

Quantitative work in HPLC

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www.forumsci.co.il/HPLC

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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

VDWZ DMUHLGXDROYHQWZ HJ KRW ?

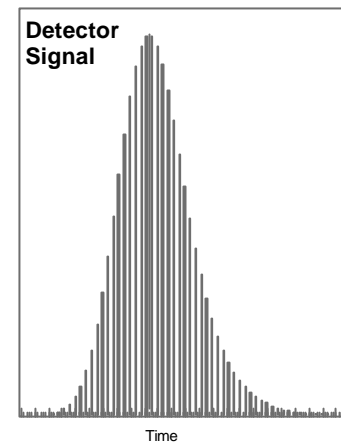
Choice of Standardization: External or Internal

Simple formulations and sample preparation: external standard

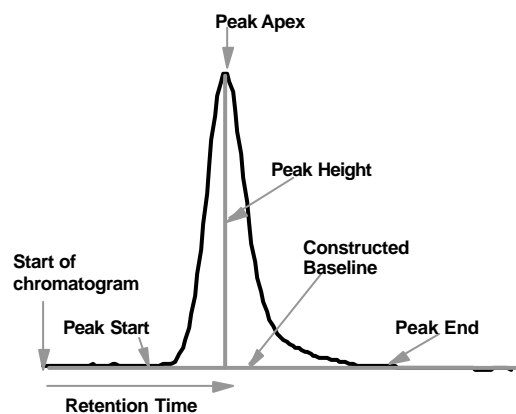
Gas chromatography, bio-studies or complex medium and complex sample preparation: internal standard

Measurement of Area - Integration

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

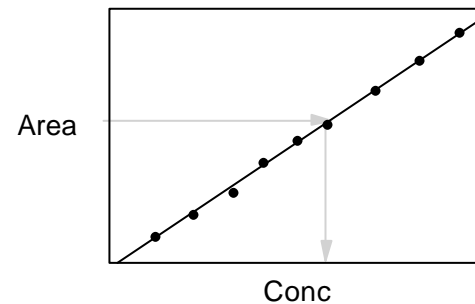


Peak Detection:



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.

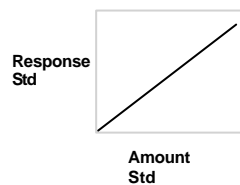
Choice of Standardization: External or Internal

External Standard

Amount Std → Response Std

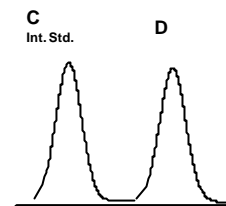
Amount Unk → Response Unk

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



Choice of Standardization: External or Internal

Internal Standard

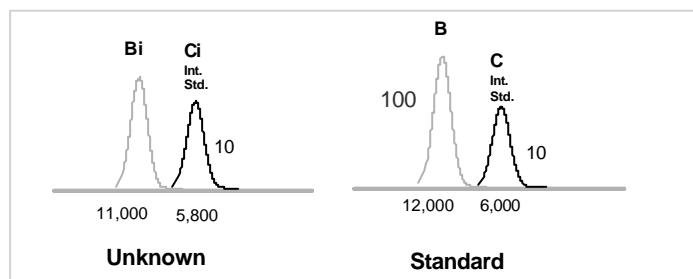


$$\frac{\text{Amount Std}}{\text{Amount Istd}} \rightarrow \frac{\text{Response Std}}{\text{Response Istd}}$$

