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Validation of Analytical Procedures

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Introduction

Validation:

> "A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting pre-determined acceptance criteria."

USP <1225>

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the **performance characteristics of the procedure meet the requirements** for the intended analytical applications.

ICH Q2 (R1)

"The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose."

Purpose of Validation

Validation of Analytical procedures are essential to prove that:

01 The method is acceptable for intended use such as evaluation of a known product for potency, and impurities

02 Identification of sources and Quantitation of potential errors w.r.t reliability and sustainability

03 Establish “Proof of Concept” that a procedure can be used for decision making throughout its life cycle

NOTE

- Satisfy regulatory requirements

Introduction

Challenges in Validation of Analytical Procedures

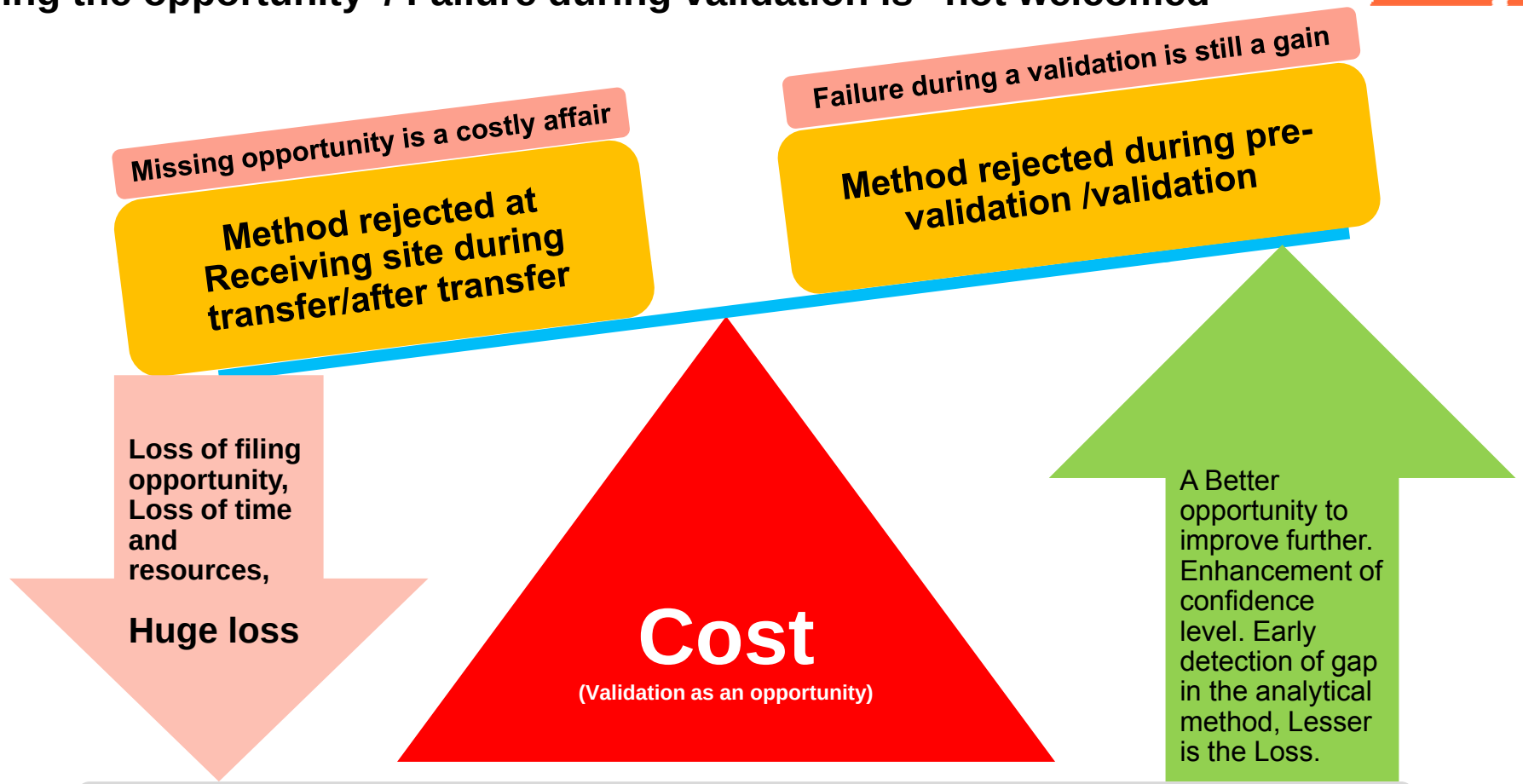
- Almost from three decades, firms are carrying out validation activity by traditional way
- Despite best efforts, method failures during regular use and transfer
- Guidelines describe the use of appropriate statistical tools but there is limited information on **how** to use these tools effectively
- Variable approaches across industry to report results and conclusions which warrants queries/observations from agencies

The analyst needs to know whether the **results of measurement can be accepted with confidence** or, on the contrary, rejected because they are wrong. Also, it is more important for the researcher to know if he **can trust a newly developed procedure** and what are the criteria to ensure the validity of new procedure.

The application of statistical tools allow us to address all these points.

Introduction

Cost of “missing the opportunity”/ Failure during validation is “not welcomed”



Challenge – Failure during validation is seen as setback, organizations never want to fail at this stage, In reality failure at this stage may add more value to technology, and it may lead to delivery of the most robust analytical method.

Validation Characteristics

Characteristics

Validation characteristics to be selected according to type of method:

- Specificity
- Precision
- Accuracy
- Detection limit (LOD)
- Quantitation limit (LOQ)
- Linearity
- Range
- Robustness (not part of the formal validation process)
- Solution Stability

Precision

The precision of an analytical procedure expresses the **closeness of agreement** (degree of scatter) between a series of measurements obtained from **multiple sampling** of the **same homogeneous sample** under the prescribed conditions.

1

Repeatability (system and method)

It is precision under the same operating conditions for a short period of time.

