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Carbon isotope ratio analysis of organic moieties from fossil mummified wood: establishing optimum conditions for off-line pyrolysis extraction using gas chromatography/mass spectrometry

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Mummified fossil wood was studied using off-line pyrolysis-gas chromatography/mass spectrometry to reveal detailed insights into the pyrolysis conditions that are needed to obtain simultaneously sufficient amounts of both cellulose and lignin markers for stable carbon isotope analyses. The off-line pyrolysis was applied at a range of temperatures (200, 250 and 300 °C) and times (1 and 2 h) to determine the optimum temperature and time that yielded the highest quantity of true markers for lignin and cellulose. Increasing the time from 1 to 2 h had no effect whereas increasing the temperature led to large differences. The products released during the low-temperature pyrolysis were mostly related to thermally labile moieties. Only at 300 °C were sufficient amounts of products released that represent true cellulose and lignin building blocks and which could be studied using gas chromatography/combustion isotope ratio mass spectrometry.

Wood is often abundantly present in the fossil record and thus can provide a valuable source of information pertaining to palaeoenvironmental and palaeoclimatic conditions.[1] This information can be based on anatomical and/or chemical characteristics (i.e. taxon-specific chemical composition or stable carbon isotope composition). In particular, the use of stable carbon isotopes of well-defined organic remains, such as wood, for palaeoenvironmental analyses has been gathering momentum in recent years.[2–4] However, various external factors affect the bulk stable carbon isotope signature of plants during the time they grew, for example, aspect, salinity, temperature and altitude.[2,5,6] Also, intrinsic variations that occur within a single plant most probably relate to differences in carbon flow.[7,8]

Another important aspect to be taken into account is that bulk carbon isotope values of these well-defined entities (e.g. wood, leaves) are still a weighted mean average of all the different organic moieties present, each of which can have entirely different isotope compositions. This is especially the case for wood where the polysaccharides are ^{13}C enriched by about 5 per mil when compared with lignin.[9,10] In addition, fossil wood material will undergo chemical changes prior to and during fossilisation, such as charring by fire, attack by fungi or diagenesis, all of which can result in the preferential degradation of one fraction over another (i.e. chemical taphonomy[4]). In particular, the selective removal of polysaccharides can lead to stable carbon isotope shifts that can be interpreted erroneously as climatic/environmental changes (cf.[4]). Hence, many studies of modern and subfossil woods have concentrated on a so-called cellulose fraction in order to avoid chemical taphonomy.

Unfortunately, cellulose is relatively labile and most fossil wood specimens (older than 2 Ma) often contain mainly lignin. Thus, in order to obtain meaningful stable carbon isotope data from both subfossil and fossil wood specimens, it would be ideal to be able to measure both cellulose and lignin simultaneously. One such method is compound-specific stable carbon isotope analyses of cellulose- and lignin-specific building blocks.[11] However, to obtain these representative markers from the same sample, destructive chemical methods must be applied because the ligno-cellulose complex in wood is macromolecular in nature and therefore requires controlled chemical 'fragmentation'. Analytical pyrolysis is one such technique that has been used extensively to study bio-and geomacromolecules including the characterisation of ligno-cellulose.[12–14] Because compound-specific stable carbon isotope analyses require relatively large amounts of organic material, off-line open-system pyrolysis was used since this has the potential to generate simultaneously sufficient amounts of cellulose and lignin markers.

The objective of this work was to determine the differences in lignin and cellulose marker patterns and quantitatively assess the pyrolysis products thereby formulating an experimental procedure resulting in the optimum yield of true lignin and cellulose moieties from fossil wood speci-

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Table 1. Summary of the yields at different temperatures and duration at that temperature

Temperature (°C)	Time (h)	Mass (mg)	Yield (%)
200	1	0.8	0.2
200	2	1.7	0.4
250	1	13.3	3.3
250	2	9.9	2.4
300	1	28.3	6.3
300	2	30.7	6.6

mens. These marker products can be derivatised and subsequently used in compound-specific stable carbon isotope analyses. Gas chromatography/mass spectrometry of the trimethylsilylated derivatised pyrolysis products is of crucial importance because this will allow the determination of the number of derivatised alcohol and acid groups, which need to be corrected for, during stable carbon isotope measurements.

