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**VYUŽITÍ VYSOCEROZLIŠOVACÍ ULTRAZVUKOVÉ SPEKTROSKOPIE PŘI
CHARAKTERIZACI HUMINOVÝCH LÁTEK**

**HUMIC SUBSTANCES CHARACTERIZATION EMPLOYING HIGH RESOLUTION
ULTRASONIC SPECTROSCOPY**

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1 INTRODUCTION

Humic substances are ubiquitous and also the most occurring organic matter on the Earth. Due to their high heterogeneity and chemical character they tend to self-aggregate forming various molecular organizations. The mechanisms and ways of aggregation are still not precisely known. The knowledge of the aggregates nature and mass is of a great importance since the mass of aggregates influences the function of HS in nature and determines application in industry.

There have been employed many different methods to study humic substances in both solid and liquid state. One of them the High Resolution Ultrasonic Spectroscopy (HRUS) is. It is a relatively new method with high potential in colloid chemistry. Since in HRUS mechanical type of waves is involved, it can succeed where classical optical methods are limited by the nature of the sample or conditions required. This nondestructive method has already been applied to monitor various physical-chemical processes of biomolecules and proved itself as a powerful tool due to its high sensitivity and broad possibilities of application. Although it was applied in the field of humic substances study several times, all its possibilities and advantages were not fully acknowledged so far.

2 STATE OF THE ART

2.1 HUMIC SUBSTANCES

Based on historical reasons, humic substances (HS) can be classified according to their solubility under acidic or alkaline conditions into three groups: humin, the insoluble fraction; humic acids (HA), the fraction soluble under alkaline but not acidic conditions (generally $\text{pH} < 2$); and fulvic acids (FA), the fraction soluble under all pH conditions 5 . Nevertheless, at least some scientists maintain a position that all the classifications and definitions in humus chemistry are only operational on the basis of procedures used for isolation of HS and that there is no ideal system of classification which would satisfy each scientist's need [2],[3].

2.1.1 Elemental composition – primary structure

Humic substances consist mainly of carbon, oxygen and hydrogen. Amounts of nitrogen, sulfur and phosphorus are often significant enough to be mentioned in the analysis reports. The rest of trace elements is usually summarized as ash content. Amounts of C, H and N are determined directly, O content is in general obtained by subtracting the sum of the other elemental contents, plus the ash content, from 100 %. This method makes the O content the least reliable since it includes the sum of all errors originating from other elements determinations and also the S and P content which is usually disregarded. However, S content can represent as much as 3 % in some samples of humic substances and even P may constitute a measurable fraction of humic substances [4].

The H/C and O/C ratios were originally developed to study the coalification process but later on adopted by organic geochemists to study diagenesis on HS to illustrate compositional differences between HA and FA, and also to show variations in HS as a function of source [5]. The magnitude of the H/C ratio indicates the degree of aromaticity or unsaturation (a small value) or aliphaticity (a large value) of a substance. In the case of HS, the situation is a little bit complicated since some degree of unsaturation is presented also in functional groups, primarily carboxyl and carbonyl groups.

In the work of Rice and MacCarthy [6], the elemental composition data (C, H, O, N, S, atomic H/C and O/C ratios) for humic acids (410 samples) and fulvic acids (214 samples), isolated from environments all over the world, were compiled from the literature. Authors analyzed the data statistically. Interestingly, the standard deviations for carbon contents were found remarkably small suggesting that perhaps an optimum composition exists for humic substances in nature. The evaluation showed that fulvic acids are statistically distinct from humic acids on the basis of its C (lower), N (lower) and O (higher) contents and its O/C (higher) and H/C (higher) ratio. Because a larger H/C ratio is indicative of a more aliphatic character, this is consistent with fulvic acid being, in general, more aliphatic than humic acid. The only parameter for which there was no significant difference between HA and FA was the hydrogen content. When segregated by source, some significant differences between humic acids isolated from freshwater, marine, and soil environments were evident. Similarly, significant differences were found between fulvic acids from freshwater and soil sources.

2.1.2 Functional groups

What makes humic substances so unique, at least to some extent, is the variety and number of functional groups. The majority of them are oxygen-containing groups like carboxyl, phenolic, enol, alcohol, quinone, hydroxyquinone, lactone, ether, ketone and anhydride groups. Also some nitrogen-containing groups (amino, amine, imine and amide) can be found. Some small amounts of sulfur and phosphorus functional groups are present as well [7].

Fulvic acids contain more acidic functional groups, particularly COOH, in comparison with humic acids. The total acidities of fulvic acids (900–1400 mmol/100 g) are considerably higher than for humic acids (400–870 mmol/100 g) [7].

2.1.3 Known constituents

Humic fractions extracted from soils contain recognizable biomolecular fragments that were specifically excluded from traditional definitions of humic substances. These fragments are derived primarily from lipids, lignin, carbohydrates and proteins and come mainly from decomposing debris of plants, animals and microorganisms. Many of these biomolecular moieties are intimately associated

(sometimes even covalently bonded) with the humic fraction and cannot be separated effectively without significant alteration of the chemical properties of the fraction [7],[8] and therefore contemporary analytical approaches consider them as indivisible part of the fraction [9].

2.1.4 In search of structure

In 1961 Kononova [10] introduced the concept of humic substances as system of polymers based on the observation that elemental composition, optical properties, exchange acidities, electrophoretic properties and molecular weight (MW) characteristics varied consistently with soil classes. The MW of presented macromolecules in different fractions was assessed in the range from several hundreds to perhaps over 300,000 Da. Nevertheless, the measurement of sedimentation coefficients of polydisperse materials that include subunits, which is the case of HS, constantly leads to erroneous values of MW as shown in [11].

Ghosh and Schnitzer [12] tried to shed light on shapes attributed to humic polymeric macromolecules by measuring surface tensions and viscosities of HS at different pH values and neutral salt concentrations. To interpret the measured results, equations developed for real polymers were employed. They explained the observed behavior of HS (uncharged matter at low pH and polyelectrolytes at high pH) on the basis of the polymeric theory. They proposed that HS are rigid spherocolloids at high sample concentration and ionic strength and at low pH; whereas at high pH values, low sample concentrations and ionic strength, they behave as flexible linear polymers. This approach had two major flaws: a) it was based on studies with whole humic extracts with full polydispersity; b) the data were evaluated using equations specifically derived for polymers. Nevertheless, this reversible coiling model for humic configurations soon became the most widely used to describe HS, although it does not explain all of the behavior of HS.

Later on, Wershaw [13] considered humic substances as ordered aggregates of amphiphils composed mainly of relatively unaltered plant polymer segments possessing acidic functional groups. These aggregates are supposed to hold together by H-bonds and hydrophobic (π - π and charge-transfer bonds) interactions. The hydrophobic parts of the molecules are hidden in the interiors while the hydrophilic parts are exposed to surrounding water molecules making up the exterior surfaces. Ordered aggregates of humus in soils were depicted to exist as bilayer membranes coating mineral grains and as micelles in solutions. This innovative concept of aggregation of small particles was in strong contrast to the traditional view of polydisperse humic polymers. This concept evoked the hypothesis of critical micelle concentration (CMC) of HS although the heterogeneity of HS is antithetic to homogeneity of molecules forming micelles as presented by classical micellar concept. Anyway, CMC of various HS was reported in the wide range 1–10 g.L⁻¹ [14],[15] and it was supposed that amphiphilic molecules exist solely as single unit species at concentrations lower than the CMC, whereas at higher concentration ordered aggregates or micelles are formed.

Huge amounts of results obtained by Piccolo et al. employing size exclusion chromatography (HPSEC) gave rise to a new concept of HS internal structure [16],[17]. This model depicts HS as supramolecular associates of relatively small molecules that self-assemble into aggregates of apparent high-molecular size. Individual constituents are bonded together by weak interactions. Results obtained by utilizing several different analyzing methods including fluorescence spectroscopy [18], NMR [19], or ultrasonic spectroscopy [20] supported these conclusions.

Tab 1: Elemental composition of IHSS samples; C, H, N, O expressed as atomic percent of dry, ash-free sample; ash expressed as weight percent of inorganic residue in a dry sample [21].

	C	H	N	O	C/O	C/H	Ash
Suwannee Siver HS (2S101H/F)							
HA	38.64	37.45	0.74	23.17	1.67	1.03	1.04
FA	38.17	37.89	0.42	23.53	1.62	1.01	0.58
Elliott soil (2S102H/F)							
HA	44.34	33.44	2.71	19.51	2.27	1.33	0.88
FA	36.77	37.41	2.36	23.46	1.57	0.98	1.00
Pahokee peat (1S103H/F)							
HA	42.36	34.20	2.38	21.06	2.01	1.24	1.12
FA	40.12	32.89	1.57	25.43	1.58	1.22	0.90
Leonardite (1S104H-5)							
HA	48.18	33.29	0.80	17.73	2.72	1.45	2.58

Tab 2: ^{13}C estimates of carbon distribution in IHSS samples, expressed as electronically integrated peak area percentages for selected ranges of chemical shift [21].

	Carbonyl 220-190 ppm	Carboxyl 190-165 ppm	Aromatic 165-110 ppm	Acetal 110-90 ppm	Heteroaliphatic 90-60 ppm	Aliphatic 60-0 ppm
Suwannee River HS (2S101H/F)						
HA	6	15	31	7	13	29
FA	5	17	22	6	16	35
Elliott soil (2S102H/F)						
HA	6	18	50	4	6	16
FA	12	25	30	1	9	22
Pahokee peat (1S103H/F)						
HA	5	20	47	4	5	19
FA	7	28	34	3	9	20
Leonardite (1S104H-5)						
HA	8	15	58	4	1	14

2.1.1 IHSS samples

The International Humic Substances Society (IHSS) was established to organize scientists interested in humics and to fulfill a concept of having a collection of standard humic and fulvic acids. Three solid-phase source materials and one blackwater river source were selected (Tab. 1 and Tab. 2). The procedure of isolation of these samples was carefully controlled, supervised and fully documented and nowadays is considered as the standard isolation procedure although it has been stated by IHSS that this is not meant to be a recommended or approved method, but a method that has been found to be satisfactory for most soil types and one which can be performed in most laboratories [21].

