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Compound-specific $\delta^{15}N$ values express differences in amino acid metabolism in plants of varying lignin content

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Abstract

Amino acid $\delta^{15}N$ values of foliage of various plant taxa, grown at the experimental farm stations of North Wyke, UK and Bad Lauchstädt, Germany were determined by GC-C-IRMS. The difference between $\delta^{15}N$ values of glutamate (Glx) and phenylalanine (Phe) were found to differ significantly between woody and herbaceous plants, with mean $\Delta^{15}N_{Glx\text{-}Phe}$ (i.e. $\delta^{15}N_{Phe} - \delta^{15}N_{Glx}$) values of -9.3±1.6‰ and -5.8±2.1‰, respectively. These differences in values are hypothesised to be due to the involvement of Phe in the phenylpropanoid pathway, by which lignin and other phenolic secondary metabolites are produced, leading to isotopic fractionation and enrichment of the remaining Phe pool available for protein biosynthesis. This results in the more negative $\Delta^{15}N_{Glx\text{-}Phe}$ values observed in woody plants relative to herbaceous plants, as the former are assumed to produce more lignin. To test this assumption, plant leaf tissue lignin concentrations were estimated by solid state ^{13}C cross-polarisation, magic-angle-spinning (CPMAS) NMR spectroscopy for a subset of plants, which showed that tree foliage has a higher concentration of lignin (12.6 wt%) than herbaceous foliage (6.3 wt%). The correlation of lignin concentration with $\Delta^{15}N_{Glx\text{-}Phe}$ values demonstrates that the difference in these values with plant type is indeed due to differential production of lignin. The ability to estimate the lignin content of plants from amino acid $\delta^{15}N$ values will, to give one example, allow refinement of estimates of herbivore diet in present and past ecosystems, enabling more accurate environmental niche modelling.

Keywords

Nitrogen isotopes; phenylpropanoid pathway; solid state NMR; GC-C-IRMS;

Introduction

As the base of the terrestrial food chain, plants are fundamental to understanding the wide range of biochemical and environmental processes that occur in these ecosystems. Bulk isotopic analysis of plant tissue is therefore widely used as a source for ecological and palaeoecological information but can obscure the specific combination of pathways and mechanisms that make up bulk tissue stable isotope ratios. Bulk $\delta^{15}N$ values are determined by the primary inorganic nitrogen sources, as well as isotope discriminations during uptake, assimilation, protein biosynthesis, metabolism and catabolism (Handley and Raven, 1992; Robinson et al., 1998). Differences in the nitrogen isotopic composition of individual plant biochemicals can provide information about both the nature of the metabolic processes occurring and the relative rates of the component reactions, and therefore investigation of $\delta^{15}N$ values at the individual amino acid (AA) level can improve understanding of the isotope signals of various nitrogen-containing biomolecules and allows access to stable isotope information inaccessible to bulk methods. The nitrogen isotope composition of plants forms a baseline which affects the $\delta^{15}N$ values of the rest of the terrestrial food web, and hence an understanding of the nitrogen cycling in plants is important for trophic level analysis in ecological and archaeological studies. Indeed, for amino acid-based methods of environmental niche and trophic position modelling (e.g. Popp et al., 2007; Chikaraishi et al., 2010; Germain et al., 2013), it is vital that the underlying isotopic composition of the base of the food web is known, as this will affect all subsequent interpretations.

Nitrogen uptake and amino acid metabolism in plants

Nitrogen is taken up from the soil as NO_3^-, which is then reduced to NH_3, or as NH_4^+, and incorporated into glutamate (Glu) to form glutamine (Gln) by glutamine synthetase (GS). The

amide N of Gln is transferred to α-ketoglutarate (α-KG) by glutamine oxoglutarate aminotransferase (GOGAT) to form Glu (Forde and Woodall, 1995). The Gln amide N is therefore the primary nitrogen source for the amino groups of all other plant amino acids, via transamination reactions between their corresponding α-keto acids and Glu (Forde and Lea, 2007). Therefore, any differences between the $\delta^{15}N$ values of Glu and the other amino acids are due to the isotopic fractionations that occur as a result of the subsequent transaminations, reductions, amidations, lyase reactions and hydrolyses that are involved in plant N metabolism. Glu and Gln play a central role in nitrogen cycling and assimilation via the GS-GOGAT pathway, using NH_4^+. Glu supplies the amino group to many other amino acids by transamination reactions with their corresponding keto acids, as well as providing the carbon skeleton and amino groups of Pro, arginine and γ-amino butyric acid (Forde and Lea, 2007). Glu is also a precursor for chlorophyll biosynthesis in photosynthetic organisms (Von Wettstein et al., 1995). As well as through transamination reactions, catabolism of Glu can occur in mitochondria via a deamination reaction by glutamate dehydrogenase to give α-KG and ammonium, which is reassimilated by the GS-GOGAT pathway.

Phe is biosynthesised in the shikimate pathway, responsible for the biosynthesis of the aromatic amino acids, by a transamination reaction of prephenate and Glu to form arogenate, which undergoes decarboxylation and dehydration by arogenate dehydratase to give Phe (Figure 1). As the amino group of Phe comes from Glu, it may be expected to be ^{15}N-depleted relative to Glx. However, high $\delta^{15}N$ values of Phe relative to other amino acids have been noted in several previous studies of cereal grains (Hofmann et al., 1995; Styring et al., 2014a), grassland plants (Bol et al., 2002; Ostle et al., 1999), mangrove trees (Smallwood et al., 2003), and apple trees and bean plants (Steffan et al., 2013). It has been hypothesised that its involvement in the phenylpropanoid pathway is responsible for its relative ^{15}N enrichment. Phe is the main

precursor in this pathway, which is responsible for the synthesis of various secondary metabolites and phenolic compounds in plants such as flavonoids, tannins, lignans and lignin.

The first step in the phenylpropanoid pathway involves the deamination of Phe to form cinnamate and ammonium, catalysed by the enzyme phenylalanine ammonia-lyase (PAL; Figure 1), a member of the aromatic amino acid lyase family. This enzyme discriminates against ^{15}N due to a kinetic isotope effect (Hermes et al., 1985): PAL is a branch point enzyme (Dixon and Paiva, 1995) between the shikimate pathway and the phenylpropanoid pathway, so any kinetic isotope effect associated with this deamination will be expressed. Cinnamate is then hydroxylated by cinnamate 4-hydroxylase to *p*-coumarate, which undergoes reduction and methoxylation reactions to give the monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These are incorporated into lignin as hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units respectively (Boerjan et al., 2003). Tyrosine (Tyr) can also be deaminated directly to *p*-coumarate, using PAL or a phenylalanine/tyrosine ammonia lyase (Appert et al., 1994; Cochrane et al., 2004; Schuster and Retey, 1995) but is a poorer substrate than Phe.

The ammonia produced by the phenylpropanoid pathway will be depleted in ^{15}N, as the lighter Phe isotopologue is preferentially deaminated. This will leave the remaining Phe pool relatively ^{15}N-enriched, and therefore Phe $\delta^{15}N$ values should be greater than those of AAs more central to N-cycling, such as Glu. Vascular plants have a high turnover of Phe, as 30-45% of plant organic matter originates from Phe, and to a lesser extent Tyr, via the phenylpropanoid pathway (Cantón et al., 2005; Razal et al., 1996).

Glycine (Gly) and serine (Ser) are important C_1 sources in one-carbon metabolism – Ser is converted to Gly using the cofactor tetrahydrofolate (THF) in the reversible reaction Ser + THF ⇌ Gly + CH_2-THF + H_2O, catalysed by serine hydroxymethyltransferase (SHMT), and this CH_2-THF enters into C_1 metabolism. In the cytosol, the equilibrium constant of this reaction favours Gly formation due to rapid use of the C_1 unit and consequent regeneration of THF, and the conversion of Gly back to Ser in the mitochondria by glycine decarboxylase (GDC) and SMHT (Hanson and Roje, 2001; Mouillon et al., 1999; Figure 2). This reaction system leads to a net loss of Gly, as two molecules of Gly are needed to regenerate one molecule of Ser, and therefore a need for production of Gly or Ser via transamination of Glu with glyoxylate or 3-phosphohydroxypyruvate, respectively. While the NH_4^+ produced in the Gly decarboxylation reaction will be ^{15}N-depleted relative to Gly, leading to the remaining Gly being relatively ^{15}N enriched, this NH_4^+ is reassimilated by the GS-GOGAT pathway locally (Cantón et al., 2005), forming the Glu which is then used to biosynthesise Gly and Ser. This leads to a net ^{15}N-depletion of Gly and Ser relative to Glu.

Data from previous studies of plant amino acid $\delta^{15}N$ values, displayed in Figure 3, shows a trend in the enrichment of Phe relative to Glx, where tree species have more enriched Phe $\delta^{15}N$ values than herbaceous plants, i.e. lower $\Delta^{15}N_{Glx\text{-}Phe}$ values. In plants in which more phenolic secondary metabolites, such as lignin, are biosynthesised, it is likely that there will be a greater ^{15}N-enrichment of Phe, as there is a higher turnover of the phenylpropanoid pathway. Hence the remaining Phe pool has even higher $\delta^{15}N$ values. It should therefore be possible to determine the relative lignin content of plants based on their $\Delta^{15}N_{Glx\text{-}Phe}$ values. This hypothesis is based upon the few plant $\delta^{15}N$ values that have been reported in the literature so far, and it is clear that a more extensive dataset is needed to test this.

