การประชุมเชิงปฏิบัติการ

เรื่อง การใช้เครื่องมือ LC-MS

ณ ห้อง Meeting Room 1 อาคารวิจัย ภาคบรรยาย

ห้อง B2107 อาคารเครื่องมือ 2 (F2) ภาคปฏิบัติ

วันที่ 4-5-6 สิงหาคม 2546

จัดโดย สาขาวิชาเคมี สำนักวิชาวิทยาศาสตร์

มหาวิทยาลัยเทคโนโลยีสุรนารี

กำหนดการประชุมเชิงปฏิบัติการ

เรื่อง "การใช้เครื่องมือ LC – MS"

ในระหว่างวันที่ 4 -5- 6 สิงหาคม 2546

สถานที่ ห้อง Meeting Room 1 อาคารวิจัย ภาคบรรยาย
ห้อง B2107 อาคารเครื่องมือ 2 (F2) ภาคปฏิบัติ
สาขาวิชาเคมี สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี

เวลา 3 วัน

วันจันทร์ที่ 4 สิงหาคม 2546

08.20 – 08.50 น.	ลงทะเบียน
08.50 – 09.00 น.	พิธีเปิดการประชุม โดย ผู้อำนวยการศูนย์เครื่องมือวิทยาศาสตร์และเทคโนโลยี ผศ. ดร.สุเทพ อุสาหะ
09.00 – 10.30 น.	บรรยายเรื่อง Basics of LC – MS วิทยากรโดย ผศ. ดร.สันติ ศักดารัตน์
10.30 – 10.45 น.	พักรับประทานอาหารว่าง
10.45 – 12.15 น.	บรรยายเรื่อง MS for Analysis and Identification of Organic Compounds วิทยากรโดย ผศ. ดร.สันติ ศักดารัตน์
12.15 – 13.15 น.	พักรับประทานอาหารกลางวัน
13.15 – 15.15 น.	บรรยายเรื่อง Application of LC – MS in medicinal , food and environmental analysis (วิทยากรโดย ผศ.ดร.สันติ ศักดารัตน์)
15.15 – 15.30 น.	พักรับประทานอาหารว่าง
15.30 – 17.30 น.	LC – MS เครื่องมือและขั้นตอนการใช้เครื่องมือ การเตรียมตัวอย่าง การวิเคราะห์

วันอังคารที่ 5 สิงหาคม 2546

08.30 – 10.-15 น.	บรรยายเรื่อง Mass spectrometry for Biomacromolecules (วิทยากรโดย PD. Dr. Heino PRINZ)
10.00 – 10.15 น.	พักรับประทานอาหารว่าง
10.15 – 12.15 น.	บรรยายเรื่อง Mass spectrometry for Proteomics Application (วิทยากรโดย PD. Dr. Heino PRINZ)

12.15 – 13.15 น.	พักรับประทานอาหารกลางวัน
13.15 – 15.15 น.	การเตรียมตัวอย่างเพปไทด์ (วิทยากรโดย PD. Dr. Heino PRINZ และ ดร.วิภา สุจินต์)
15.15 – 15.30 น.	พักรับประทานอาหารว่าง
15.30 – 17.30 น.	การเตรียมตัวอย่างเพปไทด์ (วิทยากรโดย PD. Dr. Heino PRINZ และ ดร.วิภา สุจินต์)

วันพุธที่ 6 สิงหาคม 2546

08.30 – 10.00 น.	การเตรียมตัวอย่างเพปไทด์ (ต่อ) (วิทยากรโดย PD. Dr. Heino PRINZ และ ดร.วิภา สุจินต์)
10.00 – 10.15 น.	พักรับประทานอาหารว่าง
10.15 – 12.15 น.	การวิเคราะห์หามวลโมเลกุลของโปรตีน (วิทยากรโดย PD. Dr. Heino PRINZ และ ดร. วิภา สุจินต์)
12.15 – 13.15 น.	พักรับประทานอาหารกลางวัน
13.15 – 15.15 น.	การแยกและวิเคราะห์เพปไทด์ (วิทยากรโดย PD. Dr. Heino PRINZ และ ดร.วิภา สุจินต์)
15.15 – 15.30 น.	พักรับประทานอาหารว่าง
15.30 – 17.30 น.	การแยกและวิเคราะห์เพปไทด์และการทำ peptide mass fingerprint (วิทยากรโดย PD. Dr. Heino PRINZ และ ดร.วิภา สุจินต์)

เอกสารประกอบการประชุมเชิงปฏิบัติการ

เรื่อง

"การใช้เครื่องมือ LC-MS"

1 Basics of LC - MS

2 MS for Analysis And Identification of Organic Compounds

3 Application of LC-MS

โดย ผศ.ดร.สันติ ศักดารัตน์

สาขาวิชาเคมี สำนักวิชาวิทยาศาสตร์

มหาวิทยาลัยเทคโนโลยีสุรนารี

LC – MS

Lecture

ผศ.ดร.สันติ ศักดารัตน์

Why Liquid Chromatography / Mass Spectrometry?

Liquid chromatography is a fundamental separation technique in the life sciences and related fields of chemistry. Unlike gas chromatography, which is unsuitable for nonvolatile and thermally fragile molecules, liquid chromatography can safely separate a very wide range of organic compounds, from small-molecule drug metabolites to peptides and proteins.

Mass spectrometers also generate three dimensional data. In addition to retention time and wavelength, they generate mass spectral data that can provide valuable information about the molecular weight, structure, identity, quantity, and purity of a sample. Mass spectral data add specificity that increases confidence in the results of both qualitative and quantitative analyses.

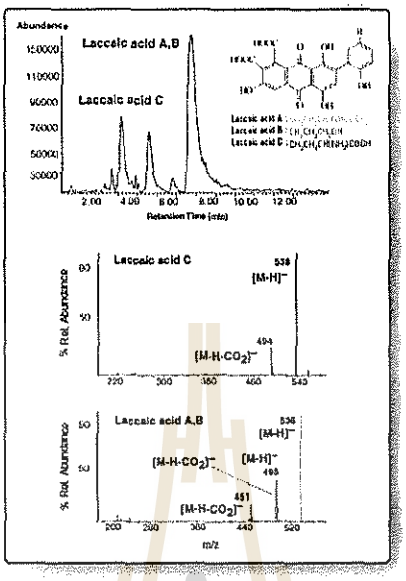
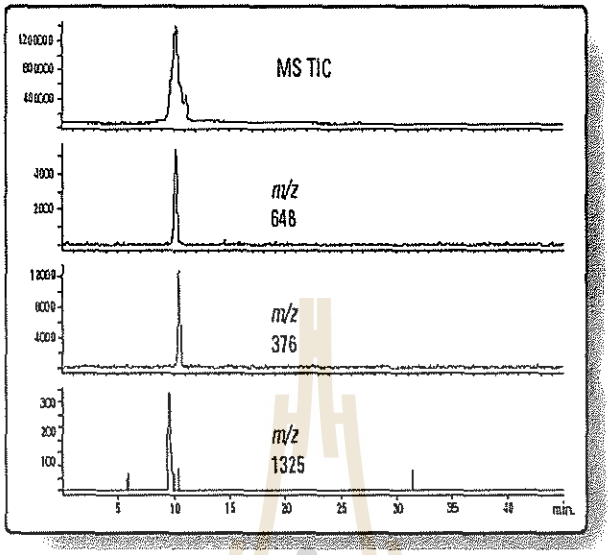


Figure 1. Two-dimensional abundance data and three-dimensional mass spectral data from a mass spectrometer

For most compounds, a mass spectrometer is more sensitive and far more specific than all other LC detectors. It can analyze compounds that lack a suitable chromophore. It can also identify components in unresolved chromatographic peaks, reducing the need for perfect chromatography.



Instrumentation

Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (*m/z*) ratios. Two key components in this process are the ion source, which generates the ions, and the mass analyzer, which sorts the ions.

Ion Sources

Earlier LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet, thermospray) or did so before ionization (particle beam). The analyte molecules were then ionized in the mass spectrometer under vacuum, often by traditional electron ionization. These approaches were successful only for a very limited number of compounds.

The introduction of atmospheric pressure ionization (API) techniques greatly expanded the number of compounds that can be successfully analyzed by LC/MS. In atmospheric pressure ionization, the analyte molecules are ionized first, at atmospheric pressure. The analyte ions are then mechanically and electrostatically separated from neutral molecules. Common atmospheric pressure ionization techniques are:

- Electrospray ionization (ESI)
- Atmospheric pressure chemical ionization (APCI)
- Atmospheric pressure photoionization (APPI)

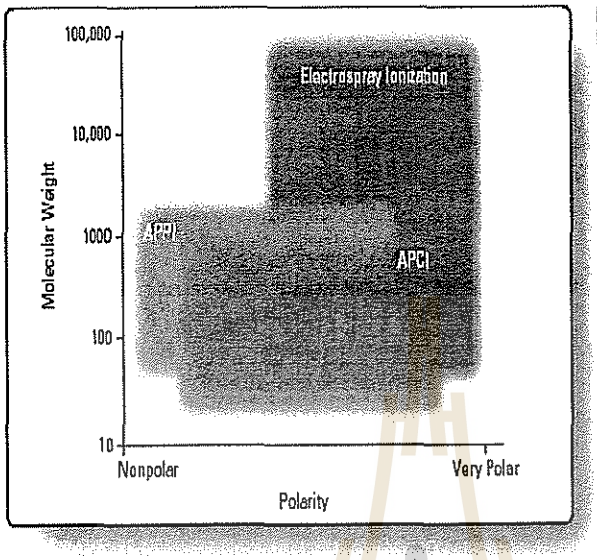
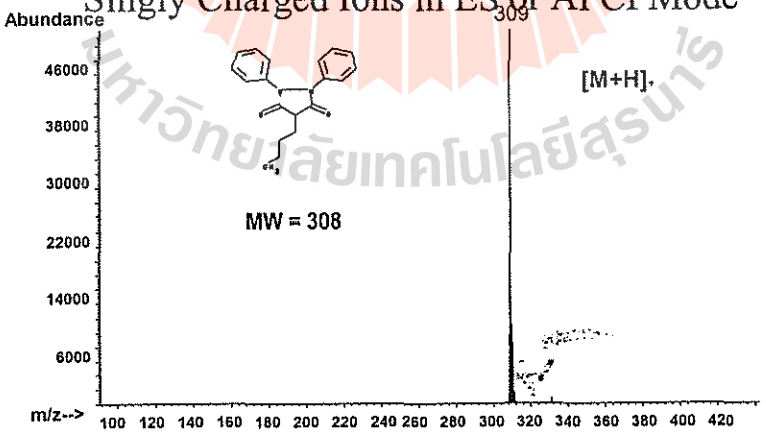


Figure 3. Applications of various LC/MS ionization techniques

What Kind of Data Do You Obtain?

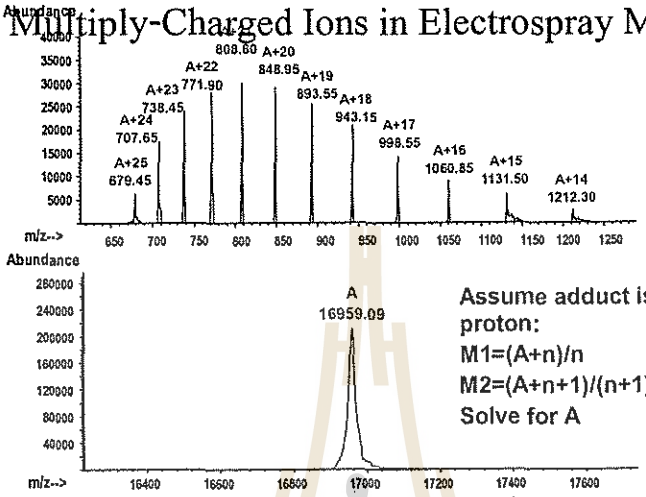
Singly Charged Ions in ES or APCI Mode



API-ES spectrum of Phenylbutazone

What Kind of Data Do You Obtain?

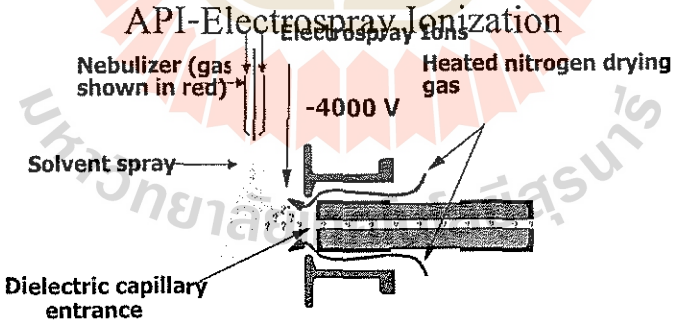
Multiply-Charged Ions in Electrospray Mode

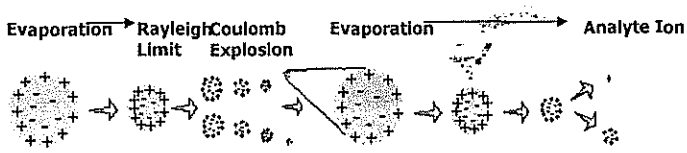


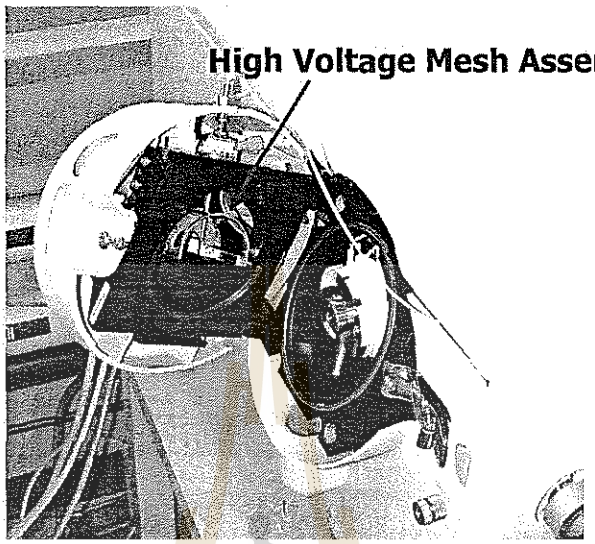
Assume adduct is a proton:
M1=(A+n)/n
M2=(A+n+1)/(n+1)
Solve for A

API-ES spectrum of Myoglobin

API-Electrospray Ionization







Electrospray ionization

Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas.

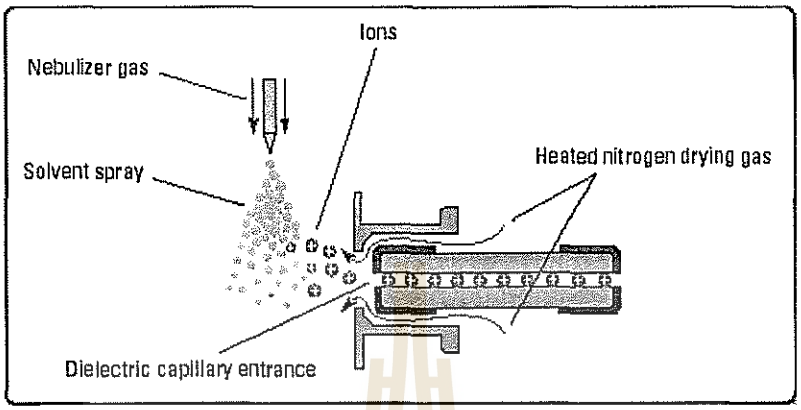


Figure 4. Electrospray ion source

Electrospray is especially useful for analyzing large biomolecules such as proteins, peptides, and oligonucleotides, but can also analyze smaller molecules like benzodiazepines and sulfated conjugates.

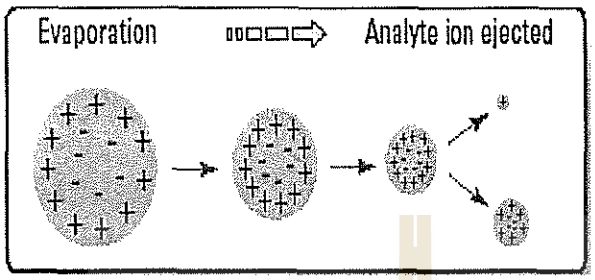


Figure 5. Desorption of ions from solution

Atmospheric pressure chemical ionization

In APCI, the LC eluent is sprayed through a heated (typically 250°C – 400°C) vaporizer at atmospheric pressure. The heat vaporizes the liquid. The resulting gas-phase solvent molecules are ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical reactions (chemical ionization). The analyte ions pass through a capillary sampling orifice into the mass analyzer.

APCI is applicable to a wide range of polar and nonpolar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500 u.

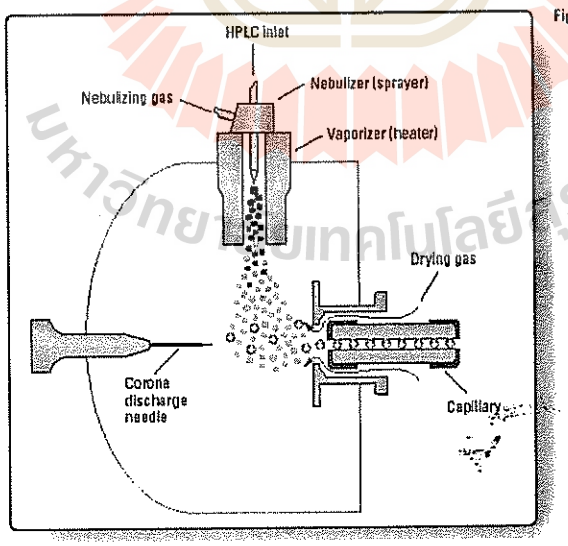


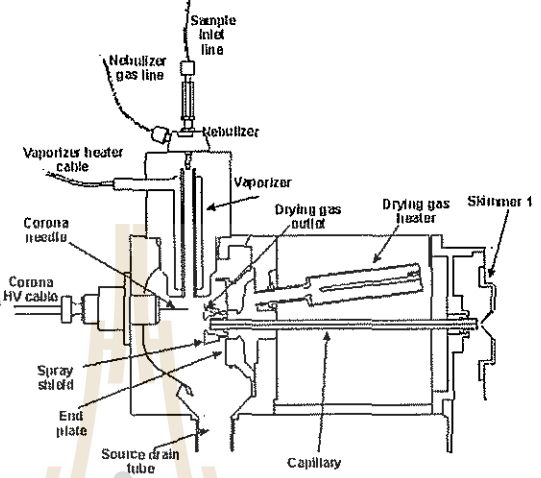
Figure 6. APCI ion source

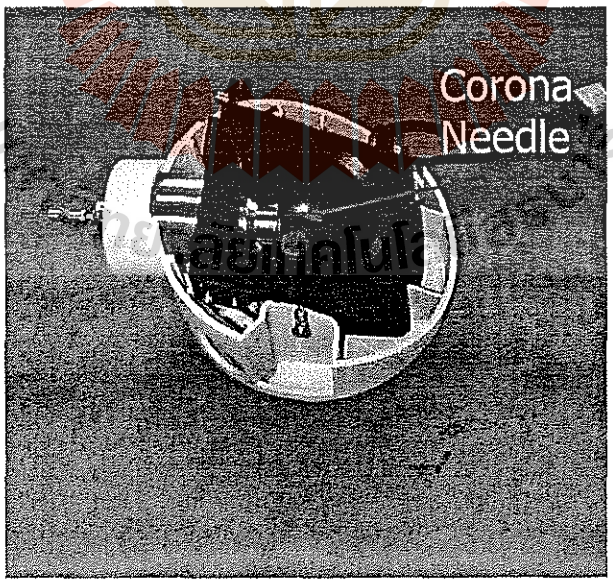
APCI Ionization Process

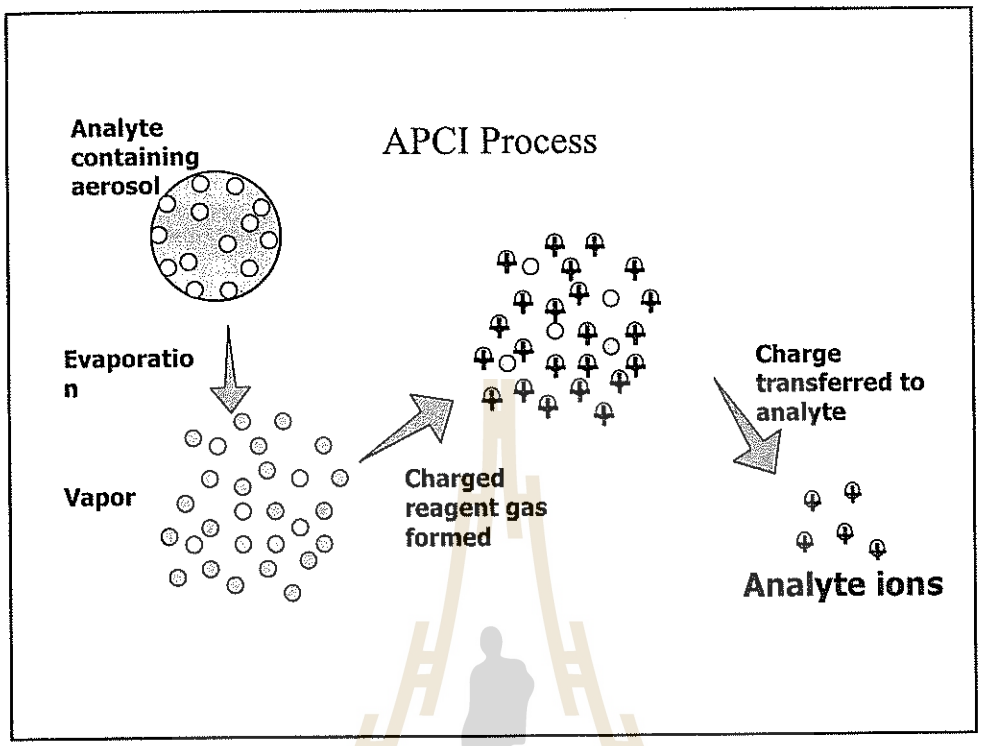
?Nebulize
?Evaporate Droplets
?Gas-phase ionization

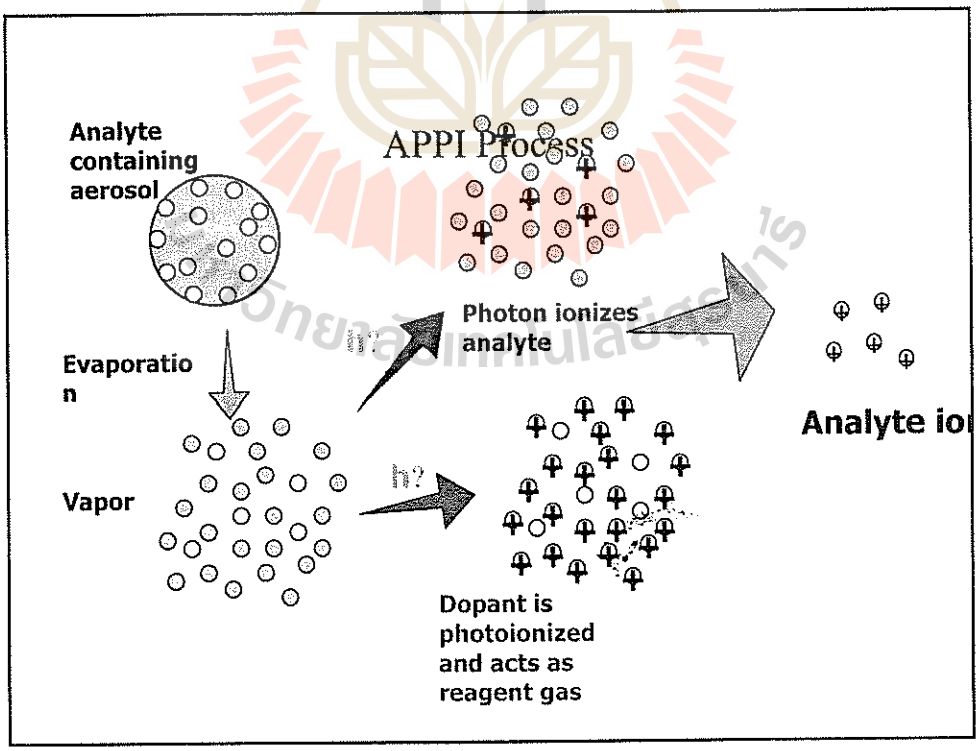
$[Solvent + H]^+ + A$

$Solvent + [A + H]^+$






APCI Process
Analyte containing aerosol
Evaporation
Vapor
Charged reagent gas formed
Charge transferred to analyte
Analyte ions


APPI Process
Analyte containing aerosol
Evaporation
Vapor
h?
Photon ionizes analyte
h?
Dopant is photoionized and acts as reagent gas
Analyte io

Atmospheric pressure photoionization

Atmospheric pressure photoionization (APPI) for LC/MS is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer.

APPI is applicable to many of the same compounds that are typically analyzed by APCI. It shows particular promise in two applications, highly nonpolar compounds and low flow rates (< 100 µl/min), where APCI sensitivity is sometimes reduced.

APPI Mechanisms

- Direct APPI
  - M + hν → $M^{\bullet+}$ + e⁻ — **Analyte molecule M is ionized to molecular ion $M^{\bullet+}$** (If analyte ionization potential is below photon energy)
  - $M^{\bullet+}$ + $SH^{\bullet}$ → $[M+H]^+$ + $S^{\bullet}$ — **Molecular ion $M^{\bullet+}$ may abstract a hydrogen to form $[M+H]^+$**

- Dopant APPI
  - D + hν → $D^{\bullet+}$ + e⁻ — **Photoionizable dopant D is in excess & yields many $D^{\bullet+}$ ions**
  - $D^{\bullet+}$ + MH → $[M+H]^+$ + D — **Analyte M ionizes by proton transfer from dopant or solvent**
  - $D^{\bullet+}$ + M → $M^{\bullet+}$ + D — **$D^{\bullet+}$ ionizes analyte M by electron transfer**

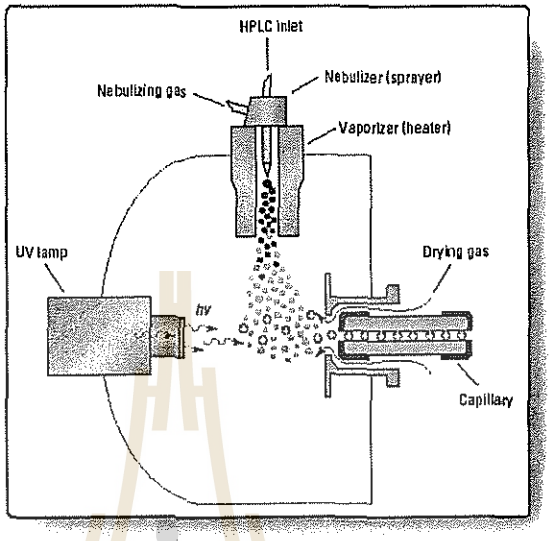
Energetics for Photoionization.

