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Coversheet

*This is the pre-peer reviewed version of the following article: Enggrob, KL, Larsen, T, Larsen, M, Elsgaard, L, Rasmussen, J. The influence of hydrolysis and derivatization on the determination of amino acid content and isotopic ratios in dual-labeled (^{13}C , ^{15}N) white clover. *Rapid Commun Mass Spectrom.* 2019; 33: 21– 30. <https://doi.org/10.1002/rcm.8300>, which has been published in final form at <https://doi.org/10.1002/rcm.8300>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions*

How to cite this publication:

Enggrob, KL, Larsen, T, Larsen, M, Elsgaard, L, Rasmussen, J. The influence of hydrolysis and derivatization on the determination of amino acid content and isotopic ratios in dual-labeled (^{13}C , ^{15}N) white clover. *Rapid Commun Mass Spectrom.* 2019; 33: 21– 30.
<https://doi.org/10.1002/rcm.8300>

Publication metadata

Title:	The influence of hydrolysis and derivatization on the determination of amino acid content and isotopic ratios in dual-labeled (^{13}C , ^{15}N) white clover
Author(s):	Enggrob, KL, Larsen, T, Larsen, M, Elsgaard, L, Rasmussen, J
Journal:	Rapid Communications in Mass Spectrometry
DOI/Link:	https://doi.org/10.1002/mnfr.201601048
Document version:	Pre-peer reviewed

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1 Original paper Date of preparation: 25-09-2018
2 Page: 12 Figures: 6 Tables: 3 Equations: 7 Supporting online material: 1
3
4 **The influence of hydrolysis and derivatization on the determination of amino acid content and isotopic**
5 **ratios in dual labeled (^{13}C , ^{15}N) white clover.**
6
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15

16 **Abstract**

17 **Rationale:** The cycling of peptide and protein-bound amino acids (AA) is important for studying the rate
18 limiting steps in soil N turnover. A strong tool is stable C and N isotopes used in combination with
19 compound specific isotope analysis (CSIA), where a prerequisite for analysis is appropriate methods for
20 peptide and protein hydrolysis and appropriate methods for derivatization of AA for analysis by gas
21 chromatography (GC).

22 **Methods:** We examined the efficiency of a standard acidic hydrolysis (6 M HCl, 20h at 110°C) and a fast
23 acidic hydrolysis (6 M HCl, 70 min at 150°C) on the recovery of AAs from a protein standard (Bovine Serum
24 Albumin). The best methods were used on dual-labeled (¹³C and ¹⁵N) clover shoot and root juice, divided in
25 four molecular weight (Mw) size fractions. We used the NAIP (*N*-acetyl isopropyl esterification)
26 derivatization for GC/C-IRMS analysis of AA standards.

27 **Results:** The NAIP derivatization gave very low LOD (< 2 pmol) and LOQ ranging from 0.55-4.89 pmol.
28 Comparing concentrations of individual AAs in hydrolyzed versus un-hydrolyzed clover juice samples of the
29 low Mw size fraction (< 1 kDa) showed a significant decline in concentration ($p < 0.03$) for seven AAs after
30 hydrolysis. Despite the decline in AA concentration, we found a linear connection between the obtained
31 atomic fraction (¹³C/total carbon and ¹⁵N/total nitrogen) for individual AAs of hydrolyzed versus un-
32 hydrolyzed samples.

33 **Conclusion:** The methodology distinguished differences in atomic fractions across AA, in individual AA in
34 Mw size fractions, and between shoot and root samples of experimentally labeled white clover. Specifically,
35 the method separated L-glutamate (Glu) and glutamine (Gln). Thus, for a broader use in plant and soil
36 ecology, we present an optimized methodology for GC/C-IRMS analysis of AAs from organic nitrogen
37 samples enriched with ¹³C and ¹⁵N, AA stable isotope probing (SIP).

38

39 *Keywords:*

40 Compound specific isotope analysis (CSIA)

41 Amino acid (AA)

42 Stable isotope probing (SIP)

43 ¹³C

44 ¹⁵N

45 IRMS

1. Introduction

Nitrogen (N) is a required nutrient for all lifeforms and a building block for the backbone of amino acids (AAs). In studies of plant-soil interactions, AAs have gained considerable interest as plant nutrient^[1], as components of root exudates^[2], and as key compounds for understanding key fluxes through soil organic N pools^[3,4]. A strong tool to study these issues is stable isotope labeling, where isotopically enriched AAs are used to trace the fate of single or a few AAs. Such studies have, for example, documented direct acquisition of asparagine by white clover^[5,6], direct uptake of glycine in a number of crop species^[7], and direct uptake of a mixture of glycine, valine, tyrosine and lysine by plantain^[8]. However, it is generally acknowledged that AAs and small peptides (<1 kDa) have a rapid turnover in soil^[3,9] and therefore does not constitute the rate limiting step of the turnover of organic N in plant-soil N cycling. However, progress in characterizing the cycling and role of more complex organic N molecules, such as AAs bound in larger peptides and proteins (>1 kDa), has been hampered by two methodological issues. One is the need for testing the optimal hydrolysis of peptides and proteins, and the other is separating as many isotopically labeled AAs as possible.

To analyze proteinogenic AAs in plant and soil samples, it is necessary to perform several analytical steps. The first step is breaking the peptide bounds between individual AAs, which is commonly done by acidic hydrolysis^[10,11]. The second step is purifying the hydrolysates and isolate the AA fraction using a cation exchange column. For gas chromatographic separation, the third and final step is making the AAs more volatile by derivatizing them.

Several protocols have been used for acid hydrolysis, which basically differs in type and strength of acids, reaction temperature and incubation time^[12,13]. Samples can be incubated in either heating blocks, oven or microwave oven. Joergensen et al.^[14] reported similar results, when comparing microwave oven incubation for between 10 and 30 min and oven incubation for 20 hours. The benefit is the reduction in incubation time. The hydrolysis of protein bonds of hydrophobic AAs, valine, isoleucine and leucine may require an extended hydrolysis time up to 72 h^[15]. However, hydrolysis is a harsh treatment that may also destroy AAs. A typical result is deamination of asparagine and glutamine into aspartate and glutamate, respectively, and the destruction of tryptophan and cysteine^[15]. In addition, residual oxygen left in the hydrolysis reaction vessel may induce thermal breakdown resulting in reduced recovery of the hydroxyl- and C-heavy AAs, such as serine, threonine, tyrosine and methionine^[12]. Thus, it is challenging to balance the release of strongly bound AAs against the loss of labile AAs. We here selected two methods differing in temperature and hydrolysis length occurring in recent literature^[13,16].

