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Lingkup Materi

- *Pengantar HPLC*
- *Pengantar MS*
- *Ion Source*
- Mass Analyser
- *Analisis Kimia dengan LC-TQD MS*
- *Analisis Kimia dengan LC-HRMS*

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Pengantar HPLC

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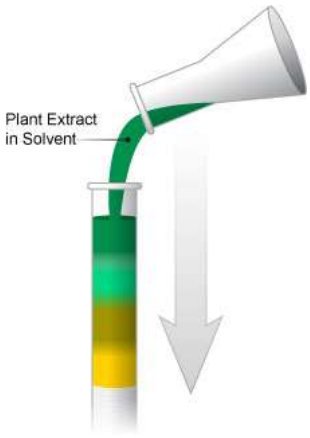
Kromatografi

- Teknik pemisahan komponen dalam campuran
- Analit terdistribusi dalam fase gerak (*mobile phase*) dan fase diam (*stationary phase*)
- Untuk analisis kualitatif, kuantitatif dan preparatif



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MOBILE PHASE	STATIONARY PHASE
GAS Gas Chromatography (GC)	LIQUID Gas-Liquid Chromatography (GLC)
	SOLID Gas-Solid Chromatography (GSC)
LIQUID Liquid Chromatography (LC)	LIQUID Liquid-Liquid Chromatography (LLC)
	SOLID Liquid-Solid Chromatography (LSC)

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Kromatografi Kolom vs HPLC

Kolom

- How does the mobile phase flow? (Gravity)
- What are the consequences?
  - Slow → **time consuming**
  - Peak broadening → **low Rs**

LOW PERFORMANCE

HPLC

- High Pressure Liquid Chromatography (HPLC) : high pressure to flow the liquid into packed columns.
- Pressure: 500 psi (35 bar, 1 bar = 0,9868 atm)
- The early 1970's
  - HPLC up to 6,000 psi (400 bar)
  - Improvement detectors
  - Improvement of columns.

Performance improvement

High Performance Liquid Chromatography (HPLC).

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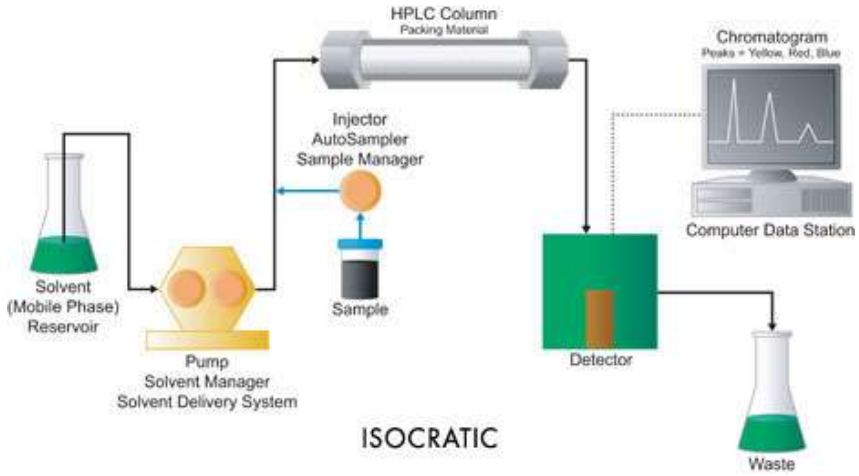

ISOCRATIC

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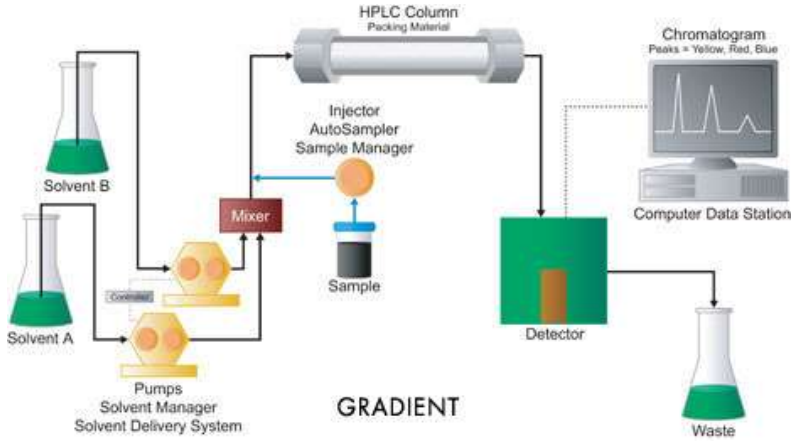

GRADIENT

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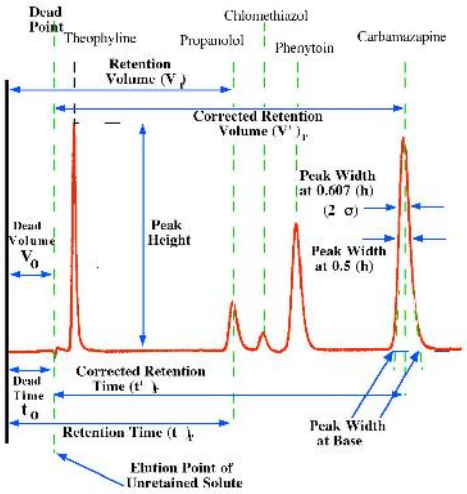
Kromatogram

- Plot respon detektor terhadap waktu
- Data: t_R: waktu retensi (kualitatif)
- Luas peak: peak area (kuantitatif)
- Tinggi puncak: peak height (kuantitatif)
- Width: lebar peak



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Selektivitas Vs Resolusi (Rs)

- The selectivity factor, α, describes the separation of band centres,
- The resolution, Rs, of two species, A and B, is defined as
- Baseline resolution is achieved when Rs = 1.5

$$\alpha = (tR)_B\text{-}tM)/(tR)_A\text{-}tM)$$

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

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Van Deemter plots

HETP = *A* + *B* / *u* + *C u*

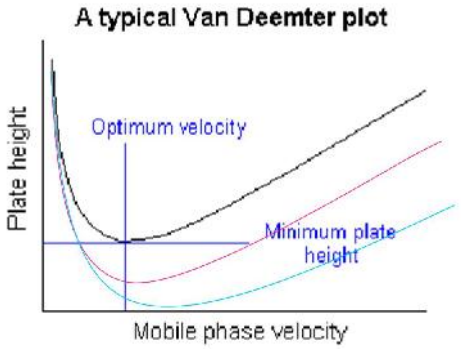
u = average velocity of the mobile phase.

A, B, and *C* = band broadening factors

***A* - Eddy diffusion**

***B* - Longitudinal diffusion**.

***C* - Resistance to mass transfer.**



HETP = *L* / *N*

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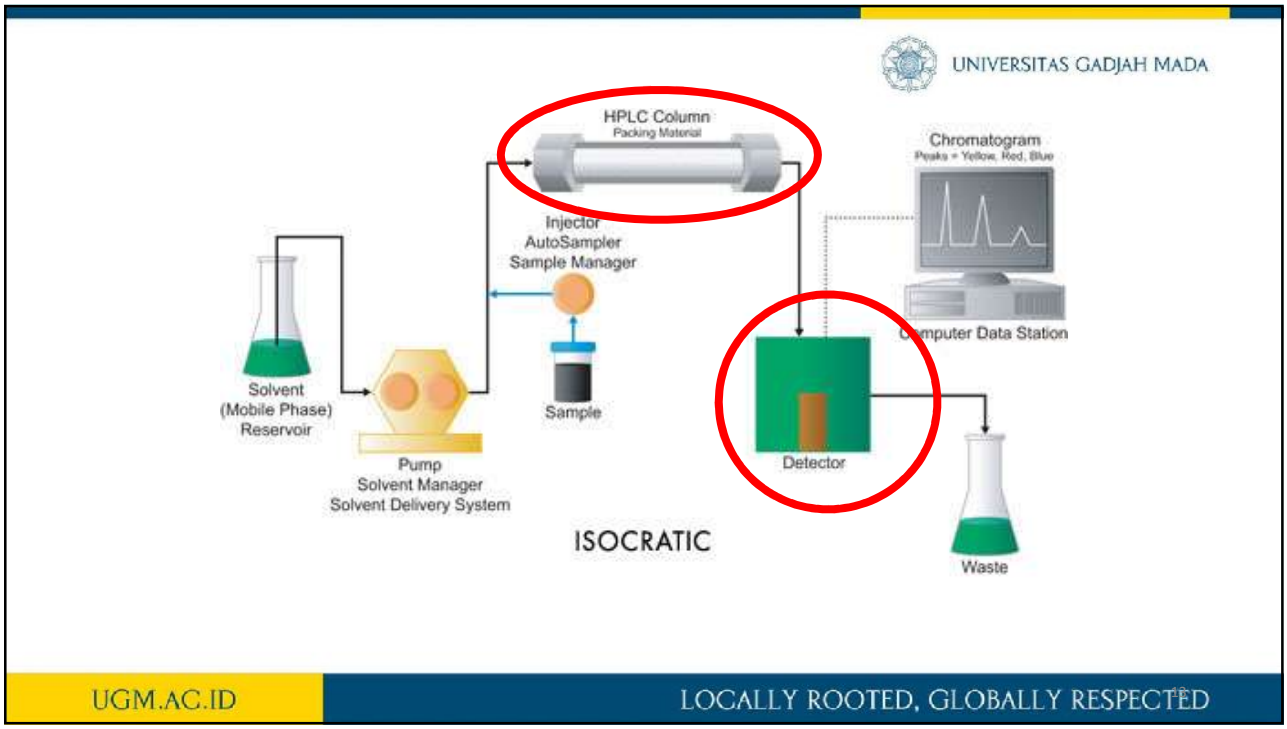
N menunjukkan Efisiensi pemisahan

Pemisahan kolom semakin efisien, jika:

1. N semakin tinggi (untuk HPLC minimal 2000)
2. H semakin rendah
3. Ukuran partikel semakin kecil → N semakin besar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HPLC Column
Packing Material
Chromatogram
Injector
AutoSampler
Sample Manager
Computer Data Station
Solvent
(Mobile Phase)
Reservoir
Sample
Pump
Solvent Manager
Solvent Delivery System
Detector
ISOCRATIC
Waste
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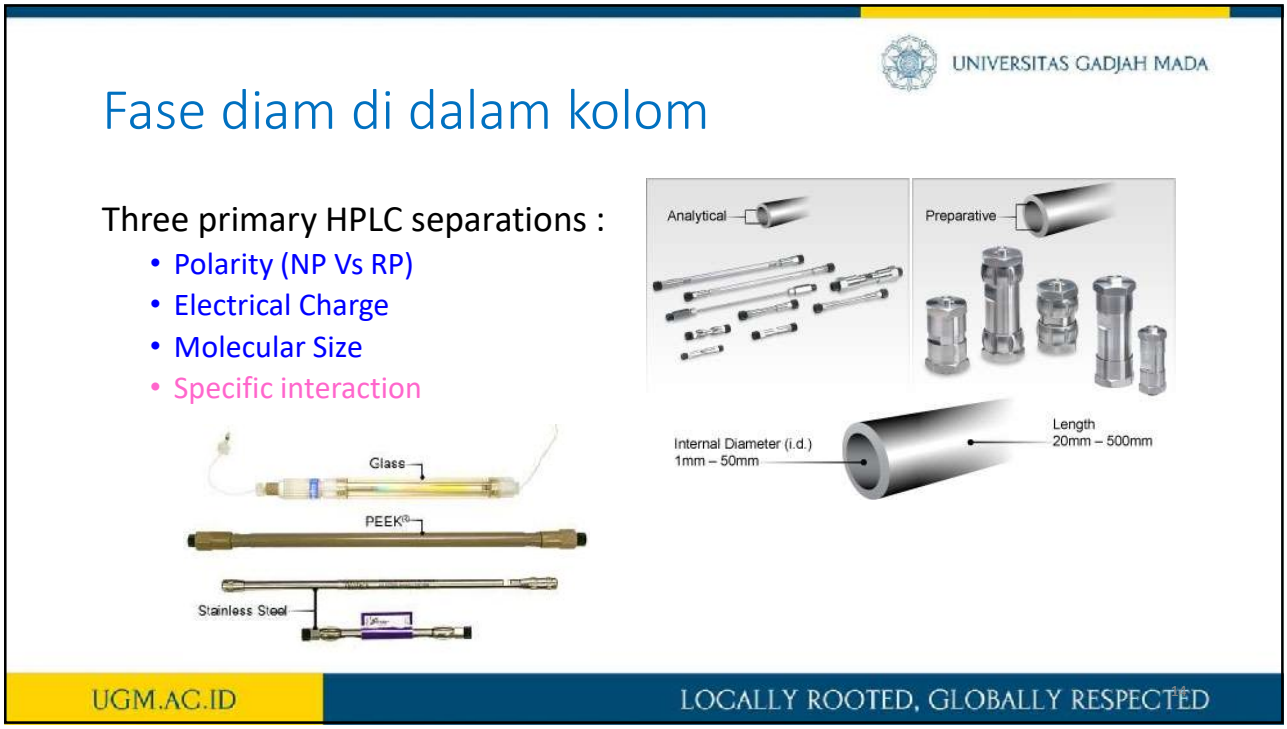
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Fase diam di dalam kolom
Three primary HPLC separations :
• Polarity (NP Vs RP)
• Electrical Charge
• Molecular Size
• Specific interaction
Analytical
Preparative
Internal Diameter (i.d.)
1mm – 50mm
Length
20mm – 500mm
Glass
PEEK®
Stainless Steel
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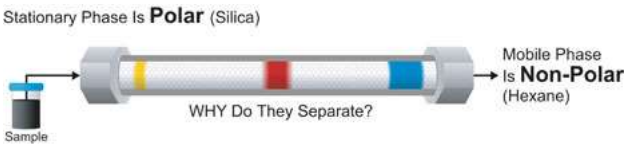
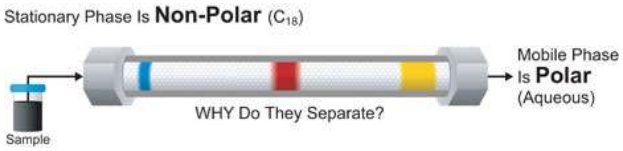
Polarity (NP Vs RP)

