Open Access

^{13}C-NMR Chemical Shift Databases as a Quick Tool to Evaluate Structural Models of Humic Substances

Christian Nyrop Albers*,[a,b] and Poul Erik Hansen*,[a]

[a] *Department Science, Systems and Models, Roskilde University, Universitetsvej 1, DK-4000, Roskilde, Denmark*

[b] *Department Geochemistry, Geological Survey of Denmark & Greenland, Ø. Voldgade 10, DK-1350, Copenhagen, Denmark*

Abstract: Models for humic and fulvic acids are discussed based on ^{13}C liquid state NMR spectra combined with results from elemental analysis and titration studies. The analysis of NMR spectra is based on a full reconstruction of the NMR spectrum done with help of ^{13}C-NMR data bases by adding up chemical shifts of all substructures from the proposed models. A full reconstruction makes sure that all carbons are accounted for and enables on the negative side to discuss structural elements identified from recorded spectra of humic substances that cannot be observed in the simulated spectrum. On the positive side missing structural elements in the models can be suggested. A number of proposed structures for humic and fulvic acids are discussed based on the above analysis.

Keywords: Humic acid, fulvic acid, ^{13}C NMR, spectral reconstruction.

INTRODUCTION

Humic substances (HS) are one of the most abundant organic materials on earth. They represent 30-75% of soil organic matter (SOM) [1, 2] and the majority of dissolved organic matter (DOM) in both fresh and salt waters. Traditionally, HS from SOM has been subdivided into three fractions; Fulvic acids (FA) soluble at all pHs, Humic acids (HA) soluble at basic pHs and humin, not soluble in aqueous solutions. In DOM, only FA and HA, and often mainly FA, represents HS [3]. Both HA and FA play a major role in binding and, hence, fate of both organic compounds, including pollutants like pesticides and polyaromatic hydrocarbons [4-7], and inorganic materials, especially cations [8]. The capacity for binding organic and inorganic pollutants as well as many other properties of HS is determined by the presence of various structural elements. Among those, aromatic structures have been shown to be important for binding of aromatic pollutants [5, 6] while carboxylic acid groups are important for the complex binding of cations [8]. The molecular structure of HS is very variable and complex, and despite many years of research, no agreement for a common structure has been reached. There seems to be a general agreement that no single structure can be found. However, during the years very different representative models for HA and FA have been proposed (see e.g. Figs. (**2** & **3**) and references therein). These models have included very different structural subunits as well as similar structural elements in very different proportions. This variety of models and substructures can be highly confusing if one seeks inspiration in the literature e.g. to understand and predict interactions between a HS-fraction and some pollutant as well as understanding other properties of HS. One possibility to explain the variety of structural models could be that they represent different environmental compartments and hence different parent material. Principal component analysis (PCA) and similar techniques have proven quite useful in distinguishing between origin based on measured functional groups and other parameters [9, 10], but in these studies it was found that while FA and HA fractions can easily be separated based on structural characteristics, the same fraction obtained from different soils are not so different. Soil and lignite HAs were clearly separated, though, so the origin does have an effect as well. The purpose of the present paper is to suggest ways of characterizing different fractions of humic substances and humic fractions from different environments using liquid state NMR and ^{13}C NMR databases and to verify the validity of the previously proposed models by such method.

In characterizing the complex humic substances it is obviously advantageous to use as many descriptors as possible. Some of these descriptors may be obtained using NMR. Both ^{1}H- and ^{13}C-NMR have been used, the latter also in the solid state. Obvious 2D techniques are HETCOR, HMQC or HSQC [11-14]. The result is a correlation between ^{1}H and ^{13}C chemical shifts. A plot of these parameters shows that the ^{1}H and ^{13}C chemical shifts are largely proportional as has been found in general [15], but in some cases such spectra have proved useful, e.g. in identifying substructures of the aromatic parts of HA and FA [11]. HMBC spectra have also been included. The extra information of such spectra is to some extent counteracted by the many extra resonances and increased overlap (see e.g. Ref. [16]).

It is essential to distinguish between liquid and solid state spectra as different rules about intensities are found. In the present paper we concentrate on 1D ^{13}C liquid state spectra, which have been used extensively to identify the presence of functional groups such as ketones, carboxylic acids, amides, oxygen substituted aromatic carbons, methoxy groups and

---

*Address correspondence to these authors at the Department Science, Systems and Models, Roskilde University, Universitetsvej 1, DK-4000, Roskilde, Denmark; Tel: +45 46742432; Fax: +45 46743011;
E-mails: calbers@ruc.dk, poulerik@ruc.dk

aliphatic side chains as well as small molecular fragments such as carbohydrates and amino acids. Since ^{13}C-NMR is a chemical shift based method, even the carbon skeleton itself will give rise to widely different chemical shifts (Fig. **1**). In a broad sense the number of terminal carbons in aliphatic chains, versus the central carbons and finally more branched situations can be estimated from the shape of the aliphatic signal. Other elements such as oxygen, nitrogen and sulphur are detected by means of their substituent effects. Except for HAs derived from lignite, very little sulphur is present so this is really not an issue. For single oxygens the substituent effects are so that both aliphatic and aromatic substituents can easily be detected, with e.g. mono-phenolic structural elements typically appearing around 160 ppm. For di- and tri-phenolic elements, the picture is less clear, and some or all of the O-substituted C-atoms will give rise to signals at much lower frequency (Fig. **1e**).

A structural model of HS may be constructed based on identified structural elements, carboxylic acids, ketones aromatic carbons etc. A more holistic approach is to identify structural elements, gather a possible model and reconstruct the entire NMR spectrum of the proposed model [10, 13]. This has been demonstrated based on data using chemical software like e.g. ChemDraw [13] or based on suggested structures and data from data bases [10]. 2D NMR data bases have also been constructed to identify structural elements and with the intent to reconstruct spectra of complex mixtures like humic substances [16]. The entire 2D spectrum is, however, not yet possible to match, and the easy access to 1D NMR data bases and the ability to find suitable data for complex structures [17] have prompted this study of a series of suggested models of HA and FA.

MATERIALS AND METHODS

Liquid State ^{13}C-NMR

HA and FA were purified from clayey agricultural soil (Orthic Luvisol, sampled from the A-horizon), sandy agricultural soil (Humic Podzol, sampled from the B_h-horizon), clayey grassland soil (Luvisol, sampled from the A-horizon) and sandy coniferous forest soil (aeolian sand in the beginning of a podzolization, sampled from organic H-horizon and B_h-horizon), largely according to the IHSS standard procedure with slight modifications [10]. Briefly, the soil was air-dried and then extracted first with 0.1M HCl followed by extraction with 0.1M NaOH under a N_2-atmposphere. HA was precipitated at pH=1, re-dissolved in KOH + KCl under a N_2-atmosphere, re-precipitated at pH=1 and inorganic impurities were then removed with HCl/HF followed by dialysis. Aldrich HA was purchased from Sigma-Aldrich (Steinheim, Germany) and purified to remove the large content of inorganic impurities and FA as described previously [10].

