Introduction to Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF MS) Mass Spectrometry

Shimadzu Axima Confidence: MALDI TOF Reflectron
I. AXIMA Overview
   a. Hardware Overview
   b. Software Overview
II. Sample Preparation
   a. Matrix selection and preparation
   b. Sample spotting techniques
III. Reflectron Mode Analysis
   a. Acquiring a Spectrum
   b. Setting Processing Parameters
   c. Calibration
IV. Linear Mode Analysis
   a. Acquiring a Spectrum
   b. Setting Processing Parameters
   c. Calibration
V. Protein Identification
   a. Peptide Mass Fingerprinting
   b. Tandem MS (performance and Resonance)
VI. Customer Samples
IX. Wrap Up and Questions
Shimadzu History

1875
Established in Nijo area of Kyoto's Kiyamachi district
Started manufacture and sales of physical and chemical instruments

1877
Succeeded in Japan's first manned balloon flight

1895
Started production of storage batteries

1896
Succeeded in taking radiographs

1909
Built Japan's first medical X-ray apparatus

Successful balloon flight (1877)

Early X-ray radiographs (1896)
Delivered X-ray apparatus to Japan Red Cross' Ohtsu hospital (1911)
Founder Genzo Shimadzu
Genzo Shimadzu Jr.
Developing Businesses Globally

Developing Marketing, R&D, Manufacturing, and Sales and Service Capabilities Throughout the World

Sales and Service Locations
Japan, USA, Germany, Austria, Italy, UK, Switzerland, France, Netherlands, Singapore, Philippines, Australia, India, China, Korea, Taiwan, Vietnam, Turkey, UAE, Russia, Brazil, etc.

Manufacturing Sites
Japan, USA, UK, China, Philippines, and Vietnam

Research and Development Centers
Japan, USA, UK, and China
Shimadzu made the first MALDI TOF instrument
### The Shimadzu connection to MALDI and TOFs

<table>
<thead>
<tr>
<th>The Five Mass Spectrometry</th>
<th>Nobel Prize Pioneers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Joseph John Thomson</strong> 1906 Nobel Prize for Physics <em>&quot;in recognition of the great merits of his theoretical and experimental investigations on the conduction of electricity by gases&quot;</em></td>
<td><strong>Francis William Aston</strong> 1922 Nobel Prize for Physics <em>&quot;for his discovery, by means of his mass spectrograph, of isotopes, in a large number of non-radioactive elements, and for his enunciation of the whole-number rule&quot;</em></td>
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<td><strong>Wolfgang Paul Fenn</strong> 1989 Nobel Prize for Chemistry <em>&quot;for the development of the ion trap technique&quot;</em></td>
<td><strong>John Bennet Fenn</strong> 2002 Nobel Prize for Chemistry <em>&quot;for the development of soft desorption ionisation methods (ESI) for mass spectrometric analyses of biological macromolecules&quot;</em></td>
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<td><strong>Koichi Tanaka</strong> 2002 Nobel Prize for Chemistry <em>&quot;for the development of soft desorption ionisation methods (MALDI) for mass spectrometric analyses of biological macromolecules&quot;</em></td>
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Shimadzu MALDI History

1988 LAMS-50k

1992 Kompact-MALDI

2000 MALDI ToF-MS AXIMA-CFR / -LNR

2002 MALDI Trap-ToF AXIMA-QIT

2006 MALDI ToF MS/MS AXIMA-TOF²

2008 AXIMA Assurance, Confidence & Performance

2009 AXIMA Resonance

2010 AXIMA MegaTOF
Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF MS) Mass Spectrometry

Theory
Matrix Assisted Laser Desorption Ionization (MALDI) is a sensitive ionization technique that uses a matrix in order to desorb and ionize analytes of interest in preparation for mass spectrometric analysis.

- The matrix compound must absorb efficiently at the laser wavelength, it must also co-crystallize and co-desorb with the analyte upon irradiation.

- Primary reactions happen in the early desorption process and lead to the formation of primary matrix ions.