79
80 The analysis of labeled AAs is performed by compound-specific stable isotope analysis (CSIA), using either
81 gas chromatography-combustion-isotope ratio mass spectrometry (GC/C-IRMS) or liquid chromatography
82 (LC) coupled to either an IRMS or a time of flight mass spectrometry (TOF-MS). GC/C-IRMS analysis
83 requires a derivatization of AAs to make them volatile, but derivatization introduces extra carbon atoms to
84 the AAs and thereby induces a potential error in measurement of $\delta^{13}\text{C}$ values, which have to be adjusted by
85 correction factors calculated for each individual AA ^[17,18]. We here used GC/C-IRMS, since it is the most
86 widely used method to rapidly separate multiple AAs.
87
88 Indeed, in selecting the most appropriate derivatization method for GC/C-IRMS, several issues must be
89 taken into account ^[19,20]. These include the number of C atoms introduced, the stability of the derivative,
90 ease of handling and use of hazardous reagents, the effect of the derivative on the durability of the
91 combustion oven, and the type of study in terms of natural isotope abundance or experimental labeling.
92 Corr et al. ^[19] compared seven derivatization methods, two of which they detailed in a subsequent study ^[21],
93 namely the N-acetyl methyl esterification (NACME) and N-acetyl isopropyl esterification (NAIP) as depicted
94 in Figure 1. Corr et al. ^[21] recommended the NACME method as it only introduces the small number of
95 additional carbon atoms and had the smallest errors associated with $\delta^{13}\text{C}$ value determinations. Yet, a
96 disadvantage of NACME compared to NAIP derivatization ^[22] is that it is harder obtaining a good baseline
97 separation ^[13,23]. The NAIP method has the disadvantage that it introduces five carbon atoms (Figure 1) and
98 is thus associated with a potentially greater error on the measured $\delta^{13}\text{C}$ values; this may be of particular
99 concern in natural abundance studies. However, when working with experimental labeling the main
100 concern is not related to (relatively small) errors on the measured $\delta^{13}\text{C}$ values, but rather to kinetic isotope
101 effects ^[18], arising from the faster reaction of lighter than heavier atoms. Thus, for experimental isotope
102 labeling studies, the most important criterion is choosing a method with superior baseline separation. For
103 this reason, we used the NAIP derivatization method.
104
105 The aim of this study was to refine and test GC/C-IRMS analysis of labeled AAs, both free and bound in
106 peptides and proteins, separating as many AAs as possible to be used in experimental labeling studies. We
107 compared the recovery of AAs from standard protein using two common acid hydrolysis protocols:
108 standard hydrolysis in 6 N HCl for 20 hours at 110°C ^[12] and fast hydrolysis in 6 N HCl for 70 minutes at
109 150°C ^[13]. Finally, we then used the best methods for acid hydrolysis on dual-labeled (^{13}C and ^{15}N) clover
110 shoot and root juice, divided in four molecular weight (Mw) size fractions, to test the power of the methods
111 in separating AA concentration and the isotopic enrichment of free and bound AAs in the clover.

112

113 **2. Materials and Methods**

114 *2.1. Amino acid preparation, derivatization and analysis*

115 *2.1.1. Standards and reagent*

116 Twenty-one individual unlabeled amino acid standards was purchased, namely L-alanine (Ala), L-valine
117 (Val), glycine (Gly), L-leucine (Leu), L-isoleucine (Ile), L-proline (Pro), L-threonine (Thr), asparagine (Asn), L-
118 aspartate (Asp), L-serine (Ser), L-glutamate (Glu), glutamine (Gln), methionine (Met), L-phenylalanine (Phe),
119 L-cysteine (Cys), hydroxy-L-proline (Hyp), tryptophan (Trp), L-tyrosine (Tyr), L-lysine (Lys) and norvaline (Avl)
120 from Sigma-Aldrich, St. Louis, USA and D-(-)-norleucine (Nle) from Alfa Aesar, Thermo Fisher Scientific,
121 Lancashire, United Kingdom. D-(-)-nor leucine and nor valine were used as internal standards (ISTD). All
122 solvents used were of HPLC grade and purchased from VWR International (Herlev, Denmark).

123

124 *2.1.2. Acid hydrolysis*

125 In order to release AA from protein and peptide bonds, samples were exposed to a standard acidic
126 hydrolysis ^[16] and a fast acidic hydrolysis ^[13]. The two methods were tested (with three replications of the
127 entire procedure) on 10 µl of a Bovine Serum Albumin (BSA) protein standard (200 mg mL⁻¹) in 16x100
128 soda-lime disposable culture tubes (Duran Group, Mainz, Germany). BSA was chosen as a protein standard
129 to test the influences of the hydrolysis because it is a relative small, water-soluble, standardized protein,
130 and therefore directly comparable to the easily degradable DON compounds found in the juice fraction of
131 the white clover. For both methods, 1 mL 6M HCl was added to the sample under an N₂ atmosphere to
132 eliminate oxygen and the culture tubes were sealed to prevent oxygen penetration. In the standard
133 hydrolysis samples were heated to 110°C for 20 hours using aluminum blocks. In the fast hydrolysis,
134 samples were heated to 150°C for 70 minutes using aluminum blocks. After hydrolysis, the AAs were
135 purified and derivatized before GC/C-IRMS analysis as described below.

136

137 *2.1.3. Sample purification*

138 Prior to derivatization, the hydrolyzed samples were purified, using a modified version of the method by
139 Amelung and Zhang ^[10]. Solids and lipophilic compounds were removed from the hydrolysate by adding 2
140 mL n-hexane/dichloromethane (6:5, v/v), vortexing for 30 sec and centrifugation at 1000 g for 2 min. The
141 aquatic phase was then transferred through a Pasteur pipette lined with glass wool into a new glass tube,
142 followed by 2 x 0.5 mL 0.1 M HCl to rinse the pipette. The aquatic sample was then added ISTD and diluted
143 to 8 mL with milliQ water (Synergy® System, Millipore, Molsheim, France) and transferred to a propylene
144 column (10 mL) containing 1 g Dowex 50W-X8 cation exchange resin (analytical grade, 100-200 mesh,

hydrogen form, Bio-Rad laboratory Inc. USA) and rinsed with 3 x 3 mL 0.1 M oxalic acid, 3 x 3 mL 0.01 M HCl and 3 x 3 mL milliQ water. Finally, the AAs were eluted with 3 x 1 mL 2.5 M ammonium hydroxide solution (Merck, Darmstadt, Germany). To avoid heating of the samples prior to derivatization, the ammonium hydroxide solution was evaporated by freeze centrifugation.

