GBS 724

# LC-MS analysis of metabolites

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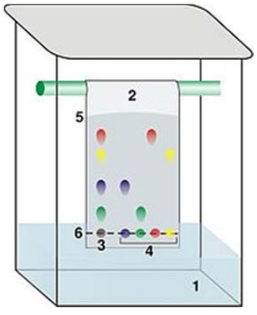
# Basis of Chromatography

- **A moving (mobile) phase passes over an inert, stationary phase**
  - **The compounds differentially interact with the stationary phase and elute at different times**

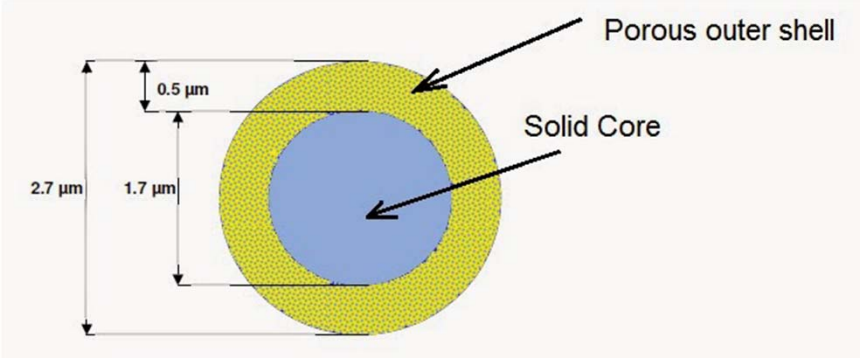



- **The *stationary phase* can be paper, silica, coated silica and derivatized silica**
- **The *mobile phase* can be a gas or a liquid (organic solvent or water)**

# LC-MS

- The stationary phase
  - Silica
    - surface can be made to be hydrophobic or hydrophilic
  - Open or pellicular
    - Having a pellicular support increases the rate of equilibrium (better performance), but lowers capacity
- Graphitized carbon (quite hydrophobic)
  - Stable to alkaline pH (unlike the alkyl silicas)
- The mobile phase is a liquid
  - Gradients of water-miscible organic solvents in water with volatile additives (0.1% formic acid or formic acid, 2-10 mM ammonium acetate or formate)
  - Trifluoroacetic acid is not used, nor are Tris or phosphate buffers

# Pellicular stationary support

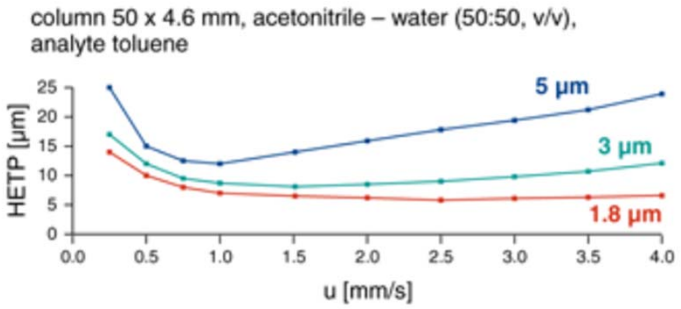


# Chemistry of the column phases

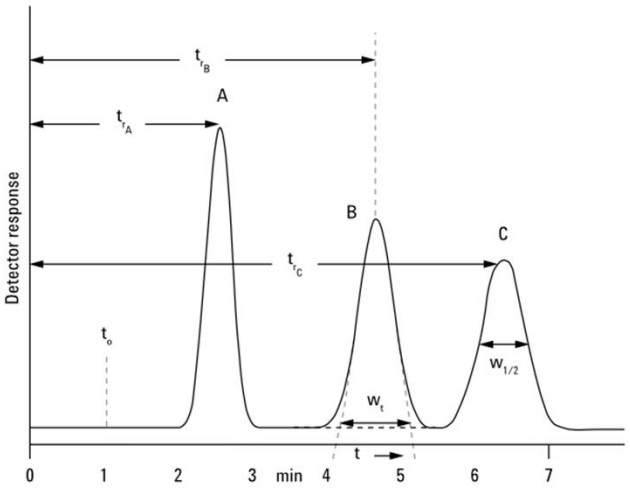
- **Reverse-phase**
  - $C_4$, $C_5$, $C_8$, $C_{18}$, phenyl-hexyl-bonded phases
- **Normal phase**
  - Bare silica, cyano and amino-bonded phases
- **Hydrophilic interaction chromatography**
  - Bare silica, polyol-bonded phase
- **Particle sizes**
  - 5, 3, 2.5, 2.2 μm and 1.7 μm (for UPLC)

