Workbook for Calibration

Excel workbook *Calibration_8.xlsm*

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Introduction

The purpose of this workbook is to select the best calibration function and to calculate the calibration function, the detection limits as well as concentrations with associated uncertainties for unknown samples.

Calibration_8.xlsm can be used for weighted and unweighted least square linear regression and polynomial regression of 2nd and 3rd degree.

It is possible to force the calibration through zero (Intercept = 0), but this is NOT recommended.

For an introduction to why and how to select the right calibration function see the PowerPoint presentation about calibration functions.

Instructions for use are also given in the sheets in the workbooks. The Add-In *Analysis ToolPack-VBA* and *Solver* must be activated before using the sheet (see troubleshooting at the end of this manual).

This workbook is intended to be used together with the quantitation workbook *chimera,* but can also be used stand alone, see Stand alone or used with chimera.

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WARNING: Never ever MOVE cells. ALWAYS use Copy-Paste Values…Do only enter (or Copy-Paste) values into WHITE CELLS

1) Stand alone or used with *chimera*

X-values as concentrations or ratios

The X-values for calibration can either be concentrations of analyte or ratios = C(analyte)/C(IS). The result Xo of the calibration is thus either the concentration, μg/ml, or the ratio, μg(analyte)/μg(IS), for the solutions that were analysed. Now in most cases, the sample solutions are produced by sample preparation starting with a known amount of solid or liquid sample (sample mass or sample volume) to which a known amount of internal standard IS is added. For both X = concentrations and ratios, the data for V/m is the same, i.e. ratio of ml of IS to g of sample for the blanks and the samples (mass of sample being the same in both cases as no mass is used for the blanks). It is assumed that the concentration in the stock solution of IS used in calibration and for the samples are identical.

The IS_{factor} for:

X = ratios: IS_{factor} = Concentration of IS in IS stock solution

X = concentrations: IS_{factor} = Volume(calibration standards)/Volume(IS added).

If 100 μl IS is added to 5 ml standard IS_{factor} = 50.

With *chimera* all input to sheet *Experimental data* are filled in automatically, and the data can be analysed directly without further user input. However, many parameters for the analysis are already entered as default values in the sheets and the user may want to change some of these parameters:

In sheet *Experimental data*:

- In Table 0: The parameter *Weighted?* is blank, meaning that the macros automatically find the best method weighted/not weighted. Change to 1 or 0 for always to use weighted/not weighted.

In sheet *Input*:

- Check all parameters in Table 1, 5 and 6, and change according to your wishes. See the instructions in the sheet and in section 3) Sheet: Input.

Stand alone:

In sheet experimental data:

- All data must be entered manually or by *Copy – Paste Values*. See the instructions in the sheet and in section 5) Sheet: Experimental data.

In sheet *Input*:

- Check all parameters in Table 1, 5 and 6, and change according to your wishes. See the instructions in the sheet and in section 3) Sheet: Input.

2) Quantitation and internal standards

The workbook calculates two results for each sample.

Xo: The concentration, μg/ml, or the ratio of analyte to IS, μg(analyte)/μg(IS), for all sample solutions (the solutions that were injected into the instrument)

Xm: The concentration of the analyte in the initial solid sample (μg/g). For liquid samples replace the mass in g with the volume in ml

Xm may be calculated with subtraction of a blank value.

To calculate Xm from Xo the following data are used:

m_{sa}: The mass of the initial sample, g

$V_{IS,sa}$: The volume of internal standard added to the initial sample, ml

IS_{factor}: The concentration of the stock IS solution (X = ratios) – or – the volume of the calibration standard divided by the volume of IS in the calibration standards (X = concentration).

The input in sheet *Experimental data* is the ratio $V_{IS,sa}/m_{sa}$ or IS_{factor}.

If a blank value should be subtracted further data is needed:

$Yo_{,bl}$ The average response for blank sample(s)

