A lignin-like polymer in the cuticle of spruce needles: Implications for the humification of spruce litter

ARTICLE *in* ORGANIC GEOCHEMISTRY · DECEMBER 1994

Impact Factor: 3.07 · DOI: 10.1016/0146-6380(94)90165-1

8 AUTHORS, INCLUDING:

Hans Kerp
University of Münster
211 PUBLICATIONS **4,515** CITATIONS

Pergamon

0146-6380(94)E0016-S

Org. Geochem. Vol. 21, No. 12, pp. 1219–1228, 1994

0146-6380/94 $7.00 + 0.00

A lignin-like polymer in the cuticle of spruce needles: implications for the humification of spruce litter

INGRID KÖGEL-KNABNER,[1]* JAN W. DE LEEUW,[2] ERIK W. TEGELAAR,[2]†
PATRICK G. HATCHER[3] and HANS KERP[4]

[1]Soil Science Group, University of Bochum, NA 6/134, D-44780 Bochum, Germany, [2]Division of Marine Biogeochemistry, Netherlands Institute of Sea Research (NIOZ), P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands, [3]Fuel Science Program, The Pennsylvania State University, 209 Academic Projects Building, University Park, PA 16802, U.S.A. and [4]Westfälische Wilhelms-Universität, Abteilung Paläobotanik, am Geologisch-Paläontologischen Institut und Museum, Hindenburgplatz 57-59, 48143 Münster, Germany

(Received 22 November 1993; returned for revision 6 January 1994; accepted in revised form 24 February 1994)

Abstract—Information on the type and amount of refractory biopolymers produced by plants is still insufficient. The aim of the present work was to determine the chemical (structural) composition of spruce cuticles as a source material for humification. Intact cuticles were isolated by conventional techniques from fully developed needles of Norway spruce (*Picea abies* (L.) Karst.). The cuticles were subjected to a series of selective treatments to remove different types of polymers. Extraction with organic solvents, to remove lipids and waxes, was followed by saponification to remove the cutin polyester. Finally, the cuticle residues were hydrolyzed to remove polysaccharides. Through investigations combining CP/MAS ^{13}C-NMR spectroscopy, analytical pyrolysis, and wet chemical methods (CuO oxidation), the chemical composition of the polymer was determined in the cuticle, and in the residues obtained by the selective chemical treatments. These data show that the isolated spruce cuticles consist of extractable lipids, polysaccharides, and cutin, biopolymers commonly found in plant cuticles. In addition, a lignin-type polymer was identified, which was selectively isolated after the treatments described above, from the final residue. In conjunction with results from a microscopic survey of the cuticles, these investigations provide evidence for the presence of a lignin-like polymer as a component of intact cuticles of Norway spruce. The consequences of this finding for the humification process of spruce litter are discussed.

Key words—plant cuticle, lignin, humification, pyrolysis, ^{13}C-NMR spectroscopy, CuO oxidation

INTRODUCTION

The quantification of biogeochemical cycles raises problems because of the complex chemistry involved, and the variability in decomposition rates of biochemicals. Lignin, as well as non-hydrolysable aliphatic biopolymers, have been found to be among the most recalcitrant compounds of vascular plants (Tegelaar *et al.*, 1989c; Hedges, 1992). The chemical composition of lignin in different plant types is well established. It is also known that lignin, in different tissues of the same plant species, shows differences in its monomeric composition as well as in the types of bonds (Higuchi, 1990).

The major aliphatic biomacromolecule of spruce cuticles is a polyester-type cutin. Little information is available on the presence and preservation potential of cutin in soils (Tegelaar *et al.*, 1989a, 1993). Cutin is observed in significant quantities in forest soils under Norway spruce (*Picea abies*) and European beech (*Fagus silvatica*) (Kögel-Knabner *et al.*, 1989; Riederer *et al.*, 1993). The highly resistant non-polyester type aliphatic biopolymers, present in cuticles from other plants (Nip *et al.*, 1986), were not encountered in spruce litter and soil organic matter derived from spruce (Kögel-Knabner *et al.*, 1992a). Lendzian *et al.* (1986) noted that lignin present in the transition zone between cell wall and cuticle of *P. abies* gave problems, when cuticles were isolated by enzyme treatment. Although the presence of lignin in this transition zone, which is the innermost part of the cuticular layer, has been observed previously, a detailed study of the lignin, present in cuticles, has not been conducted.

The chemical composition of plant tissues has implications for the recalcitrance of the litter material, and thus for humification processes, in soils and sediments (Tegelaar *et al.*, 1991; Kögel-Knabner *et al.*, 1990, 1992b). The focus of the present study is to investigate the chemical composition of spruce cuticles, by a combination of several methods, namely

*To whom all correspondence should be addressed.
†Present address: ARCO Exploration and Production Technology, 2300 West Plano Parkway, Plano, TX 75075, U.S.A.

