

Quantitative work in HPLC

Steps in Method Validation

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Statistics for Analytical Scientists:

<http://www.vam.org.uk/terp/stats/stats.htm>

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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

Significant Figures

Reporting results

12.554 g +/- 0.003 g.

Multiplication and division

Example:
8.9444 g
+18.52 g

27.46 g

Addition and subtraction

Example:
8.9 g / 12.01 g/mol = 0.74 mol

Choice of Standards

■ Standards are materials containing a known concentration of an analyte. They provide a reference to determine unknown concentrations or to calibrate analytical instruments.

Primary Standards

A primary standard is a reagent that is extremely pure, stable, has no waters of hydration, and has a high molecular weight.

Secondary Standards

A secondary standard is a standard that is prepared in the laboratory for a specific analysis. It is usually standardized against a primary standard.

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).

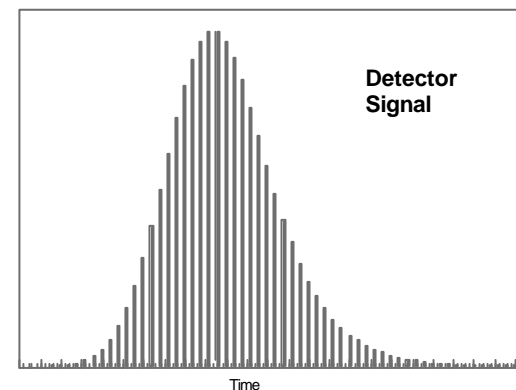
Choice of Standardization: External or Internal

Simple formulations and sample preparation: external standard

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

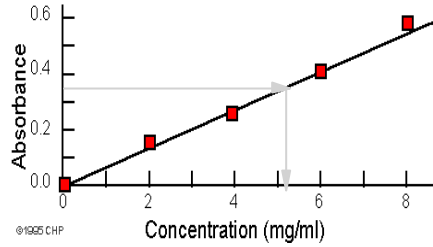
Measurement of Area - Integration

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



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.

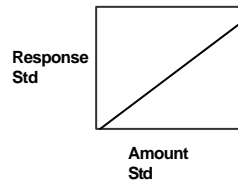
Quality Control

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
				Clear Calibration	LC Demo Method Set			
3	Std1	20.0	5	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
				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	LC Demo Method Set			
				Calibrate	LC Demo Method Set			

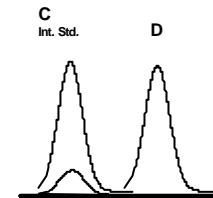
Choice of Standardization: External or Internal External Standard

Amount Std → Response Std
Amount Unk → Response Unk

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



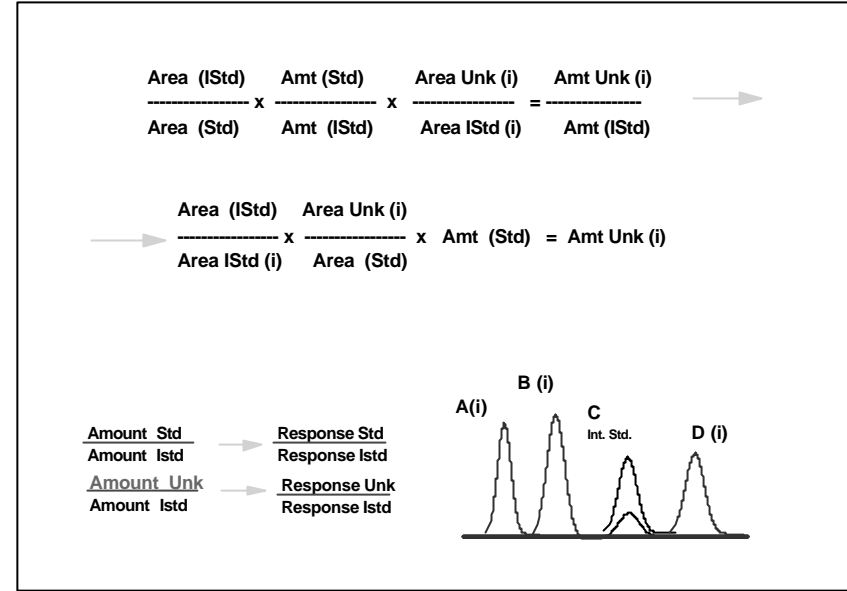
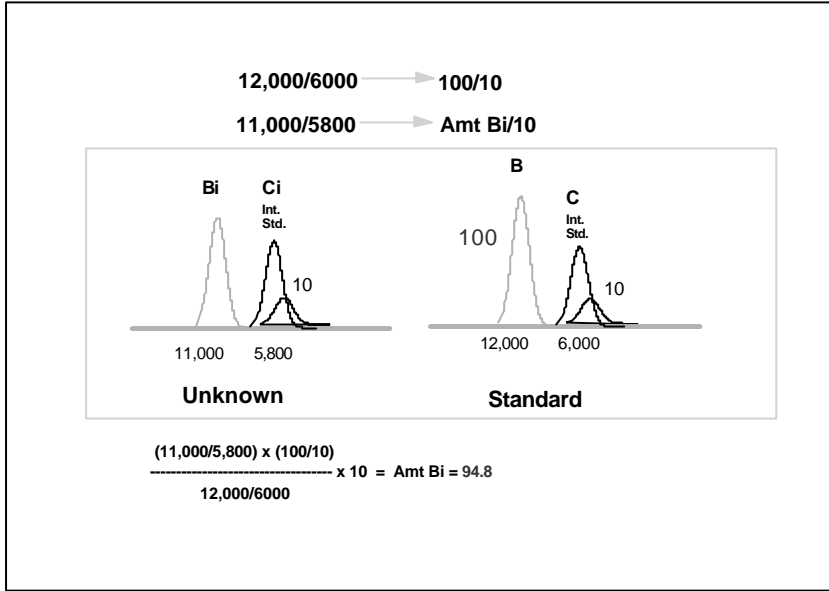
Choice of Standardization: External or Internal Internal Standard



