

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

FACULTY OF CHEMISTRY
INSTITUTE OF PHYSICAL AND APPLIED CHEMISTRY

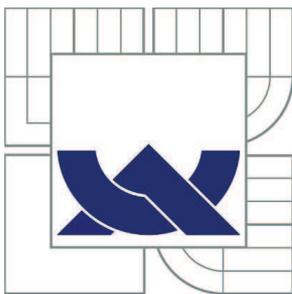
HUMIC SUBSTANCES CHARACTERIZATION EMPLOYING HIGH
RESOLUTION ULTRASONIC SPECTROSCOPY

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

AUTOR PRÁCE
AUTHOR

Ing. MARTIN DRASTÍK

BRNO 2010



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VYUŽITÍ VYSOCEROZLIŠOVACÍ ULTRAZVUKOVÉ SPEKTROSKOPIE PŘI CHARAKTERIZACI
HUMINOVÝCH LÁTEK

DIZERTAČNÍ PRÁCE

DOCTORAL THESIS

AUTOR PRÁCE

AUTHOR

Ing. MARTIN DRASTÍK

VEDOUCÍ PRÁCE

SUPERVISOR

prof. Ing. MILOSLAV PEKAŘ, CSc.

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Brno University of Technology
Faculty of Chemistry
Purkyňova 464/118, 61200 Brno 12

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Humic Substances Characterization Employing High Resolution Ultrasonic Spectroscopy

Doctoral thesis assignment:

In this work High resolution ultrasonic spectroscopy (HRUS) will be employed to study the aggregation behavior of humic substances (HS). To cover the widest spectrum of possible diversity occurring in the nature, humic substances of different origin will be used. Standards of the International humic substances society will be purchased, HS originating from different places and soil horizons will be extracted. 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. High precision densitometry will be employed to assess the extent of hydration shells.

Deadline for doctoral thesis delivery: 15.9.2010

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

Ing. Martin Drastík
Student

prof. Ing. Miloslav Pekař, CSc.
Head of thesis

prof. Ing. Miloslav Pekař, CSc.
Head of institute

In Brno, 1.9.2009

prof. Ing. Jaromír Havlica, DrSc.
Dean

ABSTRAKT

Předkládaná dizertační práce se zabývá využitím techniky vysoce rozlišovací ultrazvukové spektroskopie (HRUS) při analýze huminových látek, za účelem získání hlubšího vhledu do problematiky vztahu mezi jejich primárními charakteristikami (elementární složení a rozložení uhlíku ve funkčních skupinách) a agregačními vlastnostmi. V literární rešerši jsou shrnuty nejnovější poznatky z oblasti studia huminových látek a představeny základní principy HRUS. Dále jsou uvedeny základní informace z oblasti fraktální analýzy a její aplikace na data získaná pomocí různých metod při studiu huminových látek. První úkol experimentální části je zaměřen na výhodné využití HRUS pro výzkum huminových látek, zde reprezentovaných standardy Mezinárodní společnosti pro huminové látky (IHSS) a to sodnými solemi huminových a fulvinových kyselin. Fulvinové kyseliny v jejich protonované formě byly taktéž zkoumány a to z důvodu objasnění vlivu sodného kationu. Pro popis chování vzorků byla použita mocninná funkce, jejíž empirické parametry byly korelovány s primárními charakteristikami. Byla vytvořena metoda fraktální analýzy a následně byla aplikována na data získána ultrazvukovou spektroskopií. Data získaná pomocí ultrazvukové spektroskopie byla zpracována i alternativní metodou. Ta spočívala v globálním pohledu na závislost ultrazvukové rychlosti na koncentraci a využití lineární regrese. Druhým z cílů práce je získání informací o vlivu teploty na stabilitu agregátů HS (IHSS standardy). Byl zkoumán vliv teplotních gradientů na chování agregátů při čtyřech různých koncentracích. V třetí části práce pak byly zkoumány koncentrační závislosti u vzorků pocházejících především z lokalit příliš nezasážených lidskou činností. HRUS data byla proložena mocninnou funkcí a zkoumána pomocí fraktální analýzy. Takto získané parametry byly korelovány s primárními vlastnostmi. Ze znalosti hustoty při dané koncentraci mohly být stanoveny velikosti hydratačních obálek. Jak se v současné době ukazuje, informace o agregačním chování huminových biomolekul mohou být v budoucnu velmi důležité pro navrhování průmyslových aplikací huminových látek, zejména v zemědělství a v ochraně životního prostředí, ale také například v medicíně.

KLÍČOVÁ SLOVA

huminové látky, standardy Mezinárodní společnosti pro huminové látky (IHSS), vysoce rozlišovací ultrazvuková spektroskopie (HRUS), agregace, teplotní stabilita, fraktální analýza, hydratace

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.

KEYWORDS

humic substances, International Humic Substances Society (IHSS) standards, High Resolution Ultrasonic Spectroscopy (HRUS), aggregation, thermal stability, fractal analysis, hydration

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PROHLÁŠENÍ

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

.....
podpis studenta

DECLARATION

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

.....
student's signature

My first thank you goes to Prof. Miloslav Pekař who allowed me to have a look through the looking-glass to Wonderland commonly called Physical Chemistry. My second thank you goes to Assoc. Prof. Jiří Kučerík who was my guide in this world and without whom there would be nothing to read on these pages. I am also thankful for the bunch of young scientists who I may call friends – without them it won't be such tremendous fun. My last thank you is assigned to my family and mate for their never-ending support, love and understanding.

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INTRODUCTION

“I believe in intuition and inspiration. Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution. It is, strictly speaking, a real factor in scientific research.”

Albert Einstein, 1931

Indeed, big imagination that is exactly what one needs when dealing with such complicated heterogeneous system known under the name humic substances. Even after decades of intensive research when the number of papers is literally growing exponentially there are still unknown issues and it is hard to predict when these black spots of our unknowingness will be erased.

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 is High Resolution Ultrasonic Spectroscopy (HRUS). 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.

1 STATE OF THE ART

1.1 Humic substances

The global soil carbon pool is 3.3 times the size of the atmospheric pool and 4.5 times that of the biotic pool. Organic carbon represents approximately 62 % of global soil carbon while at least 50 % of this carbon can be categorized as the chemically resistant component known as humic substances (HS) [1,2,3] (Fig. 1).

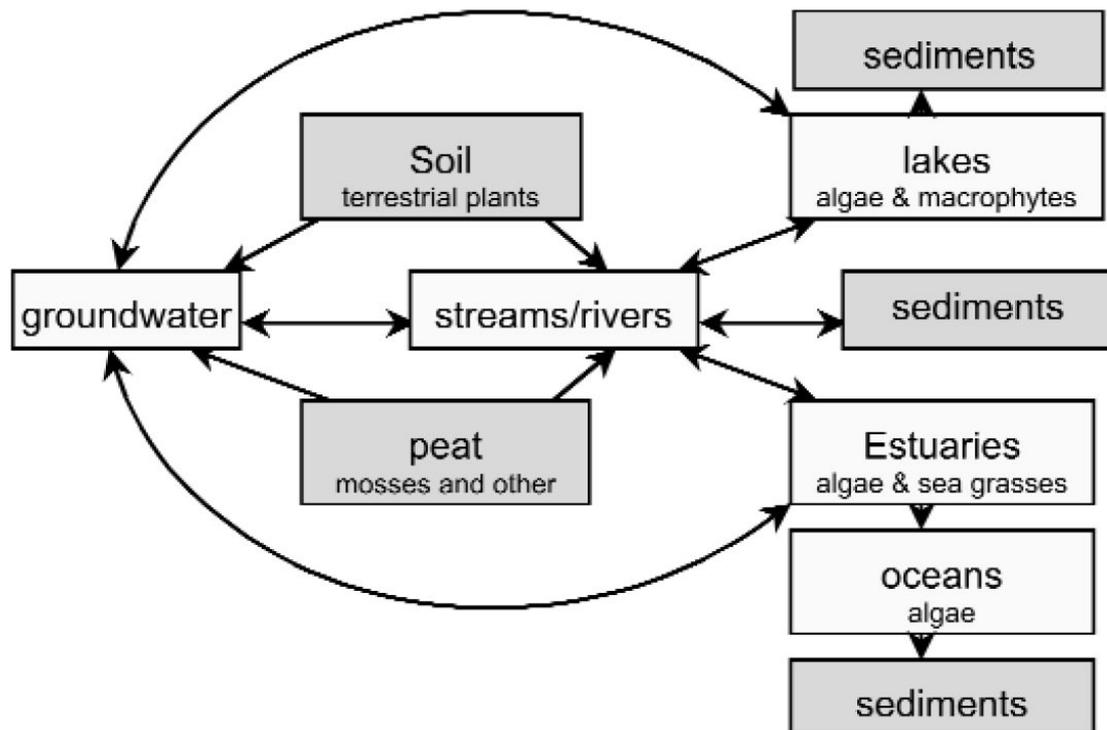


Fig. 1: Diagram of the occurrence and possible environmental flow paths of humic substances [4].

Although the word “humus” was already known by ancient Romans, there is no worldwide-accepted definition of humic substances. Stevenson [5] defines humic substances as a series of high molecular weight molecules formed by secondary synthesis reactions; they may be generally characterized as being rich in oxygen containing functional groups – carboxylic COOH, phenolic/enolic OH, alcoholic OH and carbonylic C=O of quinines. From another point of view, HS can be defined as a general category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in color and refractory [6]. Sutton and Sposito [7] defines HS as a collection of diverse, relatively low molecular mass components forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds and capable of organizing into micellar structures in suitable aqueous environments. Based on historical reason, one can still HS operationally classify 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 [2,8]. Nevertheless, no definition mentioned here nor any other was adopted by IUPAC and published in Gold Book so far.

On the other hand, at least some scientists maintain a position that all the classifications and definitions in humus chemistry are only operational on the basis of the procedures used for isolation of HS and that there is no ideal system of classification which would satisfy each scientist's need [9,10,11].

1.1.1 Elemental composition – primary structure

One of the most fundamental characteristics of a chemical substance is its elemental composition. However, determining some general elemental composition in the case of non-stoichiometric materials as humic substances is very limited and a unique, structural formula does not exist for this kind of material. Nevertheless, elemental analysis is still a useful tool for characterizing HS.

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 in the other elemental determinations and also the S and P content which is usually disregarded [12]. 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 [13].

The H/C and O/C ratio developed by van Krevelen in 1961 [14] to study the coalification process was later on adopted by organic geochemists to study diagenesis on humic substances to illustrate compositional differences between humic acids and fulvic acids, and also to show variations in humic substances as a function of source [15]. 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 [12], the elemental data (C, H, O, N, S, atomic H/C and O/C ratios) for humic acids (410 samples), fulvic acids (214 samples) and humin (26 samples), isolated from environments all over the world, were compiled from the literature. Authors analyzed the data statistically using the mean, median, mode, range, standard deviation and t-test. 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.

The composition of individual samples depends on many factors, to mention at least the most important: origin of matrix, conditions of formations and location. The isolation procedure may alter the results as well.

Tab. 1: Elemental composition of HS regardless origin, expressed as weight percent; O/C and H/C ratios are atomic percent; all values are on an ash-free basis [12].

	C	H	N	S	O	O/C	H/C
HA							
Mean	55.1	5.0	3.5	1.8	35.6	0.50	1.10
Range	37.18–75.76	1.64–11.68	0.50–10.54	0.1–8.3	7.93–56.6	0.08–1.2	0.08–1.85
Samples	410	410	410	160	410	410	410
FA							
Mean	46.2	4.9	2.5	1.2	45.6	0.76	1.28
Range	35.1–75.4	0.43–7.2	0.45–8.16	0.1–3.6	16.9–55.8	0.17–1.19	0.77–2.13
Samples	214	214	214	71	214	214	214
Humin							
Mean	56.1	5.5	3.7	0.4	34.7	0.46	1.17
Range	48.29–61.6	4.2–7.28	0.9–6.0	0.1–0.9	28.8–45.12	0.37–0.61	0.82–1.72
Samples	26	26	24	16	26	26	26

1.1.2 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 amino, amine, imine and amide are nitrogen-containing group that can be found. Some small amounts of sulfur and phosphorus functional groups are present as well [5].

There are basically two ways how to determine functional groups in HS. The first one based mainly on derivatization of functional groups by methylation or acetylation is called the wet chemical method. Second possibility is to employ some spectroscopic method – Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared Spectroscopy (FTIR), Electron Paramagnetic Resonance (EPR) for instance. Anyway, because of enormous heterogeneity,

the character of each functional group of certain class is somehow unique which is in strong contrast to character of functional groups in the case of pure substances. This means that the pKa of carboxylic groups is an average of several of them or that there cannot be found a distinct line between phenolic and alcoholic OH groups.

Nevertheless, one can conclude that 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) [5].

1.1.3 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 [5,16,17] and therefore contemporary analytical approaches consider them as indivisible part of the fraction [7]. There are several reasons for that.

When in soil, biomolecules originated from debris are easily accessible to microorganisms and are rapidly utilized as source of energy and building blocks. In contrast to this, soil humic substances typically possess average ^{14}C ages ranging from hundreds to thousands of years [5]. But when the biomolecules are bound to HS, this interconnection can lead to protection of biomolecules from microbial degradation and forming of refractory associates [18,19,20]. Alternatively to this, MacCarthy [21] posits a “two-compartment” view of HS stability. He agrees that most of humic material is persistent but he claims that there is transient part too. The persistent material stands for the long ^{14}C age, whereas the transient components play the main role in the fast carbon turnover rate. And it is possible that although strongly associated, still easily degradable biomolecules make up a large portion of the transitory and frequently replenished humic components. This theory elucidates how is possible that humic components as members of associations may protect individual molecules from microbial degradation and simultaneously that there is a minority population that is accessible to carbon turnover.

Another argument why to consider biomolecular fragments as an indivisible part of humics is that these biomolecular moieties contribute noticeably to the functional behavior of humic associations [17,22,23]. The research by del Vecchio et al. [24] indicates that fluorescence absorption and emission spectra arise from a continuum of coupled states formed through charge-transfer interactions of a few distinct chromophores, rather than from a superposition of many independent chromophores. Therefore one can speculate that biomolecules interconnected with humic material contribute to the charge-transfer interactions that lead to the complex optical properties of humic substances. Authors believe that these donor-acceptor

interactions may be a common phenomenon, occurring within all natural hydroxy- or polyhydroxy-aromatic polymers that form appropriate acceptors upon partial oxidation through either (bio)chemical or photochemical pathways. Examples of such species include lignin, polyphenols, tannins, and melanins.

1.1.4 In search of structure

In 1961 Kononova [25] 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. These results were supported by many measurements employing sedimentation-velocity and diffusion methods [26,27]. Papers presenting molecular dimensions measured by osmometry, viscometry and diffusion of about one or two thousand Daltons were ignored [28,29]. The reason for this overlooking was that scientists believed in the hypotheses that HS are products of biologically-assisted syntheses from compounds derived from degradations of lignin, polyphenols, cellulose and amino acids. Evidence for this polymeric assumption was researched in many classical laboratory experiments that indicated possibilities for either biotic or abiotic condensations of simple molecules into humic-like materials [25]. Some of these early laboratory studies were later repeated in more carefully defined conditions and no direct evidence for the occurrence of such polymer build-up processes in natural soil systems was registered [30,31]. Furthermore, the measurement of sedimentation coefficients of polydisperse materials that include subunits, which is the case of humic substances, constantly leads to erroneous values of MW as shown by Laue and Rodhes [32]. In other studies employing sedimentation-velocity ultracentrifuge studies [33] and equilibrium centrifugation [34,35] was showed that polydispersity in HS is the matter of fact and confirmed that MW values obtained by ultracentrifuge methods are ambiguous, for this reason.

Ghosh and Schnitzer [36] 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 (the Flory and Fox and the Staudinger equation) 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.

The particle sizes of the whole HA in solution and of fractions (separated by adsorption chromatography using cross-linked dextran gels) were confronted by Wershaw [37] employing small-angle X-ray scattering. It was found out that HS formed molecular aggregates in solution and their sizes were a function of pH. Based on these findings, it was concluded that the various fractions were chemically different and that the differences in aggregation behavior were a reflection of the interactions of different bonding mechanisms. Results by Hayase and Tsubota [38] suggested that humic fractions from different sources possess surface activity. All these findings led to the conclusion that there must inevitably be another explanation of HS behavior than the random coil polymeric structure theory.

This new theory proposed by Wershaw [39,40] considers humic substances as ordered aggregates of amphiphils composed mainly of relatively unaltered plant polymer segments possessing acidic functional groups. These aggregates are supposed to be held 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 is in strong contrast to the traditional view of polydisperse humic polymers.

Wershaw's 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 [38,41,42] 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 [43,44,45,46]. 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 [47], NMR [48], thermal analysis [49], mass spectrometry [50] or ultrasonic spectroscopy [51] supported these conclusions. Some other researchers accepted this theory although they do not exclude the presence of high MW fractions as the remnants of parental plant tissues protected by humic molecules from biological degradation [52]. A novel model of HA structure which takes under account all the new findings is presented in the Fig. 2.

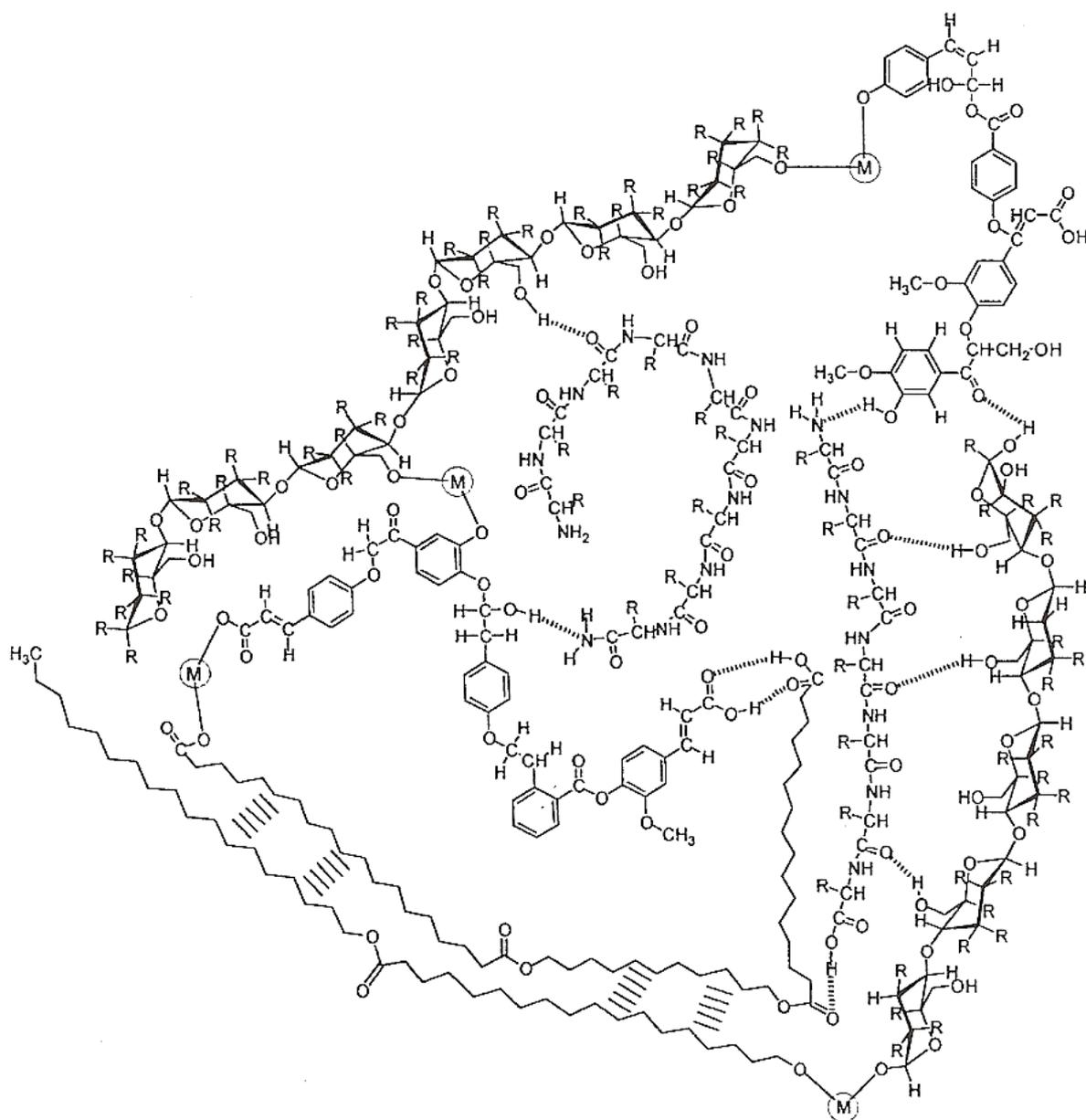


Fig. 2: Recent model of humic acid structure [53].

1.1.5 IHSS samples

In 1981 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. With substantial support from the United States Geological Survey, the IHSS selected three solid-phase source materials (a soil, a peat, and a leonardite) and one water sample (a blackwater river) and then isolated a suite of standard humic and fulvic acids from those source materials. 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 clearly 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 [54].

The Suwannee River HS (2S101H/F) originates from the Okefenokee Swamp in southern Georgia, U.S.A. The Okefenokee Swamp contains extensive peat deposits. However, decomposing vegetation is believed to provide most of the dissolved organic carbon (DOC) to its waters. At its headwaters in the Okefenokee Swamp, the Suwannee River is a blackwater river, with DOC concentrations ranging from 25 to 75 mg/L and pH values of less than pH 4.0. Suwannee River FA contains the most aliphatic carbon and also the least of nitrogen of all IHSS standards, followed by Suwannee River HA.

The Elliott soil (2S102H/F) is typical of the fertile prairie soils of the Midwest of United States. The IHSS sample was obtained from an undisturbed area on the grounds of the Joliet Army Ammunition Plant near Joliet, Illinois, U.S.A. The Elliott series consists of very deep, somewhat poorly drained soils on moraines and till plains. They formed in as much as 50 cm of loess or silty material and silty clay loam glacial till.

Pahokee peat (1S103H/F) is a typical agricultural peat soil of the Florida Everglades (U.S.A.). The IHSS sample was obtained from the University of Florida Belle Glade Research Station. The Pahokee series consists of very poorly drained soils that are 90 to 130 cm thick over limestone. Pahokee soils formed in organic deposits of freshwater marshes.

Leonardite humic acid (1S104H-5) was produced by the natural oxidation of exposed lignite and originates from North Dakota, U.S.A. Lignite is the best source of HA since the content is more than 85 % which is the highest of any natural source. Furthermore, lignite is a plentiful and inexpensive compared to other possible humic acid sources. Leonardite HA is the most aromatic IHSS standard since it contains more than four times more of aromatic than aliphatic carbon. The amount of carbon in carboxylic groups is very low which corresponds to high aromaticity [54,55].

Tab. 2: 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 [54].

	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. 3: ^{13}C estimates of carbon distribution in IHSS samples, expressed as electronically integrated peak area percentages for selected ranges of chemical shift [54].

	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

1.2 Ultrasonic spectroscopy

The basics of ultrasonic spectroscopy, in past the name ultrasonic interferometry was often used, were laid down in 1940s but only in recent years this method has become much more popular mainly because of development of new high-precision methods of measurement. Nowadays, ultrasonic measurements can provide a rapid and non-destructive analysis of wide range of samples. Moreover, running costs are usually relatively very low [56]. Unlike light in UV/VIS part of spectrum, the ultrasound waves are able to pass through opaque samples and in fact through most of materials. Another advantage is that it is easy to change the wavelength of ultrasonic waves because they are synthesized electronically which is in strong contrast to optics where the wave originates from a light source and therefore special care must be taken to ensure spectral purity [57].

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 to these compressions by intermolecular repulsion. When under decompression, the attractive forces play the role [58]. The amplitude of deformations caused by analytical ultrasound waves is extremely small and therefore making the ultrasonic spectroscopy a non-destructive technique [59].

Parameters that are traditionally measured in ultrasonic spectroscopy are ultrasonic attenuation and ultrasonic velocity. Attenuation is determined by the energy losses of the ultrasonic waves due to absorption and scattering contributions. In homogenous samples, the periodical compressions and decompressions of the molecules shift the equilibrium of the chemical reactions. A delay in the relaxation of the molecules to the equilibrium state causes absorption of energy (Fig. 3). In non-homogenous samples, the presence of particles results in scattering of ultrasonic wave. The ultrasonic attenuation is observed as the decrease of the amplitude of the output wave (Fig. 4).

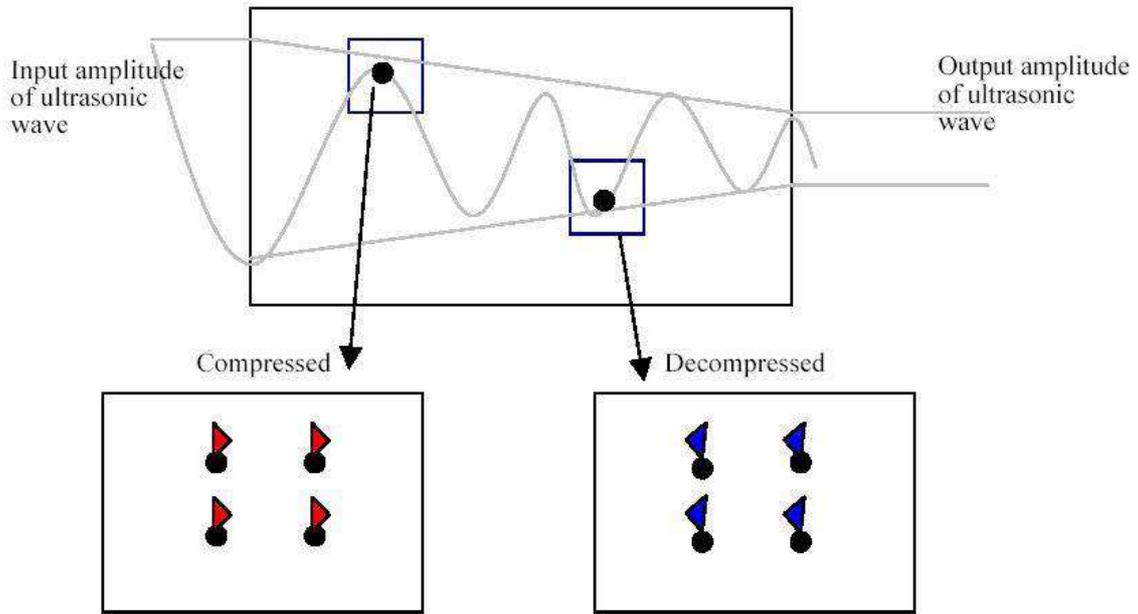


Fig. 3: Ultrasonic attenuation in homogenous samples [59]

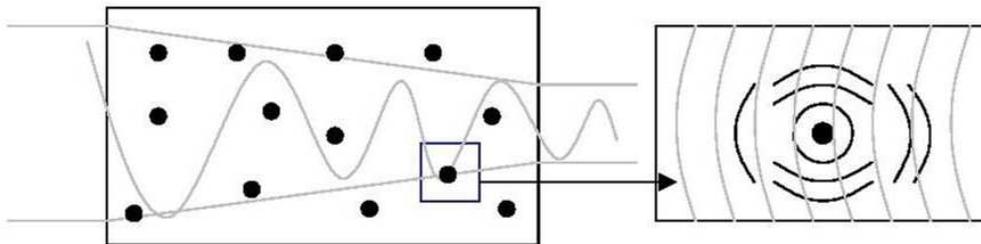


Fig. 4: Ultrasonic attenuation in heterogeneous samples [59]

The second parameter measured is ultrasonic velocity. It is determined by the density and the elasticity of the medium. The speed of ultrasound is proportional to rigidity of the sample and since the solids are more rigid (which also means having higher elasticity of molecules) then liquids and gases, respectively, the sound propagates through them faster than through liquids and gases. The rigidity of the material is determined by both density and compressibility but the contribution of the compressibility is in the majority of standard samples the leading factor [56]. 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 [60]

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

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

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

$$\beta_s = V^{-1} \left(\frac{\partial V}{\partial P} \right)_s \quad (2)$$

and

$$\beta_T = -V^{-1} \left(\frac{\partial V}{\partial P} \right)_T = \beta_s + \frac{\kappa^2 T}{\rho C_p} \quad (3)$$

where κ

$$\kappa = V^{-1} \left(\frac{\partial V}{\partial T} \right)_P \quad (4)$$

is the volume coefficient of thermal expansion and V is volume. The difference between isothermal and adiabatic compressibility for aqueous solutions is small and usually does not exceed a few percent because for water the values of C_p are large and of κ are small, on the contrary. The adiabatic compressibility K_s of a substance is defined as

$$K_s = - \left(\frac{\partial V}{\partial P} \right)_s = \beta_s V \quad (5)$$

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

$$\beta_s = \frac{1}{\rho U^2} \quad (6)$$

Measurements of ultrasound velocity and density are the only direct ways to evaluate the adiabatic compressibility coefficient of a liquid [56].

