

# Determining the reaction stoichiometry

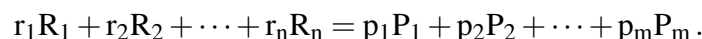
Theory: P.W. Atkins: *Physical Chemistry* (6th Ed.) Chapter 16.2.

Type: Individual.

Aim: Practice the Job's and the mole ratio methods to determine the reaction stoichiometry.

## 1 Introduction

The stoichiometric ratio is one of the most important information when a reaction is studied. If  $P_1, P_2, \dots, P_m$  products are formed in the reaction between reactants  $R_1, R_2, \dots, R_n$  then the reaction equation can be given as



To know the stoichiometry means that the ratios of  $r_1, r_2, \dots, r_n, p_1, p_2, \dots, p_m$  are known. During the practice, systems with two reactants ( $n = 2$ ) and one product ( $m = 1$ ) are investigated, therefore the



reaction equation is used for simplicity. The two most common methods are described and will be used in the practice:

**Mole ratio method:** The basis of the method that while keeping the initial concentration of  $R_1$  ( $[R_1]_0$ ) constant, the initial concentration of the other reactant ( $[R_2]_0$ ) is varied. While  $R_1$  is in stoichiometric excess, the increase of  $[R_2]_0$  results in proportionally larger product concentration at the end of the reaction ( $[P]_\infty$ ). Contrarily, if  $R_2$  is already in stoichiometric excess compared to  $R_1$  then the increase of  $[R_2]_0$  does not yield more product in an irreversible reaction. However, this statement is not valid for equilibrium processes, as seen by the comparison of Fig. 1a and b. Therefore, if the concentration of the product is measured and plotted at several  $[R_2]_0/[R_1]_0$  ratios, the points lie along two straight lines as illustrated in Figure 1a.<sup>1</sup> The  $[R_2]_0/[R_1]_0$  at the intersection point of the two lines corresponds to the stoichiometric ratio  $r_2/r_1$ .

**Job's method:** It is also called *the method of continuous variations* or *the method of isomolar solutions*. The basis of the method is to keep constant the sum of the initial concentrations of the reactants ( $[R_1]_0 + [R_2]_0$ ) while their ratio is gradually changed to measure the concentration of the product.  $[P]_\infty$  has its maximum value when the two reactants are in exact stoichiometric ratio. By plotting the product concentration against the mole fraction of the second reactant ( $x$ ) we obtain very informative graphs. Three such cases are illustrated in Figure 1c, in which the stoichiometric ratio of the two reactants are 3 : 1, 1 : 1 and 1 : 2 from left to right. It can be seen in each curve that the  $[P]_\infty - x$  function consists of two line segments, and the maximum of the function (i.e., the stoichiometric ratio) is at their intersection point (at  $x_{\max}$ ):

$$x_{\max} = \frac{r_2}{r_1 + r_2}, \quad \text{from which} \quad \frac{r_2}{r_1} = \frac{x_{\max}}{1 - x_{\max}}. \quad (2)$$

Both methods are simple and fast therefore they are widely applied. However, they also have limitations which are summarized in the following:

- For both methods, the line segments are straight and they have a sharp cut-off point only in case of an irreversible reaction. In equilibrium reactions (e.g., complex formation), the cut-off points disappear, and the lines have curvatures. This is illustrated for both methods in Figures 1b and d where the yield is about 70% when the reaction is completed at stoichiometric ratio. In this case, the stoichiometric ratio can be estimated by the intersection point of the tangent lines at  $x = 0$  and  $x = 1$ . It can also be

<sup>1</sup>Obviously, both lines are only visible if there are points corresponding to the excess of  $R_1$  and  $R_2$ , as well.

