

A new LC-O-IRMS separation strategy for $\delta^{13}\text{C}$ measurement of underivatized amino sugars from soil

Jesse Reimer

Bobbi Helgason

Soil Microbiology Lab

University of Saskatchewan



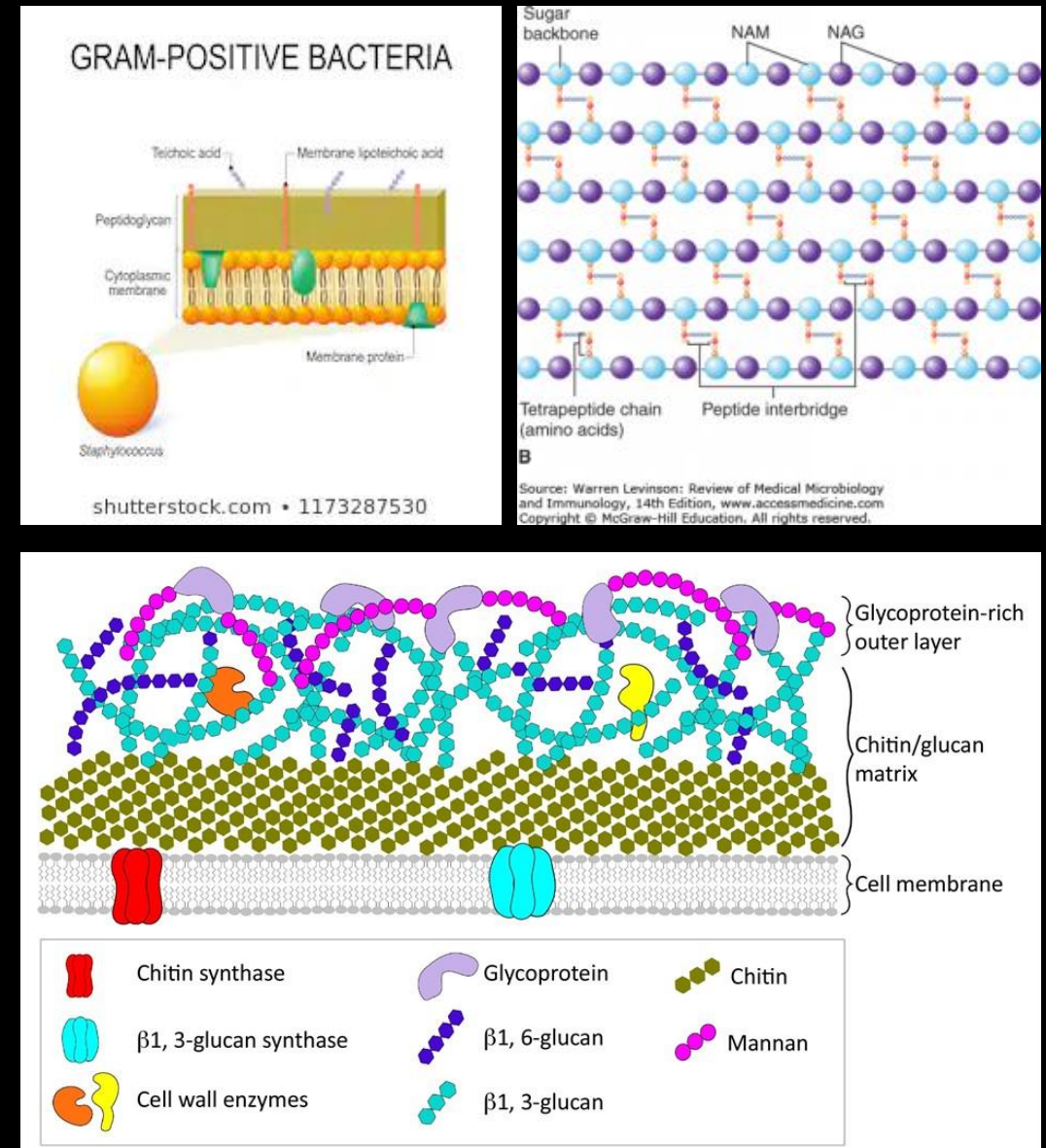
UNIVERSITY OF SASKATCHEWAN

College of Agriculture
and Bioresources

DEPARTMENT OF SOIL SCIENCE
AGBIO.USASK.CA

Utility of soil amino sugars

- Determine relative contributions of microbial necromass to mineral associated organic carbon (MAOC/MAOM)
 - Comparatively stable carbon pool
 - Peptidoglycan → GluN + MurN
 - Chitin → GluN
 - GalN + ManN from bacterial/fungal biofilms/EPS (Oliva et al., 2024)
- Basic amino sugars: GluN, ManN, GalN
- Acidic amino sugars: MurN
- ¹³C labelling can help determine dynamics of amino sugar pools under conditions of interest



