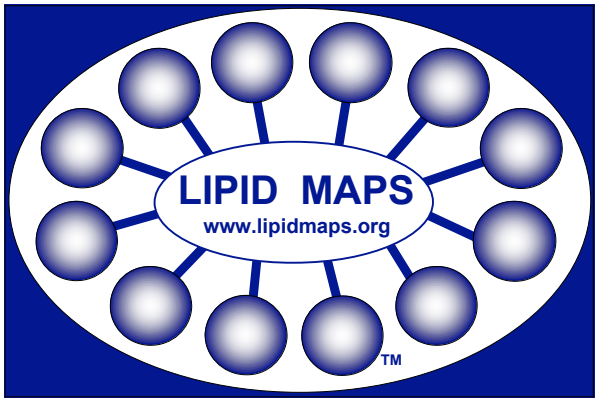
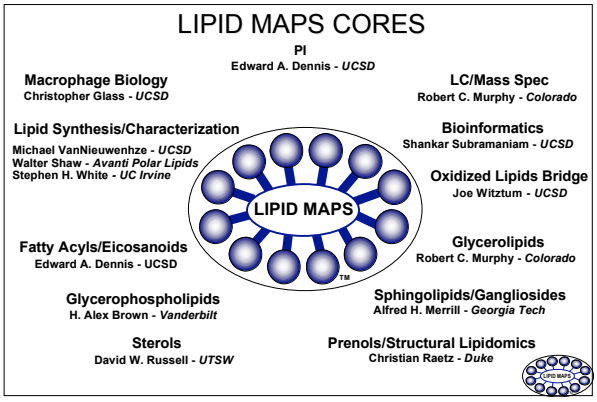


LIPID MAPS HYPOTHESIS

A systems biology approach to identify the many thousands of lipid molecular species in cells and their quantitative changes in response to cellular stress and challenge will lead us to new methodologies for monitoring lipid changes, and this in turn will lead to breakthroughs in the understanding and treatment of lipid based diseases.

LIPID MAPS CORES

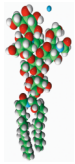


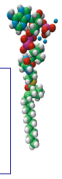
LIPID MAPS Lipidomics Workshop
April 28, 2007

www.lipidmaps.org

Lipid Analysis by Mass Spectrometry
Introduction and Challenges
Neutral and Phospholipids

Robert C. Murphy
Department of Pharmacology
University of Colorado
Health Sciences Center





Other LIPID MAPS Neutral Lipid Core members:
Robert Barkley
Miguel Gijon
Jessica Krank

Outline:

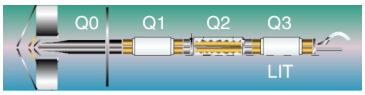
A. Electrospray Ionization of Neutral Lipids
B. NL- tandem mass spectrometry-TAGs /CE
C. Compound identification: Qualitative Analysis/Challenges/artifacts
D. Quantitation: Internal standards, etc.
E. Data analysis/visualization: Lipid Profiler
F. Phospholipids
G. LC/MS/MS Quantitation
H. Other strategies

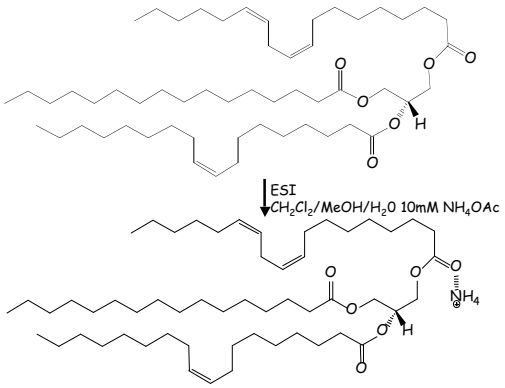
Neutral Lipids: Mass spectrometric Challenges

- Formation of gas phase ions
  - Desorption/Spray Ionization
    - Attachment of charging species
- Complex mixture – molecular species
  - Signal divided by total number of components
  - Hundreds of species

Mass Spectrometry

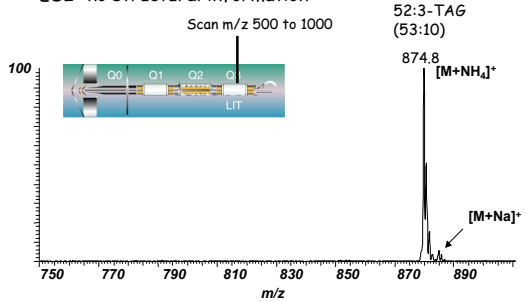
- Electrospray Ionization-Neutral Lipids
  - Tandem Mass spectrometry
    - Product ions
    - Precursor Ions
    - Neutral loss

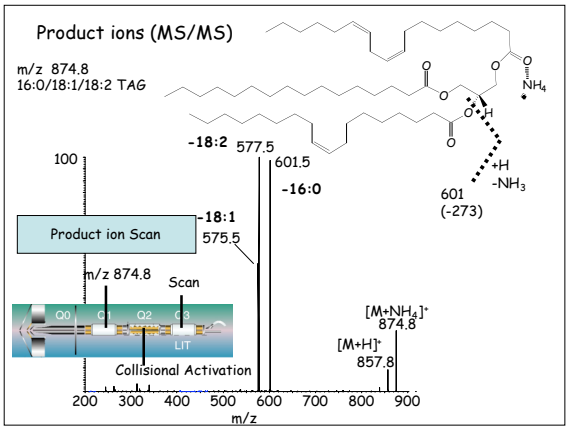


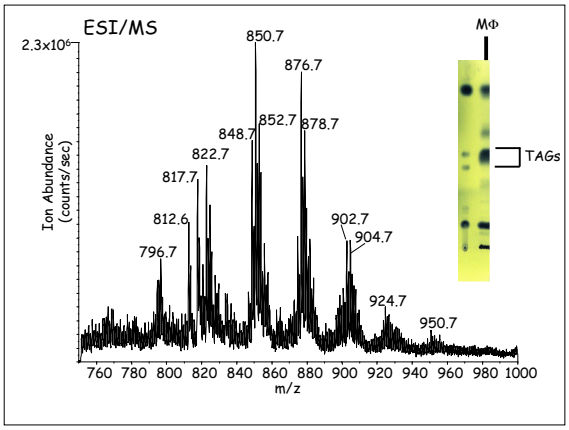


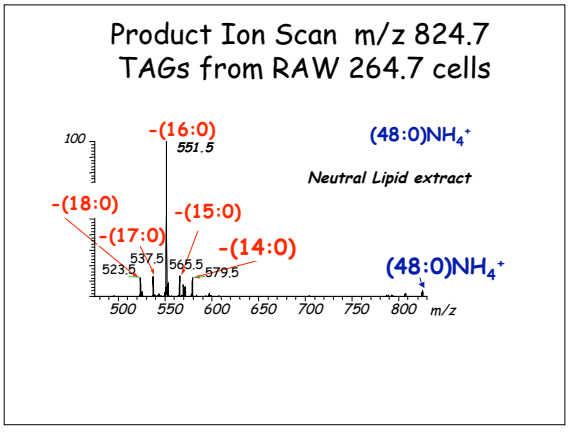
Challenge: Neutral Lipids

- ESI- no structural information




Product ions (MS/MS)
m/z 874.8
16:0/18:1/18:2 TAG
-18:2
577.5
601.5
-16:0
-18:1
575.5
Product ion Scan
m/z 874.8
Scan
Q0
Q1
Q2
Q3
LIT
Collisional Activation
[M+NH4]+
874.8
[M+H]+
857.8
601
(-273)
+H
-NH3
100
200 300 400 500 600 700 800 900
m/z


ESI/MS
2.3x10^6
Ion Abundance (counts/sec)
850.7
876.7
852.7
878.7
848.7
822.7
817.7
812.6
796.7
902.7
904.7
924.7
950.7
760 780 800 820 840 860 880 900 920 940 960 980 1000
m/z
MΦ
TAGs


Product Ion Scan m/z 824.7
TAGs from RAW 264.7 cells
100
-(16:0)
551.5
(48:0)NH4+
Neutral Lipid extract
-(18:0)
-(15:0)
-(17:0)
-(14:0)
523.5
537.5
565.5
579.5
(48:0)NH4+
500 550 600 650 700 750 800 m/z

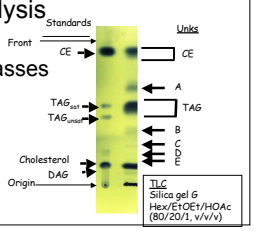
List of MS^3 identified TAGs From 6 different m/z values

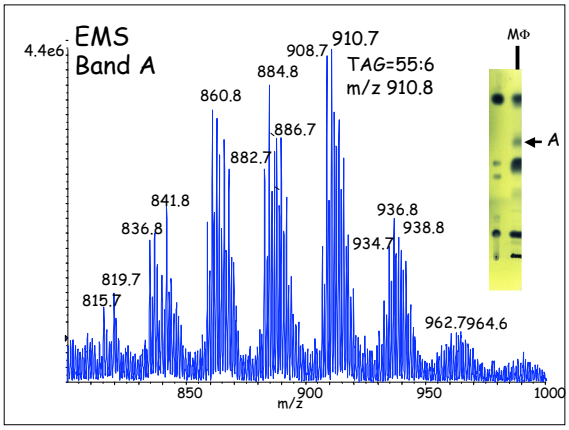
	m/z 824	*m/z 822*	*m/z 820*	*m/z 838*	*m/z 836*	*m/z 834*
	TG(48:0)	TG(48:1)	TG(48:2)	TG(49:0)	TG(49:1)	TG(49:2)
Major	16:0/16:0/16:0	16:0/16:0/16:1	16:0/16:1/16:1	16:0/16:0/17:0	15:0/16:0/18:1 16:0/16:1/17:0 16:0/16:0/17:1	15:0/16:1/18:1 16:0/16:1/17:1 16:1/16:1/17:0
Minor	14:0/16:0/18:0 15:0/16:0/17:0	14:0/16:0/18:1 14:0/16:1/18:0 15:0/16:1/17:0 15:0/16:0/17:1 15:0/15:0/18:1	14:0/16:1/18:1 14:0/16:0/18:2 15:0/16:1/17:1	15:0/16:0/18:0	14:0/17:0/18:1 15:0/16:1/18:0	15:1/16:0/18:1
Trace	15:0/15:0/18:0 12:0/18:0/18:0 14:0/17:0/17:0	14:0/17:0/17:1 14:1/17:0/17:0 15:1/16:0/17:0	12:0/18:1/18:1 14:0/17:1/17:1 14:1/16:0/18:1 15:0/15:0/18:2 15:0/15:1/18:1 15:1/16:0/17:1 14:1/17:0/17:1	12:0/17:0/18:0 13:0/18:0/18:0 14:0/16:0/19:0 15:0/15:0/19:0	13:0/16:0/20:1 14:0/16:0/19:1 14:0/17:1/18:0 14:1/17:0/18:0 15:0/15:0/19:1 15:1/16:0/18:0	13:0/16:0/20:2 14:0/15:0/20:2 14:0/17:1/18:1 14:0/17:0/18:2 14:1/17:0/18:1 15:0/16:0/18:2 15:0/17:1/17:1 15:1/17:0/17:1

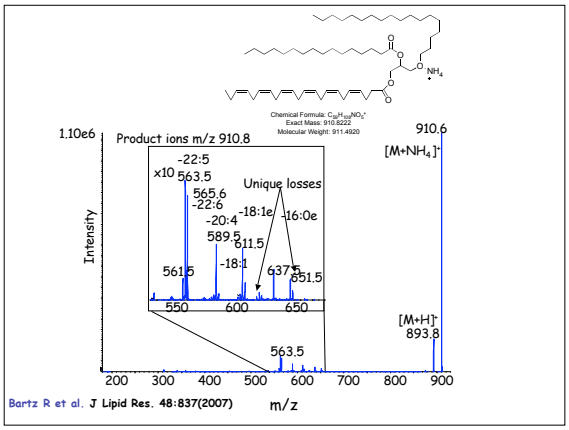
MS^3 data allowed for the unequivocal identification of 55 TAGs

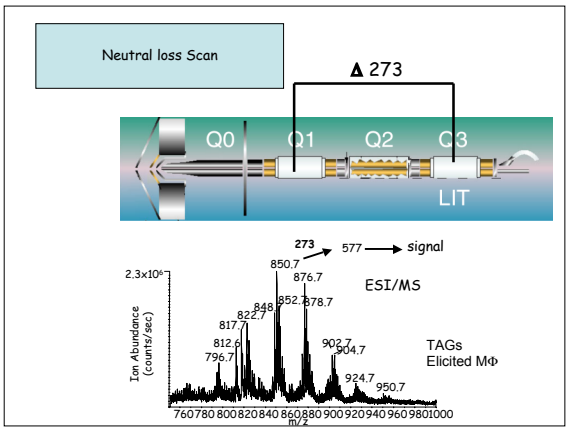
Crude Mixture or Purification of Lipids by Class

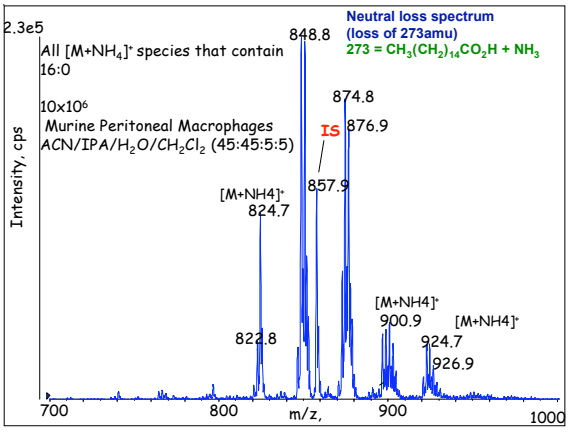
- Shotgun analysis
  - Use the power of MS to separate species
  - Unique product ions after collisional activation
- Separation prior to analysis
  - LC/MS and LC/MS/MS
  - Off line separation of classes
    - TLC (NP)
    - HPLC (NP and/or RP)
    - SPE

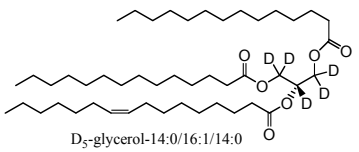





Chemical Formula: $C_{55}H_{102}NO_6^+$
Exact Mass: 910.8222
Molecular Weight: 911.4920
1.10e6
Product ions m/z 910.8
910.6
[M+NH4]+
x10
-22:5
563.5
565.6
-22:6
Unique losses
-20:4
-18:1e
-16:0e
589.5
611.5
-18:1
561.5
637.5
651.5
550
600
650
[M+H]+
893.8
563.5
Intensity
200
300
400
500
600
700
800
900
m/z
Bartz R et al. J Lipid Res. 48:837(2007)


Neutral loss Scan
Δ 273
Q0
Q1
Q2
Q3
LIT
273
577
signal
ESI/MS
2.3x10⁶
Ion Abundance (counts/sec)
850.7
876.7
852.7
878.7
848
822.7
817.7
812.6
796.7
902.7
904.7
924.7
950.7
TAGs
Elicited MΦ
760 780 800 820 840 860 880 900 920 940 960 980 1000
m/z


2.3e5
All [M+NH4]+ species that contain 16:0
10x10⁶
Murine Peritoneal Macrophages
ACN/IPA/H2O/CH2Cl2 (45:45:5:5)
Neutral loss spectrum
(loss of 273amu)
273 = CH3(CH2)14CO2H + NH3
848.8
874.8
IS
876.9
857.9
[M+NH4]+
824.7
[M+NH4]+
900.9
[M+NH4]+
924.7
822.8
926.9
Intensity, cps
700
800
m/z,
900
1000



D_5-glycerol-14:0/16:1/14:0

Avanti Polar Lipids

DAGs (7)

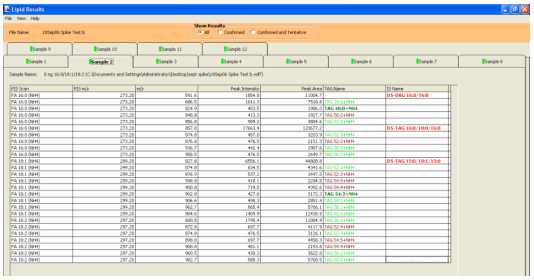
sn-1	sn-2	sn-3	M+NH4]+
14:0	OH	19:0	675.6
15:0	OH	14:0	535.5
16:0	OH	15:0	563.5
17:0	OH	16:0	591.5
20:4	OH	17:0	619.6
20:0	OH	20:4	687.5
		20:0	703.6

TAGs (9)

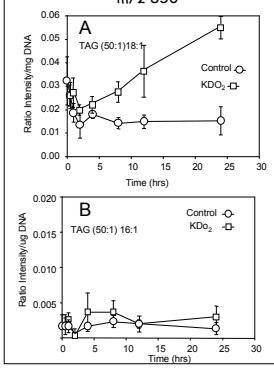
18- Fatty acyl groups

sn-1	sn-2	sn-3	M+NH4]+
19:0	12:0	19:0	857.8
14:0	16:1	14:0	771.7
15:0	18:1	15:0	827.8
16:0	18:0	16:0	857.8
17:0	17:1	17:0	869.8
20:4	18:2	20:4	949.7
20:0	20:1	20:0	995.9
20:2	18:3	20:2	955.8
20:5	22:6	20:5	993.7

