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Analisis dengan LC-MS

Tri Joko Raharjo

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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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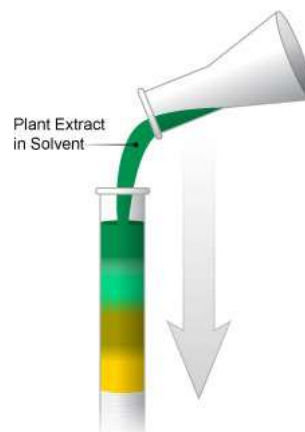
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Kromatografi

- 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

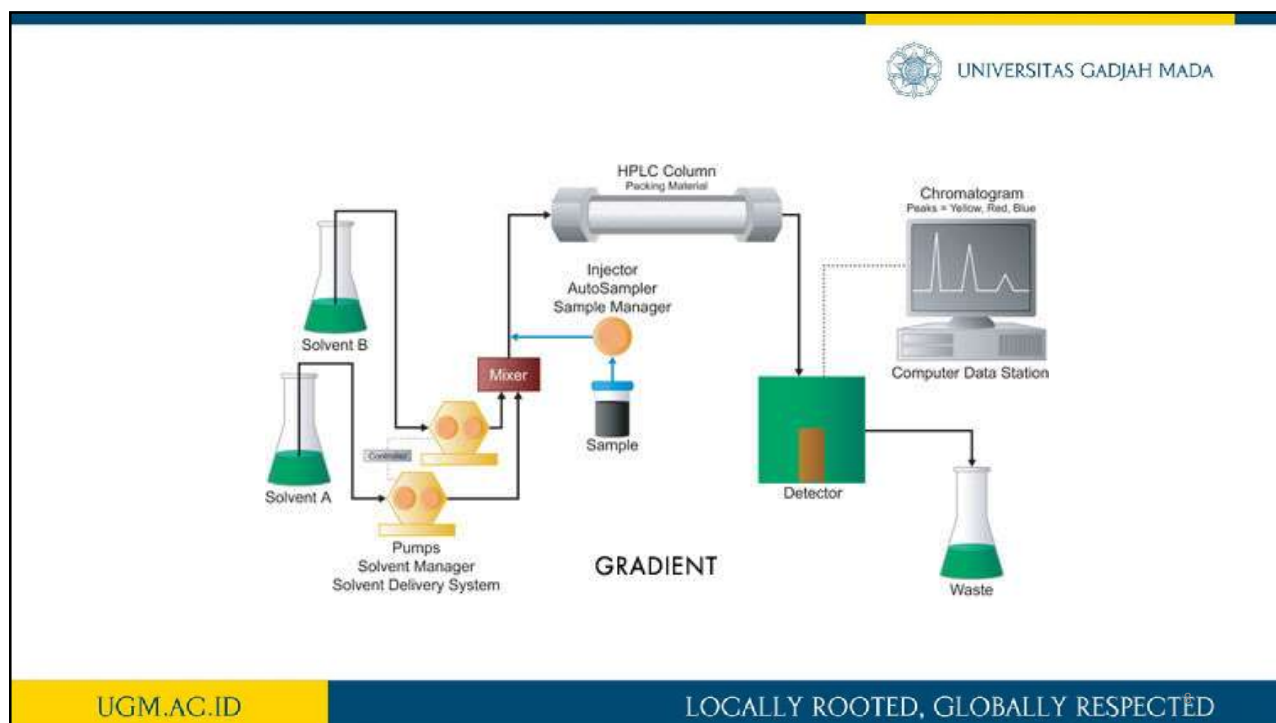
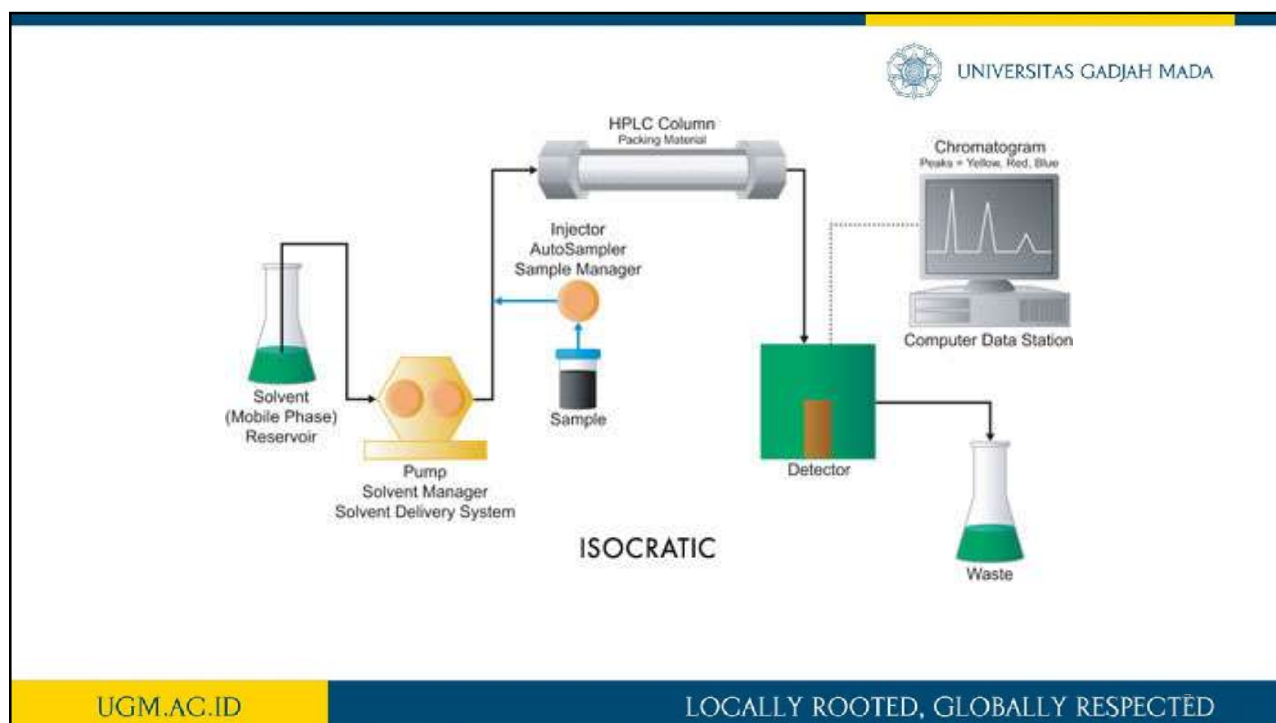
- High Pressure Liquid Chromatography (HPLC) : high pressure to flow the liquid into packed columns.
- Pressure: 500 psi (35 bar, 1 bar = 0,9868 atm)
- The early 1970's
 - HPLC up to 6,000 psi (400 bar)
 - Improvement detectors
 - Improvement of columns.



Performance improvement

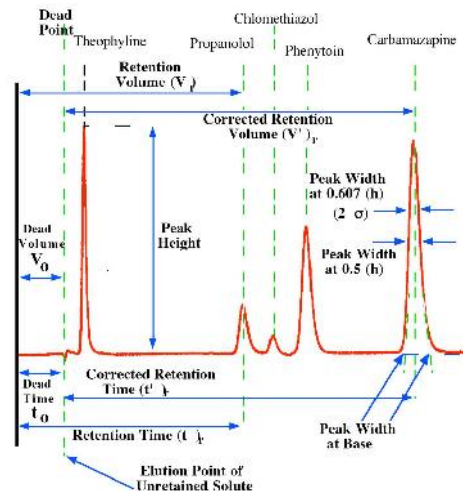
High Performance Liquid Chromatography (HPLC).

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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 (R_s)

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

$$\alpha = (t_R)_B / (t_R)_A$$

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

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Van Deemter plots

$$HETP = A + B/u + Cu$$

u = average velocity of the mobile phase.

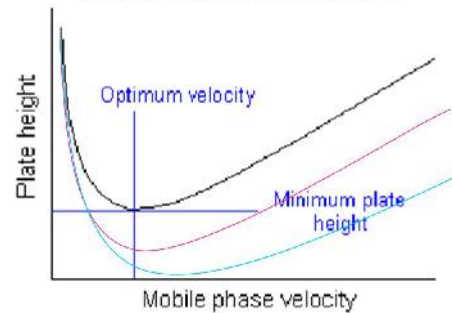
A , B , and C = band broadening factors

A - Eddy diffusion

B - Longitudinal diffusion.

C - Resistance to mass transfer.

A typical Van Deemter plot



$$HETP = L/N$$

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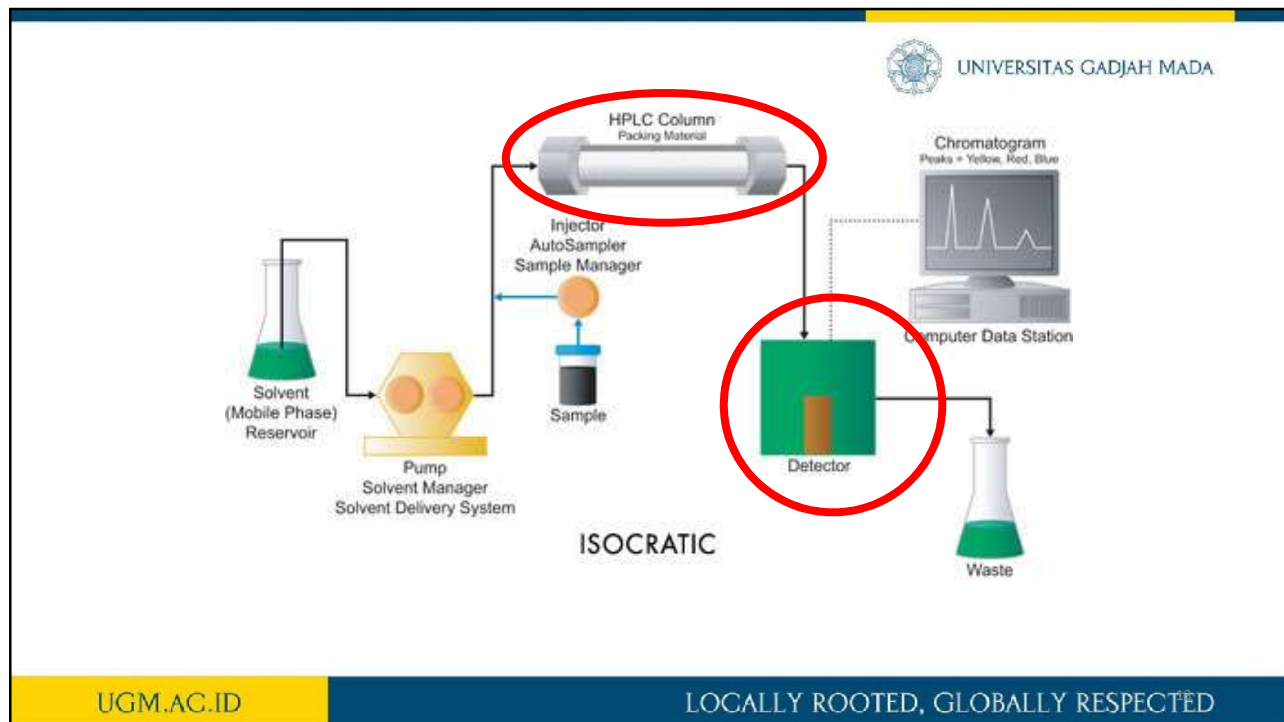
N menunjukkan Efisiensi pemisahan