MATERIALS AND METHODS

A fossilised mummified specimen was used to determine an off-line pyrolysis protocol for extracting lignin and polysaccharide moieties simultaneously from fossil wood to obtain large enough quantities for compound-specific stable carbon isotope analyses. The specimen originated from the Miocene Madre de Dios Formation[15] outcropping along the Madre de Dios River, eastern Peru. The material was originally waterlogged therefore, prior to solvent extraction, the specimen was air-dried and powdered using a solvent-cleaned pestle and mortar.

Solvent extraction

Powdered mummified material was extracted using ultrasonication [3 × methanol (MeOH), 3 × dichloromethane (DCM)/MeOH (1:1 v/v), 3 × DCM]. After each extraction step the material was centrifuged and the supernatant was removed. The final solvent-extracted residue was air-dried. All residues, containing the insoluble organic matter representing the bulk of the wood, were stored dry in the dark prior to subjecting them to off-line pyrolysis.

Off-line pyrolysis

Open-system off-line pyrolysis was undertaken using a preheated tube furnace (Carbolite® MTF 12/38/250, Hope, UK). 350–400 mg of sample were placed in a Pyrex boat and introduced into a Pyrex tube located within the preheated oven. The sample was heated for a set time (Table 1). Pyrolysis products were flushed from the glass tube using a continuous stream of nitrogen (150 mL/min). Products were collected in an ice-cooled trap containing MeOH/DCM (1:1). Samples were heated at a range of temperatures, from 200 to 300 °C, for either 1 or 2 h to determine optimum volatile production (Table 1). At the end of the experiment the glass tube was removed from the oven and the sample left to cool under a flow of nitrogen to avoid oxidation. The liquid products collected were rotary evaporated, blown down under nitrogen and weighed to determine the yield.

Derivatisation

Aliquots of extracts were derivatised using an excess of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1% TMCS) and pyridine. Samples were heated at 80 °C for 45 min. Ethyl acetate was added to the derivatised sample and the extract was subsequently filtered using a Pasteur pipette packed with silica gel to remove non-GC-amenable compounds. The filtered extract was blown down almost to dryness, redissolved in ethyl acetate and analysed using gas chromatography (GC) and GC/mass spectrometry (GC/MS).

GC and GC/MS

The derivatised extracts were analysed using a Hewlett-Packard (Wilmington, DE, USA) 6890 gas chromatograph. Each sample (1 μL) was injected on-column onto a CP-Sil 5CB capillary column (50 m × 0.32 mm, 0.12 μm film thickness) with helium as carrier gas (3 mL/min). The GC oven was temperature programmed as follows: isothermal at 40 °C for 5 min, then rising to 300 at 4 °C/min, with an isothermal period of 10 min. Compounds were detected using a flame ionisation detector (330 °C).

Compounds were identified by GC/MS using an HP 5890 series II gas chromatograph coupled to a Fisons Instruments VG platform II mass spectrometer (Manchester, UK) operating at 70 eV, scanning the range *m/z* 50–650 with a cycle time of 0.65 s. The capillary column and the temperature programme used were as described for the GC analyses. Compound identifications were based on data published in the literature,[16] authentic standards and mass spectral data and retention time comparisons. In particular, the distinct fragmentation pattern showing a loss of mass 30, which represents ethane, is characteristic for trimethylsilylated 2-methoxyphenols.[17]

RESULTS AND DISCUSSION

Yields and general observations

As expected, the yields of liquid organic products released increased with increasing temperature from about 0.3% to 6.5% of the starting material (Table 1). Increasing the duration of the experiment from 1 to 2 h at a set temperature did not yield significantly larger amounts of products (Table 1). The release of organic volatiles, seen as a dark brown-yellow deposit on the inside of the glass tube and in the solvent trap, usually occurred within the first 15 min after insertion of the sample into the oven. A certain amount of volatiles did escape based on the pungent smell at the external outflow of the cold trap.