Normal Phase (NP)	Reversed Phase (RP)
The hydrophobic compounds elute more quickly than do hydrophilic compounds	• The hydrophilic compounds elute more quickly than do hydrophobic compounds





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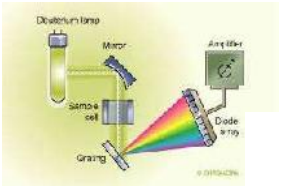
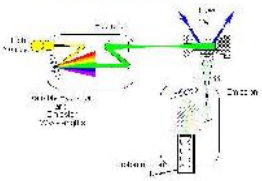
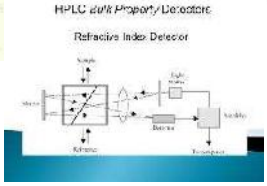
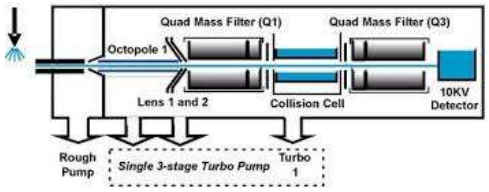
Detektor

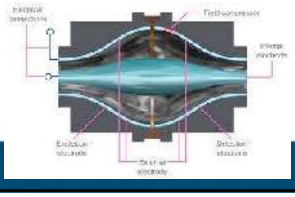
- UV - Ultraviolet
- ED - Electrical Conductivity
- RI - Refractive Index
- FD - Fluorescence
- MS - Mass Spec
  - Mass to charge ratio (m/z)
  - Allows specific compound ID
  - Several types of ionization techniques
- ELSD - Evaporative Light Scattering Detector











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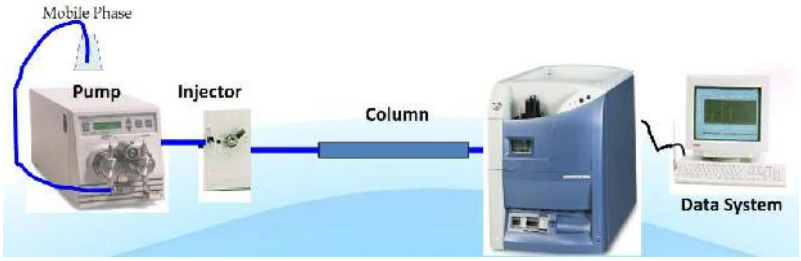
LC-MS LC-MSMS



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Pengantar MS

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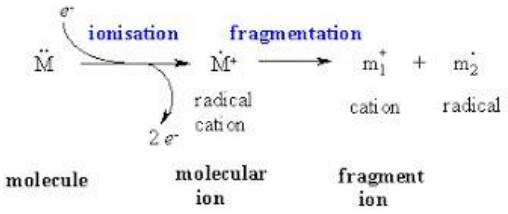
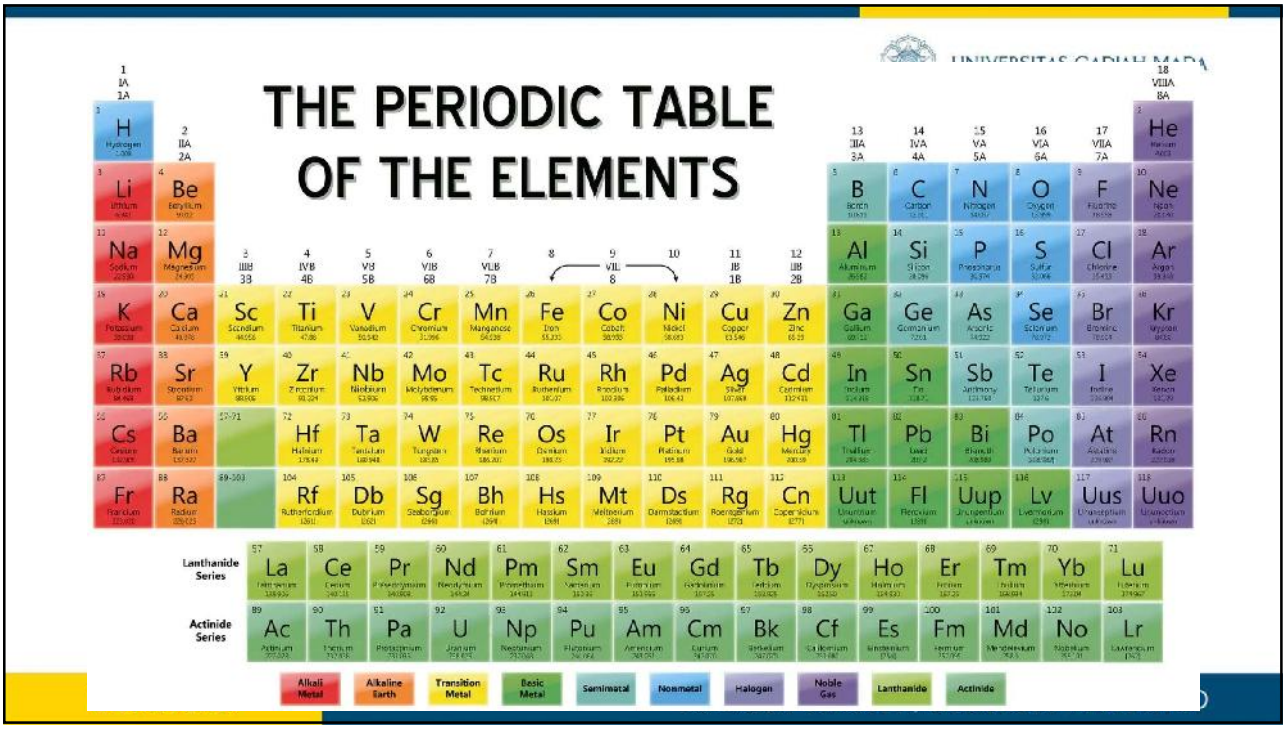
Mass Spectrometry (MS)

- Analytical technique for identification,confirmation, quantitation of compound based on the measurement of mass to charge ratio (m/z)



- **key is to be charge of the compound can be identification ms**



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MS Principles: nominal vs. exact mass

Compound	Nominal Mass	Exact Mass	
Carbon	12	12.0000	
Hydrogen	1	1.0078	
Oxygen	16	15.9949	
Nitrogen	14	14.0031	
Carbon Monoxide (CO)	28	27.9949	Toxic substance
Nitrogen Gas (N2)	28	28.0061	80% of atmosphere
Ethylene (C2H4)	28	28.0313	Flammable substance

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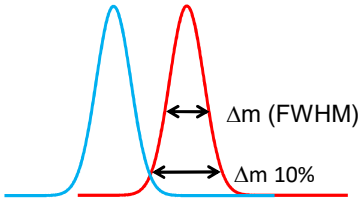
What is resolution in MS

- Resolving power

$$R = \frac{m}{\Delta m}$$



- Quadrupole MS $R = \frac{m}{\Delta m} = \frac{500}{0.6} = 833$
- Orbitrap MS $R = \frac{m}{\Delta m} = \frac{500}{0.005} = 100,000$

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What is Mass Accuracy

- Mass accuracy usually expressed as **ppm** or **mDa** error

$$\Delta m/z = \frac{m_{meas} - m_{true}}{m_{true}} \cdot 10^6$$

- Quadrupole MS $\Delta m/z = \frac{500.1 - 500.0}{500} \cdot 10^6 = 200 ppm$ 100 mDa error

- Orbitrap MS
TOF MS $\Delta m/z = \frac{500.10414 - 500.10314}{500.10314} \cdot 10^6 = 2 ppm$ 1 mDa error

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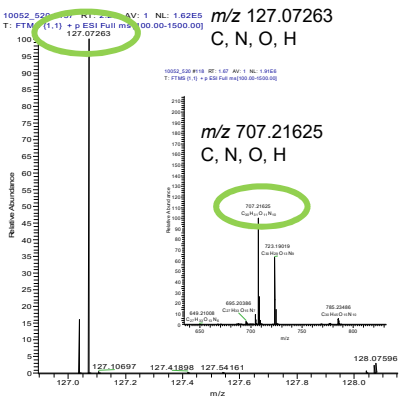
Why Do We Need Mass Accuracy: Molecular Formula



ppm error	Number of hits
150	11
50	4
10	1
5	1
2	1

ppm error	Number of hits
150	38
50	12
10	3
5	3
2	2

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Element	Exact Mass
H	1.007825
C	12.000000
N	14.003074
O	15.994915

0.1033 amu

288.0441	C9H21O2P1S3	Terbufos
288.0949	C13H21O3P1S1	Iprobenfos
288.1142	C15H17N4Cl1	Myclobutanil
288.1256	C11H20N4O3S1	Epronaz
288.1351	C11H21N4O3P1	Pirimethaphos
288.1474	C16H20N2O3	Imazamethabenz

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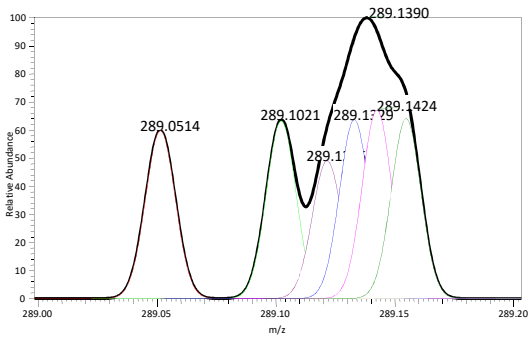
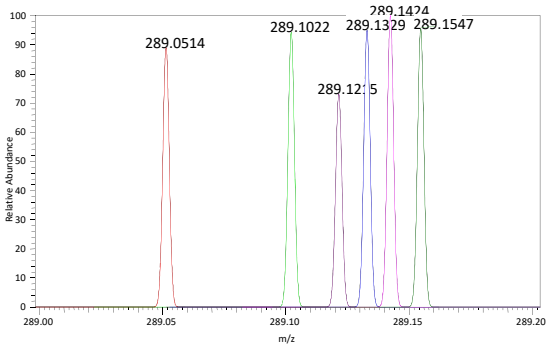
R = 20,000



R = 100,000



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- **Nominal mass**: the mass of an ion calculated using the *integer mass of the most abundant isotope of each element.*
  - ignores the mass defect, where H=1, C=12, O=16
- **Monoisotopic mass**: the mass of an ion calculated using the *exact mass of the most abundant isotope of each element.*
  - includes the mass defect, where ^{1}H=1.**0078**, ^{12}C=12.**0000**, ^{16}O=15.**9949**
- **Average mass**: the mass of an ion calculated using the *relative average isotopic mass of each element.*
  - where C=12.0111(12.000000) (0.9890) + (13.003355) (0.0110) = 12.011 Da
  - H=1.00797, O=15.9994
- **Isotopic abundance**: the naturally occurring distribution of the same element with different atomic mass.
  - ex: ^{12}C=12.0000 = 98.9%
    ^{13}C=13.0034 = 1.1%