149

2.1.4. Derivatization

The NAIP derivatization method was modified from Corr et al. and Larsen et al. ^[21,22] and tested on individual AAs standards to assess and optimize the stabilization of the derivatives. First, solutions of standard AAs were dried as described above by freeze centrifugation. Next, the dried AAs were added 0.70 mL acidified isopropanol, prepared by transferring 1:4 cooled acetyl chloride to cooled isopropanol. The reaction vessels were then flushed with N₂, closed and placed in a heating block at 75°C for 60 min, followed by cooling to room temperature (20°C) in aluminum blocks. After cooling of the sample, the excess solvent was evaporated under a gentle N₂-flow at 60°C. Any residual acetate left over from the propylation was removed by the addition and evaporation of 0.5 mL dichloromethane (DCM) to ensure a proper acetylation. The derivatized AA were acetylated by adding 0.75 mL acetylation mixture containing acetic anhydride, triethylamine and acetone (1:2:5, v/v, 10 min, 60°C). Excess solvent was evaporated under a gentle N₂-flow for 8 min at 60°C. Salts and precipitates were removed by adding ethyl acetate and a saturated NaCl solution (1:1, v/v) and centrifuged for 2 min at 1500 G. After centrifugation, the organic phase was transferred to a new test tube and gently evaporated under N₂-flow at room temperature. Residual water was removed by the addition and evaporation of 0.5 mL DCM. Finally, the derivatized AA were dissolved in ethyl acetate and transferred to GC glass vials.

166

2.1.5. Gas chromatography-combustion-isotope ratio mass spectrometry (GC/C-IRMS) analysis

GC analyses were performed on a Trace GC Ultra mounted with a TriPlus autosampler (both from Thermo Scientific, Hvidovre, DK). The derivatives were separated with a VF-23m capillary column (60 m x 0.25 mm inner diameter x 0.25 µm film thickness; Agilent Technologies, Amstelveen, Netherland). The inlet was operating with a temperature of 250°C in splitless mode, with a Helium column flow of 1.4 mL min⁻¹. The temperature gradient was shaped as followed: initial oven temperature set to 90°C for 1 min, raised to 120°C with a rate of 15°C·min⁻¹, then raised to 250°C with a rate of 3°C·min⁻¹ and held at 250°C for 5-45 min, depending on the AA retention time. The gas chromatograph was coupled via a combustion reactor (GC IsoLink, Thermo Scientific, Hvidovre, DK), oxidation at 1000°C, to an isotope ratio mass spectrometer (Delta V Plus IRMS, Thermo Scientific, Hvidovre, DK). All mass spectrometer related parameters were controlled by the Isodat software version 3.0 (Thermo Scientific, Hvidovre, DK). All δ¹³C values are reported relative to

the Vienna PeeDee Belemnite (VPDB) international isotope standard. All $\delta^{15}\text{N}$ values are reported relative to the $\delta^{15}\text{N}$ values of atmospheric N_2 . A standard curve based on analyses of Asn with an increasing percentages of dual labeled Asn ($^{13}\text{C}_4$, $^{15}\text{N}_2$), showed a strong linearity of all $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, with increasing amount of dual labeled Asn ($^{13}\text{C}_4$, $^{15}\text{N}_2$), with coefficient of determination of $R^2=0.984$ and $R^2=0.982$, respectively. The AAs were identified by the retention time of standards and the concentration calculated relative to individual standard curves.

184

2.1.6. Correction factor and kinetic isotope effect (KIE)

The obtained $\delta^{13}\text{C}$ values were converted to atomic fractions (AFs) using the following equation ^[17]:

187

$$AF = \frac{VPDB(10^{-3}\delta^{13}\text{C}+1)}{1+VPDB(10^{-3}\delta^{13}\text{C}+1)} \quad (1)$$

189

To adjust for the carbon added during the derivatization a correction factor, AF_L , was calculated for each AA according to ^[17]:

192

$$AF_L = \frac{(y+x)AF_D - xAF_U}{y} \quad (2)$$

194

where y is the number of added carbon atoms, x is the number of carbon atoms in the AA, AF_D is the $\delta^{13}\text{C}$ atomic fraction of the derivatized AA, and AF_U is the $\delta^{13}\text{C}$ atomic fraction of the un-derivatized AA.

The AF of the enriched AA can now be calculated as:

198

$$AF_{AA} = \frac{(x+y)AF_{AAD} - yAF_L}{x} \quad (3)$$

200

During derivatization, isotopic fractionation occurs when bonds involving heavier isotopes are broken, thereby creating a kinetic isotopic effect (KIE) ^[18]. The KIE can be calculated according to the following equation:

204

$$KIE = -\left(1 + \frac{AF_L(x+y)}{1000z}\right) \quad (4)$$

206

where z the number of functional groups available for acetylation.

208

2.2. Determination of Limit of Detection (LOD) and Limit of Quantification (LOQ)

209

Typically, LOD and LOQ are calculated by multiplying the signal to noise ratio by 3 for LOD, and by 10 for LOQ. This method, unfortunately, do not reflect on the stability of the signal nor background noise of the analysis. We applied the more stringent three-step method suggested by Harris ^[24] and first established a calibration curve for the AAs of interest, spanning from 0.75 to 7.5 mM, done in triplicate. Secondly, eight standard mixtures, containing all AAs of interest, were analyzed at an equal low concentration of 0.75 mM to reflect the stability of the analyses. Thirdly, eight blind samples were analyzed to reflect the stability of the background noise. The lowest detectable signal (y_{dl}), LOD and LOQ were determined using the following equations ^[24]:

$$y_{dl} = y_{blank} + 3 \cdot s \quad (5)$$

$$LoD = \frac{3 \cdot s}{m} \quad (6)$$

$$LoQ = \frac{10 \cdot s}{m} \quad (7)$$

where y_{blank} is the average of the signal obtained from blind samples, s is the standard deviation from the analysis of standards in mixtures and m is the slope of the standard curve determined for individual AAs.

2.3. Test of analytical procedure on amino acids in white clover

2.3.1. Cultivating and labeling of white clover

White clover was sown in pots containing sand and irrigated once a week for the first eight week, twice a week for the next five weeks and three times a week for the last six weeks. The water used for irrigation contained 3 at% ¹⁵N-PK fertilizer. From week eight and onwards, the clover was labeled with ¹³CO₂ prior to irrigation as previously described ^[25]. Briefly, 5 mL of a saturated solution of sodium bicarbonate (¹³C 99at%) dissolved in 1 M NaOH was placed in a beaker in each pot of clover. Then the pot was covered with a transparent plastic bag and 5 mL 2 M HCl was added to the ¹³C-bicarbonate solution, hence releasing ¹³CO₂. After 2 h the labeling was stopped by removing the plastic bags.

