



Development of Alditol Acetate Derivatives for the Determination of ¹⁵N-Enriched Amino Sugars by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry

Reay, Michaela K; Knowles, Timothy D J; Jones, Davey L; Evershed, Richard P

Analytical Chemistry

DOI:
[10.1021/acs.analchem.8b04838](https://doi.org/10.1021/acs.analchem.8b04838)

Published: 05/03/2019

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):
Reay, M. K., Knowles, T. D. J., Jones, D. L., & Evershed, R. P. (2019). Development of Alditol Acetate Derivatives for the Determination of ¹⁵N-Enriched Amino Sugars by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry. *Analytical Chemistry*, 91(5), 3397-3404. <https://doi.org/10.1021/acs.analchem.8b04838>

Hawliau Cyffredinol / General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 Development of alditol acetate derivatives for the determination of ^{15}N -
2 enriched amino sugars by gas chromatography-combustion-isotope
3 ratio mass spectrometry

4

5 Michaela K. Reay,^a Timothy D.J. Knowles,^b Davey L. Jones,^{c, d} Richard P. Evershed^{a*}

6 a. Organic Geochemistry Unit, School of Chemistry, University of Bristol, Cantock's Close,
7 Bristol, BS8 1TS, U.K.

8 b. Bristol Radiocarbon Accelerator Mass Spectrometer, University of Bristol, 43 Woodland Road,
9 Bristol, BS8 1UU, U.K.

10 c. UWA School of Agriculture and Environment, University of Western Australia, Crawley, WA
11 6009, Australia

12 d. Environment Centre Wales, Deiniol Road, Bangor University, Bangor, Gwynedd, LL57 2UW,
13 U.K.

14

15 *author for correspondence: email: r.p.evershed@bristol.ac.uk

16

17 Amino sugars can be used as indices to evaluate the role of soil microorganisms in active
18 nitrogen (N) cycling in soil. This paper details the assessment of the suitability of GC-C-IRMS
19 for the analysis of ^{15}N -enriched amino sugars as alditol acetate derivatives prior to application
20 of a novel ^{15}N -stable isotope probing (SIP) approach to amino sugars. The efficient
21 derivatisation and clean-up of alditol acetates derivatives for GC was achieved using
22 commercially available amino sugars, including: glucosamine, mannosamine, galactosamine
23 and muramic acid, as laboratory standards. A VF-23MS stationary phase was found to produce
24 optimal separations of all four compounds. The structure of the alditol acetate derivatives was
25 confirmed using GC-MS. For GC-C-IRMS determinations, implementation of a two-point
26 normalisation confirmed the optimal carrier gas flow rate to be 1.7 ml min^{-1} . Linearity of $\delta^{15}\text{N}$
27 value determinations up to $\delta^{15}\text{N}_t$ of $469 \pm 3.1 \text{ ‰}$ (where $\delta^{15}\text{N}_t$ is the independently measured
28 $\delta^{15}\text{N}$ value) was confirmed when 30 nmol N was injected on-column, with the direction of
29 deviation from $\delta^{15}\text{N}_t$ at low sample amount dependent on the ^{15}N -abundance of the analyte.

30 Observed between- and within-run memory effects were significant ($P < 0.007$) when a highly
31 enriched standard ($469 \pm 3.1 \text{ ‰}$) was run therefore analytical run order and variation in ^{15}N -
32 enrichment of analytes within the same sample must be considered. The investigated
33 parameters have confirmed the isotopic robustness of alditol acetate derivatives of amino
34 sugars for the GC-C-IRMS analysis of ^{15}N -enriched amino sugars in terms of linearity over an
35 enrichment range (natural abundance to $469 \pm 3.1 \text{ ‰}$) with on column analyte amount over 30
36 nmol N.

37

38 Amino sugars are the building blocks of structural biopolymers in many microorganisms and
39 invertebrates, constituting the second largest structurally defined pool of organic nitrogen (ON)
40 in soil, accounting for between 5-12 %.¹ The microbial source-specificity (with minor
41 contributions from other sources) of these compounds enables investigation of the size and
42 activity of bacterial and fungal pools within soil.^{1,2} For example, the bacterial contribution to
43 the glucosamine (GlcN) pool from that of fungal origin can be calculated using the conservative
44 mass ratio of GlcN and muramic acid 5-to-1 in soil bacteria.³⁻⁷ Muramic acid (MurN) is solely
45 of bacterial origin, whilst the two other dominant amino sugars quantified in soils,
46 galactosamine (GalN) and mannosamine (ManN) have both bacterial and fungal sources.^{3,8}
47 Quantification of amino sugars in soils by gas chromatography (GC) and liquid
48 chromatography (LC) have been utilised to investigate the impact of environmental controls
49 and agricultural practices on the microbial community composition.^{3,9-13} These quantification
50 techniques cannot differentiate between amino sugars within the active microbial pool and
51 those in the necromass.^{14,15} Therefore, isotopic labelling techniques can be utilised to
52 investigate the dynamics within the active bacterial and fungal communities and the role of the
53 microbial community in the soil-N cycle. Using a compound-specific ^{15}N -SIP approach
54 provides a selective method of tracing the fate of applied ^{15}N -substrates into the microbial

55 community.¹⁶ Furthermore, it is possible to elucidate differences in relative importance of N
56 transformation pathways in the soil N cycle.^{3,15-17}

57 A ¹⁵N-SIP approach has applied been to amino sugars, determining ¹⁵N-incorporation into soil
58 amino sugars using electron ionisation (EI) gas chromatography-mass spectrometry (GC-
59 MS).^{2,4,18} These investigations have revealed the differing temporal response within the
60 microbial community to substrate addition and the differing stability of amino sugar residues.^{2,4}
61 The incorporation of ¹⁵N into amino sugars was determined following acid hydrolysis of parent
62 amino polysaccharides and aldonitrile derivatisation, based on selected ion monitoring of
63 *m/z* 98.¹⁸ A major drawback of this technique is that isotopic determinations using GC-MS
64 require high ¹⁵N-enrichments and therefore high N application rates which can perturb the
65 system and potentially result in ¹⁵N isotopic discrimination.¹⁶ Furthermore, this technique
66 employs a N-containing derivative group, which adds substantial uncertainty to N-isotope
67 determinations (see below).

68 A preferred approach to determining nitrogen isotopic compositions of amino sugars would be
69 to use gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). This
70 is a much more sensitive (0.5-2.0 ‰; 0.0002 to 0.0008 atom %) method for the determination
71 of $\delta^{15}\text{N}$ values of N-containing compounds than GC-MS and can achieve far higher levels of
72 high precision and accuracy. Its use would mean that ¹⁵N-SIP experiments could use low-level
73 substrate additions, thereby minimising system perturbations and discrimination.^{16,19,20} This
74 technique has already been applied to amino acid $\delta^{15}\text{N}$ value determinations, providing
75 previously inaccessible levels of detail regarding the rate of N and C transfers within amino
76 acids and soil protein fraction^{17,21}, amino acid uptake dynamics of plants²² and evidence for
77 microbial N assimilation pathways and differences in microbial processing of N fertiliser.¹⁶
78 Hence, used in this way, the ¹⁵N-SIP approach can provide hitherto unobtainable insights into

79 N-cycling through the amino sugar pool despite the complex nature of soil organic matter
80 (SOM) and the soil N-cycle.^{16,17}

81 Extending this GC-C-IRMS approach to amino sugars and exploiting their source-specificity,
82 would enable elucidation of the role of the bacterial and fungal communities in soil N-cycling
83 with N from environmentally relevant substrates applied at low levels of ¹⁵N-enrichment at
84 environmentally relevant concentrations.¹⁶ However, the aldonitrile derivatisation strategy
85 is unsuitable for use with GC-C-IRMS due to the addition of one nitrogen atom.²³ Whilst it is
86 possible to apply a mathematical correction to the determined $\delta^{15}\text{N}$ values, large uncertainties
87 have been observed for $\delta^{13}\text{C}$ determinations where corrections are applied due to error
88 propagation.^{14,24} GC-C-IRMS therefore requires an alternative derivatisation strategy. The
89 alditol acetate derivatisation method, commonly used for sugars and has been applied to amino
90 sugars, was selected as no nitrogen is added through derivatisation, eliminating these additional
91 uncertainties and is therefore preferred for $\delta^{15}\text{N}$ determinations using GC-C-IRMS.^{25,26}

