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Ganglioside extraction, purification and profiling

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TITLE:

Ganglioside Extraction, Purification and Profiling

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SUMMARY:

Gangliosides are sialic acid-bearing glycosphingolipids that are particularly abundant in the brain. Their amphipathic nature requires organic/aqueous extraction and purification techniques to ensure optimal recovery and accurate analyses. This article provides overviews of analytic and preparative scale ganglioside extraction, purification, and thin layer chromatography analysis.

ABSTRACT:

Gangliosides are glycosphingolipids that contain one or more sialic acid residues. They are found on all vertebrate cells and tissues but are especially abundant in the brain. Expressed primarily on the outer leaflet of the plasma membranes of cells, they modulate the activities of cell surface proteins via lateral association, act as receptors in cell-cell interactions and are targets for pathogens and toxins. Genetic dysregulation of ganglioside biosynthesis in humans results in severe congenital nervous system disorders. Because of their amphipathic nature, extraction, purification, and analysis of gangliosides require techniques that have been optimized by many investigators in the 80 years since their discovery. Here, we describe bench-level methods for the extraction, purification, and preliminary qualitative and quantitative analyses of major gangliosides from tissues and cells that can be completed in a few hours. We also describe methods for larger scale isolation and purification of major ganglioside species from brain. Together, these methods provide analytical and preparative scale access to this class of bioactive molecules.

INTRODUCTION:

Gangliosides are defined as glycosphingolipids bearing one or more sialic acid residues[1]. They are expressed primarily at the cell surface with their hydrophobic ceramide lipid moiety embedded in the outer leaflet of the plasma membrane and their hydrophilic glycans extending into the extracellular space[2]. Although distributed widely in vertebrate cells and tissues, they are particularly abundant in the vertebrate brain[3], where they were first discovered and named[4].

The structures of ganglioside glycans vary and are the basis for their nomenclature (**Figure 1**). Ganglioside glycans are comprised of a neutral sugar core bearing different numbers and distributions of sialic acids. The smallest ganglioside, GM4, has only two sugars (sialic acid bound to galactose)[5]. Larger naturally occurring gangliosides may contain well over a dozen total sugars[6] or up to seven sialic acids on a single neutral core[7]. Their ceramide lipid moieties also vary, having different sphingosine lengths and a variety of fatty acid amides. In the vertebrate brain four ganglioside species, GM1, GD1a, GD1b, and GT1b predominate. Ganglioside expression is developmentally regulated, tissue specific, and cell type specific.

[Place **Figure 1** here]

Gangliosides function at the molecular level by engaging and modulating proteins in their own membranes (cis regulation) or by engaging glycan binding proteins in the extracellular milieu, including bacterial toxins and lectins on other cells (trans recognition)[3]. Specific binding of gangliosides to regulatory proteins and/or self-association with other molecules into lipid rafts results in changes in cell behavior that impact nervous system structure and function, cancer progression, metabolism, inflammation, neuronal proteinopathies, and infectious diseases[8]. Because of their diverse cellular roles, methods for their isolation and analysis can provide enhanced insights into the regulation of physiological and pathological processes. Here, validated methods for rapid small-scale extraction and analysis, and preparative scale isolation of gangliosides from brain are provided. Opportunities and challenges for application to other tissues are discussed.

PROTOCOL:

Tissue collection was performed under conditions authorized by the Johns Hopkins Animal Care and Use Committee.

1. Small scale ganglioside extraction and partial purification

CAUTION: Use appropriate ventilation when working with volatile and toxic solvents. Avoid plastic throughout; solvents will extract chemical components from many plastics that interfere with subsequent analyses. Polytetrafluoroethylene (PTFE) is an exception; PTFE-lined closures should be used to cap glass storage vials.

1.1. Extraction

1.1.1. Weigh a single fresh or thawed mouse brain (or sagittal half-brain, ~ 0.2-0.5 g) and place in a Potter-Elvehjem homogenizer prechilled in a bucket of ice.

NOTE: Previously frozen brains can be used after thawing at 0-4 °C.

1.1.2. Add 4.1 mL per g tissue wet weight of water and homogenize with 10 strokes.

NOTE: Accurate solvent ratios are key to optimal extraction and partition, the goal is chloroform-methanol-aqueous (4:8:3) assuming brain tissue is 80% aqueous.

1.1.3. Add 13 mL per g tissue wet weight of methanol, shift to ambient temperature (22 °C) and mix.

NOTE: The solution will appear cloudy. Addition of methanol at this step, without chloroform, optimizes protein precipitation. All subsequent steps are at ambient temperature (22 °C).

1.1.4. Transfer to a thick-walled glass screw-capped tube with a PTFE-lined screw cap at ambient temperature (22 °C) and mix thoroughly. Add 6.5 mL per g tissue wet weight of chloroform, cap, and mix thoroughly. Centrifuge at 450 x *g* for 15 min. Transfer the clear supernatant to a fresh screw-capped tube and measure the volume, "recovered extract volume".

1.2. Partition

1.2.1. Add 0.173x "recovered extract volume" of water to the clear supernatant, cap, vortex vigorously, and centrifuge as described in step 1.1.4.

NOTE: The goal is to have chloroform-methanol-aqueous in the ratio 4:8:5.6. The mixture will be cloudy and rapidly resolve into two phases: an upper aqueous-rich phase and a lower chloroform-rich phase at ~ 4:1 ratio. Wait for 60 min for complete phase separation.

1.2.2. Transfer the upper phase, which contains the gangliosides, into a fresh glass tube with a PTFE-lined screw cap.

1.3. Reverse phase cartridge chromatography

1.3.1. Using a 5 mL glass syringe, wash a tC18 solid phase extraction cartridge (400 mg) with 3 mL of methanol, then 3 mL of chloroform-methanol-water (2:43:55). Load the upper phase from step 1.2.2 onto the tC18 cartridge through the same glass syringe, collect the flow-through and reload it onto the column to optimize adsorption.

1.3.2. Wash the cartridge with 3 mL of chloroform-methanol-water (2:43:55) then 3 mL of methanol-water (1:1).

1.3.3. Elute the gangliosides with 3 mL of methanol into a fresh screw-capped tube. Evaporate

to dryness under a gentle stream of nitrogen at ≤ 45 °C. Dissolve in methanol at 1 mL per g of original tissue wet weight.

2. Large scale ganglioside extraction and purification

CAUTION: When working with volatile solvents, use explosion resistant blenders. Do not use plastics except PTFE. Tetrahydrofuran, chloroform, and ethyl ether are toxic volatile organic compounds. Work in a fume hood with protective gloves and safety goggles.

2.1. Extraction

2.1.1. Thaw frozen bovine brain at 4 °C for several hours. Dissect the grey matter from meninges and white matter.

NOTE: The following procedure is described for 100 ± 20 g of isolated brain grey matter and is scalable.

2.1.2. Place 100 g of brain grey matter in a blender and add 1 mL per g brain wet weight of chilled 10 mM potassium phosphate buffer pH 6.8. Homogenize on low for 20 s. Add 8 mL tetrahydrofuran per g brain wet weight and homogenize on low for 10 s. Decant into glass centrifuge bottles and centrifuge at 5,000 x *g* for 15 min at ambient temperature (22 °C).

