

# Pyrolysis–GC–MS Analysis of the Formation and Degradation Stages of Charred Residues from Lignocellulosic Biomass

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The structural transformations undergone by lignocellulosic biomass (freeze-dried rye grass, *Lolium rigidum*) subjected to progressive isothermal heating (burning at 350 °C under oxidizing conditions for 30, 45, 60, 75, and 90 s) have been monitored by Curie-point pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS). The pyrograms suggest that even charred residues after severe heating (carbon loss ca. 50%) still contain substantial concentrations of some resistant plant structural components. Several trends were observed when monitoring the relative concentrations of the different groups of pyrolysis compounds released during successive charring stages: (i) the tetrapyrrole moiety of chlorophylls is rapidly destroyed as indicated by the decreasing yields of pyrroles and pyrrolines, whereas the phytol backbone is comparatively more resistant, leading to phytadienes after dehydration and reduction; (ii) the increasing yields of imidazoles from progressively heated samples (maximum at 45 s stage) suggest accumulation of newly formed nitrogen-containing compounds that may survive natural fires; (iii) the lignin backbone shows a relative resistance, the yields of aromatic products pointing to progressive demethoxylation; and, (iv) a selective accumulation of recalcitrant alkyl material occurred, which is interpreted as the result of thermal condensation of hydrocarbons and fatty acids into macromolecular materials in the charred residue. In terms of the intensity of the isothermal heating, the yields of the different classes of alkyl compounds follow the order phytadienes < fatty acids < alkanes < wax esters < sterols.

**Keywords:** Charred residues; forest fires; heated biomass; humic substances; *Lolium rigidum*; melanoidins; browning reactions

## INTRODUCTION

Research at a molecular level of the origin and transformations of sedimentary organic matter under natural conditions is a complicated task which frequently takes advantage of laboratory experiments that simulate the formation pathways of humic substances or kerogens. The well-known formation of humic-like melanoidins by thermal treatment of sugar- and amino acid-containing mixtures (Maillard reaction) is frequently taken as a model process for the formation of recalcitrant macromolecules through abiotic reactions, and may occur in lignin-lacking sedimentary media affected by external or geothermal heat sources (1, 2). Nevertheless, most literature on the effect of heating on plant biomass can be found in the field of food chemistry, in which browning reactions occurring even during cooking of nitrogen-lacking aliphatic substrates leads to newly formed aromatic and/or unsaturated macromolecules structurally more complex than the original material subjected to heating (3–5).

The quantitative importance of the abiotic transformation of soil aliphatic macromolecules into aromatic structures has also been considered in present-day environmental conditions. It has been demonstrated that forest fires or controlled burning of crop residues

induce a series of inter- and intramolecular reactions leading to condensed heterogeneous materials with close resemblance to natural humic substances (6–8). Likewise, laboratory experiments have proved that even lipids in young sediments have the ability to polymerize into kerogen-like matter under thermal conditions (9).

Because these newly formed structures are highly resistant to biological degradation, charring processes are often considered to contribute to the long-term carbon sequestration on the Earth's surface through high-yield accumulation of black carbon in soil (10, 11). In particular, charred plant material has traditionally been studied as regards the origin of stable humus fractions (12–14) and it has been recently postulated that its presence in humic matter is associated with the release of benzenepolycarboxylic acids after laboratory degradations (10, 15).

The study of humus fractions affected by fires has provided valuable information on the contribution of abiotic reactions to the formation of humic-like material. Nevertheless, even in closed systems, there is a dynamic balance between degradation and generation of organic substances which depends on both temperature and heating time. Such processes can be simulated in the laboratory by heating lignocellulosic materials under conditions comparable to those recorded for different types of forest burning (16–19).

