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## Analysis of glycosphingolipids from animal tissues

Forked from [Analysis of glycosphingolipids from human plasma](#)

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ASAP Collaborative Research Network



Reuben Bush

### ABSTRACT

Interest in the role of cellular glycosphingolipids (GSLs) in health and disease led to us developing a sensitive method to analyse the full complement of GSL structures present in mammalian cells, fluids and tissues. The original qualitative method we developed was published in 2004 and measured the oligosaccharides selectively released from glycosphingolipids using a ceramide glycanase enzyme derived from the medicinal leech. We have now updated and refined this protocol with the focus on achieving sensitive and reproducible quantitation of GSLs in animal tissues. The method uses the fluorescent compound anthranilic acid (2-AA) to label oligosaccharides prior to analysis using normal-phase high-performance liquid chromatography. The labelling procedure is rapid, selective, and easy to perform. With the inclusion of a 2AA-labelled chitotriose calibration standard, it is possible to obtain accurate and reproducible molar quantities of individual GSL species.

### GUIDELINES

This protocol requires the use of some hazardous materials. As such, users must be appropriately trained and hazardous materials stored, used, and disposed of in accordance with your institution's health and safety policies, and approved laboratory policies, risk assessments and codes of practice.

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**Protocol status:** Working  
We use this protocol and it's working

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**PROTOCOL integer ID:** 72039

**Keywords:** Glycosphingolipids, Glucosyl Ceramide, HPLC, Oligosaccharide Analysis, ASAPCRN

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## MATERIALS

### Acetonitrile 1.00030

**Gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur**

[https://www.sigmaaldrich.com/catalog/product/mm/100030?lang=en&region=GB&gclid=EAlaIqobChMl1JyZu\\_Xa8AIVcwUGAB10IgVLEAAYASAAEgJHmfD\\_BwE](https://www.sigmaaldrich.com/catalog/product/mm/100030?lang=en&region=GB&gclid=EAlaIqobChMl1JyZu_Xa8AIVcwUGAB10IgVLEAAYASAAEgJHmfD_BwE)

### Anthranilic acid

A89855 Sigma-Aldrich

Reagent grade, ≥98%

<https://www.sigmaaldrich.com/catalog/product/sial/a89855?lang=en&region=GB>

### Bicinchoninic Acid solution

<https://www.sigmaaldrich.com/GB/en/product/sigma/b9643>

### Boric acid

ReagentPlus®, ≥99.5%

B0252 Sigma-Aldrich

[https://www.sigmaaldrich.com/GB/en/product/sigald/b0252?cm\\_sp=Insite-\\_-caSrpResults\\_srpRecs\\_srpModel\\_boric%20acid%20reagent%20plus-\\_-srpRecs3-1](https://www.sigmaaldrich.com/GB/en/product/sigald/b0252?cm_sp=Insite-_-caSrpResults_srpRecs_srpModel_boric%20acid%20reagent%20plus-_-srpRecs3-1)

### Chloroform

Suitable for HPLC, ≥99.8%, amylene stabilized

34854-M Sigma-Aldrich

<https://www.sigmaaldrich.com/catalog/product/sial/34854m?lang=en&region=GB>

### Copper(II) sulfate solution

<https://www.sigmaaldrich.com/GB/en/product/sigald/c2284>

### Discovery® DPA-6S SPE Tube

[https://www.sigmaaldrich.com/GB/en/search/dpa-6s?focus=products&page=1&perPage=30&sort=relevance&term=DPA-6S&type=product\\_name](https://www.sigmaaldrich.com/GB/en/search/dpa-6s?focus=products&page=1&perPage=30&sort=relevance&term=DPA-6S&type=product_name)

### Gibco™ DPBS, no calcium, no magnesium

14190094

<https://www.thermofisher.com/order/catalog/product/14190094#/14190094>

### Glycosphingolipid standards

<https://www.matreya.com/Departments/Product-Categories/Glycosphingolipids.aspx>

**Kinesis SPE Columns: TELOS® C18(EC) 100mg/1ml SPE Columns**

<https://kinesis.co.uk/kinesis-spe-columns-telosr-c18-ec-100mg-1ml-spe-columns-210-100m-001t.html>