Geoghegan et al. 2017 Trends in Microbiol. 25:957-967

Helgason Lab research applications

- Plants pulse-labelled using $^{13}\text{CO}_2$ in growth chamber
 - a) Plant below-ground inputs traced into microbial pools
 - b) Application of plant residues to soils to trace decomposition into microbial pools or $^{13}\text{CO}_2$



$\delta^{13}\text{C}$ amino sugar measurement challenges

LC-IRMS:
*narrow
selection of
compatible LC
column tech*

No organic eluents
can be used (e.g.
methanol,
acetonitrile)
GalN/ManN/GluN
elute closely together

GC-IRMS:
*derivatization
increases
uncertainty*

- Doubles C# for MurN
- Triples C# for GluN, ManN, GalN
- Prone to isotopic fractionation
- Uncertain derivatization efficiency (Bodé et al., 2009)

LC-O-IRMS measurement strategy

1. LC separation of underivatized amino sugars [Agilent 1260 Inf. II Bio-Inert]
2. Wet oxidation [Elementar LiquiFace]
 - i. Sample + persulfate + phosphoric acid mix
 - ii. Solution oxidized at 100 °C
 - iii. CO₂ transfers through expanded PTFE membrane into He stream
3. CO₂ measurement at IRMS [Elementar Isoprime precisION]



Challenges of IC separation strategies

- Bodé et al. (2009), Dippold et al. (2014) used CarboPac PA20 strong anion exchange column
- Strong quaternary amine stationary phase:
 - separates basic amino sugars
 - strongly binds muramic acid; requires 'pusher' ion to elute
- NaOH mobile phase "pulls" CO₂ from air
 - Carbonates form under basic conditions
 - Carbonates pass through degasser and produce CO₂ at interface
 - ∴ Eluent bottles & flowpath must be CO₂-tight
- Anion exchange = carbonates bind and breakthrough, usually with pusher ion
- Bodé et al. (2009): modified LC w/ switching valve
- Dippold et al. (2014): IC with in-line eluent generator



HPLC-RID ligand exchange/ion exclusion

- Williams et al. (1991) separated hydrolyzed *P. putida* membranes on Bio-Rad ligand exchange/ion exclusion column (Ca form)
 - Soil bacteria
 - No organic eluents: low pH CaNO₃
 - G- cultures; no GalN or ManN present in samples
- *Adapt and optimize for soil extracts containing GalN and ManN?*

