

Fachgebiet Bodenbiologie und Pflanzenernährung

Fachbereich Ökologische Agrarwissenschaften

Universität Kassel

**Optimierung der aminozucker-spezifischen $\delta^{13}\text{C}$ -Analyse
in Böden- und Pflanzenmaterialien zur Erfassung des
Umsatzes von mikrobiellen Residuen**

Dissertation

zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften

(Dr. rer. nat.)

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Die Wissenschaft, richtig verstanden, heilt den Menschen von seinem Stolz:
denn sie zeigt ihm seine Grenzen.

(Albert Schweitzer)

Die vorliegende Arbeit wurde vom Fachbereich Ökologische Agrarwissenschaften der Universität Kassel als Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.) angenommen.

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Eidesstattliche Erklärung

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Göttingen, den 19.12.2011

Caroline Indorf

Vorwort

Die vorliegende Dissertation wurde im Rahmen des DFG- Projektes JO 362/7 an der Universität Kassel im Fachbereich Ökologische Agrarwissenschaften im Fachgebiet Bodenbiologie und Pflanzenernährung erstellt, um die Anforderungen des akademischen Grades Doktor der Naturwissenschaften zu erfüllen. Die Arbeit beinhaltet 3 Artikel, von denen einer bereits bei einer international, begutachteten Fachzeitschrift veröffentlicht wurde. Der zweite und der dritte Artikel wurden im Dezember 2011 eingereicht. Diese Artikel sind in Kapitel 3, 4 und 5 eingebettet. Kapitel 1 ist eine generelle Einleitung zum Thema, während Kapitel 2 die Ziele dieser Arbeit beschreibt. In Kapitel 6 und 7 werden die Ergebnisse aus Kapitel 3, 4 und 5 auf Deutsch sowie auf Englisch zusammenfassend dargestellt. Kapitel 7 gibt einen Ausblick auf weitere Untersuchungen, während in Kapitel 8 die Quellen der Kapitel 1, 2 und 7 aufgeführt sind.

Die folgenden Artikel sind in diese Arbeit eingearbeitet worden:

Kapitel 3

Indorf, C., Dyckmans, J., Khan, K.S., Joergensen, R.G. (2011). Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates. *Biol Fertil Soils* (DOI 10.1007/s00374-011-0545-5).

Kapitel 4

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Kapitel 5

Indorf, C., Stamm, F., Dyckmans, J., Joergensen, R.G.
Determination of saprotrophic fungi turnover in different substrates by glucosamine specific $\delta^{13}\text{C}$ liquid chromatography/isotope ratio mass spectrometry. *Fungal Ecology*, submitted.

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Abkürzungsverzeichnis

ANOVA	Varianzanalyse
BaCl ₂	Bariumchlorid
BaCO ₃	Bariumcarbonat
C	Kohlenstoff
¹³ C	Kohlenstoffisotop mit der Masse 13
CaCl ₂	Calciumchlorid
CO ₂	Kohlenstoffdioxid
CV	Variationskoeffizient
DFG	Deutsche Forschungsgemeinschaft
EA	Elementaranalysator
Fl	Fluoreszenz
g	Gramm
GalN	Galaktosamin
GC	Gaschromatographie
GC-C-IRMS	Gaschromatographie-Verbrennungs-Isotopenmassenspektrometrie
GlcN	Glucosamin
Gt	Gigatonne
H ₂ O	Wasser
H ₃ BO ₃	Borsäure
H ₃ PO ₄	Phosphorsäure
HCl	Salzsäure
HNO ₃	Salpetersäure
HPAEC	Hochleistungsflüssigkeitanionenaustauschchromatographie
HPCEC	Hochleistungsflüssigkeitkationenaustauschchromatographie
HPEXC	Hochleistungsflüssigkeitanionenausschlusschromatographie
HPLC	Hochleistungsflüssigkeitchromatographie
KH ₂ PO ₄	Kaliumdihydrogenphosphat
KOH	Kaliumhydroxid
IRMS	Isotopenverhältnismassenspektrometrie
LC	Flüssigkeitchromatographie
LOD	Nachweisgrenze

LOQ	Bestimmungsgrenze
M	Molar
ManN	Mannosamin
mg	Milligramm
µg	Mikrogramm
min	Minute
ml	Milliliter
µl	Mikroliter
mmol	Millimolar
µmol	Mikromolar
MurN	Muraminsäure
mV	Millivolt
N	Stickstoff
Na	Natrium
Na ₂ HPO ₄	Natriumhydrogenphosphat
NaOH	Natronlauge
NaNO ₃	Natriumnitrat
Na ₂ S ₂ O ₈	Natriumpersulfat
(NH ₄) ₂ SO ₄	Ammoniumsulfat
ODS	Octadecylsilan
OH ⁻	Hydroxidion
OPA	ortho-Phthaldialdehyd
PAD	gepulster amperometrischer Detektor
PDB	PeeDeeBelmnite
pH	negativer dekadischer Logarithmus der Wasserstoffionenaktivität (<i>potentia Hydrogenii</i>)
R-	organischer Rest
<i>r</i>	Korrelationskoeffizient
RP	Umkehrphase
RSA	relative spezifische Allokation
s	Sekunde
SAX	starker Anionenaustauscher
SCX	starker Kationenaustauscher
SD	Standardabweichung

SOC	organischer Kohlenstoff des Bodens
SO ₃ ⁻	Sulfonsäureion
WHC	Wasserhaltekapazität

1. Einleitung

1.1 Vorkommen und Bedeutung von Aminosuktern

Aminosucker sind als Bestandteile von Zellwänden für viele Organismen von Bedeutung. So sind sie in der bakteriellen Zellwand zu finden, und in Pilzen sowie in Invertebraten liefern sie den Baustein für das strukturbildende Chitin. Aminosucker sind bis zu einem gewissen Maße aber nicht nur in der Zellwand sondern auch in anderen Zellkompartimenten nachgewiesen worden (Parsons, 1981). Bei höheren Lebewesen kommen sie insbesondere als Baustein in Mucopolysacchariden vor. Mucopolysaccharide sind Bestandteile vieler Makromoleküle. So sind sie als Faserstoffe in Zellmembranen aber auch im Blut oder Frauenmilch zu finden (Ternes et al., 2007).

Besondere Bedeutung gebührt den Aminosuktern jedoch in den Bodenwissenschaften, wo sie als Indikatoren für die mikrobielle Residualmasse (abgestorbene mikrobielle organische Substanz) fungieren. Denn Aminosucker sind im Boden überwiegend Bestandteile von Mikroorganismen (Pilze und Bakterien). Mikroorganismen spielen im Boden eine zentrale Rolle. Sie sind verantwortlich für die Bodenfruchtbarkeit und –qualität. So wandeln die Mikroorganismen organisches Material (Erntereste oder organische Dünger) in pflanzenverfügbare Nährstoffe um (Balot et al., 2003, Wu et al., 1993). Weiterhin wächst im Kontext der globalen Erwärmung die Bedeutung der C-Sequestrierung (Humusanreicherung) von Böden (Bodé et al., 2009). Böden sind in der Lage schätzungsweise 2300 Gt Kohlenstoff zu sequestrieren (IPCC, 2007). Um jedoch fundierte Aussagen über sequenzierten Kohlenstoff treffen zu können, ist ein besseres Verständnis der Kohlenstoffbiogeochemie unverzichtbar. Die Biogeochemie hängt wiederum von der mikrobiellen Gemeinschaft innerhalb eines Ökosystems ab (Amelung, 2001).

Im Boden steuern Aminosucker 5 bis 12% des Gesamtstickstoffs bei (Stevenson, 1982) und ungefähr 3% des organischen Bodenkohlenstoffs (Jørgensen und Meyer, 1990). Es gibt bis zu 26 Aminosucker (Sharon, 1965), wobei bisher nur Glucosamin, Galaktosamin, Muraminsäure und Mannosamin in Böden quantifiziert werden. Da höhere Pflanzen Aminosucker nicht in signifikanten Mengen enthalten, können sie als Indikatoren für die Akkumulation von mikrobiellen Residuen (abgestorbene organische mikrobielle Substanz) im Boden (Amelung et al., 2002; Amelung 2003; Liang et al. 2006, 2007a/b) und für die mikrobielle Biomasse (lebende organische mikrobielle Substanz) auf frisch besiedelten Substraten wie Weizenstreu herangezogen werden. So ist während des Abbaus von

1. Einleitung

Pflanzenmaterial wie zum Beispiel von Buchenblattstreu die kontinuierliche Zunahme des Aminosuckergehaltes speziell nur auf die mikrobiellen Abbauprozesse zurückzuführen (Scholle et al., 1993). Im Gegensatz zu anderen Zellwandkomponenten wie Ergosterol oder Phosphorlipidfettsäuren, die nach dem Zelltod sehr schnell abgebaut werden, haben Aminosucker die Eigenschaft zur Akkumulation in Huminstoffen (Bondietti et al., 1972) und anderen Komponenten des organischen Bodenmaterials (Jørgensen and Meyer, 1990, Coelho et al., 1997, Amelung, 2001). Somit können nur Aminosucker als Indikatoren der mikrobiellen Biomasse für die Bestimmung der mikrobiellen Besiedlung auf lebendem oder gerade abgestorbenem Pflanzenmaterial zu Rate gezogen werden. Weiterhin konnte wiederholt gezeigt werden, dass Aminosucker nützliche Indikatoren für die Akkumulation von verschiedenen Arten mikrobieller Residuen in Böden sind (Amelung et al., 1999, Guggenberger et al., 1999, Amelung et al., 2002, Amelung, 2003, Liang et al., 2007 ab).

Eine besondere Bedeutung kommt dabei den Aminosuckern Glucosamin und Muraminsäure zu. Diese Aminosucker sind spezifisch für zwei Hauptgruppen von Bodenmikroorganismen. So kommt Muraminsäure ausschließlich in der bakteriellen Zellwand insbesondere im Mureingerüst Gram-positiver Bakterien vor (Millar und Casida, 1970, Kenne und Lindburg, 1983). Glucosamin kommt hauptsächlich in der pilzlichen Zellwand vor (Appuhn und Jørgensen, 2006). Bakterien enthalten ebenfalls Glucosamin als Bestandteil des Zellwand-Peptidoglykans. Durch die von Appuhn und Jørgensen (2006) bestimmten Umrechnungsfaktoren ist es möglich den pilzlichen und den bakteriellen Anteil in mikrobiellen Residuen zu bestimmen. So wird pilzlicher Kohlenstoff durch Multiplikation des pilzlichen Glucosamins mit 9 und bakterieller Kohlenstoff durch Multiplikation mit 45 erhalten. Wobei pilzliches Glucosamin in Wurzel- und Bodenhydrolysaten durch das Subtrahieren des bakteriellen Glucosamins vom Gesamtglucosamin (mol Glucosamin – mol Muraminsäure) erhalten wird. Dabei wird angenommen, dass Muraminsäure und Glucosamin im Verhältnis 1 zu 2 in bakteriellen Zellwänden vorhanden ist (Engelking et al., 2007b). Die Funktion des theoretischen Verhältnisses von Glucosamin und Muraminsäure in der bakteriellen Zellwand von 1 zu 1 ist unbekannt (Zelles und Alef, 1995).

Wenig ist bekannt über den Ursprung von Galaktosamin im Boden, obwohl es einen Anteil von 30 bis 50% des Aminosuckergehalts ausmacht. Galaktosamin steuert durchschnittlich 4% in Bakterienkulturen und 15% in Pilzkulturen zum Gesamtaminozuckergehalt bei. Engelking et al. (2007b) konnten nach der Zugabe von Glucose zum Boden keine Veränderungen bezüglich des Galaktosamingehalts feststellen.

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Noch weniger ist über den Ursprung von Mannosamin bekannt (Amelung 2001; Amelung et al. 2008), obwohl Autoren wie Amelung et al. (1999) und Guggenberger et al. (1999) signifikante Mengen an Mannosamin in verschiedenen Böden bestimmt haben. Mannosamin ist Bestandteil vieler Organismen. So kommt es im pilzlichen Melanin (Coelho et al. 1997) und als Komponente von Sialinsäure vor. Sialinsäure ist in Pilzen wie *Aspergillus fumigatus* (Wasylnka et al., 2011) und in Bakterien (Ferrero und Aparicio. 2010, Yoneyama et al., 1982) enthalten.

1.2 Aminosuckeranalytik – Allgemeine Chemie der Aminosucker

Für ein besseres Verständnis der Aminosuckeranalytik ist es wichtig die chemische Struktur und damit auch die chemischen Eigenschaften der zu bestimmenden Aminosucker zu kennen. Aminosucker sind Monosaccharide, deren Hydroxylgruppe durch eine Aminogruppe ersetzt ist (Abb.1). Dabei wird zwischen Glycosylaminen und Aminodesoxyzuckern unterschieden. Bei Glycosylaminen ist das Stickstoffatom an das anomere Kohlenstoffatom gebunden, bei Aminodesoxyzuckern ersetzt das Stickstoffatom das Sauerstoffatom an einer anderen Stelle im Molekül. Somit handelt es sich nur dann um Aminosucker, wenn das Stickstoffatom das Sauerstoffatom nicht am anomeren Kohlenstoffatom ersetzt hat (Vollhardt und Schore, 2000).

1. Einleitung

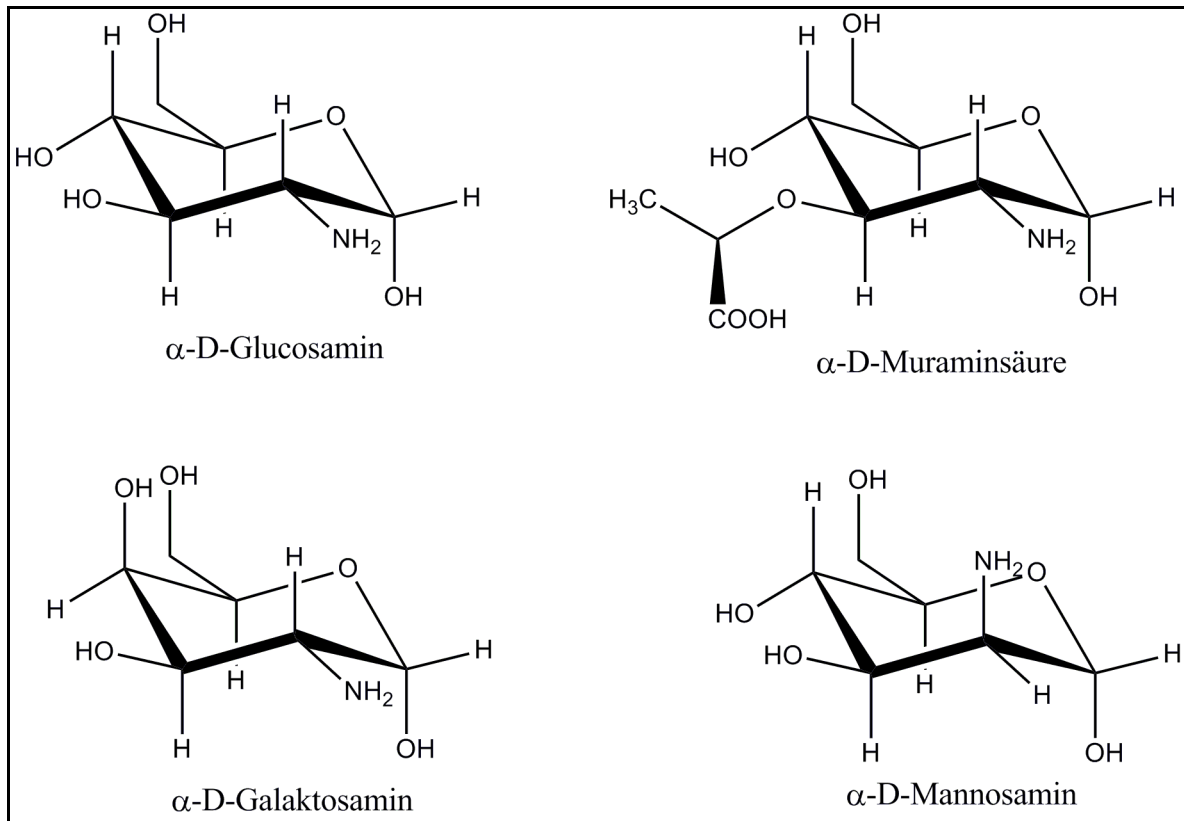


Abb. 1 Strukturformeln der Aminozucker-Monomere

In der Natur kommen Aminozucker nur gebunden vor. So sind im Chitin, einem Polysaccharid, die Acetylglucosamineinheiten β -1,4-glykosidisch gebunden (Abb.2).

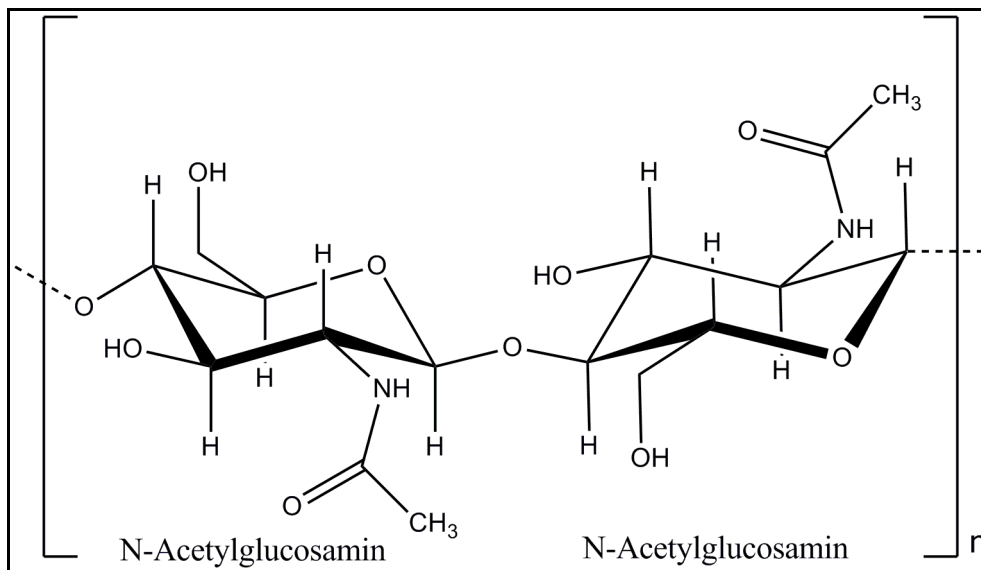


Abb. 2 Baueinheit von Chitin

Wohingegen im Peptidoglycan der bakteriellen Zellwand N-Acetylglucosamin und N-Acetylmuraminsäure β -1,4-glykosidisch miteinander verknüpft sind und lineare

1. Einleitung

Kettenmoleküle bilden. Dabei ist an N-Acetylmuraminsäure eine Kette aus 4 Aminosäuren angeknüpft (Abb.3). Bei grampositiven Bakterien ist in dem Tetrapeptid Diaminopimelinsäure durch L-Lysin ersetzt.

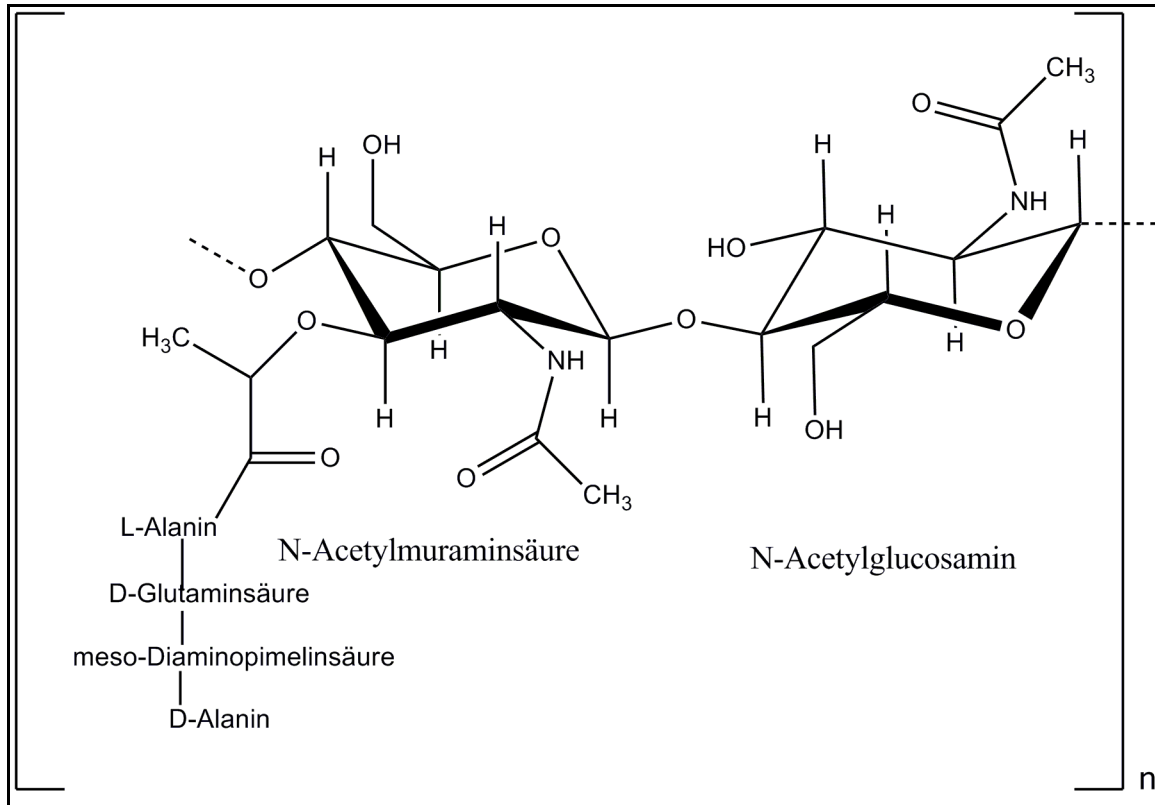


Abb. 3 Aufbau des Peptidoglycans (Murein) gramnegativer Bakterien

Um Aminosucker quantitativ bestimmen zu können, ist es notwendig, dass sie als Monomere vorliegen. Aminosucker sind im Gegensatz zu Zuckern aufgrund der Aminogruppe sehr stabil. Somit kann eine Hydrolyse, welche die Polymere in Monomere spaltet, mit Salzsäure erfolgen. Bei Verwendung von starken Mineralsäuren wie Salzsäure ist eine nahezu komplette Freisetzung der Monomere aus ihrem Polymerverband gewährleistet.

Durch ihre Aminogruppe zeigen Aminosucker ein basisches Verhalten, indem sie im sauren und neutralen Milieu ein Proton an das freie Elektronenpaar des Stickstoffs anlagern. Ein davon abweichendes chemisches Verhalten ist bei der Muraminsäure zu finden. Aufgrund ihrer Carboxylgruppe reagiert Muraminsäure nicht nur basisch sondern auch sauer. Ähnlich wie bei Aminosäuren (Vollhardt und Schore, 2000) ist davon auszugehen, dass Muraminsäure im sehr sauren Bereich ($\text{pH} < 1$) als diprotoniertes Kation (RNH_3^+ und R-COOH), im neutralen Bereich ($\text{pH} 6-7$) als monoprotoniertes Zwitterion (RNH_3^+ und R-COO^-) und im basischen Bereich ($\text{pH} > 13$) als deprotoniertes Aminocarboxylat-Ion vorliegt (RNH_2 und R-

1. Einleitung

COO^-), wobei R den Rest des Moleküls darstellt. Dieses abweichende Verhalten der Muraminsäure von den anderen Aminozuckern erschwert die Analytik der underivatisierten Aminozucker eminent.

1.3 Aminozuckeranalytik – Chromatographie der Aminozucker

In underivatisierter Form liegen die basischen Aminozucker (Glucosamin, Galaktosamin und Mannosamin) im neutralen und sauren Milieu (pH 2-7) in protonierter Form vor, wohingegen Muraminsäure monoprotiniert vorliegt. Für die Trennung von Kationen ist in der Flüssigkeitchromatographie (LC) die Kationenaustauschchromatographie vielversprechend. Bei der Kationenaustauschchromatographie sind an der stationären Phase Anionen (Abb.4a) (hier: Sulfonsäureionen) gebunden, die aufgrund der negativen Ladung positiv geladene Teilchen binden und retardieren können (Cammann, 2001). Die Aminozucker werden mit einem schwach sauren gepufferten Eluenten auf die Säule befördert und liegen dabei in positiv geladener Form vor. Eine Ausnahme bildet hier jedoch Muraminsäure. Die negativ geladene Carboxylgruppe der Muraminsäure verkürzt die Retardierung auf dem Säulenmaterial. Somit reagiert Muraminsäure nur sehr gering mit dem Säulenmaterial und eluiert kurz nach der Totzeit des Systems. Eine Trennung erfolgt aufgrund der unterschiedlichen Affinitäten der Aminozucker zu dem Säulenmaterial.

Im basischen Milieu liegen die Aminozucker deprotoniert vor. Hier erfolgt für die basischen Aminozucker, ähnlich wie bei Einfachzuckern, eine Bindung mit dem positiv geladenen Harz und den negativ geladenen Hydroxylgruppen (Abb.4b). Da die Carboxylgruppe der Muraminsäure eine viel stärkere Affinität zu dem Säulenmaterial hat als die Hydroxylgruppen des basischen Eluenten (Natriumhydroxid), verbleibt sie länger auf der Säule und kann nur durch Eluenten mit hoher Elutionskraft (z.B. Acetat) eluiert werden

1. Einleitung

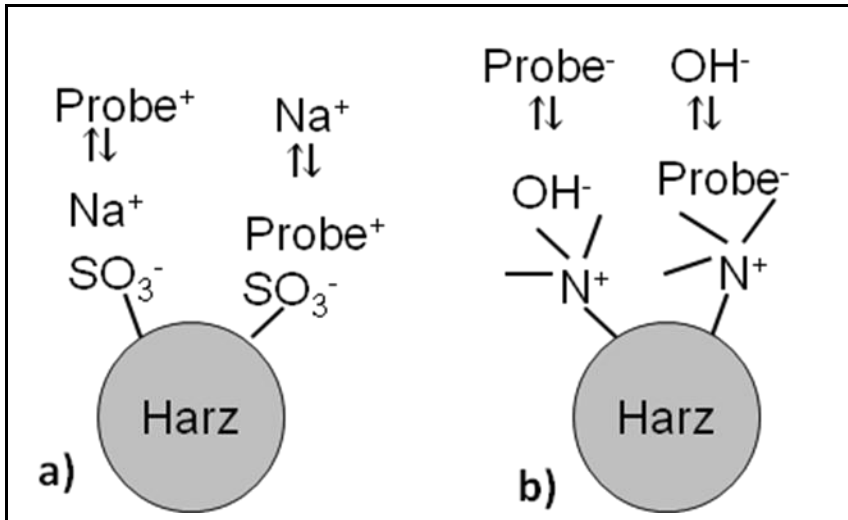


Abb. 4 a) Prinzip des Trennmechanismus Kationenaustauschchromatographie, **b)** Anionenaustauschchromatographie

Nach erfolgreicher Trennung werden die Aminosucker detektiert. Dies erfolgt entweder mittels Nachsäulenderivatisierung und Fluoreszenzdetektion oder mittels massenspektrometrischen Detektoren. Die chromatographische Trennung mittels Anionen- oder Kationenaustauschchromatographie kann auch zur Probenaufbereitung verwendet werden. So können z.B. bei Anwendung der Kationenaustauschchromatographie die Aminosucker von neutralen und negativ geladenen Verunreinigungen aus der Matrix abgetrennt werden.

