Topics

- Principle of Mass Spectrometry
- MALDI-TOF
- ESI-MS
- Determination of Mw of DNA and Proteins
- Separation by MS
- Structural Information by MS
A. Principles

• Ionization: by Ion Source
  – Production of ions in vacum ($\sim 10^{-5}$ Pa or 9.8 $10^{-11}$ atm)
  – To prevent reaction between ions and air molecules
• “Separation” of Ions: in Mass Analyzer
  – “Separation” of ions according to mass-to-charge ratio (m/z)
• Detection of ions
• Storage of Data
• Analysis
A-1 Ionization Methods

- Electron Impact (EI)
  - Heated molecular vapor of sample is bombarded with a beam of energetic electrons
  - Relaxation of highly excited molecules via extensive fragmentation produces several ions of mass less than the mass of the molecular ion (M⁺, molecular ion) called daughter ions.
- Chemical Ionization (CI)
  - Collision between analyte and gaseous ions (excess reagent: reagent to sample ratio 10³ to 10⁴) produced by electron bombardment (e.g. methane)
- Fast Atom Bombardment (FAB) (liquid secondary-ion sources)
  - Bombardment with energetic argon or xenon atoms: desorption and ionization
  - Polar high molecular mass species (up to ~10,000 Da)
- Electrospray Ionization (ESI) (1984) (Biomolecules) (~100,000 Da and higher)
- Matrix Assisted Laser Desorption Ionization (MALDI)

A-2 Mass Analyzer

- Separates ionized species according to mass-to-charge (m/z) ratio
- Magnetic Sector
- Double Focussing: ElectroStatic Analyzer (ESA) + Magnetic Sector
- Quadrupolar Magnetic Field
- Time-of-Flight Analyser (TOF)
A-3 Detector

• Faraday cup collector
  – Ion beam is directed to a collector electrode attached to ground through a large load (R). The charge of the positive ions are neutralized by a flow of electrons from ground. The voltage drop is proportional to the number of ions neutralized.

• Secondary Electron Multiplier
  – Ion beam is directed at a first dynode from which electrons are ejected. Electrons are attracted to successive dynodes maintained at increasing voltages. Number of electrons ejected is multiplied at every stage.

• Multichannel plates (Array Transducer)
  
Electrooptical Ion Detector EOID)

• Microchannel Electron Multiplier (microplate, image intensifier)
  – Plate is an array of tiny tubes (6 µm) made of lead glass with metallic electrodes deposited on both side of the array.
  – A voltage is applied between the ends of the channels
  – Each tube acts as an electron multiplier
  – A phosphorescent screen is excited with the by the electrons
  – Phosphorescent light is captured by an optical array detector via fiber optics

- MALDI makes it possible to introduce large biomolecules into vacuum without fragmentation
- Provides accurate molecular mass. Relative error of 0.1-0.01% and even smaller are possible
- Extremely sensitive (down to femtomolar quantities)
- Broad mass range
- High resolution
- Relatively tolerant of buffers and salts
- Simple mixtures can be analyzed
- Data collected can be submitted automatically for database search.

B-1 Ionization

- Low concentration analyte is dispersed in a solid or liquid matrix and deposited on a metal plate
- Typical analyte to matrix ratios: 1:10³ to 1:10⁵
- Plate is placed in vacuum chamber where a laser beam is focused onto the sample
- Matrix must strongly absorb the laser radiation
- Matrix and analyte are desorbed and ionized
- Ions are accelerated towards the drift tube (TOF mass analyser)
Proposed Mechanism of Ionization

- Absorption of laser beam energy by matrix molecules
- Transfer of energy from matrix molecules to analyte molecules
- Desorption of analyte and matrix molecules
  - Analyte molecules are desorbed as neutral molecules
- Analyte is ionized by proton-transfer with protonated matrix ions

