www.lipidmaps.org

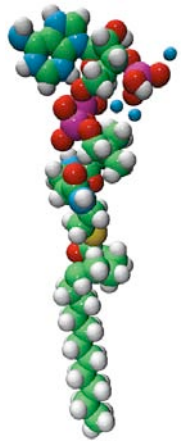
LIPID MAPS Lipidomics Workshop
EB2009

April 19, 2009

Lipidomic analysis of sphingolipids (and precursor fatty acyl-CoA's)

M. Cameron Sullards

Schools of Chemistry, Biochemistry and Biology &
Petit Institute for Bioengineering and Bioscience
Georgia Institute of Technology



Other LIPID MAPS Sphingolipid Core members:

	Mass spectrometry	Cell biology
Al Merrill	Samuel Kelly	Elaine Wang
	Chris Haynes	Ying Liu
	Rebecca Shaner	Kacee Sims
	Yanfeng Chen	Amin Momin

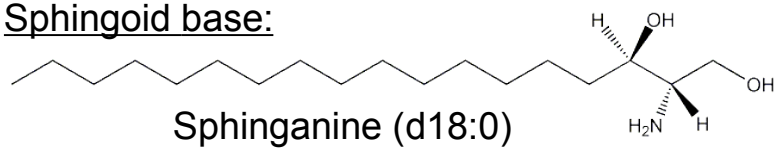
Outline:

A. Brief introduction to the lipid class: nomenclature & range of compounds to analyze
B. Sample preparation issues: solvents, chromatography, recovery, reproducibility
C. Compound identification: Characteristic fragmentations; MS/MS and MS^n (LC for isomers and isobars, etc.)
D. Quantitation: MRM, Internal standards, etc.
E. Data analysis/visualization: LIMS, Website, other
F. Remaining challenges and opportunities
G. Discoveries from sphingolipidomic analysis thus far
H. Comparison of Lipid MAPS methods with others in the literature

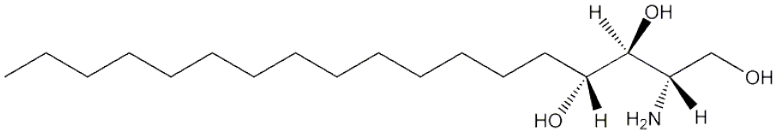
A. Brief introduction to the lipid class: nomenclature & range of compounds to analyze

Backbone variation

Sphingoid base:

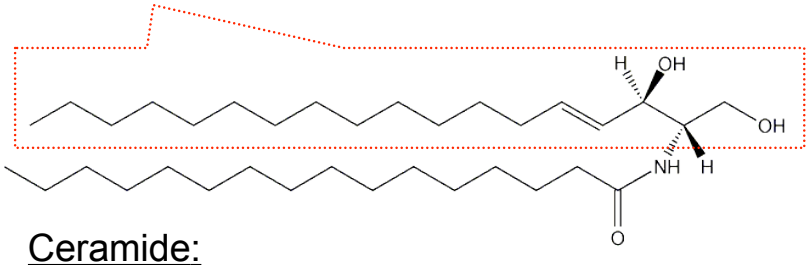


Sphinganine (d18:0)



4-Hydroxysphinganine
(phytosphingosine) (t18:0)

D-*erythro*-sphingosine (d18:1)



Ceramide:

Shown: N-palmitoylsphingosine (d18:1/16:0)
Other fatty acids-
typically C16-C26
0-1 double bond
sometimes α- or ω-hydroxy

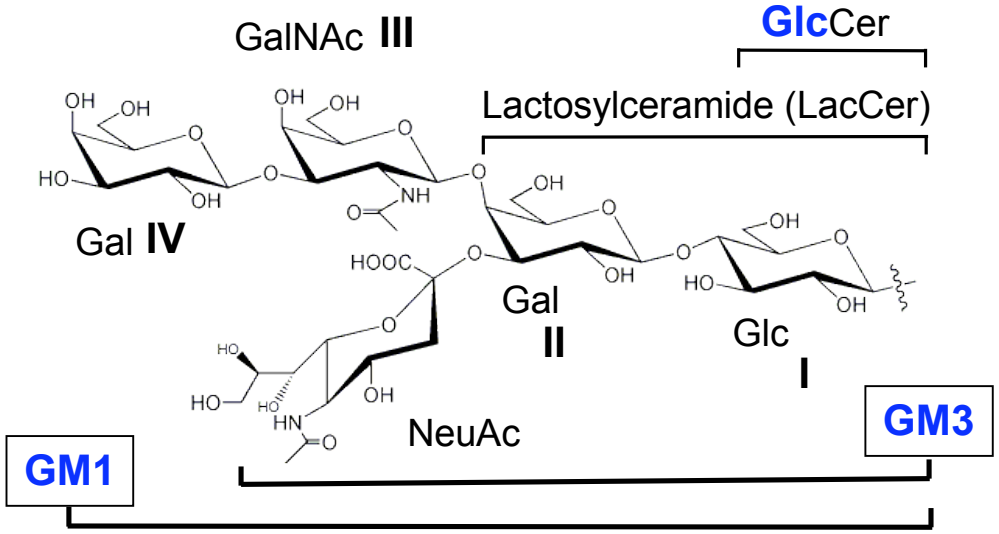
Headgroup variation

Phosphosphingolipids:

-OP(O_2^-)O-choline, Sphingomyelin

Glycosphingolipids:

Glc, Gal, Lac, Sulfatides…>400; see **www.lipidmap.org**; www.sphingomap.org



Neu5Acα2-3(Galβ1-3GalNAcβ1-4)Galβ1-4Glc1-1Cer

$II^3Neu5AcGg_4Cer$

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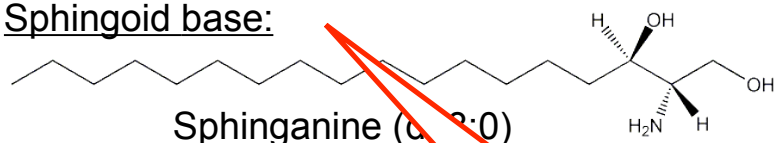
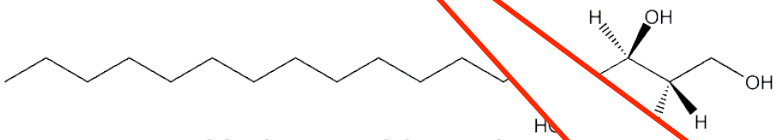


Sphinganine (d18:0)



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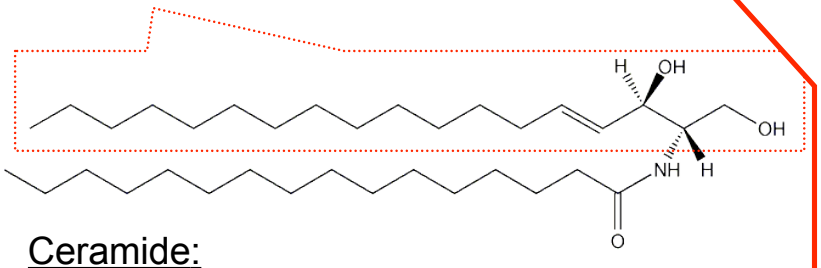

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Headgroup variation

Phosphosphingolipids:

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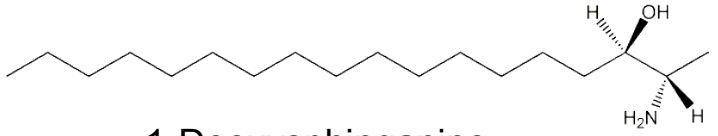
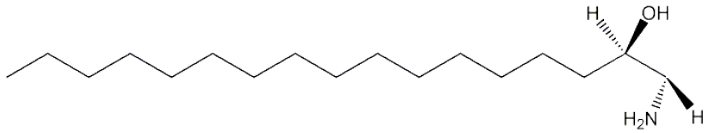
Glc, Gal, Lac, Sulfatides…>400; see

Novel sphingoid bases



1-Deoxysphinganine

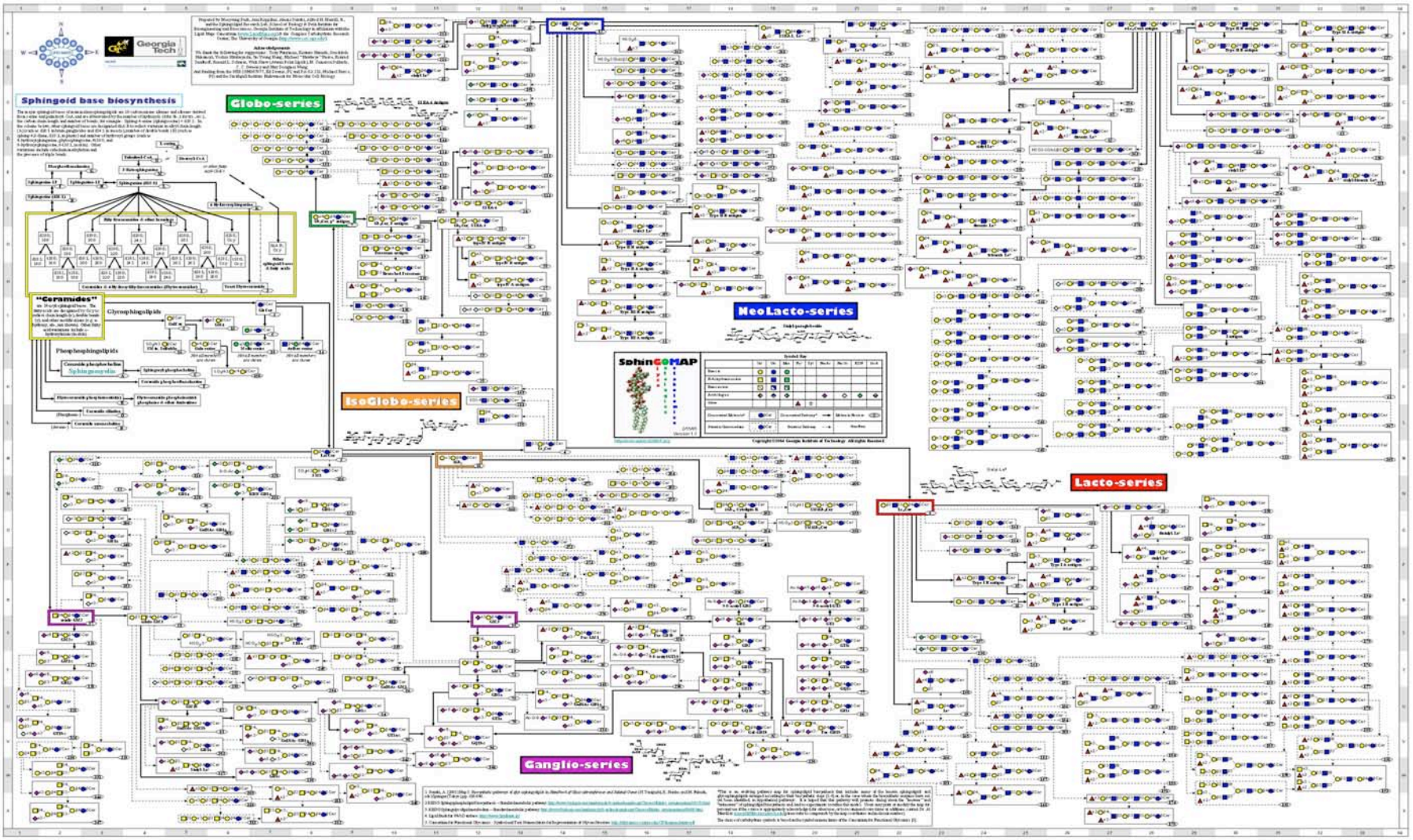


1-Desoxymethylsphinganine

Zitomer et al. *JBC* 284:4786-95 (2009)
Pruett et al. *J. Lipid Res*. 49:1621-39 (2008).

