Mechanisms of retention in HPLC

María Celia García-Álvarez-Coque

Department of Analytical Chemistry
University of Valencia
Valencia, Spain

https://sites.google.com/site/fuschrom/

Part 6

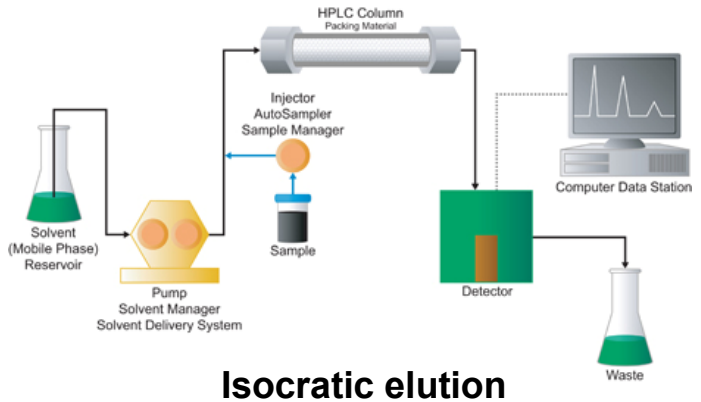
Index

6.1. Previous considerations

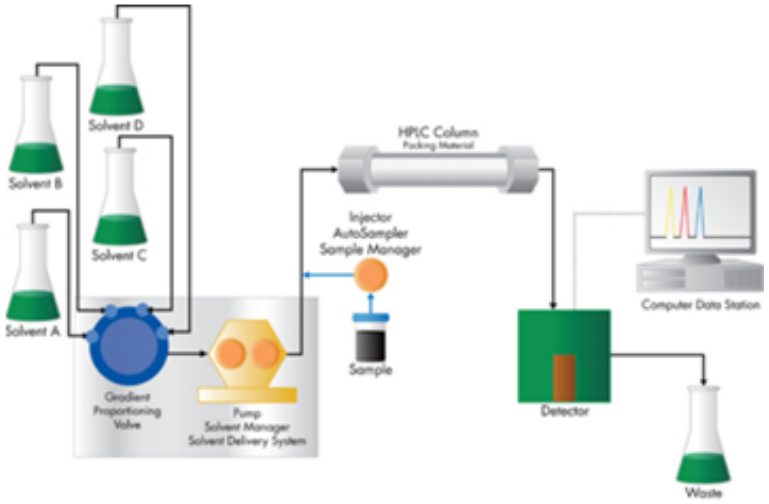
HPLC separations can be approached from two perspectives: isocratic and gradient elution.

Isocratic elution: advantages

- **Greater simplicity, simpler instrumentation, lower cost, and no need of column re-equilibration between consecutive injections.**
- **Allows the maximal capability of the system: it expands the separation among peaks as much as possible.**



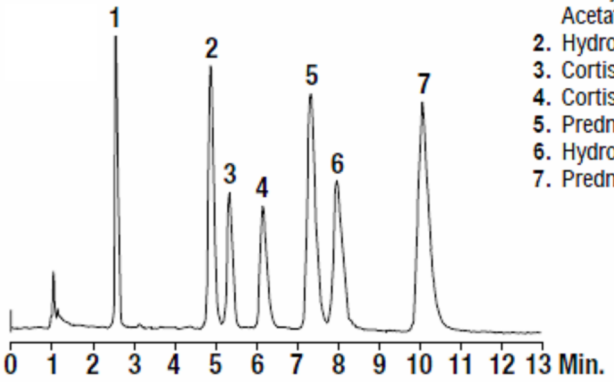
Isocratic elution



Gradient elution

Isocratic elution

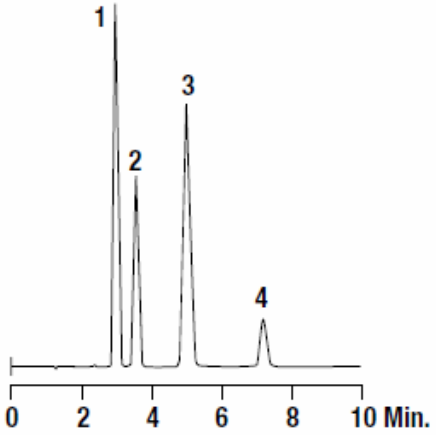
Corticosteroids



1. Deoxycorticosterone Acetate
2. Hydrocortisone Acetate
3. Cortisone Acetate
4. Cortisone
5. Prednisone
6. Hydrocortisone
7. Prednisolone

Column: Alltech® Alltima™ HP CN, 5µm, 150 x 4.6mm (Part No. **87769**)
Mobile Phase: Hexane:Isopropanol (93.7:6.3)
Flow Rate: 2.0mL/min
Detector: UV at 254nm

