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Identification of protein remnants in insoluble geopolymers using TMAH thermochemolysis/GC–MS

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Abstract

Thermochemolysis with tetramethylammonium hydroxide (TMAH) was utilized to analyze peptide-like material in insoluble residues of geochemical samples. Product identification was performed by gas chromatography/mass spectrometry using bovine albumin and di-, tri- and oligo-peptides. Most of the identified amino acid derivatives originate from the cleavage of the peptide bonds and subsequent methylation of the carboxylic and the amino groups. Some products are explained by deamination of aromatic amino acids. Only two products were identified that experienced chemical rearrangement after methylation. TMAH/thermochemolysis of algal material resulted in products comparable to those obtained from albumin. Amino acid derivatives were also identified among the TMAH/thermochemolysis products of refractory biopolymer of the alga *Scenedesmus communis* and the HCl-hydrolysis residue of the humin from an algal sapropel. These results strongly indicate that for those samples, hydrolysis fails to extract all proteinaceous and peptide-like components, possibly because these approaches rely on extraction of peptides and amino acids into solution and some of these are entrapped within a non-extractable hydrophobic network. However, alternative explanations for this behavior are possible. For example, at the higher temperatures and pressures and strongly basic conditions used in TMAH themochemolysis, penetration of this hydrophobic barrier takes place thus allowing breakdown and methylation of protein remnants.

Keywords: TMAH/thermochemolysis; Refractory organic nitrogen; GC/MS; Humin; Algae; Peptide; Amino acid; Protein

1. Introduction

From an analytical perspective, deciphering the structural details of organic nitrogen in complex insoluble geochemical samples is challenging. Common approaches involve elemental analysis, and basic and/or acid hydrolysis. However, with these techniques only 10–60% of the total organic nitrogen in soils and sediments is identified, mostly as amino acids and amino sugars (e.g. Kelly and Stevenson, 1996). The nature of the remaining nitrogen is still subject to debate. As alternatives to wet chemical analysis, degradative techniques such as pyrolysis-gas chromatography/mass spectrometry (GC/MS) (Schulten et al., 1997) and non-degradative spectroscopic techniques such as solid-state ^{15}N nuclear magnetic resonance (NMR) spectroscopy (Knicker and Kögel-Knabner, 1998) have been applied to obtain some structural information about the chemistry of refractory organic nitrogen in humified material.

Recent solid-state ^{15}N NMR spectroscopic studies on microbiologically degraded plant residues (Knicker and Lüdemann, 1995) and algal material (Knicker et al., 1996b) as well as natural soils (Knicker et al., 1993) and recent sediments (Knicker et al., 1996a,b) revealed that

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most of the nitrogen in humified samples occurs in amides that have survived microbial degradation. Solid-state ^{15}N NMR analysis of the residues obtained after acid hydrolysis of microbiologically degraded wheat (Knicker et al., 1997) and soils, before and after biowaste application (Knicker and Kögel-Knabner, 1998; Siebert et al., 1998), indicated that some of those amides are protected, even against strong acid hydrolysis with hydrochloric acid. No evidence for the formation of heteroaromatic-N was obtained. However, a serious limitation of solid-state ^{15}N NMR spectra obtained from humic material is the broadness of the signal assigned to amide structures which makes precise assignment of signals to specific chemical classes impossible.

Although previous data gave some indication that the amide signals in those spectra derive from peptide-like material (Knicker et al., 1995; Knicker and Lüdemann, 1995; Knicker, 2000), other origins could not be excluded. Therefore, an alternative analytical method is needed.

Such a technique may represent GC/MS analysis of products derived from thermochemolysis with tetramethylammonium hydroxide (TMAH). This technique, originally introduced as pyrolysis with in situ methylation (Challinor, 1989), has been used as an alternative approach to overcome the limitations of conventional pyrolysis. In this method, TMAH is used to convert polar products to less polar derivatives which are amenable to chromatographic separation (Challinor, 1989; de Leeuw and Baas, 1993; Hatcher and Clifford, 1994; Saiz-Jimenez, 1994). This procedure avoids decarboxylation and produces methyl esters of carboxylic acids and methyl ethers of hydroxyl groups, rendering many of the polar products volatile enough for gas chromatographic analysis. Thus, it is possible to separate and detect many more structurally significant products than observed previously by conventional pyrolysis-GC/MS. Since its first introduction, pyrolysis/methylation and also pyrolysis/alkylation, has been applied to many different biopolymers (Clifford et al., 1995; Martín et al., 1995; Abbas-Hawks et al., 1996), humic materials (Saiz-Jimenez, 1994; Saiz-Jimenez et al., 1994; del Rio and Hatcher, 1996), whole soils (Schulten and Sorge, 1995, Chefez et al., 2000), asphaltenes, kerogens and coals (del Río and Hatcher, 1996; Kralert et al., 1995), and resins and resinites (Anderson and Winans, 1991; Clifford and Hatcher, 1995). Previously, Schulten et al. (1997) used pyrolysis/methylation to identify uncharacterized soil nitrogen. They observed nitrogen derivatives of benzene and long-chain nitriles, not usually detected in pyrolysis-mass spectrometry of plants and microorganisms. They suggested that those compounds are characteristic of soils and result from stable transformation products of soil nitrogen. Such compounds were not detected after TMAH pyrolysis/methylation of red soybean protein powder and clover hay (Reeves and Francis, 1998). However, as known to the authors, a systematic study to identify possible amino acid-derivatives produced by TMAH/thermochemolysis has not been published recently.

Sub-pyrolysis temperatures of 300°C in the presence of TMAH were found to release from lignin a suite of products similar to those observed at higher pyrolysis temperatures (Hatcher and Clifford, 1994; Clifford et al., 1995; del Río et al., 1998), indicating that pyrolysis temperatures are not necessary for the break-down of the macromolecules in the presence of TMAH. Thus, it was suggested that chemolysis is involved rather than pyrolysis (de Leeuw and Baas, 1993; McKinney et al., 1995; Filley et al., 1999). This finding may be of great advantage if TMAH/thermochemolysis is to be used for peptide-like material. Since peptides and proteins are extensively transformed at high temperature, the lower temperatures used in TMAH/thermochemolysis may help to reduce secondary reactions making the assignment of the resulting methyl derivatives to structural units of the original macromolecular material more definitive. In order to demonstrate that this technique can be applied as a useful structural characterization tool for peptide-like material, we have applied it to a number of small peptides of known composition.

