Quantification by MALDI TOF MS

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Types of Analyses

Qualitative Analysis: Identification of the analyte(s) in a sample. This is the typical application of MALDI TOF.

Quantitative Analysis: The determination of the amount or concentration of a target analyte(s) per unit volume or mass of sample. MALDI TOF MS can be used for this application.

Types of Quantitative Analyses

Absolute Quantification: Analyte amount (or concentration) expressed in absolute terms *i.e.*, per unit mass or unit volume.

Relative (Comparative) Quantification: Analyte amount (or concentration) expressed relative to another analyte, or the same analyte in a different sample.

Profile Analysis: Generating and comparing spectral 'features' across spectra; classification of a sample based on spectral features.

Some Important Terms, Concepts & Definitions

Precision: Scatter in results obtained from replicate measurements.

Accuracy: Closeness of estimate to "true" value.

Sensitivity

Limit of detection: Lowest amount of analyte that gives a response above noise.

Limit of quantification: Limiting concentration that provides a defined level of precision and accuracy - note upper and lower limits.

Standard: Substance used as a reference for both mass and quantity.

Range: The concentration interval over which linearity, accuracy, and precision are all acceptable.

Robustness: The ability of an analytical method to be unaffected by small, deliberate changes in operating parameters.

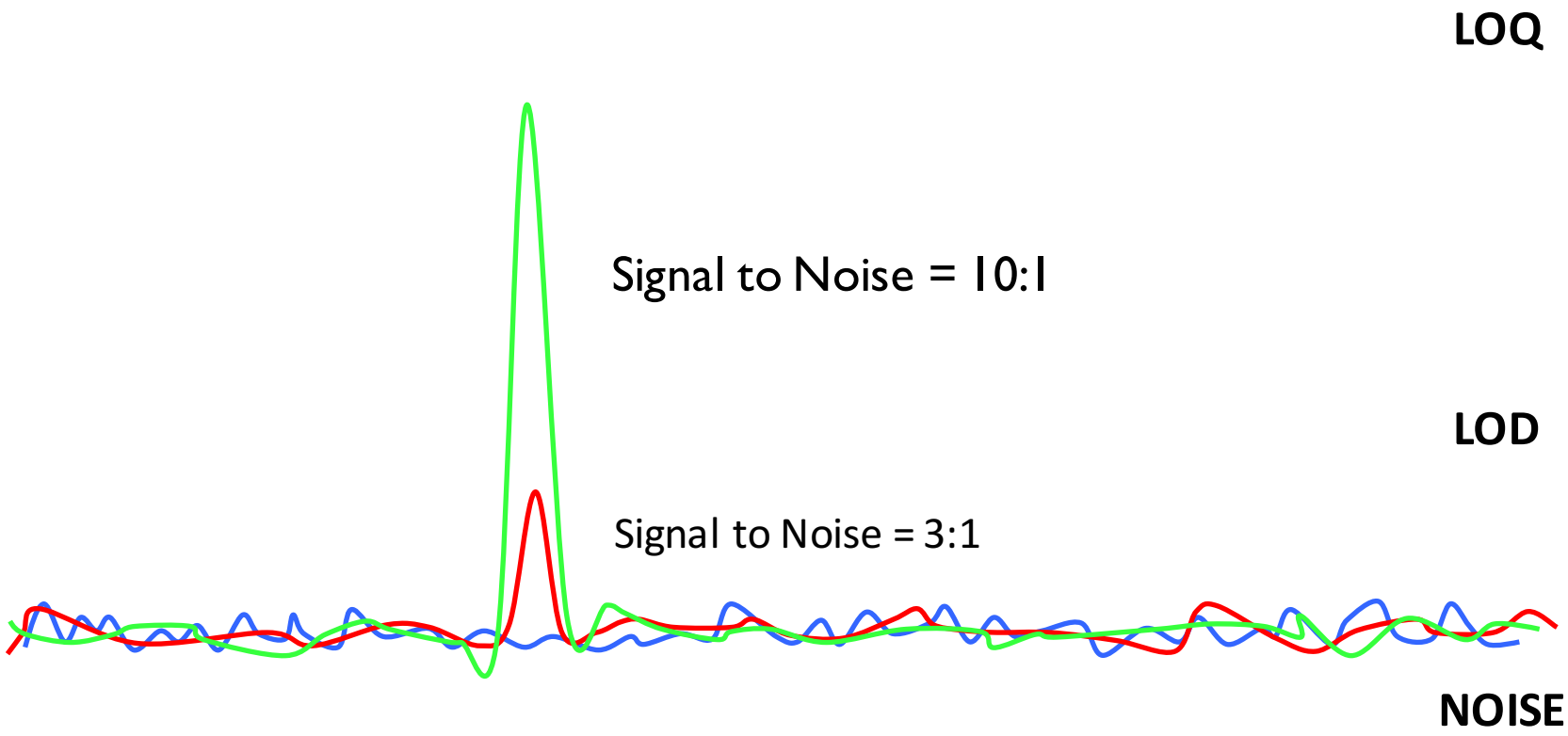
High Accuracy
High Precision

Low Accuracy
High Precision

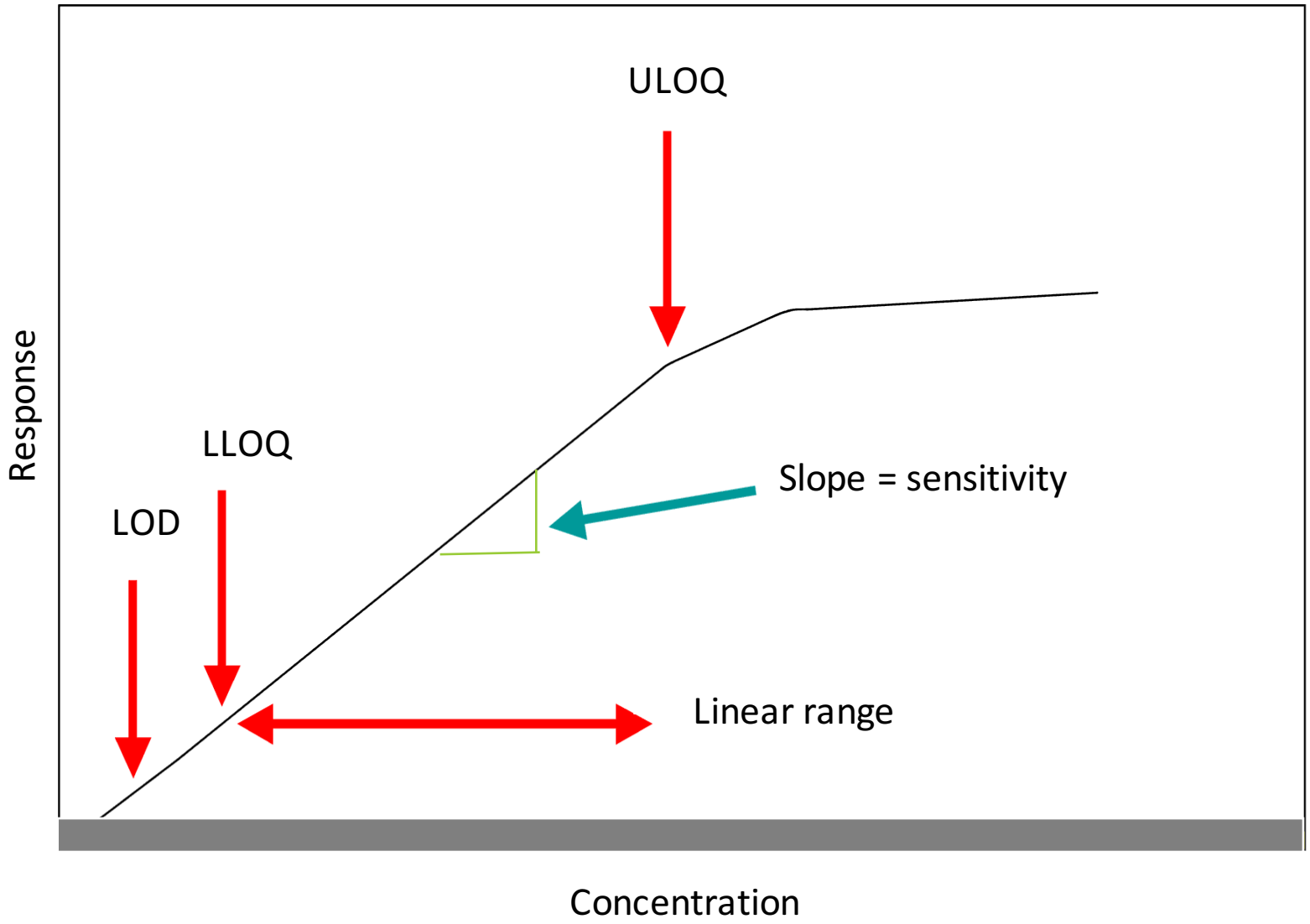
High Accuracy
Low Precision

Low Accuracy
Low Precision

LOD, LOQ and Signal-to-Noise Ratio (SNR)



Graphical Representation of Key Terms



Ways of Performing a Quantitative Analysis

Principles of Quantitative Analyses

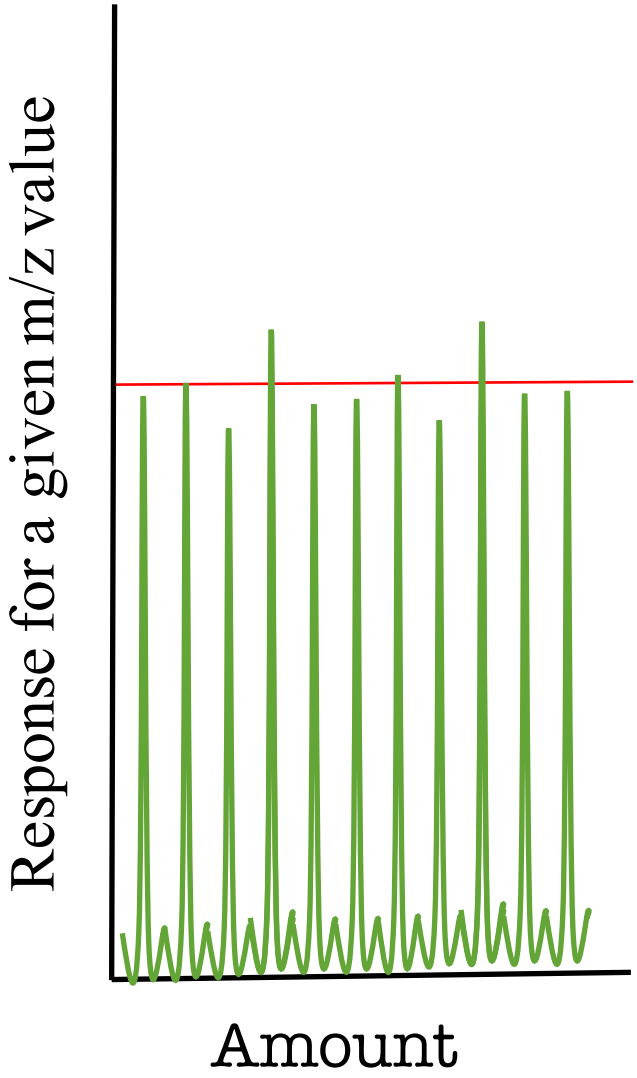
An analytical results depend on a final measurement of a physical or chemical property (X). Ideally, the measurement of the property is directly proportional to the concentration (C_A).

$$\mathbf{C_A = kX}$$

where, k is a proportionality constant. The process of determining k is an important step in most analyses. This step is called a calibration.

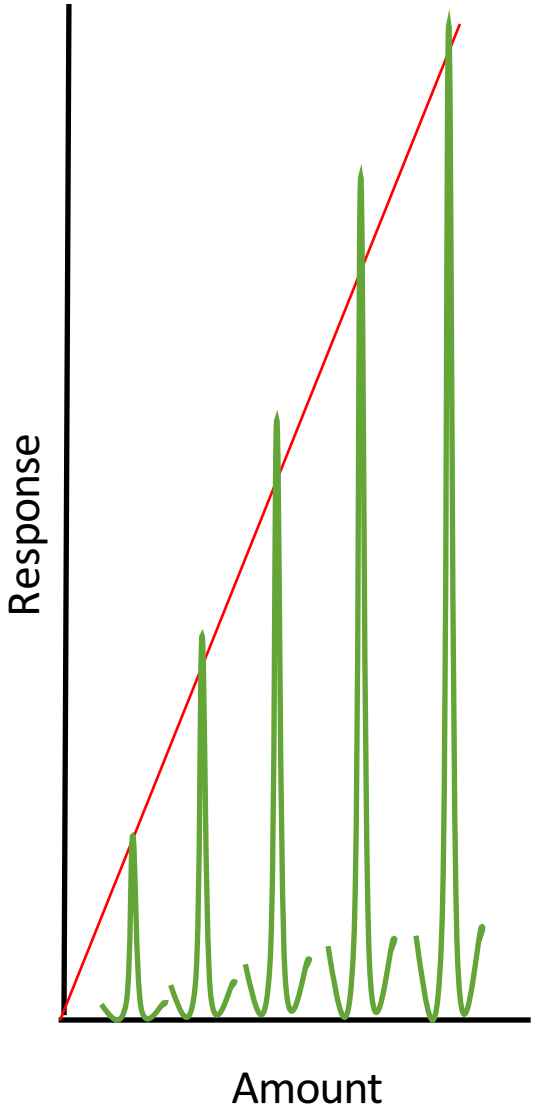
Quantification requires that:

Repeat analysis of the same sample must give reproducible response intensities. The more reproducible the spectra, the higher the degree of precision obtainable.



Quantification requires that:

The signal response for a given component (e.g., a fixed *m/z* value corresponding to specific species, or a single $[M+H]^+$) must increase proportionally as the amount of that component introduced into the system increases.



General Approaches to Quantitative Analysis

Approaches to Quantification

1. The External Standard Method

- Most commonly employed with other instrumental methods, but unsuitable for mass spectrometry.
- Calculation of analyte concentration requires reference to a calibration curve prepared and measured at the time as the unknowns.

2. Internal Standard Method

- Most frequently used in mass spectrometry and involves the incorporation of an internal standard.