Anti-Inflammatories



1. Naproxen
2. Acemetacin
3. Indomethacin
4. p-Xylene

Column: Alltech® Platinum™ C18, 5µm, 150 x 4.6mm (Part No. **32043**)
Mobile Phase: 0.02M KH_2PO_4:Methanol:Acetonitrile: pH4.5 (40:50:10)
Flow Rate: 1.0mL/min
Detector: UV at 254nm

Isocratic elution: disadvantage

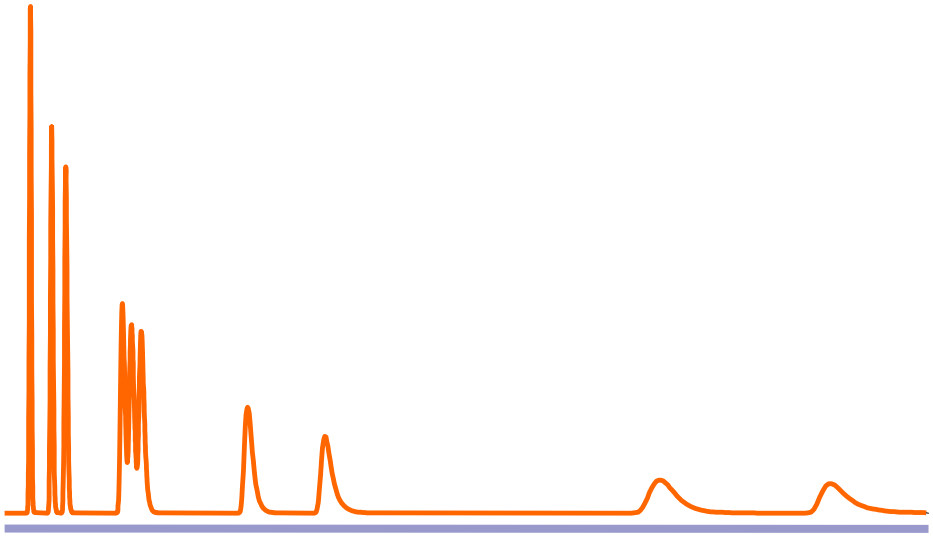
- **Owing to the dependence between the retention and solute polarity:**

$$\log k = c_0 + c_1 \log P_{o/w} \qquad (6.1)$$

if the polarity range is too wide, it will be difficult to find a set of chromatographic conditions able to balance a satisfactory separation power for the least retained solutes, and a reasonable retention time for the most retained ones.

General elution problem of chromatography: The utility of isocratic elution is seriously restricted to sets of compounds in a relatively narrow range of polarities.

General elution problem of chromatography

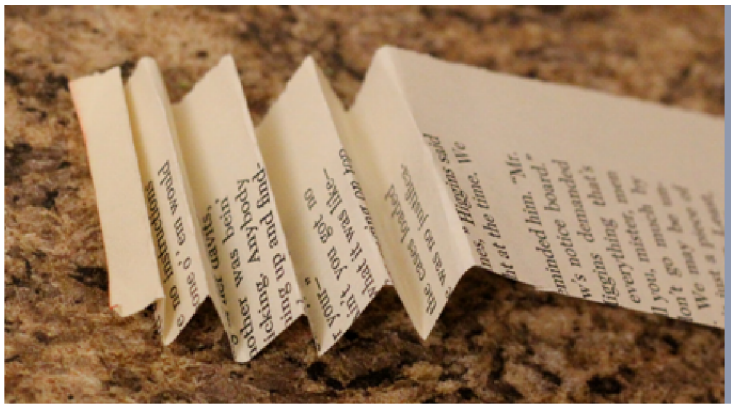


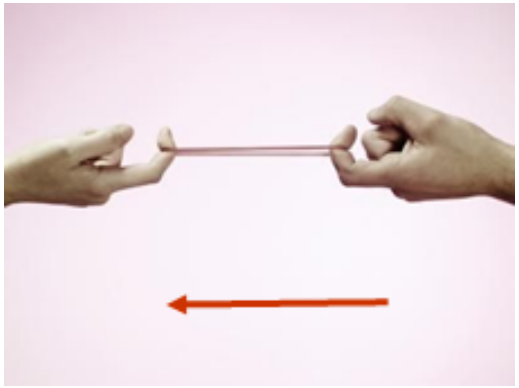
Retention time

A logical solution is gradient elution, where the elution strength is increased gradually by altering at least one experimental factor (organic solvent content, pH, temperature or flow rate) as the analysis progresses, so that the elution of the most retained compounds is expedited.