As a continuation of a previous work in which the balance between the different carbon forms in plant biomass was studied during progressive heating by NMR spectroscopy (20), in this report we apply analytical pyrolysis to provide information on the signature of

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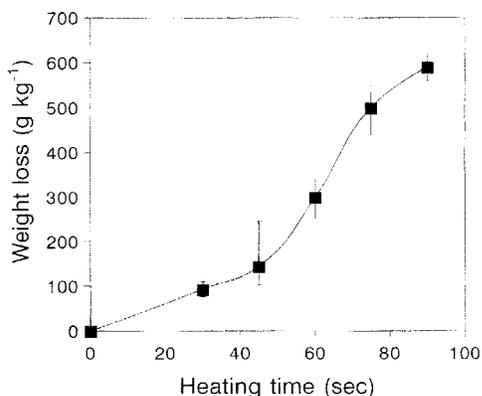
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**Table 1. Elementary Composition (g kg<sup>-1</sup>) and Calculated C and N Losses (g kg<sup>-1</sup>) of *L. rigidum* Subjected to Progressive Heating at 350 °C as a Function of Oxidation Time**

oxidation time (s)	atomic ratios							
	C	H	N	H/C	O/C	C-loss <sup>a</sup>	N-loss <sup>a</sup>	C/N
0	341	49	55	1.72	1.21	0.0	0.0	6.2
30	348	48	56	1.65	1.17	76	76	6.2
45	352	48	59	1.63	1.15	116	120	6.0
60	310	39	53	1.51	1.44	340	304	5.8
75	277	21	40	0.91	1.78	469	655	6.9
90	228	18	33	0.94	2.36	722	761	6.9

<sup>a</sup> N-loss (as g kg<sup>-1</sup> initial N) was calculated as = 1000 × (1000 × (N × (1000 - weight loss)/1000)/initial N. Similar calculation was performed in the case of C-loss.

**Figure 1.** Average weight loss values of freeze-dried rye-grass (*Lolium rigidum*) samples heated at 350 °C under air atmosphere for periods of progressive duration. For each point, error bars indicate extreme values between replicated experiments.

molecular assemblages useful to assess the effect of heating on lignocellulosic substrates.

#### EXPERIMENTAL PROCEDURES

**Sample Preparation.** Rye-grass (*Lolium rigidum*) was grown on washed sand supplemented with Hoagland's solution (21). The leaves were periodically harvested by cutting the plants to a height of 2 cm and then freeze-dried.

The standardized experimental conditions for the laboratory heating have been described elsewhere (2). Previous experiments have shown that isothermal heating is more reproducible and easier to control than alternative heating for a constant time at different temperatures (6). Consequently, samples of 300 mg of *L. rigidum* were transformed at 350 °C for variable periods of between 15 and 90 s. Triplicate samples in porcelain boats were heated in a ceramic tubular electric furnace (Wosthoff o.H.G, Bochum, Germany) operating at atmospheric pressure and a 625 mL min<sup>-1</sup> suction flow. A membrane pump connected to a soda-lime column was used to provide the oxidation tube with CO<sub>2</sub>-free air. After progressive oxidation times (30, 45, 60, 75, and 90 s), the boats were immediately removed and introduced into a desiccator with P<sub>2</sub>O<sub>5</sub>.