- PhotoMate® lamp
  - Krypton 10.0 eV, 10.6 eV
- Ionization Potentials (IP)
  - Anthracene 7.4 eV
  - Fluoranthene 7.8 eV
  - Caffeine 8.0 eV
  - 4-Nitrotoluene 9.5 eV
  - 2,4,6-Trinitrotoluene 10.59 eV

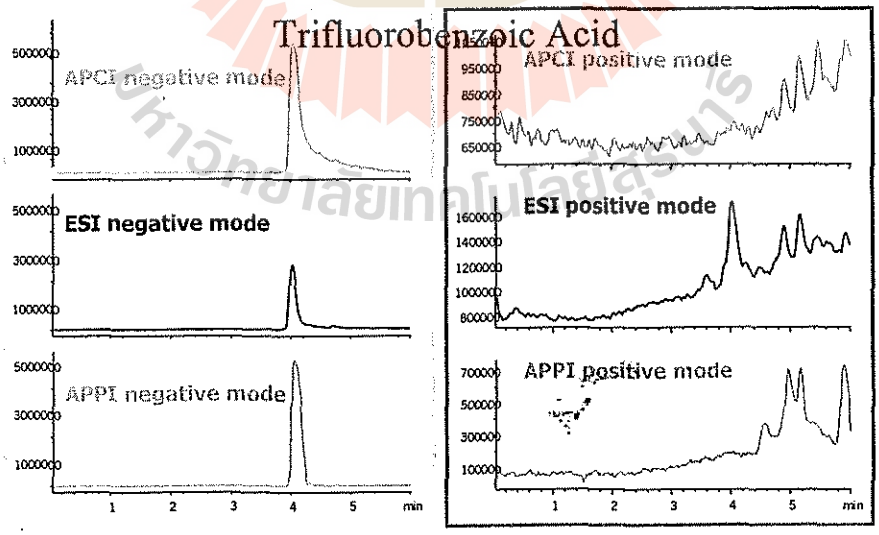
Solvent Ionization Potentials (IP)

- Toluene 8.82 eV
- Acetone 9.70 eV
- Methanol 10.85 eV
- Acetonitrile 12.19 eV
- Water 12.61 eV

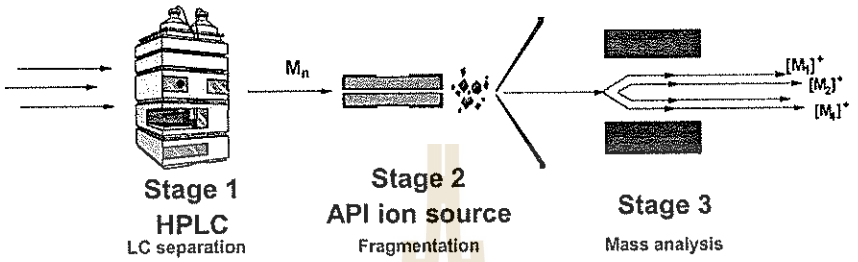
Figure 7. APPI ion source



Comparison of API Techniques for Analysis of Trifluorobenzoic Acid

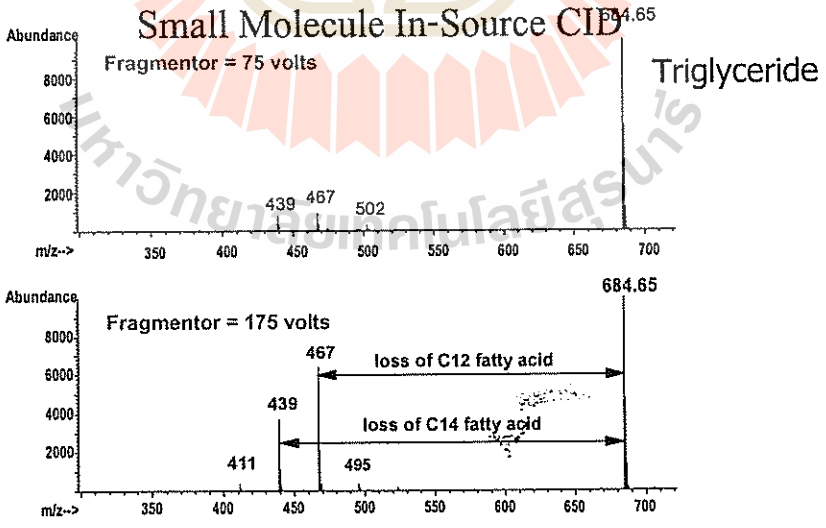


LC/CID/MS



Small Molecule In-Source CID

Triglyceride



Mass Analyzers

Although in theory any type of mass analyzer could be used for LC/MS, four types:

- Quadrupole
- Time-of-flight
- Ion trap
- Fourier transform-ion cyclotron resonance

(FT-ICR or FT-MS)

are used most often. Each has advantages and disadvantages depending on the requirements of a particular analysis.

Quadrupole

A quadrupole mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time. Quadrupoles tend to be the simplest and least expensive mass analyzers.

Quadrupole mass analyzers can operate in two modes:

- Scanning (scan) mode
- Selected ion monitoring (SIM) mode

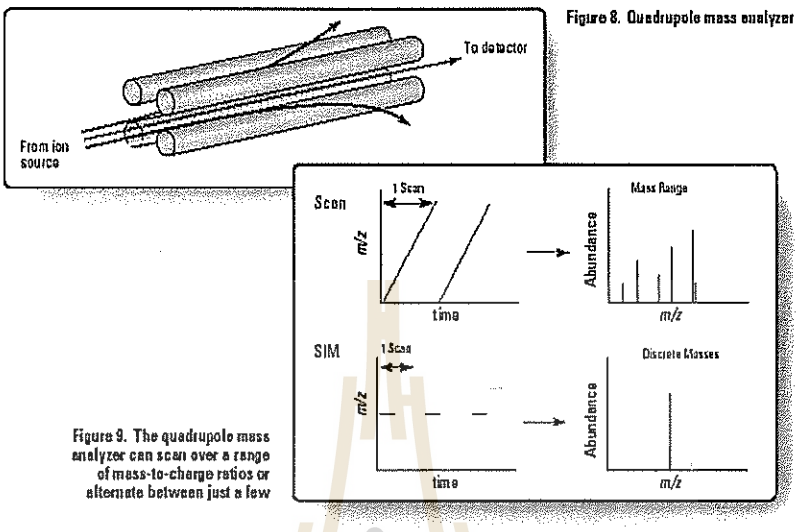


Figure 8. Quadrupole mass analyzer

Figure 9. The quadrupole mass analyzer can scan over a range of mass-to-charge ratios or alternate between just a few

Time-of-flight

In a time-of-flight (TOF) mass analyzer, a uniform electromagnetic force is applied to all ions at the same time, causing them to accelerate down a flight tube. Lighter ions travel faster and arrive at the detector first, so the mass-to-charge ratios of the ions are determined by their arrival times. Time-offlight mass analyzers have a wide mass range and can be very accurate in their mass measurements.

Ion trap

An ion trap mass analyzer consists of a circular ring electrode plus two end caps that together form a chamber. Ions entering the chamber are "trapped" there by electromagnetic fields. Another field can be applied to selectively eject ions from the trap. Ion traps have the advantage of being able to perform multiple stages of mass spectrometry without additional mass analyzers.

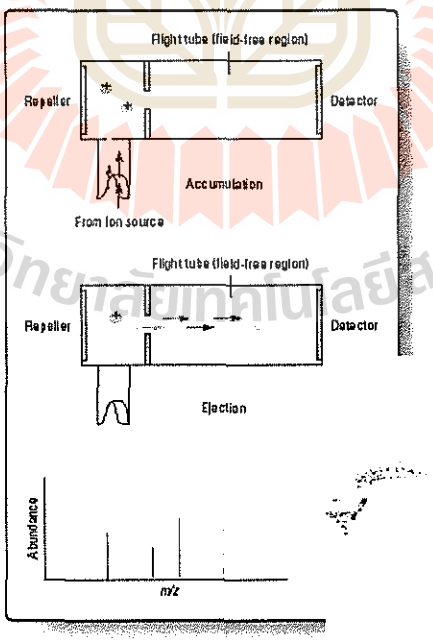


Figure 10. Time-of-flight mass analyzer

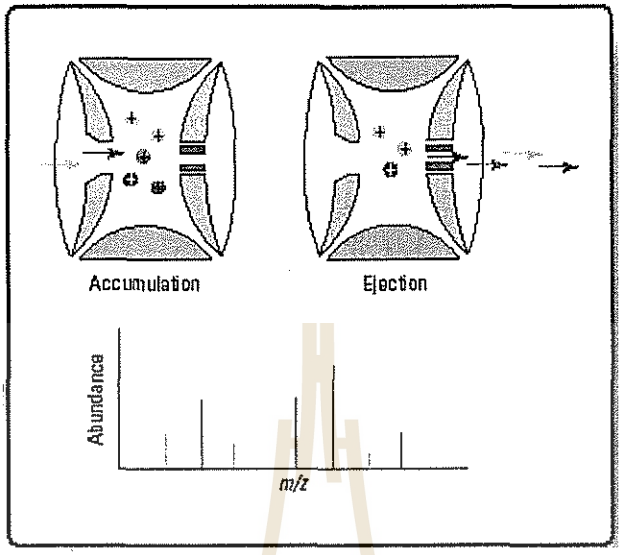


Figure 11. Ion trap mass analyzer

Fourier transform-ion cyclotron resonance (FT-ICR)

An FT-ICR mass analyzer (also called FT-MS) is another type of trapping analyzer. Ions entering a chamber are trapped in circular orbits by powerful electrical and magnetic fields. When excited by a radio-frequency (RF) electrical field, the ions generate a time dependent current. This current is converted by Fourier transform into orbital frequencies of the ions which correspond to their mass to charge ratios.

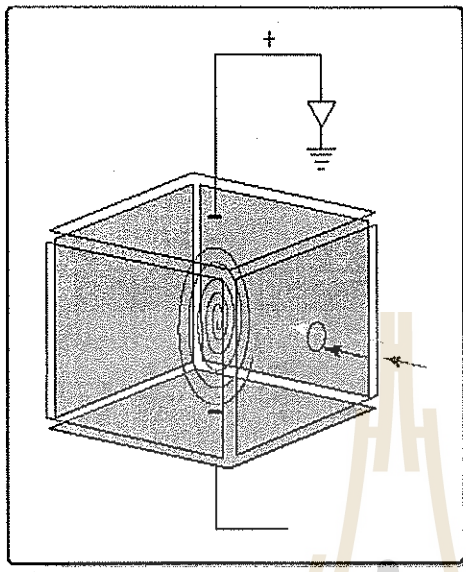


Figure 12. FT-ICR mass analyzer

Collision-Induced Dissociation and Multiple-Stage MS

The atmospheric pressure ionization techniques discussed are all relatively "soft" techniques. They generate primarily:

- Molecular ions M+ or M–
- Protonated molecules [M + H]+
- Simple adduct ions [M + Na]+
- Ions representing simple losses such as the loss of a water [M + H – H2O]+

The resulting molecular weight information is very valuable, but complementary structural information is often needed. To obtain structural information, analyte ions are fragmented by colliding them with neutral molecules in a process known as collisioninduced dissociation (CID) or collisionally activated dissociation (CAD). Voltages are applied to the analyte ions to add energy to the collisions and create more fragmentation.

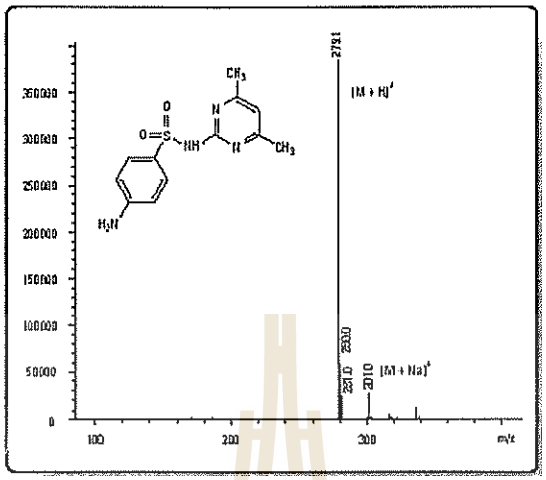


Figure 13. Mass spectrum of sulfamethazine acquired without collision-induced dissociation exhibits little fragmentation

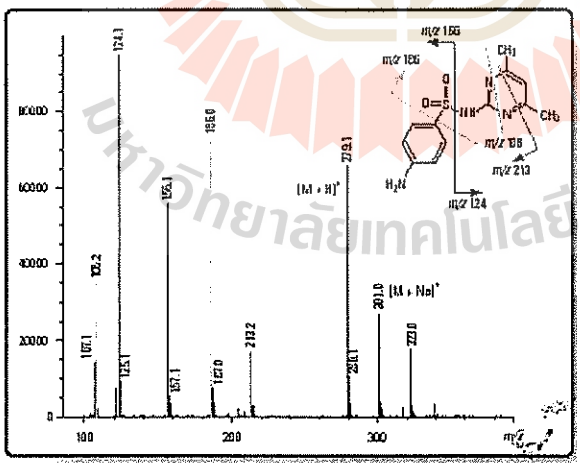


Figure 14. Mass spectrum of sulfamethazine acquired with collision-induced dissociation exhibits more fragmentation and thus more structural information

CID in single-stage MS

CID is most often associated with multistage mass spectrometers where it takes place between each stage of MS filtering, but CID can also be accomplished in single-stage quadrupole or time-of-flight mass spectrometers. In single-stage mass spectrometers, CID takes place in the ion source and is thus sometimes called source CID or in-source CID. Analyte (precursor) ions are accelerated and collide with residual neutral molecules to yield fragments called product ions.

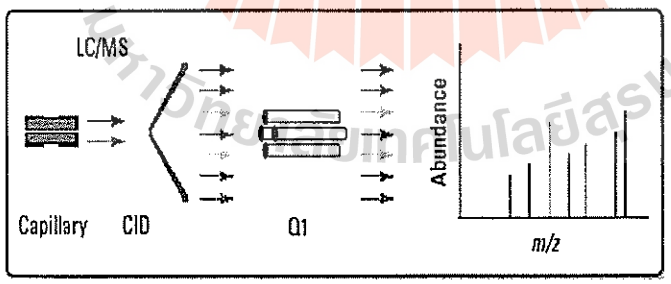
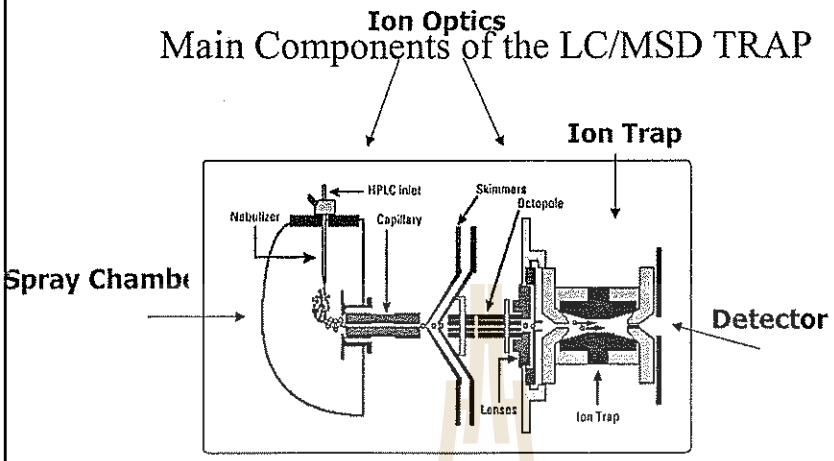


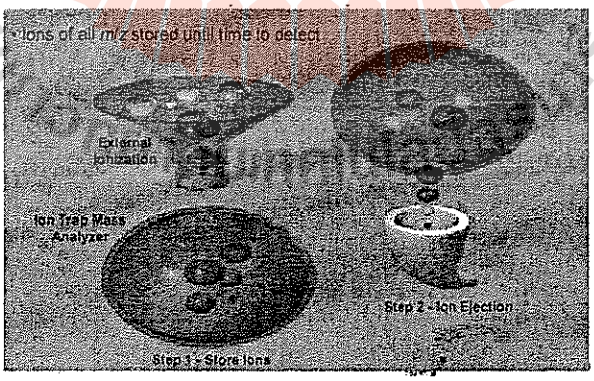
Figure 15. In-source CID with a single-quadrupole mass analyzer

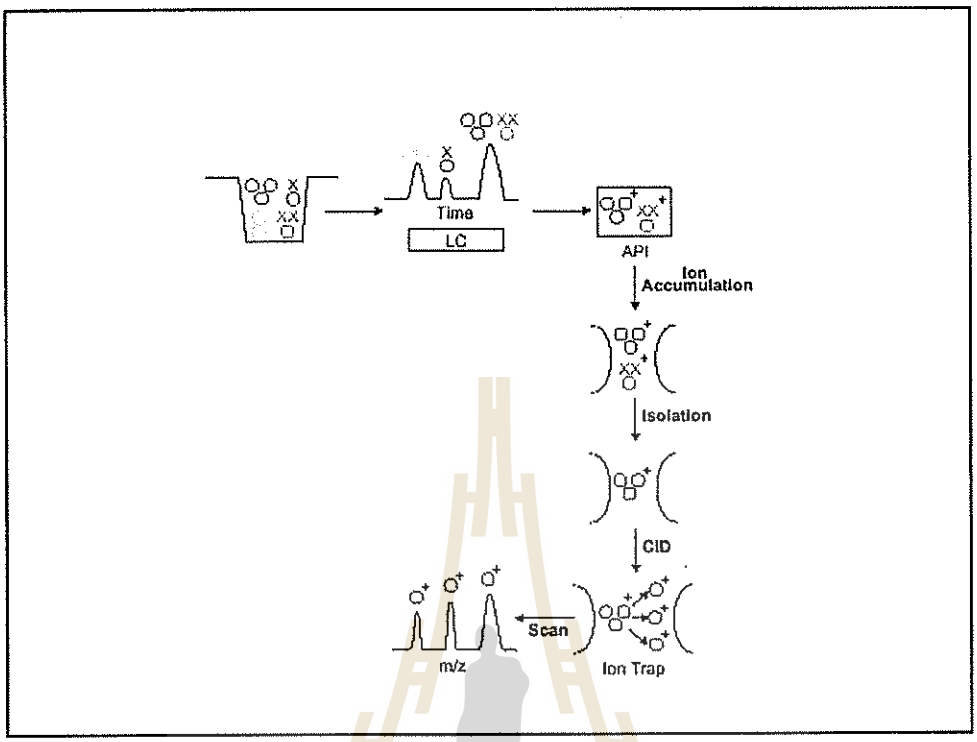
Main Components of the LC/MSD TRAP

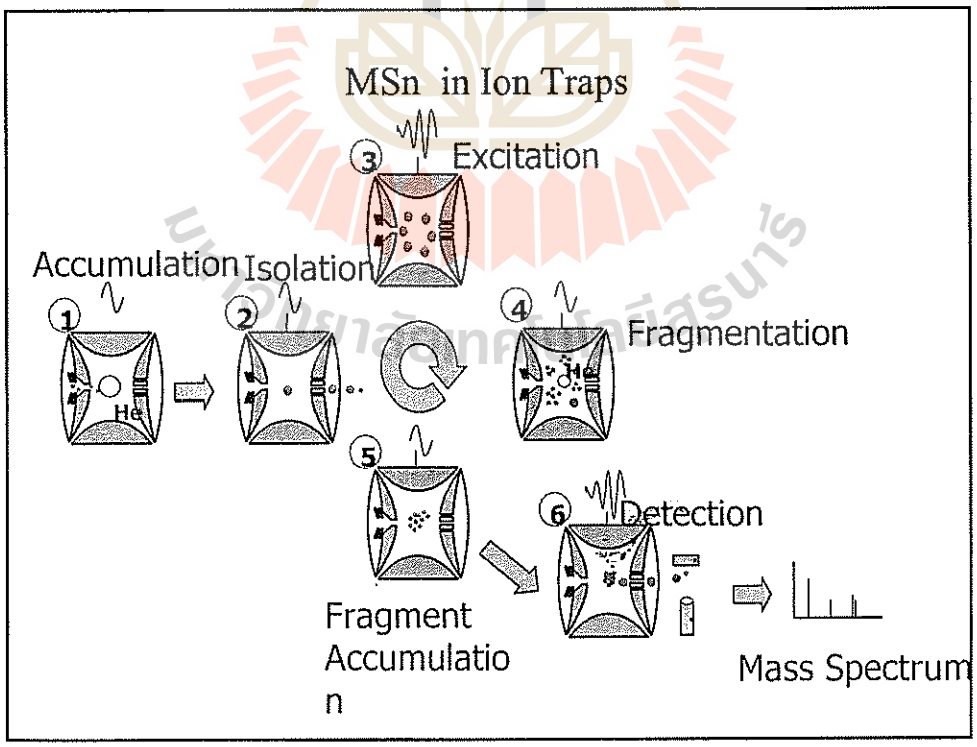


VL Model
SL Model has one Skimmer

Ion Trap concept



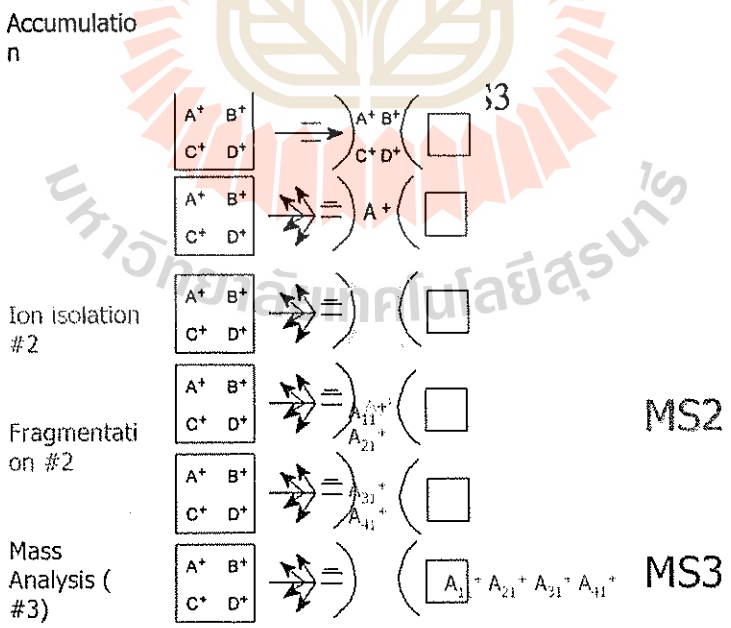

Time
LC
API
Ion Accumulation
Isolation
CID
Scan
m/z
Ion Trap

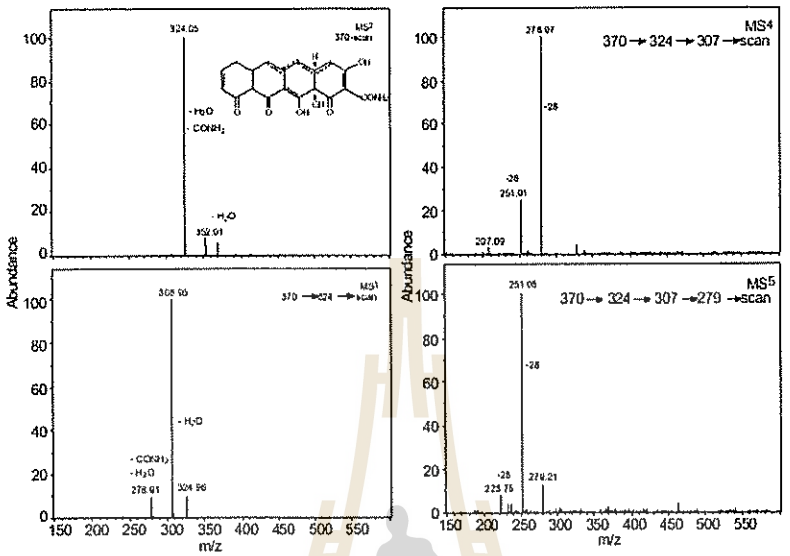

MSn in Ion Traps
Excitation
Accumulation
Isolation
Fragmentation
He
Detection
Fragment Accumulation
Mass Spectrum

MS^n Capabilities on an Ion Trap

- What is MS^n?
  - It's a way to denote multiple ion isolation and fragmentation steps to yield a product ion mass spectrum.
  - n = the number of isolation and fragmentation steps minus 1 (the last step is a full scan).

- e.g. MS^3 means
  - 1) isolation of a parent ion and fragmentation of that ion.
  - 2) Isolation of a product ion from the previous fragmentation and fragmentation of that ion.





CID and multiple-stage MS

Multiple-stage MS (also called tandem MS or MS/MS or MSn) is a powerful way to obtain structural information. In triple-quadrupole or quadrupole/quadrupole/time-of-flight instruments (see Figure 16), the first quadrupole is used to select the precursor ion. CID takes place in the second stage (quadrupole or octopole), which is called the collision cell.

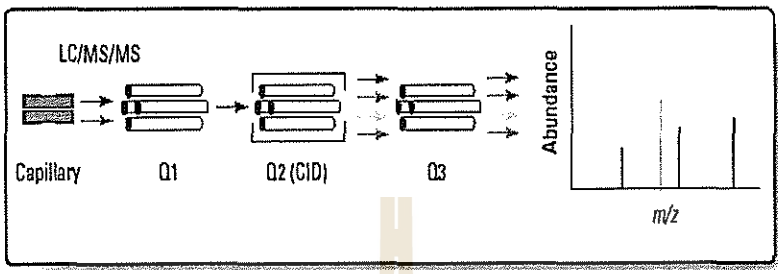


Figure 16. MS/MS in a triple-quadrupole mass spectrometer

The third stage (quadrupole or TOF) then generates a pectrum of the resulting product ions. It can also perform selected ion monitoring of only a few product ions when quantitating target compounds.

In ion trap and FT-ICR mass spectrometers, all ions except the desired precursor ion are ejected from the trap. The precursor ion is then energized and collided to generate product ions. The product ions can be ejected to generate a mass spectrum, or a particular product ion can be retained and collided to obtain another set of product ions. This process can be sequentially automated so that the most abundant ion(s) from each stage of MS are retained and collided.

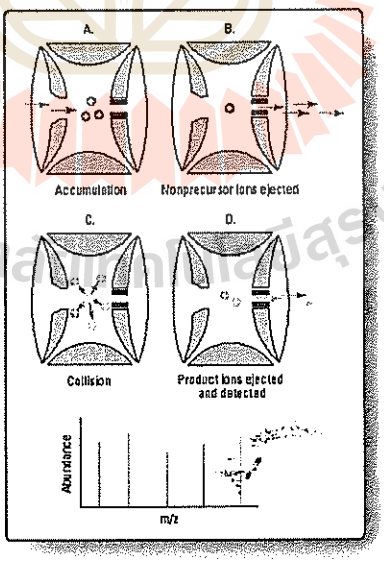
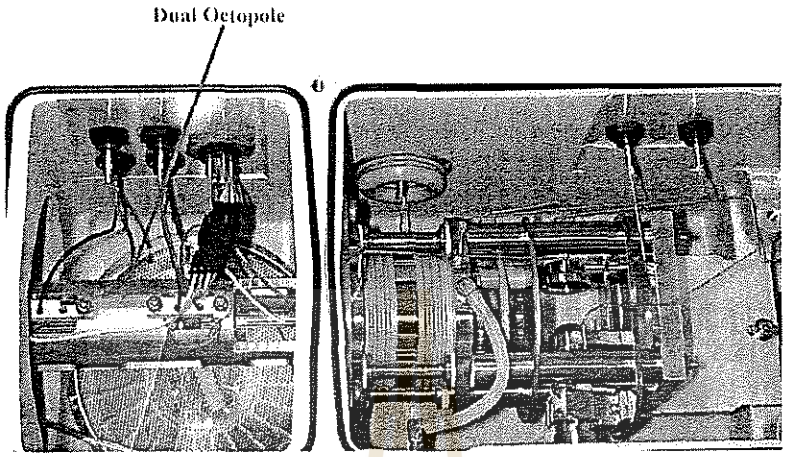


Figure 17. MS/MS in an ion trap mass spectrometer



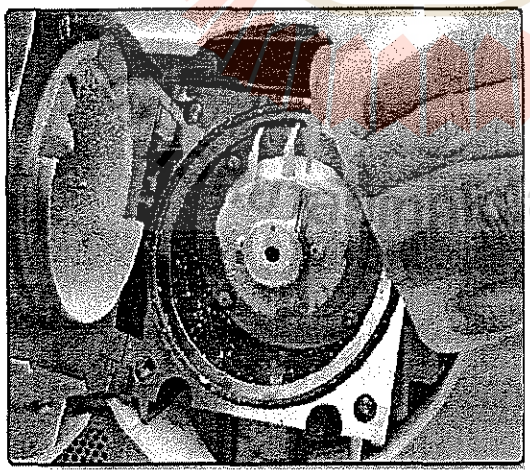


Figure 18. Salt deposits in this Agilent APCI ion source had little effect on performance thanks to orthogonal spray orientation and robust ion source design

MS for Analysis

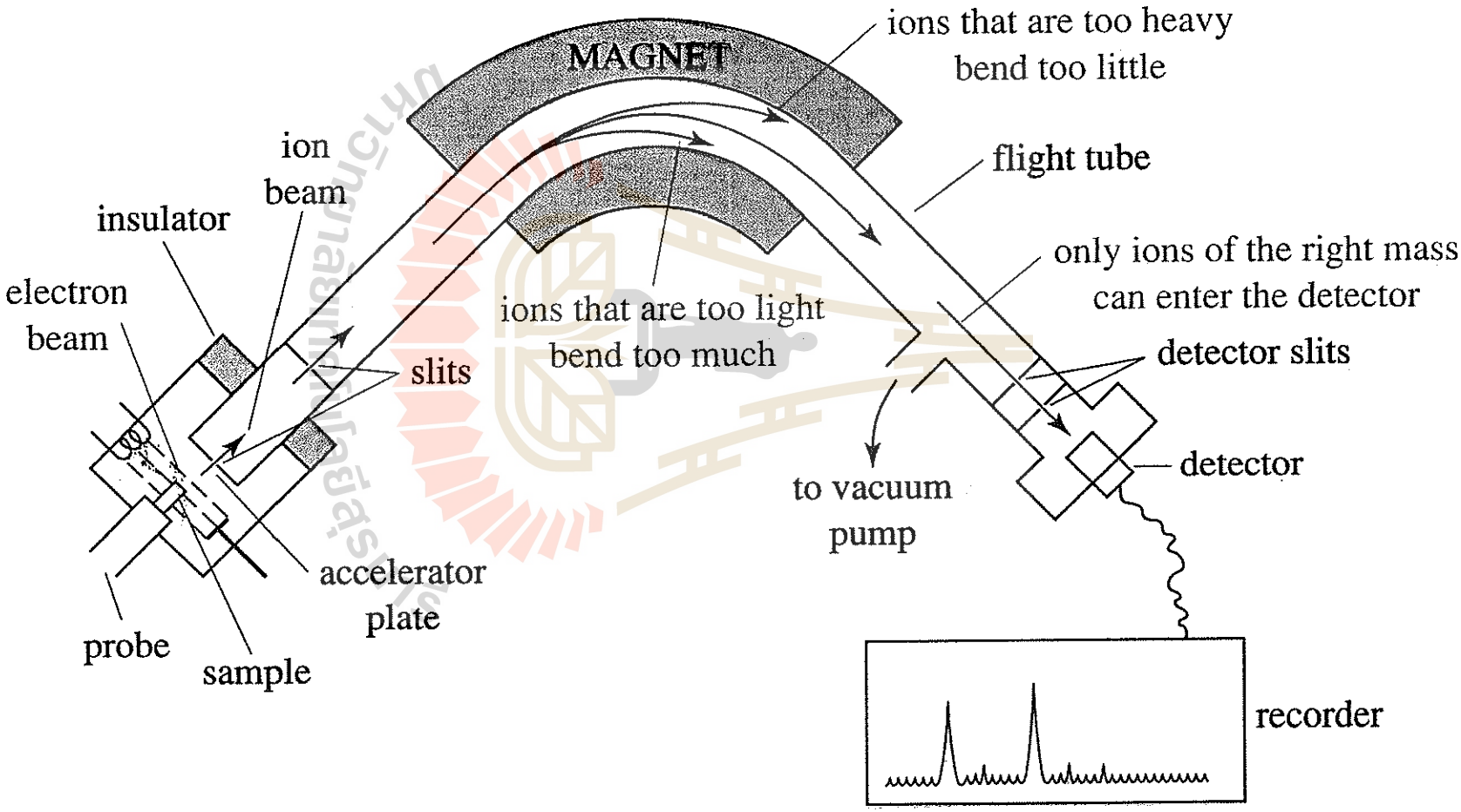
And Identification of

Organic Compounds

Lecture

ผศ.ดร.สันติ ศักดารัตน์

Fig. 12-14 Diagram of a Mass Spectrometer



n with the gas phase molecule. This distinction is shown in Figure 13-3, where a soft lision event is shown to result in molecular ions that do not fragment and a harder ctron-molecule collision yields unstable molecular ions. Since the nature of the colli- n between an electron and molecule is statistically controlled, a mixture of processes urs. Thus electron impact mass spectra often contain both intact molecular ions as ll as characteristic fragment ions. Electron impact with low-energy electrons is a ter ionization method and increases the relative abundance of intact molecular ions. wever, this result is achieved only with a great loss in ionization efficiency.

