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Rapid Commun. Mass Spectrom. 2008; **22**: 1631–1639
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3454

Off-line pyrolysis and compound-specific stable carbon isotope analysis of lignin moieties: a new method for determining the fate of lignin residues in soil†

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Received 24 January 2008; Accepted 25 January 2008

Off-line pyrolysis was used to liberate lignin moieties from dung and soil and, after trimethylsilylation, the $\delta^{13}C$ values of these derivatives were determined by gas chromatography-combustion-isotope ratio mass spectrometry. Initial $\delta^{13}C$ values determined for 4-vinylphenol, syringol, 4-vinylguaiacol, 4-acetylsyringol, 4-vinylsyringol, 4-(2-*Z*-propenyl)syringol, 4-(2-*E*-propenyl)syringol and 4-(2-propenone)syringol pyrolysis products of the lignin polyphenol structure from C_4 ($\delta^{13}C_{bulk} = -12.6\%$) and C_3 ($\delta^{13}C_{bulk} = -30.1‰$) dung confirmed the robust and reproducible nature of the off-line preparation technique. C_4 dung was used as a treatment in a randomised field experiment to assess the short-term sequestration of dung carbon in managed grasslands. Since lignin was on average 3.5‰ depleted in ^{13}C compared with bulk dung $\delta^{13}C$ values, this may have resulted in an under-estimation of dung C incorporation based on bulk $\delta^{13}C$ values. Therefore, an investigation of the compound-specific $\delta^{13}C$ values of dung-derived lignin moieties extracted from soils sampled up to 372 days was undertaken. $\Delta^{13}C$ values between lignin moieties extracted from treated and untreated soils showed that dung-derived lignin was not especially resistant to degradation and suggested that individual moieties of the lignin macromolecule must: (i) move into soil, (ii) be degraded, or (iii) be transformed diagenetically at different rates. This adds to a gathering body of evidence that lignin is not particularly stable in soils, which has considerable significance for the perceived role of different biochemical components in the cycling of C in soils.

Lignin is a phenolic biopolymer comprising a substantial component of all terrestrial plants,[1] and is the most abundant aromatic polymer on the earth,[2] accounting for an estimated 30% organic carbon (C) in the biosphere.[3] Lignin is assigned to the 'slow pool' of soil organic matter (SOM)[4] with mean residence times of 15–100 years, and is traditionally perceived as the rate-limiting step in biological C cycling.[5] The resistance of the lignin macromolecule to strong acid or base hydrolysis, such as during forage fibre analysis,[6] also supports its status as a highly recalcitrant structure resistant to depolymerisation. Lignin survives digestion, such as rumination, relatively intact[7] and is regularly used as an inert marker to calculate cell-wall digestibility in ruminants.[8] Using forage fibre analyses, Dungait *et al*.[9] found that the proportion of lignin in dung increased by 5% compared with diet due to its resistance to digestion relative to other dung constituents. Bulk $\delta^{13}C$ values of C_4 dung-treated soil had been used previously to estimate the short-term sequestration of dung C in grassland soils.[9–11] Lignin is widely reported to be 2–9‰ depleted in ^{13}C compared with bulk OM values.[12–14] Therefore, if lignin is more resistant to decomposition than other components of OM, its relative increase in proportion to more labile fractions would cause depletion in bulk $\delta^{13}C$ values. Therefore, it was postulated that the depletion in bulk $\delta^{13}C$ values of C_4 dung-treated soil over time, in an experiment investigating the incorporation of C_4 dung into a C_3 grassland soil,[9] might not necessarily imply a loss of ^{13}C-labelled OM *per se* from the soil, but provide evidence of the preferential degradation of labile, relatively ^{13}C-enriched components, e.g. carbohydrates. Thus, it was essential that the contribution of ^{13}C-depleted lignin to soil bulk $\delta^{13}C$ values was determined to avoid under-estimation of dung C incorporation in the later stages of the experiment. This could only be achieved by liberating representative gas chromatography (GC)-amenable moieties from macromol-

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†Presented at the annual meeting of the Stable Isotopes Mass Spectrometry Users' Group (SIMSUG), 20–22 June, 2007, hosted by the Institute for Research on the Environment and Sustainability (IRES) and the School of Civil Engineering and Geosciences, Newcastle University, UK.

ecular lignin structures for compound-specific carbon isotope analysis.

Lignin is an aromatic polymer of 4-hydroxyphenylpropanoids[15] with molecular weight <50 000 Da.[16] Unlike most other biological polymers, it has no defined structure, no regularly repeating sequences of any length, and an ill-defined size.[17] The C_6-C_3 chemical structure of the phenylpropanoid subunit is exceptional and essentially absent from all other living organisms.[18] The propyl side chain and methoxylated aromatic rings enable the three fundamental phenylpropanoid subunits, i.e. *p*-hydroxyphenol, syringyl and guaiacyl moieties, to bond via ether and C–C linkages with up to 20 linkages between subunits[19] forming a three-dimensional structure with a range of bond strengths. Hence, this unique biochemistry affords a range of challenges in investigations requiring isolation of individual monomers diagnostic of the original lignin structure.

