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Interspecific variation in bulk tissue, fatty acid and monosaccharide $\delta^{13}C$ values of leaves from a mesotrophic grassland plant community

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ABSTRACT

The leaves of 37 grass, herb, shrub and tree species were collected from a mesotrophic grassland to assess natural variability in bulk, fatty acid and monosaccharide $\delta^{13}C$ values of leaves from one plant community. The leaf tissue mean bulk $\delta^{13}C$ value was −29.3‰. No significant differences between tissue bulk $\delta^{13}C$ values with life form were determined ($P = 0.40$). On average, $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ constituted 89% of leaf tissue total fatty acids, whose $\delta^{13}C$ values were depleted compared to whole leaf tissues. A general interspecific (between different species) trend for fatty acids $\delta^{13}C$ values was observed, i.e. $\delta^{13}C_{16:0} < \delta^{13}C_{18:2} < \delta^{13}C_{18:3}$, although these values ranged widely between species, e.g. $C_{16:0}$ (−34.7‰, *Alisma plantago-aquatica*; −44.0‰, *Leucanthemum vulgare*), $C_{18:2}$ (−33.3‰, *Acer campestre*; −44.2‰, *L. vulgare*;) and $C_{18:3}$ (−34.3‰, *Bellis perennis*; −41.8‰, *Plantago lanceolata*). Average relative abundances of leaf monosaccharides arabinose, xylose, mannose, galactose and glucose were 12%, 13%, 5%, 12% and 54%, respectively. Mean $\delta^{13}C$ values of these monosaccharides were −26.6‰ (arabinose), −27.2‰ (xylose), −30.9‰ (mannose), −30.0‰ (galactose) and −29.0‰ (glucose). The general relationship between individual monosaccharide $\delta^{13}C$ values, $\delta^{13}C_{arabinose} > \delta^{13}C_{xylose} > \delta^{13}C_{glucose} > \delta^{13}C_{galactose}$, was consistently observed. Therefore, we have shown (i) diversity in compound-specific $\delta^{13}C$ values contributing to leaf bulk $\delta^{13}C$ values; (ii) interspecific variability between bulk and compound-specific $\delta^{13}C$ values of leaves of individual grassland species, and (iii) trends between individual fatty acid and monosaccharide $\delta^{13}C$ values common to leaves of all species within one plant community.

1. Introduction

Stable isotope methods have emerged as one of the more powerful tools for advancing understanding of relationships between plants and their environments (Dawson et al., 2002). Bulk $\delta^{13}C$ values have been are used to investigate routing of photosynthate C in plants tissues and organs (Behboudian et al., 2000; Damesin and Lelarge, 2003; Badeck et al., 2005), bulk organic matter turnover in soils (Balesdent and Marriotti, 1996; Boutton et al., 1998; Glaser, 2005) and sediments (Macko et al., 1984; Yoshii et al., 1997; Martinelli et al., 2003), and routing of C in modern (Coates et al., 1991; Dungait et al., 2005) and palaeodiets (Heaton, 1999). However, bulk isotopic determinations provide average values of a wide range of individual biochemical components, each of which have different isotope compositions and abundances (Schweizer et al., 1999; Grice, 2001; Yeh and Wang, 2001) due to (i) the C source utilized in biosynthesis, (ii) isotope effects associated with assimilation of C by the producing organism, (iii) isotope effects associated with metabolism and biosynthesis, and (iv) cellular C budgets (Hayes, 1993). The difference in natural abundance of ^{13}C between C_3 ($\delta^{13}C = -20$ to −32‰) and C_4 ($\delta^{13}C = -9$ to −17‰) detrital vegetation input to ecosystem C pools is routinely exploited to determine C provenance (Boutton, 1991), however, C_3 and C_4 photosynthetic pathways also differ in shifts in ^{13}C between different specific plant tissues, between specific compounds and at the intramolecular level (Hobbie and Werner, 2004). Therefore, information on (i) the contributions of biomolecular components to bulk $\delta^{13}C$ values and (ii) the variation between $\delta^{13}C$ values of key biochemical components within specific plant communities would enhance understanding of the constraints on C cycling between pools at organism to field scales.

Many investigations have used bulk $\delta^{13}C$ values of foliar tissues to investigate C turnover in plants (Lockheart et al., 1998; Hobbie et al., 2002; Jumpponen et al., 2005; Li et al., 2007; Zheng and Shangguan, 2007) and have revealed genotypic and spatiotemporal variation due to differences in C isotope fractionation during carbon fixation, i.e. discrimination against the heavier ^{13}C isotope by Rubisco. In C_3 plants, isotopic fractionation is linked to photosynthesis via c_i/c_a, the ratio of intercellular to atmospheric CO_2 concentrations (Farquhar et al., 1989). Thus, variation in isotopic

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fractionation is caused by physiology, i.e. leaf size and thickness, stomatal density and canopy height, and environmental factors, such as soil moisture, temperature and irradiance, which combine to influence gas exchange through morphological and functional plant responses (Dawson et al., 2002). Such factors explain observed differences in C isotope fractionation with life form (Chen et al., 2005; Jumpponen et al., 2005; Li et al., 2007), and spatial (Zheng and Shangguan, 2007) and temporal (Smedley et al., 1991) gradients. In this paper, we present a comprehensive investigation of leaf bulk $\delta^{13}C$ values within a temperate grassland plant assemblage. In addition, we have determined $\delta^{13}C$ values for fatty acid and carbohydrate components of the same leaves in order to explore the variation in $\delta^{13}C$ values of biochemical components that contribute to bulk $\delta^{13}C$ values.

In leaves, fatty acids are found as components of cell membranes and epicuticular waxes, and are reported as being ^{13}C-depleted compared to bulk tissue (Schweizer et al., 1999; Grice, 2001). Isotopic fractionation against ^{13}C occurs during the oxidation of pyruvate to acetyl-CoA by pyruvate dehydrogenase (DeNiro and Epstein, 1977), and may also occur at branch points during fatty acid modification/membrane synthesis: (i) where acyl lipids undergo elongation and desaturation; (ii) when acyl lipids are diverted to membranes to undergo transacetylation to form phospholipids; and (iii) at the point of release of acyl lipids prior to reduction or decarboxylation to form lipid classes other than fatty acids. Thus, the overall processes governing plant fatty acid $\delta^{13}C$ values appear to be far from simple and require further investigation. Long chain *n*-alkyl (*n*-alkanoic, *n*-alkane and *n*-alkanol) components of plant waxes are often the focus of ^{13}C isotope investigations due to their importance as lipid biomarkers in organic geochemical applications (Lockheart et al., 1997; Wiesenberg et al., 2004; Bi et al., 2005; Chikaraishi and Naraoka, 2007). However, the majority of leaf lipids are glycerolipids dominated by the galactolipids, monogalactosyldiacylglycerol and digalactosyldiacylglycerol, that contain high abundances of polyunsaturated *cis*-9,12-octadecadienoic acid ($C_{18:2}$) and *cis*-9,12,15-octadecatrienoic acid ($C_{18:3}$). Along with hexadecanoic acid ($C_{16:0}$), these three *n*-alkanoic (or fatty) acids are the most abundant leaf lipids (Somerville et al., 2000) and are, therefore, the lipids of focus in this paper. However, lipids comprise a relatively minor proportion of plant biomass (around 2%; Brady and Weil, 2001), and, therefore, contribute less significantly to bulk $\delta^{13}C$ values.