1.2.1 Instrumentation

Basically, there are two different arrangements of the ultrasound measuring devices – the sing-around method and the resonator method. The first one mentioned, also called a pulse technique, was developed in 1950s and became very popular because very high level of precision in measuring of ultrasound velocity can be achieved. Furthermore, the construction of the measuring cells and inner electronics is very simple which leads to lower cost of the machine [61,62]. In this method, a transmitted ultrasonic pulse passes through the solution in the measuring cell to the receiving transducer and then is used to retrigger the transmitter. The repetition frequency of the complete cycle is a function of the ultrasound velocity in the liquid. The pathway of the ultrasonic pulse should be long enough to detect the propagation time and the amplitude decay with necessary resolution. When high resolution measurements are the case, high volume cells in order of tens of milliliters are needed. These relatively high sample volumes requirements may be a big disadvantage in some cases. Obvious example is if the amount of the sample itself is low. Other problems may arise from difficulties connected with temperature since it takes a long time to equilibrate the temperature gradients in large volumes [63]. The scheme of the device can be seen in Fig. 5.

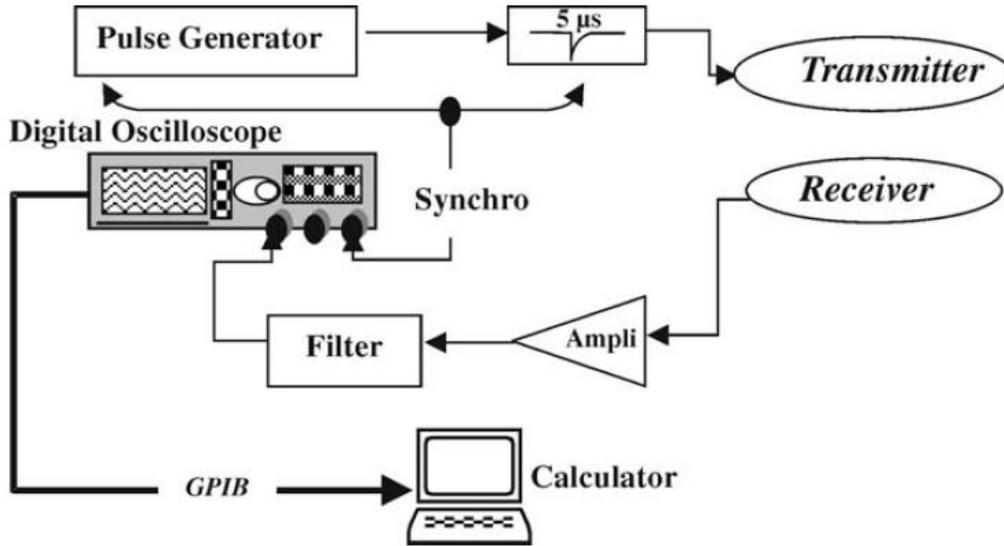


Fig. 5: The sing-around method diagram.

The second technique, the resonator method, has a precision similar to that of the sing-around method but the cell volumes may be as low as 0.1 mL. The first versions of fixed-path interferometers were described in 1939 [64]. Initially, the instruments were intended to measure ultrasonic attenuation but later on they proved themselves to be capable of measuring ultrasonic velocity with high precision as well. They started to spread more widely after the milliliter cells were developed in 1970s [65] because by utilizing this feature the resonator technique overcomes the problem of a large cell because a decrease in the volume of the resonator cell leads to an increase in the number of reflections of the ultrasonic wave at the resonator walls, while the total length of the path of the ultrasonic wave in the liquid remains nearly constant. Nowadays, this technique is increasingly used in biophysical studies.

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 resonator whose natural frequencies f_n are linearly related to the ultrasound velocity (Fig. 6). 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. A typical dependence of the amplitude ratio of the signal on the frequency of the resonator is given in Fig. 7 where the amplitude ratio is the ratio of the amplitude of the input signal applied to the emitting transducer to the amplitude of the signal detected from the receiving transducer. The value of a resonance frequency may be evaluated by finding the maximum of the output voltage amplitude or by determining the inflection point of the phase-frequency characteristic. The absolute value of U is given by this equation:

$$U = 2l(f_{n+1} - f_n)(1 + \gamma) \quad (7)$$

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} . The ultrasonic attenuation is determined from the energy losses in the resonance [56].

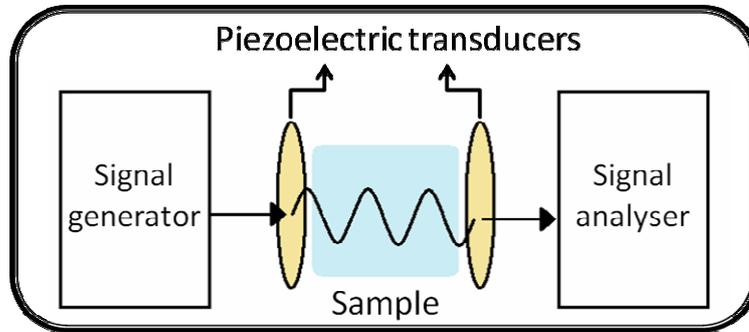


Fig. 6: Diagram of a plane parallel resonator.

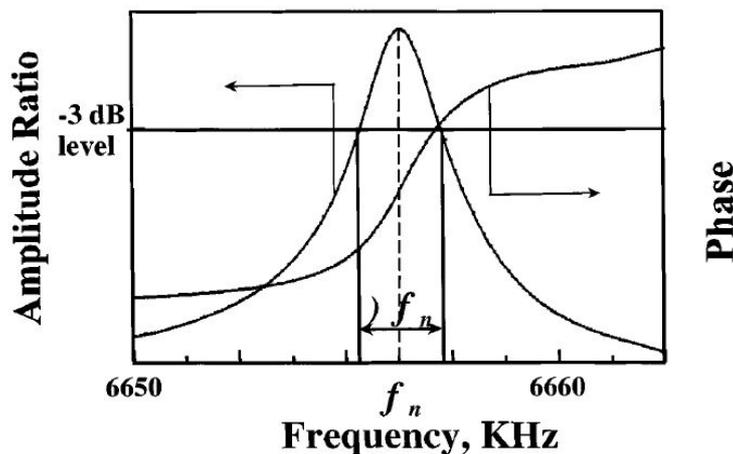


Fig. 7: Typical amplitude-frequency and phase-frequency characteristics of a resonance [63].

1.2.1.1 High Resolution Ultrasonic Spectroscopy (HRUS)

In 2004, a novel arrangement of ultrasound device based on the resonator method was patented by Buckin [66]. Employing this device, which is nowadays produced by Sonas Technologies Ltd., Ireland, it is possible to achieve the resolution of the spectrometer down to 10^{-5} % for ultrasonic velocity and 0.2 % for attenuation measurement. The machine itself consists of two independent measuring cells tempered by common water bath and stirred by electromagnetic stirrers (Fig. 8). Ultrasonic velocity is temperature-dependent. Observed changes in measured values are therefore caused both by internal physical-chemical processes and by external fluctuations of temperature. If the second cell as a reference cell is used and is loaded only by the solvent no reactions can occur there. All the changes of measured parameters are therefore caused only by external temperature fluctuations in this cell. Because common water bath secures same temperature in both cells, it will be possible to subtract

recorded values (i.e. measuring cell U_1 minus reference cell U_2 , further in text denoted as U_{12}) to obtain the values of ultrasonic velocity and attenuation free of external temperature fluctuations.

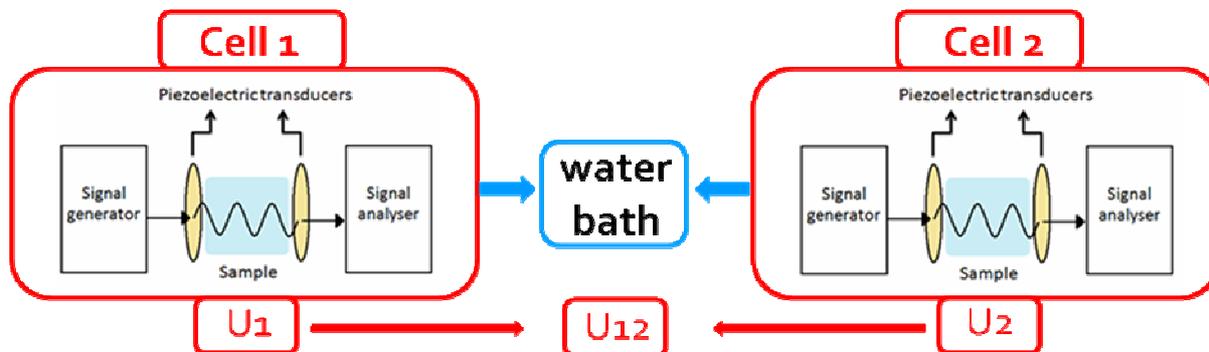


Fig. 8: Scheme of a high resolution ultrasonic spectrometer.

This kind of arrangement was already utilized on many different fields, especially in food industry, biotechnology and pharmacy. High resolution ultrasonic spectroscopy was employed for characterization of emulsions and dispersions [67], monitoring of heat-induced transitions in biopolymers such as protein denaturation, aggregation and gelation [68], monitoring of crystal formation, kinetics of this reaction, size and amount of crystals [68], studying enzyme activities, reaction mechanisms and kinetic parameters, including inhibition mechanisms and inhibition constants [69], direct real time monitoring of hydrolysis of cellulose [70] and many others.

To author's best knowledge HRUS was employed just several times in the study of humic substances. In the study using lignite humic acids, Kučerík et al. [71] 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 [51]. In 2009, Kučerík et al. [72] employed HRUS to study progressive aggregation and structural changes in both sodium salts and protonized forms of fulvic acids and sodium salts of humic acids. The standards of the International Humic Substances Society (IHSS) were used to cover wide range of possible sources of HS. The method allowing the data treatment by fractal analysis was developed and tested [72,73]. Determined dependency of fractal dimension on concentration revealed clear differences in mechanisms of aggregation of individual humic and fulvic samples.

1.3 Fractals

Most of natural objects are fractals [74]. The term “fractal” is usually associated with scale-invariant objects. The strictest kind of scale-invariance is the self-similarity. Such objects have similar features in all scales of view (i.e. they can be transformed by a single scaling factor) and their complex geometry cannot be characterized by an integer dimension. If a different scaling factor for each coordinate axis is needed the objects are called “self-affine” [75]. Deterministic fractals are exactly self-similar or self-affine, while stochastic fractals are statistically self-similar or self-affine, i.e. the scaled version is identical in all statistical respects to the original [76].

Fractals 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. A classical example to illustrate this property is the “length” of a coastline [77]. When measured at a given scale d , the total length of a coastline $L(d)$ is estimated as the sum of N straightline segments of length d . Small details of the coastline cannot be recognized when having big gauge. But they become apparent at higher resolutions. This leads to the situation that the total measured length $L(d)$ increases as the scale of measurement d increases. Thus, in fractal geometry, the Euclidean concept of “length” becomes a process rather than an event, and this process is controlled by a constant parameter. According to Richardson [78], the empirical relationship between the measuring scale d and the length L can be expressed as

$$L(d) = K d^{(1-D)} \quad (8)$$

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

1.3.1 Fractal analysis in study of HS

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 bronchial tube, river and tree branching [74].

Theory of fractals can provide a powerful mathematical tool to describe structure and properties of such random and heterogeneous systems as HS. A fractal object can be described quantitatively by a non-integral dimension D which reflects the actual space occupied by the system. 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 is often the only way how to quantitatively compare such objects or sets in reasonable amount of time.

A system wherein the mass and surface scales are the same is defined as a mass fractal and is described by a mass fractal dimension D_m . A system where surface and the pore volumes scale in similar manner are described as pore fractals D_p . And finally, if only the surface is fractal, the system is denoted as a surface fractal and is characterized by a surface fractal dimension D_s [74,79].

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 [80].

Employing 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 [81].

In another study, HA extracted from soil and peat were equilibrated for various lengths of time in aqueous suspension at different pH values. Fractal dimensions were determined by measuring their turbidity as a function of wavelength. The analysis of the power-law dependence of the turbidity on the wavelength revealed that soil and peat humic acids may exhibit either a nonfractal or a mass fractal nature. Peat humic acid could also be described as a surface fractal. The passage from one regime to another depended on the pH and equilibration time. With an increase of either factor, the mass fractal dimension of humic acids decreased from about 2.8 to values close to 1.0. These statements were supported by scanning electron microscopy observations showing that humic particles in suspension evolve from compact, almost space-filled structures with smooth surfaces, to less compact, fragmented and elongated structures with increasingly rough and irregular surfaces as the pH or time increases [82].

In a similar study by Senesi et al. [83] soil and peat humic acids diluted in aqueous suspension exhibited a mass or surface fractal nature or a non-fractal nature depending on the origin and the experimental conditions. In the absence of salt in the system, the three HA examined exhibited different fractal behavior. One of the two soil HA showed a mass fractal nature over the entire pH range examined. The other soil HA and the peat HA showed a non-fractal regime. But at low pH a surface fractal regime and on the contrary at high pH a mass fractal regime was observed. The crossover between the two regimes for these humic acids occurred at pH 5 or 6. In the presence of NaCl, the three HA exhibited a mass fractal regime at any ionic strength and over the entire pH range. However, when CaCl₂ instead of NaCl was added, one soil HA maintained a mass fractal behavior, whereas the other soil HA and the peat HA were described as surface fractals. The values of the D_m , which can be used to identify the underlying aggregation process, were relatively high at acidic pH. This probably indicates the occurrence of restructuring and/or reformation processes of HA particles connected with the diffusion-limited aggregation model (DLA). On the other hand, at about neutral pH, the low and intermediate values of the D_m would suggest a preferential reaction-limited cluster-cluster (RLA) process, which implies the existence of short-range chemical attraction between HA particles [83].

In work of Rice et al. [84] 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. [73] and Kučerík et al. [72]. 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 [51,71] about the progressive aggregation of humic substances in diluted solutions, i.e. even at concentrations lower than 0.01 g/L. An attempt was paid to develop and test a method allowing the treatment of obtained data by fractal analysis. Determined dependency of fractal dimension on concentration revealed clear differences in mechanisms of aggregation of individual humic and fulvic samples. The value of fractal dimension 2.5 was determined for the concentration 3 g/L; diluting the solution caused increasing in fractal dimension value up to 3 for concentrations around 0.01 g/L. The increase was not the same for all samples, in fact, the most significant fluctuations in this 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 that a big change in aggregates properties around the concentration of 1 g/L is observable.

2 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 [85] 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 and what is remarkable, they currently also provide the possibility to determine the thermodynamics parameters of complicated systems [86]. In fact, fractal dimension represents an added value to the traditional statistics since it reveals a trend which a system undergoes, in other words a change in entropy. Therefore, the aim of presented thesis is to combine high resolution ultrasonic spectroscopy and fractal analysis to enlarge the knowledge in these fields.

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.

3 OVERVIEW OF RESULTS AND DISCUSSION

3.1 IHSS samples – isothermal measurements

Humic substances, when dissolved, are known to form complicated structures even at low concentrations in the form of “premicellar” aggregates. At higher concentrations, there are several theories to explain their behavior. First one uses the concept adopted from chemistry of surfactants, i.e. it assumes that below a specific concentration the molecules are freely dispersed in the solution and at critical micelle concentration formation of micelles starts. This concept is in contrast to premicellar theory introduced by Wershaw and recent finding of von Wandruszka, Simpson and our group which confirmed the unique behavior of dissolved humic substances.

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. 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, increase in surface area of humic aggregates exposed to solvent and thus change in hydration of aggregates. Those parameters are responsible for change in ultrasonic velocity and thus they are detectable by HRUS. The main point in this issue represents the change in density. As indicated in previous sections, that fact 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.

3.1.1 Experimental part

3.1.1.1 Sample preparation

HS samples were purchased from the International Humic Substances Society. Four HA (Suwannee River, Elliot Soil, Pahokee Peat and Leonardite) and three FA (Suwannee River, Elliot Soil and Pahokee Peat) were delivered as powder in their H-form. To obtain sodium salts of humic (NaHA) or fulvic (NaFA) acids, each sample was dissolved in distilled water and titrated to pH 7.2 with 0.1M NaOH in automatic titrator (TitroLine® alpha plus, Schott/SI Analytics, Mainz, Germany). After reaching constant pH value 7.2, sample was kept stirred for next 60 minutes and freeze-dried. Obtained product was milled in an agate mortar and stored at room temperature in a sealed container. The non-titrated samples of FA were used as received. 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. The solutions with lower concentrations (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 (Sigma Aldrich) 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.

3.1.1.2 HRUS measurement

Ultrasonic Spectroscopy HRUS 102 device (Ultrasonic-Scientific, Dublin, Ireland) was employed. HRUS consists of two independent quartz cells in which the velocity of ultrasonic wave propagation is measured. Whole system is tempered by a 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 and under constant stirring (600 rpm). The full range of ultrasound frequencies (2370, 5110, 5480, 7850, 8220, 11950, 12200 and 14690 kHz) were utilized for half of the samples and when no change of ultrasonic velocity on applied frequency occurred, for the rest only 3 frequencies (5480, 8220, and 12200 kHz) were used (for explanation see below). 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. For easier observation of potential interactions, the concentration increment of ultrasonic velocity (I) was determined using the relation published earlier by Kankia et al. [87] and as in detail described by Kučerík et al. when applied on HS [71] i.e.

$$I = \frac{U_1 - U_2}{U_2 m \rho_2} \quad (9)$$

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.

3.1.2 Results and discussion

The chemical character of the investigated samples is summarized in Tab. 2 and Tab. 3. Generally, the origin of humic substances used in this work covers wide range of sources as pointed out in 2.1.5 IHSS samples chapter.

In Fig. 9, Fig. 10 and Fig. 11 the decreasing dependence of the concentration increment of ultrasonic velocity (I) on concentration can be observed for all humates, fulvates and fulvic acids, respectively. Similar behavior of lignitic humates was observed by Kučerík et al. [71] not only from concentration 0.001 g/L at pH 7 but also at pH 12 and at high ionic strength

(1M NaCl). These observations indicated the progressive aggregation even under conditions that are generally considered as unfavorable for aggregates formation. That conclusion was based on the observation that the increment of ultrasonic velocity in dependency on concentration is not constant but decreases with increasing concentration.

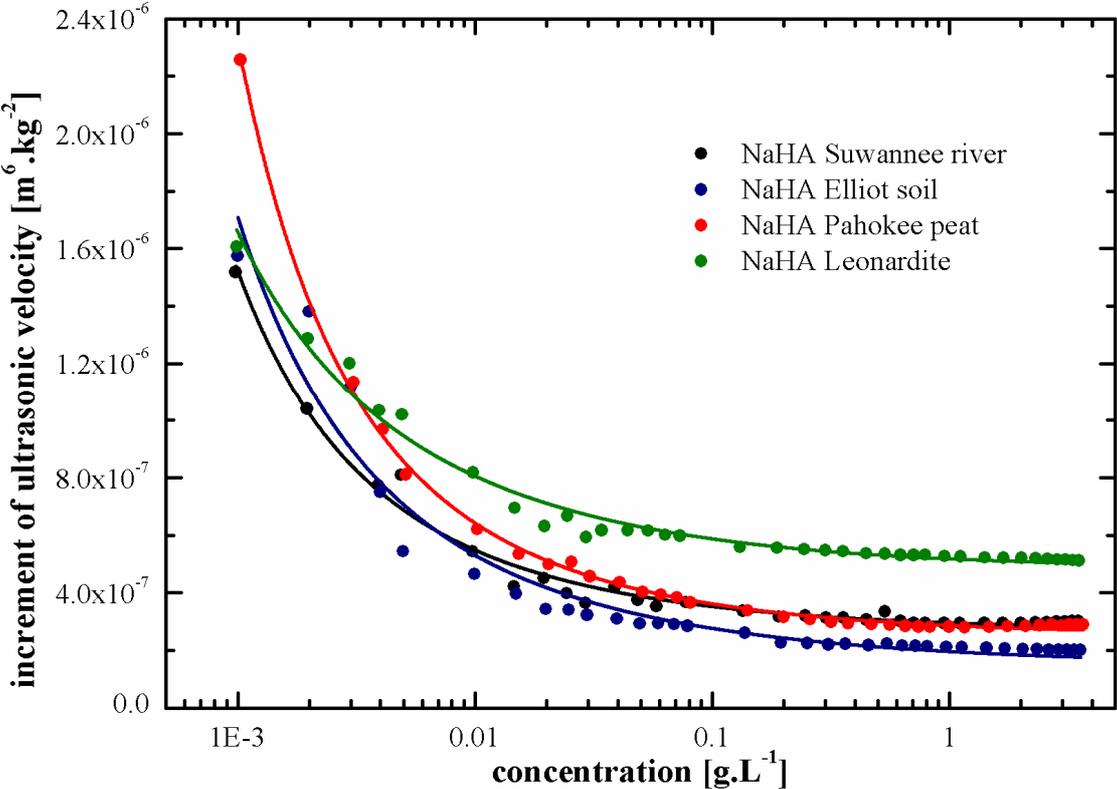


Fig. 9: Dependency of increment of ultrasonic velocity on concentration – humates.

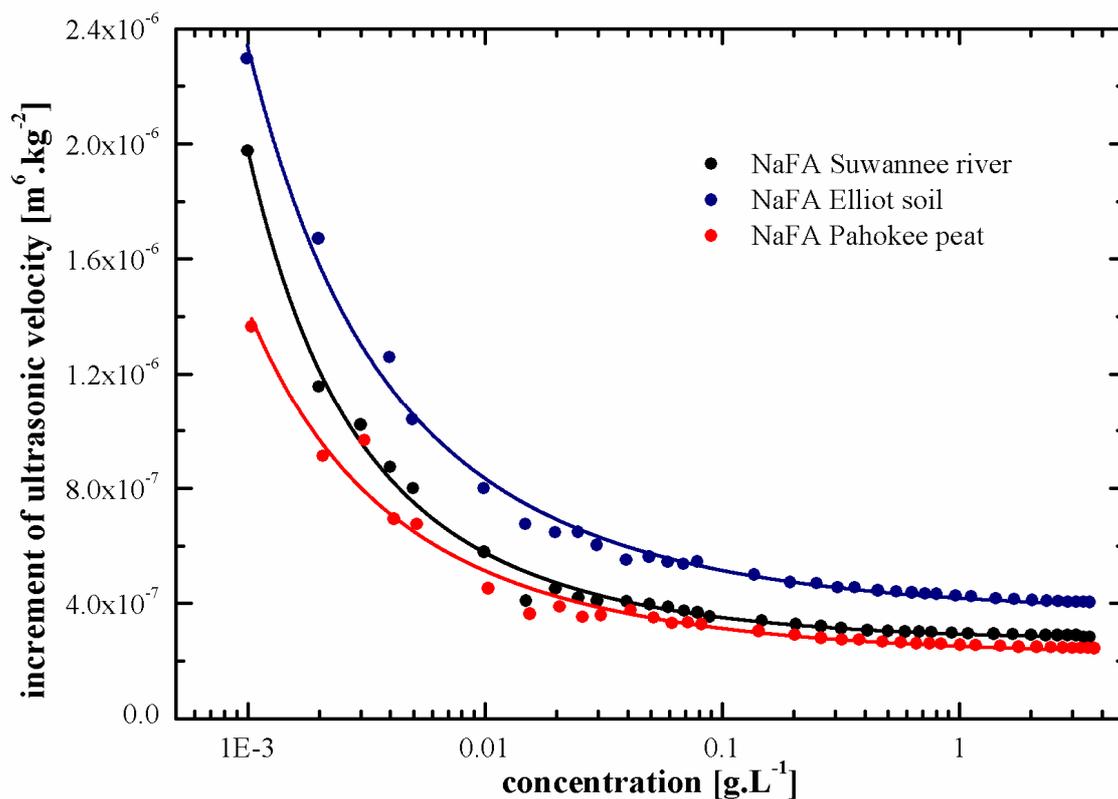


Fig. 10: Dependency of increment of ultrasonic velocity on concentration – fulvates.

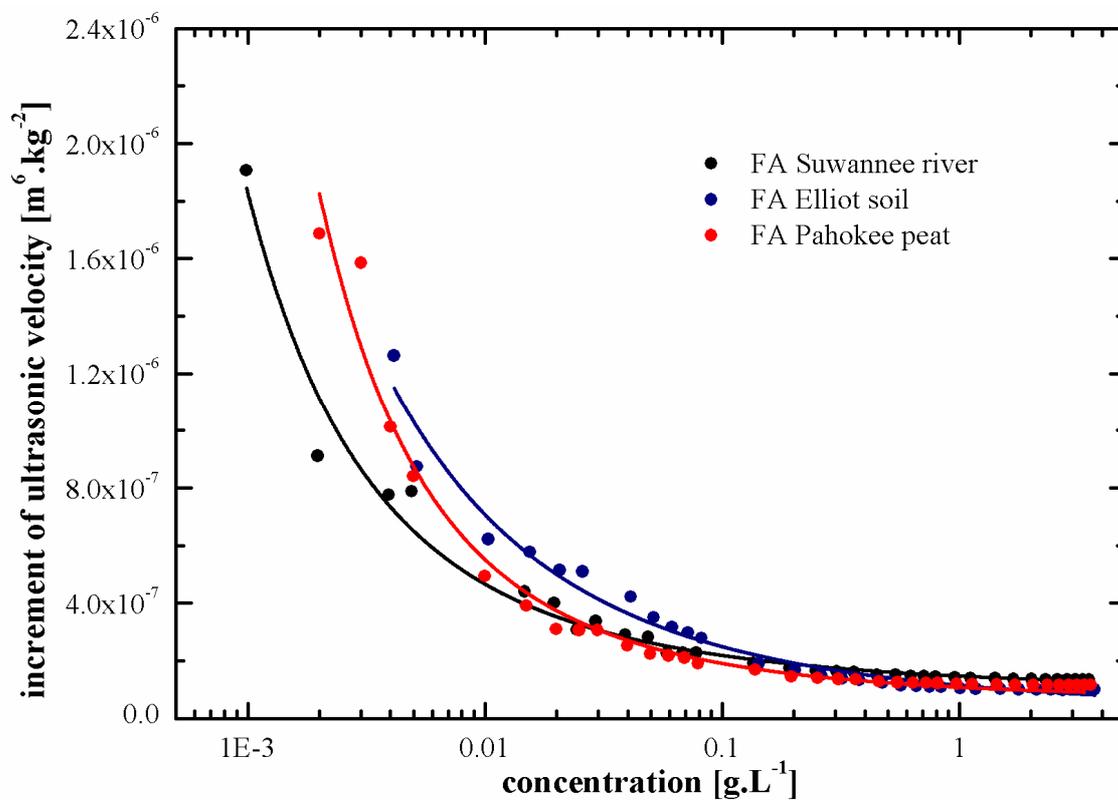


Fig. 11: Dependency of increment of ultrasonic velocity on concentration – fulvic acids.

