Calibration

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- Introduction
- Basics of Calibration
- Limits of detection, quantification
- Standard Addition Method

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What is Calibration?

- Calibration is the process of establishing how the response of a measurement process varies with respect to the parameter being measured
- The usual way to perform calibration is to subject known amounts of the parameter (e.g. using a measurement standard or reference material) to the measurement process and monitor the measurement response

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What is Calibration?..

- Calibration is an important process in
  - Establishing the link between a signal of the measuring instrument and the associated quantity (e.g. concentration) of the measurand
  - Establishing traceability
  - Method validation to get the performance characteristics

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Two Major Aims

- Establishing a mathematical function which describes the dependency of the system's parameter (e.g. concentration) on the measured value
- Gaining statistical information and characteristics of the analytical system, e.g. sensitivity, precision

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Calibration Concepts

- External standard

  Measurement of separate samples containing known amount of analyte compared to the signal from both the calibration sample and unknown sample.

- Internal standard

  Addition of known amount of substance which has properties similar to the analyte.

- Standard addition

  Known amount of analyte is added to the sample and from the increase in signal the original content is extrapolated.

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Goals of Calibration

- "Ability to calculate a (measurement) result in a secure (safe) working range"

(Funk, W., Dammann, V., and Donnevert, G.: "Quality Assurance in Analytical Chemistry")

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First Steps to the Goal

- Establishing the calibration function
  - Choosing the preliminary working range
  - Measuring several calibration standards
  - Linear regression
  - Test of non linear regression
  - Test of variance homogeneity
  - Calculate performance characteristics
  - Fix the working range

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In Routine

- Calculating the (measurement) results
  - Conversion of the calibration function into an analytical function
  - Reporting the measurement results

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Basics of Calibration

- Mathematical Functions
  - Simple linear function without intercept
    $y = m\,x$
  - Linear function
    (intercept a, slope b)
    $y = a + bx$
  - Quadratic function
    (intercept a, slope 2cx+b)
    $y = a + bx + cx^2$

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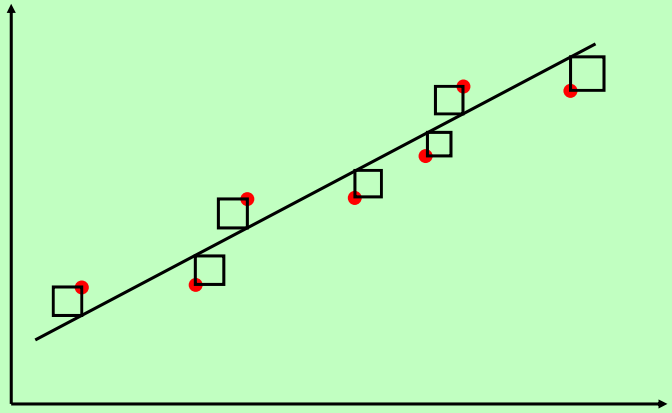
Principle of Linear Regression



- Minimizing the sum of the squares of the residuals

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International Standards

- ISO 8466 Water quality– Calibration and evaluation of analytical methods and estimation of performance characteristics
  - Part 1: Statistical evaluation of the linear calibration function
  - Part 2: Calibration strategy for non-linear second order calibration functions

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Analytical Function / Calibration Function

- With calibration standards we get a measurement result y for each content x of the analyte in the standard
  With that we get (by regression analysis) the **calibration function**

$$y = f(x)$$

- If we invert the function we can calculate the content of analyte in the unknown sample from the result of the measurement.
  This function is the **analytical function**

$$\hat{x} = f(\hat{y})$$

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Basic Calibration

- With the Basic Calibration only the measurement step itself is calibrated
- I.e. no sample preparation like extraction, digestion etc. is done
- We simply analyse standards in pure solvent

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Choosing the Preliminary Working Range

- Take into consideration
  - The practical application of the analysis (the purpose)
  - The possibilities that are technically feasible
    - Measurement results at the lower application limit must be significantly different from blanks
    - The requested analytical precision (or measurement uncertainty) has to be achieved over the whole working range
    - If a linear regression procedure has to be applied the variances have to be homogeneous over the whole range and linearity has to be assured

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Preparation of the Standard Samples

- Requirements:
  - Purity, matrix free or defined matrix
  - Homogeneity
  - Representative for real samples
    - Species similar in chemistry
    - Same oxidation state
    - etc.
  - Stability, preserveability
  - No influence on the sample from the container and external conditions

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Preparation of the Standard Samples