# Important equation in chromatography

- **Van Deemter**
  - Height of theoretical plate (HETP, smaller the better)
  - HETP = A + B/μ + Cμ, where μ is the linear flow velocity
    - Where A is the eddy diffusion term due to non-ideal flow
    - B is diffusion that occurs in the longitudinal direction
    - C is the resistance to equilibration between the stationary and mobile phases



# LC parameters




**Efficiency**
$N = 16\ (t_R/w_t)^2$
For Peak B, $16(4.5\ \text{min.}/0.9\ \text{min.})^2 = 400$ plates
$k = (t_R - t_0)/t_0$

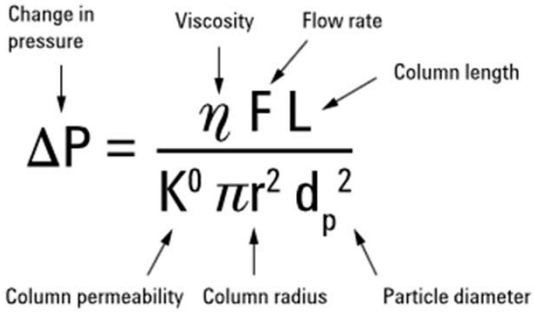
**Retention**
$k_A = (2.5 - 1)/1 = 1.5$
$k_B = (4.6 - 1)/1 = 3.6$
$k_C = (6.2 - 1)/1 = 5.2$

