Determination of arginine $\delta^{15}N$ values in plant and animal proteins by gas chromatography-combustion-isotope ratio mass spectrometry

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Abstract: Nitrogen stable isotope techniques are widely used in ecology, archaeology and forensic science to explore trophic relationships and provenances of organisms and materials, most widely using bulk $\delta^{15}N$ values of whole organisms, tissues or other materials. However, compound-specific isotope values can provide more diagnostic isotope "fingerprints" and specific information about metabolic processes. Existing techniques for nitrogen isotope analysis allow the determination of $\delta^{15}N$ values of 14 amino acids (AAs), accounting for *ca.* 75% of plant protein and collagen N. The majority of remaining N is from arginine, comprising 16% and 14% of collagen and plant protein N, respectively. We therefore aimed to develop a method to detect arginine and determine its $\delta^{15}N$ value ($\delta^{15}N_{Arg}$) by GC-C-IRMS, to further contribute to the understanding of metabolic routing of this important AA. We demonstrate that arginine, as its *N*-acetyl isopropyl ester, is amenable to GC analysis using a 15 m mid-polarity DB-35 column, eluting with baseline resolution from other AAs. The recorded $\delta^{15}N$ value by GC-C-IRMS was within error of that of the underivatized compound determined by EA-IRMS. The newly-developed GC-C-IRMS method was applied to modern plant protein and cattle collagen, enabling their $\delta^{15}N_{Arg}$ values to be related to AA biosynthesis. Determinations of archaeological cattle collagen $\delta^{15}N_{Arg}$ values confirmed the suitability of this method to provide further insights into past diets and ecosystems. Bulk collagen $\delta^{15}N$ value reconstruction including $\delta^{15}N_{Arg}$ values better reflect measured bulk values, as the isotopic ratio of 91‰ of collagen N can now be determined at the compound-specific level.

INTRODUCTION

Nitrogen (N) stable isotope analysis of whole organisms, tissues or other materials, such as soils, sediments and gaseous or dissolved nitrogen compounds, is widely used in a variety of fields, including ecology, palaeoecology, archaeology and forensic science to explore trophic relationships, past diet, and provenances of organisms and materials. These bulk $\delta^{15}N$ values are determined by the primary inorganic nitrogen sources in a food web, as well as isotope discriminations during N-uptake, assimilation, protein biosynthesis, metabolism and catabolism [1, 2], which can obscure the specific combinations of pathways and mechanisms that make up bulk tissue stable isotope ratios. Investigation of $\delta^{15}N$ values at the individual amino acid (AA) level, using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), can therefore improve understanding of the isotope signals of various nitrogen-containing biomolecules and allows access to more specific information about the nature of these metabolic processes.

The derivatisation and analytical techniques commonly employed in GC and GC-C-IRMS analyses of AAs allow the determination of between 8 and 14 of the common AAs that comprise proteins, dependent on the derivatives used [3, 4]. For collagen and plant foliage proteins, these 14 AAs represent *ca.* 90‰ and 85% of protein AAs, respectively, accounting for around 75% of protein N. The majority of remaining N comes from arginine (Arg), which while only constituting 5% of collagen and 4% of plant protein AAs, comprises 16% of collagen N and 14% of plant protein N, due to the presence of 4 N atoms in its structure.

In addition to being a proteinogenic AA, Arg serves as a precursor in the biosynthesis of important N-containing biomolecules, such as nitric oxide [5], polyamines [6], alkaloids [7], and other AAs [8], as well as in N excretion as urea in mammals [9]. The capacity to determine the $\delta^{15}N$ values of Arg would therefore significantly increase the percentage of plant protein and collagen N analysable in these tissues, which will help to illuminate bulk isotope signals, and further contribute to the understanding of metabolic routing and relationships to other biomolecules of this AA in plants and animals. In order to investigate these factors affecting the $\delta^{15}N$ value of Arg, its main biosynthetic and catabolic pathways must first be considered.

Arginine Biosynthesis

Biosynthesis of Arg in both plants and animals first requires the synthesis of ornithine (Orn). In plants this occurs via a five-step pathway from glutamic acid (Glu) [10] (Figure 1a). In mammals, Orn can be produced by ornithine aminotransferase (OAT) from glutamate-5-semialdehyde [11], which is an intermediate in proline (Pro) biosynthesis from, or catabolism to, Glu, with the additional amino group coming from another molecule of Glu (Figure 1b). In both plants and animals Orn then enters the urea cycle [10, 11], where it reacts with carbamoyl phosphate, formed from ammonia, bicarbonate, and ATP by carbamoyl phosphate synthetase, to form citrulline. This undergoes condensation with aspartate, catalysed by argininosuccinate synthetase, to form argininosuccinate, which is cleaved by argininosuccinase to fumarate and Arg. Orn can then be regenerated by the hydrolysis of Arg by arginase, also forming urea in the process, removing two N atoms from the cycle. Of the four N atoms in Arg, two are originally from Glu, one from aspartic

acid (Asp) and one from ammonia, however, there are multiple isotopic fractionation-inducing biosynthetic steps involving the creation or breaking of C-N bonds, which will likely result in a $\delta^{15}N$ value which is not simply a weighted average of the $\delta^{15}N$ values of these other compounds.