$II^3Neu5AcGg_4Cer$

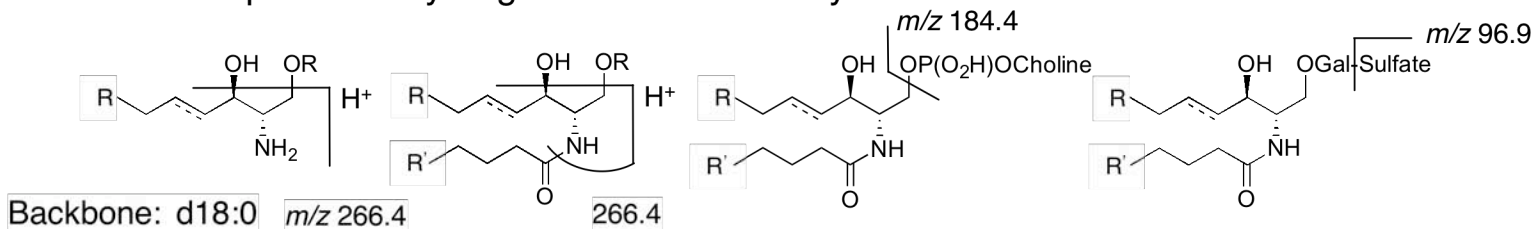
SphinGOMAP (www.sphingomap.org & Merrill et al., Trends Biochem Sci., 2007)



A. Extraction and liquid chromatography conditions

Extract fraction	Analytes	Column	Mode
Single Phase	So(1P), Sa(1P)	2.1 x 50 mm C18	(+)
Single Phase	Cer1P	2.1 x 50 mm C18 or 2.1 x 50 mm C8 or 2.1 x 50 mm LC-NH_2	(+) (+) (-)
Single Phase	Sulfatide	2.1 x 50 mm LC-NH_2	(-)
Add solvents, split: Upper (reextract, then discard)	Cer(P), HexCer, LacCer, SM	2.1 x 50 mm LC-NH_2	(+)
Add solvents, split: Lower (pooled)	GlcCer, GalCer	2.1 x 250 mm LC-Si	(+)

B. Mass spectrometry fragmentation summary

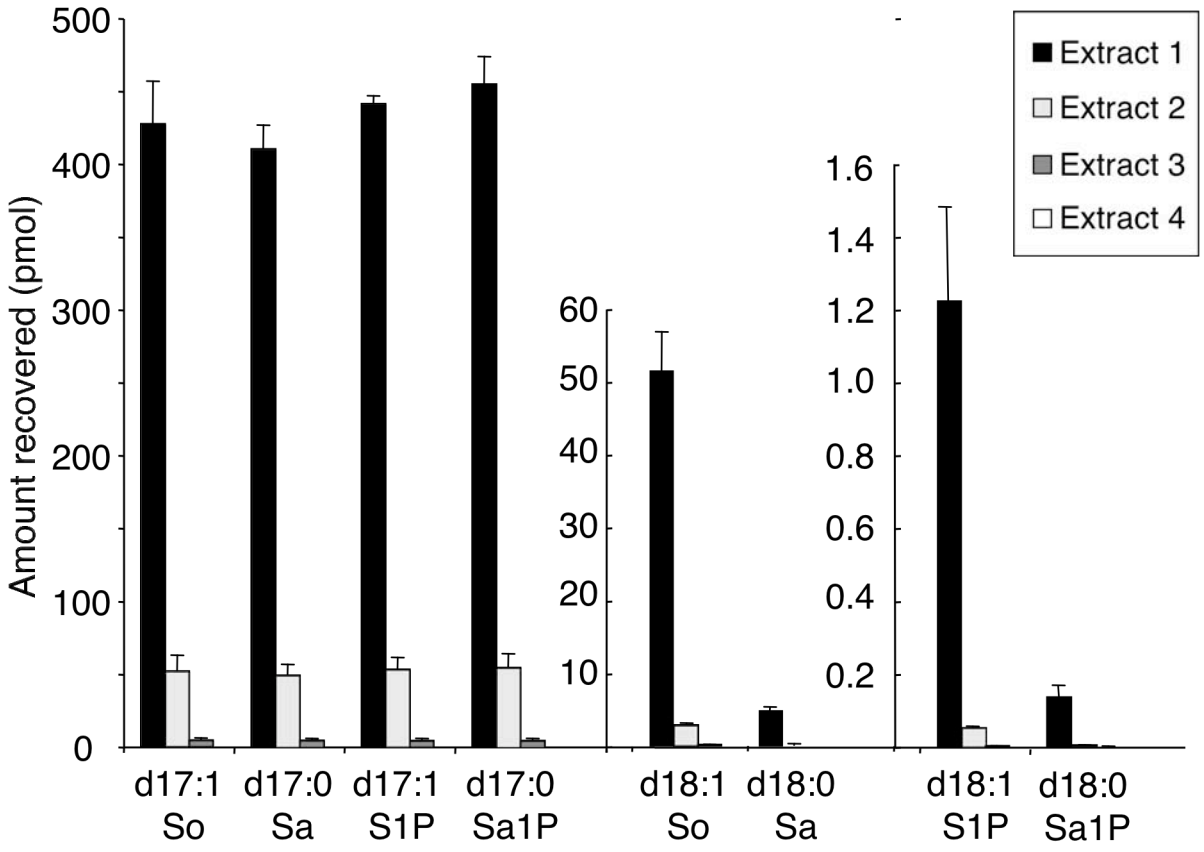


Backbone:		
d18:0	*m/z* 266.4	266.4
d18:1	*m/z* 264.4	264.4

Shaner et al. J. Lipid Res. (2009)

Validation of extraction recovery (Shaner et al., JLR 2009):

>95% recovered in Extract 1 and 2 (which are pooled for analysis)



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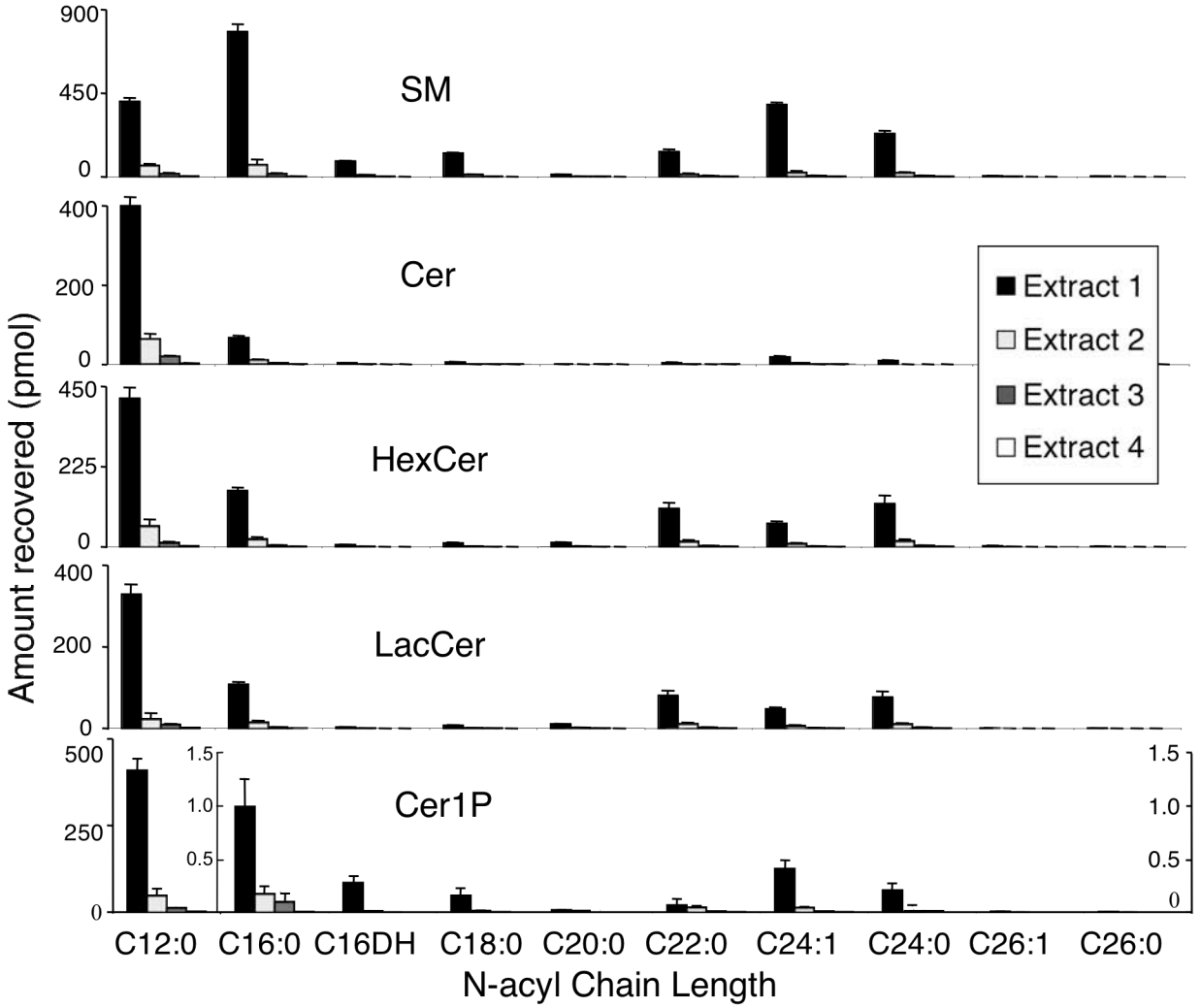


Sphingolipid analysis by LC-MS/MS

A (Polar, e.g. So, Sa, 4-HO-Sa, SoP, So-Pcholine, Psychosine, Cer-P, etc.)

B (Nonpolar, e.g. Cer, Cer-P, SM, GlcCer, GalCer, LacCer, etc.)

