

## **Lipids Analysis**

**Stephen Barnes**

**03-08-17**

### **Lipids**

- **Lipids are mostly very hydrophobic**
- **Most are conjugates of fatty acids of a variety of chain lengths, which have different degrees of unsaturation, cis-trans isomers, and chiral centers**
- **The conjugating frame to which the fatty acids bind can be quite hydrophilic**
- **This results in a very wide (evergrowing) number of lipid species**

## **Analysis of fatty acids**

- **Fractional crystallization**
- **Thin layer chromatography (TLC)**
  - **Argentation TLC (to separate according to number of double bonds)**
- **Gas liquid chromatography**
  - **Packed columns**
  - **Capillary columns**
- **LC-MS**
- **SWATH-MS**
- **Differential ion mobility**
- **DESI-MS**

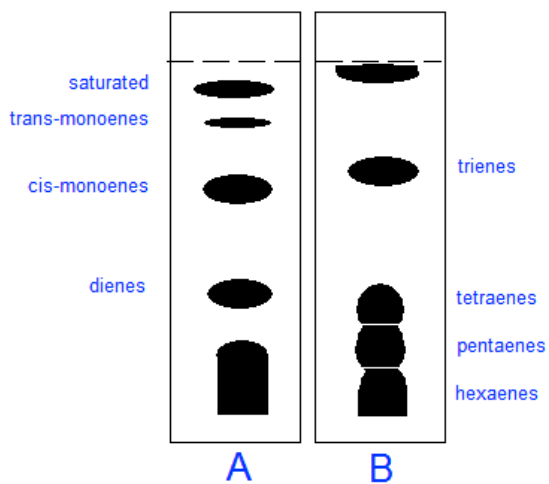
## **Fractional crystallization**

- **Still used in industry**
- **Crystallization is used to determine whether adulteration of butter fat by other lower quality fats has occurred**
- **Unsaturated fats are more soluble at lower temperatures**
  - **Division into “stearins” and “oleins”**
  - **For fatty acids, make lead salts and cool in diethyl ether or ethanol – the saturated FAs crystallize out first**

## Thin-layer chromatography

- Fatty acids or methylated fatty acids separated on alumina or silica gel TLC
- When  $\text{AgNO}_3$  is incorporated into the silica slurry before making the TLC plate, the observed separation is dependent on the degree of unsaturation ( $\pi$ -bonding)
  - Saturated
  - Mono-unsaturated
  - Di-unsaturated, etc.

## $\text{Ag}^+$ ion TLC of fatty acids

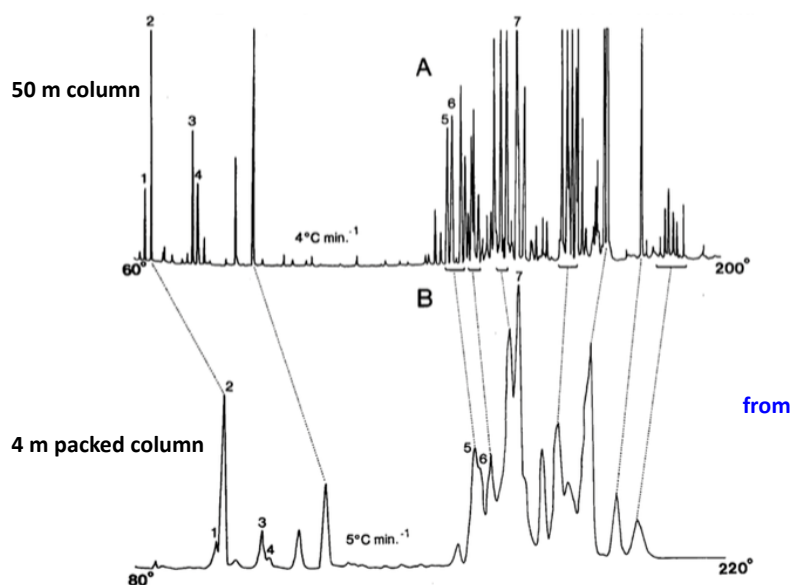


It is a class separation

## Gas-liquid chromatography

- **1952 Martin and James start GC by separating volatile fatty acids ( $C_1$ - $C_6$ )**
  - Quickly extended it to long chain FAs by methylating them
  - Used 5-6 feet x  $\frac{1}{4}$  inch glass or stainless steel packed columns
- **1955 Patent for capillary, open tubular columns awarded**
  - Did not enter commercial use until the mid-1970s