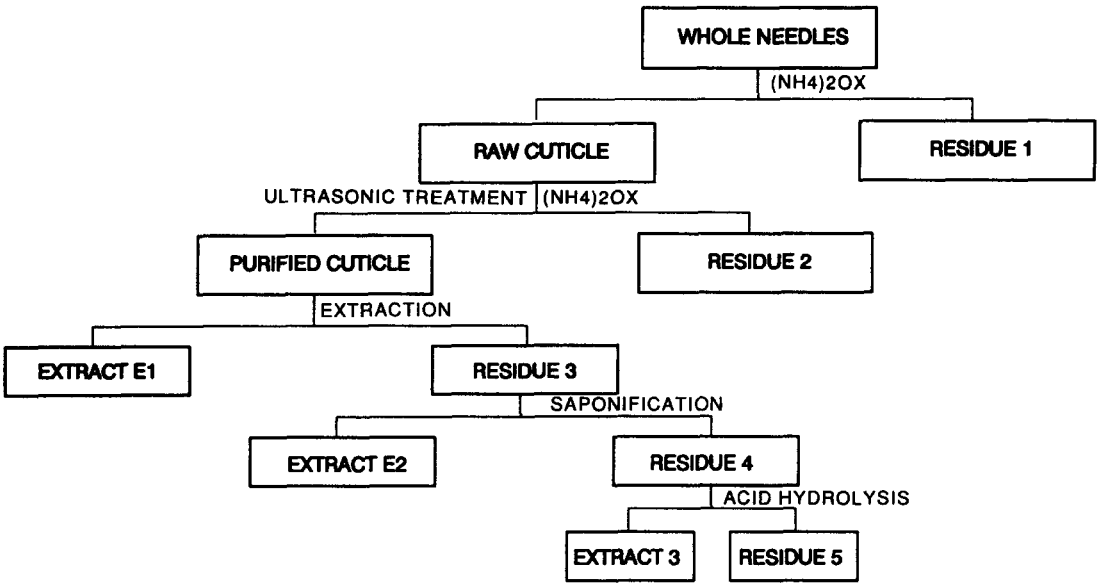

Fig. 1. Analytical flow sheet for the isolation of the purified cuticle and cuticle residues from Norway spruce (*P. abies*).

CuO oxidation, Curie-point pyrolysis–GC–MS and solid-state ^{13}C-NMR spectroscopy.

MATERIALS AND METHODS

Whole needles were obtained from Norway spruce (*P. abies*) in the surroundings of Bayreuth, NE Bavaria, Germany. Needles 2–3 years old were picked from twigs from which the apex and base were removed. Cuticles from *P. abies* were prepared by a series of treatments (Fig. 1). This sequential extraction/depolymerization treatment, or individual parts of it, are used commonly to isolate the polyester type cutin, and the non-polyester type cutan, macromolecules from cuticles (Tegelaar *et al.*, 1991). The whole needles (50 g) were boiled in a mixture of oxalic acid (4 g l^{-1}) and diammoniumoxalate (16 g l^{-1}) for 27 h. To obtain the raw cuticle, each individual needle was scraped with a knife to separate the raw cuticle from other needle material. This material yields residue 1 (Fig. 1). The raw cuticles (400 mg) were boiled again for 10 h in the same oxalic acid/diammoniumoxalate mixture, and subjected to ultrasonic treatment to remove any impurities present. The raw cuticles were allowed to cool overnight and subsequently filtered using Whatman GF/F filters. The whole procedure was then repeated once. After filtration, the cuticles were washed with doubly-distilled water and air-dried. This procedure yields the purified cuticle and residue 2. The purified cuticle was extracted with 200 ml chloroform/methanol (1:1) for 24 h in a Soxhlet apparatus to remove extractable lipids. The residue, after extraction, was washed with the chloroform/methanol mixture and dried to yield cuticle residue 3. To obtain cuticle residue 4, the material was saponified with KOH (1%) in methanol for 3 h under reflux at 70°C. This step will depolymerize the cutin polyester. The residue from saponification was filtered using Whatman GF/F filters and washed with 30 ml of doubly-distilled water, twice with 30 ml of a mixture of doubly-distilled water/methanol (1:1), 30 ml MeOH/HCl (2 M) (1:1), 30 ml methanol (twice), and finally 30 ml CH_2Cl_2 (3 ×), and dried. Residue 4 was then subjected to acid hydrolysis to remove carbohydrates. The cuticle residue was allowed to stand at room temperature in 72% H_2SO_4 for 5 h. After dilution with 2 N H_2SO_4, hydrolysis was performed for 16 h at 100°C. The hydrolysis residue was filtered using Whatman GF/F filters, washed with about 1 L doubly-distilled water and finally dried at 65°C. This treatment yielded cuticle residue 5. The yields of the different residues were determined by weighing each residue before further treatment.

Fig. 2. (*Opposite*) Microscopic observation of the purified cuticles. All photographs were taken with Differential Interference Contrast. (a) Overview of the purified cuticle with two rows of stomata (darker rows in the middle), ×250. (b) Overview of the cuticle with one row of stomata (darker row in the middle), ×250. (c 1/2) Two pictures at different focusing levels showing the cells with darker bodies; the darker areas on the side are stomata which are out of focus, ×400. (d) Details showing the darker rounded bodies, ×1000.