Normal phase HPLC

Reverse phase HPLC

Inlet System

Ion Source

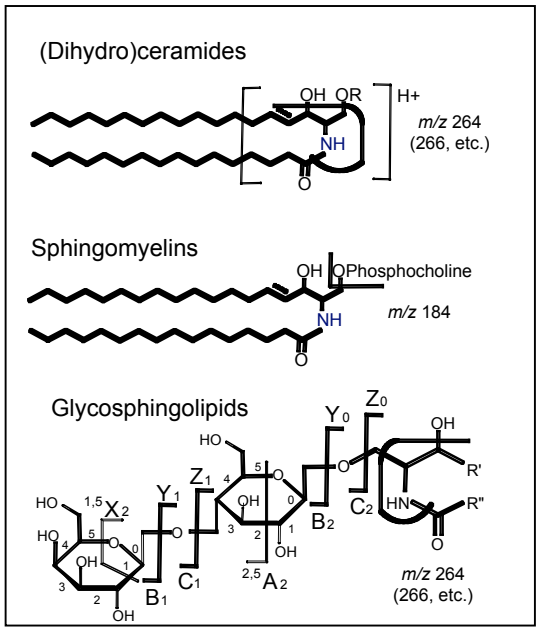
Mass Analyzer (Q1, Q2, Q3 or LIT)

Detector

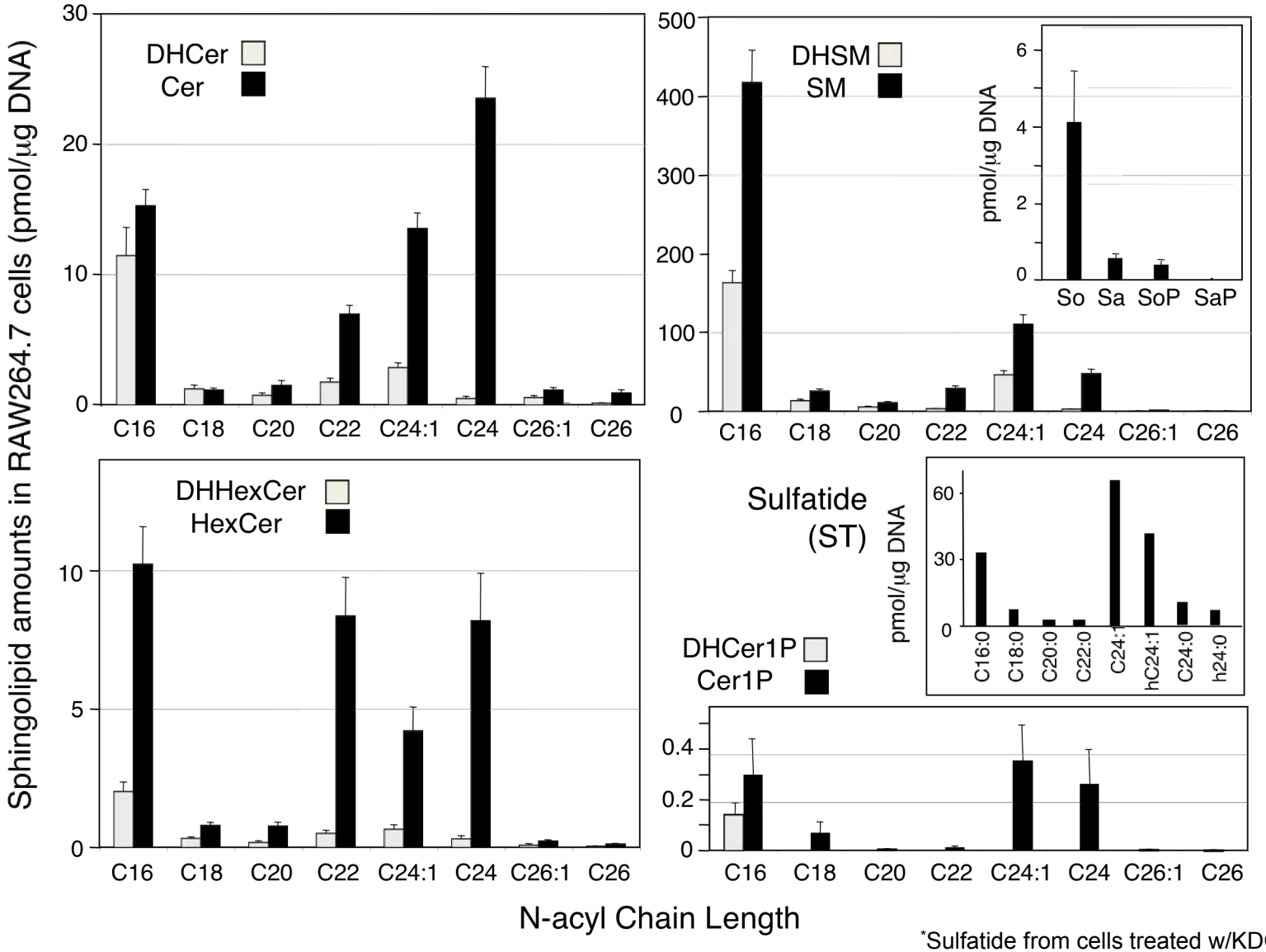
Data System for quantitation by MRM (Multiple Reaction Monitoring) w/ appropriate internal standards

Species analyzed to date (in most mammalian sphingoid base & FA variants)**:**

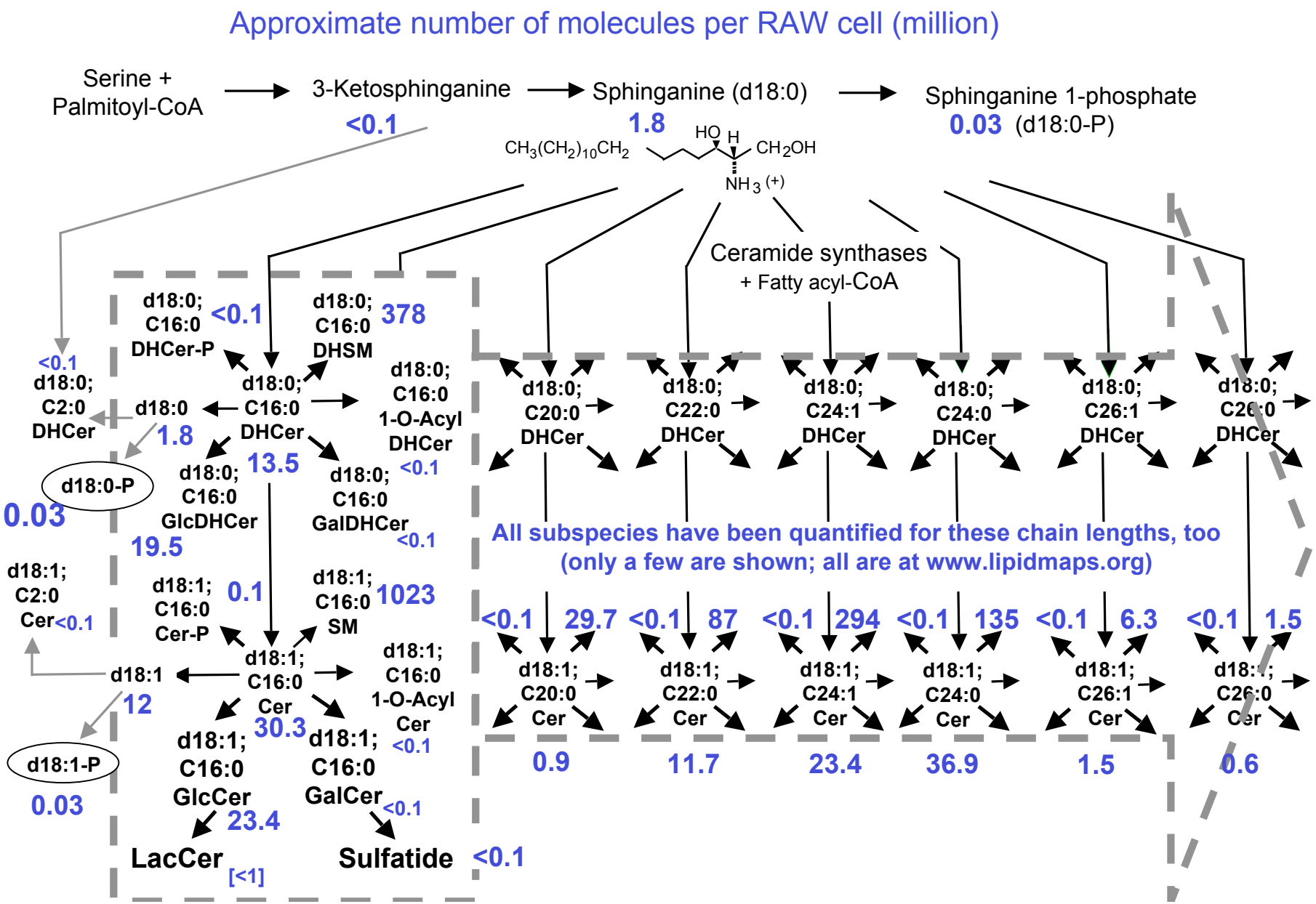
✓SM's, GlcCer, GalCer, LacCer, Sulfatide (*quanlitative* analysis of globosides and gangliosides)
✓Cer & Cer-P
✓So, Sa, 4-hydroxy-Sa, 1-DeoxySa, etc.
✓So-P, Sa-P and other derivatives (lysoSM, psychosine, N-methyl-)
✓Plus metabolites labeled with stable isotope precursors