2.3.2. Harvest and preparation of juice samples

White clover was harvested after 19 weeks of growth and divided into shoot and root biomass by cutting the shoots at the sand surface (i.e. everything containing chlorophyll was considered a part of the shoots). The shoots were rinsed with water and dabbed dry with a towel. The roots were carefully separated from the sand and rinsed in the same manner.

243

244 Juice from shoots and roots was extracted by screw pressing^[26]. The resulting juice was centrifuged at
245 10.000 g for 30 min followed by filtration through a 0.45 µm syringe CA filter (VWR International, Søborg,
246 DK).

247

248 Prior to analysis of AAs, the juice was subjected to molecular size fractionation to reduce interference from
249 larger molecules. Molecular size fractionation was performed by a modified ultrafiltration method^[27] using
250 20 mL centrifugal filter tubes (Macrosep® Advance, Pall Corporation, MI, USA) equipped with permeable
251 membranes of pore sizes 1, 10, and 100 kDa. First, each centrifugal tube was washed with 0.1 N HCl and
252 rinsed with MilliQ water three times before use. Then, the juice was added to the 100 kDa filter tubes and
253 centrifuged at 5000 g for up to 180 min. After centrifugation of the juice sample, the filter was washed two
254 times by adding 5.0 mL milliQ water to the filter and centrifuged at 5000 g for up to 60 min. The filtrate that
255 had passed through the 100 kDa filter was collected and the process was repeated for 10 kDa and 1 kDa
256 filters. The residues, which had not passed through the filters, were washed out by shaking the filter three
257 times in 5.0 mL MilliQ water. The fractionation procedure eventually resulted in four size fractions of juice
258 from both root or shoot biomass, i.e., >100 kDa, 10-100 kDa, 1-10 kDa and <1 kDa. All fractionated samples
259 were stored frozen until derivatization and analysis as described above.

260

261 *2.4. Data analysis and statistics*

262 The influence of the hydrolysis and the molecular size distribution on the atomic fraction were tested with
263 a linear mixed-effects model using the statistical analysis program R version 3.3.1 (R Core Team, 2016)^[28].
264 For statistical analysis, the datasets were divided into subsets, each subset were tested for normal
265 distribution by the Shapiro-Wilk normality test and for homogeneity of variances by the Bartlett test.
266 Models describing each subset were tested by either an ANOVA test or a pairwise test.

267

268 **3. Results**

269 *3.1. Efficiency of the hydrolysis*

270 BSA standard solutions, subjected to the two acid hydrolysis methods and GC/C-IRMS analysis, revealed
271 between 21 and 23 peaks, where 12 could be assigned to a specific AA or group of AAs, including one peak
272 from the ISTD. The total area ± SE of both identified and un-identified AA was significantly higher ($P <$
273 0.001) with the standard acid hydrolysis (1083 Vs ± 104 Vs) as compared to the fast acid hydrolysis (636 Vs
274 ± 55 Vs). The area of identified AAs were 685 Vs ± 46 Vs and 502 Vs ± 39 Vs for the standard and fast
275 hydrolysis, respectively. We then calculated that the total recovery, based on the identified AAs, were

276 35.6% ($\pm 1.3\%$) and 31.8% ($\pm 1.5\%$) of the total mass of the BSA standard for the standard and fast
277 hydrolysis, respectively. The recovery was significantly higher ($P < 0.04$) with the standard acid hydrolysis
278 for 7 of the 11 identified AAs, whereas the fast acid hydrolysis was comparable to the standard acid
279 hydrolysis for the remaining 4 identified AA (Figure 2).

280 281 *3.2. Efficiency of the NAIP derivatization method for separating amino acids*

282 Individual AA standard solutions were derivatized to optimize the gradient of the GC column for maximum
283 separation of the AA. We were able to obtain separation and stable retention time for all 21 AAs in single
284 standards with the NAIP method (Table 1).Ala eluted as the first with a retention time of 1232 s and Lys
285 eluted as the last with a retention time of 5181 s (Figure S.1). However, in mixed standards, Met and Cys
286 disappeared, whereas Pro + Thr (Pro/Thr) eluted simultaneous with retention times at 1886 s and Asn + Asp
287 (Asx) eluted simultaneous with retention times 2000 s. Also the ISTD Avl eluted simultaneous with Gly, we
288 therefore decided to use Nle as the ISTD for the sample analysis.

289 290 *3.3. Limit of detection and quantification for the C IRMS analysis*

291 Using the three-step methodology suggested by Harris ^[24] we determined the LOD and LOQ for 15 AAs or
292 co-eluting AAs using NAIP derivatization for C-IRMS analysis (Table 2). The LOD ranged from 0.17 pmol for
293 Leu to 1.47 pmol for Lys. The LOQ ranged from 0.55 pmol for Ser to 4.89 pmol for Lys. The LOD was below 1
294 pmol for seven AAs and below 2 pmol for another four AAs.

295 296 *3.4. Amino acids in white clover samples*

297 *3.4.1 Low molecular size fraction*

298 The low molecular size fraction (<1 kDa) was analyzed both for free AAs and for bound AAs after hydrolysis
299 in order to release the AAs in small peptides. We found that the un-hydrolyzed samples contained 11 single
300 eluting AA (Ala, Val, Gly, Leu, Ile, Ser, Glu, Gln, Phe, Tyr and Lys), and two pairs of co-eluting AA: Pro/Thr,
301 and Asx, respectively. The hydrolyzed sample contained the same eluting AAs, except for Glu and Tyr that
302 were completely lost. Further, the amount of seven AAs (Asx, Gln, Ser, Pro/Thr, Ala and Val) was
303 significantly lower ($p < 0.03$) after acid hydrolysis, whereas four AAs (Phe, Leu, Ile and Gly) were unaffected
304 ($p > 0.09$) by hydrolyzation (Figure 3).

305 306 *3.4.2 High molecular size fraction*

307 The three larger molecular size fractionations (>1 kDa) were all subject to hydrolysis prior to derivatization,
308 in order to release all peptide and protein bound AAs (Table S.1). For the 1-10 kDa size fraction we

309 detected, in both shoot and root juice, six single eluting AA (Ala, Val, Gly, Leu, Ile and Ser) and one pair of
310 co-eluting AAs: Pro/Thr; with an additional co-eluting pair, Asx, found in the shoot juice. For the 10-100 kDa
311 size fraction, both shoot and root juice gave four single eluting AA (Val, Leu, Ile and Phe). For the > 100 kDa
312 size fraction, both shoot and root juice gave 10 single eluting AA (Ala, Val, Gly, Leu, Ile, Ser, Gln, Phe, Tyr
313 and Lys), and two pairs of co-eluting AA: Pro/Thr, and Asx, respectively.