2. Materials and methods

2.1. Sample material

The identification of TMAH/thermochemolysis products deriving from peptides or proteins was performed by gas chromatography/mass spectrometry (GC/MS) using commercially available bovine albumin and several polypeptides with known amino acid sequences (Table 1, Appendix). To test if such compounds can be identified in heterogenous mixtures, a protein-rich algal culture from *Spirulina* sp., cultured under laboratory conditions as

Table 1
Polypeptides used for the assignment of mass spectra of TMAH/thermochemolysis products of peptide-like material

	Peptide[a]
1	Trp-Ala-Gly-Asp-Ala-Ser-Gly-Glu
2	Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg
3	Glu-Asn-Gly
4	Arg-Val-Tyr-Ile-His-Pro-Leu
5	Lys-Val-Ile-Leu-Phe
6	Thr-Val-Leu
7	Met-Leu-Phe

[a] Trp: tryptophan; Ala: alanine; Gly: glycine; Asp: aspartic acid; Ser: serine; Glu: glutamic acid; Arg: arginine; Pro: proline; Phe: phenylalanine; Asn: asparagine; Val: valine; Tyr: tyrosine; Ile: isoleucine; His: histidine; Leu: leucine; Lys: lysine; Met: methionine.

previously described (Harvey et al., 1986), was subjected to TMAH/thermochemolysis and the products were subsequently analyzed by GC/MS. The mass spectra of the products were compared to those obtained from albumin.

The algaenan of the axenic strain of the alga *Scenedesmus communis,* was isolated after a sequential series of treatments involving successive solvent extraction of the dry biomass (hexane and $CHCl_3$/MeOH, 2/1 v/v), basic hydrolysis (refluxing for 6 h in 6% KOH solution in MeOH/H_2O, 9/1 v/v) and acid hydrolysis (stirring for 13 days in 85% H_3PO_4 at 55°C) as previously described (Berkaloff et al., 1983). The final residue was dried for TMAH/thermochemolysis.

A humin fraction of the Mangrove Lake sapropel (Bermuda) collected at a depth of 3.6 m was obtained after successive extraction with benzene/methanol (1:1 v/v), 0.1 M HCl and 0.5 M NaOH (under nitrogen) (Hatcher et al., 1983). The base-extracted solid residues were then treated with a concentrated HF/HCl solution (1:1 v/v) to remove most of the mineral matter and polysaccharide-like materials. To hydrolyze labile biopolymers not removed by HF/HCl, the humin was further treated with refluxing 6 M HCl.

2.2. TMAH/thermochemolysis

TMAH/thermochemolysis was performed with 0.5 mg sample in a sealed glass tube with a measured amount (100 µl) of TMAH (25% in methanol) and heated for 30 min at 250°C, as described by McKinney et al. (1995). The thermochemolysis products were extracted three min with 2 mL methylene chloride. The volume of the extract was reduced to 1 µ1 by removing the solvent by drying under gaseous N_2. Approximately 1 µl was analyzed by capillary gas chromatography on a Hewlett Packard 5890 Series II gas chromatograph. Compounds were identified using a Kratos MS-80 RFA high resolution gas chromatograph/mass spectrometer system. The columns used for the GC separation were 30 m×0.25 mm, I.D., fused-silica capillary columns with a film thickness of 0.25 µm (DB-5, J&W Scientific). The column was heated at 15°C/min from an initial temperature of 50–100°C, at which point the rate was slowed to 6°C/min to a final temperature of 280C (temperature program I). For the analysis of the TMAH/thermochemolysis products of *Spirulina*, the temperature was increased at a rate of 8°C/min from 40–100°C, held for 2 min and then increased a rate of 6°C/min to 280°C (temperature program II). Injector and detector temperatures were set at 300°C. Mass spectra were obtained at an electron impact potential of 50 eV with a mass range of 40–510 *m/z* at a scan rate of 0.6 s/decade of mass with a 0.2 s magnet settling time added. Compounds were identified on the basis of their mass spectra by comparison with spectra in the Wiley/NIST library, supplied with the instrument software. Many of the peptide derivatives having methoxylated side-chains did not have mass spectra present in the library. In these instances, identification is tentative and based solely on analysis of the fragmentation patterns and the comparison of the mass spectra obtained for the peptide standards listed in the Appendix.

2.3. Solid-state ^{15}N NMR spectroscopy

Solid-state ^{15}N NMR spectra were obtained on a Bruker MSL-300 spectrometer at a frequency of 30.4 MHz with the standard cross-polarization magic-angle-spinning technique (Pines et al., 1973; Schaefer and Stejskal, 1976) with a contact time of 0.7 ms, a pulse delay of 200 ms, and a spinning speed of 4.5 kHz. The spectra were obtained after accumulation of approximately 500,000 to 1 million scans and applying a line broadening of 100 Hz. The chemical shifts were plotted referenced to external nitromethane (= 0 ppm), using glycine (−347 ppm) as a surrogate standard (Witanowski et al., 1993).

3. Results

3.1. TMAH/thermochemolysis products of peptides and proteins

The chromatogram of products released after the TMAH/thermochemolysis of albumin is shown in Fig. 1. The identities are listed in Table 2.