- Secondary reactions, analyte reacts with the matrix in the expanding plume: gas-phase thermodynamics apply.
MALDI Fundamentals
Primary reactions

- Solid mixture of matrix and analyte desorption / ablation results from Matrix absorption of laser energy (photons (eV))

- Desorption = smooth transition from solid to gas phase

- Laser = continuous source of photon. In MALDI 2 types of lasers: UV and IR lasers


- Matrices absorb at the laser wavelength. With UV lasers, excitation of the electronic levels. We want as much photons absorbed within the relaxation time of the matrix => Laser pulse length (N2 laser, 337nm, 3ns/pulse and ~ 3.5 eV/photon)

- Primary reactions take place in a high density / high temperature plume (600 K – 1200 K, modelisation at various laser fluence)

- Plume expands. Secondary reactions take place. Gas phase thermodynamics apply
MALDI Secondary Ionization Reactions

Gas phase proton transfer

\[ \text{MH}^+ + A \rightarrow \text{M} + \text{AH}^+ \]

\textbf{M}: α-cyano-4-hydroxycinnamic acid (CHCA); 3,5-dimethoxybenzoic acid (sinapinic acid)

\textbf{A}: peptide, proteins, any compound with acidic and basic sites

Thermodynamic constant: \textbf{Proton Affinity} (PA) or \textbf{Gas-Phase Basicity} (GB)

Exothermic reactions, thermodynamically favored:

\[ \text{PA (A)} > \text{PA (M)} \]

α-Cyano-4-hydroxycinnamic acid
MALDI Secondary Ionization Reactions

Gas phase Metal ion transfer

\[ A + m^+ \rightarrow Am^+ \]

\( m^+ \): Lithium (Lithium Chloride), Silver (Silver Trifluoroacetate)

\( A \): compounds whose PA is lower than the matrix. Polymers, such as polystyrene, class of lipids (acylglycerol)

Thermodynamic constant: **Metal Affinity (MA)**

\[ MA(A) > MA (\text{matrix}) \]

1,8,9-Anthracenetriol / Dithranol
MALDI Secondary Ionization Reactions

Gas Phase Electron Transfer

\[ \text{M}^{+\cdot} + \text{A} \rightarrow \text{A}^{+\cdot} + \text{M} \]

\( \text{M} \): Terthiophene, acenaphtene, anthracene, DCTB (Trans-2-[3-(4-t-butylphenyl)-2-methyl-2-propenylidene]malononitrile)

\( \text{A} \): compounds that lack, or hold few, polar functional groups. Compounds that can stabilize the formed radical

Thermodynamic constant: Ionization Potential (\( \text{IP} \))

Exothermic, favored reaction: \( \text{IP}(\text{A}) < \text{IP}(\text{M}) \)
MALDI matrices

• Karas et al: analyzed an amino acid with or without tryptophan using a Laser desorption technique. Noticed improvement in the Amino Acid signal when using tryptophan

• Koichi Tanaka: used Ultra Fine Metal Powder (UFMP, Japanese powder) and glycerol to desorb high MW proteins

• Choice of matrix is compound related. Each matrix has a thermodynamic constant (gas phase basicity, Ionization Potential, Metal Affinity)

• Needs to co-crystallize with compound of interest. Ideally soluble in same solvent (same polarity) but not a necessity

• Must absorb laser photons = absorbs at the laser wavelength.

• If the ionization energy requires is over a photon process (N2 laser photon is 3.5 eV, ionization energy 7.2 eV) than matrix need to absorb 2 photons. The time required for absorbing 2 photons should be smaller than the time for molecular radiationless relaxation time => > High-frequency lasers.
MALDI matrices

α-Cyano-4-hydroxycinnamic acid / CHCA

3,5 dimethoxy-benzoic Acid / Sinapinic Acid

2,5-Dihydroxy-benzoic Acid / DHB

1,8,9-Anthracenetriol / Dithranol
MALDI matrices

2,4,6-Trihydroxyacetophenone / THAP

2,5-Dihydroxyacetophenone / DHAP

3-hydroxypicolinic acid / 3-HPA
MALDI matrices

Hot Vs Cold matrices

• Influence of the choice of the matrix on analyte fragmentation

• Matrix transfers energy, which is taken up from laser. For instance, Sinapinic acid transfers more energy than DHB.