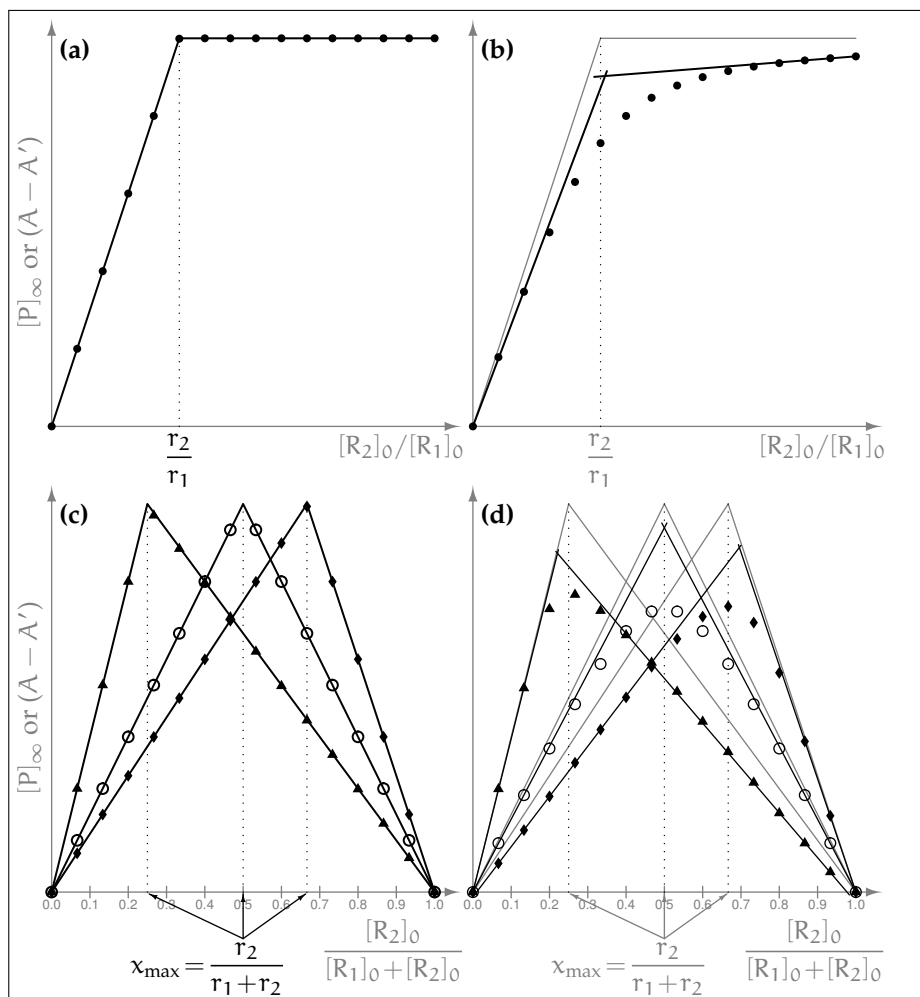


Figure 1: Illustration of the mole ratio (a, b) and the Job (c, d) method for irreversible (a, c) and equilibrium (b, d) reactions. The lines in the left graphs are also indicated on the right graphs, as gray lines. Further explanation is in the text.

seen in the figures (more clearly in (d)), however, that this estimation can significantly deviate from the real stoichiometric ratio if it differs from 1 : 1. It is generally true, that smaller maximum yield and larger deviation from the 1 : 1 ratio leads to less precise evaluation.

The curves in the figures also suggest that the Job's method performs better than the mole ratio method in cases of equilibrium reactions. Although the intersection point of the tangent lines changes more in case of the Job's method, the position of the maximum can be estimated by eye more precisely with this method than applying the mole ratio method. Moreover, the ratio of the reactants can be changed in a much larger range in the case of the Job's method. For equilibrium reactions, the mole ratio method would require unrealistically large excess of  $R_2$ .

- Both methods can give unambiguous result only if one product is formed exclusively.<sup>2</sup> If a reaction behaves differently, then the experimental curves certainly deviate from those of displayed in Figure 1.
- The reaction itself can change the  $pH$  and/or the ionic strength of the reaction mixture. They could influence the path of the reaction (mainly in equilibrium reactions) therefore constant  $pH$  and ionic strength has to be maintained for more precise measurements.

<sup>2</sup>More precisely, if more products are formed, then their quality and stoichiometric ratios must be independent of their initial concentrations.

## 1.1 Application of the Job's and mole ratio methods

In general, we do not measure directly the concentration of the product, rather a quantity is followed which is proportional to that. The Job's and mole ratio methods are frequently applied to determine the composition of water soluble complex molecules. In such cases, the product concentration can be obtained from photometric measurements carried out at the appropriate wavelength. This wavelength usually falls in the range of UV-vis. On the other hand, catchy and well-visible results can be obtained, if precipitation reactions are applied to illustrate the above methods. We now introduce these experimental procedures in more details.

