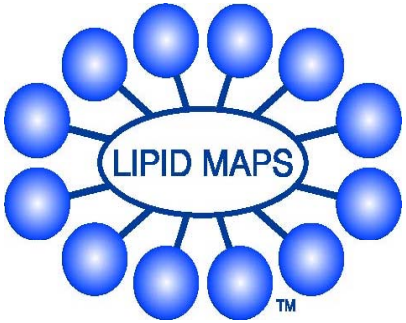


www.lipidmaps.org

LIPID MAPS Lipidomics Workshop

April 19, 2009

Lipidomic Analysis of Phosphoglycerolipids

H. Alex Brown

Departments of Pharmacology and Chemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine

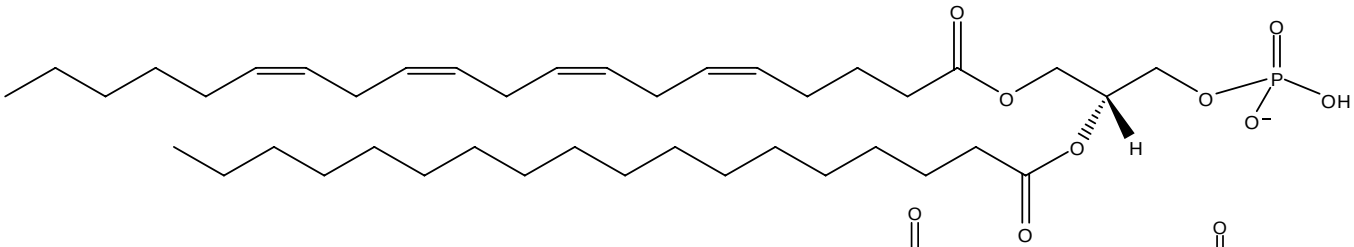
LIPID MAPS Phospholipid Core H Members:

Mass spectrometry
Stephen Milne
David Myers
Pavlina Ivanova

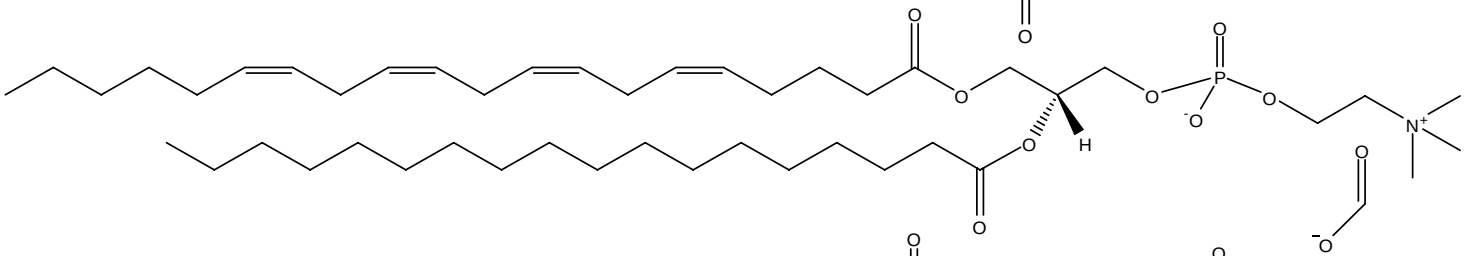
Overview

1) Phospholipid Classes Analyzed
2) Extraction Protocol
3) LC/MS Analysis
4) Internal Standards and Standard Curves
5) MS/MS Identification of Lipids
6) Online Tools for Lipid Identification
7) Phospholipid References