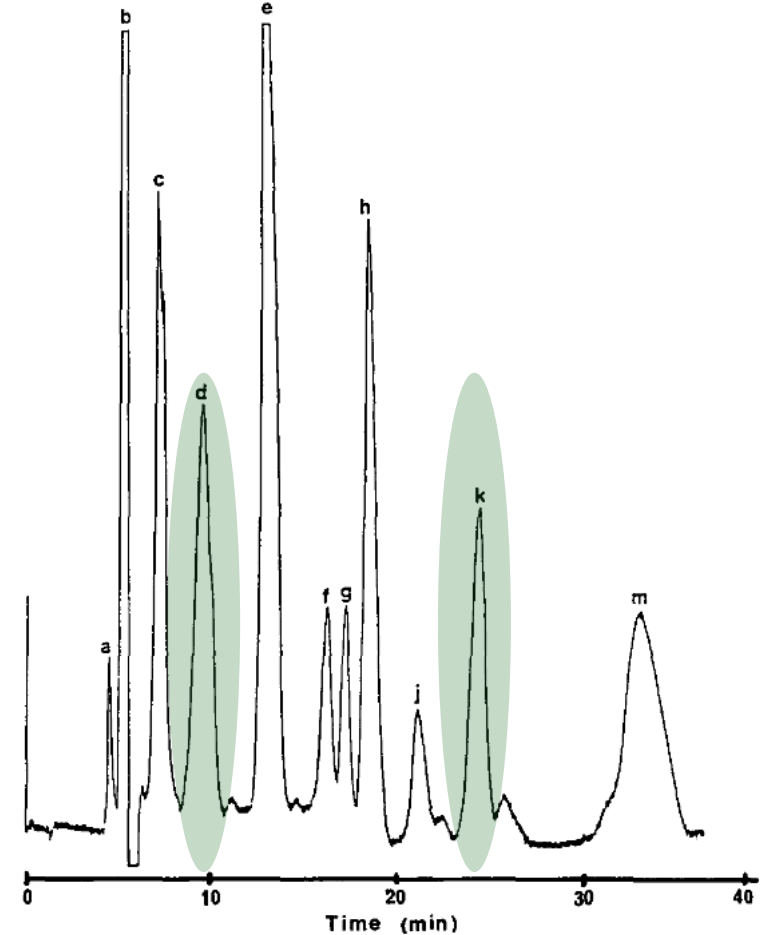


FIG. 1. HPX-87C high performance liquid chromatography of hydrolyzed peptidoglycan from *P. putida*. The peptidoglycan was treated with 6 N HCl as described under Methods. Chromatographic conditions: solvent, 50 mM calcium nitrate, pH. 3.6; flow rate, 1.0 ml/min; column temperature, 85°C; detector attenuation, 16×. Retention time scale: 1.0 cm = 2.3 min. (a) Chloride ion; (b) nitrate ion; (c) *N*-acetylglucosamine; (d) muramic acid; (e) glutamic acid; (f) threonine; (g) serine; (h) alanine; (i) glycine; (k) glucosamine; (m) diaminopimelic acid.

Method: Soil extraction

- Follows modified Dippold et al. (2014)
 - 8 h hydrolyzation of soil (6M HCl at 105 °C under N₂)
 - Filter then N-evap drydown
 - Centrifuge precipitation of Fe/Al
 - Cation exchange column purification (Bio-Rad AX50WX8)
 - Salt precipitation using dry methanol, N-evap drydown
 - Repeat if necessary

Method: Reference materials

- Standard reference materials purchased from Millipore-Sigma
 - MeGluN, Fructose, GluN, GalN, ManN, MurN
- Normalized to VPDB scale via EA-IRMS analysis (Stable Isotope Facility, Dept of Soil Science, U of Saskatchewan)
 - USGS 40 and USGS 41

Methods: LC-IRMS analysis

Anion Exchange (CarboPac PA20)

- 3x150 mm column, 3x30 mm guard column (30 °C)
- 50 min 0.4 mL/min
 - Preconditioning (25 min)
 - 15 min 200 mM NaOH
 - 10 min 8 mM NaOH
 - Analysis (35 min)
 - 11 min 8 mM NaOH
 - 4 min 9 mM NaOH + 2.5 mM NaNO₃
 - 20 min 55 mM NaOH
 - Dippold et al. (2014)
- Eluent bottle CO₂-proofing:
 - PEEK lines, CO₂ filters (Litholyme sorbent), in-line degassers (Cytiva IFD), NaOH lines bypassed degasser unit.

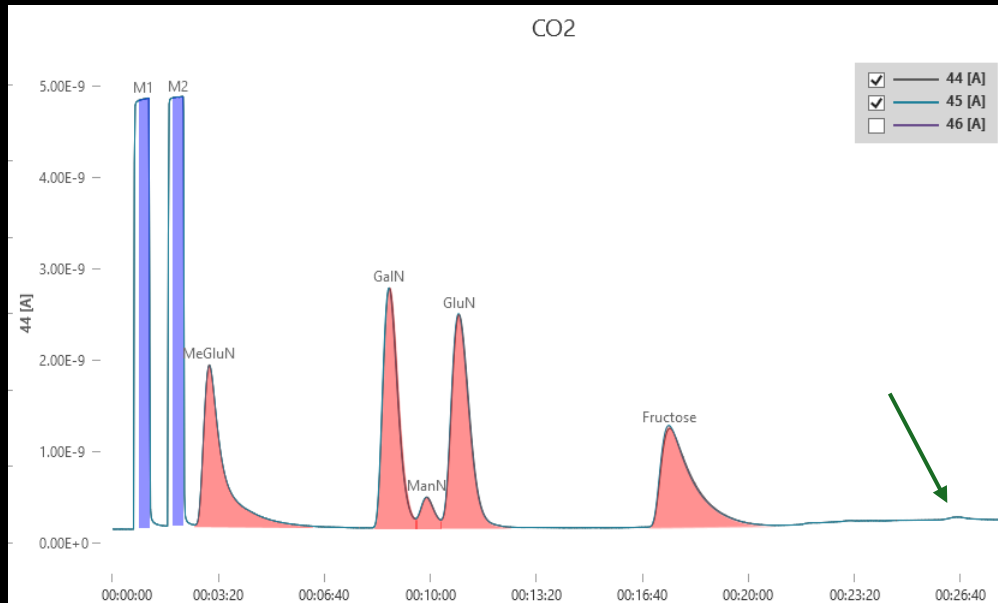
Ligand Exchange/Ion Exclusion (Hi-Plex Ca)

- 4.6x250 mm column, 3x5 mm guard cartridge (85 °C)
- 60 min 0.3 mL/min
 - 10 mM CaNO₃₍₂₎ pH 3.4
- Optimized for best separation of analytes using multichannel gradient valve (MCGV)