**Ludger - BioQuant 2AA Labelled Chitotriose Standard**

Cat. #: BQ-CAA-CHI-01 Batch: B37I-02

<https://www.ludger.com/docs/products/bq/bq-caa-chi/bq-caa-chi-01-b37i-02-cofa.pdf>

**Ludger - 2-AA Labeled Glucose Homopolymer Ladder**

Cat. # CAA-GHP-30

<https://www.glycoprofiling.com/docs/products/caa/ludger-caa-ghp-30-guide.pdf>

**Methanol**

34860 Sigma-Aldrich

Suitable for HPLC, ≥99.9%

[https://www.sigmaaldrich.com/catalog/product/sigald/34860?lang=en&region=GB&cm\\_sp=Insite--caSrpResults\\_srpRecs\\_srpModel\\_methanol%2034860\\_-srpRecs3-1](https://www.sigmaaldrich.com/catalog/product/sigald/34860?lang=en&region=GB&cm_sp=Insite--caSrpResults_srpRecs_srpModel_methanol%2034860_-srpRecs3-1)

**rEGCase I: recombinant Endoglycoceramidase custom-synthesized by GenScript**

<https://www.genscript.com/>

**Sarstedt 1.5 ml Micro tubes with screw cap and seal**

Product reference number 72.692

<https://www.sarstedt.com/en/products/laboratory/screw-cap-micro-tubes-reaction-tubes/screw-cap-micro-tubes/product/dmsarproducts/specificationPdf/Product/72.692.005/>

**Sodium acetate trihydrate**

S1304 Sigma-Aldrich

Meets USP testing specifications

[https://www.sigmaaldrich.com/catalog/product/sial/s1304?lang=en&region=GB&gclid=CjwKCAjwJ2FBhAuEiwAiku19jp2DL5CW1Ke85Y0Ksb5boyV-5zKyUkzv3uVVuZXV3abjGZbVruJhoCescQAvD\\_BwE](https://www.sigmaaldrich.com/catalog/product/sial/s1304?lang=en&region=GB&gclid=CjwKCAjwJ2FBhAuEiwAiku19jp2DL5CW1Ke85Y0Ksb5boyV-5zKyUkzv3uVVuZXV3abjGZbVruJhoCescQAvD_BwE)

**Sodium cyanoborohydride**

Reagent grade, 95%


156159 Sigma-Aldrich

<https://www.sigmaaldrich.com/catalog/product/aldrich/156159?lang=en&region=GB>

## TSKgel® Amide-80 HPLC Column

<https://www.sigmaaldrich.com/GB/en/product/supelco/813071>


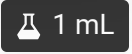






### SAFETY WARNINGS

 This protocol requires the use of some hazardous solvents, reagents and chemicals. Refer to the Safety Data Sheets (SDS) provided by supplier and applicable Control of Substances Harmful to Health (COSHH). The correct personal protective equipment must be worn, and incidents reported in line with your institution's policy and procedures.

### BEFORE START INSTRUCTIONS

Check that you have the required reagents, solvents, chemicals, equipment and PPE.



## GSL preparation from animal tissues

- 1 Homogenise tissue in ddH<sub>2</sub>O yielding tissue concentration of  25 mg tissue (wet weight) per  1 mL ddH<sub>2</sub>O.
- 2  Measure protein concentration using the Bicinchoninic acid assay (BCA) method in triplicate at 2 fold dilution (repeat if triplicate values are not in agreement).
- 3  Using determined protein concentrations, prepare sample as  200 µg protein in  200 µL ddH<sub>2</sub>O in a 1.5 ml screw-cap tube.
- 4  Add  0.8 mL of chloroform/methanol (1:2, v/v) to give (C/M/W 4:8:3 final).

## Safety information

Chloroform and methanol are very toxic, and methanol is flammable. Refer to the Safety Data Sheets.

5 Vortex.

6 Leave  Overnight at  4 °C .






7 Vortex.

8 Centrifuge at 16,000 x g for  00:10:00 at  Room temperature .

10m



9 Transfer supernatant (about  1 mL ) to new tube and separate into two phases: by adding  0.2 mL PBS and then  0.2 mL chloroform.