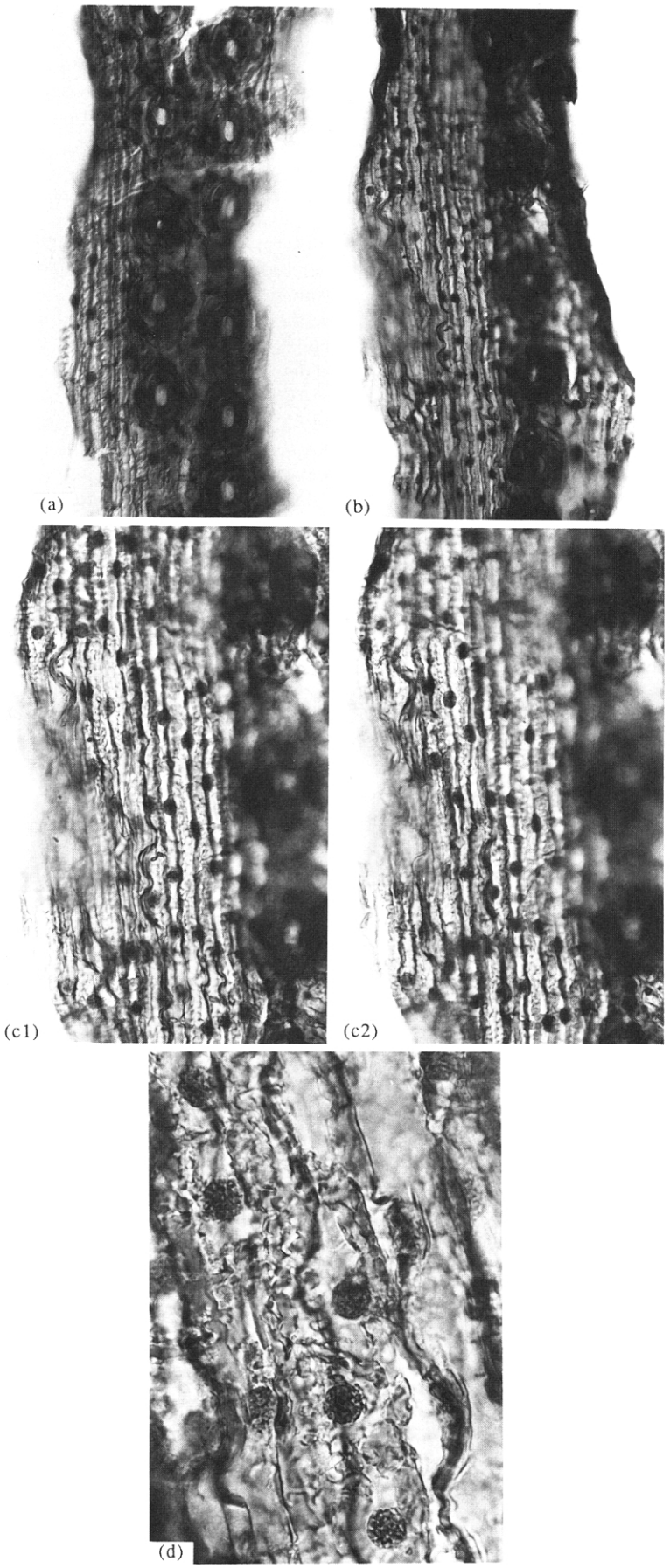

Fig. 2—*legend opposite.*

CuO oxidation

Alkaline CuO oxidation (Hedges and Ertel, 1982) was used as a diagnostic chemical degradation procedure for lignin. The procedures of oxidation, and extraction of the CuO oxidation products, were the same as described by Kögel and Bochter (1985). Briefly, the method includes oxidation of 50 mg of sample with CuO and 2 M NaOH at 170°C under N_2 for 2 h. After solid-phase extraction, phenyl acetic acid was added as internal standard and the samples were dried under N_2. The generated lignin-derived phenols (*p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, acetovanillone, vanillin, vanillic acid) were derivatized with BSTFA, and separated and quantified by gas chromatography using a Siemens Sichromat 1 as described by Guggenberger *et al.* (1994).

CP/MAS ^{13}C-NMR spectroscopy

CP/MAS ^{13}C-NMR spectra were obtained on a Chemagnetics 100S/200L instrument operating at 25.2 MHz for carbon. A 1 ms contact time and 1 s pulse delay was used with a spinning speed of 3300 Hz. A detailed description of the technique is given by Hatcher (1987) and Wilson (1987).

Analytical pyrolysis

Curie-point pyrolysis–gas chromatography (py–GC) and Curie-point pyrolysis–gas chromatography–mass spectrometry (py–GC–MS) were performed as described by Tegelaar *et al.* (1989b). The samples were applied to a flattened Fe wire with a Curie temperature of 770°C and pyrolysed for 10 s. Separation of pyrolysis products was achieved on a CP-Sil 5 fused silica capillary column. The oven of the gas chromatograph was programmed from 0°C (1 min) to 320°C (20 min) at a rate of 3°C/min.

For py–GC–MS the pyrolysis and GC conditions were the same as those for py–GC. The GC column was interfaced (interface temperature 250°C) with a VG 70-250 SE double focusing mass spectrometer operating in the electron impact mode under the following conditions: mass range m/z 45–800, cycle time 2 s, ionization energy 70 eV.

RESULTS AND DISCUSSION

Preparation and yields of cuticles and cuticle residues

A criterion for the measure of success of isolation of cuticle material is the morphology observed by light microscopy of the purified cuticle, prepared according to Fig. 1. The preparations show basically cuticles (Fig. 2). There is also material present which is not a part of the cuticle. These darker bodies are most probably remnants of cell nuclei of epidermis cells which have survived the chemical treatment used to separate the cuticles. They are regular in shape, uniform in size, and very regularly distributed at one per cell. Mostly they lie in the middle of a cell but sometimes they seem to lie against the anticlinal walls. However, it should be noted that the cuticles observed were lying slightly obliquely on the slides.

Table 1. Yields of cuticles and cuticle residues after the different chemical treatments indicated in Fig. 1

	Yield (mg)	%
Purified cuticle	409	100
Residue 3	401	98
Residue 4	338	83
Residue 5	71	17

Table 1 gives the yields of the different cuticle residues as obtained by gravimetry. Because of the low amount of cuticle material obtained, determination of carbon in the different residues was not attempted. The solvent extraction step removes only 2% of the purified cuticle. After saponification, 83% of the initial purified cuticle material is still recovered as residue 4, indicating that the polyester-type cutin only makes up a small portion of the total purified cuticle isolated. The major loss occurs after acid hydrolysis, which removed 70% of the total mass, leaving the final residue 5, corresponding to 17% of the purified cuticle.