314

315 *3.5. Enrichment of amino acids in clover samples*

316 The correction factor and KIE was calculated based on obtained $\delta^{13}\text{C}$ data from analysis of un-derivatized
317 amino acid standards on a Flash Elemental Analyzer (Thermo Scientific, Hvidovre, DK) and GC/C-IRMS
318 analysis of derivatized amino acid standards, in the present study, KIE were on average of $0.13 \pm 0.06 \%$
319 (Table 3). The correction factor reflects the contribution of ^{13}C from the added C atoms during
320 derivatization. The correction factor must therefore be significantly lower than the ^{13}C atomic fraction of
321 the samples for the results to be reliable. In the present study, the ^{13}C atomic fraction for AAs were five to
322 ten times higher than the correction factor, substantiating that for ^{13}C enriched samples, the number of C-
323 atoms added with the NAIP derivatization is not an issue of concern.

324

325 In the low Mw size fraction (< 1 kDa) we found a linear relation, with a slope close to 1, between both ^{13}C
326 and ^{15}N atomic fractions for individual AAs as free and bound AAs for both shoot and root juice (Figure 4 A -
327 D).

328 The statistical comparison of ^{13}C atomic fraction in shoot and root from un-hydrolyzed versus hydrolyzed
329 samples showed that there was no significant difference between the atomic fraction obtained from free
330 AA and bound AA in the <1 kDa fraction for both shoot ($p = 0.73$) and root ($p = 0.84$). For the ^{15}N atomic
331 fraction in shoot and root juice from hydrolyzed versus un-hydrolyzed samples there was no significant
332 difference for Ile ($p = 0.82$) and Phe ($p = 0.19$) in shoot juice and for Val ($p = 0.15$), Gly ($p = 0.08$), Leu ($p =$
333 0.27), Ser ($p = 0.41$), Phe ($p = 0.31$) and Lys ($p = 0.16$) in root juice. For the three larger Mw size fractions (>1
334 kDa) there were significant differences between the atomic fraction obtained across Mw size fractions both
335 for ^{15}N and ^{13}C , except for Leu and Ser for ^{13}C in shoot juice, and Gln, Tyr and Lys for ^{13}C in root juice (Figure
336 5 and Table S.2). The ^{13}C atomic fraction of AAs in shoot juice generally had a higher enrichment than root
337 juice compared across Mw size fractions (Figure 6).

338

339 **4. Discussion**

340 *4.1 Influence of hydrolysis and derivatization on recovery of amino acids*

341 The recovery of BSA from the two acidic hydrolysis methods, i.e., 35.6% ($\pm$ 1.3%) for standard hydrolysis
342 and 31.8% ($\pm$ 1.5%) for fast hydrolysis, are in line with previously reported recoveries of approximately 30%
343 ^[12]. Based on the total recovery and the recovery of individual AA in BSA standard solutions, the standard
344 acid hydrolysis was chosen for the further work.

345

346 The NAIP derivatization method could separate and identify 13 proteinogenic AA, one synthetic AA (Nle)
347 used as ISTD and two pairs of co-eluting AA. Compared to other methods ^[19,22] we were uniquely able to
348 separate Gln and Glu in a mixed standard and we were also able to obtain signals from Hyp and Trp and
349 separate Pro and Thr. Both Corr et al. ^[19] and Larsen et al. ^[16] showed co-elution of these two AAs with the
350 NACME derivatization method using VF-23ms GC and TG-200MS GC columns, respectively. However, Wang
351 et al. ^[29] recently separated NACME derivatized Pro and Thr using a VF-35ms GC column, but further studies
352 are warranted to test whether this GC column is suited for AAs enriched in heavy isotopes.

353

354 We obtained markedly lower LOD and LOQ than previously reported for GC/C-IRMS analysis of AAs. Walsh
355 et al. ^[20] reported a LOQ of 10-50 pmol and Sessions ^[30] stated that instrument sensitivity for GC/C-IRMS
356 typically lies between 0.1-10 nmol. Our LOQ ranged from 0.55-4.89 pmol with the lowest concentrations
357 with stable AF ranging from 0.74-2.55 pmol of the non-enriched standards. The low LOD and LOQ for C-
358 IRMS was achieved in part because at least five C atoms were added during NAIP derivatization (Figure 1).
359 As demonstrated in this study, the dilution of the ¹³C labelled atoms is a relatively low source of error
360 compared to analyzing peaks next to or below LOD and LOQ. The improvement in LOD and LOQ for C by
361 using NAIP does not apply to the N analysis since there is no addition of N atoms during the derivatization.
362 The low LOD and LOQ of the NAIP derivatization is particularly important when analyzing labeled natural
363 samples with low AA concentrations.

364

365 4.2. Content and enrichment of amino acids in Mw size fractionated clover shoot and root juice

366 As stated in the literature ^[12,15] a decline in the concentration of Asn, Gln, Ser, Thr and Tyr is expected due
367 to hydrolysis, along with an increase in Val, Ile and Leu. Yet, we were surprised to find that the AA
368 concentration from <1 kDa Mw size fraction was generally lower after hydrolysis than the concentrations of
369 free AAs (i.e., in the un-hydrolysed samples). This indicated that the hydrolysis affects the composition of
370 AAs much more than previously recognized ^[11]. This highlights the challenge of calculating the total amount
371 of AA, peptides and proteins in natural samples and the need to adjust hydrolysis procedures to the actual
372 samples under investigation ^[10,12,14]. Importantly, the atomic fraction of ¹³C proved to be independent of the

373 concentration and did not show the same tendency to decline following the hydrolysis; meaning that even
374 though AAs were lost during the hydrolysis, the ^{13}C enrichment was not affected.
375 Due to the influences of the hydrolysis and the fact that there is no significant difference between the ^{13}C
376 atomic fraction from the hydrolyzed and un-hydrolyzed <1 kDa Mw size fraction, the > 1 kDa Mw size
377 fraction was not included in the comparison of ^{13}C atomic fraction across Mw size fraction in Figure 5.
378 The atomic fraction of ^{13}C in shoot and root showed similar distribution, with a tendency to a higher
379 relative abundance of ^{13}C in the shoots than in the roots. This is coherent with the fact that ^{13}C was induced
380 into the white clover through atmospheric labeling.
381 The atomic fraction of ^{15}N in shoot showed a similar distribution in all Mw size fractions except for Tyr and
382 Lys. But the ^{15}N atomic fraction in roots was higher in the low than high Mw size fractions. This align with
383 the way of labeling, since ^{15}N was added to the white clover through irrigation.