A range of chemolytic techniques has been applied to the analysis of lignin in order to facilitate its depolymerisation to yield diagnostic monomeric (or dimeric) moieties. CuO oxidation, after Hedges and Ertel,[20] is the most widely used method for obtaining lignin-derived phenols for GC analysis.[21–23] The reaction favours the cleavage of the most commonly occurring β-O-4 linkages, yielding acid and aldehyde forms of lignin moieties and retaining nearly 30 phenolic dimers with C–C linkages at ring-ring or side-chain-ring positions; acid/aldehyde ratios are useful indicators of the extent of diagenetic alterations of lignin.[24] However, CuO oxidation is less efficient at releasing syringyl than guaiacyl moieties of plant lignins[20] such that no derivatives with side chains longer than C_1 are produced.[25]

Another common method of obtaining lignin moieties is by pyrolysis. This involves the rapid heating of samples in an inert atmosphere to release volatile fragments by reproducible bond cleavage, and these are subsequently identified using gas chromatography-mass spectrometry (GC-MS). While pyrolysis of SOM yields moieties from carbohydrates and proteins, the majority derive from lignin or esterified phenolics. Flash pyrolysis-GC-MS (py-GC-MS) is widely used in SOM research[26] for the chemical characterisation of OM at the molecular level.[27–31] Recently, py-GC-MS has been interfaced with stable isotope ratio mass spectrometry (py-GC-IRMS) to determine $\delta^{13}C$ values for lignin derivatives.[32–33] However, py-GC-MS suffers limitations for the identification of compounds comprising polar functional groups[34] and it is estimated that <25% pyrolysis products can be detected using this method.[35] A methylating agent, such as tetramethylammonium hydroxide (TMAH), may be added to a sample prior to pyrolysis;[36] however, this changes the nature of the analysis from thermolysis to thermochemolysis and the amount and nature of products generated are affected. Kuroda[37] suggests that the inherent alkalinity of TMAH results in unfavourable cleavage, or even damage, of substructures to create nonspecific products, and that insufficient alkylation prior to pyrolysis produces high abundances of dehydration products. Alternatively, off-line pyrolysis with post-pyrolysis silylation allows analysis of a wider range of components that provide more information about the parent material as silylation does not alter the nature of the pyrolysate.[35] Faix *et al.*[38] highlighted several advantages of off-line pyrolysis over py-GC-MS including: (i) predetermination of optimal sample sizes for GC analysis; (ii) possible use of internal standards; (iii) repeated injections of the same sample to test reproducibility; and (iv) potential for fractionation of components according to their functional class.

Therefore, the objectives of this work were to: (i) assess the application of an off-line pyrolysis method with subsequent compound-specific isotope analysis (CSIA), comparable with a recently developed method for the analysis of fossil mummified wood,[39,40] to an investigation of dung lignin incorporation in soil; and (ii) determine the mechanisms and proportion of dung C incorporated as lignin into the surface soil horizons to accurately interpret bulk $\delta^{13}C$ values. In this paper, we present results of the analyses of specific moieties released during off-line pyrolysis from dung and soils at two key points in a time course of a large-scale field experiment.

EXPERIMENTAL

Field site, experimental design and sampling

A field experiment was established in April 2002 on a previously grazed C_3 ($\delta^{13}C = -30.3 \pm 1.3$‰) grassland at IGER-North Wyke, Devon, UK (50°45′N and 4°53′W). The site had been ploughed and reseeded 3 years prior to the start of the experiment. The soil is of the Halstow series of non-calcareous pelocols, with an impermeable brownish clay loam or silty clay A horizon (0–17 cm) and a mean pH of 6.6 (0–5 cm horizon). The area had a mean annual temperature of 10.5°C[41] and total annual precipitation of 1269 mm (April 2004 to March 2005).

Full details of natural abundance ^{13}C-labelled dung production and field application are given in Dungait *et al.*[9] Briefly, two cows were switched from a C_3 *Lolium perenne* silage diet to a C_4 *Zea mays* silage diet to produce C_3-labelled ($\delta^{13}C = -31.3 \pm 0.1$‰) and C_4-labelled (bulk $\delta^{13}C = -12.6 \pm 0.3$‰) dung. Dung pats (1.5 kg, fresh weight, $n = 8$) and controls (no dung, $n = 4$) were deposited on a grassland plot at IGER-North Wyke in a fully randomised 7×20 experimental design (with no blocking) on 22 April 2002. The dung residues, controls and soil beneath (0–5 cm) were destructively sampled after 7, 14, 28, 56, 112, 224 and 372 days. Two key dates were selected for the analysis of dung-derived lignin incorporation: 56 days, at maximum bulk dung C incorporation,[9] and 372 days, the end of the experiment.

Bulk stable isotope analysis

Soils and dung were ground using liquid nitrogen. An aliquot was measured into a tin capsule and weighed. Triplicate $\delta^{13}C$ values were determined using continuous-flow isotope ratio mass spectrometry (CF-IRMS) using a Europa 20-20 instrument (Crewe, UK: IGER-NW) after combustion to pure CO_2. Reference standards were run every ten samples. The 1σ values were ±0.1‰ for the determination of $\delta^{13}C$ values. Bulk $\delta^{13}C$ values were used to calculate % dung C incorporation using the method of Bol *et al.*[10]

DOI: 10.1002/rcm

Off-line pyrolysis

In order to obtain lignin derivatives suitable for GC analysis, a modified off-line pyrolysis method was applied to soil samples from the 0–1 cm horizon of C_4 dung-treated and untreated soils after 56 and 372 days. All materials were dried at 30°C to a constant weight then ground in a model 12 ball mill (with 1 L ceramic pots; Pascal Engineering Co. Ltd., Crawley, UK) to achieve a homogeneous fine powder (<0.2 mm) for chemical and stable isotope analyses. All samples were lipid-extracted prior to analysis according to the method of van Bergen *et al*.[42] The lipid-extracted residues were air-dried and stored dry in the dark prior to further analysis.

Open-system off-line pyrolysis was undertaken using a preheated tube furnace (12/38/250 with 8 segment programmer, type 2416CG; Carbolite, Hope, UK). Either 400 mg of dung or 4 g of experimental soil were placed in a Pyrex boat and introduced into a Pyrex tube located within the preheated furnace in an inert atmosphere provided by a continuous stream of nitrogen. The sample was heated for 1 h at 300°C (after preliminary tests to assess the effect of heating at 400°C). Pyrolysis products were flushed from the glass tube by the nitrogen. Products were collected as char on the inside of the Pyrex tube and in a glass U-tube cooled to ~ -40°C in CO_2 in acetone. At the end of the experiment the apparatus was left to cool under a flow of nitrogen to avoid oxidation. The pyrolysate was rinsed from the Pyrex tube and U-tube using dichloromethane/methanol (1:1 v/v) The liquid products collected were rotary evaporated at 40°C.

