RAPID COMMUNICATIONS IN MASS SPECTROMETRY, VOL. 7, 488–491 (1993)

Gas Chromatography/Isotope Ratio Mass Spectrometry of Leaf Wax n-Alkanes from Plants of Differing Carbon Dioxide Metabolisms

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Individual n-alkanes isolated from the leaf-surface waxes of plants utilizing differing photosynthetic pathways were analysed using gas chromatography/isotope ratio mass spectrometry (GC/IRMS), in order to obtain the ratios of ^{13}C to ^{12}C ($\delta^{13}C$). Marked differences in the average ^{13}C-depletion of the n-alkanes relative to whole leaf tissues were observed ($C_3 = -7.7‰$; $C_4 = -9.9‰$; CAM $= -11‰$). Additionally, all three metabolic types exhibited considerable differences of ^{13}C-depletion between n-alkane homologues isolated from the same plant.

The technique of connecting a gas chromatograph to an isotope ratio mass spectrometer via a combustion interface[1] (GC/IRMS or irmGC/MS) allows for the calculation of the ratios of the stable isotopes of carbon (^{12}C and ^{13}C) for individual compounds within complex mixtures.[2,3,4] This ratio is expressed relative to a standard and calculated according to the equation:

$$\delta^{13}C = \left[\frac{^{13}C_{sample}/^{12}C_{sample}}{^{13}C_{standard}/^{12}C_{standard}} - 1 \right] \times 1000$$

where $^{13}C_{standard}/^{12}C_{standard} = 0.0112372$ based on a belemnite from the Pee Dee formation (PDB). Due to the only relatively recent availability of commercial GC/IRMS instruments, there are few $\delta^{13}C$ values for individual plant compounds, such as those within lipid mixtures. Previous research on the ^{13}C-depletion of lipids relative to source carbon (e.g., saccharides) has indicated that fatty acids have $\delta^{13}C$ values 3–5‰ more negative than the source.[5,6,7] However, work by Rieley *et al.*[3] has indicated that individual n-alkanes, and thus long-chain ($>C_{20}$) plant acyl lipids in general, could be more depeleted in ^{13}C than might be expected from these general studies, and from published measurements on total lipids of higher plants in particular.[8,9,10,11] The aim of this study has been to utilize GC/IRMS to investigate the relationship of leaf-wax lipid $\delta^{13}C$ values from plants of various metabolisms, and especially leaf-wax lipids derived from the acetate-malonate pathway,[12] to bulk leaf $\delta^{13}C$ and bulk leaf-wax $\delta^{13}C$ values. n-Alkanes are likely to provide an adequate guide to the $\delta^{13}C$ composition of the homologous families of plant acyl lipids, which are closely related biosynthetically.

EXPERIMENTAL

Plant material

The following plants were chosen for this study: *Selenecerus grandiflorus* (Cactaceae), *Tillandsia usneoides* (Bromeliaceae), and *Aechmeade albata* (Bromeliaceae) representing CAM (=crassulacean acid metabolism) species, and *Psidium cattleionum* (Myrtaceae), *Jacobinia cornea* (Acanthaceae), *Cyperus diffusus* (Cyperaceae), *Dendrocalamus strictus* (Gramineae) and *Cyperus alternifolius* (Cyperaceae) representing C_3 plants. All leaves were harvested on 7 November 1991 from a well-ventilated glasshouse with the approximate dimensions of 10 m by 40 m and 5 m in height. In addition, the following plants were collected in the open: *Saccharum officinarum* (Gramineae), *Miscanthus sacchariflorum* (Gramineae) and *Zea mays* cv. dentiformis (Gramineae), representing C_4 plants. All plants were taken from the collection at the University of Bristol Botanical Gardens, Bristol, UK.

Sample preparation and extraction

The leaves collected were dried at 80 °C for 24 hours. The leaf-surface lipids (waxes) were extracted by immersing whole leaves in dichloromethane (CH_2Cl_2). Fractions containing n-alkane homologues were isolated using silica-gel chromatography, eluting with hexane.