• Dense plume Matrix will induce internal heating / cooling due to collisions between Matrix and analyte

• In PSD experiment these are wanted and required phenomena. However, for intact analyte detection, metastable formation may decrease your sensitivity.

• MALDI matrices were characterized as “hot” or “cold” depending on their propensity to induce fragmentation of glycoproteins. Fragmentation, using PSD, was found to decrease in this order:

  CHCA > SA > 2,5-DHB > 3-HPA
Instrumentation

Axima Confidence instrument design
Time of Flight Analyzer

Ions are accelerated to a defined kinetic energy and the time required to move through a fixed / known distance is measured

Scheme from, Jonathan Karty, Indiana University
Time of Flight Analyzer

Basic TOF mass Spectrometer consist of

- Short source region (s, ~cm) with a high electric field (E)
- A long drift region of a distance D (field free, ~ m)
- Accelerating voltage: \( E = \frac{V}{s} \)

Ions are accelerated to a kinetic energy

\[
\frac{1}{2} mv^2 = eV
\]

\( m = \) ion mass; \( v = \) velocity; \( e = \) charge; \( V = \) acceleration voltage

Time of Flight Analyzer

The time, $t$, required for the ion to travel the drift region is

$$t = D / v$$

$$t = \left( \frac{m}{2eV} \right)^{1/2} * D$$

When including terms related to "initial spatial distribution" and "initial kinetic energy distribution", a more general TOF expression would be:

$$t = \frac{(2m)^{1/2}}{z * E} * \left[ (U_0 * e * E * s)^{1/2} + U_0^{1/2} \right] + \frac{(2m)^{1/2} * D}{2 * (U_0 + e * E * s)^{1/2}} + t_0$$

Initial Kinetic energy and position   Turn around time

$U_0 =$ Initial Kinetic energy  $t_0 =$ uncertainty in the time of ion formation

Starting Position Influence

Uncertainty in starting position causes the peaks to broaden
Lower Resolution

$t = t_{source} + t_{analyzer}$

Resolution

\[ R = \frac{m}{\Delta m} = \frac{t}{2 \Delta t} \]

\[ \Delta t_1 > \Delta t_2 \implies R_1 < R_2 \]
Time-lag focusing (pulsed extraction)

Wiley, W. C.; Mc Laren, I. H. Bendix time of flight mass spectrometer. Science 1956, 26, 1150-1157
There is a point in the flight tube where two ions starting at different position will arrive simultaneously. The idea behind adding a grid is to move the focus plane to the detector.

In 1955, the Wiley and McLaren instrument used a dual stage source to push the space focus plane to the detector.


The Reflectron increases the flight distance for ions with higher kinetic energy / higher speed

Similar to the source time-lag focusing, reflectron voltages will be set so that the focus plan is at the detector

Example: ACTH (Adrenocorticotropic Hormone) $m/z$ 2465
Time of Flight Analyzer

• Use *transient recorder* with a sampling rate of 20 Msample/s: bin size of 50 ns

• Microchannel plate (MCP) as a detector in the Reflectron mode
• Electron multiplier for linear.

http://www.tectra.de/_icons/MCP_magnification.jpg
Fragmentation of molecules: breaking them into pieces in order to investigate their structure

The precursor ion, parent ion, which is the ion we want to fragment is activated. Activation will use different source of energy in order to increase the internal energy of the precursor / parent ion. The intake of energy will cause covalent bonds to cleave (homolytically, heterolytically)

In Mass spectrometry we refer to this experiments as: tandem mass spectrometry or MS/MS. It requires two analyzers in tandem separated by a collision cell.
The fragmentation mode available in the Axima Confidence is called: PSD or sPSD and it stands for Seamless Post Source Decay.

