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

Published: 05/03/2019

Peer reviewed version

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

Michaela K. Reay,[a] Timothy D.J. Knowles,[b] Davey L. Jones,[c, d] Richard P. Evershed [a*]

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

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

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

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

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

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

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

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

community.[16] Furthermore, it is possible to elucidate differences in relative importance of N transformation pathways in the soil N cycle.[3,15–17]

A ^{15}N-SIP approach has applied been to amino sugars, determining ^{15}N-incorporation into soil amino sugars using electron ionisation (EI) gas chromatography-mass spectrometry (GC-MS).[2,4,18] These investigations have revealed the differing temporal response within the microbial community to substrate addition and the differing stability of amino sugar residues.[2,4] The incorporation of ^{15}N into amino sugars was determined following acid hydrolysis of parent amino polysaccharides and aldononitrile derivatisation, based on selected ion monitoring of *m*/*z* 98.[18] A major drawback of this technique is that isotopic determinations using GC-MS require high ^{15}N-enrichments and therefore high N application rates which can perturb the system and potentially result in ^{15}N isotopic discrimination.[16] Furthermore, this technique employs a N-containing derivative group, which adds substantial uncertainty to N-isotope determinations (see below).

A preferred approach to determining nitrogen isotopic compositions of amino sugars would be to use gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). This is a much more sensitive (0.5-2.0 ‰; 0.0002 to 0.0008 atom %) method for the determination of δ^{15}N values of N-containing compounds than GC-MS and can achieve far higher levels of high precision and accuracy. Its use would mean that ^{15}N-SIP experiments could use low-level substrate additions, thereby minimising system perturbations and discrimination.[16,19,20] This technique has already been applied to amino acid δ^{15}N value determinations, providing previously inaccessible levels of detail regarding the rate of N and C transfers within amino acids and soil protein fraction[17,21], amino acid uptake dynamics of plants[22] and evidence for microbial N assimilation pathways and differences in microbial processing of N fertiliser.[16] Hence, used in this way, the ^{15}N-SIP approach can provide hitherto unobtainable insights into

N-cycling through the amino sugar pool despite the complex nature of soil organic matter (SOM) and the soil N-cycle.[16,17]

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

The suitability of the GC-C-IRMS method for δ^{15}N value determinations of ^{15}N-enriched amino sugars must be established prior to application to ^{15}N-tracer studies. The suitability of GC-C-IRMS for the analysis of amino acids has previously been established over a range of ^{15}N-enrichments.[16,17,19,27–30] Instrument parameters, such as optimal carrier gas flow rate, which has a direct influence on residence time of analytes in oxidation and reduction reactors of the GC-C-IRMS interface, have been investigated to ensure accurate determination of δ^{15}N values of amino acids.[29] Furthermore, required sample amount for accurate and precise δ^{15}N value determinations have been tested, and found to range from 2 to 100 nmol N on column.[17,29,30] Importantly, for analysis of ^{15}N-enriched compounds, linearity with ^{15}N-enrichment and memory effects (both between- and within-analytical runs) must be considered.[16,17,30] Such assessments of GC-C-IRMS have led to the development of robust methods for the accurate determination of δ^{15}N values of amino acids over a range of ^{15}N-enrichments, allowing

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

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

EXPERIMENTAL

Reagents and standards

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

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

Derivatisation reagents (sodium borohydride ($NaBH_4$), acetic acid and acetic anhydride) were supplied by Sigma-Aldrich (Steinheim, Germany). All solvents were HPLC grade and supplied by Rathburn Chemicals Ltd. (Walkerburn, UK), double-distilled water (DDW) was produced using a Bibby Aquatron still.

