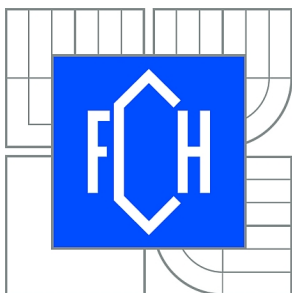


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BRNO UNIVERSITY OF TECHNOLOGY



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HYDRATACE HUMINOVÝCH LÁTEK

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ABSTRACT

This doctoral thesis studies the character of hydration water in water/humic substances system. The goal is to determine both quantitative and qualitative aspects of hydration of humic substances (HS) in solid and liquid phase and to explore the differences in properties of water surrounding humic matter with the assistance of high resolution ultrasonic spectroscopy (HRUS) and methods of thermal analysis such as differential scanning calorimetry (DSC) and thermogravimetry (TGA). The main aim of this work is to contribute to the knowledge about hydration of humic samples originating from different sources and thus having different properties and composition applying the approaches and techniques already used and reported as being successful for determination and enumeration of hydration water in hydrophilic biopolymers and hydrogels. This thesis investigates the effect of water on humic structure, its way of wetting and penetrating the surface of HS, the possible conformational changes, the retention capacity of HS, and also the influence of origin of individual humic substance on hydration properties with regard to the kinetic of those processes. Moreover, it tries to recognize the influence of degree of humification on hydration processes of humic substances as well as reversibility of these processes. The results of this thesis reveal same parallelism with the properties of non-reversible hydrogels; some similarities between biopolymers and HS were discovered as well.

ABSTRAKT

Tato disertační práce studuje charakter hydratační vody v systému voda/huminová látka. Úkolem je určit jak kvantitativní, tak i kvalitativní aspekty hydratace huminových látek (HS) v pevné i kapalně fázi a prozkoumat rozdíly ve vlastnostech vody obklopující huminovou látku s použitím vysokorozlišovací ultrazvukové spektroskopie (HRUS) a metod termické analýzy, jako je diferenční kompenzační kalorimetrie (DSC) a termogravimetrie (TGA). Hlavním cílem této práce je přispět k objasnění problému hydratace huminových látek pocházejících z různých zdrojů a majících proto odlišné vlastnosti a složení, a to s využitím postupů a technik, které se již dříve osvědčily při stanovení hydratační vody v hydrofilních polymerech. Tato práce zkoumá účinek vody na strukturu huminových látek, způsob, jakým voda smáčí jejich povrch a jak jimi proniká, způsobuje změny v konformaci HS, jejich retenční kapacitu a také vliv původu jednotlivých huminových látek na jejich hydratační vlastnosti s ohledem na kinetiku těchto procesů. Dále studuje vliv stupně humifikace na hydratační procesy huminových látek, stejně jako reverzibilitu těchto procesů. Výsledky této práce objasňují paralelu s vlastnostmi hydrogelů a podobnosti i odlišnosti mezi biopolymery a huminovými látkami.

KEYWORDS

humic substances, hydration, water, thermal analysis

KLÍČOVÁ SLOVA

huminové látky, hydratace, voda, termická analýza

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DECLARATION

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

PROHLÁŠENÍ

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

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student's signature
podpis studenta

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1. INTRODUCTION

Recent advances in analytical chemistry, especially in methods Nuclear Magnetic Resonance and Mass Spectrometry together with molecular fractionation techniques enable to take a closer look into molecular composition of complicated mixtures. Whereas the chemistry and physics of ordinary classes of biomolecules such as proteins, polysaccharides or DNA occurring in plant tissues and animal bodies have already been relatively well investigated, humic substances belong to a specific group of chemical substances. The specificity is caused by several factors: i) there is still no way to predict the structure of humic substances even if the parental material is known, ii) despite the advances in evaluation of primary structure of humic substances, there are still some undiscovered issues, iii) humic substances consist of heterogeneous supramolecular structure stabilized by weak interactions creating a vast of physical sub-structures. It is noteworthy that the information on primary humic composition is rarely combined with the character of physical (secondary) humic structure and further with its function in natural systems. Their role is, among others, closely linked with water holding capacity of soils and cell biology of soil living organisms. As they are both hydrophobic and hydrophilic in nature, their function is closely related to properties of water shell intimately bound on humic molecules and consequently on humic aggregates. Thus, the qualitative and quantitative aspects of interactions of water molecules with humic substances are crucial in order to understand how those processes work.

2. THE STATE OF THE ART

2.1 Humic substances

The term "humus" originates from the Romans, when it was familiarly used to signify the entire soil. Later the term was used to denominate soil organic matter and compost or for different parts of this organic matter, as well as for complexes created by chemical agent treatments to a wide palette of organic substances. The principal definition of humus, as decomposed organic matter, originates from 1761 [1].

The first relevant study of the origin and chemical nature of humic substances (HS) was worked out by Sprengel [1]. His comprehensive study on the acidic nature of humic acids is thought to be his most important benefit to humus chemistry. Research on the chemical properties of HS was extended by the Swedish researcher Berzelius, whose main contribution was the isolation of two light-yellow coloured HS from mineral water and slimy mud rich in iron oxides [3]. Enormous advances have been made during the last decade thanks to modern physicochemical methods. Nevertheless, the structural chemistry of lignin and HS did not advance so fast as the chemistry of animal-originated biopolymers [4]. Organic matter in the environment can be divided into two classes of compounds, non-humic material (e.g. protein, polysaccharides, nucleic acids and small molecules such as sugars and amino acids) and humic substances [5].

Among the various naturally occurring organic substances, humic substances (HS) are the most widespread. They form most of the organic component of soil, peat, lignite, natural waters, and their sediments. The most common definition is: "Humic substances are a general category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in colour and refractory." [6]

Early efforts to characterize this material resulted in the following fractionation scheme based on solubility under acidic or alkaline conditions [1]: humin, the insoluble fraction of humic substances; humic acids (HAs), the fraction soluble under alkaline conditions but not acidic conditions (generally $\text{pH} < 2$); and fulvic acids (FAs), the fraction soluble under all pH conditions. Although chemical and physical differences do underlie these variations in solubility, the separation of humic substances into three fractions is operational, and does not indicate, for example, the existence of three distinct types of organic molecule [7].

2.2 The formation of humic substances

Although the biochemistry of the formation process of HS has been studied hard and for a long time, this is still the subject of long-standing and continued research [4]. The substances are undoubtedly mixtures that develop randomly from the decay of plant tissues, from microbial metabolism-catabolism, or from both [8]. Some theories have lasted for years; for example, the sugar-amine condensation theory, the lignin theory or the polyphenol theory. These main pathways of formation of HS are shown in Fig. 1 [1]. A review of such theories can be found in a monograph of Davies and Ghabbour [9].

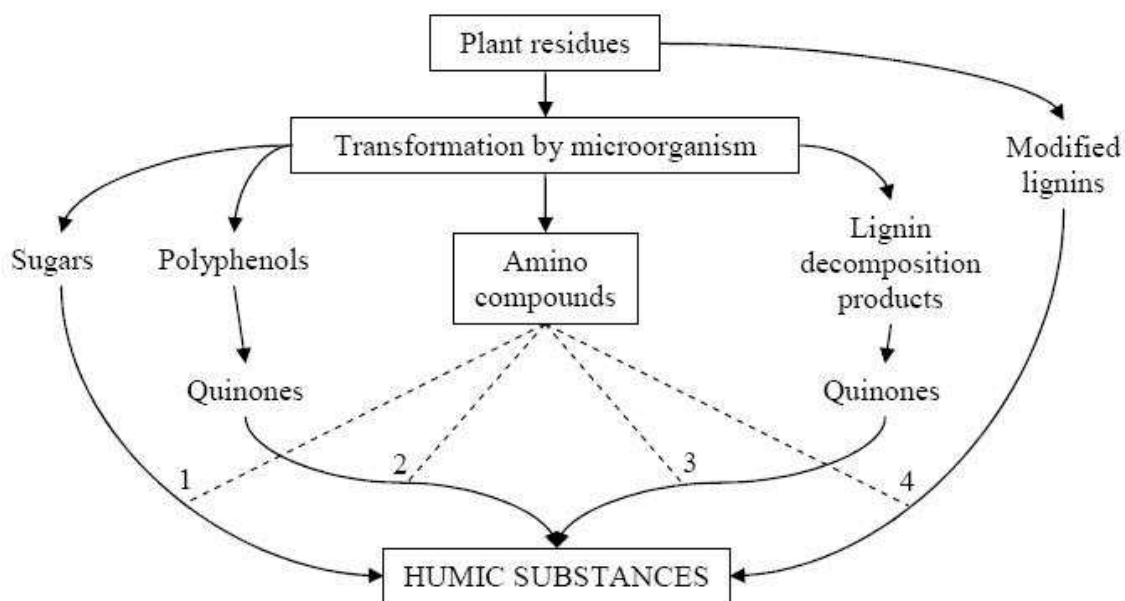


Fig. 1 Mechanisms of the formation of humic substances. Amino compounds synthesized by microorganisms are seen to react with reducing sugars (pathway 1), quinones (pathways 2 and 3), and modified lignins (pathway 4) to form complex dark-coloured polymers [1].

The classical theory, popularized by Waksman [10], is that HS represent modified lignins (pathway 1) but the majority of present-day investigators favour a mechanism involving quinones (pathways 2 and 3). In practise, all four pathways must be considered as likely mechanism for the synthesis of humic and fulvic acids in nature, including sugar-amine condensation (pathway 4).

2.2.1 The Maillard sugar-amine condensation

The notion that humus is formed from sugars (pathway 4) dates back to early days of humus chemistry. According to this concept, reducing sugars and amino acids, formed as by-products of microbial metabolism, undergo non-enzymatic polymerization to form brown nitrogenous polymers. HS are formed from purely chemical reactions in which microorganisms do not play a direct role except to produce sugars from carbohydrates and amino acids from proteins (Fig. 2).

2.2.2 The Waksman lignin theory

According to this theory, lignin is incompletely utilized by microorganisms and the residuum becomes part of the soil humus. Modifications in lignin include loss of methoxyl (OCH_3) groups with generation of *o*-hydroxyphenols and oxidation of aliphatic side chains to form COOH groups. Assuming that HS represent a system of polymers, the initial product would be components of humin, further oxidation and fragmentation would yield first humic acids and then fulvic acids. This pathway is illustrated in Fig. 3.

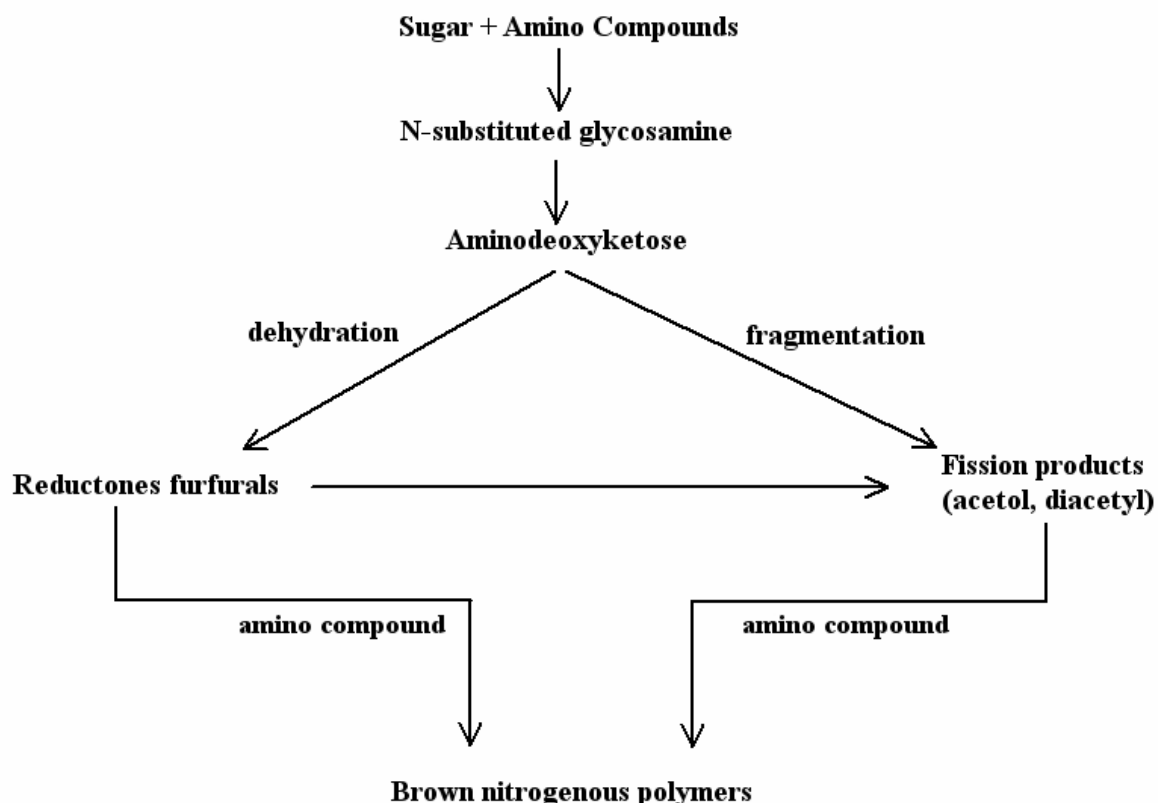


Fig. 2 The theory of sugar-amine condensation [1].

2.2.3 The Flaig polyphenol theory

Pathways 2 and 3 form the basis of the popular polyphenol theory, the starting material consists of low molecular weight organic compounds, from which large molecules are formed through condensation and polymerization. In pathway 3, lignin still plays an important role in humus synthesis, but in a different way. In this case, phenolic aldehydes and acids released from lignin during microbial attack undergo enzymatic conversion to quinones, which polymerize in the presence or absence of amino compounds to form humic-like macromolecules. Pathway 2 is somewhat similar to pathway 3 except that polyphenols are synthesized by microorganisms from non-lignin C sources (e.g., cellulose). The polyphenols are enzymatically oxidized to quinones and converted to humic substances. A schematic representation of the polyphenol theory is shown in Fig. 4.

HS in soil may be formed by all of the mentioned mechanisms. Although a multiple origin is suspect, the major pathway in most soils appears through condensation reactions involving polyphenols and quinones. According to the recent concepts, polyphenols derived from lignin, or synthesized by microorganisms, are enzymatically converted to quinones, which undergo self-condensation or combine with amino compounds to form N-containing polymers. The number of precursor molecules is large and the number of ways in which they combine is astronomical, thereby accounting for the heterogeneous nature of the humic material in any given soil [1].

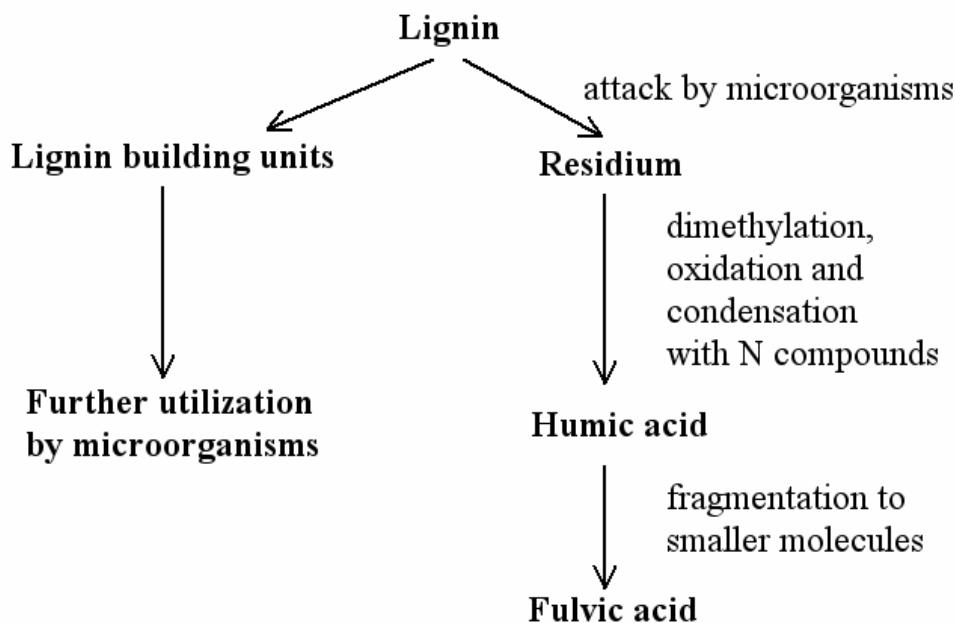


Fig. 3 Schematic representation of the lignin theory of humus formation [1].

It is evident that the mechanisms of the formation of humic substances can be slightly different, depending on geographical, climatic, physical and biological circumstances, respectively. These compounds can be formed in several ways, and the role of lignin is important in the majority of these processes [11-12]. Burdon proposed that humic organic matter consists mainly of a mixture of plant and microbial constituents plus the same constituents in various stages of decomposition (i.e. plant/microbial mixtures of carbohydrates, proteins, lipids and partially degraded lignins, tannins, melanins, etc.) [11].

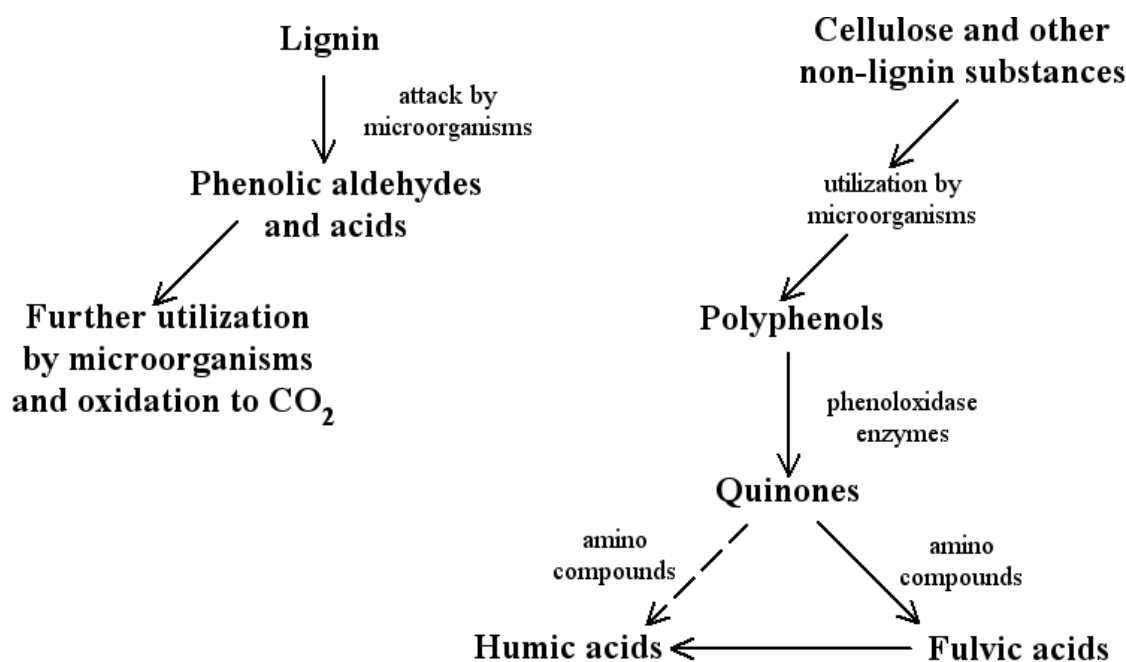


Fig. 4 Schematic representation of the polyphenol theory of humus formation [1].

According to recently introduced concept, humification in soil can be considered as a two-step process of biodegradation of dead-cells components, aggregation of the degradation products [8]. In light of the supramolecular model, one needs not to invoke the formation of new covalent bonds in the humification process that leads to the production of humus. Humification is the progressive self-association of the mainly hydrophobic molecules that resist the biodegradation. These suprastructures are thermodynamically separated by the water medium and adsorbed on the surfaces of soil minerals and other pre-existing humic aggregates. The exclusion from water means exclusion from microbial degradation and the long-term persistence of humic matter in soil.

2.3 The role of humic substances

Up to 70 % of the soil organic carbon and up to 90 % of dissolved organic carbon may occur in the form of humic substances. Humic substances influence groundwater properties and the process of formation of fossil fuels; however, they play a major role in the global carbon geochemical cycle. The global pool of humic matter is an important component in the formation of atmospheric carbon dioxide. The role of humic substances is determined by their formation during the humification (decay) process of living matter. Humic substances form an intermediate phase in the transformation process of living matter (organic carbon reservoir) that continues in the organic carbon cycle or are deposited (as fossil materials). The chemistry 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. it is not surprising that, despite the efforts of many excellent scientists in the distant and recent past (Kononova [13], Stevenson [1]), there is still much to be done to achieve an appropriate awareness of humic chemistry [8].

Despite their role in the sustainability of life, the knowledge of basic chemical nature and the reactivities of HS is still limited. The scientific community of humic scientists has so far failed to provide an unified understanding of this field of science, and there is still, therefore, a poor awareness of fundamental aspects of humic structures and reactivities. Nevertheless, the implications of the relevance of awareness of HA structure should extend far beyond the interests of a few chemists; HA structures affect the ways that the soil ecosystem work, as well as the bioavailability of organic substances in the soil environment [14]. Most of the difficulties encountered in chemically defining the structures and reactivities of HS derive from their large chemical heterogeneity and geographical variability [8].

Organic matter contributes to plant growth through its effect on the physical, chemical, and biological properties of the soil. It has a nutritional function in that it serves as a source of N, P, and S for plant growth, 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. The dark brown to black colour of soils is due to their stable humus content. Highly productive soils often have a characteristic rich odour that can be attributed to organic constituents. Many of the benefits attributed to organic matter 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 properties of soil humus and associated effects on soil are given in *Table 1* [1].

2.4 The utilization of humic substances

Nowadays, except power-producing application, applications of HS can be divided into four main categories: agricultural, industrial, environmental and pharmacological [15]. In addition to its natural occurrence in soils, humus and modified humus materials have been also added to soil for agricultural purposes. In natural systems HS act as a soil stabilizer and a nutrient and water reservoir for plants [16]. A number of different formulations have been used with the aim to increase soil fertility. The HS and their derivatives were applied with the aim either stimulate plant growth or improve physical properties of soil and in many cases the excellent results were achieved.

Table 1 General properties of humus and associated effects in the soil [1].

Property	Remarks	Effect on soil
Colour	The typical dark colour of many soils is caused by organic matter	May facilitate warming
Water retention	Organic matter can hold up 20 times its weight in water	Helps prevent drying and shrinking. Improves moisture-retaining properties of sandy soils
Combination with clay minerals	Cements soil particles into structural units called aggregates	Permits exchange of gases, stabilizes structure, increases permeability
Chelation	Forms stable complexes with Cu^{2+} , Mn^{2+} , Zn^{2+} , and other polyvalent cations	Enhances availability of micronutrients to higher plants
Solubility in water	Insolubility of organic matter is due to its association with clay. Also, salts of divalent and trivalent cations with organic matter are insoluble.	Little organic matter is lost by leaching
Buffer action	Exhibits buffering in slightly acid, neutral, and alkaline ranges	Helps to maintain a uniform reaction in the soil
Cation exchange	Total acidities of isolated fractions of humus range from 300 to 1400 cmoles/kg	Increases cation exchange capacity (CEC) of the soil. From 20 to 70 % of the CEC of many soils (e. g., Mollisols) is caused by organic matter
Mineralization	Decomposition of organic matter yields CO_2 , NH_4^+ , NO_3^- , PO_4^{3-} , and SO_4^{2-}	source of nutrients for plant growth
Combines with xenobiotics	Affects bioactivity, persistence, and biodegradability of pesticides	Modifies application rate of pesticides for effective control

So far, industrial applications of humus and humus-derived products are rare. On the contrary, the usage of coal was more abundant and essentially, it constituted the basis of the chemical industry in the second half of the 19th century and the first half of the 20th century. Petroleum was also an application and it was regarded as the main raw material for the chemical industry of the 20th century [4]. Although in 1960 Piret et al. stated, that more consideration should be given to the industrial potential of HAs, in the intervening forty years no widespread, systematic industrial application have not been developed. Nevertheless, a review of literature reveals numerous industrial applications. Several examples are given here: improving the characteristics of well-drilling fluids [1], a cement additive [17]; an agent for tanning leather [18]; a pigment in inks [19]; a plasticizer component for polyvinyl chloride [20], etc.

The role of humic substances in the environment was well approved during the last decades. HS have important environmental functions [21]. They are known to complex heavy metals and persistent organic xenobiotics. The interaction of humic substances with xenobiotics may modify the uptake and toxicity of these compounds by living organisms and affect the fate of pollutants in the environment [6]. In aqueous systems, due to their spectroscopic properties, they, among others, protect microorganisms against UV radiation. HS control the pH balance; govern the mobility of contaminants through absorption, aggregation, and sedimentation [21]. They act as a sorbent for radionuclides and organic pollutants, chemical buffers with catalytic activity, etc. [16]. These properties of HS are still a topic of debate between HS scientists. The characterization of the size, shape, conformation, structure, and composition of HS is crucial to understand their physico-chemical reactions and to predict their role in the environment [21]. Briefly, some environmental application of HS in last: removing heavy metal ions, cyanide, phosphates, oil, detergents and dyes from water with more than 98% efficiency [22], [23]; removing phenol from water [24]; sorbent for waste gases from an animal-carcass rendering [25] etc. Despite of very little anion exchange capacity, peat and other humus-based material were converted into anion exchangers [26-27].

Fuchsman stated that pharmacologically the most important components of peat humus might be steroids and terpenoids [27]. The importance of these compounds classes resides not only in their applications for topical treatments (such as cosmetic creams and therapeutic bath) but more significantly in the isolation and identification of discrete compounds from which new medicinal chemicals may be synthesized. For better information on the subject, the excellent review can be consulted [15].

2.5 The structure of humic substances

From the point of view of elementary analysis humic substances consist of carbon, oxygen, hydrogen and sometimes small amounts of nitrogen and occasionally phosphorous and sulphur. Humic molecules are composed of aromatic and/or aliphatic chains and with specific content of functional groups. Their number and position depend on the conditions of formation. Elementary analyses data of humic samples originated from miscellaneous sources differ in their elementary composition and reactivity. Although, undisputable differences exist in way of genesis, humic substances from different sources should be considered as members of the same class of chemical compounds [29].

Many model structures of HA and FA were suggested, but they should be considered only as models taking into account average composition. Therefore, in the real humic mixture, such a structure may not be necessarily present. One of the most recent HA model structure taking into account the system complexity is presented in Fig. 5.

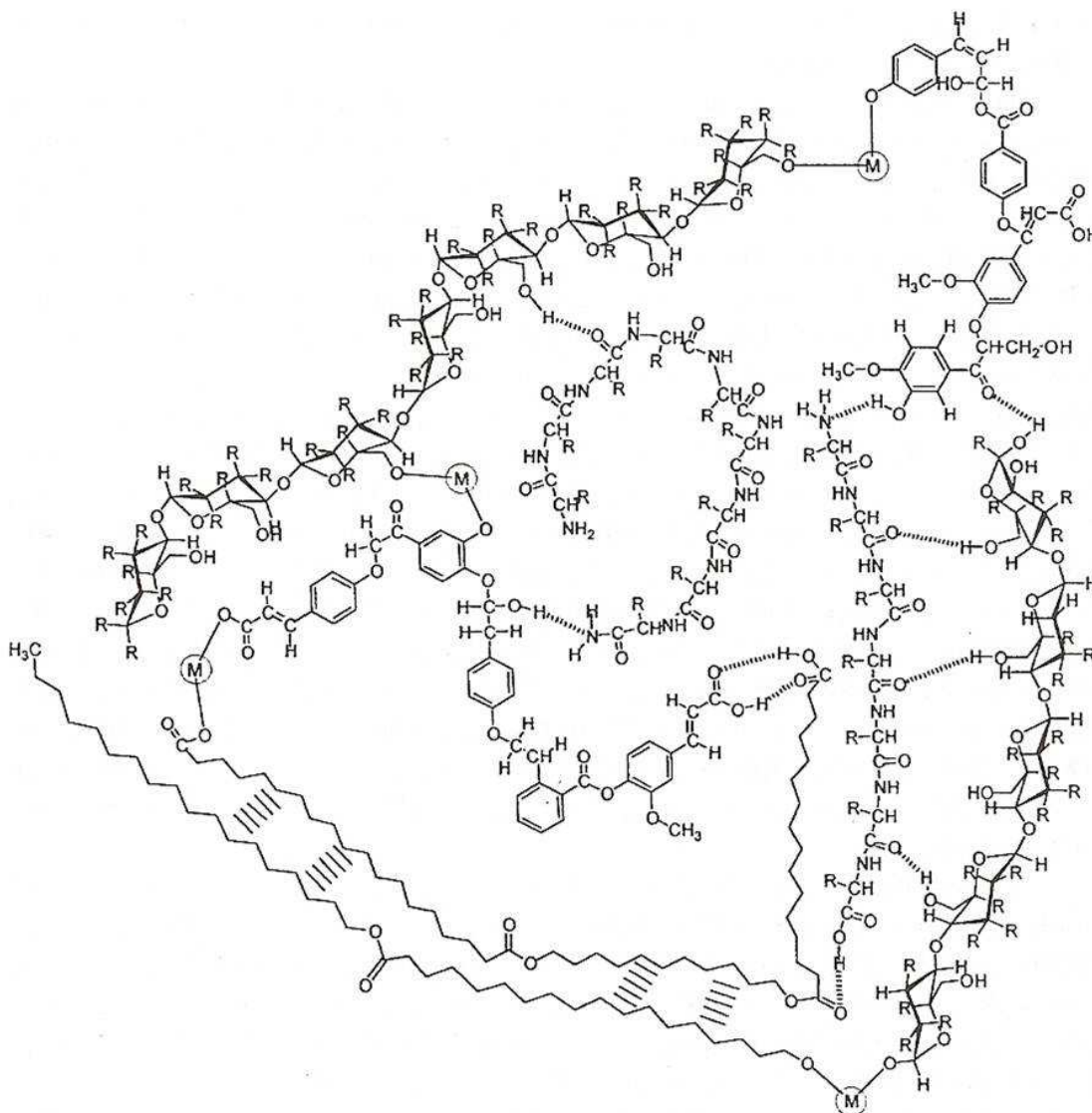


Fig. 5 Recent model structure of humic acid [41]; M - metal.

2.5.1 Functional groups and reactivity

Besides the elemental composition, group composition is used to characterize HS as it gives information about the chemistry and structural properties of HS. A variety of functional groups, including COOH, phenolic OH, enolic OH, quinone, hydroxyquinone, lactone, ether, and alcoholic OH, have been reported in humic substances [1]. Also small amounts of nitrogen, sulphur and phosphorus functional groups or bridges can be found.

Because of lack of specificity, absolute values concerning of relative distribution of functional groups in humic substances must be accepted with reservation. For instance, based on wet-chemical methods, as a result of the variability in reactivity of OH groups, values reported for “phenolic OH” and “alcoholic OH” can be somewhat misleading. In general, it has been

found that COOH and C=O groups increase in amount during humification while other functional groups (alcoholic OH, phenolic OH and OCH₃) decrease [1]. Fulvic acids contain more functional groups of an acidic nature, particularly COOH. The total acidities of fulvic acids (900 – 1400 mmol/100 g) are considerably higher than for humic acids (400 – 870 mmol/100 g). Another important difference is that while the oxygen in fulvic acids is largely in known functional groups (COOH, OH, C=O). The proportion of oxygen in humic acids seems to occur as a structural component of the nucleus [21].

Functional carboxyl and hydroxyl groups in HS were found to be related to biological activity [42] but the manner in which they act has still to be elucidated. Low molecular weight components of HS have proved to be biologically active although high molecular weight components appeared to be similarly active too [43].

2.5.2 The molecular structure

Divergent descriptions of HS defended by its proponent have been reviewed by Clapp and Hayes. First, HS are described as macromolecules and assumed to have a random coil conformation in solution. They are thought to be macromolecules due to their measured apparent molecular weight values, which can exceed 1,000,000 Da. Second, they are described as micelles or “pseudomicelles” structures in solution. Micelle assumption is based on the fact that HS consist essentially of amphiphilic molecules. That is, molecules consist of separate hydrophobic (non-polar) parts composed of relatively unaltered segments of plant polymers and hydrophilic (polar) parts composed of carboxylic acid groups. These amphiphiles form membrane-like aggregates on mineral surfaces and micelle-like aggregates in solution. The exterior surfaces of these aggregates are hydrophilic, while the interiors are composed of separate hydrophobic liquid-like phases. Third, they are described as molecular associations of relatively small molecules held together by weak interaction forces [21]. Piccolo [8] has provided several evidences on the supramolecular association of small HS based on several chromatographic and spectroscopic techniques.