**Selectivity (C-B)**
$\alpha = k_2/k_1$
$\alpha = k_c/k_b = 5.2/3.6 = 1.44$
$\alpha = 1.44$

**Selectivity (B-A)**
$\alpha = k_2/k_1$
$\alpha = k_b/k_a = 3.6/1.5 = 2.4$
$\alpha = 2.4$

Agilent handbook of LC

# The pressure equation



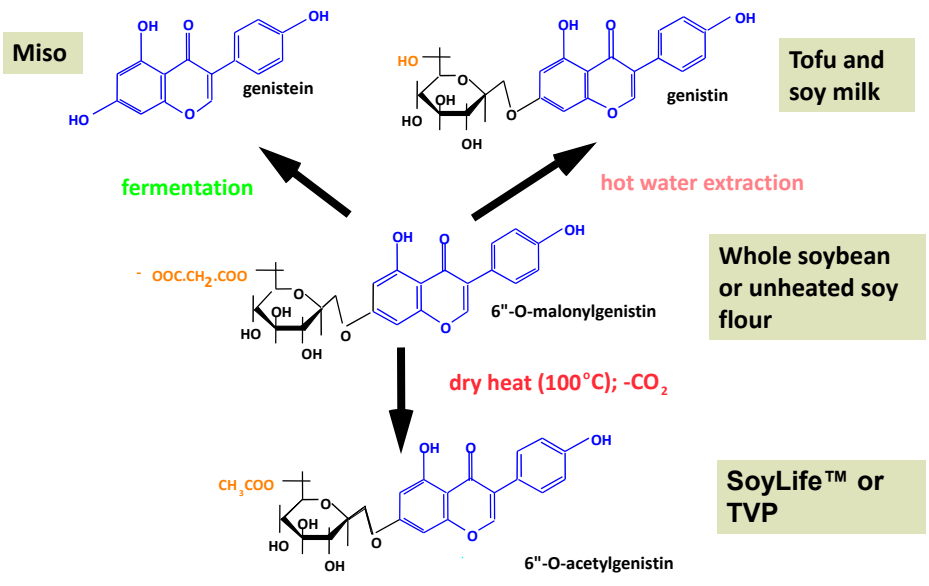

**As the particle diameter is decreased by a factor of two, the backpressure goes up by a factor of four**

Agilent Handbook of LC

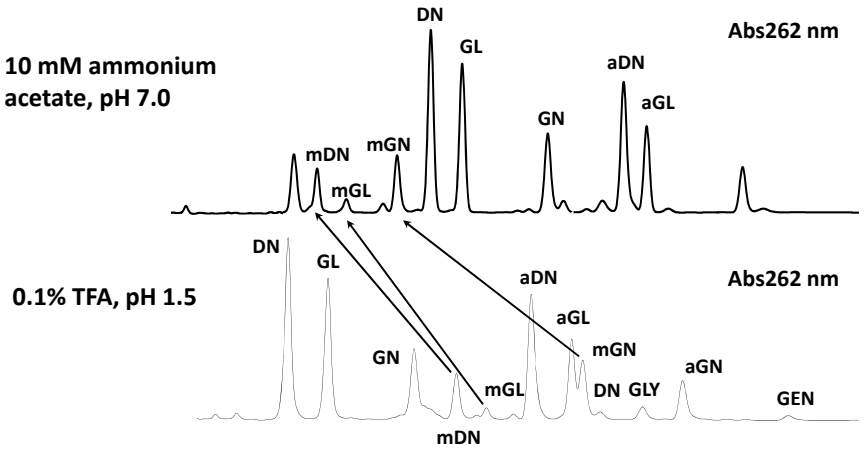
# Mobile phases

- **Acidic media**
  - Typically 0.1% formic acid
- **Neutral media**
  - 1-10 mM ammonium acetate or formate
- **Alkaline media**
  - 0.1% ammonium hydroxide (but not with $C_4$-$C_{18}$ phases)
- **Solvents (water-miscible)**
  - Methanol, acetonitrile, isopropanol (for hydrophobic metabolites)

# Genistein forms in soy products



## Effect of changing the solvent pH identifies malonylglucosides of isoflavones in extracts of a novel soy food




## LC-MS

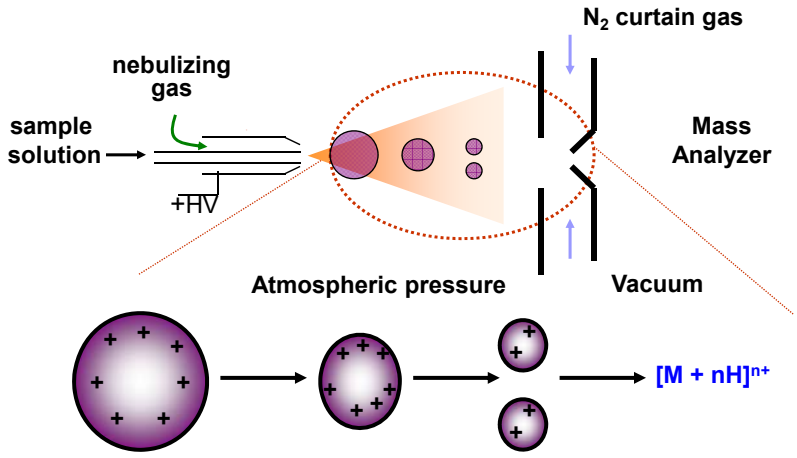
- **The advantage of an effective LC-MS system would be that it would allow thermally unstable compounds, even large ones (such as proteins), to go into the gas phase from liquid solution and into the mass spectrometer**
- **Importantly, the ionization methods used are soft in nature and molecular ions $[M+H]^+$ or $[M-H]^-$ are easily formed (see later re other molecular ions)**
- **However, there are some compounds that cannot be ionized by LC-MS**
  - polycyclic aromatic hydrocarbons, alkanes, waxes.

# LC-MS interface

**The key issue is how to transfer ions from the liquid phase into the gas phase while minimizing the transfer of solvent into the mass spectrometer**

- **For compounds that can be charged, electrospray ionization (ESI) is the principal method of choice**
- **Nebulization of the electrical charged droplets more effectively decreases the size of droplets**
  - This allows all aqueous solvents to be processed by the interface
- **Heating the spray further increases sensitivity**
  - Not needed or used in nanoelectrospray ionization

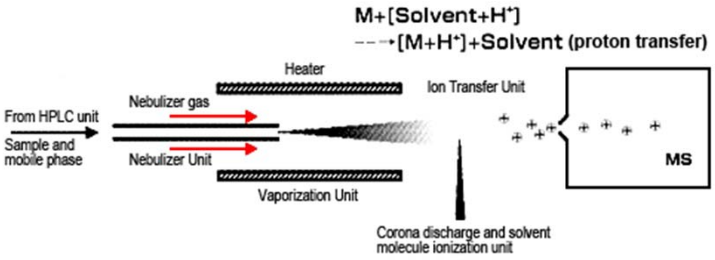
# Electrospray Ionization (ESI)




**1. Solvent evaporation**
**2. Coulombic repulsion**

# Atmospheric chemical ionization (APCI)

- Suitable for compounds that are neutral
- A corona discharge needle ionizes air molecules that transfer their energy to the solvent and hence the solutes




# Guide to LC-MS flow rates

| Type | Column ID | Flow rate | Solvent consumed* |
|---|---|---|---|
| Conventional | 1.0-4.6 mm | 0.050-1.00 ml/min | 72-1440 ml |
| Capillary | 0.3-1.0 mm | 0.005-0.050 ml/min | 7.2-72 ml |
| Nano | 0.05-0.20 mm | 100-1000 nl/min | 0.144-1.44 ml |

