Calibration

In this unit, we will review how to construct a calibration curve.

Each calibration has its own limits.

A proper understanding of those limits will help you develop the 'best' possible calibration and avoid many problems.

Constructing a calibration curve

You typically have two (or more) variables to work with.

One (or more) is set at known values.
- ✓ Your analyte
- ✓ Other experimental conditions

One is a measured response.
- ✓ Absorbance, current, area, ...

Constructing a calibration curve

For a simple two variable calibration curve we commonly assume that we are dealing with two types of variables:

Independent - the one we set
Dependent - the one we measure

In reality, both variables should be considered independent.

We rely on developing a model to show that the two variables are related.

Constructing a calibration curve

The simplest approach to developing our model is to:

- Select a series of known analyte standards.
- Hold other factors constant - or as many as possible
- Measure the response.
- Develop a model (calibration curve).

Constructing a calibration curve

Our response may actually rely on a vast number of factors:

Examples
- matrix
- interfering analytes
- random errors
- sample preparation
- sample collection method
- ...

Constructing a calibration curve

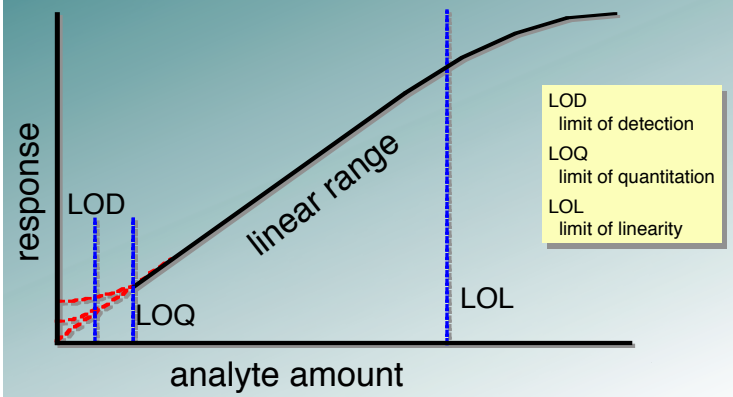
So our response is actually a measure of an entire method.

The relationship between analyte and response is a function of the type of method.

Examples

gravimetry	f(mass) = amount
chromatography	f(area) = amount
ISE	f(mV) = log[ ]

Most methods have a fixed range where the relationship between response and analyte amount is valid.



Constructing a calibration curve

To initially establish linear range, sensitivity and detection limits, we commonly rely on the external standard method.

Separately run knowns and unknowns.

Assume that the only difference in response if due to the analyte.

Develop a model to show any relationships and limits.

Linear modeling

General unweighted least squares

Assumptions

- Standards are correct and all errors come from the measurement of response.
- Variances are independent of analyte concentration.

Linear model

$$R = b_1 X + b_0 + e$$

R = response
b_1 = slope parameter
X = Standard value
b_0 = intercept parameter
e = residual error

Linear model

We've already showed how to calculate the slope and intercept.

$$b_1 = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sum X^2 - \frac{\left(\sum X\right)^2}{N}} \quad \textit{slope}$$

$$b_0 = \frac{\sum Y - b_1 \sum X}{N} \quad \text{intercept}$$

Linear model

$$s_{b_0}^2 = \frac{s_Y^2 \sum X^2}{N \sum (X_i - \overline{X})^2}$$

$$s_{b_1}^2 = \frac{s_Y^2}{\sum (X_i - \overline{X})^2}$$

We can estimate the variances via propagation of errors - produced during ANOVA analysis.

$$s_e^2 = \frac{\sum (Y_i - \overline{Y})^2 - b_1^2 \sum (X_i - \overline{X})^2}{(N-2)}$$

Linear models and uncertainty

Any linear model has some degree of uncertainty associated with it. At a given confidence level, our model actually represents a regression band.

$$slope_{range} = b_1 \pm t\, s_{b_1}$$

$$intercept_{range} = b_0 \pm t\, s_{b_0}$$

Use one sided t value

Calculating the regression bands

- Determine the desired confidence limit and look up the proper t value (df = N - 2)
- Calculate the predicted X' value based on the predicted R' (response) value. This is so you can make a more complete plot.
- For each point, calculate your interval value as:

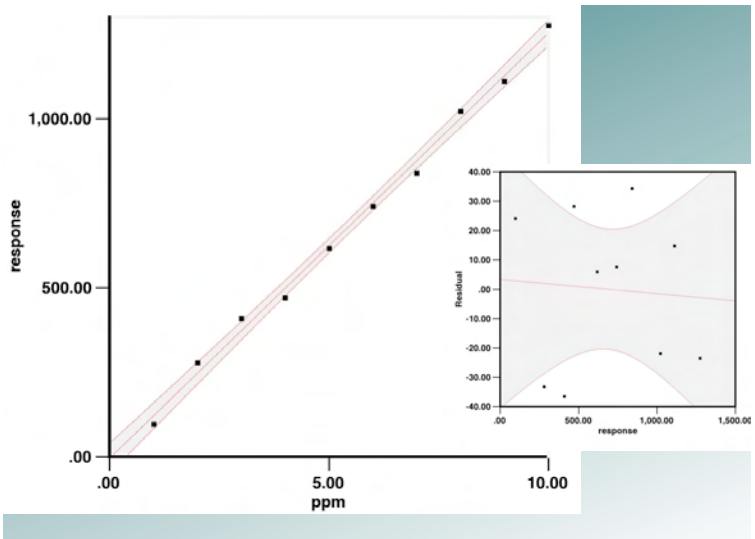
$$C = t\, s_y \left[\frac{1}{N} + \frac{(X' - \overline{X})^2}{\sum (X_i - \overline{X})^2} \right]^{1/2}$$

