Quantitative Characterization of Soil Organic Matter and Its Fractionation Products by Solid State High Resolution C-13 (CPMAS) Spectroscopy

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In a systematic study the organic carbon content of typical German soils was studied by solid state C-13 CPMAS spectroscopy.

In order to check the quantitative validity of the standard sodium hydroxide extraction procedure, which fractionates soil organic matter into humin, humic acid, and fulvic acid also the high resolution solid state spectra of these fractions were determined.

The chemical information obtained from these spectra is discussed.

Introduction

Soil organic matter is formed from dead plant material in a sequence of decomposition and conversion processes [1–3]. Attempts to characterize it by classical chemical methods have been only partly successful because a large fraction of it is insoluble in any chemically inert solvent, and because of its complex chemical nature. In the last two decades carbon-13 NMR has become the method of choice for the quantitative description of this material [3]. Especially the application of modern solid state high resolution NMR techniques like the CPMAS method (Cross Polarization Magic Angle Spinning) which permit the investigation of complete soils with carbon contents around 1% w/w have furthered the understanding of the gross chemical composition of this class of materials. In a previous paper [4] we could show, that by careful determination of all relevant relaxation times quantitative C-13 high resolution solid state spectra can be obtained, *i.e.* spectra where the relative signal intensity is proportional to the relative concentration of the carbon structures in the various ranges of chemical shift. It could be expected, that the relatively high concentration of free organic radicals in soil organic matter broadens a part of the carbon signals beyond detectability for a high resolution experiment [3]. Careful analysis of large series of spectra, however, did not reveal a significant effect.

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The aim of the following paper is on the one hand to test the classical chemical fractionation procedure for soil organic matter which classifies three fractions by their solubility in 0.5 N aqueous sodium hydroxide. The insoluble fraction found by this approach is called humin [6]. The alkali soluble part that can be precipitated by acidification with hydrochloric acid to $p_H \approx 1$ is the humic acid fraction and the substance remaining soluble in acidic water is the fulvic acid fraction. On the other hand it is attempted to learn to extract the maximum of chemical information from the integration of the spectra. It is obvious, that in C-13 spectra of compounds with the chemical complexity of the humic material no specific assignments to defined structural elements can be made. The only analysis that appears feasible is the division of the C-13 spectra into regions of chemical shifts, the comparison of the relative intensities of these regions, and their changes when soils of different composition are compared. Nine soil samples from German locations were chosen for this work. Their characteristics are given in Table I.

Materials and Methods

The extraction process is described in Scheme 1. The alkali soluble fraction was extracted by mixing 20 g of the soil with 60 g oxygen free 0.5 N aqueous sodium hydroxide. The oxygen was removed from the aqueous sodium hydroxide solution by bubbling pure nitrogen through the solution for 1 h prior to use. After sonication of the dispersion with a Branson sonifier for 5 min, the mixtures were immediately centrifuged until the supernatant liquid became free of solid material.

Tab. I. Origin and some characteristics of the soil samples (top 10 cm).

Abbreviation		Geographical Origin		Order (12)	% C	% N
POF		Pfaffenhofen	Ba[a]	Cambisol	1.2	0.1
PUS	field	Pfaffenhausen	Ba	Vertic Cambisol	1.5	0.1
MAI		Mainburg	Ba	Calcaric Regosol	1.1	0.1
HAR		Harthausen	Ba	Luvic Cambisol	3.4	0.3
OWA	grassland	Oberwarngau	Ba	Rendzina	4.6	0.4
ISA		Ismaning	Ba	"Black" Calcaric Regosol	11.5	0.9
SOL B		Solling B 1	LS[b]	Spodic Distric Cambisol	4.5	0.3
SOL D	forest	Solling D 1	LS	Spodic Distric Cambisol	3.4	0.2
GOW		Göttingen	LS	Chromo-Calcic Cambisol	4.4	0.4

[a] Ba = Bavaria.
[b] LS = Lower Saxony.

Scheme 1. Extraction procedure.

20 g ground soil
60 g 0.5 N NaOH
sonicate 5 min
centrifuge 20 min
4 repetitions (N_2)

residue
+ 60 g H_2O
sonicate
centrifuge

solution
+ HCl (pH: 1.0)
centrifuge
(N_2)

liquid
dialyze
freeze dry

residue
dry on porous
ceramics

precipitate
dissolve in
0.5 N NaOH

solution
concentrate
to 100 cm^3
(vacuum)
(N_2)

dialyze
(10–15 kDa)
freeze dry

washing water

extraction residue "humin"

humic acid

fulvic acid

In most cases the water in the dialysis tank turned brown.