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

12,000/6000 → 100/10

11,000/5800 → Amt Bi/10

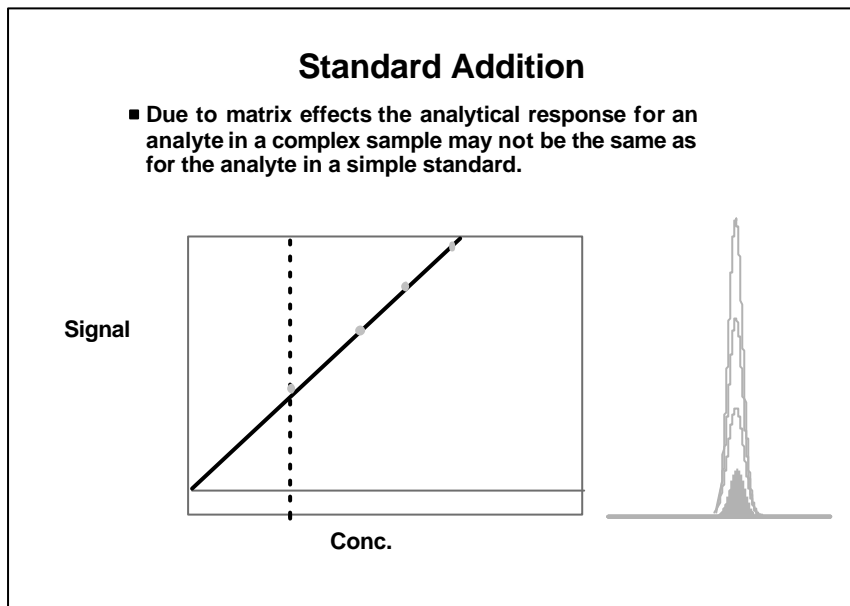


$$\frac{(11,000/5,800) \times (100/10)}{12,000/6000} \times 10 = \text{Amt Bi} = 94.8$$

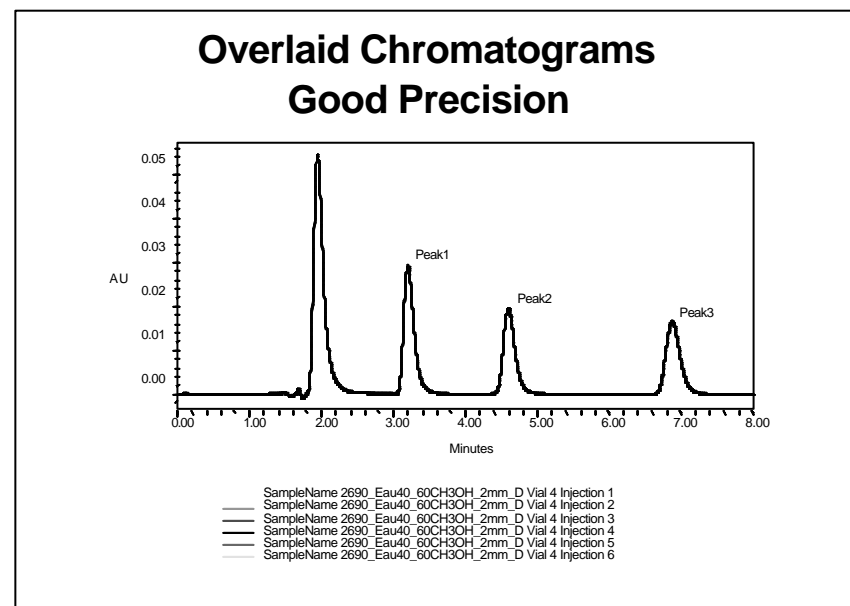
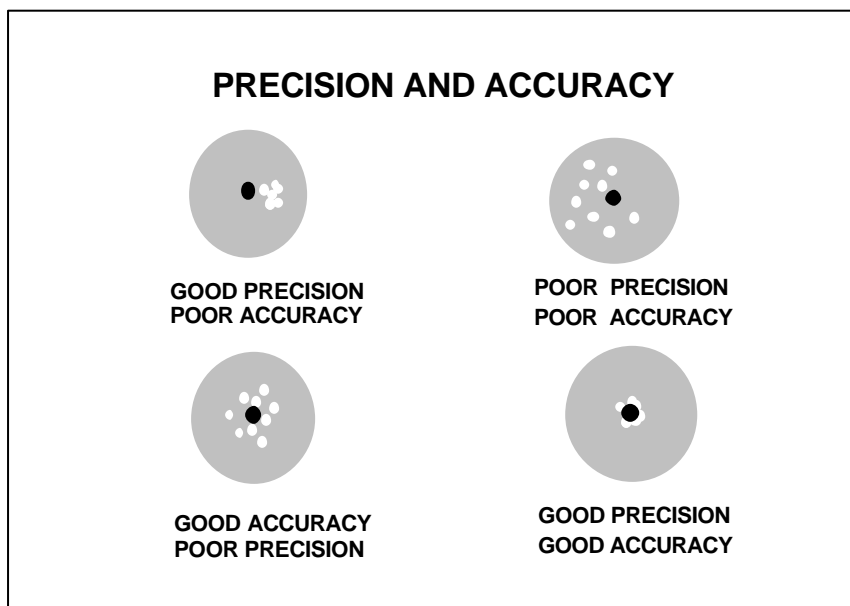
Linear Regression for the Equation:

$$y = mx + b$$

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



- ### Parameters To Monitor - Validation
- ▶ Precision (Ruggedness)
 - ▶ Accuracy
 - ▶ Limit of detection
 - ▶ Limit of quantitation
 - ▶ Linearity (range)
 - ▶ Selectivity
 - ▶ Robustness