2.2 ULTRASONIC SPECTROSCOPY

Ultrasound waves are mechanical in their nature. They probe the samples by propagating through them causing oscillating compressions and decompressions. The compressions decrease the distances between neighboring molecules and force them to respond by intermolecular repulsion. The amplitude of deformations caused by analytical ultrasound waves is extremely small and therefore making the ultrasonic spectroscopy a non-destructive technique [22].

Parameters that are traditionally measured in ultrasonic spectroscopy are ultrasonic attenuation and ultrasonic velocity. Attenuation is determined by the energy losses of the ultrasonic waves due to absorption and scattering contributions. The second parameter – ultrasonic velocity – is determined by the density and the elasticity of the medium. The speed of ultrasound is proportional to rigidity of the sample and since the solids are more rigid (which also means having higher elasticity of molecules) than liquids and gases, respectively, the sound propagates through them faster than through liquids and gases. The rigidity of the material is determined by both density and compressibility but the contribution of the compressibility is in the majority of standard liquid samples the leading factor [23]. Ultrasonic velocity is extremely sensitive to the molecular organization and intermolecular interactions in the medium. The (ultra)sound velocity U is simple function of the pressure derivative of density [24]

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

where ρ stands for density, P for pressure and S is entropy. From this, following equation, where β_S adiabatic compressibility, can be deduced [23]

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

2.2.1 High Resolution Ultrasonic Spectroscopy (HRUS)

This instrumentation is an improvement of a well-known resonator method. In the resonator method the sample liquid is loaded into the cylindrical cavity between two plane-parallel ultrasonic transducers which acts as a high quality acoustical resonators whose natural frequencies f_n are linearly related to the ultrasound

velocity. The transducers are piezoelectric crystals, actually, and by applying electrical current they perform spatial change which leads to production of mechanical stress. Based on this mechanism, one of the piezotransducers excites the ultrasonic wave which then travels through the liquid in the direction of the second piezotransducer. When reaching the second piezotransducer, the wave is redirected and comes back and then is redirected again. At the frequencies corresponding to the whole number of half wavelengths between the piezotransducers, a resonance occurs. The absolute value of U is then given by this equation

$$U = 2l(f_{n+1} - f_n)(1 + \gamma)$$

where f_{n+1} and f_n are neighboring resonating frequencies, l is the distance between transmitting and receiving transducers and γ is resonator nonideality correction parameter that depends on the reflection coefficient of ultrasonic waves at transducer-liquid interfaces and on diffractive losses in the cell. The values of γ are usually below 10^{-3} [23].

HRUS, as patented by Buckin [25], consists of two independent measuring cells tempered by common water bath and stirred by electromagnetic stirrers. Ultrasonic velocity is temperature-dependent. Observed changes in measured values are therefore caused both by internal physical-chemical processes and by external fluctuations of temperature. If the second cell as a reference cell is used and is loaded only by the solvent no reactions can occur there. All the changes of measured parameters are therefore caused only by external temperature fluctuations in this cell. Because common water bath secures same temperature in both cells, it will be possible to subtract recorded values (i.e. measuring cell U_1 minus reference cell U_2 , further in text denoted as U_{12}) to obtain the values of ultrasonic velocity free of external temperature fluctuations. By this simple trick, the resolution down to 10^{-5} % for ultrasonic velocity can be achieved.

To author's best knowledge HRUS was employed just several times in the study of humic substances. In the study using lignite humic acids, Kučerík et al. [26] stated and confirmed earlier observation about progressive aggregation of humic acids in diluted solutions. Aggregation was noticed also in environment generally considered as unfavorable to aggregation, i.e. at high ionic strength (1M NaCl) and at pH as high as 12. Several modifications of humates solutions supported supramolecular theory. In another study, a change in ultrasonic velocity in solutions exposed to a temperature program revealed significant differences in character of hydration at different concentrations [20]. In 2009, Kučerík et al. [27] employed HRUS to study progressive aggregation and structural changes in both sodium salts and protonized forms of fulvic acids and sodium salts of humic acids. The method allowing the data treatment by fractal analysis was developed and tested [27],[28]. Determined dependencies of concentration increment and fractal dimension on concentration revealed that similar mechanisms of aggregation are involved, nevertheless, clear differences among individual humic and fulvic samples was observable.

2.3 FRACTALS

Most of natural objects are fractals [29]. They can be described using a fractal measure (K) and a noninteger fractal dimension (D). Fractal measure defines in practice the magnitude of the coverage of space using the elementary cell (e.g. percentage coverage), while fractal dimension describes the trend of change of coverage as a function of size of measuring cell. In other words, in case of fractals their measured metric properties, such as length or area, are a function of the scale of measurement. The empirical relationship between the measuring scale d and the length L can be expressed as

$$L(d) = Kd^{(1-D)}$$

where K is a constant (fractal measure) and D is the fractal dimension [30].

Fractal analysis is a method designated to characterize irregular geometry patterns and to quantify patterns that are seemingly chaotic and random. It can be used not only as a tool for statistical evaluations but also to predict the future and the past of the system. Mandelbrot himself applied the concept of fractal geometry to subjects as diverse as price changes and salary distributions, word frequencies in books, the statistics of errors in telephone messages, turbulence, water level fluctuations, and branching of bronchial tubes, rivers and trees [29].

Theory of fractals can provide a powerful mathematical tool to describe structure and properties of such random and heterogeneous systems as HS. The importance of fractal dimension arises from its ability to characterize fractal as a whole rather than in terms of point by point connection which is suitable for Euclidean objects since in real world experiments the objects and sets are too complex and can be measured only approximately (snowflakes, feathers, tree roots, ferns, shore coast, borders among countries, etc.). The fractal dimension D is often the only way how to quantitatively compare such objects or sets in reasonable amount of time.

2.3.1 Fractal analysis in study of HS

Fractal analysis has been used as an evaluation tool in study of humic substances several times. The most common experimental methods that are used to obtain data needed to estimate the fractal dimensions of environmental systems include scattering techniques, turbidimetry, vapor adsorption methods, reaction rate methods and microscopy. Ultrasonic spectroscopy has been used as well.

Small-angle neutron scattering technique was used to study aggregation of two humic acids in solutions with acidity corresponding to pH 5.0 and at 0.1M ionic strength. It was concluded that HA particles can be described as building blocks of a radial size $\leq 25 \text{ \AA}$ aggregated into clusters with an average radius of 400–500 \AA . Fractal dimension of $D = 2.3 \pm 0.1$ was determined [31].

Employing the small-angle X-ray scattering it was found out that fractal dimension of HA in liquid state is 1.6 while in the solid state the same material exhibits fractal dimension of 2.5. The lower fractal dimension of the sample in

solution suggests that the dissolved aquatic humus sample has a less compact, more open form than it does when it is dried [32].

In work of Rice et al. [33] it was demonstrated that fractal characterization of humic materials can be performed by using static X-ray and light scattering, and by dynamic light scattering methods. Furthermore, it was stated that humic materials are surface fractals in the solid state and mass fractals when in solution.

High resolution ultrasonic spectroscopy was employed to study aggregation and structural changes of HS solutions by Drastík et al. [28] and Kučerík et al. [27]. Humates, fulvates and fulvic acids originating from various sources (the IHSS standards) in wide range of concentrations (0.001 g/L to 3 g/L) were subject of study. Obtained results confirmed previously published statements [20],[26] about progressive aggregation of humic substances in diluted solutions, i.e. even at concentrations lower than 0.1 g/L. An attempt was paid to develop and test a method allowing the treatment of obtained data by fractal analysis. The value of fractal dimension 2.5 was determined for the concentration 3 g/L; diluting the solution caused increasing fluctuations in fractal dimension value. The increase was not the same for all samples, in fact, the most significant fluctuations at low concentration range were observed for fulvic acids. It was found out that despite the prevalence of hydrophobic forces in stabilization of humic substances in diluted solutions, also the nature of the counterion represents one of the crucial factors playing role in conformation and stability. Similarly as in case of lignite humates, it was found out that a big change in aggregates properties in concentration range from 0.1 to 1 g/L is observable.

3 AIM OF THE WORK

The physical chemistry of mixtures can be evaluated by two main approaches. The first one can use the principles of statistical thermodynamics and try to model the possible conformations and mutual interactions or to develop a model based on experimental data. Since HA represent a highly complicated mixture composed of ten thousands of different molecular masses [34] such approach would require more advanced knowledge on the primary chemistry of individual molecules as well as the deeper insight into quaternary HA structure (distribution of crystalline and amorphous domains, hydration, charge transfer between molecules etc.). Despite the advances in analytical chemistry reached in last decades that knowledge is still far to be satisfactorily and practically employable. The advantage of the second, fractal, approach was indicated in previous paragraphs. Methods of fractal analysis offer the possibility to avoid the troubles with lack of knowledge and known parameters. In fact, fractal dimension represents an added value to the traditional statistics since it reveals a trend which a system undergoes.

In this work, humic substances of different origin will be used to cover the widest spectrum of possible diversity occurring in the nature. To reveal the potential role of

different counterions, the physical structure of sodium salts of humic acids and both Na- and H-form of fulvic acids will be studied.

Different methods have been already employed in study of humic substances. However, most of them suffer in limitations regarding experimental conditions i.e. concentration range; temperatures range; pH and ionic strength applicable; impossibility or high difficulty of changing temperature or/and concentration during the measurement; high financial demands etc. Most of these problems can be overcome by High Resolution Ultrasonic Spectroscopy and therefore it is the main method of choice in this study. Obtained data would serve to develop the fractal description of behavior of humic substances in the broad range of concentrations and under various conditions.