As part of the phenylpropanoid pathway, cinnamate is *O*-methylated to produce coniferyl and sinapyl alcohols, requiring large quantities of one-carbon units from C_1 metabolism (Cantón et al., 2005), and therefore it is also expected that Gly and Ser, which are important C_1 sources, will be more ^{15}N-depleted in woody plants than herbaceous plants.

These isotopic methods of classification are based on the assumption that woody plants produce more lignin than herbaceous plants, even in the plant foliage rather than just the woody tissues. However, this assumption has not previously been tested, and hence quantification of lignin in these different plant types will help to confirm that these isotopic differences are due to differences in the extent of lignin production as hypothesised. CP-MAS ^{13}C solid state NMR has been used for the analysis of lignin in wood since 1980 (Bartuska et al., 1980) but, like other lignin quantification techniques, it has been mostly applied to studies of wood pulp and products of the paper industry. Therefore, studies have focussed on woody plant parts, rather than other plant tissues, such as leaves, and as such there are few examples of the analysis of the lignin content of plant foliage (Toda et al., 2015).

The method uses the relative intensities of peaks attributed to carbohydrates compared to those attributed to the aromatic carbons of lignin and relies on the following assumptions: (i) the carbohydrate fraction consists solely of cellulose (ignoring hemicelluloses and other polysaccharides), so has the empirical formula $C_6H_{10}O_5$, and (ii) the empirical formula of the lignin in each sample is the same, and known. In reality, the empirical formula varies depending on the number of S, G and H units. As this has not been studied for many of the species here,

an empirical formula for softwood lignin is used as a compromise. While these assumptions may lead to this method not providing an accurate absolute concentration of lignin, it should show any *relative* differences in concentration between tree and herb leaf tissue and be able to support the hypothesis that the more enriched Phe $\delta^{15}N$ values relative to Glx in woody plants compared to herbaceous plants are due to higher lignin concentrations.

In this study, the $\delta^{15}N$ values of foliage from a variety of plant species, grown under controlled conditions at North Wyke Farm Platform and Bad Lauchstädt Research Station, as well as wild plants from the Chew Valley, were determined by GC-C-IRMS. This will help understanding of amino acid N-cycling in different plant types. Comparison of $\delta^{15}N$ values of herbaceous and woody plant foliage with lignin concentrations determined by ^{13}C cross-polarisation, magic-angle-spinning (CPMAS) NMR will help to determine the effect of lignin content on plant N metabolism.

Results

Amino acid $\delta^{15}N$ values

Typical gas chromatograms for herbaceous (*Poa annua*) and woody (*Tilia platyphyllos*) plant tissue are shown in (Figure 4). No substantial difference was found between the amino acid distributions of herbaceous and tree species.

Individual amino acid $\delta^{15}N$ values were determined for up to 13 of the 20 AAs found in plants: Glu/Gln (Glx), Ala, Asp/Asn (Asx), Pro, Val, Leu, Ile, Phe, Tyr, Thr, Gly, Ser, and Lys (Table

1). Standard deviations were all below 0.9‰. During hydrolysis, Gln and Asn are quantitatively deamidated to Glu and Asp respectively (Hill, 1965). The $\delta^{15}N$ value of Asx therefore combines the N of Asp and the amino N of Asn, while the $\delta^{15}N$ value of Glx is a mean of the N of Glu and the amino N of Gln.

In the following figures AAs are ordered according to their metabolic relationships in plants, as described in Styring et al. (2014b). Phe has a distinct metabolic pathway from the other AAs; Glx and Pro are closely related since the amino group of Pro derives from Glu; the amino group of Ala derives from Glu or γ-aminobutyric acid; the N from Asx can be exchanged with many AAs, including Glu and Ala; Val, Leu and Ile are branched-chain AAs; and Gly and Ser can be biosynthesised from each other.

The plant AA $\delta^{15}N$ values vary from a minimum of -19.3‰ for Thr to a maximum of 20.6‰ for Phe, reflecting the isotopic fractionations caused by the differences in individual AA routing and metabolism (Figure 5a).

Phenylalanine and tyrosine $\delta^{15}N$ values

Phe is the most ^{15}N enriched amino acid in all plants, and therefore has the most negative $\Delta^{15}N_{Glx\text{-}AA}$ values (Figure 5b). An unequal variance Student's t-test was conducted to compare the $\Delta^{15}N_{Glx\text{-}Phe}$ values of lignified woody plants (trees) and non-lignified plants (herbs), showing that the more negative values for trees ($n = 27$, mean = -9.3‰, standard deviation = 1.6‰) relative to herbs ($n = 26$, mean = -5.4‰, standard deviation = 2.1‰), displayed in Figure 6a, are of statistical significance at the 99% confidence interval; $p < 0.01$.

Tyr shows a minor overall enrichment in ^{15}N relative to Glx. A comparison of tree and herbaceous species (Figure 6b) shows that this enrichment is due to the woody plants, with the herbaceous plants having $\Delta^{15}N_{Glx\text{-}Try}$ values close to zero. An equal variance Student's t-test was also conducted to compare the tree and herb $\Delta^{15}N_{Glx\text{-}Tyr}$ values, showing that the more negative values for trees/shrubs (n = 14, mean = -2.3‰, standard deviation = 1.9‰) relative to herbs (n = 10, mean = -0.1‰, standard deviation = 1.8‰) are of statistical significance at the 99% confidence interval; $p < 0.01$.

Glycine and serine $\delta^{15}N$ values

Gly is the most ^{15}N depleted AA in most plants, with Ser also ^{15}N depleted relative to most AAs. An unequal variance Student's t-test was conducted to compare the tree and herb $\Delta^{15}N_{Glx\text{-}Gly}$ values, showing that the higher values for trees (n = 27, mean = 12.9‰, standard deviation = 1.4‰) relative to herbs (n = 26, mean = 9.2‰, standard deviation = 2.1‰), as shown in Figure 7a, are statistically significant at the 99% confidence interval; $p < 0.01$. However, an unequal variance Student's t-test conducted to compare the tree and herb $\Delta^{15}N_{Glx\text{-}Ser}$ values, showed that the higher values for trees (n = 26, mean = 8.8‰, standard deviation = 1.5‰) relative to herbs (n = 26, mean = 8.1‰, standard deviation = 1.6‰) are not statistically significant at the 99% confidence interval; $p = 0.27$ (Figure 7b).

^{13}C CPMAS NMR

Typical NMR spectra for herbaceous (*Poa annua*) and woody (*Quercus robor*) plant tissue are shown in Figure 8. Lignin concentrations were calculated according to Haw et al. (1984), using Equation 1.

$$\text{wt \% Lignin} = \frac{(^{183}/_{9.92})\mathrm{I}'_{\mathrm{lig}}}{(^{183}/_{9.92})\mathrm{I}'_{\mathrm{lig}} + (^{162}/_{6})\mathrm{I}'_{\mathrm{carb}}} \qquad \text{Equation 1}$$

Where I'_{lig} and I'_{carb} are the normalised intensity due to lignin and carbohydrate respectively, 183 and 162 are the formula masses of lignin ($C_9H_{7.95}O_{2.4}(OMe)_{0.92}$, 185 g mol^{-1}) and carbohydrate ($C_6H_{10}O_5$, 162 g mol^{-1}), and 9.92 and 6 are the numbers of carbon atoms in the repeating units. Derivation of this equation can be found in Haw et al. (1984). Calculated lignin concentrations as a percentage of dry, lipid extracted mass are given in Table 2.

Discussion

Amino acid $\delta^{15}N$ values

While the variation in $\delta^{15}N$ values between samples for each amino is quite large, when normalised to Glx the variation is greatly reduced, as this removes the effect of different N sources depending on location and soil type. Glx is an appropriate choice for this due to its central role in N cycling as it is the immediate recipient of soil NH_4^+ assimilated by the GS-GOGAT pathway.

Pro is formed by the cyclisation of Glu, and as this cyclisation involves the formation of a C-N bond, Pro is expected to be ^{15}N depleted relative to Glx. However, there is low demand for

Pro in plant protein – it constitutes less leaf protein AA than Glu and Gln, leading to little fractionation as this transaminase reaction is reversible.

As Glx and Asx are both of central importance in plant nitrogen cycling, their $\delta^{15}N$ values are very similar (4.8‰ and 5.6‰ respectively). Ala is formed by a transamination reaction of Glu or Asp with pyruvate, leading to slightly lower $\delta^{15}N$ values in Ala relative to Glx and Asx.

Of the BCAAs, Leu and Ile $\delta^{15}N$ values are depleted relative to Glx and have similar values (1.1‰ and 1.8‰), while Val has similar $\delta^{15}N$ values to Glx (4.6‰). The biosynthesis of Val and Leu/Ile are catalysed by two different forms of BCAT, valine aminotransferase and leucine/isoleucine aminotransferase, which, based on the $\delta^{15}N$ values obtained here, likely have different KIEs associated with them.

Thr has the largest variation in $\delta^{15}N$ values, ranging from -19.3‰ to 7.2‰, although the range is decreased in normalised $\Delta^{15}N_{Glx\text{-}Thr}$ values (from 0.6‰ to 17.9‰). Thr is biosynthesised from Asp with no C-N bond breaking or formation step, and therefore would be expected to possess very similar $\delta^{15}N$ values to Asx, but instead displayed relatively depleted values. A possible explanation for this may be the activity of the threonine aldolase enzyme, which reversibly catalyses the conversion of Thr to Gly and acetaldehyde (Joshi et al., 2006). If this reverse reaction is a contributor to Thr biosynthesis in plants, Thr $\delta^{15}N$ values should represent a weighted average of the contribution from Asx and Gly, leading to the intermediate $\delta^{15}N$ values as displayed here. While ^{13}C-labelling experiments of *Arabdiopsis thalania* indicate that Gly is not directly converted to Thr *in vivo* (Prabhu et al., 1996), metabolic profiling studies

suggest this reverse reaction does occur in *A. thalania* (Joshi et al., 2006) and other plant species (Broeckling et al., 2005).