- Production of standard samples
  - Consider precision of balance and volumetric equipment
  - Weighings are always more precise and therefore to be preferred
  - Avoid successive dilutions
  - Prepare 6...10 standard samples, equidistant over the whole working range

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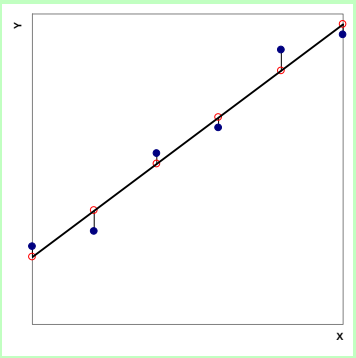
Linear Calibration



- Regression analysis delivers the calibration function
  y = a + bx
- slope (sensitivity)

$$b = \frac{\sum[(x_i - \bar{x}) \cdot (y_i - \bar{y})]}{\sum(x_i - \bar{x})^2}$$

- Intercept

$$a = \bar{y} - b\bar{x}$$

- Residual standard deviation (vertical scattering of the results around the regression line)

$$s_y = \sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{N-2}} \quad \text{with } \hat{y}_i = a + bx_i$$

- Process standard deviation $s_{x0} = \frac{s_y}{b}$
- Process variation coefficient $V_{x0} = \frac{s_{x0}}{\bar{x}} \cdot 100\%$

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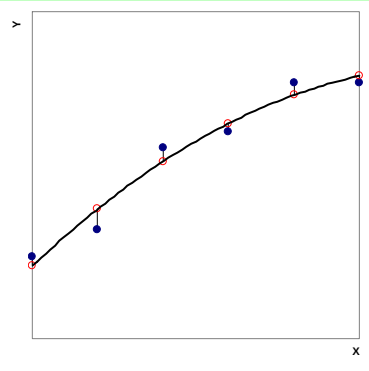
Second Order Calibration Function $y = a + bx + cx^2$

- Calculations are somewhat more difficult here
- For details see ISO 8466-2



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Second Order Calibration Function

- Residual standard deviation

  $$s_y = \sqrt{\frac{\sum (y_i - \hat{y}_i)^2}{N-3}} \quad \text{with} \quad \hat{y}_i = a + bx_i + cx_i^2$$

- Sensitivity
  - First order derivation of the calibration function $S(x) = b + 2c \cdot x$
  - In the middle of the working range $E(\bar{x}) = b + 2c \cdot \bar{x}$
- Process standard deviation

  $$s_{x0} = \frac{s_y}{E(\bar{x})}$$

- Process variation coefficient

  $$V_{x0} = \frac{s_{x0}}{\bar{x}} \cdot 100\%$$

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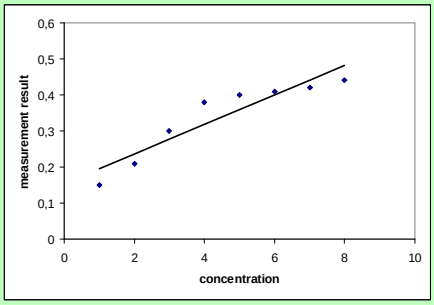
Linearity Check

- If possible, the linear calibration function should be used, only in special circumstances second order calibration should be used
- Visual linearity check
  - Graphical display incl. calibration line
  - If non-linearity is obvious you may abstain from a statistical linearity check



Linearity Check

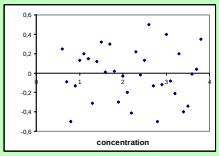
- Mandel test
  - Calculation of linear calibration function y=a+bx and the 2nd order calibration function $y=a+bx+cx^2$ including the respective residual standard deviations s_{y1} (linear) and s_{y2} (quadratic)
  - Calculation of the differences of variances DS^2:

    $DS^2 = (N-2)s_{y_1}^2 - (N-3)s_{y_2}^2$ with degree of freedom $f = 1$

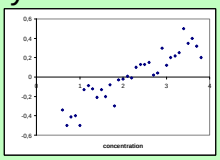
  - Check with F-test $F_{observed} = \frac{DS^2}{s_{y_2}^2}$
  - Comparison with the critical value from the table $F_{critical}$ for $f_1=1$, $f_2=N-3$, P=99%
  - If $F_{observed} < F_{critical}$, the 2nd order function does **not** give a significant better result
  - Linear calibration function should then be used

Residual Analysis

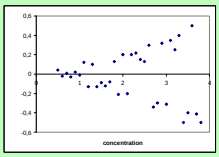
- Residuals are the vertical distances of the results from the regression line
- Residuals should be normally distributed