Amino sugar derivatisation

The alditol acetate derivatisation method for amino sugars was adapted from that reported by Whiton et al.[25] Briefly, dried amino sugar residues were reduced with sodium borohydride (500 µl, 100 mg ml^{-1} in DDW) and heated (60 °C, 2.5 h). Excess sodium borohydride was destroyed by the addition of acetic acid-methanol (2 ml; 1:200 *v*/*v*) and evaporated to dryness using a stream of N_2 at 60 °C. This was repeated four times to ensure the complete destruction of residual sodium borohydride. Acetylation was performed by addition of 500 µl of acetic anhydride and heating (100 °C, 2.5 h). The reaction was quenched by freezing at -20 °C (15 min) and acetic anhydride subsequently destroyed by the dropwise addition of 2.5 ml of double-distilled water. The alditol acetate derivatives of amino sugars were extracted with chloroform (3 x 2 ml), combined and dried under a gentle stream of N_2 at 40 °C. Residues were re-dissolved in 1.5 ml chloroform and the organic phase washed with double-distilled water (2 x 1.5 ml) to remove residual acetic acid produced during acetic anhydride hydrolysis. The alditol acetate derivatives were dissolved in ethyl acetate for subsequent analysis by GC-FID and GC-C-IRMS.

Instrumental analyses

EA-IRMS

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

GC-FID

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

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

GC-MS

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

GC-C-IRMS

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

209 The suitability of GC-C-IRMS for $\delta^{15}N$ value determinations of alditol acetate derivatives of
210 amino sugars in terms of linearity across a ^{15}N-enrichment range was investigated using six
211 ^{15}N-GlcN standards (-3.31±0.24 to 469 ±3.1 ‰; 30 nmol N on column; n=6). Sample
212 requirements for consistent $\delta^{15}N$ value determinations for alditol acetate derivatives of amino
213 sugars were assessed using the same ^{15}N-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 ^{15}N-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 ^{15}N-
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

Calculations

Two-point normalisation

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

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

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

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

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

RESULTS AND DISCUSSION

Derivatisation optimisation and chromatographic separation

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

Mass spectral identification

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

Variation in precision with column flow

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

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

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

Linearity with enrichment

Another important parameter to consider, particularly for analysis of ^{15}N-enriched analytes is linearity across a wide range of $\delta^{15}N$ values. The relationship between known $\delta^{15}N_t$ value and values determined by GC-C-IRMS ($\delta^{15}N_d$) was found to be linear ($R^2 = 0.9997$), as depicted in Figure 4. Linearity across the ^{15}N-enrichement range is important to confirm prior to application of two-point-normalisation to compound-specific $\delta^{15}N$ determination as this criteria is assumed in the normalisaiton.[42] Furthermore, across the enrichment range, the error associated with $\delta^{15}N_d$ was less than 4 % of $\delta^{15}N$ value (and the relative error decreased with increased $\delta^{15}N_t$). The relative error observed across the linear $\delta^{15}N$ scale was comparable with other studies[29] and informed subsequent criteria for evaluating fit of the two-point normalisation. This finding confirms the suitability of the GC-C-IRMS system used for the analysis of ^{15}N-enriched amino sugars up to 469 ±3.1 ‰.