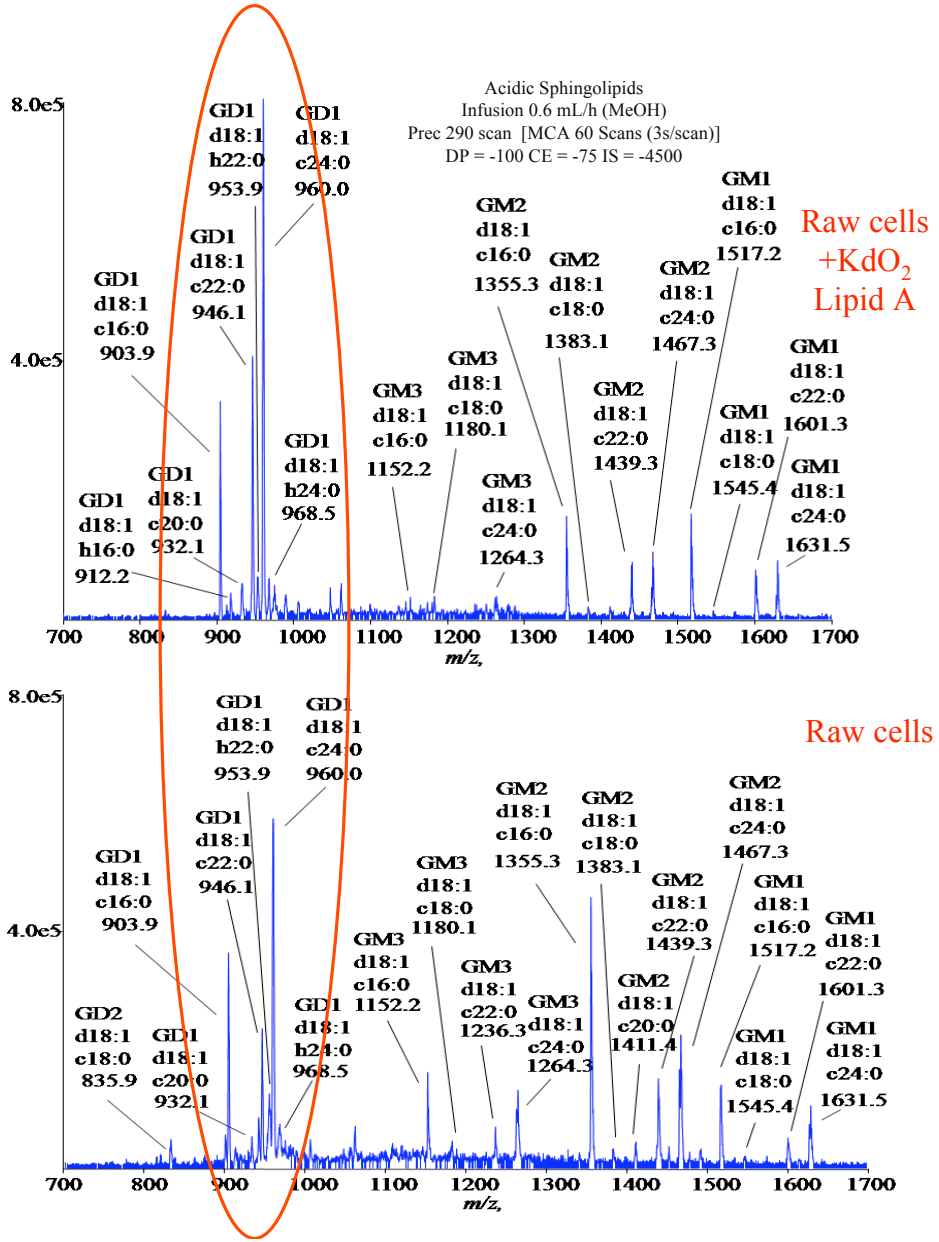


Sullards, Merrill & coworkers *STKE* (2001), *Methods* (2005), *Meth. Enzymol.* (2008) & Shaner et al. *JLR* (2009)

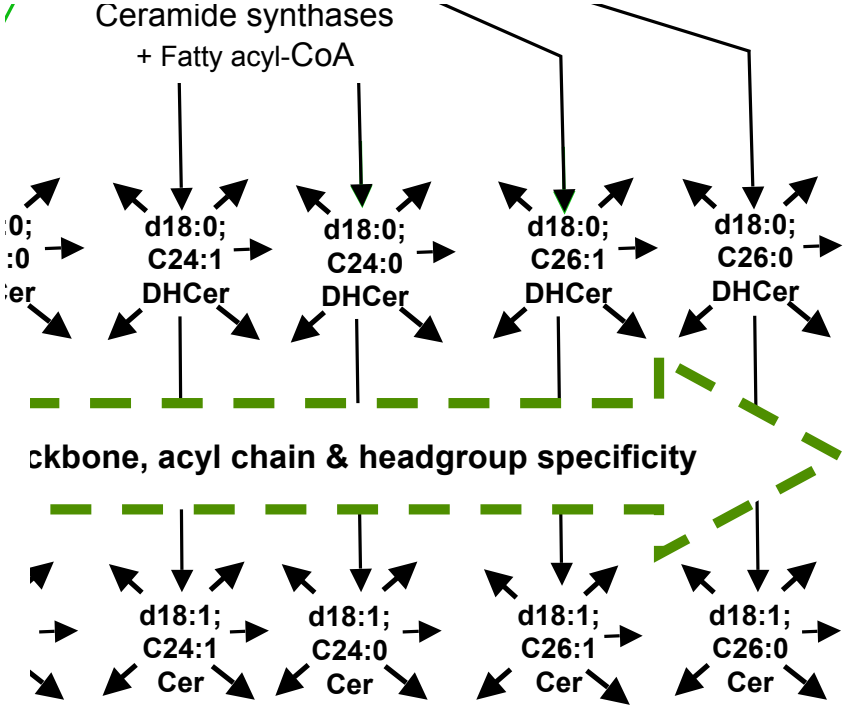


*Sulfatide from cells treated w/KDO_2 Lipid A


Approximate number of molecules per RAW cell (million)
Serine + Palmitoyl-CoA
3-Ketosphinganine
<0.1
Sphinganine (d18:0)
1.8
Sphinganine 1-phosphate
0.03 (d18:0-P)
Ceramide synthases + Fatty acyl-CoA
d18:0; C16:0 DHCer-P
<0.1
d18:0; C16:0 DHSM
378
d18:0; C2:0 DHCer
<0.1
d18:0
1.8
d18:0; C16:0 DHCer
13.5
d18:0; C16:0 1-O-Acyl DHCer
<0.1
d18:0-P
0.03
d18:0; C16:0 GlcDHCer
19.5
d18:0; C16:0 GalDHCer
<0.1
d18:0; C20:0 DHCer
d18:0; C22:0 DHCer
d18:0; C24:1 DHCer
d18:0; C24:0 DHCer
d18:0; C26:1 DHCer
d18:0; C26:0 DHCer
All subspecies have been quantified for these chain lengths, too
(only a few are shown; all are at www.lipidmaps.org)
d18:1; C2:0 Cer
<0.1
d18:1; C16:0 Cer-P
0.1
d18:1; C16:0 SM
1023
d18:1
12
d18:1; C16:0 Cer
30.3
d18:1; C16:0 1-O-Acyl Cer
<0.1
d18:1-P
0.03
d18:1; C16:0 GlcCer
23.4
d18:1; C16:0 GalCer
<0.1
LacCer
[<1]
Sulfatide
<0.1
<0.1 29.7 d18:1; C20:0 Cer
0.9
<0.1 87 d18:1; C22:0 Cer
11.7
<0.1 294 d18:1; C24:1 Cer
23.4
<0.1 135 d18:1; C24:0 Cer
36.9
<0.1 6.3 d18:1; C26:1 Cer
1.5
<0.1 1.5 d18:1; C26:0 Cer
0.6



Next category of compounds being analyzed by "Inside-out" sphingolipidomics: Gangliosides



→GM3 → GM2 → GM1 → GD1a

Work-flow for analysis of new samples using this LC-MS/MS Methodology

1. Identify structure specific dissociations unique to various classes (e.g., SM, GlcCer, GalCer, LacCer, etc.)

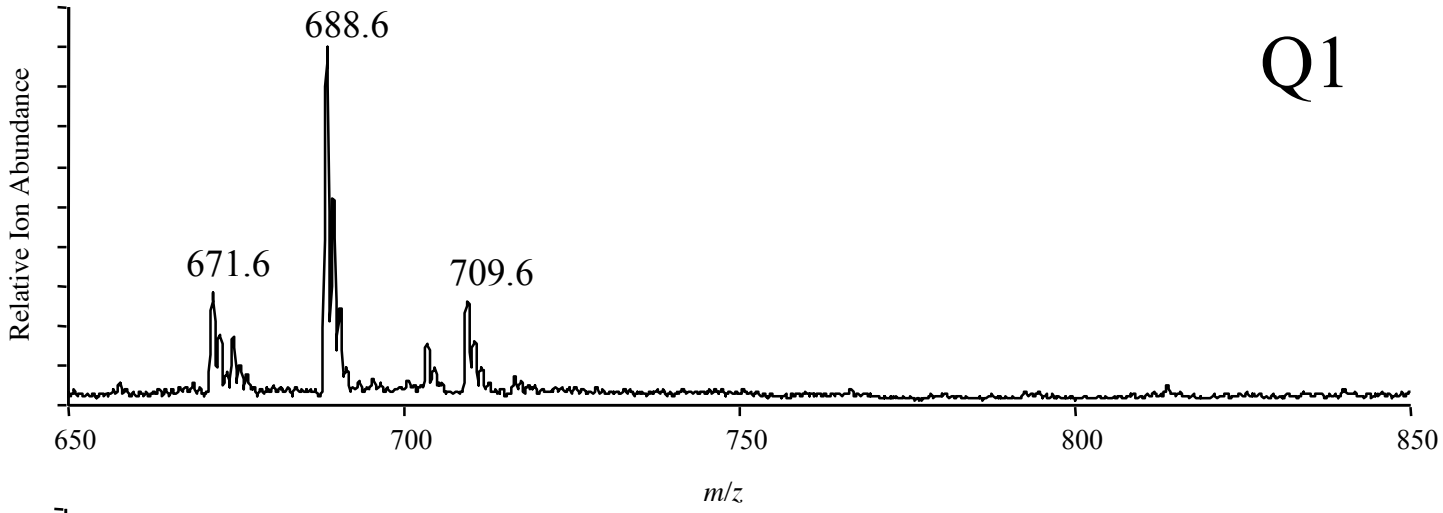
2. Utilize precursor ion and neutral loss scans to identify individual headgroup, sphingoid base, and fatty acid combinations.

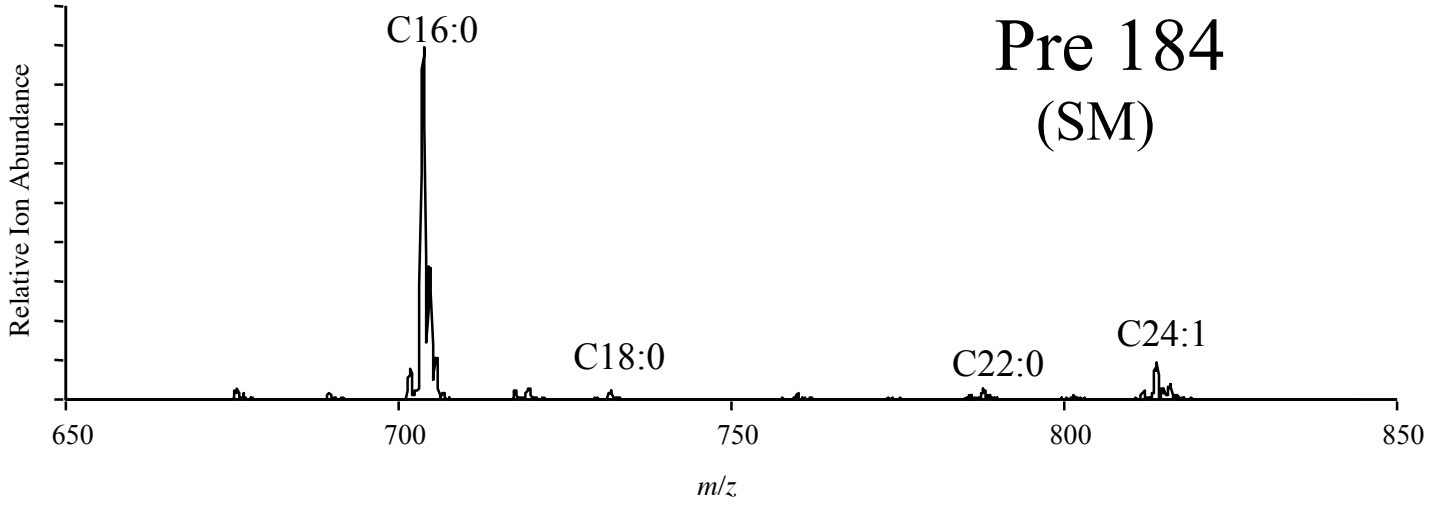
3. Optimize ionization and dissociation conditions for all species.