The structure of aggregation of small and mostly amphiphilic aggregates is similar to the surface-active substances pattern but their formation is different which is supposed to be caused by strong heterogeneity of these aggregates. In this concept, one can imagine HS to be relatively small and heterogeneous molecules of various origins that self-organize in supramolecular conformations. Humic superstructures of relatively small molecules are not associated by covalent bonds but are stabilized only by weak forces such as dispersive hydrophobic interactions (van der Waals, $\pi - \pi$, and CH – π bonds) and hydrogen bonds, the latter being progressively more important at low pH values. Hydrophilic and hydrophobic domains of humic molecules can be contiguous to or contained in each other and, in hydration water, form apparently large molecular size associations. In humic supramolecular organizations, the intermolecular forces determine the conformational structure of HS, and the complexities of the multiple non-covalent interactions control their environmental reactivity.

The FAs may be regarded as associations of small hydrophilic molecules in which there are enough acidic functional groups to keep the fulvic clusters dispersed in solution at any pH. The HAs are composed by associations of predominantly hydrophobic compounds

(polymethylenic chains, fatty acids, steroid compounds) that are stabilized at neutral pH by hydrophobic dispersive forces. Their conformations grow progressively in size when intermolecular hydrogen bonds are increasingly formed at lower pH values until they flocculate [8].

Concerning the term “supramolecular organization” the definition given by Lehn [30] may well be applied to HS: “Supramolecular assemblies (are) molecular entities that result from the spontaneous association of a large undefined number of components into a specific phase having more or less well-defined microscopic organization and macroscopic characteristics depending on its nature (such as films, layers, membranes, vesicles, micelles, mesomorphic phases, solid state structures, etc.).”

2.6 Weak interactions and supramolecular assembly

Aggregates form only when there is a difference in the cohesive energies between the molecules in the aggregated and the dispersed (monomer) states. Amphiphiles such as surfactants and lipids can associate into a variety of structures in aqueous solutions. These can transform from one to another by changing the solution conditions such as the electrolyte or lipid concentration, pH, or temperature. In most cases the hydrocarbon chains are in the fluid state allowing for the passage of water and ions through the narrow hydrophobic regions, e.g. across bilayers [31].

2.6.1 Fundamental thermodynamic equations of self-assembly

Equilibrium thermodynamics requires that in a system of molecules that form aggregated structures in solution (Fig. 6) the chemical potential of all identical molecules in different aggregates is the same.

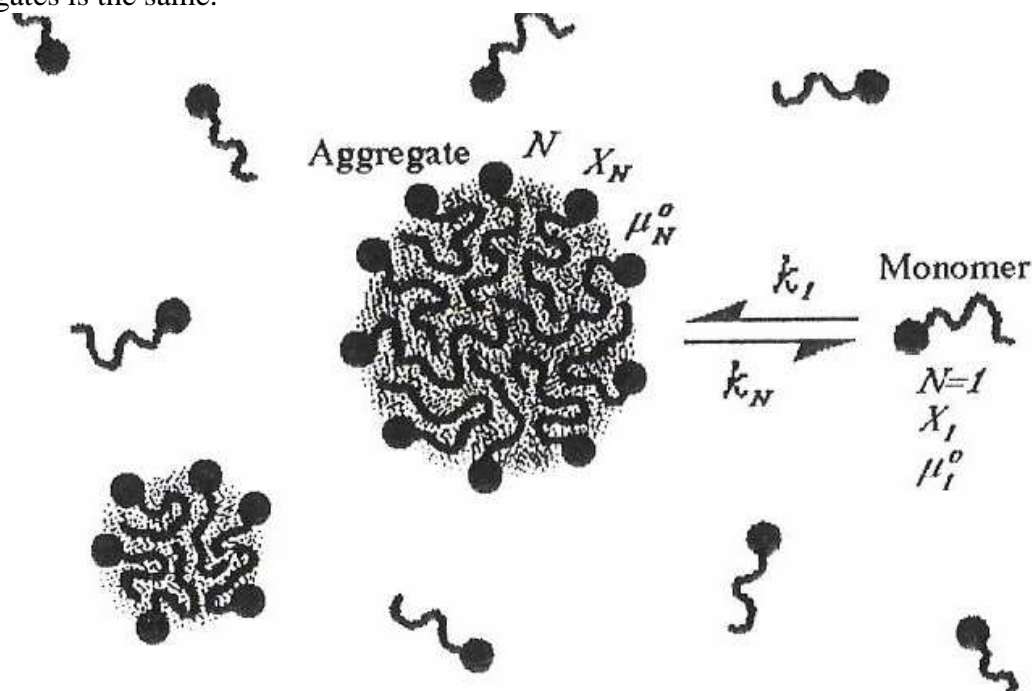


Fig. 6 Association of N monomers into an aggregate (e.g. a micelle). The mean lifetime of an amphiphilic molecule in a small micelle is very short, typically $10^{-5} - 10^{-3}$ s [31].

This may be expressed as

$$\mu = \underset{\text{monomers}}{\mu_1^0} + kT \log X_1 = \underset{\text{dimers}}{\mu_2^0} + \frac{1}{2} kT \log \frac{1}{2} X_2 = \underset{\text{trimers}}{\mu_3^0} + \frac{1}{3} kT \log \frac{1}{3} X_3 = \dots$$

or

$$\mu = \mu_N = \mu_N^0 + \frac{kT}{N} \log \left(\frac{X_N}{N} \right) = \text{constant}, \quad N = 1, 2, 3, \dots, \quad (1)$$

where μ_N is the mean chemical potential of a molecule in an aggregate of aggregation number N , μ_N^0 the standard part of the chemical potential (the mean interaction free energy per molecule) in aggregates of aggregation number N , and X_N the concentration (more strictly the activity) of molecules in aggregates of number N ($N = 1$, μ_1^0 and X_1 correspond to isolated molecules, or *monomers*, in solution). Equation (1) can also be derived using the familiar *law of mass action* [44] as follows: referring to Fig. 6 we may write

$$\text{rate of association} = k_1 X_1^N,$$

$$\text{rate of dissociation} = k_N (X_N / N),$$

where

$$K = k_1 / k_N = \exp[-N(\mu_N^0 - \mu_1^0) / kT] \quad (2)$$

is the ratio of two ‘reaction’ rates (the equilibrium constant). These combine to give Eq. (1), which can also be written in the more useful (and equivalent) forms

$$X_N = N \left\{ (X_M / M) \exp[M(\mu_M^0 - \mu_N^0) / kT] \right\}^{N/M} \quad (3)$$

and, putting $M = 1$,

$$X_N = N \left\{ X_1 \exp[(\mu_1^0 - \mu_N^0) / kT] \right\}^N \quad (4)$$

where M is any arbitrary reference state of aggregates (or monomers) with aggregation number M (or 1). Equations (3) and (4) together with the conservation relation for the total solute concentration C

$$C = X_1 + X_2 + X_3 + \dots = \sum_{N=1}^{\infty} X_N \quad (5)$$

completely defines the system. Depending on how the free energies μ_1^0, μ_N^0 are defined the dimensionless concentrations C and X_N can be expressed in volume fraction units

((mol L⁻¹)/55.5 or $M/55.5$ for aqueous solutions). In particular, note that C and X_N can never exceed unity. Equation (2) assumes ideal mixing and is restricted to dilute systems where interaggregate interactions can be ignored [31].

2.6.2 The critical micelle concentration (CMC) theory

Micelles are spherical or ellipsoid structures on whose surface the hydrophilic heads of the surfactant molecules are gathered together whereas the hydrophobic tails project inwards. An important measure for the characterization of surfactants is the critical micelle concentration (CMC). Surfactants consist of a hydrophilic "head" and a hydrophobic "tail". If a surfactant is added to water then it will initially enrich itself at the surface; the hydrophobic tail projects from the surface. Only when the surface has no more room for further surfactant molecules will the surfactant molecules start to form agglomerates inside the liquid; these agglomerates are known as micelles. The surfactant concentration at which micelle formation begins is known as the critical micelle formation concentration (CMC) [40].

The hypothesis of critical micelle concentration (CMC) of HS supposes that amphiphilic molecules exist solely as single unit species at concentrations lower than CMC whereas at greater concentrations ordered aggregates or micelles are formed (e.g., [33-35]). Methods which can be used for determination CMC are as follows: optical methods (fluorescence, light scattering) or physical (surface tension measurement, ultrasonic spectroscopy).

2.6.3 The hydrophobic effect

The hydrophobic effect is the terminology commonly used to refer to processes where non-polar molecules, or non-polar parts of molecules, are spontaneously removed from water. Micellization of surfactants is an example of the hydrophobic effect. In the micellization there are two opposing forces at work. The first is the hydrophobicity of the hydrocarbon tail, favouring the formation of micelles and the second is the repulsion between the surfactant head groups. The mere fact that micelles are formed from ionic surfactants is an indication of the fact that the hydrophobic driving force is large enough to overcome the electrostatic repulsion arising from the surfactant head groups [40].

The thermodynamic factors which give rise to the hydrophobic effect are complex and still incompletely understood. The free energy of transfer of a non-polar compound from some reference state, such as an organic solution, into water, ΔG_{tr} , is made up of an enthalpy, ΔH_{tr} , and entropy, $-T\Delta S_{tr}$, term:

$$\Delta G_{tr} = \Delta H_{tr} - T\Delta S_{tr} \quad (6)$$

At room temperature, the *enthalpy* of transfer from organic solution into aqueous solution is negligible; the interaction enthalpies are the same in both cases.

The *entropy* however is negative. Water tends to form ordered cages around the non-polar molecule and this leads to a decrease in entropy. At high temperatures these cages are no longer any stronger than bulk water, and the entropy contribution tends to zero. The enthalpy of transfer, however, is now positive (unfavourable). Because the temperature dependence of entropy and enthalpy are not the same, there is some temperature at which the hydrophobic effect is strongest, and the effect decreases at temperatures above and below this temperature [37].

Although occasionally mistaken for a force, hydrophobic effects generally relate to the exclusion from polar solvents, particularly water, of large particles or those that are weakly solvated (e.g. via hydrogen bonds or dipolar interactions). The effect is obvious in the immiscibility of mineral oil and water. Essentially, the water molecules are attracted strongly to one another resulting in a natural agglomeration of other species (such as non-polar organic molecules) as they are squeezed out of the way of the strong intersolvent interactions. This can produce effects resembling attraction between one organic molecule and another, although there are in addition van der Waals and $\pi - \pi$ stacking attractions (details see below) between the organic molecules themselves. Hydrophobic effects are of crucial importance in the binding of organic guests by cyclodextrins and cyclophane hosts in water and may be divided into two energetic components: enthalpic and entropic. The enthalpic hydrophobic effect involves the stabilisation of water molecules that are driven from a host cavity upon guest binding. Because host cavities are often hydrophobic, intracavity water does not interact strongly with the host walls and is therefore of high energy. Upon release into the bulk solvent, it is stabilised by interactions with other water molecules. The entropic hydrophobic effect arises from the fact that the presence of two (often organic) molecules in solution (host and guest) creates two “holes” in the structure of bulk water. Combining host and guest to form a complex results in less disruption to the solvent structure and hence an entropic gain (resulting in a lowering of overall free energy) [38].

2.6.4 Weak interactions

In general, supramolecular chemistry concerns non-covalent bonding interactions. The term “non-covalent” encompasses an enormous range of attractive and repulsive forces. The most important, along with an indication of their approximate energies, are explained below. When considering a supramolecular system it is vital to consider the interplay of all of these interactions and effects relating both to the host and guest as well as their surroundings (e.g. solvation, crystal lattice, gas phase etc.) [38].

Ion – ion interactions

Ionic bonding is comparable in strength to covalent bonding (bond energy is 100 - 350 kJ mol⁻¹). A typical ionic solid is sodium chloride, which has a cubic lattice in which each Na⁺ cation is surrounded by six Cl⁻ anions. It would require a large stretch of the imagination to regard NaCl as a supramolecular compound but this simple ionic lattice does illustrate the way in which a Na⁺ cation is able to organise six complementary donor atoms about it in order to maximise non-covalent ion – ion interactions. Note that this kind of lattice structure breaks down in solution because of solvation effects to give species such as labile, octahedral Na(H₂O)₆⁺. A much more supramolecular example of ion – ion interactions is the

interaction of the *tris*(diazabicyclooctane) host, which carries a 3^+ charge, with anions such as $\text{Fe}(\text{CN})_6^{3-}$ [38].

Ion – dipole interactions (50 – 200 kJ mol⁻¹)

The bonding of an ion, such as Na^+ , with a polar molecule, such as water, is an example of an ion-dipole interaction. This kind of bonding is seen both in the solid state and in solution. A supramolecular analogue is readily apparent in the structures of the complexes of alkali metal cations with macrocyclic (large ring) ethers termed crown ethers in which the ether oxygen atoms play the same role as the polar water molecules. The oxygen lone pairs are attracted to the cation positive charge. Ion – dipole interactions also include coordinative bonds, which are mostly electrostatic in nature in the case of the interactions of non-polarisable metal cations and hard bases. Coordinate (dative) bonds with a significant covalent component, as in $[\text{Ru}(\text{bpy})_3]^{2+}$, are also often used in supramolecular assembly and the distinction between supramolecular and molecular species can become rather blurred [38].

Cation – π interactions (5 – 80 kJ mol⁻¹)

Transition metal cations such as Fe_2^+ , Pt_2^+ etc. are well known to form complexes with olefinic and aromatic hydrocarbons such as ferrocene $[\text{Fe}(\text{C}_5\text{H}_5)_2]$ and Zeise's salt $[\text{PtCl}_3(\text{C}_2\text{H}_4)]^-$. The bonding in such complexes is strong and could by no means be considered non-covalent, since it is intimately linked with the partially occupied *d*-orbitals of the metals. Even species such as $\text{Ag}^+ \dots \text{C}_6\text{H}_6$ have a significant covalent component. The interaction of alkaline and alkaline earth metal cations with $\text{C}=\text{C}$ double bonds is, however, a much more non-covalent 'weak' interaction, and plays a very important role in biological systems. For example, the interaction energy of K^+ and benzene in the gas phase is about 80 kJ mol^{-1} . By comparison, the association of K^+ with a single water molecule is only 75 kJ mol^{-1} . The reason K^+ is more soluble in water than in benzene is related to the fact that many water molecules can interact with the potassium ion, whereas only a few bulkier benzene molecules can fit around it. The interaction of non-metallic cations such as RNH_3^+ with double bonds may be thought of as a form of $\text{X} - \text{H} \dots \pi$ hydrogen bond [38].

$\pi - \pi$ stacking (0 – 50 kJ mol⁻¹)

This weak electrostatic interaction occurs between aromatic rings, often in situations where one is relatively electron rich and one is electron poor. There are two general types of π -stacking; face-to-face and edge-to-face, although a wide variety of intermediate geometries are known. Face-to-face π -stacking interactions between the aryl rings of nucleobase pairs also help to stabilize the DNA double helix. Edge-to-face interactions may be regarded as weak forms of hydrogen bonds between the slightly electron deficient hydrogen atoms of one aromatic ring and the electron rich π -cloud of another. Edge-to-face interactions are responsible for the characteristic herringbone packing in the crystal structures of a range of small aromatic hydrocarbons including benzene [38].

Close packing in the solid state

In examination of solid state (i.e. crystal) structures the need to achieve a close packed arrangement is also a significant driving force. This has been summed up in the truism 'Nature abhors a vacuum' but according to the close packing theory of Kitaigorodsky [39], it is simply a manifestation of the maximisation of favourable isotropic van der Waals

interactions. Kitaigorodsky's theory tells us that molecules undergo a shape simplification as they progress towards dimers, trimers, higher oligomers, and ultimately crystals. This means that one molecule dovetails into the hollows of its neighbours so that a maximum number of intermolecular contacts are achieved, rather like the popular computer game *Tetris*. Very few solid-state structures are known to exhibit significant amounts of 'empty' space. Those which do possess a very rigid framework that is able to resist implosion under what amounts effectively to an enormous pressure differential between atmospheric pressure and the empty crystal pore channel. Such materials often exhibit very interesting and useful properties in catalysis and separation science [38].

Van der Waals forces ($< 5 \text{ kJ mol}^{-1}$; variable)

Van der Waals interactions arise from the polarization of an electron cloud by the proximity of an adjacent nucleus, resulting in a weak electrostatic attraction. They are non-directional and hence possess only limited scope in the design of specific hosts for selective complexation of particular guests. In general, van der Waals interactions provide a general attractive interaction for most 'soft' (polarisable) species. This is the force involved in interactions between the noble gases. In supramolecular chemistry, they are most important in formation of 'inclusion' compounds in which small, typically organic molecules are loosely incorporated within crystalline lattices or molecular cavities. Strictly, van der Waals interactions may be divided into dispersion (London) and exchange-repulsion terms. The dispersion interaction is the attractive component that results from the interactions between fluctuating multipoles (quadrupole, octupole etc.) in adjacent molecules. The attraction decreases very rapidly with distance (r^{-6} dependence) and is additive with every bond in the molecule contributing to the overall interaction energy. The exchange-repulsion defines molecular shape and balances dispersion at short range, decreasing with the twelfth power of interatomic separation [38].

Hydrogen bonding ($4 - 120 \text{ kJ mol}^{-1}$)

However, though dispersion forces are the ones mainly responsible for bringing molecules together, they lack the discrimination, specificity and directionality of dipolar and H-bonding interactions, and it is these that often determine the fine and subtle details of molecular and macromolecular structures, such as those of molecular crystals, polypeptides (e.g., proteins), polynucleotides (e.g., DNA and RNA), micelles and biological membranes [31].

The hydrogen bond is one of the strongest and the most common types of noncovalent bond. It is difficult to define a hydrogen bond in a way that would cover all the features ascribed to it by the different branches of science. The most common definition describes it as an attractive interaction between two species (atoms, groups, or molecules) in a structural arrangement where the hydrogen atom, covalently bound to a more electronegative atom of one species, is non-covalently bound to a place with an excess of electrons of the other species [45]. This definition does not specify the nature of the hydrogen bond, which makes the definition quite general, but not in concrete terms. The hydrogen bond plays a key role in chemistry, physics, and biology and its consequences are enormous. Hydrogen bonds are responsible for the structure and properties of water, an essential compound for life, as a solvent and in its various phases. Further, hydrogen bonds also play a key role in determining the shapes, properties, and functions of biomolecules (referred to recently published

monographs on hydrogen bonding [46-48]. The term “hydrogen bond” was probably used first by Pauling in his article on the nature of the chemical bond [49].

A hydrogen bond may be regarded as a particular kind of dipole-dipole interactions in which a hydrogen atom attached to an electronegative atom (or electron withdrawing group) is attracted to a neighbouring dipole on an adjacent molecule or functional group. Because of its relatively strong and highly directional nature, hydrogen bonding has been described as the “master key” interaction in supramolecular chemistry”. An excellent example is the formation of carboxylic acid dimers which results in the shift of the $\nu(\text{OH})$ infrared stretching frequency from about 3400 cm^{-1} to about 2500 cm^{-1} , accompanied by a significant broadening and intensifying of the absorption. Typically hydrogen bonded $\text{O}\dots\text{O}$ distances are $2.50 - 2.80\text{ \AA}$ in length, though interactions in excess of 3.0 \AA may also be significant. Hydrogen bonds to larger atoms such as chloride are generally longer, and may be weaker as a consequence of the reduced electronegativity of the larger halide acceptor, although the precise strength of the hydrogen bonds is greatly dependent on its environment. Hydrogen bonds are ubiquitous in supramolecular chemistry. In particular, hydrogen bonds are responsible for the overall shape of many properties, recognition of substrates by numerous enzymes, and for the double helix structure of DNA.

Hydrogen bonds come in a bewildering range of lengths, strengths and geometries. They can be strong, which are mainly covalent with the bond energy of $60 - 120\text{ kJ mol}^{-1}$, or moderate, which are mainly electrostatic with the bond energy of $16 - 60\text{ kJ mol}^{-1}$, and weak electrostatic interactions with the bond energy < 12 . A single, strong hydrogen bond per molecule may be sufficient to determine solid-state structure and exert a marked influence on the solution and gas phases. Weaker hydrogen bonds play a role in structure stabilisation and can be significant when large numbers act in concert.

In the case of hydrogen bonds between neutral species, it is generally accepted that there is a direct correlation between hydrogen bond strength (in terms of formation energy) and the crystallographically determined distance between hydrogen bond donor and acceptor. In the case of interanion interactions, as in potassium hydrogen oxalate (KHC_2O_4) for example, *ab initio* calculations show that the hydrogen bonding interactions between pairs of HC_2O_4^- anions are repulsive in all orientations, i.e. there is no attractive force of type $\text{O}-\text{H}^+ \dots \text{O}^-$. Despite this, the $\text{O}\dots\text{O}$ separation is an extremely short 2.52 \AA , suggesting a very strong hydrogen bond. This apparent contradiction is explained in this and a range of related systems by the strong attraction to the K^+ cations, which dominate over the anion-anion repulsions. The resemblance to a truly attractive hydrogen bond arises from the fact that the interanion potential is least repulsive in the mutual orientation in which the $\text{O}-\text{H}$ group is directed towards the oxygen atoms of the next anion in the chain [38].

Recent interest has also focused on apparent hydrogen bonding interactions involving hydrogen atoms attached to carbon, rather than electronegative atoms such as N and O (electronegativities: C: 2.55, H: 2.20, N: 3.04, O: 3.44) while these interactions are at the weaker end of the energy scale of hydrogen bonds, the presence of electronegative atoms near the carbon can enhance significantly the acidity of the $\text{C}-\text{H}$ proton, resulting in a significant

dipole. An elegant example of C–H... N and C–H ... O hydrogen bonds is the interaction of the methyl group of nitromethane with the pyridyl crown ether [38].

In general, the hydrogen bond is a noncovalent bond between the electron-deficient hydrogen and a region of high electron density. Most frequently, a hydrogen bond is of the X–H...Y type, where X is the electronegative element and Y is the place with the excess of electrons (e.g., lone electron pairs, p electrons) [45].

In the last few years several pieces of evidence were collected [50] which indicated that the X–H...Y arrangement can be accompanied by opposite geometrical and spectral manifestation. Instead of elongation of the X–H bond accompanied by a red shift of the X–H stretch vibration, the contraction of this bond and the blue shift of the respective stretch vibration were detected. Moreover, the intensity of the X–H stretch vibration often decreased upon formation of the X–H...Y contact, again in contrast to standard hydrogen bonding. The common feature of this novel type of bonding, called improper, blue-shifting hydrogen bonding, and standard hydrogen bonding is the charge transfer from the proton acceptor to the proton donor [45].

2.7 Water and its hydration properties

Water and life are closely linked. It possesses specific properties that cannot be found in other materials and that are required for life-giving processes. These properties are brought about by the hydrogen-bonded environment particularly evident in liquid water [51]. The water hydrogen bond is a weak bond, never stronger than about a twentieth of the strength of the O–H covalent bond. The attraction of the O–H bonding electrons towards the oxygen atom leaves a deficiency on the far side of the hydrogen atom relative to the oxygen atom. The result is that the attractive force between the O–H hydrogen and the O-atom of a nearby water molecule is strongest when the three atoms are in a straight line (that is, O–H...O) and when the O-atoms are separated by about 0.28 nm [52].

Hydrogen bonds play an especially prominent role in water since each oxygen atom with its two hydrogens can participate in four such linkages with other water molecules – two involving its own H atoms and two involving its unshared (lone-pair) electrons with other H atoms [31]. These four hydrogen bonds optimally arrange themselves tetrahedrally around each water molecule as found in ordinary ice [52]. In liquid water, the tendency to retain the ice-like tetrahedral network remains, but the structure is now disordered and labile. The average number of nearest neighbours per molecule rises to about five (hence the higher density of water on melting), but the mean number of H bonds per molecule falls to about 3.5. It is instructive to note that the tetrahedral coordination of the water molecule is at the heart of the unusual properties of water, much more than the hydrogen bonds themselves [31]. The Fig. 7 shows such a typical average cluster of five water molecules.

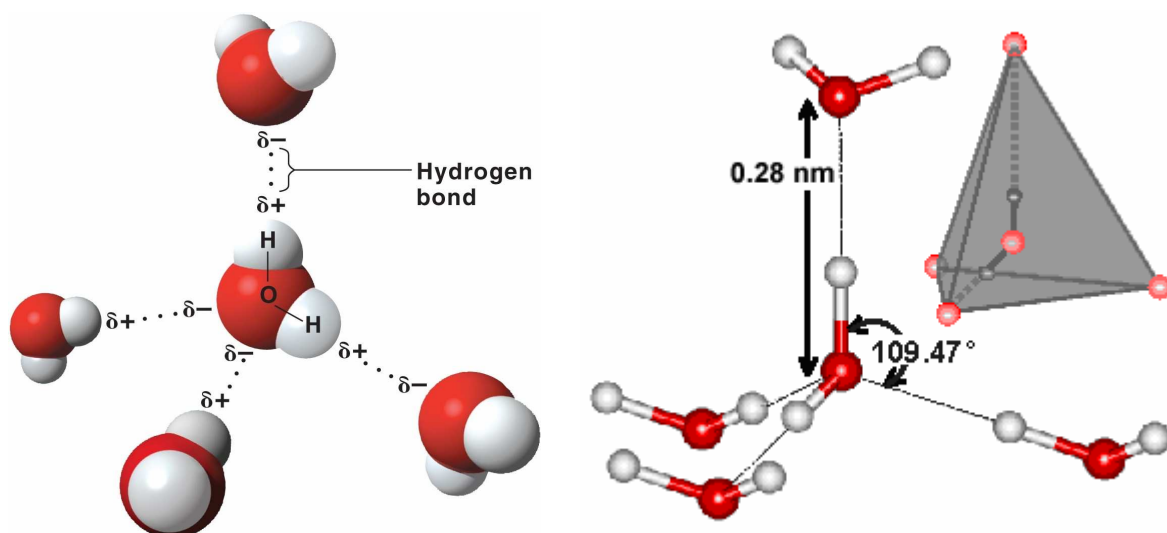


Fig. 7 The cluster of five water molecules in tetrahedrally arrangement [52].

In liquid water, the tetrahedral clustering is only locally found and reduces with increasing temperature. In ice, this tetrahedral clustering is extensive, producing its crystalline form. The stronger the bonds, the more ordered and static is the resultant structure. The energetic cost of the disorder is proportional to the temperature, being smaller at lower temperatures. This is why the structure of liquid water is more ordered at low temperatures. This increase in orderliness in water as the temperature is lowered is far greater than in other liquids, due to the strength and preferred direction of the hydrogen bonds. In ice there is the clustering during producing of crystalline form more extensive. There are several types of ice which differ in arrangement of water molecules. We distinguish twenty types of ice which differ in various ambient conditions during their formation (pressure and temperature). Many of the crystalline forms may remain metastable in much of the low-temperature phase space at lower pressures [52].

2.8 Hydration of biomolecules

Generally, the hydration is a term concerning the amount of bound water. Hydration is one of the most important factors playing the role in biological function of molecules in both living and natural systems. Water plays an important role as a medium for nutrient transport, cell membrane processes, induces biologically active conformation of biomolecules etc. Due to unique properties and strong affinity of water molecules to stick to each other via H-bonds it forms various structures having different physical-chemical properties. Formation of these specific clusters is a driving force assembling molecules into complicated organizations. Hydration of biomolecules depends importantly on the relative strength of the biomolecule-water interactions as compared with the water-water hydrogen bond interactions. Stronger water hydrogen bonding leads to the clustering of water molecules together; and therefore these molecules are no longer available for biomolecular hydration [52]. Water is involved in these processes in a variety of ways, ranging from direct bridging to collective effects (such as hydrophobic effect) [52].

Due to the presence of solids, all the water does not have similar properties in terms of vapour pressure, enthalpy, entropy, viscosity and density. In general terms, two main types of water

are considered [54]: the free water which is not influenced by the solid particles and the bound water whose properties are modified due to the presence of solid. The estimation of the amount of free water (the bound water being the complement to the whole amount of water) is based on this difference of behaviour. Consequently, supplementary types of water can also be considered. The distribution suggested by Vesilind and his fellow workers [55-58] is often used as a reference, which supposes the following four categories: 1) Free water: water non-associated with solid particles and including void water not affected by capillary force. 2) Interstitial water: water trapped inside crevices and interstitial spaces of flocs and organisms. 3) Surface (or vicinal) water: water held on to the surface of solid particles by adsorption and adhesion. 4) Bound (or hydration) water.

2.8.1 Hydration of proteins

Protein hydration is very important for their three-dimensional structure and activity [59, 61]. Indeed, proteins lack activity in the absence of hydrating water. In solution, they possess a conformational flexibility, which encompasses a wide range of hydration states, not seen in the crystal [62] or in non-aqueous environments. Equilibrium between these states will depend on the activity of the water within its microenvironment; that is, the freedom that the water has to hydrate the protein [63]. Thus, protein conformations demanding greater hydration are favoured by more (re-)active water (for example, high density water containing many weak bent and/or broken hydrogen bonds) and 'drier' conformations are relatively favoured by lower activity water (for example, low-density water containing many strong intra-molecular aqueous hydrogen bonds). Surface water molecules are held to each other most strongly by the positively charged basic amino acids. The exchange of surface water (and hence the perseverance of the local clustering and the overall system flexibility) is controlled by the exposure of the groups to the bulk solvent (that is, greater exposure correlates to greater flexibility and freer protein chain movement) [64-65]. Hydration also affects the reactions and interactions of coenzymes and cofactors; thus, the various redox potentials (and hence whether they oxidize or reduce) of some iron-sulphur proteins are accounted for by differential hydration rather than direct protein binding effects [66].

The folding of proteins depends on the same factors as control the junction zone formation in some polysaccharides; that is, the incompatibility between the low-density water and the hydrophobic surface that drives such groups to form the hydrophobic core [67]. This drive for hydrophobic groups to mostly cluster away from the protein surface (in water soluble proteins) is controlled by the charged and polar group interactions with each other and water. Interestingly, in pure water and in the absence of screening dissolved ions, some buffer-insoluble proteins are quite soluble due to the weak (here unshielded) interactions between the protein's intrinsic charges [68]. Non-ionic kosmotropes, which stabilize low-density water, consequentially stabilize the structure of proteins. In addition, water acts as a lubricant [69-70], so easing the necessary peptide amide-carbonyl hydrogen bonding changes. The biological activity of proteins appears to depend on the formation of a 2-D hydrogen-bonded network spanning most of the protein surface and connecting all the surface hydrogen-bonded water clusters [71-72]. Such a water network is able to transmit information around the protein and control the protein's dynamics, such as its domain motions [73]. Much life,

however, functions optimally at about 37°C where this spanning network is about to break down on heating, perhaps to help compensate for the biosystem's entropic changes [74].

Intramolecular peptide (amide) hydrogen bonding makes a major contribution to protein structure and stability but is only effective in the absence of accessible competing water. Even just the presence of close-by water molecules causes peptide hydrogen bonds to lengthen [75], so loosening the structure. The internal molecular motions in proteins, necessary for biological activity, are very dependent on the degree of plasticizing, which is determined by the level of hydration [76]. Thus internal water enables the folding of proteins and is only expelled from the hydrophobic central core when finally squeezed out by cooperative protein chain interactions [76]. Many water molecules (similar in amount to individual amino acids) remain behind buried in the core of the proteins, so forming structurally-important hydrogen bonded linkages.

The first hydration shell around proteins (~0.3 g/g) is ordered; with high proton transfer rates and well resolved time-averaged hydration sites; surface water showing coherent hydrogen-bond patterns with large net dipole fields [78-79]. The hydrogen bonds holding these water molecules to the protein are stronger with longer lifetimes than bulk water [80] and this water is unavailable to colligative effects. As hydration sites may be positioned close together and therefore mutually exclusive, it has been argued that the solvation is better described as a water distribution density function rather than by specific water occupied sites. The first hydration shell is also 10-20% denser than the bulk water and probably responsible for keeping the molecules sufficiently separated so that they remain in solution [81]. Although a significant amount of this density increase has been shown to be due to simple statistical factors dependent upon the way that the surface is defined in depressions [82], much is due to a protein's structure with the excess of polar hydration sites easily counteracting the remaining non-polar surface groups (tending to produce low density surface water). This water is required for the protein to show its biological function, as without it, the necessary fast conformational fluctuations cannot occur. Thus proteins have no activity (and enter a glassy state, at about 220 K) when the surrounding water becomes predominantly low-density [83-84].

Proteins are formed from a mixture of polar and non-polar groups. Water is most well ordered round the polar groups where residence times are longer, but where they will interfere with water's natural hydrogen bonding, than around non-polar groups where aqueous clathrate structuring may form. Both types of group create order in the water molecules surrounding them but their ability to do this and the types of ordering produced are very different. Polar groups are most capable of creating ordered hydration through hydrogen bonding and ionic interactions [52].

