

## Quantitative work in HPLC

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Medtechnica

[www.forumsci.co.il/HPLC](http://www.forumsci.co.il/HPLC)

## Data Handling

Analytical Chemistry -

Science of making quantitative measurement

Raw data is manipulated and reported correctly to give a realistic estimate of the uncertainty in a result.

## Chemist's Concerns

Maximize Confidence

- ▶ Accuracy of the data
- ▶ Precision or reproducibility of the data
- ▶ Sensitivity of detection
- ▶ Selectivity of the separation
- ▶ Ruggedness of the method

## REFERENCE STANDARDS:

Established source and known grade (DMF or COA)

% purity from assay will be taken into account in the calculations.

% residual compounds (GC, heavy metals, inorganic salts, water, residual solvents, weight loss).?

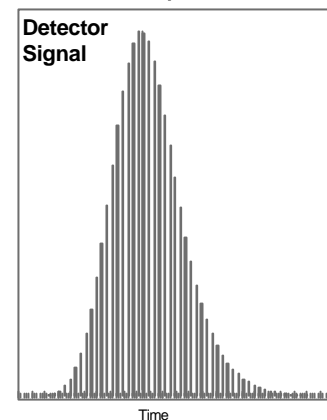
### Choice of Standardization: External or Internal

Simple formulations and sample preparation: external standard

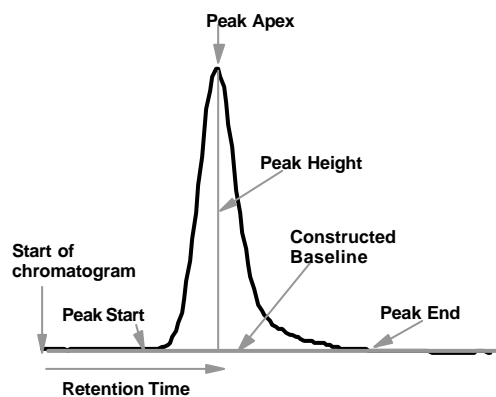
Gas chromatography, bio-studies or complex medium and complex sample preparation: internal standard

### Measurement of Area - Integration

$$\text{Area} = \int \text{Abs} \times dt$$



### Peak Detection:

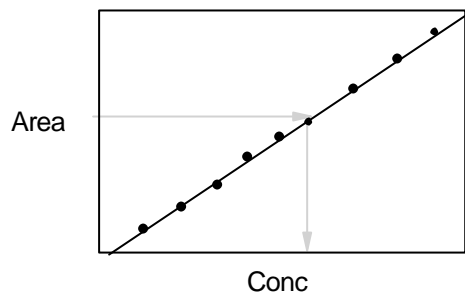


### Quality Control

VIAL	SAMPLE NAME	INJ VOL	No of Inj	Function	Method	Run Time	Sample Weight	Dilution
1	Blank	20.0	1	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
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3	Std1	20.0	5	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
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				Report	LC Calibration Report			
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				Clear Calibration	LC Demo Method Set			
				Calibrate	LC Demo Method Set			

## Working Curve

■ A plot of the analytical signal (the instrument or detector response) as a function of analyte concentration, using a series of standards of known concentration.



The working curves are then used to determine the concentration of an unknown sample or to calibrate the linearity of an analytical instrument.

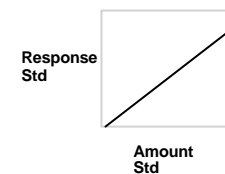
## Choice of Standardization: External or Internal

### External Standard

Amount Std → Response Std

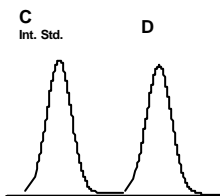
Amount Unk → Response Unk

$$\text{Amount Unk} = \frac{\text{Amount Std}}{\text{Response Std}} \times \text{Response Unk}$$



## Choice of Standardization: External or Internal

### Internal Standard

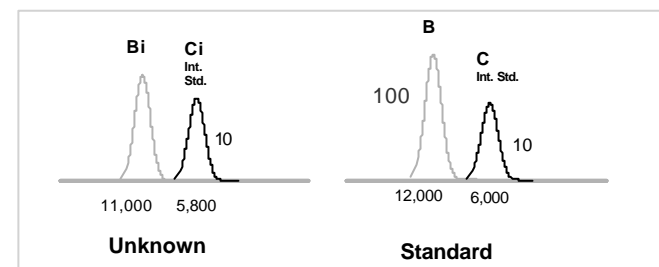


$\frac{\text{Amount Std}}{\text{Amount Istd}} \rightarrow \frac{\text{Response Std}}{\text{Response Istd}}$

