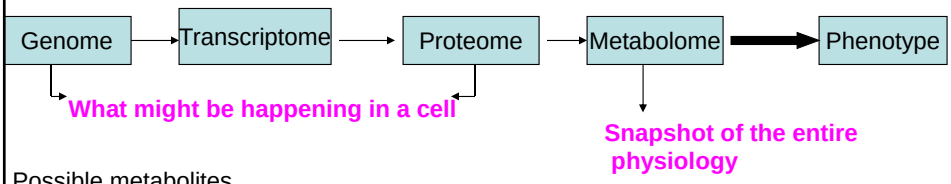
Mass Spectrometry analysis of Small molecules

Metabolomics- A realm of small molecules (<1000 Da)

Metabolomics in the context of other omics



Possible metabolites

Amino acids
Fatty acids
Phenolics
Prostaglandins
Steroids
Organic acids
Organic amines
Nucleosides
Nucleotides
Polyamines
Lipids etc.

Steps involved in metabolomic analysis

- Profiling involves finding of all metabolites detectable to a selected analytical technique with statistically significant variations in abundance within a set of experimental and control groups.
- Identification of chemical structures of metabolites of interest after profiling
- Quantification and validation
- Interpretation of data making connections between the metabolites discovered and the biological conditions

Metabolomics is complementary to the other omics and the combination of these three may provide important information about the status of a cell

Application of metabolomics

- Nutrition sciences- eg. oil seed analysis/polyphenols/food adulteration/quality control
- Herbal drug evaluation, drug discovery
- Biomarker identification- eg. Cancer
- Toxicology assessment/functional genomics

Metabolomics

- Targeted (quantitative)- measurement of defined groups of chemically characterized and biochemically annotated metabolites using optimized assay
- Untargeted- comprehensive analysis of all the measurable analytes in a sample, including chemical unknowns.

Targeted/untargeted metabolomics

- **Targeted metabolomics-** extraction procedures can be optimized for compounds of interest
- Optimized MRM or SRM can be used for quantitation when standards are available.
- Provide comprehensive understanding of a vast array of metabolic enzymes, their kinetics, and biochemical pathways.
- **Untargeted metabolomics-** single extraction method may not able to extract all compounds and important compounds may be missed during extraction. Data mining can be a problem and requires the use of metabolomic software for identification.
- Offers opportunities for discoveries of novel drug, and biomarkers.

Platform to process untargeted metabolomic data

- XCMS (developed by the Siuzdak Lab at the Scripps Research Institute) Online, is a web-based version that allows users to easily upload and process LC-MS data. It is a bioinformatics platform to identify endogenous metabolites..
- METLIN (developed by the Siuzdak Lab.) is a metabolite database for metabolomics containing over 64,000 structures and it also has comprehensive tandem mass spectrometry data on over 10,000 molecules.

Workflow for metabolome analysis

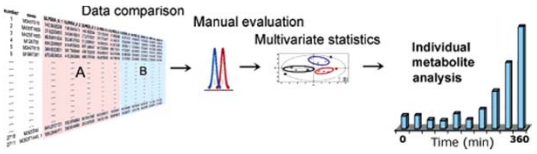
Sample collection → Treatment or Diseased group / Control group

Urine, plasma, tissue etc.

Sample preparation → Internal standard spiking for quantitative analysis
Extraction (liquid-liquid, ppt with or without hydrolysis)

Sample analysis → LC-MS and LC-MS/MS analysis
- Q1 scan using HPLC or UPLC/-TOFMS
- +ve/-ve ion mode- accurate mass measurement
- MS/MS experiments using a hybrid instrument Q-Trap

Data export → Data analysis
Multivariat analysis e.g. PCA



Marker identification

Points to be considered in LC-MS analysis

- **Choice of ionization mode- ESI Vs APCI +ve/-ve modes**
- **Choice of eluting solvent- methanol Vs acetonitrile**
- **Additives/pH in mobile phase**
- **Molecular ion recognition (adduct formation)**
- **Chromatographic separation- stationary phase C8, C18 ..**
- **Evaluation of spectral quality- what to look for in a good quality spectra**

Sample preparation

Sample collection

↓

Quenching by liquid Nitrogen or cold methanol
(stops metabolism)

↓ Prepare internal standard stock solution

Extraction of metabolites
(methanol, methanol-water for polar)
(chloroform or hexane for less polar)
(protein precipitation, supercritical fluid extraction)

↓

Concentration
(evaporation under vacuum, lyophilization, SPE)

MS acquisition strategy

Full scan (Q1 scanning) for total profiling of metabolites
(+ve and –ve ion mode) ESI/APCI

ESI- Effluent is charged and nebulized, for semi- polar or polar compounds e.g. Conjugated metabolites.