It is clear that evolutionary processes have made use of the organization in water surrounding proteins to create preferred diffusive routes and orientation for metabolites and favoured conformational changes and interactions. Such diffusive paths lead to binding sites with their own helpful hydration. It has been suggested that pressure waves formed from flickering water clusters may link protein molecular vibrations, so carrying information through the intracellular milieu [85] and powering product movement between enzymes in biochemical

pathways. The energetic optimization of mutual hydrogen bonded networks between protein, water and ligand is an intrinsic part of the molecular recognition process in enzymes, binding proteins and biological macromolecules generally [86].

2.8.2 Hydration of polysaccharides

Using a simplistic approach to polysaccharide hydration, water can be divided into 'bound water', subcategorized as being capable of freezing or not, and 'unbound water', subcategorized as being trapped or not, and with 'hydration' as a general term concerning the amount of bound water. 'Unbound' water freezes at the same temperature as normal water ($< 0^{\circ}\text{C}$ dependent on cooling rate). However, some water may take up to 24 hr to freeze. 'Bound freezable' water freezes at a lower temperature than normal water, being easily supercooled. It also exhibits a reduced enthalpy of fusion (melting). Although the inability to freeze is often used to determine bound water, freezing may not a good measure of hydration as it concerns the water content of the glassy state; not aqueous hydration [52]. In the glassy state the viscosity is extremely high (more than $10^{12} - 10^{13}$ Pa s), conformational changes are severely inhibited and the material is metastably trapped in a solid but microscopically disordered state. The segmental motion of macromolecules occurs when the temperature increases through the glass transition temperature. The value of T_g (temperature of the glass transition) depends somewhat on the method of its determination; the glass transition, unlike phase changes, occurring over a range of a few Kelvin.

There exist two different alternatives for defining bound and unbound water. The first one divides bound water into non-freezing and freezing [87] and unbound water into trapped and bulk. For this alternative, the type of water is determined by freezing and melting calorimetry using the enthalpy of the phase change [88] and thermogravimetry, measuring the weight changes due to absorption from set humidity atmospheres. However, non-freezing water may be trapped in a glassy state, lowering diffusion by several orders of magnitude and hindering crystal formation. The less precise alternative divides bound water into tightly bound (can be removed by freeze-drying) and loosely bound (can be removed by centrifugation), and defines unbound water as removable by filtration. This is used in the dietary fibre area, the firmly held water not removed by centrifugation, gives the water binding capacity whereas the loosely associated water, which is not removed by filtration gives the water holding capacity. Both schemes, however, only determine what they measure and this may be somewhat different from what they represent as measuring; that is, aqueous hydration. At the present time, the literature provides little useful explanatory theory for polysaccharide hydration, with poor predictability [52].

In practical experience, the effects of water on polysaccharide and polysaccharide on water are complex and become even more complex in the presence of other materials, such as salts. Water competes for hydrogen bonding sites with intramolecular and intermolecular hydrogen bonding, certainly will determine the carbohydrate's flexibility and may determine the carbohydrate's preferred conformation(s) [89].

2.8.3 Hydration of hydrogels

Hydrogels are hydrophilic polymer networks, which may absorb 10-20 % up to thousands of times their dry weight in water. Hydrogels may be chemically stable or they may degrade and eventually disintegrate and dissolve.

They are called ‘reversible’, or ‘physical’ gels when the networks are held together by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces [90-91]. Physical hydrogels are not homogeneous, since clusters of molecular entanglements, or hydrophobically- or ionically-associated domains, can create inhomogeneities. Free chain ends or chain loops also represent transient network defects in physical gels. When a polyelectrolyte is combined with a multivalent ion of the opposite charge, it may form a physical hydrogel known as an ‘ionotropic’ hydrogel (Fig. 8). Further, when polyelectrolytes of opposite charges are mixed, they may gel or precipitate depending on their concentrations, the ionic strength, and pH of the solution. The products of such ion-crosslinked systems are known as complex coacervates, polyion complexes, or polyelectrolyte complexes. All of these interactions are reversible, and can be disrupted by changes in physical conditions such as ionic strength, pH, temperature, application of stress, or addition of specific solutes that compete with the polymeric ligand for the affinity site on the protein [94].

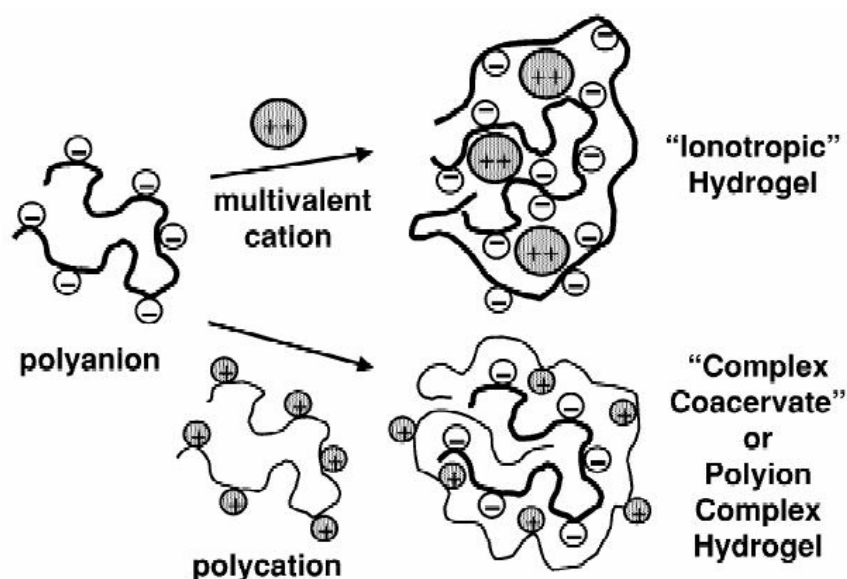


Fig. 8 Schematic of methods for formation of two types of ionic hydrogels [93].

Hydrogels are called ‘permanent’ or ‘chemical’ gels when they are covalently-crosslinked networks. Chemical hydrogels may also be generated by crosslinking of water-soluble polymers, or by conversion of hydrophobic polymers to hydrophilic polymers plus crosslinking to form a network (Fig. 9). Like physical hydrogels, chemical hydrogels are not homogeneous. They usually contain regions of low water swelling and high crosslink density, called ‘clusters’, that are dispersed within regions of high swelling, and low crosslink density. This may be due to hydrophobic aggregation of crosslinking agents, leading to high crosslink density clusters [92].

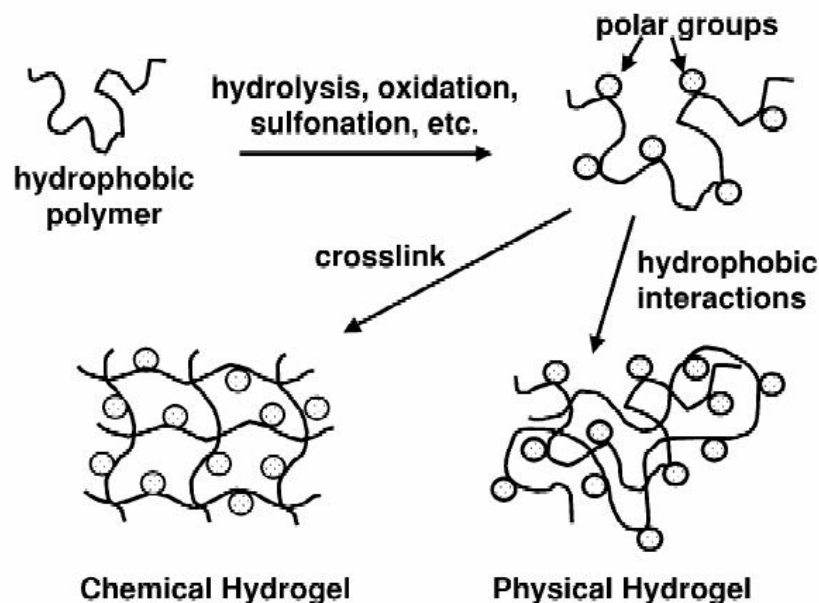


Fig. 9 Schematic of methods for formation of hydrogels by chemical modification of hydrophobic polymers [93].

In some cases, depending on the solvent composition, temperature and solids concentration during gel formation, phase separation can occur, and water-filled ‘voids’ or ‘macropores’ can form. In chemical gels, free chain ends represent gel network ‘defects’ which do not contribute to the elasticity of the network. Other network defects are chain ‘loops’ and entanglements, which also do not contribute to the permanent network elasticity. Many different macromolecular structures are possible for physical and chemical hydrogels [94]. They include the following: crosslinked or entangled networks of linear homopolymers, linear copolymers, and block or graft copolymers; polyion–multivalent ion, polyion–polyion or H-bonded complexes; hydrophilic networks stabilized by hydrophobic domains; and interpenetrating networks or physical blends. Wide and diverse range of polymer compositions have been used to fabricate hydrogels. These compositions can be divided into natural polymer hydrogels, synthetic polymer hydrogels and combinations of the two classes [95]. Many different routes have been used to synthesize hydrogels, and they are shown schematically in Figs. 8-11.

The character of the water in a hydrogel can determine the overall permeation of nutrients into and cellular products out of the gel. When a dry hydrogel begins to absorb water, the first water molecules entering the matrix will hydrate the most polar, hydrophilic groups, leading to ‘primary bound water’. As the polar groups are hydrated, the network swells, and exposes hydrophobic groups, which also interact with water molecules, leading to hydrophobically-bound water, or ‘secondary bound water’. Primary and secondary bound water are often combined and simply called the ‘total bound water’. After the polar and hydrophobic sites have interacted with and bound water molecules, the network will imbibe additional water, due to the osmotic driving force of the network chains towards infinite dilution. This additional swelling is opposed by the covalent or physical cross links, leading to an elastic network retraction force. Thus, the hydrogel will reach an equilibrium swelling level. The additional swelling water that is imbibed after the ionic, polar and hydrophobic groups

become saturated with bound water is called ‘free water’ or ‘bulk water’, and is assumed to fill the space between the network chains, and/or the centre of larger pores, macropores or voids. As the network swells, if the network chains or crosslinks are degradable, the gel will begin to disintegrate and dissolve, at a rate depending on its composition. It should be noted that a gel used as a tissue engineering matrix may never be dried, but the total water in the gel is still comprised of ‘bound’ and ‘free’ water [93].

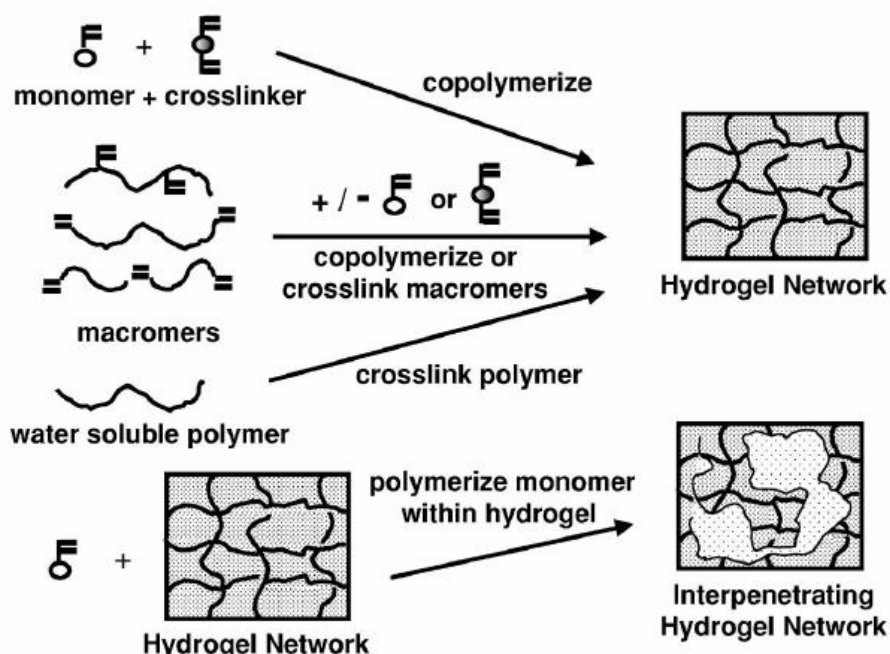


Fig. 10 Schematic of methods for formation of crosslinked hydrogels by free radical reactions, including variety of polymerizations and crosslinking of water-soluble polymers [93].

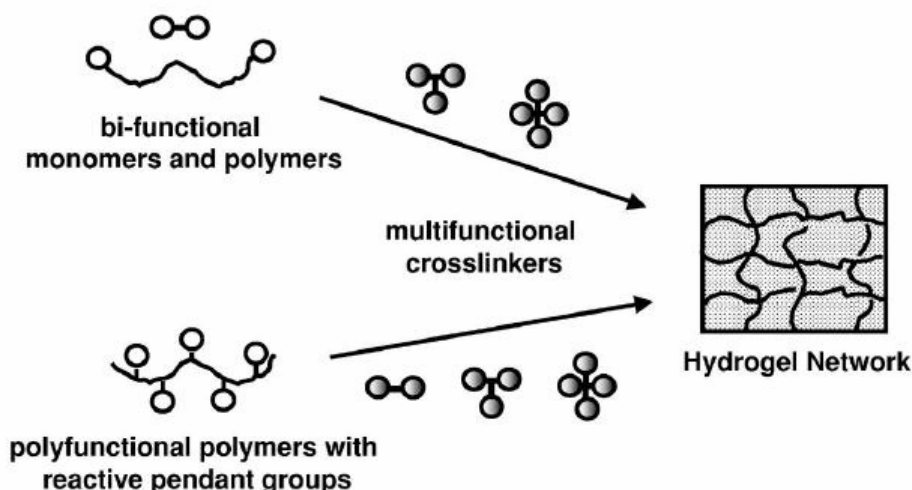


Fig. 11 Schematic of methods for formation of crosslinked hydrogels by condensation reactions of multifunctional reactants [93].

2.8.4 Hydration of soil organic matter (SOM)

Swelling of SOM is an important factor for understanding most ecologically relevant processes in upper soil layers, especially when processes in field studies are the focus of interest. Swelling has to be considered when investigating sorption and transport phenomena as well as the structure and relevance of the solid SOM. Due to this relevance, swelling of SOM opens a wide, enigmatic and challenging field of research with high demand [96].

Under field conditions, the upper soil layers are exposed to variations in moisture and temperature. In the course of moisture fluctuation, the soil organic matter (SOM) changes its water content and its state of swelling. Recent results show that with the state of swelling, SOM gradually changes its physicochemical properties, such as sorbent properties [97-98], “macromolecular” structure [99-100], thermal characteristics [101] or the binding of hydrophobic organic compounds. These changes are well known to affect sorption and transport phenomena and thus have to be taken into consideration.

Similarly as for many macromolecular substances, hydration of natural organic matter (NOM) may result in swelling, increased flexibility, alterations in conformation, and changes in ionization status of polar functional groups. These hydration-driven changes in NOM may strongly affect sorption interactions of organic compounds with NOM by influencing intraorganic matter diffusion, sorption kinetics, and extent of sorption. Since the hydration status of NOM can vary considerably under different environmental scenarios, it is clear that understanding NOM-organic pollutant sorption interactions requires insights into the effect of NOM hydration on sorption. Among processes that can potentially contribute to the overall hydration effect on sorbate interactions in the NOM phase are competition between water and sorbate molecules, a change of sorbate speciation in the NOM phase, direct interaction between complexed water molecules and sorbate molecules in the NOM phase, a change in the total volume of the NOM phase, and a change in NOM polarity. The net effect of NOM hydration on organic compound sorption interactions is far from self-evident due to the scarcity of experimental organic compound sorption data in dry, wet, and partially hydrated NOM, and the complexity of NOM hydration phenomena [102-104].

Therefore, the hydration kinetics of soil organic matter (SOM) is influential factor for transport and sorption processes in soil. Nevertheless, our knowledge about wetting and swelling processes, which both control the overall hydration kinetics, is limited [105]. Many studies have shown that water content, hydration time, and the type of water binding strongly influence the sorption of organic substances in SOM ([96-97], [106-108]). Additionally, hydration kinetics undoubtedly is very important for plant growth. Nevertheless, many aspects of hydration and drying processes are still not understood.

The first step of the hydration process of soil samples includes the wetting of mineral and organic soil components. After wetting the surfaces, the water is distributed within the pore volume. Possibly, swelling of SOM and clay minerals as well as hydration of salts may follow. If one of these processes is slower than the others, it will be the rate-limiting step of the overall hydration process. Both wetting and swelling of SOM may be slow depending on the properties of the amorphous organic soil matrix, while the penetration of water into the pore volume occurs immediately as soon as the pore walls are wettable. The wetting is

defined as the first contact between water and soil surfaces including the resulting alterations of the surface properties. Since wetting requires liquid water, water vapour cannot wet unless it condenses [105].

Water vapour uptake of isolated humic and fulvic acids was previously analyzed (e.g., [109-111]). In a relative humidity of 90%, Chen and Schnitzer (1976) [110] determined water contents (related to dry mass) of 23% for humic acids and of 51% for fulvic acids at 25°C. For 100% relative humidity and 23°C, Chiou et al. (1988) [111] calculated a comparable equilibrium water content of 25%. Few studies deal with hydration of SOM in unfractionated soil samples, however. It can be assumed that the wetting and swelling processes differ between extracted humic fractions and the naturally occurring organic soil matrix, where large macromolecules and the extractable fractions are intimately mixed. The network structure between the distinctly smaller humic and fulvic acid molecules [112-113] may be controlled by types of bonds and crosslinks that are different from those in the soil matrix, whose characteristics are responsible for the degree of swelling and retarding hydration. Where the uptake of water vapor to bulk soil samples was studied, mainly high organic- content soils were used (e.g., [114-115]). Based on sorption isotherms, Rutherford and Chiou (1992) [114] determined saturation water contents (related to dry mass) of 37% for the SOM of a peat sample from the Everglades and 39% for the SOM of a muck sample from the Michigan State University Muck Research Farm. Contrary to the results of Rutherford and Chiou (1992) [114], water sorption isotherms of a peat sample measured by Robens and Wenzig (1996) [115] indicated pronounced swelling associated with water contents about 60% (related to dry mass) for a relative humidity of 100%. Studies dealing with water vapour sorption by mineral soils have usually focused on adsorption on the soil mineral matrix [105].

Under field conditions, the water uptake from the liquid phase, rather than from the gas phase, may represent the dominant process in soil. For solid SOM, limiting water contents between 300 and 500% (related to dry mass) are cited [112]. Presumably, the water contents achieved for humic substances are distinctly higher if the supply is via the liquid phase rather than the gas phase. The differences between water adsorption via liquid and gas phases can be explained by different interfacial tensions between soil and water in the former and soil and humid air in the latter [105].

Water uptake is mainly influenced by SOM and that equilibrium water contents of the organic matrix are comparable for whole-soil samples and isolated humic fractions. Differences in the bond strength of water molecules in wettable and water-repellent soil samples are probably a result of different moisture contents under field conditions. For modelling transport and sorption processes, the hydration kinetics, which may be very slow in water-repellent soils, have to be taken into account. In some water-repellent soil regions, equilibrium conditions are hardly achieved, because the wetting process may last several weeks, rather than only some hours. Consequently, in such soil regions, only time-delayed infiltration and subsequent water storage are possible after precipitation events. By contrast, equilibrium between water and the solid soil phase may be reached immediately in wettable soil regions if no swelling processes are involved. Substances that are transported with the water phase thus may interact instantly with wettable regions, while the penetration into water-repellent parts of the soil is retarded or may not occur at all [105].

2.9 Phase transitions

The phase transitions are characterized by discontinuous change of some property of system, which occurs at certain, accurately determined values of temperature and pressure. A phase consists of a homogeneous, macroscopic volume of matter, separated by well-defined surfaces of negligible influence on the phase properties. Domains in a sample, which differ in composition or physical states, are considered as different phases. In the case where two phases are in contact and at equilibrium, the stability of both phases must be equal. This requires that the two free enthalpies per mole, G' , must be the same at given temperature and pressure. In addition, any infinitesimal change in dG' of each phase must also be equal so that the equilibrium is stable [116].

To stay in equilibrium during a transition, the change in Gibbs energy, ΔG , must be zero. The derivatives of ΔG with respect to temperature, on the other hand, do not have to be zero. This suggests a way to characterize the transitions thermodynamically. Ehrenfest suggested that a transition, for which $\partial\Delta G/\partial T = -\Delta S$ is not equal to zero, to be called as first-order transition. A second-order transition would analogously have the first derivative $\partial\Delta G/\partial T$ equal to zero, but the second derivative $\partial^2\Delta G/\partial T^2$ would not equal to zero [117]. Formally, if the Gibbs free energy function is discontinuous at the transformation temperature, it is a first order transformation; if not, it is of higher order [118].

For first order thermodynamic transitions, such as the melting or boiling points, the entropy, volume, and therefore enthalpy change markedly. This may be mathematically expressed in terms of the first derivative of the Gibbs free energy with respect to temperature (at constant pressure) or pressure (at constant temperature):

$$\begin{aligned}\left(\frac{\partial G}{\partial T}\right)_P &= -S \\ \left(\frac{\partial G}{\partial P}\right)_T &= V\end{aligned}\tag{7}$$

Entropy is a property, which may not be measured directly, therefore more convenient form of the first derivative to inverse temperature may be used [119]:

$$\left(\frac{\partial(G/T)}{\partial(1/T)}\right)_P = H\tag{8}$$

Examples of first order phase transformation would be boiling, sublimation, and solidification. Most solid-state polymorphic transformations are also first-order transitions [118]. In the range of first-order transitions such as melting and crystallization latent heats are absorbed or released [120].

Using the melting point of a solid as an example of a first order transformation, the large discontinuity in enthalpy at the melting point is due to the latent heat absorbed for the structural alterations of the transformation. During that period, the heat capacity is infinite, since all heat input contributes to the transformation and not temperature rise [118].

Regarding the crystallization, at low temperature the crystalline state is most stable. The crystals are stable up to the melting temperature, T_m . At this temperature, the crystal becomes

liquid in order to remain stable. The change of state is connected with a change in the slope of G - i.e., the transition must be of first order [117]. A large number of polymers are able to form crystalline structures, in which parts of the macromolecule are oriented parallelly to each other. Besides crystallites, amorphous regions can also be formed in these materials. In practice, one distinguishes between two kinds of amorphous regions in partially crystalline polymers based on the different types of possible molecular mobility. The mobile amorphous regions are between the crystallites. They also determine the step height of the glass transition. At the surface of the crystallites there are rigid amorphous regions that exhibit an amorphous structure. Despite of this fact, these do not take part in the glass transition because of their reduced mobility. The temperature, at which crystallization occurs, how rapidly it takes place and the degree of crystallinity achieved, depends on the molecular structure of the sample. The size of the crystallites formed during crystallization depends on how easily the polymer chains fit into the crystal structure. In general, polymer chains are less mobile at lower temperatures and only small, less stable crystals are produced. These crystals have a low melting point. The mobility of the molecules is greater at higher temperatures so that larger, more perfect crystals are formed that melt at higher temperatures. The melting curve of a partially crystalline polymer therefore contains information on the size distribution of the crystallites present in the material. The crystallinity of partially crystalline polymers can be investigated by differential scanning calorimetry (DSC) [121].

Second derivatives of the free energy equation yield expressions for second order thermodynamic transitions, including specific heat capacity (C_p), compressibility (k), and thermal expansion coefficient (α); each of which may be determined experimentally [122]:

$$\begin{aligned} \left[\frac{\partial}{\partial T} \left(\frac{\partial G}{\partial T} \right)_P \right]_P &= \left(\frac{\partial^2 G}{\partial T^2} \right)_P = \frac{\partial}{\partial T} (-S)_P = -\frac{C_p}{T} \\ \left[\frac{\partial}{\partial T} \left(\frac{\partial G}{\partial P} \right)_T \right]_T &= \left(\frac{\partial^2 G}{\partial P^2} \right)_T = \frac{\partial}{\partial T} (V)_T = -kV \\ \left[\frac{\partial}{\partial T} \left(\frac{\partial(G/T)}{\partial(1/T)} \right)_P \right]_P &= \left(\frac{\partial H}{\partial T} \right)_P = C_p \\ \left[\frac{\partial}{\partial T} \left(\frac{\partial G}{\partial P} \right)_T \right]_P &= \left(\frac{\partial V}{\partial T} \right)_P = \alpha V \end{aligned} \quad (9)$$

With the second-order transition, there is no latent heat associated; hence the change in heat capacity at the critical temperature is finite. An example of a second-order transformation is the non-superconducting to superconducting transformation at cryogenic temperatures [118].

2.9.1 The glass transitions

All macromolecules display distinct thermodynamic properties that can manifest significant differences in their physical structure and mechanical behavior. One of these characteristics is the glass transition temperature, T_g , which marks a second-order phase transition between a hard, rigid, glasslike state and a soft, flexible, rubbery state. This characteristic property may be measured using differential scanning calorimetry (DSC) [119]. The glass transition is a phenomenon that in principle can occur in all non-crystalline or semicrystalline materials [123]. In these types of material, crystallites and amorphous regions coexist. With increasing

crystallinity, the amorphous content and consequently the intensity of the glass transition decrease [121]. The requirement for the glass transition is a sufficiently large degree of molecular disorder at least in one direction [123].

A special point to note is that glass is an amorphous solid and is not in thermodynamic equilibrium. The transition to the liquid or rubbery state is a relaxation process and is therefore under kinetic control. The glass transition does not therefore occur at a specific temperature as melting, but rather over a broad temperature range [124]. Glass transition is a second-order transition, which is manifested by an abrupt, discontinuous change in specific heat capacity (C_p) and thermal expansion coefficient (α) (Equation 10) [125-126].

$$\left[\frac{\partial}{\partial T} \left(\frac{\partial G}{\partial P} \right)_T \right]_P = \left(\frac{\partial V}{\partial T} \right)_P = \alpha V \quad \left[\frac{\partial}{\partial T} \left(\frac{\partial (G/T)}{\partial (1/T)} \right)_P \right]_P = \left(\frac{\partial H}{\partial T} \right)_P = C_p \quad (10)$$

However, according to Hatakeyama and Quinn, the glass transition is neither first- nor second-order thermodynamic phase transition, since neither the glassy state nor the viscous state is an equilibrium state. The glass transition is exhibited by amorphous polymers or the amorphous regions of partially crystalline polymers when a viscous or rubbery state is transformed into a hard, brittle, glass-like state [127]. The glass transition, although superficially similar to the second-order transition, is time- and frequency-dependent, and thus, it should not be treated as thermodynamic transition [117].

Considering the facts mentioned above, glass transition behaviour could be explained as both kinetic and thermodynamic phenomenon. A thermodynamic argument derives from the observation of the change in the thermodynamics associated with the shift from glassy to rubbery behaviour, as evidenced by changes in heat capacity, viscosity, dielectric relaxation, or thermal expansion coefficients. The kinetic argument derives from observation of changes in glass transition temperatures for samples exposed to different thermal histories [119, 99].

Glass transition temperature, T_g , is the temperature that separates the glassy state from the rubbery state. T_g marks a second-order phase transition in which there is continuity of the free energy function and its first partial derivatives with respect to state variables such as temperature or pressure, but there is a discontinuity in the second partial derivatives of free energy. There is, therefore, continuity in enthalpy, entropy, or volume at the transition temperature but not in the constant-pressure heat capacity, C_p . Hence, measurements of changes in C_p with increasing temperature yield information about T_g as well as about the magnitude of change in C_p that occurs in the transition from glassy state to rubbery state. Since the rubbery state allows greater molecular motion, it exhibits a greater ability to disperse heat and thus manifests a correspondingly higher C_p [128]. Thus, if one assumes that glass transitions are fundamentally thermodynamic in nature, then T_g may be determined by evaluating the temperature at which a discontinuity occurs in C_p (via evaluation of heat capacity changes as a function of temperature through differential scanning calorimetry) or α (via measurement of dimension changes as a function of temperature through thermal mechanical analysis) [119].

2.9.2 Factors affecting the glass transition of macromolecules

Usually, the glass transition temperature depends on the polymer's architecture. There exist several factors influencing the glass transition, such as chain length, its flexibility, the character of side groups, the branching or cross-linking of the polymer's chain. Also, the presence of plasticizers or heating rate significantly affects T_g .

For example, polymer with shorter chains will have more chain ends per unit volume, so there will be more free volume. Therefore, T_g for shorter chains will be lower than T_g for long chains. A polymer with a backbone that exhibits higher flexibility has a lower T_g , since the activation energy for conformational changes is lower. Larger side groups can hinder bond rotation more than smaller ones, and therefore cause an increase in T_g (polar groups such as $-Cl$, $-CN$ or $-OH$ have the strongest effect). Polymers with more branching have more chain ends, so have more free volume, which reduces T_g , but the branches also hinder rotation, like large side groups, which increases T_g . T_g may rise or fall, it depends on the polymer which of these effects is greater [129].

Because T_g is a function of macromolecular mobility, any changes to the macromolecular structure that increase or decrease this mobility will have similar effects on T_g . For example, increased cross-linking restricts chain mobility of larger macromolecular segments, while increased attractive forces between molecules (as measured by the solubility parameter) require more thermal energy to produce molecular motion. Thus, T_g will generally increase with increased cross-linking and increased cohesive energy density σ_p [128].

Factors influencing the mobility of a macromolecule include stiffness of the macromolecule (generally, more aromatic regions are more rigid than aliphatic regions), cohesive energy density (higher values of solubility parameters generally coincide with more polar macromolecules and are responsible for increased interaction energies between individual macromolecule segments), cross-link density (increased number of cross-links reduce segment mobility), molecular weight (larger molecular weights tend to possess decreased numbers of mobile end groups) [130]. The internal mobility of a macromolecule chain is primarily affected by the size of the side-chain, or substituent functional groups attached to one of the carbon-carbon bonds of the macromolecule backbone [131]. The molecular mobility in amorphous regions is influenced by the presence of the crystallites [121].

When a rubbery, amorphous polymer is cooled fast through the glass transition region, it transforms to a glassy matrix with non-equilibrium structure. Glassy matrixes, although called rigid, undergo a process called structural relaxation or physical aging during which the macromolecular structure changes gradually with time, tending toward an equilibrium state [133]. The glass transition is thus strongly influenced by the thermal history of the sample. After sample storage below T_g or slow cooling, glass transition reveal the shape of a peak rather than of a step. This peak is called annealing peak or enthalpic overshoot. Wide distributions of structural relaxation times result in broad glass transition regions with weakly pronounced or no annealing peaks. Thus, broad step-like glass transitions are expected for heterogeneous amorphous materials such as NOM [132]. More complex thermal histories can result in an isothermal relaxation in which the response is initially to depart further from equilibrium before returning to the expected approach towards equilibrium [134]. The

relaxation rate of individual macromolecular chains decreases with increasing glassy character, and it increases with increasing temperature when approaching the glass transition temperature (T_g) [133]. A macromolecule heated at a slower rate will have a lower observed T_g than its more quickly heated counterpart. Observed glass-transition behaviour is clearly influenced by the time scale of the experiment. At a slower heating rate, the macromolecule has a chance to “keep up” with the experiment and thus display a transition that is more consistent with its true transition, compared to the faster heating rates. It is well established that higher heating rates will sharpen heat flow events (increase their intensity) and make them more obvious [119, 135].

The addition of small molecules to the polymer (typically esters), increases the chain mobility by spacing out the chains, and so reduce T_g [129]. Also, exposure of sorbents to solvents or good swelling compounds can impact the glass transition temperature [135]. For instance, water can play a special role since it may either act as a plasticizer (i.e., water removal may increase T_g above the investigated temperature range) or it may act as an antiplasticizer (i.e., the removal of water may reduce the glassy character and induce a water-content-driven continuous transition to a rubbery state). While antiplasticization is rather common in polymer chemistry, antiplasticizing effects of water are rarely reported for environmental systems [133]. The discrepancy between the magnitude in drop of the T_g for the water-wet sorbents relative to their respective “dry” states can be attributed to the interaction of water molecules with the sorbing matrices. Water interacts more favorably with chemically similar matrices (sorbents with solubility parameters closest to its own value). This increased interaction results in greater water uptake, causing additional swelling within the humic acid matrix, and hence a greater lowering of T_g . The water may not only play a role in swelling of the matrix and disruption of van der Waals forces but it may also play a role in disrupting some hydrogen bonds, thus the larger reduction in T_g . The sorbed water likely disrupts many intermolecular bonds, thus less thermal energy is required for the macromolecule to transcend to the rubbery state [135]. Glass transition behaviour in this model would represent a cooperative process of cross-link disruption and increase in side chain mobility connected with an increased mobility of the released water molecules. The slow reduction of T_g upon water uptake may point either to additional matrix plasticization or to a decrease in portions of the matrix experiencing antiplasticization effects. In terms of the suggested model, water-based cross-links become more hydrated with increasing water content, leading to increased water-water interactions at the expense of water-polymer chain interactions, resulting in reduced antiplasticization capability [133].

