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Improved $\delta^{13}C$ analysis of amino sugars in soil by ion chromatography–oxidation–isotope ratio mass spectrometry

Michaela A. Dippold[1,2,3]*, Stefanie Boesel[2], Anna Gunina[1,3], Yakov Kuzyakov[3,4] and Bruno Glaser[2]

[1]Department of Agroecosystem Research, BayCEER, University of Bayreuth, Germany
[2]Department of Soil Biochemistry, Institute of Agricultural and Nutritional Science, Martin-Luther University Halle-Wittenberg, Germany
[3]Department of Agricultural Soil Science, University of Göttingen, Germany
[4]Department of Soil Science of Temperate Ecosystems, Georg-August University of Götingen, Germany

RATIONALE: Amino sugars build up microbial cell walls and are important components of soil organic matter. To evaluate their sources and turnover, $\delta^{13}C$ analysis of soil-derived amino sugars by liquid chromatography was recently suggested. However, amino sugar $\delta^{13}C$ determination remains challenging due to (1) a strong matrix effect, (2) CO_2-binding by alkaline eluents, and (3) strongly different chromatographic behavior and concentrations of basic and acidic amino sugars. To overcome these difficulties we established an ion chromatography–oxidation–isotope ratio mass spectrometry method to improve and facilitate soil amino sugar analysis.
METHODS: After acid hydrolysis of soil samples, the extract was purified from salts and other components impeding chromatographic resolution. The amino sugar concentrations and $\delta^{13}C$ values were determined by coupling an ion chromatograph to an isotope ratio mass spectrometer. The accuracy and precision of quantification and $\delta^{13}C$ determination were assessed.
RESULTS: Internal standards enabled correction for losses during analysis, with a relative standard deviation <6%. The higher magnitude peaks of basic than of acidic amino sugars required an amount-dependent correction of $\delta^{13}C$ values. This correction improved the accuracy of the determination of $\delta^{13}C$ values to <1.5‰ and the precision to <0.5‰ for basic and acidic amino sugars in a single run.
CONCLUSIONS: This method enables parallel quantification and $\delta^{13}C$ determination of basic and acidic amino sugars in a single chromatogram due to the advantages of coupling an ion chromatograph to the isotope ratio mass spectrometer. Small adjustments of sample amount and injection volume are necessary to optimize precision and accuracy for individual soils. Copyright © 2014 John Wiley & Sons, Ltd.

The great relevance of microbial compounds within soil organic matter (SOM) has become evident within the last decade. Microbial cell wall compounds seem to be the most relevant microbial-derived compound class within slow cycling SOM, as they are (1) highly polymeric substances[1] and (2) stabilized by interaction with soil surfaces.[2,3] Thus, there is an increasing interest in investigating their turnover and accumulation in soils.[2] In addition to their contribution to the soil organic C (SOC) pool, amino sugars are – together with proteins – the compound classes linking the C and N cycles in soil and they contribute significantly to the soil organic N.[1] Amino sugars also provide information about the microbial community structure. Bacterial cell walls consist of peptidoglycan – a polymer of *N*-acetylmuramic acid and *N*-acetylglucosamine – whereas fungal cell walls consist of chitin, a *N*-acetylglucosamine polymer.[4,5] The origin of mannosamine and galactosamine, additional amino sugars found in hydrolysis extracts of soils, is still debated.

In contrast to cell membrane compounds such as phospholipids, which turn over rapidly in soils,[6] amino sugars are more stable. Information on the contribution of living biomass versus necromass in soils[4] or fungal and bacterial biomass,[7] as well as reliable and generally accepted results on their turnover time in soils, are still rare[8,9] as no methods for ^{14}C measurements of amino sugars, either in their natural abundance or as ^{14}C-labeled, have been reported to our knowledge. Recent approaches have focused on determinations of the $\delta^{13}C$ or $\delta^{15}N$ values of amino sugars. These studies started with the quantification of amino sugars by gas chromatography[10–12] and continued with gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS).[13] However, $\delta^{13}C$-determination by GC-C-IRMS has aggravating shortcomings:[14] ^{13}C fractionation occurs during measurement, although the resulting offset and amount dependence of the isotope signal can in part be corrected for by the use of an external standard.[15,16] However, the greater the amount of introduced

* *Correspondence to:* M. A. Dippold, Department of Agricultural Soil Science, Georg-August University of Goettingen, Buesgenweg 2, 97077 Goettingen, Germany.
E-mail: midipp@gmx.de

derivative C relative to the C atoms of interest, the larger the error in the $\delta^{13}C$ determination that still remains after applying correction functions.[14,17]

As amino sugars are water-soluble low molecular weight organic substances, they can also be quantified by high-performance liquid chromatography (HPLC).[18,19] Therefore, current methodological developments have focused on the establishment of liquid chromatography–oxidation–isotope ratio mass spectrometry (LC-O-IRMS) methods[20] for $\delta^{13}C$ measurement of amino sugars,[21] and these studies have already revealed the high potential of this technique for soil science applications.[22–24]

