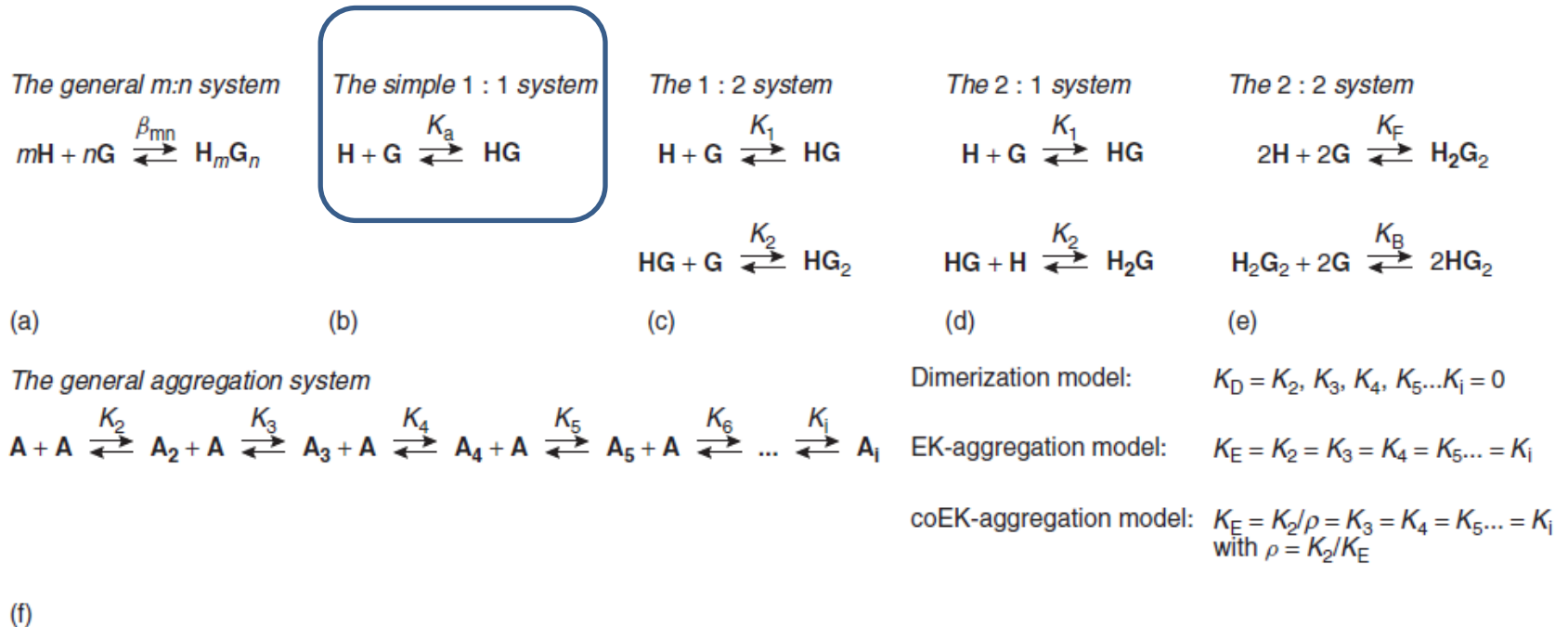


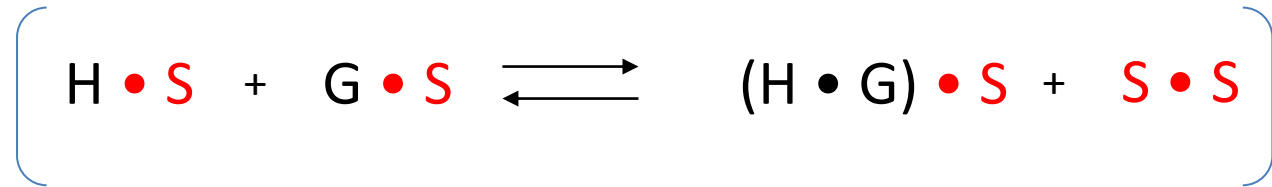
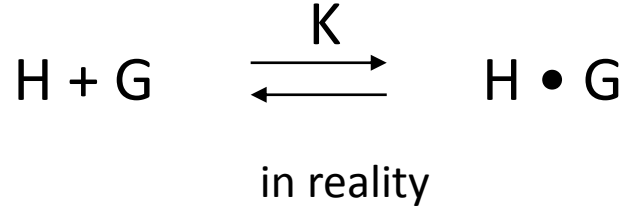
Binding Constants and Their Measurement

In general, the key step in determining binding constants involves defining some sort of a model that relates to the underlying equilibria. The model(s) is then compared to the data obtained. If the data and model are in reasonably good agreement, data analysis (fitting) can be used to extract valuable information, including the association constant (K_a).



The bimolecular binding event (1:1)

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



explicit solvent is not used because ΔG° for the association constant reflects the stability of solvated H and G relative to solvated H•G and released solvent.

Binding constants should be always tabulated reporting the solvent and temperature.

$$K_a = \frac{[\text{H} \bullet \text{G}]}{[\text{H}][\text{G}]} \quad (M^{-1}) \qquad K_d = \frac{[\text{H}][\text{G}]}{[\text{H} \bullet \text{G}]} \quad (M)$$

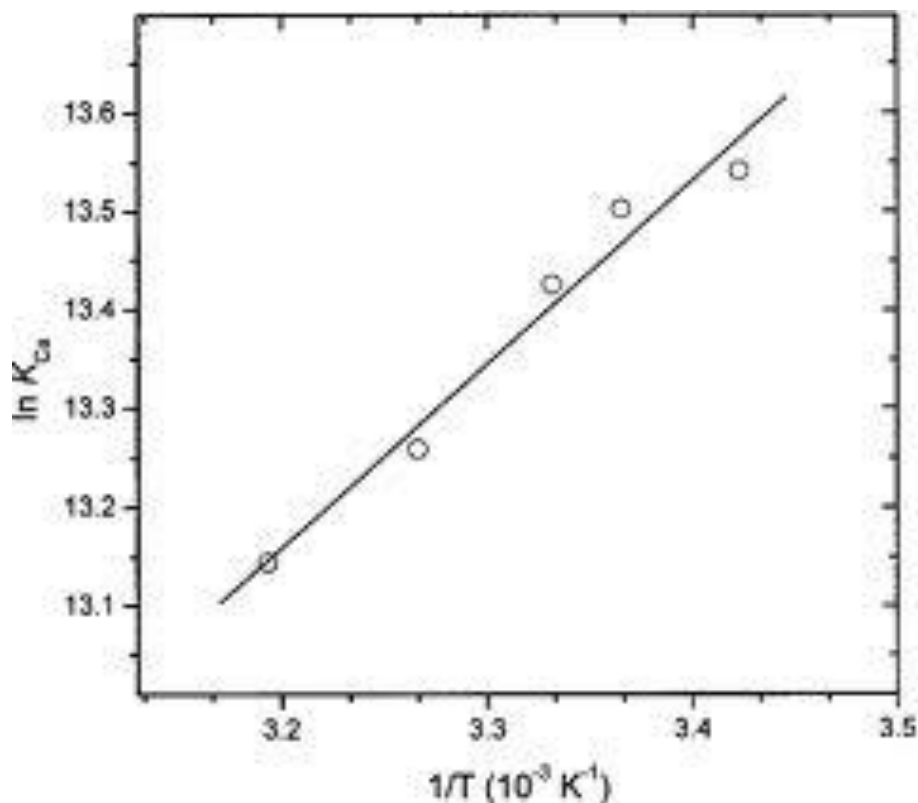
In reality, equilibrium constants are defined as ratios of activities, which are dimensionless values. Consequently, also equilibrium constants are dimensionless. Assumption that concentrations are close to activities leads to the use of units.

From K to ΔG° and ΔH° and ΔS° (Van 't Hoff analysis)

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

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

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



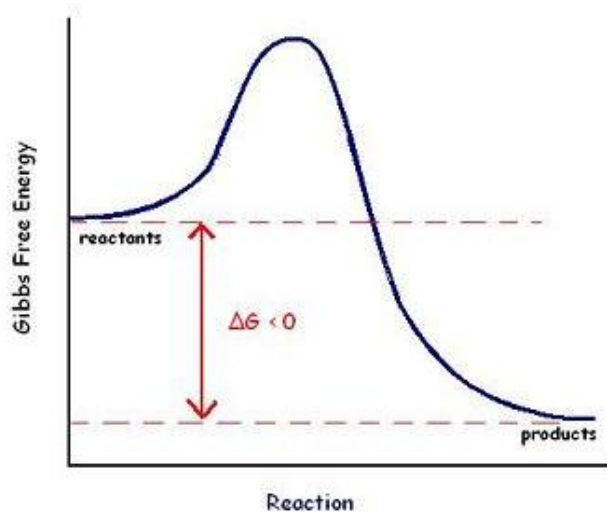
slope = $-\Delta H^\circ/R$

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

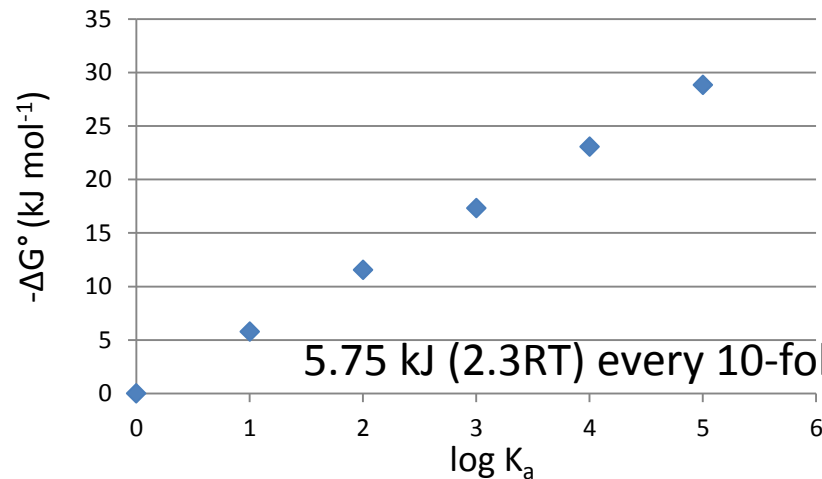
problem: often small temperature interval possible

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

K_a (M^{-1})	ΔG° ($kJ.mol^{-1}$)
1	0
10	-5.8
100	-11.5
1000	-17.3
10000	-23.0
100000	-28.8



at $T = 301$ K ($R = 8.314$ $J.mol^{-1}.K^{-1}$)

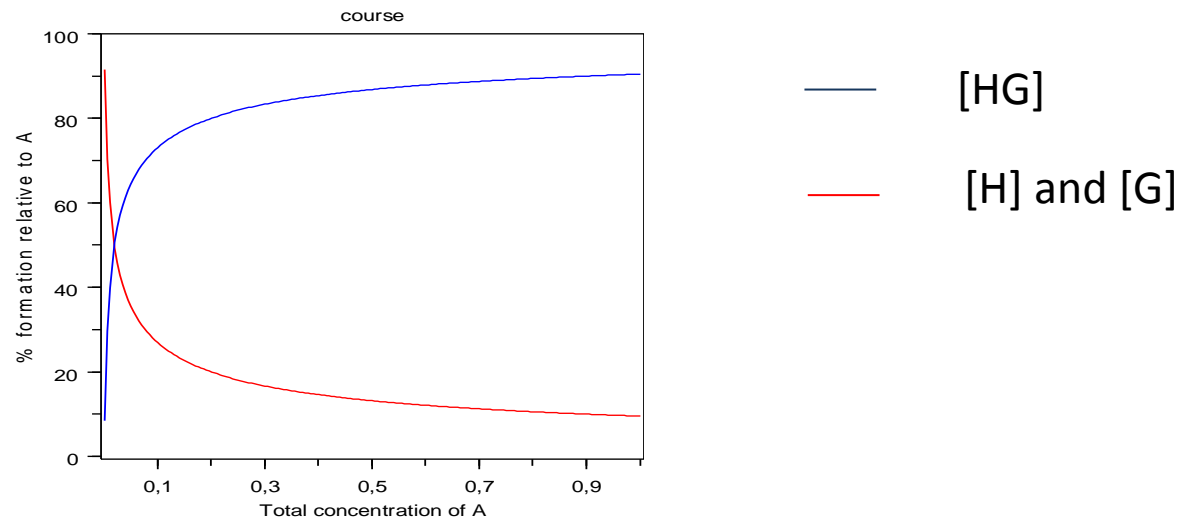


The value of K determines the position of the equilibrium

but the ratios of H, G, and HG depend also on the initial concentrations of H_0 and G_0

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

H_0 (M)	G_0 (M)	H (M)	G (M)	HG (M) /%
1×10^{-3}	1×10^{-3}	0.9×10^{-3}	0.9×10^{-3}	$0.1 \times 10^{-3} / 10$
1×10^{-2}	1×10^{-2}	6.6×10^{-3}	6.6×10^{-3}	$4.4 \times 10^{-3} / 44$
1×10^{-1}	1×10^{-1}	27×10^{-3}	27×10^{-3}	$73 \times 10^{-3} / 73$
1	1	95×10^{-3}	95×10^{-3}	$905 \times 10^{-3} / 91$

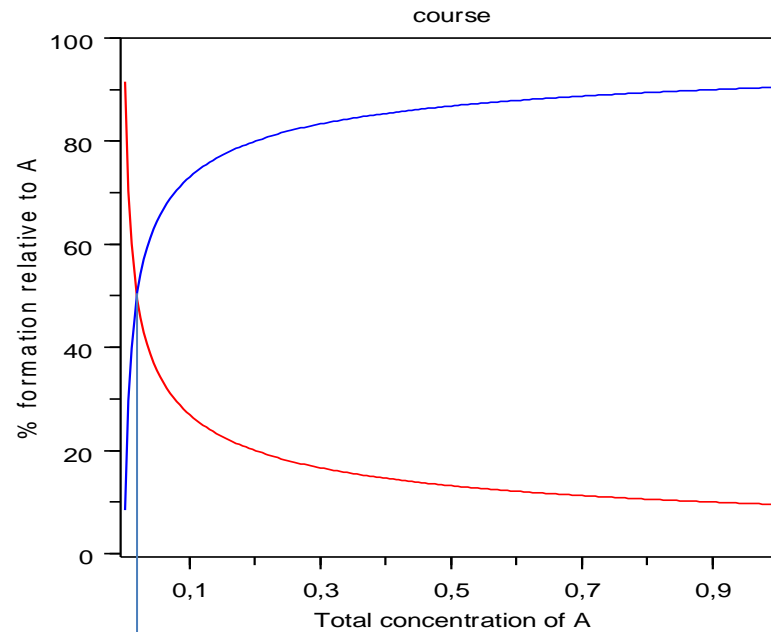


$K_d (1/K_a)$ is a convenient reference point for estimating the amount of complex

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



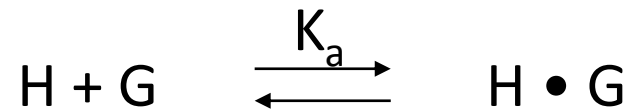
$$K_d = 1 \times 10^{-2} \text{ M} = 10 \text{ mM}$$



$$[H]_0 = [G]_0 = 20 \text{ mM} \quad \Rightarrow \quad [H] = [G] = 10 \text{ mM} \text{ and } [HG] = 10 \text{ mM}$$

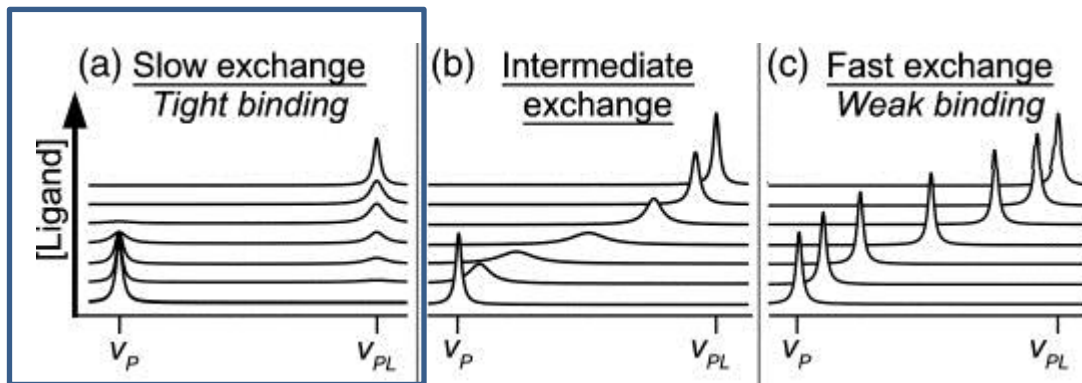
At K_d , $[H] = [G] = [HG] = K_d$ (!! only in case $[H]_0 = [G]_0$!!)

how to determine the concentration of each species ?