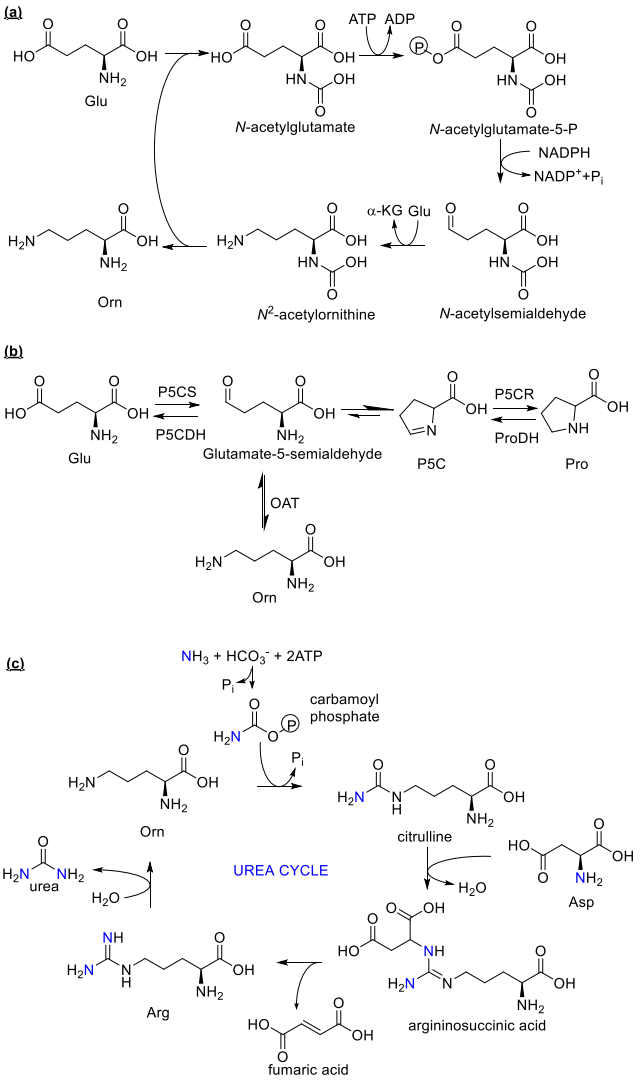

Figure 1. (a) Orn biosynthesis in plants; (b) Orn biosynthesis in mammals from glutamate-5-semialdehyde by ornithine aminotransferase (OAT); (c) biosynthesis and catabolism of Arg as part of the urea cycle, with Orn also entering the cycle from the pathways shown in (a) and (b).

Arginine analysis by gas chromatography

Commonly used analytical procedures and derivatisation methods for the stable isotope analysis of AAs by GC-C-IRMS have been unable to detect or measure the stable isotope values of Arg, e.g. [3, 4, 12, 13, 14]. The $\delta^{15}N$ value of Arg has been previously reported only once, by Hare *et al.*[15], in which AAs were separated by preparative cation-exchange column chromatography before off-line combustion and analysis by IRMS, giving a value of 4.0‰. While this negates the need to derivatise the AAs, it is a time-consuming method with many individual steps. It may also not be easily reproducible, at least for Arg, as the $\delta^{15}N$ value was reported for only one individual in a feeding experiment, unfortunately not allowing a diet-consumer comparison. It is, however, possible to determine $\delta^{13}C$ values of Arg, using on-line high performance liquid chromatography-isotope ratio mass spectrometry, with values reported for archaeological human and faunal collagen [16, 17]. While this method is not suitable for nitrogen isotope analysis, both this and the off-line column chromatography method show that Arg survives the harsh acid hydrolysis of proteins to their constituent AAs with minimal degradation, and it has previously been shown that the guanidine side chain of Arg is resistant to acid hydrolysis, with less than 1% converted to ornithine under normal acid hydrolysis conditions [18].

As Arg survives acid hydrolysis of proteins, it is therefore likely that the inability to observe this AA under previously used conditions is rather due to either the derivatisation procedure, or the chromatographic conditions employed.

While the GC and GC-MS analysis of Arg has been reported, these derivatisation methods have involved the addition of bulky groups with a large number of C atoms, such as heptafluorobutyryl isobutyl ester (HBB) derivatives [19], *N*(*O*,*S*)-*tert*-butyldimethylsilyl (TBDMS) derivatives [20], N-pentafluoropropionyl methyl (PFPM) esters [21], or even enzymatic conversion of Arg to Orn before derivatisation to *N*(*O*,*S*)-isobutoxycarbonyl methyl esters [22]. These derivatisation procedures are all problematic for GC-C-IRMS analyses, however, as fluorine released upon combustion of F-containing derivatising groups, such as in HBB and PFPM esters, irreversibly forms very stable metal fluorides with Cu and Ni, reducing the efficiency of the CuO/NiO GC-C-IRMS oxidation reactor, and poisons the Pt catalyst [23]. This can lead to incomplete combustion of the analytes, introducing a kinetic isotope effect and reducing accuracy of the results. Gaseous HF released during combustion may also affect other components downstream of the combustion reactor [4]. Baseline resolution is required for isotopic analysis, but TBDMS derivatives can hamper GC separation due to the apolar nature of the large silyl groups, and produce a mixture of silylated derivatives [24], while the large amount of carbon and silicon added may also result in incomplete combustion, which affects the accuracy of isotopic analysis [23, 25]. Enzymatic conversion to Orn before GC-C-IRMS results in the loss of two of the four nitrogen atoms, reducing the amount of retrievable isotopic information.

This study therefore aims to identify a combination of derivatisation and chromatographic methods which will enable the detection of Arg by gas chromatography and the determination of its $\delta^{15}N$ values by GC-C-IRMS.

EXPERIMENTAL

Standards and samples

An AA mixture (each 1 mg mL^{-1} in 0.1 M HCl) was prepared by the addition of 14 AA standards of known $\delta^{15}N$ values, comprising alanine (Ala), Arg, Asp, Glu, glycine (Gly), hydroxyproline (Hyp), leucine (Leu), lysine (Lys), phenylalanine (Phe), Pro, serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val), and norleucine (Nle) internal standard (Sigma-Aldrich, UK). A separate Arg standard, containing Nle was also prepared (both 1 mg mL^{-1} in 0.1 M HCl). AA standard $\delta^{15}N$ values were previously measured commercially (Iso-Analytical Limited, Cheshire, UK) in triplicate by elemental analyser-isotope ratio mass spectrometry (EA-IRMS), using an in-house reference material calibrated against IAEA-N-1 (inter-laboratory comparison standard, International Atomic Energy Agency, Vienna),

with standard deviations of reference materials, quality control check samples, and the measured AAs all below 0.1‰.