Mass spectrometry

1 mg of ground leaf tissue and 1 mg of the total leaf-wax extracts were isotopically analysed using an automated linked system consisting of an elemental analyser, a cryogenic purification unit, and a Finnigan MAT (Bremen, Germany) Delta-S isotope-ratio mass spectrometer.[13] Homologous n-alkanes were identified with a Finnigan 4500 GC/MS (Finnigan MAT), using an electron ionization energy of 70 eV.

Isotope analyses were undertaken using a Varian 3500 (Walnut Creek, CA, USA) GC attached to a Finnigan MAT Delta-S isotope ratio mass spectrometer via a Finnigan MAT combustion interface consisting of an alumina reactor (0.5 mm ID) within which copper and platinum wires are held (0.1 mm diameter). The copper wires were oxidized daily by passing oxygen through the reactor at 500 °C for a miniumum of 8 h. For analysis purposes, reactor temperature was maintained at 860 °C with mass spectrometer source pressure at 7×10^{-5} Torr. Effluent from the reactor was passed through a water trap, and dry CO_2 continuously monitored for the ions $m/z = 44$, 45 and 46. The ion $m/z = 46$ is measured in order to correct for the $^{12}C^{16}O^{17}O$ isobaric interference at $m/z = 45$.[14] Isotope ratios were assigned using supercritical fluid grade CO_2

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0951–4198/93/060488–04 $07.00

Received 21 April 1993
Accepted 21 April 1993

Table 1. Carbon isotopic compositions (‰ PDB) of total leaf tissue, total surface lipid extracts, mean weighted alkanes, and individual n-alkanes for C_3 plants.[a] Values in bold print indicate the most abundant n-alkanes in the leaf surface lipid extracts

Plant[b]	E	F	G	H	I	Mean
Total tissue	−29.7	−29.6	−28.9	−28.5	−27.6	−28.9 ± 0.9
Total wax	−32.7	−34.2	−32.1	−32.3	−32.2	−32.7 ± 0.9
Weighted mean alkanes[c]	−38.6	−36.1	−36.8	−36.3	−35.1	−36.6 ± 1.3
n-Alkane carbon no.						
27				−34.3		
28						
29	−38.6 ± 0.9	**−36.1 ± 0.1**	−36.2 ± 0.5	−35.3 ± 0.3	−34.2	−36.1 ± 1.6
30						
31	**−38.8 ± 0.1**	−36.2 ± 0.3	−36.5 ± 0.1	−36.4 ± 0.5	**−35.3 ± 1.3**	−36.6 ± 1.3
32	−38.0 ± 0.2					
33	−38.4 ± 0.2		**−36.8 ± 0.2**	**−37.8 ± 0.7**		−37.7 ± 0.8
34						
35			−37.3			

[a] Indicated uncertainties are standard deviations of duplicate analyses.
[b] Codes: E: *Psidium cattleionum* (Myrtaceae); F: *Jacobinia cornea* (Acanthaceae); G: *Cyperus diffusus* (Cyperaceae); H: *Dendrocalamus strictus* (Gramineae); I: *Cyperus alternifolius* (Cyperaceae).
[c] Weighted mean alkanes = $(\Sigma[c_i] \times d_i)/\Sigma[c_i]$; for $i = 27$ to 35, where c_i is the concentration of the n-alkane containing i carbon atoms.

(Air Products, Walton-on-Thames, UK) as a reference. Isotope-ratio data were edited after aquisition using Isodat ver. 5.x (Finnigan MAT), in order to discount peak co-elution as a factor affecting $\delta^{13}C$ values.[15] In order to obtain optimum isotope measurements, 50–200 ng of each compound of interest was combusted.

GC was undertaken upon fused-silica capillary columns (Chrompack; 50 m: ID 0.32 mm) with methyl silicone phases (CPSil 5CB; thickness 0.12 μm, GC/MS; 0.4 μm, GC/IRMS) using on-column injection and helium as carrier gas. The GC temperature program used for all analyses was 40 °C–108 °C at 10 °min^{-1} then 180 °C–300 °C at 5 °min^{-1}, isothermal for 20 min. Samples were dissolved in hexane prior to injection.

