The bimolecular binding event (1:1)

\[
H + G \quad \xrightarrow{K} \quad H \cdot G
\]

Topics

- determination of the binding constant
  - experimental conditions
  - data fitting
- binding stoichiometry
- analytical techniques: scope and limitations
- practical considerations
Definitions

\[
\begin{align*}
H + G & \quad \xrightleftharpoons[K]{\text{K}} \quad H \cdot G \\
\text{in reality} & \\
(H \cdot G) \cdot S + S \cdot S & \quad \xrightleftharpoons[\text{K}]{\text{K}} \quad (H \cdot G) \cdot S + S \cdot S
\end{align*}
\]

Explicit solvent is not used because \(\Delta G^\circ\) for the association constant reflects the stability of solvated H and G relative to solvated \(H \cdot G\) and released solvent

\[
\begin{align*}
K_a &= \frac{[H \cdot G]}{[H][G]} \quad \text{(M}^{-1}\text{)} \\
K_d &= \frac{[H][G]}{[H \cdot G]} \quad \text{(M)}
\end{align*}
\]

\(\text{preferred by chemists}\) \quad \text{\(\text{preferred by biologists}\)}

\[
\Delta G^\circ = -RT\ln(K_a)
\]

assuming activity = concentration
The ratios of $H$, $G$, and $HG$ depend on the initial concentrations of $H_0$ and $G_0$ for $K_a = 100 \text{ M}^{-1}$

<table>
<thead>
<tr>
<th>Initial concentrations</th>
<th>Effective concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_0$ (M)</td>
<td>$G_0$ (M)</td>
</tr>
<tr>
<td>1x$10^{-3}$</td>
<td>1x$10^{-3}$</td>
</tr>
<tr>
<td>1x$10^{-2}$</td>
<td>1x$10^{-2}$</td>
</tr>
<tr>
<td>1x$10^{-1}$</td>
<td>1x$10^{-1}$</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Graph: 
- $[HG]$ 
- $[H]$ and $[G]$
$K_d$ is a convenient reference point for estimating the amount of complex

$K_a = 100 \text{ M}^{-1}$

$K_d = 1 \times 10^{-2} \text{ M} = 10 \text{ mM}$

The significance of $K_d$

$\frac{[10][10]}{[10]} = 10 \text{ mM}$

all concentrations in mM

At $K_d$: $[H] = [G] = [HG] = K_d$

(!! only in case $[H]_0 = [G]_0$ !!)
\[ \Delta G^\circ = -RT \ln(K_a) \]

<table>
<thead>
<tr>
<th>( K_a ) (M(^{-1}))</th>
<th>( \Delta G^0 ) (kJ.mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>-5.8</td>
</tr>
<tr>
<td>100</td>
<td>-11.5</td>
</tr>
<tr>
<td>1000</td>
<td>-17.3</td>
</tr>
<tr>
<td>10000</td>
<td>-23.0</td>
</tr>
<tr>
<td>100000</td>
<td>-28.8</td>
</tr>
</tbody>
</table>

at \( T = 301 \text{ K} \) (\( R = 8.314 \text{ J.mol}^{-1}\cdot\text{K}^{-1} \))
Determining association constants from titration experiments in supramolecular chemistry

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How to determine the concentration of each species?

\[ \text{H} + \text{G} \xrightleftharpoons[K_a]{\text{H \cdot G}} \]

If H, G, and HG are all known, then K is easily calculated:

\[ K_a = \frac{[\text{H\cdotG}]}{[\text{H}][\text{G}]} \]

Regrettably, this is hardly ever the case.
Almost all experimental methods to measure binding constants rely on the analysis of a **binding isotherm**.

A binding isotherm gives *the change in the concentration of one component (H, G, HG) as a function of the initial concentration of one of the components (H₀, G₀)* at constant temperature and pressure.

The concentrations are experimentally determined (e.g. NMR, UV/vis, etc) and fitted to the theoretical binding isotherm.

**example**

\[ K_a = 100 \text{ M}^{-1} \]

\[ [H]_0 = 0.01 \text{M} \]
Typically a titration is performed holding the concentration of one species (H) constant while varying the concentration of the other (G).

During the course of this titration, the physical changes in the system are monitored, usually spectroscopically, and this change is then plotted as a function of the initial concentration of guest to give the binding isotherm.