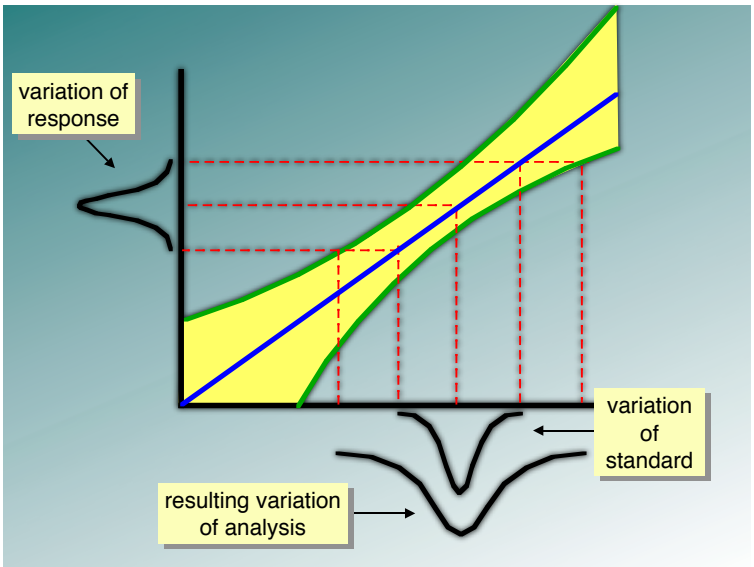
Calculating the regression band

Your regression band is then calculated as:

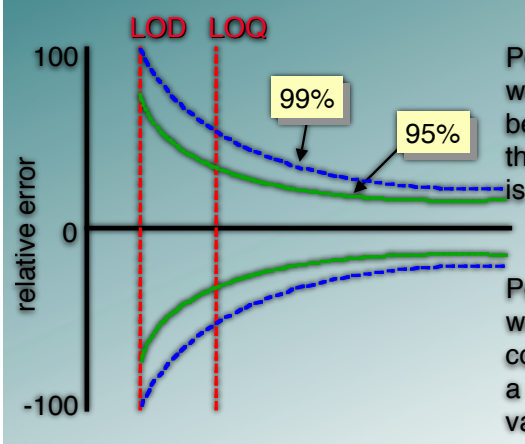
- Lower $b_1 X' + b_0 - C$
- Upper $b_1 X' + b_0 + C$

You are just plotting out the confidence limits for each data point.





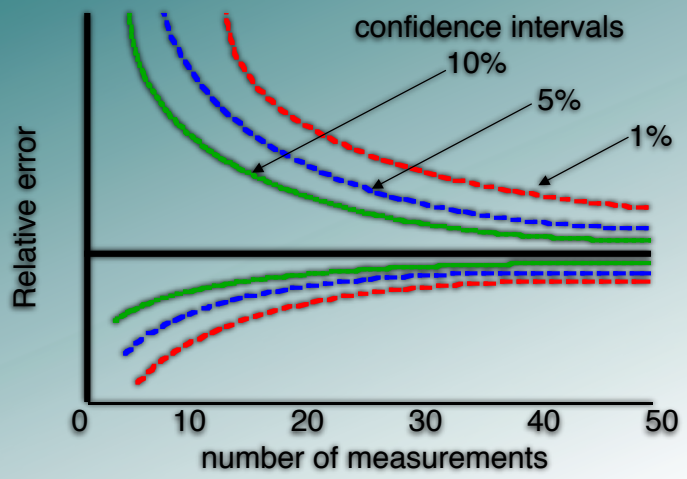
Uncertainty near the detection limit



LOD
Point below which we can no longer be confident that our analyte is present.

LOQ
Point below which we can no longer confidently report a quantitative value.

