Introduction to Metabolomics

10:00-11:00 Overview of techniques

Targeted and non-targeted metabolomics (metabolite extraction procedures, equipment GC-MS, HPLC-PDA-MS)

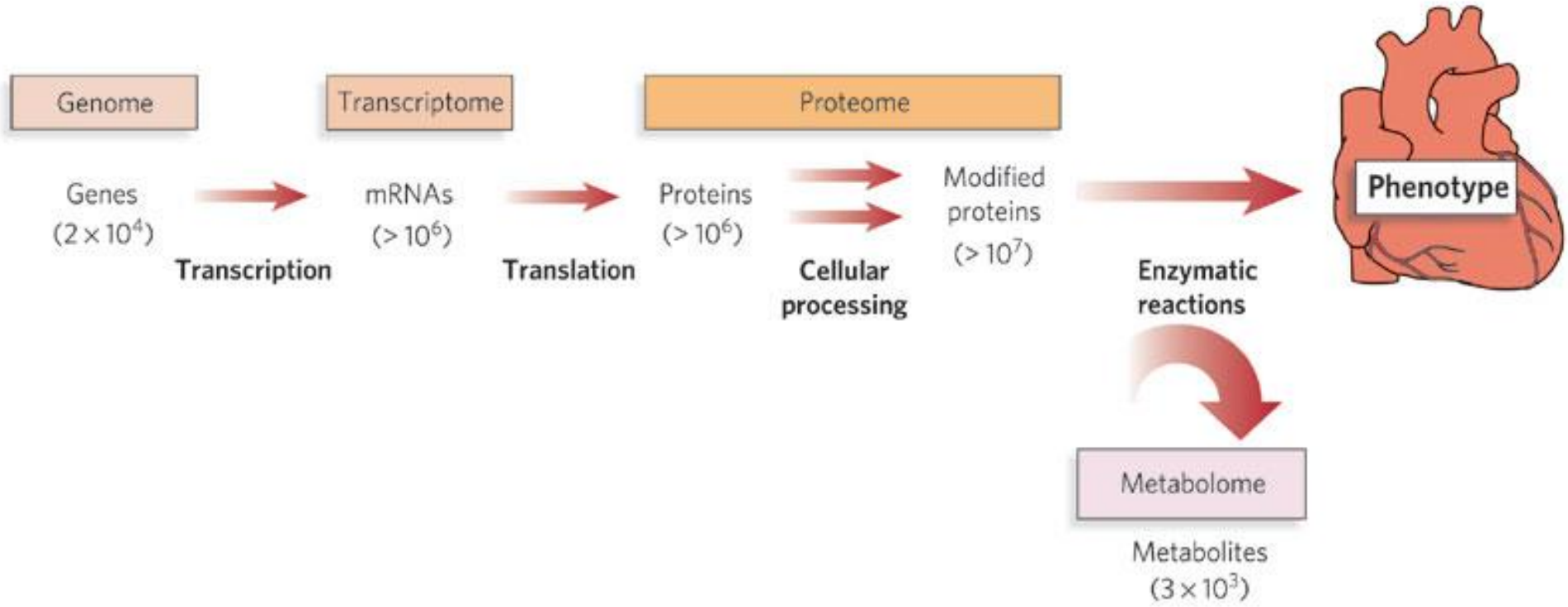
UNIVERSITY OF CAMBRIDGE

Department of Plant Sciences

AIMS:

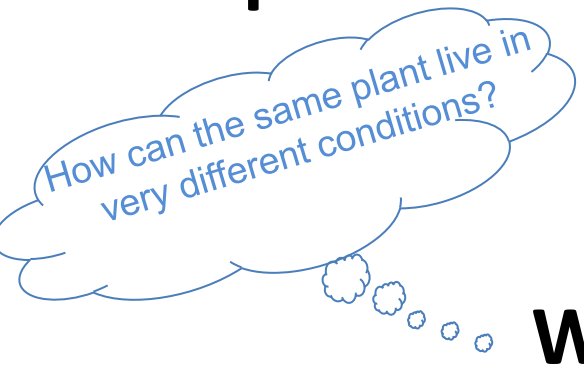
1. Experimental Design
2. Metabolite preparation: quenching and extraction
3. Metabolite separation and detection
4. Metabolite identification

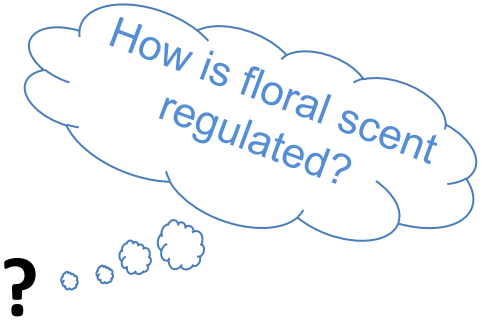
The Metabolome



R. E. Gerszten & T. J. Wang (2008) Nature **451**, 949-952

1. Experimental design: things to consider before you start.





WHAT'S THE QUESTION?

Non-targeted metabolomics

comprehensive analysis of all the measurable analytes in a sample including chemical unknowns.

Hypothesis generating

Targeted metabolomics

the measurement of defined groups of chemically characterized and biochemically annotated metabolites

Hypothesis testing

1. Experimental design: technical considerations.

Material to be analysed:

- organism, tissue, organ, serum, cell, subcellular compartment?
- Contamination of sample with microbes
- How much sample1-10mg MS
- 50-100mg NMR
- How many samples do you *need* for correct biological interpretation?
- How many samples do you *have* access to?

Sample preparation:

- How are you going to stop (**quench**) metabolism?
- Location – lab, field, hospital ward
- How are you going to extract the metabolites?

Analytical platforms:

What's available conduct the analysis?

- MS,
- HPLC,
- NMR
- GC

How are you going to **interpret** the data –

- univariate or
- multivariate statistics?

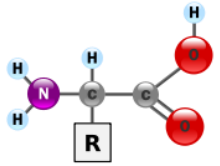
Will this answer your question?

AIMS:

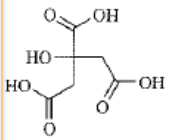
1. Experimental Design
2. **Metabolite preparation: quenching and extraction**
3. Metabolite separation and detection
4. Metabolite identification

2. Sample preparation: structural diversity of metabolites

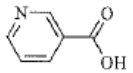
Amino acids



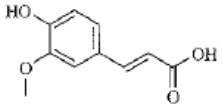
Organic acids



Citric acid

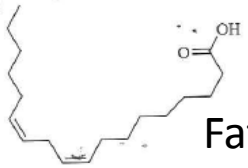


Nicotinic acid

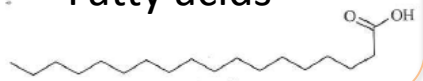


Ferulic acid

Fatty acids

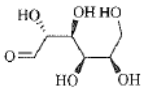


Linoleic acid

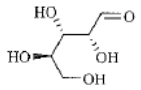


Stearic acid

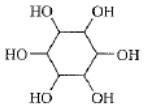
Monosaccharides



D-Glucose

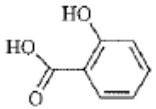


Xylose

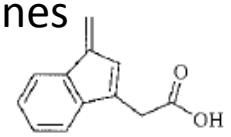


Inositol

Hormones

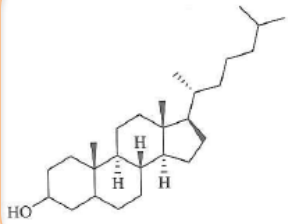


Salicylic acid

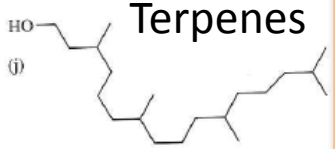


Indole-3-acetic acid

Sterols/ Terpenes

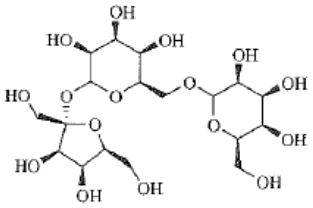


Cholesterol



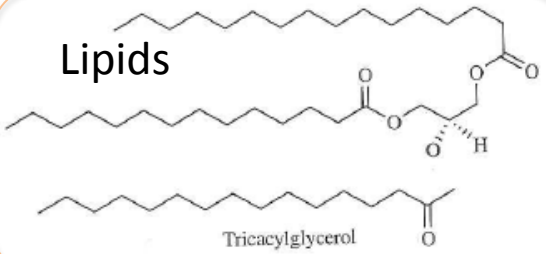
Phytol

Trisaccharides



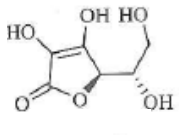
Raffinose

Lipids

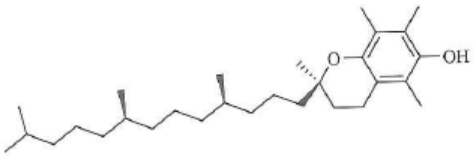


Tricacylglycerol

Vitamins



Vitamine C



Vitamine E

Solubility in water

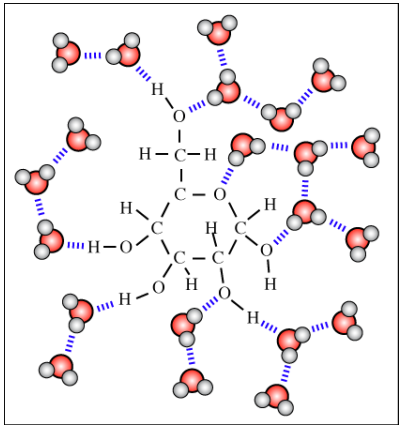
2. Sample preparation: chemical and physical properties of metabolites

Molecular weight = the sum of weights of all atoms making the molecule,
H_2O = 18 (18 g per mol); lipids = >1000 g per mol

Molecular size = the 3D size of the structure, measured as Å

Polarity = differences in electronegativity:
Polar (large difference in positive and negative charges) (**hydrophilic**)
non(a)-polar compounds (no or little difference in charge) (**hydrophobic**)
more O and H = more polar; more N = less polar

Solubility = related to polarity, temperature and size
(***like dissolves like***)
To dissolve - particles need to separate and fit between the solvent spaces
eg, in polar metabolites, a positive end of a metabolite attaches to a negative end of solvent – cannot happen if a positive charge has no negative charge to attach to



Volatility = depends on boiling and melting point – liquid to gas phase
(more polar = less volatile)

Stability = thermal or oxidative instability

2. Sample preparation: Solvents for extraction

Polarity	Highly non-polar (hydrophobic)			Highly polar (hydrophilic)	
Extract in Solvent	Heptane Hexane	Ethyl acetate Chloroform Dichloromethane		Acetone Acetonitrile Methanol	Ethanol Water Perchloric acid NaCl
Metabolites extracted	Lipids Fatty acids Waxes Terpenes	Carotenoids Chlorophylls Steroids Flavonoids	Phenolics Alcohols	Amino acids Organic acids Organic amines Alkaloids	Sugars Nucleotides Phosphates

2. Sample preparation: Quenching

Quenching - Stop enzymatic metabolism

- Turnover rate is **fast**: reaction half lives < 1s
  - glucose to glucose-6-phosphate 0.3 to 1 mM per s
  - ATP used at a rate of 1.5mM per s

- Temperature:
  - Cold (< -40^{o}C)
  - Hot (>80^{o}C)
- pH
  - Acid (pH <2.0)
  - Alkaline (pH > 10)

- Common quenching methods:
  - Liquid nitrogen with or without freeze drying
  - Hot or cold Ethanol/Methanol
  - Perchloric acid
  - Sodium Hydroxide
  - Cold NaCl

Now metabolism is stopped, how do you extract metabolites?

2. Sample preparation: Extraction methods

Each group of metabolites will have an optimal extraction method (no single method will extract them all)

Liquid phase extraction

Grind sample, extract with solvent

Liquid : Liquid biphasic extraction

Take liquid extract, extract with another solvent

Solid : Liquid Extraction

Take liquid extract, extract with solid phase material

Volatile extraction

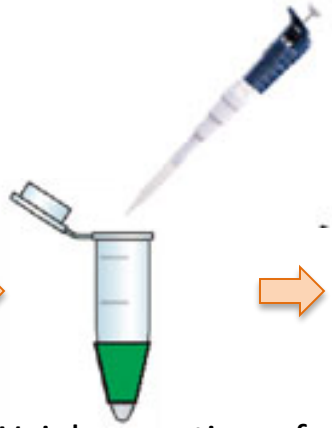
Steam distillation or headspace volatile collection

2. Sample preparation: Tissue disruption and Liquid phase extraction

Pestle and mortar

Grind tissue to fine powder in liquid nitrogen

Freeze dry material 1-2 days

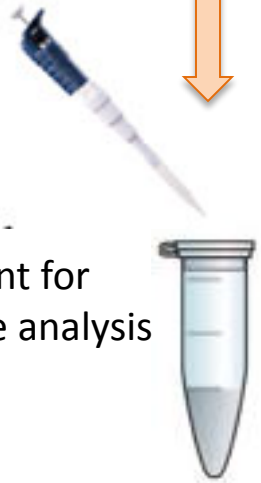


Weigh a portion of freeze-dried sample and add extraction solvent

Sonicate for 20-30 minutes

Spin down tissue debris (requirement for cooled centrifuge?)

Tissuelyser / Ball mill
Grinds frozen or freeze-dried material with or without extraction solvent



Recover supernatent for metabolite analysis

2. Sample preparation: Liquid: Liquid biphasic extraction

Biphasic partitioning allows polar and non-polar metabolites to be recovered separately

i) Extraction in polar solvent

extract in aqueous alcohols such as methanol, ethanol, and isopropanol.

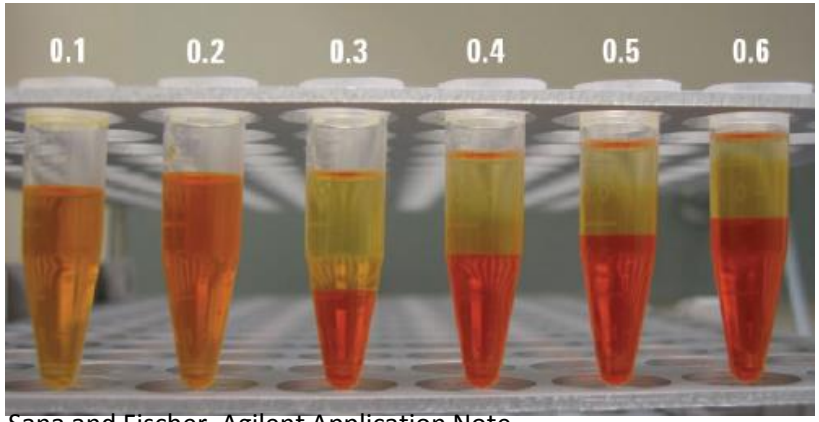
Alcohols are miscible with water and may be used as aqueous phase co-solvents to enhance the solubility of less-polar metabolites during the extraction process

ii) Mix with non-polar solvent

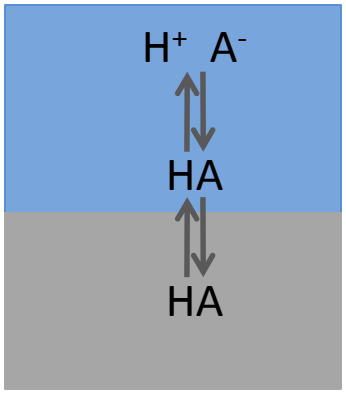
organic solvents such as chloroform

$CHCl_3$ is volatile, nonreactive, immiscible with and denser than water

to form a two-phase system for liquid-liquid extraction



Sana and Fischer, Agilent Application Note,

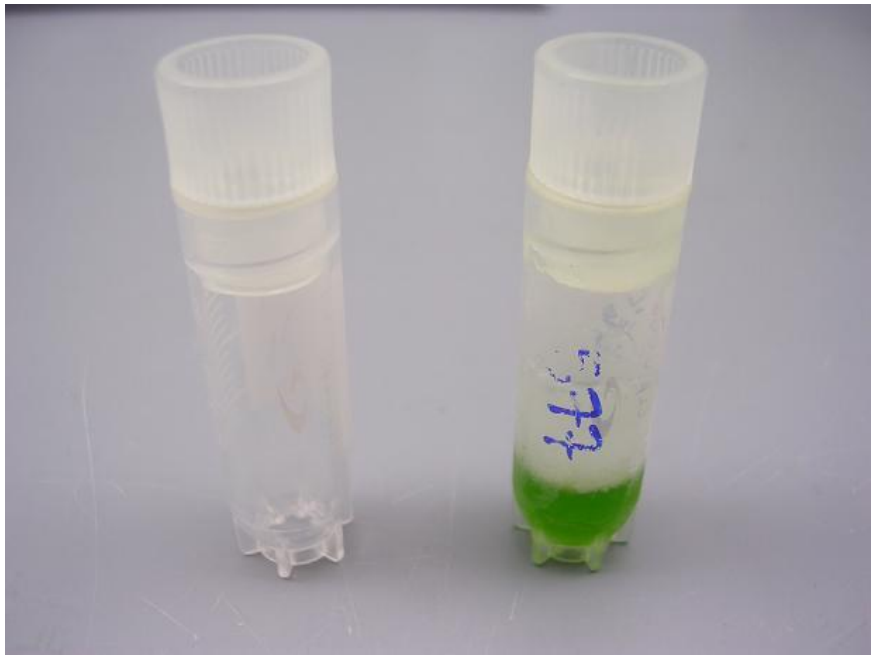


2. Sample preparation: Common bi-phasic metabolite extraction procedure

Solvent mixture A = MeOH/$CHCl_3$/H_2O, 2.5:1:1, v/v/v at –20 ^oC;

Solvent mixture B = MeOH/$CHCl_3$, 1:1, v/v at –20 ^oC

Solvent C = deionised/distilled H_2O at 4 ^oC



What next – need to analyse metabolites

2. Sample preparation: Quality control

ALWAYS validate methodologies

- **Pool** from representative samples after extraction
- **Run** at the start and end, an every 5 or 10 samples during data acquisition
- Observe **technical** reproducibility
- **Spike** (add) extract with known amount of non-interfering substance – can you recover all of that spike after your analysis?