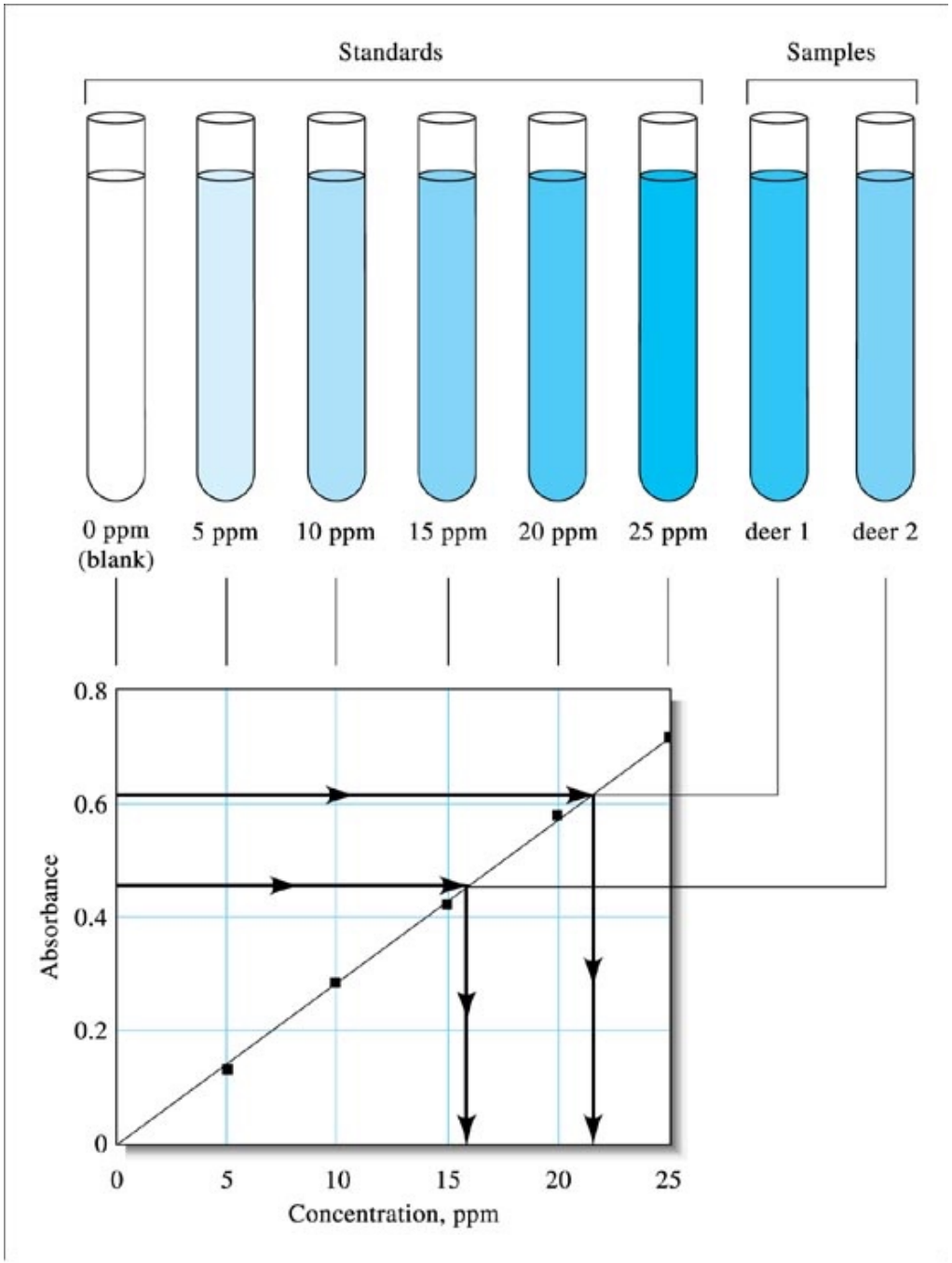
3. The Standard Addition Method

- Involves direct addition of standard to samples. Samples are measured both before and after addition. Calculation of analyte concentration requires analysis of multiple samples of each analyte (i.e., with and without known amounts of the analyte added).

Approach 1:
External Standard Method

- If the relationship between signal and amount is reproducible and rugged, then a plot of response (R) vs concentration (C) can be generated across the linear range by the analysis of a series of standards run under well-defined conditions.
- Thereafter, R for an unknown can be translated directly to a concentration or amount by reference to the calibration curve

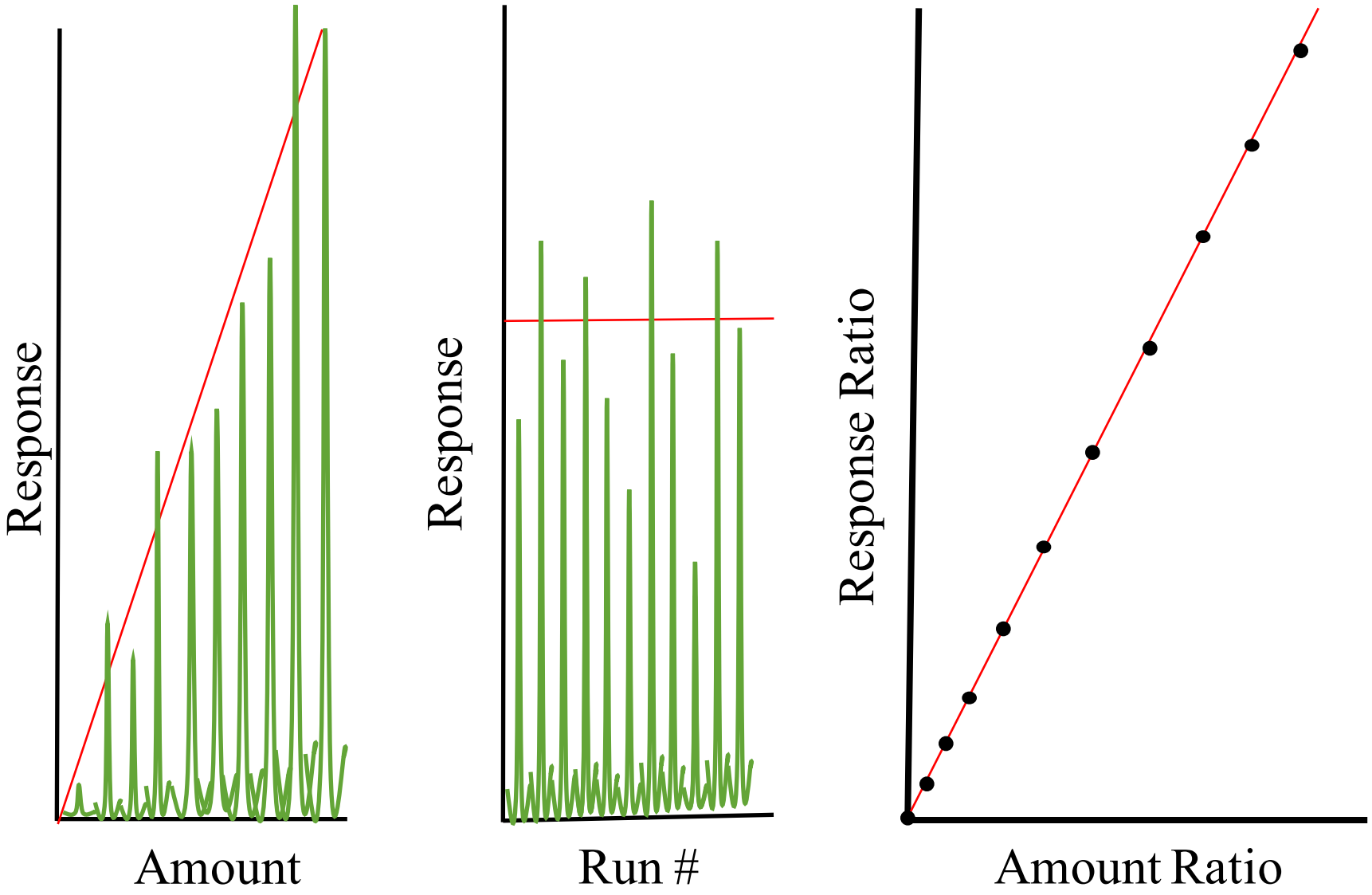
Approach 1: Quantification with External Standards



Approach 2:
Internal Standard Method

- A chemical mimic of the analyte is added to ALL samples and standards at a FIXED and KNOWN concentration. The signals for the analyte AND the internal standard are measured. The ratio R/R_{is} vs concentration is plotted for all standards to yield a calibration curve. The ratio R/R_{is} is calculated for all unknowns, and by reference to the calibration curve, is converted to concentration (or amount).
- Frequently used in mass spectrometry because the response fluctuates with time and in response to other components in the matrix.
- Requires an appropriate analyte-free sample matrix

Approach 2: Internal Standard Quantification



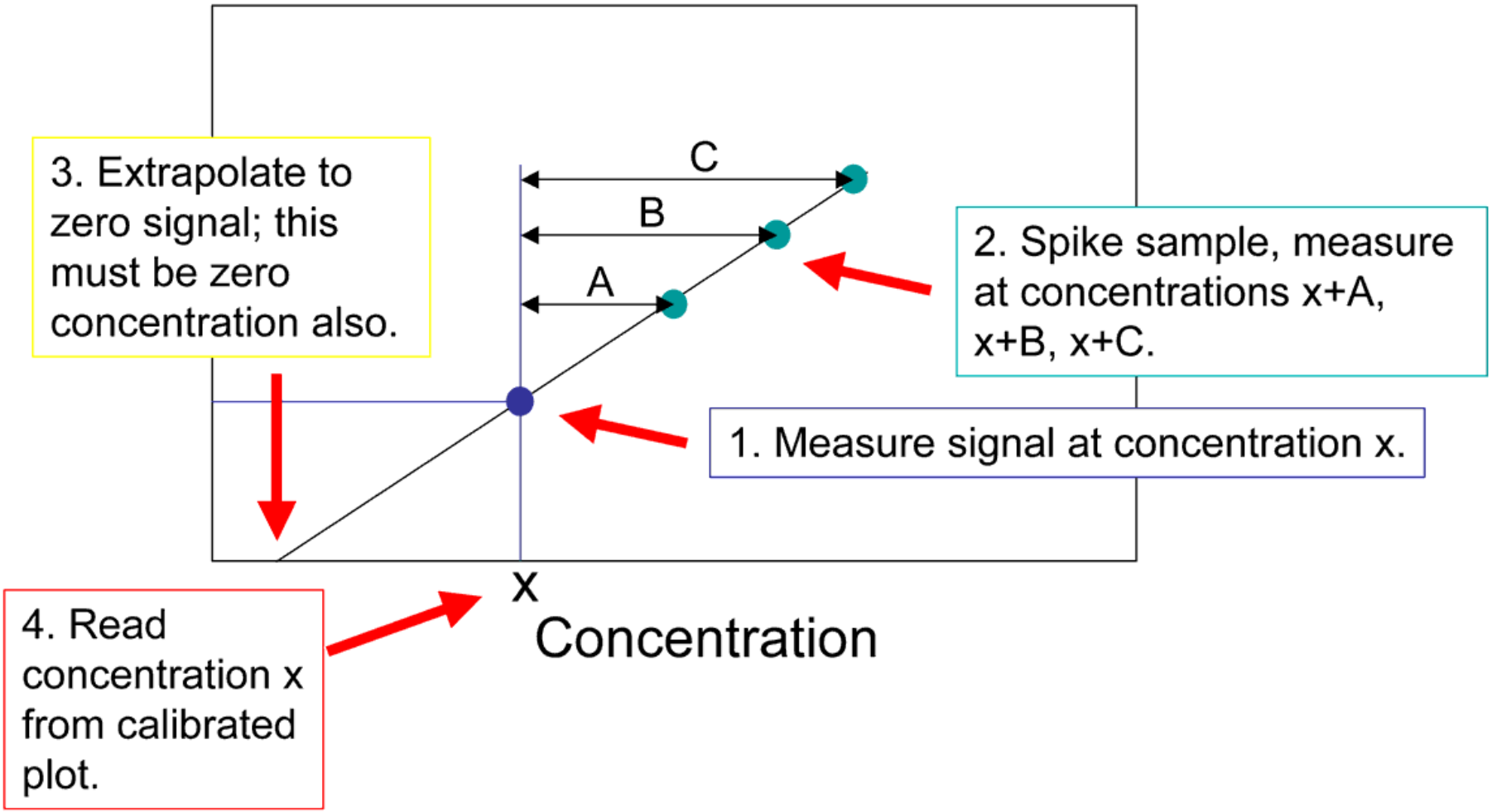
Approach 3:
Standard Addition Method

- Useful when matrix effects are pronounced, levels are near the LOD, the sample set is unique or diverse in composition, or when analyte-free matrix is unavailable.
- The unknown sample is divided into two (or more) portions and a KNOWN amount of analyte (a spike) is added to one of these. The interferent(s) in the sample will affect the standard and sample response to the same extent. Both samples are analyzed.