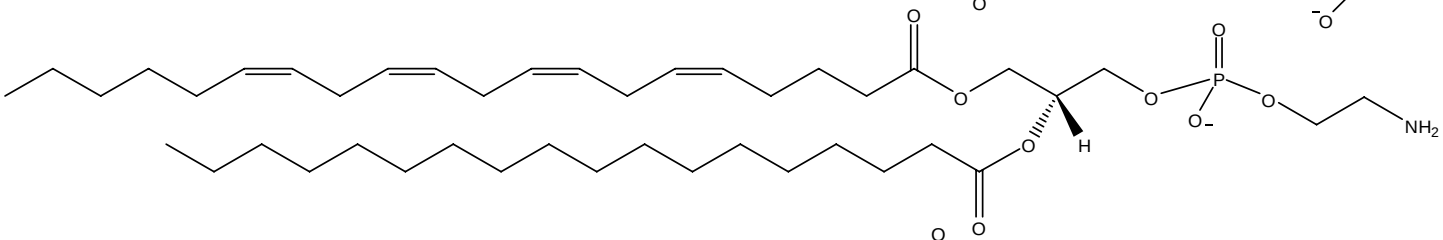
6 Major Glycerophospholipid Classes



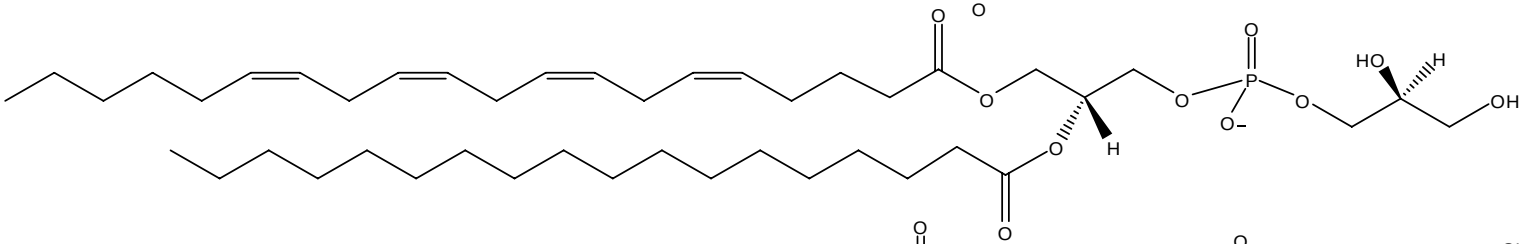
PA



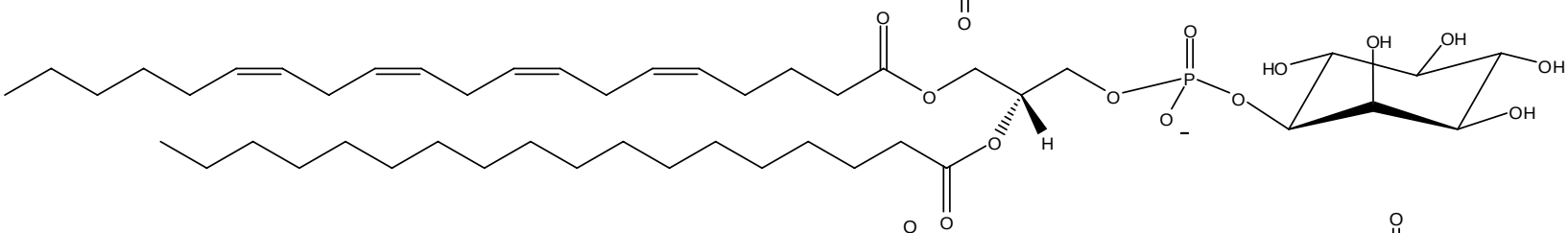
PC



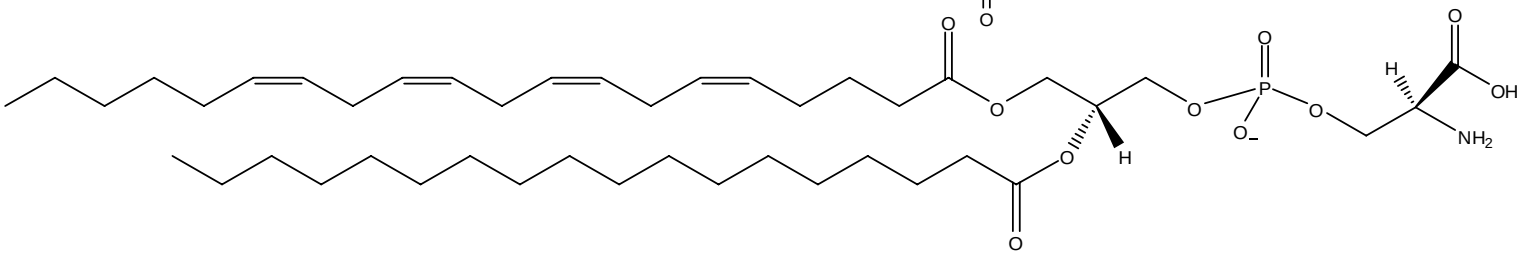
PE



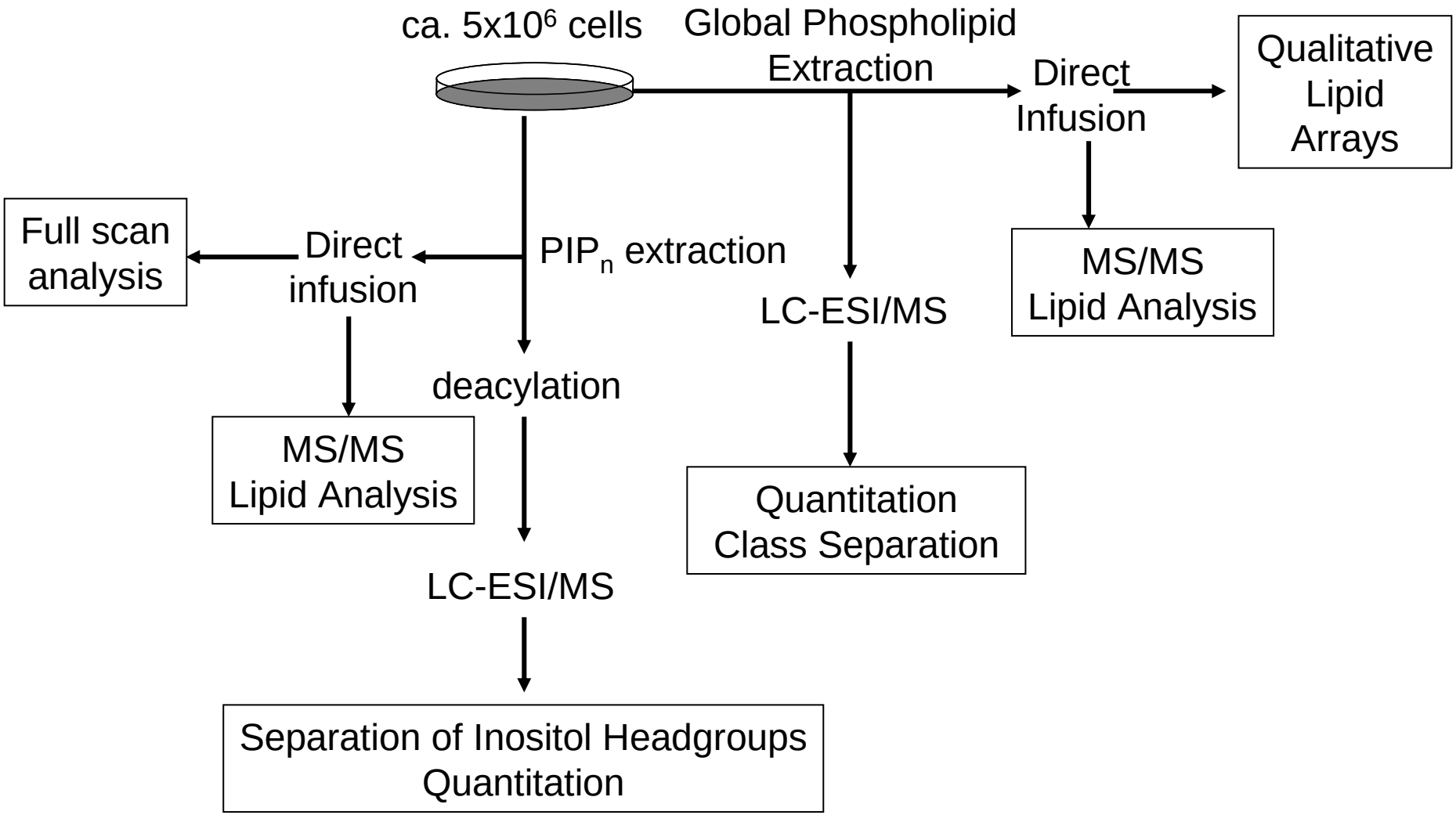
PG

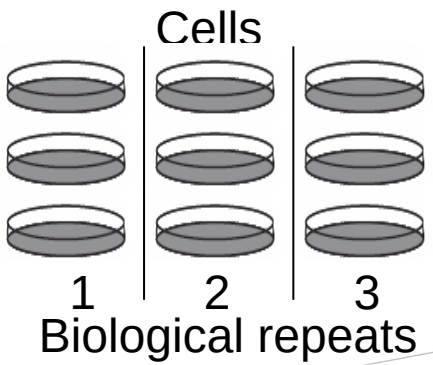


PI



PS


ca. 5x10^6 cells
Global Phospholipid Extraction
Direct Infusion
Qualitative Lipid Arrays
Full scan analysis
Direct infusion
PIPn extraction
LC-ESI/MS
MS/MS Lipid Analysis
deacylation
MS/MS Lipid Analysis
Quantitation Class Separation
LC-ESI/MS
Separation of Inositol Headgroups Quantitation



Extraction

Mass spec

Data analysis

Spectra

Direct inject pipeline

HAB lab analysis programs.
3 stds per mode (+,-)
Match peaks to ID list
Filter S/N>3
Deisotope (isotope abundance corrections)

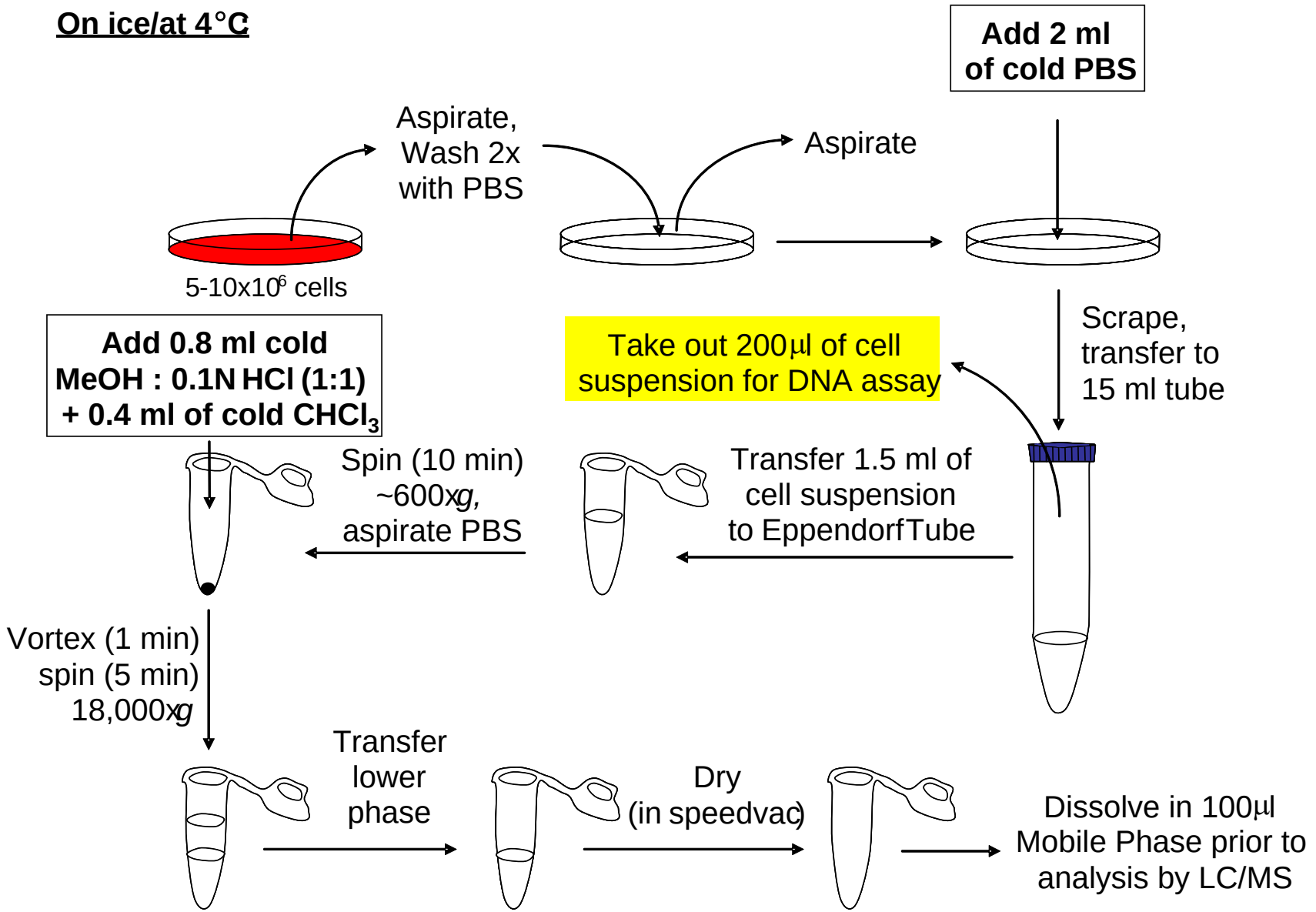
Stat analysis
Powerful 3x3 design of reps for AnoVa

