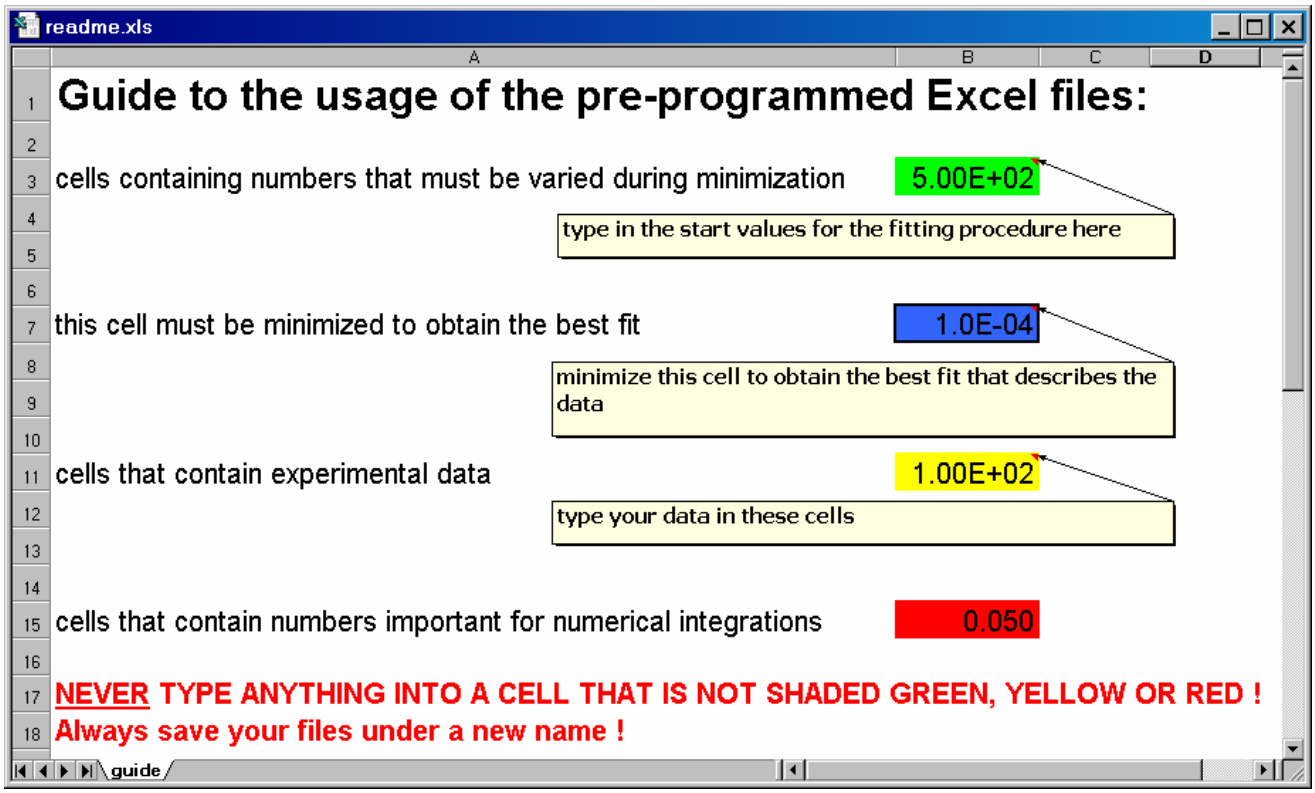
Practical exercises (text on CD only)

Contents:

9.4 Preface

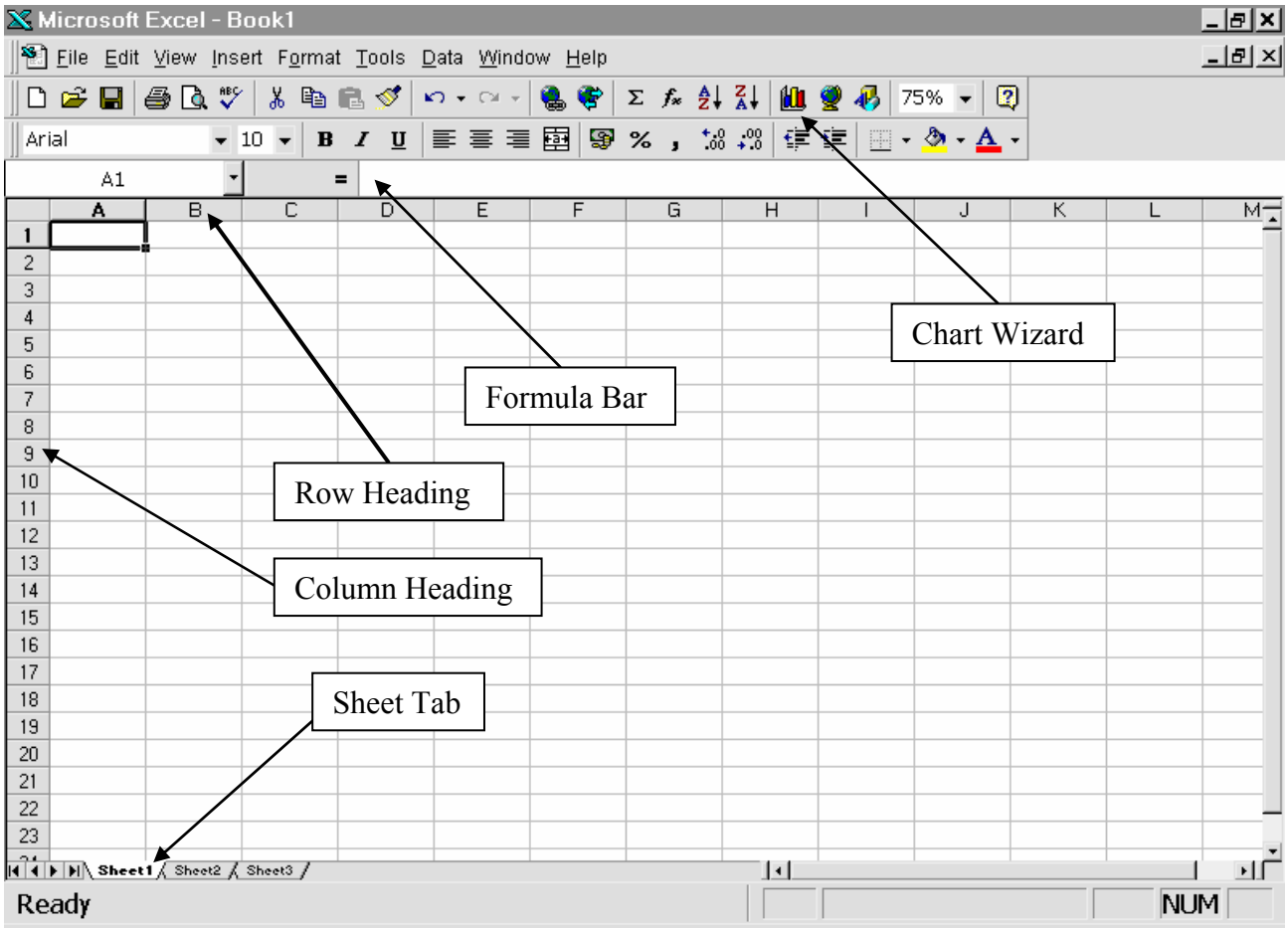
The data analysis exercises on this CD are based on the use of Excel spreadsheets. Functional sheets can be programmed in very many different forms, and it is useful to define certain rules or guidelines which will help the reader to use them more effectively and to locate potential errors. These guidelines are usually contained in the instructions for the exercises, and it is important that they should be followed carefully, even by users who are familiar with the specific data handling problem. The test data for the analyses are in the Excel file "Test Data.xls" on this CD. Pre-programmed data spreadsheets are in the folder "Solutions" stored under "Exercise XYZ.xls". These files can be used to track down errors in user programmed files, or directly to analyse experimental data.



9.5 Introduction to MS-Excel

Excel is a program that carries out mathematical operations in a spreadsheet format. When the program is opened, a spreadsheet appears which is composed of many cells organised in columns and rows; columns are denoted by letters A,B,C etc. and rows by numbers 1,2,3, etc. A cell is uniquely identified by specifying both, so E6 is the cell at the junction of column E and row 6. Cells can contain text, numbers or formulae. A cell containing a formula uses the values contained in other specified cells to calculate the value in its own cell. It should be noted that although the cell actually contains a formula, by default only the result is displayed. The use of formulae in this way enables mathematical operations to be carried out simultaneously on multiple data elements. If the number entered in a cell which is specified by a formula is altered, then the new number is automatically used in the Excel

calculation. The diagram below illustrates some of the important elements in an Excel spreadsheet (as shown here in the layout of Excel 97):



- To enter data into a cell move the cursor to the cell using the mouse or arrow keys, left-click to highlight, enter the number or formula and complete the operation by pressing the ENTER key.
- To enter complex functions, highlight the cell, then left-click with the mouse on the formula bar, and enter formulae or text, or alternatively edit the existing contents of the cell.
- Formulae can either be entered directly as text, or by selecting the desired cells using the mouse. For example, if one wished to insert the quantity =A4+1 into cell C4 (all formulae begin with the = sign), first click on C4, type "=", click on A4, type "+1" and press the ENTER key.
- "=B4+C4" written in A4 means that Excel will calculate the sum of the numbers in cells B4 and C4 and will place the result in cell A4. This operation is done easily: click on A4, type "=", click on B4, type "+" click on C4 and then press ENTER.
- Relative cell references: on normal entering of a formula, the cell references are defined by their relative position on the spreadsheet. For example, if the expression "=B4+1" is placed in cell A4, this means that Excel takes the value in the neighbouring cell on the right and adds one to it. If this

cell is copied to A5, the formula is changed by Excel to "B5+1", and similarly copying to B4 would change the formula to "C4+1".

- A fixed cell reference can be specified using the $ symbol, and in this case the cell reference is not altered on copying. For example the expression "=$A$4+5" would always take the number in cell A4 and add 5 to it. Fixed cell references can also be set up using <F4>, either by pressing the <F4> key or clicking on <F4> after entering the formula. Using a fixed cell reference for a number is easier as changing the number in the cell means that the whole spreadsheet can be changed immediately.
- Fixed and relative cell references can be combined, for example "=$A5+1" entered in A4 would be changed to "=$A6+1" on copying to B5. The <F4> function can be used to alternate between fixed and relative cell references.
- Regions of the spreadsheet can be specified as follows: "A2:C4" indicates a continuous region from A2 to C4. "A2:A6;C2:C6" indicates two separate regions from A2 to A6 and from C2 to C6. Regions can either be entered directly or highlighted using a mouse. Separated regions can be highlighted with a mouse by pressing the Ctrl key.
- In order to keep the structure of the spreadsheet clear, values of the independent variable (x) are always entered as the first column. Directly above these values there is a column label. The corresponding y values are placed in neighbouring columns, also labelled. All of the values in a particular row relate to the x value in the first column of that row.
- A file comprises (in general) several spreadsheets which can be accessed by clicking on the Sheet Tab.

Highlighting cells

Click the mouse on the corner of a region that is to be highlighted, hold down the left button and drag over the desired region. Cells can also be highlighted using SHIFT and $\leftarrow$, $\uparrow$, $\rightarrow$ or $\downarrow$. Separated areas are highlighted as follows: highlight the first area as described above, then press the Ctrl key and highlight further areas by left-clicking with the mouse.

Copying cells

To copy a formula from one cell to another cell in column A, highlight the formula and then press Ctrl-C ("C" stands for copy, and the formula is now in the clipboard). Press shift, and using$\downarrow$, $\leftarrow$, $\uparrow$ and $\rightarrow$ enlarge the highlighting over all the cells that should contain the formula. End the operation by pressing ENTER.

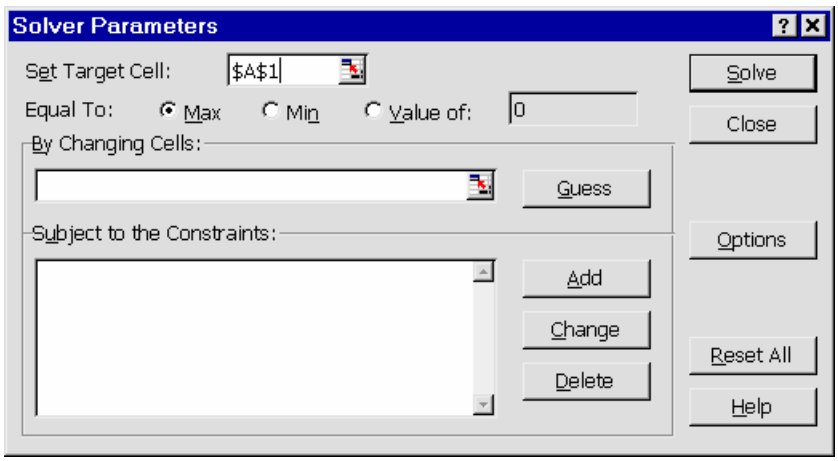
There are at least three ways of making entries in Excel: keyboard, combined keyboard and mouse, and mouse. The example just given illustrates how to copy a formula by keyboard operations. Alternatively, the contents of a cell can be copied to the clipboard (using Ctrl-C or Shift-Del, or using the mouse and the "Edit/Copy" command in the main menu), and then highlight the desired area and press ENTER. The same can be achieved by clicking on a cell, and then on the heavy corner in the cell frame, and then enlarge the highlighted area by left clicking and dragging the mouse. In what follows, only one mode of operation is described, chiefly using the keyboard.

Creating graphs and diagrams

- Graphs and diagrams can be created as follows: first the appropriate data cells are highlighted, preferably with the relevant title, and then click on the Chart Wizard and select the desired graphics, for example: XY (scatter) with unsmoothed lines**,** complete by selecting Finish.
- To remove the grid lines from the graph, click on a line and press Delete.
- To alter the grey background of the plot, double-click on the surface and from the resulting Menu select for Border "black" and Area "none".
- To change the axis labels, click on the diagram and select "Chart/Options".
- We adopt the convention here that experimental data are represented by points without lines and fitted results are lines without points. To change the appearance of the fitted curves, double-click on a curve and select "black" for Line, and "none" for Marker. For the data points select "none" for Line and "black" for Marker.
- To present the data on a logarithmic X-axis, double-click on the X-axis, select "Scaling" and then "Logarithmic".
- To generate a bar diagram, highlight the data, click on the "Chart Wizard" and select "Column" and then "Finish".

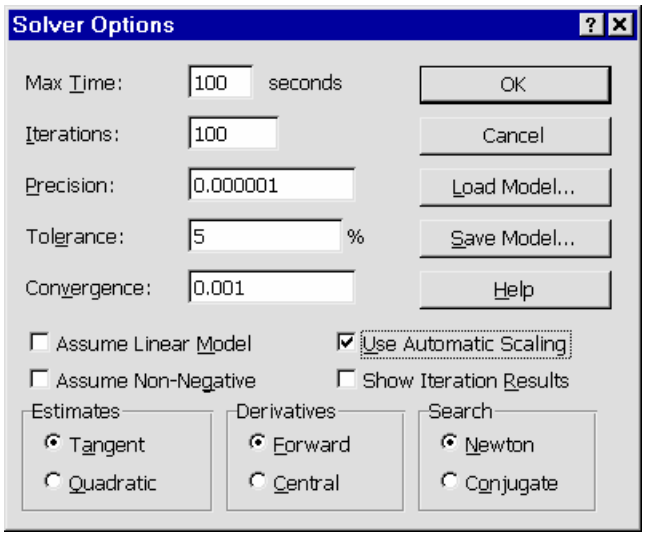
Use of the Solver Module

The Solver module can be used to optimise values in specific cells as a result of varying the values in other cells. We use it chiefly to minimise the sum of errors squared by variation of the parameters of a model. To do this click on "Solver" in the "Tools" menu. The following window appears:



- The Target Cell is the value to be minimised. To select D4 for example either enter “D4” in the box, or click on the “Set Target Cell“ box in the Solver window, and then click on the cell D4.
- Select “Min”.
- “By changing cells“ indicates the cells whose values are to be varied. For example, to select cells B1 and B2 either enter “B1;B2” (or “B1:B2”) into the box, or click on the box then click with the mouse on B1 hold down the left button and drag to B2.

Click on “Options“:

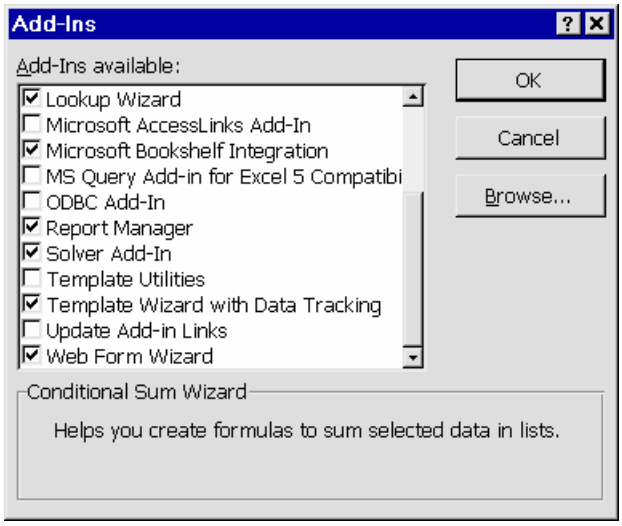


- “Use Automatic Scaling“ must be selected.
- “Precision“ is set to 0.00000001, “Convergence“ to 0.000001.
- The other fields are left at default values. If minimisation does not work, try selecting “Quadratic”, “Central” and “Conjugate”
- Click on OK to return to the previous window.
- Select “Solve“.

- If the minimisation is successful select OK and the result appears on the screen. If an error message appears select Cancel.

Checking the installation of MS-Office

The Solver option of MS-Excel used on this CD is located in the "Tools" menu. Unfortunately, Solver is not a standard option in MS Office setup, and it may therefore not be available or activated on some computers. If there is no "Solver" entry in the Tools menu of your computer, select "Tools/Add-Ins". Scroll down the list of available Add-Ins in the window, click on the "Solver Add-In" and finish with OK.



If there is no "Solver Add-In" entry in the Add-Ins window, or if "Solver" still does not appear in the "Tools" menu, re-install Excel in the full version form the MS-Office CD. If you use a different Excel version also consult the online help.

Exercise 1: Introduction to the use of MS-Excel.

Set up a spreadsheet in which the equation $y=x^3-50x-25$ is calculated in the range x=0 to x=10 with intervals of 0.5, and present the results in graphical form.

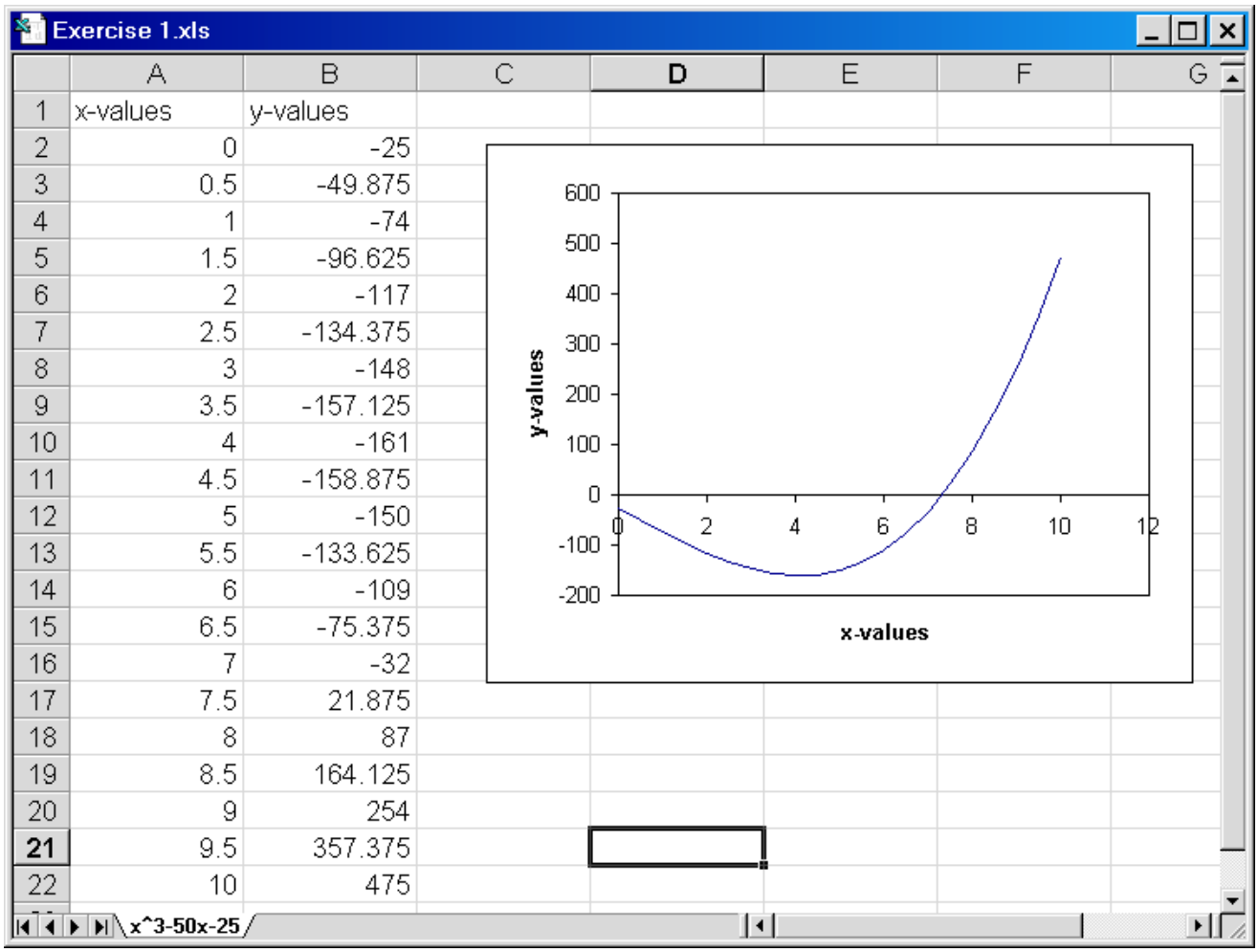
Objective of the exercise: to gain experience of using MS-Excel

Instructions:

- Open an empty spreadsheet.
- In box A1 enter "X-values", in B1 "Y-values".
- In A2 enter the value "0".
- In A3 enter the expression "=A2+0.5".

- Move to row 3 of column A (A3), and enter "=", use the up-arrow ↑ to move the cursor to cell A2 enter "0.5" and press ENTER.
- To copy the formula to the other cells in column A, return to cell A3 press Ctrl-C ("C" for copy, to place the formula in the clipboard). Press Shift and extend the highlighting using the ↓ to row 22 and press ENTER
- The y-values are entered in row B. Move to B2 and enter "=", move the cursor to cell A2 using the ← arrow, enter the expression "^3-50*", move the cursor again to A2 with the ← and enter "-25", complete by pressing ENTER. Note that the ^-symbol does not appear on the screen until the next symbol is entered.
- This has entered the formula A2^3+50*A2-25 into cell B2. This formula is copied to the other cells of the rows up to cell B22 using Ctrl-C as described above, and this generates the desired set of data.
- Create a graphics plot of the data with column A as x-values and column B as y-values. To do this, highlight the data from A1 to B22, click on Chart Wizard, select "Scatter(XY)", and chose the plot that shows only lines.

The result should look something like the following:



	A	B
1	x-values	y-values
2	0	-25
3	0.5	-49.875
4	1	-74
5	1.5	-96.625
6	2	-117
7	2.5	-134.375
8	3	-148
9	3.5	-157.125
10	4	-161
11	4.5	-158.875
12	5	-150
13	5.5	-133.625
14	6	-109
15	6.5	-75.375
16	7	-32
17	7.5	21.875
18	8	87
19	8.5	164.125
20	9	254
21	9.5	357.375
22	10	475

Exercise 2: Linear Regression.

In this example we shall determine the initial rate of an enzyme reaction using linear regression. The aim of the exercise is to programme a spreadsheet that will carry out a linear regression, and enable the results to be shown graphically. We shall also show how outliers can be treated without loosing the original data.

Objectives of the exercise: manipulations with MS-Excel functions, treatment of outliers, programming a linear regression.

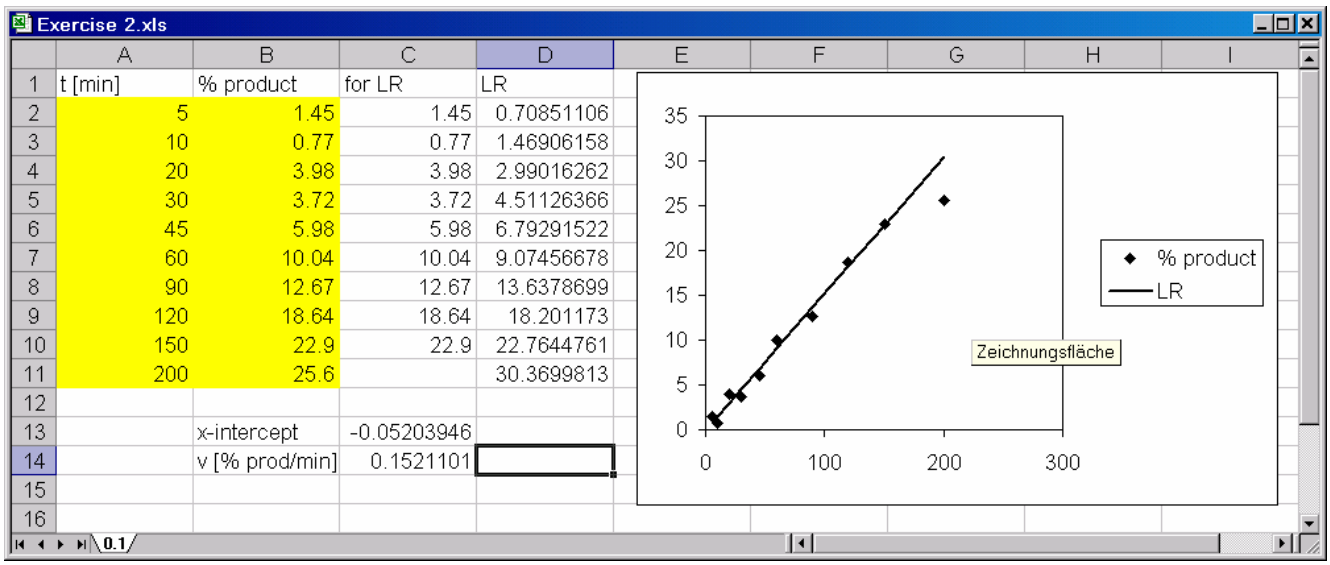
Useful background information about this exercise can be found as follows:

- Fitting data by the method of least squares (Sect. 9.1.8)
- Linear regression (Sect. 9.2.1)

Instructions:

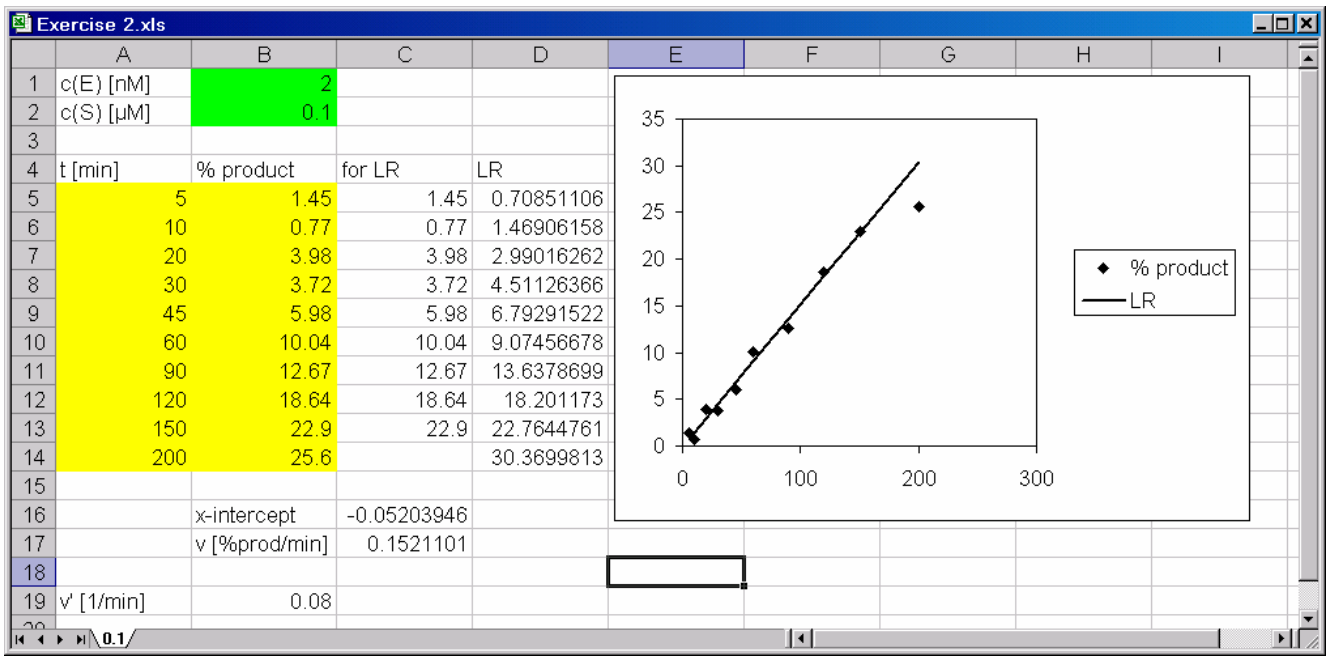
- Open a new table.
- The first row contains the titles for the two columns: “t [min]” for the x-values in column 1, and “% product” as the readings in column 2.
- Column 3 contains a copy of the data in column 2 which can be edited without deleting the original data.
- Go to the second row of column 3 (C2), enter “=” and use the left arrow “←” to move the cursor to cell B2 and press ENTER.
- To copy the formula to the other cells in column C, leave the cursor in C2 and press Ctrl-C (the formula is now held on the clipboard). Press Shift and enlarge the highlighted region with the down arrow “↓”, and complete the operation by pressing ENTER.
- The Excel functions “intercept” and “slope” are now used.
- The intercept function: move the cursor to C13 and enter “=intercept(”; highlight the y-values with the mouse (column C, without a title), enter “;”, highlight the x-values (column A, without a title), and then press ENTER.
- The slope function: move the cursor to C14, enter “=slope(” highlight the y-values with the mouse, enter “;”, highlight x-values, and press ENTER. This slope corresponds to the initial rate of the reaction (v°) expressed in %product/min.
- We now calculate the predicted data for the linear regression in column D: to do this, move the cursor to D2, enter “=”, click with the mouse on C10, press <F4>, “+” then click with the mouse on A2, enter “*”, click with the mouse on C11, press <F4> and then ENTER.
- Copy the formula into the other cells in column D (using Ctrl=C, see above).
- Create a plot of the data points and the linear fit: first highlight the results to be plotted by moving the cursor to A1, and highlighting cells to B8, press Ctrl and then highlight D1 to D8. Click on Chart Wizard, and then proceed as described above in Creating graphics.
- Data points that deviate significantly from the linear fit can be deleted from column C. This is often the case for data points occurring at the end of the set of data, where deviations from the linear initial rate can become pronounced.