$\frac{\text{Amount Unk}}{\text{Amount Istd}} \rightarrow \frac{\text{Response Unk}}{\text{Response Istd}}$

12,000/6000 → 100/10

11,000/5800 → Amt Bi/10



$$\frac{(11,000/5,800) \times (100/10)}{12,000/6000} \times 10 = \text{Amt Bi} = 94.8$$

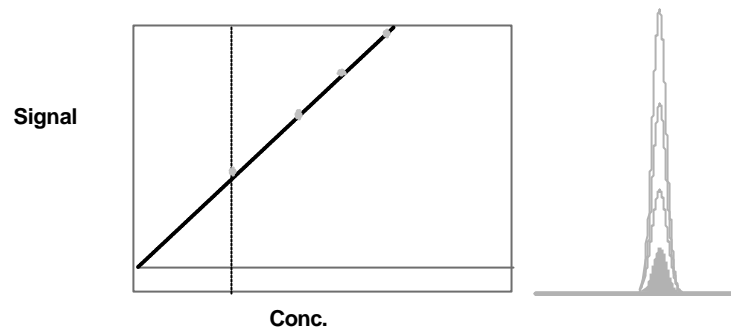
### Linear Regression for the Equation:

$$y = mx + b$$

- Linear regression uses the method of least squares to determine the best equation describing a set of x and y data points.

### Standard Addition

- Due to matrix effects the analytical response for an analyte in a complex sample may not be the same as for the analyte in a simple standard.