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

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

Quantitative HPLC



Internal Standard

Peak Name	Quantitation Basis	Amount in Standard	Peak Area	Response Factor
A	Area	20 mg	10000	$\frac{6000}{10000} \times 20$
B	Area	100 mg	12000	$\frac{6000}{12000} \times 100$
C (Int. Std.)	Area	10 mg	6000	
D	Area	5 mg	8000	$\frac{6000}{8000} \times 5$

$\frac{\text{Area (IStd)}}{\text{Area IStd (i)}} \times \frac{\text{Area Unk (i)}}{\text{Area (Std)}} \times \text{Amt (Std)} = \text{Amt Unk}$

Standard Deviation

■ A measure of the uncertainty due to random error in a set of data (also: precision of a set of measurements).

$$s = \sqrt{\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N-1}}$$

s = standard deviation,
 N = the number of data points,
 x_i = each individual measurement,
 x bar = the mean of all measurements.

The value x_i - x bar is called the residual for each measurement.

Q-test for Rejection of Outliers

■ The Q-test is a simple statistical test to determine if a data point that is very different from the other data points in a set can be rejected. Only one data point may be discarded using the Q-test.

Table of Q critical values
(90% confidence)

N	Q _C
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

$$Q = \frac{|\text{outlier} - \text{value closest to the outlier}|}{|\text{highest value} - \text{lowest value}|}$$

$$Q = (0.94 - 0.76) / (0.94 - 0.41)$$

If Q is larger than Q_C the outlier can be discarded with 90% confidence.

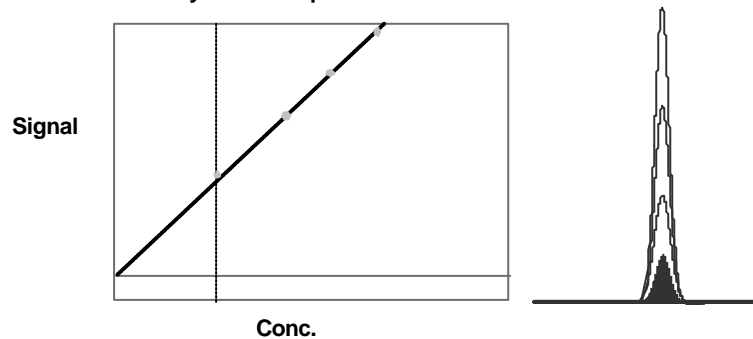
Linear Regression for the Equation:

$$y = mx + b$$

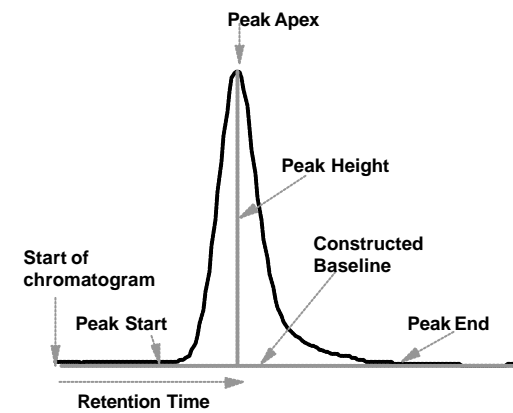
■ Linear regression uses the method of least squares to determine the best equation describing a set of x and y data points.

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.



Peak Detection:

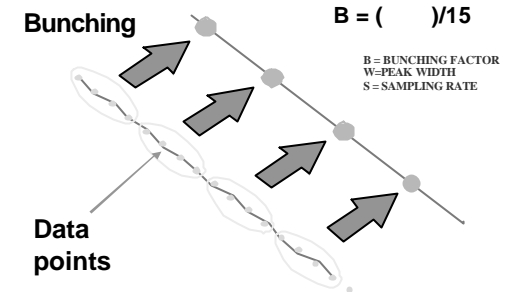


Measurement of Area: Peak Integration

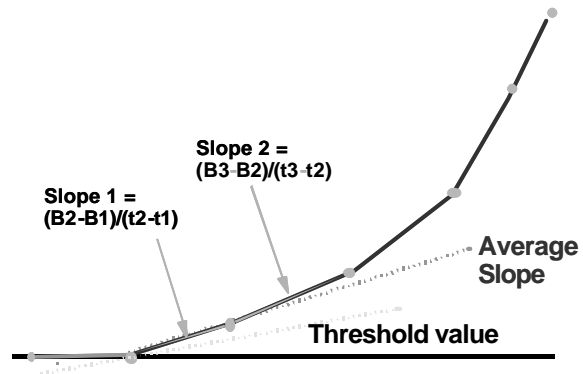
Data Bunching
Peak Start
Peak Apex
Peak End

Measurement of Area: Peak Integration: Data Bunching

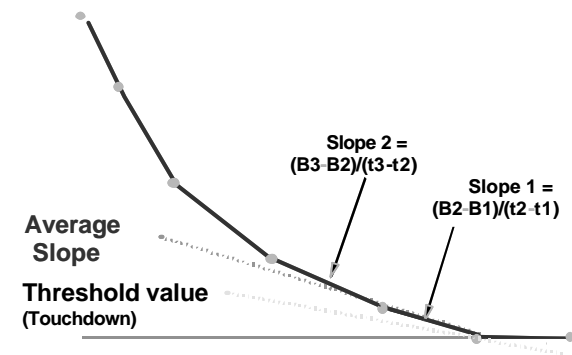
15 = Minimum Number of points to define a peak