To justify the application of I for monitoring of aggregation and/or micelle formation Triton® X-100 (oligomeric surfactant) was used. Results are reported in Fig. 12. It can be seen that before the CMC was reached, the concentration increment had practically constant value which corresponds to freely dissolved molecules in solution (i.e. no interaction among surfactant molecules). Those are hydrated and the value U_{12} is proportional mainly to the difference in compressibility of hydration shell of the molecules (lower compressibility in compare to bulk water) and bulk water itself. In other words, lower compressibility and higher density of water structures in hydration shell is more supportive to the ultrasound wave propagation than the bulk (non-interacting) water. That causes the linear increase in U_{12} in dependence on the concentration. However, when the CMC is reached, hydrophobic effect causes the aggregation of molecules in solutions (to put it in better way, micellization of those molecules which are in excess to the CMC). Thus, micelles which consist of less compressible hydration shell on the surface and more compressible hydrophobic interior are formed. As a result the increase in U_{12} becomes slower than before CMC. Mathematically speaking, there is a sharp change corresponding to the CMC in the slope of dependency of ultrasonic velocity on the concentration. In case of dependency of increment of ultrasonic velocity on the concentration, a sharp edge is observable at concentration about 0.13 g/L as indicated in Fig. 12.

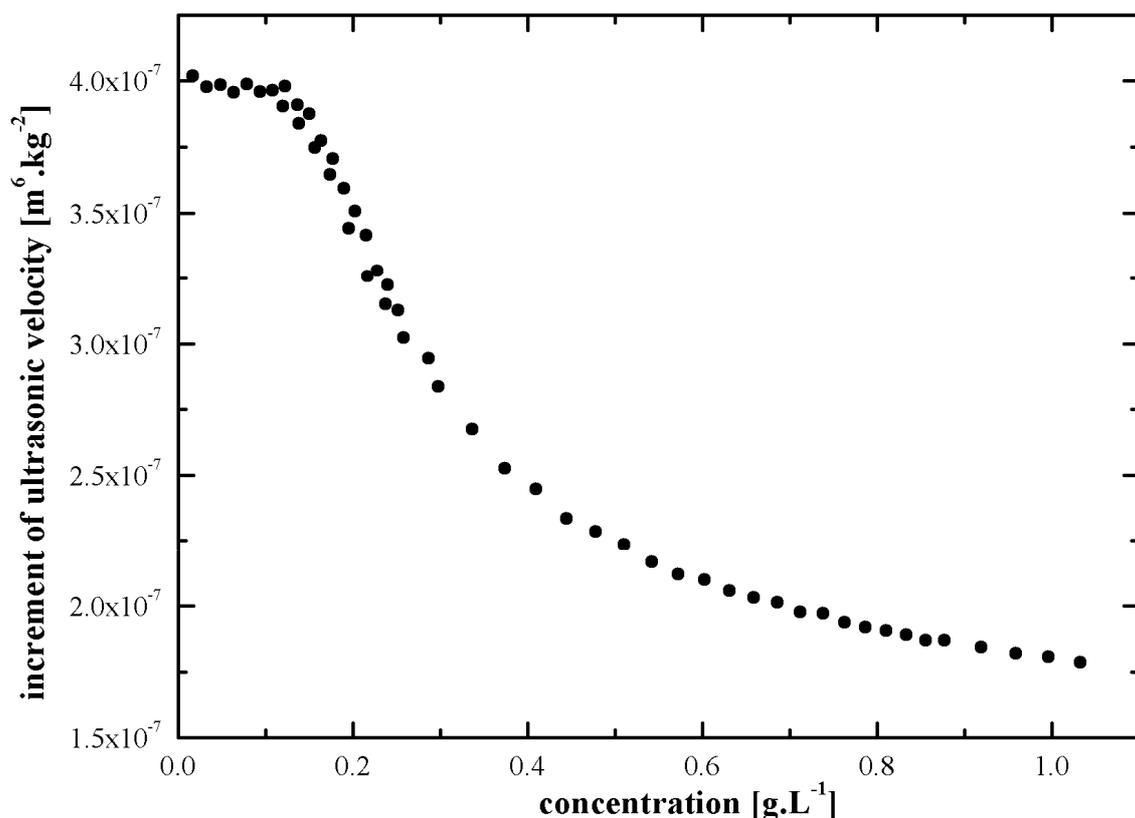


Fig. 12: Dependency of increment of ultrasonic velocity on concentration – Triton X-100.

Fig. 9, Fig. 10 and Fig. 11 report behavior of IHSS standards in concentration range 0.001–3.6 g/L. As is evident from these figures, only the decreasing, second part of above-described processes, i.e. aggregate formation, was observed. On basis of results reported in these figures several statements and hypothesis can be formulated. First of all, both humic and fulvic acids extracted from materials of different origin aggregate at very low concentrations, lower than those usually reported (1–10 g/L) [38,41,42]. Since all HS exhibit similar records one can hypothesize that this indicates similar aggregation mechanism. Furthermore, since all applied frequencies gave the same values of ultrasonic velocity (range within 2370 and 14690 kHz was used) 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. The last statement 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 is not a case of humic samples measured in this study since all the frequencies gave the same results of ultrasonic velocity at all concentrations.

To determine the influence of Na⁺ counterion, records for both H⁺ and Na⁺ forms of FA were performed. Fig. 13 reports the results for Suwannee River FA. Similar results were obtained also for Elliott Soil and Pahokee Peat. As can be seen in Fig. 13, the dependences are relatively similar, the difference between both forms exists mainly at higher concentrations. Na⁺ form showed higher values of *I* at higher concentrations while further dilution causes curves approximation. This is clearly caused by the fact that solution containing Na⁺ form is more supportive to ultrasound propagation and thus the values of *I* parameter are higher than those of H⁺ form. Further, both forms (titrated and non-titrated) show the same shape of the curve, therefore one can assume that the observation on progressive aggregation of humic acids is relevant for fulvic acids in both forms as well.

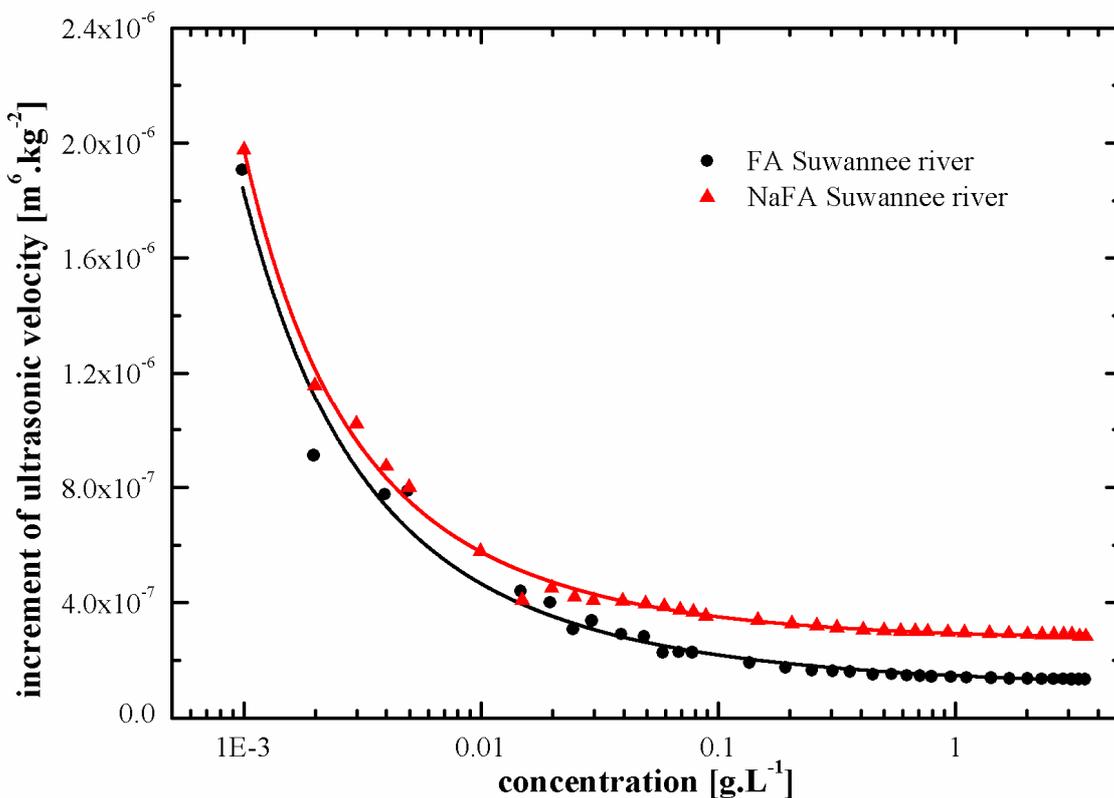


Fig. 13: Dependency of increment of ultrasonic velocity on concentration for H^+ and Na^+ form of Suwannee River FA.

As demonstrated earlier, the parameter I graphically extrapolated to zero concentration can be used as a measure of affinity of molecule to form aggregates [71]. The larger value I the higher affinity of molecules to form aggregates. In principle, it follows mathematical principle derived from the dependence of U_{12} on concentration. The highest deviation of dependency from zero at infinite dilution means higher affinity of molecules to form aggregates. As it can be seen in Fig. 9, Fig. 10 and Fig. 11, there are differences in the curvatures of the dependencies which might contain information on the details of aggregation. Therefore, the attempt was made to find the best fitting equation which can be used to describe such processes. Fig. 9, Fig. 10 and Fig. 11 show fittings gained by using (10)

$$y = ax^{bx^{-c}} \quad (10)$$

where y stands for concentration increment of ultrasonic velocity (I in $m^6.kg^{-2}$), 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”. Obtained parameters a , b and c are summarized in Tab. 4.

Tab. 4: Parameters a , b and c obtained using (10).

	IHSS No	$a \times (10^{-7})$	$b \times (10^{-2})$	$c \times (10^{-2})$
NaHA Suwannee River	2S101H	2.97±0.07	-4.26±0.55	24.81±1.81
NaFA Suwannee River	2S101F	2.94±0.06	-4.11±0.63	27.55±2.39
FA Suwannee River	2S101F	1.48±0.10	-11.75±1.95	16.38±2.15
NaHA Elliot Soil	2S102H	1.9±0.19	-11.04±2.93	15.29±3.44
NaFA Elliot Soil	2S102F	4.19±0.07	-5.41±0.46	22.11±1.18
FA Elliot Soil	2S102F	1.15±0.09	-28.50±4.11	7.01±2.22
NaHA Pahokee Peat	1S103H	2.87±0.03	-5.87±0.28	23.73±0.64
NaFA Pahokee Peat	2S103F	2.52±0.09	-5.91±1.00	20.93±2.36
FA Pahokee Peat	2S103F	1.09±0.13	-17.05±3.71	15.77±3.00
NaHA Leonardite	1S104H	5.18±0.08	-3.11±0.42	24.43±1.94

It is clear that, from the mathematical point of view, equation (10) cannot be used to extrapolate the data to zero concentration. This may suggest a possible aggregation even below the concentration 0.001 g/L and/or limitation of this equation for a range of concentrations.

To shed light on the principles of aggregation of humic substances in solution the relationship between parameters of equation (10) and composition of HS was searched. 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). For this correlations only Na⁺ forms of humic substances were used since H⁺ forms (i.e. fulvic acids), as is clearly indicated in Fig. 13, can slightly differ in aggregation principles especially at higher concentrations. Correlation analyses showed that NaHA Leonardite also 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. 5. 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. 5: Correlation of fitting parameters of equation (10) 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
a	0.685	0.461	0.171	0.005	0.426	0.643	0.081	0.330	0.102	0.335
b	0.661	0.433	0.509	0.522	0.702	0.625	0.009	0.639	0.589	0.788
c	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. 5). 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.

For the measurement and evaluation of propagation of ultrasonic waves through a sample, a mathematical description has been developed. The sound energy density w is an adequate measure to describe the sound field at a given point as a sound energy value. The formula for sound energy density of sound waves propagating through the medium is

$$w = \rho U^2 \quad (11)$$

where U is sound velocity in the solvent and ρ is mass density of the solvent. This mass density has generally fractal distribution. In papers [88,89] the density of fractal physical quantity $F(r)$ in E -dimensional Euclidean space E_n ($E = n$) was defined. Accordingly, for the density of media $\rho(r)$ ($\text{kg} \cdot \text{m}^{-E}$) one can write [90]

$$\rho(r) = k_\rho F(r) = k_\rho K r^{D-E} \quad (12)$$

where k_ρ is constant (elementary mass, kg), r is the radius of elementary quantity, K the fractal measure (in m^{-D}) and D is the fractal dimension. It is clear that in homogeneous medium ($D = E$) the mass density is constant, while for the fractal distribution of mass ($E - 2 < D \leq E$, $E = 3$, in 3-dimensional space), the mass density decreases with the distance from the source by $(D-3)$ power. For radial concentration field (in E -dimensional space) one can write the dependence of potential (proportional to quadrate of velocity) on radius using [58]

$$V(r) = U^2(r) = k_v \frac{K r^{D-E+2}}{D(D-E+2)} \quad (13)$$

where k_V is constant in $\text{m}^E \text{s}^{-2}$. This equation simply says that in the space with constant density of mass $\rho(r)$ (i.e. for $D = E$) the quadrate of velocity increases with second power of r . In the case of fractal distribution of mass density (i.e. for $E - 2 < D \leq E$, $E = 3$, in 3-dimensional space) the square of velocity (potential) decreases with the distance by $(D-1)$ power. From the mass density (12) and from the quadrate of velocity (13) the density of energy (in $\text{J} \cdot \text{m}^{-E}$) can be determined as follows

$$w(r) = \rho(r)U^2(r) = k_\rho k_V \frac{K^2 r^{2(D-E+1)}}{D(D-E+2)} \quad (14)$$

Finally it can be obtained the dependence of velocity of sound in continuum on density of media with fractal structure

$$U = \sqrt{\frac{k_V K}{D(D-E+2)}} \left(\frac{\rho}{k_\rho K} \right)^{\frac{D-E+2}{2(D-3)}} \quad (15)$$

Let's first consider the properties of mass transfer in the three-dimensional space ($E = 3$) just for the fractal dimensions $D \in (1,3)$ of concentration. In this case, the velocity (15) can be rewritten using equation

$$U = \sqrt{\frac{k_V K}{D(D-1)}} \left(\frac{\rho}{k_\rho K} \right)^{\frac{D-1}{2(D-3)}} \quad (16)$$

Since $U = U_1 - U_2$ and $F(r) = (\rho_1 - \rho_2) / k_\rho = c / k_c$ (see (12)), where U_1 is the ultrasonic speed in the sample with mass density $\rho_1(r)$ and concentration of humate in water $c(r)$ and where U_2 is speed of sound in pure water with mass density $\rho_2(r)$ one can analogically write

$$U_1 - U_2 = \sqrt{\frac{k_V K}{D(D-1)}} \left(\frac{\rho_1 - \rho_2}{k_\rho K} \right)^{\frac{D-1}{2(D-3)}} \quad (17)$$

In fact, it is a sound velocity related to the system associated with sound waving in pure solvent. Sound propagation depends on the density of the hydration shell of dissolved humates, i.e. increases with concentration. Thus, D is in this case fractal dimension of humate aggregates in water. Accordingly, equation (17) can be rewritten as follows:

$$U_1 - U_2 = \sqrt{\frac{k_V K}{D(D-1)}} \left(\frac{c}{k_c K} \right)^{\frac{D-1}{2(D-3)}} \quad (18)$$

where $(\rho_1 - \rho_2) / k_\rho = c / k_c$. Equation (18) can be formally written as

$$U_1 - U_2 = A c^a \quad (19)$$

while

$$D \approx \frac{6a-1}{2a-1} \quad (20)$$

Assuming that fractal dimension D is constant, for $k_c K = 1$ it is possible to determine the ratio k_V / k_c , i.e.

$$\frac{k_v}{k_c} = D(D-1)A^2 \quad (21)$$

In addition to ultrasound velocity and concentration increment several additional parameters were calculated utilizing these mathematical derivations. Fig. 14 reports the dependence of several calculated coefficients on concentration.

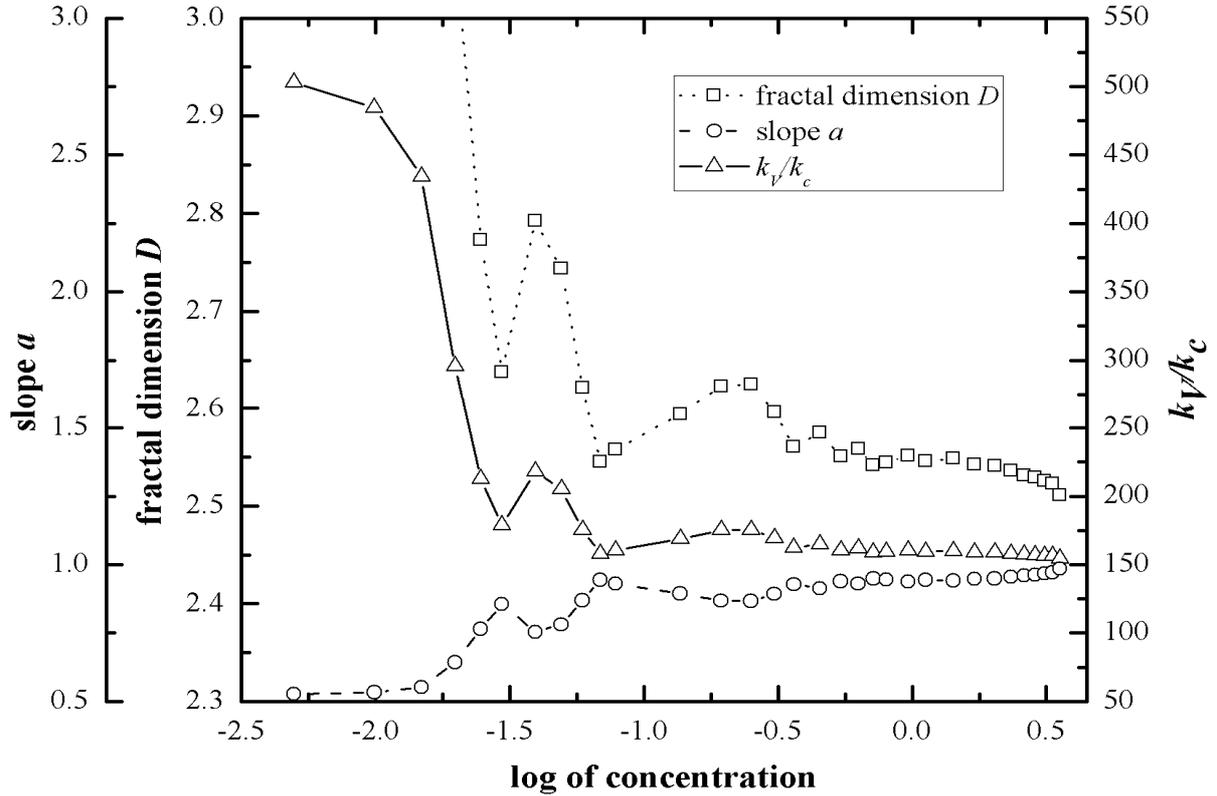


Fig. 14: Dependence of coefficients D (fractal dimension), slope a and k_v/k_c on the concentration for NaFA Elliot Soil.

All the dependences indicate that this approach is useful for concentration 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 [51]. The fraction k_v/k_c shows the relationship between elemental quantity of ultrasonic velocity k_v (Equ. (13)) and elemental quantity of concentration k_c or k_p in (Equ. (12)).

Fig. 15 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. [51] 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^+ .

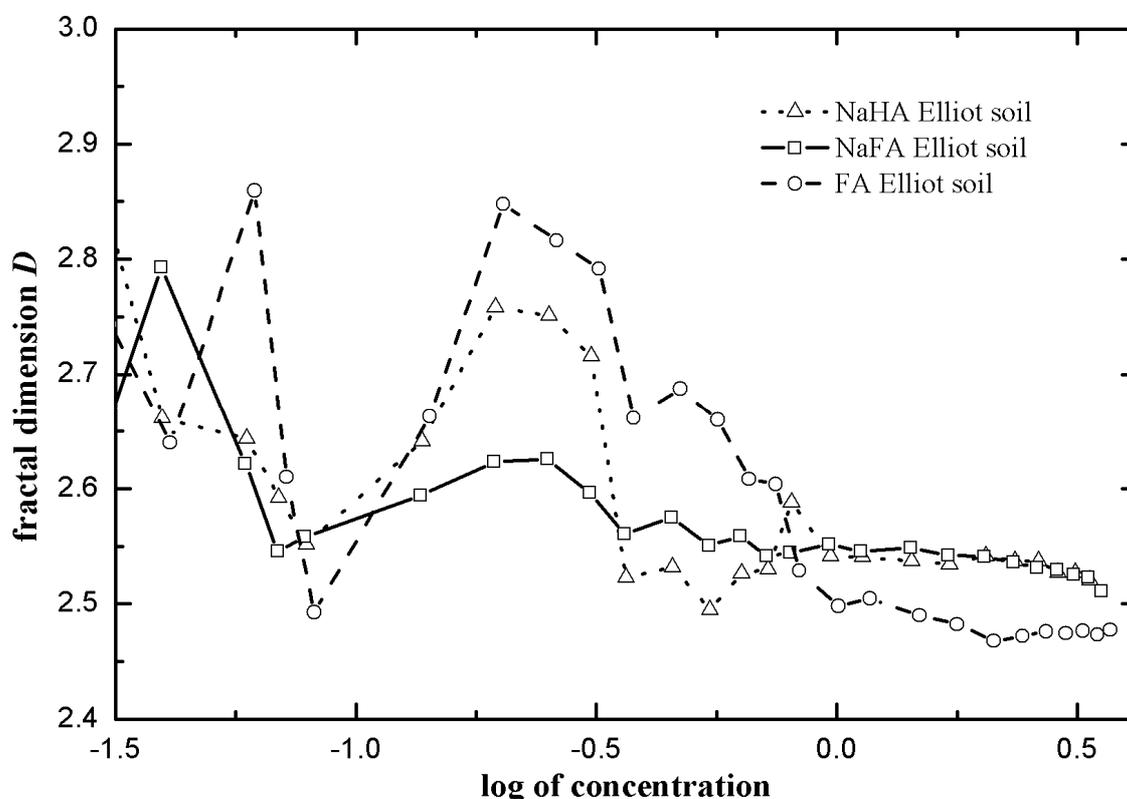


Fig. 15: Comparison of fractal dimensions of Elliott Soil humate, fulvate and fulvic acid.

3.1.3 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), a quaternary ammonium surfactant. In Fig. 16 the dependency of concentration increment on concentration of the aqueous TTAB solution is depicted. The same explanation as in the case of Triton® X-100 can be applied. In short, interval of constant dependency corresponds to premicellar state. Increase of TTAB molecules leads to linear increase in

amount of hydration shells. When concentration increment of ultrasonic velocity I was calculated according to

$$I = \frac{U_1 - U_2}{c} \quad (22)$$

where U_1 and U_2 stand for ultrasonic velocity (in m/s) in the sample and reference cell, respectively, and c stands for concentration (in g/L), clearly, constant value must be obtained 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.

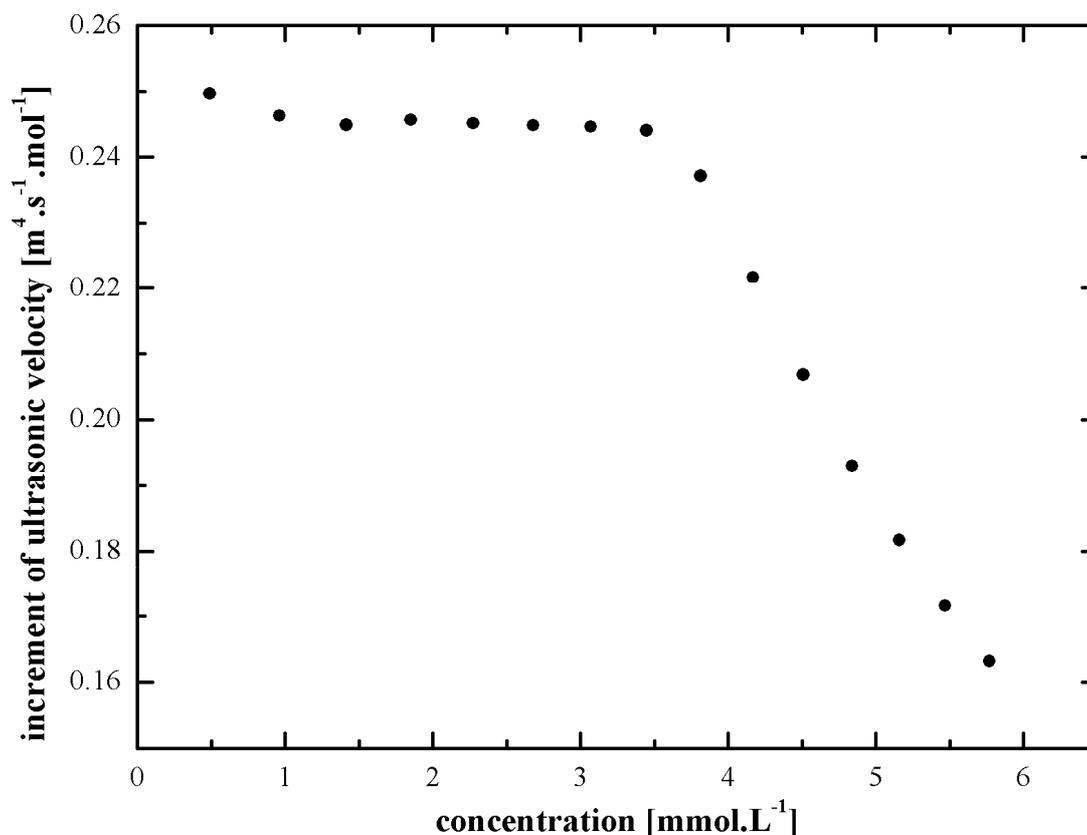


Fig. 16: Dependency of the concentration increment of ultrasonic velocity on concentration for the TTAB aqueous solution.

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 Fig. 17.

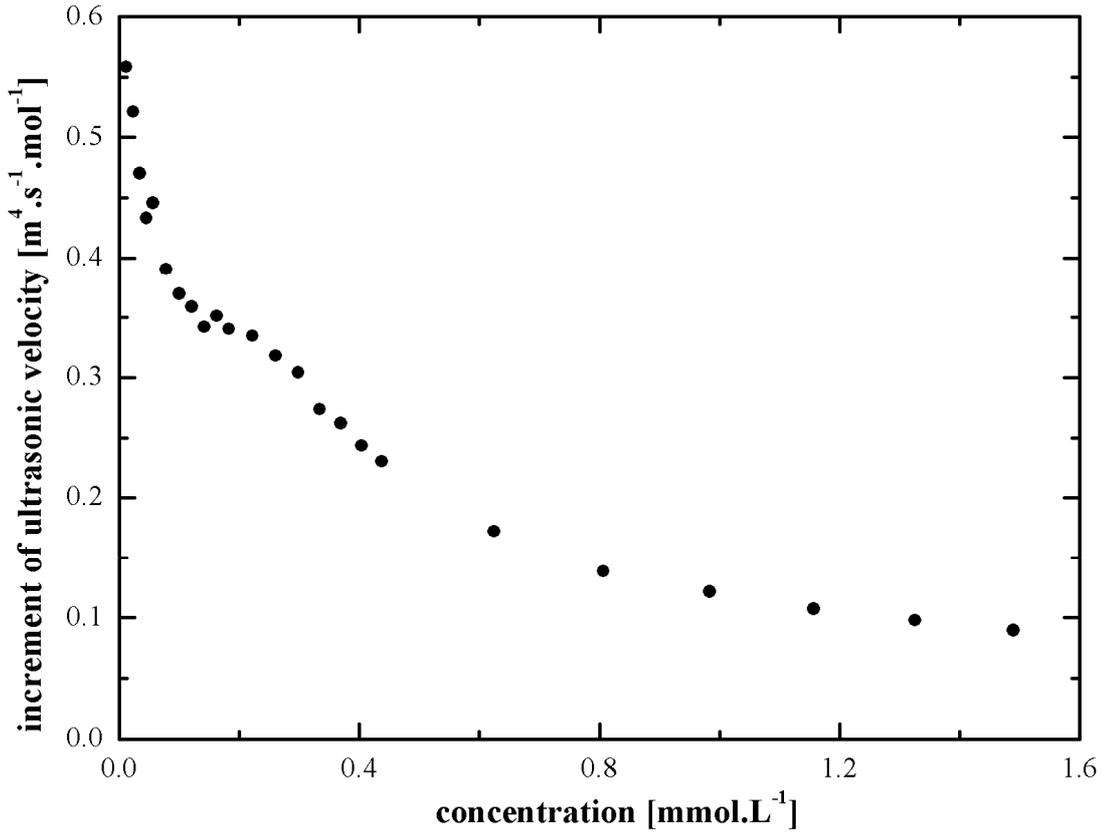


Fig. 17: Dependency of the concentration increment of ultrasonic velocity on concentration for the TTAB in 0.15M NaBr solution.