Carbohydrates, as cellulose, hemicellulose, starch and simple sugars, constitute ca. <70% of green plant tissues (Taiz and Zeiger, 1991). As such, the stable isotope composition of these major compounds will be the primary influence on bulk $\delta^{13}C$ values of plant tissues, however, relatively little is known about the $\delta^{13}C$ values of plant carbohydrates at the molecular level. Carbohydrates are reported as being enriched in ^{13}C relative to bulk tissue (Deines, 1980; Loader et al., 2003) yet carbohydrate metabolism is complex and gives rise to ranges of $\delta^{13}C$ values of monosaccharide components. Macko et al. (1991) and Teece and Fogel (2007) showed several monosaccharide components were ^{13}C-depleted compared to bulk values in *Sphagnum cuspidatum* and *Juniper virginia*. Furthermore, Gleixner et al. (1993) observed a 4‰ difference in $\delta^{13}C$ values between soluble and structural fractions of glucose in plant matter due to isotopic fractionation by aldolase and/or triose phosphate isomerase enzymes (Brugnoli et al., 1988) and branching at the level of triose phosphates inducing enrichment in positions C-3 and C-4 of hexoses (Rossmann et al., 1991). Thus, biosynthetic processes give rise to inherent heterogeneity in $\delta^{13}C$ values of specific molecular components of carbohydrates. However, this variation is rarely considered in interpretations of bulk $\delta^{13}C$ values, and a comprehensive investigation of interspecific (between different species) variation in monosaccharide $\delta^{13}C$ values of a plant community has not yet been undertaken.

In this study, the $\delta^{13}C$ values of whole tissue, individual fatty acids and monosaccharides were determined to investigate interspecific variation within the plant assemblage of a mesotrophic grassland (MG5; Rodwell, 1992) at Port Meadow (Oxford, UK), a Site of Special Scientific Interest (SSSI). The site has been pastured by horses and cattle at least since the Domesday survey of 1085 (Tansley, 1965), and has characteristic associations of plants that are considered to reflect the influence of grazing on the balance of species better than any other British grassland (English Nature, 1977). MG5 grasslands have become increasingly rare as a result of agricultural improvement which decreases herb and grass species richness (Morris, 2005). Therefore, Port Meadow was considered a prime site to investigate interspecific variability of bulk and compound-specific $\delta^{13}C$ values of individual species contributing to a well-characterised plant community.

2. Results and discussion

2.1. Bulk tissues

A variety of different grass, herb, shrub and tree species were collected from Port Meadow on one sampling date in order to assess the natural variability in $\delta^{13}C$ values among the plant community. Since all of the individual plants sampled for each species were pooled together, tissue mean bulk $\delta^{13}C$ values (Fig. 1a) represent the variation in observed $\delta^{13}C$ values of whole leaf tissues between different plant species (the corresponding species-specific $\delta^{13}C$ values are given in Table 1). The mean leaf tissue bulk $\delta^{13}C$ value of the plants collected from Port Meadow was -29.3 ± 1.2‰ which is consistent with the $\delta^{13}C$ values expected for C_3 vegetation (Brugnoli and Farquhar, 2000). No significant differences between tissue bulk $\delta^{13}C$ values with life form were determined ($P = 0.40$). The mean bulk $\delta^{13}C$ values for grass, herb, shrub and tree species were -29.4 ± 1.4‰, -29.4 ± 1.2‰, -29.5 ± 0.3‰ and -27.8 ± 0.8‰, respectively. The most ^{13}C-enriched bulk value was determined for *Urtica dioica* ($\delta^{13}C = -27.2$‰) and the least ^{13}C-enriched was for *Bellis perennis* ($\delta^{13}C = -32.0$‰), both herbaceous perennials.

Differences in bulk $\delta^{13}C$ values at the species level reflect variation in physiological responses of plants to environmental factors influencing stomatal conductance and carboxylation rates. Strong genetic influences have been found to affect bulk $\delta^{13}C$ values among different varieties of the same species (Farquhar et al., 1989), and several studies have indicated that there may be a consistent ranking of bulk $\delta^{13}C$ values of plants of different species or life forms growing in the same area (Smedley et al., 1991; Mole et al., 1994; Brooks et al., 1997; Tsialtas et al., 2001). Typically, woody plants are reported to exhibit higher bulk $\delta^{13}C$ values than herbaceous plants, and different life forms can have average bulk $\delta^{13}C$ values varying by 2‰ or more Heaton (1999). Lloyd et al. (1992) found higher bulk $\delta^{13}C$ values of evergreen trees compared to deciduous trees and proposed this was due to differences in the conductance of CO_2 to the site of carboxylation. In this study, the overall mean bulk $\delta^{13}C$ value for tree leaf tissue was 2‰ more ^{13}C-enriched than grass and herbs, but also 'woody' shrubs *Crataegus monogyna* and *Sambucus nigra*. This may be accounted for by a 'canopy effect' of the overshadowing trees. Broadmeadow and Griffiths (1993) found vertical gradients (increasing with height) of bulk $\delta^{13}C$ values of leaves in forest trees. Other possible factors that may have an influence on $\delta^{13}C$ values and variation with species are (i) plant responses to competition (Williams et al., 1991), (ii) average root depth (Tsialtas et al., 2001), (iii) lifespan and growth rate (Smedley et al., 1991) and (iv) dependence on mycorrhizae (Handley et al., 1993).