The result should look like the following. In this example the final data point is excluded from the linear regression.



We must now convert the initial rate into turnovers/min ($v'=v/c_{E,tot}$).

- First, we need to make place at the top of the spreadsheet. Click on the row heading of row 1 and highlight rows 1-3. Right click the mouse on one of the row headings and select "insert cells".
- In the new A1 cell, insert "c(E) [nM]", and in A2 "c(S) [µM]"
- The numerical values are inserted in B1 and B2.
- In A19 the term "v' [1/min]" is entered.
- Since data are entered in %product and the time increment is min, the slope of the linear regression gives the initial rate in %product/min. This has to be divided by 100, multiplied by c(S) (in µM), multiplied by 1000 and finally divided by c(E) (in nM) to yield the number of turnovers per enzyme molecule per minute (v').
- This is done as follows: in cell B19 place "=" then click on C14, type "/100*", click on B2 and insert "/", click on B1 and enter "*1000". The evaluated turnover rate is now in B19
- The result is rounded to 2 decimal places by clicking twice on the  –symbol.



Result

The fitted data give an initial turnover rate of 0.08 turnovers per enzyme molecule per min.

Exercise 3: Michaelis-Menten Kinetics I

Evaluate the specimen data by linear regression and fitting the initial rates using the Michaelis-Menten Model. Carry out an error analysis by systematic removal of individual data points in the Michaelis-Menten fitting.

Objectives of the exercise: MS-Excel programming using multiple data sheets; non-linear regression using the MS-Excel Solver module; programming a data table for Michaelis-Menten kinetics; error estimation.

Exercise 3 is linked to Exercise 2.

Background information about this exercise can be found as follows:

- Fitting data by the method of least squares (Sect. 9.1.8)
- Introduction to error estimation (Sect. 9.1.10)
- Michaelis-Menten kinetics (Sect. 9.2.2)

Instructions

Firstly, carry out linear regressions on the five kinetic data sets, each on a separate Excel spreadsheet.

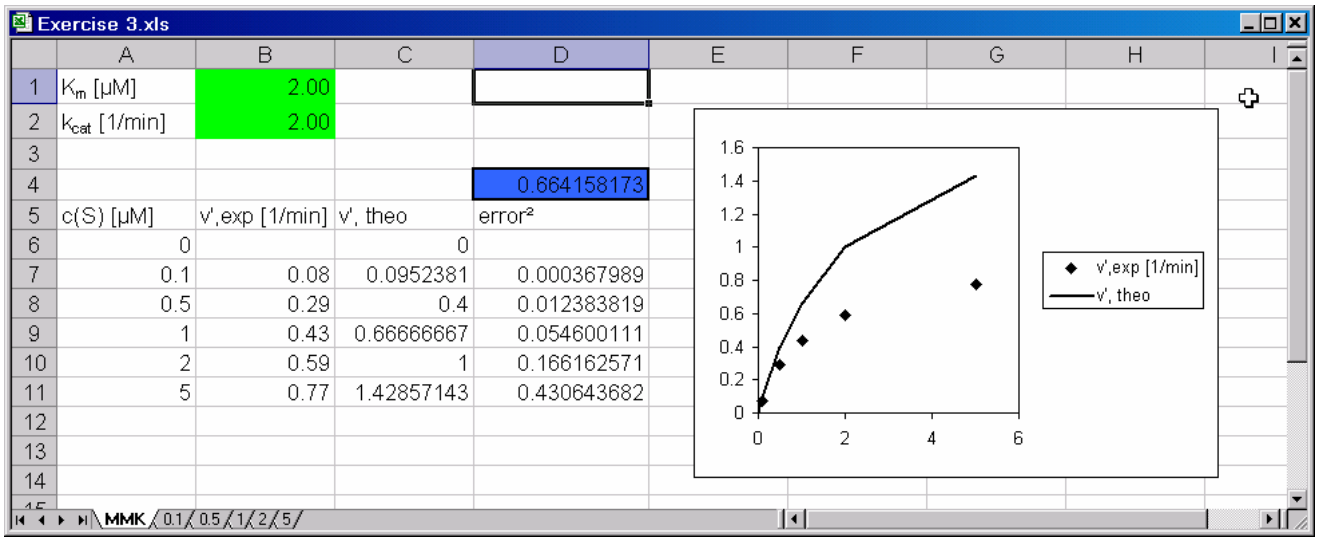
- One spreadsheet is used for each substrate concentration, and together these spreadsheets form a file.

- To copy an already existing spreadsheet, right click with the mouse on the sheet tab at the bottom, select Move/Copy, click on Copy, then OK. Alternatively, left click and hold down on the sheet tab, press Ctrl, move the mouse to right and release.
- A single click on the sheet tab changes between the various spreadsheets in a file. The "Window" menu can be used to change between the various open files.
- It is helpful to use the substrate concentrations as the spreadsheet name. To do this, double click on the sheet tab and enter the new name.

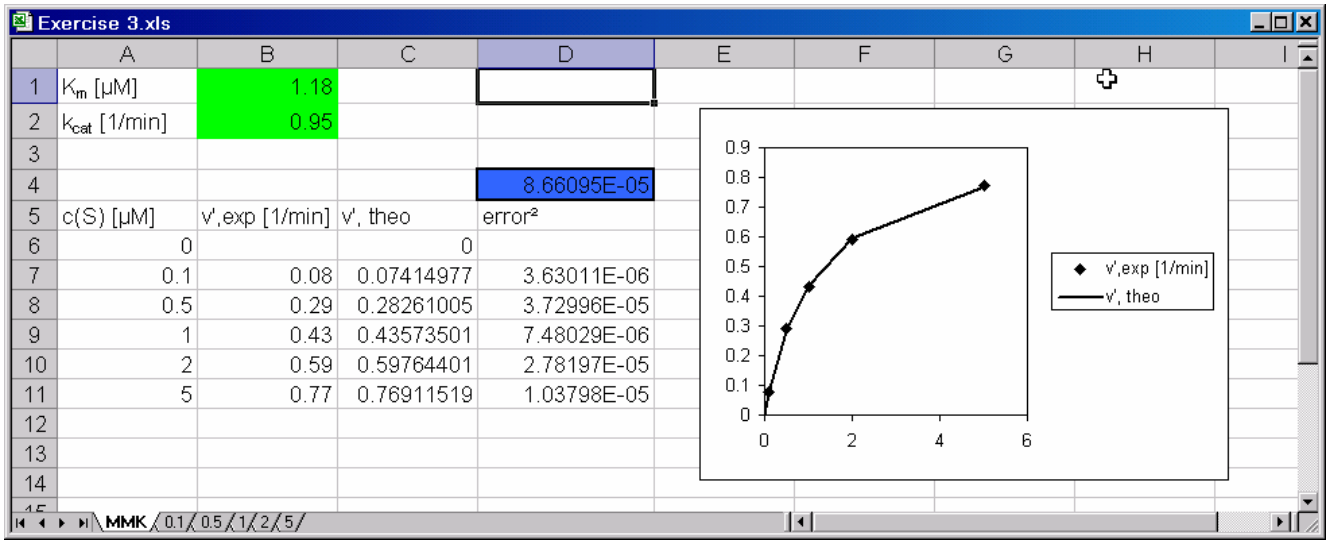
For the Michaelis-Menten evaluation, change to a new spreadsheet that we call "MMK":

- There are only two parameters: K_m und k_{cat}.
- In cell A1 enter "K_m [µM]", in A2 "k_{cat} [1/min]". Initial values of 2 are entered into B1 und B2.
- The headings of the data files are entered into row 5: "c(S) [µM]", "v' [1/min]", "v',theo", "error²". v' again is defined as $v/c_{E,tot}$.
- From row 6 onwards, the substrate concentrations are entered into column A, and the initial reaction rates from the linear regressions in column B (with units 1/min).
- The linear regressions and the evaluation are linked online: enter "=", left click on the sheet tab containing the relevant linear regression, and click on the cell containing the result (the value k/min), and press ENTER. When c(S)=0.1 µM the value shown is "='0.1'!B19" (sheet 0.1, cell B19). Now, when the linear regression changes, the values in the Michaelis-Menten table alter correspondingly.
- Column C contains the theoretical data, i.e. the calculated values $k_{cat} * c_S/[K_m + c_S]$. To calculate this, the following expression is entered into C6: "=$B$2*A6/(A6+$B$1)". This formula can either be typed in directly, or using the mouse and <F4> as described above, followed by copying the formula into the other cells of column C.
- Column D contains the squares of the deviations between the experimentally measured and calculated values, entered as: "=(B6-C6)^2". (Note that the ^-symbol does not appear until the next character is entered.)
- In cell D4 we enter the sum of deviations squared. To do this, the Excel function "sum" is used. Enter in D4: "=sum(" + then highlight the individual errors with the mouse, and press ENTER. This is the value that we will shortly minimise in the fitting step.
- Graphical presentation: highlight the area A5-C10, click on the Chart Wizard, and select XY(Scatter), etc., as before. The plot should show the data points as symbols without line, and the theoretical values a line without symbol. This is done by double-clicking on the plot, then double-click the relevant curve and select as appropriate from the menu.

So far, the results should look something like the following:



- We are now in a position to do the data fitting. Call up Solver from the tools menu, and minimise the value of D4 as B1 and B2 are varied. The result of the minimisation should look as follows, with the data fitting well to the theoretical curve.



- To remove points from the fitting process, the relevant error squared data point is simply deleted from the column.
- To restore the error squared point to the fitting, re-enter the formula in the respective cell.
- The error analysis is carried out, by removing one error squared term at a time from the data in column D and repeating the minimisation step. The system carries out the fitting omitting the selected point, and we obtain new values for the solution. On omitting the errors squared term for the last data point, we obtain the following values for K_m (1.10 µM) and k_{cat} (0.92 min^{-1}), and we

use the deviation of these values from the values determined from the other of the data points as an estimate of the error.

Result

The best values of the parameters to fit the data are: K_m = 1.18 (±0.08) μM and k_{cat} = 0.95 (±0.03) min^{-1}.

Exercise 4: Determine the apparent first order rate constant of dissociation kinetics

Objective of the exercise: Evaluation of the kinetics of a dissociation process. Establishing the robustness and accuracy of the fit by examining the effect on parameter estimates of omitting individual points from the fitting process. Programming a spreadsheet for dissociation kinetics. Error estimation.

Background information about this exercise can be found as follows:

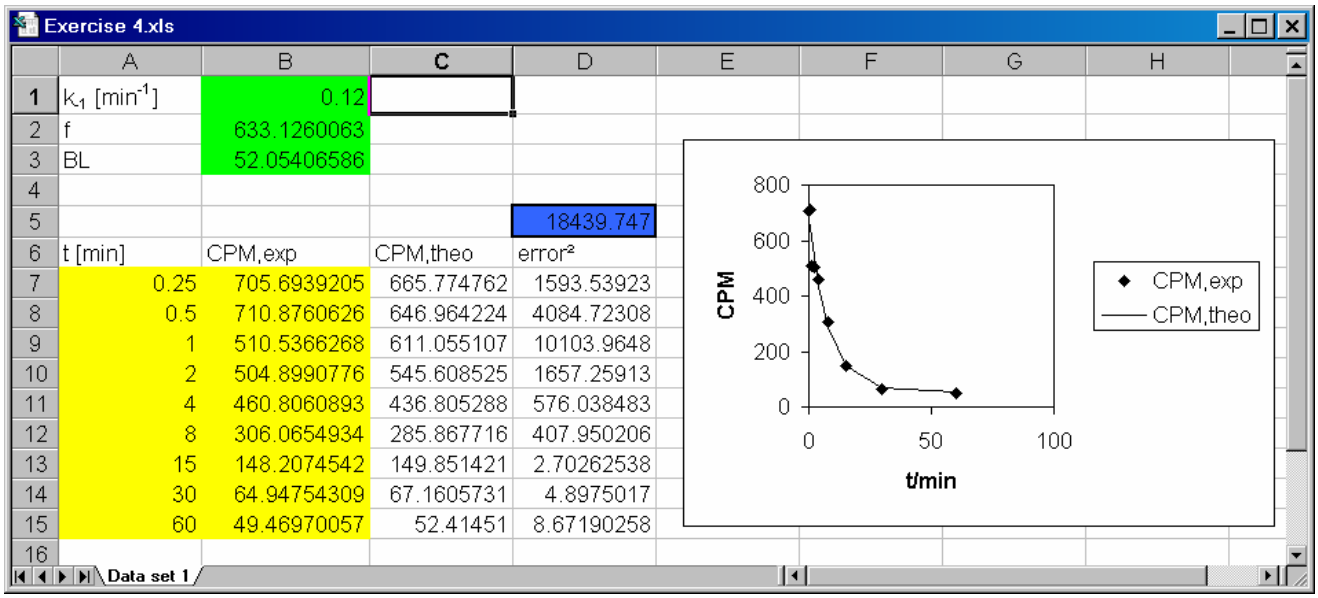
- Fitting data by the method of least squares (Sect. 9.1.8)
- Linear regression (Sect. 9.2.1)
- Dissociation kinetics (Sect. 9.2.3)

Instructions

- We make the assumption that only the species AB contributes to the measured signal, and that the there is a constant background (BL).
- The first three rows contain the parameters: A1: "k_{-1}", A2: "BL" and A3: "f".
- In cells B1-B3 we enter 1 as initial values for k_{-1}, BL and f.
- Row 6 contains the titles of the data sets: "t [min]", "CPM", "CPM,theo", "error²".
- From Row 7 onwards, the times are entered in column A, and the measured data in column B.
- Column C contains the theoretical data: enter "=$B$2+$B$3*exp(-A7*$B$1)".
- Column D contains the errors squared. The sum of errors squared is placed in cell D5.
- Define the graphics plot: highlight columns A, B und C from row 6, then select Scatter(XY) from the Chart Wizard.
- Minimise the value of the sum of error squared in D5 with respect to variation of B1-B3.
- Note that in this evaluation, rather untypical, there are no concentrations (cpm is used instead).

- To remove points from the fitting process, the relevant error squared data point is simply deleted from the column.
- To restore the error squared point to the fitting re-enter the formula in the respective cell.

The spreadsheet should look something like the following:



Result

The apparent first order rate constant of dissociation is k_{-1} = 0.12 ±0.01 min^{-1}. If, however, we assume that the points at 0.25 min and 0.5 min are incorrect and omit these from the fitting, we obtain a value for k_{-1} of 0.09 min^{-1}.

Exercise 5: Global analysis of multiple data sets

Evaluate dissociation kinetics using three independent data sets simultaneously.

Object of the exercise: global analysis of multiple data sets.

Exercise 5 is linked to Exercise 4.

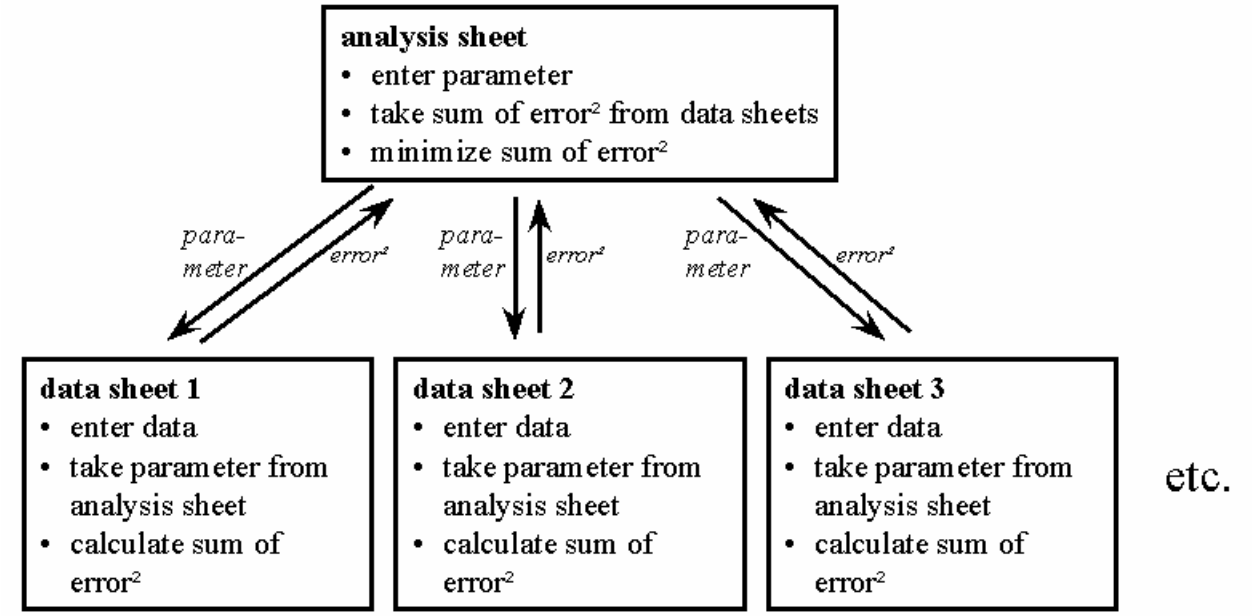
Background information about this exercise can be found as follows:

- Global fitting of multiple data sets (Sect. 9.1.9)

Instructions

- BL and f can vary from one experiment to another (they are local parameters)
- k_{-1} must be the same for all data sets because it is a physical constant (it is a global parameter)

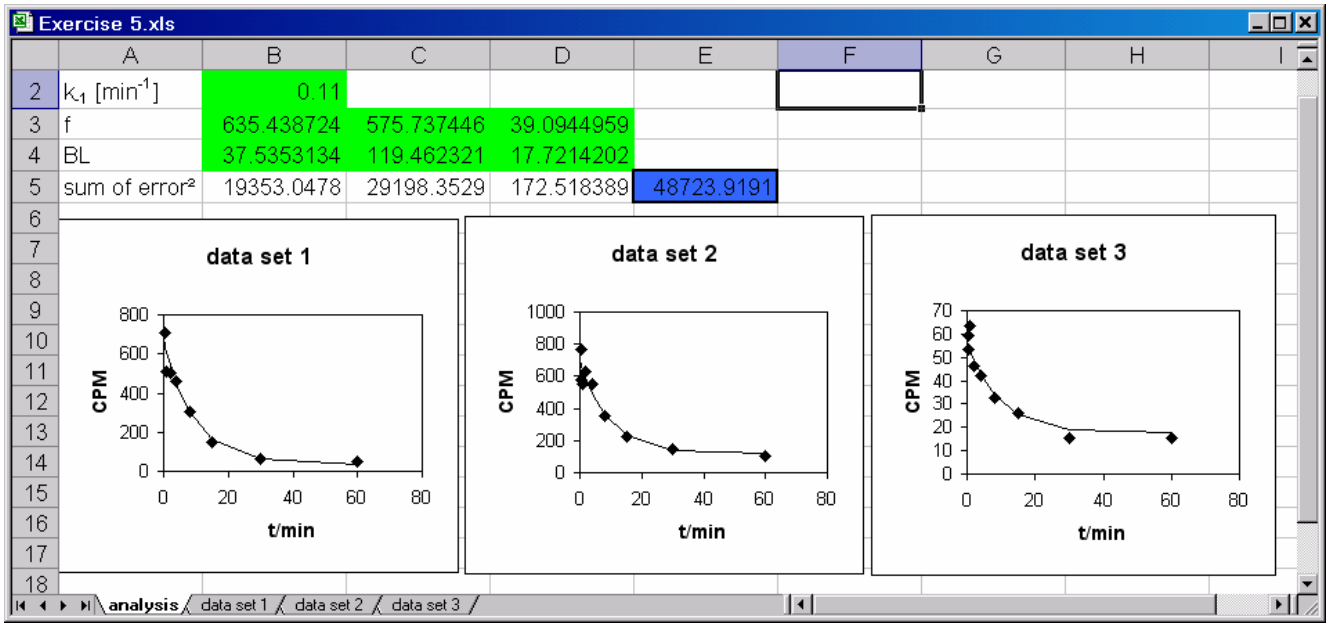
- A joint evaluation of all three data sets requires 7 parameters in total: k_{-1}, BL_1, f_1, BL_2, f_2, BL_3 and f_3. We use a separate spreadsheet to manipulate these parameters and to carry out the evaluation. The fitting is carried out using this "master" analysis spreadsheet, and it is important that the transfer of parameters is carried out correctly. All parameters are entered into the analysis spreadsheet, and transferred to the subsidiary sheets. The sums of errors squared are calculated in the subsidiary sheets, and then transferred back to the analysis sheet.



- First, each of the three data sets must be evaluated in their own data sheets. This, in brief, involves the following steps: copying the table, alter the name, enter new data, prepare graphics, and analyse.
- Then open a new empty spreadsheet, and change its name to "analysis".
- In the analysis sheet, enter "k_{-1}", "f" and "BL" in A2, A3 and A4 respectively. The titles "Data set 1", "Data set 2" and Data set 3" are entered into cells B1, C1 and D1 respectively. In B2 is the value of k_{-1} (which is valid for all three data sets), in C2 to C4 are the f-values for the three data sets, and in D2 to D4 the corresponding BL values. Initially all if these values are set equal to "1".
- Row 5 contains the errors squared for the individual data sets, and "sum of error²" is entered into cell A5.
- Now the numerical values for k_{-1}, BL and f must be transferred from the master sheet to the data sheets. To this end, go to the data sheet, select the field containing k_{-1}, BL or f, type "=", move to the analysis sheet, highlight the relevant field and press ENTER (Excel will return automatically to the original subsidiary sheet). This process should be repeated with all 3 parameters in the three subsidiary tables.
- Note: from now on, these parameters must only be entered and fitted in the analysis sheet.

- Move to the analysis worksheet. Enter the sum of errors squared in rows 8-10. Note that the direction of transfer is now reversed: the analysis sheet takes the sum of errors squared from the subsidiary sheets. To do this, go to cell B5 and enter "=", move to the appropriate subsidiary sheet and highlight the cell containing sum of errors squared and press ENTER.
- In cell E5 we enter the sum of B5...D5.
- Fitting: the value in E5 is minimised with respect to the variation of B2 to B4, C3 to C4, D3 to D4. At the same time, all of the subsidiary sheets are evaluated.
- Graphic plots can also be assimilated into the analysis worksheet. Go to the subsidiary sheets, highlight Graphics, press Ctrl-C (this places the Graphics in the clipboard), move to the analysis sheet, click in a cell and press Shift-Insert. The size and position of the plots can be altered using the mouse.

The result should look like the following:



Result

The best global fit to all data sets is given by an apparent dissociation rate constant k_{-1}=0.11 min^{-1}.

Exercise 6: Weighting of data sets

Evaluate two dissociation kinetics data sets simultaneously. Use an appropriate weighting for the errors squared.

Objective of the exercise: global data analysis; weighting factors.

Exercise 6 is linked to Exercise 5.

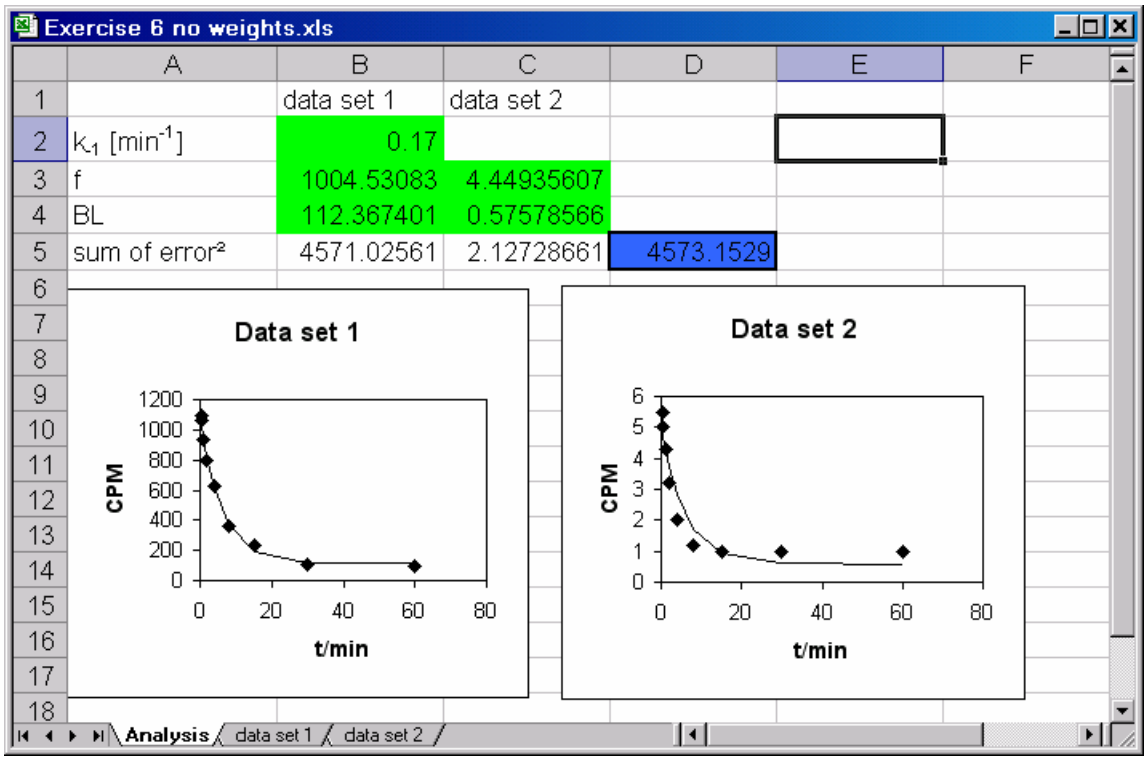
Background information about this exercise can be found as follows:

- Global fitting of multiple data sets (Sect. 9.1.9)

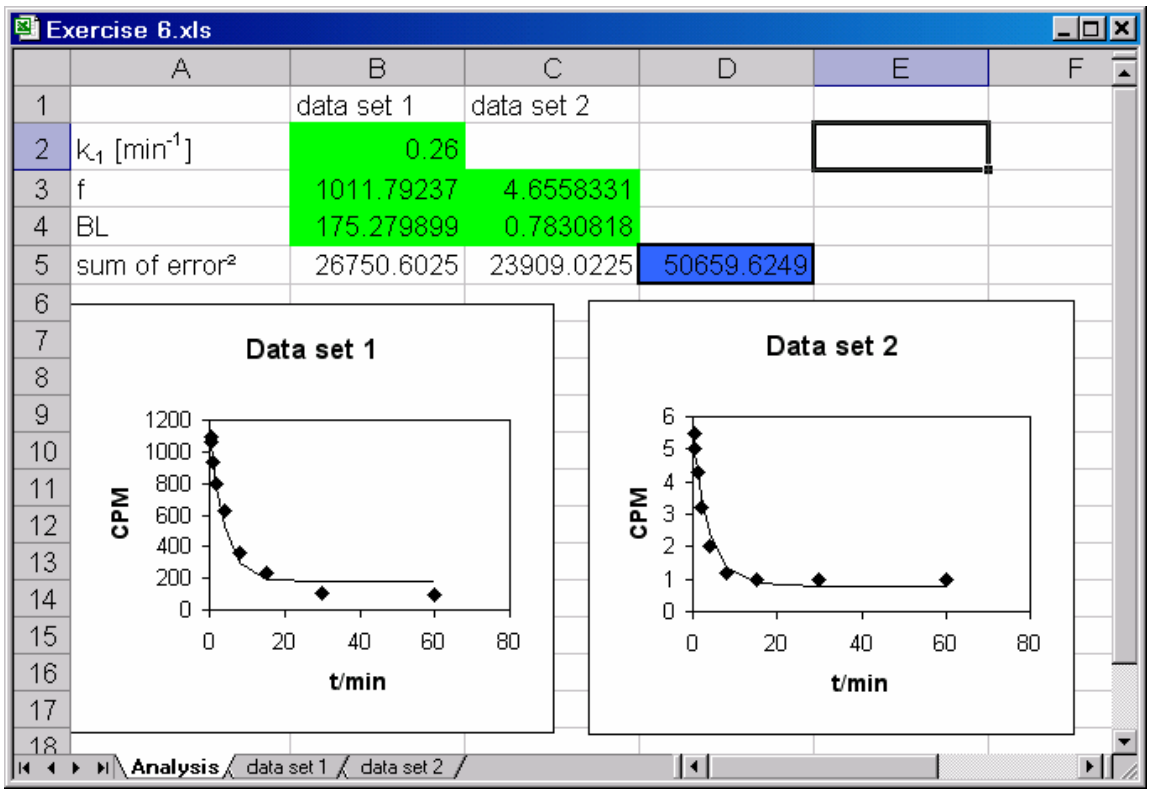
Instructions

- First, proceed as in Exercise 5.
- The analysis gives a value for k_{-1} of 0.17 min^{-1}. Checking this, however, shows that the fit to data set 2 is poor. This arises because the effect factor of this data set is about 200 fold smaller than that of data set 1. Consequently, the sum of errors squared of data set 2 is also much smaller, despite the fact that the fit to the data is not good.
- In order to give both data sets comparable weight, the weighting of the sum of errors squared of data set 2 must be increased by a factor of 40000 (=200×200). This can be done by entering "='Data set 2'!D5*40000" in cell C5 of the analysis sheet.
- A new weighted fitting leads to a value for k_{-1} of 0.26 min^{-1}. The two data sets are now of equal importance in the fitting.