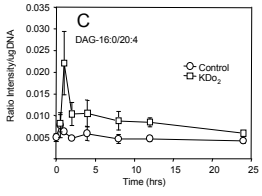
Lipid Profiler

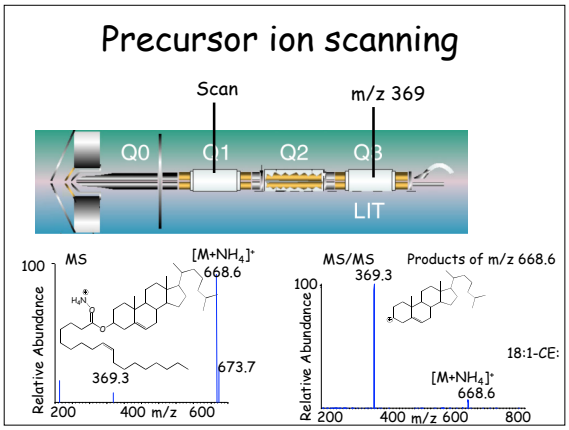


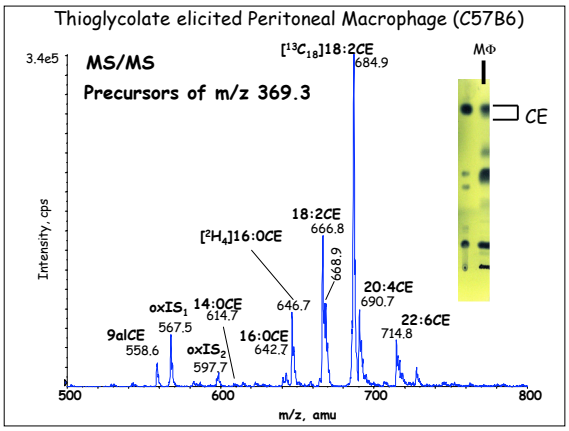
m/z 850

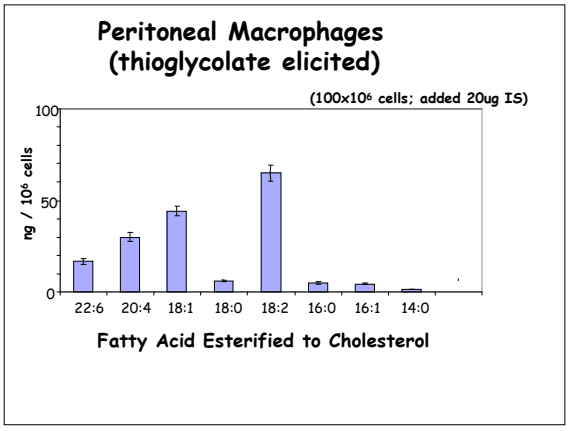


RAW 264.7 Cells
Stimulated with KDO_2-Lipid A




Precursor ion scanning
Scan
m/z 369
Q0
Q1
Q2
Q3
LIT
MS
[M+NH4]+
668.6
673.7
369.3
Relative Abundance
100
200
400 m/z 600
MS/MS
369.3
Products of m/z 668.6
18:1-CE:
[M+NH4]+
668.6
800


Thioglycolate elicited Peritoneal Macrophage (C57B6)
3.4e5
MS/MS
Precursors of m/z 369.3
[13C18]18:2CE
684.9
MΦ
CE
18:2CE
666.8
[2H4]16:0CE
668.9
20:4CE
690.7
646.7
oxIS1
14:0CE
614.7
567.5
9alCE
558.6
16:0CE
642.7
oxIS2
597.7
22:6CE
714.8
Intensity, cps
500
600
700
800
m/z, amu

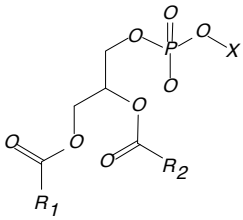

Peritoneal Macrophages
(thioglycolate elicited)
(100x10⁶ cells; added 20ug IS)
ng / 10⁶ cells
100
50
0
22:6
20:4
18:1
18:0
18:2
16:0
16:1
14:0
Fatty Acid Esterified to Cholesterol

Phospholipids

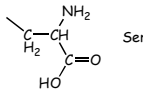
- Classes
- Quantitative and Qualitative analysis
  - Positive/negative ions
  - Isotope corrections
  - Quantity indicating ions and standard curves
- Chromatographic separation
  - Normal phase (LC)
  - Reverse phase(LC)
- Artifacts/challenges

Glycerophospholipids

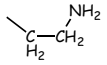
X (Polar Head Group)



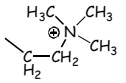
1,2-diacyl



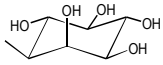
Serine (GPSer)



Ethanolamine(GPEtn)

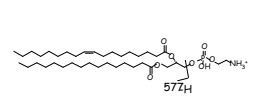


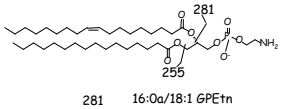
Choline (GPCho)



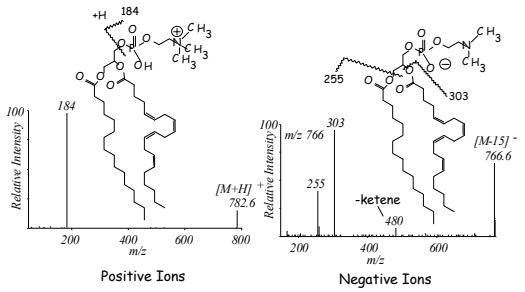
Inositol (GPIns)

CID 16:0a/18:1-GPEtn
Positive and negative product ions



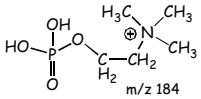


ESI-MS/MS (tandem quadrupole)



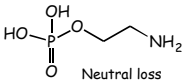
Diagnostic Product Ions from Positive $[M+H]^+$ Glycerophospholipids (CID)

GPCho



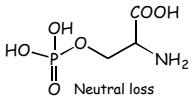
m/z 184

GPEtn



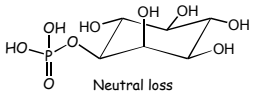
Neutral loss 141 u

GPSer



Neutral loss 185 u

GPIns

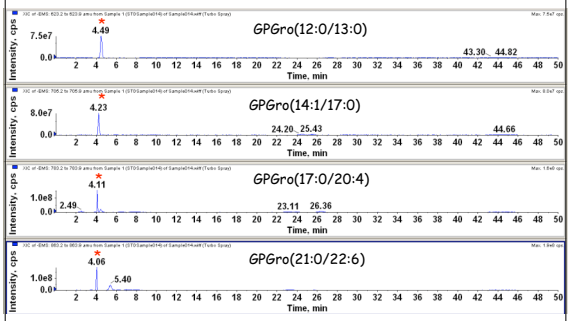


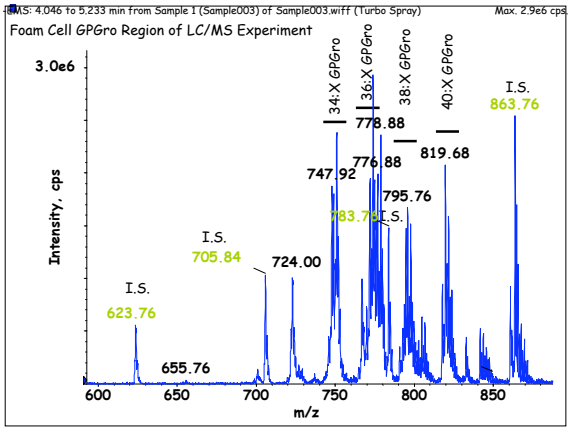
Neutral loss 260 u

Common Negative Ions of Glycerophospholipids (CID)

GPCho	***GPSer***	***GPEtn***	***GPIns***
$[M-15]^-$			
R_1COO^-	R_1COO^-	R_1COO^-	R_1COO^-
R_2COO^-	R_2COO^-	R_2COO^-	R_2COO^-
$[M-H]^- -R_2COOH$	$[M-H]^- -87$	$[M-H]^- -R_2COOH$	$[M-H]^- -R_2COOH$
$[M-H]^- -R_2C=C=O$		$[M-H]^- -R_2C=C=O$	$[M-H]^- -R_2C=C=O$
	$[M-H]^- -R_2COOH-87$		m/z 241
	m/z 153	m/z 153	m/z 153

LC/MS Analysis (A. Brown)





Avanti Lipid Maps Standards

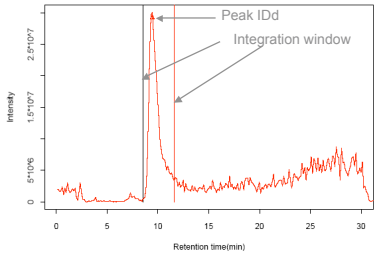
25:0 GPA	31:1 GPA	37:4 GPA	43:6 GPA
25:0 GPCho	31:1 GPCho	37:4 GPCho	43:6 GPCho
25:0 GPEtn	31:1 GPEtn	37:4 GPEtn	43:6 GPEtn
25:0 GPGro	31:1 GPGro	37:4 GPGro	43:6 GPGro
25:0 GPIns	31:1 GPIns	37:4 GPIns	43:6 GPIns
25:0 GPSer	31:1 GPSer	37:4 GPSer	43:6 GPSer
13:0 Lyso GPA	17:1 Lyso GPA		
13:0 Lyso GPCho	17:1 Lyso GPCho		
37:4 GPIns(3)P	37:4 GPIns(4)P	37:4 GPIns(5)P	
37:4 GPIns(3,4)P2	37:4 GPIns(3,5)P2	37:4 GPInsI(4,5)P2	
37:4 GPIns(3,4,5)P3			

LC-MS Computational Analysis

- XIC ASCII data is automatically generated from fullscan data for all m/z values, peaks found, integrated, and aligned across files (samples).

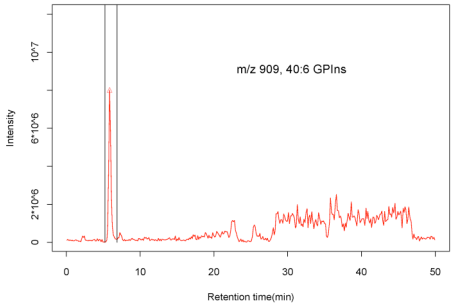
-Visual checks and error checking per *m/z* are used to confirm automated results.

XIC from *m/z* 631.06 to 631.96 (Peak fragmentation indicates 31:1 GPA standard)



LC-MS Analysis (cont'd)

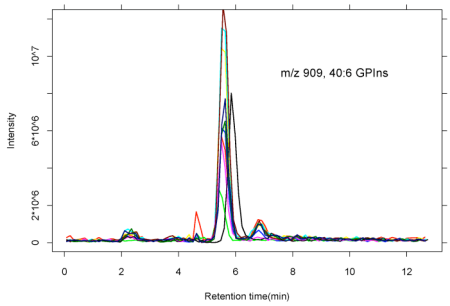
XIC from *m/z* 909.06 to 909.96 (LMAPS Raw cell)



- Peaks areas are normalized to fixed odd-carbon internal standards (when available).
- Even carbon titrations are used for estimating quantities in the extract.

Multiple XICs (10 spectra) from *m/z* 909.06 to 909.96 (LMAPS RAW cell)

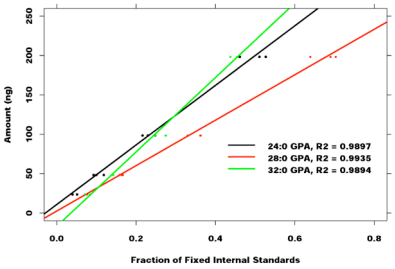
- Determine peak areas using automated integration algorithms.
- Align integrated values across samples (confirm alignment graphically).
- Normalize areas to fixed internal standard areas.
- Currently do this for ~130 glycerophospholipid analytes.



Standard curves from integrated peak XIC data

Amount(ng) = slope*(fraction of odd carbon std) + intercept

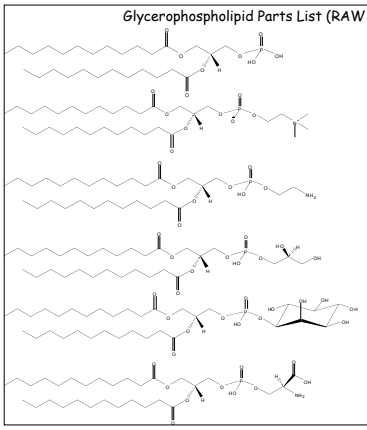
GPA



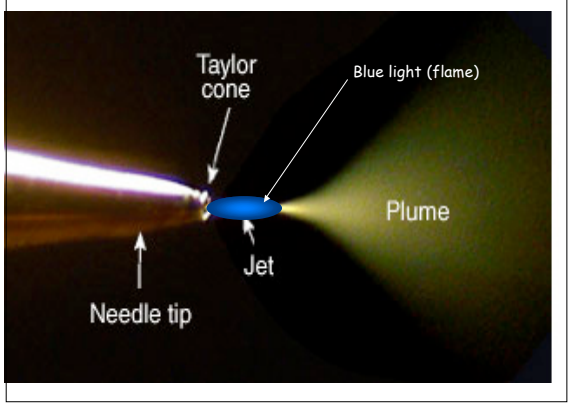
- Peak areas normalized to mean of 4 fixed odd carbon internal standard peak areas within the class (as in KDO2 time-course data set).

- 11 even carbon GPA standard curves generated.

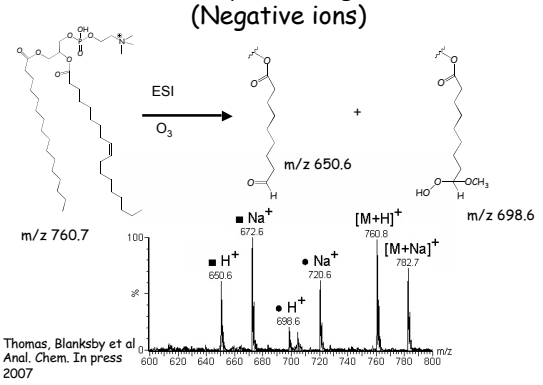
Glycerophospholipid Parts List (RAW 264.7 cells)



Lipid Class	Lipids Identified
GPA	101
GPCho	106
GPEtn	136
GPGro	105
GPIns	84
GPSer	68



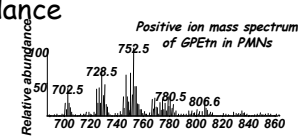
Ozonloysis during ESI (Negative ions)



Thomas, Blanksby et al Anal. Chem. In press 2007

Isotopic Abundance

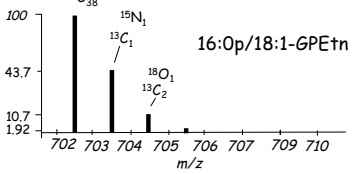
Isotope	% Natural Abun-dance
^{1}H	99.98
^{2}H	0.015
^{12}C	98.9
^{13}C	1.1
^{14}N	99.64
^{15}N	0.36
^{16}O	99.8
^{18}O	0.2
^{19}F	100
^{28}Si	92.2
^{29}Si	4.7
^{30}Si	3.1