Lys is also synthesised from Asp, with no C-N bond breaking or formation step associated with the α-amino group, but receives the ε-amino group from Glu later in its biosynthetic pathway, in a step involving a KIE. As Lys $\delta^{15}N$ values represent an average of these two N atoms, a small ^{15}N-depletion relative to Asx and Glx occurs.

Ser and Gly are the most ^{15}N-depleted AAs relative to Glx. Due to their involvement in the photorespiratory cycle and in C_1 metabolism (Hanson and Roje, 2001), there is a high turnover of these AAs, leading to a larger depletion relative to their amino group donor AAs. The biosynthesis of Ser from Gly involves no N-fractionating steps, but Ser $\delta^{15}N$ values are less depleted than those of Gly, possibly due to Ser (along with Glu, Ala and Asn) being a source of the Gly amino group by the action of serine:glyoxylate aminotransferase. This relative depletion is contrary to a previous study of plant grain AAs (Styring et al., 2014a), suggesting a difference in metabolism between photosynthetic (and photorespiratory) plant tissues, i.e. leaves, and storage tissues, i.e. seeds.

Differentiating between woody and herbaceous plants

As well as other secondary metabolites, all vascular plants produce lignin to some extent, and therefore exhibit relatively enriched Phe $\delta^{15}N$ values compared to Glx. For woody plants, which produce a higher concentration of lignin per unit biomass, there is likely to be an enhanced turnover of Phe, as more is routed into the phenylpropanoid pathway for lignin

biosynthesis. This is proposed to result in ^{15}N fractionation, leading to the remaining Phe pool, available for protein synthesis, being ^{15}N-enriched, which explains the more negative $\Delta^{15}N_{Glx\text{-}Phe}$ values seen in the woody plant samples (-9.3±1.6‰). However, herbaceous plants also produce lignin, and other phenolic secondary metabolites, and consequently there is still some ^{15}N fractionation, leading to their Phe $\delta^{15}N$ values being enriched relative to those of Glx. The lower flux of Phe into the phenylpropanoid pathway in less lignified (herbaceous) plants, compared to woody plants, is reflected in the less negative $\Delta^{15}N_{Glx\text{-}Phe}$ values observed in the former (-5.8±2.1‰; Figure 6).

Tyr is also a substrate in the phenylpropanoid pathway, where it is deaminated by aromatic amino acid ammonia lyases to form *p*-coumaric acid. As with Phe, this has the capacity to cause ^{15}N fractionation. However, Tyr is a less preferred substrate for this pathway and so, the extent of fractionation is less than for Phe, as seen in Figure 6, and occurs mainly in tree species. Consideration of the above biochemical factors and observed $\delta^{15}N$ values make the $\Delta^{15}N_{Glx\text{-}Phe}$ proxy an appropriate discriminator between plant types.

Gly, and to a lesser extent Ser, shows the opposite pattern to Phe, with $\delta^{15}N$ values for trees more depleted relative to Glx than herb $\delta^{15}N$ values. As part of the phenylpropanoid pathway, cinnamate is *O*-methylated to produce coniferyl and sinapyl alcohols, requiring large quantities of one-carbon units from C_1 metabolism (Cantón et al., 2005). Ser and Gly are a source of these C_1 units, which leads to their depleted $\delta^{15}N$ values. As there is a higher demand for C_1 units in more lignified plants due to a greater production of the methoxylated monolignols, this ^{15}N-depletion is greater in trees than in herbaceous plants. However, as well as methylation of lignols, C_1 metabolism provides one-carbon units for amino acids, proteins, nucleic acids, and

other methylated compounds (Cossins and Chen, 1997). Therefore, the $\delta^{15}N$ values of Gly and Ser will vary depending on the fluxes and rates of these C_1 metabolism reactions. This complicates the use of $\Delta^{15}N_{Glx\text{-}Gly}$ values as an indicator of lignin production, thus $\Delta^{15}N_{Glx\text{-}Phe}$ values may be a better indicator of lignin content as there are likely to be fewer factors underpinning its values.

Lignin concentration of herbaceous and woody plant foliage by ^{13}C CPMAS NMR

As hypothesised, woody plant foliage was found to display higher lignin concentrations (12.6±1.4%) than herbaceous foliage (6.3±1.2%), which interestingly shows that higher lignin production is not localised to woody tissues. While the results show a relative difference in lignin concentration between these plant types, there are a number of sources of error that may lead to inaccuracies in the absolute lignin concentrations including: (i) the exact chemical structure of lignin is not known, (ii) there are likely to be carbohydrate contributions of polysaccharides other than cellulose, such as hemicelluloses, and (iii) signals at 10-50 ppm likely arise from insoluble aliphatic lipids, such as cutin and cutan, that are not removed during the lipid extraction method employed for these samples. These would add to both the carbohydrate and lignin signals. However, the aim of this analysis was to determine the relative lignin concentrations of the plant types, and therefore exact absolute concentrations are not essential; the finding that the lignin concentration is higher in the foliage of woody plants compared to that of herbaceous plants is important and allows the comparison of the isotopic proxies with the lignin content of these plants.

The lignin content estimated from ^{13}C CPMAS NMR was compared with the $\Delta^{15}N_{Glx\text{-}Phe}$ values of these samples, as shown in Figure 9. A Pearson product-moment correlation coefficient was computed, showing a negative correlation between $\Delta^{15}N_{Glx\text{-}Phe}$ values and lignin content calculated by NMR, ($r = -0.70$, $df = 8$, $p = 0.025$), i.e. more negative $\Delta^{15}N_{Glx\text{-}Phe}$ values are correlated with higher lignin content. This supports the hypothesis that the more negative $\Delta^{15}N_{Glx\text{-}Phe}$ values in woody plant leaf tissue compared to herbaceous foliage is related to a higher lignin production, and therefore concentration, in tree foliage.

Conclusions

The $\delta^{15}N$ values of plant foliage AAs have been determined in 52 individual plants from 34 different plant species, which were related to their biosynthetic and metabolic pathways, providing the largest baseline dataset for the primary producers of the terrestrial food web reported to date. The major findings were that:

i) The difference between $\delta^{15}N$ values of Glx and Phe can be used to differentiate between woody and herbaceous plants, with $\Delta^{15}N_{Glx\text{-}Phe}$ values of -9.3‰ and -5.8‰ respectively.

ii) Analysis of plant leaf tissue lignin concentration by ^{13}C CPMAS NMR showed that tree foliage has a higher concentration of lignin (12.6 wt%) than herbaceous foliage (6.3 wt%).

iii) The difference in $\Delta^{15}N_{Glx\text{-}Phe}$ values between plant types is caused by the difference in lignin production in these plants, as demonstrated by the correlation between lignin concentrations and $\Delta^{15}N_{Glx\text{-}Phe}$ values.

These findings have important implications for the interpretation of vertebrate and invertebrate consumer $\delta^{15}N$ values and their underlying individual AA $\delta^{15}N$ values in both archaeology and ecology, as it is clear that different plant types, even within a C_3 ecosystem, can have significantly different AA $\delta^{15}N$ values.

Experimental

Plant tissue sampling strategy

Plant foliage from 27 tree specimens of 20 individual species, and 26 herb specimens of 17 individual species, was collected from the Chew Valley, Somerset, UK (n = 25, classified as tree: n = 19, or herb: n = 6), Bad Lauchstädt Experimental Research Station, Saxony-Anhalt, Germany (n = 20, classified as tree: n = 8, or herb: n = 12), and North Wyke Farm Platform, Devon, UK (herbs, n = 8), for amino acid analysis. A subset of these samples (n = 10, consisting of 5 tree samples and 5 herb samples), was analysed by ^{13}C CPMAS NMR spectroscopy.

Chew Valley tree and herb specimens were collected from areas of uncultivated land and hedges within the nitrate vulnerable zones (NVZ) around Chew Valley reservoirs and their catchments. Thus, the various plants will have been minimally impacted by inorganic fertilisers. The Bad Lauchstädt Experimental Research Station is part of the Helmholtz Centre for Environmental Research (UFZ). It aims to contribute to a better understanding of the complex relationships in the soil-plant-atmosphere system, in particular factors arising from changes in land use, climate and species. Herb samples were taken from ‘control’ plots of the Static Fertilization Experiment, in which no nitrogen fertiliser has been added since 1902. Tree

specimens were collected from the unfertilised historic park. North Wyke Farm Platform is part of Rothamsted Research in Devon, UK. It is a controlled and self-contained experiment, which aims to address questions relating to sustainable agriculture, such the improvement of grasslands for sustainable ruminant livestock production systems, including how different pastures affect livestock growth and condition. All the plant specimens for this study were collected from the 'control' permanent pasture, which comprised a mixed sward dominated by ryegrass (*Lolium perenne*).