Without error weighting, the results look as follows:



Whereas with error weighting the results are as below:



Result

When the two data sets are weighted so that they are of equal significance in the fitting, the best fit value is k_{-1}=0.26 min^{-1} ; unweighted data sets give a best fit of k_{-1}=0.17 min^{-1}, and data set 2 is very poorly fitted.

Exercise 7: Michaelis-Menten kinetics II

Evaluate kinetic data according to the Michaelis-Menten model by direct fitting of the initial rates.

Objective of the exercise: evaluation of Michaelis-Menten kinetics

In Exercise 3 we evaluated the kinetics of an enzyme reaction following the Michaelis-Menten model. We used linear regression to calculate the initial rates at different substrate concentrations, and these initial rates were then fitted by non-linear regression to the Michaelis-Menten equation. One problem with this approach is that the linear regression is carried out using somewhat arbitrary criteria, and the non-linear fitting follows using derived or secondary data. This can introduce a subjective element into the evaluation that may be particularly pronounced with noisy data. To minimise this element of subjectivity, this exercise evaluates kinetic parameters from the primary data by directly coupling linear regression of individual data sets with fitting to the Michaelis-Menten model.

Background information about this exercise can be found as follows:

- Global fitting of multiple data sets (Sect. 9.1.9)
- Michaelis-Menten kinetics (Sect. 9.2.2)

Instructions

First we calculate theoretical initial slopes for each substrate concentration using arbitrary initial values for K_m and k_{cat}.

- This step basically follows the procedure developed in Exercise 3. Using these values and a y-axis intercept that is also varied during minimisation, a straight line is calculated for each data set in a subsidiary sheet. Then the errors squared are calculated between each data point and the corresponding point on the straight lines. The sum of errors squared values are transferred back to the main sheet, where minimisation is carried out.
- K_m und k_{cat} are placed in row 1 and 2. In cell A1 enter "Km [µM]", in A2 "kcat [1/min]". Initial values of 2 are entered into B1 und B2.
- In A4 type c(E) [nM], in B4 type 1, for the enzyme concentration in nM.
- The headings of the data files are entered into row 6: "c(S) [µM]", "v',theo [1/min]", "intercept" and "error²".
- From row 7 onwards, the substrate concentrations are entered into column A.
- In column B v' values are calculated. The rate for an enzyme catalysed reaction ($v'=v/c_{E,tot}$) is given by the Michaelis-Menten equation: $v'(c_S) = k_{cat} \times \frac{c_S}{c_S + K_m}$. Therefore, for example B7 should contain the formula: "=$B$2*A7/(A7+$B$1).
- In column C "0" as starting value for the intercept is typed in any cell.

Exercise 7.xls

	A	B	C	D
2	k_{cat} [1/min]	0.63		
3				
4	c(E) [nM]	1		
5				
6	c(S) [µM]	v',theo	intercept	error²
7	0.1	0.04701391	-0.24205743	
8	0.5	0.18115782	-0.23822935	
9	1	0.2815896	-0.06116948	
10	2	0.38957849	0.11143124	
11	5	0.50601079	0.19825567	

analysis

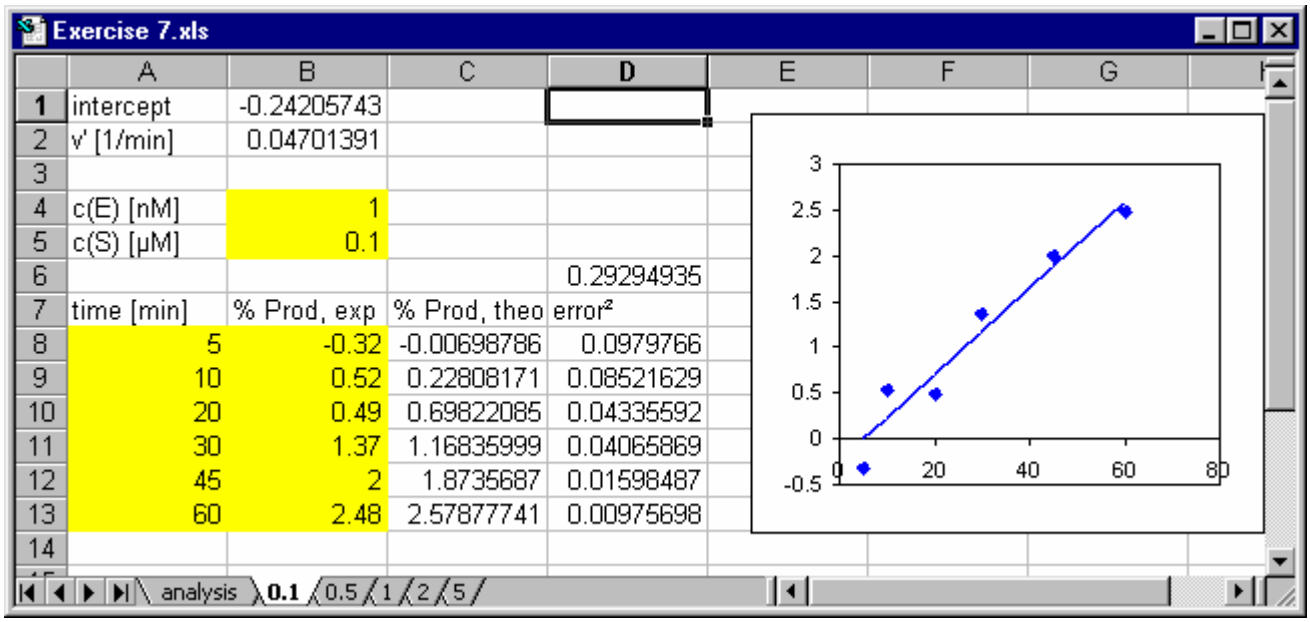
Now the subsidiary sheets need to be programmed:

- We need one sheet for each concentration. Name the sheets accordingly.

- The first two rows contain date required to calculate the straight line: Type “intercept” and “v’ [1/min]” in column A and in column B take the numbers from the analysis sheet. For example in the 0.1 sheet highlight B1, type “=”, change to the analysis sheet by clicking on the analysis sheet tab, highlight cell C5 and press ENTER. “ =analysis!C5” appears in B” of the 0.1 sheet. Put “ =analysis!B5” in B2 of the 0.1 sheet.
- We put c(E) in nM in row 4, c(S) in µM in row 5.
- In row 7 type the four the column headings: “t [min]”, and “% product, exp”, “% product, theo” and “error²”.
- Row 8 and following contain the data in columns A and B.
- In column C the theoretical straight line which represents the initial slope of the reaction at the given substrate concentration is calculated. The amout of product (in %) formed in the initial phase of the reaction during time t is given by:

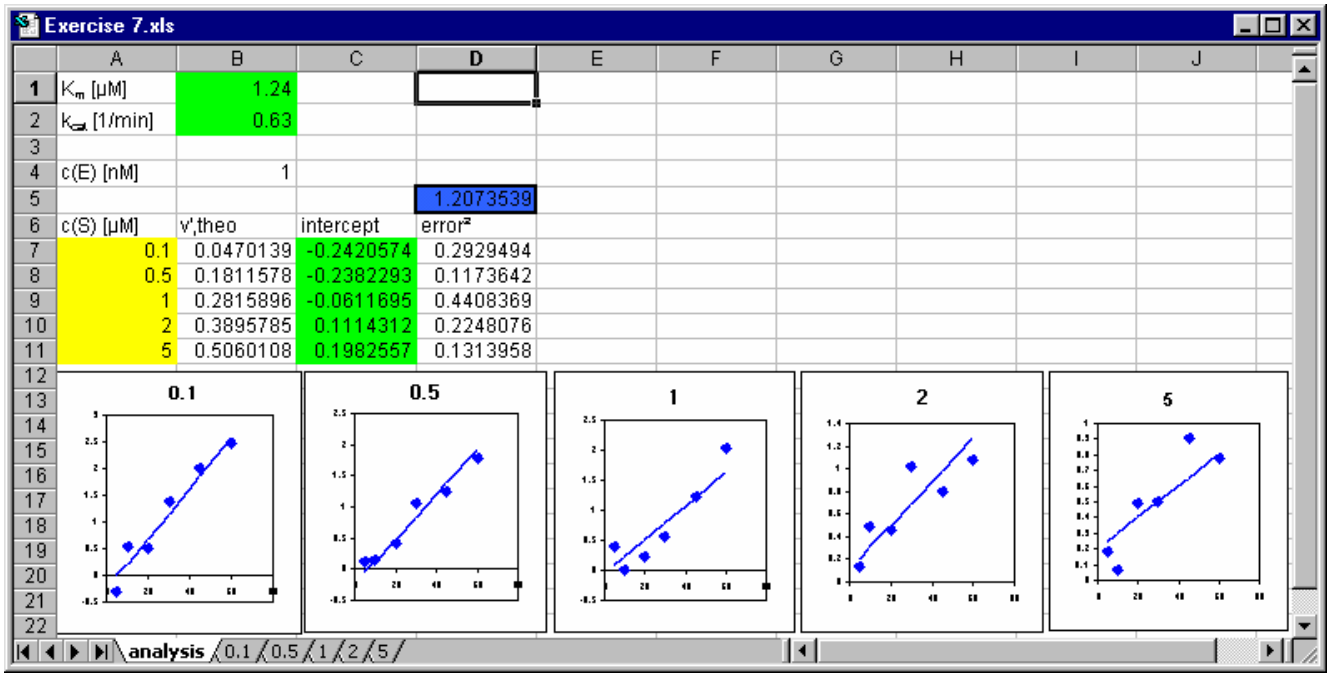
  Prod [%] = $c_{E,\,tot}$ [nM] × t [min] × v’ [1/min] × 100/$c_{S,\,tot}$ [µM] /1000

  Note, that the correction factor of 100 appears to obtain the amount of product in %, and the factor of 1000 is necessary, because we specify the enzyme concentration in nM and the substrate concentration in µM.
- We must also add the intercept to this expression. Therefore, the final formula for example in cell C8 is: =$B$4*A8*$B$2*100/$B$5/1000+$B$1.
- The errors squared are calculated in column D, the sum of errors squared is taken in cell D6.
- Create a plot of the data points and the linear fit. Copy the plots into the analysis sheet.
- The sum of errors squared of each subsidiary sheets is copied in column D of the analysis sheet, and the sum of them is placed in cell D5.



- Now D5 of the analysis sheet is minimized by variation of B1 and B2 and C7 to C11.
- Note, that in this analysis only data from the initial phase of the reaction should be used.

The result should look like the following:



Result

The data are fitted best by a K_m of 1.24 µM and a k_{cat} of 0.63 min^{-1}.

Exercise 8: Binding equilibrium

Objective of the exercise: evaluation of a binary thermodynamic binding equilibrium; programming of complex functions in MS-Excel

Background information about this exercise can be found as follows:

- Fitting data by the method of least squares (Sect. 9.1.8)
- Introduction to error estimation (Sect.9.1.10)
- Analysis of simple binding data (Sect. 9.2.6)

Instructions

This exercise does not introduce anything new in terms of creating the spreadsheet. We assume that at constant A the concentration of B is increased and that only AB contributes to the observed signal. The concentration of A is a constant in the analysis, but the more complex mathematical expression of Eqn. 9.12 requires a stepwise approach in the programming.

- The first row of the spreadsheet contains the constants, in this case only one, the total concentration of A ($c_{A,tot}$).
- The parameters are placed in the next rows (BL, f, K_{ass}). We take 0, 1 und 1×10^7 as initial values for these.
- Leave a row free.
- Then the column titles are inserted ("$c_{B,tot}$", "Signal" etc.).
- Concentrations are inserted in column A, the experimental reading in column B.
- To calculate theoretical data, a stepwise approach is recommended to enter the equation. Enter p in column C, q in column D, (p/2)^2-q in column E, and the square root of column E into column F.
- To do this we use the Excel function SQRT (type "=SQRT(" + ← + ENTER).
- Then the theoretical concentration of AB is programmed into column G (Eqn. 9.12).
- From this the theoretical signal can be calculated in column H: $signal_{theo} = BL + c_{AB,theo} \times f$
- Adjacent to this (Column I) are inserted the errors squared (from the differences between the experimental and calculated signals), and above this the sum of errors squared.

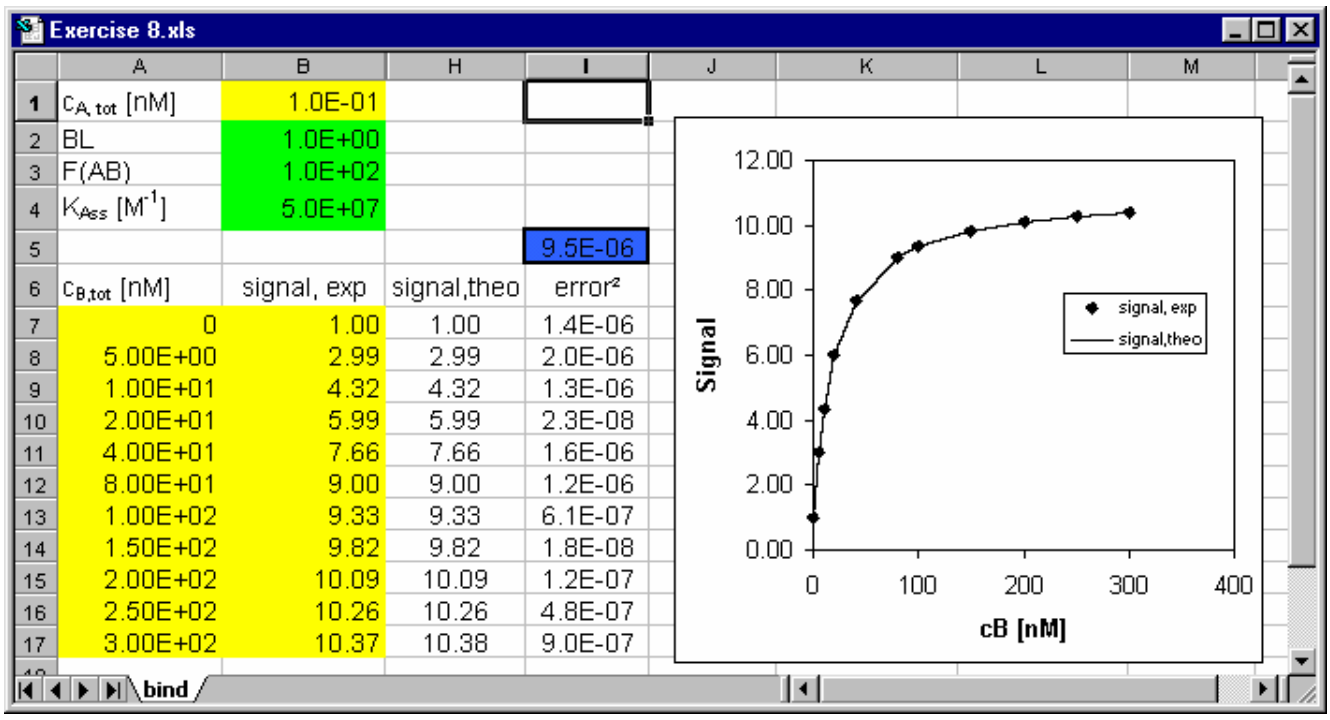
The spreadsheet should look like the following:

Exercise 8.xls

	A	B	C	D	E	F	G	H	I
1	$c_{A,tot}$ [nM]	1.0E-01							
2	BL	1.0E+00							
3	F(AB)	1.0E+02							
4	K_{Ass} [M^{-1}]	5.0E+07							
5									9.5E-06
6	$c_{B,tot}$ [nM]	signal, exp	p	q	M	SQRT(M)	$c_{AB,theo}$ [nM]	signal,theo	error²
7	0	1.00	-2.02E+01	0.00	101.58	10.08	0.00	1.00	1.4E-06
8	5.00E+00	2.99	-2.52E+01	0.50	157.72	12.56	0.02	2.99	2.0E-06
9	1.00E+01	4.32	-3.02E+01	1.00	226.37	15.05	0.03	4.32	1.3E-06
10	2.00E+01	5.99	-4.02E+01	2.00	401.15	20.03	0.05	5.99	2.3E-08
11	4.00E+01	7.66	-6.02E+01	4.00	900.73	30.01	0.07	7.66	1.6E-06
12	8.00E+01	9.00	-1.00E+02	8.00	2499.88	50.00	0.08	9.00	1.2E-06
13	1.00E+02	9.33	-1.20E+02	10.00	3599.45	60.00	0.08	9.33	6.1E-07
14	1.50E+02	9.82	-1.70E+02	15.00	7223.39	84.99	0.09	9.82	1.8E-08
15	2.00E+02	10.09	-2.20E+02	20.00	12097.32	109.99	0.09	10.09	1.2E-07
16	2.50E+02	10.26	-2.70E+02	25.00	18221.26	134.99	0.09	10.26	4.8E-07
17	3.00E+02	10.37	-3.20E+02	30.00	25595.20	159.98	0.09	10.38	9.0E-07
18									

bind

- To make the sheet easier to follow, click on C in the column heading, and highlight the columns from C to G. Right-click with the mouse in the column heading area and select Hide.
- We create a graphics plot showing c_B, experimental signal and theoretical signal.
- The fitting is carried out by minimising the sum of errors squared with respect to variation of K_{Ass}, BL and f.



Result

The best fit to the data is obtained with $K_{Ass}=5\times10^{7}$ M^{-1}.

Exercise 9: Independent identical binding sites

Analyse a binding process and evaluate the binding parameters n (number of sites) and K_{Ass} (the association constant) assuming independent binding to identical sites.

Objective of the exercise: analysis of binding to obtain optimal values of n and K_{Ass}

Exercise 9 is linked to Exercise 8:

Background information about this exercise can be found as follows:

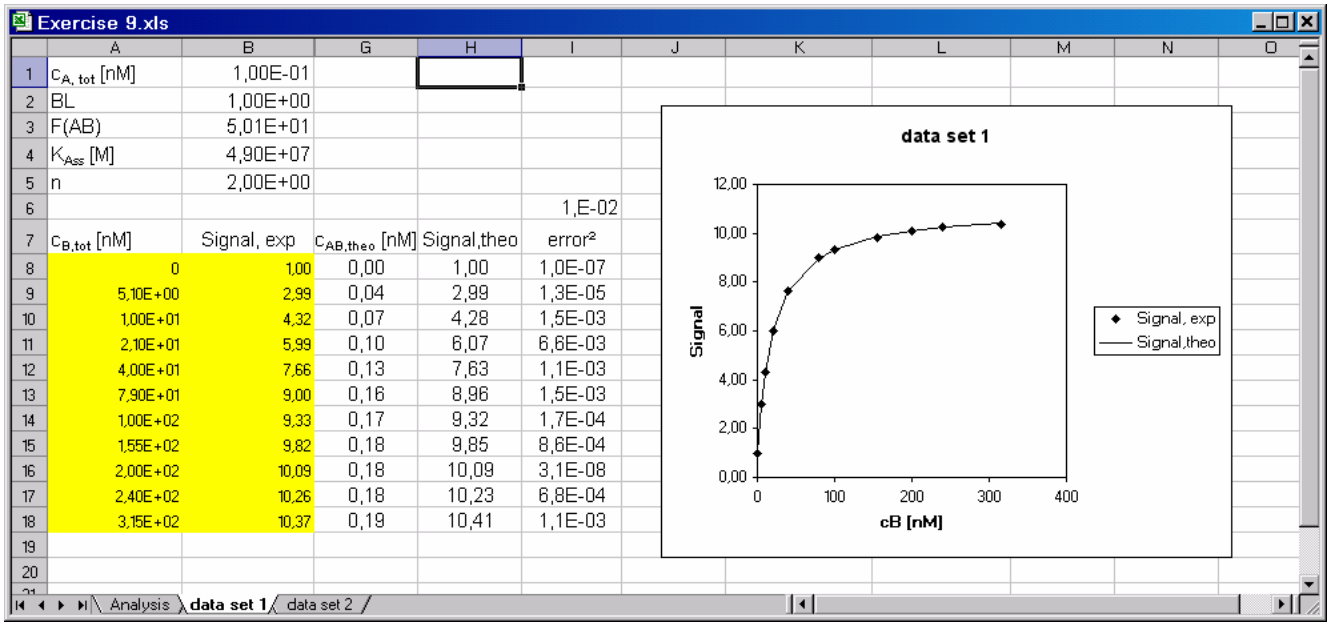
- Selection of appropriate models (Sect. 9.1.5)
- Independent identical binding sites (Sect. 9.2.5)

Instructions

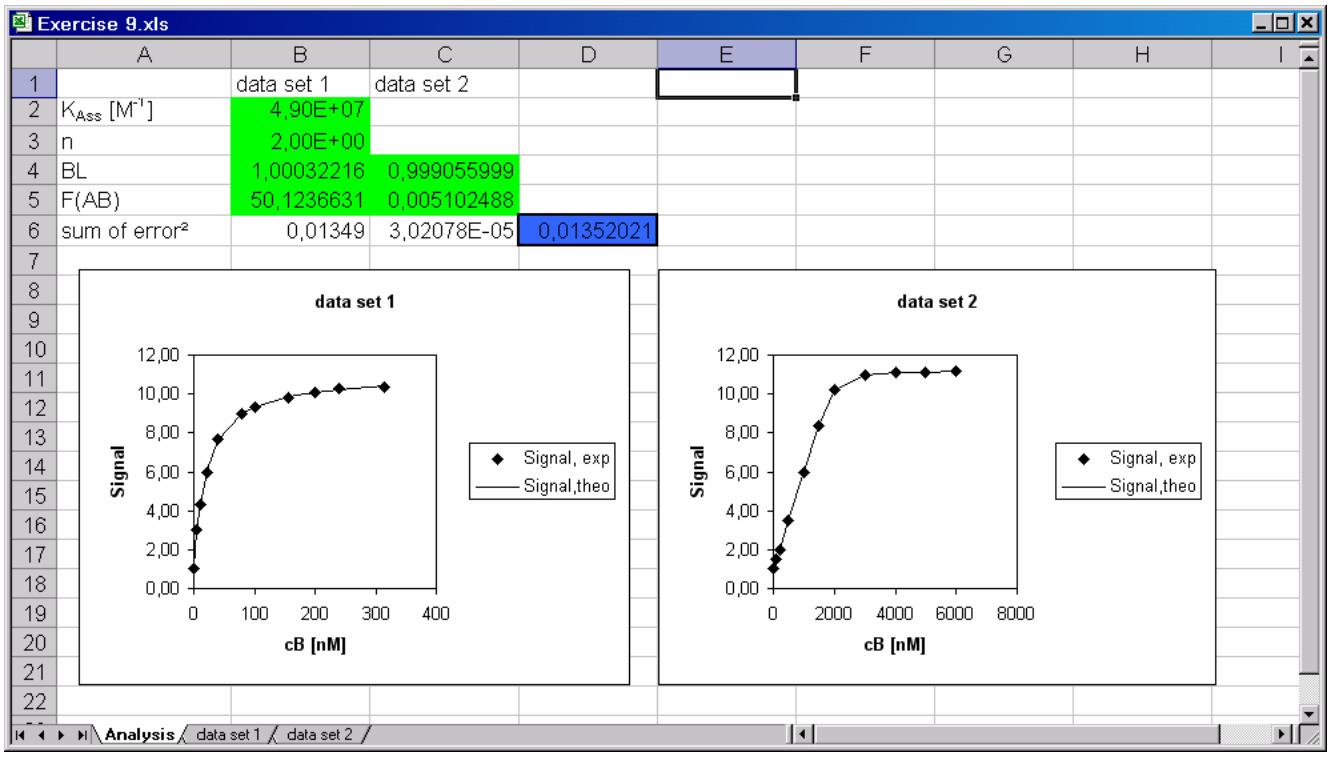
Programming a datasheet to evaluate association constants following the procedures described in Exercise 8 should be straightforward. We assume that the signal obtained in proportional to the amount of B bound to A. An additional global parameter in this exercise is n, the number of binding sites for B on A. Use Eqn. 9.13. A few tips:

- There are two data sets; the global parameters are n and K_{Ass}, and the local parameters BL_1, BL_2, f_1 and f_2.

- First, draw up two spreadsheets to evaluate the individual experiments and test these individually.
- Now programme an analysis sheet and carry out a global analysis of the two data sets.



	A	B	G	H	I
1	$c_{A,tot}$ [nM]	1,00E-01			
2	BL	1,00E+00			
3	F(AB)	5,01E+01			
4	K_{Ass} [M]	4,90E+07			
5	n	2,00E+00			
6					1,E-02
7	$c_{B,tot}$ [nM]	Signal, exp	$c_{AB,theo}$ [nM]	Signal,theo	error²
8	0	1,00	0,00	1,00	1,0E-07
9	5,10E+00	2,99	0,04	2,99	1,3E-05
10	1,00E+01	4,32	0,07	4,28	1,5E-03
11	2,10E+01	5,99	0,10	6,07	6,6E-03
12	4,00E+01	7,66	0,13	7,63	1,1E-03
13	7,90E+01	9,00	0,16	8,96	1,5E-03
14	1,00E+02	9,33	0,17	9,32	1,7E-04
15	1,55E+02	9,82	0,18	9,85	8,6E-04
16	2,00E+02	10,09	0,18	10,09	3,1E-08
17	2,40E+02	10,26	0,18	10,23	6,8E-04
18	3,15E+02	10,37	0,19	10,41	1,1E-03



	A	B	C	D	E	F	G	H	I
1		data set 1	data set 2						
2	K_{Ass} [M^{-1}]	4,90E+07							
3	n	2,00E+00							
4	BL	1,00032216	0,999055999						
5	F(AB)	50,1236631	0,005102488						
6	sum of error²	0,01349	3,02078E-05	0,01352021					

Results

The two data sets can be only be jointly fitted with values for the binding parameters of n=2 and $K_{Ass}=5\times10^7$ M^{-1}. Thus, the fact that two B molecules bind to one A was simply invisible on the basis of data set 1 alone!

Exercise 10: Independent binding sites II

Evaluate binding data according to a simplified model, and test whether the data can be fitted satisfactorily on the basis of a single binding site

Objective of the exercise: Fitting of binding data to simplified models; selection of appropriate binding models.

Background information about this exercise can be found as follows:

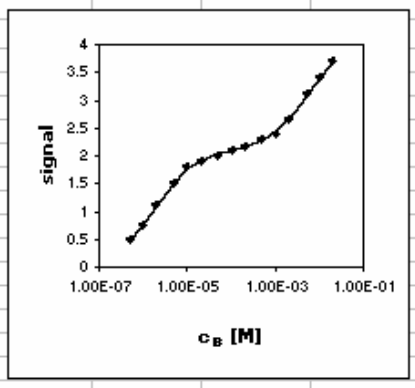
- Selection of appropriate models (Sect. 9.1.5)
- Fitting data by the method of least squares (Sect. 9.1.8)
- Analysis of simple binding data (Sect. 9.2.6)
- Independent identical binding sites (Sect. 9.2.5)

Instructions

- In this exercise we use Eqn. 9.16 for the analysis.
- The global parameters are $K_{Ass,1}$ and $K_{Ass,2}$, and the local parameters BL and f. Enter the parameters in rows 1-4.
- Row 7 contains the column headings: "c_B [M]", "signal,exp", the saturation of site 1 with B "θ_1", the saturation of site 2 with B "θ_2", the relative saturation of all binding sites "θ_{bound}", the calculated signal "signal,theo" and "error²".
- In column A enter the concentration of B, and the experimental signal in column B. Enter Eqn. 9.16 for site 1 into column C, e.g. in cell C8: "=$B$3+$B$4*$B$1*A8^$B$2/(1+$B$1*A8^$B$2)". In column D enter the corresponding expression for site 2, and in column E the total saturation of all sites (θ_1+θ_2). The calculated (theoretical) signal is entered into column F, the errors squared in column G, and in G6 the sum of errors squared.
- In the fitting we minimise G6 with respect to variation of B1 to B4.