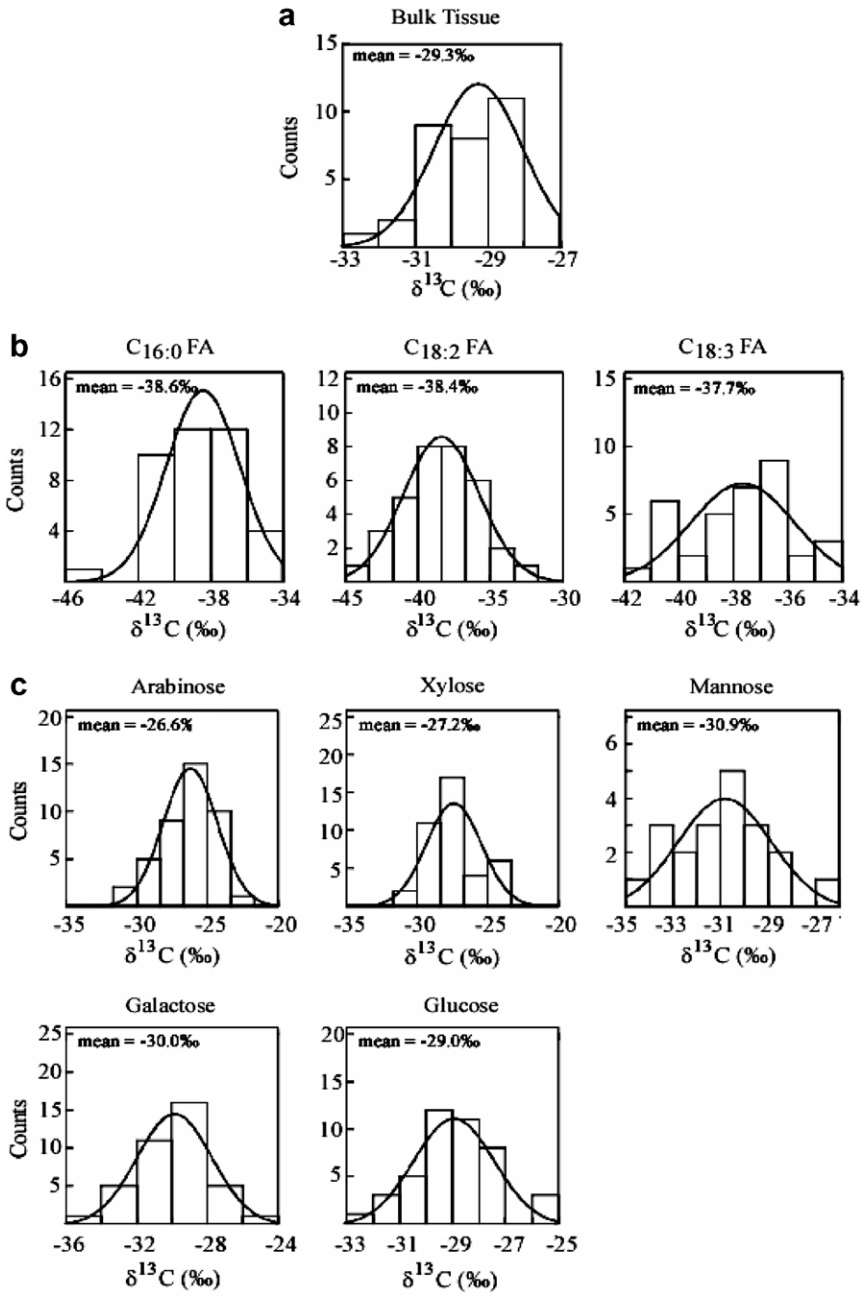

Fig. 1. Histogram representations with superimposed distribution curves of $\delta^{13}C$ values of: (a) whole leaf tissue, (b) leaf fatty acids $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$, and (c) leaf monosaccharides arabinose, xylose, mannose, galactose and glucose, from plants collected at Port Meadow.

2.2. Fatty acid analyses

Fatty acid compositions of plant leaves are highly consistent among species. Palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and α-linolenic acid ($C_{18:3}$) normally constitute over 90% of total leaf fatty acids (Padley et al., 1986) with $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ being the most abundant components (Somerville et al., 2000). Fig. 2a shows a gas chromatogram of fatty acids extracted from *Plantago media*. The high abundance of saturated C_{16} and unsaturated C_{18} fatty acids is clear. Average percentage fatty acid contents for $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ of leaf tissues from all plant species analysed were 20%, 13% and 56%, respectively (Fig. 2b). These average fatty acid compositions are in good agreement with those reported for leaves of crop species (Padley et al., 1986; Wiesenberg et al., 2004), edible wild plants (Guil et al., 1996; Guil-Guerrero and Rodriguez-Garcia, 1999; Liu et al., 2002) and tree species (Mongrand et al., 2001; Rawlins et al., 2005).

The determination of $\delta^{13}C$ values of individual fatty acids from a wide variety of plant species provides an opportunity to observe general trends between these compounds and to contribute to the knowledge base of lipid $\delta^{13}C$ values among wild plant species. As expected, in all plant species collected at Port Meadow, the $\delta^{13}C$ values of fatty acids were depleted compared to whole leaf tissues (Fig. 1b). The overall depletion of lipid $\delta^{13}C$ values are due to the kinetic isotope effect associated with decarboxylation from pyruvate to acetyl CoA (DeNiro and Epstein, 1977; Melzer and Schmidt, 1987) and the non-statistical distribution of ^{13}C in the glucose molecule (Fig. 3; Rossmann et al., 1991; Gleixner and Schmidt, 1997). $\delta^{13}C$ values of $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ were generally higher in trees and shrubs (Table 1; $\text{mean}_{C16:0+C18:2+C18:3} = \delta^{13}C$ $-36.0 \pm 3.5‰$,

Table 1
The $\delta^{13}C$ values of whole leaf tissues and major individual fatty acids of the plant species collected from Port Meadow

Species collected	Life form	Bulk tissue	$C_{16:0}$	$C_{18:2}$	$C_{18:3}$
Dactylis glomerata	Grass	−28.4	−38.0	−39.0	−36.0
Holcus lanatus	Grass	−30.4	−37.8	−37.1	−36.9
Mean grass		−29.4 (1.4)	−37.9 (0.1)	−38.1 (1.3)	−36.5 (0.6)
Achillea millefolium	Herb	−31.2	−40.3	−40.6	−37.2
Alisma plantago-aquatica	Herb	−27.8	−34.7	−33.6	−34.3
Bellis perennis	Herb	**−32.0**	−40.7	−41.7	−40.1
Centaurea nigra	Herb	−30.7	−41.2	−41.1	−39.4
Cerastium holosteoides	Herb	−28.2	−36.6	−36.4	−35.6
Cirsium arvense	Herb	−28.2	−37.4	–	–
Cirsium vulgare	Herb	−28.7	−38.4	−39.8	−37.7
Crepis sp.	Herb	−30.2	−40.8	–	−39.2
Galium verum	Herb	−29.7	−38.2	−37.2	−37.6
Leucanthemum vulgare	Grass	−29.5	**−44.0**	**−44.2**	−40.1
Lotus tenuis	Herb	−30.5	−40.3	−40.3	−40.1
Medicago lupulina	Herb	−29.0	−36.9	−37.1	−36.2
Myosotis scorpiodes	Herb	−29.5	−41.1	−43.3	−40.7
Plantago lanceolata	Herb	−28.8	−41.2	−40.9	**−41.8**
Plantago major	Herb	−30.8	−40.6	−42.1	−40.5
Plantago media	Herb	−27.4	−38.2	−39.5	−38.2
Potentilla anserine	Herb	−29.2	−39.1	−39.1	−36.9
Potentilla reptans	Herb	−29.2	−38.6	−38.5	−36.9
Prunella vulgaris	Herb	−30.2	−41.4	−40.6	−40.2
Ranunculus acris	Herb	−30.7	−40.0	−39.5	−38.9
Ranunculus repens	Herb	−28.4	−38.7	−37.7	−37.3
Rumex acetosa	Herb	−28.5	−39.1	−36.3	−37.7
Rumex conglomeratus	Herb	−28.4	−36.7	−34.3	−38.3
Rumex palustris	Herb	–	−36.3	−36.1	−35.5
Stellaria graminea	Herb	−27.7	−38.5	−35.5	−36.1
Taraxacum officinale	Herb	−30.4	−38.6	−39.5	−38.5
Trifolium pratense	Herb	−30.1	−37.8	−37.6	−36.5
Trifolium repens	Herb	−28.7	−38.5	−37.2	−37.5
Urtica dioica	Herb	−27.2	−36.7	−37.4	−36.9
Veronica serpyllifolia	Herb	−31.0	−38.3	−37.1	−38.1
Mean herb		−29.4 (1.2)	−39.0 (2.0)	−38.7 (2.6)	−38.1 (1.8)
Crataegus monogyna	Shrub	−29.7	−36.7	–	−37.3
Sambucus nigra	Shrub	−29.3	−37.0	−36.6	−36.3
Mean shrub		−29.5 (0.3)	−36.9 (0.2)	−36.6 (–)	−36.8 (0.7)
Acer campestre	Tree	−27.5	−35.1	−33.3	−35.4
Malus sylvestris	Tree	−27.3	−37.4	−39.4	−37.0
Salix fragilis	Tree	−28.7	−36.0	−36.0	−34.7
Mean tree		−27.8 (0.8)	−36.2 (1.2)	−36.2 (3.1)	−35.7 (1.2)
Total mean		−29.3 (1.2)	−38.6 (2.0)	−38.4 (2.6)	−37.7 (1.8)

Figures in bold are maximum values, and those underlined are minimum values.

and −36.7 ± 0.7‰, respectively) than herbs and grasses ($mean_{C16:0+C18:2+C18:3}$ = −38.6 ± 3.7‰ and $\delta^{13}C$ −37.5 ± 1.4‰, respectively. In particular the $\delta^{13}C$ value of $C_{16:0}$ was significantly different between life forms ($P = 0.05$). However, there was wide variation between the highest and lowest $\delta^{13}C$ values for $C_{16:0}$ (−34.7‰, *Alisma plantago-aquatica*; −44.0‰, *Leucanthemum vulgare*), $C_{18:2}$ (−33.3‰, *A. campestre*; −44.2‰, *L. vulgare*;) and $C_{18:3}$ (−34.3‰, *B. perennis*; −41.8‰, *Plantago lanceolata*).