RESULTS AND DISCUSSION

The $\delta^{13}C$ values for the leaf tissue, total surface lipid extracts, and individual n-alkane homologues from the plants examined are summarized in Tables 1 and 2. Average values for each metabolism type are also indicated in these Tables. Figure 1 shows the individual n-alkane distributions and isotope data in a graphical form for selected plants analysed.

From the results obtained it can be inferred that the CAM plants examined were utilizing night fixation, and hence a C_4 pathway, in view of their relatively heavy $\delta^{13}C$ values (−13 to −16‰).[5] The C_4 plants examined had leaf tissue values between −10.7 and −11.9‰, consistent with literature for other C_4 plants.[8,17,18] The bulk leaf $\delta^{13}C$ values for the C_3 plants analysed are all

Table 2. Carbon isotopic compositions (‰ PDB) of total leaf tissue, total surface lipid extracts, mean weighted alkanes, and individual n-alkanes for C_4 and CAM plants.[a] Values in bold print indicate the most abundant n-alkanes in the leaf surface lipid extracts

	CAM				C_4			
Plant[b]	A	B	C	Mean	X	Y	Z	Mean
Total tissue	−13.0	−14.9	−16.2	−14.7 ± 1.6	−10.7	−11.9	−11.2	−11.3 ± 0.6
Total wax	−22.1	−22.5	−24.2	−22.9 ± 1.1	−18.4	−19.2	−18.9	−18.8 ± 0.4
Weighted mean alkanes[c]	−26.8	−25.2	−25.2	−25.7 ± 0.9	−24.5	−18.5	−20.5	−21.2 ± 3.1
n-Alkane carbon no.								
21		−23.0 ± 0.2						
22								
23		**−24.0 ± 0.3**						
24								
25	−25.1 ± 0.6	−25.2 ± 0.2		−25.1 ± 0.1				
26	−26.1					−18.0		
27	−25.1 ± 0.6	−25.5 ± 0.4		−25.3 ± 0.2	−24.8 ± 0.1	−18.5 ± 0.1		−21.7 ± 4.5
28	−28.2					−19.4		
29	−27.1 ± 0.5	−26.9 ± 0.7		−27.0 ± 0.1	**−24.5 ± 0.1**	−18.4 ± 0.1		−21.5 ± 4.3
30	−29.2					−19.8		
31	−26.4 ± 0.1	−29.2 ± 1.1	**−25.2 ± 0.2**	−27.9 ± 2.1	−23.4 ± 0.1	**−18.4 ± 0.1**	**−20.5**	−20.8 ± 2.5
32	−28.4 ± 0.3							
33	**−26.2 ± 0.3**				−25.8 ± 0.1	−18.4		−22.1 ± 5.2
34	−29.2 ± 0.4							
35	−28.5							

[a] Indicated uncertainties are standard deviations of duplicate analyses.
[b] Codes: A: *Selenecereus grandiflorus* (Cactaceae); B: *Tillandsia usneoides* (Bromeliaceae); C: *Aechmeade albata* (Bromeliaceae); X: *Saccharum officinarum* (Gramineae); Y: *Miscanthus sacchariflorum* (Gramineae); Z: *Zea mays* cv. dentiformis (Gramineae).
[c] Weighted mean alkanes = $(\Sigma[c_i] \times d_i)/\Sigma[c_i]$; for $i = 21$ to 35, where c_i is the concentration of the n-alkane containing i carbon atoms.