2.1.3. Collect the supernatant, measure its volume, and transfer to a glass separatory funnel. Add 0.3 mL of ethyl ether per mL of the supernatant. Shake vigorously, then allow to sit undisturbed for 30 min during which two phases, an upper ether phase and a lower aqueous phase, separate. Collect the lower phase, which contains the gangliosides, into a glass bottle with a PTFE-lined cap.

2.1.4. To the upper (ether) phase remaining in the separatory funnel, add 0.1 mL water per mL of original supernatant (step 2.1.3). Shake vigorously, allow phases to separate, collect the lower (aqueous) phase and combine with the previous lower phase. Evaporate the combined lower phases to a dry powder and weigh.

2.2. Saponification

2.2.1. Add 10 mL of 100 mM aqueous NaOH per g powder in a sealed tube. Mix and incubate at 37 °C for 3 h. Allow to cool and adjust to pH 4.5 by dropwise addition of 100 mM aqueous HCl. Measure the volume and transfer to a glass separatory funnel.

NOTE: Sialic acids are acid labile; avoid acidification below pH 4.5.

2.2.2. Based on the aqueous volume, add 2.67 volumes of methanol, mix gently, then add 1.33 volumes of chloroform to create a single-phase solution of chloroform-methanol-aqueous (4:8:3). Mix well.

2.2.3. Based on the original aqueous volume, add 2.6 volumes of water to bring the mixture to chloroform-methanol-aqueous to a ratio of 4:8:5.6. Shake vigorously, then allow to sit undisturbed to separate two phases, a polar upper phase containing the gangliosides and a nonpolar lower phase. Collect the upper phase in a glass bottle with a PTFE-lined cap.

NOTE: Non-sialylated lipids will not appear on thin layer chromatography (TLC) plates stained using resorcinol but will appear when using a *p*-anisaldehyde stain. The purpose of this saponification is to remove the acetylated compounds such as phospholipids.

2.3. Reverse phase chromatography

2.3.1. Pre-wash a large scale (10 g) tC18 solid phase extraction cartridge by passing 50 mL of each of the following three solvents through the column using vacuum or pressure (<1 min each wash): methanol, methanol-water (1:1), then chloroform-methanol-water (2:43:55). Load the upper phase from step 2.2.3 onto the column by vacuum or pressure, collect the flow through, reload, and collect the flow through for subsequent analysis.

2.3.2. Wash the column with 30 mL of chloroform-methanol-water (2:43:55), then 30 mL methanol-water (1:1), then elute the gangliosides with 50 mL methanol, and again with 10 mL methanol, collecting each wash and each elution separately. Using TLC (below) confirm that ganglioside is absent from the flow through and washes and eluted in the first elution (50 mL methanol). Evaporate the eluted gangliosides to a dry powder and weigh.

NOTE: The purpose of tC18 chromatography is to separate gangliosides from both less and more polar contaminants. Mixed brain ganglioside yield after saponification is ~ 120 mg per g dry brain extract (step 2.1.4). Appearance of gangliosides by TLC in the flow through or washes indicates the solid phase extraction column was saturated. After methanol elution, the column may be further eluted with chloroform-methanol (1:1) to capture less polar lipids.

2.4. HPLC purification of individual gangliosides

2.4.1. Prepare HPLC Solvent A: acetonitrile-5 mM aqueous sodium phosphate buffer pH 5.6 (83:17) and Solvent B: acetonitrile-20 mM sodium phosphate buffer, pH 5.6 (1:1). Degas both solvents for 5 min.

2.4.2. Pre-equilibrate an HPLC column (20 x 250 mm column packed with amine bonded (NH_2) silica spheres, 5 µm diameter, 100 Å pore size) with 100% Solvent A for 20 min at 5 mL/min. Set a UV HPLC column effluent detector to 215 nm.

2.4.3. Dissolve the ganglioside powder from the reverse phase eluate in water at 5 mg/mL. Inject 0.5 mL of the ganglioside mixture onto the HPLC and run the solvent gradient (**Table 1**) at 5 mL/min, collecting fractions. Gangliosides will appear as A_{215} peaks with retention times (major brain gangliosides) of 25-70 min: GM1 ≈ 28 min; GD1a ≈ 38 min; GD1b ≈ 46 min; GT1b ≈ 65 min. Re-equilibrate 20 min with Solvent A after each run. Analyze fractions by thin-layer chromatography.

[Place **Table 1** here]

3. Thin layer chromatography (TLC) analysis of gangliosides

CAUTION: Chloroform is a toxic volatile organic compound. Work in a fume hood with protective gloves and safety goggles.

3.1. Running solvent and TLC plate preparation

3.1.1. Prepare a running solvent of chloroform-methanol-aqueous 0.25% KCl (60:35:8 by volume). Pour into a 10 cm x 10 cm glass TLC chamber with a stainless-steel cover so that the solvent depth is ~ 0.5 cm. Cover and allow to equilibrate in an area free of air currents for >10 min.

NOTE: An acrylic 5-sided box can be constructed or purchased for this purpose (**Figure 2**). Do not use solvent-saturated filter paper inside the chamber.

3.1.2. Place a 10 cm x 10 cm or 5 cm x 10 cm silica gel coated glass-backed high performance TLC plate in a drying oven at 125 °C for 10 min. Allow to cool. Use a dulled #2 pencil to draw 5-mm spotting lines with 2-mm separations along a line 1 cm above bottom of the plate and at least 1 cm from either side. Avoid disturbing the silica layer while marking.

3.1.3. Prepare a standard mix of pure gangliosides in methanol containing 100 µM GM1, 50 µM each of GD1a and GD1b and 33 µM of GT1b.

NOTE: This mixture contains 100 pmol of ganglioside sialic acid per µL for each of the four gangliosides, a quantity that provides a strong colorimetric signal by resorcinol staining, which is sialic acid dependent.

3.2. Ganglioside resolution

3.2.1. Wash a 10-µL Hamilton syringe with a beveled needle with methanol. Draw 1 µL methanol into a glass syringe to fill the needle dead volume and then 1 µL of sample or standard. Spot the sample evenly onto the 5-mm premarked lines until <1 µL of solvent (methanol) remains in the syringe. Allow the plate to dry at ambient temperature (22 °C) after all samples are spotted.

NOTE: Wash the syringe with methanol between sample loading. An unheated air blower set at

low can be used to accelerate drying.

3.2.2. Place the spotted and dried plate into the preequilibrated TLC chamber with the bottom edge immersed in the running solvent and cover and protect from air currents (**Figure 2**). Allow the running solvent to advance up the plate by capillary action until the solvent front reaches within 1 cm of the top of the plate. Remove and mark the solvent front at the edge of the plate with a pencil. Allow the solvents to evaporate completely either undisturbed or under mild air flow.

[Place **Figure 2** here]

3.3. Ganglioside staining

CAUTION: Reagent stains are toxic. Hydrochloric acid is corrosive and toxic. Prepare and spray reagents in a fume hood with protective gloves and safety goggles.

NOTE: The plate can be imaged for qualitative image analysis or stored by removing the clamps and securing the cover plate in place with clear tape. Quantitative analysis can be performed by measuring densitometry of ganglioside standards spotted in adjacent lanes.