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

$$K_a = \frac{[H \cdot G]}{[H][G]}$$



With slow equilibria and high association constants centrifugation or ultrafiltration may allow to isolate the complex (ELISA)

regrettable this is hardly ever the case

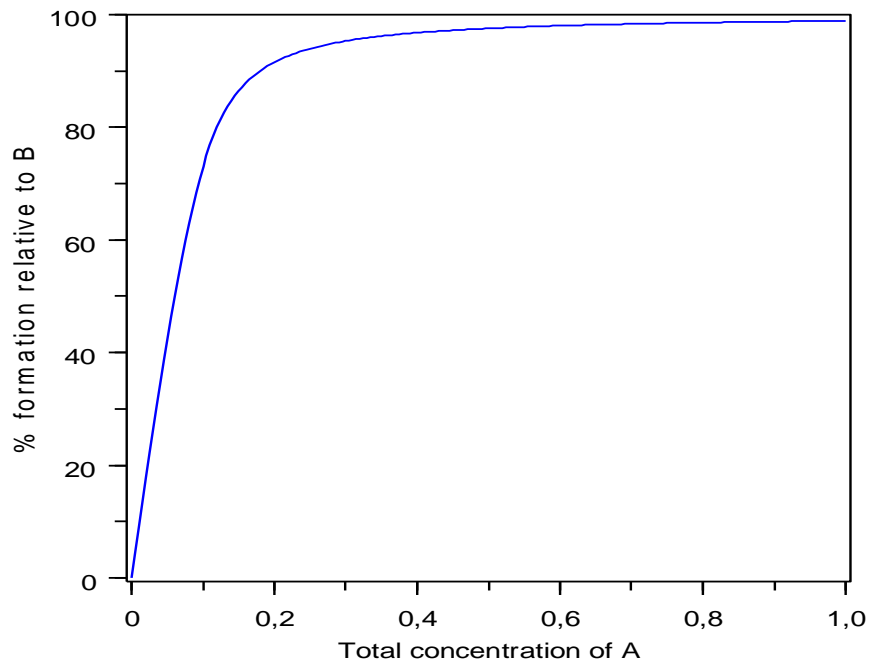
determination of the binding constant K_a

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 as a function of the concentration of another component at constant temperature.

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

example



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

$$[H]_0 = 0.01 \text{ M}$$

Titration experiments

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 guest added to host (equivalent guests) to give the binding isotherm.

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

$$\Delta Y = f[\text{HG}]$$

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

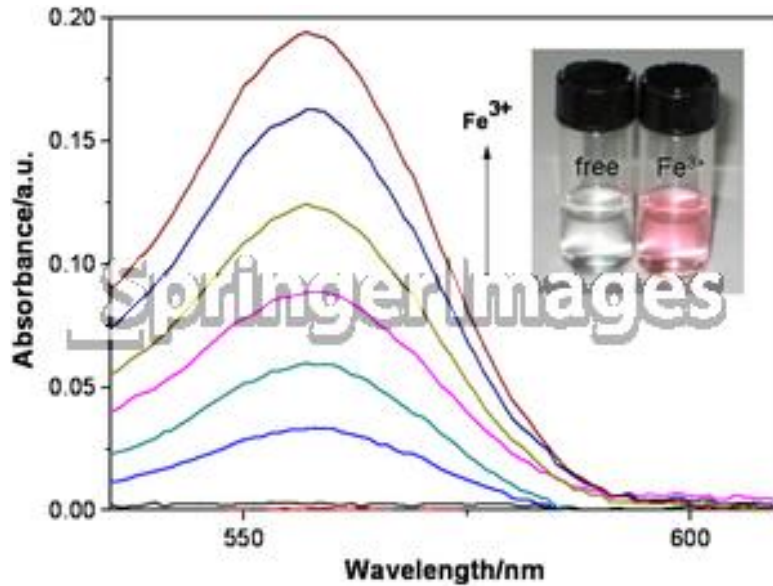
The physical change (Y) being monitored can usually be described as the aggregate of the individual components according to eqn (1) as a function of concentration (e.g., for UV-Vis spectroscopy) or eqn (2) as a function of mole fractions f_x (f_x defined as: $f_x = [X]/[X]_0$) in the special case of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

$$(1) Y = Y_H[\text{H}] + Y_G[\text{G}] + Y_{\text{HG}}[\text{HG}]$$

$$(2) Y = Y_H f_H + Y_G f_G + Y_{\text{HG}} f_{\text{HG}}$$

Example

only HG has a particular absorption band in the UV/vis spectrum.



$$\text{Thus, } A = \epsilon_{\text{HG}}[\text{HG}]$$

$$K_a = \frac{[\text{H}\cdot\text{G}]}{[\text{H}][\text{G}]}$$

$$[\text{G}] = [\text{G}]_0 - [\text{HG}]$$

$$[\text{H}] = [\text{H}]_0 - [\text{HG}]$$

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

Example

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

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

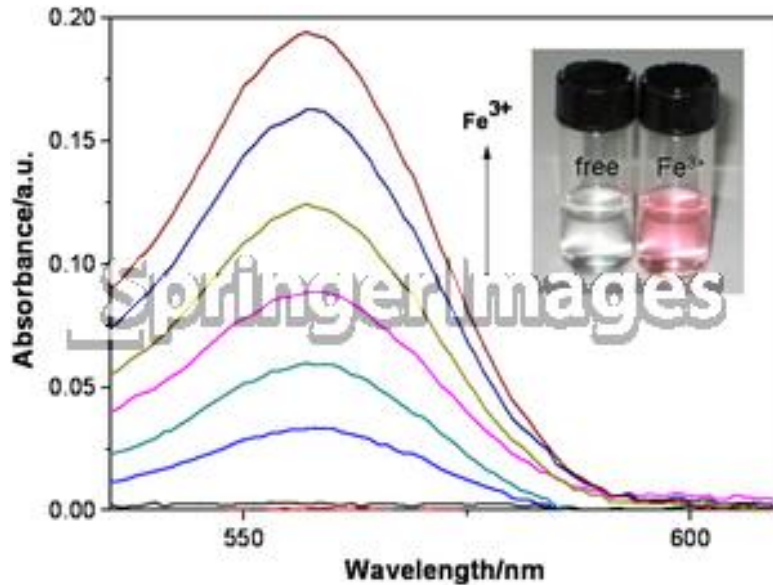
$$K_a ([\text{H}]_0[\text{G}]_0 - [\text{HG}][\text{G}]_0 - [\text{HG}][\text{H}]_0 + [\text{HG}]^2) = [\text{HG}]$$

$$[\text{HG}]^2 - ([\text{G}]_0 + [\text{H}]_0 + 1/K_a)[\text{HG}] + [\text{H}]_0[\text{G}]_0 = 0$$

$$[\text{HG}] = \frac{1}{2} \left\{ \left([\text{G}]_0 + [\text{H}]_0 + \frac{1}{K_a} \right) - \sqrt{\left([\text{G}]_0 + [\text{H}]_0 + \frac{1}{K_a} \right)^2 - 4[\text{H}]_0[\text{G}]_0} \right\}$$

this expresses [HG] as a function of K_a , which is the only unknown !!

Example



Since, $A = \epsilon_{HG}[HG]$

and

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

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

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 (ΔY) during the course of the titration.

In general for the NMR

$$Y = Y_H f_H + Y_G f_G + Y_{HG} f_{HG}$$

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 $\Delta Y = Y - Y_H$ – *this is the experimental data point*

and $Y_{\Delta HG} = Y_{HG} - Y_H$ – *this is the maximum difference of the physical parameter between HG and H*

for example: in case $[G]_0$ is titrated to $[H]_0$

then for $[G]_0 = 0$, $[HG] = 0$ and $\Delta Y = 0$; whereas for $[G]_0 \gg [H]_0$, $[HG] \gg [H]_0$ and $\Delta Y = Y_{\Delta HG}$

The binding isotherm

$$Y = Y_H f_H + Y_G f_G + Y_{HG} f_{HG}$$

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

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

$$f_{HG} = \frac{K_a [G]}{1 + K_a [G]}$$

General binding isotherm that shows a hyperbolic dependence of the mole fraction of the complex from the concentration of free G

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

Starting from this equation the same quadratic expression of [HG] seen before can be derived

In general for the UV

$$A = \varepsilon_H[H] + \varepsilon_G[G] + \varepsilon_{HG}[HG]$$

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

$$A = \varepsilon_H[H] + \varepsilon_{HG}[HG]$$

which, since $A_0 = \varepsilon_H[H]_0$ and $[H] = [H]_0 - [HG]$, can be further simplified to

$$A = \varepsilon_H([H]_0 - [HG]) + \varepsilon_{HG}[HG]$$

$$A = A_0 + (\varepsilon_{HG} - \varepsilon_H)[HG]$$

and, finally

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

in which $\Delta A_{obs} = A - A_0$ – *this is the experimental data point*

and $\varepsilon_{\Delta HG} = \varepsilon_{HG} - \varepsilon_H$ – *this is the maximum difference of the physical parameter between HG and H*

Which is the difference between the molar absorptivity between HG and H

NMR: Changes up on titration – eqn (30):

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

UV-Vis: Changes up on titration – eqn (34):

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

Fluorescence: Changes in fluorescence of host – complex up on titration

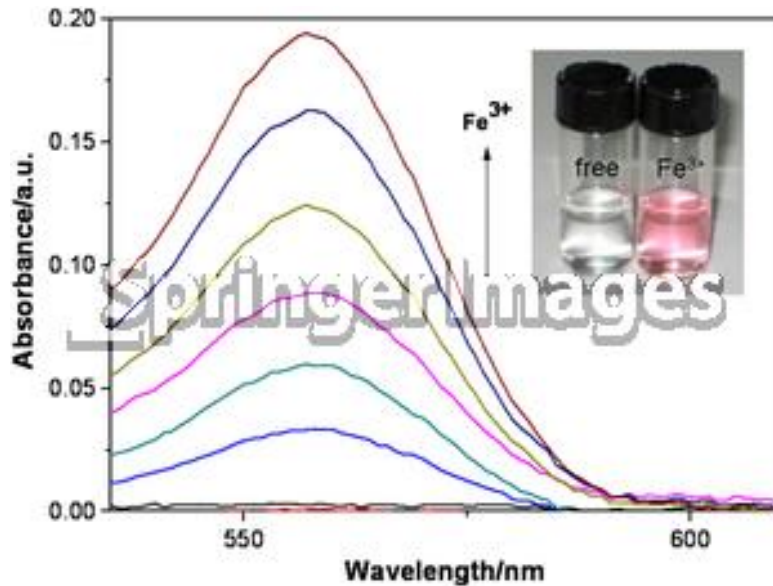
(no dynamic quenching) – eqn (42):

$$\Delta F_{\text{obs}} = k_{\Delta\text{HG}} ([\text{HG}])$$

Calorimetry: Heat of formation upon on titration – eqn (46):

$$Q = \Delta H_{\text{HG}} V ([\text{HG}])$$

One problem remains though....



Since, $A = \epsilon_{\text{HG}}[\text{HG}]$

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 $A = f(\epsilon_{\text{HG}}, K_a)$

what is ϵ_{HG} ??

or, in general, for

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

what is $Y_{\Delta \text{HG}}$??

Old-fashioned Shortcuts

(Old-fashioned) Shortcuts to the binding constant

Older references and 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 $[G]_0 \gg [H]_0$ (and thus $A^H \ll A^G$)

$$A = A^{HG} + A^G$$

with $\Delta A = A - A_0$ this gives (Lambert-Beer)

$$\Delta A = \epsilon^{HG}[HG]b + \epsilon^G[G]b - \epsilon^G[G]_0b \quad b = \text{optical path}$$

considering that $[G]_0 \gg [H]_0$ one can assume that $[G]=[G]_0$ and this

$$\Delta A = \epsilon^{HG}[HG]b$$

Old-fashioned Shortcuts

The binding isotherm can be rewritten as

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

$$[H] = [H]_0 - [HG]$$



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

and

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

which is

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

or

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

Together this gives

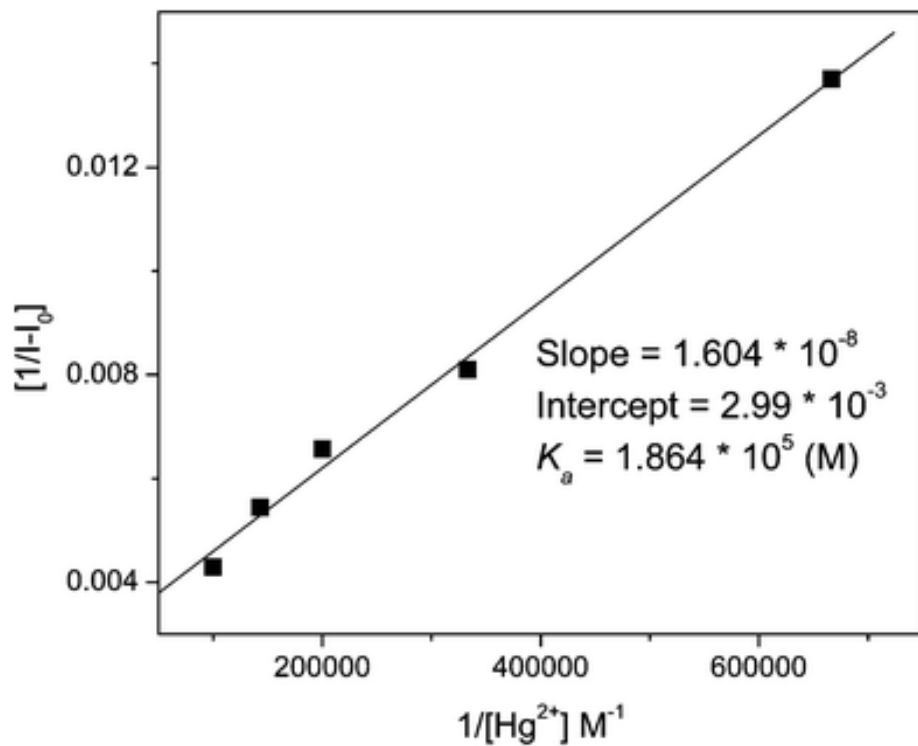
$$\Delta A = b \epsilon^{HG} \frac{[H]_0 K_a [G]_0}{1 + K_a [G]_0} \quad (\text{assuming that } [G] = [G]_0)$$

and finally

$$\frac{1}{\Delta A} = \frac{1}{b \epsilon^{HG} [G]_0 [H]_0 K_a} + \frac{1}{b \epsilon^{HG} [H]_0}$$

Old-fashioned Shortcuts

$$\frac{1}{\Delta A} = \frac{1}{b\epsilon^{HG}[G]_0[H]_0K_a} + \frac{1}{b\epsilon^{HG}[H]_0}$$



intercept $+$ $\frac{1}{b\epsilon^{HG}[H]_0}$

slope $\frac{1}{b\epsilon^{HG}[H]_0K_a}$

Other examples include Lineweaver-Burke plots, Scatchard plots, etc.