### 1.1.1 Test tube experiments with precipitation reactions

In order to determine the reaction stoichiometry, it is not necessary to know the absolute amount or concentration of the product. It is sufficient to measure a quantity being proportional to one of those descriptors, and reveal the trend how this quantity changes with the composition of the reactant solution. If the constituent ions of a sparingly soluble salt are separately contained by two reactant solutions with an appropriately high concentration (i.e., solubility product is overcome upon mixing), the solid phase (chemical precipitate) sooner or later will appear upon mixing the reactant solutions. In most cases this precipitate sediments to the bottom of the container. If the reactions of various reactant compositions are performed in identical test tubes, the height of the precipitate column is proportional to the amount of precipitate. For this reason, here we simply assume a constant particle density for the precipitate columns independently of the composition of the reactant solution. The height of the precipitate column can be determined by a simple ruler, if its amount is appropriately high and differs from one test tube to the other. In this practice, however, we will apply digital image processing for such purpose to become familiar with its concept.

### 1.1.2 Photometric measurements

To measure the concentration of a solute by photometry, we apply the Beer-Lambert law

$$\lg \frac{I_0}{I} = A = \epsilon^\lambda \ell c,$$

where  $I_0$  and  $I$  are the intensity of the light before and after passing through the solution,  $A$  is absorbance,  $\epsilon^\lambda$  is the molar absorbance at the wavelength of  $\lambda$ ,  $\ell$  is the length of the light path, and  $c$  is the concentration of the colored species. Frequently not only the products but also one or more reactants are colored or only the reactants have color. For reaction (1) the absorbance can generally be expressed as the sum of the light absorption of both the product and the reactants:

$$A = \ell (\epsilon_{R_1}^\lambda [R_1] + \epsilon_{R_2}^\lambda [R_2] + \epsilon_P^\lambda [P]),$$

where  $\epsilon_{R_1}^\lambda$ ,  $\epsilon_{R_2}^\lambda$  and  $\epsilon_P^\lambda$  are the molar absorbances of the species participating in the reaction at the wavelength of  $\lambda$ . A simple evaluation, however, requires such absorbance value which is directly proportional to only  $[P]$ . Therefore the background absorbance  $A'$  is defined as

$$A' = \ell (\epsilon_{R_1}^\lambda [R_1]_0 + \epsilon_{R_2}^\lambda [R_2]_0).$$

This value would be measured in a hypothetical case, when the reaction is absent after mixing the reactants.<sup>3</sup> By subtracting it from the absorbance of the reaction mixture, and applying the stoichiometry of Eqn. (1), one can derive

$$[R_1] = [R_1]_0 - \frac{r_1}{p} [P] \quad \text{and} \quad [R_2] = [R_2]_0 - \frac{r_2}{p} [P]$$

---

<sup>3</sup>The background absorbance can easily be calculated if the absorbance of the stock solutions of the reactants, as well as, their dilution ratios are known, since absorbance is an additive quantity.

and substituting them back:

$$\begin{aligned} A - A' &= \ell (\epsilon_{R_1}^\lambda ([R_1] - [R_1]_0) + \epsilon_{R_2}^\lambda ([R_2] - [R_2]_0) + \epsilon_P^\lambda [P]) = \\ &= \ell \left( \epsilon_{R_1}^\lambda \left( -\frac{r_1}{p} [P] \right) + \epsilon_{R_2}^\lambda \left( -\frac{r_2}{p} [P] \right) + \epsilon_P^\lambda [P] \right) = \ell \underbrace{\left( \epsilon_P^\lambda - \frac{r_1 \epsilon_{R_1}^\lambda + r_2 \epsilon_{R_2}^\lambda}{p} \right)}_{\text{constant at a given } \lambda} [P]. \end{aligned}$$

This relation shows that the quantity  $(A - A')$  is directly proportional to  $[P]$ ; the same relation is valid for  $(A - A')_\infty$  and  $[P]_\infty$ . The plots can be different because concentrations cannot be negative, while the value of  $(A - A')$  can also be negative, if the total absorbance of the reactants are larger than that of the products at the used wavelength. As a consequence, the curves in Figure 1 can also be decreasing and can exhibit minimum.

## 2 Experimental

### 2.1 Test tube experiments with precipitation reactions

*For technical reasons, start the work with these experiments.*

Place the 17 pcs (20 cm<sup>3</sup>) test tubes next to each other in the tube stand. *If the test tubes are not identical (especially considering the inner diameter), inform the technician and ask for new ones.*

Measure the appropriate volumes of the two unknown reactant solutions (Cs<sub>1</sub> and Cs<sub>2</sub>) by applying an automatic pipette. For technical reasons, always measure the volume of Cs<sub>1</sub> stock solution into the test tube and then add the appropriate amount of Cs<sub>2</sub>. Into the first test tube, measure 1 cm<sup>3</sup> of Cs<sub>1</sub> stock solution, then increase the volume by 0.5 cm<sup>3</sup> for each coming tubes, so that the last tube (# 17) contains 9 cm<sup>3</sup> of Cs<sub>1</sub> stock solution. After measuring the appropriate volumes of Cs<sub>1</sub> stock solution into each test tubes, with a clean pipette tip, add the portions of the Cs<sub>2</sub> stock solution, so that each test tubes contain 10 cm<sup>3</sup> of reactant mixture (consider the solution volumes additive).