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

$$I = \frac{U_1 - U_2}{c} = k \quad (23)$$

where k represents a constant. When rewriting this to

$$U_1 - U_2 = k \cdot c \quad (24)$$

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 some extent a shift was present. Accordingly (24) can be adjusted by adding an intercept parameter e

$$U_1 - U_2 = k \cdot c + e \quad (25)$$

Dividing both sides of (25) by concentration, concentration increment is expressed on the left side

$$I = \frac{U_1 - U_2}{c} = k + \frac{e}{c} \quad (26)$$

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 is observable for concentrations far beyond the real CMC (Fig. 17).

Nevertheless, when dependence of difference in ultrasonic velocity on concentration was used to assess the CMC a sharp edge was observed at CMC (Fig. 18). This example clearly demonstrates the inability of the concentration increment to be a precise guideline for CMC determination under every situation [91].

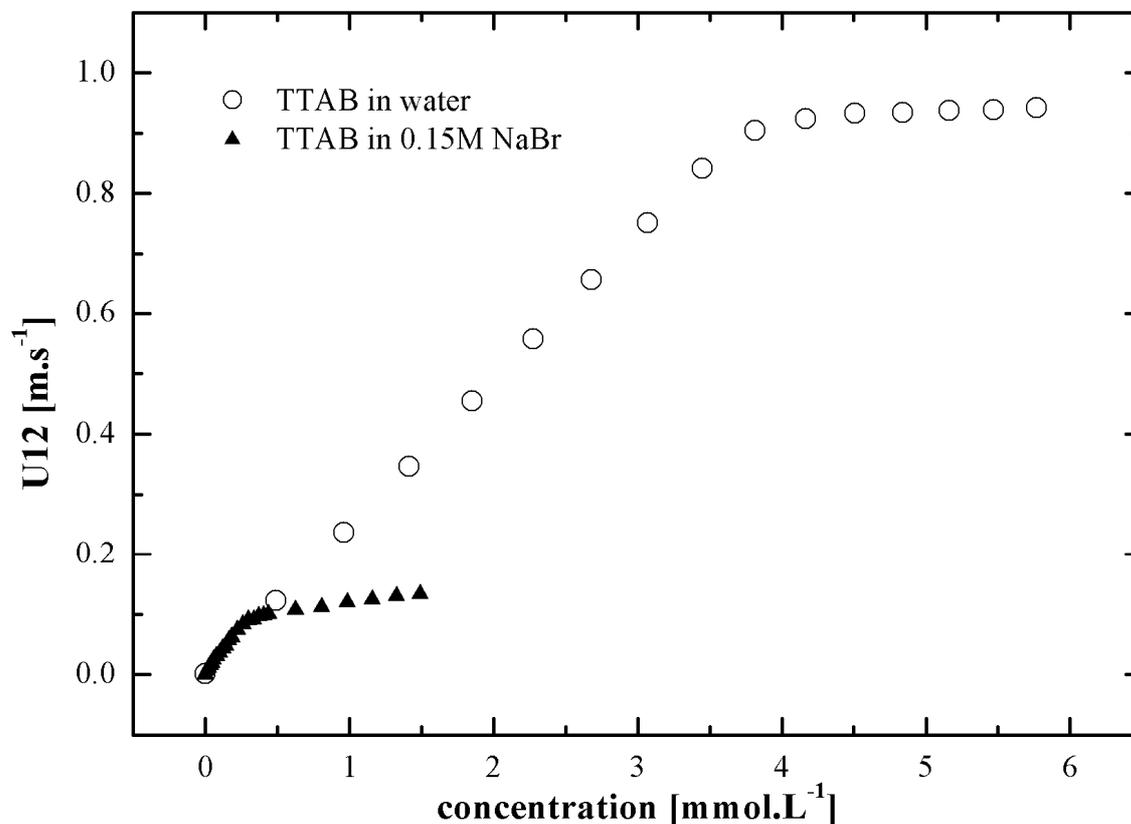


Fig. 18: Dependence of the difference of ultrasound velocity $U12$ on concentration of TTAB in water and in 0.15M NaBr.

When applied this linear approach on 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. In Tab. 6 the linear regression slopes are summarized.

Tab. 6: Calculated slope for the IHSS standards.

Name	IHSS No	Slope [$\text{m}^4 \cdot \text{s}^{-1} \cdot \text{kg}^{-1}$]
NaHA Suwannee River	2S101H	0.4452
NaFA Suwannee River	2S101F	0.4307
FA Suwannee River	2S101F	0.2015
NaHA Elliot Soil	2S102H	0.3007
NaFA Elliot Soil	2S102F	0.6033
FA Elliot Soil	2S102F	0.1611
NaHA Pahokee Peat	1S103H	0.4261
NaFA Pahokee Peat	2S103F	0.3648
FA Pahokee Peat	2S103F	0.1715
NaHA Leonardite	1S104H	0.7613

It can be noticed that slopes calculated for protonated fulvic acids are 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.

3.1.4 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 successfully 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 carbon 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 in concentration range from 0.03 to 3.5 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.

3.2 IHSS samples – non-isothermal measurements

Following the notion obtained in the previous part, 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 to i) 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 concentration as suggested in previous paragraphs where increment of ultrasonic velocity and fractal dimensions were used to detect the aggregation, 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.

3.2.1 Experimental part

3.2.1.1 Samples preparation

Standards of humic acids Suwanee River (1S101H) and Leonardite (1S104H) and fulvic acids Suwanee River (2S101F), Elliott Soil (2S102F) and Pahokee Peat (2S103F) were purchased from IHSS. To obtain sodium humates and fulvates each sample was dissolved in distilled water and titrated to pH 7.2 by 0.1M NaOH in automatic titrator (TitroLine® alpha plus, Schott/SI Analytics, Mainz, Germany). After reaching constant value at least for one hour, the solution was freeze-dried. Obtained product was homogenized and stored at room temperature in sealed containers. The measured solutions of 0.01, 0.1, 1 and 4 g/L were prepared in distilled water and well stirred.

3.2.1.2 HRUS measurement

Ultrasonic Spectroscopy HRUS 102 device (Ultrasonic-Scientific, Dublin, Ireland) was employed. HRUS consists of two independent quartz cells tempered by common water bath. Cell 1 serves as a sample cell and was loaded up by 1 ml of the sample solution whereas cell 2 is 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 on the bottom of both cells by rod stirrers. 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.

3.2.2 Results and Discussion

Rearranging equation (6), the speed of sound in liquids can be expressed as

$$U^2 = \frac{1}{\rho \beta_s} \quad (27)$$

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 speed 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 dependency is shown in Fig. 19. It is a matter of fact, that any other substances which do not undergo any transition in the temperature interval under study give only monotonic increase or decrease depending on their chemical character and nature of solvation forces.

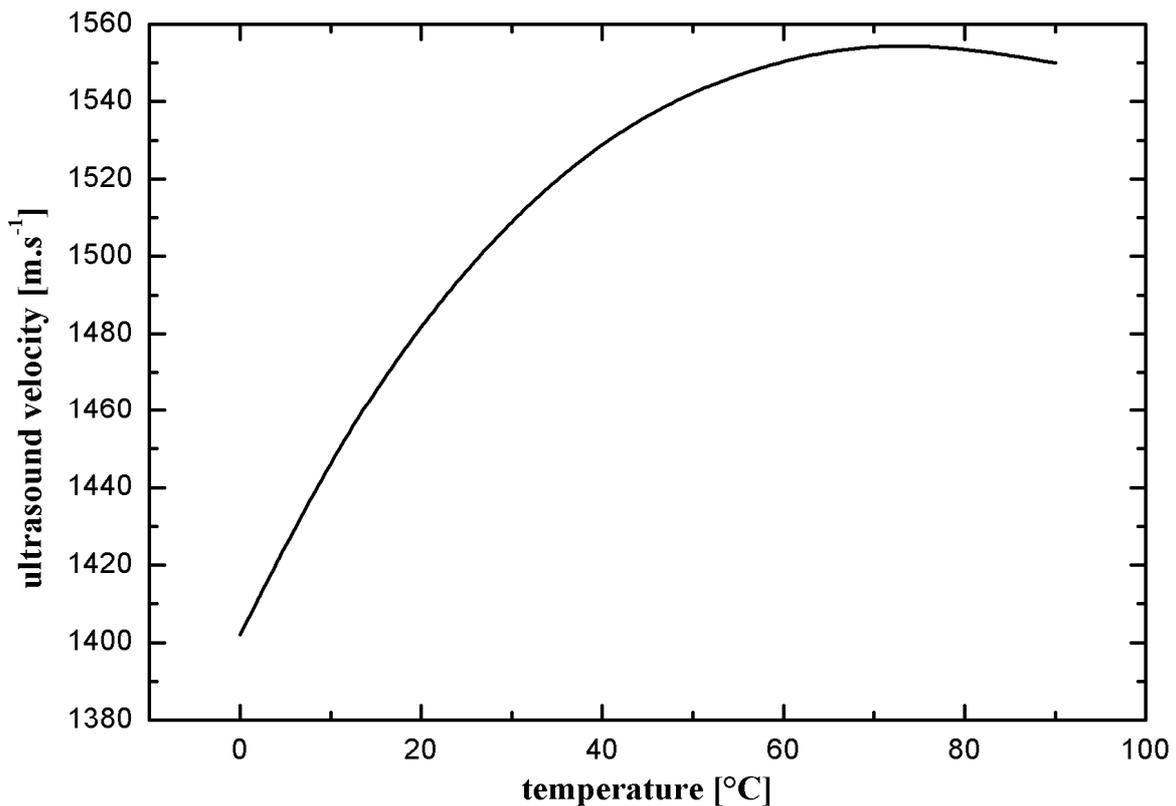


Fig. 19: Dependency of ultrasound velocity on temperature at 0.1 MPa for distilled water [92].

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. 20.

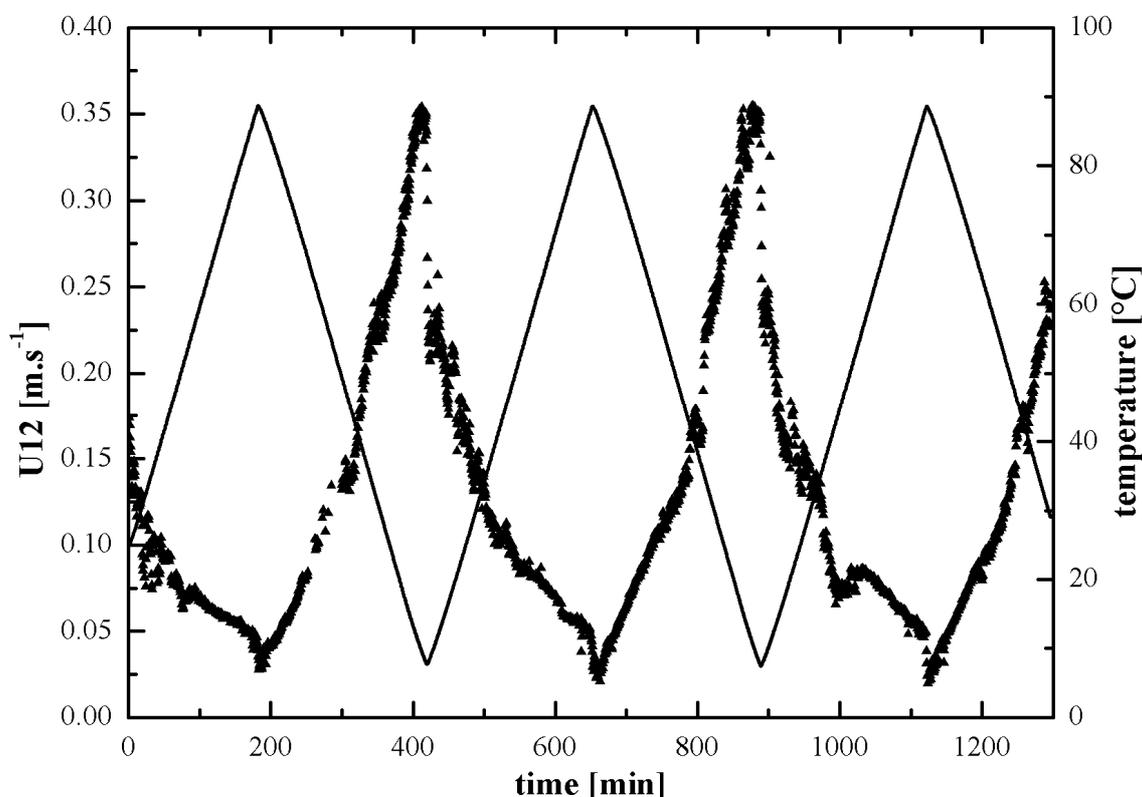


Fig. 20: Difference in ultrasonic velocity (sample–water) and temperature program (NaHA Suwannee River, 1 g/L).

The whole temperature program involved 3 steps. The first heating cycle (from 0 to 420 min in Fig. 20) 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. 20) was used for the measurement itself, i.e. 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.

First, the U_{12} records depicted in following figures represent mainly changes in hydration of humic molecules and their aggregates. In principle, higher values indicate larger hydration shell since such water layer is more supportive for (ultra)sound wave propagation – unlike the free (bulk) water molecules, water molecules present in the hydrophilic hydration shell are less compressible and thus more rigid from the mechanical wave propagation point of view. Furthermore, the changes in U_{12} can be also attributed to formation and decomposition of micelle-like humic and fulvic domains which are relatively soft and propagation of ultrasonic wave can cause their compression associated with a decrease of its amplitude and frequency.

Next four figures show the first part of the second heating cycle of sodium salt of Suwannee River fulvic acid IHSS sample (2S101F). Four different concentrations were chosen to cover wide concentration spectrum – from the lowest concentration of 0.01 g/L which is close to the edge of device resolution, 0.1 and 1 g/L as intermediate concentrations and up to 4 g/L. Higher concentrations were not used since as reported recently [51] their thermal treatment did not show any remarkable results (transitions) except a monotonic decrease of U_{12} in dependency on temperature. As it can be seen from following records the temperature dependence of differential ultrasonic velocity dramatically changes with changing of humate and/or fulvate concentrations. Concentration of 4 g/L showed more or less a monotonous exponential-like decay for all samples (Fig. 21).

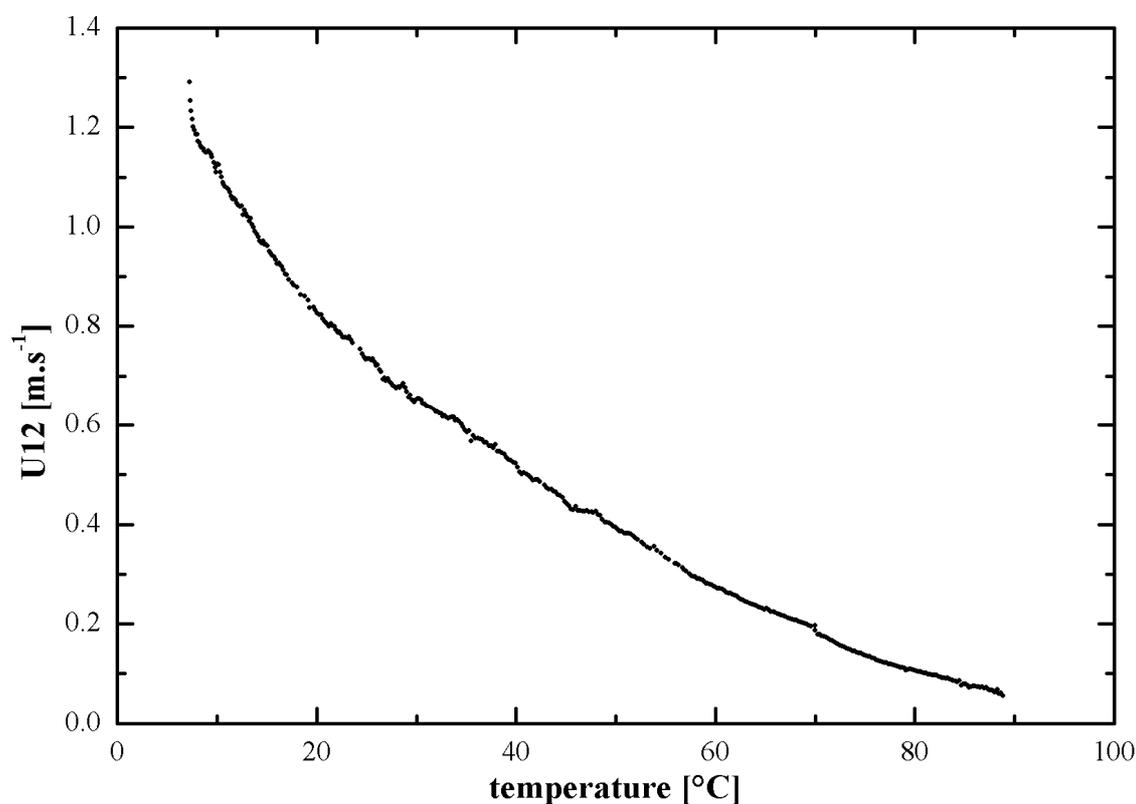


Fig. 21: Difference in ultrasonic velocity for NaFA Suwannee River (2S101F), 4 g/L.

Similarly as reported recently for lignite humates, at this concentration, aggregates with mostly hydrophilic surface are present in solution. This surface is surrounded by hydration shell that is due to the 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 fact that the hydration shell is weakened 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. Different situation is in the core of aggregates. With increasing temperature the strength of the attractive hydrophobic interactions among aggregating humic molecules slightly increases. A shorter distance between humic molecules results in higher density and lower compressibility of a compressed hydrophobic structure. In addition, a decrease in mutual

repulsion of charged head-groups induced by elevated temperature can be expected as well [51,71,93,94].

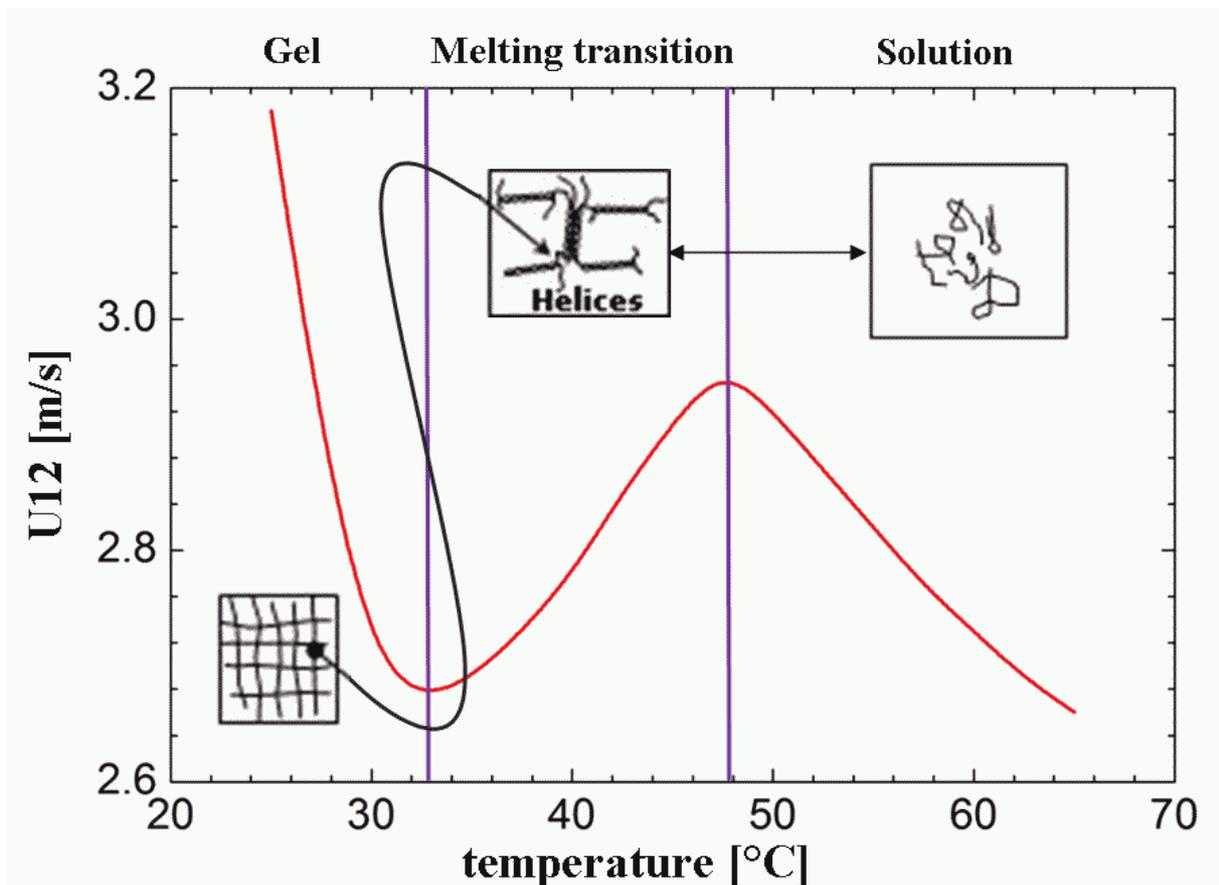


Fig. 22: Dependence of ultrasonic velocity on temperature for carrageenan; adopted from www.ultrasonic-scientific.com.

To make next paragraphs more clear an analogy is presented here. Carrageenans are linear polysaccharides extracted from seaweeds and extensively used in food industry as additives. In the first part of the graph, the difference of ultrasound velocity U_{12} decreases because of the same reasons as explained in previous paragraph. However, at the temperature of approximately 33 °C the melting and unfolding of carrageenan helices occurs and newly raised hydrophilic surface can be hydrated so new hydration shells are formed. That leads to the increase of U_{12} . But at some point – at equilibrium temperature which is in this case about 48 °C – no new surface that can be hydrated occurs and the U_{12} decrease can be observed because of the same reason as in the first part of this dependency.

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. 23).

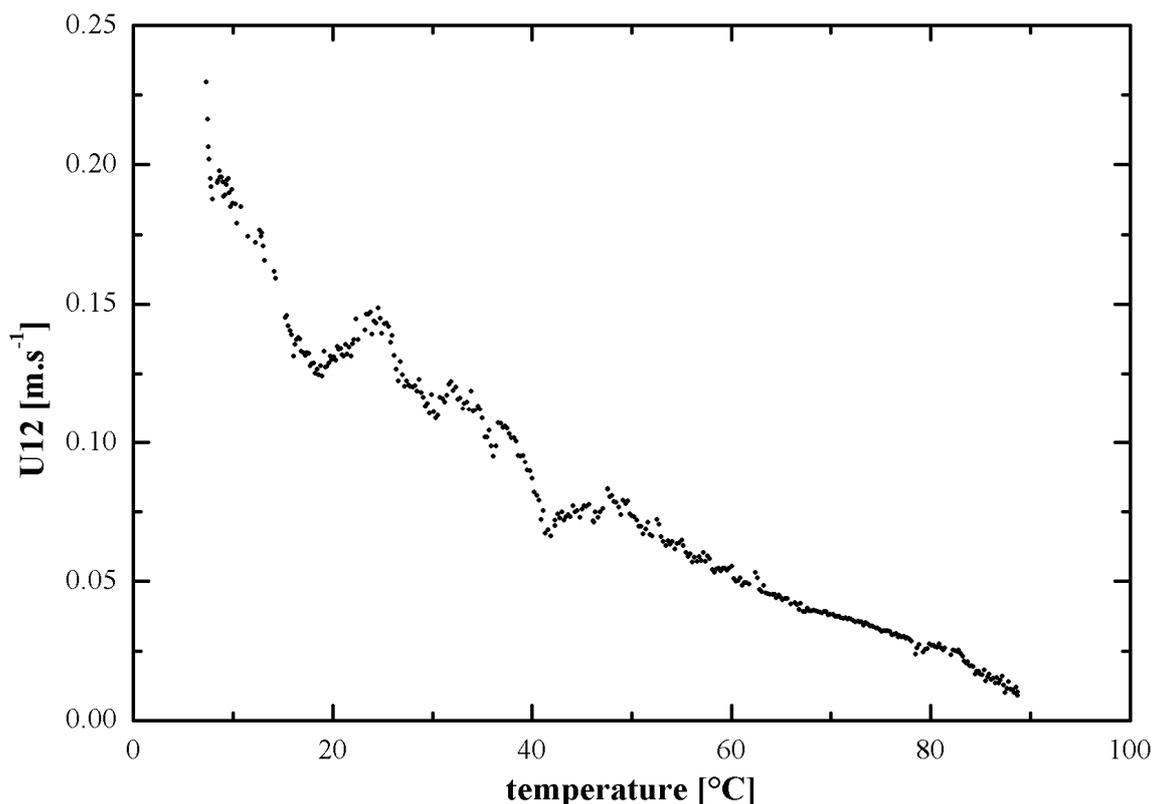


Fig. 23: Difference in ultrasonic velocity for NaFA Suwannee River (2S101F), 1 g/L.

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. [95] 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.

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. It has been already stated that at this concentration 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 [51,72,94].

Hydrophobic effect, which is responsible for aggregate formation is driven by several factors. One of them is high cohesive energy (how much energy is needed to break all interactions among one water molecule and its neighbors, i.e. infinite isolation), another is surface tension of water. This means that a lot of energy is needed to form a cavity in the bulk of water.

Furthermore, water molecules prefer to interact with each other than with non-polar organic molecules. All these aspects can be summarized as an enthalpic factor. There is another important factor – entropy. When two non-polar molecules interact in water, ΔG° would be negative. On the other side, ΔH° is in such situations usually positive, although small. This means that ΔS° must be positive although one would expect to be negative since the formation of aggregate is connected with better organization of the system. These facts altogether means that entropic factor plays the crucial role during the aggregation process.

Water is a dynamic system which means that H-bond interactions among water molecule and their four neighbors are not stable and rearrangement of molecules proceeds whole time. Only in ice one can observe ideal rigid structure of water molecules utilizing all four possible H-bonds. In ice because of higher amount of H-bonds, the enthalpy is a little bit lower in compare to liquid water but more importantly, the entropy is much lower because of high organization. Similar situation take place in water when adding hydrophobic molecules. This means that if a water molecule in contact with these non-polar molecules loses some H-bond interactions with its neighbors, it will try to compensate this loss by strengthening remaining H-bonds. Therefore, water molecules in hydrophobic hydration shells are more rigid, bonds among them are stronger and they start to resemble ice molecules. All together this leads to lowering enthalpy and entropy analogously as described for ice a few lines upward.

Summarizing all the statements, hydrophobic molecules do not aggregate because they are attracted to each other but rather water molecules repel them to minimize amount of hydration molecules and to form more H-bonds among themselves [96].