Seamless = uses a Curved Field Reflectron, which focuses all ions to the same focal point “simultaneously”

Post Source Decay: Ion of interest / parent ion / precursor ion, is activated in the source using high laser energy (photons absorption, collisions in a “denser” plume. The activated parent ion = metastable, will be selected by an “ion gate” after extraction and will decompose in the field free analyzer.
Types of Fragmentation

• Three ‘types’ of fragmentation:
  • Post source decay (PSD) - ions fragment in the flight tube after being extracted from the source
  • Collision Induced Dissociation (CID) - ions are accelerated in a collision region where they undergo collisions with gas molecules. During the collisions, energy is transferred to the analyte ion causing it to fragment. (AXIMA-TOF2)
  • In-source decay (ISD) - ions fragment before they are extracted from the ion source
MS/MS Using Mass Spectrometry

- Ion source to generate ions
- Analyser 1 to isolate precursor
- Collision gas to generate fragment ions
- Analyser 2 to separate fragment ions
- Detector at end of analyser 2 to produce MS/MS spectrum
Applications

Peptides / protein analysis

Oligosaccharides

Lipids

Oligonucleotides

Polymers
Spotting techniques

Typical sample preparation method:

Matrix concentrations vary depending on matrix selection.
   CHCA – 5 mg/ml

Mix 1 µl of sample with 1 µl of matrix solution. Dry sample completely
Spotting techniques

- **Dried Droplet**

  Introduced in 1988 by Kara and Hillenkamp. Most commonly used spotting technique. It is the original and oldest technique. A saturated solution of Matrix is mixed with a solution of analyte (in an eppendorff tube, or on the plate). Technique suffers from inhomogeneous spots (search for the hot spot) and localization of the samples in a ring around the edge. Vacuum drying was found to help.

- **Crushed Crystal**

  Similar to the dried droplet technique. Use of non-volatile solvents, glycerol, DMSO to enable the growth of analyte-matrix crystals. More uniform films, higher reproducibility but longer preparation times.

Spotting techniques

- **Overlay and Sandwich Methods / fast evaporation method**

  Sample plate is pre-coated with matrix dissolved in an organic solvent that evaporates fast at room temperature (acetone). Matrix-analyte mixture is spotted on the top.

- **Electrospray**

  Matrix analyte mixture is sprayed on the top of the plate. This technique is widely used in MALDI imaging, where the matrix is sprayed on the top of our tissue of interest.

Peptides / protein analysis

- Peptides up to 6 to 7 KDa can be analyzed in the reflectron mode and will therefore benefit from a higher resolution, better mass accuracy (300 Da – 4000 Da)
- Peptides can also be analyzed in the linear mode. The resolution and mass accuracy are lower
- Proteins over 6 KDa are analyzed in the linear mode (6000 Da – 500 KDa)

http://www.nature.com/scitable/content/ne0000/ne0000/ne0000/ne0000/14711381/U2CP4-1_PeptideBondSidechain_ksm.jpg
Peptides / protein analysis

Matrices

3-methoxy-4-hydroxy-Ferrulic Acid

α-cyano-4-hydroxy-CHCA

3.4-dihydroxy-Caffeic Acid

3,5-dimethoxy-4-hydroxy-Sinapinic Acid
Peptides / protein analysis

Benzoic Acid Derivatives

2,5-diHydroxyBenzoic Acid

2-hydroxy-5-methoxy-benzoic acid

Peptides / protein analysis

Phosphopeptides

CHCA is a hot matrix and causes β-elimination of phosphate group from Serine

- Add phosphoric Acid to the matrix
- Use different matrix such as Dihydroxyacetophenone mixed with Di-ammonium hydrogen citrate

http://www.shimadzu.com/an/industry/pharmaceuticallifesience/proteome0203003.html
Peptides / protein analysis

Phosphopeptides

Dihydroxyacetophenone (DHAP) / Di-ammonium hydrogen citrate (DAHC)

Trihydroxyacetophenone
Peptides / protein analysis

- Use of carbohydrates-containing-co matrices gave better results than single component matrices
  - 5-methoxysalicylic acid + 2,5-DHB
  - Fucose + 2,5-DHB

Peptide fragmentation

Spectrum of a seamless PSD (sPSD, using Curved Field Reflectron) of a doubly phosphorylated peptide. Unique feature on Shimadzu Axima MALDI TOF instruments

PSD deposits less energy in the parent ion. Enables the detection of labile PTMs such as Phosphorylation
Proteases cleave proteins at specific amino acids:

Example: Trypsin cleaves the carboxyl side of lysines and arginines.

The masses of proteolytic peptide products form a signature **FINGERPRINT** for a protein.