Analytical procedures for amino acid analyses

Plant leaves and stems were washed with double-distilled water (DDW), lyophilised and ground to a powder. Lipids were extracted from the powdered samples with chloroform/methanol (2:1 *v/v*, 3 × 3 mL) by ultrasonication. AA *N*-acetyl isopropyl (NAIP) ester derivatives were prepared according to established protocols (Corr et al., 2007; Styring et al., 2012). Briefly, norleucine was added as an internal standard to ca. 25 mg of dried, crushed and lipid-extracted plant tissues, which were hydrolysed (6 M HCl, 5 mL; 100°C, 24 h) and allowed to cool before centrifugation (1700 *g*, 10 min). The hydrolysates were transferred to clean culture tubes with 0.1 M HCl (2 mL), and the solutions blown to dryness under nitrogen. AAs were separated from other compounds using Dowex 50WX8 ion-exchange resin. The AAs were converted to their isopropyl esters by addition of a mixture of isopropanol and acetyl chloride (4:1 *v/v*, 1 mL; 100°C, 1 h). Reagents were evaporated under a gentle stream of N_2 (40°C). AA isopropyl esters were acetylated with a mixture of acetone, triethylamine and acetic anhydride (5:2:1 v/v/v, 1 mL; 60°C, 10 min). Reagents were removed under a gentle stream of nitrogen at room temperature, then 1 mL saturated NaCl solution added, and NAIP esters extracted into ethyl acetate (3 × 3 mL). The solvent was evaporated under nitrogen as before.

AA NAIP ester derivatives were redissolved in ethyl acetate for analysis. Asparagine and glutamine are converted into aspartic acid and glutamic acid respectively during hydrolysis. The $\delta^{15}N$ value of Asx therefore combines the N of aspartate and the amino N of asparagine, while the $\delta^{15}N$ value of Glx is a mean of the N of glutamate and the amino N of glutamine.

AAs were identified by GC-FID by comparison with AA standards, and quantified by comparison with a known amount of norleucine internal standard. Their $\delta^{15}N$ values were determined by GC-C-IRMS as described in Styring et al. (2012) with a modified GC method, using a DB-35 capillary column (30 m × 0.32 mm internal diameter; 0.5 µm film thickness; Agilent Technologies, UK). The oven temperature of the GC held at 40°C for 5 min before increasing at 15°C min^{-1} to 120°C, then 3°C min^{-1} to 180°C, then 1.5°C min^{-1} to 210°C and finally 5°C min^{-1} to 270°C and held for 1 min. A Nafion™ drier removed water and a cryogenic trap removed CO_2 from the oxidised and reduced analyte. Isotopic compositions are expressed using the delta scale as follows: $\delta^{15}N = R_{sample} / R_{standard} - 1$, where R is the $^{15}N/^{14}N$ ratio, and the standard is atmospheric N_2 (AIR). All $\delta^{15}N$ values are reported relative to reference N_2 of known isotopic composition, introduced directly into the ion source in four pulses at the start and end of each run. Each reported $\delta^{15}N$ value is the mean of triplicate determinations. A standard mixture of AAs of known $\delta^{15}N$ values was analysed every three runs to ensure acceptable instrument performance.

^{13}C CPMAS NMR

Freeze-dried, ground and lipid extracted leaf tissues (*c.a.* 300 mg) were analysed by ^{13}C CPMAS NMR spectroscopy. All solid state ^{13}C CPMAS NMR measurements were performed

at ambient temperatures using a 7.05 T Varian-Chemagnetics Infinity Plus-300 NMR spectrometer operating at a ^{13}C Larmor frequency of 75.46 MHz. These measurements were facilitated by a Bruker 4 mm dual channel (HX) MAS NMR probe which enabled MAS frequencies of 12 kHz for each experiment. A conventional CPMAS experiment was implemented in each case which used an initial ^{1}H pulse time of 3 μs, a $^{1}H/^{13}C$ Hartmann-Hahn contact period of 1 ms, a recycle delay of 5 s, and a ^{1}H rf field strength of 100 kHz during acquisition. All ^{13}C chemical shifts were calibrated against TMS (the primary IUPAC ^{13}C reference, $\delta_{iso} = 0$ ppm) via a secondary solid alanine reference ($\delta_{iso}(CH_3) = 20.5$ ppm, $\delta_{iso}(CH) = 50.5$ ppm, $\delta_{iso}(COOH) = 177.8$ ppm).

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Figure and table captions:

Figure 1 – Simplified pathway of monolignol formation from Phe in the phenylpropanoid pathway, starting with Phe biosynthesis from the shikimate pathway. TA – transaminases; ADT – arogenate dehydratase; PAL – phenylalanine ammonia-lyase; C4H – cinnamate 4-hydroxylase; GS-GOGAT – glutamine synthetase-glutamate synthase pathway.

Figure 2 – Gly and Ser biosynthesis in plants. THF – tetrahydrofolate; CH_2-THF – 5,10-methylenetetrahydrofolate; 3-P Ser – 3-phosphoserine; 3-P hydroxypyruvate – 3-phosphohydroxypyruvate; α-KG – α-ketoglutarate. SHMT – serine hydroxymethyltransferase; GDC – glycine decarboxylase; PSAT – phosphoserine aminotransferase; PSP – phosphoserine phosphatase; GS-GOGAT – glutamine synthetase-glutamate synthase pathway. After Cantón et al. (2005).

Figure 3 – $\Delta^{15}N_{Glx\text{-}AA}$ values from plant leaf proteins, from Ostle et al. (1999), Bol et al. (2002), Smallwood et al. (2003) and Steffan et al. (2013). Open markers denote tree species, filled denote herbaceous species. Numerical values estimated from graphs except from Steffan et al. (2013).

Figure 4 – Typical gas chromatograms of herbaceous (*Poa annua*) and tree (*Tilia platyphyllos*) leaf tissue. IS – norleucine internal standard.

Figure 5 – Range and variability of (a) individual AA $\delta^{15}N$ values, and (b) $\Delta^{15}N_{Glx\text{-}AA}$ values, for all plant samples. Data points are jittered to allow visualisation of overlapping values.

Figure 6 – Comparison of (a) the $\Delta^{15}N_{Glx\text{-}Phe}$ values, and (b) the $\Delta^{15}N_{Glx\text{-}Tyr}$ values, of tree and herb species. Data points are jittered to allow visualisation of overlapping values.

Figure 7 – Comparison of (a) the $\Delta^{15}N_{Glx\text{-}Gly}$ values, and (b) the $\Delta^{15}N_{Glx\text{-}Ser}$ values, of tree and herb species. Data points are jittered to allow visualisation of overlapping values.

Figure 8 – Typical CPMAS NMR spectra of herbaceous (*Poa annua*) and tree (*Quercus robor*) leaf tissue, with signals at 160-109 ppm from lignin aromatics, and at 109-20 ppm from carbohydrates and lignin propyl and methoxy carbons.

Figure 9 – Correlation between $\Delta^{15}N_{Glx\text{-}Phe}$ values and lignin content of tree (filled markers) and herbaceous (open markers) species. Pearson product-moment correlation coefficient $r = -0.70$, $df = 8$, $p = 0.025$.

Table 1 – Nitrogen isotopic composition of plant amino acids, and $\Delta^{15}N_{Glx\text{-}Phe}$ proxy values.

Table 2 – Lignin concentration as wt% of dry lipid-extracted mass, as determined by CPMAS NMR. Numbers in brackets indicate the sample number from Table 1

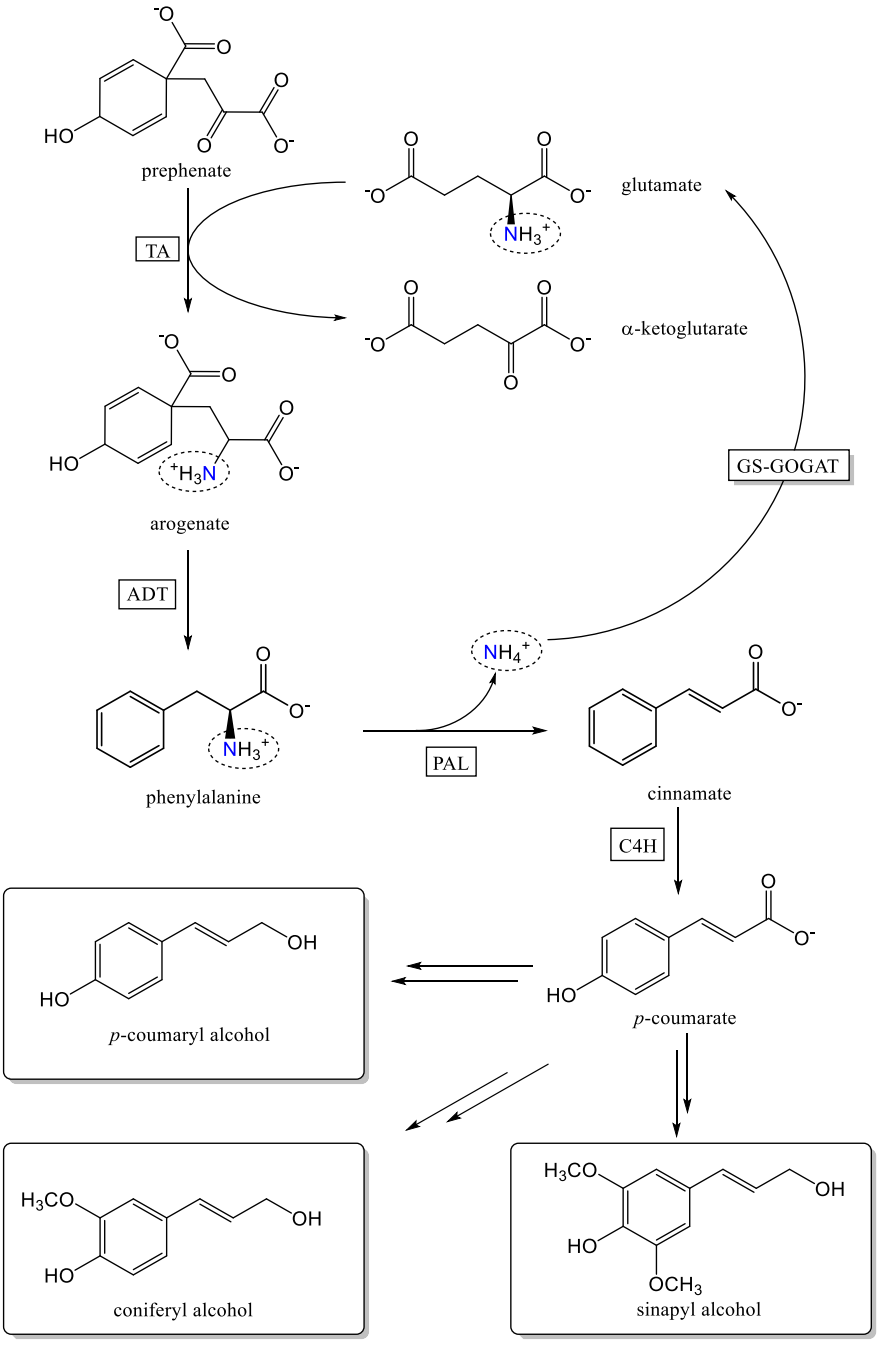
prephenate
TA
glutamate
α-ketoglutarate
arogenate
GS-GOGAT
ADT
NH4+
phenylalanine
PAL
cinnamate
C4H
p-coumaryl alcohol
p-coumarate
coniferyl alcohol
sinapyl alcohol