92 The suitability of the GC-C-IRMS method for $\delta^{15}\text{N}$ value determinations of ¹⁵N-enriched
93 amino sugars must be established prior to application to ¹⁵N-tracer studies. The suitability of
94 GC-C-IRMS for the analysis of amino acids has previously been established over a range of
95 ¹⁵N-enrichments.^{16,17,19,27-30} Instrument parameters, such as optimal carrier gas flow rate, which
96 has a direct influence on residence time of analytes in oxidation and reduction reactors of the
97 GC-C-IRMS interface, have been investigated to ensure accurate determination of $\delta^{15}\text{N}$ values
98 of amino acids.²⁹ Furthermore, required sample amount for accurate and precise $\delta^{15}\text{N}$ value
99 determinations have been tested, and found to range from 2 to 100 nmol N on column.^{17,29,30}

100 Importantly, for analysis of ¹⁵N-enriched compounds, linearity with ¹⁵N-enrichment and
101 memory effects (both between- and within-analytical runs) must be considered.^{16,17,30} Such
102 assessments of GC-C-IRMS have led to the development of robust methods for the accurate
103 determination of $\delta^{15}\text{N}$ values of amino acids over a range of ¹⁵N-enrichments, allowing

104 applications using ^{15}N -tracer^{16,17,21,22,31} and natural abundance (biological, ecological and
105 archaeological^{27,32–35} approaches. The suitability of GC-C-IRMS for the determination of $\delta^{15}\text{N}$
106 values of alditol acetate derivatives of amino sugars must be assessed before such a method
107 can be applied in similar studies.

108 Herein, we describe the results of our investigations aimed at implementing a new
109 derivatisation and GC methods compatible with GC-C-IRMS, together with a two-point linear
110 normalisation to correct measured $\delta^{15}\text{N}$ values against amino sugar standards with known $\delta^{15}\text{N}$
111 values. Investigations into the effect of carrier gas flow on precision ensure instrumental
112 parameters are optimised for $\delta^{15}\text{N}$ determinations of amino sugars. The relationship between
113 isotopic linearity, sample amount and ^{15}N -enrichment have been investigated to confirm the
114 suitability of the method for the analysis of ^{15}N -enriched amino sugars. Finally, we investigated
115 the extent to which a ^{15}N -enriched compound can affect the determined $\delta^{15}\text{N}$ value of amino
116 sugars with lower ^{15}N -abundance within the same analytical run, and within subsequent
117 analytical runs, i.e. ‘memory effects’, which have previously been reported in analyses of
118 enriched $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ compounds.^{30,36–38} Validation of the GC-C-IRMS method for $\delta^{15}\text{N}$
119 value determinations of ^{15}N -enriched amino sugars ensures subsequent studies using a ^{15}N -SIP
120 approach to probe the fate of N-substrates in environmental settings are robust.

121

122 **EXPERIMENTAL**

123 **Reagents and standards**

124 A natural ^{15}N -abundance amino sugar standard (1 mg ml⁻¹ glucosamine, muramic acid,
125 galactosamine and mannosamine, Sigma-Aldrich, Dorset, UK) was prepared. ^{15}N -enriched
126 glucosamine standards ($\delta^{15}\text{N}$ value targets between 35 to 500 ‰) were prepared by the addition

127 of 1 mg ml⁻¹ solution 98.0 ±0.3 atom % ¹⁵N-GlcN (Sigma Aldrich) to a 1 mg ml⁻¹ solution of
128 natural abundance GlcN (isotopic dilution calculations are shown in equation S1 and S2).

129 Derivatisation reagents (sodium borohydride (NaBH₄), acetic acid and acetic anhydride) were
130 supplied by Sigma-Aldrich (Steinheim, Germany). All solvents were HPLC grade and supplied
131 by Rathburn Chemicals Ltd. (Walkerburn, UK), double-distilled water (DDW) was produced
132 using a Bibby Aquatron still.

133

134 **Amino sugar derivatisation**

135 The alditol acetate derivatisation method for amino sugars was adapted from that reported by
136 Whiton et al.²⁵ Briefly, dried amino sugar residues were reduced with sodium borohydride (500
137 µl, 100 mg ml⁻¹ in DDW) and heated (60 °C, 2.5 h). Excess sodium borohydride was destroyed
138 by the addition of acetic acid-methanol (2 ml; 1:200 v/v) and evaporated to dryness using a
139 stream of N₂ at 60 °C. This was repeated four times to ensure the complete destruction of
140 residual sodium borohydride. Acetylation was performed by addition of 500 µl of acetic
141 anhydride and heating (100 °C, 2.5 h). The reaction was quenched by freezing at -20 °C (15
142 min) and acetic anhydride subsequently destroyed by the dropwise addition of 2.5 ml of double-
143 distilled water. The alditol acetate derivatives of amino sugars were extracted with chloroform
144 (3 x 2 ml), combined and dried under a gentle stream of N₂ at 40 °C. Residues were re-dissolved
145 in 1.5 ml chloroform and the organic phase washed with double-distilled water (2 x 1.5 ml) to
146 remove residual acetic acid produced during acetic anhydride hydrolysis. The alditol acetate
147 derivatives were dissolved in ethyl acetate for subsequent analysis by GC-FID and GC-C-
148 IRMS.

149

150 **Instrumental analyses**

151 ***EA-IRMS***

152 Bulk $\delta^{15}\text{N}$ values for the amino sugar standards and prepared ^{15}N -enriched GlcN standard were
153 determined using elemental analysis-isotope ratio mass spectrometry (EA-IRMS). A Flash EA
154 1112 Series NC Analyser (Thermo Electron Corporation, MA, USA) was coupled to a
155 ThermoFinnigan Delta^{Plus} XP (Thermo Electron Corporation) *via* a ConFlo III interface.
156 Standards (0.2 mg N; n=9) were weighed into tin capsules for analysis. The $\delta^{15}\text{N}$ values of N_2
157 generated by the oxidation and subsequent reduction of the samples in the EA was determined
158 in the IRMS. A two-point normalisation was employed using traceable standards to the
159 international $\delta^{15}\text{N}_{\text{air}}$ scale using caffeine ($-25.5 \pm 0.3 \text{ ‰}$), benzocaine ($-0.3 \text{ ‰} \pm 0.2 \text{ ‰}$)³⁹ and
160 IAEA-305-B (ammonium sulphate; $375.3 \pm 2.3 \text{ ‰}$ for ^{15}N -enriched GlcN)⁴⁰ as normalisation
161 standards and phenacetin ($-8.4 \pm 0.4 \text{ ‰}$)³⁹ or IAEA-305-A (ammonium sulphate; 39.8 ± 0.5
162 ‰)⁴⁰ as quality control materials. Benzocaine and phenacetin standards were distributed during
163 proficiency tests organised by the Forensic IRMS network.³⁹ Caffeine was an in-house standard
164 normalised against USGS25 ($-30.41 \pm 0.27 \text{ ‰}$)⁴¹ and benzocaine ($-0.3 \text{ ‰} \pm 0.2 \text{ ‰}$)³⁹. IAEA
165 standards were supplied by the International Atomic Energy Agency, Vienna and USGS
166 standard was supplied by the US Geological Society. Detailed error propagation for the $\delta^{15}\text{N}$
167 values for natural abundance and enriched AS measured using EA-IRMS and corrected using
168 a two-point normalisation is shown in supplementary information (Equations S3 and S4) and
169 associated error is shown in Table S1.

170

171 ***GC-FID***

172 An Agilent Technologies 7890B GC-FID (Agilent Technologies, CA, USA) fitted with a VF-
173 23ms column (60 m x 0.32 mm i.d., 0.15 μm film thickness; Agilent Technologies) was used
174 for quantification of amino sugars as their alditol acetate derivatives by comparison to an

175 internal standard (myo-inositol; Sigma Aldrich; $\geq 99\%$). Elution order was determined by GC
176 analysis of individual standards and the known elution order subsequently used for
177 identification. The carrier gas was helium (He) at a flow rate of 2.0 ml min^{-1} and the
178 temperature programme was $70\text{ }^{\circ}\text{C}$ (1 min hold) to $210\text{ }^{\circ}\text{C}$ ($30\text{ }^{\circ}\text{C min}^{-1}$) to $260\text{ }^{\circ}\text{C}$ ($10\text{ }^{\circ}\text{C min}^{-1}$;
179 18 min hold). Data was acquired and analysed using Agilent OpenLab Control Panel (version
180 1.0; Agilent Technologies). Figure 1 shows a typical chromatogram of the amino sugar
181 standard.