Once each reactant mixture is ready, shake the tubes rigorously, and note the time. Precipitation sets in immediately upon the contact of the reactant solutions. To ensure appropriate crystal growth and sedimentation, shake the test tubes again approx. 1 min later. Finally, let the precipitate settle down in rest.

After 90 mins sedimentation time, take a photo of the system by paying attention to the following:

- Test tubes must be stored vertically and make sure nothing hides the precipitate columns;
- To achieve better contrast, place a black background behind the test tubes; avoid glittering;
- Scale bar must be clearly seen on the picture;
- Take the photo in front of and in plane with the tube stand; avoid twisting;
- If possible, take a single photo which depicts all test tubes at the same time (do not show the entire test tubes, precipitate columns must be focused). Alternatively, take two photos and make sure that the scale bar is visible on both;
- Check the scale bar on the photo, it should not be blurry when zooming in.

If the Instructor approved the photo, wash all equipment.

### 2.2 Photometric measurements

After performing the experimental work described in Section 2.1, and while waiting for 90 mins sedimentation, switch on the spectrophotometer to warm up the lamp (approx. 15 mins). This is necessary to achieve intense and time-independent light intensity.

First, carry out the experiments related to the Job's method. From the stock solutions of  $R_1$  and  $R_2$  (provided as unknowns and having equal concentrations) prepare a series of nine solutions in volumetric flasks with volume of  $25\text{ cm}^3$  according to Table 1. Measure the necessary volumes of the stock solutions into the volumetric flasks with analytical precision. Then fill up to the meniscus with distilled water in each volumetric flask. The reactants must be completely mixed before the final dilution.

Table 1: The required volumes of reactants.

Sample No.	1	2	3	4	5	6	7	8	9
$R_1$ ( $\text{cm}^3$ )	9.00	8.00	7.00	6.00	5.00	4.00	3.00	2.00	1.00
$R_2$ ( $\text{cm}^3$ )	1.00	2.00	3.00	4.00	5.00	6.00	7.00	8.00	9.00

Select the solution which shows the largest color change (or difference) from the prepared nine ones. Presumably this solution has the composition closest to the stoichiometric ratio. If any of the stock solutions (or both ones) are colored, then one (or two) more dilutions must be carried out to determine  $A'$ . For example, if solution 4 is chosen from Table 1 and the stock solution of  $R_1$  is colored, then  $6.00\text{ cm}^3$  of stock solution  $R_1$  must be measured into a new volumetric flask with the volume of  $25\text{ cm}^3$ , and the flask must be filled up with distilled water without the addition of  $R_2$  (not to forget homogenization!).

After reading the user's manual of the spectrophotometer, the absorbance spectrum of both the chosen solution and the colored, appropriately diluted stock solution(s) should be recorded to determine the appropriate wavelength for the further measurements. The wavelength range to be investigated is determined by the fact that *the solution is colorful, i.e., absorbs the visible light*. Save the spectra from the instrument or, if it is not possible, take a photo of them and attach it to the lab notes.

Based on the recorded spectra, the wavelength to be used for the further experiments should be selected by considering the following:

- If there is a wavelength at which the stock solutions do not absorb light, and the product(s) ha(s/ve) a maximum absorption, then this wavelength should be selected.<sup>4</sup>
- Select a wavelength where the relative difference between the total absorbance of the reactants and that of the product is the greatest.
- The relative error of the absorbance measurements is the smallest in the absorbance range of 0.2–2.0, therefore select the wavelength so the greatest expected absorbance is in the absorbance range of 1.7–2.0.

The selected wavelength should be approved by the instructor.

Once the wavelength is selected, only a single cuvette to be used to eliminate the cuvette correction due to the absorbance differences between the cuvettes. Fill up the clean cuvette with distilled water and set its absorbance to 0 at the selected wavelength. Then measure the absorbance of the 9 solutions and the colored stock solution(s). Before each measurement, the cuvette has to be washed at least three times with  $\approx 1 - 2\text{ cm}^3$  of the sample. The cuvette then has to be filled ca.  $2/3$  of its height and measure its absorbance. Pay attention that (1) the outer surface of the cuvette has to be clean and dry before each measurement and (2) the cuvette has to be placed correctly into the spectrophotometer<sup>5</sup>.

