

PROTON HOMONUCLEAR TWO-DIMENSIONAL NMR SPECTROSCOPY OF HUMIC SUBSTANCES WITH EXTREME RESOLUTION AND SENSITIVITY

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INTRODUCTION

While one dimensional NMR spectroscopy in the solid and liquid state has contributed enormously to an understanding of structural detail of humic substances¹, two dimensional NMR spectroscopy is the most important tool to enhance the reliability of NMR resonance assignments² and references therein.

Proton homonuclear shift correlated 2D NMR spectra offer high sensitivity and long range connectivity information and enable to assemble individual proton resonances and CH cross peaks (available from one bond heteronuclear correlation spectroscopy) into humic substructures. The rather long transfer times in COSY and TOCSY NMR spectra^{3,4} imply loss of magnetization caused by transverse relaxation, so homonuclear 2D NMR spectra of humic materials are in general acquired from rather concentrated samples. High quality homonuclear 2D NMR spectra would significantly improve the currently rather limited knowledge of accurate structural detail of humic materials.

MATERIALS AND METHODS

Isolation of the peat fulvic acid. The peat fulvic acid was isolated from Pine Eriophorum highland peat (*Eriophorum vaginatum*: 45%, pine skin: 35%, Sphagnum moss: 15%, *Mengathes trifoliata*: 10%, decomposition rate: 30-35%) according to the procedure described².

NMR spectroscopy. NMR spectra were acquired with a Bruker DMX 500 NMR spectrometer (Rheinstetten, Germany) operating at 500.13 MHz proton frequency from 2.5 mg Sedge peat fulvic acid dissolved in 700 μ L DMSO- d_6 under complete exclusion of air and moisture employing vacuum line techniques in a sealed NMR tube using a cryogenic 5 mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ TXI probe, equipped with an actively shielded z-gradient coil at 303 K ($90^\circ(^1\text{H}) = 7.0 \mu\text{s}$); chemical shift values are referred to DMSO- d_6 at $\delta = 2.49$ ppm. Gradient enhanced (length: 1 ms; recovery: 450 μs) TOCSY spectra (AQ = 400 ms at SW = 5165 Hz) used MLEV-17 mixing (100 ms) and 512 echo-antiecho increments. The double quantum filtered COSY spectra (AQ = 500 ms at SW = 5482 Hz), acquired with 1024 increments, was multiplied (8k x 1k matrix) by a squared sine bell, shifted by $\pi/6$ in F2 and by a sine bell, shifted by $\pi/6$ in F1; the TOCSY spectrum (4k x 512 matrix) by a squared sine bell, shifted by $\pi/10$ in F2 and F1. ^1H chemical shift values indicated in Fig. 3 were calculated by the ACD/Labs software (Advanced Chemistry Development, Pegnitz, Germany) NMR Proton Predictor, Version 5.0, and put into display with the EXCEL format.

PROTON HOMONUCLEAR NMR SPECTRA OF A PEAT FULVIC ACID

The use of cryogenic NMR probes leads to a dramatic increase in signal to noise ratio for proton NMR detection when compared with classic inverse geometry probes^{5,6}. The relative S/N enhancement depends on sample composition, reaching a factor in excess of four in case of organic solvents, which is declining to near one for solutions with very high ionic strength⁷. Therefore humic materials, dissolved in DMSO- d_6 under complete exclusion of air and moisture are promising samples for NMR spectra of very high quality. The exclusion of oxygen obviates longitudinal relaxation from paramagnetic oxygen. The very large intrinsic sensitivity of the cryogenic probes allows the acquisition of meaningful NMR spectra from samples of low concentration, when the tendency to aggregate in solution is minimized. In addition, hydrogen bonding is severely reduced in dipolar aprotic solvents. Both effects enhance the molecular mobility, increase the longitudinal⁸ and spin-spin relaxation times and consequently the attainable resolution in 2D NMR spectra⁹, which is demonstrated with COSY and TOCSY NMR spectra showing superior resolution compared to all published spectra of humic materials so far.

The COSY NMR spectrum of a peat fulvic acid exhibits resolved cross peak fine structure in many cases. Both position and fine structure of cross peaks indicate the negligible contributions from amino acids in the fulvic acid in line with previous results². This leaves various forms of oxygenated aliphatic chains (probably ester derived structures) of intermediate length as the most likely candidates to explain the cross peaks within section E.