384

385 **5. Conclusion**

386 We investigated two hydrolysis methods for GC/C-IRMS analysis of AAs. We found that the standard
387 hydrolysis had significantly higher recovery of AAs from BSA standards than fast hydrolysis. Using mixtures
388 of AAs, we found that NAIP derivatization resulted in a very low LOD and LOQ, due to the addition of extra
389 C-atoms, with the latter ranging from 0.55-4.89 pmol for the 15 AA's determined. We tested the
390 methodology (i.e., standard hydrolysis and NAIP derivatization) on molecular size fractionated organic N in
391 juice from dual labeled white clover shoot and root and documented the ability to distinguish differences in
392 atomic fractions across AA, in individual AA in four molecular size fractions, and between shoot and root
393 samples. Of particular interest, we showed that hydrolysis of the smallest fraction (< 1 kDa), to release AAs
394 bound in small peptides, caused a notable loss of AAs, actually resulting in lower concentrations of most
395 AAs in the hydrolyzed sample than when analyzed for free AAs in the un-hydrolysed sample. The present
396 study highlighted the importance of determining the recovery efficiency of the applied hydrolysis method
397 and the great potential for using NAIP derivatization for GC/C-IRMS analysis of enriched (^{13}C and ^{15}N)
398 samples. In contrast to previous studies, the hydrolysis and NAIP procedures presently described were
399 specifically able to separate Gln and Glu.

400

401 **Acknowledgements**

402 The study was financially supported by The Independent Research Fund Denmark – Technology and
403 Production. (Project no. 1335-00760B).

404

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487

488

489 **Figure legend:**

490 **Figure 1**

491 Step by step structural information of the two amino acids derivatization methods, i.e. the N-acetyl methyl
492 esterification (A) and the N-acetyl isopropyl esterification (B)

493

494 **Figure 2**

495 Recovery of 11 identified amino acids or groups of amino acids from Bovine Serum Albumin (BSA) standard
496 solutions after standard acid hydrolysis ("black bars") and fast acid hydrolysis ("grey bars"). Data are mean
497 $\pm$ standard error ($n = 3$); Asterisks indicate significant differences in the recovery among the hydrolysis
498 methods.

499

500 **Figure 3**

501 The content of free amino acids (AAs) versus bound AAs in the <1 kDa Mw size fraction for (A) root juice
502 and (B) shoot juice ($n=3$) using the standard hydrolysis method to retrieve bound AAs.

503

504 **Figure 4**

505 The atomic fraction in free amino acids (AAs) versus bound AAs in the <1 kDa Mw size fraction for (A) ^{13}C in
506 shoot juice, (B) ^{15}N in shoot juice, (C) ^{13}C in root juice, and (D) ^{15}N in root juice of experimentally labeled
507 white clover ($n=3$).

508

509 **Figure 5**

510 Example of the ^{13}C atomic fraction of amino acids (AAs) in white clover shoot juice for different Mw size
511 fractions: free AAs (black circle), AAs bound in 1-10 kDa (dark grey triangle up), AAs bound in 10-100 kDa
512 (light grey square), and AAs bound in >100 kDa (white diamond). For free AAs both Glu and Gln were
513 measured (Glu omitted in this figure), whereas in the hydrolyzed Mw size fractions >1 kDa Glu/Gln is
514 reported as Glx. Table S.2 gives ^{13}C and ^{15}N atomic fractions for AAs in all Mw size fraction from white
515 clover shoot and root juice ($n=3$). Asterisks indicate significant differences in the obtained AF. Double
516 asterisks indicate no normal distribution.

517

518 **Figure 6**

519 Comparison of the ^{13}C atom fraction in root juice versus shoot juice in the Mw size fractions >1 kDa ($n=3$).

520

A) N-acetyl methyl esterification (NACME)

B) N-acetyl isopropyl esterification (NAIP)

Figure 1. Step by step structural information of the two amino acid derivatization methods, i.e. the N-acetyl methyl esterification (A) and N-acetyl isopropyl esterification (B).

Figure 2. Recovery of 11 identified amino acids or groups of amino acids from Bovine Serum Albumin (BSA) standard solutions after standard acid hydrolysis ("black bars") and fast acid hydrolysis ("grey bars"). Data are mean $\pm$ standard error ($n = 3$); Asterisks indicate significant differences in the recovery among the hydrolysis methods.

Figure 3. The content of free amino acids (AAs) versus bound AAs in the <1 kDa Mw size fraction for (A) root juice and (B) shoot juice ($n=3$) using the standard hydrolysis method to retrieve bound AAs.

Figure 4. The atomic fraction in free amino acids (AAs) versus bound AAs in the <1 kDa Mw size fraction for (A) ^{13}C in shoot juice, (B) ^{15}N in shoot juice, (C) ^{13}C in root juice, and (D) ^{15}N in root juice of experimentally labeled white clover ($n=3$).

Figure 5. Example of the ^{13}C atomic fraction of amino acids (AAs) in white clover shoot juice for different Mw size fractions: free AAs (black circle), AAs bound in 1-10 kDa (dark grey triangle up), AAs bound in 10-100 kDa (light grey square), and AAs bound in >100 kDa (white diamond). For free AAs both Glu and Gln were measured (Glu omitted in this figure), whereas in the hydrolyzed Mw size fractions >1 kDa Glu/Gln is reported as Glx. Table S.2 gives ^{13}C and ^{15}N atomic fractions for AAs in all Mw size fraction from white clover shoot and root juice ($n=3$). Asterisks indicate significant differences in the obtained AF. Double asterisks indicate no normal distribution.

Figure 6. Comparison of the ^{13}C atomic fraction in root versus shoot juice in the Mw size fractions >1 kDa ($n=3$).

Table 1. Obtained retention time of AA after NAIP derivatization (ISTD = internal standard).