2

Intermediate precision
(Ruggedness)
Indicates intra-laboratory variations; (different days, different analysts, different equipment)

3

Reproducibility

Indicates inter-laboratory variations (applied to standardization of methodology)

- Minimum 9 determination covering the specified range (3 conc./3 replicates); **or**
- Minimum 6 determinations at 100% of test concentration

Case Study-1

Intermediate Precision

	Analyst 1	Analyst 2
Instrument 1	99.84	100.21
	99.93	99.31
	99.50	99.86
	100.24	100.59
	101.30	100.54
	102.00	100.70
Instrument 2	98.27	99.41
	99.31	99.41
	98.26	99.23
	99.43	99.91
	100.01	99.13
	99.76	98.86
	Mean	**99.79**
	stdev	**0.857**
	%RSD	**0.859**

Case Study-1

Intermediate Precision

	Analyst 1	Analyst 2
Instrument 1		
Instrument 2		
	Mean	
	stdev	
	%RSD	

ANOVA Assumptions

Distribution should be normal

Independent observations

Equivalent Variations

Hypothesis set up

Null Hypothesis

H_0 = Population means are equal

Alternative Hypothesis

H_a = Population means are not equal

Hypothesis set up

Null Hypothesis

H_0 = Population means are equal

Alternative Hypothesis

H_a = Population means are not equal

F-test

$$F = \frac{\text{Between group variation}}{\text{Within group variation}}$$

F-Crit > *F*-Cal ; Null Hypothesis

F-Crit < *F*-Cal ; Alternative Hypothesis

Hypothesis set up

Null Hypothesis

H_0 = Population means are equal

Alternative Hypothesis

H_a = Population means are not equal

p-value

Significance value, $\alpha = 0.05$

p-value > 0.05 ; Null Hypothesis

p-value < 0.05 ; Alternative Hypothesis

α = It is the maximum acceptable level of risk for rejecting a true null hypothesis (Type I error)

The *p*-value represents the probability of incorrectly rejecting the null hypothesis when it is actually true (Type I error).

Case Study-1 cont..

Anova: Two-Factor With Replication

SUMMARY	Analyst 1	Analyst 2	Total
Instrument 1			
Count	6	6	12
Sum	602.81	601.21	1204.02
Average	100.46833	100.20167	100.335
Variance	0.9424167	0.2850967	0.577354545
Instrument 2			
Count	6	6	12
Sum	595.04	595.95	1190.99
Average	99.173333	99.325	99.24916667
Variance	0.5557867	0.12399	0.315262879
Total			
Count	12	12	
Sum	1197.85	1197.16	
Average	99.820833	99.763333	
Variance	1.138372	0.3955515	

Overall repeatability=
100*(Sqrt 0.47/99.76)= 0.69%

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F Cal*	*P-value*	*F crit*
Analyst	7.0742042	1	7.074204167	14.84	0.000995	4.351
Instrument	0.0198375	1	0.0198375	0.042	0.840439	4.351
Interaction	0.2625042	1	0.262504167	0.551	0.466727	4.351
Within	9.53645	20	**0.4768225**			
Total	16.892996	23				

Case Study-1 cont..

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Within	9.53645	20	**0.4768225**			
Total	16.892996	23				

Case Study-2

Statistical analysis to estimate Precision

For an alternative procedure to be considered to have “comparable” precision to that of a current procedure, its precision must not be worse than that of the current procedure by an amount deemed important.

Procedure:

- Estimate the variance (s^2) of each procedure
- Calculate a one-sided upper confidence interval for the ratio of (true) variances

$$Ratio = \frac{Variance\ of\ alternative\ procedure}{Variance\ of\ current\ procedure}$$

Conclusion:

If the one-sided upper confidence bound limit **is less than this upper acceptable limit**, then the precision of the alternative procedure is considered acceptable.

Case Study-2

Statistical analysis to estimate Precision

Three different quantities of reference standard were weighted to correspond to three different percentages of the test concentrations: 50%, 100%, and 150%.

The value of τ is 1000 mg/g for all three concentrations. The computed statistics from the validation data set include the sample mean (Y), the sample standard deviation (S), and the number of reportable values (n).

Test Concentration (%)	Test Solution	Reportable Value (mg/g)
50	1	996.07
50	2	988.43
50	3	995.90
100	4	987.22
100	5	990.53
100	6	999.39
150	7	996.33
150	8	993.67
150	9	987.76
Sample mean (Y)		**992.81**
Sample standard deviation (S)		**4.44**

Ref: USP <1010> Analytical data- Interpretation and treatment and USP <1210> Statistical tools for procedure validation

Case Study-2

For the standard deviation, one is concerned with only the 100(1 − α)% upper confidence bound since typically, it needs to be shown that the standard deviation is not too large.

$$U = S\sqrt{\frac{n-1}{\chi^2_{\alpha;n-1}}}$$

Where,

U = an upper $100(1-\alpha)\%$ confidence bound for σ

S = Standard Deviation from Int. precision

n = number of reportable values

From the data:

If $S=4.44, \alpha = 0.05$ and $n = 9$,

then $\chi^2_{0.05;8} = 2.73$

$$U = 4.44\sqrt{\frac{9-1}{2.73}} = 7.60 \text{mg/g}.$$

Conclusion:

Suppose the pre-defined acceptance criterion for precision requires σ to be < 20 mg/g. The computed upper bound of 7.60 mg/g in equation represents the largest value we expect for σ with 95% confidence.

7.60 mg/g is < 20 mg/g, precision has been successfully validated with a confidence of 95%.

α = Significance value (It is the maximum acceptable level of risk)

$\chi^2_{0.05;8} = 2.73$ *the value is obtained from the Chi square distribution table.*

σ = Standard deviation for population

USP Education

Ref: USP <1010> Analytical data- Interpretation and treatment and USP <1210> Statistical tools for procedure validation

Accuracy

The **closeness** of the test result obtained by the method to a value that is accepted as conventionally **true value** or **as a Reference value**.