4. Optimize LC as required to minimize ionization suppression effects, and interferences arising from isobaric, isotopic, and isomeric species (repeat #3 if necessary).

5. Optimize conditions for quantitation via ratio of peak areas vs validated internal standards for all of the species present.

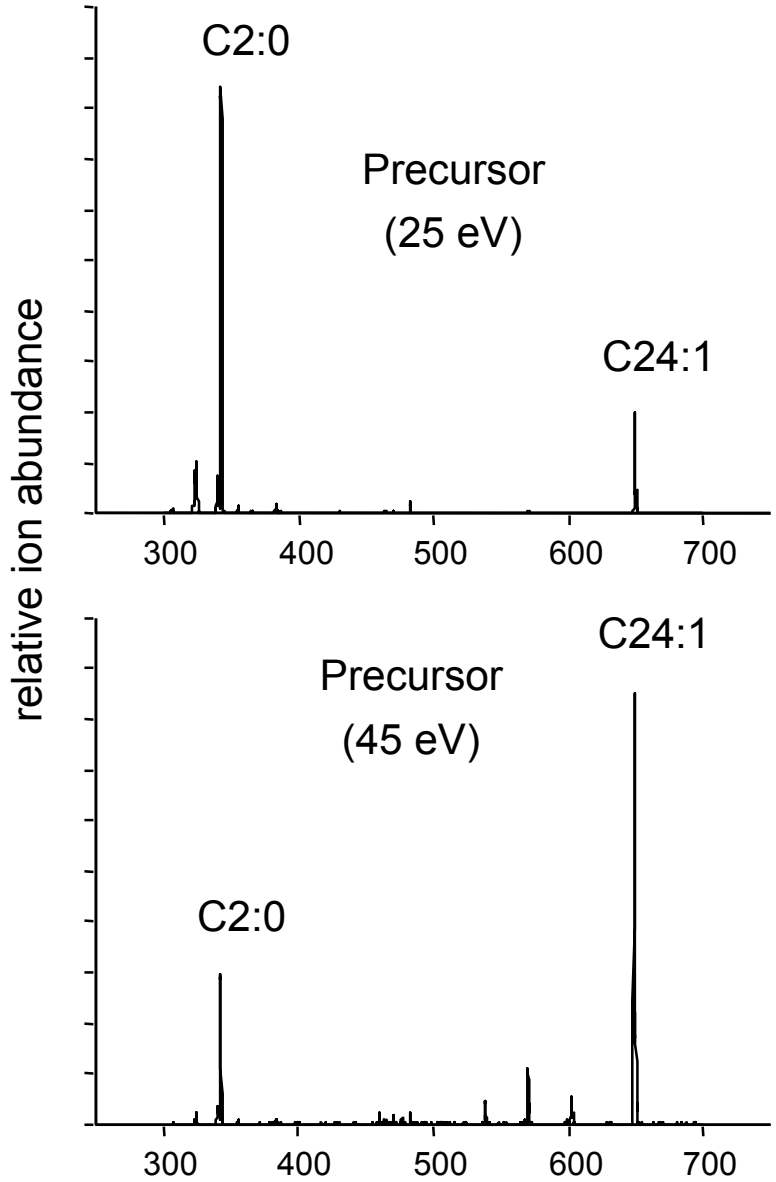
Example: Identification of sphingolipid subspecies via Neutral Loss or Precursor Ion Scans

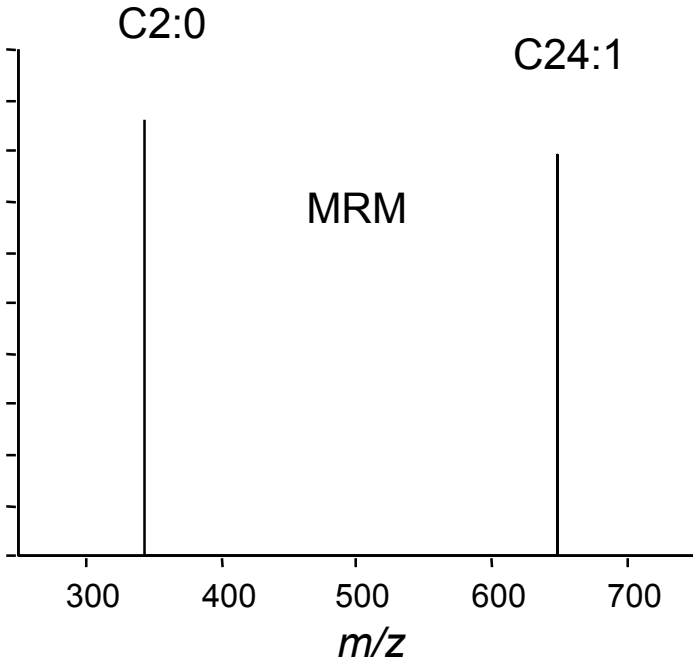




D. Quantitation of Sphingolipids

Comparison of ion abundance for ceramides of varying chain length when analyzed under single ionization and dissociation conditions vs optimized MRM





Criteria for selection of internal standards

1. Must have the same chemical and physical properties as the analyte of interest, ideally stable isotope labeled analogs.

2. Should be practical for “omic” analysis--i.e., cover as many subspecies as possible because adding an internal standard for every analyte would require 100s to 1000s of molecules to be synthesized, added and analyzed, which is too expensive, time consuming and possibly analytically impossible.

LIPID MAPS Sphingolipidomics cocktail (available from Avanti Polar Lipids): 10 uncommon sphingolipid species that are used to spike samples prior to extraction (Walt Shaw)

LIPID MAPS internal standard cocktail (cont.)

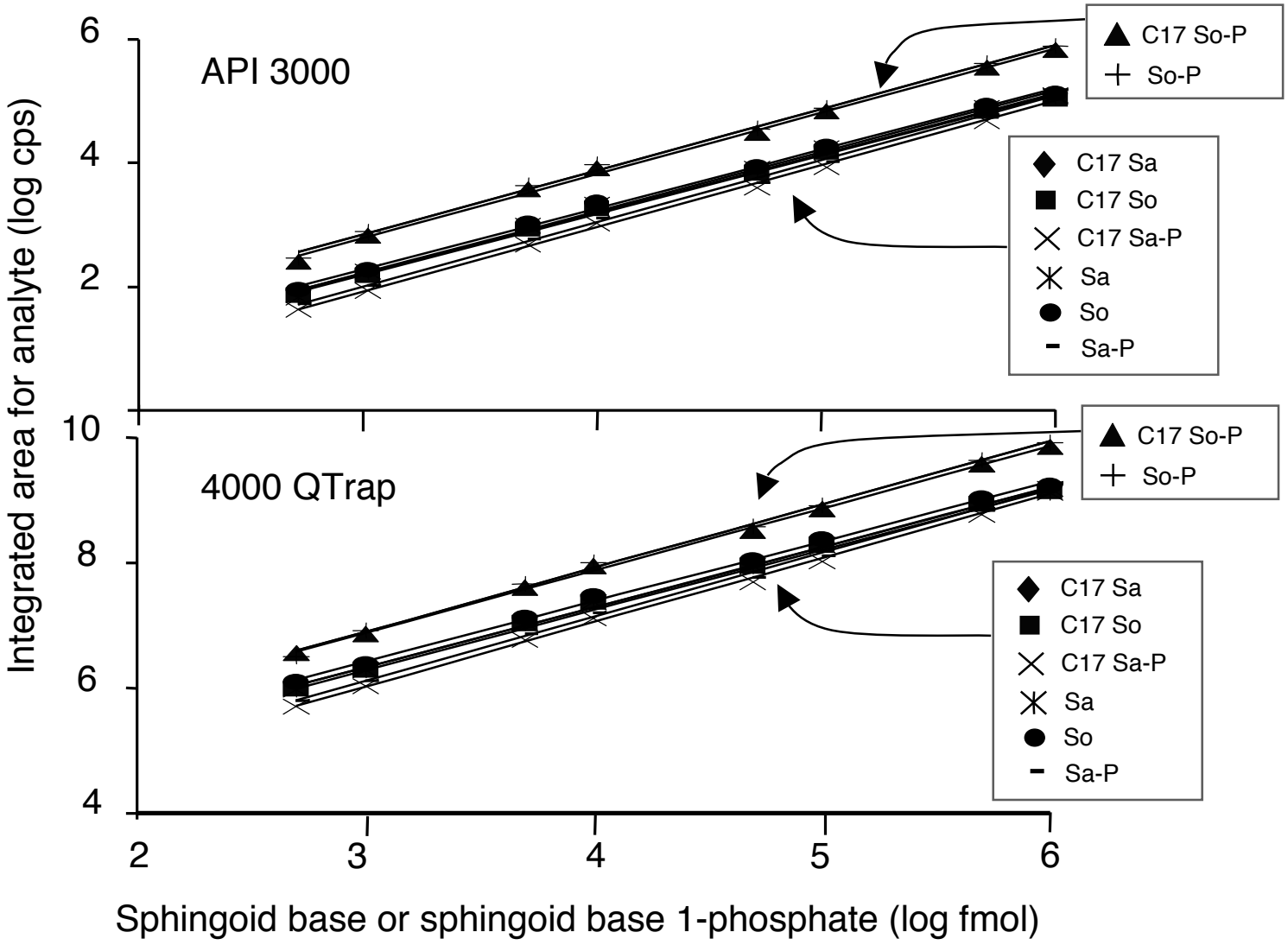
For sphingoid bases: odd chain length variants that elute under similar conditions so there is little ionization or dissociation effects and precursor and product ion masses are slightly shifted.

d17:1 “sphingosine” and “sphingosine 1-phosphate” homologs
d17:0 “sphinganine” and “sphinganine 1-phosphate” homologs