Derivatisation

The pyrolysate was dissolved in 1 mL ethyl acetate, and one-fifth aliquots of the extract derivatised at 70°C (1 h) using an excess of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1% TMCS) and pyridine. The solvent was evaporated from the extract at 40°C under nitrogen. Derivatised dung and soil pyrolysate were dissolved in ethyl acetate and analysed using GC, GC-MS and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

Instrumentation

Derivatised dung and soil pyrolysates were analysed using a Hewlett Packard 5890 Series II gas chromatograph (Stockport, UK) equipped with a fused-silica capillary column (100% dimethyl polysiloxane, Chrompack CPSil-5CB, 50 m × 0.32 mm i.d.; film thickness 0.12 μm) using hydrogen as the carrier gas (10 psi head pressure) with the following temperature programme: 5 min isothermal 40°C followed by a ramp to 300°C at 4°C min^{-1}, held for 10 min isothermal. Compounds were injected on-column and detected using a flame ionisation detector (320°C).

Compounds were identified using GC-MS analyses performed on a Trace mass spectrometer (ThermoFinnigan, Hemel Hempstead, UK), equipped with a fused-silica capillary column (100% dimethyl polysiloxane, Phenomenex ZB1, 60 m length × 0.32 mm i.d.; film thickness 0.1 μm). Samples were introduced using a programmable temperature vapourising (PTV) injector maintained at a temperature of 300°C. The source temperature was held at 200°C and ionisation energy was set at 70 eV with the quadrupole mass analyser scanning the range *m/z* 50–650 with a cycle time of 0.6 s^{-1}. The temperature program used was the same as in the GC conditions above. Helium was employed as carrier gas at a constant flow of 2 mL min^{-1}. Data were acquired and processed using the Excalibur data system version 1.2 (ThermoFinnigan). Compounds were identified based on comparison with published mass spectra and elution orders.[28,33,39,43]

GC-C-IRMS analyses were performed using a Varian 3400 gas chromatograph (Oxford, UK) coupled to a Delta XP isotope ratio mass spectrometer (Finnigan MAT, Hemel Hempstead, UK) (electron ionisation, 100 eV, 1 mA electron energy, three Faraday cup collectors – *m/z* 44, 45 and 46, CuO/Pt Finnigan MAT Mark I combustion interface maintained at 850°C) equipped with a fused-silica capillary column (Chrompack CP Sil-5CB, 50 m × 0.32 mm i.d.; film thickness 25 μm). A Nafion membrane was employed to prevent water from reaching the ion source. Sample injections were performed using a septum-equipped programmable injector (SPI). The GC conditions were the same as those described above. Samples were calibrated against reference CO_2 of known isotopic composition, which was introduced directly into the source three times at the beginning and end of every run. Each sample was run at least in duplicate to ensure reliable mean $\delta^{13}C$ values. The $\delta^{13}C$ values were expressed relative to VPDB:

$$\delta^{13}C(‰) = 1000[(R_{sample} \times R_{standard})/R_{standard}],$$

where R is the $^{13}C/^{12}C$ ratio.

The $\delta^{13}C$ values of the original, underivatised, compounds were obtained by correcting the $\delta^{13}C$ values of the trimethylsilylated compounds determined by GC-C-IRMS for the addition of the extra C during the derivatisation using the following mass balance equation:

$$n_{cd}\delta^{13}C_{cd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d \quad (1)$$

where $\delta^{13}C_c$ is the $\delta^{13}C$ value of the original compound, $\delta^{13}C_d$ the $\delta^{13}C$ value of the TMS group donated by the derivatizing agent (BSTFA), $\delta^{13}C_{cd}$ the $\delta^{13}C$ value of the derivatised compound and n_c, n_d, and n_{cd} the number of carbon atoms of the original compound, those from BSTFA, and the derivatised compound, respectively. The $\delta^{13}C$ value for the BSTFA (−37.4‰) was determined by derivatisation of a *meso*-inositol standard of known $\delta^{13}C$ value and back-calculating $\delta^{13}C_{cd}$ by rearrangement of Eqn. (1).

Relative abundance calculation

The difference (Δ) between the $\delta^{13}C$ values of the lignin moieties in the control soil and in C_4 dung-treated soil was used to estimate the incorporation and flux of dung-derived lignin in the treated soils according to the following expression:

$$p = \left(\frac{\Delta^{13}C_{ts-cs}}{\Delta^{13}C_{d-cs}}\right) \quad (2)$$

where p is the proportion of dung lignin moiety in C_4 dung-treated soil, *ts* the lignin moiety in C_4 dung-treated soil,