Figure 1

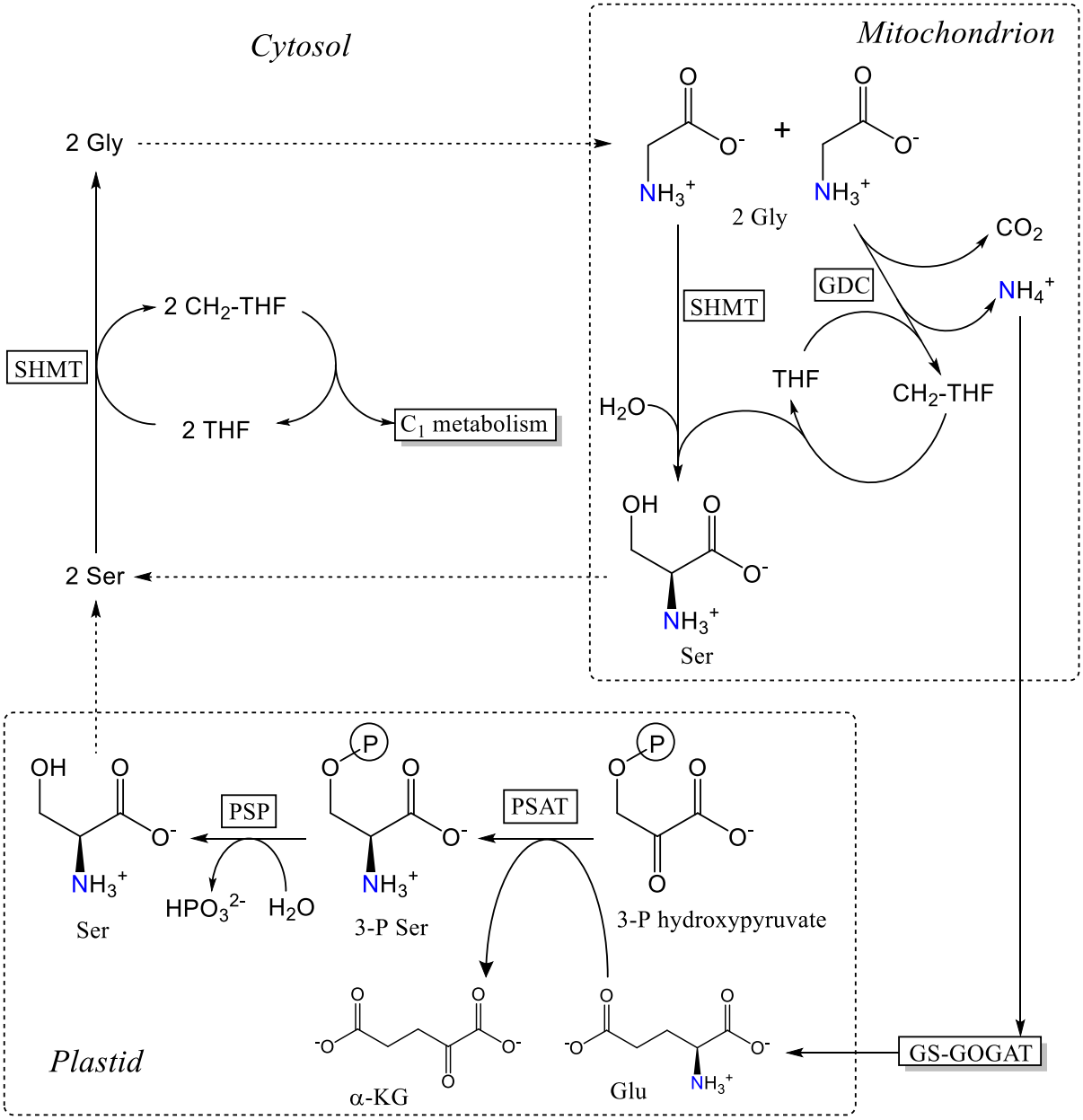
Cytosol
Mitochondrion
2 Gly
2 Gly
SHMT
2 CH2-THF
2 THF
C1 metabolism
SHMT
GDC
CO2
NH4+
THF
CH2-THF
H2O
2 Ser
Ser
PSP
PSAT
HPO3 2-
H2O
Ser
3-P Ser
3-P hydroxypyruvate
Plastid
α-KG
Glu
GS-GOGAT