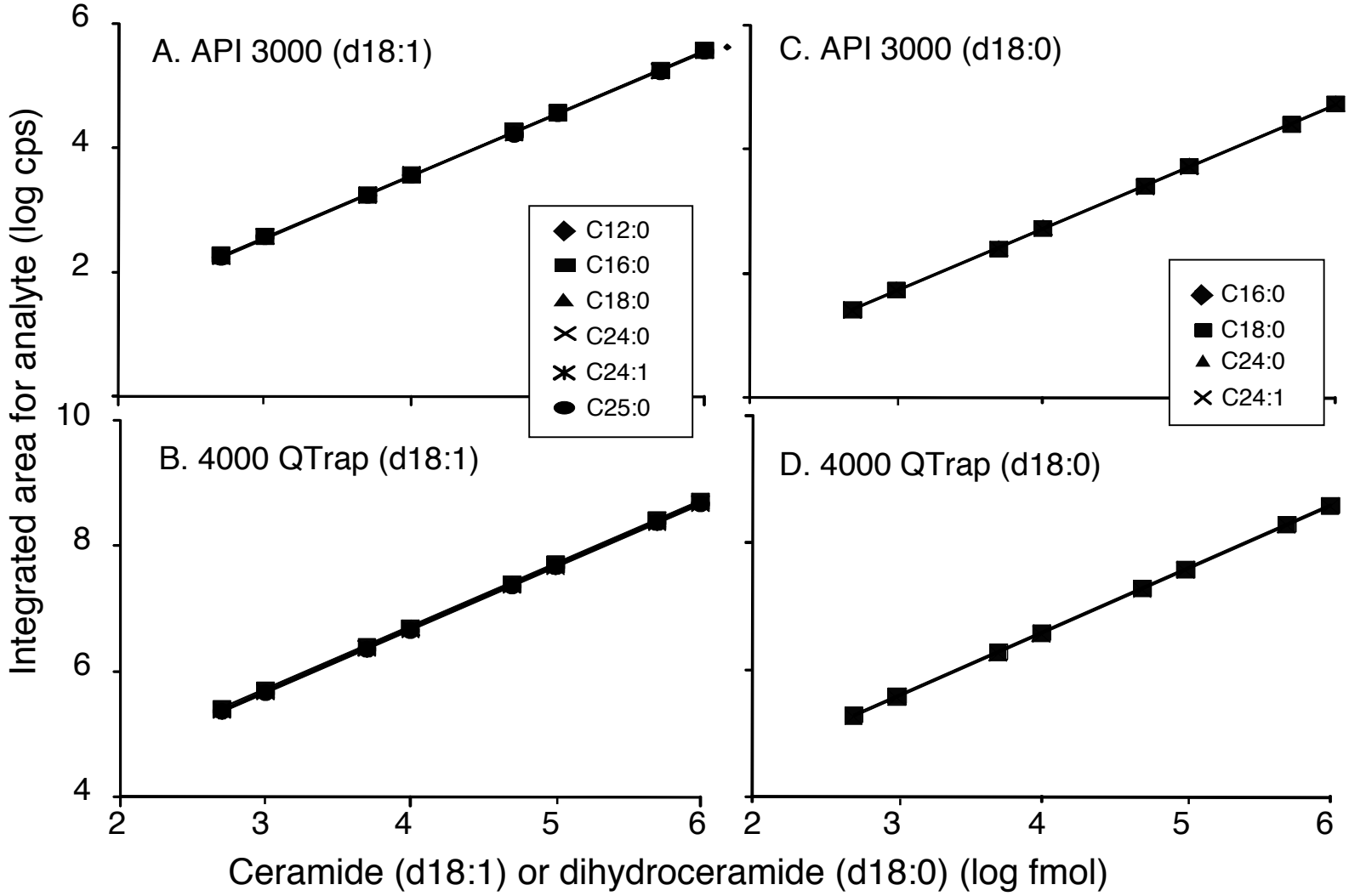
For complex sphingolipids: shorter fatty acid chain length variants (C12:0) that co-elute with analytes of interest so there are no ionization effects, and have different precursor ion masses but similar fragmentation when optimized.

C12-Cer, [C25-Cer], C12-Cer-1-P, C12-GlcCer, C12-LacCer, C12 SM

Also available:	C12-Sulfatide
Under development:	C12-GM1 and other complex glycosphingolipids
	C17:1 sphingosylphosphocholine; N-methyl-sphingoid bases
	1-deoxysphinganine



Shaner et al. JLR (2009)



Shaner et al. JLR (2009)

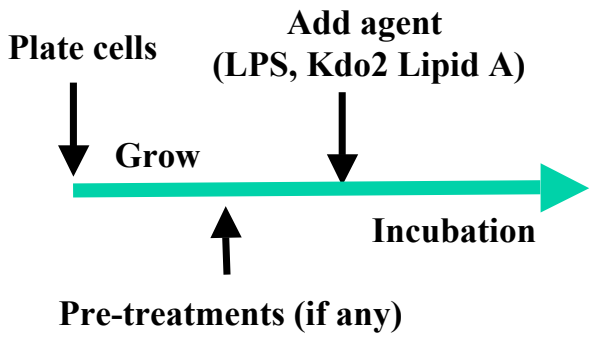
E. Data analysis/visualization:

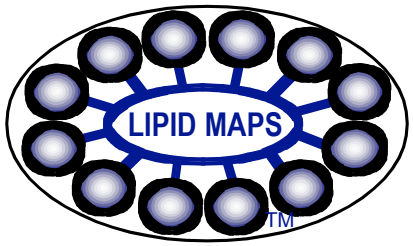
Example data from Lipid MAPS experiments:

Changes in Cer in RAW 267 cells treated with Kdo_2 Lipid A

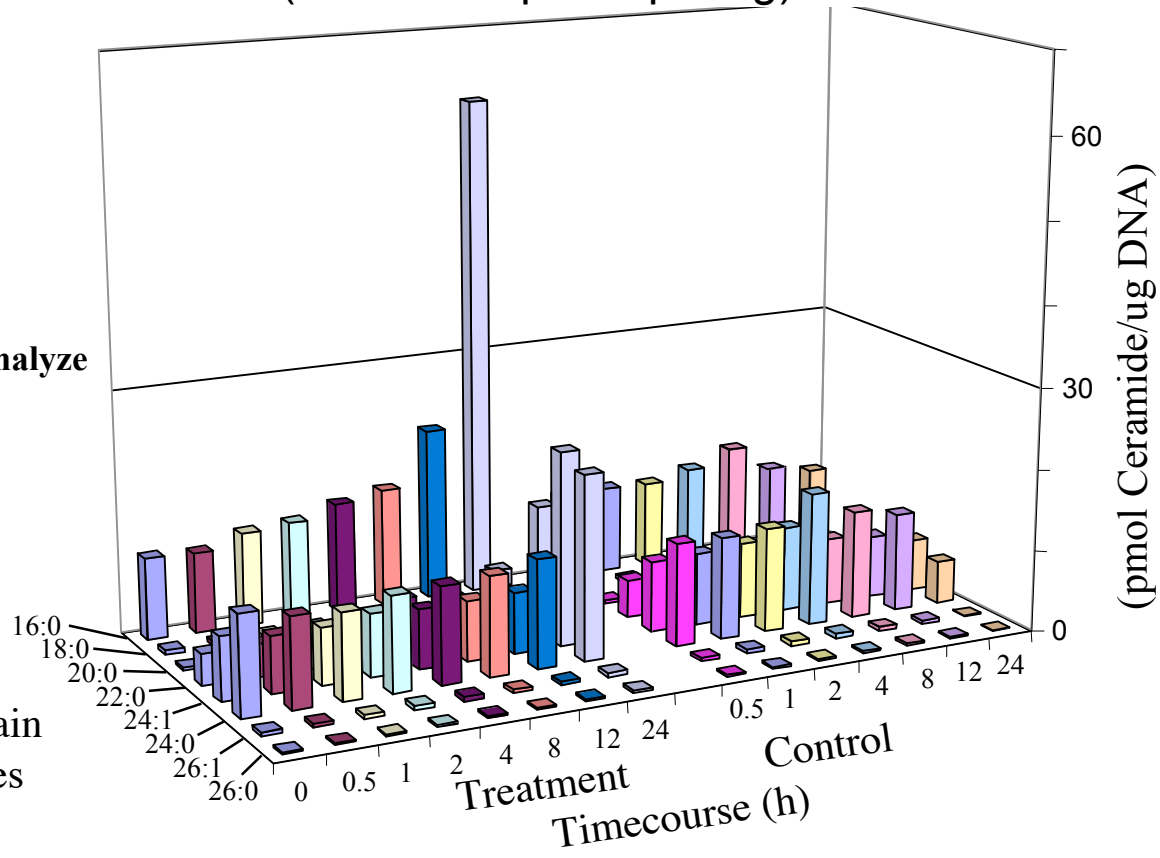
(see www.lipidmaps.org)

Basic LIPID MAPS Protocol





N-acyl chain subspecies

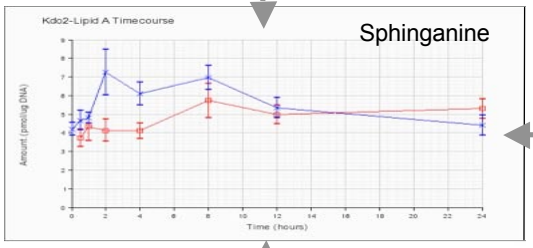


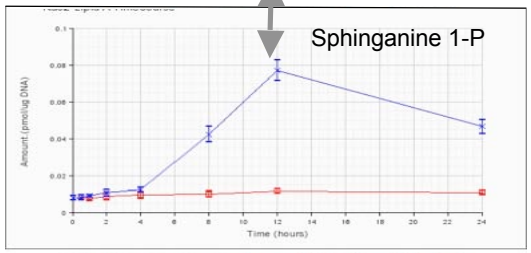
CORE I: Sphingolipids (only C16 shown)

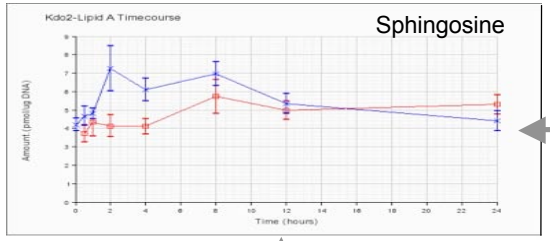
Biological Replicates +/- SE

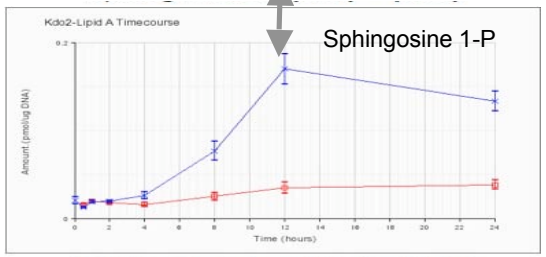
Serine + Palmitoyl-CoA

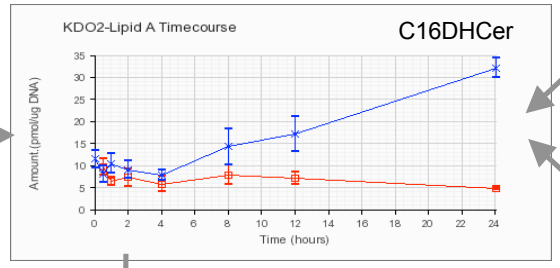
3-Ketosphinganine (below LOD)