The mathematical model used to obtain the association constant is usually developed from realising that *the physical change* ($\Delta Y$, e.g. a NMR shift or a change in UV-Vis absorbance) *observed is correlated to the concentration of the complex [HG] as*

\[ \Delta Y = f[HG] \]

or, in some cases, the free host ($\Delta Y = f[H]$) or the free guest ($\Delta Y = f[G]$).

The physical change ($Y$) being monitored can usually be described as the aggregate of the individual components according to eqn (1) as a function of concentration (e.g., for UV-Vis spectroscopy) or eqn (2) as a function of mole fractions $f_X$ ($f_X$ defined as: $f_X = [X]/[X]_0$) in the special case of NMR.

\[
\begin{align*}
(1) \ Y &= Y_H[H] + Y_G[G] + Y_{HG}[HG] \quad \text{when concentrations are used} \\
(2) \ Y &= Y_Hf_H + Y_Gf_G + Y_{HG}f_{HG} \quad \text{when mole fractions are used}
\end{align*}
\]
only \( HG \) has a particular absorption band in the UV/vis spectrum.

\[
K_a = \frac{[HG]}{[H][G]}
\]

\[
[G] = [G]_0 - [HG]
\]

\[
[H] = [H]_0 - [HG]
\]

\[
K_a = \frac{[HG]}{([H]_0 - [HG])([G]_0 - [HG])}
\]

Thus, \( A = \varepsilon_{HG}[HG] / \)
\[ K_a = \frac{[HG]}{([H]_0 - [HG])([G]_0 - [HG])} \]

\[ K_a = \frac{[HG]}{[H]_0[G]_0 - [HG][G]_0 - [HG][H]_0 + [HG]^2} \]

\[ K_a ([H]_0[G]_0 - [HG][G]_0 - [HG][H]_0 + [HG]^2) = [HG] \]

\[ K_a[HG]^2 - (K_a[G]_0 - K_a[H]_0 + 1) [HG] + K_a[H]_0[G]_0 = 0 \]

This expresses \([HG]\) as a function of \(K_a\), which is the only unknown!!

\[ [HG] = \frac{1}{2} \left( G_0 + H_0 + \frac{1}{K_a} \right) - \sqrt{\left( G_0 + H_0 + \frac{1}{K_a} \right)^2 + 4[H_0][G_0]} \]
Since, $A = \varepsilon_{HG}[HG]$ 

and

$$[HG] = \frac{1}{2} \left( G_0 + H_0 + \frac{1}{K_a} \right) - \sqrt{\left( G_0 + H_0 + \frac{1}{K_a} \right)^2 + 4[H_0][G_0]}$$

Then $A = f(\varepsilon_{HG}, K_a)$

The power of this equation should not be understated as we can now start to develop solutions that require only the knowledge of the total (or initial) concentrations of the host and guest ($[H]_0$ and $[G]_0$) in addition to the association constant ($K_a$) and the physical properties ($Y$) that are changing ($\Delta Y$) during the course of the titration.
\[ Y = Y_H f_H + Y_G f_G + Y_{HG} f_{HG} \]

If we assume that one of the components is “silent” e.g., a non-absorbing free guest \([G]\), we can simplify the equation to

\[ Y = Y_H f_H + Y_{HG} f_{HG} \]

which, since \( f_{HG} = [HG]/[H]_0 \) and \( f_H = 1 - f_{HG} \), can be further simplified to

\[ Y = Y_H + ([HG]/[H]_0)(Y_{HG} - Y_H) \]

and, finally

\[ \Delta Y = Y_{\Delta HG} \left( \frac{[HG]}{[H]_0} \right) \]

in which \( \Delta Y = Y - Y_H \) \( (\text{this is the experimental data point}) \)
and \( Y_{\Delta HG} = Y_{HG} - Y_H \) \( (\text{this is the maximum difference of the physical parameter between HG and H}) \)
**Example**

*for example:* in case \([G]_0\) is titrated to \([H]_0\)

then for \([G]_0 = 0\), \([HG] = 0\) and \(\Delta Y = 0\)

whereas for \([G]_0 \gg [H]_0\), \([HG] \sim [H]_0\) and \(\Delta Y = Y_{\Delta HG}\)
Different analytical techniques