An important part of the study will be devoted to the combination of data obtained from HRUS with primary composition such as elemental analysis and NMR results. An attempt will be paid to find possible correlation between primary characteristic of the samples (i.e. elemental composition and functional groups distribution) and parameters obtained from measured ultrasonic data. On the basis of established information the attempt to discuss mechanism of aggregation and relations between aggregation of HS (physical structure) and composition (primary structure) will be made.

4 OVERVIEW OF RESULTS AND DISCUSSION

4.1 IHSS SAMPLES – ISOTHERMAL MEASUREMENTS

In the literature several approaches to study character of humic aggregates in solutions were published. The obvious limitations of those works are mainly in the applied instrumentation. None of applied techniques except HRUS allows the study of physical character of humic solutions in such wide range of concentrations and under such variable conditions. In this part of thesis it is assumed that one of the important aspects, which can help to elucidate the humic aggregation, is the change of the physical properties of investigated solutions. In fact, the drop-wise increase in concentration is connected with formation of new intermolecular interactions among humic molecules and with increase in total surface area of humic aggregates exposed to solvent and thus with change in hydration of aggregates. Those parameters are responsible for change in ultrasonic velocity and therefore they are detectable by HRUS. The main point in this issue represents the change in compressibility and density. That can be used for determination of respective fractal dimension of aggregates which reflects the arrangement in their physical structure. Apparently that approach is not able to answer all the issues associated with humic acids conformation but it is a promising approach in determination of evolution of their aggregates. That information is important in understand of interaction of dissolved organic matter, their response to external influences and secondary also to carbon sequestration and protection of humified substrata.

4.1.1 Experimental part

HS samples were purchased from the IHSS. All four HA and three FA standards were delivered as powder in their H-form. Standards were converted to sodium salts (NaHA, NaFA) by titration with 0.1M NaOH. After reaching constant pH value 7.2 and keeping it stirred for next 60 minutes, the samples were freeze-dried. Ten HS samples were prepared altogether (4 NaHA, 3 FA and 3 NaFA). The stock solution of 10 g/L was prepared in distilled water and well stirred for at least 24 hours before preparation of other solutions. Stirring continued also during the experimental usage. Additional stock solutions with lower concentration (1 and 5 g/L) were prepared by dilution of stock solution several hours before measurement and stirred throughout their usage.

For demonstration of HRUS application potential, the commercial nonionic surfactant Triton® X-100 was used as a standard compound exhibiting critical micelle concentration. The stock solution was prepared and used in the similar way as humic and fulvic solutions.

HRUS 102 device was employed to measure the velocity of ultrasonic wave propagation. All measurements were carried out at 25.00 ± 0.02 °C and under constant stirring (600 rpm). Ultrasound frequencies in range from 2300 to 14700 kHz were utilized. Both cells of HRUS were loaded up by 1 mL of deionized and degassed water. In the cell 1, the solution of respective HS was added by Hamilton syringe stepwise every 10 min (i.e. when constant values of ultrasound velocity were achieved and recorded) using appropriate combination of stock solutions to reach desired concentrations in range from 0.001 to 3.5 g/L. The ultrasonic velocity (U) was measured in both cells. Obtained data were processed using the relation published earlier by Kankia et al. [35] and as in detail described by Kučerík et al. when applied on HS [26] i.e.

$$I = \frac{U_1 - U_2}{U_2 m \rho_2}$$

where U_1 and U_2 are the values of ultrasonic velocity in solution (cell 1) and pure solvent – water (cell 2) (the difference denoted as U_{12}), respectively, m stands for the weight concentration of the solute and ρ_2 is the density of the pure solvent at 25 °C.

4.1.2 Results and discussion

In Fig. 1 the decreasing dependence of the concentration increment of ultrasonic velocity (I) on concentration can be observed for all humates. The records of both fulvates and fulvic acids looked fairly similar – same trends were noticed. These observations indicate aggregation at concentrations much lower than those typically reported for CMC of humic substances. That conclusion was based on comparison to Triton® X-100 (and other surfactants) which exhibited constant increment of ultrasonic velocity for concentrations below CMC and a decrease after reaching CMC. Furthermore, since all applied frequencies gave the same values of ultrasonic velocity it can be assumed that the character of aggregates, i.e. size and/or

compressibility of the hydrophobic interior does not interfere with frequency applied in reported measurements. This is based on the fact that the ultrasonic wave is capable of significant mechanical compression of soft interiors of micelles and spherical aggregates. If the micelles or aggregates presented in the solution have the compression relaxation time higher than the time proportional to the frequency applied for the measurement, the change in ultrasonic velocity in dependency on frequency should be seen. Nevertheless, this was not the case of any humic sample.

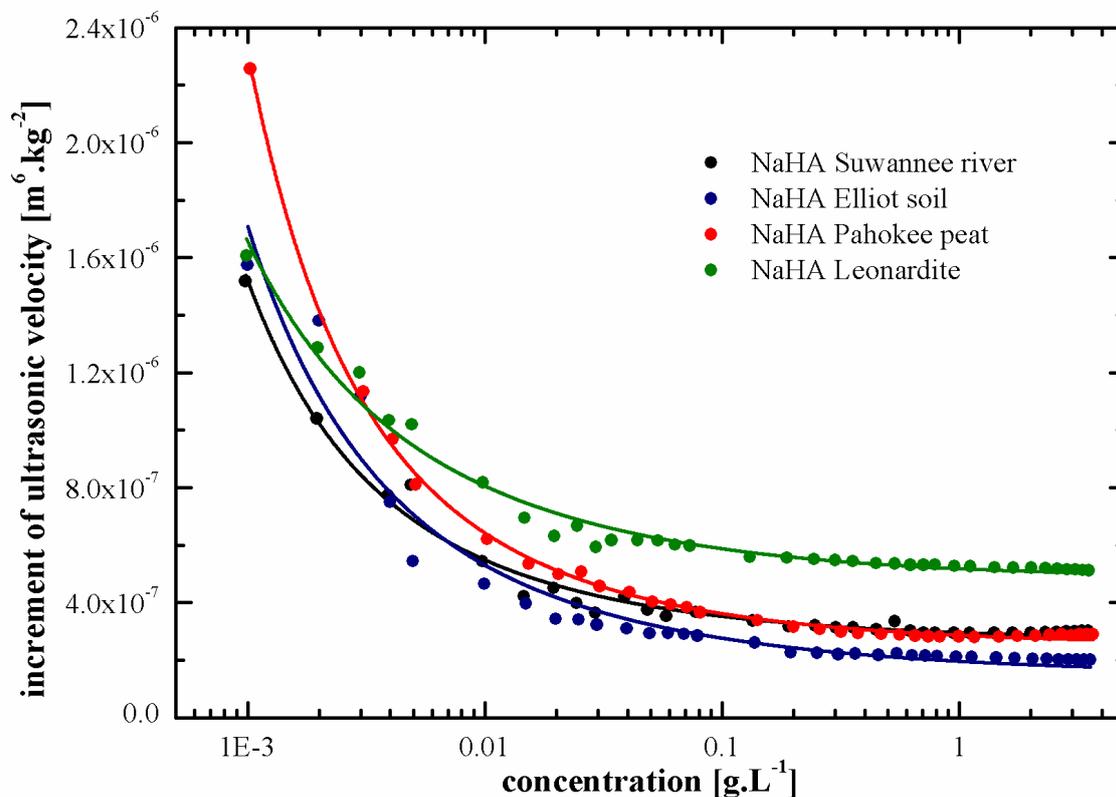


Fig. 1: Dependency of increment of ultrasonic velocity on concentration – humates.

An attempt was paid to find the best mathematical fitting of measured data as indicated in Fig. 1. This fitting can be expressed by this equation

$$y = ax^{bx^{-c}}$$

where y stands for concentration increment of ultrasonic velocity (I in $\text{m}^6.\text{kg}^{-2}$), x for sample concentration (in g/L) and a , b and c are adjustable parameters. This function was chosen from a number of fitting functions served by Origin software as the best because it provided the lowest “Chi factor”.

To shed light on the principles of aggregation, parameters a , b and c were correlated with elemental analysis and results of distribution of carbon in humic molecules as determined by solid state NMR using linear regression least square method; i.e. C, H, O, N, amount of carbon in carboxylic, aromatic and aliphatic groups and C/O, C/H and also aromatic to aliphatic carbon ratios (data taken from the IHSS web site). Correlation analyses showed that NaHA Leonardite behaved in different manner. That was probably caused by very different origin and/or way of genesis of this sample in comparison with the rest. Because of that reason data for NaHA Leonardite were omitted in this part of work. Obtained results are

summarized in Tab. 3. It is necessary to point out that correlation analysis was done to see mainly the mutual relationship between correlated parameters, the reliability of trends and to observe differences between samples, therefore, the coefficient of determination (R^2) is used as an indicator of coherence.

Tab. 3: Correlation of fitting parameters with composition of humic substances represented by coefficient of determination (R^2) (C. C – Carboxyl carbon, Ar. C – Aromatic carbon, Al. C – Aliphatic carbon, Ar/Al C – Aromatic to aliphatic carbon ratio).