182

183 ***GC-MS***

184 GC-MS analyses were performed on a Thermo Scientific ISQ Single Quadrupole GC-MS
185 (Thermo Electron Corporation) operated in electron ionisation mode (70 eV , m/z ranges of 50 to
186 650 Da). The carrier gas was He and identical GC column and conditions were employed as for
187 GC analysis.

188

189 ***GC-C-IRMS***

190 The $\delta^{15}\text{N}$ values of amino sugars as their alditol acetate derivatives were determined using a
191 ThermoFinnigan Trace 2000 gas chromatograph coupled via a ThermoFinnigan GC-III
192 interface to a ThermoFinnigan Delta^{Plus} XP isotope ratio mass spectrometer (Thermo Electron
193 Corporation). A GC Pal autosampler (CTC Analytics, Zwingen Switzerland) was used to
194 introduce samples via a programmable temperature vaporisation (PTV) inlet (Thermo Electron
195 Corporation). The GC was fitted with the same column as for GC-FID analyses and the
196 temperature ramp was from $70\text{ }^{\circ}\text{C}$ (1 min hold) to $200\text{ }^{\circ}\text{C}$ ($30\text{ }^{\circ}\text{C min}^{-1}$) to $260\text{ }^{\circ}\text{C}$ ($12\text{ }^{\circ}\text{C min}^{-1}$;
197 23 min hold). The oxidation reactor was composed of copper (Cu) and nickel (Ni) wires
198 (OEA Laboratories Ltd, Callington, UK) and maintained at $1030\text{ }^{\circ}\text{C}$. The reduction reactor was

199 composed of Cu wires and maintained at 650 °C. The carrier gas flow rate was tested over a
200 range of flow rates (1.3 to 2.0 ml min⁻¹) to optimise the carrier gas flow for δ¹⁵N value
201 determinations. The carrier gas was helium for which the optimal carrier gas flow was found
202 to be 1.7 ml min⁻¹ in constant flow mode. This was used for all subsequent δ¹⁵N value
203 determinations. Data was acquired and analysed using IsoDat NT 3.0 (Thermo Electron
204 Corporation). Figure 2 shows a typical chromatogram for the amino sugar standard mixture
205 including ion current signal for the *m/z* values recorded. It was not possible to completely
206 baseline separate ManN and GalN however determined δ¹⁵N_d of the two compounds did not
207 significantly vary when analysed as a mixture or single compounds (t-test; P>0.05). This
208 indicated the co-elution had little effect on the determined δ¹⁵N.

209 The suitability of GC-C-IRMS for δ¹⁵N value determinations of alditol acetate derivatives of
210 amino sugars in terms of linearity across a ¹⁵N-enrichment range was investigated using six
211 ¹⁵N-GlcN standards (-3.31±0.24 to 469 ±3.1 ‰; 30 nmol N on column; n=6). Sample
212 requirements for consistent δ¹⁵N value determinations for alditol acetate derivatives of amino
213 sugars were assessed using the same ¹⁵N-GlcN standards. The amount of analyte introduced on
214 column was: 8, 15, 30, 50 and 180 nmol N (equivalent to 3.2, 6, 12, 20 and 72 nmol N per
215 analyte in the ion source). All analyses were carried out in triplicate sequence runs in order of
216 increasing sample amount (where applicable) and increasing ¹⁵N-enrichment. Finally, possible
217 memory effects within the same run and between runs were investigated (see Table S2 for the
218 sequence order used). For within run memory effects, standard solutions containing ¹⁵N-
219 enriched GlcN (either 92.7 ±0.95 or 469 ±3.1 ‰) and MurN and GalN (-0.19 ±0.24 and -3.3
220 ±0.24 ‰, respectively) were prepared, derivatised and analysed in triplicate in order of
221 increasing enrichment.

222

223 Calculations

224 *Two-point normalisation*

225 A two-point normalisation was applied to correct measured $\delta^{15}\text{N}$ values using two bracketing
226 standards for both EA-IRMS and GC-C-IRMS analyses. This uses a linear regression of
227 measured and true $\delta^{15}\text{N}$ values of standards to normalise measured $\delta^{15}\text{N}$ values of unknown
228 samples and assumes any systematic error within the dynamic range is constant or linear.⁴²

229 For optimisation of carrier gas flow rate, the two-point normalisation was conducted with two
230 standards: standard-1 (Std-1, natural abundance amino sugar mixture) and standard-2 (Std-2;
231 ^{15}N -GlcN $31.9 \pm 0.4 \text{ ‰}$). Only ^{15}N -enriched GlcN standards were used due to lack of
232 commercial availability of a ^{15}N -enriched standard for other amino sugars. Due to the similar
233 chemical structures of the amino sugars, this was deemed acceptable. The two bracketing
234 standards were analysed (n=6) followed by the QC standard (same as Std-1; n=6) and $\delta^{15}\text{N}_d$
235 value of the QC standard calculated using Equation 1, where $\delta^{15}\text{N}_d$ is the measured $\delta^{15}\text{N}$ value
236 and $\delta^{15}\text{N}_t$ is true value of the standards determined independently using EA-IRMS.⁴²

237 **Equation 1:**
$$\delta^{15}N_t^{QC} = \frac{\delta^{15}N_t^{Std1} - \delta^{15}N_t^{Std2}}{\delta^{15}N_d^{Std1} - \delta^{15}N_d^{Std2}} \times (\delta^{15}N_d^{QC} - \delta^{15}N_d^{Std2}) + \delta^{15}N_t^{Std2}$$

238 The calibration was accepted if 75 % of the normalised $\delta^{15}\text{N}$ values for the QC standard were
239 within $\pm 0.75 \text{ ‰}$ and the remainder were within $\pm 1.5 \text{ ‰}$, and $1 \sigma < \pm 0.75 \text{ ‰}$. For the bracketing
240 and QC standards. The standard deviation of the standards was calculated based on the gaussian
241 error propagation outlined in Equation S4. These criteria assessed both the accuracy and
242 precision of determinations and are based on the repeated calibrations (n=5) to assess stability
243 and consistency in the instrumental set-up. The QC standard was analysed every 5 analytical
244 runs to check for drift from the two-point normalisation prepared. When QC values did not fit
245 these criteria, instrument maintenance (inlet maintenance and regeneration of oxidation

246 reactor) was conducted and the two-point normalisation repeated. Two-point normalisation
247 was not carried out when investigating linearity with sample amount and ^{15}N -enrichment and
248 during investigation of memory effects, as it was necessary to confirm these parameters before
249 a two-point normalisation could be applied for high ^{15}N -enrichments.

250

251 **RESULTS AND DISCUSSION**

252 **Derivatisation optimisation and chromatographic separation**

253 The alditol acetate derivatisation method was adapted from the procedure described by Whiton et al.
254 using the sodium acetate catalysed derivatisation.²⁵ Reduction was achieved at 60 °C, allowing a
255 shorter reduction step and the acetylation reagent was destroyed using double-distilled water,
256 as in Pettolino et al.²⁶ Due to residual acetic acid remaining from extraction of alditol acetate
257 derivatives using chloroform, an additional washing step was added prior to GC analyses.
258 Chromatographic separation was tested on non-polar (HP-5, Agilent Technologies), mid-
259 polarity (DB-35, Agilent Technologies) and high polarity (ZB-WAX (Phenomenex Zebron)
260 and VF-23ms, Agilent Technologies) columns. The high polarity cyanopropylphenyl
261 substituted stationary phase provided the best separation for GC-FID of the tested columns for
262 the four amino sugar derivatives, as shown in Figure 1, with co-elution of GlcN, GalN and
263 ManN observed on the other tested columns. This column has been previously used for $\delta^{15}\text{N}$
264 value determinations with amino acids and minimal interference from the nitrogen-containing
265 column bleed was observed.²⁹ Furthermore, during subsequent GC-C-IRMS analyses, an
266 individual background correction for each peak was applied (using 5 s of baseline history) and
267 the applied two-point normalisation will correct for any interference from low level column
268 bleed in the baseline.