These LC-O-IRMS methods, and in particular the amino sugar method, are not routinely used. Conventional liquid chromatographs are constructed for organic eluents and problems occur if they are used continuously with strong acids or bases. However, $\delta^{13}C$ determination by LC-O-IRMS does not allow the use of any organic eluents, i.e. organic C. Hence, the liquid chromatography in such methods is restricted to ion-exchange columns which implies the use of salt solutions or acids and bases as eluents.[21,25] Thus, metallic ions can be dissolved from stainless steel pumps or capillaries and salt crystallization can occur within the system.[21,26] This causes a loss in the performance of the columns as well as blockages of the system. To prevent such problems, lengthy and expensive purging steps have to be implemented between sample measurements.[21,26] In addition, any contamination by HCO_3^- has to be avoided for $\delta^{13}C$ determination as HCO_3^- increases the C background (i.e. baseline) and it will influence the $\delta^{13}C$ values of the analytes. However, liquid chromatographs are *per se* not constructed to avoid gas diffusion into the system. Thus, pre-degassing of eluents has to be performed to enable carbonate-free chromatography – especially if bases are used as eluents. In addition, basic amino sugars (glucosamine, galactosamine and mannosamine) show greatly different chromatographic behavior from the acidic muramic acid. Thus, a high gradient with the eluents has to be driven, leading to strong elution of the matrix, especially for soils.[21] In addition, the concentrations of muramic acid are 10 to 100 times lower than those of basic amino sugars. This hampers quantification due to the limited linear range of the detectors as well as $\delta^{13}C$ determination due to a limited range of peak area with reproducible results. Therefore, current methods use a double measurement with different chromatographies to measure first the muramic acid and afterwards the basic amino sugars.[21] This double measurement, as well as the additional effort required for solvent-free HPLC methods, renders routine measurement of $\delta^{13}C$ values of amino sugars nearly impossible.

The aim of this study was to establish an ion chromatography–oxidation–isotope ratio mass spectrometry (IC-O-IRMS) method for the quantification and $\delta^{13}C$ determination of soil-derived amino sugars. We hypothesized that the use of an ion chromatograph would strongly facilitate IRMS measurement of many biomarkers, as some basic requirements like carbonate-free measurement or metal-free systems are already fulfilled by the instrument. In addition, we intended to optimize amino sugar purification to reduce cationic contamination and matrix peaks originating from soil. The aim was to provide a method enabling a routine application of $\delta^{13}C$ amino sugar measurements, which are crucial to the increasing interest in microbial contributions to stable SOM.

EXPERIMENTAL

Soil

Topsoil (0–10 cm) from the Ap horizon of a silt loamy haplic Luvisol[27] was collected from a long-term cultivated field in Bavaria (49.907 N, 11.152 E, 501 m.a.s.l., mean annual temperature 6–7 °C, mean annual precipitation 874 mm). The soil had a pH_{KCl} of 4.88 and pH_{H2O} of 6.49; the TOC and TN content were 1.77% and 0.19%, respectively, and the potential cation-exchange capacity was 13.6 $cmol_c\ kg^{-1}$. Field fresh soil was sieved to 2 mm and all roots were removed with tweezers. The soil was then freeze-dried and ball-milled, and 500 mg of the resulting powder were used for each hydrolysis.

Chemicals, reagents and external and internal standards

All chemicals for hydrolysis and purification were obtained from Sigma-Aldrich (St. Louis, MO, USA) with a minimum grade of 'pro analysis' (>99.0% purity). For ion chromatography, a 50–52%, ultra-pure NaOH solution was purchased from Sigma Aldrich. A $NaNO_3$ solution (0.01 M) was produced from metal-free sodium nitrate, puratronic (99.999% purity, Alfa Aesar, Karlsruhe, Germany). For oxidation, a 0.26 M sodium persulfate solution and 10% phosphoric acid solutions were used (Sigma-Aldrich).

Methylglucamine p.a. (5 mg mL^{-1}) and fructose p.a. (1 mg mL^{-1}) (Sigma-Aldrich) were used as the first and second internal standards (IS1 and IS2), respectively. Stock solutions for external standards contained methylglucamine, glucosamine, mannosamine and galactosamine at concentrations of 5, 14, 1.5 and 20 mg L^{-1} (Sigma-Aldrich) and muramic acid (Toronto Research Chemicals Inc., Toronto, Canada) at 7.5 mg L^{-1}. The IAEA-calibrated $\delta^{13}C$ value of each external standard was determined by repeated elemental analyzer–isotope ratio mass spectrometry (Flash 2000 HT Plus elemental analyzer and Delta V Advantage isotope ratio mass spectrometer, both from Thermo Fisher Scientific, Bremen, Germany) measurement of these substances and calibrated against certified standards from the International Atomic Energy Agency (IAEA, Vienna, Austria: IAEA-CH6: –10.4‰, IAEA-CH7: –31.8‰ and USGS41: 37.8‰) versus Pee Dee Belemnite (PDB).