Mean $\delta^{13}C$ values for the $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ of fatty acids of leaf tissues collected from Port Meadow were −38.6 ± 2.0‰, −38.4 ± 2.6‰ and −37.7 ± 1.8‰, respectively (Fig. 1b). This shows a trend towards ^{13}C-enrichment with increasing acyl chain length and unsaturation. This trend, i.e. $\delta^{13}C_{16:0} < \delta^{13}C_{18:2} < \delta^{13}C_{18:3}$, is similar to that described by Ballentine et al. (1998) and Huang et al. (1999) for C_3 and C_4 species. This suggests the $\delta^{13}C$ values of individual plant fatty acids are likely to be controlled by specific biological mechanisms that are common to all higher plants. Further depletions in the ^{13}C content of lipids has been shown to be caused, in unicellular organisms, by the C branching ratios at pyruvate, alternate sources of acetyl-CoA, and downstream isotope effects (Blair et al., 1985) including: (i) elongation and desaturation, (ii) diversion to membranes to undergo transacetylation to form phospholipids; and, (iii) at the point of release of acyl lipids prior to reduction or decarboxylation (Monson and Hayes, 1980, 1982a,b; Hayes, 2001). Collister et al. (1994) and Lockheart et al. (1997) determined depletion in the $\delta^{13}C$ values of plant *n*-alkanes with increasing chain length (1‰ from C_{28} to C_{30} alkane), and Dungait (2005) found similar depletions in very long chain fatty acids (VLCFAs; $>C_{21}$) in *Lolium perenne* and *Zea mays* silages. However, a more comprehensive survey of plant fatty acids in different ecosystems, and at different spatiotemporal scales, is required to explore the ubiquity and mechanistic basis for this phenomenon.

2.3. Carbohydrate analysis

Reported $\delta^{13}C$ values of individual monosaccharides from plants are relatively scarce and are for individual species from a range of ecosystems (Table 2). Thus, our study is the first to determine $\delta^{13}C$ values of these compounds among a wide variety of species within a single plant community. Fig. 4a shows a typical gas chromatogram of monosaccharides extracted from *Taraxacum officinale*. This shows that the production of the alditol acetates of monosaccharides from the hydrolysate of total carbohydrate has the advantage of producing single, well-resolved peaks for each sugar. Average relative abundances for arabinose, xylose, mannose, galactose

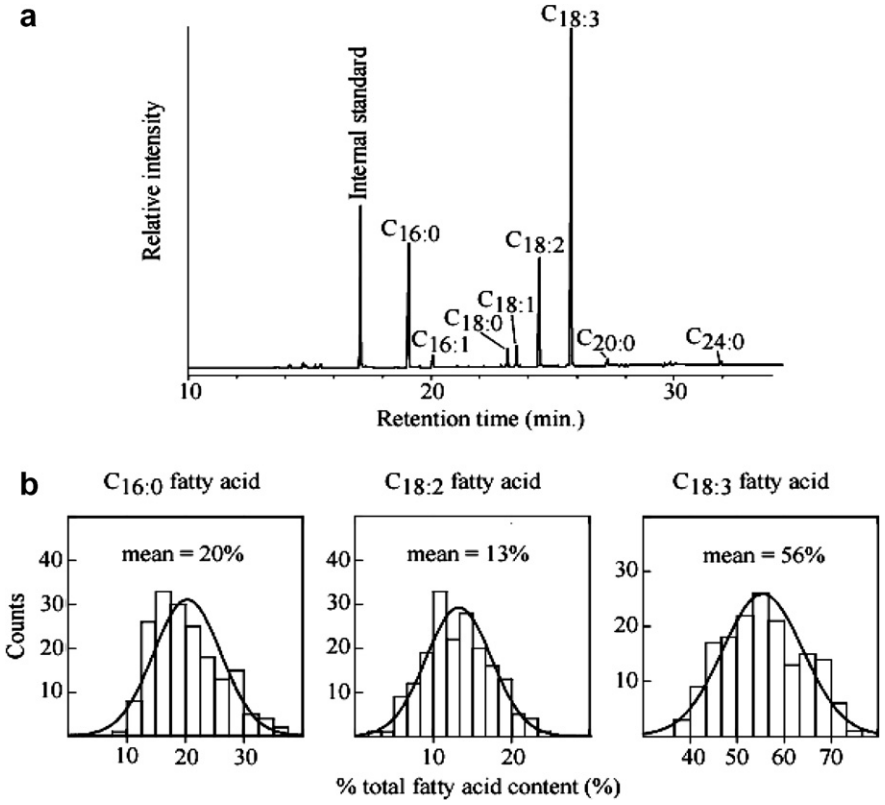

Fig. 2. (a) 'Typical' partial gas chromatogram of fatty acids isolated from *Plantago media* leaf tissue as their respective FAMEs; internal standard = *n*-pentadecanoic acid ($C_{15:0}$); (b) Histogram representations with superimposed distribution curves of percentage total leaf fatty acid content of $C_{16:0}$, $C_{18:2}$, and $C_{18:3}$ fatty acids from leaves of plants sampled at Port Meadow and Manor Farm (Dungait et al., in preparation).

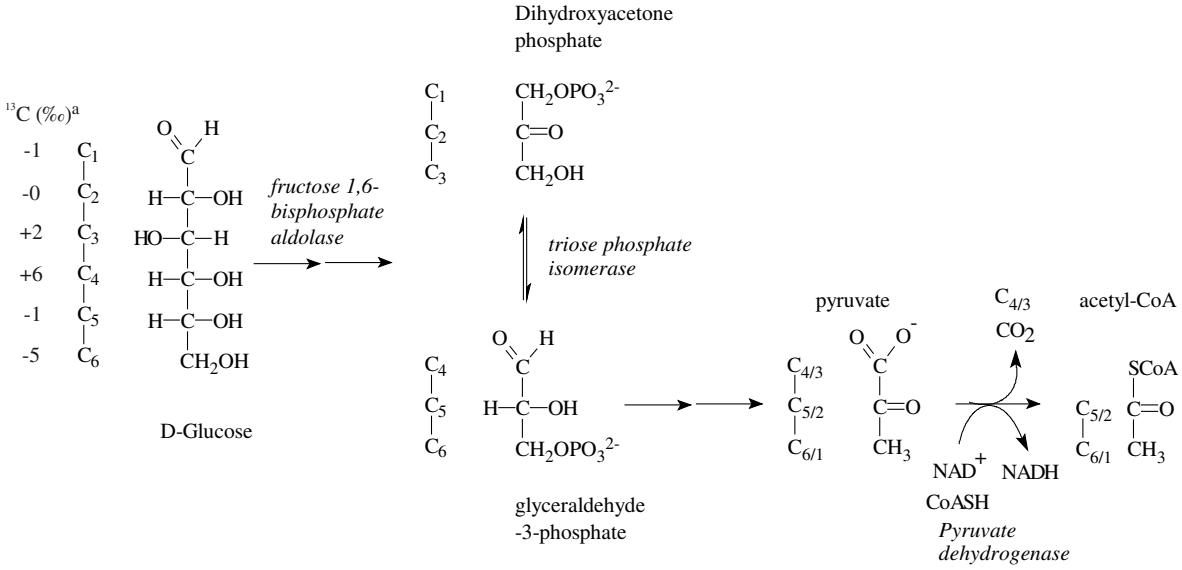

Fig. 3. The biosynthetic fate of individual C atoms within glucose and the non-uniform distribution of C isotopes among the individual C atoms of glucose (adapted from Rossmann et al., 1991). Numbers on the C skeleton on the left hand side indicate the numbering of the glucose carbons. [a]The $\Delta^{13}C$ values (‰) indicate the difference in isotopic composition of each individual glucose C from the mean $\delta^{13}C$ value of the whole glucose molecule.