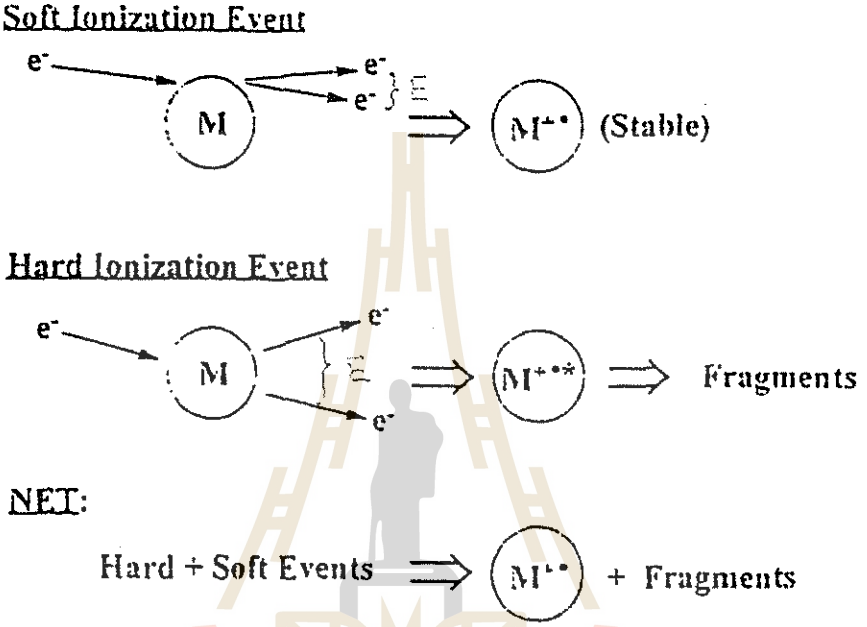


Figure 13-3 Electron ionization accompanied by different degrees of excitation of the molecular ion. Soft ionizing events transfer little excess energy to the ionized molecule, which is observed intact. Harder collisions also occur and give rise to the fragment ions frequently seen in EI mass spectra.

measuring compounds containing any combination of P, S, Cl, and F, as well as some classes of aromatic compounds, all of which can be ionized selectively in the presence of large concentrations of other types of organic compounds. Many compounds of environmental interest (including many pesticides and herbicides) undergo electron capture. Certain groups, notably fluorinated groups such as pentafluorobenzyl, have high electron capture cross sections and hence are introduced into molecules for this purpose. Chemical derivatization in this fashion is often worthwhile if trace analysis is required. In addition to its high selectivity, electron capture also is more sensitive than most other ionization methods. Electrons have high velocities, which increase the likelihood of electron-molecule collisions leading to electron capture and increase the $M^{-\cdot}$ abundance. Detection limits often are one or two orders of magnitude lower than those for other positive or other negative ion CI experiments.

Figure 13-11 summarizes some chemical ionization processes and contrasts the rich CI chemistry with the much simpler EI process. In addition, a summary of some of

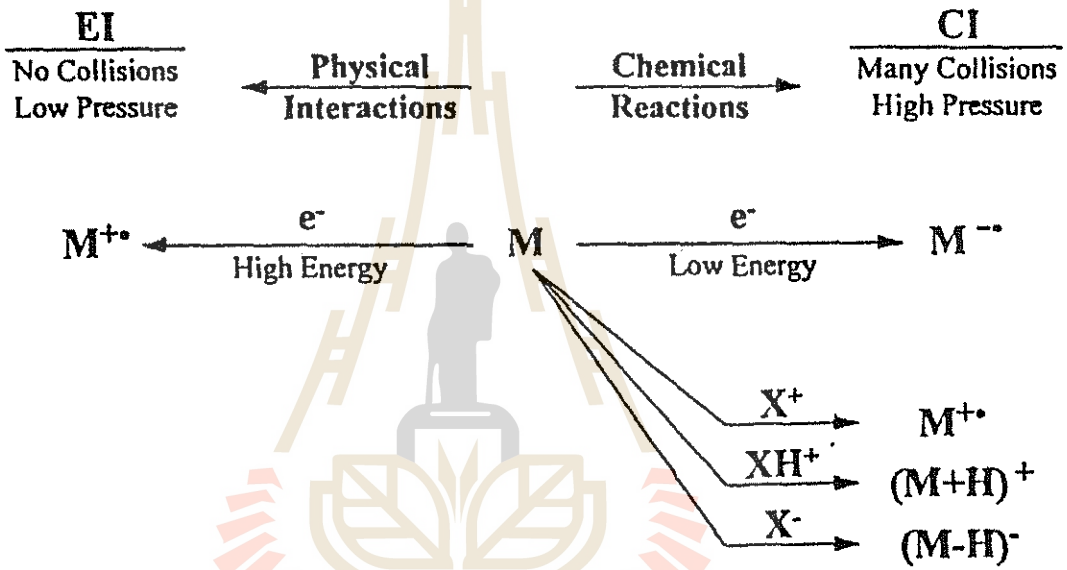


Figure 13-11 Contrasts between EI and CI, emphasizing greater versatility of the latter in terms of the type of molecular ion formed and the control achieved over its internal energy.

3.1.1 Reactions in chemical ionization [2]

Reagent ions may be molecular ions, fragment ions or the product of ion–molecule reactions between these ions and reagent gas molecules. For example, the principal reagent ions formed by electron bombardment of methane at 1 torr are CH_5^+ and $C_2H_5^+$ [3]:

$$CH_4 \longrightarrow CH_4^{+\cdot}, CH_3^+, CH_2^{+\cdot} \quad (3.1)$$

$$CH_4^{+\cdot} + CH_4 \longrightarrow CH_5^+ + CH_3^{\cdot} \quad (3.2)$$

$$CH_3^+ + CH_4 \longrightarrow C_2H_5^+ + H_2 \quad (3.3)$$

Other reactions that do not lead to useful ions also occur:

$$CH_2^{+\cdot} + CH_4 \longrightarrow C_2H_3^+ + H_2 + H^{\cdot} \quad (3.4)$$

$$C_2H_3^+ + CH_4 \longrightarrow C_3H_5^+ + H_2 \quad (3.5)$$

The subsequent chemical reactions between positively charged ions and sample molecules can conveniently be grouped into four major categories, viz.:

(a) Proton transfer	$M + BH^+$	$\rightarrow MH^+ + B$
(b) Charge exchange	$M + X^{+\cdot}$	$\rightarrow M^{+\cdot} + X$
(c) Electrophilic addition	$M + X^+$	$\rightarrow MX^+$
(d) Anion abstraction	$AB + X^+$	$\rightarrow B^+ + AX$

For example the CH_5^+ and $C_2H_5^+$ ions formed from methane react with and ionize sample molecules principally by proton transfer:

$$M + C_2H_5^+ \longrightarrow MH^+ + C_2H_4 \quad (3.6)$$

Each type of reaction is discussed in more detail in the following sections, with examples chosen to illustrate the use of the more common reagent gases. Published applications of these same reagent gases listed by compound type are documented later in Appendices 3.4 and 3.5.

tendency for a reagent ion BH^+ to protonate a particular sample molecule may assessed from a knowledge of proton affinity values. The proton affinity (PA) ompound B, [PA(B)], is the negative of the change in enthalpy or heat content mpanying reaction:

$$B + H^+ \rightarrow BH^+, \qquad PA(B) = -\Delta H \tag{3.7}$$

s, observation of the reaction

$$M + BH^+ \rightarrow MH^+ + B \tag{3.8}$$

ies that PA(M) > PA(B), i.e. the reaction is exothermic.

Table 3.1 Selected proton affinities

Conjugate base (B)	Reagent ion	PA(B) kJ mole^{-1}[b]
H_2	H_3^+	423
CH_4	CH_5^+	551
C_2H_6	$C_2H_7^+$	601
H_2O	H_3O^+	697
CH_3OH	$CH_3OH_2^+$	761
CH_3CN	CH_3CNH^+	787
$(CH_3)_2C{=}CH_2$[a]	$(CH_3)_3C^+$[a]	824
NH_3	NH_4^+	854
CH_3NH_2	$CH_3NH_3^+$	896
$NH_2(CH_2)_2NH_2$	$NH_2(CH_2)_2NH_3^+$	945

[a] Reagent ion from isobutane.
[b] 100 kJ mole^{-1} ≡ 23.9 kcal mole^{-1} ≡ 1.04 eV.

resentative proton affinity values taken from the very extensive data lations now available [4] are listed in Table 3.1. From the data given in the

4.2.2 Ion formation by ion–molecule reactions [12]

These reactions can conveniently be grouped into four major categories, viz.

(a) Proton transfer $M + X^- \rightarrow (M - H)^- + XH$
(b) Charge exchange $M + X^- \rightarrow M^- + X$
(c) Nucleophilic addition $M + X^- \rightarrow MX^-$
(d) Nucleophilic displacement $AB + X^- \rightarrow BX + A^-$

ABLE 13-9

ome Reagents Used in Negative Ion and Electron Capture CI

Reagent Gas	*Reagent Ion/e⁻*	*PA(X⁻)* kJ m*
IH_3	NH_2^-/e^-	1689
H_4	e^-	
H_4 and N_2O	OH^-	1635
or CH_4, N_2O and He		
or H_2, N_2O and He		
N_2O or $N_2O + N_2$	$O^{-\cdot}$	1599
CH_4 (at 1 torr) + 0.1% methyl nitrite	CH_3O^-	1592
$CH_3O^- + CH_3CN$	CH_2CN^-	1560
NF_3 (0.1 torr) or CHF_3	F^-	1554
O_2 (1 torr, Townsend discharge)	$O2^{-\cdot}$	1476
or mixture of (O_2 and H_2)		
$F^- + CH_3CH_2CN$	CN^-	1469
$OH^- + CH_3COOCH_3$	$CH_3CO_2^-$	1459
CH_2Br_2	Br^-	1356
CH_2Cl_2 or $CHCl_3$ or CF_2Cl_2 or ($CH_2Cl_2 + CH_4$)	Cl^-	1395

* Proton affinity of X anion (4.18 kJ mol^{-1} = 1 kcal mol^{-1}).

TABLE 13-10

Summary of Characteristics of CI

Sample must be volatile	Limited to sample molecular weight appro mately 800 Da or less
Hard or soft ionization	Molecular weight determination or fragm tion for structural information
Variety of ionization processes	Multiple checks of molecular weight, struc features
elective method	Either is available, depending on choice o

One method of confirming that a particular peak corresponds to a molecular ion is to vary the energy of the ionizing electron beam. If the energy of the beam is lowered, the tendency of the molecular ion to fragment lessens. As a result, the intensity of the molecular ion peak should increase with decreasing electron potential, while the intensities of the fragment ion peaks should decrease.

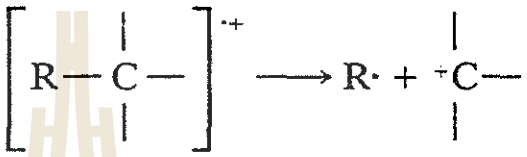
Certain facts must apply to a molecular ion peak:

1. The peak must correspond to the ion of highest mass in the spectrum, excluding isotopic peaks that occur at even higher masses. The isotopic peaks are usually of much lower intensity than the molecular ion peak. At the sample pressures used in most spectral studies, the probability that ions and molecules will collide to form heavier particles is quite low.
2. The ion must have an odd number of electrons. When a molecule is ionized by an electron beam, it loses one electron to become a radical-cation. The charge on such an ion is one, thus making it an ion with an odd number of electrons.
3. The ion must be capable of forming the important fragment ions in the spectrum, particularly the fragments of relatively high mass, by loss of logical neutral fragments. Section 7.6 will explain these fragmentation processes in detail.

The observed abundance of the suspected molecular ion must correspond to expectations based on the assumed molecule structure. Highly branched substances undergo fragmentation very easily. Observation of an intense molecular ion peak for a highly branched molecule thus would be unlikely. The lifetimes of molecular ions vary according to the following generalized sequence.

Aromatic compounds > conjugated alkenes > alicyclic compounds > organic sulfides > unbranched hydrocarbons > mercaptans > ketones > amines > esters > ethers > carboxylic acids > branched hydrocarbons > alcohols

1. The relative height of the molecular ion peak is greatest for the straight-chain compound and decreases as the degree of branching increases (see rule 3).
2. The relative height of the molecular ion peak usually decreases with increasing molecular weight in a homologous series. Fatty esters appear to be an exception.
3. Cleavage is favored at alkyl-substituted carbon atoms; the more substituted, the more likely is cleavage. This is a consequence of the increased stability of a tertiary carbocation over a secondary, which in turn is more stable than a primary.

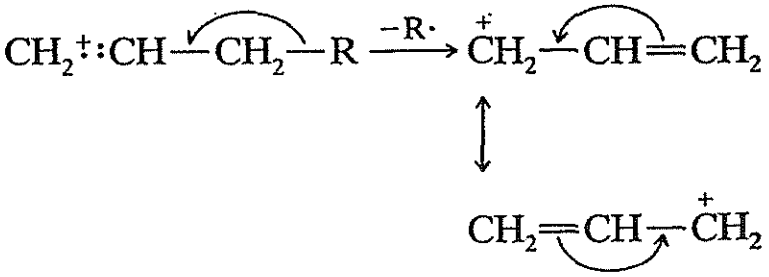


Cation stability order:

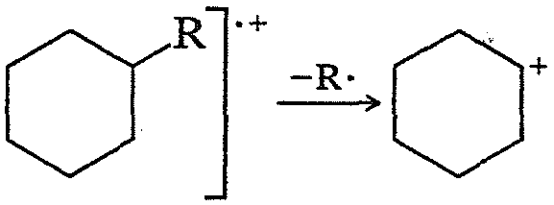
$$CH_3^+ < R'CH_2^+ < R_2'CH^+ < R_3'C^+$$

Generally, the largest substituent at a branch is eliminated most readily as a radical, presumably because a long-chain radical can achieve some stability by delocalization of the lone electron.

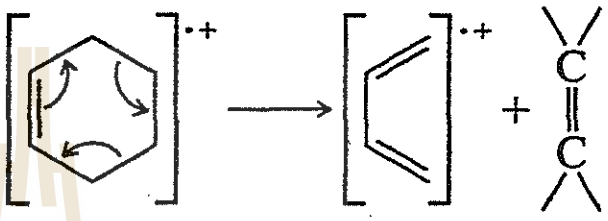
4. Double bonds, cyclic structures, and especially aromatic (or heteroaromatic) rings stabilize the molecular ion and thus increase the probability of its appearance.
5. Double bonds favor allylic cleavage and give the resonance-stabilized allylic carbocation. This rule does not hold for simple alkenes because of the ready migration of the double bond, but it does hold for cycloalkenes.



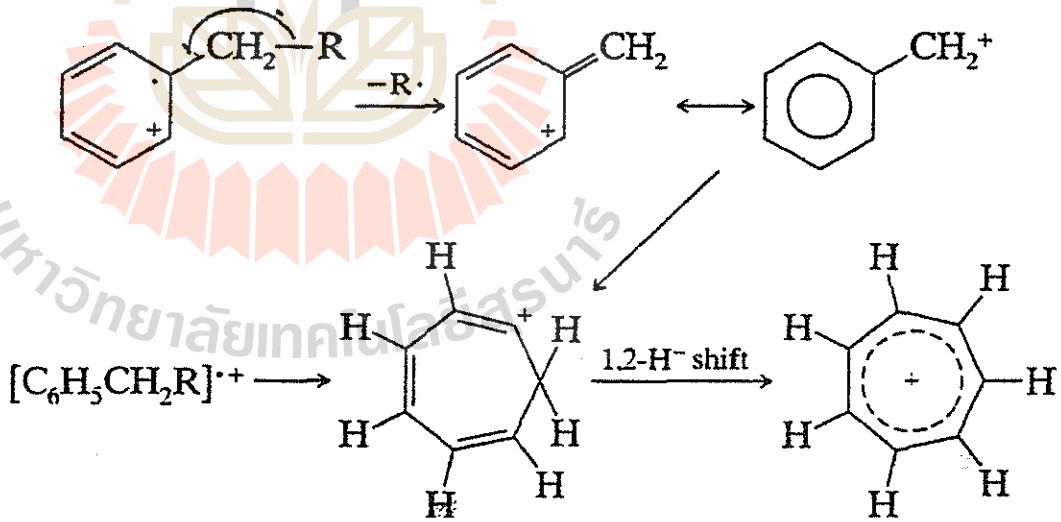
6. Saturated rings tend to lose alkyl side chains at the α bond. This is merely a special case of branching (rule 3). The positive charge tends to stay with the ring fragment.



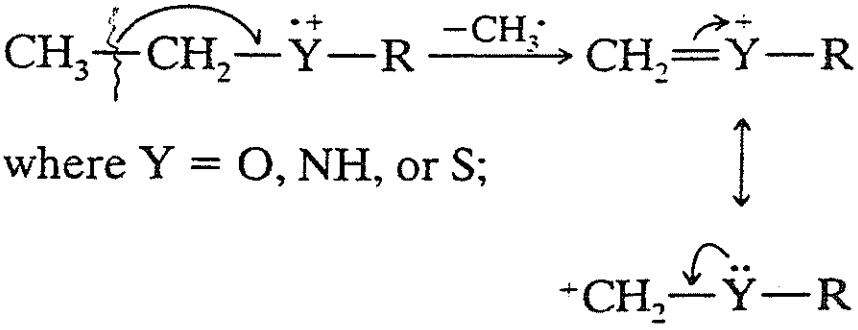
Unsaturated rings can undergo a *retro*-Diels-Alder reaction:



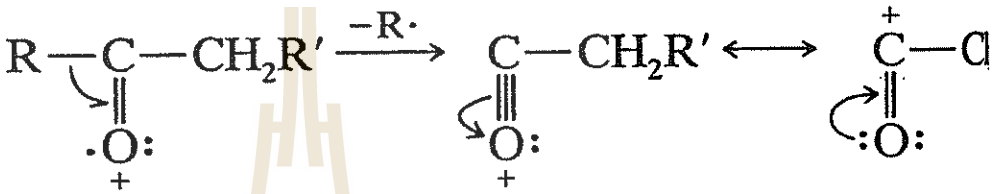
7. In alkyl-substituted aromatic compounds, cleavage is very probable at the bond β to the ring, giving the resonance-stabilized benzyl ion or, more likely, the tropylium ion:



8. The C—C bonds next to a heteroatom are frequently cleaved, leaving the charge on the fragment containing the heteroatom whose nonbonding electrons provide resonance stabilization.



where Y = O, NH, or S;



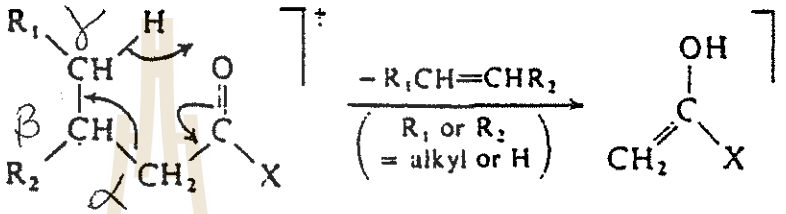
9. Cleavage is often associated with eliminati small, stable, neutral molecules, such as c monoxide, olefins, water, ammonia, hydroge fide, hydrogen cyanide, mercaptans, ketené, cohols, often with rearrangement (Section 2.8

It should be kept in mind that the fragmen rules above apply to EI mass spectrometry. Since ionizing (CI, etc.) techniques often produce mole ions with much lower energy or quasimolecular with very different fragmentation patterns, diff rules govern the fragmentation of these molecular

Table 4.9 Useful ion series

Functional group	*Simplest ion type*	*Ion series (m/z)*
Amine	$CH_2{=}\overset{+}{N}H_2$ *m/z* 30	30, 44, 58, 72, 86, 100, . . .
Ether / Alcohol	$CH_2{=}\overset{+}{O}H$ *m/z* 31	31, 45, 59, 73, 87, 101, . . .
Ketone	$CH_3C{\equiv}\overset{+}{O}$ *m/z* 43	43, 57, 71, 85, 99, 113, . . .
[Hydrocarbon]	$C_2H_5^+$ *m/z* 29	29, 43, 57, 71, 85, 99, 113, . . .

Table 4.10 *m/z* values of some rearrangement ions found in the mass spectra of carbonyl compounds



Compound	X	*m/z*
Aldehyde	H	44
Ketone (methyl)	CH_3	58
Ketone (ethyl)	C_2H_5	72
Acid	OH	60
Ester (methyl)	OCH_3	74
Ester (ethyl)	OC_2H_5	88
Amide	NH_2	59

Examples of spectra of aliphatic compounds

Ketones

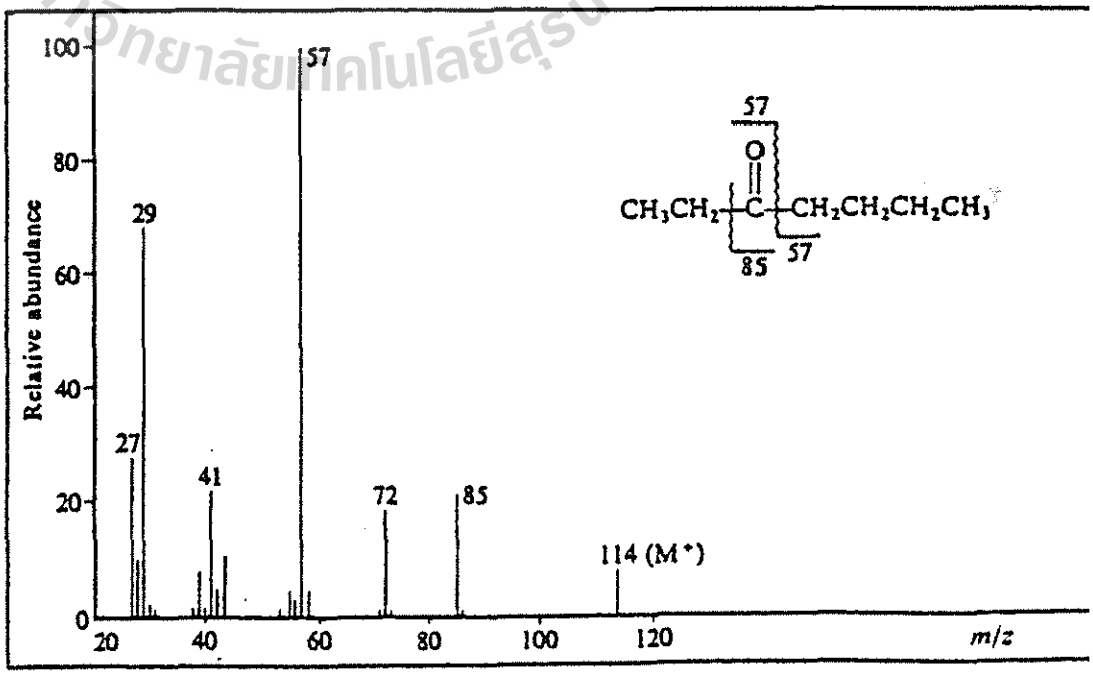
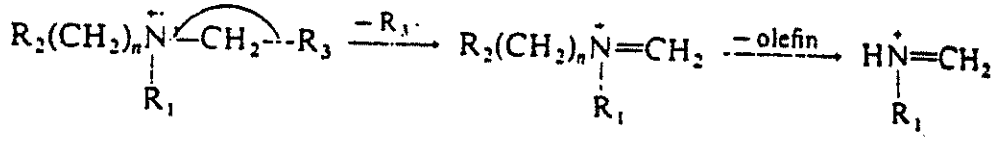
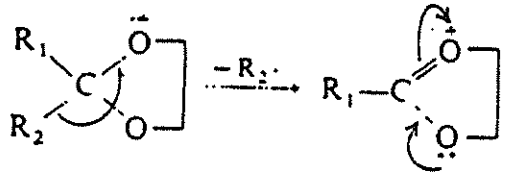
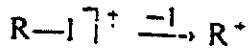
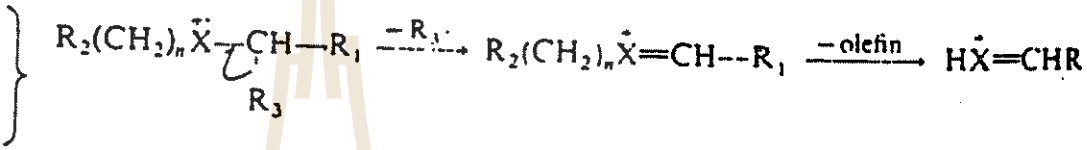
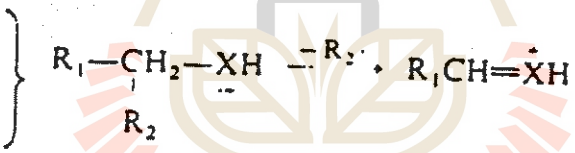
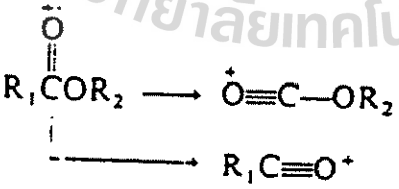


Fig. 4.21

Table 4.8 Primary single-bond cleavage processes associated with some common functional groups†

Functional group	*Fragmentation*
Amine	
Ketal	
Iodide	
Ether (X=2O) Thioether (X=2S)	
Ketone	
Alcohol (X=2O) Thiol (X=S)	
Bromide	
Ester	

† In polyfunctional aliphatic molecules, cleavages associated with functional groups higher up the tabl are preferred over those cleavages associated with lower entries.