**Sensitivity in LC-ESI-MS increases in proportion to the inverse of the flow rate. Therefore, there is value in going to lower flow rates – it also saves money on solvents.**

# Engineered microflow LC

**Chromatography at flow rates of 5-50 μl/min using 0.3-0.5 mm ID columns**

**Very low dead volumes between the sample injection valve and the ESI interface despite the low flow rate**

**Enables short, reproducible gradients (1-2 min) or up to a 20 min gradient (for metabolomics) at 5 μl/min**

# ChipLC versus nanoLC

- **A nanoLC column is so thin (75-200 μm i.d.) it has very little thermal capacity – this leads to variable retention times due to temperature fluctuations in the lab**

**A column etched in a block of silica can be engineered to have greater physical reproducibility and it has far greater thermal capacity. The CHIP can be placed in temperature-controlled chamber – we operate ours at 45°C – to recover more hydrophobic metabolites**

# Open tubular columns

- Provide the opportunity for high resolution chromatography
- Have to be hand-made, not commercially available
  - Opportunity to make specific stationary phases
- Most suited to discovery metabolomics until a standardized product becomes available

# Detector types

| Type | Mass range (*m/z*) | Resolution | Accuracy (ppm) | Time for MSMS (msec) |
|---|---|---|---|---|
| Quadrupole | 20-3000 | 2,000 | 50 | 1000 |
| TOF | unlimited | 30,000-40,000 | 2-3 | 50 or less |
| Orbi-trap* | 50-6000 | 80,000-200,000 | 1-3 | 200+ |
| FT-ICR* | 100-1,500 | Up to 1,000,000 | <1 | 1000 |

*These detectors depend on ion motion and therefore their performance declines as the acquisition time is shortened. Using a 80 msec MSMS acquisition, mass resolution on an Orbi-trap falls to 17,000. The TOF detector is the preferred one for untargeted analysis. The Orbi-trap and FT-ICR instruments are important for follow-up high mass accuracy experiments.

# Detector combinations (targeted)

- Traditionally, a targeted method uses a combination of two quadrupole filters
  - Another quadrupole (between the other two) focuses the selected precursor ion so it collides with a jet of gas and forms fragments
  - Known as a *reaction monitoring* and **triple quadrupole method**
  - Recently, quadrupole-TOF combinations have been introduced - pseudoMRM
- Many reaction (ion) monitoring steps can be observed per sec
  - Multiple reaction monitoring (**MRM**)

# Primer for selecting ions for MRM

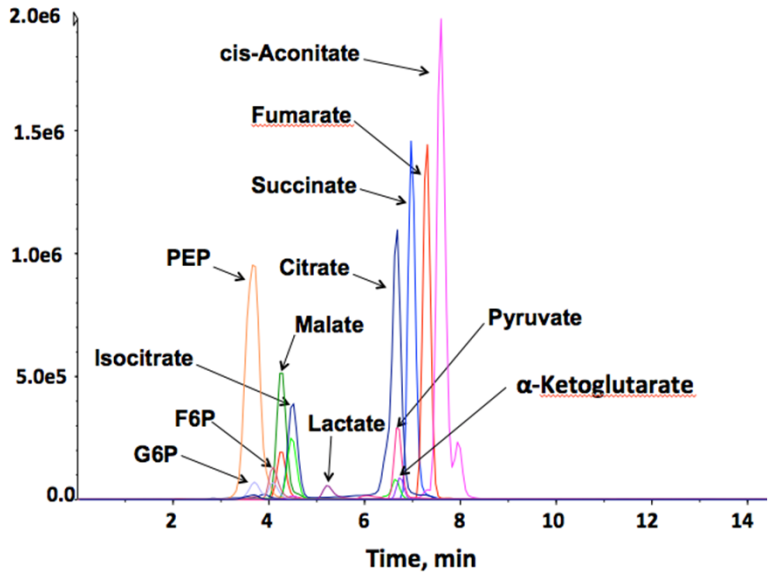
| Peak width (sec) | Cycle time (sec) | Dwell time (msec) | Number of channels |
|---|---|---|---|
| 5 | 0.5 | 20 | 25 |
| 10 | 1.0 | 20 | 50 |
| 5 | 0.5 | 10 | 50 |
| 10 | 1.0 | 10 | 100 |
| 5 | 0.5 | 5 | 100 |
| 10 | 1.0 | 5 | 200 |
| 5 | 0.5 | 2 | 250 |
| 10 | 1.0 | 2 | 500 |

