

# **Variability in the carbon isotope composition of individual amino acids in plant proteins from different sources: 1 Leaves**

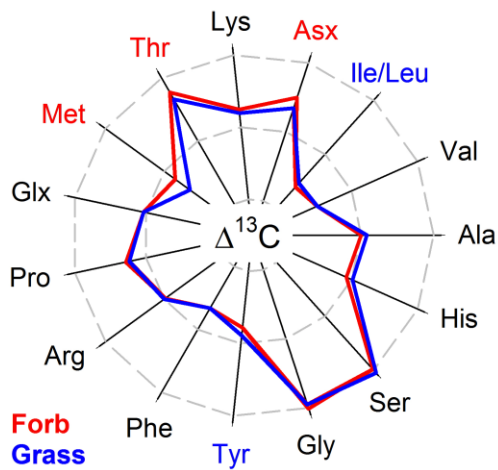
Anthony H. Lynch<sup>a,c</sup>, Nicholas J. Kruger<sup>b</sup>, Robert E.M. Hedges<sup>a</sup> and James S.O. McCullagh<sup>c\*</sup>

<sup>a</sup> Laboratory for Archaeology and the History of Art, South Parks Road, University of Oxford, Oxford  
OX1 3QY, UK

<sup>b</sup> Department of Plant Sciences, South Parks Road, University of Oxford, Oxford  
OX1 3RB, UK

<sup>c</sup> Department of Chemistry, Mansfield Road, University of Oxford, Oxford OX1 3TA, UK

\*Corresponding author: email [james.mccullagh@chem.ox.ac.uk](mailto:james.mccullagh@chem.ox.ac.uk), tel +44 1865 275657, fax: +44 1865  
285002.



Radar plot shows normalised natural-abundance  $^{13}\text{C}$  isotope values of individual amino acids from plant leaf proteins vary consistently between plant groups, but are little influenced by season, environment or species. Values that are significantly greater in either forbs or grasses are indicated in red and blue, respectively.

## ABSTRACT

The natural carbon isotope composition of individual amino acids from plant leaf proteins has been measured to establish potential sources of variability. The plant leaves studied, taken from a range of plant groups (forbs, trees, grasses, and freshwater aquatic plants), showed no significant influence of either season or environment (water and light availability) on their  $\Delta\delta^{13}\text{C}$  values. Plant groups did, however, differ in carbon isotope composition, although no consistent differences were identified at the species level. A discriminant analysis model was constructed which allowed leaves from (1) nettles, (2) Pooideae, (3) other Poales, (4) trees and (5) freshwater higher plants to be distinguished from each other on the basis of their natural abundance  $^{13}\text{C}/^{12}\text{C}$  ratios of individual amino acids. Differences in carbon isotope composition are known to be retained, to some extent, in the tissues of their consumers, and hence an understanding of compound-specific variation in  $^{13}\text{C}/^{12}\text{C}$  fractional abundance in plants has the potential to provide dietary insights of value in archaeological and ecological studies.



## Keywords

Nettle (*Urtica dioica* L.); Grass; Tree; Aquatic plant;  $^{13}\text{C}/^{12}\text{C}$  isotope fractionation; Amino acid; Leaf, metabolism.

## 1. Introduction

Variability in the carbon isotope composition of whole plant tissue has long been known (Wickman 1952) and the basis for a major component of this variation is relatively well understood in terms of the physiology of photosynthesis (Farquhar et al., 1989). The major source of carbon isotope discrimination is due to the mechanism involved in the fixation of carbon by photosynthesis. In  $C_3$  plants, fixation leads to a depletion in  $\delta^{13}C$  of ca. 20‰ in the carbon of Calvin cycle intermediates compared with source  $CO_2$ , itself at ca. -8‰ (Farquhar et al., 1982). In  $C_4$  plants, this is much lower, giving depletions of ca. 4‰. In both types of plant, however, further isotope discrimination takes place during subsequent metabolic processes. The reaction rates of many enzymes differ according to the isotope composition of their substrates, giving rise to a change in isotope composition between reactant and product (e.g. Smith and Epstein, 1971). The conversion of only a small proportion of the reactant into a product will generally result in isotope fractionation, leading to a product that is modified in  $^{13}C/^{12}C$  isotope ratio (compared with more complete conversion), while extensive conversion tends to leave the residual reactant more enriched in  $^{13}C$ . Further, different fluxes through enzymes in reaction networks will also influence the isotope compositions of the metabolites in these networks (Hayes, 2001; Schmidt, 2003; Schmidt et al., 2015). Therefore, whilst the major differences in  $^{13}C/^{12}C$  of plant metabolites are explained by common metabolic processes such as photosynthesis, genetic and environmental variation between plants will potentially have an additional significant and measurable effect on isotopic composition.

Thus, differences in the carbon isotopic composition of individual plant compounds can provide information about both the nature of the metabolic processes occurring and the relative rates of the component reactions (Schmidt et al., 2015). For example, acetogenic lipids become more depleted in  $^{13}C$  than their precursors (DeNiro and Epstein, 1977) due, at least partially, to the kinetic isotope effect of pyruvate dehydrogenase (Melzer and Schmidt, 1987) and the relative fluxes of pyruvate into lipid synthesis and alternative pathways. Hence, the study of isotope compositions

of individual compounds at natural abundance presents additional levels of information on the underlying physiological and biochemical processes (Tcherkez et al., 2011).

It is also known that different degrees of isotope fractionation occur in different species, and indeed in different tissues from the same species. Thus, the pattern of  $^{13}\text{C}$  fractionation associated with metabolites should provide information on the nature and extent of the metabolic processes taking place in individual plant species. This variability is then reflected in the carbon isotope composition of the tissues of animals consuming these plants, a correlation widely exploited in contemporary ecological studies of the diet, migration of animals, or palaeodietary analysis (Gannes et al., 1998). The isotope composition of bulk plant tissue represents a weighted mean of the differing isotope composition of the various chemical entities of which the tissue is composed, principally a variable mix of protein, lipid and carbohydrate (Dungait et al., 2008). Pioneering work by Abelson and Hoering (1961) showed that the carbon isotope composition varies between amino acids in individual organisms and also, often to a greater extent, within the same amino acid obtained from different organisms. More recent work has shown variability in isotope fractionation between autotrophic, heterotrophic and acetoclastic organisms (Scott et al., 2006), and among plants, bacteria and fungi (Larsen et al., 2009, 2013).

However, currently, information on individual amino acid  $\delta^{13}\text{C}$  values from plants, and how these vary both within and between plant groups, is limited. This is in part due to the technical challenges of performing compound specific isotope analysis (CSIA). Until recently, this involved either separation of the amino acids prior to individual analysis of their  $\delta^{13}\text{C}$  values by isotope ratio monitoring by mass spectrometry following combustion in an elemental analyser (irm-EA/MS), or their conversion to volatile derivatives for analysis by irm-MS linked to gas chromatography (irm-GC/MS) (Larsen et al., 2013). The former is tedious and risks incomplete recovery of the analytes, whereas the latter requires introduction of correction factors for the added carbon, hence a source

of inaccuracy. However, an online liquid chromatography method for the CSIA of amino acids from physiological tissues based on irm-LC/MS has been developed (McCullagh et al., 2006) which, in conjunction with a new sample extraction protocol, can be extended for the  $\delta^{13}\text{C}$  analysis of amino acids in plant leaves (Lynch et al., 2011).

Previous studies have already established that the relative isotope composition of amino acids from proteins of photosynthetic (leaf) tissue differs between individual amino acids (Fogel and Tuross, 2003), and that it also differs between leaf and seed (heterotrophic) tissues of the same plant (Lynch et al., 2011). However, almost no data exist comparing natural abundance  $\delta^{13}\text{C}$  values of amino acids between different terrestrial plant species. The identification of any inter-specific differences in these  $\delta^{13}\text{C}$  values could be particularly informative; as such differences would provide evidence of metabolic and/or environmental variation between species and offer proof of principle that the contribution of different plant species to the palaeodiet may be detected through differing carbon isotope compositions of protein.

Recent studies have concluded that the  $\delta^{13}\text{C}$  values of aquatic plants (Larsen et al., 2013) and algae (Larsen et al., 2015) are rather insensitive to environmental parameters. However, as there is a paucity of information on the variation of natural abundance isotope composition in terrestrial plants, we decided to exploit recently-developed techniques for the  $\delta^{13}\text{C}$  analysis of free and protein-bound amino acids (Lynch et al., 2011) to investigate inter-species variation in the isotope composition of amino acids in plants of (palaeo)dietary importance. In the present study, leaves from three main plant groups were analysed: forbs (a single species, nettle), grasses (Poales including reeds, rushes and sedges) and deciduous trees (Fagales and Sapindales). These plants were selected for their potential dietary significance for animal consumers in a northern European archaeological context, notably with the aim of being able to distinguish the habitats of domesticated cattle and aurochs. Nettle was selected as the model forb as it has been proposed to have been used as a human food in, for example, Mesolithic Denmark (Kubiak-Martens, 1999). For the selected species,  $\delta^{13}\text{C}$  (‰) values of individual amino acids from proteins were measured and Lynch *et al.* (2016)

analysed in relation to environmental (light/shade and adequate/abundant water availability), seasonal (spring/autumn) and species influences. Limited sampling of other plants (brambles and freshwater aquatic plants) was undertaken to provide an indication of broader variability between species and to allow cross-comparison with existing data on aquatic species (Larsen et al., 2013, 2015).

## **2. Results and Discussion**

### **2.1. Variation in $^{13}\text{C}$ isotope composition of leaf protein**

The range of  $\delta^{13}\text{C}$  (‰) determined for the total proteins extracted from the plants under consideration was ca. -25 to -35‰ (Table 1). To facilitate comparison of carbon isotope composition from different plants, the  $\delta^{13}\text{C}$  values for the amino acids obtained by the acid hydrolysis of plant proteins are expressed as normalized  $\Delta\delta^{13}\text{C}$  (the difference between  $\delta^{13}\text{C}$  (expressed in parts per thousand) of an individual amino acid and that of the weighted mean of all measured amino acids in the sample adjusted for the relative C atom abundance of amino acyl residues within the plant protein extract; see Section 4.2). Considerable variation is seen in the individual values of carbon isotope composition (Tables 2 & 3). However, the pattern in  $\Delta\delta^{13}\text{C}$  is relatively consistent from one species to another. Positive values are found for Thr, Gly and Ser in all cases, while Val, Ile/Leu, Phe and Tyr always give negative values. Such consistency is to be expected, considering that the main biosynthetic pathways remain the same across the species, with the notable exception of the role of photorespiration in Gly and Ser metabolism (see below). Similar  $\delta^{13}\text{C}$  amino acid patterns have been observed in plants by others (Abelson and Hoering, 1961; Larsen et al., 2009, 2013).