Chemical composition of the off-line pyrolysates

All off-line pyrolysates contained both polysaccharide- and lignin-derived pyrolysis products, but their relative contributions varied significantly between pyrolysates from different temperatures (Fig. 1; Table 2). In contrast, the distribution patterns of the compounds in the pyrolysates of the 1 h and 2 h experiments showed very little variation. This is consistent with the observations of the similar yields and the fact that most visual products appear to be released within the first 15–20 min of the experiment.

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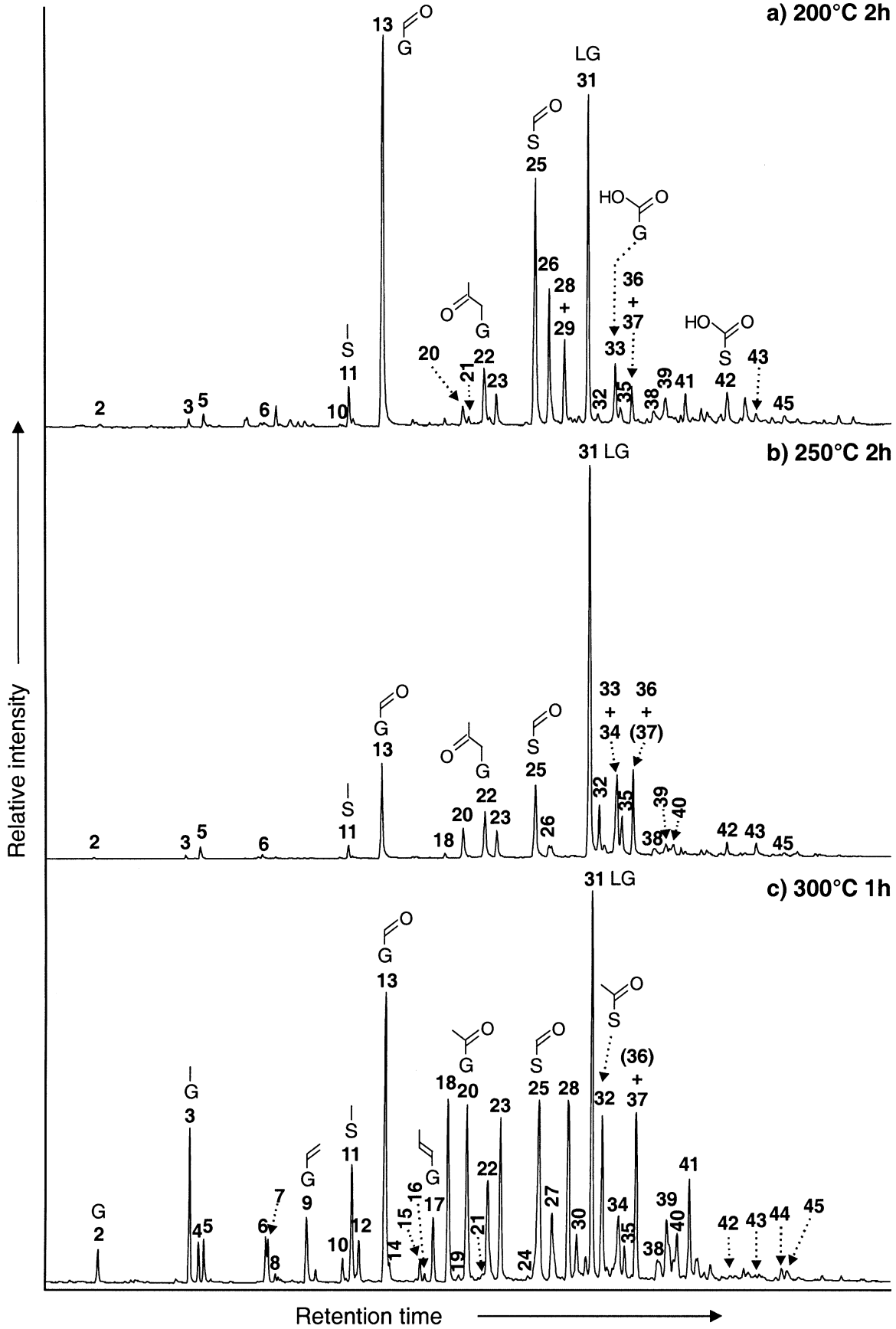