At concentration 0.1 g/L (Fig. 24) and below it, obtained records showed significantly different feature. Unlike the higher concentrations, the increase of U_{12} with increasing temperature was observed. Several breaks disrupted monotonousness of the recorded increase, mainly at lower temperatures. The only exception of this behavior gave Pahokee Peat fulvate (2S103F). In this case the initial increase up to 20 °C was followed by a decrease. This diminution to almost initial level stopped at 38 °C and it was changed by a small increase up to 45 °C. Though little fluctuations were still present the almost horizontal trend was obtained and continued up to 88 °C which represents the upper limit of the measurement.

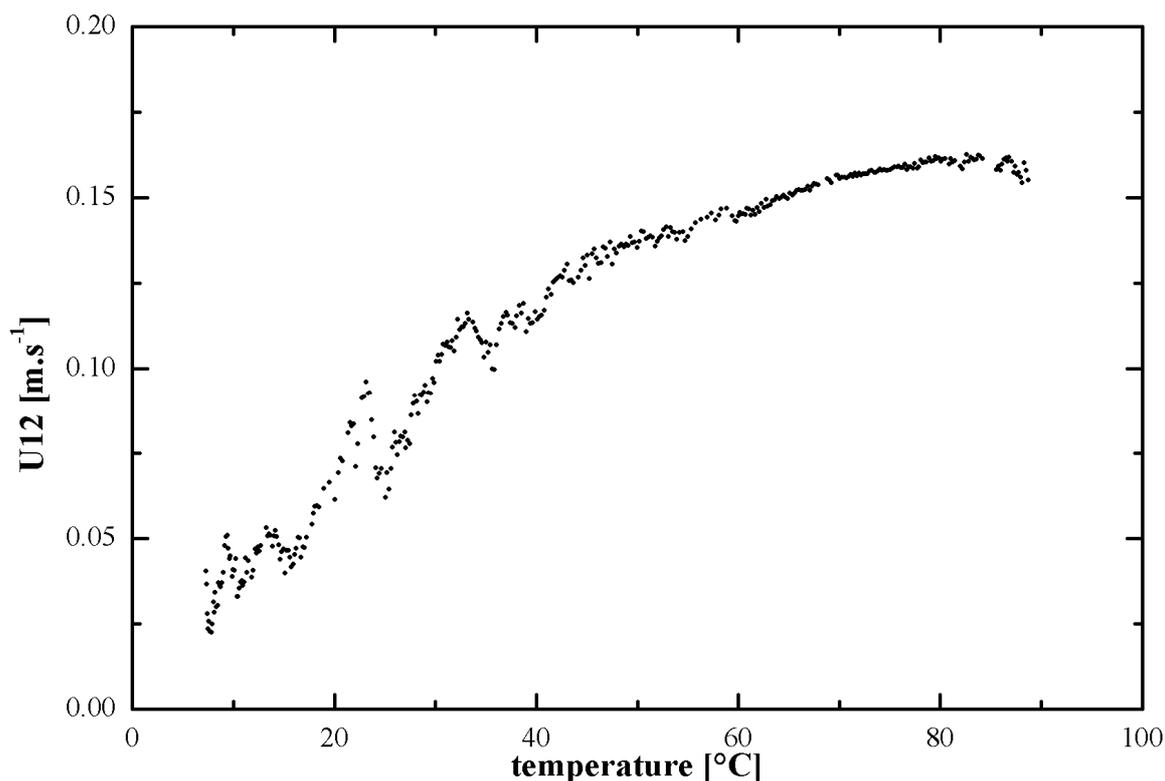


Fig. 24: Difference in ultrasonic velocity for NaFA Suwannee River (2S101F), 0.1 g/L.

The explanation of such behavior was published recently [51]. The dilution of humates solutions at 0.1 g/L leads to formation of aggregates with predominantly hydrophobic exterior. At some concentration or more likely range of concentrations, the nature of prevailing interactions holding together humic assemblies is dramatically changed [95]. 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. As tested in separate experiments, the temperatures of those transitions are independent of temperature regime, therefore, their nature is of thermodynamic origin and thus they can be considered as phase transitions. Since there is no information about the heat capacity of such processes, the order of transition is still unknown.

With further dilution to 0.01 g/L the situation remains similar as described for 0.1 g/L (Fig. 25). 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. This notion is important for understanding of reactivity of humic substances in natural systems and explains the higher sorption ratio between humic substance and organic hydrophobic pollutants at higher concentrations in comparison with lower ones. Further, it also better explains the transport of dangerous compounds such as DDT and PAHs in nature using fulvic acids since those occur in nature in low concentrations which rarely extend hundreds of ppm.

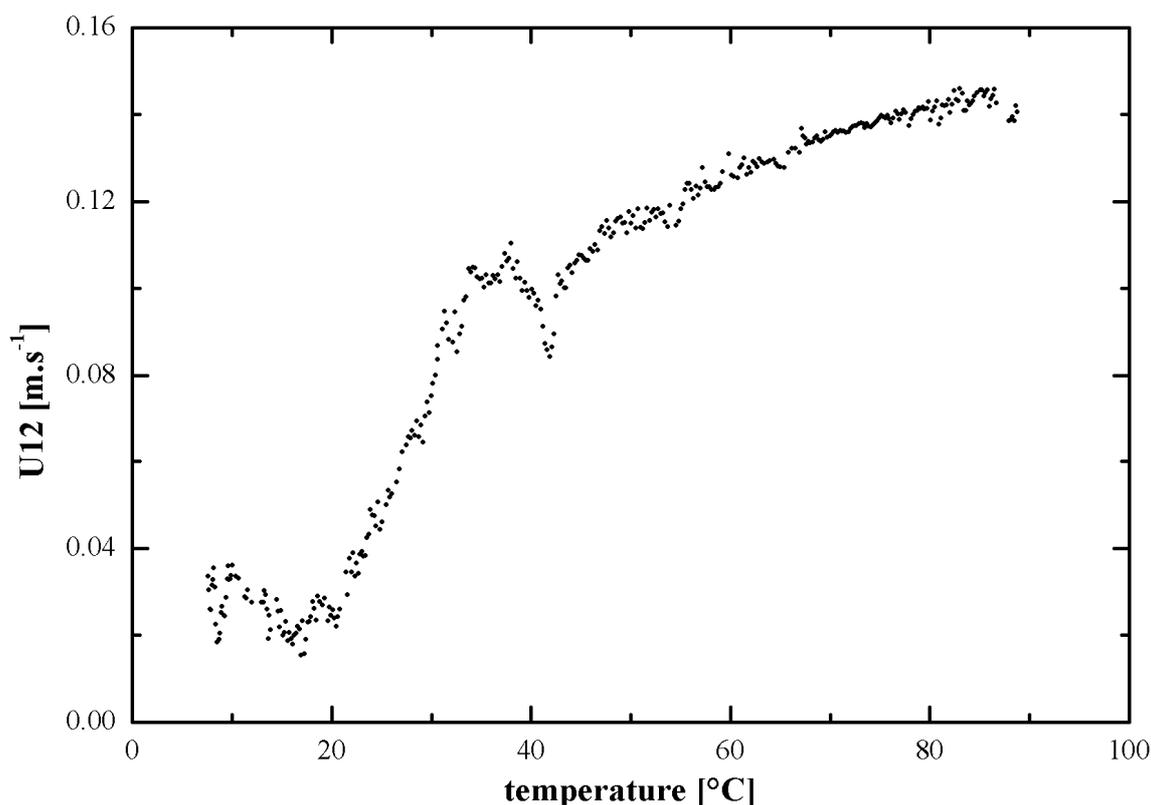


Fig. 25: Difference in ultrasonic velocity for NaFA Suwannee River (2S101F), 0.01 g/L.

Furthermore, the basic chemical composition (elemental analysis and carbon distribution in functional groups as revealed by liquid state ^{13}C NMR) was compared with the slope of ultrasonic velocity decrease of IHSS standards at concentration of 4 g/L as measured by HRUS (Tab. 7). In fact, the decrease in U_{12} is associated with a weakening of hydration shell surrounding the hydrophilic surface of aggregates. Its change without breaking of aggregates structure should be proportional to the polarity of the aggregate itself while for the charge density and distribution are responsible functional groups containing O and N. Those electronegative atoms should also influence the stability of aggregates reflected by the temperatures in which the breaks in U_{12} occurred. The slope between 10 and 80 °C was observed steeper in line: NaFA Elliott Soil, NaHA Leonardite, NaHA Suwannee River, NaFA Pahokee Peat and NaFA Suwannee River.

Tab. 7: Elemental composition (atomic percent) and carbon distribution (peak area percentage) of HA and FA standards (C. C – Carboxyl carbon, Ar. C – Aromatic carbon, Al. C – Aliphatic carbon, Ar/Al C – Aromatic to aliphatic carbon ratio) [54].

Name	C	H	O	N	C/O	C/H	C. C	Ar. C	Al. C	Ar/Al C
NaFA Elliott Soil	36.8	37.4	23.5	2.4	1.57	0.98	25	30	22	1.36
NaHA Leonardite	48.2	33.3	17.7	0.8	2.72	1.45	15	58	14	4.14
NaHA Suwannee River	38.6	37.5	23.2	0.7	1.67	1.03	15	31	29	1.07
NaFA Pahokee Peat	40.1	32.9	25.4	1.6	1.58	1.22	28	34	20	1.70
NaFA Suwannee River	38.2	37.9	23.5	0.4	1.62	1.01	17	22	35	0.63

In Tab. 7 the samples are arranged according to the slope of ultrasonic velocity decrease. The sample with the most gradual slope is on the top, the sample showing the steepest slope is on the bottom of the table. As it can be seen basic chemical composition does not show any clear correlation with the slope of ultrasonic velocity decrease. Neither the carbon distribution does. One can hypothesize that either there is no correlation at all or the connections among data are on much more complex level.

Further, the attempt was paid to elucidate the nature of transitions which occur around 20 and 42 °C in humic samples at concentration 1 g/L (Fig. 23). As noted previously these two breaks were assigned to reaggregation connected with temperature induced disruption of forces stabilizing aggregates at particular conformation. Thermal agitation causes higher amplitude of vibration of those forces which along with the weakening of hydrophilic hydration lead to their destabilization and previously hidden parts are exposed to water. This increases amount of water in hydration shells reflected by the increase in U_{12} . Unlike the transition occurring at T1, the processes of hydration are kinetically driven and take a certain time period which is proportional to the temperature increase (ΔT). At the endset of this process (T2), the decrease of U_{12} starts again due to continuous decrease of relative permittivity of water. The question appears which types of bonds are broken at those specific temperatures. In order to clarify this issue, several parameters characterizing the transitions such as initial and terminal temperatures (T1 and T2) and values of differences in ultrasonic velocity (U1 and U2) were assessed as depicted in Fig. 26. Measured values together with their differences are summarized in Tab. 8 and Tab. 9. Again, the attempt was paid to correlate them with primary characteristics of humic substances (Tab. 2 and Tab. 3).

Tab. 8: Values of $T1$, $T2$, $U1$ and $U2$ and their differences for the 1st break at 20 °C.

Name	IHSS	T1	T2	dT	U1	U2	dU
NaHA Suwannee river	2S101H	18.47	21.29	2.82	0.183	0.208	0.025
NaHA Leonardite	1S104H	19.27	26.47	7.20	0.110	0.173	0.063
NaFA Suwannee river	2S101F	18.56	24.62	6.06	0.125	0.144	0.019
NaFA Elliott soil	2S102F	21.11	25.17	4.06	0.147	0.163	0.016
NaFA Pahokee Peat	2S103F	18.90	22.13	3.23	0.243	0.257	0.014

Tab. 9: Values of $T1$, $T2$, $U1$ and $U2$ and their differences for 2nd break at 42 °C.

Name	IHSS	T1	T2	dT	U1	U2	dU
NaHA Suwannee river	2S101H	43.77	46.74	2.97	0.102	0.106	0.004
NaHA Leonardite	1S104H	42.15	48.57	6.42	0.101	0.133	0.032
NaFA Suwannee river	2S101F	41.79	45.38	3.56	0.067	0.076	0.009
NaFA Elliott soil	2S102F	41.19	43.95	2.76	0.076	0.100	0.024
NaFA Pahokee Peat	2S103F	43.46	47.69	4.23	0.139	0.149	0.010

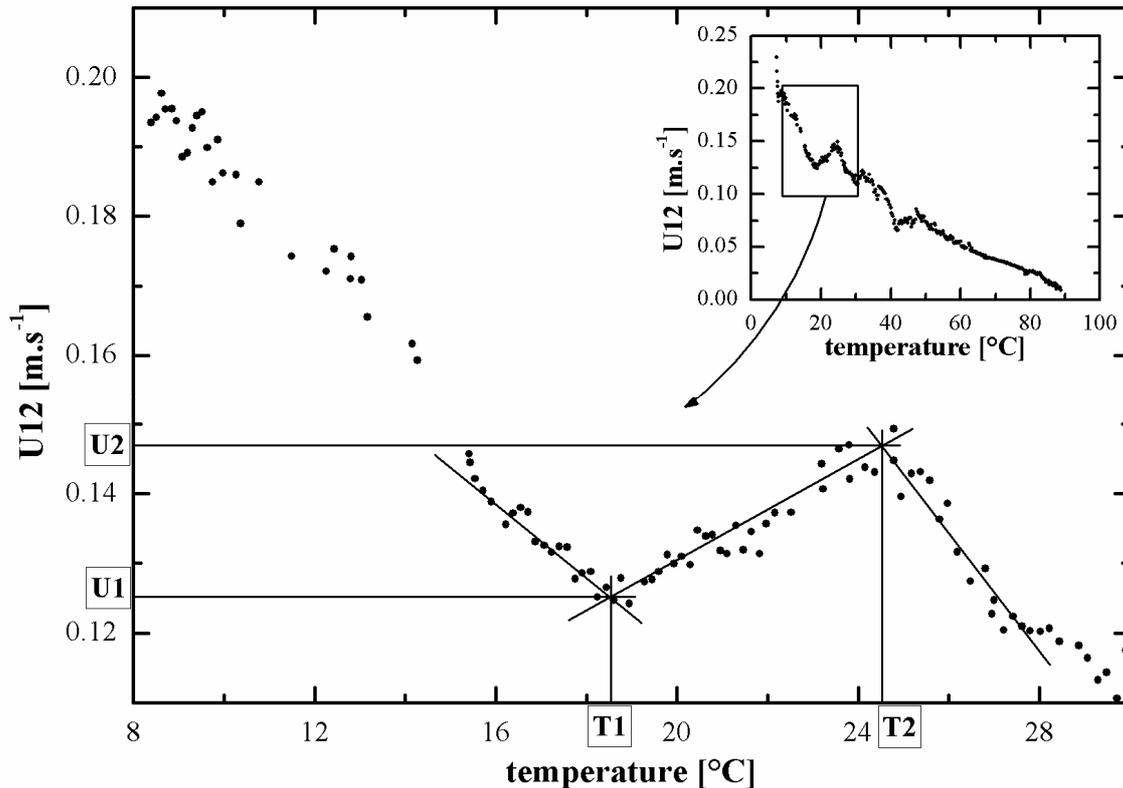


Fig. 26: Detail of NaFA Suwannee river (2S101F) record at concentration 1 g/L. Assessment of values $U1$, $U2$ and $T1$ and $T2$.

Considering this part of thesis as a preliminary study of the possible relationship between parameters describing the transitions and primary characteristics of the samples, only linear

correlation was performed. Results represented by the values of the coefficient of determination (R^2) are summarized in Tab. 10. Nevertheless, it seems that more important than the absolute values describing the initiation or termination of the reaggregation processes are the differences in values, i.e. ΔT and ΔU , since they better express the trends. The difference in temperatures stands for the duration of the process while the difference of ultrasound velocities represents the intensity of the process, in other words the quantity of water in hydration shell at the beginning and at the end of hydration processes.

Tab. 10: Correlation of parameters describing breaks of HS samples at concentration 1 g/L with their primary characteristics (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
1 st break at 20 °C	T1	0.033	0.014	0.002	0.740	0.002	0.028	0.220	0.004	0.158	0.012
	T2	0.234	0.009	0.476	0.004	0.385	0.142	0.035	0.224	0.139	0.318
	ΔT	0.434	0.031	0.583	0.177	0.547	0.280	0.233	0.258	0.040	0.342
	U1	0.115	0.109	0.527	0.100	0.302	0.012	0.424	0.077	0.002	0.105
	U2	0.000	0.356	0.179	0.040	0.042	0.056	0.247	0.007	0.100	0.001
	ΔU	0.883	0.205	0.952	0.152	0.984	0.670	0.378	0.832	0.337	0.820
2 nd break at 42 °C	T1	0.003	0.099	0.069	0.093	0.014	0.023	0.002	0.001	0.000	0.005
	T2	0.664	0.654	0.211	0.202	0.432	0.752	0.066	0.529	0.275	0.475
	ΔT	0.951	0.600	0.595	0.110	0.829	0.942	0.080	0.786	0.450	0.836
	U1	0.089	0.634	0.032	0.017	0.004	0.256	0.172	0.116	0.266	0.086
	U2	0.076	0.005	0.013	0.903	0.033	0.047	0.362	0.000	0.168	0.001
	ΔU	0.419	0.154	0.573	0.091	0.554	0.359	0.001	0.571	0.582	0.656

In the Tab. 10 some remarkably strong correlations can be seen. However, some of them originate from the non-uniform distribution of parameters, i.e. in case when the parameter of one sample significantly differs in comparison with others. In this case, the sample NaHA Leonardite (1S104H) is the disruptor because its parameters are out of the dimensions valid for the rest of the samples. This is probably caused by the totally different origin and genesis of the sample.

However, some of the correlations are not influenced by the Leonardite sample and. In case of the first break at 20 °C, it seem that the intensity of the reaggregation process (ΔU) correlates with amount of oxygen ($R^2=0.952$) and the C/O ration ($R^2=0.984$). The dependence is that the higher is the ΔU the lower is amount of O and the higher is C/O ration. The amount of carbon in carboxylic groups is also decreasing with the increase in ΔU , but not linearly. The correlation indicates that O-containing groups are involved in processes of hydration but in inverse manner than it would be expected. Their presence decreases the intensity of hydration which is connected with the ability of formation of H-bonds stabilizing humic molecules.

In case of hydrophilic surfaces the increase should be proportional to the content of O containing groups, however in this case, an inverse trend is observed. Therefore, the processes in which hydrophobic surface is exposed to water take place causing U_{12} increase.

In principal, in aqueous solutions the H-bonds formation competes between water/water and water/hydrophilic surface interactions. Hydrophobic moieties attached to polar molecules give higher tendency to molecules to aggregate in comparison with polar molecules. Present polar groups can be both protonized and deprotonized (at pH 7 phenolic and carboxylic groups, respectively). While between two carboxylic groups the repulsive forces would take place, other combinations, i.e. -OH/-OH and -COO⁻/-OH, has the potential of H-bonds formation. The determined correlation implies that those interactions are responsible for stabilization of supramolecular structure of humic substances and processes occurring at 20°C are associated more with the reorientation of molecules in aggregates than the expected reversible destruction of aggregates [51]. In other words, hydrogen bonds are the main stabilizing factor at lower temperatures and the higher is their amount the lower the intensity of reaggregation is.

The second break occurs approximately at 42 °C and correlation of two pairs of parameters was observed; ΔT correlates with the C content ($R^2=0.951$) and with the C/H ratio ($R^2=0.942$). For both pairs the direct proportion was observed. In fact, the higher aromaticity the longer the reaggregation is. It suggests itself that at these higher temperatures the hydrophobic interactions such as π - π , CH- π and van der Waals forces play a crucial role. This theory can be supported by the fact that with increasing temperature the water relative static permittivity decreases [97] and hydration of hydrophobic surfaces is therefore enhanced [98 and references therein].

Nevertheless, only five samples were involved in this study, besides that the Leonardite sample disrupts the statistics. Therefore, using more samples covering wide range of values of primary characteristics is in need. In future, samples studied in the chapter 3.3 of this thesis will be used to fulfill this task.

3.2.3 Conclusions

- Results obtained by HRUS revealed similarities and differences in ultrasonic records for all samples.
- Only reversible processes occurred in the temperature interval from 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.
- No correlation between primary characteristics and slope of U_{12} at concentration 4 g/L was observed.
- Breaks observable at 20 and 42 °C for all samples at concentration 1 g/L contain information about their character and are interrelated with the primary composition of humic acids.

3.3 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.

Application of fractal analysis on data gained from several different methods including scattering techniques, turbidimetry, vapor adsorption methods, reaction rate methods and microscopy was performed many times [80–84]. As demonstrated recently [72,73], data obtained from HRUS measurement can be processed in this way as well; a specially developed fractal analysis method was successfully applied on the IHSS standards in the wide range of concentrations. 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.

3.3.1 Experimental part

3.3.1.1 Samples preparation

Humic (HA) and fulvic (FA) acids were isolated from individual soil horizons of long-term research stands (Tab. 11). 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 [99]. The sodium humate and fulvate were converted into H⁺ form and freeze-dried.

Prior to HRUS measurement the samples were suspended in distilled water and titrated to pH 7.2 with 0.1M NaOH in automatic titrator (TitroLine® alpha plus, Schott/SI Analytics, Mainz, Germany) to obtain sodium salts of HS. After reaching constant pH value 7.2, samples were kept stirred for next 60 minutes and freeze-dried. Obtained products were milled in an agate mortar. Then the stock solutions of 10 g/L were prepared by dissolving HS powder in distilled water. Samples were well stirred for at least 24 hours prior to preparation of other

stock solutions of concentration 1 g/L. All stock solutions were stirred during the carrying out of the experiment.

3.3.1.2 ^{13}C NMR spectroscopy

Quantitative ^{13}C liquid-state nuclear magnetic resonance (^{13}C LS NMR) spectra of HA and FA were measured. Samples were dissolved in 0.1M NaOD and measured on a Bruker Avance DRX 500 spectrometer operating at 125.75 MHz using the following parameters: recycle delay = 5 seconds, number of scans = 20 000. Inverse gated decoupling was applied during acquisition. The free induction decay was processed with line broadening of 20 Hz. The ^{13}C chemical shifts were referenced to tetramethylsilane (TMS). Quantification was achieved by instrumental integration of NMR peaks. The local minimum at 108 ppm was used as the border between aromatic and O-alkyl regions. 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 [100]. Aromaticity was calculated as the ratio between aromatic and the sum of aromatic plus aliphatic (alkyl + O-alkyl) carbons [101].

3.3.1.3 HRUS measurement

Ultrasonic Spectroscopy HRUS 102 device (Ultrasonic-Scientific, Dublin, Ireland) was employed to obtain ultrasonic data. HRUS consists of two independent quartz cells in which the velocity of ultrasonic wave propagation is measured. The whole system is tempered by a 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 and under constant stirring (600 rpm). To perform the set up three different initial frequencies (5480, 8220 and 12200 kHz) were used. Both cells of HRUS were loaded up by 1 mL of deionized and degassed water. In the cell 1, using the Hamilton syringe, the solution of respective HS was added stepwise every 10 min (i.e. when constant values of ultrasound velocity were achieved and recorded) to reach desired concentrations from 0.001 to 3.5 g/L using appropriate combination of stock solutions. The ultrasonic velocity U was measured in both cells. For easier observation of potential interactions, the concentration increment of ultrasonic velocity I was determined using the relation published earlier by Kankia et al. [87] and as in detail described by Kučerík when applied on HS [71], i.e.

$$I = \frac{U_1 - U_2}{U_2 m \rho_2} \quad (9)$$

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.

3.3.1.4 High precision density measurements

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 (Austria) was employed. The stock solutions of 1 and 10 g/L were prepared in distilled water

at least 24 hours before measurement. Sample solutions in concentration range from 0.003 to at least 1.1 g/L (in some case to 3.5 g/L) were prepared by dilution of stock solutions 24 hours prior to measurement. Both stock and sample solutions were under constant stirring. In addition to that all samples were well-shaken and degassed before injection into the device. 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.

3.3.2 Results and Discussion

Soil represents the upper layer of Earth's crust, bordering with the atmosphere. It is a natural reservoir of water, minerals and organic matter and an indispensable condition for terrestrial life preservation. Part of that organic matter can be isolated and is known as humic substances. Properties of a soil sample and amount of present humic substances, respectively, depend enormously not only on the geographical location of origin but also on the depth of sampling. When observing a vertical soil profile, several layers – soil horizons – can be differentiated as indicated in Fig. 27.

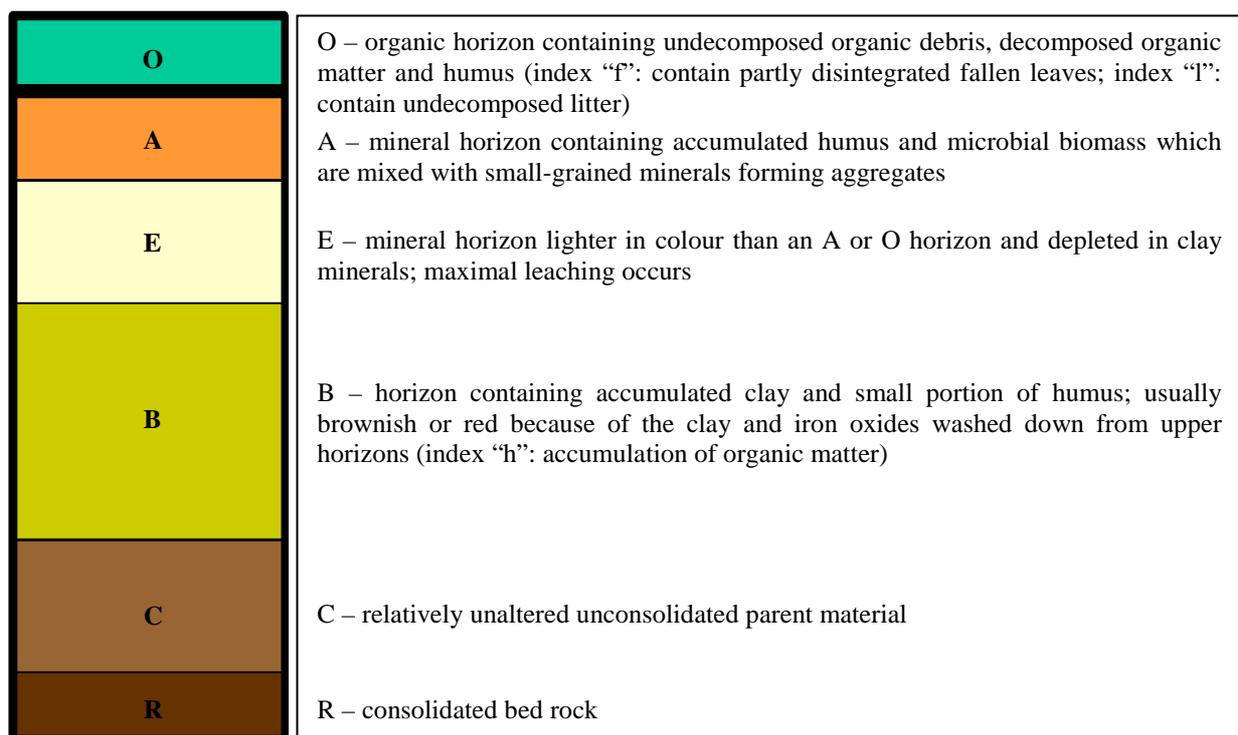


Fig. 27: A hypothetical soil profile showing all of the principle soil horizons [102,103,104].

Several samples originating mainly from human activities untouched sites were collected; two samples originated from horizon O, one from horizon A and one from horizon B. The exceptions are Fluka HA, purchased from Sigma-Aldrich, and Oxyhumolite, Břilina which

represents a low-rank coal mined in North Bohemia. Primary structure characteristics are summarized in Tab. 11.

Tab. 11: 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 comparison with IHSS standard samples (Tab. 2 and Tab. 3) and to the average values of hundreds of HS samples (Tab. 1) by Rice and MacCarthy [12], 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 [5,105]. 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 [12]. This seems to be the situation of this sample. As can be seen in the Tab. 11, 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)).

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.