### **2.2. Seasonal and environmental impact on variation in carbon isotope composition of leaves**

With changing season plants are subjected to variation in light, temperature and day-length, all of which might impact on the carbon isotope composition of amino acids. This was examined using nettle (*Urtica dioica* L.), a model forb that is readily identifiable, and found growing naturally Lynch *et al.* (2016)

in environments that vary in light intensity and water availability. These environmental factors both affect the  $\delta^{13}\text{C}$  value of bulk plant tissue (Pearcy and Pfitsch, 1991; Bloch et al., 2006) and may differentially influence the metabolism of individual amino acids. Nonetheless, for nettle leaves, the general pattern of  $\Delta\delta^{13}\text{C}$  differences between individual amino acids shows little seasonal variation (Fig. 1A), although Leu/Ile and Tyr were significantly more relatively enriched in  $^{13}\text{C}$  in spring (March to May) than summer (June to August) by 0.6‰ (s.e. 0.14) and 0.8‰ (s.e. 0.23) respectively, whereas Lys was significantly more depleted in  $^{13}\text{C}$  by 0.5‰ (s.e. 0.15). Leu/Ile and Phe were significantly more depleted in  $^{13}\text{C}$  in summer than in autumn (September to November) by 0.5‰ (s.e. 0.18) and 1.2‰ (s.e. 0.44) respectively.

Similarly, leaves from deciduous trees displayed limited seasonal variability (Fig. 1B). The only significant differences were relative  $^{13}\text{C}$  enrichments in Val, Asp and Met in the autumn, by 1.0‰ (s.e. 0.17), 1.7‰ (s.e. 0.37) and 7.4‰ (s.e. 2.17) respectively, compared with the spring. However, the pattern of amino acid isotope variation between seasons was not significant as assessed by discriminant function analysis.

Shading has been shown to reduce the  $\delta^{13}\text{C}$  value of bulk leaf tissue by ca. 5 ‰, compared with full sunlight, because the rate of carbon fixation is more dependent on the activity of the isotopically highly-fractionating enzyme Rubisco than on the less-discriminating diffusional processes (Farquhar et al., 1982). The degree of shading had only a small influence on the relative carbon isotope composition of individual amino acids of leaf proteins of nettles harvested from open or shaded locations in this study (Supplementary Fig. S1). The  $\Delta\delta^{13}\text{C}$  values for Ile/Leu, Arg and Tyr in nettles in an open environment are each significantly more depleted, by ca. 1.1‰ (s.e. 0.10 to 0.25), than those in shaded nettles. Conversely, the  $\Delta\delta^{13}\text{C}$  value for Phe is ca. 1.1‰ (s.e. 0.30) higher in nettles in an open site. It is not clear why this is so but it could relate to the depletion in Tyr. Of interest is the larger difference between shade and sun for Ser of about 3‰ (s.e. 2.1), possibly related to photorespiration, which might also explain the larger variation in the values. In contrast,

for leaves of trees harvested from open and shaded environments, there is no indication that the relative isotope compositions of individual amino acids of leaf proteins differ.

### 2.3. *Variability in carbon isotope composition of the amino acid pool between species*

Since there is little variation in the relative  $^{13}\text{C}/^{12}\text{C}$  isotope ratios of individual amino acids attributable to seasonal factors or to the limited range of environmental influences studied, differences between species were examined using pooled data from plants taken at different times of year (Table 2 and Supplementary Fig. S2).

The amino acids for which the  $^{13}\text{C}/^{12}\text{C}$  isotope ratios differ highly significantly between pairs of plant types are shown in Table 3. These differences were sufficient to allow statistical discrimination between the plant groups examined. There were also some significant differences in the  $^{13}\text{C}/^{12}\text{C}$  isotope ratio of individual amino acids within plant groups (results for grasses are shown in Supplementary Table S1 and for trees in Supplementary Table S2). However, it was not possible to construct discriminant analysis models which were robust enough to resolve different species within the identified plant functional groups.

The discriminant analysis model constructed to distinguish between leaves from nettle, Pooideae, reeds, trees and freshwater higher plants comprised four statistically significant discrimination functions (Supplementary Table S3). The first two functions accounted for 61.1% and 17.6%, respectively, of inter-group variability. Individuals for which full isotope information is available are plotted using the first two discriminant functions in Fig. 2. The model gives 81% correct group classification for samples used for modelling and correctly assigned 71% of the samples withheld during modelling. These results indicate that plant types can be differentiated on the pattern of variation in carbon isotope ratios of their amino acids, and discrimination at this level is not compromised by fluctuations arising from either seasonality or shading.

### 2.4. *Variability in carbon isotope composition of individual amino acids*

In an actively photosynthesising leaf, the majority of protein is likely to be formed using amino acids generated de novo from assimilated CO<sub>2</sub>. Consequently we might anticipate that the carbon isotope abundance of a protein-derived amino acid will reflect that of the principal metabolic intermediate(s) from which it is produced. Hence any difference in the pattern of isotopic labelling between individual amino acids will be due to variation in the isotopic composition of the precursors, or differences in the pathways of amino acid biosynthesis or their subsequent turnover. Despite this, within our data there is no obvious relationship between the  $\delta^{13}\text{C}$  values of amino acids produced from the carbon skeleton of a mutual metabolic precursor. A similar lack of correlation between  $\delta^{13}\text{C}$  values of amino acids of shared metabolic origin has been observed in cyanobacteria (Hayes, 2001). Thus, the majority of variation in  $\delta^{13}\text{C}$  between amino acids cannot be explained by a simple linear model of isotope fractionation during biosynthesis. This lack of equivalent isotope fractionation in biosynthetically-related amino acids presumably reflects the organization of the metabolic network, in particular (i) the existence of branch points within the pathways to groups of amino acids, and (ii) the potential for alternative routes of biosynthesis for an amino acid. Such complications make it difficult to provide a precise interpretation of the  $\delta^{13}\text{C}$  values of individual amino acids. However, comparisons of the relative <sup>13</sup>C isotope abundance of individual amino acids within and between plant groups do reveal several notable features.

#### 2.4.1. *Glycine and serine*

The <sup>13</sup>C enrichments of Gly and Ser are generally ca. 10‰ greater than the mean for all amino acids across the samples studied. This is likely to be due to photorespiration. In the mitochondria of C<sub>3</sub> leaves, the photorespiratory loss of <sup>13</sup>C-depleted CO<sub>2</sub> due to the glycine decarboxylase complex fractionates against <sup>13</sup>C (Tcherkez, 2006), leaving the remaining glycine molecules <sup>13</sup>C-enriched in C-1: so is serine. This can have far-reaching effects since C-1-enriched serine can be recycled to the Calvin cycle via glyceraldehyde-3-phosphate hence to the C-4 position in glucose (Gilbert et al., 2012).

The extent of enrichment will depend on the size of the photorespiratory flux. Thus, although there are some strong positive correlations between the isotope composition of Gly and that of Ser and/or Thr (Table 6), this is not the case for all plants. The correlation between Gly and Ser is not significant in grassy plants and trees, for example, suggesting that other metabolic pathways involving these amino acids are important here. The correlation between the  $\Delta\delta^{13}\text{C}$  values of Gly and Thr, especially in freshwater plants, could be explained by a large proportion of the Gly in these plants being derived from the catabolism of Thr (Joshi et al., 2006). Whether a similar pattern is found in freshwater algae is unknown since data for Gly are not presented in the relevant publications (Larsen et al., 2013, 2015).

#### 2.4.2. Valine

In most plants Val, in common with other branched-chain amino acids, is typically ca. 5-6 ‰ more depleted in  $^{13}\text{C}$  than the mean for all amino acids. However, this effect is less pronounced in freshwater plants in which Val is enriched in  $^{13}\text{C}$  (by ca. 2.5‰) relative to leaves of terrestrial plants (Table 1 and Supplementary Fig. S2). Since this amino acid is synthesised solely from pyruvate, factors influencing the isotope composition of pyruvate will contribute directly to that of Val. Pyruvate is also a precursor of lipid synthesis, principally the production of alkanolic acids via acetyl-CoA. In freshwater plants n-alkanoic acids from are ca. 8.4‰ more depleted in  $^{13}\text{C}$  than their bulk tissue, while those from  $\text{C}_3$  plants are only ca. 3.7‰ more depleted (Chikaraishi et al., 2004). In contrast, sterols (also synthesised from pyruvate, but via the mevalonic acid pathway) are only ca. 0.9‰ depleted in  $^{13}\text{C}$  relative to bulk tissue in freshwater plants, and 1.1‰ more depleted in  $\text{C}_3$  plants.

These observations suggest that n-alkanoic acid biosynthesis in aquatic plants may discriminate against  $^{13}\text{C}$  more strongly than in terrestrial  $\text{C}_3$  plants, thus contributing to a  $^{13}\text{C}$  enrichment of the remaining pyruvate pool through mass balance considerations. Val synthesised from this pyruvate will reflect its relative enrichment in  $^{13}\text{C}$ . While attractive, these arguments are

not conclusive. For example, the lower extent of impoverishment of sterols in aquatic plants compared with terrestrial C<sub>3</sub> plants remains to be explained. Nevertheless, small (statistically non-significant) enrichments in the  $\Delta\delta^{13}\text{C}$  values of the other pyruvate-derived amino acids Ala and Leu/Ile (+1.1‰ and +0.5‰ respectively) in freshwater plants relative to terrestrial C<sub>3</sub> plant leaves support the explanation based on preferential <sup>13</sup>C-enrichment of pyruvate.