APCI- Effluent is heated but not charged- a corona discharge is needed. Good for neutral or less polar compound.

ESI is the most common ionization method

Advantage: non-selective and most ionizable ions are detected
Disadvantage: low sensitivity and detection of minor metabolites is compromised.

MS analysis

- **Direct MS analysis**

- Without chromatographic separations- HTS possible.

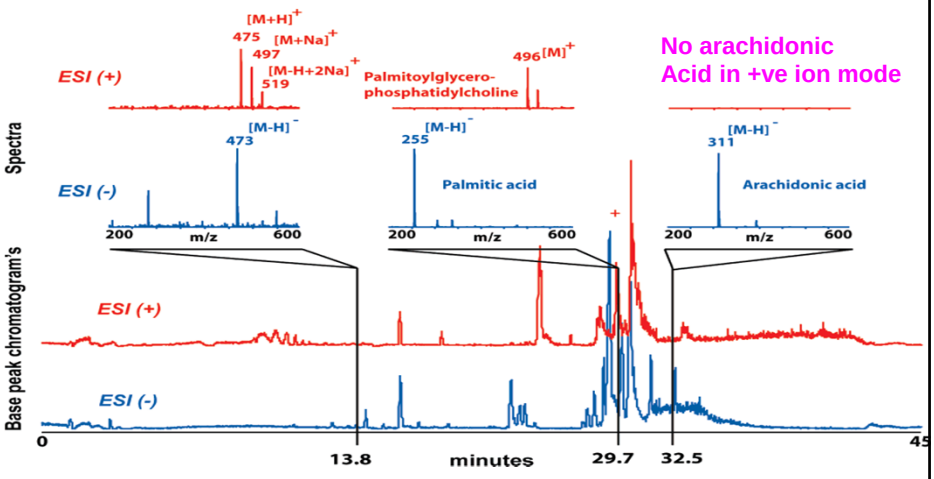
- High resolution MS – FTICR-MS high resolution >1,000,000 and mass accuracy (<1 ppm)

- Problems- difficult to interpret the data
- **LC-MS and MS/MS**
- **- GC-MS (volatile metabolites) and LC-MS- normal phase, reverse phase (C8/C18) and HILIC. UPLC-QTOF-MS for highly complex plant metabolomics.**

Quantification

- **Relative or absolute quantification**.
- Relative- normalizes the metabolite signal that of an internal standard signal intensity in large scale un-targated profiling (eg. Non-naturally occurring lipid standards- Cer 17, stable isotope labeling through metabolism- AA-d_8.
- Absolute quantification- based on external standards or internal isotopically labeled standards- targeted metabolomics.
- Matrix effects- signal suppression or enhancement are major issues. Stable isotope labeled standards are needed.

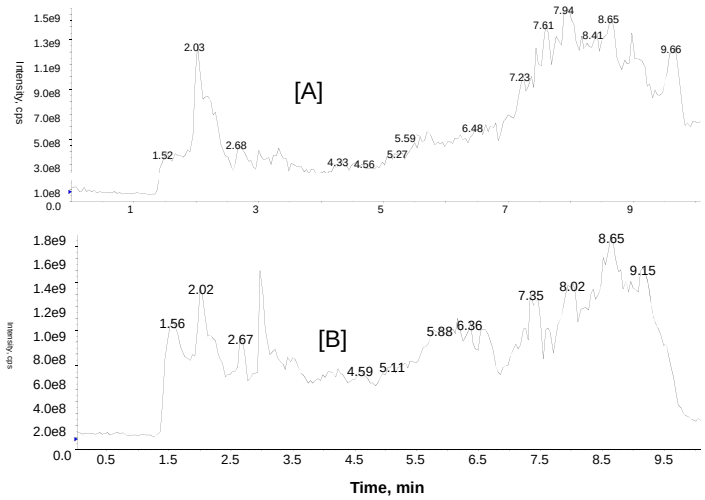
Increasing metabolite coverage using +ve and –ve ion mode