and glucose in plants from Port Meadow were 12%, 13%, 5%, 12% and 54%, respectively (Fig. 4b). Glucose was the most abundant monosaccharide; arabinose, xylose and galactose were present in roughly similar abundances, with a smaller proportion of mannose. However, in the grass species analysed, a relatively higher concentration of xylose and lower concentration of galactose was observed, reflecting differences in the hemicellulosic polysaccharides between monocotyledonous and dicotyledonous species. Therefore, the $\delta^{13}C$ values of these five most abundant monosaccharides are reported in this paper.

Fig. 1c represents the variation in observed $\delta^{13}C$ values of individual monosaccharides among different plant species. Significant differences between life forms were found for arabinose ($P = 0.03$), galactose ($P = 0.01$) and glucose ($P = 0.05$), particularly between

Table 2
Reported $\delta^{13}C$ values for monosaccharides and bulk $\delta^{13}C$ values from plant material

Species	$\delta^{13}C$ (‰)								Reference
	Life form	C_3/C_4	Bulk	Arabinose	Xylose	Mannose	Galactose	Glucose	
Triticum aestivum leaf	Grass	C_3	−28	−24	−27			−26	Derrien et al., 2006
Zea mays leaf	Grass	C_4	−12	−10	−12			−12	Derrien et al., 2006
Erica tetralix	Herb	C_3	−26	−18	−21			−24	van Dongen et al., 2002
Mangrove leaf	Tree	C_3	−28	−19	−20	−25	−26	−24	Moers et al., 1993
Juniper virginia wood	Tree	C_3	−24	−24	−28	−30	−32	−30	Teece and Fogel, 2007
J. virginia wood NDF	Tree	C_3	−25		−21	−22	−25	−18	Teece and Fogel, 2007
J. virginia wood ADF	Tree	C_3	−25		−21	−26		−27	Teece and Fogel, 2007
Sphagnum sp.	Moss	C_3	−26	−28	−26	−27	−26	−26	Macko et al., 1991
S. cuspidatum	Moss	C_3	−26		−20			−21	van Dongen et al., 2002

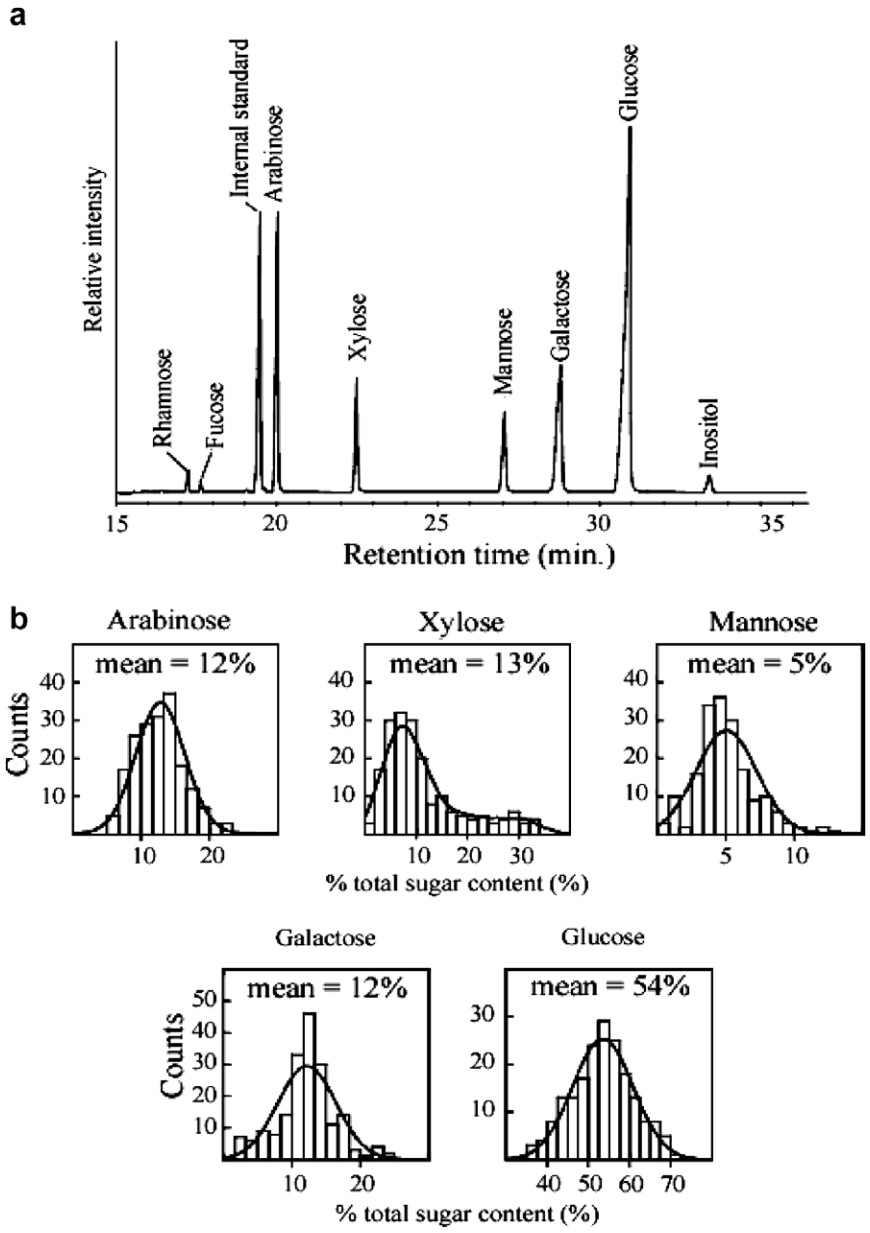

Fig. 4. (a) 'Typical' partial gas chromatogram of total monosaccharides extracted from lipid-extracted *Taraxacum officinale* leaf tissue as their respective alditol acetates. Internal standard = pentaerythritol. (b) Histogram representations with superimposed distribution curves of percentage total leaf monosaccharide content of arabinose, xylose, mannose, galactose and glucose from leaves of plants sampled at Port Meadow and Manor Farm (Dungait et al., in preparation).

herbs and trees. The mean $\delta^{13}C$ values of the major monosaccharides across all of the plant species analysed were −26.6 ± 2.1‰ (arabinose), −27.2 ± 2.4‰ (xylose), −30.9 ± 2.0‰ (mannose), −30.0 ± 2.0‰ (galactose) and −29.0 ± 1.6‰ (glucose). The species-specific values contributing to this mean are given in Table 3. This shows that non-woody herbs and grasses generally had

Table 3
The $\delta^{13}C$ values of major individual monosaccharides of the plant species collected from Port Meadow