#### 2.4.3. Aspartate/asparagine

During acid hydrolysis of proteins, Asn is de-amidated to Asp and thus  $\delta^{13}\text{C}$  values of the latter will reflect the isotopic composition of a combination of Asn and Asp. However, given their close metabolic origins, the  $\delta^{13}\text{C}$  values of the component residues in the Asn/Asp couple are likely to be very similar, even though this has yet to be established experimentally. (Similar considerations apply to Gln and Glu, both of which yield Glu during analysis.) The  $\Delta\delta^{13}\text{C}$  value of Asp as measured from nettles (+4.9‰) and bramble (+5.0‰), is significantly more positive than that in Pooideae (+3.4‰), reeds (+2.8‰), or trees (+2.8‰). Asp is produced by transamination of oxaloacetate which is formed from, *inter alia*, malate generated in the tricarboxylic acid cycle in mitochondria and also by carboxylation of phosphoenolpyruvate in the cytosol. The later (anaplerotic) route is catalysed by PEP carboxylase, which leads to a relative enrichment in <sup>13</sup>C in the product (Melzer and O’Leary, 1987). Thus, the relative contributions of these two sources, which are likely to vary depending on the metabolic state of the cell and the plant species, will have a significant influence of the  $\delta^{13}\text{C}$  value of Asp. Other factors that will influence the <sup>13</sup>C/<sup>12</sup>C ratio are its further metabolism – it is a precursor for Thr, Ile and Lys – and its relative flux. It is noteworthy that in nettle Asn is the principal free amino acid in xylem, accounting for 40-60% of the total, while Gln accounts for only ca. 10-20% at low levels of nitrogen nutrition (Rosnitschek-Schimmel, 1985). It is probable that the relative importance of these amino acids in N transport affects their <sup>13</sup>C isotope ratios in these plants.

#### 2.4.4. Lysine, threonine and methionine

These three amino acids, together with Asp, are all derived from oxaloacetate. Despite their common biosynthetic origin, the only significant isotope correlation between them (among individual plant groups) is the negative correlation between Thr and Met (Table 4), providing support for the view that metabolic fractionation plays a more significant role in influencing the resulting  $\delta^{13}\text{C}$  value than biosynthetic proximity. Some of these amino acids correlate positively with Asp within plant groups, although other significant correlations are negative.

#### 2.4.5. *Proline and arginine*

Pro and Arg are both derived from Glu and may therefore be expected to have similar carbon isotope compositions (Hayes, 2001). Comparisons of the relative isotope abundance of all three amino acids in grasses and, more widely, between Pro and Arg are broadly consistent with this expectation (Table 5). However, as seen with the oxaloacetate group of amino acids (see 2.4.4), the correlations between normalised  $\Delta\delta^{13}\text{C}$  values of Glu, Pro and Arg are generally weak and frequently inconsistent across the plant groups. These comparisons again suggest that further metabolic features are contributing to the relative carbon isotope composition of these amino acids..

Pro is synthesised from Glu by reduction with NADH or NADPH. Variation in the availability of reducing power from these sources in different plant species or under different environmental conditions will affect the kinetics of Pro biosynthesis, and thus potentially alter the relationship of its isotope composition to that of its source Glu. Although both of these coenzymes are also used in reductive steps in the biosynthetic pathways of other amino acids, the relative impact of variation in their availability may be reduced by differences in the relative demand for individual amino acids and the inherently greater opportunities for regulation within the longer biosynthetic pathways.

Arg is synthesised from Glu by a long sequence of steps via the non-proteinogenic amino acids ornithine and citrulline (Slocum, 2005). Citrulline plays a particularly important role in trees as a major form in which nitrogen is remobilised (Barnes, 1963; Millard et al., 1998; Bertani et al.,

2006). This may influence the relationship between the  $^{13}\text{C}$  isotope abundance of Glu and Arg in such plants.

#### 2.4.6. *Aromatic amino acids*

The  $\delta^{13}\text{C}$  values for Phe and Tyr are ca. 1-4‰ negative relative to the mean value for all amino acids (Figs. 1A,B and S2). The aromatic amino acids Phe and Tyr are both synthesised via the shikimic acid pathway, with their routes of synthesis diverging only towards the end of the pathway. Despite this, their  $^{13}\text{C}$  isotope abundances are not strongly correlated. The correlation coefficients for comparison of  $\Delta\delta^{13}\text{C}$  values of Phe and Tyr across all plants studied and within the individual groupings of nettles, reeds, Pooideae, trees and freshwater aquatic plants are +0.120, +0.239, -0.524, +0.098 and +0.040, respectively. Of these, the only significant positive correlation is within nettles ( $p = 0.027$ ) whereas the negative correlation within Pooideae is highly significant ( $p < 0.001$ ).

This lack of a consistent correlation is likely to be linked to the catabolic fates of these two amino acids. Both serve as precursors for products other than proteins following an initial deamination: Phe by phenylalanine ammonia lyase to cinnamate, and Tyr by tyrosine ammonia lyase to 4-hydroxycinnamate. These reactions lead to marked depletion in  $^{13}\text{C}$  at the  $\alpha$ -carbon atom of the product (Schmidt and Gleixner, 1998), resulting in a corresponding  $^{13}\text{C}$  enrichment of the remaining amino acid.

The role of Phe as a precursor of further metabolites is much greater than that of Tyr. Cinnamate is the precursor for a wide range of plant products, notably lignin and flavanoids. Lignin represents a major component of global net primary production (said to constitute 25% (Graham, 1993, p219)), second in importance only to cellulose. Lignin is depleted in  $^{13}\text{C}$  by between 4 and 7‰ relative to cellulose (Benner et al., 1987). In plants that synthesize large quantities of lignin, this reaction pathway will represent a major fate for Phe, and the residual pool of free Phe (available for protein synthesis) will be isotopically enriched in  $^{13}\text{C}$ . In contrast, although Tyr may be incorporated

into lignin in grasses (Higuchi et al., 1967), this is likely to be a quantitatively far less significant drain and thus not result in such a pronounced  $^{13}\text{C}$  enrichment of the Tyr pool.

By extension, the relative enrichment in  $^{13}\text{C}$  of Phe in protein from leaves of nettles grown in an open, rather than a shaded, environment (Fig. S1) may be attributable to differences in lignin content. Lignin content in nettle leaves increases with maturity (Sultan et al., 2009) and is greater in the (older) leaves towards the base of the plant (Bacci et al., 2009). Nettles in a shaded environment are likely to mature more slowly than those from an open environment and thus have lower lignin content. As a result of decreased demand for cinnamate for lignin production, the extent of enrichment of  $^{13}\text{C}$  in the pool of free Phe is likely to be reduced in shaded plants.

### 3. Conclusions

The analyses of the carbon isotope composition of leaf-protein amino acids from a range of plants indicates that carbon isotope fractionation associated with biosynthesis and metabolism in plants leads to significant differences in  $\delta^{13}\text{C}$  values between amino acids in the same tissue. The  $\delta^{13}\text{C}$  values obtained suggest that isotope compositions are influenced to a greater extent by differential partitioning of metabolic flux at branching points than the fractionation *per se* associated with *de novo* biosynthesis (i.e. isotope fractionation is most commonly associated with  $^{13}\text{C}$  discrimination during enzymatic conversions leading to products enriched or depleted in  $^{13}\text{C}$  relative to the substrates of the reaction).

Amino acid  $\delta^{13}\text{C}$  (‰) values therefore have the potential to provide a rich source of information about amino acid metabolism and the relative contribution of competing metabolic processes in plants. By expressing data as the  $\Delta\delta^{13}\text{C}$  (‰), the relationship between isotope abundance of individual amino acids and that of the sum of all proteogenic amino acids for a particular species is emphasized, since fluctuations in  $\delta^{13}\text{C}$  (‰) values arising from variations in external conditions such as season, light intensity or water status, are factored out. One of the most

Lynch *et al.* (2016)

interesting features to emerge when the data are expressed in this way is that individual amino acid  $\delta^{13}\text{C}$  values in plants are highly variable; arguably, more so than we might have anticipated. This variation is advantageous since it increases the potential range of isotope biomarkers of the variation in metabolic activity of different plant types, and may even be used to differentiate between plants (Fig. 2). Thus, while this study has examined only a limited range of plant species, it establishes the principle that variation in the amino acid isotope signature of proteins can be used to delimit the botanical origin of the material, and this paves the way for more comprehensive systematic studies.

Such variation has implications for other areas of investigation including analysis of food chains in ecosystem studies and paleodietary reconstruction. The carbon isotope compositions of individual amino acids in the protein of bone collagen reflect those in the diet (Jim et al., 2006; Dunn et al., 2011), and in particular, the isotope signatures of the ‘essential’ amino acids, which cannot be synthesised in animals (Reeds, 2000), are strongly retained. Ultimately these compositions originate in plants and may represent plant values relatively unchanged. Thus the  $\delta^{13}\text{C}$  values of individual amino acids in the protein of the tissues of consumers should more precisely reflect the composition of their diet than does the  $\delta^{13}\text{C}$  value of total (bulk) tissue protein, and this offers the potential to improve the resolution of the information provided by analysis of amino acid  $^{13}\text{C}/^{12}\text{C}$  isotope signatures.

## **4. Experimental**

### *4.1. Plant sampling*

#### *4.1.1. Nettles*

Samples of (apparently healthy) nettles were taken from locations in Oxfordshire (Harpsden Wood, SU763807 and Cold Bath, Mill Meadows, Henley, SU768819) and Wiltshire, grown in different situations of subjectively-assessed water and light availability. Samples were taken at different times

Lynch *et al.* (2016)

of year (April, May, June, July and October 2008) in order to identify potential influences of plant growth stage on measured  $\delta^{13}\text{C}$  values.

#### 4.1.2. *Deciduous trees*

Samples of leaves were taken from individual trees throughout Oxfordshire. These were: hazel, *Corylus avellana* L. (Henley SU763818); oak, *Quercus robur* L. (Henley SU747842); horse chestnut, *Aesculus hippocastanum* L., and beech, *Fagus sylvatica* L. (Nuffield SU656889 and SU673879) at different times during the autumn (September and October 2007). Further leaf samples were taken from different individual trees at the same sites and times of year (2008) used for nettle sampling in Oxfordshire, to establish seasonal and inter-individual variation (see Section 4.2.1). Tree leaf samples were taken at heights between 1 and 2 m, to reflect likely accessibility by browsing animals. At Harpsden Wood, all trees were in shaded locations. At the Cold Bath site, hazel samples were taken from trees in both open and shaded environments, while oak leaves were taken from trees in open environments only.