The mass spectrum of peak 6 shows intensities at *m/z* 42, 58 and 117 (**I**). Comparable spectra around the same retention time were obtained from peptide 1, 2 and 3 but were not observed for any of the other peptides. Based on the mass spectral fragmentation ($M^+ = 117$, *m/z* 58 $= (CH_3)_2N{=}CH_2^+$) and the fact that only peptides 1, 2, and 3 contain glycine, peak 6 is assigned to the TMAH

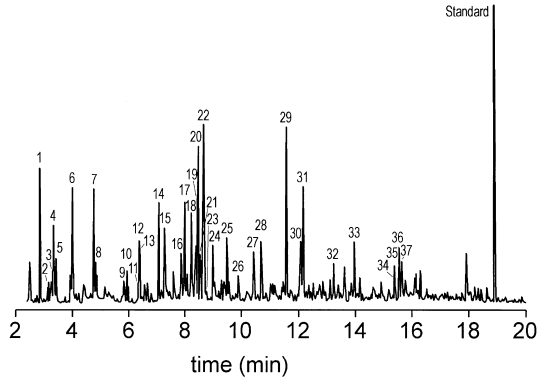

Fig. 1. TIC chromatogram of TMAH/thermochemolysis products released from albumin. For peak identification refer to Table 2.

Table 2
TMAH/thermochemolysis products of albumin numbered from 1 to 37

No.	Min	Ions (m/z)[a]	Compound	Peptide	Origin
1	2.2		Not identified		?
2	2.8	45	Propanoic acid, 2-hydroxy-methyl ester[b]		?
3	3.31	59, 44, 43	Acetamide[b]		?
4	3.32	73, 44	*N,N*-Dimethylformamide[b]		?
5	3.42	59, 43, 73	Not identified		?
6	3.99	58, 42, 117	*N,N*-Dimethylglycine methyl ester[c]	1, 2, 3	Gly
7	4.79	72, 42, 131	*N,N*-Dimethylalanine methyl ester[c]	1	Ala
8	4.81	72, 74, 87, 99, 131	Not identified	1	?
9	5.80	86, 102, 116	Not identified		?
10	5.92	106, 77, 51, 50	Benzaldehyde[b]		Phe
11	6.30	100, 58, 42, 116, 159	*N,N*-Dimethylvaline methyl ester[b]	1	Val
12	6.36	91, 122, 121, 77	Benzyl alcohol methyl ester[b]		Phe
13	6.54	59, 86, 57, 101	Butanamide, 3-methyl-ester[b]		?
14	7.02	115, 55, 59, 114, 87	Butanedioic acid dimethyl ester[b]		?
15	7.27	84, 42, 143	*N*-Methyl-L-proline methyl ester[c]	2, 4	Pro
16	7.82	58, 42,114, 116, 173	*N*, *N*-Dimethylleucine (or isoleucine) methyl ester[c]	4, 5, 6	Leu or
17	7.92	113, 56	*N*-Methyl-2,5-Pyrolidinedione [d,e]	7	Ile Asp[e]
18	8.18	42, 127	Not identified		?
19	8.32	59, 72, 44	Not identified		?
20	8.41	74, 87, 127, 158	Not identified		?
21	8.50	139, 54, 110	Not identified		?
Numbers 22 to 37					
22	8.57	59, 100, 129, 101	Pentanedioic acid dimethyl ester[b,c]		?
23	8.66	98, 70, 42, 157	*N*-Methyl-2-aminohex-5-enoic acid methyl ester[b]		Lys
24	8.96	57, 113, 114,	1 Methyl-piperidinone[b,e]		?
25	9.42	42, 127, 98, 70	*N*-Methylglutaramide[b,e]		Gln*
26	9.95	142. 127	Not identified		?
27	10.39	135, 136, 77, 92,104	Benzaldehyde, 4-methoxyl[b]		Tyr
28	10.67	104, 91, 105, 164	3-Phenylpropanoic acid methyl ester[b]		Phe
29	11.54	98, 42, 70, 157	L-Proline, 1 methyl-5-oxo-methyl ester[c]		Glu
30	12.01	84, 56, 41, 143, 166	Not identified		
31	12.12	131, 103, 162, 77, 51	3-Phenyl propenoic acid methyl ester[b]		Phe
32	13.19	116, 148, 133, 91	*N,N*-Dimethylphenylalanine methyl ester[d]	2, 5, 7	Phe
33	13.96	121, 194, 134	4-Methoxybenzenepropanoic acid methyl ester[b]		Tyr
34	15.36	138, 194	Not identified		?
35	15.56	152, 165, 137	Not identified		?
36	15.65	161, 192, 133	3(4-Methoxyphenyl)-propenoic methyl ester[b]		Tyr
37	15.78	156, 127, 126, 58	Not identified		?

[a] Masses are listed in decreasing abundance.
[b] Identified according to the Wiley or NIST libraries supplied with the instrument software.
[c] Identified by comparison to the mass spectra of the peptides listed in Table 1.
[d] Identified by interpretation of the mass spectrum of the corresponding compound.
[e] Tentative assignment.

thermochemolysis product of glycine (*N,N*-dimethylglycine methyl ester).

Other compounds that originate from cleavage of the peptide and methylation of the carboxylic acid and amino groups are *N,N*-dimethylalanine methyl ester (peak 7) (**II**), *N,N*-dimethylvaline methyl ester (peak 11) (**III**), *N*-methyl-L-proline methyl ester, (peak 15) (**IV**), *N,N*-dimethylleucine methyl ester or *N,N*-dimethylisoleucine methyl ester (peak 16) (**V**) and *N,N*-dimethylphenylalanine methyl ester (peak 32) (**VI**).

N,N-Dimethylalanine methyl ester was only identified for albumin and peptide 1. The mass spectrum of *N,N*-dimethylvaline methyl ester was found for the peptides 4, 5, 6 and albumin. The mass spectrum obtained at a retention time of 7.3 min and having peaks at m/z 42, 84 and 143 was found for albumin and peptides 2 and 4, all containing proline and thus allowing an assignment to *N*-methyl-L-proline methyl ester.

The mass spectrum of peak 16 (**V**) with abundant ions at m/z 42, 58, 114, and 116 may derive both from dimethylleucine methyl ester or its isomer dimethylisoleucine methyl ester and was observed for peptides 4, 5, 6, 7 and albumin. After a retention time of 13.2 min, a mass spectrum of the peak 32 with abundant ions at m/z 116, 148, 91 and 133 was obtained for peptides 2, 5 and 7 and albumin. All three peptides contain phenylalanine

allowing an assignment of this peak to *N,N*-dimethylphenylalanine methyl ester (**VI**). Other methylated amino acids that were identified for the peptides but not in albumin are *N,N*-dimethylarginine methyl ester (peptide 2, 20.3 min, **VII**), *N,N,O*-trimethyltyrosine methyl ester (peptide 4, 20.8 min, **VIII**) and *N,N,N′*-trimethyltryptophan methyl ester (peptide 1, 29.5 min, **IX**).