**The number of channels can be increased by using timed windows**

# Detector combinations (untargeted)

- **Each detector can record a MS spectrum**
  - Not sufficient even with the highest mass accuracy to ***uniquely*** identify the metabolite
    - 100s of metabolites can have the same empirical formula (and identical mass)
- **Fragmentation of selected ions creates a MSMS spectrum to distinguish isobaric metabolites**
  - In IDA analysis, molecular ions detected in a quick Hi-Res MS, are "selected" by the quadrupole filter one at a time
  - The ion is fragmented and a MSMS spectrum recorded
    - TOF instruments can record 20 MSMS spectra per second

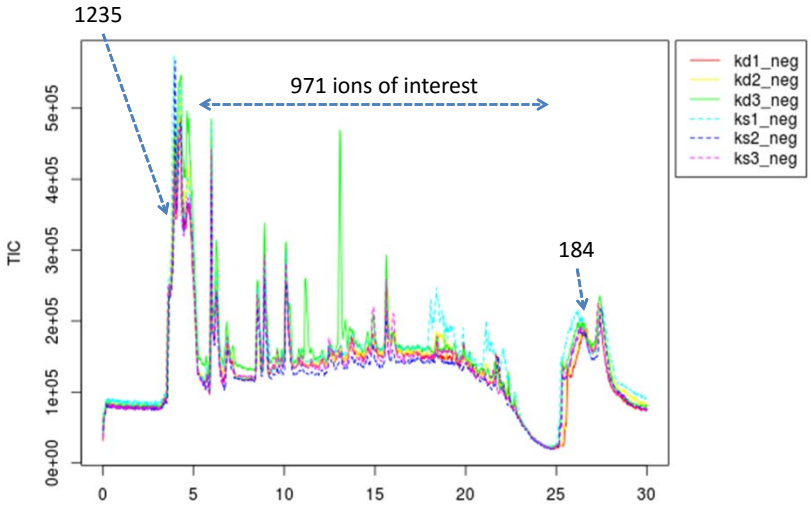
# Combined channels for Krebs cycle

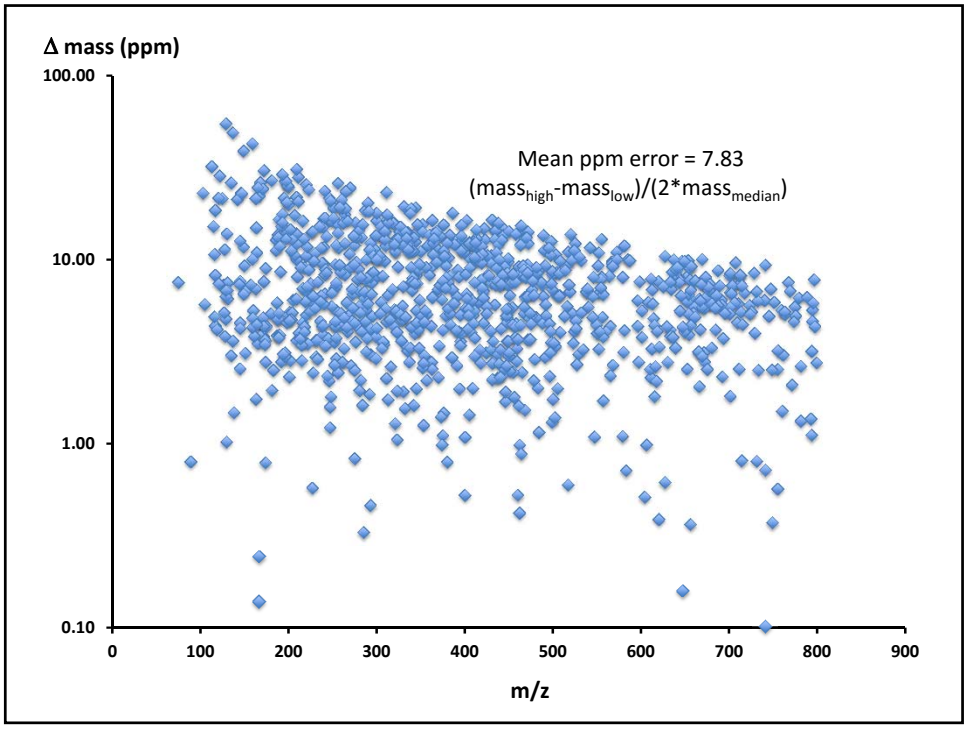


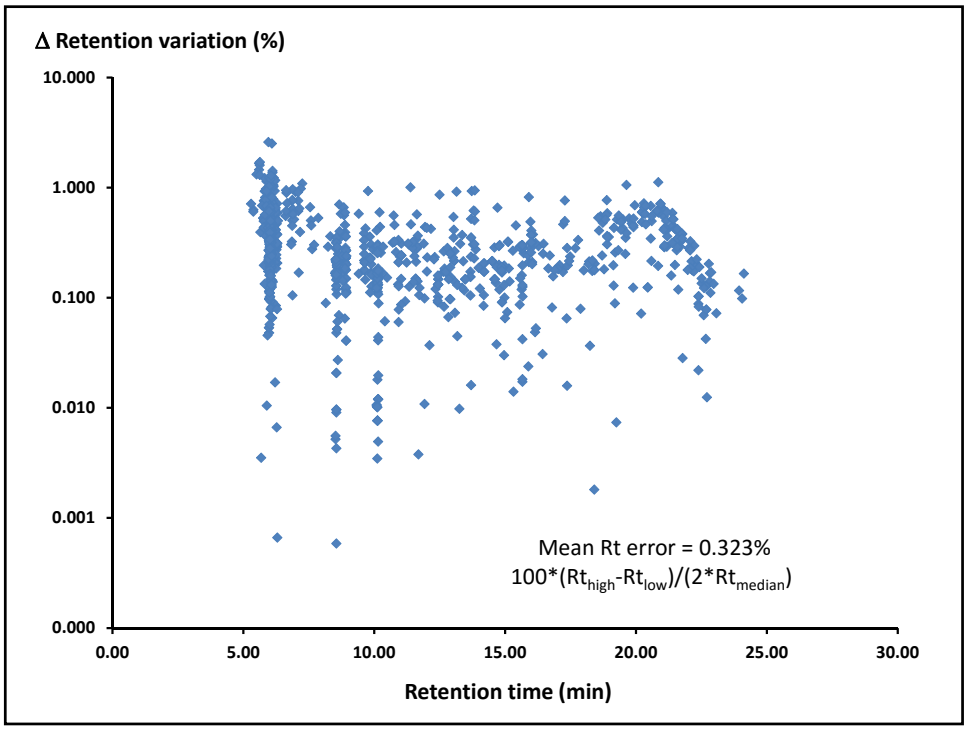