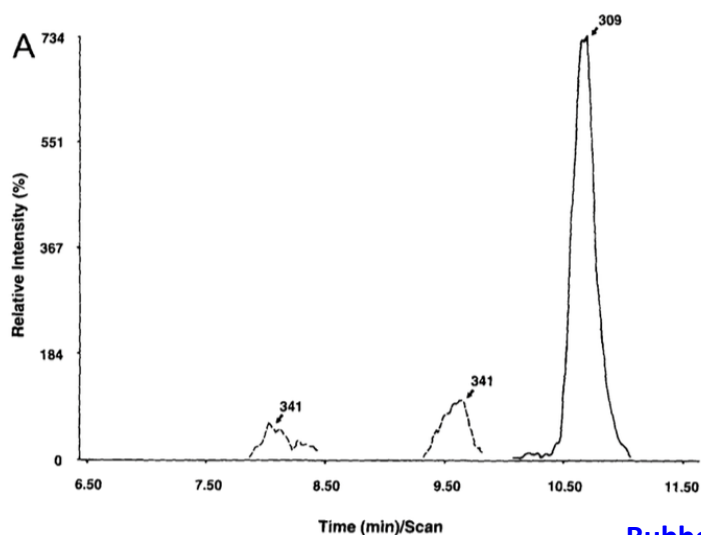
## Packed vs Capillary GC of FAME



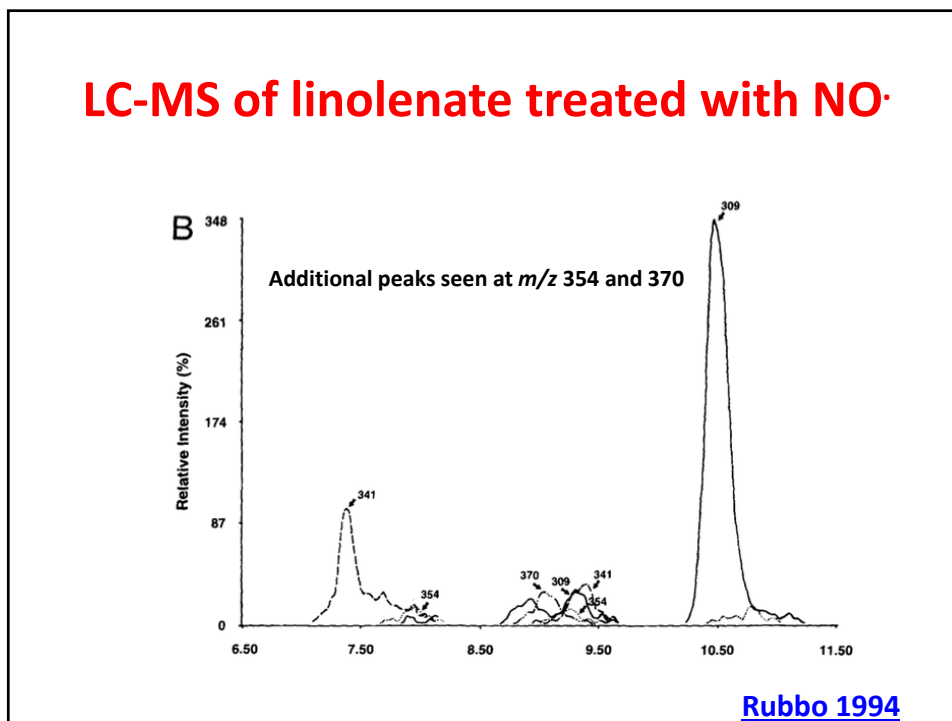
## (HP)LC

- Reverse-phase LC
  - Can be used for lipid class separation based on hydrophobicity
  - Again,  $\text{Ag}^+$  can be introduced into the medium to enhance the separation of unsaturated fatty acids
  - Very difficult to detect lipids spectroscopically
  - LC-MS is the preferred method