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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Fase diam di dalam kolom

Three primary HPLC separations :

- Polarity (NP Vs RP)
- Electrical Charge
- Molecular Size
- Specific interaction

Glass

PEEK®

Stainless Steel

Analytical

Preparative

Internal Diameter (i.d.)
1mm – 50mm

Length
20mm – 500mm

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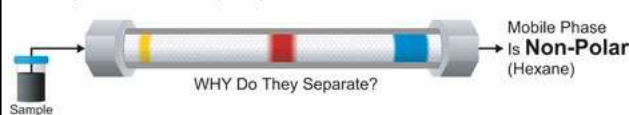
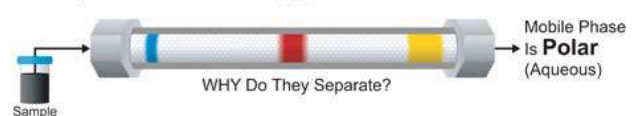
Polarity (NP Vs RP)

Normal Phase (NP)

The hydrophobic compounds elute more quickly than do hydrophilic compounds

Reversed Phase (RP)

- The hydrophilic compounds elute more quickly than do hydrophobic compounds

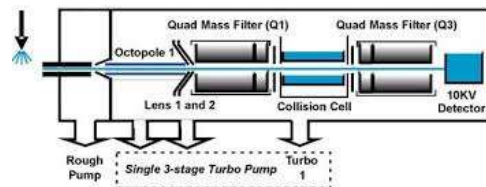
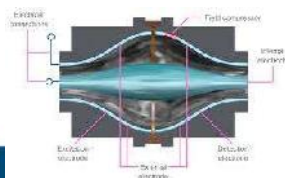
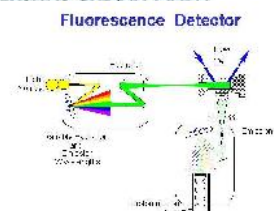
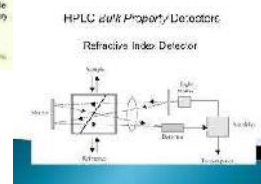
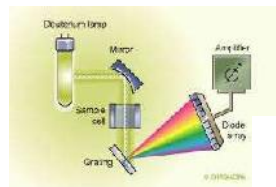
Stationary Phase Is **Polar** (Silica)Stationary Phase Is **Non-Polar** (C₁₈)

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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



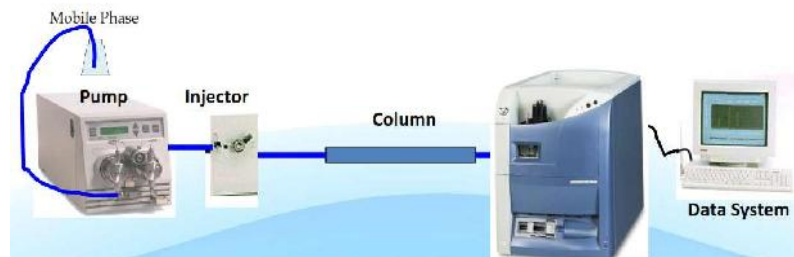
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LC-MS LC-MSMS



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Pengantar MS

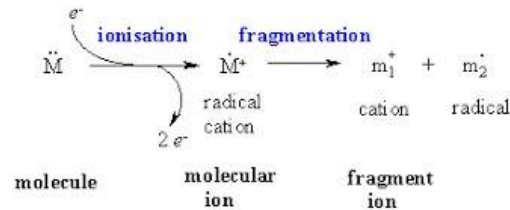
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Mass Spectrometry (MS)

- 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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THE PERIODIC TABLE OF THE ELEMENTS

1 IA 1A	2 IIA 2A																	13 IIIA 3A	14 IVA 4A	15 VA 5A	16 VIA 6A	17 VIIA 7A	18 VIIIA 8A																												
1 H Hydrogen 1.008		3 Li Lithium 6.941	4 Be Beryllium 9.012																	5 B Boron 10.81	6 C Carbon 12.01	7 N Nitrogen 14.01	8 O Oxygen 16.00	9 F Fluorine 18.99	10 Ne Neon 20.18																										
11 Na Sodium 22.99	12 Mg Magnesium 24.31	13 Al Aluminum 26.98	14 Si Silicon 28.09	15 P Phosphorus 30.97	16 S Sulfur 32.06	17 Cl Chlorine 35.45	18 Ar Argon 39.95																	19 K Potassium 39.10	20 Ca Calcium 40.08	21 Sc Scandium 44.96	22 Ti Titanium 47.88	23 V Vanadium 50.94	24 Cr Chromium 52.00	25 Mn Manganese 54.94	26 Fe Iron 55.85	27 Co Cobalt 58.93	28 Ni Nickel 58.69	29 Cu Copper 63.55	30 Zn Zinc 65.38	31 Ga Gallium 69.72	32 Ge Germanium 72.64	33 As Arsenic 74.92	34 Se Selenium 78.96	35 Br Bromine 79.90	36 Kr Krypton 83.80										
37 Rb Rubidium 85.47	38 Sr Strontium 87.62	39 Y Yttrium 88.91	40 Zr Zirconium 91.22	41 Nb Niobium 92.91	42 Mo Molybdenum 95.94	43 Tc Technetium 98.01	44 Ru Ruthenium 101.07	45 Rh Rhodium 102.91	46 Pd Palladium 106.42	47 Ag Silver 107.87	48 Cd Cadmium 112.41	49 In Indium 114.82	50 Sn Tin 118.71	51 Sb Antimony 121.76	52 Te Tellurium 127.60	53 I Iodine 126.91	54 Xe Xenon 131.29																	55 Cs Cesium 132.91	56 Ba Barium 137.33	57-71 Lanthanide Series	72 Hf Hafnium 178.49	73 Ta Tantalum 180.95	74 W Tungsten 183.84	75 Re Rhenium 186.21	76 Os Osmium 190.23	77 Ir Iridium 192.22	78 Pt Platinum 195.08	79 Au Gold 196.97	80 Hg Mercury 200.59	81 Tl Thallium 204.38	82 Pb Lead 207.2	83 Bi Bismuth 208.98	84 Po Polonium 209	85 At Astatine 210	86 Rn Radon 222
87 Fr Francium 223	88 Ra Radium 226	89-103 Actinide Series	104 Rf Rutherfordium 261	105 Db Dubnium 262	106 Sg Seaborgium 266	107 Bh Bohrium 264	108 Hs Hassium 277	109 Mt Meitnerium 268	110 Ds Darmstadtium 271	111 Rg Roentgenium 272	112 Cn Copernicium 285	113 Uut Ununtrium 284	114 Uuq Ununquadium 289	115 Uup Ununpentium 288	116 Uuh Ununhexium 292	117 Uus Ununseptium 294	118 Uuo Ununoctium 294																																		

Lanthanide Series

Actinide Series

57 La Lanthanum 138.91	58 Ce Cerium 140.12	59 Pr Praseodymium 140.91	60 Nd Neodymium 144.24	61 Pm Promethium 145	62 Sm Samarium 150.36	63 Eu Europium 151.96	64 Gd Gadolinium 157.25	65 Tb Terbium 158.93	66 Dy Dysprosium 162.50	67 Ho Holmium 164.93	68 Er Erbium 167.26	69 Tm Thulium 168.93	70 Yb Ytterbium 173.05	71 Lu Lutetium 174.97
89 Ac Actinium 227	90 Th Thorium 232.04	91 Pa Protactinium 231.04	92 U Uranium 238.03	93 Np Neptunium 237	94 Pu Plutonium 244	95 Am Americium 243	96 Cm Curium 247	97 Bk Berkelium 247	98 Cf Californium 251	99 Es Einsteinium 252	100 Fm Fermium 257	101 Md Mendelevium 258	102 No Nobelium 259	103 Lr Lawrencium 262

Alkali Metal

Alkaline Earth

Transition Metal

Basic Metal

Semimetal

Nonmetal

Halogen

Noble Gas

Lanthanide

Actinide



MS Principles: nominal vs. exact mass

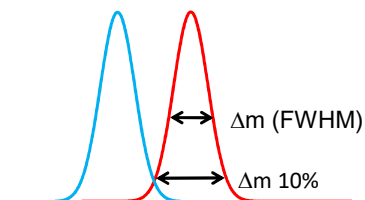
Compound	Nominal Mass	Exact Mass	
Carbon	12	12.0000	
Hydrogen	1	1.0078	
Oxygen	16	15.9949	
Nitrogen	14	14.0031	
Carbon Monoxide (CO)	28	27.9949	Toxic substance
Nitrogen Gas (N ₂)	28	28.0061	80% of atmosphere
Ethylene (C ₂ H ₄)	28	28.0313	Flammable substance



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 $\Delta m / z = \frac{500.1 - 500.0}{500} \cdot 10^6 = 200 \text{ ppm}$ **100 mDa error**
- Orbitrap MS TOF MS $\Delta m / z = \frac{500.10414 - 500.10314}{500.10314} \cdot 10^6 = 2 \text{ ppm}$ **1 mDa error**

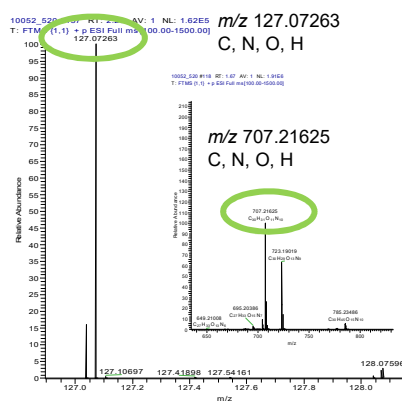
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Why Do We Need Mass Accuracy: Molecular Formula



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	C ₉ H ₂₁ O ₂ P ₁ S ₃	Terbufos
288.0949	C ₁₃ H ₂₁ O ₃ P ₁ S ₁	Iprobenfos
288.1142	C ₁₅ H ₁₇ N ₄ Cl ₁	Myclobutanil
288.1256	C ₁₁ H ₂₀ N ₄ O ₃ S ₁	Epronaz
288.1351	C ₁₁ H ₂₁ N ₄ O ₃ P ₁	Pirimethaphos
288.1474	C ₁₆ H ₂₀ N ₂ O ₃	Imazamethabenz

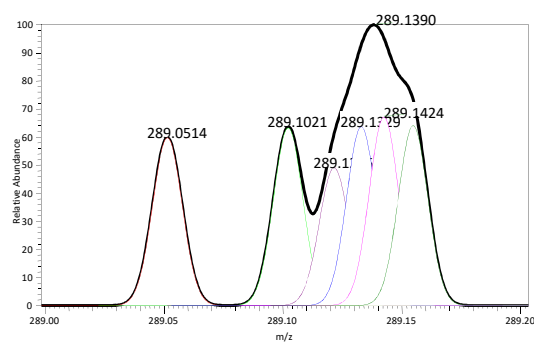
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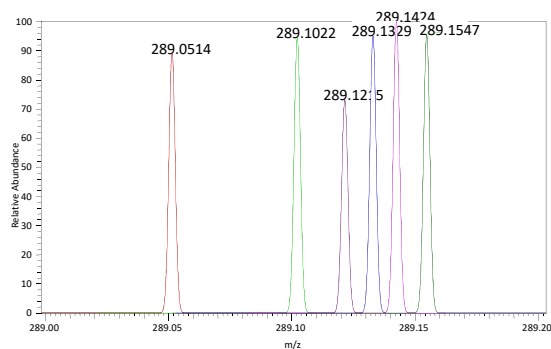