269

270 **Mass spectral identification**

271 Following chromatographic separation of the AS derivatives, the structures of the alditol
272 acetate derivatives was confirmed using GC-MS. The mass spectra observed for GlcN, ManN
273 and GalN were identical, with characteristic carbon-chain bond cleavage (e.g. [M-73]⁺, [M-
274 289]⁺) and subsequent cleavage of acetylated hydroxyl (e.g. [M-115]⁺ and [M-331]⁺) and
275 amine ([M-349]⁺) groups from these fragments allowing identification. The presence of all
276 fragment ions arising from carbon-chain bond cleavage indicated the alditol acetate derivative
277 was acetylated in all hydroxyl and amine positions.^{25,43} MurN was derivatised to muramicitol
278 pentaacetate (MPA) in the lactam form, shown by the presence of [M-42]⁺, indicating the loss
279 of a ketene.^{25,43} The lactam containing fragments [M-277]⁺ and [M-217]⁺ dominate the spectra
280 and are characteristic of muramic acid.⁴⁴ A second alditol acetate derivative of MurN (to
281 muramicitol tetraacetate (MTA) co-elutes with the dominant isomer which has been previously
282 observed.²⁵

283

284 **Variation in precision with column flow**

285 The carrier gas flow rate controls the residence time of amino sugar derivatives in the
286 combustion and reduction reactors during GC-C-IRMS analyses, therefore this parameter is
287 important to optimise for GC-C-IRMS analyses. At low flow rates (1.3 to 1.5 ml min⁻¹), $\delta^{15}\text{N}_d$
288 values determined using GC-C-IRMS compared to independently measured $\delta^{15}\text{N}_t$ value are
289 depicted in Figure 3 for three AS. The deviation from $\delta^{15}\text{N}_t$ (i.e. depleted or enriched relative
290 to $\delta^{15}\text{N}_t$ value) was inconsistent and was not significant (t-test; $P>0.5$). Importantly, determined
291 $\delta^{15}\text{N}_t$ values at low flow rate have high associated standard deviation (1 σ ca. 2.1 ‰), as
292 depicted in Figure 3. The $\delta^{15}\text{N}$ values obtained following two-point normalisation at higher
293 flow rates (1.7 to 2.0 ml min⁻¹) have lower associated error (1 σ between 0.5 to 0.8 ‰) and were

294 consistent with offline $\delta^{15}\text{N}_t$ values. For subsequent analyses, the carrier gas flow rate was set
295 to 1.7 ml min^{-1} , equating to a residence time in both the oxidation and reduction reactor of 2.2
296 s. The implementation of the two-point normalisation when analysing unknown samples to
297 correct against known $\delta^{15}\text{N}$ values for amino sugars helps to improve reproducibility and
298 precision of $\delta^{15}\text{N}$ values compared to routinely used single point anchoring techniques.⁴²

299 The observed oxidation reactor residence time in this study is comparable to that observed in a
300 previous study for amino acids (more than 2.1 s; flow rate of between $0.8\text{-}1.4 \text{ ml min}^{-1}$).²⁹
301 Residence time is the critical parameter to consider when optimising the instrumental set-up
302 for $\delta^{15}\text{N}$ values determinations, adjusting carrier gas flow rate to provide both accurate $\delta^{15}\text{N}$
303 value determinations with high precision ($1\sigma < 0.5 \text{ ‰}$ Pv/iPr ester derivatives of amino acids
304 and $1\sigma < 0.6 \text{ ‰}$ for alditol acetate derivatives of amino sugars in the present study).²⁹

305

306 **Linearity with enrichment**

307 Another important parameter to consider, particularly for analysis of ^{15}N -enriched analytes is
308 linearity across a wide range of $\delta^{15}\text{N}$ values. The relationship between known $\delta^{15}\text{N}_t$ value and
309 values determined by GC-C-IRMS ($\delta^{15}\text{N}_d$) was found to be linear ($R^2 = 0.9997$), as depicted in
310 Figure 4. Linearity across the ^{15}N -enrichment range is important to confirm prior to
311 application of two-point-normalisation to compound-specific $\delta^{15}\text{N}$ determination as this
312 criteria is assumed in the normalisation.⁴² Furthermore, across the enrichment range, the error
313 associated with $\delta^{15}\text{N}_d$ was less than 4 % of $\delta^{15}\text{N}$ value (and the relative error decreased with
314 increased $\delta^{15}\text{N}_t$). The relative error observed across the linear $\delta^{15}\text{N}$ scale was comparable with
315 other studies²⁹ and informed subsequent criteria for evaluating fit of the two-point
316 normalisation. This finding confirms the suitability of the GC-C-IRMS system used for the
317 analysis of ^{15}N -enriched amino sugars up to $469 \pm 3.1 \text{ ‰}$.

318

319 **Required analyte amount**

320 At $\delta^{15}\text{N}_t$ values up to 68.6 ‰, at low analyte amounts (below 30 nmol N), $\delta^{15}\text{N}_d$ values appeared
321 enriched compared to offline $\delta^{15}\text{N}_t$ values (Figure 5a). At higher analyte amount, (above 30
322 nmol N on column; 12 nmol N entering the ion source) $\delta^{15}\text{N}_d$ were both consistent with
323 measured $\delta^{15}\text{N}_t$ values for GlcN standards and were more precise ($1 \sigma < 0.7 \text{ ‰}$). Consistency
324 between replicates could be further increased at higher sample amounts ($1 \sigma < 0.5 \text{ ‰}$), however,
325 this must be balanced with chromatographic performance and oxidation and reduction reactor
326 capacity.

327 At enrichments above 92.7 ‰, low sample amount (15 nmol N on-column) resulted in depleted
328 $\delta^{15}\text{N}_d$ values compared to offline $\delta^{15}\text{N}_t$ values (for example 469 ‰ shown in Figure 5b).
329 Deviation from $\delta^{15}\text{N}_t$ increased with a greater proportion of ^{15}N in the analyte and consistency
330 between replicates was low ($1 \sigma > 5.0 \text{ ‰}$ for $92.7 \pm 0.95 \text{ ‰}$ and $1 \sigma > 50 \text{ ‰}$ for $469 \pm 3.1 \text{ ‰}$).
331 This observation was the same as for amino acids across a ^{15}N -enrichment range.³⁰ This has
332 been hypothesised to be due to the relative sizes of peaks in the m/z 28 and m/z 29 traces and
333 sensitivity of Faraday cups used in the IRMS.³⁰ The m/z 29 cup is 2 orders of magnitude more
334 sensitive than that measuring the m/z 28 ion abundance. The $\delta^{15}\text{N}$ values are subsequently
335 calculated by the data acquisition (Isodat NT) based on the relative areas of the m/z 28 and m/z
336 29 traces, and the relative contribution of these ions can be overestimated at low sample amount
337 (Figure 6). At low ^{15}N -enrichments, low analyte amount causes overestimation of m/z 29
338 abundance and deviation towards $\delta^{15}\text{N}$ enriched values, as shown in Figure 5a. At high ^{15}N -
339 enrichments and low analyte amount, m/z 28 ion abundance is overestimated due to high error
340 associated with this small peak, yielding depleted $\delta^{15}\text{N}_d$ values (Figure 5b). Based on these
341 results, it is recommended between 30 nmol and 50 nmol N are introduced on column for each

342 analyte. This range balances the requirement for sufficient analyte amount to ensure true and
343 precise $\delta^{15}\text{N}$ value determinations, whilst considering reactor life span and the need for optimal
344 chromatographic performance.