Approach 3:
Standard Addition Method Approach

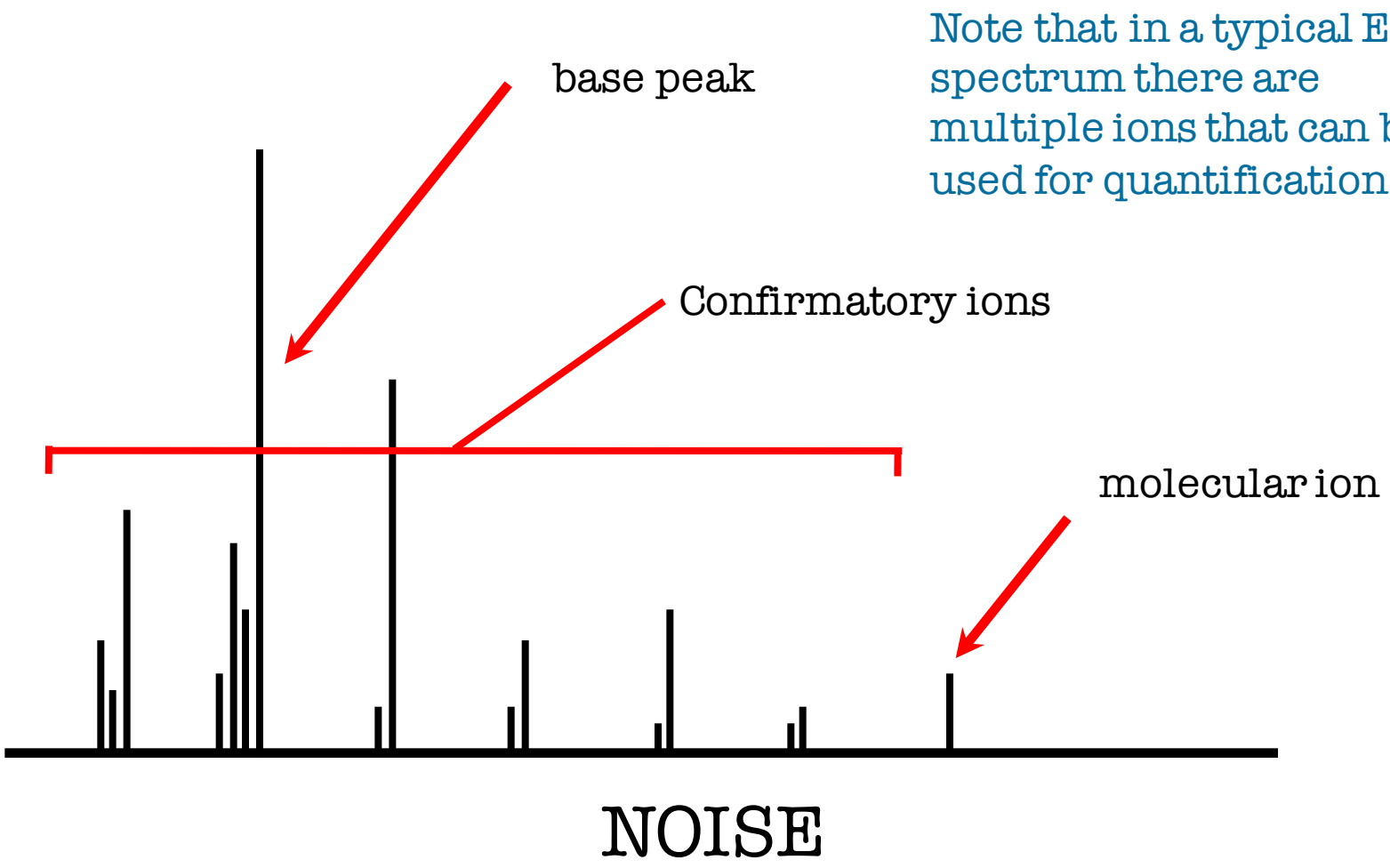
- The spiked sample shows a larger analytical response and the difference in response between the spiked and unspiked samples is due to the (additional) amount of analyte in the spike. This provides a calibration point to determine the analyte in the original sample.
- A linear response with concentration (or amount) is assumed with a 2-point (or more) determination.

Approach 3:
Standard Addition Method



Approaches to Quantitative Analysis by Mass Spectrometry

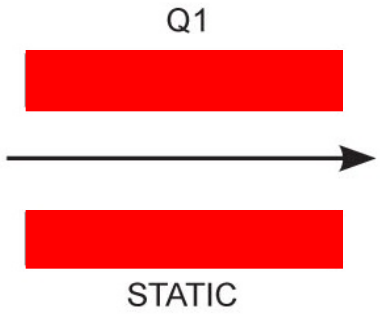
A Typical EI Mass Spectrum



Note that in a typical EI spectrum there are multiple ions that can be used for quantification

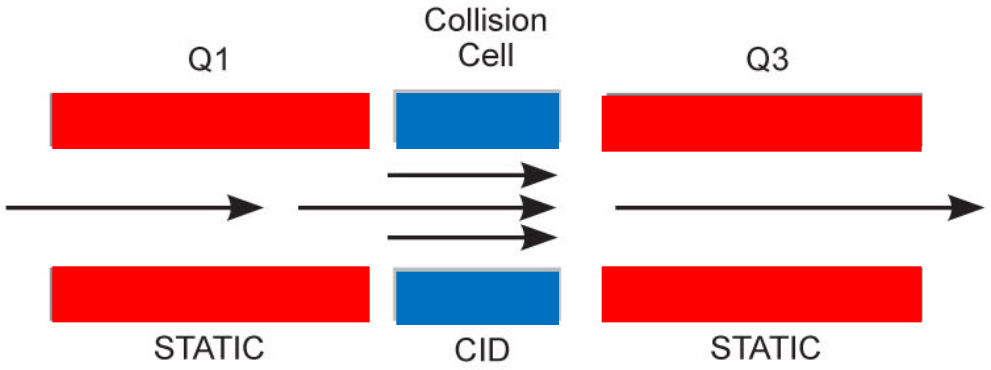
Conventional Approaches to Quantification by Mass Spectrometry

Selected Ion Monitoring



One or more ions can be 'selected' and used for quantification

Selected Reaction Monitoring

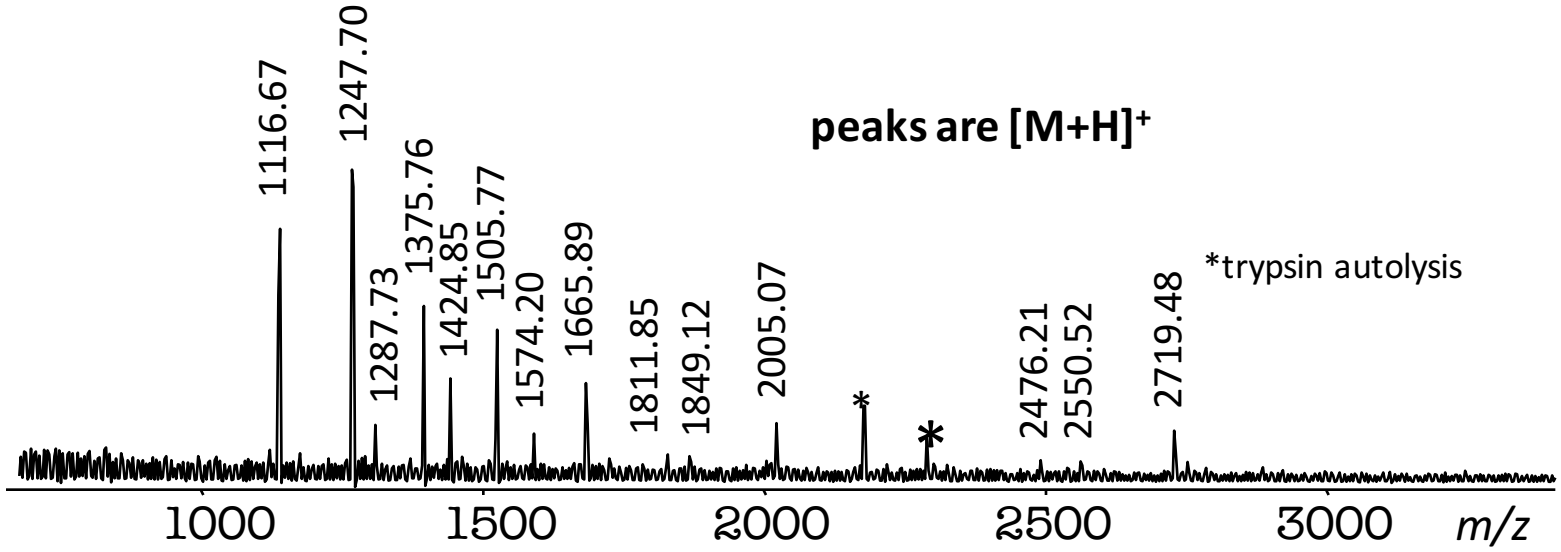


A selected ion can be selected, fragmented, and one or more of its product ions monitored.

Mass Spectrometry is not Inherently Quantitative

Components that are of the same concentration may give drastically different signal intensities

For example, if a protein is digested into peptides, different peptides give very different signal intensities (and some peptides may not be detected at all). Results are further compromised by other components of the sample matrix.



Why we use the Internal Standard Approach for Quantification by MS, including MALDI TOF MS

Ion current for a compound are influenced by multiple factors – ease of ionization, amount, and other components of the sample matrix - so that there may not be a direct relationship between amount and signal.

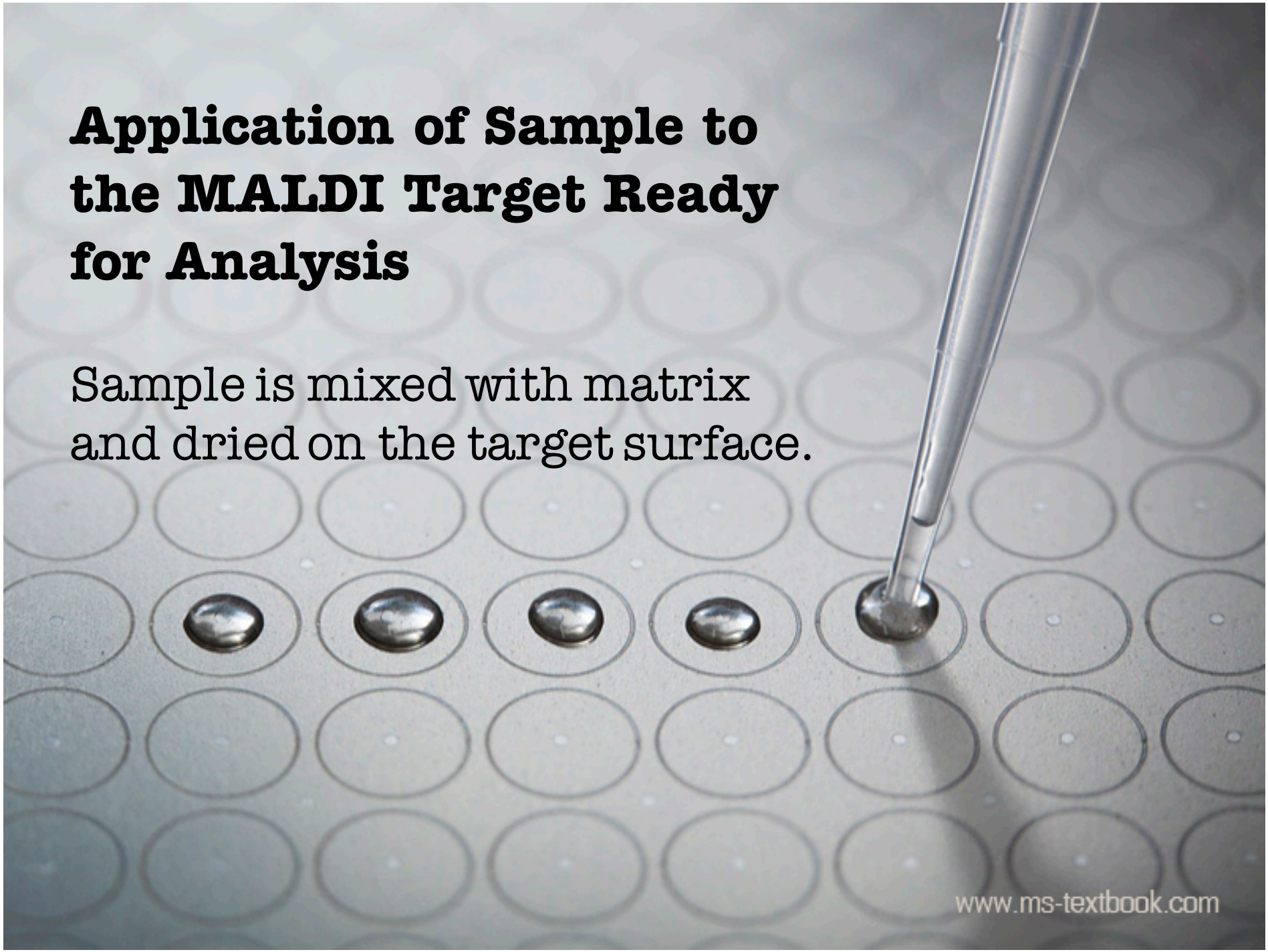
$$\text{Ion Current (EI): } dI = n\,\sigma l dI_e$$

However, given two compounds have similar chemical and physical properties, when both are present in the ion source at the same time:

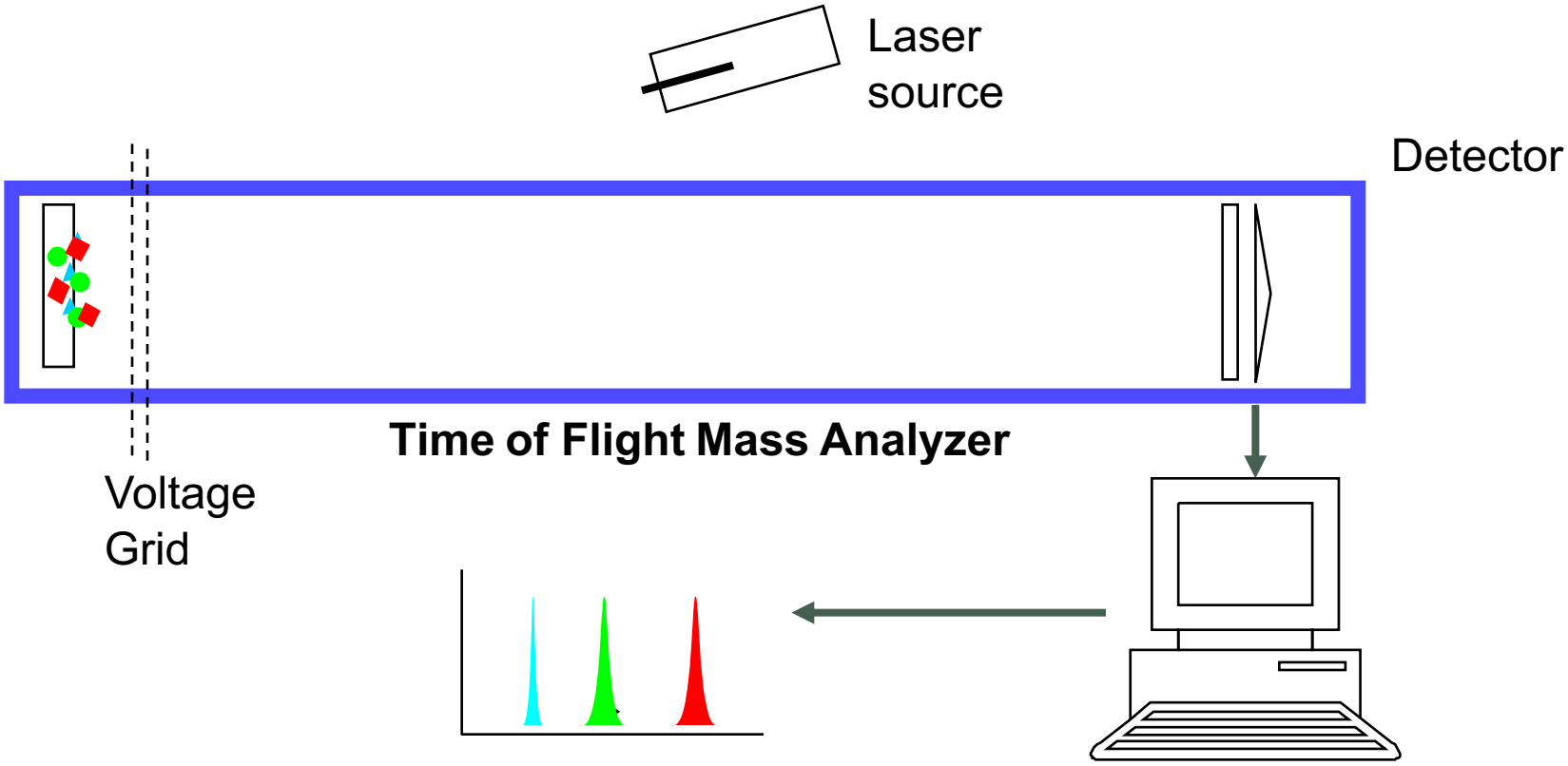
$$I_1 / I_2 = c_1 / c_2$$

Therefore, if we can measure two ion currents, one per compound, and we know one concentration, we can calculate the second.