Accordion effect

The **effect of gradient elution on retention** can be graphically expressed as **closing an accordion,** or **contracting an elastic rubber hold by only one extreme.**



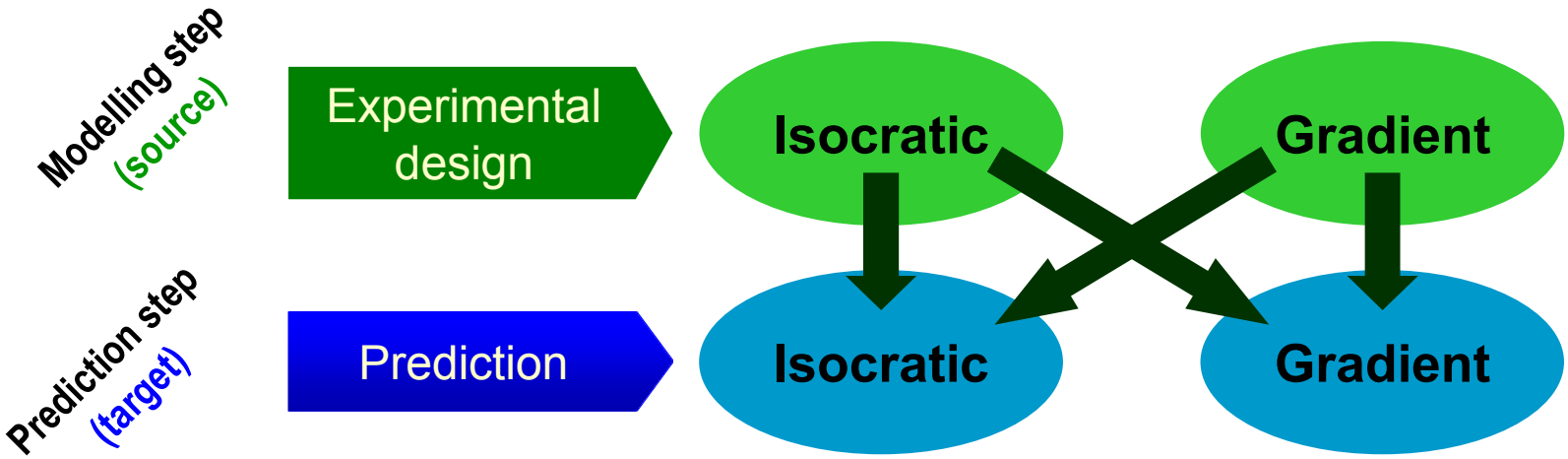


Gradients of organic modifier

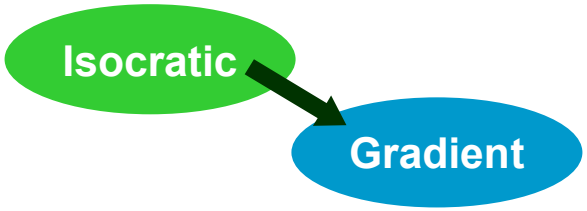
In RPLC, the use of gradients of organic modifier has experienced a large development in the separation of solutes in complex samples.

Transference of data between elution modes

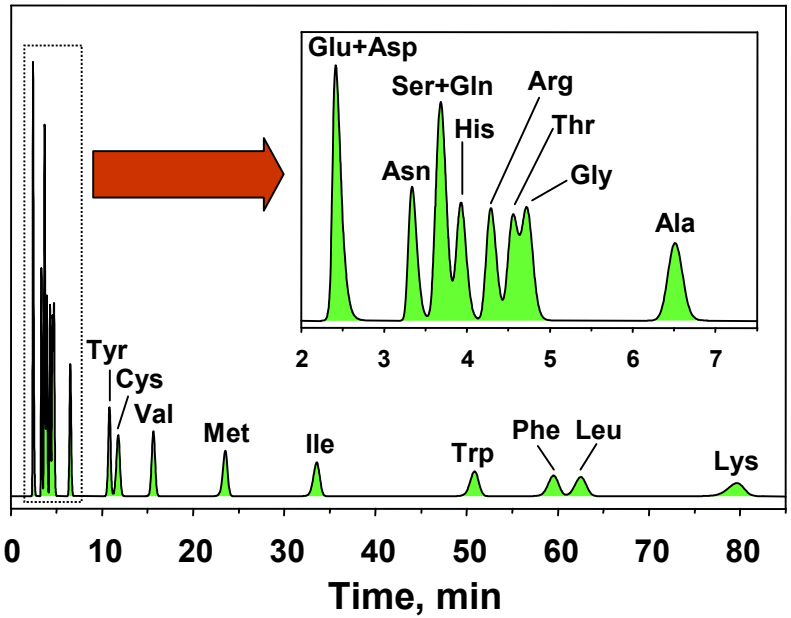
- **The prediction of retention under gradient conditions is usually made from training sets involving gradient experiments.**
- **Crossing the information between both elution modes is also possible:**
  data coming from isocratic experiments can be used to anticipate the retention in gradient runs, and *vice versa*.