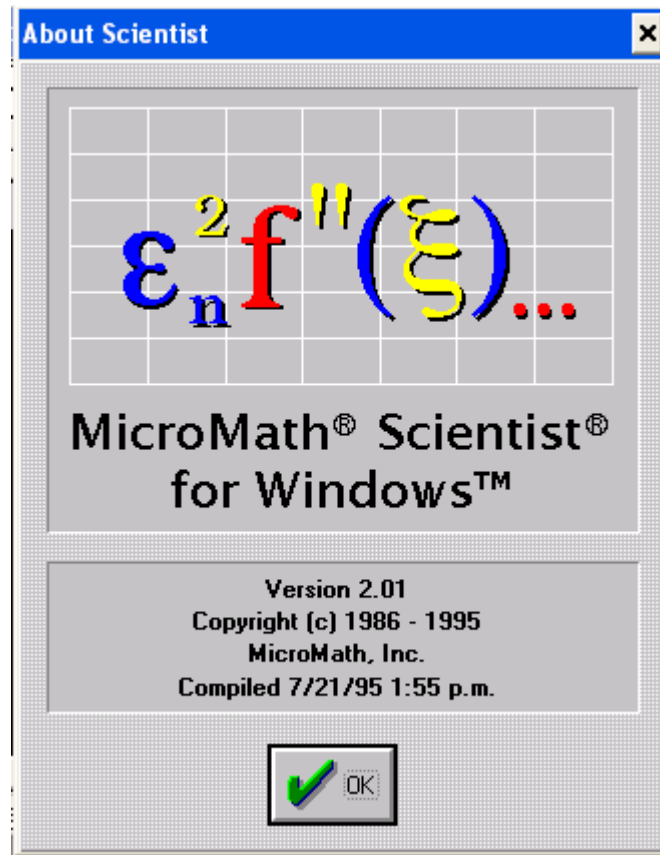
Limitations

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 $[G]_0 \gg [H]_0$ 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 and distort the results.

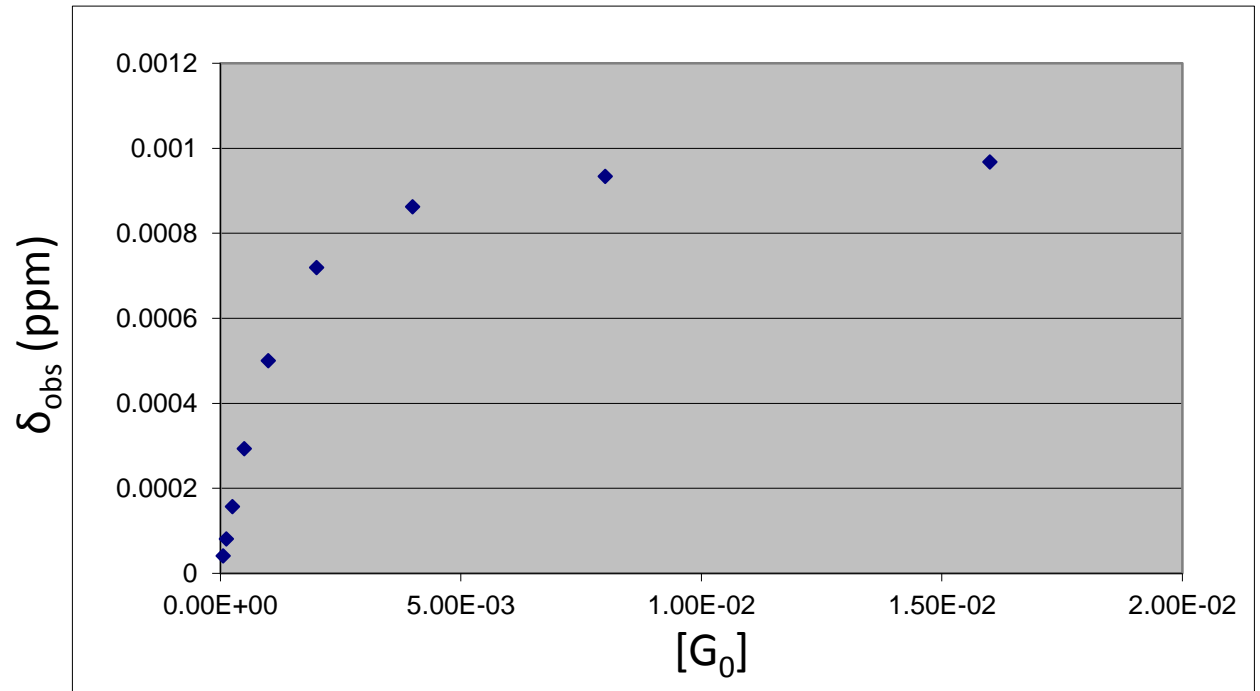
The non-linear regression approach with exact solutions of the quadratic equation (see before) produces the most accurate results. This approach is not difficult with modern computer technology and *there is no real excuse for using old-fashion linear transformations anymore!*

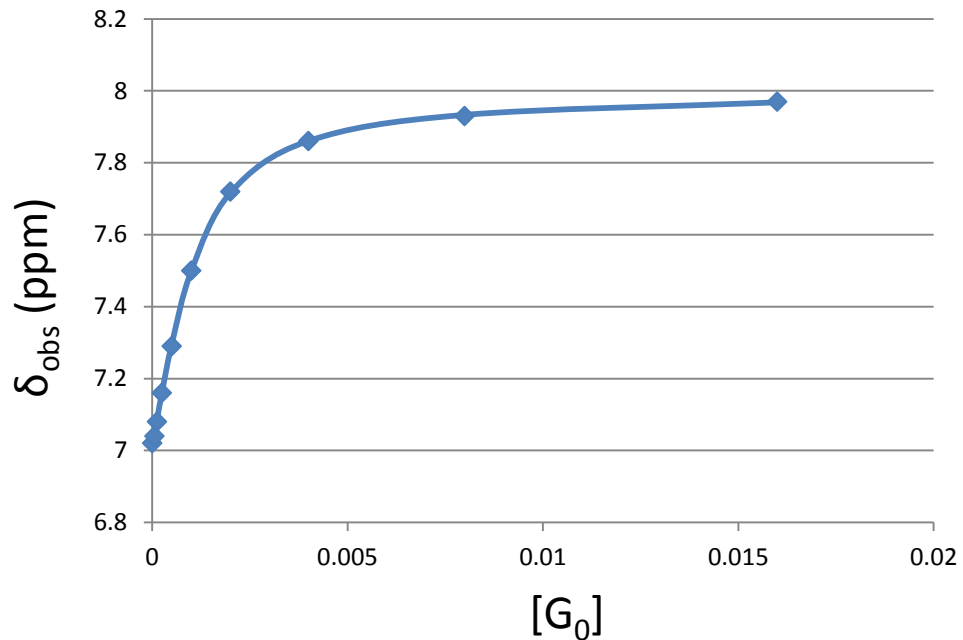
Scientist



Example: NMR titration

G_0	Dobs (ppm)
6.25E-05	7.04E+00
1.25E-04	7.08E+00
2.50E-04	7.16E+00
5.00E-04	7.29E+00
1.00E-03	7.50E+00
2.00E-03	7.72E+00
4.00E-03	7.86E+00
8.00E-03	7.93E+00
1.60E-02	7.97E+00





K		1938.702	78	M ⁻¹
DH		7.00624	0.004	ppm
DHG		8.000867	6.00E-03	ppm

// Titolazione spettrometrica 1:1

IndVars: G0

DepVars: D

Params: K, H0, DMAX

H0=H*(1+K*G)

G0=G*(1+K*H)

HG=K*G*H

D=(HG/H0)*DMAX

0<G<G0

0<H<H0

How to chose the experimental conditions ?

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 (ΔY) in the system during the course of experiment. The two key concerns here should be:

- i. The expected association constant(s).
- ii. The expected physical changes (ΔY) 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

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

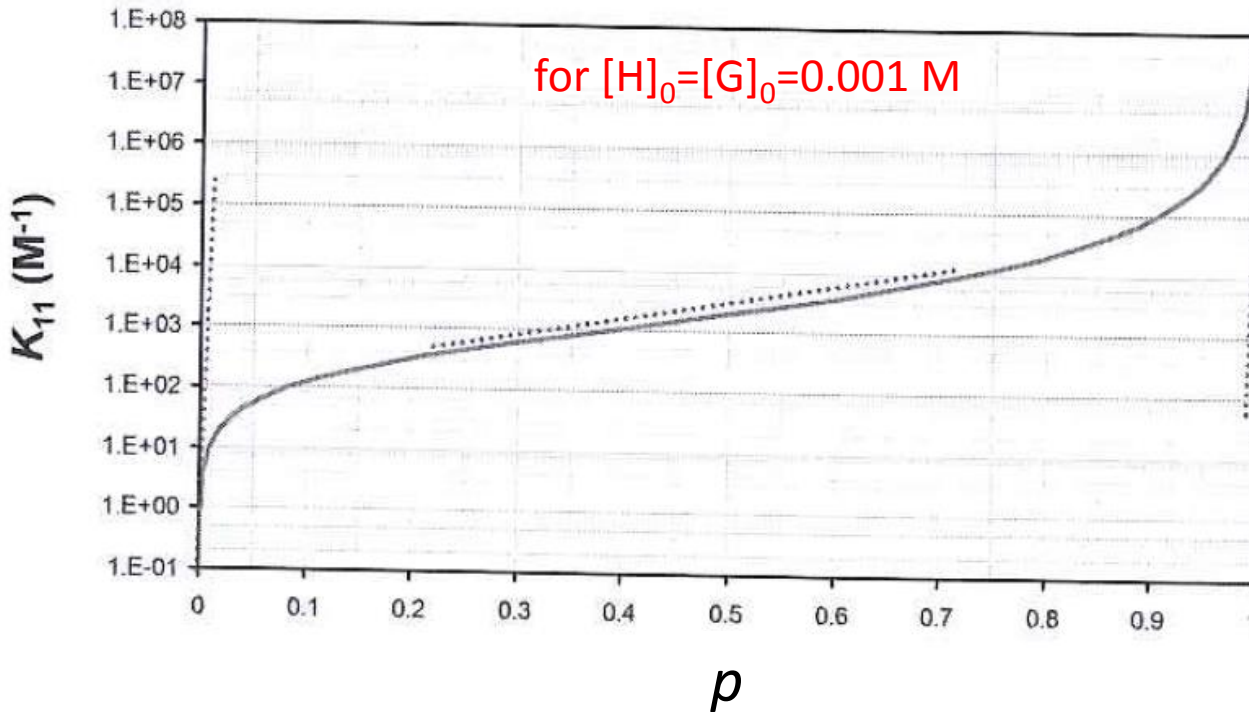
$$p = \frac{[HG]}{[H]_0} \text{ when } [H]_0 < [G]_0$$

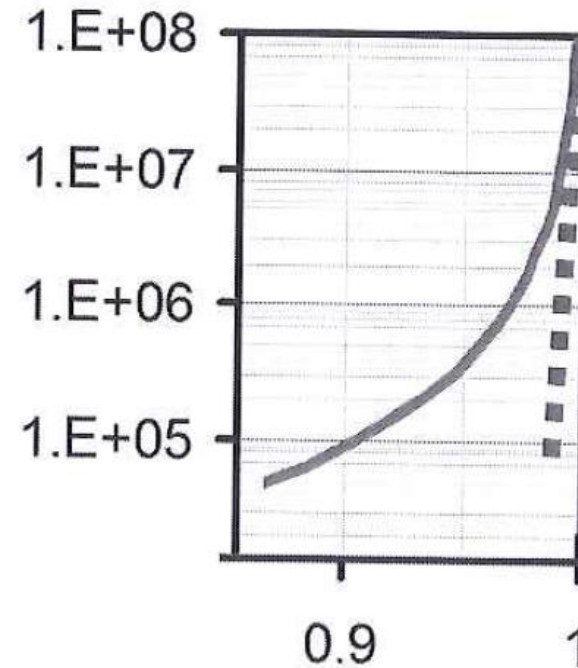
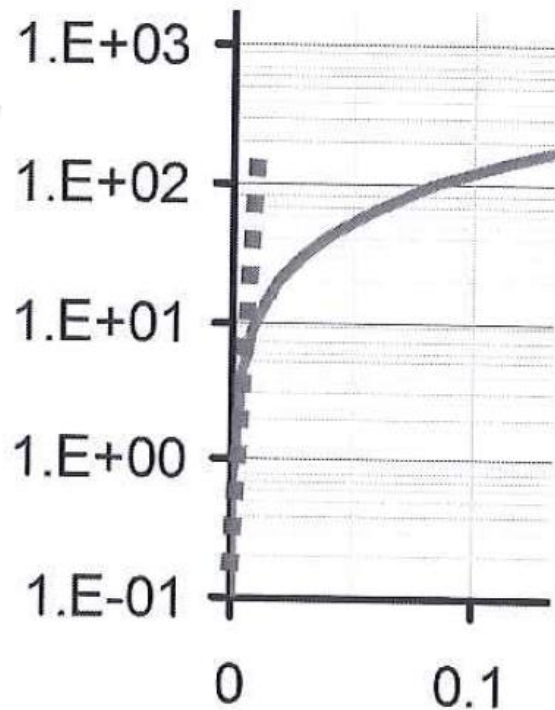
$$K_a = \frac{[\text{HG}]}{([\text{H}]_0 - [\text{HG}])([\text{G}]_0 - [\text{HG}])}$$

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

gives

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



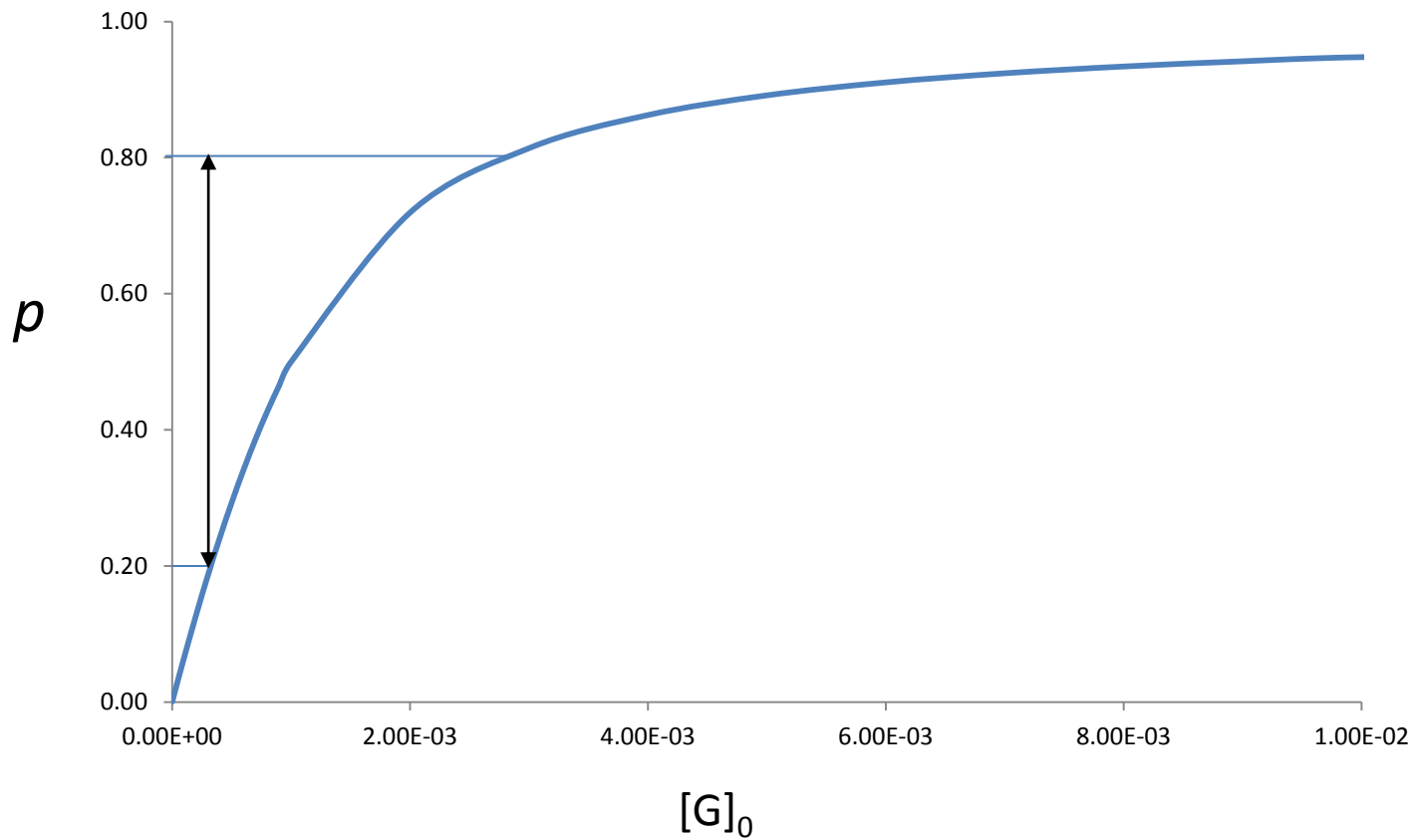


near $p = 0$ and $p = 1$, small errors in p (experimental error in determination of the concentrations !!) gives large variations in K_a .