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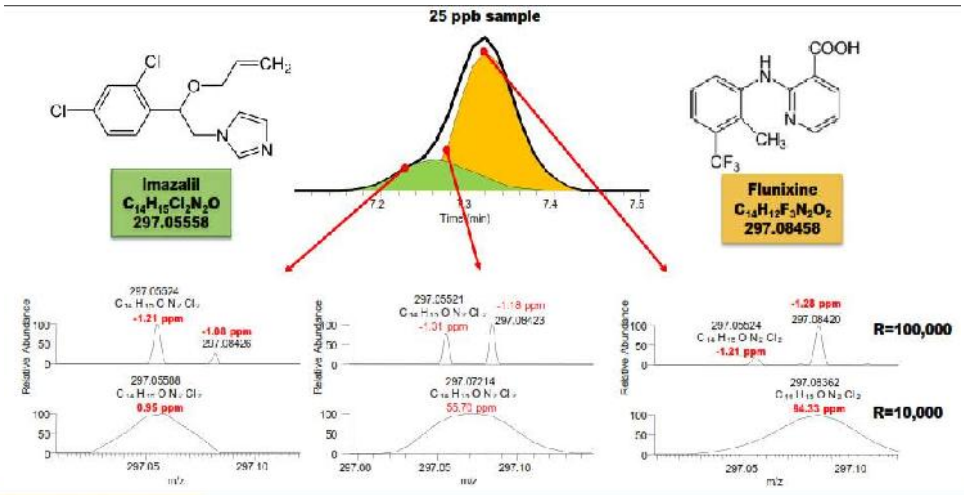
Importance of Mass Accuracy and Resolving power



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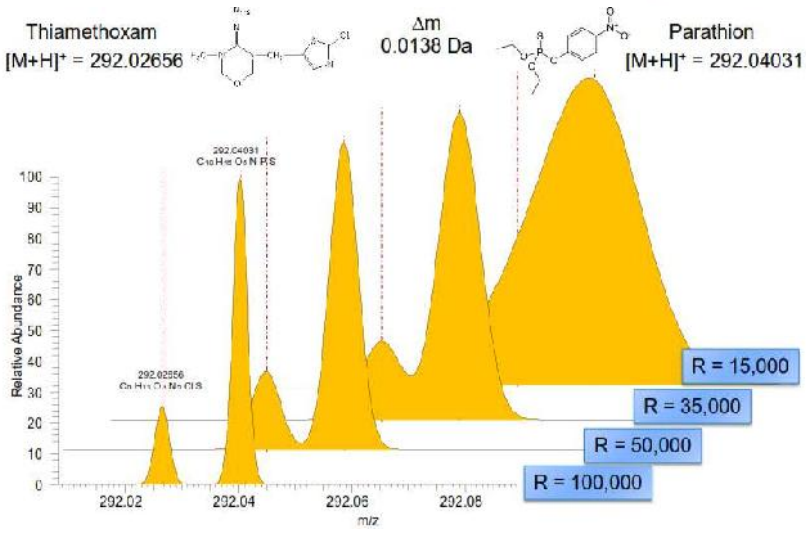

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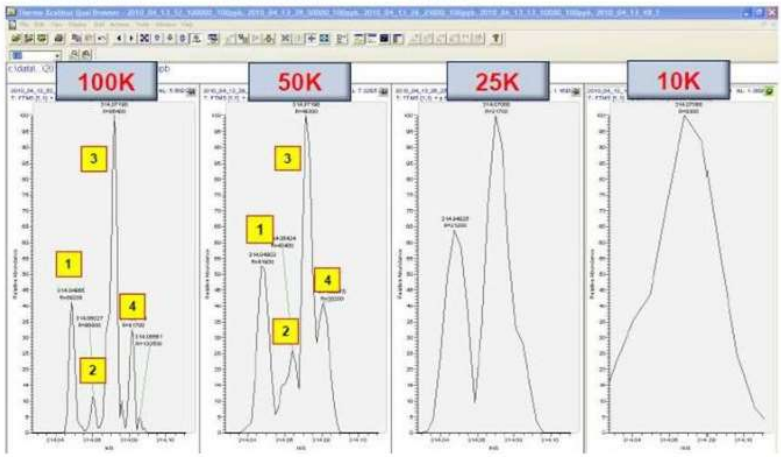
Isazofos	C9H17ClN3O3PS	314.04895
Isoxathion	C13H16NO4PS	314.06104
Triazophos	C12H16N3O3PS	314.07228
Hexaconazole	C14H17Cl2N3O	314.08214



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Type	Mass Accuracy
FT-ICR-MS	0.1 - 1 ppm
Orbitrap	0.5 - 1 ppm
Magnetic Sector	1 - 2 ppm
TOF-MS	3 - 5 ppm
Q-TOF	3 - 5 ppm
Triple Quad	3 - 5 ppm
Linear IonTrap	50-200 ppm (10 ppm in Ultra-Zoom)

Type	Resolving Power (FWHM)
FT-ICR-MS	1,000,000
FT-Orbitrap	100,000
High-Res-TOF	60,000
TOF	10,000
Quadrupole / IonTrap in UltraZoom mode	10,000
Quadrupole / Iontrap	1,000

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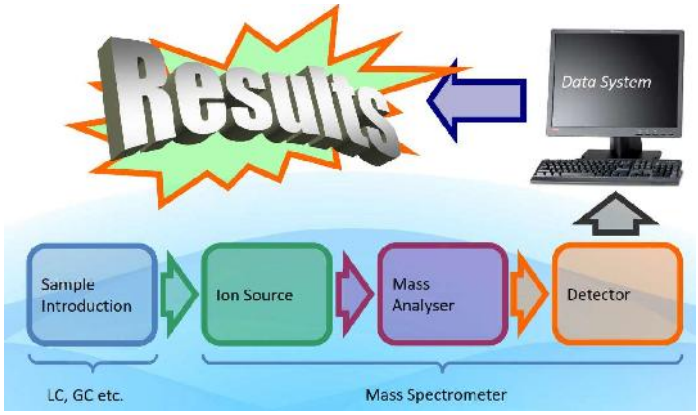
Mass Spectrometer Intrumentation



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Ion Source

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Hard vs Soft Ionisation

Hard

- GC-MS
- Electron impact
- Fragmentation

Soft

- LC-MS
- Ion molecular

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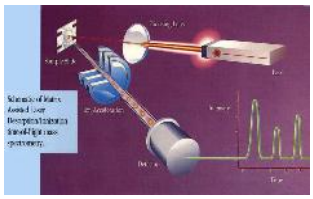
Soft Ionization

- Several common modes differing by method of ion formation:
  - Electrospray (ESI)
  - Atmospheric Pressure Chemical Ionization (APCI)
  - Atmospheric Pressure Photo-Ionization (APPI)
  - New dual sources (ESI/APCI) or (APCI/APPI)
  - Matrix Assisted Laser Adsorption (MALDI)



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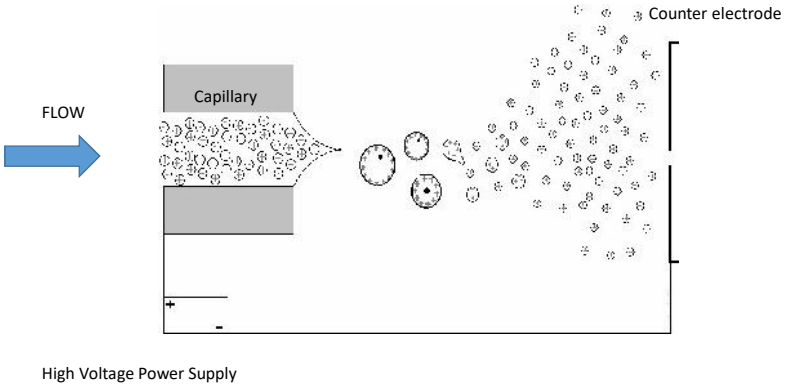
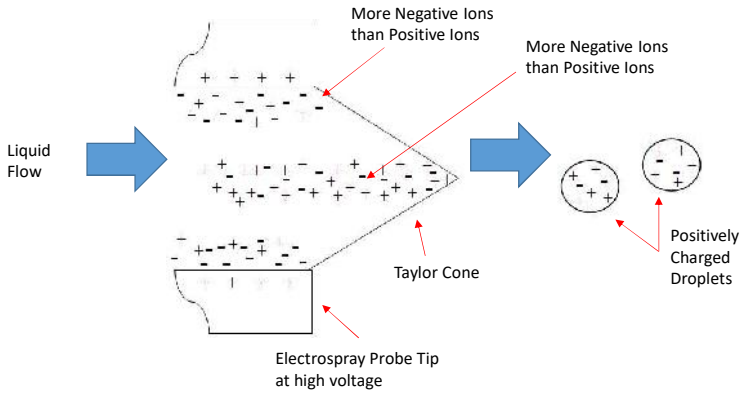
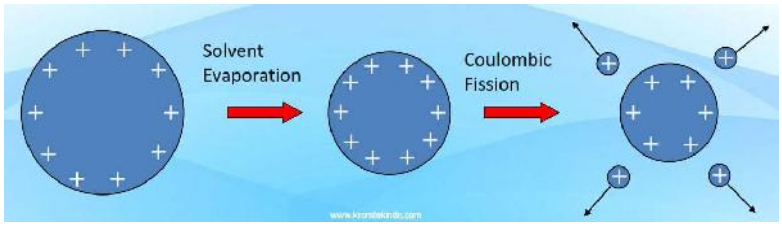
Electrospray (ESI)



High Voltage Power Supply



- Droplets produced from the spray have a surface charge
- Surface charged droplets undergo solvent evaporation and droplet fission to produce smaller droplets
- Like charge repulsion becomes greater than droplet surface tension and fission occurs to produce smaller charged droplets



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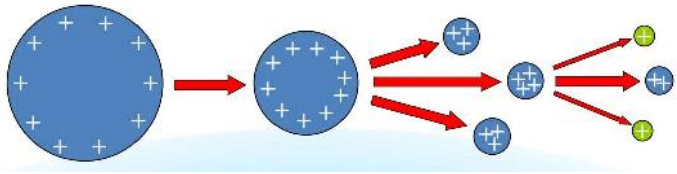
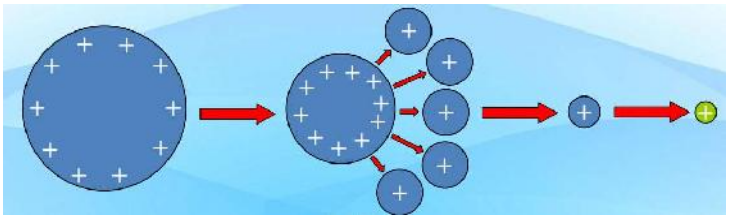
Ion Desorption Mechanism



Charged Residue Mechanism



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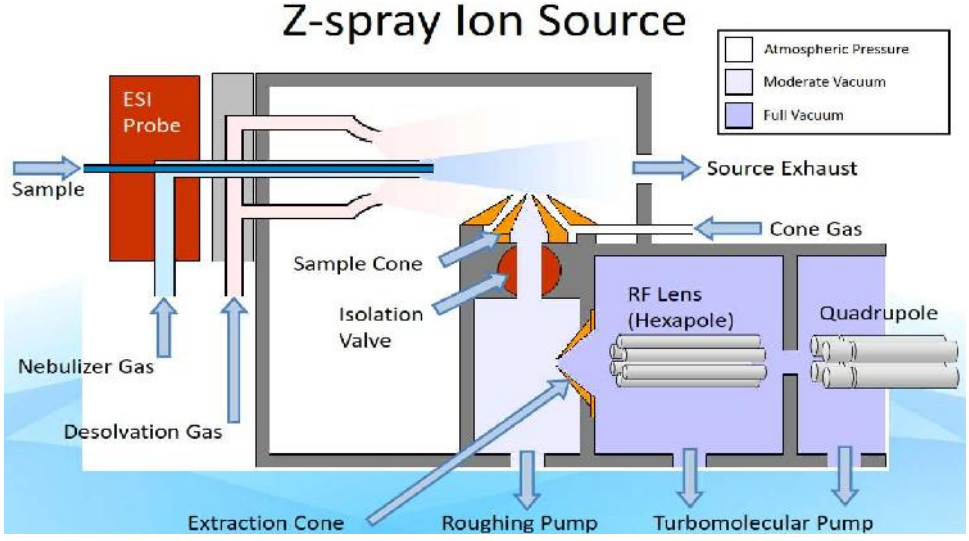
Z-spray Ion Source