#### 4.1.3. *Leaves from other plants*

Plants with different growth habits and from different environments were selected to provide a broader basis for inter-specific comparison. Dryland plants included several grasses (*Melica uniflora* Retz., *Deschampsia cespitosa* (L.) P. Beauv., *Dactylis glomerata* L.), and bramble (*Rubus fruticosus* L.), taken from Harpsden Wood (SU763807) at the same time as nettles were sampled (see Section 4.2.1). Wetland terrestrial plants included pondside rush, sedge and reed (*Juncus inflexus* L., *Carex flacca* Schreb. and *Phragmites australis* (Cav.) Steud.) from Mill and Marsh Meadows, Henley (SU768819 and nearby). Aquatic plants were also taken from this area: *Cladophora* sp. and *Zannichellia palustris* L. (freshwater ponds, depth ca. 0-10 cm), *Lemna minor* L. and *Riccia fluitans* (stagnant pool, depth ca. 0-2 cm), and *Nuphar lutea* Sibth. & Sm. and *Sparganium* sp. (River Thames, depth ca. 10-20cm). *M. uniflora*, *D. cespitosa*, *D. glomerata*, *J. inflexus*, *C. flacca*,

*Z. palustris* and *Sparganium* were identified by Dr Stephen Harris (Department of Plant Sciences, University of Oxford).

#### 4.2. Experimental procedures

Plant protein was extracted and hydrolysed using the methods described previously (Lynch et al., 2011). Briefly, crushed freeze-dried leaves (ca. 3 g) were extracted with aq. Buffer (pH 9-11) containing 0.125 M sodium tetraborate, 3% (v/v) 2-mercaptoethanol and 1% (w/v) sodium dodecyl sulphate. Proteins precipitated from these extracts using 10% (w/v) trichloroacetic acid were hydrolyzed using 6 M HCl at 110°C for 24 h. Preliminary analysis established that although this procedure can result in loss of up to 20% of the Met and Arg, the isotope values obtained for these amino acids were not affected.

The carbon isotope composition of individual amino acids was determined by irm-MS coupled on-line to HPLC, as described previously (McCullagh et al., 2006, Lynch et al., 2011). Separation is achieved using a mixed-mode Primesep A HPLC column and compounds eluted with a mobile phase comprising a gradient of water to 0.03M H<sub>2</sub>SO<sub>4</sub>. Following elution, the amino acids were oxidized quantitatively in the aqueous phase to carbon dioxide using peroxodisulphate in a Finnigan Isolink LC stage and the carbon isotope ratio of the resulting CO<sub>2</sub> measured with a Delta V Plus irm-MS (Thermo Fisher Scientific). The  $\delta^{13}\text{C}$  value of each of the amino acids in the mixture was measured using an internal standard (2-aminoisobutyric acid (Smith et al., 2009)), with  $\delta^{13}\text{C}$  of -32.85‰ ( $\pm 0.3\text{‰}$ , n=3) measured using an irm-EA/MS. Amino acid isotope composition is expressed as a normalised  $\Delta\delta^{13}\text{C}$ , defined as the difference between  $\delta^{13}\text{C}$  (expressed in parts per thousand) of an individual amino acid and that of the weighted mean of all measured amino acids in the sample adjusted for the relative C atom abundance of amino acyl residues within the plant protein extract. Protein amino acid compositions were derived from analysis of representative samples of protein or obtained from the literature for the same, or closely related, species (see Lynch et al., 2011). Current methodology is unable to resolve Leu and Ile chromatographically; therefore their combined  $\delta^{13}\text{C}$

value was calculated giving a average atom weighted mean of their individual isotope abundances. This value may vary because the amount or  $\delta^{13}\text{C}$  one, or either, amino acid varies, either singly or in combination.

#### 4.3. Statistical procedures

All statistical procedures were performed using SPSS 16 (IBM, <http://www.ibm.com>). Differences between groups of samples were assessed using Student's t-test (two-tailed, unequal variance) and ANOVA. Correlation between pairs of variables was evaluated using Pearson's product-moment correlation coefficient. Discriminant function analysis was conducted on untransformed values of normalized  $\Delta\delta^{13}\text{C}$  measurements and the statistical significance of each extracted function (equivalent to a weighted combination of the dependent variables used in the analysis) was assessed using Wilks' lambda and  $\chi^2$  values. Discriminant functions having  $p < 0.01$  or  $p < 0.001$  were considered significant or highly significance, respectively. In all other statistical tests,  $p$  values  $< 0.05$  are deemed significant, and  $p$  values  $< 0.01$  are regarded as highly significant.

#### Acknowledgments

The authors thank the Woodland Trust and Henley-on-Thames Town Council for permission to collect plant samples from natural sites, and are grateful to Dr Stephen Harris, Department of Plant Sciences, University of Oxford for identification of plant species. Thanks are also given to Prof. Carmichael Wallace, Department of Biochemistry and Molecular Biology, Dalhousie University, for providing plant samples from Wiltshire, and to several anonymous reviewers who have helped improve the manuscript.

#### References

- Abelson, P.H. and Hoering, T.C., 1961. Carbon isotope fractionation in formation of amino acids by photosynthetic organisms. *Proc. Natl. Acad. Sci. U.S.A.*, 47, 623-632
- Araus, J.L., Brown, H.R., Febrero, A., Bort, J. and Serret, M.D., 1993. Ear photosynthesis, carbon isotope discrimination and the contribution of respiratory CO<sub>2</sub> to difference in grain mass in durum-wheat. *Plant, Cell Environ.*, 16, 383-392.
- Bacci, L., Baronti, S., Predieri, S. and di Virgilio, N., 2009. Fiber yield and quality of fiber nettle (*Urtica dioica* L.) cultivated in Italy. *Ind. Crops Prod.*, 29, 480-484.
- Balmer, Y., Vensel, W.H., DuPont, F.M, Buchanan, B.B. and Hurkman, W.J., 2006. Proteome of amyloplasts isolated from developing wheat endosperm presents evidence of broad metabolic capability. *J. Exp. Bot.*, 57, 1591-1602.
- Barnes, R.L., 1963. Organic nitrogen compounds in tree xylem sap. *For. Sci.*, 9, 98-102.
- Benner, R., Fogel, M.L., Sprague, E.K. and Hodson, R.E., 1987. Depletion of <sup>13</sup>C in lignin and its implications for stable carbon isotope studies. *Nature*, 329, 708-710.
- Bertani, A., Brambilla, I. and Mapelli, S., 2006. Nitrogen storage and translocation in walnut plant: Free amino acids. *Acta Hortic.*, 705, 261-267.
- Bloch, D., Hoffmann, C.M. and Märkländer, B., 2006. Impact of water supply on photosynthesis, water use and carbon isotope discrimination of sugar beet genotypes. *Eur. J. Agron.*, 24, 218-225.
- Blum, A., 1985. Photosynthesis and transpiration in leaves and ears of wheat and barley varieties. *J. Exp. Bot.*, 36, 432-440.
- Butzenlechner, M., Thimet, S., Kempe, K., Kexel, H. and Schmidt, H.-L., 1996. Inter- and intramolecular isotope correlations in some cyanogenic glycosides and glucosinolates and their practical importance. *Phytochemistry*, 43, 585-592.
- Caputo, C. and Barneix, A.J., 1997. Export of amino acids to the phloem in relation to N supply in wheat. *Physiol. Plant.*, 101, 853-860.

- Chikaraishi, Y., Naraoka, H. and Poulson, S.R., 2004. Hydrogen and carbon isotope fractionations of lipid biosynthesis among terrestrial (C<sub>3</sub>, C<sub>4</sub> and CAM) and aquatic plants. *Phytochemistry*, 65, 1369-1381.
- Chikov, V. and Bakirova, G., 1999. Relationship between carbon and nitrogen metabolisms in photosynthesis. The role of photooxidation processes. *Photosynthetica*, 37, 527.
- Choo, Y.-S., Lee, C.-B. and Albert, R., 2002. Effects of nitrogen nutrition on the pattern of ions and organic solutes in five sedges (*Carex* spp.). *Flora*, 197, 56-66.
- Davies, D.D. and Humphrey, T.J., 1978. Amino acid recycling in relation to protein turnover. *Plant Physiol.*, 61, 54-58.
- Deniro, M.J. and Epstein, S., 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science*, 197, 261-263.
- Dungait, J.A.J., Doherty, G., Straker, V. and Evershed, R.P., 2008. Interspecific variation in bulk tissue, fatty acid and monosaccharide  $\delta^{13}\text{C}$  values of leaves from a mesotrophic grassland plant community. *Phytochemistry*, 69, 2041-2051.
- Dunn, P.J.H., Honch, N.V. and Evershed, R.P., 2011. Comparison of liquid chromatography-isotope ratio mass spectrometry (LC/IRMS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) for the determination of collagen amino acid  $\delta^{13}\text{C}$  values for palaeodietary and palaeoecological reconstruction. *Rapid Commun. Mass Spectrom.*, 25, 2995-3011.
- Farquhar, G.D., Ehleringer, J.R. and Hubick, K.T., 1989. Carbon isotope discrimination and photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 40, 503-537.
- Farquhar, G.D., O'Leary, M.H. and Berry, J.A., 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Aust. J. Plant Physiol.*, 9, 121-137.

- Fisher, D.B. and MacNicol, P.K., 1986. Amino acid composition along the transport pathway during grain filling in wheat. *Plant Physiol.*, 82, 1019-1023.
- Fogel, M.L. and Tuross, N., 2003. Extending the limits of paleodietary studies of humans with compound specific carbon isotope analysis of amino acids. *J. Archaeol. Sci.*, 30, 535-545.
- Gannes, L.Z, del Rio, C.M. and Koch, P., 1998. Natural abundance variations in stable isotopes and their potential uses in animal physiological ecology. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.*, 119, 725-737.
- Gilbert, A., Silvestre, V., Robins, R.J., Remaud, G.S. and Tcherkez, G., 2012. Biochemical and physiological determinants of intramolecular isotope patterns in sucrose from C<sub>3</sub>, C<sub>4</sub> and CAM plants accessed by isotopic <sup>13</sup>C NMR spectrometry: A viewpoint. *Nat. Prod. Rep.*, 29, 476-486.
- Graham, L.E., 1993. *Origin of Land Plants*. Wiley, New York.
- Hayes, J.M., 2001. Fractionation of carbon and hydrogen isotopes in biosynthetic processes. *Rev. Mineral. Geochem.*, 43, 191-277.
- Higuchi, T, Ito, Y. and Kawamura, I., 1967. p-Hydroxyphenylpropane component of grass lignin and role of tyrosine-ammonia lyase in its formation. *Phytochemistry*, 6, 875-881.
- Hobson, K.A., 1999. Tracing origins and migration of wildlife using stable isotopes: A review. *Oecologia*, 120, 314-326.
- Jander, G. and Joshi, V., 2010. Recent progress in deciphering the biosynthesis of aspartate-derived amino acids in plants. *Mol. Plant*, 3, 54-65.
- Jim, S., Jones, V., Ambrose, S.H. and Evershed, R.P., 2006. Quantifying dietary macronutrient sources of carbon for bone collagen biosynthesis using natural abundance stable carbon isotope analysis. *Br. J. Nutr.*, 95, 1055-1062.