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

EXAMPLE

for $[H]_0 = 0.001 \text{ M}$
 $K_a = 2000 \text{ M}^{-1}$

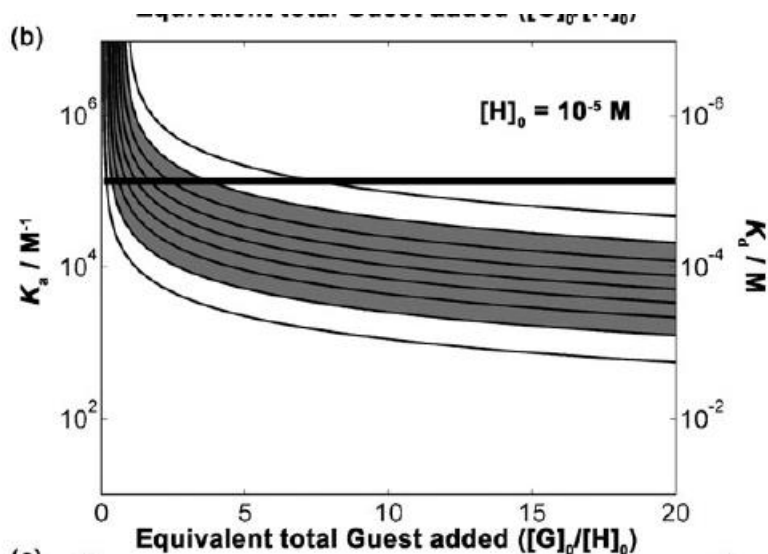


Using

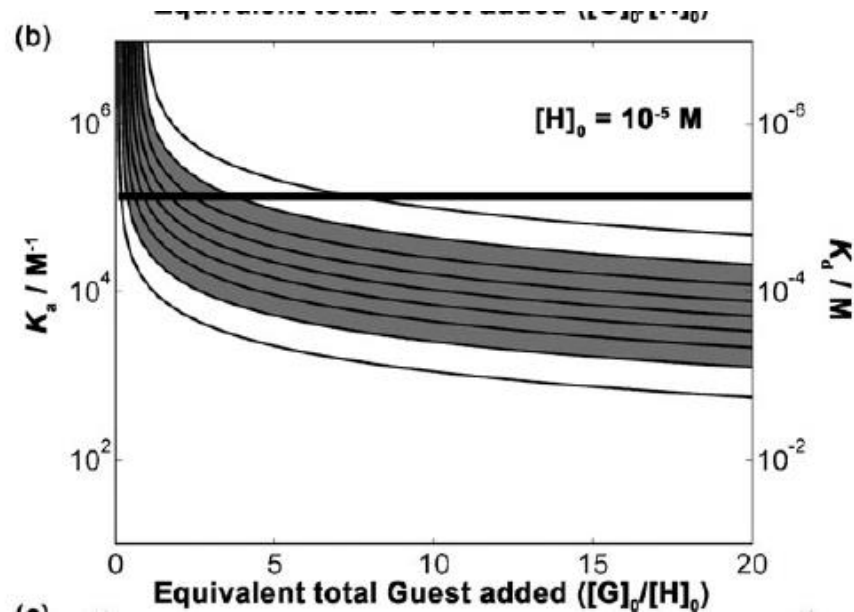
$$[HG] = \frac{1}{2} \left(G_0 + H_0 + \frac{1}{K_a} \right) - \sqrt{\left(G_0 + H_0 + \frac{1}{K_a} \right)^2 + 4[H_0][G_0]}$$

it is possible to calculate p for a range of $[H]_0$, $[G]_0$ and K_a values.

When the results are plotted for a fixed $[H]_0$ concentration (here 10^{-5} M) as a function of K_a and $[G]_0/[H]_0$ (equivalents of guest added) typically employed in UV-Vis spectroscopy studies a revealing pattern appears with the shaded areas indicating p in the range of 0.2–0.8 (note that this applies only to 1 : 1 binding systems).



The thick horizontal line indicates where $[H]_0 = K_d = 1/K_a$. The contour lines are 0.1 units of p apart with the lowest one shown at $p = 0.1$ and with p in the range of 0.2–0.8 shaded grey. See text for details.

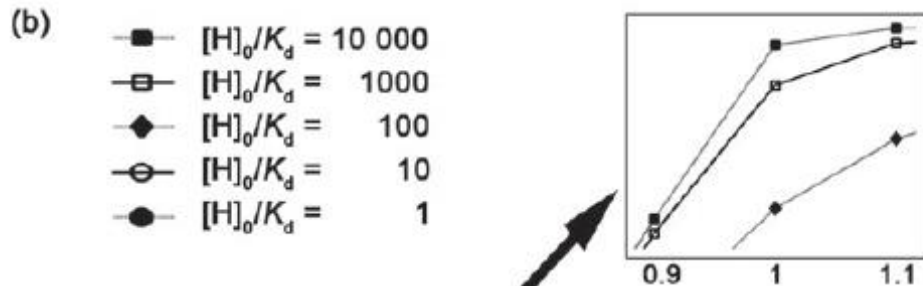


if $K_d > [H]_0$ (hence K_a fairly low) then a relatively large excess of $[G]_0$ is required to obtain good p -values. In this situation it would be advisable to collect several data points in the range of 1–50 equivalents of G added.

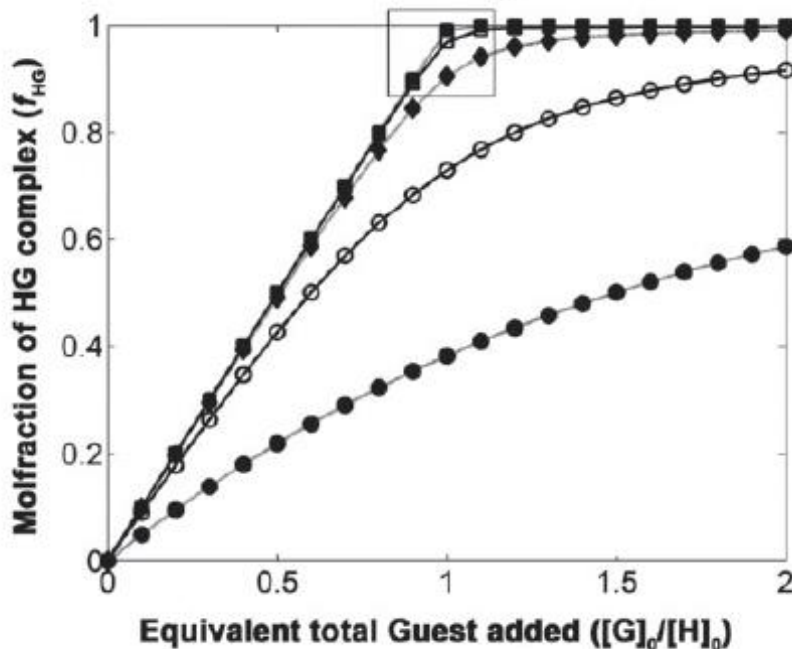
If $K_d < [H]_0$ (hence K_a fairly high) the only data points with good p -values are within the range of $[G]_0 \leq [H]_0$. In other words, it is essential to obtain as many points as possible between 0–1 equivalents of G added.

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 $p = 0.38$.

The fourth scenario to consider is when $K_d \ll [H]_0$, *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

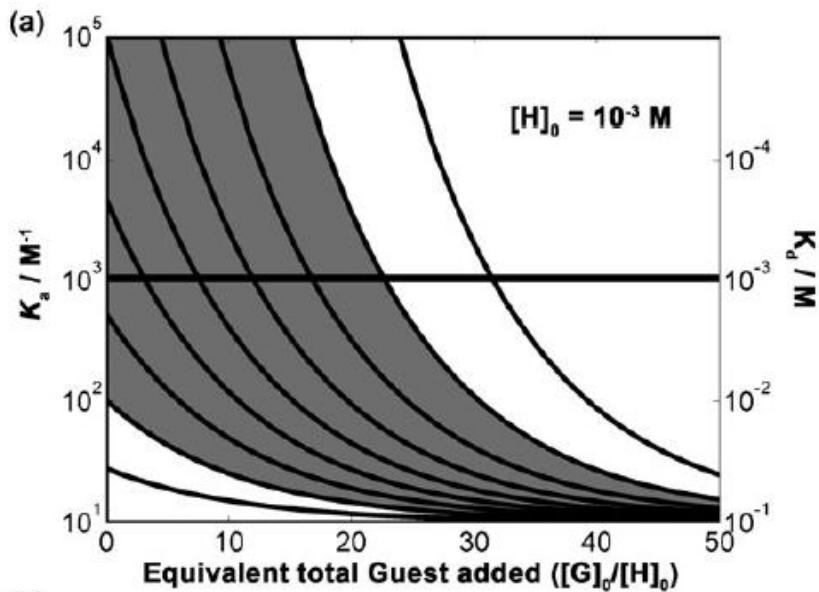


Binding isotherms for different $[H]_0/K_d$ ratio's from 1–10 000. The inset shows the region around 0.9–1.1 equivalents added for $[H]_0/K_d = 1000$ –10 000 only.

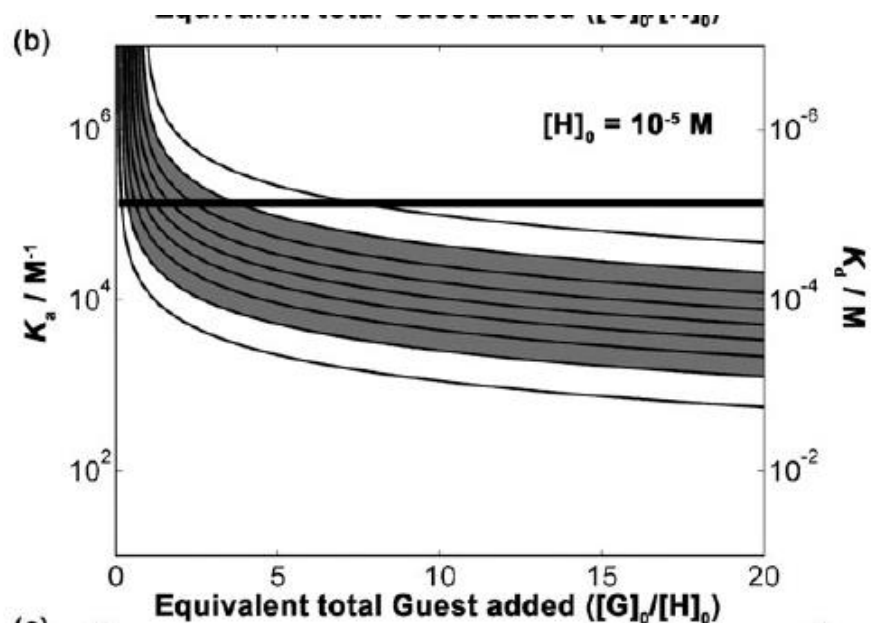


Here it becomes clear that once $[H]_0/K_d > 100$, the nonlinear portion of the resulting binding isotherms is restricted to a small region around 1 equivalent of guest added. With $[H]_0/K_d > 1000$, it is clear that there is very little “information” content in the isotherms.

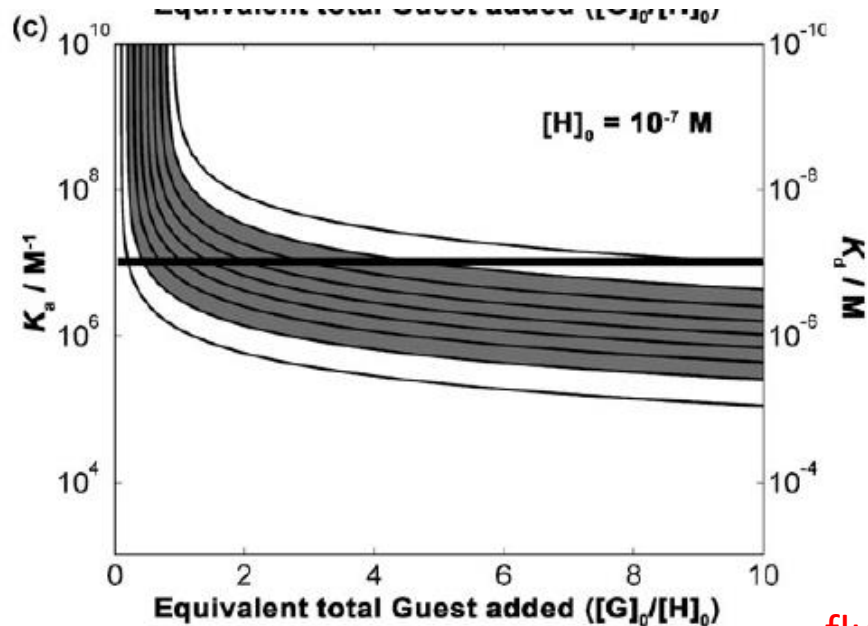
When binding occurs under saturation conditions, this implies that the experimental conditions are NOT adequate for determination of K .



NMR concentrations



UV-Vis concentrations



fluorescence concentrations

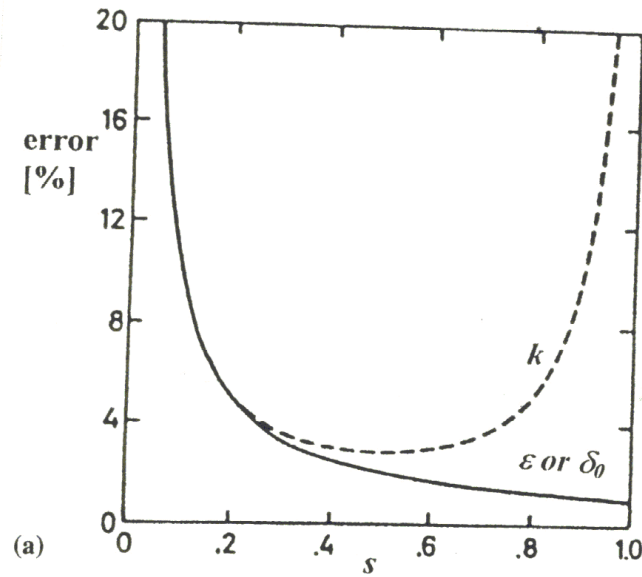
The suitable analytical technique for determination of K_a depends on its value.

$$\text{limit: } [H]_0/K_d < 100$$

$$\text{and thus } K_{d,\text{lim}} > [H]_{0,\text{lim}}/100$$

The lower limit is dictated by the solubility of H and G and by their spectroscopic properties

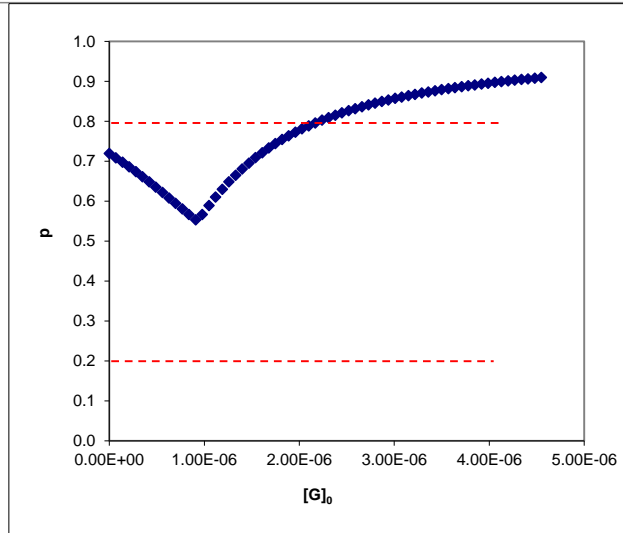
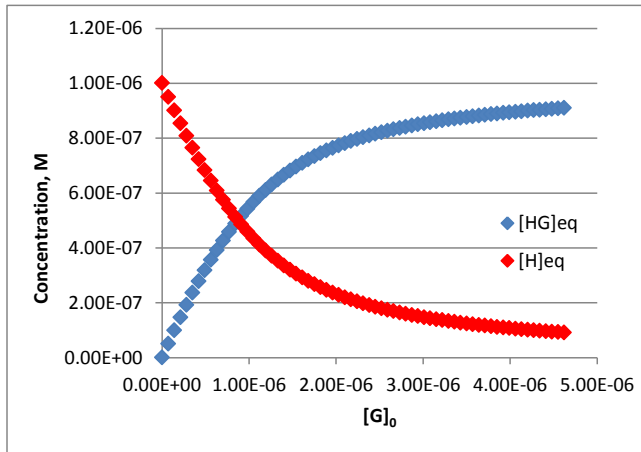
The error in the parameter determination is minimum for K at low saturation and for ε (or δ) at high saturation.