3.3.2.1 HRUS measurement

The purpose of this part of thesis is to extend and support the results from 3.1 part 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 3.1 part of the thesis, the high resolution ultrasonic spectroscopy was used to observe the aggregation behavior of several HS samples. The same approach as published in [71,72,73,106] was applied. Briefly speaking, to make the changes of difference of ultrasonic velocity more clear, the increment of ultrasonic velocity was calculated according the theory published by Kankia et al. [87] and already successfully applied on HS samples by Kučerík et al. [51,72]. Gained data were fitted by (10)

$$y = ax^{bx^{-c}} \quad (10)$$

where y stands for concentration increment of ultrasonic velocity (I in $\text{m}^6 \cdot \text{kg}^{-2}$), x for sample concentration (in g/L) and a , b and c are adjustable parameters. Obtained parameters a , b and c are summarized in Tab. 12. Fig. 28 reports the fitted dependencies for all samples.

Tab. 12: Parameters a , b and c obtained using (10).

Locality, substrate	Sample	$a \times (10^{-7})$	$b \times (10^{-2})$	$c \times (10^{-1})$
Fluka, purified	XF150HK	2.34 ± 0.09	-7.66 ± 0.92	2.08 ± 0.16
Bohemian Forest, Trojmezí	T55HK	2.00 ± 0.10	-6.09 ± 1.09	2.30 ± 0.24
Bohemian Forest, Boubín	B2HK	2.86 ± 0.06	-0.40 ± 0.10	6.05 ± 0.33
Oxyhumolite, Bílina	XM1HK	2.62 ± 0.14	-3.53 ± 0.91	3.04 ± 0.36
Bohemian Forest, Trojmezí	T15HK	2.05 ± 0.11	-1.56 ± 0.67	4.64 ± 0.67
Ore Mts., Alžbětinka	A65FK	3.22 ± 0.13	-5.48 ± 0.77	2.60 ± 0.19

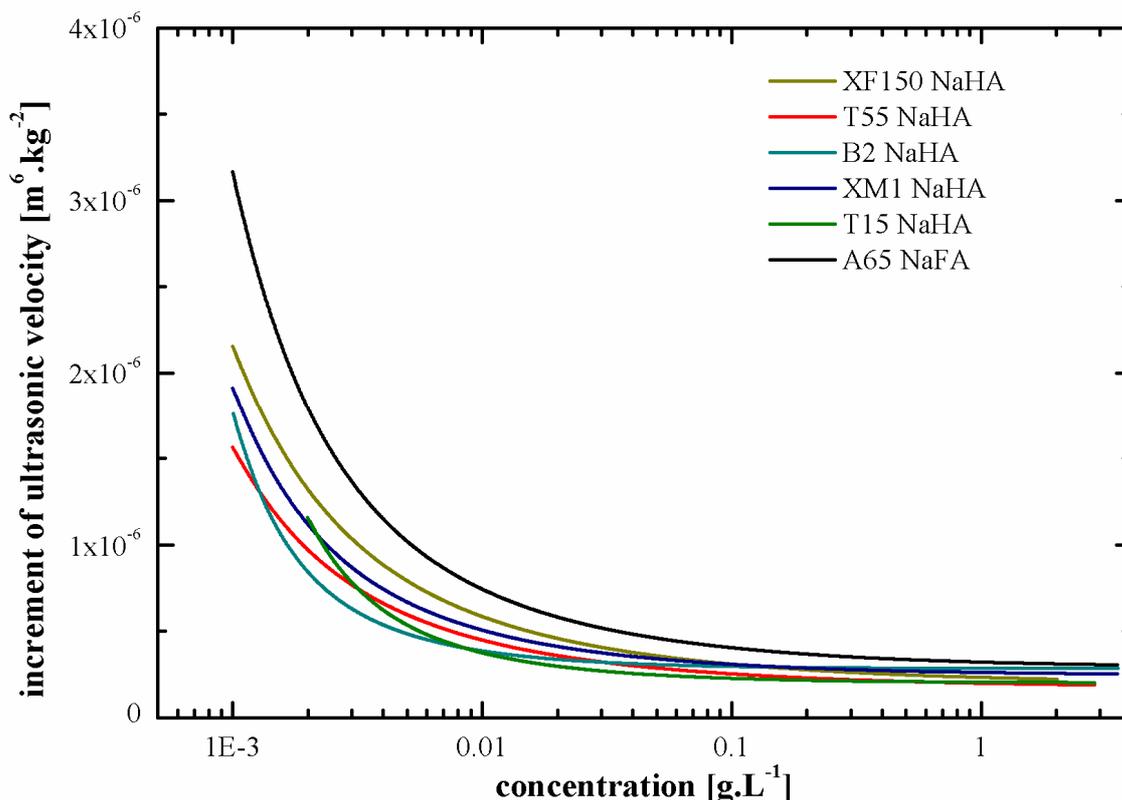


Fig. 28: Dependency of increment of ultrasonic velocity on concentration.

When compared with results reported in Tab. 4 (IHSS samples), numbers in the same order of magnitude were obtained. Trends similar to those depicted in Fig. 9, Fig. 10 and Fig. 11 can be observed in Fig. 28. 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, based on the results obtained at different frequencies where no influence of the applied frequency on the ultrasonic velocity was observed, it can be assumed, that the character of aggregates, i.e. size and/or compressibility of the hydrophobic interiors, does not resemble classical micelles. Again, it is demonstrated that the mechanisms of aggregation of fulvates and humates is quite similar and can be fitted by the same mathematical apparatus and therefore the differentiation of humic substances based only on their solubility can be considered only as operational.

According to theory of Zmeškal et al. [88,89,90] and its applications on ultrasonic data [72,73], as already showed in 3.1 part of this thesis, the fractal analysis was applied on measured data. The dependency of fractal dimension D on the concentration is reported in Fig. 29.

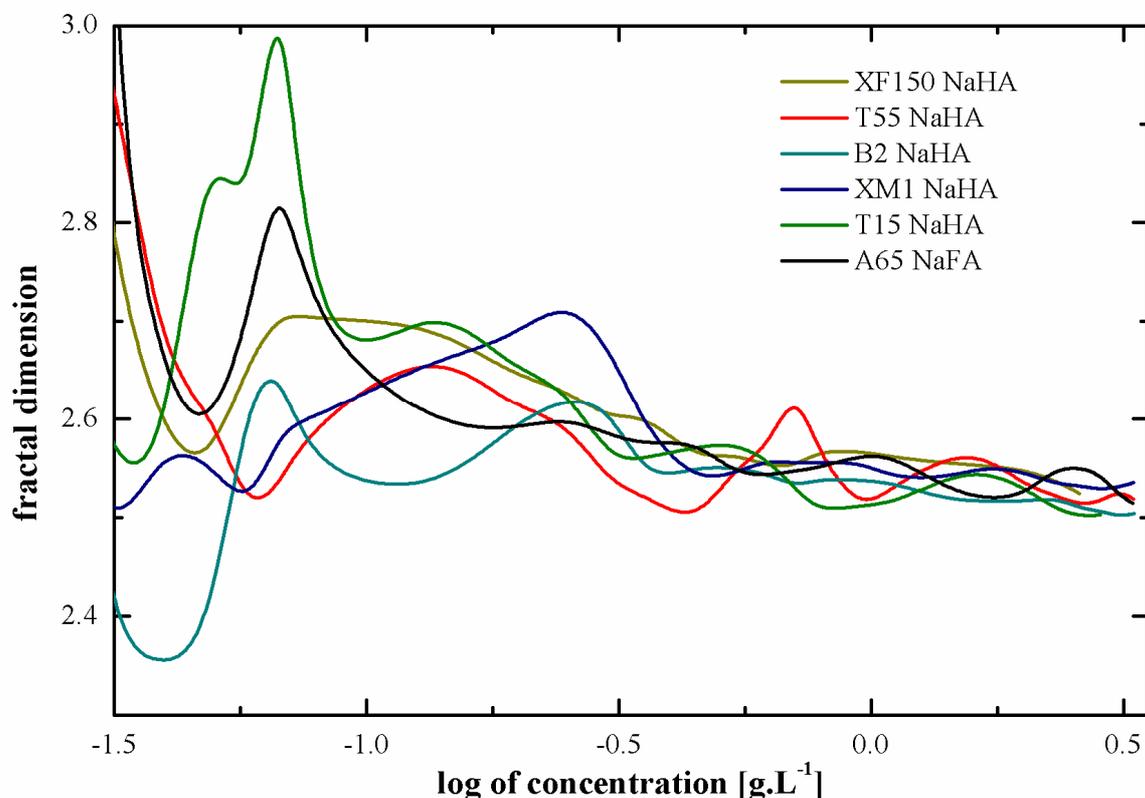


Fig. 29: Dependency of fractal dimension on concentration.

As it is indicated in Fig. 29, this approach is useful for concentration above approximately 0.03 g/L. The fractal dimension D can be used as a “measure of changes” and reveals the details in mechanisms of aggregation. Around the concentration 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 proposed in [51]. 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 were correlated with elemental analysis and distribution of carbon in humic molecules using linear regression least square method; i.e. C, H, O, N; C/O, C/H ratios, carbon content in carboxylic groups and aromaticity degree. Results are summarized in Tab. 13. The data of the sample Bohemian Forest, Trojmezí (T15HK) were omitted in this correlation since the difference in ultrasonic velocity input data at very low

concentration are missing (as can be seen in Fig. 28) which might lead to misinterpretation and invalid calculation of a , b and c parameters.

Tab. 13: Correlation of fitting parameters of equation (10) 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.
a	0.687	0.626	0.194	0.118	0.022	0.772	0.111	0.018
b	0.428	0.508	0.329	0.196	0.015	0.418	0.009	0.000
c	0.518	0.569	0.334	0.202	0.004	0.469	0.007	0.103
$a \times b$	0.168	0.239	0.219	0.395	0.036	0.144	0.057	0.014
a^b	0.415	0.451	0.265	0.180	0.006	0.386	0.000	0.017
$(a^b)^c$	0.431	0.521	0.347	0.224	0.016	0.412	0.020	0.046
D end	0.381	0.029	0.430	0.097	0.836	0.000	0.775	0.511
D average *	0.546	0.079	0.025	0.590	0.430	0.092	0.372	0.234
D slope *	0.295	0.068	0.616	0.000	0.912	0.009	0.950	0.060
D intercept *	0.120	0.277	0.826	0.105	0.945	0.124	0.796	0.368
D at 0.95 g/L	0.021	0.284	0.362	0.890	0.214	0.329	0.091	0.085
D at 0.44 g/L	0.002	0.112	0.228	0.687	0.250	0.139	0.084	0.610

As indicate data reported in Tab. 13, 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 the IHSS standard samples [72,73]. 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.

The fluctuation of fractal dimension D as depicted in Fig. 29 indicates important notion. Unlike in binary solutions, in solutions of humic substances the physical properties of solutions are strictly concentration dependent with no apparent dependency and the nature of solution is therefore unpredictable. This is associated with heterogeneity of humic substances, generally at specific concentration, specific interaction prevail influencing actual conformation and thus the reactivity of dissolved organic matter. Such notion is of great importance, first similarly as in biological systems higher conformation variability of biomolecules implies higher variability in biological activity; and second variability in conformation of humic substances allows solubilization of wider range of both organic and inorganic compounds, their transport or sequestration. This seems to represent one of the most crucial points in versatility of humic materials.

3.3.2.2 High precision density measurements

As has been mentioned several times in this thesis, 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.

Rearranging equation (6), the speed of sound in liquids can be expressed as

$$U^2 = \frac{1}{\rho \beta_s} \quad (27)$$

where U stands for sound velocity (m/s), ρ for density (kg/m³) and β_s (m.s²/kg) for adiabatic compressibility. If we assume that humic molecules and its Na⁺ counterions are incompressible in comparison with the bulk solvent, the volume fraction of non-interacting solvent V_{0,H_2O} can be calculated according to (28)

$$V_{0,H_2O} = \frac{\beta_{HS}}{\beta_{H_2O}} \quad (28)$$

where β_{HS} and β_{H_2O} represent the compressibilities of the humic solutions and pure solvent, respectively [107]. Since both ultrasonic velocity U and density of the solution ρ from (27) are known, both adiabatic compressibilities β_{HS} and β_{H_2O} can be calculated.

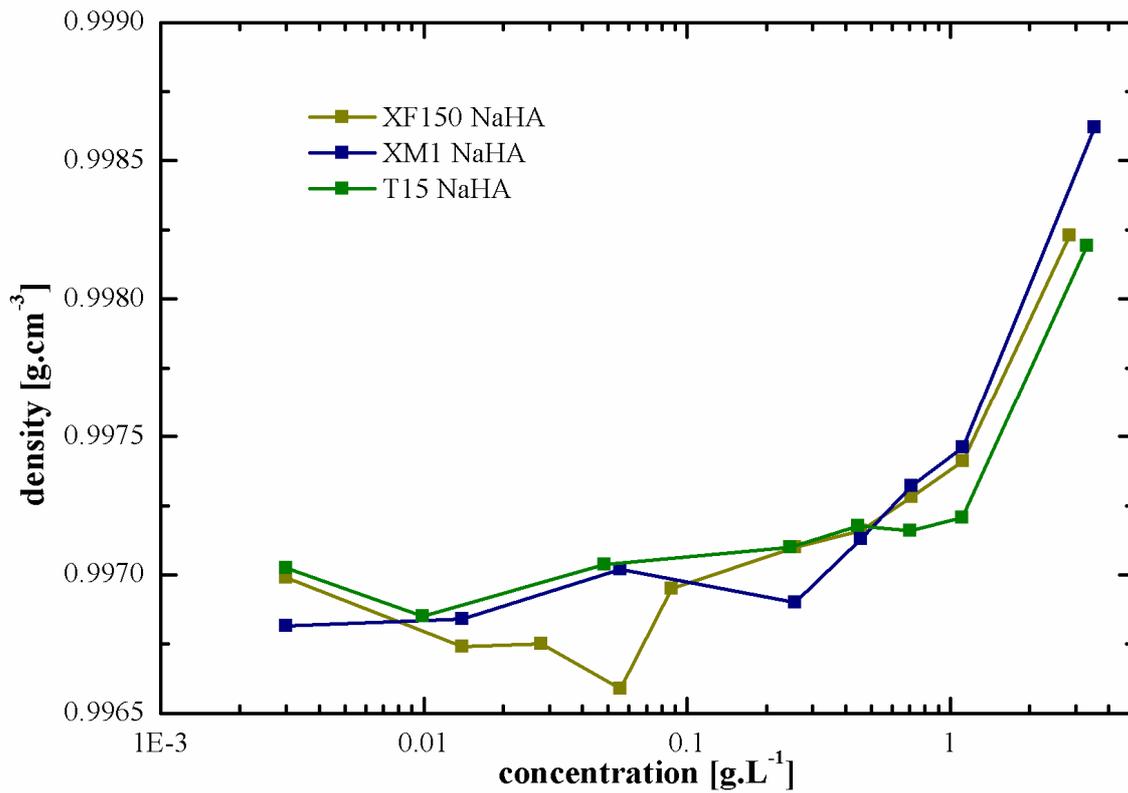


Fig. 30: Dependency of density on concentration at 25 °C.

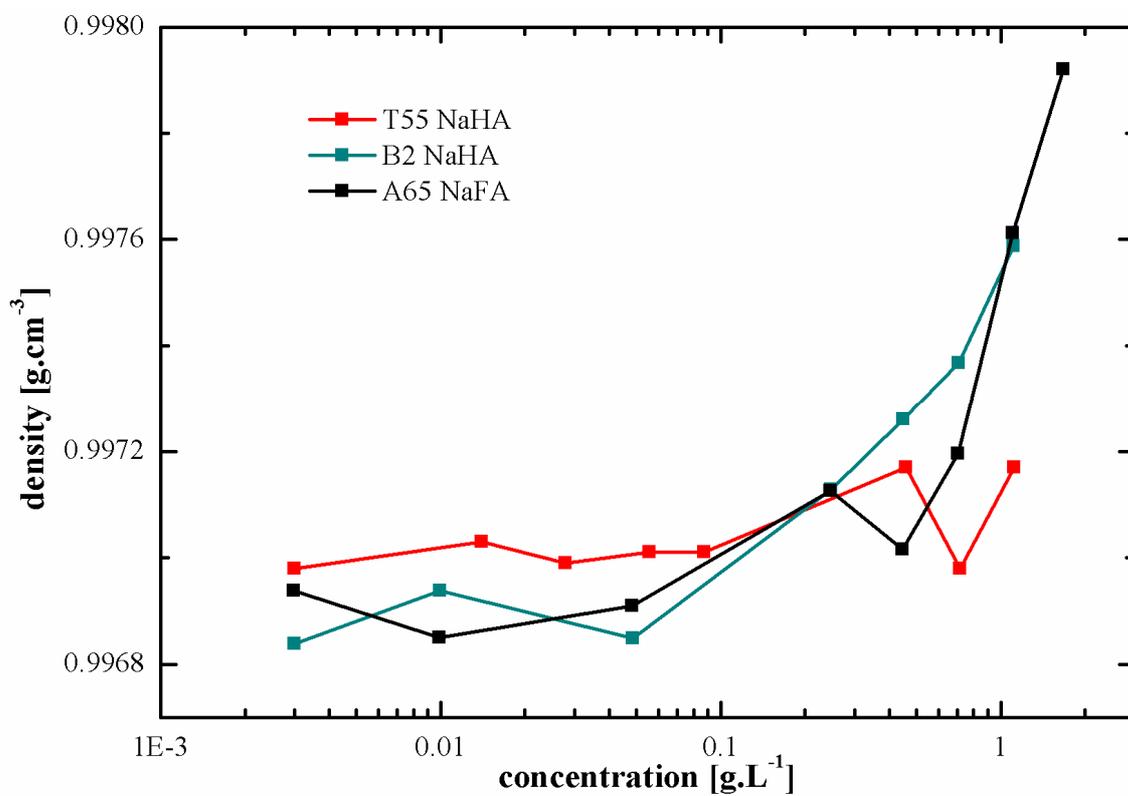


Fig. 31: Dependency of density on concentration at 25 °C.

Densities of six samples (Tab. 11) were measured in concentration range from 0.003 to at least 1.1 g/L. The dependencies of their density on concentration are depicted in Fig. 30 and Fig. 31.

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 [108,109,110]. At low concentrations, the exterior of very small aggregates is predominantly hydrated by hydrophobic hydration [51,94]. 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 [96]. Another explanation might be the higher error of measurement at lower concentration of humic substances. Nevertheless, because of lack of samples repeating of the measurements was not possible and in following paragraphs it is assumed that the correctness of the measurement is indisputable.

The relationship between concentration of a humic solution and ultrasound velocity in it is known for several years [71]. So it was not a surprise that the increase in U_{12} with increasing concentration was observed (Fig. 32 and Fig. 33). In this case the ultrasonic velocity depends mainly on hydration of humic aggregates (in the meaning of humic molecules together with their counterions) and on its inner compressibility. Since the measurements at different frequencies showed that there are no micelle-like compressible cores which would significantly decrease the U_{12} , it can be assumed that the concentration depended increase is caused predominantly by extent of the total surface leading to increase of hydration shells which are less compressible than the bulk of water and therefore more supportive to ultrasound wave propagation.

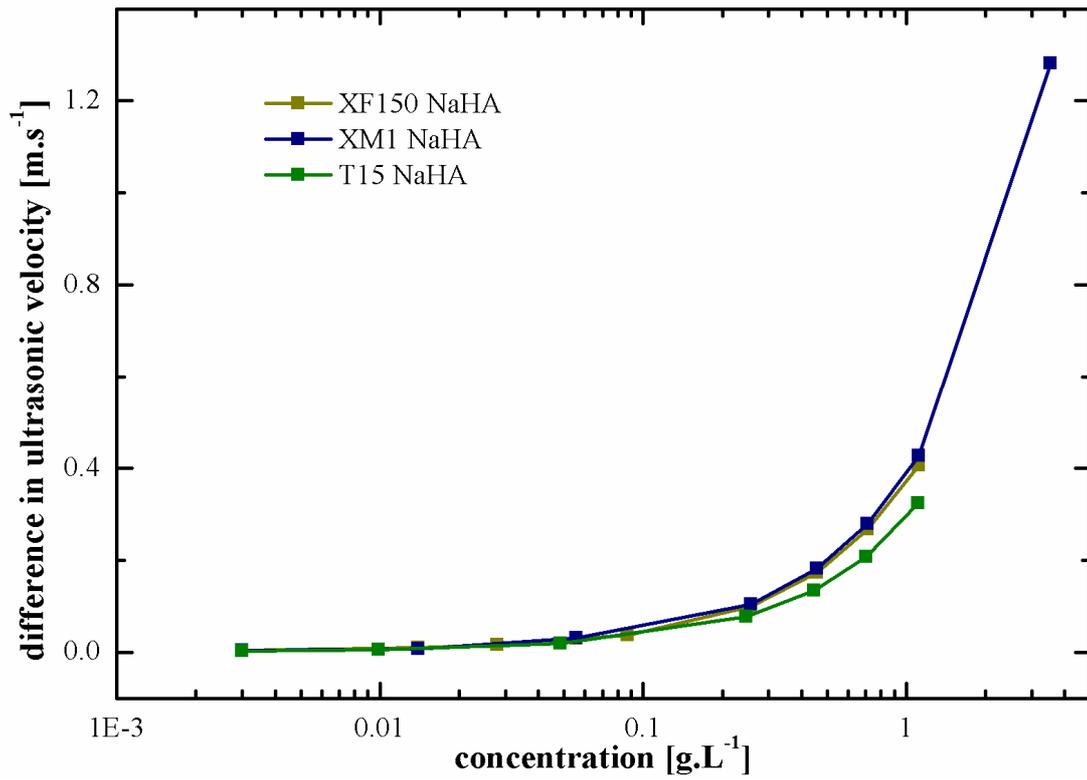


Fig. 32: Dependency of difference in ultrasonic velocity U_{12} on concentration at 25 °C.

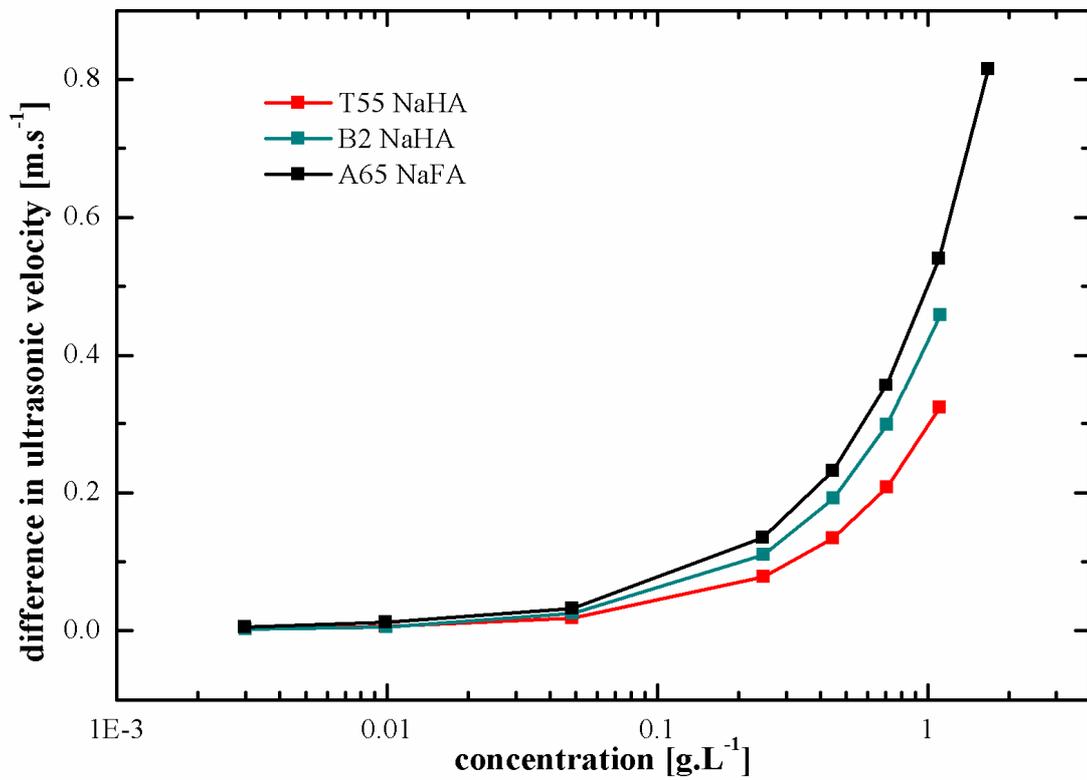


Fig. 33: Dependency of difference in ultrasonic velocity U_{12} on concentration at 25 °C.

Utilizing equation (27), the adiabatic compressibilities of pure water and humic samples were calculated. Subsequently, equation (28) was used to calculate the volume fraction of non-interacting solvent which provides information about the hydration of the humate aggregates. This volume fraction represents water molecules of bulk solution, i.e. all water molecules except those being part of hydration shells of the counterions and humic assemblies. Results are depicted in Fig. 34 and Fig. 35. It is clear from the equations that the concentrations for which lower densities than of pure water were measured gave the value of volume fraction of non-interacting solvent bigger than one. The speculation explaining these observations is discussed upward. Because for the lack of explanation for this behavior, these results were not depicted in following figures and were not considered in further calculations.

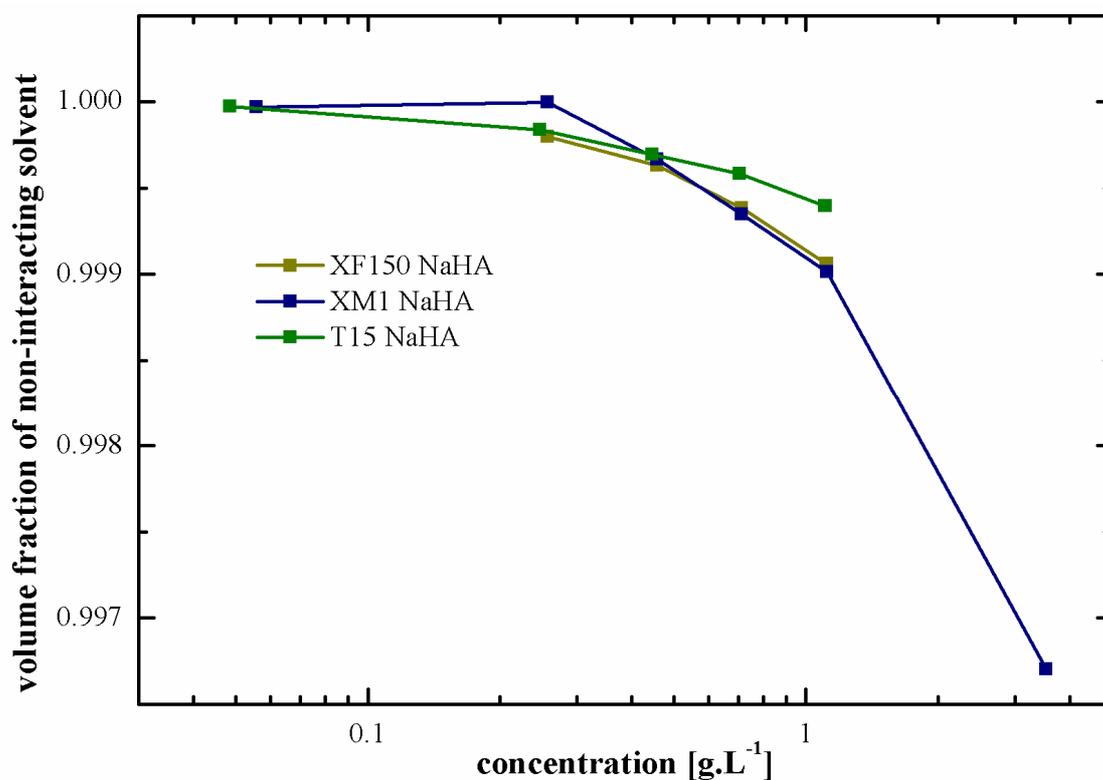


Fig. 34: Dependency of volume fraction of non-interacting solvent on concentration at 25 °C.