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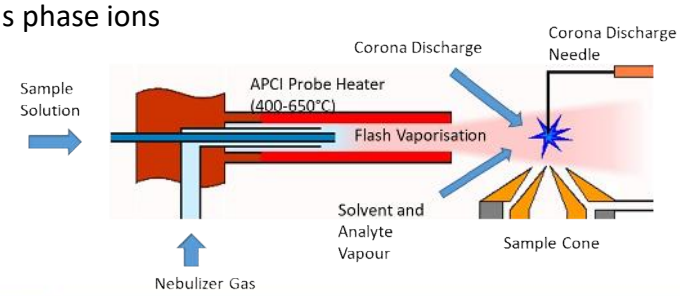
Atmospheric Pressure Chemical Ionisation (APCI)

- Liquid flow is forced through a narrow capillary to give it a high linear velocity
- The APCI Probe heater combined with nebulizer gas then vaporises the liquid flow
- The solvent and analyte vapour passes though the corona discharge region to produce gas phase ions



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ESI Electro Spray Ionization uses ESI Probe

ESI +

ESI -

APCI Atmospheric Presuure Chemical Ionization uses APCI Probe

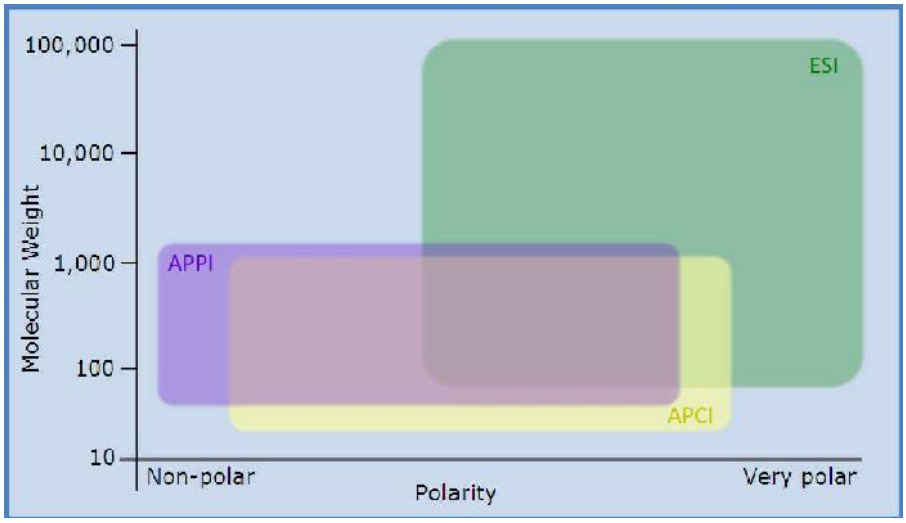
APCI +

APCI -

ESCI Electrospray Chemical Ionization → ESI + APCI Using ESI Probe

ESCI +

ESCI -



- Electrospray can operate in either positive or negative mode.
- Positive mode:
  - Best suited to basic compounds that form a stable HCl salt.
  - $[M+H]^+$ is the primary ion formed
  - $[M+nH]^{n+}$ and $[M+Na^+]^+$ can also be formed.
- Negative mode
  - Best suited to acidic compounds that form stable Na salts.
  - $[M-H]^-$, $[M-nH]^{n-}$and $[M+I^-]^-$

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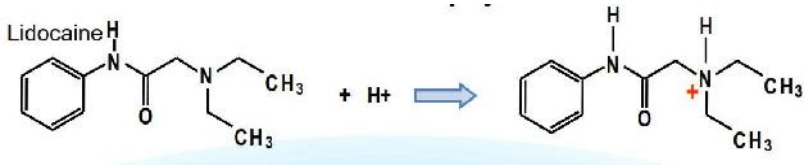
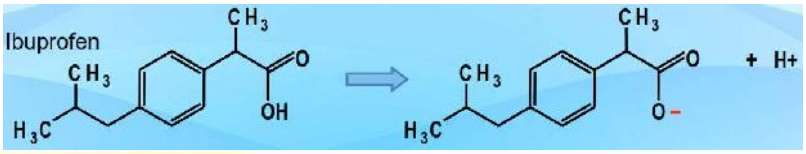
Mechanisms of Ion Formation

Positive Electrospray Ions



Negative ElectroSpray Ions



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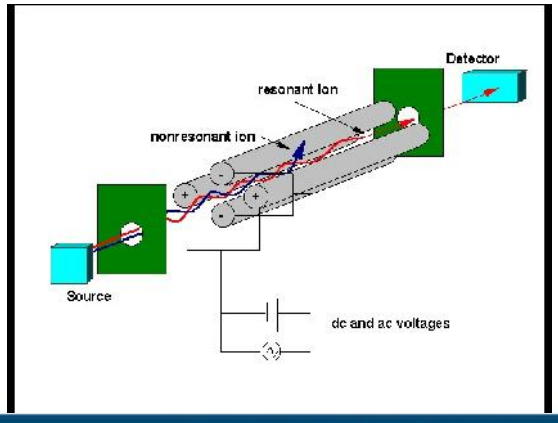
Mass analyser

- Low Resolution
  - Quadrupole (Mass Filter, Ion Trap)
  - Quadrupole Tandem
- High Resolution
  - Time-of-Flight (Linier TOF, Reflectron)
  - Double Focusing Magnetic Sector
  - Orbitrap
- Ultra high resolution
  - ICR ion cyclotron resonance

Type	Resolving Power (FWHM)
FT-ICR-MS	1,000,000
FT-Orbitrap	100,000
High-Res-TOF	60,000
TOF	10,000
Quadrupole / IonTrap in UltraZoom mode	10,000
Quadrupole / Iontrap	1,000

Quadrupole

- **Suitable for Quantitation and known compound confirmation Good linearity**
- **Structural information**
- **Single Quad**
  - **Mass confirmation**
  - **Get molecular ion**
  - **NO fragmentation**



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When to use Single Quads

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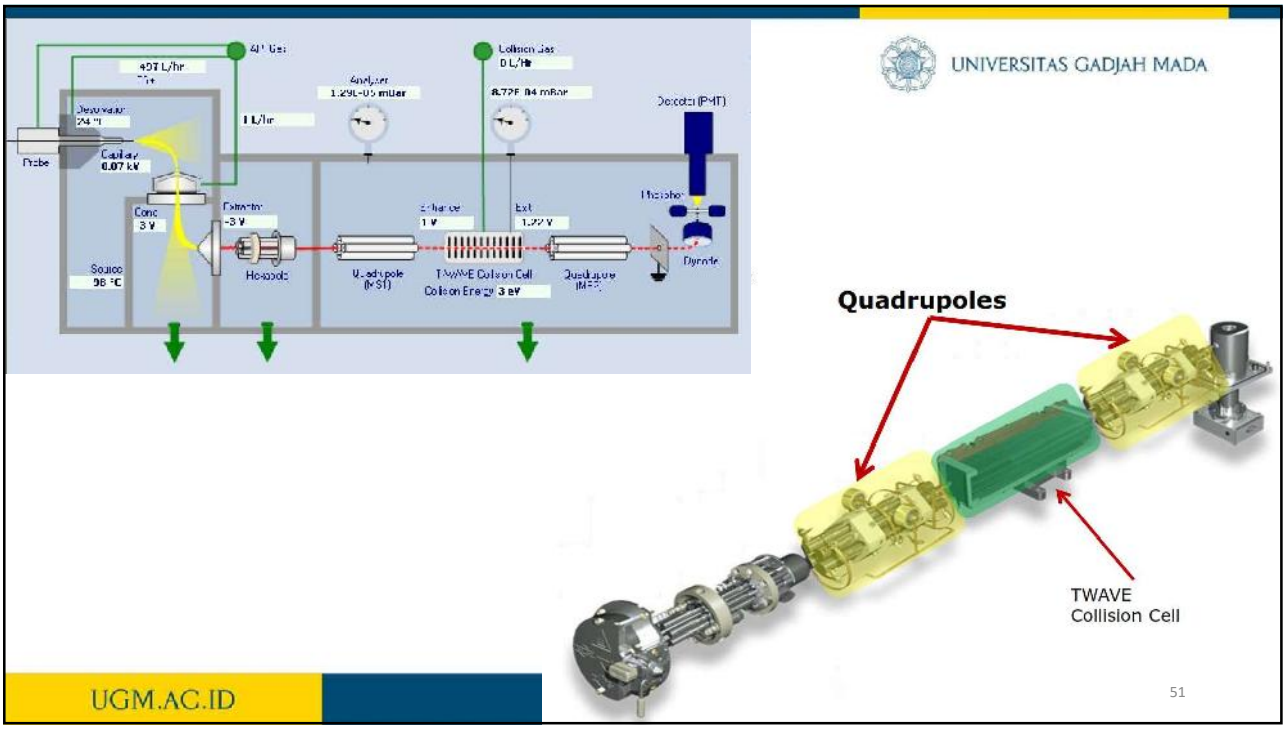
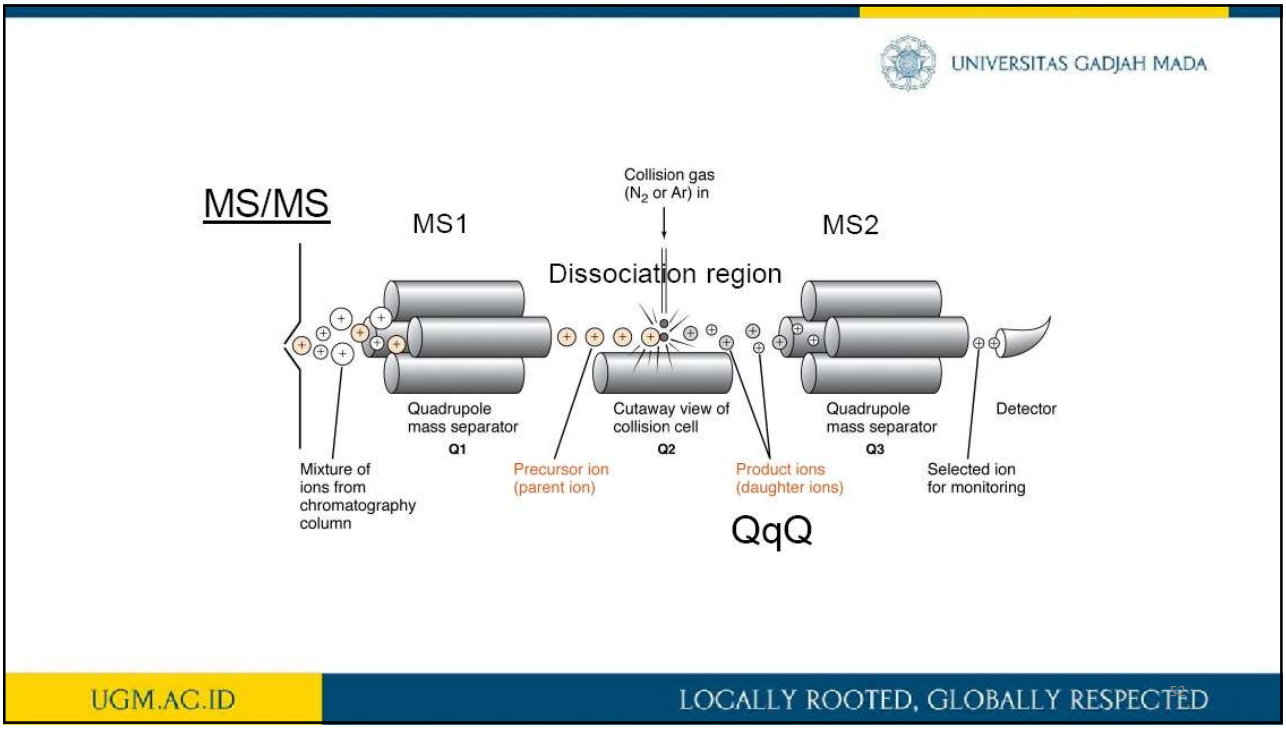
Tandem Quad (MS/MS)

- **Mass confirmation**
- **Fragmentation- for some structural information**
- **Structural confirmation**
- **More selectivity than Single Quad**


UNIVERSITAS GADJAH MADA
Quadrupoles
TWAVE
Collision Cell
UGM.AC.ID
51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MS/MS
MS1
MS2
Collision gas
(N_2 or Ar) in
Dissociation region
Quadrupole
mass separator
Q1
Cutaway view of
collision cell
Q2
Quadrupole
mass separator
Q3
Detector
Mixture of
ions from
chromatography
column
Precursor ion
(parent ion)
Product ions
(daughter ions)
Selected ion
for monitoring
QqQ
UGM.AC.ID
LOCALLY ROOTED, GLOBALLY RESPECTED