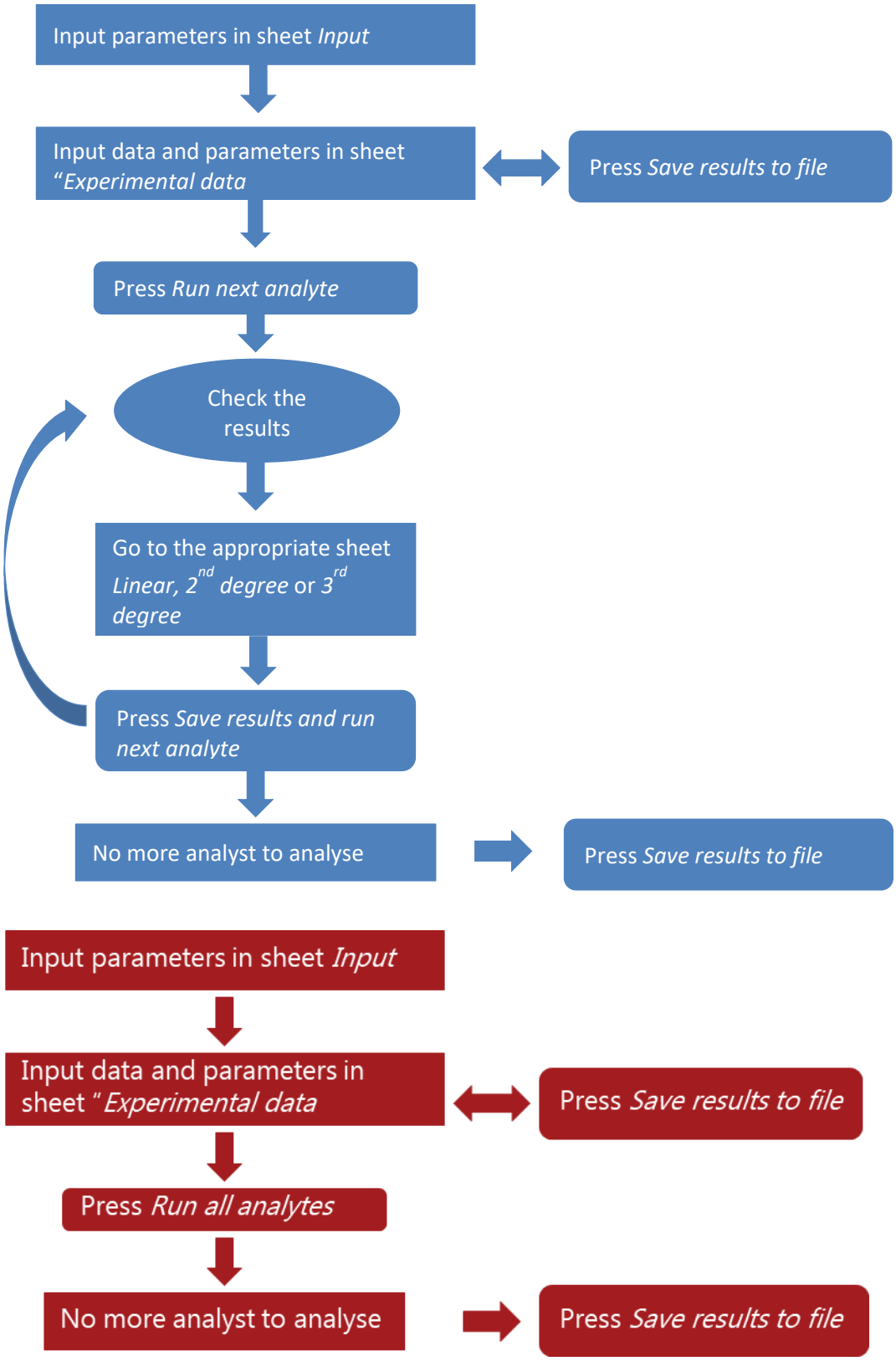
$V_{IS,bl}$: The volume of internal standard added to the blank samples, ml

The input in sheet *Experimental data* is $Yo_{,bl}$ and the ratio $V_{IS,bl}/m_{sa}$.

If no internal standard is used:

In this case the X-values in the calibration is concentrations, and the results Xo are concentrations. The parameters $V_{IS,sa}/m_{sa}$ and $V_{IS,bl}/m_{sa}$ should both be replaced by V_{final}/m_{sa} and the IS_{factor} set to 1. Here V_{final} is the total volume of the final extract (not the volume in the vial. If an extract of 10 ml were evaporated to 1.0 ml and 0.1 ml were put in the vial, V_{final} is 1.0 ml)).

3) Workflow for semi-automatic runs or automatic runs



If you want to save the whole workbook, you do not need to press *Save results to file*

You can press *Save data to file* anytime to save the actual results. See next page for detailed instructions.

Go to sheet *Input* and set statistical parameters for calibration and output

a. Table 1: Set parameters for:
    i. Low limit: Indication for results below LOQ or below calibration standards
    ii. Output of uncertainty (none, s, RSD % or confidence interval CI)
    iii. Values below detection limit (none, below DL(s), below DL(calibration))
    iv. No of digits in addition to significant digits
    v. Significance levels for detection limit etc. (it is recommended to use the default values)
b. Table 5: Set parameters for weighted regression
c. Table 6: Run # is the index for the next output. Start with Run # = 1
d. Table 2-4 are filled automatically from sheet *Experimental data*

Go to sheet *Experimental data* and input experimental value for

a. Calibration: X = concentrations or ratios and Y = responses
b. Results for repeated measurements for determination of detection limits (if any)
c. Unknown samples, names and responses Yo
d. Volume to mass ratios for samples and blanks: Volume(IS)/mass of sample
e. Internal standard factor, see section 2) Quantitation and internal standards
f. Parameters for calibration: force weighted, force degree or automatic
g. Execution key: 1: to be analysed, 0: to be skipped

Press *Save results to file* and a file dialog opens. Enter the name and path for a file to save the results.

Start execution by pressing "Run next analyte" Results will be analysed and displayed

a. Check that the results look ok, and go to the sheet *Linear, 2nd degree* or *3rd degree*
b. Calibration through zero can now be performed manually (not recommended)
c. If results looks ok*, press "Save results and analyse next analyte".
a. Otherwise reanalyse data

Automatic run of all analytes:

It is possible to run all analytes automatically by pressing *Run all analytes*. In this case, all results are saved in sheet "Output" by the macros. Only data for the last analyte can be seen in the other sheets. Please be extremely careful when this function is used, it is fast, but results may not be the best possible.