Species collected	Life form	Arabinose	Xylose ‰	Mannose	Galactose	Glucose
Dactylis glomerata	Grass	−24.4	−27.4	–	−29.0	−28.9
Holcus lanatus	Grass	−26.0	−29.2	–	−27.8	−29.8
Mean grass		−25.2 (1.1)	−28.3 (1.3)	–	−28.4 (0.8)	−29.4 (0.6)
Achillea millefolium	Herb	−30.4	−28.4	−30.6	−32.3	−31.0
Alisma plantago-aquatica	Herb	−27.2	−25.3	–	−26.9	−27.7
Bellis perennis	Herb	−32.3	−31.0	−30.6	−33.9	−32.2
Centaurea nigra	Herb	−25.5	−28.6	−28.9	−31.8	−29.2
Cerastium holosteoides	Herb	−26.9	−28.9	−31.3	−30.3	−29.4
Cirsium arvense	Herb	−26.4	−26.8	–	−29.8	−27.6
Cirsium vulgare	Herb	−26.6	−26.8	−30.3	−31.4	−28.5
Crepis sp.	Herb	−31.4	−31.2	−33.7	**−34.3**	−30.9
Galium verum	Herb	−25.5	–	–	−30.3	−29.1
Leucanthemum vulgare	Herb	−28.5	−25.6	−30.2	−30.4	−29.2
Lotus tenuis	Herb	−26.4	−27.4	−33.7	−31.2	−30.6
Medicago lupulina	Herb	−26.7	−27.6	–	−29.2	−28.2
Myosotis scorpiodes	Herb	−28.4	−29.8	−29.6	−31.3	−29.8
Plantago lanceolata	Herb	−27.8	−29.5	−29.2	−29.5	−29.4
Plantago major	Herb	−29.3	−25.6	–	−28.8	−31.0
Plantago media	Herb	−25.4	−23.5	−28.7	−28.6	−28.0
Potentilla anserine	Herb	−25.7	−26.8	–	−32.2	−30.4
Potentilla reptans	Herb	−25.5	−27.7	–	−29.5	−29.2
Prunella vulgaris	Herb	−25.4	−29.8	−31.6	−29.7	−30.7
Ranunculus acris	Herb	−27.9	−29.2	–	–	−31.9
Ranunculus repens	Herb	−27.9	−28.2	–	−30.6	−29.2
Rumex acetosa	Herb	−26.7	−24.1	**−26.5**	−30.7	−29.8
Rumex conglomeratus	Herb	−24.9	**−20.4**	−29.0	−28.9	−28.3
Rumex palustris	Herb	−24.3	−24.3	–	−28.8	−27.0
Stellaria graminea	Herb	−28.4	−28.6	−33.1	−31.2	−28.8
Taraxacum officinale	Herb	−27.5	−28.6	−31.4	−32.4	−29.2
Trifolium pratense	Herb	−25.5	−27.4	–	−33.2	–
Trifolium repens	Herb	−25.7	−27.9	−32.2	−28.9	−27.7
Urtica dioica	Herb	−24.3	–	–	**−26.2**	−25.9
Veronica serpyllifolia	Herb	−28.4	−27.9	−32.0	−31.8	−30.4
Mean herb		−27.1 (2.0)	−27.4 (2.4)	−30.7 (1.9)	−30.5 (1.9)	−29.3 (1.5)
Crataegus monogyna	Shrub	−23.7	−24.6	–	−28.3	−28.0
Sambucus nigra	Shrub	−25.7	−27.7	−30.1	−29.5	−28.0
Mean shrub		−24.7 (1.4)	−26.2 (2.2)	−30.1 (–)	−28.9 (0.8)	−28.0 (0.0)
Acer campestre	Tree	−23.9	−23.8	−34.3	−27.6	−25.9
Malus sylvestris	Tree	**−22.2**	−23.4	–	−26.5	**−25.8**
Salix fragilis	Tree	−24.6	−27.4	–	−28.9	−27.4
Mean tree		−23.6 (1.2)	−24.9 (2.2)	−34.3 (–)	−27.7 (1.2)	−26.4 (0.9)
Total mean		−26.6 (2.1)	−27.2 (2.4)	−30.9 (2.0)	−30.0 (2.0)	−29.0 (1.6)

MWcpd*m*, weighted mean $\delta^{13}C$ values calculated using Eq. (1). Figures in bold are maximum values, and those underlined are minimum values.

more ^{13}C-depleted monosaccharides than trees and shrubs, in a similar fashion to fatty acids. However, the overall similarity of individual monosaccharide $\delta^{13}C$ values to whole leaf bulk $\delta^{13}C$ values is in contrast to the large difference determined for individual fatty acid $\delta^{13}C$ values relative to bulk $\delta^{13}C$ values of whole leaf tissue. This reflects the generally observed difference between primary (e.g. monosaccharides) and secondary (e.g. lipids) metabolic products that arise primarily from the isotopic fractionation associated with the enzymes fructose-1,6-bisphosphate isomerase and pyruvate dehydrogenase (Fig. 3; Gleixner and Schmidt, 1997).

Like the fatty acids described above, a general relationship between individual monosaccharide $\delta^{13}C$ values, $\delta^{13}C_{arabinose} > \delta^{13}C_{xylose} > \delta^{13}C_{glucose} > \delta^{13}C_{galactose}$, was observed (mannose abundance was below limits that would provide robust isotope values in seventeen species). This trend was also observed by Moers and Larter (1993) and Teece and Fogel (2007) for monosaccharides derivatised as alditol acetates from mangrove leaves and juniper wood, respectively (Table 2). However, $\delta^{13}C$ values for each monosaccharide represent a weighted average value of the $\delta^{13}C$ values of the monosaccharide liberated from each class of polysaccharide. For instance, although the majority of the glucose component consists of glucose liberated from cellulose, it will also derive in part from free glucose, and as constituents of starch, some fructans and hemicellulose. Teece and Fogel (2007) determined a 12‰ range of $\delta^{13}C$ values for glucose from whole juniper wood (−30‰; Table 2), its neutral detergent fraction (−18‰; NDF: lignin, cellulose and hemicellulose) and acid detergent fraction (−27‰; ADF; cellulose and lignin).

The major monosaccharide glucose, that constituted 54% leaf sugars, was on average 0.5‰ more ^{13}C-enriched relative to whole leaf tissues (Fig. 5). This is in good agreement with previous studies which suggested that sucrose and starch were *ca.* 1‰ ^{13}C-enriched compared with whole leaf tissues (Brugnoli et al., 1988) and cellulose was <2‰ more enriched than whole leaf tissue (Ehleringer, 1991). Derrien et al. (2006) determined ^{13}C-enrichment of 2‰ for glucose from *Triticum aestivum* leaves compared to bulk tissue, and van Dongen et al. (2002) observed a 2‰ ^{13}C-enrichment in glucose from *Erica tetralix* tissues. However, a wide range of $\delta^{13}C$ values have been reported for glucose from the same species. van Dongen et al. (2002) determined a 5‰ ^{13}C-enrichment in glucose of *Sphagnum cuspidatum* tissues, compared with Macko et al. (1991) who found no difference in glucose and bulk $\delta^{13}C$ values in *Sphagnum* sp. Moers and Larter (1993) determined a 4‰

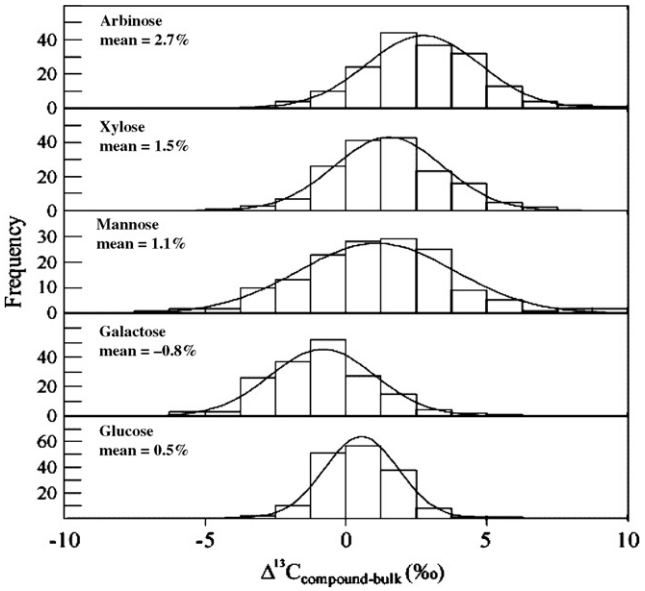

Fig. 5. The relationship of individual monosaccharide $\delta^{13}C$ values to the whole leaf tissue bulk $\delta^{13}C$ value. A value of 0‰ indicates coincidence with the bulk $\delta^{13}C$ value of the leaf.