### Matrices

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>Wavelength, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitroprusside</td>
<td>Protein, oligonucleotides</td>
<td>395</td>
</tr>
<tr>
<td>2-Amino-6-methyl-5-nitropyridine</td>
<td>Protein, oligonucleotides</td>
<td>395</td>
</tr>
<tr>
<td>2-Amino-5-nitropyridine</td>
<td>Oligonucleotides</td>
<td>395</td>
</tr>
<tr>
<td>Natrium acid</td>
<td>Protein, glycosides, oligonucleotides</td>
<td>366, 230–290</td>
</tr>
<tr>
<td>Benzene and derivatives</td>
<td>Protein</td>
<td>290–293</td>
</tr>
<tr>
<td>2,5-Dihydroxybenzoic acid</td>
<td>Protein</td>
<td>366, 377, 355, 390</td>
</tr>
<tr>
<td>Vicilic acid</td>
<td>Protein</td>
<td>386</td>
</tr>
<tr>
<td>2-Aminobenzonic acid</td>
<td>Protein</td>
<td>395, 366, 290–293</td>
</tr>
<tr>
<td>2-(1-Hydroxyphenyl)benzoic acid</td>
<td>Protein, glycosides, peptides</td>
<td>366, 377</td>
</tr>
<tr>
<td>2-Pyrinolactone acid</td>
<td>Protein</td>
<td>290</td>
</tr>
<tr>
<td>3-Aminoacryloyl-3-oxobutyric acid</td>
<td>Protein</td>
<td>395</td>
</tr>
<tr>
<td>Cinnamate acid derivatives</td>
<td>Protein</td>
<td>366, 377, 355, 488</td>
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<tr>
<td>Stypric acid</td>
<td>Protein, industrial polymer</td>
<td>377, 338</td>
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<tr>
<td>Caffeic</td>
<td>Protein, oligonucleotides</td>
<td>366, 377, 355, 390–393</td>
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<tr>
<td>2-Cyclohexene-1-carboxylic acid</td>
<td>Protein, oligomeric acid</td>
<td>335</td>
</tr>
<tr>
<td>3-Nitroanilid</td>
<td>Protein</td>
<td>395</td>
</tr>
<tr>
<td>3-Nitrobenzyl alcohol with 1,4-diethylnyl-1,3-butadiene</td>
<td>Protein</td>
<td>322</td>
</tr>
<tr>
<td>3-Hydroxyacetic acid</td>
<td>Oligonucleotides, glycoprotein</td>
<td>395, 393, 355</td>
</tr>
<tr>
<td>Sotacic acid</td>
<td>Protein</td>
<td>290, 368, 393</td>
</tr>
</tbody>
</table>

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Lasers

- Nitrogen: 337 nm
- Nd-YAG: Neodymium-Yttrium Aluminium Garnet: 266 and 355 nm
- Pulse length: 1-5 nanoseconds

B-2 Mass Analysis in TOF Analyzer

- The DRIFT TUBE

Theoretically, MALDI TOF is limitless in its ability to measure m/z

Practically: can accurately measure masses up to ~300 kDa
(1) $E_{kin} = \frac{1}{2} mv^2 = zeV$

$m$: mass  
$v$: velocity  
$z$: charge  
$e$: elementary charge  
$V$: voltage

(2) $v = \frac{L}{t_F}$

$L$: length of drift tube

(3) $m \frac{2eV}{L^2} = \frac{t_F^2}{z}$

(4) $t_F = L \sqrt{\frac{m}{2zeV}}$

**Calibration:**
- Measure time of flight of standards of known m/z to obtain calibration constants
- Measure t for “unknown
- Calculate mass

**Calibration and Determination of Mass**

(3) $\frac{m}{z} = \frac{2eV}{L^2} t_F^2 = Const t_F^2$

(5) $t_F = Const \sqrt{\frac{m}{z}}$

(6) $t_F = Const_a + Const_b \sqrt{\frac{m}{z} + Const_c \frac{m}{z}}$

$Flex - control: t_F = C_o + \frac{m}{C_1 \sqrt{\frac{m}{z} + C_2 \frac{m}{z}}}$

Solve for $-\frac{m}{z}$

- Constant a accounts for uncertainties in the start time
- Variations in Constant b account differences in the energy of the ions due mainly to the topology of the matrix-preparation and to a lesser extent to the geometric variations of the target
- Constant c: correction for "higher order errors"
MALDI TOF Hardware

Laser, Attenuator and Prism
Nitrogen laser at 337 nm, 3 ns wide pulses, 20 Hz.
Laser attenuator varies the intensity of the laser hitting the sample.
Prism deflects the laser beam into the ion source.