Liquid state ^{13}C-NMR of humic fractions was performed as follows: ~80 mg HS was dissolved in 680 µl $H_2O{:}D_2O$ (4:1) and pH was adjusted with 10 M NaOH to pH=7 (FA) or pH=11 (HA). Spectra were recorded on a Varian 600 Inova Spectrometer (Varian, Palo Alto, California), working at 150 MHz on ^{13}C, using a 5 mm BB-probe. Spectral width was set to 40000 Hz, and 50000-100000 transients were recorded with gated decoupling to suppress Nuclear Overhauser Effects with: Delay between experiments = 1000 ms, pulse width = 6 µs (corresponding to a flip angle of 52^{o}), acquisition time = 438 ms. Spectra were also recorded with a delay of 2000 ms between experiments but this gave no differences in intensities. A line broadening (LB) of 50 Hz was applied to all spectra and 3-trimethylsilyl propionate (TSP) was used as an external reference. One spectrum of freshwater FA and marine HA was taken from the literature in order to compare across a wider range of environmental compartments.

Simulated Spectra

The spectral data base Modgraph NMRPredict (MestreLab Research) [17] was used to predict chemical shifts. The software predicts chemical shifts with protonated COOH and phenolic OH groups, while spectra of FA fractions were recorded at pH=7 where COOH groups are deprotonated, and HA fractions were recorded at pH=11 where most phenolic OH groups would also be deprotonated. In order to compare recorded and simulated spectra, the predicted COOH chemical shifts were changed by adding 9 ppm to the values obtained for the Aromatic COOH groups and 6 ppm to those calculated for the Alkyl COOH systems. 9 and 6 ppm are

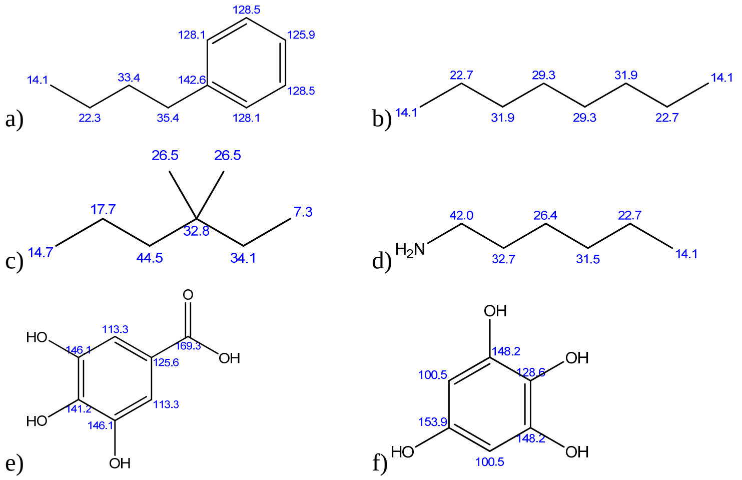

Fig. (1). ^{13}C chemical shifts of model structures.

reported to be the average chemical shift changes occurring when deprotonation of carboxylic acids arises following the reactions: Aromatic COOH (Ar-COOH) → Aromatic COO- (Ar-COO-) and Alkyl-COOH → Alkyl-COO-, respectively [18]. Chemical shifts of phenols are also affected by the deprotonation that occurs at pH=11, where the HA fractions were dissolved. The number of phenolic structures for which such a change in chemical shift has been reported, is scarce and more important, very different changes in chemical shifts have been observed, depending on numbers and types of substituents on the ring [18-20]. Therefore no attempts were made to correct this. For the predicted spectra of HA, this gives a slight error around 160-170 ppm, while for the FA fractions (analyzed at pH=7) this was not of concern. Spectra were simulated on the assumption of Lorentz-formed peaks with a line broadening (LB) of 400 Hz at half peak-height.

RESULTS AND DISCUSSION

Models of HS

A large number of model structures have been suggested over the years. For the present study, we have chosen 11 structures, which present a wide range of the most recent models within different humic fractions and deriving from several environmental compartments (Figs. **2** & **3**). The models were chosen on the basis that they have been cited often and/or are the newest representatives for their class of HS. It is obvious that no single structure as such can be constructed for neither HS in general nor even for a specific fraction of HS from a specific environmental compartment. Most if not all of the authors who have proposed the structural models in Figs. (**2** & **3**), have emphasized that their model is not the final solution to humic structures, but that the structures should rather be considered as assemblies of structural elements. Despite these reservations, the authors have presented such very different structures, that they obviously cannot all be representative of some "average structure" of HAs or FAs.

^{13}C-NMR Spectra

Numerous ^{13}C-NMR spectra have been published during the years. Spectra have been recorded with the HS either in aqueous solutions or in the solid state. The intensities are clearly different in the liquid and solid state spectra as pointed out by Conte *et al.* [28] and as seen from Fig. (**4**).

