A method for quantifying heterocyst glycolipids in biomass and sediments

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ABSTRACT

Heterocyst glycolipids (HGs) are biomarkers for heterocystous N_2 fixing cyanobacteria and consist of a sugar moiety bound to an even-numbered C_{26}-C_{32} alkyl chain, containing various alcohol or ketone groups. They have been reported in ancient sediments but, due to a lack of standards, they were quantified in terms of integrated peak area response. Here we describe the use of a commercially available glycolipid (n-dodecyl-ß-D-glucopyranoside) as an internal standard (IS) for reliable quantitative analysis. The common HG 1-(O-hexose)-3,25-hexacosanediol was isolated using both normal and reversed phase chromatography and used to assess the relative response factor compared with the IS, allowing correction of difference in mass spectral response between the IS and the target HGs.

Keywords: Heterocyst, Cyanobacteria, heterocyst glycolipids, semi-preparative

high performance liquid chromatography, quantification

1. Introduction

In all heterocyst-forming cyanobacteria, the heterocyst cell envelope contains heterocyst glycolipids (HGs), comprising either a hexose or pentose head group, bound to long chain diols, triols, or hydroxyketones (Gambacorta et al., 1995, 1999; Bauersachs et al., 2009, 2011; Woermer et al., 2012; Schouten et al., 2013; Bale et al., 2015). Recent studies have applied specific high performance liquid chromatography-multistage mass spectrometry (HPLC-MS^2) methods to detect and characterize HGs in cyanobacterial cultures and samples from the natural environment (Bauersachs et al., 2009, 2014; Woermer et al., 2012; Schouten et al., 2013; Bale et al., 2015, 2016). However, the methods were not used for absolute quantification of the HGs due to the lack of authentic standards. Here we describe the application of a commercially available short chain glycolipid as an internal standard (IS), which allows absolute quantification of HGs in biomass and sediments. To correct for differences in response factor between the IS and naturally occurring HGs, we isolated a HG from an extract of mixed biomass material of heterocystous cyanobacteria. This was used with the IS to constrain the relative response factor (RRF). Furthermore, instead of HPLC-MS^2 in multiple reaction mode (MRM) we used a UHPLC (ultra high pressure liquid chromatography) separation combined with high resolution mass spectrometry (HRMS) in full scan mode to quantify HGs using this novel approach.

2. Experimental

The IS was n-dodecyl-ß-D-glucopyranoside (≥ 98%; Sigma-Aldrich,

Zwijndrecht, The Netherlands). For isolation of a hexose-containing (C_6) HG standard, freeze dried cyanobacterial biomass (0.14 g dry wt; comprising a mixture of cultivated *Anabaena* sp. (CCY 9922) and *Nostoc* sp. (CCY 1933); culture conditions detailed by Bauersach et al. 2009) and was extracted using a modified Bligh-Dyer extraction as described by Bale et al. (2015). Initial analysis of the extract and all further analyses, unless specified otherwise, were carried out using UHPLC-HRMS (Q-Exactive Orbitrap; Thermo Scientific, Waltham, MA), as described by Moore et al. (2016) with some modifications: (i) An Acquity UPLC BEH HILIC column (1.7 µm, 2.1 x 150 mm; Waters) was used; (ii) the elution program was 100% A at 5 min, 66% A at 25 - 40 min, 40% A at 55 min, 30% A at 65 min, where A = hexane/propan-2-ol/HCO_2H/14.8 M NH_{3aq} (79:20:0.12:0.04, v/v) and B = propan-2-ol/H_2O/HCO_2H/14.8 M NH_{3aq} (88:10:0.12:0.04, v/v). The total run time was 70 min with a re-equilibration period of 20 min between runs.