Sample Plate and Sample Stage
An accelerating voltage is applied to the sample plate in the range 15-25 kV.

Variable Voltage Grid
A grid 1-2 mm above the sample plate with an additional voltage to fine-tune ion acceleration

Ground Grid
Grounded surface defines end of acceleration region

Grounded Aperture
Entrance to flight tube

MALDI TOF Hardware

Vacuum System
High vacuum is required to avoid ion collisions

Flight tube
A field free region where ions drift at a velocity inversely proportional to the square root of their mass/charge.

Linear Detector
Measures the ion abundance in linear mode (no reflector used) and sends a signal to the digitizer.
The problem: Peaks are inherently broad in MALDI-TOF spectra (poor mass resolution).

The cause: Ions of the same mass coming from the target have different speeds. This is due to uneven energy distribution when the ions are formed by the laser pulse.

Can we compensate for the initial energy spread of ions of the same mass to produce narrower peaks?

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Delayed Extraction

Reflector TOF Mass Analyzer
Delayed Extraction (DE) improves performance

Step 1: No applied electric field. Ions spread out.

Step 2: Field applied. Slow ions accelerated more than fast ones.

Step 3: Slow ions catch up with faster ones.

What is a reflector TOF analyzer?

A single stage gridded ion mirror that subjects the ions to a uniform repulsive electric field to reflect them.

The reflector or ion mirror compensates for the initial energy spread of ions of the same mass coming from the ion source, and improves resolution.
A reflector focuses ions to give better mass resolution

Resolution & mass accuracy on mellitin

26 amino acid peptide: 50 % of dry weight of bee venom
Isotope effect on MALDI spectrum

<table>
<thead>
<tr>
<th>Element</th>
<th>Mass %abund A</th>
<th>Mass %abund A+1</th>
<th>Mass %abund A+2</th>
<th>Element Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>1</td>
<td>100</td>
<td>2</td>
<td>A+1</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>100</td>
<td>13</td>
<td>A+1</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>100</td>
<td>15</td>
<td>A+1</td>
</tr>
<tr>
<td>O</td>
<td>16</td>
<td>100</td>
<td>17</td>
<td>A+1</td>
</tr>
<tr>
<td>F</td>
<td>19</td>
<td>100</td>
<td>18</td>
<td>A+2</td>
</tr>
<tr>
<td>P</td>
<td>31</td>
<td>100</td>
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<td>A+2</td>
</tr>
<tr>
<td>S</td>
<td>28</td>
<td>100</td>
<td>33</td>
<td>A+2</td>
</tr>
<tr>
<td>Cl</td>
<td>35</td>
<td>100</td>
<td>37</td>
<td>A+2</td>
</tr>
</tbody>
</table>

B-3 Detection of Ions

- Microchannel Plates:
  - Ion currents are converted to electrical current, digitized and stored
B-4 Post Source Decay (PSD) (MS/MS)

1. PSD refers to a method of detecting and measuring the masses of fragment ions that are formed from a selected precursor ion.

2. Fragment ions are mainly formed by unimolecular decomposition after the precursor ions are fully accelerated (after they exit the source—hence post-source decay).

3. Fragment ions are separated and detected in the reflector.

Decomposition occurs in the flight tube

![Diagram of Post Source Decay (PSD) (MS/MS)]
Internal energy of precursor ions

Only a small fraction of the precursor ions have enough energy to fragment during their lifetimes.