Protein

Trypsin

Peptides

MALDI

Database Search

Protein ID
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<th>Start - End</th>
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<th>Mr(calc)</th>
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Protein Mixtures – The PMF Foil!

Protein Mixtures

- Trypsin
- Protein Peptides
- MALDI
- Database Search
- Protein ID

Protein Mixture

- Trypsin
- Peptides
- MALDI
- Database Search
- No Protein ID
When PMF Fails! Try MS/MS

Trypsin

MALDI (PMF)

Isolate Precursor

Introduce collision gas

b'y'y_b'y_b'

precursor

Database Search

Protein ID

No Protein ID
Applications
Protein Database Searching

Evaluating the “false discovery” rate for *Fundulus grandis* proteome expression project
Introduction

- *Fundulus grandis* occurs in coastal marshes of the Northern Gulf of Mexico

- This species displays a broad tolerance for variation in salinity, temperature and oxygen, combined with availability and ease to maintain

  => A useful model for proteomic studies of fish

- Dedicated protein database is unavailable
Introduction

Extract proteins from five tissues
Brain, gill, heart, liver and muscle

Separation using 2D gel electrophoresis

Protein spots cut and in-gel digested using trypsin

MALDI MS and MS/MS analysis

Protein database searching
Protein Database Searching

Protein database

In-silico generated tryptic peptides

In-silico generated MS spectra

Sequence database matching

Peptide sequence assignment

Peptide sequence confirmation

MALDI MS

MALDI MS/MS

Protein separated using 2D gel electrophoresis

Peptides resulting from in-gel tryptic digest

In-silico generated MS spectra

In-silico generated MS/MS spectrum
MASCOT Database Searching

- Probability based identification

- Protein is identified as a match when given a score above threshold: less than 5% probability that the match is a random event

\[ S = -10 \times \log_{10}(P) \quad P = \frac{0.05}{\text{number of protein sequences in database}} \]

(score threshold will vary with database size)

- Number of submitted protein for DB searching as well as the use of a non-model species prompted us to evaluate the false discovery rate (FDR)

- Use a MASCOT script in-order to conduct a “target-decoy” database search and evaluate the FDR
A false positive is an identification that has been given a score above the threshold, ranked first amongst all possible matches, but resulted from a random match.

Original database:
370,000 protein sequences

Decoy database:
370,000 protein with randomized sequences

Amino-acid sequences of protein in the database are randomized, creating new protein sequences that have the same molecular weight but different sequence.

The two databases are merged into a unique database.

Download Actinopterygii protein database from NCBI.
Result of the original database for the protein expression project

405 protein identified out of 864 gel spots digested and submitted to analysis, 47% identification rate
93 in liver / 49 in muscle / 100 in brain / 92 heart / 71 in gill

9.7 peptide per identified protein
29.7% sequence coverage
Result of the original database search for the protein expression project

Scores ranged from 67 to 757
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<th>Plate</th>
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<th>% identification</th>
<th># of False positive identification</th>
<th>False discovery rate (FDR, %)</th>
<th>Maximum False Positive score</th>
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Conclusion

- Three of the five tissues submitted to DB searching returned an FDR of 0%
- Liver and gill both recorded a False positive identification for an FDR rate of 1%
- Overall FDR rate is 0.2%
- Mascot probability scoring scheme is set so there is less than 5% chance that a match is a random event. In our case random events represented less than 1% of all the events
- MALDI MS and MS/MS spectra quality can affect the identification. Liver and gill were sample that had a low amount of peptides. Difficult to run and interpret
- Proteins that had scores below the scores of the random matches (72 for liver plate 2 and 68 for liver plate 1) which represents four proteins need to be carefully reviewed
Protein analysis
Linear Mode Intact Protein Analysis

• Typically use 10-20 mg/ml Sinapinic acid as matrix
• Direct path from source to detector
• Capable of detecting ions < 500 kDa
• Average mass reported
• Linear mode is typically associated with high molecular weight analysis
Standard Linear Detection of IgG Aggregates