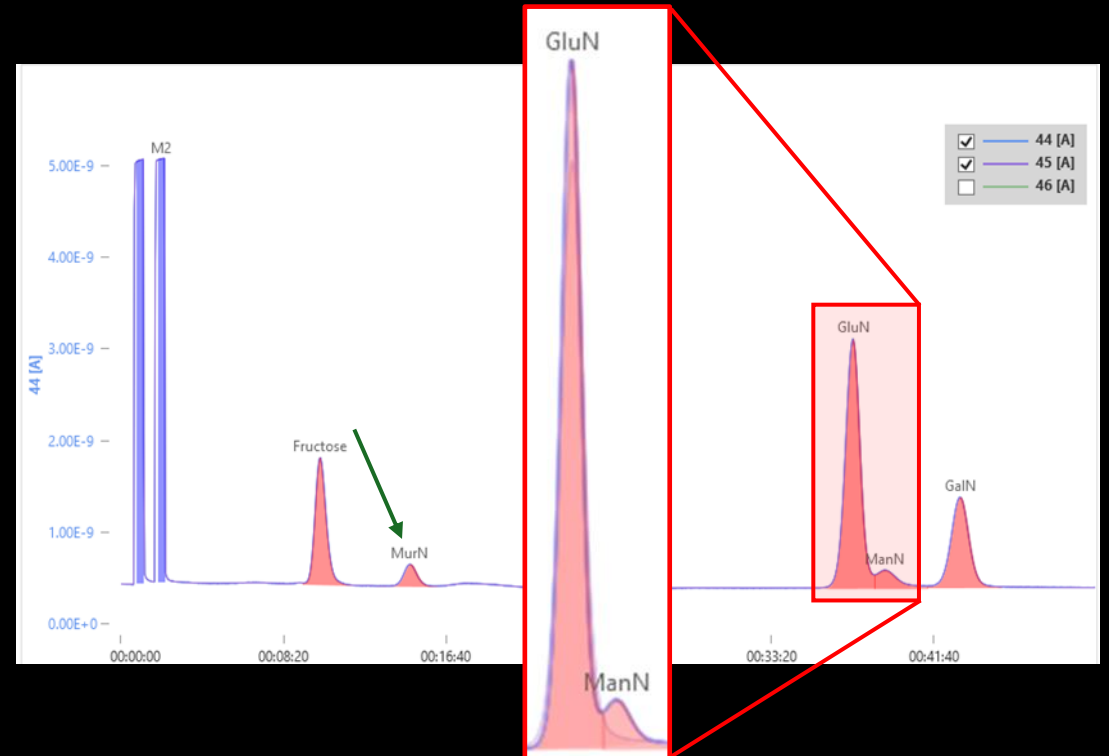
Results: LC-O-IRMS

Standard reference material chromatography

Anion Exchange



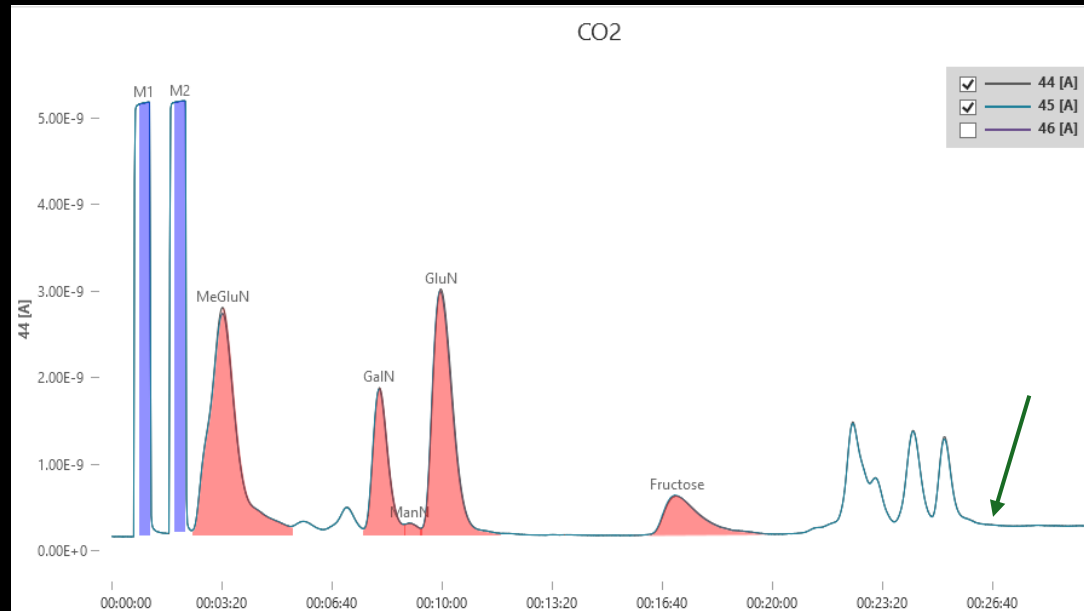
Ligand Exchange/Ion Exclusion



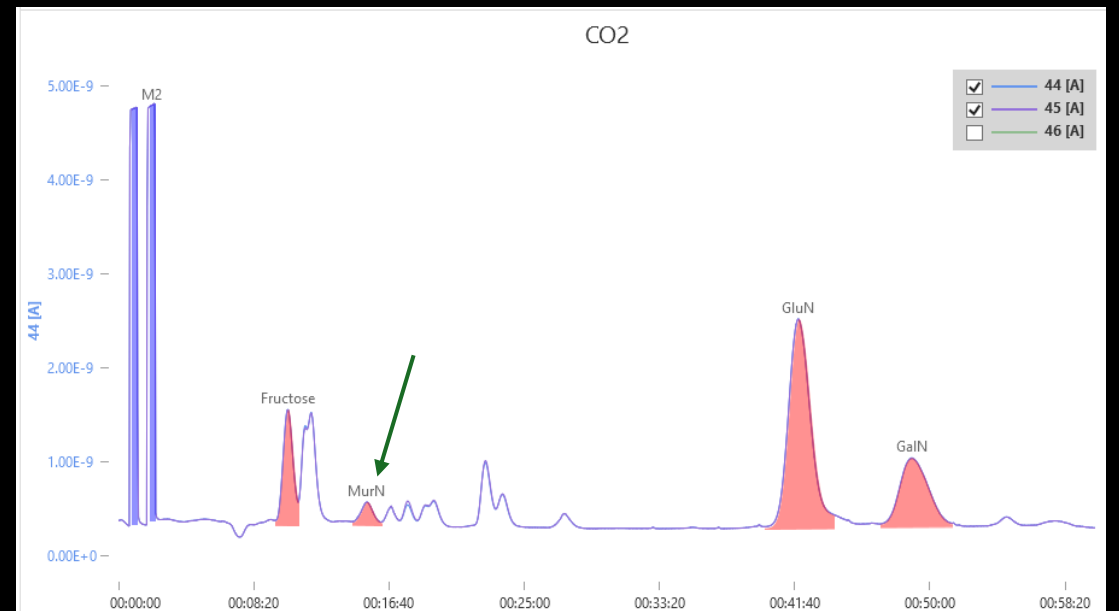