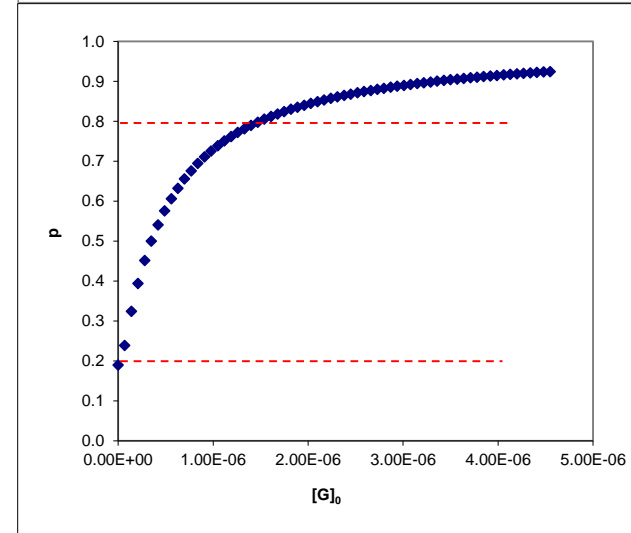
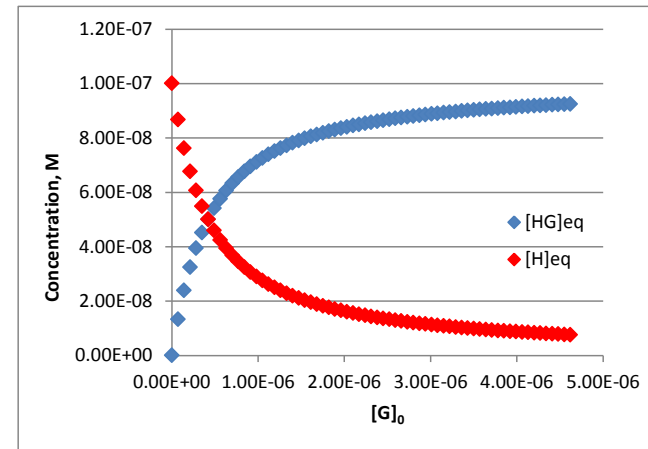
Percentage relative errors
($100\Delta k/k$ and $100\Delta\varepsilon/\varepsilon$) as a
function of the saturation
fraction

When $[H]_0 \approx [G]_0$ p has to be calculated on the component in defect.

$K = 2.7 \cdot 10^{-6} \text{ M}^{-1}$, $[H]_0 = 1.0 \cdot 10^{-6} \text{ M}$



$K = 2.7 \cdot 10^{-6} \text{ M}^{-1}$, $[H]_0 = 1.0 \cdot 10^{-7} \text{ M}$



analytical techniques: scope and limitations

NMR spectroscopy

The most informative technique in most situations is ^1H 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 **how** the host and guest(s) are interacting and the stoichiometry of interaction. 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 (δ) 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

$$\delta_{\text{obs}} = \chi_{\text{H}}\delta_{\text{H}} + \chi_{\text{HG}}\delta_{\text{HG}} \quad \text{since} \quad X_{\text{H}\cdot\text{G}} = \frac{K_{\text{a}}[\text{G}]}{1 + K_{\text{a}}[\text{G}]}$$

$$\text{since } \chi_{\text{H}} = 1 - \chi_{\text{HG}}$$

$$\delta_{\text{obs}} - \delta_{\text{H}} = \chi_{\text{HG}}(\delta_{\text{HG}} - \delta_{\text{H}}) \quad \text{this gives} \quad \Delta\delta = \frac{\Delta\delta_{\text{tot}} K_{\text{a}}[\text{G}]}{1 + K_{\text{a}}[\text{G}]} \quad \text{and then}$$

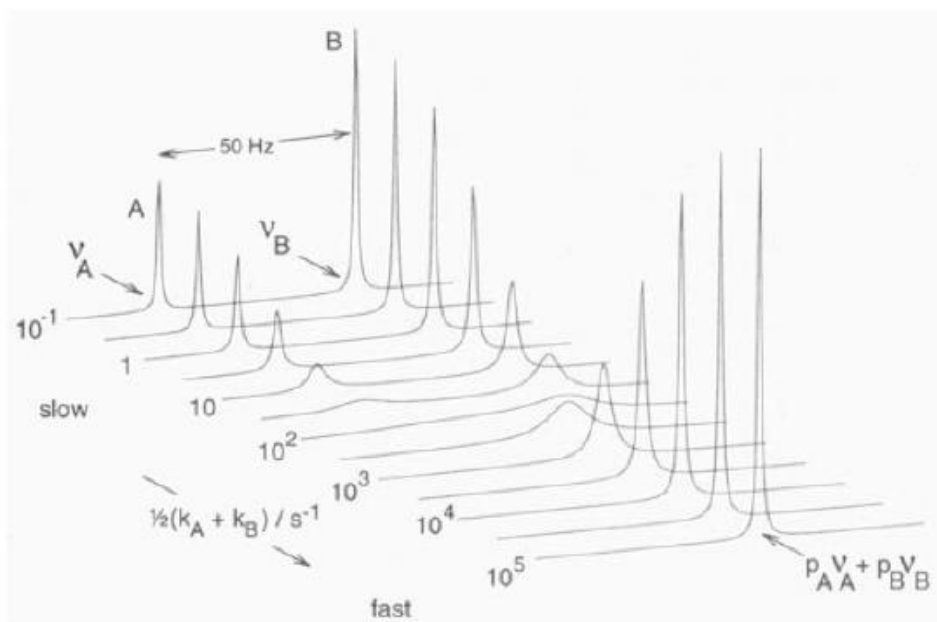
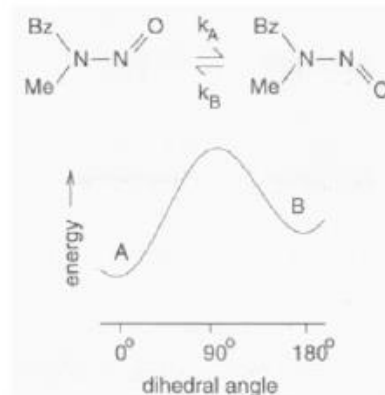
$$\Delta\delta = \frac{\delta_{\Delta\text{HG}}}{[\text{H}]_0} \left(\frac{1}{2} \left\{ \left([\text{G}]_0 + [\text{H}]_0 + \frac{1}{K_{\text{a}}} \right) - \sqrt{\left([\text{G}]_0 + [\text{H}]_0 + \frac{1}{K_{\text{a}}} \right)^2 + 4[\text{H}]_0[\text{G}]_0} \right\} \right)$$

NMR spectroscopy

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 the relationship between the equilibrium association constant and the kinetics 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, 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.



- Two different conformations appear as resolved peaks in the NMR only if their interconversions rate constant is smaller than 1000 s^{-1} (55 kJ/mol a 25°C)

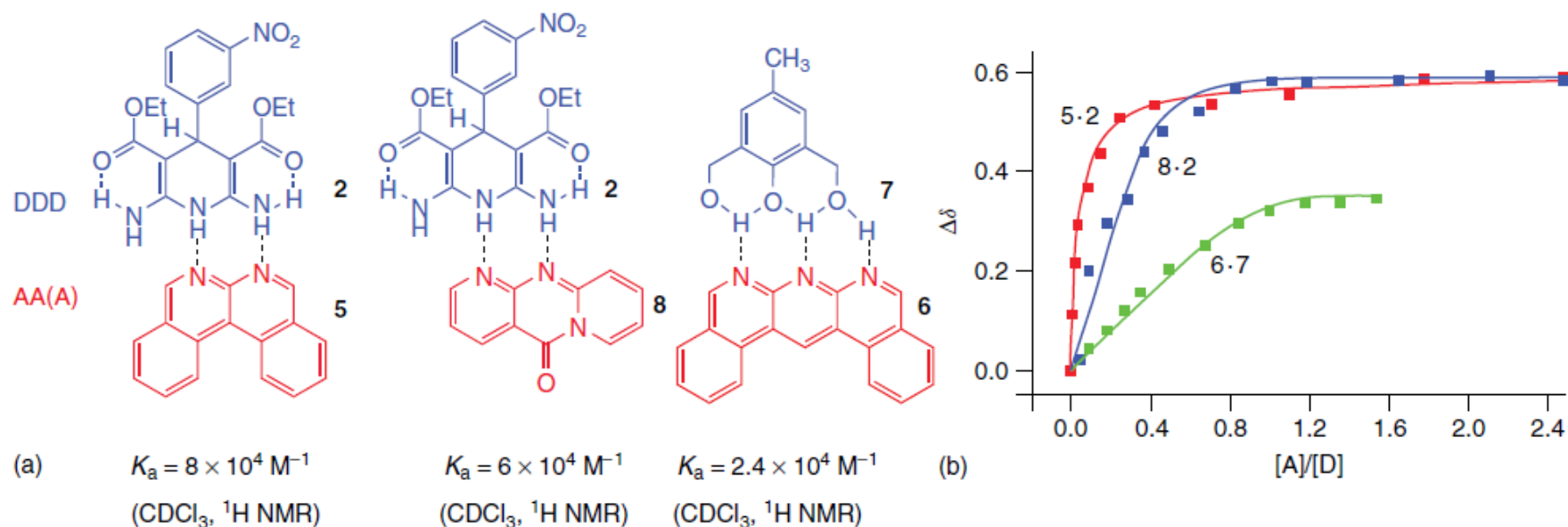


Figure 2 (a) Structures, association constants, and (b) binding isotherms of receptor pairs 5-2, 8-2, and 6-7. ¹H NMR titration analyses performed in CDCl₃ using the change in chemical shift ($\Delta\delta$) of the amino NH₂ groups of 2 (10^{-3} M) upon addition of 5 or 8 and the hydroxyl groups of 7 (10^{-3} M) upon addition of 6. The lines indicate best-fitting K_a s for 5-2 (red), 8-2 (blue), and 6-7 (green).¹² (Reproduced with permission from Ref. 12. © American Chemical Society, 2009.)

UV-Vis spectroscopy

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

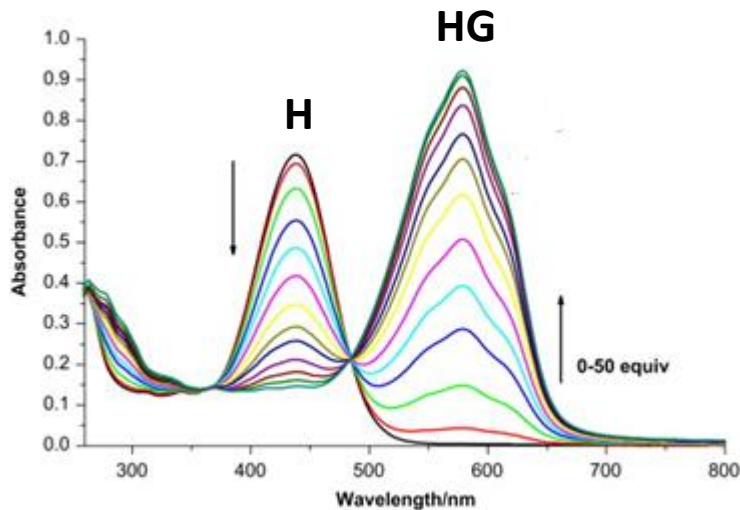
Advantages:

- rapid. Absorption and related phenomenon (fluorescence) occur on ps time scale or faster, in all cases faster than complex dissociation rate. Therefore, the system is always in the slow exchange regime. This implies that observed absorption spectra are a sum of the spectra of the species in solution ($A_{obs} = A_H + A_G + A_{HG}$).
- straightforward to correlate signal intensity to concentration (linear regime; Lambert-Beer)
- presence of isosbestic points confirms the 1:1 binding model
- 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

UV-Vis spectroscopy: isosbestic point



When $[H] = [HG]$ at the point of intersection (isosbestic) of the two spectra:

$$A_H = \epsilon_H [H] = A_{HG} = \epsilon_{HG} [HG] \quad \text{then} \quad \epsilon_H = \epsilon_{HG}$$

At the isosbestic point the two species have the same molar extinction coefficient

In a titration we start from a fixed concentration of host ($[H]_0$) and we increase the concentration of guest ($[G]$)

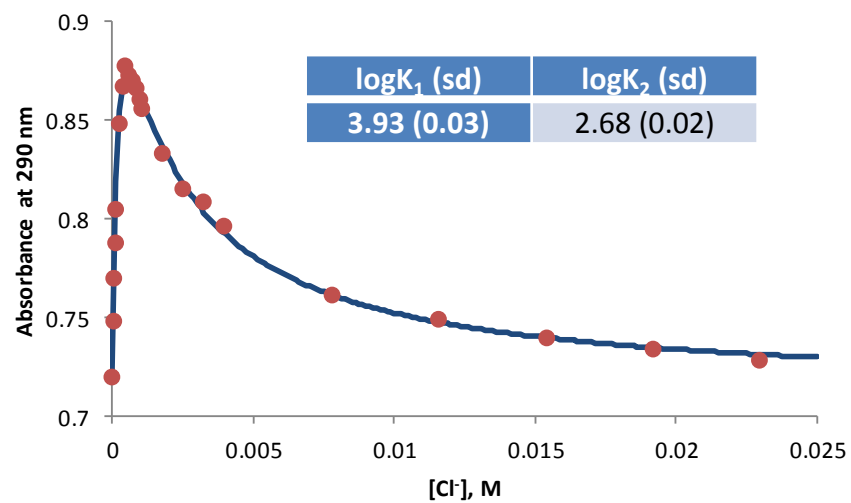
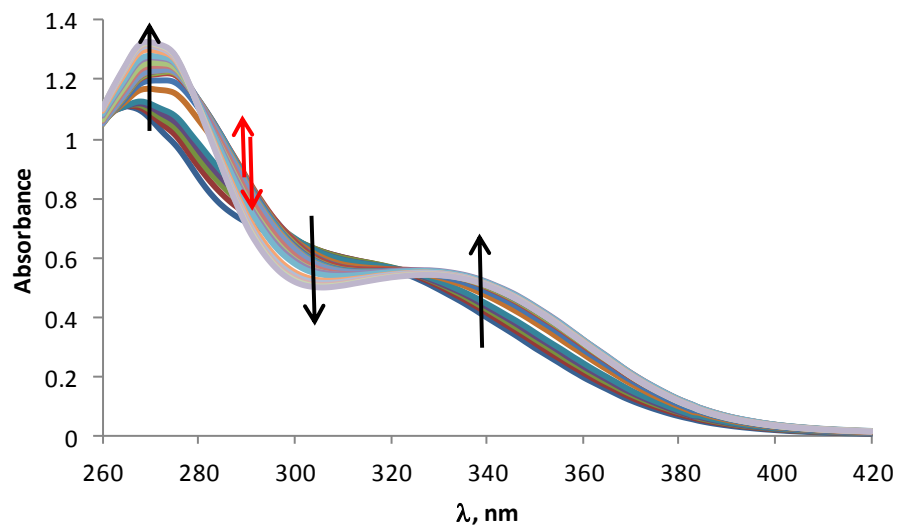
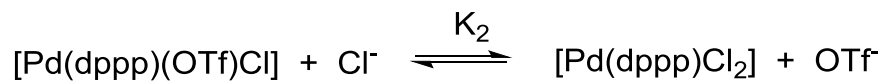
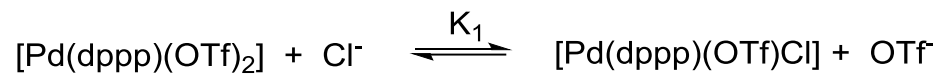
At the i addition of G the amount of complex formed is $[HG]^i = [H]_0 - [H]^i$ and the absorbance is :