T-67

Fig. 12-15 Mass Spectrum of 2,4-dimethylpentane

m/z	*Abundance (% of base peak)*
39	62
41	100 (base peak)
42	24
43	90
56	23
57	50
85	11
100 (M^+)	10

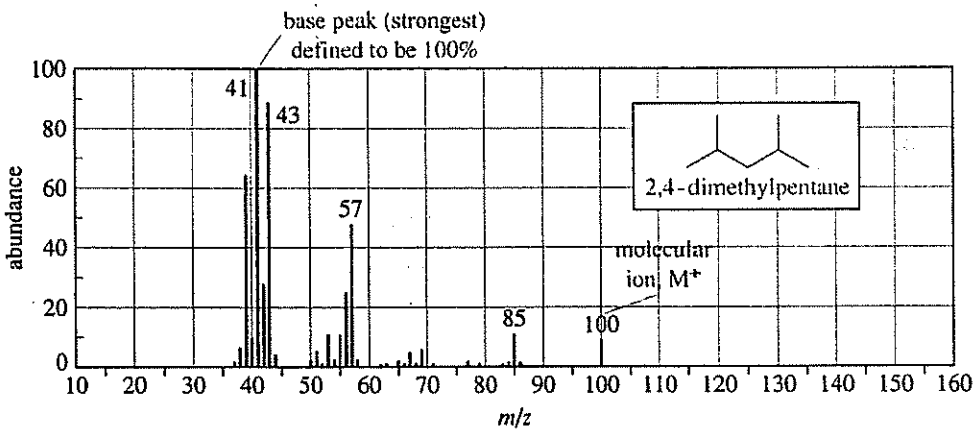
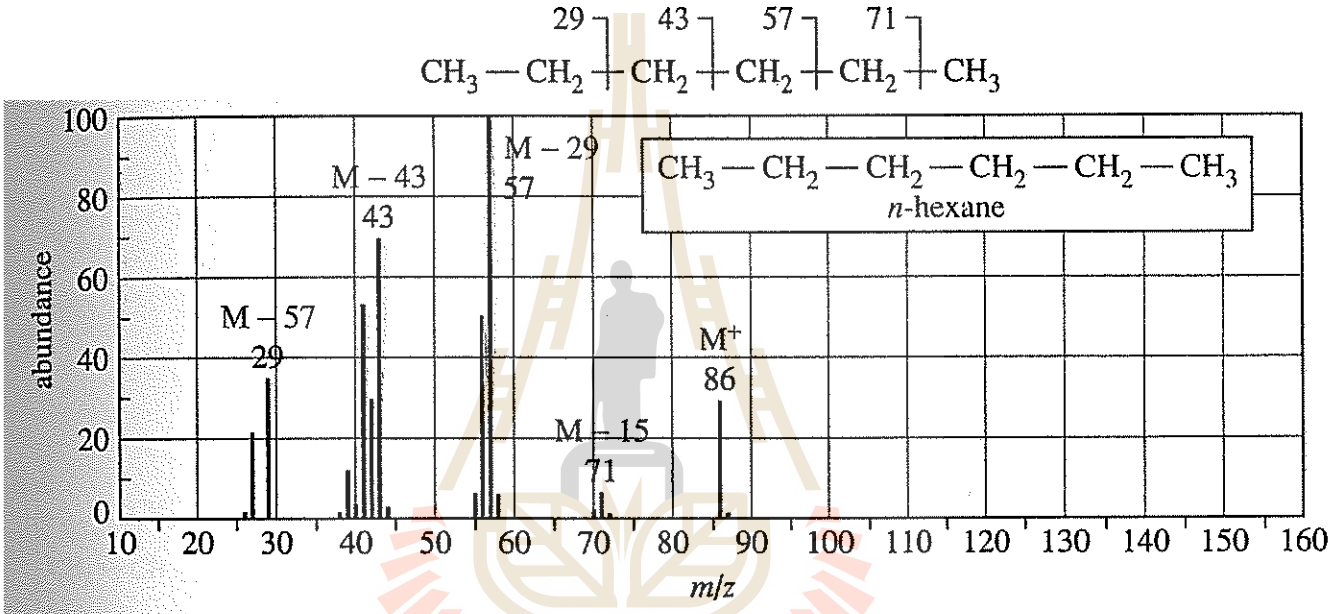


Fig. 12-16 Mass Spectrum of *n*-hexane



Prob. 12-23 Mass Spectrum of 1-octyne

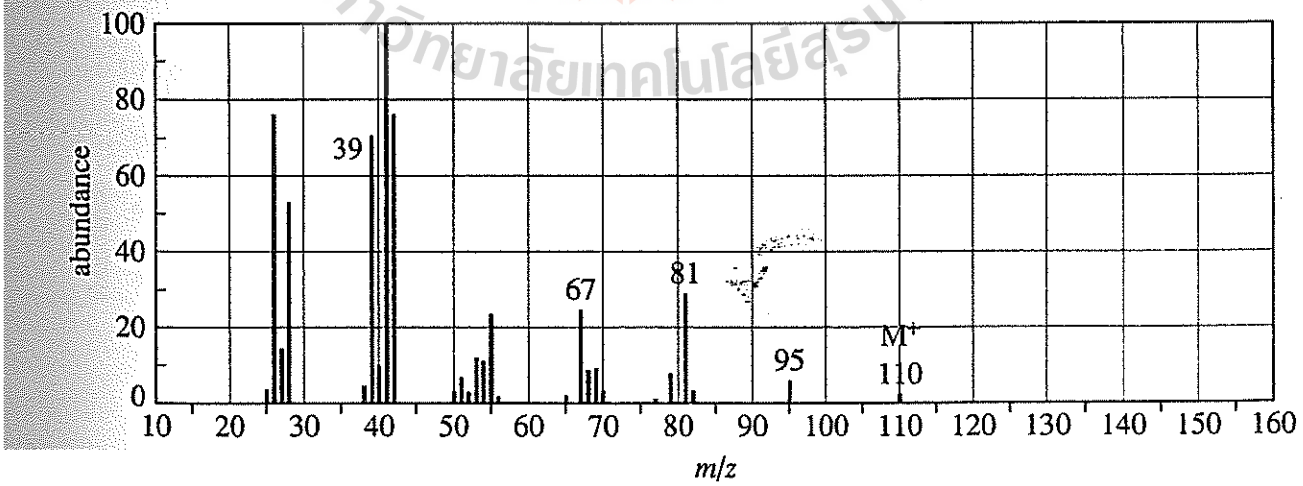
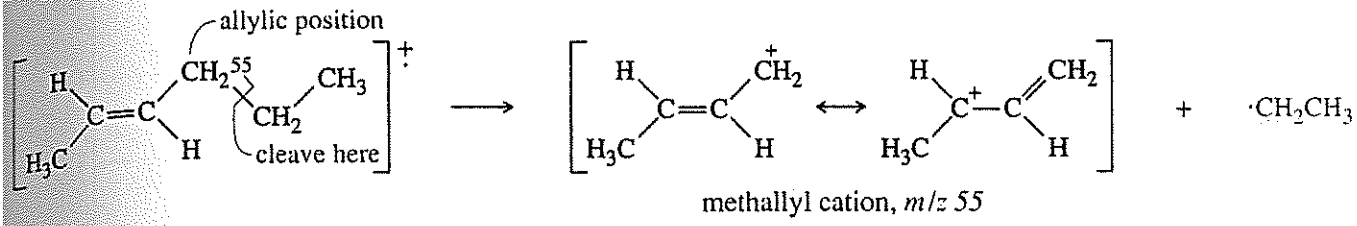


Fig. 12-18 Mass Spectrum of *trans*-2-hexene



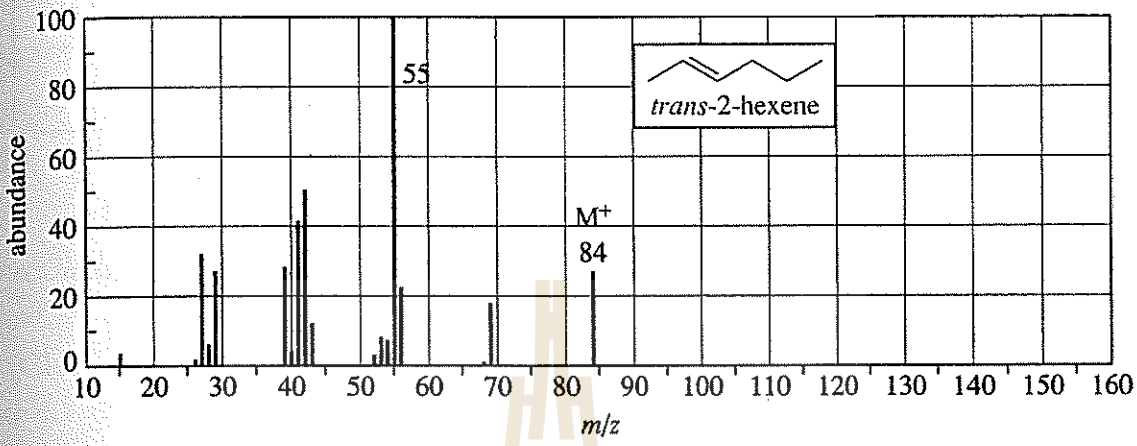
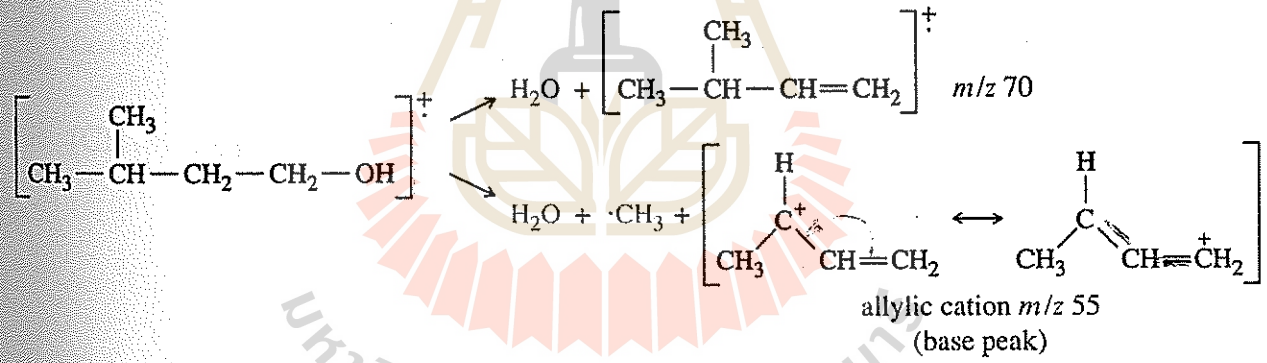
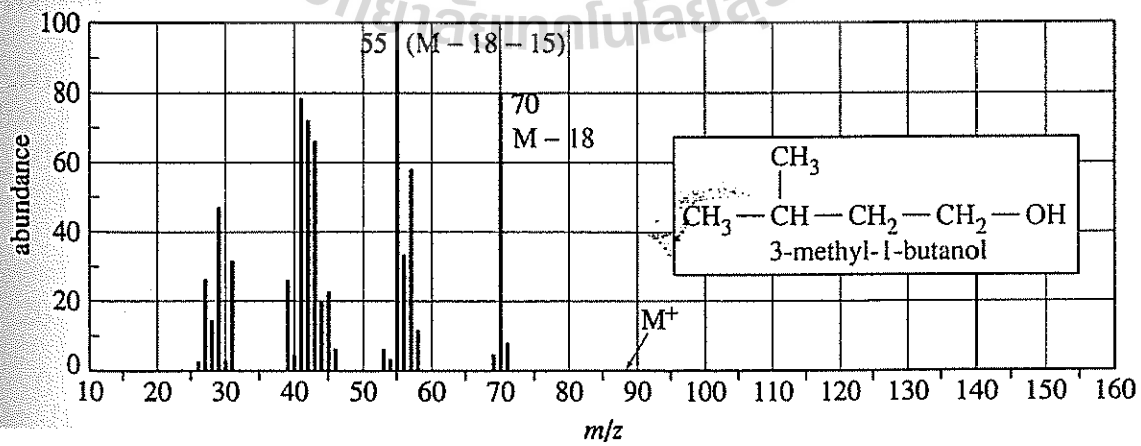


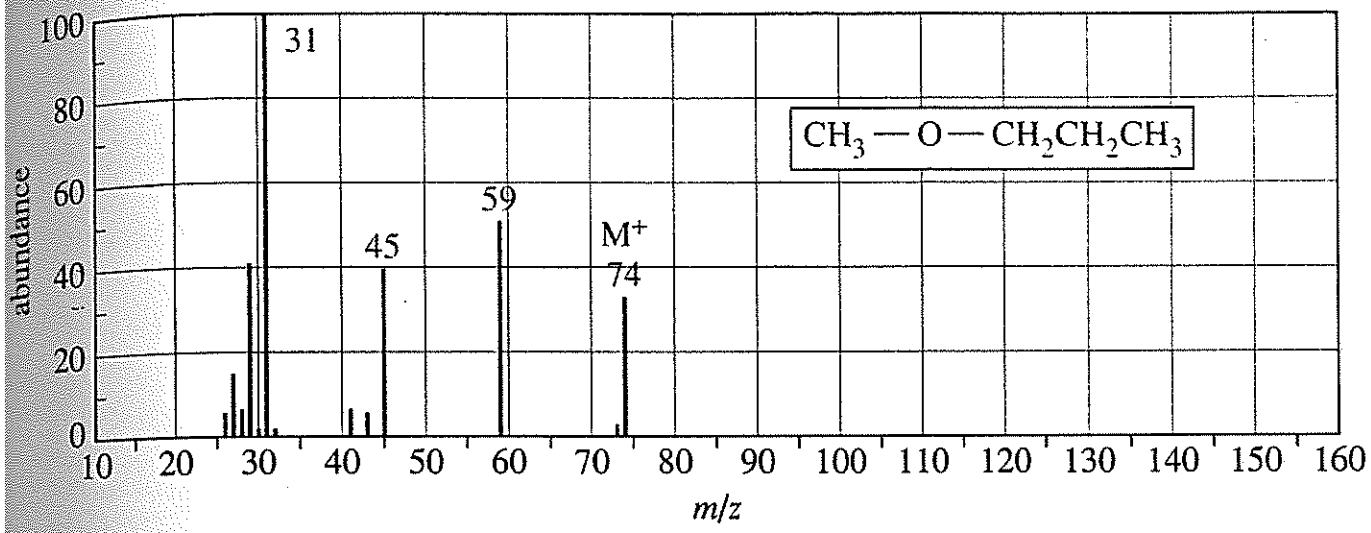
Fig. 12-19 Mass Spectrum of 3-methyl-1-butanol



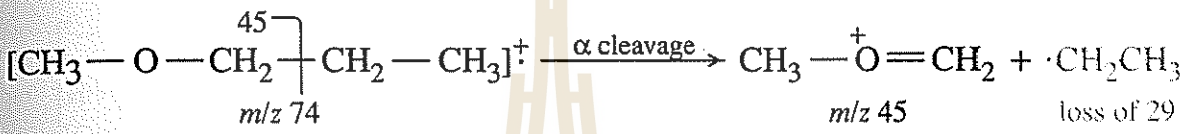


T-96

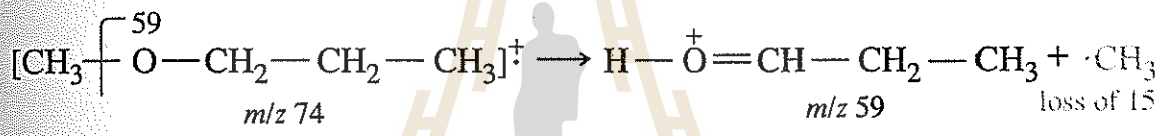
Fig. 14-5 Mass Spectrum of Methyl *n*-Propyl Ether



α Cleavage



Loss of methyl group



Loss of propyl group

Prob. 14-7 Mass Spectrum of Butyl Isopropyl Ether

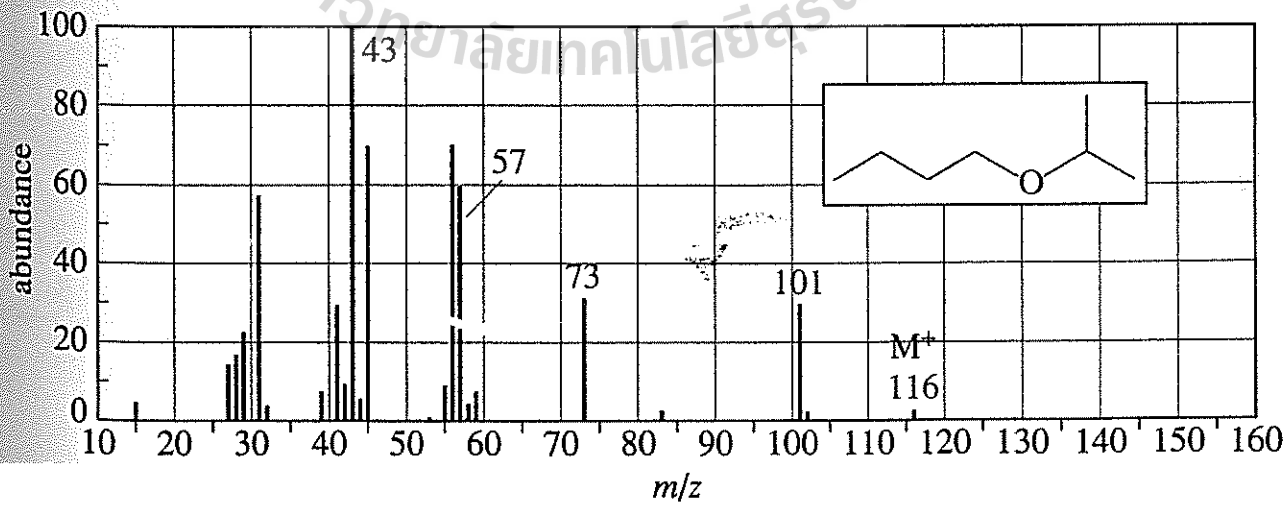
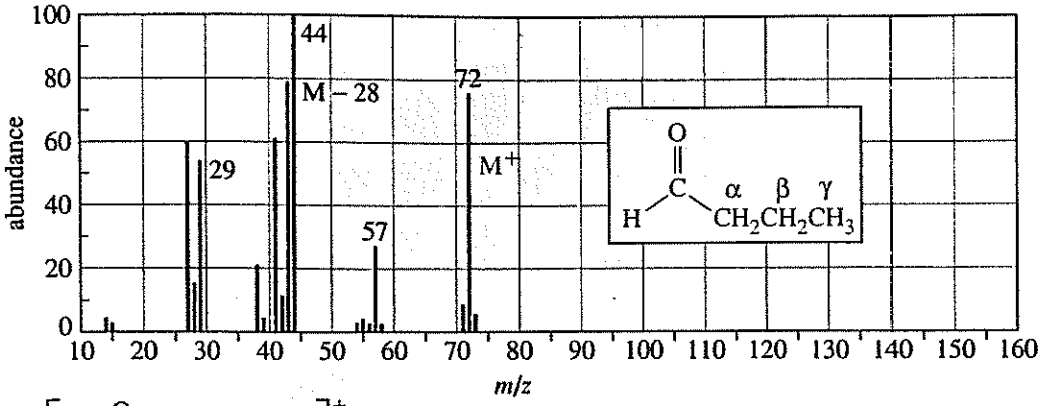
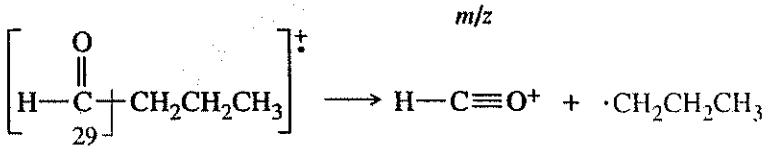
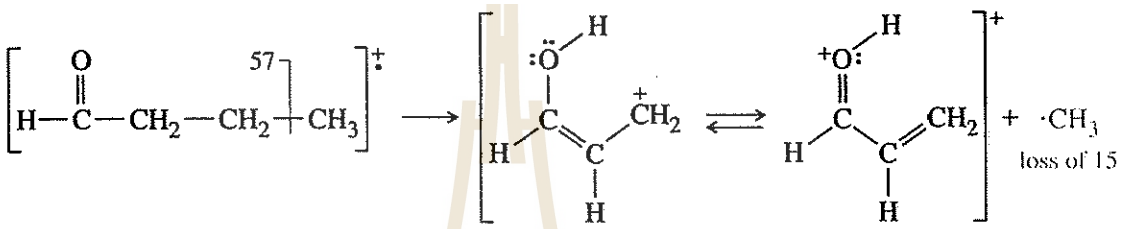


Fig. 18-4 Mass Spectrum of butyraldehyde





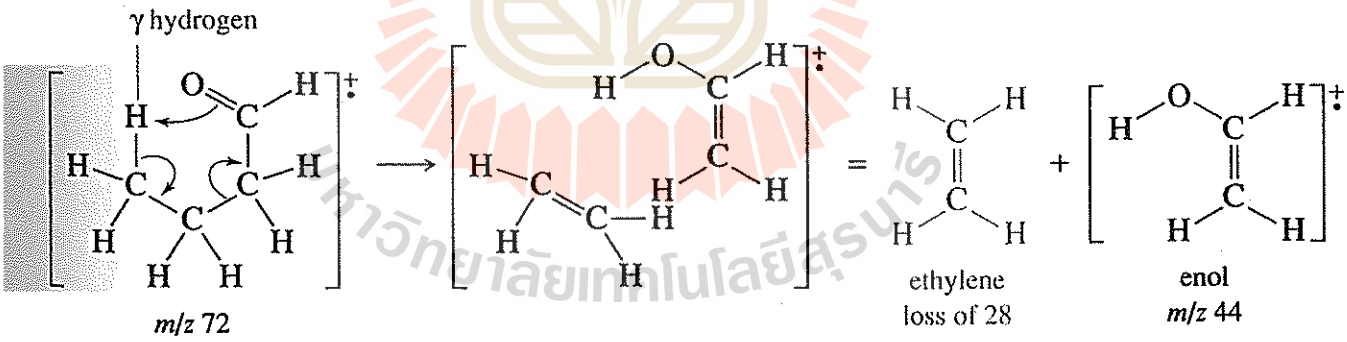
m/z 72 *m/z* 29 loss of 43



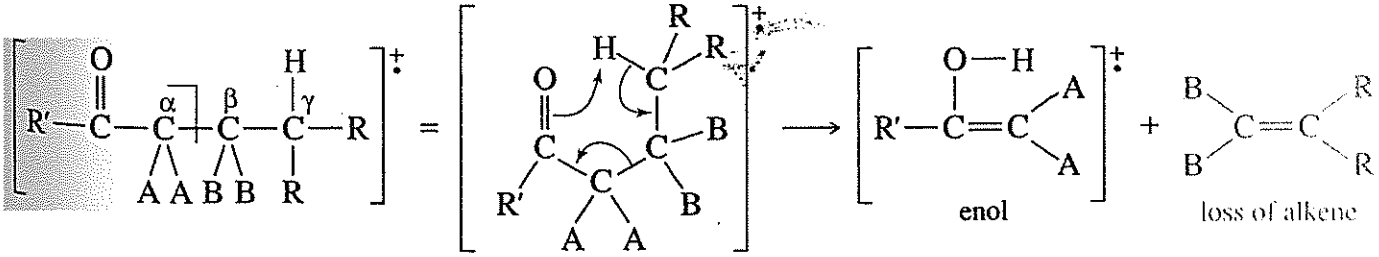
β, γ cleavage stabilized cation *m/z* 57 loss of 15

McLafferty rearrangement *m/z* 72 ⟶ *m/z* 44 (base peak) + loss of 28

Fig. 18-5 The McLafferty Rearrangement

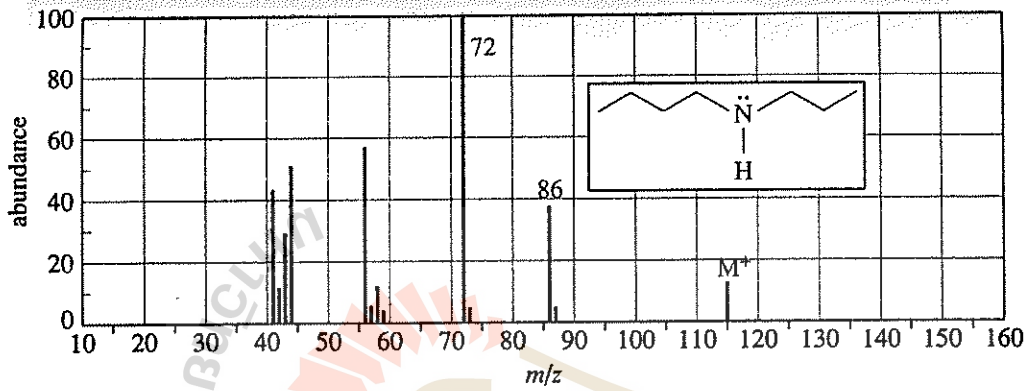


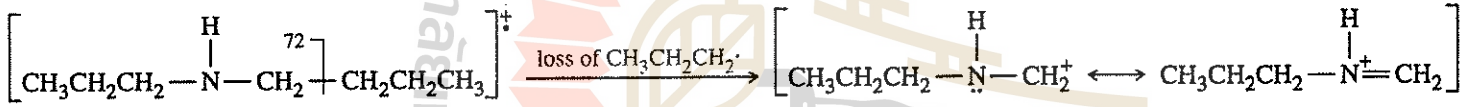
McLafferty rearrangement of butyraldehyde



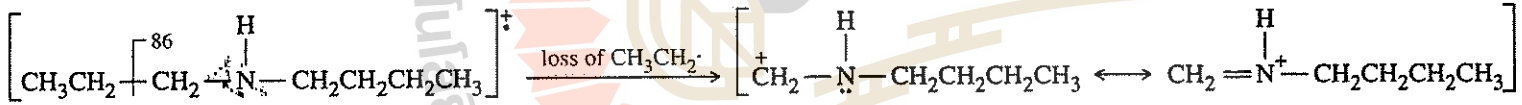
McLafferty rearrangement of a general ketone or aldehyde

Fig. 19-9 Mass Spectrum of butyl propyl amine





butyl propyl amine, *m/z* 115 — iminium ion, *m/z* 72



butyl propyl amine, *m/z* 115 — iminium ion, *m/z* 86

Prob. 19-10 Mass Spectrum of ethyl propyl amine

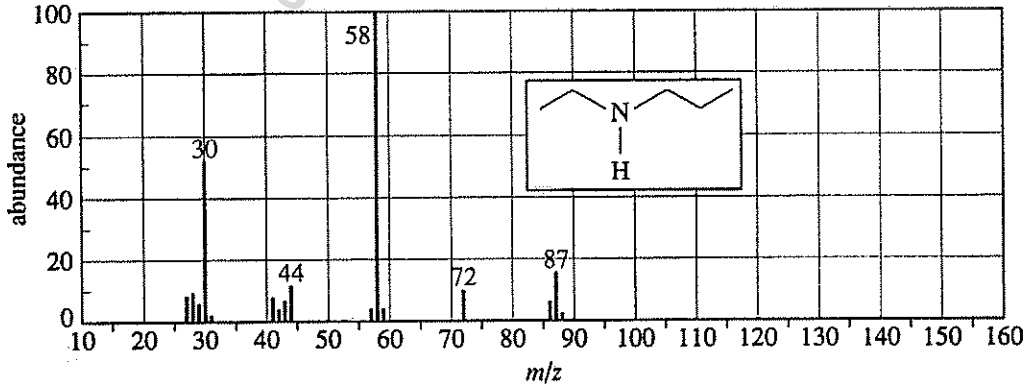


TABLE 7.3

Natural Abundances of Common Elements and Their Isotopes

Element	Relative Abundance					
Hydrogen	^{1}H	100	^{2}H	0.016		
Carbon	^{12}C	100	^{13}C	1.08		
Nitrogen	^{14}N	100	^{15}N	0.38		
Oxygen	^{16}O	100	^{17}O	0.04	^{18}O	0.20
Fluorine	^{19}F	100				
Silicon	^{28}Si	100	^{29}Si	5.10	^{30}Si	3.35
Phosphorus	^{31}P	100				
Sulfur	^{32}S	100	^{33}S	0.78	^{34}S	4.40
Chlorine	^{35}Cl	100			^{37}Cl	32.5
Bromine	^{79}Br	100			^{81}Br	98.0
Iodine	^{127}I	100				

abundances of the isotopes of each element are calculated by setting the abunda common isotopes equal to 100.

To demonstrate how the intensities of the $M + 1$ and $M + 2$ peaks provide a u given molecular formula, consider two molecules of mass 42, propene (C_3H_6) a (CH_2N_2). For propene, the intensity of the $M + 1$ peak should be (3×1.08) 3.34%, and the intensity of the $M + 2$ peak should be 0.05%. The natural ab isotopes of nitrogen is 0.38% of the abundance of ^{14}N atoms. In diazomethane relative intensity of the $M + 1$ peak to be $1.08 + (2 \times 0.016) + (2 \times 0.38) = 1.8$ sity of the molecular ion peak, and the intensity of the $M + 2$ peak would be 0.01% of the molecular ion peak. Table 7.4 summarizes these intensity ratios. It sho molecules have nearly the same molecular weight, but the relative intensities of $M + 2$ peaks which they yield are quite different. As an additional illustration, Tab the ratios of the molecular ion, $M + 1$, and $M + 2$ peaks for three substances of monoxide, nitrogen, and ethene. Again, notice that the relative intensities of the M peaks provide a means of distinguishing among these molecules.