Figure 2

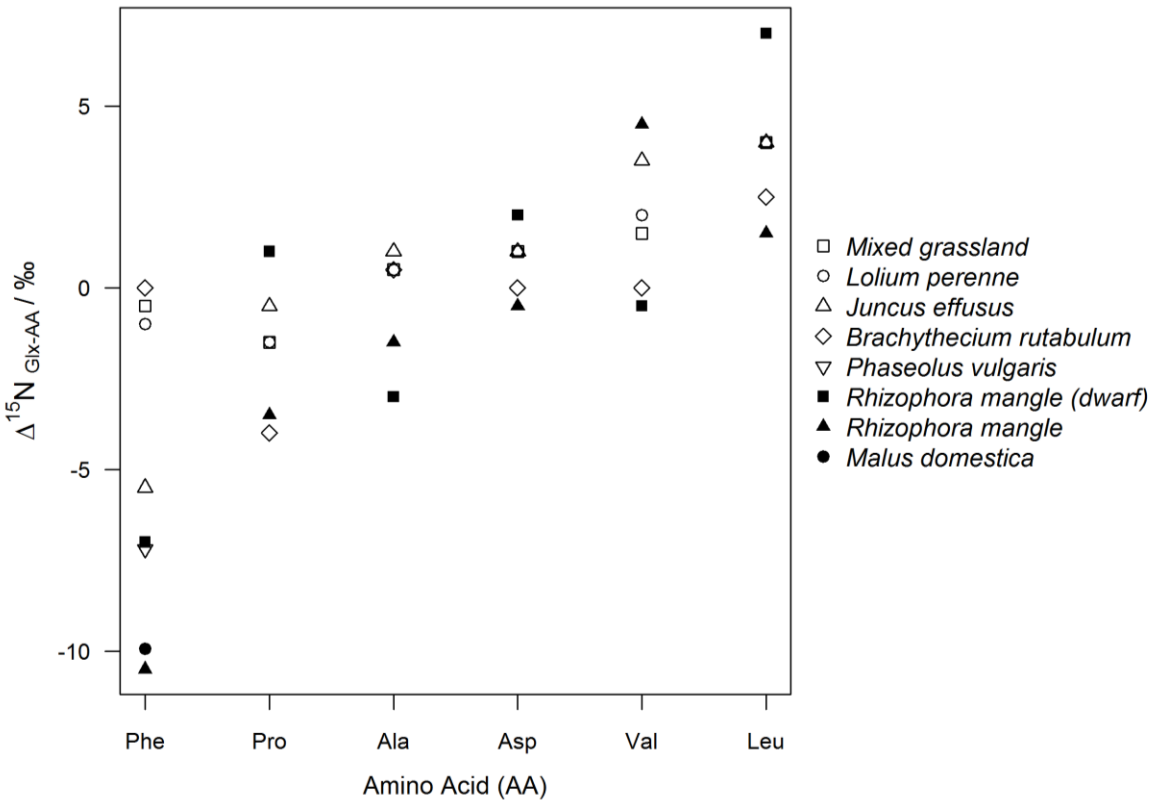

Figure 3

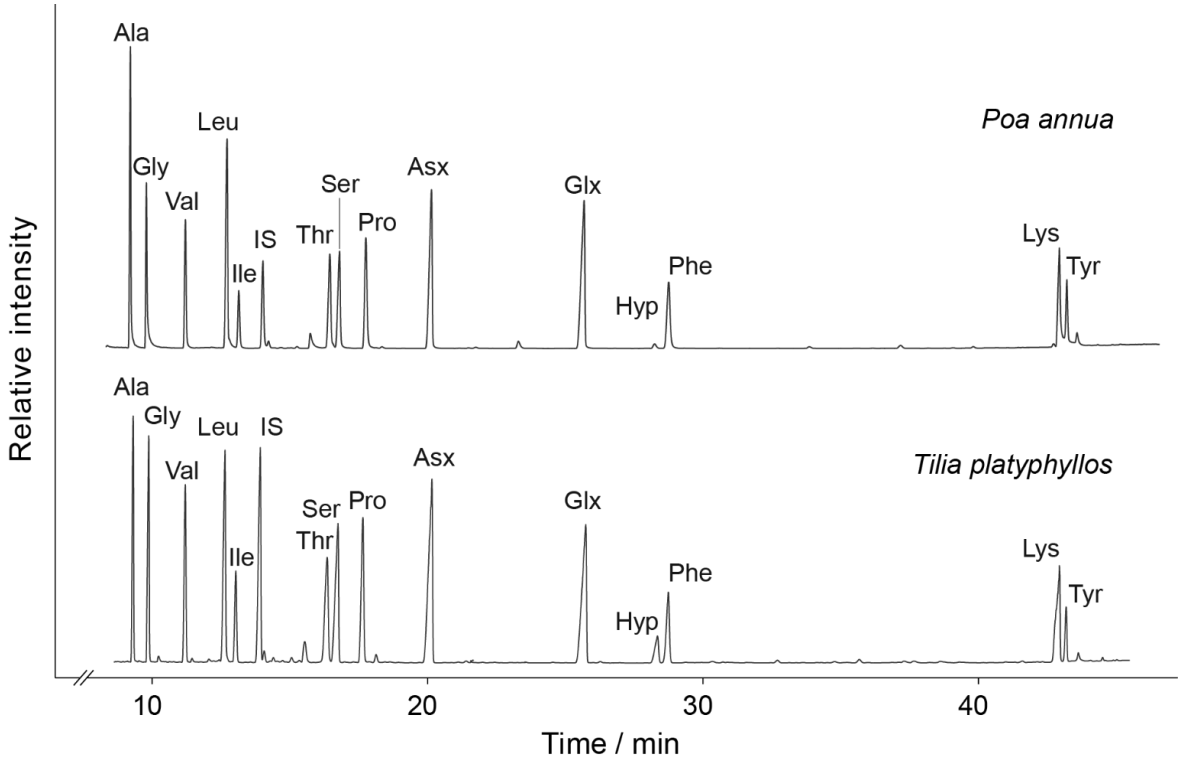

Figure 4

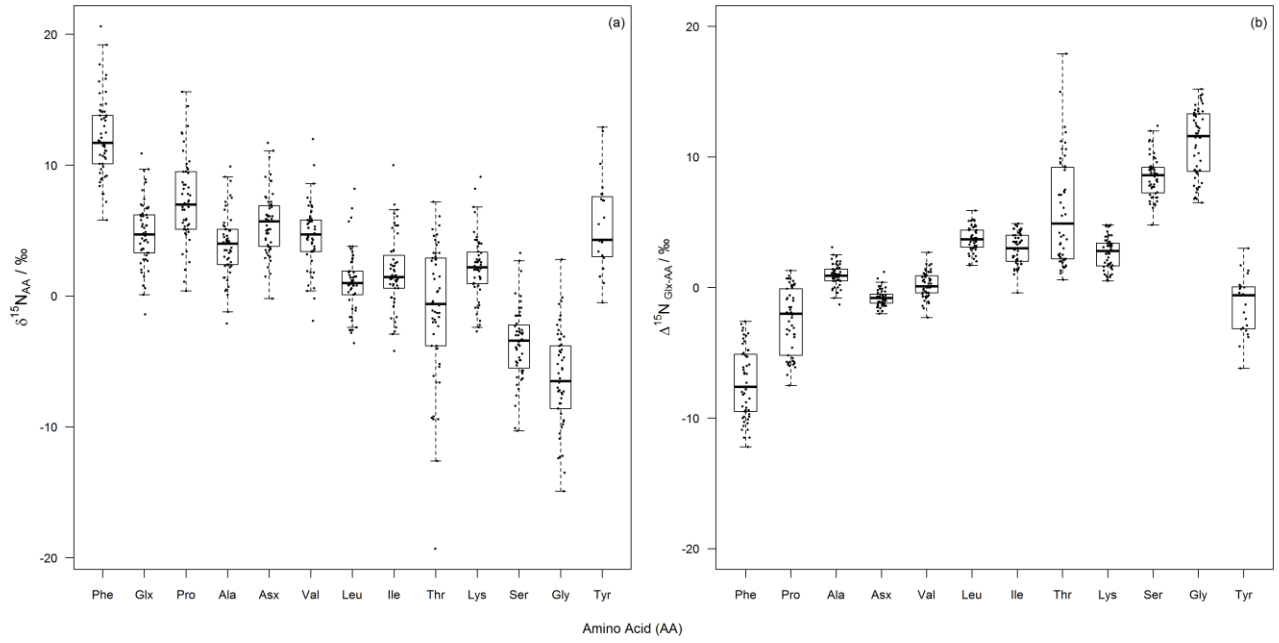

Figure 5

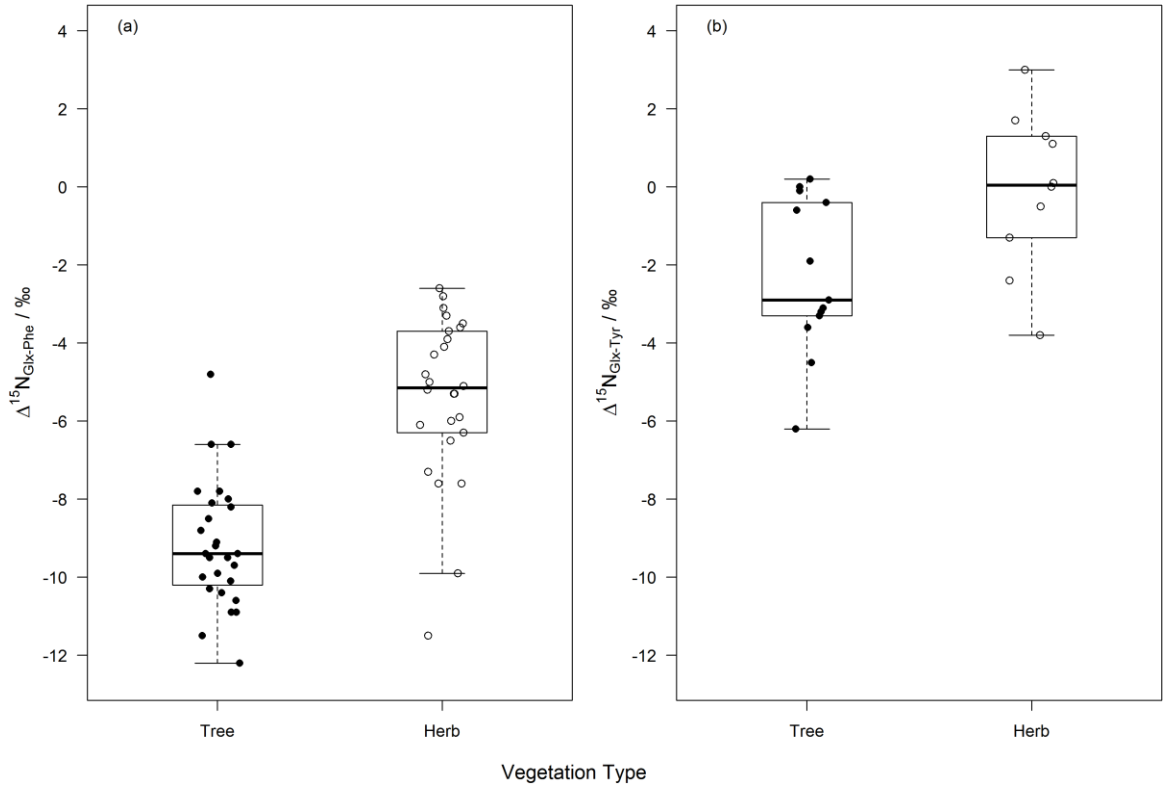

Figure 6

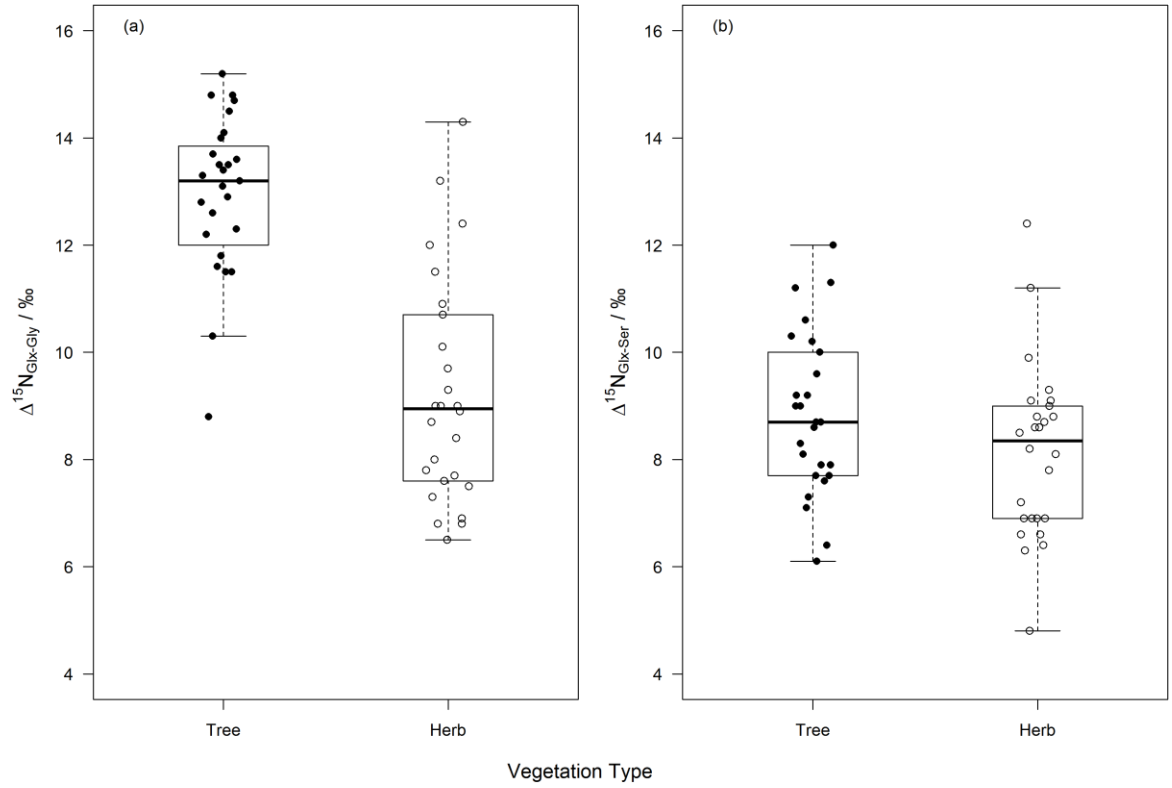
(a)
(b)
16
14
12
10
8
6
4
Δ 15NGlx-Gly / ‰
Δ 15NGlx-Ser / ‰
Tree
Herb
Vegetation Type