Representative Q1 scans of a methanolic extract of human blood serum

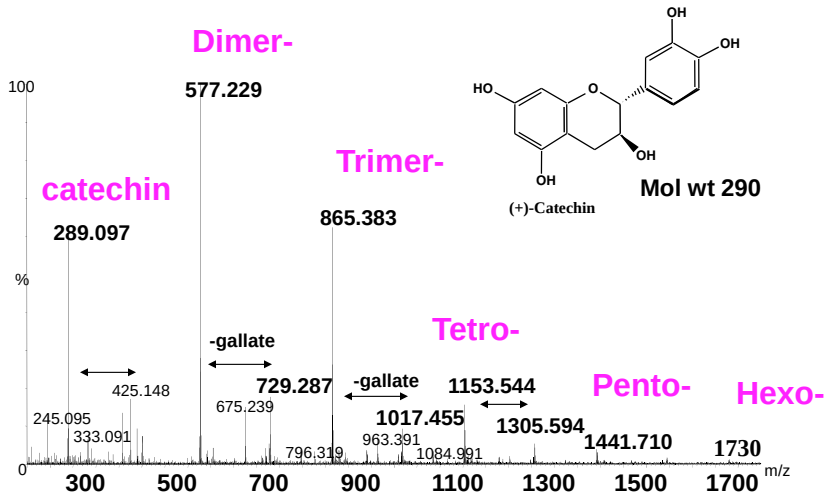
Source: Nordstrom et al. Analytical Chemistry, 2007

TIC obtained from grape seed extract treated urine operated in -ve Q1 [A] and +ve Q1 [B] modes



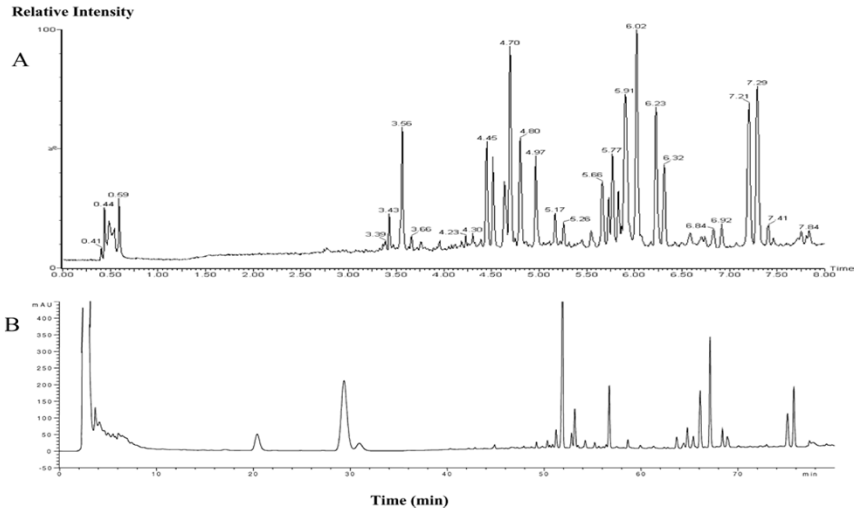
Visual inspection of the two TIC plots show that the two modes of ionization will generate different metabolomic information based on their ionization difference

Profiling of grape seed extract metabolitesin ESI-MS Q-TOF –ve ion mode



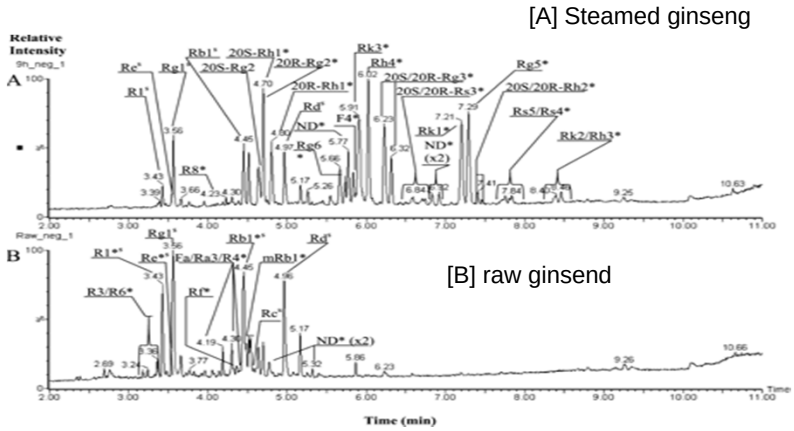
Metabolomics of raw and steamed *P. notoginseng*

[A] UPLC/TOF-MS total ion current chromatogram (TIC) and HPLC-UV Chromatogram of steamed *P. notoginseng*