Assay of Drug substance (DS)	Assay of Drug Product (DP)	Impurities
• Application of an analytical procedure to an analyte of known purity e.g.; Reference material • Comparison to a second well-characterized procedure	• Evaluation by analyzing synthetic mixture of known amount **or** sample spiked with known quantities of component • Comparison to a second well-characterized procedure	• The accuracy should be assessed on samples (DS/DP) spiked with known amount of impurities • In cases where it is impossible to obtain **impurities or degradation products**, comparison of results with results obtained by independent procedure is acceptable

Recommendations:

Accuracy should be evaluated using a minimum 9 determinations over minimum 3 concentration levels (3 concentrations /3 replicates) from LOQ to 120% of specification.

Accuracy

Assessment

Assessment of accuracy can be accomplished in a variety of ways -

- Evaluating the recovery of the analyte (% recovery) across the range of the assay,
- Evaluating the linearity of the relationship between amount found and amount added

The statistically preferred criterion is that In the confidence interval for the **slope** be contained in an interval around 1.0, or alternatively, that the slope be close to 1.0. either case, the interval or the definition of closeness should be specified in the validation protocol.

Note: An unbiased analysis has slope of 1 and an intercept of zero.

Accuracy

General acceptance criteria

The acceptance criteria for the recovery of the accuracy samples are usually based on an acceptable range for the mean

From linear regression of actual concentration (amount added) v/s estimated amount (amount found), then the acceptance criteria may be based on the slope and intercept.

Recovery of the accuracy samples:

- **Assay :** Between 98 to 102%
- **Impurities** : Between 90 to 110%

- **Assay :**
  - Slope between 0.98 to 1.02
  - 95% CI should include 1
- **Impurities :**
  - Slope between 0.9 to 1.1
  - 95% CI should include 1

Accuracy Case Study

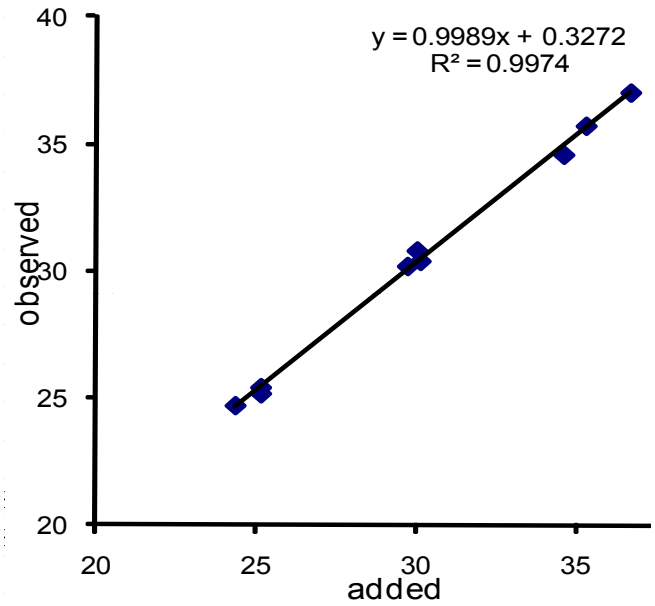
Recovery of impurity 1:

Amount added (mg)	Amound observed (mg)	Recovered
24.35	24.70	101.4%
25.15	25.41	101.0%
25.15	25.17	100.1%
30.04	30.79	102.5%
29.74	30.18	101.5%
30.14	30.38	100.8%
35.33	35.70	101.0%
34.63	34.56	99.8%
36.73	37.01	100.8%
	Mean:	101.0%
	SD:	0.7945

Regression analysis

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99870599
R Square	0.997413654
Adjusted R Square	0.997044177
Standard Error	0.253568809
Observations	9



ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	173.57152	173.5715	2699.521582	2.56314E-10
Residual	7	0.450079988	0.064297		
Total	8	174.0216			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.327238102	0.585575609	0.558832	0.593694893	-1.057428184	1.711904388
X Variable 1 (slope)	0.998888347	0.019225319	51.95692	2.56314E-10	0.953427693	1.044349002

Typical acceptance criteria:

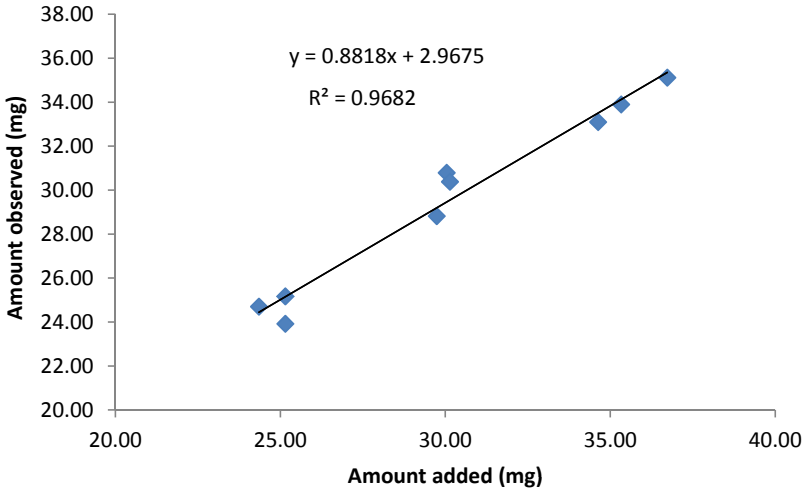
- Mean Recovery
- Individual recovery
- Regression analysis of known vs estimated
  - Slope within 0.9-1.1
  - 95% confidence interval of slope includes 1

Accuracy Case Study

Recovery of impurity 2:

Amount added (mg)	Amound observed (mg)	% Recovery
24.35	24.70	101.43
25.15	23.92	95.11
25.15	25.17	100.08
30.04	30.79	102.50
29.74	28.82	96.91
30.14	30.38	100.80
35.33	33.90	95.95
34.63	33.10	95.58
36.73	35.12	95.63
	Mean	98.22