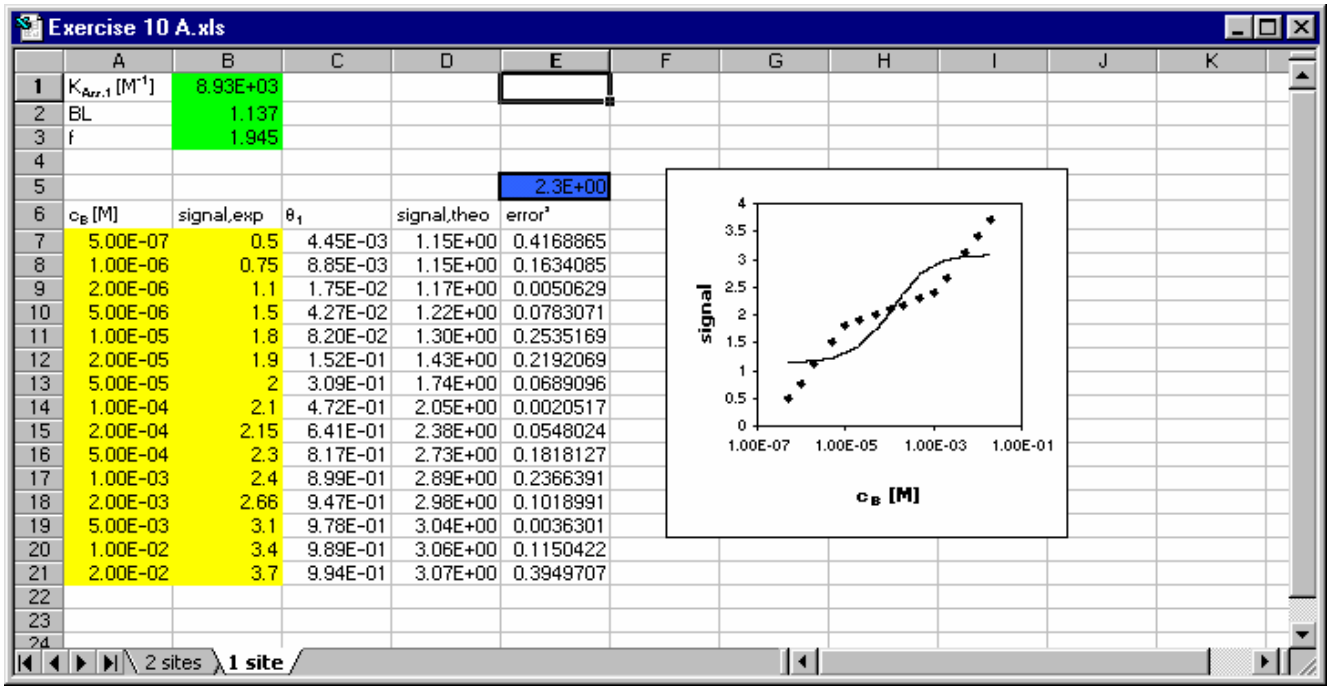
Exercise 10 A.xls

	A	B	C	D	E	F	G	H	I	J	K
1	$K_{Ass,1}$ [M^{-1}]	4.96E+05									
2	$K_{Ass,2}$ [M^{-1}]	2.00E+02									
3	BL	0.098									
4	f	1.994									
5											
6							6.0E-03				
7	c_B [M]	signal,exp	θ_1	θ_2	θ_{bound}	signal,theo	error²				
8	5.00E-07	0.5	1.99E-01	1.00E-04	1.99E-01	4.94E-01	3.548E-05				
9	1.00E-06	0.75	3.31E-01	2.00E-04	3.32E-01	7.59E-01	8.112E-05				
10	2.00E-06	1.1	4.98E-01	4.01E-04	4.98E-01	1.09E+00	7.698E-05				
11	5.00E-06	1.5	7.13E-01	1.00E-03	7.14E-01	1.52E+00	0.000419				
12	1.00E-05	1.8	8.32E-01	2.00E-03	8.34E-01	1.76E+00	0.0015241				
13	2.00E-05	1.9	9.08E-01	3.99E-03	9.12E-01	1.92E+00	0.0002877				
14	5.00E-05	2	9.61E-01	9.92E-03	9.71E-01	2.03E+00	0.0011663				
15	1.00E-04	2.1	9.80E-01	1.96E-02	1.00E+00	2.09E+00	7.326E-05				
16	2.00E-04	2.15	9.90E-01	3.85E-02	1.03E+00	2.15E+00	1.917E-06				
17	5.00E-04	2.3	9.96E-01	9.11E-02	1.09E+00	2.27E+00	0.0012073				
18	1.00E-03	2.4	9.98E-01	1.67E-01	1.16E+00	2.42E+00	0.0004205				
19	2.00E-03	2.66	9.99E-01	2.86E-01	1.29E+00	2.66E+00	1.225E-08				
20	5.00E-03	3.1	1.00E+00	5.00E-01	1.50E+00	3.09E+00	0.0001269				
21	1.00E-02	3.4	1.00E+00	6.67E-01	1.67E+00	3.42E+00	0.0004557				
22	2.00E-02	3.7	1.00E+00	8.00E-01	1.80E+00	3.69E+00	0.0001646				
23											



2 sites / 1 site

- The evaluation of these data provides our first example of the occurrence of local minima in data fitting. If we take identical initial values for $K_{Ass,1}$ and $K_{Ass,2}$, the program may attempt to fit the data with two identical binding constants. That works satisfactorily for data set B, but not at all well for data set A. In this case, it is advisable to start with significantly different initial values for the binding constants, e.g.100 and 10000.
- To evaluate the data with one binding site, we make a copy of the spreadsheet, named in "1 site", calculate the theoretical signal for θ_1 and delete columns D and E. After deleting row 2, E5 (sum of errors squared) can be minimised with respect to variation of B1:B3. The best fit is significantly worse than that possible with the assumption that n=2.



	A	B	C	D	E
1	$K_{Arr.1}$ [M^{-1}]	8.93E+03			
2	BL	1.137			
3	f	1.945			
4					
5					2.3E+00
6	c_B [M]	signal,exp	θ_1	signal,theo	error²
7	5.00E-07	0.5	4.45E-03	1.15E+00	0.4168865
8	1.00E-06	0.75	8.85E-03	1.15E+00	0.1634085
9	2.00E-06	1.1	1.75E-02	1.17E+00	0.0050629
10	5.00E-06	1.5	4.27E-02	1.22E+00	0.0783071
11	1.00E-05	1.8	8.20E-02	1.30E+00	0.2535169
12	2.00E-05	1.9	1.52E-01	1.43E+00	0.2192069
13	5.00E-05	2	3.09E-01	1.74E+00	0.0689096
14	1.00E-04	2.1	4.72E-01	2.05E+00	0.0020517
15	2.00E-04	2.15	6.41E-01	2.38E+00	0.0548024
16	5.00E-04	2.3	8.17E-01	2.73E+00	0.1818127
17	1.00E-03	2.4	8.99E-01	2.89E+00	0.2366391
18	2.00E-03	2.66	9.47E-01	2.98E+00	0.1018991
19	5.00E-03	3.1	9.78E-01	3.04E+00	0.0036301
20	1.00E-02	3.4	9.89E-01	3.06E+00	0.1150422
21	2.00E-02	3.7	9.94E-01	3.07E+00	0.3949707
22					
23					
24					

- Data set B is evaluated similarly.

Result

Data set A can be fitted significantly better with two binding constants (K_1=50000 M^{-1}, K_2=200 M^{-1}) than with one. Data set B also gives a better fit with two binding constants (K_1=10000 M^{-1}, K_2=1000 M^{-1}), but the fit with a single binding constant (K_1 = 3450 M^{-1}) is also quite reasonable. The results of this exercise make two important points: 1) it is difficult to establish unequivocally that two binding sites are present if their binding constants differ by only an order of magnitude, or less; 2) if the binding constants do differ significantly, accurate analysis requires data in which ligand concentrations are varied over several orders of magnitude, which can be experimentally very difficult to accomplish.

Exercise 11: Independent binding sites III

Evaluate binding data according to a simplified model. Assume that there are two sites and that the only species producing a signal is A fully saturated with B, i.e. BAB.

Objective of the exercise: Fitting of binding data to simplified models; how to avoid local minima.

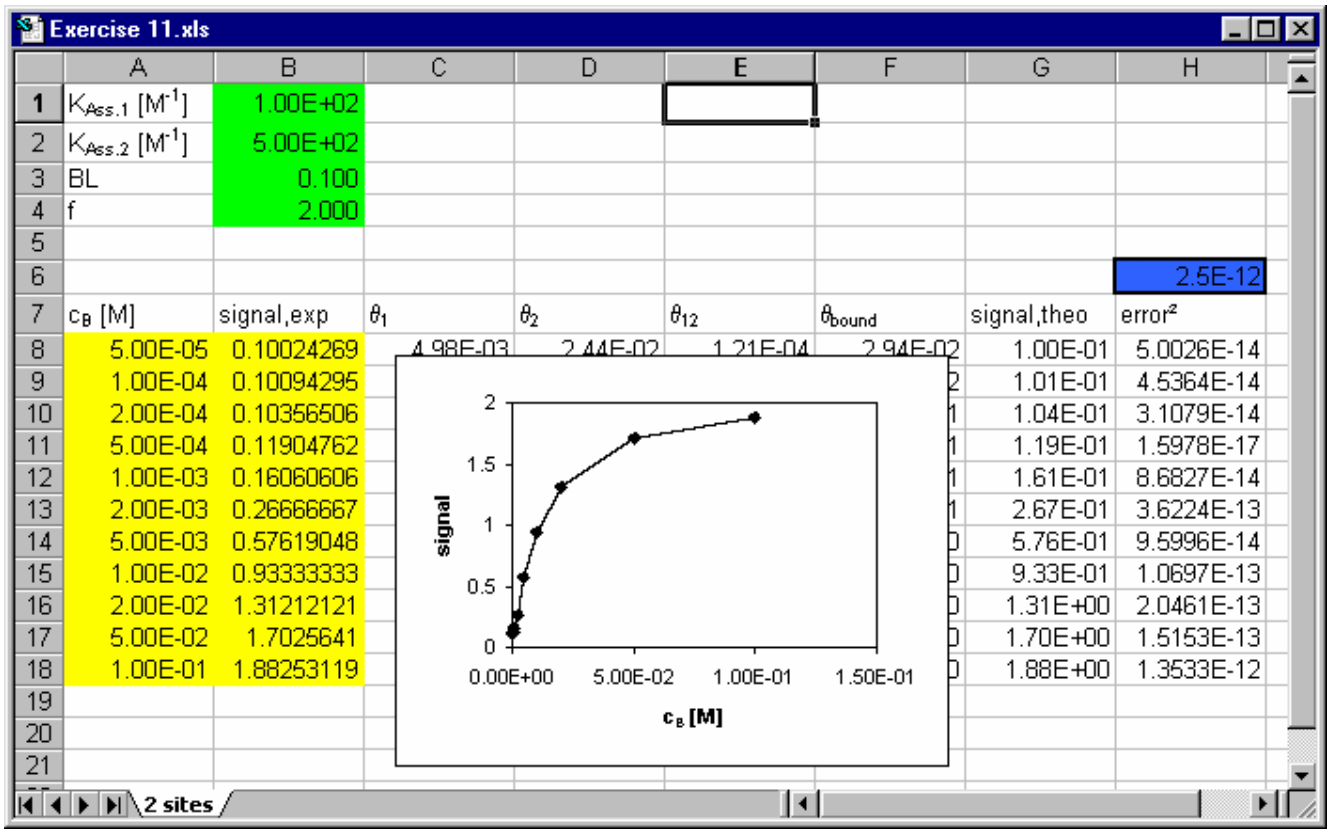
Background information about this exercise can be found as follows:

- Fitting data by the method of least squares (Sect. 9.1.8)
- Analysis of simple binding data (Sect. 9.2.6)
- Independent identical binding sites (Sect. 9.2.5)

Instructions

- In this exercise we use Eqns. 9.16 and 9.17 in the analysis.
- The global parameters are $K_{Ass,1}$ and $K_{Ass,2}$, and local parameters BL and f. First, enter the parameters into rows 1-4.
- Row 7 contains the column headings: "c_B [M]", "signal,exp", "θ_1"the saturation of site 1 with B, "θ_2" the saturation of site 2 with B, and "θ_{12}" the fraction of A in which sites 1 and 2 are both occupied by B. The other headings are "θ_{bound}" the relative saturation of all binding sites, and "signal,theo" and "error²", which have their previous meanings.
- Enter the concentrations of B in column A, and the measured signal in column B. Eqn. 9.16 is entered into column C , for site 1 for example, the following expression is entered into cell C8: "=A8*$B$1/(1+$B$1*A8)". The corresponding expression for site 2 is entered into column D. Column E contains the proportion of AB_2 ($\theta_1 \times \theta_2$) and column F to total site saturation ($\theta_1+\theta_2$). The theoretical signal is evaluated in column G, the errors squared in column H, and the sum of errors squared in cell H6.
- In the fitting, the value in H6 (sum of errors squared) is minimised with respect to variation of B1 to B4.
- To check that θ_{bound} does in fact rise hyperbolically as the ligand concentration is increased, highlight F7 to F18, press Ctrl-C, highlight the graphics and press SHIFT+INS. The curve for θ_{bound} appears on the plot. To remove this curve, highlight the graphics, then the curve, and press DELETE.
- Local minima are also prone to occur in this analysis. The data were generated with the following binding parameters: $K_{Ass,1}$=500 M^{-1}, $K_{Ass,2}$=100 M^{-1}, BL=0.1 and F=2. However, from the following initial values $K_{Ass,1}$=100 M^{-1}, $K_{Ass,2}$=100 M^{-1} (the same for the two K_{Ass}), BL=0.1 and F=1, the final best fit result is $K_{Ass,1}$=$K_{Ass,2}$=187 M^{-1}, BL=0.107 and F=1.959. The sum of errors squared for this fit was 8.6×10^{-4}. If, however, the initial values for the two K_{Ass} are chosen to be very different, e.g. $K_{Ass,1}$=100 M^{-1}, $K_{Ass,2}$=1000 M^{-1}, BL=0.1 and F=1 the best fit solution is $K_{Ass,1}$=100 M^{-1}, $K_{Ass,2}$=500 M^{-1}, BL=0.1 and F=2. The sum of errors squared is 2.5×10^{-12}, much smaller than the value for the local minimum identified above.
- As a general rule, it is advisable to carry out the minimisation several times using different sets of initial values, to exclude, as far as possible, the chance of returning results on a local minimum rather than the global minimum.
- For real data, which are subject to error "noise", it is difficult to extract reliable values of multiple K_{Ass} constants from binding curves, in part because it is hard to be sure which are the local and

which the global minima. In such cases, it is important to have sufficient data sets, evaluated globally, to increase the accuracy of the analysis.



	A	B	C	D	E	F	G	H
1	$K_{Ass.1}$ [M^{-1}]	1.00E+02						
2	$K_{Ass.2}$ [M^{-1}]	5.00E+02						
3	BL	0.100						
4	f	2.000						
5								
6								2.5E-12
7	c_B [M]	signal,exp	θ_1	θ_2	θ_{12}	θ_{bound}	signal,theo	error²
8	5.00E-05	0.10024269	4.98E-03	2.44E-02	1.21E-04	2.94E-02	1.00E-01	5.0026E-14
9	1.00E-04	0.10094295				2	1.01E-01	4.5364E-14
10	2.00E-04	0.10356506				1	1.04E-01	3.1079E-14
11	5.00E-04	0.11904762				1	1.19E-01	1.5978E-17
12	1.00E-03	0.16060606				1	1.61E-01	8.6827E-14
13	2.00E-03	0.26666667				1	2.67E-01	3.6224E-13
14	5.00E-03	0.57619048				0	5.76E-01	9.5996E-14
15	1.00E-02	0.93333333				0	9.33E-01	1.0697E-13
16	2.00E-02	1.31212121				0	1.31E+00	2.0461E-13
17	5.00E-02	1.7025641				0	1.70E+00	1.5153E-13
18	1.00E-01	1.88253119				0	1.88E+00	1.3533E-12
19								
20								
21								

Result

The optimal fit of the binding data is with two binding constants: K_1=100 M^{-1}, K_2=500 M^{-1}. There is a local minimum at the following values: K_1=K_2=187 M^{-1}.

Exercise 12: Cooperative binding

Analysis of binding data according to an all-or-nothing model. Investigate whether, bearing in mind the accuracy of the data, it can be decided whether one or two Bs bind to A.

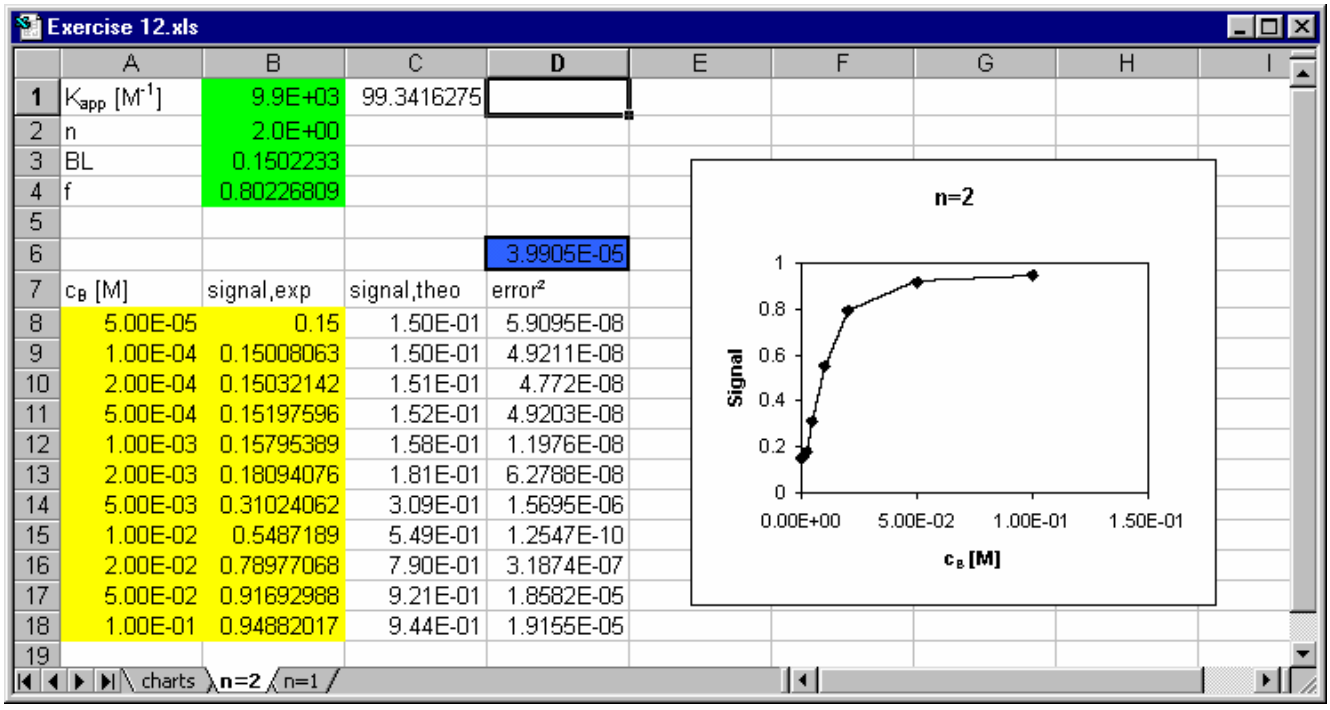
Objective of the exercise: Evaluation of all-or-nothing binding equilibria; choice of models.

Background information about this exercise can be found as follows:

- Selection of appropriate models (Sect. 9.1.5)
- Fitting data by the method of least squares (Sect. 9.1.8)
- Binding equilibria (Sect. 9.2.4)
- Cooperative binding (Sect. 9.2.8)

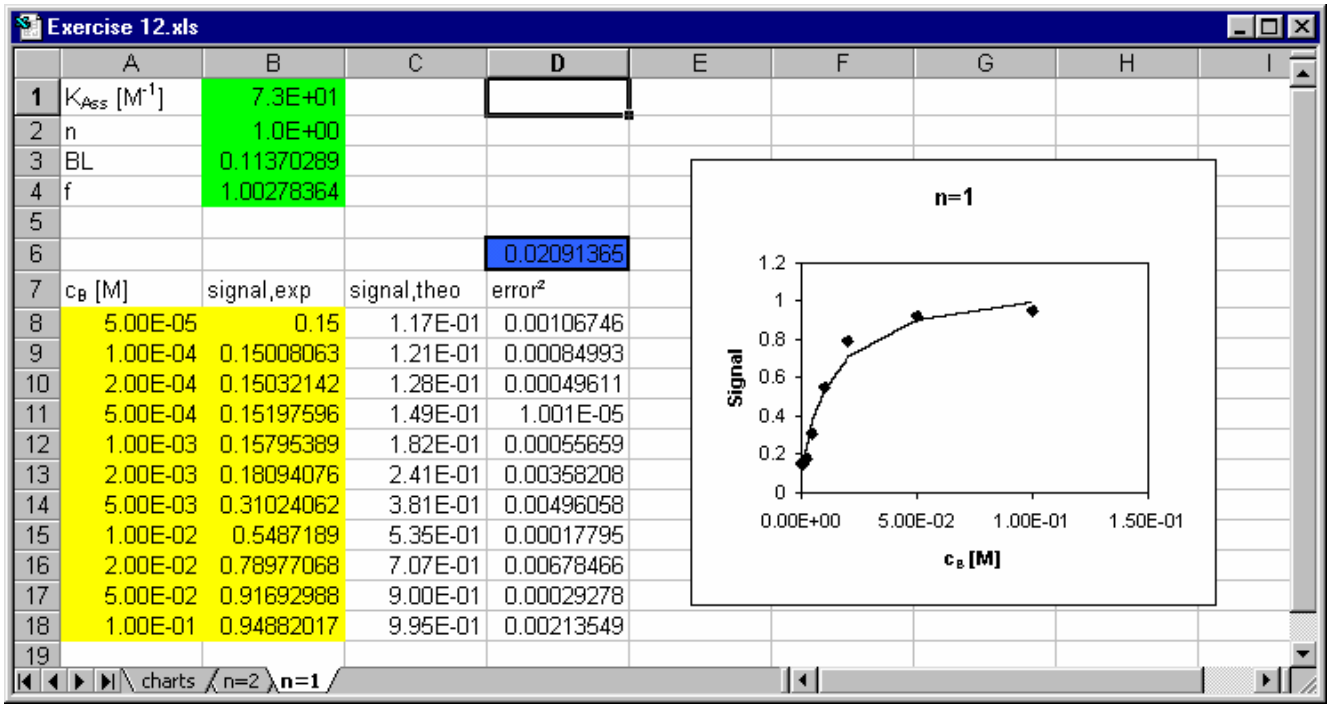
Instructions

- In this exercise we use Eqn. 9.19 for the analysis.
- The global parameters are K_{Ass} and n, and local parameters BL and f. Enter these parameters into rows 1-4.
- The column headings are entered into row 7: "c_B [M]", "signal,exp", "signal,theo" and "error²".
- Concentrations are entered into column A, and the measured signal into column B. Eqn. 9.19 is entered into column C, e.g. in cell C8: "=$B$3+($B$4*$B$1*A8^$B$2)/ (1+$B$1*A8^$B$2)". The error squared terms are in D, and the sum of errors squared in cell D6.
- The fitting minimises the sum of error squared (D6) with respect to variation of B1 to B4.



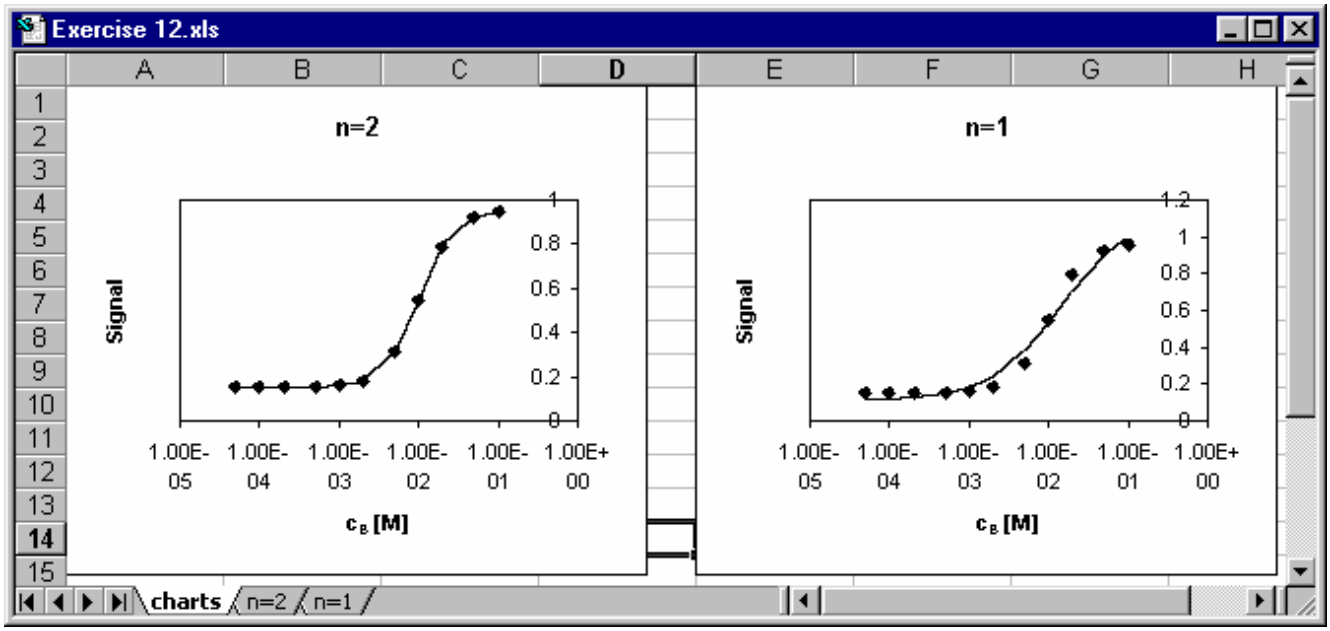
	A	B	C	D
1	K_{app} [M^{-1}]	9.9E+03	99.3416275	
2	n	2.0E+00		
3	BL	0.1502233		
4	f	0.80226809		
5				
6				3.9905E-05
7	c_B [M]	signal,exp	signal,theo	error²
8	5.00E-05	0.15	1.50E-01	5.9095E-08
9	1.00E-04	0.15008063	1.50E-01	4.9211E-08
10	2.00E-04	0.15032142	1.51E-01	4.772E-08
11	5.00E-04	0.15197596	1.52E-01	4.9203E-08
12	1.00E-03	0.15795389	1.58E-01	1.1976E-08
13	2.00E-03	0.18094076	1.81E-01	6.2788E-08
14	5.00E-03	0.31024062	3.09E-01	1.5695E-06
15	1.00E-02	0.5487189	5.49E-01	1.2547E-10
16	2.00E-02	0.78977068	7.90E-01	3.1874E-07
17	5.00E-02	0.91692988	9.21E-01	1.8582E-05
18	1.00E-01	0.94882017	9.44E-01	1.9155E-05
19				

- We check first whether the data can be fitted with an n value of 1 (with n=1 the all-or-nothing model becomes a simple binding model). To do this, we create a copy of the spreadsheet, called n=1, to distinguish it from the original sheet which is called n=2. We then set n equal to 1 and 2 respectively in the two speadsheets and minimise both sheets with respect to K_{Ass}, BL and f.



	A	B	C	D
1	K_{Ass} [M^{-1}]	7.3E+01		
2	n	1.0E+00		
3	BL	0.11370289		
4	f	1.00278364		
5				
6				0.02091365
7	c_B [M]	signal,exp	signal,theo	error²
8	5.00E-05	0.15	1.17E-01	0.00106746
9	1.00E-04	0.15008063	1.21E-01	0.00084993
10	2.00E-04	0.15032142	1.28E-01	0.00049611
11	5.00E-04	0.15197596	1.49E-01	1.001E-05
12	1.00E-03	0.15795389	1.82E-01	0.00055659
13	2.00E-03	0.18094076	2.41E-01	0.00358208
14	5.00E-03	0.31024062	3.81E-01	0.00496058
15	1.00E-02	0.5487189	5.35E-01	0.00017795
16	2.00E-02	0.78977068	7.07E-01	0.00678466
17	5.00E-02	0.91692988	9.00E-01	0.00029278
18	1.00E-01	0.94882017	9.95E-01	0.00213549
19				

- To compare the quality of the two fits, we copy both plots to a new spreadsheet.
- It is evident that the fit to the data is much better for the n=2 analysis. This is much clearer when the curves are presented with a logarithmic x-axis. Click on the plot, double click on the x-axis, chose “Scaling”, then “Logarithmic”.



Result

The data can be fitted to an all-or-nothing model with n=2 and K_{app}=6700 M^{-2}. This corresponds to a K_{Ass} of 99.3 M^{-1}. An equivalently good fit is not possible with the assumption of a single binding site.

Exercise 13: Analysis of binding data by different models

Analyse the data sets according to several different models: n=1, n=2 with independent binding sites, n=2 cooperative binding. Which model fits the data best? For the various fits, plot the deviations between experimental and theoretical data, and compare the absolute magnitude and scattering of the deviations.