NMR (chemical shift)

$$\Delta \delta = \delta_{\Delta HG} \left( \frac{[HG]}{[H]_0} \right)$$

UV-Vis (absorbance)

$$\Delta A_{obs} = \varepsilon_{\Delta HG} [HG]$$

just absorbance from complex

fluorescence (intensity)

$$\Delta F_{I_{obs}} = k_{\Delta HG} [HG]$$

just fluorescence from complex

no dynamic quenching (see later)

calorimetry (heat of formation)

$$Q = \Delta H_{HG} V [HG]$$
There is one problem though

Since, \( A = \varepsilon_{\text{HG}}[\text{HG}] \)

and

\[
[\text{HG}] = \frac{1}{2} \left( G_0 + H_0 + \frac{1}{K_a} \right) - \sqrt{\left( G_0 + H_0 + \frac{1}{K_a} \right)^2 + 4[H_0][G_0]}
\]

Then \( A = f(\varepsilon_{\text{HG}}, K_a) \)

I need to know \( \varepsilon_{\text{HG}} \) to correlate \( A \) and \([\text{HG}] \) !!

what is \( \varepsilon_{\text{HG}} \) ??

or, in general, for

\[
\Delta Y = Y_{\Delta HH} \left( \frac{[\text{HG}]}{[H]_0} \right)
\]

what is \( Y_{\Delta HH} \) ??

\[
\Delta \delta = \delta_{\Delta \text{HG}} \left( \frac{[\text{HG}]}{[H]_0} \right) \quad \Delta A_{\text{obs}} = \varepsilon_{\Delta \text{HG}}[\text{HG}] \quad \Delta F I_{\text{obs}} = k_{\Delta \text{HG}}[\text{HG}] \quad Q = \Delta H_{\text{HG}} V[\text{HG}]
\]
Older references and (biochemistry) textbooks are full of examples on how some of the above expressions and equations can be simplified or transformed to linear equations \((y = a + bx)\) which could then be plotted by hand to obtain the \(K_a\) and other parameters of interest by inspection of the slope and intercepts.

**Benesi–Hildebrand plot**

(determination of binding constants based on absorbance)

\[
A = A^{HG} + A^G + A^H
\]

assuming that \([G]_0 >> [H]_0\) (or that \(A^H << A^G\))

\[
A = A^{HG} + A^G
\]

with \(\Delta A_{obs} = A - A_0\) (experimental points; Lambert - Beer) this gives

\[
\Delta A_{obs} = \varepsilon^{HG}[HG]l + \varepsilon^G[G]l - \varepsilon^G[G]_0l
\]

\(l = \text{optical path}\)

considering that \([G]_0 >> [H]_0\) one can assume that \([G]=[G]_0\) and thus

\[
\Delta A_{obs} = \varepsilon^{HG}[HG]l
\]
The binding isotherm can be rewritten as

\[ K_a = \frac{[HG]}{[H][G]} \]

\[ [H] = [H]_0 - [HG] \]

and

\[ K_a = \frac{[HG]}{[G]([H]_0 - [HG])} \]

which is

\[ K_a ([G]([H]_0 - [HG])) - [HG] = 0 \]

or

\[ K_a [G][H]_0 - K_a [G][HG] - [HG] = 0 \]

Together this gives

\[ \Delta A_{obs} = \varepsilon^{HG} [HG]l \]

\[ [HG] = \frac{[H]_0 K_a [G]}{1 + K_a [G]} \]

(assuming that \([G] = [G]_0\))

and finally

\[ \frac{1}{\Delta A} = \frac{1}{l \varepsilon^{HG} [G]_0 [H]_0 K_a} + \frac{1}{l \varepsilon^{HG} [H]_0} \]
(Old-fashioned) Shortcuts to the binding constant: Benesi-Hildebrandt plot

\[ \frac{1}{\Delta A} = \frac{1}{l\varepsilon^H [G]_0 [H]_0 K_a} + \frac{1}{l\varepsilon^H [H]_0} \]

Other examples include Lineweaver-Burke plots, Scatchard plots, etc. (see later when we speak on cooperativity)
There are **two key problems** associated with using these linear transformations that make their use highly questionable:

(i) they violate some of the fundamental assumption of linear regression by distorting the experimental error.

(ii) they frequently involve assumptions and shortcuts (such as assuming that $[G_0] >> [H_0]$ or that $Y_{HG} = Y$ at the end of titration (i.e., the complex is fully formed at the end of titration - which would then help to give $Y_{\Delta HG}$). These assumptions are often not valid.