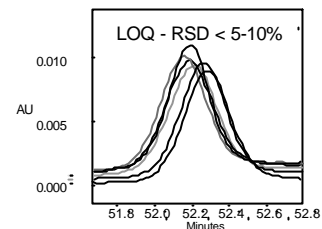
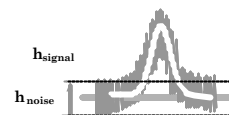
Quality Control

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
2	System Suitability	20.0	1	Inject Samples	SST Method Set	10.00	1.00000	1.00000
3	Std1	20.0	5	Clear Calibration Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
4	Std2	20.0	2	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
Accuracy check				Report	LC Calibration Report			
				Report	Standard Comparison			
				Clear Calibration	LC Demo Method Set			
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
2	Unk.1	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
3	Unk.2	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
4	Unk.3	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
5	Unk.4	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
6	Unk.5	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
7	Unk.6	20.0	2	Inject Samples	LC Demo Method Set	10.00	1.00000	1.00000
1	Std1	20.0	1	Inject Standards	LC Demo Method Set	10.00	1.00000	1.00000
				Clear Calibration Calibrate	LC Demo Method Set			

Parameters To Monitor - Validation

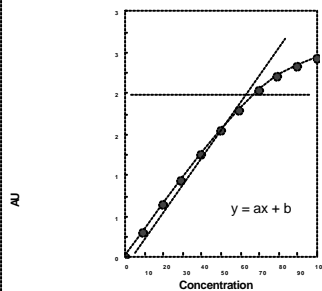
Limit of Detection: $h_{\text{signal}} = 2 \times h_{\text{noise}}$

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

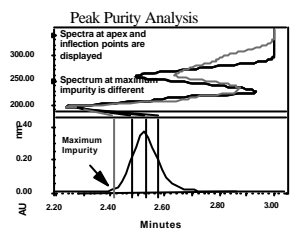
LINEARITY

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

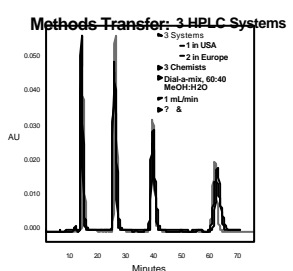
Stability Indicating Method

■ Stress Studies

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

- Acid
- Base
- Oxidizer (H₂O₂)
- UV radiation
- Heat.