Review of the MALDI Process


Application of Sample to the MALDI Target Ready for Analysis
Sample is mixed with matrix and dried on the target surface.
www.ms-textbook.com

MALDI-ToF MS


Laser
source
Detector
Time of Flight Mass Analyzer
Voltage
Grid

Quantification by MALDI TOF MS

Why Use MALDI TOF for Quantitative Analysis?

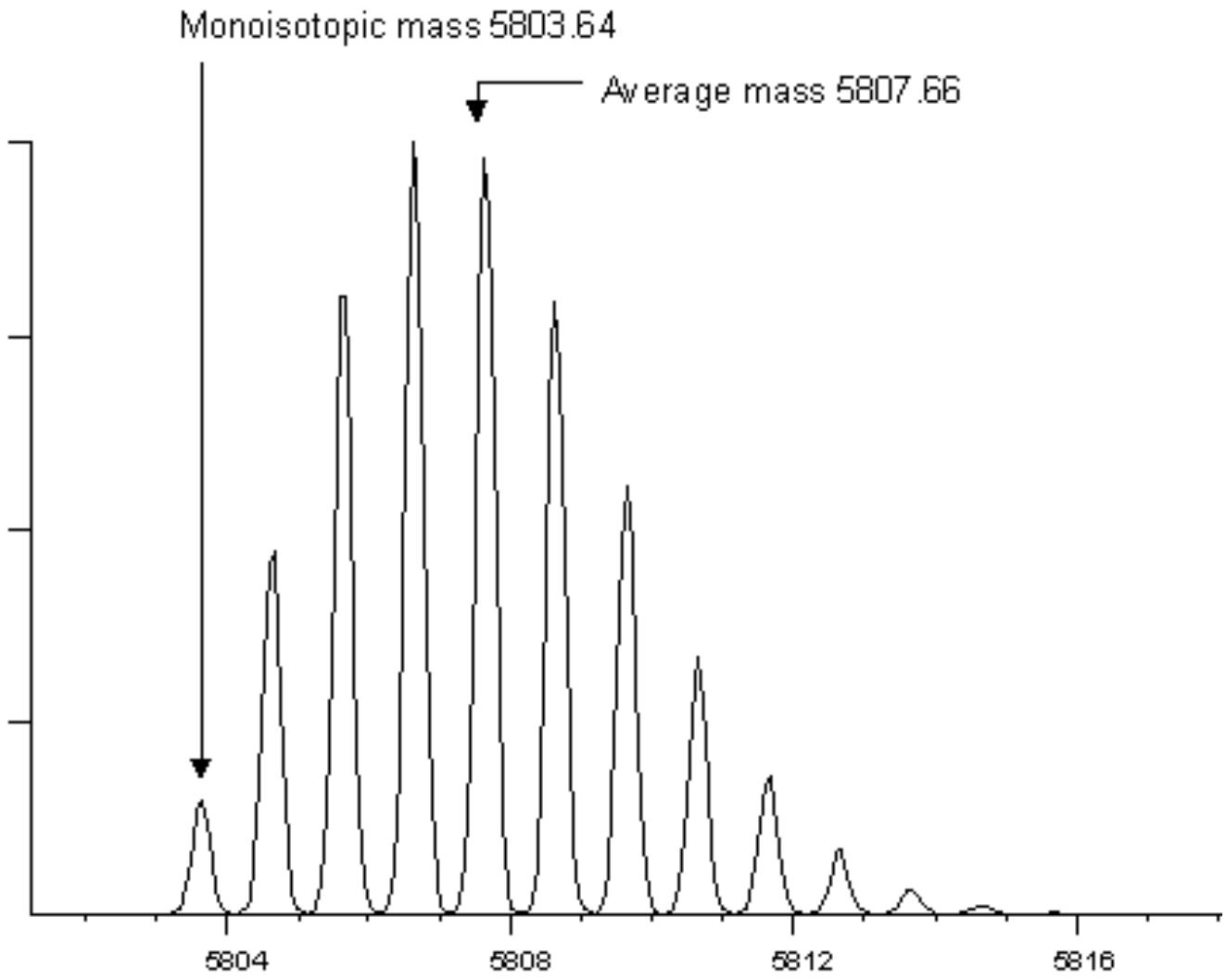
- *Fast*
- *Precise*
- *Affordable*
- *Selective*
- *Sensitive*
- *Versatile*
- *Easy to use*
- *Reliable*

In the lectures that follow we will demonstrate these and other features of MALDI that make it an ideal choice for quantification.

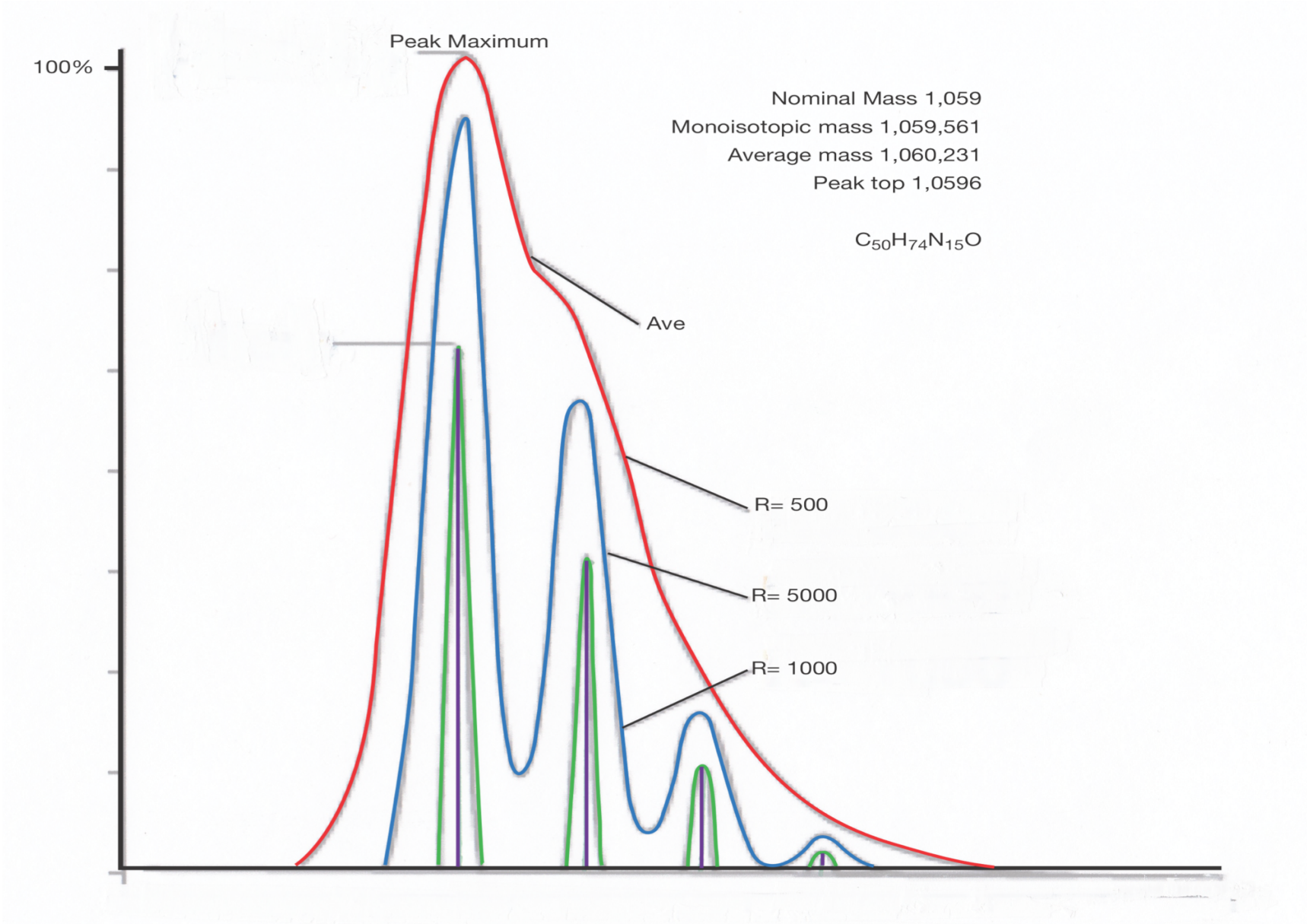
MALDI TOF Quantification

Typically, quantification by MALDI TOF MS uses a *single* signal, the peak intensity for the protonated molecule, $[M+H]^+$. Additional ions, notably multiply charged ions, can also be used. This is in contrast to other MS approaches (e.g., EI and ESI MS/MS) where multiple signals are used increase selectivity.