Analysis of the HGs in the cyanobacterial extract revealed that 1-(O-hexose)-3,25-hexacosanediol (hereafter C_6 HG_{26} diol) was the most abundant HG and was therefore targeted for isolation. It was isolated, first using the semi-preparative HPLC system, fraction collector, column, eluent composition and elution program described by Bauersachs et al. (2009). The column effluent was collected in 1 min fractions and the fractions containing the target HGs were pooled and purified in reverse phase with a semi-preparative Symmetry C_{18} column (250 x 10 mm, 7 µm; Waters, Milford, MA). Typical injection volume was 200 µl, containing up to 1 mg of extract. The gradient and conditions were adapted from Wörmer et al. (2013): eluent A was MeOH/H_2O (85:15, v/v) and

73 eluent B MeOH/propan-2-ol (50:50, v/v), at 3 ml/min. The elution program was
74 100% A for 2 min, then an increase to 15% B at 2.10 min, then to 43.5% B at 15
75 min (held to 16 min), and subsequent washing with 100% B from 16 min to 24
76 min before returning to 100% A for 5 min. The effluent was collected in 0.25 min
77 fractions, between 7 and 15 min.

78 Fractions from semi-preparative HPLC were screened for the presence of
79 the target HGs with flow injection analysis using linear ion trap MS (Thermo
80 Scientific LTQ XL, Ion Max source) with electrospray ionization (ESI); source
81 settings were as described by Moore et al. (2016) in selected reaction monitoring
82 (SRM) mode, targeting the dominant parent→ product transition for the C_6 HG_{26}
83 diol: m/z 577 → m/z 415.

84 Final combined fractions were analyzed using UHPLC-HRMS, with
85 conditions as described above for initial extract analysis. This suggested that the
86 final combined fraction was pure, i.e.no other peaks were present in the base
87 peak chromatogram. C_6 HG_{26} diol was present as two isomers (annotated as HG
88 and HG' in Fig. 1a), with the same exact mass and same fragmentation in MS^2,
89 so it was surmised that their hexose moieties represented two diastereoisomers.
90 The ratio of the isomer peak areas remained constant at 1.9 ± 0.13 in all
91 analyses. Their peak areas were combined and they were treated as a single
92 component. The relative response factor (RRF) of the C_6 HG_{26} diol vs. the
93 synthetic glycolipid IS was determined using the same analytical method by
94 injection of mixtures of the C_6 HG_{26} diol and IS (1:1, w/w) in amounts ranging
95 from 1 to 10 ng on column.

96 A microbial mat was sampled from a beach on the North Sea Dutch

97 barrier island of Schiermonnikoog in April 2010 (Station III in Fan et al., 2015)).
98 Extraction of the mat was carried out as described for sediments by Bale et al.
99 (2015) with addition of the IS (2 µg/ml) before filtration of the extract through a
100 0.45 µm regenerated cellulose syringe filter (4 mm diameter; Grace Alltech,
101 Deerfield, IL). Analysis of the sample was carried out using UHPLC-HRMS as
102 described above.

103 **3. Results and discussion**

104 N-dodecyl-ß-D-glucopyranoside was selected as an internal standard as it
105 is a commercially available analogue of the naturally occurring glycolipids, yet
106 contains a carbon chain not observed in nature. The MS^2 spectrum (Fig. 1b),
107 resulting from fragmentation of $[M+NH_4]^{+\cdot}$ at m/z 366 also contained the $[M+H]^+$
108 protonated molecule at m/z 349 and exhibited characteristic fragment ions at
109 m/z 180 ($[M+NH_4$-dodecanol$]^{+\cdot}$) and m/z 163 ($[M+H$-dodecanol$]^{+\cdot}$). Sequential
110 losses from m/z 163 of OH groups as water, at m/z 145 and m/z 127, were also
111 observed. The pattern is in contrast to that observed for HGs (Bauersachs et al.,
112 2009), where the dominant fragment ions in MS^2 correspond to loss of a hexose
113 moiety and further losses of water therefrom. Hence, while the IS is suitable for
114 quantification in full MS, its fragmentation pattern does make it less suitable for
115 quantification of HGs in MS^2, e.g. for a selected reaction monitoring (SRM)
116 method. However, the use of HRMS affords similar specificity and sensitivity in
117 full scan mode compared with MS^2-assays on mass spectrometers with nominal
118 mass resolution. Furthermore, quantification in full scan has the benefit that
119 factors such as differing fragment yield, as is the case with MRM, no longer play
120 a role.