Several non-nitrogen-containing products were also detected. Examples are benzenepropanoic acid methyl ester (peak 28, 10.7 min, peaks at *m/z* 91, 104,105 and 164 (M^+)); 3-phenyl-2-propenoic acid methyl ester (peak 31; 12.1 min, peaks at *m/z* 51, 77, 103, 131 and 162 (M^+)); 4-methoxybenzenepropanoic acid methyl ester (peak 33, retention time 14 min, peaks at *m/z* 121 and 134 and 194 (M^+)); 3(4-methoxylphenyl)-2-propenoic acid methyl ester (peak 36, retention time 15.7 min, peaks at *m/z* 133, 161, and 192 (M^+)). These products can be explained by deamination of the two aromatic amino acids, phenylalanine and tyrosine, during thermochemolysis. Further degradation of these amino acids during thermochemolysis may result in benzaldehyde (peak 10), benzyl alcohol methyl ether (peak 12), and 4-methoxy benzaldehyde (peak 27).

Some of the products formed during thermochemolysis of the peptides can be explained by methylation and subsequent rearrangement. The mass spectrum of peak 23 obtained at a retention time of 8.66 min with peaks at *m/z* 70, 98, and 157 was found for albumin, and peptide 5 (**X**). This mass spectrum is assigned to *N*-methyl-2-aminohex-5-enoic acid methyl ester, derived from an elimination reaction of methylated lysine. Cyclization of glutamic acid may have resulted in 1-methyl-5-oxo-L-proline methyl ester (peak 29) (**XI**) with a mass spectrum with peaks at *m/z* 98, 157 and, relative to that of the compound 22, a smaller peak at *m/z* 70. This spectrum was obtained for peptides 1and 3. The products corresponding to peaks 18 and 22 could not be identified. Those compounds were also observed among the products obtained after methylation of soybean powder with TMAH in conjugation with pyrolysis (Reeves and Francis, 1998).

3.2. TMAH/thermochemolysis products obtained from algal residues

At elevated temperatures, proteins and peptides are known to be very susceptible to recondensation reactions if compounds containing carbonyl groups e.g. carbohydrates or quinones are present (Ikan, 1996). Such reactions with amino acids, known as Maillard reactions (Maillard, 1916) are often discussed as possible mechanisms for the stabilization of organic nitrogen: in particular, for lignin-depleted environments such as sapropels or marine sediments. In order to test if such reactions may obscure the identification of amino-acid-derived TMAH/thermochemolysis products in a heterogeneous mixture containing proteins, carbohydrates and lipids, a dried powder of an algal culture from *Spirulina* sp. was analyzed. Most of the compounds identified for albumin were also found for the algae (Fig. 2). Additional N-containing products, such as pyridine- or pyrrole-like compounds indicating the formation of condensation products between amino acids and carbohydrates were not identified. This observation leads to the conclusion that after the cleavage of the amide bonds, methylation of the amino groups with TMAH represents an efficient means to protect those groups from further involvement in recondensation reactions during thermochemolysis. The results further demonstrate that peptide-derived TMAH/thermochemolysis products can be distinguished in a heterogeneous mixture.

The CPMAS ^{15}N NMR spectrum of the algaenan of *Scenedesmus communis* in Fig. 3a shows a feature already observed for those of the algaenans isolated (i) from the same species of *Scenedesmus* grown with $Na^{15}NO_3$ as a sole nitrogen source (Derenne et al., 1993) and (ii) from a ^{13}C- and ^{15}N-enriched algae (Knicker et al., 1996b). As indicated by the dominant signal at −260 ppm, most of the nitrogen that survived rigorous extraction with various organic solvents, plus alkaline and acid hydrolysis, is comprised of amide structures. Beside the strong resonance at −260 ppm, a further clear signal can be identified at −340 ppm, pointing to the presence of aliphatic amines. In a previous study, this signal at −340 ppm in the CPMAS ^{15}N NMR spectrum of the ^{15}N-enriched *Scenedesmus* algaenan was shown to disappear upon heating at 300°C (Derenne et al., 1993). possibly due to heat-induced destruction of aliphatic amines.

These features of the spectrum in Fig. 3 indicate that amides are a common component of the algaenan under study. Because amides are normally highly susceptible to hydrolysis, a physical protection mechanism can be inferred. Previously it was suggested that the amides may act as links between the various alkyl chains of the algaenan (Derenne et al., 1993). Alternatively, such amides may

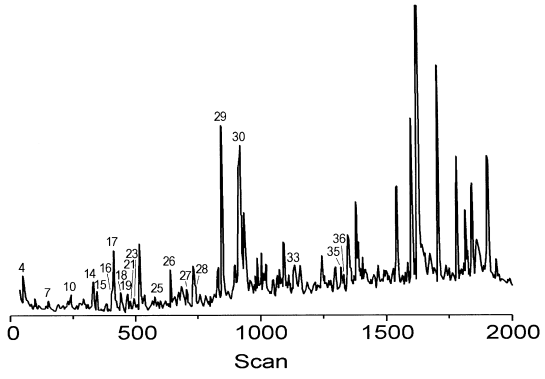

Fig. 2. TIC chromatogram of the TMAH/thermochemolysis products released from the algae Spirulina. For peak identification refer to Table 2.