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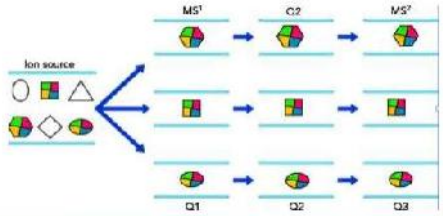
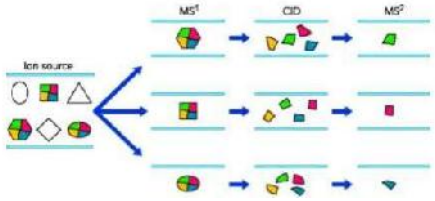
MS/MS

- When the Collision Cell is **OFF**
  - The Twave guides ions through the cell
  - Generally no collision gas
  - No Collision Energy



- When Collision Cell is **ON**
  - Break ions
  - Twave guides ions through the cell
  - Collision gas is ON
  - Collision energy is ON



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Quadrupole

Advantages

- Inexpensive
- Easily Interfaced to Many Ionization Methods

Disadvantages

- Low Resolution (<4000)
- Low Accuracy (>100ppm)
- MS/MS requires multiple analyzers
- Low Mass Range (<4000)
- Slow Scanning

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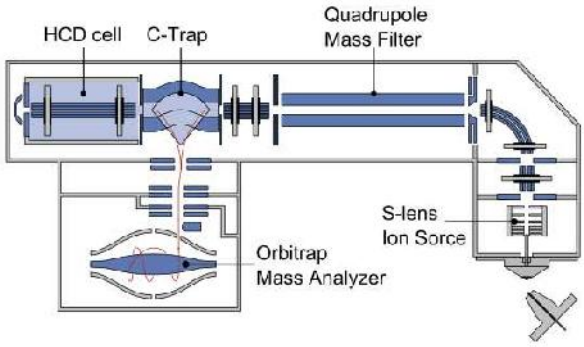
Orbitrap (High resolution)



- orbitrap

Orbitrap 2

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Analisis Kimia dengan LC-TQD MS

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Aplikasi MS

Qualitative

- Identification of unknown : HRMS or TOF
  Quadruple : MS Scan (limited only m/z information)
- Confirmation of targeted compound:
  Quadrupole : MS Scan, SIR, MS/MS, MRM

Quantitation

- Quadrupole SIR (LCMS)
- Quadrupole SIR, MRM (LC-MSMS)
- HRMS : (SIM, PRM)

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Confirmation of targeted compound

- *Identification point (IP) : 4 IP*

LC-MS/MS

- Ion molecular: 1 IP
- Each Transition (daughter ion) of MS/MS : 1.5 IP

Confirmed as a compound if m/z of ion molecular and 2 m/z of daughter ion confirmed

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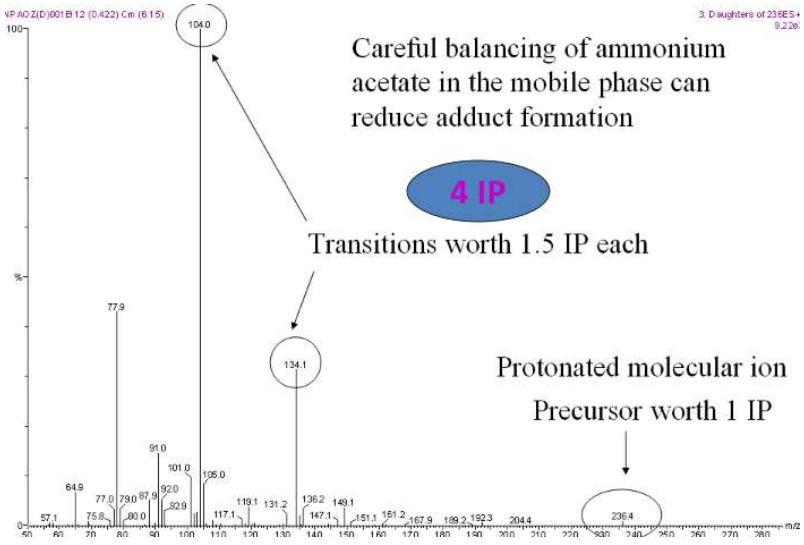

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Mode Analysis TQD

- **MS Scan**
- **SIR (Selected Ion Recording)**
- **MRM (Multiple Reaction Monitoring)**
- **Daughter Scan**

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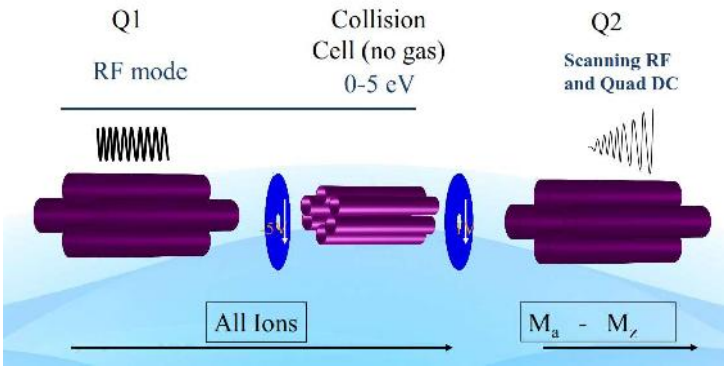
Scanning (Operating like a single quad)



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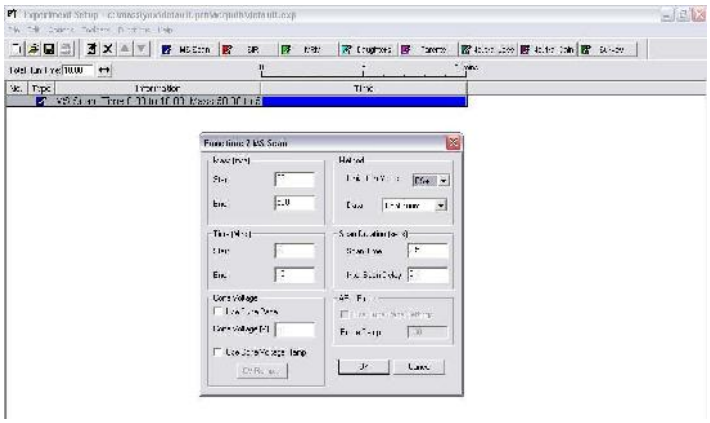
Setting parameters for MS Scan Mode



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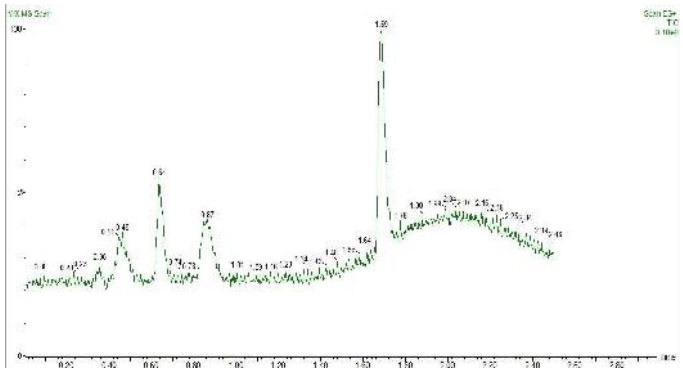
Contoh Chromatogram MS Scan Mode



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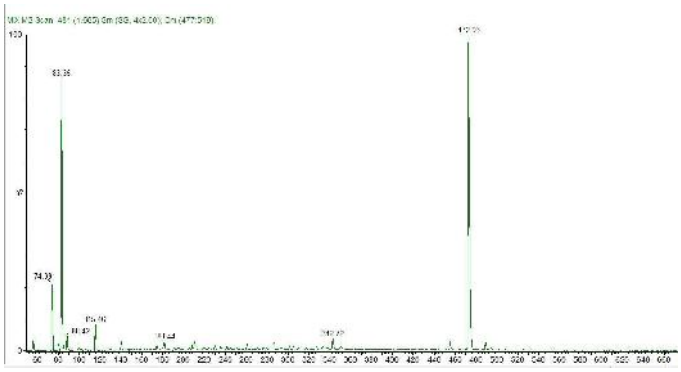
Contoh Spectrum MS Scan Mode



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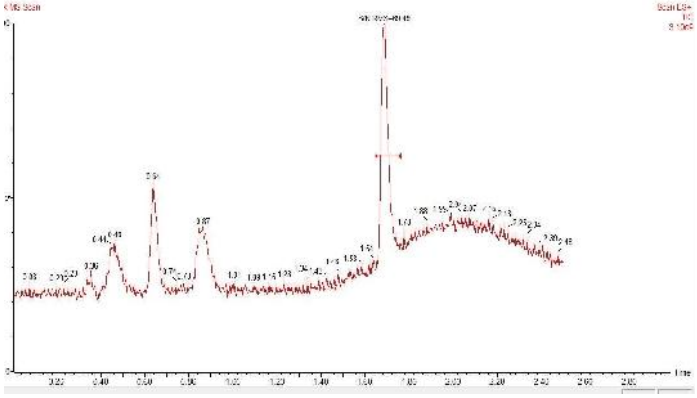
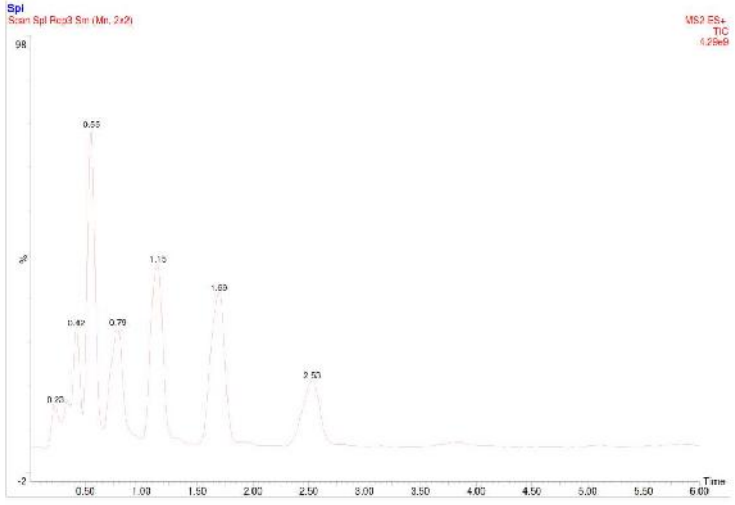
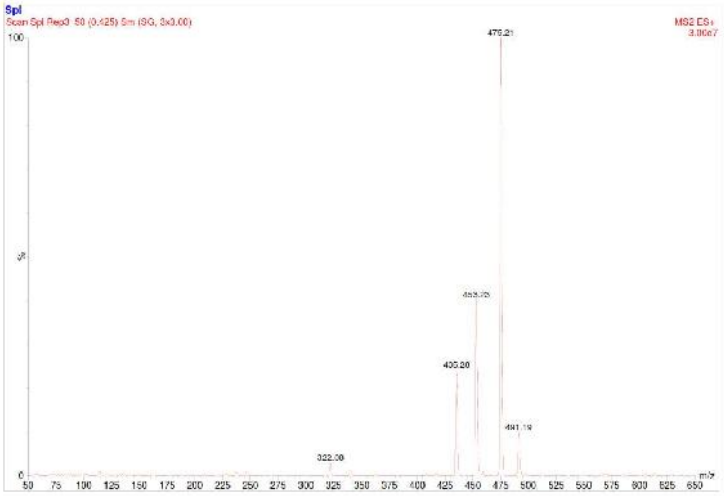
S to N pada MS Scan Mode