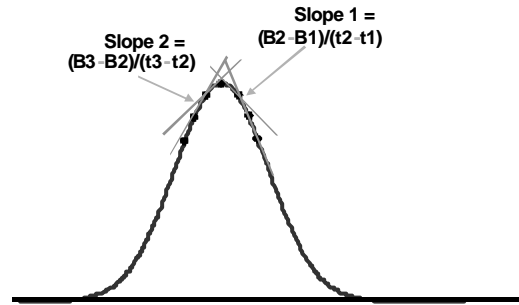
Measurement of Area: Peak Integration - Peak Start



Measurement of Area: Peak Integration - Peak End

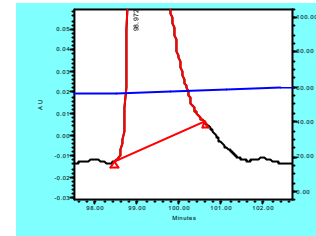


Measurement of Area: Peak Integration - Peak Apex

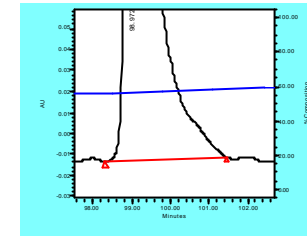


Set Peak Width

Peak Width = 30

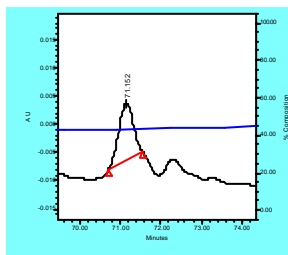


Peak Width = 120

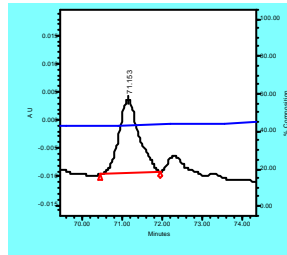


Peak Threshold

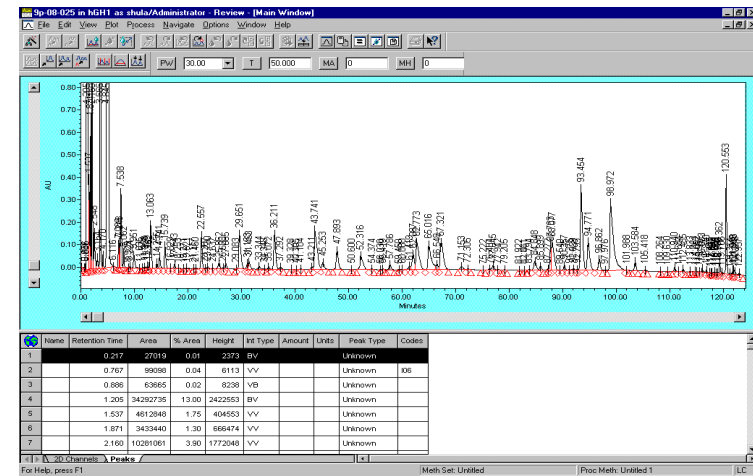
Peak Threshold=300



Peak Threshold=75

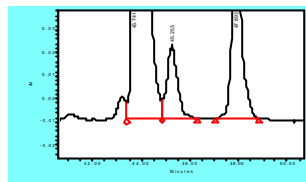


Proper Integration Events are Required

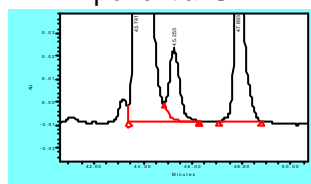


Quantitative HPLC

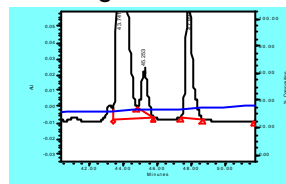
When a riding peak needs to be skimmed from the slop there are two options, exponential or tangential skim:



Exponential Skim



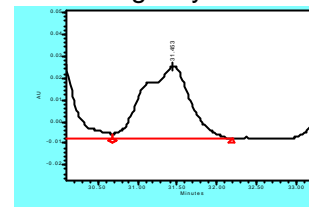
Tangential Skim



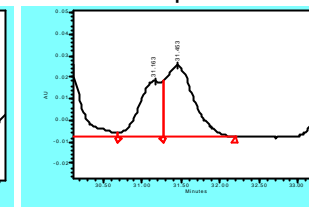
Force Drop line

For example force drop line at 31.26 min to force fused peaks to split

Originally



Force Drop line

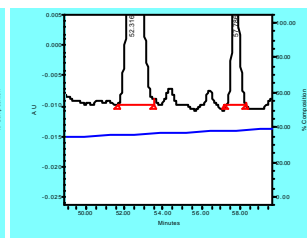
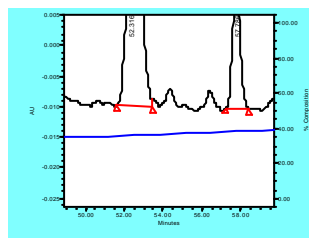


Force Baseline by Time & Force Baseline by Peak

A gradient chromatogram, where the baseline is drifting:

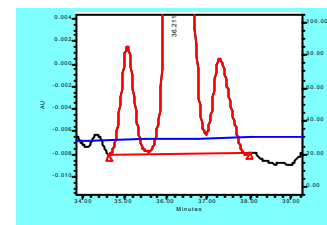
Event start and end times (Force Baseline by Time)

Peak start and end points within this range (Force Baseline by Peak)

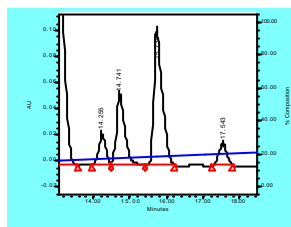


Force Peak

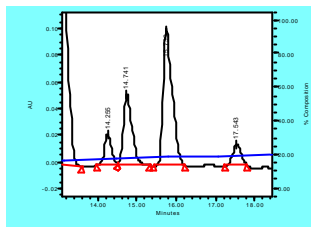
When adjacent peaks have to be integrated together and reported as a sum of areas:



Forward Horizontal by time

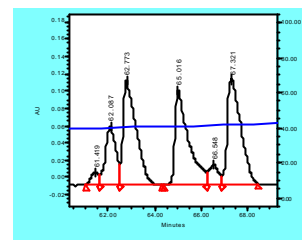


Forward Horizontal by peak

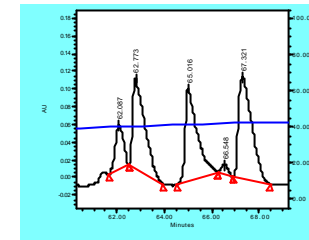


Valey to Valey

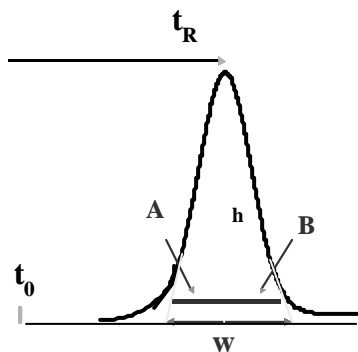
Original



Valey to Valey



Asymmetric factor or Tailing factor



ASYMMETRY FACTOR

$$A_f = \frac{B_{(10\% h)}}{A_{(10\% h)}}$$

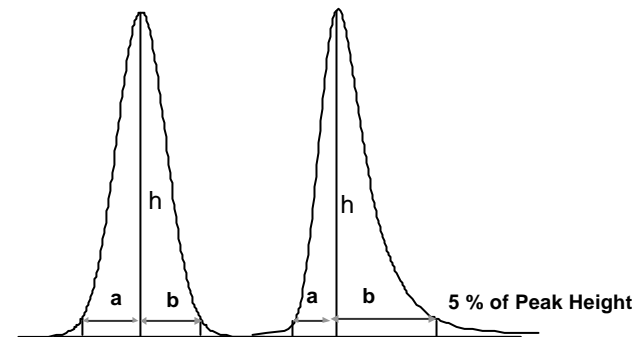
TAILING FACTOR

$$T = \frac{A_{5\% h} + B_{5\% h}}{2 \times A_{5\% h}}$$

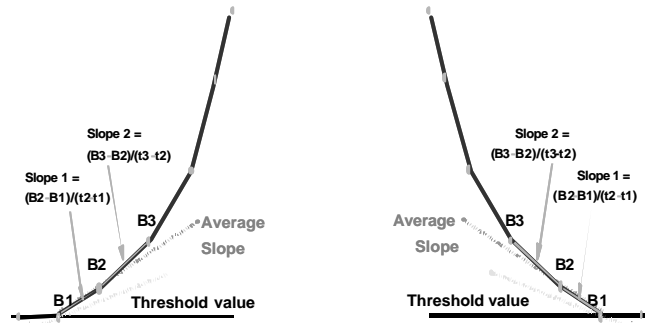
Asymmetric factor or Tailing factor

Symmetric

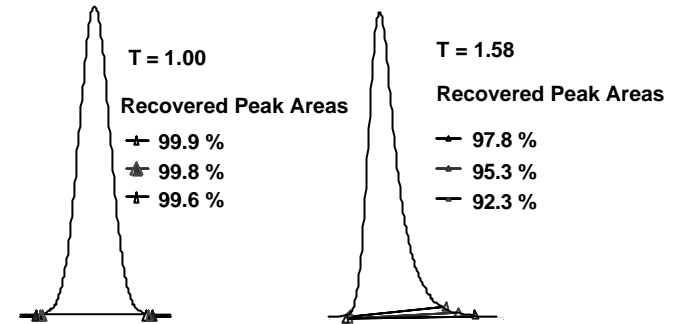
Asymmetric



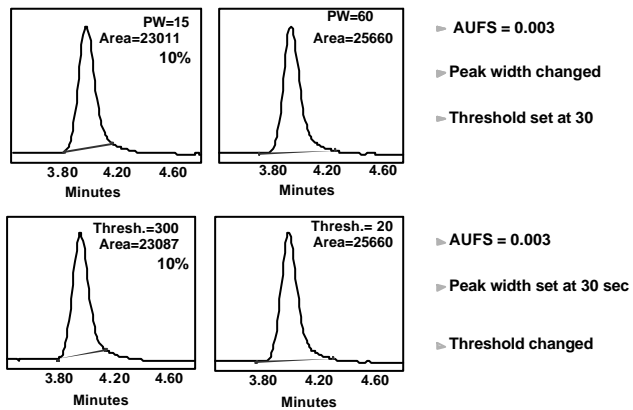
Sources of Errors in Integration of Asymmetric Peaks



Integration Error Caused by Tailing of Peaks



Integration of Small Peaks



Validation of Chromatographic Method

Final method is defined by the following parameters:

- Column type: chemistry, geometry and brand
- Mobile phase composition and mode (isocratic or gradient).
- Flow rate and pressure
- Temperature (column and autosampler)
- Sample volume
- Detection (wavelength and/or resolution in UV)
- Sample ID.

METHOD VALIDATION

- **Analysis time: $k_{first}' > 2$ and $k_{last}' < 40$**

(flow-rate, column length and solvent composition can be adjusted to speed up the separation).

Parameters To Monitor

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

COLUMN AND INSTRUMENT PERFORMANCE:

- Number of theoretical plates, $N \geq 2000$. [$16 (tR/w)^2$]
- Resolution, $R_s \geq 2$ between adjacent peaks; [$(tR_2 - tR_1)/0.5(w_1 + w_2)$]
- Tailing factor $0.5 \leq T \leq 2$.
- Precision; $RSD \leq 1-2\%$
(otherwise: the specified value in literature method) in 10 injections of the standard.