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R = 20,000



R = 100,000



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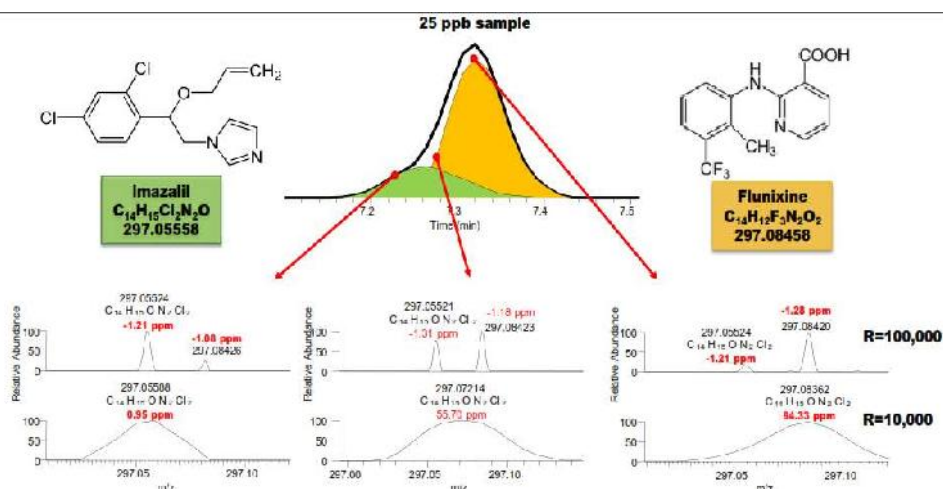
- **Nominal mass:** the mass of an ion calculated using the integer mass of the most abundant isotope of each element.
 - ignores the mass defect, where $H=1$, $C=12$, $O=16$
- **Monoisotopic mass:** the mass of an ion calculated using the exact mass of the most abundant isotope of each element.
 - includes the mass defect, where $^1H=1.0078$, $^{12}C=12.0000$, $^{16}O=15.9949$
- **Average mass:** the mass of an ion calculated using the relative average isotopic mass of each element.
 - where $C=12.0111(12.000000)(0.9890) + (13.003355)(0.0110) = 12.011 \text{ Da}$
 - $H=1.00797$, $O=15.9994$
- **Isotopic abundance:** the naturally occurring distribution of the same element with different atomic mass.
 - ex: $^{12}C=12.0000 = 98.9\%$
 $^{13}C=13.0034 = 1.1\%$

TED

Importance of Mass Accuracy and Resolving power

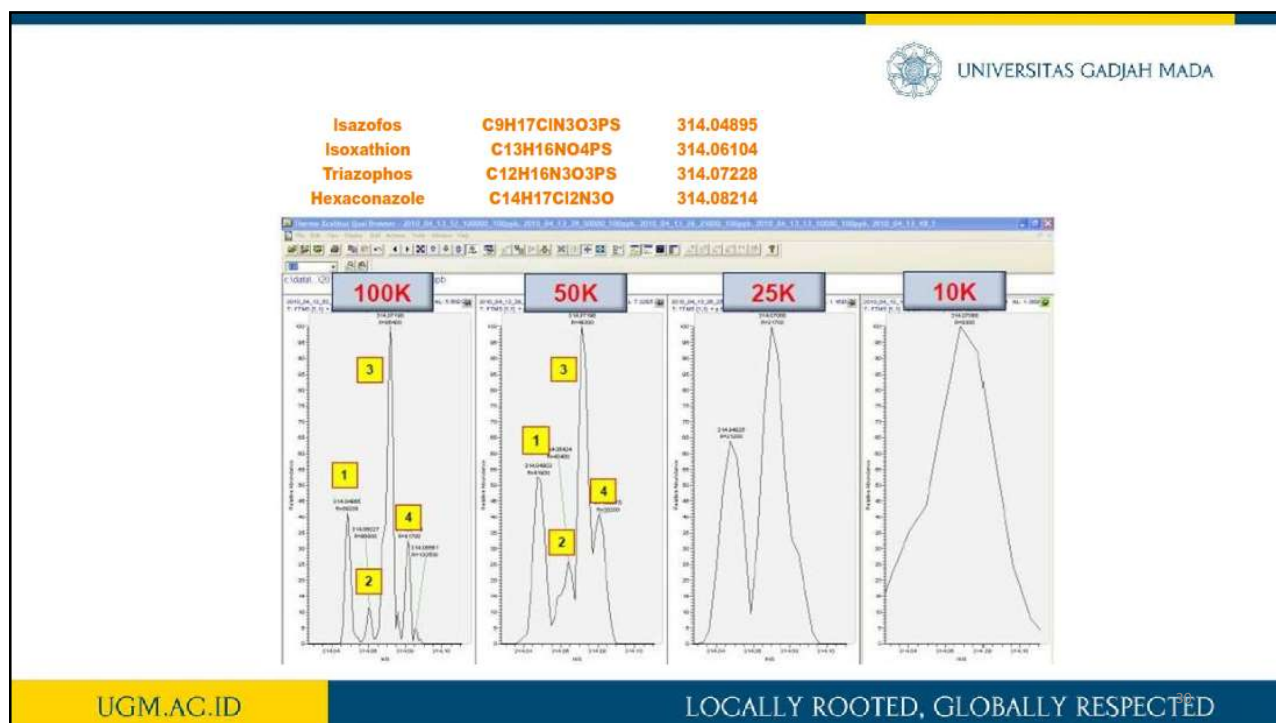
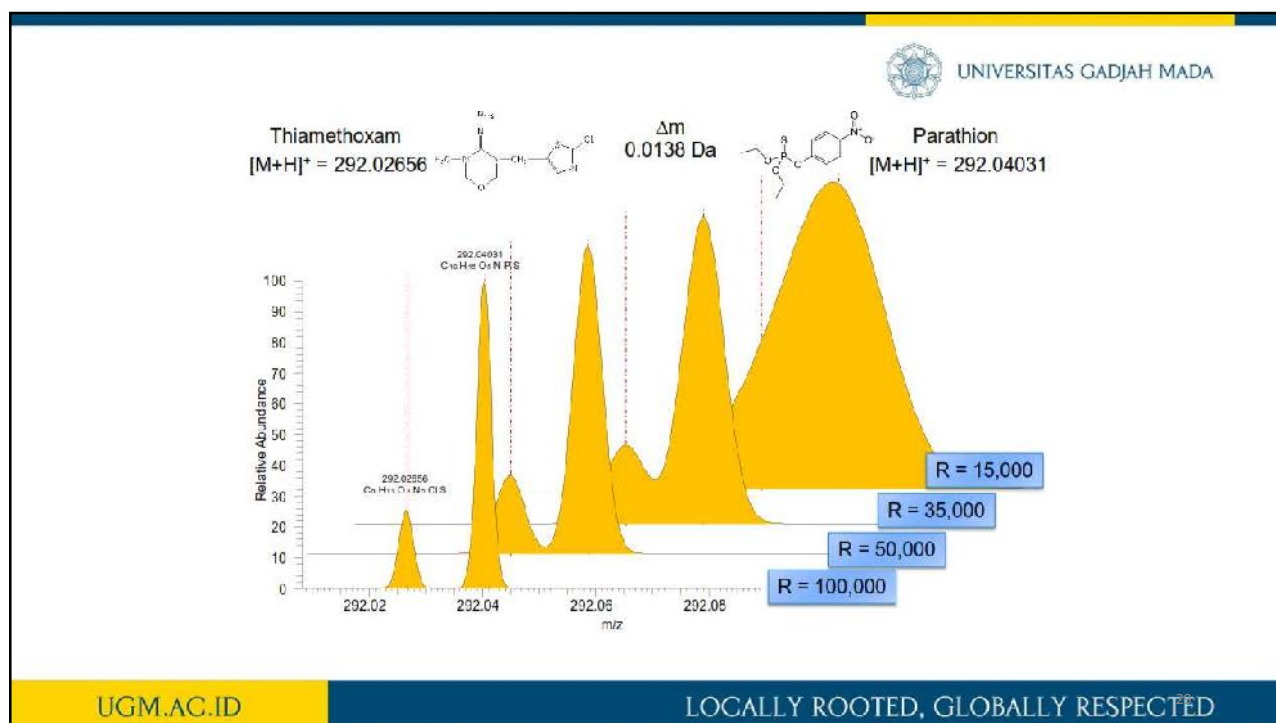


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Type	Mass Accuracy
FT-ICR-MS	0.1 - 1 ppm
Orbitrap	0.5 - 1 ppm
Magnetic Sector	1 - 2 ppm
TOF-MS	3 - 5 ppm
Q-TOF	3 - 5 ppm
Triple Quad	3 - 5 ppm
Linear IonTrap	50-200 ppm (10 ppm in Ultra-Zoom)

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

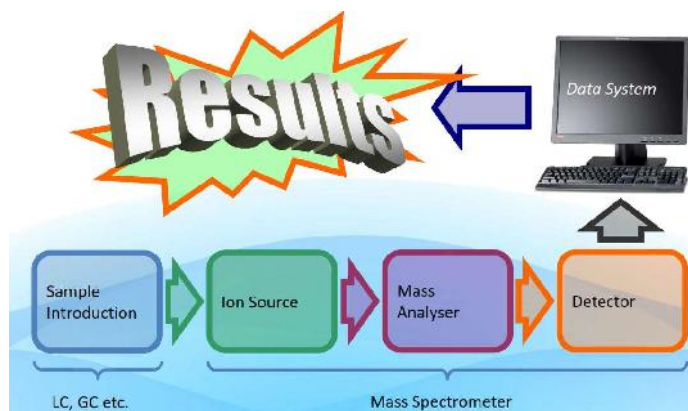
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Mass Spectrometer Instrumentation



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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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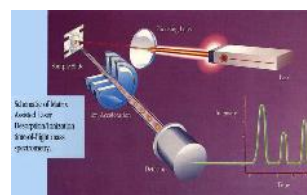
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Soft Ionization

- 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)