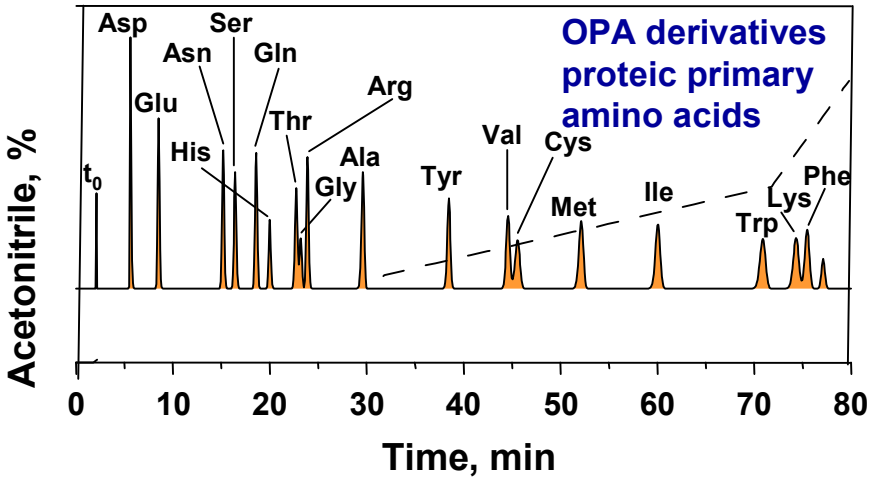


Isocratic versus gradient prediction



- In gradient elution, there is always an underlying model isocratically relating retention to mobile phase composition.
- Modelling gradient retention from wisely planned isocratic experiments may be safer and also faster, since no re-equilibration step is needed.
- Isocratic data may be available from the literature, or from previous data sets, and the chromatographer may wish to explore whether a gradient separation can give satisfactory results before carrying out any gradient experiment.



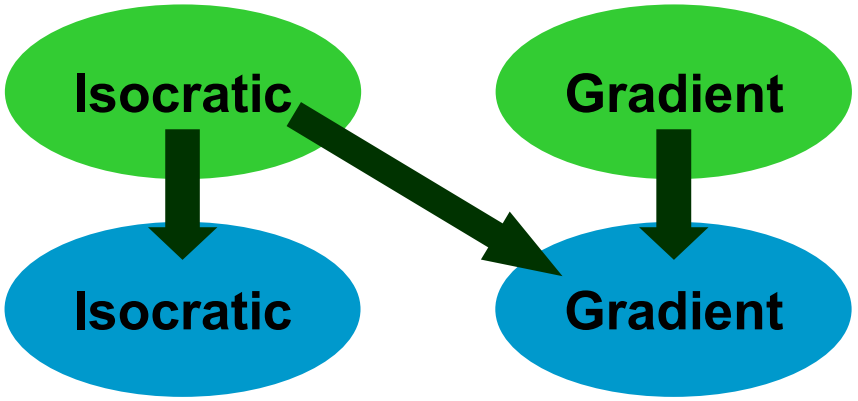


Isocratic versus gradient prediction

- The quadratic logarithmic model describes wide ranges of organic solvent concentrations in both isocratic-to-isocratic and isocratic-to-gradient predictions. This contrasts with the results offered by the linear model, which yields unsatisfactory predictions.

- The picture in gradient-to-gradient predictions may be different: both retention models may perform similarly, unless the modifier concentration range is significantly wide.

$\log k = c_0 + c_1 \varphi + c_2 \varphi^2$



$\log k = c_0 + c_1 \varphi + c_2 \varphi^2$

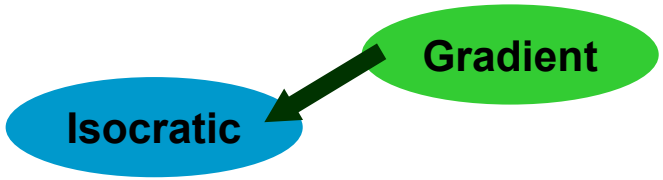
$\log k = c_0 + c_1 \varphi$

Isocratic versus gradient prediction

- Gradient elution concerns narrower solvent concentration domains compared to the isocratic case.
- The migration of each solute under gradient elution is affected by a rather narrow fraction of the concentrations scanned by the gradient program, which has been called