The method described herein was applied to reference plant foliage proteins, as well as modern reference and archaeological cattle dentinal collagen (Table S1), for which the other AA $\delta^{15}N$ values have previously been reported [26-28]. These were chosen to provide example applications in ecologically and archaeologically relevant samples. Briefly, *L. perenne* plant (*n*=5) and reference cattle (*n*=4) samples were sourced from the Biotechnology and Biological Sciences Research Council (BBSRC) National Capability - North Wyke Farm Platform, Rothamsted Research, Devon, UK (www.rothamsted.ac.uk/north-wyke-farmplatform), to enable comparisons between cattle and the plants that constituted their diet. Additional plant samples (*n*=3) were obtained from Bad Lauchstädt Experimental Research Station, Saxony-Anhalt, Germany. All plants were from locations to which no fertiliser had been added, to eliminate any effect of artificial fertilisation on nitrogen cycling in soils and the plants, and hence are suitable for comparison with the diets of archaeological animals. Archaeological cattle tooth samples (*n*=7) were obtained from three Linearbandkeramik (LBK) sites from across Europe: Apc-Berekalja, Hungary (APC); Bischoffsheim, France (BIS); and Ludwinowo, Poland (LUD); these sites are dated to between 5470 and 4800 cal BCE.

Amino acid derivatisation

AA *N*-acetyl isopropyl (NAIP) ester derivatives were prepared according to established protocols [4, 12]. 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.

Instrumental analyses

AAs were identified by GC-FID by comparison with AA standards, and quantified by comparison with a known amount of Nle internal standard. Several combinations of GC column and oven temperature program were tried and are described below: 1) The standard GC method for analysis of AA NAIP ester derivatives [27] using a mid-polarity column, with an extended isothermal hold at the end of the temperature program; 2) A non-polar stationary phase; 3) a mid-polarity stationary phase, but with a shorter column length; 4) a mid-polarity with a shorter column length and a single temperature ramp.

GC methods:

1. DB-35 coated column (35%-phenyl-methylpolysiloxane, 30 m × 0.32 mm internal diameter; 0.5 µm film thickness; Agilent Technologies, UK): the oven temperature of the GC was held at 70°C (2 min), increased to 150°C at 15°C min^{-1}, to 210°C at 2°C min^{-1}, to 270°C at 8°C min^{-1}, and then held isothermally (15 min).
2. DB-1 coated column (100% dimethylpolysiloxane, 50 m × 0.32 mm internal diameter; 0.52 µm film thickness; Agilent Technologies, UK): the oven temperature of the GC was held at 50°C (2 min), increased at 10°C min^{-1} to 300°C, and held for 10 min.
3. DB-35 coated column (15 m × 0.32 mm internal diameter; 0.5 µm film thickness; Agilent Technologies, UK): the oven temperature of the GC was held at 70°C (2 min), increased at 30°C min^{-1} to 150°C, then 2°C min^{-1} to 210°C and held isothermally (25 min).
4. DB-35 coated column (15 m × 0.32 mm internal diameter; 0.5 µm film thickness; Agilent Technologies, UK): the oven temperature of the GC was held at 70°C (2 min), increased at 30°C min^{-1} to 210°C and held isothermally (25 min).

GC-MS analysis was performed using a Thermo Scientific Trace 1300 gas chromatograph coupled to an ISQ single quadrupole mass spectrometer. Solutions were introduced via a PTV injector set to splitless mode onto a DB-35 column (30 m × 0.32 mm × 0.5 µm film thickness; Agilent Technologies, UK), using GC method 1 described above. The MS was operated in electron ionisation (EI) mode at 70 eV, with a GC interface temperature of 270°C and a source temperature of 300°C. The MS was set to acquire in the range of m/z 50-950 at 2 scans s-1 in full scan mode.

The $\delta^{15}N$ values of Arg were determined by GC-C-IRMS as described in Styring *et al.* [12], but with a modified GC method, as described above (*GC method 4*), altered to backflush compounds eluting before 11 min in order to not overload the oxidation reactor. 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 at least triplicate determinations. An Arg standard of known $\delta^{15}N$ value was analysed every three runs to ensure acceptable instrument performance.

RESULTS AND DISCUSSION

Derivatisation and Chromatography

Chromatographic separation of the NAIP ester derivatives of a standard mixture of AAs was initially achieved using previously described methods [27], altered to include a longer isothermal hold (15 min) at the end of the GC oven program. Under these conditions the late eluting Arg NAIP ester was observed, confirming that Arg is, at least partially, derivatised to its NAIP ester (Figure 3a). This was confirmed by the GC-MS spectrum of the derivatised Arg standard Arg (Figure 2). The molecular ion, *m/z* 342, matches the expected mass of the tris-*N*-acetyl *O*-isopropyl ester of Arg (Figure 2b). Other characteristic ions at *m/z* 283 $[M\text{-}OC_3H_7]^+$, *m/z* 255 $[M\text{-}COOC_3H_7]^+$, *m/z* 184 (the acetylated Arg side chain), and others shown in Figure 2b, confirm the structure of this derivative. However, as indicated by the poor chromatographic peak shape, on-column thermal decomposition of Arg appears to be occurring during the chromatographic run (Figure 2a). This is likely a consequence of the length of time spent in the column, and therefore the length of time spent at elevated temperatures, leading to thermal decomposition. Further testing and optimisation were therefore required to minimise on-column thermal decomposition during separation by GC, and to confirm that the derivatisation reactions proceed quantitatively. These criteria are required to ensure that isotopic fractionation is avoided [29], and that the

determined $\delta^{15}N$ values are representative of the true values of the protein AAs.

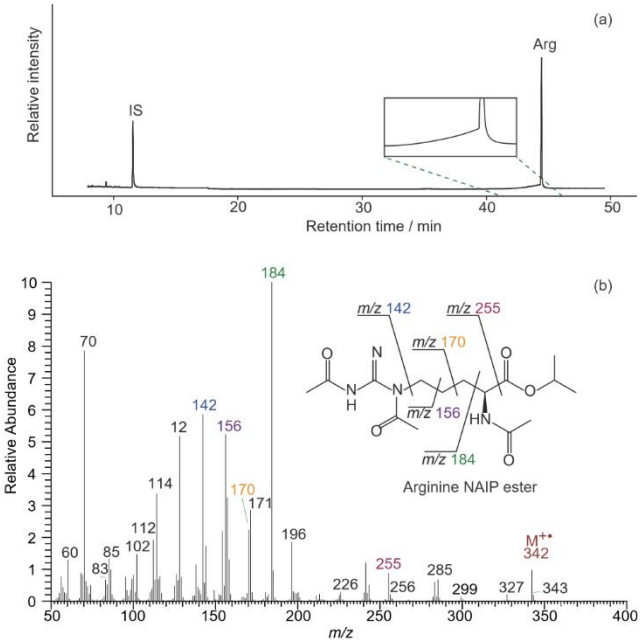

Figure 2. (a) Gas chromatogram of derivatised Arg standard, displaying on-column thermal degradation of the Arg NAIP ester (inset); (b) Mass spectrum (EI mode, 70 eV) of this compound, identified as Arg as its tris-N-acetyl isopropyl ester (inset structure). IS – Nle internal standard.