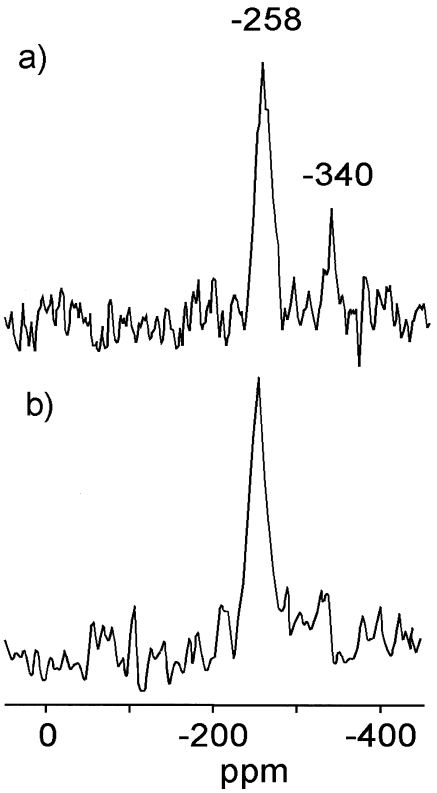

Fig. 3. Solid-state ^{15}N NMR spectrum of (a) the algaenan of *Scenedesmus communis* and the hydrolysis residue of a humin fraction obtained at a depth of 3.6 m from the sapropel from Mangrove Lake, Bermuda.

derive from peptide structures that are entrapped within the algaenan network and are thus physically protected against chemical extraction or degradation. Such a model could also explain the occurrence of the signal of aliphatic amines at −340 ppm in the ^{15}N NMR spectrum.

To obtain more information about the chemical nature of those amides, TMAH/thermochemolysis was applied to the algaenan. Derivatized amino acids, specifically products of proline, lysine and glutamic acid, were identified (Fig. 4). In spite of the low intensity of their signals in the chromatogram, their identification indicates that some amino acids have been sterically or physically protected in the algaenan from the drastic acid and basic hydrolyse used for isolation. Their release after TMAH/thermochemolysis may be explained by the fact that methanolic TMAH solution acts as a swelling agent, that is able to widen the pores in the hydrophobic network of the algal remains and thus increase the accessibility of the amide bonds to thermochemolysis with subsequent methylation. However, it seems also likely that rapid penetration of TMAH from the external surface inward toward the core of the matrix may be responsible for the rapid reaction.

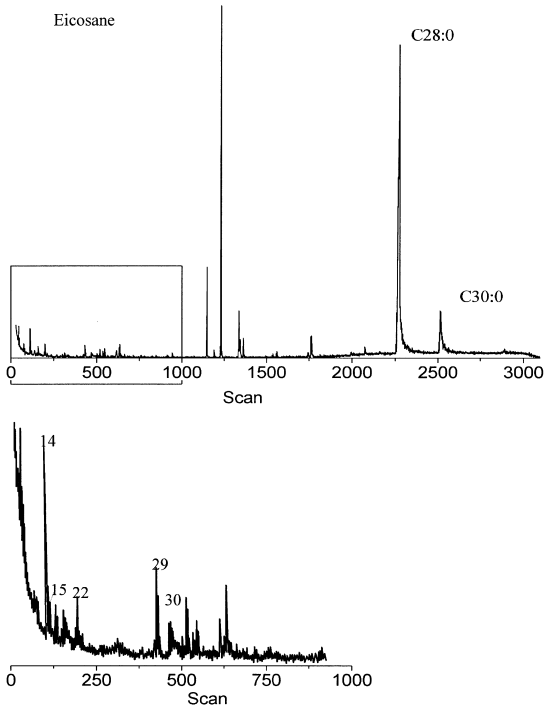

Fig. 4. TIC chromatogram of the TMAH/thermochemolysis products from the algaenan of *Scenedesmus communis*. For peak identification refer to Table 2. Eicosane represents an internal standard.

3.3. TMAH/thermochemolysis products obtained from the hydrolysis residue of a humin fraction of an algal sapropel

In a previous study it was shown that amide functional groups comprise the main fraction of the organic nitrogen in a 4000 year old algal sapropel (Knicker et al., 1996b). As depicted in Fig. 3 showing the solid-state ^{15}N NMR of the HCl-hydrolysis residue of a humin fraction extracted from the sapropel at depth of 3.6 m, some of those amides even survive chemical degradation. In order to reveal if some of these amides can be assigned to peptide-like material, the hydrolysis residue of this sample was analyzed for its TMAH/thermochemolysis products.

The chromatogram in Fig. 5 shows peaks marked by numbers that indicate the presence of amino acid derivatives in the hydrolysis residue of the sapropel produced by the method. Comparable compounds were also identified in a previous preliminary study of the hydrolysis residue of the humin fraction from the freshwater unit of the same sapropel but obtained at 11.5 m depth (Knicker and Hatcher, 1997). Other signals are assigned to alkanes and fatty acid methyl esters, confirming the highly paraffinic nature of the insoluble

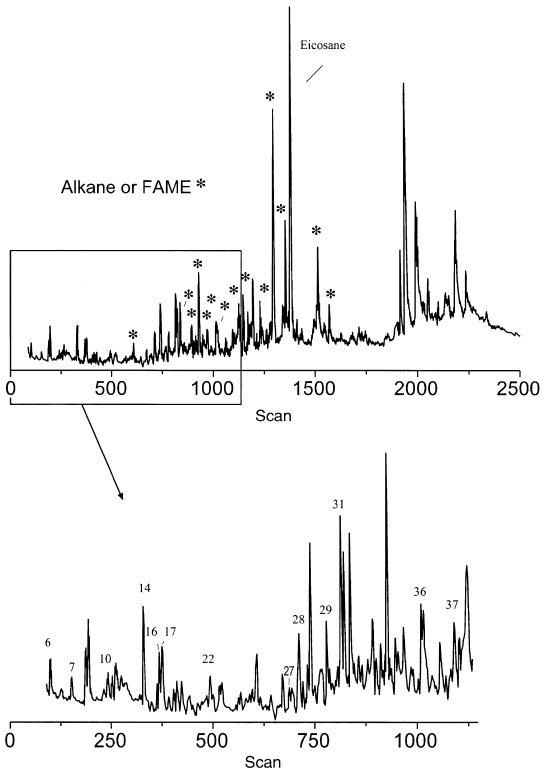

Fig. 5. TIC chromatogram of TMAH/thermochemolysis products released from the hydrolyzed humin residue of the sapropel obtained at a depth of 3.6 m from Mangrove Lake, Bermuda. or peak identification refer to Table 2. Asterisks indicate peaks of alkane and fatty acid methyl esters. Eicosane represents an internal standard.

sapropelic humin previously revealed by solid-state ^{13}C NMR spectroscopic studies (Hatcher et al., 1983). The identities of released compounds with retention times greater than 1750 scans (36 min) were not determined due to their complexity. Products of lignin are not observed. These results support the assumption that at least some of the amide-N in the CPMAS ^{15}N NMR spectra of the hydrolysis residues originates from peptide-like structures that have survived (1) diagenetic alteration in the sapropel and (2) treatment with dilute alkali followed by 6 M HCl.