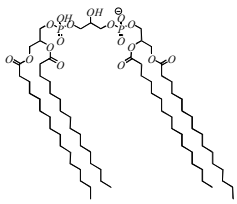
64.0% Total Ion current

[M+H]$^+$
m/z 702.5437
$C_{38}H_{77}O_7NP$

16:0p/18:1-GPEtn



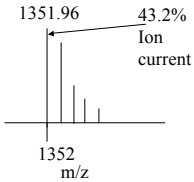
Exact mass and Isotopes



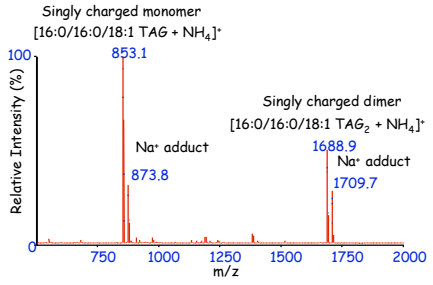
$C_{73}H_{141}O_{17}P_2^-$

Exact Mass: 1351.96

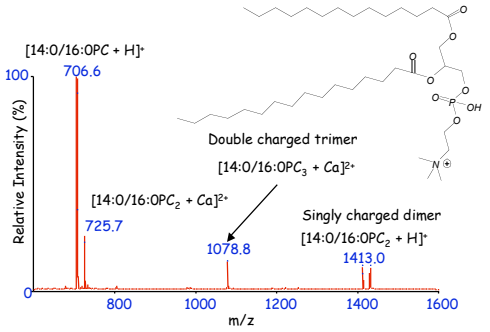
Cardiolipin
Mitochonrial lipid

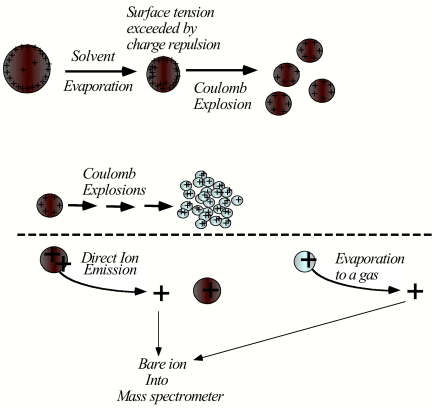


16:0/16:0/18:1-TAG ESI-MS



14:0a/16:0 GPCho ESI-MS





References

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  - Qualitative MS/MS and MS^3
    - McAnoy et al. J Amer Soc Mass Spectrom. 16:1498 (2005)
  - Quantitation by neutral loss scanning
    - Murphy et al. Anal. Biochem. In press 2007
- Phospholipid analysis
  - Qualitative analysis by ESI Mass Spectrometry
    - Pulfer and Murphy, Mass Spectrom. Rev. 22:332 (2003)
    - RCMurphy Mass Spectrometry of Phospholipids (2002).
  - Quantitation
  - Rouzer, R.A., Ivanova, P.T., Byrne, M.O., Milne, S.B., Marnett, L.J., and Brown, H.A. , (2006) Lipid profiling reveals arachidonate deficiency in RAW264.7 cells: Structural and functional implications., ***Biochemistry*** 45: 14795-14808.
  - Milne, S.B., Ivanova, P.T., Forrester, J.S., and Brown, H.A., (2006) Lipidomics: Analysis of cellular lipids by ESI-MS. **Methods** 39: 92-103. Edited by V. Bankaitis. Elsevier Press.
  - Callender, H.L., Forrester, J.S., Ivanova, P., Preininger, A., Milne, S., and Brown, H.A., (2007) Quantification of diacylglycerol species from biological extracts by electrospray ionization mass spectrometry. **Analytical Chemistry** 79: 263-272.
  - 4. Ivanova, P.T., Milne, S.B., Byrne, M.O., Xiang, Y., and Brown, H.A. (2007) Glycerophospholipid Identification and Quantitation by Electrospray Mass Spectrometry. **Methods in Enzymology:** Lipidomics and Bioactive Lipids. Voume 1. Edited by H. Alex Brown, Elsevier Press. In press.
- Web sites for tools:
- www.lipidmaps.org
- www2.uchsc.edu/pharmacology/RCMweb1—use Microsoft Internet Explorer

Acknowledgements

- Analysis of neutral lipids
  - Jessica Krank
  - Miguel Gijon
  - Patrick Hutchins
- Analysis of Phospholipids
  - Alex Brown (Vanderbilt University)
- Lipid Profiler
  - Eva Duchoslav (Applied Biosystems, Canada)
- Support: National Institutes of Health
  LipidMaps GM069338

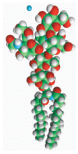
LIPID MAPS Lipidomics Workshop
April 28, 2007

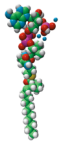
www.lipidmaps.org

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	Jeremy Allegood	Elaine Wang
	Chris Haynes	Ying Liu
	Samuel Kelly	Jia Wei

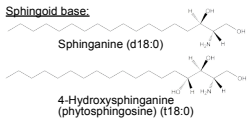
Outline:

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

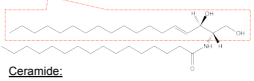
Sphingoid base:



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-hydroxy

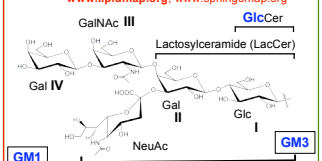
Headgroup variation

Phosphosphingolipids:

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

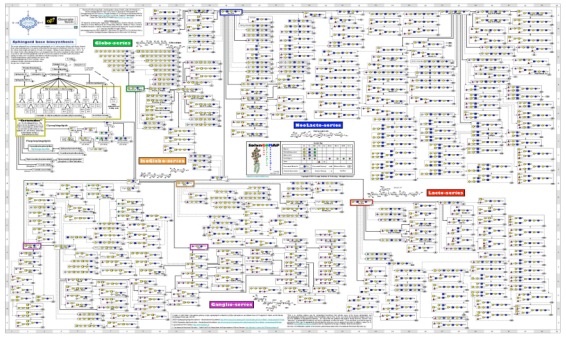
Glycosphingolipids:

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

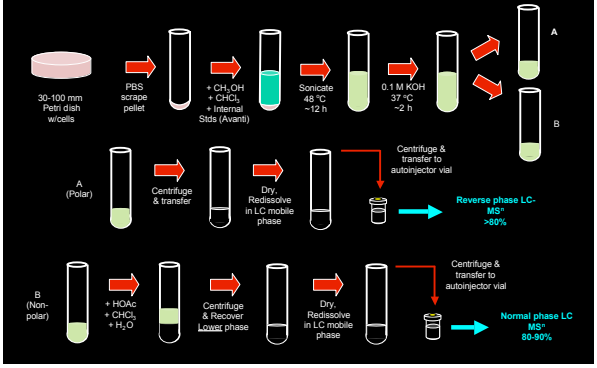


Neu5Acα2-3(Galβ1-3GalNAcβ1-4)Galβ1-4Glc1-1Cer
$II^3Neu5AcGg_4Cer$

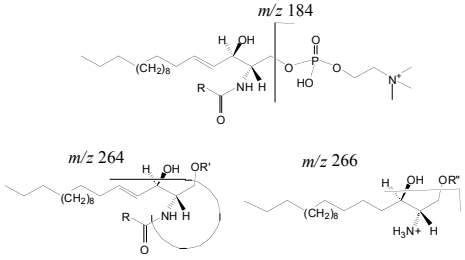
SphinGOMAP (www.sphingomap.org)



B. Sample preparation issues: solvents, chromatography, recovery, reproducibility:



C. Compound Identification: Characteristic fragmentations of sphingolipids



R = n-alkyl chain, R' = H, Glu/Gal, Lac, and R" = H, PO_3

Sphingolipid analysis by LC-MS/MS

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

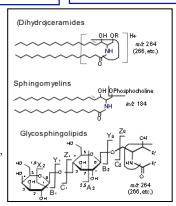
B (Nonpolar, e.g. Cer, Cer-P, SM, GlcCer, GalCer, LacCer, etc.)

Normal phase HPLC

Reverse phase HPLC → Inlet System → Ion Source → Mass Analyzer (Q1, Q2, Q3 or LIT) → Detector

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 and 4-hydroxy-Sa
✓So-P, Sa-P and other derivatives (lysoSM, psychosine, N-methyl-)
✓Plus metabolites labeled with stable isotope precursors



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

Sullards, Merrill & coworkers *STKE* (2001), *Methods* (2005), *Meth. Enzymol.* (in press) & www.sphingomap.org

Summary of conditions used for Liquid Chromatography

Reversed Phase

- LCB analysis: So, Sa, phyto So, So-1-P, Sa-1-P, and standards

- 2.1 x 50mm Supelco Discovery C18, 5 μm, 120 Å

- A: 74:25:1 $CH_3OH/H_2O/HCOOH$
  B: 99:1 $CH_3OH/HCOOH$

- Flow rate 1 mL/min, 0.6 min. 80:20 A/B, 1.8 min. to 100% B, 0.6 min. hold 100% B

Normal Phase

- Complex SL's: Cer, GlcCer, GalCer, LacCer, SM, and standards

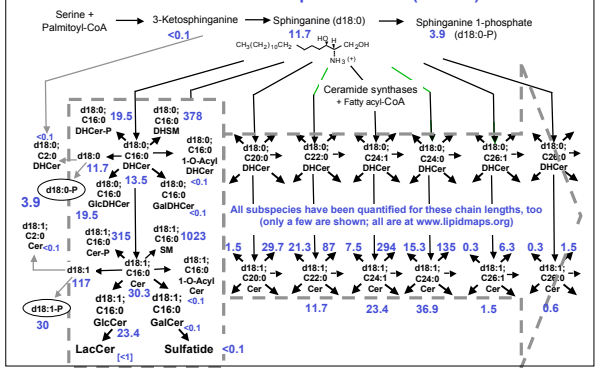
- 2.1 x 50 mm Supelco NH_2, 3 μm, 120 Å

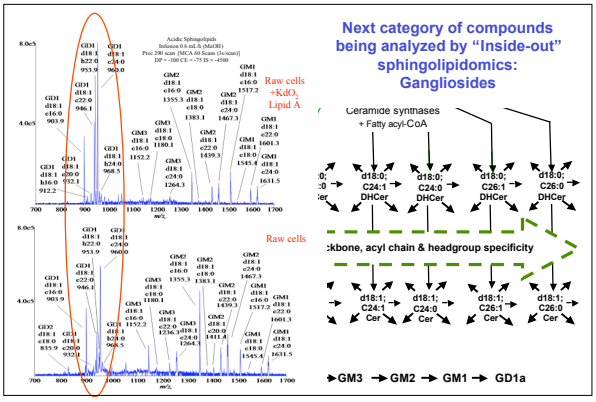
- A: 97:2:1 $CH_3CN/CH_3OH/CH_3COOH$
  B: 99:1 CH_3OH/CH_3COOH
  both 5mM Ammonium Acetate

- Flow rate 1.5 mL/min, 0.5 min. 100% A, 0.2 min. gradient to 90:10 A/B, 0.5 min. hold, 0.4 min. to 82:18 A/B, 0.6 min hold, 0.4 min. to 100% B

For more information, see Sullards, Merrill & coworkers: *STKE* (2001), *Methods* (2005), *Meth. Enzymol.* (in press) & www.sphingomap.org

Number of molecules per RAW cell (million)

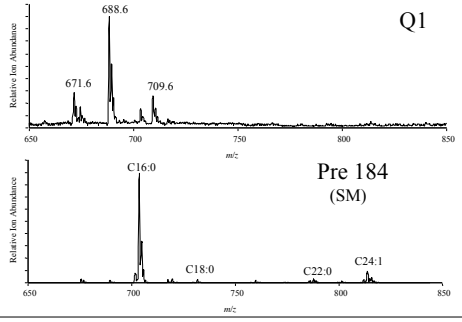


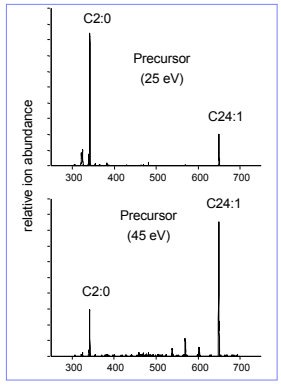


Work-flow for analysis of new samples using this LC-MS/MS Methodology

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.

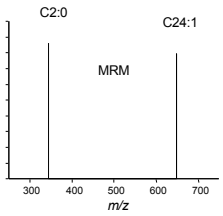
Example: Identification of sphingolipid subspecies via Neutral Loss or Precursor Ion Scans





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.)

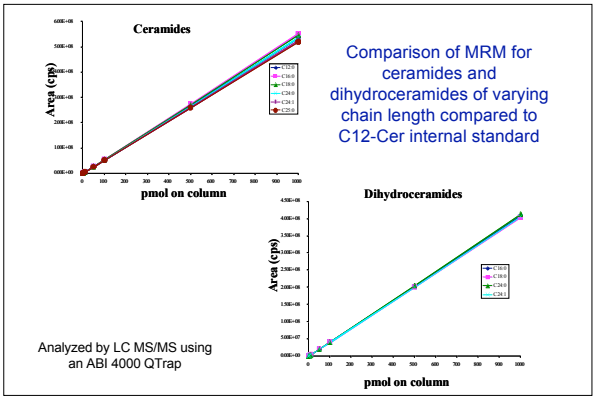
For sphingoid bases: odd chain length variants that elute under similar conditions so there is little ionization or dissociation effects and precursor and product ion masses are slightly shifted.

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.

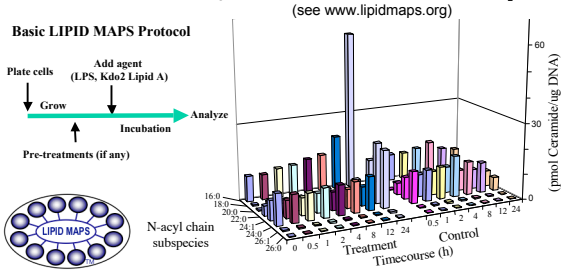
C12-Cer, C25-Cer, C12-Cer-1-P, C12-GlcCer, C12-LacCer, C12 SM

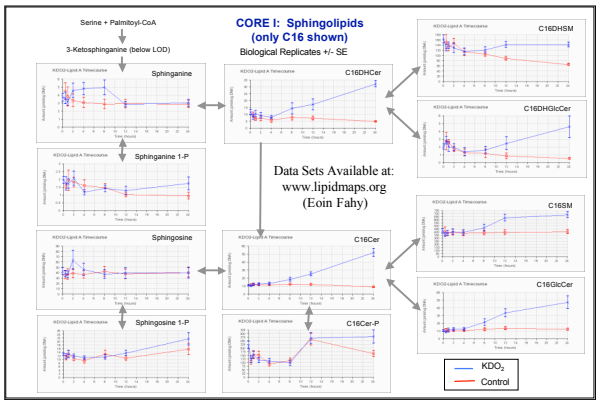
Also available: C12-Sulfatide
Under development: C12-GM1 and other complex glycosphingolipids
C17:1 sphingosylphosphocholine; N-methyl-sphingoid bases



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)





F. Remaining challenges (and opportunities)

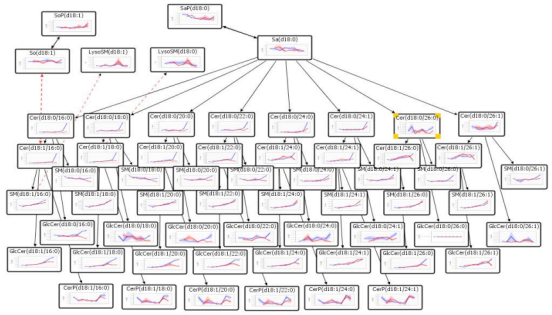
Discovery of new subspecies (and new functions for known subspecies)--such as N-methylsphingoid bases

Obtain standards for glycosphingolipids (and new sphingolipids)

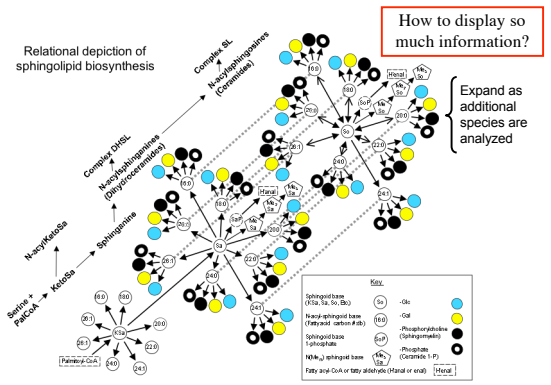
Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.

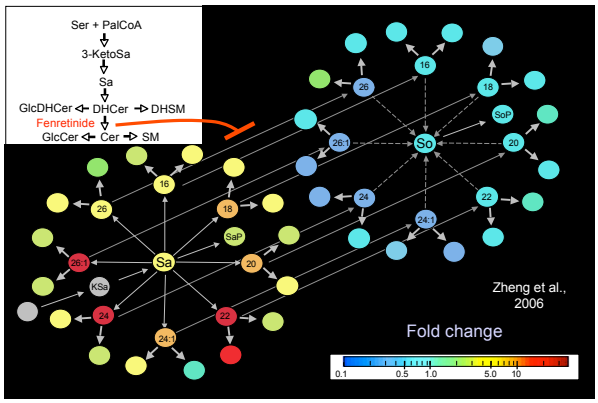
Develop methods to differentiate appearance/disappearance of particular subspecies via de novo biosynthesis vs turnover.

Example of Lipid MAPS timecourse data set for sphingolipids
(see www.lipidmaps.org)



How to display so much information?





F. Remaining challenges (and opportunities)

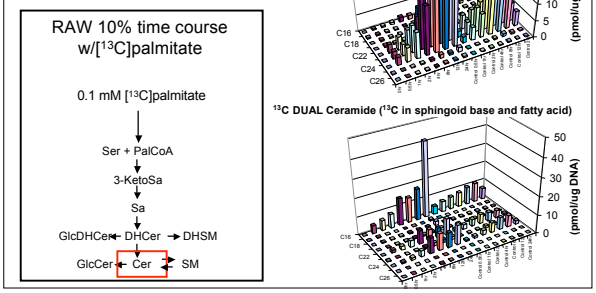
Discovery of new subspecies (and new functions for known subspecies).

Obtain standards for glycosphingolipids.

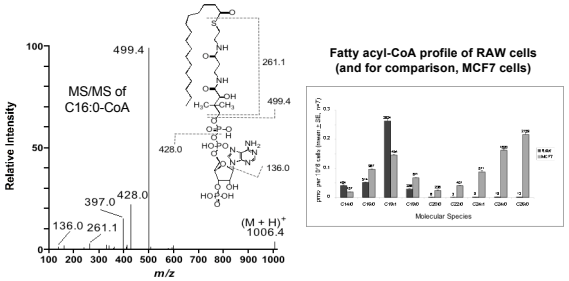
Determine how to better visualize changes in abundances in multiple classes of sphingolipids over time.