Several approaches were attempted to shorten the chromatographic run time to reduce the on-column degradation of this compound: (i) altering the temperature program of the chromatographic run while using the same GC column as previously; (ii) Altering the column stationary phase to provide better compatibility with the Arg derivative; (iii) Using the same column stationary phase, with a shortened column length. A thick film (0.5 µm) stationary phase was employed in each case to enable the analyte loading of AAs required to achieve the necessary signal intensity, due to the relatively low sensitivity of N isotopic analyses, without impairing chromatographic separations [30]. Using a non-polar DB-1 GC column also resulted in thermal decomposition of the Arg derivative (Figure 3b). However, the short, mid-polarity DB-35 column method was successful, with Arg NAIP ester eluting with minimal degradation, but at the expense of the chromatographic separation of the other AAs (Figure 3c). We concluded that it was unlikely to be possible to achieve sufficient separation of all AAs to reliably measure their $\delta^{15}N$ values, using a short enough column for Arg to elute without thermal decomposition, so method (iii) was optimised to provide the shortest chromatographic run time, using a single temperature ramp, resulting in Arg eluting within 23 min (Figure 3d).

Determination of arginine $\delta^{15}N$ values

Using this method, adapted for use with the GC-C-IRMS, an Arg NAIP ester standard containing Nle as an internal standard was prepared, using Arg of known isotopic composition, previously determined in its underivatised form by EA-IRMS. The Arg standard was measured by GC-C-IRMS 47 times, over multiple analytical runs across multiple days, giving a mean $\delta^{15}N$ value of -5.5‰, with standard deviation 0.6‰. This was within one standard deviation of the known off-line measured value of -5.81 ± 0.08‰, and implies both that Arg is quantitively converted to its NAIP derivative, and that on-column thermal decomposition is negligible, as both phenomena would negatively affect the accuracy of $\delta^{15}N$ value determinations. As samples are typically analysed in triplicate, three-point moving standard deviations were calculated for consecutive analyses of the Arg standard, to provide expected errors for triplicate analyses. These ranged between 0.0‰ and 0.9‰, with an average of 0.5‰. Typical GC-C-IRMS chromatograms for the Arg standard and each sample type can be seen in Figure 4.

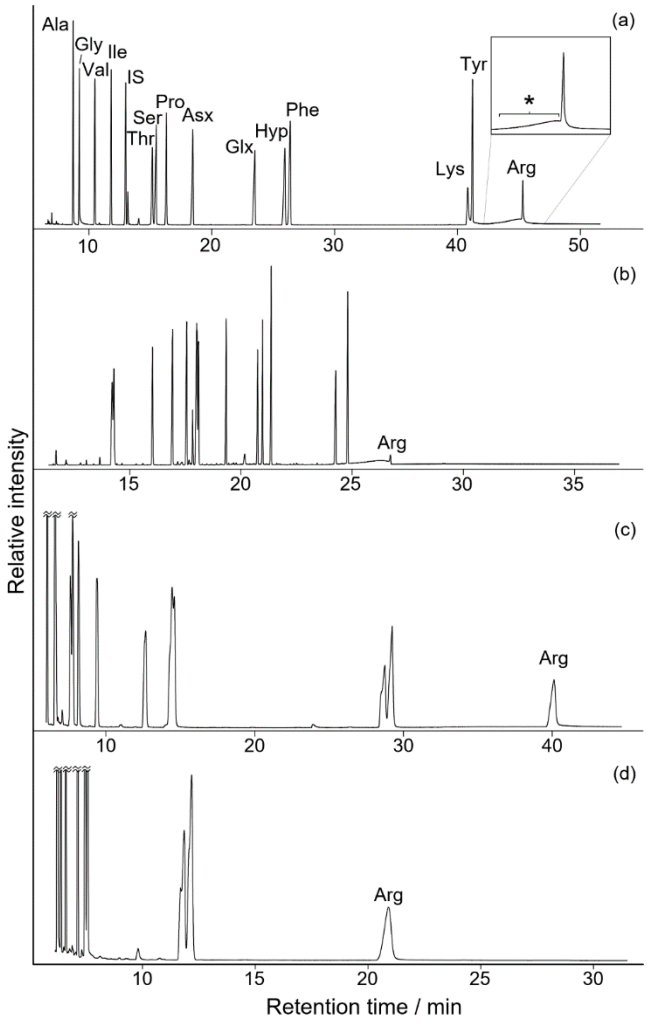

Figure 3. Gas chromatograms for each of the methods described in the experimental section: (a) method 1, 30 m DB-35 column; (b) method 2, 50 m DB-1 column; (c) method 3, 15 m DB-35 column; (d) method 4, 15 m DB-35 column with shortened temperature program. Inset partial chromatogram shows thermal decomposition of Arg derivative (*) using method 1. No thermal decomposition is displayed for method 4.

Arginine $\delta^{15}N$ values in plants

Arginine $\delta^{15}N$ value determinations were then performed for modern reference plant specimens for which the other AA $\delta^{15}N$ values have previously been determined and reported [26, 27]. The Arg $\delta^{15}N$ values for all plant foliage protein analysed herein varied from 2.6 to 7.1‰ (mean = 5.5‰, sd = 1.4‰; Figure 5), reflecting the values for several different plant species from different locations. The North Wyke *L. perenne* specimens alone had a smaller range, varying from 3.8 to 6.5‰ (mean = 5.6‰,

sd = 0.9‰), reflecting the biological variation of a single species from a single location.