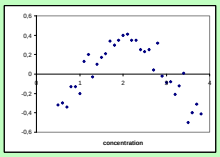
Normally distributed residuals
correct modelling approach



linear trend
wrong approach or calculation error



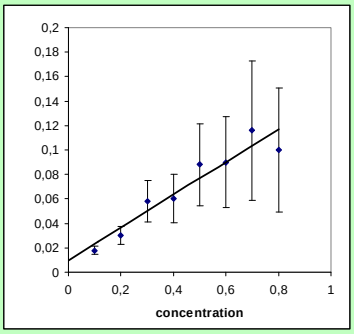
Increasing variances
inhomogeneity of variances



Non linear
wrong regression function

Homogeneity of Variances

- Linear regression assumes constant (homogeneous) imprecision (variance of the results) over the whole working range
- Inhomogeneous variances:



- Inhomogeneity of variances not only leads to increased imprecision, but also may affect bias due to a changed slope of the regression line

Homogeneity Check for Variances

- Measure the lowest and the highest standard ten times
- Calculate variances for both data sets

$$s_i^2 = \frac{\sum (y_{ij} - \bar{y}_i)^2}{n_i - 1}$$

- Check with F-test

$$F_{observed} = \frac{s_N^2}{s_1^2}$$

- If $F_{observed} > F_{critical}$, variances are not homogeneous
- Possible consequences:
  - Reduced working range
  - Weighted regression

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Outlier Test

- Calibration data always have to be free from outliers
- The outlier test assumes that the chosen regression approach is correct
- Potential outliers can be identified from the residual analysis
- First calculate the residual standard deviation $s_{y,A1}$ using all results; than eliminate the potential outlier and calculate again $s_{y,A2}$
- Check with F-test or t-test

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Outlier Test using the F-Test

- The residual standard deviations are checked for significant differences
- Calculate

$$F_{observed} = \frac{(N_{A1}-2)s_{y_{A1}}^2 - (N_{A2}-2)s_{y_{A2}}^2}{s_{y_{A2}}^2}$$

- And compare with the critical value from a statistical table for f_1=1, f_2=N_{A2}-2, P=95%
- If $F_{observed} < F_{critical}$, no outlier is identified

Outlier Test using the t-Test

- Calculate the prognostic interval of the regression line <u>without</u> the potential outlier

$$PI(\hat{y}_A) = \hat{y}_A \pm t \cdot s_{y_{A2}} \cdot \sqrt{1+\frac{1}{N_A}+\frac{(x_A-\bar{x})^2}{\sum x_i^2 - \frac{1}{N_A}(\sum x_i)^2}}$$

$$= a_2 + b_2 \cdot x_A \pm t \cdot s_{y_{A2}} \cdot \sqrt{1+\frac{1}{N_A}+\frac{(x_A-\bar{x})^2}{\sum x_i^2 - \frac{1}{N_A}(\sum x_i)^2}}$$

t = tabulated value from the t distribution ($P = 95\%$, $f = N_A$-2)
N_A = N-1
x_A = concentration of the potential outlier
$\bar{x}$ = mean of all x_i (without x_A)

- If the potential outlier is located within the prognostic interval, it‘s no outlier
- **If an outlier is identified statistically, the error source <u>has</u> to be found and eliminated. Than the <u>complete</u> calibration has to be repeated.**

Calibration Strategies in Routine Analysis

- The basic calibration as described up to here is part of the (re-)validation of an analytical method
- For routine use calibration strategies with less effort are used
- The effort made depends on the demands of the customer and the stability of the method

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Calibration Strategies in Routine Analysis

- Number of calibration points in routine
  - Where the calibration of the method is very stable a one-point calibration to verify a previous multi-point calibration may be sufficient
  - In other, less stable circumstances a 3- or 5-point calibration may be needed
- Frequency of calibration
  - Also depends on the stability of calibration
  - Some analytical methods need a daily calibration whereas other calibrations may last for months. At least check of the calibration is adviceable in any case

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Internal Standard

- Advisable for methods including a complex sample preparation procedure like extraction and clean-up
- Addition of a known amount of a substance different from the analyte and not present in the sample, but chemically behaving in the same way as the analyte
- Correction of the measurement result for the analyte with the recovery rate of the internal standard
- In mass spectrometry isotope marked analytes are often used for this purpose

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Limits of detection, quantification

Limit of Detection (LoD)
Limit of Quantification (LoQ)

- Are used to quantify detection and quantification capabilities (the lower end of the working range)
- Limit of detection (LoD)
  - The lowest concentration that can be detected with a certain level of confidence
- Limit of quantification (LoQ)
  - the minimum content that can be quantified with a certain confidence