Results: LC-O-IRMS

Sample chromatography

Anion Exchange

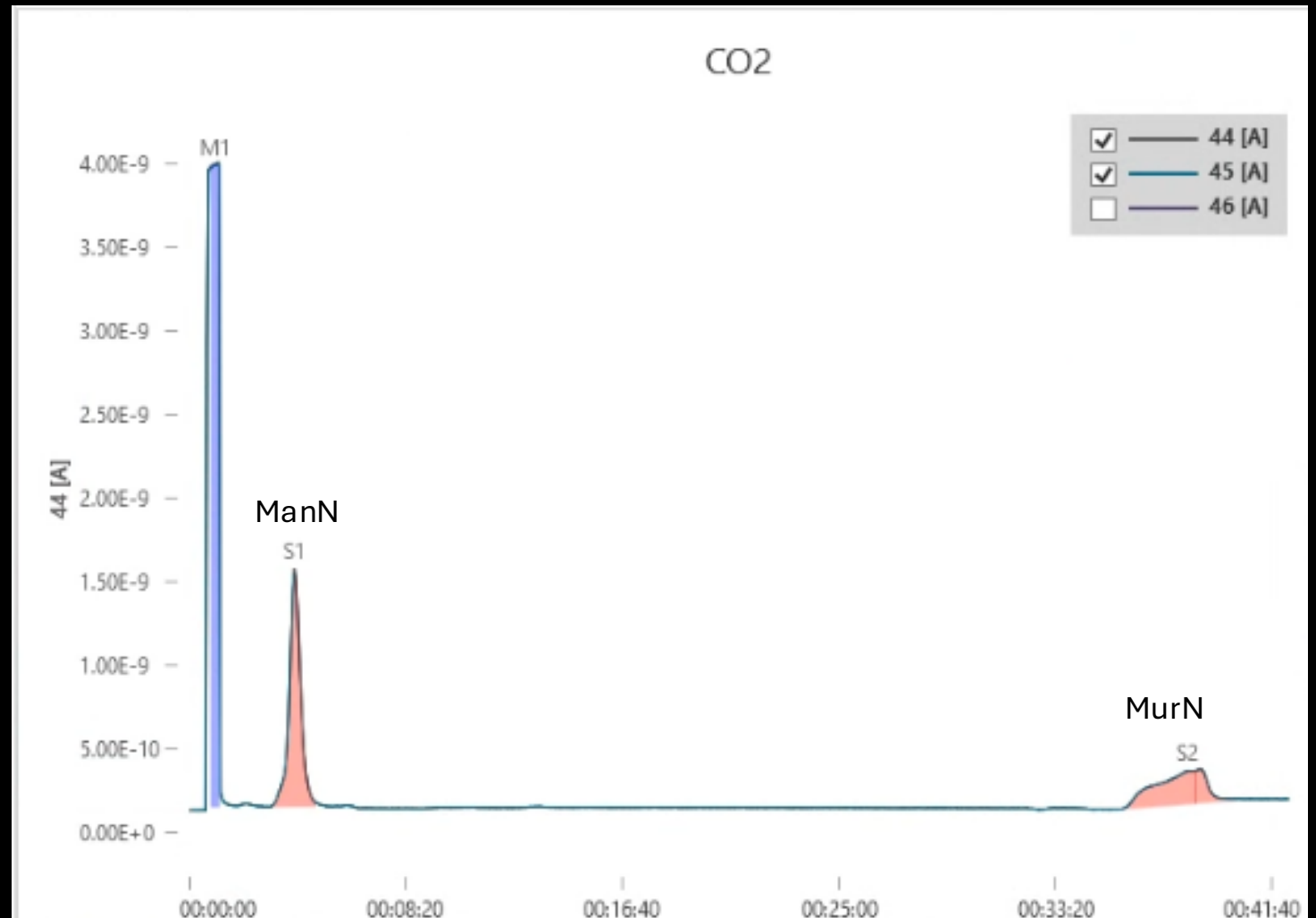


Ligand Exchange/Ion Exclusion



'Trap and release' of MurN on anion exchange guard column using switching valve?

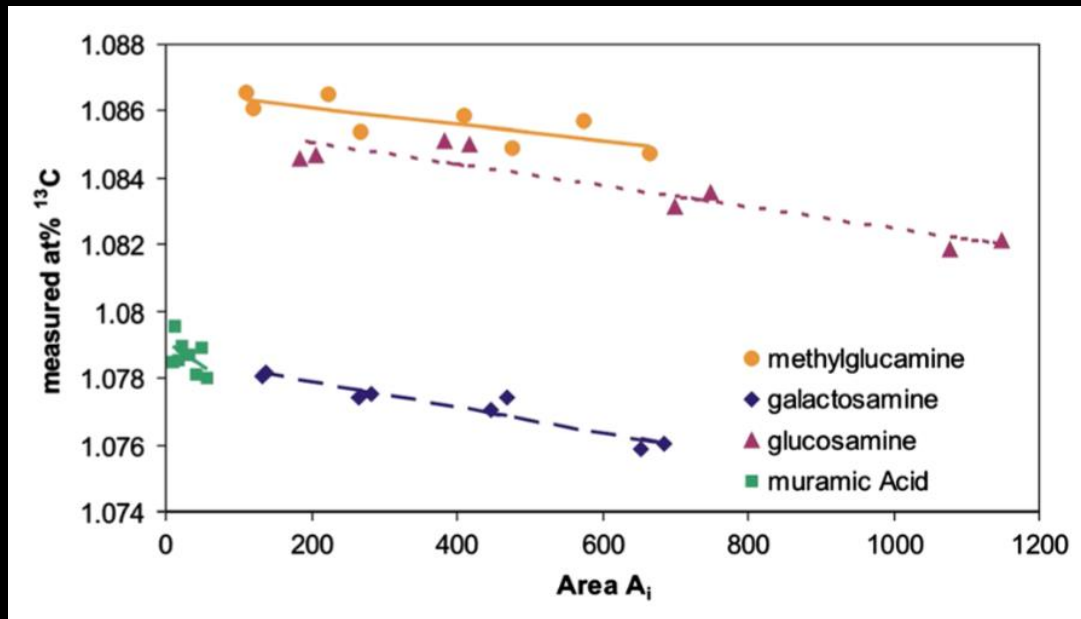
- Tested Bodé et al. (2009) approach using guard column only
 - Equal mass of ManN & MurN injected
- More MurN recovered by bypassing analytical column route
- Smears even with strong ion pusher
- Carbonate breakthrough still occurs