Figure 1. Gas chromatograms of three off-line pyrolysates derived from mummified wood samples heated at different temperatures: (a) 200 °C, (b) 250 °C, and (c) 300 °C. Compounds indicated were analysed as their fully TMS-derivatised counterparts. Side chains indicated are attached to carbon atom 4 in G and S. G = guaiacols (2-methoxyphenols), S = syringols (2,6-dimethoxyphenols), LG = levoglucosan. Numbers refer to compounds indicated in Table 2.

Both pyrolysates obtained at 200°C (Fig. 1(a)) were dominated by the lignin products 4-formylguaiacol (**13**) and 4-formylsyringol (**25**), and the polysaccharide product levoglucosan (**31**). Other relatively abundant lignin products included 4-(2-propanone)guaiacol (**22**), and acids, i.e. vanillic acid (**33**) and syringic acid (**42**). An interesting distribution pattern (Fig. 2) was also noted for the three propenyl derivatives of both the guaiacol (**10**, **14**, **17**) and syringol series (**21**, **24**, **30**). Of the three isomers, the 1-propenyl isomer dominated (Figs 2(a) and 2(c)), with the *Z*-2-propenyl isomer being least abundant among the 2-propenyl isomers. Also noteworthy was the detection of two other polysaccharide pyrolysis products, **26** and **29**, with characteristic fragments at *m/z* 73, 75, 147, 204, 205, 217, 218, 333, implying a structure similar to that of the abundant anhydrosugar, levoglucosan (Fig. 3(a), Table 2). Based on their retention times, eluting earlier than levoglucosan, and comparisons with the literature,[18] it is suggested that these two products represent levogalactosan (**26**) and levomannosan (**29**). In particular, the first product had relative abundance quite similar to that of levoglucosan (Fig. 3(a)).

All the abundant lignin pyrolysis products can be related to the original lignin structure and are derived from either β-O-4 or α-O-4 linkages. The abundance of the 4-formyl derivatives (**13**, **25**) at these low temperatures must indicate that these are released from units bound into the macromolecular matrix by relatively thermally labile bonds. Alternatively, they are more easily released from oxidised

Table 2. Main pyrolysis products shown in Figs 1–3. The characteristic MS fragmentation pattern, as TMS derivatives, for the products is given where the $M^{+\cdot}$ is underlined and the base peak is in bold with relative percentages in parentheses. PS: polysaccharide product, LP: lignin product