Polymers having hydrophilic components such as hydroxyl or amide groups form intermolecular bonds in the presence of water, which strongly affect the characteristics of the glass transition. Main-chain motion is restricted owing to these intermolecular interactions and the glass transition temperature is higher than that of the hydrophilic polymer in the completely dry state or a similar hydrophobic polymer. In certain kinds of proteins and polysaccharides no glass transition or melting is observed until decomposition of the main chain occurs because intramolecular and intermolecular hydrogen bonds stabilize the high order structure of these polymers. On the other hand, introducing a small amount of water to a hydrophilic polymer may disrupt the intermolecular bonds, thereby enhancing the main-chain motion. In this case T_g shifts to lower temperatures in the presence of water [127].

2.9.3 Glass transitions in Natural Organic Matter and Soil Organic Matter

Implications of the existence of T_g in natural organic matter are limited not only to yield information about the macromolecular structure of humic substances but also can provide valuable insights into the sorptive behaviour of these substances [119, 99].

Macromolecules of relatively homogeneous composition are more likely to form crystalline regions than macromolecules with irregular chain structures and protruding side functional groups or so-called *atactic* macromolecules. Given the relative heterogeneity of humified soil or sediment organic macromolecules, one would thus expect the occurrence of total crystallinity in these natural (likely atactic) systems to be relatively infrequent, with only very small or so-called “microcrystalline regions” forming under glassy state conditions. However, for more homogeneous biopolymers or largely diagenetically altered natural organic matter (e.g. coals) it may be possible to find significantly larger regions of crystallinity. While regions of microcrystallinity may influence observed equilibrium sorption behaviour, it is likely that such crystalline areas do not contribute significantly to sorption capacity and do not play significant roles in non-equilibrium sorption behaviour due to the inability of most solutes to penetrate these regions. It is more likely that the void spaces between the crystallites in the amorphous rubbery and glassy regions of the macromolecule control non-equilibrium sorption behaviour [99]. Organic and other “poorly crystallizing” compounds form a solid glass on cooling [121]. Such amorphous material can then crystallize on heating to temperatures above the glass transition temperature (devitrification, cold crystallization) and recrystallize to form larger, more stable crystals that then melt at higher temperatures [136, 123]. Cold crystallization can often occur in two steps. On further heating, polymorphic transitions can occur before the solid phase finally melts [121].

Both the rubbery and glassy states of natural organic molecules are amorphous in nature, the distinction being one of degree. Thus, while all structural features influence the mobility of such molecules, only those of the amorphous (non-crystalline) regions influence the magnitude of the glass transition temperature [135]. If the molecular weight decreases, molecular motion of the end groups begins to affect the main chain motion, i.e. rotation of the end groups cannot be ignored and enhances the whole chain movement. In that situation, the C_p of the glassy state is high and a small ΔC_p value at T_g can be observed [137]. Molecules possessing aromatic (e.g. coals) or parallel bonds in their backbones have extremely stiff bonds, resulting in a reduction of molecular mobility and increased T_g s [131]. In addition, an increase in the free volume of a macromolecule allows more room for molecular movement and thus yields an accompanying reduction in T_g . Swelling of a macromolecular sorbent by thermodynamically compatible solutes will therefore tend to increase the free volume and lower T_g [128]. The mobility of polymer chains may be important for the transport of sorbed organic compounds, which can be transferred from one site to another by movement of polymer chains [101].

Glass transition behaviour is an inherent property of amorphous solids. Two mechanisms of glass transitions have been distinguished in humous soil samples. One of them is closely related with water binding and may be explained by the formation and disruption of cross-linking water bridges between individual strands. Due to its slowly reversing character, this transition does not fully represent a classical glass transition [139]. In order to distinguish this

transition type from the classical one, it is referred to as glass transition-like step transition with the transition temperature T_g^* [138]. The glass transition-like step transitions occur at higher temperatures and reveal higher intensities than the classical glass transitions of SOM [132]. Classical glass transition is connected with a decrease in matrix rigidity [139], characterizes structural properties of the water-free organic matrix and may be related to organic matter aromaticity [132]. Water in this transition surprisingly acts in an antagonistic way as short-term plasticizer and long-term antiplasticizer in soil organic matter. The second transition can be detected in water-free systems only and indicates classical glass transition behaviour [139].

The two types of glass transition behaviour in SOM can be explained by the hydrogen bond-based cross-linking (HBCL) model proposed by Schaumann and LeBoeuf [138, 133, 140]. This model supposes hydrogen bond-based cross-links by individual water molecules between the molecular chains of SOM, resulting in an antiplasticization of the SOM structure for low water contents. If these cross-links are disrupted by the removal of water out of the system, the chain mobility increases, and consequently the glass transition-like step transition shifts to significantly lower temperatures and becomes a reversible classical glass transition [138, 133, 140].

For a simple polymer chain to change its conformation, individual C-C bonds must twist from *trans* to *gauche* position – i.e. the torsion angles must change. This is a thermally activated process. At low temperatures, there is not enough thermal energy available to allow torsion angle changes, so the conformation becomes “frozen”. The temperature above which the torsion angles can change is called glass transition temperature. The changes in conformation depend on time-scale [129]. A general observation is that carbonaceous SOM (e.g. kerogen, black carbon), with higher degree of diagenetic/anthropogenic alteration, possess higher glass transition temperatures than humic-based SOM (e.g. humic acid, fulvic acid) due to higher degree of cross-linking, chain stiffness from an atomic-level perspective and higher average molecular weight, higher aromatic character, leading to different SOM macromolecular mobility and sorption/desorption behaviours [128, 125].

Although identified in some soil samples, it is still uncertain whether glass transition behaviour is representative of SOM. Furthermore, it is unknown which properties of the organic substances control temperature and intensity of the transitions. The SOM content, as well as structural properties such as cross-linking density, degree of decomposition, and degree of swelling may represent important influencing factors [138].

3. THE AIM OF THE WORK

The aim of this work is to contribute to the knowledge of the character of hydration water in water/humic substances systems. Humic substances originate from different sources, which cause the variability in their chemical composition and physical-chemical properties. The goal is to determine both quantitative and qualitative aspects of hydration of humic substances both in solid and liquid phases. The attempt was made to recognize the differences in properties of water surrounding humic molecules and aggregates with the assistance of physical methods and quantify it by combinations of various techniques and approaches. Methods of choice were mainly methods of thermal analysis such as differential scanning calorimetry and thermogravimetry and high resolution ultrasonic spectroscopy.

In our recent work [141] we revealed that hydrophobic hydration plays a significant role in hydration processes of HS. That statement is quite understandable since HS consist of hydrophobic, hydrophilic and amphiphilic(-like) molecules arranged in a kind of planar structures resembling open-layer assemblies variably orientated in solutions. We assume that such assemblies can persist when water/HS system is completely dry [141], and forms variety of hydrophobic domains (amorphous and/or crystalline [7]). Therefore, the simple similarity between HS and polysaccharides or proteins can be barely expected [142]. Nevertheless, there is still a certain parallelism with the properties of hydrogels; however the structure of HS does not seem to be reversible and flexible. The reason can be seen in the supramolecular character of their structure, i.e. mostly non-covalent nature of bonds stabilizing their structure, differences in polarity of individual domains or in mechanical properties (elasticity) of individual components.

In addition, in line with the literature [105] we assume that kinetics play an important role in hydration processes. Last but not least, reversibility of those processes is also a question since hydration of biopolymers is associated with their structural and functional role. It is desirable to keep the original structure after rewetting and drying cycles. Hydration of HS, on the other hand, cannot be fully reversible since at least small redistribution of particular moieties can be expected after those cycles. As already mentioned, it seems that the processes of humification can take part in this way. Accordingly, the former kind of hydration can be considered as reversible while the latter (not fully understood yet) includes progressive humic material transformation, physical and potentially chemical.

In light of above discussion, the approaches and techniques already used and reported as being successful for determination and enumeration of hydration water in biopolymers will be applied for HS, but the attention will be also paid to reveal possible similarities and dissimilarities between those classes of organic compounds.

4. RESULTS AND DISCUSSION

4.1 Study on properties of water surrounding humic aggregates in aqueous humic solutions [141]

The thermodynamic stability and molecular feature of lignite humic acids aggregates were studied in recently published work using high resolution ultrasonic spectroscopy [141]. In fact, humic acids solutions of different concentrations were heated and cooled within the temperature interval from 5 to 90 °C and changes in ultrasonic velocity were monitored. The motivation of this part of our work was to clarify the character of weak dispersive forces assembling humic molecules together. The aim was to observe the change in the nature of water structure surrounding humic aggregates and the influence of increasing temperature on hydration of humic aggregates in aqueous humic solutions within the concentration range from 0.005 to 10 g L⁻¹.

A number of studies give excellent accounts of the present state of knowledge regarding the secondary structure of humic substances. It is generally recognized that humic molecules are surface active and can solubilize a wide variety of hydrophobic species [143-145]. This behaviour of humic molecules is usually attributed to micelle-like organizations of HA in aqueous solutions. However, high concentrations required for the formation of such organizations rarely correspond to environmental situations [145]. It is therefore of practical interest to study the character of humic organizations also in diluted solutions. In fact, the solubilization capacity of humic substances has been reported also at significantly lower concentration than the critical micelle concentration reported for humic substances (i.e. from 2-10 g L⁻¹). Wershaw [146] proposed two possible explanations: (i) partition of hydrophobic molecules into interiors of aggregates of amphiphilic molecules at concentrations lower than the CMC (premicellar aggregates), or (ii) association of hydrophobic molecules with the non-polar parts of unassociated amphiphilic molecules. Evidence for micelle-like organizations which does not feature a critical micelle concentration was found by Engebretson et al. [147-148]. A decade ago, spectroscopic evidence for existence of humic pseudomicelles was reviewed [149]. It has been stated that the prevailing interactions holding humic aggregates/molecules together in diluted solutions are in particular hydrophobic weak interactions. Micelle-like character of humic molecules indicates the hydrophobic-hydrophilic nature of molecules and implies presence of charged sites at neutral pH. Those are in diluted solutions separated due to repulsive forces and the conditions for H-bridges formation are limited.

Recent results obtained by high resolution ultrasonic spectroscopy (HRUS) indicated that the humic aggregation instead of being described as a micellization should be better viewed as a kind of hydrotropic process [150]. According to the theory of hydrotropy, small molecules, with hydrophobic chains shorter than eight carbons, tend to self-aggregate as well, but at concentrations different from the critical micelle concentration reported for surfactants. Most of methods, which can be carried out to elucidate the secondary structure of humic substances, have several limitations, such as composition of mobile phase for the applications of size exclusion chromatography or concentration of humic solutions limiting a large number of spectroscopic methods. Such limitations can be overcome by a recently developed

analytical technique, a high resolution ultrasonic spectroscopy measuring the velocity and attenuation of ultrasonic wave propagating through the liquid sample. In principle, the ultrasonic wave interacts with the sample's interior, causes compression and decompression and thereby enables the analysis of its physical and chemical properties including the information on both inter and intramolecular nature of molecular organizations and their hydration [151]. One of the advantages of that method is a bimodal arrangement (independent sample and reference cell) when samples properties can be monitored either under isothermal conditions or under non-isothermal regime [152].

The purpose of this work was to monitor changes in ultrasonic parameters (ultrasonic velocity and attenuation) induced by heating of HA solutions at different concentrations, covering the range from 0.005 to 10 g L⁻¹. The conformation rearrangement of secondary structure of humic molecules was further evaluated upon addition of selected modifiers to humic solutions.

4.1.1 Experimental

Humic substances

Humic acids (HA) (a part of humic substances soluble in alkali media) were isolated from the South Moravian lignite collected from the Mír mine in the area of Mikulčice, near Hodonín, Moravia, the Czech Republic [153] using standard alkali extraction with 0.5M NaOH. Full details on extraction procedure and HA characterization can be found elsewhere [154-159]. The ash content was approximately 2 %. HA sample was titrated to pH 7 with 0.1M NaOH employing an automatic titrator (Schott, TitroLine Alpha Plus) under constant stirring. After reaching the constant value for 60 min, the sodium humate (NaHA) solution was filtered, freeze-dried and lyophilized. The product was milled in an agate mortar and stored. Details on the elemental and composition analysis of HA sample is given in refs [150] and [158].

HRUS measurement

To monitor ultrasonic velocity and attenuation, HRUS 102 device (Ultrasonic-Scientific, Dublin, Ireland) was employed. HRUS consists of two independently measuring quartz cells tempered by the same water bath; cell 1 serves as a sample cell and cell 2 as a reference. All measurements were carried out at 25.00 ± 0.02 °C, under constant stirring (600 rpm) and at ultrasound frequency of 5480 kHz. NaHA was dissolved in deionized water to desired concentration in the range of 0.005 to 10 g L⁻¹. Cell 1 was loaded up by 1 mL of a sample whereas cell 2 by 1 mL of deionized water. Thermal behaviour of NaHA solutions was investigated using following temperature regime: step 1 - from 25 °C to 90 °C for 3 hours, back to 5 °C for 4 hours; step 2 - from 5 °C to 90 °C for 4 hours and back to 5 °C for 4 hours; step 3 - from 5 °C to 90 °C for 4 hours and back to 25 °C for 3 hours. Then the influence of HCl and propionic acid on the solution character was tested. The sample of concentration of 1 g L⁻¹ was modified by addition of HCl or propionic acid to obtain pH 3.5. Temperature regime was the same as for original sample. The amount of added compounds was in the comparison with the amount of HAs negligible and therefore the ionic strength of the solution has been changed only infinitesimally. During the temperature programs velocity (U) was measured in both cells. The results were expressed as a difference of measured values between cell 1 and cell 2, i.e. U₁₂. Simultaneously with U also the attenuation was measured,

however, no valuable result was obtained and therefore, data are not presented. The resolution of the spectrometer is down to 10^{-5} % for ultrasonic velocity and 0.2 % for attenuation measurement. All experiments were carried out in duplicate and no significant deviations between experimental results have been observed.

4.1.2 Results and discussion

Concentration influence

To avoid the influence of anomalies of water in the presented work (such as dependence of velocity of sound in water on temperature), results are given as a difference of ultrasonic velocity (U12) in cell 1 (sample) and cell 2 (reference). The sample is 1 g L^{-1} humic solution while reference is the water solvent. The changes induced by elevated temperatures from 5 to 90 °C were reversible which implies that processes were of physical, rather than chemical i.e. irreversible character. The first heating cycle was used to adjust humic samples having the same “thermal history” mainly to be completely dissolved since dissolution processes in humic solutions can be rather slow [159]. The first part of second heating cycle has been used for monitoring of changes in humic solutions induced by heat while the third one for the controlling of reproducibility of the experiment.

A strong affinity of water molecules to form structures stabilized by H-bonds is a driving force promoting aggregation of amphiphilic and hydrophobic humic molecules. Next force, although less strong, that governs self-assembly of amphiphiles into organized structures derives from hydrophobic attraction at the hydrocarbon-water interface and hydrophilic, ionic and steric repulsion of the head-groups, which imposes the opposite requirement that they remain in contact with water. Two thermodynamic factors play crucial roles in the forming of water structure: entropy and enthalpy. In fact, at the room temperature the enthalpy contribution of transfer from organic solution into aqueous solution is practically negligible. The entropy however is negative. Water tends to form ordered cages around the non-polar (i.e. hydrophobic) molecule and this leads to a decrease in entropy. At high temperatures (~ 110 °C) these cages are no longer stronger than bulk water, and the entropy contribution tends to zero. The enthalpy of transfer, however, is now positive (unfavourable). Because the temperature dependence of entropy and enthalpy are not the same, there is some temperature at which the hydrophobic effect is strongest, and the effect decreases at temperatures above and below this temperature [160].

Figures 12-14 report examples of temperature dependence of ultrasonic velocity difference of humic solutions at $10\text{-}0.005 \text{ g L}^{-1}$. As can be seen, the temperature dependence of differential ultrasonic velocity dramatically changes with changing HA concentration. Concentration of 10 g L^{-1} (Fig. 12A) showed a monotonous exponential decay while the slope of the dependency slowly but gradually increased. The exception in monotonous decrease can be seen at low temperature up to 8 °C – this artefact was observed during each heating cycle including low concentrations and it can be attributed to the switch in temperature program from cooling to heating ramp. Therefore, in our considerations it is not taken into account.

In an ideal case, i.e. in case of incompressible dissolved materials, the dependence of U12 on temperature is linearly decreasing for most hydrophilic materials; it is a result of the decrease

in storage modulus of water with temperature [161]. Such linear decrease is associated with the decrease in dielectric constant of water at elevated temperatures causing a decrease in the strength of water H-bonds which is the main driving force assembling the humic molecules/aggregates together. The water in close vicinity of solute molecules (water shell) has been reported to be more supportive for ultrasonic wave propagation [161-162]. It is in accordance with the knowledge that the first hydration shell is 10-20 % denser than the bulk water [163]. The exponential-like curvature given in Fig. 12 can be explained by the fact that the strength of the attractive hydrophobic interactions among aggregating humic molecules slightly increases with temperature. Closer distance among humic molecules in aggregates with increasing temperature causes higher density and supports the elastic response of compression caused by propagating US wave. As a result, the relaxation time of a compressed hydrophobic structure decreases. Moreover, an increased strength of hydrophobic interactions can partially result from the increased hydrophobicity of some hydrophilic headgroups that occurs at higher temperatures [168].

Slightly different record can be seen in Fig. 12B where the ultrasonic velocity for concentration 5 g L^{-1} is reported. The decrease of U12 is accompanied by several breaks or perhaps better transitions: a small increase within 27 to 29 °C and increase at 48 °C followed by decrease at 49 °C. On the other hand, ultrasonic records of solution 4 g L^{-1} reported in Fig. 12C resulted in the shift of break – decrease to 47-48 °C. Moreover, new disruptions appeared at 15, 21, 33 and 53 °C. That is the consequence of continuous dilution of humic solutions, which causes the decrease in stability of present aggregates.

Fig. 13A-C shows that the continuous disruption of humic aggregates at 3 g L^{-1} did not result in any changes in comparison with 4 g L^{-1} except of three breaks in the temperature interval between 21-36 °C. In contrast, 2 g L^{-1} exhibited the intensification of disruption in the interval 20-24 °C whereas from 40 °C the decrease showed a monotonous shape.

As stated before, at elevated temperature the importance of entropic factor is diminished which causes the weakening of hydration shell surrounding humic molecules, disrupting the water structure, which consequently leads to an aggregate destabilization. In thermoanalytical regime, the HRUS has been already employed for instance for studying of phase transitions of carbonic anhydrase, thermal transitions in carrageenan solutins or coagulation in calcium fortified milk [161, 164, 165]. The decrease in velocity over the course of the break for carbonic anhydrase was attributed to the formation of highly compressible hydrophobic core in which hydrophobic residues stack to each other. The increase of US in carrageenan solution, on the other hand, was explained as a phase transition (melting of its helical structure and breaking of intermolecular connections) accompanied by increase in hydration level of atomic groups of carrageenan. Thus the sudden increase in velocity in the lower concentrations records can be associated with disruption of weak interactions assembling humic aggregates together. It has been demonstrated that diluting of humates is associated with decreasing in the number of relatively stable H-bonds and humic molecules are loosely bound by hydrophobic interactions such as $\text{CH} - \pi$, $\pi - \pi$ interactions or van der Waals forces [166]. Within the whole concentration range under investigation, i.e. from 5 to 2 g L^{-1} the temperature of the break is slowly shifted to lower temperatures depending on the solution

concentration. This implies the diminishing of the role of an interaction stabilizing humic aggregates with decreasing of humic acids concentration.

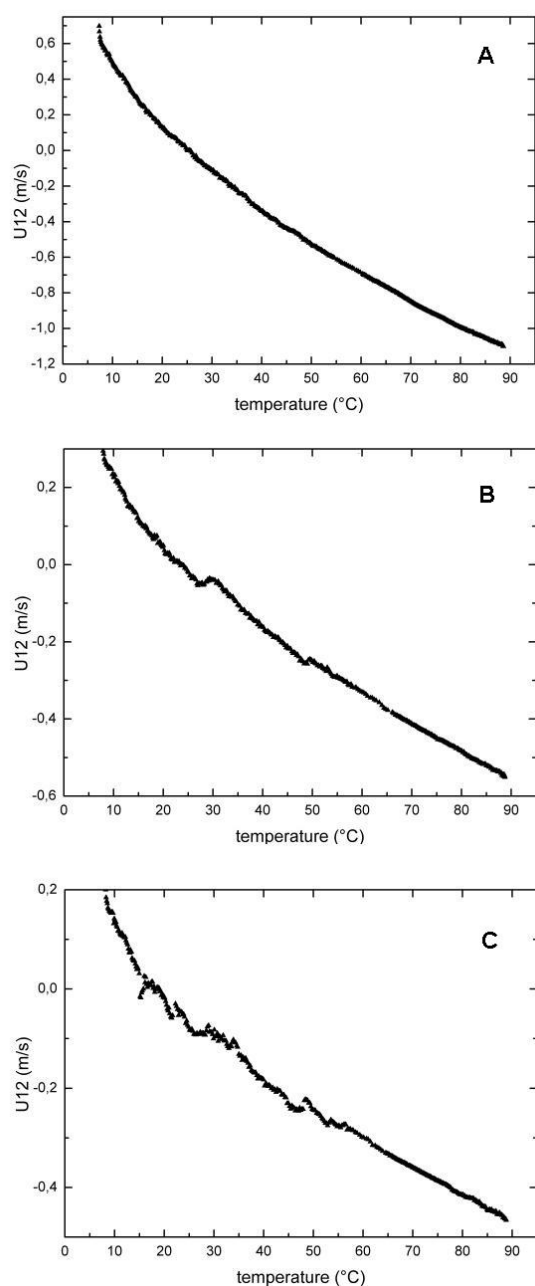


Fig. 12 Difference in ultrasonic velocity for 10 g L^{-1} (A), 5 g L^{-1} (B) and 4 g L^{-1} (C)

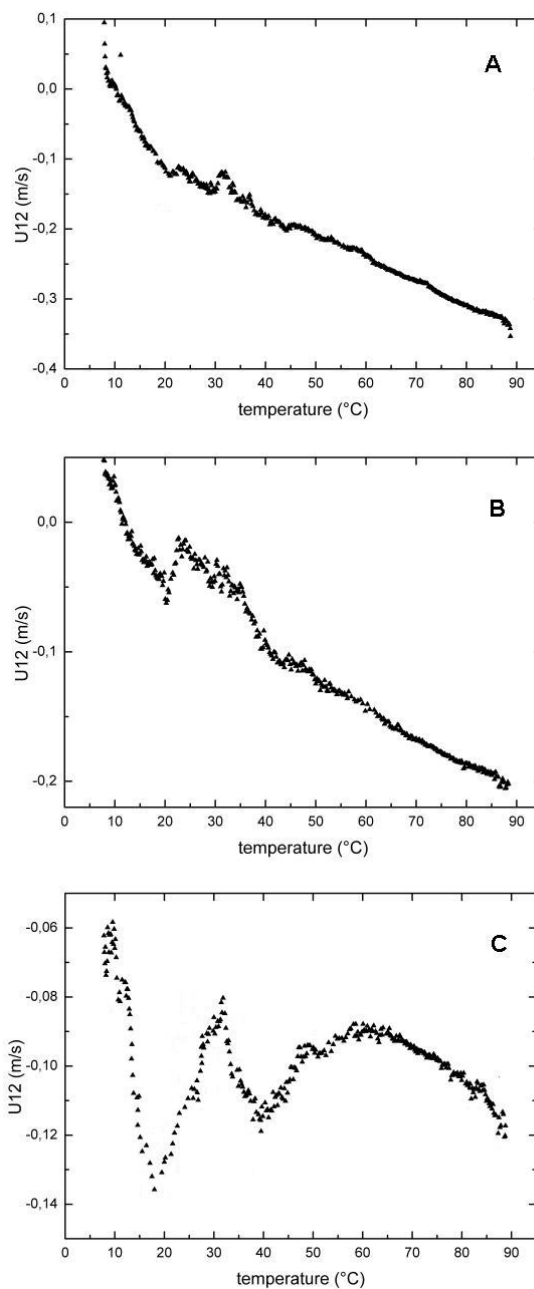


Fig. 13 Difference in ultrasonic velocity for 3 g L^{-1} (A), 2 g L^{-1} (B) and 1 g L^{-1} (C)

A dramatic change in ultrasonic profile showed 1 g L^{-1} concentrated solution. Due to several intensive transitions associated with increasing of ultrasonic wave velocity, values at 5°C did not differ significantly from that at 90°C as previously seen for higher concentrations and also later for lower concentrations. Records of HRUS of concentrations $0.5\text{--}0.005 \text{ g L}^{-1}$ are reported in Fig. 14. Main difference of the low concentration record is associated with the switching into “negative” dependency of U12 on temperature in the low temperature interval and in turn to an increasing tendency at elevated temperatures. Decreasing of concentration

brought the reduction of number of transitions while for 0.005 g L^{-1} transitions at 10, 15, from 35 to 45 °C and at 52 °C were still visible. Again, in comparison with concentrations 0.5 and 0.05 g L^{-1} the shift to lower temperature and reduction in intensity were recorded. From 60 °C the U12 parameter stayed practically constant up to 90 °C.

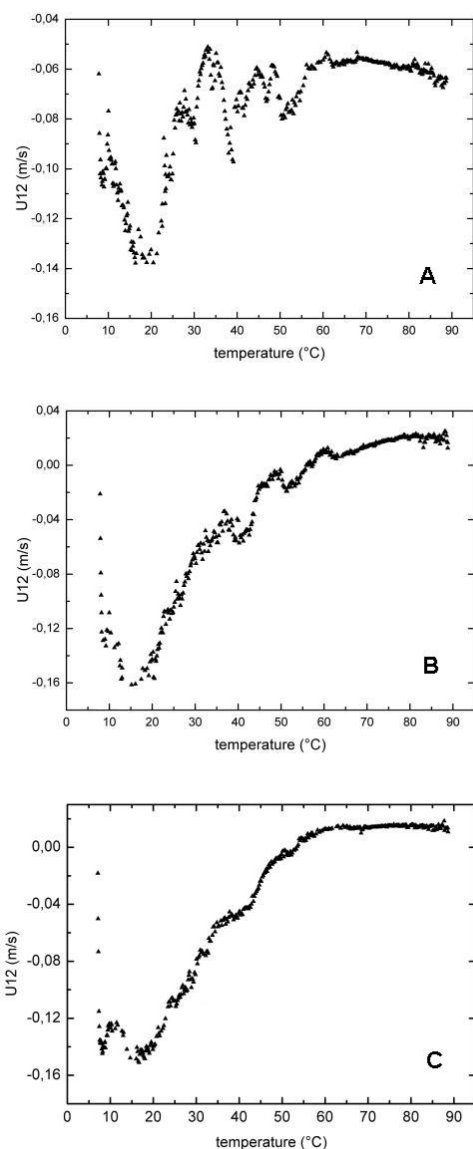


Fig. 14 Difference in ultrasonic velocity for 0.5 g L^{-1} (A), 0.05 g L^{-1} (B) and 0.005 g L^{-1} (C)

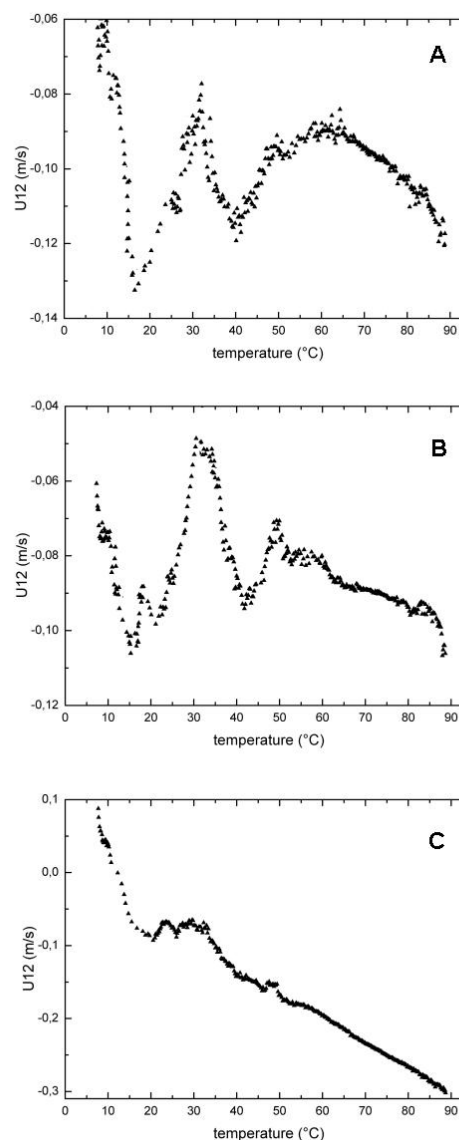


Fig. 15 Difference in ultrasonic velocity for 1 g L^{-1} (A) and the same concentration modified by HCl (B) and by propionic acid (C) at pH 3.5

These results are in line with previous observation that character of prevailing interactions among humic molecules strongly depends on concentration [166, 150]. The transitions can be attributed to the weakening of bonds among hydrophilic moieties of humic acids and water leading to the collapse of molecular organizations.

Basically, hydrophobic hydration produces a reduction in density [167] and an increase in the heat capacity [168]. As ultrasonic velocity depends on the state of water in hydration shell and

in a special case also on intrinsic compressibility of a solute [162], indeed, the contribution of highly hydrated atomic groups would increase the U12, while the contribution of hydrophobic hydration is close to zero. The expanded network causes the density decrease whereas the ordered bonds must be bent on increasing the temperature, so affecting the heat capacity. Thus, hydrophobic hydration behaves in an opposite manner to polar hydration, which increases the density and decreases heat capacity due to their associated disorganized hydrogen bonds being already bent or broken. Hydrophobic hydration is accompanied by a negative enthalpy change, due in part to the multiple van der Waals interactions between water and the hydrophobic material, a negative entropy change due to the increased order in the surrounding water and positive heat capacity change due to the negative enthalpy change (i.e. the stronger hydrogen bonds at the surface). As a result, in our case the decreasing concentrations lead to a decrease in U12 parameter while around 1 g L^{-1} U12 switched to opposite feature. According to von Palmer and von Wandruszka [169] elevated temperatures can promote the clouding effect which has been demonstrated for a set of humic and fulvic acids using dynamic light scattering. In light of our observations, it seems that elevated temperatures increase also the density of hydration shell around hydrophobic molecules/aggregates, which can be interpreted as an increase in humic aggregate dimensions. That can be seen in the temperature interval 15-35 °C in Fig. 14B, which is the same temperature interval and more or less the same concentration which was reported in ref. [169]. Since humic acids generally consist of a huge number of molecules possessing different polarity and polarizability, the temperature induced aggregation promoted by weakening of hydration shell around hydrophilic moieties is probable.

The question remains if changes in temperatures of breaks within 1 °C are significant and relevant. Although records are reproducible and heating and cooling programs are very slow, HRUS 102 is not primarily constructed as a thermoanalytical device and thus resulted temperatures can be slightly biased by the (unknown) heat transfer coefficient and potentially by the kinetics of processes under study.

Modified humic solutions

In order to test the previous assumptions [150], 1 g L^{-1} humic solution was treated either by propionic or hydrochloric acid (decrease in pH from 7 to 3.5) to see changes in stability induced by protonization of COOH groups and consequently a change in the stability of humate aggregates. It is important to note that the amount of additives was very low in comparison with the volume of humic solutions (microliters of additives to milliliters of humic acids) and therefore, the addition of modifiers significantly influenced neither the solution composition nor the solutions ionic strength. In addition, the water in reference cell was modified by the same amount of modifiers as humic solution. Therefore i) the changes in structure stability can be attributed only to the action of modifiers, ii) the concentration and composition of humic solution stayed practically constant. Fig. 15 reports the HRUS records of modified 1 g L^{-1} humate solution. As demonstrated earlier, the modification of humate solutions by hydrochloric acid causes the protonization of humic molecules which decreases the strength of repulsive forces of charged sites and promotes the aggregation and formation of larger humic aggregates [8, 150]. As can be seen from Fig. 15, the modification had a slight effect on the stability of secondary humic structure. The most important differences, if compared with non-modified sample, can be seen in the shift of the break from the previous

29 to 31 °C and from 39 to 41 °C, which indicates the employment of higher number of H-bridges stabilizing humic structure. Values of U12 are higher in modified sample, which can indicate either better hydration, lower compressibility of resulted aggregates or a decrease in the relaxation time of present hydrophobic cores. Since the former is in contrast with enhanced capability of humic substances to interact with hydrophobic compounds at lower pH induced by hydrochloric acid [143], we suppose a slight modification of the compressibility of hydrophobic domains. The protonization of polar head-groups of molecules involved into micelle-like structures lead to the decrease of their mutual repulsions which can, although only slightly, decrease the compressibility of the aggregates or influence the relaxation time [162]. Assuming the spherical geometry of a micelle-like aggregate, a higher affinity of polar head-groups to each other, is supposed to be a barrier of hydrophobic compound to interact with hydrophobic core as mentioned above. It invokes a view in which humic aggregates are arranged in a kind of planar structures or open-layer assemblies variably oriented in solutions. That is in line with recently proposed model of aggregation of humic acids based on the similarity with hydrotropic compounds [150]. The planar structure formed by some hydrotropic compounds is well recognized [170]. Moreover, the strength of weak hydrophobic interactions strongly depends on their orientation [171] and repulsion of deprotonized polar groups at neutral pH maintains the structure unfolded. On the other hand, the hydrotropy model does not suit to humic self-assembly mechanisms perfectly. Further, it has been demonstrated no mutual stacking among aromatic hydrotropic molecules, while that is probable in case of highly heterogeneous humic mixture [8, 158].