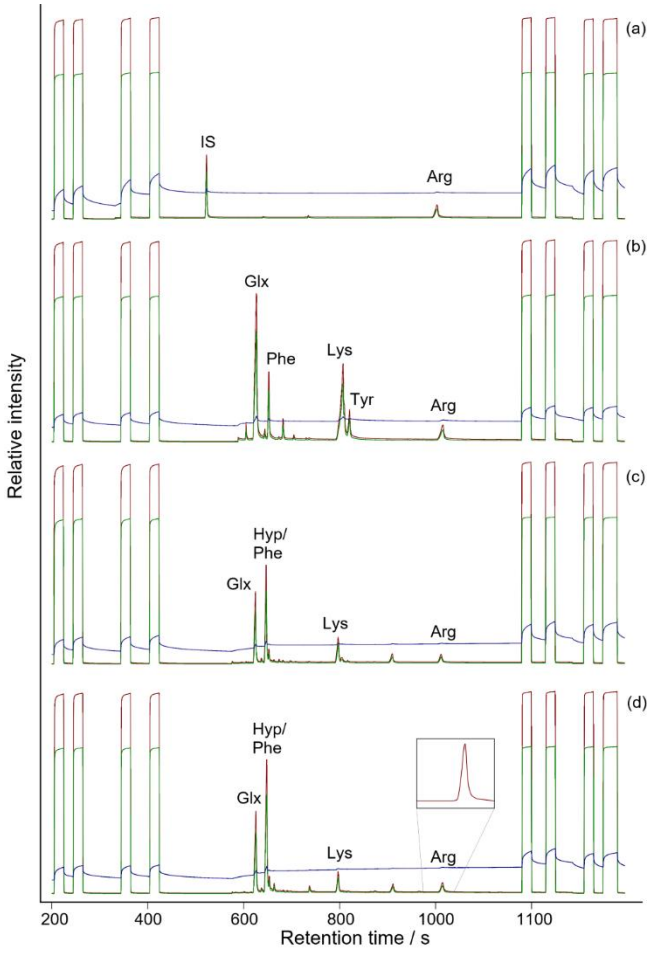

Figure 4. Typical GC-C-IRMS chromatograms, displaying the ion current signals recorded for *m/z* 28 (red), 29 (green), and 30 (blue), for (a) Arg standard, (b) reference plant, (c) modern reference tooth dentine collagen, and (d) archaeological cattle tooth dentine collagen. IS – Nle internal standard. The Arg peak shape indicates that no on-column thermal degradation of the Arg derivative is evident (inset). For all except standards (a), compounds eluting before 580 s were backflushed to avoid overloading the oxidation reactor.

As Arg N is derived from Asp, Glu, and ammonia from AA deamination (Figure 1), it would be expected that its $\delta^{15}N$ value would be lower than for these other compounds, due to the intervening C-N bond breaking and forming biosynthetic steps favouring the lighter isotope. Indeed, the plant $\delta^{15}N_{Arg}$ values were, on average, 1.6‰ lower than those of Glx, and 2.6‰ lower than Asx (Asn and Gln are converted into Asp and Glu respectively during protein hydrolysis. 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). These values are, however, quite variable, with depletions of between -0.9 and 3.7‰ relative to Glx, and between 0.4 and 4.1‰ relative to Asx (Table S2). This may simply be a result of natural biological variations in nitrogen isotope values between the individual plants, with the standard deviation of the plant $\delta^{15}N_{Arg}$ values of a similar magnitude to other plant AAs [26].

Arginine $\delta^{15}N$ values in cattle

The values of the modern reference cattle collagen $\delta^{15}N_{Arg}$ values ranged from 5.7 to 6.5‰ (Figure 5), with a mean value of 6.2‰ (sd = 0.3‰). This is within one standard deviation of the mean $\delta^{15}N_{Arg}$ value of the North Wyke reference plants (5.6‰, sd = 0.9‰; Table S2), and therefore Arg may initially be considered to be a source group amino acid here, i.e. an AA whose $\delta^{15}N$ value changes little with increased trophic position, due to being directly incorporated from the diet [31]. This is surprising due to the central role of Arg in N metabolism and excretion in mammals, which results in high turnover of the metabolic pool of Arg, and therefore its constituent N. This would be expected to create a larger overall $\delta^{15}N$ fractionation between plants and the animals consuming them. Arg has been shown to be a "conditionally essential" AA in young mammals [32, 33], meaning that in periods of metabolic stress or rapid growth, Arg requirements are higher than are able to be biosynthesised *de novo* or through protein turnover, and therefore uptake from dietary protein is required. If Arg is routed directly from the diet to tissue protein biosynthesis, there will be minimal N-fractionation. However, the cattle from which the teeth were sampled were not in a state of rapid muscle or bone growth during the formation of the third molar (M3) tooth dentine analysed herein [27], and therefore it is likely that most protein Arg was biosynthesised *de novo*, using N derived from Glu, Asp and ammonia, as Arg has been shown to be non-essential in cattle with sufficient metabolic precursors [34].

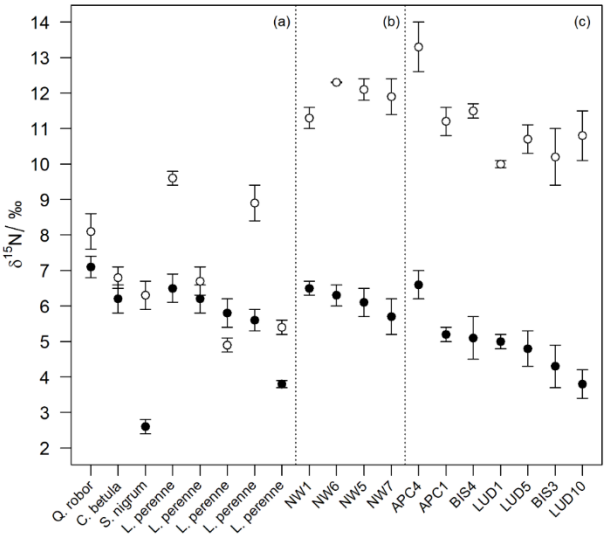

Figure 5. $\delta^{15}N$ values of Arg (filled points) compared to Glx (open points) for (a) modern plants, (b) modern reference cattle and (c) archaeological cattle. The *L. perenne* specimens, from North Wyke, were used for the diet-consumer comparisons with the North Wyke modern reference cattle. Error bars represent the standard deviation associated with triplicate measurements.