Reanalyse of data:

1. Change the *Execution key* for the analyte to 1 in sheet *Experimental data* and press *Run next analyte*
2. In *Test of calibration function* find the best calibration function as explained here
    a. When you found the best calibration function, set the parameter for weighted regression in cell C26 (1 or 0) and go to the appropriate sheet and proceed by either:
    b. Pressing "Save results and analyse next analyte" to continue analysis - or
    c. Pressing "Save results". You can now do what you like, e.g. analyse data with another function and save a new set of results to sheet *Output*

*For 3rd degree polynomial regression be extremely careful to check the concentrations Xo of the unknown samples, because there are 3 solutions to the equations, which is solved by iterations in Excel *Solver*. If results look wrong, try to enter new reasonable start values for Xo in the cells B19:B118 and run “Update Xo” again.

5) Sheet: Input

Here you enter most parameters for the calibration and the detection limits.

Table1: Enter parameters common for all analytes (see below for an explanation of detection limits)

It is strongly recommended to use the default values for these statistical parameters in Table 1, unless you know what you are doing!

α(DL) %: The risk in % for a false positive, i.e. to detect a compound even if it is not present (1 %)

β(LOD) %: The risk in % for a false negative, i.e. to not detect a compound that is present (5 %)

s(LOQ)%: The smallest standard deviation we want for quantification (10 %)

CI(LOQ)%: The smallest confidence interval we want for quantification (20 %)

α(LOQ) %: Significance level for LOQ (5 %)

α(Blank) %: Significance level for *Blank limit* (10 %)

m: Number of repeat measurements for unknown concentrations if it is different from the number of measurements used in the calibration, else *m* = 1 (see section 8).

r: Number of repeat measurements in calibration (see section 8)

CI %: The confidence interval for the uncertainty for unknown concentrations

These parameters may be changed according to what kind of output you need:

Low limit: Code for color marking of concentrations below:

- 0: No color marking
- 1: LOQ from standard deviation
- 2: LOQ from confidence interval
- 3: The lowest standard concentration

Unc. Key: Code for what kind of uncertainty should be copied to the sheet Output

- 0: No uncertainty given
- 1: Standard deviation *s* (same unit as concentration)
- 2: Relative standard deviation in %, *RSD %*
- 3: Confidence interval *CI* (same unit as concentration)

DL limit: Code for Detection Limit:

- 0: No notification about detected/not detected – all concentration printed
- 1: Concentrations below *DL*(s) are shown as *<99*, where *99* is the *DL*-concentration
  - If there is no *DL(s)*, *DL(calibration)* will be used as detection limit
- 2: Concentrations below *DL*(calibration) are shown as *<DL*.

Digits: Digits in addition to significant digits. The output of concentrations in sheet *Output* are rounded so only statistical significant digits are shown. It is recommended to use 0 here.

OBS: Raw concentration with all digits are always printed in a separate table.

Table 2: Parameters for the analyte (filled automatically in automatic runs)

Analyte: The name of the analyte

n(DL): The number of repeat measurements for the Detection Limit (could be 0 for no data)

s(DL): Standard deviation (concentration units) for repeat measurements for calculation of the Detection Limit.
n(Blank): Number of observations for a blank
Mean(Bl): Mean of responses for the blanks
s(Blank): Standard deviation for the blanks

Table 3: Calibration measurements for the analyte (filled automatically in automatic runs)
X: Concentrations (in **ascending order!** followed by blank cells)
Y: Either 1 to 4 individual measurements in Y1-Y4.
Or
Mean *Y* in *Y1* and standard deviation of *Y* in *s(Y)obs.*

If you have *statistically independent* measurements for each concentration, i.e. if 3 independent solutions were prepared and put into 3 different vials, the 3 measurements are independent and should be entered in 3 lines with the same X-value and the response in Y1. See section 8)

Table 4: Unknown samples: (filled automatically in automatic runs)
Sample: Name of sample
Yo: Response (One measurement - or mean of measurements)

Table 5: Parameters for weighted regression (**Must be controlled or changed also for automatic runs)**
Selection of weights is quite tricky, and they will in most cases be set somewhat arbitrarily. The weights are a function of the uncertainty *s(Y)* of the responses, which in general will have the following contributions:

s0: Constant standard deviation, independent of concentration.
s½: Standard deviation proportional to the square root of the concentration.
s1: Standard deviation proportional to the concentration.

The cells in the table are:

- C(i): Coefficients in the expression for *s(Y)* vs *X* calculated from the manual values
- Estimated: Calculated from repeat measurements. In most cases not used (0, 0, 0)
- Manual s0: The constant uncertainty (*s,* standard deviation) at the lowest concentration in the calibration
- Manual s½: The uncertainty *s* proportional to Sqrt(*X*) at the lowest concentration
- Manual s1: The uncertainty *s* proportional to *X* at the lowest concentration
- s0, s½ and s1 must be positive or zero

The reason why the manual input is expressed at the lowest concentration in the calibration is that it is the uncertainty at the lowest response, which is important for the detection limits for weighted regression.

Simple selection of weights
If you do not have experimental data for the uncertainty as function of concentration, it is suggested to use the default values **(s0, s½, s1) = (1, 1, 0).** This corresponds to a weight of 1/X where X is analyte

concentration, but with a small constant contribution to the uncertainty. These values do often give quite good results for the calibration functions and the detection limits.