The non-linear regression approach with exact solutions of the quadratic equation (see before) produces the most accurate results. This approach is not difficult with modern computer technology and **there is no real excuse for using old-fashion linear transformations anymore!**
Computer now permit non-linear regression
Example: NMR titration

<table>
<thead>
<tr>
<th>H</th>
<th>1.00E-03</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>δ (ppm)</td>
</tr>
<tr>
<td>M</td>
<td>ppm</td>
</tr>
<tr>
<td>0</td>
<td>7.02</td>
</tr>
<tr>
<td>6.25E-05</td>
<td>7.04</td>
</tr>
<tr>
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<tr>
<td>1.60E-02</td>
<td>7.97</td>
</tr>
</tbody>
</table>
// MicroMath Scientist Model File
IndVars: G0
DepVars: D, H, G, HG
Params: K, DH, DHG, H0
HG=K*H*G
H=H0-HG
G=G0-HG
D=(H/(H+HG))*DH+(HG/(H+HG))*DHG
// boundaries
0<H<H0
0<HG<H0
0<G<G0
***
### least-squares fit

<table>
<thead>
<tr>
<th>All</th>
<th>G0</th>
<th>D</th>
<th>D_CALC</th>
<th>H_CALC</th>
<th>G_CALC</th>
<th>HG_CALC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0000</td>
<td>7.0200</td>
<td>7.0086</td>
<td>0.0010785</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>2</td>
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<td>7.0400</td>
<td>7.0478</td>
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<td>4.2761E-5</td>
</tr>
<tr>
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</table>
**Confidence Intervals:**

<table>
<thead>
<tr>
<th>Parameter Name</th>
<th>Estimate Value</th>
<th>Standard Deviation</th>
<th>95% Range (Univar)</th>
<th>95% Range (S-Plane)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K</strong></td>
<td>1813.51142</td>
<td>102.930810</td>
<td>1576.15255</td>
<td>2050.87029</td>
</tr>
<tr>
<td><strong>DHG</strong></td>
<td>8.00495406</td>
<td>0.00947210623</td>
<td>7.98311134</td>
<td>8.02679677</td>
</tr>
</tbody>
</table>

**K**

- Estimate Value: 1813.51142
- Standard Deviation: 102.930810
- 95% Range (Univar): 1576.15255 to 2050.87029
- 95% Range (S-Plane): 1506.12996 to 2120.89288

**DHG**

- Estimate Value: 8.00495406
- Standard Deviation: 0.00947210623
- 95% Range (Univar): 7.98311134 to 8.02679677
- 95% Range (S-Plane): 7.97666758 to 8.03324053
When a supramolecular titration study is carried out one has to first make a **decision on what technique is going to be used** to follow the physical changes ($\Delta Y$) in the system during the course of experiment.

The two key issues that need to be evaluated are:

i. The expected association constant.

ii. The expected physical changes ($\Delta Y$) upon association.

The expected association constant determines what concentration should be chosen for the host system, which in turn will have an influence on the choice of technique.

Wilcox, using a parameter defined as probability of binding ($p$), showed that it is vital to collect as many data point as possible within the range: $0.2 < p < 0.8$

with $p$ defined according to

$$p = \frac{[HG]}{[G]_0} \quad \text{when} \quad [H]_0 \geq [G]_0$$

**or**

$$p = \frac{[HG]}{[H]_0} \quad \text{when} \quad [H]_0 < [G]_0$$
The probability factor $p$

\[
K_a = \frac{[HG]}{([H]_0 - [HG])([G]_0 - [HG])}
\]

\[
p = \frac{[HG]}{[H]_0}
\]

gives

\[
K_a = \frac{p}{[G]_0 - ([H]_0 + [G]_0)p + [H]_0 p^2}
\]

for $[H]_0 = [G]_0 = 0.001$ M
near $p = 0$ and $p = 1$, small errors in $p$ (experimental error in determination of the concentrations !!) gives large variations in $K_a$.

The best results are obtained in the region $0.2 < p < 0.8$
Example

for $[H]_0 = 0.001$ M

$K_a = 2000$ M$^{-1}$
A systematic exploration of optimal conditions

\[
[H_G] = \frac{1}{2} \left( G_0 + H_0 + \frac{1}{K_a} \right) - \sqrt{\left( G_0 + H_0 + \frac{1}{K_a} \right)^2 + 4[H_0][G_0]}
\]

Using it is possible to calculate \( p \) for a range of \([H]_0\), \([G]_0\) and \(K_a\) values (for a 1:1 complex !!).