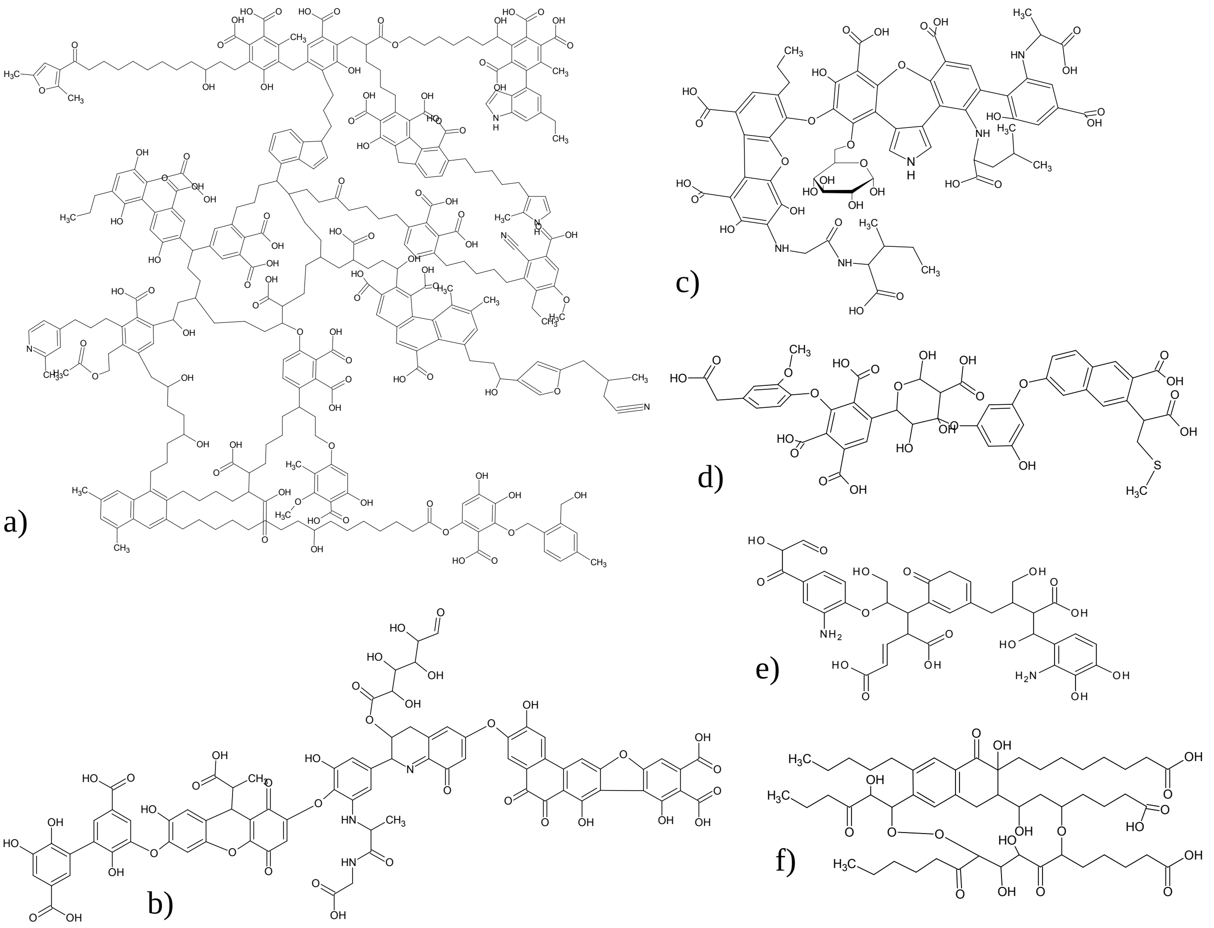

Fig. (2). Structural models for humic acids (HAs), taken from the literature. **a**) Not further defined HA (Schulten & Schnitzer, 1993) [21]. **b**) Not further defined HA (Stevenson, 1994) [1]. **c**) Agricultural soil HA (Albers *et al.*, 2008) [10]. **d**) Agricultural soil HA. One of five substructures (Diallo *et al.*, 2003) [13] **e**) Terrestrial HA from commercial Acros Organics Humic Substance (Sein *et al.*, 1999) [22]. **f**) Marine HA (Harvey *et al.*, 1983) [23].

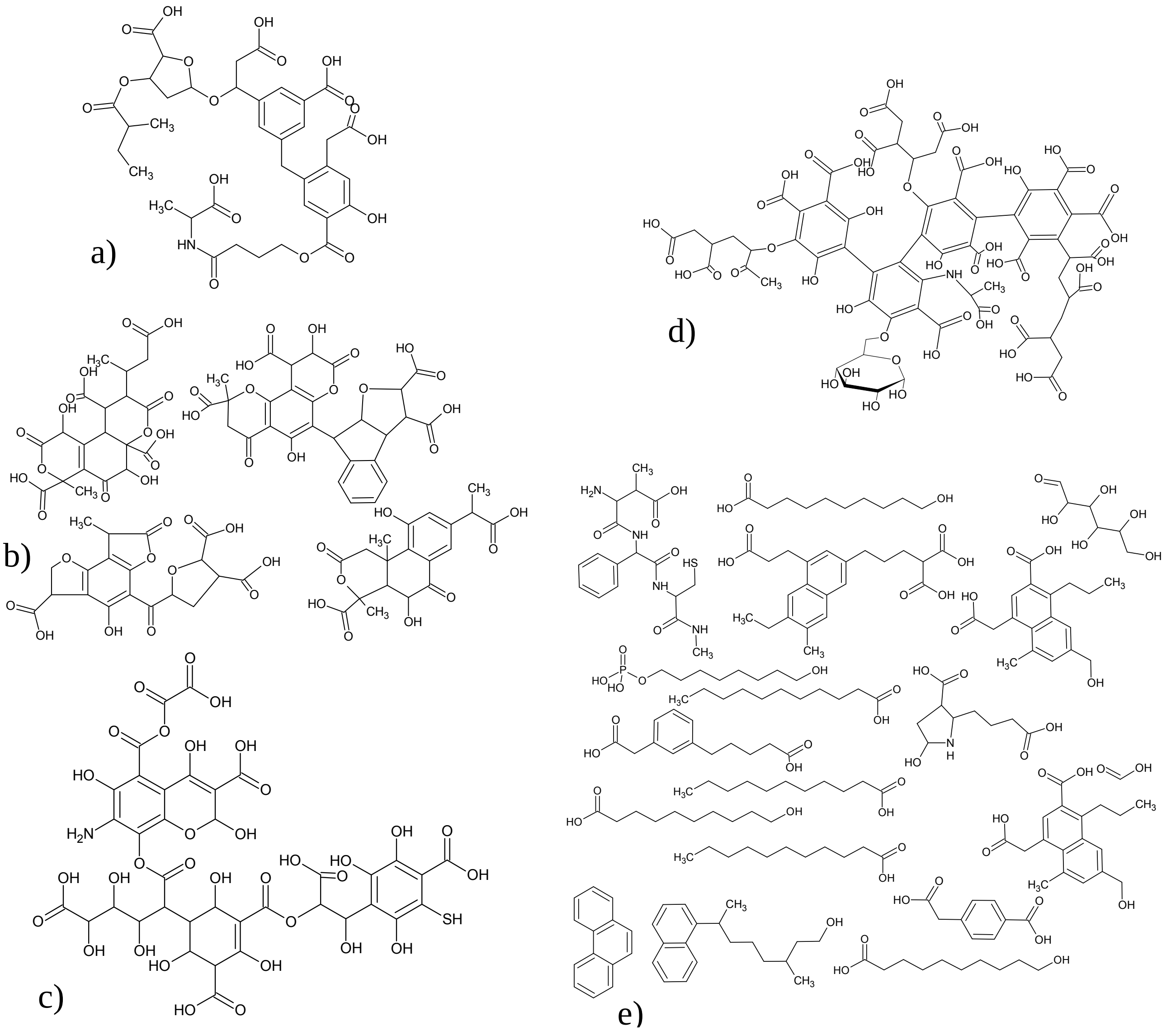

Fig. (3). Structural models for fulvic acids (FAs) and whole soil HS from literature. **a**) Soil FA (Shin & Moon, 1996) [24]. **b**) Four structural units representing Suwannee River FA (Leenheer & Rostad, 2004) [25]. **c**) Terrestrial FA from commercial Acros Organics Humic substance (Alvarez-Puebla *et al.*, 2006) [26]. **d**) Agricultural soil FA (Albers *et al.*, 2008) [10]. **e**) Assembly of the partial structures included in a conceptual model for whole soil SOM (Kleber *et al.*, 2007) [27].