Parameters To Monitor

► Precision

(Ruggedness)

► Accuracy

► Limit of detection

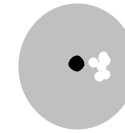
► Limit of quantitation

► Linearity (range)

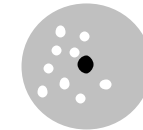
► Selectivity

► Robustness

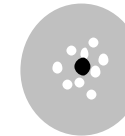
PRECISION AND ACCURACY



GOOD PRECISION
POOR ACCURACY



POOR PRECISION
POOR ACCURACY



GOOD ACCURACY
POOR PRECISION



GOOD PRECISION
GOOD ACCURACY

Peak Area Precision

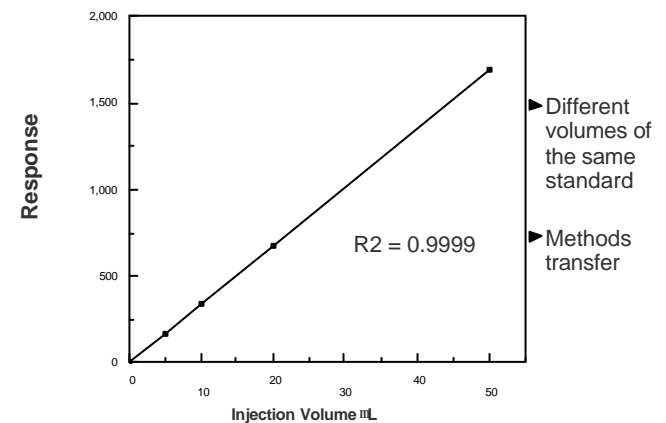
► Autoinjector

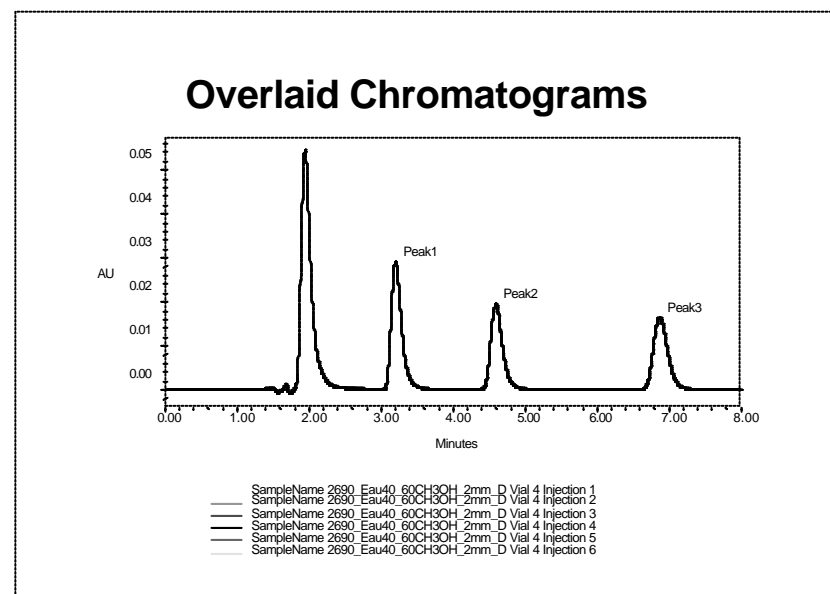
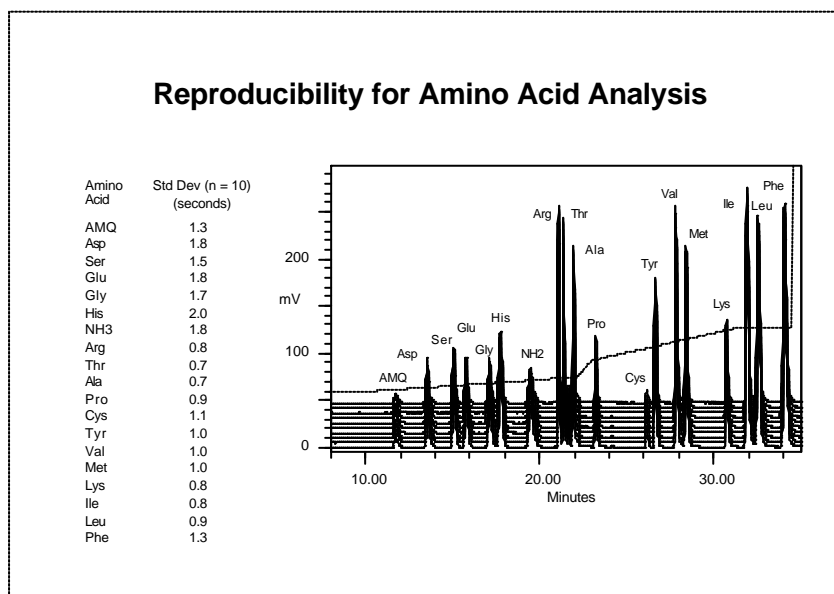
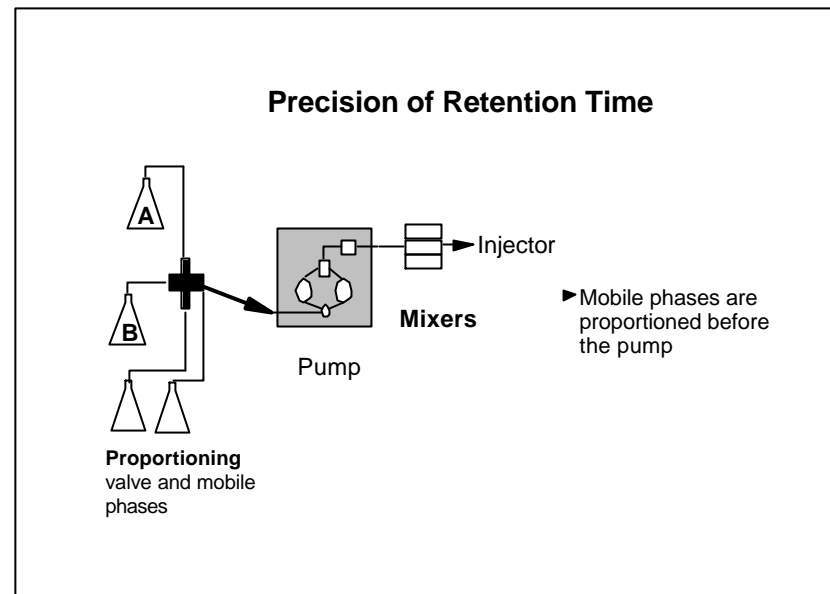
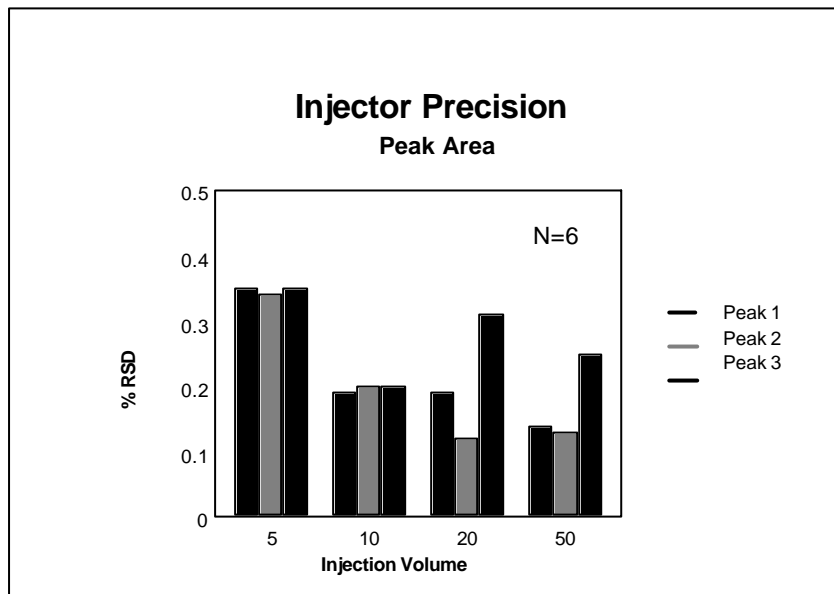
- Injection volume
- Injector design