# Untargeted LC-MS

- The instrument of choice is a quadrupole-orthogonal TOF
  - We use a Sciex 5600 TripleTOF
- Collection of data
  - Duty cycle of 2 secs (to allow enough data points across a 20 sec wide chromatography peak)
  - 0-100 msec high accuracy MS in TOF analyzer (to identify the most intense ions)
  - 100 msec – 2 sec
    - 100 msec MSMS spectra of up to 19 different peaks

# A LC-MS TIC

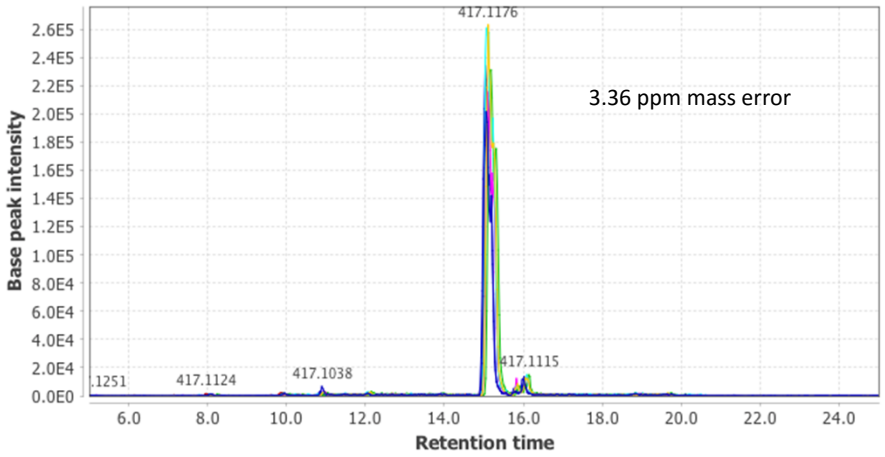



Δ mass (ppm)
100.00
10.00
1.00
0.10
0
100
200
300
400
500
600
700
800
900
m/z
Mean ppm error = 7.83
(mass$_{high}$-mass$_{low}$)/(2*mass$_{median}$)


Δ Retention variation (%)
10.000
1.000
0.100
0.010
0.001
0.000
0.00
5.00
10.00
15.00
20.00
25.00
30.00
Retention time (min)
Mean Rt error = 0.323%
100*(Rt$_{high}$-Rt$_{low}$)/(2*Rt$_{median}$)