LC-MS pipeline

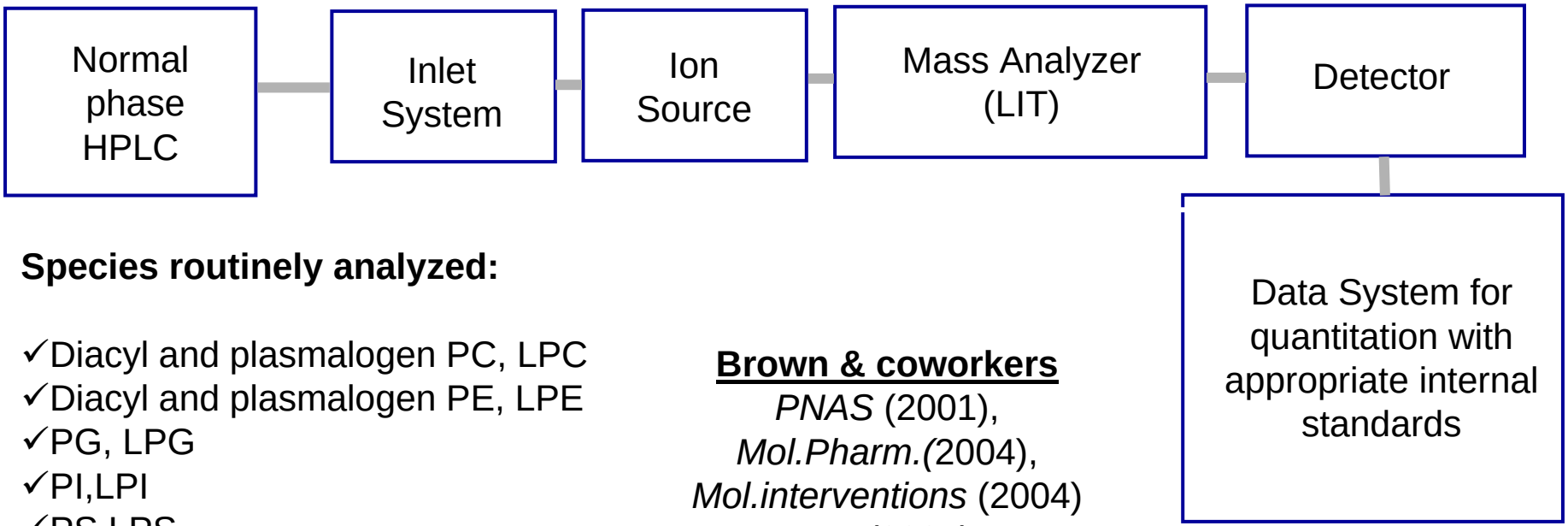
Open source converter

4 odd carbon standards per class.
Match peaks to ID list
Filter S/N>3
Deisotope
Apply nearest neighbor standard curve slope

Mammalian Cell Glycerophospholipid Extraction Procedure



Glycerophospholipid analysis by LC-MS/MS



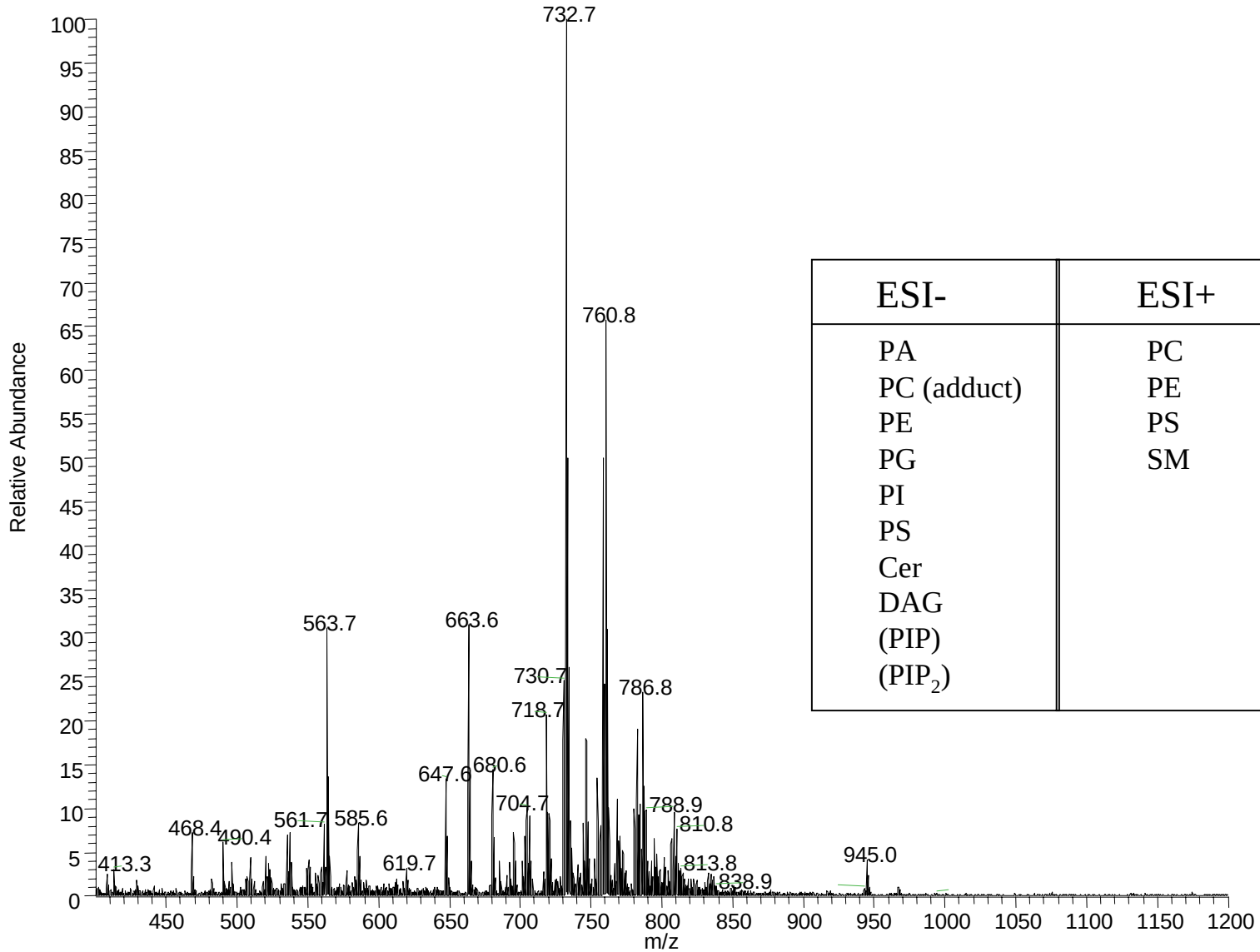
Species routinely analyzed:

- ✓Diacyl and plasmalogen PC, LPC
- ✓Diacyl and plasmalogen PE, LPE
- ✓PG, LPG
- ✓PI,LPI
- ✓PS,LPS
- ✓PA, LPA
- ✓PIP, PIP_2
- ✓SM

Brown & coworkers
PNAS (2001),
*Mol.Pharm.(*2004),
Mol.interventions (2004)
JLR (2005),
Methods (2006),
Meth. Enzymol. (2008)
Nature Chem Bio (2009)

ESI+

There are > 1000 Phospholipids in a mammalian cell



ESI-	ESI+
PA	PC
PC (adduct)	PE
PE	PS
PG	SM
PI	
PS	
Cer	
DAG	
(PIP)	
(PIP_2)	

The majority fall in the 700 and 900 *m/z range*

Quantitation Via Direct Infusion MS Isn't Possible for Most Phospholipid Classes

Every *m/z* between 700 and 900 has either a parent or isotopic peak from two or more lipid classes. As an example, lipids from 4 classes are present between *m/z* 758-762 in ESI^- mode. When considering different fatty acid combinations, there are 28 different phospholipids present in this mass range. Quantitation in regions this complex isn't possible.

m/z	PC	PE	PG	PS
758		38:1e		34:2
759			35:2	
760				34:1
761			35:1	
762	32:1e (form)	38:6		34:0

LC/MS Analysis of Phospholipids

Instrument Used: 4000 QTrap MS

Luna Silica Column, reconstituted to 100 uL, 20 uL injection,
hexane, IPA, ammonium formate solvent system. 350 to 1200 *m/z* scan range

HPLC parameters:

Phenomenex Luna Silica column 2 x 250 mm 5 micron

Mobile phase A: IPA:Hexane: 100 mM $NH_4CO_2H_{(aq)}$ 58:40:2

Mobile phase B: IPA:Hexane: 100 mM $NH_4CO_2H_{(aq)}$ 50:40:10

Flow rate: 300 uL/min

Initial %B 50

Gradient program:

Time	Event	
0.01	Controller	Start
5.00	Pump B	50%
30.00	Pump B	100%
40.00	Pump B	100%
41.00	Pump B	50%
50.00	Controller	Stop