Required analyte amount

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

At enrichments above 92.7 ‰, low sample amount (15 nmol N on-column) resulted in depleted $\delta^{15}N_d$ values compared to offline $\delta^{15}N_t$ values (for example 469 ‰ shown in Figure 5b). Deviation from $\delta^{15}N_t$ increased with a greater proportion of ^{15}N in the analyte and consistency between replicates was low ($1\ \sigma > 5.0$ ‰ for 92. 7 ±0.95 ‰ and $1\ \sigma > 50$ ‰ for 469 ±3.1 ‰). This observation was the same as for amino acids across a ^{15}N-enrichment range.[30] This has been hypothesised to be due to the relative sizes of peaks in the *m*/*z* 28 and *m*/*z* 29 traces and sensitivity of Faraday cups used in the IRMS.[30] The *m*/*z* 29 cup is 2 orders of magnitude more sensitive than that measuring the *m*/*z* 28 ion abundance. The $\delta^{15}N$ values are subsequently calculated by the data acquisition (Isodat NT) based on the relative areas of the *m*/*z* 28 and *m*/*z* 29 traces, and the relative contribution of these ions can be overestimated at low sample amount (Figure 6). At low ^{15}N-enrichments, low analyte amount causes overestimation of *m*/*z* 29 abundance and deviation towards $\delta^{15}N$ enriched values, as shown in Figure 5a. At high ^{15}N-enrichments and low analyte amount, *m*/*z* 28 ion abundance is overestimated due to high error associated with this small peak, yielding depleted $\delta^{15}N_d$ values (Figure 5b). Based on these 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 $\delta^{15}N$ 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 $\delta^{15}N$ value determinations of amino acids at high accuracy (2 to 15 nmol N on
349 column for high precision 1 σ < 0.5 ‰)[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 $\delta^{15}N$ 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 $\delta^{15}N_d$ following analysis of an enriched standard.
358 There was no significant difference between $\delta^{15}N_d$ for the natural abundance standards run
359 between and after one and three 92.7 ±0.95 ‰ standards (P = 0.085; Figure 7a and 7b). There
360 was, however, a significant difference in the determined $\delta^{15}N_d$ of natural abundance standards
361 before and after the analysis of the standard with a $\delta^{15}N_t$ value of 469 ±3.1 ‰ (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 ‰ and 16.7 ‰ enriched compared to $\delta^{15}N_t$
364 following analysis of one and three enriched standards with a $\delta^{15}N_t$ of 469 ±3.1 ‰, 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 $\delta^{15}N_d$ of the 92.7 ±0.95
368 ‰ GlcN standard following the analysis of the standard with a $\delta^{15}N_t$ 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 $\delta^{15}N_t$ value of 92.7 ±0.95 ‰ were required before no significant difference in $\delta^{15}N_d$ 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 $\delta^{15}N_d$. When
376 GlcN with a $\delta^{15}N_t$ 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 $\delta^{15}N_d$ when compared to $\delta^{15}N_d$
378 of GalN in the same run as natural abundance GlcN. However, with a standard containing GlcN
379 with a $\delta^{15}N_t$ value of 469 ±3.1 ‰, there was a significant difference in the $\delta^{15}N_d$ of GalN
380 compared to $\delta^{15}N_d$ 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 $\delta^{15}N_d$ GlcN and
384 GalN before and after the analysis of the ^{15}N-enriched standard. When the enriched GluN ($\delta^{15}N_t$
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 $\delta^{15}N_d$ of MurN when analysed in the same run as GlcN standards with a $\delta^{15}N_t$ 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.

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

CONCLUSIONS

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

414 ^{15}N-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.

(9) Liang, C.; Fujinuma, R.; Balser, T. C. Comparing PLFA and Amino Sugars for Microbial Analysis in an Upper Michigan Old Growth Forest. *Soil Biol. Biochem.* **2008**, *40* (8), 2063–2065.

(10) Van Groenigen, K.-J.; Six, J.; Harris, D.; Van Kessel, C. Elevated CO_2 Does Not Favor a Fungal Decomposition Pathway. *Soil Biol. Biochem.* **2007**, *39* (8), 2168–2172.

(11) Indorf, C.; Dyckmans, J.; Khan, K. S.; Joergensen, R. G. Optimisation of Amino Sugar Quantification by HPLC in Soil and Plant Hydrolysates. *Biol. Fertil. Soils* **2011**, *47* (4), 387–396.

(12) Joergensen, R. G.; Mäder, P.; Fließbach, A. Long-Term Effects of Organic Farming on Fungal and Bacterial Residues in Relation to Microbial Energy Metabolism. *Biol. Fertil. Soils* **2010**, *46* (3), 303–307.

(13) Khan, K. S.; Mack, R.; Castillo, X.; Kaiser, M.; Joergensen, R. G. Microbial Biomass, Fungal and Bacterial Residues, and Their Relationships to the Soil Organic Matter C/N/P/S Ratios. *Geoderma* **2016**, *271*, 115–123.