This could be a serious problem in an attempt to reconstruct spectra. Recent experiments have shown that a lignite humic acid could be separated into fractions with varying sizes and aromatic and aliphatic contents [29]. This is explained by the recently growing view that humic substances are supramolecular assemblies of compounds having relatively low molecular weights [30, 31]. This again could lead to loss of signal in the aliphatic region and therefore underestimation of the aliphatic region (0-45 ppm) if parts of the aliphatic chains are in a hydrophobic core of very large micelles or similar macro ensembles. However, as seen from our previous experiments combining NMR, elemental analysis and titration studies a good balance was actually found between the different types of data [10]. This can be elaborated as follows for a typical agricultural field humic acid (SlA HA). From elemental analysis the formula was determined as $C_{65}H_{65}O_{32}N_5$ [10]. This leads to 36 degrees of unsaturation (DoU). Eight carboxylic acid groups could be identified either from integration of liquid state ^{13}C-NMR or a little less (5-6) from titration studies and in addition six carbohydrate carbons and four amino acid αC carbons could be determined from the NMR experiments [10]. Subtracting the average of the two estimates of carboxylic acids as well as carbohydrate and amino acid carbons, the identified units we end up with ~48 carbons and ~28 DoU left for aromatic and aliphatic (0-45 ppm) structures. If we assume that all rings are benzene types this requires $^2/_3$ DoU pr. carbon. However, if also heterocyclic aromatic rings are present like furans, benzofurans etc. it is closer to ¾ DoU pr. carbon. 28 DoU corresponds in the case of $^2/_3$ DoU pr. carbon to 42 carbons and in the case of ¾ DoU pr. carbon to 37 carbons of aromatic type leaving only 6 respectively 11 carbons for the

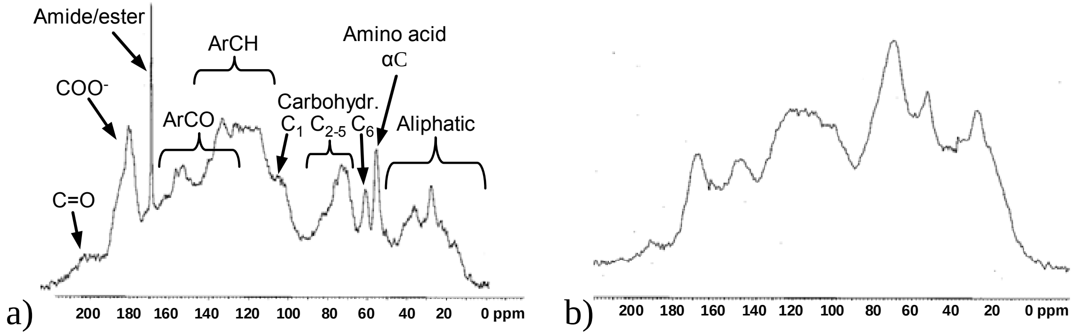

Fig. (4). Comparison of **a**) liquid and **b**) solid state ^{13}C-NMR spectra of a forest HA. Peaks "assigned" according to Albers *et al.*, 2008 [10].

aliphatic region. Integration of liquid state ^{13}C-NMR gave 18% aliphatic (0-45 ppm) carbon [10] corresponding to 11.7 aliphatic carbons in the proposed structure with totally 65 carbon atoms. Since only 6-11 carbons were left to aliphatic structures in the above calculation, this even leaves room for a number of non-aromatic ring structures. Furthermore it shows that the aliphatic part in humic acids of field type is most likely not underestimated using liquid state ^{13}C-NMR. On that basis we have decided to use this NMR method for our reconstructions or in other words as our tool to evaluate the various models. Another good reason for this is the narrower line widths of liquid state spectra, and hence more insight into the presence of various structural elements (Fig. **4**), as well as the possibility to distinguish amides/esters from carboxylic acids [10].

Spectra of various HAs and FAs are presented in Figs. (**5** and **6**). The presented spectra are typical for a range of HAs and FAs, and should largely cover those fractions and environmental compartments that the HS-models in Figs. (**2** and **3**) are suggested to represent. Spectra of HA from widely different soils are somewhat similar, reflecting that despite the differences in parent material, HS-fractions from different geographical and environmental compartments are most often surprisingly similar. This is the case regarding structural elements as well as various physical behaviors. Also between soil horizons, the differences can be quite minimal, as seen for forest HA extracted from the organic horizon (Fig. **5d**) and from a subhorizon (Fig. **5e**). PCA analyses have previously demonstrated HAs extracted from lignite, like Aldrich HA, to be in a class of their own when compared to soil HA [9, 10]. This is due mainly to the lack of some specific groups like amino acids and carbohydrates (Fig. **5a**), but despite this, Aldrich HA does share some similarities with the field and forest HAs. The marine HA (Fig. **5f**), although less aromatic than the other HAs, also shares most of the structural elements of the soil HAs.

The FA-fractions (Fig. **6**) are in general less aromatic than the HA-fractions from the same environments. Beside this, they do however carry most of the same structural elements and show somewhat similar shapes of the ^{13}C-NMR spectra, also when comparing the terrestrial FAs (Fig. **6a** & **b**) with the aquatic FA (Fig. **6c**). Significant differences are lower amounts of amino acids in the FA-fraction (seen as a less pronounced peak ~55 ppm) and a shift away from long chain unbranched aliphatics (seen as a peak ~30-35 ppm) towards peaks in the aliphatic area ~40 and/or 20-30 ppm. This does not really seem to be the case for the Suwannee River FA, though (Fig. **6c**).

Simulated ^{13}C-NMR Spectra of HS-Models

Predicted ^{13}C-NMR spectra of all 11 HS-models in Figs. (**2** and **3**) are presented in Figs. (**7** and **8**).

Humic Acids

The authors who suggested the molecularly large soil HA polymer model in Fig. (**2a**) did not ascribe their model to HA from any specific environmental compartment [21]. Since the model lacks amino acids and carbohydrates, it is not immediately a very good model for soil HAs. This is clearly reflected in the simulated spectrum (Fig. **7a**) and furthermore, there seem to be too many aliphatic subunits and too few aromatic and carboxylic subunits. Also, the aromatic area is too narrow around the typical chemical shifts of unsubstituted aromatic carbons, which indicates too few O-substituted aromatics compared to typical HAs. Nevertheless, compared to any of the other model simulations, this model does show the best fit in the unsubstituted aromatic region (115-145 ppm), with a clear peak around 128 ppm, which is typically observed in ^{13}C-NMR spectra of both HAs and FAs. HAs from lignite do not contain significant amounts of amino acids and carbohydrates, and in comparison with Aldrich HA (Fig. **5a**), the simulated ^{13}C-NMR spectrum of the model proposed by Schulten & Schnitzer [21] is rather good. Looking at other properties of the model, the fit of this HA is less perfect, with regards to e.g. the CHNO content, which is far from lignite HAs like Aldrich HA (Table **1**), and also different from what is typically observed for soil HAs [1, 34], Table **1**.