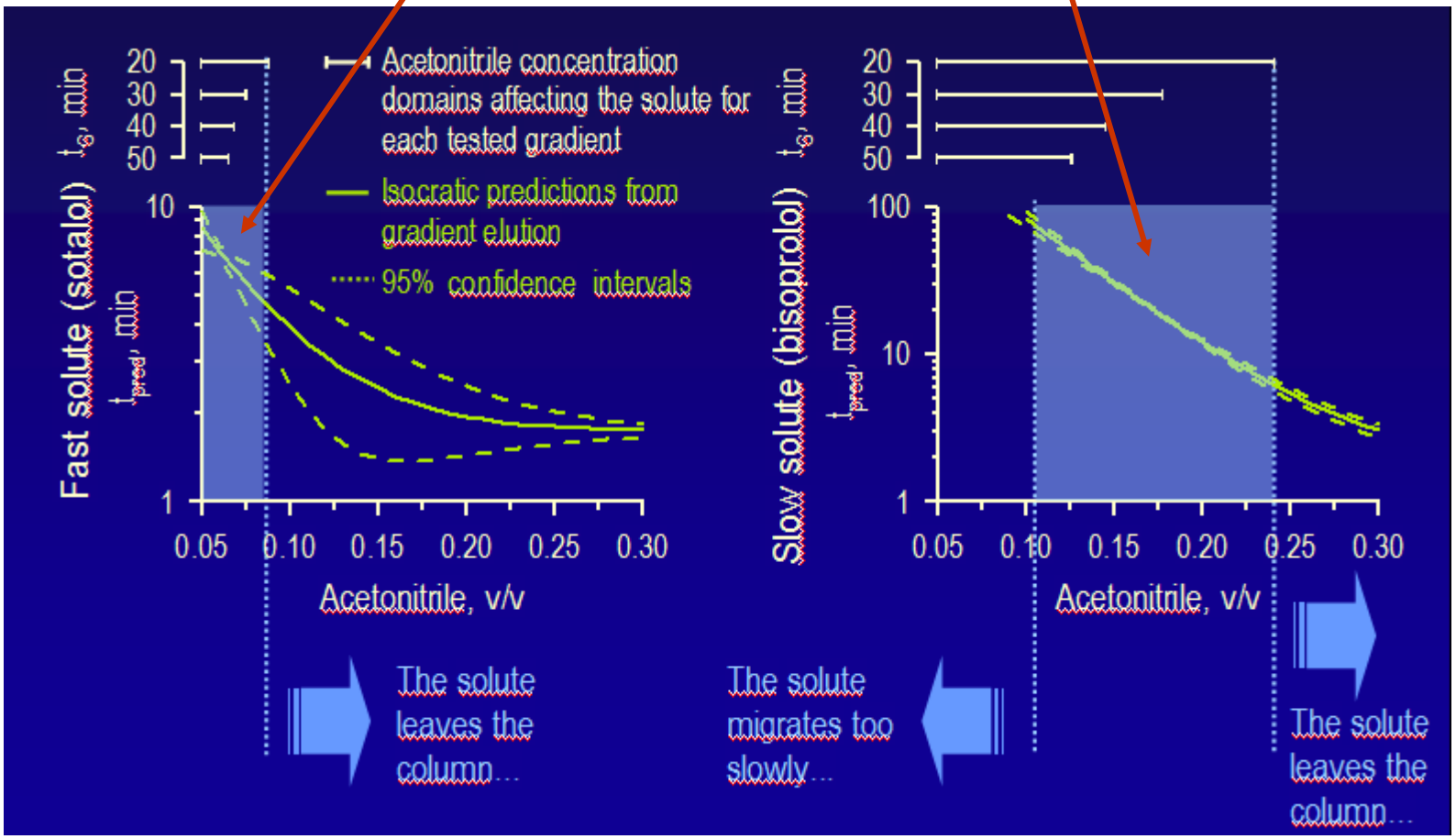
  solvent concentration informative range

  the solute starts to migrate significantly once a certain modifier concentration is reached, and can leave the column before the completion of the gradient.
- This explains the poor results yielded in gradient-to-isocratic predictions, which may imply extrapolations.

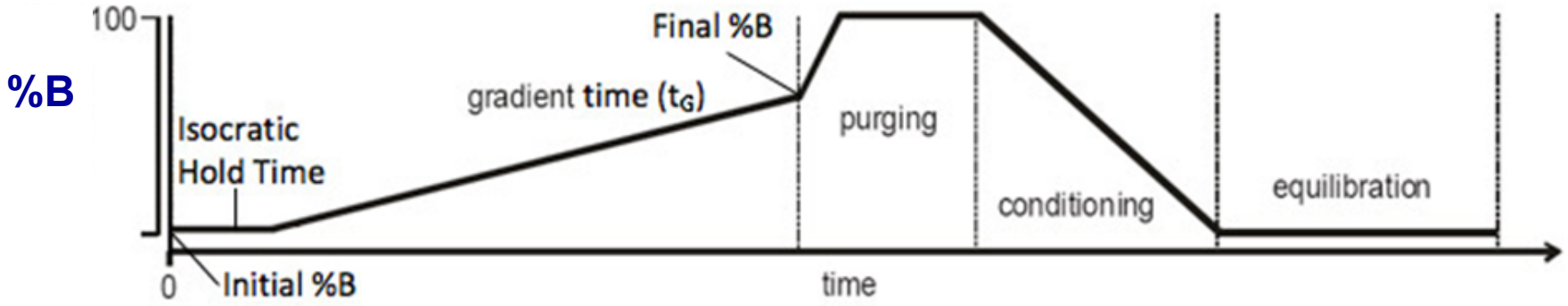


Isocratic experiments are considerably richer in information !!!