345 This is the first study investigating such sample requirements for amino sugars, although we
346 can compare our findings to the analyte amounts for amino acids. Importantly, the
347 recommended analyte amount determined in the present study is higher than that for natural
348 abundance $\delta^{15}\text{N}$ value determinations of amino acids at high accuracy (2 to 15 nmol N on
349 column for high precision $1\ \sigma < 0.5\ \text{‰}$)²⁹, but comparable to the amount recommended for
350 ^{15}N -enriched amino acids (100 nmol N on column).³⁰ With different instrumental set-ups,
351 analyte amount required for accurate determination of $\delta^{15}\text{N}$ values for amino acids varied,
352 therefore it is recommended required sample amount for amino sugars is determined when
353 using a different instrumental configuration.^{19,29,30}

354

355 **Memory effects**

356 The between-run memory effect was investigated using multiple run sequences (Table S2).
357 Table 1 shows the observed difference in $\delta^{15}\text{N}_d$ following analysis of an enriched standard.
358 There was no significant difference between $\delta^{15}\text{N}_d$ for the natural abundance standards run
359 between and after one and three $92.7 \pm 0.95\ \text{‰}$ standards ($P = 0.085$; Figure 7a and 7b). There
360 was, however, a significant difference in the determined $\delta^{15}\text{N}_d$ of natural abundance standards
361 before and after the analysis of the standard with a $\delta^{15}\text{N}_t$ value of $469 \pm 3.1\ \text{‰}$ (both one and
362 three enriched standards analysed; $P=0.007$ and $P < 0.001$ respectively; Figure 7c and 7d). The
363 natural abundance GlcN standard was $3.19\ \text{‰}$ and $16.7\ \text{‰}$ enriched compared to $\delta^{15}\text{N}_t$
364 following analysis of one and three enriched standards with a $\delta^{15}\text{N}_t$ of $469 \pm 3.1\ \text{‰}$, respectively,
365 and 5 and 7 subsequent analyses of the natural abundance standards were required to achieve

366 no significant difference compared to analyses before the 469 ± 3.1 ‰ standard; depicted in
367 Figures 7c and 7d. A significant difference was also observed between $\delta^{15}\text{N}_d$ of the 92.7 ± 0.95
368 ‰ GlcN standard following the analysis of the standard with a $\delta^{15}\text{N}_t$ value of 469 ± 3.1 ‰ in
369 triplicate ($P < 0.001$), with an enrichment on 47.6 ‰ in the standard analyses immediately after
370 the analysis of the ^{15}N enriched standards. Six analytical runs of the GlcN standard with a
371 $\delta^{15}\text{N}_t$ value of 92.7 ± 0.95 ‰ were required before no significant difference in $\delta^{15}\text{N}_d$ values was
372 achieved compared to analyses performed before the analysis of the ^{15}N enriched standards
373 (Figure 7e).

374 Within-run memory effects were also investigated, to determine if the analysis of an ^{15}N -
375 enriched analyte within the same run as a natural abundance analyte influenced $\delta^{15}\text{N}_d$. When
376 GlcN with a $\delta^{15}\text{N}_t$ value of 92.7 ± 0.95 ‰ was analysed in the same analytical run as natural
377 abundance GalN, there was no significant difference ($P > 0.3$) in $\delta^{15}\text{N}_d$ when compared to $\delta^{15}\text{N}_d$
378 of GalN in the same run as natural abundance GlcN. However, with a standard containing GlcN
379 with a $\delta^{15}\text{N}_t$ value of 469 ± 3.1 ‰, there was a significant difference in the $\delta^{15}\text{N}_d$ of GalN
380 compared to $\delta^{15}\text{N}_d$ of GalN eluting after natural abundance GlcN ($P < 0.01$). Memory effects,
381 with an enrichment of 5.6 ‰ and 3.1 ‰ for GlcN and GalN (both natural abundance)
382 respectively, were also observed in subsequent analyses and 5 repeated injections of the natural
383 abundance standard was required to confirm no significant difference in the $\delta^{15}\text{N}_d$ GlcN and
384 GalN before and after the analysis of the ^{15}N -enriched standard. When the enriched GluN ($\delta^{15}\text{N}_t$
385 value of 469 ± 3.1 ‰) was vented and the instrument returned to straight mode for the natural
386 abundance GalN, there were no significant memory effect was observed, indicating carry-over
387 effects were due the oxidation reactor. Furthermore, there was no significant difference ($P >$
388 0.1) in the $\delta^{15}\text{N}_d$ of MurN when analysed in the same run as GlcN standards with a $\delta^{15}\text{N}_t$ value
389 of 92.7 ± 0.95 ‰ and 469 ± 3.1 ‰ compared to a standard containing natural abundance GlcN,
390 indicating no memory effects for analytes eluting before an enriched analyte.

391 The observed between-run memory effect indicates analyses should be carried out in order of
392 increasing ^{15}N -enrichment to avoid these. Furthermore, when selecting ^{15}N -enriched standards
393 for use as part of the two-point normalisation, care should be taken to ensure there are no
394 between-run memory effects for subsequent standards used in the two-point normalisation and
395 subsequent sample analysis. To avoid within-run memory effects, which occur after a ^{15}N -
396 enriched analyte, it is recommended to vent column effluent at the time of elution of ^{15}N -
397 enriched analytes to accurately determine the $\delta^{15}\text{N}_d$ of the later eluting analytes of interest. This
398 is not necessary if all analytes of interest elute prior to the ^{15}N -enriched analyte.

399

400 **CONCLUSIONS**

401 The work described herein has assessed the suitability of alditol acetate derivatives of amino
402 sugars for GC-C-IRMS, negating the need to add additional N atoms, improving the accuracy
403 of $\delta^{15}\text{N}$ determinations. Following optimisation of GC and GC-C-IRMS conditions
404 particularly carrier gas flow, $\delta^{15}\text{N}$ values can be determined within $\pm 0.75\text{‰}$ ($1\sigma < 0.7\text{‰}$)
405 following correction using two-point normalisation. We have demonstrated $\delta^{15}\text{N}$
406 determinations are linear up to $469 \pm 3.1\text{‰}$ and the required sample amount is between 30 to
407 50 nmol N injected on-column to balance $\delta^{15}\text{N}$ determination accuracy alongside
408 chromatographic performance and oxidation reactor lifetime. At low on-column N, between
409 replicate error is high and determined $\delta^{15}\text{N}$ values systematically deviated from $\delta^{15}\text{N}_t$
410 depending on ^{15}N -abundance. Between- and within-run memory effects necessitate analysis in
411 order of increasing enrichment and venting column flow during the elution of the ^{15}N -enriched
412 component. Following the confirmation of the suitability of the derivatives for the GC-C-IRMS
413 determination of $\delta^{15}\text{N}$ values for ^{15}N -enriched amino sugars, this method can be applied to a

414 ¹⁵N-SIP approach to investigate the role of the bacterial and fungal communities in N-
415 assimilation in the environment.

416

417 **ACKNOWLEDGEMENTS**

418 This work was funded under the NERC DOMAINE (Characterisation of the nature, origins and
419 ecological significance of dissolved organic matter in freshwater ecosystems) Large Grant programme
420 which is supported by a Natural Environment Research Council Large Grant (split award codes
421 NE/K010689/1; NE/K010603/1; and NE/K010522/1). We thank the NERC for partial funding of the
422 mass spectrometry facilities at the Bristol node of the Life Sciences Mass Spectrometry Facility
423 (LSMSF; Contract No. R8/H10/63) and A. Kuhl and H. Gruszczynska for assistance with GC-C-IRMS
424 analyses. We thank Simon Hammann for assistance with EA-IRMS calculations and two anonymous
425 reviews for their suggestions for improvements to the manuscript.

426

427 **REFERENCES**

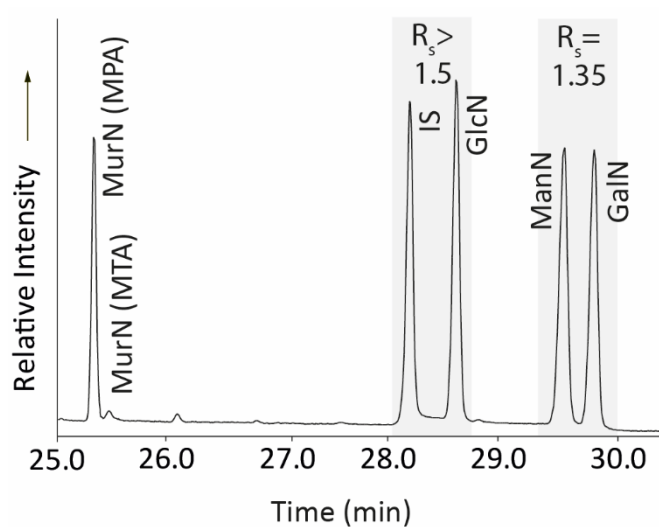
- 428 (1) Schulten, H.-R.; Schnitzer, M. The Chemistry of Soil Organic Nitrogen: A Review. *Biol.*
429 *Fertil. Soils* **1998**, *26* (1), 1–15.
- 430 (2) He, H.; Li, X. B.; Zhang, W.; Zhang, X. Differentiating the Dynamics of Native and Newly
431 Immobilized Amino Sugars in Soil Frequently Amended with Inorganic Nitrogen and Glucose.
432 *Eur. J. Soil Sci.* **2011**, *62* (1), 144–151.
- 433 (3) Glaser, B.; Turrión, M.-B.; Alef, K. Amino Sugars and Muramic Acid—biomarkers for Soil
434 Microbial Community Structure Analysis. *Soil Biol. Biochem.* **2004**, *36* (3), 399–407.
- 435 (4) He, H.; Zhang, W.; Zhang, X.; Xie, H.; Zhuang, J. Temporal Responses of Soil
436 Microorganisms to Substrate Addition as Indicated by Amino Sugar Differentiation. *Soil Biol.*
437 *Biochem.* **2011**, *43* (6), 1155–1161.
- 438 (5) Appuhn, A.; Joergensen, R. G. Microbial Colonisation of Roots as a Function of Plant Species.
439 *Soil Biol. Biochem.* **2006**, *38* (5), 1040–1051.
- 440 (6) Lin, Y.; Liu, J.; Hu, Y.; Song, X.; Zhao, Y. An Antioxidant Exopolysaccharide Devoid of pro-
441 Oxidant Activity Produced by the Soil Bacterium *Bordetella* Sp. B4. *Bioresour. Technol.*
442 **2012**, *124*, 245–251.
- 443 (7) Mikusová, K.; Mikus, M.; Besra, G. S.; Hancock, I.; Brennan, P. J. Biosynthesis of the
444 Linkage Region of the Mycobacterial Cell Wall. *J. Biol. Chem.* **1996**, *271* (13), 7820–7828.
- 445 (8) Rogers, H. J. (Howard J.; Perkins, H. R.; Ward, J. B. *Microbial Cell Walls and Membranes*;
446 Chapman and Hall, 1980.