CP/MAS ^{13}C-NMR spectroscopy

Figure 3(a)–(d) show the CP/MAS ^{13}C-NMR spectra of the purified cuticle and cuticle residues 3, 4 and 5. Signal assignment is based on previous work with bulk litter material of spruce (Kögel *et al.*, 1988; Wilson *et al.*, 1983). The NMR spectrum of the purified cuticle [Fig. 3(a)] is dominated by well-resolved signals at 66, 72, 74, 82, 88, and 105 ppm, which are characteristic for polysaccharides. The presence of polysaccharides in cuticles has been described previously. According to Holloway (1984) polysaccharides are a common component of cuticles. Nip *et al.* (1986) also found polysaccharides in plant cuticles after similar chemical treatment. In addition to the signals assigned to polysaccharides, a rather broad band of signals is found in the chemical shift-region 0–50 ppm, which is most probably due to carbon atoms in long-chain and branched aliphatic structures associated with fatty acids, lipids, waxes, and cutin acids. Signals around 175 ppm are due to carboxyl groups of fatty acids and hemicelluloses. Because of the low signal-to-noise ratio, the aromatic structures observed in the chemical-shift region 110–160 ppm cannot be assigned properly. The spectrum of residue 3 [Fig. 3(b)] is that of the cuticle after exhaustive extraction to remove lipids and waxes. Major signals at 66, 72, 88, and 105 ppm are again from polysaccharides. Due to the removal of impurities during extraction, and also the higher number of acquisitions for this spectrum, the signal at 175 is more clearly attributable to carboxyl groups. The

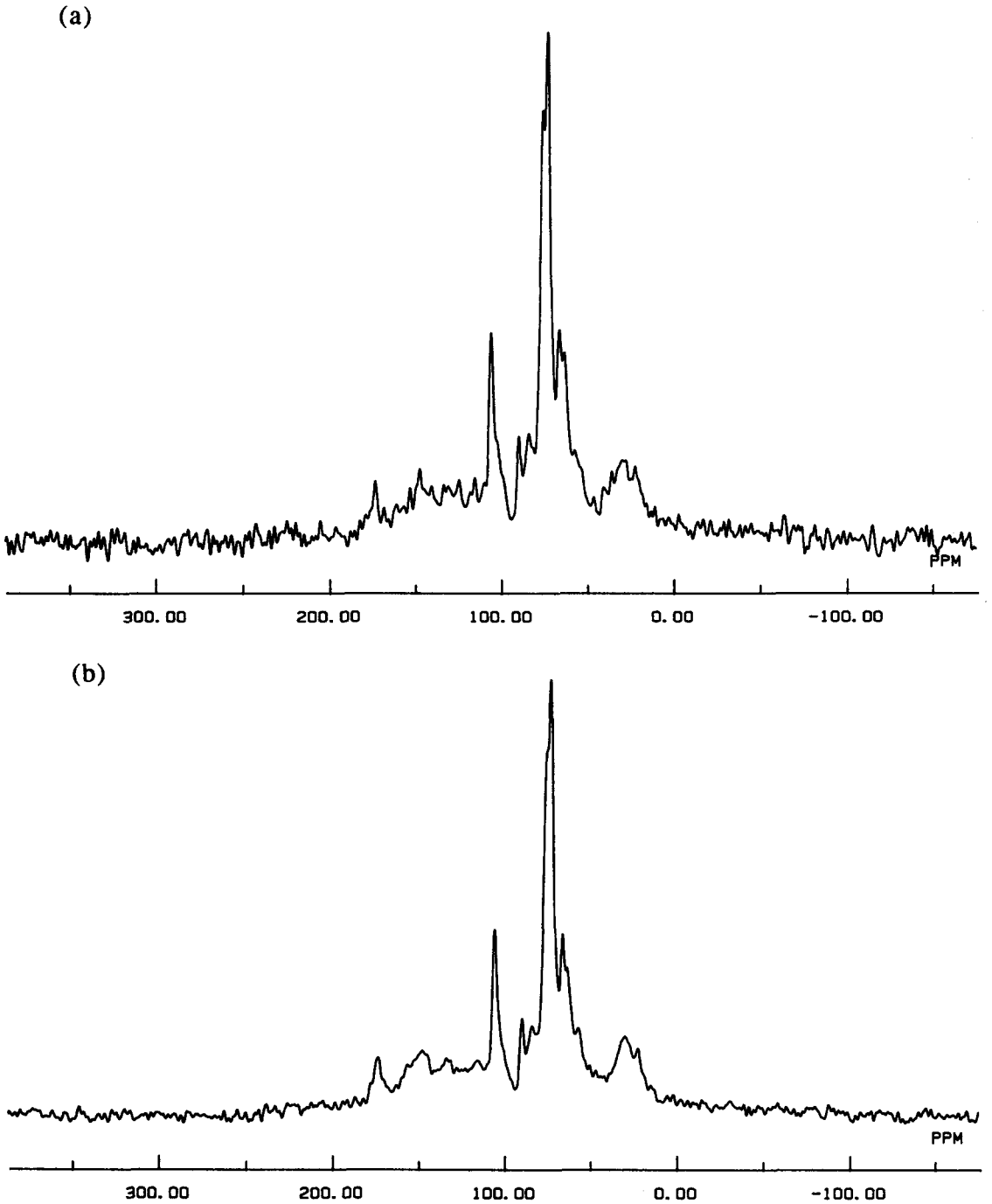

Fig. 3(a) and (b)—*legend overleaf.*

aromatic region shows distinct signals centred around 150 ppm, 130 ppm and 110 ppm, which are characteristic for O-aryl, C-substituted and protonated C in aromatic rings of lignin structures. After removal of extractable lipids and waxes, the signals in the alkyl-C region can be assigned to paraffinic C in cutin acids for the signal centred around 30 ppm, and to C in acetyl groups of hemicelluloses for the distinct signal at 22 ppm. Also, the shoulder at 55 ppm can now be assigned to methoxyl groups in lignin. The NMR spectrum of the cuticle residue 4 [Fig. 3(c)] is mainly attributable to polysaccharide structures, although some signal intensity in the aromatic region for lignin, and in the alkyl carbon region, is observed also. The polysaccharide signals at 66, 72, 84, 88, and 105 ppm are assigned to the C-6, C-2/C-3/C-5, C-4