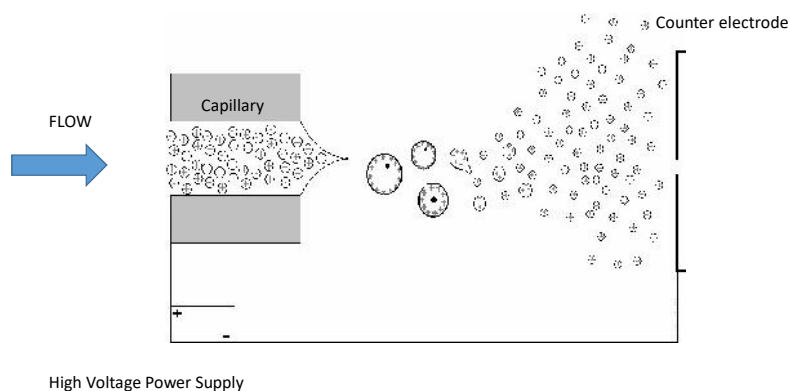
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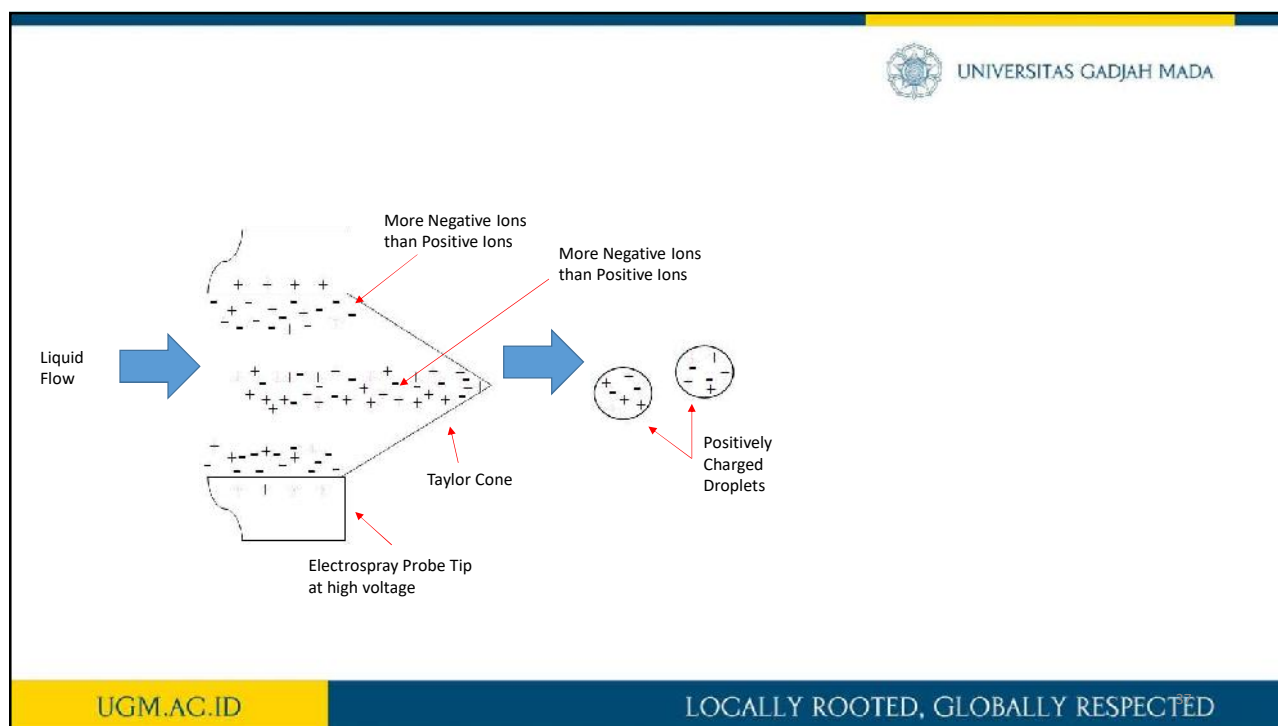
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Electrospray (ESI)



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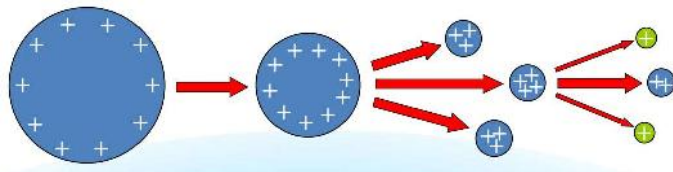
- Droplets produced from the spray have a surface charge
- Surface charged droplets undergo solvent evaporation and droplet fission to produce smaller droplets
- Like charge repulsion becomes greater than droplet surface tension and fission occurs to produce smaller charged droplets

The diagram illustrates the process of solvent evaporation and Coulombic fission. It shows a sequence of three stages. In the first stage, a large blue circle with '+' signs represents a charged droplet. A red arrow labeled "Solvent Evaporation" points to the second stage, where the droplet is smaller. A second red arrow labeled "Coulombic Fission" points to the third stage, where the droplet has split into several smaller droplets, each with '+' signs. The background is light blue with a darker blue border at the top and bottom. The top right corner features the logo of Universitas Gadjah Mada (UGM) and the text "UNIVERSITAS GADJAH MADA". The bottom left corner has the text "UGM.AC.ID" and the bottom right corner has the text "LOCALLY ROOTED, GLOBALLY RESPECTED".

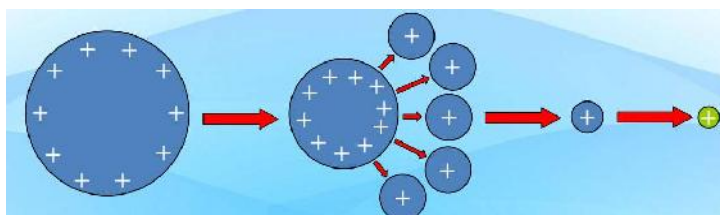


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Ion Desorption Mechanism



Charged Residue Mechanism



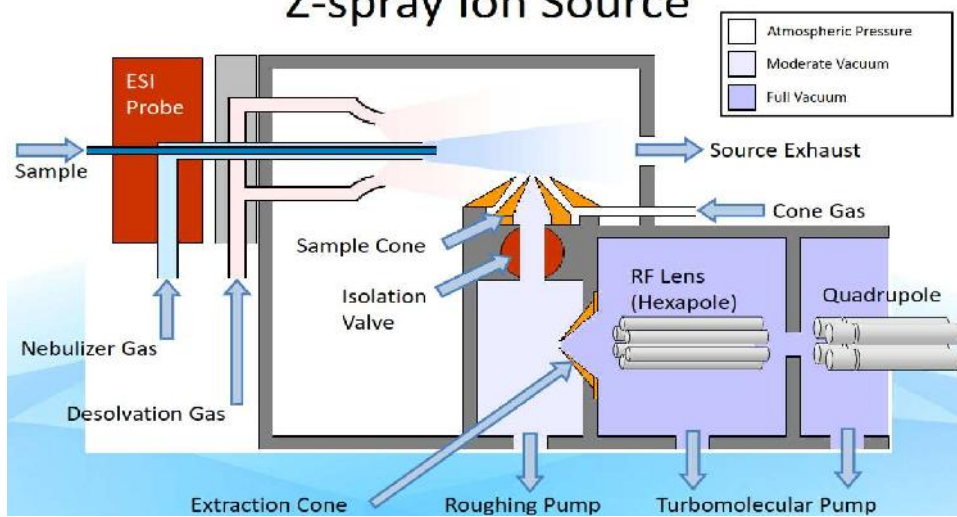
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Z-spray Ion Source



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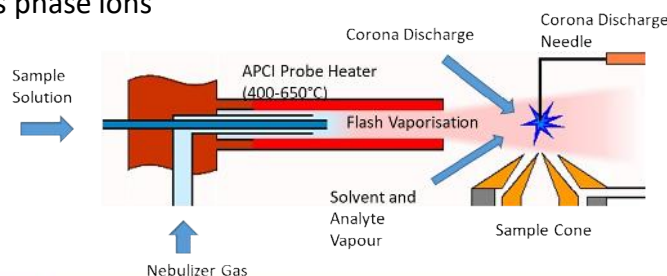
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Atmospheric Pressure Chemical Ionisation (APCI)

- 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 through the corona discharge region to produce gas phase ions



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ESI Electro Spray Ionization uses ESI Probe

ESI +

ESI -

APCI Atmospheric Pressure Chemical Ionization uses APCI Probe

APCI +

APCI -

ESCI Electrospray Chemical Ionization

ESCI +

ESCI -



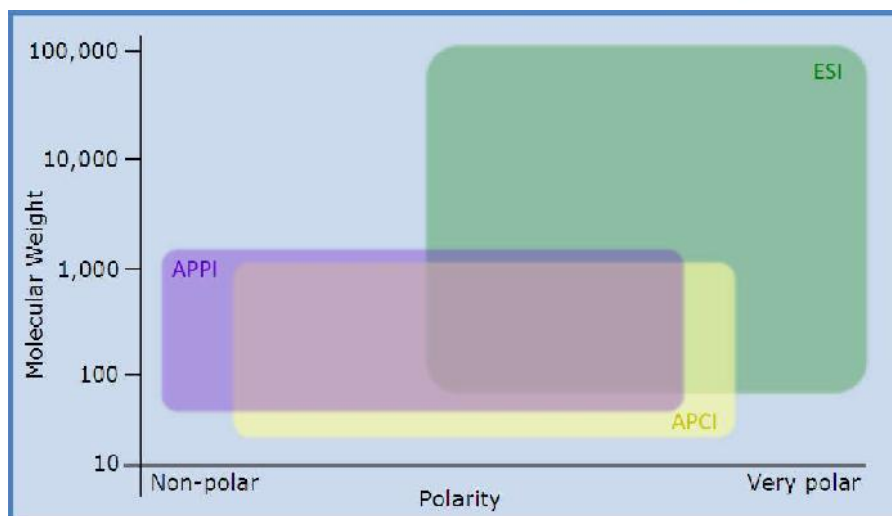
ESI + APCI Using ESI Probe

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- 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 $[M+I]^-$

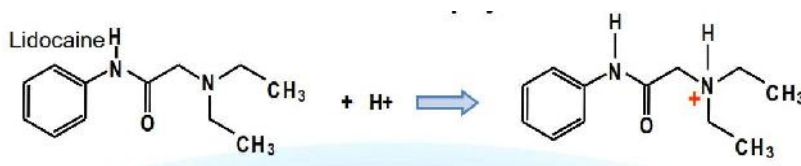
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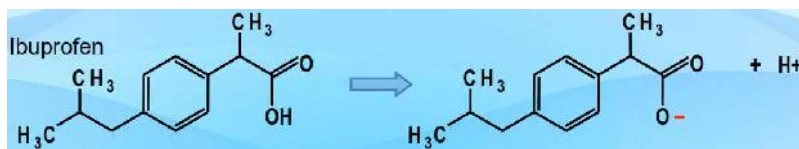


Mechanisms of Ion Formation

Positive Electrospray Ions



Negative ElectroSpray Ions



Mass analyser



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- **Low Resolution**
 - Quadrupole (Mass Filter, Ion Trap)
 - Quadrupole Tandem
- **High Resolution**
 - Time-of-Flight (Linear 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

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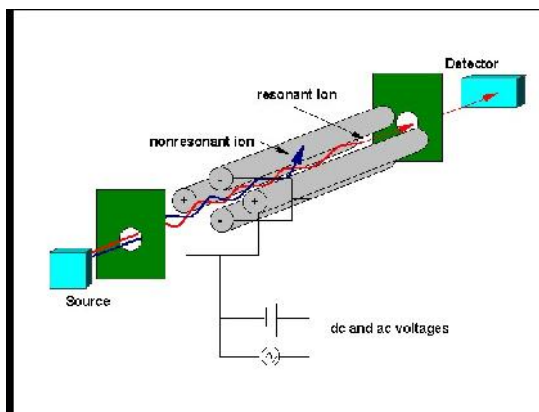
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Quadrupole