- Joshi, V., Laubengayer, K.M., Schauer, N., Fernie, A.R. and Jander, G., 2006. Two Arabidopsis threonine aldolases are nonredundant and compete with threonine deaminase for a common substrate pool. *Plant Cell*, 18, 3565-3575.
- Keppler, F., Kalin, R.M., Harper, D.B., McRoberts, W.C. and Hamilton, J.T.G., 2004. Carbon isotope anomaly in the major plant C1 pool and its global biogeochemical implications. *Biogeosciences*, 1, 123-131.
- Kruger, N.J and Ratcliffe, G.R., 2009. Insights into plant metabolic networks from steady-state metabolic flux analysis. *Biochimie*, 91, 697-702.
- Krummen, M., Hilkert, A.W., Juchelka, D., Duhr, A., Schlüter, H.-J. and Pesch, R., 2004. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.*, 18, 2260-2266.
- Kubiak-Martens, L., 1999. The plant food component of the diet at the late Mesolithic (Ertebølle) settlement at Tybrind Vig, Denmark. *Veg. Hist. Archaeobotany*, 8, 117-127.
- Larsen, T., Taylor, D.L, Leigh, M.B. and O'Brien, D.M., 2009. Stable isotope fingerprinting: A novel method for identifying plant, fungal, or bacterial origins of amino acids. *Ecology*, 90, 3526-3535.
- Larsen T, Ventura M, Andersen N, O'Brien DM, Piatkowski U, McCarthy MD, 2013. Tracing Carbon Sources through Aquatic and Terrestrial Food Webs Using Amino Acid Stable Isotope Fingerprinting. *PLoS ONE* 8: e73441.
- Larsen T, Bach L.T., Salvatecci R, Wang YV, Anderson N, Ventura M and McCarthy MD, 2015. Assessing the potential of amino acid <sup>13</sup>C patterns as a carbon source tracer in marine sediments: Effects of algal growth conditions and sedimentary diagnosis. *Biogeosciences*, 12, 4979-4992.

- Leusing, P. and Holtum, J.A.M., 1987. The partitioning of photosynthetically assimilated  $^{14}\text{C}$  into amino acids in wheat. 1. Partitioning during transport to the grain. *Ann. Bot.*, 60, 459-467.
- Lopes, M.S., Cortadellas, N., Kichey, T., Dubois, F., Habash, D.Z. and Araus, J.L., 2006. Wheat nitrogen metabolism during grain filling: Comparative roles of glumes and the flag leaf. *Planta*, 225, 165-181.
- Lu, C., Hawkesford, M.J., Barraclough, P.B., Poulton, P.R., Wilson, I.D., Barker, G.L. and Edwards, K.J., 2005. Markedly different gene expression in wheat grown with organic or inorganic fertiliser. *Proc. R. Soc. London, Ser. B*, 272, 1901-1908.
- Lynch, A.H., McCullagh, J.S.O. and Hedges, R.E.M., 2011. Liquid chromatography - isotope ratio mass spectrometry measurement of  $\delta^{13}\text{C}$  of amino acids in plant proteins. *Rapid Commun. Mass Spectrom.*, 25, 2981-2988.
- Maltais, J.B. and Auclair, J.L., 1962. Free amino acid and amide composition of pea leaf juice, pea aphid haemolymph, and honeydew, following the rearing of aphids on single pea leaves treated with amino compounds. *J. Insect Physiol.*, 8, 391-399.
- Melzer, E. and O'Leary, M.H., 1987. Anapleurotic  $\text{CO}_2$  fixation by phosphoenolpyruvate carboxylase in  $\text{C}_3$  plants. *Plant Physiol.*, 84, 58-60.
- McCullagh J.S.O., Hedges R.E.M and Juchelka, D., 2006. Analysis of amino acids  $^{13}\text{C}$  abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. *Rapid Commun. Mass Sp.*, 20, 2761-2768.
- Melzer, E. and Schmidt, H.-L., 1987. Carbon isotope effects on the pyruvate dehydrogenase reaction and their importance for relative carbon-13 depletion in lipids. *J. Biol. Chem.*, 262, 8159-8164.
- Millard, P., Wendler, R., Hepburn, A. and Smith, A., 1998. Variations in the amino acid composition of xylem sap of *Betula pendula* Roth. trees due to remobilization of stored N in the spring. *Plant, Cell Environ.* 21, 715-722.

- Näsholm, T., Kielland, K. and Ganeteg, U., 2009. Uptake of organic nitrogen by plants. *New Phytol.* 182, 31-48.
- Patrick, J.W. and Offler, C.E., 1995. Post-sieve element transport of sucrose in developing seeds. *Aust. J. Plant Physiol*, 22, 681-702.
- Patrick, J.W. and Offler, C.E., 2001. Compartmentation of transport and transfer events in developing seeds. *J. Exp. Bot.*, 52, 551-564.
- Pearcy, R.W. and Pfitsch, W.A., 1991. Influence of sunflecks on the  $\delta^{13}\text{C}$  of *Adenocaulon bicolor* plants occurring in contrasting forest understory microsites. *Oecologia*, 86, 457-462.
- Raggi, V., 1994. Changes in free amino acids and osmotic adjustment in leaves of water-stressed bean. *Physiol. Plant.*, 91, 427-434.
- Reeds, P.J., 2000. Dispensable and indispensable amino acids for humans. *J. Nutr.*, 130, 1835S-1840S.
- Rosinger, C.H., Wilson, J.M. and Kerr, M.W., 1984. Changes in the soluble protein and free amino acid content of chill-sensitive and chill-resistant plants during chilling and hardening treatments. *J. Exp. Bot.*, 35, 1460-1471.
- Rosnitschek-Schimmel, I., 1985. The influence of nitrogen nutrition on the accumulation of free amino acids in root tissue of *Urtica dioica* and their apical transport in xylem sap. *Plant Cell Physiol.*, 26, 215-219.
- Sato, T., Harada, T. and Ishizawa, K., 2002. Stimulation of glycolysis in anaerobic elongation of pondweed (*Potamogeton distinctus*) turions. *J. Exp. Bot.*, 53, 1847-1856.
- Sauer, U., 2006. Metabolic networks in motion:  $^{13}\text{C}$ -based flux analysis. *Mol. Syst. Biol.*, 2, article 62.
- Savidge, W.B. and Blair, N.E., 2004. Patterns of intramolecular carbon isotope heterogeneity within amino acids of autotrophs and heterotrophs. *Oecologia*, 139, 178-189.
- Schmidt, H.-L., 2003. Fundamentals and systematics of the non-statistical distribution of isotopes in natural compounds. *Naturwissenschaften* 90, 537-552.
- Lynch *et al.* (2016)

- Schmidt, H.-L. and Gleixner, G., 1998. Carbon isotope effects on key reactions in plant metabolism and  $^{13}\text{C}$ -patterns in natural compounds, in: Griffiths, H. (Ed.), *Stable isotopes: integration of biological, ecological and geochemical processes*. BIOS Scientific Publishers, Oxford, pp. 13-25.
- Schmidt, H.-L., Robins, R.J., Werner, R.A. 2015 Multi-factorial in vivo stable isotope fractionation: causes, correlations, consequences and applications. *Isotop. Environ. Health Stud.*, 51, 155–199.
- Schnyder, H., 1993. The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling: a review. *New Phytol.*, 123, 233-245.
- Scott, J.H., O'Brien, D.M., Emerson, D., Sun, H., McDonald, G.D., Salgado, A. and Fogel, M.L., 2006. An examination of the carbon isotope effects associated with amino acid biosynthesis. *Astrobiology*, 6, 867-880.
- Slocum, R.D., 2005. Genes, enzymes and regulation of arginine biosynthesis in plants. *Plant Physiol. Biochem.*, 43, 729-745.
- Smith, B.N. and Epstein, S., 1971. Two categories of  $^{13}\text{C}/^{12}\text{C}$  ratios for higher plants. *Plant Physiol.*, 47, 380-384.
- Smith, C.I., Fuller, B.T., Choy, K. and Richards, M.P., 2009. A three-phase liquid chromatographic method for  $\delta^{13}\text{C}$  analysis of amino acids from biological protein hydrolysates using liquid chromatography-isotope ratio mass spectrometry. *Anal. Biochem.*, 390, 165-172.
- Sultan, J.I., Inam-ur-Rahim, Yaqoob, M., Mustafa, M.I., Nawaz, H. and Akhtar, P., 2009. Nutritional evaluation of herbs as fodder source for ruminants. *Pakistan J. Bot.*, 41, 2765-2776.
- Tcherkez, G., 2006. Viewpoint: How large is the carbon isotope fractionation of the photorespiratory enzyme glycine decarboxylase? *Funct. Plant Biol.*, 33, 911-920.
- Tcherkez, G., Mahé, A. and Hodges, M., 2011.  $^{12}\text{C}/^{13}\text{C}$  fractionations in plant primary metabolism. *Trends Plant Sci.*, 16, 499-506.