	C	H	O	N	C/O	C/H	C. C	Ar. C	Al. C	Ar/Al C
<i>a</i>	0.685	0.461	0.171	0.005	0.426	0.643	0.081	0.330	0.102	0.335
<i>b</i>	0.661	0.433	0.509	0.522	0.702	0.625	0.009	0.639	0.589	0.788
<i>c</i>	0.451	0.489	0.248	0.590	0.421	0.535	0.013	0.519	0.723	0.681
$a \times b$	0.977	0.526	0.596	0.897	0.849	0.867	0.001	0.570	0.794	0.961
$a \times c$	0.777	0.670	0.781	0.273	0.537	0.818	0.299	0.536	0.395	0.610
$b \times c$	0.797	0.495	0.664	0.603	0.837	0.730	0.370	0.797	0.638	0.908
a^b	0.353	0.784	0.000	0.604	0.711	0.646	0.604	0.534	0.933	0.754
$(a^b)^{-c}$	0.828	0.521	0.618	0.558	0.841	0.762	0.011	0.793	0.617	0.903
b^{-c}	0.346	0.538	0.148	0.835	0.285	0.490	0.072	0.676	0.960	0.720

It was found out that parameters obtained from fitting correlate quite well with C content and C/H ratio (Tab. 3). Other humic composition characteristics did not show any remarkable R^2 values. Greater correlations were observed when correlated empiric parameters in different combinations as multiplied and powered, i.e. $a \times b$, $a \times c$, $b \times c$, a^b , $(a^b)^{-c}$ and b^{-c} . Generally, best correlation was achieved for C/H and aromatic to aliphatic carbon ratios followed by carbon content and C/O ratio.

Based on papers by Zmeškal et al. [36],[37] and [38] the fractal analysis approach was developed and tested. The final derived equation

$$U_1 - U_2 = \sqrt{\frac{k_v K}{D(D-1)}} \left(\frac{c}{k_c K} \right)^{\frac{D-1}{2(D-3)}}$$

was formally rewritten as

$$U_1 - U_2 = A c^a$$

and fractal dimension was expressed

$$D \approx \frac{6a-1}{2a-1}$$

In Fig. 2 a typical concentration behavior of dependence of fractal dimension and several additional parameters which were calculated utilizing above-stated mathematical derivations are reported. All dependences indicate that this approach is useful for concentrations down to 0.03 g/L. The meaning of D is a “measure of changes” and reveals the mechanisms of aggregation. Around 1 g/L it seems that the constant value $D \approx 2.5$ is reached. That suggests the switch in mechanisms of aggregation similarly as reported in [20].

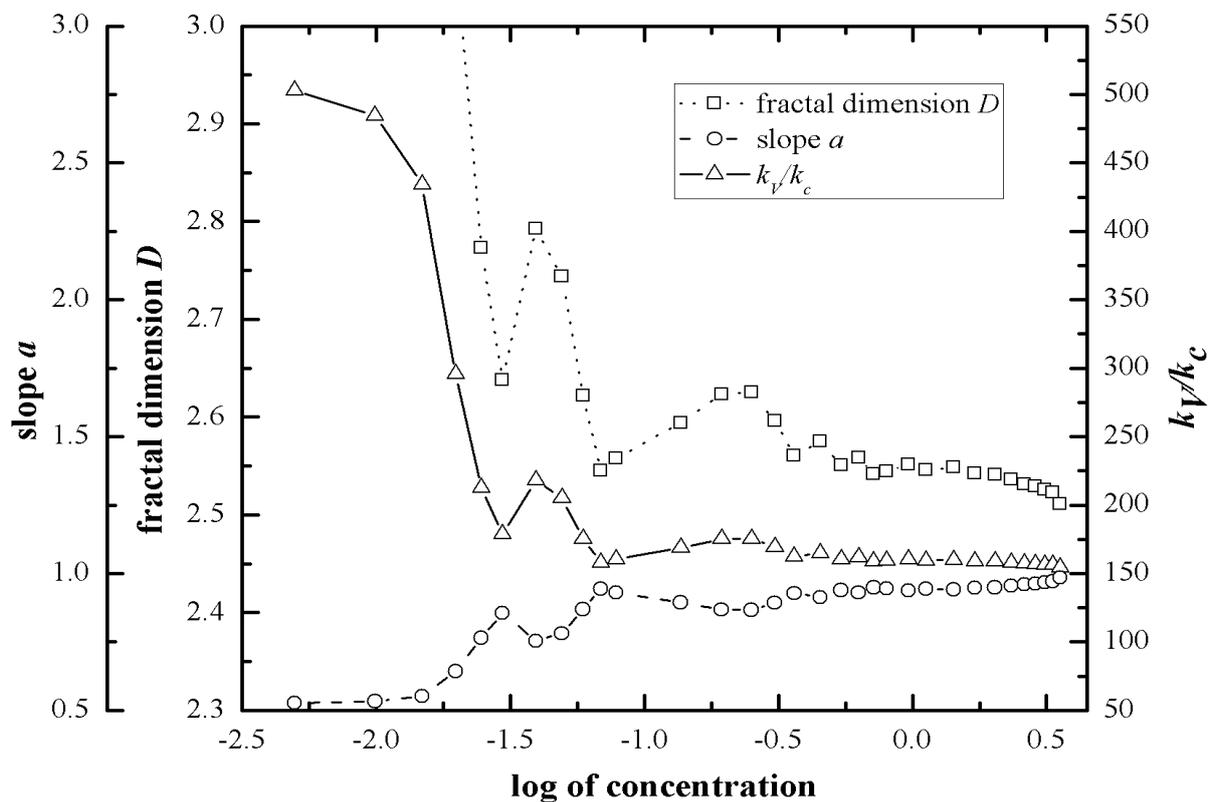


Fig. 2: Dependence of coefficients D (fractal dimension), slope a and k_v/k_c on the concentration for NaFA Elliot Soil.

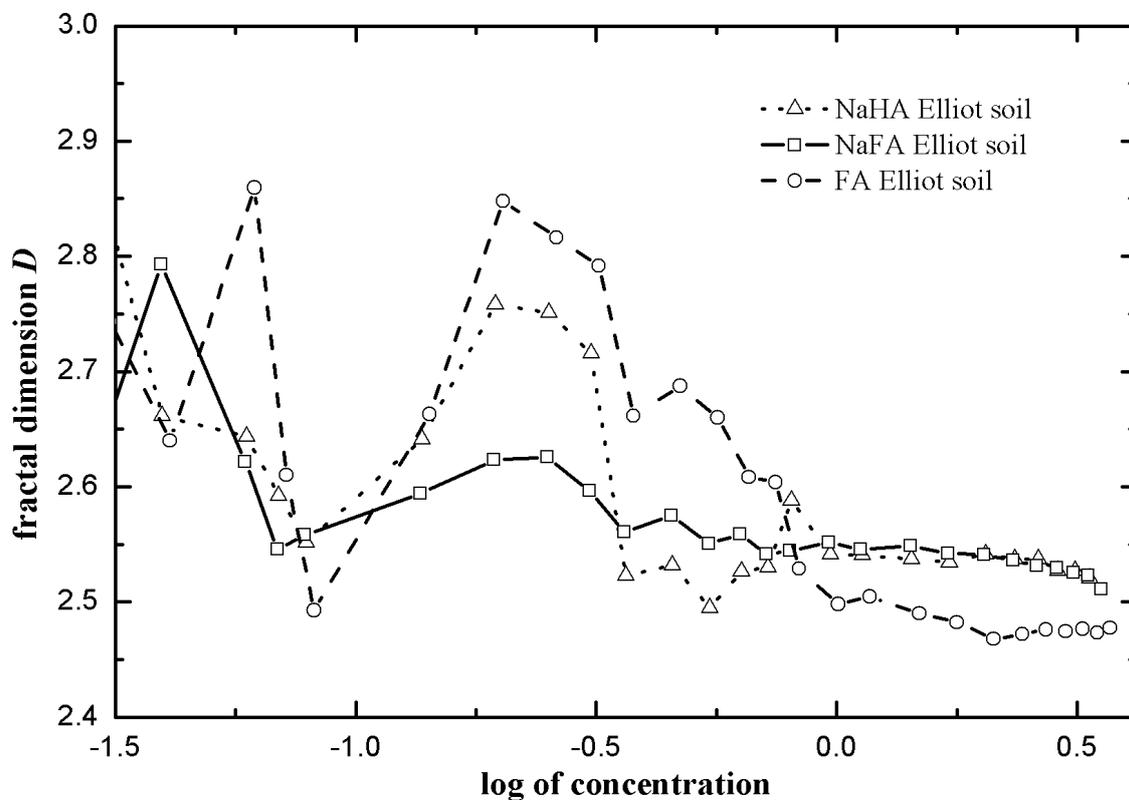


Fig. 3: Comparison of fractal dimensions of Elliott Soil humate, fulvate and fulvic acid.

Fig. 3 reports the comparison of fractal dimensions among fulvic acid, sodium fulvate and sodium humate. Dependency clearly shows that the introduction of Na^+ ion into the fulvic structure changes the aggregation profile of the sample. As can be seen, the D profile is relatively stable at higher concentration and fluctuates only slightly. But the dilution brings about significant changes mainly in case of H^+ form of FA. The fractal dimension in Na^+ fulvate solution slowly increases with only weak fluctuation. It seems that the presence of the Na^+ cation causes stabilization of the structure and steady formation of the structure. However, this is not truth in the case of humic acids where fluctuations similar to non-nitrated FA were observed. It can be hypothesized that the H^+ in fulvic acids is due to its dimension less capable to compensate the mutual repulsion of polar groups such as mainly deprotonized carboxylic groups. Therefore, with increasing concentration the weak interactions stabilizing system must constantly change their geometry and consequently the conformation of whole supramolecular system. In work of Kučerík et al. [20] it was stated that humic substances are stabilized mainly by hydrophobic interactions in diluted systems. It is well known that those interactions are strongly dependent on their mutual orientation. In contrast, the interaction of cation with other moieties can be seen as a charge interaction which has no orientation demand. As a result the stabilization of the system is more efficient by Na^+ than by small H^+ .