NAIP	Amino acid	Retention time individual standards [s]	Retention time mixed standards [s]
1	Ala	1232,3	1230
2	Val	1360,2	1360
3	Avl ^{ISTD}	1438,5	1444
4	Gly	1443,8	1444
5	Leu	1465,3	1464
6	Ile	1477,6	1478
7	Nle ^{ISTD}	1556,2	1554
8	Pro	1874	1886
9	Thr	1883	1886
10	Asn	1996,8	2000
11	Asp	2001	2000
12	Ser	2058	2051
13	Glu	2197	2190
14	Gln	2295,9	2297
15	Met	2347,3	-
16	Phe	2434	2436
17	Cys	2443 - 2445	-
18	Hyp	2523,9	2523
19	Trp	2840,3	2979
20	Tyr	3553,6	3589
21	Lys	5188,6	5181

Table 2. Listing the standard equation, the LOD and LOQ, lowest detectable signal (y_{dl}) and ^{13}C AF stability for 15 AAs or co-eluting AAs after NAIP derivatization of non-enriched standard mixtures for C-IRMS analysis. (ISTD = internal standard). AF stability of ^{13}C is listed as the lowest concentration with stable AF ($P>0.05$).

Amino acid	Molar mass [g/mol]	Slope	Intercept	R ²	y_{dl} [signal intensity]	LOD C [pmol]	LOQ C [pmol]	AF stability [pmol]
Ala	89,09	7,714	3,284	0,9527	2,117	0,23	0,77	0.74
Val	117,15	2,949	1,039	0,9599	1,213	0,19	0,63	1.23
Gly	75,07	5,512	3,122	0,9489	2,100	0,25	0,85	0.74
Leu	131,17	9,443	3,733	0,9697	2,544	0,17	0,58	2.55
Ile	131,17	3,957	-0,473	0,9239	1,828	0,29	0,96	2.48
Nle ^{ISTD}	131,17	9,653	3,898	0,9691	3,947	0,34	1,12	0.75
Pro/Thr	115,13	6,224	9,858	0,9459	6,900	0,92	3,07	1.51
Asx	132,12	5,558	5,792	0,9691	3,138	0,28	0,92	2.52
Ser	105,09	7,081	-1,144	0,8589	3,013	0,16	0,55	0.74
Glu	147,13	4,464	1,485	0,9439	5,734	0,76	2,53	2.5
Gln	146,14	14,801	7,417	0,9731	8,358	0,35	1,18	0.75
Phe	165,19	7,813	6,359	0,9574	6,631	0,36	1,19	2.51
Hyp	131,13	4,662	7,828	0,8245	5,641	0,64	2,12	0.74
Tyr	181,19	5,231	7,937	0,9194	4,324	0,60	1,98	1.24
Lys	182,65	9,195	0,167	0,9081	18,88	1,47	4,89	2.5

Table 3. Listing the correction factor and KIE for individual AA for the IRMS analysis of ^{13}C .

Amino acid	Correction factor	KIE [‰]
Ala	0,0106229	0,08
Val	0,0105033	0,11
Gly	0,0106524	0,07
Leu	0,0104997	0,12
Ile	0,0104222	0,11
Nle	0,0105710	0,12
Pro	0,0104943	0,10
Thr	0,0106344	0,13
Asn	0,0106472	0,23
Asp	0,0106316	0,13
Ser	0,0105209	0,12
Glu	0,0109726	0,14
Gln	0,0105238	0,00
Phe	0,0103794	0,15
Hyp	0,0105120	0,14
Tyr	0,0102663	0,14
Lys	0,0105582	0,30
Average	0,0105537	0,13
SE	0,0001444	0,03

Supporting material for:

The influence of hydrolysis and derivatization on the determination of amino acid content and isotopic ratios in dual labeled (^{13}C , ^{15}N) white clover.

Figure S.1

Chromatogram showing the analysis of AAs after NAIP derivatization of a standard mixture containing 21 AA: L-alanine (Ala), L-valine (Val), glycine (Gly), L-leucine (Leu), L-isoleucine (Ile), L-proline (Pro), L-threonine (Thr), asparagine (Asn), L-aspartate (Asp), L-serine (Ser), L-glutamate (Glu), glutamine (Gln), methionine (Met), L-phenylalanine (Phe), L-cysteine (Cys), hydroxy-L-proline (Hyp), tryptophan (Trp), L-tyrosine (Tyr), L-lysine (Lys) and D-(-)-nor-leucine (Nle). The retention times are listed in table 1.

Table S.1:

Average values of the obtained concentration calculated as ng AA/g fresh weight plant material, across mw size fraction. We see a higher concentration of AA in the lower mw size fraction (<1kDa) and in the highest mw size fraction (>100kDa), than in the two middle fractions (1-10 kDa and 10-100 kDa).

Shoot [ng/g]	Ala	Val	Gly	Leu	Ile	Pro	Thr	Asn	Asp	Ser	Glu	Gln x	Phe	Tyr	Lys
Free	60.52 ± 4.58	63.69 ± 2.56	10.57 ± 0.62	57.79 ± 3.44	34.34 ± 0.96	95.62 ± 7.7	98.93 ± 7.97	300.91 c	303.15 c	77.52 ± 16.29	44.02 ± 3.19	79.48 ± 1.75	36.03 ± 2.2	157.97 ± 90.71	37.69 ± 7.72 a
< 1 kDa	17.42 ± 1.86	31.12 ± 2.82	8.2 ± 1.16 a	59.37 ± 3.39	39.88 ± 2.08	37.5 ± 3.11 a	38.8 ± 3.21 a	38.05 ± 9.07	38.33 ± 9.14	10.34 ± 0.4	nd	12.05 ± 1.56 a	59.29 ± 3.88	nd	128.7 ± 5.86
1-10 kDa	3.18 ± 0.57	6.15 ± 0.89	3.9 ± 0.61	10.09 ± 1.01	7.43 ± 0.38	12.68 ± 1.93	13.12 ± 2	4.3 ± 1.33	4.33 ± 1.34	2.08 ± 0.16	nd	nd	nd	nd	nd
10-100 kDa	nd	2.52 ± 0.04 a	nd	5.78 ± 2.24	6.47 ± 0.12	nd	nd	nd	nd	nd	nd	nd	6.54 ± 0.87 a	nd	nd
>100 kDa	18.06 ± 1.75	36.09 ± 4.21	13.39 ± 1.92	84.14 ± 9.47	50.07 ± 5.96	38.57 ± 4.48	39.91 ± 4.63	nd	nd	6.28 ± 0.63		6.97 ± 1.7	85.94 ± 11.31	34.76 ± 4.34	53.05 ± 11.25 a
Root [ng/g]	Ala	Val	Gly	Leu	Ile	Pro	Thr	Asn	Asp	Ser	Glu	Gln x	Phe	Tyr	Lys
Free	50.13 ± 2.47	48.17 ± 2.04	13.97 ± 1.24	50.74 ± 1.55	32.12 ± 2.06	47.65 ± 2.59	49.3 ± 2.68	172.69 ± 13.77	173.98 ± 13.87	49.89 ± 29.71	16.12 ± 1.31	17.8 ± 3.23	29.46 ± 1.46	18.42 ± 3.23 a	45.51 ± 2.87 b
< 1 kDa	14.95 ± 1.88	27.74 ± 2.43	7.2 ± 1.55	53.2 ± 4.47	36.48 ± 3.05	17.45 ± b.c	18.06 ± b.c	9.62 b,c	9.69 b,c	7.3 ± 0.86 a	nd	nd	45.54 ± 6.46	nd	101.35 ± 16.78 b
1-10 kDa	0.8 ± 0.18 a	2.4 ± 0.34	1.27 ± 0.07 a	4.45 ± 0.67	4.23 ± 0.46	3.62 ± 0.24 a	3.75 ± 0.25 a	nd	nd	1.14 ± 0.1 a	nd	nd	nd	nd	nd
10-100 kDa	nd	1.61 ± 0.15 a	nd	6.97 ± 0.37	4.4 ± 1.21 a	nd	nd	nd	nd	nd	nd	nd	7.25 ± 0.45 a	nd	nd
>100 kDa	31.52 ± 7.08	54.01 ± 8.88	22.31 ± 5.19	121.64 ± 18.67	73.39 ± 9.83	63.89 ± 13.37	66.1 ± 13.84	6.52 ± 1.19 a	6.56 ± 1.2 a	12.19 ± 1.18		14.41 ± 3.13	129.27 ± 26.92	45.76 ± 19.2	56.25 c