Importance of weights

The parameters have a high influence on the detection limits (DL and LOD) determined from the weighted regression. If you do not have repeat measurements at a low concentration for determination of DL and LOD, it is extremely important to examine whether or not DL and LOD looks reasonable. So it may be necessary to iterate by setting parameter in Table 5, looking at DL and LOD for the selected calibration function, go back and change parameters in Table 5 and repeat this until it looks correct.

OBS: Weights are normalized in the calculations, so it is only the relative magnitude of *s0 ,s½, s1*, that is important; the absolute value is not important.

Estimation of weights from experiments

If you have determined the standard derivation of the response as function of concentration (from repeated independent measurements for each concentrations) the spreadsheet will fit a curve for *s(Y)obs* vs *X* and give the results in the *Estimated* cells in Table 5. Here you can just enter these values in the manual cells in the table. Never use a negative or zero s0 (a warning will be given).

But quite often this curve is not very good, and there is a great spread of the points, because experimental determination of s(Y) is *often quite inaccurate (and time consuming).* So it may be a good idea to try different values for the parameters and look at the result, the green curve in the graph, as well as DL and LOD as explained above.

Weights explained

Least square regression do minimize the Chi-square sum:

$$\chi^2 = \sum_{i=1}^{n} \left[\frac{y_i - \hat{y}_i}{\sigma_i} \right]^2$$

Assume that the standard deviation for each concentration has 3 contributions:

Constant: $\sigma_i(const) = C_0$

Proportional to the square root of the concentration: $\sigma_i(sqrt) = C_{½} \cdot \sqrt{X_i}$

Proportional to concentration: $\sigma_i(prop) = C_{½} \cdot X_i$

Then: $\sigma_i^2 = C_0^2 + C_{½}^2 X_i + C_1^2 X_i^2$

The weights are scaled, so that the sum of all weights is equal to the number of points, *n:*

Weights: $w_i = \frac{\sigma_i^{-2}}{\sum \sigma_i^{-2} / n}$

Please note that the residual standard deviation of regression, *s(y/x)*, is calculated from the deviations from the calculated calibration line, and is *NOT* directly related to the standard deviations used to calculate the weights.

6) Save results to file

In sheet “Experimental data” press the button *Save results to file.*

- This opens a file dialogue where it is possible to enter a filename and choose a folder.
  - Default filename: Workbook-name_Results_YYMMDD_HHMM.xlsx
  - Default folder: The same folder as the calibration workbook
  - Files cannot be overwritten, use a new name
  - The filename including the path will be written in cell G28 in sheet “Input”

Next a checkbox opens where the sheets to include in the file can be selected. Choose from:

- Experimental data
- Plot
- Output
- Conc RAW
- Conc Rounded
- Standard deviations

- Finally the selected sheets are copied to the save-file and the file is saved

The user can now continue to use the calibration workbook.

To generate a new file for output:

- Press *Save results to file* and data will be copied, and the file saved.
- Delete the filename in cell G28 in sheet “Input”.
- Press *Save results to file* in sheet “Experimental data”, and generate a new file

When the calibration workbook is closed, data will be copied to the save file and it will be saved. If no save file is present (blank G28 in sheet “Input”), the user will be asked to save a copy of the whole workbook.

7) Save the workbook

The Calibration workbook itself cannot be saved.

- First time you try to save the workbook, at COPY is saved with a new name.
- Default name of the copy: Workbook-name_YYMMDD_HHMM.xlsm
- You can change the filename of the copy
- Subsequent Saves will save the workbook in the copy file

8) Sheet: Experimental data

Enter or Copy-Paste experimental data for all analytes here:

Table 1: Concentrations X of standards for the standard curve + data for detection limits
Table 2: Responses Y for the standard curve
Table 3: Names and responses Yo for the unknown samples
Table 0: Keys for automatic runs:
Weighted: 1: Use weighted regression, 0: Use ordinary regression: Blank: Use the statistically best method
Degree: 1, 2 or 3: Maximum degree for polynomial regression. Blank: Use the statistically best degree up to 3rd degree*.
Execution key: Determines which analytes to analyse. The macros searches the execution keys from left to right:

- 0: Analyt should not be analysed
- 1: Analyt should be analyses
- After analysis the macro changes 1 to 2, i.e. 2 this means that the analyt has been analysed. Change 2 to 1 in order to analyse the analyt again
-

Press the button Press *Save results to file* and a file dialog opens. Enter the name and path for a file to save the results.

Using macros activated by buttons it is possible to process all the analyst in 2 different ways:

1. Automatic one-by-one, button “Run next analyte”
2. Automatic all analyst, button “Run all analytes”

Automatic one-by-one: The next analyte is analyzed automatic. After the run, it is easy to check the calculations by looking in the relevant sheets with the data for the present analyte. Furthermore it is easy to repeat the calculation with new parameters if needed.

Automatic all analyst: All analytes are processed until the end of the list, and it is only possible to see the intermediate results for the last analyte.

It is recommended to use **automatic one-by-one** for all analytes. **Automatic all analytes** should only be used for batch runs when no check of intermediate results is needed.

For Automatic runs the workbook can find the statistically best calibration function, but be careful and check the results, the automatic selection may not be good under all conditions.

A manual for automatic runs is given in sheet “Experimental data”

*For analysis *one-by-one* this is only suggested limits. The user can use all degrees 1 to 3 after looking at the statistics. For batch runs of all analytes, the automatic optimization will be limited to the degree selected here. 3rd degree may be to high even if statistics allows it, especially for calibration standards with increasing intervals like 1, 2, 5, 10, 20, 50, 100….10000.

