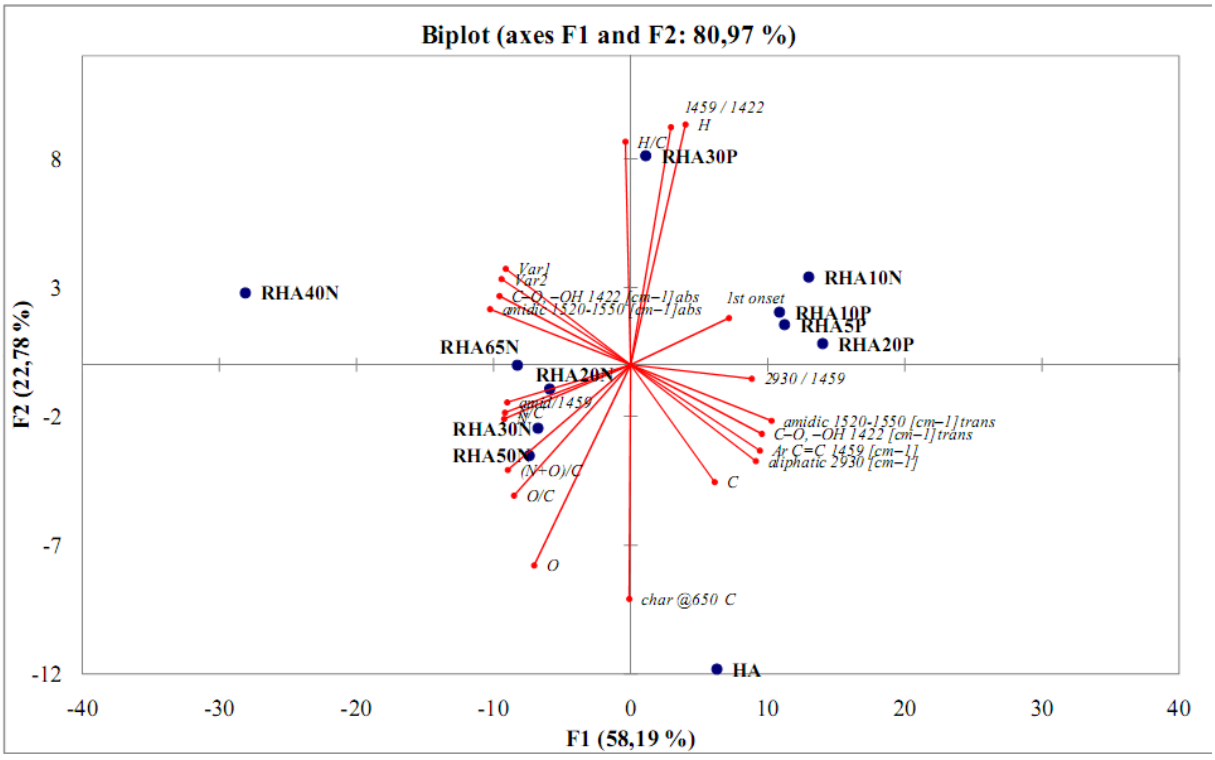


Figure 61: *Principle Component Analysis of solid humic acid samples.*

In the graph of liquid samples (**Figure 62**) the similarities of samples KHA + KRHA20N and further of KRHA5P + KRHA10P samples can be seen. The other samples are generally different from each other, the KRHA65N sample mainly for its average aggregate particle size, and the KRHA30N sample for its hydration value, the KRHA30P sample distinct in absorptivity (DAD peak area) and in biological activity. The KRHA40N sample presents clearly visible complete distinctness in the DAD obtained M_W, which is the most reasonable explained by its artefactuality.