The model proposed by Stevenson [1], contains amino acid and carbohydrate structural elements and therefore could be a likely candidate for soil HA, but as quickly recognized from the simulated spectra there are some deficiencies in the aliphatic part of the spectrum. The rather recent models proposed by Diallo *et al.* [13] and Albers *et al.* [10] are both constructed to be models of soil HA. While the models are clearly better representatives of soil HA than many other published models, they also have some deficiencies. In the Albers *et al.* HA, despite having the right proportions with regards to various structural elements and also the elemental content (Table **1**) [10], some aliphatic signals in the region 30-35 ppm are missing. Furthermore, the aromatic

105

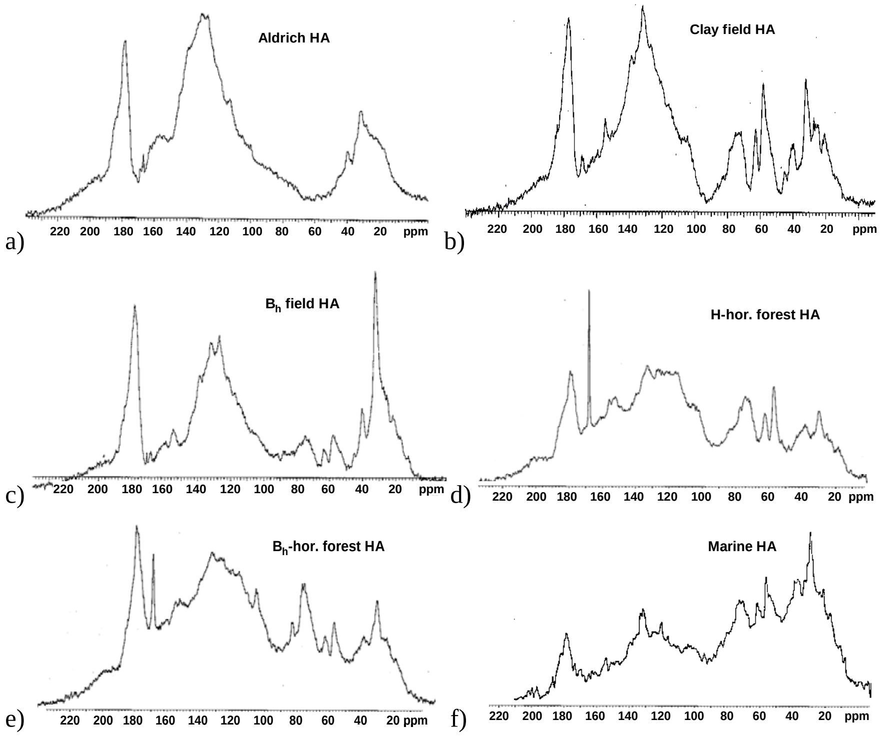

Fig. (5). Liquid state ^{13}C-NMR spectra of humic acids (HAs). **a**) Aldrich HA, commercial HA extracted from lignite. **b**) Field HA, extracted from clayey soil. **c**) Field HA, extracted from the B$_h$-horizon of an acidic sandy podzol. **d**) Forest HA, extracted from the organic H-horizon. **e**) Forest HA, extracted from the B$_h$ horizon of a forest podzol. **f**) Marine HA [32].

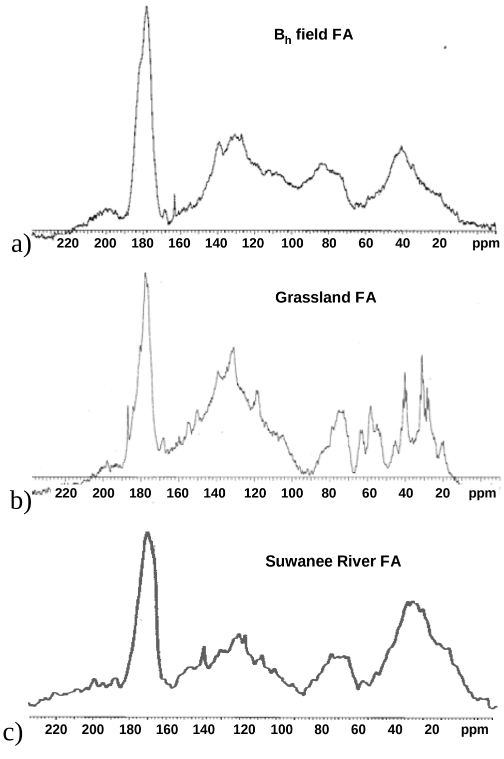

Fig. (6). Liquid state ^{13}C-NMR spectra of fulvic acids (FAs). **a**) Field FA, extracted with acid from the B$_h$-horizon of an acidic sandy podzol. **b**) Grassland FA, extracted from a clayey soil. **c**) Aquatic FA, the IHSS standard FA from Suwannee River [33].

region is not shaped, with a clear peak around 128 ppm, as seen for recorded spectrum of soil HA. The same is the case for the model proposed by Diallo *et al.* [13] and furthermore this model clearly lacks aliphatic structures which will give rise to chemical shifts around 30 ppm. Likely candidates for this would be simple unsubstituted aliphatic chains, which might be incorporated in the proposed model. Furthermore, as previously pointed out [10], the N- and S-content should be changed in order to make this model in better agreement with typical soil HAs.

Sein *et al.* [22] proposed a model of terrestrial HA (Fig. **2e**), but it was not clear if the HA used as a model HA, was derived from soil or lignite. The proposed model could possibly be one structural element in HA, since, apart from a too intense carbonyl-signal, its simulated spectrum contains no wrong signals (Fig. **7e**). It does however miss amino acids, which is reflected in the lack of signal ~55 ppm and furthermore it contains no unsubstituted aliphatics, which is also reflected in the simulated spectrum as the lack of signal below ~32 ppm. It can therefore not stand alone as a representative structure of HA.

We have included a model of a marine HA, since, compared to soil HAs, these HAs have very different parent material, which would also be expected to be reflected in their final structure and hence ^{13}C-NMR spectra (Fig. **5f**). One of the characteristics of marine HAs is their low aromatic contents, and this feature is well captured by the model of Harvey *et al.* [23]. Furthermore, the model contains rather long aliphatic chains, which give rise to the intense signal at ~30 ppm, which is also seen in the recorded ^{13}C-NMR spectrum.