Regression analysis



SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.983948158
R Square	0.968153978
Adjusted R Square	0.963604546
Standard Error	0.797254008
Observations	9

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	135.2635246	135.2635	212.8076703	1.69854E-06
Residual	7	4.449297672	0.635614		
Total	8	139.7128222			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	2.967469467	1.841127473	1.611768	0.151047627	-1.386105205	7.32104414
X Variable 1(slope)	0.881795876	0.060446954	14.58793	1.69854E-06	0.738861541	1.02473021

Typical acceptance criteria:

- Mean Recovery
- Individual recovery
- Regression analysis of known vs estimated
  - Slope within 0.9-1.1
  - 95% confidence interval of slope includes 1

Accuracy

Statistical analysis to estimate Accuracy

Comparison of the accuracy of procedures provides information useful in determining if the new procedure is equivalent, on the average, to the current procedure.

A simple method for making this comparison is by calculating a confidence interval for the difference in true means.

Difference = Mean of alternative procedure – Mean of current procedure

This approach is often referred to as **TOST (two one sided t-test)**

Conclusion:
If the confidence interval falls entirely within this acceptable range, then the two procedures can be considered equivalent.

Ref: USP <1010> Analytical data- Interpretation and treatment and USP <1210> Statistical tools for procedure validation

Case Study-3 : Confidence Interval on Bias

Statistical analysis to estimate Accuracy

Three different quantities of reference standard were weighted to correspond to three different percentages of the test concentrations: 50%, 100%, and 150%.

The value of τ is 1000 mg/g for all three concentrations. The computed statistics from the validation data set include the sample mean (Y), the sample standard deviation (S), and the number of reportable values (n).

Test Concentration (%)	Test Solution	Reportable Value (mg/g)
50	1	996.07
50	2	988.43
50	3	995.90
100	4	987.22
100	5	990.53
100	6	999.39
150	7	996.33
150	8	993.67
150	9	987.76
Sample mean (Y)		**992.81**
Sample standard deviation (S)		**4.44**

Case Study-3 : Confidence Interval on Bias

Set the confidence interval at 90% because it is equivalent to a 95% Two One-Sided Test (TOST)

A 100 (1-2α) % two-sided confidence interval for the bias (β) is:

$$(\bar{Y} - \tau) \pm t_{1-\alpha;n-1}\frac{S}{\sqrt{n}}$$

where,

β = 100(1 − 2α)% two-sided confidence interval of bias

S = std dev from int. precision

n = number of reportable values

$t_{1-\alpha;n-1}$ = the percentile of a central t- distribution with area 1- α to the left and n-1 degree of freedom

From the data:

If τ=1000, S=4.44, $\bar{Y}$=992.81

$\alpha = 0.05$ and $n = 9$,

then $t_{0.95;8} = 1.860$

The 90% confidence interval on β is:

$$(\bar{Y} - \tau) \pm t_{1-\alpha;n-1}\frac{S}{\sqrt{n}}$$

$$(992.81 - 1000) \pm 1.86\frac{4.44}{\sqrt{9}}$$

$$[-9.94 \; to \; -4.44] \; mg/g$$

Conclusion:

The accuracy requirement is validated if evidence demonstrates that the absolute value of β is NMT 15 mg/g.

Since the computed confidence interval from −9.94 to −4.44 mg/g falls entirely within the range from −15 to +15 mg/g, the bias criterion is satisfied.

Linearity

The linearity of an analytical procedure is its ability to elicit test results that are **directly**, or by a well-defined mathematical transformation, **proportional to the concentration** of analyte in samples within a given range

Typical concentrations:

- For the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration
- For impurities, reporting level (LOQ) of impurity to 120% of the specification
- For content uniformity, covering a minimum of 70 to 130 percent of the test concentration
- For dissolution testing: +/-20 % over the specified range