(14) Liang, C.; Balser, T. C. Mass Spectrometric Characterization of Amino Sugar Aldononitrile Acetate Derivatives Used for Isotope Enrichment Assessment of Microbial Residues. *Soil Biol. Biochem.* **2010**, *42* (6), 904–909.

(15) Joergensen, R. G. Amino Sugars as Specific Indices for Fungal and Bacterial Residues in Soil. *Biol. Fertil. Soils* **2018**, 1–10.

(16) Charteris, A. F.; Knowles, T. D. J.; Michaelides, K.; Evershed, R. P. Compound Specific Amino Acid ^{15}N Stable Isotope Probing of Nitrogen Assimilation by the Soil Microbial Biomass Using Gas Chromatography/combustion/isotope Ratio Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2016**, *30* (16), 1846–1856.

(17) Knowles, T. D. J.; Chadwick, D. R.; Bol, R.; Evershed, R. P. Tracing the Rate and Extent of N and C Flow from ^{13}C,^{15}N-Glycine and Glutamate into Individual de Novo Synthesised Soil Amino Acids. *Org. Geochem.* **2010**, *41* (12), 1259–1268.

(18) He, H.; Xie, H.; Zhang, X. A Novel GC/MS Technique to Assess 15N and 13C Incorporation into Soil Amino Sugars. *Soil Biol. Biochem.* **2006**, *38* (5), 1083–1091.

(19) Merritt, D. A.; Hayes, J. M. Nitrogen Isotopic Analyses by Isotope-Ratio-Monitoring Gas Chromatography/mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **1994**, *5* (5), 387–397.

(20) Metges, C. C.; Petzke, K.-J.; Hennig, U. Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometric Comparison of N -Acetyl- and N-Pivaloyl Amino Acid Esters to Measure ^{15}N Isotopic Abundances in Physiological Samples: A Pilot Study on Amino Acid Synthesis in the Upper Gastro-Intestinal Trac. *J. Mass Spectrom.* **1996**, *31* (4), 367–376.

(21) Redmile-Gordon, M. A.; Evershed, R. P.; Hirsch, P. R.; White, R. P.; Goulding, K. W. T. Soil Organic Matter and the Extracellular Microbial Matrix Show Contrasting Responses to C and N Availability. *Soil Biol. Biochem.* **2015**, *88*, 257–267.

(22) Sauheitl, L.; Glaser, B.; Weigelt, A. Advantages of Compound-Specific Stable Isotope Measurements over Bulk Measurements in Studies on Plant Uptake of Intact Amino Acids. *Rapid Commun. Mass Spectrom.* **2009**, *23* (20), 3333–3342.

(23) Zhang, X.; Amelung, W. Gas Chromatographic Determination of Muramic Acid, Glucosamine, Mannosamine, and Galactosamine in Soils. *Soil Biol. Biochem.* **1996**, *28* (9), 1201–1206.

(24) Decock, C.; Denef, K.; Bodé, S.; Six, J.; Boeckx, P. Critical Assessment of the Applicability of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry to Determine Amino

Sugar Dynamics in Soil. *Rapid Commun. Mass Spectrom.* **2009**, *23* (8), 1201–1211.

(25) Whiton, R. S.; Lau, P.; Morgan, S. L.; Gilbart, J.; Fox, A. Modifications in the Alditol Acetate Method for Analysis of Muramic Acid and Other Neutral and Amino Sugars by Capillary Gas Chromatography—mass Spectrometry with Selected Ion Monitoring. *J. Chromatogr. A* **1985**, *347*, 109–120.

(26) Pettolino, F. A.; Walsh, C.; Fincher, G. B.; Bacic, A. Determining the Polysaccharide Composition of Plant Cell Walls. *Nat. Protoc.* **2012**, *7* (9), 1590–1607.