- **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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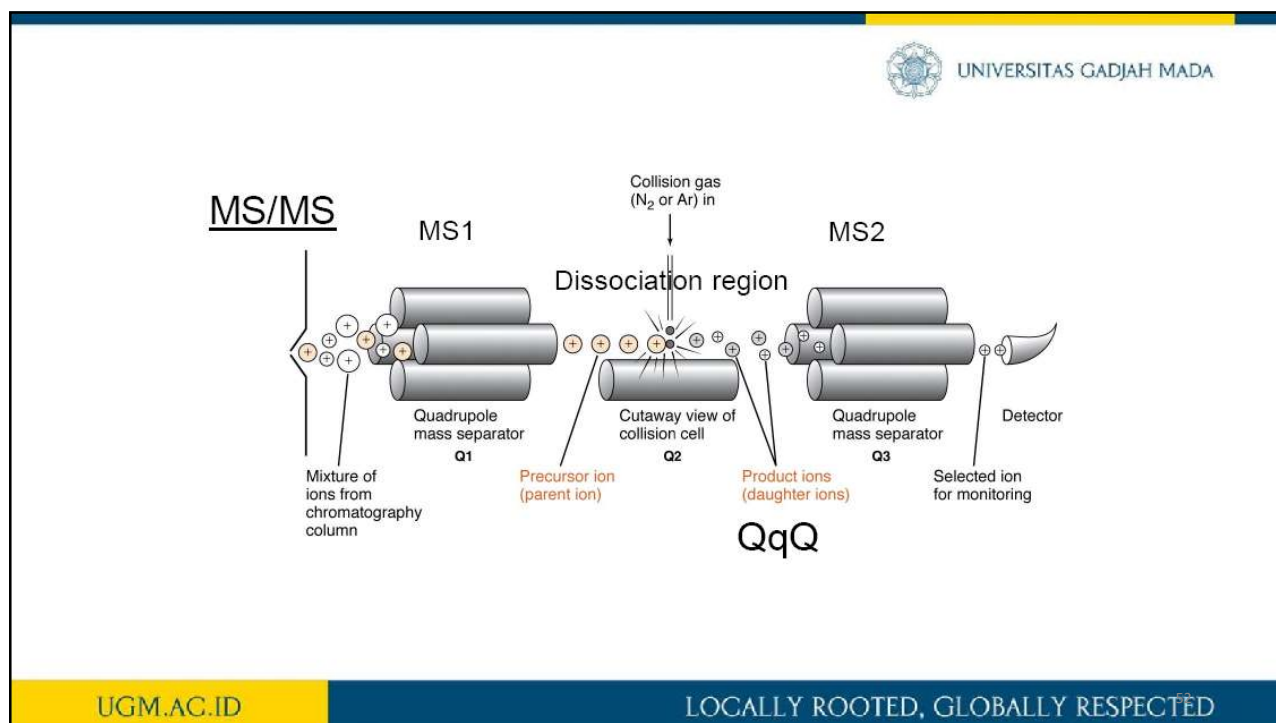
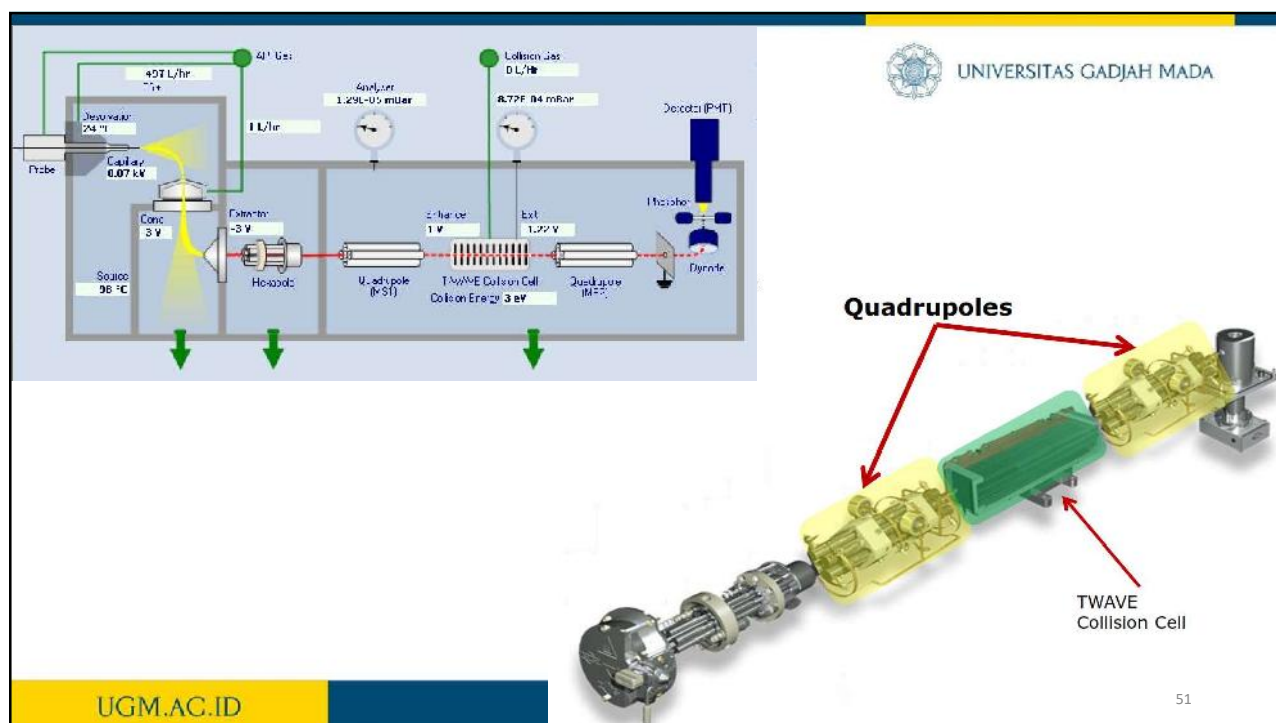
Tandem Quad (MS/MS)

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



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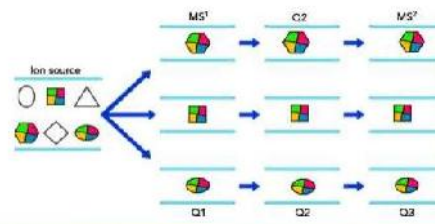


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MS/MS

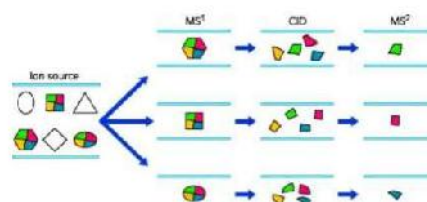
■ When the Collision Cell is **OFF**

- 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

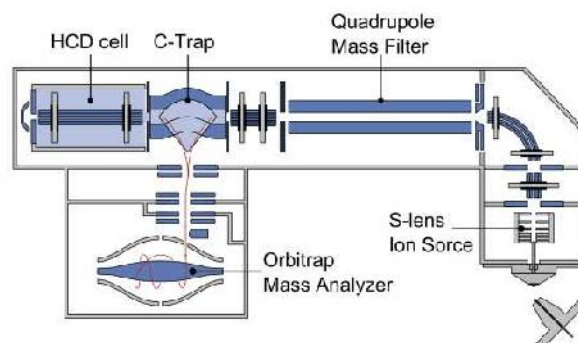
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Orbitrap (High resolution)



- [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)



Confirmation of targeted compound

- *Identification point (IP) : 4 IP*

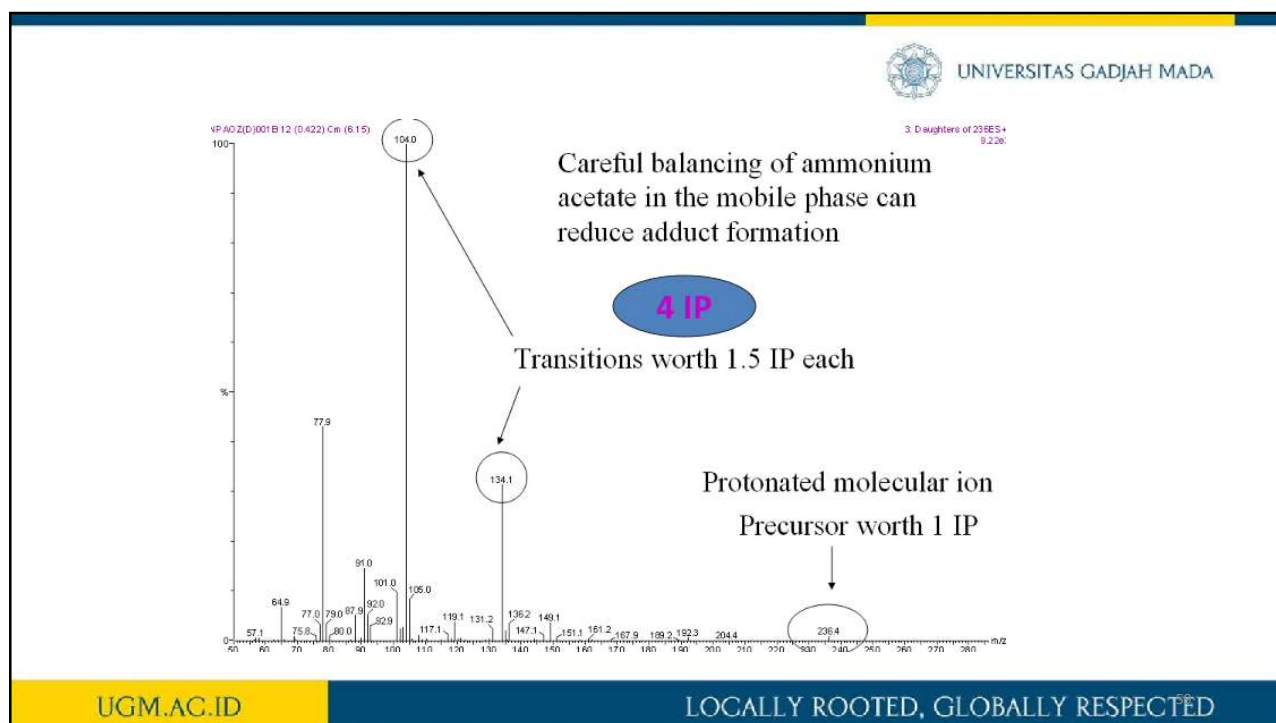


LC-MS/MS

- 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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Mode Analysis TQD

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

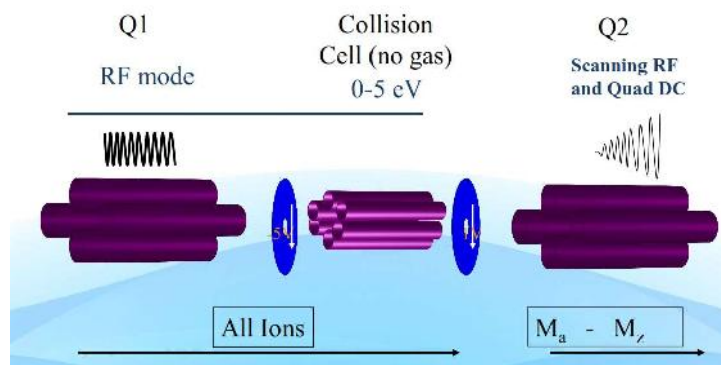
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Scanning (Operating like a single quad)