3.3.1. Prepare resorcinol spray reagent for the detection of gangliosides based on their sialic acids. Dissolve 6 g of resorcinol in 100 mL water for a 6% resorcinol stock. Dissolve 1 g of $CuSO_4$ in 100 mL of water to make a 1% stock. To 64.7 mL of water add 5 mL of the 6% resorcinol stock, 0.31 mL of the 1% $CuSO_4$ stock then slowly add 30 mL of concentrated HCl and stir gently. May be stored at 4 °C for a month.

3.3.2. In a chemical fume hood, place the TLC plate with resolved gangliosides, origin end upside down, in a cut-away cardboard box to protect the walls of the hood from acid spray. Place resorcinol spray reagent in a glass TLC sprayer, attach to a source of pressurized nitrogen, and lightly spray the plate diagonally in the vertical and horizontal directions. Spray the TLC sorbent surface uniformly, but lightly.

3.3.3. Immediately cover the plate with a clean dry glass cover plate of the same dimensions and secure the cover plate in place with binder clips (**Figure 3**). Heat the covered plate at 125 °C for 20 min. Gangliosides will appear dark purple against a white background.

NOTE: The plate should not appear wet when spraying is complete. Cover plates can be fashioned by scraping the sorbent from previously used TLC plates using a single-edge razor blade.

CAUTION: Silica powder is toxic to lungs. Use a mask and dispose silica in a sealed container.

[Place **Figure 3** here]

3.4. General lipid staining for gangliosides and phospholipids.

CAUTION: Sulfuric acid is toxic and corrosive. Addition of concentrated acid to ethanol is exothermic and must be done slowly. Prepare staining reagent in a fume hood with protective gloves and safety goggles.

3.4.1. Prepare *p*-anisaldehyde stain by slowly adding 15 mL of concentrated sulfuric acid to 500 mL of ethanol. Stir for 30 min to allow the solution to cool before proceeding. Add 15 mL *p*-anisaldehyde and stir gently. This may be stored at room temperature (22 °C) up to six months.

3.4.2. In a chemical fume hood, dip the TLC plate with resolved gangliosides, origin side down, into a beaker containing the *p*-anisaldehyde stain. Submerge to the running front for ≥ 2 s. Remove TLC from stain and allow to drain. Heat the TLC plate on a hot plate at low temperature to develop.

NOTE: Lipids will appear dark against a purple background. Stain may be recovered for repeated use.

REPRESENTATIVE RESULTS:

The methods described in section 1 (small scale) provide gangliosides at sufficient quantity and purity for qualitative and quantitative determination of major brain gangliosides. Recovery from mouse brain is ~ 1 µmol ganglioside per g brain wet weight (1 nmol/µL) when prepared as described. TLC resolution of 1 µL (1 nmol) using section 3 provides ample material for resorcinol detection and resolves all of the major brain gangliosides as shown for wild type and genetically modified mice in **Figure 4**. Although mixed gangliosides prepared using section 1 are not free of other major lipids, gangliosides are of sufficient purity for mass spectrometric (MS) determination as shown in **Figure 5**, either as native purified gangliosides in negative mode or after permethylation in positive mode[9] Since section 1 avoids alkaline hydrolysis to remove phospholipids, it retains alkali-sensitive natural modifications, such as *O*-acetylated sialic acids (see GT1b-OAc, **Figure 4**)[9].

[Place **Figure 4** here]

[Place **Figure 5** here]

Large scale purification (section 2) includes extraction, saponification (to remove phospholipids) and HPLC resolution to provide purified major brain gangliosides GM1, GD1a, GD1b and GT1b suitable for biological experiments and for further chemical and enzymatic modifications. An exemplary HPLC profile and subsequent TLC analysis are shown in **Figure 6**. Alkali treatment (saponification) is necessary in this protocol to hydrolyze and remove contaminating phospholipids (**Figure 7**) but will also hydrolyze natural modifications of gangliosides, such as *O*-acetylated sialic acids, which may be important in some contexts[10]. For these applications, alternate effective methods for removal of phospholipids from isolated gangliosides have been published[6].

[Place **Figure 6** here]
[Place **Figure 7** here]

FIGURE LEGENDS:

Table 1: Solvent gradient for HPLC.

Figure 1: Major brain gangliosides and their biosynthetic precursors. Structures are shown using Symbol Nomenclature for Glycans[11].

Figure 2: Ganglioside TLC equipment and set up. A twin trough chamber is filled to ≈ 0.5 cm on both sides with running solvent. The plate is placed against one side with the origin end immersed in the running buffer. The chamber is covered with an acrylic box to avoid air currents. Panel A, side view prior to plate insertion. The solvent level is visible a few mm above the chamber bottom; Panel B, front view during development. The solvent front is visible at about 40% of the way up the plate.

Figure 3: TLC plate of resolved mixed ganglioside. TLC plate of resolved mixed ganglioside standards (left lane) and purified mixed bovine grey matter gangliosides (right lane) after resorcinol staining and heating with glass cover plate clipped in place. Standard gangliosides (top to bottom) are GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b and GQ1b. After cooling, the plate can be imaged and/or the cover plate taped in place for storage.

Figure 4: TLC of mouse brain gangliosides. TLC of mouse brain gangliosides from wild-type (WT) and *St3gal* single and double-null mice purified as in Protocol 1. This figure has been modified from Sturgill et al, 2012[9].

Figure 5: MS of permethylated wild-type and *St3gal2/3*-double-null mouse brain gangliosides purified as in section 1. Note that while GD1a and GD1b resolve by TLC (**Figure 4**) but have the same mass so are not distinguishable by one-dimensional MS. This figure has been modified from Sturgill et al, 2012[9].

Figure 6: Representative HPLC of bovine brain gangliosides. The elution gradient (%B, dotted line) is overlaid on the absorbance (A_{215}, solid line) for the first 75 min of the cycle. Peaks (A_{215}) were collected (numbers in brackets) and subjected to TLC as in Protocol 3. Lane numbers refer to the peak numbers on the chromatogram.

Figure 7: TLC plate of resolved mixed ganglioside. TLC plate of resolved mixed ganglioside standards (lane 1) along with post-saponification partitioned gangliosides (lane 2) and released fatty acids (lane 3). Lipids, including gangliosides, are detected with *p*-anisaldehyde stain. Standard gangliosides (top to bottom) are GM3, GM2, GM1, GD3, GD1a, GD1b, and GT1b.

DISCUSSION:

The methods for small and large scale ganglioside extraction and isolation reported here are not

unique – there are many different solvent extraction and purification approaches that provide excellent results[12]. The methods reported here for small scale purification from brain, from Fredman and Svennerholm[13], were shown to optimize recovery and have proven to be robust and straightforward over many years in our laboratory. Isolation and purification suitable for TLC and MS can be readily completed, from intact tissue to isolated gangliosides, in a few hours. MS can be performed on native purified gangliosides in the negative mode or after permethylation in the positive mode (**Figure 7**, e.g., see Sturgill et al.[9]). The yield is very consistent, ≈ 1 µmol ganglioside (≈ 2 µmol ganglioside-bound sialic acid) per g fresh brain tissue for most mammals. The method for large scale extraction and partition from brain reported here, introduced by Tettamani et al.[14], is selected to minimize the volume of ganglioside-containing solvent at the first partition (ether-tetrahydrofuran-water). This simplifies subsequent steps that can become cumbersome with techniques that generate large volumes of partitioned gangliosides at the first steps. The HPLC method described, from Gazzotti et al.[15], has relatively high capacity and good resolution of the major brain gangliosides.