Note: ICH recommends minimum of five (05) concentrations

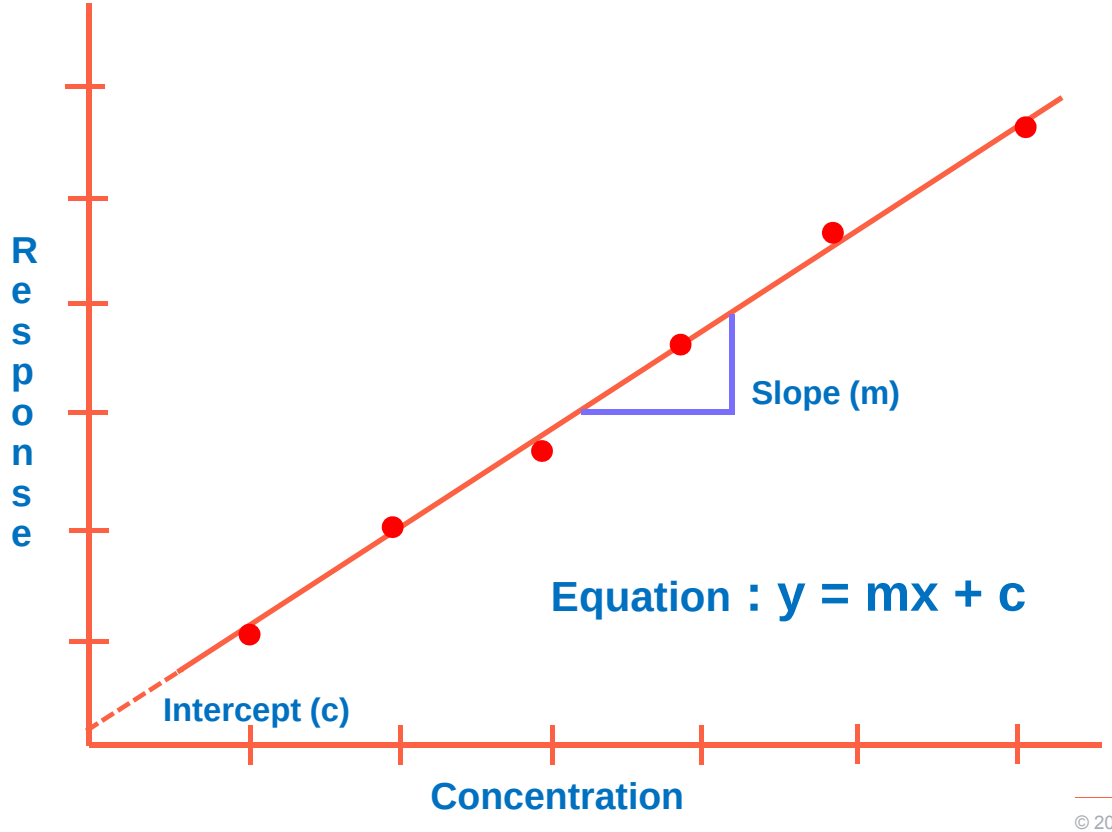
Linearity

Regression Plot

The linear relationship between the analyte response and the corresponding concentration is evaluated by statistical or mathematical approach. One common procedure is the generation of **regression plot using the least squares method** and calculation of the correlation coefficient (*r*).

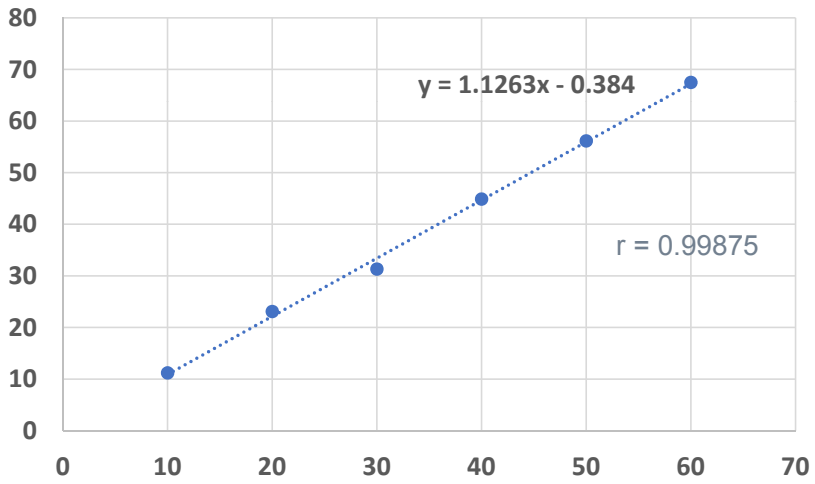
The acceptance criteria should balance scientific rigor with practical needs

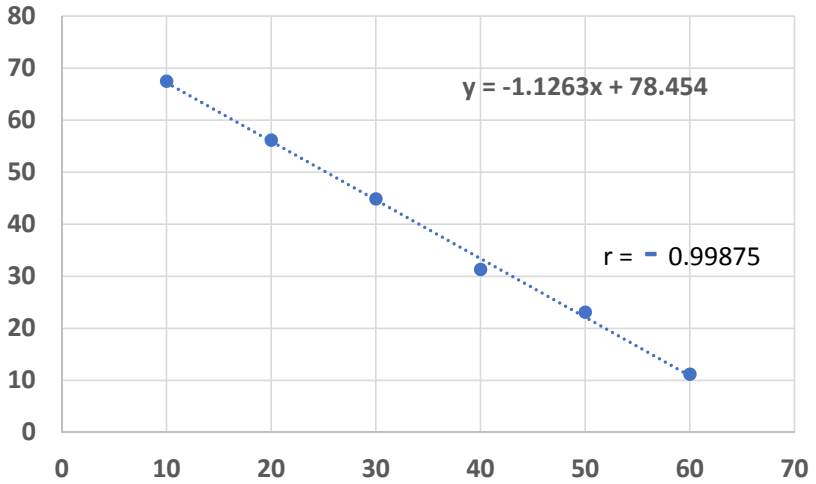
- Minimum *r* and r^2 value- ≥ 0.99 up to ≥ 0.9999
- Y intercept- statistically insignificant, within n% of the response of the standard solution.



The correlation coefficient (*r*)

The strength of the relationship is quantified by the Correlation Coefficient, or Pearson Correlation Coefficient. It can range from -1 to +1.





If there is no correlation, the coefficient is zero, or close to zero.

It is important to understand that **the correlation coefficient is not a measure of linearity but rather a measure** of how well the data fits the model.

The coefficient of determination (r^2)

It is equivalent to the ratio of the regression SS and total SS and thus is an expression of how much variability in the response is fitted by the regression.

$$r^2 = \frac{R}{}$$

Where

- **Regression sum of squares** is the amount of variability in the response
- **Total sum of squares** is the sum of squares explained by the regression line + sum of squares not explained by the regression line i.e. residual sum of squares.

r^2 = 0.969 means that 96.9% of variation in observed values is explained by the equation.

Ideally, r^2 should be equal to one, which would indicate zero error.

Linearity

Significance of Intercept:

Option 1

95% confidence interval of intercept includes zero

Intercept: It is the value of y when x = 0

- If 95% confidence intervals includes zero, the true intercept can also be assumed to be zero and a single point calibration is justified.

Option 2

% y-intercept should be statistically insignificant

An alternative approach is to express the intercept as a % of the analytical response at the target concentration for e.g. 100% concentration level in the assay.