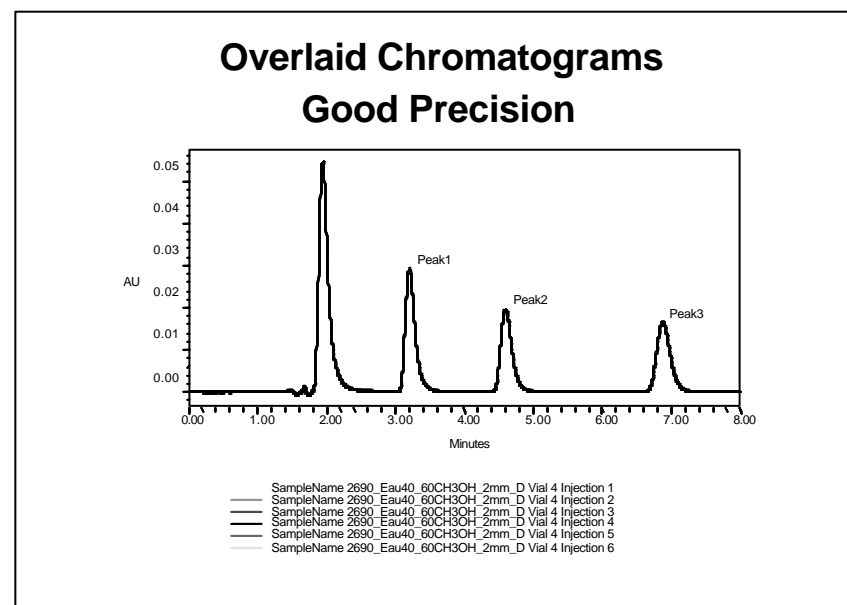
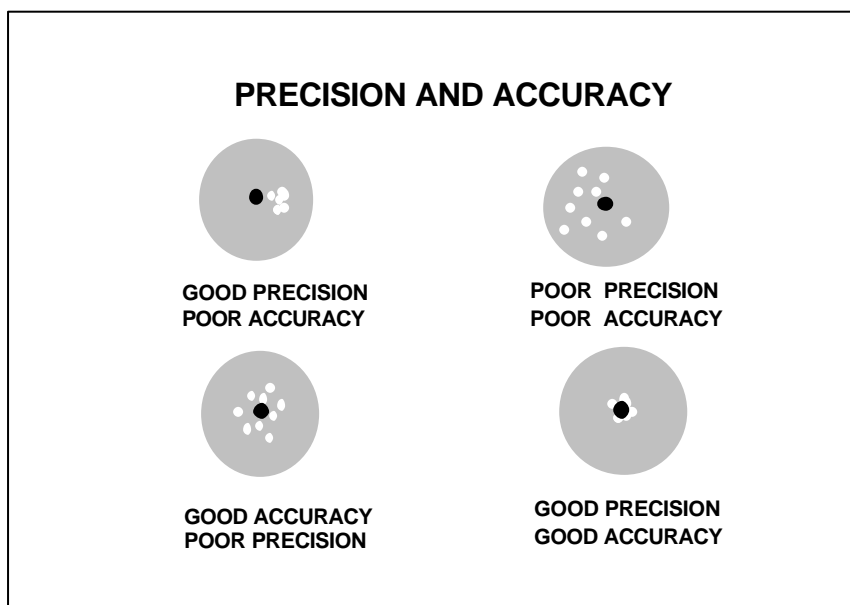
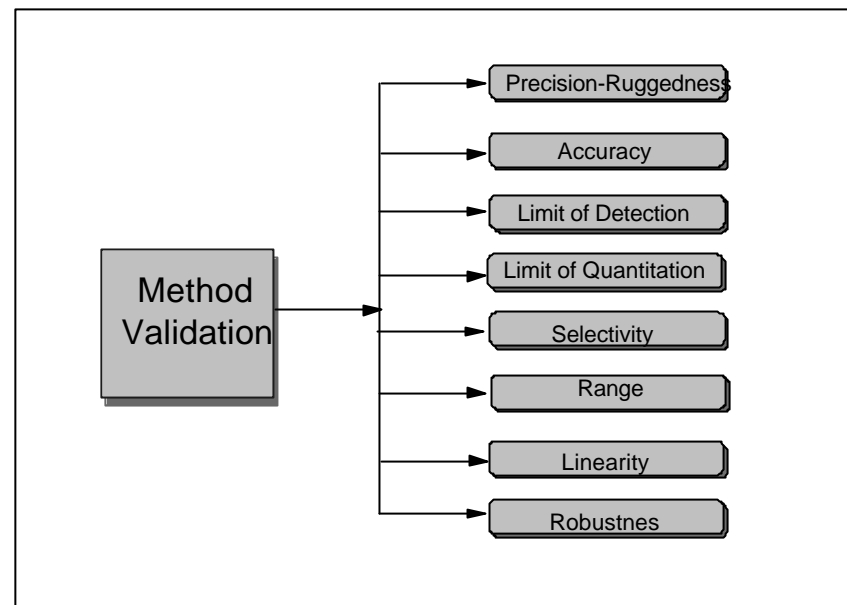
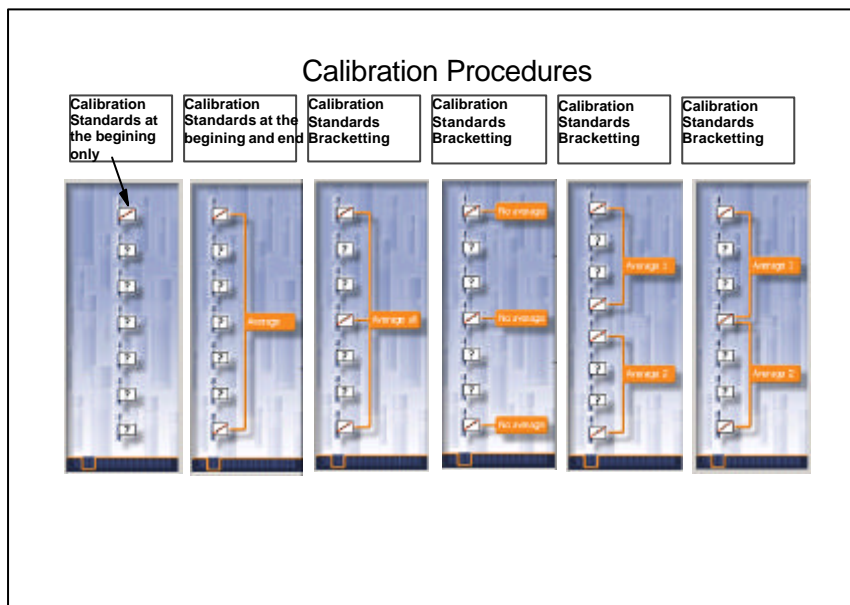


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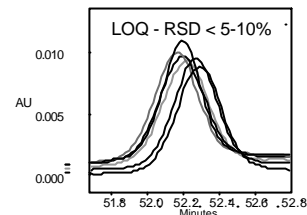
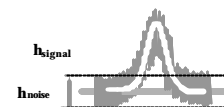
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### Parameters To Monitor - Validation