In contrast to hydrochloric acid, addition of propionic acid into humic sample gave a completely different result (Fig. 15C). All the temperatures of breaks registered in non-treated sample were shifted to higher temperatures, diminished or even disappeared. The slope of the record is similar to those at higher concentrations. Therefore, it is clear that propionic acid action causes more intensive stabilization effect which is associated with the increase in number of H-bonds and probably re-aggregation of secondary structure [150]. In light of above-discussion, the slope of the dependence, if compared with the non-treated sample, indicates the prevalence of hydrophilic hydration. In fact, the protonization of dissociated charged sites such as carboxylic groups promotes the attraction among polar groups and structure strengthening.

Recently, Šmejkalová and Piccolo [172] used diffusion drift NMR for evaluation of humic and fulvic acids aggregates treated by various organic acids. Authors observed the shift in diameter of humic aggregates towards lower molecular dimensions, which was attributed to the separation of aggregates from each other. The same conclusion was reported previously using Size Exclusion Chromatography [8]. It has been stated that the hydrophobic core of humic micelle-like aggregates was after addition of propionic acid reorganized forming assemblies stabilized mostly by H-bonds. We cannot also exclude the hypothesis that humic hydrophobic molecules were separated from each other and surrounded by propionic acids molecules. That phenomenon again reflects the consequences of effect of hydrotropy [173]. In this case, water shell surrounds the polar part of propionic acid while hydrophobic is oriented towards humic molecules. Due to H-bridging between propionic acid and water, such arrangement is more thermodynamically stable and enhances the solubilization of hydrophobic humic molecules.

4.2 Study on hydration properties of biomolecules using thermal analysis

Published works focused on study of hydration properties of biomolecules employing Differential Scanning Calorimetry goes back to the seventies of the 20th century. For example, Pouchlý et al. investigated the heat capacities of water swollen poly[2-(2-hydroxyethoxy)ethyl methacrylate] (so called PHEOEMA) [174] and poly(2-hydroxyethyl acrylate) (PHEA) [175]. The authors stated that at temperatures above 0 °C the partial specific heat capacity of water in gel is concentration independent and equal to the specific heat capacity of pure liquid water. They suggested that water does not form stable ice-like structures near polymer chains and that the non-freezing water need not to be identical with “strongly bound” water. Furthermore, they assumed that if some water did not freeze below 0 °C it could be probably ascribed to the fact that the system was not in equilibrium. They observed that at low water content or at temperatures below 250 K, the crystallization of water from gel was inhibited by kinetic factors originating probably in the reduced diffusivity of water in gel, due to the reduced mobility of polymer chains. They also observed that the heating phase transition peak area belongs to the sum of melting enthalpy of water and heat of admixture. The crystallization and melting of adsorbed water on cellulose samples was studied by Nakamura et al. [176]. They evaluated two exothermic peaks of crystallization of adsorbed water on the cellulose samples. One was a sharp peak observed at about 255 K in a DSC curve ascribed to the free water; the other was a broad peak observed at about 230-250 K attributed to the freezing bound water. Concluding from the amounts of water, calculated from the results obtained by the DSC study, they assumed that there was present also some non-freezing water, which did not crystallize at all.

In the nineties of the 20th century, many works regarding study on hydration properties of water were published. For example, Yoshida et al. in [177] determined the amounts of freezing and non-freezing water depending on water content in a sample (W_c) of hyaluronan (HYA). They observed that with increasing W_c the temperature of melting increased while the heat capacity decreased. The higher the concentration of water in a sample was, the more heat capacity value approximates the value generally accepted for pure water. They were judging from the results either that the freezing water was free or bound while the bound water was due to HYA mainly in glassy state because the structure of HYA kept water from crystallization. In the follow-up study [179], Yoshida et al. observed water molecules in hydrogels of hyaluronic acid and xanthan. They classified water molecules into three categories according to phase transition behaviour; non-freezing, freezing bound and free water. It was observed that melting, crystallization, and glass transition of water in hydrogels reflected the state of water interacting with polysaccharides. They obtained the nucleation rate and crystal grow rate from the isothermal crystallization measurements, and found out that the crystal grow rate of freezing bound water was about ten times slower than that of free water. The hydration in hyaluronic acid and its ethyl and benzyl esters was investigated in work [178]. The DSC results revealed that while the amount of freezing water was decreasing with the increasing degree of esterification, the amount of non-freezing water was higher. In work [180] the ability of hylan, the formaldehyde cross-linked derivative of hyaluronan to interact with water has been studied. Again, three types of water (non-freezing, freezing-bound and free) were distinguished. The authors assumed from the results that freezing bound water was thermodynamically different from free water. When the water content of the system was increased, even by up to 10 %, almost all the water remained in the freezing bound state, with

the value of enthalpy change lesser than that of free water. The large capacity shown by hyaluronan entangled networks to build water into their structure, which could also be responsible for their unusually high viscosity and elasticity. Hatakeyama et al. calculated the number of bound water molecules restricted by mono-, di-, and trivalent cations in polyelectrolytes, such as polystyrene sulphonate, carboxymethylcellulose and alginic acid. [181]. The results indicated that the higher order structure of polyelectrolytes strongly influenced the number of water molecules tightly bound by cations. The number of bound water molecules decreased with increasing ionic radius in the series of mono- and divalent cations when polyelectrolytes formed the liquid crystalline state. However, when polyelectrolytes formed rigid junction zones in the presence of cations, the number of bound water molecules was maintained at a constant value regardless of ionic radius. Norinaga et al. [182] analyzed water sorbed in eight coals of different ranks. The water was classified into three types as was in works mentioned above. The contents of the free and bound water were experimentally determined from their respective heats of con-gelation, and the non-freezable water was then calculated by difference. The authors stated that the water classified as bound was actually water condensed in pores. The authors in their follow-up work [183] studied the interactions between water and hydrophilic polymers. They stated that various natural and synthetic polymers with hydrophilic groups, such as hydroxyl, carboxyl and carbonyl groups, have either a strong or weak interaction with water, and thermal properties of polymers and water are both markedly influenced through this interaction. Again, they described three types of water using DSC. They assumed that bound water in the water-insoluble hydrophilic polymers, such as cellulose, lignin and poly(hydroxystyrene) derivatives, breaks hydrogen bonding between the hydroxyl groups of the polymers. The bound water content depends on the chemical and high-order structure of each polymer. Aqueous solutions of water-soluble polyelectrolytes, such as hyaluronic acid, gellan gum, xanthan gum and poly(vinyl alcohol) form gels above a threshold concentration. In the above gels, water mostly exists as the freezing bound water, playing an important role in the junction zone formation. Cowman et al. in [184] investigated the interactions of water with HYA. Engaging DSC, they investigated the influence of HYA on ice nucleation. According to the results, HYA inhibited the formation of ice, mainly for higher W_c . They also observed that water presented in pores of hydrogels played important role because of having different behaviour without being bound. They supposed that the size of pores descended with increasing concentration of HYA. Thereafter, Cowman et al. continued in research on HYA with follow-up study regarding the freezing and melting of water in semi-dilute solutions of the polysaccharide hyaluronan [185]. Their results showed that semi-dilute hyaluronan had significantly altered freezing and melting transitions for water. The effects were greater than could be accounted for the presence of a small amount of non-freezing water, strongly bound to the polymer. So that, they propose a larger boundary layer of water, with slightly altered thermodynamic properties and its size was estimated. They suggested that this boundary layer water behaved similarly to water trapped in small pores in solid materials and crosslinked hydrogels.

In 2001, Liu et al. [186] explored the origin of unfrozen bound water in gelatine samples with various water contents. The results revealed that the content of unfrozen bound water increased upon increasing water content up to 3.75 g water/g gelatine, above which, it declined. Moreover, at 50 g water/g gelatine, no unfrozen bound water was detected. They proposed that the ‘nanocavity’ in polymers was an important reason for the formation of

unfrozen water. Nakamura et al. in their next work [191] studied bound water in carboxymethylcellulose–polylysine complexes and measured their non-freezing water content (W_{nf}) by DSC. W_{nf} of sodium carboxymethylcellulose (NaCMC) increased with increasing degree of substitution, since hydrophilic groups restrict water molecules. W_{nf} of carboxymethylcellulose–polylysine (CMC–PLys) complexes decreased with increasing PLys/CMC ratio due to cross-linking formation. They observed that the amounts of W_{nf} calculated from melting enthalpies (W_{nfm}) were larger than those calculated from crystallization enthalpies (W_{nfc}). The difference between W_{nfm} and W_{nfc} ($\Delta W_{nf} = W_{nfc} - W_{nfm}$) of CMC–PLys increased with increasing degree of substitution and PLys/CMC ratio. They concluded that W_{nf} is markedly affected by degree of substitution and cross-linking density. In the recent study [192], the glass transition, cold crystallization, melting of eutectic crystal, water and poly(ethylene glycol) (PEG) crystal were investigated depending on water content. The particular attention received the cold crystallization of the system, which is thought to be used as an index of biocompatibility of polymer–water interaction. The authors found that cold crystallization and glass transition were observed in a wide water content range from 0.05 to 10 g g⁻¹. They confirmed that cold crystallization was attributable to the molecular rearrangement of PEG molecules associated with amorphous ice. When four water molecules were attached to one repeating unit of PEG, the heat capacity difference at glass transition temperature attained the largest value and the enthalpy of cold crystallization showed the maximum value.

Chaplin in his study [187] noted that all methods for measuring ‘bound’ water only determine the water according to the method used and should not be generalised to include other binding classes. For example, ‘non-freezing’ water indicates only that the water does not freeze. It indicates nothing about the intrinsic binding, as it is generally considered that even ice has a layer of unfrozen water at its surface [188], and such water may simply be present as a glass without any specific ‘binding’. There are two ‘flavours’ for unbound water, trapped and free. Generally, as particle size increases so does the trapped volume, due to imperfect packing, and consequential apparent water binding [189]. Trapped water has decreased freedom of movement (entropy). In order to keep the water activity constant, therefore, the water has to form bonds with a more negative enthalpy. This process results in stronger, more linear hydrogen bonds, causing greater structuring and lower density. The same effect is caused by capillary action; the reduced pressure ‘stretching’ the water into a more open tetrahedral structure [190].

Furthermore, the hydration properties of biomolecules can be also studied using other techniques besides DSC, e.g. X-ray diffractometry [194], FT-IR spectroscopy [195, 193] or NMR spectroscopy [196] etc.

4.2.1 A comparative study on hyaluronan and humic substances hydration [142]

Hyaluronan (HYA) is naturally occurring high molecular weight biopolymer consisting of glucuronic acid and N-acetyl-glucosamine. One of the most important functions of the HYA is hydration (the hydration of epidermis or the hydration of ocular cornea) [197]. HYA binds water very strongly but there are some differences between the various types of binding water, which are based on structure and arrangement of water in liquid state. In contrast, humic substances (HS) represent a complicated mixture of amphiphilic, hydrophobic and hydrophilic molecules developed as a by-product of biomass decomposition. Chemically, HS can be visualized as a mixture of both substituted aromatic and aliphatic molecules (up to 1500 Da) forming due to intermolecular interactions various structures. The primary structure reflects the conditions of formation such as parental material, climate conditions and character of present microorganisms. Their role is, among others, closely linked with water holding capacity of soils and cell biology of soil living organisms. The aim of this part of the work was to study hydration properties of two different sorts of biomolecules classes. The enumeration of water molecules is crucial in order to understand how biomolecular processes work. The ability of various hydration shells to freeze and melt in broad temperature range was used to determine these different types of water using the DSC method.

Experimental:

HYA isolated from *Streptococcus zooepidemicus* was obtained from CPN Company. The molecular weight of hyaluronan was 1.39 MDa. The concentration range water/HYA was from 50-300 % (w/w). HS, i.e. humic and fulvic acids were purchased from International Humic Substances Society. The set consisted of HS of soil, peat and leonardite. The concentration range was from 10-150 %. Samples of selected concentrations were prepared by addition of water to a sample previously placed into an aluminum pan, weighted until certain water content was reached. The pan was hermetically sealed, let 24 hours under the room temperature and measured by DSC Q200 TA Instruments. The temperature program was cooling down to $-90\text{ }^{\circ}\text{C}$ and heating up to $40\text{ }^{\circ}\text{C}$. Heating and cooling rate was $3\text{ }^{\circ}\text{C}$ per minute under the nitrogen flow.

Results and Discussion:

Generally, the hydration is a term which refers to the amount of bound water. It has been also defined as a 'non-bulk' water. As already mentioned, water surrounding molecules can be divided into 'bound water' [197], subcategorized as being capable of freezing or not, and 'unbound (bulk) water'. Bulk water freezes at the same temperature as normal water ($< 0\text{ }^{\circ}\text{C}$ dependent on cooling rate). 'Freezing-bound' water freezes at lower temperature than normal water, being easily supercooled. It also exhibits a reduced enthalpy of fusion (melting) and the 'non-freezing' water does not freeze even at low temperature.

Fig. 16 reports the representative record of heating fulvic acid/water system. At $-25\text{ }^{\circ}\text{C}$, there can be seen a weak endotherm corresponding to the melting of freezing-bound water while at higher temperatures is observed an intensive peak attributable to the overlapping of melting of freezing-bound and freezing water. The existence of both mentioned types of water can be deduced from the temperature at which the processes started and at which the process terminated. In addition, considering the temperature range of the peak and the number of shoulders, the overlapping of several melting processes corresponding to various types of freezing-bound water can be assumed.

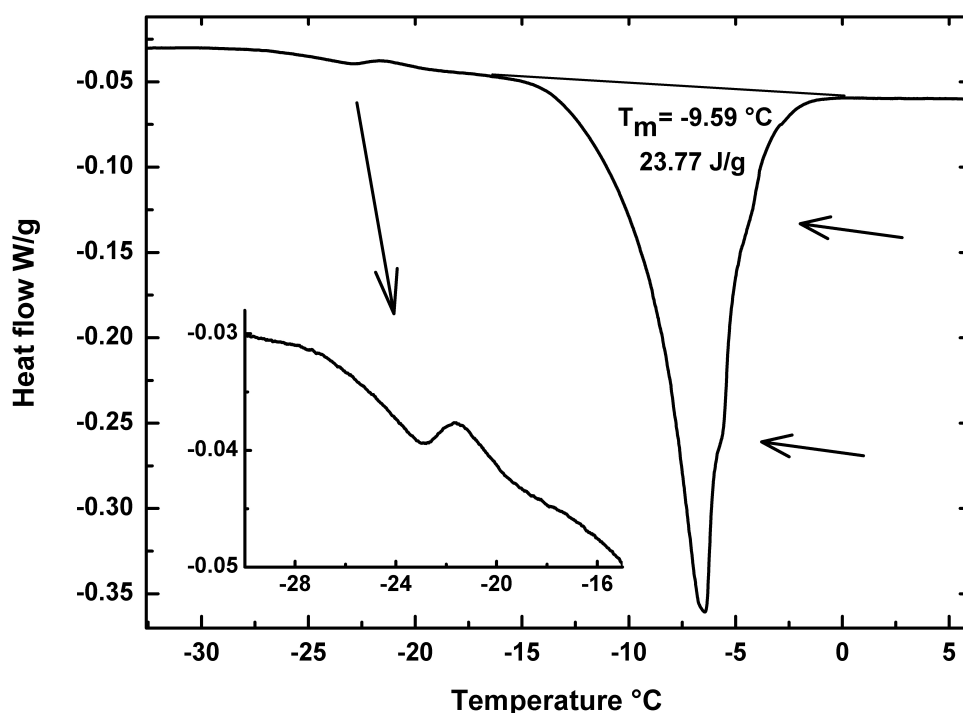


Fig. 16 DSC heating curves for Elliot Soil Fulvic acid/water system.

All molecular fractions of hyaluronan showed greater water retention capacity than that of humic substances. It is reasonable because of higher hydrophilicity of hyaluronan and probably also due to its polymeric feature. The biggest difference in records comparison was in the presence of cold crystallization (Fig. 17). When the frozen hyaluronan-water system was heated, the glassy water cold crystallized to give ice and a broad exothermic transition appeared. In the glassy state, the conformational changes are severely inhibited and the material is meta-stably trapped in a solid but microscopically disordered state. That phenomenon was observed in records of humic substances only slightly, probably because of their lower affinity to water molecules and unique secondary structure.

Recently, DSC was used to determine the number of water molecules in the hydration shell of hyaluronan of different molecular weights and counterions [198]. It was found out that the dependency of melting enthalpy change on W_c remains constant after 24 hours from preparation of the sample. Non-freezing water was determined using extrapolation to zero enthalpy. For sodium hyaluronan within the molecular weight range between 100 and 740 kDa the hydration shell was determined as 0.74 g g^{-1} HYA. A larger hydration shell containing 0.84 and 0.82 g g^{-1} HYA was determined for hyaluronan of 1390 kDa in its sodium and protonized form, respectively.

Unlike results of HYA, the situation for HS was different. The dependency of melting enthalpy change on W_c was not constant after 24 hours from sample preparation, most likely because of its incomplete hydration. DSC represents a technique, which can recognize the differences in properties of water surrounding biomolecules. Although same DSC technique

limitations associated with processes of ice formation should be taken into account (e.g. supercooling effect) that method can bring valuable information regarding the function of hyaluronan and humic substances. Nevertheless, the time modification of this technique was needed when it was used for study of HS because of their complexity of hydration processes. Therefore, next and deeper study was necessary. It was established that experiments described above were just pilot ones, and this course of study requires higher attention.

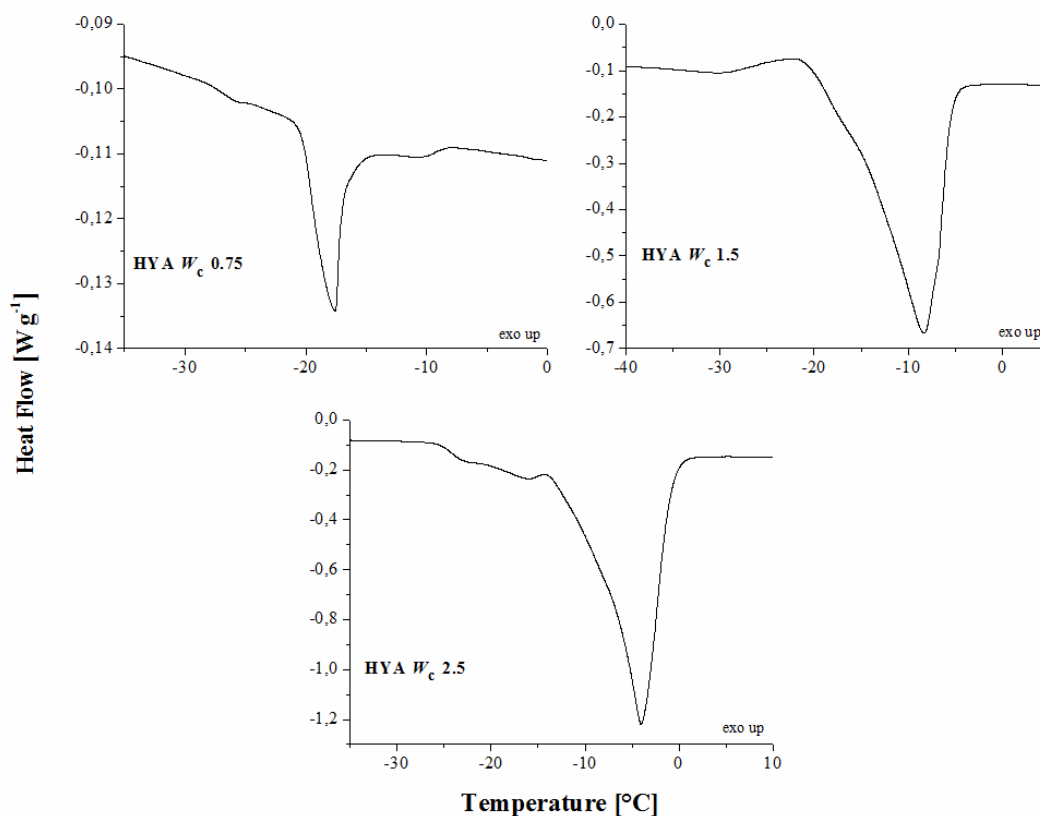


Fig. 17 DSC heating curves for HYA/water system of W_c 0.75, 1.50 and 2.00 (g/g).

4.3 Study on hydration properties of humic substances using thermal analysis

This part of the work explores hydration properties of HS from slightly different point of view than commonly reported for hydrophilic biopolymers. Both extension and kinetics of hydration processes of humic substances were studied. The approaches and techniques already used and reported as being successful for determination and enumeration of hydration water in biopolymers were partially modified and applied for HS. The effect of water on humic structure, its way of wetting and penetrating from the surface into the structure of HS, the possible conformational changes caused by water, the water retention capacity of HS, and also the influence of origin of individual HS on hydration properties with regard to the time scale were investigated. We assume that the degree of humification should have a strong influence on hydration processes of humic substances. Last but not least, the reversibility of hydration processes is also a question, as well as parallelism with the properties of hydrogels or similarities and dissimilarities between biopolymers and HS.

4.3.1 Experimental part

Material

In this work, standard samples were purchased from the International Humic Substances Society (IHSS). Following samples were measured as obtained: Suwannee River II HA 2S101H (SRHA), Suwannee River II FA 2S101F (SRFA), Elliot Soil HA 1S102H (ESHA), Elliot Soil II FA 2S102F (ESFA), Pahokee Peat HA 1S103H (PPHA), Pahokee Peat II FA 2S103F (PPFA), Leonardite HA 1S104H (LHA).

DSC Measurements

A sample (typically around 2 mg) was placed in an aluminium pan and excess of water was added. Surplus water was allowed to evaporate slowly at room temperature until the desired water content was obtained. Subsequently, the pans were hermetically sealed. Differential Scanning Calorimetry (DSC) was performed using the TA Instruments Q200 equipped by the RCS system in order to measure phase transitions of present water/ice. An empty hermetically sealed pan was used as a reference. The measurements were conducted ranging from 40 °C to –90 °C at 3 °C min^{–1} and then from –90 °C to 30 °C at 5 °C min^{–1} under the flow of nitrogen (50 mL min^{–1}). The temperature scale and heat of transition were calibrated using distilled water and indium as standards, respectively.

Water content determination

The Thermogravimetry Analysis (TGA TA Instruments Q5000IR) was used to measure the moisture content of purchased IHSS standard samples to obtain the precise concentration of water (W_c) in the samples. Experiments were carried out in open Pt pans. The temperature range of the experiments was from 25 °C to 150 °C at 3 °C min^{–1} under the flow of nitrogen. The W_c was determined as follows:

$$W_c = \frac{\text{grams of water}}{\text{grams of dry sample}} \quad (\text{g/g}).$$

Glass transition determination

A hermetically sealed aluminium pan with the sample of known W_c was carefully perforated with a sharp needle and sample was immediately dried using the thermogravimetry analysis with the thermal range from 25 °C to 170 °C at 3 °C min⁻¹ under the flow of nitrogen. Thereafter, the Temperature-modulated Differential Scanning Calorimetry (TMDSC) was employed. The sample was replaced into DSC measuring cell and put through a sinusoidal temperature oscillation, which was overlaid on the traditional linear ramp with the modulation of ± 2 °C every 90 seconds and heating rate 2.5 °C min⁻¹. The measurement was conducted in the temperature modulation mode from -90 °C to 150 °C and two heating and cooling cycles were performed in one TMDSC experiment. All thermo-analytical scans were analyzed using the TA Instruments Universal Analysis 2000 software.

4.3.2 DSC of water/humic substance system after 1 day

IHSS samples from different sources with W_c range between 0.1 and 2.0 (g/g) were observed. Representative DSC heating curves of fulvic and humic acid originating from Suwannee River of different W_c , measured approximately 24 hours after preparation are given in Figs. 18-19. The figures of DSC heating curves reported in this work are displayed in the temperature range from -40 to 10 °C to increase the quality of picture; in fact below -40 °C no obvious events occurred, only baseline shifts which are discussed later.

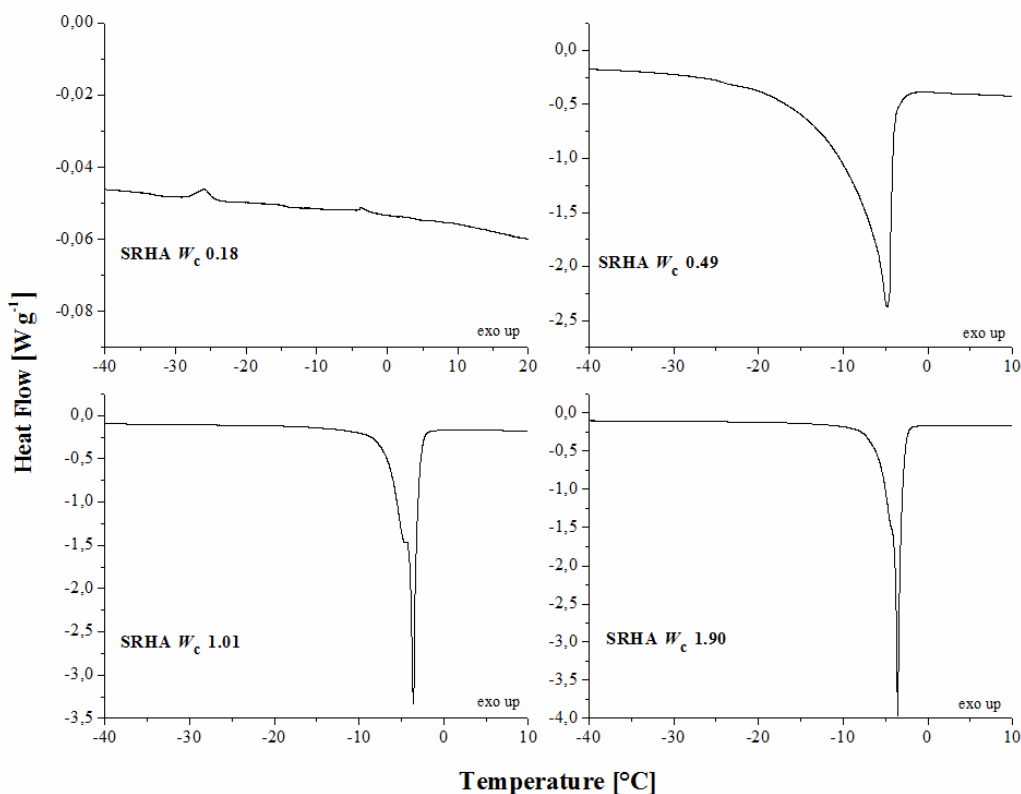


Fig. 18 DSC heating curves of SRHA measured after 24 hours.

When W_c is about 0.18 no significant phase transition can be observed in the record. In fact, at very low water content, all water molecules are present in the form of non-freezing water (W_{nf}) in this system, they are associated closely to the humic molecules and thus they cannot participate in ice formation. Increasing content of water in samples ($W_c = 0.49$ for SRHA and 0.30 for SRFA) brought about the appearance of an endothermal events below 0 °C. These events (around –10 °C and –25 °C for SRFA and around –5 °C for SRHA) indicates ice melting and therefore the presence of water in the form of freezing-bound water (W_{fb}).

As mentioned earlier, W_{fb} represents a kind of water/ice whose structure is affected by the interaction with humic molecules. Its crystalline structure (clusters) differs from those of bulk water and is physically recognizable by shifted temperature of melting (around –10 °C). Moreover, also the enthalpy of melting of such ice differs from that developed from bulk water (334 J g^{–1}) [183]. There exists a theory that due to the different structure of ice such enthalpy can drop down to 312 J g^{–1} and perhaps even lower values [183].

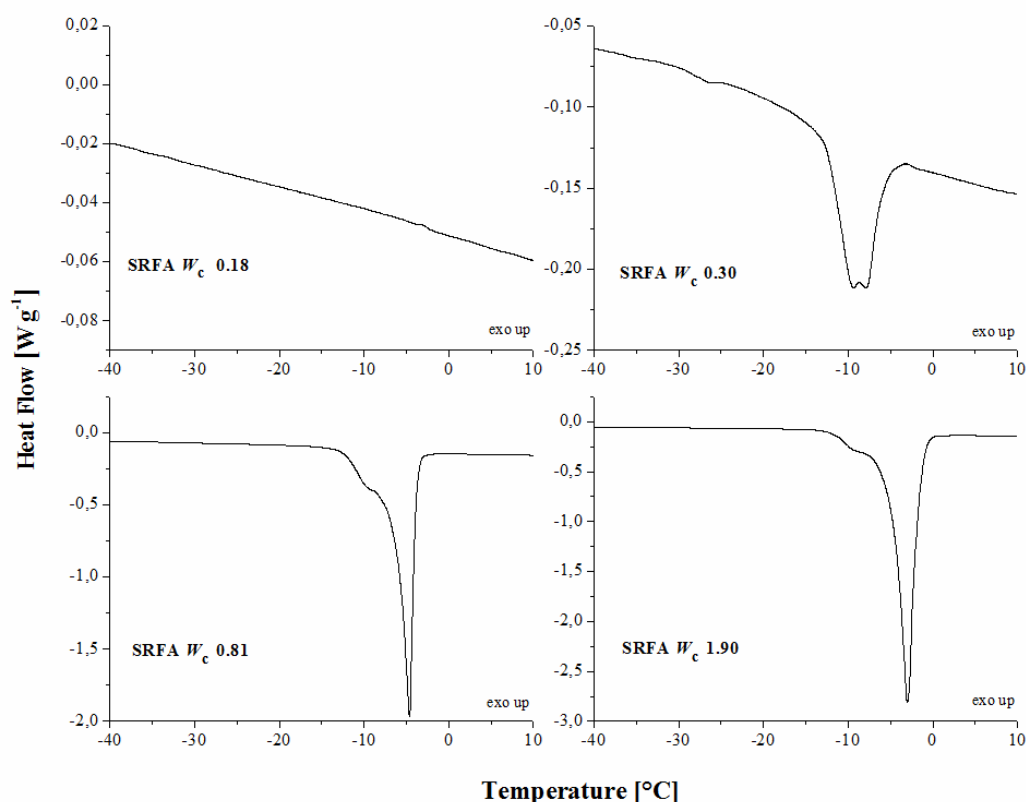


Fig. 19 DSC heating curves of SRFA measured after 24 hours.

As W_c increased to 1.01 (SRHA) or 0.81 (SRFA), the transitions increased in intensity (i.e. peak area) and their peak temperatures moved to higher values, a large heating endotherm appeared in the region around –6 °C. This endotherm was ascribed to the melting of the free and freezing bound water in the hydrated HS-water system [179]. Samples of SRFA and SRHA showed more distinct endothermic fusion peaks on heating curves. Shoulders, which can be identified in those records, reveal overlapping of several different peaks, which reflects the presence of several different types of freezing-bound water. Some authors also speculated about gradual restructuralization of ice crystalline structure in the course of such endothermal

event. Since cubic structure of freezing-bound water is thermodynamically metastable, such assumption cannot be rejected [179]. At higher water content (1.90) the endothermic transitions are progressively moved to higher temperatures (around $-3\text{ }^{\circ}\text{C}$).

Other IHSS samples of humic and fulvic acids measured in this work show similar behaviour (see Appendix I). The measurements were running through three following identical cycles to prove their reproducibility. In this way it was also verified that sample pans were well hermetically sealed so no water was evaporating and that formation of ice, due to its volume expansion, does not cause the HS supramolecular destruction. Contrary to Prawitwong [200], no differences between individual cycles, such as elimination of T_{cc} by cyclic measurements, were observed.

4.3.3 Hydration kinetics

Unlike the hydrophilic biopolymers, where the hydration processes are usually finished within hours to days, the kinetics of hydration in HS plays more important role as well as the mechanisms seem to be different. When the identical DSC measurement of hermetically sealed sample was repeated after longer period and compared with the sample measured after 24 hours after preparation, the significant decrease in enthalpy of heating was observed. It is in line with the recent results [105] according which the kinetic factor plays an important role in hydration processes of SOM. Therefore, the phase transition behaviour was monitored over the period of 21 days, as recommended by Schaumann et al. [105]. The IHSS samples of six selected W_c (around 0.5, 0.75, 1.0, 1.25, 1.5 and 1.9 g/g) were prepared and subjected to the same DSC experiment as described in part 4.3.2.