10 Vortex.

11 Centrifuge at 16,000 x g for  00:10:00 at room temperature.

10m






- 12 Remove very carefully the lower phase to a new tube and retain the upper phase.
- 13 Dry down the lower phase under a stream of (oxygen-free) nitrogen in heating block (  42 °C ).
- 14 When dry, re-suspend the lower phase in  20 µL chloroform/methanol (1:3).
- 15 Add upper phase to lower phase and vortex.
- 16 Pre-equilibrate C18 columns (telos, Kinesis, UK) with 4 x  1 mL methanol and 3 x  1 mL deionised water.
- 17 Load lower/upper phase mix onto column, let drip through gravity flow.
- 18 Rinse sample tube with  1 mL water, apply to column to wash.
- 19 Wash column with 3 x  1 mL water.





20 Elute GSLs into a new tube with:

20.1  2 mL chloroform/methanol (98:2). Push through first  0.5 mL. You can use syringe with adapter that fits into top of column.

20.2  2 mL chloroform/methanol (1:3).



20.3  1 mL methanol.

21 Vortex and leave  Overnight at  4 °C or carry on to enzymatic digestion.




## GSL digestion with EGC'ase I

16h


22 Vortex ( 5 mL, C18) and dry down samples under a stream of nitrogen in heating block ( 42 °C ).




23 When about  150 µL sample remaining, transfer to  1.5 mL screw-cap tube.

24 Rinse sample tube with  200  $\mu\text{L}$  C:M 2:1, vortex and combine with the rest of the sample in the screw-cap tube.



25 Rinse sample tube with  200  $\mu\text{L}$  chloroform, vortex and combine with the rest of the sample in the screw-cap tube.



26 Dry down, under a slow stream of nitrogen in heating block ( 42  $^{\circ}\text{C}$ ).



27 Re-suspend in  50  $\mu\text{L}$  C:M 2:1, vortex, dry down under a very slow stream of nitrogen.

#### Note


NB It is essential that ALL the sample is dried in the bottom of the tube.

28 Add  90  $\mu\text{L}$  enzyme/buffer to each sample and vortex:



28.1 rEGCase :  5  $\mu\text{L}$  enzyme (stored in freezer) plus  85  $\mu\text{L}$  buffer per sample.

#### Note

rEGCase buffer (stored at  4  $^{\circ}\text{C}$ ): [M] 0.6 % (v/v) Triton in [M] 50 millimolar (mM) sodium acetate pH 5.2.

29 Incubate at  37  $^{\circ}\text{C}$  for  16:00:00 .

16h





#### Note

rEGCaseI: recombinant Endoglycoceramidase I was custom synthesised by GenScript.

## 2AA labelling of glycans released from GSLs

1h 30m

30



#### Note

**Labelling mix:** 30 mg/mL 2AA and 45 mg/mL sodium cyanoborohydride, in labelling buffer  
**Labelling buffer:** 4% sodium acetate and 2% boric acid in methanol

Dissolve 2AA in labelling buffer first with vortexing, then add the sodium cyanoborohydride and vortex.

#### Safety information

Sodium cyanoborohydride is very toxic. Refer to the Safety Data Sheet.

31



Add  310  $\mu$ L labelling mix to the  90  $\mu$ L sample (digest) in  1.5 mL screw-cap tube.

32



Incubate in oven at  80  $^{\circ}$ C for  01:00:00, vortexing at  00:30:00.


1h 30m

33

Allow to cool to  Room temperature.

34 Add  1 mL acetonitrile:water (97:3).



35 Transfer from screw-cap tube to  15 mL tubes.


36 Rinse screw-cap tube with 2 x  1 mL acetonitrile:water (97:3) and add to  15 mL tube.

37 Pre-equilibrate 50 mg Discovery® DPA-6S SPE Tube (supplied by Sigma-Aldrich) with:

 1 mL acetonitrile

2 x  1 mL water

2 x  1 mL acetonitrile

38 Apply samples (  3.4 mL ) to equilibrated DPA-6S columns, let drip through gravity flow.

39 Wash columns with acetonitrile:water (95:5) → add  1 mL into the  15 mL tubes to wash and add to columns, and then add 3 x  1 mL acetonitrile:water (95:5) directly onto the columns.