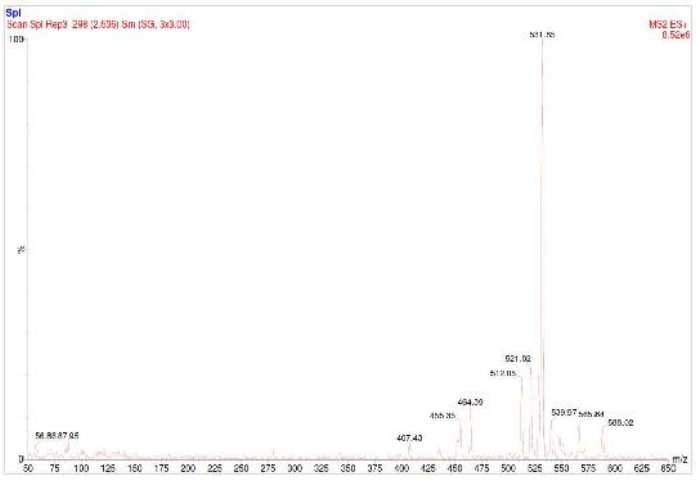
UNIVERSITAS GADJAH MADA



UNIVERSITAS GADJAH MADA



UNIVERSITAS GADJAH MADA



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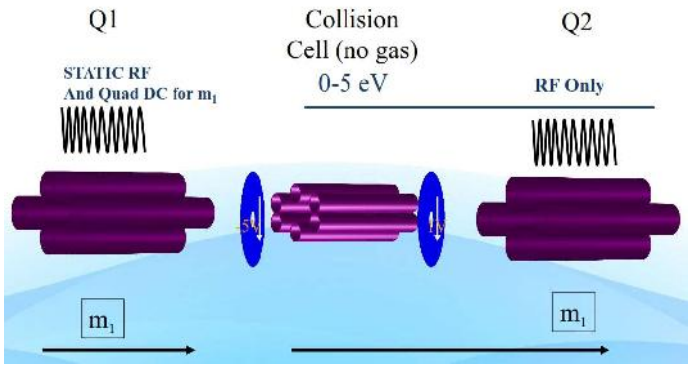
SIR (Selected Ion Recording) Single Quad



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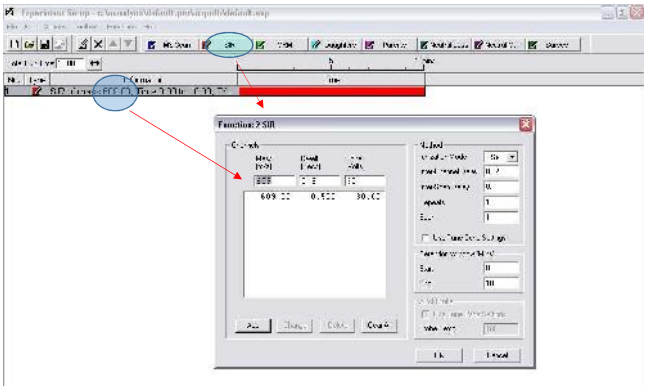
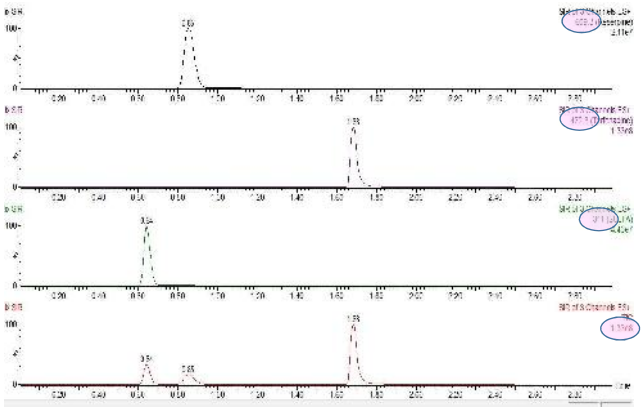
Setting parameters for SIR Mode



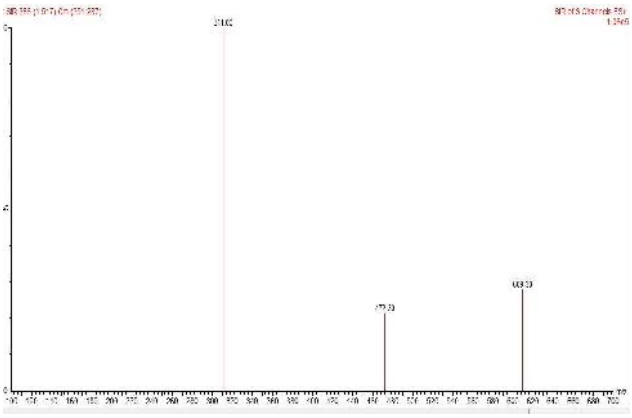
UNIVERSITAS GADJAH MADA

Contoh Chromatogram SIR Mode



UNIVERSITAS GADJAH MADA

Contoh Spectrum SIR Mode



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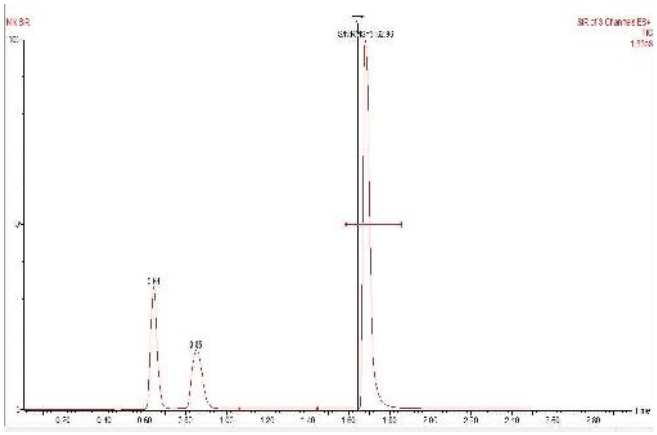
S to N pada SIR Mode



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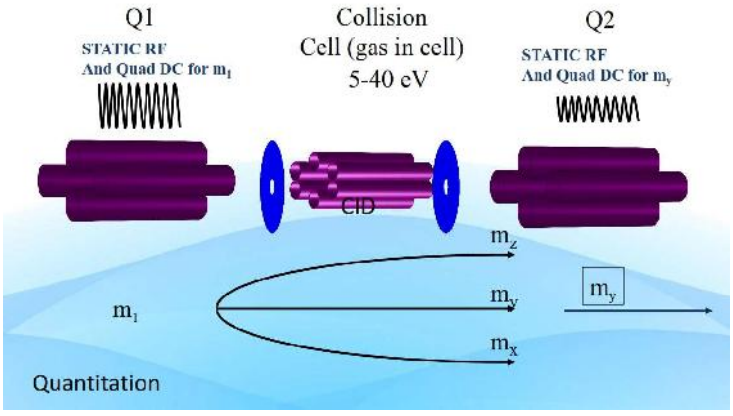
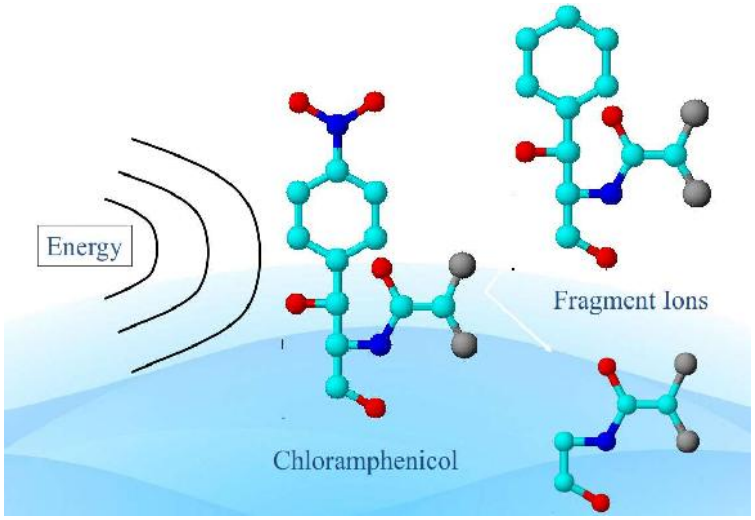
MRM (Multiple Reaction Monitoring)





Contoh kondisi MRM

Vitamin compound	RT	MRM transition	Cone voltage (V)	Collision energy (eV)
Ascorbic acid (C)	0.40	177 > 141	24	8
Thiamine (B1)	0.48	265 > 122	24	17
Nicotinic acid (B3)	0.54	124 > 80	38	20
Pyridoxal (B6)	0.67	168 > 150	27	15
Pyridoxine (B6)	0.89	170 > 152	28	14
Nicotinamide (B3)	0.93	123 > 80	40	20
Calcium pantothenate (B5)	2.77	242 > 153	30	15
Cyanocobalamin (B12)	3.03	678 > 147	36	34
Folic acid (B9)	3.03	442 > 295	23	17
Riboflavin 5' phosphate (B2)	3.08	457 > 439	41	18
Biotin (B7)	3.15	245 > 227	28	13
Riboflavin (B2)	3.20	377 > 243	42	22

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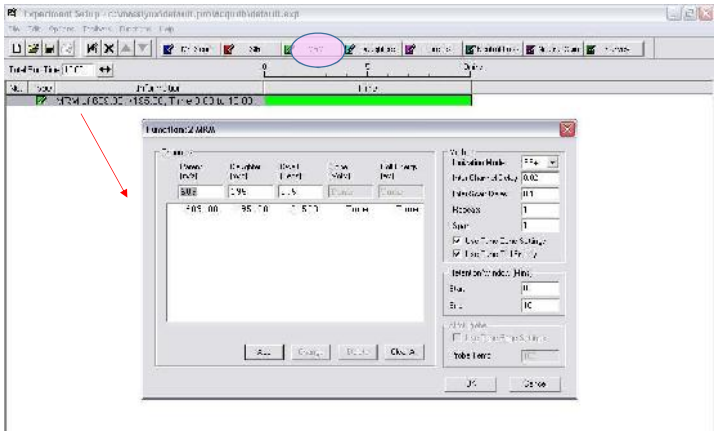
Setting parameters for MRM Mode



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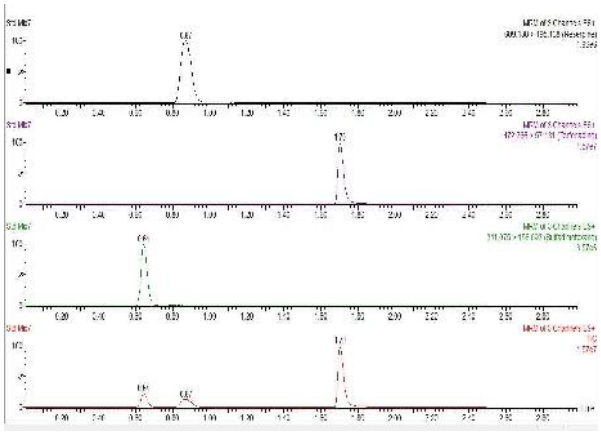
Contoh Chromatogram MRM Mode



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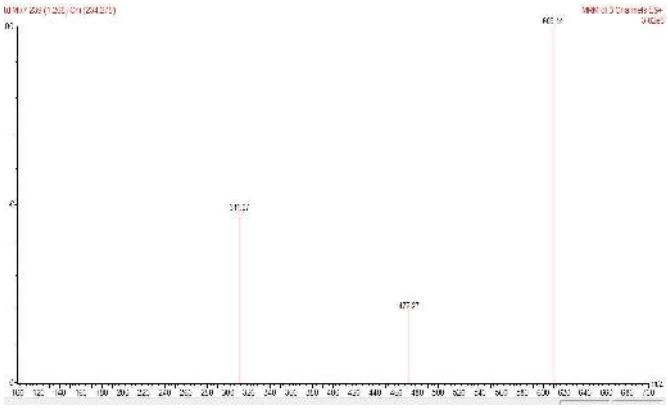
Contoh Spectrum pada MRM Mode



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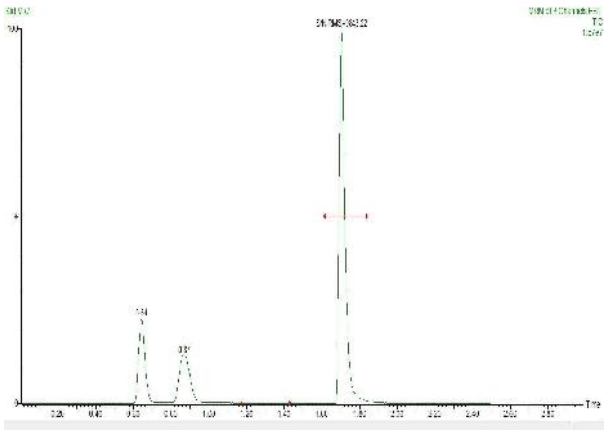
S to N pada MRM Mode