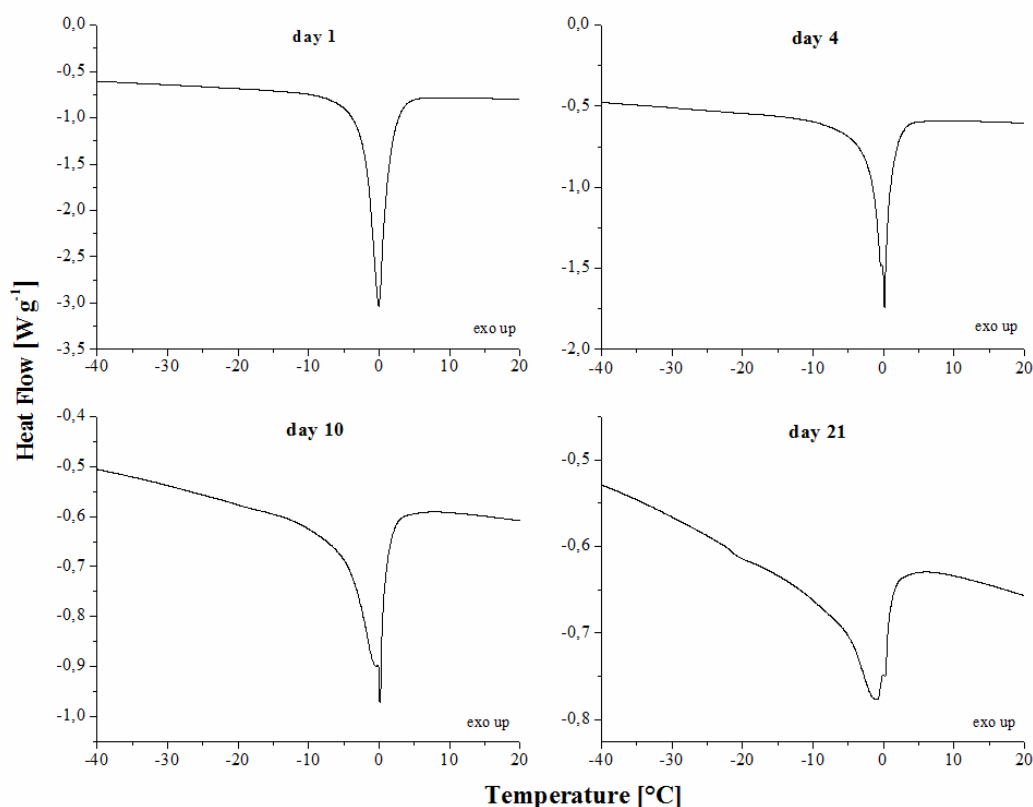


Fig. 20 DSC heating curves of LHA of W_c 0.73 measured during the period of 21 days.

In Fig. 20 there can be seen representative DSC heating curves of LHA sample of W_c 0.73 measured during the period of 21 days. At the beginning of hydration process, a narrow and sharp endothermic peak can be observed in the area around 0 °C, which can be attributed to the melting of freezable water in hydrated HS-water system. During the measuring period, this endotherm gradually increased in width and decreased in intensity, which caused a decrease in enthalpy of melting. The onset of the endothermic peak was shifted to the lower temperatures, and from the day 10 there can be seen an appearance of another small endothermic peak around -20 °C. This peak became more obvious on day 21 and due to its temperature, it can be described to the melting of some type of freezing-bound water. Further, a splitting of main peak (appearance of a small “shoulder”) on day 21 reveals the overlapping of two processes characterized by two peaks. It implies a slow and gradual penetration of water into the LHA physical structure, hydration of larger surfaces associated with a decrease of amount of freezable water (peak area). The peak broadening suggests an increasing distribution of inner places and surfaces in LHA such as cavities and holes in which water molecules experience a variety of interactions and physical states.

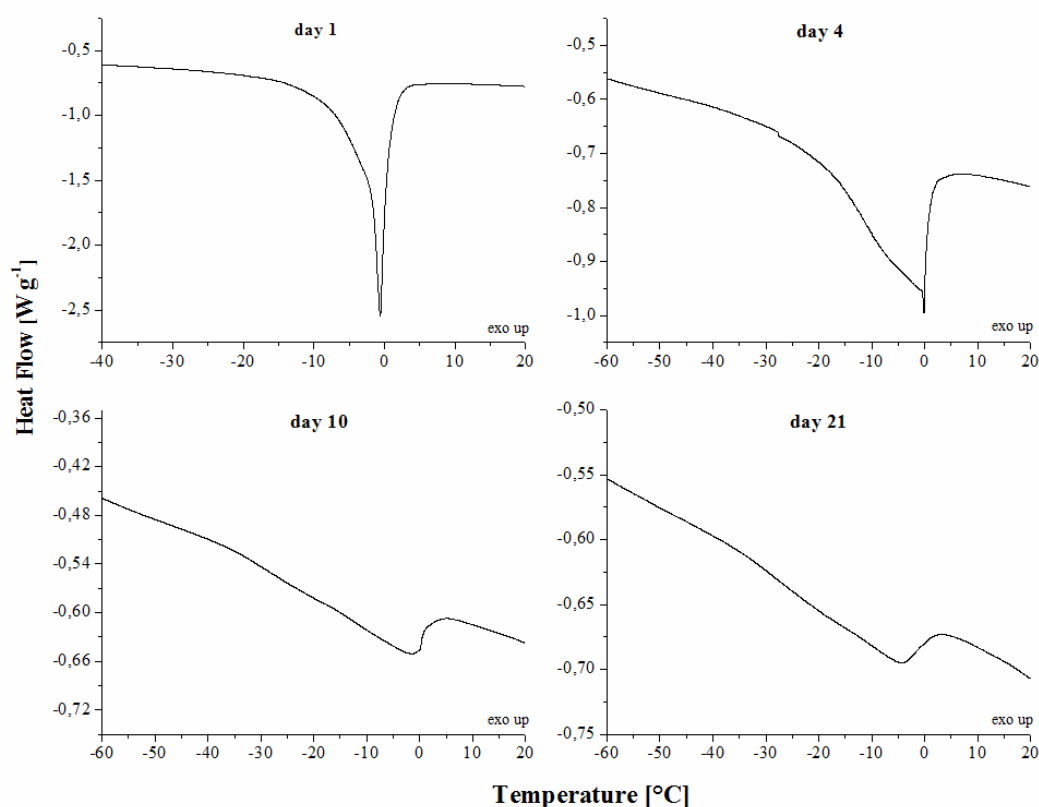


Fig. 21 DSC heating curves of SRHA of W_c 0.50 measured during the period of 21 days.

The situation for SRHA sample is similar as for LHA. The DSC heating curves of SRHA sample of W_c 0.50 measured during the period of 21 days are reported in Fig. 21. The record from the first day displays a sharp and quite narrow endothermic peak with maximum around 0 °C. Nevertheless, the widening and intensity diminishing of this peak can be observed already on the day 4. It can be seen that the process of melting begins around -35 °C and

develops slowly. This tendency is even more evident on days 10 and 21 where the enthalpy of melting endotherm is much smaller and the maximum of the endotherm shifts to $-5\text{ }^{\circ}\text{C}$.

Fig. 22 reports the SRFA sample of W_c 0.51. The W_c is similar as for SRHA, but the DSC records are different. The shape of the SRFA heating endotherms is almost the same as for SRHA, only the overlapping and intensity decrease of peaks is more pronounced for days 10 and 21, and the maximums of melting peaks can be found already around $-5\text{ }^{\circ}\text{C}$ from the first day of the experiment. That fact can be explained with respect to higher polarity of SRFA than SRHA, the surface of SRFA is more hydrophilic and the processes of wetting are generally quicker. The presence of polar groups has greater influence on the water molecules in comparison with less polar, more hydrophobic SRHA.

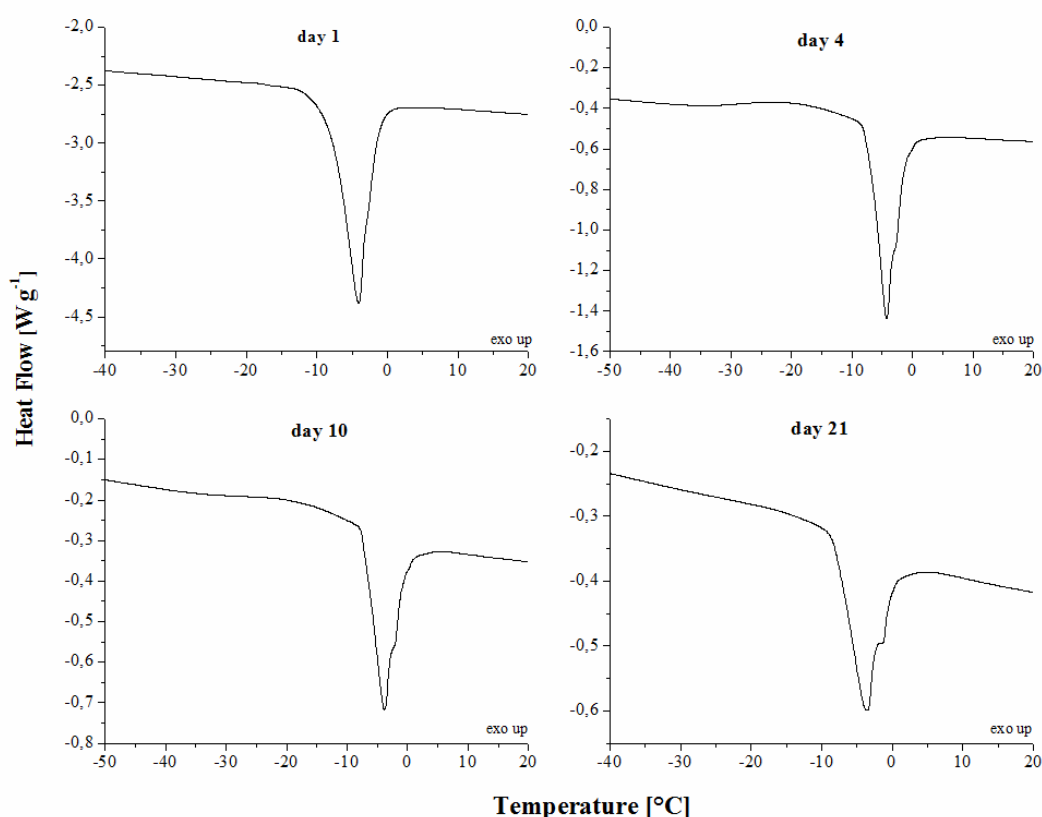


Fig. 22 DSC heating curves of SRFA of W_c 0.51 measured during the period of 21 days.

As reported in our recent work [141], hydrophobic hydration plays a role in humic acids; fulvic acids are more polar and their influence on polar water molecules is generally larger. The motion of water confined in polar cavity or bound on the polar surface is more restricted and therefore, the formation of ice is associated with formation of less perfect crystals or even amorphous ice than in case of bulk water or water on hydrophobic surface. This explanation is in line with the presence of cold crystallization for the sample of SRFA. The endothermic peaks are preceded by small broad exothermic transitions, which can be ascribed to the cold crystallization (T_{cc}) of glassy water [201, 177]. When the HS-water system is cooled rapidly, part of water might remain in a supercooled state due to above-mentioned restrictions. Such metastable water is termed as ‘glassy water’. On heating, the molecular motion of glassy

water is enhanced and water changes to crystal ice [200]. That phenomenon was not observed in records of the first day, which points to the importance of kinetic factor while considering hydration processes of HS, i.e. wetting and swelling.

The results for hydration kinetics of Elliot Soil humic acid sample of W_c 0.74 can be found in Fig. 23. The heating endotherms remain very sharp and narrow with the maximum around 0 °C during over the whole measuring period. From the day 10 there can be also seen an appearance of another small endothermic peak around –15 °C which is more distinct on the record of day 21.

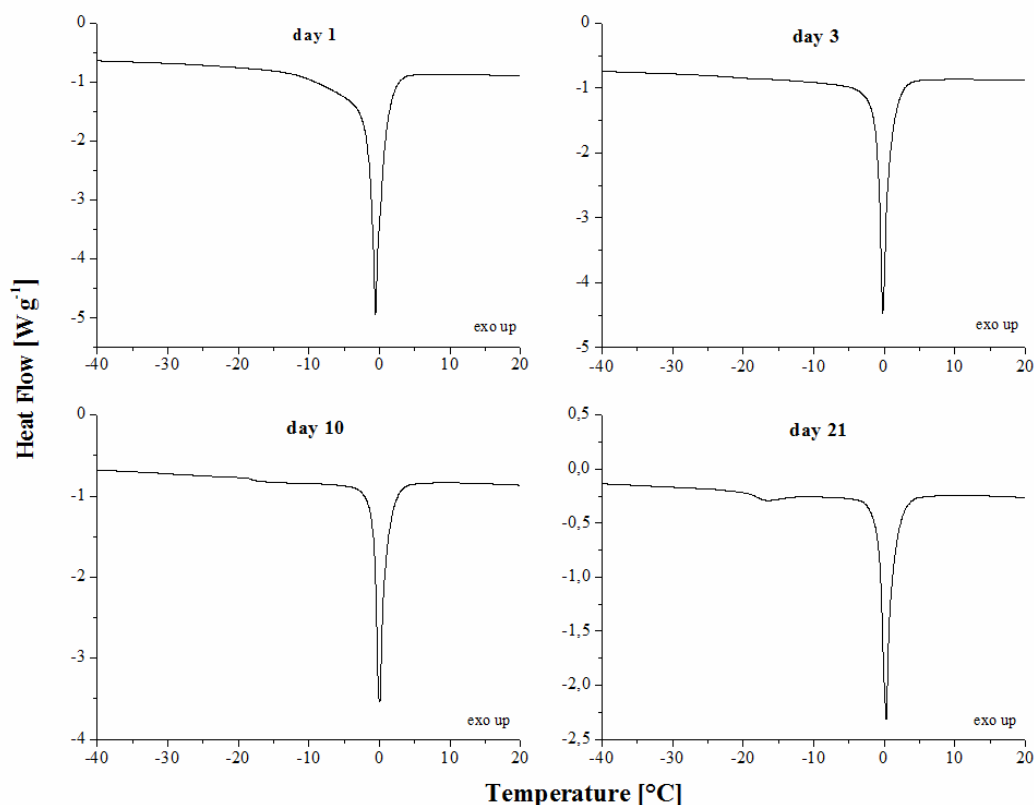


Fig. 23 DSC heating curves of ESHA of W_c 0.74 measured during the period of 21 days.

The DSC heating curves of Elliot Soil fulvic acid sample of W_c 0.44 measured during the period of 21 days are shown in Fig. 24. Unlike the results for ESHA, the endotherms of melting are rather wide with the maximum around –5 °C. From the day 3 there can be seen a cold crystallization characterized by a broad exothermic transition on the heating curve. As the time proceeded (day 10) the endothermic peak occurring in the temperature region between –5 °C and 0 °C, was separated into two individual endotherms (day 21). In due time, the value of melting enthalpy of ESHA and ESFA samples decreased, similarly as observed for all other samples. As can be seen in Appendix II, the samples of PPHA and PPFA exhibit comparable results.

The selected representative samples of smaller W_c described in the part above were chosen on purpose since for the records of the samples with higher W_c the effect of melting of freezing water prevailed and only the large intensive heating endotherm can be observed.

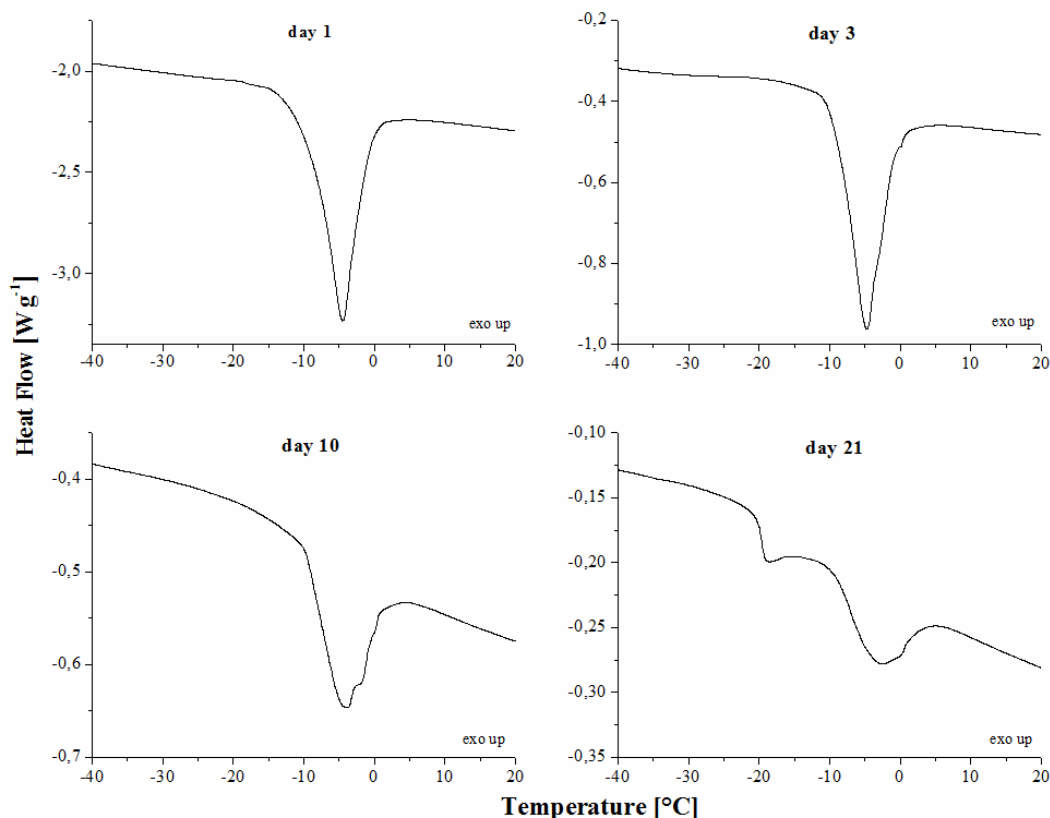


Fig. 24 DSC heating curves of ESFA of W_c 0.44 measured during the period of 21 days.

In the next step, the endothermic peaks were integrated and the change of melting enthalpy was plotted as a function of time. Obtained records of four selected W_c of LHA, SRHA and SRFA are reported in Fig. 25. The results suggest that the hydration of humic substances is not a straightforward process. As depicted in Fig. 25, around approximately day 7 the rapid decrease in change of melting enthalpy occurred. After that time period, the diminution became more moderate. This situation is in line with recent statement [105] that the wetting of SOM proceeds in two steps. The first step of the hydration process of soil samples includes the wetting of the surfaces (slow process) and after that, the water is distributed to the pore volume. Since the enthalpy dropped significantly mainly in the first phase and in the second phase only slightly, such assumption seems to be reasonable.

After the integration of endothermic peaks, the dependency of melting enthalpy change was plotted as a function of W_c . In Fig. 26 there are shown results for this dependency for LHA, SRHA and SRFA samples. In fact, SRFA sample showed a linear dependency, while both humic acids showed some kind of steps. The dependency of SRHA sample demonstrates unusual decrease in change of enthalpy around W_c 1.40. This decrease was verified by measuring of two another samples with similar W_c and can be attributed to slower penetration of water into the humic structure, followed by swelling (constant surface, higher W_c), breaking of weak bonds stabilizing a cavity, its breaking and further water penetration, i.e. increase in content of non-freezing water. Further explanation of this phenomenon can be found in next paragraphs. For results of ESHA, ESFA, PPHA and PPFA samples see Appendix III.

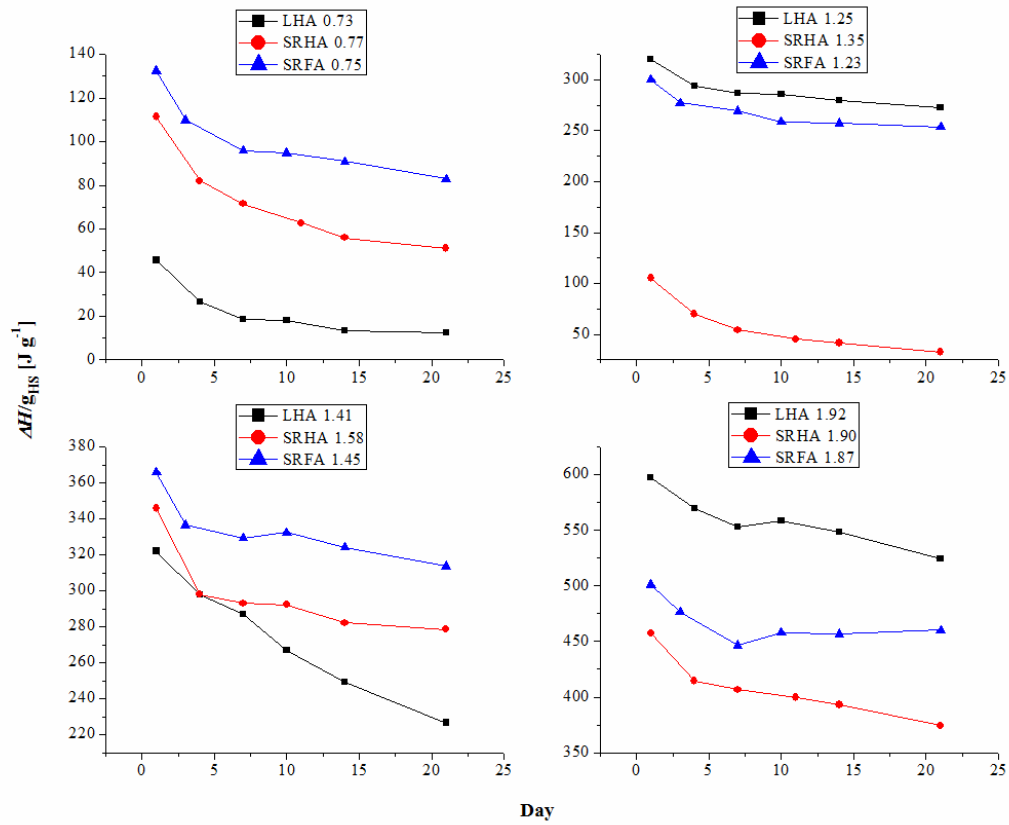


Fig. 25 The dependency of melting enthalpy change on time.

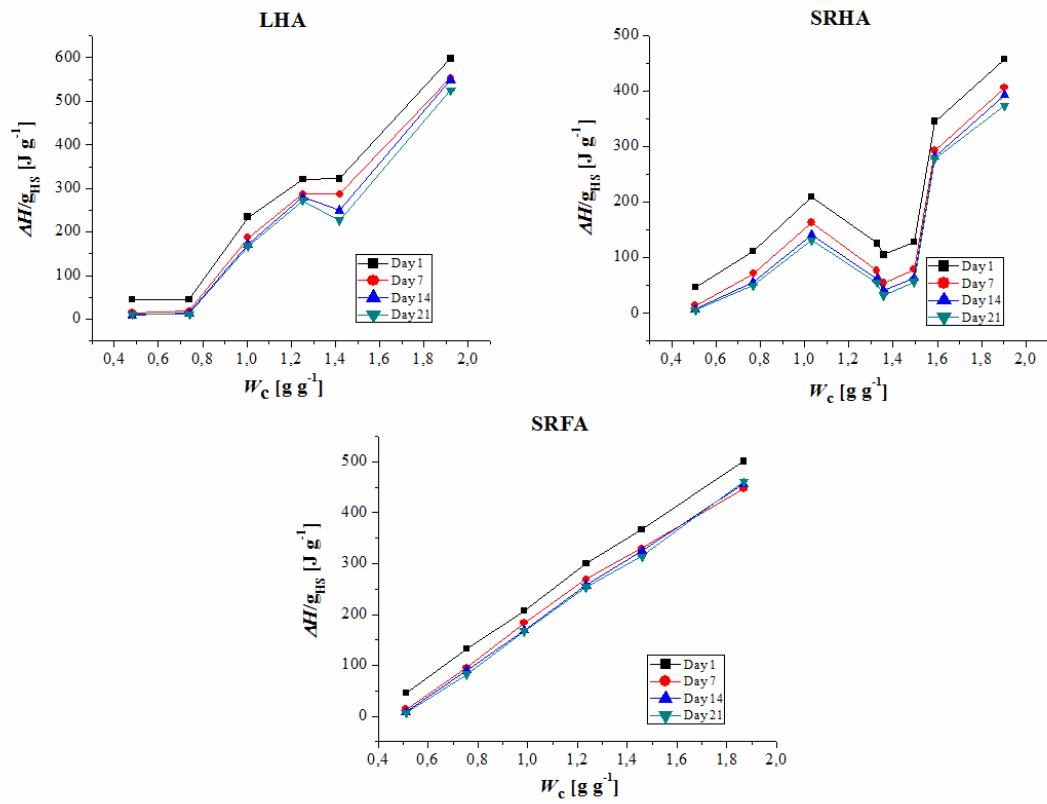


Fig. 26 The kinetics of dependency of melting enthalpy change on W_c for LHA, SRFA and SRHA samples during the period of 21 days.

Other samples showed different mechanism of water absorption, which is represented by nearly straight dependencies, similar as can be seen for hydrophilic polymers [183]. In fact, they showed lower tendency to form a rigid structure (characterized by a step-like dependency of enthalpy on water content) since the environment does not support such “scaffolding”. Generally, fulvic acids reflect higher affinity to water than their respective humic acids. The more complicated inner structure of humic acids with higher porosity allows the water molecules to hydrate higher surface than in FA.

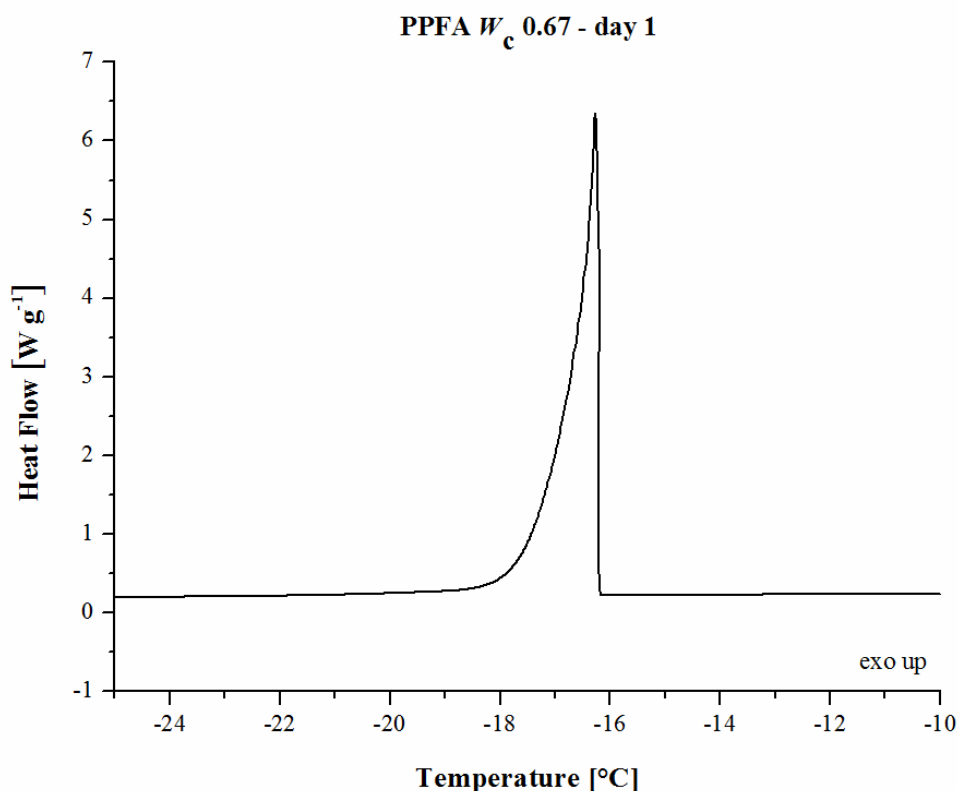


Fig. 27 DSC cooling curves for PPFA/water system of W_c 0.67 measured on day 1.

In Fig. 27 there is shown representative cooling curve of PPFA/water system of W_c 0.67 measured on day 1. As can be seen, the exothermic transition due to the freezing of water in the cooling curve began at lower temperature in the PPFA/water system than it is typical for pure water. This phenomena was earlier observed in the work [179] where was found out that the crystal grow rate of freezing bound water was about ten times slower than that of free water. Under the conditions used in this work, i. e. cooling rate at 3 °C min^{-1} , ice formation began at approximately -16 °C . This inhibition of ice nucleation is partly caused by the presence of HS in water. This effect was previously observed also in hyaluronan/water system [184]. Generally, the enthalpy change associated with the cooling curve was always smaller than the one associated with the heating curve. In our work, that phenomenon reflects the water restriction by humic samples and difference in heat capacity of liquid water and ice [185].

The Fig. 28 depicts the differences between kinetics of dependency of melting and crystallization enthalpy change on W_c for LHA, SRHA and SRFA samples. The exothermic

phase transitions of crystallization were integrated as well and the change of crystallization enthalpy was plotted as a function of W_c . The dependencies of melting and crystallization were compared with regard to the kinetic factor of hydration processes. In the Fig. 28 there can be seen that the value of change of crystallization enthalpy is smaller during whole hydration period than that one of melting. If one neglects the difference in heat capacity of liquid water and ice, it implies that when the water in humic matter is frozen, there is still some additional exothermal process in which ice crystals re-conform (similar to crystal perfection). It is our experience, that this process takes part in the heating run of the DSC experiment and as a rule it is hidden in the baseline shift immediately when the heating starts (i.e. at -90°C). Therefore, it is difficult to quantify this process and it is always underestimated. According to the literature, this process is caused by the presence of freezing bound water which has tendency to form anomalous crystalline and amorphous structures [205]. Thus the difference in melting and crystallization enthalpy in Fig. 28 shows the presence of W_{fb} and reveals an important notion, that at low water content and at $W_c = 1.4$ the amount of W_{nf} is very low. It confirms our theory about the disruption of the structure and wetting of new surfaces, i.e. less water is present in swollen structures (pressed and with restricted self-diffusion). For results of ESHA and PPHA samples see Appendix III.

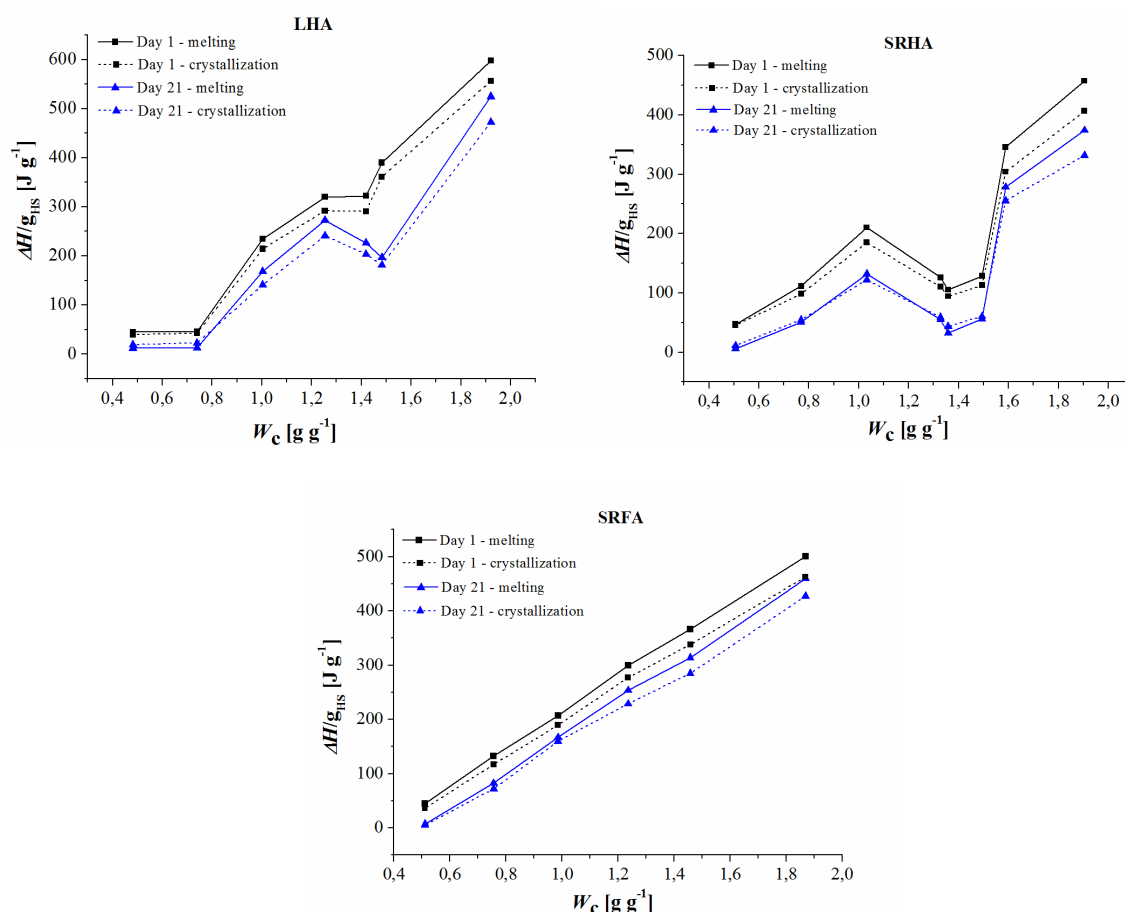


Fig. 28 The kinetics of dependency of melting and crystallization enthalpy change on W_c for LHA, SRHA and SRFA samples.