Example for \([H]_0 = 10^{-5} \text{ M} \) (a typical concentration used in UV-Vis spectroscopy studies).

The results are plotted for a fixed \([H]_0\) concentration (here \(10^{-5} \text{ M}\)) as a function of \(K_a\) and \([G]_0/[H]_0\) (equivalents of guest added).

The shaded areas indicating \( p \) in the range of 0.2–0.8.
Four different situations can be distinguished

I: If $K_d > [H]_0$ (hence $K_a$ fairly low) then a relatively large excess of $[G]_0$ is required to obtain good $p$-values. In this situation it would be advisable to collect several data points in the range of 1–50 equivalents of G added.

II: If $K_d < [H]_0$ (hence $K_a$ fairly high) the only data points with good $p$-values are within the range of $[G]_0 \circ [H]_0$. In other words, it is essential to obtain as many points as possible between 0–1 equivalents of G added.

III: If $K_d \approx [H]_0$, good $p$-values are obtained almost anywhere within the range of 0 to >10 equivalent of G added. Note that when $K_d = [H]_0 = [G]_0$, then $p = 0.38$. 

IV: If $K_d << [H]_0$
IV: $K_d << [H]_0$: The fourth scenario to consider is when $K_d << [H]_0$, i.e., by a factor of at least 100. Here, it is not enough to look just at the $p$ values obtained. Consider instead what is happening with the non-linear portion of the resulting binding isotherms.

Here it becomes clear that once $[H]_0/K_d > 100$, the nonlinear portion of the resulting binding isotherms is restricted to a small region around 1 equivalent of guest added. With $H]_0/K_d > 1000$, it is clear that there is very little 'information' content in the isotherms.

When binding occurs under saturation conditions, this implies that the experimental conditions are NOT adequate for determination of $K$. 

![Graph showing binding isotherms for different $[H]_0/K_d$ ratio's from 1–10 000. The inset shows the region around 0.9–1.1 equivalents added for $[H]_0/K_d = 1000–10 000$ only.](image-url)
fluorescence concentrations

The suitable analytical technique for determination of $K_a$ depends on its value.

limit: $[H)_0/K_d < 100$
and thus $K_{d,lim} > [H)_0,lim/100$

or $K_{a,lim} < 100/[H)_0,lim$

$[H)_0,lim$ is determined by the sensitivity of the technique
scope and limitations
The most informative technique in most situations is $^1$H NMR. Other forms ($^{13}$C, $^{19}$F etc.) of NMR are also applicable. Apart from the **quantitative information** that an NMR titration can yield, the relative shifts and changes in symmetry can often give valuable information about **structure** of the host-guest complex (mode of interactions and stoichiometry). This information can be of significant benefit even in situations where complete quantitative data cannot be obtained from the NMR titration.

Classical approaches for data analysis of NMR titrations assume that the resonance ($\delta$) of interest is the weighted average of the free host (H) and the bound host in the complex (HG) in the experiment for a simple 1 : 1 system

$$\delta_{\text{obs}} = \chi_H \delta_H + \chi_{HG} \delta_{HG}$$

since $\chi_H = 1 - \chi_{HG}$

$$\delta_{\text{obs}} - \delta_H = \chi_{HG} (\delta_{HG} - \delta_H)$$

since

$$X_{HG} = \frac{[HG]}{[H]_0} = \frac{K_a[G]}{1 + K_a[G]}$$

see slide 18

this gives

$$\Delta \delta = \frac{\delta_{HG}K_a[G]}{1 + K_a[G]}$$
With modern NMR instruments it is possible to obtain good quality spectra with sub-millimolar concentrations (routinely now as low as $10^{-4}$ M), suggesting that NMR is suitable for $K_a$ up to and even above $10^6$ M$^{-1}$. Many literature references will state that $10^5$ M$^{-1}$ is the limit for NMR titration experiments.

With NMR one has also to take into account the relative exchange rates within the host–guest, i.e. the on/off rates ($K_a = k_1/k_{-1}$), and the timescale of the NMR experiment. The real limiting factor for NMR titrations is therefore whether the system of interest is in the fast or slow exchange region under the conditions used.