	Compound name	Characteristic fragment ions
1	Levoglucosenone	**98** (100), 96 (90), 68 (75), 53 (70)
2	Guaiacol	196 (15), 181 (20), **166** (100), 151 (25), 136 (20)
3	4-methylguaiacol	210 (20), 195 (15), **180** (100), 165 (10)
4	Catechol	254 (40), 239 (20), 166 (10), 151 (15), 136 (8), **73** (100)
5	Unknown PS	186 (5), 171 (10), 170 (20), 155 (15), 129 (25), 103 (10), 75 (40), **73** (100)
6	Syringol	226 (15), 211 (20), **196** (100), 181 (15)
7	4-ethylguaiacol	224 (20), 209 (30), **194** (100), 179 (75)
8	3- or 4-methylcatechol	268 (25), 253 (10), 180 (10), 179, (10), 165 (5), 150 (5), **73** (100)
9	4-vinylguaiacol	222 (20), 207 (10), **192** (100), 177 (10), 162 (15)
10	4-(1-propenyl)guaiacol	236 (25), 221 (20), **206** (100), 205 (40), 179 (15), 73 (80)
11	4-methylsyringol	240 (20), 225 (15), **210** (100), 195 (10), 167 (15)
12	3-methoxycatechol	284 (40), 269 (50), 254 (20), 239 (15), 196 (10), 73 **(100)**
13	4-formylguaiacol	224 (20), 209 (40), **194** (100), 193 (50)
14	4-(2-*Z*-propenyl)guaiacol	236 (25), 221 (10), **206** (100), 205 (25)
15	4-ethylsyringol	254 (20), 239 (20), **224** (100), 209 (50), 73 (90)
16	Methyl-3-methoxycatechol	298 (30), 283 (20), 268 (35), 253 (5), **73** (100)
17	4-(2-*E*-propenyl)guaiacol	236 (25), 221 (10), **206** (100), 205 (25)
18	Unknown PS	320 (4), 305 (5), 273 (10), 247 (10), 129 (20), 127 (60), 103 (20), 89 (40), 75 (50), **73** (100) 59 (70)
19	4-vinylsyringol	252 (30), 237 (20), **222** (100), 179 (15)
20	4-acetylguaiacol	238 (25), 223 (65), 208 (50), **193** (100), 73 (85)
21	4-(1-propenyl)syringol	266 (20), 251 (20), **236** (100), 221 (10), 205 (15)
22	4-(2-propanone)guaiacol	252 (15), 222 (5), **209** (100), 179 (20), 73 (50)
23	Vanillic acid methyl ester	254 (40), 239 (50), **224** (100), 209 (5), 193 (50)
24	4-(2-*Z*-propenyl)syringol	266 (40), 251 (5), **236** (100), 221 (10), 205 (20)
25	4-formylsyringol	254 (20), 239 (30), **224** (100), 223 (30)
26	Levogalactosan?	378 (2), 333 (4), 218 (20), **217** (100), 204 (20), 147 (15), 75 (30), 73 (80)
27	4-(3-propanone)guaiacol	252 (20), 237 (10), 224 (20), **223** (100), 193 (40), 73 (90)
28	Unknown LP	252 (30), 237 (10), 222 (50), 209 (15), 207 (15), 179 (30), 133 (30), **73** (100)
29	Levomannosan?	378 (–), 334 (5), 333(4), 218 (10), 217 (80), 204 (90), 147 (20), **73** (100)
30	4-(2-*E*-propenyl)syringol	266 (30), 251 (10), **236** (100), 221 (10), 205 (20)
31	Levoglucosan	378 (1), 333 (10), 218 (15), 217 (70), 204 (80), 147 (40), 75 (60), **73** (100)
32	4-acetylsyringol	268 (30), 253 (40), 238 (70), 223 (80), **73** (100)
33	Vanillic acid	312 (40), 297 (60), 282 (20), 267 (50), 253 (40), 239 (30), 223 (60), 193 (30), 126 (30), **73** (100)
34	4-(2-propanone)syringol	282 (20), 267 (10), 252 (10), 239 (80), 209 (40), **73** (100)
35	Unknown PS	M^+ (–), 319 (5), 218 (20), **217** (100), 147, (10), 75 (70), 73 (90)
36	Unknown LP	310 (70), 280 (80), **73** (100)
37	Syringyl acid methyl ester	284 (20), 269 (30), **254** (100), 223 (25)
38	Coniferylaldehyde	250 (70), 235 (40), **220** (100), 219 (80), 204 (20), 192 (50)
39	Dihydroconiferyl alcohol	326 (20), 311 (10), 236 (20), 209 (30), **206** (100), 205 (50), 179 (30)
40	4-(3-propanone)syringol	282 (30), 267 (25), 253 (55), 223 (70), **73** (100)
41	Unknown LP (S equivalent of #28)	282 (60), 267 (10), 252 (80), 239 (20), 237 (20), 222 (15), 209 (25), 126 (15), **73** (100)
42	Syringyl acid	342 (30), 327 (40), 312 (30), 297 (25), 253 (25), 223 (20), 156 (10), **73** (100)
43	Unknown LP (S equivalent of #36)	340 (55), 310 (50), **73** (100)
44	Sinapyl alcohol	354 (15), 324 (15), 296 (10), 234 (20), **73** (100)
45	Sinapylaldehyde	280 (40), 265 (25), 250 (45), 234 (20), **222** (100), 179 (20)