4.1.3 Linear approach

The main purpose of using concentration increment of ultrasonic velocity in previous paragraphs was to process data and make the changes of ultrasonic velocity more visible. The very same approach was applied also on data of tetradecyltrimethylammonium bromide (TTAB) in both pure water and 0.15M NaBr solution. The dependency of concentration increment on concentration of the aqueous TTAB solution looks the same way as in the case of Triton® X-100 and other surfactants. In short, interval of constant dependency corresponds to premicellar state since the change of ultrasonic velocity is linearly proportional to concentration. But when CMC was reached an intensive change of increment was observed. This is caused by sudden presence of micelles for which very compressible conformation is typical. In additional experiments 0.15M NaBr solution of TTAB sample was prepared to simulate strong ionic environment. Similar results were expected. Nevertheless, the decreasing change of increment was observed even at concentrations under CMC and no constant dependency was recorded even at the lowest concentrations.

An attempt was paid to elucidate this behavior. The cause was found to be in the mathematical behavior of reciprocal function. In the interval of constant increment this equation must be valid

$$I = \frac{U_1 - U_2}{c} = k$$

where k represents a constant. When rewriting this to

$$U_1 - U_2 = k \cdot c$$

it is clear that the difference of ultrasonic velocity must be a straight line passing through the zero under some slope. In reality, no such observations were done and to some extent a shift was always present, in the following equation represented by adding an intercept parameter e

$$U_1 - U_2 = k \cdot c + e$$

Dividing both sides by concentration, concentration increment is expressed on the left side

$$I = \frac{U_1 - U_2}{c} = k + \frac{e}{c}$$

The fraction e/c can be very large, especially when assessing very low concentrations and more importantly, can totally overlap the constant part and the decreasing trend typical for over-CMC concentration can be then observable for concentrations far beyond the real CMC [39]. Nevertheless, when dependence of difference in ultrasonic velocity on concentration was used to assess the CMC a sharp edge was observed at CMC for TTAB dissolved both in water and in 0.15M NaBr. This example clearly demonstrates the inability of the concentration increment to be a precise guideline for CMC determination under every situation.

When applied this linear approach on the IHSS standards data very high degree of agreement between data and their linear regression was observed. Coefficient of determination very close to 1 was calculated for all samples. It was noticed that slopes of linear regression abscissas calculated for protonated fulvic acids were smaller when compared with their Na-form and also with humates. Smaller increase in slope means that the sample's environment is less supportive to the spread of ultrasound waves. Higher slope in case of NaFA can be attributed predominantly to the presence of the Na^+ ion which is only a little bit compressible and is surrounded by a big hydration shell. Both these factors enhance the ultrasonic velocity. On the other hand, no clear trend was observed for the corresponding pairs of humate and fulvate. The extremely high slope of the NaHA Leonardite sample cannot be attributed solely to the hydration shell since no extremely spacious shells can be expected due to the low content of polar groups. A possible explanation might be the extraordinary high content of aromatic moieties which are almost incompressible resulting in high values of ultrasonic velocity.

The biggest advantage of this approach is that original data are used and therefore one can be sure that no artifacts connected to mathematical manipulations are involved. On the other hand, tiny curvatures can be easily overlooked. The situation is reverse in the case of concentration increment. The advantage is that even small changes can be significantly emphasized. But in some cases this might lead to the situation when small fluctuations caused by imperfection of the device are overestimated and misinterpreted.

4.1.4 Conclusions

- All humic substances under study exhibited aggregation at concentrations as low as 0.001 g/L.

- Fulvic acids and fulvates showed similar aggregation patterns as humates.
- Fulvate solutions were more supportive to ultrasound propagation, most likely due to presence of Na^+ which led to strengthening of hydration shell.
- Stability of aggregates can be enhanced by presence of Na^+ ions which compensate the carboxylic group repulsions.
- Power-based fitting can be applied on all samples under study.
- Empirical parameters gained by non-linear fitting correlate quite well with C content and C/H ratio.
- Combinations of gained parameters correlated significantly with C content and C/H, C/O and aromatic to aliphatic carbon ratios.
- HRUS is a unique method for acquiring data needed for fractal analysis since it allows run experiments in wide range of concentrations and variable conditions.
- Fractal analysis brought another evidences that the nature of prevailing interactions holding together humic assemblies is dramatically changed under the concentration 1 g/L.
- Developed fractal analysis is applicable on data down to concentration 0.03 g/L.
- At concentrations higher than 1 g/L almost constant value of fractal dimension ($D \approx 2.5$) is reached.
- Using of concentration increment might introduce big mistakes when not applied with caution.
- Linear approach represents an alternative way how to evaluate data gained by HRUS.

4.2 IHSS SAMPLES – NON-ISOTHERMAL MEASUREMENTS

In this part of the work the stability of aggregates was studied. In principle, heat is used as a probe to investigate the stability of intermolecular interaction, hydrophobic effect responsible for humic molecular assembling and hydration shell surrounding both hydrophilic and hydrophobic parts of aggregates. Stability, both thermodynamic and kinetic, is a key factor reflecting the nature of humic assemblies and it is supposed to reflect their primary composition, reactivity and genesis. In fact, elevated temperatures are responsible for higher amplitude of vibration of weak interaction stabilizing humic aggregates and for weakening of interactions between water and polar moieties. As shown recently, elevated temperatures caused several transitions in lignite humates solutions which were attributed to breaking of specific bonds followed by aggregates reformation or destruction of their physical structure. The main aim of this part is i) to repeat recent experiments and confirm the behavior of humic aggregates at elevated temperatures using samples originating from various sources, ii) to confirm the presence of aggregates formed at low concentrations, iii) to try to attribute the transitions to specific interactions and iv) to

find a possible relationship between primary composition of tested humic substances and their response to thermal treatment.

4.2.1 Experimental part

Sodium salts of the IHSS standards were prepared in the same way as described in the preceding part. Solutions of 0.01, 0.1, 1 and 4 g/L were prepared in distilled water and well stirred.

HRUS 102 device was employed. Cell 1 served as a sample cell and was loaded up by 1 ml of the sample solution whereas cell 2 was a reference cell and was loaded up by 1 ml of degassed distilled water. All measurement set ups were performed at 25.00 ± 0.02 °C and at initial ultrasound frequency of 5480 kHz. Intensive stirring (600 rpm) was enabled. Temperature regime was as follows: step 1 – from 25 °C to 90 °C for 3 hours then down 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 then back to initial 25 °C for 3 hours. It follows that the heating and cooling rates were about 0.36 °C/min.

4.2.2 Results and Discussion

The speed of sound in liquids can be expressed as

$$U^2 = \frac{1}{\rho \beta_s}$$

where U stands for sound velocity (m/s), ρ for density (kg/m^3) and β_s ($\text{m}\cdot\text{s}^2/\text{kg}$) for adiabatic compressibility. Both density and adiabatic compressibility are influenced by temperature among others. In case of water the impact of temperature is enormous and, in comparison with other liquids, it is non-linear. At low temperatures both compressibility and density are high and therefore lower values of sound velocity are observed. As the temperature increases the compressibility drops and goes through a minimum whereas the density goes through a maximum and then drops. Combination of these two properties leads to the maximum in the speed of sound at about 74 °C.

The differential arrangement of the HRUS device allowed avoiding the influence of anomalous behavior of bulk (non-interacting) water molecules by simple subtraction of a reference. Depicted curves are free of this influence and represent the response only of the sample and its hydration shell. An example of temperature dependence in the whole temperature regime is given in Fig. 4.

The whole temperature program involved 3 steps. The first heating cycle (from 0 to 420 min in Fig. 4) was used to adjust humate and fulvate solutions to have same ‘thermal history’ and to ensure the complete dissolution of samples. The first part of second heating cycle (from 420 to 660 min in Fig. 4) was used for monitoring of changes in humic solutions induced by heat. And finally, the third cycle served as a control of the reproducibility. As can be seen, all three temperature steps gave similar result which can be concluded that only the physical (i.e. reversible) and no

chemical changes of the humate and fulvate structure occurred in the temperature interval from 5 to 90 °C.

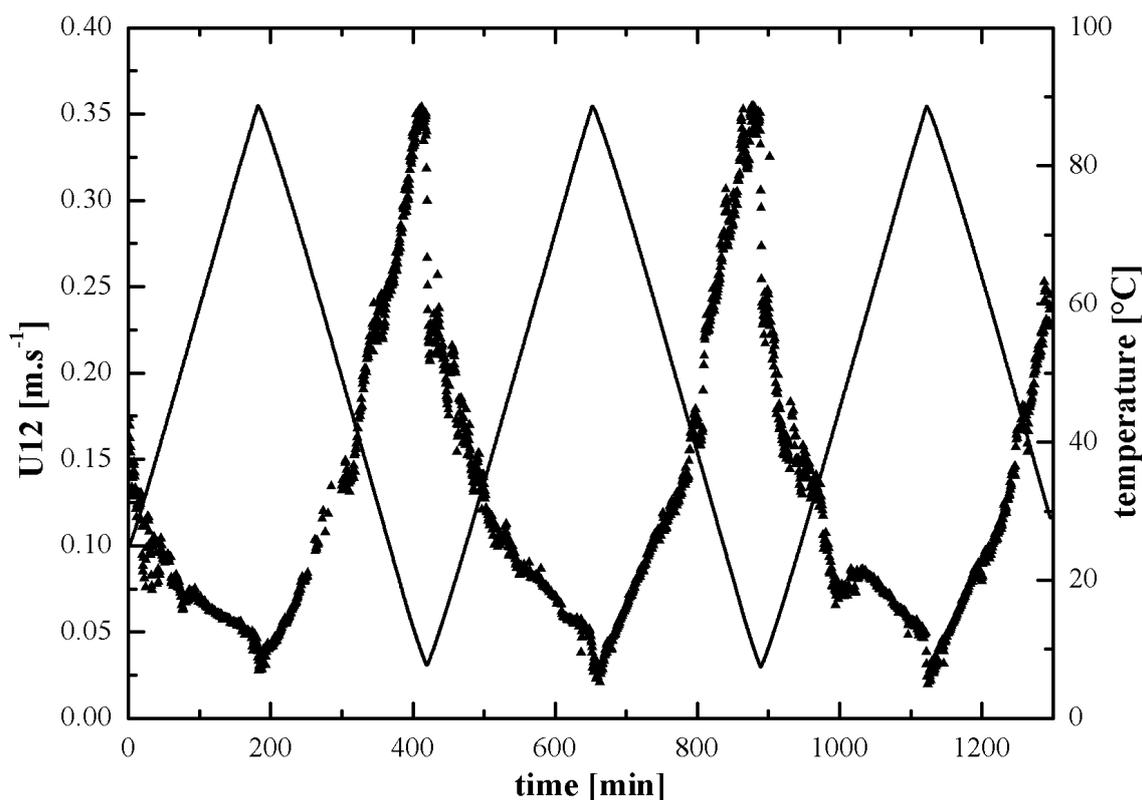


Fig. 4: Difference in ultrasonic velocity (sample–water) and temperature program (NaHA Suwannee River, 1 g/L).