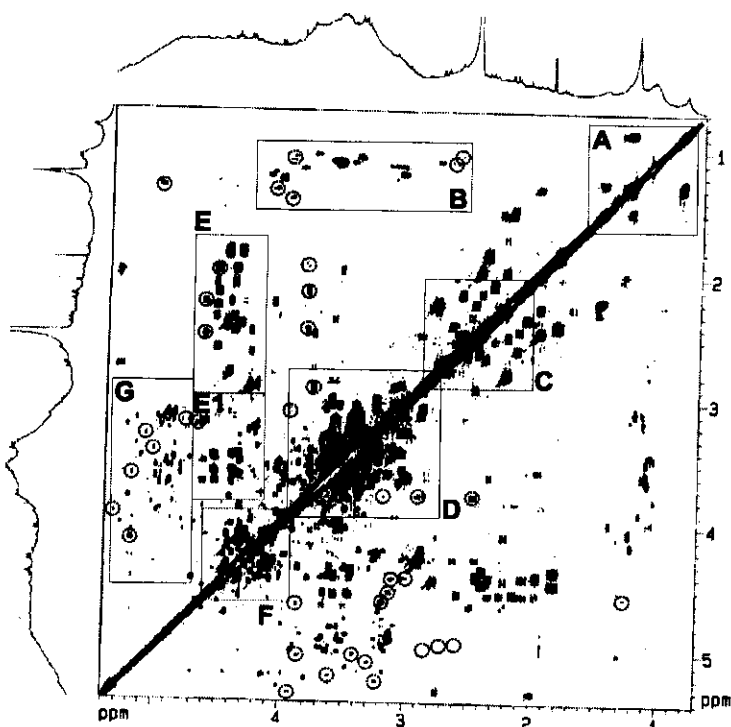


Figure 1 *DQF-COSY-NMR-spectrum (aliphatic section) of a peat fulvic acid (label: cross peaks visible only in COSY, but not in TOCSY NMR spectra); sections of chemical shift according to humic substructures, A: $-\text{CH}_{1,3}-\text{CH}_{1,2}-\text{CH}_{1,2}-\text{CH}_{1,3}$, B: $-\text{C}-\text{CH}-\text{CH}-\text{O}$, C: $-\text{C}_\alpha-\text{CH}-\text{C}_\beta-\text{C}$, D: $-\text{CH}(\text{O})-\text{CH}(\text{O})-$, E: $-\text{C}(=\text{O})-\text{O}-\text{CH}-\text{CH}-\text{CH}-\text{O}$, E1: $-\text{C}(=\text{O})-\text{O}-\text{C}_\alpha-\text{C}_\beta-\text{O}$, F: $-\text{C}(=\text{O})-\text{O}-\text{C}_\alpha-\text{C}_\beta-\text{O}$, G: $-\text{O}-\text{CH}(\text{O})-\text{CH}(\text{O})-$.*

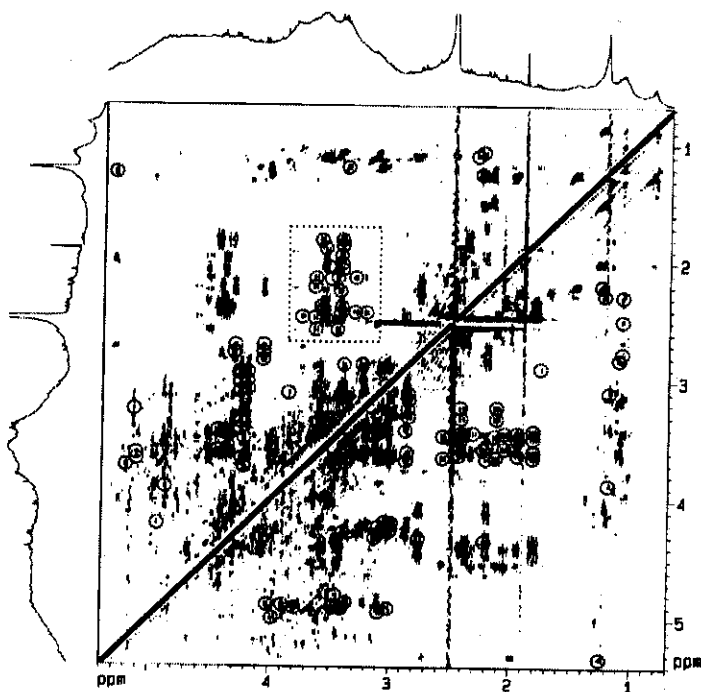


Figure 2 *TOCSY-NMR-spectrum (aliphatic section) of a peat fulvic acid (label: cross peaks visible only in TOCSY, but not in COSY NMR spectra; dotted box: cf. text)*