Objective of the exercise: choosing between models

This exercise is linked to Exercises 8-12.

Background information about this exercise can be found as follows:

- Selection of appropriate models (Sect. 9.1.5)

Instructions

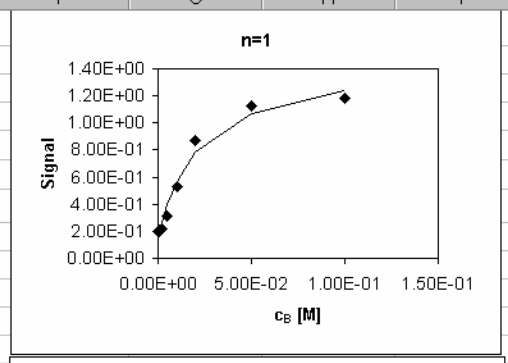
- For each data set programme a separate spreadsheet for n=1, n=2 independent binding (Exercise 10) and cooperative binding (Exercise 12) and carry out the fitting.
- Prepare a plot of the deviations of the best fit of each of the models. To do this, we calculate the deviations (expressed as: Signal,exp-Signal,theo) in the column next to "error²" . In the title row (row 7) of this column, enter the model descriptor: "n=1", "n=2" or "coop", as appropriate. Highlight the relevant x-values and the deviations with the column heading, click on Chart Wizard, and select bar diagram.
- Compare the plots of the deviations. The best fit should have the smallest deviations, which should be distributed randomly.

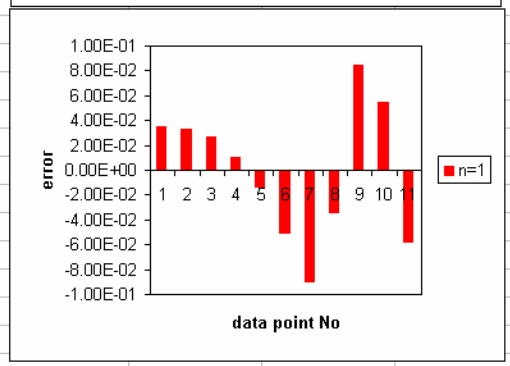
For data set A, we obtain the following spreadsheets:

D1 =

	A	B	C	D	E
1	K_{Ass} [M^{-1}]	4.4E+01			
2					
3	BL	0.1616329			
4	f	1.3181521			
5					
6				0.02852161	
7	c_B [M]	signal,exp	signal,theo	error²	n=1
8	5.00E-05	2.00E-01	1.65E-01	0.00125663	3.54E-02
9	1.00E-04	2.00E-01	1.67E-01	0.0010591	3.25E-02
10	2.00E-04	2.00E-01	1.73E-01	0.00071672	2.68E-02
11	5.00E-04	2.01E-01	1.90E-01	0.00011569	1.08E-02
12	1.00E-03	2.04E-01	2.18E-01	0.00018604	-1.36E-02
13	2.00E-03	2.19E-01	2.69E-01	0.00250808	-5.01E-02
14	5.00E-03	3.11E-01	4.01E-01	0.00809758	-9.00E-02
15	1.00E-02	5.33E-01	5.67E-01	0.00114066	-3.38E-02
16	2.00E-02	8.66E-01	7.81E-01	0.00715381	8.46E-02
17	5.00E-02	1.13E+00	1.07E+00	0.00299686	5.47E-02
18	1.00E-01	1.18E+00	1.24E+00	0.00329044	-5.74E-02
19					
20					
21					
22					
23					
24					
25					

Charts | n=1 | n=2 | coop

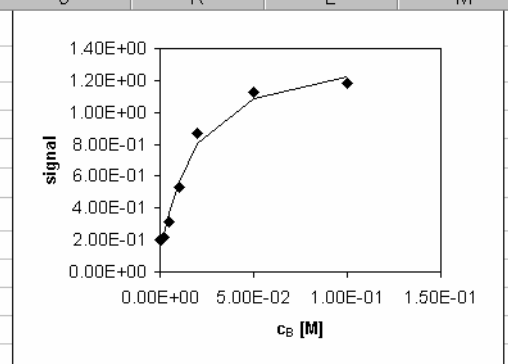


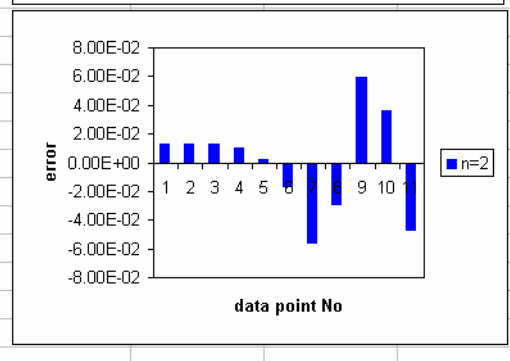


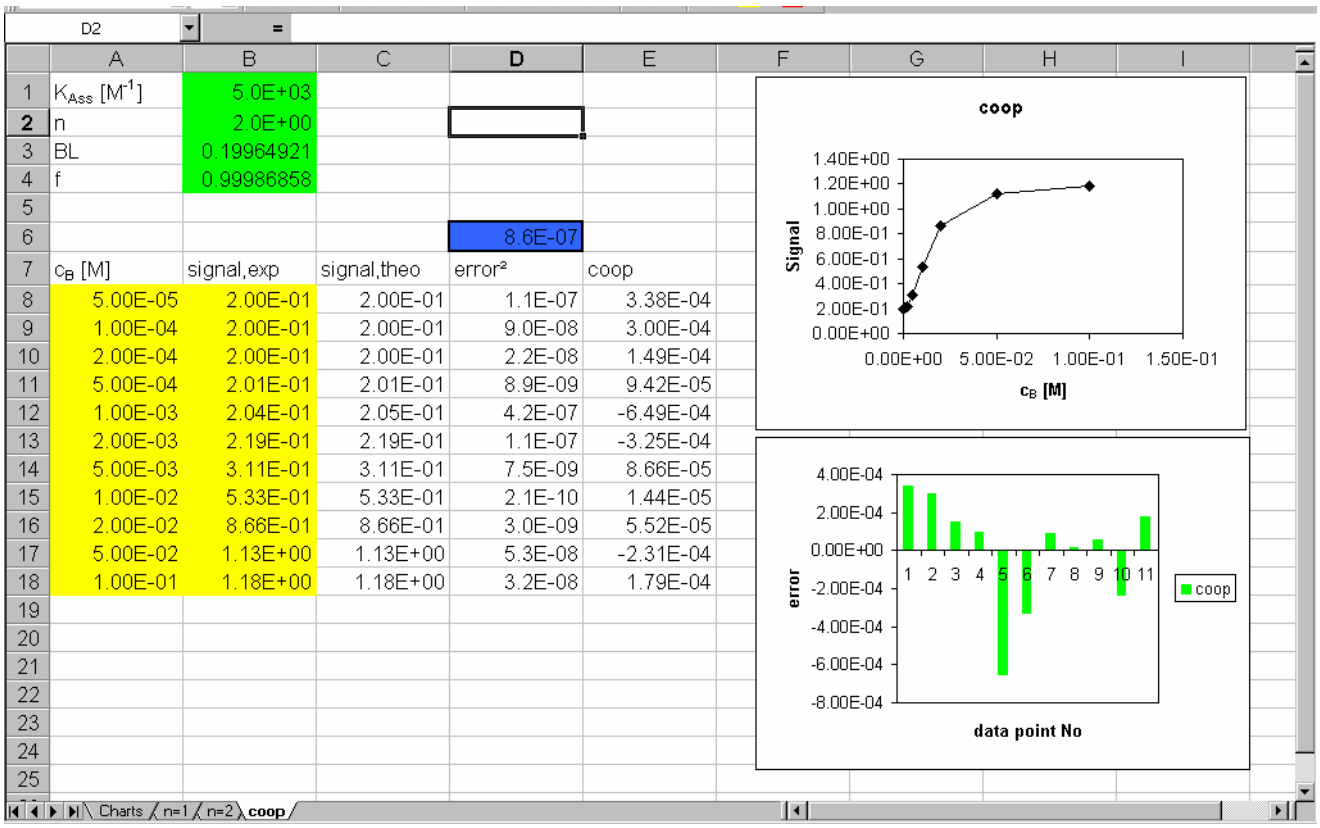
H1 =

	A	B	G	H	I
1	$K_{Ass.1}$ [M^{-1}]	1.25E+02			
2	$K_{Ass.2}$ [M^{-1}]	1.25E+02			
3	BL	0.187			
4	f	1.212			
5					
6				1.2E-02	
7	c_B [M]	signal,exp	signal,theo	error²	n=2
8	5.00E-05	2.00E-01	1.87E-01	0.00017513	1.32E-02
9	1.00E-04	2.00E-01	1.87E-01	0.00017147	1.31E-02
10	2.00E-04	2.00E-01	1.87E-01	0.00015764	1.26E-02
11	5.00E-04	2.01E-01	1.91E-01	0.00010129	1.01E-02
12	1.00E-03	2.04E-01	2.02E-01	5.0208E-06	2.24E-03
13	2.00E-03	2.19E-01	2.35E-01	0.00026979	-1.64E-02
14	5.00E-03	3.11E-01	3.67E-01	0.00309875	-5.57E-02
15	1.00E-02	5.33E-01	5.62E-01	0.0008306	-2.88E-02
16	2.00E-02	8.66E-01	8.06E-01	0.00356933	5.97E-02
17	5.00E-02	1.13E+00	1.09E+00	0.001338	3.66E-02
18	1.00E-01	1.18E+00	1.23E+00	0.00217141	-4.66E-02
19					
20					
21					
22					
23					
24					
25					

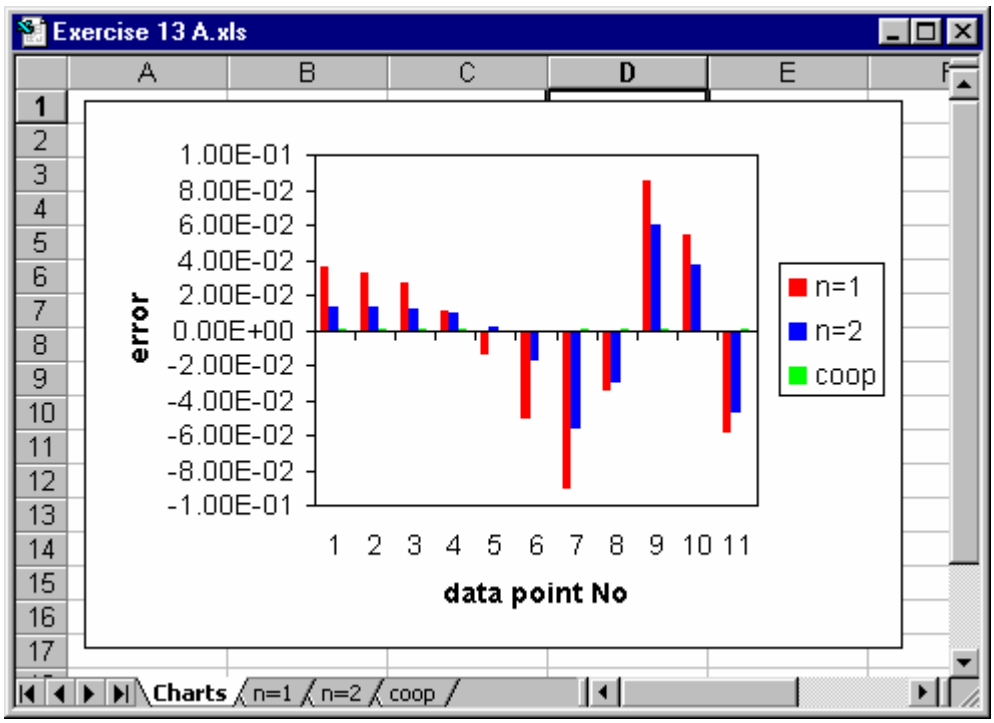
Charts | n=1 | n=2 | coop



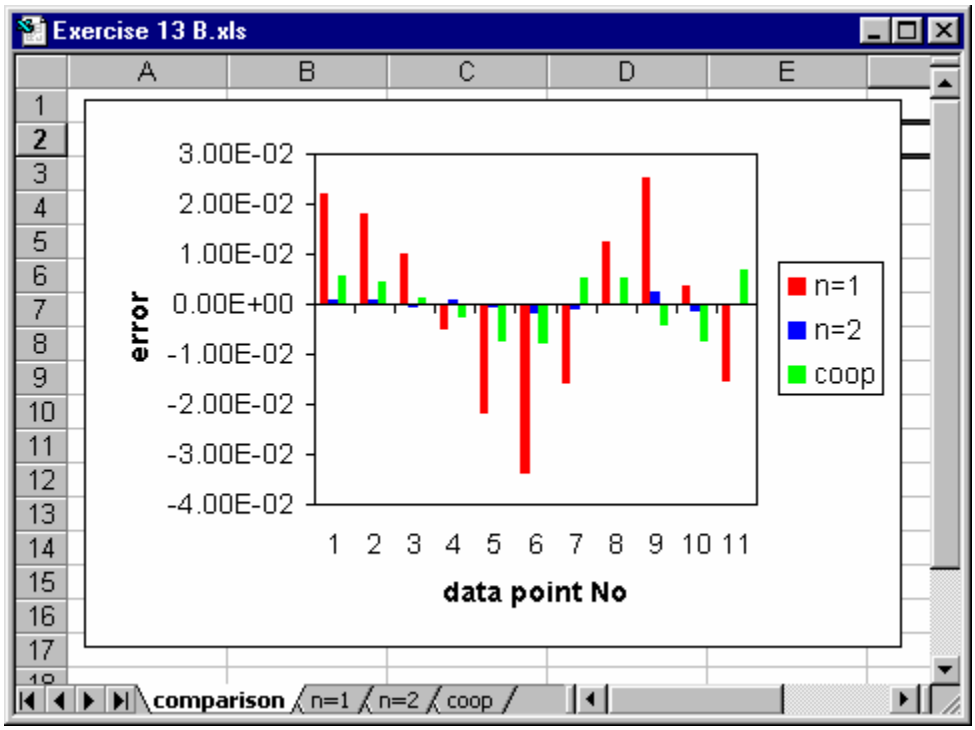




The clearest way of comparing the quality of the fits is to show all of the errors in one diagram. To do this, create a new spreadsheet, called Charts. Go to the spreadsheet n=1, click on the bar diagram, press Ctrl-C, go to the Charts Table, highlight a cell with the mouse, and press Shift+INS. Then move to the n=2 sheet, click on the bar diagram, press Ctrl-C, go to the Charts Table, highlight the diagram that is already there, and press Shift+INS. The data for n=2 will be transferred into to diagram. Go to the coop spreadsheet, and proceed as before, to present all three bar diagrams on one plot.



Similar analysis for data set B gives the following picture:



Result

Data set A fit best to a cooperative model, whereas data set B are best described by independent sites with n=2. It should be noted that real data usually show more errors (noise) than the present test data, and consequently it can be more difficult in practice to make decisions about which model is the best.

Exercise 14: Fitting rapid reaction data to exponential functions.

Fit the kinetic data to one or two exponential functions (Eqn. 9.25). Examine which model fits the data better, and decide whether the difference is significant.

Objective of the exercise: programming exponential functions to fit kinetic data; selection of appropriate models.

Background information about this exercise can be found as follows:

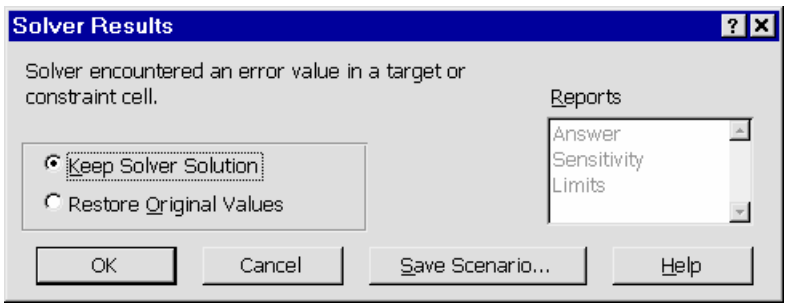
- Selection of appropriate models (Sect. 9.1.5)
- Fitting data by the method of least squares (Sect. 9.1.8)
- Global fitting of multiple data sets (Sect. 9.1.9)
- Pre-steady state kinetics, (Sect. 9.2.10)

Instructions

The theoretical data for the two models are calculated from the following equations:

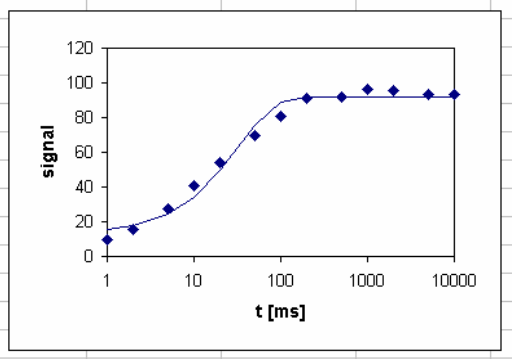
$S(t) = BL + A \times [1\text{-}\exp(-t/\tau)]$ and $S(t) = BL + A_1 \times [1\text{-}\exp(-t/\tau_1)] + A_2 \times [1\text{-}\exp(-t/\tau_2)]$.

- The parameters τ_1 [ms], A_1, τ_2 [ms], A_2 and BL are entered in the first 4 rows. Initial values are taken to be 100, 100, 100 and 0, respectively.
- Row 7 contains the titles of the of the columns: “t [ms]”, “signal,exp”, “signal,theo” and “error²”.
- The times and experimental signals are entered into columns A and B respectively, from row 8 onwards.
- Eqn. 9.25 is entered into column C, e.g. for cell C8: “=$B$5+(1-EXP(-A8/$B$1))*$B$2+(1-EXP(-A8/$B$3))*$B$4”.
- The errors squared are entered into column D, e.g. for D8: “=(B8-C8)^2”
- The sum of errors squared: “=SUM(D8:D20)” are entered into cell D2.
- We produce plots with logarithmic x-axes, and the values “Signal,exp” and “Signal,theo”.
- The fitting is carried out by minimising the sum of errors squared (D2) with respect to variation of B1 to B5.
- We obtain an error message because the algorithm is trying to use a negative value for τ.



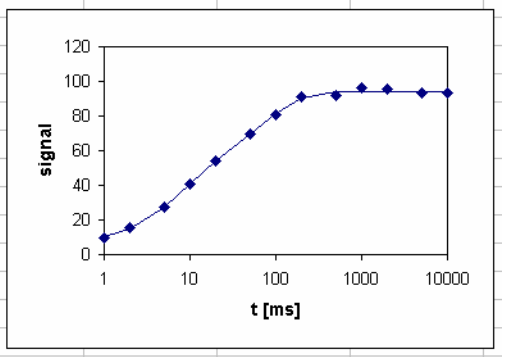
- Select Cancel.
- The occurrence of this error means that we must select more realistic initial values for the parameters. We chose 10 for the two times τ, and 50 for the two amplitudes, and try the fitting again.
- We obtain a fit with $\tau_1 = \tau_2 = 32.2$ ms. The sum of errors squared has the value 158.5 and the fit looks a lot worse that we are used to. It appears that are systematic errors in the fit.

	A	B	C	D
1	τ_1 [ms]	32.2488097		sum of error²
2	A_1	39.573949		226.930102
3	τ_2 [ms]	32.2488099		
4	A_2	39.5739486		
5	BL	13.0697606		
6				
7	t [ms]	signal,exp	signal,theo	error²
8	1	10	15.4863874	30.1004472
9	2	15.2	17.8292273	6.91283619
10	5	27.1	24.4372125	7.09043736
11	10	40.6	34.1720378	41.3186984
12	20	54	49.6480619	18.9393654
13	50	69.8	75.4261481	31.6535427
14	100	80.9	88.6552792	60.1443553
15	200	90.8	92.0573186	1.58085007
16	500	91.7	92.2176436	0.26795491
17	1000	96	92.2176582	14.3061093
18	2000	95.5	92.2176582	10.7737675

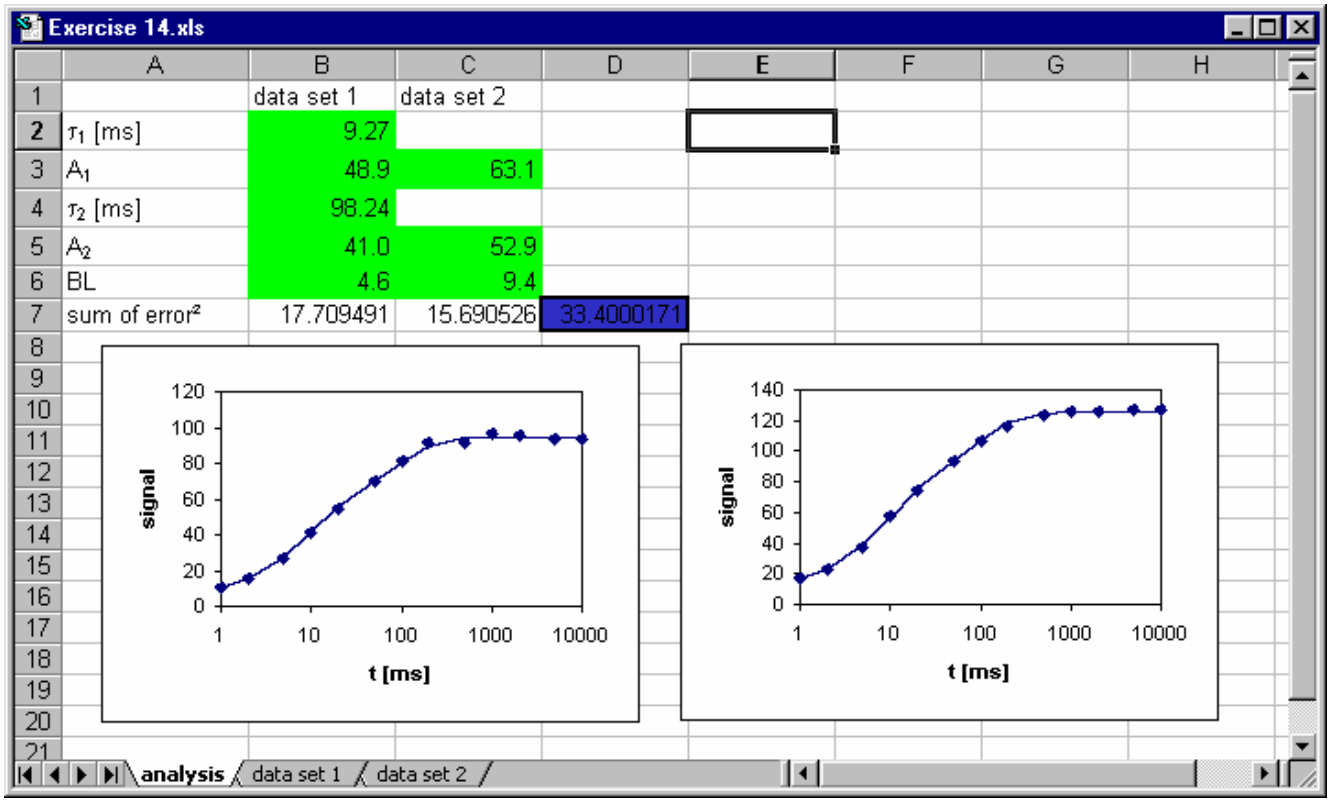


- Because of this poor result, we chose new initial values, 10 and 100 for the two τ values, 10 for the amplitudes and 0 for the background parameter BL, and try again.
- This time the sum of errors squared is 11.9 and the solution is $\tau_1 = 8.2$ ms and $\tau_2 = 78$ ms. The fit looks much better, as we would expect from the lower value of 11.9. It appears that the solution $\tau_1 = \tau_2 = 32.2$ ms corresponds to a local minimum.

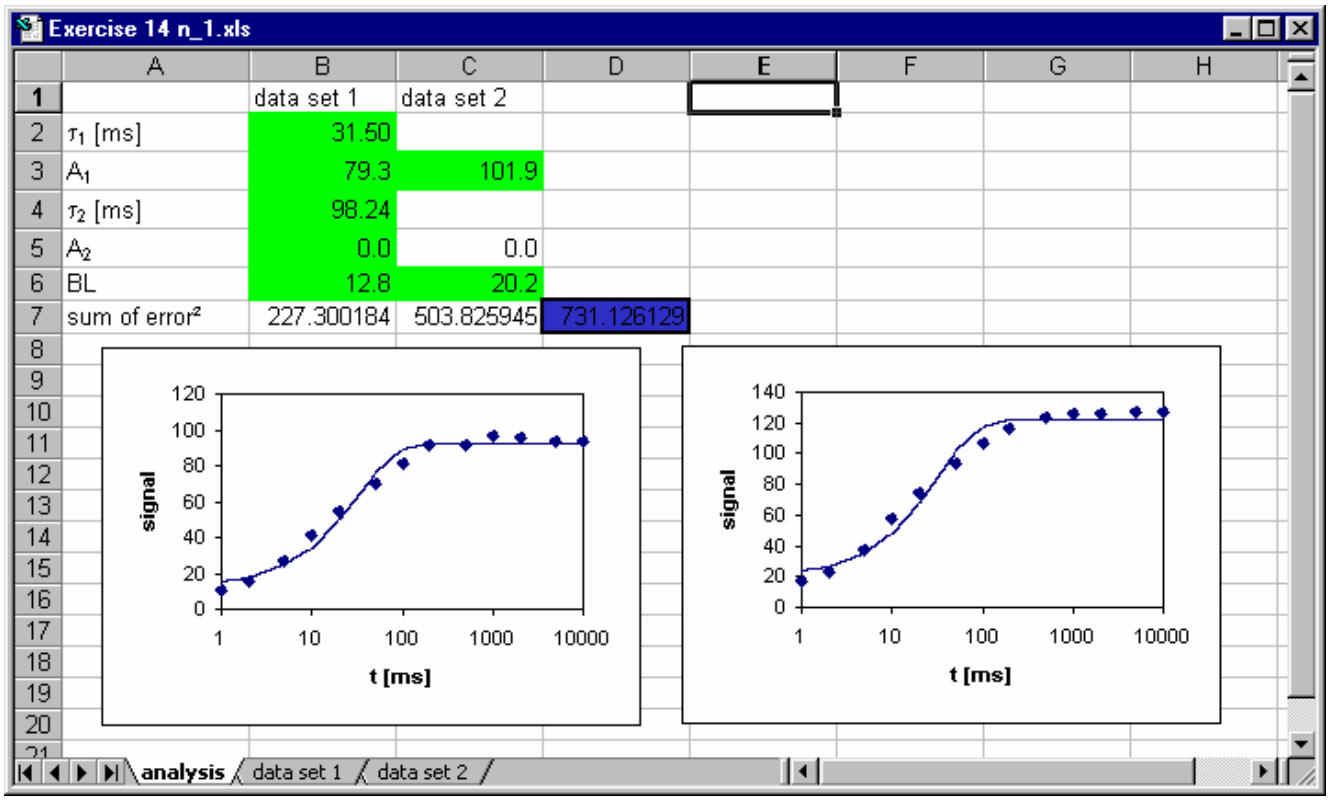
	A	B	C	D
1	τ_1 [ms]	8.18414716		sum of error²
2	A_1	42.8858124		11.9042781
3	τ_2 [ms]	78.0238621		
4	A_2	46.641726		
5	BL	4.5994569		
6				
7	t [ms]	signal,exp	signal,theo	error²
8	1	10	10.1260506	0.01588874
9	2	15.2	15.0777423	0.01494695
10	5	27.1	27.1002376	5.6449E-08
11	10	40.6	40.4584479	0.02003699
12	20	54	54.3076803	0.09466715
13	50	69.8	69.458271	0.1167787
14	100	80.9	81.1801427	0.07847994
15	200	90.8	90.5333137	0.07112156
16	500	91.7	94.0501376	5.52314682
17	1000	96	94.1268686	3.50862121
18	2000	95.5	94.1269953	1.88514203
19	5000	93.5	94.1269953	0.39312305
20	10000	93.7	94.1269953	0.18232495



- In order to analyse both data sets globally, copy the spreadsheet that has just been made, and enter the second data set into column B.
- We then create a new spreadsheet to evaluate the data sets and call this “analysis”.
- The two τ values (which are valid for both data sets) are in cells B2 and B4, for each data set a value for A_1 (in B3 and C3), for A_2 (in B5 and C5) and a background BL (in B6 and C6). The parameters are transferred from the “analysis” spreadsheet to the subsidiary tables (e.g. we enter in the filed for τ1 for data set 1: “=analysis!B2”).
- The error squared values from the subsidiary tables are transferred to the analysis table. This is done by entering in B7 the sum of errors squared of data set 1: “='data set 1'!D2” and in C7 that of data set 2: “='data set 2'!D2”. In D7 we enter the sum of B7 and C7.
- The plots from the subsidiary spreadsheets are copied into the analysis spreadsheet. Move to the subsidiary spreadsheet, highlight the plot, press Ctrl-C, move to the analysis spreadsheet, highlight any cell, and press SHIFT+INS. The plot can be move to a convenient position with the mouse.
- Take initial values of 10 and 100 for the two τ parameters, 10 all of the amplitudes and 0 for the background BL, and minimise the value of D7 (the combined sum of errors squared) with respect to variation of B2 to B6, C3, C5 and C6.
- We can reduce the number of variables, for example, it is reasonable to assume that the amplitude ratio A_1/A_2 is the same for both data sets. In the analysis spreadsheet, we can write for A_2 of data set 2: $A_1(2)/A_1(1)\times A_1(1)$ (“=B5/B3*C3”) and not include this value in the fitting.