DOI: 10.1002/rcm

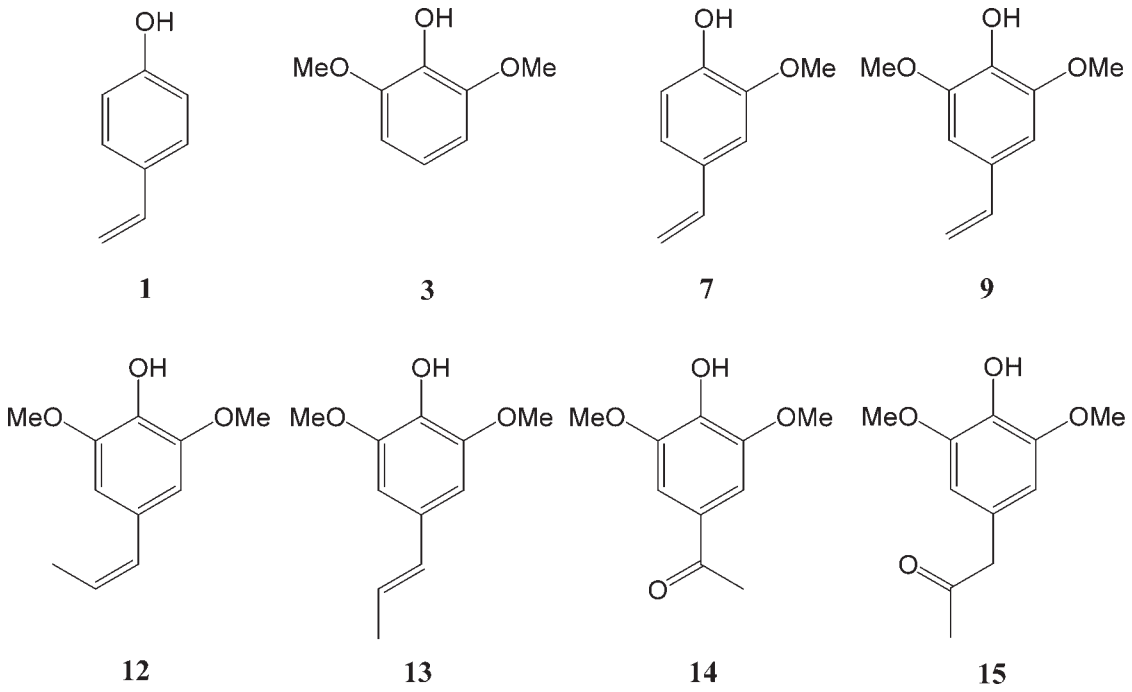
cs the lignin moiety in control soil and *d* the lignin moiety in C_4 dung. The calculation assumed that total replacement of native C_3 lignin moieties would give $\delta^{13}C$ values equivalent to the $\delta^{13}C$ values of the lignin moieties in the fresh C_4 dung.

RESULTS AND DISCUSSION

Optimisation of aspects of the protocol

Dung (400 mg) was pyrolysed three times to assess the reproducibility of the off-line pyrolysis technique described previously.[39,40] This revealed that, although the range of lignin pyrolysis products was similar, the relative abundances of individual pyrolysis products differed between replicates. The sample residue remaining from pyrolysis under normal conditions (300°C, 1 h) was repyrolysed and yielded an insignificant levoglucosan peak but no lignin moieties. Therefore, dung was pyrolysed at 300°C under a range of conditions with the aim of reducing differences in relative abundance between the pyrolysis products and to optimise the yield of pyrolysate. Initially, six masses of dung were pyrolysed (50, 100, 200, 400, 800 and 1000 mg); relative abundance calculations showed that the abundance of each pyrolysis product was highest in the 400 mg samples. The effects of sample compaction were also investigated: 400 mg of dung was compacted into a volume of 2.4 cm^3 and the mean ($n = 3$) yield of pyrolysate compared with that more loosely dispersed in the sample boat (volume 26.3 cm^3). The compacted sample yielded 300% more pyrolysate than the dung pyrolysed at normal volume and suggests that the production of less volatile higher molecular weight pyrolysis products was favoured by less efficient transfer of heat through the compacted sample. These higher molecular weight components would be more prone to condensation and, therefore, less liable to loss via the carrier gas outflow. Loss of volatile constituents by this route was raised as a concern by Poole and van Bergen.[39] In order to increase the recovery of pyrolysis products, the original Dreschel bottles, which contained the ice-cooled dichloromethane/methanol solvent to trap the pyrolysis products, were replaced with a glass U-tube (i.d. 4 mm, length 120 mm). The U-tube was cryogenically cooled by placing it in a flask containing CO_2 in acetone (~ -40°C). Yields were increased by 400% and this modification also reduced the volume of solvent used and avoided two solvent evaporation steps; the latter were found to affect reproducibility and yield.

Due to the biochemically complex nature of organic matter comprising dung and soil, their pyrolysis yielded a wide range of products (Fig. 1). Poole van Bergen[39] used off-line pyrolysis to study fossil wood, which is biochemically less complex than soil; however, similar pyrolysis products were identified as in the C_4 dung and C_4 dung-treated soil (Table 1). Although several of the pyrolysis products were not base-line resolved (e.g. **9**), and others co-eluted with both lignin and carbohydrate pyrolysis products (e.g. **2** and **13**), they were used to tentatively estimate $\delta^{13}C$ values.[40] Similar resolution problems are evident in previous studies that used both off-line pyrolysis with subsequent silylation[40] and on-line py-GC-IRMS (using a 5% phenyl polysiloxane column).[32,44] $\delta^{13}C$ values reported from such incompletely resolved components must be used with considerable caution. However, the prospect of developing methodology for post-pyrolysis fractionation, to remove interfering non-phenolic products prior to GC analysis, is a significant advantage of the off-line pyrolysis[38] approach, which holds considerable promise for accurate determinations of $\delta^{13}C$ values of lignin moieties.