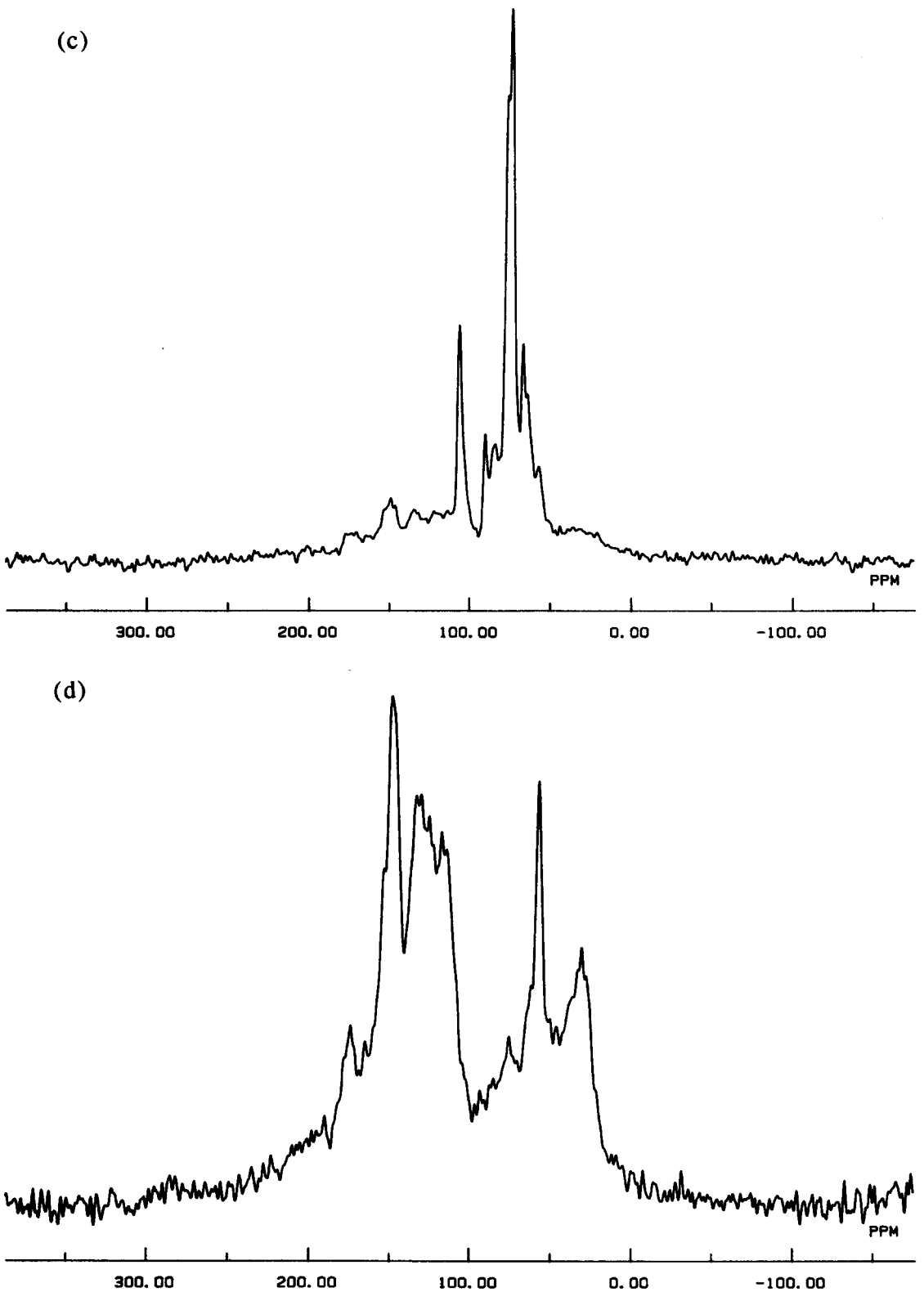

Fig. 3(c) and (d).

Fig. 3. CP/MAS ^{13}C-NMR spectra of cuticle residues; (a) purified cuticle, (b) residue 3, (c) residue 4, (d) residue 5.

Table 2. Yields of phenolic CuO oxidation products

Sample	Phenol	Concentration ($g\ kg^{-1}$)
Purified cuticle	*p*-Hydroxybenzaldehyde	0.64
	p-Hydroxybenzoic acid	0.86
	Acetovanillone	4.44
	Vanillin	19.85
	Vanillic acid	5.33
Residue 3	*p*-Hydroxybenzaldehyde	0.52
	p-Hydroxybenzoic acid	0.65
	Acetovanillone	4.09
	Vanillin	16.76
	Vanillic acid	4.90
Residue 4	*p*-Hydroxybenzaldehyde	0.35
	p-Hydroxybenzoic acid	0.37
	Acetovanillone	5.22
	Vanillin	26.10
	Vanillic acid	5.72
Residue 5	*p*-Hydroxybenzaldehyde	2.38
	p-Hydroxybenzoic acid	1.37
	Acetovanillone	4.97
	Vanillin	11.70
	Vanillic acid	11.41

(amorphous), C-4 (crystalline) and C-1 carbons in cellulose, respectively (Lindberg and Hortling, 1985). Cutin has been removed from the sample by saponification with KOH/methanol. Therefore, the signals for C in long-chain alkyl structures at 30 ppm are almost completely absent in the spectrum. This confirms the presence of cutin and the absence of cutan (Tegelaar *et al.*, 1989a) in the samples analyzed. Also, the signal intensity for carboxyl groups at 175 ppm is considerably diminished after saponification. Obviously, most of the carboxyl groups in the cuticle residue 3 were associated with the cutin polyester, and only a small amount is associated with hemicelluloses (acetyl groups) and lignin. Fig. 3(d) shows the NMR spectrum of the cuticle residue 5 after the ultimate acid hydrolysis step. Cellulose has been removed completely from the sample, as indicated by the loss of signal intensities at 72 and 105 ppm. The spectrum closely resembles the ^{13}C-NMR spectra of Norway spruce lignin, as far as the signals at 55 ppm (methoxyl groups), 147, around 130, and around 115 to 120 ppm are concerned (Maciel *et al.*, 1981). However, additional signals are observed at 30 and 175 ppm, which can not be attributed to lignin. They are probably due to cutin acid remains. The spectrum of the final residue can therefore be attributed mainly to lignin and another substance rich in alkyl moieties.