- Valle, E.M. and Heldt, H.-W., 1991. Ala synthesis by bundle sheath cells of maize. *Plant Physiol.*, 95, 839-845.
- Weichert, N., Saalbach, I., Weichert, H., Kohl, S., Erban, A., Kopka, J., Hause, B., Varshney, A., Sreenivasulu, N., Strickert, M., Kumlehn, J., Weschke, W. and Weber, H., 2010. Increasing sucrose uptake capacity of wheat grains stimulates storage protein synthesis. *Plant Physiol.*, 152, 698-710.
- Weilacher, T., Gleixner, G. and Schmidt, H.-L., 1996. Carbon isotope pattern in purine alkaloids a key to isotope discriminations in C1 compounds. *Phytochemistry*, 41, 1073-1077.
- Wickman, F.E., 1952. Variations in the relative abundance of the carbon isotopes in plants. *Geochim. Cosmochim. Acta*, 2, 243-254.
- Xu, X.-L., Zhang, Y.-H. and Wang, Z.-M., 2004. Effect of heat stress during grain filling on phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase activities of various green organs in winter wheat. *Photosynthetica*, 42, 317-320.
- Zamboni, N., 2011.  $^{13}\text{C}$  metabolic flux analysis in complex systems. *Curr. Opin. Biotechnol.*, 22, 103-108.
- Zhang, W.-H., Zhou, Y., Dibley, K.E., Tyerman, S.D., Furbank, R.T. and Patrick, J.W., 2007. Review: Nutrient loading of developing seeds. *Funct. Plant Biol.*, 34, 314-331.

Plant species	Number of samples	$\delta^{13}\text{C}$ (‰) range	
		Max	Min
Nettle: open environment	35	-23.8	-30.4
Nettle: shaded environment	8	-27.0	-31.6
Deciduous tree leaves: open	6	-23.8	-27.4

environment			
Deciduous tree leaves: shaded	16	-25.8	-30.9
environment			
Grasses	13	-28.6	-31.4
Bramble	3	-28.2 (mean)	
Wetland terrestrial plants	8	-24.1	-28.8
Aquatic plants	9	-28.4	-36.4

---

**Table 1.** Carbon isotope composition ( $\delta^{13}\text{C}$  (‰)) for the various protein samples analysed in the present study (bulk protein, prior to hydrolysis).

Amino acid	Normalised $\Delta\delta^{13}\text{C}$ value (‰)					
	Nettle [43]	Pooideae [13]	Reed [8]	Tree [22]	Bramble [3]	Freshwater plant [9]
Ala	+0.0 (0.4)	+0.8 (0.4)	+0.1 (0.4)	-0.2 (0.3)	+0.3 (0.3)	+1.2 (0.5)
Val	-5.2 (0.2)	-5.2 (0.2)	-5.3 (0.3)	-5.8 (0.2)	-6.1 (0.5)	-2.9 (0.9)
Ile/Leu	-6.1 (0.1)	-5.4 (0.2)	-6.1 (0.2)	-5.2 (0.2)	-6.5 (0.4)	-5.3 (0.4)
Asp/Asn	+4.9 (0.1)	+3.4 (0.2)	+2.8 (0.3)	+2.8 (0.2)	+5.0 (0.6)	+3.9 (1.1)
Lys	+2.4 (0.1)	+1.9 (0.3)	+1.0 (0.2)	+1.4 (0.2)	+1.5 (0.2)	+1.3 (0.7)
Thr	+7.7 (0.2)	+6.6 (0.3)	+5.8 (0.8)	+7.9 (0.8)	+9.2 (0.2)	+9.4 (1.3)
Met	-1.9 (0.5)	-4.4 (0.7)	+1.5 (1.3)	-2.8 (0.9)	-4.8 (0.7)	+0.1 (0.8)
Glu/Gln	+0.3 (0.2)	+0.3 (0.3)	-0.3 (0.3)	+0.8 (0.3)	-0.2 (0.7)	-0.1 (2.1)
Pro	+2.8 (0.4)	+2.3 (0.5)	+1.3 (0.3)	+1.7 (0.3)	+2.2 (0.8)	+3.2 (0.6)
Arg	-0.2 (0.2)	-0.4 (0.3)	-1.0 (0.5)	-2.0 (0.4)	-2.7 (1.3)	-1.0 (1.4)
Phe	-3.4 (0.2)	-3.4 (0.3)	-2.0 (0.3)	-3.0 (0.3)	-4.1 (0.9)	-4.5 (0.6)
Tyr	-2.2 (0.1)	-1.1 (0.2)	-2.5 (0.4)	-3.2 (0.3)	-4.1 (0.4)	-5.6 (1.2)
Gly	+10.1 (0.3)	+9.5 (0.2)	+11.0 (0.3)	+6.8 (0.3)	+9.2 (1.0)	+8.2 (1.0)
Ser	+9.8 (0.4)	+10.5 (0.5)	+7.8 (0.7)	+8.6 (0.9)	+10.3 (1.1)	+5.4 (2.5)
His	-0.8 (0.3)	+0.1 (0.5)	-0.5 (0.3)	-1.9 (0.4)	-1.8 (0.6)	-0.2 (1.0)
Mean value $\delta^{13}\text{C}$ (‰)	-29.5	-32.0	-28.4	-28.6	-29.8	-35.5

**Table 2.** Variation in normalised  $\Delta\delta^{13}\text{C}$  for individual amino acids of proteins extracted from leaves of different plant groups. Each value is the mean of measurements taken from samples throughout the year (with corresponding s.e. in parentheses). Values in brackets in column headings indicate the number of independent plant samples analysed. The numbers of measurements obtained for individual amino acids, when less than the total number of samples analysed, were as follows.

Nettle: Val, 38; Lys, 41; Thr, 41; Met, 37; Glu, 35; Ser, 36; His, 41. Pooideae: Val, 12; Lys, 12; Met, 12; His, 12. Trees: Val, 18; Lys, 21; Thr, 21; Met, 18; Glu, 21; Ser, 21; His – 21. Freshwater plants: Met, 5; Phe, 8; Ser, 8; His, 8. Normalised  $\Delta\delta^{13}\text{C}$  values are the difference between  $\delta^{13}\text{C}$  (expressed in parts per thousand) of an individual amino acid and that of the weighted mean of all measured amino

acids in the sample adjusted for the relative C atom abundance of amino acyl residues within the plant protein extract.

Plant group	Pooideae	Reeds etc	Trees	Bramble	Freshwater
Nettle	Ile/Leu, Asp, Thr, Met, Tyr	Asp, Lys, Thr, Met, Phe, Ser	Val, Ile/Leu, Asp, Lys, Pro, Arg, Tyr, Gly, His	Thr, Arg, Tyr	Val, Ile/Leu, Asp, Lys, Thr, Tyr, Gly, Ser
Pooideae	-	Ile/Leu, Lys, Met, Phe, Tyr, Gly, Ser	Arg, Tyr, Gly, His	Ile/Leu, Asp, Thr, Arg, Tyr	Val, Thr, Met, Tyr, Ser
Reeds etc	-	-	Ile/Leu, Met, Gly	Asp, Thr, Met, Phe, Tyr, Gly	Val, Thr, Pro, Phe, Tyr, Gly
Trees	-	-	-	Ile/Leu, Asp, Gly	Ala, Val, Pro, Phe, Tyr
Bramble	-	-	-	-	Met

**Table 3.** Comparison of normalised  $\Delta\delta^{13}\text{C}$  (‰) values of individual amino acids from leaf protein of different plant groups. The table lists amino acids showing highly significantly different normalised  $\Delta\delta^{13}\text{C}$  values ( $p < 0.01$ ) in cross-comparisons between plant groups analysed in Table 1.

Plant group	Correlation coefficient for comparison of $\Delta\delta^{13}\text{C}$ between:				
	Asp and:			Lys and:	Thr and:
	Lys	Thr	Met	Thr	Met
All plants	+0.150	+0.091	+0.075	-0.004	-0.229**
Nettle	+0.008	+0.404**	+0.010	-0.081	-0.138
Pooideae	+0.332*	-0.008	-0.256	-0.088	-0.432**
Trees	-0.015	-0.002	+0.390*	-0.294	-0.391*
Freshwater	-0.408	-0.178	+0.384	+0.196	+0.196

**Table 4.** Correlation between annual  $\Delta\delta^{13}\text{C}$  values of individual amino acids within leaf protein.

Comparisons are made between aspartic acid, lysine, threonine and methionine for all plants and within plant groups for which more than 3 measurements were available. Each value is the linear correlation coefficient and is identified as \* significant ( $p < 0.05$ ), and \*\* highly significant ( $p < 0.01$ ) as appropriate. There are no significant correlations between lysine and methionine.

Plant group	Correlation coefficient for comparison of $\Delta\delta^{13}\text{C}$ between:		
	Glu and:		Pro and:
	Pro	Arg	Arg
All plants	-0.161	-0.461**	+0.405**
Nettle	-0.362**	-0.277*	+0.438**
Pooideae	+0.523**	+0.568**	+0.503**
Trees	-0.163	-0.234	+0.435**
Freshwater	-0.336	-0.718**	+0.572*

**Table 5**

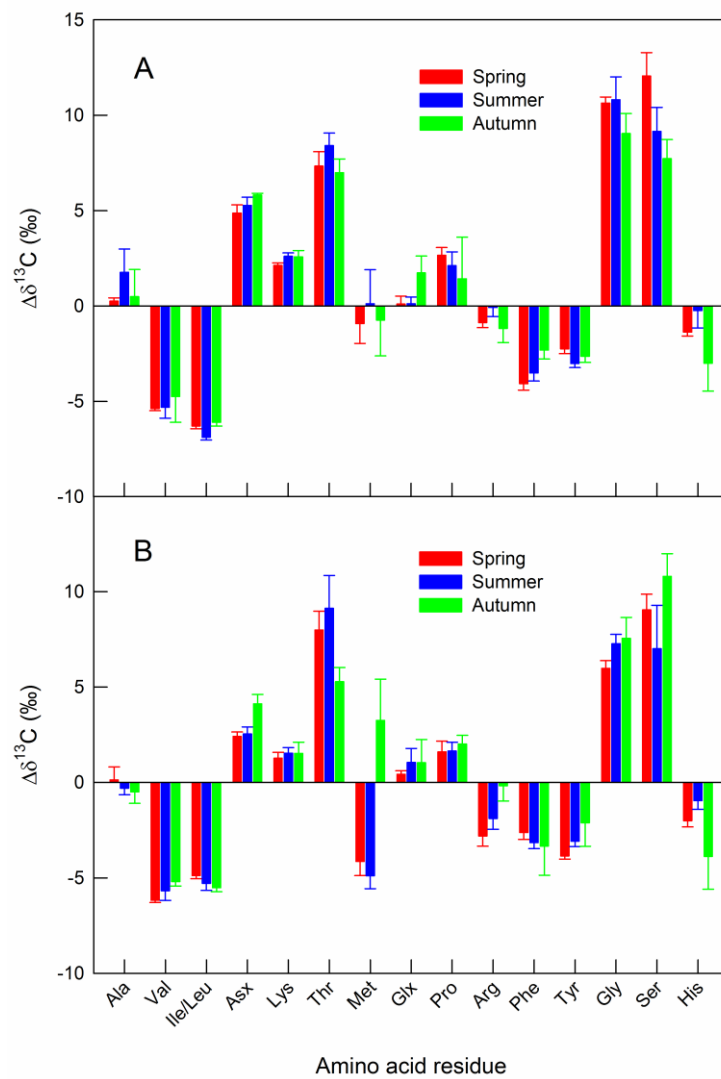
Correlation between annual  $\Delta\delta^{13}\text{C}$  values of Glu, Pro and Arg within leaf protein. Comparisons are made for all plants and within plant groups for which more than three measurements were available. Each value is the linear correlation coefficient and is identified as \* significant ( $p < 0.05$ ), and \*\* highly significant ( $p < 0.01$ ) as appropriate.