Due to the cyclical nature of Arg biosynthesis and catabolism, and the number of N atoms in its structure, it is difficult to predict the effects of metabolism on its nitrogen isotope values, as this will be affected by the relative rates of the biosynthetic and catabolic steps. However, as the direct precursor to urea, which is ^{15}N-depleted relative to bulk dietary N [35], it may be expected that Arg would also be depleted. In the modern cattle, Arg is indeed ^{15}N-depleted relative to Glx and Asx, the source of three of the N atoms, by an average of 5.7‰ and 4.1‰,

respectively. This confirms that Arg is not metabolically a source group AA, incorporated directly from the diet with no fractionation. Rather, Arg is biosynthesized from these molecules via multiple C-N bond-forming and bond-breaking steps, which all have the potential to cause fractionation, favouring the lighter isotope, and therefore the ^{15}N depleted Arg values relative to Glx and Asx $\delta^{15}N$ values are logical in terms of this AAs biosynthetic pathway in these animals. These relative depletions are greater than seen in the plants studied herein, which is likely a reflection of the different biosynthetic route to Orn between these organisms, while the different enzymes involved also likely exhibit different kinetic isotope effects.

The $\delta^{15}N_{Arg}$ values for the archaeological cattle varied between 3.8 and 6.6‰ (mean = 5.0‰, sd = 0.8‰). This is a lower mean value, by 1.2‰, with a wider range than for the modern reference cattle. While the modern cattle are from one location and had a consistent diet, the archaeological cattle were from three different locations across Europe, with unknown, variable diets, and thus the increased variation is likely a reflection of this. As with the modern cattle, the archaeological cattle Arg is ^{15}N-depleted relative to Glx and Asx, by an average of 6.1‰ for each. The ^{15}N-depletion relative to Asx is larger than in the modern cattle, due to Asx being closer in value to Glx in the archaeological cattle, although the reason for this is unclear. However, the ^{15}N-depletion relative to Glx is not significantly different to that of the modern cattle, with similar variation in values (sd = 0.6 for both), again suggesting that there is biosynthesis of Arg occurring in these animals, rather than total direct incorporation of dietary Arg into tissue proteins, as would be the case in periods of physiological stress. This also demonstrates that $\delta^{15}N_{Arg}$ values determined by this method are no more affected by diagenetic effects than previously measured AA $\delta^{15}N$ values, demonstrating the suitability of applying this method to archaeological samples.

Reconstructing bulk collagen $\delta^{15}N$ values

It is possible to reconstruct bulk collagen $\delta^{15}N$ values from individual amino acid $\delta^{15}N$, values using the following mass balance equation:

$$\delta^{15}N_{Coll} = \sum_{i=1}^{n} A_i B_i$$

where A_i = amino acid $\delta^{15}N$ value, B_i = percentage nitrogen contribution of the amino acid to the collagen molecule, and n = the number of AAs determined [30].

Reconstruction of bulk values was performed for the modern reference cattle collagen, both excluding and including the newly determined $\delta^{15}N_{Arg}$ values. These were compared to the previously reported measured $\delta^{15}N$ values [27] and can be seen in Table 1 The percentage N contribution of each AA to collagen was calculated from protein sequence data for type I collagen of *Bos taurus* [36]. There is no detailed protein sequence data for total plant foliar proteins available, so bulk reconstructions could not be performed for these.

Table 1. Modern reference cattle bulk collagen $\delta^{15}N$ values measured by EA-IRMS, and reconstructed collagen $\delta^{15}N$ values, excluding and including $\delta^{15}N_{Arg}$ values, with associated errors in brackets.

	Measured collagen $\delta^{15}N$ / ‰	Reconstructed collagen $\delta^{15}N$ / ‰	
		Excluding Arg	Including Arg
NW1	9.3	6.4 (1.3)	7.4 (1.3)
NW5	9.6	6.9 (0.8)	7.9 (0.9)
NW6	10.0	7.3 (0.9)	8.3 (0.9)
NW7	9.5	6.8 (1.2)	7.7 (1.3)
Mean	**9.6**	**6.8 (1.0)**	**7.8 (1.1)**

Using previous methods, 75% of collagen N could be accounted for, however with the ability to measure $\delta^{15}N_{Arg}$ values, this is increased to 91% of collagen N, an improvement of 16%. Hence the values calculated including Arg better represent the measured bulk collagen $\delta^{15}N$ values, with a smaller underestimation of around 1.8 ± 1.1‰, rather than 2.8 ± 1.0‰ without Arg. This underestimation of bulk $\delta^{15}N$ values has been seen in previous attempts to reconstruct bulk values of human collagen [30], and was originally ascribed to the missing Arg N. However, there is still an underestimation when including Arg in reconstructed values. Using the difference in reconstructed and measured bulk values, it is possible to calculate the average $\delta^{15}N$ value of the approximately 9% of remaining AA N contributing to collagen, which was found to be *ca.* 20‰ for these samples. This is unusually high compared to the other AA $\delta^{15}N$ values determined, but this is likely a reflection of the error associated with the reconstructed bulk values, a combination of the individual errors associated with 14 AAs. This leads to errors of only around ± 1‰ for the reconstructed values, using a linear propagation of uncertainty. However, using this to calculate the $\delta^{15}N$ value of the remaining N leads to possible $\delta^{15}N$ values of the remaining N of between 7‰ and 33‰, as the associated error of the reconstructed value must be divided by the fraction of the remaining N (i.e. 9%, 0.09), leading to an uncertainty of *ca.* ± 13‰. It is therefore not possible to calculate this value to a useful level of precision.