Werden die Aminosucker jedoch vor der chromatographischen Trennung zu neutralen und hydrophoben Molekülen (fluoreszierende Isoindolderivate) mittels Ortho-phthaldialdehyd (OPA) in fluoreszierende Isoindolderivate derivatisiert (Abb.5), ist eine Trennung durch die Umkehrphasenchromatographie (Abb.6a) sehr geeignet.

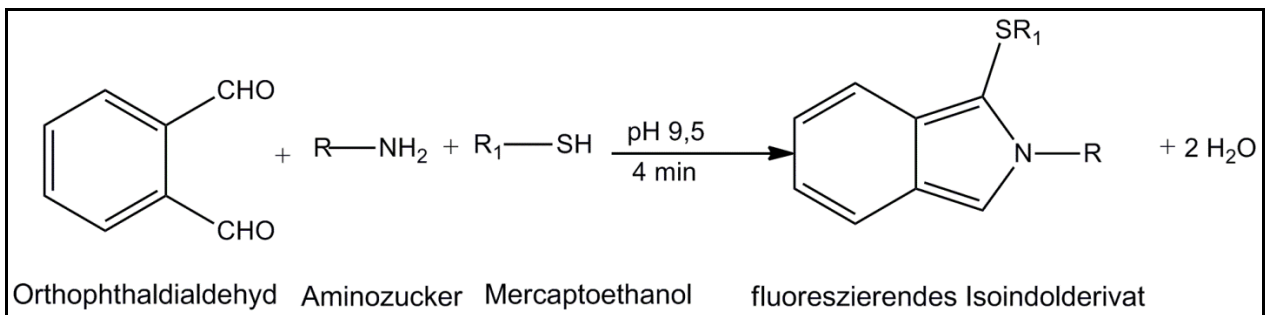


Abb. 5 Reaktionsmechanismus der OPA- Derivatisierung

Die Trennung erfolgt dabei an einer Umkehrphase (RP-Phase) (hydrophobe feste stationäre Phase mit C18-Ketten und aktiven Silanolgruppen; hier: Hypersil Octadecylsilan (ODS) –

1. Einleitung

Säule) (Abb.6b), wobei der Analyt Wechselwirkungen mit der stationären Phase eingeht (Abb. 6a). Die Wechselwirkungen sind in erster Linie polare Wechselwirkungen wie Dipol-Dipol-Reaktionen und Wasserstoffbrückenbindungen) sowie sterische Aspekte. Weiterhin spielen hydrophobe Wechselwirkungen eine mögliche jedoch untergeordnete Rolle für die Trennung an einer RP-Phase (Cammann, 2001). Bei dieser Methode übt die Derivatisierung der Analyten mit OPA auch Einfluss auf die Güte der Trennung aus. Durch die Derivatisierung der Aminosucker mit OPA werden fluoreszierende, hydrophobe, neutrale Isoindolderivate erhalten, die aufgrund ihrer Molekülstruktur zu Wechselwirkungen basierend auf sterischen Aspekten mit dem Säulenmaterial befähigt sind. Demzufolge ist für diese Methode eine Nachsäulenderivatisierung eher ungeeignet

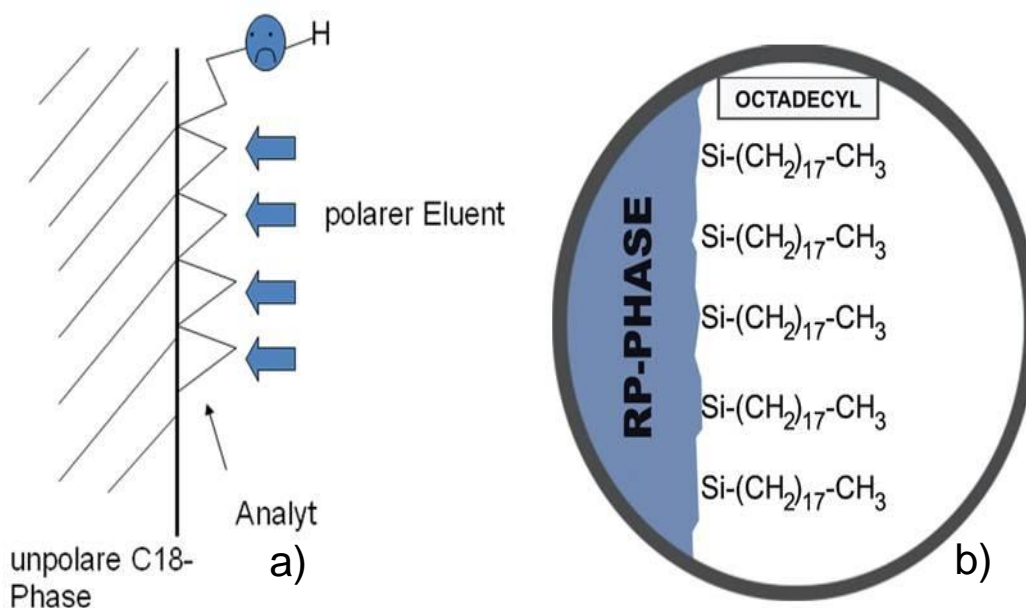


Abb. 6 a) Prinzip des Trennmechanismus Umkehrphasen(RP)-Chromatographie, **b)** Säulenmaterial Umkehrphasen(RP)-Chromatographie

1.4 Aminosuckeranalytik – Isotopenmassenspektrometrie

Die Bestimmung stabiler Isotope ist ein wichtiger Bestandteil innerhalb der Umweltforschung und gewinnt zunehmend an Bedeutung für die Grundlagenforschung in Bereichen wie Lebensmittelchemie, Biochemie sowie Klinische- und Pharmazeutische Chemie (Monsadl et al., 2007). Kohlenstoff hat drei natürlich vorkommende Isotope (¹²C, ¹³C, und ¹⁴C), wobei ¹²C und ¹³C stabile und ¹⁴C radioaktive Isotope sind. Die natürliche Abundanz in der Umwelt beträgt für ¹²C ca. 98,89%, für ¹³C 1,11% (Boutton, 1996) und für ¹⁴C <10⁻¹⁰ % (Goh, 1991). Die unterschiedliche Anzahl an Neutronen im Kern stabiler Isotope eines Elements haben Auswirkungen auf physikalische und chemische Eigenschaften von

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Molekülen, die diese Isotope enthalten. Diese sogenannten Isotopeneffekte machen sich in der Kinetik biochemischer Effekte, welche an der Entstehung und am Abbau organischer Substanz beteiligt sind, bemerkbar. So führt beispielsweise die Diskriminierung des reaktionsträgeren Kohlenstoffisotops (^{13}C) zu einer Abreicherung in gebildeter organischer Substanz im Vergleich zu dem atmosphärischen CO_2 . Diese Abreicherung bzw. Defizit an CO_2 ist nicht für alle Pflanzen gleich, sondern vom Photosynthesemechanismus der Pflanzen abhängig. Bei C_3 Pflanzen stellt das erste fassbare Produkt der CO_2 -Fixierung eine C_3 -Verbindung (3-Phosphoglycerinsäure) dar. Dabei wird das CO_2 an die Ribulose-1,5-Bisphosphat gebunden, wobei ein Zwischenprodukt mit sechs C-Atomen entsteht (C_6 -Körper), das sofort in zwei C_3 -Körper (PGS = 3-Phosphoglycerinsäure) zerfällt. Diese Reaktion wird durch das Enzym Ribulose-Bisphosphat-Carboxylase/Oxygenase (RuBisCo) katalysiert. Bei C_4 -Pflanzen ist das erste fassbare Produkt der CO_2 -Fixierung eine C_4 -Verbindung (Oxalessigsäure). Dabei wird CO_2 durch die Phosphoenolpyruvat (PEP)-Carboxylase in das Phosphoenolpyruvat eingebaut, wodurch nach Abspaltung des anorganischen Phosphats Oxalessigsäure entsteht (Nultsch, 2000). So diskriminieren C_4 Pflanzen weniger gegen $^{13}\text{CO}_2$ als C_3 Pflanzen und haben daher auch einen höheren delta-Wert (Abb.7). Der delta (δ)- Wert ist ein häufig verwendetes Maßsystem zur Angabe von Isotopenverhältnissen. Er beschreibt die Abweichung des Isotopenverhältnisses relativ zu einem Standard. Der Standard zur Bestimmung des $\delta^{13}\text{C}$ - Wertes ist auf den international anerkannten PDB (PeeDeeBelmnite)-Standard festgelegt. PDB ist ein fossiler Kohlenstoff aus der Kreidezeit *Bellefornitella americana* aus den Peedee Formation in South Carolina, USA (Carle, 1991). Aufgrund der unterschiedlichen Photosynthesemechanismen liegen die $\delta^{13}\text{C}$ -Werte für C_4 -Pflanzen zwischen -9 und -17‰ PDB und für C_3 -Pflanzen zwischen -22 und -32 ‰ PDB (Boutton, 1996). So können, das System Boden-Pflanze betrachtend, bei einer Vegetationsänderung (C_3 zu C_4 -Pflanzen) Veränderungen im C_4 -bürtigen Kohlenstoff im Boden, in der mikrobiellen Biomasse sowie im CO_2 des Bodens gemessen werden (Gregorich et al., 1996, Collins et al., 1999, Ludwig et al., 2003).

1. Einleitung

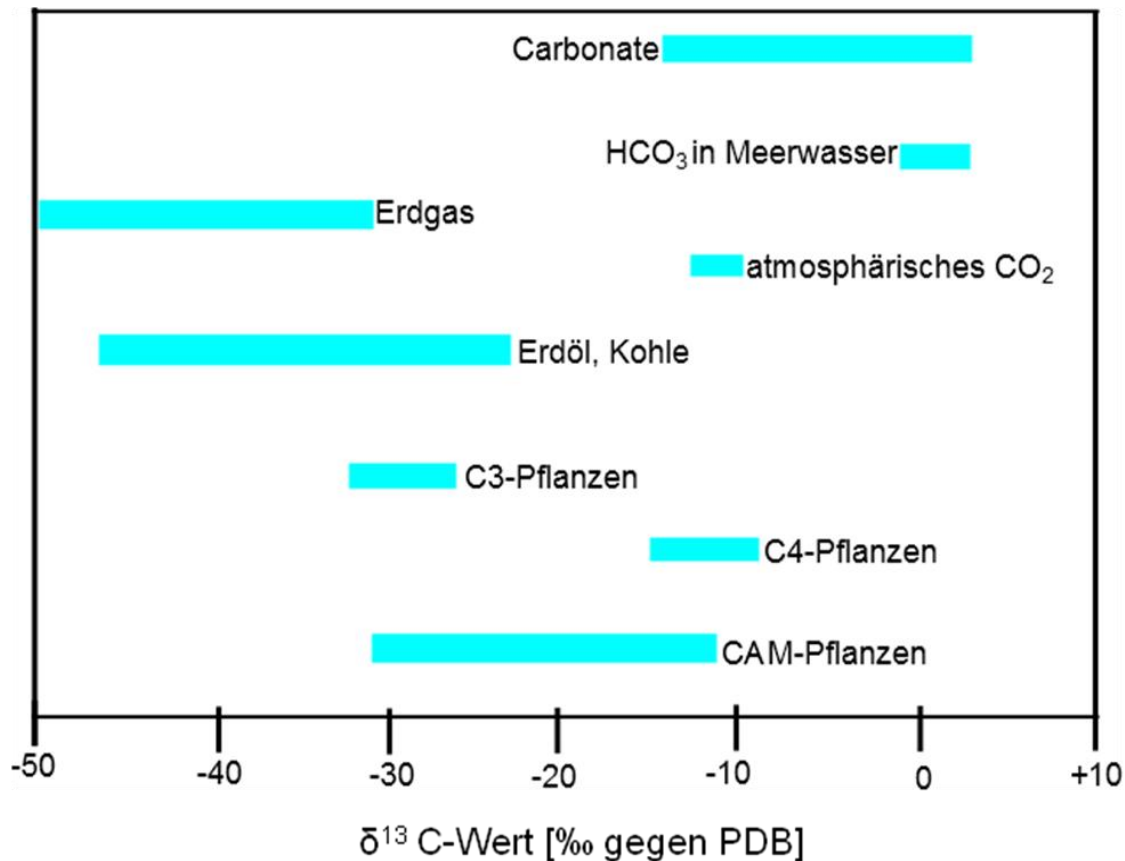


Abb. 7 ¹³C-Isotopengehalt der wichtigsten Kohlenstoff reservoirs der Erde (Carle, 1991)

Auch Mikroorganismen sind in der Lage die $\delta^{13}\text{C}$ zu diskriminieren. In einem ungestörten Boden-Pflanze- System hat die organische Substanz des Bodens ungefähr den gleichen $\delta^{13}\text{C}$ -Wert wie die Pflanzengesellschaft (Boutton, 1996). Die mikrobielle Biomasse nimmt die in Boden eingearbeiteten Materialien auf und wandelt sie in neue Komponenten um. Bei dieser Umwandlung kommt es zu Isotopeneffekten. Zum Beispiel veratmen Mikroorganismen CO_2 mit geringerem ¹³C-Anteil, wohingegen schwere Moleküle in der mikrobiellen Biomasse und der restlichen organischen Bodensubstanz angereichert werden (Nadelhofer und Frey, 1988). Andere Autoren wie Agren et al. (1996) gehen aber davon aus, dass bei einer Mineralisation von komplexen Komponenten (z.B. Lignin) mit hohem ¹²C-Anteil die verbleibende organische Substanz ¹³C angereichert ist, während bei Verbrauch von leicht verfügbaren Komponenten, die ¹³C angereichert sind, das verbleibende Material ¹³C abgereichert ist.

Mit der Isotopenverhältnismassenspektrometrie (IRMS) ist es möglich diese Effekte zu bestimmen. Die Kopplung eines Isotopenverhältnismassenspektrometers mit einem Hochleistungsflüssigkeitschromatographie (HPLC) - System bietet die Möglichkeit die

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Isotopie einzelner Analyte zu bestimmen. Dadurch wird es möglich die Umsatzdynamiken bakterieller und pilzlicher Mikroorganismen direkt zu erfassen.

2. Ziele der Arbeit

Ziel der Arbeit ist es, eine aminozucker-spezifische $\delta^{13}\text{C}$ Methode mit hoher Genauigkeit zur Erfassung der Bildung und Nutzung von mikrobiellen Residuen in Böden- und Pflanzenmaterialien zu etablieren und anzuwenden. Die von Glaser und Gross (2005) beschriebene aminozucker-spezifische $\delta^{13}\text{C}$ -Methode mittels Gaschromatographieverbrennung (GC-C)-IRMS ist aufgrund der vorherigen offline-Derivatisierung der Aminozucker zeitaufwendig und ungenau. Die Ungenauigkeit der Methode ist auf die Derivatisierung zurückzuführen, da diese zu Fraktionierung und zur Einbringung zusätzlicher C-Atome führt. Dafür soll zunächst die HPLC-RP-Methode auf der Basis von (Appuhn et al., 2004) modifiziert und an die verfügbare HPLC adaptiert werden. Nach erfolgreicher Validierung dieser HPLC-RP-Methode ist eine weitere Methode zur Bestimmung von Aminozuckern an einer Hochleistungsanionenaustauschchromatographie (HPAEC) (Bodé et al., 2009) zu optimieren und zu validieren. Die Einführung der HPAEC-Methodik ist erforderlich, um kohlenstofffreie Messungen durchzuführen, die wiederum für die spätere Messung der $^{13}\text{C}/^{12}\text{C}$ - Verhältnisse mit einem IRMS notwendig sind. Nach der Kalibrierung der HPAEC-Methode soll diese mit der RP-HPLC-Methode verglichen werden. Weiterhin sind andere chromatographische Methoden mit kohlenstofffreien Eluenten zu testen (Kationenaustausch- und Ionenausschlusschromatographie) in Hinblick auf eine schnellere und effizientere Trennung der Aminozucker. Die verschiedenen HPLC-Methoden sollen dann mit der validierten RP-HPLC- Methode verglichen werden.

Zusätzlich sind verschiedene Purifikationsmethoden zur Eliminierung von Matrixsubstanzen und zur Konzentrierung von Muraminsäure in den Probenhydrolysaten zu testen. Die Konzentrierung von Muraminsäure ist notwendig, da die LC-IRMS im Vergleich zur RP-HPLC eine um ca. 400 fach geringere Empfindlichkeit bezüglich Muraminsäure hat. Als Purifikationsmethoden können unter anderem Kationen- und Anionenaustauscherharze verwendet werden.

Nach erfolgreicher Kalibrierung der HPAEC-IRMS wird mit der neu eingeführten Methodik ein Pilzwachstumsversuch durchgeführt, um die Bildung und den Umsatz saprotropher Pilze zu bestimmen. Der Hintergrund des Pilzwachstumsversuchs sind folgende Fragestellungen:

- a) Welches Substrat wird bevorzugt von den saprotrophen holzzeretzenden Pilzen abgebaut?

2. Ziele der Arbeit

- b) Worauf lässt sich die in der Literatur beschriebene $\delta^{13}\text{C}$ -Anreicherung in den saprotrophen Pilzen während des Pilzwachstums zurückführen? Inwiefern spielen kinetische Isotopenfraktionierung und Inkorporation des Substrat-Kohlenstoffs dabei eine Rolle?

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

Biology and Fertility of Soils

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Abstract

Amino sugars are increasingly used as indicators for the accumulation of microbial residues in soil and plant material. A reverse-phase high-performance liquid chromatography (HPLC) method was improved for the simultaneous determination of muramic acid, mannosamine, glucosamine, and galactosamine in soil and plant hydrolysates via ortho-phthaldialdehyde (OPA) pre-column derivatisation and fluorescence detection. The retention time was reduced and the separation of muramic acid and mannosamine was optimised by modifying the mobile phase. The effects of excitation wavelength, OPA reaction time, tetrahydrofuran concentration and pH value of the mobile phase on the amino sugar separation were tested. Quantification limits were in the range of 0.13 to 0.90 $\mu\text{g ml}^{-1}$. No interferences exist from amino acids or other primary amines, occurring in soil and plant hydrolysates.

Key words: amino sugars / HPLC / ortho-phthaldialdehyde / microbial residues

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3.1. Introduction

Amino sugars make a significant contribution of 5 to 12% to soil organic N (Stevenson 1982) and roughly 3% to soil organic C (Joergensen and Meyer 1990). Up to 26 amino sugars have been found in microorganisms (Sharon 1965), whereas four of them have been quantified in soil. These amino sugars are glucosamine, galactosamine, muramic acid and mannosamine (Amelung et al. 2008). It has repeatedly been shown that amino sugars are useful indicators for the accumulation of different types of microbial residues in soil (Amelung et al. 2002; Amelung 2003; Liang et al. 2006, 2007a/b). Fungal cell walls are the major source of glucosamine in soils (Appuhn and Joergensen 2006). Muramic acid occurs exclusively in bacterial cell walls, especially in the murein skeleton of Gram-positive species (Millar and Casida 1970; Kenne and Lindburg 1983). Also bacteria contain glucosamine in their peptidoglycan cell wall, but only the glucosamine that occurs in excess to muramic acid may be attributed to fungal sources (Chantigny et al. 1997; Guggenberger et al. 1999; Amelung 2001). Appuhn and Joergensen (2006) determined average conversion factors of 9 to convert fungal glucosamine to fungal C and 45 to convert muramic acid to bacterial C.

Galactosamine contributes roughly one third to the total sum of amino sugars observed in soil and is also nearly exclusively of microbial origin (Engelking et al. 2007). Galactosamine contributed on average 4% to the total amino sugar concentration in cultured bacteria and 15% in cultured fungi (Engelking et al. 2007). However, the function of galactosamine within bacterial and fungal cells and consequently the processes behind galactosamine formation during decomposition processes are still unknown. However, galactosamine in soil is still attributed to be mainly of bacterial origin (Amelung et al. 2008), which has been sometimes supported by correlation analysis (Rottmann et al. 2010). Virtually nothing is known about the origin of mannosamine (Amelung 2001; Amelung et al. 2008), although Amelung et al. (1999) and Guggenberger et al. (1999) found significant amounts in different soils. Mannosamine has been found in fungal melanin (Coelho et al. 1997) and as component of sialic acids of *Aspergillus fumigatus* on their conidial surface (Wasylnka et al. 2001). Mannosamine, i.e. N-acetyl D-mannosamine containing sialic acids are present as protective capsular components of bacteria, invading mammals (Ferrero and Aparicio 2010). However, small amounts of mannosamine may be also present as common linkage units between peptidoglycan and other bacterial cell wall components such as glycerol teichoic acid (Yoneyama et al. 1982).

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

Several methods for the determination of amino sugars have been published. Highly specific gas chromatographic analyses require difficult off-line derivatisation steps, e.g. derivatisation of the hydrolysis products in volatile aldonitrile acetates (Zhang and Amelung 1996). High performance liquid chromatography (HPLC) methods need either derivatisation steps (Diaz et al. 1996; Ekblad and Näsholm 1996; Appuhn et al. 2004) or special equipment for anion exchange chromatography combined with pulsed amperometric detection (Kaiser and Benner 2000; Benner and Kaiser 2003). This means that HPLC methods are generally less time consuming as they employ no or automated on-line derivatisation.

Appuhn et al. (2004) were the first to describe a method to determine the four amino sugars simultaneously. However, this methodological approach was hampered by two drawbacks: (1) The method was reliably working only on the Agilent 1100 HPLC equipment and (2) mannosamine was insufficiently separated in most soil hydrolysates, which resulted in erroneous high values usually omitted in further publications (Appuhn and Joergensen 2006; Engelking et al. 2007). The objective of the present paper was improving the method of Appuhn et al. (2004) to give reliable results for all four amino sugars in soil and plant hydrolysates using different HPLC equipments.

3.2. Materials and methods

3.2.1. Soil and plant samples

Method optimisation was implemented using six different soil samples (0-10 cm) taken from four arable and two forest sites in Germany (Hessia and Lower Saxony) differing in physical, chemical and microbial properties (Table 1) and five different plant litter samples (Table 2). Soil physical, chemical and biological properties of the soil samples and total C and N of litter samples were determined as described by Probst et al. (2008).

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

Table 1 Physical, chemical and microbiological properties of the soils used in this investigation

Soil	Clay	Silt	Sand	pH-H ₂ O	Soil		Microbial	
					organic C	Total N	biomass C	Ergosterol
					mg g ⁻¹ soil		μg g ⁻¹ soil	
Forest 1	15	77	8	3.9	59.9	4.3	610	3.2
Forest 2	6	39	55	3.9	58.0	2.4	520	5.4
Arable 1	35	55	10	7.0	18.6	1.8	450	1.6
Arable 2	34	56	10	7.3	15.1	1.4	360	1.1
Arable 3	18	66	16	7.8	14.0	1.2	200	0.5
Arable 4	8	8	84	7.4	7.8	0.7	180	0.6

Table 2 Chemical properties of the plant litter materials used in this investigation

Litter	Total C	Total N
	μg g ⁻¹ dry weight	
	Sugarcane filter cake	448
Maize leaves	440	22.0
Pea leaves	443	14.3
Amaranth straw	397	8.2
Wheat straw	440	4.1

The amino sugar extraction was based on the method described by Appuhn et al. (2004) with minor modifications. Sieved (< 2 mm) and air-dried soil (400 mg) or oven-dried (40 °C) and steel ball-milled (Retsch, Haan, Germany) plant material (700 mg) was mixed with 10 ml of 6 M HCl. After 6 h (soil) or 3 h (plant material) hydrolysis at 105°C the samples were filtered over glass filters (Whatman GF/A). For the determination of the recovery rate, 0.3 ml of a 150 μmol l⁻¹ standard solution was added to 0.3 ml of a quartz sand hydrolysate. A 0.3 ml aliquot was evaporated to dryness at 40-45 °C to remove HCl, re-dissolved in water, evaporated a second time and re-dissolved in 1 ml water. To test the effect of sample pH on the amino sugar amounts another set of samples was prepared using 1 ml phosphate buffer solution pH 7 (containing 40.8 mmol l⁻¹ Na₂HPO₄ × 2H₂O and 25.9 mmol l⁻¹ KH₂PO₄) to re-dissolve samples. After centrifugation at 5000 g, the supernatant was frozen and stored at -18 °C until analysis.

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3.2.2. Solutions

All solutions were prepared with Milli-Q water produced via a Direct-Q 3 system (Millipore, Billerica, MA, USA). All other reagents were of high purity ($\geq 95\%$). The buffer solution (pH 11) was prepared by dissolving 50 g H_3BO_3 in 900 ml water, adjusted to pH 11 with KOH (47% solution) and diluted to 1 l with water. This solution was stable for up to 12 months at 4 °C. The reducing solution was prepared by adding 2.5 ml 2-mercaptoethanol to 100 ml buffer solution. This solution was stable for up to 6 months at 4 °C in the dark. The derivatisation reagent was prepared by dissolving 25 mg ortho-phthaldialdehyde (OPA) in 2 ml methanol, mixed with 2 ml of reducing solution and diluting to 44 ml with buffer solution. The reagent was stable for up to 7 days at 4 °C in the dark.