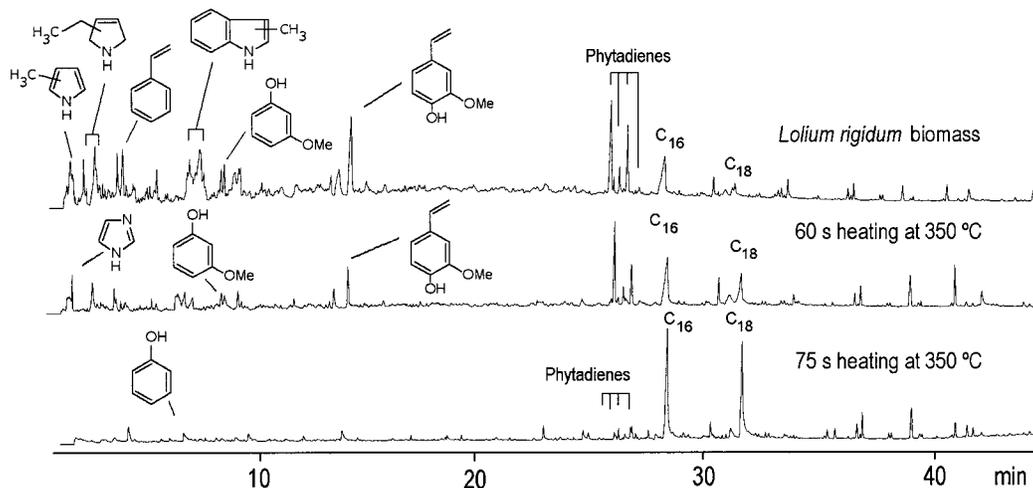
**Py-GC-MS.** Pyrolysis experiments were carried out using a Curie-point pyrolyzer (Horizon Instruments) attached to a Varian Saturn 2000 GC-MS system. The samples were heated on ferromagnetic wires at 510 °C of Curie-point temperature. The interface temperature of the pyrolysis unit was set at 250 °C and the pyrolysis time was 5 s. The injector, equipped with a liquid CO<sub>2</sub> cryogenic unit, was programmed from -30 °C (1 min) to 300 °C at 20 °C min<sup>-1</sup>. In both cases the GC oven was programmed from 50 °C to 100 °C at 32 °C min<sup>-1</sup> and then up to 320 °C at a rate of 6 °C min<sup>-1</sup>. For the chromatographic separation a fused-silica capillary column (25 m × 0.32 mm) coated with CPSil (film thickness 0.4 μm) and He as carrier