► Integration of peaks

- Lloff and touchdown
- Threshold
- Peak width

Injector Linearity





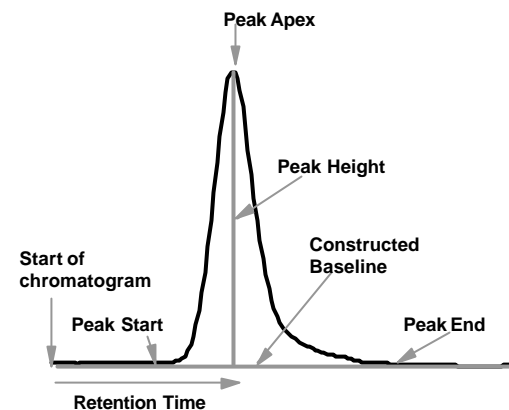
Detector Criteria



Refractive Index

- ▶ Selectivity
- ▶ Sensitivity and detection limit
- ▶ Stability
- ▶ Linear range
- ▶ Dynamic Range
- ▶ Reproducibility
- ▶ Effect on peak shape
- ▶ Maintenance

Peak Definition and processing



Summary

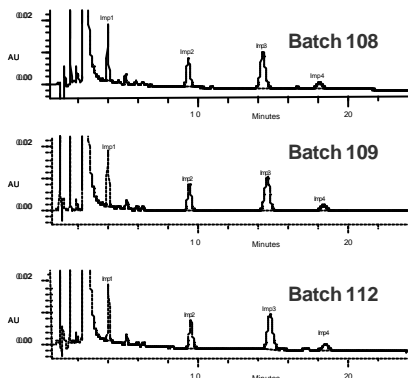
Factors Affecting Precision -
Area and Retention Time

- ▶ Pump performance
- ▶ Injector performance
- ▶ Integration parameters
- ▶ Detector performance

Method Ruggedness

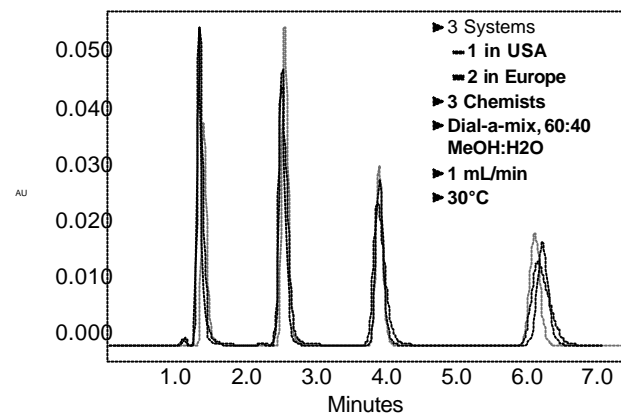
- Analyst to analyst
- Instrument to instrument
- Lab to lab
- Column to column
- Batch to batch

Column Batch-to-Batch Reproducibility



Sample: AZT
 Injection: 150 µL of 0.5 mg/mL solution
 Column: Symmetry C18, 3.9 mm x 150 mm
 Temperature: 45 °C
 Mobile Phase: 6% MeOH/ 6% THF/ 88%
 10 mM potassium phosphate buffer, pH 2.5
 Flow rate: 1.7 mL/min
 Detector: UV at 268 nm