As molecules become larger and more complex, the number of possible comb yield $M + 1$ and $M + 2$ peaks grows. For a particular combination of atoms, the inte peaks relative to the intensity of the molecular ion peak are unique. Thus, the isotop can be used to establish the molecular formula of a compound. Tables of possible c

Compound	Molecular Mass	M	$M+1$	$M+2$
CO	28	100	1.12	0.2
N_2	28	100	0.76	
C_2H_4	28	100	2.23	0.01

carbon, hydrogen, oxygen, and nitrogen and intensity ratios for the $M + 1$ and $M +$ each combination have been developed. Appendix 7 and Beynon (in the reference list a this chapter) contain extensive tables of this sort. For a given molecular weight, you c the tables to find the molecular formula that corresponds to the isotope ratios obser

For atoms other than carbon, hydrogen, oxygen, and nitrogen, it is necessary to c expected intensities of the $M + 1$ and $M + 2$ peaks for a particular molecular formul establish the presence of these other elements by means other than mass spectromet

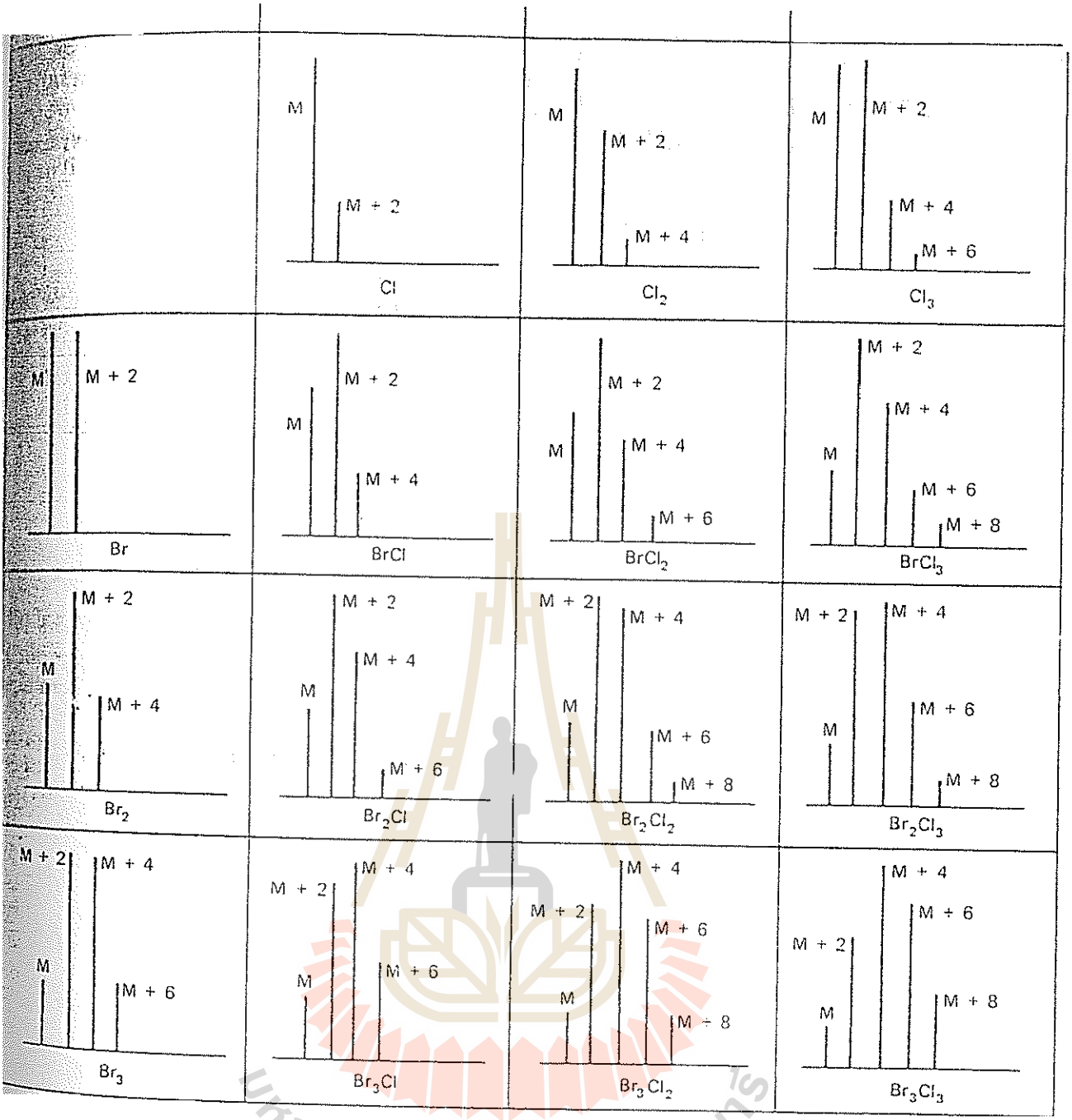
When chlorine or bromine is present, the $M + 2$ peak becomes very significant. isotope of each of these elements is two mass units heavier than the lighter isotope. abundance of ^{37}Cl is 32.5% that of ^{35}Cl; the natural abundance of ^{81}Br is 98.0% that of either of these elements is present, the $M + 2$ peak becomes quite intense. If a compou two chlorine or bromine atoms, a quite distinct $M + 4$ peak, as well as an intense M should be observed. In such a case, it is important to exercise caution in identifying th ion peak in the mass spectrum. Section 7.6M will discuss the mass spectral properties of halogen compounds in greater detail. Table 7.6 gives the relative intensities of isotop various combinations of bromine and chlorine atoms, and Figure 7.4 illustrates them

Examination of the intensity of the $M + 2$ peak is also useful for obtaining inform elements which may be present in the molecular formula. An unusually intense $M +$

TABLE 7.6

Relative Intensities of Isotope Peaks for Various Combinations of Bromine and Chlorine

	Relative Intensities			
Halogen	M	$M+2$	$M+4$	$M+6$
Br	100	97.7		
Br_2	100	195.0	95.4	
Br_3	100	293.0	286.0	93.4
Cl	100	32.6		
Cl_2	100	65.3	10.6	
Cl_3	100	97.8	31.9	3.47
BrCl	100	130.0	31.9	
Br_2Cl	100	228.0	159.0	31.2
Cl_2Br	100	163.0	74.4	10.4



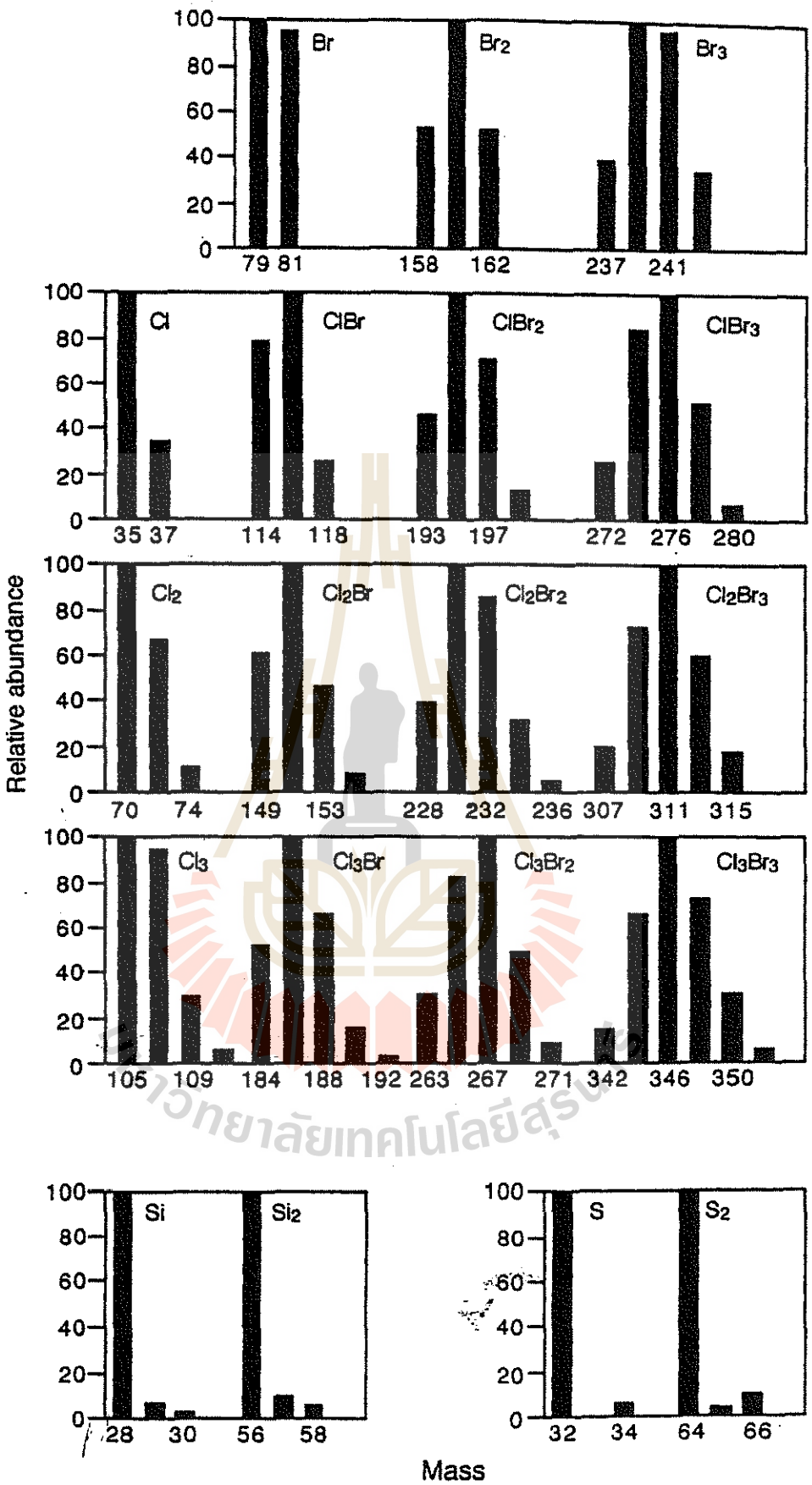
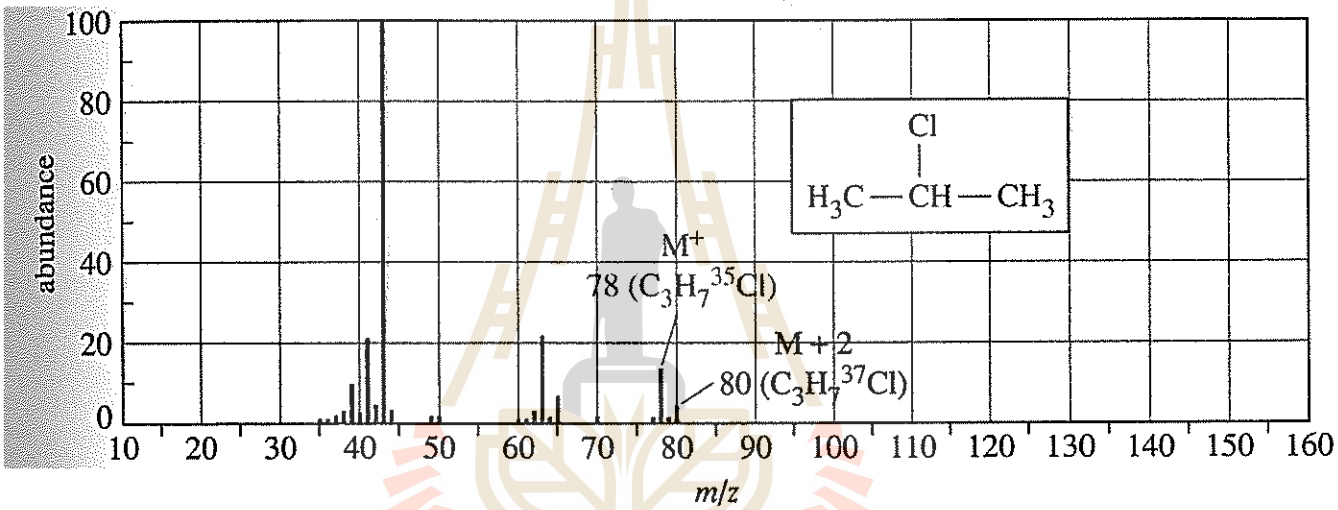


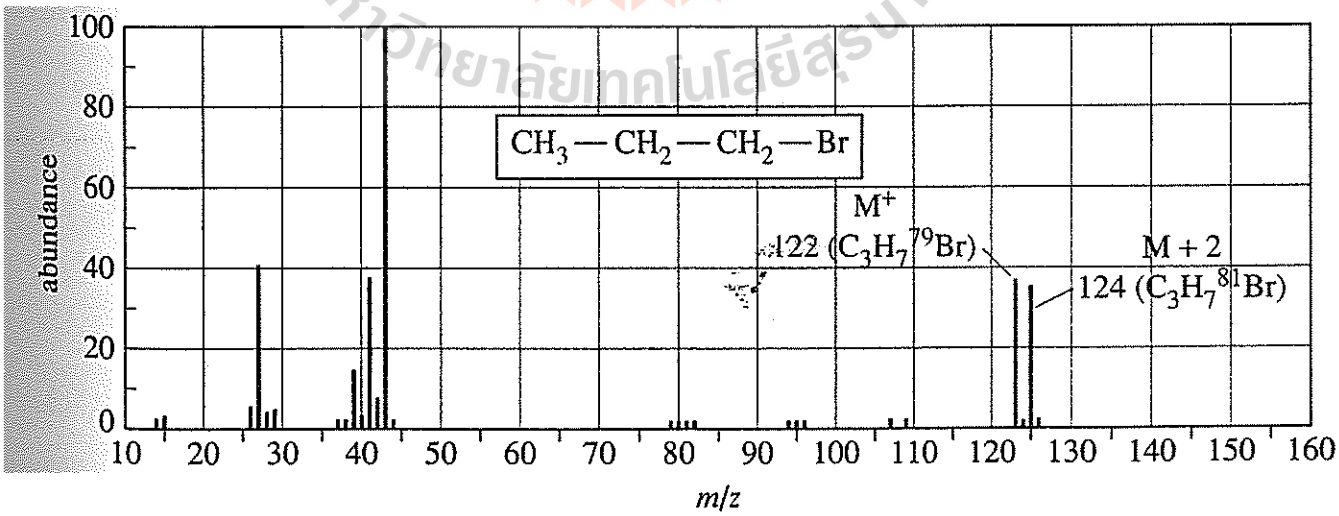
TABLE 12-4 Isotopic Composition of Some Common Elements

Element	*M+*		*M+1*		*M+2*	
hydrogen	^{1}H	100.0%				
carbon	^{12}C	98.9%	^{13}C	1.1%		
nitrogen	^{14}N	99.6%	^{15}N	0.4%		
oxygen	^{16}O	99.8%			^{18}O	0.2%
sulfur	^{32}S	95.0%	^{33}S	0.8%	^{34}S	4.2%
chlorine	^{35}Cl	75.5%			^{37}Cl	24.5%
bromine	^{79}Br	50.5%			^{81}Cl	49.5%
iodine	^{127}I	100.0%				

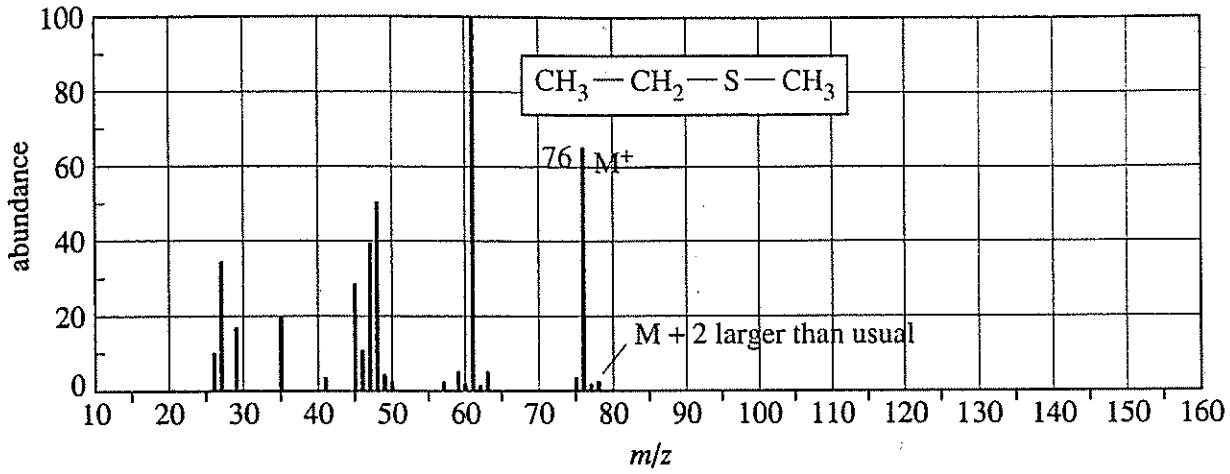
Pg. 530 Mass Spectrum of 2-chloropropane



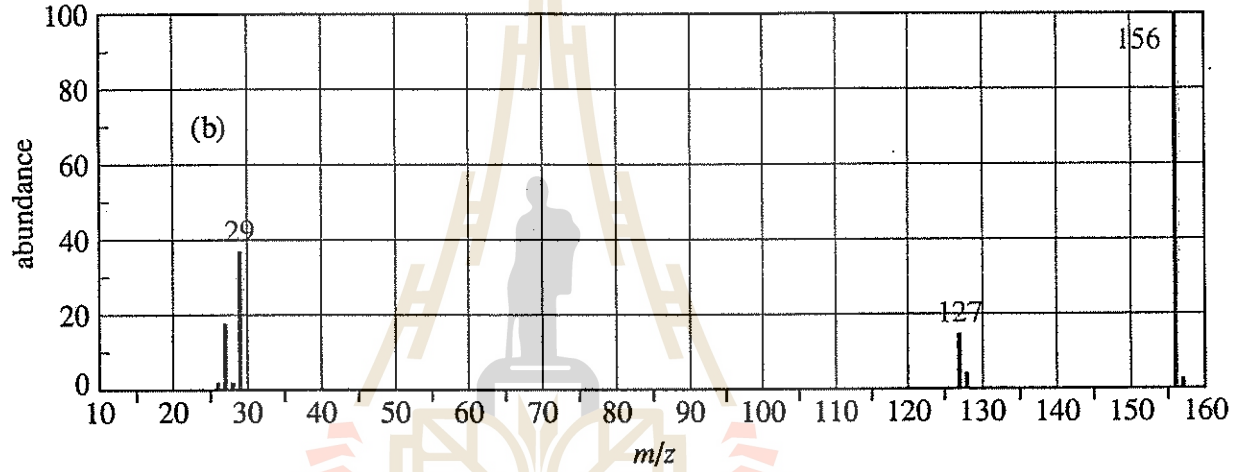
Pg. 530 Mass Spectrum of 1-bromopropane



Pg. 530 Mass Spectrum of Ethyl Methyl Sulfide



Prob. 12-7(b) Mass Spectrum of Iodoethane



Prob. 12-20 Mass Spectrum of 1-bromobutane

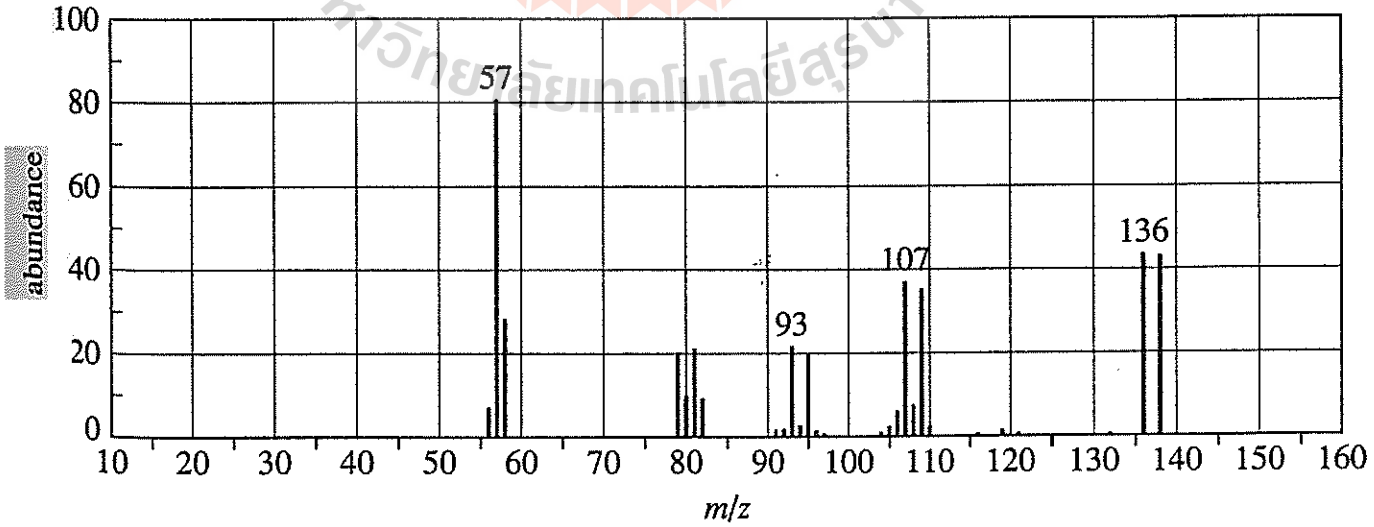


Table B2 Some common neutral losses from molecular ions

Mass	Groups Commonly Associated with the Mass Lost	Possible Inference
M–1	H	—
M–2	H_2	—
M–14	—	Homologue
M–15	CH_3	—
M–16	O	Nitro compound, *N*-oxide, sulphoxide
M–16	NH_2	$ArCONH_2$, $ArSO_2NH_2$
M–17	OH	$ArCO_2H$
M–18	H_2O	Alcohol, ketone, ether, etc.
M–19	F	Fluoro compound
M–20	HF	Fluoro compound
M–26	C_2H_2	Aromatic hydrocarbon, ArF
M–27	HCN	Aromatic amine or nitrile, nitrogen heterocycle
M–28	C_2H_4	Ethyl ester, aromatic ethyl ether, *n*-propyl ketone
M–28	CO	Quinone, phenol, oxygen heterocycle
M–29	C_2H_5	Ethyl group
M–29	CHO	Aromatic aldehyde, phenol
M–30	CH_2O	Aromatic methyl ether
M–30	NO	Aromatic nitro compound
M–31	CH_3O	Methyl ester, methyl ether
M–32	CH_3OH	Methyl ether, some methyl esters
M–33	SH	Thiol
M–33	H_2O+CH_3	—
M–34	H_2S	Thiol
M–35 & M–37	Cl	Chloro compound
M–36 & M–38	HCl	Chloro compound
M–41	C_3H_5	Propyl ester
M–42	CH_2CO	Acetamides, aromatic acetates
M–42	C_3H_6	Butyl ketone, aromatic propyl ether
M–43	C_3H_7	Propyl ketone
M–43	CH_3CO	Methyl ketone
M–44	CO_2	Anhydride, unsaturated ester
M–45	CO_2H	Carboxylic acid
M–45	C_2H_5O	Ethyl ester, ethyl ether
M–46	C_2H_5OH	Ethyl ether, some ethyl esters
M–46	NO_2	$ArNO_2$
M–48	CH_3SH	Methyl thioether
M–55	C_4H_7	Butyl ester
M–56	C_4H_8	Aromatic butyl ether, amyl ketone

Application of

LC - MS

Lecture

ผศ.ดร.สันติ ศักดารัตน์

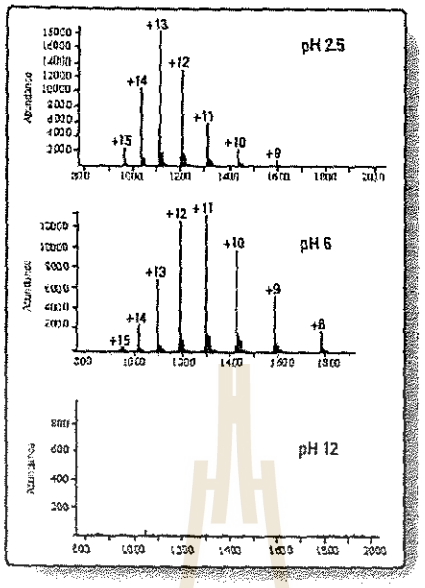


Figure 19. Effect of solvent pH on the abundance of multiply charged ions of the protein lysozyme in electrospray mode

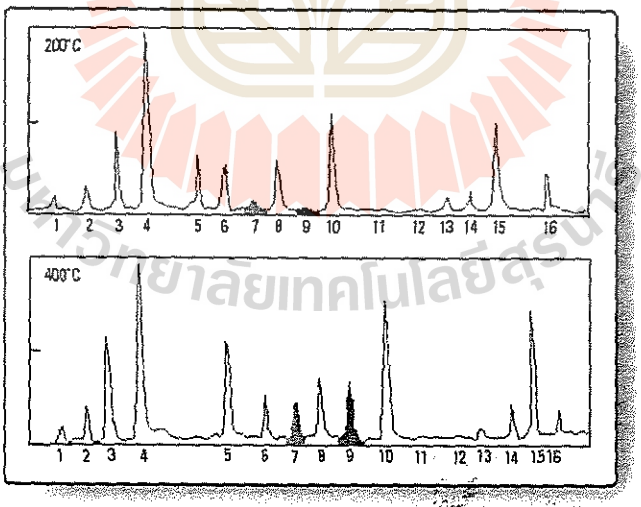


Figure 20. APCI analysis with an inadequate vaporizer temperature (200°C) yields poor results for some compounds compared to a more typical vaporizer temperature (400°C)

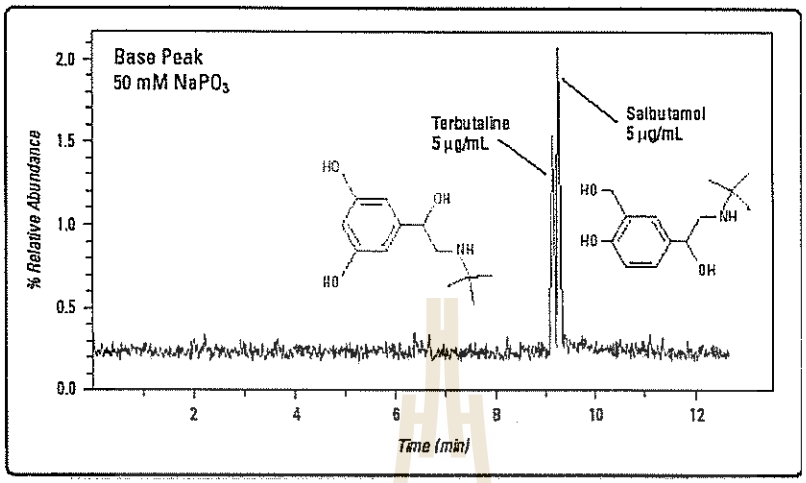


Figure 21. CE/MS/MS analysis of terbutaline and salbutamol shows good chromatographic separation and good signal even in the presence of a high concentration of sodium phosphate salt

Structural determination of ginsenosides using MSn analysis

Ginseng root, a traditional Chinese herbal remedy, contains more than a dozen biologically active saponins called ginsenosides. Since most ginsenosides contain multiple oligosaccharide chains at different positions in the molecule, structural elucidation of these compounds can be quite complicated.