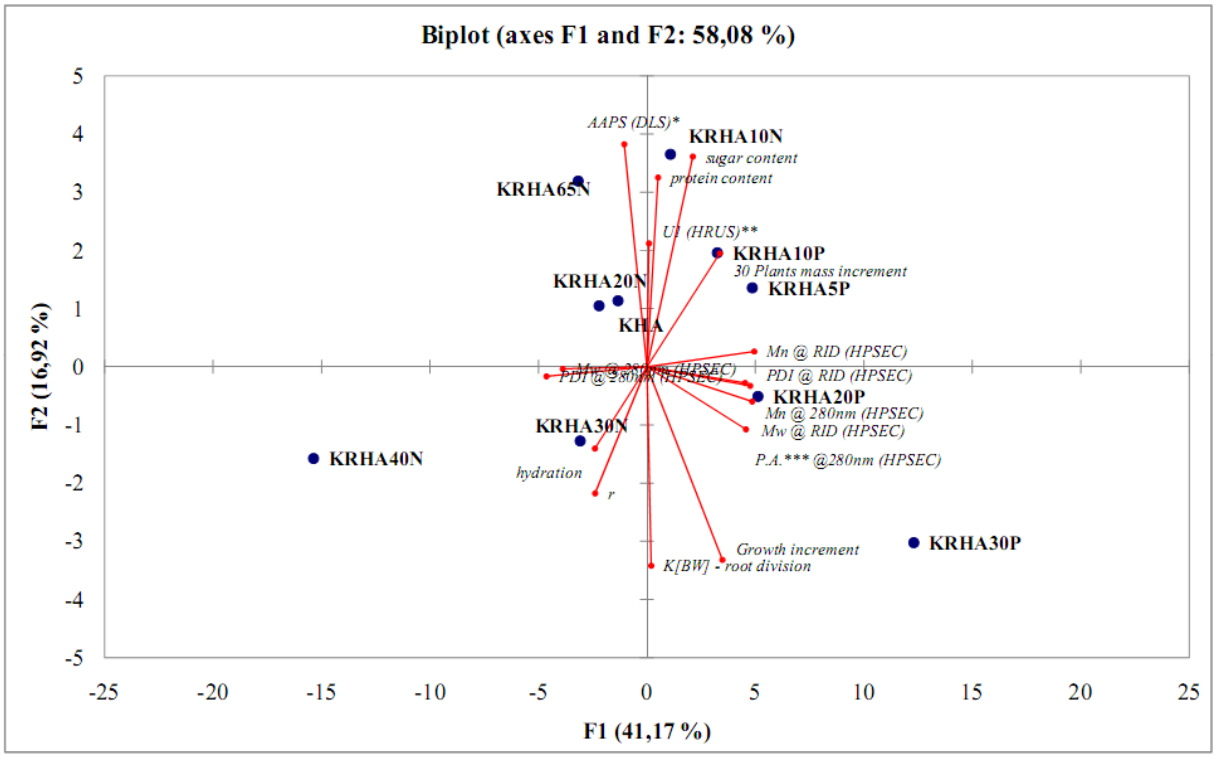


Figure 62: *Principle Component Analysis of liquid potassium humate samples.*

As a general synthesis of the statistical approach, the assumption of humic substances as complex structures has been proved, while no clear identifications of interconnections between their physical, chemical and biological properties can be found. However, the PCA showed, that the respective regenerations of lignite (by means of various regeneration agents and their concentrations) allows further to extract a set of humic acid samples, which are very distinct from each other.

6. CONCLUSIONS

- Based on oxidation of lignite by means of HNO_3 and H_2O_2, novel regenerated humic acids and potassium humates were successfully prepared. In general, the regeneration with nitric acid increases significantly the further yield of humic acid extraction (with the exception of RHA10N sample) while regeneration with hydrogen peroxide slightly decreases the further yield of humic acid extraction (with the exception of RHA20P sample). Yields of regenerated filtrates were very low or negligible, therefore no significant amount of desired humic material is lost during the lignite regeneration.

- Regenerated humic acids presented higher amount of H and O, as well in the terms of H/C ratio, therefore it can be assumed, that the regeneration of lignite increases resulting humic acids aliphaticity. With the exception of RHA10N sample, regeneration with HNO_3 brought N moieties into the samples and increased the N content. As for the elemental composition, the RHA_N samples are comparable to the soil humic acids rather than to lignite humic acids. With HNO_3 as agent, the quality of the humic sample therefore can be enhanced twice. Elemental analysis results were supported employing FTIR, where all the spectra are generally comparable, containing common humic moieties, but the increased aliphaticity and incorporated N moieties are confirmed.

- FFC NMR Relaxometry showed relaxation times of water protons in studied humic acids in the ranges maximally up to hundreds of milliseconds, therefore, the present water is considered adsorbed or confined. For respective humic acids, relaxation times are generally rising with rising concentration of applied regeneration agent, but in case of HNO_3 only up to 40 vol%., ergo, the regeneration of lignite generally increases the surface of the consequently extracted humic acids. This technique showed a great potential for further research, not only of soil samples, but also of the particular humic acids.