Concentrate by
vacuum distillation
(40 °C) to 100 cm^3
dialyze
freeze dry
humic acid 2
fulvic acid 2

This extraction process was repeated 4 times. The last extraction yielded a pale yellow liquid phase only, indicating that most of the alkali soluble material had been dissolved. In order to remove the surplus of sodium hydroxide from the solid residue it was washed once with distilled water, sonicated and centrifuged. For most soils this yielded a brownish liquid which after exhaustive dialysis (Serva standard hose 10–15 kDa) and freeze drying yielded a black solid material with an ash content of 30 to 80%.

This fraction must thus contain complexes of the soil organic matter with alumosilicates. It carried between 0.3 to 9% of the total carbon. This

fraction is called washing water in the following. The sodium hydroxide solution was acidified with hydrochloric acid to $p_H \approx 1$ and centrifuged. The precipitate, the humic acid fraction, was redissolved in 0.5 N sodium hydroxide and dialyzed against water, until the dialysis water remained neutral. Also the fulvic acid fraction, the organic fraction, soluble in aqueous hydrochloric acid at $p_H \approx 1$, was dialyzed until it was free of chloride.

In most cases the first and second water filling of the dialysis beaker for the humic as well as for the fulvic acid fraction turned brown. These waters were concentrated by vacuum distillation on a rotary evaporator (bath temperature: 50 °C, p: 60 mbar) to approx. 200 ml and dialyzed. These fractions contained together between 2 to 10% of the total organic carbon. In the following they are named fulvic acid 2 resp. humic acid 2. After dialysis all fractions were concentrated under vacuum to ~ 15 ml and freeze dried. In a second series of fractionation procedures the sodium hydroxide extracts were dialyzed without any prior treatment. These fractions are called sodium hydroxide extracts or soluble humic material in the following.

NMR Methods

The spectra were obtained at a Bruker MSL 100 spectrometer operating at 2.3 Tesla. (C-13 resonance frequency 25.2 MHz). The spinning rate was 4 KHz. A commercial Bruker double bearing probe with 7 mm o.d. and phase stabilized zirconium dioxide rotors were used. The chemical shift scale was calibrated with neat glycine. A conventional cross polarization pulse program with a contact time of 1 ms was used. To improve the signal to noise ratio a line-broadening of 100 Hz was applied. For further details see [7]. One of the principal aims of this work was to learn which subdivision of the chemical shift range produced data most useful for the classification of humic material. In previous work [4] four ranges of chemical shift were used for integration. The chemical shift range $\delta > 160$ ppm was assigned to carboxyl/carbonyl group. 160 ppm $\geq \delta \geq$ 110 was ascribed to the aromatic carbons. Between 110 ppm $\geq \delta \geq$ 45 ppm carbohydrate derived structures but also ethers or c-α of α-amino acids should yield signals. Finally $\delta \leq 45$ ppm is the region of chemical shift where aliphatic structures are found.

Spectra of the quality given in Fig. 1 and 2 obviously would allow a much finer subdivision of the chemical shift range, and thus permit to extract more chemical information from the spectra. The integration of the spectra was therefore finally performed by dividing the chemical shift ranges into 11 regions. They are given together with possible assignment in Table II.

However, the analysis of the 70 spectra taken in this investigation as well as the analysis of other sets of data [8] revealed, that for instance the

Tab. II. Ranges of chemical shift and plausible assignments to structures in the starting material and the bioconversion products.

Preliminary range for integration	Final choice	Chemical shift range	Structural elements
1		0–25	methyl groups bound to carbon
2	1	25–35	methylene groups in aliphatic rings or chains
3		35–45	sp_3 carbons in branched chains or rings
4	2	45–60	methoxyl groups and C-6 of some polysaccharides
5	3	60–80	carbohydrate derived structures (C-2 to C-5) in hexoses. C-α of α-amino acids, higher alcohols, aliphatic part of lignin structures
6	4	80–100	C-α of amino acids, carbohydrate derived structures
7		100–110	anomeric carbon of carbohydrates, C-2, C-6 of syringyl units
8	5	110–120	aromatic C-H carbons in guajacyl C-2, C-6
9		120–140	aromatic C-H carbons
10		140–160	aromatic COR groups in lignin derived structures
11	6	160–210	carboxyl/carbonyl groups

intensities in the ranges between 160–140 ppm, 140–120 ppm and 120–110 ppm changed in parallel. Also in the aliphatic region variations in intensity in the range between 25 and 0 ppm occurred within the limits of experimental error in parallel with variations between 45 and 25 ppm. In the final Table III and IV therefore only 6 intensities are compiled. We will return to more detailed chemical evaluation of these findings in the results and discussion section. In previous experiments the reproducibility of the integration procedure was checked by measuring one humic material probe under complete retuning of the spectrometer. The reproducibility in the individual regions was better than 0.5%.