For the standard stock solutions of the four amino sugars, standards (Sigma Aldrich, St. Louis, MO, USA) were dissolved in water to a concentration of 1000 $\mu\text{mol l}^{-1}$ (mannosamine, galactosamine, glucosamine) and 100 $\mu\text{mol l}^{-1}$ (muramic acid) respectively and stored at -18°C . Standard working solutions were prepared by diluting 4 aliquots of the stock solutions to a concentration range between 210 $\mu\text{mol l}^{-1}$ and 5 $\mu\text{mol l}^{-1}$ (mannosamine, galactosamine, glucosamine) and 21 $\mu\text{mol l}^{-1}$ and 0.5 $\mu\text{mol l}^{-1}$ (muramic acid), respectively. The working standard solutions were stable for over 12 months at -18°C .

For evaluating the interference of common amino acids and related compounds which are abundant in soil and plant hydrolysates, we measured two different amino acids standards (Sigma Aldrich, St. Louis, MO, USA). Amino acid standard solution 1 contained the following components at a concentration of 25 $\mu\text{mol l}^{-1}$ and 12 $\mu\text{mol l}^{-1}$ for L-cysteine, respectively: L-alanine, ammonium chloride, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-thyrosin, L-valine. Amino acid standard solution 2 contained the following components at a concentration of 50 $\mu\text{mol l}^{-1}$: γ -amino-n-butyric acid, ammonium chloride, L-anserine, L-arginine, L-carnosine, creatinine, ethanolamine, L-histidine, L-homocystine, δ -DL-hydroxylysine, L-lysine, 1-methyl-L-histidine, 3-methyl-L-histidine, L-ornithine and L-tryptophan.

3.2.3 Chromatographic conditions

Chromatographic separations were performed on a Phenomenex (Aschaffenburg, Germany) Hyperclone C_{18} (ODS) column (125 mm length \times 4 mm diameter, 5 μm particle size, 12 nm pore size), protected by a Phenomenex C_{18} security guard cartridge

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

(4 mm length × 2 mm diameter). The column was placed in a column oven set at 35 °C. The HPLC system consisted of a Dionex (Germering, Germany) P 580 gradient pump, a Dionex Ultimate WPS – 3000TSL analytical autosampler with in-line split-loop injection and thermostat and a Dionex RF 2000 fluorescence detector set at 445 nm emission and 330 nm excitation wavelength with medium sensitivity.

An autosampler designed for automated pre-column derivatisation was used because of the need of a high injection precision and an effective external needle wash for eliminating carryover. Another autosampler tested (Dionex ASI 100) yielded poor reproducibility. Vials with OPA reagent and samples as well as vials for preparation were stored in the autosampler at 15 °C. For derivatisation, 50 µl OPA reagent and 30 µl sample were mixed in a preparation vial and after 120 sec reaction time 15 µl of the indole derivatives were injected.

The mobile phase consisted of two eluents. Eluent A was a 97.8/0.7/1.5 (v/v/v) mixture of a water phase, methanol and tetrahydrofuran (THF). The water phase contained 52 mmol sodium citrate and 4 mmol sodium acetate, adjusted to pH 5.3 with HCl. Then methanol and THF were added. Eluent B consisted of 50% water and 50% methanol (v/v). For degassing and sterilisation both eluents were filtered over 0.2 µm pore size hydrophilic propylene membrane filters. The mobile phase was delivered at a flow rate of 1.5 ml min⁻¹. The amino sugar separation was performed isocratically but a gradient was used for cleaning the column after every run. Every run was starting at an eluent A/B v/v composition of 93/7 for 19 min. A linear gradient was run to reach 80% B after 3 min and remaining isocratic for 3 min. A reverse gradient to 93/7 within 3 min was followed by 2 min isocratic run after which the column is preconditioned for the next sample. It is important to start the OPA derivatisation only at the beginning of every run and not during column preconditioning of the previous run. Otherwise, the equilibration time is not long enough for a good reproducibility.

3.3. Results

3.3.1 Method optimisation

The chromatographic conditions used in the optimised method provided a good separation within approximately 19 min (Fig. 1). Retention times of examined amino sugars were: (1) muramic acid $t_R = 8.4$ min, (2) mannosamine $t_R = 9.4$ min, (3) galactosamine $t_R = 15.4$ min and (4) Glucosamine $t_R = 16.6$ min.

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

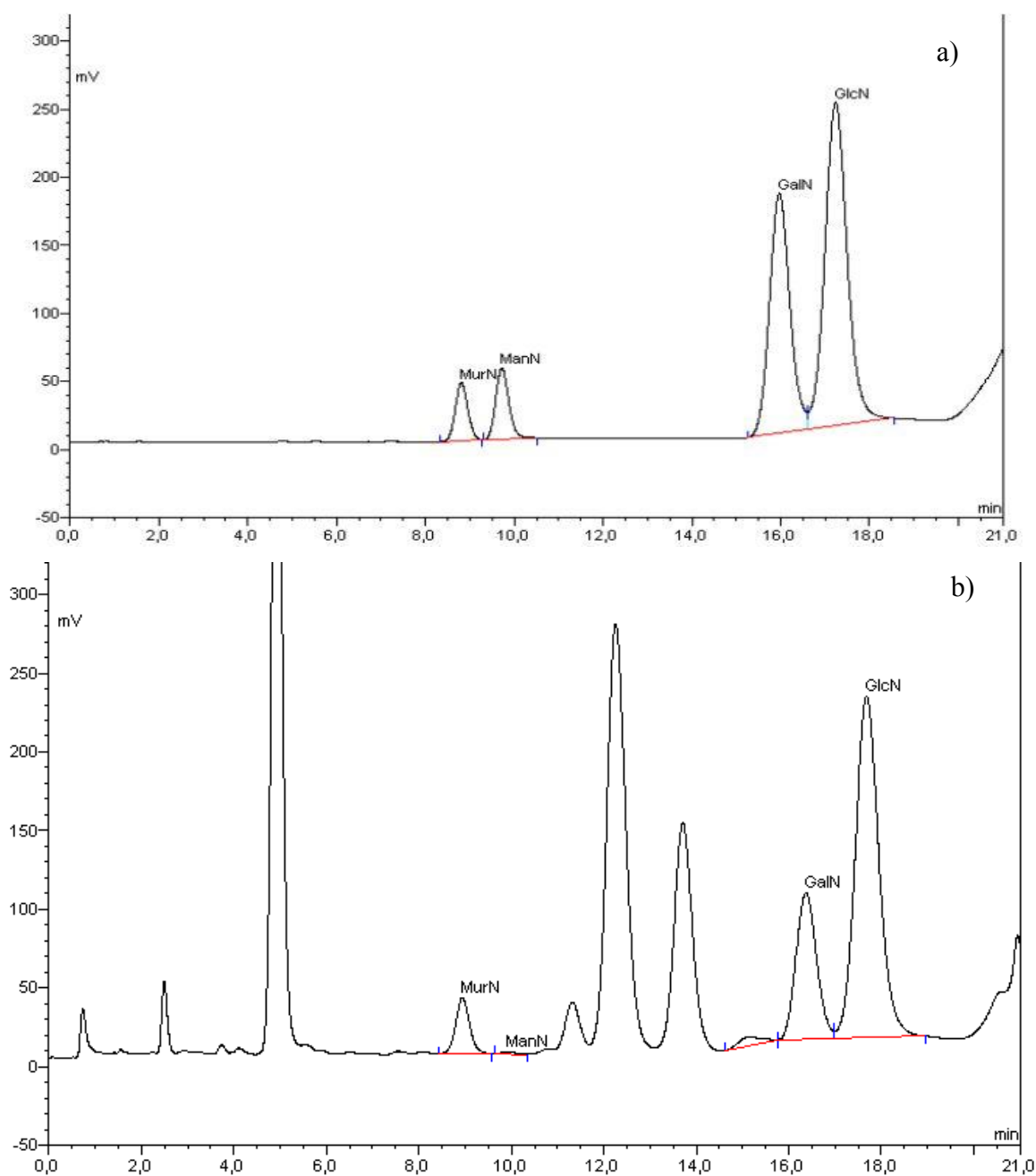


Fig.1 Chromatograms of (a) a standard mixture consisting of 13 μ mol l-1 muramic acid, 130 μ mol l-1 mannosamine, 130 μ mol l-1 galactosamine, 130 μ mol l-1 glucosamine and of (b) a soil hydrolysate (arable 1).

The components of the amino acid standard solution 1 occurred at 4.7 min, 10.5 min, 11.7 min, 12.6 min and > 20.0 min, respectively (Fig. 2b). The retention times of the amino acid standard solution 2 were at 10.5 min, 11.8 min, 18.1 min and > 20.min, respectively (Fig. 2c).

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

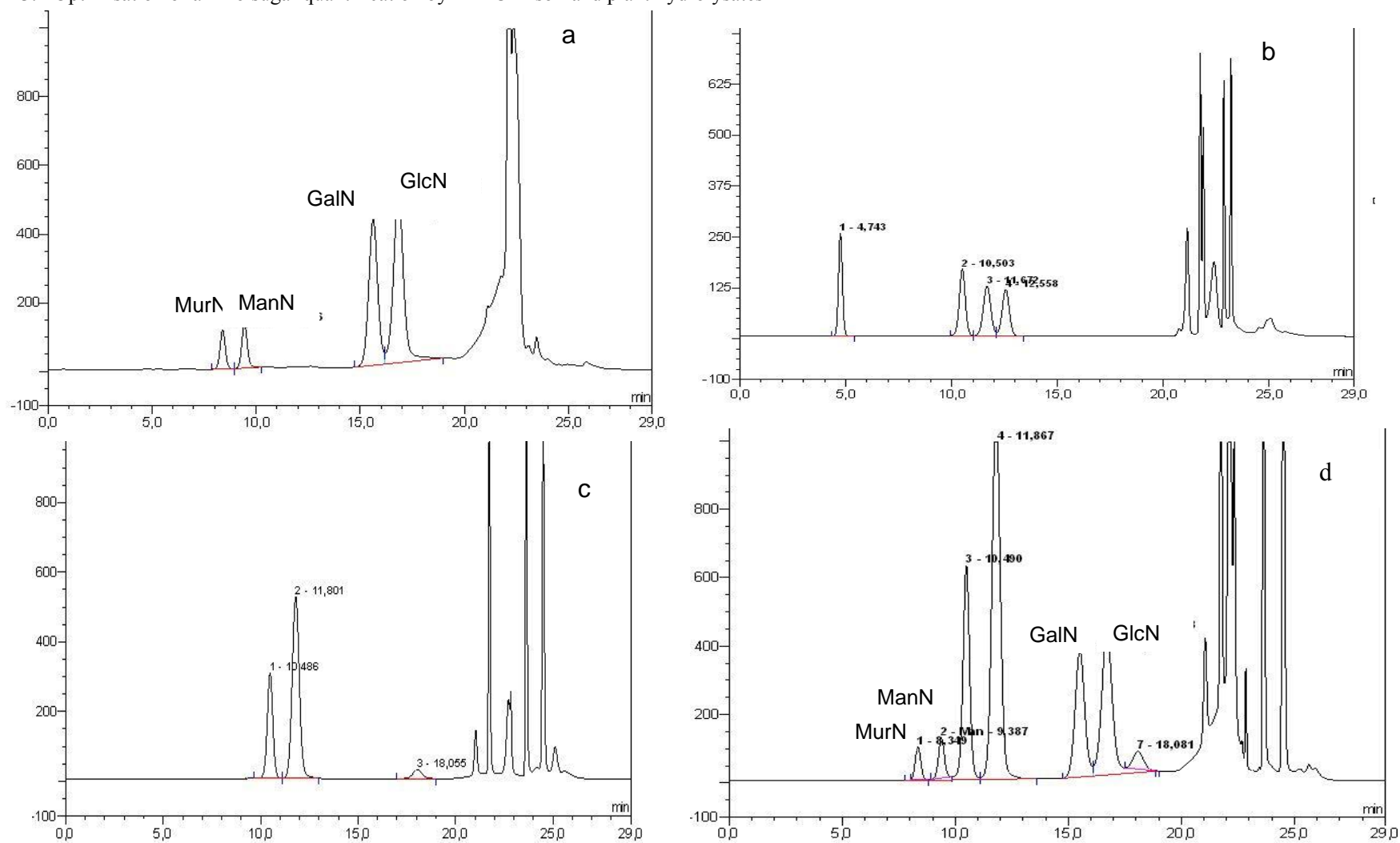


Fig. 2 Chromatograms of (a) an amino sugar standard mixture consisting of $17\mu\text{mol l}^{-1}$ muramic acid, $170\mu\text{mol l}^{-1}$ mannosamine, $170\mu\text{mol l}^{-1}$ galactosamine, $170\mu\text{mol l}^{-1}$ glucosamine and of (b) an amino acid standard 1, (c) an amino acid standard 2 and (d) an amino sugar standard spiked with an amino acid standard 2, components of amino acid standards (see materials and methods).

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For testing the absence of any interference potential, an amino sugar standard ($170 \mu\text{mol l}^{-1}$) was mixed with the amino acid standard 2 ($500 \mu\text{mol l}^{-1}$) at the ratio of 5:1. All four amino sugar peaks were well separated from those of the amino acids (Fig. 2d).

Analysis of a standard mixture at different excitation wavelengths, different OPA reaction times and different pH values of the eluent revealed maxima for the area of all four amino sugar peaks at an excitation wavelength of 330 nm, an OPA reaction time of 120 sec an eluent pH of 5.3 (with the exception of mannosamine) respectively (Fig. 3). Increasing THF concentration in the eluent from 0.75% to 1.50% yielded a better resolution between muramic acid and mannosamine as well as to shorter retention times for all four amino sugars (Fig. 3d).

The calibration curves (Fig. 4) were linear in the range from 5 to $210 \mu\text{mol l}^{-1}$ (mannosamine, galactosamine, glucosamine) and from 0.5 to $21 \mu\text{mol l}^{-1}$ (muramic acid), respectively, with good correlation coefficients and standard deviations (Table 3). The coefficient of variation was roughly 2% for intraday and 5% for interday precision, respectively (Table 3). This reflects the reproducibility and the precision of the method excluding sample preparation. The accuracy (expressed as recovery) for the four analytes was determined by spiked quartz sand hydrolysate with the standard mixture solution. The results of the recovery of mannosamine, galactosamine and muramic acid ranged from 105 to 115%. The recovery rate for glucosamine was 81%. The limit of quantification (LOQ) depended on the amino sugar and varied from 0.5 to $5 \mu\text{mol l}^{-1}$ for the standard mixture solution (Table 3).

The content of the different amino sugars increased in the order mannosamine < muramic acid < galactosamine < glucosamine (Table 4). In three arable soils mannosamine was below the LOQ value. The glucosamine content in the soil samples ranged from 980 to $2730 \mu\text{g g}^{-1}$ soil and contributed between 55 to 62% to the total amino sugar content. Galactosamine and muramic acid contributed on average 35% and 5%, respectively, mannosamine, if present, only 2%. The glucosamine content in the plant litter material ranged from 36 (maize leaves) to $1550 \mu\text{g g}^{-1}$ dry weight (amaranth straw) and contributed 58 (maize leaves) to 93% (wheat straw) to the total amino sugar content (Table 5). Muramic acid was below the LOQ values in green pea and maize leaves. Mannosamine was below LOQ in maize leaves and wheat straw, but contributed roughly 20% to the total amino sugar content in pea leaves. Soil samples dissolved in water had significantly higher amino sugar contents than those dissolved in buffer solution (Table 4). In contrast, the sample solvent had no effect on the amino sugar amount in plant material (Table 5).

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

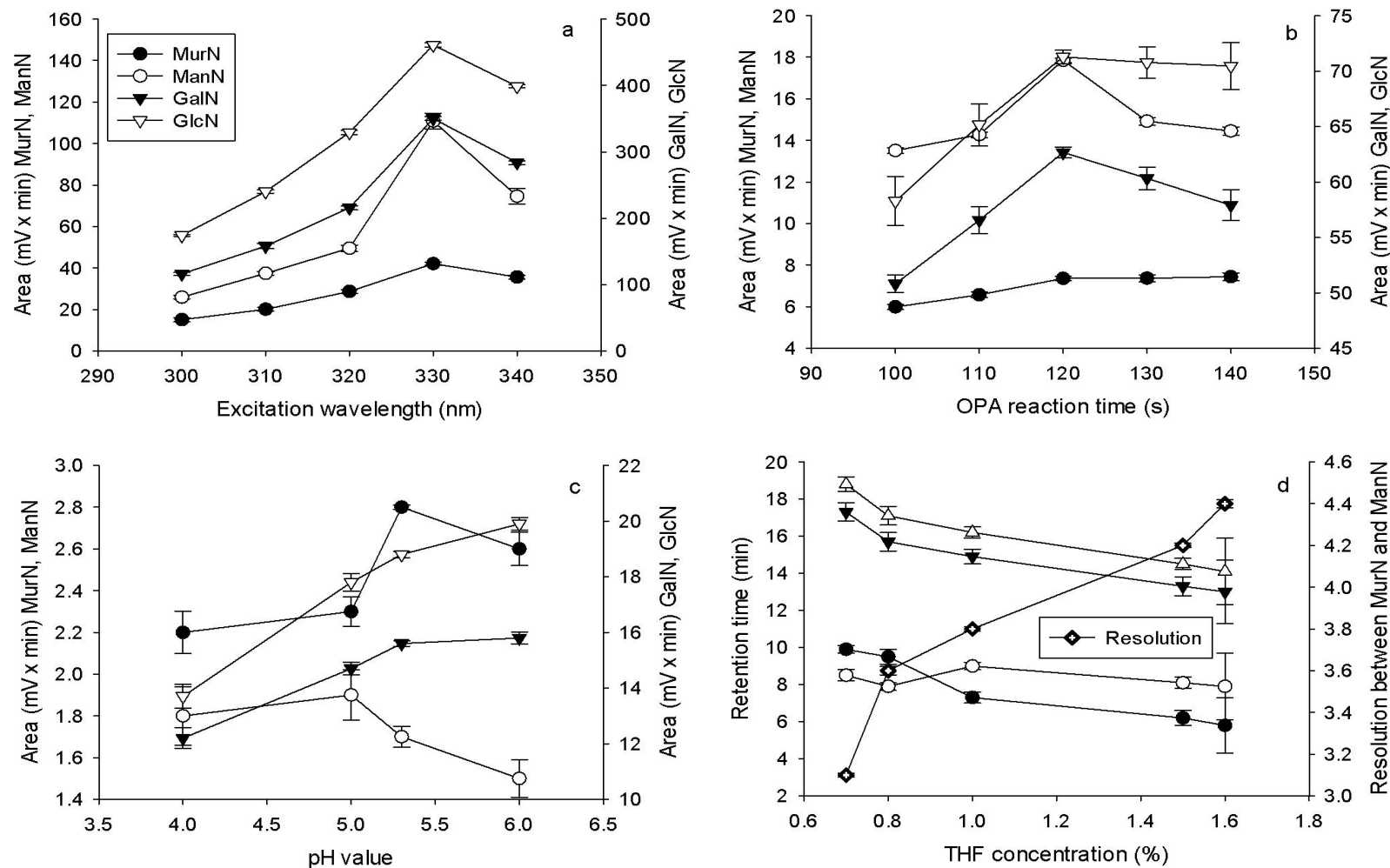


Fig. 3 Optimisation of HPLC parameters: (a) sensitivity of the amino sugar determination as a function of OPA reaction time; (b) optimisation of the excitation wavelength; (c) changes in area as a function of eluent pH value; (d) Change in resolution between muramic acid (MurN) and mannosamine (ManN) peaks and in retention time of MurN, ManN, galactosamine (GalN) and glucosamine (GlcN) as a function of tetrahydrofuran (THF) concentration in eluent (error bars represent \pm standard deviation).

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

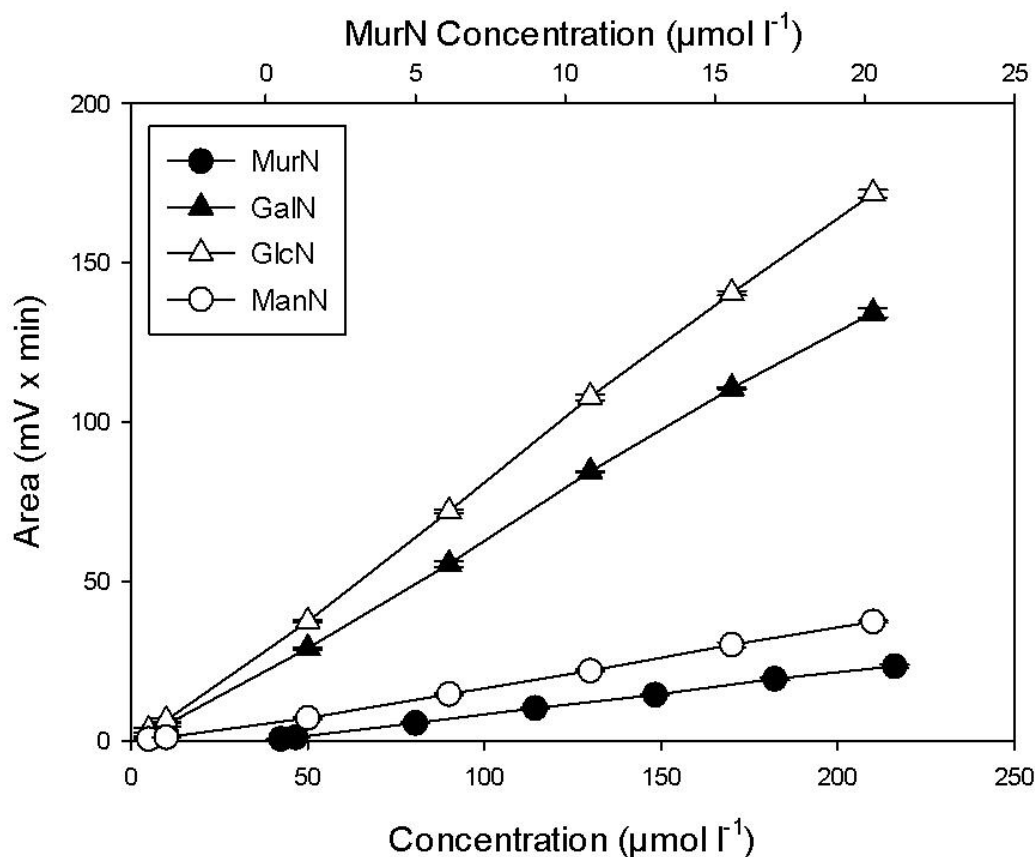


Fig. 4 Calibration curves of reference amino sugars (error bars represent \pm standard deviation).

Table 3 Validation parameters of the HPLC method; intraday precision values are means of six measurements of a $90 \mu\text{mol l}^{-1}$ (muramic acid: $9 \mu\text{mol l}^{-1}$) standard mixture analysed on the same day; inter-day precision values are means of six measurements of $90 \mu\text{mol l}^{-1}$ (muramic acid: $9 \mu\text{mol l}^{-1}$) standard mixtures analysed on six days; recovery rates are mean values of each three test samples consisting of amino sugar standard mixture added to quartz sand samples after hydrolysis.

Component	Intraday	Interday	Correlation coefficient	Recovery rate	LOQ	LOD
	precision	precision				
	Area (mV \times min) (\pm SD)		r	% (\pm SD)	mg kg ⁻¹	
Muramic acid	9.13 (0.13)	9.85 (0.55)	0.999	115 (2)	4.2	1.7
Mannosamine	12.74 (0.24)	11.83 (0.75)	0.998	105 (3)	29.9	9.0
Galactosamine	55.15 (0.98)	52.83 (1.47)	0.999	112 (1)	11.9	3.6
Glucosamine	63.31 (0.68)	64.67 (3.67)	0.999	81 (1)	11.9	3.6

SD = standard deviation, LOQ = limit of quantification, defined as a signal ten times higher than blank. Limit of detection (LOD) defined as a signal three times higher than blank.

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

Table 4 Effect of sample solvent on the contents of muramic acid, mannosamine, galactosamine and glucosamine in soil samples

Soil	Muramic acid		Mannosamine		Galactosamine		Glucosamine	
	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer
$\mu\text{g g}^{-1}$ soil								
Forest 1	210	96	160	63	1840	800	2730	1340
Forest 2	140	110	49	16	1550	1410	2640	2300
Arable 1	140	130	33	34	690	650	1180	1170
Arable 2	117	116	<LOQ	<LOQ	840	890	1190	1360
Arable 3	110	100	<LOQ	<LOQ	910	800	1400	1280
Arable 4	67	52	<LOQ	<LOQ	540	430	980	850
Probability values								
Solvent	<0.01		<0.01		0.01		0.02	
Soil	<0.01		<0.01		<0.01		<0.01	
Soil \times solvent	<0.01		<0.01		<0.01		<0.01	
CV ($\pm\%$)	14	24	12	33	16	23	15	24

CV = mean coefficient of variation between replicate samples (n = 3); LOQ = limit of quantification; samples < LOQ were excluded from the ANOVA.

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

Table 5 Effect of sample solvent on the contents of muramic acid, mannosamine, galactosamine and glucosamine in plant litter samples.

Plant litter	Muramic acid		Mannosamine		Galactosamine		Glucosamine	
	Water	Buffer	Water	Buffer	Water	Buffer	Water	Buffer
	$\mu\text{g g}^{-1}$ soil							
Sugarcane filter cake	55	68	20	17	210	210	1070	880
Maize leaves	<LOQ	<LOQ	<LOQ	<LOQ	26	31	36	36
Pea leaves	<LOQ	<LOQ	34	24	16	19	98	100
Amaranth straw	42	51	23	31	170	180	1440	1550
Wheat straw	27	25	<LOQ	<LOQ	76	83	1290	1350
Solvent	0.34		0.98		0.49		0.28	
Litter	0.07		<0.01		<0.01		<0.01	
Litter \times solvent	0.59		0.01		<0.01		0.86	
CV ($\pm\%$)	14	27	14	18	24	18	6	10

CV = mean coefficient of variation between replicate samples ($n = 3$); LOQ = limit of quantification; samples $< \text{LOQ}$ were excluded from the ANOVA.