121 The IS would be expected to have a different MS response factor vs.
122 naturally occurring HGs due the lack of OH groups on the dodecyl chain. To
123 correct for the difference, we isolated a natural HG from an extract of mixed
124 biomass material of heterocystous cyanobacteria. Following a two-step procedure
125 using semi-preparative HPLC, 0. 506 mg of the C_6 HG_{26} diol was isolated. This
126 HG commonly occurs in sediments (Bauersachs 2010) and we assume that other
127 HGs have similar MS response. Response curves for the IS and the C_6 HG_{26} diol
128 were determined at a range of concentration (1 – 10 ng on column; Fig. 1c) and
129 the slopes used to determine the RRF for the two HGs. The concentration range
130 was chosen firstly to reflect the peak areas in samples from the natural
131 environment when analyzed using UHPLC-HRMS (N.B., unpublished) and
132 secondly as this was range which produced a linear response. In order to
133 examine its variability RRF, was determined 3x over a period of a month and fell
134 in the range 2.1 – 3.0. The use of our newly proposed IS for quantification of HGs
135 in samples from the natural environment should be of benefit vs. e.g. external
136 standards or ISs with a different polarity and structure (such as the platelet
137 activating factor (PAF) standard (1-O-hexadecyl-2-acetyl-sn-glycero-3-
138 phosphocholine; Lipp and Hinrichs (2009); Moore et al. (2013)), as it allows
139 correction for sample loss, e.g. during pre-analysis filtration of samples or during
140 sample injection.
141 To test its application, the IS was used during UHPLC-HRMS analysis of
142 a microbial mat from a beach on the North Sea Dutch barrier island of
143 Schiermonnikoog. It was found to contain 22.7 ng/g dry sediment of C_6 HG_{26} diol.
144 For comparison, the microbial mat was found to contain 3.74 μg/g dry sediment

of the sulfoquinovosyldiacylglycerol (SQDG) with fatty acid constituents 16:0 and 18:3 (Bale et al., unpublished data).

Our method therefore allows quantification of HGs in biomass and sediments and thereby quantitative comparison with other lipids and inference of the importance of past heterocystous N_2 fixation in ancient environments.