Depending on the experimental data, two routes are possible to continue the work:

1. If the stoichiometric ratio is expected to differ from 1 : 1, perform 8 more measurements with the Job's method around the expected maximum (minimum) according to Table 2. In the table,  $z$  is the value

<sup>4</sup>It can also be reversed, i.e., the reactant(s) ha(s/ve) absorbance maximum, and the product(s) do(es) not have any absorption.

<sup>5</sup>Cuvette has to stand the same way in the instrument throughout the practice. It has to be vertical and the light should enter the same wall of the cuvette and that wall should not be opaque.

of the volume of the  $R_1$  stock solutions in  $\text{cm}^3$  which is expected to have the composition closest to the stoichiometric ratio. The solutions and the measurements should be prepared and carried out as described earlier. The reproducibility of the reaction can be checked as solution 14 is the exact repetition of the selected solution of the first series.

Table 2: The required volumes of the stock solutions for the continuation of the Job method.

Sample No.	10	11	12	13	14	15	16	17	18
$R_1$ ( $\text{cm}^3$ )	$z+0.80$	$z+0.60$	$z+0.40$	$z+0.20$	$z$	$z-0.20$	$z-0.40$	$z-0.60$	$z-0.80$
$R_2$ ( $\text{cm}^3$ )	$10.00 - (\text{the volume of the } R_1 \text{ stock solution in } \text{cm}^3)$								

- If the stoichiometric ratio is expected to be 1 : 1, then a solution series corresponding to the mole ratio method according to Table 3 has to be prepared. The solution preparation and the absorbance measurements are to be performed as described earlier.

Table 3: The required volumes of the reactant stock solutions for the mole ratio method.

Sample No.	I	II	III	IV	V	VI	VII	VIII	IX
$R_1$ ( $\text{cm}^3$ )	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
$R_2$ ( $\text{cm}^3$ )	1.00	2.00	3.00	4.00	5.00	6.00	8.00	10.00	12.00

## 3 Evaluation

### 3.1 Test tube experiments with precipitation reactions

- Hand in the photo(s) together with the calculations.
- Determine the height of the precipitate column ( $h$  / mm) by digital image processing for each test tube (reactant solution composition); see *Appendix* for a step-by-step image processing guide. The obtained distance calibration (e.g., px / mm) must appear in the labnote. In addition, attach a printscreen showing the evaluation (e.g., similar to Figure 5a; no need to format the style of printscreen). Tabulate your results according to Table 4. To calculate  $x_{\text{Cs}_2}$  for the reaction mixtures, consider that no need to know the molarity of the stock solutions. Since  $[\text{Cs}_1]_0 = [\text{Cs}_2]_0$ , only the dilution must be taken into account.<sup>6</sup>

Table 4: Results of digital image processing and subsequent calculations.

No. test tube	$x_{\text{Cs}_2}$	$h$ / mm	$h'$

<sup>6</sup>Since  $x_{\text{Cs}_2} = \frac{n_{\text{Cs}_2}}{n_{\text{Cs}_2} + n_{\text{Cs}_1}} = \frac{c_{\text{Cs}_2} V_{\text{Cs}_2}}{c_{\text{Cs}_2} V_{\text{Cs}_2} + c_{\text{Cs}_1} V_{\text{Cs}_1}}$ , where  $c_{\text{Cs}_1} = c_{\text{Cs}_2}$  due to experimental design, the equation simplifies and mole fractions can be calculated as dilution.

- Plot the  $h - x_{\text{CS}_2}$  data pairs.
- To determine the reaction stoichiometry, look for the mole fraction ( $x_{\text{max}}$ ) where the height of the precipitate column is maximal, *i.e.*, find the maximum position of the function described by the  $h - x_{\text{CS}_2}$  data set. This might not be equivalent with the solution composition of any test tube. To find  $x_{\text{max}}$ , numerically differentiate the data set with a spreadsheet editor, *e.g.*, MS Excel (*i.e.*, compute the difference quotients,  $h' = \frac{\Delta h}{\Delta x_{\text{R}_2}}$  for each measured point (from three points for those which has lower and upper neighbors and from two points for the endpoints); see Fig. 2). Tabulate the difference quotients in Table 4, and plot the  $h' - x_{\text{R}_2}$  data sets in the previous graph; use separate y-axis for better visibility.
- The difference quotient is positive for increasing precipitate heights, while it is negative for decreasing tendency. In the region where, based on the cellphone image, the maximum height of the precipitate column is expected, look for the two  $h' - x_{\text{R}_2}$  data pairs, where the different quotient changes sign. To do so, apply linear interpolation and calculate  $x_{\text{max}}$  (*i.e.*, where  $h' = 0$ ).
- With the aid of eqn. (2) and  $x_{\text{max}}$ , find the stoichiometry of the reaction.