Results and Discussion

Fig. 1 and 2 contain the spectra collected for all fractions of the soils from Ismaning (grassland) and Solling (forest). The effects observed there are typical for all soils studied hitherto. The sodium hydroxide extract has a slightly higher intensity in the carboxyl/carbonyl range and a much more pronounced increase in the spectral area around 70 ppm (carbohydrate derived structures) the content of aromatic carbons is slightly reduced and also aliphatic structures remain preferably in the insoluble fraction. (Most clearly visible in the spectra from the forest soil.) However, differences between the three classical fractions (NaOH extract, insoluble residue, complete soil) and the washing water are not very pronounced and cast considerable doubt upon the standard classification of the humic material. It appears that differences in the solubility of the complete humic material are rather caused by physical accessibility of the organic matter, *i.e.* by the complexation or binding with the mineral phases of the soil, than by principal chemical differences between the three fractions. The same conclusion must be drawn from an in-

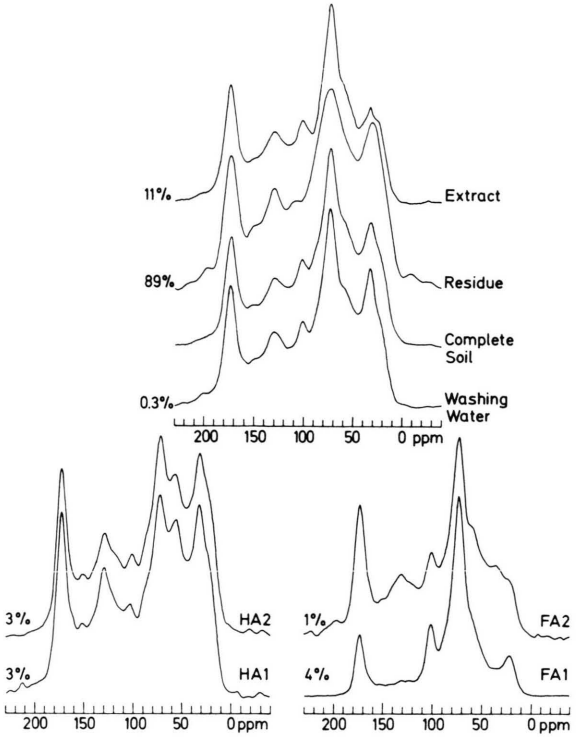

Fig. 1. 25 MHz C-13 CPMAS spectra of all soil fractions obtained from the sample Isa (Ismaning/grassland/ Black Calcaric Regosol). The percent values given in the figure indicate the contribution of the individual fractions to the total organic carbon.

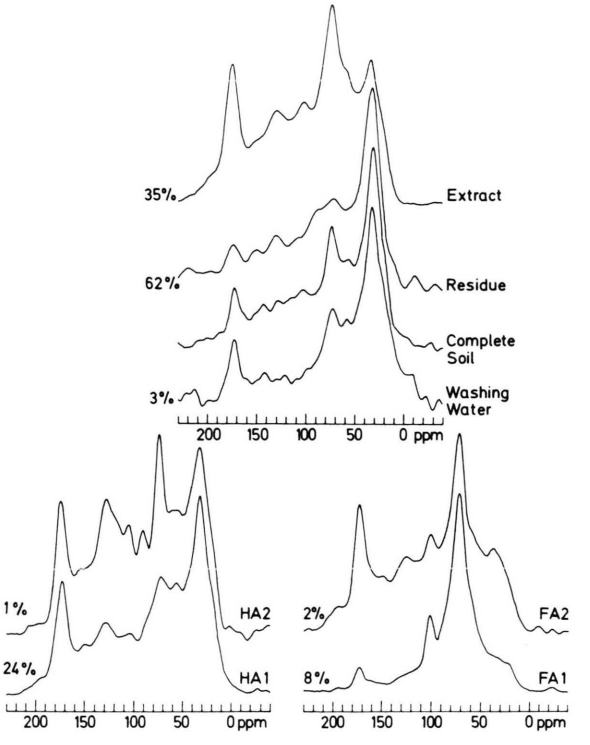

Fig. 2. 25 MHz C-13 CPMAS spectra of all soil fractions obtained from the sample Sol B (Solling B1/forest/ Spodic Distric Cambisol). The percent values given in the figure indicate the contribution of the individual fractions to the total organic carbon.