Plant group	Correlation coefficient for comparison of $\Delta\delta^{13}\text{C}$ between:		
	Gly and:		Ser and:
	Ser	Thr	Thr
All plants	+0.399**	+0.070	-0.279**
Nettles	+0.538**	+0.206	-0.129
Pooideae	-0.066	+0.096	+0.147
Trees	+0.036	+0.263	-0.658**
Freshwater	+0.721**	+0.453	+0.212

**Table 6**

Correlation between annual  $\Delta\delta^{13}\text{C}$  values of Gly, Ser and Thr within leaf protein. Comparisons are made for all plants and within plant groups for which more than three measurements were available. Each value is the linear correlation coefficient and is identified as \*\* highly significant where ( $p < 0.01$ ).

## Figures



**Fig. 1.** Influence of season on normalised  $\Delta\delta^{13}\text{C}$  value for individual amino acids in leaf proteins from (A) nettles in a dry, open environment, (B) deciduous trees. Plants were sampled in spring, summer and autumn, as indicated. Each value is the mean with s.e. bars. Normalised  $\Delta\delta^{13}\text{C}$  values are the difference between  $\delta^{13}\text{C}$  (expressed in parts per thousand) of an individual amino acid and that of the weighted mean of all measured amino acids in the sample adjusted for the relative C atom abundance of amino acyl residues within the plant protein extract.

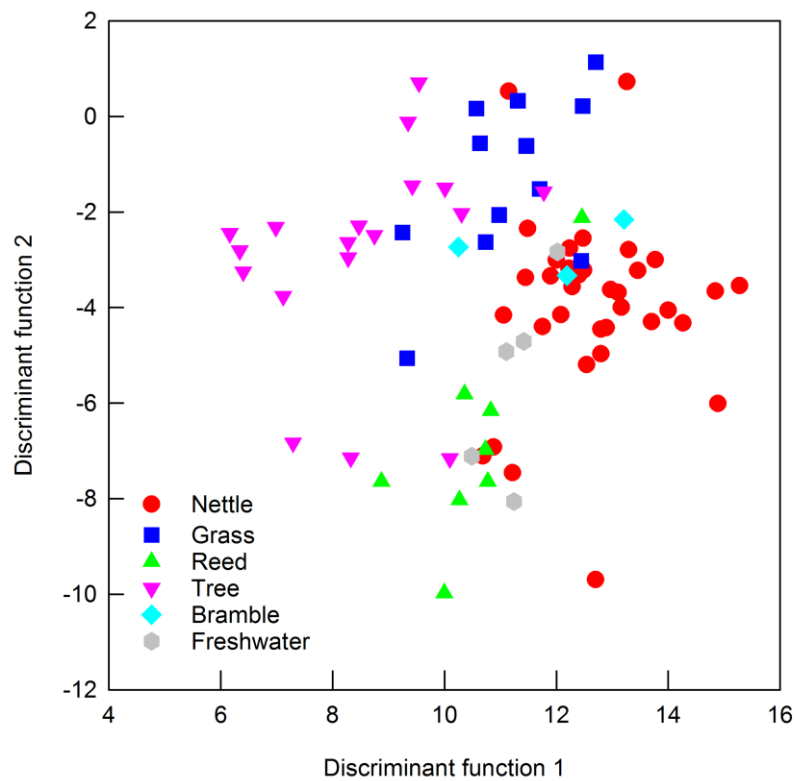


Fig. 2. Discriminant function analysis score plot of normalised  $\Delta\delta^{13}\text{C}$  values for individual amino acids in leaf proteins from different plant groups. Values are the discriminant function scores (for the top two significant principal functions) for individual samples obtained throughout the year from nettle (●), grasses (■), reeds (▲), deciduous trees (▼), bramble (◆) and freshwater plants (●) growing in natural habitats. The amino acid analysed were the same as for Figure 1. Normalised  $\Delta\delta^{13}\text{C}$  values are the difference between  $\delta^{13}\text{C}$  (expressed in parts per thousand) of an individual amino acid and that of the weighted mean of all measured amino acids in the sample adjusted for the relative C atom abundance of amino acyl residues within the plant protein extract.

# Variability in the carbon isotope composition of individual amino acids in plant proteins from different sources: 1 Leaves

Anthony H. Lynch, Nicholas J. Kruger, Robert E.M. Hedges and James S.O. McCullagh

## SUPPLEMENTARY DATA

		Page
Table S1	$\Delta\delta^{13}\text{C}$ values for individual amino acids of proteins extracted from leaves of different genera of Pooideae	2
Table S2	$\Delta\delta^{13}\text{C}$ values for individual amino acids of proteins extracted from leaves of deciduous trees	3
Table S3	Standardised canonical function coefficients for discrimination between plant groups	4
Figure S1	Effect of shading on $\Delta\delta^{13}\text{C}$ values for individual amino acids in leaf proteins from nettles in a dry environment	5
Figure S2	Variation in $\Delta\delta^{13}\text{C}$ values for individual amino acids in leaf proteins from different plant groups	6

### Supplementary Table S1

$\Delta\delta^{13}\text{C}$  values for individual amino acids of proteins extracted from leaves of different genera of Pooideae. Each value is the mean of measurements taken from samples throughout the year (with corresponding s.e. in parentheses). Values in brackets in column headings indicate the number of independent plant samples analysed, with the exception that  $\Delta\delta^{13}\text{C}$  values for Val, Lys, Met and His in *Dactylis* were derived from only three measurements. Identical superscript letters in the same row indicate a significant difference ( $p < 0.05$ ) between pairs of genera.

Amino acid	$\Delta\delta^{13}\text{C}$ value of amino acid from protein hydrolysate (‰)					
	Deschampsia [5]	Melica [4]	Dactylis [4]	Carex [3]	Juncus [2]	Phragmites [3]
Ala	+1.5 (0.7)	+0.5 (0.6)	+0.1 (0.9)	-0.5 (0.5)	+1.5 (0.7)	-0.4 (0.6)
Val	-4.9 <sup>b</sup> (0.3)	-4.5 <sup>ad</sup> (0.2)	-6.0 <sup>abc</sup> (0.3)	-5.6 <sup>d</sup> (0.5)	-5.3 (0.1)	-4.5 <sup>c</sup> (0.4)
Ile/Leu	-5.6 (0.3)	-4.6 <sup>ab</sup> (0.3)	-5.9 (0.5)	-6.4 <sup>a</sup> (0.4)	-5.7 (0.4)	-6.0 <sup>b</sup> (0.4)
Asp/Asn	+3.9 (0.6)	+3.3 (0.4)	+3.1 (0.5)	+2.6 (0.3)	+2.5 (0.2)	+3.6 (0.8)
Lys	+1.6 (0.2)	+2.2 (0.3)	+1.9 (0.8)	+0.9 (0.3)	+0.9 (0.3)	+1.7 (0.4)
Thr	+7.0 (0.7)	+7.4 (0.5)	+5.7 (0.3)	+5.0 (1.5)	+6.2 (2.1)	+5.0 (1.7)
Met	-5.4 <sup>a</sup> (0.7)	-2.9 (1.3)	-4.9 <sup>bc</sup> (1.3)	+1.3 <sup>b</sup> (1.1)	+1.8 <sup>ac</sup> (0.5)	+1.5 (3.3)
Glu/Gln	+0.7 (0.3)	+0.1 (0.3)	-0.1 (0.6)	-0.1 (0.2)	+0.0 (0.6)	+0.7 (1.5)
Pro	+3.2 (0.6)	+2.3 (0.6)	+1.7 (0.9)	+1.1 (0.4)	+2.0 (0.3)	+1.3 (0.6)
Arg	+0.3 <sup>abc</sup> (0.3)	-0.6 <sup>c</sup> (0.3)	-1.2 <sup>b</sup> (0.6)	-0.6 (0.9)	-0.2 (0.9)	-1.3 <sup>a</sup> (0.6)
Phe	-4.0 <sup>ab</sup> (0.3)	-2.6 (0.6)	-2.9 (0.9)	-1.8 (0.3)	-1.5 <sup>a</sup> (0.4)	-2.6 <sup>b</sup> (0.4)
Tyr	-1.2 (0.6)	-0.6 <sup>ab</sup> (0.2)	-1.7 (0.6)	-2.5 <sup>a</sup> (0.3)	-3.5 <sup>b</sup> (0.8)	-1.8 (1.3)
Gly	+10.2 <sup>aef</sup> (0.2)	+9.3 <sup>ceg</sup> (0.4)	+9.1 <sup>abd</sup> (0.3)	+10.3 (0.8)	+11.3 <sup>bef</sup> (0.5)	+11.0 <sup>dg</sup> (0.7)
Ser	+10.7 (1.1)	+10.1 <sup>a</sup> (0.6)	+9.7 (0.9)	+6.8 <sup>a</sup> (1.3)	+7.6 (1.4)	+8.1 (1.0)
His	-0.8 (1.1)	+0.5 (0.7)	-0.2 (0.4)	-0.4 (0.8)	-0.1 (0.9)	-0.8 (0.4)