AIMS:

1. Experimental Design
2. Metabolite preparation: quenching and extraction
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4. Metabolite identification

3. Metabolite separation

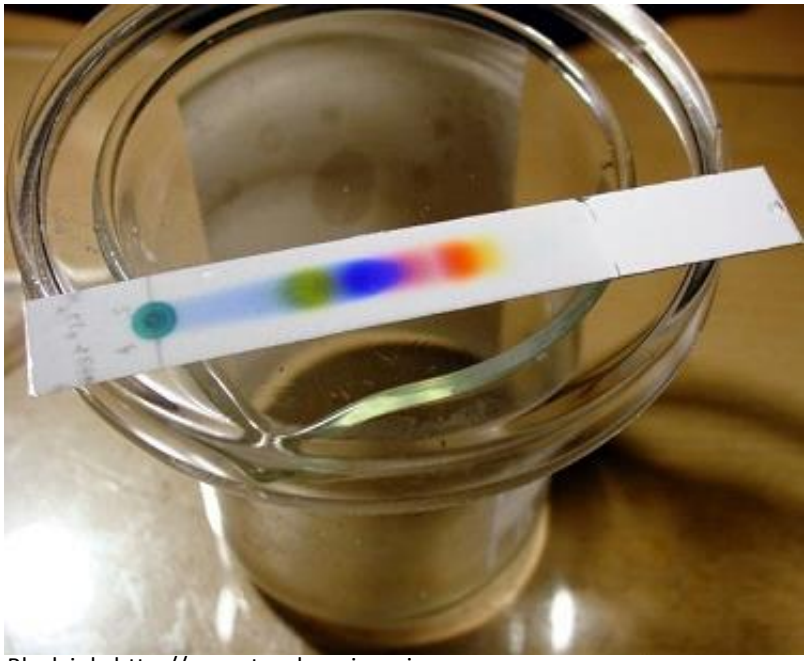
Separation Methods

- Thin layer chromatography
- High Performance Liquid Chromatography (HPLC)
- Gas chromatography (GC)
- Capillary electrophoresis (CE)

3. Metabolite separation : The basics

Thin Layer Chromatography using Paper or Silica Gel

- Aim is to separate (**resolve**) different metabolites in a mixture
- Maximum number of peaks that can be resolved is called '**peak capacity**'
- Can be increased by changing ratio of liquids/solvents or temperature



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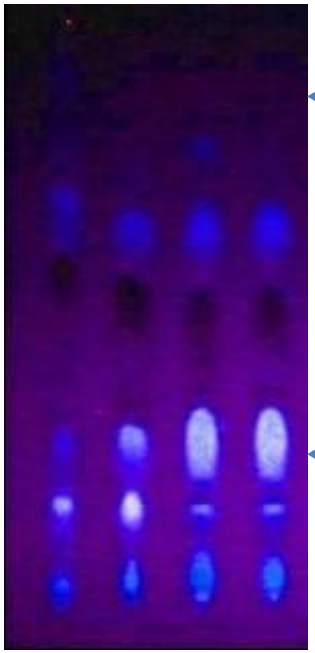
254nm



Direction of solvent flow

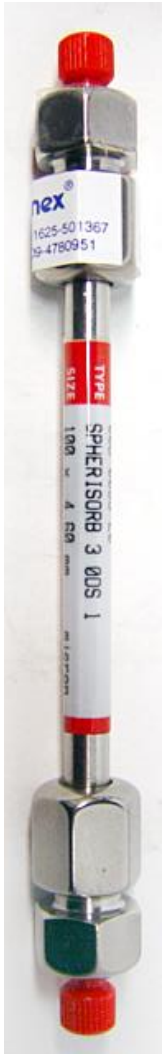
aluminium foil coated with a thin layer of adsorbent material (silica gel, =polar) = stationary phase

Compounds in the extract **adsorb** to stationary phase with differing affinities

Solvent = mobile phase (non-polar)

3. Metabolite separation : HPLC High Performance Liquid Chromatography

Same principle as TLC but **stationary phase** (resin) is packed tight in a narrow column and needs pumps to push the **mobile phase (**solvents) around the resin



Normal phase liquid chromatography

-column is packed full of a ***polar*** compound (eg. alkyl nitrile)

-***non-polar mobile phase*** such as hexane

-good for lipids

Reverse-phase liquid chromatography

-column is packed with a ***non-polar*** silica compound

(eg C8 octasilane or C18 octadecylsilane)

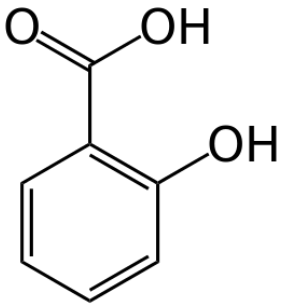
-***polar mobile*** phases such as water/methanol/acetonitrile

-changes in pH, salts, solvent affect retention times

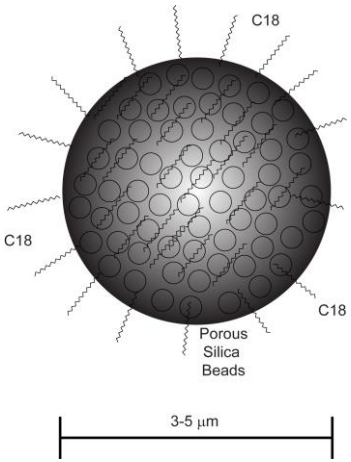
-good for phenolics, sugars, amino acids, drugs, pesticides

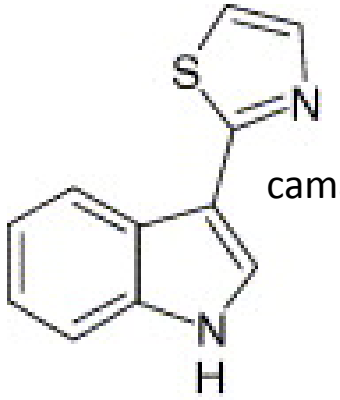
3. Metabolite separation : Reverse Phase HPLC

Different metabolites will have different retention times

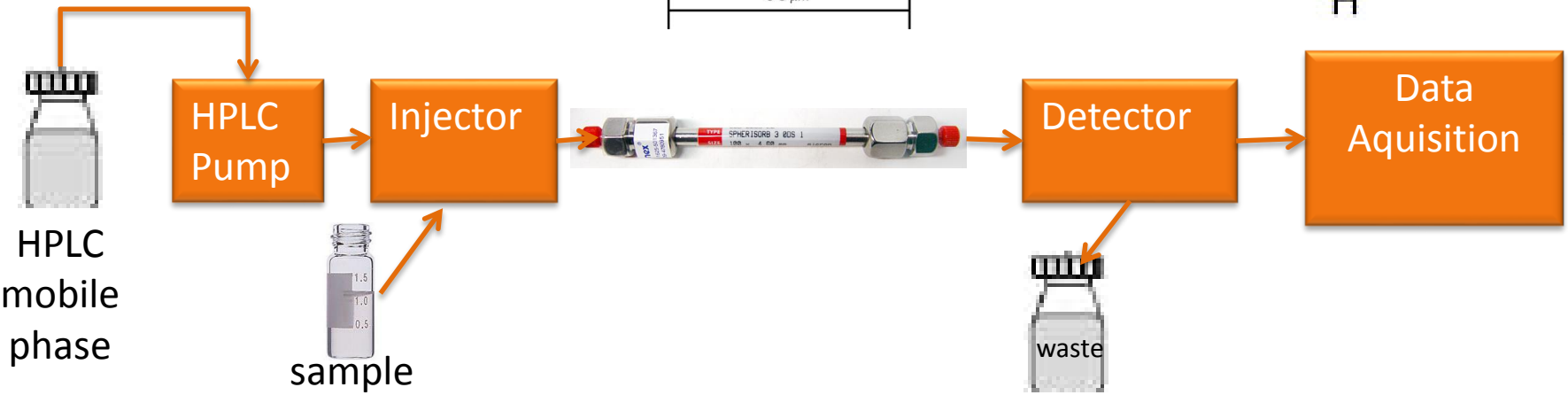


Salicylic acid
2 hydroxy
benzoic acid





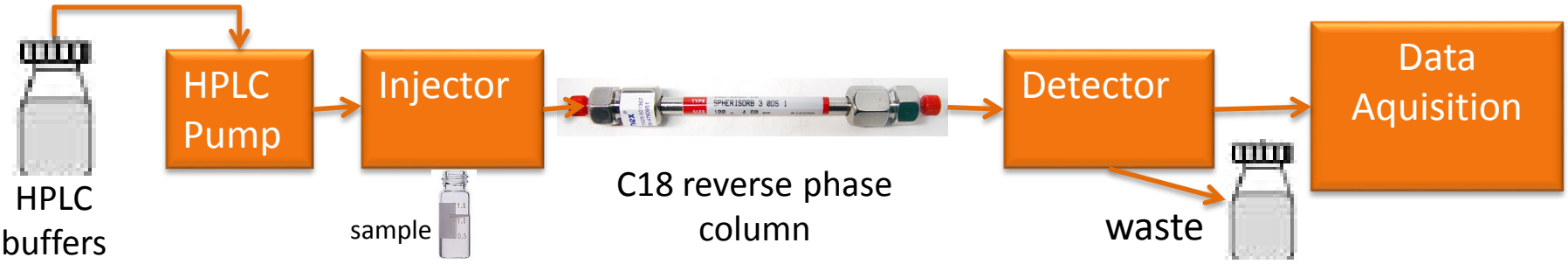
camalexin



Isocratic – same solvents ratios running through column eg. 100% Methanol

Gradient - change in solvent ratios over time
eg. start at 10 % MeOH
finish at 60% MeOH
over 20 minutes

3. Metabolite separation : HPLC The hardware



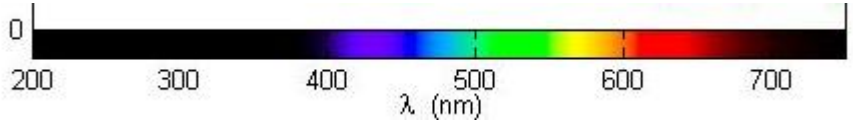
3. Metabolite separation : Detectors for HPLC

1. **UV/VIS:**
   - Fixed wavelength
   - Variable wavelength
   - Diode array

2. Refractive index
3. Fluorescence
4. Conductivity
5. Antioxidant
6. Evaporative light scattering
7. Electrochemical
8. **Mass Spectrometer**

3. Metabolite separation : UV/VIS Detectors for HPLC

SPECTROPHOTOMETRY - Absorption of electromagnetic radiation



HPLC separation – set wavelength in UV range (284 nm)

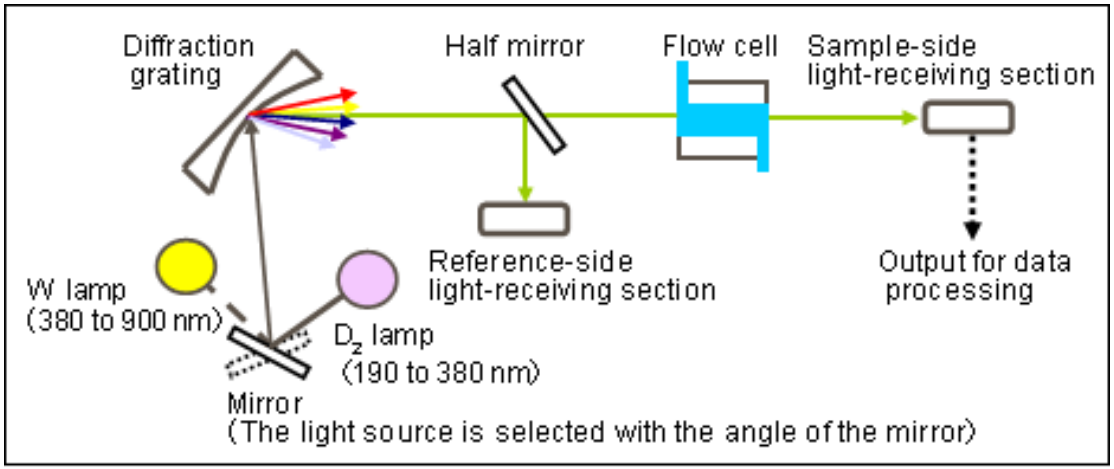
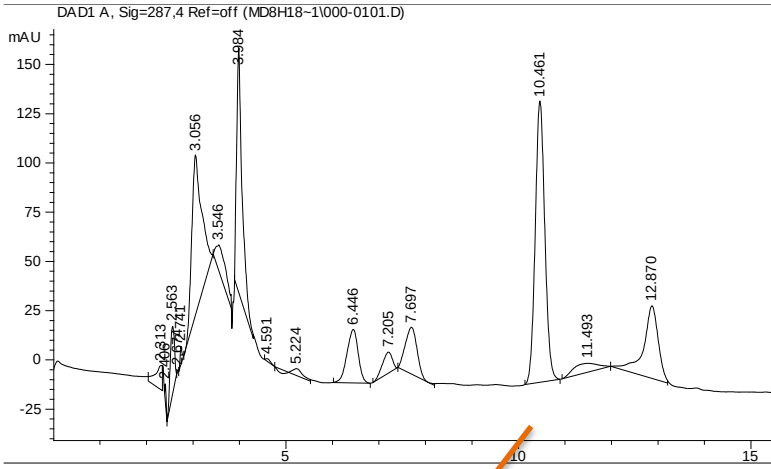