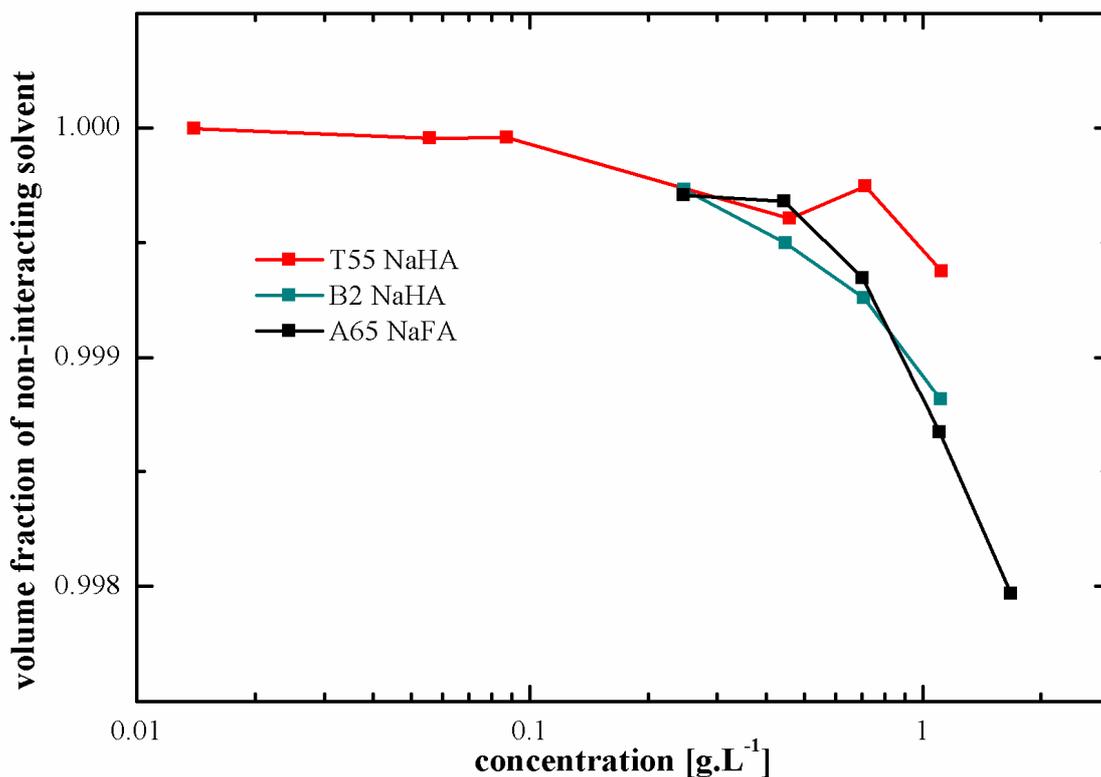


Fig. 35: Dependency of volume fraction of non-interacting solvent on concentration at 25 °C.

As can be seen, a decreasing trend of the volume fraction of non-interacting solvent with increasing concentration was recorded. As expected, with increasing concentration the volume fraction of non-interacting solvent is decreasing. This means that the amount of interacting water molecules is increasing. The explanation is similar to that one indicated above. It 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 [111].

From known concentrations the weight of water molecules in hydration shells was calculated and expressed as grams of H₂O per 1 g of HS. Results are reported in Fig. 36. 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 [112,113,114], saccharides [115] or nucleic acids [116]. Further, hydration results justify the above-discussion about unpredictable character of humic aggregates and their reactivity. Probably, some prediction can be done only above approximately 1 g/L when aggregates are stable enough to do not undergo additional and relatively simple reformation. In addition, such notion can encourage further hypotheses about dimension of humic molecules and aggregates with respect to their restricted motion in concentrated solutions.

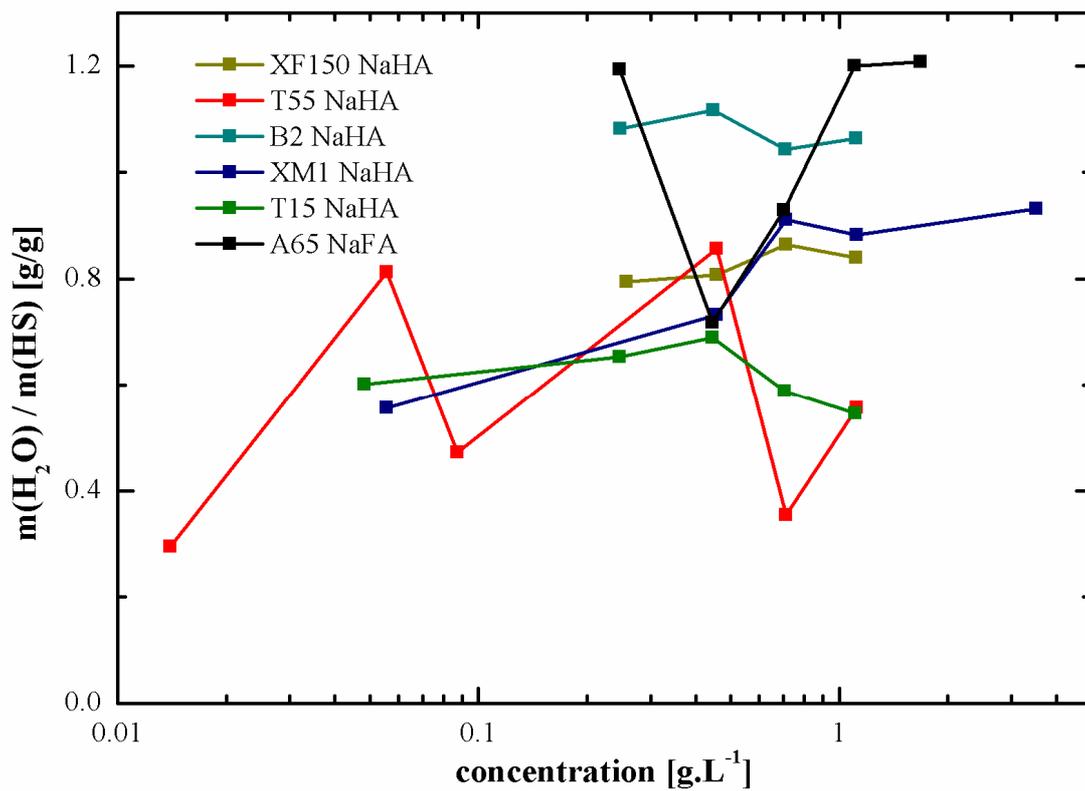


Fig. 36: Amount of water molecules per 1 g of HS in dependency on concentration.

3.3.3 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 successfully 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.
- Fractal analysis developed by our team 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”.

4 OVERALL CONCLUSIONS

- HRUS is a unique method for acquiring data needed for characterization of humic substances since it allows run experiments in wide range of concentrations and variable conditions.
- 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.
- Power-based fitting can be applied on all IHSS standard samples and gained empirical parameters correlate quite well with C content and C/H ratio and combinations of gained parameters correlated significantly with C content and C/H, C/O and aromatic to aliphatic carbon ratios.
- In strong contrast, no significant connections between parameters obtained from mathematical fitting and chemical composition were noticed for soil-originating samples.
- Specially developed fractal analysis method applicable in concentration range from 0.03 to 3.5 g/L showed that interactions holding together humic assemblies dramatically change under the concentration 1 g/L.
- At concentrations higher than 1 g/L almost constant value of fractal dimension ($D \approx 2.5$) is reached.
- In case of soil samples it was found out that fractal dimension 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.
- 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.
- Non-isothermal scans revealed some similarities and also differences in ultrasonic records for the IHSS samples. Observed differences may be explained by differences in the samples origin.
- On the other hand, 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.
- Breaks observable at 20 and 42 °C for all samples at concentration 1 g/L contain information about their character and are interrelated with the primary composition of humic acids.

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7 LIST OF ABBREVIATIONS

CMC	critical micelle concentration
DDT	dichlorodiphenyltrichloroethane (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane)
DLA	diffusion-limited aggregation model
EPR	Electron Paramagnetic Resonance
FA	fulvic acid
FTIR	Fourier Transform Infrared Spectroscopy
HA	humic acid
HPSEC	High Performance Size Exclusion Chromatography
HRUS	High Resolution Ultrasonic Spectroscopy
HS	humic substances
IHSS	International Humic Substances Society
IUPAC	The International Union of Pure and Applied Chemistry
MW	molecular weight
NaFA	sodium salt of fulvic acid
NaHA	sodium salt of humic acid
NMR	Nuclear Magnetic Resonance
PAHs	polycyclic aromatic hydrocarbons
RLA	reaction-limited cluster-cluster aggregation model
TMS	tetramethylsilane
TTAB	tetradecyltrimethylammonium bromide
UV/VIS	Ultraviolet-visible Spectroscopy

8 LIST OF PUBLICATIONS AND ACTIVITIES

Drastík, M.; Kučerík, J.; Zmeškal, O.; Čtvrtníčková, A.; Novák, F.: Influence of aromaticity degree on the aggregation of humic substances. In *Advances in Natural Organic Matter and Humic Substances Research 2008-2010 (Vol. 1, 2 & 3)*. Sevilla & Puerto de la Cruz, Spain: International Humic Substances Society, 2010, 3, pp. 153–154.

Kučerík, J., Drastík, M., Zmeškal, O., Čtvrtníčková, A.: Ultrasonic spectroscopy and fractal analysis in the study on progressive aggregation of humic substances in diluted solutions. *WSEAS Transactions on Environment and Development*, 2009, 5, pp. 705–715. ISSN 1790-5079.

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Drastík, M., Bursáková, P., Pekař, M., Kučerík, J.: High resolution ultrasonic spectroscopy in study of humic substances structure. In *12th Nordic-Baltic IHSS Symposium on Natural Organic Matter in Environment and Technology*. Tallinn, Estonia, Tallinn University of Technology Press, 2009, pp. 33–33. ISBN 978-9985-59-913-6.

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Kučerík, J., Kislinger, J., Drastík, M., Majzlík, P.: Correlation of degradation kinetics parameters of humic substances with their chemical properties. In *Livro de resumos do VII EBSH*. Florianopolis, Brazil, Brazilian humic substances society, 2007, pp. 6–6.

Given speeches:

12th Nordic-Baltic IHSS Symposium on Natural Organic Matter in Environment and Technology. Tallinn, Estonia, 2009.

Proceedings of the 14th Meeting of International Humic Substances Society. From Molecular Understanding to Innovative Applications of Humic Substances. Russia, 2008.

4th Meeting on Chemistry and Life. Brno, Czech Republic, 2008.

Posters

XV Meeting of the International Humic Substances Society. Tenerife, Spain, 2010.

Trainings and courses:

Erasmus Programme – Finland, University of Turku, Department of Chemistry (2009)

“Interpretation of IR vibration spectra” course (2008)

METTLER TOLEDO Workshop – thermal analysis (2007)

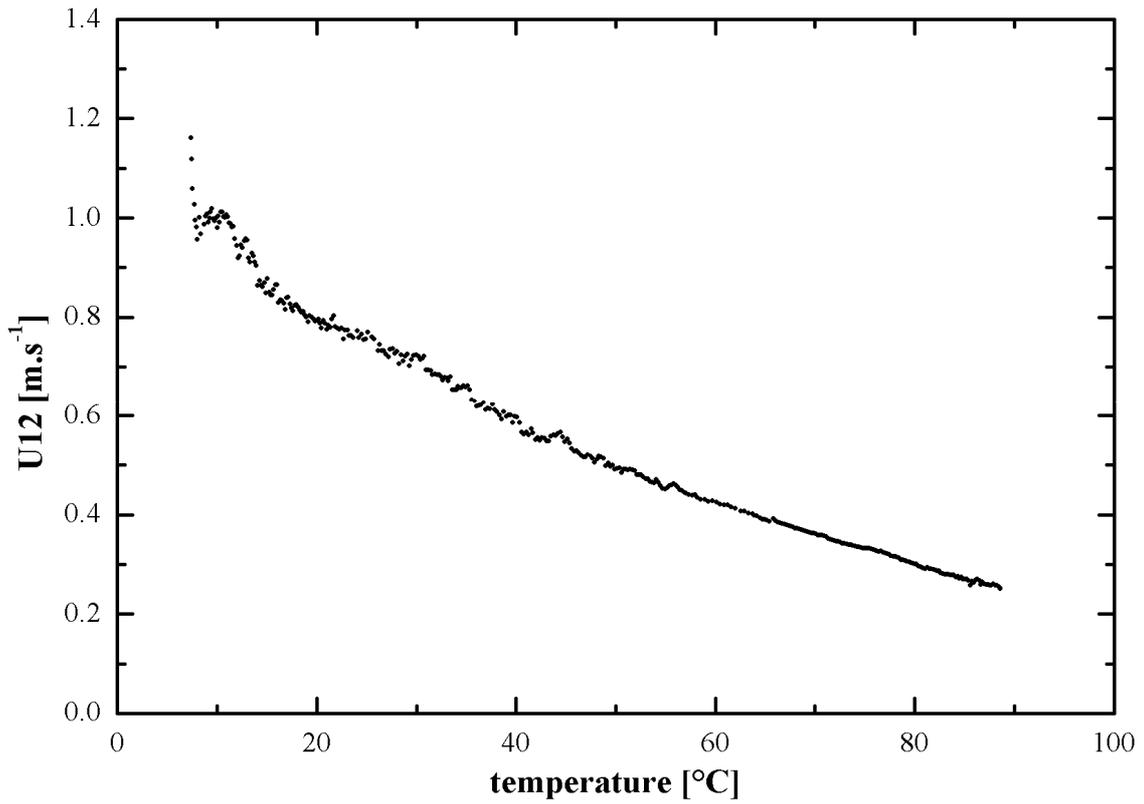
NETZSCH Workshop – thermal analysis (2006)

9 APPENDIX

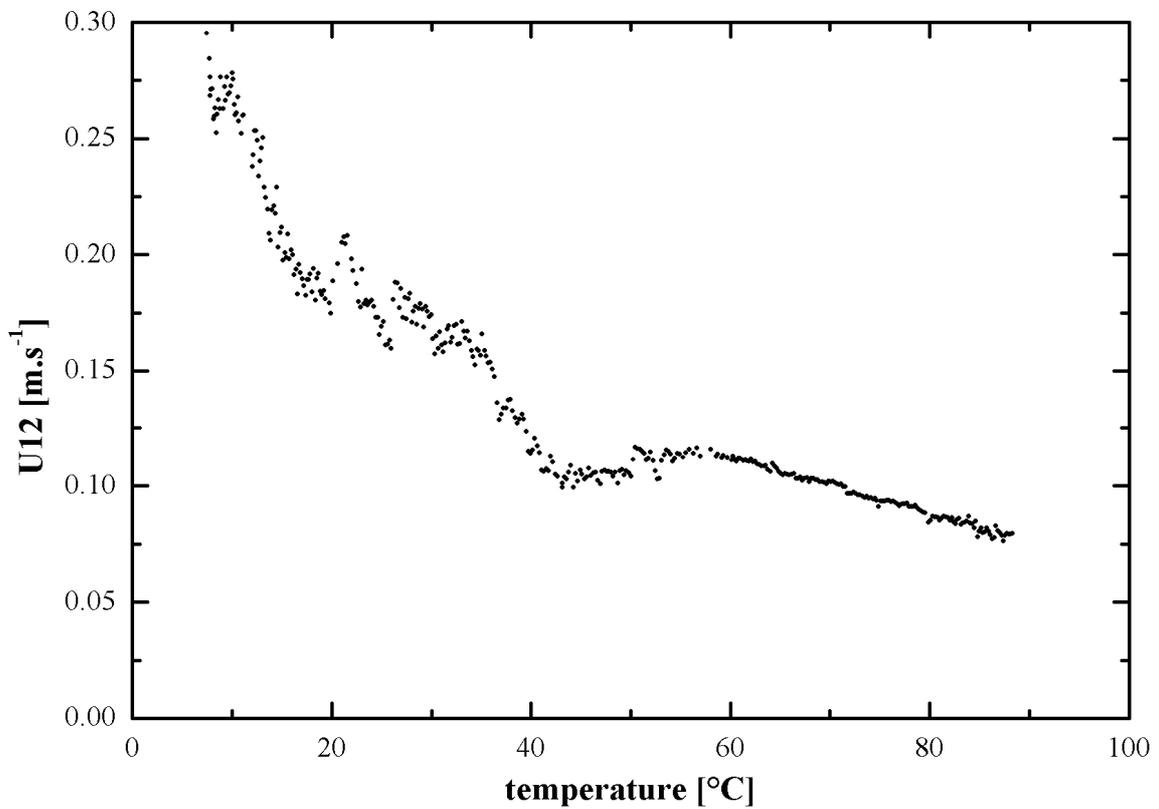
9.1	HRUS device.....	90
9.2	Dependency of difference in ultrasonic velocity U_{12} on temperature for NaHA Suwannee River.....	91
9.3	Dependency of difference in ultrasonic velocity U_{12} on temperature for NaHA Leonardite.....	93
9.4	Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Elliott Soil.....	95
9.5	Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Pahokee Peat.....	97