9) Sheet: Test of calibration function

Selection of polynomial degree for regression
A higher degree will always give a better fit to the points – but if the degree is too high, the curve will be fitted to the noise, and not to the proper relation.

Statistically this is very easy to test, and the results are given in the green and blue table for unweighted and weighted regression respectively under the heading *Polynomial fit* cells G12:H19. The statistically best calibration function is denoted by an *Ok.*

Below the table with the statistics, there is a box with the recommended calibration function. This is the calibration function which is selected by the automatic optimization (may be limited to degree 1 or 2 according to the input for the parameter *Degree?*). The user may follow the suggestions or use another function after looking at the statistics and the plots in this sheet.

Selection of weighted or unweighted regression
The purpose of the calibration is to be able to determine concentrations of unknown samples in the whole range of concentrations of the calibration. This spreadsheet uses the accuracy of the calibration to compare weighted and unweighted regression. The accuracy is calculated by comparing the known concentrations *X* to the concentrations *Xo* predicted from the experimental *Y*-values in the calibration function. If the calibration function cannot estimate the calibration concentrations correctly, it may neither be able to predict unknown concentrations correctly. The error is calculated by an approximation and may be slightly off for 2nd and 3rd degree polynomial regression.

So look at the *Error of X %* in the plots and the table below the calibration function plots – or look again in the green and blue table, where the average and max error is given in cell J12:K19. Choose the calibration function that gives the distribution of error in *X* that best fits the purpose of your calibration. In general weighted regression is best at low concentrations, and simple regression is best at high concentrations.

You must also look at the plots of the calibration functions themselves (*Y vs X* for 1st, 2nd and 3rd degree polynomial regression), especially at the lowest 3 concentrations.

Leverage
Leverage shows how important the data points are for the calibration function. As an example, for ordinary linear regression, a point will affect the mean by a value 1/n, where n is the number of points in the calibration, and the effect on the slope will be proportional to $\left(X_i - \bar{X}\right)^2$, i.e. the points around the mean will have a low influence on the line – a low leverage – and the points at ends of the range will have a high leverage. Leverage can thus help to find the best calibration function and to optimize the weights in weighted regression. See the PowerPoint lecture *Intercepts_in_regression_2023* for examples.

$$Leverage = \frac{\partial \hat{Y}_i}{\partial Y_i}$$

With exponentially increasing concentrations of the calibration standards (or similar series like 1- 2 – 5 -10 -20 – 50 -100 – etc.) leverage shows that unweighted regression is very bad and that weighting 1/X will in

general give very good results. Use **(s0, s½, s1) = (1, 1, 0)** in Table 5 in sheet *Input* in order to get good results for detection limits too.

Final selection of the best regression method

Remember that the statistics are only a guide to the best calibration function. In some cases, the statistics may be misleading, e.g. a 3rd degree calibration function may be ok, even if the statistics say "Modelling noise", in the case where the instrument actually has a true 3rd degree relation between response and concentration**. So the final decision should always be based mainly on an evaluation of the plots!**

- Enter 1 or 0 in cell C26 to choose unweighted or weighted regression
- Go to the sheet for the selected degree of regression (*Linear, 2nd degree* or *3rd degree*)

10) Sheets: Linear, 2nd degree and 3rd degree

Use the sheet that corresponds to the polynomial degree that you found to be best.

Sheet *Linear, 2nd degree* or *3rd degree*:

a. Check that detection limits look correct. It may be necessary to update the calculations. If you want to change the level of significance and other parameters in non-white cells, you must go back to sheet *Input* and make the corrections here. For *3rd degree* you also need to update the calculations of Xo.
b. Check that the plots look ok. There are 4 plots:
    i. Calibration function with prediction intervals
    ii. Calibration function at low concentrations with indication of the detection limit DL
    iii. Plot of unknown concentrations Xo including confidence intervals
    iv. Plot of accuracy, % error in X
c. Check that the calculations look correct. If input or weights or other parameters need to be corrected, go back to the first sheets and do the corrections here.
d. Click *Save results and analyse next analyte* (automatic runs) or *Save results* (manual runs), and the most important data will be copied to the sheet *Output*. You may add 1 or 2 lines of comments to the results before clicking.

There are a lot of results and plots in the sheets, but hopefully most of them should explain themselves, or be explained in the sheet. A few comments may be helpful:

Calculation of unknown concentrations

For calculation of confidence intervals and standard deviation for *Xo* check that the parameters in cells C16 and D16 are correct (or go to sheet *Input* and correct them). You can enter more *Yo*-values manually directly in the table to calculate new concentrations (but they will not be saved in the sheet *Output).*

For 3rd degree polynomial you must run a macro by pressing the *Update Xo* button whenever *Please update estimates* are shown in cell A17. Be extremely careful to check the concentrations Xo of the unknown samples, because there are 3 solutions to the equations, which is solved by iterations in Excel *Solver*. If results look wrong, try to enter new reasonable start values for Xo in the cells B19:B118 and run "Update Xo" again. The same problems can occur for LOQ , DL and LOD, i.e. check result carefully and give good new starting values if results looks odd.