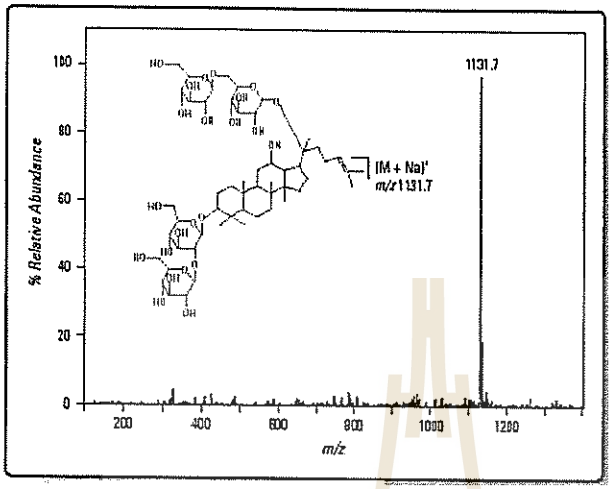


Figure 24. Full scan mass spectrum of ginsenoside Rb1 showing primarily sodium adduct ions

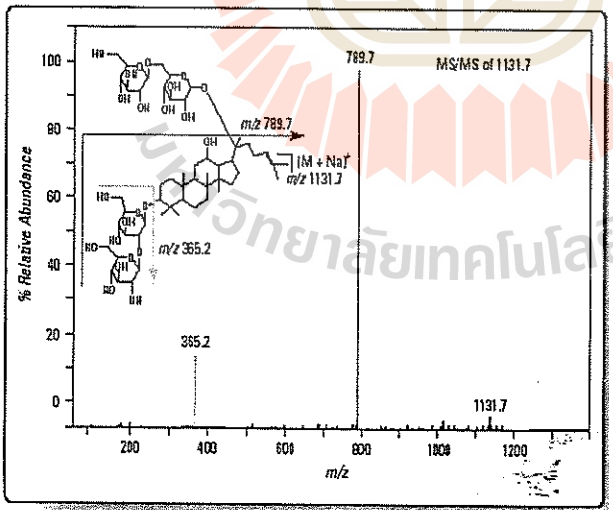
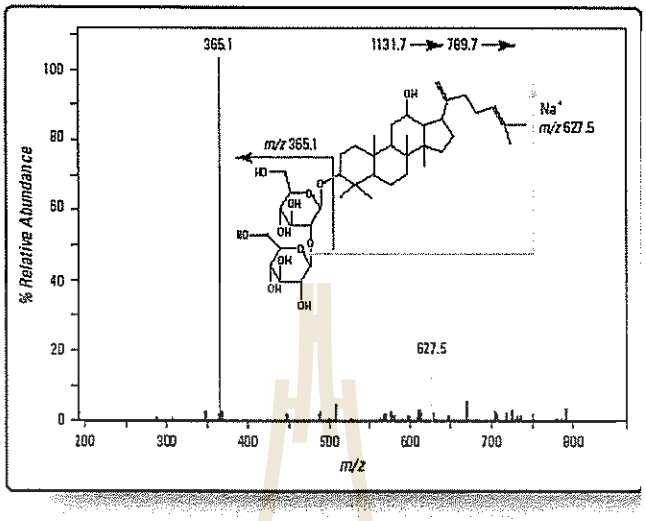


Figure 25. Full scan product ion (MS/MS) spectrum from the sodium adduct at m/z 1131.7

Figure 26. Subsequent full scan product ion spectrum (MS^2) from the ion at *m/z* 789.7



Pharmaceutical Applications

Rapid chromatography

of benzodiazepines

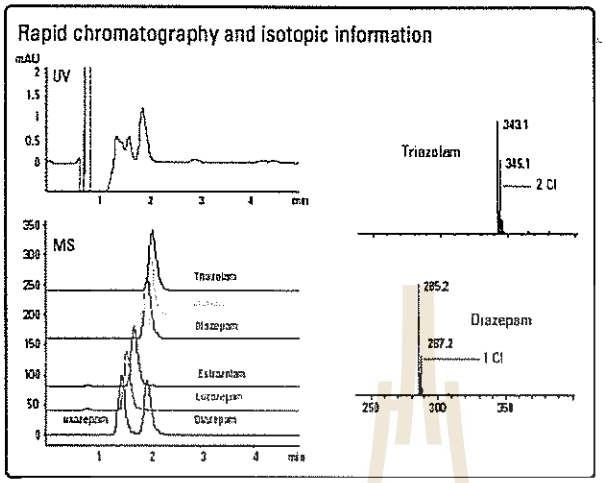


Figure 27. MS identification and quantification of individual benzodiazepines in an incompletely resolved mixture

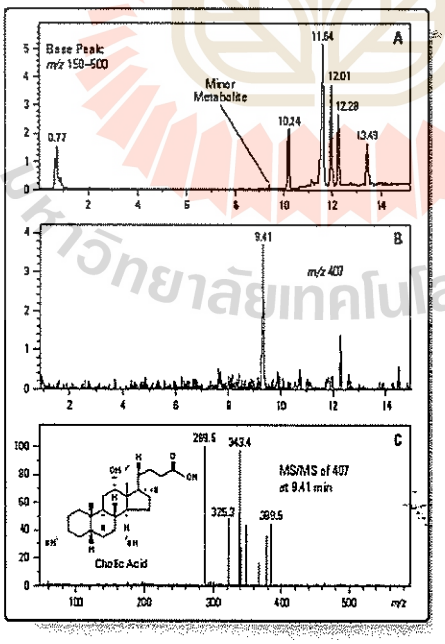


Figure 28. Identification of a minor metabolite of deoxycholic acid through MS/MS

Clinical Applications

High-sensitivity detection of

trimipramine and thioridazine

Food Applications

Identification of aflatoxins in food

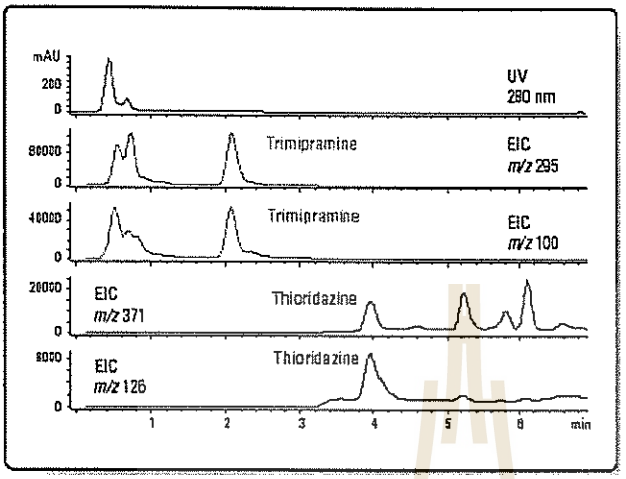


Figure 31. Trimipremine and thioridazine in a urine extract

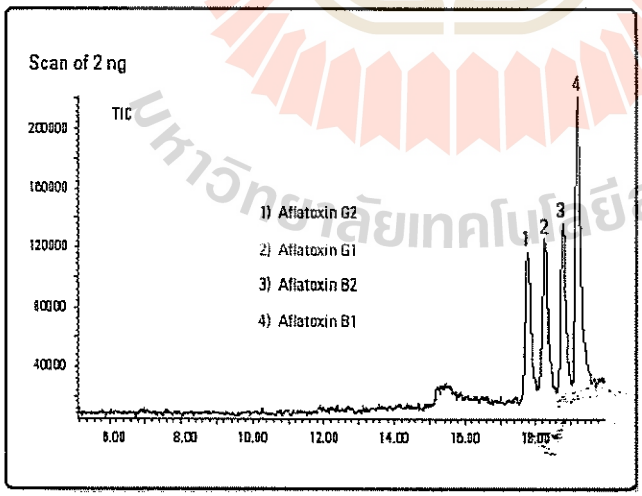
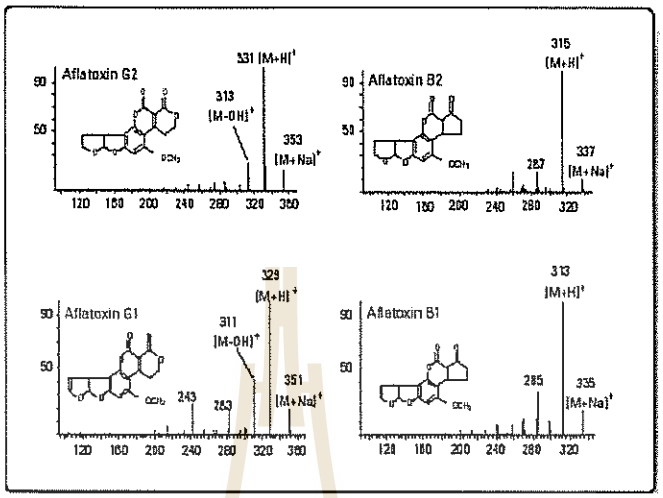


Figure 32. Total ion chromatogram of a mixture of aflatoxins

Figure 33. Unique mass spectra allow positive identification of structurally similar aflatoxins



Determination of vitamin D3 in

poultry feed supplements using MS3

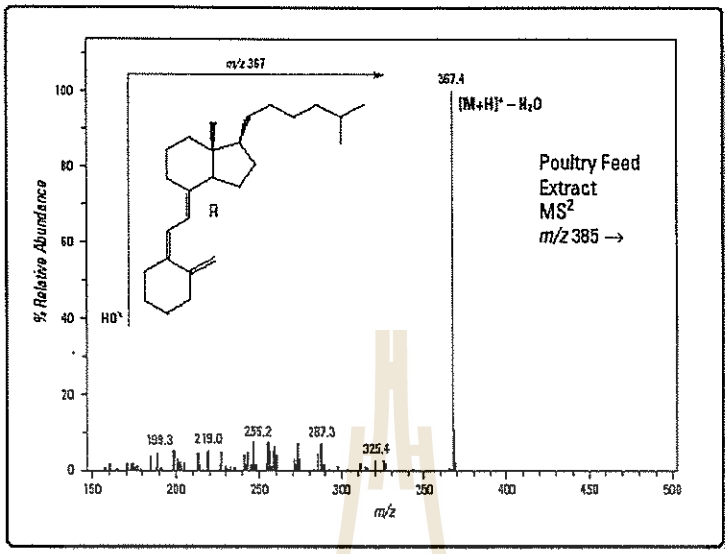


Figure 34. Full scan MS/MS product ion spectrum from the precursor ion at *m/z* 385 showing primarily the nonspecific loss of a water molecule

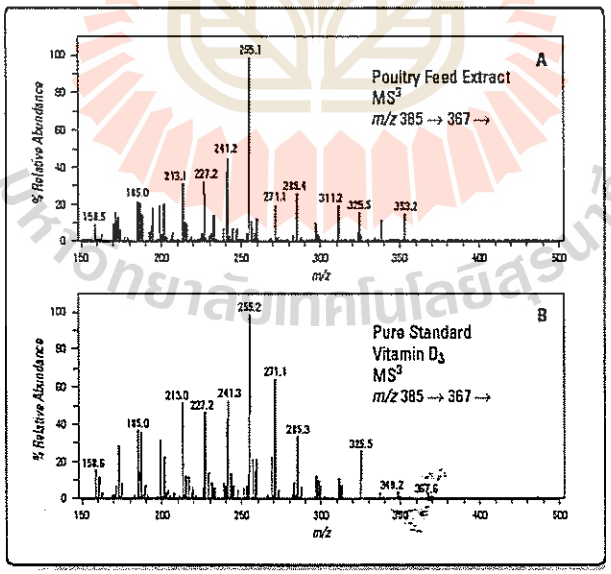


Figure 35. Full scan MS^3 product ion spectra show much more structural information

Environmental Applications

Detection of phenylurea herbicides

Detection of low levels

of carbaryl in food

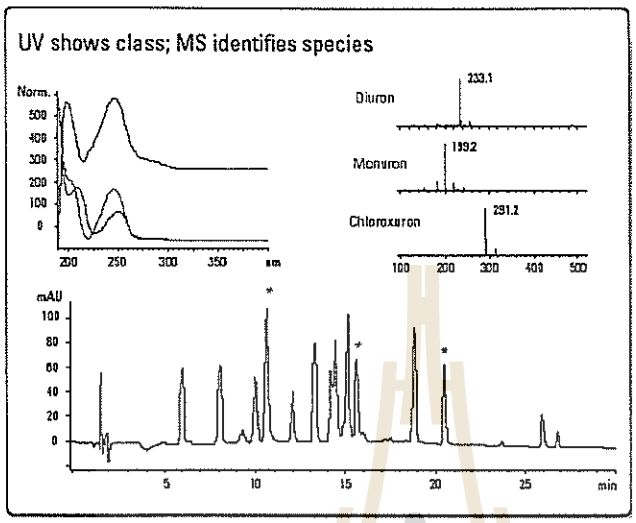


Figure 36. Chromatogram of phenylurea herbicide with UV and MS spectra

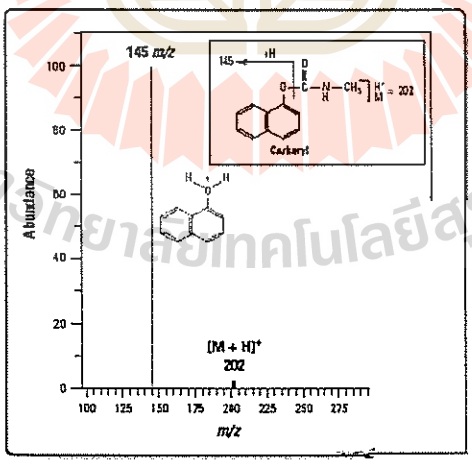


Figure 37. Full scan product ion spectrum generated by collision-induced dissociation of the $(M + H)^+$ carbaryl ion at *m/z* 202

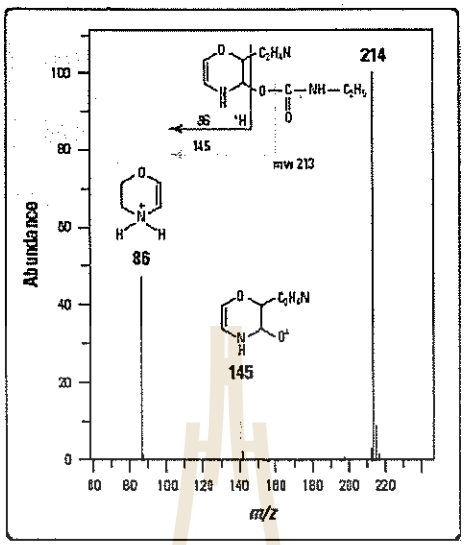


Figure 38. Full scan product ion spectrum of coeluting compound that produced false positives in HPLC fluorescence analysis

Determination of Digoxin in Human Serum by LC/MS with Online Sample Preparation

Digitalis glycosides are the classic drugs for the treatment of congestive heart failure.

Of these, digoxin (Figure 1) is one of the most prescribed cardiac glycosides.

Although digoxin has been used for more than 200 years for the therapy of heart failure, it is still not possible to prepare digoxin synthetically.

Digoxin is extracted from the leaves and seeds of the Digitalis lanata (foxglove).

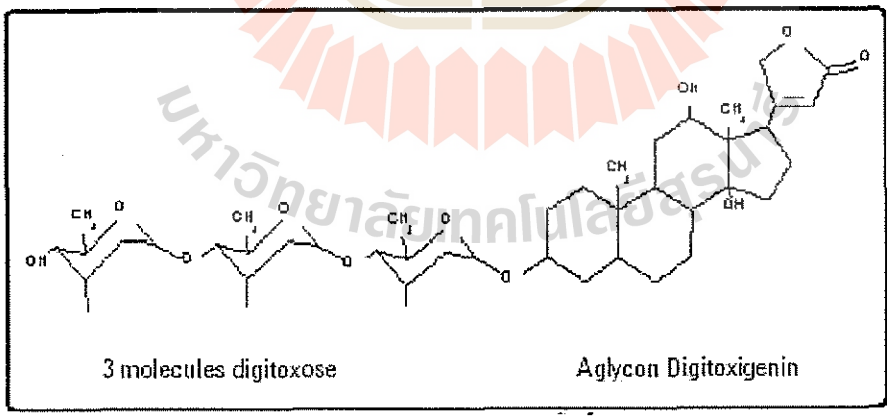


Figure 1. Chemical structure of digoxin

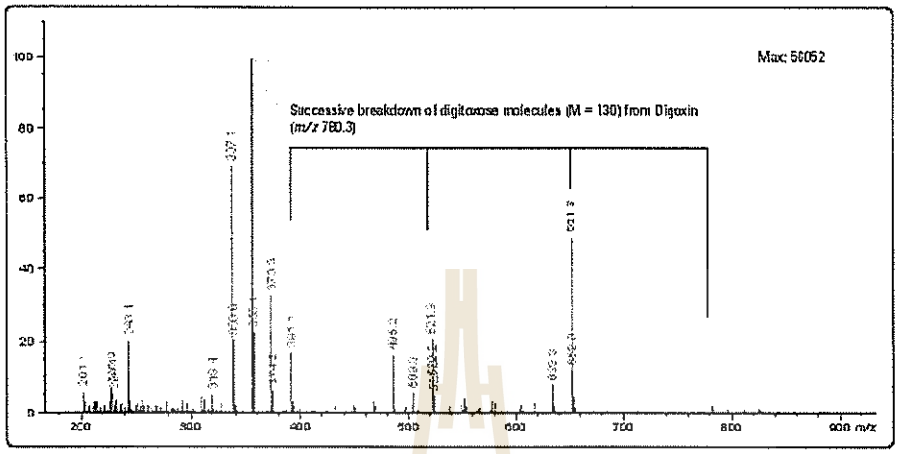


Figure 4. Mass spectra of digoxin acquired using atmospheric pressure chemical ionization

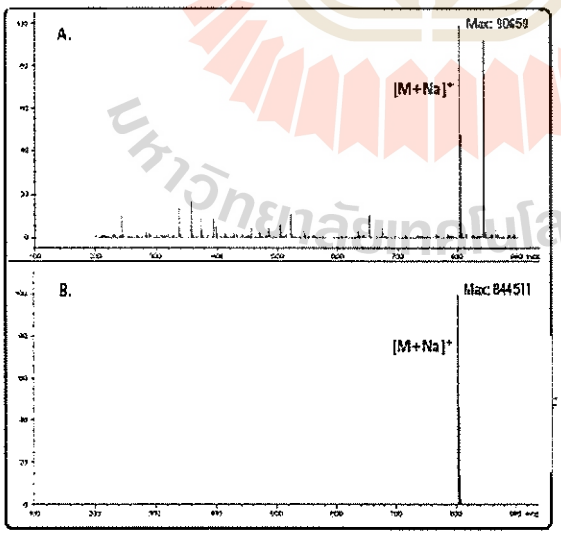
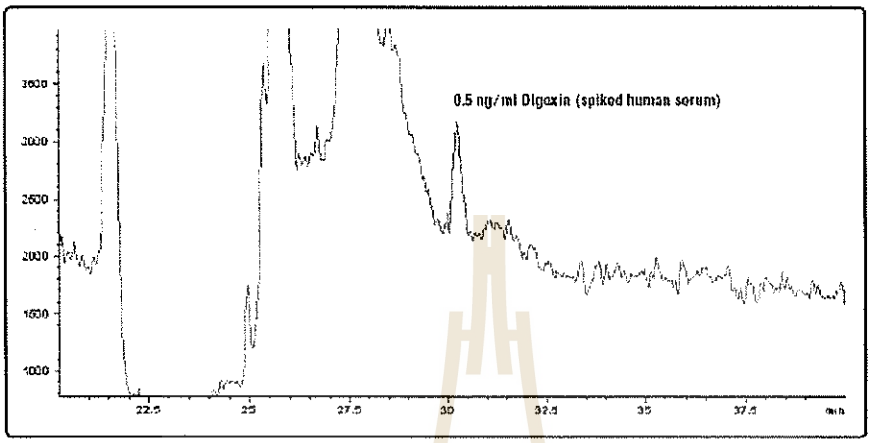


Figure 5. Mass spectra from electrospray ionization of digoxin using: (A) 1 mM ammonium acetate buffer and (B) 1 mM sodium citrate buffer

Figure 6. Chromatogram of digoxin in human serum at the limit of quantification



Development of an LC/MS Method for the Analysis of Rodenticides

Rodents such as rats and mice must be controlled because they destroy food supplies and serve as vector hosts for human diseases such as hantavirus.

Although individual animals or small groups can be removed by trapping, rodenticides are frequently used in rodent control.

Most rodenticides are also toxic to humans and domesticated animals such as dogs.

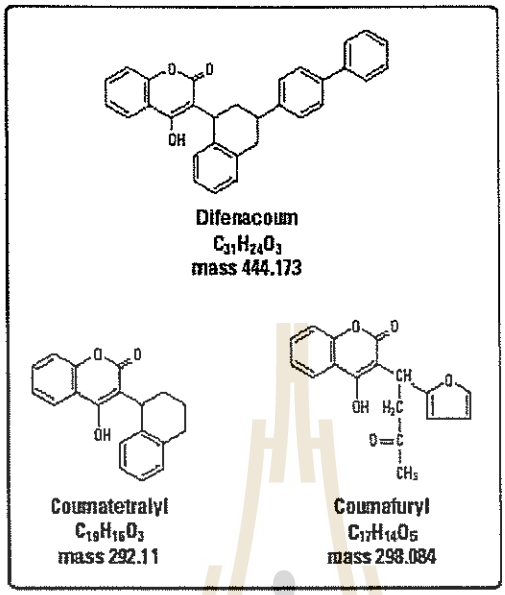


Figure 1. Coumarin rodenticides.

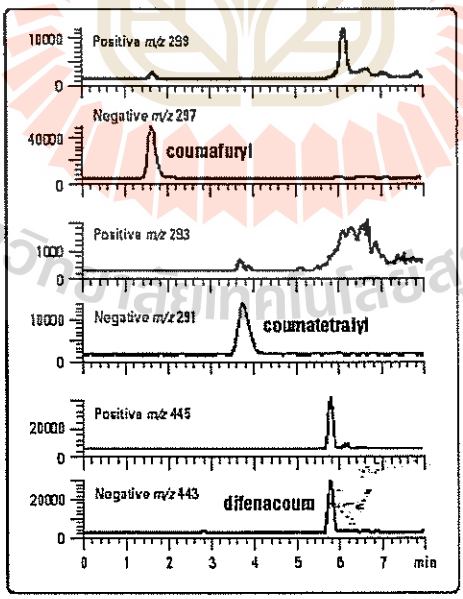


Figure 2. Comparison of positive and negative ionization modes for electrospray LC/MS.

ANALYSIS METHOD

Chromatographic Conditions

Column:	150 × 2.1 mm Zorbax® XDB-C18, 5 µm (p/n 993700-902)
Mobile phase:	A = 2 mM ammonium acetate in water B = methanol
Gradient:	start with 30% B at 2 min 50% B at 4 min 100% B
Flow rate:	0.4 ml/min from 0 to 2 min, then 0.5 ml/min
Column temp:	50°C
Injection vol:	2 µl
Diode-array detector:	signal: 280, 18 nm; reference: 550, 100 nm

ESI-MS Conditions

Source:	ESI
Drying gas flow:	10 l/min
Nebulizer:	40 psig
Drying gas temp:	350°C
Vcap:	2500 V (positive and negative)
Stepsize:	0.1
Peakwidth:	0.2 min
Time filter:	On
Scan:	*m/z* 150–500
SIM ions (negative mode):	296, 297, 298 Coumafuryl 290, 291, 292 Coumatetralyl 442, 443, 444 Difenacoum
Fragmentor:	variable 180 V (50–275) 100 V (280) 120 V (400)

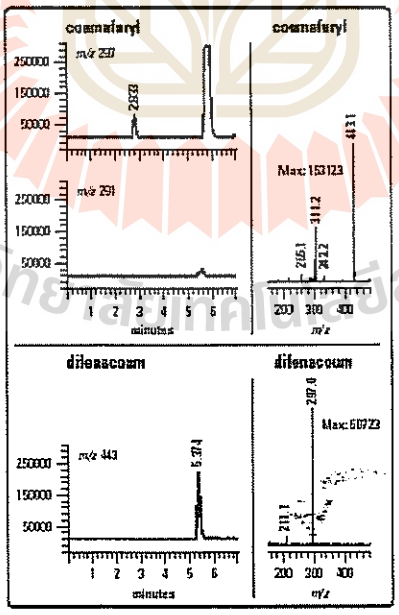


Figure 3. Extracted ion chromatograms (left) and mass spectra (right) from the analysis of sausage extract spiked with 5 ppm of coumafuryl and difenacoum. Data was collected in negative ion using scan mode.

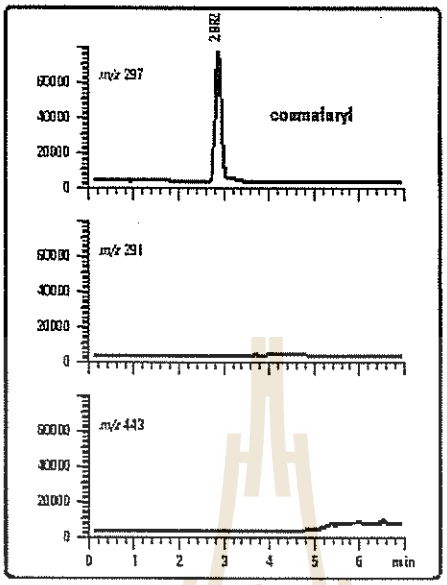


Figure 4. Extracted ion chromatograms from the analysis of dog stomach extract spiked with 0.5 ppm of coumafuryl. Data was collected in negative ion using SIM mode.

Determination of Cocaine and Metabolites in Urine Using Electrospray LC/MS

A rapid, simple, and sensitive electrospray LC/MS method has been developed for the quantitative analysis of cocaine and benzoylecgonine in urine using electrospray. Urine samples were extracted using solid phase extraction cartridges, and the drug and metabolite were analyzed without derivatization using an isocratic separation and selected ion monitoring (SIM).

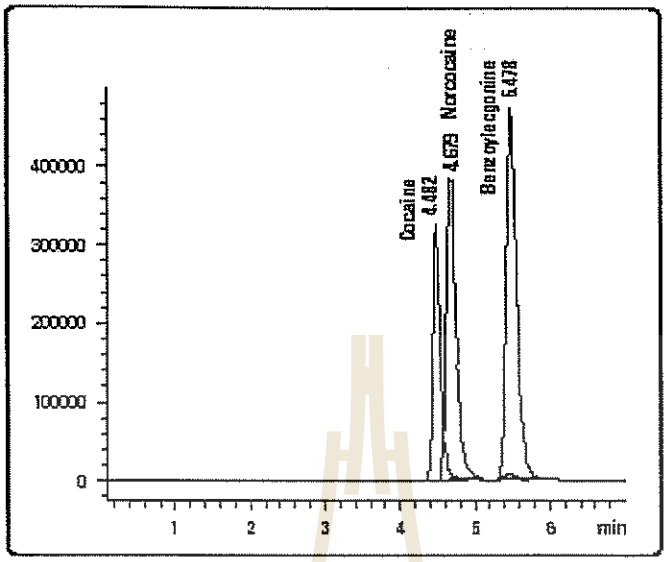


Figure 1. Isocratic separation of cocaine, norcocaine and BE.

Chromatographic Conditions	
Column:	Metasil Basic 3 µm, 3 × 150 mm (Metachem)
Mobile phase:	A = 0.1% formic acid in water
	B = methanol
Isocratic:	51% B
Flow rate:	0.2 mL/min
Column temp:	40°C
Injection vol:	20 µl
Diode-array detector:	signal: 234, 8 nm; reference: 360, 100 nm
MS Conditions	
Source:	ESI
Ionization mode:	positive
Vcap:	1500 V
Nebulizer:	20 psig
Drying gas flow:	10 L/min
Drying gas temp:	300°C
SIM ions:	*m/z* 290.1 (BE and norcocaine)
	m/z 293.1 (BE-d3)
	m/z 304.1 (cocaine)
	m/z 307.1 (cocaine –d3)
Peak width:	0.10 min
Time filter:	On
Fragmentor:	70 V

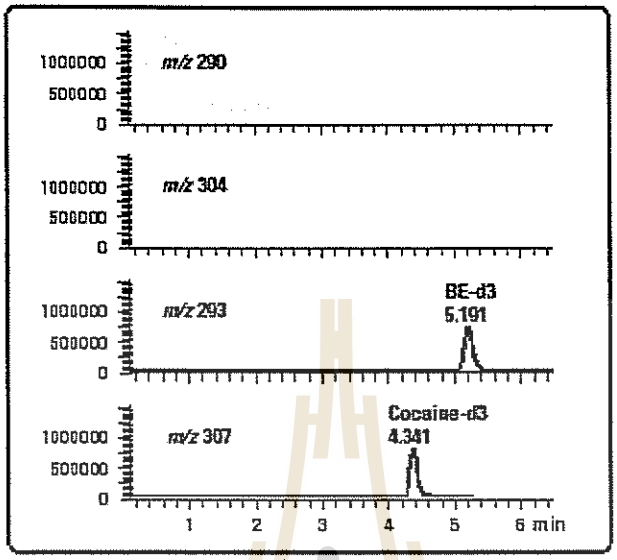


Figure 2. Extracted ion chromatograms of blank urine extract.

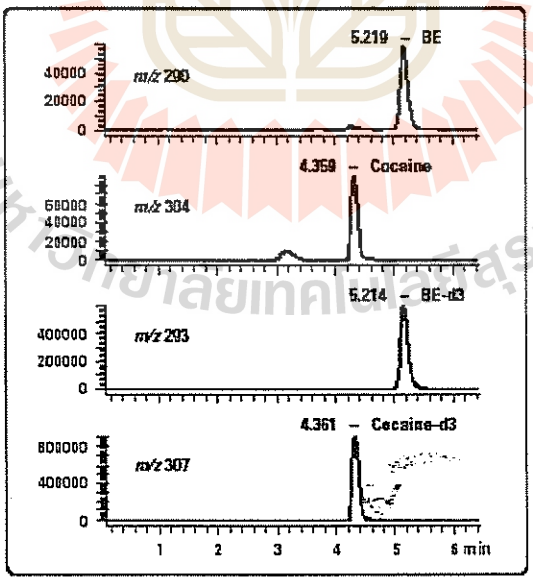


Figure 3. Extracted ion chromatograms of fortified urine extract (25 ng/mL).

Table 1. Method accuracy and precision. Target concentrations were 50 ng/mL for cocaine and 150 ng/mL for BE.

	Cocaine	BE
	48.25	146.47
	47.06	155.69
	47.41	158.97
	46.21	148.50
	38.80	147.29
	40.89	146.57
	41.38	167.06
	42.08	159.81
Mean	44.085	153.795
Std Dev	3.570	7.734
C.V.*	7.1%	5.1%

*coefficient of variation = (mean/target)*100

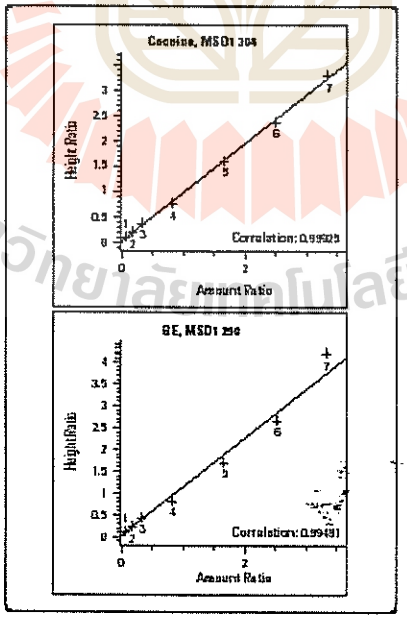


Figure 4. Calibration curves for cocaine and BE.

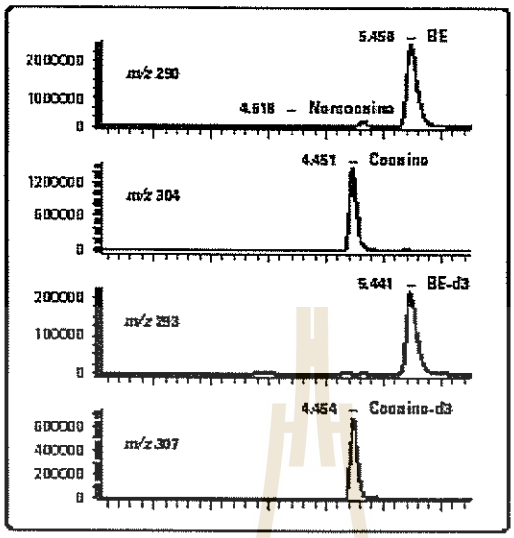


Figure 5. Extracted ion chromatograms from the extract of a positive urine sample.