3.4 Discussion

3.4.1 Optimisation of the mobile phase

Studies by Appuhn et al. (2004) and Zelles (1988) described a mobile phase with 0.75% THF. However, Zelles (1988) had no satisfactory separation and Appuhn et al. (2004) noted long retention times (32.4 min for glucosamine). In our study, we tested increased THF concentrations and found an optimum concentration of 1.5% (Fig. 1). This yielded in shorter retention times for all amino sugars and a slightly better resolution between muramic acid and mannosamine in comparison to Appuhn et al. (2004). Both, Hodgkin (1979) and Jarret et al. (1986) had already studied the impact of THF on the amino acid separation by reversed phase chromatography. In our study, we could observe similar complex effects caused by THF. On the one hand, an increasing THF concentration caused muramic acid to change its elution position with mannosamine. On the other hand, increasing THF concentration caused better separation and shorter retention times (Fig. 3d). Roggendorf and Spatz (1981) found that THF

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

accelerated compounds containing ether groups. This is probably the explanation for a better resolution between muramic acid and mannosamine and why muramic acid eluted faster than mannosamine with increased THF concentration, as muramic acid contains two ether groups and mannosamine only one. However, increasing THF concentration beyond 1.6%, we could not achieve reproducible peak separation anymore. For example, an occasional retention time shift for up to two minutes was observed, which caused an overlay of matrix- and amino sugar peaks. For this reason we decided to use a THF concentration of 1.5%.

Lindroth and Mopper (1979) showed that a decreasing pH value of the mobile phase yields better separation between the amino acids. However, they tested only a pH value range between 5.9 and 7.9. To examine whether a lower mobile phase pH shows a better separation, we tested a pH value range from 4 to 6 in the mobile phase. We obtained no better separation at low pH but we did observe that pH values below 5 reduced the fluorescence response (Fig. 3c) as already reported by Jarrett et al. (1986). Our results confirm the pH value chosen by Zelles (1988) and Appuhn et al. (2004).

3.4.2 Excitation wavelength optimisation

The optimal excitation wavelength is a compromise between increasing the fluorescence intensity of amino sugar peaks and decreasing fluorescence intensity of other primary amines of the matrix. For instance, at a wavelength of 280 nm a high fluorescence signal for all primary amines is observed, unfortunately also for interfering peaks like ammonia (Jarrett et al. 1986). Several studies about amino sugar determination (Mimura and Delmas 1983; Zelles 1988; Appuhn et al. 2004) use an excitation wavelength of 340 nm except for Rönköö et al. (1994), who suggested an excitation wavelength of 310 nm. But opposed to these studies, other authors like Lindroth and Mopper (1979) and Jarrett et al. (1986) recommended an excitation wavelength of 330 nm for amino acid methods. Jarrett et al. (1986) confirmed this adjustment by testing several excitation wavelengths for amino acid determination. We tested excitation wavelengths in a range between 300 nm and 340 nm to find the optimal excitation wavelength for amino sugar determination (Fig. 3a). With an optimum excitation wavelength of 330 nm, our result is consistent with that reported by Jarrett et al. (1986).

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

3.4.3 Ortho-phthaldialdehyde (OPA) reaction time

To obtain a high fluorescence response we tested different reaction times for the OPA-derivatisation. The fluorescence intensity of the muramic acid-isoindole-derivate was relatively constant whereas the other three amino sugars derivatives decompose after 120 sec reaction time (Fig. 3b). Lindroth and Mopper (1979) suggested that electron donating groups (in our case the carboxyl-group of muramic acid) have a stabilizing effect on the isoindole group. This assumption is supported by Roth (1971), who showed in his study notably high fluorescence intensity for amino acids with two carboxyl-groups (aspartic acid, glutamic acid). Moreover, it would explain the stability and the 10 times higher fluorescence response of the muramic acid derivatives. We presume that the low fluorescence response of mannosamine is closely related to the conformation of mannosamine. The primary amino-group of this molecule is located in an axial conformation, whereas the other three amino sugars contain the amino group in an equatorial conformation. Steric effects hinder the OPA reaction in mannosamine, which proceeds more easily in an equatorial conformation.

3.4.4 Validation parameters

In general, our validation parameters (Table 3) of this HPLC method are similar with validation parameters reported earlier (Zelles 1988; Appuhn et al. 2004). Zhang and Amelung (1996) described a reliable and sensitive amino sugar determination via gas chromatography. Their LOQ ranged from 10 $\mu\text{g ml}^{-1}$ (muramic acid) to 20 $\mu\text{g ml}^{-1}$ (other three amino sugars). In contrast to that we reached a higher sensitivity with a LOQ of 0.5 $\mu\text{mol l}^{-1}$, which is equal to 0.13 $\mu\text{g ml}^{-1}$ for muramic acid, and 5.0 $\mu\text{mol l}^{-1}$, which is equal to 0.90 $\mu\text{g ml}^{-1}$ for the other three amino sugars. Furthermore, with gas chromatographic methods a time-consuming off-line derivatisation procedure is needed for obtaining volatile components.

Our LOQ is similar to that of other HPLC methods described by Kaiser and Benner (2000), Diaz et al. (1996) or Ekblad and Näsholm (1996). Kaiser and Benner (2000) used a pulsed amperometric detector (PAD) whereas Diaz et al. (1996) applied a fluorescence detector with 6-aminoquinolyl-N-hydroxysuccinmydyl-carbamate (AQC) as derivatisation reagent. Ekblad and Näsholm (1996) used also fluorometric detection and 9-fluorenylmethylchloroformate (FMOC) for derivatisation. Diaz et al. (1996) determined only glucosamine and galactosamine. The peaks were well separated but each amino sugar yielded two peaks. Ekblad and Näsholm (1996) examined only glucosamine, which eluted in three

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

peaks. Kaiser and Benner (2000) determined all four amino sugars with two different methods based on anion exchange chromatography.

3.4.5 Effect of sample solvent

Dorresteyn et al. (1996) reported that the OPA derivatisation is a very complex reaction with three possible products: hydrolysis of OPA, stabilization of OPA by 2-mercaptoethanol to yield an ortho-phthalaldehyde-2-mercaptoethanol complex and reaction of OPA with 2-mercaptoethanol and amino sugars yielding the isoindole-derivate needed for amino sugar analysis. This complexity makes it difficult to find the optimal conditions for the derivatisation. However, pH of the reaction mixture, the concentration of 2-mercaptoethanol and the reaction time are probably the most important variables to control. The effect of the reaction time is discussed above.

With regard to method accuracy, Dorresteyn et al. (1996) suggested Milli-Q water to be best suited. Appuhn et al. (2004) also used water as sample solvent in their study. However, since sample pH is between 2 and 3 in the sample extract, we examined if better accuracy for this method is possible with higher pH during OPA reaction. Sample extract pH was increased to values about 5 with phosphate-buffer. In general, buffered samples showed higher standard deviations. Significant differences between water and buffer were only observed for the soil samples, particularly for the basalt-derived clayey soil forest 1, which contained high contents of aluminium and iron oxides. The hydrolysis products of these oxides presumably form as central ions complexes in the presence of ligands like phosphate. If these complexes bind amino sugars, they may not be completely available for OPA derivatisation anymore. With respect to the higher coefficients of variation and the lower amino sugar content using buffer as sample solvent, we recommend to use water as more reliable sample solvent.

3.4.6 Concentrations of amino sugars in soil and plant material samples

Our amino sugar contents in soil (Table 4) compare well with those reported in the literature from arable and forest soils (Zelles 1988, Guggenberger et al. 1999, Joergensen et al. 2010). Guggenberger et al. (1999) obtained in different arable soils a total amino sugar composition of 64% glucosamine, 31% galactosamine, 3% muramic acid, and 2% mannosamine. Amelung et al. (1999) found in different grassland soils 60% glucosamine,

3. Optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates

33% galactosamine, 5% muramic acid and 2% mannosamine. Liang et al. (2007a) measured in different forest soils 67% glucosamine, 21% galactosamine, 8% muramic acid, and 4% mannosamine. Ding et al. (2010) detected 55% glucosamine, 40% galactosamine, and 5% muramic acid, but no mannosamine in soil samples amended with different amounts maize residues after 38 weeks of incubation. Also Glaser et al. (2004) did not find mannosamine in all samples. Liang et al. (2006, 2007b) measured 72% glucosamine, 8% galactosamine, 13% muramic acid and 7% mannosamine in soybean straw and maize stalks. Amelung et al. (1999), Guggenberger et al. (1999), Liang et al. (2006, 2007a/b), and Ding et al. (2010) all used after conversion of the amino sugars to aldonitrile acetates the gas chromatographic method described in detail by Zhang and Amelung (1996). Benner and Kaiser (2003) measured 63% glucosamine, 17% galactosamine, 5% mannosamine, and 16% muramic acid in marine particulate organic matter, with pulsed amperometric detection and ion exchange chromatography, a method without a derivatisation step.

It is not clear whether the absence of mannosamine in the low-biomass arable soils is due to the missing sensitivity as the majority of the data reported for mannosamine has been obtained in high biomass soils (Guggenberger et al. 1999; Turrión et al. 2002; Liang et al. 2007b). In our three soils with detectable mannosamine data, the concentration is in the range obtained by others (Amelung et al. 1999; Guggenberger et al. 1999). High mannosamine percentages, as in our pea leaves, were also one time observed in litter layer (Turrión et al. 2002).

3.5 Conclusions

Our method allows fast, quantitative, and reproducible HPLC determination of the four most important amino sugars in soil and plant litter. The separation between muramic acid and mannosamine was optimised in combination with shorter retention times in comparison with previously published methods. No interferences exist from amino acids or other primary amines, occurring in soil and plant hydrolysates.

Acknowledgments

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4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

Soil Biology & Biochemistry

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Abstract

A study on the suitability of chromatographic techniques such as high performance anion exchange chromatography (HPAEC) via fluorescence detection (FI) and pulsed amperometric detection (PAD), respectively, and reversed phase (RP) chromatography for the analysis of galactosamine (GalN), glucosamine (GlcN), mannosamine (ManN) and muramic acid (MurN) in soil hydrolysates was carried out. The RP-FI method was rapid, provided good validation parameters and relatively inexpensive instrumentation. The HPAEC methods had a slightly higher limit of quantification (LOQ) 0.6 – 5.0 $\mu\text{mol l}^{-1}$ (HPAEC-FI) and 1.0 – 10.0 $\mu\text{mol l}^{-1}$ (HPAEC-PAD) in comparison to the RP-FI method 0.5-5.0 $\mu\text{mol l}^{-1}$. Various sample pretreatment methods and some further chromatographic methods were investigated and the advantages and disadvantages of the HPLC methods are discussed.

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Key words: amino sugars / HPLC / OPA / PAD / microbial residues/ HPAEC / soil analysis/ derivatisation

4.1 Introduction

The determination of amino sugars, especially MurN and GlcN, is highly suited for quantifying the fungal and bacterial contribution in soil (Joergensen and Wichern, 2008). Also Liang et al. (2007) and Amelung et al. (2008) regarded amino sugars to be the most important biomarkers for the presence of microbial residues. As plants do not contain amino sugars and soil animals contribute only minor amounts of amino sugars to soil, the majority of amino sugars are assumed to be of microbial origin (Dai et al., 2002; Appuhn and Joergensen, 2006). Amino sugars in soil are usually measured by gas chromatographic (GC) analysis (Amelung and Zhang, 2001) or reversed phase HPLC, using OPA (ortho-phthaldialdehyde) for fluorescing derivatisation (Zelles, 1988; Appuhn et al., 2004; Indorf et al., 2011). The amino sugar data published are apparently not affected by methodological differences, giving confidence in the reliability of the methods used (Joergensen and Wichern, 2008).

However, the use of compound-specific $\delta^{13}\text{C}$ analysis of amino sugars would additionally make it possible to investigate the differences in turnover between bacterial and fungal residues. Such a compound-specific $\delta^{13}\text{C}$ analysis of amino sugars is available for GC separation (Amelung and Zhang, 2001; Glaser and Amelung, 2002; Glaser, 2005; Glaser and Gross, 2005). However, the derivatisation procedure of the hydrolysis products into volatile components, e.g. alditol acetates is time consuming and needs considerable experience in laboratory work (Benzing-Purdie, 1984; Zhang and Amelung, 1996; Glaser, 2005). Moreover, the derivatisation reaction has the potential to introduce fractionation, and the introduction of external C atoms into the analysed molecule leads to a decrease in accuracy of the isotope ratio determination. An HPLC method directly linked to an isotope ratio mass spectrometer could markedly increase the sample throughput and accuracy (Bodé et al., 2009).

The RP-FI method described by Indorf et al. (2011) is reliable, but it is not suited for adaptation with isotope ratio mass spectrometry (IRMS), due to the organic mobile phase. However, a variety of other HPLC methods are free of organic solvents, such as (1) HPAEC, (2) high performance cation exchange chromatography (HPCEC, Watanabe, 1984), and (3) high performance anion exclusion chromatography (HPEXC). Bodé et al. (2009) were the first to describe an HPAEC-method for isotope amino sugar analysis. However, this method has several drawbacks: (1) The NaOH of the mobile phase is sensitive to CO_2 contamination, (2) this alkaline mobile phase ideally requires a metal-free liquid handling system, (3) the method of Bodé et al. (2009) consisted of two separate runs, one for the basic amino sugars

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and one for MurN, and (4) the limit of quantification for MurN is too high for the low MurN concentrations in soil.

For these reasons, the objectives of the present study were (1) to find a reliable purification and concentration procedure for amino sugars in HCl hydrolysates, (2) to optimise the method of Bodé et al. (2009) by testing different chromatographic separation mechanisms, and (3) to test HPCEC and HPEXC as organic solvent-free alternatives for the RP-FI method described by Indorf et al. (2011).

4.2 Materials and methods

4.2.1 Soils samples

Method evaluation was implemented using six different soil samples (0-10 cm) taken from four arable and two forest sites in Germany (Hessia and Lower Saxony), differing in physical, chemical and microbial properties (Table 1). Soil physical, chemical and biological properties of the soil samples were determined as described by Probst et al. (2008).

4.2.2 Chemicals

The reference materials (D-(+)-glucosamine (GlcN) hydrochloride, D-(+)- galactosamine (GalN) hydrochloride, D-(+)- mannosamine (ManN) hydrochloride and muramic acid (MurN) and KOH were purchased from Sigma Aldrich (St. Louis, MO, USA). The low carbonate 50 % NaOH stock solution, OPA (ortho-phthaldialdehyde), 2-mercaptoethanol, H₃BO₃, sodium acetate, sodium citrate, KH₂PO₄ and NaNO₃ were obtained from Merck (Darmstadt, Germany). The 6 M HCl solution, methanol and tetrahydrofurane (THF) were from VWR (West Chester PA, USA). All solutions were prepared with Milli-Q water produced via a Direct-Q 3 system (Millipore, Billerica, MA, USA). All other reagents were of high purity ($\geq 95\%$).

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Table 1 Physical, chemical and microbiological properties of the soils used in this investigation

Soil	Clay	Silt	Sand	pH-H ₂ O	Soil	Total N	Microbial	Ergosterol
	%				organic C		biomass C	
					mg g ⁻¹ soil		μg g ⁻¹ soil	
Forest 1	15	77	8	3.9	59.9	4.3	610	3.2
Forest 2	6	39	55	3.9	58.0	2.4	520	5.4
Arable 1	35	55	10	7.0	18.6	1.8	450	1.6
Arable 2	34	56	10	7.3	15.1	1.4	360	1.1
Arable 3	18	66	16	7.8	14.0	1.2	200	0.5
Arable 4	8	8	84	7.4	7.8	0.7	180	0.6

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

The borate buffer solution (pH 11) was prepared by dissolving 50 g of H₃BO₃ in 900 ml of water, adjusted to pH 11 with KOH (47% solution) and diluted to 1 l with water. This solution was stable for up to 12 months at 4 °C. The reducing solution was prepared by adding 2.5 ml of 2-mercaptoethanol to 100 ml of buffer solution. This solution was stable for up to 6 months at 4 °C in the dark. The OPA-reagent for pre-column derivatisation was prepared by dissolving 25 mg of OPA in 2 ml of methanol in a brown flask, mixed with 2 ml of reducing solution and diluting to 44 ml with buffer solution. The reagent was stable for up to 7 days at 4°C in the dark. For post-column derivatisation the OPA-reagent was prepared by using the same solutions but multiplied by ten to produce 480 ml of OPA-reagent, which was covered with nitrogen during chromatography.

The standard stock solutions were prepared according to Indorf et al. (2011).

4.2.3 Amino sugar extraction

The amino sugar extraction was based on a modified method described by Appuhn et al. (2004). Air-dried soil (400 mg, sieved at 2 mm) was mixed with 10 ml of 6 M HCl. After 6-h hydrolysis at 105°C, the samples were filtered over glass filters (Whatman GF/A).

4.2.4 Purification and concentration of amino sugars

To have higher amino sugar concentration and less impurity in the soil hydrolysates, we tested the following amino sugar purification methods: (1) extraction based on Appuhn et al. (2004) as described by Indorf et al. (2011), (2) extraction based on Zhang and Amelung (1996), (3) extraction via strong cation exchange resin (AG 50W-X8, hydrogen form, 100-200 dry mesh size, Biorad, Munich, Germany), (4) solid phase extraction via strong cation exchange (SCX) cartridges (Strata SCX 500µm, 70Å, 200 mg/3 ml, Phenomenex, Aschaffenburg, Germany), (5) Extraction via strong anion exchange (SAX) cartridges (Strata SAX 500 µm, 70Å, 200 mg/3 ml, Phenomenex, Aschaffenburg, Germany) to concentrate and separate MurN from the basic amino sugars.

(1) A 0.3 ml aliquot of the filtered hydrolysate was evaporated to dryness at 40-45 °C to remove HCl, re-dissolved in water, evaporated a second time and re-dissolved in 1 ml of water. After centrifugation at 5.000 g the supernatant was frozen and stored at -18 °C until analysis. A 0.3 ml aliquot was evaporated to dryness at 40-45 °C to remove HCl, re-dissolved in water, evaporated a second time and re-dissolved in 1 ml of water.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

(2) For the method described by Zhang and Amelung (1996), 6 ml of the filtered hydrolysate were evaporated to dryness, re-dissolved in 20 ml of water and adjusted to a pH between 6.6 and 6.8 with KOH(aq). After centrifugation at 5000 g for 10 min the supernatant was lyophilised. To reduce the amount of inorganic salt the residue was re-dissolved in 3 ml of dry methanol and centrifuged at 5000 g for 10 min. The clear supernatant was dried under a stream of air, re-dissolved in 5 ml of water, filtered over a 45 µm filter and stored in vials at – 18 °C until analysis.

(3) The sample preparation via cation exchange resin was performed by using a strong cation exchange resin. 2 ml of the protonated resin suspended in water were filled into plastic tubes (Biorad), the resin was activated with 2x 5 ml of 0.1M HCl and washed with 5 ml of water. 5 ml of the filtered sample hydrolysate was evaporated to dryness and re-dissolved in 1 ml of water. Both steps were repeated once and the sample was subsequently transferred into 2 ml tubes (Eppendorf) and centrifuged at 5.000 g. Then the resin was loaded with the complete supernatant. To obtain a quantitative transfer of the complete sample, the pellet was re-dissolved in 1 ml of water, centrifuged again and the supernatant was added onto the resin. For elution of the neutral and negatively charged compounds, the resin was washed with 3 x 4 ml of water. Subsequently the analytes were eluted with 3 x 4 ml of 0.5 M HCl. The eluate was evaporated to dryness and re-dissolved in 1 ml of water. After centrifugation at 5.000 g, the supernatant was transferred into vials and stored at – 18 °C.

(4) For the SCX extraction the SPE cartridges were conditioned with 2.4 ml of methanol and equilibrated with 2.4 ml of a 0.1 M KH₂PO₄ buffer solution at pH 6. After loading the sample hydrolysate (5 ml, adjusted to a pH between 2 and 4 with KOH_(aq)) onto the SPE cartridge, the cartridge was washed with an 8 % aqueous methanol solution. The analytes were eluted with 3 x 2 ml of 5 % methanolic NH₃. The eluate was evaporated to dryness at 40 °C, re-dissolved in water, evaporated a second time and re-dissolved in 1 ml of water. After centrifugation at 5.000 g, the supernatant was frozen and stored at -18 °C until analysis.

(5) For the SAX-extraction, the SPE cartridge was conditioned with 2.4 ml of methanol and equilibrated with 2.4 ml of 300 mM NaOH followed by 2.4 ml of 2 mM NaOH. Then 5 ml of the sample (adjusted to a pH between 5 and 7 with KOH_(aq)) were loaded onto the cartridge. After washing the cartridge with 3 x 2.4 ml of 2 mM NaOH the analytes were eluted with 3 x 2.4 ml of a 4 mM NaNO₃ + 4 mM NaOH (50/50 v/v) solution. The eluate was lyophilised, re-dissolved in 1 ml of water and kept at -18 °C.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

4.2.5 Reversed phase-HPLC and pre-column derivatisation

The amino sugar determination via reversed phase and pre-column derivatisation was carried out as described by Indorf et al. (2011). Chromatographic separations were performed on a Phenomenex (Aschaffenburg, Germany) Hyperclone C₁₈ (ODS) column (125 mm length × 4 mm diameter, 5 µm particle size, 12 nm pore size), protected by a Phenomenex C₁₈ guard cartridge (4 mm length × 2 mm diameter). The HPLC system consisted of a Dionex (Germering, Germany) P 580 gradient pump, a Dionex Ultimate WPS – 3000TSL analytical autosampler with in-line split-loop injection and thermostat and a Dionex RF 2000 fluorescence detector set at 445 nm emission and 330 nm excitation wavelengths with medium sensitivity. Further chromatographic conditions are shown in Table 2.

For the automated pre-column derivatisation, 50 µl of OPA and 30 µl of sample were mixed in the preparation vial and after 120 sec reaction time 15 µl of the indole derivates were injected. The mobile phase consisted of two eluents. Eluent A was a 97.8/0.7/1.5 (v/v/v) mixture of a buffer solution, methanol and tetrahydrofuran (THF). The buffer solution contained 52 mmol sodium citrate and 4 mmol sodium acetate, adjusted to pH 5.3 with HCl. Then methanol and THF were added. Eluent B consisted of 50% water and 50% methanol (v/v). The amino sugar separation was performed isocratically, but a gradient was used for cleaning the column after each run. Every run was started at an eluent A/B v/v composition of 93/7 for 19 min. A linear gradient was run to reach 80% B after 3 min and remaining isocratic for 3 min. A reverse gradient to 93/7 within 3 min was followed by 2 min isocratic run after which the column was preconditioned for the next sample.

4.2.6 HPAEC and post column derivatisation

The chromatographic conditions were based on those of Bodé et al. (2009) and consisted of two different methods for the basic amino sugars and for the acid amino sugar MurN, respectively. The anion exchange separations for both methods were performed using a Dionex CarboPac PA20 column (150 mm length x 3 mm diameter, 6.5 µm particle size) protected by a Dionex AminoTrap guard column (30 mm length x 3 mm diameter). The HPAEC system consisted of a metal-free PEEK gradient pump (Sykam S2100, Fürstfeldbruck, Germany), a PEEK autosampler (Sykam S5200) and a Dionex RF 2000 fluorescence detector set at 445 nm emission and 330 nm excitation wavelength with medium sensitivity. For the post-column derivatisation a knitted reactor was placed between the

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

column outlet and the detector inlet. The OPA derivatisation reagent was delivered by a Sykam S1122 micro pump. By means of a Y-piece, which was placed between column outlet and knitted reactor, the OPA reagent was continuously mixed with the column eluent. In the knitted reactor the separated amino sugars reacted with the OPA to form the indole derivatives that were detected by fluorescence.

The mobile phase for GalN, ManN and GlcN consisted of 2 mM NaOH. Since carbonate negatively affects the amino sugar separation, the NaOH eluent was degassed by sonication under vacuum for 15 min and was covered with nitrogen during chromatography. After every run the column was cleaned with 200 mM NaOH for 5 minutes and equilibrated for the next sample with 2 mM NaOH for 15 minutes. In addition, after every six runs, the column had to be cleaned with 200 mM NaOH for 30 min and then equilibrated with 2 mM NaOH for a further 30 minutes.

To obtain reproducible results for MurN it was necessary to use the on-line purification described by Bodé et al. (2009). Therefore the injected sample was first passed to a purification column (Dionex CarboGuard, 30 mm length x 3 mm diameter). There, the sample was washed with 2 mM NaOH for 3 min. Then MurN was eluted from the purification column with an eluent consisting of 25 % 100 mM sodium acetate + 100 mM NaOH / 70 % H₂O / 5 % 200 mM NaOH onto the analytical column (CarboPac PA 20) protected by a Dionex AminoTrap guard column (30 mm length x 3 mm diameter) for chromatographic separation. MurN eluted after approx. 60 min and subsequently the purification column was cleaned with 200 mM NaOH for 2 min and equilibrated with 2 mM NaOH for 3 min before the next sample was injected. It was necessary to clean the column with 200 mM NaOH for 30 min und equilibrate with 25 % 100 mM sodium acetate + 100 mM NaOH / 70 % H₂O / 5 % 200 mM NaOH for 30 min after every six runs. Further chromatographic conditions are listed in Table 2.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

Table 2 Chromatographic conditions of the different methods

Methods	HPAEC-PAD	HPAEC-FI (basic amino sugars)	HPAEC-FI (MurN)	HPCEC-FI	RP-FI
Separation column	CarboPac PA20	CarboPac PA20	CarboPac PA20	Luna SCX	Hyperclone C ₁₈ (ODS) column
Guard column	AminoTrap + Carbopac PA 20 guard column	AminoTrap	AminoTrap + Carbopac PA 20 guard column as purification column	SecurityGuard	C ₁₈ guard cartridge
Eluent composition	Gradient (NaOH/ sodium acetate/ water)	Isocratic (2 mM NaOH)	Isocratic (NaOH/ sodium acetate/ water)	Isocratic (10 mM KH ₂ PO ₄ , pH 7)	Gradient (buffer/methanol/THF)
Column temperature (°C)	20	15	30	50	35
Flow rate (ml min ⁻¹)	0.30	0.35	0.40	0.50	1.5
Injection volume (µl)	15	15	15	10	15 (indole derivate)
Derivatisation	No derivatisation	Post column derivatisation (OPA-flow : 0.10 ml min ⁻¹)	Post column derivatisation (OPA-flow : 0.16 ml min ⁻¹)	Post column derivatisation (OPA-flow : 0.10 ml min ⁻¹)	Pre- column derivatisation
t _R (min)					
GalN	20.1	15.8	-	14.8	15.4
ManN	22.9	17.9	-	16.8	9.4
GlcN	26.3	20.6	-	17.2	16.6
MurN	42.3	-	60.1	8.2	8.4

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

4.2.7 HPAEC and pulsed amperometric detection (PAD)

Amino sugar analysis based on Meyer et al. (2008) was performed with a Dionex ion chromatography system consisting of an autosampler AS 50, a gradient pump GP 40 and an electrochemical detector ED 40 equipped with a thin-layer-type amperometric cell. The detector cell integrated a gold working electrode and an Ag / AgCl reference electrode. Chromatographic separation was achieved using a CarboPac PA20 column (150 mm length × 3 mm diameter, 6.5 µm particle size) coupled with a CarboPac PA 20 guard column (30 mm length × 3 mm diameter) and an AminoTrap guard column (30 mm length × 3 mm diameter). Sample and standard vials were held at 10 °C in the autosampler.