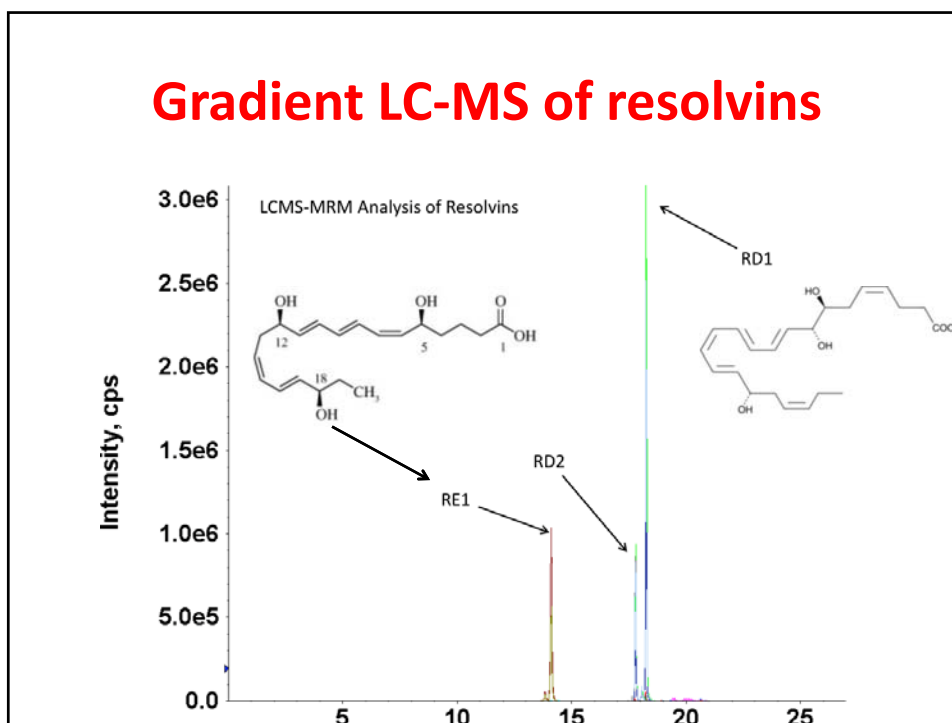
## LC-MS of oxidized linolenate



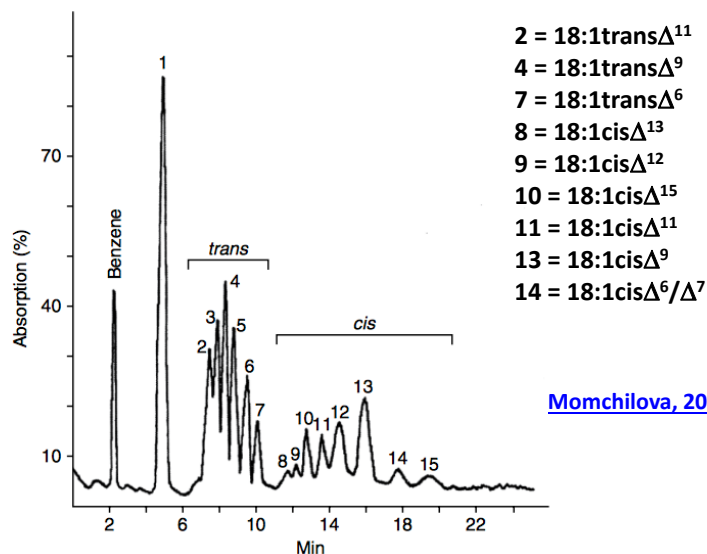
## LC-MS of linolenate treated with NO



## Gradient LC-MS of resolvins

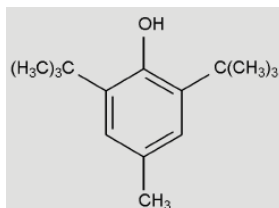


## Silver ion-HPLC of unsaturated fatty acids



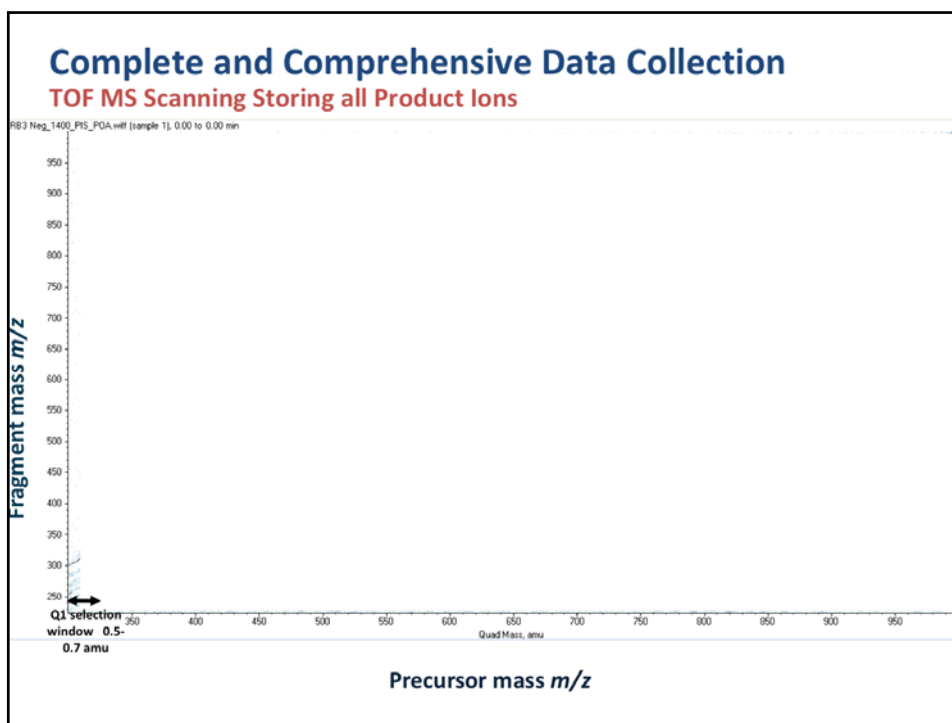
## Modern lipidomics

- Use of the SWATH-MS approach
- Preceded by total lipid extraction using a two-phase partition by adding  $\text{CHCl}_3:\text{MeOH}$ 
  - Bligh-Dyer and Folch extractions
    - Crucial to do so in an atmosphere of argon and in the presence of butylated hydroxytoluene to prevent oxidation

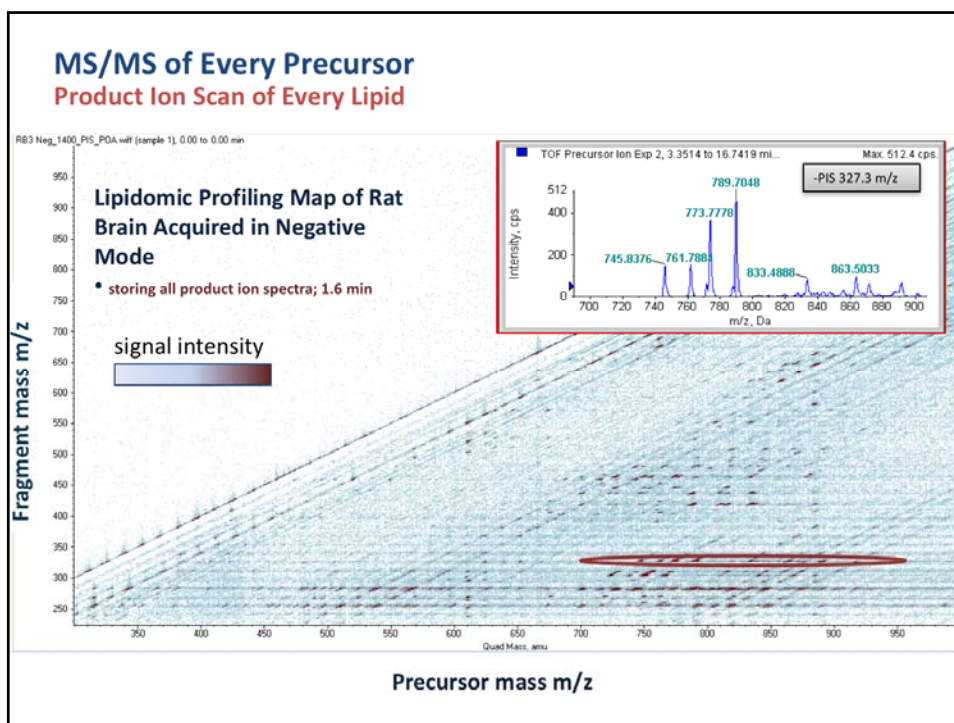
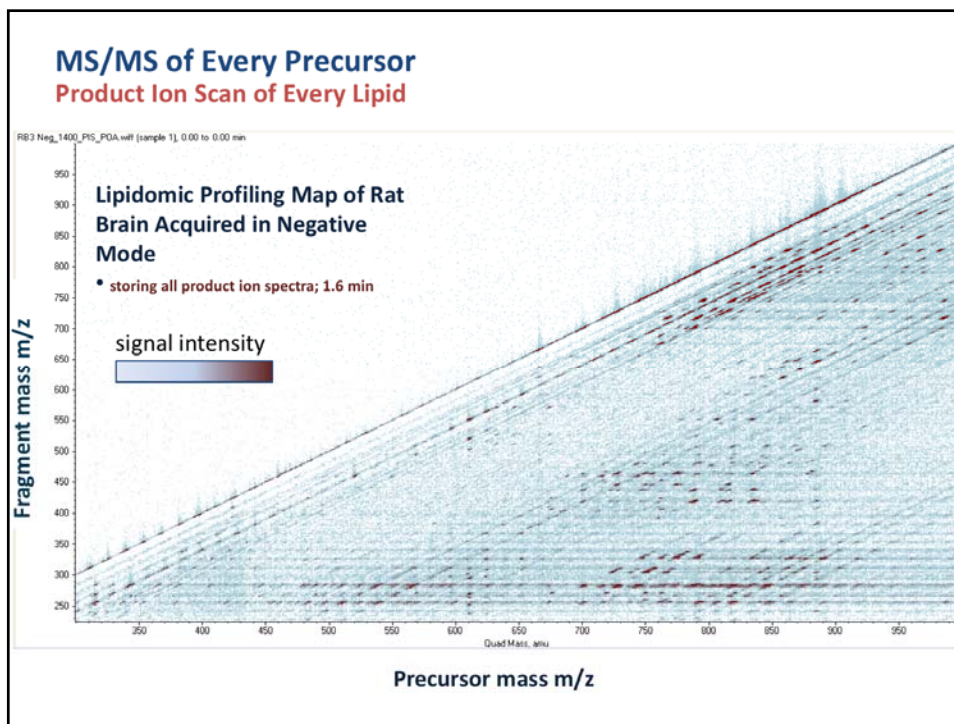


