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

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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 $\Delta\delta^{13}C$ 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 $^{13}C/^{12}C$ 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 $^{13}C/^{12}C$ fractional abundance in plants has the potential to provide dietary insights of value in archaeological and ecological studies.

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), whilst 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

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the same species. Thus, the pattern of ^{13}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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ values of amino acids between different terrestrial plant species. The identification of any inter-specific differences in these $\delta^{13}C$ 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 amino acids in protein.

Recent studies have concluded that the $\delta^{13}C$ 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 $\delta^{13}C$ 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, $\delta^{13}C$ (‰) values of individual amino acids from proteins were measured and 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}C isotope composition of leaf protein

The range of $\delta^{13}C$ (‰) 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 $\delta^{13}C$ values for the amino acids obtained by the acid hydrolysis of plant proteins are expressed as normalised $\Delta\delta^{13}C$ (the difference between $\delta^{13}C$ (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 and 3). However, the pattern in $\Delta\delta^{13}C$ is relatively consistent from one species to another. Positive values are found for Thr, Gly and Ser in all cases, whilst 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 $\delta^{13}C$ 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 in environments that vary in light intensity and water availability. These environmental factors both affect the $\delta^{13}C$ 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 $\Delta\delta^{13}C$ differences between individual amino acids shows little seasonal variation (Fig. 1A), although Leu/Ile and Tyr

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

Plant species	Number of samples	$\delta^{13}C$ (‰) range	
		Max	Min
Nettle: open environment	35	−23.8	−30.4
Nettle: shaded environment	8	−27.0	−31.6
Deciduous tree leaves: open environment	6	−23.8	−27.4
Deciduous tree leaves: shaded environment	16	−25.8	−30.9
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

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