DOI: 10.1002/rcm

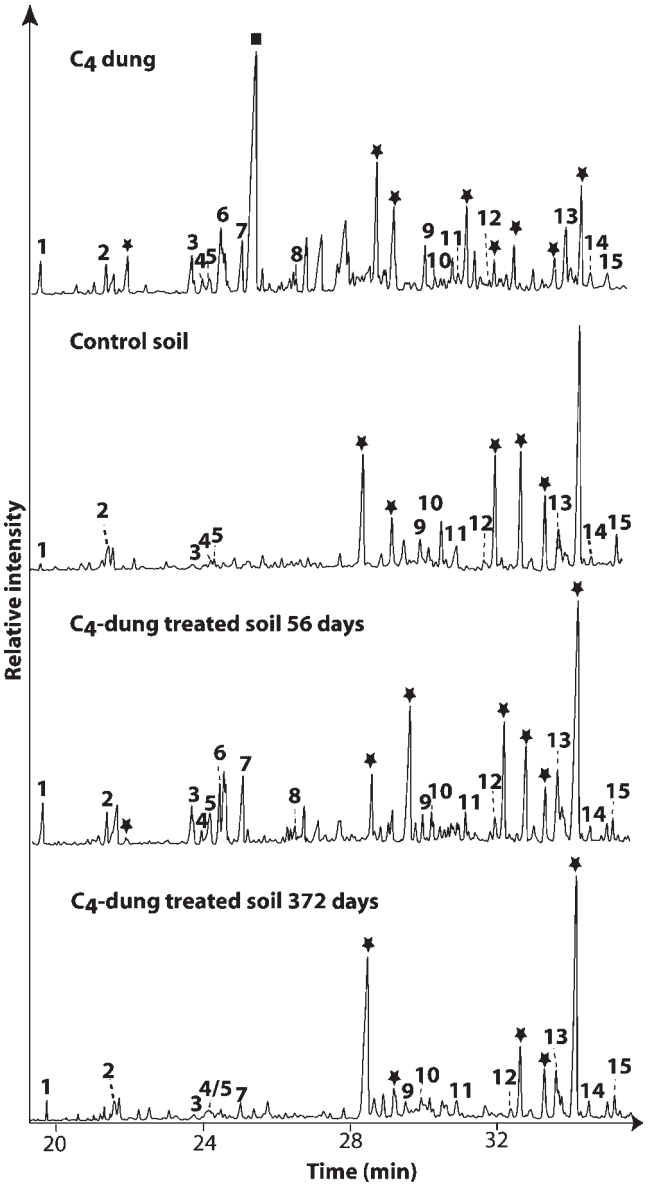


Figure 1. Partial TIC chromatograms of trimethylsilylated pyrolysate (300°C, 1 h) of lipid-extracted C_4 dung and 0–1 cm horizons of control soil and C_4 dung-treated soils after 56 and 372 days. Numbers refer to compounds detailed in Table 1; ■ = phytol; ★ = carbohydrate, pyrolysis products.

GC analyses

GC analysis of the pyrolysates of lipid-extracted dung and experimental soils yielded similar complex mixtures of lignin, organic acids and carbohydrates (Fig. 1). The various components were identified based on their mass spectra (Table 1). The C_4 dung-treated soil contained a noticeably increased abundance of lignin-derived moieties, although these were minor compared with the pyrolysis products derived from carbohydrates. Eight lignin moieties with known lignin provenance, derived from cleavage of either the β-O-4 or the α-O-4 linkages in the lignin macromolecule,[39] were observed in both dung and soil: 4-vinylphenol (**1**), syringol (**3**), 4-vinylguaiacol (**7**), 4-vinylsyringol (**9**), 4(2-*Z*-propenyl)syringol (**12**), 4(2-*E*-propenyl)syringol (**13**), 4-acetylsyringol (**14**) and 4(2-propenone)syringol (**15**). These compounds were, therefore, selected for further analysis. Catechol and methyl catechol pyrolysis products were not selected as they are known to be produced by pyrolysis of invertebrate cuticles.[45]

Stable carbon isotope analysis

The $\delta^{13}C$ values of trimethylsilylated lignin monomers were determined using GC-C-IRMS (Fig. 2). The $\delta^{13}C$ values for lignin moieties extracted from C_3 and C_4 dung varied from syringol (**3**; $C_3 = -22.4 \pm 1.6‰$; $C_4 = -8.6 \pm 0.5‰$) to 4-(2-propenone)syringol (**15**; $C_3 = -34.1 \pm 2.3\%$; $C_4 = -16.7 \pm 0.2‰$; Fig. 3). The standard deviation (1σ; $n = 5$) indicates the acceptable reproducibility of the isotope values. The majority of the lignin moieties in either dung type were up to 6.5‰ depleted in ^{13}C relative to bulk dung. However, syringol (**3**) was 4‰ and 9‰ ^{13}C-enriched compared with bulk $\delta^{13}C$ values in C_4 and C_3 dung, respectively, and 4-vinylguaiacol (**7**) was 2‰ ^{13}C-enriched compared with bulk C_4 dung. The depleted values are in agreement with earlier studies, which indicated that plant tissues yield lignin products 2–7‰ depleted in ^{13}C compared with whole tissue,[12–14] although Kracht and Gleixner[46] determined up to 5‰ ^{13}C-enrichment of lignin moieties compared with bulk values in lignin pyrolysates of peat. Differences in $\delta^{13}C$ values between compounds may be ascribed to their different biosynthetic origin,[47] and the significant differences in the pattern of ^{13}C-enrichment of 4-vinylguaiacol (**7**) in the C_3 and C_4 dungs may be due to metabolic differences between *Lolium perenne* and *Zea mays*, the plant species from which the dung was derived. For example, it is well documented that grasses incorporate tyrosine into lignin as well as phenylalanine,[48,49] so differences between the $\delta^{13}C$ values of the two amino acids may account for the observed differences in the $\delta^{13}C$ values of the lignin moieties. An 18‰ variation has been shown to exist in the intramolecular C isotope composition of ^{13}C-depleted methyl and ^{13}C-enriched carboxyl groups of acetic acid[50] and a 2‰ difference between 4-formylguaiacol and its component methoxy group has been determined.[51] These observations would seem to accord with the trends observed for C_4 dung, as the syringyl compounds with two methoxy groups were more depleted in ^{13}C than guaiacyl compounds with one methoxy group. Keppler *et al.*[52] found that methoxy groups from lignin have C isotope signatures exceptionally depleted in ^{13}C, e.g. maize lignin = −47.3‰. In addition, Poole *et al.*[40] reported that compounds with a β carbon were more depleted in ^{13}C, and observed a 7‰ difference between compounds bearing acetyl and formyl substituents.