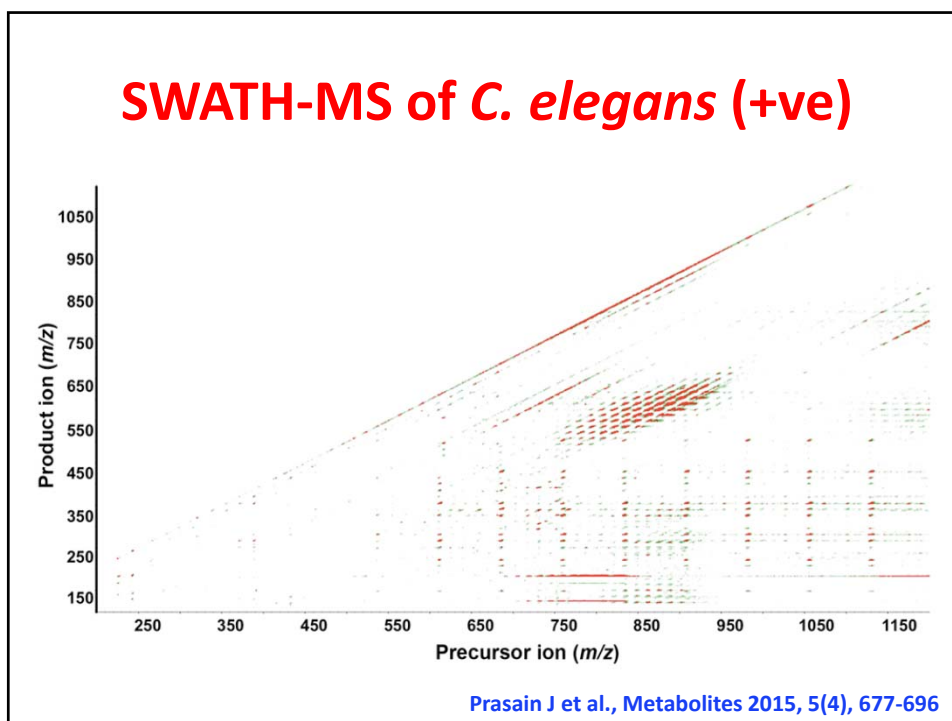
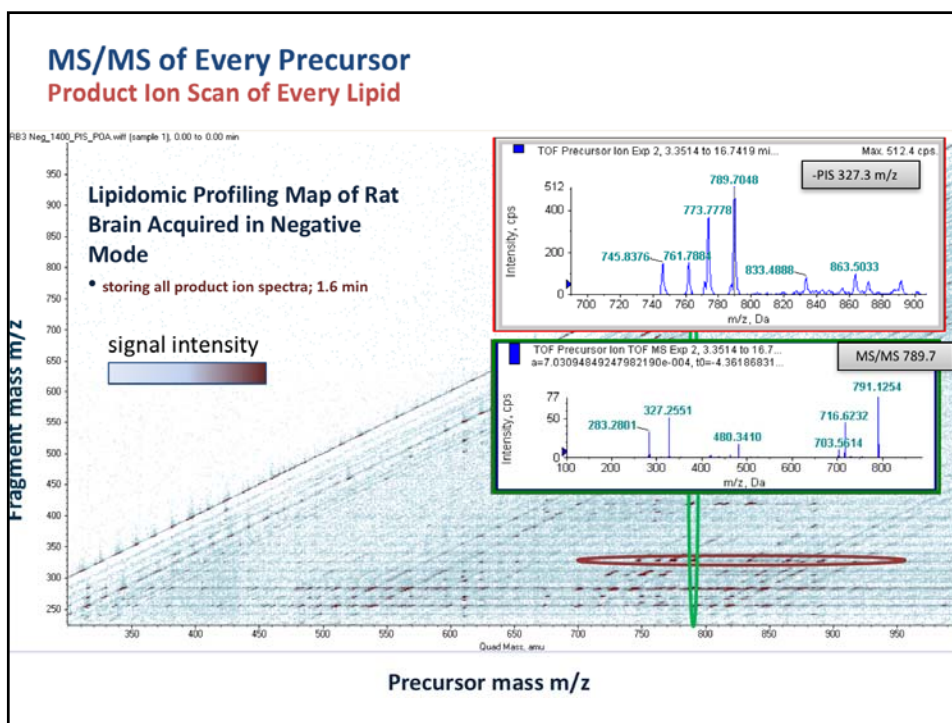
## SWATH-MS

- Based on an infusion strategy on a 5600 TripleTOF
  - Ions are filtered 1.2  $m/z$  at a time in the quadrupole over a  $m/z$  range of 200-1200
  - The filtered ions are collisionally dissociated and fragment ions analyzed by the TOF analyzer
  - MSMS spectra collected for 500 msec for each  $m/z$ , i.e., infusion for 500 sec (8.33 min)







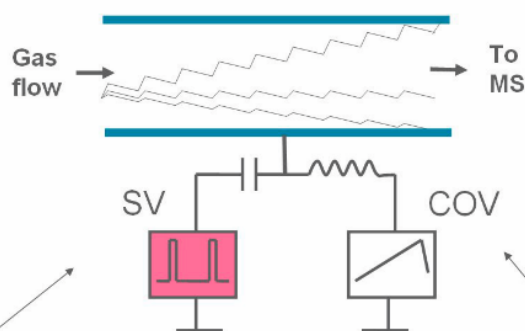


## Other MS methods for lipids

- SWATH-MS is comprehensive – no stone unturned
- However, many lipids overlap in mass and there are also isomers with the same mass
- To observe more individual lipids, it is necessary to resolve lipids before analyzing them in the mass spectrometry
- Even then, isomers can be a problem
- A form of ion mobility may be the answer to this

## SelexIon™

• Differential Mobility Spectrometry (DMS) is the term used for planar geometry



Separation waveform (SV):

Radially displaces ions towards one or the other electrode, depending upon high and low field mobility characteristics

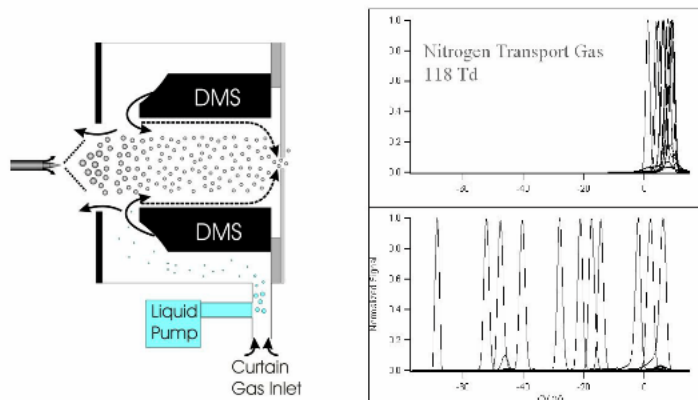
Compensation voltage (COV):