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

Analysis of sphingolipid biosynthesis using stable isotope labeled precursors



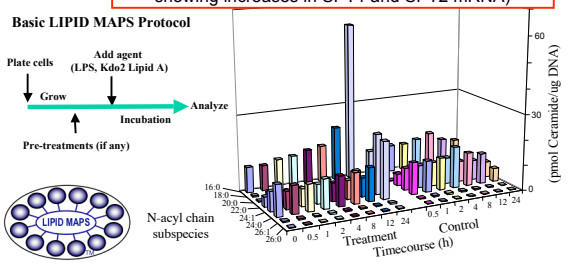
To understand sphingolipid biosynthesis one must also know the availability of the co-substrate fatty acyl-CoA's.



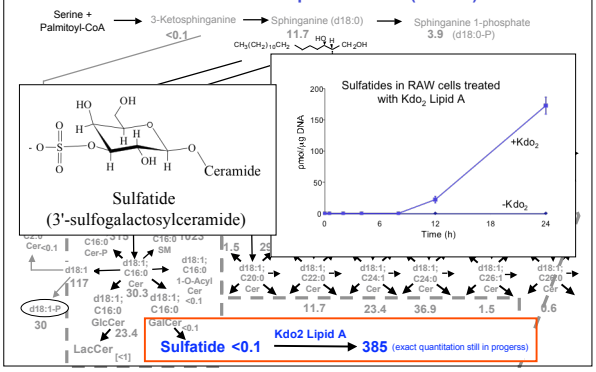
C16:0-CoA (10 pmol/μL in methanol 10 mM triethylammonium acetate) was infused at 5 μL/min. 4000QTrap parameters: IS = 5400 V, Gs1 = 12 psi, CUR = 10 psi, DP = 180 V, CE = 52 V, CXP = 14.3 V, CAD = Med

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)



Number of molecules per RAW cell (million)



G. Examples of additional discoveries from sphingolipidomic analysis thus far

Sphingosine-1-phosphate phosphohydrolase regulates endoplasmic reticulum-to-golgi trafficking of ceramide. Giussani P, Maceyka M, Le Stunff H, Mikami A, Lepine S, Wang E, Kelly S, Merrill AH Jr, Milstien S, Spiegel S. Mol Cell Biol. 2006 Jul;26(13):5055-69.

Glucosylceramide synthase is an essential regulator of pathogenicity of Cryptococcus neoformans. Rittershaus PC, Kechichian TB, Allegood JC, Merrill AH Jr, Hennig M,Luberto C, Del Poeta M. J Clin Invest. 2006 Jun;116(6):1651-9.

Effects of sphingosine-1-phosphate and ceramide-1-phosphate on rat intestinal smooth muscle cells: implications for postoperative ileus. Dragusin M, Wehner S, Kelly S, Wang E, Merrill AH Jr, Kalff JC, vanEchten-Deckert G. FASEB J. 2006 Sep;20(11):1930-2.

Ceramide kinase utilizes ceramide provided by ceramide transport protein. Localization to subcellular compartments of eicosanoid synthesis. Lamour NF, Stahelin RV, Wijesinghe DS, Maceyka M, Wang E, Allegood JC, Merrill AH Jr, Cho W, Chalfant CE. J Lipid Res. 2007 Mar 27; [Epub ahead of print]

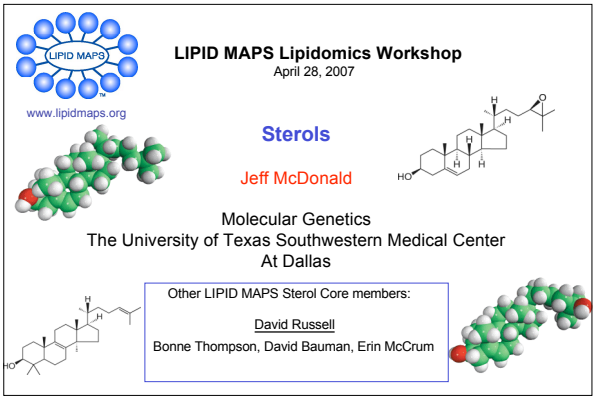
Sphingomyelinase Restricts the Lateral Diffusion of CD4 and Inhibits HIV Fusion. Finnegan CM, Rawat SS, Cho EH, Guiffre DL, Lockett S, Merrill AH Jr, Blumenthal R. J Virol. 2007 Mar 7; [Epub ahead of print]

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

"Shotgun" 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.

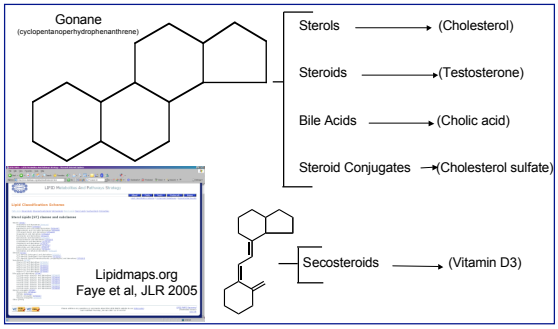
Ultra high resolution mass analysis: allows differentiation of isobaric / isotopic interferences and alternative fragmentation techniques.



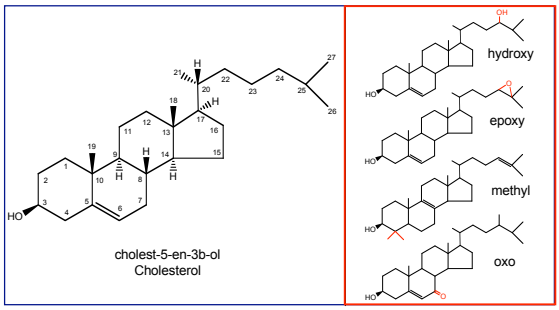
Outline:

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

Nomenclature and Range of Compounds to Analyze

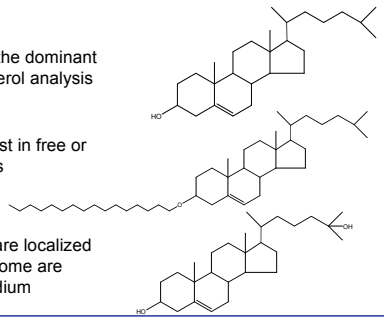


Nomenclature and Range of Compounds to Analyze

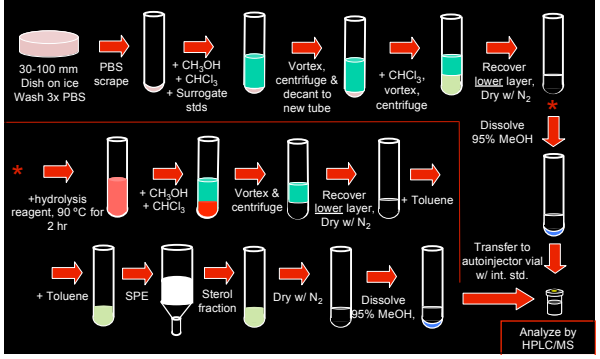


Nomenclature and Range of Compounds to Analyze

- Cholesterol is the dominant species in a sterol analysis
- Sterols can exist in free or esterified forms
- Many sterols are localized in cells only; some are located in medium



Sample Preparation: Extraction of Sterols



Sample preparation issues: solvents, chromatography, recovery, reproducibility

- Only saponify and perform SPE if necessary
  - SPE increases oxidation of cholesterol
  - Introduces "plastics" into samples
  - Increases time and effort
  - Columns may be used to process complex samples
- Extraction efficiency for basic extraction ~75%
- Lower phase is "organic" phase
- Dry solvents are necessary

See McDonald et al. In *Methods In Enzymology* (forthcoming) for detailed description of extraction methods

Compound identification: HPLC MS/MS; GC/MS

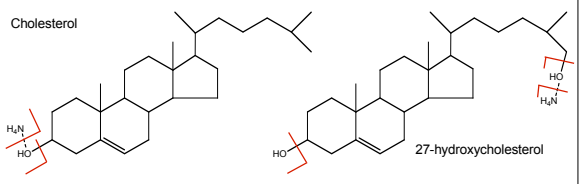
Reverse Phase HPLC

- Phenomenex Luna C_{18} (250 × 2 mm; 3 µm)
- A: 85% MeOH
  B: 100% MeOH
  (both with 5mM NH_4acetate)
- 30 ºC column temp
- 0.25 mL/min, 1 min 100%A to 100%B 15 min, 100%B 10 min, 100%A 5 min

Mass Spectrometry

- Applied Biosystems 4000QTrap
- TurboV Electrospray Ion Source
- Positive (+) mode
- Multiple Reaction Monitoring
- Optimized collision energy, declustering potential

Compound identification: HPLC MS/MS; GC/MS

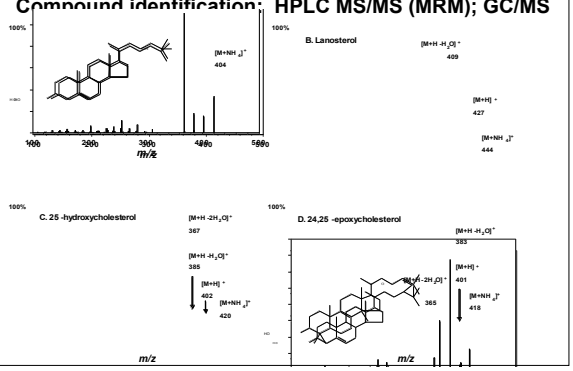


Common Ions Observed by Scan (in-source decay) and Product Ion

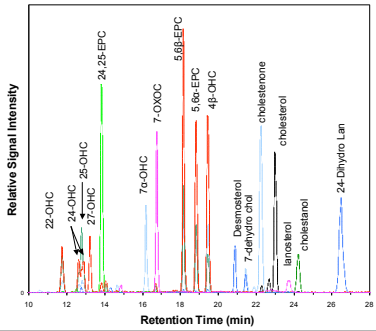
$[M+NH_4]^+$ $[M+NH_4\text{-}NH_3]^+$ (M+H) $[M+H\text{-}H_20]^+$ $[M+NH_4\text{-}H_20]^+$?	$[M+NH_4]^+$ $[M+NH_4\text{-}NH_3]^+$ (M+H) $[M+H\text{-}H_20]^+$ $[M+H\text{-}2H_20]^+$ $[M+NH4\text{-}H20]^+$?

No abundant disassociation products beyond loss of waters

Compound identification: HPLC MS/MS (MRM); GC/MS



Compound identification: HPLC MS/MS (MRM); GC/MS

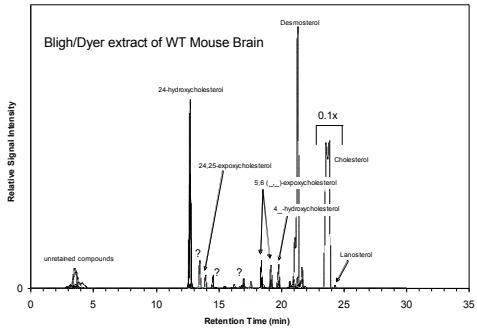


- Multiple Reaction Monitoring
- Most sterols well-resolved
- Peak widths between ~15 and 20 s @ FWHM

LOD

- 5-100 fmol on-column for oxysterols
- 200-2000 fmol on-column for sterols

Compound identification: HPLC MS/MS (MRM); GC/MS



Quantitation: Internal standards, etc.

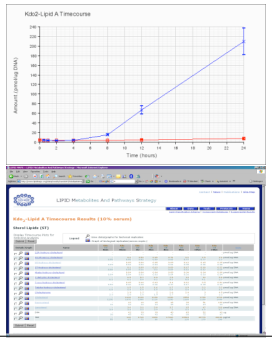
COMMON NAME	MW	SOURCE
22*R*-hydroxycholesterol	402.7	Sigma Aldrich
24-hydroxycholesterol(*R*+*S*)	402.7	Avanti Polar Lipids
25-hydroxycholesterol	402.7	Avanti Polar Lipids
27-hydroxycholesterol-(25*R*)	402.7	Avanti Polar Lipids
24,25-epoxycholesterol	400.6	Avanti Polar Lipids
7α-hydroxycholesterol	402.7	Avanti Polar Lipids
7-ketocholesterol	400.6	Avanti Polar Lipids
5,6β-epoxycholesterol	402.7	Avanti Polar Lipids
5,6α-epoxycholesterol	402.7	Avanti Polar Lipids
4β-hydroxycholesterol	402.7	Avanti Polar Lipids
Zymosterol	384.7	*
Desmosterol	384.7	Avanti Polar Lipids
7-dehydrocholesterol	384.7	Sigma Aldrich
Cholestenone	384.7	Avanti Polar Lipids
Lathosterol	386.7	Steraloids
Cholesterol	386.7	Avanti Polar Lipids
Lanosterol	426.7	Steraloids
Cholestanol	388.7	Avanti Polar Lipids
24-dihydrolanosterol	428.7	Avanti Polar Lipids

* = not available at this time

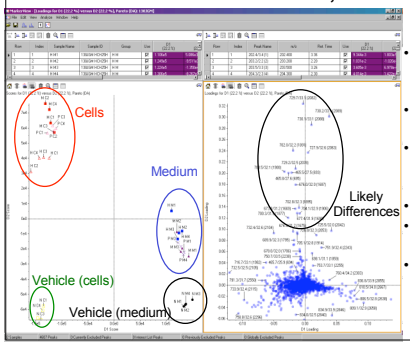
- **BOLD** = deuterated analog available from Avanti
- Avanti Polar Lipid Standards
  - Extensive characterization
    - NMR, LC, MS
    - On-going stability
- Additional deuterated and primary standards in progress
- www.avantilipids.com

Data analysis/visualization: LIMS, Website, Marker View, other

- Quantitation by isotope dilution with known standards
- Data normalized to mass of DNA
- Time course data for all sterols measured located at:
  - lipidmaps.org



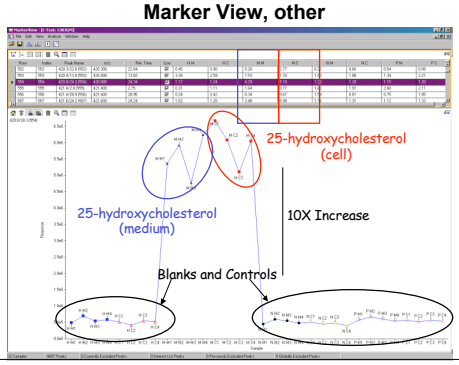
Data analysis/visualization: LIMS, Website, Marker View, other



Multivariate analysis

- Principal component and statistical analysis of LC/MS data for changes
- Compare samples and ask "what's different?"
- MarkerView, Metabolynx, MZMine, XCMS
  - Cravatt et al
- Unbiased, global profiling
- Products/substrates of (orphan) enzymes
- Identifying potential unknowns

Data analysis/visualization: LIMS, Website, Marker View, other



Comparison of Lipid MAPS methods with others in the literature

Gas-Chromatographic Mass Spectrometry (GC-MS)

Pros	Cons
• Cost	• Rigorous sample clean-up
• Superior chromatographic resolution	• Samples require derivatization
• Fragmentation	• Limited injection volume
• Compound databases	• Run time
• Established methods	• Generally limited to electron impact (EI), single quadrupole MS
• Good signal intensities	

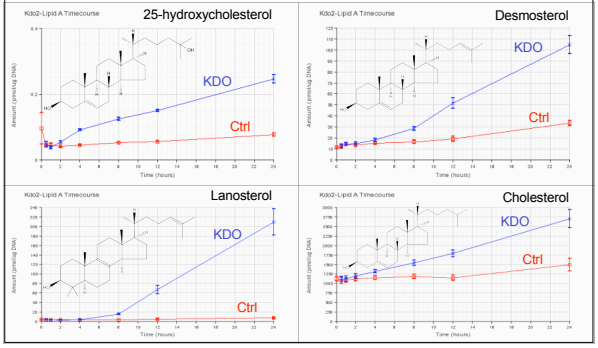
Sterol analysis ideally utilizes both LC-MS and GC-MS

Remaining challenges and opportunities

Identification of Novel Sterols

- No precursor, product, or neutral loss scans for native sterols
  - Derivitize
    - Griffiths, Sjövall, and company
- GC/MS (EI) lacks sensitivity and flexibility
- Supplement with labeled (^{2}H, ^{13}C) substrates and monitor mass shift
  - Acetate, mevalonate
  - Collect fractions, analyze with Triversa NanoMate (nanospray)
- Use predicted MRM pairs
  - ~75 MRM pairs covers most logical sterols
- Continue to increase compound library by acquiring standards

Discoveries from sterol analysis thus far



LIPID MAPS Lipidomics Workshop
April 28, 2007

www.lipidmaps.org

Eicosanoids

Richard Harkewicz

Departments of Pharmacology, Chemistry and Biochemistry
The University of California, San Diego
La Jolla, CA

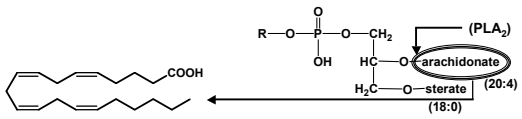
Other LIPID MAPS Eicosanoid Core Members:

Alexander Andreyev
Matthew Buczynski
Raymond Deems
Darren Stephens
Edward A. Dennis

Outline

- Brief description of eicosanoids
- Sample preparation/extraction
- Analytical methodology
- Library of eicosanoid standards
- Chiral chromatography – *enzymatic vs. nonenzymatic*
- DIMPLES/MS: A stable isotope labeling strategy enabling the search for novel eicosanoids
- Comparison of LIPID MAPS eicosanoid approach with others in literature
- Future work

stimulation (LPS or Kdo_2-Lipid A) → release of phospholipase A_2 (PLA_2) → membrane phospholipid (R = choline or ethanolamine)



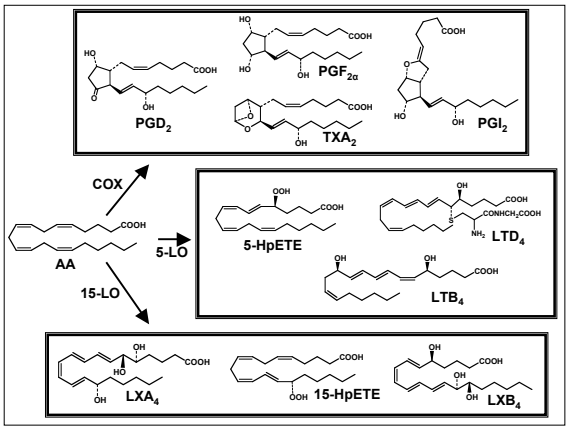
free eicosatetraenoic acid (from the Greek *eicosa* for 20)
AKA arachidonic acid (20:4, n=6)

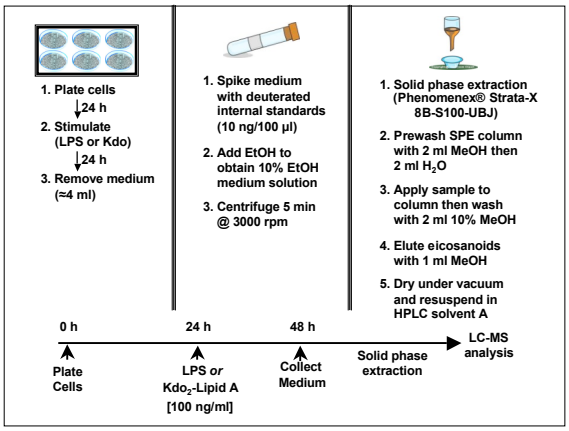
Eicosanoids *– powerful inflammatory mediators that are derived from arachidonic acid and act in autocrine and paracrine fashion (signal at or immediately adjacent to their site of synthesis)*

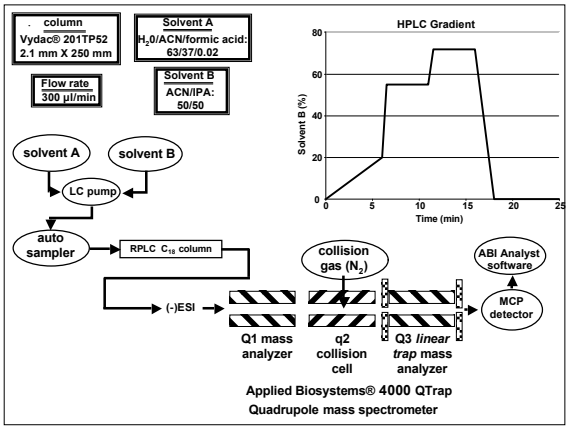
Once they are made, they are quickly secreted from the cell

Transcription factor: Can also enter cell nucleus, binding to nuclear receptors

Sometimes referred to as "*autocoids* " or *local hormones*


PGD2
PGF2α
TXA2
PGI2
COX
AA
5-LO
15-LO
5-HpETE
LTD4
LTB4
LXA4
15-HpETE
LXB4


1. Plate cells
24 h
2. Stimulate (LPS or Kdo)
24 h
3. Remove medium (≈4 ml)
1. Spike medium with deuterated internal standards (10 ng/100 µl)
2. Add EtOH to obtain 10% EtOH medium solution
3. Centrifuge 5 min @ 3000 rpm
1. Solid phase extraction (Phenomenex® Strata-X 8B-S100-UBJ)
2. Prewash SPE column with 2 ml MeOH then 2 ml H2O
3. Apply sample to column then wash with 2 ml 10% MeOH
4. Elute eicosanoids with 1 ml MeOH
5. Dry under vacuum and resuspend in HPLC solvent A
0 h
24 h
48 h
Plate Cells
LPS or Kdo2-Lipid A [100 ng/ml]
Collect Medium
Solid phase extraction
LC-MS analysis

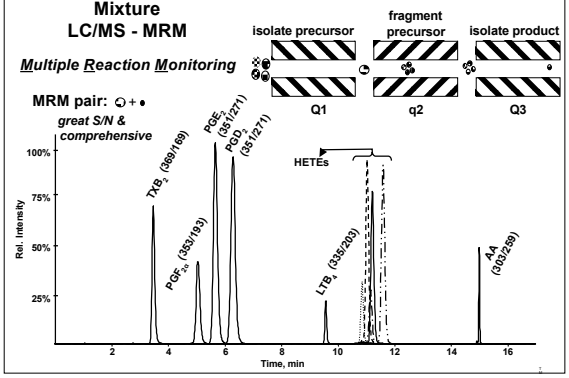

column
Vydac® 201TP52
2.1 mm X 250 mm
Solvent A
H20/ACN/formic acid:
63/37/0.02
Flow rate
300 µl/min
Solvent B
ACN/IPA:
50/50
HPLC Gradient
Solvent B (%)
Time (min)
solvent A
solvent B
LC pump
auto sampler
RPLC C18 column
(-)ESI
collision gas (N2)
ABI Analyst software
MCP detector
Q1 mass analyzer
q2 collision cell
Q3 linear trap mass analyzer
Applied Biosystems® 4000 QTrap
Quadrupole mass spectrometer

Eicosanoid Standard Mixture LC/MS - MRM

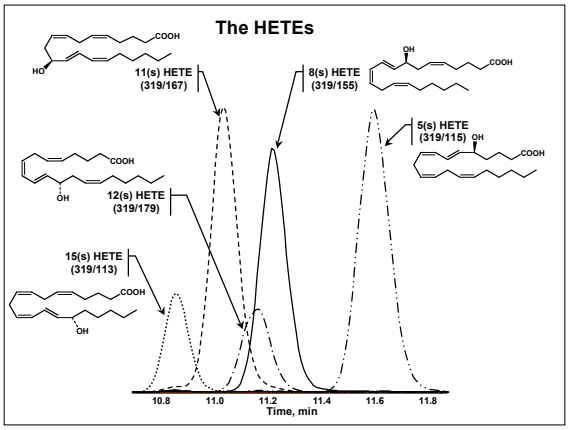
Multiple Reaction Monitoring

MRM pair: ⬭ + ●

great S/N & comprehensive



The HETEs

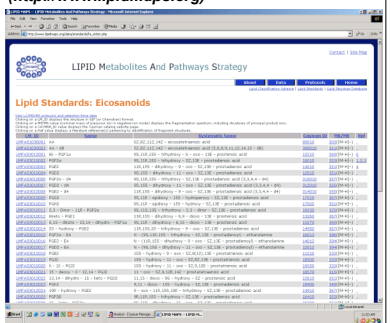


Library of Eicosanoid Standards

(http://www.lipidmaps.org)

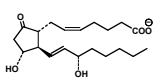
Provides:

- **Chemical structure in ChemDraw® format**
- **LC and MS protocols**
- **MSMS fragmentation spectra**
- **LC retention times for given set of conditions**
- **Web-link to Cayman Chemical for each eicosanoid**

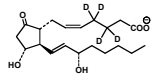


The eicosanoid library provides information from which a comprehensive method (LC-MRM-MS) is created and used to survey eicosanoid release from stimulated cells

Use of deuterium labeled internal standards allows absolute quantitation for a number of eicosanoids

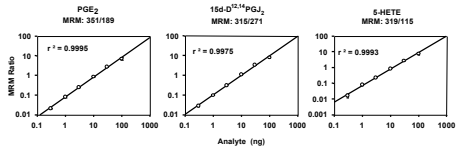


PGE_2 $[M-H]^- = 351$

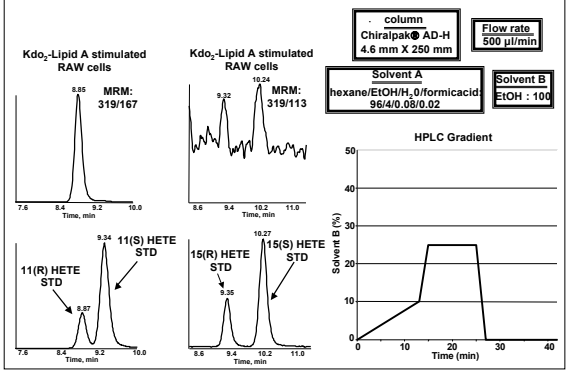


PGE_2 - d4 $[M-H]^- = 355$

example of internal standard calibration curves used for quantitation



Chiral chromatography aids in determining enzymatic vs. nonenzymatic origin



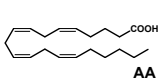
Are biologically significant eicosanoids being overlooked?

Are there species for which we have no prior knowledge of or expectation of their presence and, hence, <u>no available MRM pairs</u> or chromatography retention times that would be required for their detection?

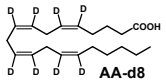
To address these concerns a mass spectral based stable isotope labeling strategy has been developed

***DIMPLES/MS*: Diverse Isotope Metabolic Profiling of Labeled Exogenous Substrates using Mass Spectrometry**

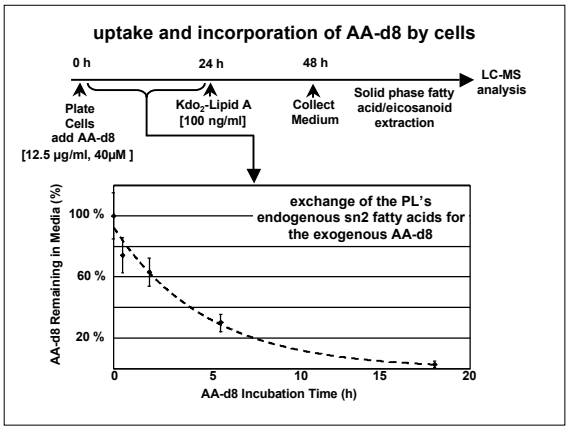
Incubation of cells in medium supplemented with deuterium-labeled arachidonic acid (AA-d8)

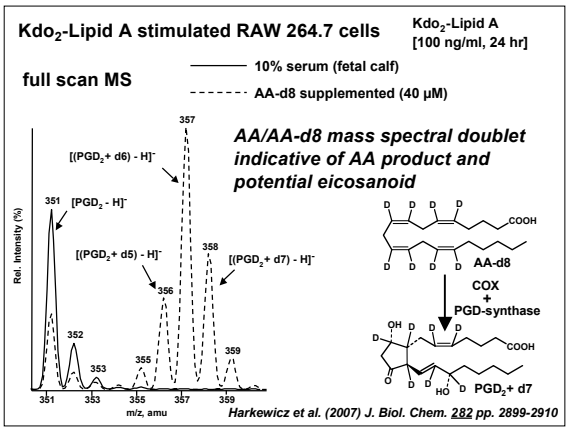


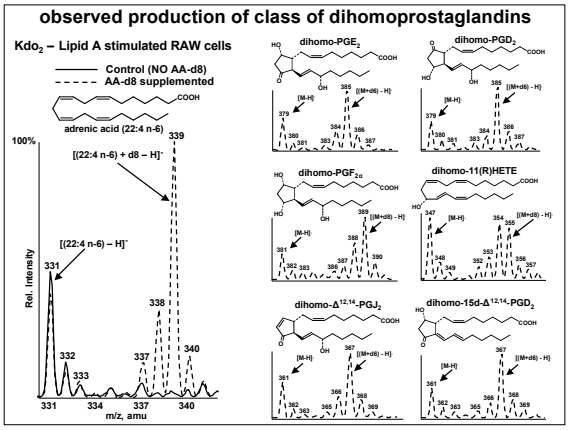
AA



AA-d8

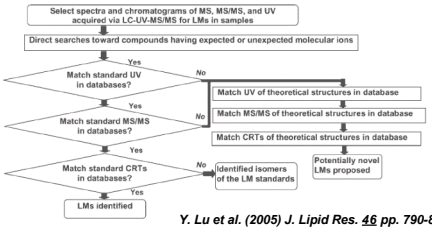

uptake and incorporation of AA-d8 by cells
0 h
24 h
48 h
LC-MS analysis
Plate Cells add AA-d8 [12.5 µg/ml, 40µM]
Kdo2-Lipid A [100 ng/ml]
Collect Medium
Solid phase fatty acid/eicosanoid extraction
exchange of the PL's endogenous sn2 fatty acids for the exogenous AA-d8
AA-d8 Remaining in Media (%)
100 %
60 %
20 %
0
5
10
15
20
AA-d8 Incubation Time (h)


Kdo2-Lipid A stimulated RAW 264.7 cells
Kdo2-Lipid A [100 ng/ml, 24 hr]
full scan MS
10% serum (fetal calf)
AA-d8 supplemented (40 µM)
AA/AA-d8 mass spectral doublet indicative of AA product and potential eicosanoid
Rel. Intensity (%)
m/z, amu
[(PGD2+ d6) - H]-
[PGD2 - H]-
[(PGD2+ d5) - H]-
[(PGD2+ d7) - H]-
AA-d8
COX + PGD-synthase
PGD2+ d7
Harkewicz et al. (2007) J. Biol. Chem. 282 pp. 2899-2910


observed production of class of dihomoprostaglandins
Kdo2 – Lipid A stimulated RAW cells
Control (NO AA-d8)
AA-d8 supplemented
adrenic acid (22:4 n-6)
[(22:4 n-6) + d8 – H]-
[(22:4 n-6) – H]-
Rel. Intensity
m/z, amu
dihomo-PGE2
dihomo-PGD2
dihomo-PGF2α
dihomo-11(R)HETE
dihomo-Δ12,14-PGJ2
dihomo-15d-Δ12,14-PGD2

another approach to eicosanoid lipidomics and the search for novel eicosanoids

Serhan Lab developing theoretical databases and algorithms based on virtual LC-UV spectroscopy-tandem mass spectrometry and chromatograms for identifying potential eicosanoids <u>without</u> synthetic or authentic products as standards



Y. Lu et al. (2005) J. Lipid Res. <u>46</u> pp. 790-802

future work

- **Continue to expand eicosanoid library**
- **Incorporate UV detection and analyses in eicosanoid surveys**
- **Analytical methodology**
- **Similar studies with Ω-3 fatty acid supplementation**
  EPA (20:5 n-3) and DHA (22:6 n-3)

www.lipidmaps.org

LIPID MAPS Lipidomics Workshop
April 28, 2007

Novel Lipid Analysis

Teresa A. Garrett

Department of Biochemistry
Duke University School of Medicine
Durham, NC

Other LIPID MAPS Core K members:

Chris Raetz
Ziqiang Guan
Reza Kordestani
Andrea Ryan

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Novel Lipid Analysis

Teresa A. Garrett

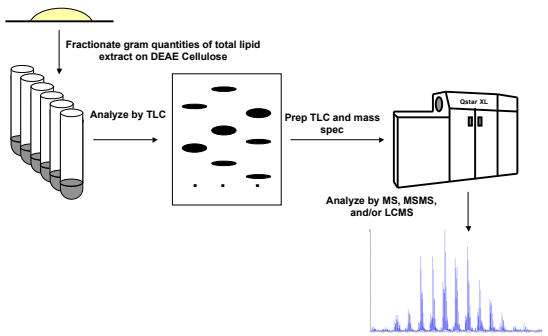
Department of Biochemistry
Duke University School of Medicine
Durham, NC

Other Core K Objectives

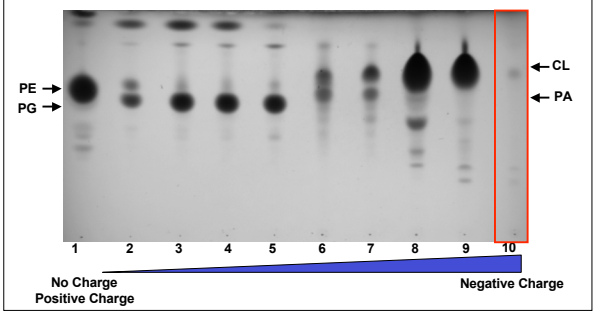
Quantification of cardiolipin, dolichols, and coenzyme Q

Methods in Enzymology. 2007. Volume 424.
"Lipidomics and Bioactive Lipids".
Edited by H. Alex Brown.