Proportions of dung moieties in soil

The proportion of dung-derived lignin moieties present in the soil, calculated using the $\Delta^{13}C$ values between compound-specific $\delta^{13}C$ values (Eqn. (2)), clearly shows that the representation of dung-derived lignin moieties in C_4 dung-treated soil changed between 56 and 372 days (Fig. 4), and that rates of incorporation, degradation or transformation between individual 4-hydroxyphenols differed markedly. 4-Vinylphenol (**1**), syringol (**3**), 4-vinylguaicol (**7**), 4-vinylsyringol (**9**), and the two isomers of 4-(2-propenyl)syringol (**12** and **13**), were highly abundant at peak dung incorporation

DOI: 10.1002/rcm

Table 1. $M^{+\cdot}$ and characteristic MS fragment ions of trimethylsilyl derivatives of pyrolysis products of C_4 dung with relative abundance (%) in parentheses. **Bold** numbers refer to structures used to assess dung lignin incorporation

Peak no.	$M^{+\cdot}$	Fragment ions (*m*/*z*)	Compound
1	192	177 (100), 192 (80)	4-vinylphenol
2	254	239 (20), 254 (10)	catechol
3	226	196 (100), 211 (40)	syringol
4	268	268 (20), 253 (5)	3-methylcatechol
5	268	268 (15), 253 (7)	4-methylcatechol
6	254	239 (100), 254 (80)	4-ethylsyringol
7	222	192 (100), 222 (40)	4-vinylguaiacol
8	284	269 (32), 284 (35)	3-methoxycatechol
9	252	222 (100), 252 (40)	4-vinylsyringol
10	238	223 (65), 193 (60)	4-acetylguaiacol
11	266	236 (70), 266 (40)	4-(1-propenyl)syringol
12	266	206 (70), 236 (50)	4-(2-*Z*-propenyl)syringol
13	266	236 (100), 266 (60)	4-(2-*E*-propenyl)syringol
14	268	223 (80), 238 (70)	4-acetylsyringol
15	282	239 (100), 209 (40)	4-(2-propanone)syringol

(56 days), after heavy rainfall,[9] and all except 4-vinylsyringol (**9**) and 4-(2-*Z*-propenyl)syringol (**12**) showed significant reduction in abundance by the end of the experiment (372 days). Gleixner *et al*.[32] found that 4-vinylphenol (**1**), the decarboxylation product of *p*-coumaric acid, and 4-vinylguaiacol (**7**) were amongst several lignin pyrolysis products identified in crop tissues but not in arable soils,

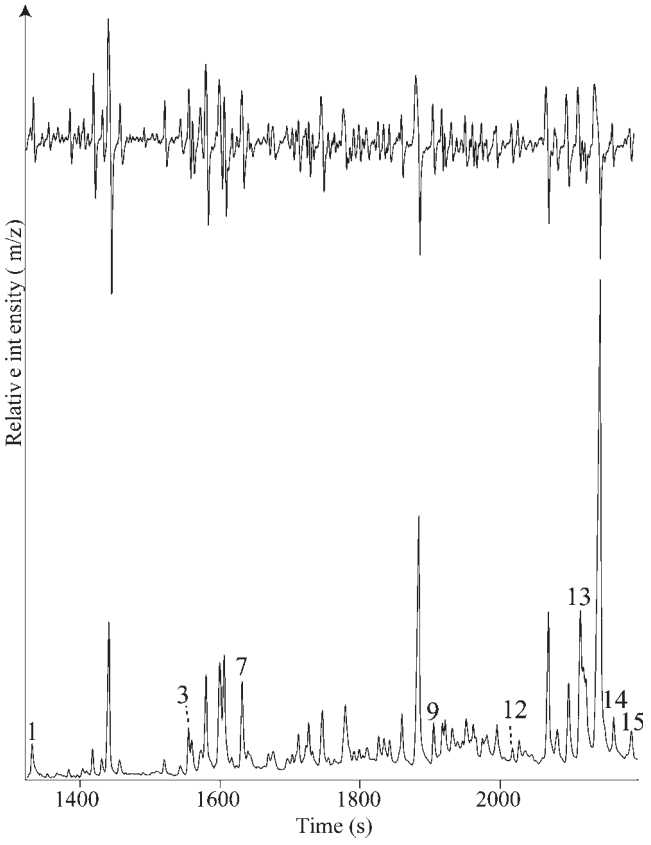


Figure 2. The *m/z* 44 ion current (above) and instantaneous ratio of *m/z* 45/44 ions (below) recorded for off-line pyrolysate of lipid-extracted C_4 dung. Numbers refer to specific lignin moieties detailed in Table 1.

suggesting that these lignin components are rapidly degraded in soils. 4-Vinylguaiacol (**7**) and 4-vinylsyringol (**9**) are the side-chain oxidation products of coniferyl alcohol and sinapyl alcohol, respectively.[53] However, 4-vinylguaiacol (**7**), one of the primary products of lignin oxidation in non-woody vascular plant tissues,[54] is also the decarboxylation product of ferulic acid that forms covalent cross-linkages between hemicellulose and core lignin in the plant cell wall[55] and, therefore, may have been more vulnerable to degradation than the constituents of core lignin.

It has been proposed that efficient lignin decomposition is linked to the presence of polysaccharides and their degradation products[56,57] on the basis that unbound carbohydrates would be utilised preferentially to lignin by decomposing lignolytic fungi; the former constitute a more accessible energy source. Thus, the loss of carbohydrates from the dung pats after heavy rainfall[58] may have triggered enhanced degradation of the lignin polymer as the white rot fungi switched from labile carbohydrate to lignocellulosic degradation. In addition, the penetration of fungal hyphae may have been retarded by the presence of the resistant crust formed on the dung pats after deposition until disturbed by the heavy precipitation after 31 days.[9] The degradation of ferulic acid and *p*-coumaric acids may also have been accelerated by activity in the rumen where significant decarboxylation of hydroxycinnamic acids to their phenolic forms occurs.[59]