The separation of GalN, ManN, GlcN and MurN was carried out using 1 M sodium acetate + 25mM NaOH (eluent A), 250 mM NaOH (eluent B), 10 mM NaOH (eluent C) and water (eluent D). Table 3 describes the gradient used for separation of amino sugars. The analytes were detected by applying a quadrupole-potential waveform on the gold electrode. The potentials were E1 = 0.1 V from 0 to 0.4 ms, E2 = 2.0V from 0.41 to 0.42 ms, E3 = 0.6V from 0.42 to 0.43 ms and E4 = - 0.1V from 0.44 to 0.50 ms. Table 2 shows further chromatographic conditions.

4.2.8 HPCEC and post-column derivatisation

The cation exchange separations, based on Watanabe [16], were conducted on a Phenomenex (Aschaffenburg, Germany) LUNA SCX column (150 mm length × 4.66 mm diameter, 5 µm particle size) which was preceded by a Phenomenex SCX security guard cartridge (4 mm length × 2 mm diameter). The HPCEC system and the post column derivatisation methodology were identical to those described above for the HPAEC method with post column derivatisation. 10 µl of the sample aliquots were reacted with OPA after chromatographic separation and quantified by fluorescence as described above. Further chromatographic conditions are shown in Table 2.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

Table 3 Gradient profile for amino sugar separation by HPAEC-PAD

Time (min)	% A (1 M sodium acetate / 25 mM NaOH)	% B (250 mM NaOH)	% C (10 mM NaOH)	% D (water)
0	0	0	20	80
27	0	0	20	80
28	5	20	0	75
29	5	20	0	75
42	30	20	0	50
44	60	20	0	20
46	60	20	0	20
48	0	50	0	50
50	0	50	0	50
52	0	100	0	0
60	0	100	0	0
68	0	0	20	80
90	0	20	0	80

4.2.9 HPEXC and post column derivatisation

For obtaining an alternative method of the MurN determination described by Bodé et al. (2009) we also tested the HPEXC. This method has the advantage of running with an acidic buffer eluent. The HPEXC analyses were performed on the chromatographic fluorescence system as described for the HPAEC method. The separation of MurN was accomplished on a Phenomenex Rezex Roa-Organic acid (300 mm length \times 7.8 diameter, 8 μ m particle size) using 1% H₃PO₄ as mobile phase and a column temperature of 25°C. The flow rate was set to 0.6 ml min⁻¹ and the injection volume was 15 μ l. The post-column derivatisation methodology was according to the one described above. After chromatographic separation and OPA reaction the indole derivatives were detected by fluorescence as described above.

4.3 Results

4.3.1 Sample purification methods

We investigated the purification and the concentration of soil and tested different sample preparation methods. For four purification methods tested the interfering matrix substances could not be substantially eliminated from the amino sugars. However, amino sugar concentrations could be increased without increasing the matrix concentrations by using strong cation exchange purification. The strong cation exchange purification via Biorad *AG50W X8* resin (method 3) and the SPE-SCX purification (method 4) gave the highest amounts for all four amino sugars, without an increase in impurities (Fig. 1). Both purification methods are based on the strong cation exchange mechanisms. The resins of these methods have the same functional groups (sulfonic acid) but the matrix of the resins is different. At method 3 the sulfonic group is attached to a styrene divinyl benzene copolymer matrix, whereas at method 4 the benzene sulfonic acid group is bonded to the surface of silica particle. Further, method 3 and 4 differ in eluent solutions used. For evaluating the purification of the different sample purification methods we determined the area of the matrix in a defined section relative to the area of the analyte peaks. The matrix area reached 34 % (method 1) and 32 % (method 2) of that of the analytes, respectively. A very low matrix area (2 % and 4 %, respectively, related to the signal sizes of the basic amino sugars) results from the use of the extraction methods 3 and 4. The matrix percentage of the MurN chromatogram is 93% and 98% for method 1 and 2, respectively, and 92% for method 3 and 4. No amino sugars were recovered via method 5.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

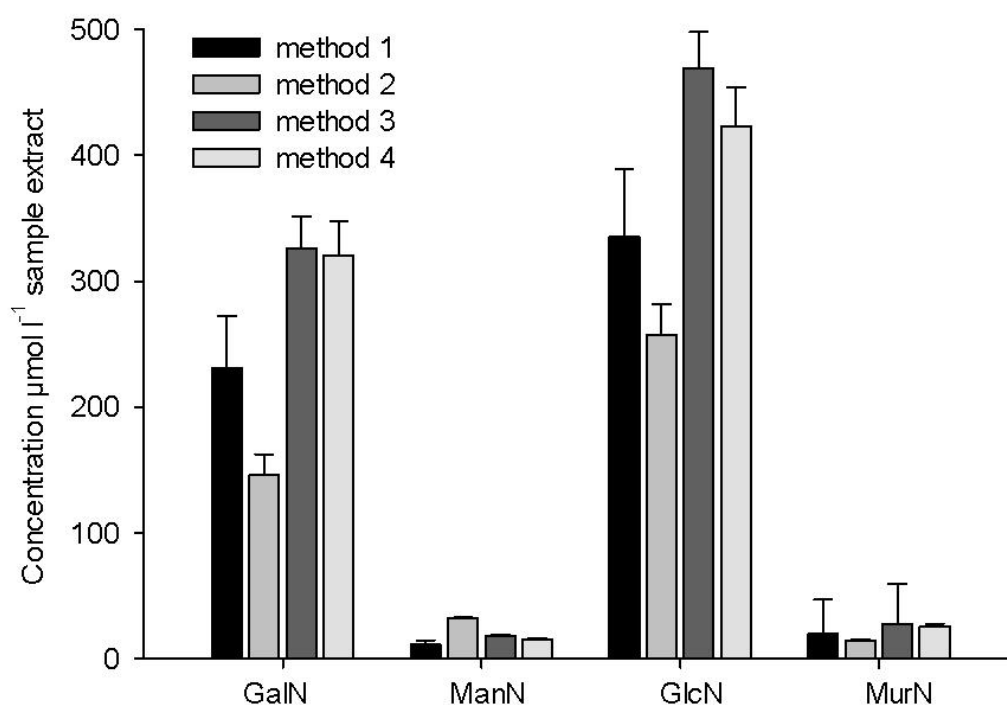


Fig. 1 Comparison of four different sample purification methods (1) based on Appuhn et al. (2004), (2) based on Zhang and Amelung (1996), (3) sample purification via a strong cation exchange resin and (4) solid phase extraction via strong cation exchange cartridges, respectively of GalN, ManN, GlcN and MurN in a soil extract (forest 1) (error bars represent \pm standard deviation). *Note:* Amino sugar concentrations presented are soil extract concentrations and not sample concentrations. The concentrations were measured via HPAEC-Fl.

4.3.2 Chromatographic methods

4.3.2.1 HPAEC analysis with fluorescence detection

As described by Bodé et al. (2009), the HPAEC separation mechanism made it possible to determine GalN, ManN, GlcN and MurN in standard solutions as well as in soil extracts. Fig. 2c shows the resulting chromatogram with baseline resolution between each of the basic compounds, whereas Fig. 2d shows the optimum result for the MurN method. Table 2 shows the retention times (t_R) by HPAEC fluorescence detection for the basic amino sugar method as well as for the acidic amino sugar method.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

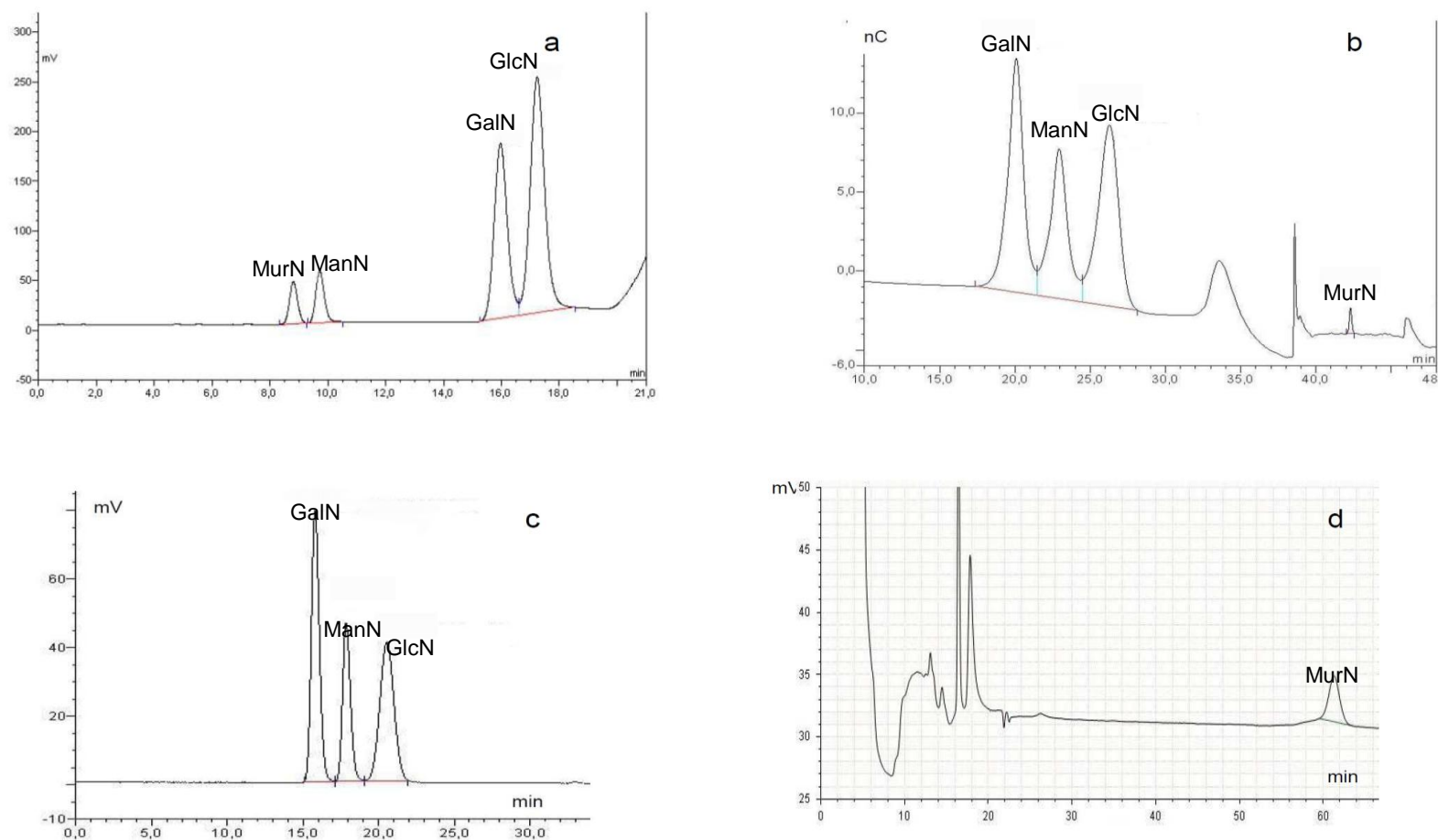


Fig. 2 Chromatograms of a standard mixture consisting of $130 \mu\text{mol l}^{-1}$ GalN, $130 \mu\text{mol l}^{-1}$ ManN, $130 \mu\text{mol l}^{-1}$ GlcN and $13 \mu\text{mol l}^{-1}$ MurN (a) obtained from the RP-FI method and (b) obtained from the HPAEC-PAD method; chromatograms of (c) a standard mixture consisting of $130 \mu\text{mol l}^{-1}$ GalN, $130 \mu\text{mol l}^{-1}$ ManN, $130 \mu\text{mol l}^{-1}$ GlcN measured by HPAEC-FI for basic amino sugars and of (d) a standard solution containing $13 \mu\text{mol l}^{-1}$ muramic acid obtained from the acidic HPAEC-FI method.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

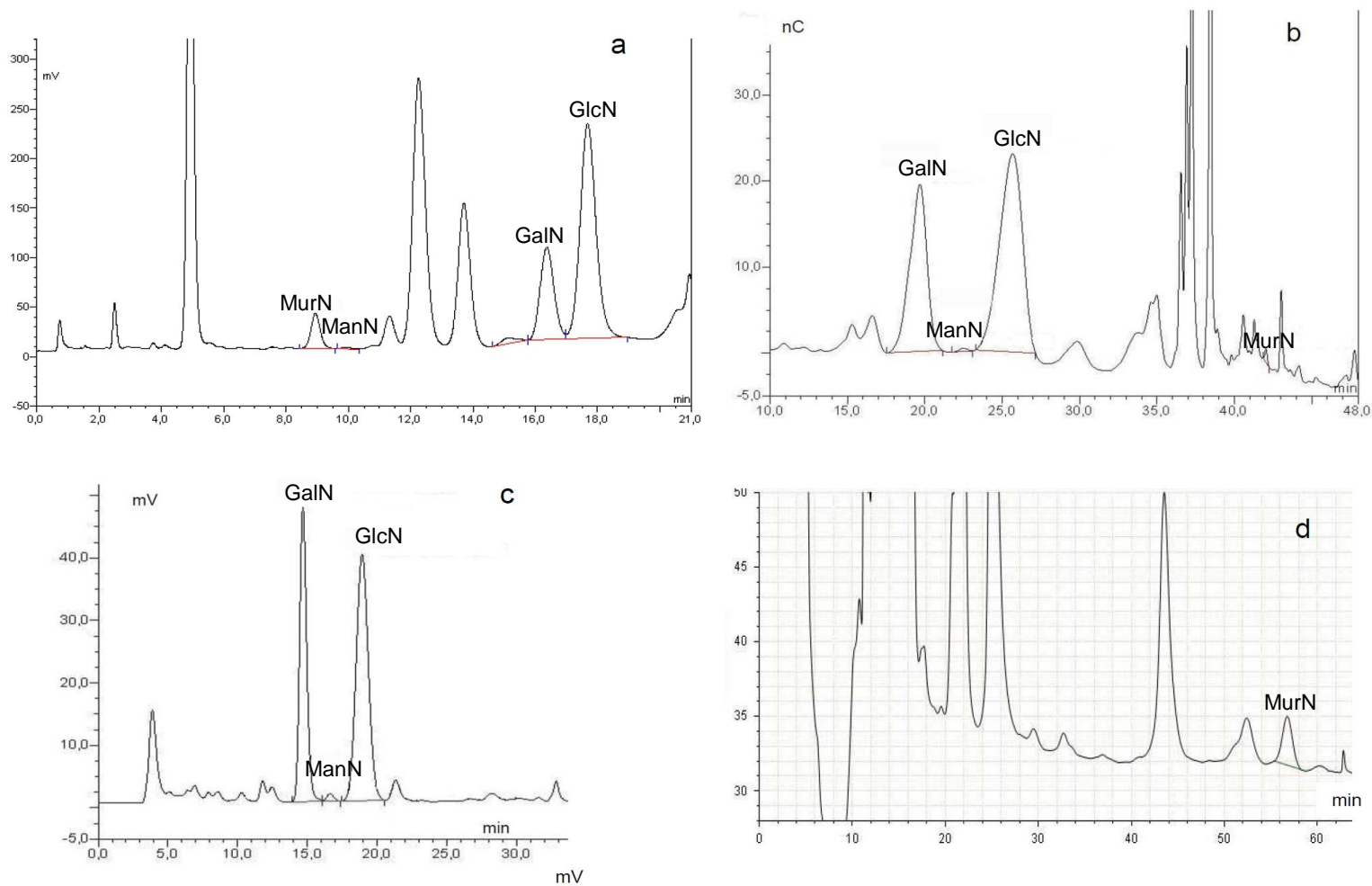


Fig. 3 Chromatograms of a soil hydrolysate (forest 1) (a) measured by RP-FI , (b) obtained from the HPAEC-PAD method (c) obtained from the HPAEC-FI method for basic amino sugars and (d) obtained from the acidic HPAEC-FI method.

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

The highest fluorescence response for the amino sugar standards was yielded at an OPA flow rate of 0.10 ml min⁻¹ for the basic amino sugars and 0.16 ml min⁻¹ for MurN, respectively. The coefficient of variation (CV) was roughly between 3% and 6% for intraday and between 3% and 6% for interday precision, respectively (Table 4a). The calibration curves were linear in a range from 5 to 210 µmol l⁻¹ for the basic amino sugars and 1 to 21 µmol l⁻¹ for MurN, with good correlation coefficients and standard deviations (Table 4b, Fig. 4). This reflects the reproducibility and the precision of the analytical method excluding sample preparation. The lowest quantification levels (LOQ, 10 times higher than noise) of the amino sugar standards were between 0.6 µmol l⁻¹ for MurN and 5 µmol l⁻¹ for ManN (Table 4b).

Table 4a Validation parameters of the RP-FI, HPAEC-FI and HPAEC-PAD methods; intraday precision values are mean coefficient of variation (CV) between replicate samples (n = 3) of three measurements of a 90 µmol l⁻¹ (MurN: 9 µmol l⁻¹) standard mixture analysed on the same day; interday precision values are mean CV of three measurements of 90 µmol l⁻¹ (MurN: 9 µmol l⁻¹) standard mixtures analysed on three days

Component	Intraday Precision (Coefficient of variation)			Interday Precision (Coefficient of variation)		
	RP-FI	HPAEC-FI	HPAEC-PAD	RP-FI	HPAEC-FI	HPAEC-PAD
GalN	1.8%	2.8%	1.0%	2.8%	2.6%	44.5%
ManN	1.9%	6.3%	1.7%	4.2%	3.4%	40.9%
GlcN	1.1%	5.7%	1.6%	3.1%	2.8%	48.4%
MurN	0.1%	5.6%	6.1%	9.2%	5.9%	48.4%

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

Table 4b Validation parameters of the RP-FI, HPAEC-FI and HPAEC-PAD methods

Component	LOQ ($\mu\text{mol l}^{-1}$ sample extract)			LOD ($\mu\text{mol l}^{-1}$ sample extract)			R ² (Correlation Coefficient)			Slope of calibration line		
	RP-FI	HPAEC-FI	HPAEC-PAD	RP-FI	HPAEC-FI	HPAEC-PAD	RP-FI	HPAEC-FI	HPAEC-PAD	RP-FI	HPAEC-FI	HPAEC-PAD
GalN	2.0	3.0	5.0	0.6	0.9	1.5	0.999	0.999	0.995	0.65	0.35	0.11
ManN	5.0	5.0	10.0	1.5	1.5	3.0	0.998	0.999	0.992	0.18	0.25	0.07
GlcN	2.0	3.0	6.0	0.6	0.9	1.8	0.999	0.999	0.998	0.83	0.37	0.11
MurN	0.5	0.6	1.0	0.2	0.3	0.3	0.999	0.997	0.998	1.12	0.76	0.05

LOQ = limit of quantification, defined as a signal ten times higher than noise. Limit of detection (LOD) defined as a signal three times higher than noise. SD = standard deviation

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

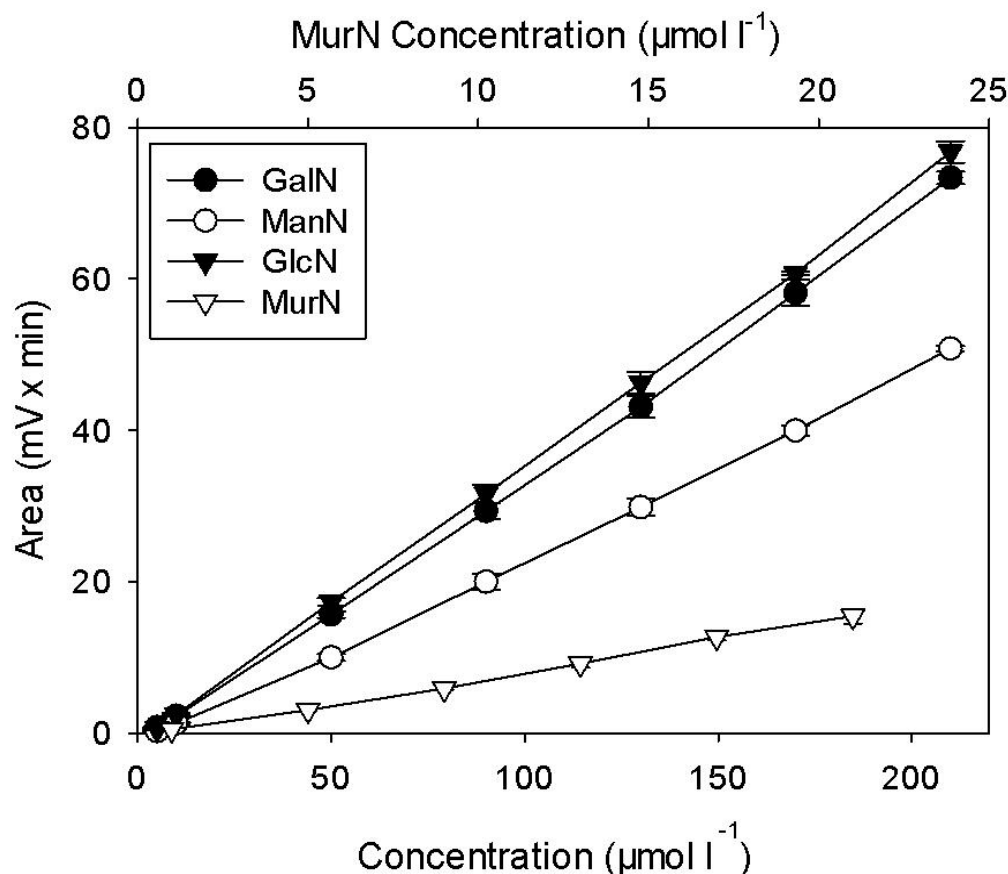


Fig. 4 Calibration curves of reference amino sugars obtained from HPAEC-FI (error bars represent \pm standard deviation).

4.3.2.2 HPAEC analysis and pulsed amperometric detection

The HPAEC-PAD method system provided reproducible separation and quantification of GalN, ManN, GlcN and MurN (Fig. 2b). The elution order and retention times by HPAEC-PAD are shown in Table 2. The intraday CV for the amino sugars was between 2% and 6%. The interday CV was between 41% and 48% (Table 4a). Fig. 5 shows the curves and demonstrates the linearity of response for each of the analytes ($R^2 \geq 0.992$) (Table 4b). The calibration curves were linear between $10 \mu\text{mol l}^{-1}$ and $210 \mu\text{mol l}^{-1}$ for the basic amino sugars and between $1 \mu\text{mol l}^{-1}$ and $21 \mu\text{mol l}^{-1}$ respectively (Fig. 5). The LOQ depended on the amino sugar and varied from $1 \mu\text{mol l}^{-1}$ for MurN to $10 \mu\text{mol l}^{-1}$ for ManN (Table 4b).

4. Comparison of HPLC methods for determination of amino sugars in soil hydrolysates

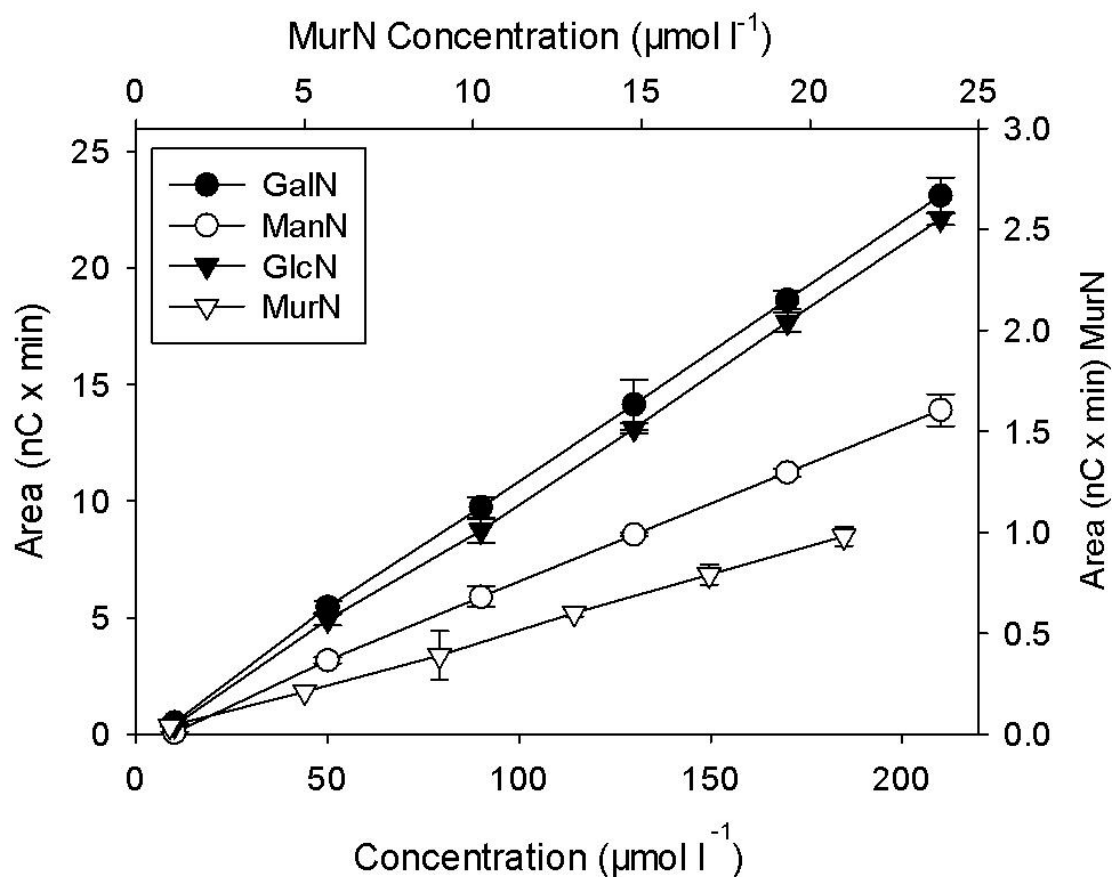


Fig. 5 Calibration curves of reference amino sugars obtained from HPAEC-PAD (error bars represent \pm standard deviation).