^{13}C-enrichment in glucose $\delta^{13}C$ values in mangrove leaves. In this study, the mean $\delta^{13}C$ value for glucose from all species was −29.0‰ (Fig. 1c), but $\delta^{13}C$ values ranged from −32.2‰ (*B. perennis*) to −25.8‰ (*M. sylvestris*; Table 3). However, a maximum difference of 1.7‰ was observed between glucose and bulk tissue for *Crataegus monogyna* in this study.

Hemicelluloses are reported as having enriched $\delta^{13}C$ values compared to whole leaf tissues and the cellulose fraction (Deines, 1980), and, on average, arabinose, xylose and mannose were more ^{13}C-enriched than whole leaf tissue by 2.7‰, 1.5‰ and 1.1‰, respectively (Fig. 5). Maximum $\delta^{13}C$ values were determined for arabinose from *M. sylvestris* (−22.2‰), xylose from *Rumex conglomeratus* (−20.4‰) and mannose from *Rumex acetosa* (−26.5‰). Relative ^{13}C-enrichment has also been determined for arabinose and xylose (Moers and Larter, 1993; van Dongen et al., 2002; Derrien et al., 2006) and mannose (Moers and Larter, 1993). The higher $\delta^{13}C$ values of the xylose and arabinose relative to glucose could be due to the loss of an isotopically light C, i.e. C-6 (Fig. 2) during the inter-conversion from glucose. This explanation has been proposed for the observed enrichment in the ^{13}C isotope of xylose relative to glucose isolated from the photoautotrophic bacterium, *Chloroflexus aurantiacus* (van der Meer et al., 2001) and for aquatic and terrestrial plants (van Dongen et al., 2002). An even higher ^{13}C-enrichment of arabinose relative to xylose may, therefore, be due to isotopic fractionation during the C-4 epimerase reaction or as a result of the different salvage pathways that operate for these two sugars. However, Teece and Fogel (2007) and Moers and Larter (1993) reported ^{13}C-depleted values compared with bulk leaf values (Table 2). In this study, a minority of species also displayed $\delta^{13}C$ values for mannose, arabinose and xylose, e.g. −34.3 ± 0.1‰ (mannose; *A. campestre*), −31.4 ± 0.1‰ (arabinose; *Crepis* sp.) and −31.2 ± 0.1‰ (xylose, *Crepis* sp.), showing that the pathways of C recruitment into carbohydrates is far from predictable. *Crepis* sp. also exhibited the most depleted $\delta^{13}C$ value for galactose (−34.3 ± 0.3‰). Overall, in all plants investigated (with the exception of *Plantago major*) galactose was more ^{13}C-depleted than bulk leaf tissue. This is similar to the findings of Teece and Fogel (2007); Table 2. This depletion in ^{13}C is probably a result of isotopic fractionation specific to the biosynthesis of galactose. The more negative $\delta^{13}C$ value of galactose could account, at least in part, for the wide variation of the $\delta^{13}C$ value of hemicellulose described by Deines (1980). In addition to the sources of variation already mentioned above for glucose, the wide variety of hemicelluloses from which arabinose, xylose, mannose and galactose derive is also likely to cause differences between $\delta^{13}C$ values of these sugars due to variation between biosynthetic pathways, and during the incorporation of monosaccharides into polysaccharides (Macko et al., 1991). However, despite these complexities, the trend: $\delta^{13}C_{arabinose} > \delta^{13}C_{xylose} > \delta^{13}C_{glucose} > \delta^{13}C_{galactose}$, was consistent across a wide range of species. More work is required to elucidate the precise mechanisms responsible for this regular pattern among the $\delta^{13}C$ values of individual monosaccharides comprising the leaf tissues of plants.

3. Conclusions

The mean leaf tissue bulk $\delta^{13}C$ value was −29.3‰. $\delta^{13}C$ values for woody species sampled from the hedgerow at Port Meadow ranged from −27.2‰ to −32.0‰, and there were no significant differences between bulk tissue $\delta^{13}C$ values and life form in this study. Fatty acids $C_{16:0}$, $C_{18:2}$ and $C_{18:3}$ constituted nearly 90% of the total fatty acid composition of leaf tissues and possessed depleted $\delta^{13}C$ values relative to bulk tissue. Although the variation in $\delta^{13}C$ values of different plant fatty acids obtained from different species was large, there was a general trend between $\delta^{13}C$ values where $\delta^{13}C_{16:0} < \delta^{13}C_{18:2} < \delta^{13}C_{18:3}$. Monosaccharides arabinose, xylose, mannose, galactose and glucose constituted 96% of total leaf carbohydrate. A trend was also seen between monosaccharide $\delta^{13}C$ values at species level where $\delta^{13}C_{arabinose} > \delta^{13}C_{xylose} > \delta^{13}C_{glucose} > \delta^{13}C_{galactose}$.

Thus, we have shown (i) important differences in compound-specific $\delta^{13}C$ values contributing to leaf bulk $\delta^{13}C$ values; (ii) interspecific variability between bulk and compound-specific $\delta^{13}C$ values of the leaves of individual grassland species; and (iii) general trends between individual fatty acid and monosaccharide $\delta^{13}C$ values common to leaves of all species within one plant community. Further work is required to explore whether the identified trends differ spatiotemporally between and within mesotrophic grasslands and other ecosystems.

4. Experimental

4.1. Sample handling and preparation

Plants were collected from the highest and driest part of Port Meadow, Oxford, UK (National Grid Reference SP495083) in June 2000. The average monthly temperature was 15.1 °C and average monthly rainfall was 35 mm. The site is neutral grassland developed on calcareous gravels deposited on Oxford Clay, and is an unimproved permanent pasture with continuous grazing giving rise to MG5 *Cynosaurus cristatus-Centaurea nigra* grassland (Rodwell, 1992). An area of 20 m^2 was sampled and several individuals of thirty-seven plant species (Tables 1 and 3) were collected and pooled. Representative samples of leaves from trees and shrubs growing in adjacent hedgerows were taken; to minimise variation in $\delta^{13}C$ values all tree and shrub samples were taken at approximately the same height, and compass orientation, from the outermost branches. Leaves free from disease and insect damage were wrapped individually in aluminium foil pouches and stored at −20 °C before freeze-drying immediately prior to extraction. Several leaves from each plant species were dried and ground to a fine powder in a mortar and pestle with liquid nitrogen. This britalises the tissues and disrupts cell membranes, and allows woody tissues to be ground to a very fine powder, ideal for subsequent solvent and acid extractions.