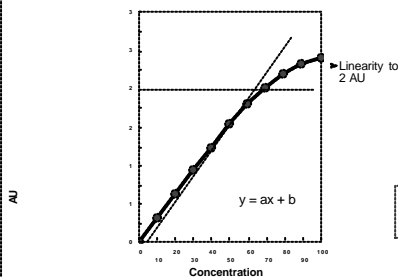
Limit of Detection:  $h_{\text{signal}} = 2 \times h_{\text{noise}}$

Limit of Quantitation:  $h_{\text{signal}} = 10 \times h_{\text{noise}}$



- ▶ Precision (Ruggedness)
- ▶ Accuracy
- ▶ Limit of detection
- ▶ Limit of quantitation
- ▶ Linearity (range)
- ▶ Selectivity
- ▶ Robustness

### Parameters To Monitor - Validation



- ▶ Precision (Ruggedness)
- ▶ Accuracy
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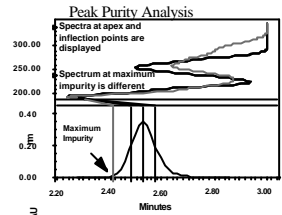
### LINEARITY

■ 5-6 concentrations of the reference standards (in duplicates or triplicates) below and above the expected concentration of the samples (20% - 120%).

#### RESULTS:

- Slope
- Intercept
- Correlation coefficient
- Range of linearity in concentration units.

### Parameters To Monitor - Validation



**Peak Purity Analysis**  
 Spectra at apex and inflection points are displayed  
 Spectrum at maximum impurity is different  
 Maximum impurity

**Photodiode Array Technology**  
 Spectral Analyses  
 Library Matching  
 Compound identification  
 Coelution detection

**Peak Purity Analysis**  
 Peak purity/peak homogeneity  
 Coelution detection

- ▶ Precision (Ruggedness)
- ▶ Accuracy
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- ▶ Linearity (range)
- ▶ Selectivity
- ▶ Robustness

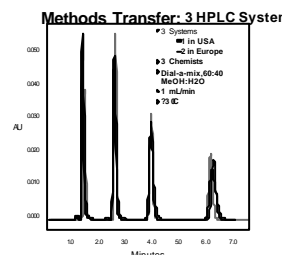
### Stability Indicating Method

■ **Stress Studies**

The drug substance, the dosage form and the placebo are stressed, using the following stress agents:

- Acid
- Base
- Oxidizer (H<sub>2</sub>O<sub>2</sub>)
- UV radiation
- Heat.

### Parameters To Monitor - Validation

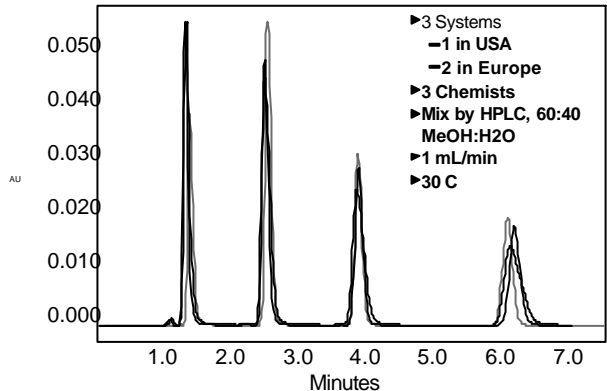


**Methods Transfer: 3 HPLC Systems**

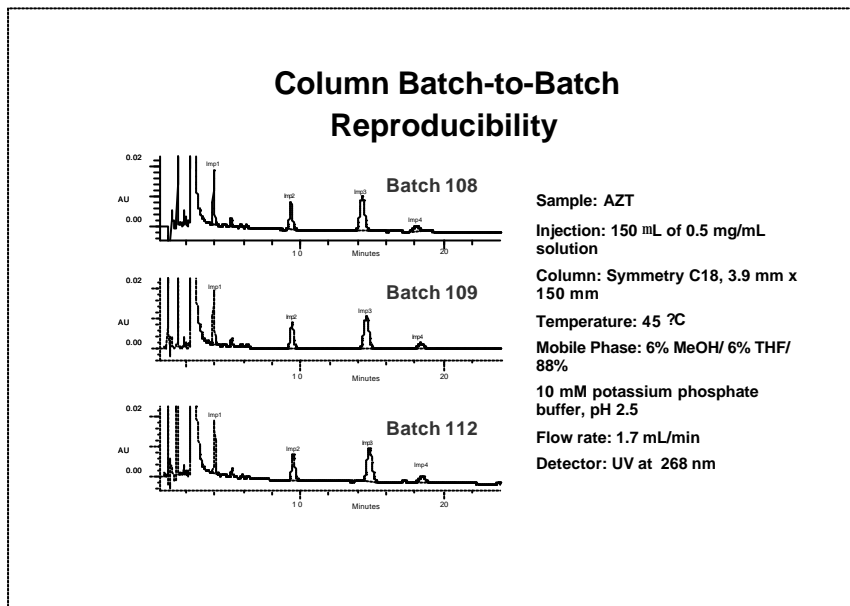
- ▶ Precision (Ruggedness)
- ▶ Accuracy
- ▶ Limit of detection
- ▶ Limit of quantitation
- ▶ Linearity (range)
- ▶ Selectivity
- ▶ Robustness - Reproducibility