Source: Chan et al. Rapid Commun. Mass Spectrom. 2007, 21, 519-528

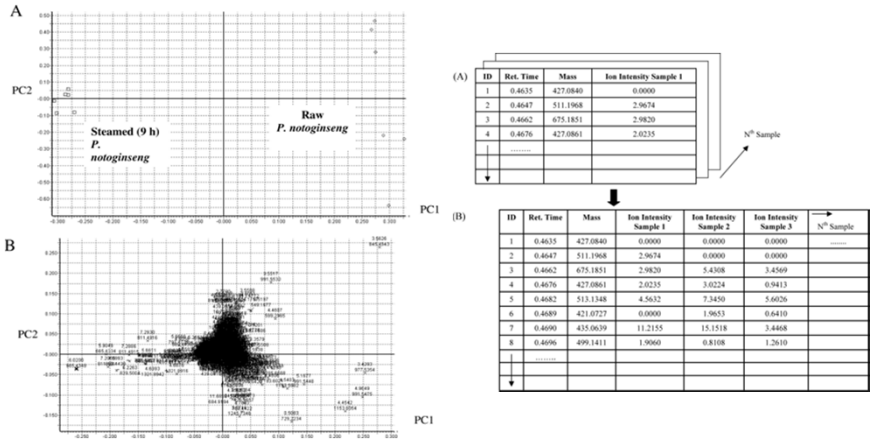
TIC of UPLC/TOF-MS analysis of [A] steamed ginseng and [B] raw ginseng



The concentration of Rg1, Re, Rb1, Rc and Rd in steam ginseng was less than that of raw ginseng

Source: Chan et al. Rapid Commun. Mass Spectrom. 2007, 21, 519-528

[A] Score plot of raw and steamed groups and [B] loadings Plot obtained using pareto scaling with mean centering



Conclusion- MS based metabolomic study is able to discriminate differentially processed herbs such as raw and streamed P. notoginseng

Source: Chan et al. Rapid Commun. Mass Spectrom. 2007, 21, 519-528

Lipidomics

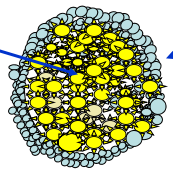
Lipidomics- A comprehensive analysis of lipid molecules in response to cellular pathophysiology

Why measure lipids?

Lipids are important- as a membrane bilayer
- **provides hydrophobic environment for protein function**
- **reservoir of energy**
- **signaling molecules**

Storage lipids

Glycerolipids
Cholesteryl esters

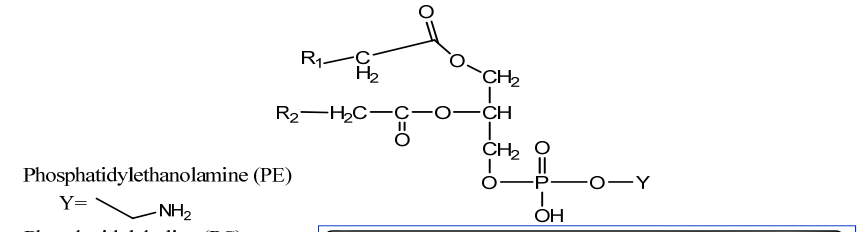


Membrane lipids

Phospholipids
Sphingolipids
Sterols

Lipidomics can perhaps best be defined as a comprehensive analysis of lipids on the systems-level scale together with their interacting factors

Structures of major phospholipids



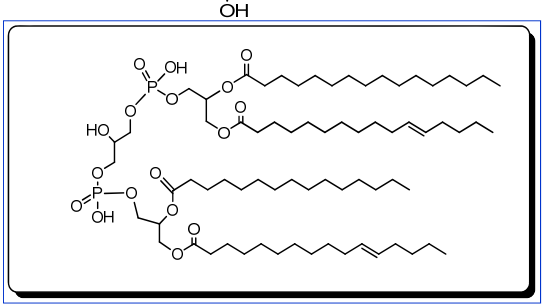
Phosphatidylethanolamine (PE)
Y= NH_2

Phosphatidylcholine (PC)
Y= $N(CH_3)_3$

Phosphatidylglycerol (PG)
Y= OH, OH

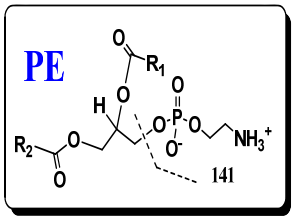
Phosphatidylserine (PS)
Y= O, OH, NH_2

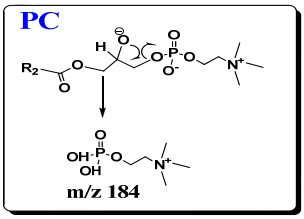
Phosphatidylinositol (PI)
Y= HO, HO, OH, OH, OH

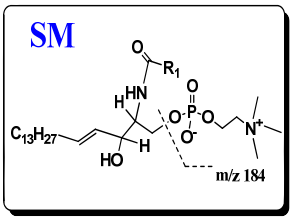


Cardiolipin (diphosphatidylglycerol)

How to profile phospholipids and sphingosines in a complex mixture using MS/MS?