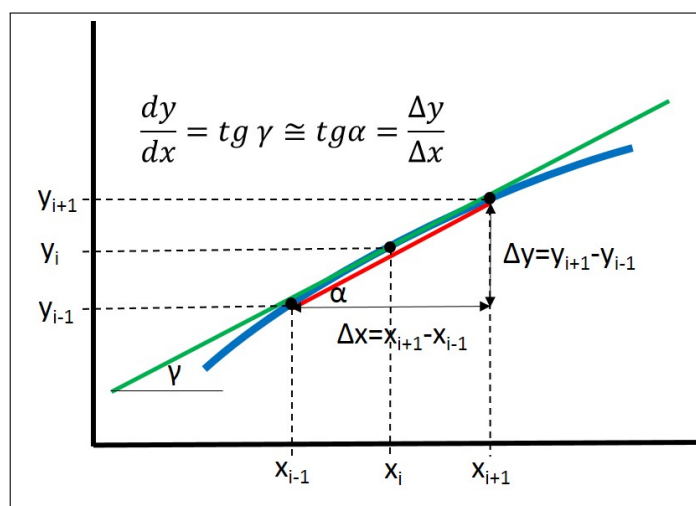


Figure 2: Schematics of the calculation of difference quotients with the three-point formula.

### 3.2 Photometric measurements

- Attach the absorbance spectra to the lab notes. Give the wavelengths used for the measurements; explain your choice.
- Summarize the measurements and calculations related to the Job's method according to Table 5. Take into account that to determine the initial concentration ratios and  $A'$ , the exact dilutions should be considered but the concentrations of the stock solutions (which are equal) are not needed.

That is, consider only the volume of the reactant solutions for the calculation of  $x_{\text{R}_2}$ ; do not consider the added water because  $x_{\text{R}_2}$  directly corresponds to the reactant mixture and not to its dilution. On the contrary, to obtain  $A'$ , consider the final volume of the solution because the measured value corresponds to this dilution.

If only Job method is used in the practice, all the data points should be included in one table.

- Plot the  $(A - A') - x_{\text{R}_2}$  function. If only Job's method is used in the practice, all the data points should be plotted in one graph. Determine  $x_{\text{max}}$  by calculating the intersection of the lines. By knowing  $x_{\text{max}}$ , give the stoichiometry of the reaction.

Table 5: Summary of the experimental results for the Job method.

$$\lambda = \dots \text{ nm}, A_{\text{diluted R}_1} = \dots, A_{\text{diluted R}_2} = \dots,$$

Sample No.	$x_{\text{R}_2}$	A	A'	A - A'

4. If the mole ratio method is used, the results should be summarized according to Table 6. The evaluation is the same as used for the Job method, except that the value of  $r_2/r_1$  can be determined immediately from the graph.

Table 6: Summary of the experimental results for the mole ratio method.

$$\text{wavelength} = \dots \text{ nm}, A_{\text{R}_1\text{-stock solution}} = \dots, A_{\text{R}_2\text{-stock solution}} = \dots,$$

Sample No.	$[\text{R}_2]_0/[\text{R}_1]_0$	A	A'	A - A'

5. Based on the graphs obtained, discuss the reversibility of the reaction.



## Questions



1. Describe the mole ratio method in 3 – 5 sentences.
2. Describe the Job method in 3 – 5 sentences.
3. Draw schematically a curve for (a) an irreversible reaction and (b) an equilibrium reaction using the mole ratio method. Assign the position of the stoichiometric ratio.
4. Draw schematically a curve for (a) an irreversible reaction and (b) an equilibrium reaction using the Job method with 2 : 1 stoichiometric ratio.
5. Give the significance of  $x_{\max}$  and its relation with the stoichiometric ratio of the two reactants.
6. What are the limits of the Job's and mole ratio methods?
7. Give the Beer – Lambert law, define each symbol.
8. Define the background absorbance, give its symbol and explain its importance for using the Job's and mole ratio methods.
9. What has to be considered on selecting the appropriate wavelength?
10. Explain in 3 – 5 sentences, how you can determine  $x_{\max}$  from the precipitation column height measurements.
11. The maximum of a Job's plot is located at  $x_{\max} = 0.25$ . Calculate the stoichiometric coefficients of the reactants for an  $r_1R_1 + r_2R_2 = pP$  type reaction.
12. From the experiments, you obtained the mole fraction – precipitate column height data set presented in the table below. By applying numerical differentiation, calculate the slope of the  $h - x_{\text{Cs}_2}$  function a) either for the first or for the last point of the data set (two-point formula), and b) for an arbitrarily selected mole fraction excluding the two above (three-point formula).