**Table 2. Pyrolysis Products<sup>a</sup> from *L. rigidum* Samples Subjected to Different Heating Periods at 350 °C**

compound	heating time (seconds)					
	0	30	45	60	75	90
methylpyrrole I	3.5	0.0	0.0	0.0	0.0	0.0
imidazole	1.8	1.3	4.5	2.3	0.0	0.0
C <sub>2</sub> -alkylpyrroline I	1.0	0.0	0.0	0.0	0.0	0.0
C <sub>2</sub> -alkylpyrroline II	5.6	0.0	0.0	0.0	0.0	0.0
methylpyrrole II	2.0	0.0	0.0	0.0	0.0	0.0
methylpyrrole III	0.6	0.0	0.0	0.0	0.0	0.0
dimethylbenzene	0.4	0.0	0.0	0.0	0.0	0.0
dimethylpyrrole	0.3	0.0	0.0	0.0	0.0	0.0
methylimidazole	0.0	6.4	6.2	0.0	0.0	0.0
methylfuran	0.0	0.4	0.6	0.8	0.0	0.0
furanmethanol	3.0	3.0	4.0	2.2	0.0	0.0
styrene	1.8	1.7	1.4	1.7	3.4	2.1
dimethylcyclohexanol	0.0	1.7	1.1	1.1	0.0	0.0
dimethylfuran	0.0	0.4	0.4	0.3	0.0	0.0
phenol	0.0	4.2	1.1	2.3	1.7	0.8
dimethyldihydropyranone I	0.0	0.6	0.0	0.0	0.0	0.0
C <sub>1</sub> -phenol I	0.0	0.5	0.5	6.5	0.8	0.3
methoxyphenol	1.1	2.9	3.3	2.2	1.1	0.0
C <sub>1</sub> -phenol II	0.0	3.2	4.4	1.5	0.0	0.5
indole	0.0	3.5	0.0	0.0	0.0	0.0
C <sub>1</sub> -phenol III	0.0	0.0	0.0	0.0	0.0	0.4
methylfurfuraldehyde	0.5	0.0	0.0	0.0	0.0	0.0
dimethylcyclohexanone	1.6	0.0	0.0	0.0	0.0	0.0
dimethyldihydropyranone II	0.0	0.8	0.0	0.0	0.0	0.0
C <sub>2</sub> -phenol I	0.0	0.2	0.9	0.9	0.7	0.0
C <sub>2</sub> -phenol II	0.0	1.3	0.6	0.4	0.0	0.2
dimethoxybenzene	0.0	0.5	0.5	0.0	0.0	0.0
butenylbenzene	0.0	1.0	1.1	0.0	0.9	0.0
C <sub>3</sub> -phenol I	0.0	0.2	0.5	0.7	0.0	0.0
C <sub>3</sub> -phenol II	0.0	0.4	0.0	0.2	0.0	0.0
C <sub>3</sub> -phenol III	0.0	0.4	0.0	0.0	0.0	0.0
dimethoxymethylbenzene I	0.4	1.6	1.1	0.7	0.0	0.0
benzofuran	0.0	4.0	3.9	3.1	2.2	1.9
methylindole I	3.9	0.8	1.1	0.0	0.0	0.0
methylindole II	3.0	0.0	0.0	0.0	0.0	0.0
C <sub>3</sub> -alkylindole	1.7	0.0	0.0	0.0	0.0	0.0
C <sub>4</sub> -alkylindole	0.6	0.0	0.0	0.0	0.0	0.0
methoxymethylphenol	1.1	0.0	0.0	0.0	0.0	0.0
ethenylmethylbenzene	1.6	0.0	0.0	0.0	0.0	0.0
2-methoxy-4-vinylphenol	9.3	7.6	7.8	5.0	0.5	0.2
C <sub>4</sub> -phenol I	0.0	0.0	0.0	0.0	0.0	0.2
tetradecanoic acid	0.0	0.0	0.0	0.0	1.7	0.5
propyl tetradecanoate	0.0	0.0	0.0	0.0	0.0	0.3
dimethoxyphenol	1.0	0.0	0.0	0.0	0.0	0.0
methoxypropenylphenol	0.0	1.1	1.0	0.0	0.0	0.0
phytadiene I	7.9	7.4	9.4	10.6	1.1	1.2
phytadiene II	0.9	1.0	1.5	1.9	0.6	0.5
phytadiene III	3.5	3.3	4.1	4.6	0.0	0.0
hexadecanoic acid	6.5	11.9	9.2	14.6	29.6	10.1
propyl hexadecanoate	0.2	0.4	0.3	0.3	0.3	3.3
phytadiene IV	1.1	1.1	1.2	3.7	0.0	0.0
heptadecane	0.0	0.0	0.0	0.0	0.0	0.5
octadecanoic acid	0.2	3.9	3.1	7.8	23.0	1.8
nonadecane	0.1	0.0	0.0	0.0	0.0	0.6
eicosane	0.0	0.0	0.0	0.0	0.0	0.6
propyl octadecanoate	0.1	0.0	0.0	0.0	0.0	0.4
heneicosane	0.0	0.0	0.0	0.0	0.6	0.9
octyl hexadecanoate	0.6	0.7	0.8	1.2	2.0	8.1
docosane	0.0	0.0	0.0	0.0	0.0	0.8
tricosane	1.0	1.5	0.4	4.4	5.1	24.3
octyl octadecanoate	0.1	0.2	0.2	0.4	0.5	1.1
squalene	0.9	1.5	0.1	4.4	0.0	0.0
cholestadiene	0.0	0.3	0.3	0.4	1.3	2.8
pentacosane	1.0	0.0	0.0	0.0	0.0	0.0
heptacosane	1.9	0.0	0.0	0.0	0.0	0.0
stigmasta-3,5-dien-7-one	0.0	0.0	0.0	0.4	0.4	0.0
total unidentified	28.1	17.1	23.3	13.5	22.4	35.5

<sup>a</sup> Percentages of the total chromatographic area. Roman numbers indicate different isomers.

gas were used. Tentative compound identification was based on literature data and comparisons with mass spectral libraries (NIST, Wiley).