Owing to different mechanisms of magnetization transfer both common and different resonances will appear in COSY and TOCSY NMR spectra of a humic substance. TOCSY cross peaks indicate extended spin systems rather than vicinal and geminal connectivities alone as in standard COSY NMR spectra. Differential relaxation within the polydisperse and molecularly inhomogeneous humic materials discriminate in favor of TOCSY (optimum transfer time at $1/2J$) relative to COSY cross peaks (optimum transfer time at $1/J$). Therefore cross peaks visible in TOCSY spectra only represent long range couplings in extended spin systems, but also may indicate short range couplings in spin systems of restricted flexibility, where self cancellation of COSY cross peaks has occurred (at linewidths > 15 Hz). Cross peaks showing up in COSY spectra only may indicate either small spin systems of very high flexibility (with long transverse relaxation times, sharp lines and large S/N ratio) or parts of extended spin systems, where resonance integrals are distributed across many cross peaks, therefore declining the S/N ratio of individual TOCSY cross peaks beyond recognition.

Interestingly, both groups of mutually exclusive homonuclear cross peaks are not statistically distributed, but fall into specific and relatively narrow spectral regions (Figs. 1 and 2). The TOCSY cross peaks, depicted in the dotted box of Fig. 2 most likely are part of extended spin systems, connecting an ether or hydroxy substituted CH (δ : 3.3-3.8 ppm) with an ester bonded CH (δ : 4.2-4.6 ppm), separated by a functionalized aliphatic position (δ : 1.7-2.7 ppm).

Nowadays NMR spectra of extended spin systems can be calculated with reasonable accuracy on desk top computers. An iterative adjustment of model and experimental NMR spectra, corroborated by the current understanding of humic bio- and geosynthetic pathways, allows to propose model structures conforming to spectral data. Even when used only as the first step, which requires verification by independent analytical data, this approach provides extreme resolution in structural detail at atomic resolution which is not available by other analytical techniques. The hypothetical model structure $C_{30}H_{50}O_{16}$ demonstrates the effects of ester, ether and hydroxy substitution in aliphatic chains of intermediate length on COSY and TOCSY cross peak positions and provides evidence for the occurrence of oxygenated ester type structures, like those found in microbial polyesters¹⁰, in Sedge peat fulvic acid.

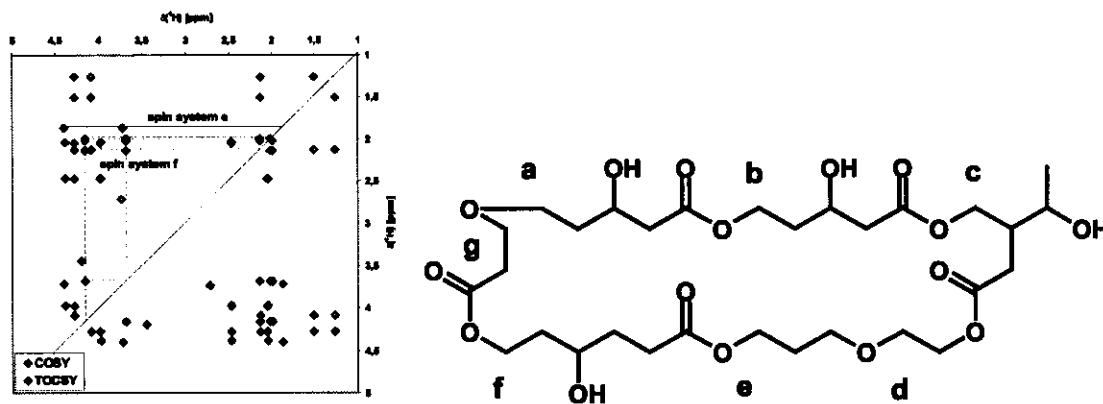


Figure 3 Calculated homonuclear NMR spectrum of a model oxygenated aliphatic humic substructure $C_{30}H_{50}O_{16}$ (spin system labels: a-g; cf. text)

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