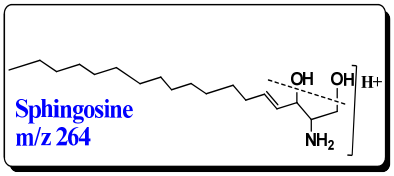




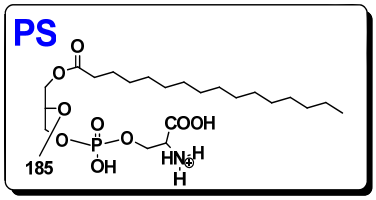


PE
Neutral Loss scan 141

PC & SM
Precursor ion scan 184

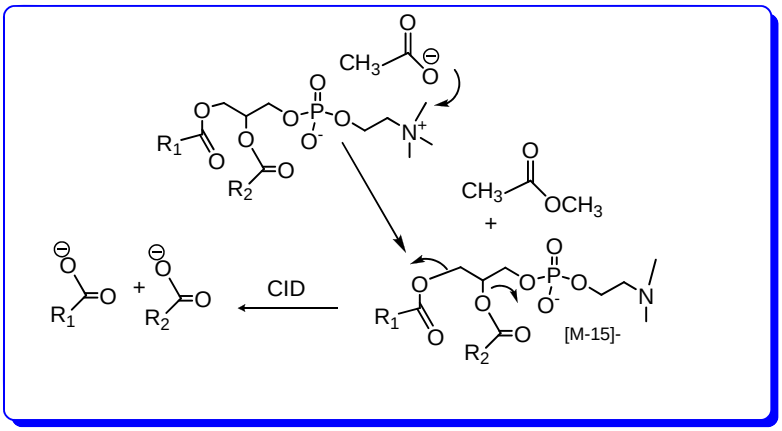


Ceramides and sphingosins
Precursor ion scan 264



PS
Neutral Loss scan 185

Tandem mass spectrometry has the ability to characterize the fatty acyl chain in -ve ion mode



Phospholipids may undergo demethylation and then the loss of the fatty acyl groups from glycerophosphocholine backbone.

Shotgun lipidomics: intrasource separation of lipids for quantitative lipidomics

Group	Electrical Propensity	Lipid Classes
Anionic lipids	Carry net negative charge(s) at physiological pH	Cardiolipin, acylCoA, sulfatide, PtdIns (PtdInsP, $PtdInsP_2$, $PtdInsP_3$), PtdGro, PtdSer, PtdH, etc.
Weak anionic lipids	Carry a net negative charge at alkaline pH	PE, lysoPE, ceramide, NEFA, eicosanoids, etc.
Neutral polar lipids	Neutral at alkaline pH	PC, lysoPC, SM, glycolipid, TAG, etc.
Special lipids	Vary	Acylcarnitine, sterols, etc.

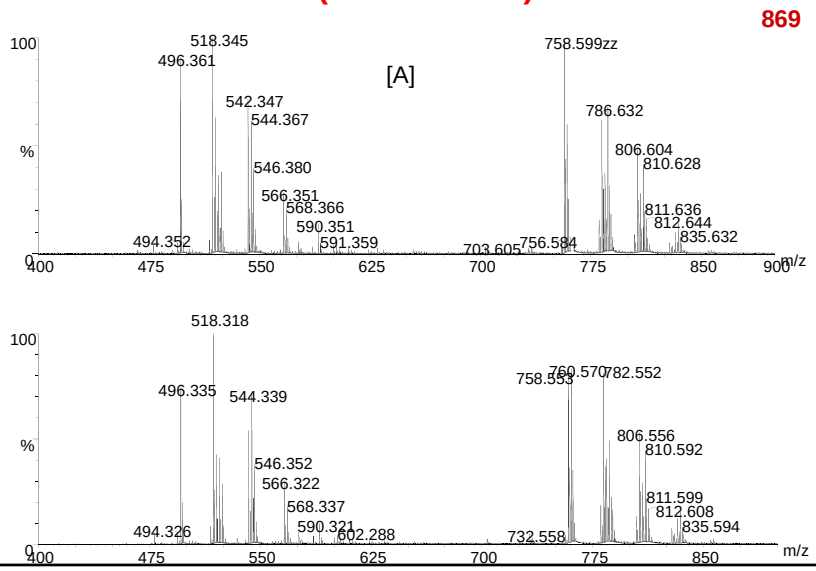
The ionization efficiency of an analyte greatly depends on the electrical propensity of an individual analyte in its own microenvironment to lose or gain a charge