Restores the trajectory for a given ion to allow them to transmit through the DMS device and enter the mass spectrometer

AB Sciex

## Liquid modifiers and SelexION™

- Liquid modifiers can be added to the curtain gas flow
- Improves separations
- More options for separation in difficult cases

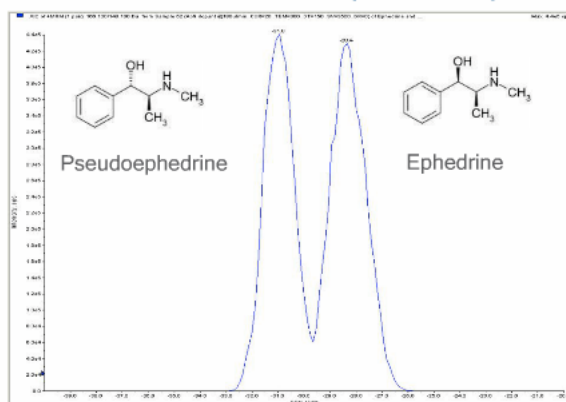


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11 compounds: methylphenamine, amoxicillin, ephedrine, norfenbutyl, acyclovir, clemastine, tramadol, quazifan, ranitidine, ranitidine, bismarck  
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## Separating isobaric isomers

### Separation of Isomers - Pseudoephedrine/Ephedrine



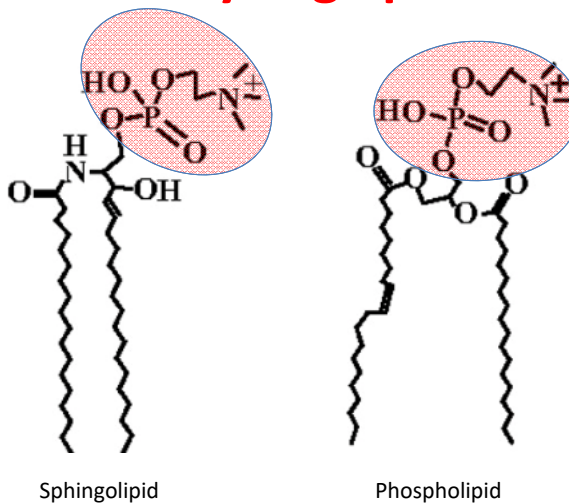
•Separation of pseudoephedrine and ephedrine with SelexION™

•Indistinguishable by MS or MS/MS

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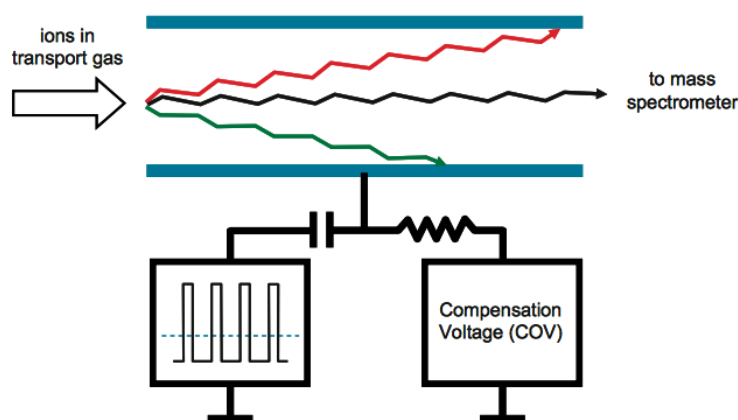
## The problem of analyzing lipids

- Despite the sheer number of lipids, the *units* comprising them are closely related and therefore they have similar masses
- Sphingolipids may only be different in mass by 1 Da from their PC analog
  - $^{13}\text{C}$ -isotope profiles overlap
  - Head groups are the same



## Differential mobility MS is an answer

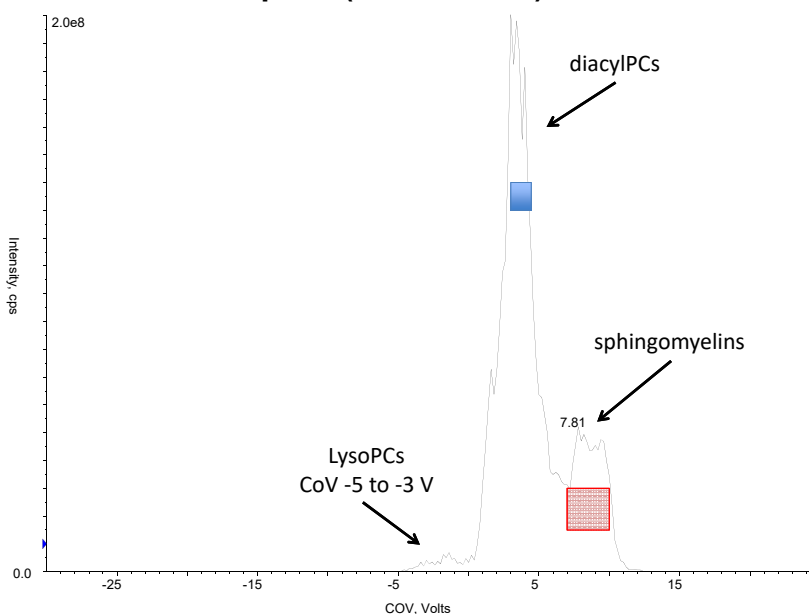
Innovative Planar Design; SelexION™ Ion Mobility Cell.