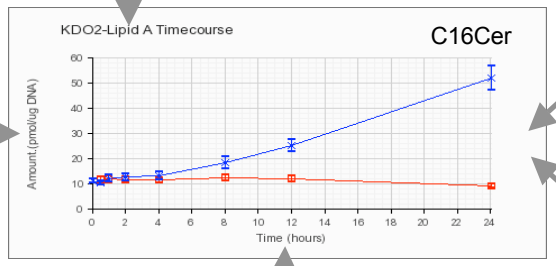


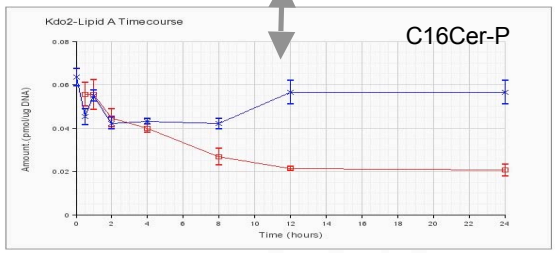




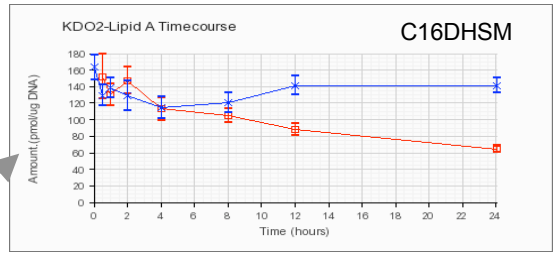


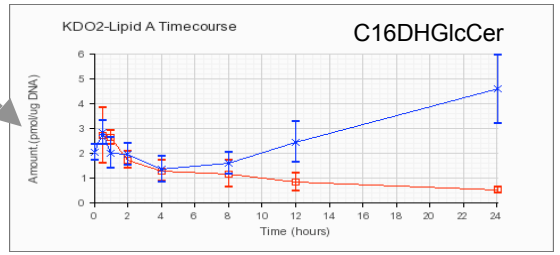


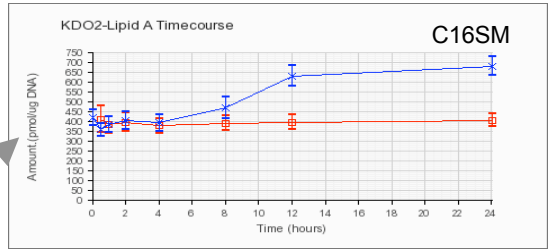


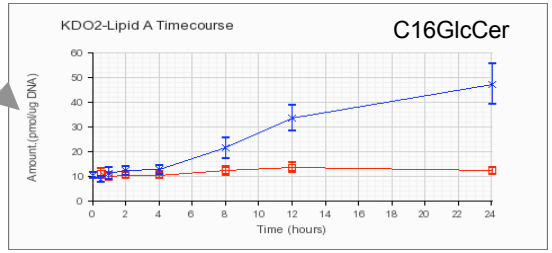


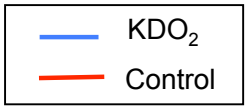
Data Sets Available at:
www.lipidmaps.org
(Eoin Fahy)











F. Remaining challenges (and opportunities)

Discovery of new subspecies (and new functions for known subspecies)--such as 1-deoxy-sphingoid bases

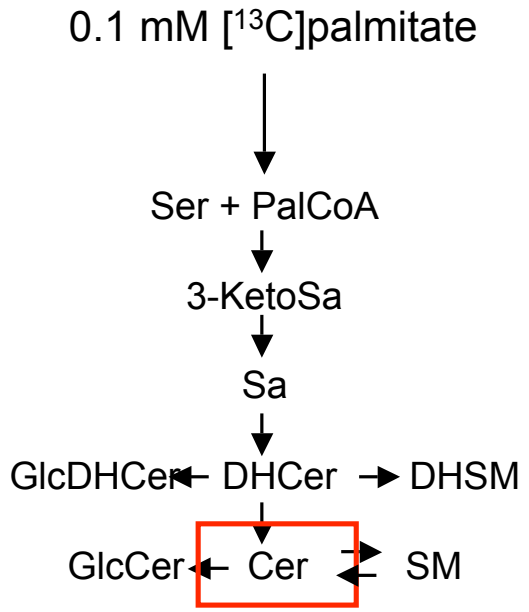
Obtain standards for glycosphingolipids (and new sphingolipids)

Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.

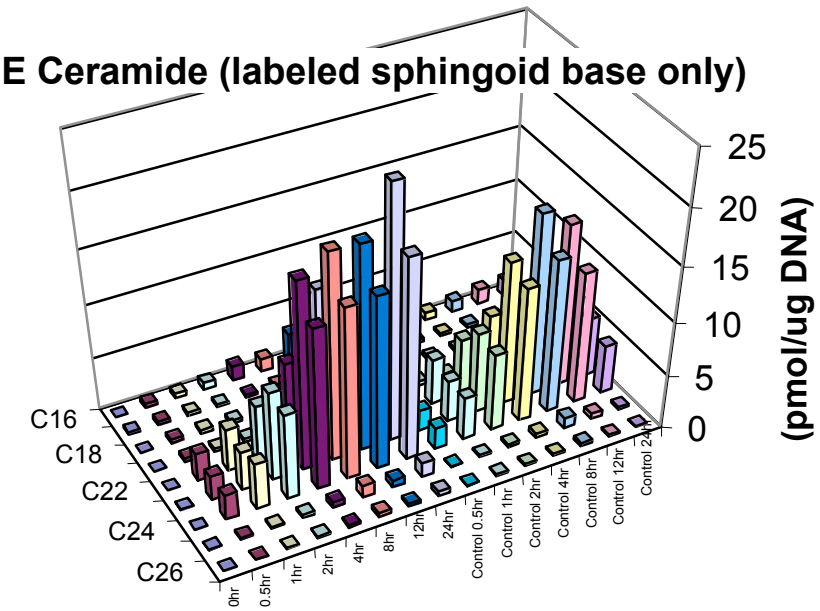
Develop methods to differentiate appearance/disappearance of particular subspecies via de novo biosynthesis vs turnover.

Analysis of sphingolipid biosynthesis using stable isotope labeled precursors

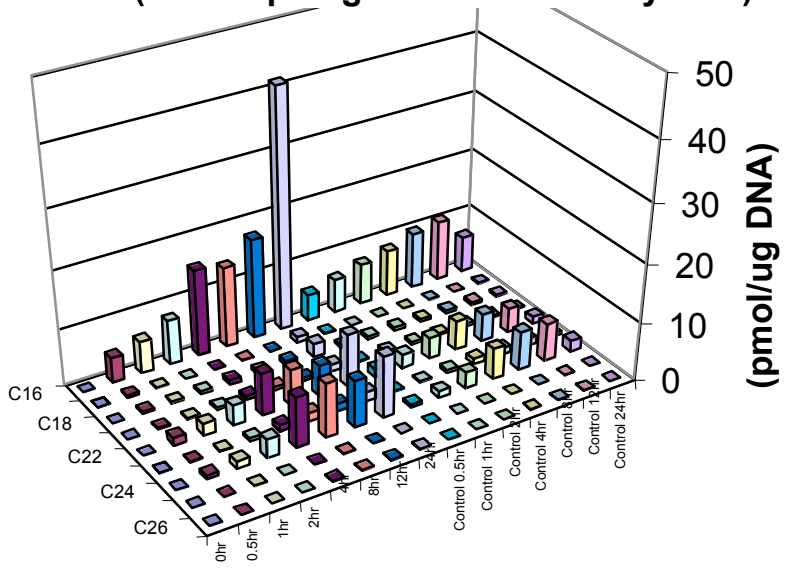
RAW 10% time course w/[^{13}C]palmitate



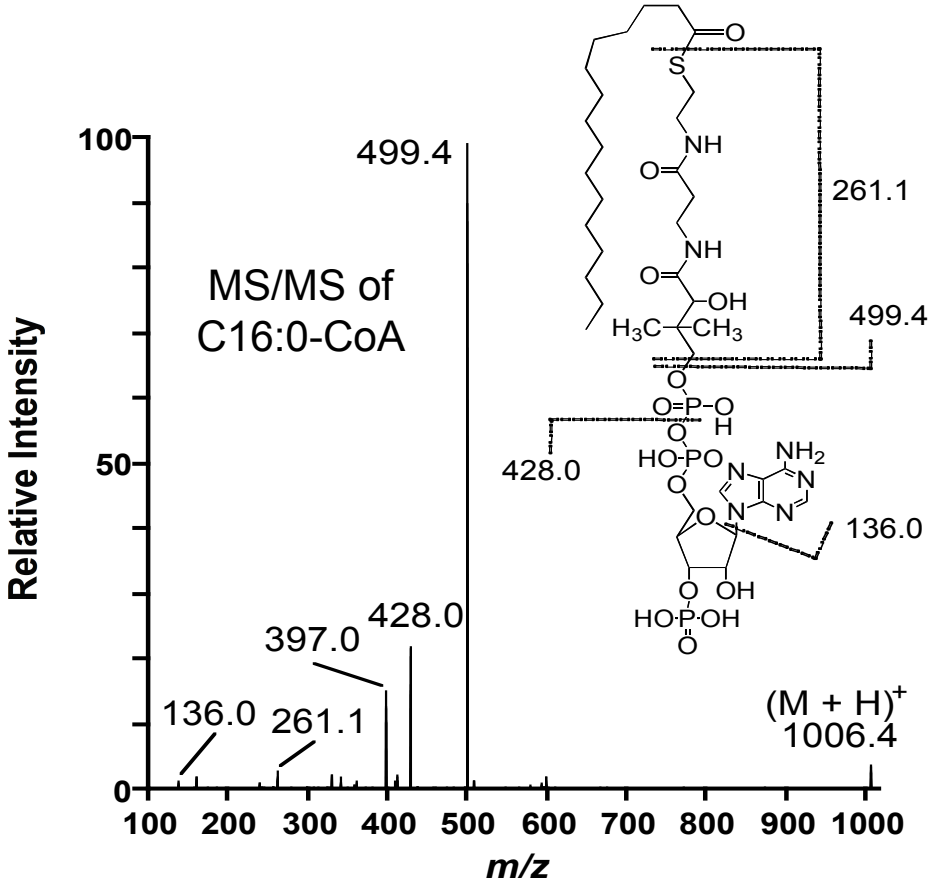
^{13}C BASE Ceramide (labeled sphingoid base only)



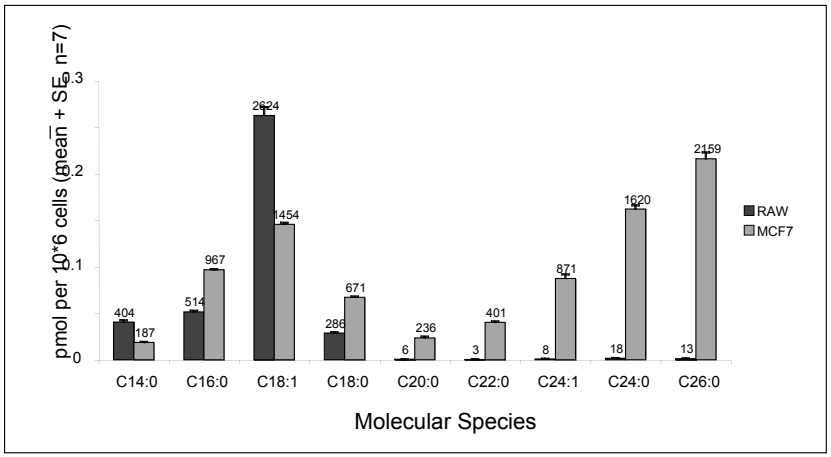
^{13}C DUAL Ceramide (^{13}C in sphingoid base and fatty acid)



To understand sphingolipid biosynthesis one must also know the availability of the co-substrate fatty acyl-CoA's.