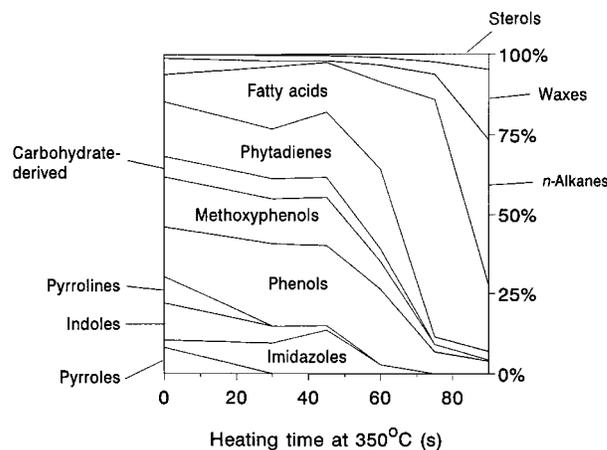


**Figure 2.** Chromatographic separation of pyrolytic compounds from progressively heated rye-grass biomass. Structures of the major cyclic compounds are indicated on the peaks. Fatty acids are indicated by C-number. Major peaks after 35 min correspond to waxes (Table 2).

## RESULTS AND DISCUSSION

The elemental composition, and carbon and nitrogen losses, of the progressively heated plant material are shown in Table 1, whereas the loss of weight values obtained in replicated heating experiments are shown in Figure 1. The values suggest that large structural transformation is expected after a heating time over 45 s. When subjected to mild thermal treatments (up to approximately 20% weight loss at 350 °C) the major changes were the removal of the constitutional water and some oxygen-containing groups in the outermost surfaces. The classical observation that the aliphatic material is comparatively less resistant than the aromatic material (22) is supported by the clear changes in the H/C atomic ratios. The values for the biomass heated above 75 s are similar to those for humic substances, but the latter incorporates much more oxygen-containing products.

Table 2 shows the chemical identity of pyrolysis products from progressively heated rye-grass biomass. The comparison of the pyrograms (Figure 2) shows intense transformations during the progressive burning. First, the molecular assemblages released from the samples heated to 60 s resemble those from the original biomass, although the peaks arising from carbohydrates and proteins progressively decrease with heating time. This means that the carbon backbone of the main plant biomacromolecules remains relatively stable in this torrefied biomass. Second, this pattern changes drastically after heating for 75 s; in this case, the pyrograms are dominated by aromatic compounds, free fatty acids, and small but significant amounts of esters and sterols. To all appearances, plant macromolecules have undergone progressive rearrangements resulting in a relative concentration of aromatic pyrolysis products with a progressively decreasing methoxyphenol/phenol ratio. This can be interpreted as an effect of the accumulation of newly formed aromatic structures, demonstrated by  $^{13}\text{C}$  NMR analyses of the same lignocellulosic material (20). The above differences are more clearly illustrated in Figure 3, which shows the relative yields of the main individual pyrolysis compounds released during the progressive burning calculated as percentages of the total volatile products. They can be grouped into three main classes of pyrolysis compounds: N-containing, aromatic, and aliphatic.



**Figure 3.** Relative yields (percentages relative to total chromatographic areas) of the major groups of pyrolysis compounds from rye-grass samples subjected to isothermal heating for periods of progressive duration.

Four phytadiene isomers are among the most abundant alkyl pyrolysis compounds. Phytadienes are intermediate products in the degradation of chlorophylls (23), and they lead to phytene and pristene, which are typical pyrolysis products of kerogen (9, 24). The alkyl products show characteristic yields in terms of heating time; the major changes were observed in the phytadienes and fatty acids, whereas the yields of paraffinic structures, waxes, and sterols were comparatively stable in terms of the charring degree. The pyrolytic data illustrate that progressive heating cannot be monitored through the presence of newly formed aromatic structures in the volatile phase.

Concerning the evolution of the different families of nitrogen-containing pyrolysis compounds, it is plausible that in the final stages, the amide nitrogen is incorporated into heterocyclic structures such as pyrroles, imidazoles, and indoles. Some evidence for pyridines and phenazines was also found. This is in agreement with previous observation (20) that the  $^{15}\text{N}$  NMR spectra of heated lignocellulosics contain large concentrations of heterocyclic nitrogen compounds; although, in the biogenic organic matter, amide nitrogen-forms dominate in both the readily hydrolyzable as well as the recalcitrant fractions (21, 25, 26).