The small-scale protocols described are well suited for both cells and tissues and are scalable. Since the final steps are reverse-phase capture, evaporation and redissolving in methanol, they can be applied at an arbitrary scale to small samples, such as cultured nerve cells. In this case, we scrape and homogenize cells into 1 mL of water for convenience and proceed with methanol and chloroform addition to generate the appropriate ratios for extraction and then partition. The final dried gangliosides after reverse phase can be redissolved in just a few microliters and analyzed by TLC and MS.

Attention to solvent ratios is critical to success in extraction and solvent partitioning steps for ganglioside isolation. For small scale extraction and partitioning, different chloroform-methanol-water ratios were tested and the ones that generated near quantitative ganglioside isolation are reported here[13]. Variations from the described ratios will diminish recovery and/or purification. Likewise, attention to solvent ratios in TLC developing solvents is critical. The chloroform-methanol-water ratios reported result in a single clear phase. Small variations may yield cloudy developing solutions which should not be used but can be clarified by dropwise addition of methanol with swirling. Although different extraction and partition solvents are common, solvent ratios should be carefully followed for each variation[12]. Alterations in TLC solvents are also common to optimize separation of specific gangliosides[16]. A relatively simple way to modify TLC migration of gangliosides is to change the aqueous phase of the solvent mixture. Using aqueous ammonium hydroxide or calcium chloride instead of potassium chloride alters the ganglioside salt form and relative TLC migration[17].

Whereas all vertebrate cells and tissues express gangliosides, the brain and nerve cells are unusual in the high amounts expressed. The protocols described here are applicable to other tissues and cells, but modifications may be required due to the lower abundance and potential contamination with other lipids. These methods as applied to human neutrophils[6] and mouse adipose[18] provide examples.

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DISCLOSURES:

The authors claim no competing interests.