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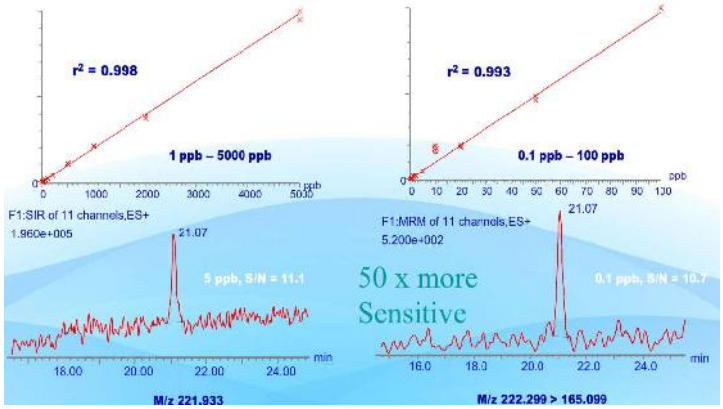
Sensitivity of SIR vs MRM Carbofuran



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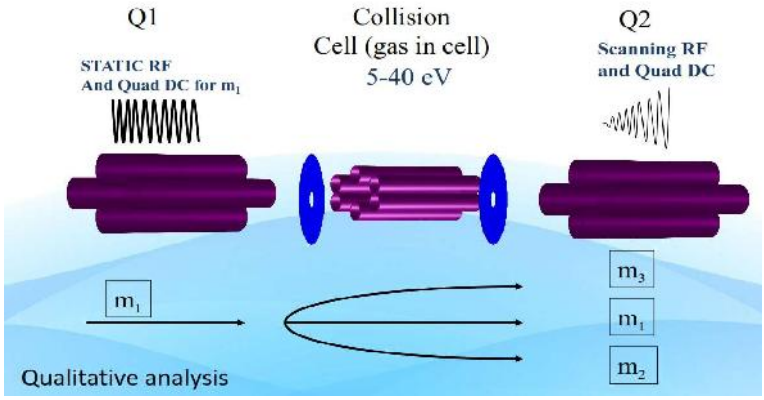
Product (Daughter) Ion Analysis



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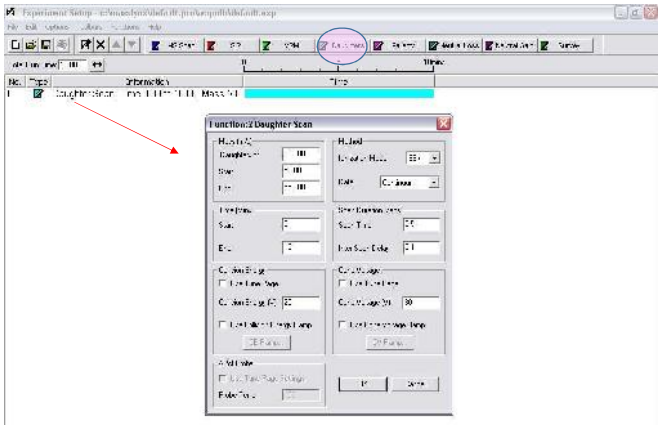
Setting parameters for Daughter Mode



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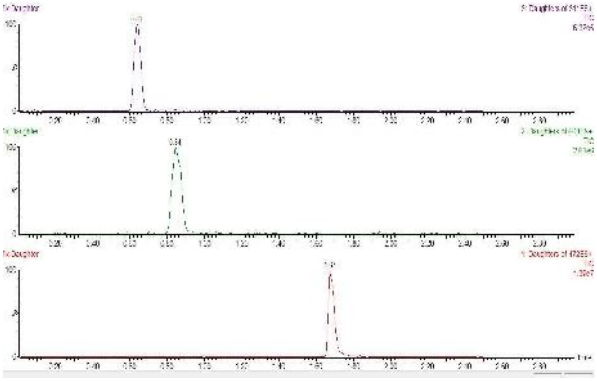
Contoh Chromatogram Daughter Ion Mode



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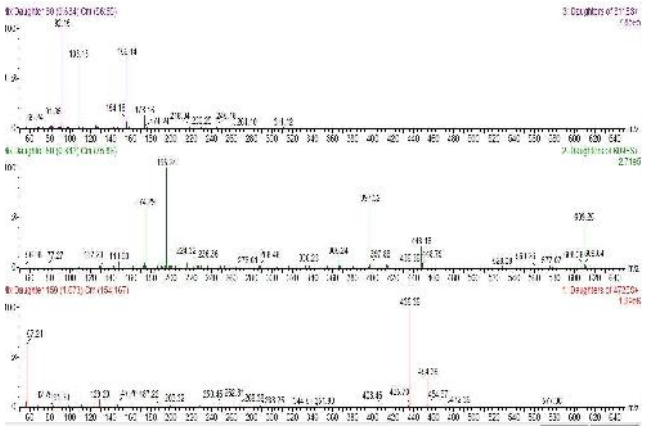
Contoh Spectrum Daughter Ion Mode



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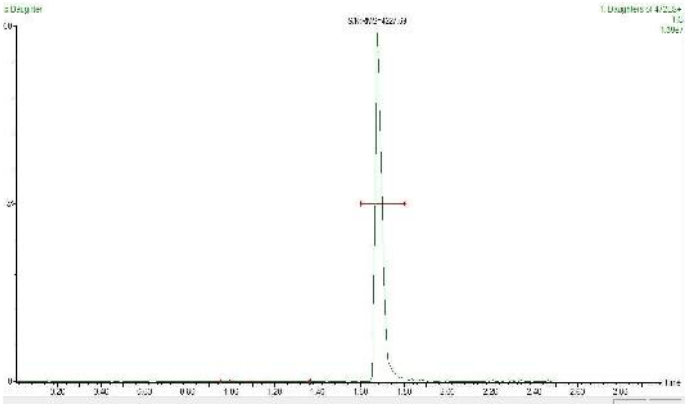
S to N pada Daughter Ion Mode



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Analisis Kimia dengan LC-HRMS

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Scan Mode

- Full scan (R=70,000, Max: 140,000)

  Metabolomics / Lipidomics / Small molecule screening / Intact protein

- Full scan (R=70,000) + data dependent MS^2 (R=17,500) (DDA)

  Proteomics

- Targeted MS^2 (R=17,000/35,000) (PRM)

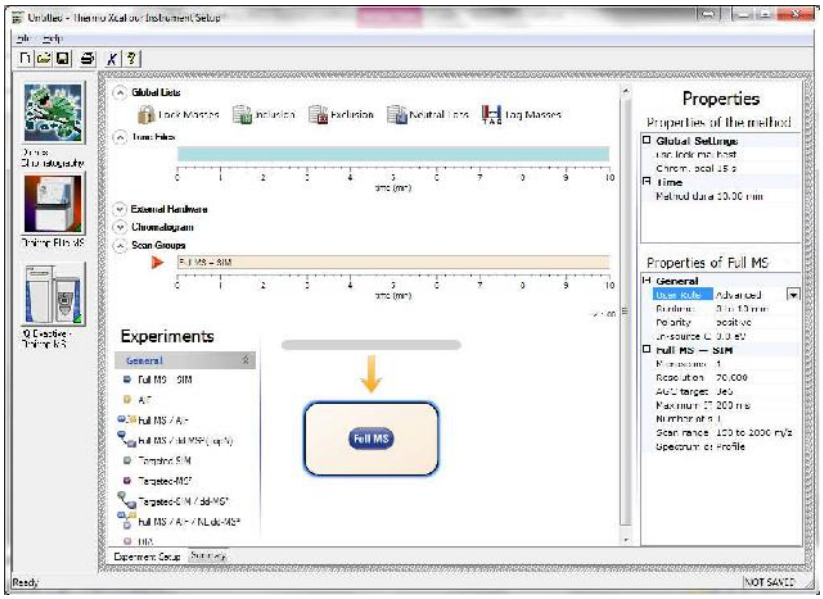
  Targeted quantitation / Quanformation

- Targeted SIM (Max: 140,000)

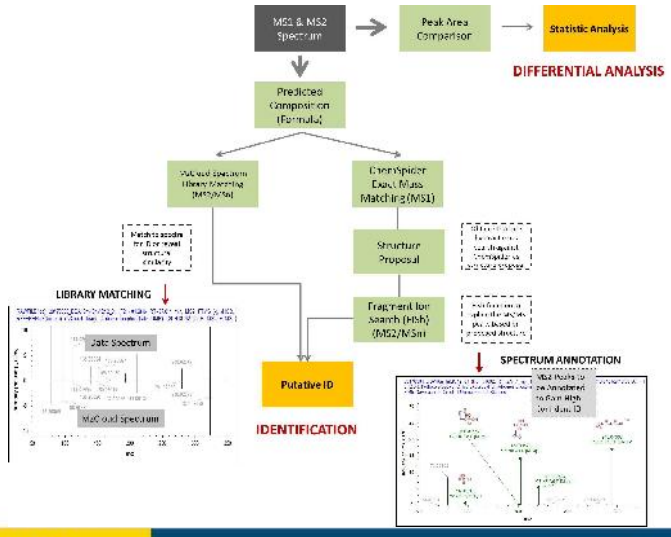
  Targeted quantitation

- Data independent acquisition (R=17,500/35,000) (DIA)

  High throughput targeted quanformation



Workflow For Unknown Screening



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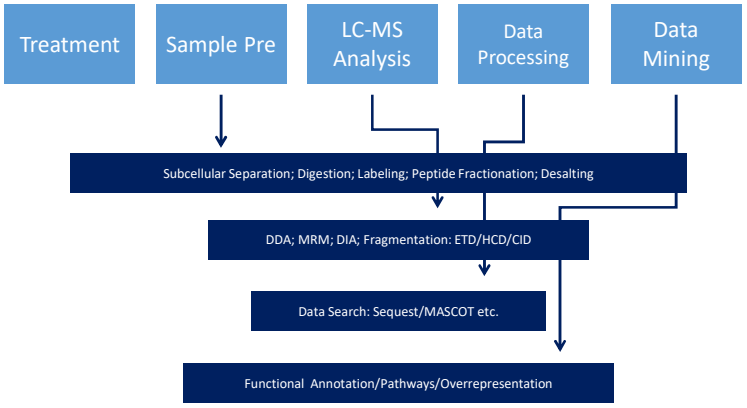
Workflow of Proteomics



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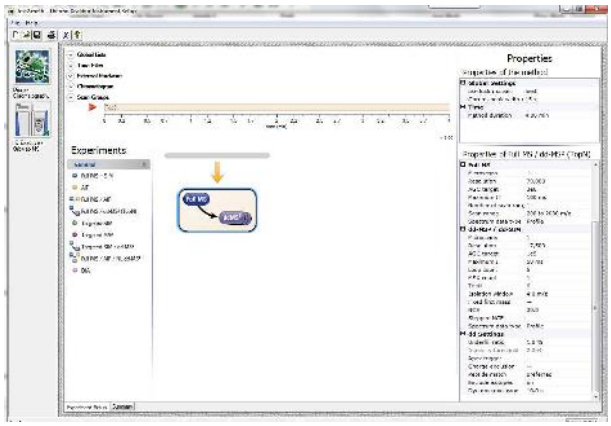
DDA Method