4.3.2.3 HPCEC and HPEXC analysis and fluorescence detection

We did not obtain satisfactory separation with the HPCEC mechanism for the four amino sugars. Because of the acidic group, which is fully deprotonated at pH 7, MurN was very poorly retained on the cation exchange column. However, it was well separated from the other amino sugars in the standard solution but it coeluted with matrix peaks in sample extracts.

As expected, the HPEXC mechanism was only applicable for MurN. The basic amino sugars did not elute from the column because of their high affinity to the HPEXC column material. The determination of MurN (peak area and retention time) was not reproducible, due to the low affinity of MurN to the stationary phase.

4.3.2.4 Amino sugar contents of soil with regard to the different HPLC methods

The content of the different amino sugars in soils increased in the order ManN < MurN < GalN < GlcN (Table 5), regardless of the HPLC method used. In three soils the ManN content was below the LOQ for all methods tested (Table 5). On average, the highest amino sugar

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values were measured using HPAEC-FI, except for ManN. The GlcN content contributed between 57% (HPAEC-FI) and 62% (HPAEC-PAD) to the total amino sugar content. The contribution of GalN and MurN to the total amino sugar content varied between 34% (HPAEC-PAD) and 36% (HPLC-RP), and 3% (HPAEC-PAD) and 5% (HPAEC-FI), respectively, whereas ManN, if present in measurable concentration, contributed between 1% (HPAEC-FI) and 2% (HPLC-RP) to the total amino sugar content. This shows that all chromatographic methods resulted in a similar pattern of amino sugars in soil samples.

The different HPLC methods had no significant effect on the concentration of ManN and GlcN measured in soil samples. However, the concentrations measured by the HPAEC-PAD were significantly smaller than those measured by the RP-FI and HPAEC-FI methods (Table 5).

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Table 5 Comparison of amino sugar results in soil samples obtained by RP-HPLC, HPAEC-Fl and HPAEC-PAD

Soil	ManN			GalN			GlcN			MurN		
	RP-HPLC	HPAEC-FL	HPAEC-PAD	RP-HPLC	HPAEC-FL	HPAEC-PAD	RP-HPLC	HPAEC-FL	HPAEC-PAD	RP-HPLC	HPAEC-FL	HPAEC-PAD
	$\mu\text{g g}^{-1}$ soil											
Forest 1	160	120	160	1840	2340	1920	2730	3120	3350	210	230	180
Forest 2	49	23	14	1550	1570	1080	2640	2750	2320	140	170	52
Arable 1	34	31	14	690	890	860	1180	1680	1570	140	150	99
Arable 2	<LOQ	<LOQ	<LOQ	840	1020	680	1190	1400	1130	120	120	64
Arable 3	<LOQ	<LOQ	<LOQ	910	920	440	1400	1280	750	110	120	34
Arable 4	<LOQ	<LOQ	<LOQ	540	350	310	980	620	580	67	70	33
Probability values												
Method	0.30			<0.01			0.20			<0.01		
Soil	<0.01			<0.01			<0.01			<0.01		
Soil \times Method	0.37			0.01			0.07			<0.01		
CV ($\pm\%$)	12	30	25	16	18	9	15	21	9	14	7	10

4.4 Discussion

4.4.1 Sample purification methods

The amino sugar extraction method described by Appuhn et al. (2004) (method 1) provided good results for the reversed-phase method (Indorf et al., 2011). However, our objective was to concentrate MurN and not the matrix substances, in order to determine MurN using detectors with a lower sensitivity for MurN, such as an isotope ratio mass spectrometer after HPLC. First we tried to concentrate the sample extract obtained from method 1 by taking 1 ml sample hydrolysate instead of 0.3 ml. But this led to a higher matrix concentration, which aggravated the separation and degraded the column material. Owing to these problems, we tested different extraction methods (Fig. 1) and found the strong cation exchange extraction (method 3) to be best suited for methods with a high LOQ for MurN. Method 3 is most suitable for reducing the matrix in sample extracts. Previous studies (Amelung and Zhang, 2000; Kaiser and Benner, 2000) have indicated that cation exchange resins are useful for eliminating interference compounds and obtaining higher amounts of amino acids and amino sugars, respectively. For the extraction we used a strong cation exchange resin, which was composed of sulfonic acid functional groups attached to a styrene divinylbenzene copolymer lattice. The negatively charged sulfonic acid groups of the resin bound the positively charged amino groups of the amino sugars until a stronger eluent removed the amino sugars. Interfering compounds that are neutral or negatively charged were eliminated beforehand by washing the resin with water. With this method we obtained a higher concentration of amino sugars without an increase of the matrix substances in the analytical aliquot, particularly for the basic amino sugars (Fig. 1). The concentration of MurN in the analytical aliquot could only be increased marginally. This is caused by the carboxyl group of muramic acid, which leads to a low affinity of MurN to the resin material.

4.4.2 HPAEC-FI

A baseline separation for the three basic amino sugars was achieved using the HPLC parameters suggested by Bodé et al. (2009). As described earlier (Bodé et al., 2009), other concentrations of the NaOH eluent, other column temperatures and other flow rates decreased the selectivity of the amino sugar chromatography. Previous studies (Bodé et al., 2009; Boschker et al., 2008; Meyer et al., 2008; Yu et al., 2003) have indicated that carboxylic acids like MurN do not elute with NaOH as the mobile phase because its carboxyl group has a very

high affinity to the column material. Thus an eluent with a higher ionic strength is needed for the elution of MurN.

Several authors (Cataldi et al., 1999; Lee, 1996; Lu et al., 1997; Rocklin et al., 1983; Wong and Jane, 1995) have suggested sodium acetate and NaNO_3 , respectively, as an applicable “pressing agent”, whereas sulfate and carbonate are not suitable (Lee, 1996; Rocklin et al., 1983). In addition, we tested phosphate as a pushing anion, which even at low concentrations led to a co-elution of all compounds, due to its high ionic strength. Cataldi et al. (1999) have claimed that nitrate as an additional competing ion for the elution of sugar acids leads to shorter retention times at millimolar concentrations, but acetate salts perform better, due to their lower ionic strength. We could not use nitrate as an eluent because Calhoun et al. (1983) and Chen (1973) showed that nitrate acts as a quencher affecting the FI detection. Consequently, we chose acetate as a pressing agent.

The use of a gradient for obtaining MurN together with the basic amino sugars in one run was not possible, due to the fact that MurN was overlaid by peaks of the matrix. Only the online purification described by Bodé et al. (2009) provided good and reproducible results for MurN. However, similarly to the method for the basic amino sugars, a loss of activity of the column was observed after 4 h, which was shown by insufficient separation. Therefore, regeneration of the column with a mobile phase consisting of 25% 100 mM sodium acetate and 75% 200 mM NaOH for 15 min and a subsequent re-equilibration for 40 min was necessary after every 3 runs. As already mentioned by Bodé et al. (2009), the decreasing activity of the column is due to the accumulation of stronger binding ions like carbonates on the column. The post column derivatisation performed well.

4.4.3 HPAEC-PAD

The advantages of the HPAEC-PAD methodology are the lack of need for derivatisation, the simultaneous determination of all 4 analytes in one run and the robustness against matrix interferences. We used this method for testing whether the OPA derivatisation had an effect on the measured amino sugar concentrations. For a comparison of the methods, it was necessary to use a similar mobile phase and, consequently, we used the method based on Meyer et al. (2008). This method had to be modified to obtain a baseline separation of the three basic amino sugars at the beginning of the run. Therefore, we reduced the column temperature from 35°C to 20°C. The pressure and the retention times increased by lowering

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the column temperature, but the separation of the three basic amino sugars significantly improved.

4.4.4 HPCEC and HPEXC

Watanabe (1984) described a determination of MurN via cation exchange chromatography and amperometric detection. He used KH_2PO_4 at pH 2.7 as the eluent. At this low pH-value we obtained two peaks for each amino sugar analyte. We obtained single peaks from pH 5 up to 7. Through an increase of the pH to 7 and of the column temperature to 50 °C a better but not optimal resolution of the basic amino sugars was achieved. A decrease of the KH_2PO_3 concentration from 30 mM to 20mM increased the retention times (Watanabe, 1984) but did not improve the resolution of the amino sugars, whereas an increase of the KH_2PO_3 concentration from 30 mM to 40 mM led to poor separation. This method is not suitable for amino sugar determination in soil hydrolysates, because the MurN peak could not be separated from matrix substances.

HPEXC was tested as an additional alternative for the determination of MurN. The Rezex ROA anion exclusion column was only applicable with water or mineral acids as the eluent. We tested water, H_3PO_4 , and H_2SO_4 at different column temperatures (20-86 °C) and different concentrations (0-250 mM H_2SO_4). MurN eluted only with 1% phosphoric acid as the mobile phase, whereas the basic amino sugars did not even elute after five hours. The separation of the ion exclusion mechanism is mainly based on the Donnan membrane exclusion principle (Hicks, 1985; Fischer, 2002). Hick et al. (1985) described ion exclusion as a process where ionic solutes pass through the interstitial volume of the resin, but are prevented from entering the pore volume of the sulfonated divinybenzene column material, because of the Donnan membrane effects. At an anion exclusion stationary phase, only sample anions are excluded from the resin phase by the fixed charges of the sulfonate groups of the cation exchange resin (Fritz, 1991). In mineral acids, basic amino sugars are protonated and positively charged, which would explain the high affinity to the column material. Probably a stronger cationic eluent, such as K_2HPO_4 , would elute the basic amino sugars. However, the column material is only suitable for mineral acids and water. The amino group of MurN is also protonated, whereas the carboxyl group is probably partially negatively charged, which causes a very low affinity of MurN to the column material. So we assume that MurN was probably not retained at all, because the retention time is almost identical to the dead time of the HPLC system. In conclusion, the anion exclusion separation mechanism is not suitable either for MurN or for

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basic amino sugar determination. For determining amino sugars via HPEXC a derivatisation, e.g. an acetylation of the amino group, would probably be necessary.

4.4.5 Comparison of RP-FI, HPAEC-FI and HPAEC-PAD

On RP-FI, it was possible to determine all four amino sugars simultaneously within 26 min (Indorf et al., 2011), whereas HPAEC-PAD analysis required 90 min. A simultaneous determination of all four amino sugars via HPAEC-FI was not achievable. The reversed phase separation with pre-column derivatisation is more suitable for a simultaneous determination of the basic amino sugars and MurN, because after derivatisation all four amino sugars are neutral indole derivatives with similar chemical properties, which makes it possible to use an isocratic method. In HPAEC without pre-column derivatisation the deprotonated carboxyl group of MurN has a much stronger affinity towards the positively charged column material than the basic amino sugars. This makes the use of a gradient or the implementation of two separate runs indispensable. Furthermore, because of the many matrix substances contained in soil, a gradient with a very slight slope has to be used. Other studies using anion exchange chromatography show similar results. Cataldi et al. (1999) and Meyer et al. (2008) determined amino sugars, sugars and uronic acids simultaneously by using a gradient, whereas Benner and Kaiser (2000) as well as Bodé et al. (2009) determined MurN in a separate chromatographic run.

For HPAEC-FI a gradient could not achieve a separation between MurN and the matrix peaks. This is probably due to the significantly broader matrix peaks in comparison to the small matrix peaks detected by HPAEC-PAD. In HPAEC-FI a separate run for MurN is necessary, because the MurN peak was otherwise completely overlapped by matrix peaks.

RP-FI of amino sugar analysis was rapid and relatively inexpensive compared with the HPAEC method, which required expensive columns, permanent helium or nitrogen stripping of the eluent, a time-consuming column cleaning and ideally a metal-free PEEK-consisting HPLC system. Although HPAEC is more time consuming and expensive than RP-FI, the validation parameters of these methods were basically the same for the three methods. The sensitivity was good for the RP-FI method, with a LOQ between $0.5 \mu\text{mol l}^{-1}$ sample extract (MurN) and $5.0 \mu\text{mol l}^{-1}$ sample extract (ManN), the LOQ of the HPAEC-FI was slightly higher, whereas the LOQ of HPAEC-PAD was even higher than the HPAEC-FI LOQ (Table 3b). This shows that the sensitivity of fluorescence detection in combination with OPA derivatisation is similar to that of PAD.

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The better correlation coefficients and the lower standard deviations of the precision values (Table 4a) of the fluorescence methods in comparison with the PAD-method are probably caused by the good baseline separation. A full baseline separation was not obtained for the HPAEC-PAD method. For achieving a baseline separation, we tried lowering the column temperature to 15°C. As a consequence, the retention times increased, but without obtaining an improvement in separation. Theoretically, a longer equilibration (e.g. 60 min) at the end of each run would improve separation.

There were no significant differences between the HPAEC-FI and the RP-FI method with regard to the amounts of all four amino sugars in soil hydrolysates (Table 5). As described above, we obtained partly significantly different amino sugar amounts in soil hydrolysates by using HPAEC-PAD. The reason for that is the different detection, not the chromatography. Probably the remaining matrix has an influence on the quantification by adsorbing on the PAD electrode surface. This would explain the lower sensitivity as well as the very low interday precision (Table 4a). As a result, both separation mechanisms (RP and HPAEC) provided similar results for amino sugars in soil samples. Thus, we have confirmed that the HPAEC separation mechanism gives valid results for amino sugar specific $\delta^{13}\text{C}$ -analysis in soil samples, as shown by Bodé et al. (2009).

4.5 Conclusions

On the basis of the data from this study, we conclude that the reversed phase method described by Indorf et al. (2011) is a reliable, rapid, inexpensive method best suited for amino sugar determination in plant and soil hydrolysates. When using HPAEC as the chromatographic system, a pulsed electrochemical detection is recommended. In comparison to fluorescence detection and post-column derivatisation, the HPAEC-PAD method has 3 advantages. (1) No derivatisation is necessary, (2) the matrix interferences are lower and (3) no separate run for determining MurN is required. However, a disadvantage of the HPAEC-PAD method is the somewhat high LOQ in comparison to the HPAEC-FI method (Table 4b). The sample purification method 1 based on Appuhn et al. (2004) and modified by Indorf et al. (2011) is rapid and provide good results for sensitive chromatographic systems. For samples with very low amino sugar content or a less sensitive chromatographic system, such as LC-IRMS, sample purification via strong cation exchange resin is recommended. However, the HPAEC method as described by Bodé et al. (2009) is suitable for the determination of amino

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sugars in soil hydrolysates and can be used for ^{13}C -specific amino sugar detection. Further research is required on increasing concentration and purification of MurN in soil samples.

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5. Determination of saprotrophic fungi turnover in different substrates by glucosamine-specific $\delta^{13}\text{C}$ liquid chromatography/isotope ratio mass spectrometry

Fungal ecology

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ABSTRACT

A high performance anion exchange chromatography (HPAEC) isotopic ratio mass spectrometry (IRMS) method was developed for amino sugar-specific $\delta^{13}\text{C}$ analysis in plant hydrolysates. The HPAEC-IRMS method provided good validation parameters and the amino sugar concentrations were similar to those obtained by reversed phase (RP) high performance liquid chromatography (HPLC) and fluorescence (FI) detection. The limit of quantification (LOQ) was $150 \mu\text{mol l}^{-1}$. This optimised HPAEC-IRMS method opens up the possibility of a glucosamine (GlcN) specific $\delta^{13}\text{C}$ analysis in plant material. Thus, it was possible to determine the $\delta^{13}\text{C}$ values in newly formed fungal GlcN for the first time. The formation and turnover of saprotrophic fungi was investigated by using the improved HPAEC-IRMS method for GlcN-specific $\delta^{13}\text{C}$ analysis. The cultivation of saprotrophic fungi (*Lentinula edodes* and *Pleurotus* species) showed the preferred formation of fungal biomass from maize-derived (80%) rather than from beech wood-derived C. The results indicate a faster formation of fungal biomass from maize than from wheat straw as co-substrate.

Key words: amino sugars / HPAEC / HPLC-IRMS / isotope / saprotrophic fungi

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5. Determination of saprotrophic fungi turnover in different substrates by glucosamine-specific $\delta^{13}\text{C}$ liquid chromatography/isotope ratio mass spectrometry

5.1 Introduction

The contribution of saprotrophic fungi to the biogeochemistry of terrestrial ecosystems is enormous (Högberg *et al.*, 1999). Owing to their decomposer properties, these fungi have a high impact in biotechnological degrading processes. The cultivation of saprotrophic, wood-decomposing fungi on organic waste is an efficient technique for recycling waste products like agricultural residues (Stamets 2000). Further, edible saprotrophic fungi play an important role in food industries (Leatham 1982, Stamets 2000) and traditional medicine (Lee & Balick 2006).

Little is known about the turnover of saprotrophic fungi. For a faster growth and a more efficient use of the profitable decomposer properties of the saprotrophic fungi, a better understanding of the formation and turnover of these fungi is indispensable. The natural abundance of stable isotopes expressed as δ -value is particularly suitable for performing tracer experiments and therefore for determining the formation and turnover of microorganisms. Based on the fact that C_3 and C_4 plants have different $\delta^{13}\text{C}$ values due to their different photosynthetic mechanisms, the use of C_4 and C_3 substrates as suitable tracers is presented in various studies (Engelking *et al.* 2007ab, Engelking *et al.* 2008, Semenina & Tiunov 2010). Advanced analytical methods like compound-specific $\delta^{13}\text{C}$ analysis by HPLC-IRMS simplify the determination of fungal turnover. The GC-C-IRMS method often described in the literature for amino sugar-specific $\delta^{13}\text{C}$ analysis is time consuming and causes isotope fractionation, due to derivatisation. Decock *et al.* (2009) suggested HPAEC-IRMS as a promising alternative, since derivatisation is not necessary for this method. Glucosamine (GlcN) is an indicator for fungal biomass (Ekblad & Näsholm 1995; Appuhn & Joergensen 2006) and therefore GlcN-specific $\delta^{13}\text{C}$ analysis facilitates a determination of the origin of newly formed fungal biomass.

The objectives of the present paper were (1) to adapt the HPAEC-IRMS method for amino sugar determination in soil hydrolysates based on Bodé *et al.* (2009) to an amino sugar analysis in plant and fungi samples and (2) to determine the formation and turnover of saprotrophic fungi, particularly the origin of the newly formed fungal biomass by HPAEC-IRMS.

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5.2 Materials and methods

5.2.1 Material

The optimisation of the HPAEC-IRMS method was implemented using three different plant litter samples (Table 1). The fungal growth experiment was carried out using three different saprotrophic wood-decomposing fungi (*Lentinula edodes* P., *Pleurotus ostreatus* K. and *Pleurotus citrinopileatus* S.). All fungi were obtained as grain spawn from Mycelia bvba (Nevele, Belgium). A mixture of beech (*Fagus sylvatica* L.) wood sawdust + maize (*Zea mays* L.) straw and a mixture of beech wood sawdust and wheat (*Triticum aestivum* L.) straw, respectively, were used as fungal substrates. The chemical properties of fungi and substrates are shown in Table 2.

Table 1 HPAEC-IRMS method optimisation: Comparison of galactosamine (GalN) and glucosamine (GlcN) results in plant samples obtained by RP-HPLC and HPAEC-IRMS. Main effects of the one-way ANOVA using plant material as independent factor and method as repeated measures for amino sugar amounts.

Plant material	GalN		GlcN	
	RP-HPLC	HPAEC-IRMS	RP-HPLC	HPAEC-IRMS
	($\mu\text{g g}^{-1}$ dry matter)			
Amaranth straw	170	170	1440	1490
Wheat straw	76	82	1290	1210
Maize leaves	26	15	36	66
Probability values				
Method	NS		NS	
Plant material	<0.01		<0.01	
Plant material \times method	NS		NS	
CV ($\pm\%$)	24	27	6	6

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Table 2 Chemical properties of the material used in the fungus experiment

Material	MurN	ManN	GalN	GlcN	$\delta^{13}\text{C}$ GlcN	$\delta^{13}\text{C}$ bulk	Total N	Total C
	$\mu\text{g g}^{-1}$ dry matter				‰		%	
Wheat	39	26	99	1100	-26.6	-27.5	0.8	43.0
Maize	4	7	3	245	-12.5	-12.7	1.3	43.4
Beech wood	20	16	13	250	-26.1	-26.0	0.4	47.0
<i>Lentinula edodes</i> .	-	82	3	19400	-27.0	-26.0	4.4	37.3
<i>Pleurotus ostreatus</i> .	-	103	9	22900	-26.6	-26.9	4.5	41.5
<i>P. citrinopileatus</i>	-	175	2	16600	-26.8	-26.8	4.5	41.2
CV	13	7	40	5	1	1	12	2

CV = pooled coefficient of variation

5.2.2 Solutions

All solutions were prepared with Milli-Q water produced via a Direct-Q 3 system (Millipore, Billerica, MA, USA). All other reagents were of high purity ($\geq 95\%$). The derivatisation reagents and standard stock solutions were prepared according to Indorf *et al.* (2011). The low carbonate 50% NaOH solution was purchased from Merck.

5.2.3 Substrate preparation and spawning

The experiment was carried out with 6 treatments (2 different substrates \times 3 different fungal strains) in 4 replicates, sampled at five time points. Consequently, 120 cultivation substrates were prepared for this experiment, 60 substrates consisted of beech wood and maize straw and the other 60 substrates were made of beech wood and wheat straw. The substrate components were mixed in special experimental jars (Microbox 0118/80+OD 118, Combiness, Eke-Nazareth, Belgium), each containing 15 g beech wood sawdust and 15 g maize straw or 15 g beech wood sawdust and 15 g wheat straw. All jars were supplied with 30 g water, that of the wood-wheat mixture additionally contained 41.06 mg $(\text{NH}_4)_2\text{SO}_4$ to obtain the same C/N ratio as the wood-maize-mixture. After mixing, the substrate-containing jars were autoclaved at 121 °C for 20 min.

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The two different substrates were both inoculated with 3 g of grain spawn using 3 different fungal species (*Lentinula edodes* P., *Pleurotus ostreatus* K. and *Pleurotus citrinopileatus* S.). Then, the samples were weighed and incubated at 24 °C for 4 weeks in a 0.48 m³ incubator, provided with 0.24 m³ h⁻¹ water filtered fresh air. The samples in the incubator were randomly distributed, each replicate was assigned to a separate grid. Twenty-four were taken initially and after every week.

5.2.4 Amino sugar extraction

The amino sugar extraction was based on the method described by Appuhn *et al.* (2004). Oven-dried (60°C for 4-5 days) plant material (800 mg, ball-milled) was mixed with 10 ml of 6 M HCl. After 3 h hydrolysis at 105°C, the samples were filtered over glass filters (Whatman GF/A). A 0.3 ml aliquot was evaporated to dryness at 40-45 °C to remove HCl, re-dissolved in water, evaporated a second time and re-dissolved in 1 ml water. After centrifugation at 5000 g, the supernatant was frozen and stored at -18 °C until analysis.

5.2.5 Amino sugar determination

The amino sugar determination by reversed phase and pre-column derivatisation was based on the method described by Indorf *et al.* (2011). Chromatographic separations were performed on a Phenomenex (Aschaffenburg, Germany) Hyperclone C₁₈ (ODS) column (125 mm length × 4 mm diameter, 5 µm particle size, 12 nm pore size), protected by a Phenomenex C₁₈ guard cartridge (4 mm length × 2 mm diameter). The column was kept at 35°C. The HPLC system consisted of a Dionex (Germering, Germany) P 580 gradient pump, a Dionex Ultimate WPS-3000TSL analytical autosampler with in-line split-loop injection and thermostat and a Dionex RF 2000 fluorescence detector set at 445 nm emission and 330 nm excitation wavelengths with medium sensitivity. For the automated pre-column derivatisation, 50 µl OPA and 30 µl sample were mixed in the preparation vial and after 120 sec reaction time 15 µl of the indole derivatives were injected. The mobile phase consisted of two eluents. Eluent A was a 97.8/0.7/1.5 (v/v/v) mixture of a water phase, methanol and tetrahydrofuran (THF). The water phase contained 52 mmol sodium citrate and 4 mmol sodium acetate, adjusted to pH 5.3 with HCl. Then methanol and THF were added. Eluent B consisted of 50% water and 50% methanol (v/v).

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5.2.6 Amino sugar specific $\delta^{13}\text{C}$ analysis by HPAEC-IRMS

The chromatographic conditions described by Bodé *et al.* (2009) had to be modified for the analysis of plant material. The anion exchange separation was performed using a Dionex CarboPac PA20 column (150 mm length x 3 mm diameter, 6.5 μm particle size) protected by a Dionex AminoTrap guard column (30 mm length x 3 mm diameter). The HPLC system consisted of a metal-free PEEK gradient pump (Sykam S2100, Fuerstenfeldbruck), a metal-free Peek autosampler (Sykam S5200), a LC Isolink interface (Thermo Electron, Bremen) and an isotope ratio mass spectrometer (IRMS, DELTA^{PLUS} XP, Thermo Electron).

The mobile phase consisted of 1 mM NaOH and the flow rate was set to 0.30 ml min⁻¹. The NaOH eluent was degassed by sonication under vacuum for 15 min and was covered with N₂ during chromatography. GlcN and galactosamine (GalN) analysis was performed isocratically. The columns were maintained at 38 °C. The injection volume was 20 μl . After chromatographic separation, the eluent was delivered to the LC Isolink interface, where all organic components were quantitatively oxidised to CO₂ (Krummen *et al.* 2004). This oxidation was performed by mixing Na₂S₂O₈ (50 μl min⁻¹) and H₃PO₄, (50 μl min⁻¹) into the eluent, directed to an oxidation reactor set at 99.9 °C. Then, a helium stream stripped the CO₂ produced from the eluent into a capillary gas exchanger. The uncovered gas was directed over a gas dryer to the IRMS. After every run the column was cleaned with 200 mM NaOH for 15 min and equilibrated for the next sample with 1 mM NaOH for 30 min.