$x_{\text{Cs}_2}$	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
h / mm	3.2	5.9	7.7	11.6	15.0	22.4	22.5	13.4	6.5

13. We measured  $6.00 \text{ cm}^3$  of  $\text{Cs}_1$  and  $4.00 \text{ cm}^3$  of  $\text{Cs}_2$  stock solutions into a test tube. The molarity of the stock solutions is equal. Express the composition of the reactant solution according to the mole fraction of the second reactant,  $x_{\text{Cs}_2}$ .
14. We have measured  $7.00 \text{ cm}^3$   $\text{R}_1$  stock solution,  $3.00 \text{ cm}^3$   $\text{R}_2$  stock solution into a volumetric flask. The solution then was diluted to  $25.00 \text{ cm}^3$ . The absorbance of the  $\text{R}_1$  stock solution is 0.327, while that of  $\text{R}_2$  is 0.015. The concentrations of the stock solutions are the same. Calculate  $[\text{R}_2]_0/[\text{R}_1]_0$  and  $\frac{[\text{R}_2]_0}{[\text{R}_1]_0 + [\text{R}_2]_0}$  in the solution.
15. Calculate  $A'$  for the solution given in question 14.

## Appendix: Digital image processing

The digital photo of the experiment is to be processed by the freely available *ImageJ* software in order to determine the height of the precipitate columns.

1. If not available at the PC, download and extract the .zip file into a selected location; link: <https://imagej.net/ij/download.html>
2. When a vertical line is drawn along the precipitate column, the software displays its grayscale profile. By numerically differentiating this profile, the end points of the precipitate column can be determined, since a sharp change marks the transition from the background to the precipitate, and *vice versa*. Expecting a dark background and a bright precipitate, the latter one is characterized by higher grayscale value. The minimum and maximum of the differentiated grayscale profile denotes the end points of the precipitate column. *ImageJ* uses a *macro* to differentiate the grayscale profiles which can be downloaded here for free: [https://github.com/MontpellierRessourcesImagerie/imagej\\_macros\\_and\\_scripts/wiki/MRI\\_Plot\\_Tool](https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/MRI_Plot_Tool)  
Open the *plot\_tools.ijm* file at the website of the *macro* (*Getting Started* chapter) and copy its whole content into *Notepad*, save it as *derivative.txt*. Copy this file into *ImageJ macros/toolsets* folder. If needed, copy as administrator. Run *ImageJ*, click on  icon in the toolbar and select the *derivative macro*. The icon, , inviting the *macro* appears in the toolbar. By right-clicking on the icon, inactivate *show zero crossings* option.
3. Open the experimental image(s) (*File/Open*) and correct any deviation from the horizontal plane; *Image/Transform/Rotate* menu (Fig. 3a). Set *Angle* to 0.0 and select *Preview*. Increase the value of *Grid lines* until they are numerous enough to judge whether, e.g., a certain part of the test tube holder or the test tube itself is in parallel with the grid lines. Set *Angle* ( $\pm$  value) until the parallel alignment is satisfactory, then select *OK*.