## Supplementary Table S2

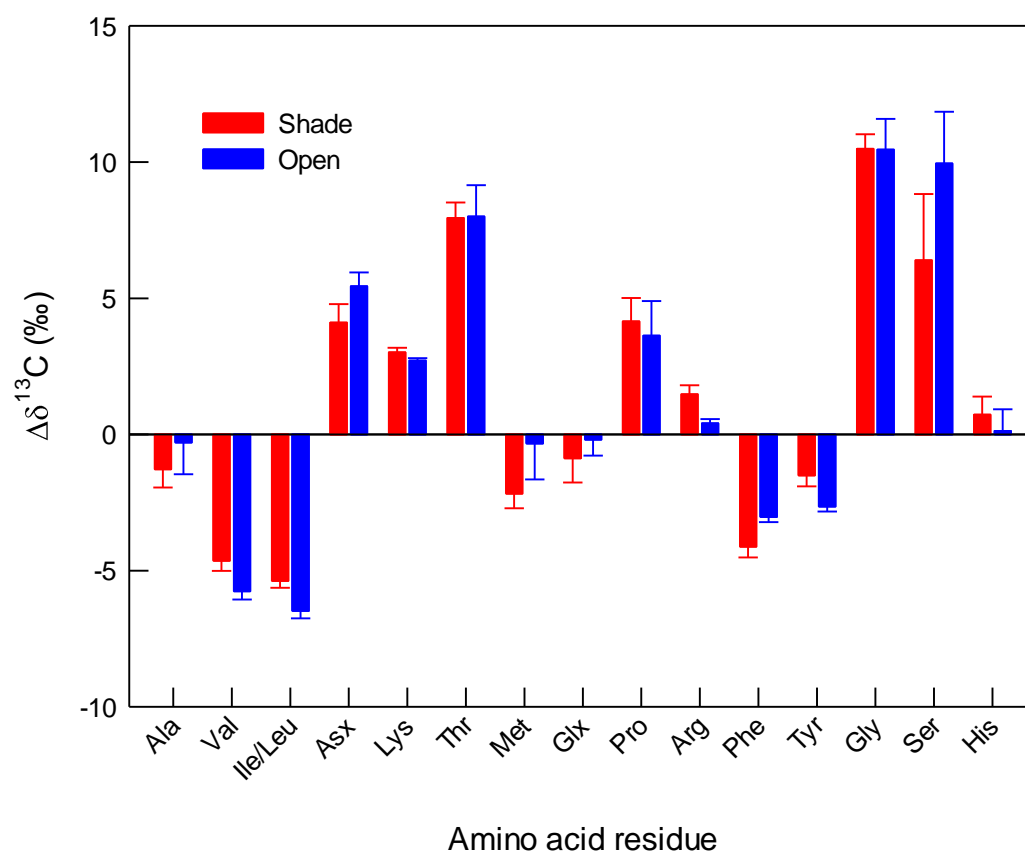
$\Delta\delta^{13}\text{C}$  values for individual amino acids of proteins extracted from leaves of deciduous trees. Each value is the mean of measurements taken from samples throughout the year (with corresponding s.e. in parentheses). Values in brackets in column headings indicate the number of independent plant samples analysed. The numbers of measurements obtained for individual amino acids, when less than the total number of samples analysed, were as follows. Hazel: Val, 3; Lys, 5; Thr, 5; Met, 3; Glx, 5; Ser, 5; His, 5. Horse chestnut: Val, 4; Met, 4. Identical superscript letters in the same row indicate a significant difference ( $p < 0.05$ ) between pairs of genera.

Amino acid	$\Delta\delta^{13}\text{C}$ values of amino acids from protein hydrolysate (‰)				
	Beech [3]	Birch [3]	Hazel [6]	Horse chestnut [5]	Oak [5]
Ala	-0.8 (0.6)	+0.7 (0.1)	+0.8 (0.8)	+0.8 (0.7)	-0.5 (0.4)
Val	-5.1 (0.4)	-4.1 <sup>ab</sup> (0.3)	-5.8 <sup>a</sup> (0.2)	-5.1 <sup>b</sup> (0.3)	-5.8 (0.5)
Ile/Leu	-4.9 (0.2)	-4.1 (0.4)	-5.0 (0.3)	-4.8 (0.3)	-4.5 (0.5)
Asp/Asn	+3.7 <sup>a</sup> (0.5)	+2.8 (0.4)	+2.6 <sup>a</sup> (0.2)	+3.3 (0.7)	+3.2 (0.5)
Lys	+1.5 (0.5)	+1.0 (0.4)	+1.4 (0.2)	+1.8 (0.6)	+1.4 (0.4)
Thr	+6.4 (1.7)	+8.0 (1.1)	+7.2 (0.4)	+8.2 (1.8)	+9.1 (2.6)
Met	-3.8 (1.6)	-0.1 (3.5)	-4.3 (1.0)	-3.8 (2.2)	-2.2 (1.6)
Glu/Gln	+1.0 (0.6)	+0.5 (0.9)	+0.3 (0.3)	+0.9 (0.8)	+1.2 (1.0)
Pro	+1.2 (0.5)	+1.6 (0.2)	+1.8 (0.9)	+1.7 (0.6)	+1.9 (0.4)
Arg	-3.4 (0.9)	-0.6 (1.4)	-1.4 (0.7)	-1.6 (0.7)	-2.9 (0.7)
Phe	-2.0 (0.5)	-1.9 (0.8)	-2.3 (0.6)	-2.1 (0.4)	-4.0 (0.8)
Tyr	-2.4 (0.6)	-1.8 (1.3)	-3.7 (0.4)	-3.6 (0.4)	-3.6 (0.3)
Gly	+8.1 (0.8)	+9.1 <sup>ab</sup> (0.5)	+6.8 <sup>a</sup> (0.3)	+6.0 <sup>b</sup> (0.5)	+7.7 (1.1)
Ser	+10.9 <sup>a</sup> (1.0)	+12.5 <sup>bc</sup> (0.2)	+8.0 <sup>b</sup> (1.0)	+7.2 <sup>ac</sup> (0.6)	+8.9 (3.5)
His	-0.9 (0.2)	-1.7 (0.2)	-0.2 (0.7)	-2.6 (1.6)	-1.7 (0.3)

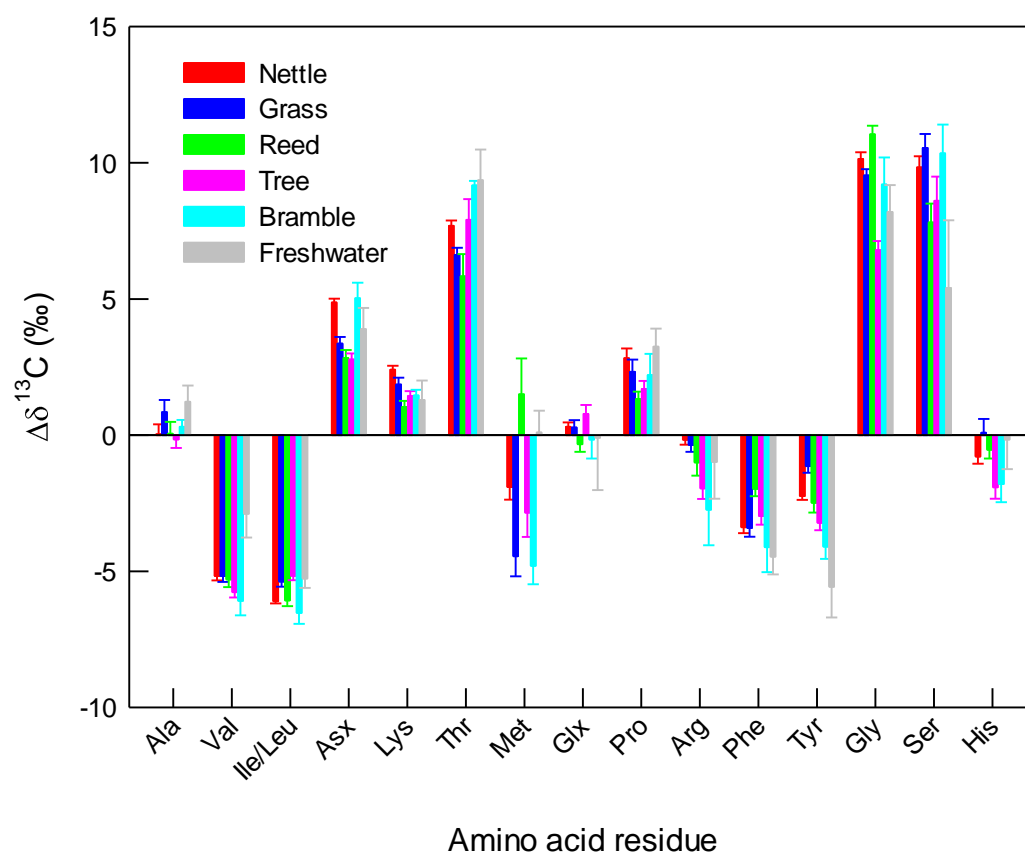
### Supplementary Table S3

Standardised canonical function coefficients for discrimination between plant groups. Discriminant function analysis was based on normalised  $\Delta\delta^{13}\text{C}$  values for individual amino acids of proteins from leaves of nettle, grasses, reeds, deciduous trees, bramble and freshwater higher plants. The first four discriminant functions yielded

Amino acid	Discriminant function			
	1	2	3	4
Ile/Leu	+0.015	+0.216	-0.209	+0.812
Asp/Asn	+0.616	+0.212	-0.579	-0.315
Lys	+0.435	+0.213	-.056	+0.468
Met	-0.193	-0.612	+0.129	-0.195
Arg	+0.039	-0.093	-0.068	+0.581
Tyr	-0.064	+0.658	+0.595	-0.564
Gly	+0.779	-0.348	+0.338	+0.380



**Supplementary Fig. S1.** Effect of shading on  $\Delta\delta^{13}\text{C}$  values for individual amino acids in leaf proteins from nettles in a dry environment. Leaves were obtained from plants growing in a shaded (●) or open (■) location. Each value is the mean with s.e. bars.



**Supplementary Fig. S2.** Variation in  $\Delta\delta^{13}\text{C}$  values for individual amino acids in leaf proteins from different plant groups. Leaves were obtained throughout the year from nettle (■), grasses (■), reeds (■), deciduous trees (■), bramble (■) and freshwater plants (■) growing in natural habitats. Each value is the mean with s.e. bars.