Number of measurements and uncertainty



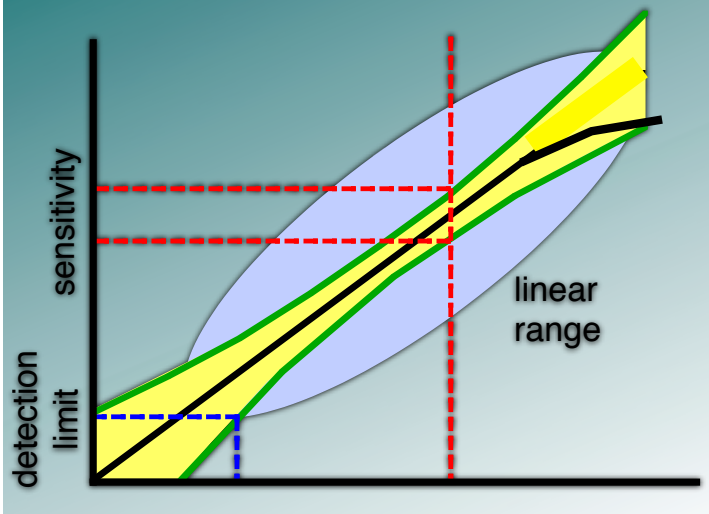
Detection limit, sensitivity & linear range

The calibration curve and regression band can determine{

Detection limit. Smallest amount we can see with a known level of confidence. The upper CL at Y=0, then converted to concentration (X).

Sensitivity. Smallest change in amount we can see with a known level of confidence. Often based on smallest change that an instrument can display.

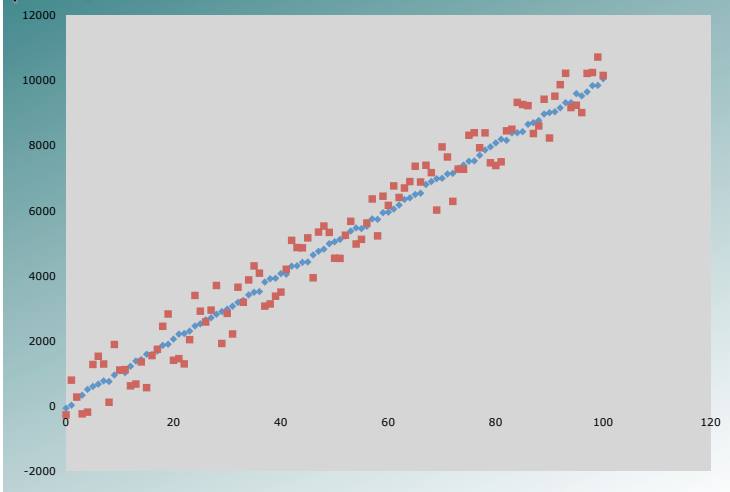
Linear range. Range where we can quantify with a known level of confidence. (lower = DL, upper = where curve intersects upper/lower CL.



Example

<>	A	B	C	D	E	F	G
1	Concentration	Noise +/- 100	Noise +/- 2000				
2	0	-81	-285				
3	1	11	781		Generated by		
4	2	247	262		Response = 100 Conc +(rand()-0.5)*factor		
5	3	319	-255				
6	4	497	-201				
7	5	588	1263				
8	6	659	1514				
9	7	758	1277				
10	8	740	102				
11	9	940	1877				
12	10	1072	1091				
13	11	1013	1105				
14	12	1206	606				
15	13	1368	662				
16	14	1415	1341				
17	15	1576	551				
18	16	1568	1535				
19	17	1675	1729				
20	18	1849	2436				
21	19	1884	2813				
22	20	2042	1390				

Example



Example

Variable	Observations	Minimum	Maximum	Mean	Std. deviation
Noise +/- 200	101	-285.000	10706.000	5103.139	3061.177
Concentration	101	0.000	100.000	50.000	29.300

Regression of Noise +/- 2000:

Analysis of variance:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	1	904260255.219	904260255.219	2727.657	< 0.0001
Error	99	32820022.840	331515.382		
Corrected Tota	100	937080278.059			

Computed against model Y=Mean(Y)

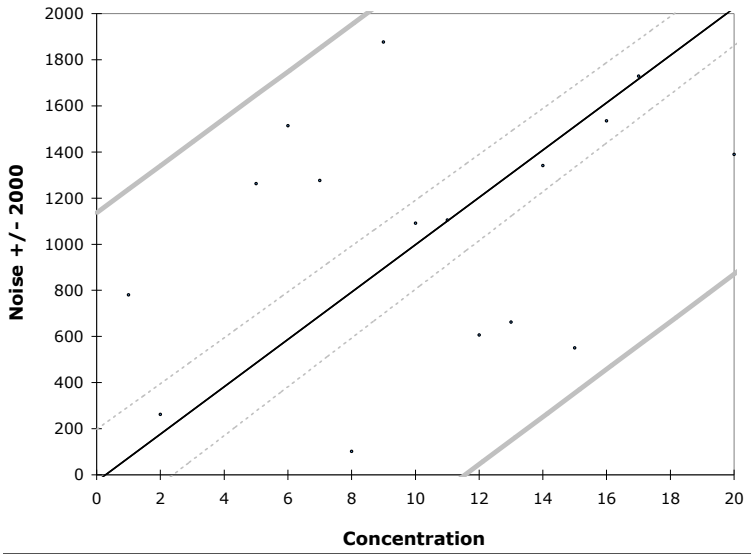
Type III Sum of Squares analysis:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Concentration	1	904260255.219	904260255.219	2727.657	< 0.0001

Model parameters:

Source	Value	Standard error	t	Pr > |t|	wer bound (95%	per bound (95%)
Intercept	-28.388	113.738	-0.250	0.803	-254.068	197.292
Concentration	102.631	1.965	52.227	< 0.0001	98.731	106.530