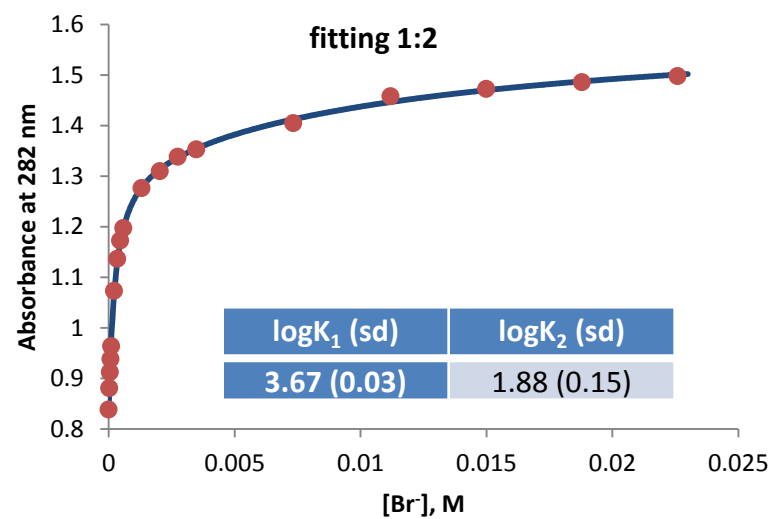
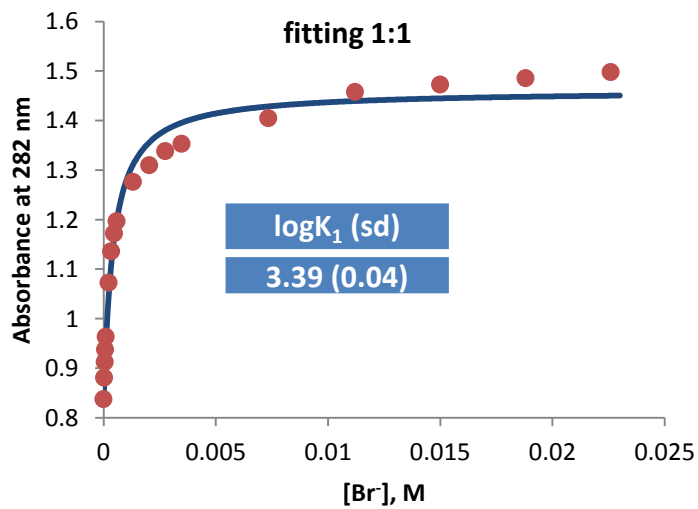
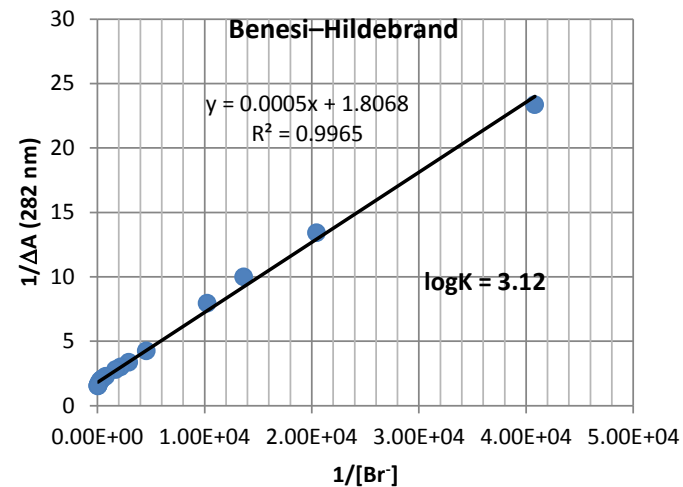
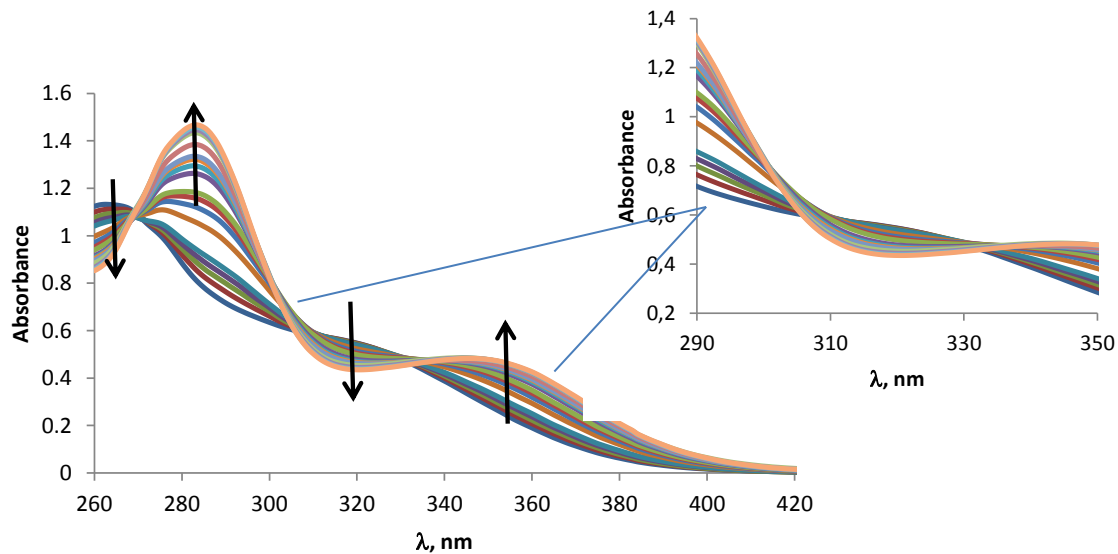
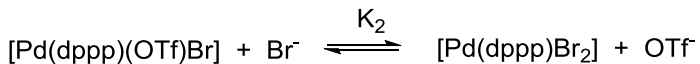
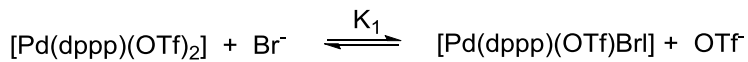
$$A^i = A^i_H + A^i_{HG} = \epsilon_H [H]^i + \epsilon_{HG} [HG]^i = \epsilon_H [H]^i + \epsilon_{HG} ([H]_0 - [H]^i)$$

At the isosbestic point $\epsilon_H = \epsilon_{HG}$ then $A^i = \epsilon_H ([H]^i + [H]_0 - [H]^i) = \epsilon_H [H]_0 = A_0$

At the isosbestic point the absorbance does not change during the titration!

Because it is unlikely that three compounds have the same ϵ at a given wavelength the observation of an isosbestic point in a UV-Vis titration is an evidence of the formation of a 1:1 complex.





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 ($K_a > 10^{10} \text{ M}^{-1}$).

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.

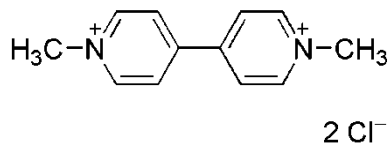
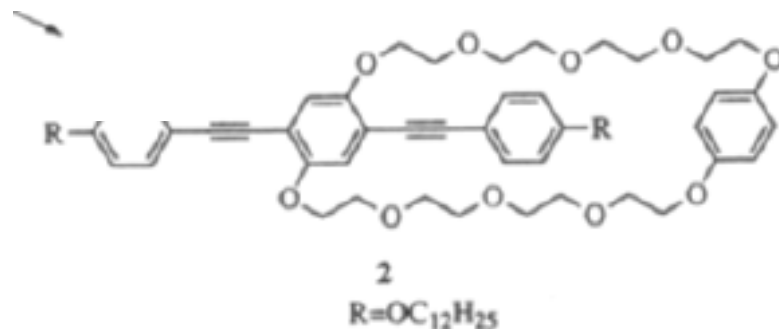
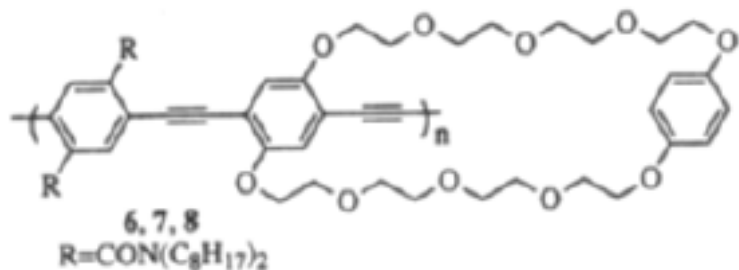
If quenching plays a role, it is necessary to differentiate between static and dynamic (collisional) quenching, with only the former of real significance for supramolecular binding studies.

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.

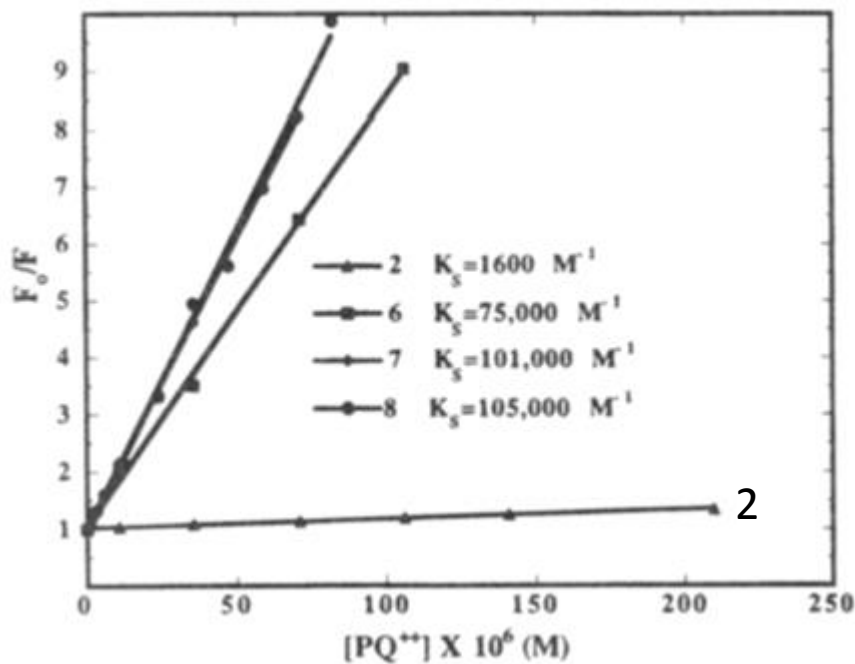
Unfortunately, pure 1 : 1 static quenching follows a nearly identical relation: $F_0/F = 1 + K_a[Q]$, with $[Q]$ = the free concentration of the quencher (guest) and K_a is the association constant of interest in supramolecular binding studies.

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.

Small molecule sensing



control



Mean molecular weight (PDI)

6: 31 100 (1.6)

7: 65 400 (1.6)

8: 122 500 (1.8)

Stern-Volmer plots ($F_0/F=1+K[PQ^{2+}]$)

fluorescence spectroscopy

In certain conditions the fluorescence emission is proportional to the emitting species concentration

$I_f = \varphi I_{abs}$ where φ is the quantum yield and I_{abs} is the intensity of absorbed light

At low absorbance ($A < 0.05$) it derives that:

$$I_f = 2.3\varphi I_0 \varepsilon l [S] = k[S]$$

where k is a proportional coefficient between I_f and the emitting species (S) concentration.

The range of linearity is usually confined to very dilute solution and at higher concentration deviations due to the inner filter effect are frequently observed.

However at low concentration and in the absence of dynamic quenching the linear relationship holds true and the same approach used for UV-Vis titration can be applied.

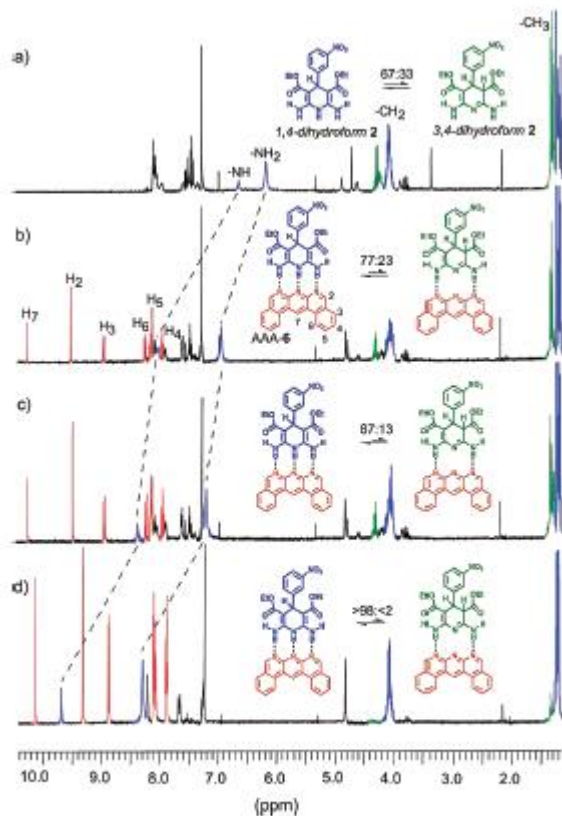


Figure 5. ^1H NMR (400 MHz, CDCl_3 , 298 K) spectra of (a) **2**, (b) **2** + 0.13 equiv of **6**, (c) **2** + 0.25 equiv of **6**, and (d) **2** + 0.5 equiv of **6**. Experiments carried out at 1×10^{-3} M concentration of **2**.

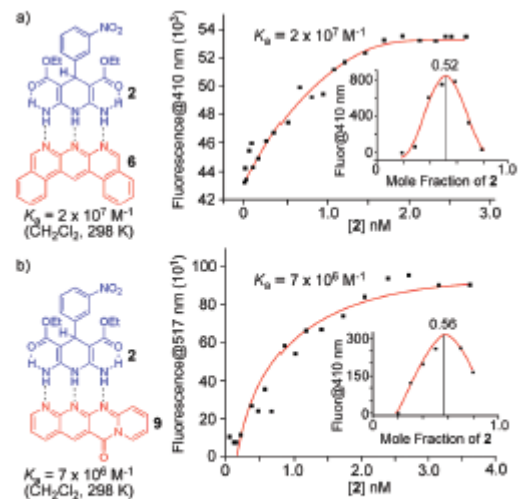


Figure 6. Change in the fluorescence intensities upon addition of aliquots of **2** to (a) **6** at 410 nm ($\lambda_{\text{excitation}} = 395$ nm) and (b) **9** at 517 nm ($\lambda_{\text{excitation}} = 480$ nm) in CH_2Cl_2 at 293 K. The red lines show the best fitting. (Insets) Job plots under the same conditions as the titration experiments.