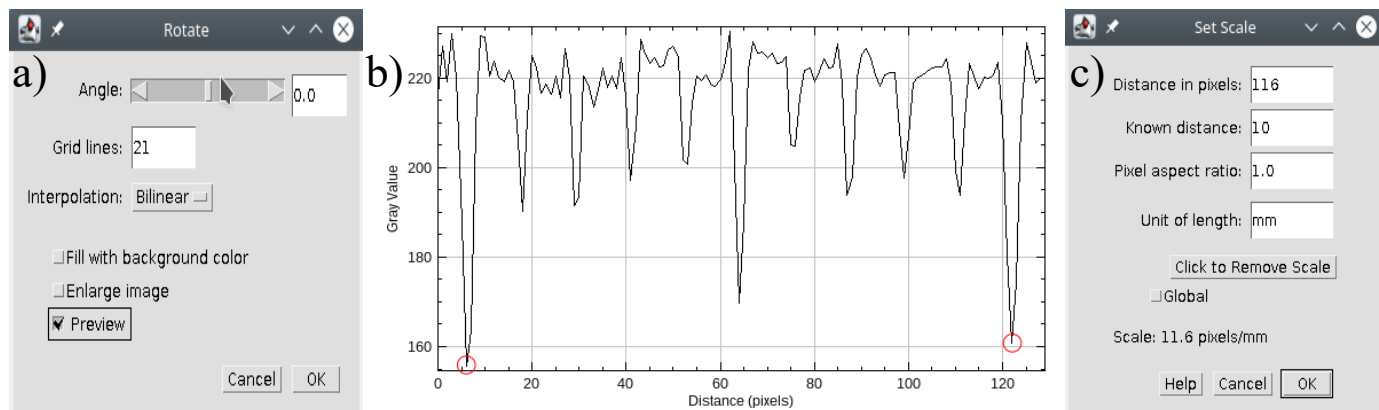





Figure 3: Menu to set image tilt (a), and the graph (b) and menu (c) to set scale.

4. To set scale, zoom in (*Ctrl+scroll*) to the mm-paper fixed on the bottom section of the tube stand. Select straight line from the tool bar (). By holding *Shift* key and moving the mouse, draw a line along the mm-paper which crosses several main grids of the mm-paper (more reliable calibration needs longer measured distance). Pay attention to that the line passes in between two grid lines of the mm-paper. Plot the gray scale profile along the line (*Ctrl+K*, see 3b).

The deep minima correspond to the main grid lines of the mm-paper, the shallower minima belong to the mm scale. Select two minima located far from each other, and read out the *x* coordinates by mousing over them. Make a note of those data and close the gray scale profile window. Note: Closing

the window is not optional before moving to the next step. Perform the calibration in *Analyze/Set Scale* menu with the aid of the above coordinates and by knowing the factual distance between them (i.e., between the selected grid lines). Type the difference of the  $x$  coordinates in *Distance in pixels* bracket ( $122 - 6 = 116$  in case of Fig. 3c, red circles in Fig. 3b). In the present case, we wrote 10 for *Known distance*, since two consecutive main grid lines of the mm-paper were selected, which are separated by 10 mm. Set mm for *Unit of length* and select OK. After this calibration, if a distance is measured on the digital photo, we will obtain the result in factual size instead of pixel. Double-check the success of the calibration. For example, draw a 1 cm long line along the mm-paper and press *Ctrl+M* to show the results of the measurement. In the *length* column of the table, a number close to 10 should appear which is the length of the line in mm. If not, repeat the calibration. If *ImageJ* is closed and re-opened, or a photo taken from different position is imported, new calibration is required.

- After setting up the scale, navigate to the first test tube by  icon. Draw a vertical line along the test tube by holding *Shift* key, as shown in Fig. 5a. Display the gray scale profile along the vertical line (*Ctrl+K*), and differentiate it by clicking on ; a graph similar to Fig. 5b will appear. The height of the precipitate columns is obtained as the difference of the  $x$  coordinates of the minimum and maximum positions seen in the plot. (If those extrema are not well-recognized, attempt to draw a new vertical line near the first one.) Make a note of the height of the precipitate ( $h/mm$ ) in the spreadsheet used to evaluate the results, close the plots, and continue with the next test tube.

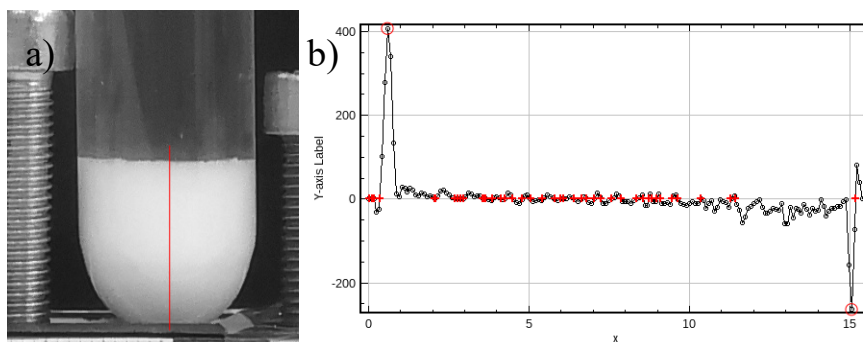


Figure 4: Determination of the precipitate column height by digital image processing: close-up of the experimental image (a) and the derivative of the gray scale profile (b).