spection of the results for all soils given in Table III. The separation of the soluble fraction by acidic precipitation into humic acids and fulvic acids yields two really different fractions. In the humic acids carboxylic groups are seen in a significantly higher concentration than in the parent material while the aliphatic region has become slightly more pronounced. This enrichment occurs at the expense of the spectral region between 110 and 60 ppm *i.e.* of the carbohydrate derived structures. In the fulvic acid fraction the reverse is true. Here the range between 110 ppm and 60 ppm is strongly enhanced while all other regions appear to be suppressed. Table IV contains the averages obtained for the various fractions. Considering the diverging origin of the samples and the limited number of samples studied, only the differences discussed above appear to be highly significant. Whether the small changes seen in the other regions of chemical shift for all fractions compiled there are significant must await further studies on series of soils were either the cultivation methods have been varied systematically or were systematic sequences of the different soil types are studied. This work is in progress.

It must however be kept in mind, that these two standard fractions contain at most 40% (compare Table III) of the total organic carbon of the humic material and experience shows that studies confined to the soluble fraction of the humic material

Tab. III. Quantitative characterization of the organic carbon of the complete soils, the sodium hydroxide extracts and the residues by the six integration intervals described in the text.

		210–160	160–110	110–80	80–60	60–45	45–0
		Chemical shift range [ppm]					
		Complete soil					
HAR		14.5	18.3	18.5	21.5	9.3	17.8
OWA		12.5	16.8	18.5	22.2	11.3	18.6
ISA		13.1	16.4	16.6	21.9	10.9	21.1
POF		11.2	22.2	19.2	18.3	9.8	19.4
MAI		10.2	21.2	18.2	21.2	10.6	18.7
PUS		12.1	20.1	18.6	18.6	11.7	18.9
SOL B		8.3	14.6	12.0	15.8	10.0	39.1
SOL D		8.0	14.8	11.4	15.4	10.3	40.3
GOW		11.4	19.9	18.9	20.6	10.0	19.2
	Fraction of organic carbon*	NaOH extract					
HAR	31%	14.1	16.2	17.6	26.3	10.4	16.5
OWA	39%	15.1	16.2	16.5	25.0	10.2	17.0
ISA	11%	14.8	17.4	15.3	23.3	10.2	19.0
POF	29%	13.9	17.0	15.3	25.1	10.3	18.5
MAI	31%	13.6	14.7	16.8	29.3	11.2	14.4
PUS	19%	12.6	14.2	14.9	29.3	11.8	17.2
SOL B	35%	16.2	19.2	15.9	18.8	9.5	20.4
SOL D	36%	16.3	19.3	14.2	17.6	9.4	23.2
GOW	28%	14.0	12.4	18.1	29.6	10.0	15.9
		Residue					
HAR	60%	16.3	24.2	16.9	17.1	9.6	16.0
OWA	55%	12.9	16.8	20.0	20.5	10.7	19.3
ISA	89%	14.2	18.0	16.8	18.3	10.2	22.6
POF	67%	10.0	27.7	19.3	14.7	8.3	20.0
MAI	65%	15.2	21.4	20.4	16.3	10.0	16.7
PUS	79%	11.3	17.4	18.0	19.7	11.0	23.5
SOL B	62%	4.8	11.9	13.4	13.2	9.5	47.4
SOL D	61%	7.1	13.7	13.5	12.5	11.0	42.2
GOW	66%	9.8	17.7	18.0	19.3	10.2	24.6

* In these two fractions the "washing water" is missing.

Tab. IV. Average chemical composition of the carbon content of the fractions obtained from the separation procedures described in Scheme 1.

	A	B %	C %	Chemical shift range 210–160	160–110	110–80 [ppm]	80–60	60–45	45–0
Complete soil	9	100	–	11.3	18.3	16.9	19.5	10.4	23.7
NaOH extract	9	29	11–45	14.5	16.3	16.1	24.9	10.3	18.0
Insoluble residue	9	67	89–55	11.3	18.8	17.4	16.8	10.1	25.8
Humic acid 1	9	19	26–3	14.0	18.9	13.3	17.0	11.9	24.9
Humic acid 2	4	2	3–0	12.9	23.2	12.6	16.2	10.9	24.3
Fulvic acid 1	9	7	10–2	9.5	8.2	20.2	38.6	10.9	12.6
Fulvic acid 2	8	2	4–0	15.4	15.6	15.5	25.7	11.1	16.8
Washing water	7	4	9–0	13.7	15.6	14.2	18.6	10.6	27.3

A: Number of samples studied.
B: Average contribution of this fraction to the total carbon content.
C: Variation of the contribution of this fraction to the total carbon content.

can lead to erroneous conclusions, since chemical variations found in this part and its subfraction may rather be the result of differences in unspecific accessibility, caused by changes in the mineral composition and grain size of the soil samples, than stem from significant chemical differences of the organic material in the various complete soils.