Equation of the model:
Noise +/- 2000 = -28.39+102.63*Concentration



Noise +/- 2000 = -28.39+102.63*Concentration		
t	1.98397147	tinv(0.05,100)
mean	5103.14	
Y Int	-28.39	
Total SS	9370880278	
STD	5103.14	
DL =	t * STDy * [1/N + (Y_int - mean)^2 / Total SS]^.5	
DL =	1155.51556	
Conc DL	11.5356134	

Example

Regression of Noise +/- 100:

Analysis of variance:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	1	853226523.267	853226523.267	277449.093	< 0.0001
Error	99	304450.179	3075.254		
Corrected Tot	100	853530973.446			

Computed against model Y=Mean(Y)

Type III Sum of Squares analysis:

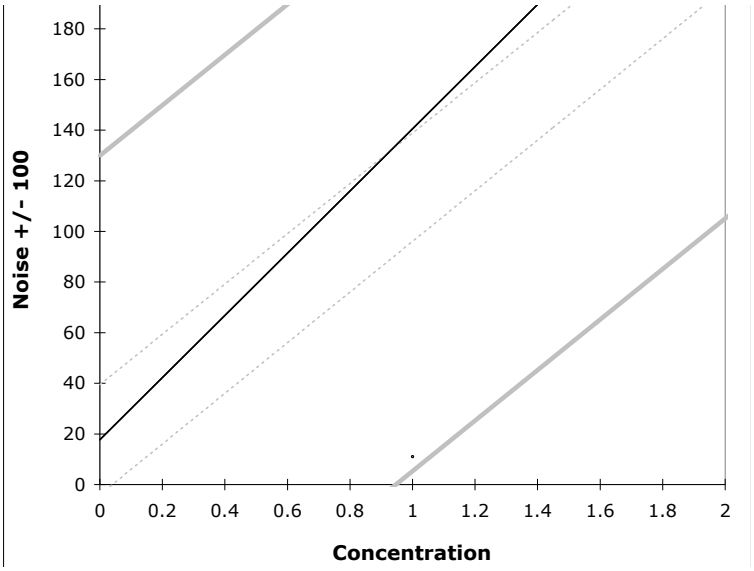
Source	DF	Sum of squares	Mean squares	F	Pr > F
Concentratior	1	853226523.267	853226523.267	277449.093	< 0.0001

Model parameters:

Source	Value	Standard error	t	Pr > |t|	ver bound (95	er b
Intercept	17.747	10.955	1.620	0.108	-3.989	
Concentratior	99.692	0.189	526.734	< 0.0001	99.317	

Equation of the model:

Noise +/- 100 = 17.75+99.69*Concentration



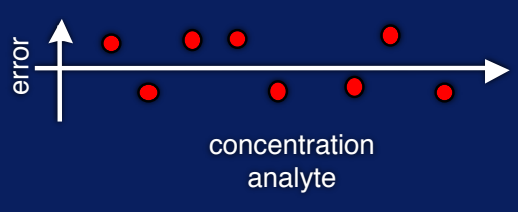
Noise +/- 100 = 17.75+99.69*Concentration	
DL =	129.91
DL Conc =	1.29961985

Saved time by using upper 95% confidence limit value that XLStat provided for [ ] = 0

Using the residuals

A plot of the residuals can give you an idea of how well your model fits the data.

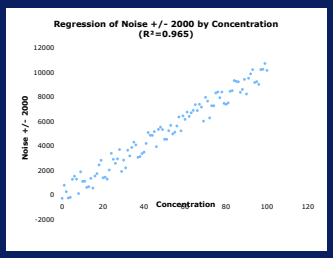
Residual = measured - predicted

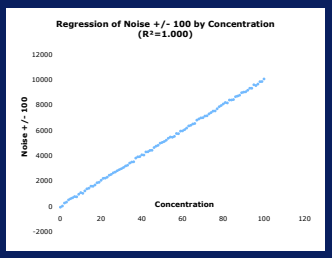


This residual plot indicates a reasonable fit of the data to the model.

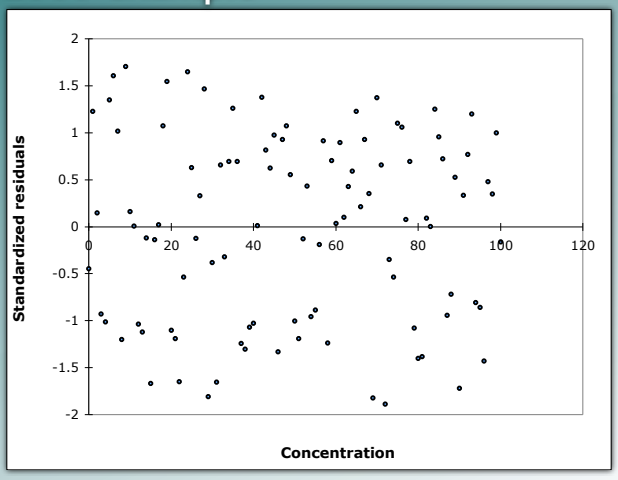
Using the residuals

OK, these are reasonable fits of a linear model but we should check the residuals to be sure