CuO oxidation

Table 2 shows the results from CuO oxidation of the purified cuticle and the residues 3, 4, and 5. CuO oxidation is used for the characterization of lignin in plant materials and geochemical samples. In all samples, significant amounts of lignin oxidation products are found. The yields of CuO oxidation products are rather similar for the purified cuticle and residues 3 and 4. They are dominated by large proportions of vanillin, followed by vanillic acid and acetovanillone. Small amounts of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid are also found. The lignin composition of spruce needles or litter, as observed with CuO oxidation, has been described previously (Kögel, 1986; Johansson *et al.*, 1986). The chemical composition, as observed with CuO oxidation, is in accordance with the monomeric composition of spruce lignin, which is composed almost exclusively of *p*-coniferyl and small amounts of *p*-coumaryl units (Higuchi, 1990).

The final residue 5 shows higher yields for the *p*-hydroxy phenols as expected for a lignin-rich material, as observed in the NMR spectrum [Fig. 3(d)]. However, low yields are observed for acetovanillone and especially for vanillin and vanillic acid, if we take into account the fact that the sample is composed almost exclusively of aromatic components, as indicated by the results from ^{13}C-NMR spectroscopy. Large amounts of vanillic acid are found with respect to vanillin. During hydrolysis with 72% sulphuric acid a small amount of lignin is solubilized, the so-called acid-soluble lignin, and the lignin residue has undergone partial hydrolysis of the beta-O-4 linkages (Leary *et al.*, 1986). This could explain the differences found in CuO oxidation products before and after the final hydrolysis step.

Table 3. Major compounds identified by GC–MS in the pyrolysates of spruce cuticle residues

Peak number	Pyrolysis product	Residue 1	Purified cuticle	Residue 3	Residue 4	Residue 5
1	2-Methylphenol					×
2	Guaiacol	×	×	×	×	×
3	4-Methylphenol	×	×	×	×	×
4	Dimethylphenol		×			×
5	4-Methylguaiacol	×	×	×	×	×
6	4-Vinylphenol		×			×
7	4-Ethylguaiacol	×	×	×	×	×
8	4-Vinylguaiacol	×	×	×	×	×
9	Eugenol	×	×	×	×	×
10	Vanillin		×	×	×	×
11	*cis*-Isoeugenol	×	×	×	×	×
12	*trans*-Isoeugenol	×	×	×	×	×
13	Homovanillin		×	×	×	
14	Acetovanillone	×		×	×	×
15	Levoglucosan	×	×	×	×	