Table II contains the 11 intervals of integration initially chosen in order to extract the possible maximum of chemical information from the spectra. The three intervals of the area of the aliphatic carbons were taken, in order to learn, whether significant structural changes in these moieties would occur in humic material and also rather recent composts. However, inspection of several hundred spectra showed, that the intensity in these three ranges changed in unisono. Indicating in our opinion, that these structures once formed do not undergo large chemical modification and are formed from fairly uniform starting material. It is also worth mentioning, that in hardly any humic material, definite C-methyl-signals can be found. This type of carbon should, because of the high mobility of terminal methylgroups, show distinct and well resolved narrow resonances [5]. Their absence appears to indicate that most of the aliphatic moieties consist either of saturated ring structures or of longer open chain molecules, in which the terminal groups have been chemically altered. Waxes as found in the cuticula of higher plants and their conversion products are likely to constitute a significant part of this fraction [9].

The aromatic range between 160 and 110 ppm was initially subdivided into three areas with the intention, to learn about a variation of the average number of oxygen carrying carbon atoms per benzene ring in the different humic material fractions (compare Table II). However, the intensity ratios I(160–140)/I(140–110) is nearly constant ≈ 0.4 for all samples studied, thus indicating that only minor modifications of the aromatic structures derived from lignin occur in the humification process. This finding puts severe limits upon any chemical model that aims to describe the chemical modifications occurring during the bioconversion of plant matter into humic material.

In this chemical shift ranges also the signals from sp^2 carbons of olefinic structures should be found. It is generally agreed upon, that these structures constitute at most a minor fraction of the total intensity in this range [3]. The total content of aromatic carbons is correlated inversely with the concentration of oxygen carrying sp_3 carbons from carbohydrate derived structures that are found predominantly in the chemical shift range between 80–60 ppm. This appears to be a strong indication, that the state of humic material is best described by a dynamic distribution between carbohydrate derived structures and aromatic structures of lignin origin. The concentration of the aliphatic moiety appears to be correlated in a very unspecific manner only to the intensities observed in the other ranges of chemical shift. As stated above this seems to indicate that this fraction originates from waxlike material not involved in any of the biochemical conversions. Due to its origin all humic material must contain a certain amount of proteinaceous compounds, of which the aliphatic moieties

should be seen in the chemical shift range between 45 to 0 ppm. The α-carbons would be found between 80 and 110 ppm. In both areas the intensity of the spectra of the main fractions of soil organic matter is remarkably constant and does not appear to correlate with any other chemical shift range, although the relativly high nitrogen content of all humic material (*cf.* Table I) demands a fairly large concentration of these compounds.

The relativly constant intensity of the three main fractions of soil organic matter in the areas between 110 and 80 ppm and 60–45 ppm seems to show, that in these two ranges several classes of compounds, most prominently carbohydrate derived structures and proteinaceous material do make a significant contribution to the total intensity.

In the range between 110–140 ppm one can in addition expect contributions from the aromatic C-2, C-6 carbons from modified syringyl-units [10, 11].

Conclusions

Carbon-13-CPMAS spectra allow the most reliable and most detailed characterization of soil organic matter. It proved possible to obtain quantitative information from the integration of specific regions of chemical shift [4]. The data show, that studies of complete soils only permit a meaningful comparison between the soil organic matter from soils with widely different mineral composition. The comparison of a large number of spectra showed, that the aromatic range (160–110 ppm) and the "carbohydrate" range (80–60 ppm) are inversely correlated. This intensity ratio is thus a reliable physical parameter for the extent of humification in soil organic matter.

The intensity ratios I(160–140)/(140–110) and also I(45–25)/I(25–0) are constant within experimental error for all samples from middle European soils studied hitherto. The first ratio seems to indicate the absence of any major chemical modification for the aromatic moieties of soil organic matter in the humification process, and also the second ratio shows that the gross chemical structure of the aliphatic material remains unchanged.

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