Soil hydrolysis and ion removal

Soil hydrolysis and ion removal were performed according to the method of Zhang and Amelung,[10] which was optimized for $\delta^{13}C$ determination by Glaser and Gross.[13] Briefly, hydrolysis was performed with 10 mL of 6 M HCl at 105 °C for 8 h. The filtrate extract was dried completely and redissolved in 20 mL H_2O. Then 100 µL of the IS1 methylglucamine (i.e. 50 µg) were added. The pH was adjusted to 6.6–6.8 with 0.6 M KOH and precipitated iron was removed by centrifugation (4000 rpm for 15 min). After freeze-drying the residue was redissolved in 5 mL of dry methanol and salt precipitates were removed by centrifugation (4000 rpm for 10 min). The supernatant was dried under a gentle stream of N_2 and stored frozen until column purification.

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Purification by a cation-exchange column

Liquid chromatography requires column purification to remove hydrolysable non-cationic compounds such as monosaccharides and carboxylic acids from the extract. A cation-exchange column (AG 50 W-X8 resin, H^+ form, mesh size 100–200; Biorad, Munich, Germany) was used as suggested by Indorf *et al.*,[28] and a thin layer of clean glass wool was installed under 4 cm of cation-exchange resin in the glass column (inner diameter: 0.8 cm). The resin was filled in by rinsing with ~10 mL of 0.1 M HCl solution to ensure the H^+ form of the sorbent, covered with a thin layer of glass wool and preconditioned with 5 mL of water. The dried extracts were redissolved in ~1 mL of water with one drop of 0.1 M HCl to ensure the cationic form of muramic acid. After transferring the sample onto the column, the neutral and anionic compounds were eluted with 8 mL water. The cationic fraction containing the amino sugars was eluted by 15 mL 0.5 M HCl, freeze-dried and transferred with 5 mL of dry methanol. After evaporation of the methanol by a gentle stream of dried N_2, the sample could be stored frozen (–20 °C) for at least 1 month. For subsequent measurement, the samples were re-dissolved in 200 µL water with the addition of 50 µL of IS2 solution and measured within 24 h after re-dissolving.

Development of the measurement by IC-O-IRMS

All measurements were performed using a Dionex ICS-5000 SP ion chromatography system coupled by an LC IsoLink to a Delta V Advantage isotope ratio mass spectrometer (see Supplementary Fig. 1, Supporting Information; all components from Thermo Fisher Scientific). The chromatographic conditions were optimized with the aim of achieving baseline separation and a resolution factor Rs greater than 1.

$$Rs = \frac{t_2 - t_1}{0.5 \cdot (w_2 + w_1)} \quad (1)$$

where t_1 and t_2 are the retention times of two neighboring peaks and w represents their respective peak width at the tangent's baseline (Fig. 1).

Volumes of 9 µL of the water-dissolved sample or external standard were injected via a 25 µL injection loop and the injection time was defined as 0 s. Chromatography was performed using a CarboPac™ PA 20 analytical anion-exchange column (3 × 150 mm, 6.5 µm) which was preceded by a PA 20 guard column[21] (both from Dionex, Amsterdam, The Netherlands). The elution sequence contained a preconditioning step before injection (15 min with 200 mM NaOH and 10 min