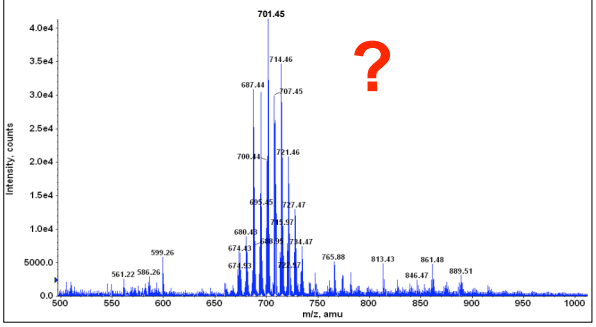
Identifying Novel Lipids



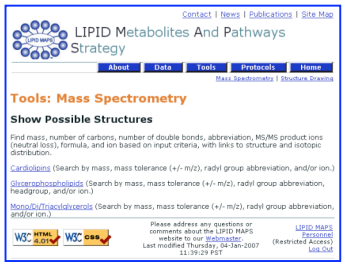
Anion Exchange Chromatography of *E. coli* Total Lipids



ESI-MS of Fraction #10

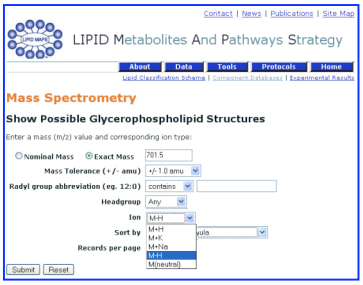


Lipid MAPS Mass Spectrometry Tools

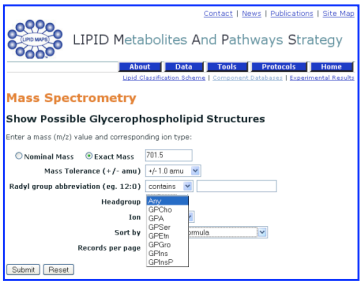


http://www.lipidmaps.org/tools/ms

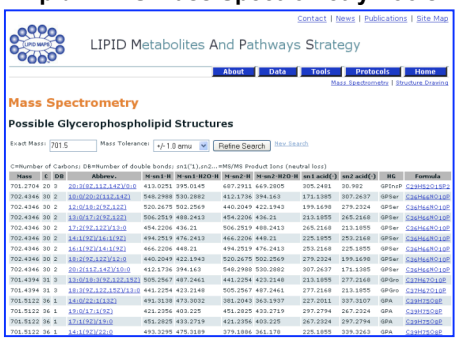
Lipid MAPS Mass Spectrometry Tools



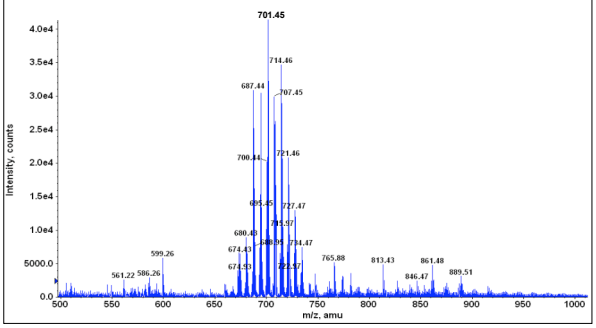
Lipid MAPS Mass Spectrometry Tools



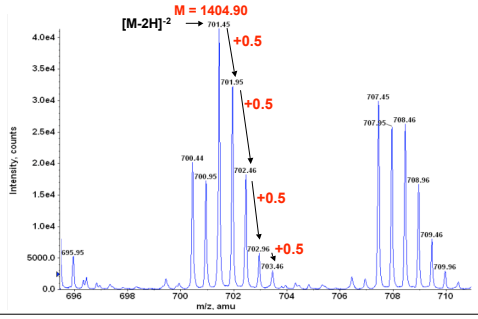
Lipid MAPS Mass Spectrometry Tools



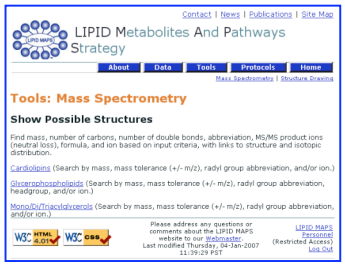
ESI-MS of Fraction #10



Major Ions of Fraction #10

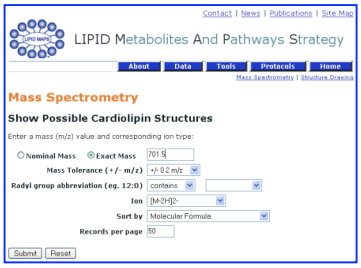


Lipid MAPS Mass Spectrometry Tools

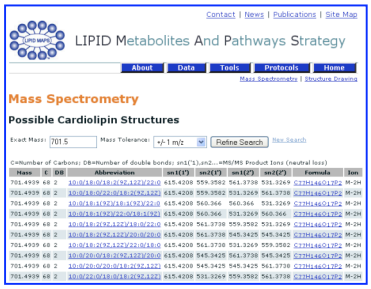


http://www.lipidmaps.org/tools/ms

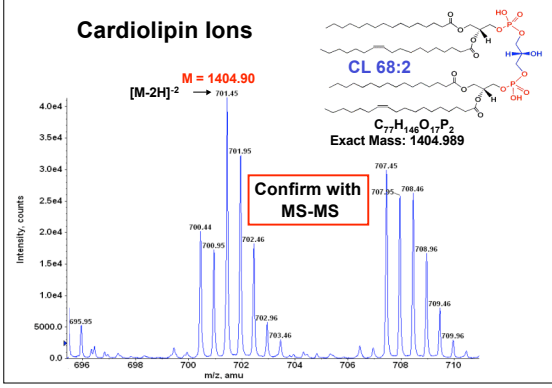
Lipid MAPS Mass Spectrometry Tools



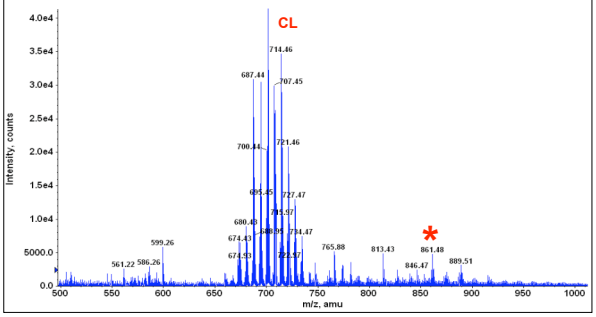
Lipid MAPS Mass Spectrometry Tools



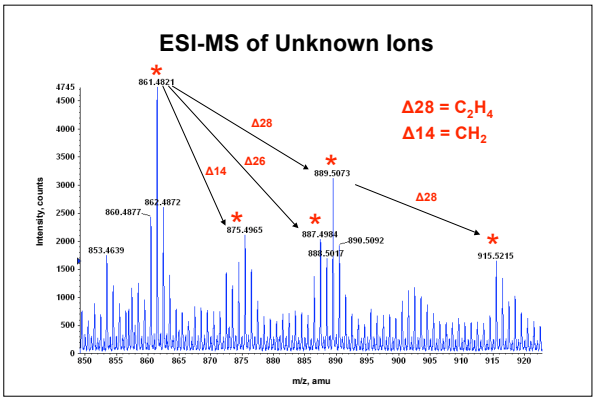
Cardiolipin Ions



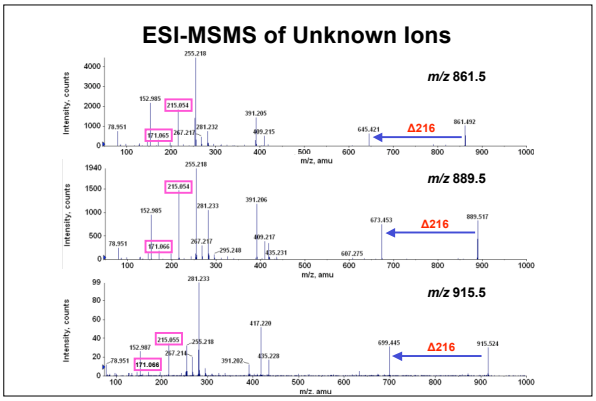
ESI-MS of Fraction #10



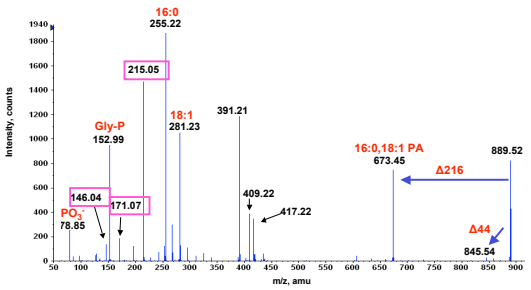
ESI-MS of Unknown Ions



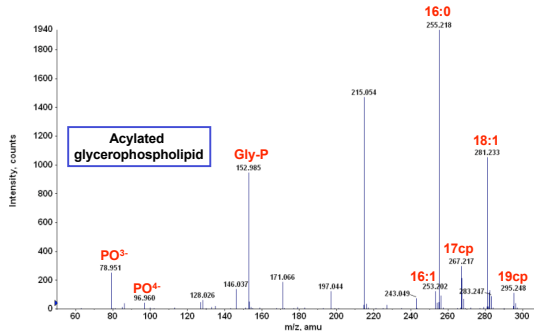
ESI-MSMS of Unknown Ions



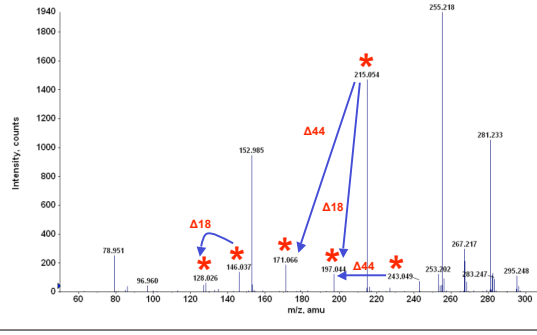
MSMS of Unknown Ion 889.52



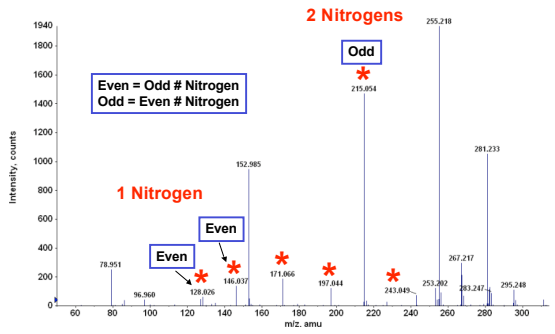
MSMS of Unknown Ion 889.52



MSMS of Unknown Ion 889.52



MSMS of Unknown Ion 889.52



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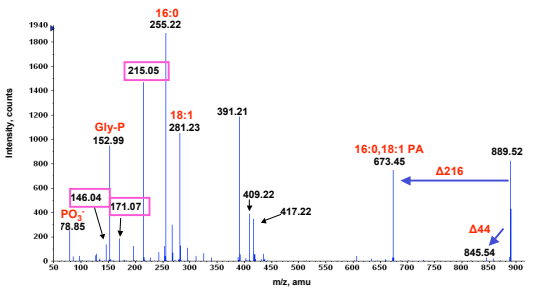
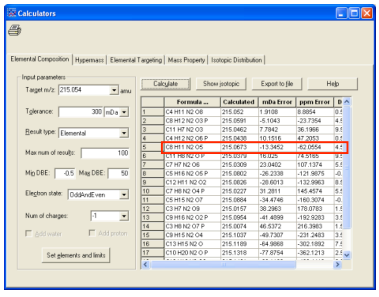
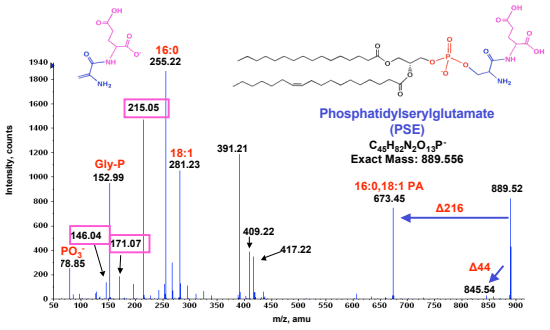


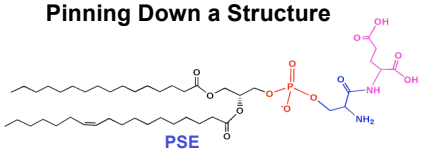
Elemental composition Calculator



MSMS of Unknown Ion 889.52



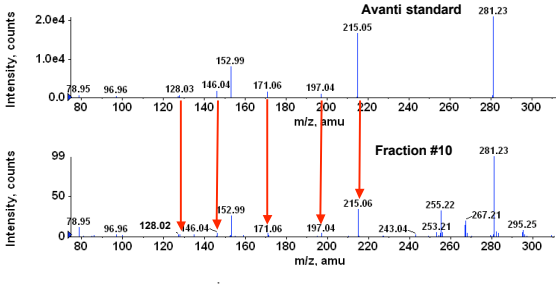
Pinning Down a Structure



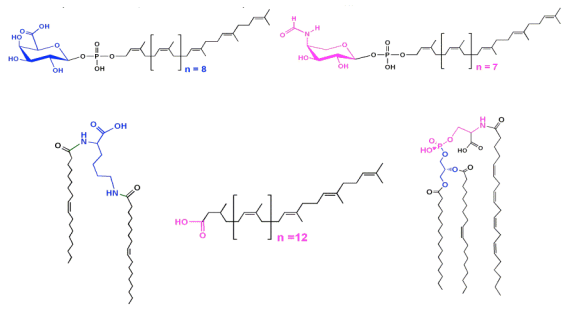
PSE

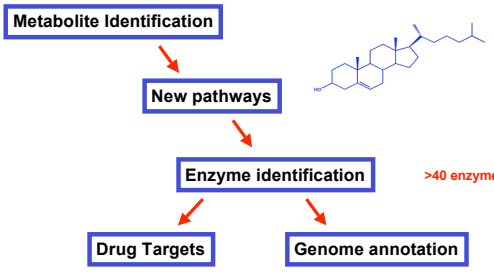
- Isotopic labeling
- Purify and characterize by NMR and GC-MS
- Compare MS, MSMS and LC-MS with synthetic standard
- Develop an *in vitro* biosynthetic assay

MSMS of PSE
Standard v. Natural Product



New Structures Identified Using ESI-MS





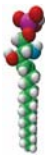
Bob Murphy – UCHSC
Walt Shaw – Avanti Polar Lipids

NIH
1U54GM069338
Lipidmaps.org

www.lipidmaps.org

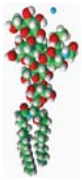
LIPID MAPS Lipidomics Workshop
April 28, 2007

Internal Standards for Lipidomic Analysis



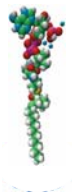
Walt Shaw

Avanti® Polar Lipids, Inc.