### Methods Transfer:

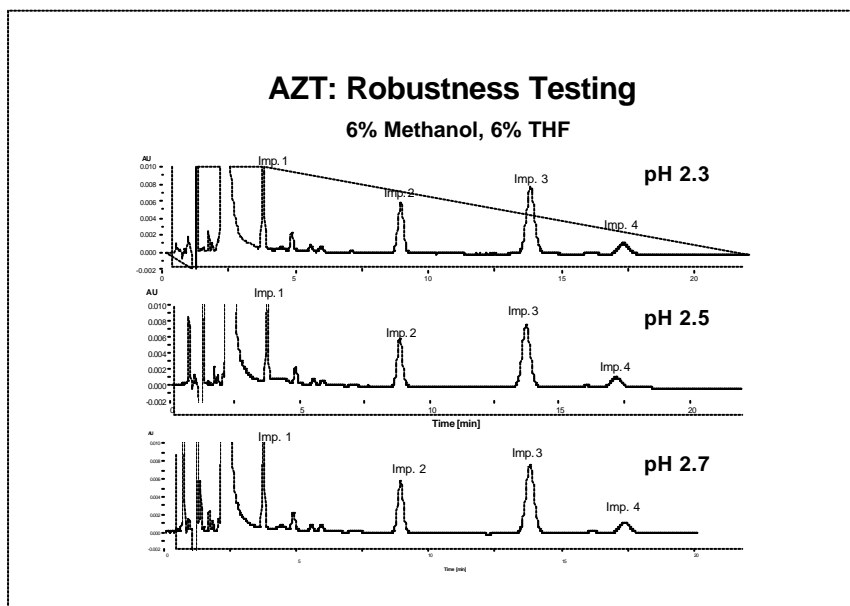
3 HPLC Systems



- ▶ 3 Systems
  - 1 in USA
  - 2 in Europe
- ▶ 3 Chemists
- ▶ Mix by HPLC, 60:40 MeOH:H<sub>2</sub>O
- ▶ 1 mL/min
- ▶ 30 C



- **Robustness**
- **Parameters Varied :**
    - Solvent strength in the mobile phase,
    - Temperature,
    - Flow rate,
    - pH of the mobile phase,
    - Ionic strength in the mobile phase,
    - Sample diluent,
    - Injection volume,
    - Wavelength of detection.
- The parameter measured:**
- Response (area/amount)
  - Retention time,
  - Selectivity and/or resolution.



- **Robustness**
- **PARAMETERS CHANGED:**
    - Duration of extraction,
    - Extraction medium,
    - Filtration type,
    - Temperatures.
- PARAMETER MEASURED:**
- Accuracy.

## METHOD VALIDATION

### REFERENCE STANDARDS:

Established source and known grade (DMF or COA)

% purity from assay will be taken into account in the calculations.

% residual compounds (GC, heavy metals, inorganic salts, water, residual solvents, weight loss).

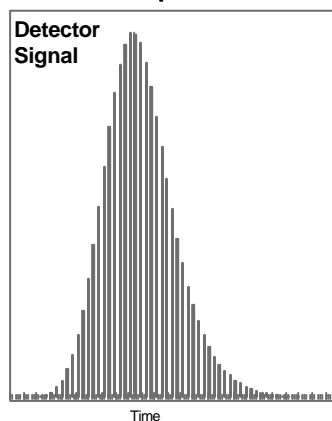
## METHOD VALIDATION

### Summary

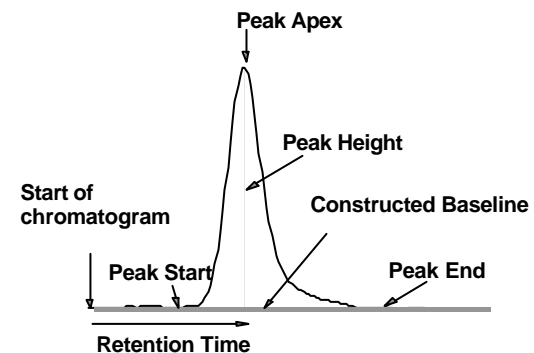
- CATEGORY I
  - Drug substance
  - VALIDATION:
    - Method suitability without LOD or LOQ
  