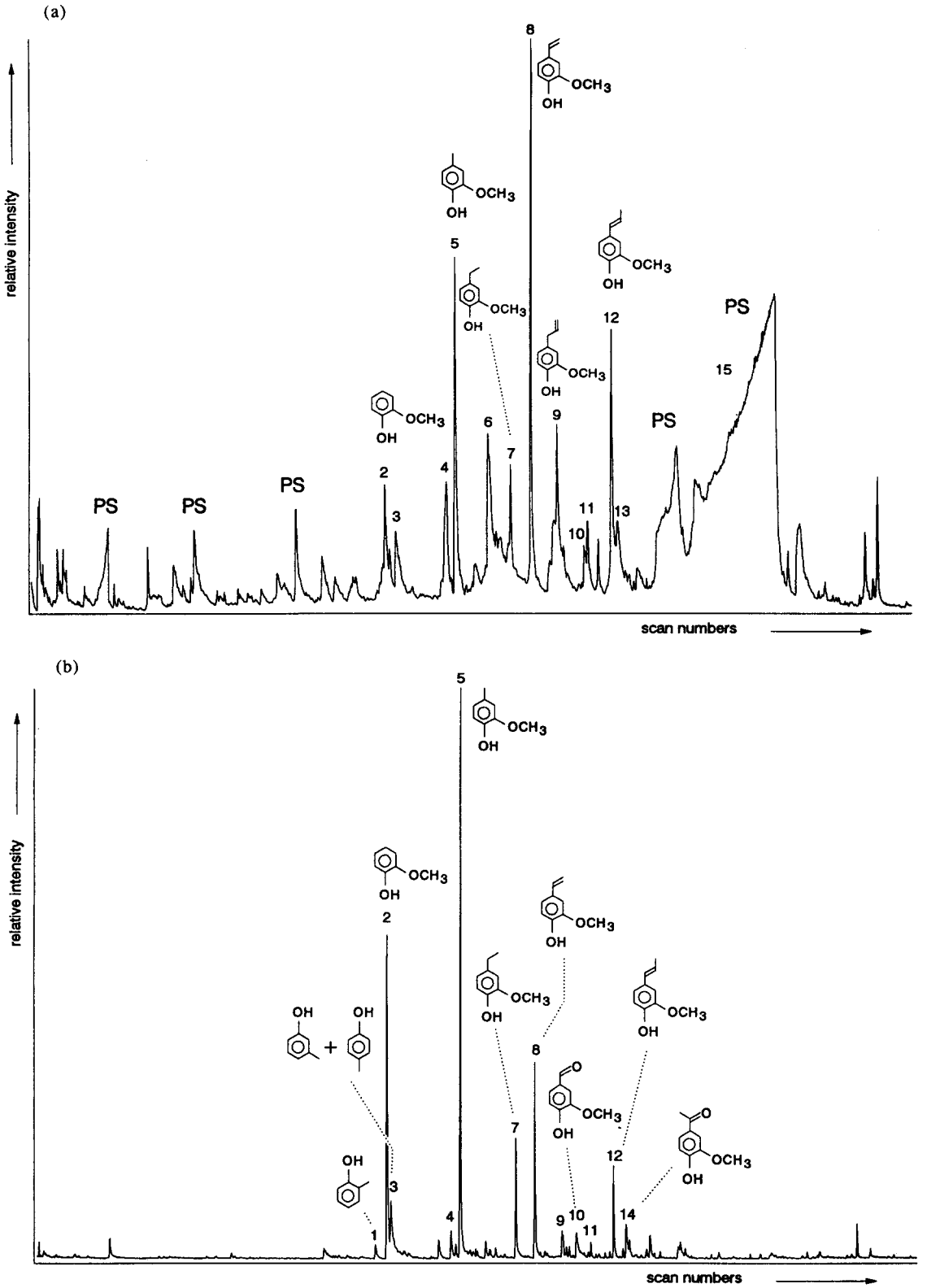

Fig. 4. Total ion current traces of pyrolysates of (a) purified cuticle, (b) cuticle residue 5. Peak numbers refer to Table 3. PS polysaccharide pyrolysis product.

Analytical pyrolysis

Table 3 lists the products identified in the pyrolysates of the purified spruce cuticle and the residues 3, 4, and 5. Figure 4 shows the py–GC–MS traces for the purified cuticle and residue 5. Residue 1 was also analyzed to compare its data with the pyrolysis data of the vein lignin. Identification of peaks is based on mass spectral data and comparisons with literature data (Saiz-Jimenez and De Leeuw, 1984; Ralph and Hatfield, 1991). All chromatograms are dominated by peaks corresponding to lignin. This is characteristic for most lignified plant materials (Ralph and Hatfield, 1991). Major peaks in the chromatograms of the pyrolysis products of the purified cuticle correspond to guaiacol, *p*-cresol, dimethylphenol, 4-methylguaiacol, 4-vinylphenol, 4-ethylguaiacol, 4-vinylguaiacol, homovanillin, and three different isomers of eugenol. Although these compounds have been identified as characteristic lignin pyrolysis products the distribution pattern is different from that of spruce milled wood lignin as reported by Saiz-Jimenez and De Leeuw (1984). Major pyrolysis compounds of spruce milled wood lignin are 4-methylguaiacol, 4-vinylguaicol, vanillin, *trans*-isoeugenol, coniferaldehyde, and *trans*-coniferyl alcohol. Supposedly, the lignin in the spruce cuticle is different with respect to the types of bonds to the lignin in wood. This is also the case with the vein lignin, which shows more similarities with the cuticle lignin.

Pyrolysis products derived from polysaccharides, such as levoglucosan, are also observed in the purified cuticle and in the residues 3 and 4. Due to the acid hydrolysis procedure applied to residue 4, levoglucosan is not observed in the final residue 5. The major pyrolysis products identified in the purified cuticle are also present in the residues 3 and 4. Differences in the distribution patterns observed in the final residue, with respect to the other samples, are attributed to changes caused by the acid hydrolysis step.

The absence of homologous series of n-alkanes and n-alkenes in all samples analysed clearly indicates that another highly aliphatic resistant biopolymer often present in higher plant cuticles (Tegelaar *et al.*, 1989a, 1993) does not occur in *P. abies* cuticles.

CONCLUSIONS

Cuticles from *P. abies* contain a lignin-like biomacromolecule, which can be isolated after a series of chemical treatments. This lignin-like material makes up about 17% of the isolated purified cuticle. The morphological structure of the isolated cuticle was confirmed by microscopy.

The presence of this lignin-like component in spruce cuticles was demonstrated unambiguously by several independent methods, including chemical degradation, analytical pyrolysis, and solid-state ^{13}C-NMR spectroscopy. These methods provide complementary results. The use of a combination of methods is necessary to avoid drawing biased conclusions on the chemical composition of complex samples containing unknown components, such as spruce cuticles.

The presence of lignin in cuticles of spruce, and possibly also in cuticles of other plant species, might have a protective function against biodegradation and lead to a selective preservation of cutin during humification in soils and sediments.

Associate Editor—M. VANDENBROUCKE

Acknowledgements—We thank C. Stein, D. Klem and G. Badewitz for assistance during preparation of the cuticles and G. Guggenberger, University of Bayreuth, Germany, for analysis of the CuO oxidation products. This work was supported by a NATO collaborative research grant (0799/87). We also appreciate helpful discussions with P. van Bergen and the technical assistance of M. Baas (NIOZ). Helpful reviews by Dr M. Wilson and Dr C. Largeau are gratefully acknowledged.

This is Delft Organic Geochemistry Contribution 126.

REFERENCES

Guggenberger G., Zech W. and Schulten H.-R. (1994) Formation and mobilization pathways of dissolved organic carbon: evidence from chemical structural studies of organic carbon fractions in acid forest floor solutions. *Org. Geochem.* **21,** 51–66.

Hatcher P. G. (1987) Chemical structural studies of natural lignin by dipolar dephasing solid-state ^{13}C nuclear magnetic resonance. *Org. Geochem.* **11,** 31–39.

Hedges J. I. (1992) Global biogeochemical cycles: progress and problems. *Mar. Chem.* **39,** 67–93.

Hedges J. I. and Ertel J. R. (1982) Characterization of lignin by gas capillary chromatography of cupric oxide oxidation products. *Anal. Chem.* **54,** 174–178.