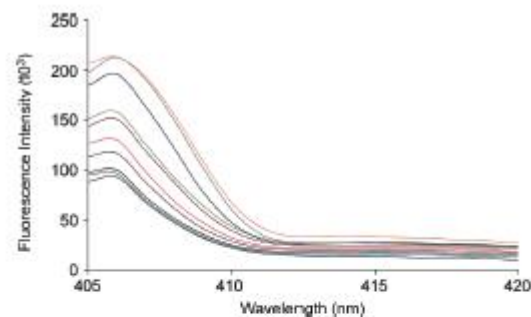


Figure 7. Fluorescence spectra of **6** (ca. 1×10^{-10} M) upon addition of $10^{-10}[\text{B}(3,5\text{-(CF}_3)_2\text{C}_6\text{H}_3)_4^-]$ (0 \rightarrow 2.5 equiv), maintaining the concentration of **6** constant, in CH_2Cl_2 at 293 K upon excitation at 395 nm.

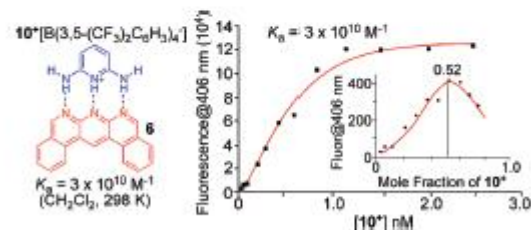
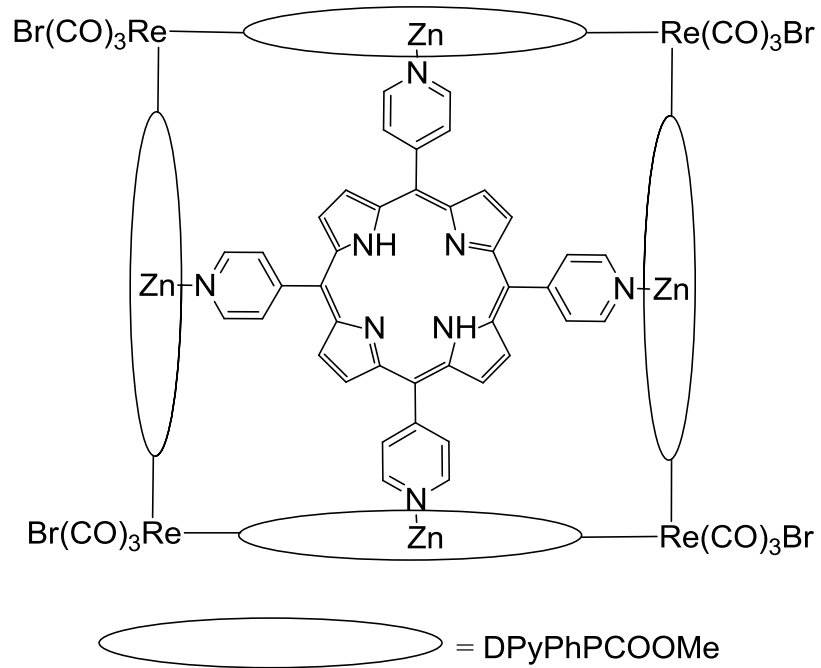
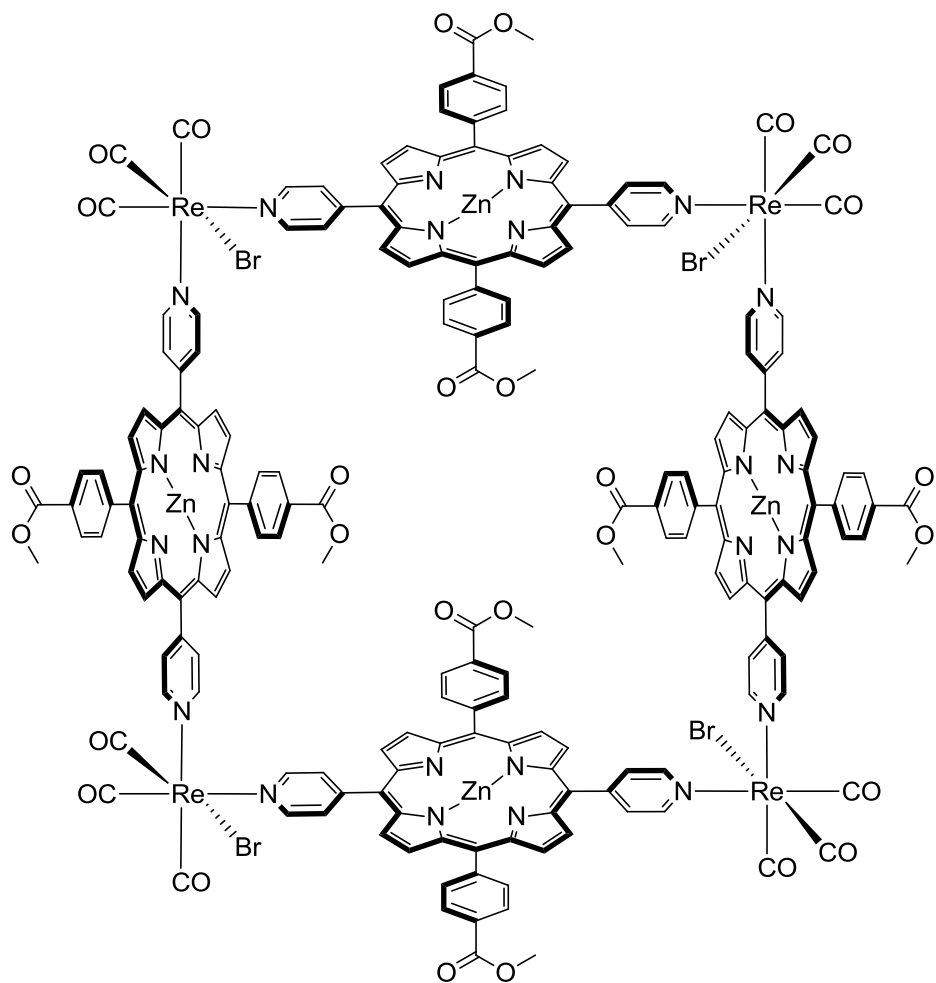


Figure 8. Fluorescence intensities of **6** (1×10^{-10} M) at 406 nm in CH_2Cl_2 at 293 K ($\lambda_{\text{excitation}} = 395$ nm) upon addition of $10^{-10}[\text{B}(3,5\text{-(CF}_3)_2\text{C}_6\text{H}_3)_4^-]$ (0 \rightarrow 2.5 equiv.), maintaining the concentration of **6** constant, using a 1:1 complexation model. (Inset) Job plot under the same conditions as the titration experiment.



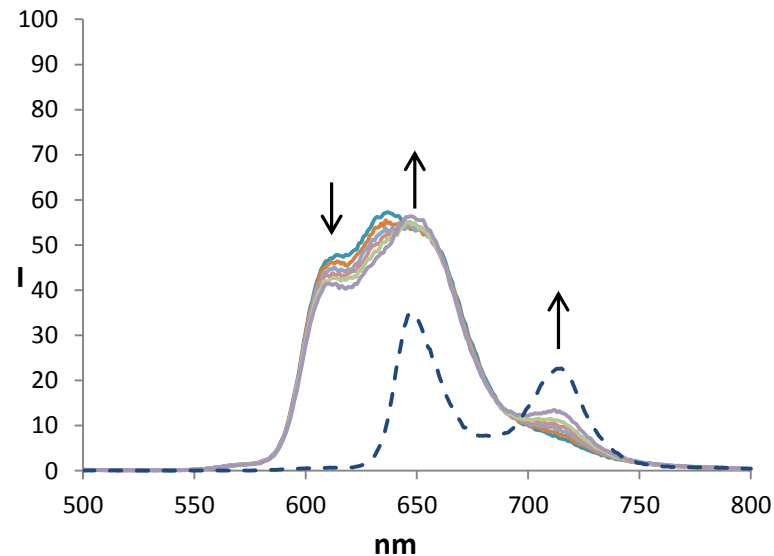
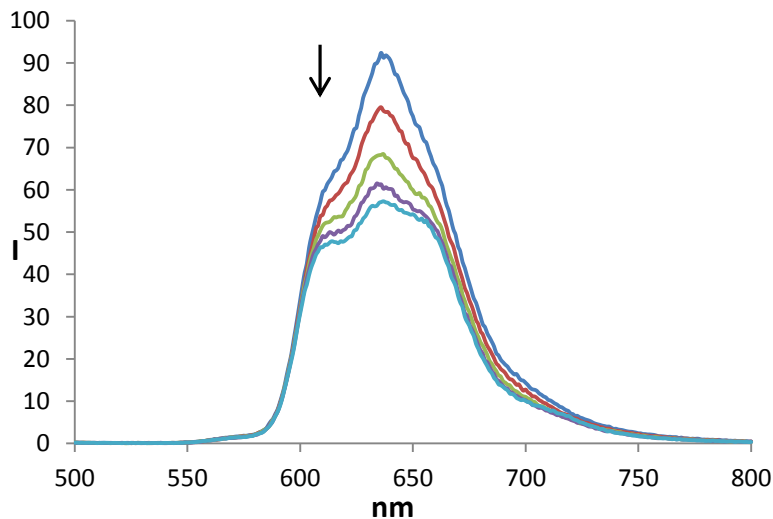


Figure S5. Fluorimetric titration of **2** (CHCl_3 , 1×10^{-6} M) with $\text{H}_2\text{-TPyP}$ (CHCl_3 , 2×10^{-5} M), $\lambda_{\text{exc}} = 425$ nm. Top: $\text{H}_2\text{-TPyP}/\mathbf{2}$ molar ratio from 0 to 1; bottom: $\text{H}_2\text{-TPyP}/\mathbf{2}$ molar ratio from 1 to 2.5. The blue dashed curve in the bottom panel corresponds to the emission spectra of $\text{H}_2\text{-TPyP}$ (CHCl_3 , 1×10^{-6} M, $\lambda_{\text{exc}} = 425$ nm).

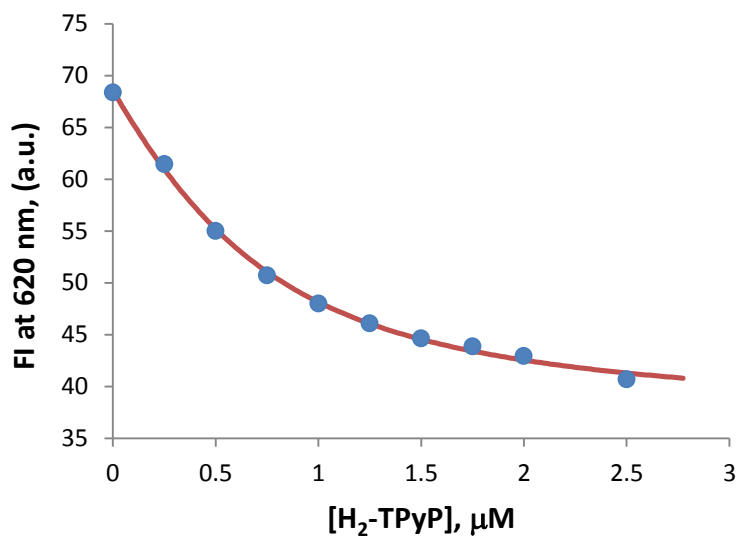


Figure S6. Fluorescence emission of **2** (1mM, CHCl_3) at 620 nm (blue dots) with increasing concentration of $\text{H}_2\text{-TPyP}$, excitation at 425 nm (data from Figure S5). The red curve is the calculated titration curve on the basis of a 1:1 binding model and $\log K_{\text{ass}} = 6.44$.

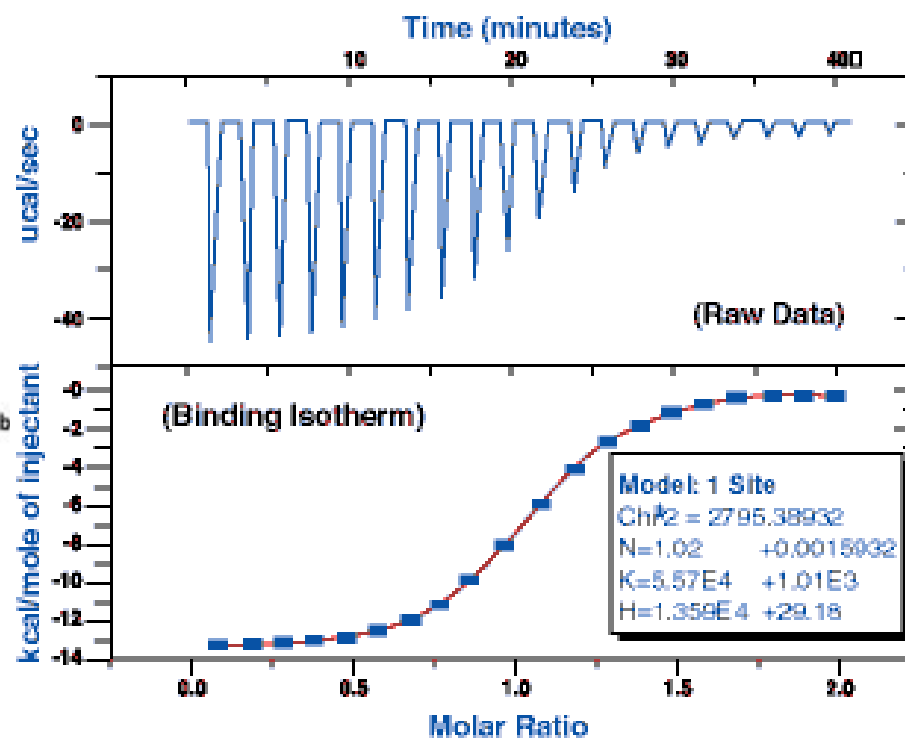
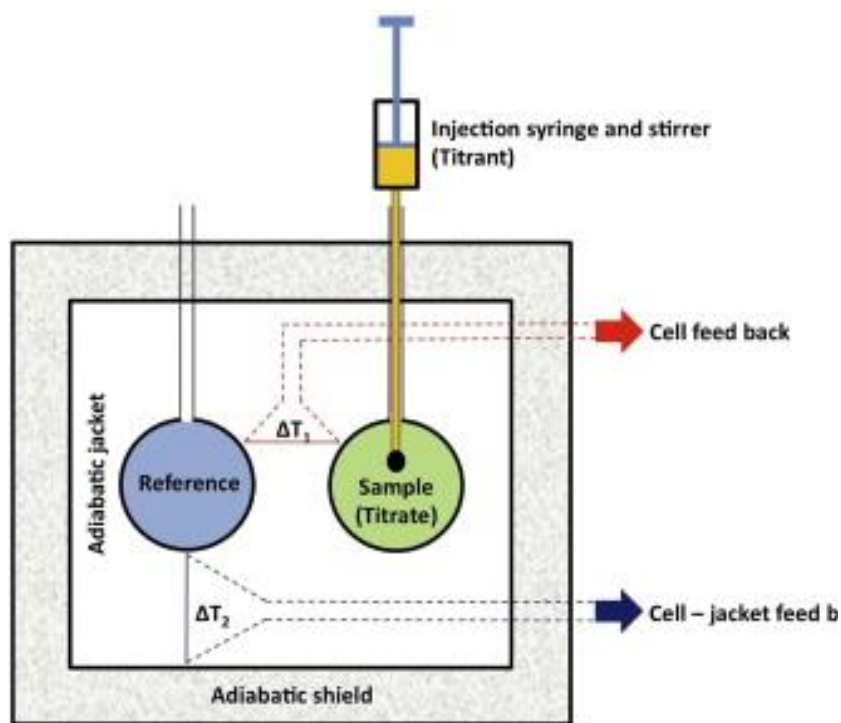
```
// MicroMath Scientist Model File
// Binding 1:1 con entrambe le specie che
// assorbono (o emettono), aggiungendo il
// metallo
// Parameter: LKML logKf; LTot. concentrazione
// fissa; E epsilon fissa; E1 epsilon complesso
IndVars: MTOT
DepVars: A
Params: LKML, LTOT, E, E1
KML=10^LKML
LTOT=L*(1+KML*M)
MTOT=M*(1+KML*L)
ML=KML*M*L
A=E*L + E1*ML
0<M<MTOT
0<L<LTOT
***
```

Isothermal titration calorimetry (ITC)

$$Q = V\Delta H^\circ [\text{HG}]$$

or

$$dq = V\Delta H^\circ \Delta[\text{HG}]$$



fitting provides K and ΔH°

Determination of stoichiometry

The other aim of a supramolecular titration experiment is the determination of the stoichiometry of the system.