- CATEGORY II
  - Impurities or degradation compounds
  - VALIDATION:
    - Complete procedure of method-suitability. If limit of purity is needed: only specificity, LOD and ruggedness.
  
- CATEGORY III
  - Performance and potency of the drug product (dissolution).
  - VALIDATION:
    - Only precision and ruggedness are needed.

### Measurement of Area - Integration

$$\text{Area} = \int \text{Abs} \times dt$$

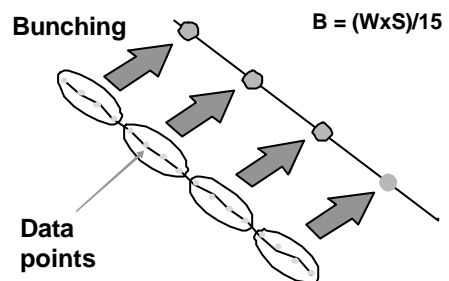


## Measurement of Area: Peak Integration: Peak Detection

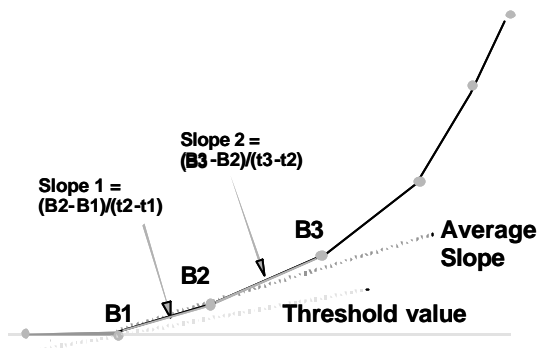


### Measurement of Area: Peak Integration: Data Bunching

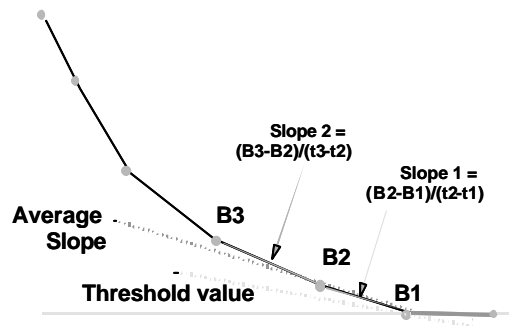
15 = Minimum Number of points to define a peak



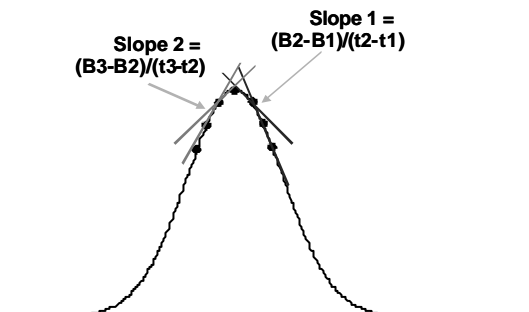
### Measurement of Area: Peak Integration - Peak Start



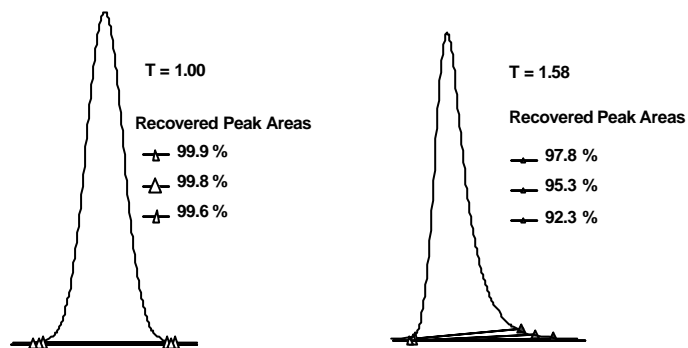
### Measurement of Area: Peak Integration - Peak End



### Measurement of Area: Peak Integration - Peak Apex



### Integration Errors Caused by Tailing



### Integration of Small Peaks