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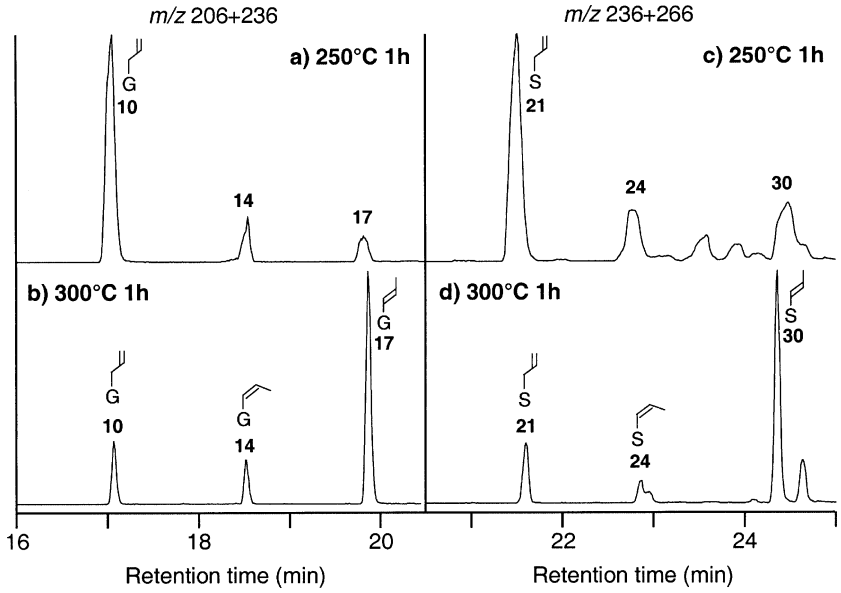

Figure 2. Summed mass chromatogram of *m/z* 206 + 236 (a) and (b) and *m/z* 236 + 266 (c) and (d) revealing the differences in the propenyl isomers of both G and S released at different temperatures, (a) and (c) 250 °C and (b) and (d) 300 °C. Numbers refer to the compounds indicated in Table 2.

moieties because this fossil wood specimen has already undergone a certain degree of chemical alteration, as witnessed by the acid derivatives (**33**, **42**). Important in this respect is the well-known oxidative formation of vanillin (= 4-formylguaiacol) from wood, which is quite possibly a thermal evaporation product rather than a true pyrolysis product. It is believed that the different anhydrosugars are derived from easily released polysaccharide moieties such as hemicelluloses, which are known to contain mannose and galactose units in dicotyledonous angiosperms.

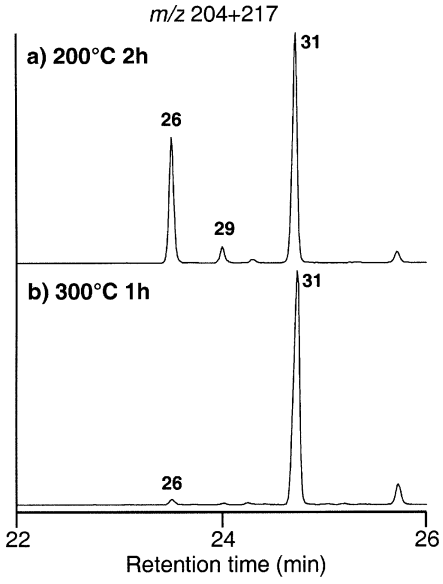

Figure 3. Summed mass chromatogram of *m/z* 204 + 217 revealing the differences in the anhydrosugars released at different temperatures: (a) 200 °C and (b) 300 °C. Numbers refer to the compounds indicated in Table 2.