4. Summary and discussion

The nature of refractory organic nitrogen in geochemical samples is still of great interest, in particular if a better understanding of nitrogen cycling is desired. Recent studies applying solid-state ^{15}N NMR spectroscopy indicated that amide is the predominant form of nitrogen in biologically humified material (Knicker et al., 1996b, 1997). Comparison of these spectra with those obtained from protein-rich material led to the assumption that those amides derive from peptide-like material. Until this study, however, direct proof for this assumption has not been obtained. A more detailed analysis of the refractory amides is necessary. Some of the techniques used involve pyrolytic degradation of the macromolecule into small fragments that are analyzed. Applying this approach, one has to consider the chemical lability of proteins and peptides. Secondary reactions (rearrangement, cracking, hydrogenation and polymerization) are very likely under pyrolytic conditions. Thus, conclusions regarding the original nitrogen-containing structures in the macromolecular phase are difficult and studies employing pyrolysis to ascertain the nature of humic N species must be viewed with caution.

In the present work, thermochemolysis with TMAH is introduced as an alternative approach to identify N-containing species in humic substances. Subpyrolysis temperatures and protection of the reactive sites of the thermochemolytically cleaved peptides by methylation seem to prevent extensive rearrangement of most amino acids identified. Many of the products exhibit typical amino acid-derived mass spectral features that allow their unequivocal assignment. Their presence among the reaction products of the TMAH/thermochemolytically-treated hydrolysis residue of the sapropelic humin further demonstrates that such products can also be revealed in heterogeneous mixtures of complex geochemical samples. Based on the results presented, such products may act as biomarkers for the presence of proteinaceous material in such samples, if their insolubility and heterogeneity circumvent complete removal and extraction of nitrogeneous compounds with the commonly used hydrolysis techniques.

The fact that products derived from amino acids can be released from the hydrolysis residue of the sapropelic humin and the algaenan, both of which were subjected to reflux under 6 M HCl, points to another important conclusion. It is clear that the sapropel from Mangrove Lake harbors peptide-like materials that resist both early diagenesis and harsh chemical treatment. This observation demonstrates that acid hydrolysis was not able to release all peptide-like components (e.g. algal- or bacterial-derived peptides, proteins or remains of bacterial peptidoglycan). Insolubility in aqueous solutions or association with a non-extractable phase may be partly responsible for the resistance of some peptide-like material toward hydrophilic enzymes and harsh chemical treatment. Such associations of peptide-like compounds with insoluble macromolecular phases are likely to represent a common component of the organic nitrogen fraction in geochemical samples. Resistance to hydrolysis of peptides tied up in such associations may explain at least a part of the so-called ''unidentified nitrogen'' in soils and recent sediments.

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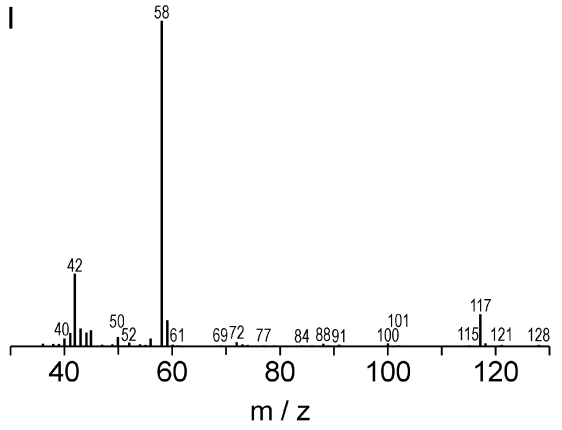
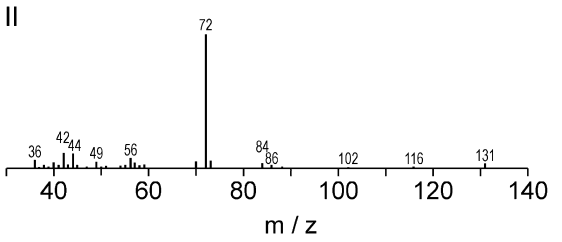
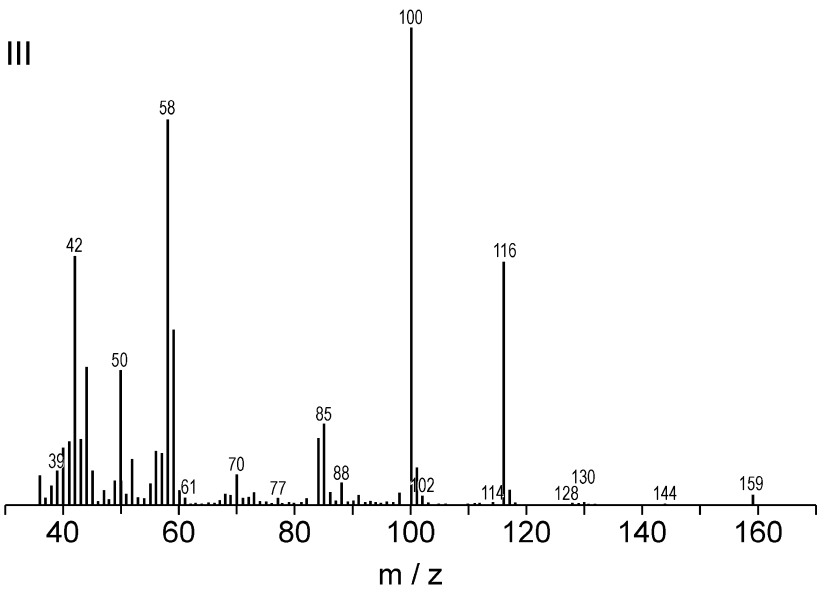
Appendix

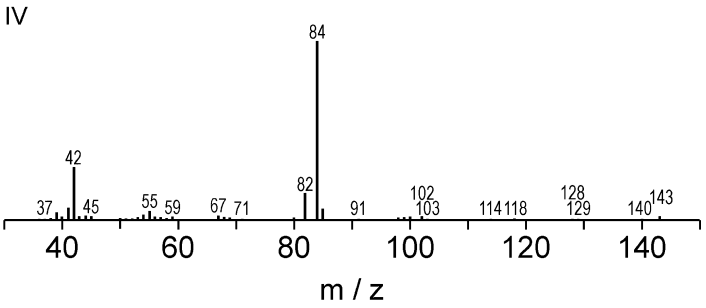
Mass spectra of products released by TMAH/thermochemolysis of albumin and peptides listed in Table 1.