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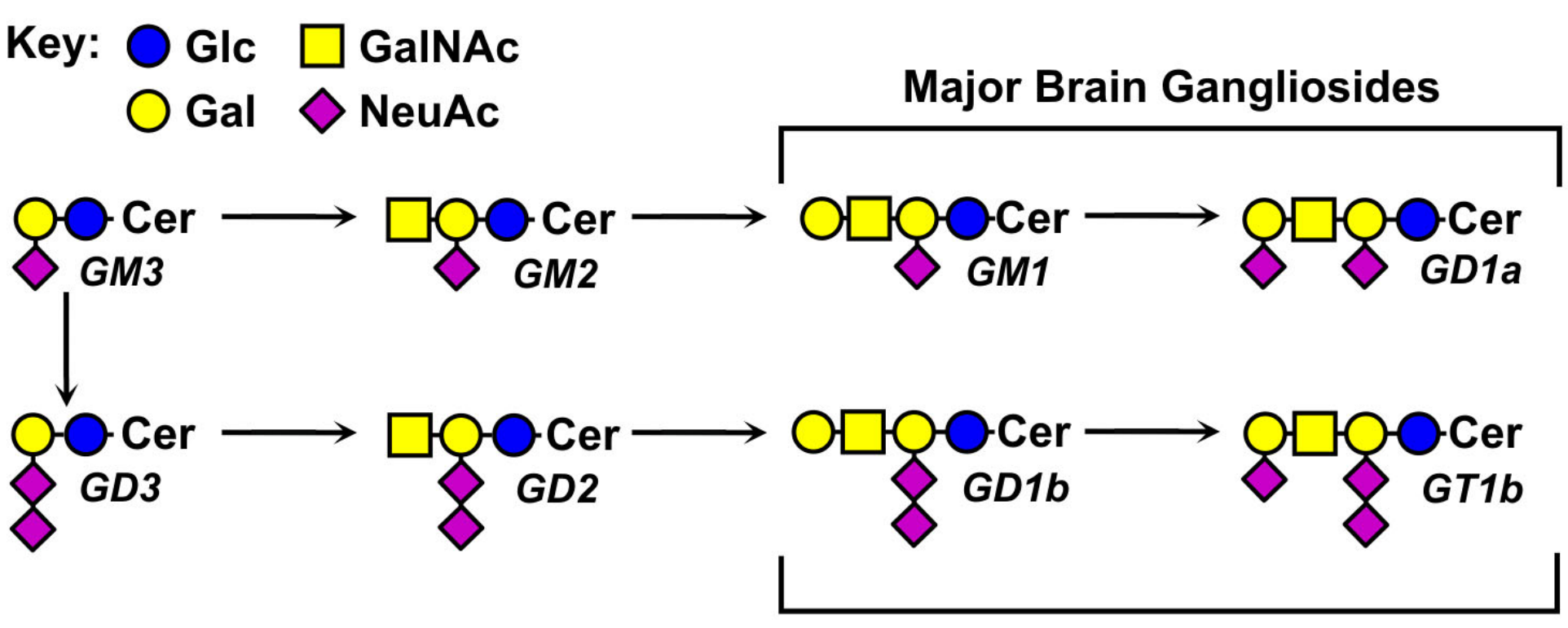
Key:
Glc
GalNAc
Gal
NeuAc
Major Brain Gangliosides
Cer
GM3
Cer
GM2
Cer
GM1
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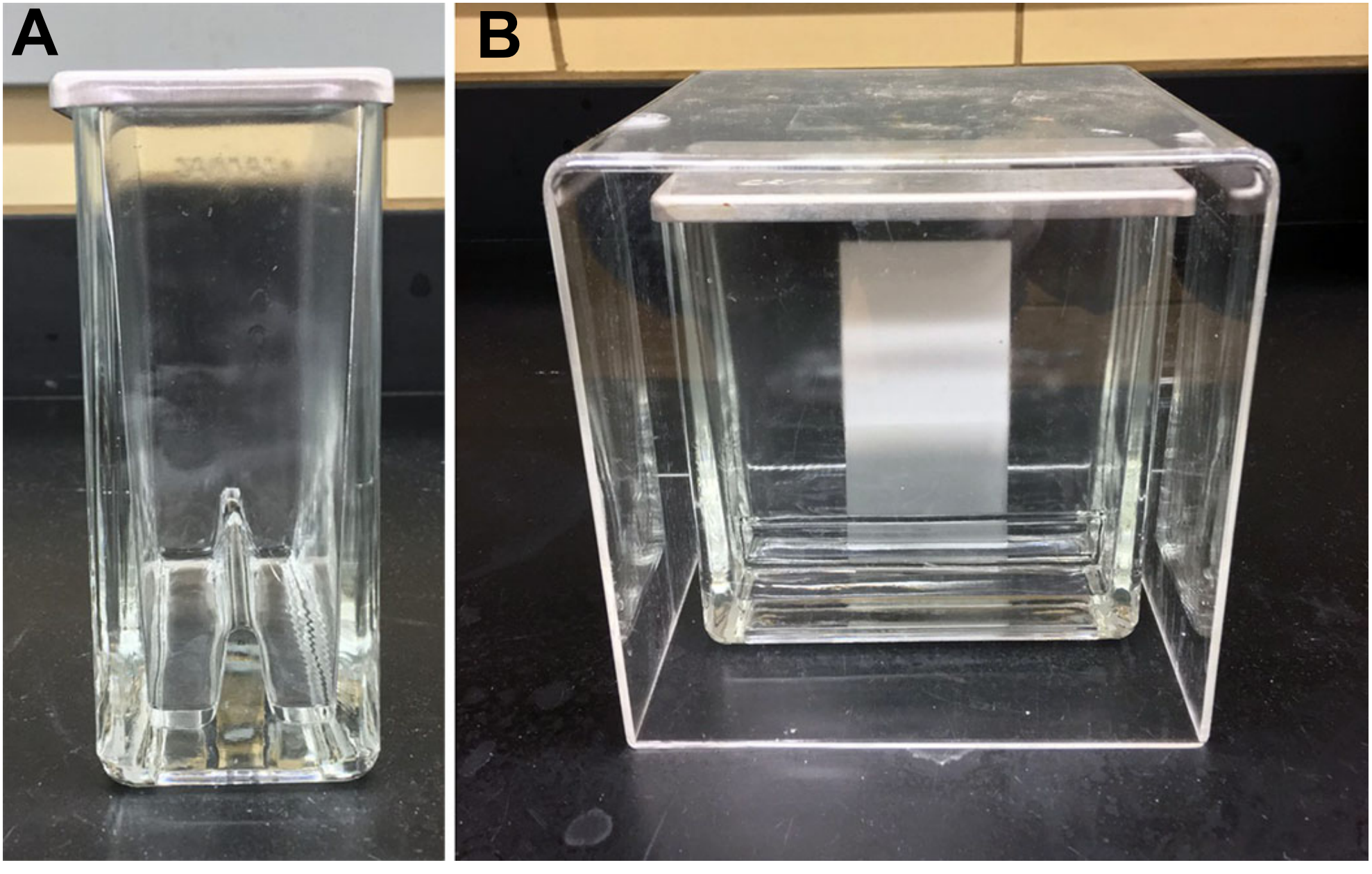
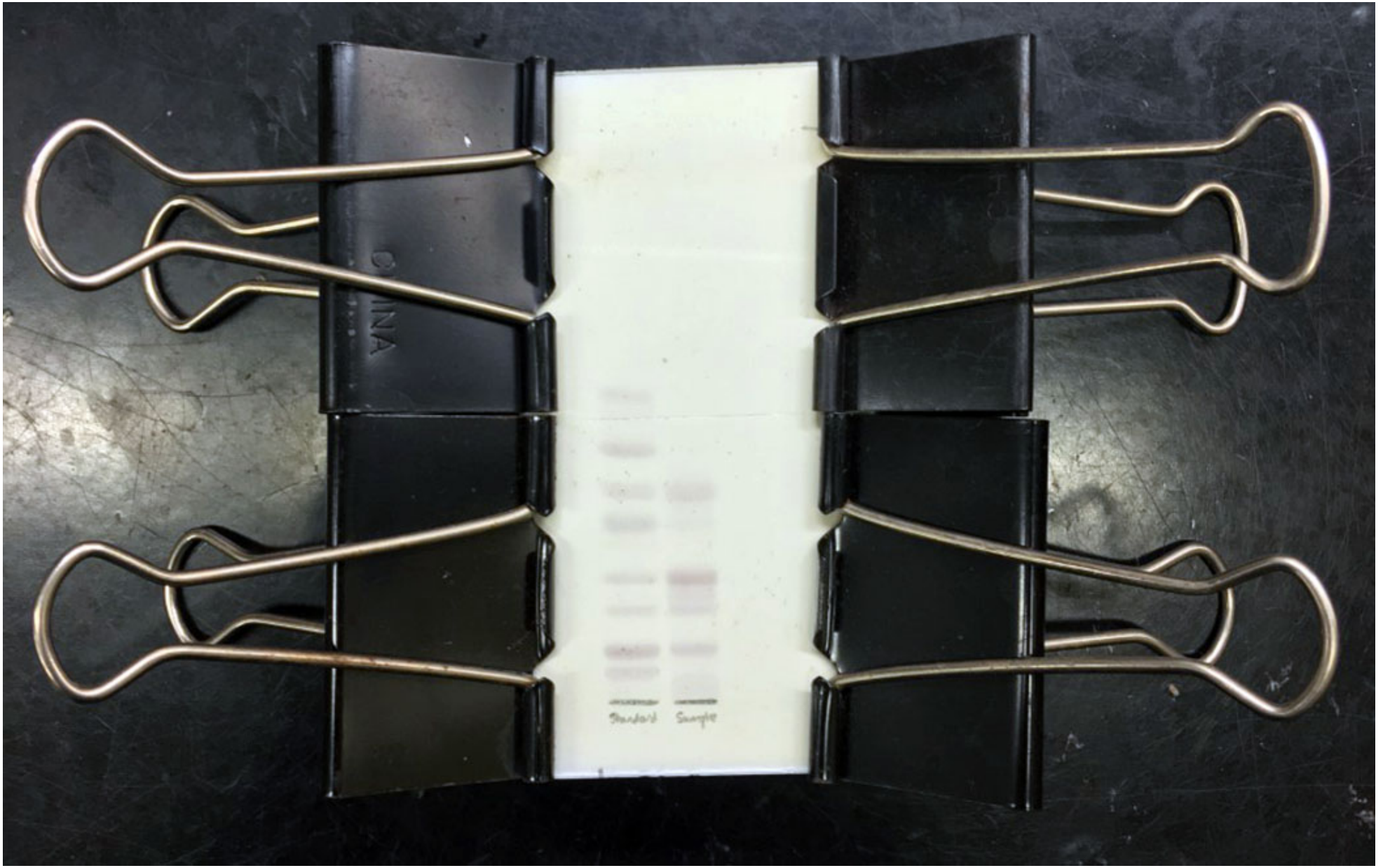
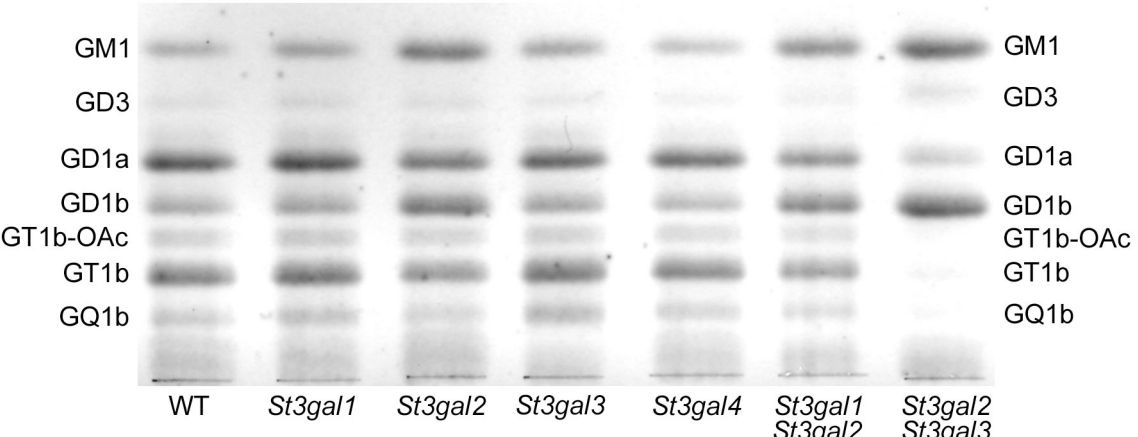
Cer
GD3
Cer
GD2
Cer
GD1b
Cer
GT1b