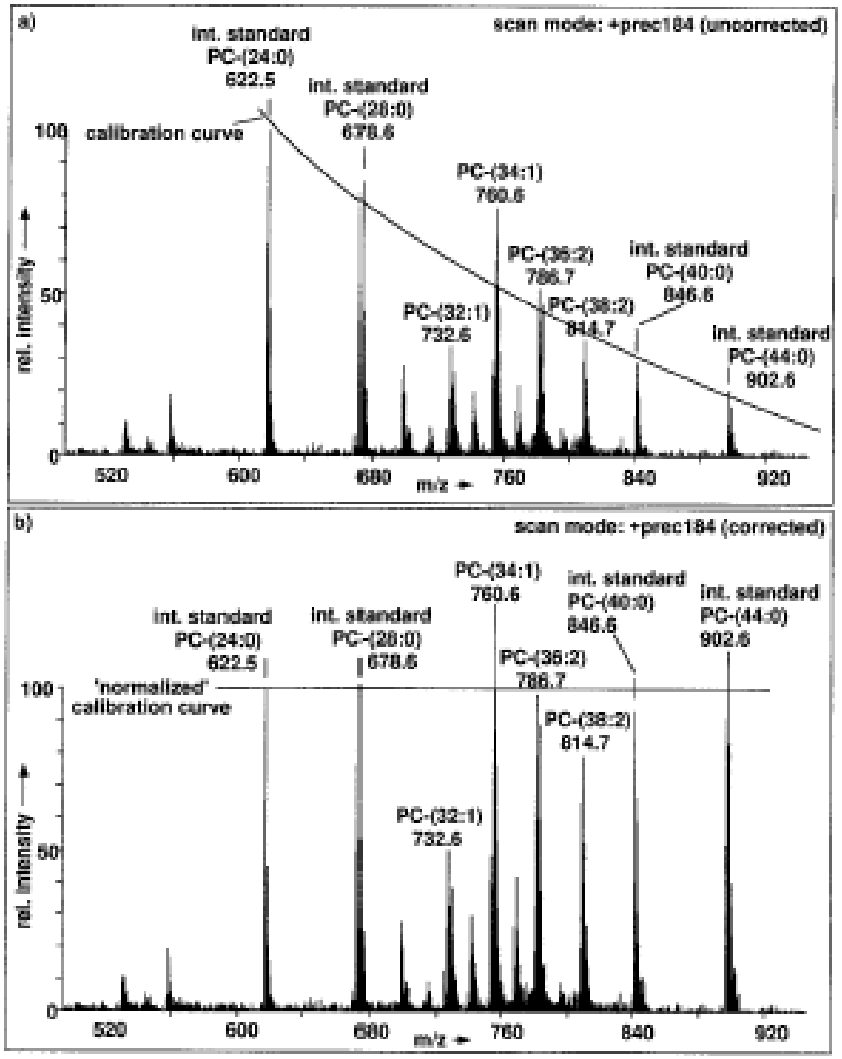


FIG. 7. An unprocessed total lipid extract of 5000 CHO cells containing equimolar amounts of PC-(24:0), -(28:0), -(40:0), and -(44:0) was analyzed by parent ion scanning for m/z 184. (*a*) Uncorrected ion intensities. The signal intensities of the internal standards were used for generation of the calibration plot insert. (*b*) Corrected ion intensities of the PC signals so that the monoisotopic signals represent the true molar abundances of the corresponding PC molecular species.

Standard Curves Should be Generated for as Many Analytes as Possible. Curves for Other Lipids can be Approximated from their Nearest Neighbors. At Least 2-4 Internal Standards per Class Should be Added to Every Sample.

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2339–2344, March 1997
Cell Biology

Cell Biology: Brügger *et al.*

Selection of internal standards

- **It is essential to use IS with similar instrument response**
- **Use several IS for each class**
  - Allows greater number of low abundance species to be detected and quantified at higher total PL concentration
  - Loosens the requirements for control the total PL concentration (low, to use fewer or 1 IS)
  - Helpful with peak assignments

LIPID MAPS internal standard cocktail

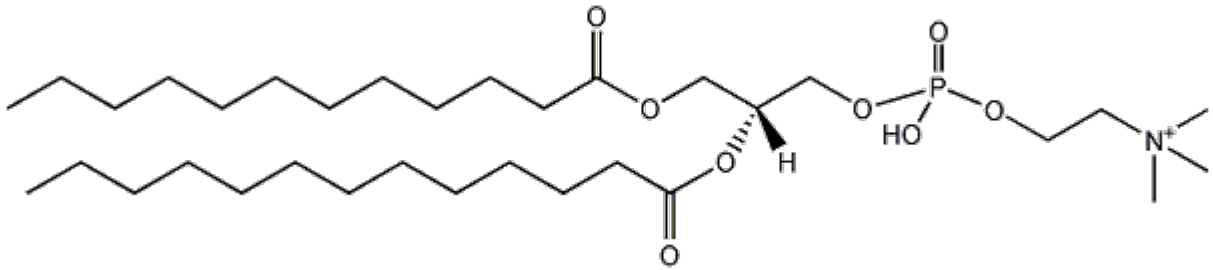
4 Odd-Carbon different length FA standards are used

for each class, containing different number of

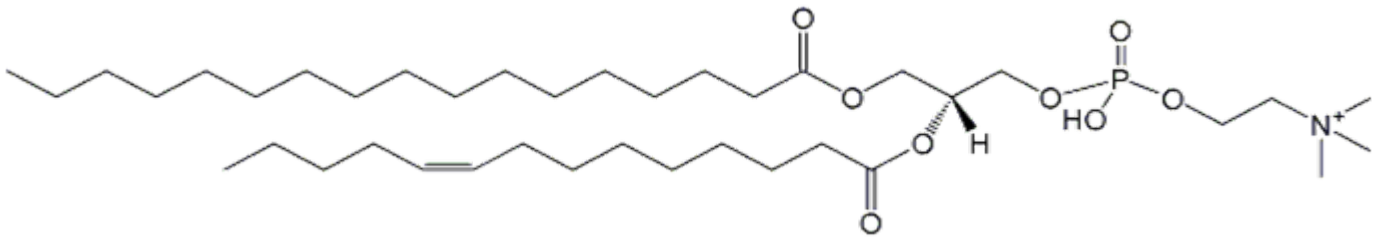
double bonds (25:0,31:1,37:4 and 43:6)

LIPID MAPS MS standards (available from Avanti Polar Lipids): 28 uncommon phospholipid species that are used to spike samples prior to analysis