Source: Gross and Han, 2004

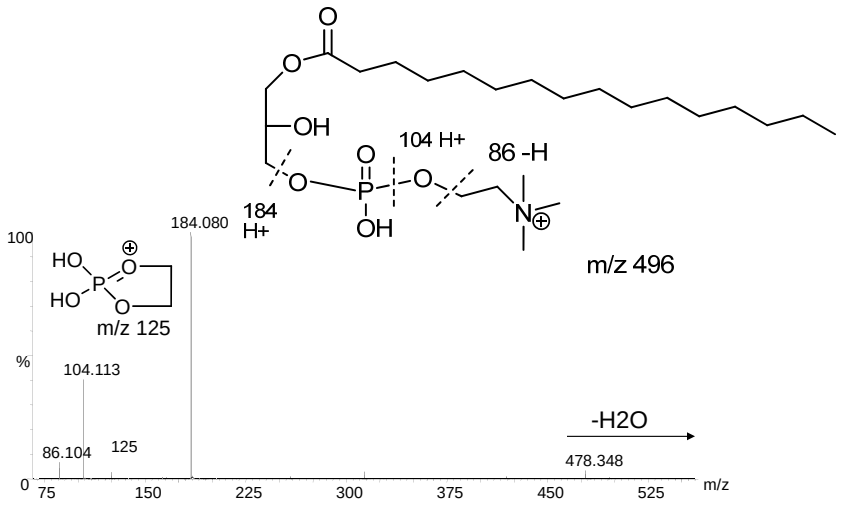
How to extract lipids?
Extraction of lipids by Bligh/Dyer method

- To a homogenized sample (1 ml containing internal standards) add methanol (2.5 ml) and chloroform (1.25 ml), sonicate by 4-5 bursts; extra 1.0 ml water and 1.25 ml chloroform added and vigorously shaken.
- Centrifuge (1,000 x g) for 2 min and separate the chloroform layer (bottom layer) and repeat the process twice.
- Combine the chloroform soluble phases and evaporate to dryness and store at -20^{o}C until analysis.

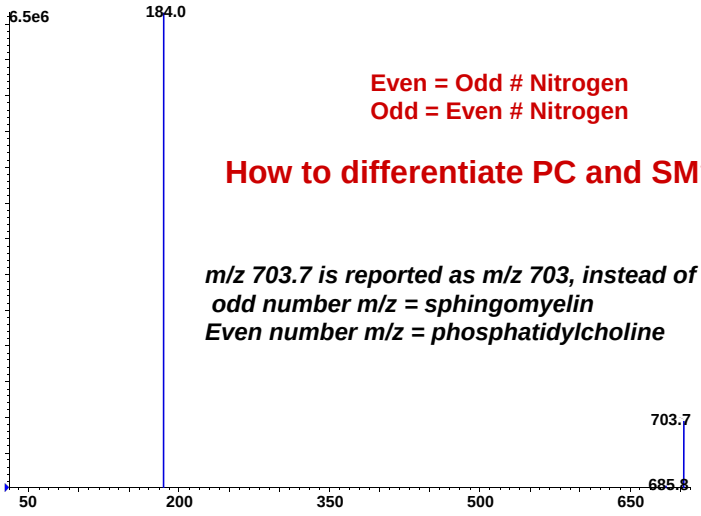
Survey scan of metabolites (+ ion mode) for a plasma sample from lean mouse [A]; ob/ob mouse [B]. Plasma lipidomes of obese mice are higher than lean littermates (1.5e3 vs. 863)



MSMS fragmentation of *m/z* 496 obtained from plasma of obese mouse identified to be LPC 16:0



MS/MS of sphingomyelin standard (2S,3R,4E)-2-acylaminooctadec-4-ene-3-hydroxy-1-Phosphocholine