- If the % intercept is not significant, then single point calibration may be used.
- If the % intercept value is not negligible, then multilevel calibration is normally used.

$$\%\,\text{intercept} = \frac{\text{y-intercept x 100}}{\text{Response at 100\%}}$$

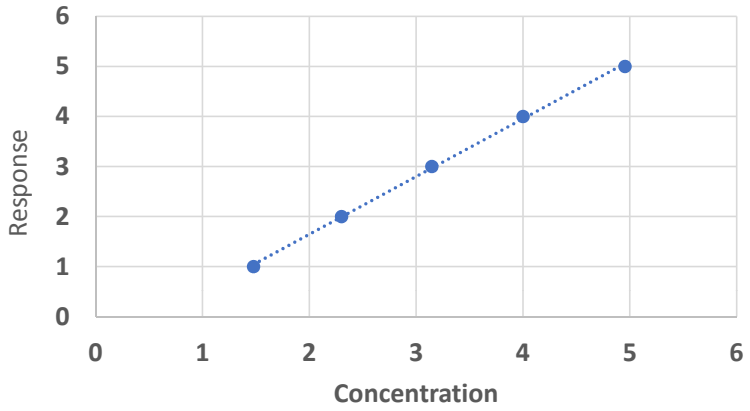
General Limits:

- Assay : ± 2%
- Impurities : ± 5%

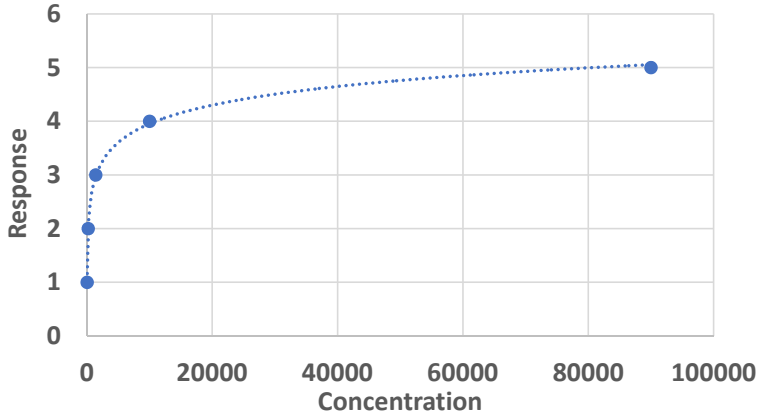
Linearity

Homoscedasticity and Heteroscedasticity :

Homoscedasticity is the term for calibration data having about equal variability over the whole calibration range.



If the data's variability changes from one end of the range to the other the data is called to be **Heteroscedastic**.

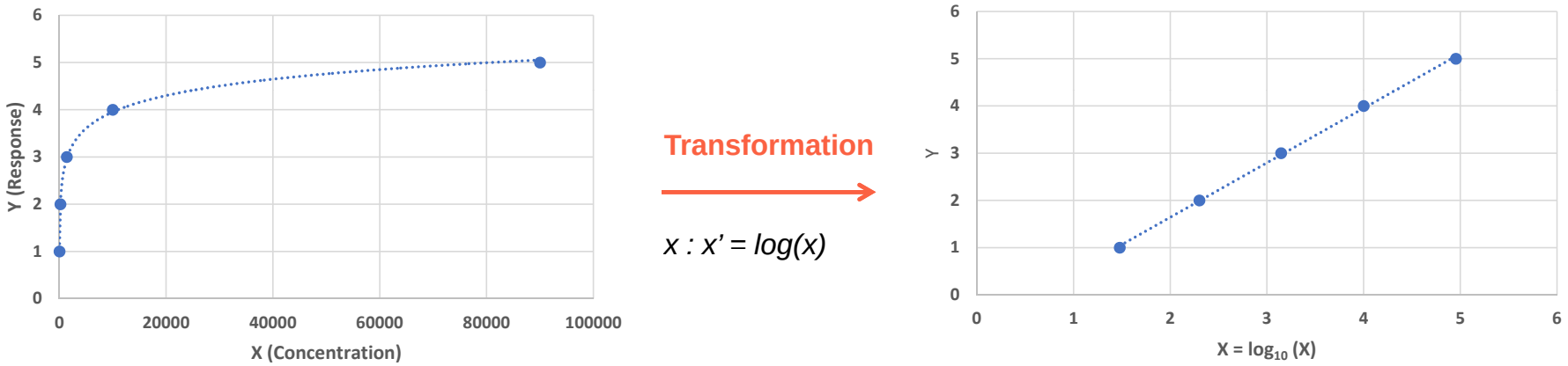


In some cases, to attain linearity, the concentration and/or the measurement may be transformed.

The weighting factors used in the regression analysis may change when a transformation is applied.

Linearity

Transformation may be performed to the response data as well as to the concentration data.