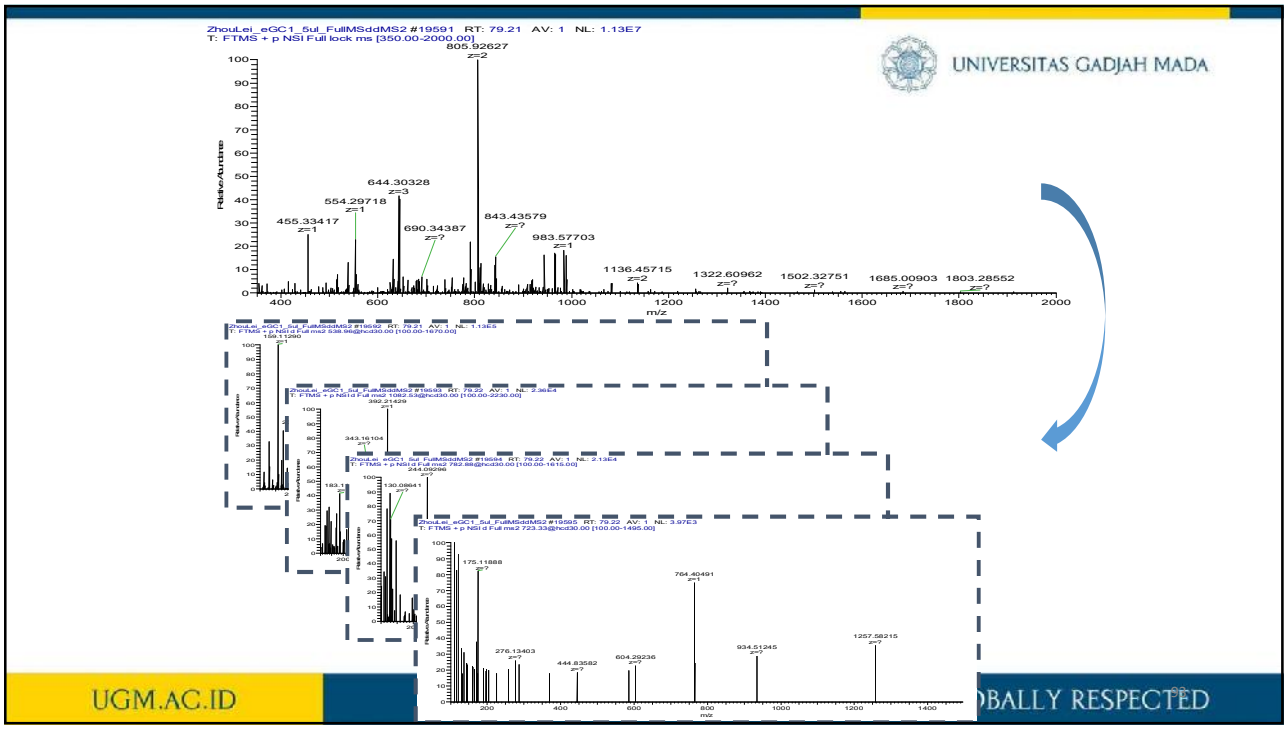
MS^1 → Top N peaks from MS^1 Selected

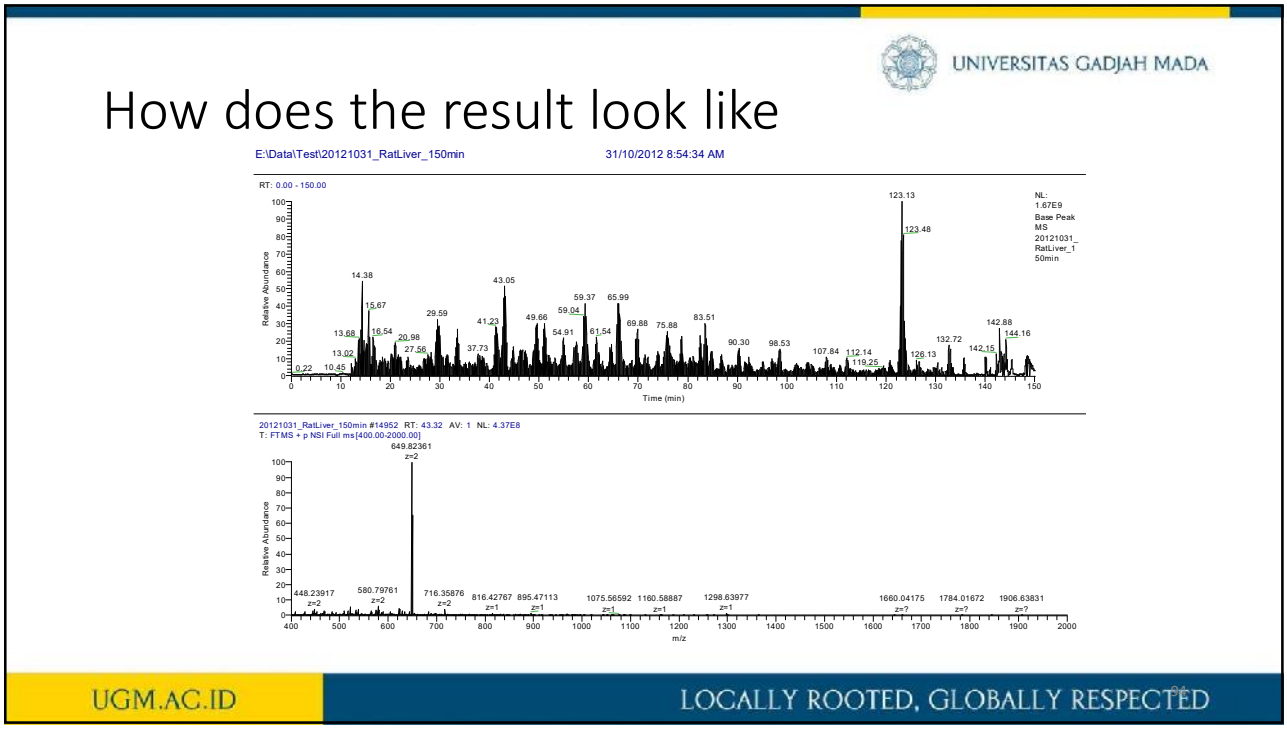
↓

1^{st} MS^2
2^{nd} MS^2
3^{rd} MS^2
4^{th} MS^2
...

[Acquire MS^2 data depends on the MS^1 intensity

For discovery/non-targeted experiment]


UNIVERSITAS GADJAH MADA
UGM.AC.ID
LOCALLY ROOTED, GLOBALLY RESPECTED


UNIVERSITAS GADJAH MADA
How does the result look like
UGM.AC.ID
LOCALLY ROOTED, GLOBALLY RESPECTED

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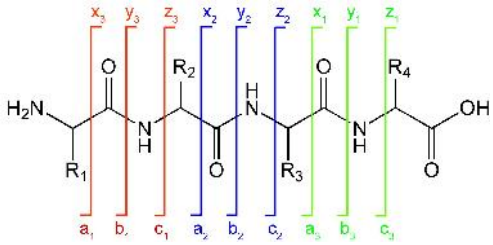
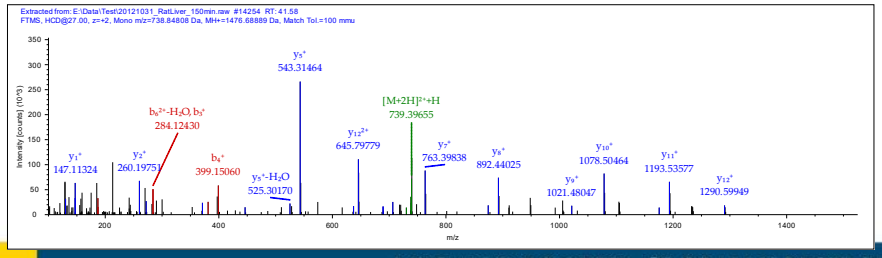
Peptide sequence





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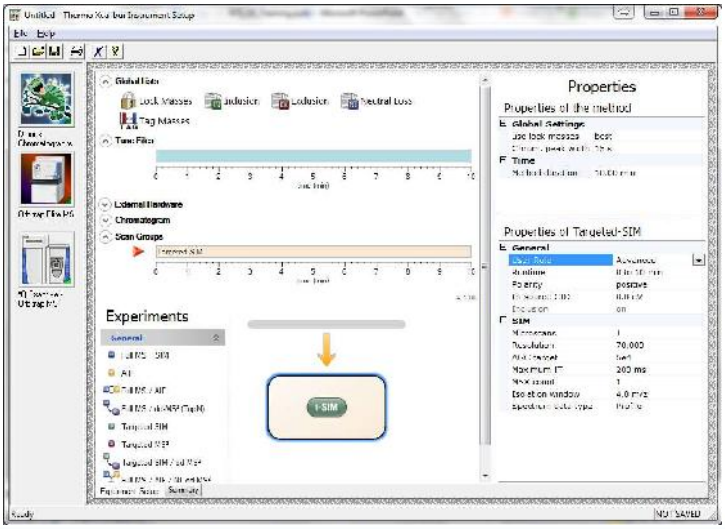
Targeted SIM



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Method editor Inclusion List (modified)

File Edit Help Done

	Mass [m/z]	Formula [M]	species	CS [z]	Polarity	Start [min]	End [min]	NCE	Comment
1	655.6525	CH3CH2Cl	+H	1	Positive				
2	155. 56 1	C8H23Cl	+H	1	Positive				
3					Positive				

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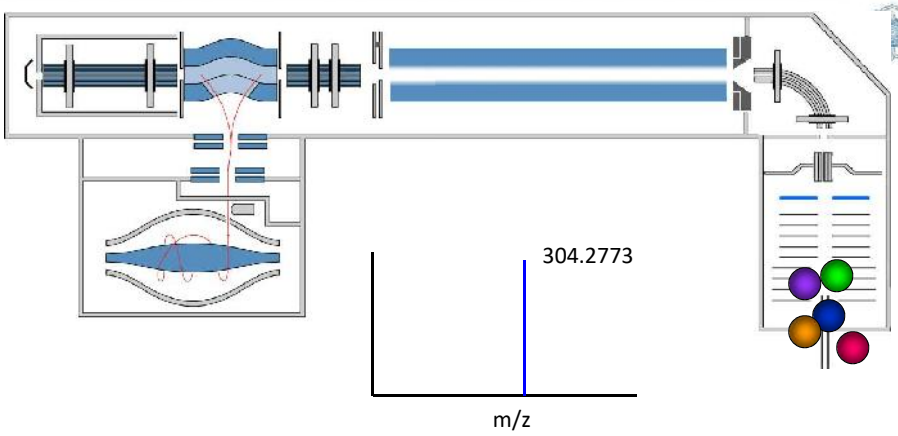

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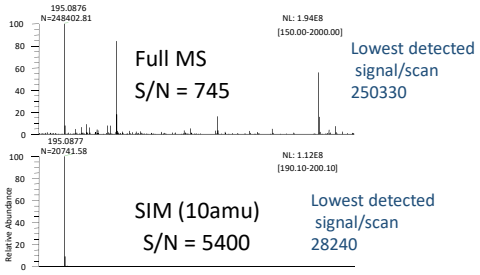
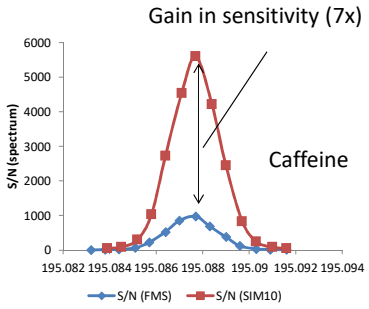
- By collecting a narrower range of ions, more target ions can be collected within same time



Sensitivity gain 5 – 10 x with SIM mode



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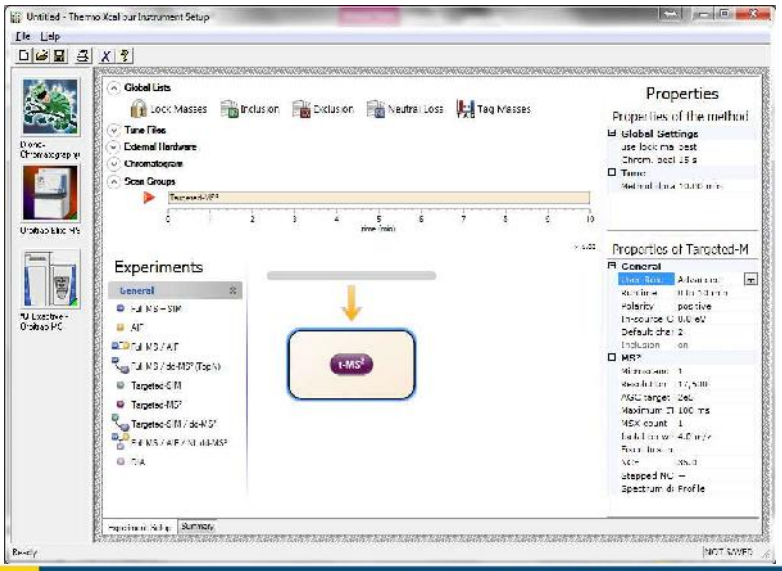
PRM



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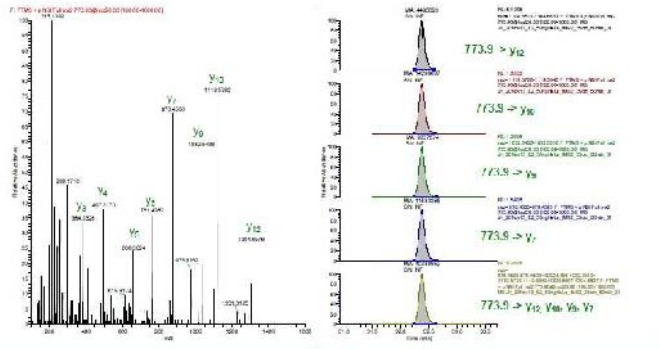
Quantitation using PRM

Peak areas are calculated from the extracted ion chromatograms of parent -> fragment ion



11 $[M+2H]^{2+}$ of ELGQSGVDTYLQTK[$^{13}C_6$,$^{15}N_2$]

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TERIMA KASIH