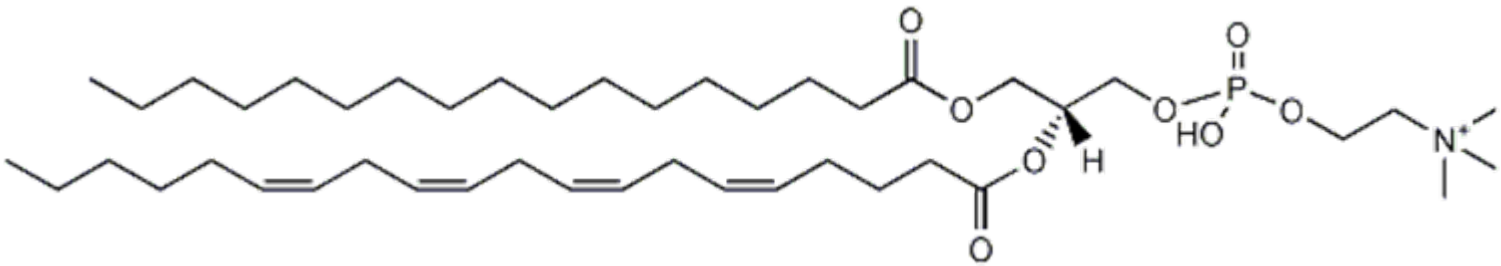
Odd-Carbon PC Internal Standards



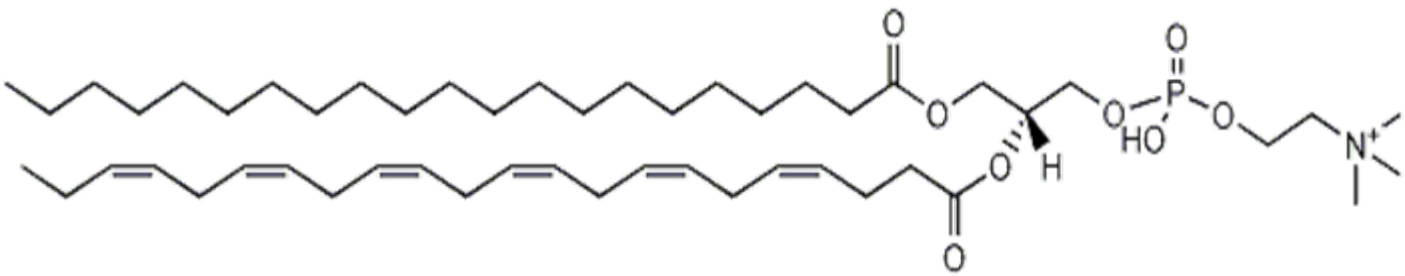
25:0 PC



31:1 PC

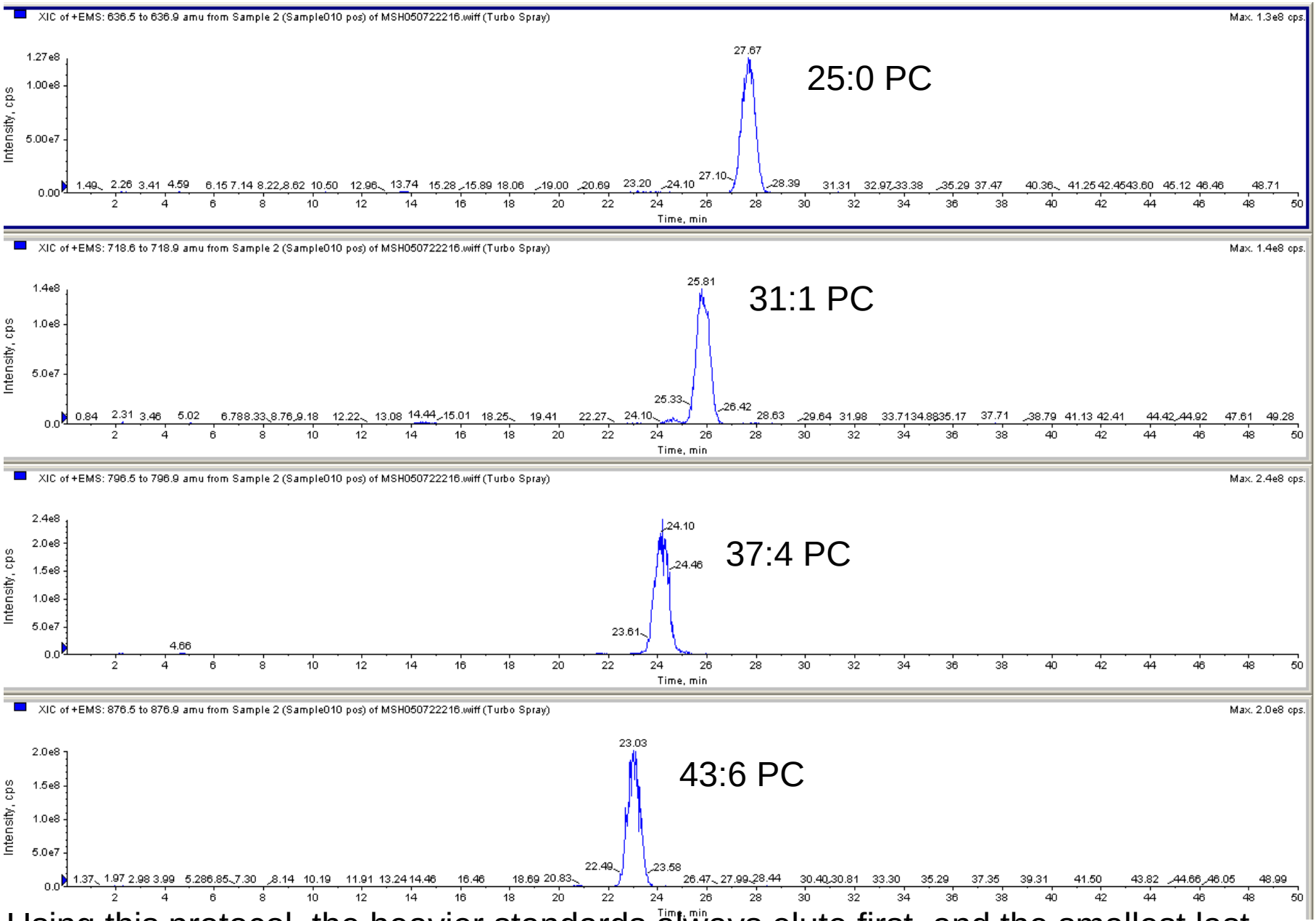


37:4 PC



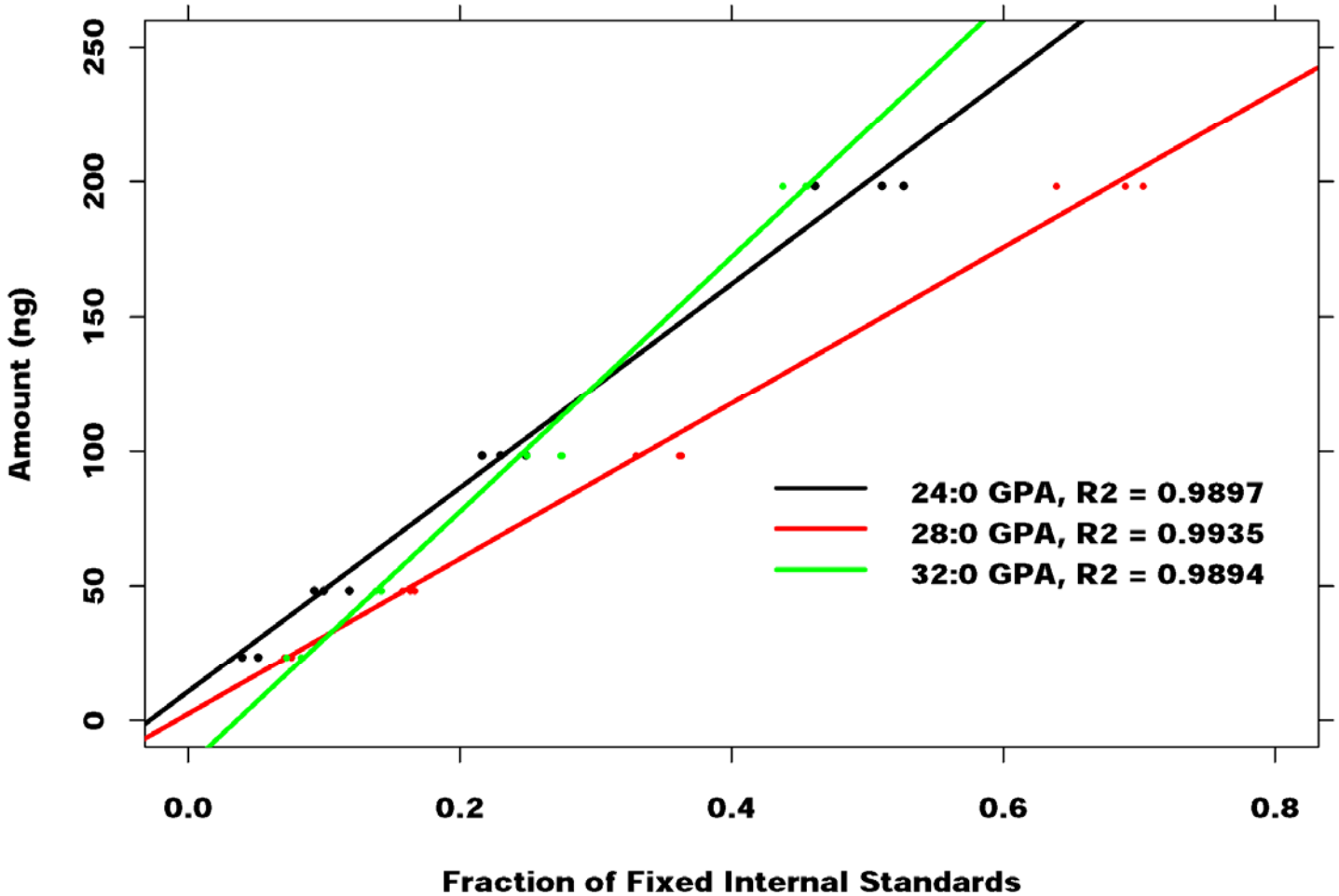
43:6 PC

HPLC Elution Pattern for PC Standards



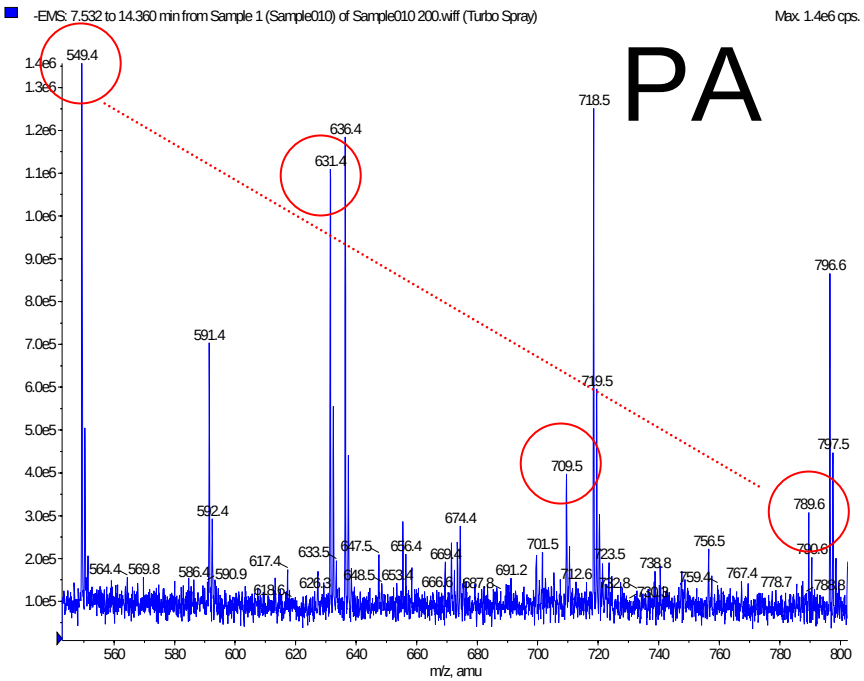
Using this protocol, the heavier standards always elute first, and the smallest last. Carbon number has greater impact on RT than does degree of unsaturation.

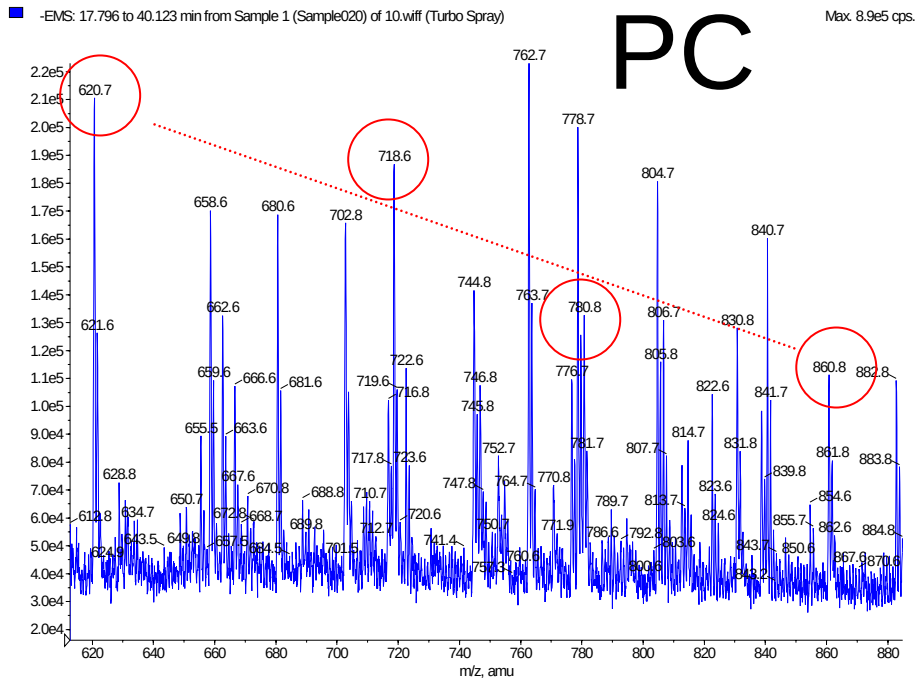
Example of 3 Saturated PA Standard Curves



The above curves were generated using even carbon PA standards and fixed amounts of 4 odd-carbon PA internal standards.