The record of samples at concentration 4 g/L showed more or less a monotonous exponential-like decrease of U_{12} with increasing temperature. At this relatively high concentration, aggregates with mostly hydrophilic surface are present in solution. This surface is surrounded by hydration shell that is due to its smaller compressibility more supportive for ultrasonic wave propagation than bulk water. With increasing temperature the relative static permittivity of water decreases which leads to the hydration shell weakening and therefore U_{12} decreases. Furthermore, at higher temperatures the hydrophilicity of some amphiphilic head-groups decreases which results in additional weakening of aggregate protecting hydration shell. On the other hand, in the core of aggregates the strength of the attractive hydrophobic interactions among aggregating humic molecules slightly increases with increasing temperature. A shorter distance between humic molecules results in higher density and lower compressibility. In addition, a decrease in mutual repulsion of charged head-groups induced by elevated temperature can be expected as well.

At concentration 1 g/L samples still showed a descendent trend but the dependencies are not as monotonous as for higher concentrations. Two substantial breaks at about 20 and 42 °C occurred accompanied by several minor breaks along the whole record (Fig. 5). At this concentration humates and fulvates still form aggregates with hydrophilic exterior but with lower stability caused probably by temperature-induced hydration shell weakening and by a decrease of number of

weak interactions stabilizing aggregates. Increasing temperature causes their destabilization and series of collapses can be identified on the record as a wave-like shape of the curve. These conclusions are in line with recent statements of Conte et al. [40] who demonstrated that diluting of humates was accompanied by a decrease in the number of relatively stable H-bonds and humic molecules are loosely bound predominantly by hydrophobic interactions such as CH- π and π - π interactions or van der Waals forces.

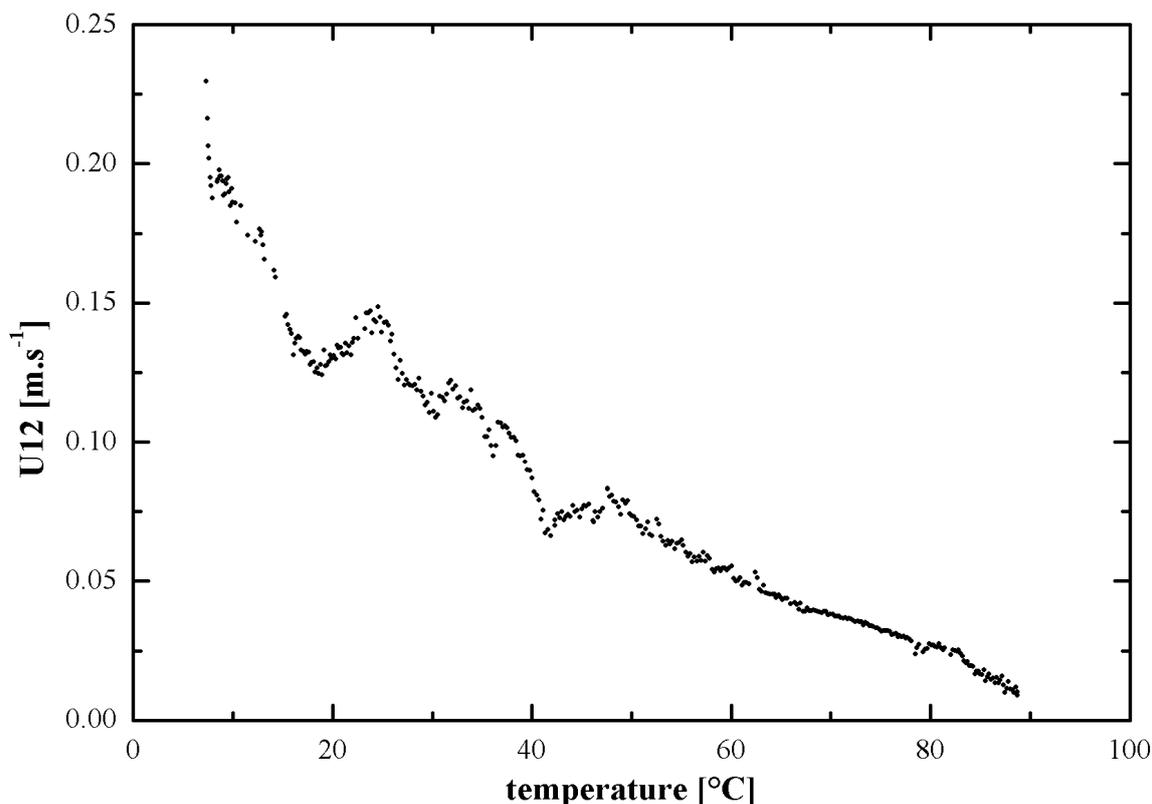


Fig. 5: Difference in ultrasonic velocity for NaFA Suwannee River (2S101F), 1 g/L.

Further dilution below concentration of 1 g/L makes the situation completely different as it can be easily identified in the slope of dependency of U_{12} on temperature. At these concentrations the hydrophobic hydration starts to play more significant role in aggregate formation and stabilization. In fact, there does not exist any edge concentration at which a switch from hydrophilic to hydrophobic hydration occurs; instead the change is gradual and takes place in the concentration range approximately from 0.1 to 1 g/L. At concentration 0.1 g/L (Fig. 6) and below it, obtained records showed increase of U_{12} with increasing temperature. Several breaks disrupted monotonousness of the recorded increase, mainly at lower temperatures.

The dilution of humates solutions to concentration 0.1 g/L leads to formation of aggregates with predominantly hydrophobic exterior, the disintegration of big aggregates into small ones or even single molecules is associated with the increase of number of water molecules in the so-called hydrophobic hydration shells. With increasing temperature, the relative static permittivity of water decreases which is supportive for hydrophobic hydration thus the hydration shell strengthens with increasing temperature. The enhancement of hydrophobic hydration shells then leads

to increase of U_{12} . The origin of breaks which appeared in records was attributed to weakening of weak interactions among involved molecules stabilizing the whole supramolecular structure.

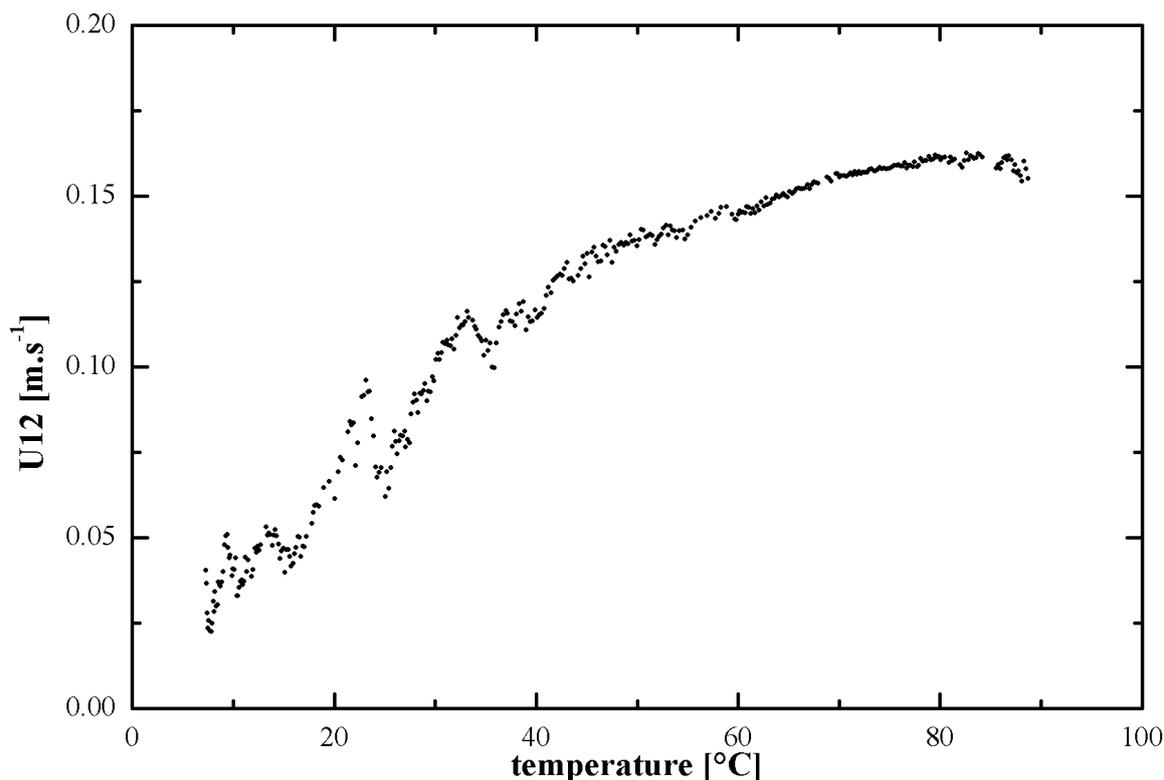


Fig. 6: Difference in ultrasonic velocity for NaFA Suwannee River (2S101F), 0.1 g/L.