Higuchi T. (1990) Lignin biochemistry: biosynthesis and biodegradation. *Wood Sci. Technol.* **24,** 23–63.

Holloway P. J. (1984) Cutins and suberins, the polymeric plant lipids. In *CRC Handbook of Chromatography, Lipids*, (Edited by Mangold H. J.) Vol. 1, pp. 321–345. CRC Press, Boca Raton, FL.

Johansson M.-B., Kögel I. and Zech W. (1986) Changes in the lignin fraction of spruce and pine needle litter during decomposition studied by various chemical methods. *Soil. Biol. Biochem.* **18,** 611–619.

Kögel I. (1986) Estimation and decompositon pattern of the lignin component in forest soils. *Soil. Biol. Biochem.* **18,** 589–594.

Kögel I. and Bochter R. (1985) Characterization of lignin in forest humus layers by high-performance liquid chromatography of cupric oxide oxidation products. *Soil Biol. Biochem.* **17,** 637–640.

Kögel I., Hempfling R., Zech W., Hatcher P. G. and Schulten H.-R. (1988) Chemical composition of the organic matter in forest soils. I. Forest litter. *Soil Sci.* **146,** 124–136.

Kögel-Knabner I., Ziegler F., Riederer M. and Zech W. (1989) Distribution and decomposition pattern of cutin and suberin in forest soils. *Z. Pflanzenernaehr. Bodenk.* **152,** 409–413.

Kögel-Knabner I., Hatcher P. G. and Zech W. (1990) Decomposition and humification processes in forest soils: implications from structural characterization of forest soil organic matter. *Trans. 14th Int. Congress Soil Sci.*, Kyoto. Vol. V, pp. 218–223.

Kögel-Knabner I., Hatcher P. G., Tegelaar E. W. and de Leeuw J. W. (1992a) Aliphatic components of forest soil organic matter as determined by solid-state ^{13}C-NMR and analytical pyrolysis. *Sci. Total Environ.* **113,** 89–106.

Kögel-Knabner I., de Leeuw J. W. and Hatcher P. G. (1992b) Nature and distribution of alkyl carbon in forest soil profiles: implications for the origin and humification of aliphatic biopolymers. *Sci. Total Environ.* **117/118,** 175–185.

Leary G. J., Newman R. H. and Morgan K. R. (1986) A carbon-13 nuclear magnetic resonance study of chemical processes involved in the isolation of Klason lignin. *Holzforschung* **40,** 267–272.

Lendzian K. J., Nakajima A. and Ziegler H. (1986) Isolation of cuticular membranes from various conifer needles. *Trees* **1,** 47–53.

Lindberg J. J. and Hortling B. (1985) Cross polarization—magic angle spinning NMR studies of carbohydrates and aromatic polymers. *Adv. Polymer Sci.* **66,** 1–22.

Maciel G. E., O'Donnell D. J. O., Ackerman J. J. H., Hawkins B. H. and Bartuska V. J. (1981) A ^{13}C-NMR study of four lignins in the solid and solutions states. *Makromol. Chem.* **182,** 2297–2304.

Nip M., Tegelaar E. W., de Leeuw J. W., Schenck P. A. and Holloway P. J. (1986) A new non-saponifiable highly aliphatic and resistant biopolymer in plant cuticles. Evidence from pyrolysis and ^{13}C-NMR analysis of present-day and fossil plants. *Naturwissenschaften* **73,** 579–585.

Ralph J. and Hatfield R. D. (1991) Pyrolysis–GC–MS characterization of forage materials. *J. Agric. Food Chem.* **39,** 1426–1437.

Riederer M., Matzke K., Ziegler F. and Kögel-Knabner I. (1993): Inventories and decomposition of the lipid plant biopolymers cutin and suberin in temperate forest soils. *Org. Geochem.* **20,** 1063–1076.

Saiz-Jimenez C. and de Leeuw J. W. (1984) Pyrolysis–gas chromatography–mass spectrometry of isolated, synthetic and degraded lignins. *Org. Geochem.* **6,** 417–422.

Tegelaar E. W., de Leeuw J. W. and Saiz-Jimenez C. (1989a) Possible origin of aliphatic moieties in humic substances. *Sci. Total Environ.* **81/82,** 1–17.

Tegelaar E. W., de Leeuw J. W., Largeau S., Derenne S., Schulten H.-R., Müller R., Boon J. J., Nip M. and Sprenkels J. C. M. (1989b) Scope and limitation of several pyrolysis methods in the structural elucidation of a macromolecular plant constituent in the leaf cuticle of Agave americana L. *J. Anal. Appl. Pyrol.* **15,** 29–54.

Tegelaar E. W., Derenne S., Largeau C. and de Leeuw J. W. (1989c) A reappraisal of kerogen formation. *Geochim. Cosmochim. Acta* **53,** 3103–3107.

Tegelaar E. W., Kerp H., Visscher H., Schenck P. A., and de Leeuw J. W. (1991) Bias of the palaeobotanical record as a consequence of the variations in the chemical composition of higher vascular plant cuticles. *Palaeobiology* **17,** 133–144.

Tegelaar E. W., Wattendorf J. and de Leeuw J. W. (1993) Possible effects of chemical heterogeneity in higher plant cuticles on the preservation of its ultrastructure upon fossilization. *Rev. Paleobot. Palynol.* **77,** 149–170.

Wilson M. A., Heng S., Goh K. M., Pugmire R. J. and Grant D. M. (1983) Studies of litter and acid insoluble soil organic matter fractions using ^{13}C-cross polarization nuclear magnetic resonance spectroscopy with magic angle spinning. *J. Soil Sci.* **34,** 83–97.

Wilson M. A. (1987) *NMR Techniques and Applications in Geochemistry and Soil Chemistry.* Pergamon Press, Oxford.