Figure 7

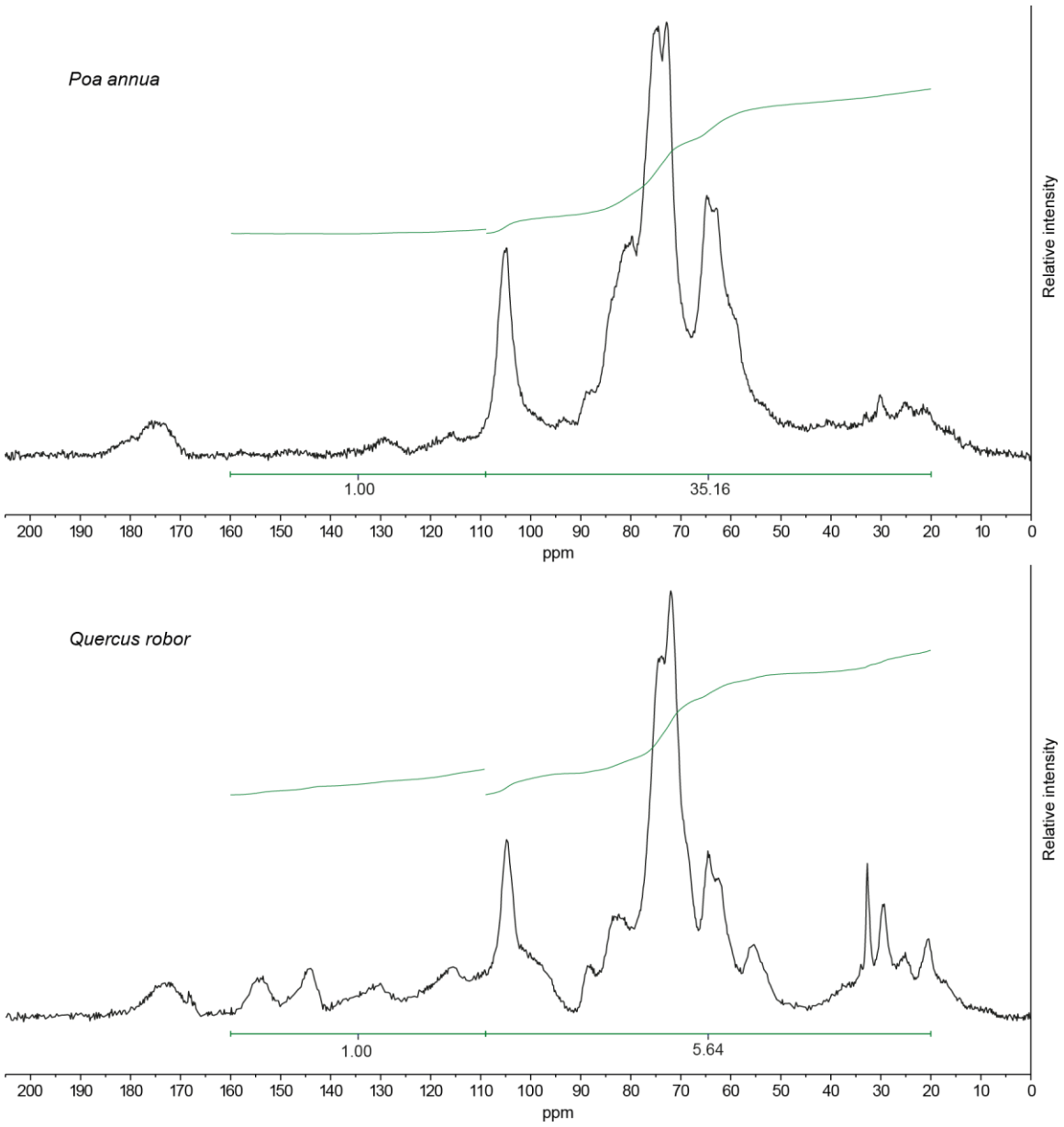
Poa annua
1.00
35.16
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm
Relative intensity
Quercus robor
1.00
5.64
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm
Relative intensity

Figure 8

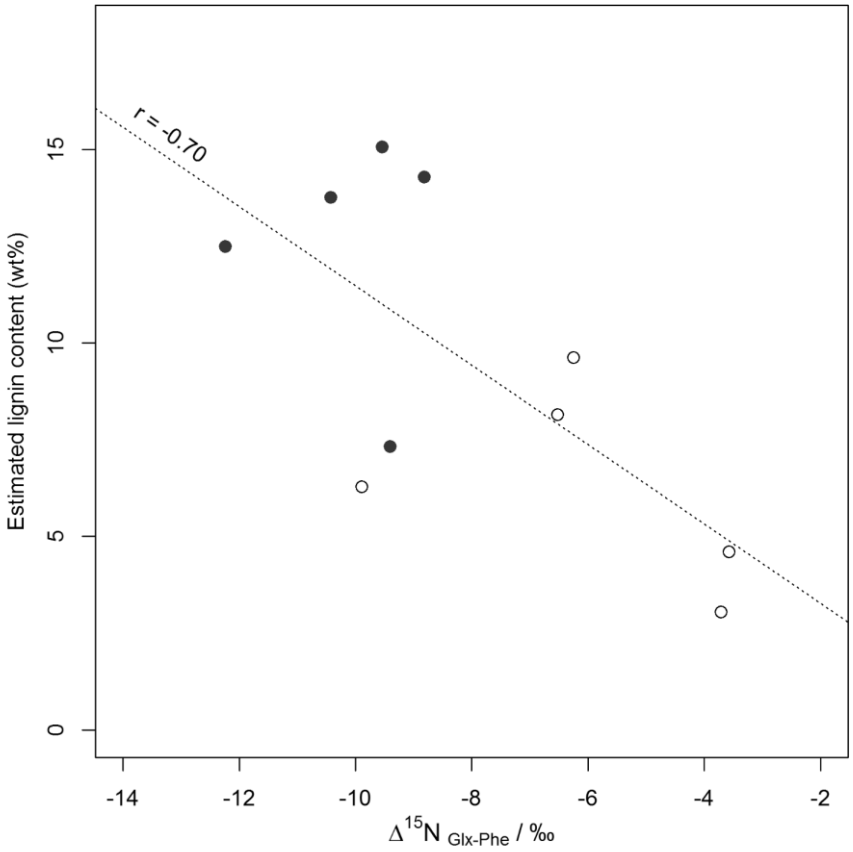
r = -0.70
Estimated lignin content (wt%)
0
5
10
15
-14
-12
-10
-8
-6
-4
-2
Δ15N Glx-Phe / ‰