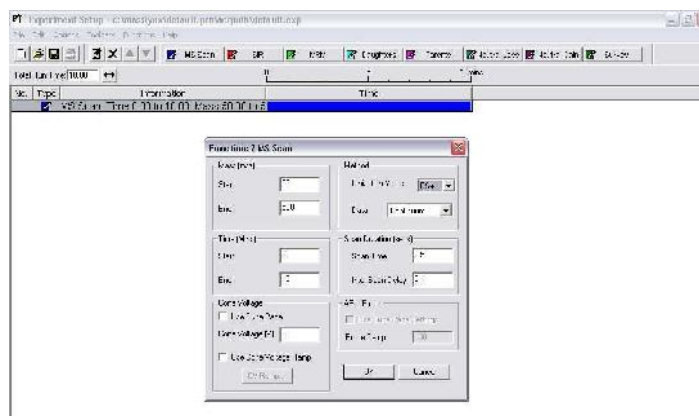
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Setting parameters for MS Scan Mode



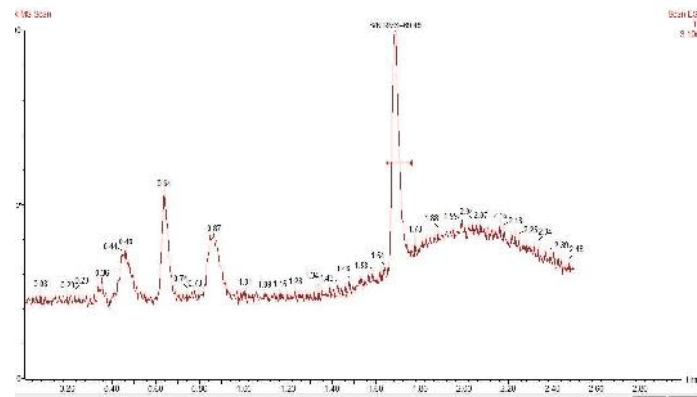
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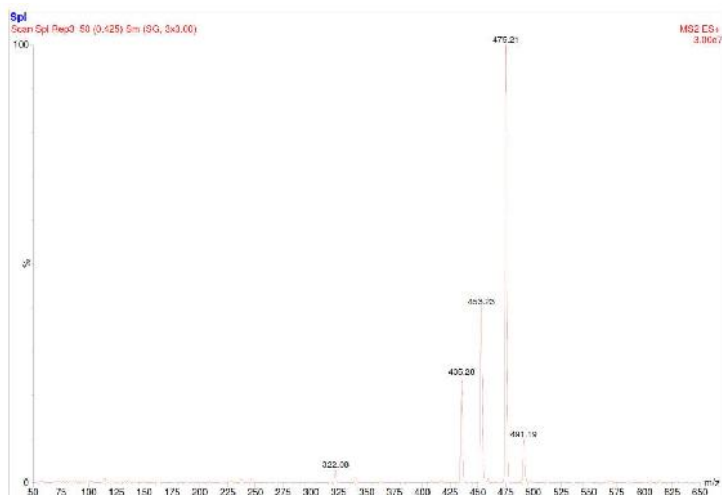
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S to N pada MS Scan Mode





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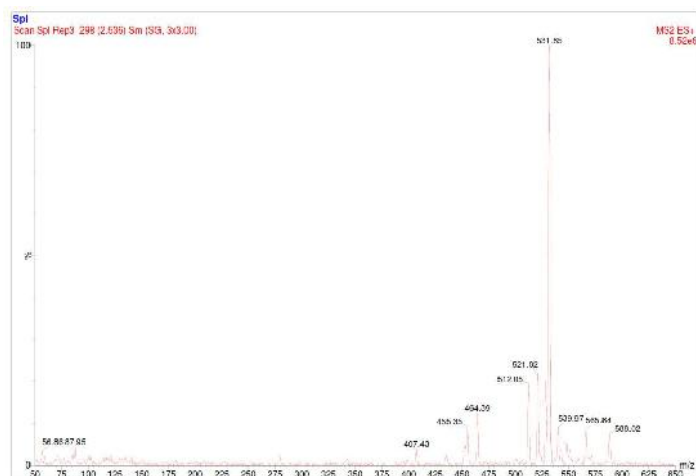


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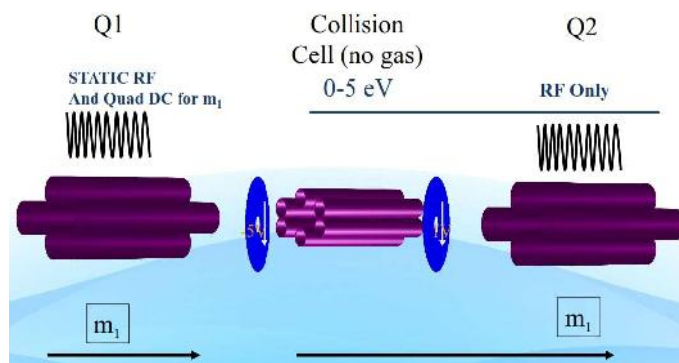
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SIR (Selected Ion Recording) Single Quad



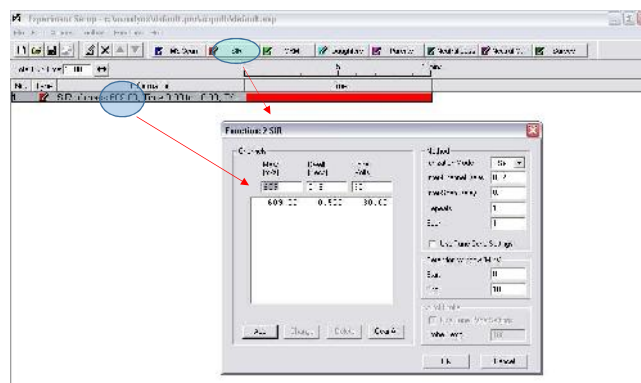
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Setting parameters for SIR Mode



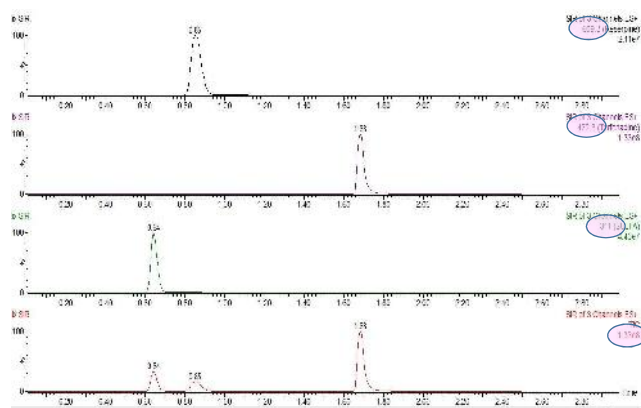
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Contoh Chromatogram SIR Mode



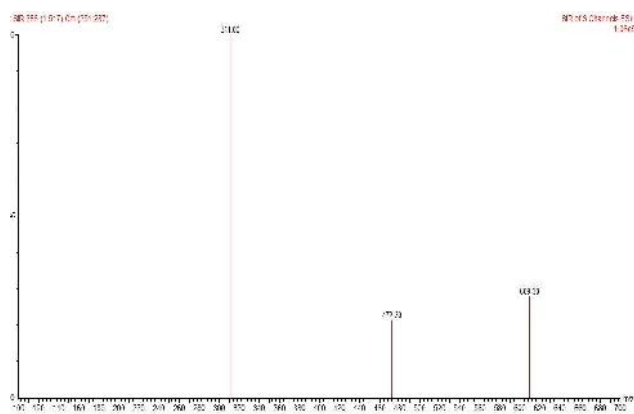
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Contoh Spectrum SIR Mode



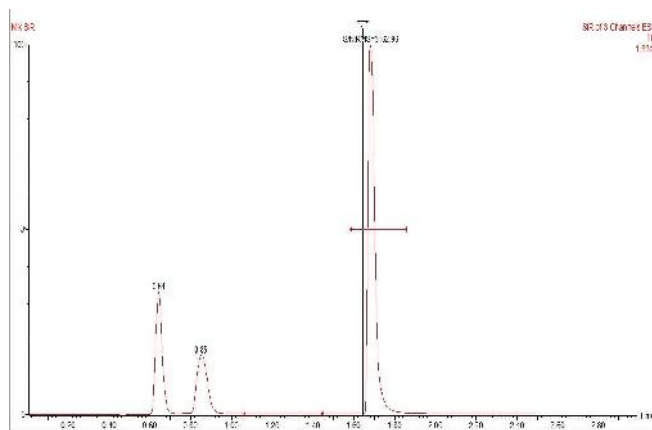
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S to N pada SIR Mode



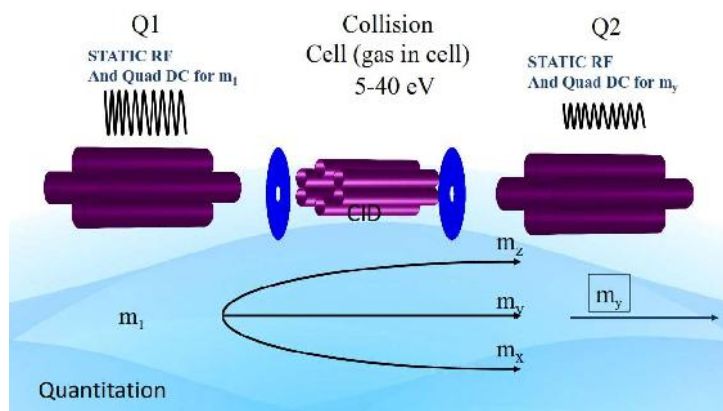
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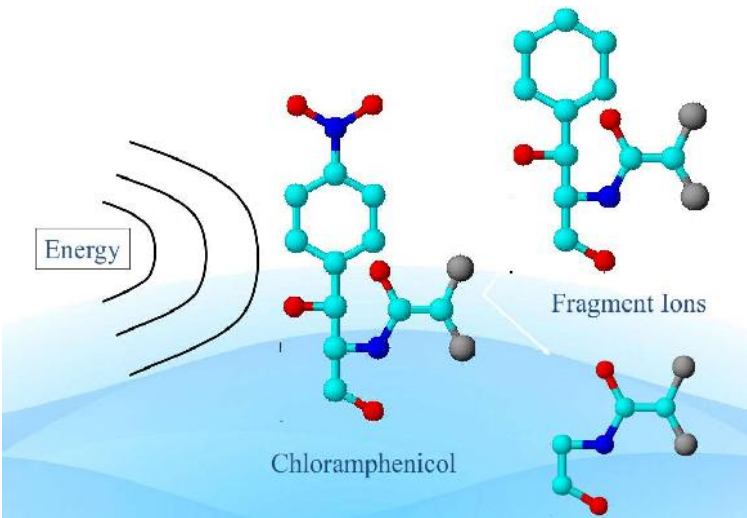
MRM (Multiple Reaction Monitoring)