Figure 1. Diagrammatic illustration of a UV-VIS detector optical system



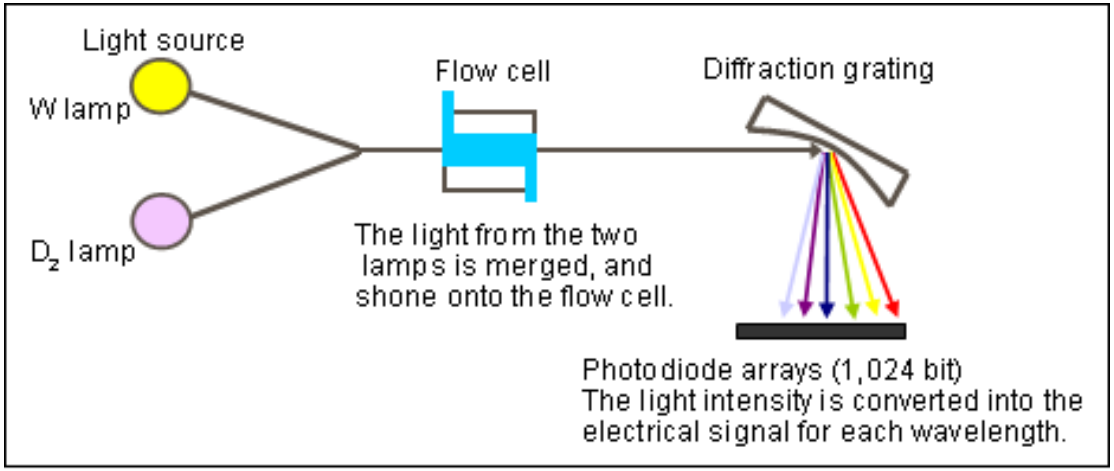
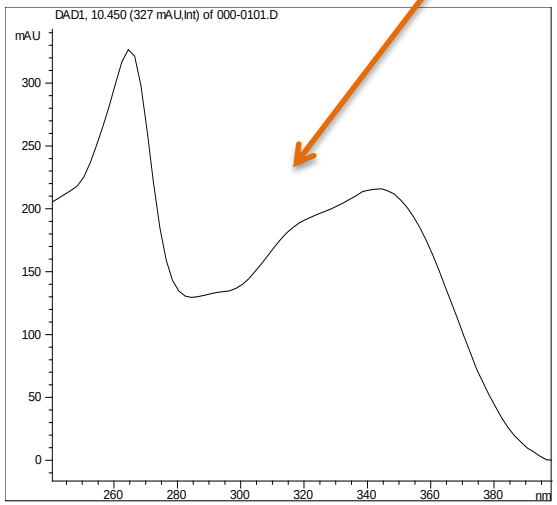
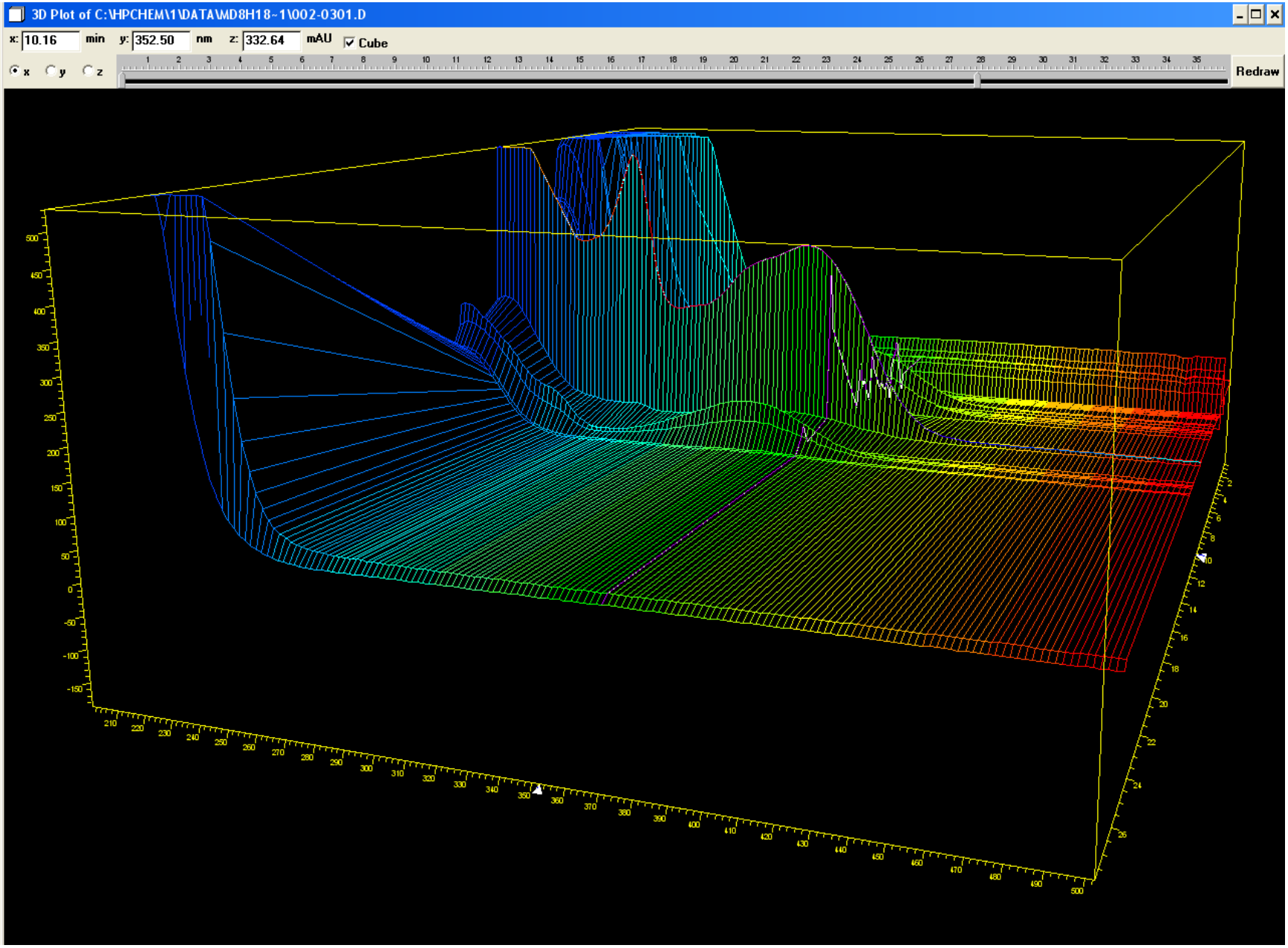


Figure2. Diagrammatic illustration of a DAD optical system



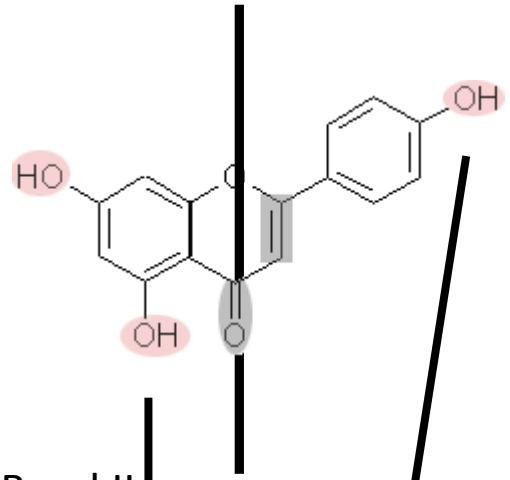
UV-absorbance Multi-scan wavelength (200 to 500nm)

Photodiode Array Detection



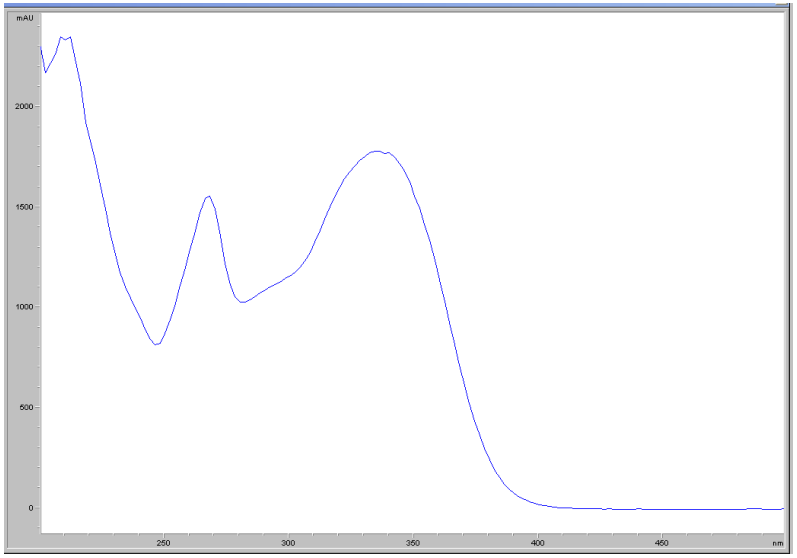
What does this UV spectrum tell us about the compound?

Flavone – eg. Apigenin



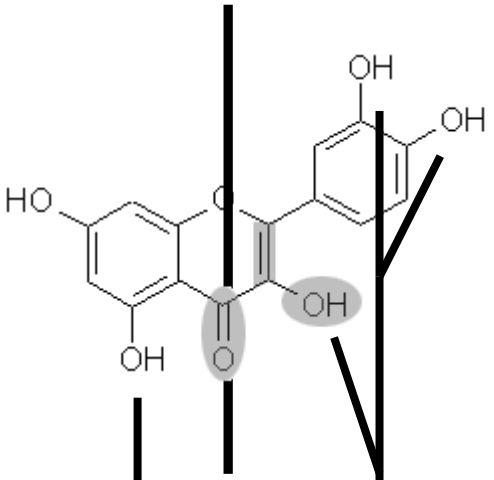
Band II
240-280nm

Band I
at 304-350nm



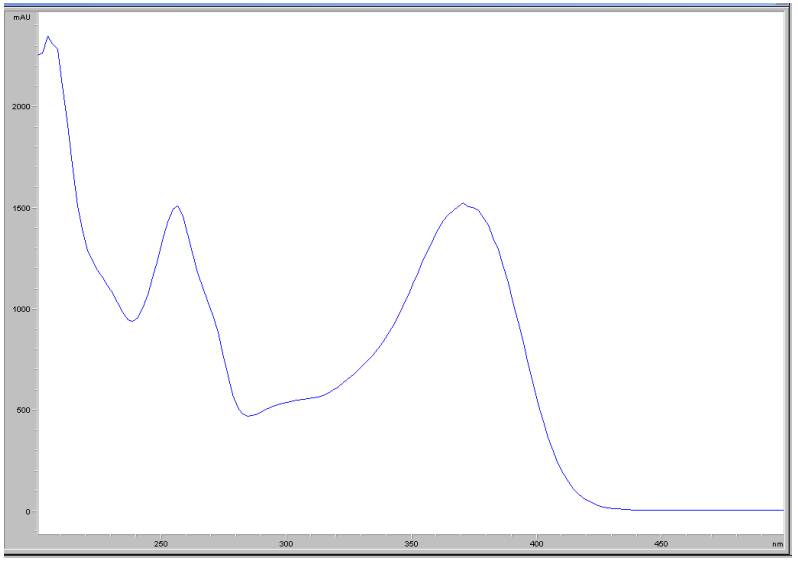
Band I and II close

Flavonol – eg. Quercitin



Band II

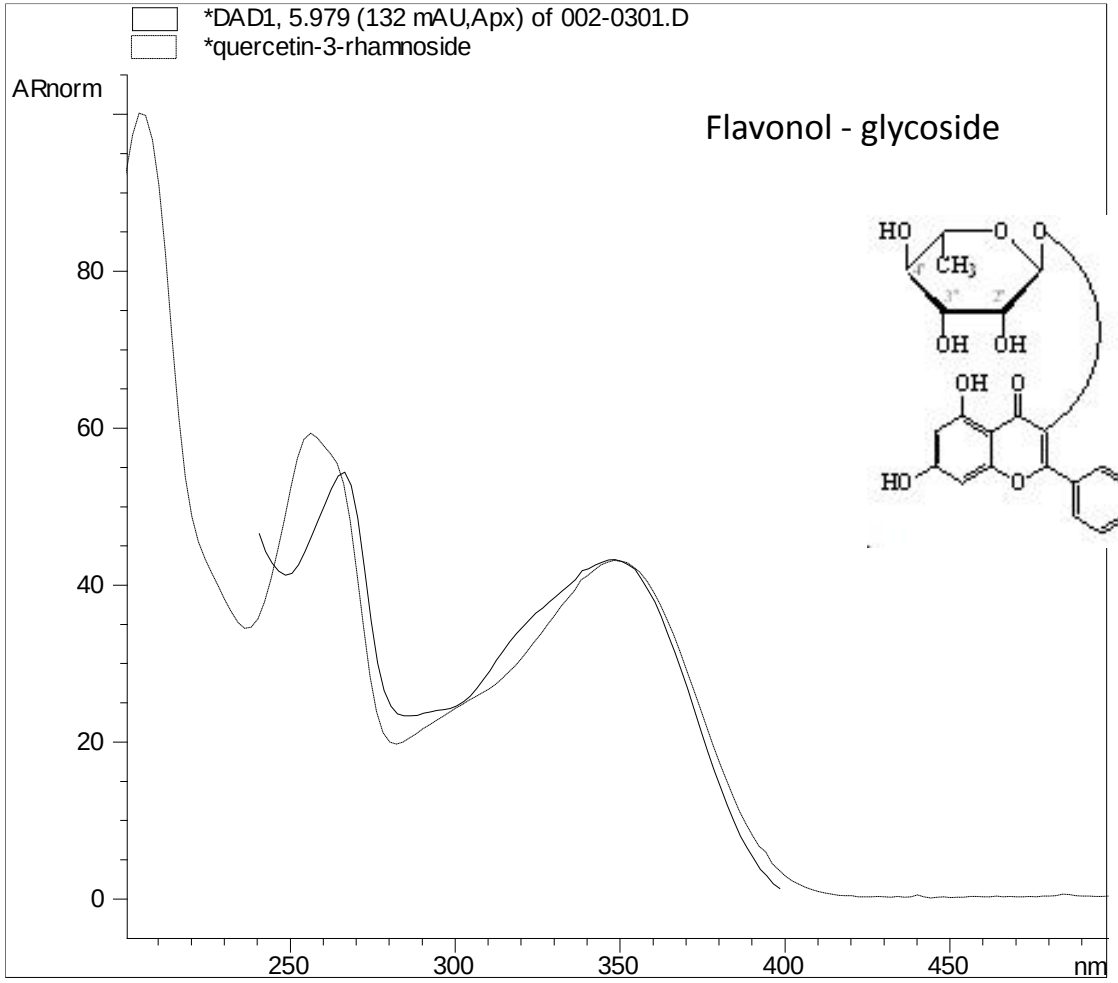
Band I
at 352-385nm



Band I more defined and further away from band II

Characteristic UV absorption spectra can be used to i.d. flavonoids

Overlay unknown sample peak (eg, possible flavonol-glycoside) with known standard peak



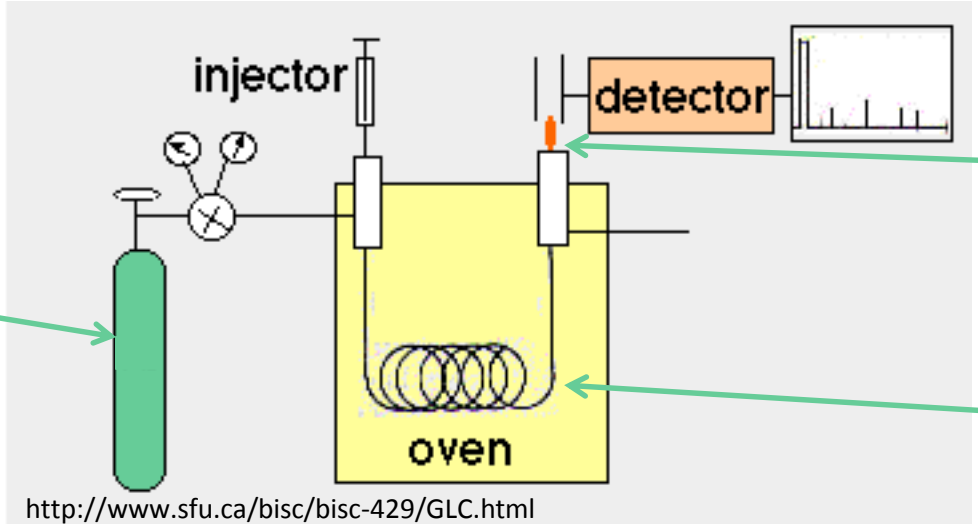
Flavonol - glycoside

Quercitin-3-Rhamnoside

3. Metabolite separation : GC-FID

Gas Chromatography – Flame Ionisation Detection

Same principle as HPLC but use **GAS** rather than Liquid to separate metabolites