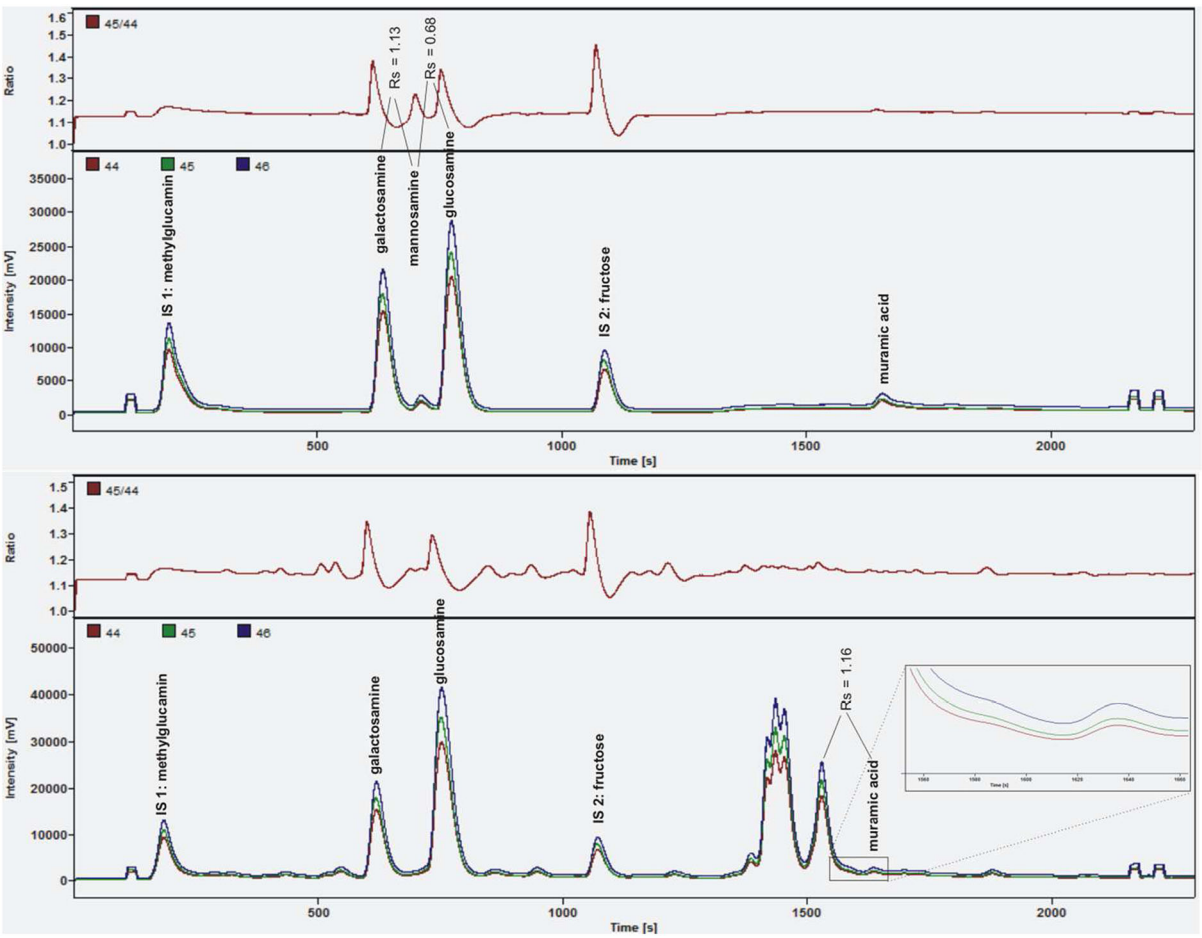

Figure 1. Chromatogram of external standard (top) and non-spiked sample (bottom). First and second internal standards as well as basic amino sugars (galactosamine, mannosamine and glucosamine) and acidic muramic acid are marked. Peak resolution Rs is included for the triplet of basic amino sugars in the upper chromatogram of the external standard and Rs for muramic acid and its preceding matrix peak is shown in the chromatogram of the sample.

Table 1. Recovery (%), relative standard deviation (RSD) and parameters of regression analysis as well as the detection (LoD) and quantification limits (LoQ) for the amino sugars assessed from the standard addition experiment

Substance	Recovery (%)	RSD (%)	R^2	$p_{slope\neq0}$	$p_{RunsTest}$	LOD (mg/vial)	LOQ (mg/vial)
Methylglucamine	67.9 ± 2.4	n.d.	0.996	< 0.001	≥ 0.05	0.006	0.019
Galactosamine	56.9 ± 2.0	3.2	0.996	< 0.001	≥ 0.05	0.005	0.066
Glucosamine	58.4 ± 4.8	2.9	0.986	< 0.001	≥ 0.05	0.001	0.021
Muramic acid	65.5 ± 1.9	5.9	0.997	< 0.001	≥ 0.05	0.018	0.057

with 8 mM NaOH). The elution sequence lasted for 35 min in total and was performed at a constant temperature of 30 °C and a flow rate of 0.4 mL min^{-1}.The NaOH concentration was increased after 11 min from 8 mM NaOH to 8 mM NaOH with a pulse of 2.5 mM $NaNO_3$ until the 15th minute. The addition of $NaNO_3$ was then ceased and the NaOH concentration increased for the final 20 min of the chromatogram (details in Supplementary Table 1, Supporting Information).

The external standards were measured at four concentrations (viz. 50, 100, 175 and 250 µL of the stock solution) at least once before and once after a sample batch. A sample batch consisted of 4–6 samples, each measured four times. A sample batch was always measured once in its entirety and the measurement was then repeated three times.

Integration was performed by Isodat 3.0 (Thermo Fisher Scientific) with the following parameters: start slope 1 mV/s, end slope 2 mV/s, peak minimum 50 mV, peak resolution 50%, and an individual background.

Evaluation of amino sugar quantification via IC-O-IRMS

To validate the method by standard addition, the standard mixture serving as the external standard was added to the hydrolysis extracts. The amounts of substance added were in the range of 0, 1.3, 1.7, 2.1 and 3 times the expected concentrations.

The data from the standard addition experiment were statistically evaluated according to Birk *et al.*[29] For each substance (including IS1) a linear regression was fitted by the method of least squares to the measured amounts as a function of the added amounts per sample (Supplementary Fig. 2, Supporting Information). The y-intercept represented the fitted amount of substance in soil and the slope gave the mean recovery of a substance. The significance of regression was tested and Steven's Runs Test was performed to identify deviations from linearity. Significant differences between recoveries were detected by covariance analysis (ANCOVA) of the slopes. All regression parameters were calculated with GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). The Relative Standard Deviation (RSD) was calculated from the standard addition experiment according to Birk *et al.*[29]

$$RSD = \sqrt{\frac{\sum_{i=1}^{i=n} (X_i - \overline{X})^2}{(n-1)\cdot\overline{X}^2}} \cdot 100 \qquad (2)$$

where X_i represents the difference between the quantified amount of a substance (corrected for recovery) and the spiked amount of the standard added to each of the n samples, and $\overline{X}$ is the mean of these differences.