With further dilution to 0.01 g/L the situation remains similar as described for 0.1 g/L. It is necessary to point out that the records of other humic substances, both humates and fulvates, behaved in the same manner confirming the knowledge about aggregation or presence of premicellar aggregates in diluted humate solutions. The only difference was observed in position of temperatures of transitions (breaks) and slope of the decrease or increase of U_{12} in dependency on temperature.

4.2.3 Conclusions

- Results obtained by HRUS revealed similarities and differences in ultrasonic records for all samples.
- Only reversible processes occurred in the temperature interval between 5 to 90°C.
- At the same concentration, samples originating from different sources showed similar trends, nevertheless, some easily distinguishable differences were observed.
- Observed differences may be explained by differences in the samples origin.
- Diluted and concentrated samples showed completely different records.
- At higher concentrations the difference of ultrasound velocity showed decreasing tendency with increasing temperature which was explained as a

dominance of hydrophilic hydration which is reduced by higher temperatures.

- At lower concentrations the change of difference of ultrasound velocity was positive with increasing temperature which was attributed to the prevalence of hydrophobic hydration and its enhancing by the increase of temperature.
- Summarizing foregoing statements by other words – at high concentrations the humic aggregates are stabilized mainly by H-bonds but at low concentrations the aggregates and/or single molecules are loosely bound predominantly via hydrophobic interactions.

4.3 SOIL SAMPLES – ISOTHERMAL MEASUREMENTS AND HYDRATION

In previous paragraphs, it was shown that all IHSS standard samples originating from different sources exhibit similar aggregation behavior; when studied by ultrasonic spectroscopy, it can be fitted by a power-based equation. Surprisingly, the fitting parameters, and especially their combinations, showed correlation with primary structure of the IHSS humic substances represented by their elemental composition and distribution of carbon in functional groups. It was also demonstrated that data obtained from HRUS measurement can be processed by a specially developed fractal analysis method. Nevertheless, it was stated that more samples covering different environments are in need to prove the correctness and applicability of developed fractal analysis approach. Therefore, in this part of work samples originating predominantly from bohemian forests were chosen. The reason for selection of such samples was their chemical composition which covers a wide range of aromaticity degree, offering a broader potential for statistically more significant correlations than samples with only narrow range of the values. Thus, the purpose of the following chapter is to continue in research described in our previous work using different set of samples and also extend the understanding of the fractal dimension parameter and its connection with the self-assembling processes taking place in the humic solutions. Furthermore, attempt will be paid to study the extent of hydration shells of humic substances to support and extend the knowledge of their stabilities and conformation postulated in previous chapter.

4.3.1 Experimental part

Humic and fulvic acids were isolated from individual soil horizons of long-term research stands. Several samples originated mainly from human activities untouched sites; two samples originated from horizon O, one from horizon A and one from horizon B. Another 5 soil samples were used as well but since data of elemental and functional groups analysis were missing in the time of writing of this thesis, the HRUS results were not included to statistical evaluation. The exceptions are Fluka HA, purchased from Sigma-Aldrich, and Oxyhumolite, Bilina which represents a

low-rank coal mined in North Bohemia. Soil samples were air-dried and sieved (<2 mm). Gained fine soil was decalcified by 0.1M hydrochloric acid. HA and FA were extracted using 0.1M NaOH at a solution/soil ratio of 20:1. FA was isolated from the mother liquor after HA precipitation using the standard method with a XAD8 resin column. The sodium humate and fulvate were converted into H⁺ form and freeze-dried.

Quantitative ¹³C liquid-state nuclear magnetic resonance measurements were performed. Quantification was achieved by instrumental integration of NMR peaks. The chemical shift regions 0–45, 45–108, 108–160 and 160–220 ppm were assigned to alkyl carbon (C), O-alkyl C, aromatic C, and carboxylic C, respectively [41]. Aromaticity was calculated as the ratio between aromatic and the sum of aromatic plus aliphatic (alkyl + O-alkyl) carbons [42].

The very same HRUS measurement set up as described in 4.1.1 was applied.

To measure the densities of the samples, high-precision (the repeatability standard deviation given by the manufacturer is 10×10^{-6} g/mL) oscillatory density meter Anton Paar DMA 4500 was employed. Sample solutions in concentration range from 0.003 to at least 1.1 g/L (in some cases up to 3.5 g/L) were prepared. Before injection into the device all samples were well-shaken and degassed. When loaded about 2.5 mL of the sample, the solution was let to be tempered by the device to 25 °C. 30 seconds after reaching desired temperature, the density was measured. Measurements were carried out for five times. Average value was used to perform subsequent calculations. The average of density standard deviations for all samples and concentrations was 4.2×10^{-6} g/mL and never exceeded 11.6×10^{-6} g/mL for individual concentration.

4.3.2 Results and Discussion – HRUS

Primary structure characteristics of all samples are summarized in Tab 4. In comparison with IHSS standard samples (Tab. 1 and Tab. 2) and with average values of hundreds of HS samples [6], the majority of composition parameters of each sample fit the common limits. Nevertheless, some exceptions can be seen. For example, the hydrogen content in the Bohemian Forest, Trojmezí (T55HK) sample is very low and is partly compensated by unusually high amount of oxygen. This leads to the highest C/H ratio of all samples. The C/H ratio is frequently used for quick evaluation of the aromaticity/aliphaticity degree of humic samples. In fact, the larger value, the higher is the overall aromaticity and the lower is the aliphaticity of the sample [7],[43]. However, this approach has been questioned since it does not consider the contribution of carbon-to-oxygen double bonds to the C/H ratio (both -COOH and -HC=CH- have the same C/H ratio). The presence of a high concentration of -COOH groups would adversely affect the use of C/H as a measure of -HC=CH- groups [6]. This seems to be the situation of this sample. As can be seen in the Tab. 4, high content of oxygen is responsible for relatively high content of carboxylic groups (above-average 21 % of carbon is part of -COOH groups). As revealed by the NMR analysis, the aromaticity is much lower than expected

according to the C/H ratio and, in fact, just slightly higher in comparison to the rest of the samples (except for the oxyhumolite sample because of its completely different origin). The C/O ratio reflects the degree of carbon oxidation; the higher C/O value the lower is the oxidation degree. As already mentioned, the amount of oxygen in this sample is very high. Consequently, the C/O ratio is very low reminiscent the values typical for FA (compare the oxygen and C/O ratio with the FA sample Ore Mts., Alžbětinka (A65FK)).

Tab. 4: Primary structure characteristics; C, H, N, O expressed as atomic percent of dry, ash-free sample; ash expressed as weight percent of inorganic residue in a dry sample; Car. C – Carboxylic carbon in %; Arom. – Aromaticity in %.

	C	H	O	N	C/O	C/H	Ash	Car. C	Arom.
Fluka HA (XF150HK), purified									
HA	41.1	32.4	25.9	0.6	1.6	1.3	4.8	13.8	34.6
Bohemian Forest, Trojmezí (T55HK), mountain spruce forest, Soil, podzol, A horizon									
HA	41.2	24.6	32.6	1.6	1.3	1.7	2.8	21.0	37.0
Bohemian Forest, Boubín (B2HK) mountain spruce forest, Soil, Spodo-Dystric Cambisol, Of horizon									
HA	34.0	41.0	23.5	1.4	1.4	0.8	2.8	17.4	30.2
Oxyhumolite, Bílina (XM1HK)									
HA	40.6	34.5	24.0	0.8	1.7	1.2	3.4	13.0	60.1
Bohemian Forest, Trojmezí (T15HK), mountain spruce forest, Soil, podzol, Ol horizon									
HA	34.9	43.0	20.6	1.5	1.7	0.8	1.7	14.4	23.7
Ore Mts., Alžbětinka (A65FK), mountain spruce forest, Soil, podzol, Bh horizon									
FA	35.2	35.5	28.5	0.8	1.2	1.0	8.2	24.4	43.0

In contrast, the C/H ratio rule can be applied on the Bohemian Forest, Trojmezí (T15HK) sample. High hydrogen content is responsible for very low C/H ratio indicating high level of aliphaticity and low level of aromaticity. As implies high C/O ratio and low carboxylic carbon content, the -COOH groups do not interfere with the C/H ratio. And indeed, as revealed by the NMR analysis, the aromaticity was calculated to only 23.7 %, the lowest degree of all samples. Furthermore, this sample clearly shows the truthfulness of the first paragraph of this chapter. Although it originates from the same place as the Bohemian Forest, Trojmezí (T55HK) sample (which comes from just a few centimeters bigger depth), the chemical composition and primary structure is extremely different.

Sample Oxyhumolite, Bílina (XM1HK) completely differs from the rest of the samples. High amounts of carbon, low of oxygen and carboxylic carbon and high aromaticity can be easily attributed the origin of the sample – coal strip mine Bílina, the Czech Republic.

Summarizing previous paragraphs, one must take in mind that none of the mentioned parameters is capable to characterize humic sample comprehensively. All parameters must be taken into consideration when interpreting measured data and attributing properties. As outlined upwards even the origin of the sample takes important role. Not to mention that sampling and isolation can alter the sample significantly.