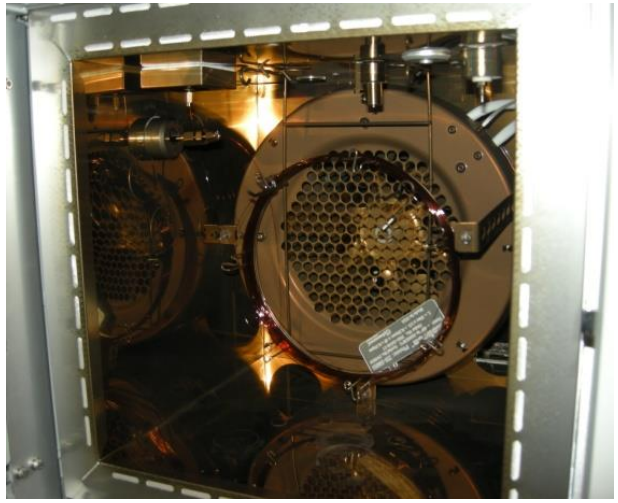


http://www.sfu.ca/bisc/bisc-429/GLC.html

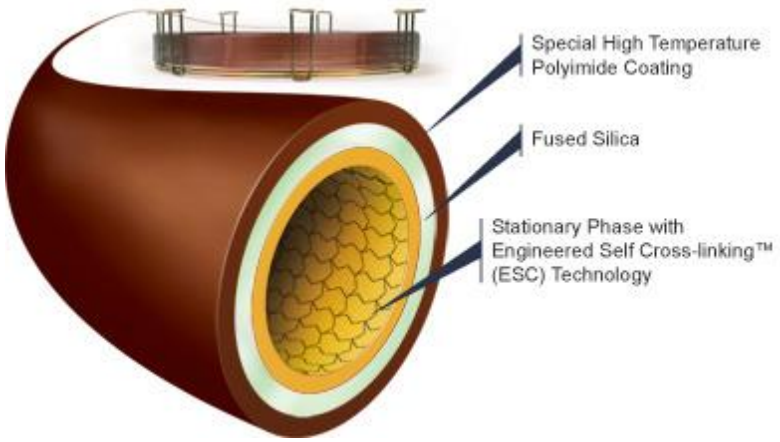
MOBILE phase
Typically Helium
(inert)

Hydrogen is
used for flame

Non-polar Fused
Silica is the
STATIONARY phase



3. Metabolite separation : GC-FID



chemwiki.ucdavis.edu

Samples (about 1µL) are injected into a hot (250 ºC) glass tube where the sample is vapourised

Vapour goes into the column

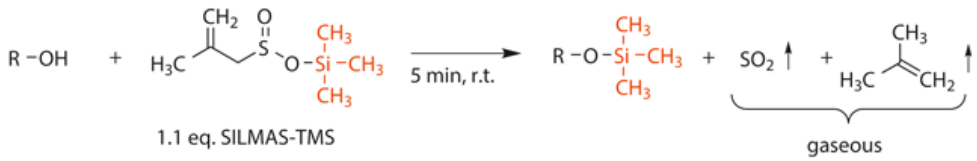
Separation based on the difference in partition coefficients between the solid (liquid) stationary phase and the mobile gas phase

Increasing temperature biases compounds to leave the stationary phase and enter the gas phase

Many polar compounds are NOT volatile
Sugars, amino acids, organic acids

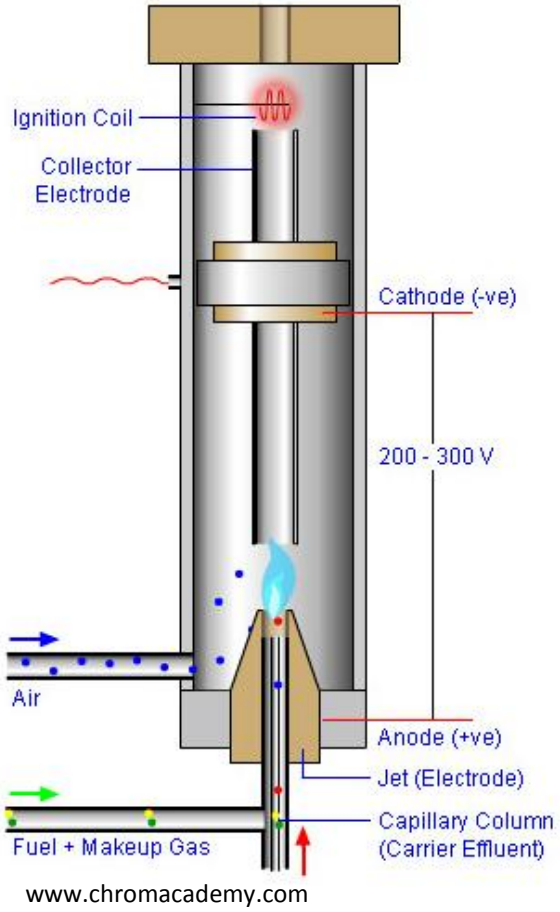
Derivatisation – make compounds volatile by making them more apolar

Silylation is the most widely used technique.
acidic hydrogen replaced with with an alkylsilyl group (eg. $SiMe_3$)
to form tri-methyl silyl (TMS) derivatives



3. Metabolite separation : GC-FID

Detection: Flame Ionisation Detection



6. FID to give picogram-level detection

5. Ionization current between the two electrodes is directly proportional to the hydrocarbon concentration in the eluate that is burned by the flame

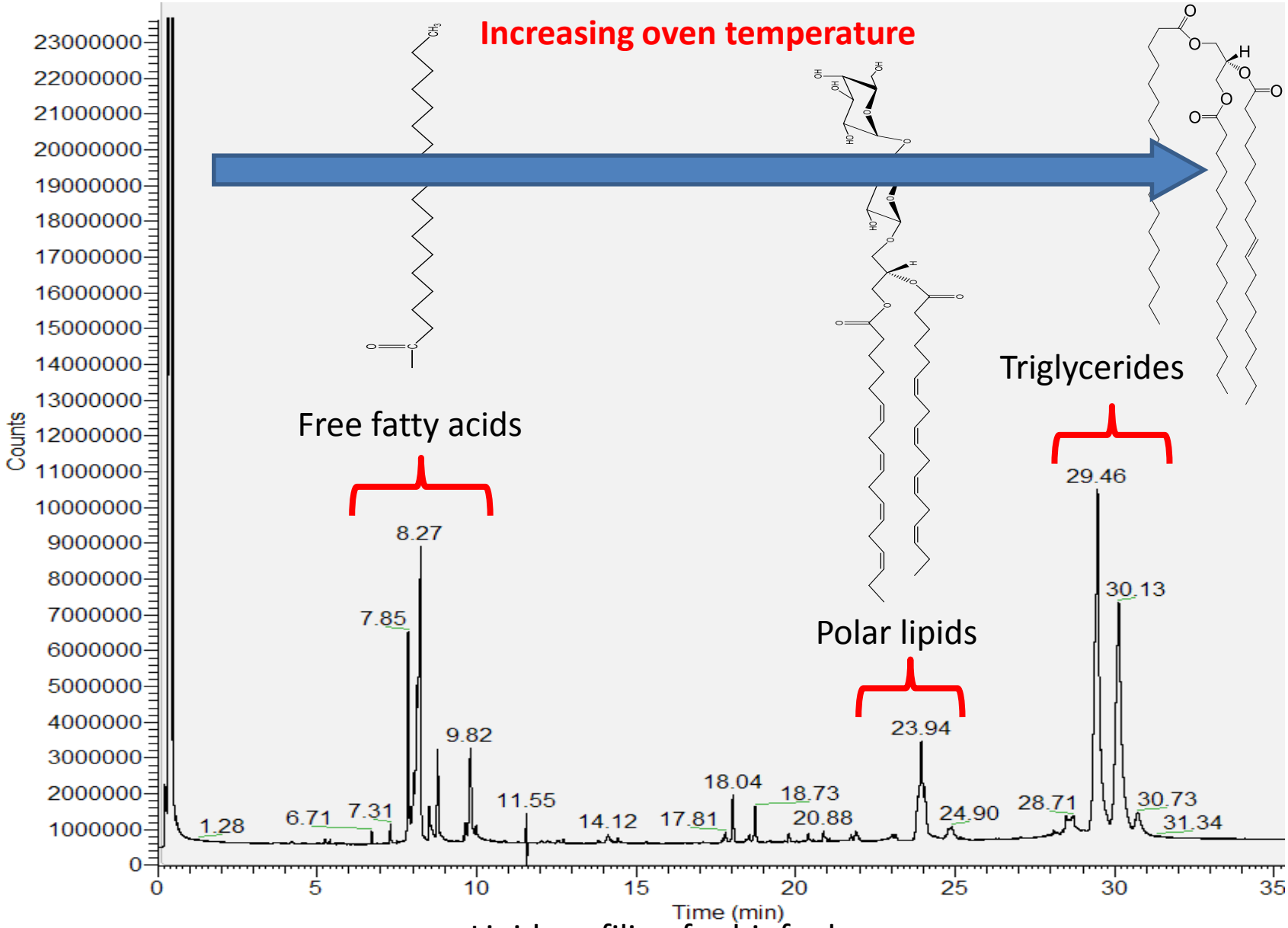
4. +ve ions migrate to the negative collector electrode and electrons migrate to the jet and are grounded

3. A high polarizing voltage is applied between the two electrodes

2. When sample emerges from column it is ionised in the flame producing +ve ions and electrons.

1. Mixture of Hydrogen and Air is used for flame. Burning hydrocarbon-free hydrogen in hydrocarbon-free air produces a negligible number of ions

Gas Chromatography (GC) – Flame Ionisation Detector (FID)



Lipid profiling for biofuels

AIMS:

1. Experimental Design
2. Metabolite preparation: quenching and extraction
3. Metabolite separation and detection
4. **Metabolite identification**

4. **Metabolite identification**
   Mass Spectrometry

- Direct Injection Mass Spectrometry (DIMS)
- HPLC-PDA-MS
- GC-MS

4. Metabolite identification

What is Mass Spectroscopy?

Determination of the molecular weight of atoms and molecules

This is useful because the knowing the mass of a molecule is sometimes sufficient, often necessary and always useful in identifying it.

How is Mass Spectroscopy done?

A charge is put on the molecules of interest, (i.e., the analyte). Then measures how the trajectories of the resulting ions respond (in vacuum) to various combinations of electric and magnetic fields

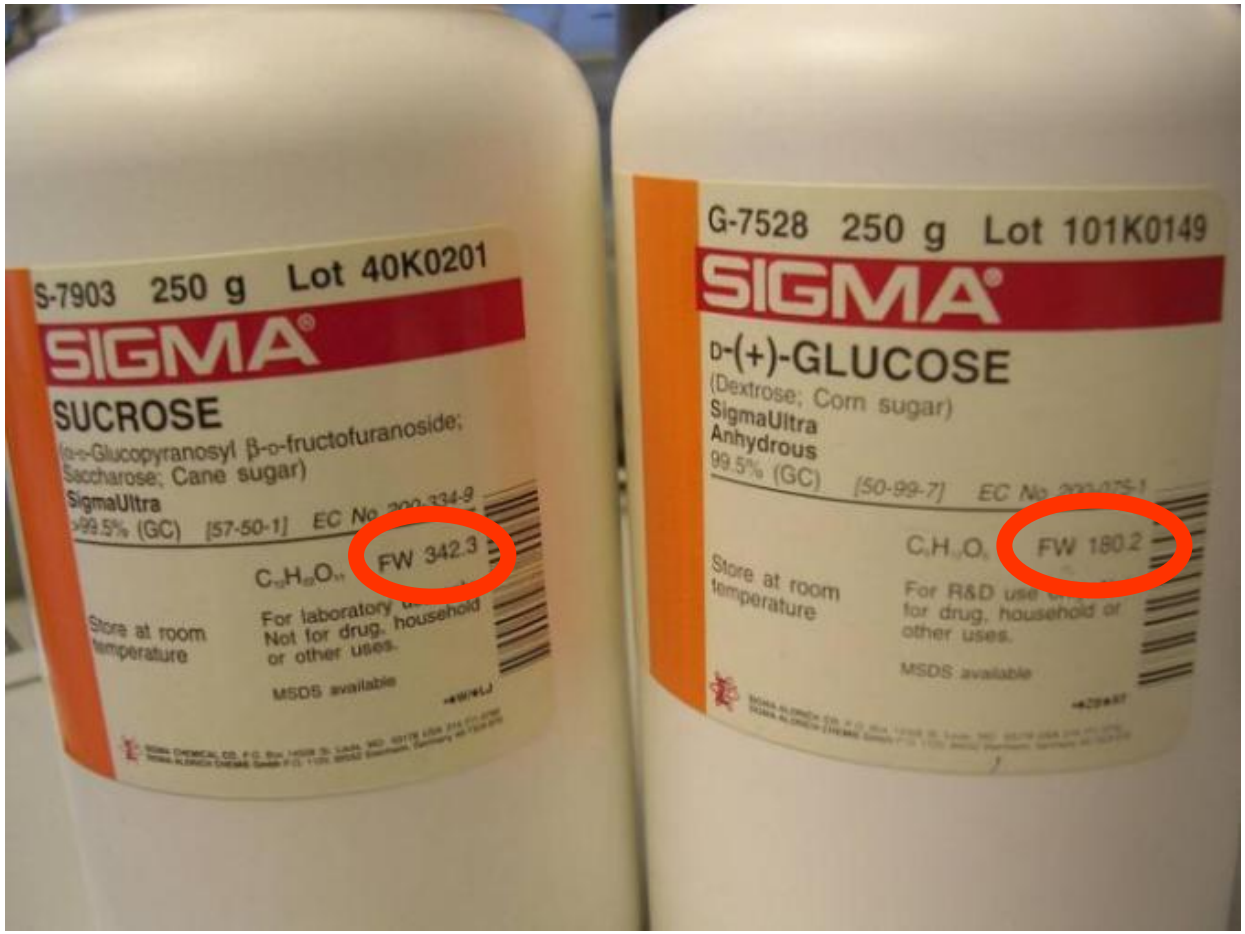
What does Mass Spectroscopy measure?

measure the *m/z ratio,* not the mass

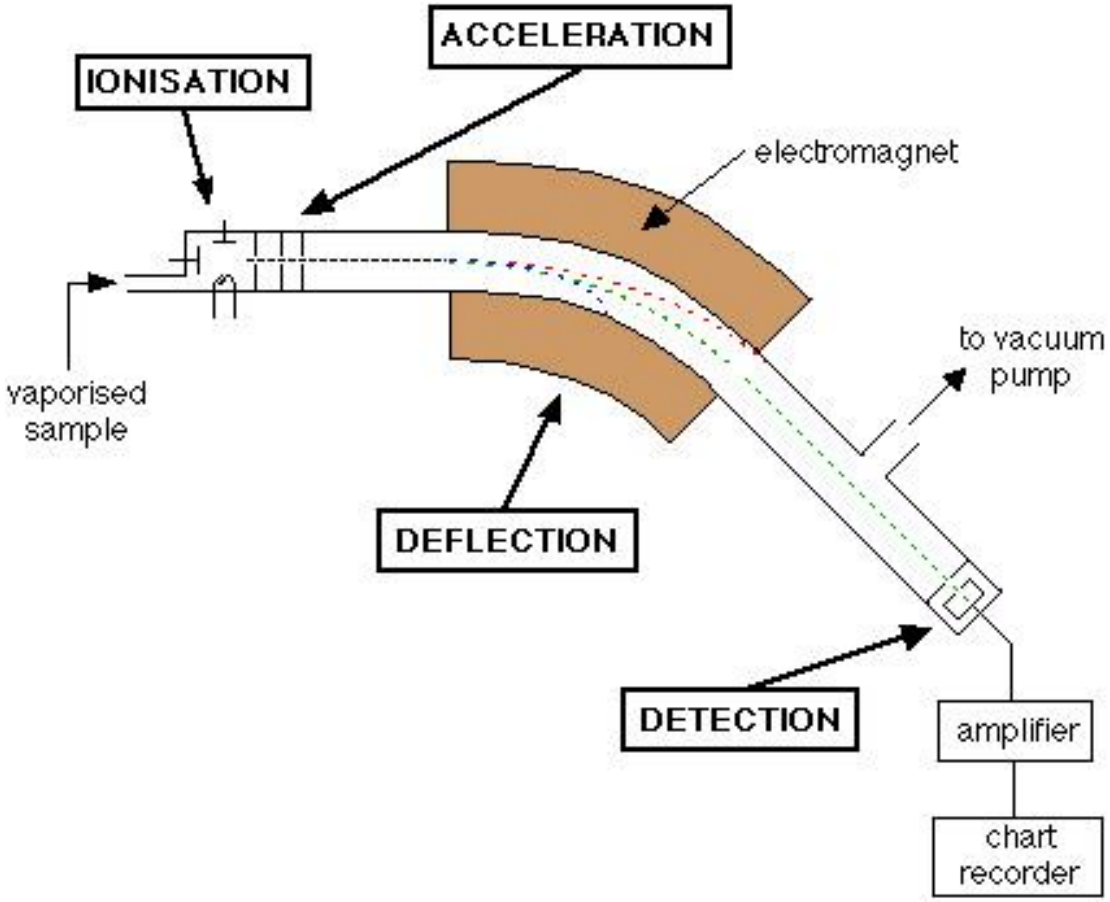
if an ion has multiple charges, the *m/*z will be significantly less than the actual mass

Knowing the mass of a molecule can help us to identify it.