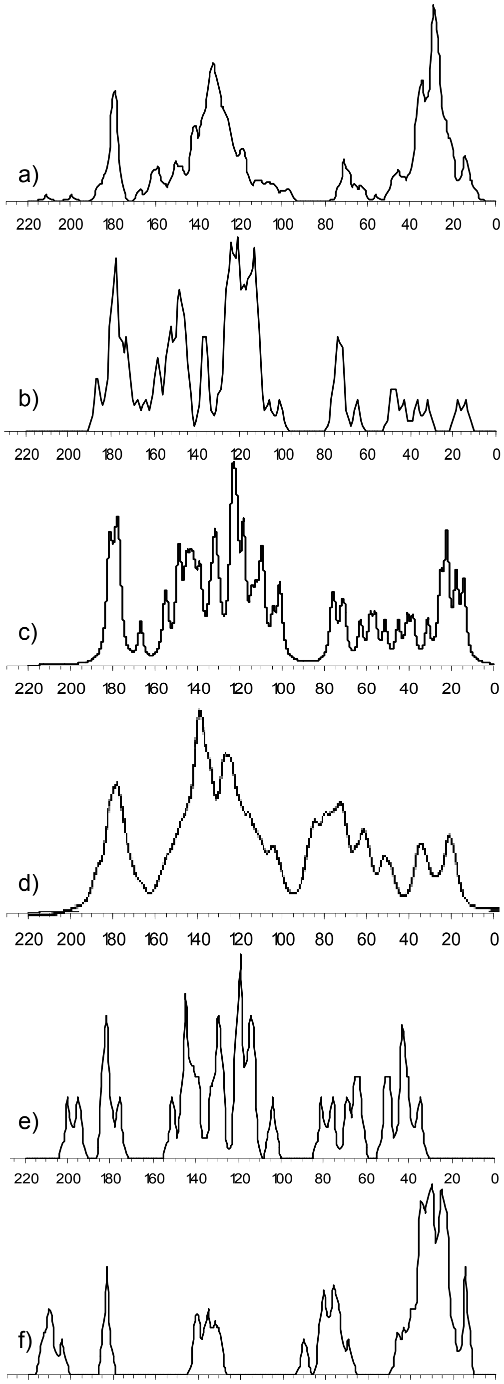

Fig. (7). Simulated ^{13}C-NMR spectra of models of humic acids (HAs). The units on the x-axes are all in ppm. **a**) Not further defined HA (Schulten & Schnitzer, 1993) [21]. **b**) Not further defined HA (Stevenson, 1994) [1]. **c**) Agricultural soil HA (Albers *et al.*, 2008) [10]. **d**) Agricultural soil HA taken from Diallo *et al.* (2003) [13]. The spectrum was based on five proposed substructures of which one is shown in Fig. (**2d**). **e**) Terrestrial HA from commercial Acros Organics Humic Substance (Sein *et al.*, 1999) [22]. **f**) Marine HA (Harvey *et al.*, 1983) [23].

The two distinct peaks at ~55 and 63 ppm, attributed to amino acid αC and C_6 of carbohydrates, respectively, is however not seen in the simulated spectra, and looking at the model structure (Fig. **2f**), no carbohydrate or amino acid structural elements are seen. Since also a peak at ~105 ppm (anomeric carbohydrate signal) is seen in the recorded spectrum of a marine HA, most of the signal around 75-80 ppm most likely derives from carbohydrate, and the model should probably contain less aliphatic alcohols and instead include carbohydrate structures. Furthermore, there are four ketonic carbons in the model, which gives rise to a rather intense signal around 205-210 ppm, while the ketonic signal in the recorded spectrum is low.

Fulvic Acids and Whole Soil HS

The simulated spectra of the four FA-models and the single HS-model are shown in Fig. (**8**). The soil FA model proposed by Shin & Moon [24], is a further development of models previously proposed for Suwanee River FA and the simulated NMR-spectra (Fig. **8a**) is not too different from the Suwanee River FA model, proposed by Leenheer & Rostad [25] (Fig. **8b**). No amino acids are included in neither of these models, which is immediately seen as the lack of signal ~55 ppm where you would usually see a signal from amino acid αCs. Aquatic FAs often do not contain significant amounts of amino acids but, although in lower amounts than the corresponding HA fractions, terrestrial FAs most often do and amino acids should probably be included in this model. The lower amounts of long-chain aliphatics is reflected in a less pronounced peak ~30-35 ppm, in Fig. (**8a** & **b**). The shape of the aliphatic area in Fig. (**8b**) fits well with the recorded spectrum of some soil FA-fractions (Fig. **6b**), but it fits the aquatic FA (Fig. **6c**), which it was constructed for, less well.

Furthermore the intense peak at ~170 ppm (derived from esters in the model) is not really seen in any of the recorded spectra for FAs. The Suwanee River FA seems to be an illustrative example, where an integration of the areas assigned to certain overall structural elements (e.g. aromatics or aliphatics) would lead to the conclusion that the model fits very good, while looking at the simulated spectra, it is obvious that the specific nature of the structural elements, cannot be more than partly correct. This is also the case for the model of terrestrial FA proposed by Alvarez-Puebla *et al.* [26]), in which the aliphatic fraction of C-atoms is so branched and substituted with O-atoms, that, besides being too small overall, it shows most of the aliphatic signals >60 ppm and no aliphatic signals <40 ppm, which is obviously a deficiency. This shape in the aliphatic area is more pronounced but still somewhat similar to the HA proposed by Sein *et al.* [22] (Fig. **2e** & **7e**), which was actually the basis for developing this model of FA [26].

The FA-model proposed by Albers *et al.* [10] (Fig. **8d**) reveals the best shape compared to the recorded spectra, especially to the grassland FA in Fig. (**6b**). The shape of the aromatic peak is not perfect though, with signals missing especially ~130 ppm. This is probably caused by the fact that no unsubstituted aromatic C-atoms exist in the model (Fig. **3d**). Furthermore, some fill-in between aromatic peaks is missing in general, which supports the concept that a purified HS-fraction is a mixture of several more or less similar molecules.