- Begin the minimisation again with initial values of 10 ms for both τ parameters and 50 for the two amplitudes. Under these conditions, we located a local minimum with data set 1. However, with the two data sets, the algorithm locates the correct minimum, even with these starting parameters. This result indicates that the analysis is more stable with two data sets.
- To fit the data to a single relaxation time, we set A_2=0 and exclude τ_2 and A_2 from the fitting.



Result

The analysis with two relaxation times gives τ_1=9.3 ms, τ_2=98 ms, and the sum of errors squared was 33.4. Fitting to a single relaxation time is much worse; the sum of errors squared was 731, and the fitting showed systematic deviations from the data.

Exercise 15: Error estimates for Exercise 14.

Evaluate the errors in the time constants determined in Ex. 14.

Objective of the exercise: error analysis

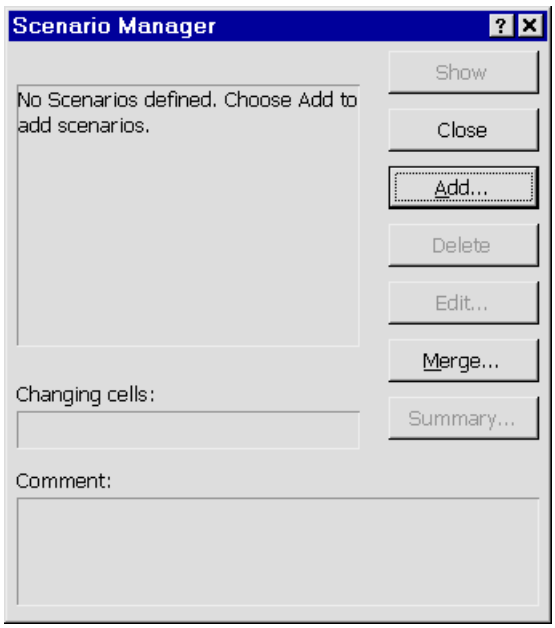
This Exercise is linked to Exercise 14.

Background information about this exercise can be found as follows:

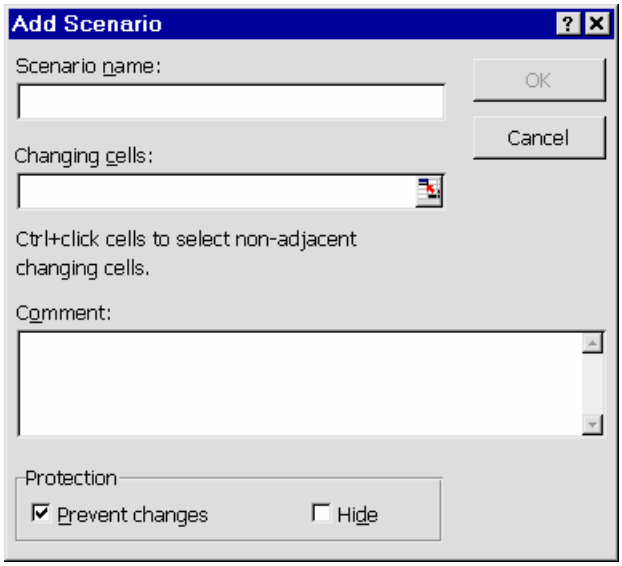
- Fitting data by the method of least squares (Sect. 9.1.8)
- Introduction to error estimation (Sect. 9.1.10)

Instructions

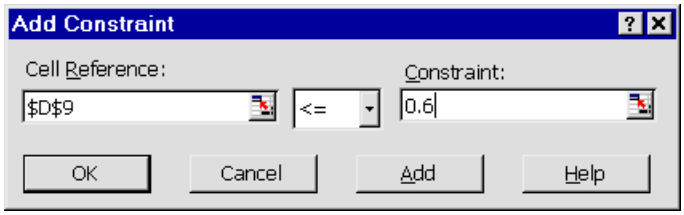
- First, we calculate the mean error of the measurement, using the square root of the sum of errors squared in cell D7 of the analysis spreadsheet ("=SQRT(SUM(B7:C7))"). This value corresponds to the total error of all data points.
- For comparison, we determine the sum of the measurements in the two subsidiary spreadsheets, and transfer these to row 8 of the analysis spreadsheet.
- We calculate the relative mean error of all points in % in cell D9 (=D7/D8*100).
- The mean error amounts to only 0.4 %, which indicates that the fit was excellent.
- In D1 and E1 we enter "min" and "max".
- From the Tools menu select: Scenarios



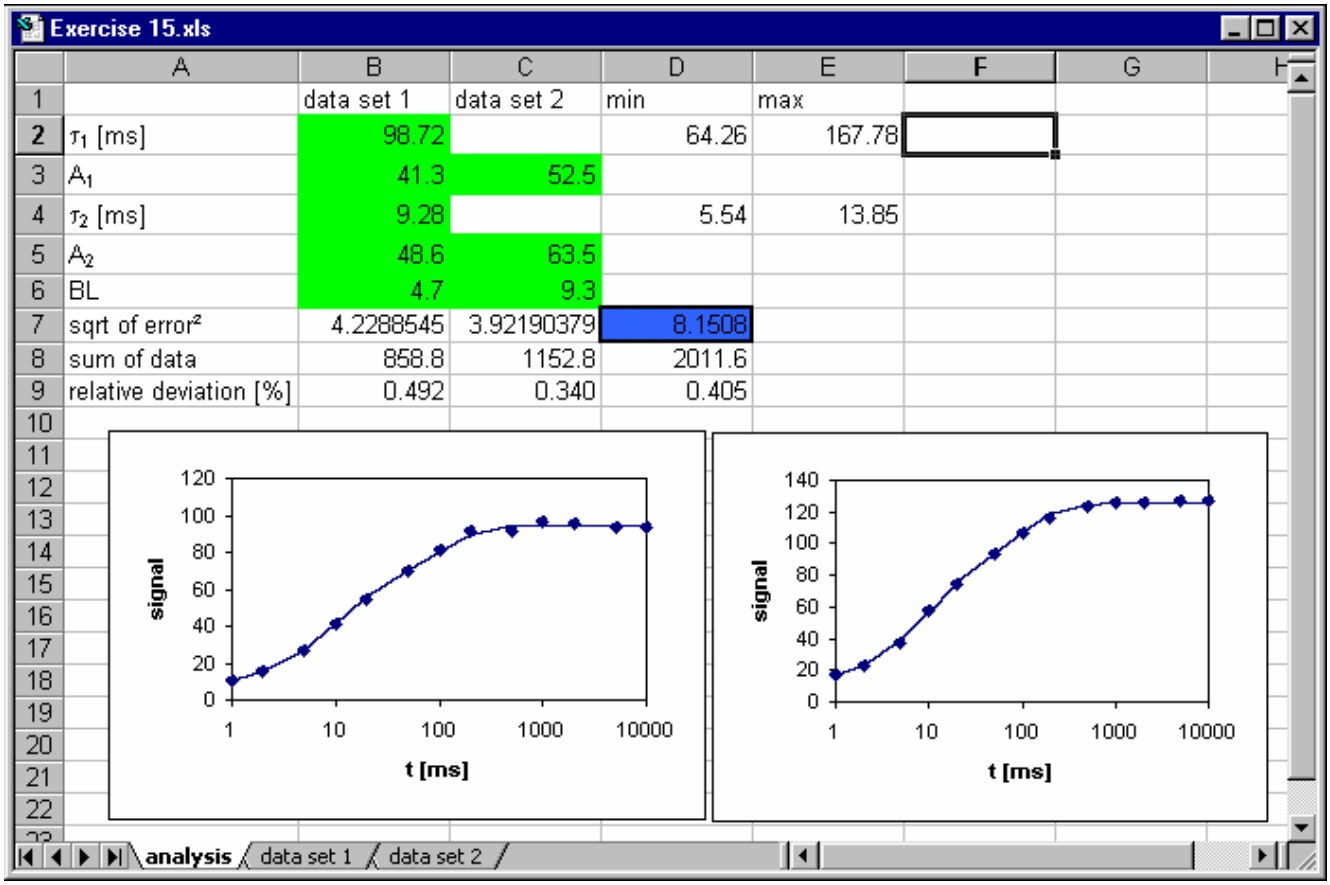
- Select Add.



- Enter under Scenario name: “best fit”, “Changing cells”: $B$2:$B$6;$C$3;$C$6 (these cells can be highlighted with the mouse), OK, OK, close
- Minimise and then maximise the two τ values. Enter the condition that D9<=0.6. This corresponds to an increase of 50% in all of the deviations. Select in the Solver window: “Subject to constraints”, “Add”.
- In the following window enter D9, select “<=” in the pull down menu and enter 0.6.



- Chose τ_1 for minimisation. As usual, all of the variables are allowed to float (τ_1, τ_2, A_1, BL_1, BL_2). Carry out the minimisation repeatedly until the result is constant. Start the minimisation again with an initial value of 60. The result cannot be further reduced, so record the result of the minimisation (62.62) in D2.
- In the Tools menu, select Scenarios, best fit, Show.
- Now maximise τ_1, and then find the minimum and maximum of τ_2. Go from the limiting values back to the best fit values, and note down all of the results.



Result

The best fit value of τ_1 was 98.3. This value could, however, lie between 64.26 and 167.78, although we have only allowed an increase in the error of 50% over the best fit error. The best values for τ_2 were: best fit time 9.3, error range: 5.54-13.85. The very large error range in the τ estimates is explained by the fact that the two parameters are correlated, and that an increase in τ_1 can therefore at least in part be compensated by a reduction in the value of τ_2 .

Exercise 16: pH-Dependence of enzyme-catalysed reactions

Analyse the pH dependence of an enzyme catalysed reaction, with the assumption that it is affected by up to 3 protonation equilibria.

Objective of the exercise: analysis of pH-effects; selection between models.

Background information about this exercise can be found as follows:

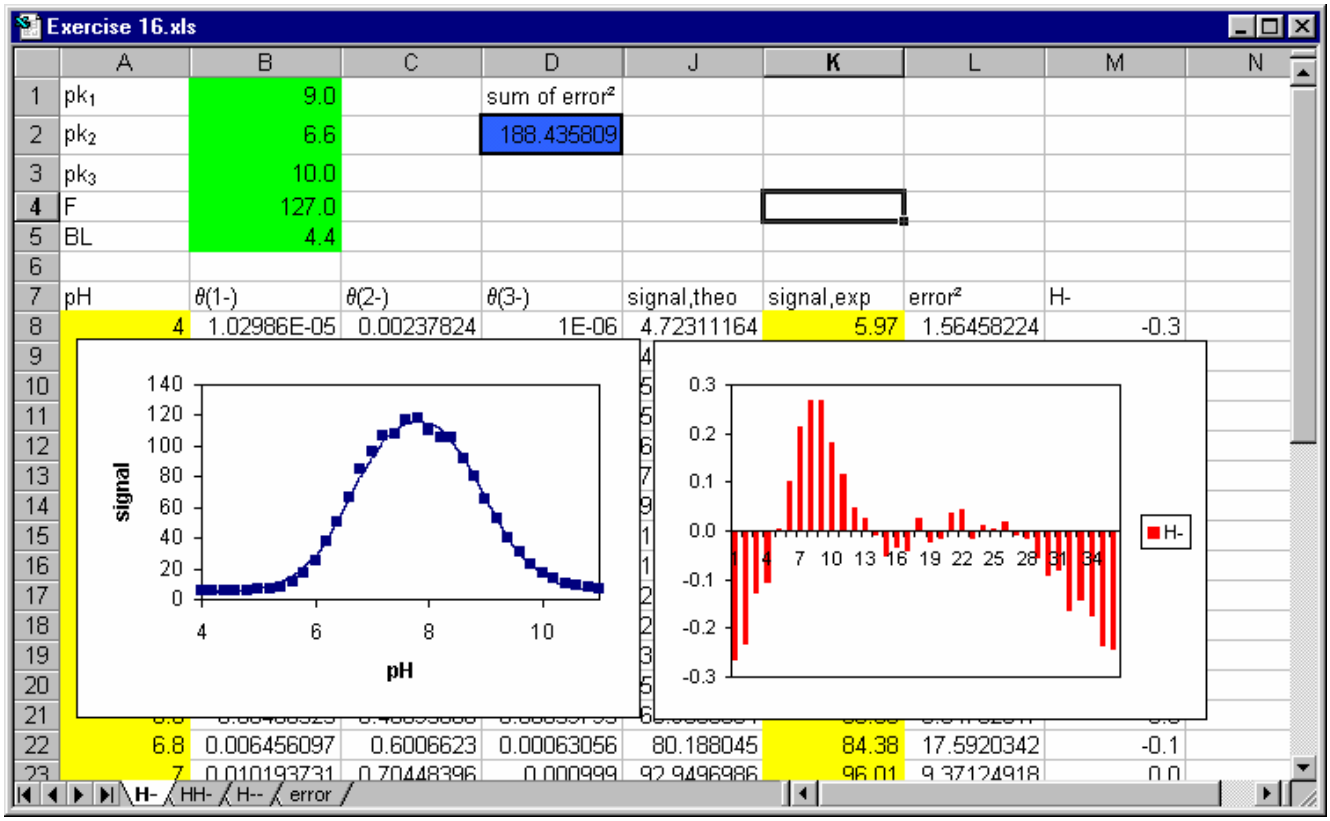
- Fitting data by the method of least squares (Sect. 9.1.8)
- Selection of appropriate models (Sect. 9.1.5)
- pH dependence of enzyme-catalysed reactions (Sect. 9.2.11)

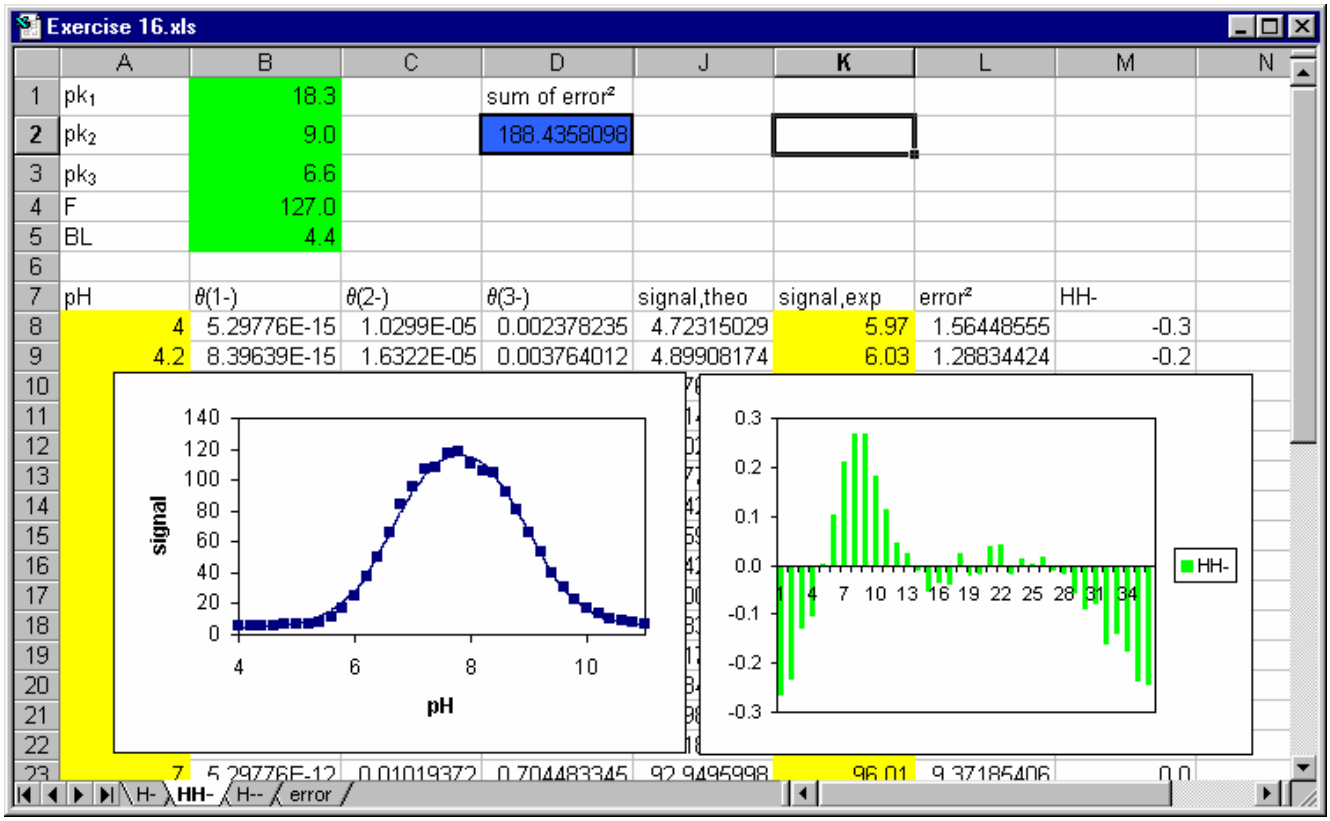
Instructions

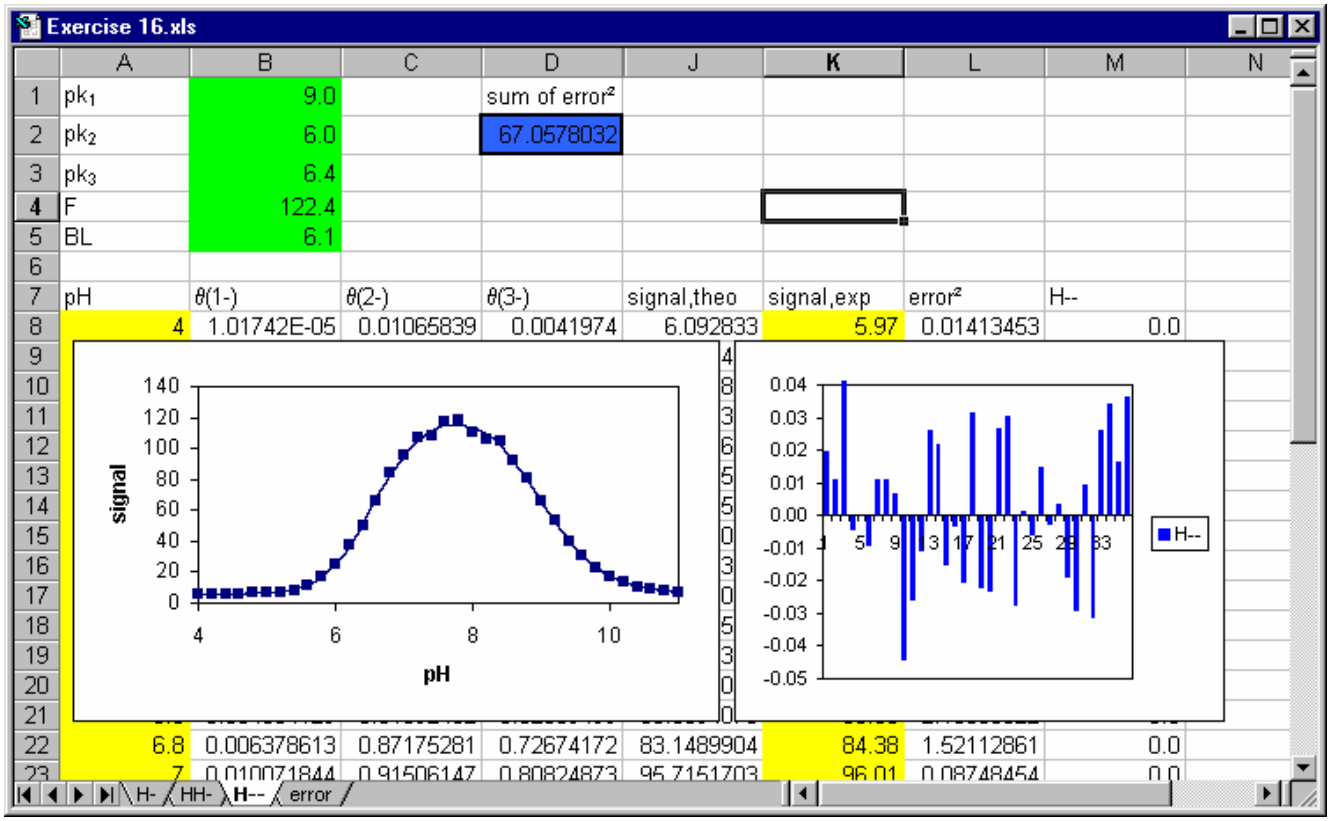
This exercise uses a general model to analyse thermodynamic measurements. In this model, ligands (protons in the present case) bind to a macromolecule, which itself produces a signal (in this case a rate) depending on the occupation of its ligand binding sites.

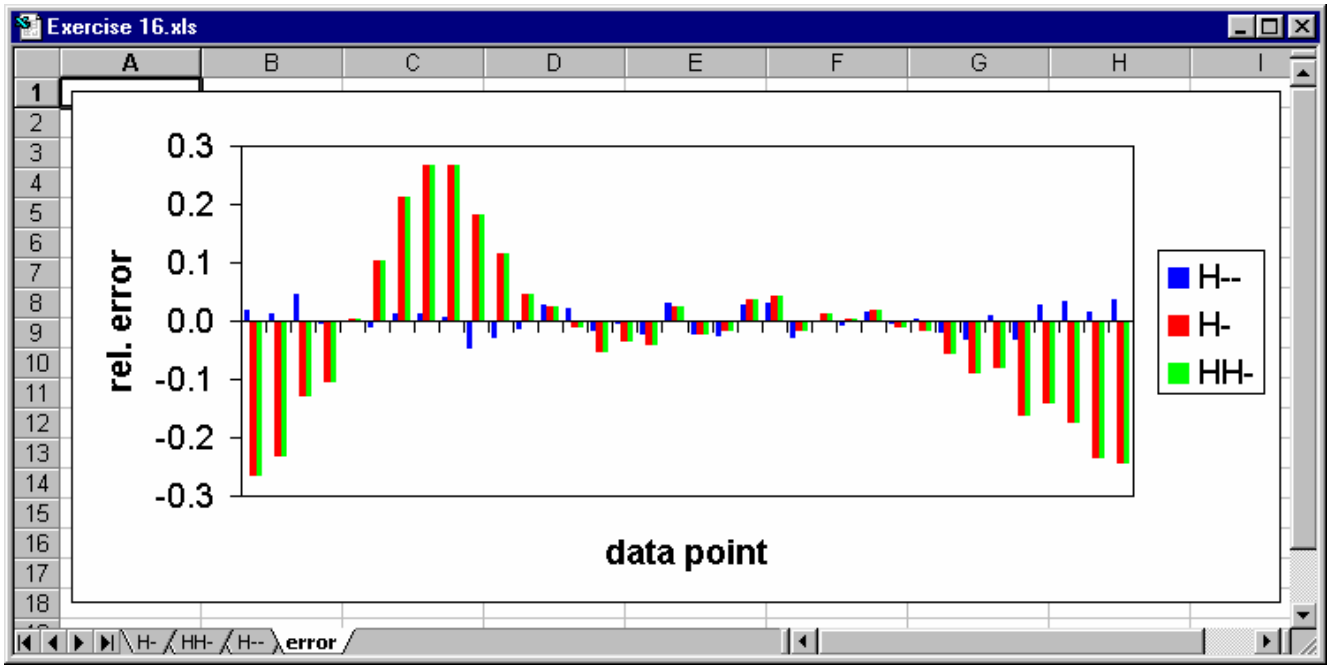
- If the enzyme has 3 protonatable groups, a total of 8 ($=2^3$) different species can exist in solution: HHH, HH-, H-H, H--, -HH, -H-, --H und ---, where H signifies the presence of a proton, and – the absence. The probability of forming these species, and hence their relative concentrations, is given by the product of the relevant probabilities that each group exists in a particular state, e.g. P(HHH) = P(group 1 is protonated) * P(group 2 is protonated) * P(group 3 is protonated); or for P(H-H) = P(group 1 is protonated) * P(group 2 is deprotonated) * P(group 3 is protonated).
- However, since we cannot correlate any of the pK_a values to a group, their order is arbitrary. Therefore, the number of possible species can be reduced to HHH, HH- (equivalent to H-H and -HH), H-- and ---.
- The probability of an acidic group being in the deprotonated state is given by Eqn. 9.27:
  $\theta(-) = c(A^-)/c(A_{ges}) = 10\text{^}(-pK_a) / [10\text{^}(-pH)+10\text{^}(-pK_a)]$
- The probability of being protonated is then simply: P(protonated)= 1-P(deprotonated).
- From these conditions it is possible to simulate the concentration dependence of all 4 species on pH. To do this, make a spreadsheet with the parameters pK_1, pK_2, pK_3, F and BL in the first 5 rows. Row 7 contains the headings of the columns: “pH”, “θ(1-)”, “θ(2-)”, “θ(3-)”, “HHH”, “HH-“,“H--“, “---“, “H-“, “signal,theo”, “signal,exp”, “error²”. H- represents an easier model with only two protonation steps, that ignores pK_3.
- In column A (“pH”) we enter the pH values between 4 and 11 (at intervals of 0.2). Columns B, C and D contain the probabilities that sites 1, 2 or 3 are deprotonated.
- So, for example, in cell B8 we enter: “=10^(-$B$1)/(10^(-A8)+10^(-$B$1))”.

- The concentrations of the species are given by the products of the probabilities that the groups are in the required state: P(HHH) = P(group 1 is protonated) * P(group 2 is protonated) * P(group 3 is protonated). So for cell E8 (HHH) we enter "=(1-B8)*(1-C8)*(1-D8)".
- We can interpret the observed enzyme turnover rate as an "effect", and therefore the results of the kinetic experiments can be used as a reporter to analyse the thermodynamic protonation equilibria. In general, one would assign each of the possible species an effect factor, and the signal would be the sum of all these factors. To make the problem more tractable, we assume that only one species contributes to catalytic activity and that there is in addition a baseline rate (BL).
- Since the activity rises in the transition from low to moderate pH, at least one of the groups in the active species must be deprotonated, and since the activity falls again at high pH, at least one group must be protonated. There are then three possible models: a model with two protonation equilibria (H-) and two models with three protonation equilibria (HH-) or (H--).
- In the (HH-) model, the theoretical signal is given by: F×(HH-)+BL. In the two protonation model (H-) we ignore the third protonation equilibrium and the signal is given by: F×(H-H + H--)+BL.
- Enter the theoretical signal in column M, the measured signal in column N, and the errors squared in column O. Column P contains the deviation between the theoretical and measured signals. The sum of errors squared is entered into cell D2.
- Prepare a XY-plot of the experimental and theoretical data, and also a column diagram of the deviations.
- We make a spreadsheet for each model and, as usual, minimise the sum of errors squared (D2) with respect to variation of pK_1, pK_2 and pK_3.
- To compare the results we place all of the error columns on a single diagram.


Exercise 16.xls
pk1 9.0
pk2 6.6
pk3 10.0
F 127.0
BL 4.4
sum of error² 188.435809
pH θ(1-) θ(2-) θ(3-) signal,theo signal,exp error² H-
4 1.02986E-05 0.00237824 1E-06 4.72311164 5.97 1.56458224 -0.3
6.8 0.006456097 0.6006623 0.00063056 80.188045 84.38 17.5920342 -0.1
signal
pH
H-
H- HH- H-- error


Exercise 16.xls
pk1 18.3
pk2 9.0
pk3 6.6
F 127.0
BL 4.4
sum of error² 188.4358098
pH θ(1-) θ(2-) θ(3-) signal,theo signal,exp error² HH-
4 5.29776E-15 1.0299E-05 0.002378235 4.72315029 5.97 1.56448555 -0.3
4.2 8.39639E-15 1.6322E-05 0.003764012 4.89908174 6.03 1.28834424 -0.2
signal
pH
HH-
H- HH- H-- error





Result

The best fit is obtained with a three protonation model (H--) with pK_a-values of 6.0, 6.4 und 9.0. The fit with the other models (H- and HH-) give 10-fold greater values of the sum of errors squared. In addition, these models show systematic variation in the deviations between the experimental and theoretical values. The data were in fact generated with a three protonation model with pK_a-values of 5.9, 6.4 and 9.0.

Note: This form of analysis is much less clear when non-optimal protonation states also have catalytic activity.

Exercise 17: Simulation of association kinetics using numerical integration

Use Eqn. 9.22 to programme the simulation of a bimolecular association reaction: (A+B→AB).

Objective of the exercise: programming by numerical integration.

The rate of a bimolecular association reaction A+B → AB is given by the following equation:

$dc_{AB}/dt = k_1 \times c_A \times c_B$ (cf. Eqn.9.21)

from which it follows that the flux from A and B to AB can be written:

$F1 = k_1 \times c_A \times c_B \times \Delta t$ (cf. Eqn.9.22)

Background information about this exercise can be found as follows:

- Introduction to numerical integration (Sect. 9.1.11)
- Association kinetics (Sect. 9.2.9)

Instructions

- We have one parameter (k_1) and one constant (Δt). Enter in A1 "k_1 [M^{-1} s^{-1}]", in A2 "Δt [s]"; in B1 place 1 and in B2 0.1 as values for k_{-1} and Δt.
- The titles to the columns are placed in row 4: "t", "cA [nM]", "cB [nM]", "cAB [nM]" and "F_1".
- The initial values for the concentrations and the time are entered into row 5 A-D: 0 (t), 0.2 (c_A), 0.2 (c_B) und 0(c_{AB}).
- The flux from A and B to AB is entered into column E. This flux is determined by the value of k_1, and is therefore defined as F_1. This flux is from Eqn., 9.22, and in our case we place in E5: "=$B$2*B5*C5*$B$3/1e9". $1x10^9$ is a factor that corrects for differences in units, because the concentrations are given in nM, but the units of k_1 should be given as s^{-1} M^{-1} .
- The integration begins in row 6:
- The time is given by the old time + Δt; so in A6 we write:"=A5+$B$2".
- c_A and c_B are given by the old values minus the flux. Thus we write in B6: "=B5-E5", and analogously for C6.
- c_{AB} is the old concentration plus the flux, thus in D6: "=D5+E5".