Molecular mass or molecular weight refers to the mass of a molecule.
It is calculated as the sum of the mass of each constituent atom



4. Metabolite identification: The basic hardware



Ionisation
Individual metabolites in the sample are ionised and either become positively or negatively charged.

Acceleration (Separation)
These ions are then accelerated so that they all have the same amount of energy.

Deflection (Separation)
The ions are then deflected by a magnetic field according to their masses. The lighter and more charged they are, the more they are deflected.

Detection
The ions passing through the machine are detected electrically.

inlet | Ionisation source | Mass analyser | Ion detector

4 basic components of all mass spectrometers

4. Metabolite identification: Ionisation sources

The sine qua non of such a method (mass spectroscopy) is the conversion of neutral analyte molecules into ions.

For small and simple species the ionization is readily carried by gas-phase encounters between the neutral molecules and electrons, photons, or other ions.

In recent years, the efforts of many investigators have led to ***new techniques for producing ions of species too large and complex to be vaporized without substantial, even catastrophic, decomposition****.*

http://en.wikipedia.org/wiki/John_Fenn

John B. Fenn, the originator of electrospray ionization for biomolecules and the 2002 Nobel Laureate in Chemistry
http://masspec.scripps.edu/mshistory/whatisms_details.php#Basics

4. Metabolite identification: Ionisation sources

Many different types:

•Electron Impact (EI) (hard)

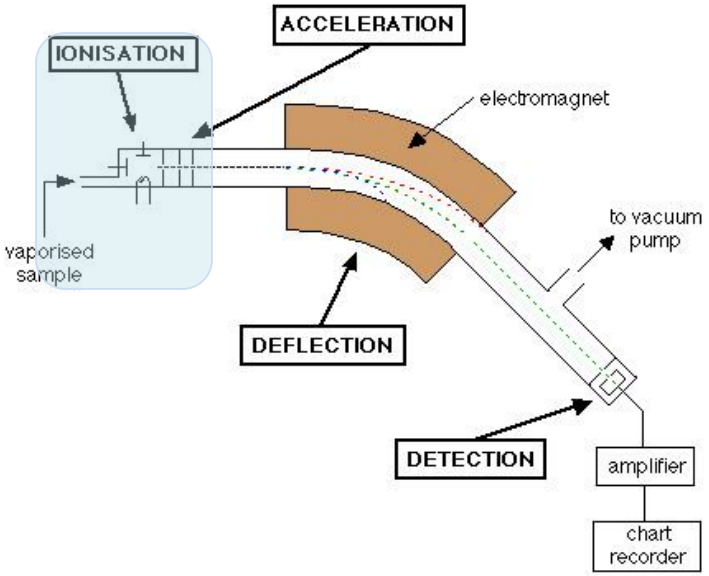
•Atmospheric Pressure Chemical Ionisation (APCI) (good for polar compounds)

•Chemical Ionisation (CI)

•Electrospray Ionisation (ESI) (good for polar compounds) (soft)

•Fast Atom Bombardment (FAB)

•Matrix Assisted Laser Desorption Ionisation (MALDI)

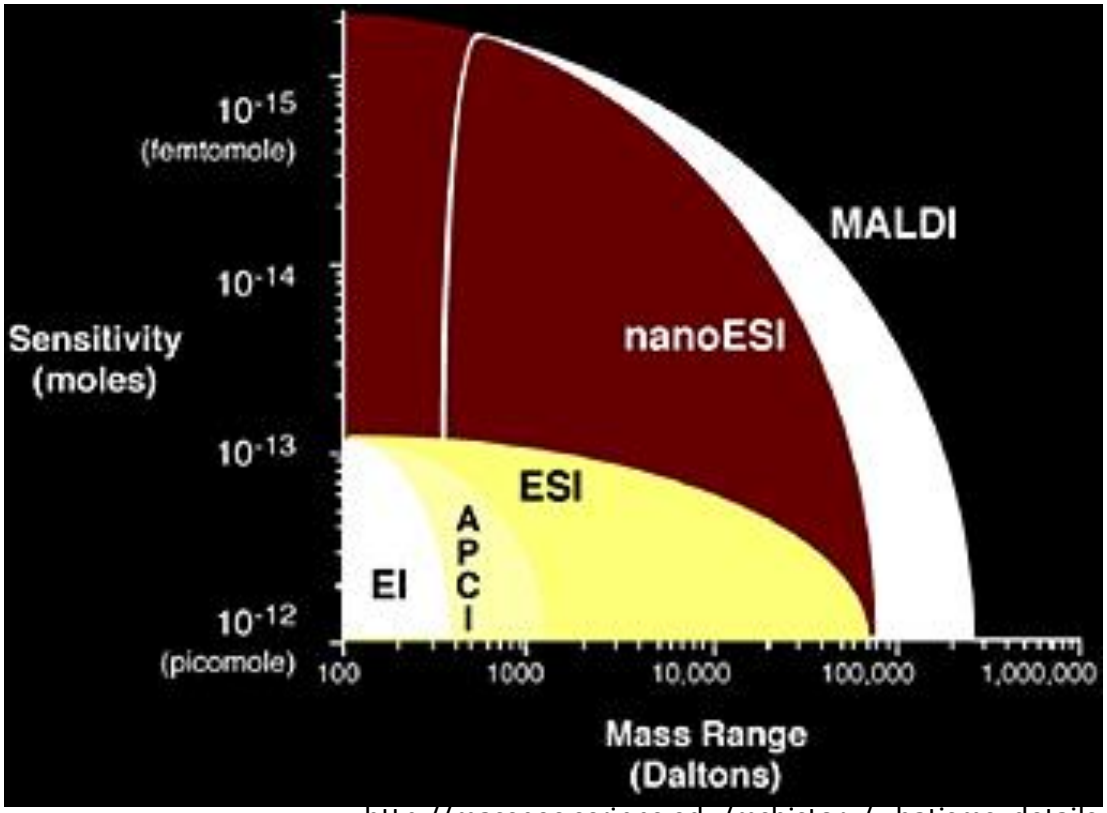


ESI and MALDI are the ionisation sources of choice for biomolecular analysis.

http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm

4. Metabolite identification:

Typical sensitivity and mass ranges allowed by different ionization techniques

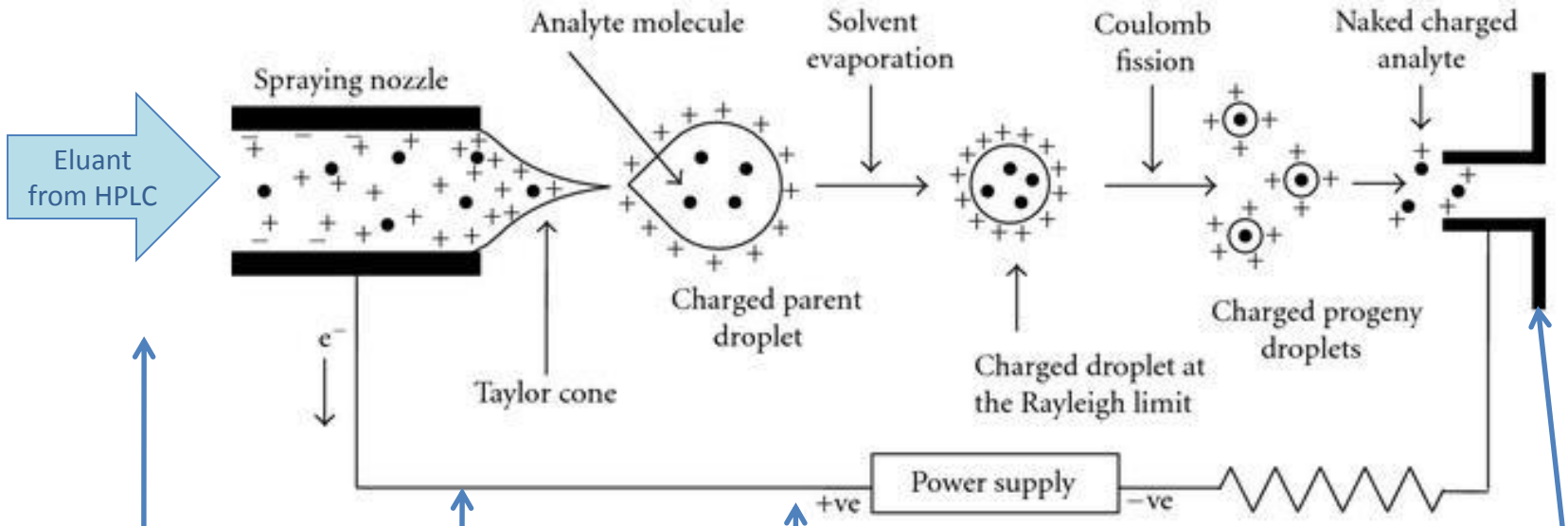


http://masspec.scripps.edu/mshistory/whatisms_details

- electron ionization (EI),
- atmospheric pressure chemical ionization (APCI) while
- electrospray ionization (ESI), nanoelectrospray ionization (nanoESI),
- matrix-assisted laser desorption ionization (MALDI) have a high practical mass range.

4. Metabolite identification: Electrospray Ionisation (ESI)

evaporation of charged droplets



Eluant from HPLC is sprayed through narrow bore steel capillary (0.2mm), atmospheric pressure

High voltage on the capillary generates a plume of highly charged droplets of analyte + solvent. This is aided by nebulising gas N_2

Solvent evaporated by warm N_2 (**drying gas**)

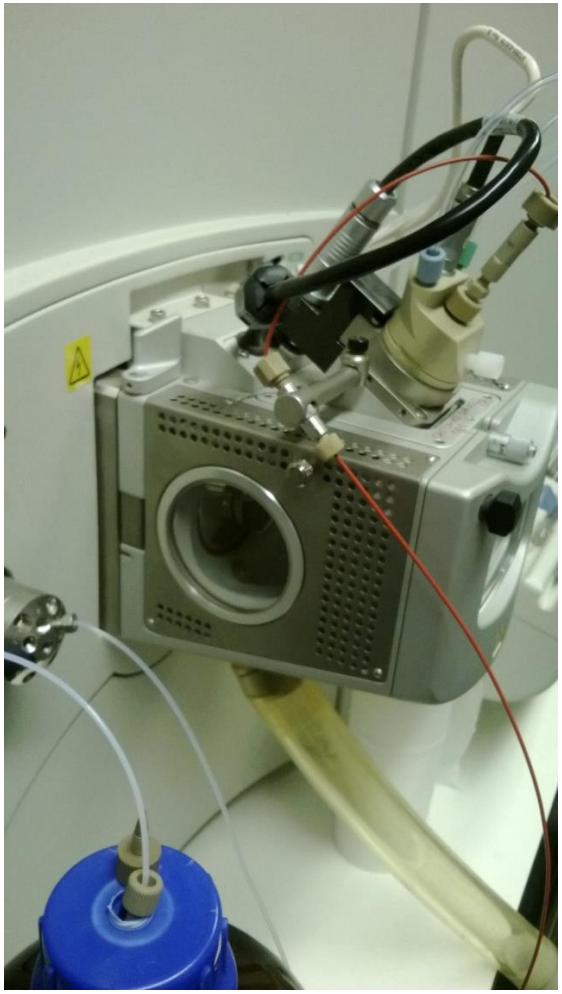
As evaporation continues, charge on droplet surface concentrates

Surface tension can no longer overcome the repulsion ➔ coulomb explosion

Eventually gas phase **ions**, free from solvent, are released from the droplets, some of which pass through the **sampling cone** and into the MS under **high vacuum**

Image: http://www.hindawi.com/journals/ijac/2012/282574/fig6/

4. Metabolite identification: Electrospray Ionisation (ESI)

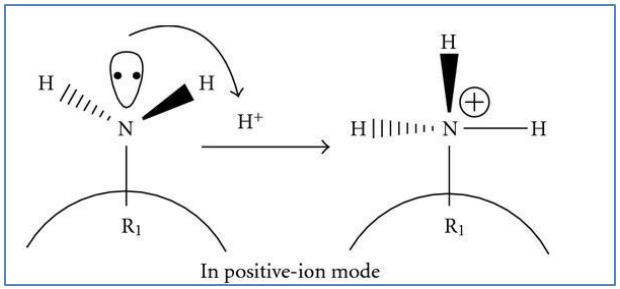


4. **Metabolite identification:** Positive or Negative Ionisation

Positive ion mode: often by addition of H+, Na+, K+
Negative ion mode: often by loss of H+ or addition of Cl-

If the sample has functional groups that readily accept a proton (H^+) then ***positive*** ion detection is used

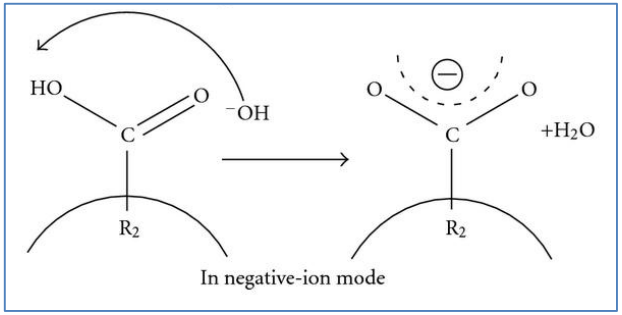
e.g. amines R-NH_2 + H^+ = R-NH_3^+ as in proteins or peptides.



In positive-ion mode

If the sample has functional groups that readily lose a proton then ***negative*** ion detection is used

e.g. carboxylic acids R-COOH = R-CO_2^- and alcohols R-OH = R-O- as in saccharides or oligonucleotides



In negative-ion mode

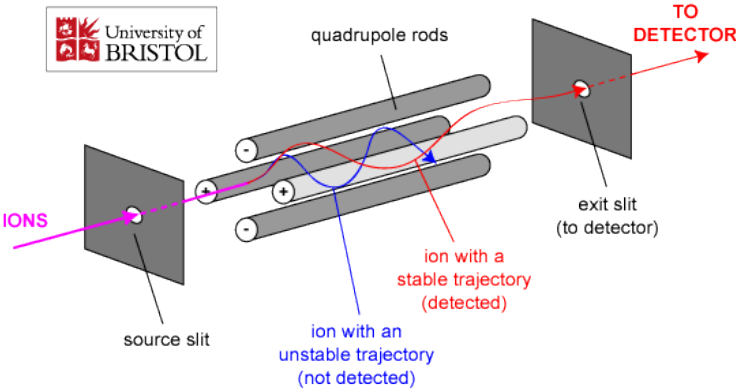
http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm

4. Metabolite identification: Separation

•The **mass analyser separates**, or **resolves**, the ions formed in the ionisation source of the MS according to their **mass-to-charge (m/z)** ratios.