- 447 (9) Liang, C.; Fujinuma, R.; Balser, T. C. Comparing PLFA and Amino Sugars for Microbial
448 Analysis in an Upper Michigan Old Growth Forest. *Soil Biol. Biochem.* **2008**, *40* (8), 2063–
449 2065.
- 450 (10) Van Groenigen, K.-J.; Six, J.; Harris, D.; Van Kessel, C. Elevated CO₂ Does Not Favor a
451 Fungal Decomposition Pathway. *Soil Biol. Biochem.* **2007**, *39* (8), 2168–2172.
- 452 (11) Indorf, C.; Dyckmans, J.; Khan, K. S.; Joergensen, R. G. Optimisation of Amino Sugar
453 Quantification by HPLC in Soil and Plant Hydrolysates. *Biol. Fertil. Soils* **2011**, *47* (4), 387–
454 396.
- 455 (12) Joergensen, R. G.; Mäder, P.; Fließbach, A. Long-Term Effects of Organic Farming on Fungal
456 and Bacterial Residues in Relation to Microbial Energy Metabolism. *Biol. Fertil. Soils* **2010**,
457 *46* (3), 303–307.
- 458 (13) Khan, K. S.; Mack, R.; Castillo, X.; Kaiser, M.; Joergensen, R. G. Microbial Biomass, Fungal
459 and Bacterial Residues, and Their Relationships to the Soil Organic Matter C/N/P/S Ratios.
460 *Geoderma* **2016**, *271*, 115–123.
- 461 (14) Liang, C.; Balser, T. C. Mass Spectrometric Characterization of Amino Sugar Aldononitrile
462 Acetate Derivatives Used for Isotope Enrichment Assessment of Microbial Residues. *Soil Biol.*
463 *Biochem.* **2010**, *42* (6), 904–909.
- 464 (15) Joergensen, R. G. Amino Sugars as Specific Indices for Fungal and Bacterial Residues in Soil.
465 *Biol. Fertil. Soils* **2018**, 1–10.
- 466 (16) Charteris, A. F.; Knowles, T. D. J.; Michaelides, K.; Evershed, R. P. Compound Specific
467 Amino Acid ¹⁵N Stable Isotope Probing of Nitrogen Assimilation by the Soil Microbial
468 Biomass Using Gas Chromatography/combustion/isotope Ratio Mass Spectrometry. *Rapid*
469 *Commun. Mass Spectrom.* **2016**, *30* (16), 1846–1856.
- 470 (17) Knowles, T. D. J.; Chadwick, D. R.; Bol, R.; Evershed, R. P. Tracing the Rate and Extent of N
471 and C Flow from ¹³C, ¹⁵N-Glycine and Glutamate into Individual de Novo Synthesised Soil
472 Amino Acids. *Org. Geochem.* **2010**, *41* (12), 1259–1268.
- 473 (18) He, H.; Xie, H.; Zhang, X. A Novel GC/MS Technique to Assess ¹⁵N and ¹³C Incorporation
474 into Soil Amino Sugars. *Soil Biol. Biochem.* **2006**, *38* (5), 1083–1091.
- 475 (19) Merritt, D. A.; Hayes, J. M. Nitrogen Isotopic Analyses by Isotope-Ratio-Monitoring Gas
476 Chromatography/mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **1994**, *5* (5), 387–397.
- 477 (20) Metges, C. C.; Petzke, K.-J.; Hennig, U. Gas Chromatography/Combustion/Isotope Ratio Mass
478 Spectrometric Comparison of N -Acetyl- and N-Pivaloyl Amino Acid Esters to Measure ¹⁵N
479 Isotopic Abundances in Physiological Samples: A Pilot Study on Amino Acid Synthesis in the
480 Upper Gastro-Intestinal Trac. *J. Mass Spectrom.* **1996**, *31* (4), 367–376.
- 481 (21) Redmile-Gordon, M. A.; Evershed, R. P.; Hirsch, P. R.; White, R. P.; Goulding, K. W. T. Soil
482 Organic Matter and the Extracellular Microbial Matrix Show Contrasting Responses to C and
483 N Availability. *Soil Biol. Biochem.* **2015**, *88*, 257–267.
- 484 (22) Sauheitl, L.; Glaser, B.; Weigelt, A. Advantages of Compound-Specific Stable Isotope
485 Measurements over Bulk Measurements in Studies on Plant Uptake of Intact Amino Acids.
486 *Rapid Commun. Mass Spectrom.* **2009**, *23* (20), 3333–3342.
- 487 (23) Zhang, X.; Amelung, W. Gas Chromatographic Determination of Muramic Acid,
488 Glucosamine, Mannosamine, and Galactosamine in Soils. *Soil Biol. Biochem.* **1996**, *28* (9),
489 1201–1206.
- 490 (24) Decock, C.; Denef, K.; Bodé, S.; Six, J.; Boeckx, P. Critical Assessment of the Applicability
491 of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry to Determine Amino