A
B


CHINA
Standard
Sample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GD3
GD1a
GD1b
GT1b-OAc
GT1b
GQ1b
GM1
GD3
GD1a
GD1b
GT1b-OAc
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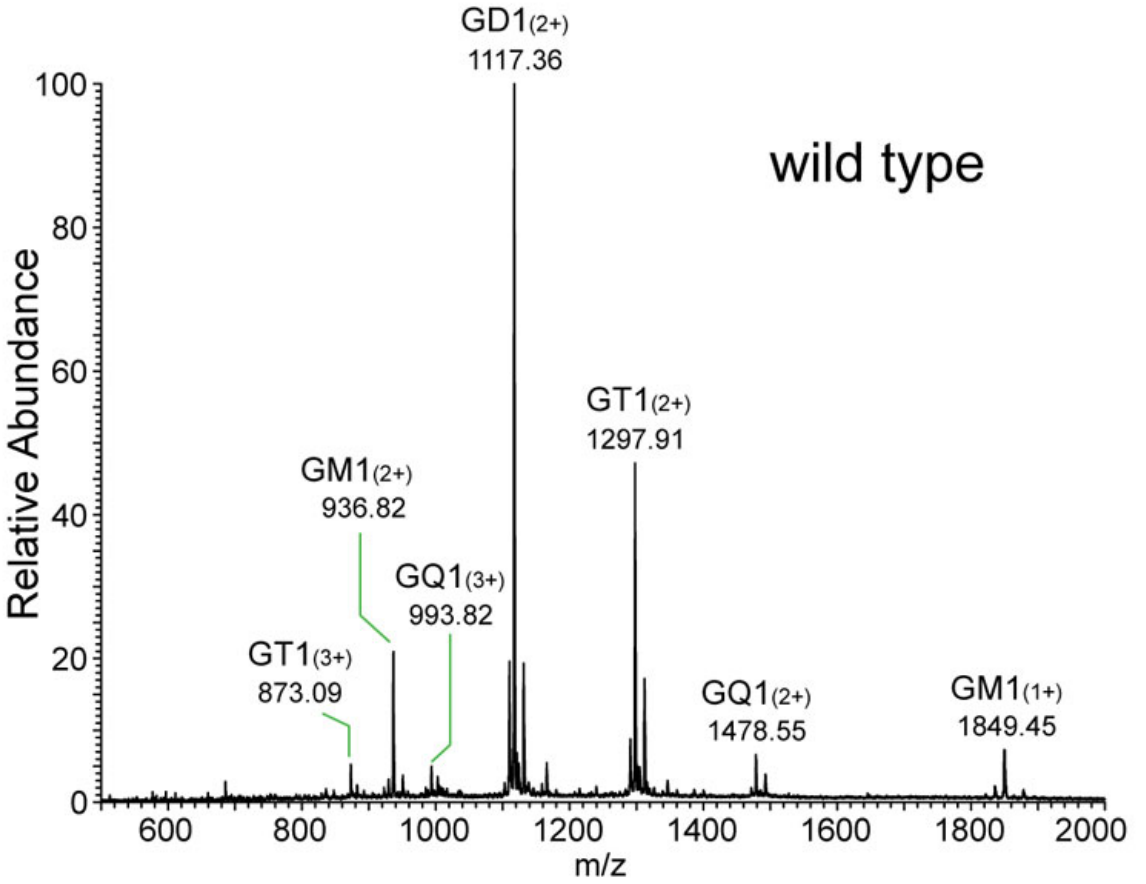
St3gal2
St3gal3
St3gal4
St3gal1 St3gal2
St3gal2 St3gal3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wild type
GT1(2+)
1297.91
GM1(2+)
936.82
GQ1(3+)
993.82
GT1(3+)
873.09
GQ1(2+)
1478.55
GM1(1+)
1849.45
Relative Abundance
m/z
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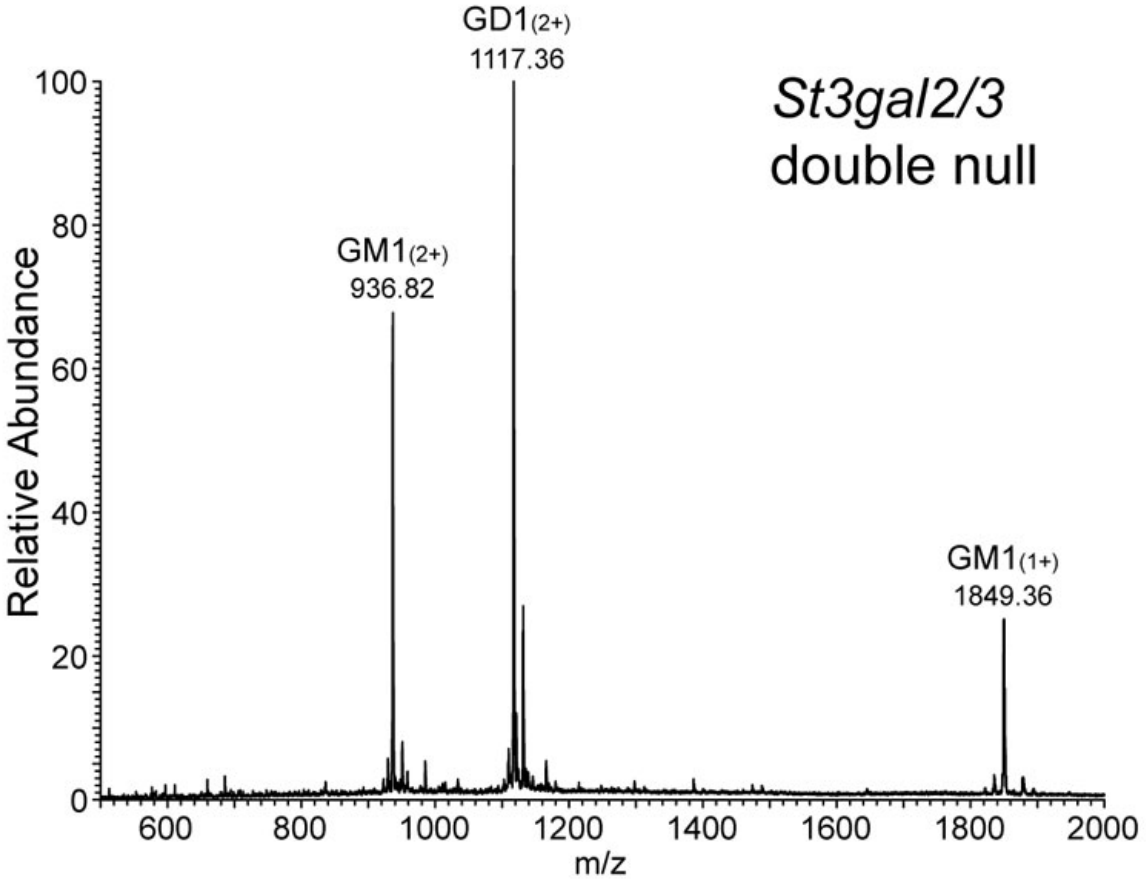
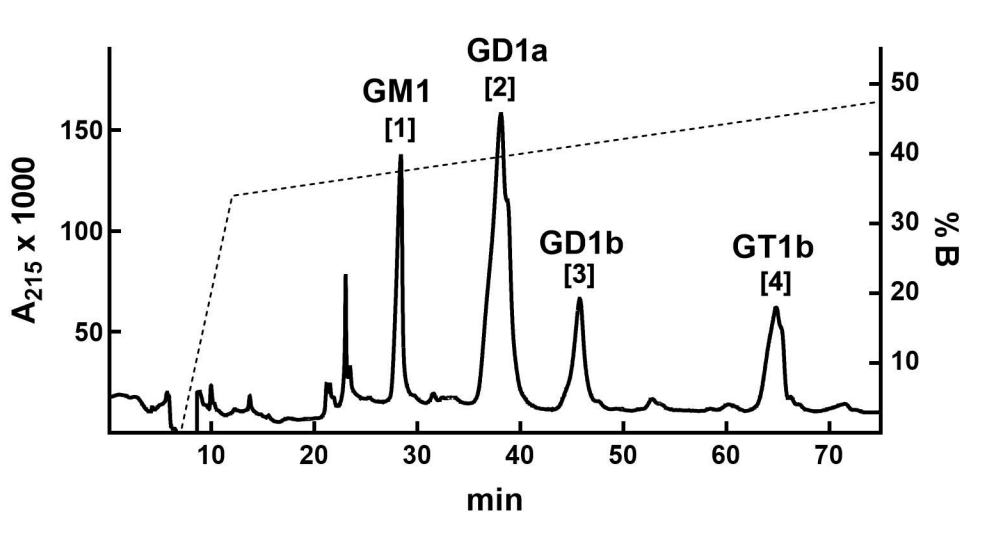
80
60
40
20
0
600
800
1000
1200
1400
1600
1800
2000