Multisignal LC/MS Analysis for Compound Screening

Acquiring MS information using different acquisition modes within a single sample analysis is a powerful way to improve productivity in LC/MS compound screening and method development.

It speeds analyses and makes it easier to use a single, generic method for LC/MS screening.

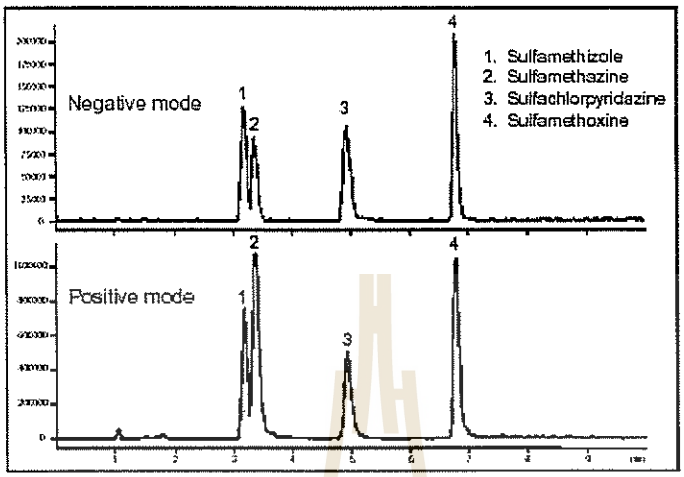


Figure 1. ESI-LC/MS analysis of sulfonamide antibiotics using positive/negative switching mode.

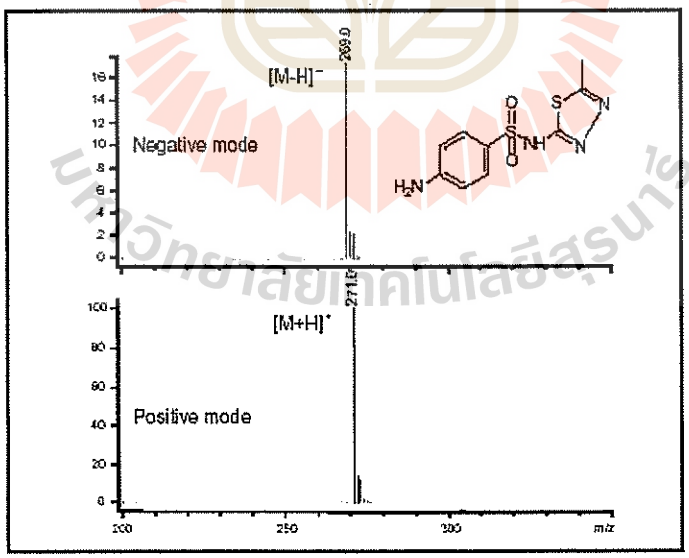


Figure 2. Mass spectra of sulfamethizole from the chromatogram in Figure 1.

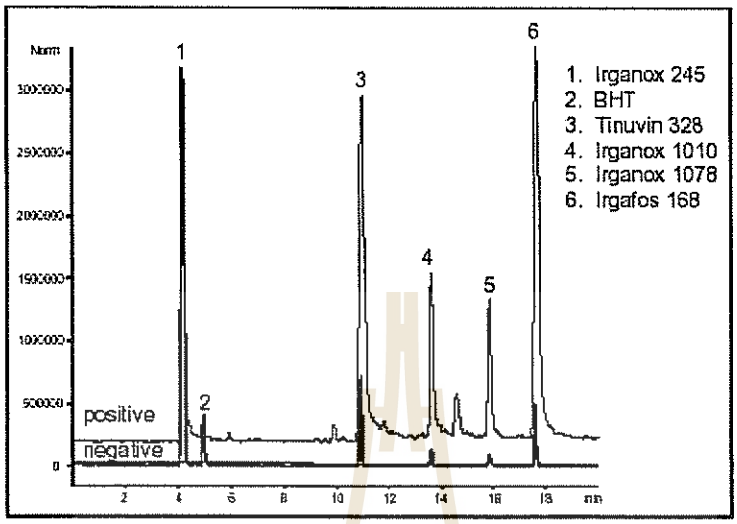


Figure 3. APCI-LC/MS analysis of a mixture of polymer additives.

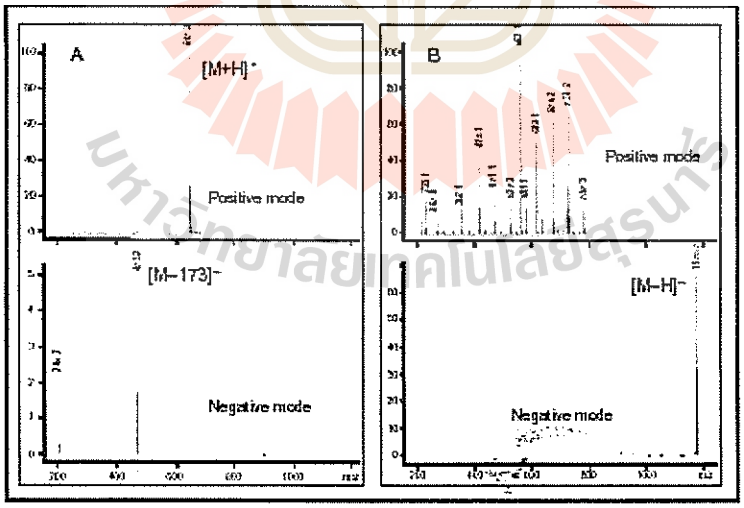


Figure 4. Mass spectra for (A) Irgafos 168 and (B) Irganox 1010 from chromatogram in Figure 3.

ANALYSIS METHOD:

Chromatographic Conditions

Column:	15 × 3 mm Zorbax® SB-C18, 3.5 µm (p/n 863954-302)
Mobile phase:	A = water B= methanol
Gradient	start with 75% B at 5 min, 90% B at 14 min, 100%B
Flow rate:	0.8 ml/min
Column temperature:	50°C
Injection volume:	5 µl of 400 ppm per component
Diode-array detector:	Signal 220, 10 nm

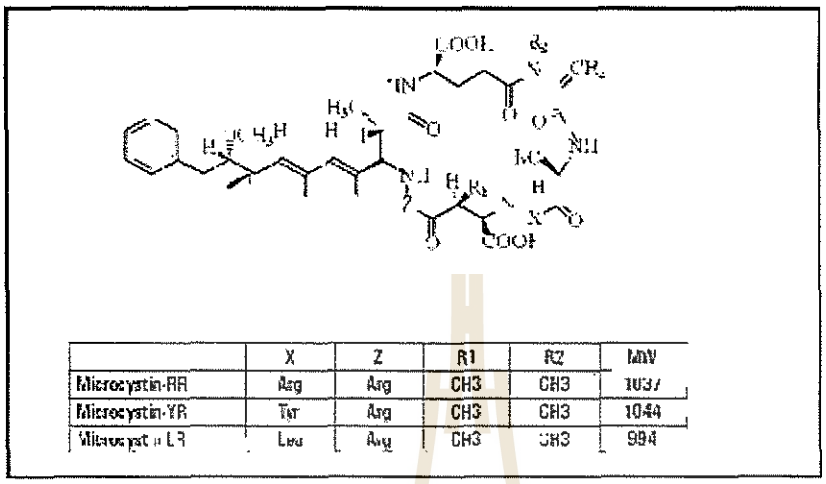
MS Conditions

Source:	APCI
Drying gas flow:	5 l/min
Nebulizer:	60 psig
Drying gas temperature:	350°C
Vaporizer:	400°C
Vcap:	3000 V (positive); 3000 V (negative)
Corona:	4 µA (positive); 20 µA (negative)
Stepsize:	0.1
Peakwidth:	0.15 min
Time filter:	On
MS Signal 1:	Ion mode: Negative Scan: 200–1200 amu Fragmentor: 150 V
MS Signal 2:	Ion mode: Positive Scan: 200–1200 amu Fragmentor: 80 V

LC/MS Analysis of Microcystins in Freshwater by Electrospray Ionization

The occurrence of toxic fresh water blooms of cyanobacteria (blue-green) has been reported in many countries.

These toxic waterblooms have cause the deaths of domestic animals and wildlife and illnesses in humans.



	X	Z	R1	R2	MW
Microcystin-RR	Arg	Arg	CH3	CH3	1037
Microcystin-YR	Tyr	Arg	CH3	CH3	1044
Microcystin-LR	Leu	Arg	CH3	CH3	994

Figure 1. Structure of microcystins.

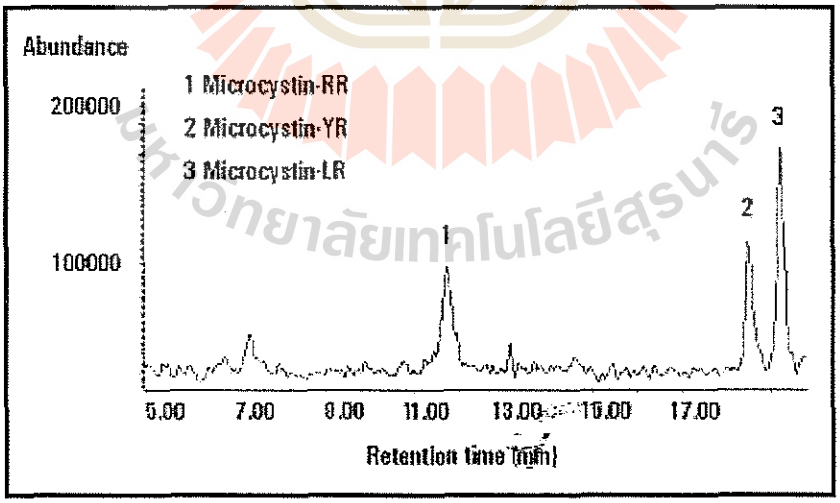


Figure 2. Total ion chromatogram of microcystin standards at 1 ng/uL

Chromatographic Conditions	
Guard column:	Mytisil ODS (10 mm x 2.1mm 5 μm)
Column:	10 x 2.1 mm Mytisil ODS, 5 μm
Mobile phase:	A – 0.2% formic acid in water B – acetonitrile
Gradient conditions:	Start with 10% B at 20 min 55% B
Flow rate:	0.8 ml/min
Injection vol:	100 μl

MS Conditions	
Source:	ESI
Ion mode:	Positive
Vcap:	4000 V
Nebulizer:	50 psig
Drying gas flow:	10 l/min
Drying gas temp:	350 °C
Scan range:	100-1200 amu
SIM target ions:	520, 1045, 995
Fragmentor:	Variable 120 V (520); 160 V (995, 1045)
Step size:	0.1 amu
Peak width:	0.15 min

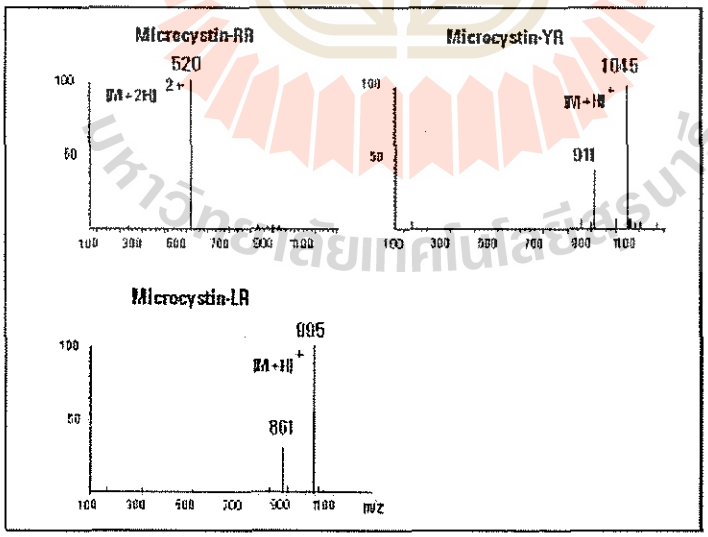


Figure 3. Mass spectra of microcystin standards at 1 ng/ul.

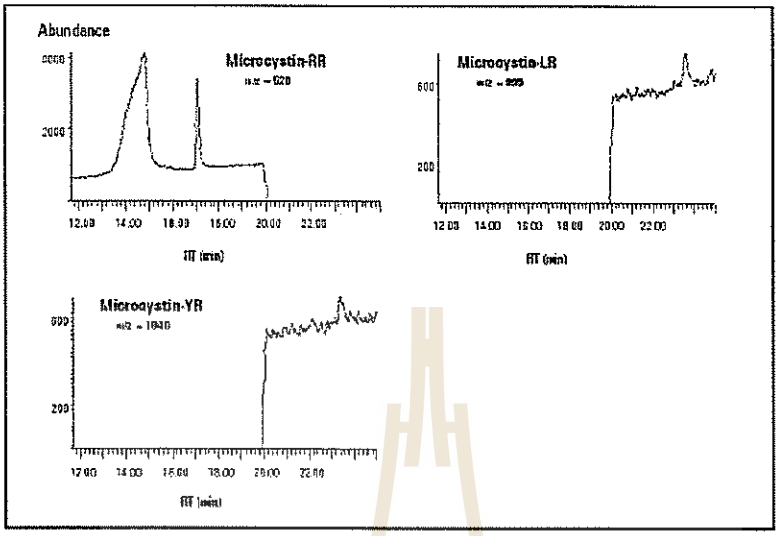


Figure 4. Extracted ion chromatograms of microcystins spiked into blank freshwater (2 pg/ml).

Table 1. Recovery of microcystins from freshwater

	Spike level (pg/ml)	Recovery %	RSD % (n = 5)
Microcystin-RR	50	93.1	2.1
	20	90.3	3.3
	5	88.2	3.9
Microcystin-YR	50	94.1	2.9
	20	89.2	3.7
	5	85.5	5.3
Microcystin-LR	50	90.3	3.2
	20	87.3	4.7
	5	86.2	5.1

Analysis of Paclitaxel by LC/MS

Paclitaxel is a natural diterpenoid that is isolated from the bark of yew trees.

It is an antitumor and antileukemic agent that is effective against ovarian and breast carcinomas.

Paclitaxel interferes with cell division by blocking the ability of the cell to break down the mitotic spindle during mitosis.

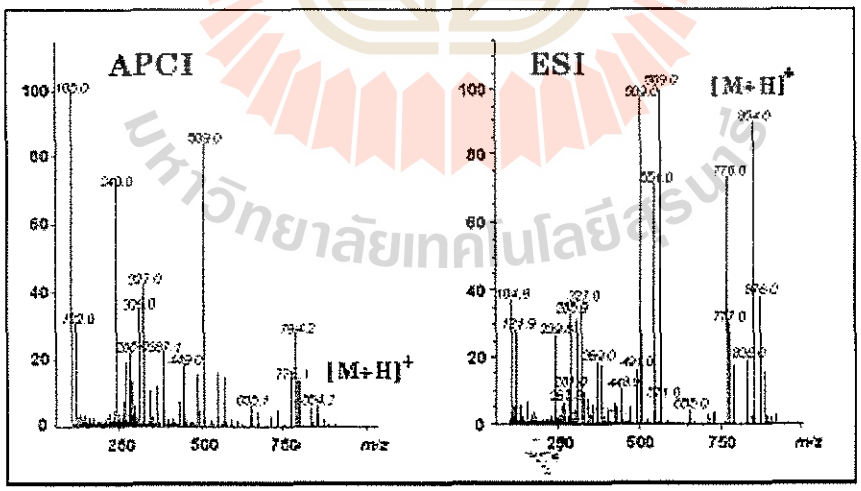


Figure 1. Mass spectra of paclitaxel by APCI and ESI-LC/MS

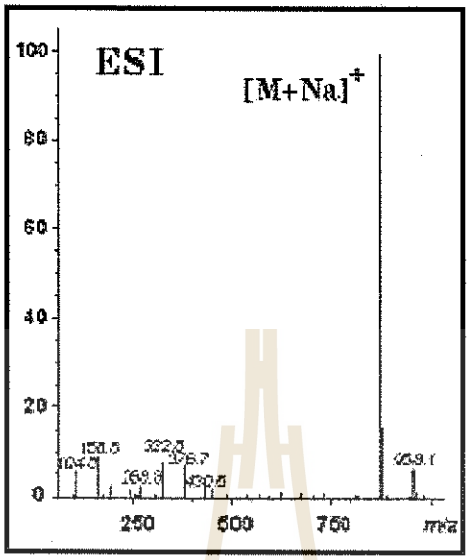


Figure 3. Mass spectrum of paclitaxel with sodium adduction.

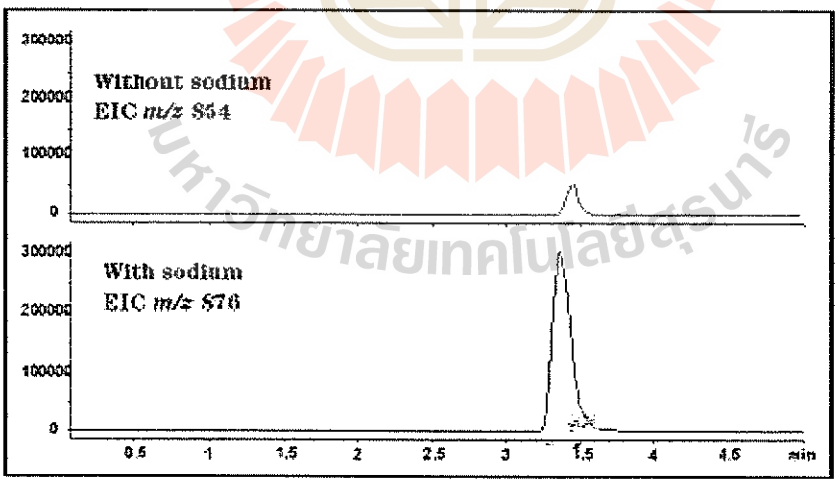


Figure 4. Chromatography of paxlitaxel with and without sodium acetate in the mobile phase.

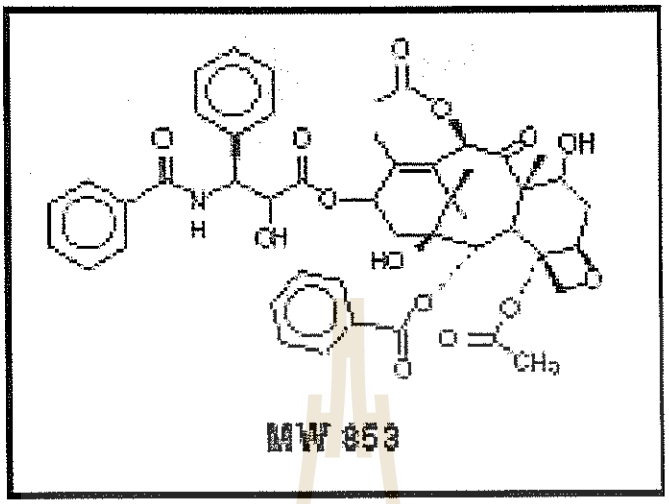


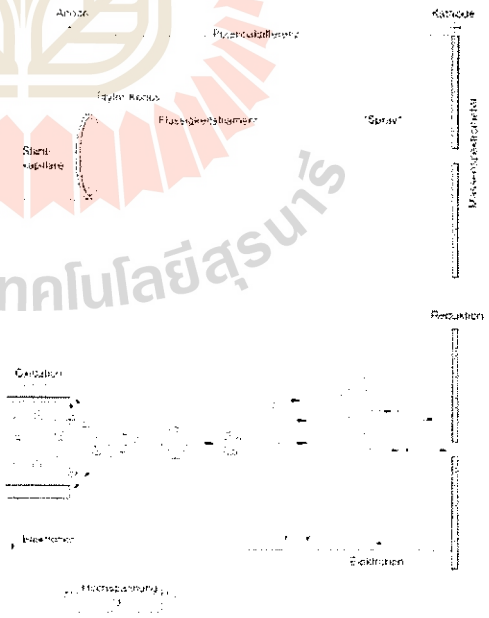
Figure 2. The structure of paclitaxel.

Mass Spectrometry of Biomolecules

- Electrospray: Principles
- Isotope Pattern of peptides and proteins
- Deconvolution
- Quadrupole and ion trap
- Collision induced fragmentation
- MALDI

Elektrospray: Ionisation

- Droplets are formed
- They loose solvent
- They decay (Coulomb Explosion)
- Proteins are desorbed from small droplets



Features relevant for Biomolecules

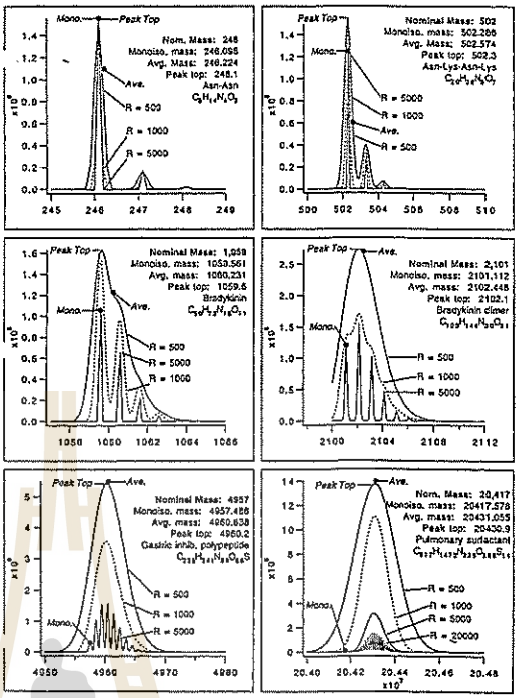
- Evaporation has to be assisted by volatile **organic solvents** (=> denaturation)
- High **charge density** prior to desorption
- All ions (paticularly **Na^+**) are concentrated inside the droplets leading to salt adducts
- **Detergents**, particularly ionic ones, would prevent desorption of proteins
- **Ion clusters** such as (M + Na^+ + M) can be formed

Sample preparation

- Use low salt
- Use volatile salts like NH_4HCO_3
- No Ionic detergents
- Add volatile organic solvents like MeOH or acetonitrile
- Electrospray is concentration dependent, therefore use relatively high (μM) concentrations with low flow rate.

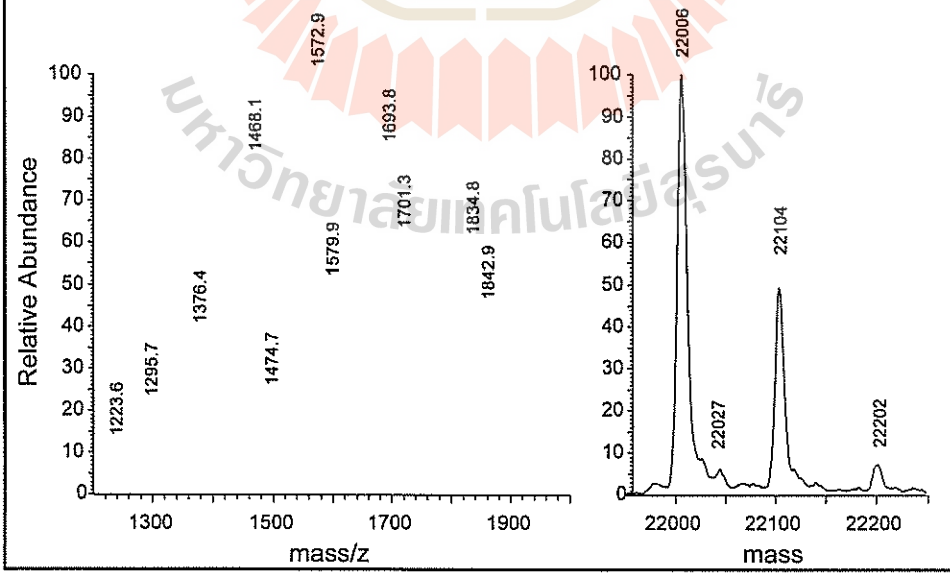
Isotope distribution for peptides and proteins

- **Monoisotopic mass** for Peptides, sugars and oligonucleotides
- **Average mass** for proteins and large biopolymers



ESI-MS: No Ionisation

Ion distribution from solution; No decay of macromolecules



Deconvolution of macromolecules

Proteins usually are charged by protonation

The proton concentration can be exremely high during droplet formation

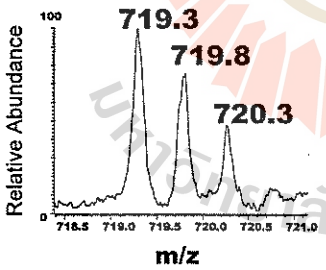
M => $M+nH^+$;

Any mass spectrometer can only detect m/z

The neutral mass can be computed as:

$M = (M_{observed} * n) - n$

Charge state



Isotopes of one element differ by at least 1 mass unit

How do you explain isotpe differences <1 ?

All mass spectromes detect m/z (Masse / Ladung), not the mass!

For positively charged peptides or proteins:

Neutral Mass = m/z * z (Ladung) - z (Protonen)

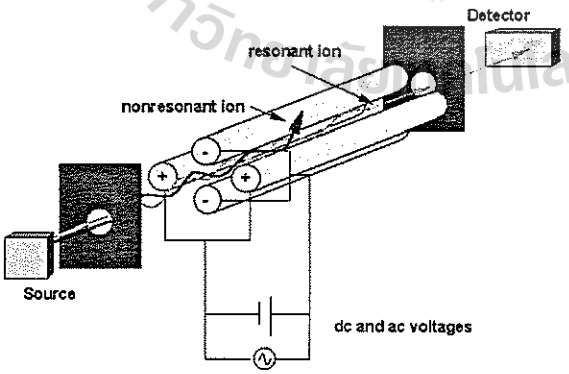
z = 1 / ?m

Ion detectors

- Quadrupole
- Ion trap
- Triple quadrupole
- Collision induced fragmentation
- MS/MS, MS^n

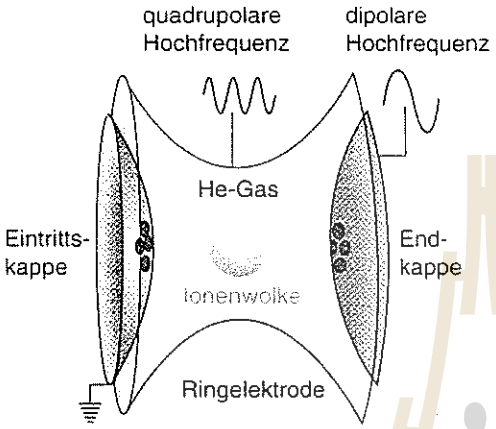
A quadrupole mass filter consists of four parallel metal rods

Two opposite rods have an applied potential of (U+Vcos(wt)) and the other two rods have a potential of -(U+Vcos(wt)), where U is a dc voltage and Vcos(wt) is an ac voltage.



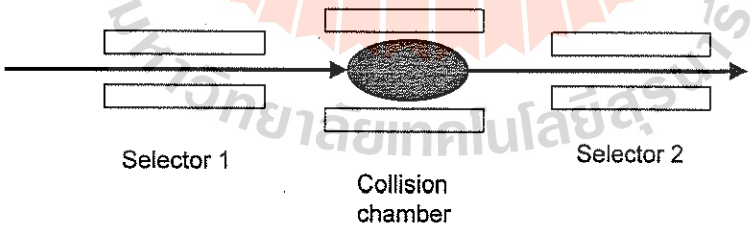
A mass spectrum is obtained by monitoring the ions passing through the quadrupole filter as the voltages on the rods are varied. There are two methods: varying w and holding U and V constant, or varying U and V (U/V) fixed for a constant w.

Ion Trap



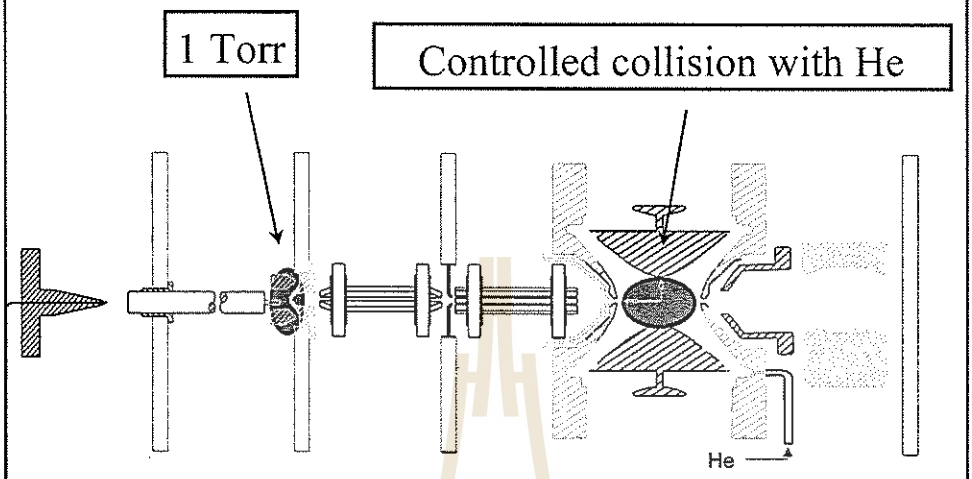
An ion trap can be regarded as a quadrupole with rods bent upon themselves.