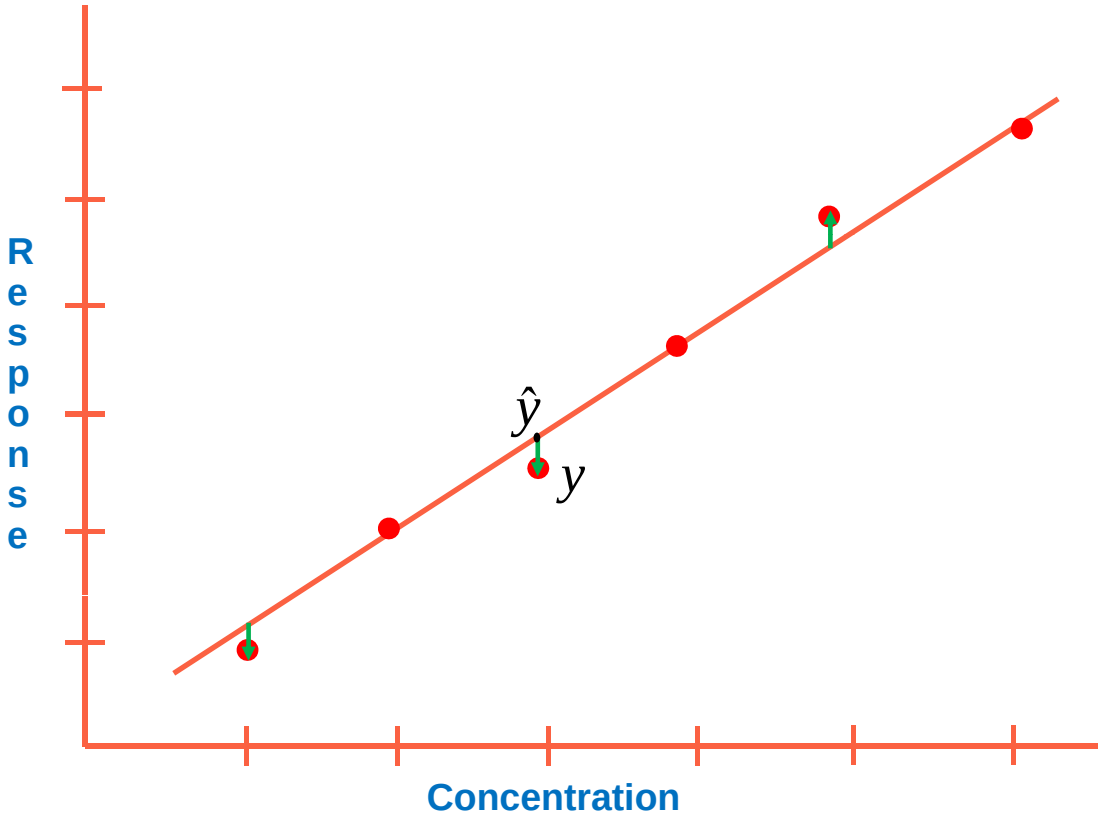


Common choices for a transformation of the response include, but not limited to,

- Log
- Natural Log
- Square root
- Reciprocal

Linearity

Residuals:



Residual:
The distance in the y-direction from the point to the regression line.

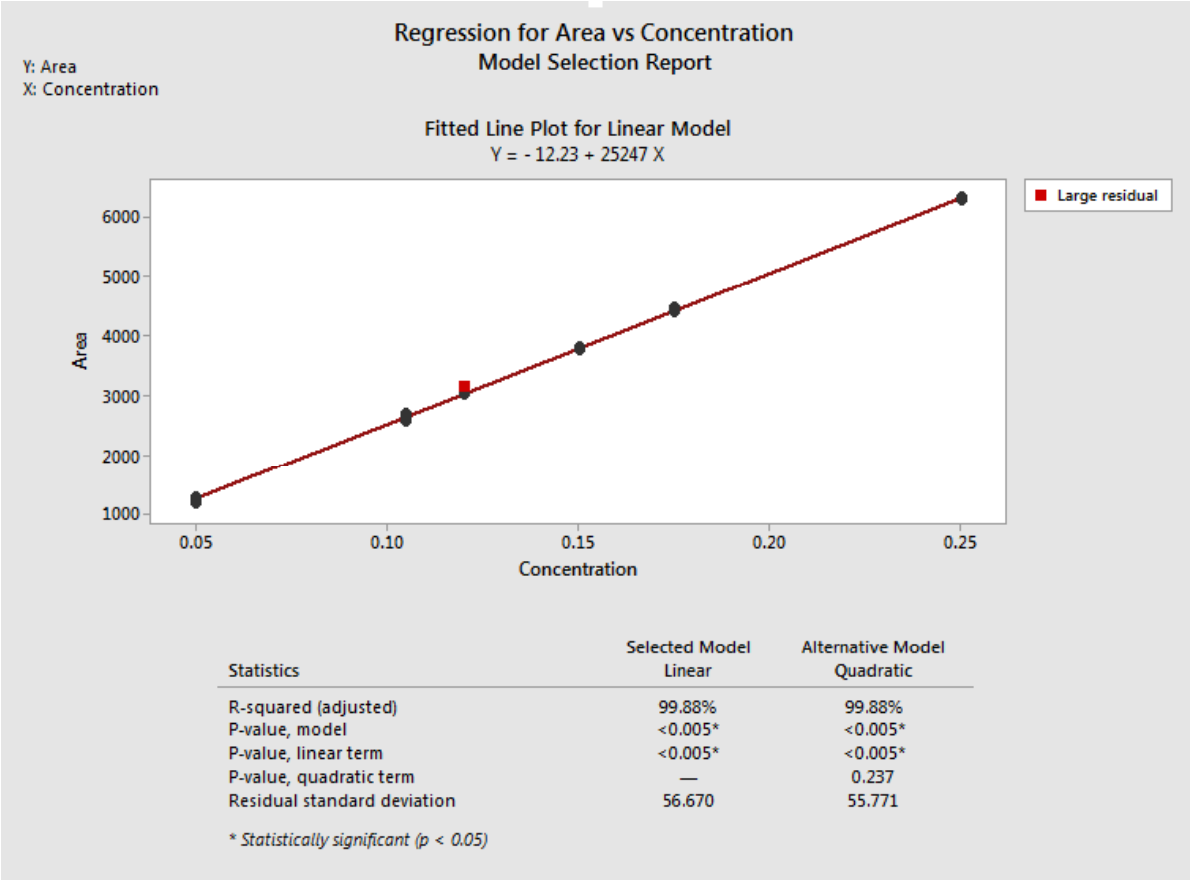
Deviation of an observed data point (y) from the corresponding predicted data point ($\hat{y}$)

Each residual : $y - \hat{y}$

Case study- 4

Regression analysis:

Sr. No	Conc.	Area
1	0.050	1250
	0.050	1260
	0.050	1155
2	0.105	2625
	0.105	2550
	0.105	2700
3	0.120	3000
	0.120	3150
	0.120	3090
4	0.150	3750
	0.150	3800
	0.150	3755
5	0.175	4375
	0.175	4402
	0.175	4450
6	0.250	6250
	0.250	6311
	0.250	6288



Statistics	Selected Model Linear	Alternative Model Quadratic
R-squared (adjusted)	99.88%	99.88%
P-value, model	<0.005*	<0.005*
P-value, linear term	<0.005*	<0.005*
P-value, quadratic term	—	0.237
Residual standard deviation	56.670	55.771

** Statistically significant (p < 0.05)*

Ref: Graph is generated by using Minitab

Case study- 4 cont..