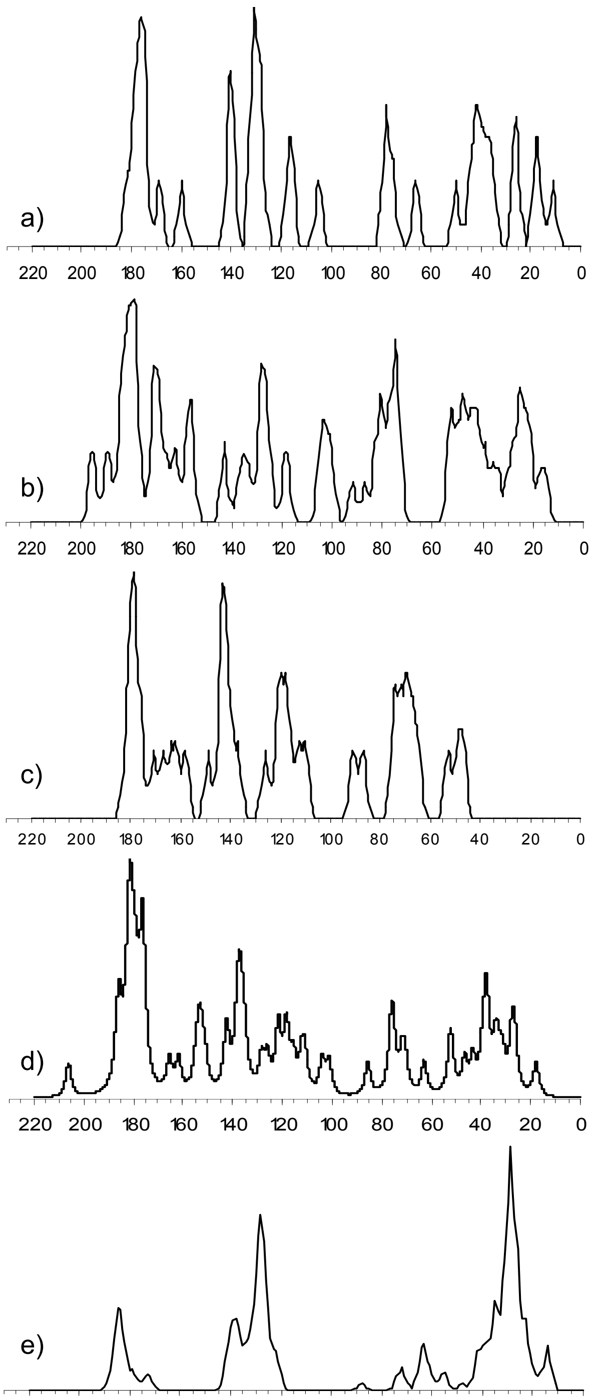

Fig. (8). Simulated ^{13}C-NMR spectra of fulvic acids (FAs) and whole soil HS. The units on the x-axes are all in ppm. **a**) Soil FA (Shin & Moon, 1996) [24]. **b**) Four structural units representing Suwannee River FA (Leenheer & Rostad, 2004) [25]. **c**) Terrestrial FA from commercial Acros Organics Humic substance (Alvarez-Puebla *et al.*, 2006) [26]. **d**) Agricultural soil FA (Albers *et al.*, 2008) [10]. **e**) Assembly of the partial structures included in a conceptual model for whole soil SOM (Kleber *et al.*, 2007) [27].

The model proposed by Kleber *et al.* [27] is a model of SOM in general, that is both FA, HA, humin as well as non-HS SOM, the latter typically making up 25-60% of SOM [1, 2]. The simulated spectrum of this model can therefore not be expected to make up more than ~50% of a mixed spectrum of HA and FA. We have nevertheless included this model, since it is the first model constructed according to the supramolecular structural view of HS/SOM. Furthermore, since it is a model of SOM, which includes HA and FA, it should be possible to choose parts of the model, which would then fit the recorded spectra of HA and/or FA. Since the model contains no O-substituted aromatic C-atoms, this is however not possible, and the simulated spectrum is quickly recognized as very different from the recorded spectra of HA and FA. Since we use liquid state ^{13}C-NMR as our tool to evaluate models of HA and FA, it is not wise to make too many conclusions of this SOM-model. We can nevertheless say, that it will be extremely difficult to choose structural elements of the SOM-model that will fit either HA- or FA-fractions, which are, after all, important fractions of SOM, quantitatively as well as regarding several properties of SOM. This conclusion would be supported by an elemental analysis, which for this model would give 64% C, 8% H, 1.7% N and 24% O (Table **1**) and a titration of acid groups, which would also reveal the lack of phenolic structures, which are always part of the HA- and FA-fraction of SOM. Kleber *et al.* [27] have some very interesting discussions on organo-mineral interactions on soil, but the model, which they propose, seems to contain very little of the compounds, which would normally be extracted into the HA- and FA-fractions.

GENERAL REMARKS

The advantage of using a ^{13}C-NMR simulation is that all carbons within a structure are taken into account, and can be immediately compared to recorded NMR-spectra. Although several overlaps will occur in a 1D-NMR spectrum of humic substances and it therefore not always is possible to be conclusive on the exact structural elements, the inclusion of all structural elements makes possible a better balance between the various parts of the spectrum. This balance should of course be checked in relation to an elemental analysis. A titration of acid groups to divide these into carboxylic acids and phenols likewise makes a good check when compared to the NMR results. Finally, both nitrogen and in the case of lignite HS also sulphur content play a role. The former can in some cases, when being part of amino acids, be correlated to the NMR findings [10].

The aromatic region in general plays an important role as many humic substances contain large proportions of aromatic carbons. The very broad region assigned to aromatic structures, clearly shows that a vast combination of structures are required not the least to explain the low frequency wing (100-115 ppm) generally found in ^{13}C-NMRspectra of HS-fractions. In HS-fractions containing significant amounts of carbohydrates, this region can to some extent, but not fully, be ascribed to anomeric carbons of carbohydrates (~105 ppm). The oxygen substituted aromatic carbons can to a good degree be determined as some of these signals will appear around 160 ppm. To help understand, which aromatic structures are present in a given HS-fraction, a check between oxygen content, number of titrated phenolic and carboxylic acid groups and NMR parameters, is necessary since

Table 1. Elemental Analysis Data of HA and FA (wt%). The Top Three Lines are General Data on HA and FA. Then the Calculated CHNOS-Content for the 11 Structural Models of HS are Shown, and Just Below each Model, Elemental Analysis Data are Included for the HA or FA for which the Model was Proposed, in the Cases where Such Data were Reported.

Type of HS	C	H	N	O	S	References
HA in general	54-59	3.2-6.2	0.8-4.3	33-40	<1	[1, 34]
FA in general	41-51	3.8-7.0	0.9-3.3	40-50	<1	[1, 34]
Lignite HA (Aldrich)	55	4.3	0.7	37	3.5	[10]
Schulten & Schnitzer HA model	67	6.0	1.5	26	0	[21]
Stevenson HA model	57	3.4	2.6	37	0	[1]
Albers *et al.* soil HA model	56	4.9	5.0	34	0	[10]
Albers *et al.* soil HA	55	4.6	4.9	36	<0.5	[10]
Diallo *et al.* Soil HA model	53	4.3	1.4	38	3.2	[13]
Diallo *et al.* Soil HA	51	4.0	4.0	40	0.9	[13]
Sein *et al.* HA model	57	5.2	3.8	34	0	[22]
Harvey *et al*. Marine HA model	63	8.1	0	29	0	[23]
Shin & Moon soil FA model	56	5.5	1.8	36	0	[24]
Alvarez-Puebla FA model	42	3.2	1.3	50	3	[26]
Albers *et al.* soil FA model	47	3.7	0.8	48	0	[10]
Albers *et al.* soil FA	47	3.8	1.0	48	<0.5	[10]
Kleber *et al.* SOM model	64	8	1.7	24	0	[27]

part of the oxygen substituted aromatic carbons are buried in the main signal due to structural elements as exemplified in Fig. (**1f**). Parameters such as bulk densities and solubility could also be taken into account [13]. Mass spectroscopic studies are clearly very useful. Diallo *et al.* [13] showed that most of the fragments of soil HA do not exceed 1200 Dalton. This fits well with most of the proposed structures evaluated in this paper, which are all below 1650 Dalton except the HA proposed by Schulten & Schnitzer (1993), which is significantly larger (~5800 Dalton). The simulated spectra have shown that even with very broad line widths, the resulting spectra of such small molecules do always miss some fill-in, especially in the aromatic region. This supports the idea that even a purified HS fraction is a mixture of several molecules and that one final structure of a HA or FA cannot be identified.

As indicated here, there are numerous considerations, which have to be taken into account before it is possible to construct a reasonable model structure, which makes a good representation of either whole soil SOM or of selected fractions like HA and FA. In the present paper, apart from having evaluated some existing proposed models, we have shown that using simulated ^{13}C-NMR spectra, in the process of constructing such model structures, will help avoiding wrong structural elements and hopefully likewise be a help in choosing the right ones.