Of the N still unaccounted for in collagen (*ca.* 9%), most is from hydroxylysine (Hly; 3.6% of collagen N). More work is required to determine if this AA quantitatively survives collagen hydrolysis and is fully derivatised using the methods described herein. However, Hyl is formed through a post-translational modification of Lys within the collagen fibril [37], in a similar way to Hyp from Pro, and therefore should have the same $\delta^{15}N$ value as Lys. Hence, it is unlikely to provide significant additional information about N metabolism or cycling. Hyl does not occur in plant proteins. The other AAs contributing to collagen N are either destroyed during the acid hydrolysis step, such as the amide groups of Asn and Gln, or occur at very low abundances in collagen (e.g. tyrosine, methionine, histidine), resulting in being below the limit of detection for GC and GC-C-IRMS analyses at concentrations for which the more abundant AAs are not markedly overloaded. Tryptophan and cysteine do not occur in collagen at all, and hence do not contribute any N. Although these do occur in plant proteins, they, along with methionine, are partially or fully degraded during acid hydrolysis, particularly in the presence of carbohydrates [38], and so $\delta^{15}N$ values cannot be reliably determined for these AAs. Due to the small individual N contributions of many of these remaining low-abundance AAs, and the analytical difficulty that would be involved in developing the new methodologies required to detect each of them, it seems that the benefit of determining their $\delta^{15}N$ values may be outweighed by the considerable additional work required to achieve this. However, with the addition of Arg, it is now possible to measure up to 91‰ of

collagen N and 89% of plant protein N at the compound-specific level.

CONCLUSIONS

A new method was developed to reliably determine the $\delta^{15}N$ values of Arg by GC-C-IRMS. The instrumental analytical method developed herein has the benefit of using the same NAIP ester derivatives as used for other AAs, and hence the same derivatised sample can be used for both, which has advantages both for required sample size and sample preparation time. However, different chromatographic conditions, with a shorter GC column, were found to be required to prevent on-column thermal degradation of the Arg derivative. This method enabled the investigation of Arg $\delta^{15}N$ values in plant and animal tissues, allowing comparisons between animals and their diet for the first time.

While Arg $\delta^{15}N$ values are very similar in the reference cattle as in plants, it is likely that this is actually due to Arg being ^{15}N-depleted relative to its biosynthetic precursors, Glu and Asp, rather than direct incorporation of plant protein Arg into body tissues from the diet, and hence Arg is not, metabolically, a "source group" AA. Arg $\delta^{15}N$ values of archaeological cattle were similar to those of the reference cattle, suggesting that, as with other AAs, the $\delta^{15}N$ values of Arg are not significantly diagenetically altered over these timescales. Reconstructed bulk collagen $\delta^{15}N$ values were found to be approximately 1‰ closer to measured bulk values when including Arg $\delta^{15}N$ values in the mass balance equation than without. However, these reconstructed values were still found to underestimate the measured bulk values, suggesting that the remaining collagen N still unaccounted for is ^{15}N-enriched relative to the bulk $\delta^{15}N$ value.

ASSOCIATED CONTENT

Supporting Information

Details of reference plants, reference cattle and archaeological cattle analysed, $\delta^{15}N$ values of Arg determined in this study, with previously determined $\delta^{15}N$ values of its metabolic precursor AAs. (PDF)

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Author Contributions

The manuscript was written through contributions of all authors.

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REFERENCES

1. Handley, L. L.; Raven, J. A., The use of natural abundance of nitrogen isotopes in plant physiology and ecology. Plant, Cell & Environment 1992, 15 (9), 965-985.
2. Robinson, D.; Handley, L. L.; Scrimgeour, C. M., A theory for 15N/14N fractionation in nitrate-grown vascular plants. Planta 1998, 205 (3), 397-406.
3. Metges, C. C.; Petzke, K.-J.; Hennig, U., Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometric Comparison of N-Acetyl- and N-Pivaloyl Amino Acid Esters to Measure 15N Isotopic Abundances in Physiological Samples: A Pilot Study on Amino Acid Synthesis in the Upper Gastro-intestinal Tract of Minipigs. Journal of Mass Spectrometry 1996, 31 (4), 367-376.
4. Corr, L. T.; Berstan, R.; Evershed, R. P., Optimisation of derivatisation procedures for the determination of δ13C values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry. Rapid Communications in Mass Spectrometry 2007, 21 (23), 3759-3771.
5. Moncada, S.; Palmer, R. M. J.; Higgs, E. A., Biosynthesis of nitric oxide from l-arginine: A pathway for the regulation of cell function and communication. Biochemical Pharmacology 1989, 38 (11), 1709-1715.
6. Bagni, N.; Tassoni, A., Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. Amino Acids 2001, 20 (3), 301-317.
7. Tiburcio, A. F.; Galston, A. W., Arginine decarboxylase as the source of putrescine for tobacco alkaloids. Phytochemistry 1985, 25 (1), 107-110.
8. Wu, G. Y.; Morris, J. S., Arginine metabolism: nitric oxide and beyond. The Biochemical journal 1998, 336, 1-17.
9. Krebs, H. A.; Henseleit, K. J. H.-S. s. Z. f. p. C., Untersuchungen uber die Harnstoffbildung im Tierkörper. 1932, 210 (1-2), 33-66.
10. Slocum, R. D., Genes, enzymes and regulation of arginine biosynthesis in plants. Plant Physiology and Biochemistry 2005, 43 (8), 729-745.
11. Morris, S. M., Jr., Regulation of enzymes of the urea cycle and arginine metabolism. Annual Review of Nutrition 2002, 22 (1), 87-105.
12. Styring, A. K.; Kuhl, A.; Knowles, T. D. I.; Fraser, R. A.; Bogaard, A.; Evershed, R. P., Practical considerations in the determination of compound-specific amino acid δ15N values in animal and plant tissues by gas chromatography-combustion-isotope ratio mass spectrometry, following derivatisation to their N-acetyl isopropyl esters. Rapid Communications in Mass Spectrometry 2012, 26 (19), 2328-2334.
13. Macko, S. A.; Uhle, M. E.; Engel, M. H.; Andrusevich, V., Stable Nitrogen Isotope Analysis of Amino Acid Enantiomers by Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry. Analytical Chemistry 1997, 69 (5), 926-929.
14. Silfer, J. A.; Engel, M. H.; Macko, S. A.; Jumeau, E. J., Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography/isotope ratio mass spectrometry. Analytical Chemistry 1991, 63 (4), 370-374.
15. Hare, P. E.; Fogel, M. L.; Stafford, T. W.; Mitchell, A. D.; Hoering, T. C., The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. Journal of Archaeological Science 1991, 18 (3), 277-292.
16. Dunn, P. J. H.; Honch, N. V.; Evershed, R. P., 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 δ13C values for palaeodietary and palaeoecological reconstruction. Rapid Communications in Mass Spectrometry 2011, 25 (20), 2995-3011.
17. McCullagh, J. S. O.; Juchelka, D.; Hedges, R. E. M., Analysis of amino acid 13C abundance from human and faunal bone collagen using liquid chromatography/isotope ratio mass spectrometry. Rapid Communications in Mass Spectrometry 2006, 20 (18), 2761-2768.