- From the physico-chemical point of view, samples of humates showed aggregation even in diluted solutions, therefore they are not behaving like common surfactants. Average particle sizes ranged from 100 to 500 nm, which is comparable to other humic samples, e.g. the IHSS standards. In non-isothermal conditions, the regenerated samples presented modified behavior similar to loam or soil humates. Ultrasonic velocity, density and therefore compressibility showed oscillatory behavior, while mainly hydrophobic hydration may be the driving contributor here. All the studied humates presented high levels of hydration, from 0.45 to 0.95 grams of water per gram of humate, which is even comparable to the most hydrated hydrophilic polysaccharide – hyaluronan. This can presuppose the humates not only for the soil conditioner and remediation applications, but in the future maybe also on the field of biomedicine.

- When analyzed using fluorescence spectrometry, the samples showed common humic EEM spectra with one or two maxima. The regenerated samples presented lower fluorescence intensity. The slight maxima shifts can be also ascribed to the oxidative regeneration of samples. These results are currently being an inspiration for further humic research in the field of transport and conducting systems.

- Employing the HPSEC, on both DAD and RID detectors the humates shown bimodal distributions of the first high and narrow peak ascribed to the long chains of aliphatic moieties, and the second broad peak of later retention time ascribed to the shorter

chains, fragments and unsaturated structures. In terms of rt, both detectors responded with similar chromatograms. Nitric acid regeneration enhanced the both detectors signal responses therefore it probably brought any additional chromophores into the humate samples. Regeneration with HNO_3 (in particular by the KRHA40N and KRHA65N samples) enhanced the second – broad peak of smaller molecular weight moieties, while regeneration with H_2O_2 *vice versa*.

- As expected and already written, regeneration increases the humic system absorptivity, while the absorptivity is decreasing with the increasing wavelength. The detectors showed significant distinctions in observed molar weights. These differences are ascribed to the distinct signal generation in the detectors. In terms of RID detection, the KRHA_N samples showed higher molecular weights while KRHA_P samples the lower molecular weights.

- The regeneration of lignite, in further extracted humic acids, favors the middle sized molecular weights (30–100 kg mol^{-1}) when performed by HNO_3. For 40 vol% HNO_3, a unique sample with enhanced both low and high (0–15 and > 100 kg mol^{-1} respectively) molar weights was obtained, while in the H_2O_2 regenerated samples, generally the higher molecular weights were favored.

- Simple, quick, undemanding and reliable hydroponic method for determination of biological activity of humates has been derived. The observed properties were the root growth increment (best results obtained with KRHA50N, KRHA30P, KRHA 20P and KRHA30N samples), the mass increment of the whole set of 30 whole plants (best results gained from the AtonikPro mixtures with humates, AtonikPro itself and KRHA5P sample) and the root division, where the best results were achieved by the KRHA50N, KRHA30N and KRHA30P samples. The mass increment is important for the agricultural yield, while the root growth and root division are crucial for the plant survival and prosperity in possible worsen conditions. Both lower molecular weights samples (e.g. KRHA50N) and higher molecular weight samples (e.g. KRHA30P) showed highly positive biological activity effects. In general, all of the humates presented beneficial effect onto the *Zea mays* roots and whole plants

- **As for the future activities, the biological activity assessment of humates according to *Aguirre et al.* [96] utilizing the real time reverse transcription DNA is hereby proposed, while the future research of biological activity phenomenon may contain the synergistic way of physical-chemical aspects, analytical techniques, plant growth assessment as well as the molecular biology approach together.**

- In this thesis, two future applications of humic materials are proposed by means of pilot studies. Crude lignite has shown a potential to be excellent sorbent for tetracycline antimicrobial, from low and environmental to high (manure or some accidental spills) concentrations. For these sorbent applications, there is no need of humic material regeneration or extraction of pure humic acid, since these two samples presented worse results. For future research, the sorption test may be modified according to *Borisover et al.* [275] to include and explain the effect of humic material hydration and swelling.

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- The Principal component analysis classified the solid humic acids and liquid humates into the groups according to their similarity and proved the distinctnesses in the prepared samples.