Figure 9

Table 1

Species	Site [a]	Amino acid $\delta^{15}N$ values (‰)													$\Delta^{15}N$ [b]
		Phe	Glx	Pro	Ala	Asx	Val	Leu	Ile	Thr	Lys	Ser	Gly	Tyr	
Trees:															
Aesculus hippocastanum	CV	9.0	-1.4	0.4	-2.1	-0.2	-1.9	-3.6	-4.2	-19.3	-2.2	-10.1	-14.9	n.d.	-10.4
Corylus avellana (1)	CV	15.5	4.0	10.1	3.5	5.4	4.6	0.8	0.6	-1.6	2.6	-4.1	-10.0	7.3	-11.5
Crataegus monogyna (1)	CV	9.9	0.8	3.4	-1.2	2.2	-0.2	-2.8	-2.7	-6.6	-2.4	-8.4	-12.3	1.5	-9.1
Crataegus monogyna (2)	CV	12.5	4.7	6.6	3.7	6.7	5.8	0.5	2.8	-1.7	1.9	-4.9	-8.6	n.d.	-7.8
Fagus silvatica (1)	CV	13.5	2.8	9.5	2.6	3.5	3.2	0.8	1.1	-9.4	2.3	-4.9	-9.0	n.d.	-10.6
Fraxinus excelsior (1)	CV	11.9	1.8	4.9	1.2	2.7	3.4	-1.1	n.d.	-9.3	-0.9	-10.3	-13.5	n.d.	-10.1
Hedera helix	CV	11.7	3.6	8.2	4.1	3.8	4.8	0.6	1.3	-5.4	2.6	-6.4	-9.8	7.3	-8.1
Malus domestica	CV	14.1	3.3	9.4	2.4	4.8	4.5	0.8	0.9	-6.1	2.4	-4.7	-8.2	n.d.	-10.9
Populus nigra	CV	13.8	4.6	10.3	2.8	4.8	5.9	1.5	2.3	-6.6	1.3	-5.7	-8.2	7.8	-9.2
Prunus spinosa	CV	11.1	0.1	5.9	0.4	1.5	0.4	-1.9	-1.6	-9.4	-0.5	n.d.	-12.4	3.1	-10.9
Quercus robor (1)	CV	9.7	1.5	7.3	0.7	3.1	0.6	-2.6	-2.9	-9.2	-1.4	-7.1	-12.2	1.5	-8.2
Rosa canina (1)	CV	11.1	4.4	10.1	4.4	5.0	4.4	1.2	1.9	2.4	2.6	-3.3	-5.9	4.2	-6.6
Rosa canina (2)	CV	10.9	6.2	8.5	5.1	6.9	5.3	1.4	2.0	4.1	4.5	-2.1	-8.6	n.d.	-4.8
Rubus plicatus	CV	10.6	4.1	5.1	4.1	4.8	3.8	1.0	1.2	-2.0	2.2	-2.3	-7.8	6.0	-6.6
Salix alba (1)	CV	11.6	2.8	6.6	2.9	3.5	5.1	1.0	-0.2	-0.6	2.0	-6.2	-9.5	n.d.	-8.8
Salix alba (2)	CV	20.6	10.9	14.5	9.9	11.7	12.0	8.2	7.0	5.4	9.1	3.3	-3.8	n.d.	-9.7
Sambucus nigra	CV	14.2	2.0	1.1	2.4	2.9	1.4	-1.0	-0.1	-1.3	-1.9	-5.9	-10.9	2.1	-12.2
Tilia platyphyllos (1)	CV	13.6	3.6	7.2	1.8	4.3	4.3	-0.9	2.6	-1.3	2.1	-3.5	-9.6	n.d.	-10.0
Ulmus minor	CV	11.8	3.3	8.5	3.4	4.4	4.0	0.9	1.2	-3.8	1.5	-2.8	-8.2	n.d.	-8.5
Carpinus betula	BL	16.6	6.7	9.8	7.5	7.8	6.4	1.9	2.3	-0.4	4.1	-3.9	-4.9	12.9	-9.9
Corylus avellana (2)	BL	14.1	6.1	11.8	4.7	6.2	5.7	0.2	2.1	-4.0	2.2	-2.6	-7.3	n.d.	-8.0
Fagus silvatica (2)	BL	12.1	4.3	9.7	5.6	5.1	3.6	0.8	0.9	-5.2	2.7	-3.0	-4.5	n.d.	-7.8
Fraxinus excelsior (2)	BL	19.2	9.7	13.0	9.1	10.6	8.6	6.0	5.2	0.6	8.2	-1.5	-4.3	n.d.	-9.4
Gingko biloba	BL	16.9	7.4	8.3	6.6	8.8	6.5	3.8	6.1	0.3	4.0	-2.8	-6.3	7.8	-9.5
Liriodendron tulipifera	BL	15.6	5.2	5.8	4.3	6.1	5.2	1.5	1.3	-2.3	4.3	-4.0	-7.0	8.3	-10.3
Quercus robor (2)	BL	17.7	8.1	15.6	7.2	9.3	6.9	3.8	6.6	-3.8	6.8	-0.9	-6.6	12.6	-9.5
Tilia platyphyllos (2)	BL	16.4	7.0	12.5	4.6	5.8	5.8	2.6	2.5	-2.9	3.4	-4.4	-7.5	n.d.	-9.4
Mean		**13.5**	**4.4**	**8.3**	**3.8**	**5.2**	**4.4**	**1.0**	**1.5**	**-3.7**	**2.4**	**-4.3**	**-8.6**	**6.3**	**-9.2**
Herbs:															

Species	Site [a]	Amino acid $\delta^{15}N$ values (‰)													$\Delta^{15}N$ [b]
		Phe	Glx	Pro	Ala	Asx	Val	Leu	Ile	Thr	Lys	Ser	Gly	Tyr	
Dryopteris felix-mas	CV	14.7	8.7	12.4	7.7	9.1	6.0	5.7	6.3	-1.8	4.0	-1.2	-5.6	10.1	-5.9
Plantago major	CV	13.7	6.1	5.4	5.0	5.9	4.4	1.4	1.6	3.9	2.6	-3.0	-2.9	n.d.	-7.6
Rumex obtusifolius	CV	12.4	4.8	4.3	2.3	6.1	3.4	-0.3	-0.1	2.8	1.4	-3.7	-4.2	n.d.	-7.6
Taraxacum officinale	CV	10.1	3.6	9.5	2.2	3.6	1.8	-1.6	-0.5	1.5	0.5	-3.0	-2.9	7.4	-6.5
Trifolium repens	CV	7.8	0.6	2.6	0.1	1.7	0.8	-2.6	-2.4	-1.1	-2.7	-7.6	-7.2	1.0	-7.3
Urtica dioica	CV	8.7	6.0	4.7	4.2	5.7	4.3	1.2	1.3	-1.2	1.4	-5.1	-7.2	n.d.	-2.6
Amaranthus lividus (1)	BL	11.5	5.3	5.1	3.5	5.9	4.7	1.6	0.8	1.3	2.1	-3.8	-3.7	4.2	-6.3
Amaranthus lividus (2)	BL	11.7	6.7	6.8	5.4	7.0	7.2	3.4	3.4	-0.5	2.8	-5.8	-1.8	n.d.	-5.0
Anagallis arvensis	BL	14.6	3.1	5.1	3.3	5.0	3.4	1.4	1.4	1.7	2.1	-5.7	-5.5	n.d.	-11.5
Cirsium arvense (1)	BL	9.4	4.2	7.1	3.5	4.1	3.4	0.3	0.8	2.9	0.9	-2.7	-6.5	2.9	-5.2
Cirsium arvense (2)	BL	8.9	5.8	7.8	4.0	5.1	4.7	1.5	1.4	3.3	1.0	-2.9	-6.3	4.1	-3.1
Lathyrus tuberosus	BL	5.8	2.5	3.2	1.4	3.3	1.9	-2.6	-2.0	-12.6	n.d.	-6.8	-7.3	-0.5	-3.3
Poa annua (1)	BL	9.2	5.5	4.8	4.1	6.1	4.1	0.9	1.5	3.0	0.8	-3.0	-3.3	5.5	-3.7
Poa annua (2)	BL	7.8	2.7	2.0	0.5	3.7	2.7	-2.4	-0.8	0.4	-0.9	-6.3	-4.1	n.d.	-5.1
Polygonum aviculare	BL	13.4	3.5	5.6	3.0	3.9	3.5	0.1	0.6	-0.7	0.7	-5.3	-7.4	3.4	-9.9
Solanum nigrum (1)	BL	7.2	1.9	7.7	1.4	3.0	1.8	-1.6	-1.7	-2.0	-0.7	-5.0	-10.5	4.3	-5.3
Solanum nigrum (2)	BL	9.1	6.3	7.8	5.1	6.8	5.2	1.8	1.5	3.3	2.0	-1.5	-5.2	n.d.	-2.8
Stellaria media	BL	10.7	5.4	5.3	2.3	6.1	5.8	1.8	1.6	4.3	3.3	-2.7	-4.7	n.d.	-5.3
Lolium perenne (1)	NW	11.5	6.8	6.8	5.3	7.5	6.8	2.9	5.4	6.1	3.8	1.9	-0.1	n.d.	-4.8
Lolium perenne (2)	NW	13.0	8.9	8.7	6.9	8.8	7.5	3.6	5.4	5.1	4.9	0.2	-0.4	n.d.	-4.1
Lolium perenne (3)	NW	10.8	4.7	5.5	4.2	6.1	5.5	1.0	2.4	2.8	2.8	-2.5	-2.6	n.d.	-6.1
Lolium perenne (4)	NW	8.4	4.9	4.7	4.0	6.2	5.6	1.1	3.5	3.3	2.6	-1.5	-3.1	n.d.	-3.5
Lolium perenne (5)	NW	13.8	9.6	11.1	8.8	11.1	10.0	6.7	10.0	7.2	6.4	2.7	2.8	n.d.	-4.3
Lolium perenne (6)	NW	10.1	6.2	5.8	5.0	7.2	6.1	2.6	4.8	4.6	3.1	-0.4	-1.5	n.d.	-3.9
Lolium perenne (7)	NW	10.5	6.9	6.6	5.8	7.6	6.9	3.1	5.6	4.7	4.3	0.0	-0.6	n.d.	-3.6
Lolium perenne (8)	NW	11.4	5.4	7.0	4.5	7.2	5.7	2.8	3.4	2.7	2.0	-0.9	-2.2	n.d.	-6.0
Mean		**10.6**	**5.2**	**6.3**	**4.0**	**5.9**	**4.7**	**1.3**	**2.1**	**1.7**	**2.1**	**-2.9**	**-4.0**	**4.2**	**-5.4**

(a) CV – Chew Valley, UK; BL – Bad Lauchstädt, Germany; NW – North Wyke, UK

(b) $\Delta^{15}N_{Glx\text{-}Phe}$

Table 2

Tree species	Lignin concentration (wt%)	Herb species	Lignin concentration (wt%)
Aesculus hippocastanum	13.8	*Taraxacum officinale*	8.2
Sambucus nigra	12.5	*Amaranthus lividus* (1)	9.6
Salix alba (1)	14.3	*Polygonum aviculare*	6.3
Tilia platyphyllos (2)	7.3	*Poa annua* (1)	3.0
Quercus robor (2)	15.1	*Lolium perenne* (7)	4.6
Mean (±S.D.)	12.6 (±1.4)	Mean (±S.D.)	6.3 (±1.2)