Methods Transfer: 3 Alliance Systems



Parameters To Monitor

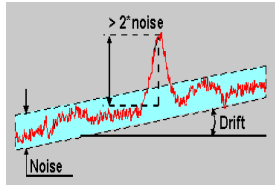
- ▶ Precision (Ruggedness)
- ▶ Accuracy
- ▶ Limit of detection
- ▶ Limit of quantitation
- ▶ Linearity (range)
- ▶ Selectivity
- ▶ Robustness

Accuracy:

$$\frac{\text{Amount Measured}}{\text{Amount Claimed}}$$

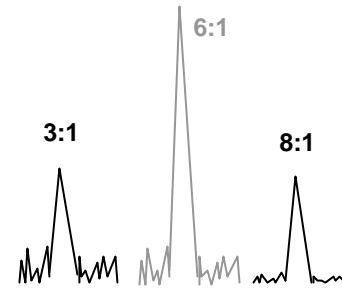
- In formulations:
- A known quantity of the standard is added to the medium (80%, 100%, 120%), and the recovery is measured.
- Recovery from sample preparation procedures:
- Known concentrations of standards before and after sample preparation (dilution, extraction or filtration) are measured.
- Solution stability of the samples:
- A fresh sample is compared to one which was left in the solution for the entire duration of the test procedure.

Parameters To Monitor



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

Increase Signal-to-Noise Ratio



- ▶ Signal-to-noise (S/N) is peak height to noise
- ▶ Increase S/N by increasing peak height
- ▶ Increase S/N by decreasing noise

Limits of Quantitation and Detection

■ LOD

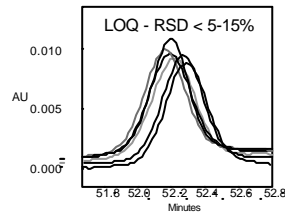
– the lowest concentration of an analyte in a sample that can be detected under the experimental conditions.

» S/N ratio of 2:1 or 3:1

■ LOQ

– the lowest concentration of the analyte that can be determined with acceptable precision and accuracy under the experimental conditions.

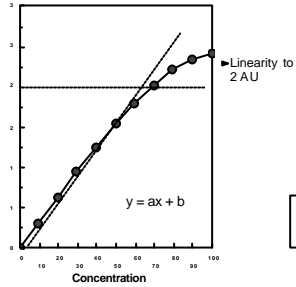
» S/N ratio generally 10:1



Strategies in Sensitivity Enhancement

- Packing Material
 - Good Peak Shape
 - High Efficiency
- Column Dimensions
 - Smaller Diameter
 - Shorter Length
 - Smaller Particle Size

Parameters To Monitor



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

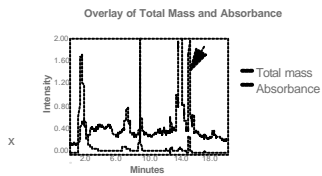
LINEARITY

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

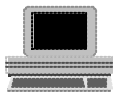
RESULTS:

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

Parameters To Monitor



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

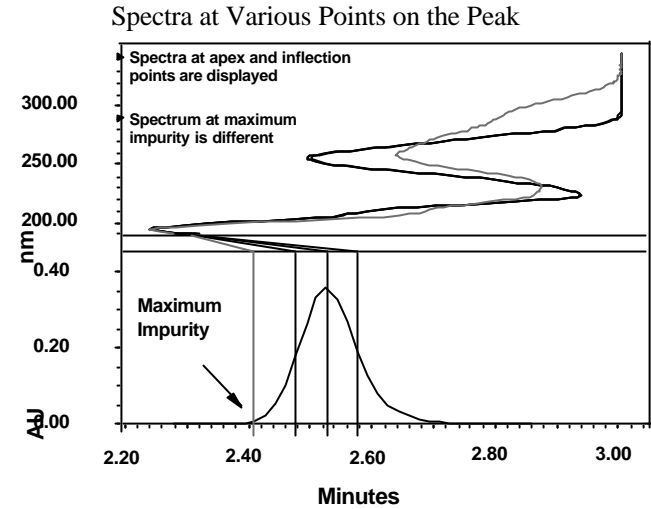
■ Stress Studies

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

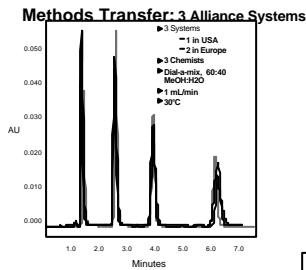
- Acid
- Base
- Oxidizer (H₂O₂)
- UV radiation
- Heat.

Stability Indicating Contd.

- **Stress:** up to 20% degradation of the drug substance (Peak area => 80%).
- The degradation products are identified and quantitated against Reference Standards.
- If there are no commercial Reference Standards, the major degradation products should be collected, purified and serve as In-House Reference Standards.
- The amount of sample taken for analysis should vary to make sure that degradation products that are approximately 0.1% of the major peak will be detected.



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▶ Robustness

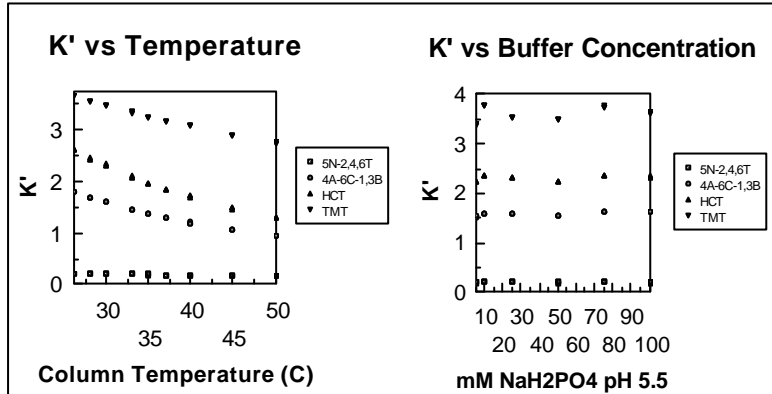
- **Variables :**
 - Solvent strength in the mobile phase,
 - Temperature,
 - Flow rate,
 - pH of the mobile phase,
 - Ionic strength in the mobile phase,
 - Sample diluent,
 - Injection volume,
 - Wavelength of detection.