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999419737
R Square	0.998839811
Adjusted R Square	0.998767299
Standard Error	56.67013031
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	44238000.44	44238000.44	13774.85595	6.4499E-25
Residual	16	51384.05872	3211.50367		
Total	17	44289384.5			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-12.22610471	33.2736504	-0.36744104	0.718105085	**-82.76309252**	**58.31088311**
X Variable 1 (slope)	25247.47839	215.1168729	117.3663323	6.450E-25	24791.45099	25703.50579

Typical acceptance criteria:

- Valid calibration model e.g. r and r^2
- Residual plots shows random scatter and no systematic trends
- 95% confidence interval of intercept includes zero

Equation ($y=mx+c$) : $25247x$ -12.23

Intercept (c) = -12.23 (The value of y when x=0)

Standard Error, (SE intercept) = 33.27

95% CI of SE (intercept)= -82.76 to 58.31

Slope (m) = 25247

Standard error (SE of slope) = 215.11

95% CI of SE (slope) = 24791 to 25703

Coefficient of determination (r^2)=0.9988

Correlation coefficient (r)= 0.9994

(This will be between 0 to 1, the closure the value 1, the better the correlation)

Regression SS = 44238000

(Regression sum of squares is the amount of variability in the response)

Residual SS = 51384

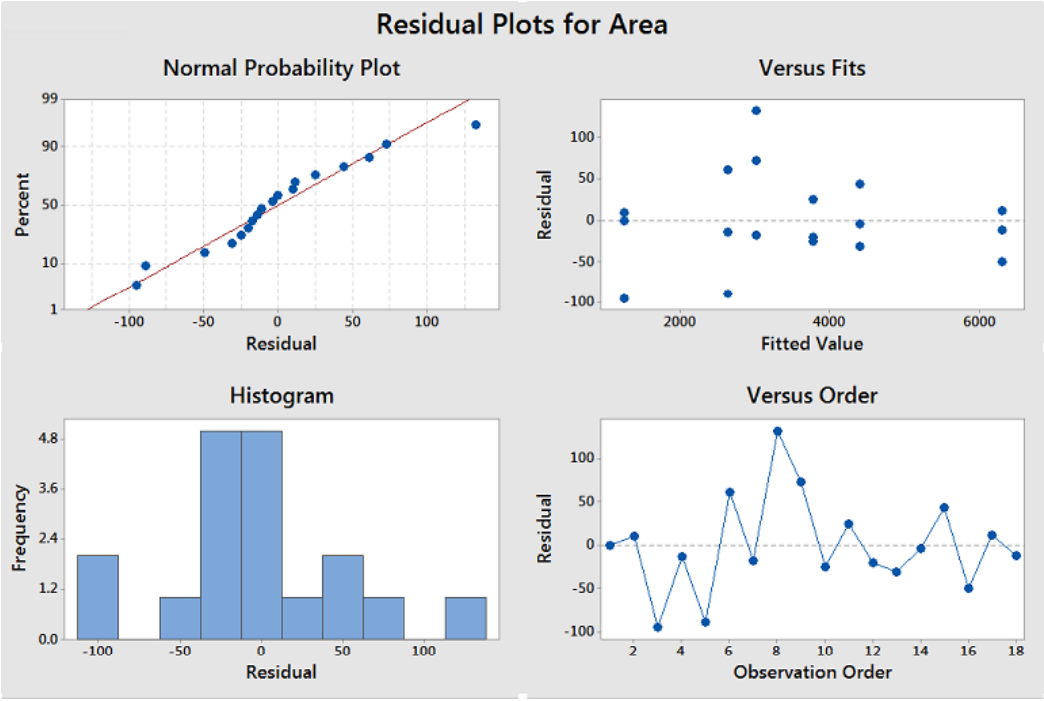
(Residual sum of squares is the variability about regression line, the amount of uncertainty remains)

Total SS = 44289384

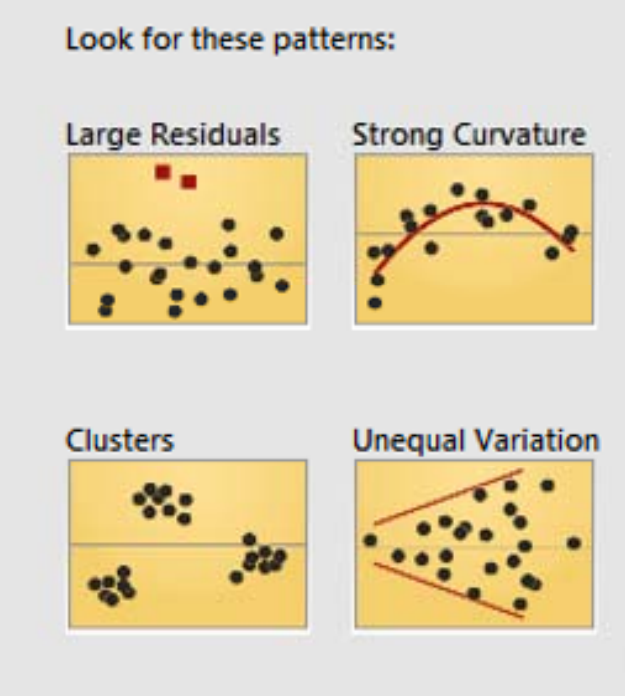
(The total sum of squares is the total amount of variability in the response)

Case study – 4 cont..

Regression analysis:



Residual pattern:



- **Normal probability plot:** To verify the assumption that the residuals are **normally distributed**
- **Histogram:** To determine whether the data are skewed or whether **outliers exist** in the data
- **Versus Fits:** To verify the assumption that the residuals have a **constant variance**
- **Versus Order:** To verify the assumption that the residuals are **uncorrelated** with each other

Ref: Graph is generated by using Minitab

Questions

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Thank You

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