(27) Metges, C. C.; Petzke, K. J. Measurement of15N/14N Isotopic Composition in Individual Plasma Free Amino Acids of Human Adults at Natural Abundance by Gas Chromatography–Combustion Isotope Ratio Mass Spectrometry. *Anal. Biochem.* **1997**, *247* (1), 158–164.

(28) Meier-Augenstein, W. GC and IRMS Technology for ^{13}C and ^{15}N Analysis on Organic Compounds and Related Gases. In *Handbook of Stable Isotope Analytical Techniques*; Elsevier, 2004; pp 153–176.

(29) Chikaraishi, Y.; Takano, Y.; Ogawa, N. O.; Ohkouchi, N. Instrumental Optimization for Compound-Specific Nitrogen Isotope Analysis of Amino Acids by Gas Chromatography/combustion/isotope Ratio Mass Spectrometry. In *Earth, Life and Isotopes*; Ohkouchi, N., Tayasu, I., Koba, K., Eds.; Kyoto University Press: Koyoto, 2010; pp 367–386.

(30) Knowles, T. D. J. Following the Fate of Proteinaceous Material in Soil Using a Compound-Specific ^{13}C- and ^{15}N-Labelled Tracer Approach, University of Bristol, 2009.

(31) Sauheitl, L.; Glaser, B.; Weigelt, A. Uptake of Intact Amino Acids by Plants Depends on Soil Amino Acid Concentrations. *Environ. Exp. Bot.* **2009**, *66* (2), 145–152.

(32) Styring, A. K.; Kuhl, A.; Knowles, T. D. J.; Fraser, R. A.; Bogaard, A.; Evershed, R. P. Practical Considerations in the Determination of Compound-Specific Amino Acid $\delta^{15}N$ Values in Animal and Plant Tissues by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry, Following Derivatisation to Their N-Acetylisopropyl Esters. *Rapid Commun. Mass Spectrom.* **2012**, *26* (19), 2328–2334.

(33) Ohkouchi, N.; Chikaraishi, Y.; Close, H. G.; Fry, B.; Larsen, T.; Madigan, D. J.; McCarthy, M. D.; McMahon, K. W.; Nagata, T.; Naito, Y. I.; et al. Advances in the Application of Amino Acid Nitrogen Isotopic Analysis in Ecological and Biogeochemical Studies. *Org. Geochem.* **2017**, *113*, 150–174.

(34) Nielsen, J. M.; Popp, B. N.; Winder, M. Meta-Analysis of Amino Acid Stable Nitrogen Isotope Ratios for Estimating Trophic Position in Marine Organisms. *Oecologia* **2015**, *178* (3), 631–642.

(35) Paolini, M.; Ziller, L.; Laursen, K. H.; Husted, S.; Camin, F. Compound-Specific $\delta^{15}N$ and $\delta^{13}C$ Analyses of Amino Acids for Potential Discrimination between Organically and Conventionally Grown Wheat. *J. Agric. Food Chem.* **2015**, *63* (25), 5841–5850.

(36) Wong, W. W.; Hachey, D. L.; Zhang, S.; Clarke, L. L. Accuracy and Precision of Gas Chromatography/combustion Isotope Ratio Mass Spectrometry for Stable Carbon Isotope Ratio Measurements. *Rapid Commun. Mass Spectrom.* **1995**, *9* (11), 1007–1011.

(37) Guo, Z. K.; Luke, A. H.; Lee, W. P.; Schoeller, D. Compound-Specific Carbon Isotope Ratio Determination of Enriched Cholesterol. *Anal. Chem.* **1993**, *65* (15), 1954–1959.

(38) Mottram, H. R.; Evershed, R. P. Practical Considerations in the Gas Chromatography/ Combustion/ Isotope Ratio Monitoring Mass Spectrometry of ^{13}C-Enriched Compounds: Detection Limits and Carryover Effects. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2669–2674.

(39) Carter, J. F.; Hill, J. C.; Doyle, S.; Lock, C. Results of Four Inter-Laboratory Comparisons Provided by the Forensic Isotope Ratio Mass Spectrometry (FIRMS) Network. *Sci. Justice* **2009**, *49* (2), 127–137.