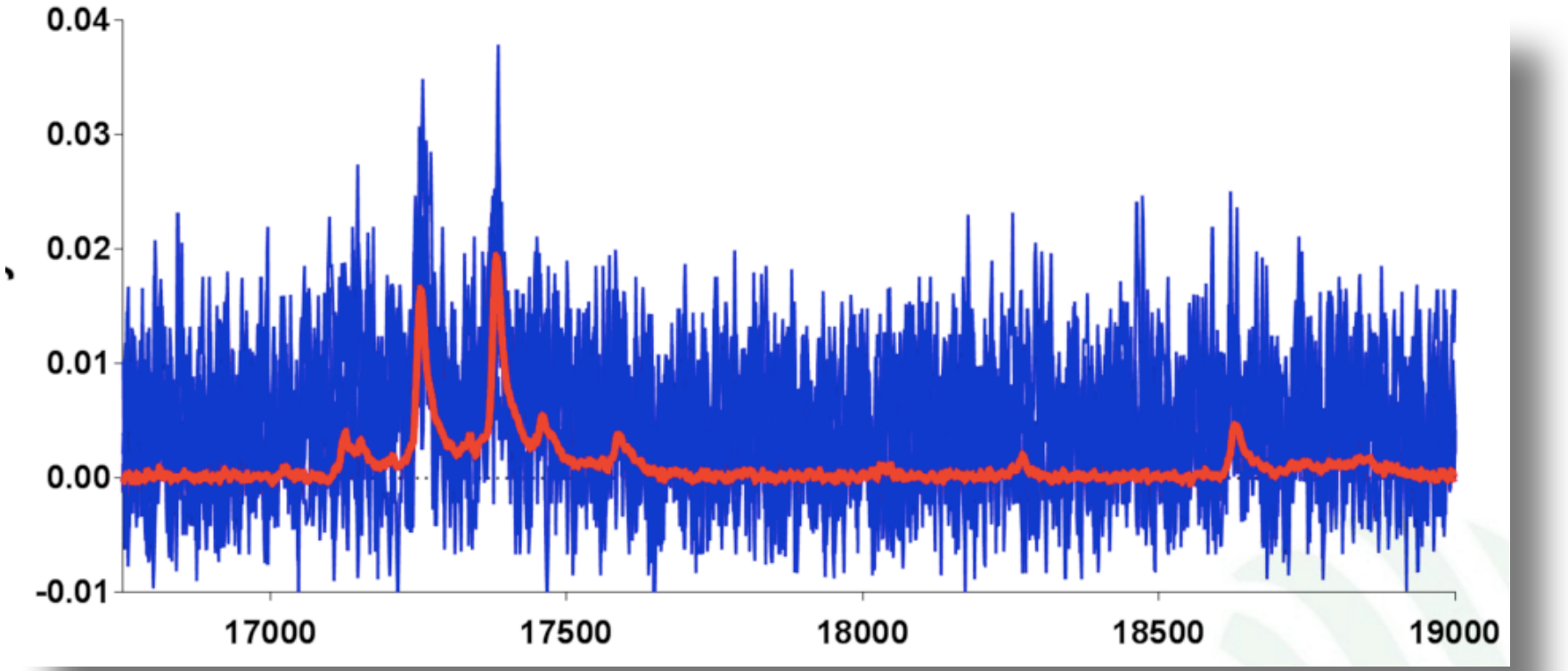
Selectivity/Specificity and Resolution


Monoisotopic mass 5803.64
Average mass 5807.66
5804
5808
5812
5816

Mass Resolution and Quantification

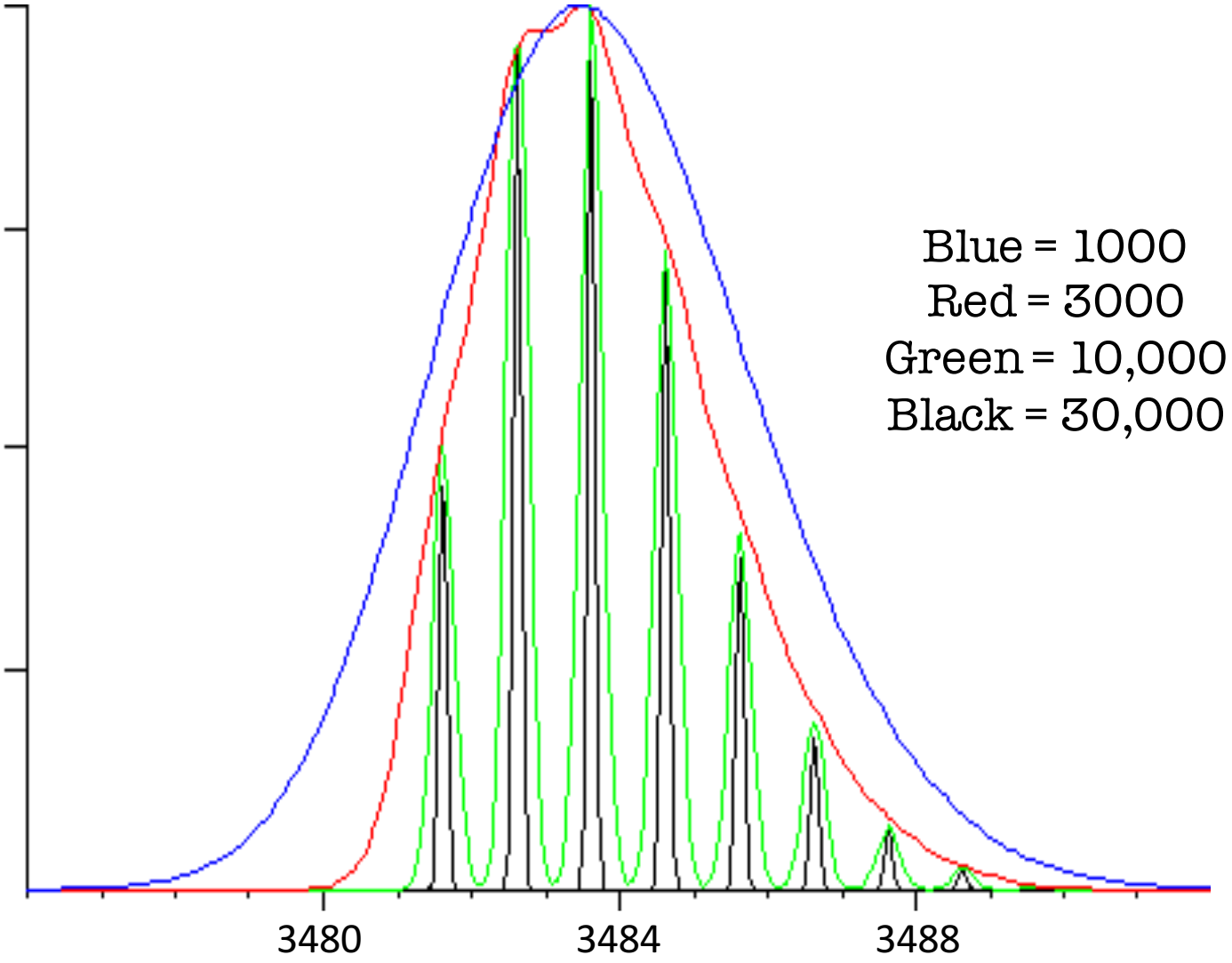


Number of Laser Shots and Signal-to-Noise



Glucagon

Various Resolutions



Specificity in Quantification by MALDI TOF Come From?

***m/z* values**

- specific structures

mass differences

- loss of specific structure

ion abundance ratios

- determined by structure

spectral pattern

- unique, but irrespective of structure

exact mass

- molecular formula

MS/MS transition

- linked structures

isotopes ratios

- specific elements

protonated molecular ion

- molecular weight info

Combined with the selectivity of the work up steps!

Unlike other MS approaches there are rarely confirmatory ions.

The Basic Steps in Quantitative Analysis

(by MALDI TOF MS)

1. Preparation of Standards

These contain differing, known amounts of the analyte of interest.

2. Preparation of Samples and Addition of Internal Standard

A fixed amount of internal standard is added to each sample, standard, QCs, and unknowns alike.

3. Sample Work-up

4. Sample Analysis

At least two separate signals are monitored for all samples: one characteristic of the analyte, the other characteristic of the internal standard.

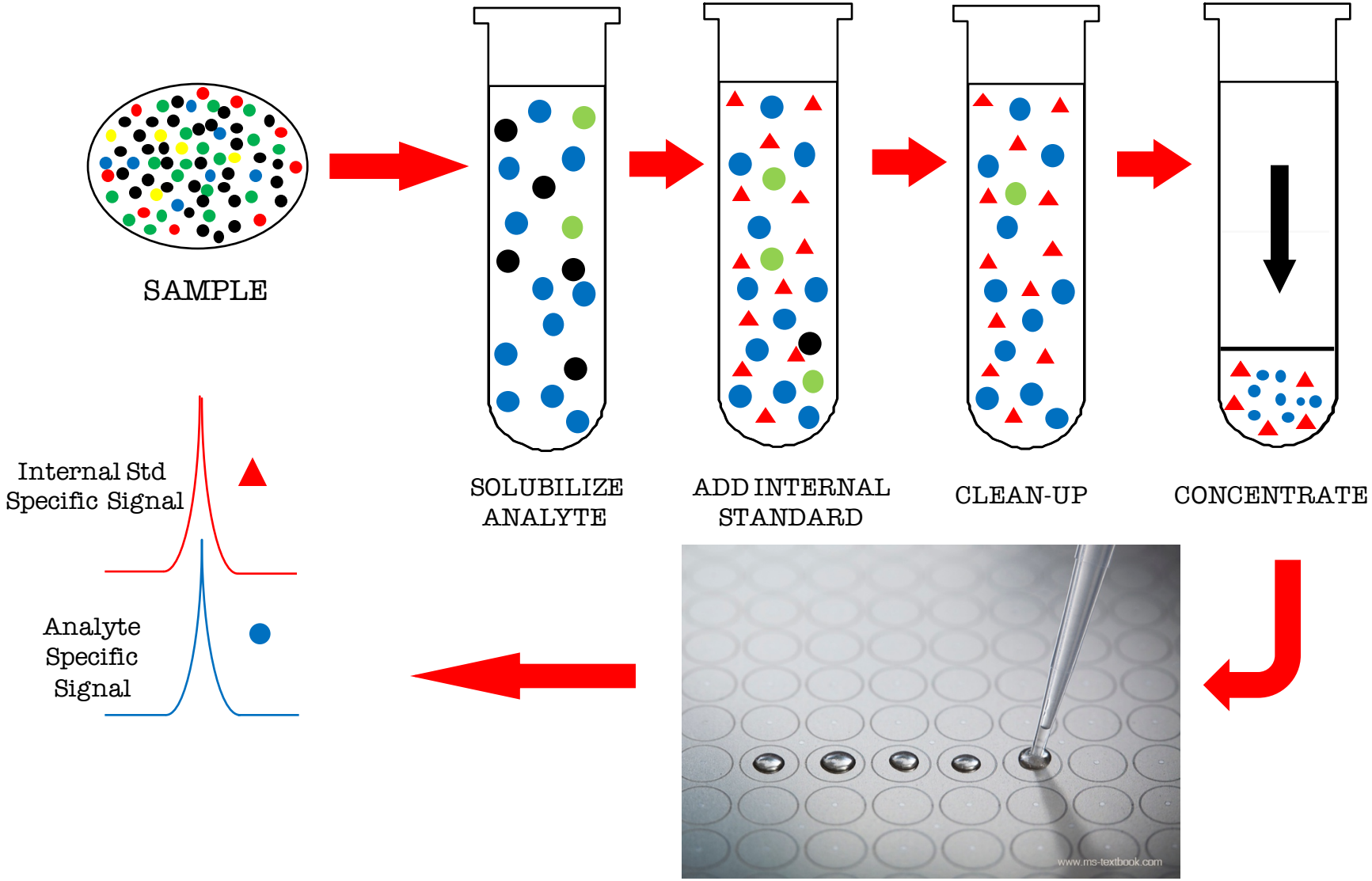
5. Regression of Calibrator Responses

6. Calculation of Concentrations

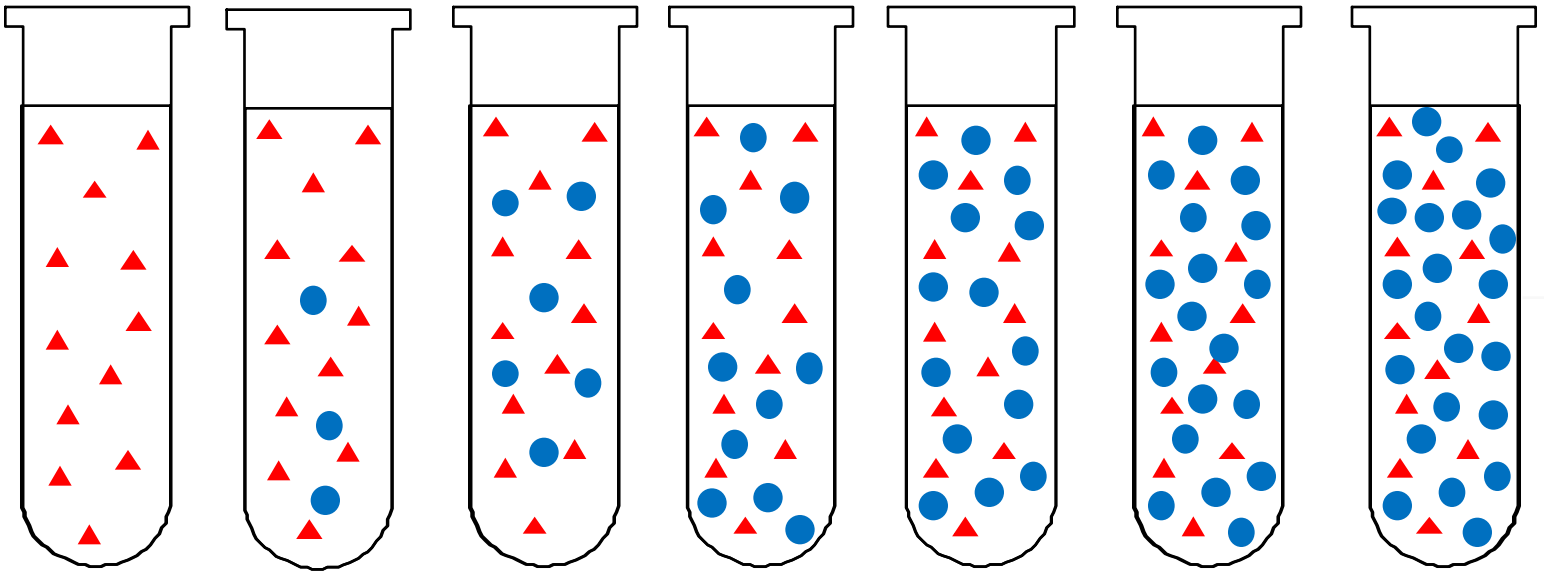
The amount of analyte in the unknown samples is determined by reference to the calibration curve.