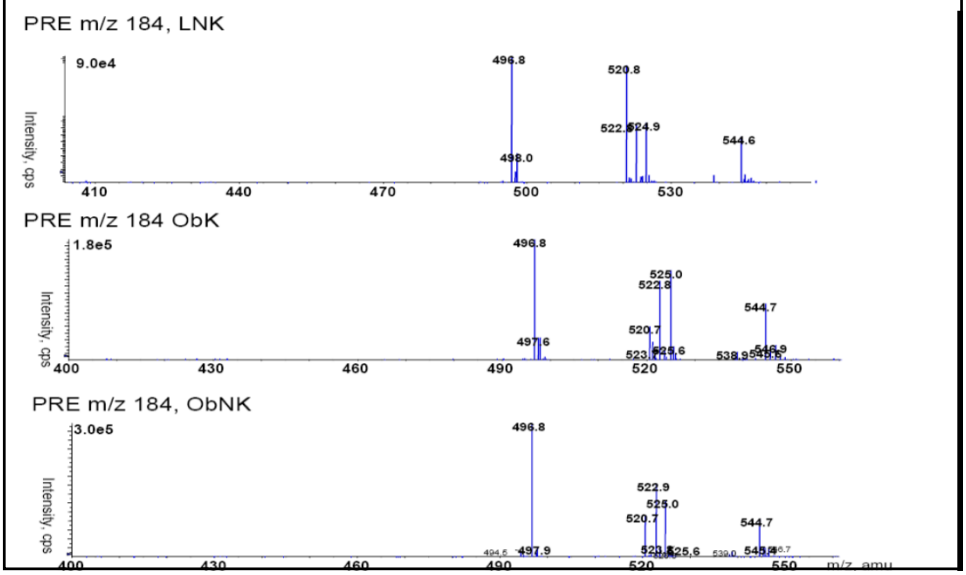


Even = Odd # Nitrogen
Odd = Even # Nitrogen

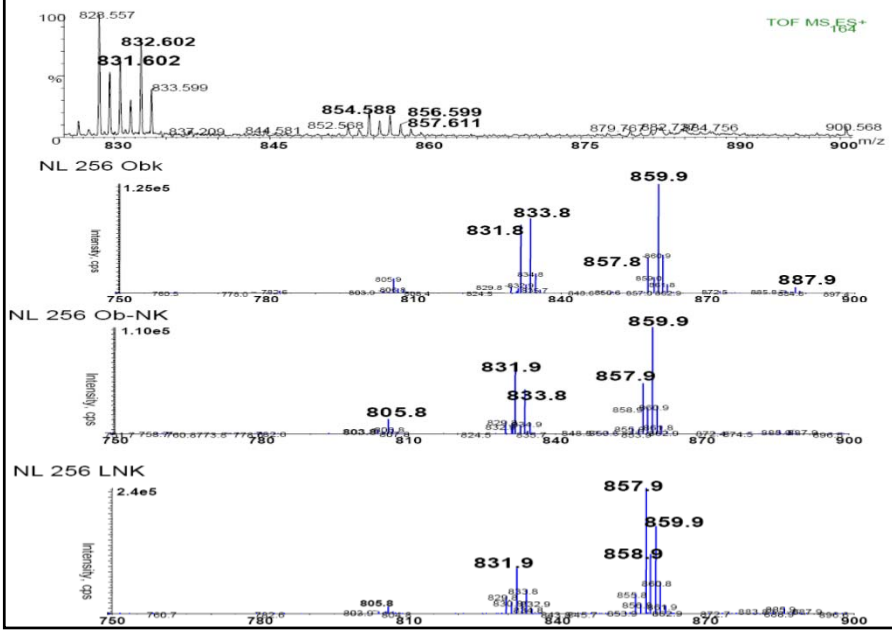
How to differentiate PC and SM?

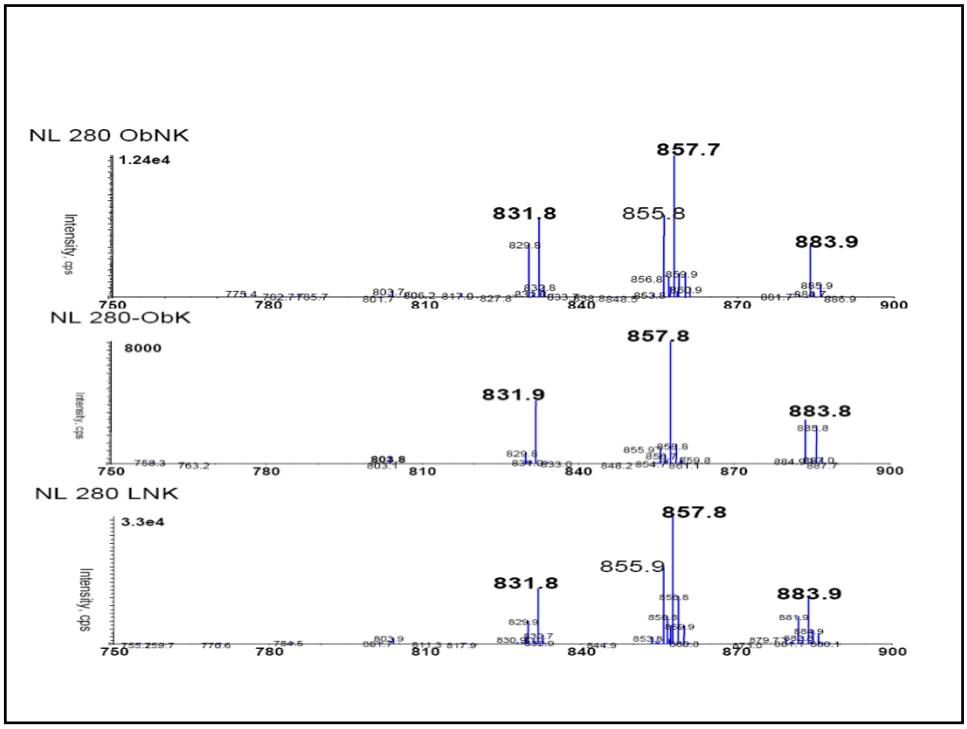
m/z 703.7 is reported as m/z 703, instead of m/z 704
odd number m/z = sphingomyelin
Even number m/z = phosphatidylcholine