The Limit of Detection (LoD) and Limit of Quantification (LoQ) were calculated based on the signal-to-noise ratio (S/N): for LoD the S/N has to exceed 3:1 and for LoQ a value of 10:1 is necessary.

Evaluation of $\delta^{13}C$ determination via IC-O-IRMS

First, the measured $\delta^{13}C$ values were drift corrected based on the reference gas drift according to GC-C-IRMS methods.[30] Thereafter, corrections for offset and amount dependence were performed.[16] We tested for linear, exponential and partial linear amount dependence by fitting the following functions to the measured data $at\%_{corrected}$:

$$\begin{aligned} &\text{(a) Linear :} \quad at\%_{corrected}(A_i) = a\cdot A_i + b \\ &\text{(b) Exponential :} \quad at\%_{corrected}(A_i) = c\cdot\exp(A_i) + d \\ &\text{(c) No amount dependence :} \quad at\%_{corrected}(A_i) = b \end{aligned} \qquad (3)$$

In these correction functions, a, b, c and d are parameters fitted to the plot of measured at% values against peak area A_i (Fig. 2). The function with the best fit was used to correct the measured $at\%_{measured}$ values of the sample, dependent on the peak area. The difference between the amount-dependent correction value $at\%_{corrected}(A_i)$ and the measured and calibrated value of the substance $at\%_{EA}$ was subtracted from the measured value to gain the PDB-calibrated ^{13}C enrichment ($at\%_{sample}$):

$$at\%_{sample} = at\%_{measured} - (at\%_{EA} - at\%_{corrected}(A_i)) \qquad (4)$$

Each substance and sample batch were corrected individually by the correction function that best described the behavior of the external standards. All corrections and calculations were performed in at% to avoid errors due to the nonlinearity of the $\delta^{13}C$ values.

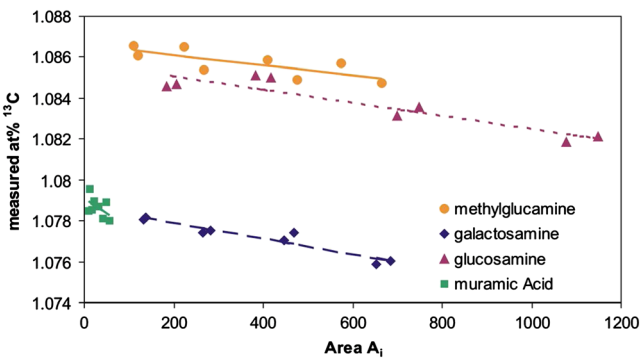

Figure 2. Linear functions adapted to the $at\%^{13}C$ values of the external standard line to correct for the amount dependency of these values.

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The accuracy of the IC-O-IRMS determination of $\delta^{13}C$ values was assessed from the standard addition experiment by using the mixing model to calculate back to the original values of the spiked substances:[21]

$$\delta^{13}C_{Sample} = \frac{N_{soil} \cdot \delta^{13}C(soil)_{fitted} - N_{Std} \cdot \delta^{13}C(Std)_{fitted}}{N_{soil} + N_{Std}} \quad (5)$$

The $\delta^{13}C_{Sample}$ value reflects the PDB-calibrated, measured $\delta^{13}C$ value (derived from $at\%_{sample}$ in Eqn. (4)), N_{soil} is the quantified amount of amino sugar in the soil and N_{Std} is the amount of standard added (% of total amino sugar per vial). Using a nonlinear fit based on the least-squares regression algorithm the $\delta^{13}C(soil)_{fitted}$ and $\delta^{13}C(Std)_{fitted}$ values were calculated by Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA).

The precision was determined (1) as the measured standard error of the four measurement replications of non-spiked soil and (2) as an area-dependent function for the standard error of the $\delta^{13}C(soil)_{sample}$ values gained by Gaussian error propagation of the standard errors of each term contributing to Eqn. (4)[31] (see Supplementary Eqn. (1), Supporting Information).

The isotopic LoQ was defined as the milligrams of amino sugar per vial needed to reach a standard error $\sigma_{final}(A_i)$ of less than 0.5‰ according to that equation.