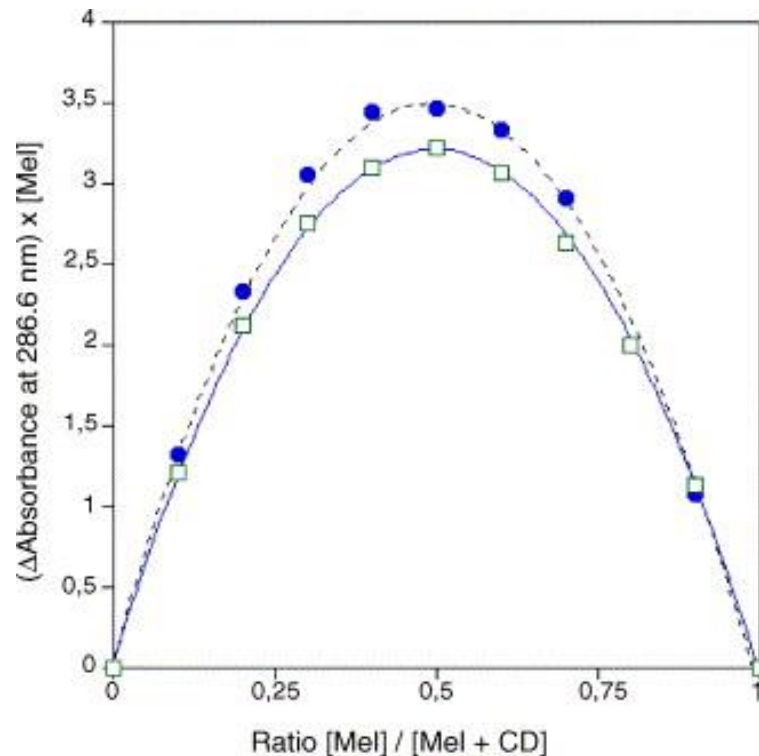
Methods

- (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.

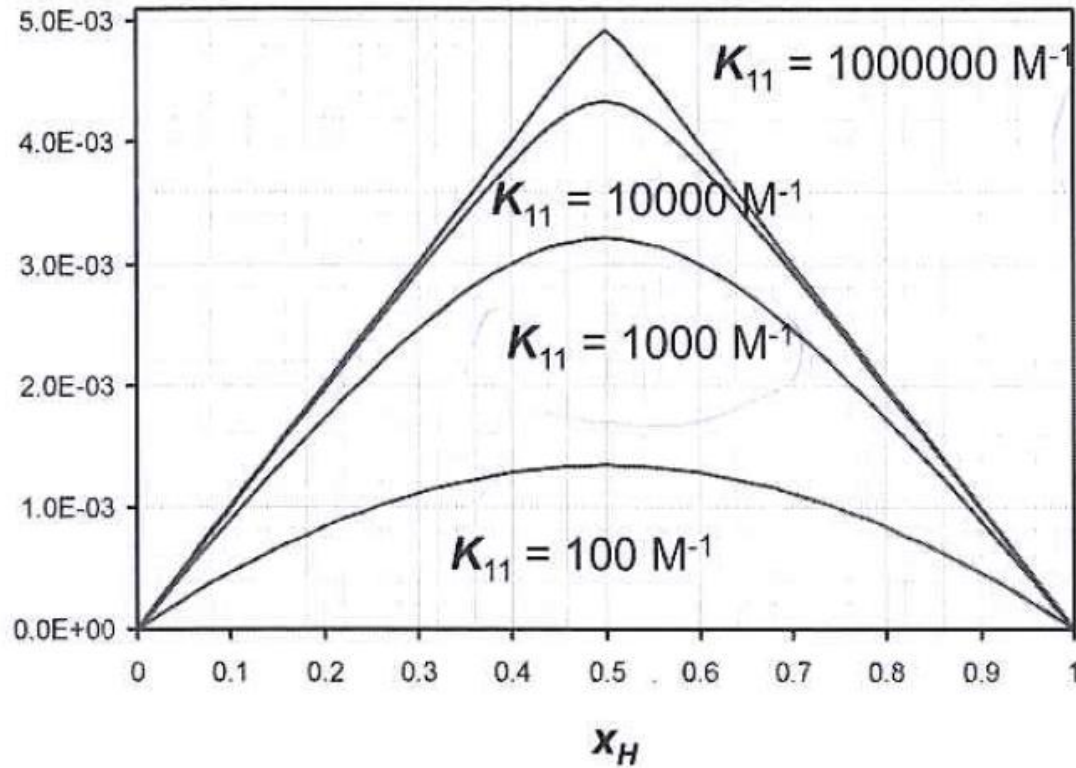
Job's method

The idea behind it is simple; the concentration of a H_mG_n ($[H_mG_n]$) complex is at maximum when the $[H]/[G]$ ratio is equal to m/n .

To do this, the mole fraction (f_G) of the guest is 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 $[H_mG_n]$ 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 $f_G = 0.5$



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



$$[H]_t + [G]_t = 0.01 \text{ M}$$

$$K_{11} = 1000 \text{ M}^{-1}$$

Limitations

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., δ_{obs}) is linearly dependent may not be valid. For similar reasons, the Job's method is likely to fail when either the host or guest aggregates in solution.

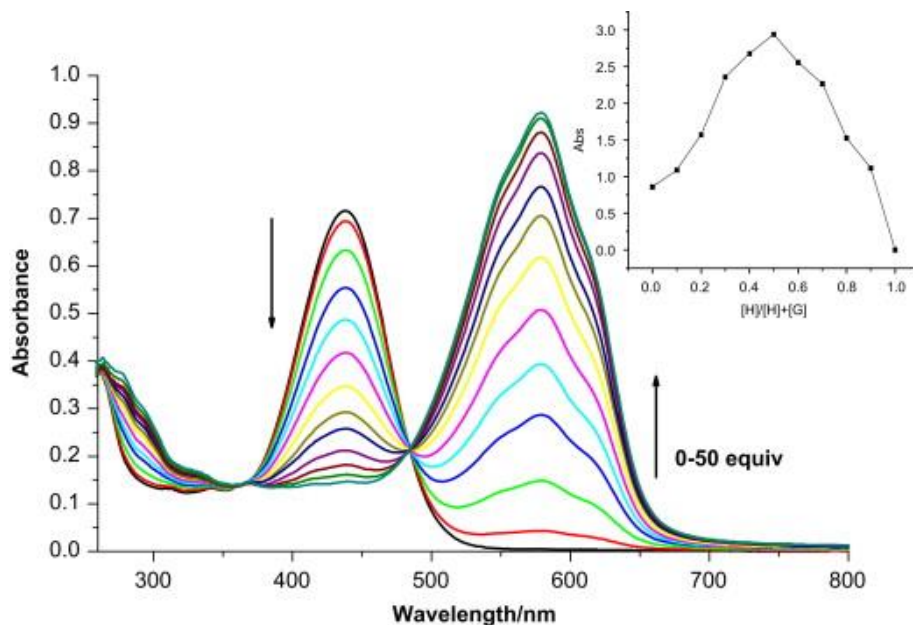
- (ii) Consistency with the host structure and available information on the host–guest complex structure.

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.

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

This relies on specific evidence such as isosbestic points which can be used to confirm that more than one type of complex is present and hence 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, i.e. the absence of more than one isosbestic point cannot be used to rule out more complex stoichiometry such as 1 : 2 complex formation, especially in cases where the cooperative (positive or negative) processes play a significant role.



- (iv) Constancy of stability concentration as the concentration is varied, that is, the success of a stoichiometric model to account for the data.

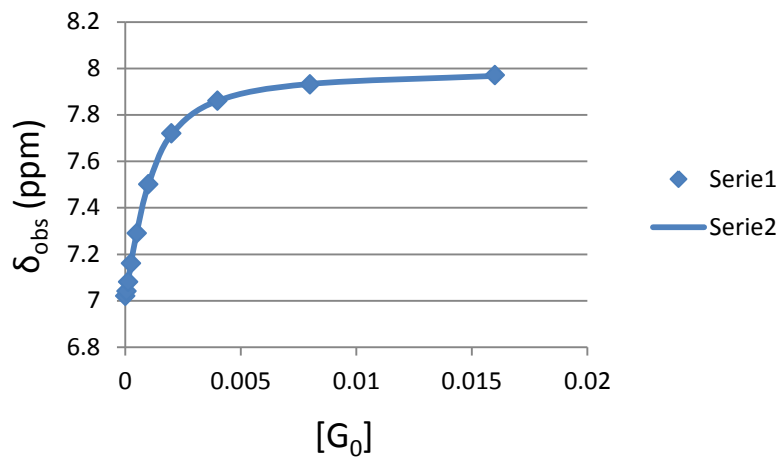
This method is probably the most generally applicable method for determining stoichiometry.

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.

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.

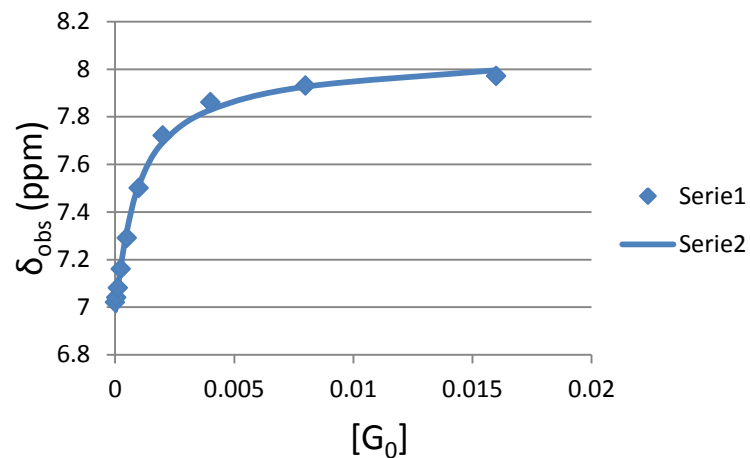
Thirdly, the fitting should be made for more signal as possible (NMR peaks, different wavelengths) and the results should be consistent

1:1 model



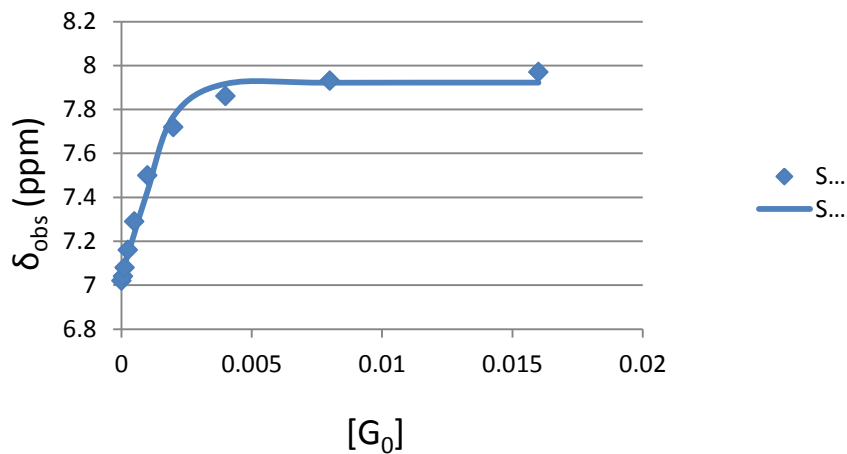
K		1938.702	78	M ⁻¹
DH		7.00624	0.004	ppm
DHG		8.000867	6.00E-03	ppm

2:1 model
HHG



K		2615630	5.00E+05	M ⁻²
DH		6.98578	0.02	ppm
DHG		8.169739	0.04	ppm

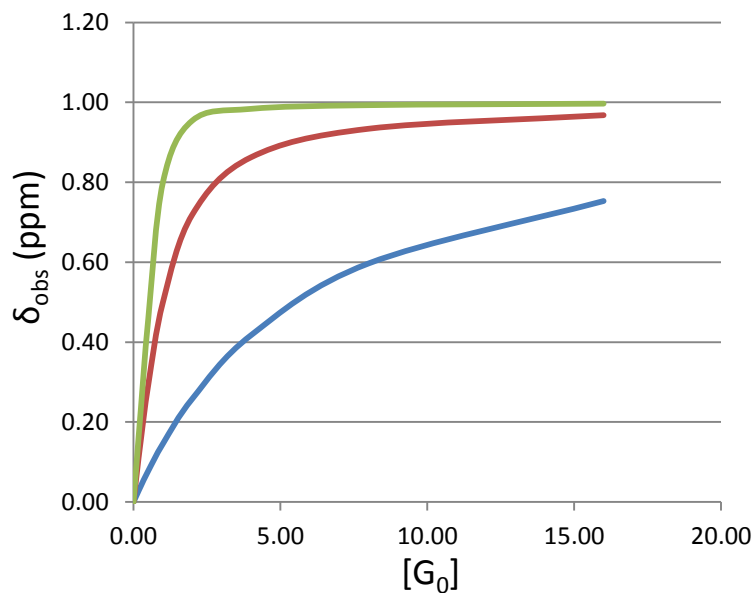
1:2 model
HGG



K		3566244	1.20E+06	M ⁻²
DH		7.05578	0.02	ppm
DHG		7.92317	0.03	ppm

dilution

1:1 model
HG

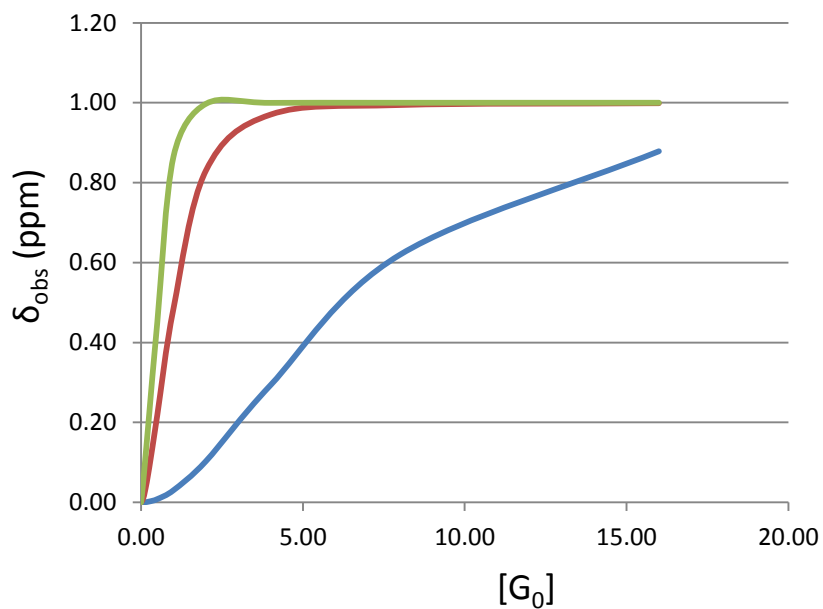


— H0=0.1 mM
— H0=1 mM
— H0=10 mM

K		1938.702	78	M ⁻¹
DH		7.00624	0.004	ppm
DHG		8.000867	6.00E-03	ppm

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

1:2 model
HGG



— Serie1
— Serie2
— Serie3

K		3566244	1.20E+06	M ⁻²
DH		7.05578	0.02	ppm
DHG		7.92317	0.03	ppm

Setting up a titration experiment