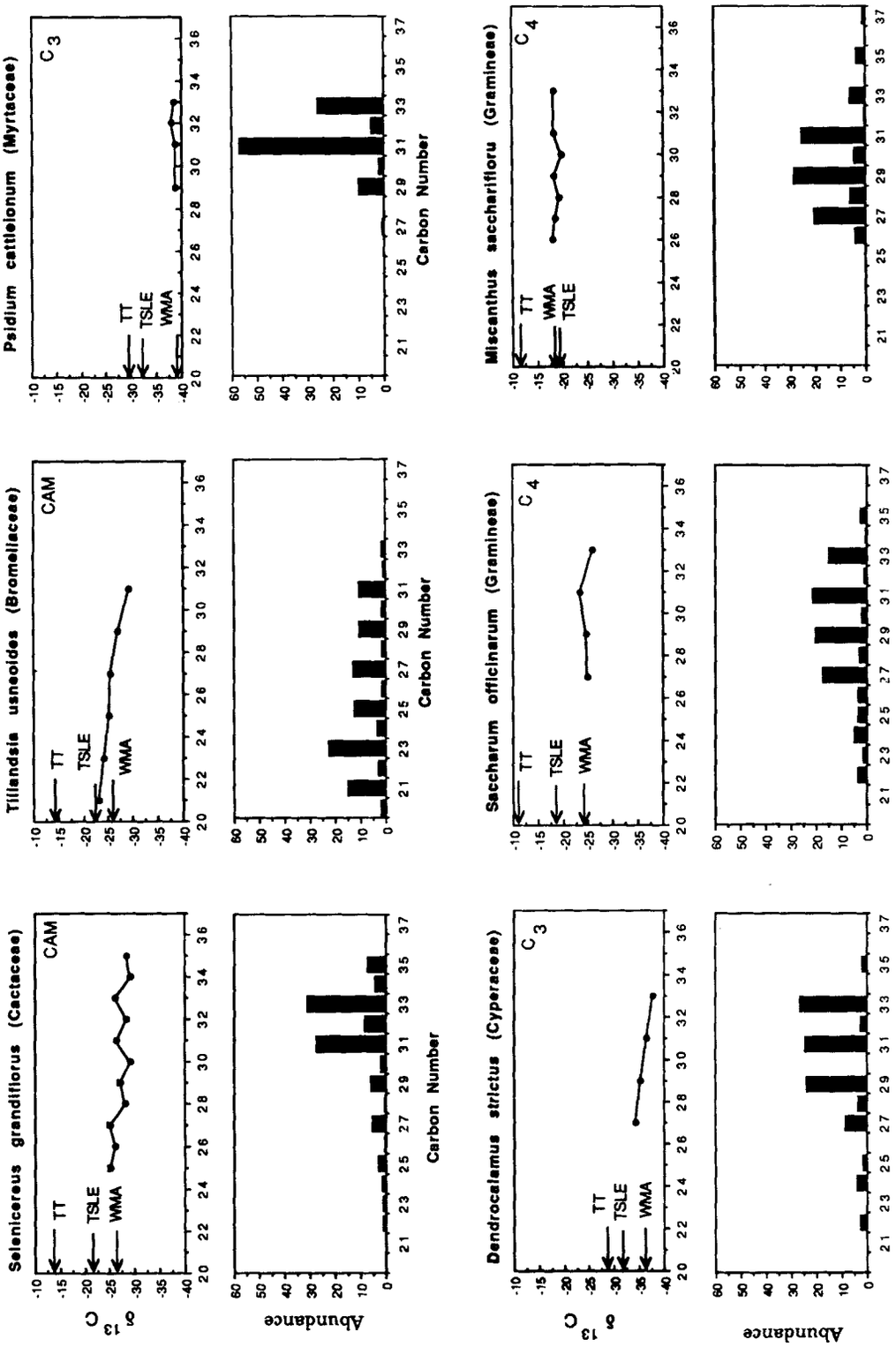

Figure 1. Individual $\delta^{13}C$ values (‰ PDB) and relative abundances (as percentage of total n-alkane fraction) vs carbon number, for individual n-alkanes extracted from leaf waxes of plants utilizing different carbon dioxide metabolisms. TT = $\delta^{13}C$ values for total leaf tissues; TSLE = $\delta^{13}C$ values for total surface lipid extract; WMA = weighted mean average $\delta^{13}C$ values of n-alkanes from a single plant.