With an increase in temperature to 250 °C the overall yields increased (Table 1) and the pyrolysates became dominated by levoglucosan (**25**) in both experiments (Fig. 1(b)). Lignin-related products were almost identical to those released in experiments at 200 °C with the 4-formyl derivatives (**13**, **25**) dominating. For the propenyl derivatives the same distribution pattern was observed as with the pyrolysates released at 200 °C, indicating that 4-(1-propenyl) isomers (**10**, **21**) are preferentially released at lower temperatures (Figs 2(a) and 2(c)). The two additional anhydrosugars (**26**, **28**) were hardly present, indicating that levoglucosan released at this temperature is mainly derived from the cellulose rather than hemicellulose. This implies that, in order to release sufficient products derived from cellulose for stable carbon isotope analyses, the pyrolysis temperature should be at least 250 °C.

At 300 °C the yield was further increased to about 20 times higher than that derived at 200 °C. Apart from levoglucosan (**31**), which is still the most abundant pyrolysis product, numerous lignin-derived products were released (Fig. 1(c)). The 4-formyl derivatives were the most abundant members (**13**, **25**) but other oxygenated, 4-acetyl-(**20**, **32**), 4-carboxylic (**23, 33, 37, 42**) and non-oxygenated products (**3, 9, 11, 17, 30**) also increased in abundance. Noteworthy is the change in distribution of the propenyl isomers with the *E*-isomer of 2-propenyls (**17**, **30**) becoming the most abundant isomers (Figs 2(b) and 2(d)). This isomer is most probably released upon the breakage of the β-O-4 link. The types of products released and their distribution pattern were very similar to data obtained by on-line flash pyrolysis-GC/MS of fossil wood specimens,[14] implying that true pyrolysis, rather than thermal evaporation, is the main process involved. Addi-

tional products, not detected in the off-line pyrolysates obtained at low temperatures (Figs 1(a) and 1(b)), were the diagenetic lignin degradation products catechols (**4**, **8**) and 3-methoxycatechols (**12**, **16**).[14] These latter products clearly provide additional evidence that at this temperature the true macromolecular ligno-cellulose structure is broken down.

Thus, off-line pyrolysis at 300 °C for 1 h is sufficient to pyrolytically degrade mummified fossil wood into chemically informative building blocks. However, older permineralised fossil wood specimens, i.e. calcified or silicified specimens, which are often found in the fossil record,[11] first need HCl and HF treatment to remove the mineral matrix.[19] For these samples higher temperatures are needed to release sufficient amounts of products in order to undertake compound-specific stable carbon isotope measurements.[11] The off-line temperatures needed for these materials ranged from 300 to 400 °C (1 h), but with this material mainly lignin degradation products were released, such as phenols and catechols, in addition to minute relative amounts of original lignin units (i.e. guaiacols).[11] These products provide evidence for enhanced cross-linking within the chemically altered lignin macromolecular matrix[20,21] explaining the need for higher off-line pyrolysis temperatures.

Implications for compound-specific stable carbon isotope measurements

These off-line pyrolysis data show that at 300 °C significant amounts of both cellulose- and lignin-derived products are released from mummified fossil wood samples. These amounts are sufficient for all compounds to be used in compound-specific stable carbon isotope studies using gas chromatography/combustion-isotope ratio mass spectrometry (GC/C-IRMS).[11] For example, compound-specific stable carbon isotope measurements of the 300 °C/1 h pyrolysate of this specimen have already revealed that meaningful data can be obtained with the compound-specific isotope signal still preserved, i.e. $\delta^{13}C$ values of −23.1‰ for levoglucosan derived from cellulose versus approx. −30 to −38‰ for the lignin-derived products (cf. 11). The difference of about 5–7‰ between the $\delta^{13}C$ values of cellulose and lignin markers is in accordance with previous analyses of bulk fractions,[9,10] although the spread in the $\delta^{13}C$ values of the lignin markers is relatively large. This therefore clearly indicates that this off-line pyrolysis method at 300 °C for 1 h is sufficient to provide meaningful compound-specific stable carbon isotope data that can subsequently be used for palaeoenvironmental and palaeoclimatic interpretations.

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