It may be tempting to think that in the (very) slow exchange region of NMR, one could obtain an association constant directly from the relative ratios of the free and bound host. However, this can be difficult in practice due to complications that arise in the intermediate-to-slow region with the size (amplitude) of the observed resonances and the usual limitation of obtaining accurate (quantitative) integration from NMR experiments.
NMR spectroscopic detection of chirality and enantiopurity in referenced systems without formation of diastereomers

Jan Labuta, Shinsuke Ishihara, Tomáš Šikorský, Zdeněk Futera, Atsuomi Shundo, Hanyková, Jaroslav V. Burda, Katsuhiro Ariga & Jonathan P. Hill

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**a**
Achiral host H:

**b**

**c**

**d**

**Titration with (-)-3**

\[ \delta_{\text{NH}, \text{p.p.m.}} \]
Multiplying the electron storage capacity of a bis-tetrazine pincer ligand†

The second most common method for the supramolecular titration experiment is probably UV-Vis spectroscopy. With the right chromophore, host concentrations in the sub-micromolar range \(10^{-7} \text{ M}\) can be applied, making the determination of association constants as high as \(10^9 \text{ M}^{-1}\) in simple 1:1 systems possible (albeit difficult) with \(K_d/[H]_0 = 100\) as discussed above.

Advantages
- rapid. Absorption and related phenomenon (fluorescence) occur on ps time scale or faster in all cases faster than complex dissociation rate slow exchange
- straightforward to correlate signal intensity to concentration (linear regime; Lambert-Beer)
- sensitive

Disadvantage
- titration by UV-Vis spectroscopy is particularly vulnerable to dilution and temperature effects (all supramolecular titration experiments need some temperature control) and the presence of impurities in either host or guest solutions.
- requires chromophoric hosts or guests
Fig. 3 UV-Vis titration to form Fe(btzp)$_2^{2+}$ in solution (purple trace) from the free ligand (pink trace), demonstrating the emergence of both n→π* and d→π* transitions in the complex.

metal-to-ligand charge transfer (MLCT) transitions
Fluorescence spectroscopy

The phenomenal sensitivity of this technique makes routine measurements in the sub-micromolar, even nanomolar (nM) range possible and hence, fluorescence spectroscopy is ideal for the determination of very large association constants ($K_a > 10^{10}$ M$^{-1}$).

Fluorescence is a particularly useful technique in the case when only one of the species in solution is fluorescently active, i.e. when either the free host or guest is fluorescent "silent" or inactive and the fluorescence of the remaining species is either turned "off" (quenched) or "on" upon complexation.

If quenching plays a role, it is necessary to differentiate between static and dynamic (collisional) quenching, with only the former of real significance for supramolecular binding studies.

Dynamic quenching is usually measured by plotting the ratio of the initial ($F_0$) and measured ($F$) fluorescence intensity ratio ($F_0/F$) against the concentration of the quencher [Q] according to the Stern–Volmer relation $F_0/F = 1 + K_{SV}[Q]$, with $K_{SV}$ = the Stern–Volmer constant.

Unfortunately, pure 1 : 1 static quenching follows a nearly identical relation: $F_0/F = 1 + K_a[Q]$, with [Q] = the free concentration of the quencher (guest) and $K_a$ is the association constant of interest in supramolecular binding studies.

In many cases the observed quenching is a mixture of both static and dynamic quenching which can lead to some complication in the analysis of the titration data.
Dynamic quenching is usually measured by plotting the ratio of the initial \((F_0)\) and measured \((F)\) fluorescence intensity ratio \((F_0/F)\) against the concentration of the quencher \([Q]\) according to the Stern–Volmer relation

\[
\frac{F_0}{F} = 1 + K_{SV}[Q]
\]

with \(K_{SV}\) = the Stern–Volmer constant.

**Example**

Quenching of fluorescein by iodine
Static quenching

A second type of quenching is static quenching, in which the quenching agent forms a non-fluorescent complex with the quenching agent.

\[
\text{Fluor} + Q \xrightarrow{K_S} \text{Fluor} \cdot Q \text{ (Quenched)} \quad K_S = \frac{[FQ]}{[F][Q]}
\]

\[
K_S = \frac{[F]_0 - [F]}{[F][Q]}
\]

\[
\frac{[F]_0}{[F]} = 1 + K_S[Q]
\]

which, regrettably, is identical to the Stern-Volmer equation for dynamic quenching.
How to distinguish dynamic and static quenching?