Use multiple odd internal standards per class (25:0, 31:1, 37:4, 43:6) covers the diversity of heterogenous, chemically defined space





LC/MS analysis

- **Elution Order of Phospholipid Classes: PG<PE<PI<PA<PS<<PC**
- **Least Polar< Most Polar**
- **Lyso Lipids Elute a Few Minutes After Diacyl Variants.**

Identification of Phospholipids by MS/MS Fragmentation

1) All six classes can be analyzed in ESI negative mode.

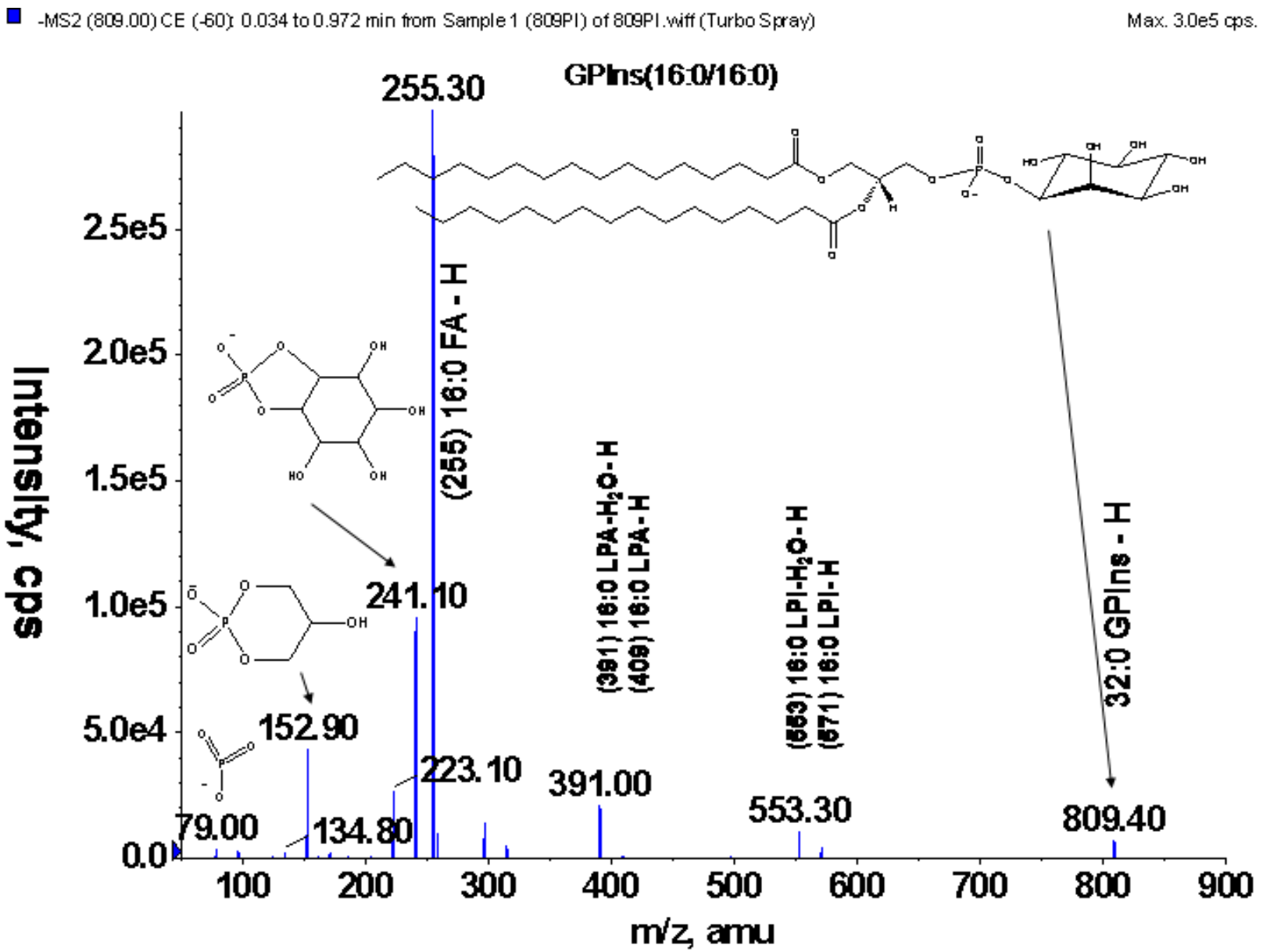
2) ESI negative mode is best for gathering structural information.

3) *sn*-1 and *sn*-2 fatty acid positions in mixtures of lipids can not be determined.

4) Each lipid class (except PA) has characteristic headgroup MS/MS fragments.

	Best Method for Detection	Characteristic Headgroup Fragments	
		ESI (-)	**ESI (+)**
PA	ESI (-)	no unique fragments	
PC	ESI (+)	224 (PC detected as adduct with anion)	184
PE	ESI (-)	196	NL 141
PG	ESI (-)	227	
PI	ESI (-)	223, 241, 259, 297, 315	
PS	ESI (-)	NL 87	NL 185

Fragmentation of a PI(16:0/16:0) standard



Number of species quantified from a typical LC/MS scan

PA	PC(p)	PE(p)	PG	PI	PS	PThr
18	51(15)	37(13)	18	16	31	3

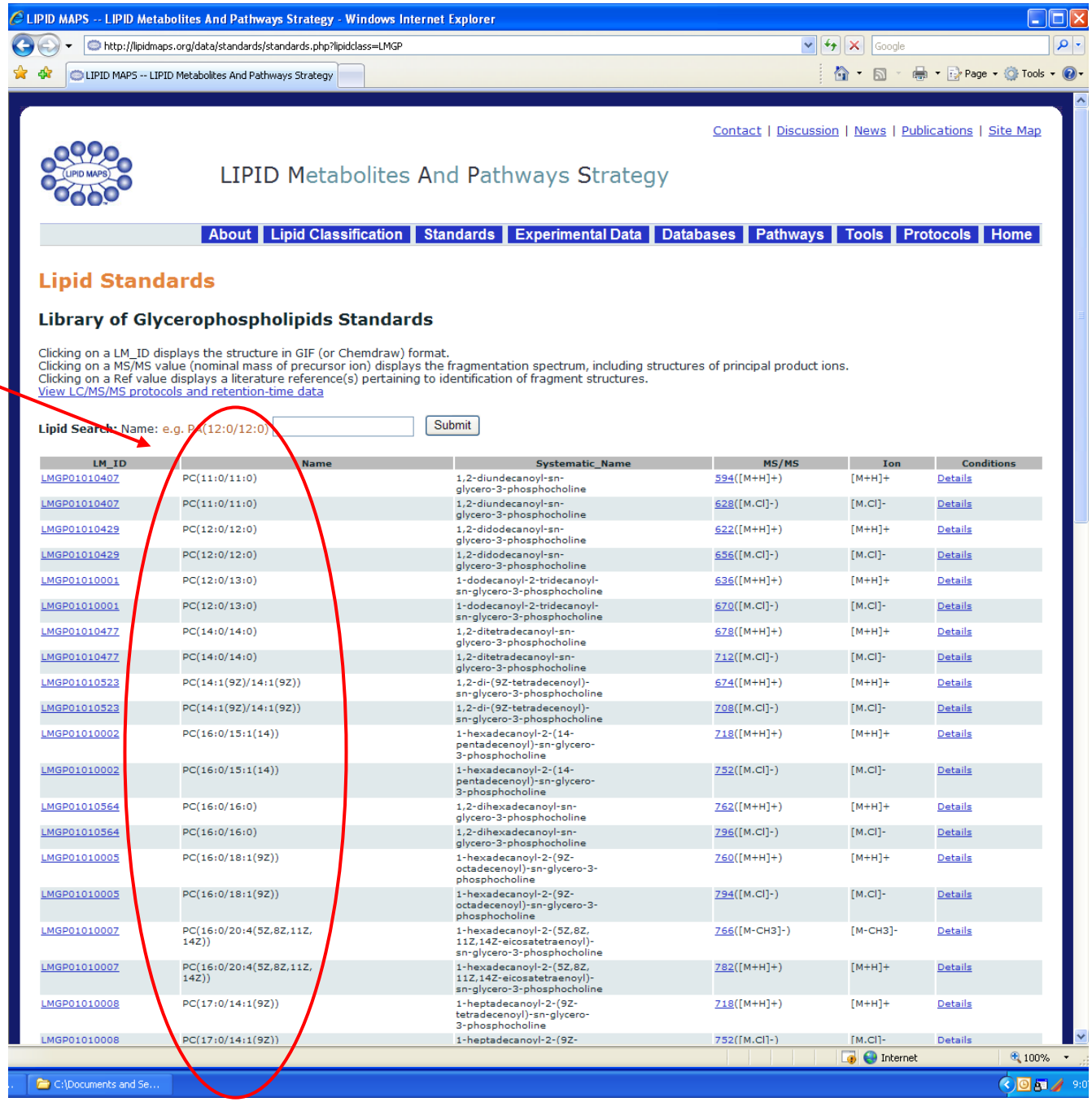
(e.g., total = 174 from this sample).