40 Elute with  600 µL water into new tubes.



41 Add  60  $\mu\text{L}$  :  140  $\mu\text{L}$  (sample:acetonitrile) to HPLC vials and vortex.



42 For HPLC, inject  5  $\mu\text{L}$  -  50  $\mu\text{L}$  .

43 DPA-6S eluate is stable stored at  -20  $^{\circ}\text{C}$  .

## HPLC protocol

1h


44 Purified 2AA-labeled oligosaccharides are separated and quantified by normal-phase high-performance liquid chromatography (NP-HPLC) as previously described (Neville *et al.*, 2004, see the reference below).


### Note

[Analysis of fluorescently labeled glycosphingolipid-derived oligosaccharides following ceramide glycanase digestion and anthranilic acid labeling](#)

DCA Neville, V Coquard, DA Priestman, DJM te Vrugte, DJ Sillence, Raymond A Dwek, Frances M Platt, Terry D Butters  
Analytical biochemistry 331 (2), 275-282, (2004).  
doi: 10.1016/j.ab.2004.03.051.

45 The HPLC system consists of a Waters Alliance 2695 separations module and an in-line Waters 2475 multi  $\lambda$ -fluorescence detector set at Ex  $\lambda$ 360 nm and Em  $\lambda$ 425 nm.


46 The solid phase used is a 4.6  $\times$  250 mm TSK gel-Amide 80 column maintained at  30  $^{\circ}\text{C}$  (Anachem, Luton, UK).


47 The chromatographic flow rate is 0.8 mL/min, and run time was 60 min. The total run time is  01:00:00 1h


Time (min)	0	6	35	37	39	41	42	54	60
Flow (mL/min)	0.8	0.8	0.8	0.8	0.8	0.8	1.2	1.2	0.8
% Solvent A	71.6	71.6	52.8	23.0	23.0	71.6	71.6	71.6	71.6
% Solvent B	8.4	8.4	27.2	57.0	57.0	8.4	8.4	8.4	8.4
% Solvent C	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0


**Gradient conditions for Normal Phase HPLC.** All chromatography was controlled and data were collected and processed using Waters Empower software.


48 The mobile phases are acetonitrile (solvent A), de-ionised water (solvent B) and [M] 100 millimolar (mM) ammonium acetate, pH 3.85 (solvent C).


49 Starting conditions are 71.6% solvent A. 8.4% solvent B and 20% solvent C at 0.8 mL/min constant flow-rat 6m for 6 mins.  00:06:00

50 The gradient is developed from 6 min to 35 min by increasing solvent B from 8.4% to 27.2% with concomitant decrease in solvent A from 71.6% to 52.8 %.  00:29:00 29m

51 From 35 to 37 min solvent B is increased to 57% and solvent A reduced to 23%, then maintained for 2 min 4m up until 39 min.  00:04:00

52 Between 39 and 41 min the solvent ratios are returned to the starting conditions and then maintained for 1 3m min.  00:03:00

**53** At 42 mins the flow-rate is increased to 1.2 mL/min and maintained for 12 min up to 54 min.  00:12:00 **12m**

**54** Between 54 and 60 min the flow-rate is gradually returned to 0.8 mL/min.  00:06:00 **6m**






**55** Individual GSL species are identified by their glucose unit values (GUs), calculated with the HPLC Empower software using a homopolymer dextran ladder (Fig. 1).




**55.1** To help identify GSL peaks in the samples, compare the GUs of the peaks in the samples with those in a GSL standard mix.

**55.2** Prepare a mixture of commercially available authentic GSLs in a screw-cap tube:



 50 $\mu$ L	Matreya Neutral GSL mix	Cat No 1505 (Glc-Cer, Lac-Cer, Gb3, Gb4)	1.0
mg/ml			
 100 $\mu$ L	Matreya Ganglioside mix	Cat No 1510 (Lac-Cer, GM3, GD3)	0.5
mg/ml			
 100 $\mu$ L	Matreya Ganglioside mix	Cat No 1511 (GA1, GM1a, GD1a, GD1b, GT1b)	0.5
mg/ml			
 30 $\mu$ L	Matreya GM2 Ganglioside	Cat No 1502 (GM2)	0.5
mg/ml			
 30 $\mu$ L	Sigma Asialo-GM2	Cat No G9398 (GA2)	1.0
mg/ml			