Solvent concentration informative range

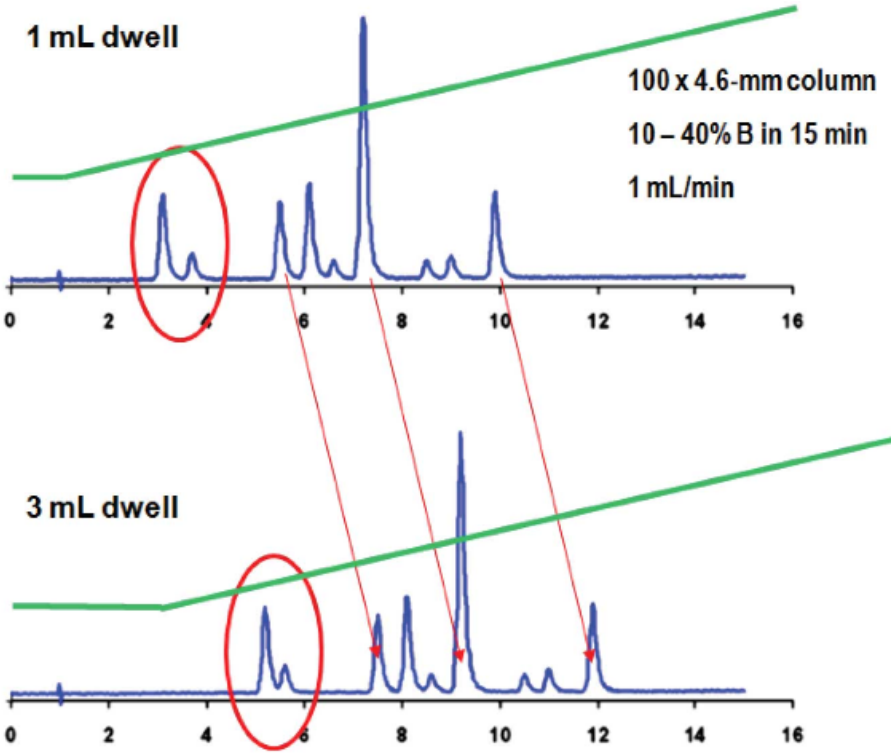


6.2. General equation for gradient elution



Steps along a gradient: organic solvent concentration at the column inlet

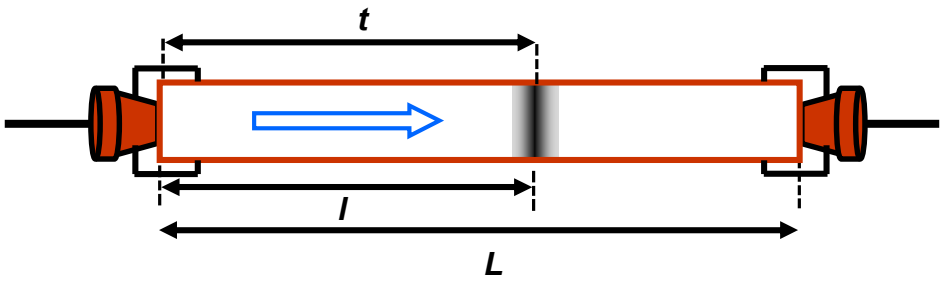
Dwell time

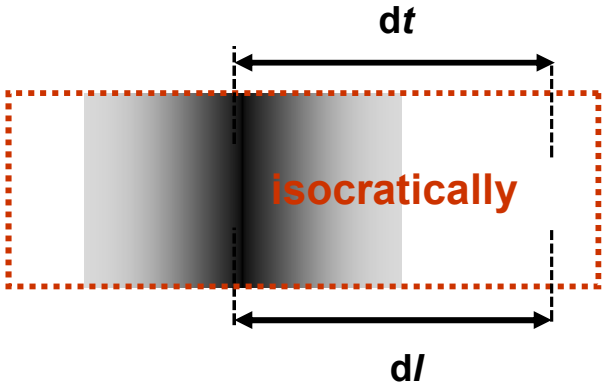


For simplicity, we will first consider:

- **linear gradients**
- **the gradient reaches instantaneously the column inlet (i.e. there is no delay associated to the dwell time)**

Solute linear velocity





- When a solute is eluted in the gradient mode, it can be considered that it moves isocratically a *dl* distance inside the column in an infinitesimal time range, *dt*.

$$u = \frac{dl}{dt} = \frac{L}{t_R(\varphi(t,l))} \qquad (6.2)$$

u : mobile phase linear velocity

t : time from the beginning of the gradient

l : distance from the column inlet to the position where the solute is at time *t*

L : total column length

t_R : retention time that the solute would present if it were eluted isocratically along the whole column, using the instantaneous mobile phase composition

φ : instantaneous mobile phase composition (organic solvent content at the column point where the solute is located), which depends on *t* and *l*