(40) Parr, R. M.; Clements, S. A. Intercomparison of Enriched Stable Isotope Reference Materials for Medical and Biological Studies. 1991.

(41) Boehlke, J. K.; Coplen, T. B. Interlaboratory Comparison of Reference Materials for Nitrogen-Isotope-Ratio Measurements. *IAEA TECDOC* **1995**, *825*, 51.

(42) Paul, D.; Skrzypek, G.; Fórizs, I. Normalization of Measured Stable Isotopic Compositions to Isotope Reference Scales – a Review. *Rapid Commun. Mass Spectrom.* **2007**, *21* (18), 3006–3014.

(43) Biermann, C. J.; McGinnis, G. D. *Analysis of Carbohydrates by GLC and MS*; CRC Press, 1989.

(44) Fox, A.; Krahmer, M.; Harrelson, D. Monitoring Muramic Acid in Air (after Alditol Acetate Derivatization) Using a Gas Chromatograph-Ion Trap Tandem Mass Spectrometer. *J. Microbiol. Methods* **1996**, *27* (2–3), 129–138.

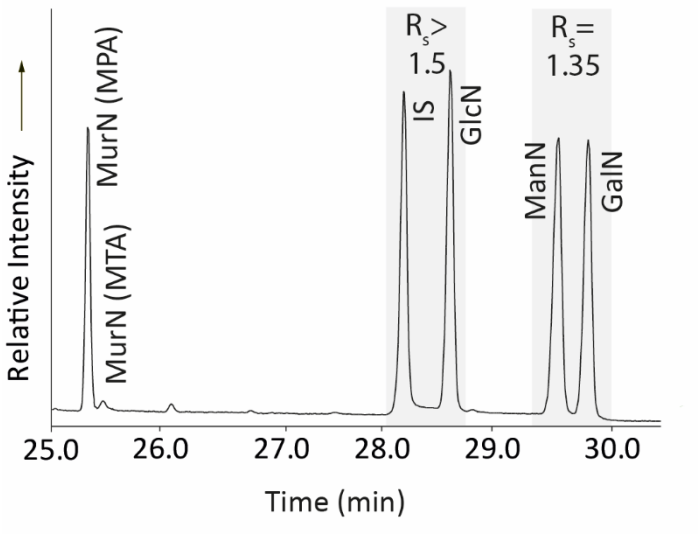


Figure 1: Typical GC chromatogram of alditol acetate derivatives of an amino sugar standard between 25.0 to 30.5 min on the VF-23ms column. IS denotes internal standard. R_s denotes resolution of the peaks.

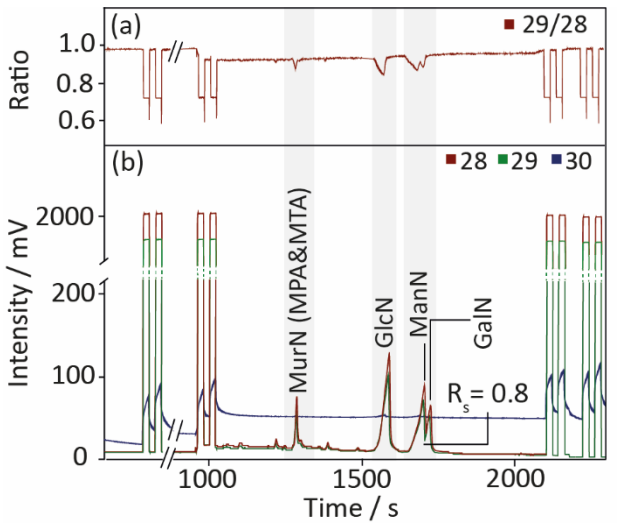
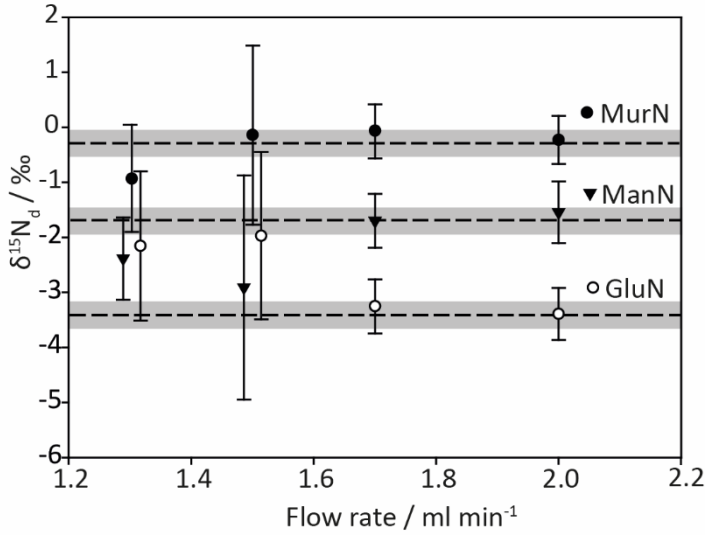