- Row 6 is highlighted and copied to row 250. To extend the highlighting use the Pg-Down key.

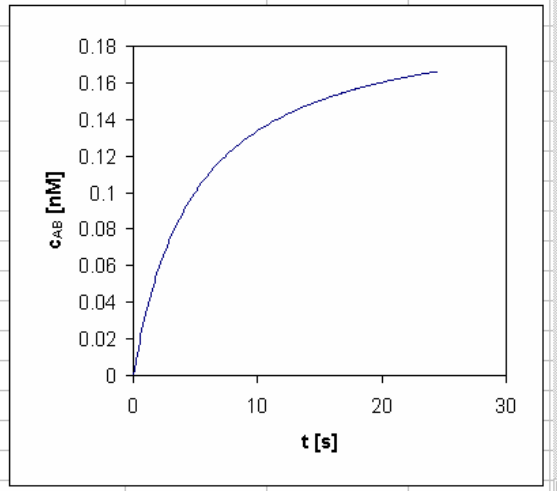
Exercise 17.xls

	A	B	C	D	E	F	G	H
1	k_1 [M^{-1} s^{-1}]	1000000000						
2	Δt [s]	1						
3								
4	t [s]	c_A [nM]	c_B [nM]	c_{AB} [nM]	F1			
5	0	0,2	0,2	0	4,00E-02			
6	1	0,16	0,16	0,04	2,56E-02			
7	2	0,1344	0,1344	0,0656	1,81E-02			
8	3	0,11633664	0,11633664	0,08366336	1,35E-02			
9	4	0,10280243	0,10280243	0,09719757	1,06E-02			
10	5	0,09223409	0,09223409	0,10776591	8,51E-03			
11	6	0,08372696	0,08372696	0,11627304	7,01E-03			
12	7	0,07671676	0,07671676	0,12328324	5,89E-03			

kass fit

- We now need to work with a graph. Go to cell A1 of the table (Ctrl+Home). Highlight the column titles and the visible data in columns A-D. Press SHIFT+END, then Shift+↓. This extends the highlighting to all of the data. Select XY(Scatter) from the Chart Wizard.

	A	B	C	D	E	F	G	H	I
1	k_1 [M^{-1} s^{-1}]	1.00E+09							
2	dt [s]	0.1							
3									
4	t [s]	c_A [nM]	c_B [nM]	c_{AB} [nM]	F1				
5	0	0.2	0.2	0	4.00E-03				
6	0.1	0.196	0.196	0.004	3.84E-03				
7	0.2	0.1921584	0.1921584	0.0078416	3.69E-03				
8	0.3	0.18846591	0.18846591	0.01153409	3.55E-03				
9	0.4	0.18491397	0.18491397	0.01508603	3.42E-03				
10	0.5	0.18149466	0.18149466	0.01850534	3.29E-03				
11	0.6	0.17820063	0.17820063	0.02179937	3.18E-03				
12	0.7	0.17502508	0.17502508	0.02497492	3.06E-03				
13	0.8	0.1719617	0.1719617	0.0280383	2.96E-03				
14	0.9	0.16900462	0.16900462	0.03099538	2.86E-03				
15	1	0.16614836	0.16614836	0.03385164	2.76E-03				
16	1.1	0.16338784	0.16338784	0.03661216	2.67E-03				
17	1.2	0.16071828	0.16071828	0.03928172	2.58E-03				
18	1.3	0.15813524	0.15813524	0.04186476	2.50E-03				
19	1.4	0.15563456	0.15563456	0.04436544	2.42E-03				



- Try out different simulations. Set the values of k to be small (1e5) and large (1e12).When the k value is very large, it can happen that the flux in a time interval can be larger than the amount of A and B available; that leads to negative concentrations and error messages. This problem can be avoided by using smaller values of Δt. When the k value is too small, the simulation is very slow, and 250 iteration steps are not enough to reach the end of the reaction. In this case, either use more iteration or a larger value for the time interval.

Exercise 18: Analysis of association kinetics.

Use numerical integration to analyse the kinetics of an association reaction.

Objective of the exercise: analysis of association kinetics.

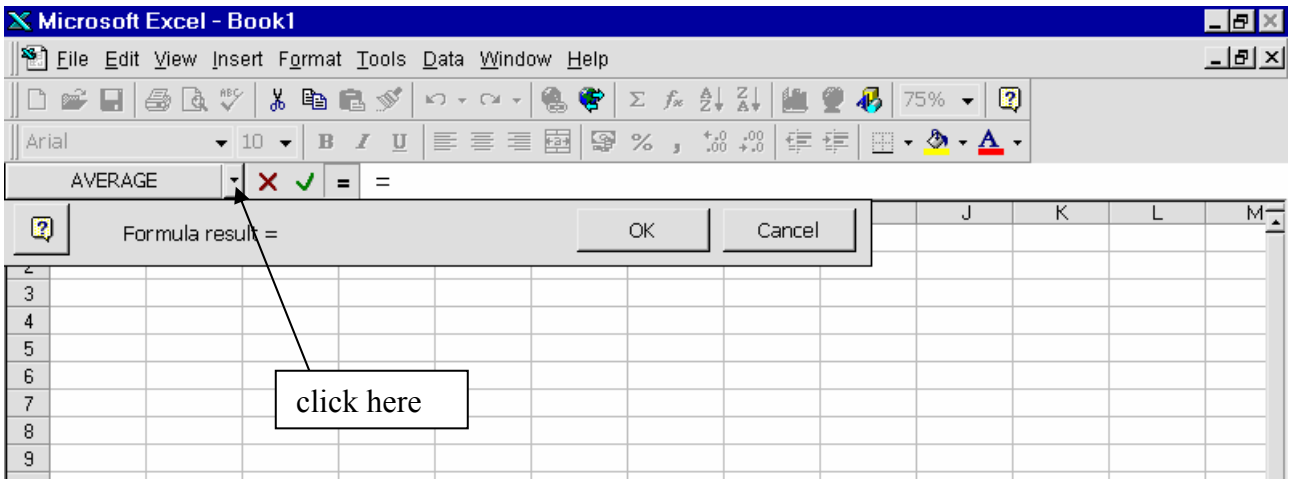
Exercise 18 is linked to Exercise 17

Background information about this exercise can be found as follows:

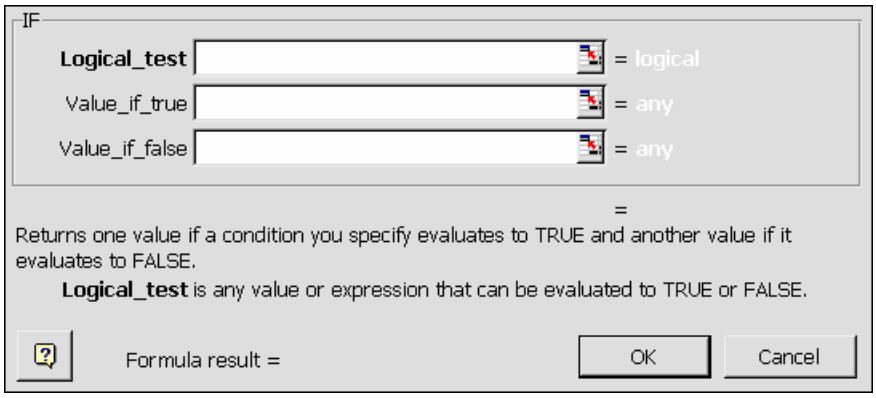
- Introduction to numerical integration (Sect. 9.1.11)
- Association kinetics (Sect. 9.2.9)

Instructions

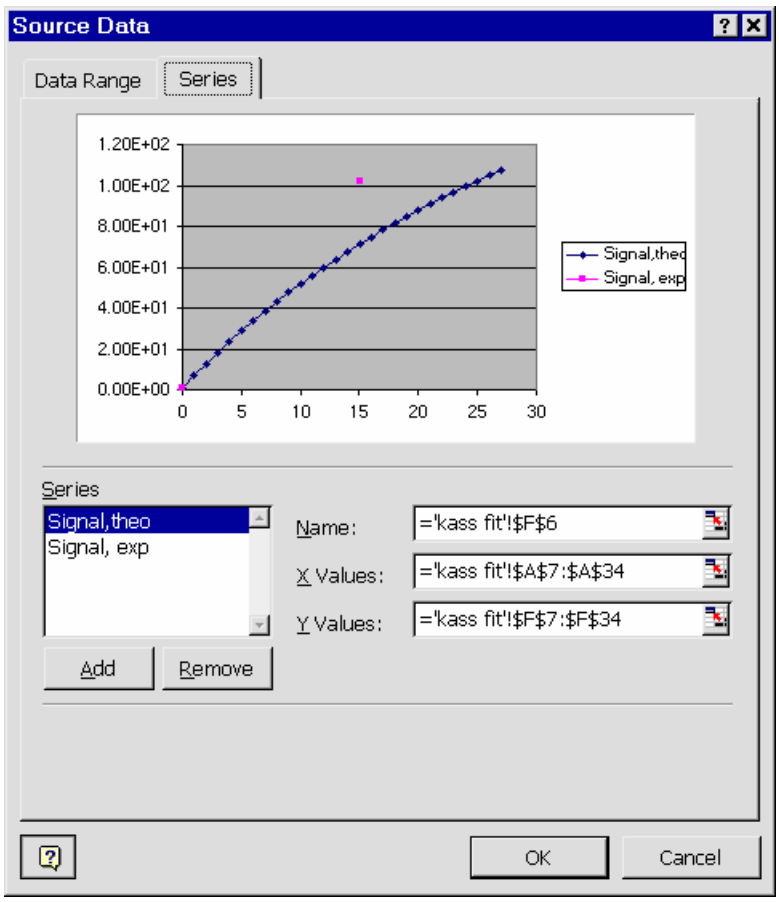
- We use the spreadsheets created in Exercise 17.
- First, we have to take account of the baseline and effect factors, and to do this we need space in the spreadsheet. Right-click on the "2" in row headings and select Insert. Repeat; there are now two new rows 2 and 3.
- Enter "BL" in A2 and "f" in A3. As initial values, chose "1" and "100" in B2 and B3.
- The theoretical signal is entered in column F (column title: "signal,theo", and in cell E7 for example enter "=D7*$B$3+$B$2").
- Now, by fixing the size of Δt, select a time scale that covers all of the available data (final value >200, so $\Delta t \approx 1$).
- In column F (column title: "signal,exp"), enter the data in the correct rows. It is an uncomfortable consequence of this way of programming that the Δt cannot be changed without re-entering the data.
- The error squared values are entered in column G. We are practising with intelligent programming, which will allow us to use this spreadsheet with other data. This involves using the Excel function: "if".
- Go to H7. Click with the mouse on the "=" next to the Formula Bar. A new pulldown menu appears which we open.



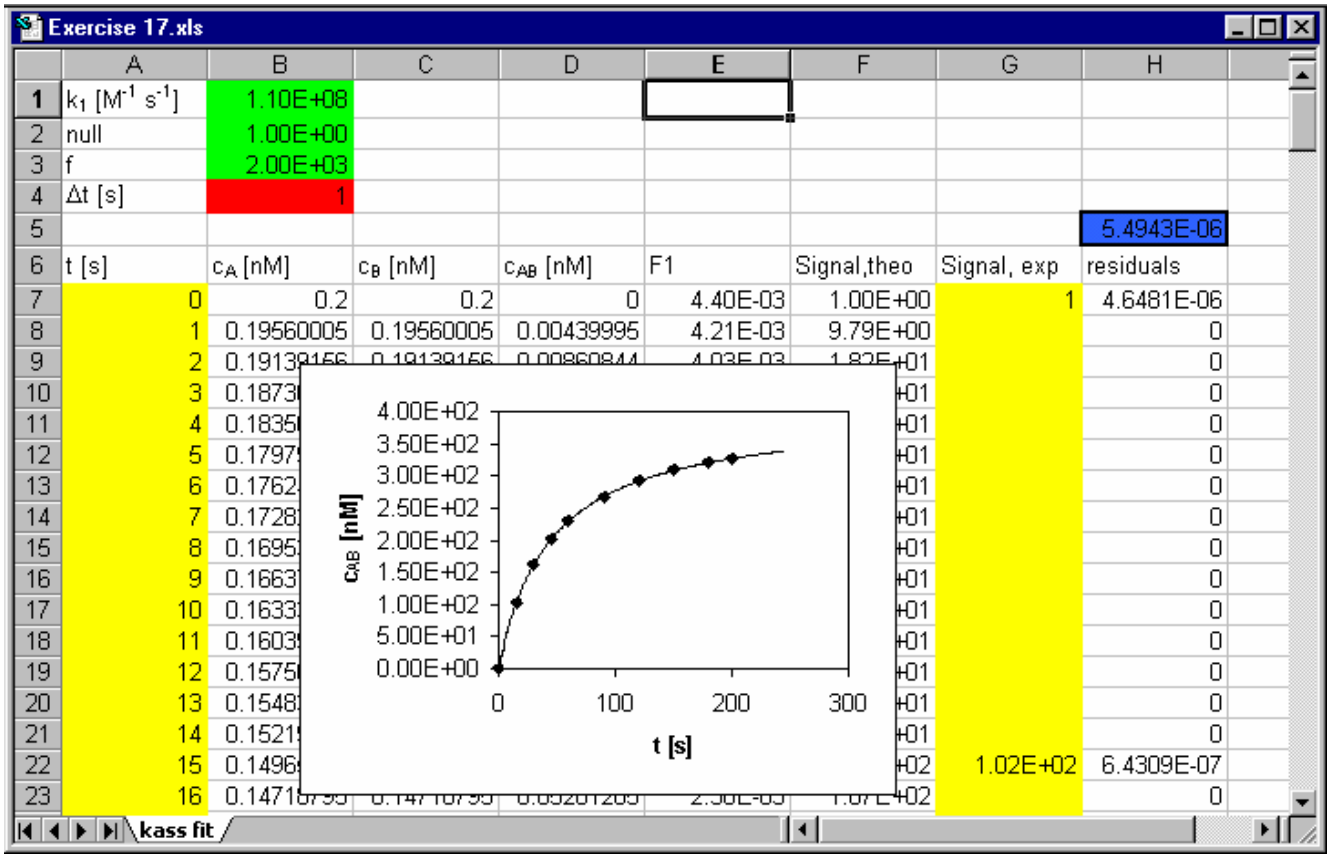
- Select: “More Functions”, “Logical” and “if”.



- Enter G7=0 in “Logical_test”. This can be done by clicking on G7 with the mouse, then entering “=0”, and finish by pressing ENTER. When the condition is true, no data are in G, and thus no calculation of error squared can be made.
- For this reason, enter “0” in “Value_if_true” (the unavailable “error squared” is 0).
- In “Value_if_false“ is the error squared, i.e. “=(F7-G7)^2”.
- Press OK. This formula will calculate the error² whenever there are data in column G; if there are no data it will return zero.
- We now copy the formula in H7 in the column H, and enter the sum into H5 (“=Sum(H7..H252)”).
- We generate a new figure. Go to A1, and highlight all the visible rows in columns A, F and G (use Ctrl). Select an XY(Scatter) from the Chart Wizard XY(Scatter).
- Highlight the graph. Select “Chart, and then “Source Data”.
- In the following window, select “Series”, and for both rows enter 7-252 for the X and Y values. In the example shown below, both 34 are altered to 252. Click on Signal,exp to select the other curve, and change the 34 into 252. Select OK.



- For the experimental points, turn the line off, and the points on. For the fitted data, the other way round: switch off points.
- The sum of errors squared is now minimised with respect to variation of k_1, BL and f.
- Sometime there are problems in obtaining association rate constants if the initial values are set too high. Chose a value for k_1 of 1×10^7 and try the fitting again.



	A	B	C	D	E	F	G	H
1	k_1 [M^{-1} s^{-1}]	1.10E+08						
2	null	1.00E+00						
3	f	2.00E+03						
4	Δt [s]	1						
5								5.4943E-06
6	t [s]	c_A [nM]	c_B [nM]	c_{AB} [nM]	F1	Signal,theo	Signal, exp	residuals
7	0	0.2	0.2	0	4.40E-03	1.00E+00	1	4.6481E-06
8	1	0.19560005	0.19560005	0.00439995	4.21E-03	9.79E+00		0
9	2	0.19139156	0.19139156	0.00860844	4.03E-03	1.82E+01		0
10	3	0.1873				+01		0
11	4	0.1835				+01		0
12	5	0.1797				+01		0
13	6	0.1762				+01		0
14	7	0.1728				+01		0
15	8	0.1695				+01		0
16	9	0.1663				+01		0
17	10	0.1633				+01		0
18	11	0.1603				+01		0
19	12	0.1575				+01		0
20	13	0.1548				+01		0
21	14	0.1521				+01		0
22	15	0.1496				+02	1.02E+02	6.4309E-07
23	16	0.1471				+02		0

Result

The best fit of the data is to an association rate constant of 1.1×10^{8} M^{-1} s^{-1} .

Using modern computers such simulations involving 2000 rows are no problem. It is recommended, therefore, to program the sheet with more rows than in this example, because fast association rates require a small Δt, and in general the errors inherent in numerical integration are smaller with smaller steps.

Exercise 19: Simulation of approach to equilibrium

Programme the simulation of an approach to equilibrium following Eqn. 9.23. Use initial concentrations of 0.2 µM and 0.1 µM for A and B respectively, rate constants $k_1 = 1\times10^{7}$ M^{-1} s^{-1} and $k_{-1} = 1.0$ s^{-1}, with a time interval $\Delta t = 0.01$ s.

Objective of the exercise: programming numerical integrations.

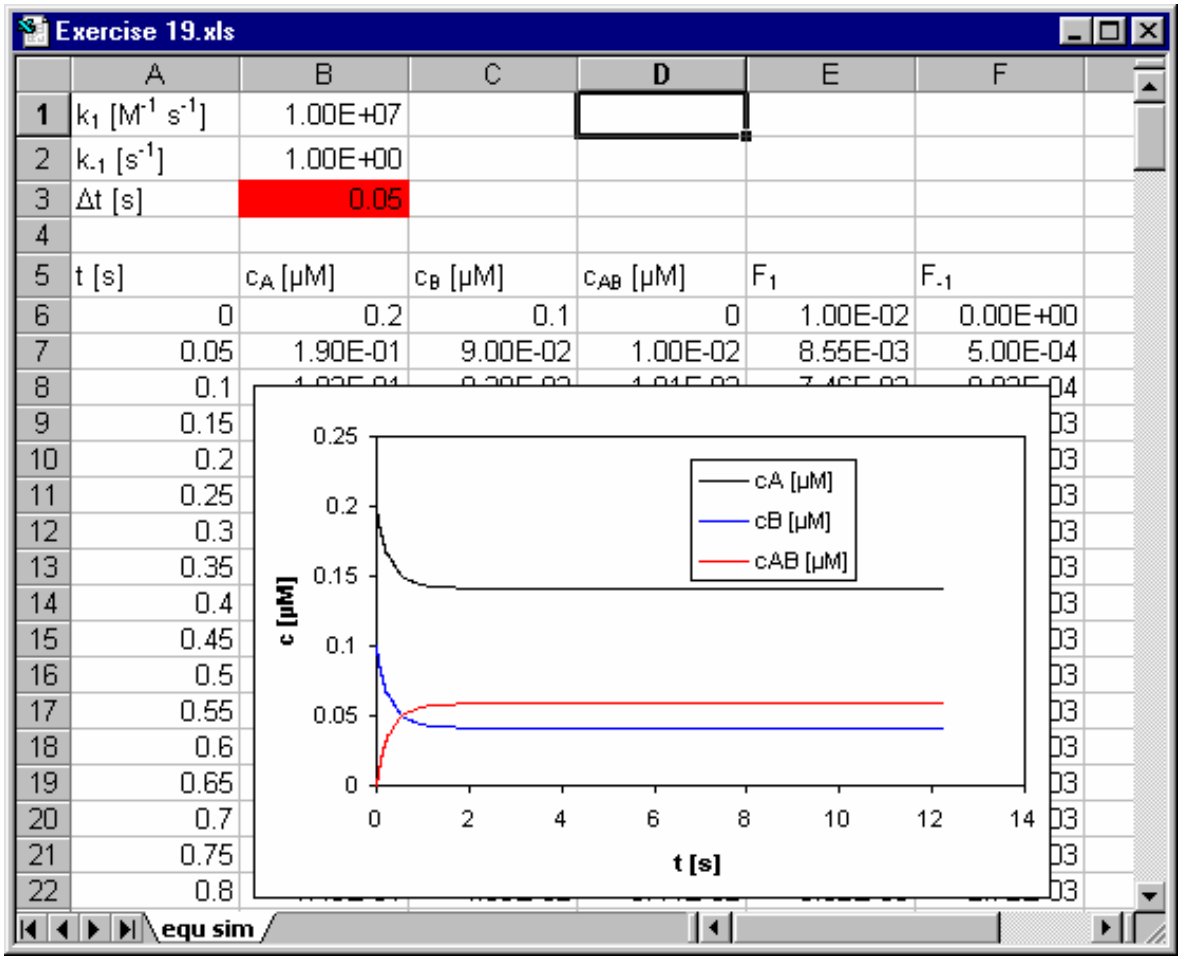
Background information about this exercise can be found as follows:

- Introduction to numerical integration (Sect. 9.1.11)
- Association kinetics (Sect. 9.2.9)

- Programming numerical integrations and fitting experimental data to these are covered in Exercises 17 and 18.

Instructions

- The rate constants k_1 and k_{-1} and the time increment Δt are in rows 1-3
- Use columns A-F for the following: t, c_A, c_B, c_{AB}, F_1, F_{-1}
- F_1 was defined in Exercise 18: Analysis of association kinetics. Remember that there was a correction factor of 1×10^6 to allow for the difference in the units (concentrations are given in µM but the units of the bimolecular rate constant k_1 should be s^{-1} M^{-1}), i.e. $F_1 = \Delta t \times k_1 \times c_E \times c_S$ /1e6.
- F_{-1} is defined by the equation $F_{-1} = dt \times k_{-1} \times c_{ES}$. No correction factor is needed for F_{-1}.
- After entering the initial values we prepare a graph for c_A, c_B and c_{AB} .
- To recognise more clearly when equilibrium is reached, we alter Δt to 0.05.



Result

With the initial values of the concentrations used and with $k_1=1\times10^7$ M^{-1} s^{-1} and $k_{-1}=1$ s^{-1} equilibrium is established after ca. 3-4 s.

Exercise 20: Simulation of a complex enzyme-catalysed reaction.

Programme the simulation of an enzyme reaction involving a bimolecular association of enzyme and substrate to form an ES complex which undergoes a conformational change preceding turnover. Use initial concentrations of 5 μM for both enzyme and substrate, rate constants as follows: $k_1=1\times10^7$ M^{-1} s^{-1}, $k_{-1}=0.1$ s^{-1}, k_2, k_{-2} and k_3 =1 s^{-1} and a time interval Δt= 0.01 s. Find the combinations of rate constants that lead to: 1) marked accumulation of ES; 2) marked accumulation of ES#; and 3) accumulation of neither enzyme-substrate complex.

Objective of the exercise: programming of numerical integrations

We assume that the enzyme reaction follows Eqn. 9.24:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES\# \xrightarrow{k_3} E + P$$

In this model there are 5 different fluxes:

- F_1: E+S → ES with $F_1 = k_1 \times c_E \times c_S \times \Delta t$
- F_{-1}: ES → E+S with $F_{-1} = k_{-1} \times c_{ES} \times \Delta t$
- F_2: ES → ES# with $F_2 = k_2 \times c_{ES} \times \Delta t$
- F_{-2}: ES# → ES with $F_{-2} = k_{-2} \times c_{ES\#} \times \Delta t$
- F_3: ES# → E+P with $F_{-3} = k_3 \times c_{ES\#} \times \Delta t$

The concentration changes are given by the following relationships:

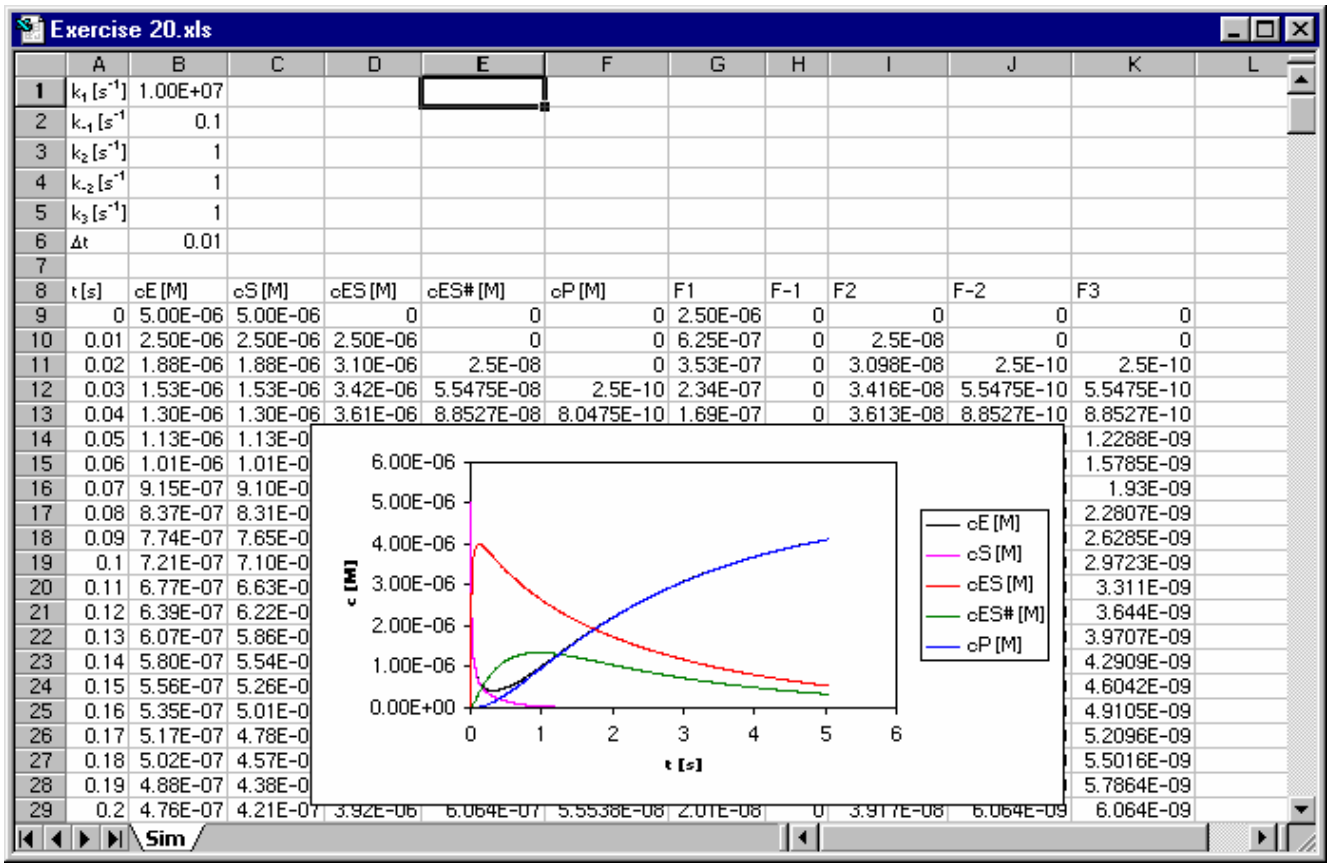
- $\Delta c_E = - F_1 + F_{-1} + F_3$
- $\Delta c_S = - F_1 + F_{-1}$
- $\Delta c_{ES} = - F_{-1} – F_2 + F_1 + F_{-2}$
- $\Delta c_{ES\#} = - F_3 – F_{-2} + F_2$
- $\Delta c_P = + F_3$

Background information about this exercise can be found as follows:

- Introduction to numerical integration (Sect. 9.1.11)
- Pre-steady state kinetics (Sect. 9.2.10)
- Programming numerical integrations and fitting experimental data to these are covered in Exercises 17 and 18.

Instructions

- The spreadsheet is programmed as in Exercise 19.
- The quantities k_1, k_{-1}, k_2, k_{-2}, k_3 and Δt are placed in the first six rows.
- Use columns A-K for the following: t, c_S, c_E, c_{ES}, $c_{ES\#}$, c_P, F_1, F_{-1}, F_2, F_{-2}, F_3
- Concentrations are given in molarity (M), and therefore no correction factor is needed as in Exercise 19.