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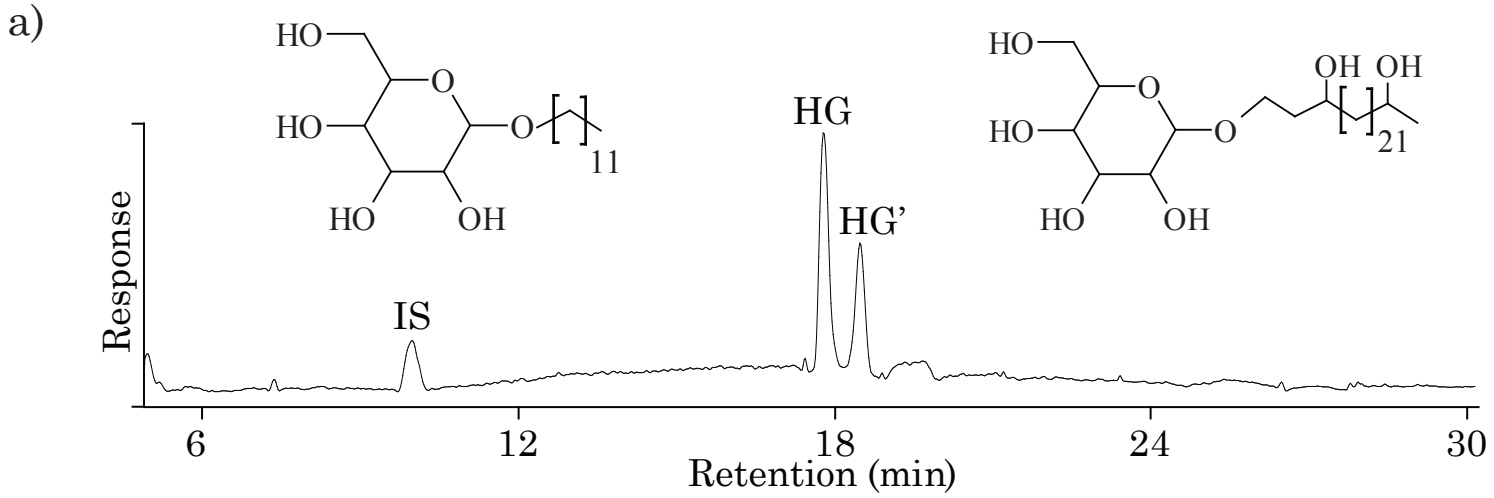
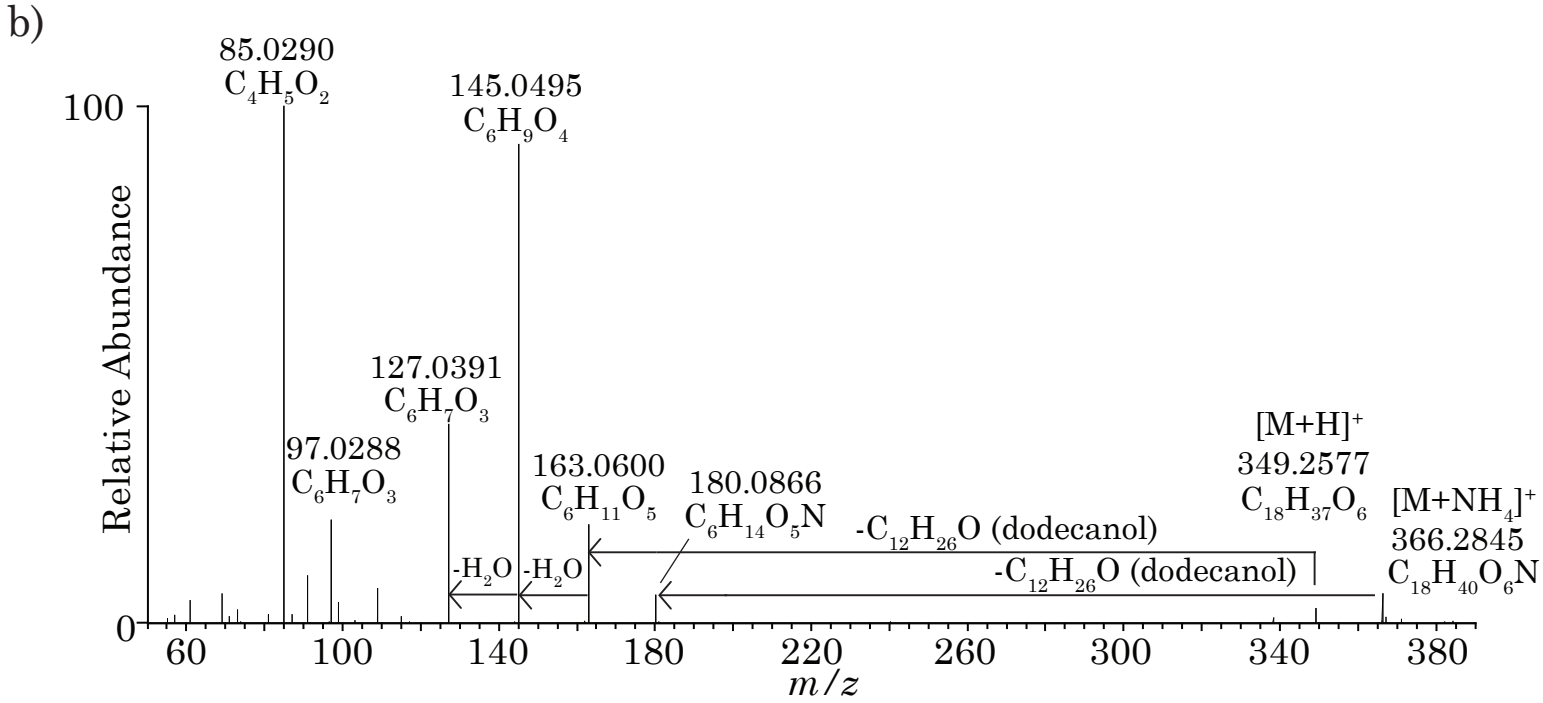
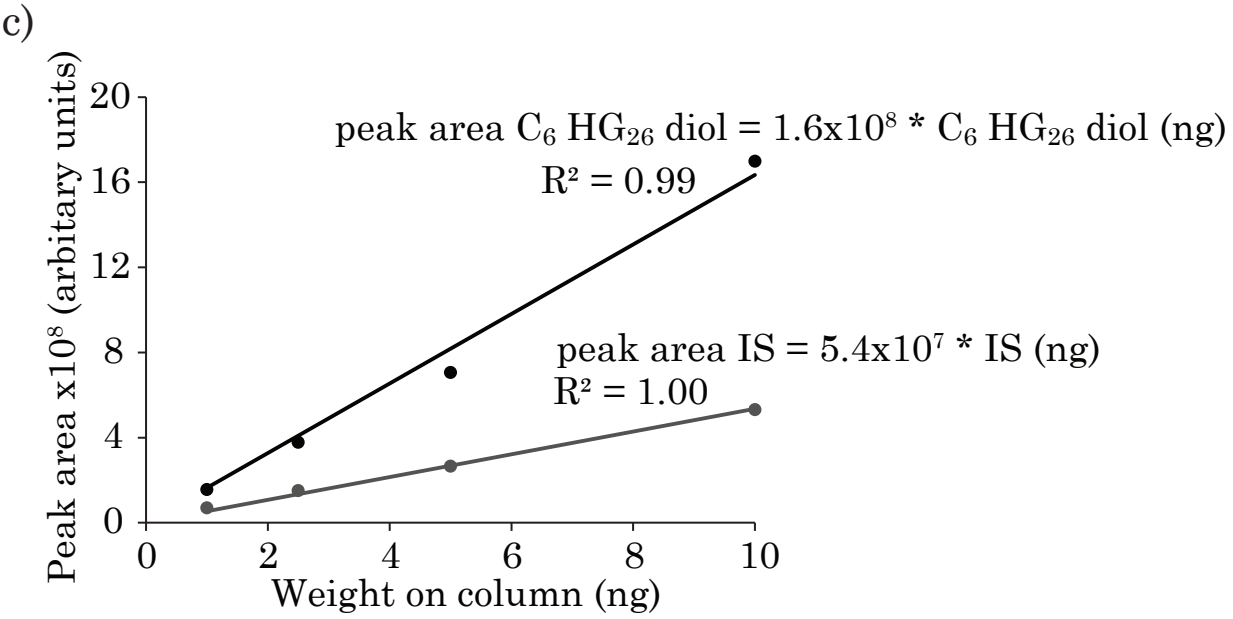
Fig. 1 (a) UHPLC AM MS partial base peak chromatogram of mixture (1:1 w:w) containing the IS and the two C_6 HG_{26} diol isomers (HG and HG'). Insert: structures of the IS (n-dodecyl-ß-D-glucopyranoside) and C_6 HG_{26} diol. (b) MS^2 spectrum of IS. For the MS^2 spectrum of C_6 HG_{26} diol, see Bauersach et al. (2009). (c) Example of standard curves of weight on column vs. peak area for the IS and the C_6 HG_{26} diol.


a)
HO
HO
HO
HO
O
O
11
HG
HG'
IS
OH OH
21
Response
6
12
18
24
30
Retention (min)


b)
85.0290
C4H5O2
145.0495
C6H9O4
127.0391
C6H7O3
97.0288
C6H7O3
163.0600
C6H11O5
180.0866
C6H14O5N
[M+H]+
349.2577
C18H37O6
[M+NH4]+
366.2845
C18H40O6N
-H2O
-H2O
-C12H26O (dodecanol)
-C12H26O (dodecanol)
100
0
Relative Abundance
60
100
140
180
220
260
300
340
380
m/z


c)
peak area C6 HG26 diol = 1.6x10^8 * C6 HG26 diol (ng)
R² = 0.99
peak area IS = 5.4x10^7 * IS (ng)
R² = 1.00
Peak area x10^8 (arbitary units)
20
16
12
8
4
0
0
2
4
6
8
10
Weight on column (ng)