**Dynamic quenching** originates from collisions between the excited state and the quencher. Higher diffusion coefficients at higher temperatures cause an increase in $K_D$.

**Static quenching** requires the formation of a complex between fluorophore and quencher. Higher temperatures typically cause dissociation and thus a decrease in the observed $K_S$. 
A non-linear plot is a sign that both static and dynamic quenching occurs

\[
\frac{F_0}{F} = (1 + K_D[Q])(1 + K_S[Q]) = 1 + (K_D + K_S)[Q] + K_D K_S[Q]^2
\]
Example

Mean molecular weight (PDI)
6: 31 100 (1.6)
7: 65 400 (1.6)
8: 122 500 (1.8)

Stern-Volmer plots (F0/F=1+K[PQ^{2+}] )
Discovery of a Structural-Element Specific G-Quadruplex “Light-Up” Probe

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Table 1: Oligonucleotide sequences used in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Structural Elements</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>93del</td>
<td>GGGG TGGG AGGA GGTT</td>
<td>parallel, interlocked dimeric</td>
<td>Aptamer, HIV-1 integrase</td>
</tr>
</tbody>
</table>

A. G-tetrad structure

R = \( 1 \text{ GQR} \quad 2 \quad 3 \quad 4 \quad 5 \)

Fluorescence intensity (a.u.) vs. Wavelength (nm)

\( \Delta \lambda \) [93del]

\( (F_{\text{max}} - F_{\text{f}})/F_{\text{f}} \) vs. 93del (μM)
Example

- $d_{Au-MPC} \approx 6-8$ nm
- $d_{Au} \approx 2$ nm
- $\sim 80-100$ head groups

$[TACN \cdot Zn(II)] = 10 \mu$M

$[HEPES] = 10$ mM; pH = 7.0, T = 25 °C.

$ATP_F$

$(\lambda_{ex} = 305$ nm, $\lambda_{em} = 370$ nm)
Beyond free energy

From K to $\Delta G^\circ$ to $\Delta H^\circ$ and $\Delta S^\circ$ (Van ’t Hoff analysis)

$$\Delta G^\circ = -RT\ln(K_a)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

$$\ln(K_a) = -\Delta H^\circ/RT + \Delta S^\circ/R$$

slope = $-\Delta H^\circ/R$

intercept = $+\Delta S^\circ/R$

problem: often small temperature interval possible + inversed temperature
Isothermal calorimetry

\[ Q = V \Delta H^0_{HG}[HG] \]

or

\[ dQ = V \Delta H^0 \Delta[HG] \]

fitting provides \( K \) and \( \Delta H^\circ \)
The second major aim of a supramolecular titration experiment is the determination of the stoichiometry of the system.

Methods

(i) The method of continuous variations (Job’s method).

(ii) Consistency with the host structure and available information on the host–guest complex structure.

(iii) Specific experimental evidence such as isosbestic point(s).

(iv) Constancy of stability concentration as the concentration is varied, that is, the success of a stoichiometric model to account for the data.
The idea behind it is simple; the concentration of a $H_m G_n ([H_m G_n])$ complex is at maximum when the $[H]/[G]$ ratio is equal to $m/n$.

To do this, the mole fractions of guest ($f_G$) and host ($f_H$) are varied while keeping the total concentration of the host and guest constant ($[H]_0+[G]_0=\text{constant}$). The concentration of the host–guest complex $[H_m G_n]$ is then plotted against the mole fraction $f_G$ yielding a curve with a maxima at $f_G = n/(m + n)$, which in the case of $m = n$ (e.g., 1 : 1) appears at $f_G = 0.5$
Dependence on $K$

\[ [H]_t + [G]_t \geq \frac{1}{K_{11}} \]

- $K_{11} = 1000000 \text{ M}^{-1}$
- $[H]_t + [G]_t = 0.01 \text{ M}$
- $K_{11} = 1000 \text{ M}^{-1}$
- $K_{11} = 100 \text{ M}^{-1}$
When there is more than one complex present, the Job’s method becomes unreliable.

This includes many situations with $m/n = 1:2$ or $2:1$ as these usually include two forms of complexes (e.g., HG and HG$_2$) that have different physical properties. Hence the assumption that the physical property of interest (e.g., $\delta_{\text{obs}}$) is linearly dependent on the final product may not be valid.