- 492 Sugar Dynamics in Soil. *Rapid Commun. Mass Spectrom.* **2009**, *23* (8), 1201–1211.
- 493 (25) Whiton, R. S.; Lau, P.; Morgan, S. L.; Gilbert, J.; Fox, A. Modifications in the Alditol Acetate
494 Method for Analysis of Muramic Acid and Other Neutral and Amino Sugars by Capillary Gas
495 Chromatography—mass Spectrometry with Selected Ion Monitoring. *J. Chromatogr. A* **1985**,
496 *347*, 109–120.
- 497 (26) Pettolino, F. A.; Walsh, C.; Fincher, G. B.; Bacic, A. Determining the Polysaccharide
498 Composition of Plant Cell Walls. *Nat. Protoc.* **2012**, *7* (9), 1590–1607.
- 499 (27) Metges, C. C.; Petzke, K. J. Measurement of $^{15}\text{N}/^{14}\text{N}$ Isotopic Composition in Individual
500 Plasma Free Amino Acids of Human Adults at Natural Abundance by Gas Chromatography–
501 Combustion Isotope Ratio Mass Spectrometry. *Anal. Biochem.* **1997**, *247* (1), 158–164.
- 502 (28) Meier-Augenstein, W. GC and IRMS Technology for ^{13}C and ^{15}N Analysis on Organic
503 Compounds and Related Gases. In *Handbook of Stable Isotope Analytical Techniques*;
504 Elsevier, 2004; pp 153–176.
- 505 (29) Chikaraishi, Y.; Takano, Y.; Ogawa, N. O.; Ohkouchi, N. Instrumental Optimization for
506 Compound-Specific Nitrogen Isotope Analysis of Amino Acids by Gas
507 Chromatography/combustion/isotope Ratio Mass Spectrometry. In *Earth, Life and Isotopes*;
508 Ohkouchi, N., Tayasu, I., Koba, K., Eds.; Kyoto University Press: Koyoto, 2010; pp 367–386.
- 509 (30) Knowles, T. D. J. Following the Fate of Proteinaceous Material in Soil Using a Compound-
510 Specific ^{13}C - and ^{15}N -Labelled Tracer Approach, University of Bristol, 2009.
- 511 (31) Sauheitl, L.; Glaser, B.; Weigelt, A. Uptake of Intact Amino Acids by Plants Depends on Soil
512 Amino Acid Concentrations. *Environ. Exp. Bot.* **2009**, *66* (2), 145–152.
- 513 (32) Styring, A. K.; Kuhl, A.; Knowles, T. D. J.; Fraser, R. A.; Bogaard, A.; Evershed, R. P.
514 Practical Considerations in the Determination of Compound-Specific Amino Acid $\delta^{15}\text{N}$ Values
515 in Animal and Plant Tissues by Gas Chromatography-Combustion-Isotope Ratio Mass
516 Spectrometry, Following Derivatisation to Their N-Acetylisopropyl Esters. *Rapid Commun.*
517 *Mass Spectrom.* **2012**, *26* (19), 2328–2334.
- 518 (33) Ohkouchi, N.; Chikaraishi, Y.; Close, H. G.; Fry, B.; Larsen, T.; Madigan, D. J.; McCarthy,
519 M. D.; McMahan, K. W.; Nagata, T.; Naito, Y. I.; et al. Advances in the Application of Amino
520 Acid Nitrogen Isotopic Analysis in Ecological and Biogeochemical Studies. *Org. Geochem.*
521 **2017**, *113*, 150–174.
- 522 (34) Nielsen, J. M.; Popp, B. N.; Winder, M. Meta-Analysis of Amino Acid Stable Nitrogen
523 Isotope Ratios for Estimating Trophic Position in Marine Organisms. *Oecologia* **2015**, *178* (3),
524 631–642.
- 525 (35) Paolini, M.; Ziller, L.; Laursen, K. H.; Husted, S.; Camin, F. Compound-Specific $\delta^{15}\text{N}$ and
526 $\delta^{13}\text{C}$ Analyses of Amino Acids for Potential Discrimination between Organically and
527 Conventionally Grown Wheat. *J. Agric. Food Chem.* **2015**, *63* (25), 5841–5850.
- 528 (36) Wong, W. W.; Hachey, D. L.; Zhang, S.; Clarke, L. L. Accuracy and Precision of Gas
529 Chromatography/combustion Isotope Ratio Mass Spectrometry for Stable Carbon Isotope
530 Ratio Measurements. *Rapid Commun. Mass Spectrom.* **1995**, *9* (11), 1007–1011.
- 531 (37) Guo, Z. K.; Luke, A. H.; Lee, W. P.; Schoeller, D. Compound-Specific Carbon Isotope Ratio
532 Determination of Enriched Cholesterol. *Anal. Chem.* **1993**, *65* (15), 1954–1959.
- 533 (38) Mottram, H. R.; Evershed, R. P. Practical Considerations in the Gas Chromatography/
534 Combustion/ Isotope Ratio Monitoring Mass Spectrometry of ^{13}C -Enriched Compounds:
535 Detection Limits and Carryover Effects. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2669–
536 2674.

- 537 (39) Carter, J. F.; Hill, J. C.; Doyle, S.; Lock, C. Results of Four Inter-Laboratory Comparisons
538 Provided by the Forensic Isotope Ratio Mass Spectrometry (FIRMS) Network. *Sci. Justice*
539 **2009**, *49* (2), 127–137.
- 540 (40) Parr, R. M.; Clements, S. A. Intercomparison of Enriched Stable Isotope Reference Materials
541 for Medical and Biological Studies. 1991.
- 542 (41) Boehlke, J. K.; Coplen, T. B. Interlaboratory Comparison of Reference Materials for Nitrogen-
543 Isotope-Ratio Measurements. *IAEA TECDOC* **1995**, 825, 51.
- 544 (42) Paul, D.; Skrzypek, G.; Fórizs, I. Normalization of Measured Stable Isotopic Compositions to
545 Isotope Reference Scales – a Review. *Rapid Commun. Mass Spectrom.* **2007**, *21* (18), 3006–
546 3014.
- 547 (43) Biermann, C. J.; McGinnis, G. D. *Analysis of Carbohydrates by GLC and MS*; CRC Press,
548 1989.
- 549 (44) Fox, A.; Krahmer, M.; Harrelson, D. Monitoring Muramic Acid in Air (after Alditol Acetate
550 Derivatization) Using a Gas Chromatograph-Ion Trap Tandem Mass Spectrometer. *J.*
551 *Microbiol. Methods* **1996**, *27* (2–3), 129–138.
- 552

553



554

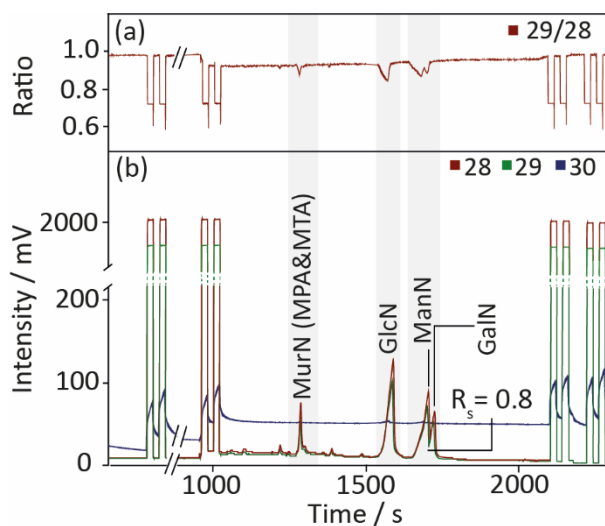
555

556 **Figure 1:** Typical GC chromatogram of alditol acetate derivatives of an amino sugar standard

557 between 25.0 to 30.5 min on the VF-23ms column. IS denotes internal standard. R_s denotes

558 resolution of the peaks.

559



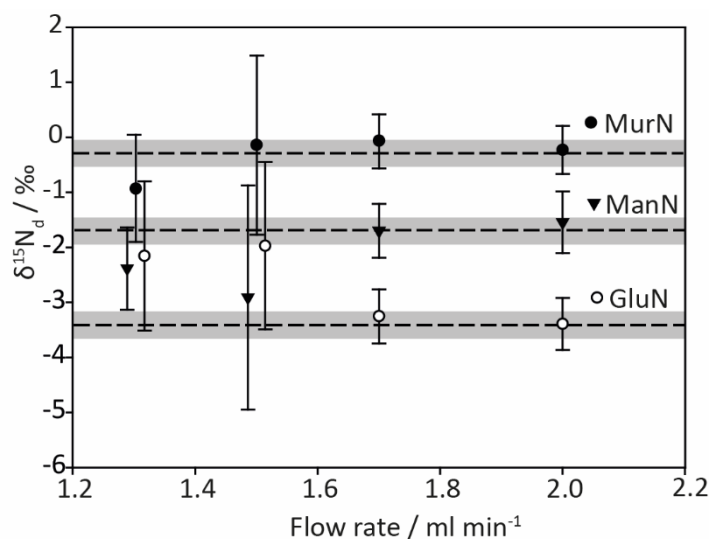
560

561

562 **Figure 2:** GC-C-IRMS chromatogram of alditol acetate derivatives of an amino sugar standard

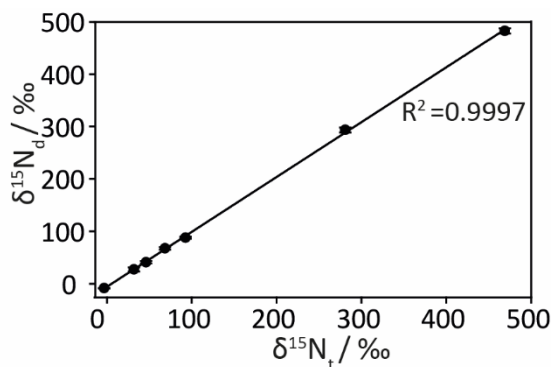
563 on a VF-23ms column, showing (a) the ratio m/z 29/28 used to generate $^{15}\text{N}/^{14}\text{N}$ isotope ratios

564 and (b) the ion current signals recorded for m/z 28, 29 and 30. R_s denotes resolution of the
565 peaks.