4-(2-*Z*-Propenyl)syringol (**12**) was present at 53% and 50% of initial dung lignin abundances in the 0–1 cm soil after 56 and 372 days, respectively. This suggests that this dung lignin product existed in a resistant form, perhaps as a constituent of a stable lignin structure, but once cleaved from the main structure and leached into the soil behaved in a fashion analogous to 4-vinylsyringol (**9**). However, the 4-(2-*E*-propenyl)syringol (**13**) lignin moiety showed a different pattern of incorporation from 4-(2-*Z*-propenyl)syringol (**12**), with its maximum abundance of 65% being reached after 56 days, but then declining to 5% after 372 days. Possible causes of this disparate activity are the physical and biochemical accessibility of these lignin

DOI: 10.1002/rcm

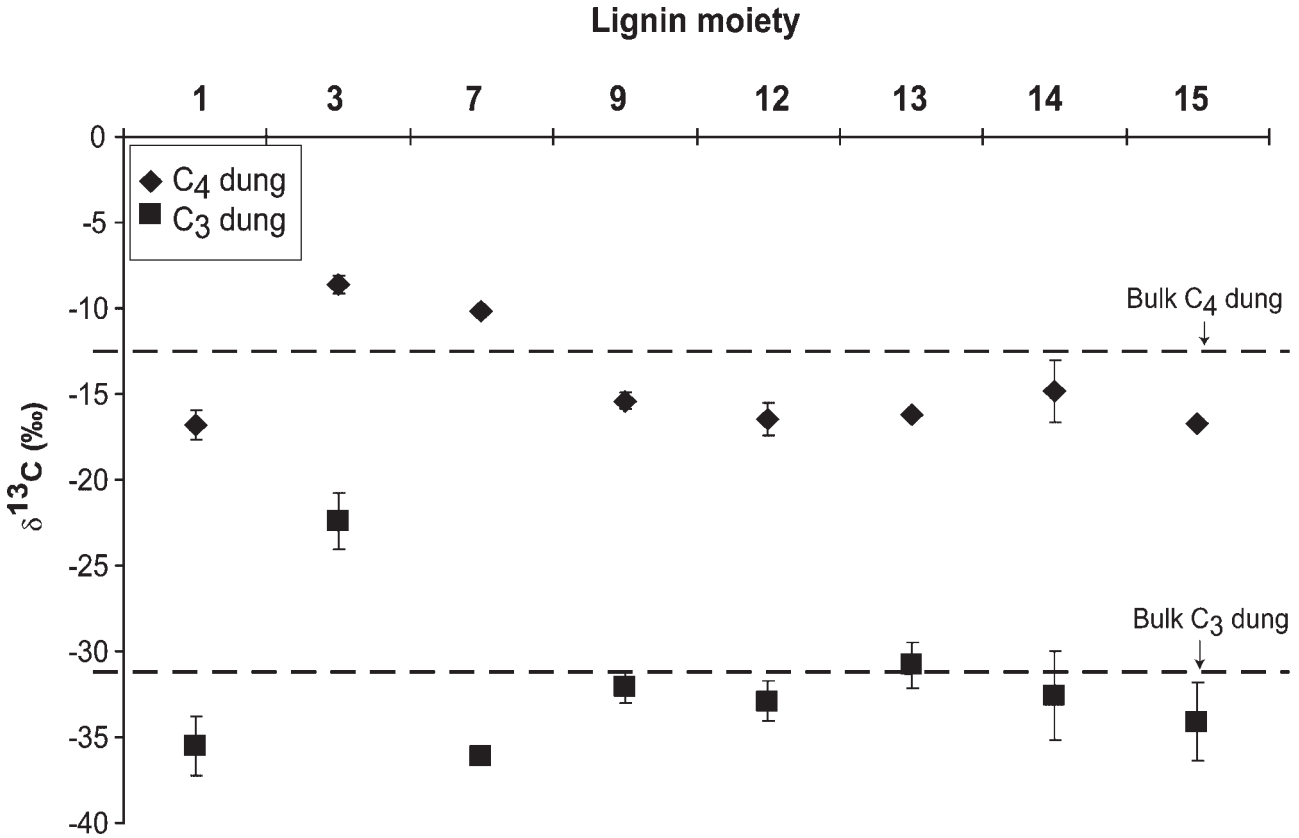


Figure 3. Compound-specific $\delta^{13}C$ values for a range of lignin moieties extracted from C_3 and C_4 dung by off-line pyrolysis (300°C, 1 h) plotted with bulk $\delta^{13}C$ values. Key: see Table 1.