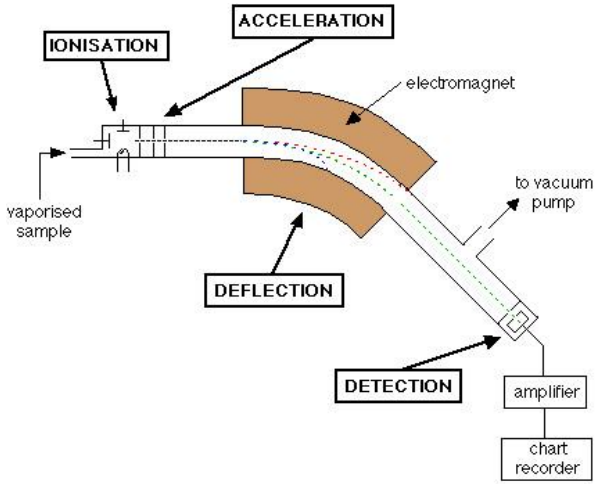
•**Quadrupole**

- •Four solid rods connected in parallel to a radio frequency (RF) generator and a **fixed** Direct Current (DC)
- •Ions travel in between the four rods
- •Trajectory of ion travel can be changed by changing RF.
- •Unstable ions do not travel but selected stable ones do continue to travel to the detector.
- •By scanning the RF field a broad m/z range (typically 100 to 4000) can be achieved in approximately one second.
- •quadrupole analyzers typically scan up to *m/z 3000*



•**Magnetic sectors** –

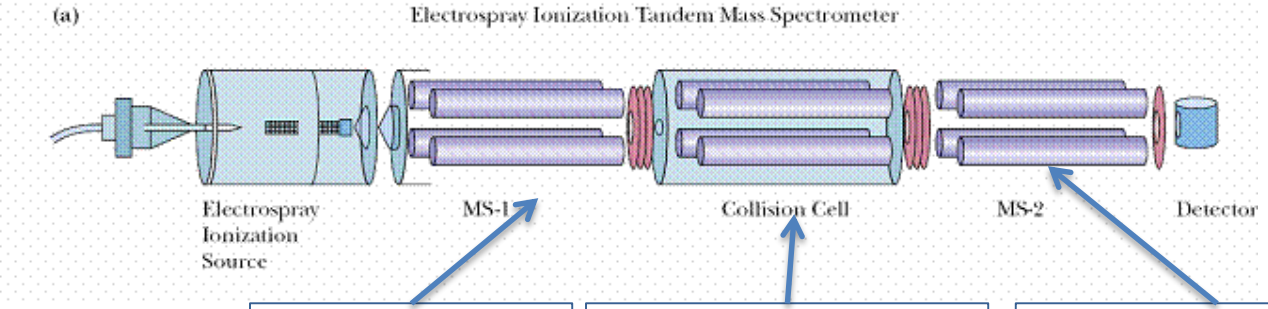
•a charged molecule propelled through a magnetic field can be deflected by that field in a manner that depends on the mass and its charge



4. Metabolite identification: Separation Tandem Mass Analysis (MS/MS or MS^n)

Tandem mass analysis is used to sequence peptides, and structurally characterize carbohydrates, small oligo-nucleotides, and lipids

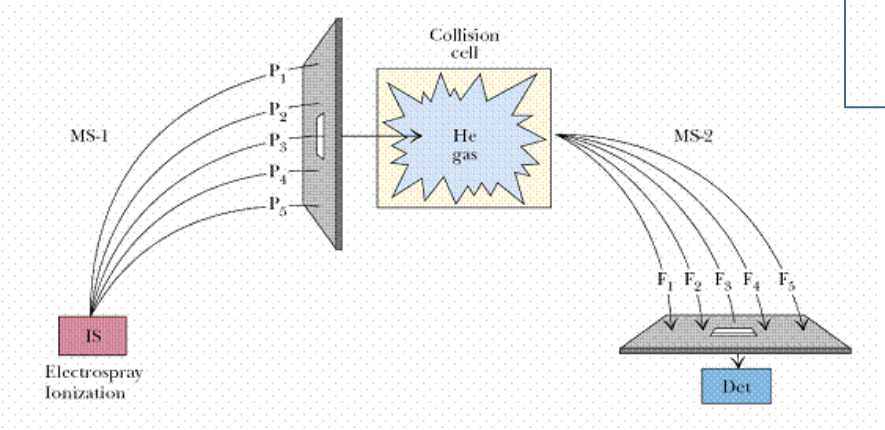
-A "triple-quad" is three of these put together in series.



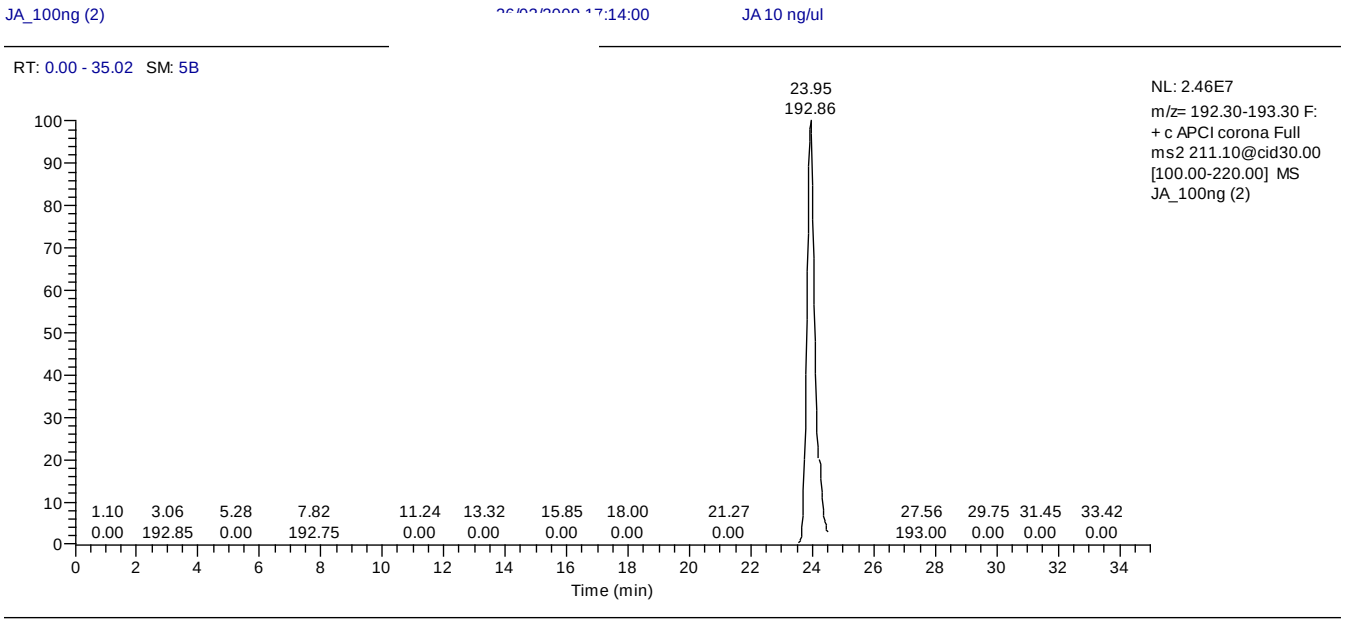
Q1 scans across a preset m/z range and selects the ion of interest

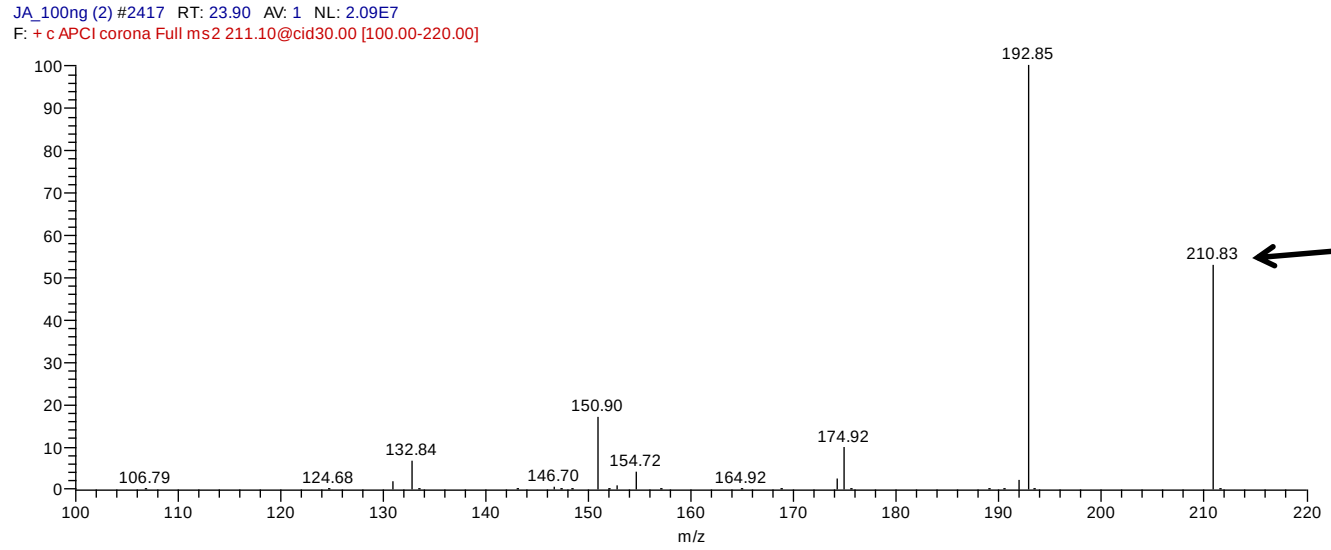
Q2 focuses and transmits the ion while introducing a collision gas (argon or helium) into the flight path of the selected ion to generate fragments

Q3 analyzes the fragment ions generated in the collision cell

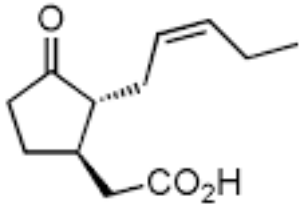


4. Metabolite identification: MS/MS on Jasmonic acid





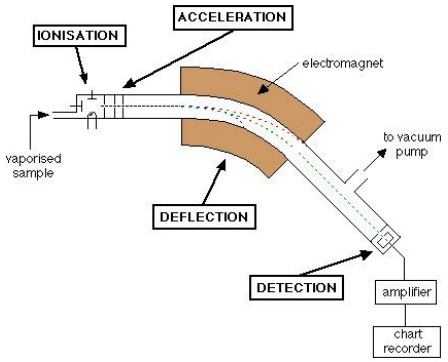
SIM
Selective ion monitoring for daughter fragment ion from JA

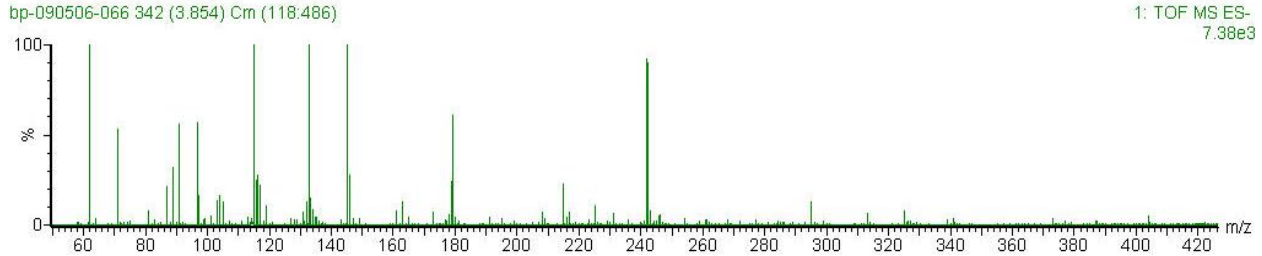


$C_{12}H_{18}O_3$
Molecular Weight 210

JA ion

4. **Metabolite identification:** Detection





The **detector** monitors the ion current, amplifies it and the signal is then transmitted to the data system where it is recorded in the form of **mass spectra** .

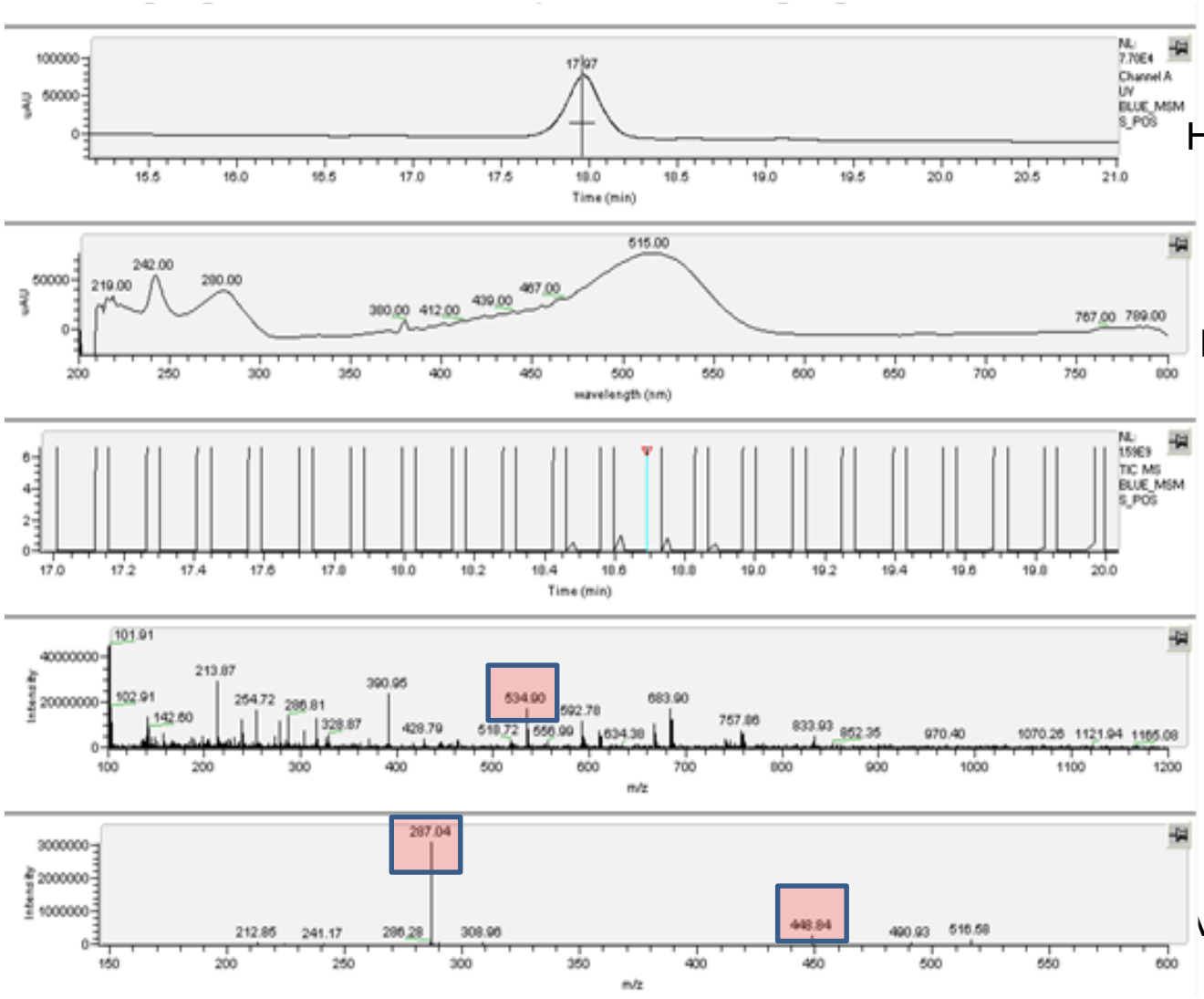
The **m/z** values of the ions are plotted against their **intensities** to show the **number of components** in the sample, the **molecular mass** of each component, and the **relative abundance** of the various components in the sample.