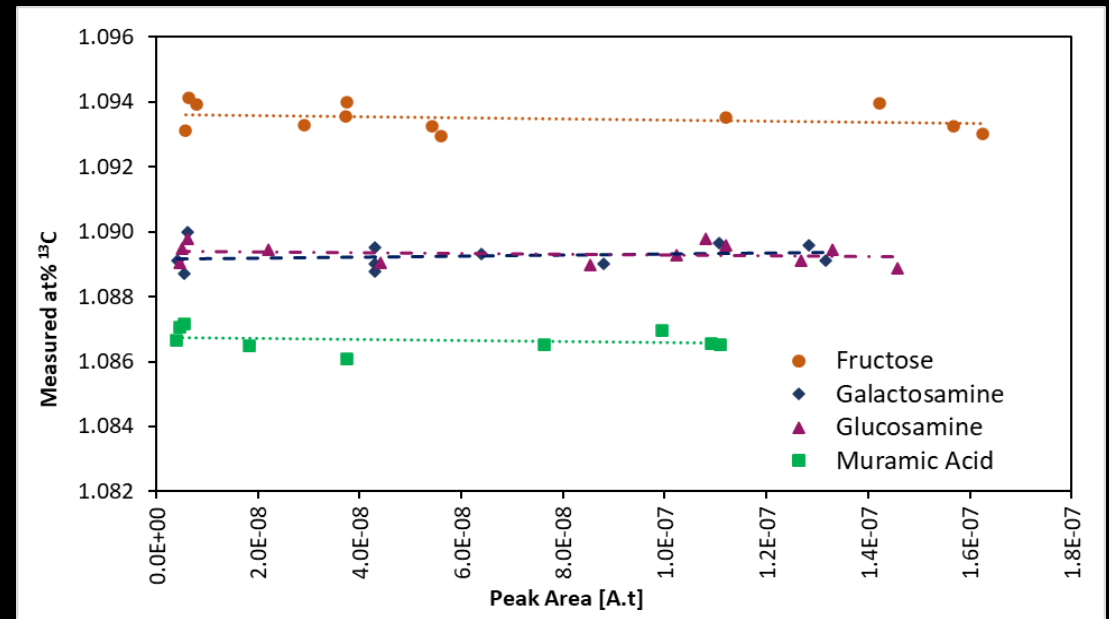
Results: LC-O-IRMS

Analyte linearity

Anion Exchange



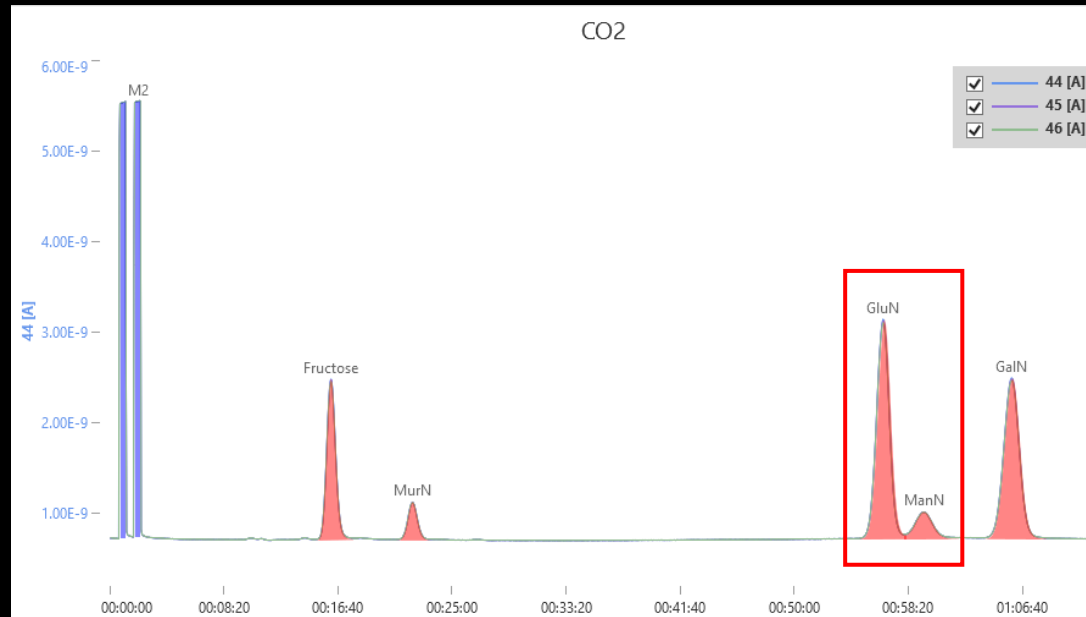
Ligand Exchange/Ion Exclusion



Dippold et al., (2014): Fig. 2

Results: LC-O-IRMS

Longer HiPlex Ca column (7.7x300 mm)