within the range −25 to −30‰ (Table 2), well within the literature range for C_3 plants.[5] We assume the $\delta^{13}C$ of CO_2 utilized by the glasshouse plants was approximately the global average of −7.8‰,[16] thus the $\delta^{13}C$ values of bulk tissue for the glasshouse plants approximate to those reported in the Literature. However, it should be noted that since this study is concerned with the stable isotope abundances of different plant components relative to each other, the $\delta^{13}C$ value of the glasshouse CO_2 is largely irrelevant. This argument assumes that the depletion of individual plant lipids relative to total tissues is independent of the $\delta^{13}C$ value of source CO_2.

The $\delta^{13}C$ values for the total surface lipid extract (leaf-wax lipids) for the CAM and C_4 plants were depleted by around −8‰ more than leaf tissue (Tables 1 and 2), whereas the $\delta^{13}C$ values of the C_3 plant total leaf-wax extracts were depleted by −4‰ relative to leaf tissue.

The metabolic types differed in ^{13}C-depletion of their surface wax n-alkanes relative to total leaf tissue (Table 1 and 2). The average difference was −11‰ for the CAM plants examined, −9.9‰ for the C_4 plants, and −7.7‰ for the C_3 plants. Depletion in ^{13}C of lipids relative to leaf tissue has been previously observed,[11,20] though the magnitude of ^{13}C depletion observed in this study is much greater than expected.

It has been demonstrated that the ^{13}C-depletion in lipids relative to biomass is due to isotopic fractionation during the oxidation of pyruvate to acetyl coenzyme-A.[6,7,21] Monson and Hayes[7] demonstrated that a secondary point for isotopic fractionation exists in lipid biosynthesis. In that, the hydrolysis of the thioester bond attaching the acyl chain to the carrier protein during chain elongation allows fatty acids with ^{13}C-depleted carboxyl groups to be incorporated more rapidly into complex lipids. However, the large ^{13}C-depletion of n-alkanes relative to plant tissue in the CAM and C_4 plants as compared to the C_3 plants is surprising, and may be related to the different carbon fixation pathways utilized (C_3 vs C_4). Species-specific differences in the magnitude of ^{13}C-depletion of lipids can arise both from different kinetic isotope effects during the pyruvate dehydrogenase reaction, and from different flow rates of pyruvate to other metabolic intermediates.[6] Comparative studies of biosynthetic pathways in C_3, C_4 and CAM plants and of the flow rates of metabolites through these pathways need to be carried out before our observations can be adequately explained in relation to such studies.

In general, the individual n-alkanes from each plant were ^{13}C-depleted relative to the total surface lipid extract (Tables 1 and 2). The corresponding n-alcohols and n-acids, which are closely related biosynthetically, can be expected to have similar isotopic compositions.[22] Possible ^{13}C-enriched components of the surface lipid extracts may include compounds derived from different biosynthetic pathways, for example triterpenoids.

Significant variations in $\delta^{13}C$ values were observed within the n-alkane homologues for each plant examined, in some cases giving a range of 6‰ and averaging around 2–3‰ (Tables 1 and 2). As can be observed from Fig. 1, $\delta^{13}C$ values were not systematically related to the n-alkane distributions. In the case of one CAM plant (A), the even carbon numbered homologues were consistently depleted in ^{13}C by as much as −3‰ relative to the neighbouring odd carbon number alkanes (Table 2). However, an opposite trend of variation was observed in one of the C_3 species (E, Table 2). A lack of data, due to the very low abundance of the even numbered homologues, prevents us from observing a trend.

The large possible variations of $\delta^{13}C$ values (up to 6‰) between n-alkane homologues isolated from the same plant emphasize the need for caution when interpreting small variations in the isotopic compositions of compounds isolated from modern and ancient sedimentary environments. Thus, where previous studies have interpreted differences of less than 5‰ within $\delta^{13}C$ values for n-alkanes as deriving from multiple sources[3,23,24,25] this study indicates that such conclusions must at least be qualified.