Fatty acyl-CoA profile of RAW cells (and for comparison, MCF7 cells)



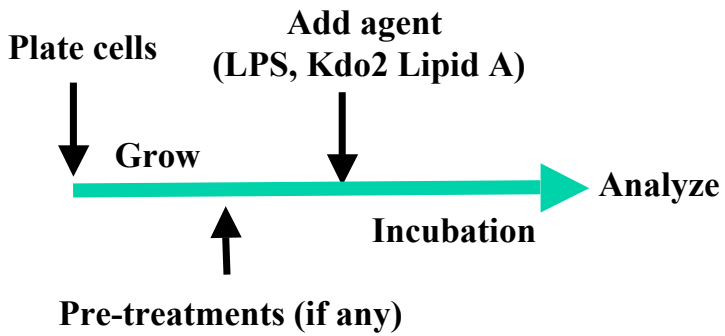
Haynes et al. J. Lipid Res. (2008)

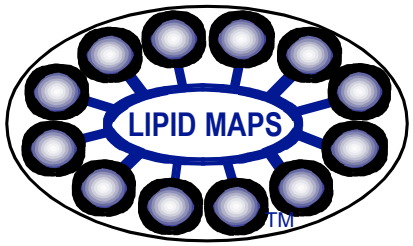
C16:0-CoA (10 pmol/μL in methanol 10 mM triethylammonium acetate) was infused at 5 μL/min. 4000QTrap parameters: IS = 5400 V, Gs1 = 12 psi, CUR = 10 psi, DP = 180 V, CE = 52 V, CXP = 14.3 V, CAD = Med

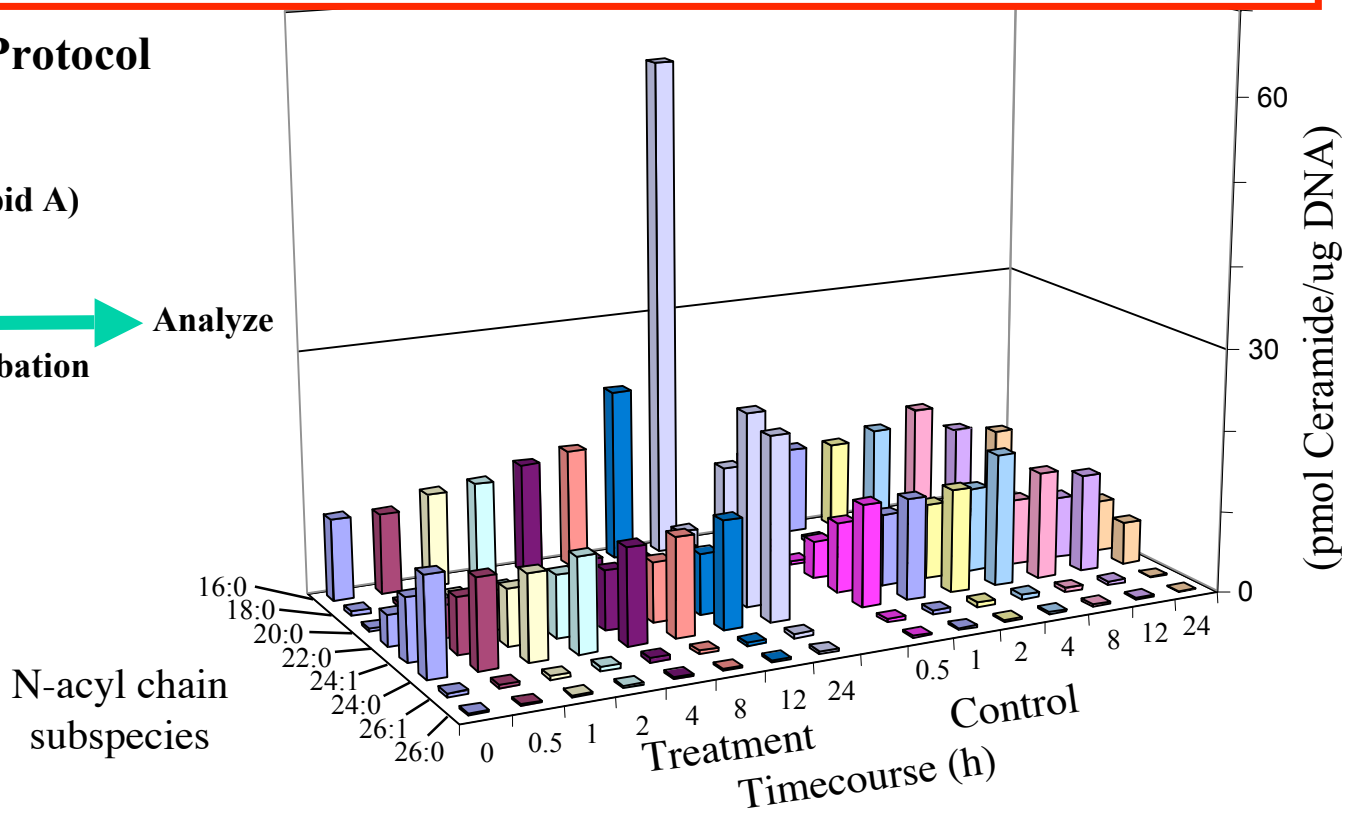
G. Examples of discoveries from sphingolipidomic analysis thus far

de novo sphingolipid biosynthesis is induced by Kdo_2 Lipid A (**has been correlated with gene array data** showing increases in SPT1 and SPT2 mRNA)

Basic LIPID MAPS Protocol

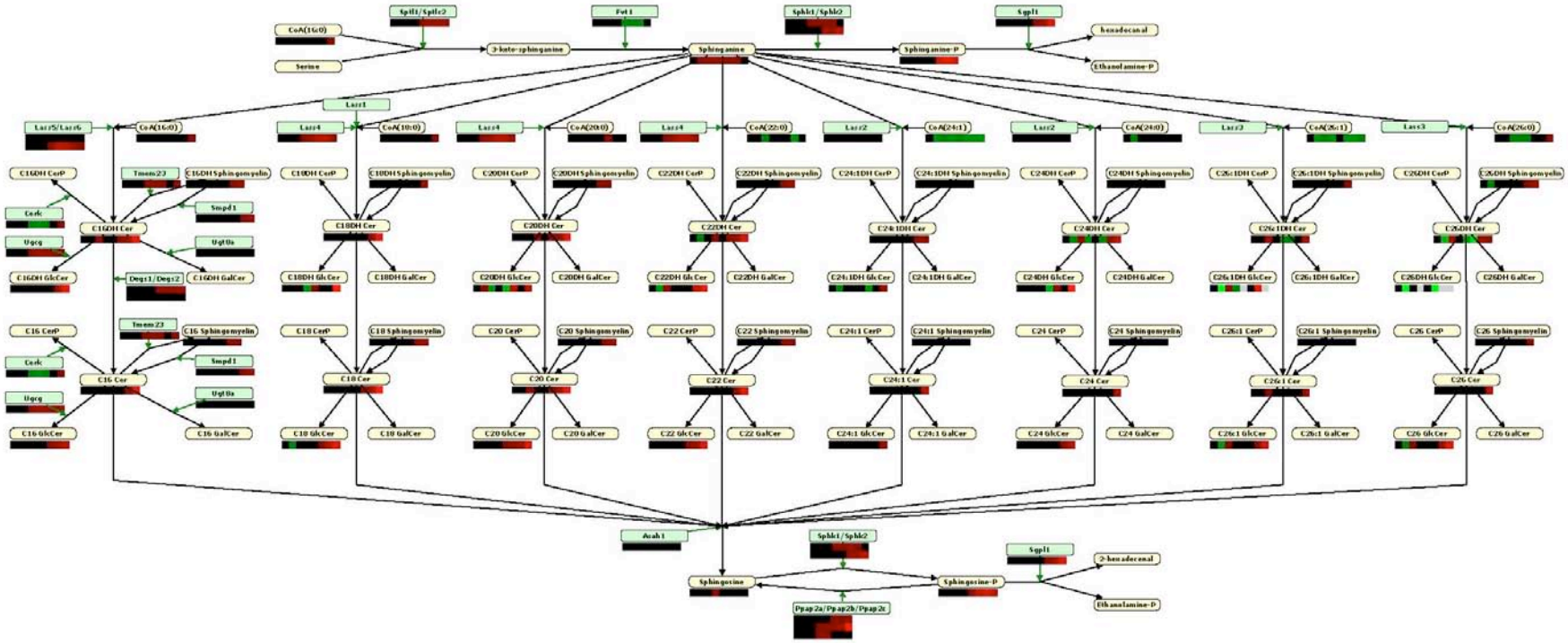






G. Examples of discoveries from sphingolipidomic analysis thus far

de novo sphingolipid biosynthesis is induced by Kdo_2 Lipid A **has been correlated with gene array data** and for other steps of the pathway, see www.lipidmaps.org)



G. Examples of additional (recent) discoveries from application of these sphingolipidomic methods

Ceramide synthase inhibition by fumonisin B1 causes accumulation of 1-deoxysphinganine: a novel category of bioactive 1-deoxysphingoid bases and 1-deoxydihydroceramides biosynthesized by mammalian cell lines and animals. (2009) Zitomer NC, Mitchell T, Voss KA, Bondy GS, Pruett ST, Garnier-Amblard EC, Liebeskind LS, Park H, Wang E, Sullards MC, Merrill AH Jr, Riley RT. J Biol Chem. 284(8):4786-95.

Acid Sphingomyelinase Deficiency Prevents Diet-induced Hepatic Triacylglycerol Accumulation and Hyperglycemia in Mice (2009) Deevska GM, Rozenova KA, Giltiay NV, Chambers MA, White J, Boyanovsky BB, Wei J, Daugherty A, Smart EJ, Reid MB, Merrill AH Jr, Nikolova-Karakashian M. J Biol Chem. 284(13):8359-68.

Human cytomegalovirus regulates bioactive sphingolipids (2008) Machesky NJ, Zhang G, Raghavan B, Zimmerman P, Kelly SL, Merrill AH Jr, Waldman WJ, Van Brocklyn JR, Trgovcich J. J Biol Chem. 283(38):26148-60.

Imaging MALDI mass spectrometry using an oscillating capillary nebulizer matrix coating system and its application to analysis of lipids in brain from a mouse model of Tay-Sachs/Sandhoff disease (2008) Chen Y, Allegood J, Liu Y, Wang E, Cachón-Gonzalez B, Cox TM, Merrill AH Jr, Sullards MC. Anal Chem. 80(8):2780-8.

H. Comparison of these methods with other sphingolipidomic techniques in the literature

“Infusion" techniques: Use the same precursor ion / neutral loss scans - Great for profiling, not quantitation, suffer from ionization suppression, isotopic, isobaric, and isomeric interferences especially without hydrolysis and extraction.

Nanospray ionization: Greatly improved sensitivity and reduced chemical noise: allows detection of low abundance species, detailed structural analyses on numerous species, chip-based systems can be coupled to LC, and fraction collection.

Ultra high resolution mass analysis: allows differentiation of isobaric / isotopic interferences and alternative fragmentation techniques.