No of ions

Internal energy

For peptides the efficiency of PSD fragmentation is amino acid composition and sequence dependent.

Increasing PSD Fragmentation

There are two ways to increase the amount of fragmentation: both act to increase the precursor ions’ internal energy.

• Use higher laser intensity*

• Use a collision cell
PSD fragment ion velocities are the same as their precursors

All three of these species travel at the same velocity in the flight tube until they reach the reflector.

Why? Velocity is determined by initial acceleration. Initial energy = 20 keV. Bond energies = ~ 10 eV, so breaking a bond has a very minor effect on velocities.

Timed Ion Selector (TIS)/PreCursor Ion Selector (PCIS)

The TIS is a Bradbury-Neilson gate, which is a type of velocity selector. It allows only selected precursor ions and their fragments to pass through to the reflector.

<table>
<thead>
<tr>
<th>Gate closed:</th>
<th>Gate open:</th>
</tr>
</thead>
<tbody>
<tr>
<td>alternating potentials on wires</td>
<td>wires at ground potential</td>
</tr>
</tbody>
</table>

Ions
Timed Ion Selector operation

TIS off
"Gate open"

TIS on
"Gate closed"

Effect of the timed ion selector
The intact molecular ion has translational kinetic energy equal to:

$$KE = \frac{1}{2} M v^2$$

where:

- $KE = \text{kinetic energy (}= z \text{ eV})$
- $M = \text{mass}$
- $v = \text{velocity}$

Before fragmentation

Post source fragmentation

The translational kinetic energy of a fragment ion is

$$KE_m = KE_M \left( \frac{m}{M} \right)$$

where

- $KE_M = \text{precursor kinetic energy}$
- $KE_m = \text{fragment kinetic energy}$
- $M = \text{precursor mass}$
- $m = \text{fragment mass}$
Precursor and PSD fragment ions take different paths in the “normal” reflector

How are PSD fragment ions that are traveling at the same speed as the precursor ion but contain reduced kinetic energy made to arrive at the detector so that they are focused?

By varying the “steepness” of the voltage gradient in the reflector across the fragment ion mass range.
Consider an ion (MH⁺) that can decompose into two fragments, A and B.

Either of the following reactions can occur:

\[
\begin{align*}
\text{MH}^+ & \rightarrow \text{AH}^+ + \text{B} \\
\text{MH}^+ & \rightarrow \text{A} + \text{BH}^+
\end{align*}
\]

Assume MH⁺ = 1,000 Da, AH⁺ = 700 Da, and BH⁺ = 300 Da.

At mirror ratio = 1.00
At mirror ratio = 0.7

- MH+ (1,000) not focused
- AH+ (700) correctly focused
- BH+ (300) Poorly focused

At mirror ratio = 0.3

- MH+ (1,000) not focused
- AH+ (700) not focused
- BH+ (300) correctly focused
A PSD spectrum is taken in “stitches”

PSD spectrum of 1 pmol of angiotensin I

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

formed by the action of renin on angiotensinogen. Renin is produced in the kidneys in response to both decreased intra-renal blood pressure

B-4 Resolution

\[ R_s = \frac{m}{\Delta m} = \frac{m}{m_2 - m_1} \]

\( \Delta m: \text{full width at half maximum (FWHM)} \)

Typically: \(~15,000\) and higher
B.5 Applications of MALDI

- Analysis of Proteins and Peptides
  - MW
  - Structural information
  - Post-translational processes
  - Sequencing
  - Identification of a protein based on analysis of a digest finger print using “proteins digest finger prints” data base

- Analysis of Mixtures of Proteins and Peptides
  - Eliminates need for separation

Whole Cell Preparations MALDI-TOF Spectra

Fig. 8. MALDI-TOF mass spectra of whole cell preparations. A. isolate 106, B. isolate 207, C. Isolate 102, D. isolate 104. Cells were prepared for mass spectrometry using a thin smear of cells on the target, and saturated alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/1.0% TFA was added.

Courtesy of Prof. Ouellette