Humic substances consist of structures containing both non-polar and polar inner space. As suggested in our previous work [141], the structure is stabilized by separated hydrophobic domains connected by non-specific van der Waals interactions forming kind of hydrophobic scaffold. The surface of humic aggregates seems to be rather hydrophobic which is reflected by low water sorption capacity depicted the mostly in the first stage of the dependency of LHA sample. Further, when water penetrates into humic structure, the polar interactions stabilizing the structure are broken and swelling takes part. Thus, humic acids structure resembles a porous structure, the combination of hydrophobic and hydrophilic domains. The disruption of the structure is rather slow since it is associated with the hydration of surfaces of different hydrophobicity/hydrophilicity. Moreover, it seems that progressive hydration brings about not only bridges formation but at the same time, the inner vacancies are formed containing relatively large volumes of water. Each cavity has its own inner volume – or capacity – where evolves a pressure causing the expansion of whole volume due to the hydrophobic effect. The water presses the hydrophobic parts and the volume of inner cavity is increasing until it bursts so that the scaffold made of hydrophobic parts is being pushed out by the hydrophobic effect.

Table II The elemental composition in % (w/w) of dry, ash-free IHSS samples [203].

IHSS sample	C	H	O	C/O	C/H
LHA	63.81	3.7	31.27	2.04	17.25
SRHA	52.63	4.28	42.04	1.25	12.30
SRFA	52.34	4.36	42.98	1.22	12.00
ESHA	58.13	3.68	34.08	1.71	15.80
ESFA	50.12	4.28	42.61	1.18	11.71
PPHA	56.37	3.82	37.34	1.51	14.76
PPFA	51.31	3.53	43.32	1.18	14.54

It is also important to take into account the different solubility of molecules; in fulvic acids, most of molecules are hydrophilic, therefore, their hydration resembles the hydrophilic biopolymers (cf. Fig. 26 and Figures reported for example in [199]). In contrast, humic acids consist of a mixture of molecules of various polarity and therefore different wettability. Hydrophilic molecules are hydrated very quickly while hydrophobic molecules are repelled from water to decrease the energy demand on the hydration of hydrophobic surface. This seems to be a crucial factor in distinguishing between humic and fulvic acids. That can be easily identified in Fig. 26. Further, Fig. 26 also reports an interesting phenomenon, that for both humic acids (LHA and SRHA), around $W_c = 1.4$ a sharp and reproducible decrease of enthalpy. In fact, this break is more intensive for SRHA sample. We hypothesise, that this is associated with the fact, that the water amount exceeded a critical mass in relation to dry mass, and the above mentioned cavity is broken and water can penetrate other humic compartments. Thus, the number of W_{nf} increases and the total enthalpy of ice melting (from freezable water) suddenly decreased. As it can be seen, the LHA showed within the concentration range under investigation the highest tendency to form layers or better speaking it preserved its original structure unlike the other humic samples. This situation persisted even after several months, i.e. the lignite HS remained its physical structure and water was not able to break the porous scaffold. This begs the question: How is the degree of humification reflected in this dependency? It seems that the younger (or less humified) HS are, the easier

and faster their change is. This issue is intimately bound with the C/O and C/H ratios of individual IHSS samples presented in the Table II. This implies a new look into the problem associated with humic acids development called humification.

According to theories which were published in the first part of 20th century [1], humic acids were formed as a product of secondary synthesis of building blocks which were formed as a by-product of biomass decay caused by an extracellular enzymatic activity of soil microorganisms. The theories assumed the presence of other soil microorganism catalyzing the back-polymerization of such building blocks, which is followed by the formation of humic macromolecules. By the end of 20th century, those views were questioned since there is no reason for microorganism to spend energy on the re-synthesis of new molecules which would serve neither as a part of organism bodies nor as a source of energy [8]. Moreover, to our knowledge, proposed soil enzymes were not found, and laboratory synthesis with model enzymes (metal-porphyrines) were able to produce only bi- or tetra-mers of polyphenol models [202]. Together with a supramolecular theory and results given in this work, it seems that the processes of humification can be considered also from the point of view of physical chemistry, i.e. as a phenomenon caused purely or partially by water and hydrophobic effect.

In accordance with Piccolo's concept [8], it can be agreed, that humification in soil can be considered as a two-step process of (1) biodegradation of dead-cells components, (2) aggregation of the degradation products. In light of the supramolecular model, it is no need to invoke the formation of new covalent bonds in the humification process that leads to the production of humus. Therefore, humification can be considered as the progressive self-association of the mainly hydrophobic molecules that resist the biodegradation. These suprastructures are thermodynamically separated by the water medium and adsorbed on the surfaces of soil minerals and other pre-existing humic aggregates. The exclusion from water means exclusion from microbial degradation and the long-term persistence of humic matter in soil.

4.3.4 Glass transitions of IHSS samples

After finishing of kinetic experiments (when an IHSS sample was exposed to interaction with water during the hydration period over 21 days), three samples of each IHSS standard of W_c around 0.5, 1.0 and 1.5 g/g were measured using TMDSC in order to determine glass transitions. The aim of this part of the work was to observe the change of physical structure of HS after their hydration, and the influence of water content on the shift of temperatures of phase transition such as crystallization, melting or glass transitions, and to compare the differences between the original samples and hydrated ones.

At first, when the period of 21 days expired, the sample was dried using TGA (the temperature range was from 25 to 170 °C at 3 °C min⁻¹ under the flow of nitrogen). Immediately when the drying finished, TMDSC was carried out in the temperature range from -90 to 150 °C in two cycles. It is a general knowledge that all physi-sorbed water is removed during the drying and first heating and cooling cycle, and therefore, in our case it did not affect the phase transitions occurring in the samples. Such a temperature program, i.e. up to 150°C was applied due to the reversibility of structural changes in HS, in contrast higher

temperatures would cause irreversible changes, which could objectionably influence the experiment. Fig. 29 reports the measured data for SRHA sample. As can be seen, the total heat flow (green line) is separated into reversing (blue line) and non-reversing heat flow (red line). This separation enables to distinguish between individual processes occurring in the sample. The reversing heat flow denotes the change in heat capacity, whereas the non-reversing heat flow reveals the presence of irreversible (kinetic) processes and non-equilibrium phase transitions. Usually, the specific heat capacity change at the glass transition is overlapped by effects such as vaporization, enthalpy relaxation, phase transitions and chemical reactions. However, TMDSC allows to separate changes in heat capacity from the other effects and to determine the glass transition temperature, so that the glass transitions can be evaluated from the reversing heat flow without being disturbed by the enthalpy relaxation.

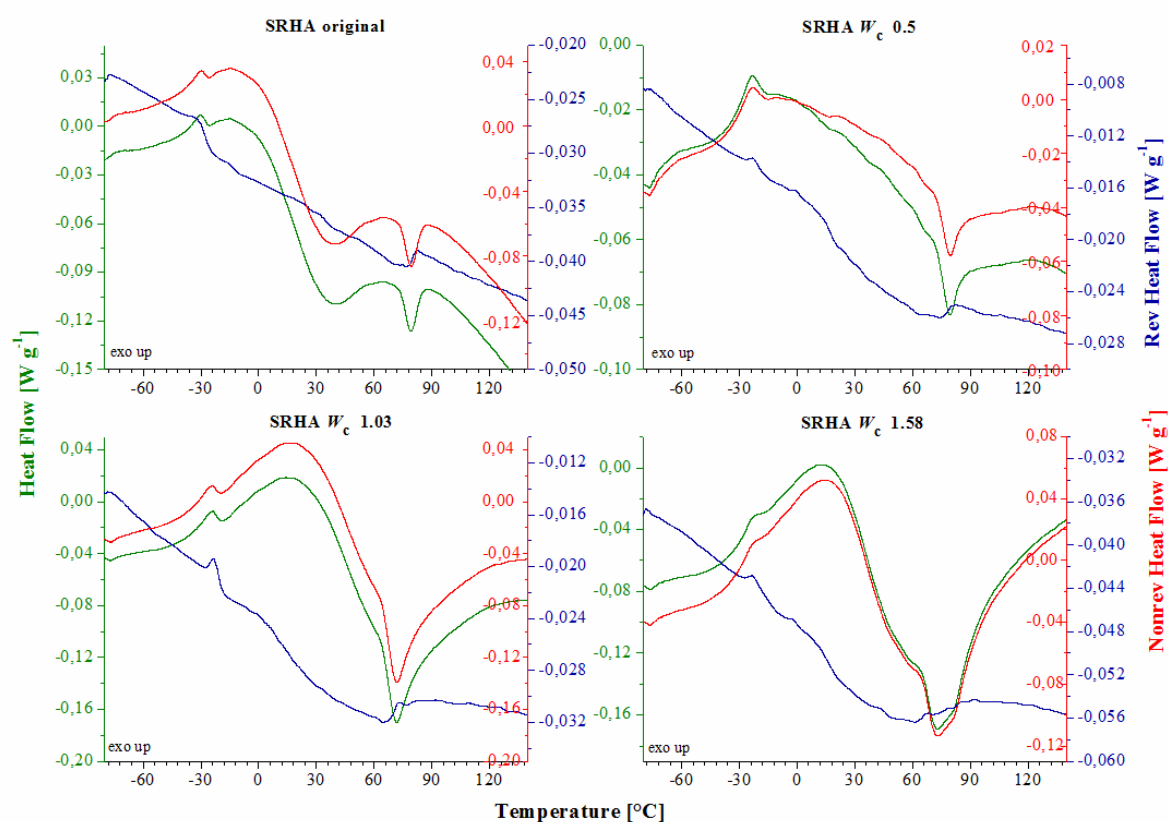


Fig. 29 TMDSC records of SRHA sample.

The glass transition for original SRHA sample can be found at $-27.2\text{ }^{\circ}\text{C}$. In the total and non-reversing signal, there can be seen an exothermic peak in this area which is attributed just to the enthalpy relaxation and complicates the detection of T_g . The broad endothermic and exothermic peaks occurring in both total and non-reversing signals between approximately $0\text{ }^{\circ}\text{C}$ and $90\text{ }^{\circ}\text{C}$ can be considered as the simultaneous crystallization/crystalline reorganization of lipid structures [206] similar to the process of crystal perfection well known from polymer chemistry. The first broad endothermic peak around $40\text{ }^{\circ}\text{C}$ was ascribed to the gradual melting of smaller crystallites of great size distribution. Subsequently, the larger, more perfect and even more stable crystallites of similar size are formed. For melting of those the higher temperature is needed, and due to similarity of these newly formed crystalline

domains the sharper and narrower endotherm around 80 °C can be observed. According to recent results, water may surprisingly act in an antagonistic way as short-term plasticizer and long-term antiplasticizer in soil organic matter [207]. The T_g of hydrated SRHA samples was shifted to higher temperatures which indicates the antiplasticizing effect of water hydration and dehydration on SRHA. The simultaneous crystallization/crystalline reorganization shows very similar trend for all W_c . There exhibits only one broad endothermic peak with little “shoulder” at the beginning in the area of 75 °C which can be observed in total and non-reversing signals. With increasing water content, it is getting even greater and more intensive and the temperature of this transition is slightly shifted to lower values.

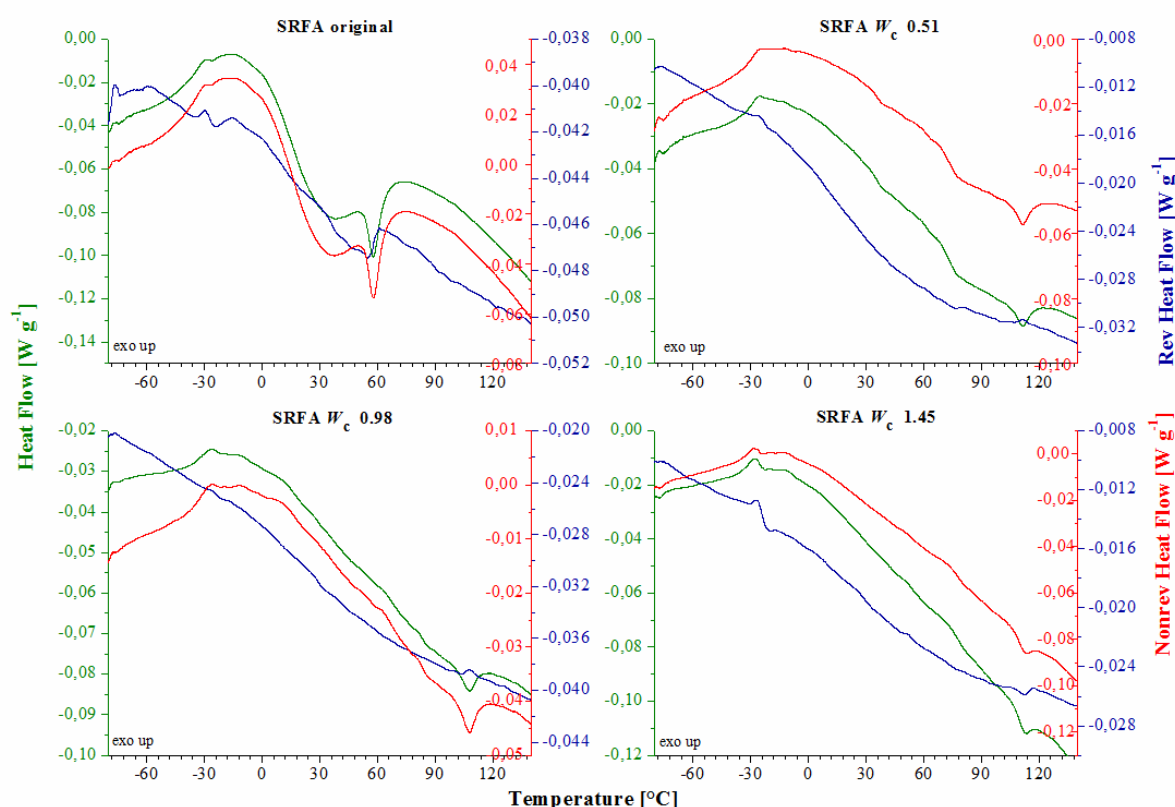


Fig. 30 TMDSC records of SRFA samples.

The Fig. 30 shows TMDSC records of SRFA sample. The temperature of glass transition of original sample is the same as it was for SRHA, nevertheless the second endothermic peak belonging to the melting of crystallites of similar size is very sharp and narrow which signifies certain resemblance to polymer materials. The glass transitions of hydrated SRFA samples occur also at higher temperatures, so that the water after re-hydration plays here a role of an antiplasticizer as well. As can be seen from the total and non-reversing signals, the endothermic transitions for simultaneous crystallization/crystalline reorganization of hydrated SRFA samples are much less intense compared to the original sample and the transition temperature is higher.

The glass transition temperature for original LHA sample (Fig. 31) is moderately lower than it was for humic and fulvic acids originating from Suwanee River, and it almost does not

change with the re-hydration. It seems that water does not get inside of the humic structure to influence the amorphous domains responsible for glass transitions. The transitions ascribed to the simultaneous crystallization/crystalline reorganization of original LHA sample is performed by one broad endotherm of medium intensity around 75 °C. The hydrated LHA samples of W_c 0.48 and 1.41 shows subtle progressive melting and crystallization of crystallite fractions. For the sample of W_c 0.48 there can be observed an exothermic transition on reversible signal at 116 °C which could be attributed to some kind of independent crystallization similar to that of synthetic materials (e.g. PETF). The records of W_c 1.00 indicated resemblance to polymeric materials as well since the endotherm around 75 °C is quite sharp and narrow.

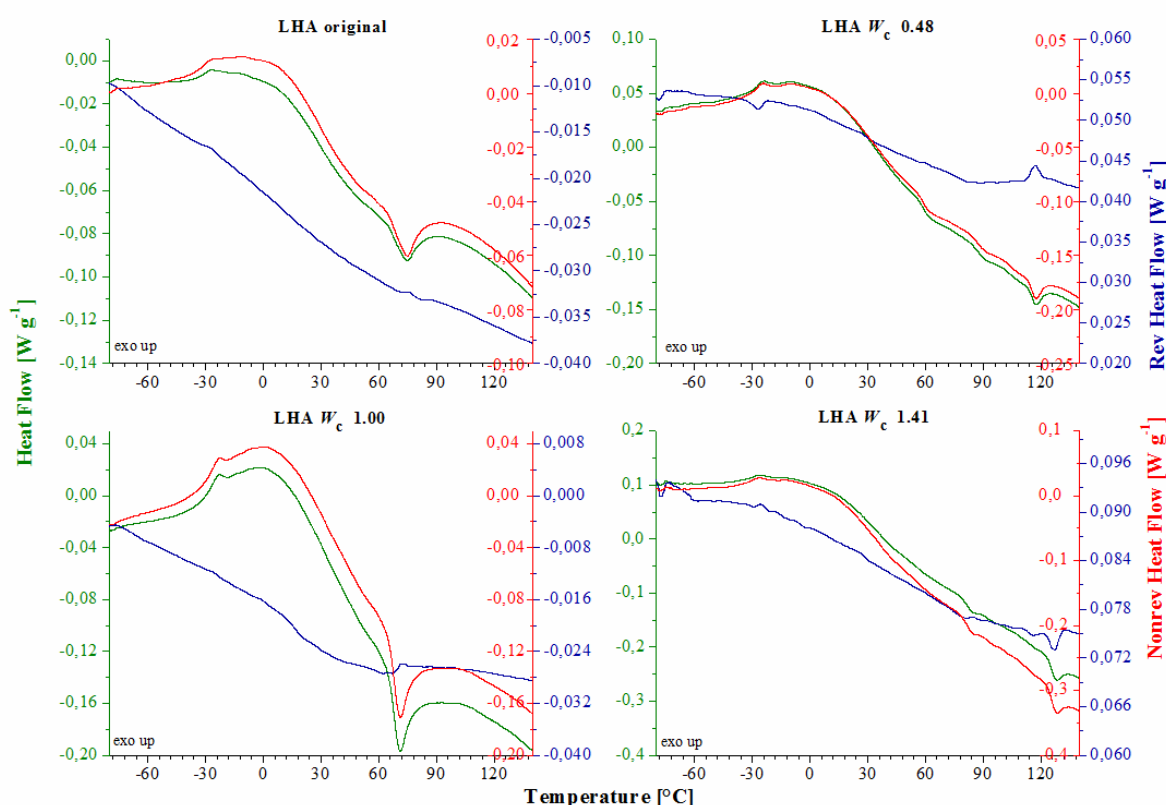


Fig. 31 TMDSC records of LHA samples.

The TMDSC records of Elliot Soil and Pahokee Peat IHSS samples can be found in Appendix IV. It is worth mentioning that the glass transition temperatures of Elliot Soil samples are evidently higher than those of other IHSS samples and water acts here as a plasticizer (see Table III). Also samples of LHA with W_c 0.48 and PPHA with W_c 1.50 seems to have slightly plasticizing effect but the enhancement of values of glass transition temperatures is hardly noticeable.

Table III The glass transition temperatures of IHSS humic and fulvic substances and the effect of water on HS structure.

W_c [g/g]	T_g [°C]	Temperature Range [°C]	ΔT [°C]	Plasticizing/ antiplasticizing effect of water
SRHA				
original	-27.2	-30.3-(-24.5)	5.8	-
0.50	-20.5	-22.7-(-19.7)	3.0	antiplasticizing
1.03	-21.0	-23.0-(-18.8)	4.2	antiplasticizing
1.58	-20.2	-22.5-(-18.0)	4.5	antiplasticizing
SRFA				
original	-27.2	-29.3-(-25.7)	3.6	-
0.51	-23.0	-24.1-(-21.7)	2.4	antiplasticizing
0.98	-24.8	-24.9-(-22.2)	2.7	antiplasticizing
1.45	-24.3	-25.9-(-22.0)	3.9	antiplasticizing
ESHA				
original	-33.6	-34.1-(-32.7)	1.4	-
0.50	-31.5	-32.3-(-29.6)	2.7	antiplasticizing
1.00	-27.8	-29.5-(-25.6)	3.9	antiplasticizing
1.48	-30.3	-31.5-(-28.9)	2.6	antiplasticizing
ESFA				
original	-32.9	-33.6-(-31.8)	1.8	-
0.43	-19.0	-20.2-(-16.9)	3.3	antiplasticizing
0.99	-27.0	-29.8-(-25.9)	3.9	antiplasticizing
1.44	-34.1	-38.8-(-33.3)	5.5	plasticizing
PPHA				
original	-26.5	-26.6-(-24.7)	1.9	-
0.50	-21.6	-23.3-(-20.4)	2.9	antiplasticizing
0.97	-26.2	-27.6-(-26.8)	0.8	antiplasticizing
1.50	-26.7	-28.2-(-24.7)	3.5	plasticizing
PPFA				
original	-28.7	-29.4-(-26.6)	2.8	-
0.48	-22.9	-24.5-(-19.7)	4.8	antiplasticizing
0.94	-20.7	-20.7-(-18.6)	2.1	antiplasticizing
1.49	-19.1	-21.3-(-17.5)	3.8	antiplasticizing
LHA				
original	-24.2	-25.4-(-22.9)	2.5	-
0.48	-25.0	-26.4-(-23.1)	3.3	plasticizing
1.00	-23.3	-24.7-(-22.2)	2.5	antiplasticizing
1.41	-22.8	-23.3-(-21.4)	1.9	antiplasticizing

5. CONCLUSION

In this work, the study on the character of hydration water in water/humic substances system is reported. Both the qualitative and quantitative aspects of hydration of HS were determined and the differences in properties of water surrounding humic matter were explored. The approaches and techniques already used and reported as being successful for determination and enumeration of hydration water in hydrophilic biopolymers were slightly modified and applied for HS originating from different sources and thus having different properties and composition.

The DSC measurements of HS/water revealed that the kinetics plays a significant role in hydration processes of HS. Unlike most hydrophilic biopolymers the dependency of melting enthalpy change of freezable water (ice) on W_c was not constant after 24 hours from preparing the sample because of its incomplete hydration. Therefore, the phase transition behaviour was monitored over the period of 21 days. During this period the enthalpy change of melting decreased for all samples. The results suggest that the hydration of humic substances is not a straightforward process. Mainly until approximately day 7 the rapid decrease in change of melting enthalpy occurred. After that time period, the diminution became more moderate. This situation is in line with recent statement that the wetting of SOM proceeds in two steps [105]. The first step of the hydration process of soil samples includes the wetting of the surfaces and after that, the water is distributed to the pore volume. A crucial factor in distinguishing between humic and fulvic acids seems to be the different solubility of particular molecules. In fulvic acids, most of molecules are hydrophilic; therefore, their hydration resembles the hydrophilic biopolymers. In contrast, humic acids consist of a mixture of molecules of various polarity and therefore different wettability.

Humic substances consist of structures containing both non-polar and polar inner space. As suggested in our previous work [141], the structure is stabilized by separated hydrophobic domains connected by non-specific van der Waals interactions forming kind of hydrophobic scaffold. The surface of humic aggregates seems to be rather hydrophobic which is reflected by lower water sorption capacity. Further, when water penetrates into humic structure, the polar interactions are stabilizing the broken structure and swelling takes part. Thus, humic acids structure resembles a porous structure, a random combination of hydrophobic and hydrophilic domains. The disruption of the structure is rather slow since it is associated with the hydration of surfaces of different hydrophobicity/hydrophilicity combined with their variability in shape and dimension. Moreover, it seems that progressive hydration brings about not only bridges formation, but at the same time, the inner vacancies are formed containing relatively large volumes of water. Each cavity has its own maximal inner volume where due to the hydrophobic effect therein creates a pressure causing the gradual expansion of whole volume. The water presses the hydrophobic parts and the volume of inner cavity is increasing until it bursts so that the scaffold made of hydrophobic parts is being pushed out by the hydrophobic effect.

Generally speaking, fulvic acids reflect higher affinity to water than their respective humic acids. The more complicated inner structure of humic acids with higher porosity allows the water molecules to hydrate higher surface than in FA. It seems that the younger (or less humified) HS are, the easier and faster their change is.

The TMDSC records of IHSS samples imply that the T_g i.e. a temperature of amorphous part transition of almost all hydrated samples was shifted to higher temperatures which indicates the antiplasticizing effect of water hydration and dehydration. Exceptional were samples of ESFA with W_c 1.44 and LHA with W_c 0.48 whose T_g was slightly shifted to higher temperatures, hence the water played a role here as a plasticizer. From TMDSC results of LHA was observed that water does not get inside of the humic structure to influence the amorphous domains responsible for glass transitions. Such observation complies with the results obtained from kinetic experiments.

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7. LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

HS	Humic substances
HAs	Humic acids
FAs	Fulvic acids
CEC	Cation exchange capacity
CMC	Critical micelle concentration
DNA	Deoxyribonucleic acid
SOM	Soil Organic Matter
NOM	Natural Organic Matter
HBLC	Hydrogen bond-based cross-linking
NMR	Nuclear Magnetic Resonance
HRUS	High Resolution Ultrasonic Spectroscopy
US	Ultrasonic
PHEOEMA	Poly[2-(2-hydroxyethoxy)ethyl methacrylate]
PHEA	Poly(2-hydroxyethyl acrylate)
HYA	Hyaluronan
FT-IR	Fourier transform infrared spectroscopy
IHSS	International Humic Substances Society
DSC	Differential Scanning Calorimetry
TGA	Thermogravimetry Analyses
TMDSC	Temperature-modulated Differential Scanning Calorimetry
SRHA	Suwannee River humic acid
SRFA	Suwannee River fulvic acid
ESHA	Elliot Soil humic acid
ESFA	Elliot Soil fulvic acid
PPHA	Pahokee Peat humic acid
PPFA	Pahokee Peat fulvic acid
LHA	Leonardite humic acid

Symbols

μ	Chemical potential
μ_N	Mean chemical potential
μ_N^0	Standard part of the chemical potential
X_N	Activity
K	Equilibrium constant

C	Total solute concentration
ΔG_{tr}	Free energy of transfer
ΔH_{tr}	Enthalpy
$-T\Delta S_{tr}$	Entropy
ΔG	Change in Gibbs energy
U	Ultrasonic velocity
T_g	Glass transition temperature
T_m	Melting temperature
C_p	Heat capacity
k	Compressibility
α	Thermal expansit coefficient
σ_p	Cohesive energy density
W_c	Water kontent
W_{nf}	Non-freezing water
W_f	Freezing water
W_{fb}	Freezing-bound water

8. LIST OF PUBLICATIONS AND ACTIVITIES

Impacted publications:

1. Kučerík, J., Čechlovská, H., Bursáková, P., Pekař, M.: The thermodynamic stability and molecular feature of lignite humic acids aggregates studied by high resolution ultrasonic spectroscopy. *J. Therm. Anal. Cal.*, 2008. ISSN: 1388-6150.
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Trainings and courses:

1. 10th School of Mass Spectrometry (Seč-Ústupky, 2009)
2. 'Interpretation of IR vibration spectra' course (Institute of Chemical Technology Prague, 2008)
3. NETZSCH Workshop – thermal analysis (Brno, 2007)

9. LIST OF APPENDICES

- APPENDIX I: DSC heating curves of selected ESHA, ESFA, PPHA and PPFA samples measured after 24 hours
- APPENDIX II: DSC heating curves of selected W_c of PPHA and PPFA samples measured during the period of 21 days
- APPENDIX III: The kinetics of dependency of change of melting and crystallization enthalpy on W_c for ESHA, ESFA, PPHA and PPFA samples
- APPENDIX IV: TMDSC records of ESHA, ESFA, PPHA and PPFA samples

APPENDIX I

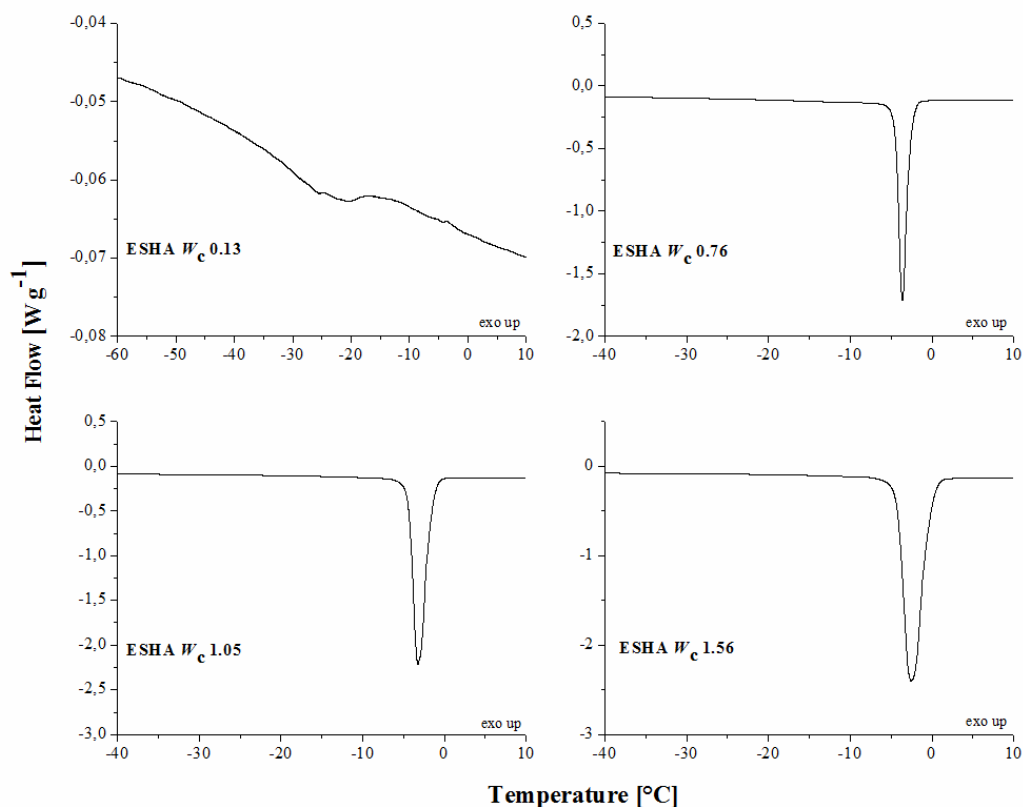


Fig. 32 DSC heating curves of ESHA measured after 24 hours.

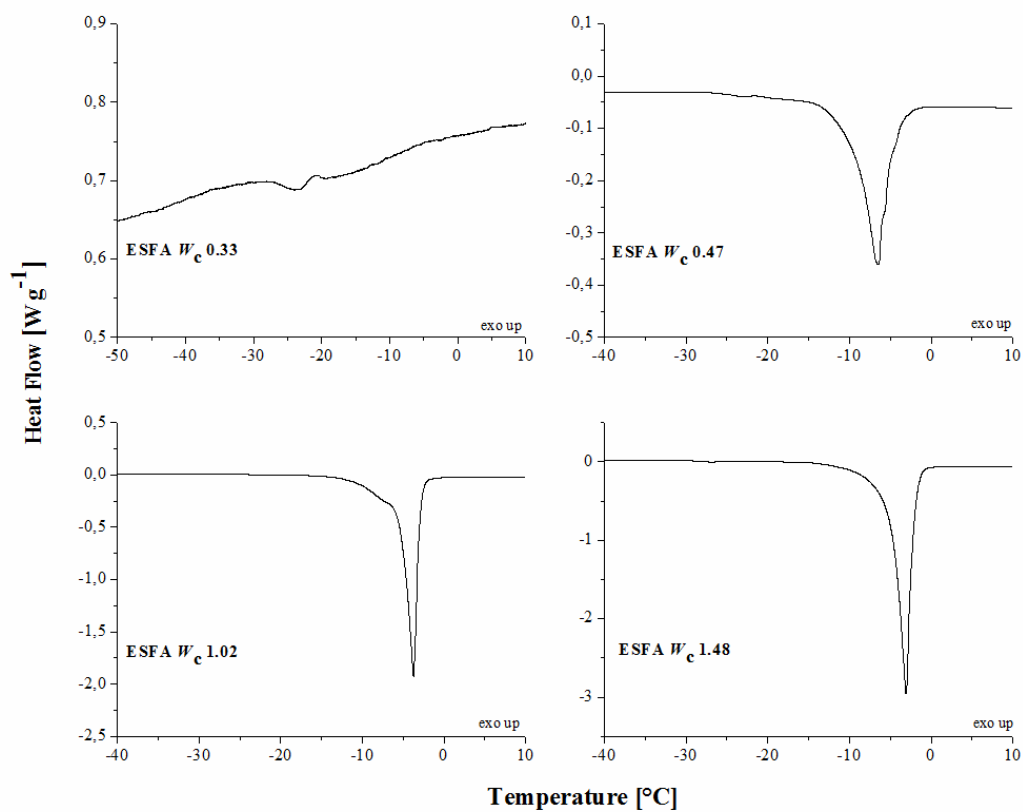


Fig. 33 DSC heating curves of ESFA measured after 24 hours.