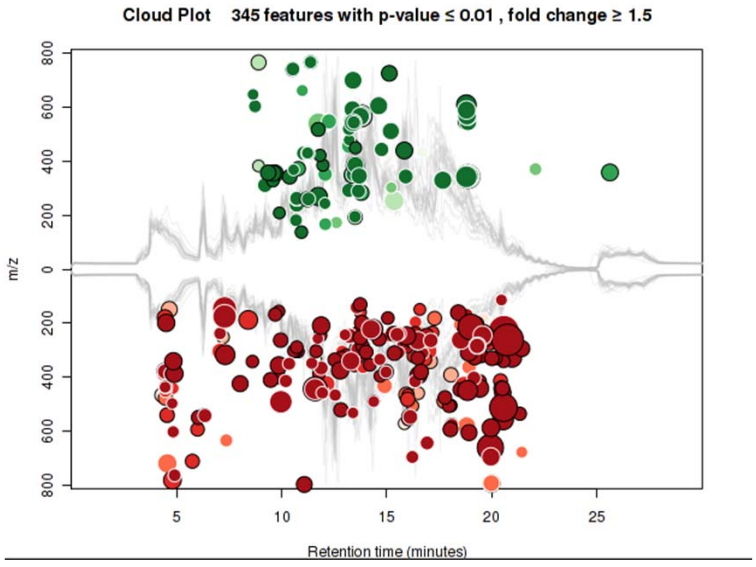
# Selected ion chromatograms of rat urine