a: The obtained data were below the LoQ

b: the obtained data were below the LoD

c: only one data point

nd: no data available.

Free: Free AA in Mw size fraction < 1 kDa

1-10 kDa: AA bound in Mw size fraction 1-10 kDa

10-100 kDa: AA bound in Mw size fraction 10-100 kDa

>100 kDa: AA bound in Mw size fraction >100 kDa

Table S.2:

Average value of the obtained atomic fraction across mw size fraction. For ^{13}C shoot and root the hydrolyzed mw size fraction < 1 kDa is excluded because there were no significant differences between the hydrolyzed and the un-hydrolyzed mw size fraction < 1 kDa. Different lettering on the values indicate that the values are significantly different, $p > 0.05$.

^{13}C shoot	Ala	Val	Gly	Leu	Ile x	Pro/Thr	Asx	Ser	Glu x	Gln	Phe	Tyr	Lys
Free	0.09228 a	0.07956 a	0.08798 a	0.08252 a	0.08309	0.09664 a	0.08950 a	0.08038 a	0.12837	0.07920 a	0.06803 a	0.05455 a	0.08268 a
1-10 kDa	0.08454 b	0.08420 b	0.07938 b	0.08505 a	0.08851	0.09728 a	0.08050 b	0.07871 a	nd	nd	nd	nd	nd
10-100 kDa	0.08150 c	0.08339 c	0.07624 c	0.08469 a	0.08660	0.08968 b	nd	nd	nd	0.07384 b	0.07836 b	nd	nd
>100 kDa	0.08361 d	0.08335 d	0.07907 d	0.08539 a	0.08703	0.09826 a	nd	0.07663 a	nd	0.07542 c	0.07597 c	0.06228 a	0.08837 b
^{13}C root	Ala	Val	Gly	Leu	Ile x	Pro/Thr	Asx	Ser x	Glu x	Gln	Phe	Tyr	Lys
Free	0.08343 a	0.07252 a	0.07704 a	0.07156 a	0.07222	0.07803 a	0.09637 a	0.08478	0.11713	0.07015 a	0.06035 a	0.05181 a	0.07475 a
1-10 kDa	0.07635 b	0.07080 b	0.07197 b	0.07473 b	0.07782	0.08418 b	0.07426 b	0.07962	nd	nd	nd	nd	nd
10-100 kDa	0.07333 c	0.07036 c	0.06689 c	0.07298 c	0.07230	0.07978 b	nd	nd	nd	0.06762 a	0.07099 b	nd	nd
>100 kDa	0.07667 d	0.07294 d	0.07525 d	0.07440 c	0.07575	0.08567 b	0.07829 c	0.07708	nd	0.06826 a	0.06499 a	0.05293 a	0.07775 a
^{15}N shoot	Ala	Val x	Gly	Leu x	Ile	Pro/Thr x	Asx	Ser	Glu	Gln x	Phe	Tyr x	Lys x
Free	0.01537 a	0.01530	0.01421 a	0.01452	0.01509 a	0.01434	0.01560 a	0.01557 a	0.01560 a	0.01580	0.01411 a	0.01451	0.01318
< 1 kDa	0.01513 b	0.01471	0.01443 b	0.01466	0.01508 a	0.01425	0.01519 b	0.01535 b	nd	0.01548	0.01409 a	nd	0.01372
1-10 kDa	0.01386 c	0.01231	0.01384 c	0.01453	0.01479 b	0.01373	0.01417 c	0.01463 c	nd	0.01484	0.01343 b	0.00852	0.01308
10-100 kDa	0.01213 d	nd	0.01231 d	0.01313	nd	0.01306	0.01344 d	0.01353 d	nd	0.01351	0.01258 c	0.00649	0.00649
>100 kDa	0.01415 e	0.01364	0.01398 e	0.01441	0.01499 b	0.01383	0.01371 e	0.01407 e	nd	0.01431	0.01396 d	0.01328	0.01330
^{15}N root	Ala	Val	Gly	Leu	Ile	Pro/Thr	Asx x	Ser	Glu x	Gln	Phe	Tyr	Lys
Free	0.01514 a	0.01928 a	0.01408 a	0.01391 a	0.01411 a	0.01402 a	0.02103	0.01886 a	0.02111	0.01862 a	0.01374 a	0.01954 a	0.01369 a
< 1 kDa	0.01437 b	0.01358 a	0.01372 a	0.01385 a	0.01424 b	0.01372 b	0.01961	0.01904 a	nd	0.01697 b	0.01365 a	nd	0.01480 a
1-10 kDa	nd	nd	nd	nd	nd	0.01200 c	0.00683	nd	nd	0.01362 c	nd	nd	0.00675 b
10-100 kDa	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
>100 kDa	0.01304 c	nd	0.01307 b	0.01360 b	nd	0.01303 d	0.01339	0.01344 b	nd	0.01346 d	0.01329 b	0.01271 b	0.01327 c

x: the data sub set was not normally distributed

nd: no data available.

Free: Free AA in Mw size fraction < 1 kDa

1-10 kDa: AA bound in Mw size fraction 1-10 kDa

10-100 kDa: AA bound in Mw size fraction 10-100 kDa

>100 kDa: AA bound in Mw size fraction >100 kDa