RESULTS AND DISCUSSION

Chromatography

Measurement of both basic and acidic amino sugars was possible in a single run (Fig. 1). Methylglucamine showed only low retention by the column and it was followed by the triplet of the basic amino sugars. The peak resolution Rs within the triplet is shown in Fig. 1 for an external standard. If a soil contains a large amount of mannosamine, there might not be complete baseline peak separation between mannosamine and glucosamine. Whether the $\delta^{13}C$ value of mannosamine is influenced by glucosamine in this case has to be evaluated for soils with a higher mannosamine content. Mannosamine was not detected in this soil (Fig. 1), even when GC-MS analysis was used to achieve a lower detection limit (data not presented). The mannosamine values reported in the literature are also very low.[1,4,10,13]

Several substances were tested from a broad spectrum of monosaccharides and uronic acids, both substance classes that did not elute in the amino sugar fraction. Fructose was chosen as IS2 as it elutes in the middle of the chromatogram and there is no matrix peak close to it. However, the second internal standard can be changed if soils with other matrix peaks are being investigated. Muramic acid was the last peak to elute from the PA 20 column and it needed a nitrate pulse as a pusher to become mobile on the column.[21] Muramic acid occurred in samples directly after a large matrix peak. However, although the matrix peak was much higher than that for muramic acid, it did not tail into the muramic acid peak and the resolution between the peaks was sufficient (Fig. 1).

Column performance was maintained only by pre-purging (Supplementary Table 1, Supporting Information) and no further purging steps between the samples were needed. Carryover from sample to sample, as described previously for LC-O-IRMS,[21] could not be detected in any blank. Thus, the sample run time and purging time were strongly reduced compared with previous methods.[21] This can be attributed to the smaller amounts of metal ions and carbonates accumulating on the column due to the advantages of IC over liquid chromatography.

Recovery, linearity, precision and detection and quantification limits

Evaluation of the quantification by standard addition revealed linearity over a wide range of concentrations (Supplementary Fig. 2, Supporting Information): R^2 was higher than 0.99, the slope was, significantly, not zero and Steven's Runs Test revealed no deviation from linearity (Table 1). As we exceeded the soil concentrations by a factor of 3, we conclude that the linear range for quantification by IRMS is sufficient to cover the range of naturally occurring amino sugar concentrations in soil (even for soils with much higher SOC contents such as chernozems), especially if the amount of soil used is adapted to the SOC content. In particular, quantification of muramic acid and glucosamine, which occur in soils in vastly different concentrations, is possible in one run irrespective of the soil type.

The calculated recoveries ranged from 57 to 68%. ANCOVA revealed that recoveries of the first internal standard and basic amino sugars as well as of muramic acid did not differ significantly. Thus, correcting the dataset with the recoveries gained by IS1 sufficiently corrects for the loss of the other analytes during analysis. The recoveries were slightly less than those observed by Bode *et al.*,[21] which can mainly be attributed to the additional column purification step included here. However, this column purification improved peak shape and reduced chromatographic noise and thus enhanced the LoD and LoQ. Correcting the amounts of analytes by the IS1 recovery will compensate for these losses. However, if strongly different soil types are compared in one study, recovery of amino sugars should be checked for these particular soils before analysis to ensure similar recoveries irrespective of matrix type.

The precision of quantification was calculated by the RSD. Whereas basic amino sugars revealed precisions <2.3%, the precision for muramic acid was less, at 6.7% (Table 1). This can be attributed to the small amount of muramic acid, which is close to the limit of quantification. However, all the RSDs were in an acceptable range and precise quantification of amino sugars was possible by IC-O-IRMS.

The LoDs ranged from 0.001 to 0.02 and the LoQs from 0.02 to 0.07 mg per vial, depending on the noise surrounding the peaks. Thus, detection is possible even at low concentrations. However, for accurate detection (especially of low concentrations of muramic acid), the injection volume or amount of hydrolyzed soil used should be adapted to the respective soil.

To conclude, quantification of basic and acidic amino sugars is possible in a single run. However, depending on the ratio of glucosamine to muramic acid, an adjustment in sample amount or injection volume may be needed to reach optimum precision. Dilution of the final sample or adaptation of the injection volume may be necessary to obtain muramic acid concentrations above the LoQ and at the same time glucosamine concentrations that are still in the linear range of quantification.

Amount dependence and correction factors of $\delta^{13}C$ values

The external standards measured parallel to each batch of samples were used to adapt the amount dependence and offset correction. For each compound a decrease in at%^{13}C with increasing area following a linear equation was observed (Fig. 2). However, the function with the best fit (Eqn. (3)) changed between individual measurement batches and between days. Therefore, (1) measurement of external standards in the concentration range of the samples and (2) individual correction functions derived from these external standards per sample batch are obligatory in order to achieve reliable determination of $\delta^{13}C$ values. This has also been observed in other compound-specific isotope studies.[15–17,31]

Accuracy, precision and isotopic LoQ of $\delta^{13}C$ determination

The accuracy was assessed by comparing the fitted $\delta^{13}C$ values (from Eqn. (5), illustrated in Supplementary Fig. 3, Supporting Information) to the measured $\delta^{13}C$ values (Table 2). Deviations in the corrected $\delta^{13}C$ $(Std)_{fitted}$ values of the added standard from those measured by EA-IRMS were less than 1‰ for the basic amino sugars but higher for muramic acid (~1.5‰). These deviations from the true $\delta^{13}C$ values were slightly higher than those observed by Bode *et al.*,[21] who also had the greatest deviation for muramic acid. This can be attributed to the small amount of muramic acid present and the fact that the $\delta^{13}C(Std)$ value of the standard spiked to the sample was quite close to the $\delta^{13}C$ value of soil muramic acid which leads to a high uncertainty in the estimation of the fitted parameters. Choosing a soil with higher $\delta^{13}C(soil)$ value (e.g. by input of C4 plants) would presumably reveal higher and more realistic accuracies for muramic acid. However, fitted values for $\delta^{13}C(soil)$ deviated by less than 0.5‰ from those of direct measurement of non-spiked soil, reflecting that the determination of amino sugar $\delta^{13}C$ values under non-spiked conditions is reliable.