566

567 **Figure 3:** $\delta^{15}N_d$ values of alditol acetate derivatives of muramic acid (●), glucosamine (○) and
568 mannosamine (▼) at various carrier gas flow rates. The dashed line represents the $\delta^{15}N_t$ values of the
569 amino sugar standards independently determined by EA-IRMS and shaded box indicates $\pm 1\sigma$. Error
570 bars indicates $\pm 1\sigma$ ($n = 12$). 30 nmol of each standard was injected on-column.

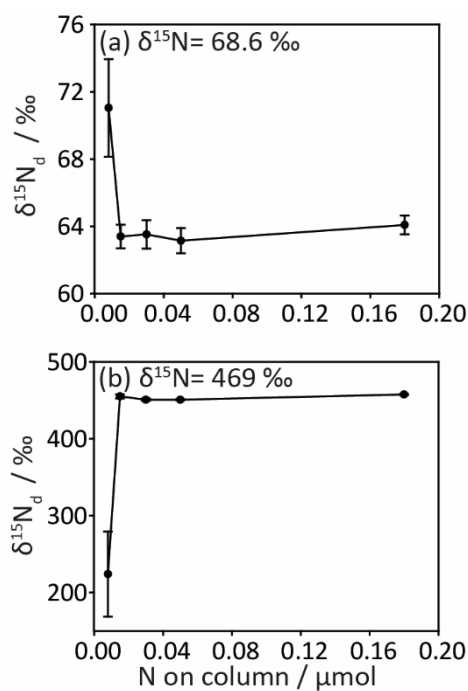


571

572

573 **Figure 4:** Linearity of $\delta^{15}N_d$ values (a) and relative error (b) with over a range of increasing ^{15}N -
574 enrichment. Each data point is the average of 12 repeat analyses with 30 nmol N injected on-column
575 and the solid line is a linear regression. Error bars are included for each point but they are same
576 magnitude as the size of the points.

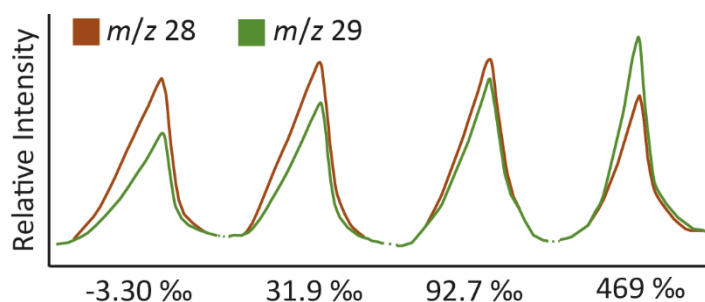
577



578

579 **Figure 5:** $\delta^{15}\text{N}_d$ values for GlcN standards with a $\delta^{15}\text{N}_t$ value of (a) 68.6 ± 0.55 ‰ and (b) 469 ± 3.1 ‰
 580 analysed at a range of analyte amounts injected on-column. Individual replicates are plotted as open
 581 circles and the mean is plotted as a filled circle connected with a solid line. Error bars are included for
 582 all points but some are so small that they appear the same magnitude as the size of the points.

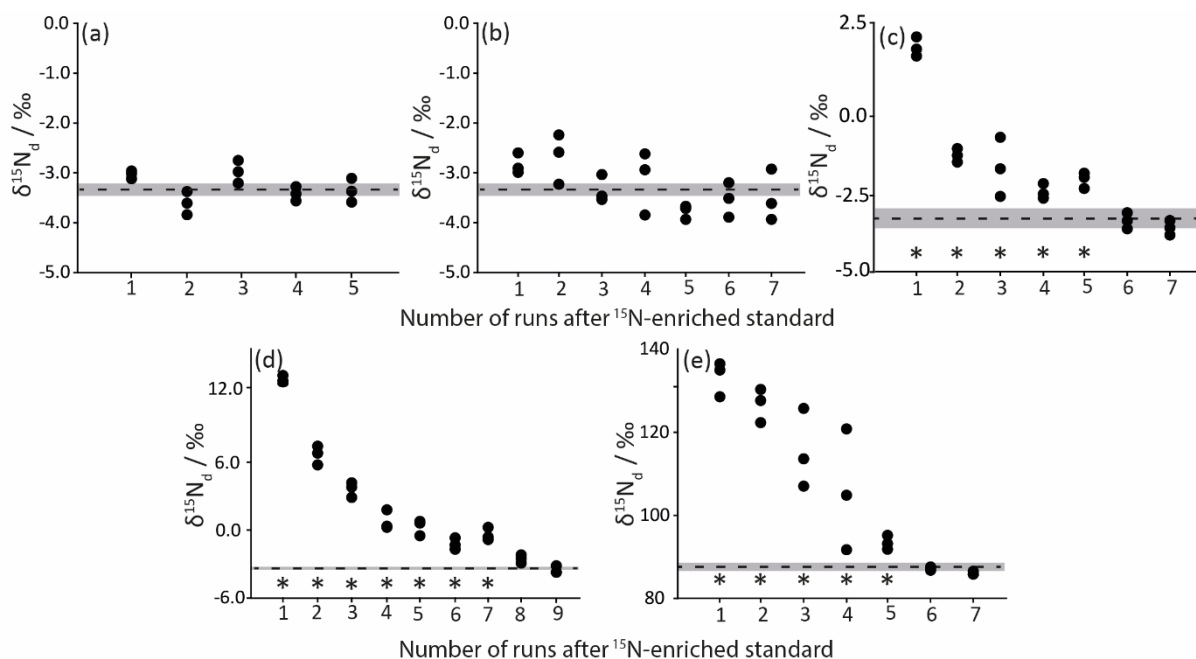
583



584

585 **Figure 6:** m/z 28 and 29 traces for glucosamine alditol acetate derivatives analysed at 0.015 μmol N on
 586 column and at a range of ^{15}N enrichments. The peaks drawn on the same scale.

587



588

589 **Figure 7:** Effect of enriched standards on $\delta^{15}\text{N}_d$ for values of GlcN standards at natural abundance after
 590 (a) 92.7 ‰ (n=1); (b) 92.7 ‰ (n=3); (c) 469 ‰ (n=1); (d) 469 ‰ (n=3) and for the 92.7 ‰ standard (e)
 591 after 469 ‰ (n=3). The dashed line is the $\delta^{15}\text{N}_t$ values for the natural abundance standard (a-d) and for
 592 the 92.7 ‰ standard in (e). The grey box represents $\pm 1\sigma$. • denotes $\delta^{15}\text{N}_d$ values from triplicate
 593 sequence runs. * denotes the average of the three analytical runs is significantly different to the $\delta^{15}\text{N}_t$
 594 (paired t-test; significance level set at $P < 0.05$).

595

596 **Table 1:** Observed change in $\delta^{15}\text{N}_d$ values after analysis of ^{15}N -enriched GlcN standard for inter-run
 597 memory effects and significance of this difference (determined using a paired t-test comparing analyses
 598 before and immediately after the analysis of an enriched standard). * denotes a significant P value. The
 599 significance level was set at $P < 0.05$.

| Analytical Sequence | $\Delta^{15}\text{N}_d / \text{‰}$ | P-Value |
|-------------------------------|------------------------------------|---------|
| 92.7 \pm 0.95 ‰ (n=1) to NA | +0.17 | 0.263 |
| 92.7 \pm 0.95 ‰ (n=3) to NA | +0.18 | 0.085 |
| 469 \pm 3.1 ‰ (n=1) to NA | +5.72 | 0.007 * |

| | | |
|---|-------|-----------|
| $469 \pm 3.1 \text{ ‰ (n=3) to NA}$ | +16.7 | < 0.001 * |
| $469 \pm 3.1 \text{ ‰ (n=3) to } 92.7 \pm 0.95$ | +40.0 | < 0.001 * |

600

601

602

603

604

605

606

607

608

609

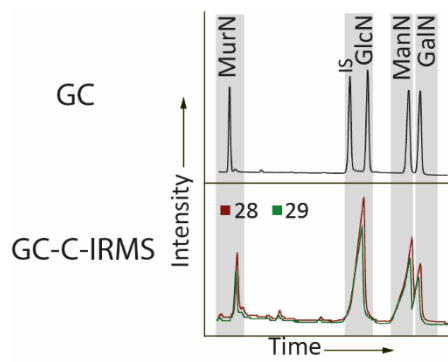
610

611

612

For TOC only

613



614