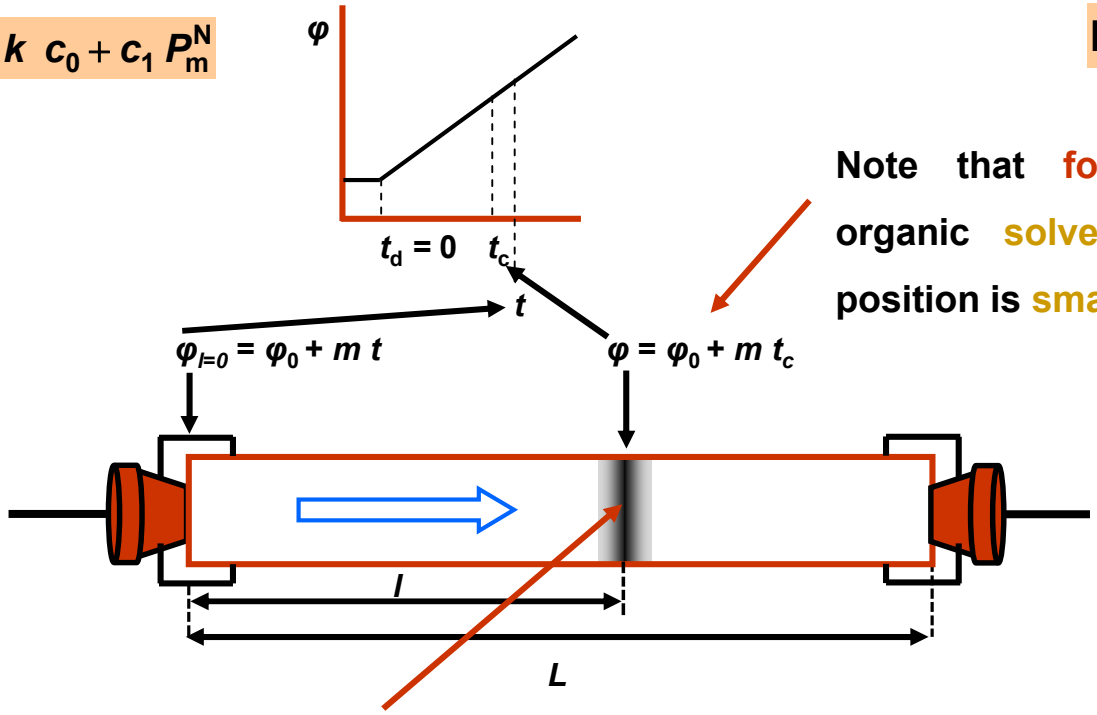
$$\frac{1}{k} = [a + b\varphi]^n$$

$$u = \frac{dl}{dt} = \frac{L}{t_R(\varphi(t,l))}$$

$$\ln k = \ln k_w - S\varphi$$

$$\log k \; c_0 + c_1 P_m^N$$

$$\ln k = \ln k_w - a\varphi + b\varphi^2$$



Note that for positive gradients, the organic solvent content at the solute position is smaller than at the column inlet.

$$m = \frac{\Delta\varphi}{t_G} \qquad (6.4)$$

$$\varphi(t,l) = \varphi_0 + m\left(t - \frac{l}{u}\right) = \varphi_0 + mt - m\frac{l}{u} = \varphi_{l=0}(t) - m\frac{l}{u} \qquad (6.3)$$

$t_{0,l} = l\,u^{-1}$: time needed by the gradient to go through the distance l to reach the position where the solute is at time t

(i.e. the dead time for the corresponding column segment)

$$u = \frac{dl}{dt} = \frac{L}{t_R(\varphi(t,l))} \qquad (6.2)$$

$$k = \frac{t_R - t_0}{t_0} \qquad (6.5)$$

$$u = \frac{L}{t_0(1 + k(\varphi(t,l)))} \qquad (6.6)$$

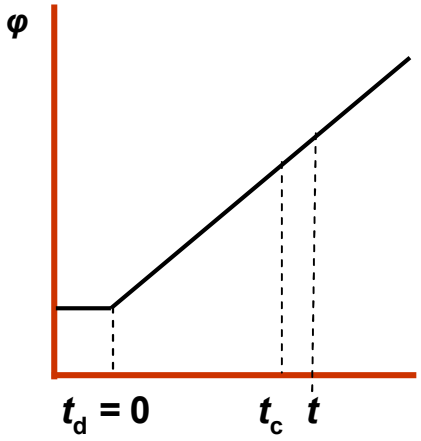
$$dl = u\,dt = \frac{L}{t_0\left(1 + k(\varphi(t,l))\right)}dt \qquad (6.7)$$

$$\int_0^L dl = L = \int_0^{t_g} \frac{L}{t_0\left(1 + k(\varphi(t,l))\right)}dt \qquad (6.8)$$

t_g : retention time under gradient conditions

(time a solute requires to reach the column outlet during the gradient)