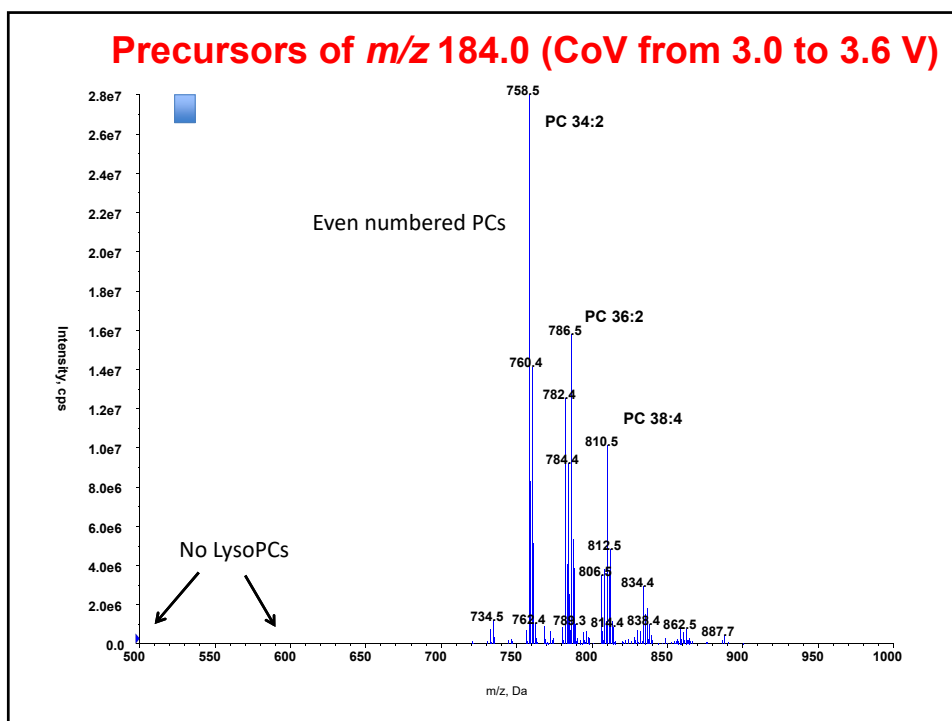
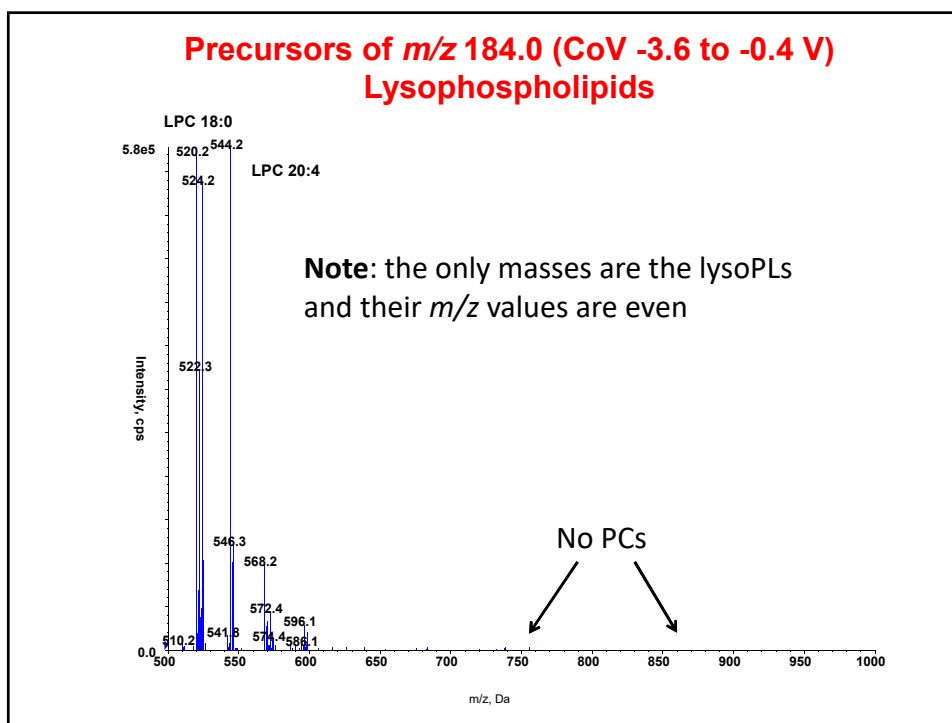


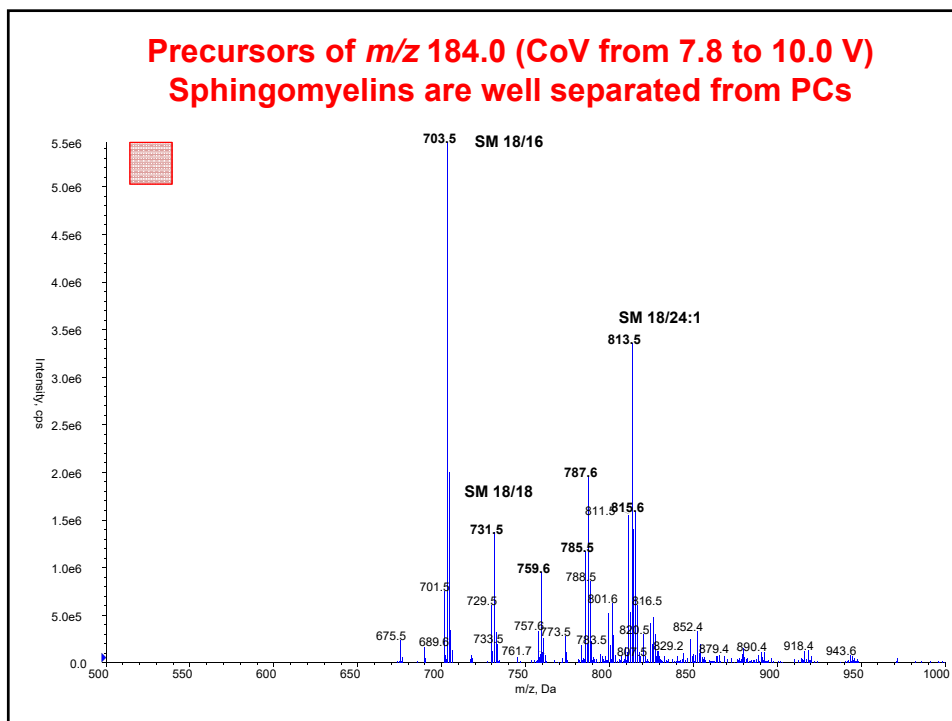
## Differential mobility mass spectrometry

- A fragment ion may have multiple precursor ions
- The precursor ions may be separable by DMS before they enter the mass spectrometer
- By scanning with the compensating voltage, the precursor ions enter the mass spectrometer at different CoVs
- (Note: Further separation is possible using resolving agents, e.g., isopropanol)