To date we have identified > 1200 species of GPL in macrophages (spectra and fragmentation available at

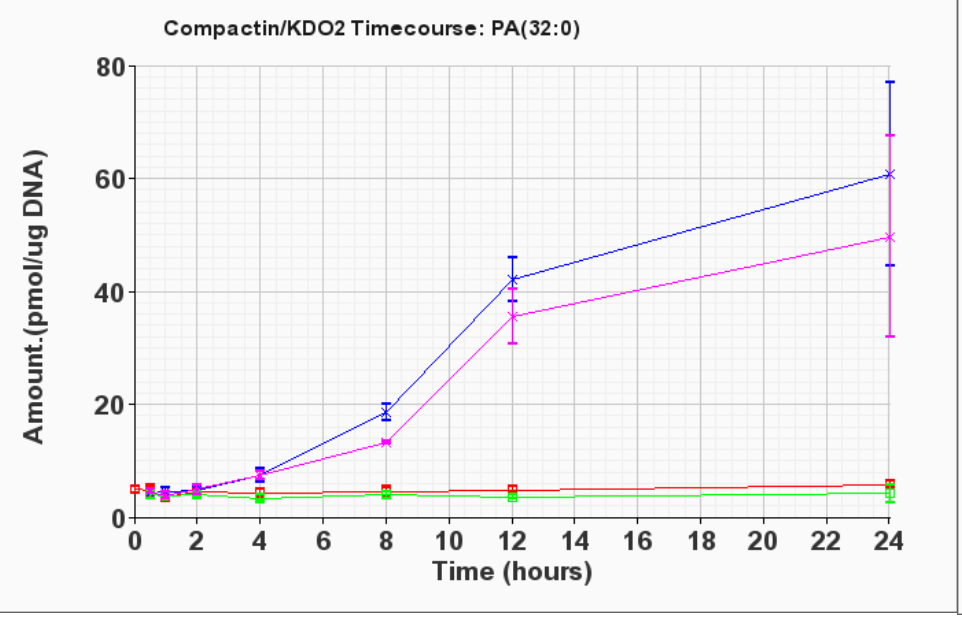
http://www.lipidmaps.org/ and publications available at http://www.alexbrownlab.org).