The standard error of the four measurement replications was calculated for all samples of the standard addition line ($\sigma_{IC\text{-}O\text{-}IRMS}(A_i)$) (Table 2). The area dependence of this standard error $\sigma_{IC\text{-}O\text{-}IRMS}(A_i)$ followed a parabolic function for the basic amino sugars (Supplementary Fig. 4, Supporting Information), which resulted from the broad range of areas covered by the standard addition approach: With decreasing peak area a loss in precision occurs due to approaching the isotopic detection limit, i.e. the error $\sigma_{IC\text{-}O\text{-}IRMS}(A_i)$ increases. Increasing the peak area can lead

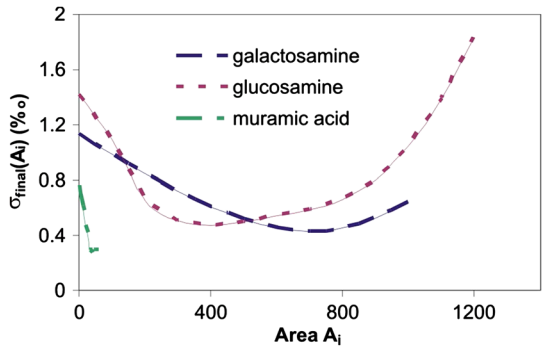

Figure 3. Amount-dependent function for estimation of the standard error of $\delta^{13}C$ values ($\sigma_{final}(A_i)$) calculated according to Supplementary Eqn. (1).

Table 2. Comparison of $\delta^{13}C(Std)_{EA\text{-}IRMS}$ (EA-IRMS PeeDeeBe calibrated $\delta^{13}C$ value of standard substances spiked to the sample) and $\delta^{13}C(Std)_{IC\text{-}O\text{-}IRMS}$ (fitted $\delta^{13}C$ value of the spiked standards from the mixing model of the standard addition method) reflecting the accuracy of IC-O-IRMS measurement. Fitted $\delta^{13}C$ values for soil from mixing model ($\delta^{13}C(soil)_{calculated}$) and real measurement of non-spiked soil $\delta^{13}C(soil)_{IC\text{-}O\text{-}IRMS}$ are also presented. Precision is shown (1) by the standard deviation of the measurement repetitions and (2) by calculating the area-dependent standard deviation according to Eqn. (6) for the measured peak area. The isotopic LoQ value reflects the minimum amount per vial needed to receive a standard error of the measurement repetitions lower than 0.5‰

Substance	$\delta^{13}C(Std)_{EA\text{-}IRMS}$ (‰)	$\delta^{13}C(Std)_{fitted}$ (‰)	$\delta^{13}C(soil)_{fitted}$ (‰)	$\delta^{13}C(soil)_{IC\text{-}O\text{-}IRMS}$ (‰)	$\sigma(\delta^{13}C(soil)_{measured})$ (‰)	$\sigma(\delta^{13}C(soil)_{final})$ (‰)	Isotopic LoQ (mg/vial)
Galactosamine	–28.42	–27.60 ± 0.14	–24.58 ± 0.12	–25.02	0.26	0.64	0.237
Glucosamine	–22.58	–21.65 ± 0.25	–26.64 ± 0.10	–26.87	0.20	0.87	0.250
Muramic acid	–20.54	–19.04 ± 0.06	–19.51 ± 0.09	–19.65	0.08	0.51	0.048

to an overload of the system with subsequent imprecise isotope determination. In contrast, muramic acid showed a linear decrease in the standard error with increasing areas reflecting that even the highest spiked samples were far from overload conditions. The error of the amount dependence (derived from the external standards) $\sigma_{correction}(A_i)$ showed a similar dependency on area as the sample-derived error: the $\sigma_{correction}(A_i)$ of basic amino sugars had a parabolic area dependence whereas the $\sigma_{correction}(A_i)$ of muramic acid showed linear behavior for the same reasons as in the samples (Supplementary Fig. 4, Supporting Information). These functions were used to sum the amount-dependent standard error $\sigma_{final}(A_i)$ (Fig. 3). For the basic amino sugars, the standard error followed a function close to a parabolic function and there was a broad range of areas enabling reliable determination of the $\delta^{13}C$ values. For muramic acid, this function showed a sharp increase in standard deviation if areas became too small. Sample preparation should be optimized to reach the isotopic LoQ, i.e. to have >0.048 mg muramic acid in the final lyophilized sample. If the amount is less, either the volume, in which the sample is finally dissolved, has to be decreased or the injection volume has to be increased to achieve a sufficient muramic acid peak area. The agricultural soil used for this method evaluation had a relative high proportion of bacteria compared with fungi. In soils with a strong preference for fungal growth, e.g. podzols, the amount of muramic acid may be too low to reach the needed LoQ without having an overload in the glucosamine peak. Under such special conditions a double measurement with a highly concentrated sample for the determination of the muramic acid $\delta^{13}C$ values and a diluted sample for the determination of the glucosamine $\delta^{13}C$ values might be necessary.