7. Evaluation of Data

Steps in a Typical Quantitative MALDI Process

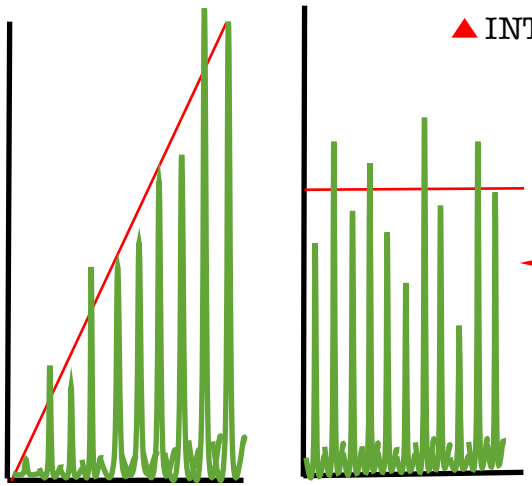


Calibration Curve



▲ INTERNAL STANDARD

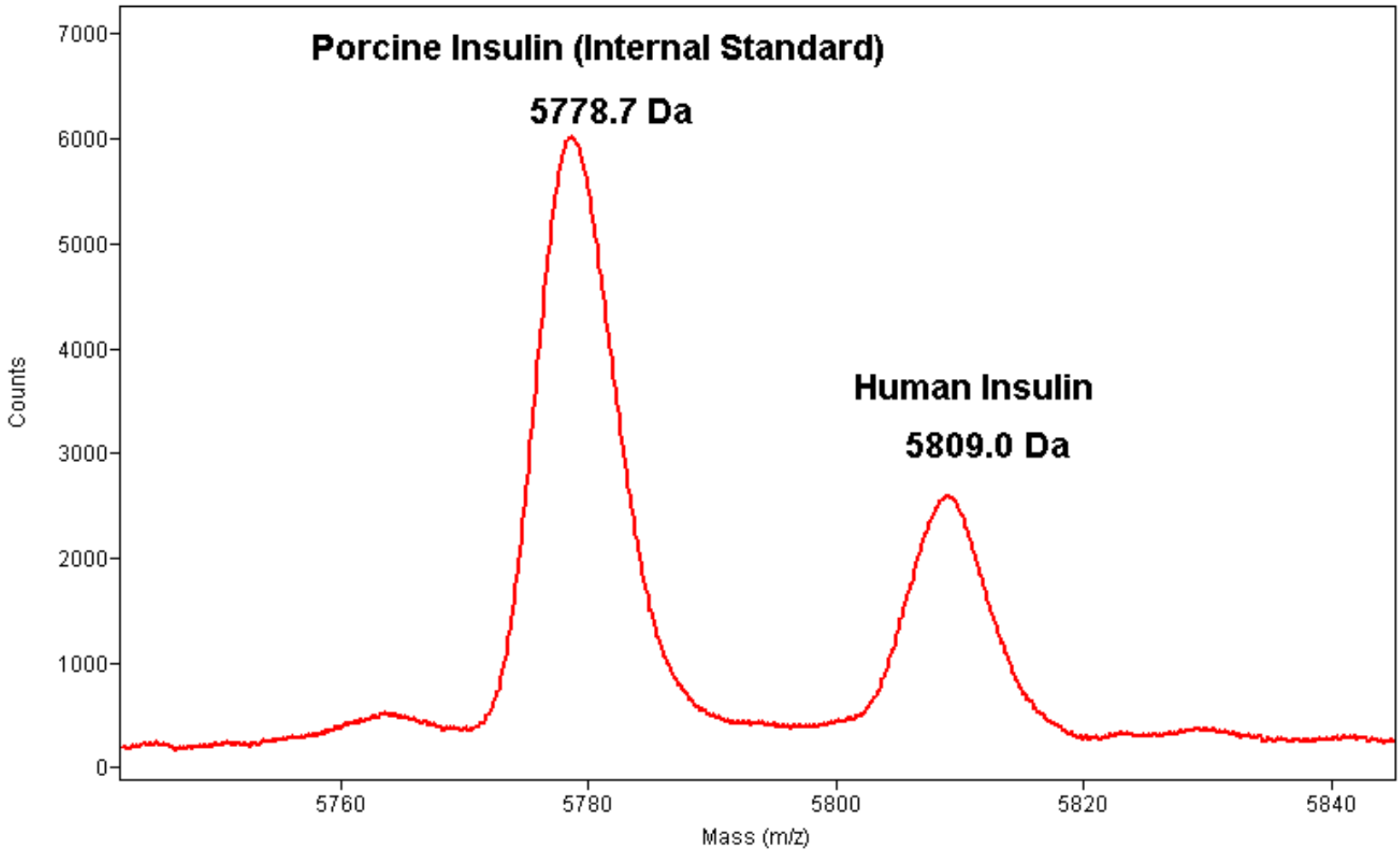
● ANALYTE



Examples of Quantification by MALDI TOF MS

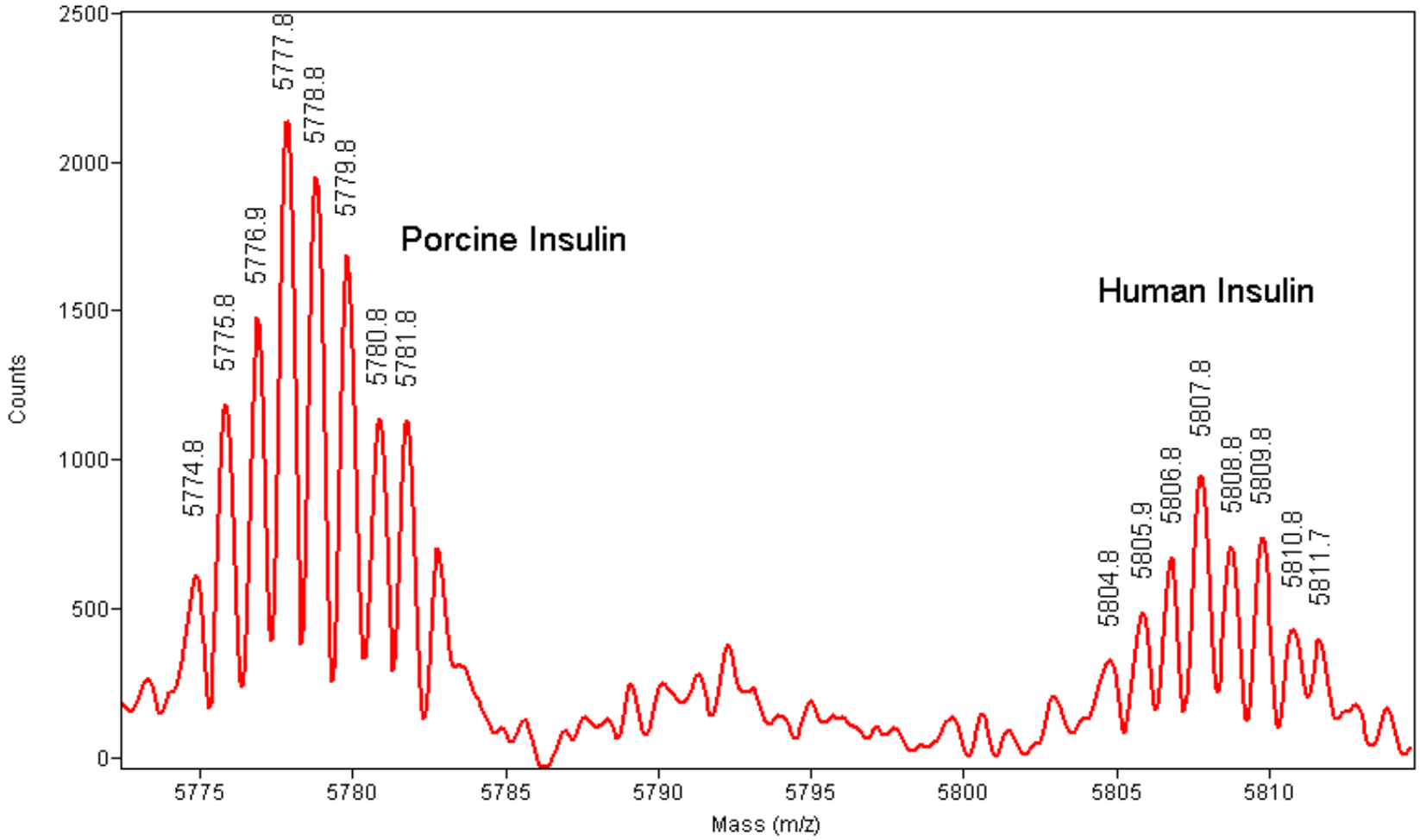
Spectrum of Pancreatic Extract

(Linear Delayed Extraction; LDE)



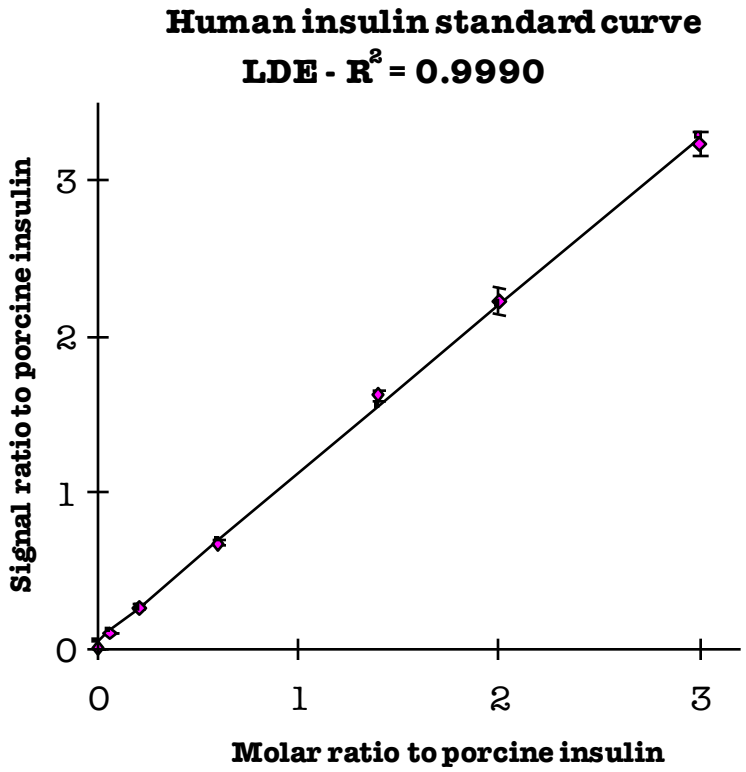
Spectrum of Pancreatic Extract

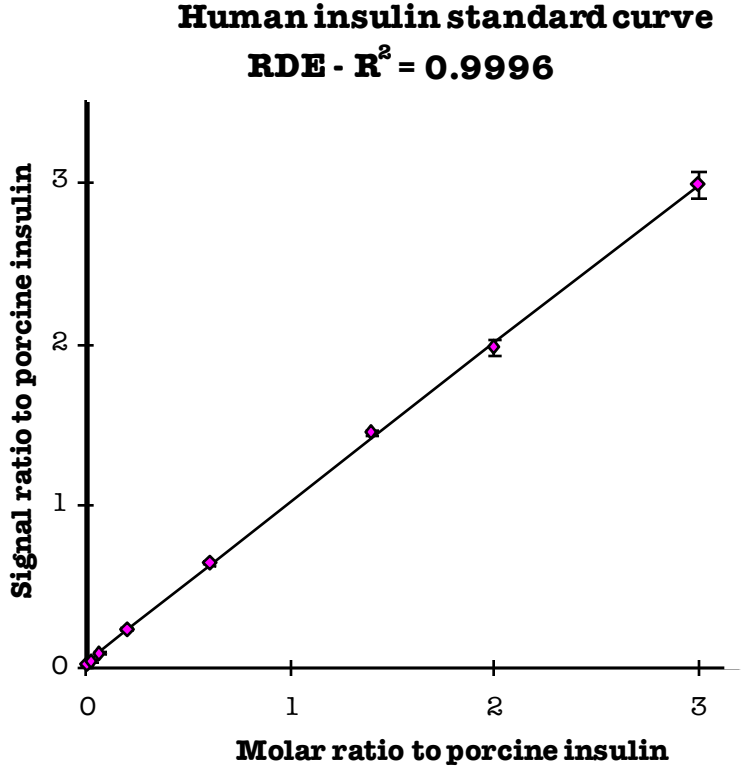
(Reflectron Delayed Extraction; RDE)



Standard Curves for Human Insulin: Linear and Reflector Modes

(Reflectron mode curve compiled using summed isotopic peak intensities.)





Insulin Quantification in Human Pancreas

(Based on both LDE and RDE Mode)

	Linear DE Mode Determinations		Reflection DE Mode Determinations	
Sample	Human Insulin nmol/g tissue	CV % (n = 10)	Human Insulin nmol/g tissue	CV % (n = 10)
1A	20.9	1.6	19.7	6.1
1B	18.7	1.4	18.2	7.1
1C	16.8	2.9	16.1	6.8
2A	12.6	3.3	12.1	6.4
2B	15.2	2.3	13.6	11
2C	12.9	3.0	10.7	6.6

Accurate Mass Measurement

Epinephrine, 10 measurements (internally calibrated)

Mean measured mass = 184.0971 Da

Standard deviation = 0.00048 Da (= 2.6 ppm)

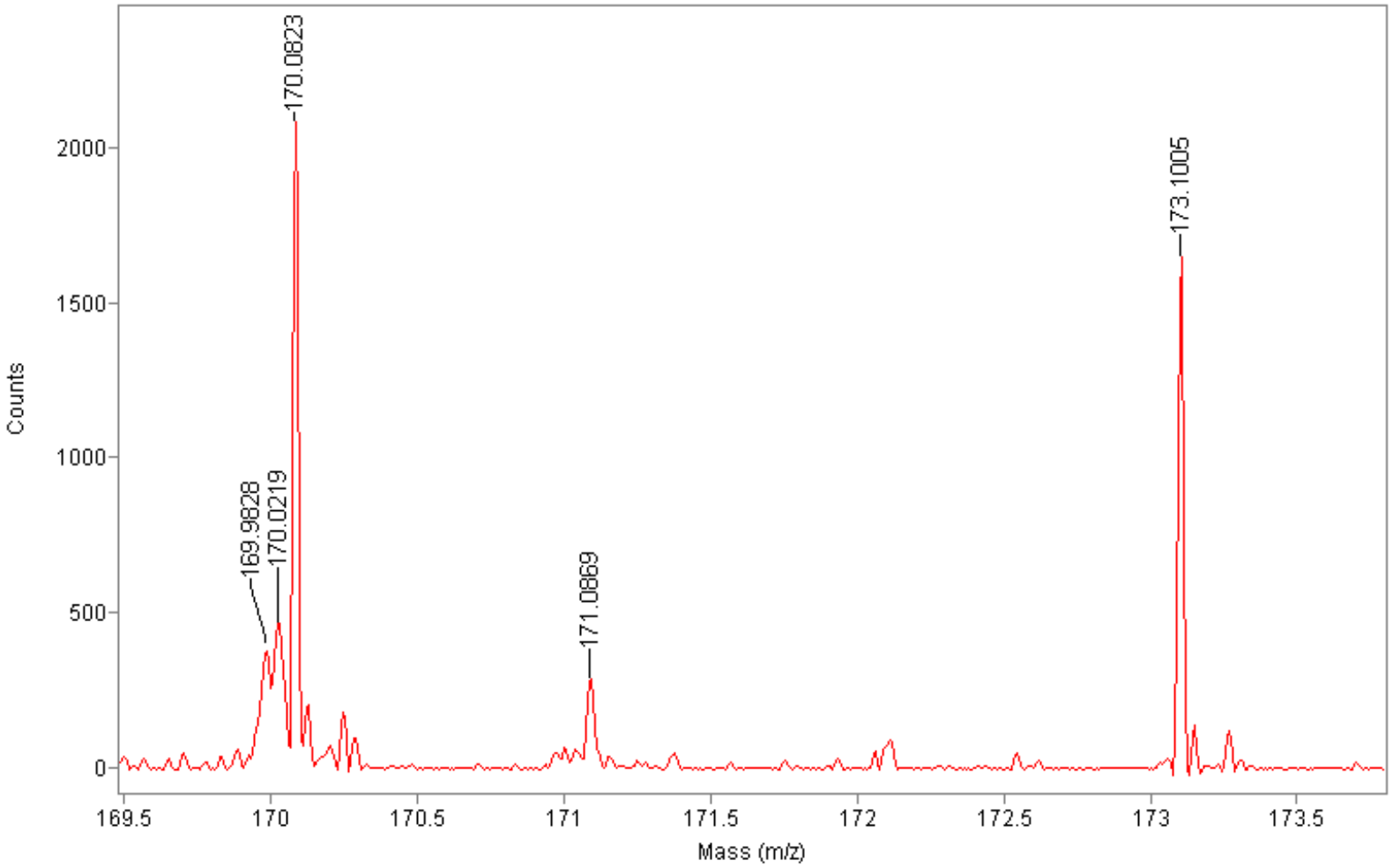
Possible elemental compositions:

Composition	Mass	Difference (ppm)
$C_{12}H_{12}N_2$	184.10005	-16.0
$C_9H_{14}NO_3$	184.09737	-1.5
$C_7H_{12}N_4O_2$	184.09603	5.8