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Chloramphenicol

Fragment Ions

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Contoh kondisi MRM

Vitamin compound	RT	MRM transition	Cone voltage (V)	Collision energy (eV)
Ascorbic acid (C)	0.40	177 > 141	24	8
Ithiamine (B1)	0.48	765 > 172	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 > 430	41	18
Biotin (B7)	3.15	245 > 227	28	13
Riboflavin (B2)	3.20	377 > 243	42	22

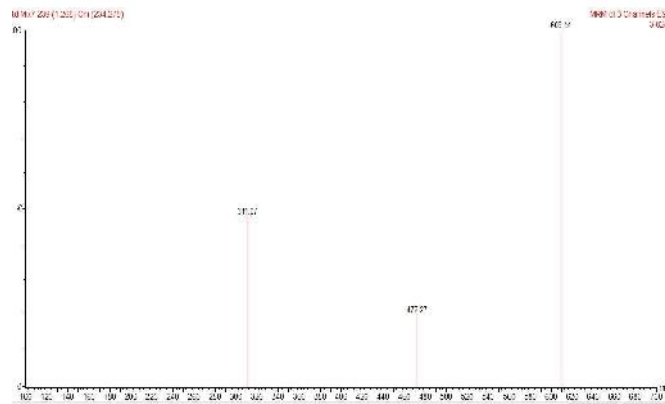
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Contoh Spectrum pada MRM Mode



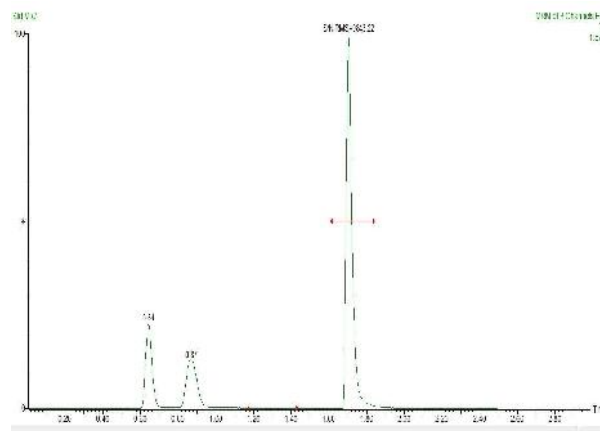
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S to N pada MRM Mode



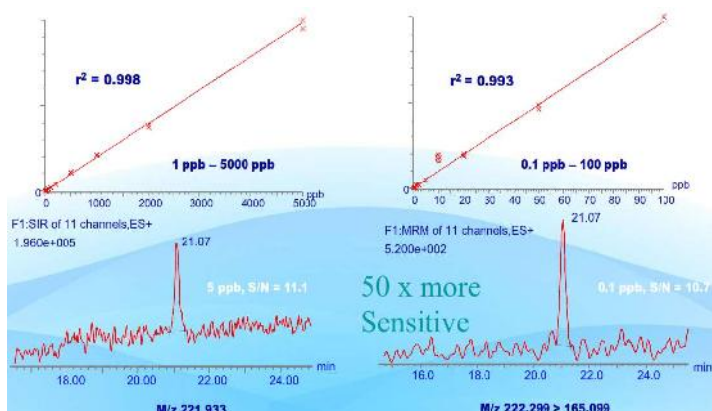
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Sensitivity of SIR vs MRM Carbofuran



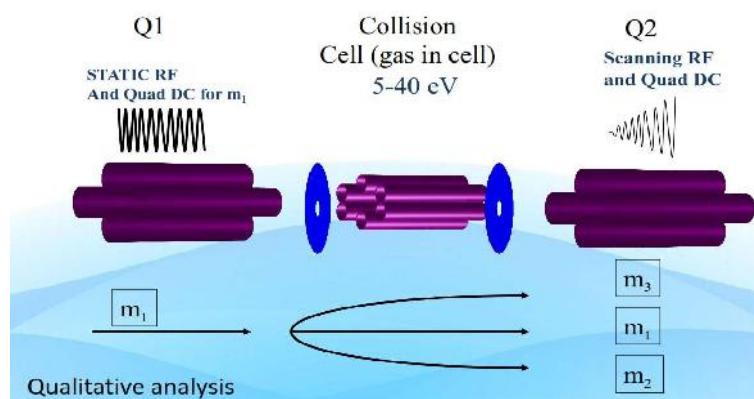
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Product (Daughter) Ion Analysis



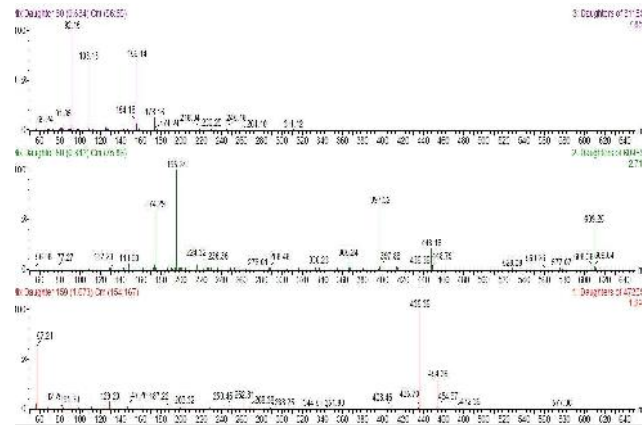
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Contoh Spectrum Daughter Ion Mode



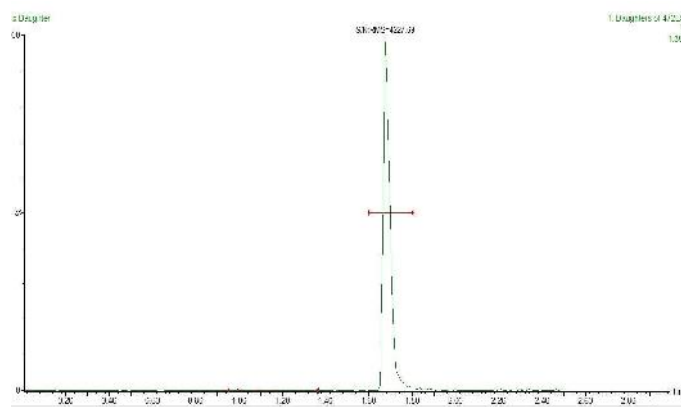
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S to N pada Daughter Ion Mode



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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 MS² (R=17,500) (DDA)
Proteomics
- Targeted MS² (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

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Workflow For Unknown Screening

MS/MS Spectrum → Peak Area Comparison → Statistic Analysis (DIFFERENTIAL ANALYSIS)

MS/MS Spectrum → Predicted Composition (Formula)

Predicted Composition (Formula) → Library Matching (MS/MS) → Putative ID (IDENTIFICATION)

Predicted Composition (Formula) → ChemSpider Data (Mass Matching (MSI)) → Structure Proposal → SPECTRUM ANNOTATION

ChemSpider Data (Mass Matching (MSI)) → Fragmentation search (MS/MS) → SPECTRUM ANNOTATION

Library Matching (MS/MS) → Putative ID (IDENTIFICATION)

Structure Proposal → SPECTRUM ANNOTATION

Fragmentation search (MS/MS) → SPECTRUM ANNOTATION

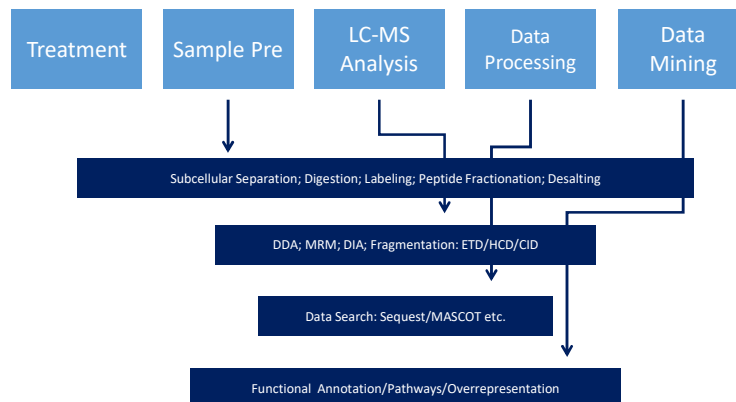
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Workflow of Proteomics



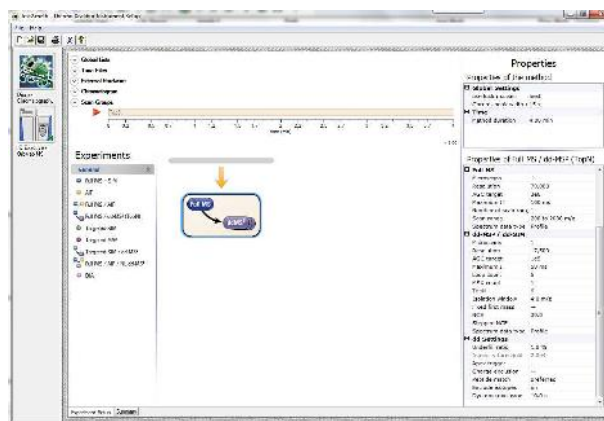
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DDA Method