GD1(2+)
1117.36
St3gal2/3
double null
GM1(2+)
936.82
GM1(1+)
1849.36
Relative Abundance
m/z
100
80
60
40
20
0
600
800
1000
1200
1400
1600
1800
2000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[1]
GD1a
[2]
GD1b
[3]
GT1b
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A215 x 1000
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100
50
10
20
30
40
50
60
70
min
B %
50
40
30
20
10

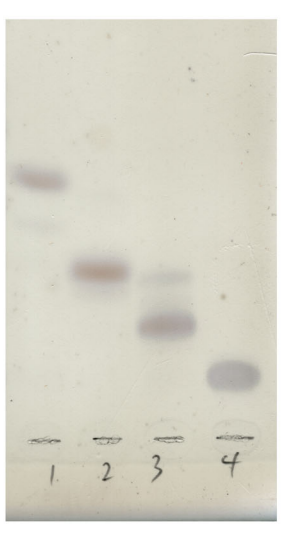
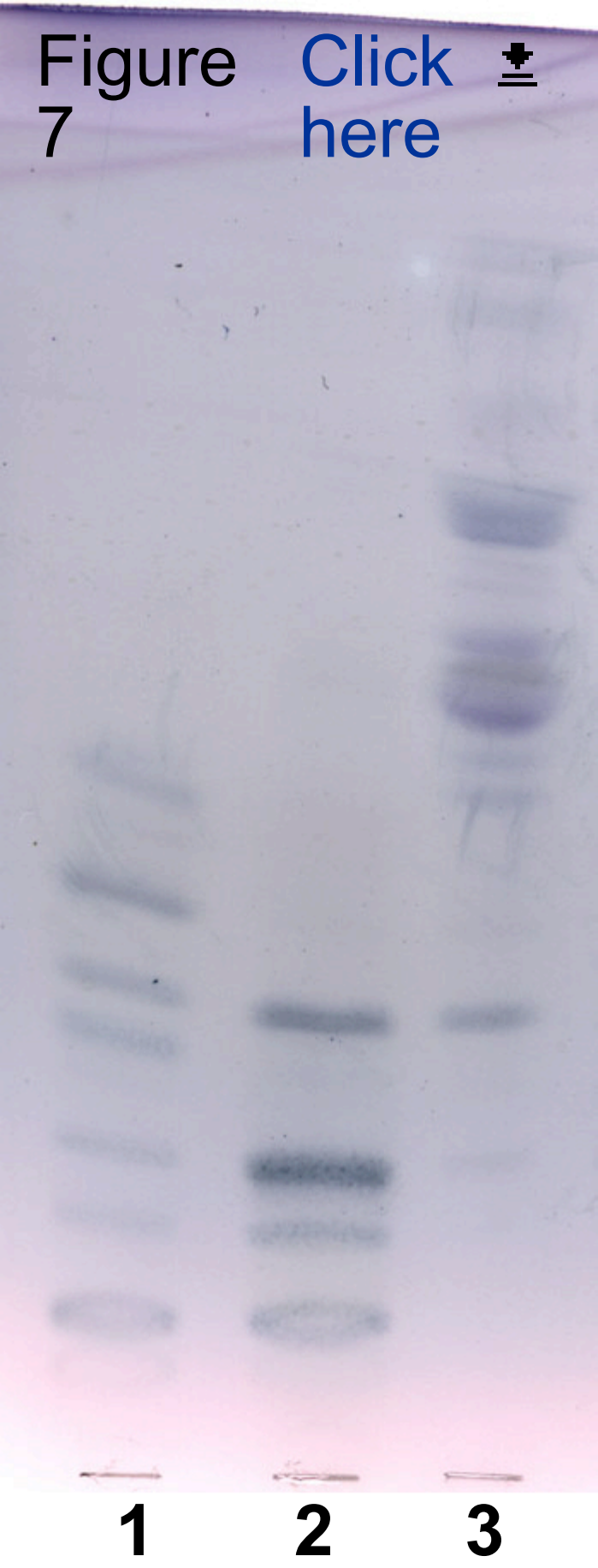