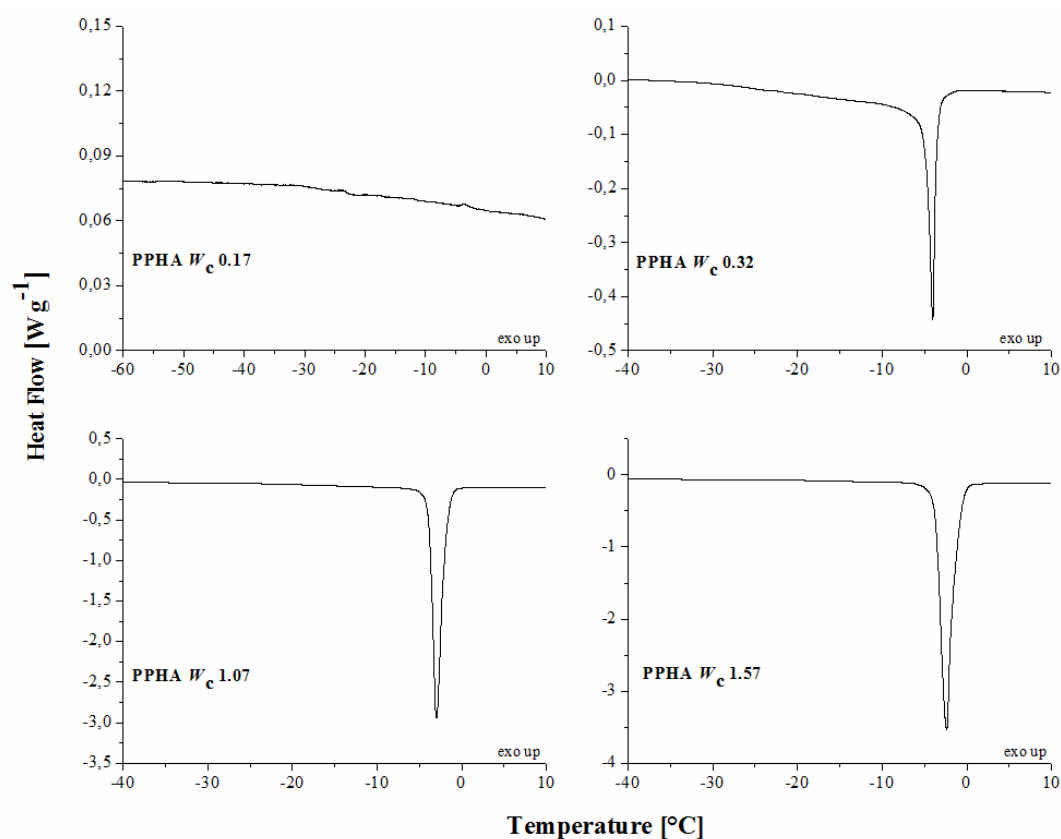


Fig. 34 DSC heating curves of PPHA measured after 24 hours.

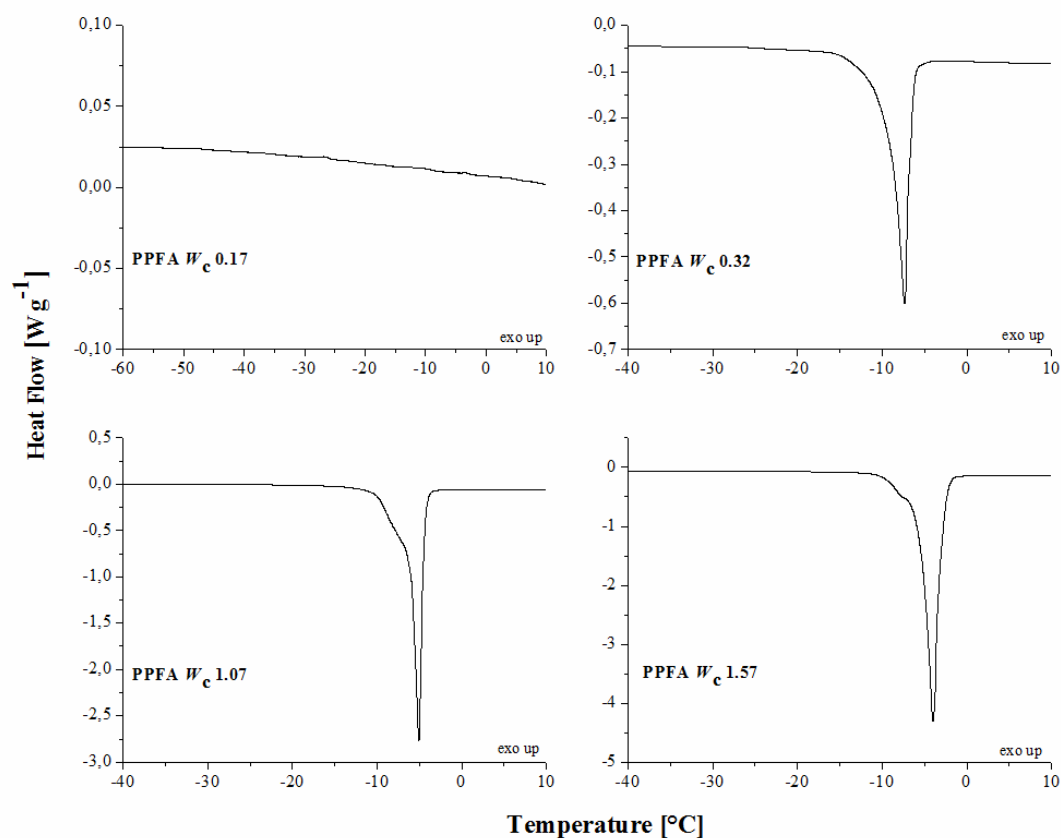


Fig. 35 DSC heating curves of PPFA measured after 24 hours.

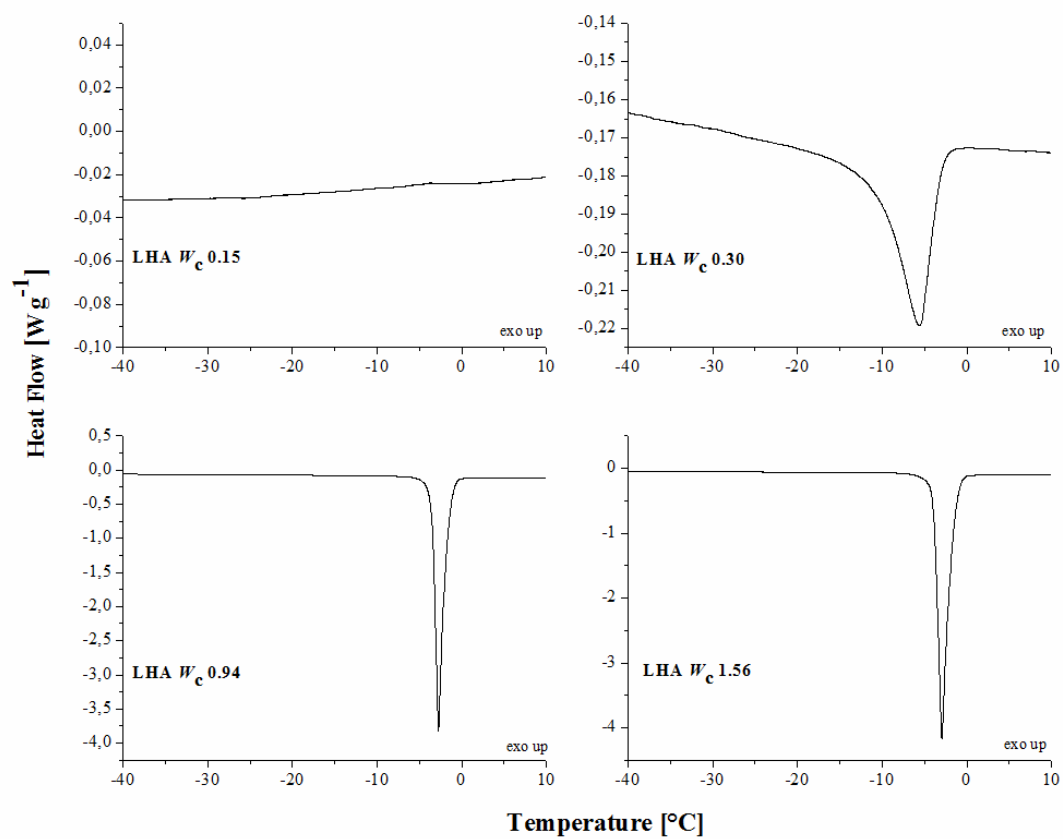


Fig. 36 DSC heating curves of LHA measured after 24 hours.

APPENDIX II

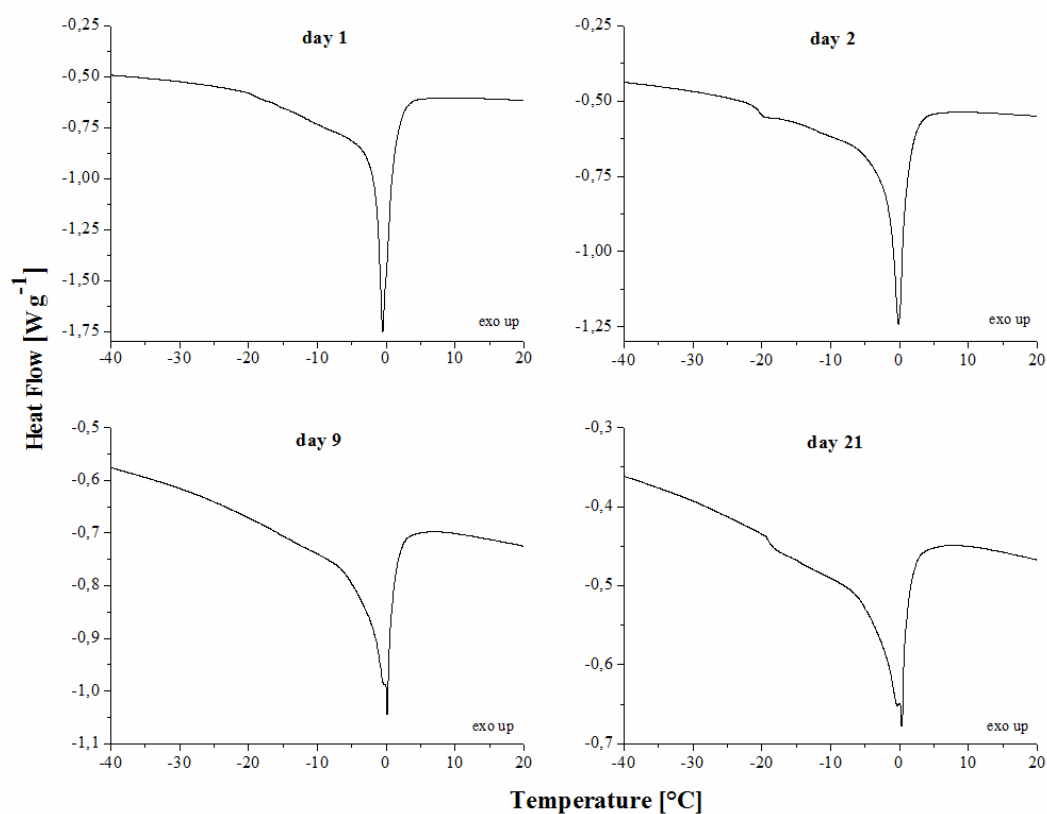


Fig. 37 DSC heating curves of PPHA of W_c 0.51 measured during the period of 21 days.

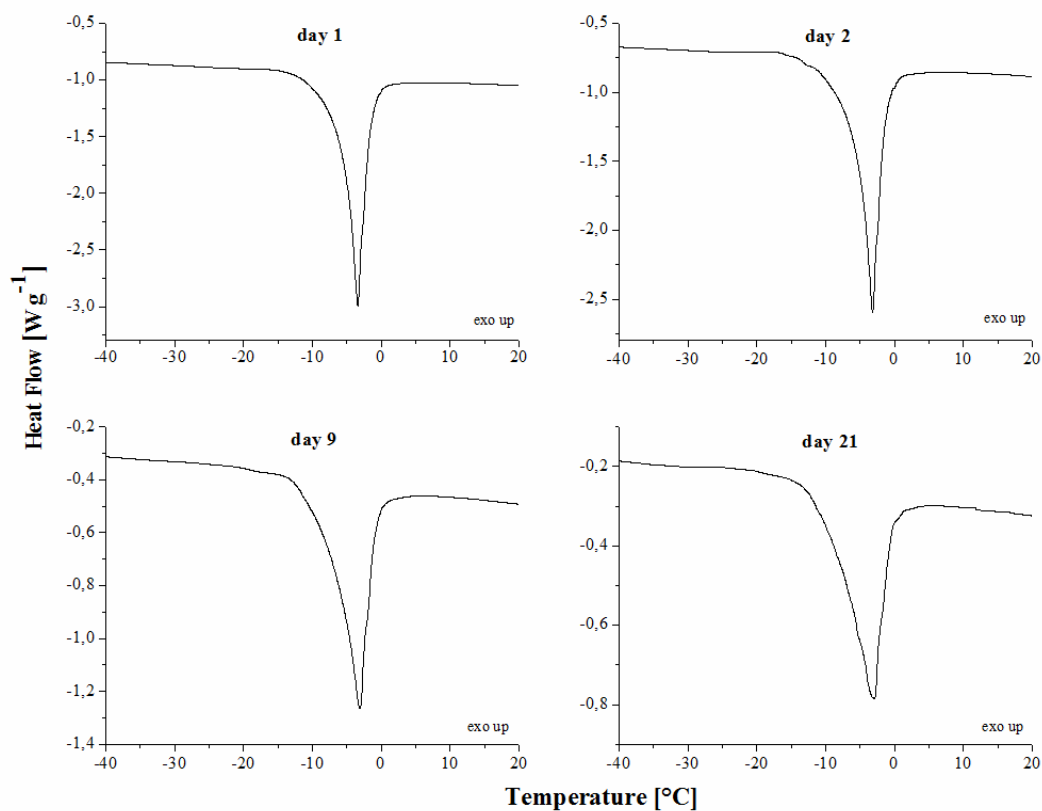


Fig. 38 DSC heating curves of PPFA of W_c 0.48 measured during the period of 21 days.

APPENDIX III

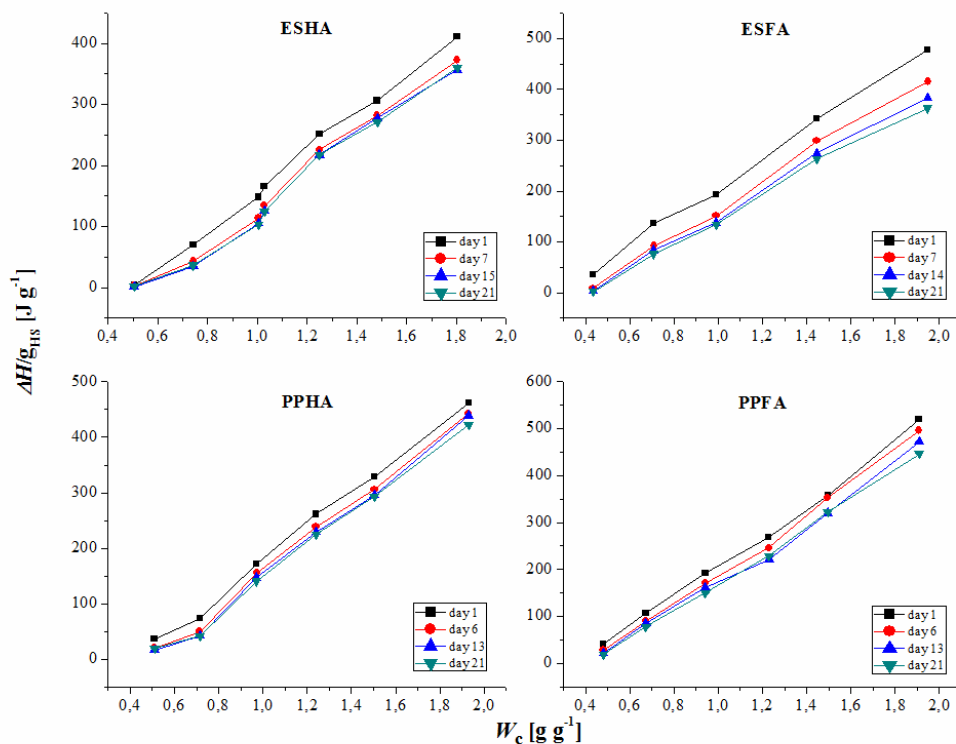


Fig. 39 The kinetics of dependency of melting enthalpy change on W_c for ESHA, ESFA, PPHA and PPFA samples during the period of 21 days.

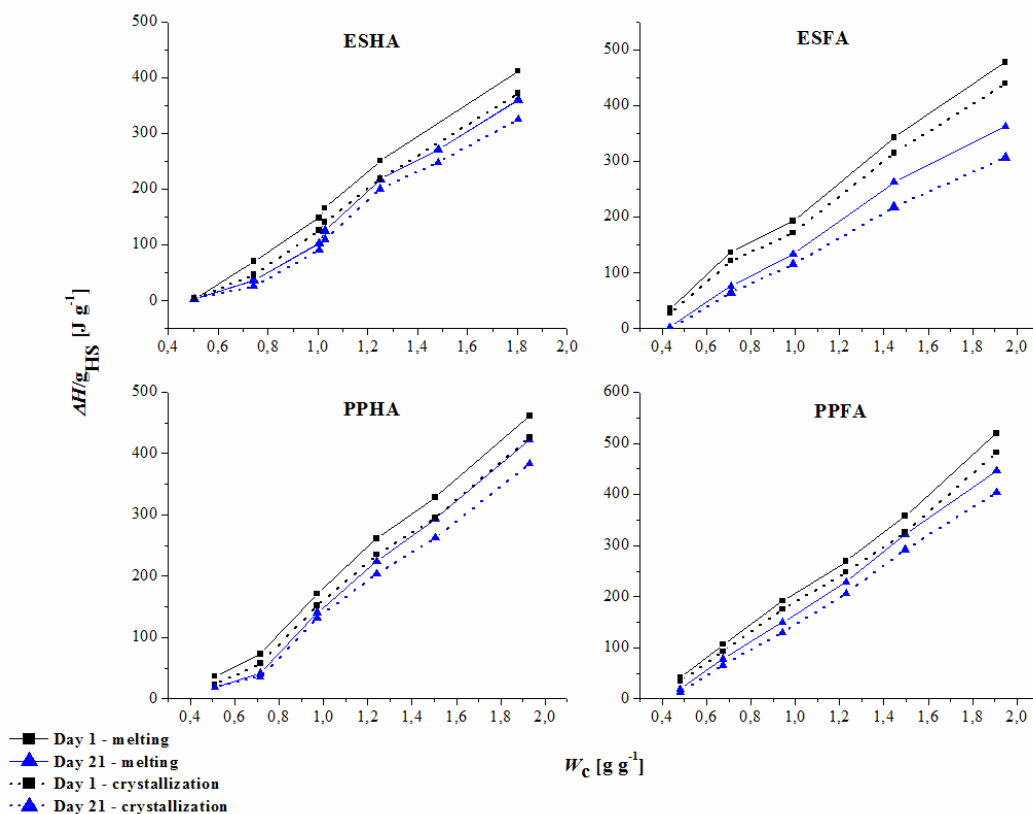


Fig. 40 The kinetics of dependency of change of melting and crystallization enthalpy on W_c for ESHA, ESFA, PPHA and PPFA samples.

APPENDIX IV

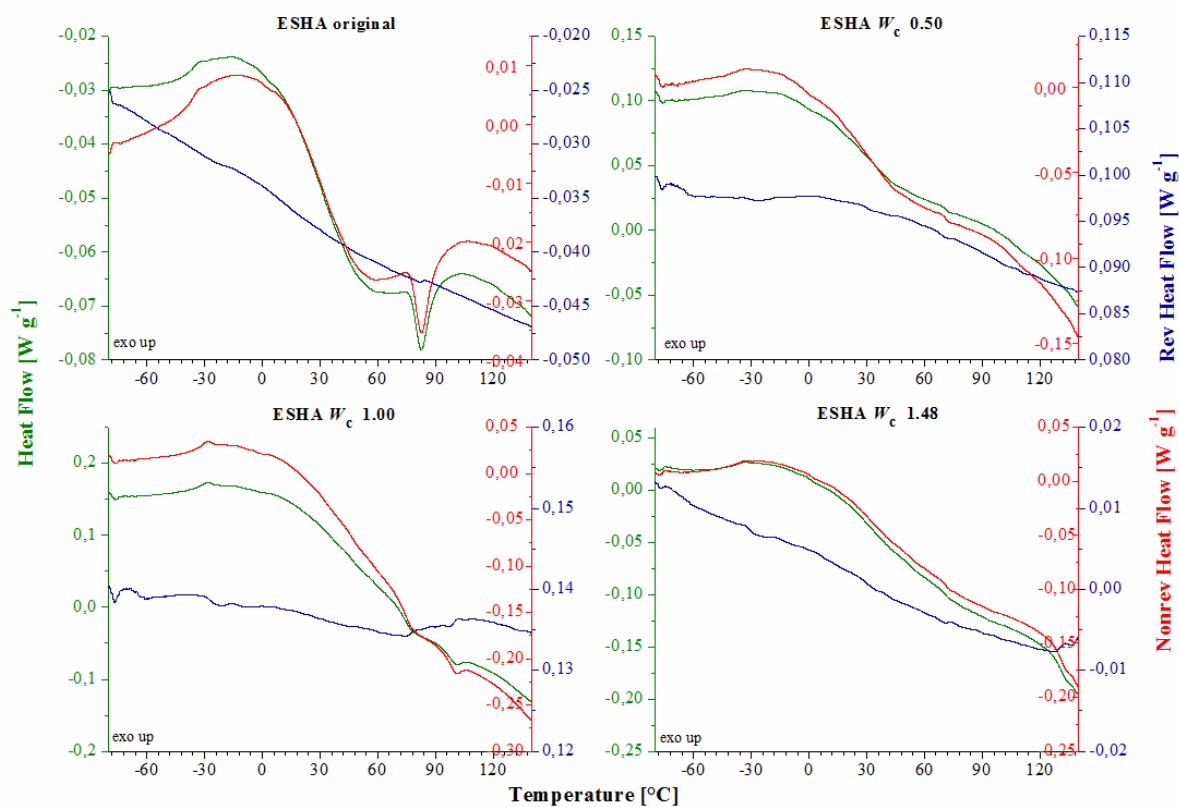


Fig. 41 TMDSC records of ESHA samples.

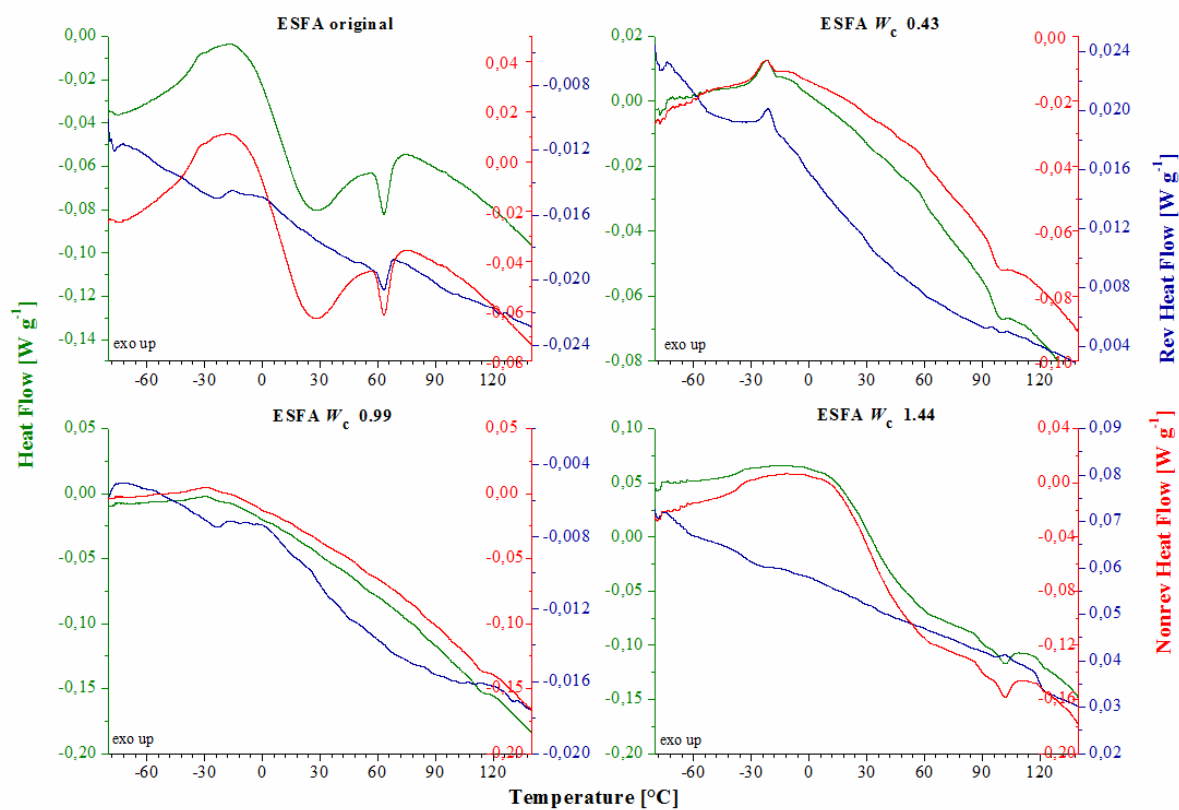


Fig. 42 TMDSC records of ESFA samples.

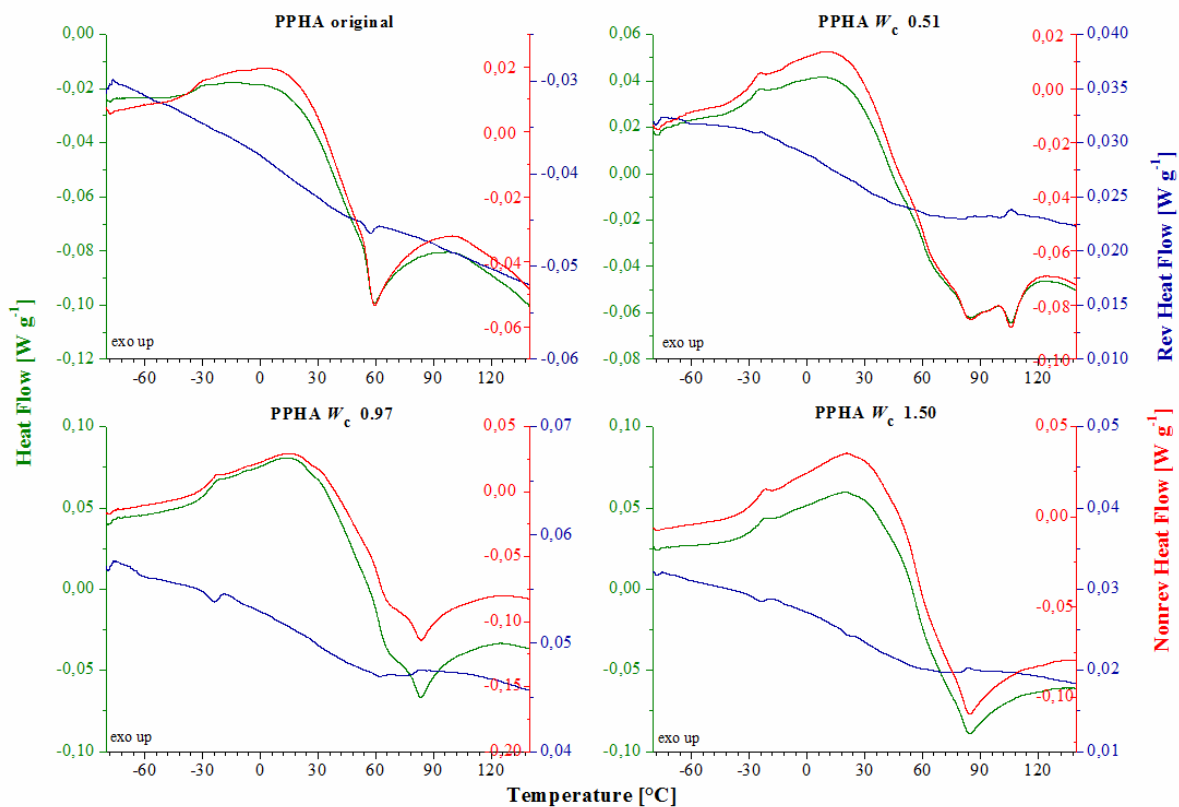


Fig. 43 TMDSC records of PPHA samples.

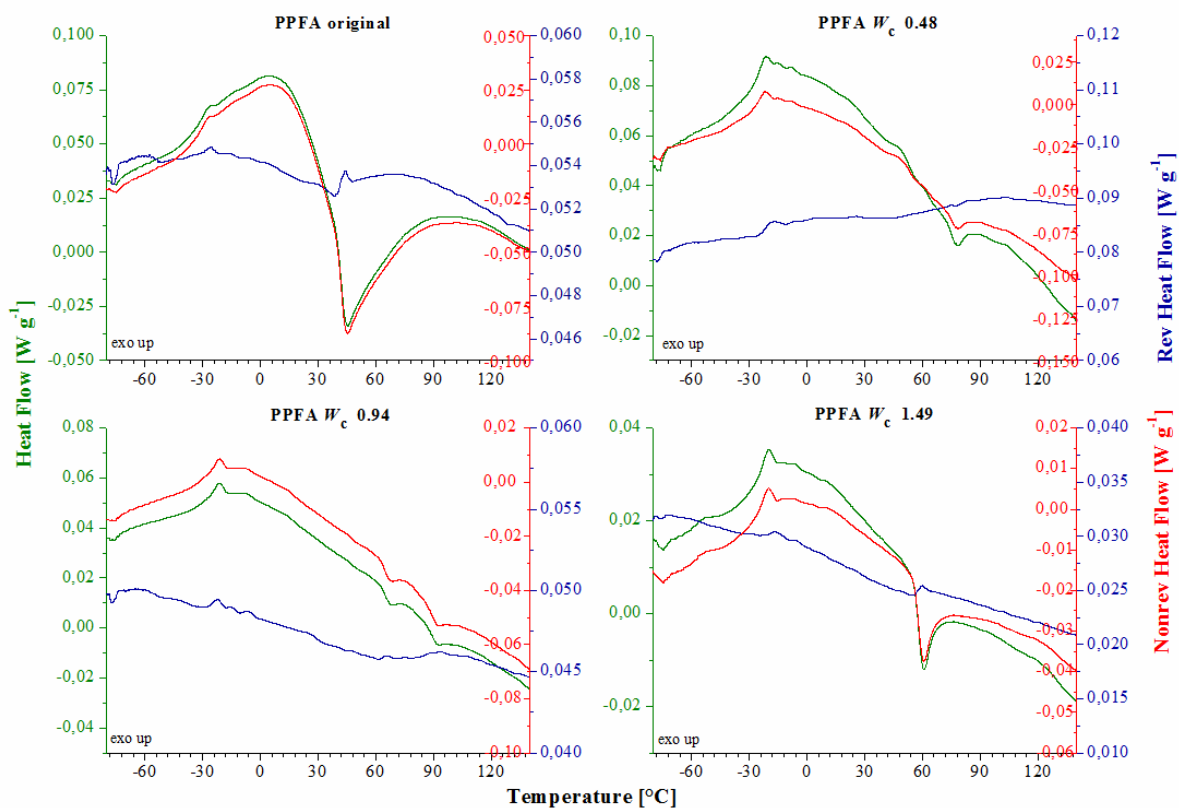


Fig. 44 TMDSC records of PPFA samples.