MS^1 → Top N peaks from MS^1 Selected

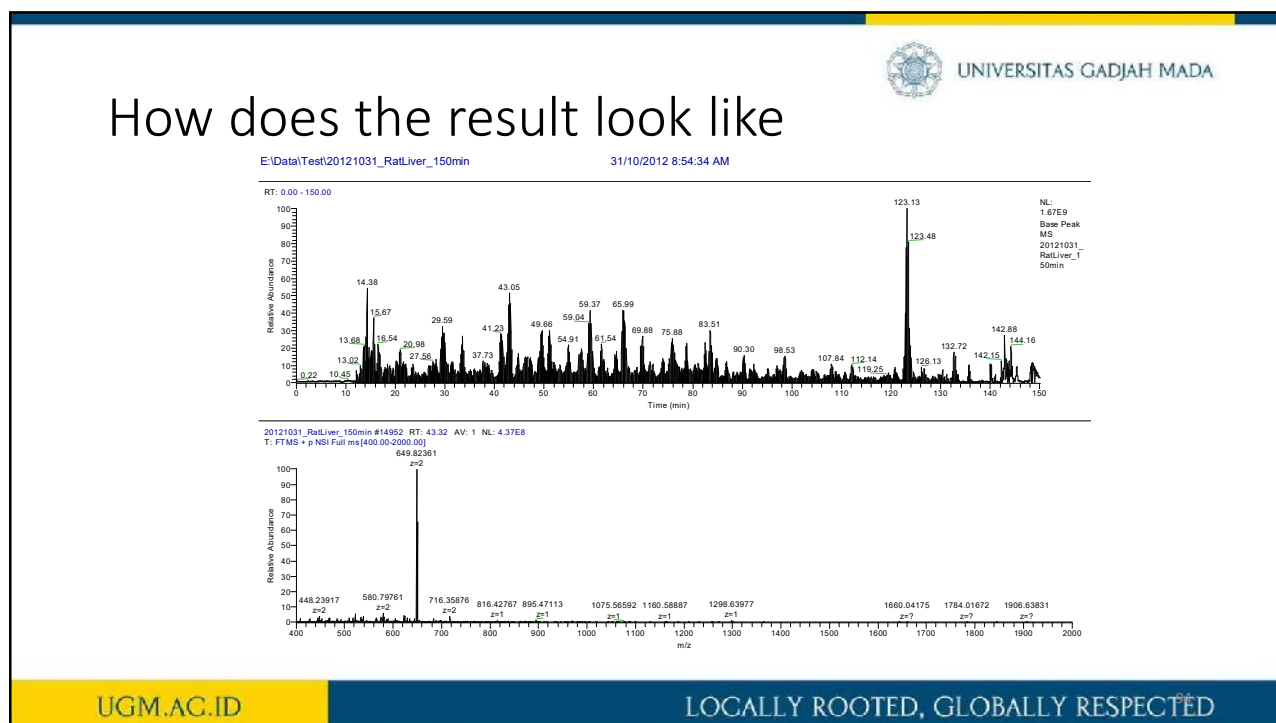
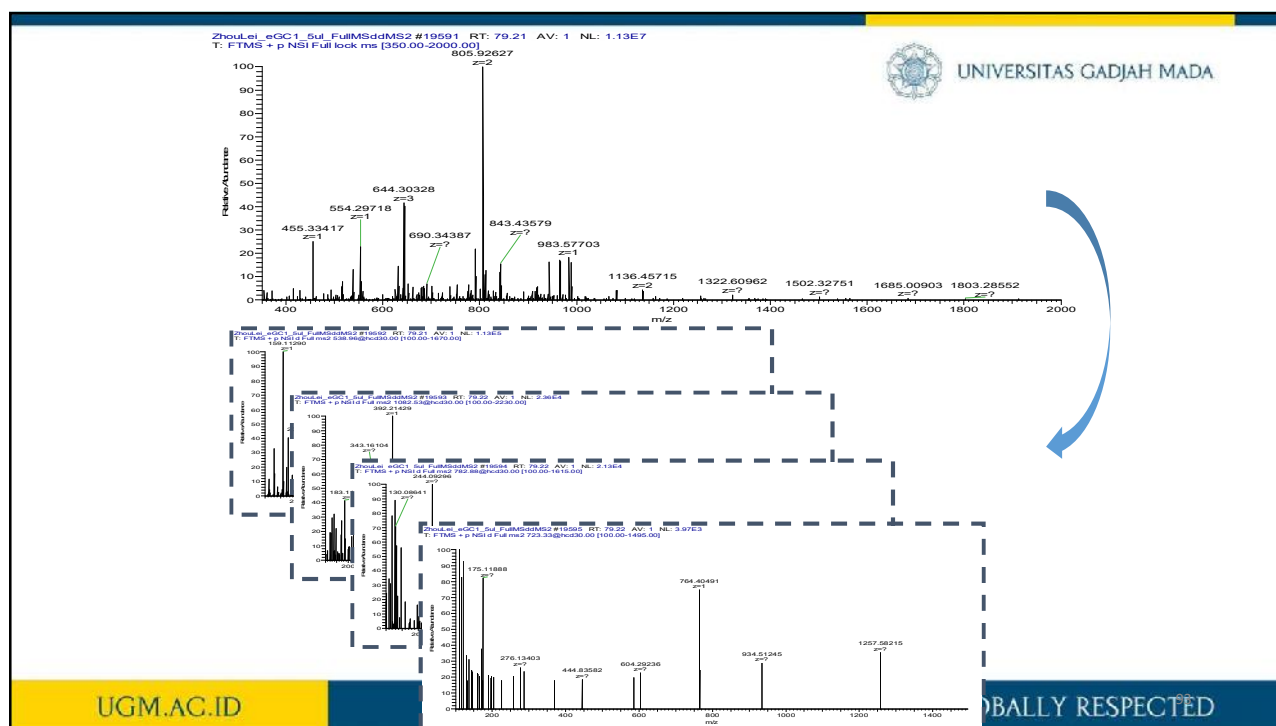
↓
 1st MS^2
 2nd MS^2
 3rd MS^2
 4th MS^2
 ...

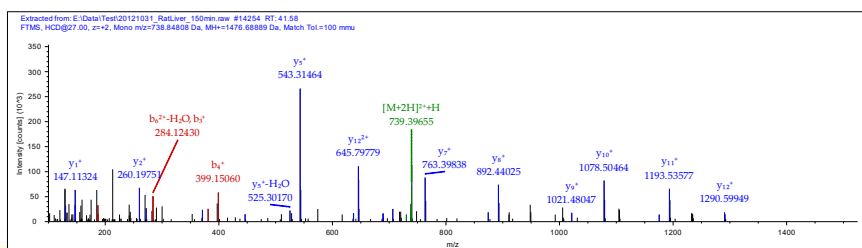
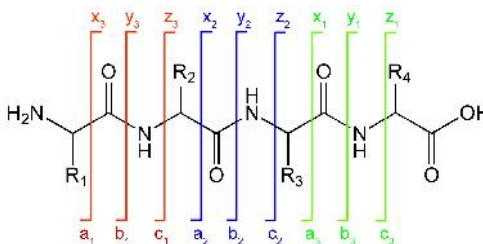
[Acquire MS^2 data depends on the MS^1 intensity

For discovery/non-targeted experiment]

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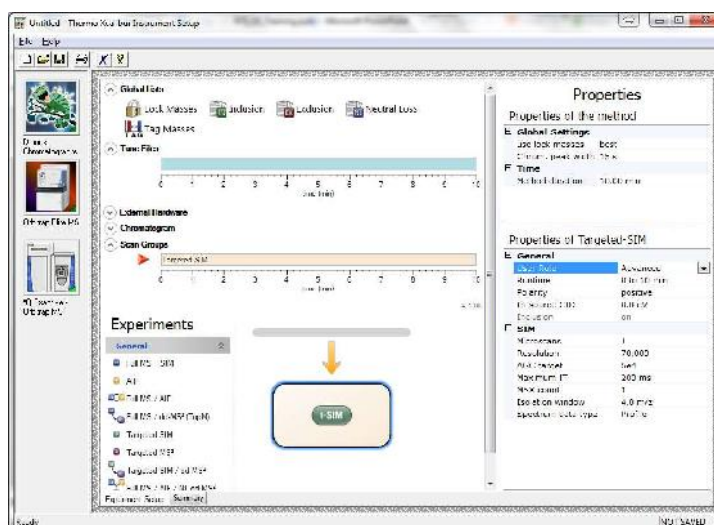


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Targeted SIM



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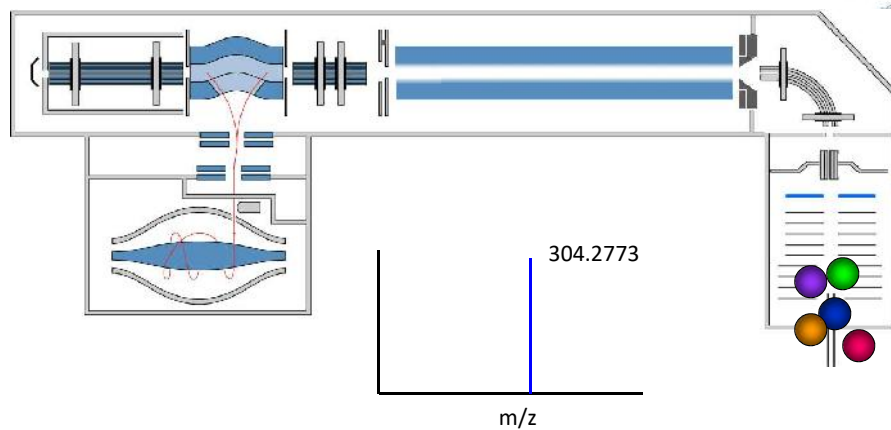
Method editor Inclusion List (modified)

File Edit Help Done ✓

	Mass [m/z]	Formula [M]	Species	CS [s]	Polarity	Start [min]	End [min]	NCE	Comment
1	65.07525	CH ₃ C ₁₀ H ₂₁	+H	1	Positive				
2	155.061	C ₈ H ₂ O	+H	1	Positive				
3					Positive				

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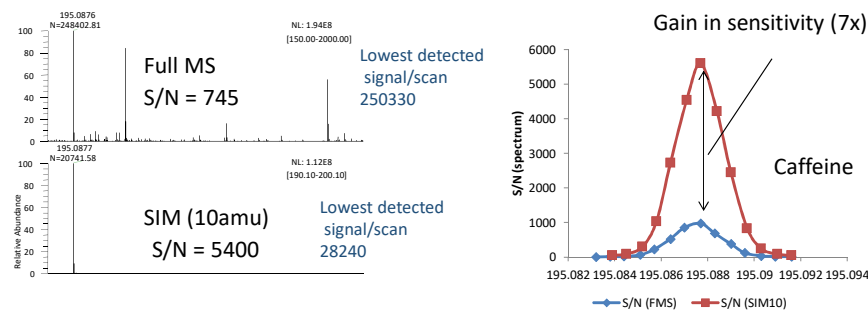
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- By collecting a narrower range of ions, more target ions can be collected within same time



Sensitivity gain 5 – 10 x with SIM mode

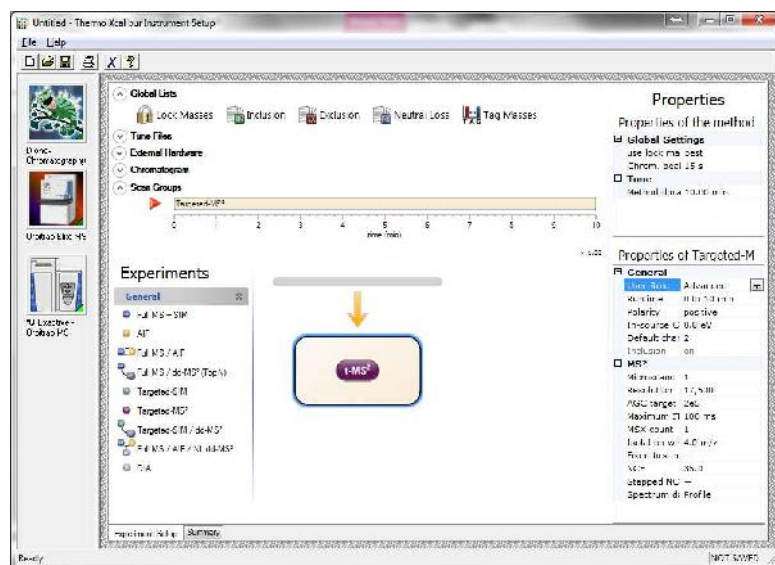
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PRM



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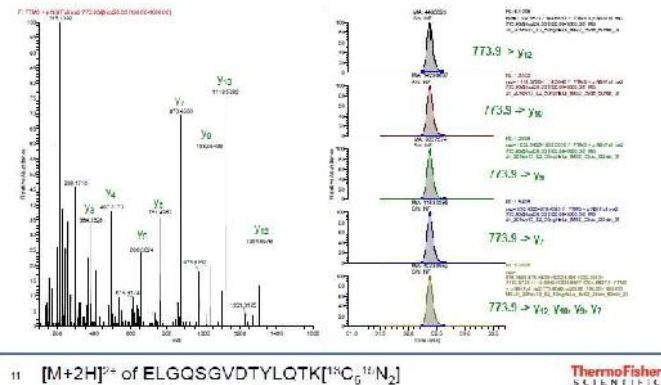
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Quantitation using PRM

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



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