Prediction intervals

The plot and the table starting in N17 shows the *Prediction intervals* (*PI*) for the calibration, i.e. the predicted confidence interval for an unknown response *Yo* (mean of *m* measurements) predicted from *Xo*.

Detection Limit (*DL*) and Limit of Detection (*LOD*)

- *DL* is the lowest concentration, which is statistically significant different from zero. A result lower than this can be printed as *<99* where *99* is the numeric value of *DL*.
- *LOD* is the lowest concentration which will give a result higher than *DL* in 100-β % of the cases. For 5% in cell J24, this concentration will be detected (i.e. > *DL*) in 95% of the cases. A concentration between *DL* and *LOD* should be treated as detected in all respects.

- *DL* and *LOD* can be calculated from the calibration function, *DL(calibration)*, or from repeat measurements at a low concentration *DL(s)*. The last method is the most correct, but the first method *may be ok*, if the calibration is correct and the weights in weighted regression are good.

DL and *LOD* are defined from the response *Y* according to IUPAC and calculated according to Funk et al. 2007[1]

$$DL_Y = t_{\alpha,d.f.,1} \cdot s + Y_{blank}$$
$$LOD_Y = \left(t_{\alpha,d.f.,1} + t_{\beta,d.f.,1}\right) \cdot s + Y_{blank}$$

Y_{blank} is the response for a blank sample. In the spreadsheet it is assumed, that Y_{blank} is identical to the *intercept* of the calibration function, thus the standard deviation of the intercept should also be included in the standard derivation *s*. To express the detection limits in concentration units, *DL* and LOD are calculated by:

$$DL \text{ or } LOD = \frac{Y_{Limit} - Intercept}{Slope}$$

So the final result is:

$$DL = \frac{t_{\alpha,d.f.,1} s}{Slope} \sqrt{\frac{1}{m} + \frac{1}{N} + \frac{\bar{x}^2}{\sum\left(x - \bar{x}\right)^2}} \text{ and } LOD = \frac{\left(t_{\alpha,d.f.,1} + t_{\beta,d.f.,1}\right) \cdot t_{\alpha,d.f.,1} s}{Slope} \sqrt{\frac{1}{m} + \frac{1}{N} + \frac{\bar{x}^2}{\sum\left(x - \bar{x}\right)^2}}$$

α is the significance level, the risk a false positive, i.e. of obtaining a result > *DL* for a blank sample.
β is the risk of obtaining a false negative, i.e. a result < *DL* for a sample with a concentration of *LOD*.

The significance level α should be less than half the significance level for the confidence interval for unknown concentrations, i.e. if a 95 % confidence interval is used, α must be less than 2.5 %. Standard values for α and β are 1 % and 5 % respectively. Then - in most cases - detected concentration will have a confidence interval *not* including 0. In a few cases with weighted regression the confidence interval for very low detected concentrations may include 0 because the uncertainty at *DL* is higher than at a concentration of 0 from which *DL* is estimated.

The standard deviation *s* in the formula is the standard deviation at a concentration close to *DL.* The best method to determine *s* is to do repeated measurements at a concentration < 5 x *DL.* Normally 7 or more measurements are preferred. If the concentration used was > 5 x *DL* a new set should be obtained at a

[1] W. Funk, V. Dammann and G. Donnevert, *Quality Assurance in Analytical Chemistry: Applications in Environmental, Food, and Materials Analysis, Biotechnology, and Medical Engineering,* Second Edition. Wiley-VCH, Weinheim, 2007. See page 29-35 for detection limits. The detection limit *DL* is called the *Decision limit* X_{DL} and the limit of detection *LOD* is called the *Minimum Detectable Value* X_{MDV} in Funk et al..

lower concentration. For repeated measurements enter the results in cell B24 and B25 in sheet *Input* for manual runs.

When *s* is determined from repeated measurements, the formulas for *DL* and *LOD* are slightly more complicated, because the number of degrees of freedom are different for the repeated measurements and for the intercept.

You can also estimate *s* and the detection limits from the calibration function, but the results may not be reliable, so be careful. Two types of errors may occur:

- Too high detection limits – mainly a problem using non-weighted regression. If the standard deviation increases with concentration, the standard deviation will be determined from the whole concentration range, which gives a much too high standard deviation resulting in an unrealistic high detection limit. Also deviations from linearity at higher concentrations will be converted into a too high standard deviation about the regression line. These problems are much less for weighted regression.
- Too low detection limits – mainly a problem for weighted regression. If you use too low a value for *s0* (relative to s½ and s1) in Table 5 in sheet *Input*, the standard deviation at a concentration of zero will be too low giving an unrealistic low detection limit. This is not an issue for non-weighted regression.

Still, with weighted regression and a reasonably good weighting function detection limits may be ok.

Follow the instructions in the sheet and look at the graph at the lowest concentrations, where *DL* and DL_Y estimated from the calibration function are indicated.

A plot of the estimated concentrations including the confidence interval for unknown samples is also given.

Limit of Quantitation (*LOQ*)

LOQ is the concentration, where the uncertainty is acceptable. What is acceptable is up to you or the application, and can be given as a relative standard deviation *s %* or as a confidence interval % (these values are entered in Table 1, sheet *Input*). The results are found by the Excel *Solver* tool when you press the button *Update LOQ*. Look at the instructions in the sheet before you press the button, because *Solver* may find local minima or may be unable to find a solution in some cases. If this happens, change the start values to more realistic settings and press update again.