Acknowledgements

We acknowledge the Natural Environment Research Council, UK, for provision of GC/MS and GC/IRMS facilities (Grants GR3/2951, GR3/3748 and GR3/7731), and the support of Shell International Petroleum Mij BV. G.R. acknowledges a Science and Engineering Research Council Studentship. Dr Brian Fry is thanked for bulk isotope determinations and for discussions during manuscript preparation. Nick Wray is gratefully thanked for assistance in plant collection and identification, and the University of Bristol Botanical Gardens, Bristol, UK, for access to their collection. Luiz Madureira is thanked for his advice during this project. Dr Iain Gilmour is thanked for contributing to an improved manuscript during the referee stage.

REFERENCES

1. D. E. Matthews and J. M. Hayes, *Anal. Chem.* **50**, 1465 (1978).
2. K. H. Freeman, J. M. Hayes, J. M. Trendel and P. Albrecht, *Nature* **343**, 254 (1990).
3. G, Rieley, R. J. Collier, D. M. Jones, G. Eglinton, P. A. Eakin and A. K. Fallick, *Nature* **352**, 425 (1991).
4. J. W. Collister, PhD Thesis, Indiana University, Bloomington (1992).
5. P. H. Abelson and T. C. Hoering, *Proc. Nat. Acad. Sci. USA* **47**, 623 (1961).
6. M. J. DeNiro and S. Epstein, *Science* **197**, 261 (1977).
7. K. D. Monson and J. M. Hayes, *Geochim. Cosmochim. Acta*, **46**, 139 (1982).
8. B, N, Smith and S. Epstein, *Plant Physiol.* **47**, 380 (1971).
9. P. Deines, in *Handbook of Environmental Geochemistry: The Terrestrial Environment, Vol. 1*, pp. 329–406, ed. by P. Fritz and J. C. Fontes, Elsevier, Amsterdam (1980).
10. D. J. Des Marais, J. M. Mitchell, W. G. Meinschein and J. M. Hayes, *Geochim. Cosmochim. Acta* **44**, 2075 (1980).
11. M. C. Kennicutt II, R. R. Bidigare, S. A. Macko and W. L. Keeney-Kennicutt, *Chem. Geol.* **101**, 235 (1992).
12. P. E. Kolattukudy, R. Croteau and J. S. Buckner, in *Chemistry and Biochemistry of Natural Waxes*, pp. 289–347, ed. by P. E. Kolattukudy, Elsevier, Amsterdam (1976).
13. B. Fry, W. Brand, F. J. Mersch, K. Tholake and R. Garritt, *Anal. Chem.* **64**, 288 (1992).
14. J. M. Hayes, K. H. Freeman, B. N. Popp and C. H. Hoham, *Org. Geochem.* **16**, 1115 (1990).
15. E. Lichtfouse and J. W. Collister, *J. Chromatog.* **585**, 177 (1992).
16. W. A. van Hook, in *Isotope Effects in Chemical Processes*, pp. 99–118, *Adv. in Chem. Series*, ed. by R. F. Gould, ACS Publications (1969).
17. B. N. Smith and W. V. Brown, *Am. J. Bot.* **60**, 505 (1973).
18. B. N. Smith and B. L. Turner, *Am. J. Bot.* **62**, 541 (1975).
19. U. Siegenthaler, *Nature* **345**, 295 (1990).
20. R. Park and S. Epstein, *Plant Physiol.* **36**, 133 (1961).
21. M. H. O'Leary, *Biochem. Biophys. Res. Comm.* **73**, 614 (1976).
22. Y. Huang, University of Bristol, Bristol, UK (unpublished results).
23. M. C. Kennicutt II and J. M. Brooks, *Org. Geochem.* **15**, 193 (1990).
24. K. H. Freeman, PhD Thesis, Indiana University, Bloomington (1991).
25. J. W. Collister, R. E. Summons, E. Lichtfouse and J. M. Hayes, *Org. Geochem.* **19**, 265 (1993).