## reversed-phase, negative ion nanoLC-MSMS



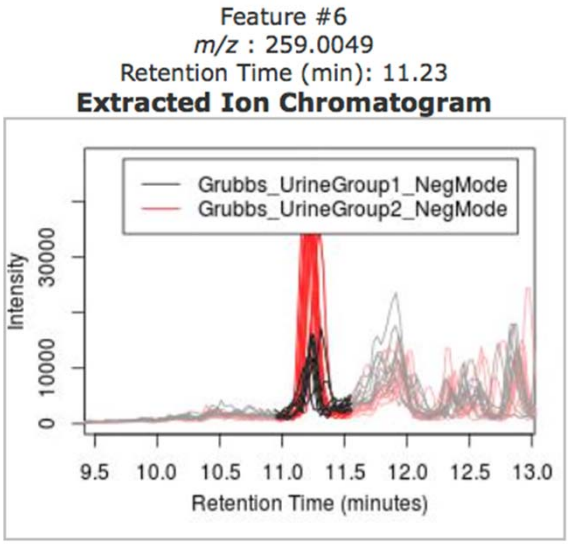

TIC library of ions was searched for *m/z* 417.119, S-equol β-glucuronide

# XCMS Cloud plot – negative ions

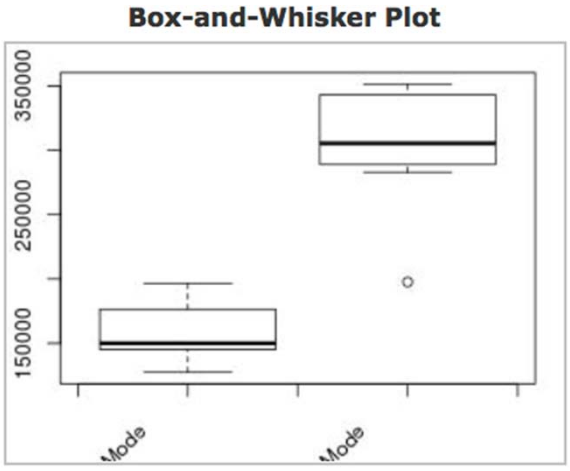


# Negative ion chromatogram

**fold change 1.9, p <6 x $10^{-6}$**

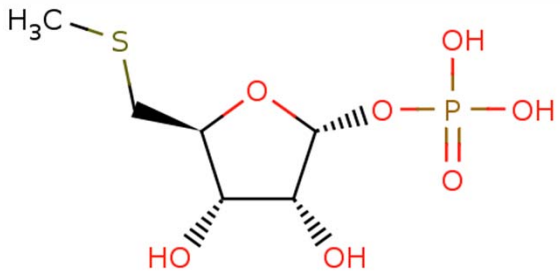



# Ion #6

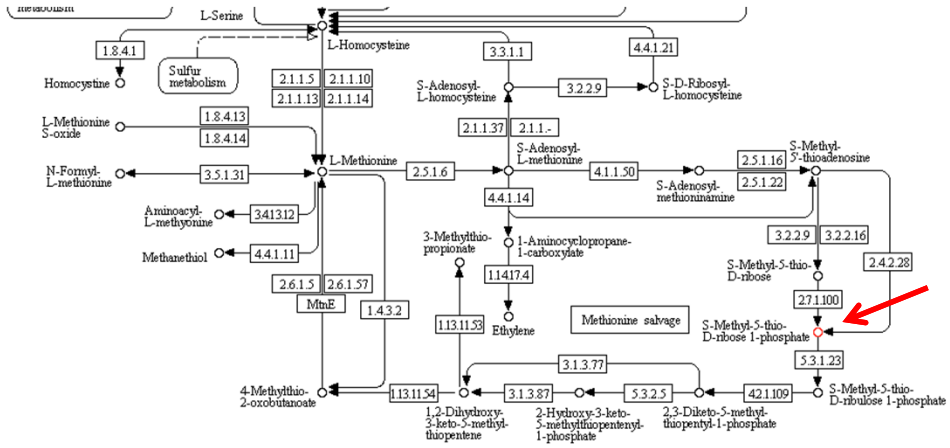


# Possible ID of ion #6

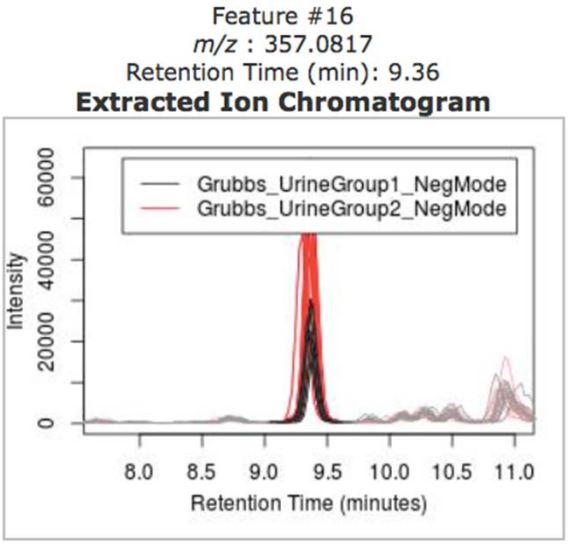
| PPM | Name | Adduct | METLINID |
|---|---|---|---|
| 1 | S-Methyl-5-thio-D-ribos | M-H | 63428 |
| 1 | 5-Methylthioribulose 1- | M-H | 6143 |
| 1 | Methylthioribosyl phosp | M-H | 6458 |
| 3 | PHORATE | M-H | 44563 |



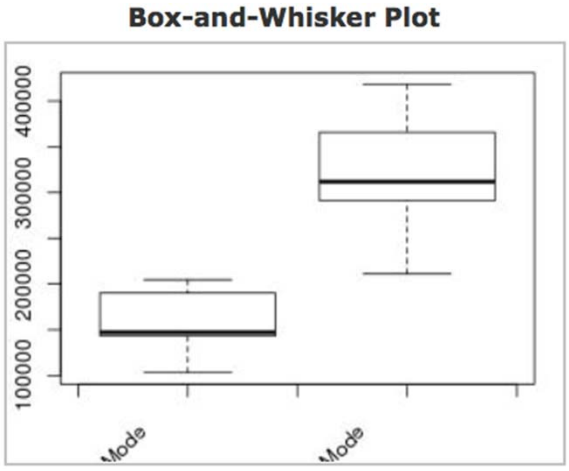

# KEGG pathway for 5-methylthio-D-ribulose-1-phosphate

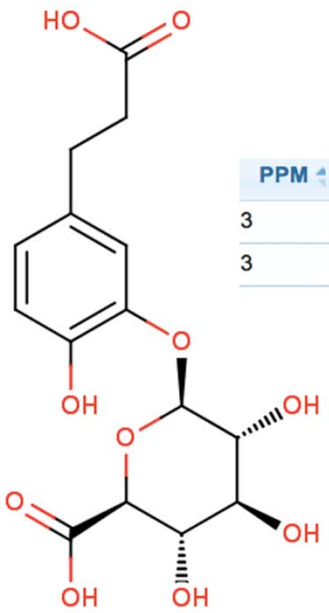


# Chromatograms for feature #16




# Quantitative change in #16






| PPM | Name | Adduct | METLINID |
|---|---|---|---|
| 3 | Dihydrocaffeic acid 3-C | M-H | 96078 |
| 3 | Mebeverine metabolite | M-H | 1082 |

# Download Mzmine 2

- Go to http://mzmine.github.io/
- Download
- Unzip the file and move the folder into Applications
  - There are three starting methods
    - Linux - startMZmine_Linux.sh
    - Mac - startMZmine_MacOSX.command
    - Windows - startMZmine_Windows.bat
  - Double click to start the program