In conclusion, the results suggest that two stages can be defined in terms of the composition of the molecular assemblages, which coincides with the proposal of Wang and Low (27) based on pyrolysis of lignins. In the early heating stages, with carbon loss up to 12%, the results are in accord with the pyrolytic behavior of chars as seen by  $^{13}\text{C}$  NMR data, their aromaticity increasing in terms of heating (28). The decrease, in advanced stages, of the yields of aromatic pyrolysis products defined the second transformation stage. The simultaneous increase in the yields of paraffinic compounds can be explained considering that a typical feature of charred materials is the thermal conversion of alkanes into nonvolatile products (29). From the viewpoint of the fire-related mechanisms involved in the accumulation of stable carbon and nitrogen forms in soils, the early stage is mainly destructive and leads to concentration of aromatic structures as well as of recalcitrant, newly formed nitrogen compounds, whereas the advanced stage probably involves free-radical condensation reactions of heat-resistant materials with paraffinic structures tightly bound to a recalcitrant charred residue.

## LITERATURE CITED

- (1) Dennis, L. W.; Maciel, G. E.; Hatcher, P. H.; Simoneit, B. R. T.  $^{13}\text{C}$  Nuclear magnetic resonance studies of kerogen from Cretaceous black shales thermally altered by basaltic intrusions and laboratory simulations. *Geochim. Cosmochim. Acta* **1982**, *46*, 901–907.
- (2) Almendros, G.; González-Vila, F. J.; Martín, F. Fire-induced transformation of soil organic matter from an oak forest. An experimental approach to the effects of fire on humic substances. *Soil Sci.* **1990**, *149*, 158–168.
- (3) Feather, M. S.; Harris, J. F. Dehydration reactions of carbohydrates. *Adv. Carbohydr. Chem. Biochem.* **1973**, *28*, 161–224.
- (4) Benzing-Purdie, L. M.; Ripmeester J. A.; Ratcliffe C. I. Effects of temperature on Maillard reaction products. *J. Agric. Food Chem.* **1985**, *33*, 31–33.
- (5) Huang, R. D.; Feather, M. S.  $^{13}\text{C}$  NMR study of some Maillard reaction products arising from D-glucose–DL-alanine interactions. *J. Agric. Food Chem.* **1988**, *36*, 673–676.
- (6) Almendros, G.; González-Vila, F. J.; Martín, F.; Fründ, R.; Lüdemann, H.-D. Solid-state NMR studies of fire-induced changes in the structure of humic substances. *Sci. Total Environ.* **1992**, *117/118*, 63–74.
- (7) Golchin, A.; Baldock, J. A.; Clarke, P.; Higashi, T.; Oades, J. M. The effects of vegetation and burning on the chemical composition of soil organic matter of a volcanic ash soil as shown by  $^{13}\text{C}$  NMR spectroscopy. II. Density fractions. *Geoderma* **1997**, *76*, 175–192.
- (8) Yanagita, T.; Jiang, Y.; Matsumoto, S. Carbohydrate and microbial decomposition of the rice-hull charred to different degrees. *Nippon Dojo Hiriyogaku Zasshi* (Japanese) **1997**, *68*, 435–437.
- (9) Shyoya, M.; Ishiwatari, R. Laboratory thermal conversion of sedimentary lipids to kerogen-like matter. *Org. Geochem.* **1983**, *5*, 7–12.
- (10) Haumaier, L.; Zech, W. Black carbon – possible source of highly aromatic components of soil humic acids. *Org. Geochem.* **1995**, *23*, 191–196.
- (11) Kuhlbusch, T. A. J.; Andreae, M. O.; Cachier, H.; Goldammer, J. G.; Lacaux, J.-P.; Shea, R.; Crutzen, P. J. Black carbon formation by savanna fires: measurements and implications for the global carbon cycle. *J. Geophys. Res. [Atmos.]* **1996**, *101*, 23651–23665.