4.2. Extraction and derivatisation

4.2.1. Preparation of fatty acid methyl esters (FAMES)

Two hundred microlitres of *n*-pentadecanoic acid solution (1 mg ml^{-1}) was added to 50 mg of dried and ground plant material as an internal standard. Lipids were extracted by ultra-sonication with a DCM/Methanol solution (2:1 v/v, 3 × 2 ml) to give a total lipid extract (TLE). The remaining lipid-extracted residue was retained for carbohydrate analysis. A portion of the TLE (2 ml) was transferred to a Teflon capped test tube and the solvent evaporated under a gentle stream of nitrogen. The TLE was saponified with 0.5 M sodium hydroxide in methanol at 75 °C for 1 h. The saponified mixture was allowed to cool and neutral components removed with repeated hexane washes (3 × 2 ml). The remaining solution was acidified to pH 3 with 2 M hydrochloric acid and fatty acids extracted with hexane (3 × 2 ml). The hexane was removed under a gentle stream of nitrogen and FAMES prepared with BF_3/methanol (14% w/v, 100 μl, 30 min, 80 °C). The reaction was halted by the addition of double distilled water (2 ml) and FAMES extracted with *n*-hexane (3 × 2 ml). After evaporation of the solvent, the FAMES were re-dissolved in 0.2 ml hexane prior to analyses by GC, GC/MS and GC/C/IRMS.

4.2.2. Preparation of alditol acetates of monosaccharides

The method of Blakeney et al. (1983) was followed with modifications. Ten milligrams of lipid-extracted plant material was dissolved in 0.1 ml 72% sulphuric acid in a Young's tube. The mixture was held at room temperature with periodic vortexing to facilitate cellulose hydrolysis. After 1 h the acid was diluted with 0.9 ml water to give approximately 1 M sulphuric acid and then heated at 100 °C for between 6 and 8 h. After cooling, the solution was made neutral and then slightly alkaline by the addition of 0.3 ml 18 M ammonia solution. Ten microlitres of pentaerythritol solution (20 mg ml^{-1}) was added as an internal standard. A 0.4 ml portion of the hydrolysate was transferred into a Teflon capped test tube and the liberated monosaccharides reduced to their corresponding alditols by reaction with 2 ml of sodium borohydride solution (2 g $NaBH_4$ in 100 ml dimethyl sulfoxide; 40 °C, 90 min). Excess $NaBH_4$ was destroyed by the addition of 0.2 ml glacial acetic acid. The alditols were acetylated by reaction with 0.2 ml *N*-methylimidazole and 1 ml acetic anhydride. Excess acetic anhydride was destroyed by the addition of 5 ml water and the alditol acetates recovered by extracting with 2 ml diethyl ether. After drying over magnesium sulphate, the diethyl ether was evaporated under a stream of nitrogen and the alditol acetates dissolved in 0.2 ml DCM prior to analysis by GC, GC/MS and GC/C/IRMS.

4.3. Instrumental analyses

4.3.1. Gas chromatography (GC)

All GC analyses were performed on a Hewlett–Packard 5890 Series II gas chromatograph fitted with a dedicated on-column capillary inlet. Separation of compounds was achieved using a fused silica capillary column coated with a stationary phase appropriate for the nature of the compounds being analysed (described below). Hydrogen was used as a carrier gas at a pressure of 10 psi, FID detection was employed and sample peaks were acquired and integrated using Hewlett–Packard Chemstation software. Quantification of components present in the fatty acid and monosaccharide GC profiles were performed relative to the appropriate internal standard.

4.3.1.1. Separation of FAMES. A Chrompack CP-wax 52 CB column (polyethylene glycol, 60 m × 0.32 mm, 0.25 μm film thickness) was used with the following temperature program: initial temperature 50 °C held for 1 min, then to 150 °C at 20 °C min^{-1}, then to 230 °C at 4 °C min^{-1} and then holding for 14 min.

4.3.1.2. Separation of alditol acetates. An SGE BPX-70 column (70% cyanopropyl polysilphenylene-siloxane, 60 m × 0.32 mm, 0.25 μm film thickness) was used with the following temperature program: initial temperature 50 °C held for 1 min, then to 200 °C at 20 °C min^{-1}, then to 230 °C at 4 °C min^{-1} and then holding for 22 min.

4.3.2. GC/mass spectrometry (GC/MS)

GC/MS analyses were performed on a Carlo Erba 5160 GC, using the same capillary columns and conditions as above but with He as a carrier gas and on-column injection. The GC was coupled, via a heated transfer line, to a Finnigan MAT 4500 quadrupole mass spectrometer (electron voltage 70 eV, filament current 0.35 mA, electron multiplier 2 kV).

4.3.3. Elemental analyser IRMS

On-line bulk $\delta^{13}C$ analysis of dried and ground leaf tissues were performed using a Carlo Erba NC2500 elemental analyser coupled to a Finnigan MAT DELTA S stable isotope ratio mass spectrometer. Depending on the C content of the sample, between 1 and 3 mg of homogeneous material was placed in a tin capsule and loaded into the elemental analyser for analysis. Samples were analysed in triplicate and the mean value determined. Standard compounds with known $\delta^{13}C$ values were run frequently in between batches of compounds to test instrument reliability.

4.3.4. GC/combustion/ isotope ratio mass spectrometry (GC/C/IRMS)

$\delta^{13}C$ values determined by injecting 1 μl of diluted sample into a Varian 3400 gas chromatograph fitted with an appropriate column using He as a carrier gas. Sample peaks were eluted through a combustion reactor (Cu/Pt, 850 °C) and passed via a water separator to a Finnigan MAT DELTA S IRMS (electron ionisation 80 eV, electron energy 1 mA, faraday cups for masses 44, 45 and 46). 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 each run. All $\delta^{13}C$ values are reported relative to the Pee Dee Belemnite international isotope standard. Each sample was run three times to obtain reliable mean $\delta^{13}C$ values. Co-injected standard compounds with known $\delta^{13}C$ value ($C_{11:0}$, $C_{13:0}$, $C_{21:0}$ and $C_{23:0}$; Sigma–Aldrich, UK) were included in every run in order to determine the performance of the GC/C/IRMS system. The standards were chosen due to the rare occurrence of these compounds in plants and the fact that they have sufficiently different retention times than the fatty acids of interest.

4.3.4.1. Correction for the isotopic composition of exogenous derivative carbon. Mass balance (Eq. (1)) was used to correct for the isotopic composition of the exogenous C introduced in the methyl group of derivatised fatty acids. The $\delta^{13}C$ value of the C added during derivatisation was determined by analysing a sample of the BF_3/MeOH derivatising reagent by IRMS (−43.3 ± 0.1‰).

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

where *n* is the number of C atoms, *c* is the underivatised compound, *d* is the derivatising agent, and cd is the derivatised compound.

A kinetic isotope effect occurs during the acetylation of monosaccharides as a bond to the carbonyl C of the acetic anhydride is broken in the reaction intermediate during the rate-limiting step. Therefore, the $\delta^{13}C_d$ term of Eq. (1) was replaced with $\delta^{13}C_{corr}$ (Docherty et al., 2001) to represent the specific correction factor for each molecule of interest.

4.5. Statistical treatment of data

Statistical analysis of the data throughout this study was carried out using Systat 7.0 for Windows (Systat, Inc.). Either paired or unpaired *t*-tests were applied, depending on the level of correspondence between data points, to test the null hypothesis that the mean $\delta^{13}C$ values of two groups were equal. One-way Analysis of Variance (ANOVA) was used to test for a difference among groups in a particular variable. The Bonferroni pair-wise procedure was used to protect both multiple *t*-tests and ANOVA.

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