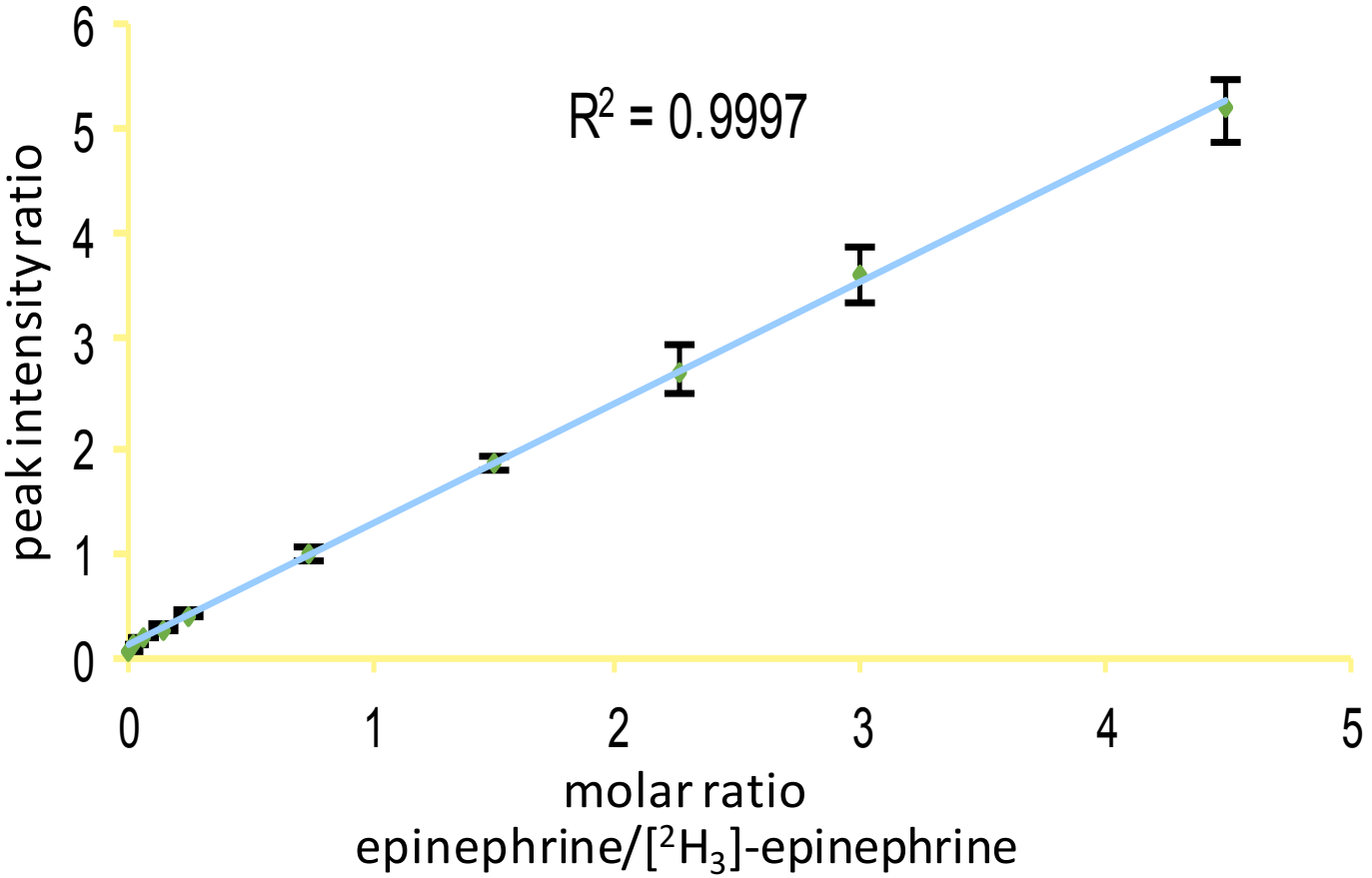
Norepinephrine & $^{2}[H]_{3}$-Norepinephrine

Adrenal Extracts

(RDE Mode)



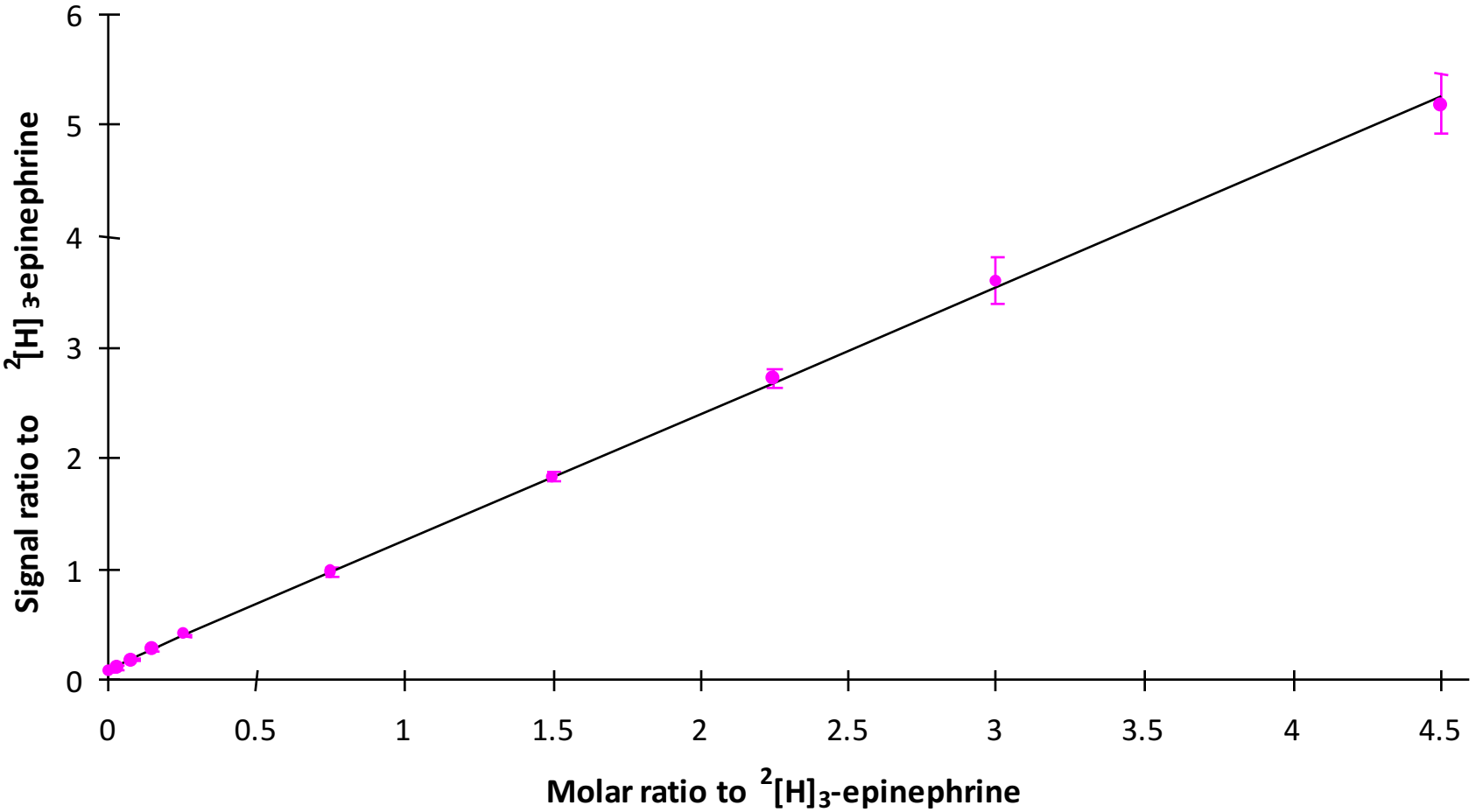
Epinephrine Standard Curve



Standard Addition Curve:

Epinephrine in Extract of an Adrenal Cortex

R^2=0.9996



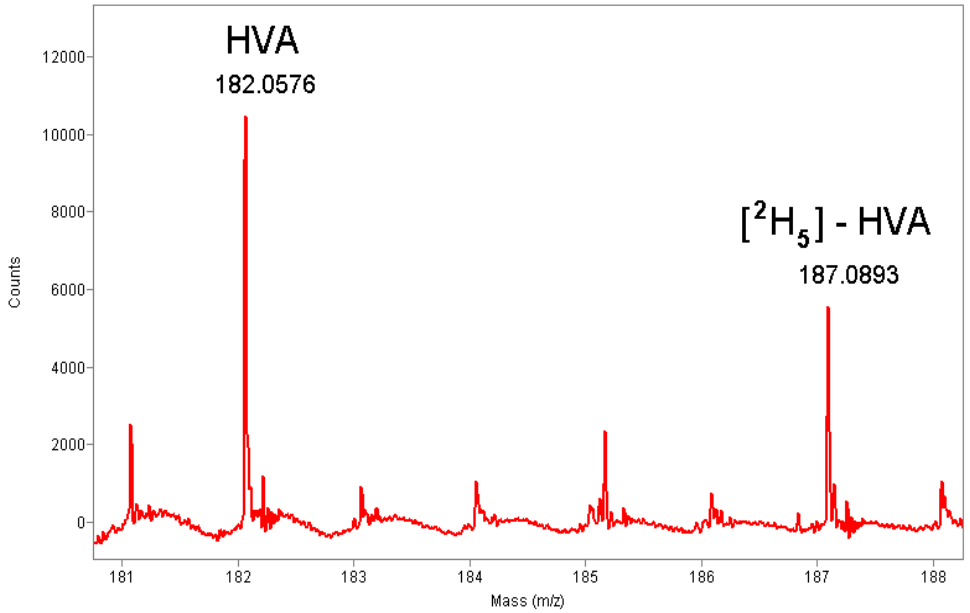
Concentration of Catecholamines in Tissue

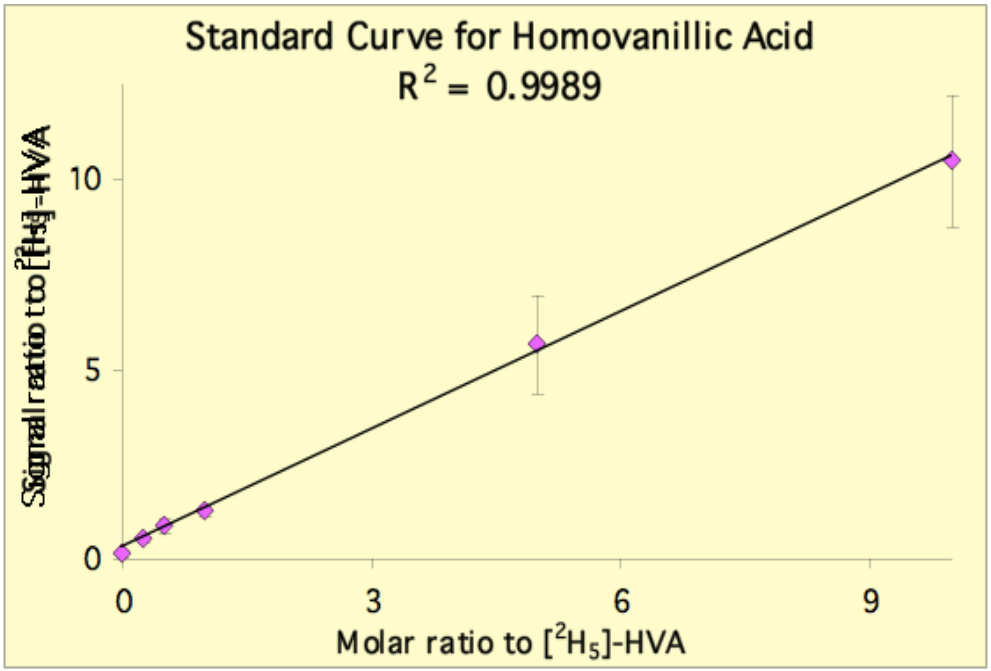
	NOREPINEPHRINE		EPINEPHRINE	
BIOLOGICAL TISSUE	CONCENTRATION (nmol/g tissue)	CV (%)	CONCENTRATION (nmol/g tissue)	CV (%)
Adrenal Gland A	978	9	4143	7
Adrenal Gland B	233	16	279	6
Adrenal Gland C	510	13	582	10
Adrenal Gland D	499	17	690	19
Pheo Tissue 1	3,989	7	184	16
Pheo Tissue 2	6,708	8	2,854	8
Pheo Tissue 3	59,940	5	5,735	4

Quantification of HVA in Human Urine

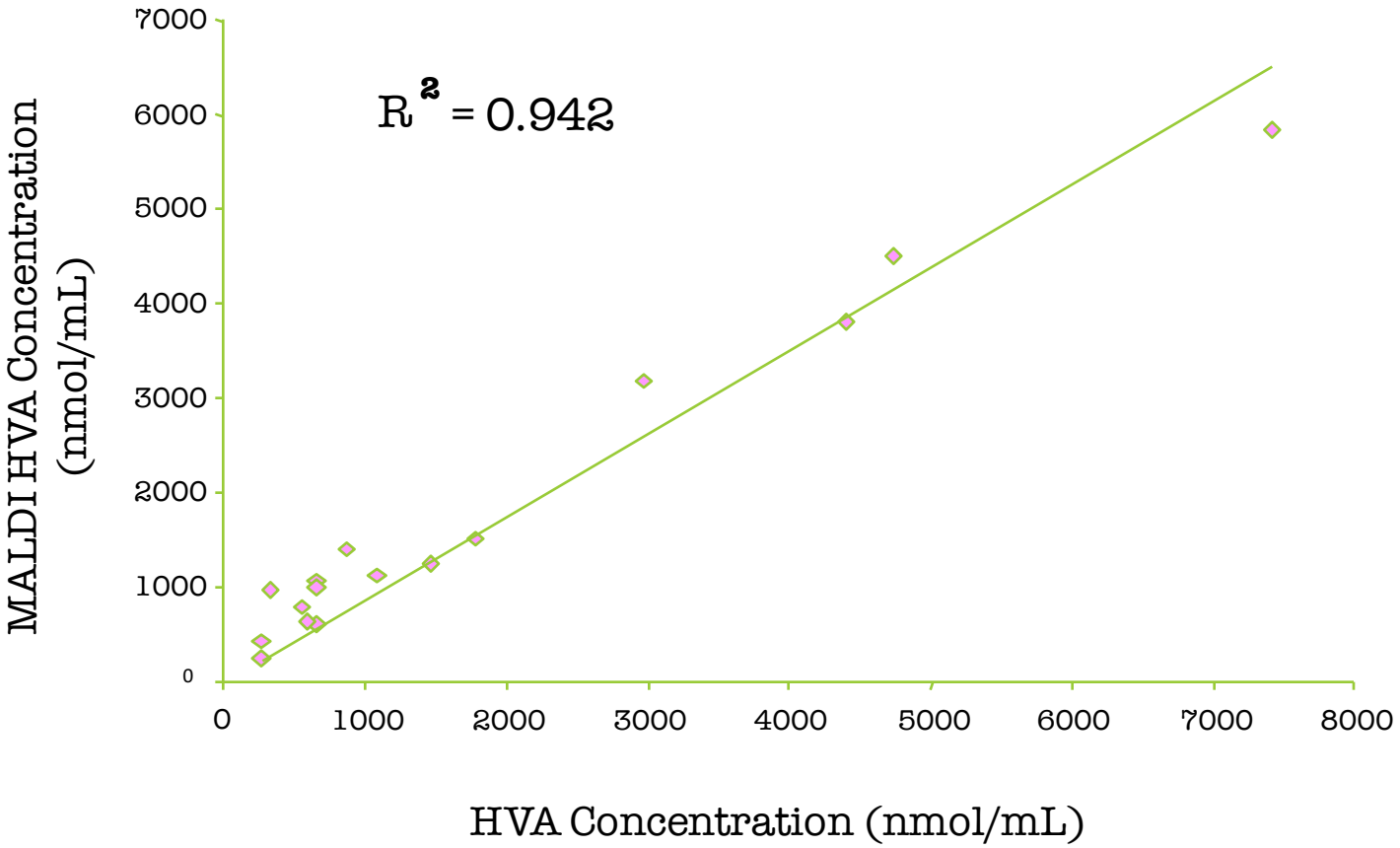
Crude urine samples were spiked with [$^2H_5$]-HVA as internal standard, mixed with DHBA matrix and assayed directly by automated MALDI-TOF MS.