### Total ion current of precursors of $m/z$ 184.0 Sample 1 (HS 07-30-15)



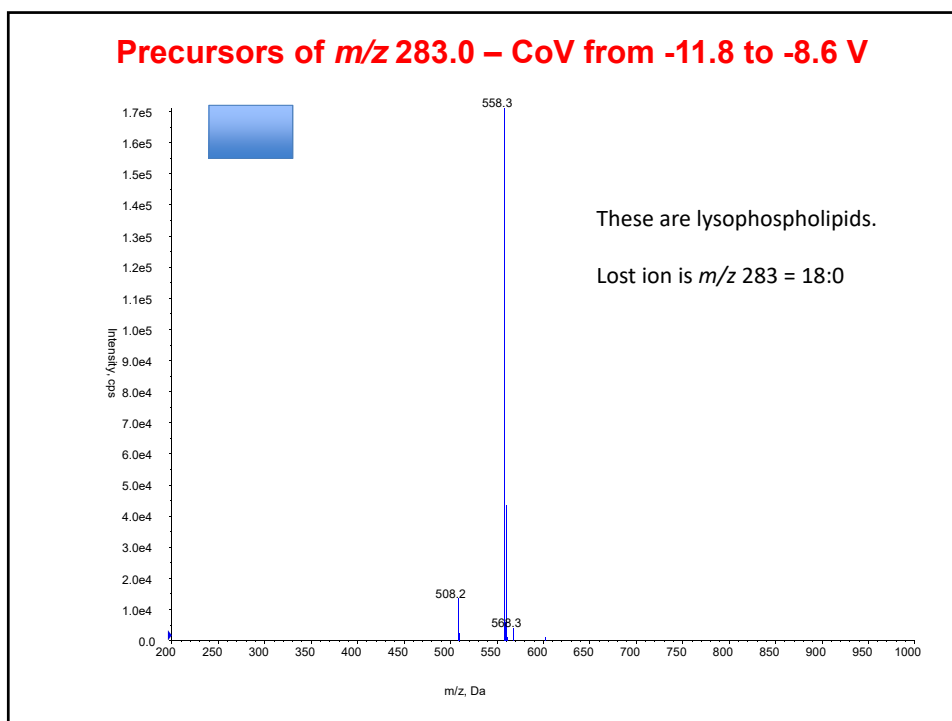
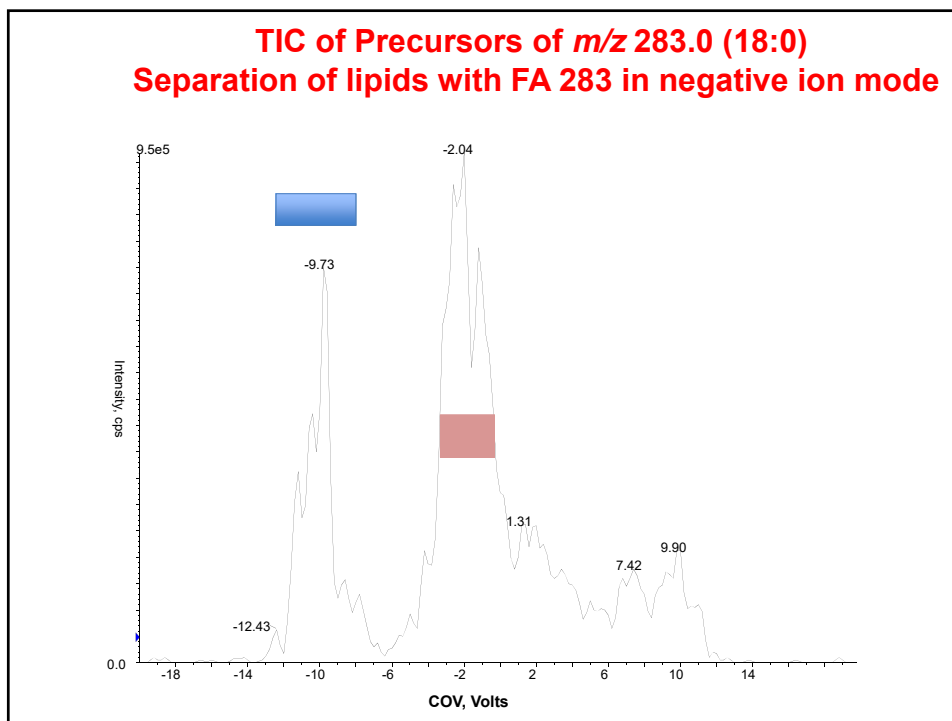


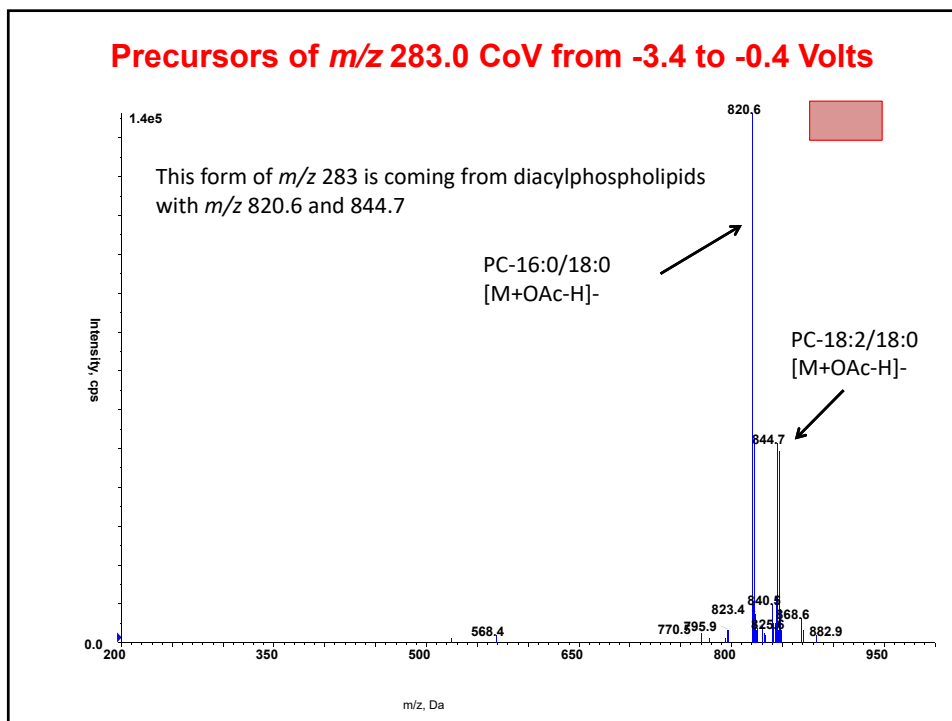


## Origins of negatively charged product ions

Precursors of  $m/z$  283 (stearate, 18:0) studied at different compensation voltages