5.2.7 Total C and total $\delta^{13}\text{C}$

Total C and total $\delta^{13}\text{C}$ in the oven-dried, ball milled bulk samples were measured on a Delta plus IRMS 251 (Finnigan Mat, Bremen, Germany) after combustion using a Carlo Erba NA 1500 elemental analyser.

5.2.8 Calculations and statistical analysis

The fungal C was calculated by multiplying fungal GlcN by 9 (Appuhn & Joergensen 2006). The new fungal C was obtained by subtracting the fungal C from the fungal C contained in the sample of week 0.

$$\text{Fungal } C_{\text{new}} = (\text{fungal GlcN}_0 \times 9) - (\text{fungal GlcN} \times 9) \quad [1]$$

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The relative specific allocation (RSA) is the proportion of newly incorporated maize derived GlcN (f_{new}) relative to total glucosamine in the sample (Cliquet *et al.* 1990; Dyckmans *et al.* 2000). The RSA and the maize-derived fungal GlcN were calculated as follows:

$$f_{\text{Maize}} = \frac{(\delta \text{GlcN}_{\text{SubstrateM}} - \delta \text{GlcN}_{\text{SubstrateW}})}{(\delta \text{GlcN}_{\text{Maize}} - \delta \text{GlcN}_{\text{Wheat}})} \quad [2]$$

$$\text{RSA}(\%) = \frac{(f_{\text{Maize}} \times \text{total GlcN} (g)) - (f_{\text{Maize}} f_{\text{Maize}} \text{GlcN}_{\text{T0}} \times \text{total GlcN}_{\text{T0}} (g))}{\text{Total GlcN} (g) - (\text{total GlcN}_{\text{T0}} (g) - \text{fungal GlcN}_{\text{T0}} (g))} \quad [3]$$

where $\delta\text{-GlcN}_{\text{maize}}$ was the GlcN-specific $\delta^{13}\text{C}$ value of pure maize straw, $\delta\text{-GlcN}_{\text{wheat}}$ was the GlcN-specific $\delta^{13}\text{C}$ value of pure wheat straw, whereas $\delta\text{-GlcN}_{\text{substrateM}}$ was the GlcN-specific $\delta^{13}\text{C}$ value of the maize-wood-fungi mixture and $\delta\text{-GlcN}_{\text{substrateW}}$ was the GlcN-specific $\delta^{13}\text{C}$ value of the wheat-wood-fungi mixture. Total GlcN is the total GlcN concentration of the maize-wood-fungi mixture, total GlcN_{T0} is the total GlcN concentration of the maize-wood-fungi sample at week 0. The fungal GlcN is the part of GlcN in the sample that was provided by the fungi at week 0.

The significance of differences between substrates and fungi was tested by a week-specific two-way ANOVA or by two-way ANOVA using sampling week as repeated measures. Statistical analyses were carried out using SPSS statistical software (SPSS 19.0). The results presented in tables and figures are arithmetic means and are given on an oven-dry basis.

5.3 Results

5.3.1 HPAEC-IRMS method optimisation

The GlcN peak of a standard mixture, using the HPAEC-IRMS method described by Bodé *et al.* (2009), eluted at 1680 sec (Fig. 1a), which moved to 720 sec after changing the eluent concentration and the column temperature (Fig. 1b). The GlcN peak of a wheat straw hydrolysate, using the HPAEC-IRMS method described by Bodé *et al.* (2009), coeluted with a matrix peak of the plant material (Fig. 1c), whereas the GlcN peak was clearly separated from the matrix by our optimised method (Fig. 1d). The $\delta^{13}\text{C}$ values of GalN and GlcN in a standard solution were reproducible from a concentration of $200 \mu\text{mol l}^{-1}$. In a concentration range between $200 \mu\text{mol l}^{-1}$ and $900 \mu\text{mol l}^{-1}$ the coefficients of variation (CV) were between

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0.2 and 1.4 for the $\delta^{13}\text{C}$ values of GalN and GlcN in a standard solution mix, whereas the lowest CV were obtained at concentrations between 300 and 700 $\mu\text{mol l}^{-1}$. The calibration lines for GalN and GlcN resulted in correlation coefficients (r^2) between 0.998 and 0.999, in a linear concentration range from 200 $\mu\text{mol l}^{-1}$ to 900 $\mu\text{mol l}^{-1}$. The lowest quantification levels (LOQ, 10-times higher than noise) of the GalN and GlcN standard solution were 50 $\mu\text{mol l}^{-1}$. However, to obtain reliable $\delta^{13}\text{C}$ values, a concentration of 200 $\mu\text{mol l}^{-1}$ is necessary.

A comparison of this optimised HPAEC-IRMS method with the validated reversed phase HPLC method provided similar results for the concentrations of GalN and GlcN in different plant materials (Table 1). The CV for the GalN- and GlcN-specific $\delta^{13}\text{C}$ values ranged between 1 for GalN and 2 for GlcN.

5.3.2 The fungal growth experiment

The concentration of total C generally decreased during the incubation, without any significant effect of the fungal species, but with a significantly stronger decline in the maize-wood mixture (Table. 3). The GlcN concentration increased significantly from 1070 $\mu\text{g g}^{-1}$ dry matter (wheat-wood mixtures) and 620 $\mu\text{g g}^{-1}$ dry matter (maize-wood mixtures), respectively, at week 0 to 2200 $\mu\text{g g}^{-1}$ dry matter (wheat-wood mixtures) and 4000 $\mu\text{g g}^{-1}$ dry matter (maize-wood mixtures) at week 4, respectively. Nevertheless, the maize-wood mixtures, especially those inoculated with *Lentinula edodes*, showed a higher increase in GlcN than the wheat-wood mixtures (Table 4). The maize-wood mixtures revealed a continuous increase of newly formed fungal C over the weeks (Fig. 2).

The average bulk $\delta^{13}\text{C}$ values over the whole incubation period were -27 ‰ for the wheat-wood and -21 ‰ for the maize-wood mixtures. Significant differences were observed between the substrates but not between the weeks or the different fungi by a two-way ANOVA using the week as repeated measures (results not shown). The situation was different for the GlcN-specific $\delta^{13}\text{C}$ values of the maize-wood mixtures, which increased significantly on average from -17.5 to -14.2‰ (Table 4). This means that they were considerably higher than the bulk $\delta^{13}\text{C}$ values. After 1 week, 60% of the newly formed fungal GlcN was maize derived, and roughly 80% after 2 weeks. No further increase in maize-derived GlcN was observed after 3 and 4 weeks (Fig. 3).

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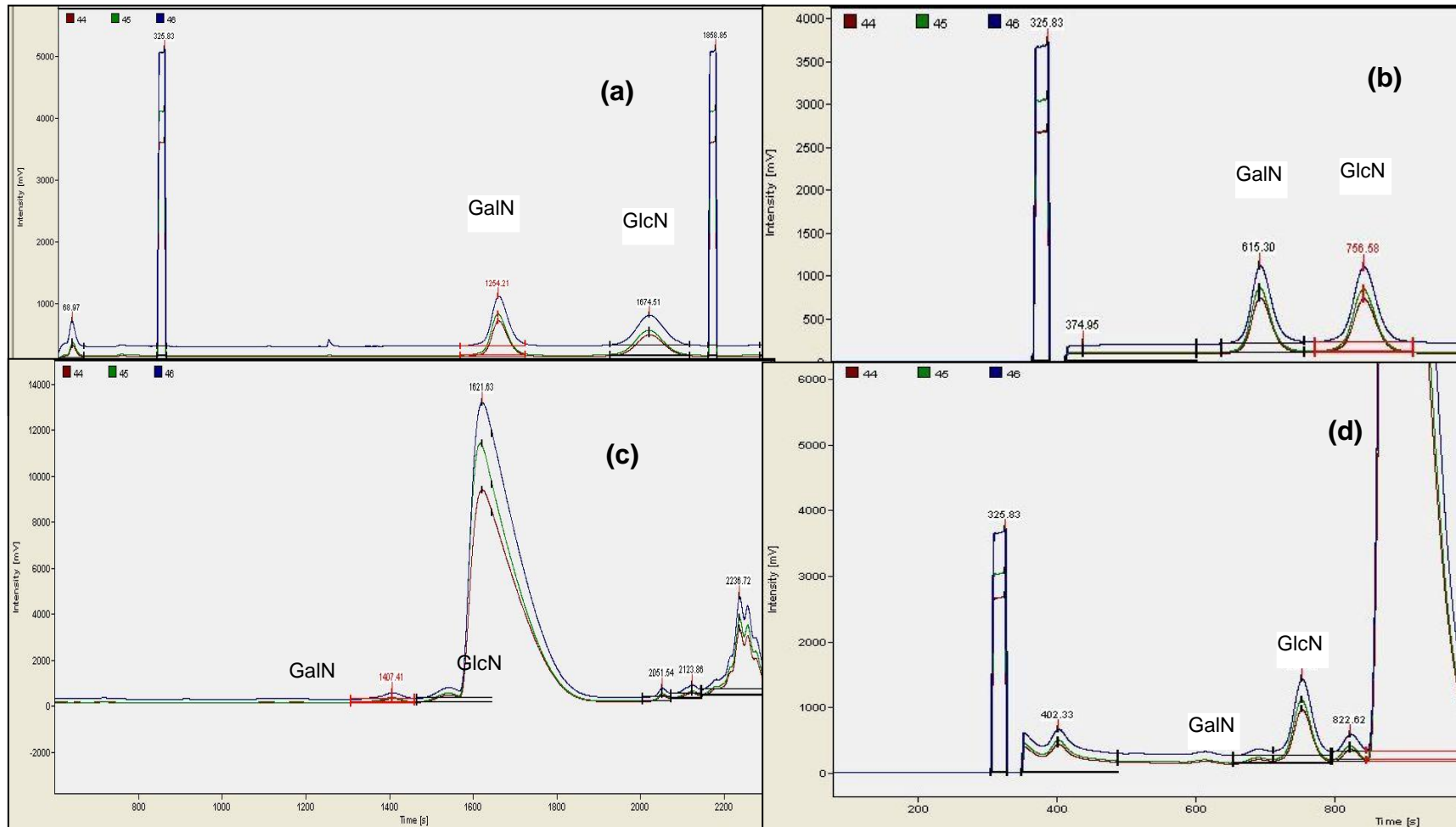


Fig. 1 Chromatograms of a standard mixture consisting of $200 \mu\text{mol l}^{-1}$ GalN and $200 \mu\text{mol l}^{-1}$ GlcN (a) obtained from the HPAEC-IRMS method based on Bodé *et al.* (2009) and (b) obtained from the optimised HPAEC-IRMS method (c) a wheat straw hydrolysate measured by HPAEC-IRMS method described by Bodé *et al.* (2009) and (d) a wheat straw hydrolysate obtained from the optimised HPAEC-IRMS method.

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Table 3 Total C in wheat-wood and maize-wood mixtures inoculated with *Lentinula edodes*, *Pleurotus ostreatus*, and *Pleurotus citrinopileatus*, respectively, during 4 weeks' incubation; main effects of the 2-way week specific ANOVA, using substrate and fungi as independent factors and week as repeated measures.

Week	Total C (%)
0	45.66
1	45.22
2	45.04
3	44.98
4	44.75
Probability values	
Substrate	<0.01
Fungi	NS
Week	0.01
Substrate \times fungi	0.01
Substrate \times week	0.03
Fungi \times week	NS
CV ($\pm\%$)	1

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Table 4 Glucosamine (GlcN) amounts and GlcN-specific $\delta^{13}\text{C}$ values in wheat (W) and maize (M) samples inoculated with *Lentinula edodes* (LE), *Pleurotus ostreatus* (PO) and *Pleurotus citrinopileatus* singer (PC), respectively, during 4 week incubation; main effects of the 2-way week specific ANOVA using substrate and fungi as independent factors.

Week	GlcN ($\mu\text{g g}^{-1}$ dry matter)					$\delta^{13}\text{C}$ GlcN (‰)				
	0	1	2	3	4	0	1	2	3	4
Sample										
LE-W	1020	1100	1370	2090	2520	-26.5	-27.4	-26.7	-26.3	-26.3
PO-W	1110	1220	1800	2000	2070	-26.7	-26.6	-26.3	-26.2	-26.2
PC-W	1090	1300	1680	1870	1970	-26.3	-26.4	-26.0	-25.9	-25.8
LE-M	660	970	2000	3490	5030	-18.6	-17.6	-15.6	-14.7	-14.1
PO-M	660	1430	2920	3050	3370	-18.9	-17.8	-15.4	-14.7	-14.5
PC-M	550	1240	2780	3250	3610	-17.7	-16.6	-14.5	-14.1	-13.9
Substrate	<0.01	NS.	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Fungi	NS	<0.01	<0.01	NS	<0.01	<0.01	<0.01	<0.01	0.02	<0.01
Substrate \times fungi	NS	NS	0.03	NS	<0.01	NS	0.03	NS	0.04	NS
CV ($\pm\%$)	10	17	17	10	17	3	3	3	2	2

CV = pooled coefficient of variation; NS = not significant.

5. Determination of saprotrophic fungi turnover in different substrates by glucosamine-specific $\delta^{13}\text{C}$ liquid chromatography/isotope ratio mass spectrometry

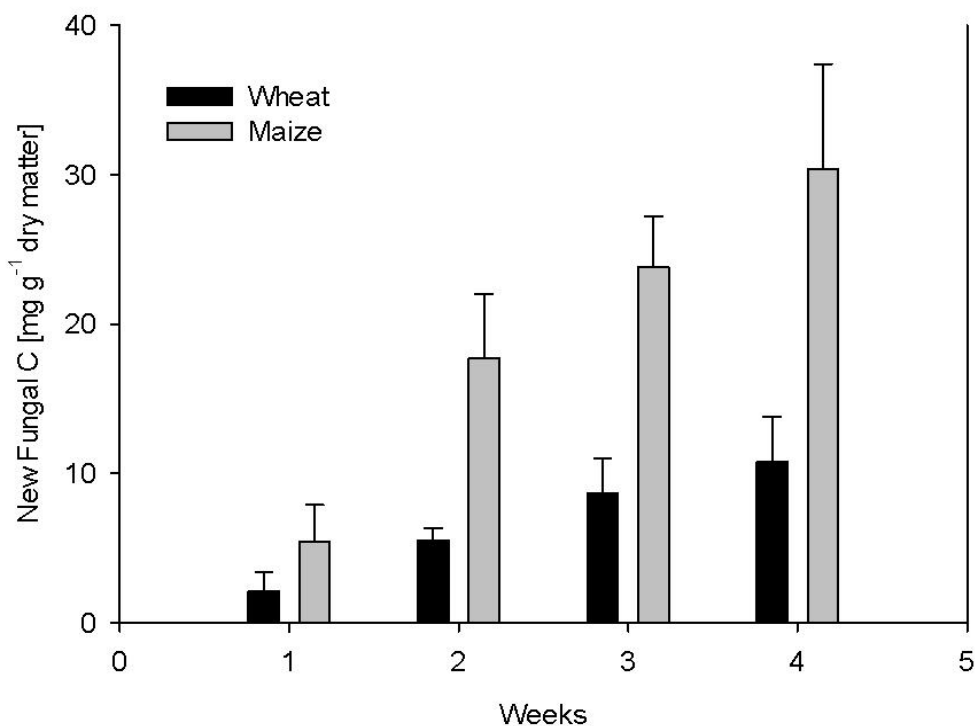


Fig. 2 Formation of new fungal C (calculated according equation (1); one bar represents the mean value of all three fungi strains) in wheat-wood and maize-wood samples inoculated with fungi during 4 week incubation at 24°C; error bars represent the standard deviation, n=12.

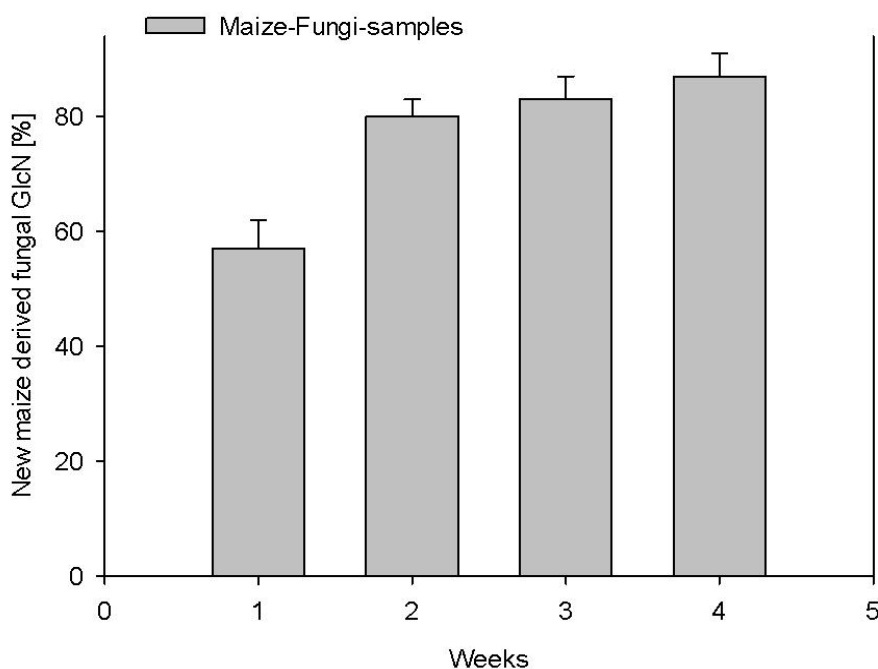


Fig. 3 Formation of new maize derived GlcN (calculated according equation (2) and (3); one bar represents the mean value of all three fungi strains) in maize-wood samples inoculated with fungi during 4 week incubation at 24°C; error bars represent the standard deviation, n=12.

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5.4 Discussion

5.4.1 HPAEC-IRMS optimisation

The optimised amino-sugar specific $\delta^{13}\text{C}$ HPAEC-IRMS method is suitable for the determination of GalN and GlcN in soil and plant hydrolysates. This is an advantage in comparison with the method described by Bodé *et al.* (2009), which performed well for soil samples, but not for plant material, as the matrix of the plant samples strongly interfered with GlcN. This advantage is of special importance as the amino sugar dynamics are certainly stronger in freshly colonised plant samples than in the bulk soil (Appuhn *et al.* 2006).

A determination of amino sugars by HPLC-Fl and post-column derivatisation with ortho-phthaldialdehyde (OPA) of the different plant samples also revealed the presence of this enormous matrix peak for all tested plant materials. Since OPA only reacts with primary amines, this interfering matrix must be a compound that, similarly to the amino sugars, contains an amine as a functional group. Amino acids can be excluded as interfering compounds, due to the use of an amino trap. All amino acids remained on this trap and were only eluted if a stronger eluent like nitrate was used and, even in this case, not before 60 min on the column. The interfering plant matrix components may be amines that originate from transamination during the decomposition of plant materials (Mannheim *et al.* 1996). The interfering components might be products of the Maillard reaction, a complex series of non-enzymatic reactions leading to hundreds of end products (melanoidins) and many stable intermediates like glycosylamines (Pigman *et al.* 1959, Nursten 2005). The Maillard reaction occurs between an amine (e.g. protein, peptide or amino acid) and a carbonyl compound (e.g. reducing sugar). The formation of some Maillard products is acid catalysed (Nursten 2005), which implies that the acid hydrolysis of the samples may have accelerated some steps of the Maillard reaction.

A purification and elimination of the matrix peaks with cation exchange resins resulted in no improvement. Only the increase of the column temperature from 15°C to 38°C yielded a clearly better separation. Horvath *et al.* (1967) already described the effect of temperature on the separation by using ion exchange chromatography. They showed in their studies that the column performance improved with temperature, due to the increased solute diffusivity in the stationary as well as in the mobile phase. They also observed a significant reduction in tailing. The increase in temperature causes a reduction of the capacity factor, which is a criterion for how much longer the sample molecules stay in the stationary phase than in the mobile phase.

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A low capacity factor reduces retention time but decreases selectivity (Camman 2001). Consequently, the analyte determines which temperature gives the best results. In our case, a higher temperature improved the separation of GlcN from the plant matrix components, but impaired the separation between GalN and GlcN. At 38°C column temperature, a good compromise was achieved between an acceptable baseline separation of GalN and GlcN and a baseline separation between GlcN and the huge matrix peak (Fig. 1d).

According to Pohl *et al.* (1997), some functional groups or solutes may change their degree of ionisation at elevated temperatures, which can lead to changes in solute retention and selectivity. It is probable that the matrix peak changed its degree of ionisation, explaining the improved selectivity for this peak at elevated temperatures. Pohl *et al.* (1997) further described the effects of eluent concentrations on the separation. An increase of concentration leads to a decrease of selectivity. For this reason, we decreased the NaOH concentration from 2 mM (Bodé *et al.* 2009) to 1 mM NaOH. However, this change only had a small effect on the separation, compared with the change of temperature, but it improved the separation between matrix peak and GlcN, without impairing the selectivity between GalN and GlcN.

Considering the amino sugar concentrations, there were no significant differences between the new HPAEC method and the HPLC-Fl method. A comparison with results of the literature and the results obtained for the chitin concentration in fungi using the optimised HPAECC-IRMS method showed no significant deviations. We determined an average GlcN concentration of about 20 mg g⁻¹ dry matter (Table 2). This is in agreement with chitin concentrations of between 10 to 60 mg g⁻¹ dry matter for saprotrophic fungi (Vetter & Siller 1990; Nitschke *et al.* 2011).

5.4.2 The fungal growth experiment

The maize-wood mixtures were preferably decomposed by the fungal species in comparison with the wheat-wood mixture. It was repeatedly shown that decomposition and formation of fungal biomass depend on the substrate quality (Fernandez & Cadish 2003; Kohzu *et al.* 2005; Pant *et al.* 2005). After N addition to the wheat-wood mixture there was initially no difference of the C/N ratio between the two substrates. Therefore, the N concentration and the C/N ratio, respectively, of the substrate may not be responsible for the faster degradation of the maize-wood mixture in the present experiment, contrasting the suggestions by Fernandez and Cadish (2005) and Liang *et al.* (2009).

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However, Roger *et al.* (1993) obtained a faster degradation of maize in comparison with wheat substrate by rumen fungi. They explained this observation with the higher hemicellulose concentrations in the maize in comparison with the wheat substrate. Liang *et al.* (2009) tested different plant-wood substrates as a growth medium for *Pleurotus citrinopileatus* and showed a preferred degradation of maize-wood mixture, due to better substrate use efficiency. The formation of GlcN and therefore that of a new fungal biomass is significantly higher in maize-wood than in the wheat-wood mixture (Table 4, Fig.2). This is in line with Leatham (1985), who observed a stimulation of ligninolytic activity by an addition of easily decomposable carbohydrates such as xylose to the wood substrate. However, the newly formed fungal GlcN is maize and not wood derived in the present experiment (Fig. 3).

Several authors (Gleixner *et al.* 1993; Henn *et al.* 2004; Kohzu *et al.* 2005; Hart *et al.* 2006; Boström *et al.* 2007; Zeller *et al.* 2007) observed a $\delta^{13}\text{C}$ enrichment in the saprotrophic fungi sporophores of between 1.2 to 6.3‰ relative to the wood decomposed. Zeller *et al.* (2007) assumed that cellulose and lignin degradation could be involved in $\delta^{13}\text{C}$ enrichment of sporocarps of saprotrophic fungi. Kohzu *et al.* (2005) explained the $\delta^{13}\text{C}$ enrichment of saprotrophic fungi relative to their substrate as being due to (1) a selective incorporation of ^{13}C enriched fraction in the substrate by fungi and (2) fungal metabolic processes. They mentioned dark fixation of ambient CO_2 and the kinetic fractionation during assimilation and dissimilation reactions. However, the $\delta^{13}\text{C}$ values of the wheat-wood substrate show no $\delta^{13}\text{C}$ enrichment. Furthermore, Semenina and Tiunov (2010) showed that there were no significant differences between the shift in $\delta^{13}\text{C}$ -values of C_4 and C_3 samples during the incubation, using C_4 and C_3 sucrose as substrate.

Consequently, $\delta^{13}\text{C}$ fractionation during formation of fungal biomass seems mainly to depend on the substrate. Most likely, the high shifts in the GlcN-specific $\delta^{13}\text{C}$ values of maize-wood mixtures were caused by the formation of maize-derived GlcN and a small part might be additionally caused by $\delta^{13}\text{C}$ fractionation. The GlcN-specific $\delta^{13}\text{C}$ values differed among the fungal species (Table 4), which is in line with Trudell *et al.* (2004), who observed significant differences between $\delta^{13}\text{C}$ values of bulk saprotrophic fungi among genera and species.

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5.5 Conclusions

The optimised HPAEC-IRMS method provided reliable results for the concentrations and the $\delta^{13}\text{C}$ values of GlcN and GalN in plant samples. The fungal growth experiment indicated that maize-wood mixture was the preferred substrate of saprotrophic fungi in comparison with the wheat-wood mixture. The shifts in $\delta^{13}\text{C}$ values were mainly due to the incorporation of maize-derived material into fungal biomass and less due to kinetic isotope fractionation. In the near future, the optimised HPAEC-IRMS method will make it possible to investigate differences in the turnover of, e.g. ecto-mycorrhizal and saprotrophic fungal species in the presence of different organic substrates.

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6. Zusammenfassung

In der vorliegenden Arbeit wurde eine LC-IRMS Methode zur aminozucker-spezifischen $\delta^{13}\text{C}$ -Analyse in Pflanzenmaterialien optimiert und etabliert, um die Bildung und den Umsatz von mikrobiellen Residuen in Boden- und Pflanzenmaterialien mit hoher Genauigkeit erfassen zu können. Weiterhin wurden mit der etablierten Methode zwei Experimente durchgeführt. Der Fokus dieser Arbeit lag jedoch auf der Methodenentwicklung. So wurden verschiedene Aufarbeitungstechniken getestet und optimiert. Außerdem wurde an der HPLC die bereits verfügbare Methode basierend auf Appuhn et al. (2004) optimiert. Zur Etablierung der aminozucker-spezifischen $\delta^{13}\text{C}$ -Analyse wurden weitere chromatographische Trennmechanismen, mit dem Hintergrund eine möglichst schnelle und effiziente Trennung der Aminozucker mit einem kohlenstofffreien Eluenten zu erzielen, getestet. Weiterhin wurde ein Pilzwachstumsversuch mit saprotrophen Pilzen an Mais-Holz- und Weizen-Holz-Substraten durchgeführt.