I: *N*,*N*-Dimethylglycine methyl ester (peak 6)
II: *N*,*N*-Dimethylalanine methyl ester (peak 7)
III: *N*,*N*-Dimethylvaline methyl ester (peak 11)
IV: *N*-Methyl, L-proline methyl ester (peak 15)
V: *N*,*N*-Dimethylleucine (isoleucine), methyl ester (peak 16)
VI: *N*,*N*-Dimethylphenylalanine, methyl ester (peak 32)
VII: *N*,*N*-Dimethylariginine, methyl ester
VIII: *N,N,O*-Trimethyltyrosine, methyl ester
IX: *N,N,N*-Trimethyltryptophan, methyl ester
X: Product released after TMAH/thermochemolysis of peptide 5 and possibly derived from rearrangement of methylated lysine.
XI: L-Proline, 1-methyl-5-oxo-, methyl ester.





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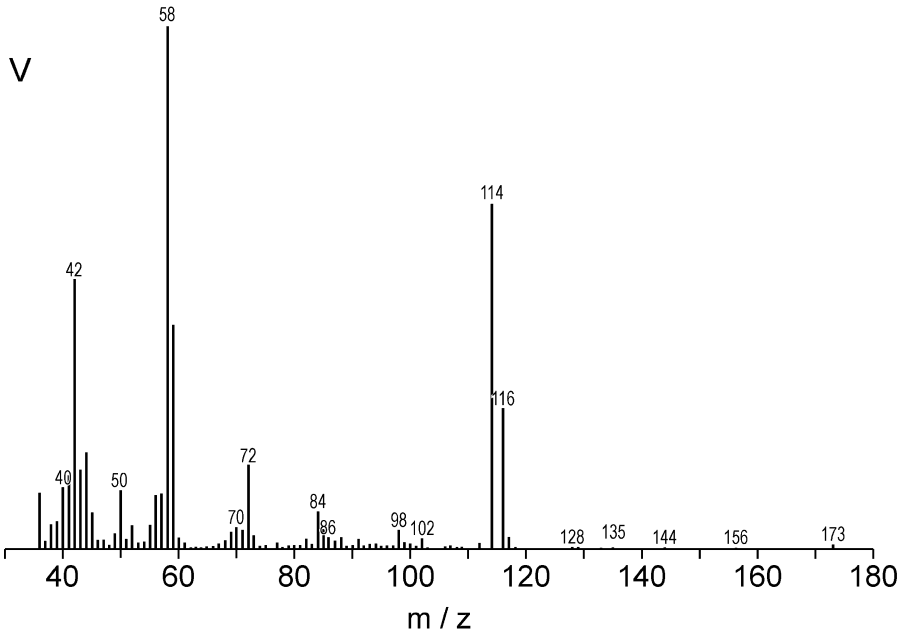
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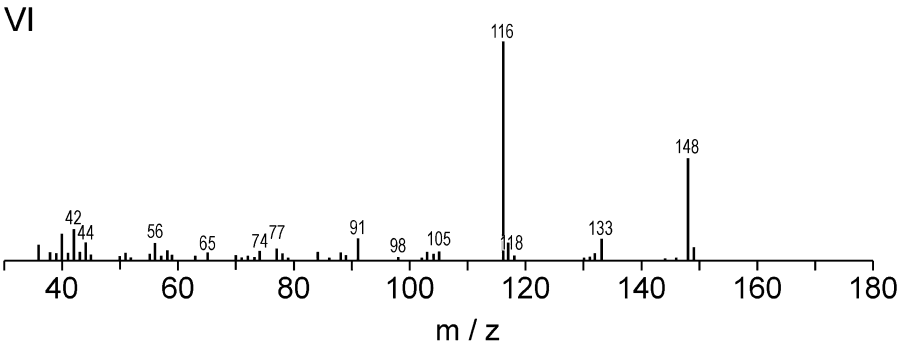
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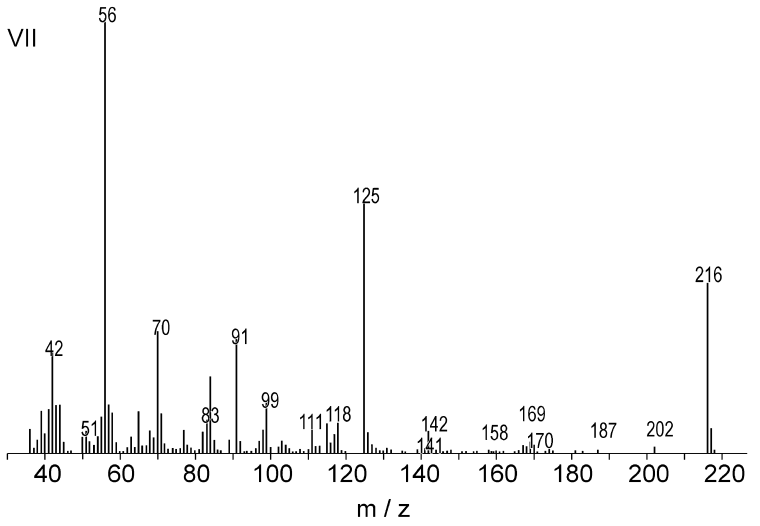
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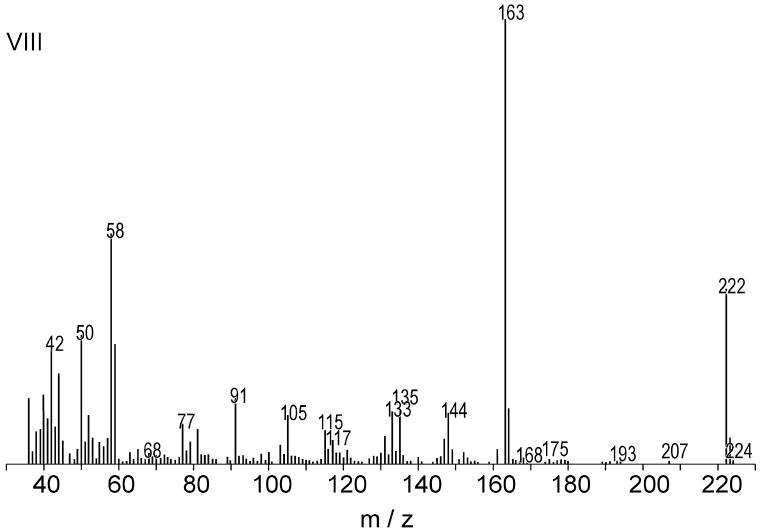
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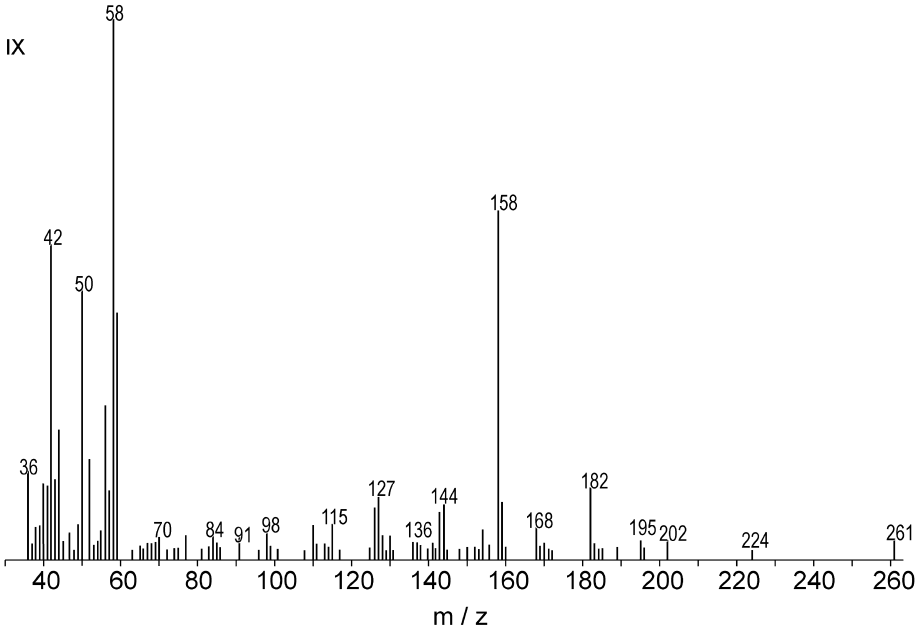
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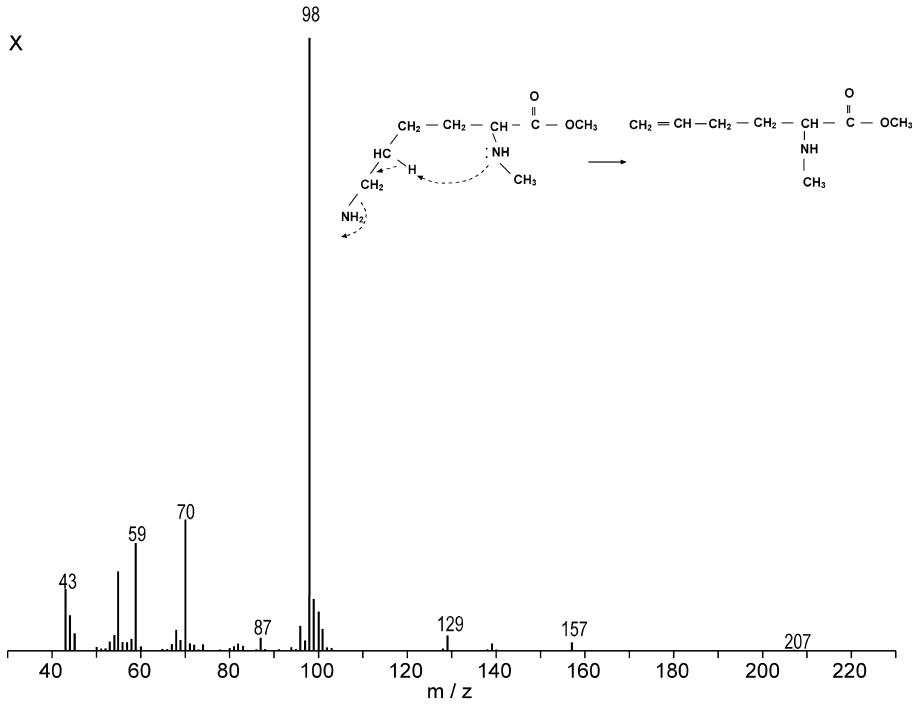
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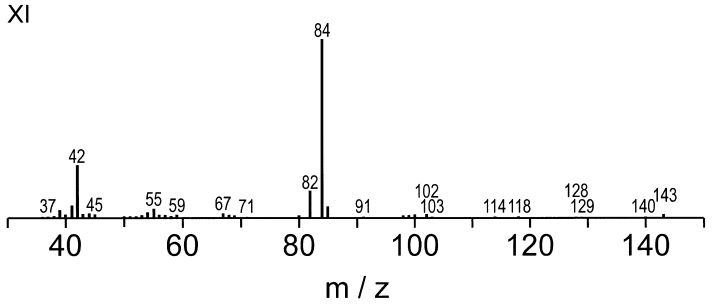

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