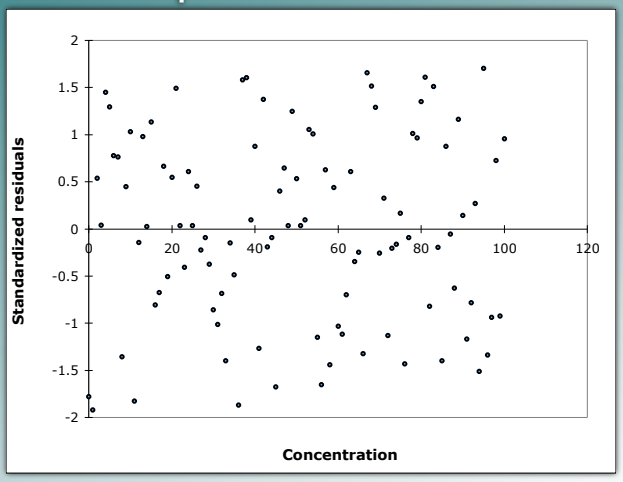




+/-2000 example



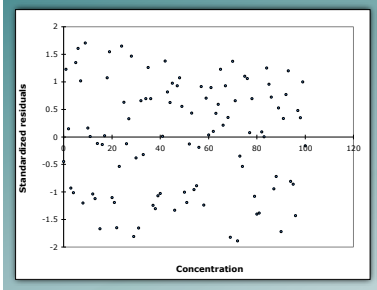
+/-100 example

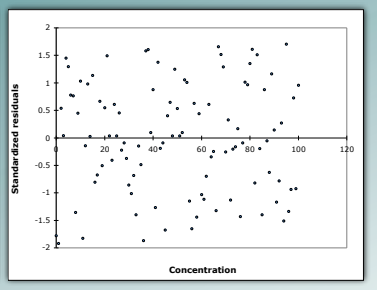


Standardized residual

- Residuals are normalized by the standard error.
- This results in both of our plots looking pretty much the same since the error introduced was random (even if it varied by a large factor.
- Typically, expect to see residual values to be randomly distributed around ± 2 units for a good model.
- ± 3 would indicate a problem. ± 4 shouldn't happen.

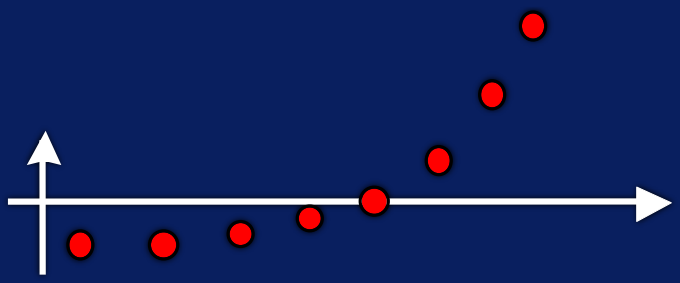
Standardized residual.





So, by using standardized residuals, we can directly compare the two data sets - even though the actual noise level is significantly different.

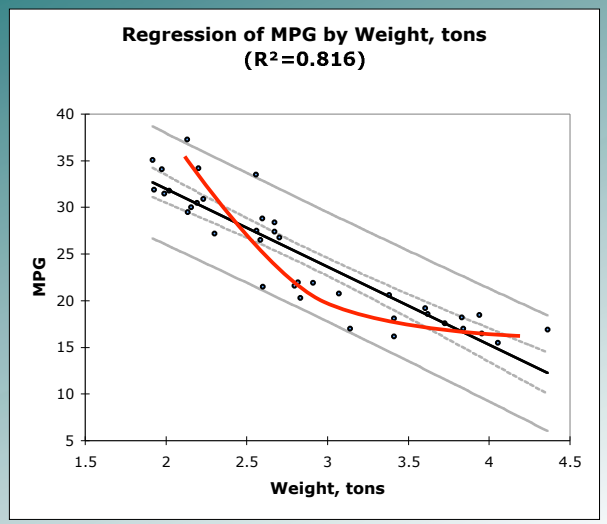
Using the residuals

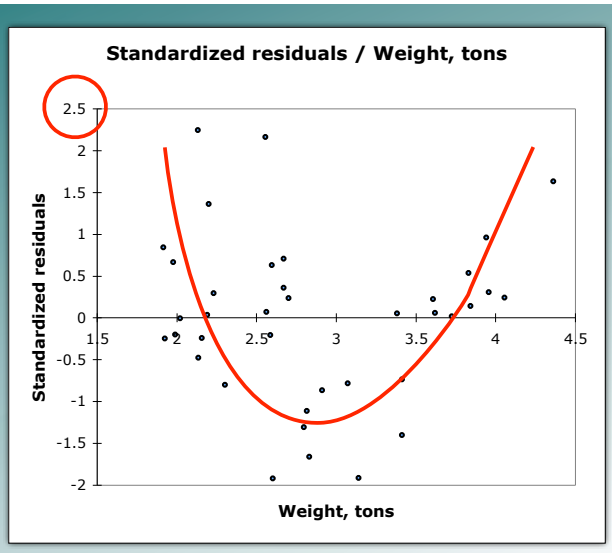


This residual plot indicates that an improper model was used.