Der erste Artikel beschäftigt sich mit der Optimierung einer HPLC-Methode zur Aminozuckerquantifizierung (Appuhn et al., 2004) in Boden- und Pflanzenhydrolysaten. Die Methode von Appuhn et al. (2004) hat zwei Nachteile. (1) Die Methode erbrachte nur zuverlässige Ergebnisse an der Agilent 1100 HPLC und (2) wurde Mannosamin in den meisten Bodenhydrolysaten nur ungenügend getrennt, was zu fehlerhaften hohen Werten in weiteren Publikationen (Appuhn und Jörgensen 2006; Engelking et al. 2007b) zur Folge hatte. Ziel des ersten Artikels war es deshalb die Methode von Appuhn et al. (2004) so zu verbessern, dass an verschiedenen HPLC-Systemen zuverlässige Ergebnisse für alle vier Aminozucker in Boden- und Pflanzenhydrolysaten erhalten werden. Dafür wurde zunächst die mobile Phase optimiert. So wurde der Tetrahydrofurananteil in der mobilen Phase von 0,75% (Appuhn et al., 2004) auf 1,5% erhöht. Durch die Erhöhung des Tetrahydrofurananteils der mobilen Phase konnte deswegen eine kürzere Retentionszeit für alle vier Aminozucker und eine bessere Trennung zwischen Muraminsäure und Mannosamin erzielt werden. Weiterhin wurde die Extinktionswellenlänge des Fluoreszenzdetektors zur Detektion der durch Derivatisierung der Aminozucker erhaltenen Isoindolderivate von 340 nm (Appuhn et al., 2004) auf 330 nm herabgesetzt. Bei dieser Wellenlänge wurde das höchste Signal für die Aminozuckerderivatpeaks detektiert, ohne dass das Signal der Matrixpeaks erhöht wurde. Außerdem hat die OPA-Derivatisierungsreaktionszeit Einfluss auf die Höhe des Fluoreszenzsignals. Bei 120 Sekunden konnte das Maximum für alle vier Aminozucker erzielt werden. Nach Optimierung der genannten Parameter erfolgte die Validierung der Methode.

Für Muraminsäure wurde eine Bestimmungsgrenze (LOQ) von $0,5 \mu\text{mol l}^{-1}$ was $0,13 \mu\text{g ml}^{-1}$ entspricht erhalten. Für die drei anderen Aminosucker wurden Bestimmungsgrenzen von $5,0 \mu\text{mol l}^{-1}$ (entspricht $0,90 \mu\text{g ml}^{-1}$) erhalten. Der lineare Arbeitsbereich lag zwischen $5,0$ und $210 \mu\text{mol l}^{-1}$ (Mannosamin, Galaktosamin und Glucosamin) bzw. zwischen $0,5$ und $21 \mu\text{mol l}^{-1}$ (Muraminsäure). Die relative Standardabweichung betrug durchschnittlich 2% innerhalb eines Tages und 5% innerhalb von sechs Tagen für die Aminosuckerstandards. Weiterhin wurden Wasser und Phosphatpuffer als Probenlösungsmittel getestet, um den Einfluss des pH-Wertes auf die OPA-Reaktion zu testen (Dorresteijn et al., 1996). Es wurde angenommen, dass in Phosphatpuffer gelöste Proben stabiler sind aufgrund des höheren pH-Wertes (pH=5) im Vergleich zu in Wasser gelösten Proben (pH=2-3). Jedoch erzielten die in Wasser aufgenommenen Proben geringere Standardabweichungen, da vermutlich die in der Bodenhydrolysatmatrix enthaltenen Aluminium- und Eisenoxide mit Phosphat Komplexe bilden. Diese Komplexe binden einen Teil der Aminosucker, so dass die gebundenen Aminosucker nicht mehr bestimmt werden können.

Die in Boden- und Pflanzenhydrolysaten mit dieser optimierten HPLC-Methode gemessenen Aminosuckergehalte waren mit den Werten aus der Literatur vergleichbar.

Im zweiten Artikel wurden mehrere HPLC Methoden zur Bestimmung von Aminosuckern in Bodenhydrolysaten getestet und miteinander verglichen. Außerdem sind diverse Aufarbeitungsmethoden zur Purifikation und Konzentrierung der Aminosucker getestet worden. Zur aminozucker-spezifischen $\delta^{13}\text{C}$ –Analyse am IRMS ist eine HPLC-Methode mit einer kohlenstofffreien mobilen Phase notwendig, andernfalls kann aufgrund des hohen Hintergrundrauschens kein vernünftiges Signal mehr detektiert werden. Da die im ersten Artikel beschriebene Umkehrphasenmethode einen kohlenstoffhaltigen Eluenten enthält, musste eine ebenso zuverlässige Methode, die jedoch keine organischen Lösungsmittel benötigt, getestet und mit der schon etablierten Methode verglichen werden. Es gibt eine Reihe von HPLC-Methoden, die ohne organische Lösungsmittel auskommen, wie z. B. (1) HPAEC, (2) Hochleistungskationenaustauschchromatographie (HPCEC) und (3) die Hochleistungsanionenausschlusschromatographie (HPEXC). Die von Bodé et al. (2009) beschriebene HPAEC- Methode für die Isotopenaminosuckeranalyse hat einige Nachteile: (1) Das NaOH in der mobilen Phase reagiert schnell mit CO_2 aus der Luft zu Carbonat und führt so zu einem hohen Hintergrundrauschen und verschlechtert die Trennung der Aminosucker auf der Analysensäule, (2) die alkalische mobile Phase setzt im Idealfall ein metallfreies System voraus, (3) die von Bodé et al. (2009) beschriebene Methode besteht aus zwei separaten Läufen (Muraminsäure und basische Aminosucker), und (4) die Nachweisgrenze

von Muraminsäure ist zu hoch für die geringe Muraminsäurekonzentration in den Probenhydrolysaten. Aufgrund dieser Faktoren waren die Ziele der Studie (1) eine zuverlässige Purifikations- und Konzentrierungsmethode für Aminosucker in HCl-Hydrolysaten zu finden und (2) die Methode von Bodé et al. (2009) zu optimieren durch das Testen verschiedener Trennungsmechanismen. Es wurden folgende fünf Aufarbeitungsmethoden zur Purifikation und Konzentrierung der Probenhydrolysate getestet: (1) Extraktion basierend auf Appuhn (2004), (2) Extraktion basierend auf Zhang und Amelung (1996), (3) Extraktion mittels eines starken Kationenaustauscherharzes auf Polymerbasis, (4) Extraktion mittels eines starken Kationenaustauscherharzes auf Silicabasis und (5) Extraktion mittels eines starken Anionenaustauscherharzes zur Konzentrierung und Separation von Muraminsäure. Die besten Ergebnisse wurden mit den Kationenaustauscherharzen erzielt, wobei die dritte Methode noch ein wenig besser geeignet war als die vierte. Nach Anwendung der dritten Methode konnten die Matrixeinflüsse leicht reduziert und die Aminosucker konzentriert werden. Zur weiteren Trennung der Aminosucker mittels HPLC konnte für die HPCEC und die HPEXC keine zufriedenstellende Trennung der Aminosucker erreicht werden. Die von Bodé et al. (2009) beschriebene HPAEC-Methode erwies sich als geeignet und es konnten mit der Umkehrphasenmethode vergleichbare Ergebnisse für Bodenhydrolysate erzielt werden. Die HPAEC-Methode wurde zunächst mittels OPA-Nachsäulenderivatisierung und Fluoreszenzdetektion durchgeführt. Bei der Muraminsäuremethode wurde als Eluent Natriumnitrat durch Natriumacetat ersetzt, da Nitrat die Fluoreszenz gelöscht hat. Aufgrund der mittels Fluoreszenzdetektion erhaltenen breiten Matrixpeaks, konnte bei dieser Methode kein Gradient angewendet werden, so dass Muraminsäure in einem separaten Lauf bestimmt werden musste. Weiterhin wurde ein elektrochemischer Detektor zur Detektion getestet. Hier waren die Peaks schmaler, so dass es möglich war alle vier Aminosucker mit Hilfe eines Laufmittelgradienten in einem Lauf zu bestimmen. Schlussfolgernd kann zusammengefasst werden, dass für Detektoren mit geringer Empfindlichkeit (z.B. IRMS) eine Konzentrierung und Purifikation insbesondere von Muraminsäure über ein Kationenaustauscherharz sinnvoll ist. Eine Basislinientrennung für alle Aminosucker war nur mit der HPAEC möglich. Da mit dieser Methode gute Validierungsdaten erzielt wurden und die Aminosuckergehalte mit der Umkehrphasenmethode vergleichbar waren, stellt die HPAEC die Methode der Wahl zur aminosucker-spezifischen $\delta^{13}\text{C}$ -Analyse am IRMS dar.

Der dritte Artikel befasst sich mit der Optimierung der aminosucker-spezifischen $\delta^{13}\text{C}$ – Analyse mittels HPAEC-IRMS in Pflanzenhydrolysaten sowie mit der Bestimmung des

Umsatzes von saprotrophen Pilzen in verschiedenen Substraten. Die von Bodé et al. (2009) beschriebene HPAEC-IRMS- Methode ist für die aminozucker-spezifische $\delta^{13}\text{C}$ -Analyse in Bodenhydrolysaten jedoch nicht in Pflanzenhydrolysaten geeignet. In Pflanzenhydrolysaten wird der Glucosaminpeak von Peaks aus der Matrix interferiert. Folglich war das erste Ziel dieses Artikels, die Methode so zu optimieren, dass eine aminozucker-spezifische $\delta^{13}\text{C}$ – Analyse in Pflanzenhydrolysaten möglich ist. Weiterhin sollten mit der optimierten HPAEC-IRMS-Methode die Bildung und der Umsatz von saprotrophen Pilzen bestimmt werden. Durch Änderung der Säulentemperatur von 15 °C auf 38 °C und durch Herabsetzung der NaOH- Konzentration von 2 mM auf 1 mM konnte der störende Matrixpeak aus dem Pflanzenhydrolysat von dem Glucosaminpeak basisliniengetrennt werden. Die Validierungsparameter waren gut und die bestimmten Aminozuckergehalte waren mit der Umkehrphasen-HPLC-Methode vergleichbar. Zur Bestimmung der Bildung und des Umsatzes von saprotrophen Pilzen auf verschiedenen Substraten wurden *Lentinula edodes* P., *Pleurotus ostreatus* K. und *Pleurotus citrinopileatus* S. auf Mais-Holz- und auf Weizen-Holz-Substrat für vier Wochen bei 24 °C kultiviert. Dieser Pilzwachstumsversuch zeigte, dass 80% des neu gebildeten pilzlichen Glucosamins maisbürtig und nicht holzbürtig waren. Weiterhin wurde der bevorzugte Abbau von Maissubstrat im Vergleich zu Weizensubstrat an diesem Versuch verdeutlicht. Außerdem lassen die Ergebnisse darauf schließen, dass die beobachtete zunehmende $\delta^{13}\text{C}$ Anreicherung in dem neu gebildeten pilzlichen Glucosamin während der vier Wochen auf die Inkorporation des angereicherten ^{13}C aus dem Substrat und eher weniger auf kinetische Isotopeneffekte zurückzuführen ist.

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In the present thesis a method for amino sugar- specific $\delta^{13}\text{C}$ -analysis via LC-IRMS in plant material was optimised and established for determining the formation and turnover of microbial residues in soil- and plant hydrolysates with high accuracy. Further an experiment was implemented by the established LC-IRMS method. However, the main focus of the thesis was on method development. Thus, different pretreatment techniques were tested and optimised. Moreover, the already available HPLC method based on Appuhn et al. (2004) was optimised. For establishing the amino sugar- specific $\delta^{13}\text{C}$ -analysis additional chromatographic separation mechanisms were tested with the background to yield a fast and efficient separation of amino sugars with a carbon-free eluent.

The implemented experiment was a fungi growth experiment with saprotrophic fungi on maize-wood- and wheat-wood-substrates.

The first article deals with the optimisation of amino sugar quantification by HPLC in soil and plant hydrolysates. The already available HPLC method based on Appuhn et al. (2004) was hampered by two drawbacks: (1) The method was reliably working on the Agilent 1100 HPLC equipment and (2) mannosamine was insufficiently separated in most soil hydrolysates, which resulted in erroneous high values usually omitted in further publications (Appuhn and Jörgensen, 2006; Engelking et al., 2007b). The objective of the first article was improving the method of Appuhn et al. (2004) to give reliable results for all four amino sugars in soil and plant hydrolysates using different HPLC equipments. At first the mobile phase was optimized. Therefore the tetrahydrofurane (THF) concentration was increased from 0.75% to 1.5%. This yielded in shorter retention times for all four amino sugars and a slightly better resolution between muramic acid and mannosamine. Moreover, the excitation wavelength of the fluorescence detector was reduced from 340 nm (Appuhn et al. 2004) to 330 nm. At 330 nm the highest fluorescence response for the amino sugar peaks was detected without increasing the fluorescence response of the matrixpeaks. Besides the OPA reaction time has an effect on the fluorescence response. A maximum height of fluorescence response for all four amino sugars was yielded at 120 sec. After optimisation of the mentioned parameters the validation of the method ensued. A limit of quantification (LOQ) of $0.5 \mu\text{mol l}^{-1}$ which is equal to $0.13 \mu\text{g ml}^{-1}$ for muramic acid and a LOQ of $5.0 \mu\text{mol l}^{-1}$ (equal to 0.90mg ml^{-1}) for the other three amino sugars were obtained. The calibration curves were linear in the range from 5.0 to $210 \mu\text{mol l}^{-1}$ (mannosamine, galactosamine, glucosamine) and from 0.5 to $21 \mu\text{mol l}^{-1}$ (muramic acid), respectively. The coefficient of variation was roughly 2% for

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intraday and 5% for interday precision, respectively. Furthermore water and phosphate buffer as sample solvent were tested, because the pH value of the sample solvent has an effect of the OPA reaction (Dorresteyn et al., 1996). Since sample pH is between 2 and 3 in the sample extract using water, it was examined if better accuracy for this method is possible with higher pH during OPA reaction. Therefore the sample extract pH was increased to values about 5 with phosphate buffer. However, buffered samples showed higher standard deviations, particularly for the basalt-derived clayey soil forest, which contained high contents of aluminium and iron oxides. The hydrolysis products of these oxides presumably form as central ion complexes in the presence of ligands like phosphate. If these complexes bind amino sugars, they may not be completely available for OPA derivatisation anymore.

The amino sugar contents in soil- and planthydrolysates measured by this optimised HPLC method compare well with those reported by the literature from arable and forest soils.

In the second article HPLC methods for the determination of amino sugars in soil hydrolysates were tested and compared. Moreover various pretreatment methods for purification and concentration of amino sugars were tested. For amino sugar-specific $\delta^{13}\text{C}$ analysis a HPLC method with a carbon-free eluent is necessary, otherwise a high background noise would be occurred. The reversed phase HPLC method (mentioned in the first article) is reliable, but is not suited for adaption with IRMS, due to the organic mobile phase. However, a variety of other HPLC methods are free of organic solvents, such as (1) HPAEC, (2) high performance cation exchange chromatography (HPCEC) and (3) high performance anion exclusion chromatography (HPEXC). The already available HPAEC method for amino sugar isotope analysis (Bodé et al., 2009) has several drawbacks: (1) The NaOH of the mobile phase is sensitive to CO_2 contamination, (2) the alkaline mobile phase ideally requires a metal free liquid handling system (3) the method of Bodé et al. consisted of two separate runs, one for the basic amino sugars and one for muramic acid, and (4) the limit of quantification for muramic acid is too high for the low muramic acid concentrations in soil. For these reasons the objectives of the second article were (1) to find a reliable purification and concentration procedure for amino sugars in HCl hydrolysates, (2) to optimise the method of Bodé et al. (2009) by testing different chromatographic separation mechanisms. To have higher amino sugar concentration and less impurity in the soil hydrolysates, the following amino sugar purification methods were tested: (1) extraction based on Appuhn et al. (2004), (2) extraction based on Zhang and Amelung (1996), (3) extraction via polymer based strong cation exchange resin, (4) extraction via silica based strong cation exchange resin and (5) extraction via strong anion exchange resin to concentrate and separate muramic acid from the basic

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amino sugars. The strong cation exchange purification methods gave the highest amounts for all four amino sugars, without an increase of impurities, whereas the polymer based cation exchange purification method (3) gave slightly higher amount than the silica based purification method (4). For the further separation of amino sugars no satisfactory separation with the HPCEC and the HPEXC mechanisms was obtained. The HPAEC method described by Bodé et al. (2009) showed a baseline separation and the determined amino sugar amounts in soil hydrolysates were comparable with the reversed phase HPLC method described in the first article. First the HPAEC method was implemented via post column OPA derivatisation and fluorescence detection. For the determination of muramic acid sodium acetate was substituted for sodium nitrate as component of the mobile phase because nitrate acted as a quencher and affected the fluorescence detection. The use of a gradient for obtaining muramic acid together with the basic amino sugars in one run was not possible, due to the fact that muramic acid was overlaid by peaks of the matrix. Additionally a pulsed amperometric electrochemical detector (PAD) was used for detection. The advantage of the PAD is the lack of derivatisation. The obtained peaks were smaller and therefore the peaks were not interfered by peaks of the matrix. Thus, the use of a gradient was possible and therefore the four amino sugars were determined in one run. In summary, a concentration and purification particularly of muramic acid is recommended for detection methods with low sensitivity. A baseline separation for the amino sugars was only obtained by the HPAEC mechanism. Good validation data were yielded with the HPAEC method and the amino sugar amount in the measured soil hydrolysates were comparable with those determined via reversed phase HPLC. Therefore the HPAEC mechanism is at the moment the best choice for amino sugar- specific $\delta^{13}\text{C}$ analysis.

The third article deals with the optimisation of amino sugar-specific $\delta^{13}\text{C}$ analysis via HPAEC-IRMS in plant hydrolysates as well as with the determination of saprotrophic fungi turnover in different substrates. The HPAEC-IRMS method described by Bodé et al. (2009) performed well for soil samples but for the plant material the matrix of the plant samples interfered with glucosamine. Consequently the first objective was to adapt the HPAEC-IRMS method described by Bodé et al. (2009) to an amino sugar analysis in plant and fungi samples. A further objective was the determination of the formation and turnover of saprotrophic fungi, particularly the substrate of the newly formed fungal biomass by the optimized HPAEC-IRMS. By increasing the column temperature from 15 °C to 38 °C and by decreasing the NaOH concentration of the eluent from 2 mM to 1 mM the interfering peak of the matrix was

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separated from the glucosamine peak. The validation data were good and measured amino sugar amounts were comparable with those determined by reversed phase HPLC.

For determining the formation and the turnover of saprotrophic fungi on different substrates *Lentinula edodes* P., *Pleurotus ostreatus* K. und *Pleurotus citrinopileatus* S. were cultivated on maize-wood- and wheat-wood-substrates for four weeks at 24 °C. The fungi growth experiment showed that 80% of the new formed fungal glucosamine was maize-derived and not wood-derived. Furthermore this experiment indicated the preferred decomposition of maize substrate in comparison to wheat substrate. Also, the results showed that the increasing $\delta^{13}\text{C}$ enrichment in the new formed fungal glucosamine during the incubation stems from the incorporation of the enriched ^{13}C of the substrate und less from kinetic isotope effects.

8. Schlussfolgerung und Ausblick

Die Ergebnisse dieser Arbeit haben gezeigt, dass die aminozucker-spezifische $\delta^{13}\text{C}$ Analyse in Pflanzen- und Bodenhydrolysaten mittels LC-IRMS möglich ist und eine höhere Genauigkeit im Vergleich zum GC-C-IRMS aufweist. So ist es mit der GC-C-IRMS nur möglich $\delta^{13}\text{C}$ Veränderungen die größer als 2 ‰ sind genau zu bestimmen (Glaser und Gross, 2005). Mit der in dieser Arbeit beschriebenen LC-IRMS Methode können auch kleine Veränderungen des $\delta^{13}\text{C}$ im Boden präzise bestimmt werden. Die Standardabweichungen der Matrixkalibrierung (mit Boden gespikte Proben) lagen unterhalb von 0,35 ‰ für die $\delta^{13}\text{C}$ -Werte. Durch diese erlangte Genauigkeit kann bei Anwendung dieser Methode zwischen frisch gebildeten mikrobiellen Residuen und älterem sich im Boden akkumuliertem Material mikrobiellen Ursprungs unterschieden werden. Die LC-IRMS Methode erlaubt es auch, Unterschiede zwischen den Umsätzen von bakteriellen und pilzlichen Residuen zu bestimmen. Weiterhin wäre die Methode hilfreich um die Bedeutung von Galaktosamin zu klären. Da der Galaktosamingehalt während des von Engelking et al. (2007b) durchgeführten Inkubationsversuchs keine Veränderungen zeigte, bietet LC-IRMS die Möglichkeit zu klären, ob die Mikroorganismen kein Galactosamin produzieren, oder ob der Abbau und die neue Bildung von Galaktosamin gleich groß sind. Darüber hinaus ist die Methode dafür geeignet viele Hypothesen zu klären. Eine davon ist beispielsweise:

- (1) Die Bildung von mikrobiellen Residuen aus C4-Saccharose ist abhängig von der Qualität der mikrobiellen Residuen und der mikrobiellen Gemeinschaftsstruktur im Boden. Es werden in Böden mit einem hohen Gehalt an pilzlichem Glucosamin mehr mikrobielle Residuen gebildet.

Außerdem besteht die Aussicht mittels LC-IRMS den Einfluss von N auf die Bildung der mikrobiellen Residuen zu bestimmen. So kann in Zukunft Fragestellungen nachgegangen werden, ob nach Zugabe von ^{13}C markiertem Streu mehr pilzliche als bakterielle Residuen bei einem sinkenden N Gehalt und einem zunehmenden Ligningehalt gebildet werden. Weiterhin ist es möglich, den Einfluss der Temperatur auf die Bildung mikrobieller Residuen zu bestimmen. Wird beispielsweise bei niedrigen Temperaturen mehr Substrat-C in die mikrobielle Biomasse und weniger in die mikrobiellen Residuen eingebaut?

Die im ersten Artikel optimierte Umkehrphasen-HPLC-Methode zur Aminozuckerbestimmung ist relativ schnell und einfach durchzuführen und liefert zuverlässige Ergebnisse. Somit sollte es kein Problem darstellen, diese Methode an verschiedenen HPLC-Anlagen durchzuführen, vorausgesetzt es ist ein für die Online-

Derivatisierung vorgesehener Probengeber vorhanden. Weiterhin sollte die im ersten Artikel beschriebene Aufarbeitung den jeweiligen Proben angepasst werden. So sollte bei Böden mit sehr hohem Carbonatgehalt das nach der Hydrolyse erhaltene Hydrolysat vollständig zur Trockne eingengt werden und nach Aufnahme in Wasser neutralisiert werden, damit die Carbonate gefällt werden. Diese könnten ansonsten bei der Derivatisierung stören.

Die im zweiten Artikel vorgestellten Ergebnisse, zeigen dass die HPAEC- Methode zuverlässig für die aminozucker spezifische ^{13}C –Analyse in Bodenhydrolysaten ist. Falls keine hohen Mengen an Mannosamin (Nachweisgrenze: $50\ \mu\text{mol l}^{-1}$ Bodenextrakt) in Böden enthalten sind, ist die im dritten Artikel vorgestellte optimierte HPAEC-IRMS- Methode vorzuziehen, da es mit dieser Methode möglich ist neben Bodenhydrolysaten auch Pflanzenhydrolysate bei einer kürzeren Retentionszeit zu messen. In den meisten Bodenextrakten ist der Mannosamingehalt mit $0\text{-}20\ \mu\text{mol l}^{-1}$ Bodenextrakt weit unter der Nachweisgrenze der HPAEC-IRMS Methode. Die im zweiten Artikel beschriebene Aufarbeitungsmethode zur Purifikation und Konzentrierung der Aminozucker ist insbesondere für die Konzentrierung der basischen Aminozucker erfolgreich. Weiterer Forschungsbedarf besteht aber bei der Konzentrierung von Muraminsäure, so dass nicht, wie im dritten Kapitel beschrieben, die Probenextrakte nach vollständiger Aufarbeitung noch zusätzlich durch Einengung konzentriert werden müssen. Ein nächster Versuch sollte sein die bereits sauren pH (2-3) Bodenextrakte vor Verwendung des im zweiten Artikel beschriebenen Kationenaustauscherharzes auf einen pH-Wert von <1 einzustellen. Theoretisch liegt bei diesem pH-Wert Muraminsäure als diprotoniertes Kation (siehe Einleitung) vor und dürfte erst nach Zugabe der Salzsäure von dem Austauscherharz eluieren. Bei einem pH von 2-3 kann die protonierte Aminogruppe zwar anziehend, die deprotonierte Carboxylgruppe jedoch abstoßend auf die Sulfonsäuregruppe des Harzes reagieren, so dass ein Teil der Muraminsäurefraktion schon während des Waschens mit Wasser von dem Harz eluiert.

Die im dritten Artikel optimierte LC-IRMS Methode lieferte sehr zuverlässige Ergebnisse für die Gehalte so wie auch für die $\delta^{13}\text{C}$ -Werte von Galaktosamin und Glucosamin in Pflanzen- und Bodenhydrolysaten. Die Methode kann somit zur Bestimmung von Galaktosamin und Glucosamin für Pflanzen- und Bodenproben angewandt werden. Der Pilzwachstumsversuch hat verdeutlicht, dass die ^{13}C Anreicherung in dem neu gebildeten pilzlichen Glucosamin auf die Inkorporation von maisbürtigem Material und weniger auf kinetische Isotopenfraktionierung zurückzuführen ist. Der Versuch hat außerdem gezeigt, dass die saprotrophen Pilze besonders schnell auf Mais-Holz-Substrat wachsen und 80% des neuen pilzlichen Glucosamin maisbürtig sind. Daraus folgt, dass für eine schnelle

8. Schlussfolgerung und Ausblick

Kultivierung von saprotrophen Pilzen Mais als Substrat verwendet werden sollte. Weiterhin könnten mit dieser Methode Unterschiede zwischen den Umsätzen von saprotrophen Pilzen und Ektomykorrhiza auf verschiedenen Substraten bestimmt und beurteilt werden.

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