18. Murray, K.; Rasmussen, P. S.; Neustaedter, J.; Luck, J. M., The hydrolysis of arginine. Journal of Biological Chemistry 1965, 240 (2), 705-709.
19. Labadarios, D.; Shephard, G. S.; Botha, E.; Jackson, L.; Moodie, I. M.; Burger, J. A., Determination of plasma amino acids by gas chromatography. Journal of Chromatography B: Biomedical Sciences and Applications 1986, 383, 281-295.
20. Chaves Das Neves, H. J.; Vasconcelos, A. M. P., Capillary gas chromatography of amino acids, including asparagine and glutamine: sensitive gas chromatogaphic—mass spectrometric and selected ion monitoring gas chromatographic—mass spectrometric detection of the N,O(S)-tert.-butyldimethylsilyl derivatives. Journal of Chromatography A 1987, 392, 249-258.
21. Tsikas, D.; Schubert, B.; Gutzki, F.-M.; Sandmann, J.; Frölich, J. C., Quantitative determination of circulating and urinary asymmetric dimethylarginine (ADMA) in humans by gas chromatography–tandem mass spectrometry as methyl ester tri(N-pentafluoropropionyl) derivative. Journal of Chromatography B 2003, 798 (1), 87-99.
22. Matsumura, S.; Kataoka, H.; Makita, M., Capillary gas chromatographic analysis of protein amino acids as their N(O,S)-isobutoxycarbonyl methyl ester derivatives. Biomedical Chromatography 1995, 9 (5), 205-210.
23. Meier-Augenstein, W., Applied gas chromatography coupled to isotope ratio mass spectrometry. Journal of Chromatography A 1999, 842 (1–2), 351-371.
24. Corso, G.; Esposito, M.; Gallo, M.; Russo, A. D.; Antonio, M., Transformation of arginine into ornithine during the preparation of its tert-butyldimethylsilyl derivative for analysis by gas chromatography/mass spectrometry. Biological Mass Spectrometry 1993, 22 (12), 698-702.
25. Shinebarger, S. R.; Haisch, M.; Matthews, D. E., Retention of carbon and alteration of expected 13C-tracer enrichments by silylated derivatives using continuous-flow combustion-isotope ratio mass spectrometry. Analytical Chemistry 2002, 74 (24), 6244-6251.
26. Kendall, I. P.; Woodward, P.; Clark, J. P.; Styring, A. K.; Hanna, J. V.; Evershed, R. P., Compound-specific $\delta^{15}N$ values express differences in amino acid metabolism in plants of varying lignin content. Phytochemistry 2019, 161, 130-138.
27. Kendall, I. P.; Lee, M. R. F.; Evershed, R. P., The effect of trophic level on individual amino acid $\delta^{15}N$ values in a terrestrial ruminant food web. STAR: Science & Technology of Archaeological Research 2017, 3 (1), 135-145.
28. Gillis, R. E.; Kendall, I. P.; Roffet-Salque, M.; Balasse, M.; Marton, T.; Oross, K.; Domboróczki, L.; Marciniak, A.; Arbogast, R.-M.; Vigne, J.-D.; Evershed, R. P., The first forest farmers: A multistable isotope perspective of cattle stock herding during the Linearbandkeramik. in prep.
29. Rieley, G., Derivatization of organic compounds prior to gas chromatographic–combustion–isotope ratio mass spectrometric analysis: identification of isotope fractionation processes. Analyst 1994, 119 (5), 915-919.
30. Styring, A. K.; Sealy, J. C.; Evershed, R. P., Resolving the bulk $\delta^{15}N$ values of ancient human and animal bone collagen via compound-specific nitrogen isotope analysis of constituent amino acids. Geochimica et Cosmochimica Acta 2010, 74 (1), 241-251.
31. Popp, B. N.; Graham, B. S.; Olson, R. J.; Hannides, C. C. S.; Lott, M. J.; López-Ibarra, G. A.; Galván-Magaña, F.; Fry, B., Insight into the Trophic Ecology of Yellowfin Tuna, *Thunnus albacares*, from Compound-Specific Nitrogen Isotope Analysis of Proteinaceous Amino Acids. In *Stable Isotopes as Indicators of Ecological Change*, Todd, E. D.; Rolf, T. W. S., Eds. Academic Press: Cambridge, MA, USA, 2007; Vol. 1, pp 173-190.
32. Flynn, N. E.; Meininger, C. J.; Haynes, T. E.; Wu, G., The metabolic basis of arginine nutrition and pharmacotherapy. Biomedicine & Pharmacotherapy 2002, 56 (9), 427-438.
33. Wu, G.; Jaeger, L. A.; Bazer, F. W.; Rhoads, J. M., Arginine deficiency in preterm infants: Biochemical mechanisms and nutritional implications. The Journal of Nutritional Biochemistry 2004, 15 (8), 442-451.
34. Sultana, H.; Inada, M.; Wadud, S.; Takahashi, T.; Morita, T.; Onodera, R., A quantitative study on arginine synthesis from argininosuccinic acid and citrulline by crude enzymes of cattle kidney. Animal Science Journal 2003, 74 (4), 289-294.
35. Steele, K. W.; Daniel, R. M., Fractionation of nitrogen isotopes by animals: a further complication to the use of variations in the natural abundance of ^{15}N for tracer studies. J Agric Sci 1978, 90 (1), 7-9.
36. UniProt Consortium, T., UniProt: a worldwide hub of protein knowledge. Nucleic Acids Research 2018, 47 (D1), D506-D515.
37. Piez, K. A.; Likins, R. C., The conversion of lysine to hydroxylysine and its relation to the biosynthesis of collagen in several tissues of the rat. *Journal of Biological Chemistry* **1957,** *229* (1), 101-109.
38. Schram, E.; Dustin, J. P.; Moore, S.; Bigwood, E. J., Application de la chromatographie sur échangeur d'ions a l'étude de la composition des aliments en acides amines. *Analytica Chimica Acta* **1953,** *9*, 149-162.

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