Country	Car	MPG	Weight, tons	Drive_Ratio	HP	Disp.	Cyl.
U.S.	Buick Estate Wagon	16.9	4.36	2.73	155	350	8
U.S.	Ford Country Squire Wagon	15.5	4.054	2.26	142	351	8
U.S.	Chevy Malibu Wagon	19.2	3.605	2.56	125	267	8
U.S.	Chrysler LeBaron Wagon	18.5	3.94	2.45	150	360	8
U.S.	Chevette	30	2.155	3.7	68	98	4
Japan	Toyota Corona	27.5	2.56	3.05	95	134	4
Japan	Datsun 510	27.2	2.3	3.54	97	119	4
U.S.	Dodge Omni	30.9	2.23	3.37	75	105	4
Germany	Audi 5000	20.3	2.83	3.9	103	131	5
Sweden	Volvo 240 GL	17	3.14	3.5	125	163	6
Sweden	Saab 99 GLE	21.6	2.795	3.77	115	121	4
France	Peugeot 694 SL	16.2	3.41	3.58	133	163	6
U.S.	Buick Century Special	20.6	3.38	2.73	105	231	6
U.S.	Mercury Zephyr	20.8	3.07	3.08	85	200	6
U.S.	Dodge Aspen	18.6	3.62	2.71	110	225	6
U.S.	AMC Concord D/L	18.1	3.41	2.73	120	258	6
U.S.	Chevy Caprice Classic	17	3.84	2.41	130	305	8
U.S.	Ford LTD	17.6	3.725	2.26	129	302	8
U.S.	Mercury Grand Marquis	16.5	3.955	2.26	138	351	8
U.S.	Dodge St Regis	18.2	3.83	2.45	135	318	8
U.S.	Ford Mustang 4	26.5	2.585	3.08	88	140	4
U.S.	Ford Mustang Ghia	21.9	2.91	3.08	109	171	6
Japan	Mazda GLC	34.1	1.975	3.73	65	86	4
Japan	Dodge Colt	35.1	1.915	2.97	80	98	4
U.S.	AMC Spirit	27.4	2.67	3.08	80	121	4
Germany	VW Scirocco	31.5	1.99	3.78	71	89	4

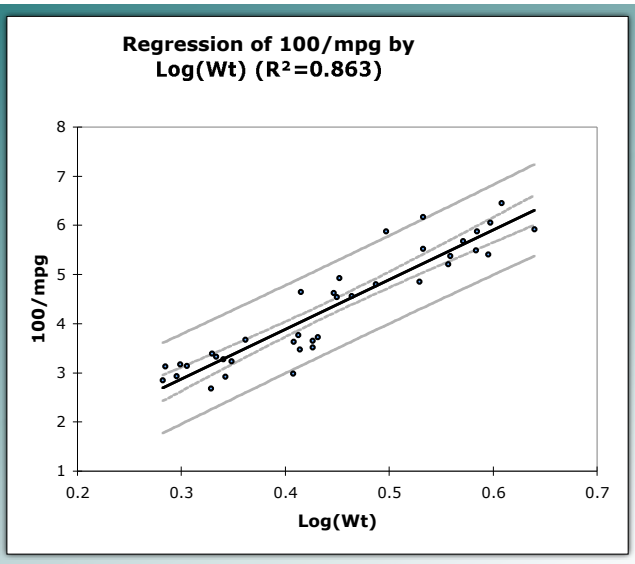
Here is an example that demonstrates a 'bad' model. We're going to try and model MPG vs. car weight.

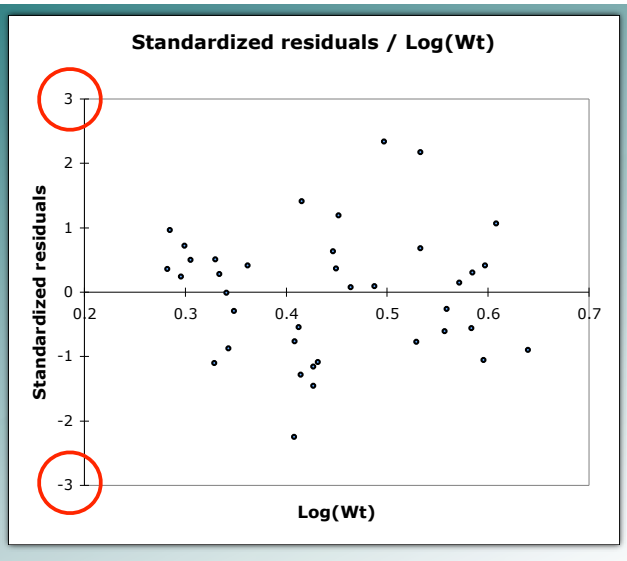




Now what?