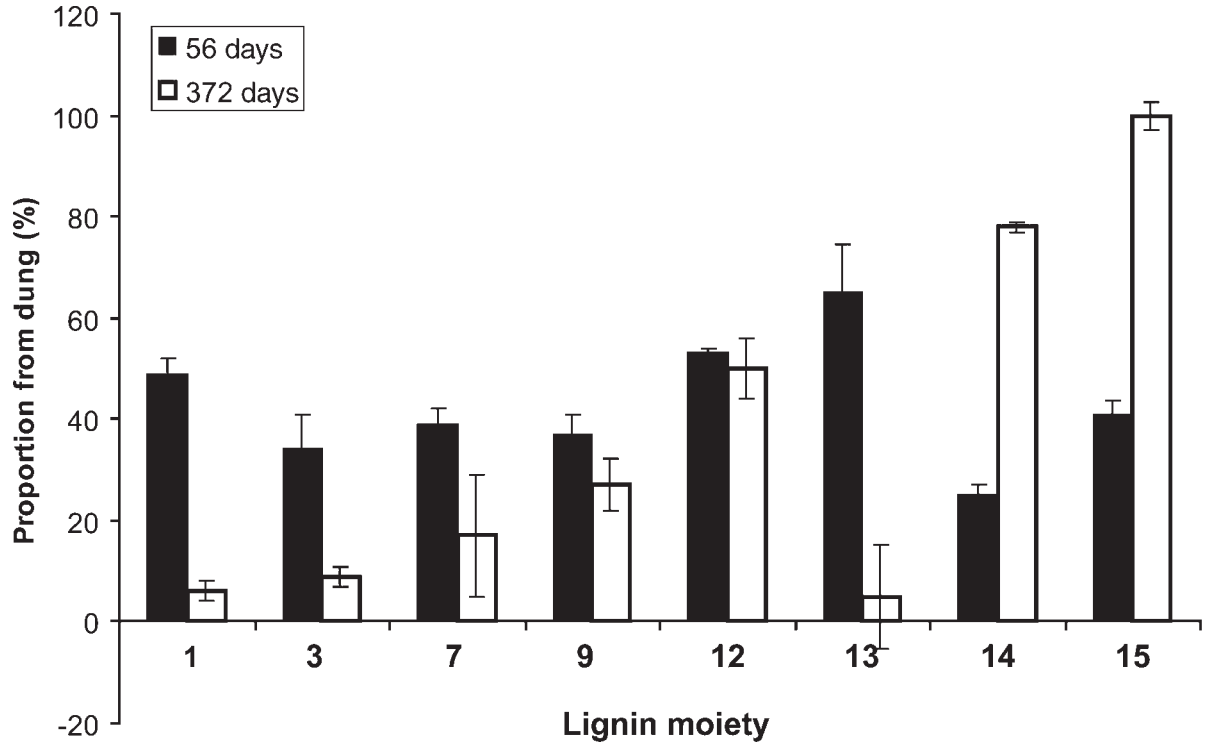


Figure 4. Proportion of dung-derived lignin moieties in pyrolysate (300°C, 1 h) of 0–1 cm soil horizon beneath C_4 dung pats 56 and 372 days after deposition. Key: see Table 1.

DOI: 10.1002/rcm

moieties to the agents of decomposition. The *Z* conformation may favour stronger C–C linking and, therefore, be derived from the mid-lamella and primary cell wall in close association with polysaccharides, whilst the *E* isomer may be more associated with the more labile β-O-4 linkages in the secondary cell wall.[60] Other lignin stereoisomers have also been observed to have different stabilities. The formation of β-O-4 ethers between monolignols is known to produce e*rythro*- and *threo*-isomers with different chemical reactivities: etherified *erytho*-isomers are cleaved more rapidly in the alkaline pulping of wood.[15] The pyrolysis products with oxygenated side chains, i.e. 4-acetylsyringol (**14**) and 4-(2-propanone)syringol (**15**), seem to derive from a resistant source of dung lignin as both lignin moieties increased significantly in abundance after 372 days, e.g. 4-(2-propanone)syringol (**15**) was determined at 41% abundance at 56 days which increased to 100% abundance at $t = 372$ days. This suggests an origin within the fibrous or hydrophobic constituents of plant material in the dung that was resistant to movement into the soil. Huang *et al*.[53] similarly observed abundant pyrolysis products, possessing a propene side chain, in surface soil horizons. However, Gleixner *et al*.[33] reported that more oxidized pyrolysis products, of a slightly higher molecular weight, were undetectable in the soil after a few years, and postulated that this was associated with on-going humification processes.

Overall, the $\delta^{13}C$ values of individual C_4 dung-derived lignin moieties revealed inhomogeneity between degradation rates of individual constituents of lignin incorporated from dung into soil, providing further evidence for the complexity of the lignin polyphenol, and supporting the recent work of Bahri *et al*.,[61] who concluded that lignin turnover in soils was monomer-specific. Although lignin moieties with propene side chains appear to be relatively stable after 372 days, the majority showed significant reduction in abundance compared with peak dung C incorporation at 56 days based on bulk $\delta^{13}C$ values.[9] Therefore, the hypothesis that lignin might contribute significantly more to bulk $\delta^{13}C$ values with time was rejected. van Bergen *et al*.[42] found that Rothamsted soils did not contain lignin biomarkers for overlying vegetation and that mineral soils contained more lignin biomarkers than arable soils,[62] concluding that lignin must be rapidly altered diagenetically in soils, a conclusion also drawn by Gleixner *et al*.[33] Similar studies estimated that lignin is lost at similar rates to bulk OM[63,64] or preferentially lost[65,66] with increasing contributions of lignin degradation products associated with finer soil fractions.[67,68] More recently, it has been determined that lignin has a faster turnover rate than total SOM using compound-specific $\delta^{13}C$ values of CuO oxidation products of lignin in an arable soil that had undergone a transition from wheat (C_3) to maize (C_4) cultivation.[22,69,70]

CONCLUSIONS

A number of aspects of a previously published protocol[39] for the generation of lignin moieties by off-line pyrolysis have been optimised. Application of the improved protocol has confirmed that:

(i) off-line pyrolysis can be successfully applied to the extraction of lignin moieties from contemporary organic matter and soils for CSIA, producing robust and reproducible $\delta^{13}C$ values that reflect the origin of the lignin.
(ii) $\delta^{13}C$ values of individual C_4 dung-derived lignin moieties revealed that the incorporation, degradation or transformation of lignin in soils varied between individual constituents of lignin providing further evidence for the complexity of the lignin polyphenol, and supporting the hypothesis that lignin turnover in soils is monomer-specific.[61]
(iii) although lignin moieties with propene side chains appear to be relatively stable after 372 days, the majority of dung-derived lignin moieties showed significant reduction in abundance compared with peak dung C incorporation at 56 days based on bulk $\delta^{13}C$ values. Therefore, the hypothesis that lignin would contribute significantly more to bulk $\delta^{13}C$ values with time was rejected.

Our findings contribute to a gathering body of evidence that lignin is not particularly stable in soils which has considerable significance for the perceived role of different biochemical components in the cycling of C in soils.

Acknowledgements

This study was undertaken while JAJD was in receipt of a BBSRC/IGER CASE studentship. The production and supply of C_3- and C_4-labelled dung were managed by Roger Evans at IGER-Aberystwyth, and the use of the NERC Life Sciences Mass Spectrometry facilities at the University of Bristol, are gratefully acknowledged.

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DOI: 10.1002/rcm