- **Overall, this thesis successfully showed how to prepare a series of distinct regenerated humic acid and humate samples from one crude lignite material. The regeneration is the way how to at least partially control the resulting humate properties and tune them according to the future application. The regeneration reaction yields humates different in both physic-chemical and biological properties and with potential industrial or agriculture applicability. From the industrial point of view, the regeneration is easy to perform, and for the regeneration agents' price or waste management question, they can be easily recycled either in the next lignite regeneration process or in some downcycling inorganic chemistry application, such as liquid fertilizer production.**

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8. ABBREVIATIONS

ADP	=	Adenosine diphosphate.
ATP	=	Adenosine triphosphate.
Ar	=	Aromatic moiety sign, mainly used in the FTIR terminology.
CHNS/O	=	Elemental Analysis; analytical technique, see EA.
Co.	=	Company; business entity.
CP-MAS NMR	=	Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance; analytical technique.
DAD	=	Diode Array Detector (detector for chromatographic techniques).
DDT	=	Dichlorodiphenyltrichloroethane (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane.
DLS	=	Dynamic Light Scattering; analytical technique.
DTG	=	Derivative ThermoGravimetry; technique in Thermal Analysis.
dpi	=	dots per inch, a measure of computer picture sharpness.
EA	=	Elemental Analysis; analytical technique.
EDTA	=	Ethylenediaminetetraacetic acid or its sodium salt.
EEM	=	Excitation-Emmission Matrix, technique in fluorescence Spectrometry.
EPDM	=	Ethylene Propylene Diene Monomer Rubber.
EPR, ESR	=	Electron Paramagnetic (Spin) Resonance; analytical technique.
ex.	=	For example.
FA, FAs	=	Fulvic acid, fulvic acids.
FIDE	=	Free industion decay.
FFC NMR	=	Fast Field Cycling Nuclear Magnetic Resonance Relaxometry; analytical technique.
FTIR	=	Fourier Transform Infrared Spectrometry; analytical technique.
GC	=	Gas Chromatography; analytical technique.
GC-MS	=	Gas Chromatography-Mass Spectrometry; analytical technique.
GmbH	=	Gesellschaft mit beschränkter Haftung; limited liability company; Business entity in Austria, Germany and Switzerland.
HA, HAs	=	Humic acid, humic acids.
HARFA	=	Harmonic and Fractal Image Analyzer, an image science software.
HL	=	Huminová látka, huminové látky.
HS	=	Humic substance, humic substances.
HPLC	=	High Performance Liquid Chromatography; analytical technique.
HPLC-MS/MS	=	High Performance Liquid Chromatography with mass spectrometry detection; analytical tandem technique
HPSEC	=	High Performance Size Exclusion Chromatography; analytical technique.
HRUS	=	High Resolution Ultrasonic Spectrometry; analytical technique.
IHSS	=	International Humic Substances Society.
Inc.	=	Incorporated; business entity.
K_{OW}	=	Octanol – water partition coefficient.
K(R)HA	=	parental or regenerated (R) potassium humate.
LHA	=	Leonardite Humic Acid.

L.L.C.	=	Limited Liability Company; business entity.
Ltd.	=	Limited Liability Company; business entity.
mRNA	=	messenger ribonucleic acid.
NMR	=	Nuclear Magnetic Resonance; analytical technique.
NMRD	=	Nuclear Magnetic Resonance Dispersion; analytical technique.
PCB, PCBs	=	polychlorinated biphenyl(s).
RHA, RFA		
RFILT	=	regenerated humic acids, fulvic acids, filtrates.
RT PCR	=	Reverse Transcription Polymerase Chain Reaction; molecular biology analytical technique.
SEM	=	Scanning Electron Microscopy; analytical technique.
SOM	=	Soil Organic Matter.
SPE	=	Solid Phase Extraction – sample preconcetration technique in analytical chemistry.
s.r.o.	=	s ručením omezeným; limited liability company – a business entity in the Czech Republic.
ST	=	Surface Tension; analytical technique.
S/V	=	Surface to volume ratio.
TA	=	Thermal analysis; set of analytical techniques.
TC	=	Tetracyclines; class of antimicrobial drugs.
™	=	Trade Mark; indicator of intellectual property.
TMDSC	=	Temperature Modulated Differential Scanning Calorimetry; analytical technique.
UV	=	Ultra-violet.
UV-VIS	=	Ultra-violet Visible Spectrometry; analytical technique.

All the symbols and magnitudes are explained in the text.