Average amino sugar $\delta^{13}C$ values differ by ~0.1 to 1.1‰ within the basic amino sugars,[21,23] by more than 3–5‰ between the basic and acidic amino sugars,[21,23,32] and by around 7‰ from those of bulk SOC.[13] The achieved accuracies of individual amino sugars enable amino sugars to be distinguished from their C sources even under natural abundance conditions. The resulting precision (0.5‰) is lower than the differences between basic and acidic amino sugars and this thus enables microbial group specifics to be identified in amino sugar formation (e.g. specifics in the used substrates or the fractionations in biochemical formation pathways). Especially in experiments leading to higher $\delta^{13}C$ differences in amino sugars such as C3 to C4 C source changes,[22] FACE experiments[13] or application of labeled substrates,[23] this method can fully distinguish C sources and individualities in the cell wall formation of fungi and bacteria.

In summary, this method enables a combined determination of $\delta^{13}C$ values of amino sugars for the majority of soils. However, adjustments to new sample types are necessary to identify the optimum amount of sample to hydrolyze or the final volume to inject so that the optimum ranges for accuracy and precision of the $\delta^{13}C$ values are met.

Advantages of IC-O-IRMS

Many previous studies reported severe problems with LC-O-IRMS, e.g. the impossibility of measuring muramic acid in non-spiked samples due to very low peak areas or the requirement for time-consuming purging steps to maintain the performance of the PA 20 column.[21] The absence of these issues in the currently proposed method can mainly be attributed to the advantages of IC over HPLC. Ion chromatographs are free of metals: all parts of the system that are in contact with sample or eluents are made from polyether ether ketone (PEEK). Thus, metal contamination can originate only from the sample. However, our method contains iron and salt precipitation steps, removing all (potentially column destroying) cations. This not only reduces measurement time but also reduces costs as, e.g., in-line high-pressure filters protecting the column from colloids and metal ions are not needed. Even after 600 injections, no decrease in performance of the PA 20 column was detected and the pre-column did not need to be exchanged.

In addition, the CO_2-tight construction of the ion chromatographs is a great advantage for $\delta^{13}C$ determination as no shifts in the $\delta^{13}C$ value due to increasing carbonate background occurred. Therefore, even CO_2-binding eluents, such as NaOH, do not cause problems for chromatography and isotope ratio mass spectrometry. In addition, ion chromatographs are routinely equipped with a degasser, which keeps the eluents and oxidizing reagents of the Isolink CO_2-free. Thus, although acquisition costs may be higher, the improved performance, higher sample throughput and lower follow-up costs reflect the clear advantages of ion chromatographs for improving LC-O-IRMS.

CONCLUSIONS

Amino sugars are important biomarkers for research on bacterial and fungal contribution to SOM. This new method enables parallel quantification and $\delta^{13}C$ determination of the most frequent amino sugars in soils and thus sets the preconditions for wider adoption of $\delta^{13}C$ amino sugar determination in soil science.

The combination of iron and salt removal from gas chromatography protocols with purification via cation-exchange resins adapted from liquid chromatography methods proved to be an optimal sample preparation for ion chromatography, including chromatographic separation, system stability and longevity of system components. In addition, ion chromatography has clear advantages over HPLC as metal and carbonate exclusion from the system avoids column contamination as well as disturbance of $\delta^{13}C$ determination by a carbonate background.

These improvements over previous methods enabled parallel quantification and $\delta^{13}C$ determination of high concentration basic amino sugars and low concentration muramic acid. Recoveries ranged from 57 to 66% and could be corrected by using methylglucamine as the first internal standard. The quantification limit of muramic acid, the compound with the lowest concentration, was around 0.05 mg per vial for quantification and for isotope measurement. When the muramic acid concentration exceeded that value, glucosamine, the most concentrated compound, was still in a linear range for quantification and $\delta^{13}C$ measurement. The accuracy of IC-O-IRMS was better than 1‰ for basic amino sugars and better than 1.5‰ for muramic acid relative to calibrated EA-IRMS values. The precision was amount-dependent and less than 0.5‰ over a comparatively broad range of areas. However, the dependence on the matrix and the ratio of muramic acid to glucosamine in individual samples necessitated adjustment in soil amount or injection volume to achieve the optimal accuracy and precision of $\delta^{13}C$ values.

The quality of the quantification and $\delta^{13}C$ determination as well as sample throughput of this method should enable this method to be used routinely in soil science. The advantages of IC-O-IRMS over HPLC-O-IRMS are evident and IC-O-IRMS might also bring advantages for the analysis of other biomarkers.

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