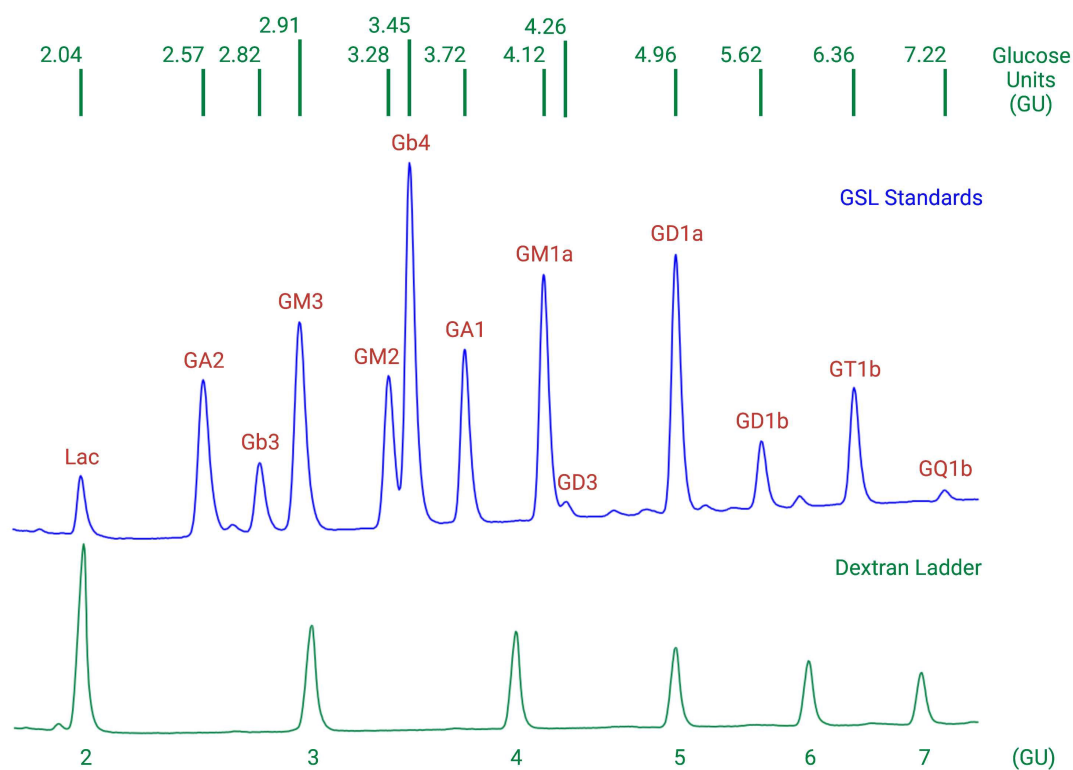
**55.3** Dry down the mixture under nitrogen and then digest, label and clean up as done for the samples in steps 25-41.