LIPID MAPS: Core F(a)
Lipid Standards and Production members:

	Mass spectrometry	Synthesis
Walt Shaw	Bill Caufield	Shengrong Li
	Jeff Moore	Dale Smith



Discussion Points

- Production of MS standards
  - Synthesis
  - Packaging
- Why bad things happen to good lipids
  - Surfaces
  - Reactive chemicals
- Available standards

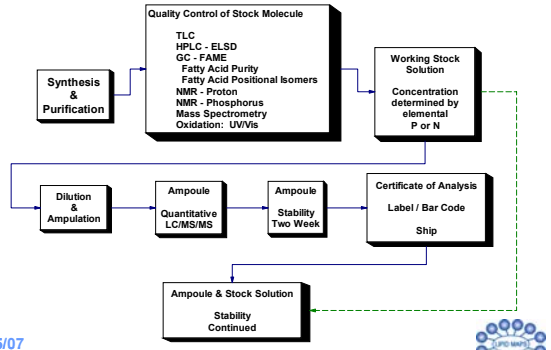
5/07

Synthesis of Lipid Standards

- Two tenets that govern synthesis of Avanti's molecules
  - It is not possible to analyze **quality** into a product!
  - **Quality** must be synthesized into a product!
    - Quality is defined as
      - (1) a molecule of defined purity
      - (2) ability to reproduce the molecule batch after batch after batch ……

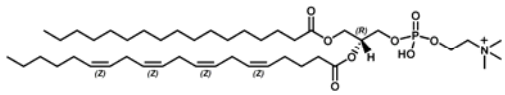
5/07

Production of MS Standards



5/07

Product Example
17:0-20:4 GPCho



LM_ID: LMGP01010003
SYS_NAME: 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine
Exact Mass: 796.59
FORMULA: $C_{45}H_{83}NO_8P$
CATEGORY: Glycerophospholipids
MAIN_CLASS: Glycerophosphocholines [01]
SUB_CLASS: Diacylglycerophosphocholines

5/07

Lipid MAPS Product Release Specification — Page 1 of 1

Mixed Acyl Phosphatidylcholine

Origination Date: January 28, 2004
Revision Date: June 28, 2004

Lipid MAPS ID No.: LMGP01010001 – LMGP01019999
Avanti Product No.: LM-1000 to LM-1099 **Specification No.: LMP-001a**
QC Sample Quantity: Not less than 200 mg

ANALYSIS	SPECIFICATION
TLC	Ninhydrin spray, negative Iodine, 1 major spot Phosphorus, positive Charring, positive (unsaturated species) negative (saturated species) Water dip, 1 major spot Rf consistent with structure
HPLC	Not less than 95% (AUC)
FAME (GC/FID)	Not less than 98% (AUC) components of interest
NMR (proton)	Consistent with theoretical structure
NMR (phosphorus)	Not less than 97% of total integrated area for compound of interest
Mass Spectrometry	Consistent with theoretical structure
Verification of sn-1 and sn-2 fatty acid structure (GC)	Not less than 90% of theoretical sn-1, sn-2 structure
Analysis to be performed on polyunsaturated samples only	
UV/VIS Oxidation	25% Oxidation at 234, 268, 278nm = not more than 5%

Approved By: _____ Date: _____
Avanti Polar Lipids Representative

Approved By: _____ Date: _____
Lipid Maps Core Representative

5/07

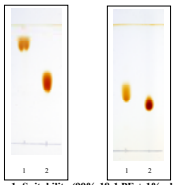
Thin layer Chromatography

Conditions: **Plate A Mobile Phase: 65:25:4 $CHCl_3$:MeOH:H_2O**
Plate B Mobile Phase: 65:25:4 $CHCl_3$:MeOH:NH_4OH

TLC Plate: **Whatman Partisil K6: 10-12 µm silica, pore size 60Å (1 inch X 3 inch)**

Data:

Plate A **Plate B**



Lane 1: Suitability (99% 18:1 PE + 1% oleic acid)
Lane 2 (17:0-20:4 PC)

Interpretation: **One spot in iodine, ninhydrin (-), phosphorus (+), char (+) and One Spot after water dip**

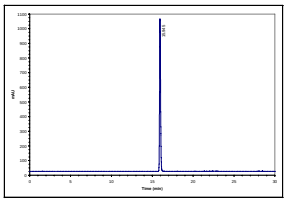
5/07

Reversed Phase HPLC-ELSD

Conditions:

Mobile Phase A:	1:1 MeOH:H_2O + 20 mM NH_4OAC
Mobile Phase B:	100 % MeOH + 20 mM NH_4OAC
Gradient:	(Linear) 100% A to 100% B over 20 minutes
Column:	C8, 4.6 mm i.d X 150 mm, 5µm
Flow rate:	1 ml/min
Detector:	Sedex 75, 40°C, 3.5 bar, gain of 5
Injection Volume:	10 µl @ 1 µg/µl

Data:



Interpret: Purity = 100% AUC

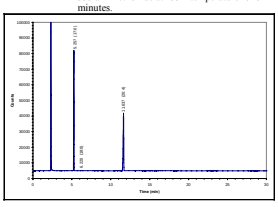
5/07

Fatty acid Methyl Esters by GC

Conditions:

Column:	DB-225, 30M X 0.25 mm, i.d.
Temperatures:	Isocratic Oven Temp. = 220° C Injection = 220°C, Detector = 250°C
Carrier:	Grade 5 Helium at 1 ml/min
Injection:	1 µl @ 1:20 split
Sample:	5 mg/ml in hexane after reaction with 25% sodium methoxide at room temperature for 5 minutes.

Data:



Interpret: Purity = 47.1% 17:0 FAME, 52.8% 20:4, 0.1% 18:0 FAME (AUC)

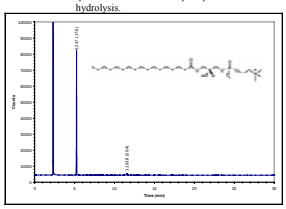
5/07

Positional Isomeric Purity by GC

Conditions:

Column:	DB-225, 30M X 0.25 mm, i.d.
Temperatures:	Oven = 220°C isocratic Injection = 250°C, Detector = 250°C
Carrier:	Grade 5 Helium at 1 ml/min
Injection:	1 µl @ 1:20 split
Sample:	Fatty acid methyl ester analysis with GC/FID of lyso PC fraction after Phopholipase A2 hydrolysis.

Data:



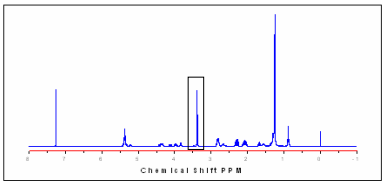
Interpretation: 97.1% sn-1, 17:0: 2.9% sn-1, 20:4 PC

5/07

Proton NMR

Conditions:

Sample Conc:	5 mg/ml in $CDCl_3$ + TMS (shift reference)
Temp:	300 K
Field Strength:	300 M Hz
Pulse:	30 ° C for 5.24 sec.
Relaxation time:	5 sec
# of scans:	32
Line Broadening	0.00 Hz
Shift standard:	TMS - 0.00 ppm



Interpretation:

Shift (ppm)	Actual Protons	Theoretical Protons	Assignment
5.20954 - 5.38665	9.107	9	olefinic protons + (proton on the 2nd carbon of the glycero backbone
4.33978 - 4.43004	2.954	3	(protons near the O on the choline) + (proton on the 1° carbon of
4.09607 - 4.15986	1	1	(proton on the 3° carbon of the glycero backbone)
3.94257 - 3.99892	2.008	2	(protons on the 3° carbon of the glycero backbone
3.83241	1.983	2	(protons near the N on the choline)
3.38089	9.111	9	(3 methyl groups on he N)
2.81104 - 2.85339	6.433	6	("interior" allylic protons)
~2.65	2.307	0	water
2.4520 - 2.34770	4.282	4	alpha protons
2.04214 - 2.11303	4.401	4	allylic protons on both "ends"
1.54489 - 1.69769	4.776	4	beta protons
1.2511	34.621	32	remaining -CH2 on the fatty acid chains
0.885563 - 0.91054	6.682	6	methyls on the fatty acids

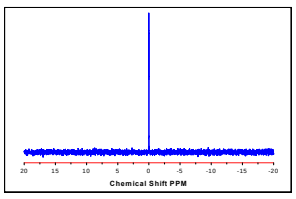
5/07

Phosphorus NMR

Conditions:

Sample conc.:	5 mg/ml in $CDCl_3$
Temp.:	300 K
Field Strength:	121.5 MHz
Pulse:	30° for 0.79 sec.
Relaxation time:	2 sec.
# of scans:	64
Line Broadening:	1.0 Hz

Data:



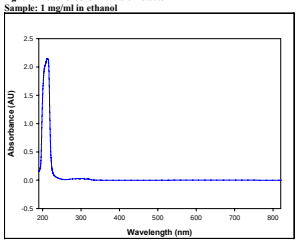
Interpret: One shift under described conditions

5/07

Oxidation by UV/Visible Spectrophotometry

Conditions: **Agilent Model 8453 UV Visible Detector**
Sample: 1 mg/ml in ethanol

Data:



Interpretation: Peroxide @ 234 nm = 0.04%
Conjugated Dienes @ 268 nm = 0.03%
Conjugated Dienes @ 278 nm = 0.05%

5/07

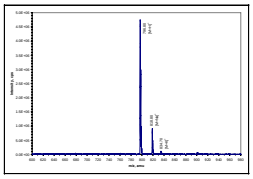
Mass Spectrometry (Q1 scan)

Qualitative Conditions: **Flow infusion Scan**

Period 1 Experiment 1:
Instrument: Applied Biosystems 4000 Q Trap
Scan Type: Q1 MS (Q1)
Polarity: Positive
Scan Mode: Profile
Ion Source: Turbo Spray

Start (amu)	Stop (amu)	Time (sec)
596.00	996.00	1.00

Data:



Interpretation: +Q1: $[M+H]^+$ = 796.80 u, $[M+Na]^+$ = 818.80 u, $[M+K]^+$ = 834.70 u.

5/07

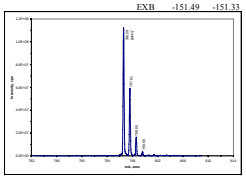
Mass Spectrometry (Ion Trap)

Qualitative Conditions: **Flow infusion Q Trap**

Period 1 Experiment 2:
Instrument: Applied Biosystems 4000 Q Trap
Scan Type: Enhanced Resolution (ER)
Polarity: Positive
Scan Mode: Profile
Ion Source: Turbo Spray

Start (amu)	Stop (amu)	Time (sec)	Param	Start	Stop
781.80	811.80	0.1201	AF3	0.30	0.31
			EXB	-151.49	-151.33

Data:



Interpret: +ER: $[M+H]^+$ = 796.59 u.

5/07

LC/MS/MS
17:0-20:4 GPCho Ampoules

Quantitative Conditions: HPLC/MS/MS Acquisition Info

Comment: Mercury Luna C18 2x20mm 3 micron
Mobile Phase A: 9:1 H20:MeOH + 10 mM NH4OAc
Mobile Phase B: 100% MeOH + 10 mM NH4OAc
Gradient: (Linear) 100% A to 100% B over 3.5 min

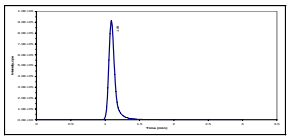
MRM Transition

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
796.70	184.30	1000.00

Step Table

Step	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0	1000	5	95
1	3	1000	5	95

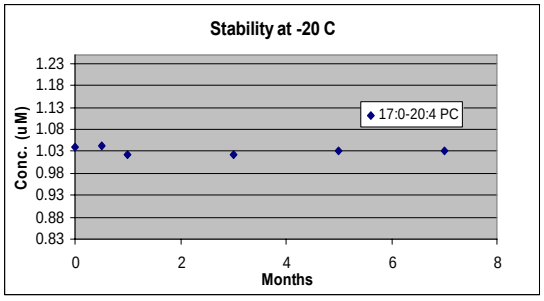
Data:



Interpret: Total 17:0-20:4 PC = 1.041 µM

5/07

Stability in Ampuoles



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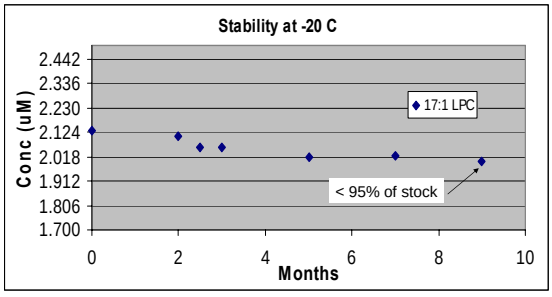
Stability in Ampuoles
Failed

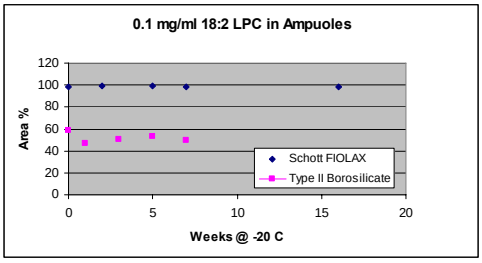


5/07

why bad things happen to good lipids!

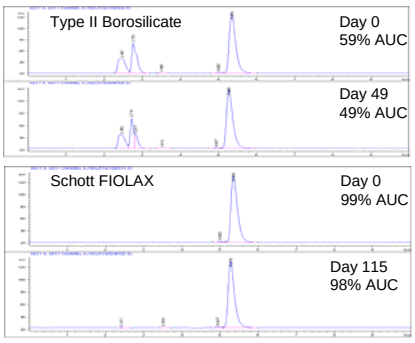
5/07

Packaging Glass Matters !



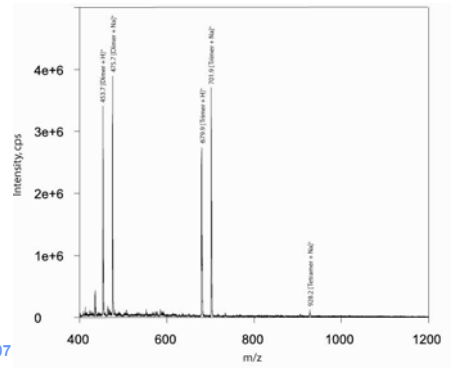
5/07

Packaging Glass Matters !



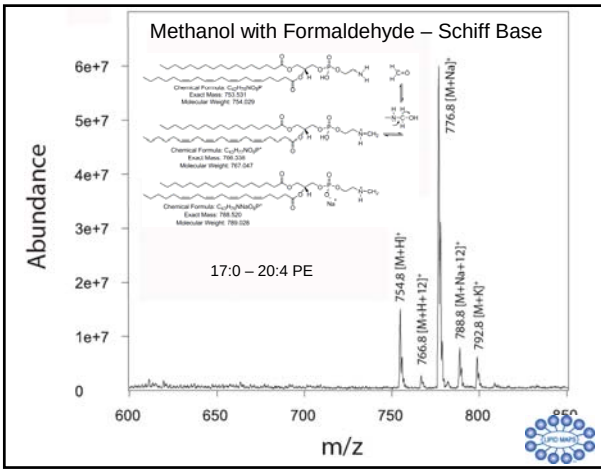
5/07

Nylon Filter Additives

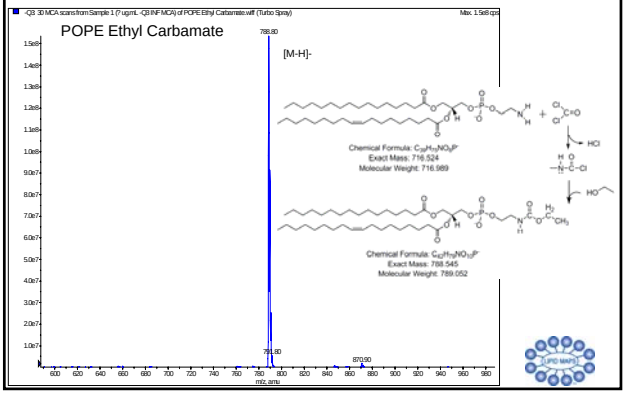


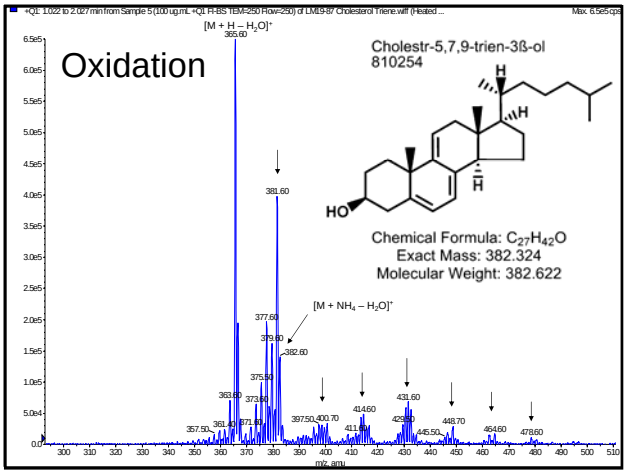
5/07

Methanol with Formaldehyde – Schiff Base



Phosgene from Chloroform





Available Standards

5/07

Sphingolipid Mixture, LM-6002

- C17 Sphingosine
- C17 Sphinganine
- C17 Sphingosine-1-phosphate
- C17 Sphinganine-1-phosphate
- C12 Sphingomyelin
- C12 Ceramide
- C12 Glucosyl Ceramide
- C12 Lactosyl Ceramide
- C12 Ceramide-1-phosphate
- C25 Ceramide

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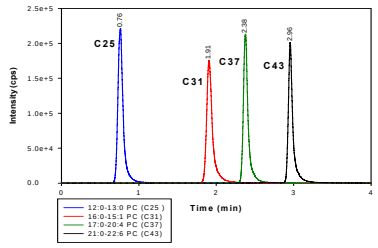
Glycerophospholipids

• LM-1000	12:0-13:0 PC	624	• LM-1400	12:0-13:0 PA
• LM-1004	17:0-14:1 PC	+ 82	• LM-1404	17:0-14:1 PA
• LM-1002	17:0-20:4 PC	+160	• LM-1402	17:0-20:4 PA
• LM-1003	21:0-22:6 PC	+240	• LM-1403	21:0-22:6 PA
• LM-1100	12:0-13:0 PE		• LM-1500	12:0-13:0 PI
• LM-1104	17:0-14:1 PE		• LM-1504	17:0-14:1 PI
• LM-1102	17:0-20:4 PE		• LM-1502	17:0-20:4 PI
• LM-1103	21:0-22:6 PE		• LM-1503	21:0-22:6 PI
• LM-1200	12:0-13:0 PG		• LM-1600	13:0 LPC
• LM-1204	17:0-14:1 PG		• LM-1601	17:1 LPC
• LM-1202	17:0-20:4 PG		• LM-1700	13:0 LPA
• LM-1203	21:0-22:6 PG		• LM-1701	17:1 LPA
• LM-1300	12:0-13:0 PS		• LM-1802	14:1(3)-15:1 CA
• LM-1304	17:0-14:1 PS		• LM-1803	15:0(3)-16:1 CA
• LM-1302	17:0-20:4 PS		• LM-1804	22:1(3)-14:1 CA
• LM-1303	21:0-22:6 PS		• LM-1801	24:1(3)-14:1 CA

5/07

Application: Mixture of GPCho ISTDs

1,2 diacyl-*sn*-glycero-3-phosphocholine species separation



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PIP's

- **LM-1900 17:0-20:4 PI(3)P**
- **LM-1901 17:0-20:4 PI(4)P**
- **LM-1902 17:0-20:4 PI(5)P**
- **LM-1903 17:0-20:4 PI(3,4)P2**
- **LM-1905 17:0-20:4 PI(3,5)P2**
- **LM-1904 17:0-20:4 PI(4,5)P2**
- **LM-1906 17:0-20:4 PI(3,4,5)P3**

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Sterols

- LM-4100 cholest-5-en-3β-ol(d7)
- LM-4101 cholest-5-en-3β,25-diol(d3)
- LM-4102 cholest-5-en-3β,4β-diol(d7)
- LM-4103 cholest-5-en-3β,7?-diol(d7)
- LM-4104 cholest-5-en-3β,7β-diol(d7)
- LM-4105 5,6α-epoxy-5α-cholestan-3β-ol(d7)
- LM-4106 5α-cholestan-3β,6α-diol(d7)
- LM-4107 7-oxo-cholest-5-en-3β-ol(d7)
- LM-4108 cholest-5,24-dien-3β-ol(d6)
- LM-4109 24,25-epoxy-cholest-5-en-3β-ol(d6)
- LM-4110 cholest-5-en-3β,24-diol(d6)

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Neutral Lipids (DG, TG)

- LM-3107 1,3-20:5 DG-d5
- LM-3108 1,3-14:0 DG-d5
- LM-3109 1,3-15:0 DG-d5
- LM-3110 1,3-16:0 DG-d5
- LM-3111 1,3-17:0 DG-d5
- LM-3112 1,3-19:0 DG-d5
- LM-3113 1,3-20:0 DG-d5
- LM-3114 1,3-20:2 DG-d5
- LM-3115 1,3-20:4 DG-d5
- LM-3116 1,3-16:1 DG-d5
- LM-3117 1,3-18:0 DG-d5
- LM-3118 1,3-18:1 DG-d5
- LM-3119 1,3-18:2 DG-d5
- LM-3207 20:5-22:6-20:5 TG-d5
- LM-3208 14:0-16:1-14:0 TG-d5
- LM-3209 15:0-18:1-15:0 TG-d5
- LM-3210 16:0-18:0-16:0 TG-d5
- LM-3211 17:0-17:1-17:0 TG-d5
- LM-3212 19:0-12:0-19:0 TG-d5
- LM-3213 20:0-20:1-20:0 TG-d5
- LM-3214 20:2-18:3-20:2 TG-d5
- LM-3215 20:4-18:2-20:4 TG-d5

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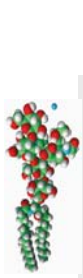
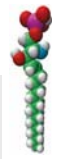
Packaging



Avanti POLAR LIPIDS, INC.