Sometimes LOQ is outside the calibration range and the warning *Please update* will not disappear. The only solution to this problem is to increase the limits for LOQ in Table 1 in sheet *input.* If *Please update* is indicated, LOQ values in the sheet "Output" will be set to 0, i.e. no concentration will be below LOQ.

- When you are satisfied with the estimation of the unknown concentrations and detection limits, you can save the results to the sheet *Output,* by pressing *Save results and analyse next analyte* (automatic runs) or *Save results* (manual runs).

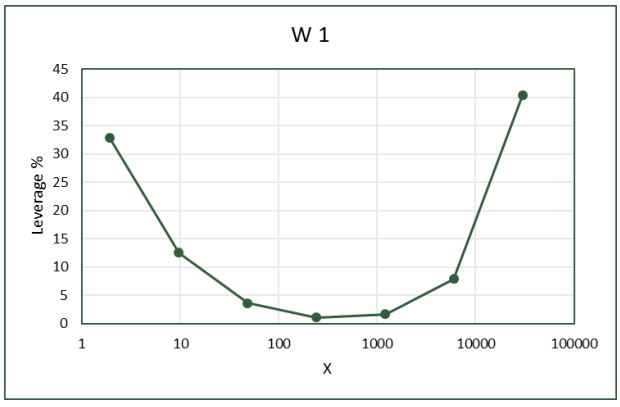
Calibration function through zero ?

If the intercept is not statistically significant different from zero, the intercept may be forced to zero, see the result of the test in cell K12:K19 (significance level 5 %). If the result is *Yes* you may use calibration forced through zero. This may be better for low concentrations. The normal procedure (Method A) is to set the intercept to zero and then find the slope by least square regression. But for weighted regression in case of exponentially increasing calibration concentrations (1-2-5-10-50-100… or similar) this may give weird results for the uncertainty and detection limits.

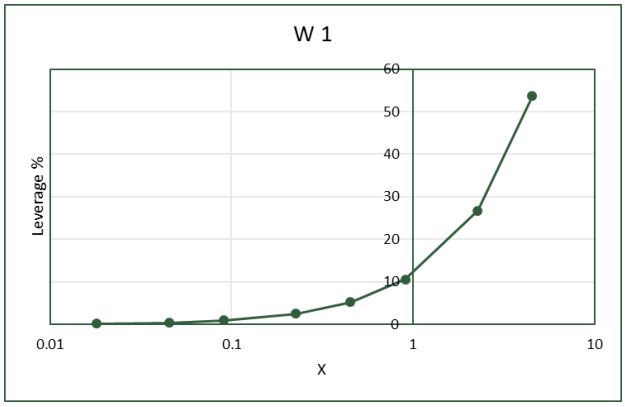
The method used here (Method B) for calibration through zero is to subtract the calculated intercept from all responses in the calibration. In this way the intercept will be zero and the slope, detection limits and uncertainties will be unchanged. Method B will affect high concentrations more than Method A, but since both methods in a way are wrong (to discard experimental results and replace them with something else) the argument for using Method B is as follows:

The reason for forcing the calibration through zero is in most cases to avoid negative concentrations for very low concentrations. The reason for negative concentrations must be a difference between the response Y in the calibration solutions and in the sample with matrix. And this effect may well have the same magnitude for high as for low concentrations. Actually a lower slope in the sample matrix could give negative concentration, but Method A will result in a higher slope for the calibration, i.e. the opposite effect, and the high concentrations will be more wrong in Method A than in Method B.

Method A is very close to be a one point calibration, actually it could be called a 1½ point calibration if calibration concentrations increases as 1-2-5-10-20… In this case the 2 highest concentrations have approximately 55 + 25 = 80 % of the leverage, i.e. the slope is independent of all the low concentrations. For Method B both low and high concentrations contribute to the slope.



~Leverage Method B



Leverage Method A

By Method B all calculated concentrations will increase by an amount dX = Intercept/Slope. For positive intercepts, the intercept could be explained by a contamination in the calibration, and Method B will correct this perfectly.

Anyway: Forcing the calibration through zero is not recommended.
For this reason you can only force the calibration through zero manually by pressing the button “Through zero” when you inspect the results in the sheets *Linear, 2nd degree* or *3rd degree*. Force zero is not destructive, it can be undone very easily be pressing the button now named “Calculate intercept”.

When results are saved to sheet “Output” the text “ZERO” is added to the calibration function name when the intercept was set to zero.

11) Sheet: Output

The spreadsheets can save results in the sheet *Output*. The results from one run will be given in one column containing:

Headings	Analyte name and comments
Statistics	Calibration function, statistics, input for weights and detection limits
X	Concentrations for the calibration
Y	Responses for the calibration
Yo	Responses for unknown samples
Xo	Calculated concentrations for the unknown samples
Uncertainty Xo	Uncertainty of Xo (could be *none, sXo*, *RSD %* or *±CI*)
Xo raw	Unformatted concentrations, i.e. all calculated digits also for Xo below DL

Format of concentrations Xo:

- Concentrations are given with significant digits, i.e. the last digit is the first digit which is uncertain. As an example for *Xo* = 12345 *sXo* = 23.3 the output will be Xo = 12350 and sXo = 23. More digits can be added with the parameter *Digits* in sheet *input.* For a nice looking table you may want to reformat the numbers.
- Concentrations below *DL* will be given as <99 where 99 is the actual detection limit
- Negative concentrations are rounded to zero (which will be shown as <DL)
- *na* means *Not Applicable* and will be used for parameters, concentrations and uncertainties with errors.