Targeted lipidomics- Precursor ion spectra (PRE *m/z* 184) from LNK, ObK and ObNK hepatocytes.

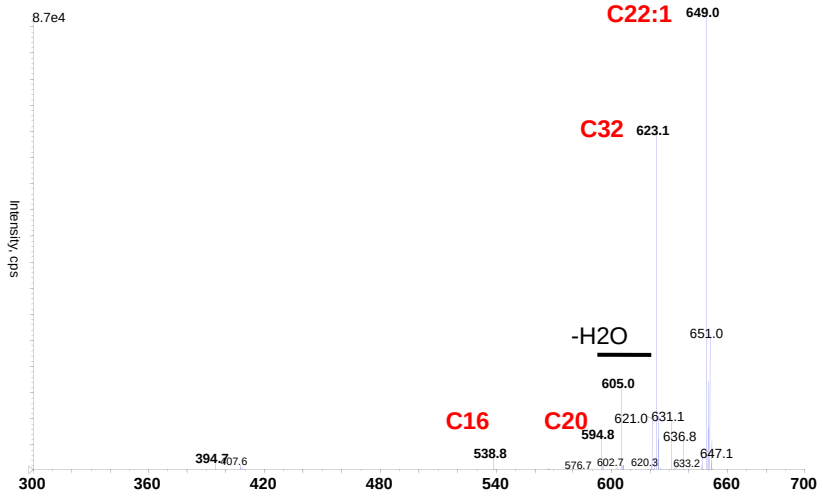


A 2D ESI mass spectrometric finger print for TG molecules

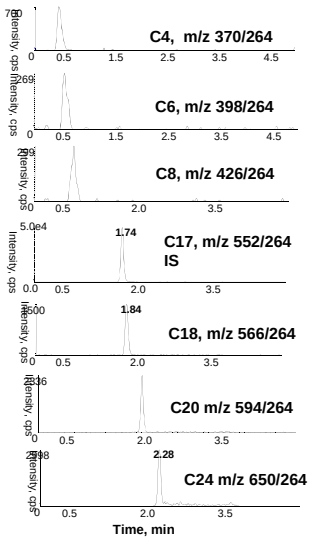


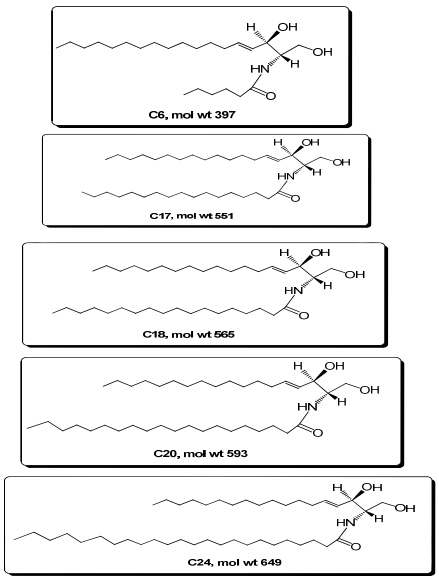


Precursor ion scan m/z 264 in +ve ion mode is specific to identify ceramides in a sample



MRM chromatograms showing simultaneous determination of ceramides (C4-C24)





Conclusions

- LC-MS-based metabolomic approach is promising for the quality control of dietary supplements, and discovery of novel markers in biomedical research.
- Tandem mass spectrometry analysis of phospholipids in +ve ion mode characterizes phospholipid polar head groups, whereas –ve ion mode provide fatty acid chain structural information.
- Shotgun lipidomics can be used for rapid and reproducible global analysis of lipids in biological samples.
- Identification of metabolites (lipids or any other metabolites) at a molecular level present a great challenge due to their structural diversity (isobars and isomers) and dynamic metabolism.