LIPID MAPS Product Labels

- PDF417 2D Barcode Information:
  - a) LIPID MAPS Reagent ID
  - b) Avanti Lot Number



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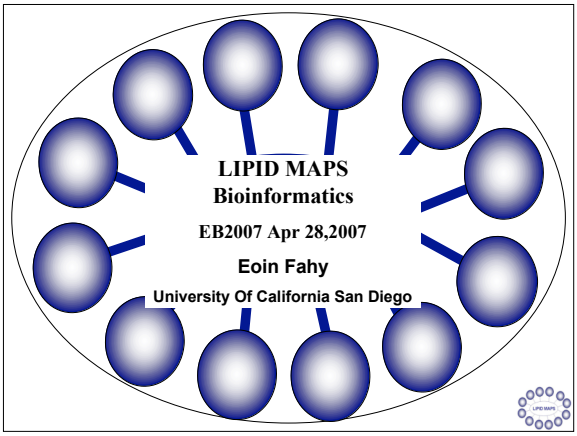
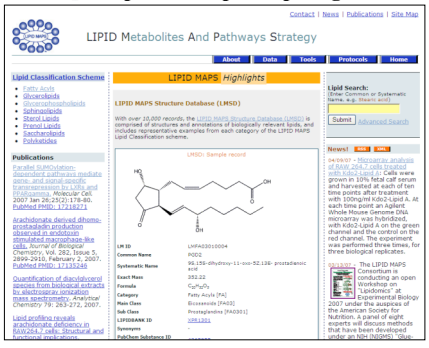


LIPID MAPS Bioinformatics Overview

1. Lipid classification, nomenclature and structure representation
2. Lipid Molecular Databases
3. Lipid-associated Protein/DNA databases
4. Lipidomic Experimental Data Display
5. Laboratory information management systems (LIMS)
6. Bioinformatics tools for lipidomics
7. Lipid-associated Pathways and Networks

LIPID MAPS Website

http://www.lipidmaps.org/

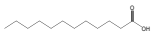


LIPID MAPS Classification System
Categories and Examples

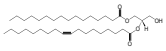
	Category	Abbrev	Example
	Fatty Acyls	FA	dodecanoic acid
	Glycerolipids	GL	1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycerol
	Glycerophospholipids	GP	1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine
	Sphingolipids	SP	N-(tetradecanoyl)-sphing-4-enine
	Sterol Lipids	ST	cholest-5-en-3β-ol
	Prenol Lipids	PR	2E,6E-farnesol
	Saccharolipids	SL	UDP-3-O-(3R-hydroxy-tetradecanoyl)-αD–N–acetylglucosamine
	Polyketides	PK	aflatoxin B_1

LIPID MAPS: Recommendations for drawing structures
Consistent structure representation across classes

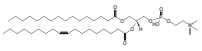
Fatty Acyls(FA)



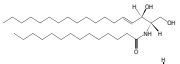
Glycerolipids (GL)



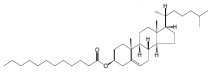
Glycerophospholipids (GP)



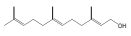
Sphingolipids (SP)



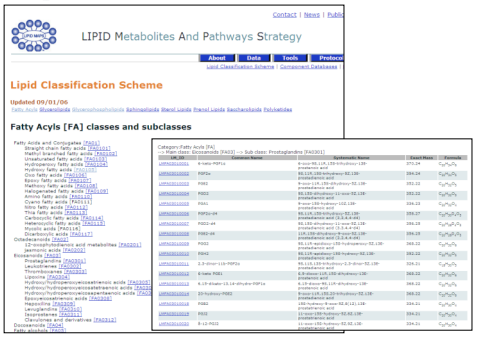
Sterol Lipids (ST)



Prenol Lipids (PR)

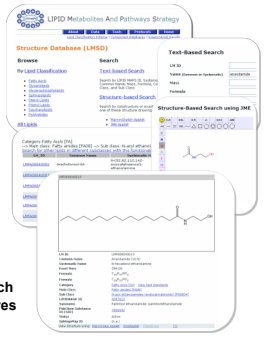


LIPID MAPS Online Classification System
http://www.lipidmaps.org/classification/



LIPID MAPS Structure Database (LMSD)

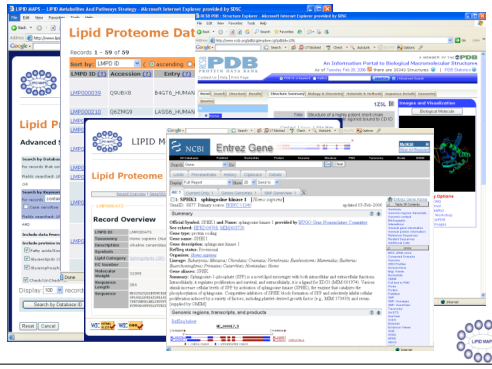
http://www.lipidmaps.org/data/structure/



Online features:
Browse
Text search
Substructure search
Download structures

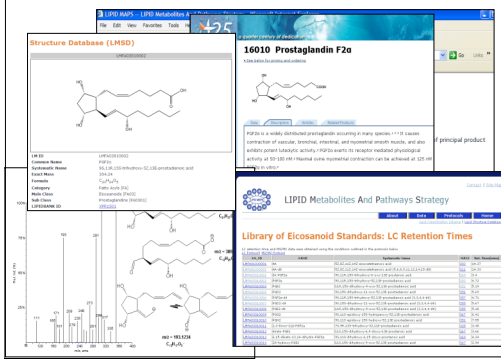
Lipid Proteome Database (LMPD)

http://www.lipidmaps.org/data/proteome/



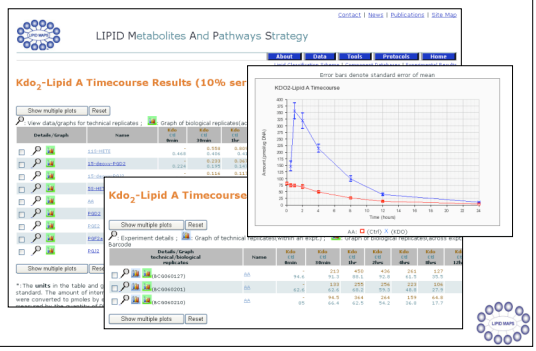
Lipidomics Data Display : MS Standards library

http://www.lipidmaps.org/data/standards/



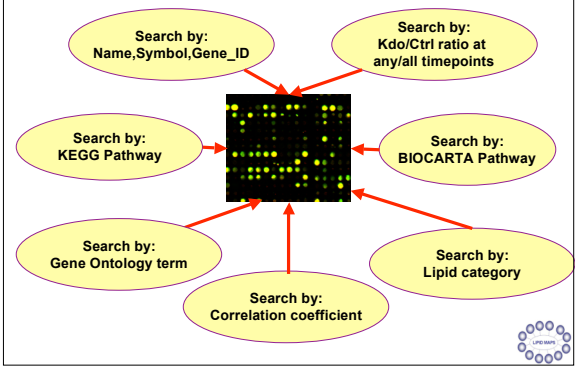
Lipidomics Data Display : MS Experimental results

http://www.lipidmaps.org/data/results/raw2647/kdo2lipidatimecourse/



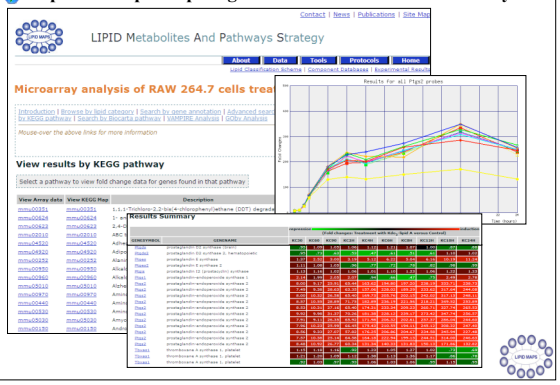
Lipidomics Data Display : Gene array data

http://www.lipidmaps.org/data/results/raw2647/microarray/

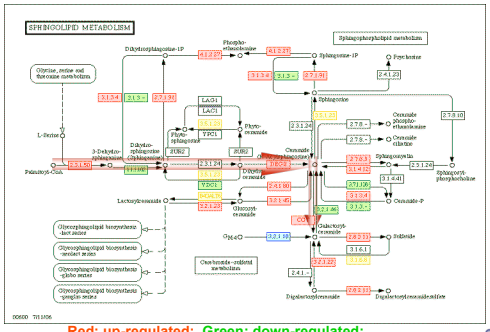


Lipidomics Data Display : Gene array-search by pathway

http://www.lipidmaps.org/data/results/raw2647/microarray/

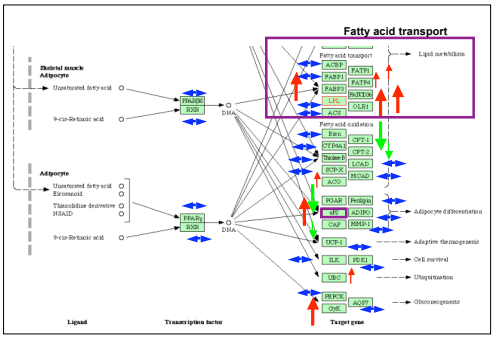


Lipidomics Data Display : Gene array data
Sphingolipid pathway(KEGG)

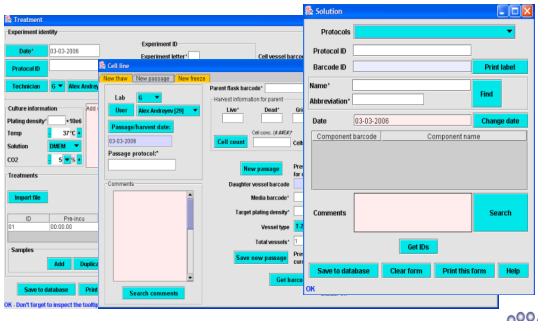


Red: up-regulated; Green: down-regulated;
Blue: up/down; Yellow: p > 0.01

Lipidomics Data Display : Gene array data
Ppar-γ signaling pathway(KEGG)



Capturing the Metadata: LIMS Data Input GUI's
(WebStart Java application communicates with Oracle database)

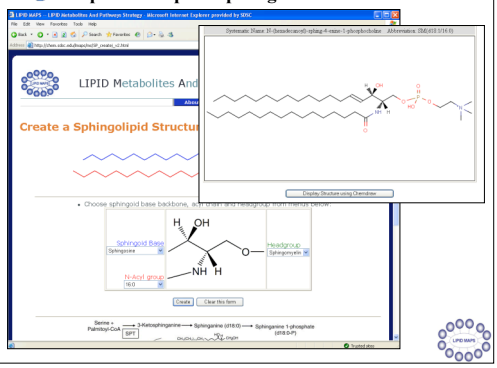


Bioinformatics tools for lipidomics

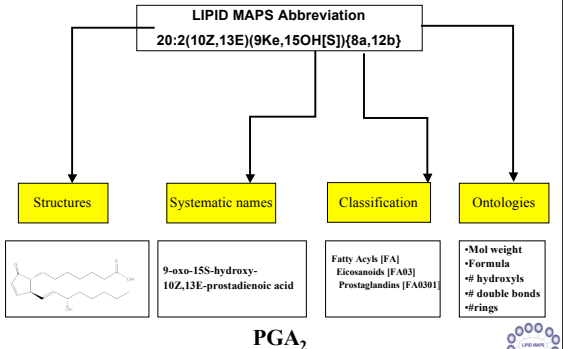
http://www.lipidmaps.org/data/tools/

Bioinformatics tools : Automated structure drawing

http://www.lipidmaps.org/data/tools/



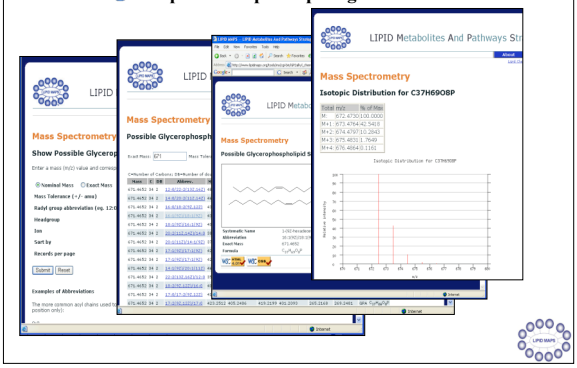
Using abbreviations to automate structure, nomenclature and ontology generation



PGA_2

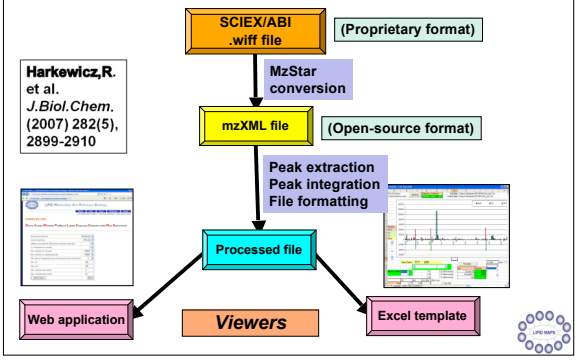
Bioinformatics tools :MS prediction

http://www.lipidmaps.org/data/tools/



Lipidomic profiling tools using stable isotopes

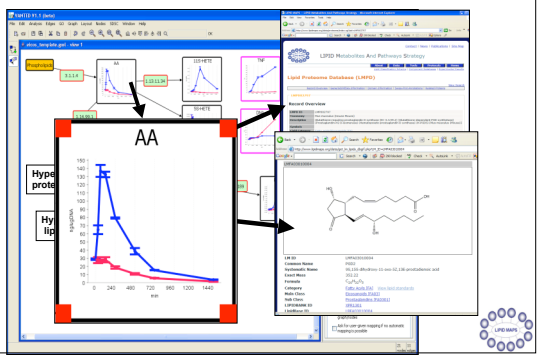
Comparative analysis using deuterated precursors



Lipid Pathways and Networks:Visualization tools

VANTED: Displaying lipidomic timecourse data

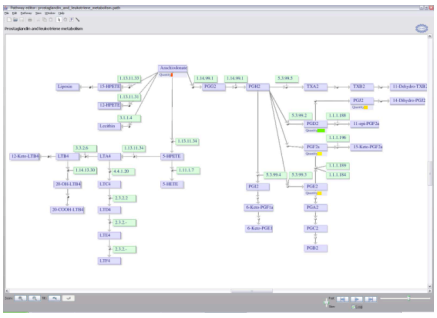
http://vanted.ipk-gatersleben.de/



Lipid Pathways and Networks:Visualization tools

Pathway Editor: Biopathways Workbench

http://www.BiopathwaysWorkbench.org



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