REFERENCES

[1] Stevenson FJ. Humus chemistry: genesis, composition, reactions. New York: John Wiley & Sons 1994.

[2] Swift RS. Organic matter characterization. In: Sparks DL, Ed. Madison: Soil Science Society of America Inc. 1996; Vol. 3: pp. 1011-71.

[3] Reckhow DA, Makdissy G, Rees PS. Disinfection by-product precursor content of natural organic matter extracts. In: Karanfil, T. Krasner SW, Westerhoff P, Xie Y. Eds. Washington: American Chemical Society 2008; Vol. 995: pp. 80-94

[4] Piccolo A, Celano G, Conte P. Adsorption of Glyphosate by Humic Substances. J Agric Food Chem 1996; 44: 2442-46.

[5] Albers CN, Banta GT, Hansen PE, Jacobsen OS. Effect of different humic substances on the fate of Diuron and its main metabolite 3,4-dichloroaniline in soil. Environ Sci Technol 2008; 42: 8687-91.

[6] Perminova IV, Grechishcheva NY, Petrosyan VS. Relationships between structure and binding affinity of humic substances for polycyclic aromatic hydrocarbons: relevance of molecular descriptors. Environ Sci Technol 1999; 33: 3781-7.

[7] Senesi N. Binding mechanisms of pesticides to soil humic substances. Sci Total Environ 1992; 123/124: 63-76.

[8] Tipping E. Cation binding by humic substances. Cambridge: Cambridge University Press 2002.

[9] Thomsen M, Lassen P, Dobel S, Hansen PE, Carlsen L, Mogensen BB. Characterisation of humic material of different origin: a multivariate approach for quantifying the latent properties of dissolved organic matter. Chemosphere 2002; 49: 1327-37.

[10] Albers CN, Banta GT, Jacobsen OS, Hansen PE. Characterization and structural modelling of humic substances in field soil displaying significant differences from previously proposed structures. Eur J Soil Sci 2008; 59: 693-705.

[11] Haiber S, Herzog H, Buddrus J, Burba P, Lambert J. Quantification of substructures of refractory organic substances by means of nuclear magnetic resonance. In: Frimmel FH, Abbt-Braun G, Heumann KG, Hock B, Lüdemann H-D, Spiteller M, Eds. Weinheim: Wiley-VCH, 2002; pp. 115-28.

[12] Simpson AJ, Burdon J, Graham CL, Hayes MHB, Spencer N, Kingery WL. Interpretation of heteronuclear and multidimensional NMR spectroscopy of humic substances, Eur J Soil Sci 2001; 52: 495-509.

[13] Diallo MS, Simpson A, Gassman P, Johnson JH, Goddard WA, Hatcher PG. 3-D structural modelling of humic acids through experimental characterization, computer assisted structure elucidation and atomistic simulations. 1. Chelsea soil humic acid. Environ Sci Technol 2003; 37: 1783-93.

[14] Mao J-D, Schmidt-Rohr K. Absence of mobile carbohydrate domains in dry humic substances proven by NMR, and implications for organic-contaminant sorption models. Environ Sci Technol 2006; 40: 1751-56.

[15] Macomber RS. Proton-carbon chemical shift correlations. J Chem Educ 1991; 68: 284-5.

[16] Simpson AJ, Lefebre B, Moser A, *et al*. Identifying residues in natural organic matter through spectral prediction and pattern matching of 2D NMR datasets. Magn Reson Chem 2004; 42: 14-22.

[17] NMR Predict, Mestrelab research. Available at: http://mestrelab.com

[18] Kalinowski H-O, Berger S, Braun S. Carbon-13 NMR spectroscopy. Chichester: John Wiley & Sons 1987.

[19] Hansen PE, Thiessen H, Brodersen R. Bilirubin acidity. Titrimetric and ^{13}C NMR studies. Acta Chem Scand B 1979; 33: 281-93.

[20] Kato-Toma Y, Iwashita T, Masuda K, Oyama Y, Ishiguro M. pK_a measurements from nuclear magnetic resonance of tyrosine-150 in class C b-lactamase. Biochem J 2003; 375: 175-81.

[21] Schulten H-R, Schnitzer M. A state of the art structural concept for humic substances. Naturwissenschaften 1993; 80: 29-30.

[22] Sein LT, Varnum JM Jansen SA. Conformational modeling of a new building block of humic acid: approaches to the lowest energy conformer. Environ Sci Technol 1999; 33: 546-52.

[23] Harvey GR, Boran DA, Chesal LA, Tokar JM. The structure of marine fulvic and humic acids. Mar Chem 1983; 12: 119-32.

[24] Shin HS, Moon H. An "average" structure proposed for soil fulvic acid aided by depth/quat ^{13}C NMR pulse techniques. Soil Sci 1996; 161: 250-6.

[25] Leenheer JA, Rostad C. Tannins and Terpenoids as Major Precursors of Suwannee River Fulvic Acid. U.S. Geological Scientific Investigations Report 2004-5276, U.S. Geological Survey, Reston, Virginia 2004.

[26] Alvarez-Puebla RA, Valenzuela-Calahorro C, Garrido JJ. Theoretical study on fulvic acid structure, conformation and aggregation A molecular approach. Sci Total Environ 2006; 358: 243-54.

[27] Kleber M, Sollins P, Sutton R. A conceptual model of organo-mineral interactions in soils: self-assembly of organic molecular fragments into zonal structures on mineral surfaces. Biogeochemistry 2007; 85: 9-24.

[28] Conte P, Piccolo A, van Lagen B, Buurman P, de Jager PA. Quantitative difference in evaluating soil humic substances by liquid- and solid-state ^{13}C-NMR spectroscopy. Geoderma 1997; 80: 339-52.

[29] Conte P, Spaccini R, Smejkalová D, Nebbioso A, Piccolo A. Spectroscopic and conformational properties of size-fractions separated from a lignite humic acid. Chemosphere 2007; 69: 1032-9.

[30] Piccolo A. The supramolecular structure of humic substances: a novel understanding of humus chemistry and implications in soil science. Adv Agron 2002; 75: 57-134.

[31] Sutton R, Sposito G. Molecular structure in soil humic substances: the new view. Environ Sci Technol 2005; 39: 9009-15.

[32] Saito Y, Haiano S. Characterization of humic and fulvic acids isolated from marine sediments of Sagami and Suruga Bays with C-13 and proton nuclear magnetic resonance J Oceanogr Soc 1981; 36: 286-92.

[33] Leenheer JA, Brown GK, MAccarthy P, Cabaniss SE. Models of metal binding structures in fulvic acid from the Suwannee River, Georgia. Environ Sci Technol 1998; 32: 2410-16.

[34] Tan KH. Humic Matter in soil and the environment – principles and controversies. New York: Marcel Dekker 2003.

Received: January 15, 2010 Revised: February 02, 2010 Accepted: February 02, 2010