Parameters To Monitor - Validation

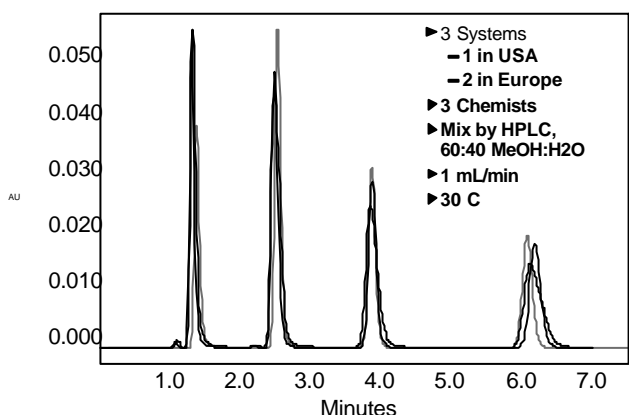


Methods Transfer: 3 HPLC Systems

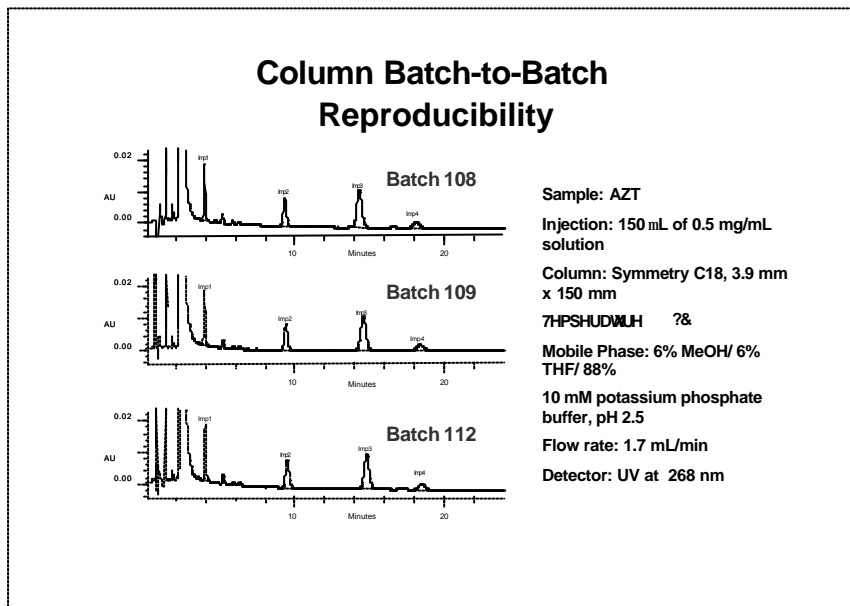
- ▶ Precision (Ruggedness)
- ▶ Accuracy
- ▶ Limit of detection
- ▶ Limit of quantitation
- ▶ Linearity (range)
- ▶ Selectivity
- ▶ Robustness - Reproducibility

Methods Transfer:

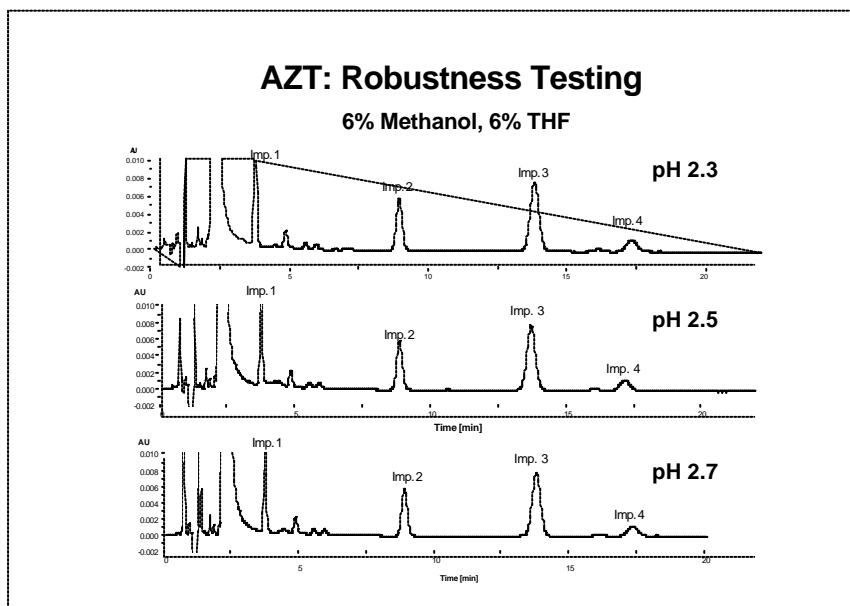
3 HPLC Systems



- ▶ 3 Systems
 - 1 in USA
 - 2 in Europe
- ▶ 3 Chemists
- ▶ Mix by HPLC, 60:40 MeOH:H₂O
- ▶ 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

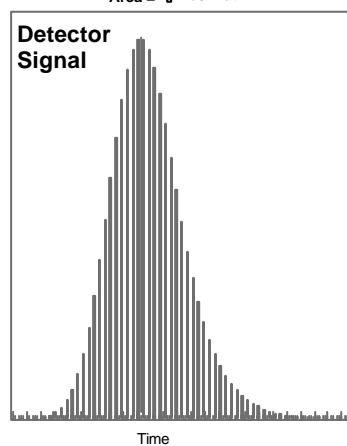
- CATEGORY I
 - Drug substance
 - VALIDATION:
 - Method suitability without LOD or LOQ

- CATEGORY II
 - Impurities or degradation compounds VALIDATION:
 - Complete procedure of method-suitability. If limit of purity is needed: only specificity, LOD and ruggedness.