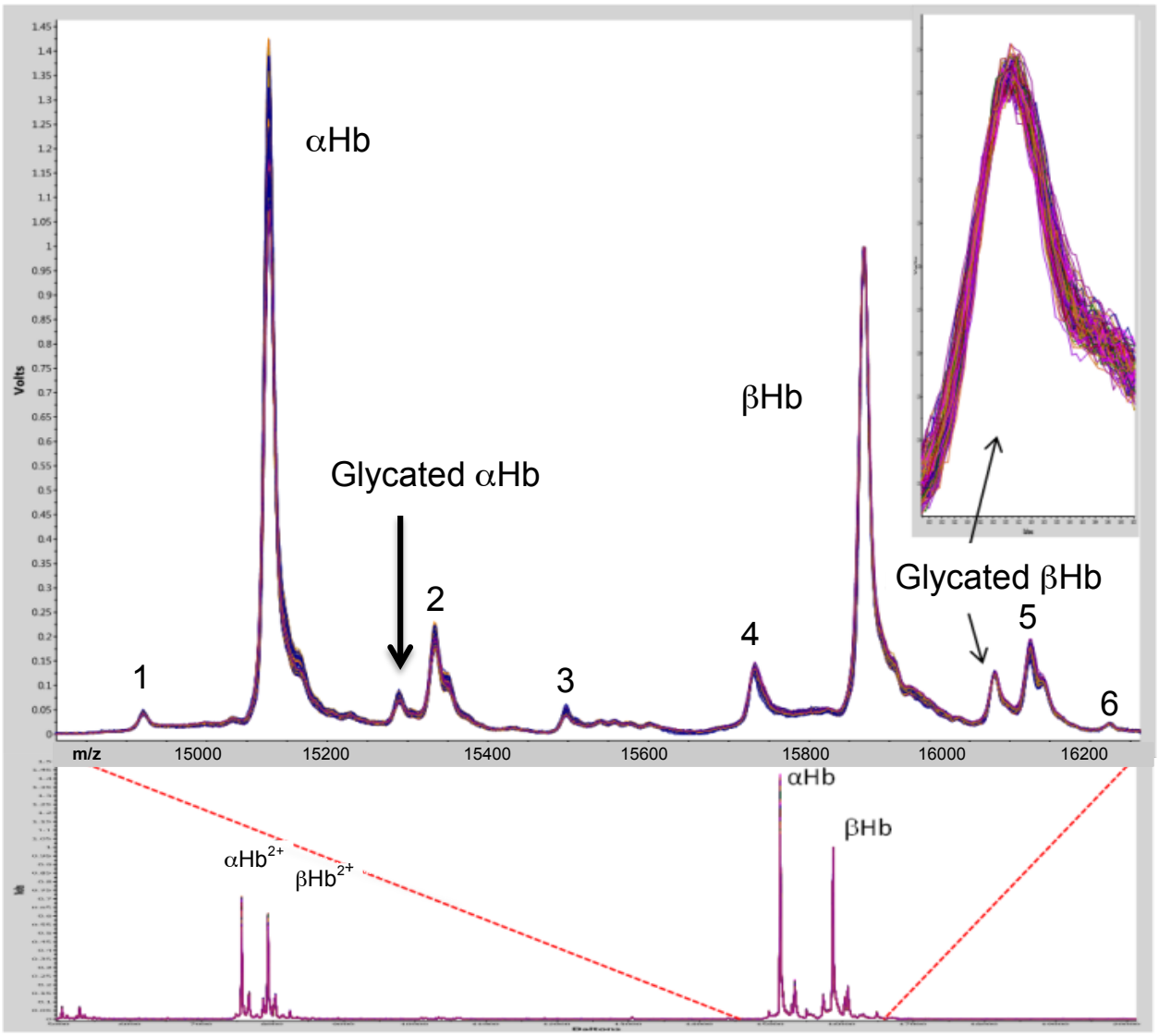
A standard curve for HVA in water gave R^2=0.9989 over the concentration range 2.5 to 100 nmol/mL.





Correlation Between HVA Results by MALDI-TOFMS and HPLC




αHb
βHb
Glycated αHb
Glycated βHb
1
2
3
4
5
6
Volts
m/z
15000
15200
15400
15600
15800
16000
16200
αHb
βHb
αHb2+
βHb2+

Method Validation

The Validation Process

- Prescription for demonstrating the ability of the assay *in toto* to achieve its purpose: i.e., to quantify analyte concentrations with a degree of accuracy and precision appropriate to the task at hand.
- The process of validation certifies that the analytical method performs as intended, i.e., that concentration data produced with the method will fit the purpose for which they are being generated.

Goals of Validation

- Demonstrate the ability of the assay to predict the concentration of unknowns accurately and reproducibly
- Determine sample storage conditions necessary to assure analyte stability
- Define conditions for sample work-up that maintain the integrity of the sample
- Set criteria that can be used to accept or reject analytical data in subsequent applications of the assay

USP defines eight steps for validation

- Accuracy
- Precision
- Specificity
- Limit of detection
- Limit of quantification
- Linearity and range
- Ruggedness
- Robustness

Validation Parameters

Primary Parameters

- Accuracy: Describes the deviation from the expected result
- Precision: Indicates the scatter in analytical results. Usually described by relative standard deviation (RSD) or coefficient of variation (CV)

Secondary Parameters

- Range (*i.e.*, upper and lower limits of quantification,
- Selectivity (*i.e.*, assess interferences)
- Recovery
- Stability
- Acceptance criteria
- Comparison with other existing methods (where possible)

Essential Documentation

A written procedure (or protocol):

- Details of all experimental steps.

A method validation report:

- Includes both the experimental design and the data justifying the claim that the method, as written, performs as intended.

System suitability criteria:

- Defining the minimum acceptable performance criteria prior to each analysis.

Criteria for the acceptance and/or rejection of the analytical data:

- Define minimum acceptable performance for standards & QCs for subsequent application of method

MALDI TOF MS Profiling

MALDI-ToF Profiling of Clinical Samples

Goal: To Develop Clinically Useful Tests

Why MALDI profiling?

- No *a priori* knowledge of specific proteins or their function is necessary.
- High throughput, good reproducibility.
- Not just a discovery platform: Discovery, development, validation and commercialization can all be performed on the same platform.

Why serum/plasma?

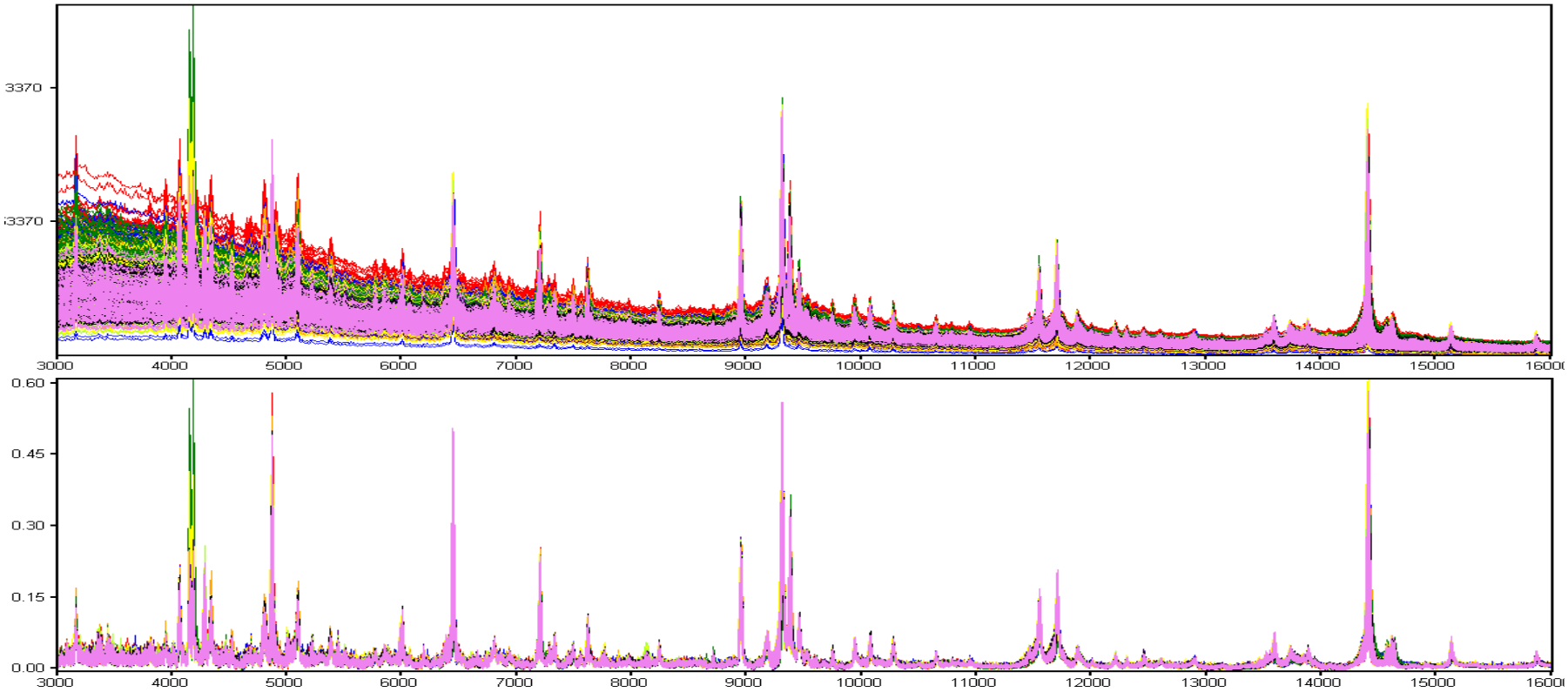
- It is readily available from many retrospective studies.
- Contains many circulating proteins that *should* characterize disease and define disease characteristics.
- Allows rapid and simultaneous multi-component determinations. Analyses can be easily repeated and give reproducible results (*cf.* tissue samples).
- Sample collection and logistics are straightforward and routine.

Criticisms of MALDI-ToF Profiling

- SELDI profiling failed spectacularly (Ciphergen).
- Few other profiling studies attempted, and most have failed to generate validated results.
- Conventional wisdom is that MALDI profiling is:
  - ✓ Intrinsically irreproducible
  - ✓ Unsuitable for quantitative determinations
  - ✓ Only detects high-abundance proteins
- The focus has switched to profiling of fractionated samples by LC-MS (either pre or post proteolysis), but this is:
  - ✓ Is time, sample and labor intensive
  - ✓ Offers poor reproducibility because of variable retention times, carryover, variable proteolysis …
  - ✓ Only suitable for discovery. Too slow, irreproducible and impractical for validation or routine use

MALDI-ToF MS Is Reproducible and Can Be Made More Reproducible

Data processing algorithms involving background subtraction, spectral alignment and normalization can be used to generate more reproducible data. Shown below: 9 groups of raw spectra, each 100 technical replicates of a single serum sample. CVs: 2-40%. (Data from Biodesix Inc.)



Additional Considerations

Peptide Standards & MS/MS Quantification

- For a typical peptide (12-20 AA's), the peptide content can vary from 50-85% because of waters of hydration and counter ions (e.g., trifluoracetate, but also formate, acetate, chloride, ammonium, etc.).
- Peptide content in a reference standard can change over time due to changes in humidity during storage or while being weighed out.
- Because of these factors, the weight of a peptide standard does not give an accurate measure of the amount of peptide: quantitative errors can be substantial.

Sample Selection

Liquids	Cerebrospinal fluid (CSF) Tears Sweat Saliva BAL Sinus lavage Urine Bile
Mixed	Blood, plasma, serum Sputum Feces
Solids	Breath condensate Tissue, eg, brain, heart, kidney, liver, lung, muscle ... Hair Nails (finger & toe) Bone

Biological Relevance, Ease of Collection & Ease of Analysis

Modified from: Maickel, Drug Determination on Therapeutic and Forensic Contents (1984)

Precisely Wrong

US laboratories participating in a proficiency testing programs

- One laboratory reported significantly higher results for urinary metanephrines than all others (3 successive surveys).
- This lab did not use commercial calibrators; all other labs did.
- At the time, only ONE commercial source of urine metanephrine calibrators (supplied as lyophilized urine).
- Analysis of the calibrators by LC-MS/MS indicated *33% higher values for normetanephrine and 40% higher values for metanephrine in the commercial calibrator than in the in-house calibrator*.
- Values assigned to the commercial calibrator were 24–33% lower than their real values. Values for commercial calibrators were inaccurate.

Singh et al., Clinical Chemistry 51, No. 2, 2005

Sources of Contaminants

- Solvents are frequently used in large volume and can contain contaminants and these can be troublesome.
- Plasticware of all kinds contains plasticizers (diesters of phthalic acid usually) or elements of the polymers *per se are very easily leached out.*
- *Fine chemicals can contain impurities that may cause problems in analytical procedures*
- *Contaminants can arise from fingerprints and from a host of materials peripheral to the sample.*
- *Cosmetics, hair preparations, hand creams, soaps, polishes, lubricants and greases*