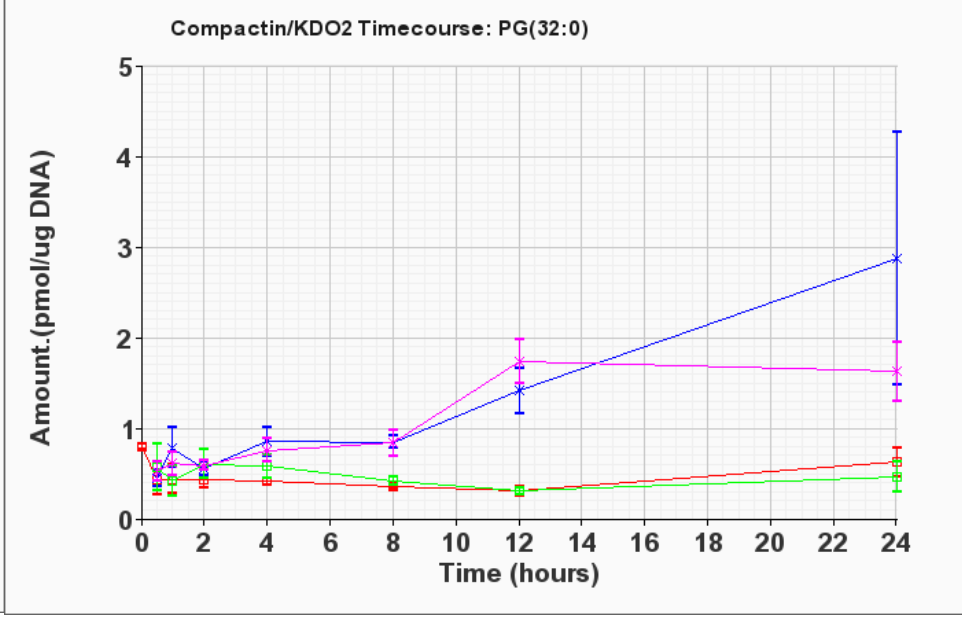
lipidmaps.org

Standards for over 200 glycerophospholipids

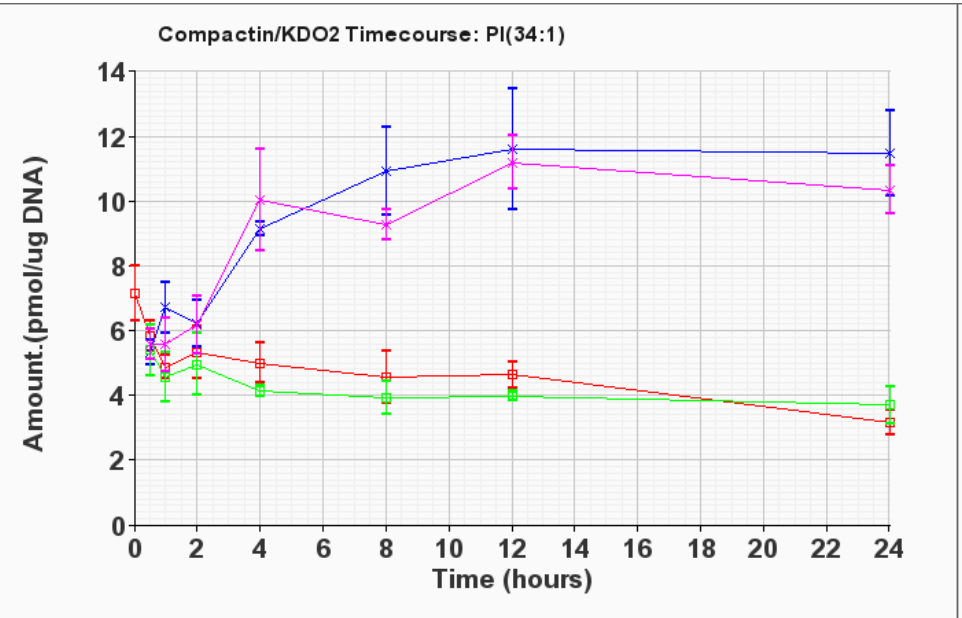


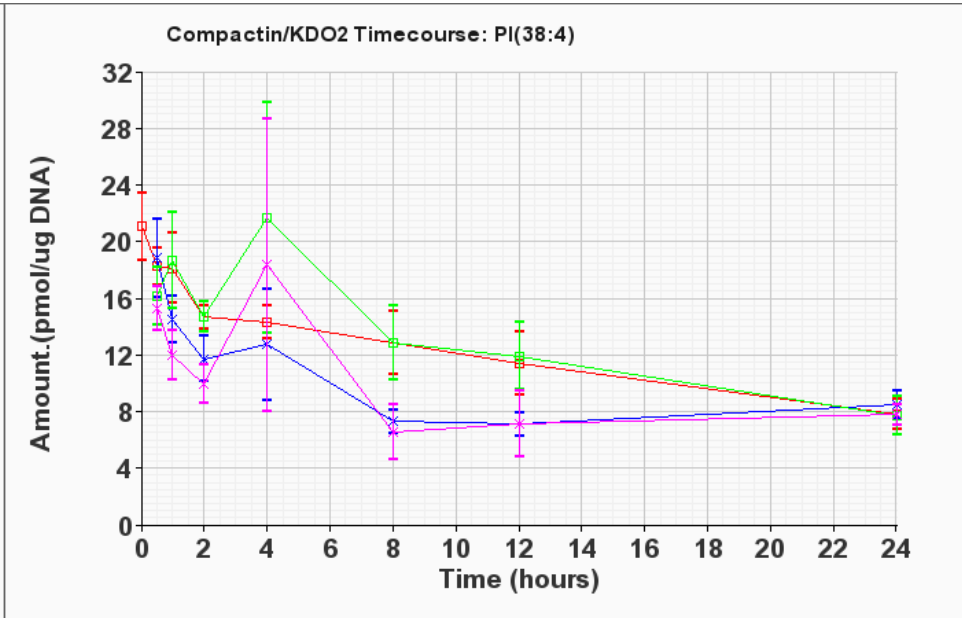
LM_ID	Name	Systematic_Name	MS/MS	Ion	Conditions
LMGP01010407	PC(11:0/11:0)	1,2-diundecanoyl-sn-glycero-3-phosphocholine	594([M+H]+)	[M+H]+	Details
LMGP01010407	PC(11:0/11:0)	1,2-diundecanoyl-sn-glycero-3-phosphocholine	628([M.Cl]-)	[M.Cl]-	Details
LMGP01010429	PC(12:0/12:0)	1,2-didodecanoyl-sn-glycero-3-phosphocholine	622([M+H]+)	[M+H]+	Details
LMGP01010429	PC(12:0/12:0)	1,2-didodecanoyl-sn-glycero-3-phosphocholine	656([M.Cl]-)	[M.Cl]-	Details
LMGP01010001	PC(12:0/13:0)	1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphocholine	636([M+H]+)	[M+H]+	Details
LMGP01010001	PC(12:0/13:0)	1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphocholine	670([M.Cl]-)	[M.Cl]-	Details
LMGP01010477	PC(14:0/14:0)	1,2-ditetradecanoyl-sn-glycero-3-phosphocholine	678([M+H]+)	[M+H]+	Details
LMGP01010477	PC(14:0/14:0)	1,2-ditetradecanoyl-sn-glycero-3-phosphocholine	712([M.Cl]-)	[M.Cl]-	Details
LMGP01010523	PC(14:1(9Z)/14:1(9Z))	1,2-di-(9Z-tetradecenoyl)-sn-glycero-3-phosphocholine	674([M+H]+)	[M+H]+	Details
LMGP01010523	PC(14:1(9Z)/14:1(9Z))	1,2-di-(9Z-tetradecenoyl)-sn-glycero-3-phosphocholine	708([M.Cl]-)	[M.Cl]-	Details
LMGP01010002	PC(16:0/15:1(14))	1-hexadecanoyl-2-(14-pentadecenoyl)-sn-glycero-3-phosphocholine	718([M+H]+)	[M+H]+	Details
LMGP01010002	PC(16:0/15:1(14))	1-hexadecanoyl-2-(14-pentadecenoyl)-sn-glycero-3-phosphocholine	752([M.Cl]-)	[M.Cl]-	Details
LMGP01010564	PC(16:0/16:0)	1,2-dihexadecanoyl-sn-glycero-3-phosphocholine	762([M+H]+)	[M+H]+	Details
LMGP01010564	PC(16:0/16:0)	1,2-dihexadecanoyl-sn-glycero-3-phosphocholine	796([M.Cl]-)	[M.Cl]-	Details
LMGP01010005	PC(16:0/18:1(9Z))	1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine	760([M+H]+)	[M+H]+	Details
LMGP01010005	PC(16:0/18:1(9Z))	1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine	794([M.Cl]-)	[M.Cl]-	Details
LMGP01010007	PC(16:0/20:4(5Z,8Z,11Z,14Z))	1-hexadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine	766([M-CH3]-)	[M-CH3]-	Details
LMGP01010007	PC(16:0/20:4(5Z,8Z,11Z,14Z))	1-hexadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine	782([M+H]+)	[M+H]+	Details
LMGP01010008	PC(17:0/14:1(9Z))	1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phosphocholine	718([M+H]+)	[M+H]+	Details
LMGP01010008	PC(17:0/14:1(9Z))	1-heptadecanoyl-2-(9Z-	752([M.Cl]-)	[M.Cl]-	Details



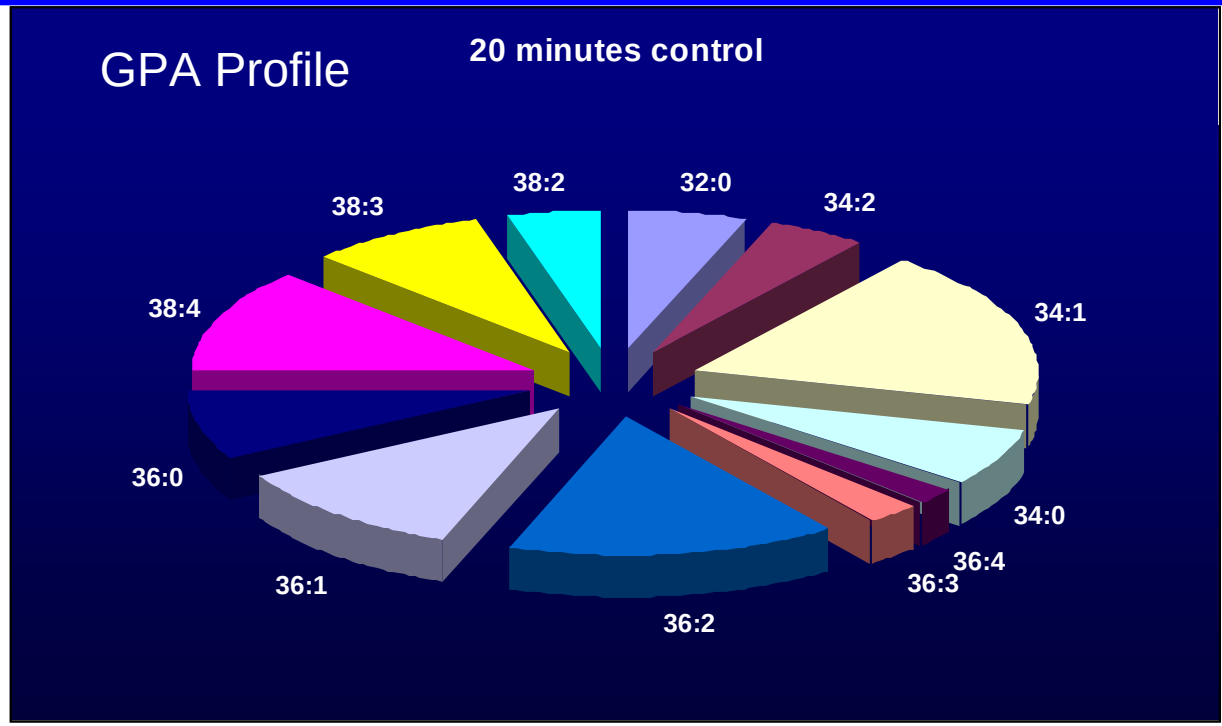


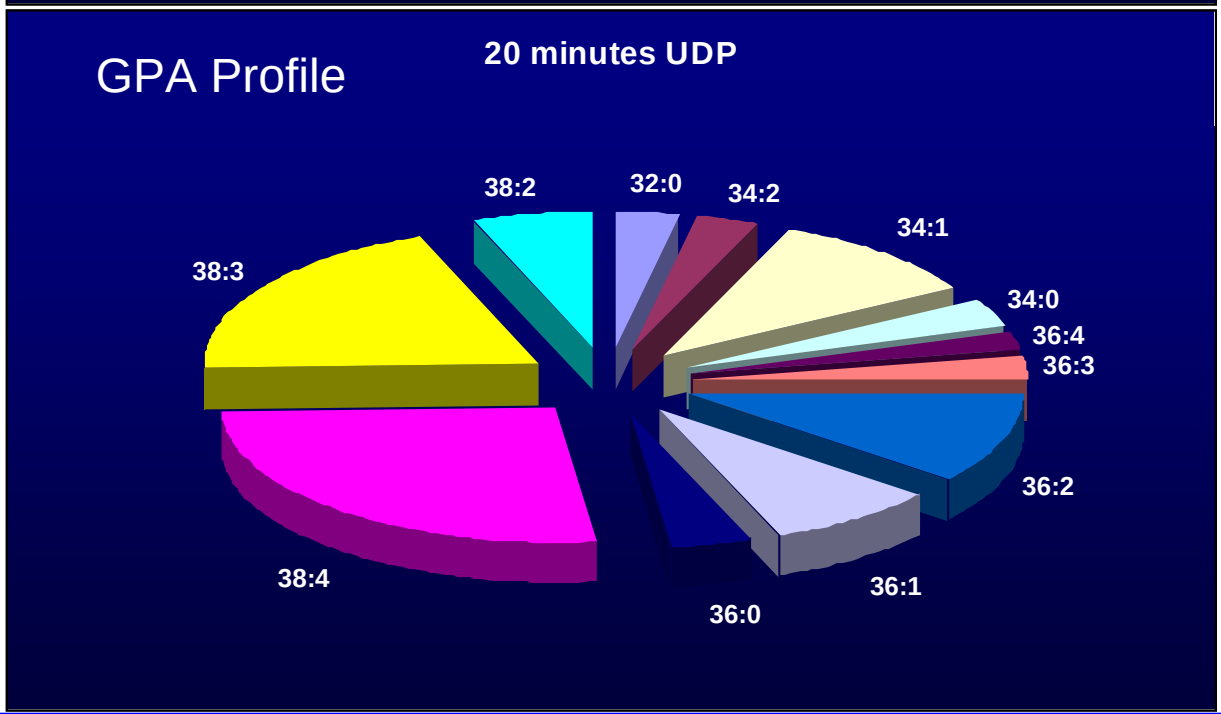
KDO/Compactin experiments in RAW cells (ctrl kdo compactin kdo+compactin)





UDP





LIPID MAPS

“Challenges and opportunities”

- Novel and Atypical lipids (e.g., ether PI) discovery.
- New MS based assay for PLD activity (PtdBuOH measurements by deuterated BuOH transesterification).
- Define lipome of cells & organisms (e.g., viruses, bacteria, macrophages, tumors).
- Substrate-product relationships (signaling and metabolic networks).