For similar reasons, the Job’s method is likely to fail when either the host or guest aggregates by itself in solution.
This method is perhaps the simplest, but often the most effective of all the approaches available to determine the stoichiometry in host–guest complexes.

In modern supramolecular chemistry it is now rare not to have detailed information through X-ray crystallography, 2D-NMR and Molecular Modelling about the structure of the host and guest and, in some cases, even the host–guest complex itself. This structural information can make the prediction of stoichiometry quite straightforward and accurate.
In spectroscopy, an **isosbestic point** is a specific wavelength, wavenumber or frequency at which the total absorbance of a sample does not change during a chemical reaction or a physical change of the sample.

\[
A_{tot} = \varepsilon_H [H]l + \varepsilon_{HG} [HG]l
\]

for \( \varepsilon_H = \varepsilon_{HG} \) this becomes

\[
A_{tot} = \varepsilon l ([H] + [HG])
\]

considered that \([H] = [H]_0 - [HG]\), this leads to

\[
A_{tot} = \varepsilon l [H]_0 = \text{constant}
\]
However...

The absence of an isosbestic point or the presence of more than one isosbestic point confirms that simple 1 : 1 complexation is not appropriate to describe the system if more than one isosbestic point is observed.

The converse is not necessarily true. That is, the presence of an isosbestic point or the absence of more than one isosbestic point do not per se rule out more complex stoichiometry such as 1 : 2 complex formation, especially in cases where cooperative (positive or negative) processes play a significant role.

Example

\[
\begin{align*}
H + G & \quad \xrightleftharpoons[K_{HG}]{K_{HG2}} HG + G & \quad \xrightleftharpoons[K_{HG}]{K_{HG2}} HG_2
\end{align*}
\]

and the absorbance is given by

\[
A_{tot} = \varepsilon_H[H]l + \varepsilon_{HG}[HG]l + \varepsilon_{HG2}[HG_2]l
\]

in case \(K_{HG2} \gg K_{HG}\) (positive cooperativity) then \([HG]\) will be negligible

\[
A_{tot} = \varepsilon_H[H]l + \varepsilon_{HG2}[HG_2]l
\]

and with \(\varepsilon_H = \varepsilon_{HG2}\) and \([H] = [H_0] - [HG_2]\) this gives

\[
A_{tot} = \varepsilon_H[H_0]l = \text{constant, but no 1:1 complex}
\]
This method is probably the most generally applicable method for determining stoichiometry.

Firstly, if anything other than 1 : 1 stoichiometry is suspected, the data should be fitted to other plausible models (e.g., 1 : 2) and the quality of fit of the different models compared in details, taking into account factors such as the increase in parameters in the fitting process and the standard deviations.

Secondly, and more importantly, it is strongly advisable to carry out the titration at different concentrations and even with different techniques (e.g., NMR and UV-Vis). If a particular model is successful at explaining the data at different concentrations then it can be taken as very strong evidence for that model.
### 1:1 model

<table>
<thead>
<tr>
<th>Substance</th>
<th>$K$ (M$^{-1}$)</th>
<th>$DH$ (ppm)</th>
<th>$DHG$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG</td>
<td>1938.702</td>
<td>7.00624</td>
<td>8.000867</td>
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</tbody>
</table>

### 2:1 model

<table>
<thead>
<tr>
<th>Substance</th>
<th>$K$ (M$^{-1}$)</th>
<th>$DH$ (ppm)</th>
<th>$DHG$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHG</td>
<td>2615630</td>
<td>6.98578</td>
<td>8.169739</td>
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</table>

### 1:2 model

<table>
<thead>
<tr>
<th>Substance</th>
<th>$K$ (M$^{-1}$)</th>
<th>$DH$ (ppm)</th>
<th>$DHG$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGG</td>
<td>3566244</td>
<td>7.05578</td>
<td>7.92317</td>
</tr>
</tbody>
</table>
Example: effect of dilution

1:1 model
HG

at lower concentrations the difference between the binary and ternary complex become more evident.

1:2 model
HGG
Finally – a practical consideration

1) measure initial spectrum
2) add aliquots of G
3) measure after each addition

weigh H
stocksolution H
use to fill

weigh G
(stocksolution G
(contains H at the same concentration !)
(concentrated solution)