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Specification of Measurement Process

- LoD and LoQ cannot be specified in the absence of a fully defined measurement process including interferences and type of sample matrix
- "Interference free detection limits" and "Instrument detection limits", for example, do not specify the measurement capabilities of a complex measurement process including sample preparation

(IUPAC Orange Book)

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Calculation of LoD

- There is no uniform way to calculate LoD values in the scientific community
- Two possibilities are described in the following slides (both taken from IUPAC Orange Book)

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LoD – Simple Approach

- The value of the LOD is given by

$$LoD = \bar{x}_{bl} + k \cdot s_{bl}$$

with

$\bar{x}_{bl}$ = mean of blanks

s_{bl} = standard deviation of blanks

k = numerical factor defining the confidence level

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LoD – Simple Approach

- The mean of measurements of blanks and their standard deviation must be found experimentally by making a sufficiently large number of measurements
- A value of 3 for k is strongly recommended
  - At low concentrations non-Gaussian distributions are more likely
  - The mean and standard deviations are only estimates of the population characteristics
  - So the 3 s_{bl} value usually corresponds to a confidence level of about 90%

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Limit of Quantification (LoQ)

- Quantification limits are performance characteristics that mark the ability of a analytical method to adequately "quantify" an analyte (IUPAC Orange book)

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LoQ

- The ability to quantify is generally expressed in terms of the signal or analyte (true) value that will produce estimates having a specified relative standard deviation (RSD), commonly 10 %

$$LoQ = k \cdot s_{LoQ}$$

with

s_{LoQ} = standard deviation at the LoQ concentration

k = multiplier (reciprocal = selected quantifying RSD)

- The IUPAC default value for k is 10
- If the variances are homogeneous $s_{LoQ} = s_{blank}$

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Standard Addition Method

- Standard addition is calibration in the real sample by stepwise addition of known amounts of the analyte

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When should the Standard Addition Method be Used?

- If the composition of the sample matrix has high influence on the accuracy of analysis
- If no matrix-adjusted calibration standards are available
- If only few samples have to be analysed

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Requirements

- Analytical results that are corrected for blank and background
- Linear relation between concentration x and signal y
- Homogeneity of variances
- Possibility to homogeneously divide samples into sub-samples
- Analyte can precisely be added to the sample

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Procedure

- Take n sample aliquots
- Add linear increasing amounts of the analyte to (n-1) sample aliquots in equidistant steps
- Apply linear least square regression to the pairs of values

  $\rightarrow\ y = a + b \cdot x$

- Extrapolite to the intersection with the x-axis
- This value delivers the sought content

  $\rightarrow\ x_A = -x_{(y=0)} = -a/b$

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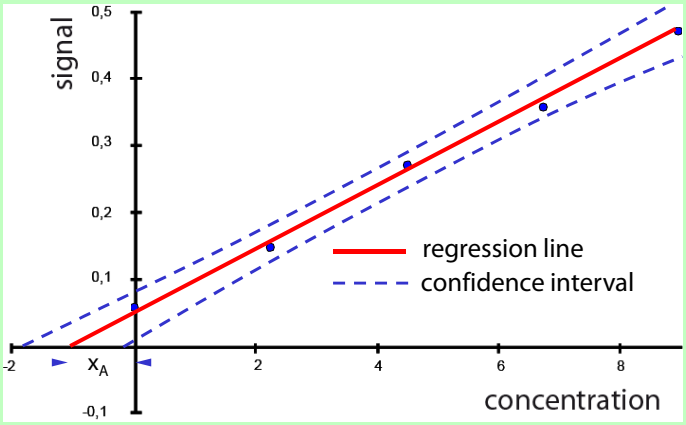
Graphical Presentation



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Uncertainty

- The uncertainty of the calculated value x_A may be quantified from the half-width of the confidence interval

$$\Delta x_A = \frac{t_{f,\alpha} \cdot s_{y,x}}{b} \cdot \sqrt{\frac{1}{n \cdot n_A} + \frac{\left[\bar{x}_z - (-x_A)\right]^2}{\sum \left(x_{z_i} - \bar{x}_z\right)^2}}$$

x_A = half - width of the confidence interval of x_A
$t_{f,\alpha}$ = two - tailed quantile of the t - distribution (probability α)
$\bar{x}_z$ = arithmetic mean of all x_{z_i}
$\sum \left(x_{z_i} - \bar{x}_z\right)^2$ = sum od squares of deviations of all x_{z_i}

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THANK YOU

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