Color codes for concentrations Xo:

Red:	Not detected
Magenta:	Below blank limit
Grey:	Below low limit (LOQ or lowest concentration of the standards)
Black:	Ok
Blue:	Higher than the highest concentration of the standards.
Green:	Concentrations with errors, *na.*

To generate the output:

- Enter the keys for the output of uncertainties and detection limits in Table 1, sheet *Input*
- In sheet *Linear, 2nd degree* or *3rd degree,* check that the results look ok, write a comment in cells K5:K6 (optional) and press the button *Save Results and run next analyte* (automatic runs) or *Save Results* (manual runs).
- In sheet *Output* you can also write any headings or comments in free format in rows 2-12.
- The output is numbered 1, 2, 3…. If you want to set the counter manually, enter the Run # in Table 6 in sheet *Input.* This number is the number of the *next* output. Always start the first run with Run # = 1.
- Set Run # =1 in Table 6 to start a new series – old output in sheet *Output* will be deleted from Run 1 until a column with blank values are found.

OBS: "Save results" do only save results in sheet *Output.* Remember to **Save the result to file** in sheet "Experimental data".

12) Sheets: Conc RAW, Conc Rounded and Standard deviation

All concentrations for unknown samples are saved in sheet “Output” and in these 3 sheets with a simplified layout. All 3 sheets include headings with the sample names (rows) and the analyte names (columns) and contains the calculated concentrations with color codes in 3 different layouts:

Sheet: Conc RAW:

Raw concentrations Xo and Xm with 4 digits.

Sheet: Conc Rounded:

Concentration Xo and Xm rounded to significant number of digits and with values below detection limits given as <DL.

Sheet: Standard deviation;

Standard deviations of Xo and Xm with 2 significant digits.

If you don’t like the color codes, select all numbers and set the font color to *automatic* or *black.*

13) Notes on repeat measurements

If you have more than one measurement at each concentration, say 3 determinations at 6 concentrations, you should decide to set *r* in table 2 equal to 1 or 3. This depends on how the replicates are produced:

1) If they are 3 injections from the same vial for the 6 concentrations, the 3 measurements are not statistically independent, because they are from the same solution. Use the average for Y in sheet *Experimental data*. For manual runs, in sheet *Input* enter the average for Y1 or enter Y1, Y2…Y4 in Table 3 and set *r* = 1.

2) If the 3 measurements for each concentration are independent, i.e. if 3 independent solutions were prepared and put into 3 different vials, the 3 measurements are independent. Enter the mean and set *r* = 3, or enter each measurement on a single line with the same X-value for the repetitions and set *r* = 1 (both automatic and manual runs).

The difference between 1) and 2) is the error structure and in the statistics, because the number of degrees of freedom (for 3 determinations at 6 concentrations) are different: 1*6 – *k* for case 1) and 3*6 - *k* for case 2), where *k* is the number of parameters in the calibration function.

Almost all experimental comparison of random uncertainty and systematic error show that the systematic derivations are (much) bigger than the random variations, i.e. it is quite likely that the error in X is bigger than the error in Y, so a major source of variation in calibrations is the concentrations of the standards and not the measurements of the response. Therefore there is only ~6-*k* degrees of freedoms for the errors in X in case 1).

In both cases, we should actually not use regression of Y on X, but regression on X on Y or better orthogonal regression…!

In both cases you should not use serial dilution (making the lower concentration standards by direct dilution from the higher concentration standards), and all the concentrations and replicates *should be run in random order*.

14) Troubleshooting

If you get an error in the macros, the Visual Basic Editor opens with an error massage:

First you must reset the error:

Click *End* in the error message window - or
Click *Debug* in the error message window
With *Debug* you can see the line in the macro with the error. Click *Reset* (the blue square in the toolbar above the code in the VBA Editor) or *Run -> Reset.*

- Compile Error: *Sub or Function not defined*.
- Run-time error '*1004*'

This error occur when a macro is called, and the Add-In *Analysis ToolPack - VBA* or the Add-In *Solver* is not activated.

To fix it:
Go to the spreadsheet and click *File -> Options -> Add-ins*
Now a window opens. Go to the bottom and click *Go…* next to *Manage: Excel Add-ins*
In the Add-ins window be sure to check the boxes next to the following Add-ins:

- Analysis ToolPack
- Analysis ToolPack – VBA
- Solver Add-in

If you get an error again:

Click *Ok* or *Debug*
Click *Reset* (the blue square in the toolbar above the code)
Click *Tools -> Reference* and a new window opens
Check the box next to *Solver*
Check the box next to *atpvbaen.xls*
Click *Ok*, go to the spreadsheet and try to click the button causing the error again

Sheets do not react properly on changes:
Sometimes the sheets go into *manual calculation mode* by an unknown error. This means that the sheets are not recalculated when cells are changed. To fix this go to the menu *Formulas*, click the down arrow for *Calculation Options*, and click on *Automatic*. In manual mode the sheets will recalculate each time F9 are pressed.