Triple Quadrupole

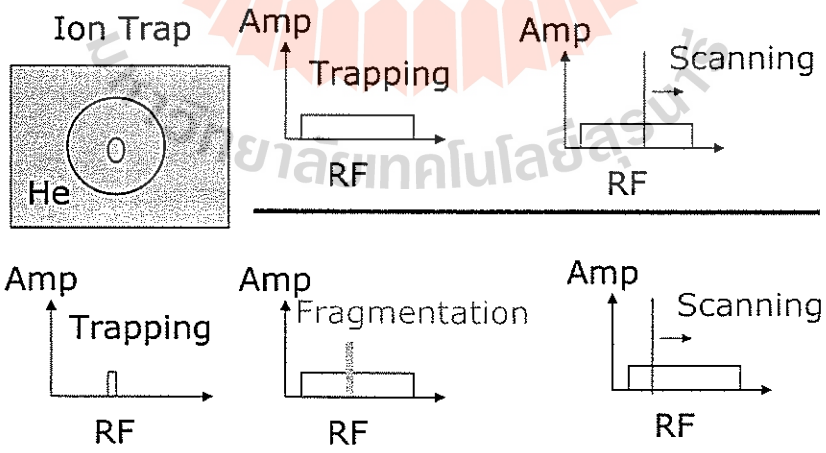


Daughter Scan:	1 fixed,	2 varied
Parent Scan:	1 varied,	2 fixed
Neutal Loss	1 varied,	2 varied, but constant difference

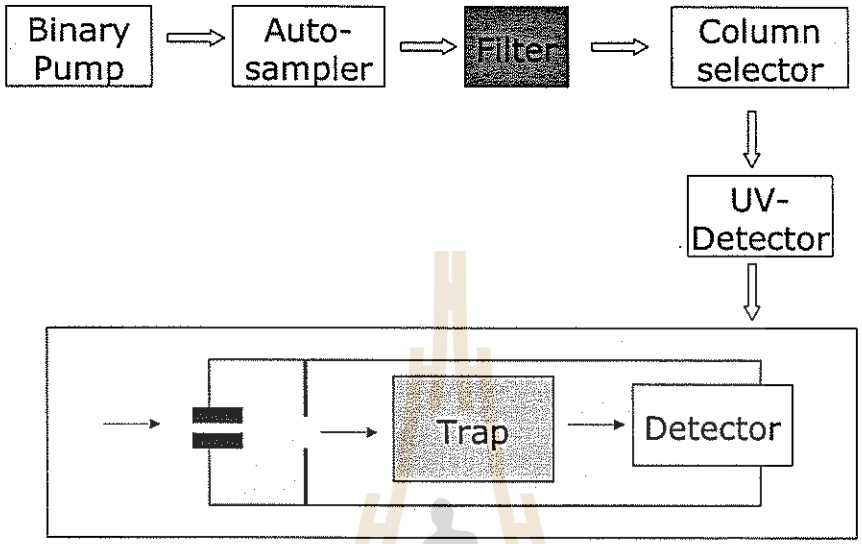
Collision induced decay (CID)



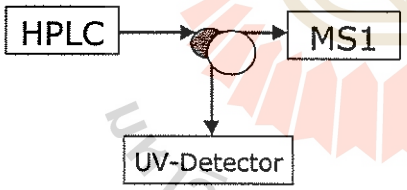
MS-MS: Kontrollierte Fragmentierung



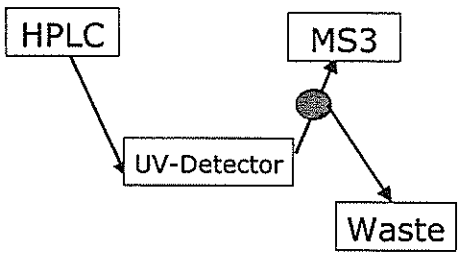
HPLC-MS (general set-up)



Splitter or linear flow



- „Waste" = Split
- High flow rates
- Selector for ESI
- Low back pressure
- High UV sensitivity

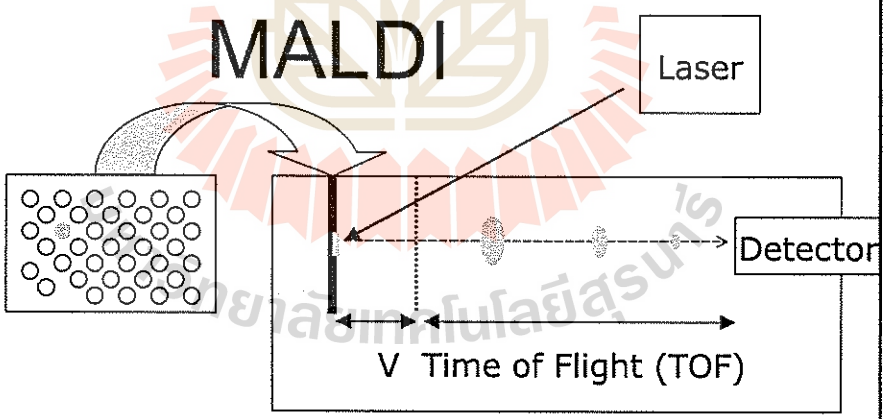


- „Waste" = Waste
- Low flow rates
- No salt for MS
- High back pressure
- low UV sensitivity

Calibrated HPLC-MS

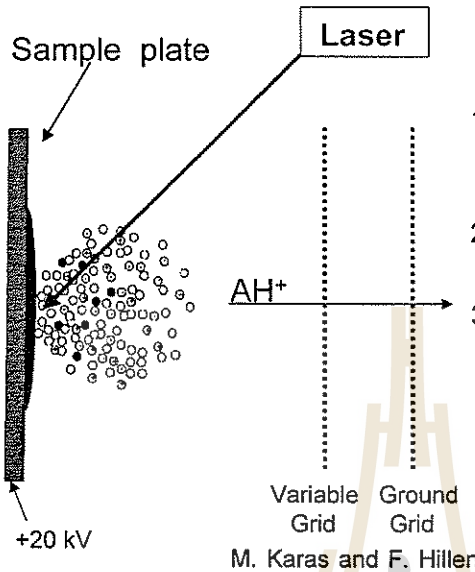
The concentration of drugs within a sample (blood, etc.) can be determined by means of HPLC-MS:

- A series of known Standards has to be run in HPLC-MS. Different drugs can be used within a series. Any signal (even the mass of MS^n) can be used for calibration!
- A set of samples of unknown drug concentrations is run under the same conditions
- The set of standards is run again
- Drug Concentrations are determined from integration of the signals by means of the „Quan" browser (xCalibur)



- Samples (and Matrix) are spotted to MALDI Plate
- Plates are transferred into MS
- Samples are ionized by light absorption to Matrix
- Ions are accelerated in the electric field
- Time of flight: t^2 is proportional to m/z

MALDI: Matrix Assisted Laser Desorption Ionization

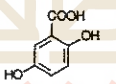
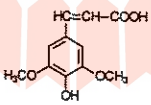
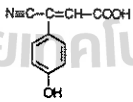
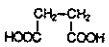
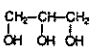


1. Sample (A) is mixed with excess matrix (M) and dried on a MALDI plate.
2. Laser flash ionizes matrix molecules.
3. Sample molecules are ionized by proton transfer from matrix:

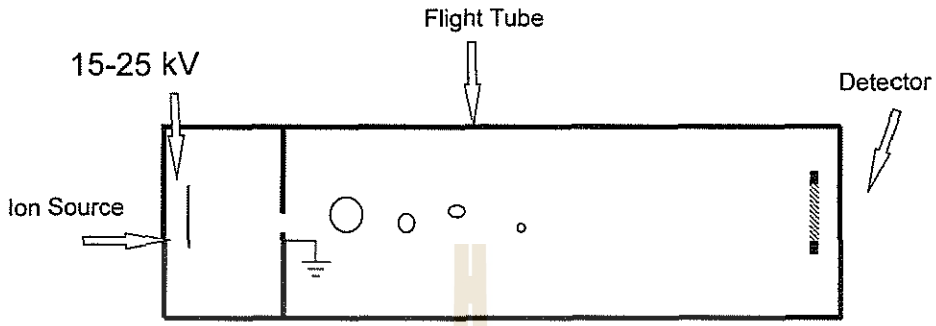
$MH^+ + A \rightleftharpoons M + AH^+$.

M. Karas and F. Hillenkamp. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal.Chem.* 60 (20):2299-2301, 1988.

MALDI Matrices

2,5-Dihydroxybenzoesäure (DHB)		266 nm 337 nm, 355 nm	Proteine, Peptide
3,5-Dimethoxy-4-hydroxyzimtsäure (Sinapinsäure)		266 nm 337 nm, 355 nm	Proteine
α-Cyano-4-hydroxyzimtsäure		337 nm, 355 nm	Peptide
4-Hydroxypicolinsäure		337 nm, 355 nm	Oligonucleotide
Bernsteinsäure		2,94 µm, 10,6 µm	Proteine, Peptide
Glycerin		2,94 µm, 10,6 µm	Proteine, Peptide

Time Of Flight (TOF)

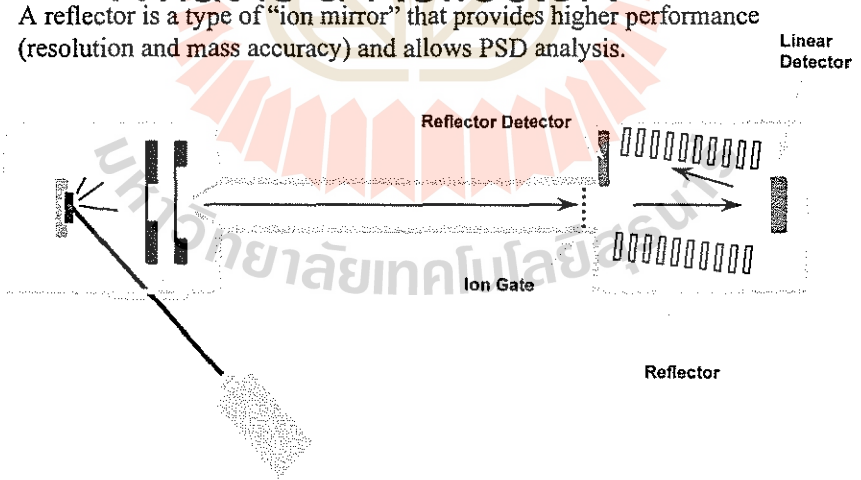


Principle: If ions are accelerated with the same potential at fixed point and a fixed initial time and are allowed to drift, the ions will separate according to their mass to charge ratios.

Post Source Decay (PSD) leads to peak broadening

What is a Reflector?

A reflector is a type of "ion mirror" that provides higher performance (resolution and mass accuracy) and allows PSD analysis.



The reflector increases the overall path length for an ion and it corrects for minor variation in the energy spread of ions of the same mass. Both effects improve resolution.

Konzept der Massenspektrometrie am MPI Dortmund: MALDI

MALDI: Selbstbedienung

Comparision MALDI <=> ESI

- Fast
- High sensitivity
- Robust towards salts
- High accuracy for small molecules
- Mixtures of proteins
- Singly charged molecules
- Individual calibration required for TOF

- Automatic HPLC-MS
- Nanospray
- Sensitive towars salts
- Same accuracy for all molecules
- Deconvolution
- Charge state has to be verified
- Stable mass accuracy

Mixtures of Compounds:

- MALDI: Rapid Identification (<5 min/sample)
- ESI: Relative concentrations of components cannot be determined

- HPLC-MS: Long experiments (>1 hour/run)
  HPLC allows separation of mixtures
  ESI (and MS^n) allows identification
  The concentration of known components can be determined. (Separate calibration required)

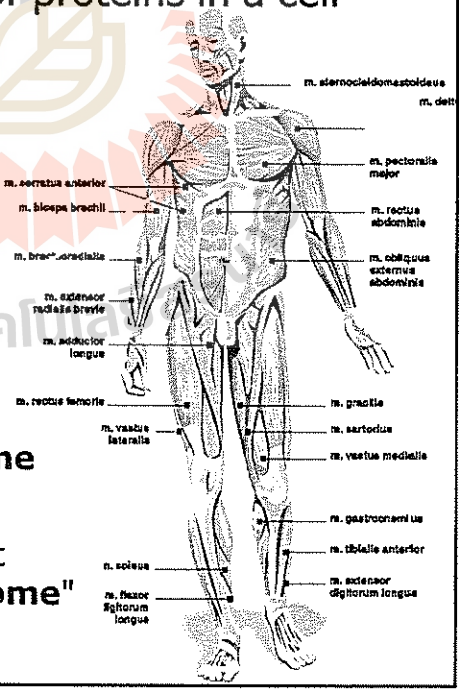
Proteom Analysis

- Running 2-D gels for protein separation
- In-Gel Digest
- „Mass fingerprint" (Ensemble of peptides)
- MS/MS for partial sequence information
- New Approaches for automated proteom analysis
- Capillary HPLC-MS

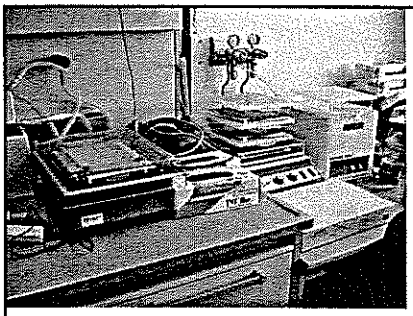
Proteom: The ensemble of proteins in a cell

All cells have the same **Genome** (Ensemble of genes).

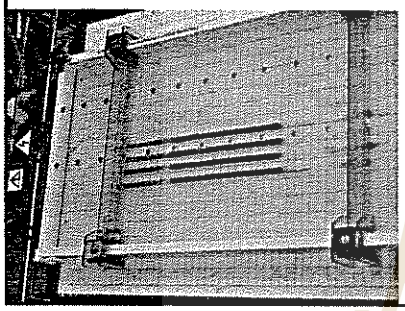
Different tissues have different ensembles of proteins "**Proteome**"

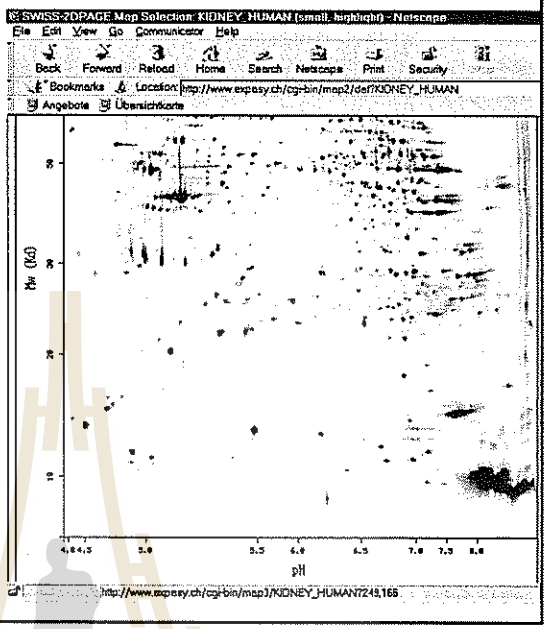


2D-PAGE



Proteins of a cell (tissue) can be separated and quantified by means of 2-D PAGE



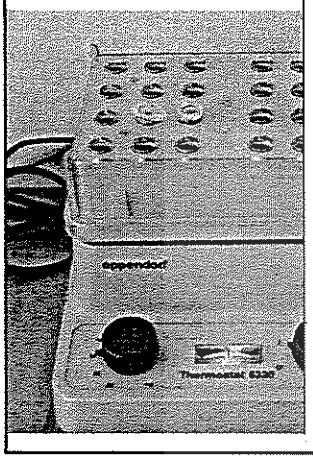


In-Gel digest of an **isolated** protein

- Run Gel, stain without Cross-linker
- Cut spots
- Wash, reduce S-S bonds and react with iodoacetamide
- Incubate with Trypsin overnight
- Extract Peptides

Shevchenko *et al.* Anal. Chem. **68**, 850-858

Enzymatic digest by trypsin



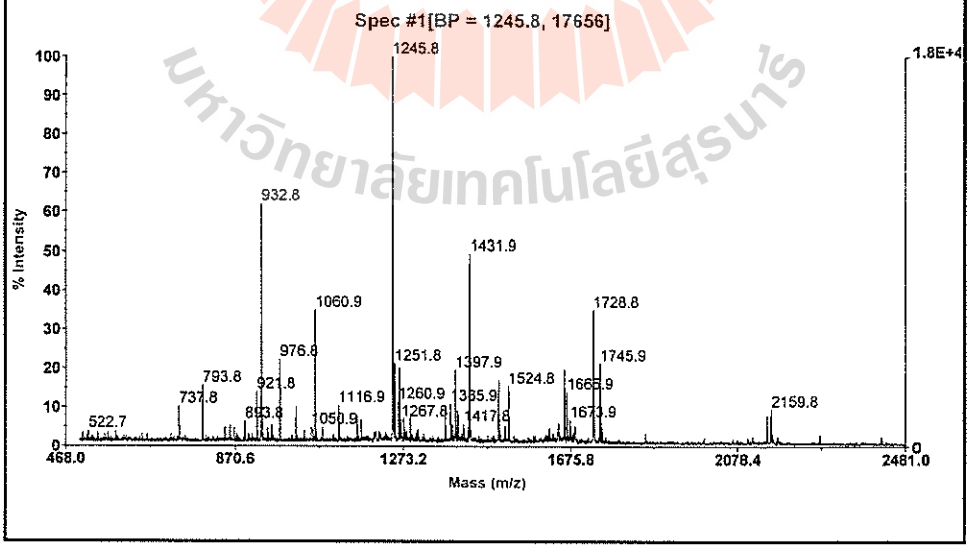
Keratin, type I cuticular HA2

[1-448] mass = 50319.0
Cleavage at KR

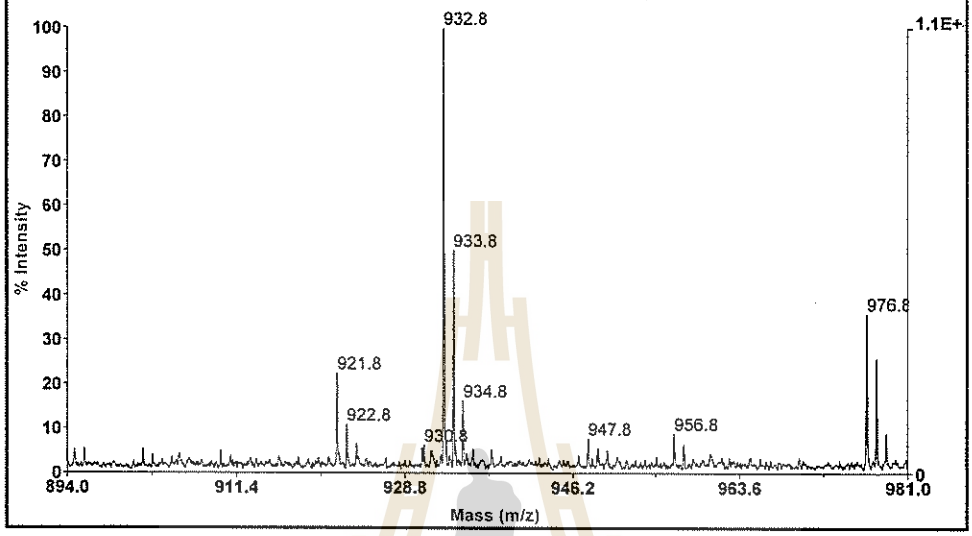
Small polar	D(19)	E(44)	N(23)	Q(32)			
Large polar	K(12)	R(31)	H(4)				
Small non-polar	S(39)	T(26)	A(33)	G(14)			
Large non-polar	L(49)	I(14)	V(27)	M(15)	F(6)	Y(12)	W(2)
Special	C(26)	P(20)					

```
  1 MTSSCCVTNNLQASLK                                  30
 31                                                   60
 61                                                   90
 91 AFNGNEK                                           120
121 NAELESR                                           150
151 DQLQQK                                            180
181 FR                                                210
211                                                   240
241                                                   270
271                                                   300
301                                                   330
331 SLRDSLENTLTESEAR                                  360
361                                                   390
```

MALDI or ESI „Mass Fingerprint"

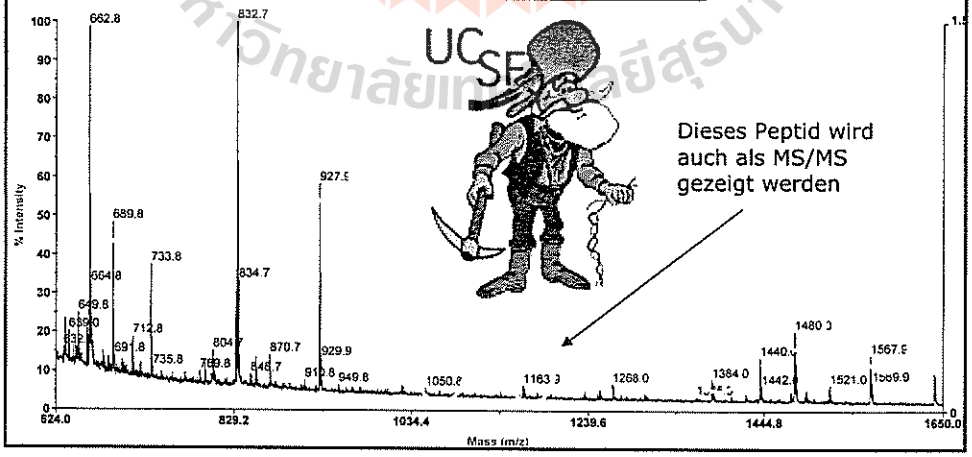


Peptides (and charge state) can be identified by the isotope pattern

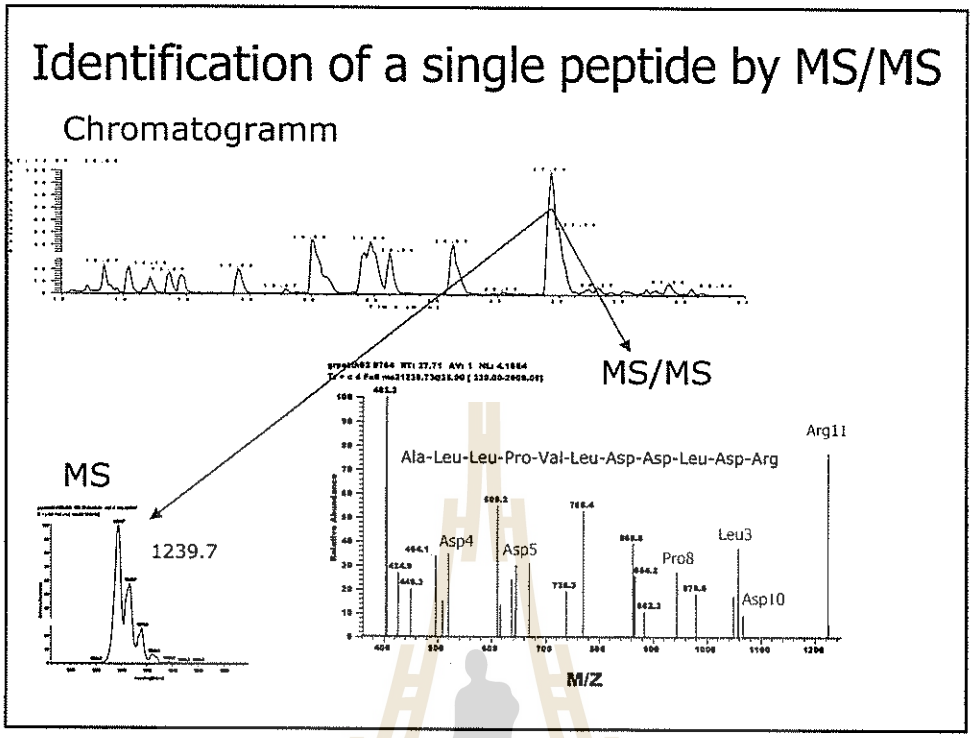


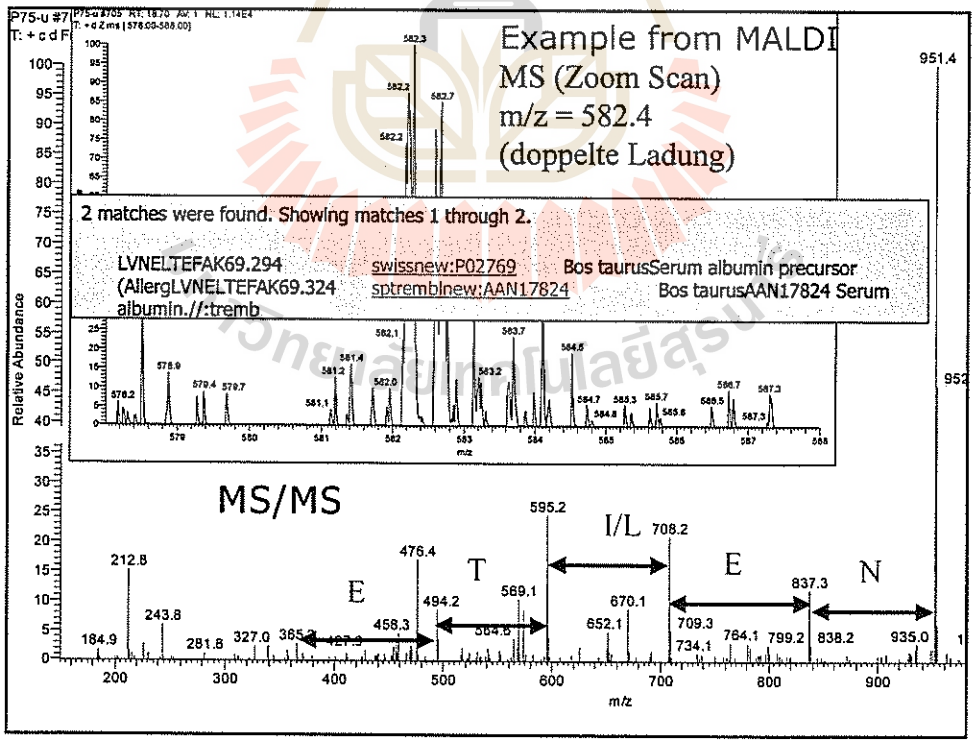
Identified peptides by MALDI Mass fingerprint

http://prospector.ucsf.edu/



Dieses Peptid wird auch als MS/MS gezeigt werden


Identification of a single peptide by MS/MS
Chromatogramm
MS/MS
MS
1239.7
Ala-Leu-Leu-Pro-Val-Leu-Asp-Asp-Leu-Asp-Arg
Arg11
Asp4
Asp5
Leu3
Pro8
Asp10
M/Z


Example from MALDI
MS (Zoom Scan)
m/z = 582.4
(doppelte Ladung)
951.4
2 matches were found. Showing matches 1 through 2.
LVNELTEFAK69.294
(AllergLVNELTEFAK69.324
albumin.//:tremb
swissnew:P02769
Bos taurusSerum albumin precursor
sptrembInew:AAN17824
Bos taurusAAN17824 Serum
Relative Abundance
m/z
MS/MS
595.2
I/L
708.2
476.4
T
E
E
N
837.3
212.8
243.8
494.2
569.1
670.1
458.3
652.1
709.3
734.1
764.1
799.2
838.2
935.0
184.9
281.8
327.0
365.2
m/z

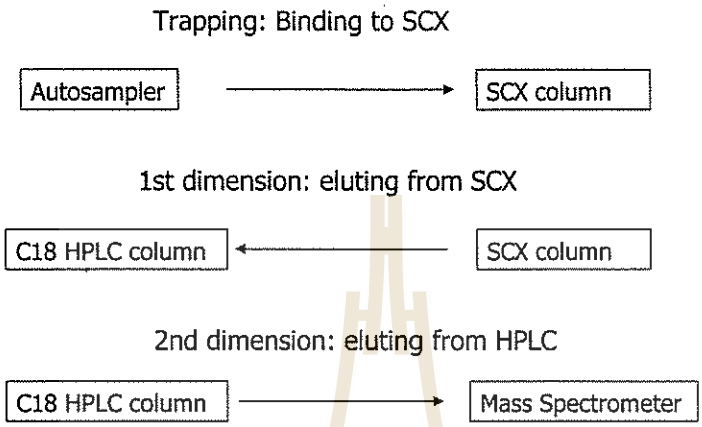
Summary

- Mass Fingerprint is sufficient to identify proteins
- HPLC-MS is a method which allows a complete coverage of almost all proteolytic enzymes.
- One single peptide is sufficient for the identification of ist parent protein.

New strategies (how to avoid 2-D gels)

- Isotope label of whole protein populations
- Proteolytic digest
- Enrichment of desired peptides (Phosphopeptide)
- Separation of peptides by two dimensional HPLC (SCX followed by reverse phase HPLC („ MudPit“))

2-D HPLC (Trapping the sample)

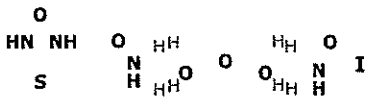


One expensive commercial strategy

ICAT™ Reagents

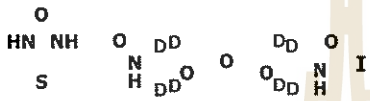
Isotope
Coded
Affinity
Tags

Struktur der Affinitätsmarker



Biotin Tag | Linker (heavy or light) | Reactive Group

Light Reagent (D0)



Biotin Tag | Linker (heavy or light) | Reactive Group

Heavy Reagent (D8)

Binding to Cystein

Heavy Reagent: d8-ICAT (X=deuterium)

Light Reagent: d0-ICAT (X=hydrogen)