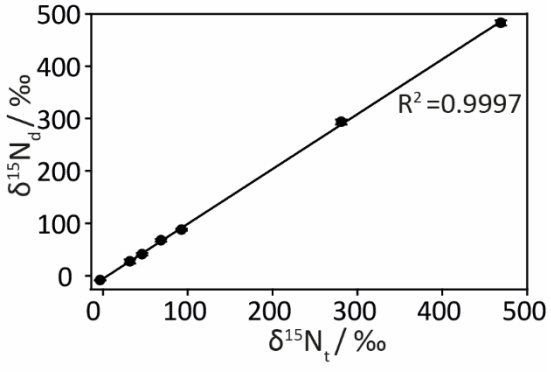
Figure 2: GC-C-IRMS chromatogram of alditol acetate derivatives of an amino sugar standard on a VF-23ms column, showing (a) the ratio *m*/*z* 29/28 used to generate $^{15}N/^{14}N$ isotope ratios

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 ± 1σ. Error
570 bars indicates ± 1σ (n = 12). 30 nmol of each standard was injected on-column.

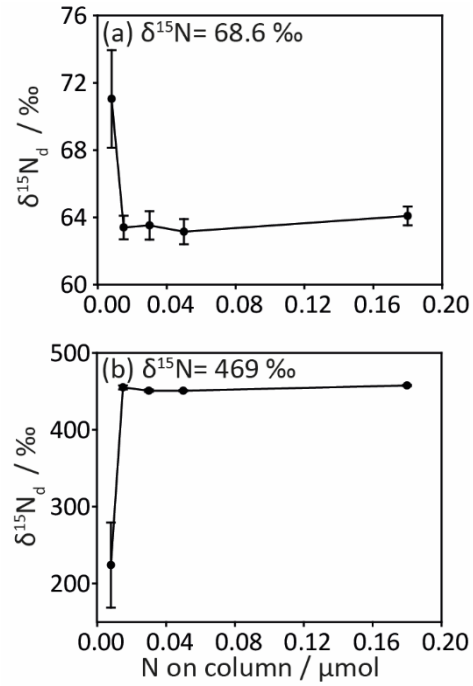


571

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.

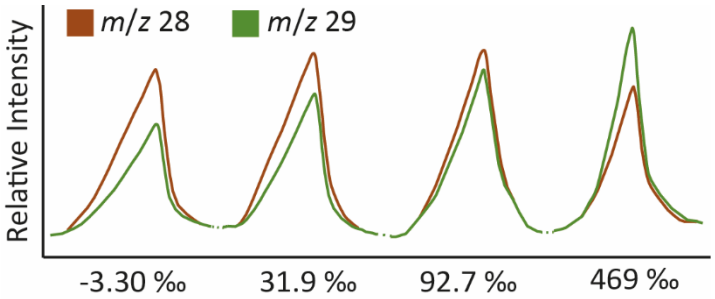
577



578

579 **Figure 5:** $\delta^{15}N_d$ values for GlcN standards with a $\delta^{15}N_t$ 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.