1
2
3
4

Figure 7

Click here



Table

Time (min)	%A	%B
0	100	0
7	100	0
12	63	37
82	54	46
82.01	0	100
92	0	100

Name of Material/ Equipment	Company	Catalog Number
Bovine brain, stripped	PelFreez	57105-1
Ganglioside standards	Matreya	GM1, 1061; GD1a, 1062; GD1b, 1501; GT1b, 1063
Glass bottle with PTFE-lined cap	Fisher Scientific	02-911-739
Glass centrifuge bottle	Fisher Scientific	05-586B
Glass culture tubes, 16 x 125 mm	VWR	60825-430
Glass separatory funnel (2 L)	Pyrex	6400-2L
Injection syringe - Hamilton 1750 gastight 500 µl	Hamilton	81265
p-Anisaldehyde, 98%	Sigma-Aldrich	A88107
Potter-Elvhjem Homogenizer	Fisher Scientific	08-414-14A
Reprosil 100 NH2 10µm 5x4mm guard columns	Analytics-Shop	AAVRS1N-100540-5
Reprospher 100 NH2, 5 µm, 250 mm x 20 mm HPLC column	Analytics-Shop	custom packed
Resorcinol	Sigma-Aldrich	30752-1
Rotary evaporator	Buchi	R-300
Sample loop for Model 7725 Injector (5 ml)	Sigma-Aldrich	57632
Sep-Pak tC18 Cartidges Vac 35 cc (10 g)	Waters	WAT043350
Sep-Pak tC18 Plus Short Cartridge, 400 mg	Waters	WAT036810
Spotting syringe - Hamilton 701N 10 µl	Hamilton	80300
Thick-walled 13-mm diameter test tubes with PFTE lined caps	Fisher Scientific	14-933A
Threaded 2-ml vials with PFTE lined caps	Fisher Scientific	14-955-323
TLC plates, HPTLC Silica gel 60 F254 Multiformat	Fisher Scientific	M1056350001
TLC reagent sprayer	Fisher Scientific	05-723-26A
TLC running chamber for 10 x 10 cm plates	Camag	22.5155
Waring 1-Liter Stainless Steal Explosion Resistant Blender	Waring	E8520

Comments/Description
for collecting HPLC fractions
Choose appropriate volume option other sizes available
For ganglioside storage Fluorescence impregnation (F254) stabilizes the sorbent surface

The authors sincerely thank the reviewers and editor for their time and useful comments to improve the manuscript. Much appreciated.

Responses to review of: JoVE62385 "Ganglioside extraction, purification and profiling." Responses in *italic underline*.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. *Completed.*

2. Please define the abbreviations before use (TLC). *Completed.*

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Sep-Pak, etc. *Completed. Generalized Sep-Pak to C18 solid phase extraction cartridges.*

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. *Completed.*

5. Please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2(lines: 77, 80, 86, 98, 99,102, 118, 122, etc.). *Completed. Changed "ml" to "mL", "µl" to "µL", and "10 x 10 cm" to "10 cm x 10 cm".*

6. Line 80/86/196: Please mention the ambient temperature. *Completed. Added (24 °C) to each reference of ambient temp.*

7. Line 117/118: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks. *Completed.*

8. Line 146: Please elaborate on the pre-washing steps. How long is it performed? *Completed.*

9. Line 152: Please mention the volume of each fraction collected. *Completed (note: each fraction was the entire wash or elution listed).*

10. Line 161: Please mention the duration for degassing the solvents. *Completed.*

11. Line 215-223/230-231: Please ensure that the actions are described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Completed: (i) moved statement "The plate should not appear wet when spraying is complete" to the corresponding note; (ii) moved 3.3.3. to a note at the top of 3.3; (iii) added the safety statement "work in fume hood" under 3.4 caution; (iv) added a safety statement under 2 and 3 about tetrahydrofuran, chloroform, and ethyl ether toxicity and to work in a fume hood.

12. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. *Completed.*

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: *Completed.*
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

14. Please Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations. *Completed.*

15. Figure 2: Please consider including labels for the figures in the panel to make them more informative. *Completed.*

16. Figure 6: Please specify what the numbers on the image on the right represent. Please consider defining it in the Figure Legend. *Completed.*

17. Figure: Please consider relabeling the panes to improve the clarity of the figure. *Completed.*

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_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The following manuscript provides a detailed description of ganglioside extraction which will be well received by the field. I have no major concerns

Minor Concerns:
1.2. Partition section can use more details in the method.
o It was unclear to me if the water is added to the clear supernatant or remaining contents after centrifugation.
Response: The water is added to the clear supernatant. Line 93, added "to the clear supernatant".

o It would be helpful to state which phase contains gangliosides like the authors did for the large scale extraction. *Response: line 98, changed to "Transfer the upper phase, which contains the gangliosides, into a fresh glass tube".*

o Wait time for phase partition? Centrifugation needed? *Response: Time (60 min) added. Centrifugation not needed.*

1.3.1 section: I think the authors meant to state "load the upper phase from step 1.2.1" *Response: Thank you. Changed "1.1.7" to "1.2.2".*

* In figure legends 3 and 5, Sturgil et al, 2012 is misspelled (supposed to be Sturgill et al, 2012). *Response: Great catch. Thanks. Fixed.*

* figure 5 and the discussion section both commented that the purified gangliosides can be used for mass spectrometry analysis - it would be helpful to describe any further preparation required for MS. *Response: MS is performed on both native purified gangliosides and their permethylated derivatives – as now added to the text.*

Reviewer #2:

Manuscript Summary:

Ganglioside analysis is quite challenging because of their structural heterogenicity, their amphiphatic nature and relatively low abundance, especially compared to other polar lipids in nervous tissues. The efficiency of the prior extraction and purification of gangliosides from biological materials makes a critical point. The present manuscript describes different methods for sample preparation and preliminary qualitative and quantitative analysis of gangliosides from nervous tissues.

These methods are clearly described. Sufficient details are provided so that the protocols can be successfully performed by others. Cautions to be taken are also mentioned. Yields are specified, which is a useful piece of information for readers. The presentation of both a small scale procedure for analytical purposes and a large scale procedure for preparative purposes makes a useful and interesting point. The limits and issues of the protocols are well mentioned (hydrolysis of O-acetylated gangliosides, contamination of ganglioside extracts with other major lipids…).

Major Concerns:

One major concern is related to the purification step using reverse phase cartridge chromatography. Have the authors verified that the washing steps of the C18 cartridge (C:M:H2O and M:H2O) do not result in losses of gangliosides, especially the more polar ones, considering the mixture of solvents used. *Response: As stated in the revised text, the flow through, each wash fraction, and the elution fractions are tested by TLC and confirmed to be free of gangliosides. If the column is saturated, gangliosides will appear in the flow through.*

Minor Concerns:

While I understand that the accuracy of the solvent ratio is important, I doubt the volume of water has to be as precise as 4.08 ml per g (1.1.2), especially because the aqueous content of the starting material is approximate. *Response: Excellent point, thank you. Changed to 4.1 mL.*

Although the purpose of the different steps of the protocols is specified at some point in the manuscript, it would be more useful to place it next to each step. For example, the purpose of the saponification step (to remove

phospholipids) should be moved from line 254 to section 2.2. Also specify the purpose of the C18 cartridge step. *Response: Completed.*

Line 100: the step 1.1.7 is referred to. This step does not seem to exist in this version of the manuscript. It should be step 1.2.2. *Response. Good catch, thank you. Corrected to 1.2.2.*

In section 3.3.3, the authors should precise that quantification of resorcinol-stained gangliosides by densitometry requires spotting ganglioside standards on the TLC plate to generate calibration curves. *Response. Added sentence indicating that for quantitative analysis, researchers must include standards to generate a calibration curve.*

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Requestor type	Author of this OUP content
Pharmaceutical support or sponsorship for this project	No

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Circulation/distribution	1000
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Lead author	Ronald Schnaar
Title of targeted journal	JoVE
Publisher	Myjove Corporation
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