The parameter measured:

- Response (area/amount)
- Retention time,
- Selectivity and/or resolution.

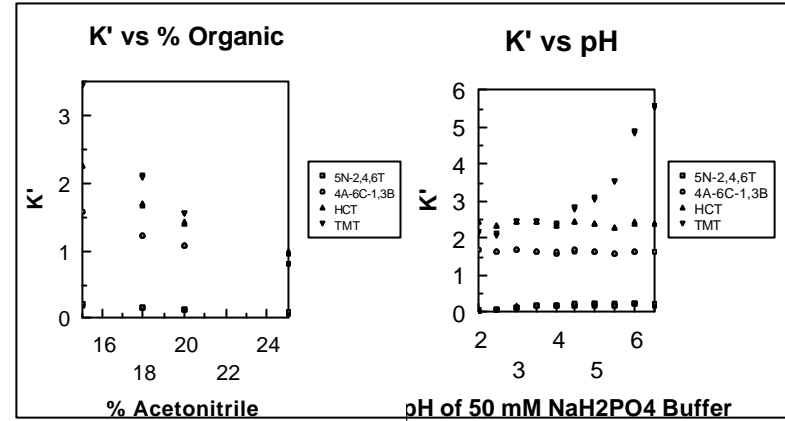
Robustness Study

k' Versus Temperature and Buffer Concentration



HPLC Method Robustness Study

k' Versus % Organic and pH



Examples of Robustness Testing

Acetaminophen Analysis Using Method Variations A and B

▶ Example of Robustness Testing

▶ Sample: Acetaminophen Tablets

▶ Method A :

▶ Mobile Phase = 16% methanol,

▶ Flow Rate = 1.0 mL/min,

▶ Temperature = 25°C

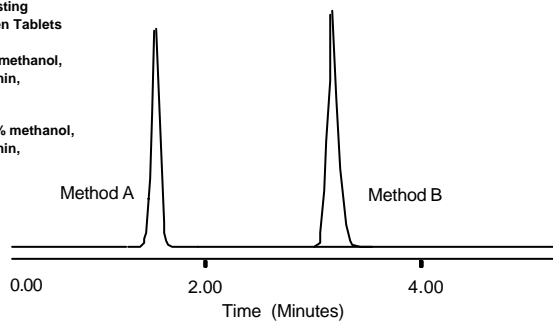
▶ Method B :

▶ Mobile Phase = 12.5% methanol,

▶ Flow Rate = 0.8 mL/min,

▶ Temperature = 30°C

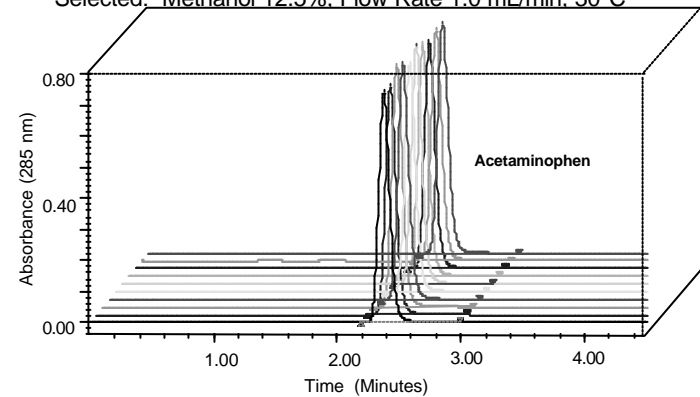
▶ IQ and OQ completec

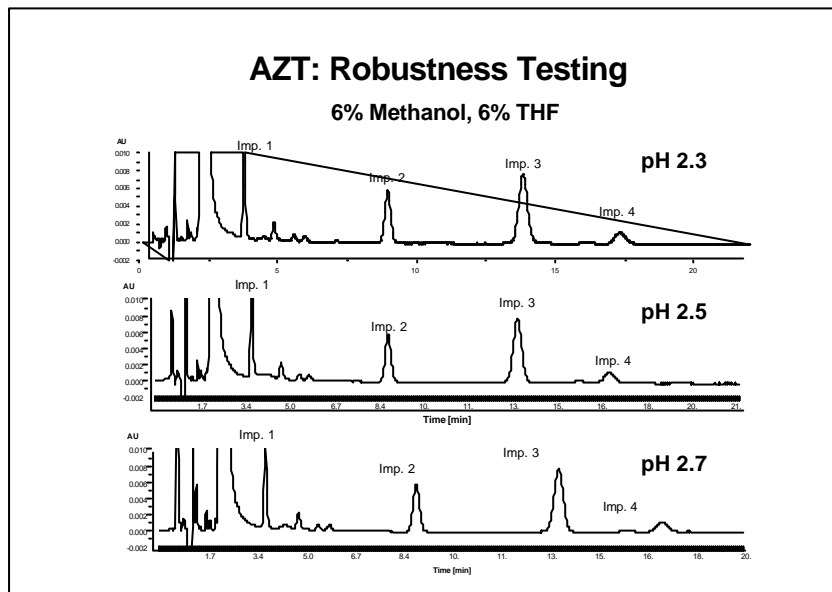


Parameter	Method A	Method B
Retention Time Average (Minutes Std. Dev.)	1.533 + 0.000	3.637 + 0.0322
Percent Label Claim (% Std. Dev.)	100.347 + 0.3961	99.766 + 0.5794

Robust Method

For a Rapid, Reproducible Analysis of Active Method C was Selected: Methanol 12.5%, Flow Rate 1.0 mL/min, 30°C





► Robustness of Sample Preparation

■ 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.