The purpose of this part is to extend and support the results presented in 4.1 part of thesis which suggested the correlation between chemical composition of sodium humates and fulvate and their aggregation behavior and its representation by fractal dimensions. Similarly to the 4.1 part, HRUS was used to observe the aggregation behavior and same power-based fitting was applied. It was noticed that values of parameters a , b and c were in the same order of when compared with those of IHSS samples. Furthermore, trends similar to those depicted in Fig. 1 were observed for all samples. This leads to the conclusions that similar mechanisms of aggregation can be expected and that self-assembling of the molecules dissolved in solution takes place at concentrations as low as 0.001 g/L. Furthermore, the fractal analysis was applied on measured data. It seems that this approach is useful for concentration above approximately 0.03 g/L. The constant value $D \approx 2.5$ is reached around concentration 1 g/L. That suggests the switch in mechanisms of aggregation similarly as proposed in [20]. For concentrations below 0.03 g/L the fractal dimension data become too scatter which might be consequence of higher error and scattering in measured data.

The application of the fractal dimension D as an indicator of aggregation behavior and the relationship between D and primary characteristics was investigated. For that reason the relationship between several parameters describing aggregation behavior and composition of HS was searched. Parameters a , b , c and D and their modifications were correlated with elemental analysis and distribution of carbon in functional groups (i.e. C, H, O, N; C/O, C/H ratios, carbon content in carboxylic groups and aromaticity degree) using linear regression least square method. Results are summarized in Tab. 5. As can be seen, no significant connections between parameters obtained from mathematical fitting and chemical composition can be observed. This is in strong contrast to previous correlations performed on IHSS standard samples. A possible explanation for this observation could be that samples were sampled in different horizons which predestine them to undergo different genesis at different time periods. Nevertheless, in case of fractal dimension parameter the situation is slightly different. Some strong correlations can be observed. It seems that the value of the D parameter is influenced predominantly by the amount of oxygen and consequently also by C/O ratio and carboxylic carbon content. This can be associated with the theory beyond the fractal analysis developed for this purpose. It supposes the local mass density fluctuations which in case of oxygen fulfill the criterion. Higher polarity on oxygen causes hydrophilic hydration causing locally higher water density (more than 10 %) as discussed in further paragraphs. Similar conclusion can be done also for nitrogen atoms.

From the point of view of overall ability to predict properties of the sample, the parameters “ D end”, “ D slope” and “ D intercept” seems to be the most successful; “ D end” stands for the end value, i.e. at concentration about 3.5 g/L, while “ D slope” and “ D intercept” stand for the slope and intercept of the linear regression of the values in concentration range 0.13–3.5 g/L. Some high correlations ($R^2 > 0.9$) can be noticed for pairs “ D slope” – carboxylic carbon, “ D intercept” – C/O ratio and “ D

slope” – C/O ratio. From “*D* slope” and “*D* intercept” data it can be concluded that the higher amount of oxygen (and amount of -COOH groups, respectively) in the sample the smaller slope and intercept which means that faster stabilization occurred. This implies that at higher concentrations the -COOH groups play an important role in aggregate stabilization probably due to ability to form H-bonds with other moieties.

Tab. 5: Correlation of parameters describing aggregation behavior with composition of humic substances represented by coefficient of determination (R^2); Car. C – Carboxylic carbon in %; Arom. – Aromaticity in % (* calculated from data in concentration range 0.13–3.5 g/L).

	C	H	O	N	C/O	C/H	Car. C	Arom.
<i>a</i>	0.687	0.626	0.194	0.118	0.022	0.772	0.111	0.018
<i>b</i>	0.428	0.508	0.329	0.196	0.015	0.418	0.009	0.000
<i>c</i>	0.518	0.569	0.334	0.202	0.004	0.469	0.007	0.103
<i>a</i> × <i>b</i>	0.168	0.239	0.219	0.395	0.036	0.144	0.057	0.014
<i>a</i> ^{<i>b</i>}	0.415	0.451	0.265	0.180	0.006	0.386	0.000	0.017
(<i>a</i> ^{<i>b</i>}) ^{−<i>c</i>}	0.431	0.521	0.347	0.224	0.016	0.412	0.020	0.046
<i>D</i> end	0.381	0.029	0.430	0.097	0.836	0.000	0.775	0.511
<i>D</i> average *	0.546	0.079	0.025	0.590	0.430	0.092	0.372	0.234
<i>D</i> slope *	0.295	0.068	0.616	0.000	0.912	0.009	0.950	0.060
<i>D</i> intercept *	0.120	0.277	0.826	0.105	0.945	0.124	0.796	0.368
<i>D</i> at 0.95 g/L	0.021	0.284	0.362	0.890	0.214	0.329	0.091	0.085
<i>D</i> at 0.44 g/L	0.002	0.112	0.228	0.687	0.250	0.139	0.084	0.610

4.3.3 Results and Discussion – Densitometry

As already mentioned, hydration of humic assemblies is one of the most important factors involved in physical character of their aqueous solutions. To understand more this phenomenon, additional density measurements were performed since known density and ultrasonic velocity can be used to determine compressibility and subsequently also the extent of hydration shells. An attempt was paid to go down as low as possible with concentrations of the samples to approximate the conditions occurring in nature.

Densities of six samples (Tab. 4) were measured in concentration range from 0.003 to at least 1.1 g/L. As expected, more or less increasing trend was recorded. The most interesting fact is, that at very low concentrations the density of the humic solution was in some cases even lower than the density of pure solvent – water ($\rho_{\text{H}_2\text{O}, 25^\circ\text{C}} = 0.99704 \text{ g/mL}$). Hypothetically, this could be explained by the nature of hydration of humic molecules as reported recently [44],[45],[46]. At low concentrations, the exterior of very small aggregates is predominantly hydrated by hydrophobic hydration [20],[47]. That means that water shell is formed of water molecules creating structure more rigid than the bulk water molecules form but on the other hand lower density in compare to bulk water can be expected [48]. Another explanation might be the higher error of measurement at lower concentration of

humic substances. Nevertheless, because of lack of samples it was not possible to repeat the measurements and in following paragraphs it is assumed that the correctness of the measurement is indisputable.

Based on knowledge of compressibilities of both pure water and humic solution, the calculation of volume fraction of non-interacting solvent was possible [49]. As expected, with increasing concentration the volume fraction of non-interacting solvent was decreasing. This means that the amount of interacting water molecules is increasing. This is caused predominantly by extent of the total hydration capable surface. Results of these experiments performed on humic samples are in line with those presented in doctoral thesis by Vlčková [50].

From known concentrations the weight of water molecules in hydration shells was calculated and expressed as grams of H₂O per 1 g of HS. It seems that there is need of about 0.4–1.2 g of water / 1 g of HS. Assuming that the apparent HS aggregate dimension is in order of a few tens of kDa, so this represents several hundreds of water molecules per one “HS molecule”. This seems as a reasonable number if compare to hydration of proteins [51],[52], saccharides [53] or nucleic acids [54]. Further, hydration results demonstrate the above-discussed unpredictableness of humic aggregates and their reactivity. Probably, some prediction can be done only above approximately 1 g/L when aggregates are stable enough to not undergo additional and relatively simple reconformations. In addition, such notion can encourage further hypotheses about dimension of humic molecules and aggregates with respect to their restricted motion in concentrated solutions.

4.3.4 Conclusions

- All humic samples under study exhibited aggregation at concentrations as low as 0.001 g/L.
- Fulvates showed similar concentration behavior as the humates.
- Similarly as in the case of the IHSS standards, the same power-based fitting can be applied on all samples under study.
- No significant connections between parameters obtained from mathematical fitting and chemical composition were noticed which is in strong contrast to the previous study using the IHSS standards.
- The developed fractal analysis can be applied under the same conditions as in the case of the IHSS standards.
- Fractal dimension of a sample is influenced predominantly by the amount of oxygen and consequently also by the C/O ratio and carboxylic carbon content.
- Coefficients of determination (R^2) higher than 0.9 were noticed for pairs “*D* slope” – carboxylic carbon, “*D* intercept” – C/O ratio and “*D* slope” – C/O ratio.
- At higher concentrations the carboxylic groups play an important role in aggregate stabilization.

- Density of the samples showed increasing trend with increasing concentration.
- At very low concentrations the density of humic solutions was lower than the density of pure solvent (water).
- Increase of U_{12} with increasing concentration was assigned to the extent of the total surface leading to increase of hydration shells. The same process is also observable from the dependency of volume fraction of non-interacting solvent on concentration records.
- Approximately 0.4–1.2 g of water forms the hydration shells of 1 g of HS meaning several hundreds of water molecules per one “HS molecule”.

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7 ABSTRACT

This doctoral thesis deals with the application of high resolution ultrasonic spectroscopy (HRUS) on studying of humic substances (HS). The goal is to obtain deeper insight to the field of relations between primary characteristics (elemental composition and distribution of carbon in functional groups) and aggregation properties. In the literature review the contemporary information about HS and HRUS are presented. Furthermore, basic information about fractal analysis as well as its application on data obtained by various techniques is mentioned. In the first section of the experimental part of this thesis the advantageous application of HRUS in the research of HS is discussed. International Humic Substances Society (IHSS) standards were selected to represent wide spectrum of samples, both humates and fulvates were used. To determine the influence of sodium counterion also fulvic acids in their protonized form were used. Power-based fitting was applied on all samples data and gained empirical parameters were correlated with primary characteristics. Fractal analysis method was developed and applied on the HRUS data. An alternative approach was applied on data gained by HRUS. From a global point of view, the dependency of ultrasonic velocity on concentration shows a linear trend and therefore a linear regression method can be applied. The second purpose of this thesis is to find information about thermal stability of HS aggregates (IHSS standards). Samples at four different concentrations were treated by temperature gradients and aggregation behavior was assessed and discussed. In the third part of thesis the concentration dependencies of the samples were studied. Unlike the first goal, samples originating mainly from locations unaffected by anthropogenic activity were used. Power-based fitting and fractal analysis were applied and acquired parameters were correlated with primary characteristics. To elucidate the extent of hydration shells, the high precision density measurements were performed. As the state-of-the-art research shows, the aggregation properties of humic biomolecules may play an important role in the future designing of humic substances applications, mainly in the agriculture and environmental protection, but even also in medicine.