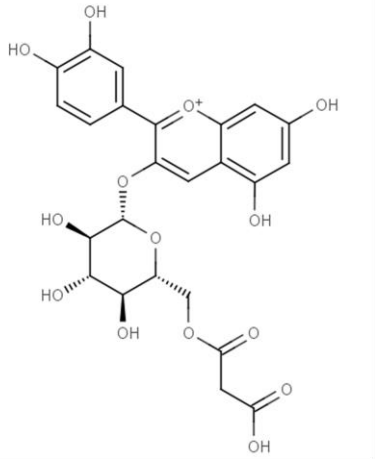
Accuracy: **The ppm (parts per million) mass accuracy** is a percent error quoting the difference between the measured and calculated mass for a particular ion. 0.1% would be equivalent to 1000 ppm error.

Standard 5ppm error is equivalent to 0.0005% - eg: for a mass 100 m/z the error would be +/- 0.05 and on mass 1000 error would be +/- 0.5

4. **Metabolite identification:** metabolic profiling

High Performance Liquid Chromatography (HPLC) – Photodiode array (PDA) – Mass spectrometry (MS)



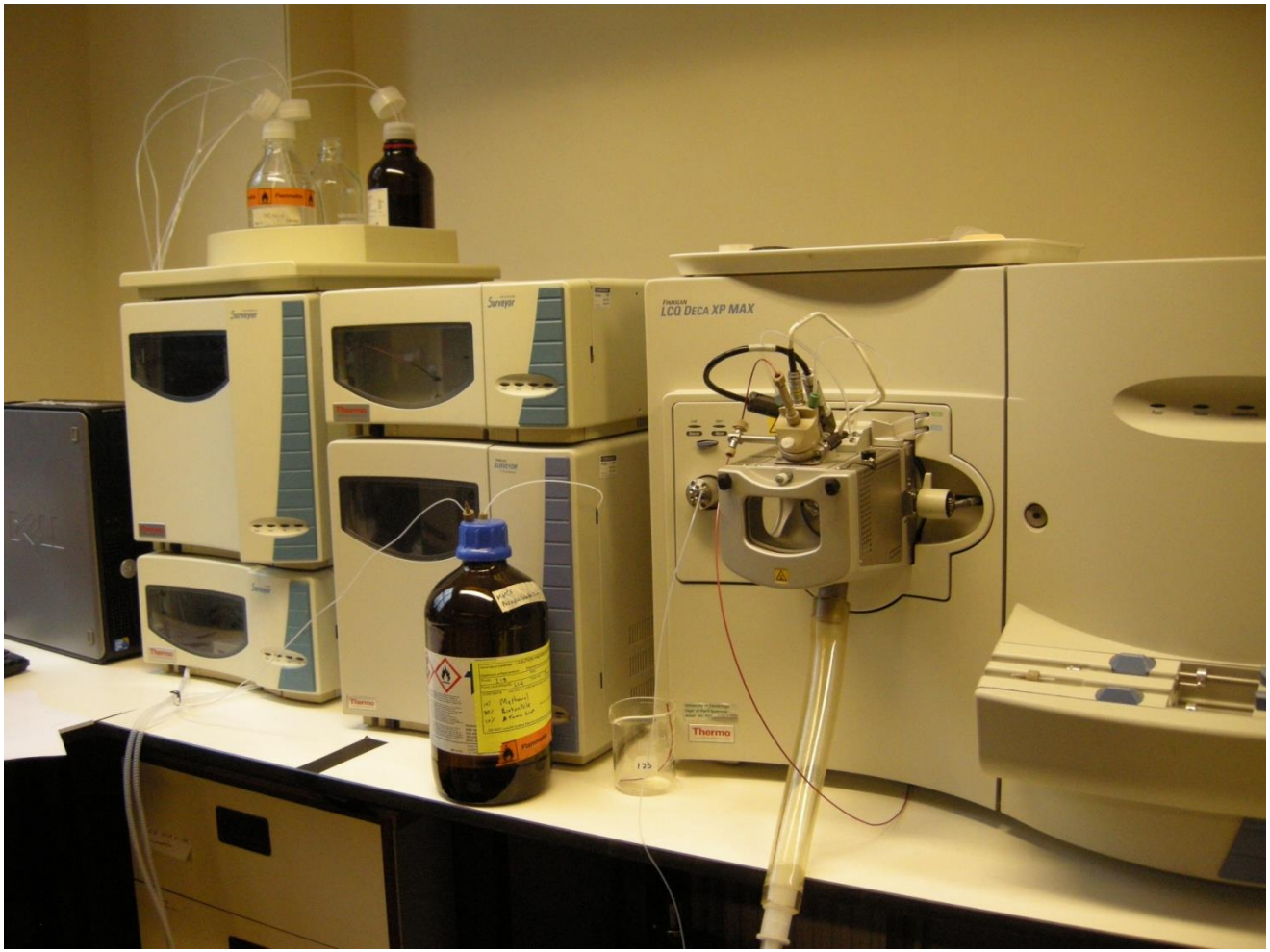


Cyanidin 3-(3-malonyl glucoside)
Mr=534.90

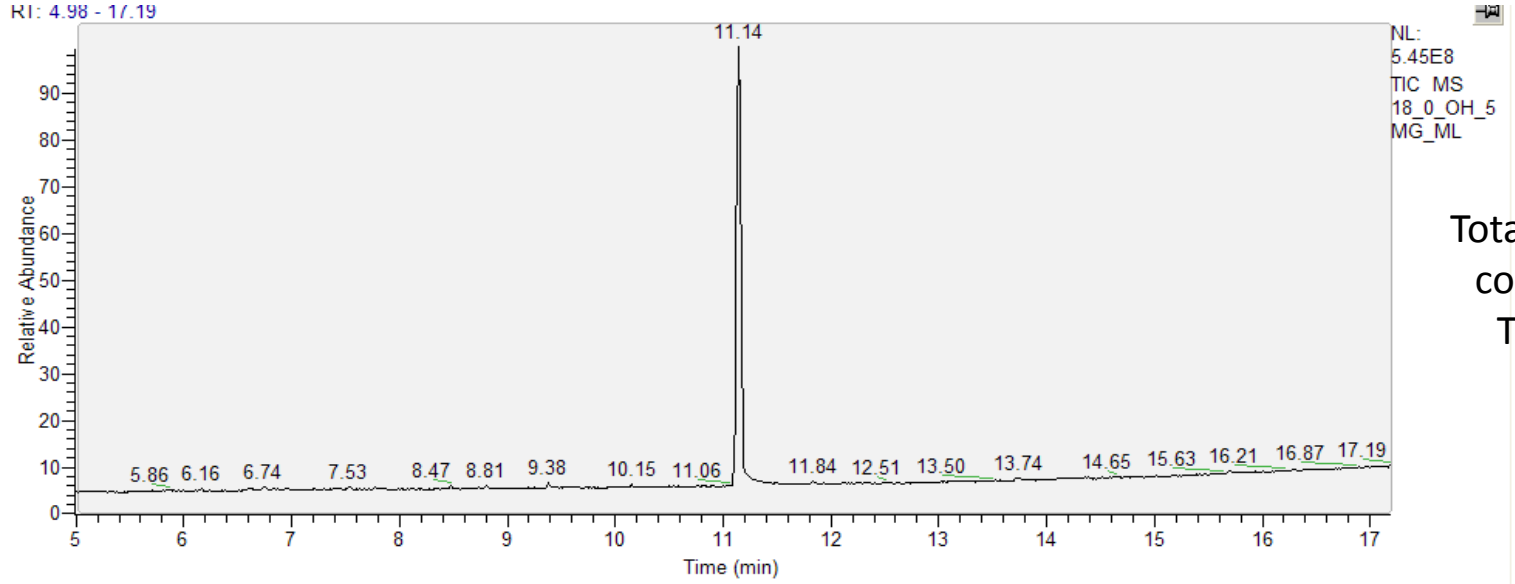
448.9 (-malonyl)
287.04 (-hexose and malonyl)

Matt Davey

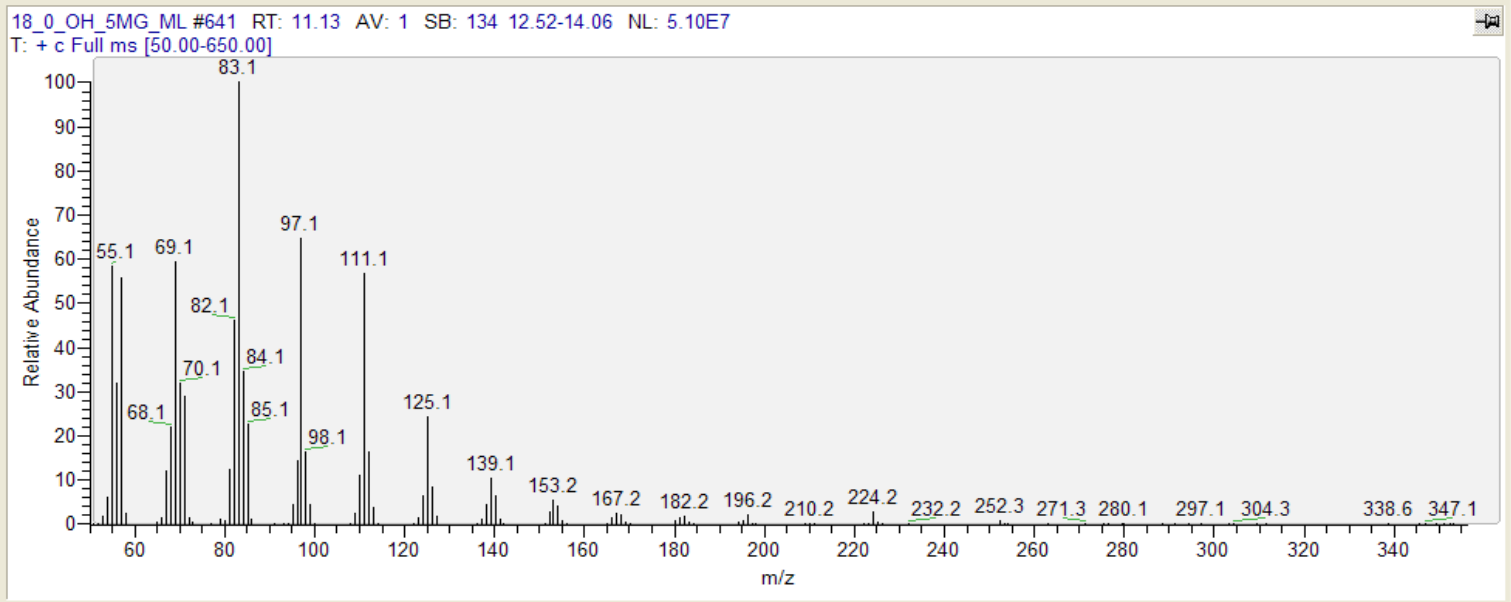
High Performance Liquid Chromatography (HPLC) – Photodiode array (PDA) – Mass spectrometry (MS)



Metabolic Profiling - Gas Chromatography (GC) – Mass spectrometry (MS)