- The model clearly has a problem.
- Researchers attempted different models and found that log(weight) gave a better fit.
- Also, MPG is determined differently in other countries - USA uses miles traveled/gallon. It is often full consumed per fixed distance (100 miles).
- So, they used log(wt) vs 100/MPG





Still not perfect.

- Residual range is excessive.
- Ultimately, the best model had to include other factors.
- Including Drive Ratio turned out to be the key in developing the best model.

Regression of variable 100/mpg:

Observations	38.000
Sum of weigh	38.000
DF	35.000
R^2	0.918
Adjusted R^2	0.913

Analysis of variance:

Source	DF	um of square	Mean squares	F	Pr > F
Model	2	45.368	22.684	194.664	< 0.0001
Error	35	4.079	0.117		
Corrected Tot	37	49.446			

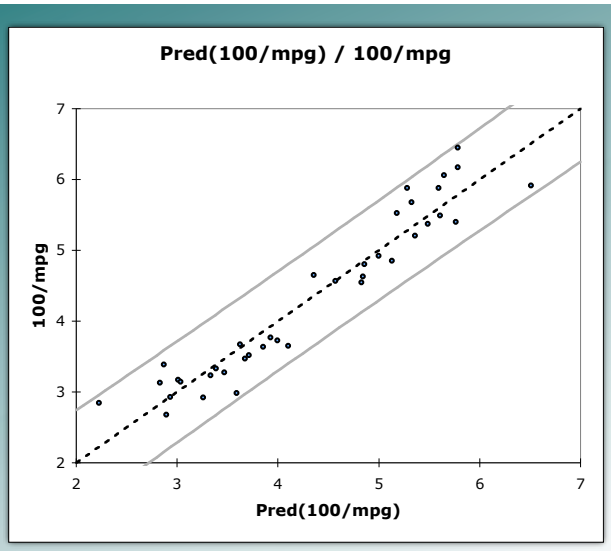
Type III Sum of Squares analysis:

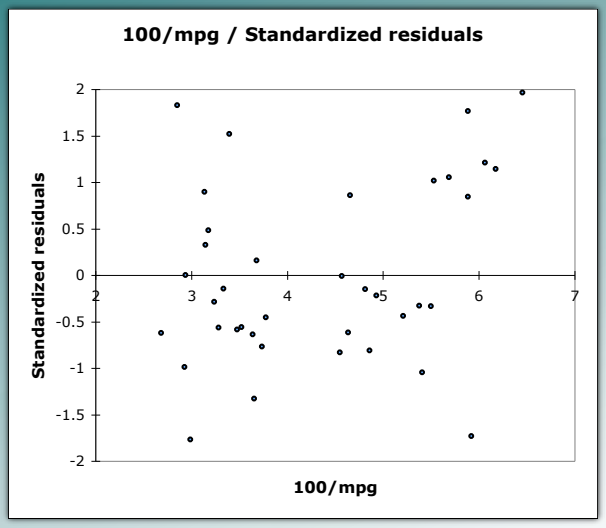
Source	DF	um of square	Mean squares	F	Pr > F
Log(Wt)	1	35.089	35.089	301.123	< 0.0001
Drive_Ratio	1	2.715	2.715	23.296	< 0.0001

Model parameters:

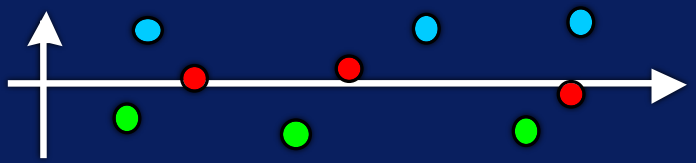
Source	Value	Standard erro	t	Pr > |t|	wer bound (95	per bound (95%)
Intercept	-3.404	0.713	-4.771	< 0.0001	-4.852	-1.956
Log(Wt)	12.466	0.718	17.353	< 0.0001	11.007	13.924
Drive_Ratio	0.711	0.147	4.827	< 0.0001	0.412	1.010

Equation of the model:
100/mpg = -3.4+12.47*Log(Wt)+0.711*Drive_Ratio



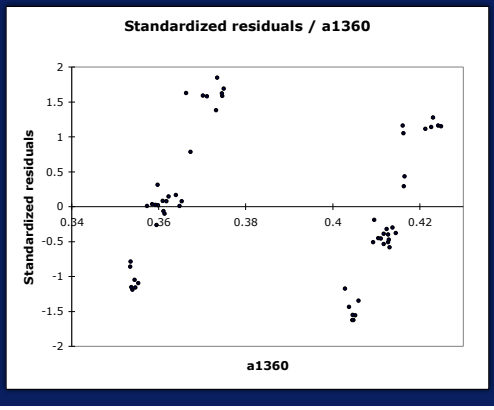


Using the residuals



This residual plot indicates that there is some sort of response dependency based on the sample used. Consider using Analysis of Covariance to confirm.

From the 'Octane' summer/winter blend example.