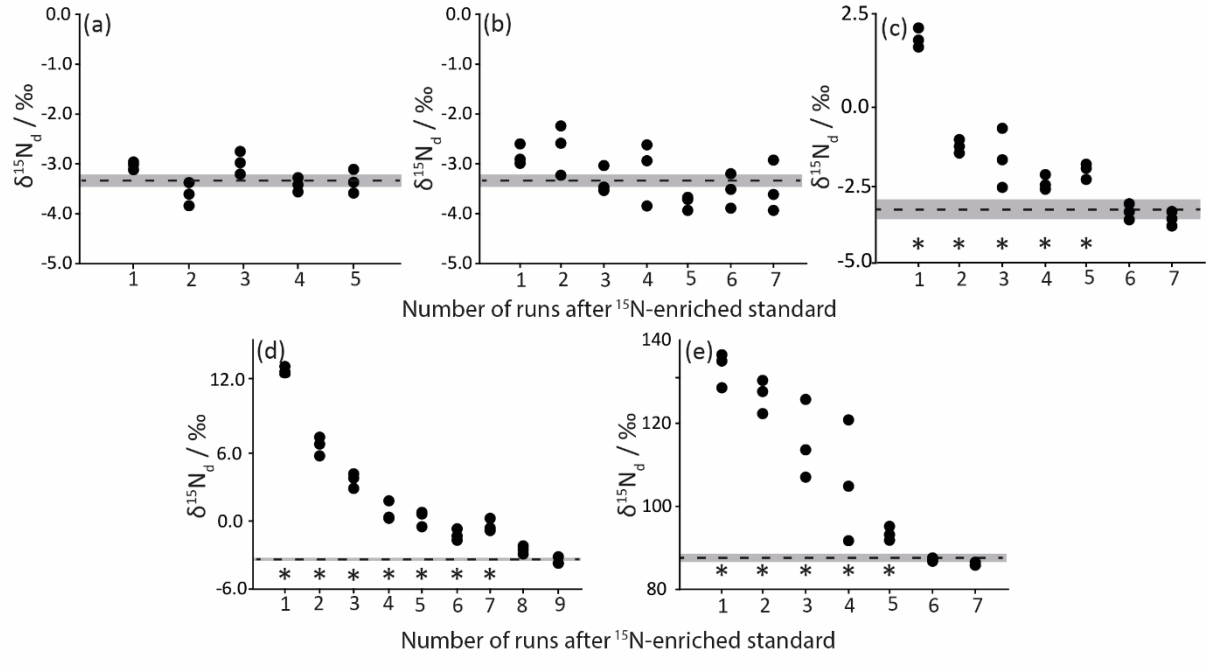
583



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 $\delta^{15}N_d$ 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 $\delta^{15}N_t$ values for the natural abundance standard (a-d) and for
592 the 92.7 ‰ standard in (e). The grey box represents ± 1σ. ● denotes $\delta^{15}N_d$ values from triplicate
593 sequence runs. * denotes the average of the three analytical runs is significantly different to the $\delta^{15}N_t$
594 (paired t-test; significance level set at $P < 0.05$).

595

596 **Table 1:** Observed change in $\delta^{15}N_d$ 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	$\Delta^{15}N_d$ / ‰	P-Value
92.7 ±0.95 ‰ (n=1) to NA	+0.17	0.263
92.7 ±0.95 ‰ (n=3) to NA	+0.18	0.085
469 ±3.1 ‰ (n=1) to NA	+5.72	0.007 *

469 ± 3.1 ‰ (n=3) to NA	+16.7	< 0.001 *
469 ± 3.1‰ (n=3) to 92.7 ±0.95	+40.0	< 0.001 *

For TOC only