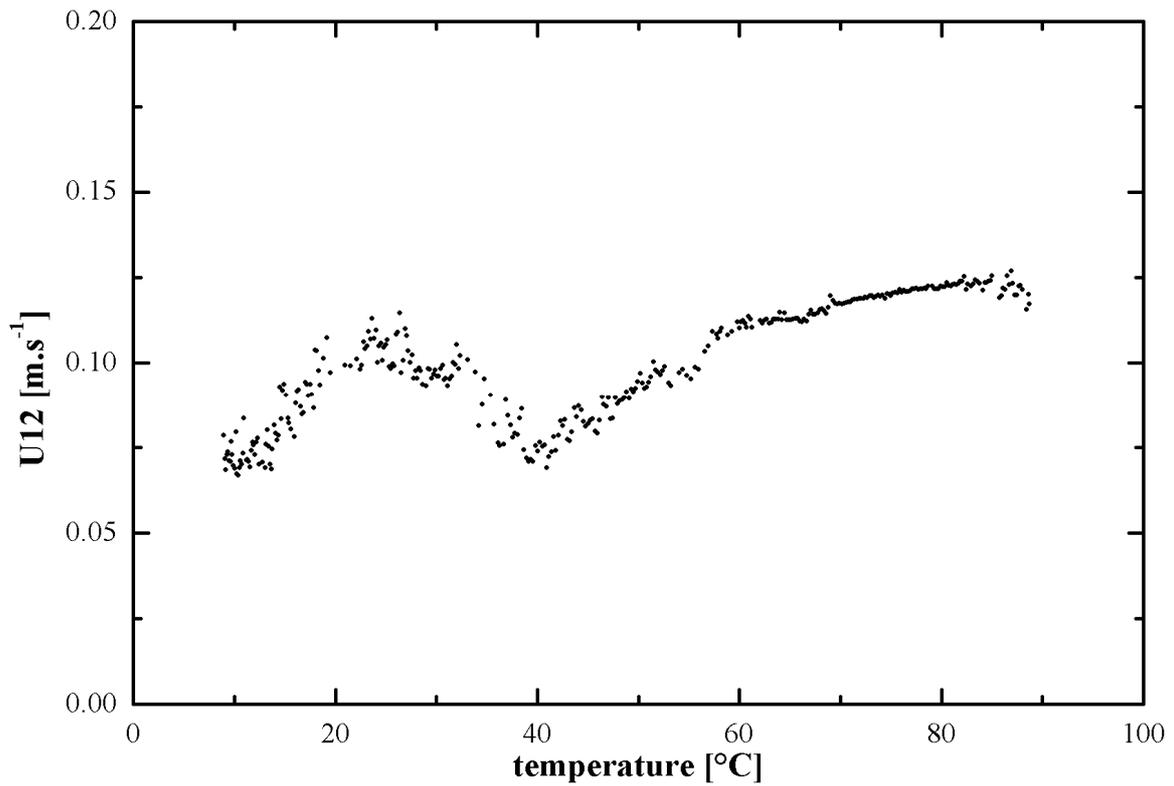
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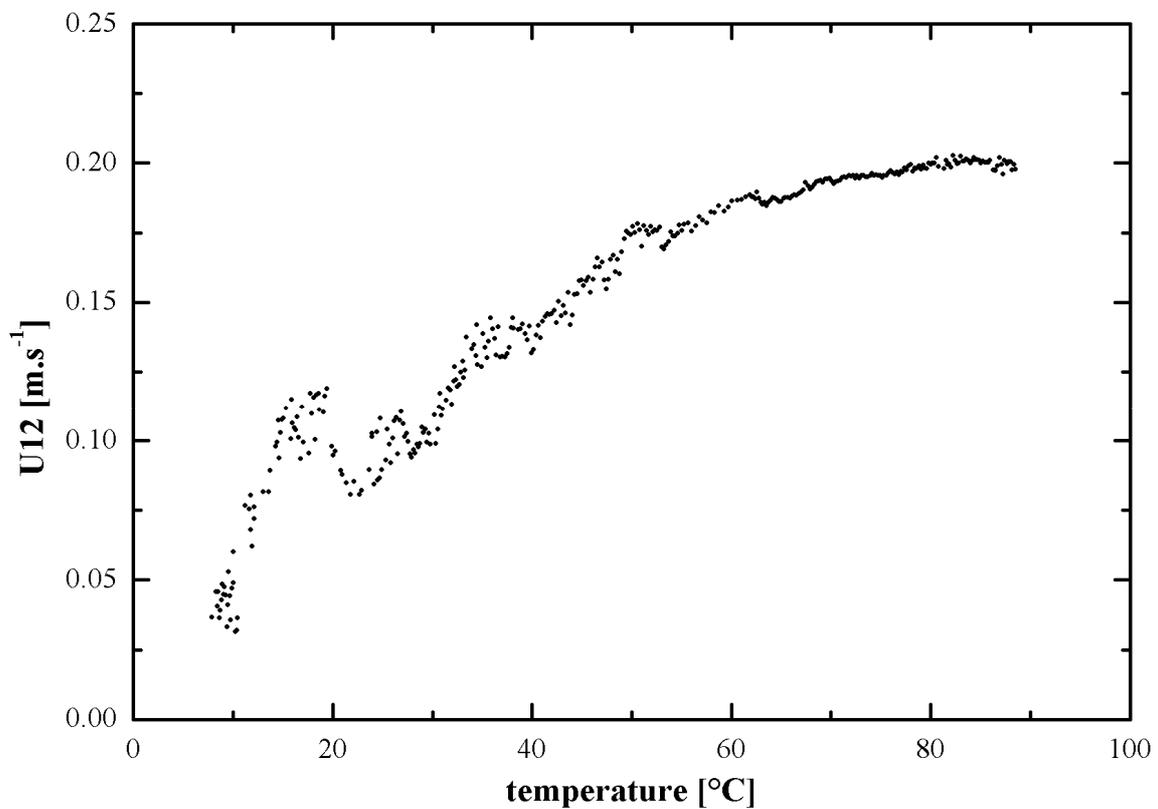
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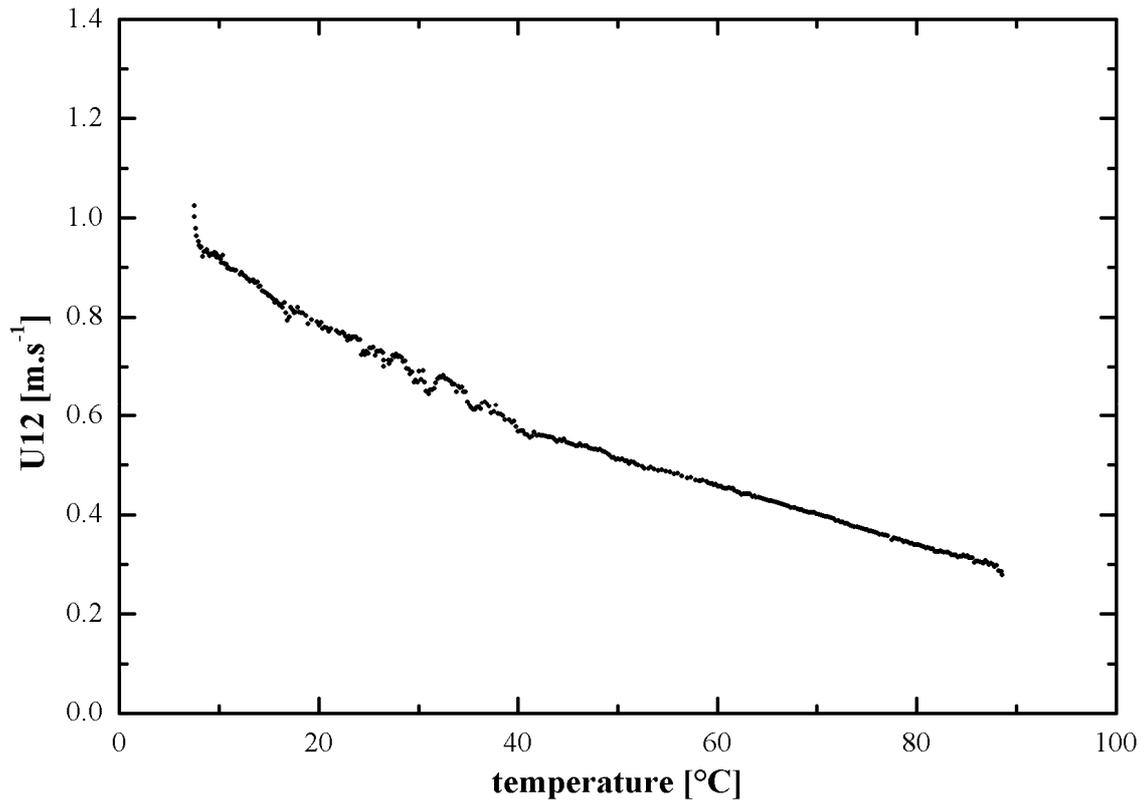
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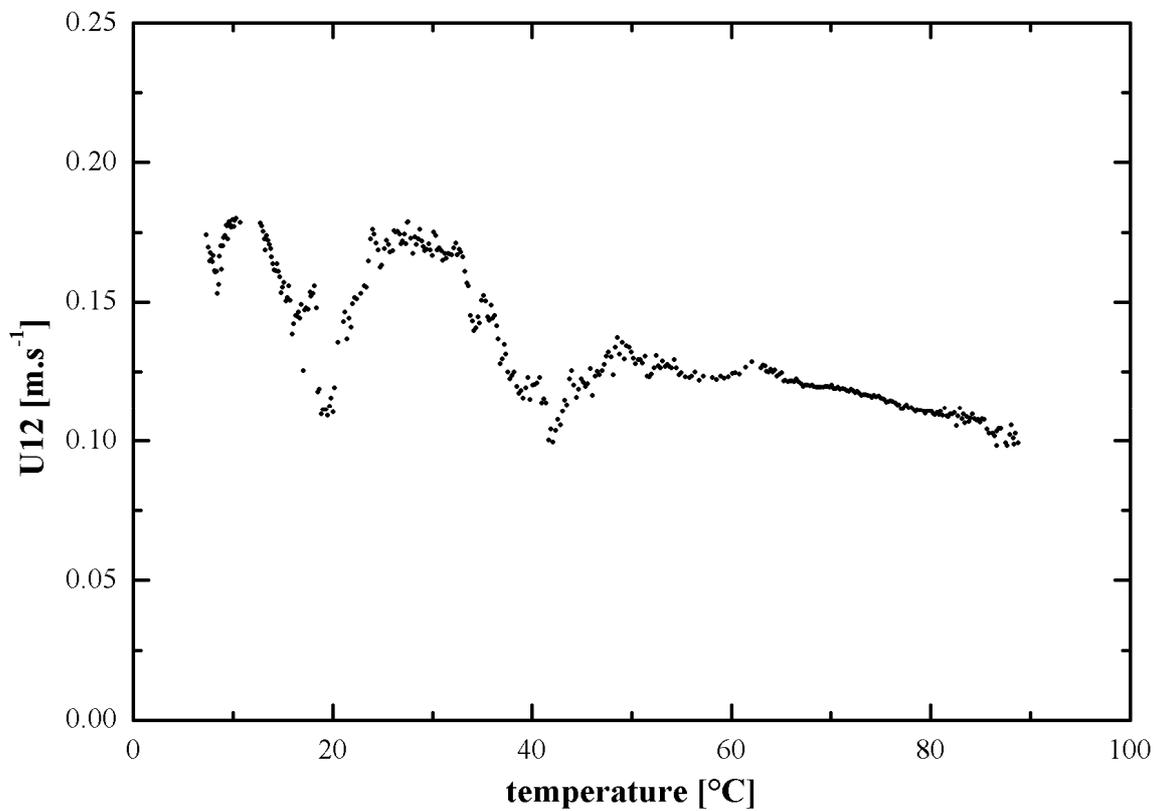
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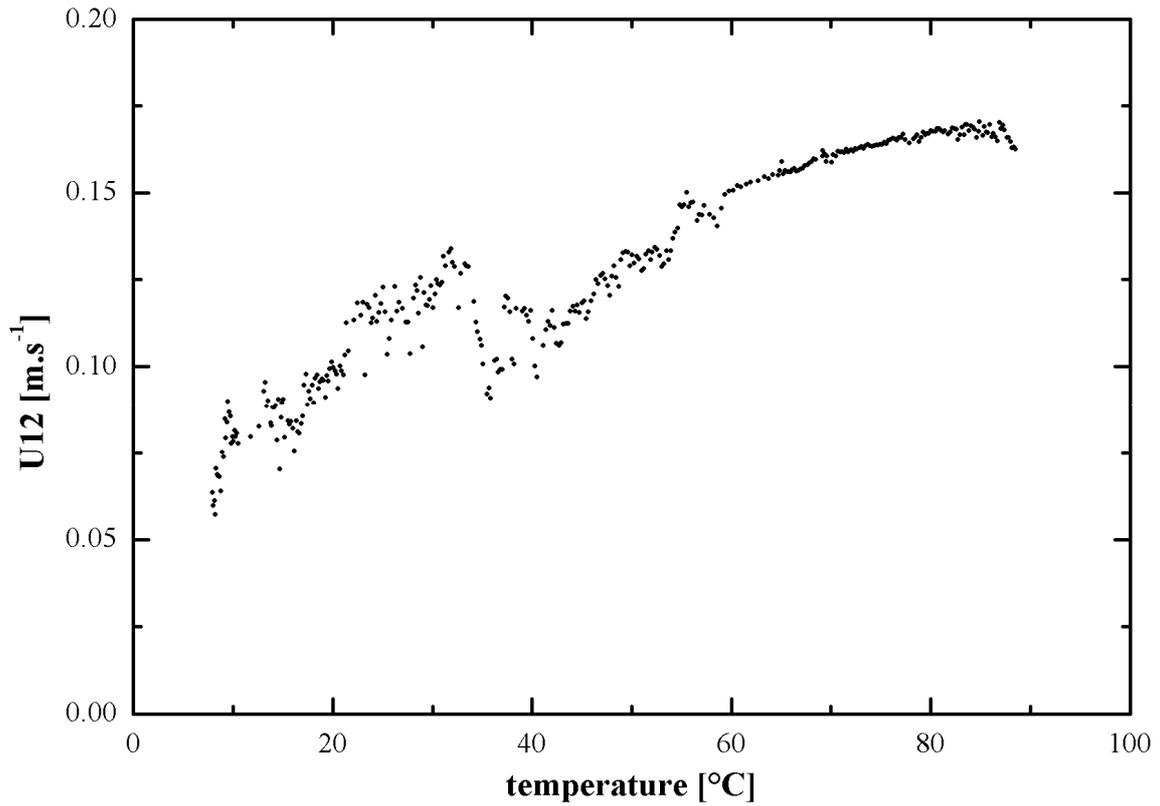
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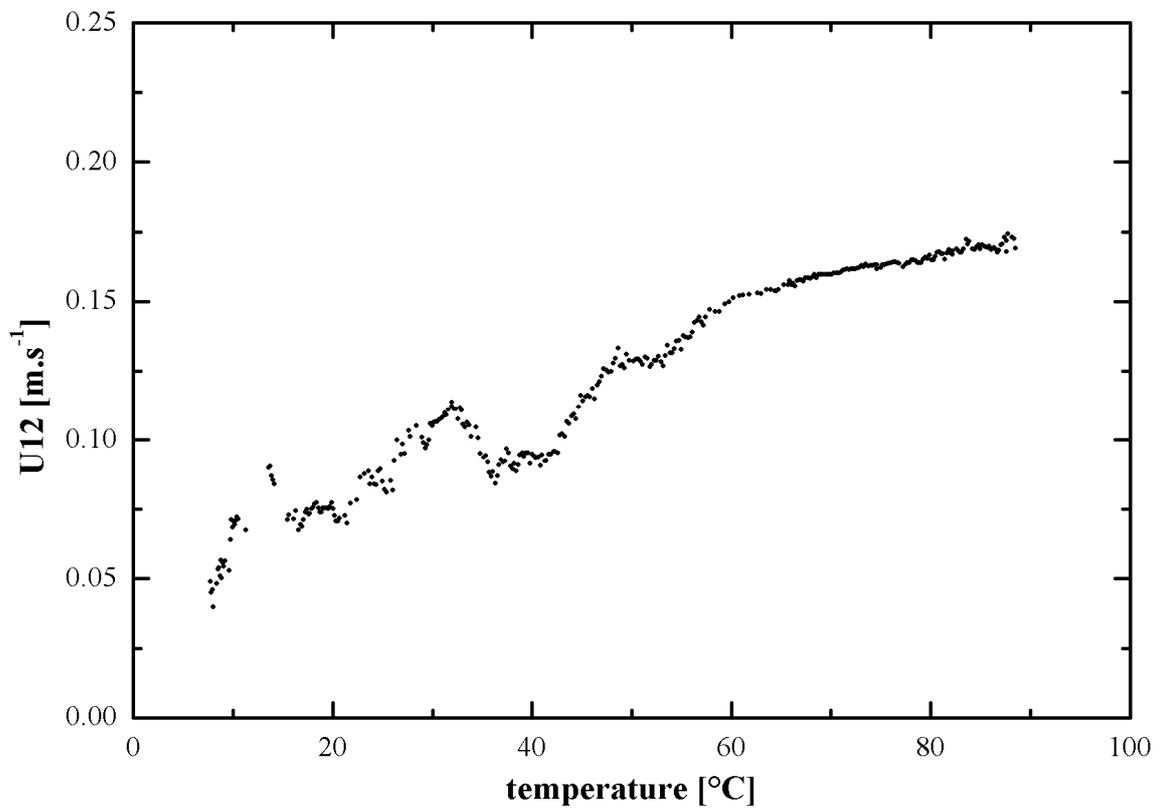
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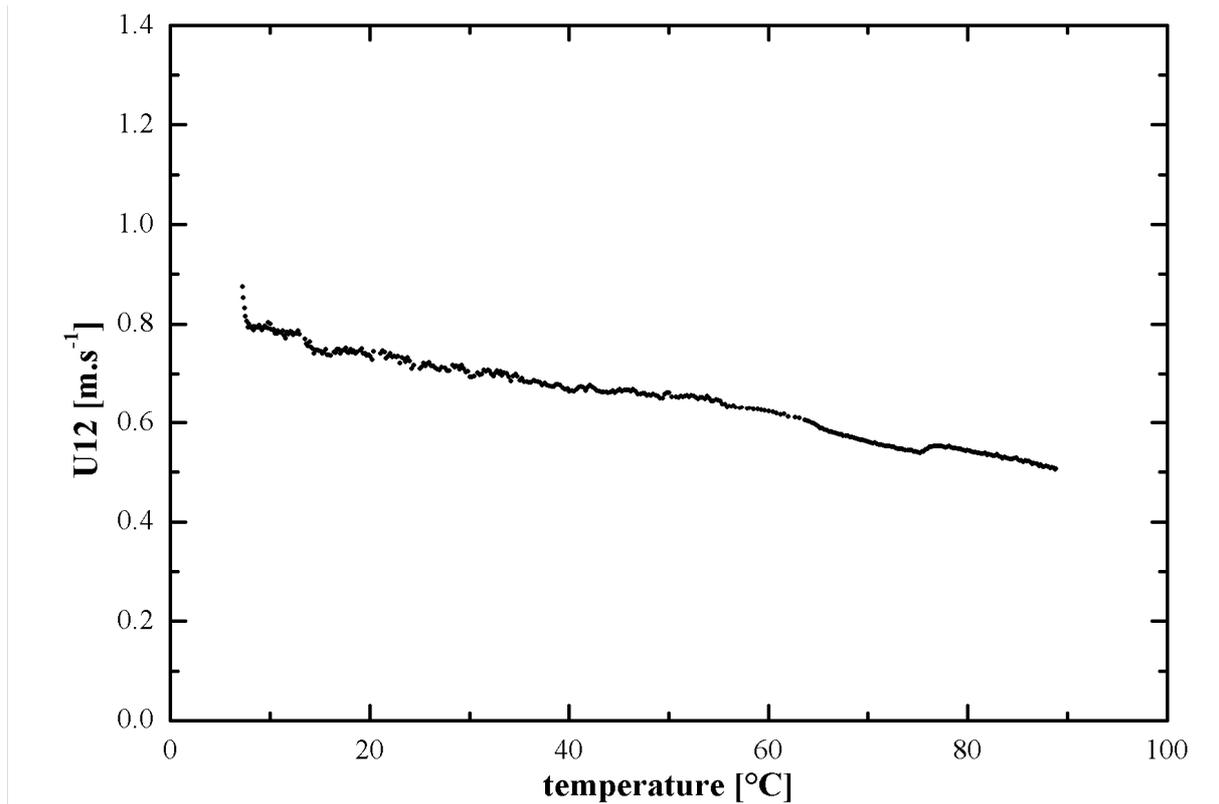
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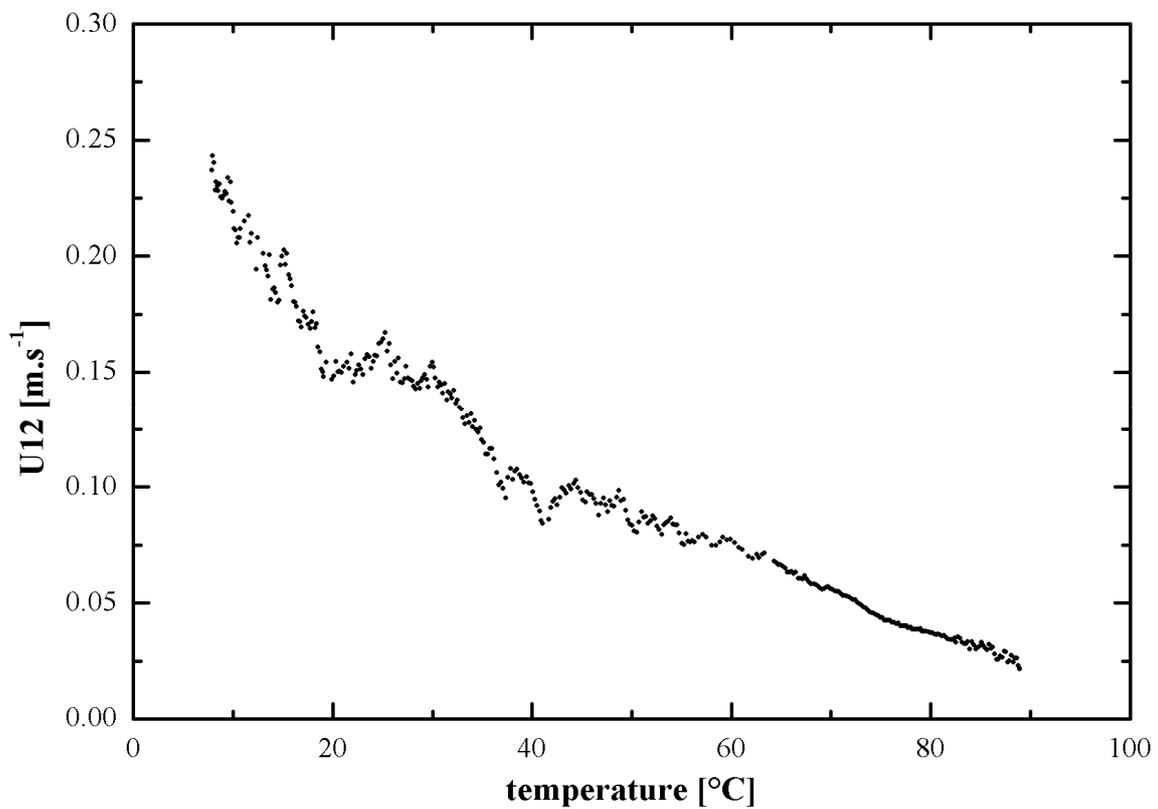
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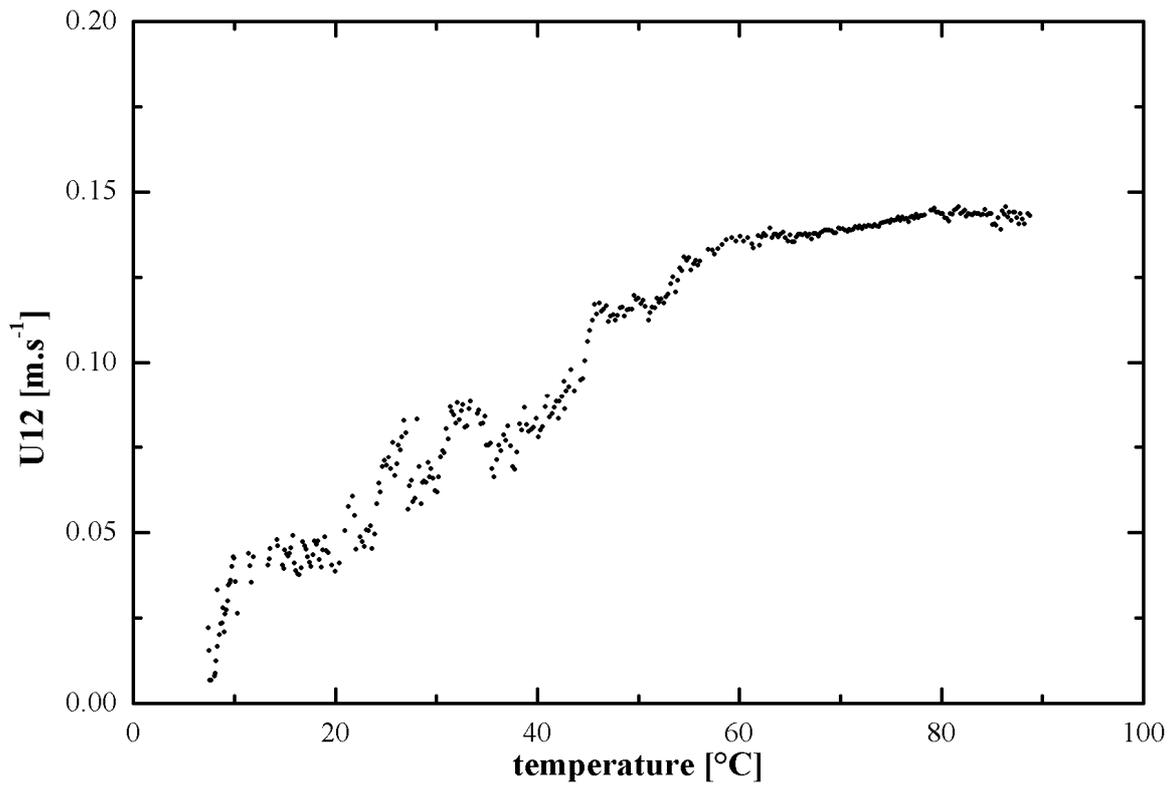
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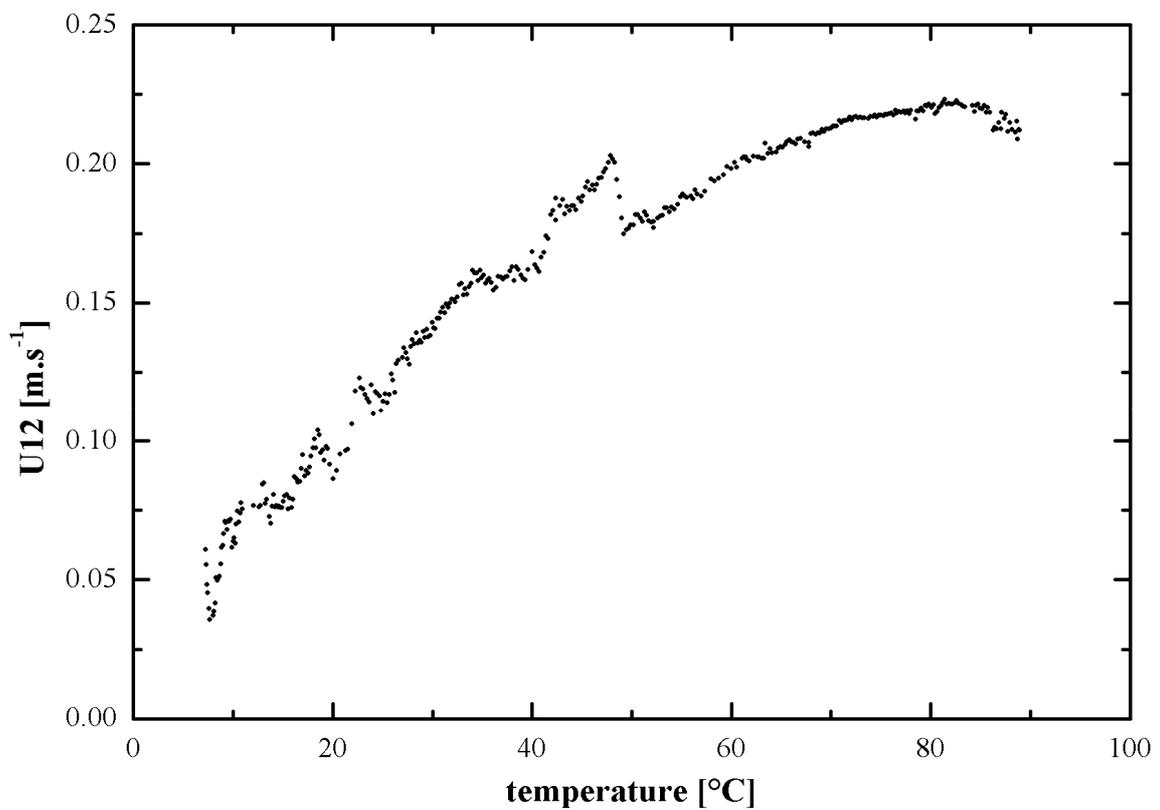
Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Elliott Soil at concentration 4 g/L.



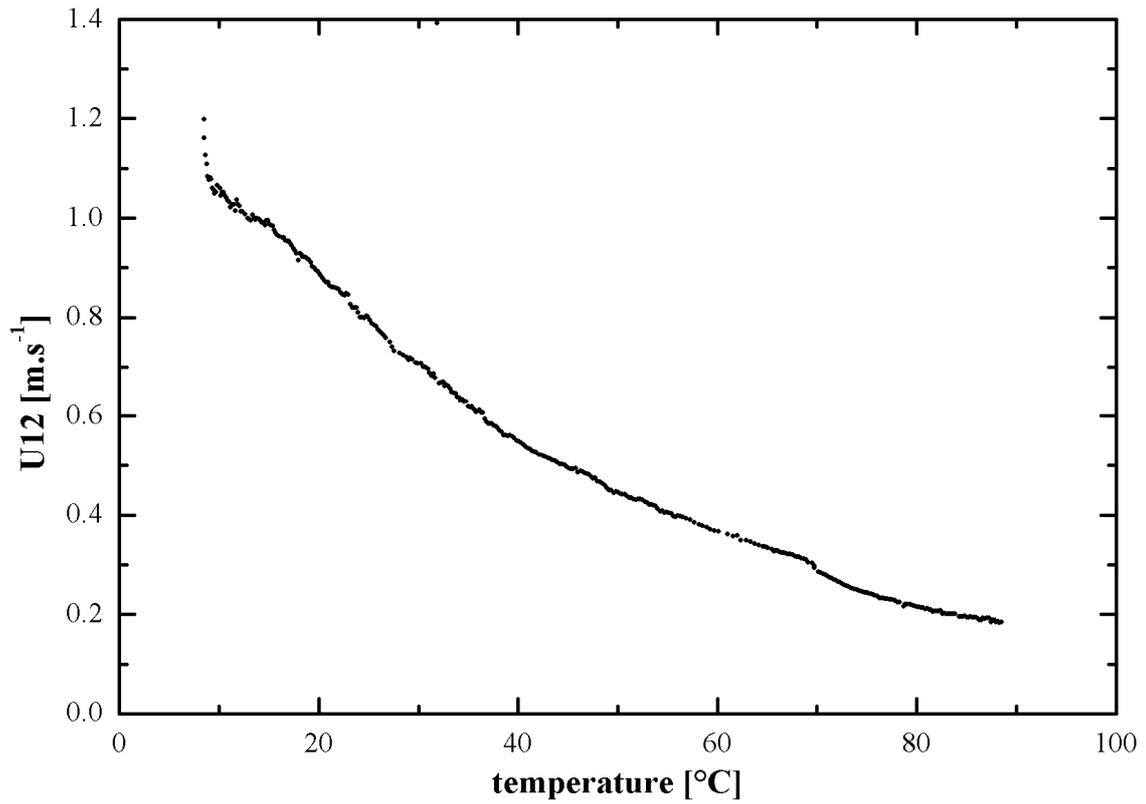
Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Elliott Soil at concentration 1 g/L.



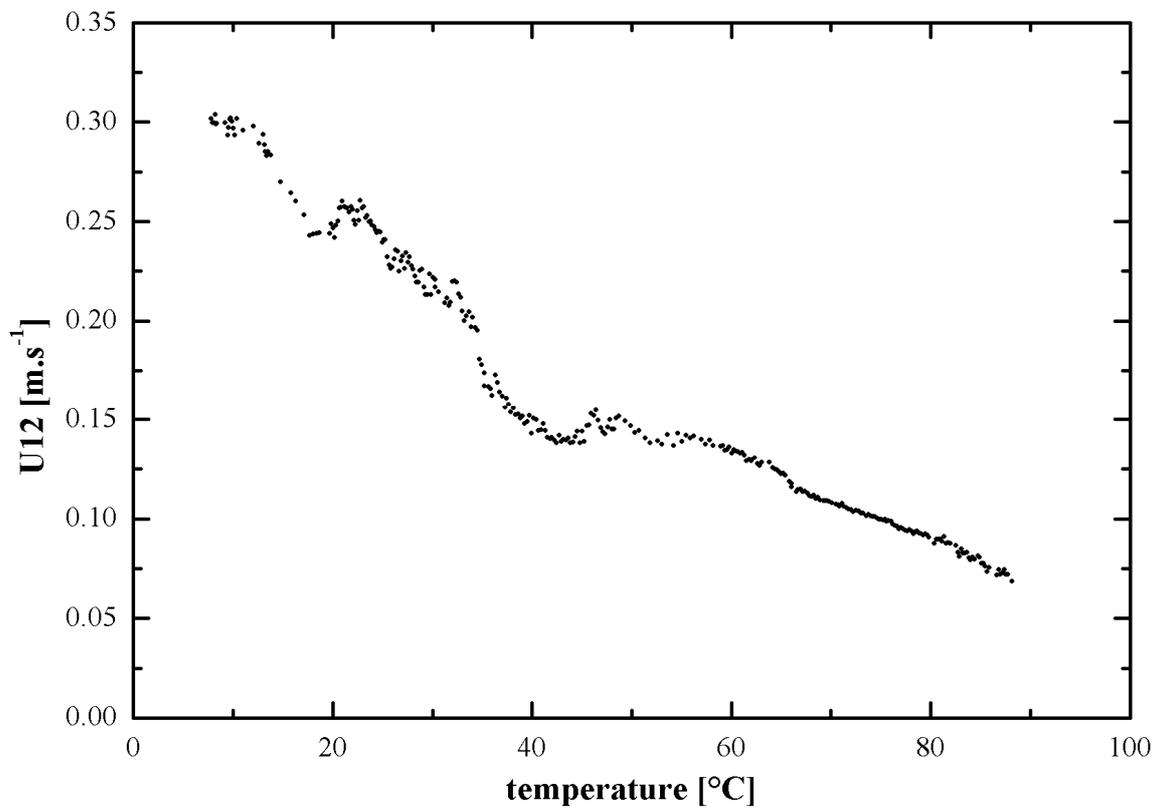
Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Elliott Soil at concentration 0.1 g/L.



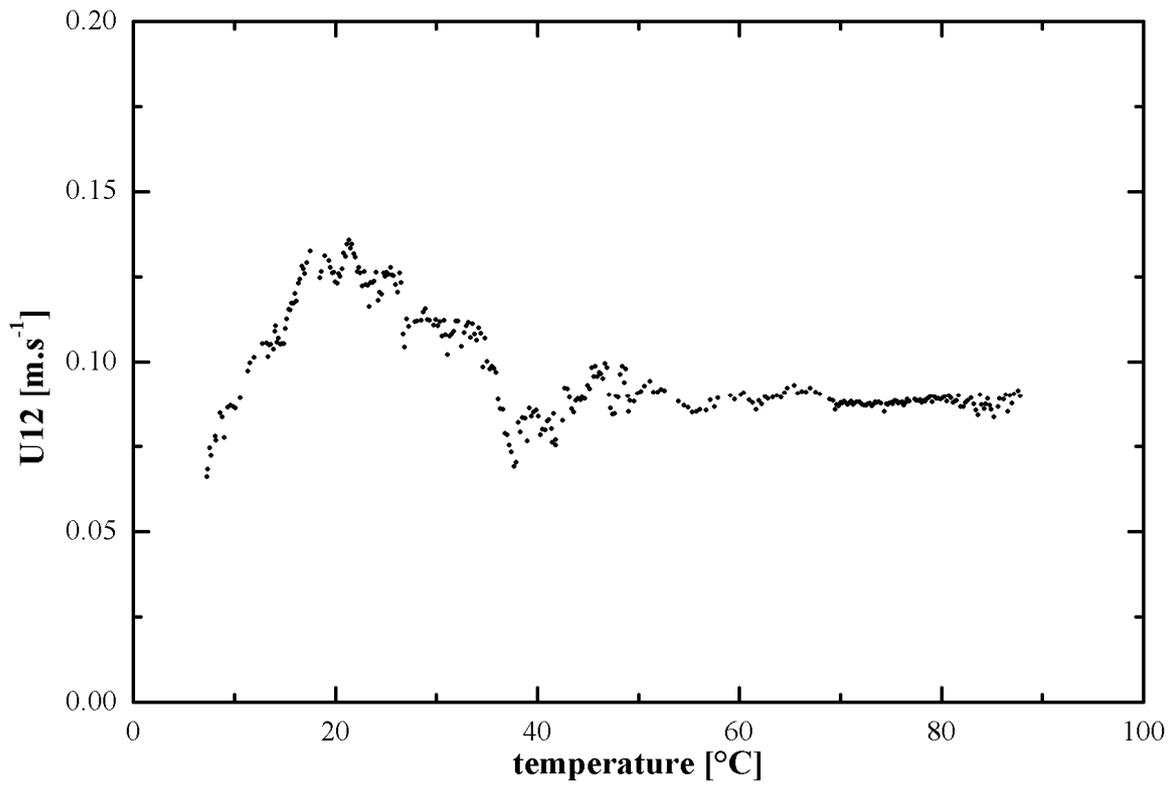
Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Elliott Soil at concentration 0.01 g/L.



Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Pahokee Peat at concentration 4 g/L.



Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Pahokee Peat at concentration 1 g/L.



Dependency of difference in ultrasonic velocity U_{12} on temperature for NaFA Pahokee Peat at concentration 0.1 g/L.