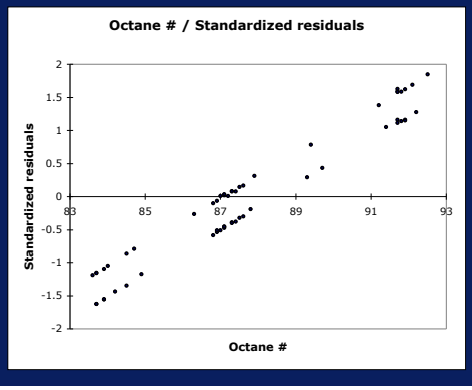


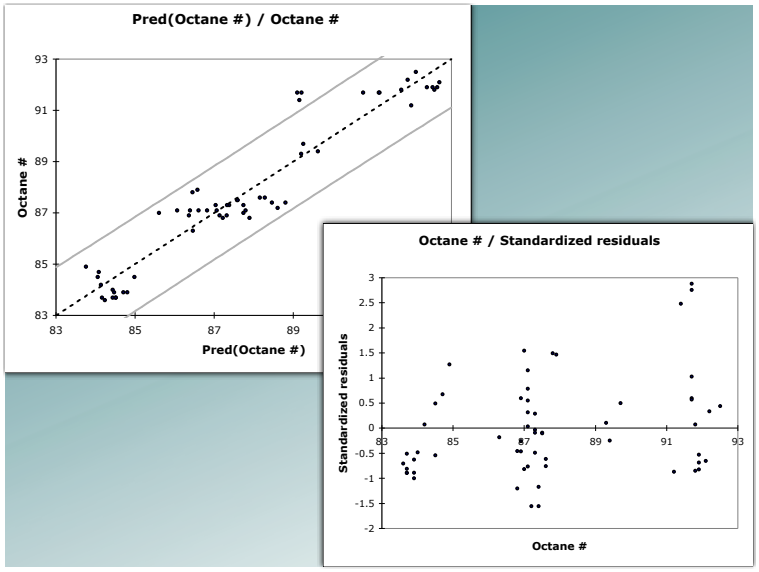
It's clear that there are two different types of samples so a simple calibration model won't work.

This was a candidate for ANCOVA,

From the 'Octane' summer/winter blend example.

ANCOVA can then be used to build a model that includes the 'season' factor as a way of merging what are clearly two different (but related) models.





Standard addition

- A calibration method where standards are added to replicates of your sample.
- You then measure the total response.
- The matrix is near identical for all samples.
- You can measure the response away from LOD and LOQ values.

Standard addition

Initial response. No standard addition.

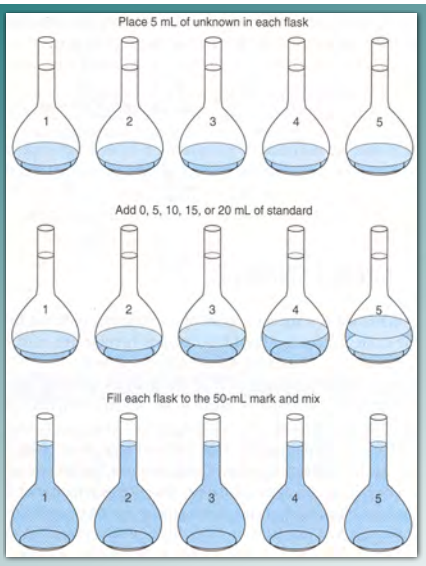
$$R_0 = k\,C_0 = k\left(\frac{n_0}{V_0}\right)$$

Response when a standard is added.

$$R_T = R_0 + R_S = k\left(\frac{n_0 + n_S}{V_0 + V_S}\right)$$

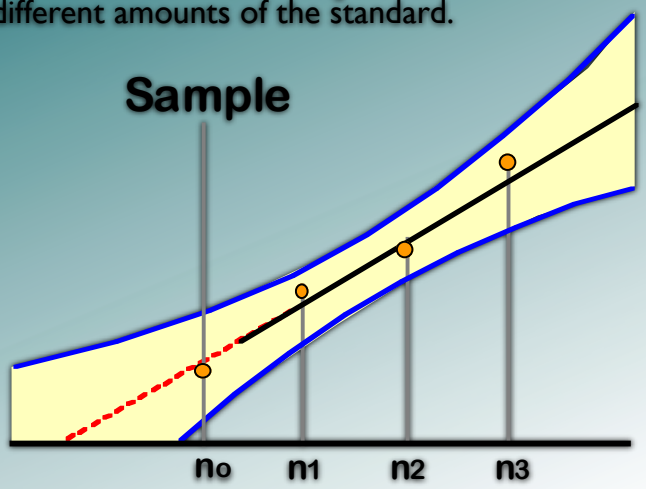
We can then set Q as:

$$Q = R_T(V_0 + V_S) = k\,n_0 + k\,n_S$$



With an approach like this, Vn+Vs would be the same for all standards.

We can then plot Q vs. n_s added using different amounts of the standard.



Standard addition

By extrapolating to the x intercept, where Q=0, we have

$$k\,n_0 = -\,k\,n_1 \qquad and \qquad n_0 = -\,n_i$$

The primary advantage of this method is that you can move your measurement from an area of high relative error.