55.4 Aliquot and store at  -20 °C.

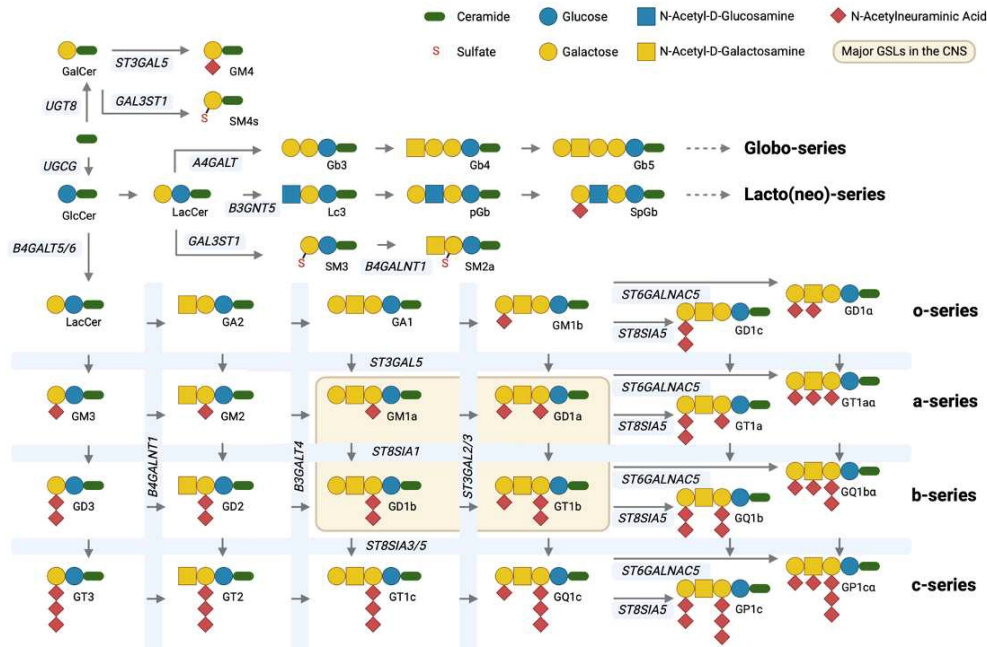
55.5 In order to calculate molar quantities from integrated peaks in the chromatogram, inject a calibration standard containing 2.5 pmol 2AA-labelled chitotriose (Ludger) with each sample set (not shown).

## Figures 1- 4: HPLC profile and sugar structures for GSLs in animal tissues...

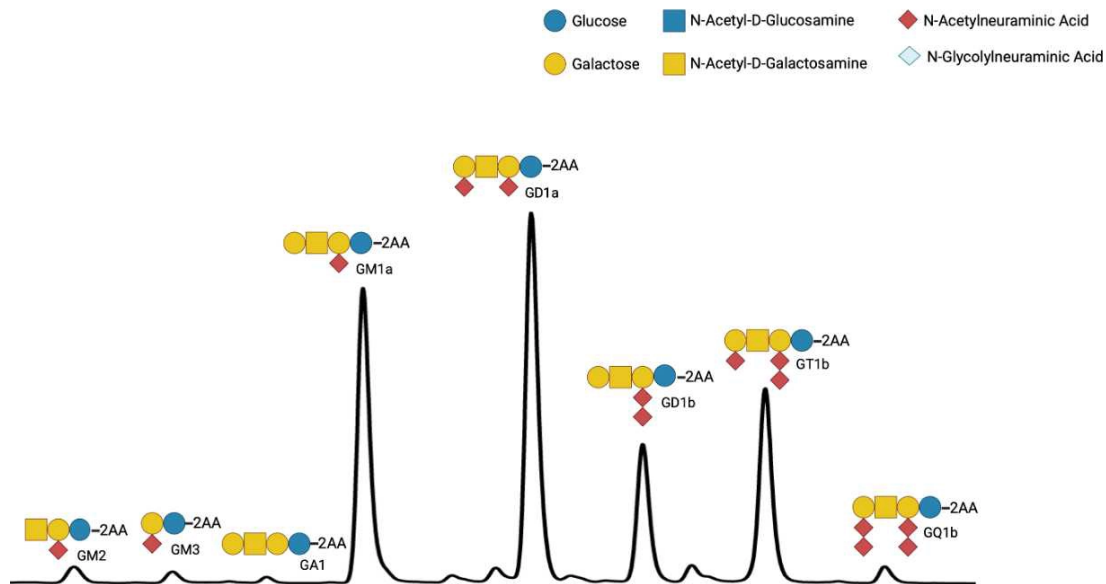
56



**Figure 1** HPLC profiles for GSL standards and 2AA-labelled homopolymer dextran ladder. Glucose Units were calculated using the retention times of a 2AA-labelled homopolymer dextran ladder and are shown for each of the GSL standard peaks.