9. AUTHOR'S UP TO DATE ACTIVITIES

9.1. PUBLICATIONS

9.1.1. Research Papers

1) David, J.; Weiter, M.; Vala, M.; Vyňuchal, J.; Kučerík, J. Stability and structural aspects of diketopyrrolopyrrole pigment and its N-alkyl derivatives. *Dyes and Pigments*, 2011, 89, 1, pp. 137–143. ISSN: 0143-7208.

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9.1.2. Conference Proceedings

1) DAVID, J.; KUČERÍK, J.: Physical-Chemical Properties and Application Potential of Humates Prepared from Regenerated Lignites. In *Advances in natural organic matter and humic substances research 2008-2010 - XV Meeting of the International Humic Substances Society Proceedings vol. 3.* Sevilla & Puerto de la Cruz, Španělsko: International Humic Substances Society, Institutional Repository of Consejo Superior de Investigaciones Científicas (CSIC), 2010. pp. 262-265.

2) Obruča, S., Márová, I., Vojtová, L., David, J., Ondruška, V., Babák, L.: Bacterial biodegradation of modified polyurethane foams: comparison of single and mixed culture. In *THE THIRD INTERNATIONAL MEETING ON ENVIRONMENTAL BIOTECHNOLOGY AND ENGINEERING: BOOK OF ABSTRACTS.* 1. Palma de Mallorca, Govern de les Illes Baleares. 2009. p.74. ISBN 978-84-692-4948-2.

3) David, J., Vojtová, L., Bočková, J., Kučerík, J, Mravcová, L., Vávrová, M., Jančář, J.: Synthesis, Characterization and Modification of PLGA-PEG Biocompatible Hydrogels. In *8th World Biomaterials Congress 28 May - 1 June 2008 Amsterdam RAI The Netherlands Abstracts2View Abstracts on CD-ROM PC & Mac CD-ROM.* Amsterdam, Abstracts2View - Marathon Multimedia. 2008. p.1186.

4) Ondruška, V., Márová, I., David, J., Vojtová, L., Kotlík, J.: Influence of modified biocomposites on production of extracellural polysaccharides by immobilized Aureobasidium Pullulans. In *Zem v pasci? 2008.* Vyhne, Zvolen, Technical University in Zvolen. 2008. pp. 534–535. ISBN 978-80-228-1848-3.

5) Vojtová, L., David, J., Obruča, S., Márová, I., Vávrová, M., Jančář, J.: Bio-polyol Based Polyurethane Foams. In *Sborník příspěvků.* 1. Zlín, UTB. 2008. p. 1 - 5. ISBN 978-80-7318-687-6.

6) Zlámalová Gargošová, H., Vávrová, M., Čáslavský, J., Vránová, J., David, J.: Ecotoxicological Evaluation of Polyurethane Foams. In *8th European Meeting on Environmental Chemistry: Book of Abstracts and Final Programme.* 1. Inverness, Environmental Research Institute. 2007. p.34.

7) David, J. Characterization Of Newly Prepared Biodegradable Polyether Based Flexible Polyurethane Foams. In *Sborník konference Chemie a společnost CD-ROM.* 1. Brno, Czech Republic, Faculty of Chemistry, Brno University of Technology. 2007. pp. 38–39. ISBN 978-80-214-3555-1.

8) David, J., Vojtová, L., Bednařík, K., Kučerík, J., Vránová, J., Vávrová, M., Jančář, J.: Study of new flexible polyurethane foams with biomass polyols. In *1st Bratislava Young POlymer Scientists Workshop Book.* 1. Bratislava, Slovakia, Slovak Academy of Sciences. 2007. p.23. ISBN 978-80-968433-4-3.

9) Vávrová, M., Čáslavský, J., Mácová, D., David, J.: Sledování průniku produktů degradace polymerů. In *XVI. medzinárodné vedecké sympózium O EKOLÓGII VO VYBRANÝCH AGLOMERÁCIÁCH JELŠAVY – LUBENÍKA A STREDNÉHO SPIŠA.* 1. Košice, Ústav geotechniky SAV. 2007. pp. 74–79. ISBN 978-80-8077-070-9.

10) Márová, I., Duroňová, K., Obruča, S., Ondruška, V., Mikulcová, A., Kučerík, J., David, J., Vojtová, L.: Analysis of genotoxicity of biocomposite degradation products using Saccharomyces cerevisiae D7 test system. In *Book of abstracts.* Slovakia, SAS. 2007. p. 57. ISBN 0-01-336483-9.