	A	B	C	D	E	F	G	H	I	J	K	L
1	k_1 [s^{-1}]	1.00E+07										
2	k_{-1} [s^{-1}]	0.1										
3	k_2 [s^{-1}]	1										
4	k_{-2} [s^{-1}]	1										
5	k_3 [s^{-1}]	1										
6	Δt	0.01										
7												
8	t [s]	cE [M]	cS [M]	cES [M]	cES# [M]	cP [M]	F1	F-1	F2	F-2	F3	
9	0	5.00E-06	5.00E-06	0	0	0	2.50E-06	0	0	0	0	
10	0.01	2.50E-06	2.50E-06	2.50E-06	0	0	6.25E-07	0	2.5E-08	0	0	
11	0.02	1.88E-06	1.88E-06	3.10E-06	2.5E-08	0	3.53E-07	0	3.098E-08	2.5E-10	2.5E-10	
12	0.03	1.53E-06	1.53E-06	3.42E-06	5.5475E-08	2.5E-10	2.34E-07	0	3.416E-08	5.5475E-10	5.5475E-10	
13	0.04	1.30E-06	1.30E-06	3.61E-06	8.8527E-08	8.0475E-10	1.69E-07	0	3.613E-08	8.8527E-10	8.8527E-10	
14	0.05	1.13E-06	1.13E-0								1.2288E-09	
15	0.06	1.01E-06	1.01E-0								1.5785E-09	
16	0.07	9.15E-07	9.10E-0								1.93E-09	
17	0.08	8.37E-07	8.31E-0								2.2807E-09	
18	0.09	7.74E-07	7.65E-0								2.6285E-09	
19	0.1	7.21E-07	7.10E-0								2.9723E-09	
20	0.11	6.77E-07	6.63E-0								3.311E-09	
21	0.12	6.39E-07	6.22E-0								3.644E-09	
22	0.13	6.07E-07	5.86E-0								3.9707E-09	
23	0.14	5.80E-07	5.54E-0								4.2909E-09	
24	0.15	5.56E-07	5.26E-0								4.6042E-09	
25	0.16	5.35E-07	5.01E-0								4.9105E-09	
26	0.17	5.17E-07	4.78E-0								5.2096E-09	
27	0.18	5.02E-07	4.57E-0								5.5016E-09	
28	0.19	4.88E-07	4.38E-0								5.7864E-09	
29	0.2	4.76E-07	4.21E-07	3.92E-06	6.064E-07	5.5538E-08	2.01E-08	0	3.917E-08	6.064E-09	6.064E-09	

Result

It is observed that ES accumulate if k_{-1} and $k_2 << k_1 \times c_{av}$ and k_{-2}. ES# accumulates if k_{-2}, $k_3 << k_3$. Neither intermediate accumulates if k_3 and $k_2 >> k_1 \times c_{av}$ (in which: $c_{av}=(c_{E,tot}+c_{S,tot})/2$).

Exercise 21: Analysis of the kinetics of a complex enzyme-catalysed reaction.

Analyse the data from model programmed in Exercise 20. Assume that the signal depends only on the product concentration, and that we know from preliminary experiments that $k_1=1\times10^7$ s^{-1} M^{-1}, $k_{-2} = 0.1$ s^{-1} and $k_{-1} = 1$ s^{-1}.

Objectives of the exercise: analysis of complex reactions.

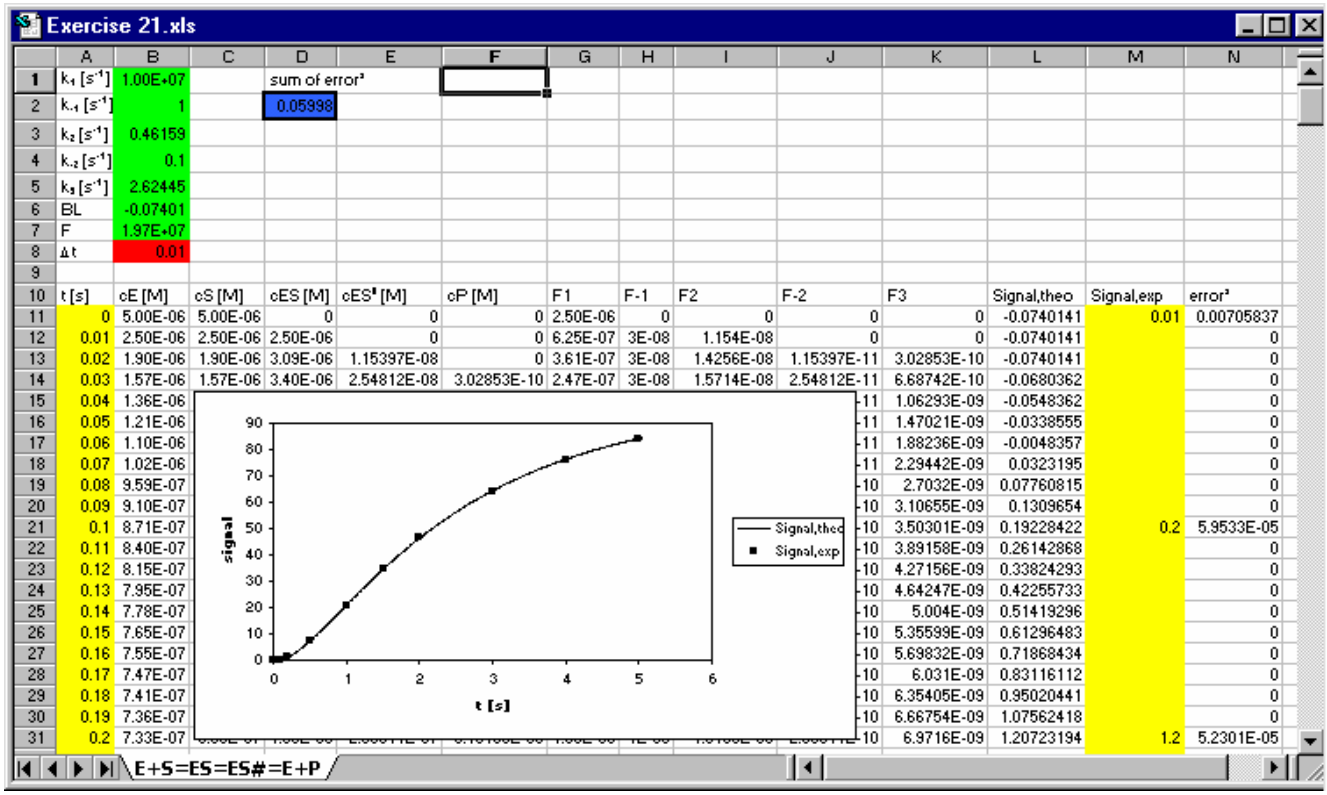
Exercise 21 is linked to Exercise 20

Background information about this exercise can be found as follows:

- Introduction to numerical integration (Sect. 9.1.11)
- Pre-steady state kinetics (Sect. 9.2.10)
- Programming numerical integrations and fitting experimental data to these are covered in Exercises 17 and 18.

Instructions

- Convert $c_{P,theo}$ into Signal,theo. To do this, introduce rows for BL and F.
- Define the error² and the sum of errors squared.
- Minimise the sum of errors squared with respect to variation in k_2, k_3, BL and F.
- Important note: for a rigorous analysis, it is necessary to obtain data spanning a range of different enzyme and substrate concentrations. In these circumstances, it may be possible, using global data analysis, to fit all of the rate constants in the kinetic scheme.



Result

The data set can be fitted satisfactorily to the scheme in Eqn.9.24 with the following rate constants: $k_1=1\times10^7$ M^{-1} s^{-1}, $k_{-1}=1$ s^{-1}, $k_2=0.46$ s^{-1}, $k_{-2}=0.1$ s^{-1}, $k_3=2.6$ s^{-1}.

Exercise 22: Analysis of Michaelis-Menten kinetics III.

Use numerical integration of Eqn. 9.7 to evaluate kinetic data according to the Michaelis-Menten model.

Objective of the exercise: analysis of Michaelis-Menten kinetics.

The rate of an enzyme reaction following the Michaelis-Menten model is given by Eqn. 9.7:

$$k(c_S) = k_{cat} \times \frac{c_S}{c_S + K_m}$$

At a given substrate concentration, the rate is given by the expression:

$$\frac{\partial c_P}{\partial t} = c_{E,tot} \times k_{cat} \times \frac{c_S}{c_S + K_m}$$

and in a finite time interval Δt the following amount of product Δc_P is formed:

$$\Delta c_P = c_{E,tot} \times k_{cat} \times \frac{c_S}{c_S + K_m} \times \Delta t$$

Background information about this exercise can be found as follows:

- Introduction to numerical integration (Sect. 9.1.11)
- Michaelis-Menten kinetics (Sect. 9.2.2)
- Programming numerical integrations and fitting experimental data to these are covered in Exercises 17 and 18.

Instructions

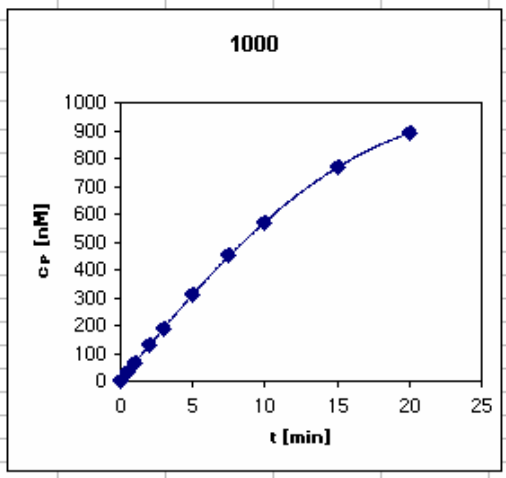
- The two parameters K_m [nM] and k_{cat} [min^{-1}], are entered in rows 1 and 2. We take initial values to be 100 nM and 20 min^{-1}.
- The constant terms $c_{S,tot}$, $c_{E,tot}$ and Δt are entered in rows 4-6. We take a time increment Δt= 0.05, and the enzyme and substrate concentrations are given in the data.
- Row 8 contains the column headings: “t [min]”, “c_S [nM]”, “$c_{P,theo}$ [nM]”, “F”, “$c_{P,exp}$ [nM]” and “error²”.
- Columns A-C of row 9 contain the initial values of the concentrations and the time: 0 (t), “=B5” (c_S) and 0 (c_P).

- Column D contains the flux of E to P. This flux is given by the above equation. In this case for D9: "=B9/(B9+$B$1)*$B$2*$B$4*$B$6".
- The experimentally determined product concentrations are entered into column E.
- The errors squared are entered in column F. We again must use the conditional programming with the IF-function, so for F9: "=IF(E9=0;0;(C9-E9)^2)".
- The integration begins in row 10:
- The time is given by the old time + Δt. So enter in A10: "=A9+$B$6".
- c_S is given by the old value minus F. So enter in B10: "=B9-D9".
- c_P is the old concentration plus F. So enter in C10: "=C9+D9".
- Row 10 is highlighted and copied to all rows up to no. 409. Use the Pg-down key to enlarge the highlighting.
- The sum of errors squared is entered in cell D2.
- Make a graph of $c_{P,theo}$ and $c_{P,exp}$ vs. t. Use a line for $c_{P,theo}$ without data points, and only data points for $c_{P,exp}$.

The spreadsheet should look like the following:

Exercise 22.xls

	A	B	C	D	E	F
1	K_m [nM]	490.33774		sum of error²		
2	k_{cat} [min^{-1}]	19.734964		26.808215		
3						
4	$c_{E,tot}$ [nM]	5				
5	$cS_{,tot}$ [nM]	1000				
6	Δt	0.05				
7						
8	t [min]	c_S [nM]	c_P [nM],theo	F	c_P [nM],exp	error²
9	0	1000	0	3.3104851	0.001	0.000001
10	0.05	996.68951	3.3104851	3.3068713		0
11	0.1	993.38264	6.6173565	3.3032454		0
12	0.15	990.0794	9.9206019	3.2996073		0
13	0.2	986.77979	13.220209	3.295957		0
14	0.25	983.48383	16.516166	3.2922943		0
15	0.3	980.19154	19.808461	3.2886194		0
16	0.35	976.90292	23.09708	3.2849321		0
17	0.4	973.61799	26.382012	3.2812323		0
18	0.45	970.33676	29.663244	3.2775202		0
19	0.5	967.05924	32.940764	3.2737955	30	8.6480958
20	0.55	963.78544	36.21456	3.2700583		0
21	0.6	960.51538	39.484618	3.2663086		0

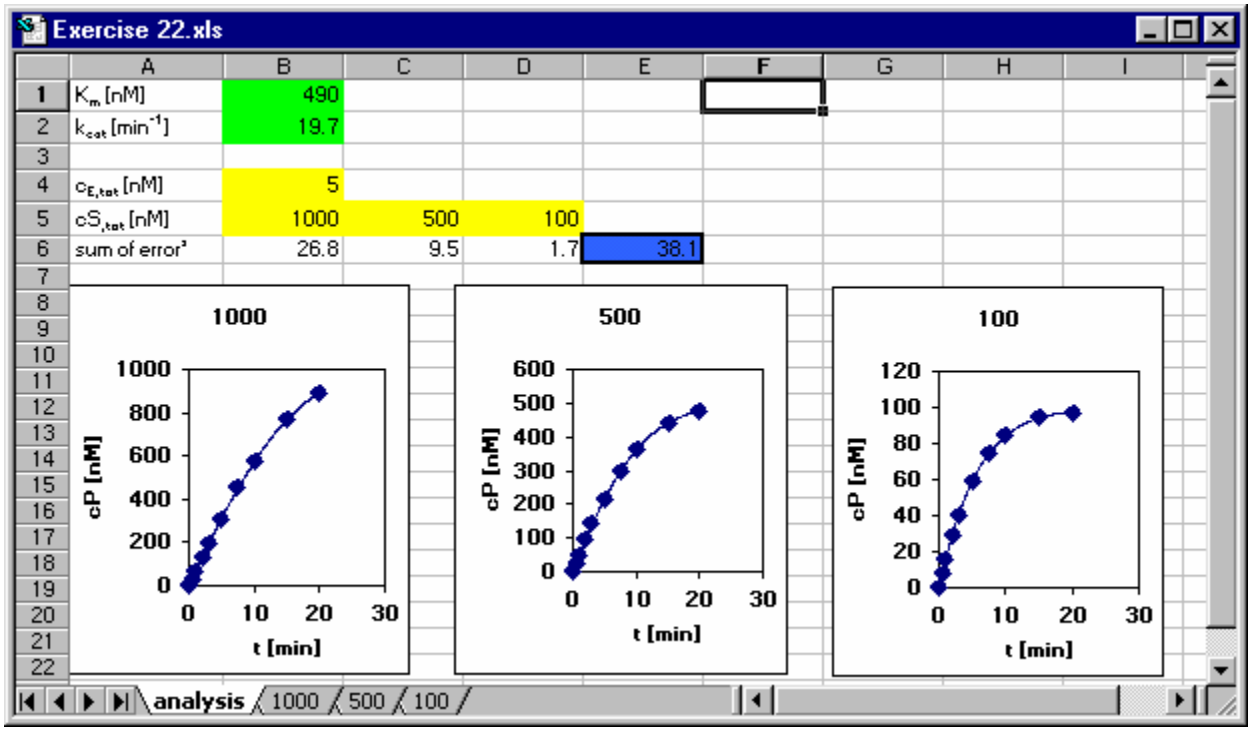


analysis | 1000 | 500 | 100

- We create a spreadsheet for each concentration.
- The global analysis is carried out as before using an analysis spreadsheet. K_m, k_{cat} and $c_{E,tot}$ are entered in the analysis spreadsheet in cells B1, B2 and B4.
- Substrate concentrations are entered in B5 to D5.

- K_m, k_{cat}, $c_{E,tot}$ and $c_{S,tot}$ are transferred from the analysis spreadsheet to subsidiary sheets. To do this, enter in place of the K_m value in the subsidiary sheet: "=analysis!$B$1". We proceed analogously with k_{cat}, $c_{E,tot}$ and $c_{S,tot}$.
- The sum of errors squared are transferred from the subsidiary sheets to the analysis sheet. They are located in B6 to D6. So, for example in B6 write: "='1000'!$D$2".
- Enter the sum of errors squared in E6.
- Copy the graph from the subsidiary sheet to the analysis sheet.
- Minimise E6 (sum of errors squared) with respect to variation of K_m and k_{cat}.

The result should look something like the following:



Result

The data can be fitted to the Michaelis-Menten model with a K_m value of 490 nM and a k_{cat} value of 19.7 min^{-1}.

Exercise 23: Analysis of competitive binding equilibria.

Analyse the data according to the binding model in Eqn. 9.28. We assume that the signal arises solely from the species AB. K_1 is taken to be 1×10^8 M^{-1}.

Objective of the exercise: analysis of competitive binding processes.

$$AB + C \underset{}{\overset{K_1}{\rightleftharpoons}} A + B + C \overset{K_2}{\rightleftharpoons} A + BC$$

Since analytical solutions of processes even as simple as that shown above (Eqn. 9.28) are intractable, we will employ numerical methods. From an arbitrary initial state (e.g. only free A, B and C present) we calculate for both isolated equilibria the final equilibrium concentrations, and the concentration changes needed to establish equilibrium. We then use these fluxes. Since, in every step, there are several fluxes, whose coupling is not taken into account, this approximate procedure takes us towards the equilibrium, but not directly to it. So the equilibrium has to be located iteratively.

We may write:

$K_1 = c_{AB,Equil.}/(c_{A,Equil.} \times c_{B,Equil.})$

or:

$c_{AB,Equil.} = K_1 \times c_{A,Equil.} \times c_{B,Equil.}$

in which: $c_{i,Equil}$ is the equilibrium concentration of i.

We are looking for the flux F, necessary to take us from the present concentrations to the equilibrium concentrations:

$c_{AB,Equil.} = c_{AB} + F$

$c_{A,Equil.} = c_A - F$

$c_{B,Equil.} = c_B - F$

Thus, we have:

$(c_{AB} + F) = K_1 \times (c_A - F) \times (c_B - F)$

and:

$$F^2 - (c_A + c_B + 1/K_1) \times F + c_A \times c_B - c_{AB}/K_1 = 0$$

$$F = -p/2 - [(p/2)^2 - q]^{1/2}$$

(Only the negative root is physically meaningful).

We calculate these fluxes for the two equilibria, and use these. We reach a new concentration state, which is not the true equilibrium state, since we have not taken the coupling of the equilibria into

account. So the operation is repeated with newly calculated fluxes, and after ca. 50 iterations, we reach a stable equilibrium. The concentrations correspond to the joint equilibrium specified by the two thermodynamic association constants K_1 and K_2.

For practical applications it is important that the calculated fluxes are not too large so that calculation does not overshoot the target. So, for example, if K_1 and K_2 are very large, and $c_A > c_B$ and $c_C > c_B$, our initial analysis of the first equilibrium will show that almost all of the B must flow to AB. At the same time, analysis of the second equilibrium will show that all of the B should flow to BC. When we take account of the two fluxes, we obtain negative concentrations in the first iteration. Since in this example, there are at most two fluxes for a single species (free B), this complication can be avoided by reducing every flux by 50%.

Background information about this exercise can be found as follows:

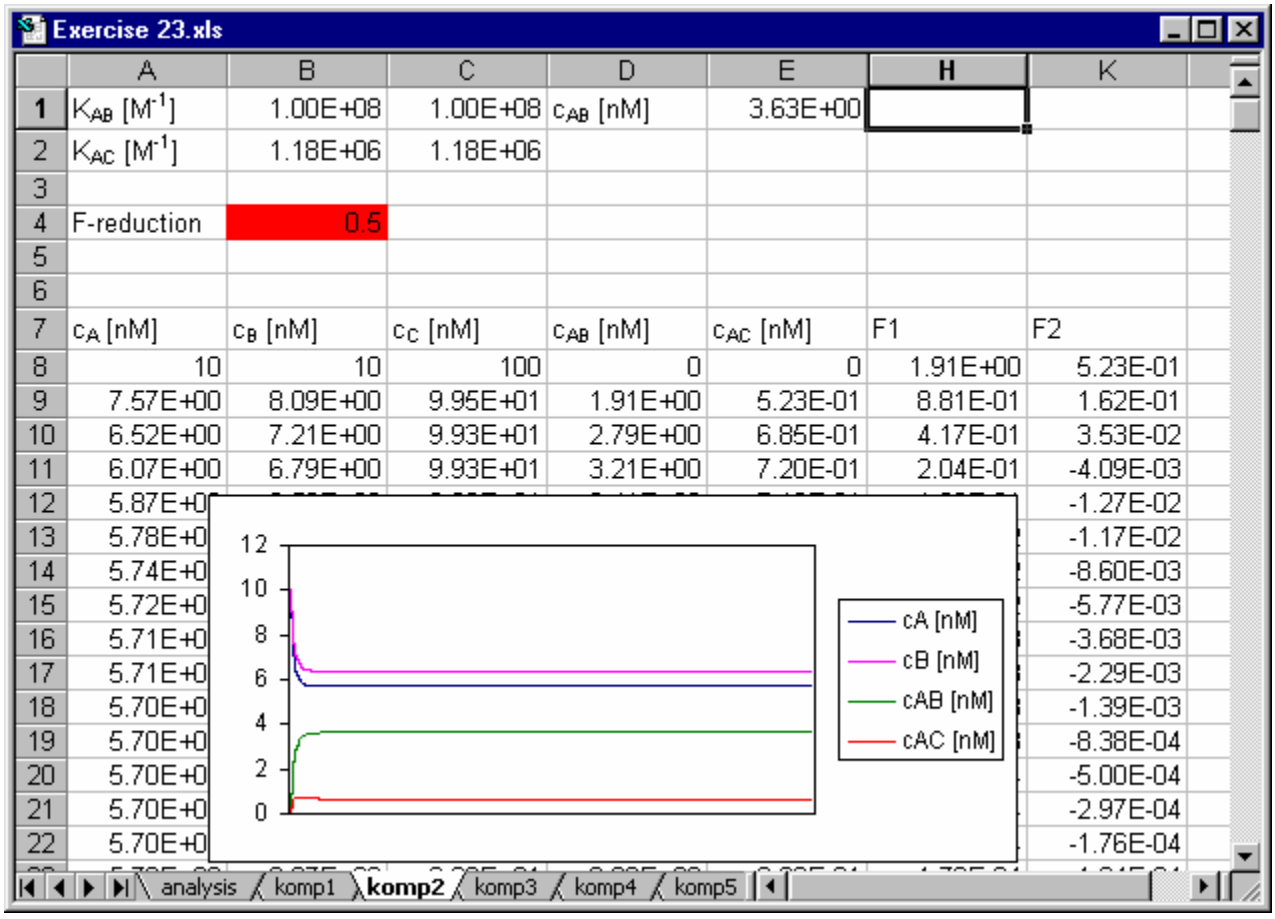
- Introduction to numerical integration (Sect. 9.1.11)
- Analysis of competition experiments (Sect. 9.2.12)
- Programming numerical integrations and fitting experimental data to these are covered in Exercises 17 and 18.

Instructions

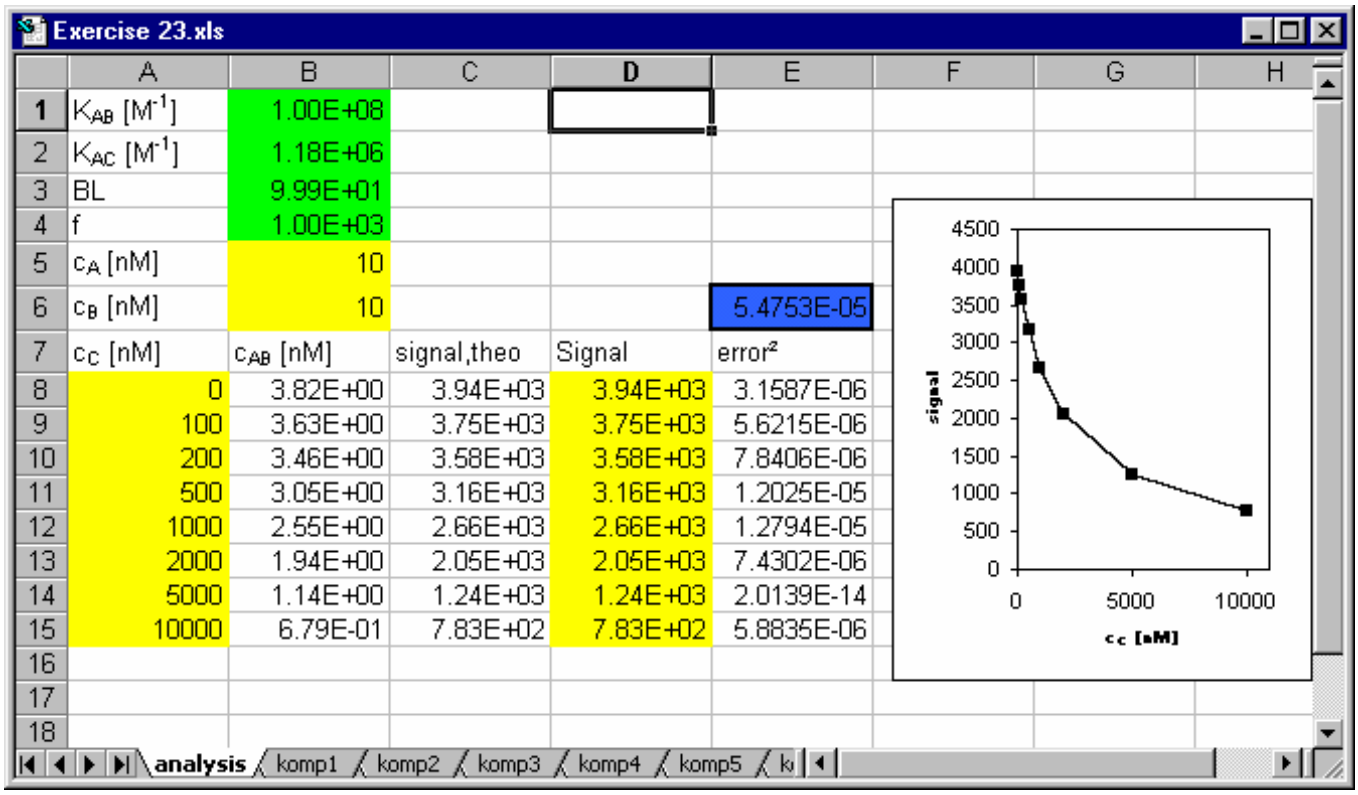
- We need an analysis spreadsheet for each $c_{C,total}$, in which the dependence of the concentrations c_A, c_B, c_C, c_{AB}, and c_{BC} on the constants K_1 und K_2 are evaluated for given values of $c_{A,total}$, $c_{B,total}$ and $c_{C,total}$.
- Rows 1 and 2 contain the variables K_1 and K_2. We use 1e9 for K_1 and 1e6 for K_2.
- In row 4 we enter the analysis parameter “F-reduction”, which is not going to be fitted; we use a value of 0.5.
- In row 7 we enter the column headings: “c_A [nM], “c_B [nM]”, “c_C [nM]”, “c_{AB} [nM]”, “c_{AC} [nM]”.
- The fluxes are then entered; for flux 1: in F6 we write “F_1“, in F7 “p“, in G7 “q“ and in H7 “F_1“.
- Flux 2 is entered similarly in columns I-K.
- In row 8 we enter the initial concentrations. We use 10, 10, 1000, 0 and 0 nM for A, B, C, AB and AC.
- The fluxes are given by the equation shown above, so in row 8 p: “=-(A8+B8+1000000000/$B$1)” (the factor of 10^9 is needed because concentrations are given in nM whereas binding constants are M^{-1}), q: “=A8*B8-1000000000*D8/$B$1” and F1 “=$B$4*(-F8/2-SQRT((F8/2)^2-G8))“, where multiplication with B4 introduces the flux reduction. The entries for flux 2 are entered analogously.
- This block (row 8, columns F-K) is highlighted and copied to row 9.

- The concentrations in row 9 are given by the simple expressions: cA "=A8-H8-K8", cB "=B8-H8", cC "=C8-K8", cAB "=D8+H8" and cAC "=E8+K8".
- Columns F and G, and I and J are now hidden.
- Highlight row 9 down to row 500.
- We create a graph of the columns A to E. On this occasion we use a simple line (not XY) because there are no X-values. Do not apply data symbols.
- The graph is of limited value because there is too much C present. Highlight the graph, click once on the legend, then once on the line for C in the legend. The line is now highlighted. Press DEL and C disappears from the plot. We can now see that equilibrium has in fact been achieved.
- In E1 we enter the value of c_{AB} at the end of the simulation: "=D200"
- To make the graph more attractive, click on the X-axis and press DEL.
- To check whether everything has been done correctly, we enter in C1 and C2 the equilibrium concentrations that were reached at the end of the iterations: K_1 (in C1) "=D200/A200/B200" and K_2 (in C2) "=E200/A200/C200". We should now have the same numbers in B1 and C1 and in B2 and C2 .

The result so far should look something like the following:



- Now set a flux reduction value of 0.1. It takes significantly longer to reach equilibrium. With a value of 1.0, we observe oscillations, but the system does become stable and converges on the "correct" solution. A flux reduction value of 0.5 seems to be optimal.
- The rest is straightforward: a spreadsheet is created for every $c_{C,total}$. In an analysis spreadsheet, K_2 is defined and transferred to the subsidiary spreadsheets. The analysis spreadsheet also contains the background BL and the effect factor f. The concentrations of AB for each value of $c_{c,total}$ are taken from cell E1 of the relevant subsidiary spreadsheet. The theoretical signal is calculated from the c_{AB} values using the values of BL and F. Then errors squared are defined, and minimised with respect to variation in K2, BL and f.



Result

The binding constant for C to A is about 1.18×10^{6} M^{-1}.