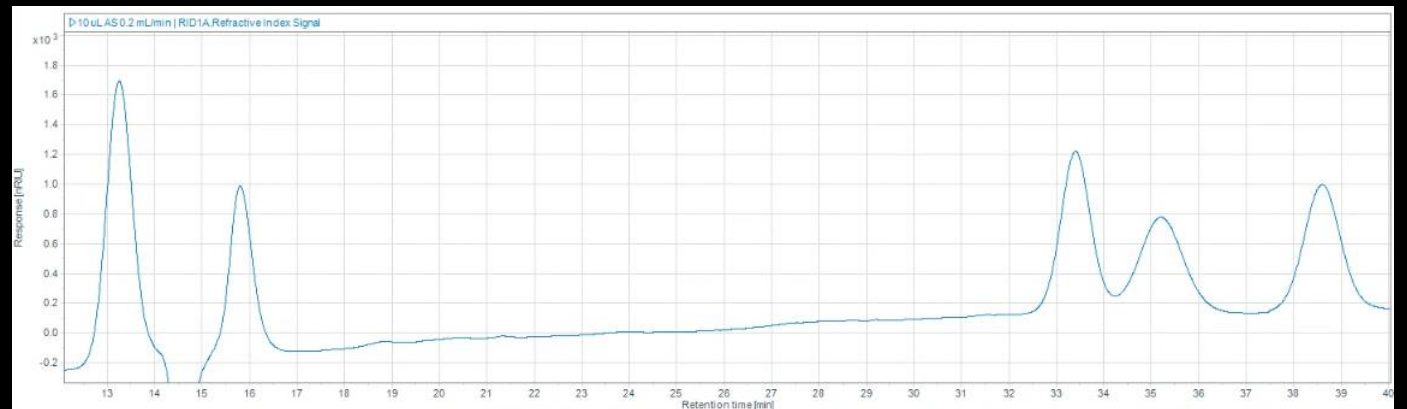


- Further improved separation of basic amino sugars
- Currently optimizing:
 - Split flows to LF/RID
 - Eluent [Ca²⁺]

Summary of Benefits

Technical

- No CO₂ proofing required
- Degasser removes dissolved CO₂
- Simpler preparation of mobile phases
- Can optimize if soils are “dirtier”, i.e., more extracted compounds
 - MCGV mixing of CaNO₃ solution, dilute acid, water possible to narrow down new pH and Ca molarity if co-eluters appear
 - MurN moves with pH; could theoretically separate from potential co-eluters (depending on co-eluter)
- Isocratic: can work with RID



Summary of Benefits

Sample Analysis

- Improved separation of basic amino sugars
 - GalN fully resolved from ManN
 - GluN and ManN separated at sample concentrations
- Better MurN elution
 - Not strongly bound to stationary phase
 - Better recovery, no smearing
 - No carbonate breakthrough
- No amount-dependent ^{13}C linearity adjustment needed

Research application example

(Mostafa Hojati thesis)

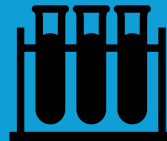
Bobbi presenting this experiment in more detail tomorrow morning



^{13}C pulse-labelled field pea, canola, and wheat grown in two field soils in growth chamber.



Observed MAOC losses (priming) under field pea and canola



^{13}C amino sugar analysis showed:

Loss of pre-existing bacterial necromass under all three crops

Concurrent accumulation of fungal necromass

Acknowledgements

- George Gachumi
- Kim Janzen
- Sergio Jaramillo-Tatis (Agilent)
- Kyle Taylor, Alejandra Cartegena-Sierra, Rob Berstan (Elementar)



UNIVERSITY OF SASKATCHEWAN
College of Agriculture
and Bioresources
DEPARTMENT OF SOIL SCIENCE
AGBIO.USASK.CA



Literature cited

- Bodé, S., Deneff, K., Boeckx, P., 2009. Development and evaluation of a high-performance liquid chromatography/isotope ratio mass spectrometry methodology for $\delta^{13}\text{C}$ analyses of amino sugars in soil. *Rapid Communications in Mass Spectrometry* 23, 2519–2526. doi:[10.1002/rcm.4093](https://doi.org/10.1002/rcm.4093)
- Dippold, M.A., Boesel, S., Gunina, A., Kuzyakov, Y., Glaser, B., 2014. Improved $\delta^{13}\text{C}$ analysis of amino sugars in soil by ion chromatography-oxidation-isotope ratio mass spectrometry: $\delta^{13}\text{C}$ analysis of amino sugars in soil by IC-O-IRMS. *Rapid Communications in Mass Spectrometry* 28, 569–576. doi:[10.1002/rcm.6814](https://doi.org/10.1002/rcm.6814)
- Oliva, R.L., Vogt, C., Bublitz, T.A., Camenzind, T., Dyckmans, J., Joergensen, R.G., 2024. Galactosamine and mannosamine are integral parts of bacterial and fungal extracellular polymeric substances. *ISME Communications* 4(1), 1-7. doi:[10.1093/ismeco/ycae038](https://doi.org/10.1093/ismeco/ycae038)
- Williams, J.W., Saravolac, E.G., Taylor, N.F., 1991. The use of high performance liquid chromatography in the identification and quantification of cell wall and lipopolysaccharide components in gram negative bacteria. *Microchemical Journal* 43, 2–9. doi:[10.1016/0026-265x\(91\)90032-k](https://doi.org/10.1016/0026-265x(91)90032-k)