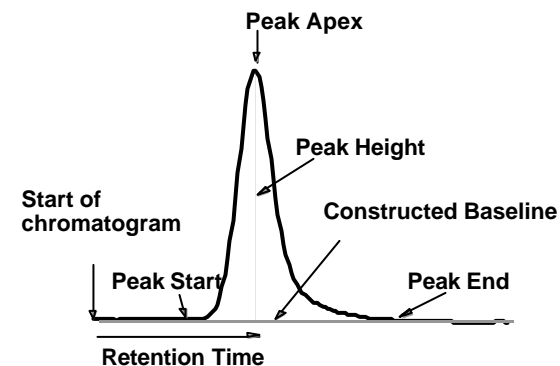
- CATEGORY III
 - Performance and potency of the drug product (dissolution).
 - VALIDATION:
 - Only precision and ruggedness are needed.

Measurement of Area - Integration

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

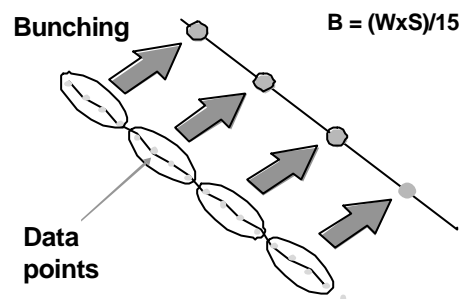


Measurement of Area: Peak Integration: Peak Detection

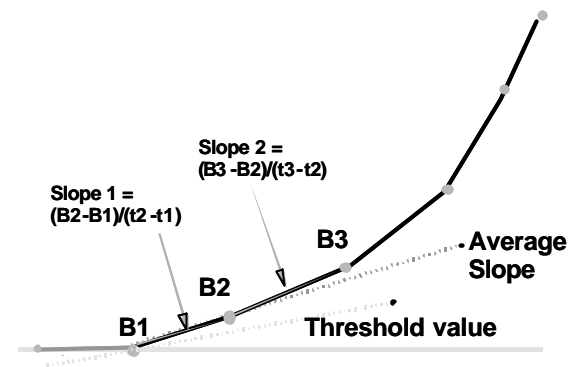


Measurement of Area: Peak Integration: Data Bunching

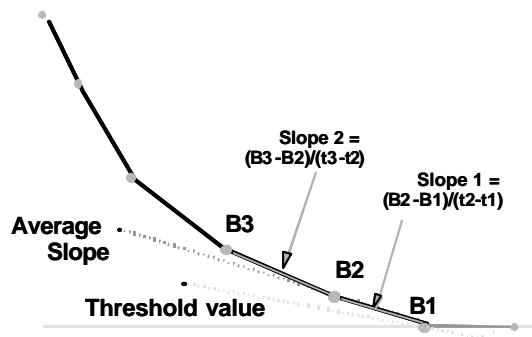
15 = Minimum Number of points to define a peak



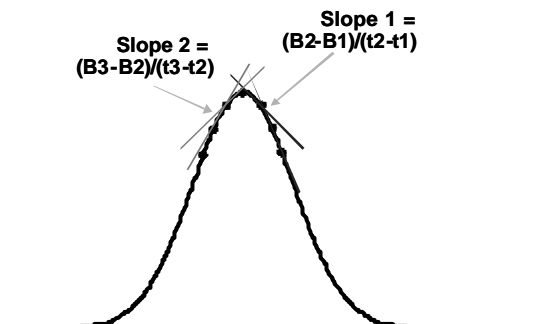
Measurement of Area: Peak Integration - Peak Start



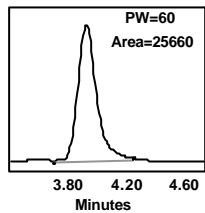
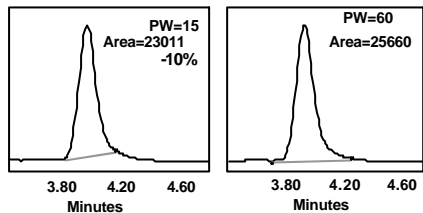
Measurement of Area: Peak Integration - Peak End



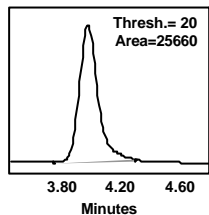
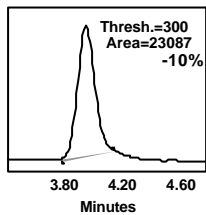
Measurement of Area: Peak Integration - Peak Apex



Integration of Small Peaks



- ▶ AUFS = 0.003
- ▶ Peak width changed
- ▶ Threshold set at 30



- ▶ AUFS = 0.003
- ▶ Peak width set at 30 sec
- ▶ Threshold changed