Total ion count TIC

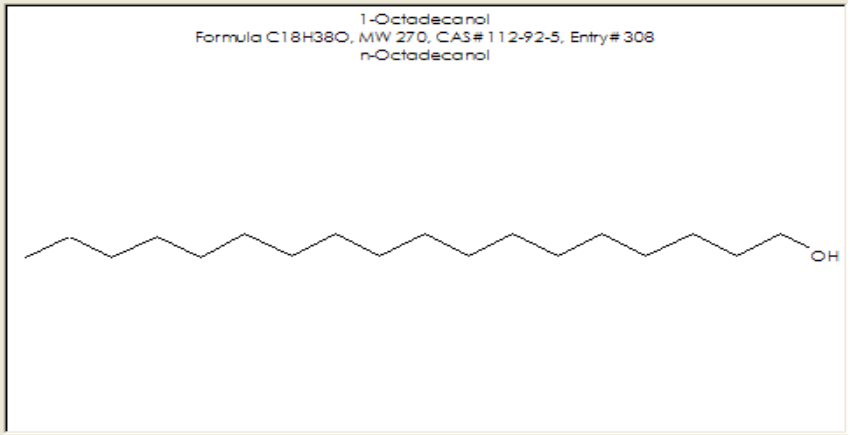


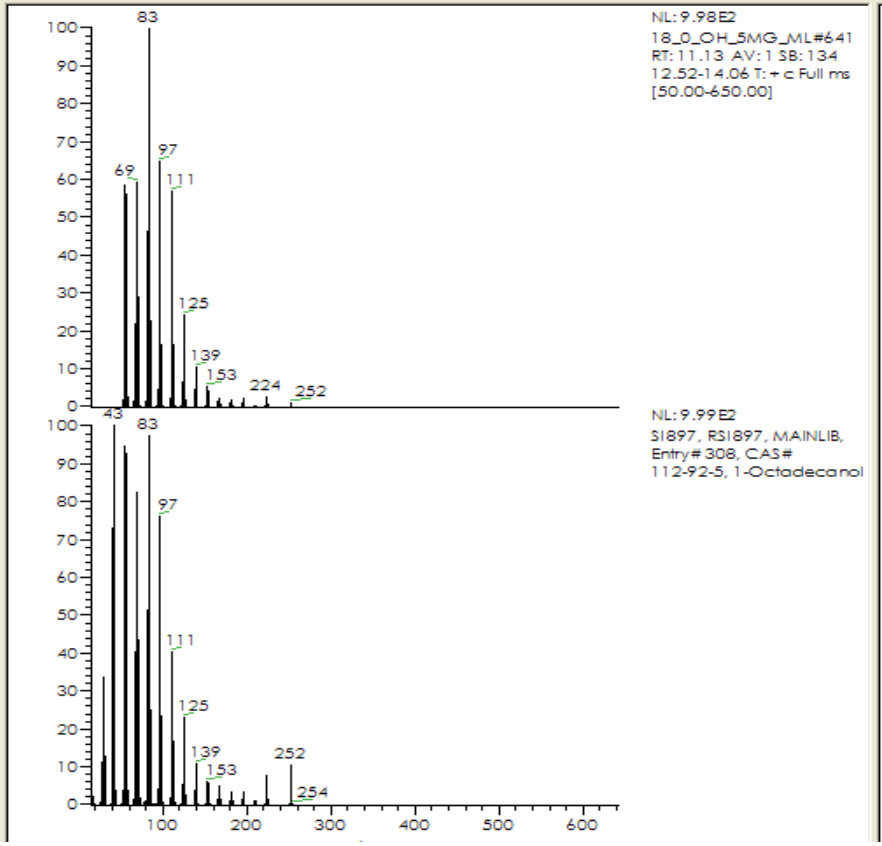
Unique fragmentation patterns

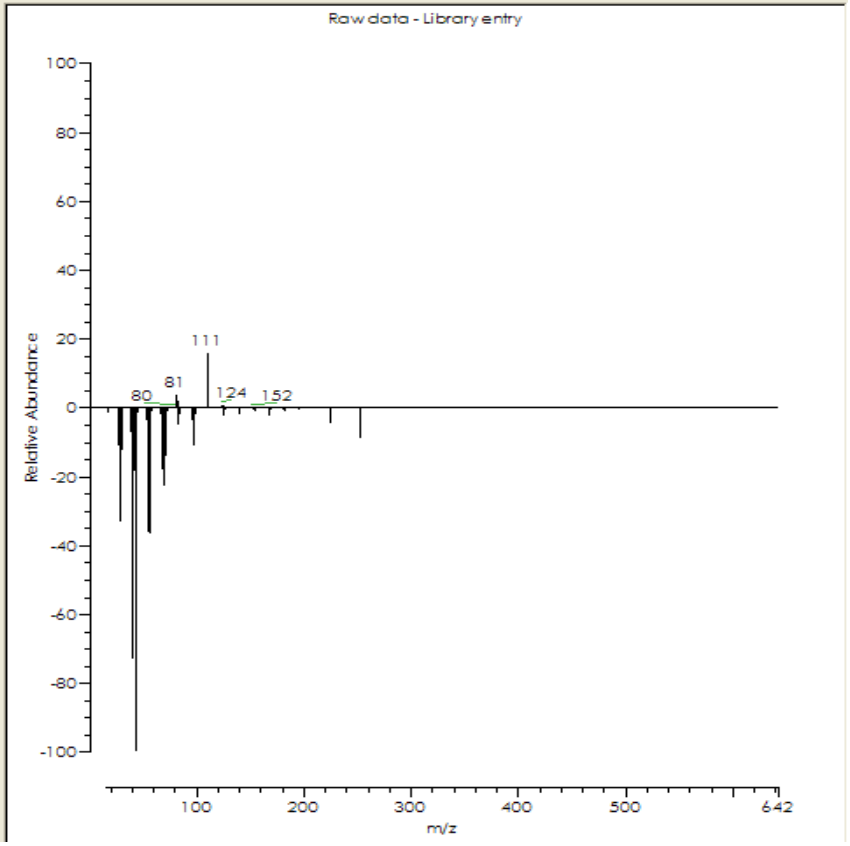
Matt Davey

GC-MS NIST fragment metabolite library – eg. fatty alcohol

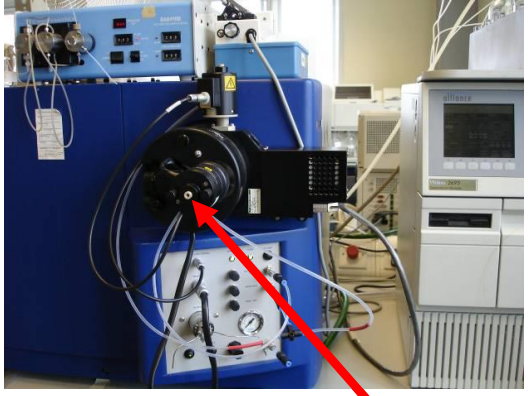
Hit	SI	RSI	Prob	Name	Library Name
1	897	897	46.93	1-Octadecanol	MAINLIB
2	875	876	18.55	1-Nonadecene	MAINLIB
3	857	865	9.56	1-Pentadecanol	MAINLIB
4	850	856	7.32	1-Heptadecanol	MAINLIB
5	844	854	5.76	1-Tetradecanol	MAINLIB
6	838	886	4.52	1-Tridecanol	MAINLIB
7	818	832	2.06	1-Tetradecene	MAINLIB
8	814	834	1.74	1-Tridecene	MAINLIB
9	806	816	1.29	1-Pentadecene	MAINLIB
10	794	822	0.86	Cyclododecane	MAINLIB
11	782	817	0.57	1-Dodecanol	MAINLIB
12	766	840	0.33	1-Undecanol	MAINLIB
13	765	798	0.31	1-Dodecene	MAINLIB
14	734	777	0.08	1-Undecene	MAINLIB
15	710	758	0.02	1-Decanol	MAINLIB

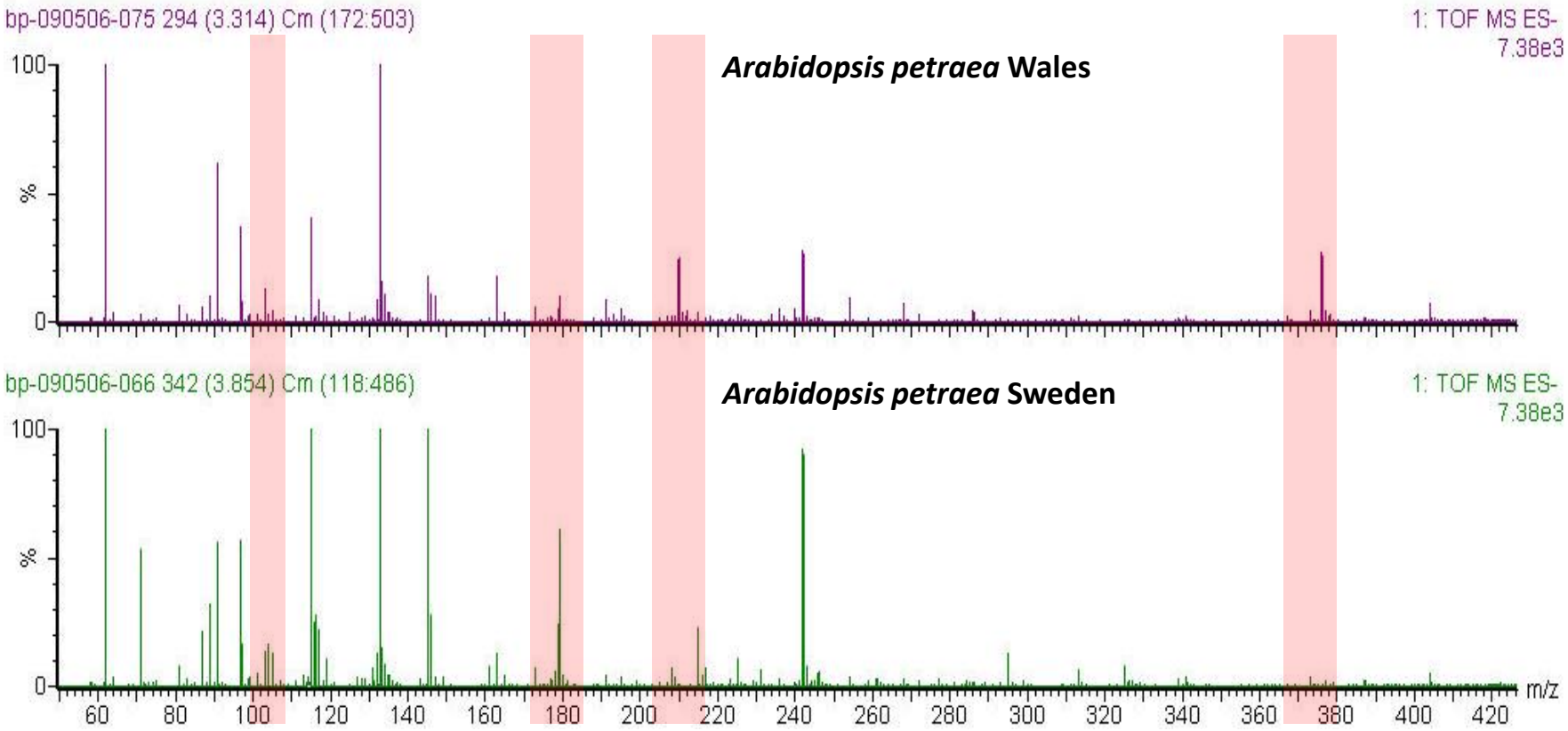






Metabolite fingerprinting





Matt Davey

(m/z)

Could be mass/1 or mass/2

NMR

FTIR

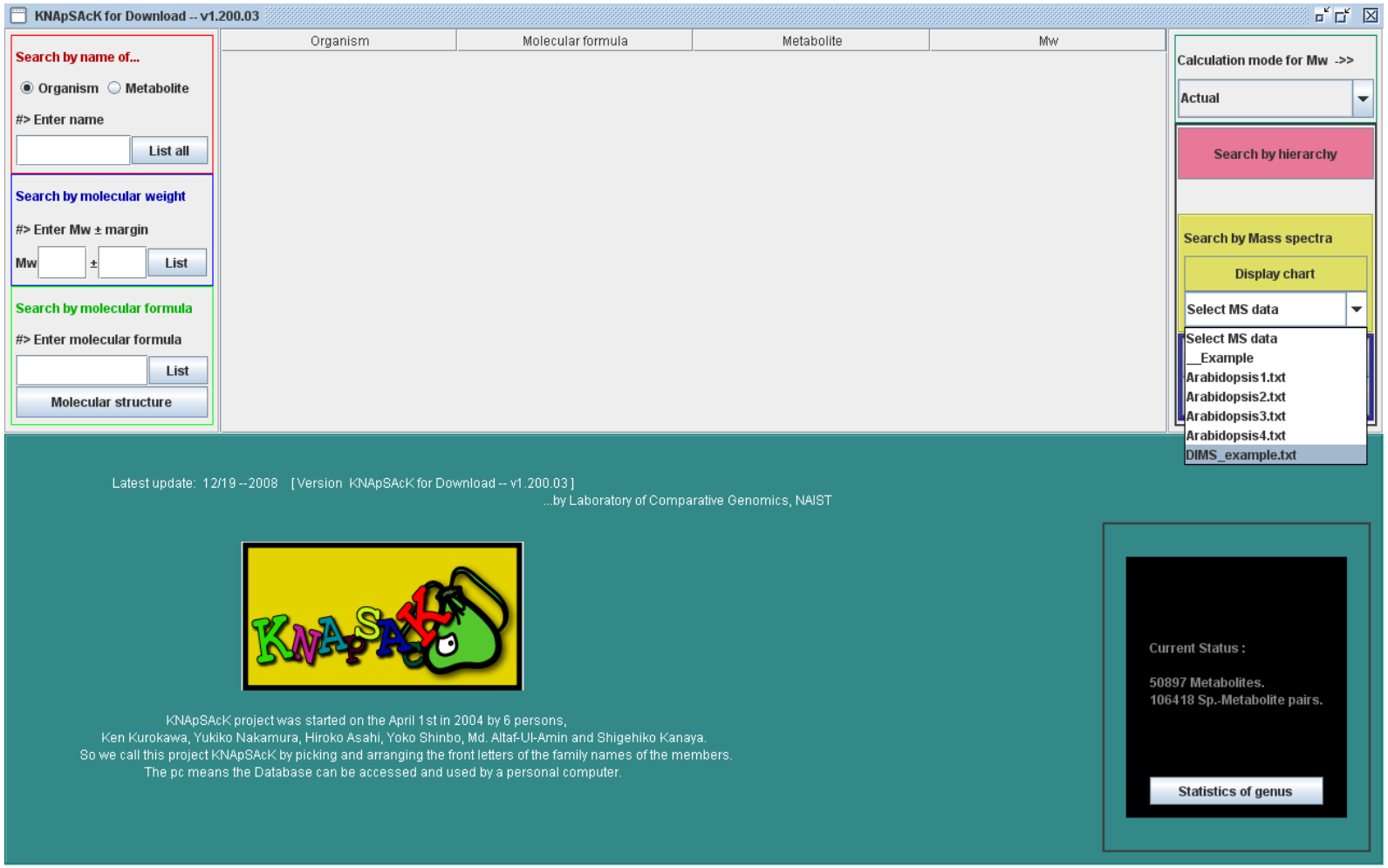
RAMAN

11:20-12:30 How to identify a metabolite

Online practical

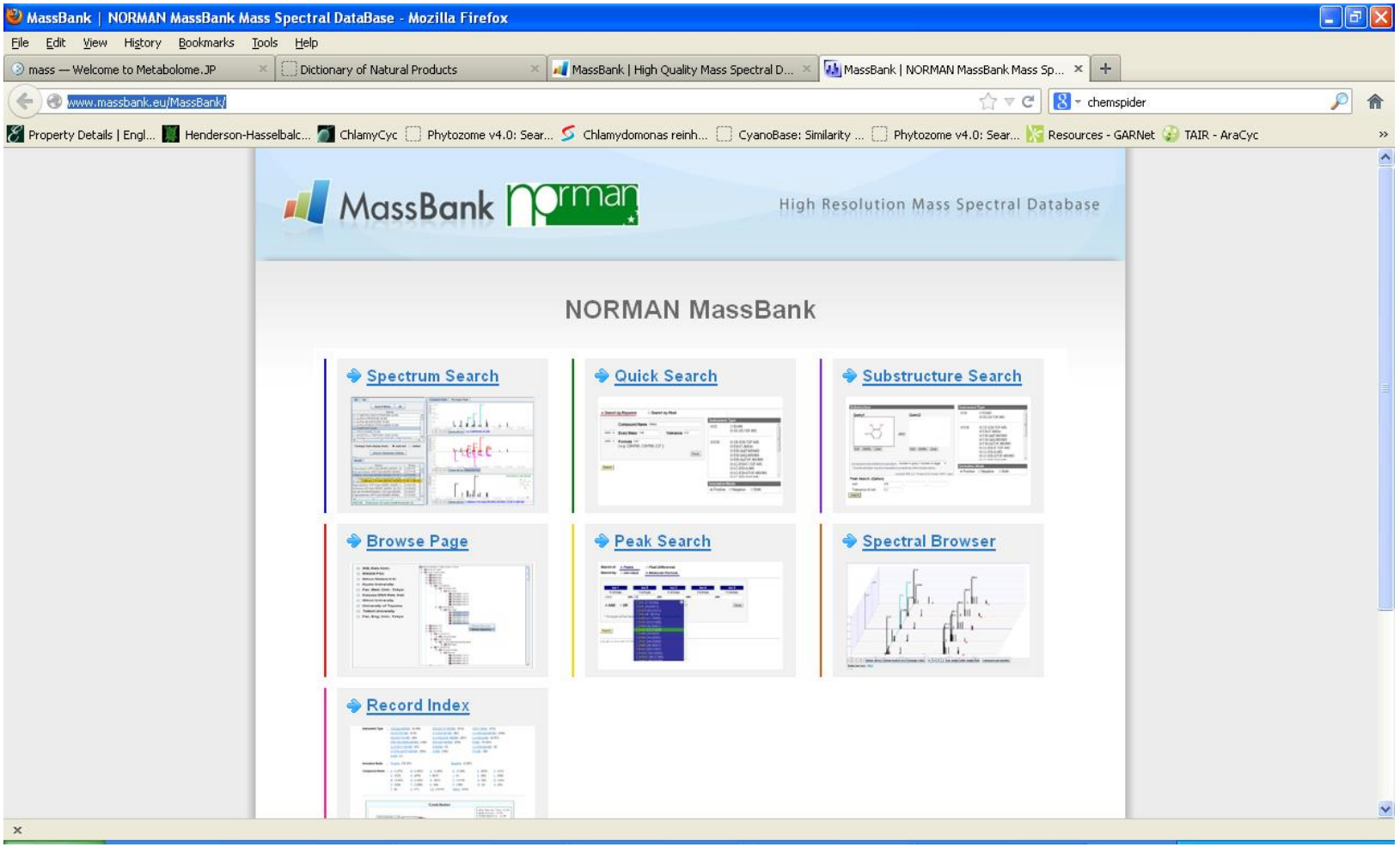
1: How to identify metabolites from a mass spectrum

•KNApSAcK



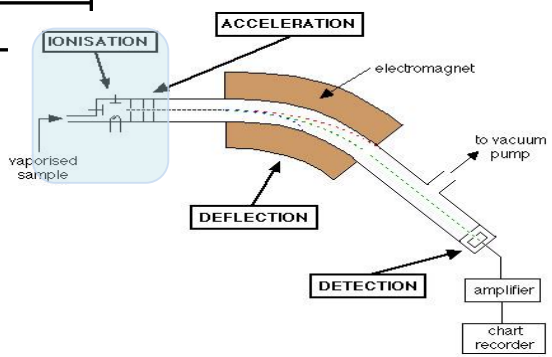
MassBank

http://www.massbank.eu/MassBank/



- **Kegg tab (www.genome.jp/kegg)**

Ionization method		Ionization source
Protonation	proton is added to a molecule, producing a net positive charge of 1+ for every proton added.	MALDI, ESI, and APCI
Deprotonation	Proton is removed from a molecule producing the net negative charge of 1-	MALDI, ESI, and APCI
Cationization	produces a charged complex by non-covalently adding a positively charged ion to a neutral molecule	MALDI, ESI, and APCI
Transfer of a charged molecule to the gas phase	transfer of compounds already charged in solution is normally achieved through the desorption or ejection of the charged species from the condensed phase into the gas phase	MALDI or ESI
electron ejection	ejection of an electron to produce a 1^+ net positive charge	electron ionization (EI)
electron capture	a net negative charge of 1- is achieved with the absorption or capture of an electron	



http://www.astbury.leeds.ac.uk/facil/MStut/mutorial.htm