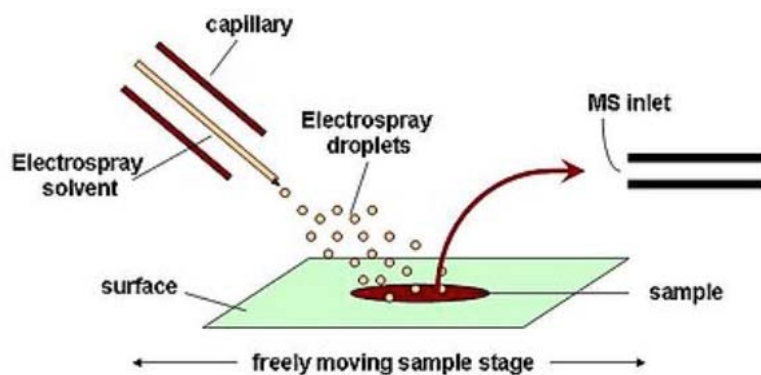




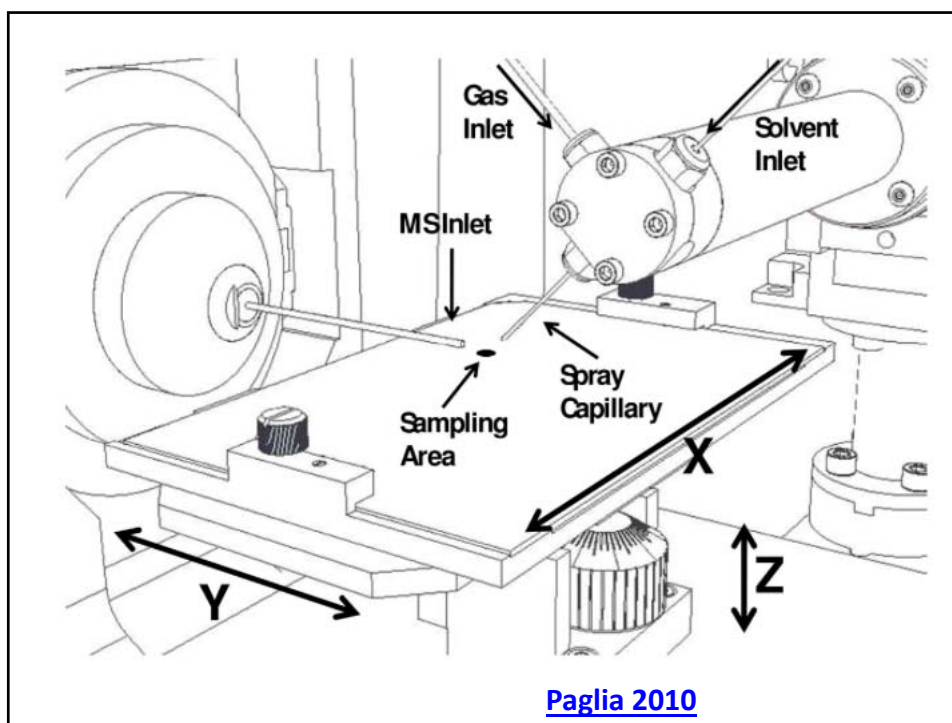
## Summary

- **Differential mobility mass spectrometry (DMS) is an important new tool in the study of lipids**
  - It overcomes many of the problems that beset the analysis of lipids with overlapping masses
- **Further separation (not exploited yet) comes from differential resolution with specific solvents**
- **Metabolon has introduced a kit for the analysis of lipids where the extraction solvent contains ~1,000 deuterated lipid internal standards enabling absolute quantification of a wide range of lipids using DMS**

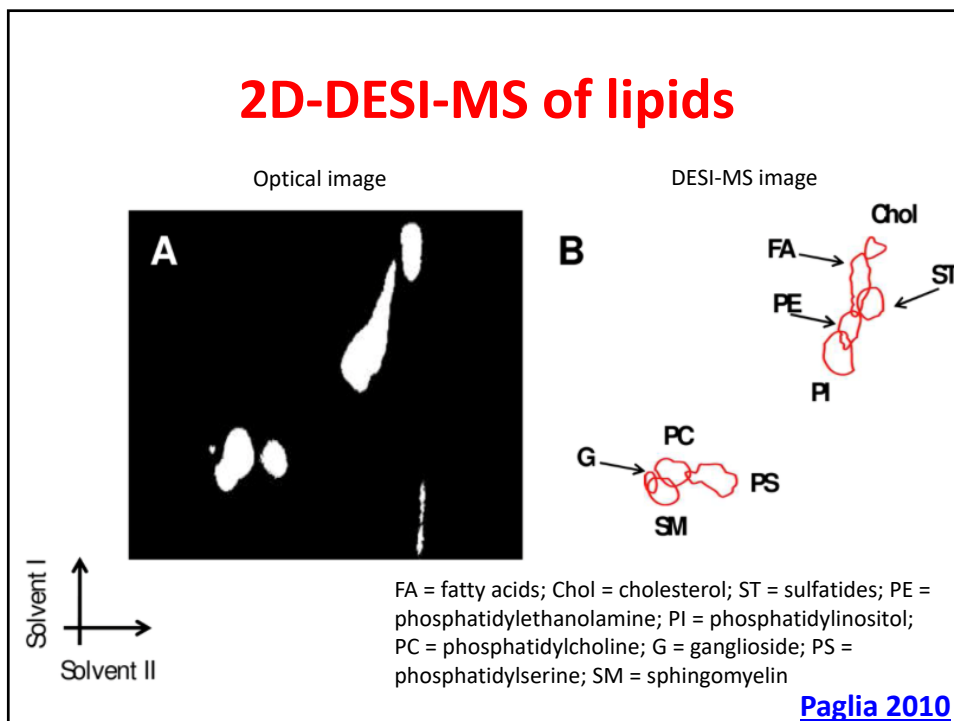
## DESI-MS TLC of lipids



Desorption electrospray ionization mass spectrometry of the lipids on the TLC plate without extraction



## 2D-DESI-MS of lipids



## References

- Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J Biol Chem.* 1994 Oct 21;269(42) 26066-75.
- Paglia G, Ifa DR, Wu C, Corso G, Cooks RG. Desorption electrospray ionization mass spectrometry analysis of lipids after two-dimensional high-performance thin-layer chromatography partial separation. *Anal Chem.* 2010 Mar 1;82(5):1744-50.
- Momchilova SM, Nikolova-Damyanova BM. Separation of isomeric octadecenoic fatty acids in partially hydrogenated vegetable oils as p-methoxyphenacyl esters using a single-column silver ion high-performance liquid chromatography (Ag-HPLC). *Nat Protoc.* 2010 Mar;5(3):473-8.
- [AB Sciex Webinar](#) on SelexION technology