- (12) Shindo, H.; Matsui, Y.; Higashi, T. A possible source of humic acids in volcanic ash soils in Japan – Charred residue of *Miscanthus sinensis*. *Soil Sci.* **1986**, *141*, 84–87.
- (13) Shindo, H.; Matsui, Y.; Higashi, T. Humus composition of charred plant residues. *Soil Sci. Plant Nutr.* **1986**, *32*, 475–478.
- (14) Shindo, H. Elementary composition. Humus composition and decomposition in soil of charred grassland plants. *Soil Sci. Plant Nutr.* **1991**, *37*, 651–657.
- (15) Glaser, B.; Haumaier, L.; Guggenberger, G.; Zech, W. Black carbon in soils: the use of benzenecarboxylic acids as specific markers. *Org. Geochem.* **1998**, *29*, 811–819.
- (16) De Bano, L. F.; Mann, L. D.; Hamilton, D. A. Translocation of hydrophobic substances by burning organic litter. *Soil Sci. Soc. Am. Proc.* **1970**, *34*, 130–133.
- (17) Savage, S. M. Mechanism of fire-induced water repellency in soil. *Soil Sci. Soc. Am. Proc.* **1974**, *38*, 652–657.
- (18) Savage, S. M.; Osborn, J.; Letey, J.; Heaton, C. Substances contributing to fire-induced water repellency in soil. *Soil Sci. Soc. Am. Proc.* **1972**, *36*, 674–678.
- (19) Scholl, D. G. Soil wettability and fire in Arizona chaparral. *Soil Sci. Soc. Am. Proc.* **1975**, *39*, 356–361.
- (20) Knicker, H.; Almendros, G.; González-Vila, F. J.; Martín, F.; Lüdemann, H.-D.  $^{13}\text{C}$  and  $^{15}\text{N}$  NMR spectroscopic examination of the transformation of organic nitrogen in plant biomass during thermal treatment. *Soil Biol. Biochem.* **1996**, *28*, 1053–1060.
- (21) Almendros, G.; Fründ, R.; González-Vila, F. J.; Haider, K.; Knicker, H.; Lüdemann, H.-D. Analysis of  $^{13}\text{C}$  and  $^{15}\text{N}$  CPMAS NMR-spectra of soil organic matter and compost. *FEBS Lett.* **1991**, *282*, 119–121.
- (22) Van Krevelen, D. W. Graphical-statistical method for the study of structure and reaction processes of coal. *Fuel* **1950**, *29*, 269–284.
- (23) Grossi, V.; Baas, M.; Schogt, N.; Breteler, W. C. M.; de Leeuw, J. W.; Rontani, J. F. Formation of phytadienes in the water column: myth or reality? *Org. Geochem.* **1996**, *24*, 833–839.
- (24) Ishiwatari, M.; Ishiwatari, R.; Sakashita, H.; Tatsumi, T.; Tominaga, H. The effects of preheating treatment on the pyrolysis of chlorophyll a. Simulation of diagenetic processes in kerogen formation. *Chem. Lett.* **1990**, *6*, 875–878.
- (25) Knicker, H.; Fründ, R.; Lüdemann, H.-D. The chemical nature of nitrogen in native soil organic matter. *Naturwissenschaften* **1993**, *80*, 219–221.
- (26) Knicker, H.; Lüdemann, H.-D. N-15 and C-13 CPMAS and solution NMR studies of N-15 enriched plant material during 600 days of microbial degradation. *Org. Geochem.* **1995**, *23*, 329–341.
- (27) Wang, N.; Low, M. J. D. The pyrolysis of a lignin. (Report CA Section: 43 Cellulose, Lignin, Paper, Other Wood Products). *Energy Res. Abstr.* **1990**, *15*, 13.
- (28) Solum, M. S.; Pugmire, R. J.; Grant, D. M.; Fletcher, T. H.; Solomon, P. Solid-state carbon-13 NMR studies of coal char structure evolution. *Soc. Div. Fuel Chem.* **1989**, *34*, 1337–46.
- (29) Remmler, M.; Kopinke, F. D. Thermal conversion of hydrocarbons on solid matrixes. *Thermochim. Acta* **1995**, *263*, 113–121.

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