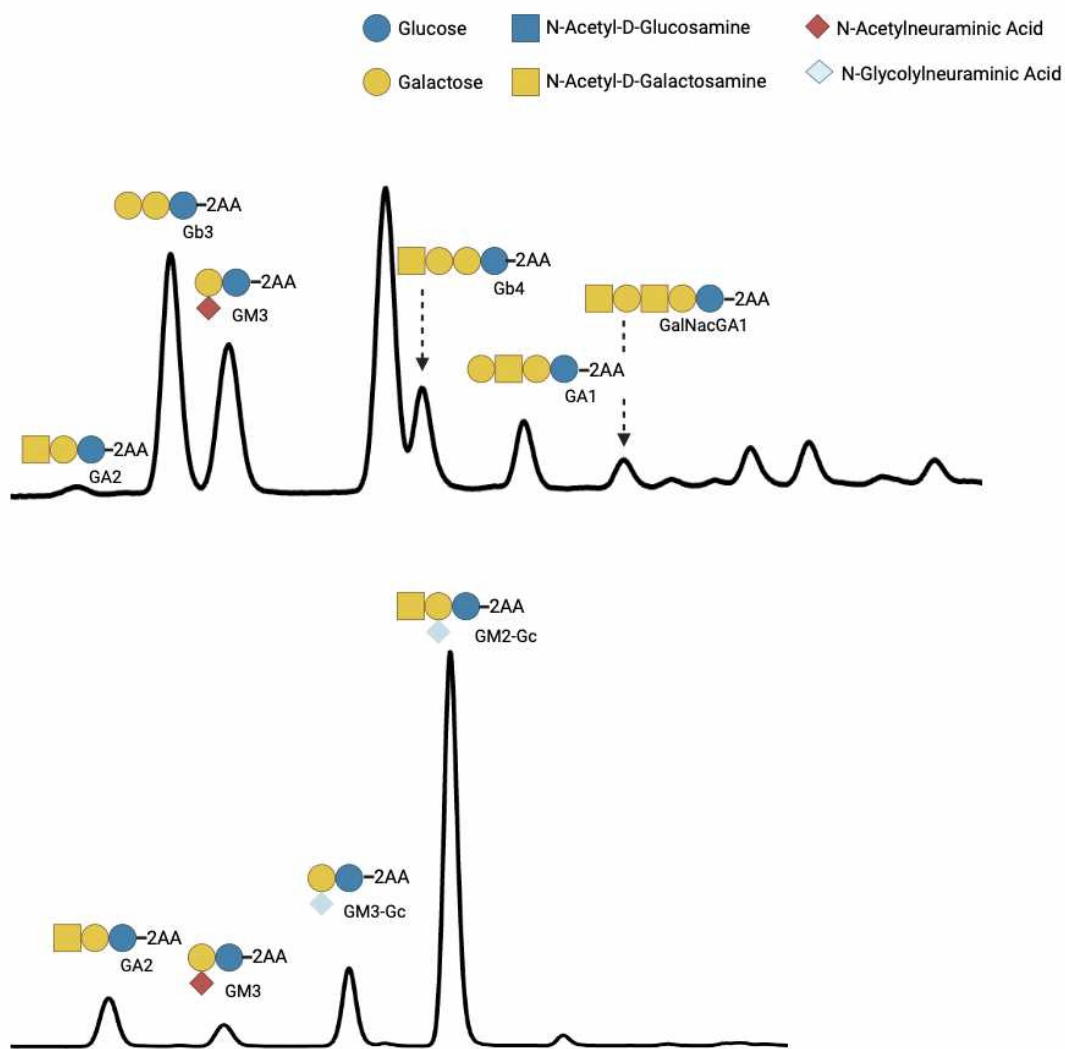


**Figure 2** GSL biosynthesis. Biosynthetic enzymes genes are indicated in the blue grid. Ganglioside names are abbreviated according to Svennerholm [1]. [1] L. Svennerholm, Designation and schematic structure of gangliosides and allied glycosphingolipids, Prog Brain Res 101 (1994) XI-XIV.



**Figure 3** Typical HPLC profile for 2AA-labelled glycans released from murine brain tissue glycosphingolipids with sugar structures indicated for each peak. In order to calculate values for GSL concentrations, the peaks are integrated and compared with a quantitative standard of known concentration.





**Figure 4** Typical HPLC profile for 2AA-labelled glycans released from murine kidney (top panel) and liver (bottom panel) glycosphingolipids with sugar structures indicated for each peak. In order to calculate values for GSL concentrations, the peaks are integrated and compared with a quantitative standard of known concentration.

#### Note

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