11) Márová, I., Ondruška, V., Obruča, S., Trčková, M., Vojtová, L., David, J.: Biodegradation of modified biocomposites by Aureobasidium pullulans. In *Book of abstracts.* Slovakia, SAS. 2007. p.98. ISBN 0-01-336483-9.

13) Vojtová, L., Vávrová, M., Bednařík, K., Šucman, E., David, J., Jančář, J.: Preparation and ecotoxicity assessment of new biodegradable polyurethane foams. *Journal of Environmental Science and Health, Part A.* 2007. A42(5). pp. 677–683. ISSN 1093-4529. (IF=0,967).

14) Hrdličková, J, Obruča, S., Ondruška, V., David, J., Márová, I., Vojtová, L., Jančář, J.: Use of bacterium Arthrobacter globiformis to biodegradation of selected biomaterials. In *Book of abstracts.* 1., Brno. 2007. p. 54. ISBN 978-80-210-4234-6.

15) Vojtová, L., Jančář, J., Babák, L., Márová, I., David, J., Vávrová, M.: Biodegradable polyurethane foams. In *Polymery 2006, Nezařazené články.* 1. Třešť, Ústav makromolekulární chemie AVČR. 2006. p. KS14 (3 p.). ISBN 80-85009-54-4.

16) Márová, I., Obruča, S., Ondruška, V., David, J., Vojtová, L., Babák, L., Jančář, J.: Biodegradation of modified polyurethane foams by Aureobasidium pullulans and thermophillic bacteria:a pilot comparative study. In *34th Annual Conference on Yeasts, Book of abstracts. Smolenice, Slovakia*, Institute of Chemistry, SAS. 2006. p. 84. ISSN 1336-4839.

9.2. ATTENDED EVENTS

9.2.1. Internships Abroad

1) 05–08/2009: von Wandruszka Laboratory, Department of Chemistry, College of Science, University of Idaho, Moscow, ID, U.S.A.

2) 10/2005 – 02/2006: Faculty for Technical Chemistry, Vienna University of Technology, Vienna, Austria.

9.2.2. Scientific Conferences

1) 06–07/2010: *XV Meeting of International Humic Substances Society*, Puerto de la Cruz, Tenerife, Canary Islands, Spain.

2) 06/2009: *64th Northwestern Regional Meeting of American Chemical Society*, Pacific Lutheran University, Tacoma, WA, U.S.A.

3) 09/2008: *4th Meeting on Chemistry and Life*, Faculty of Chemistry, Brno University of Technology, Czech Republic.

4) 05/2008: *8th World Biomaterials Congress*, Amsterdam RAI, The Netherlands.

5) 08/2007: *1st Bratislava Young POlymer Scientists Workshop*, Smolenice Chateau, Slovakia.

6) 05/2007: *2nd International Conference on Polymeric Materials in Automotive*, Technopol Congress Centre, Bratislava, Slovakia.

7) 12/2006: *7th European Meeting on Environmental Chemistry*, Faculty of Chemistry, Brno University of Technology, Brno, Czech Republic.

9.2.3. Student Scientific Conferences

1) „7. Studentská odborná konference Chemie a Společnost", Faculty of Chemistry, Brno University of Technology, 2007; special prize.

2) „8. Slovenská študentská vedecká konferencia v odbore chémie a chemickej a potravinárskej technológie", Bratislava, Slovakia, 2006; special prize – Dean's Diploma.

3) 6th year of „Student FCH", Faculty of Chemistry, Brno University of Technology, 2006; 3rd place – Dean's Prize.

9.2.4. Workshops

1) Moravian Library seminaries on various national and international patent databases searching, 10–12/2010, Brno, Czech Republic.

2) BioMedCentral workshop on "open access" scientific publishing, Academy of Sciences of the Czech Republic, Prague, Czech Republic, 04/2008.

3) Mettler Toledo workshop on thermal analysis, Brno, Czech Republic, 04/2008.

4) Netzsch / Malvern Bohlin / Anamet workshop on thermal analysis and rheology, Brno, Czech Republic, 11/2007.

5) Agilent Atomic force microscopy / Scanning probe microscopy workshop, Prague, Czech Republic, 09/2007.

9.2.5. Faculty of Chemistry, Brno University of Technology Events

Participating at events like „Noc vědců", „Den otevřených dveří", „Den Chemie", ChemPoint – Vědci pro chemickou praxi, etc.