- IgG
- 147535.26
- 73927.03
- +2
- dimer

© 2009 SHIMADZU
High Mass detection of IgG Aggregates

IgG

dimer

+2

72660.8

22470.8

145011.1

291858.4

122262.6

trimer

tetramer
MegaTOF MALDI MS: up to 1500 kDa

IgG

High-Mass detection

standard detection

452.009 kDa

3IgG

Zoom

High-Mass detection

Standard detection
High Mass Detection Using MALDI

- **HM2 detector:** outstanding sensitivity 10kDa – 1500 kDa
- **Standard detector remains for lower masses 0-10kDa**
- **15 second changeover time**
- **Low saturation for complex mixtures**
High-Mass Applications

Protein complexes characterization

Therapeutic Protein Aggregates

Antibody Characterization
  • Antibody-Antigen interactions
  • Epitope mapping
  • Sandwich assays

Inhibitors of Protein-protein interactions

High-Mass MALDI Imaging

PEG Protein Characterization

Polymer Analysis

Plasma Screening
Protein Interactions and MALDI

Dissociation Problem

Source  Flight Tube  Detector

Complex AB  UV laser 337 nm  

Proposed Solution: Chemical Stabilization

Source  Flight Tube  Detector

Complex AB  UV laser 337 nm  

Mass Spectrum  m/z  

A  B

Mass Spectrum  m/z  

AB
Protein Interactions and MALDI

**Dissociation Problem**
- **Source**: Complex AB
- **Flight Tube**: Detector

**Detection Problem**
- **Standard detector**

**Proposed Solution**
- **Source**: Complex AB
- **Flight Tube**: Detector

**Proposed Solution**
- **High Mass detector**
MegaTOF MALDI MS: up to 1500 kDa

IgM

High-Mass detection
standard detection

1016.033 kDa

Zoom

Standard detection
High-Mass detection
Antibody Characterization

Antibody/Antigen interactions

6H4 + bPrP ↔ 6H4

[6H4•bPrP] + [6H4•2bPrP]

Control
HM1 High-Mass MALDI

After cross-link
HM1 High-Mass MALDI

Nazabal et al. Anal. Chem.; 2006; 78(11); 3562-3570
Biopharmaceuticals Aggregates

IgG Hab41
SEC fraction 13
Monomer: 150 kDa
Temperature stress: 10 days, 45°C
2 μM final concentration
Linear Mode Summary

• Linear mode analysis
  – Mass range extends up to 500kDa for electron multiplier detectors
  – Useful for determining molecular weight of intact proteins

• High mass linear mode analysis
  – Mass range extends up to 1.5-2 MDa using CovalX detector
  – Use of cross-linking reagents enables analysis of protein complexes and aggregates

• Low resolution, average MH+

• Unable to provide structural information such as peptide sequence* or post translational modifications.

* In source decay discussed later
MALDI-TOF MS ISD – Top Down Sequencing

In Source Decay

In source decay fragmentation occurs at the source prior to extraction into the time of flight.

“Top-Down” approach

Requires well characterized, purified protein

matrix DAN

sample PROTEIN

Linear or reflectron mode acquisition

Increase laser power!!!
In source decay is a side affect of laser induced fragmentation and metastable fragmentation resulting during ionization.

Can be used for “Top Down” sequencing of proteins

Preferred matrix is diaminonapthalene (DAN)

Must have purified protein in order to interpret ISD spectrum

Generates primarily c- and z-ions
Bovine Serum Albumin In Source Decay Spectrum
Bovine Serum Albumin Mascot Identification

1. **ALBU_BOVIN**  Mass: 69293  Score: 164  Queries matched: 1
   Serum albumin OS=Boos taurus GN=ALB PE=1 SV=1

   □ Check to include this hit in error tolerant search or archive report

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## Bovine Serum Albumin Mascot Identification

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MALDI-TOF MS Disadvantages

- Samples are heterogeneous
- Not thought of as quantitative Quantitative (relative quantitation possible)
- Complicated samples must be separated offline
- Interfering matrix peaks in low molecular weight region.
MALDI-TOF MS Advantages

- Fast, sensitive and reproducible
- Provides high mass accuracy data
- Capable of analyzing high molecular weight compounds
- Generates predominantly singly charged species
  - Simpler spectra
- Applicable to a wide variety of applications:
  - Proteins and peptides - Microorganism Identification
  - Lipids - Tissue Imaging
  - Glycans
  - Polymers
  - Small molecules
Acknowledgements

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