Change of time variable



$$\int_0^L dl = L = \int_0^{t_g} \frac{L}{t_0\left(1 + k(\varphi(t,l))\right)} dt \qquad (6.8)$$

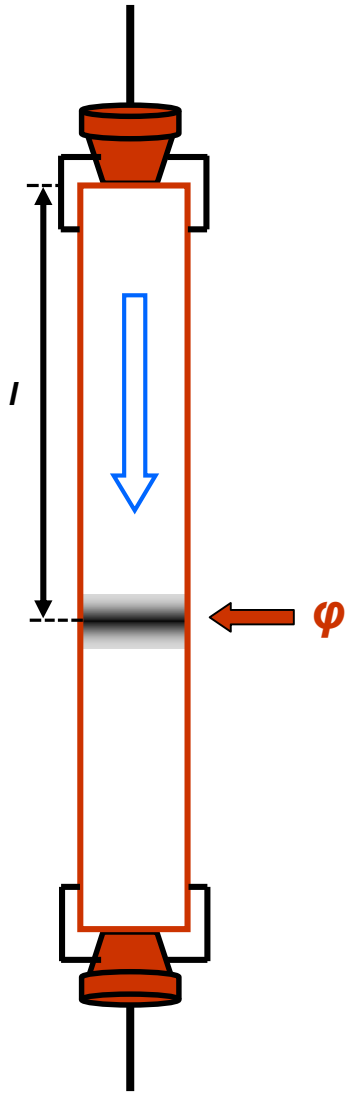
$$t_0 = \int_0^{t_g} \frac{dt}{1 + k(\varphi(t,l))} \qquad (6.9)$$

corrected time at the point where the solute is located inside the column at time *t*

$$t_c = t - \frac{l}{u} \qquad (6.10)$$

$$\varphi = \varphi_0 + m\,(t - l/u) = \varphi_0 + m\,t_c$$

$$dt_c = dt - \frac{dl}{u} \qquad (6.11)$$



General equation for gradient elution

$$dt_c = dt - \frac{dl}{u} \qquad dl = u\,dt = \frac{L}{t_0\left(1 + k(\varphi(t,l))\right)}dt \qquad t_0 = \frac{L}{u}$$

$$dt_c = dt - \frac{L/u}{t_0\left(1 + k(\varphi(t_c))\right)}dt = \left(1 - \frac{1}{1 + k(\varphi(t_c))}\right)dt = \frac{k(\varphi(t_c))}{1 + k(\varphi(t_c))}dt \qquad (6.12)$$

$$dt = \frac{1 + k(\varphi(t_c))}{k(\varphi(t_c))}dt_c$$

$$t_0 = \int_0^{t_g} \frac{dt}{1 + k(\varphi(t,l))}$$

$$t_0 = \int_0^{t_g - t_0} \frac{\left[1 + k(\varphi(t_c))\right]/k(\varphi(t_c))}{\left[1 + k(\varphi(t_c))\right]}dt_c = \int_0^{t_g - t_0} \frac{dt_c}{k(\varphi(t_c))} \qquad (6.13)$$

$t_g - t_0$ replaces t_g

Eq. (6.13) has been called fundamental or general equation for gradient elution, and is most extensively used, since it describes in a practical way the retention times for a solute eluted under a given gradient program. The retention time can be calculated provided the composite function $k(\varphi(t_c))$ is known.

6.3. Integration of the general equation

6.3.1. Equations based on integrable models: Linear solvent strength theory

$$t_0 = \int_0^{t_g - t_0} \frac{dt_c}{k(\varphi(t_c))} \qquad (6.13)$$

- Two nested equations:

  retention model $k(\varphi)$: dependence of the retention factor on the concentration of organic solvent

  gradient program $\varphi(t_c)$: change in the concentration of organic solvent with time

- The integral can be solved analytically only in a few cases.

  the most simple case: linear log $k(\varphi)$ and $\varphi(t_c)$ ⟹ linear solvent strength model

- This approach is the most widely used and can be easily extended to the construction of several consecutive linear segments (multi-linear gradients).

Linear solvent strength model

$$t_0 = \int_0^{t_g - t_0} \frac{dt_c}{k(\varphi(t_c))}$$

$$\ln k = \ln k_w - S\varphi \quad (6.14) \qquad \varphi = \varphi_0 + m t_c \quad (6.15) \qquad m = \frac{\Delta\varphi}{t_G} \quad (6.4)$$

$$k = k_w e^{-S\varphi} = k_w e^{-S(\varphi_0 + m t_c)} = k_w e^{-S\varphi_0} e^{-S m t_c} = k_0 e^{-S m t_c} \quad (6.16)$$

k_w : retention factor in water

S : elution strength

φ_0 : initial concentration of modifier at the column inlet

m : gradient slope

$\Delta\varphi$: difference between final and initial concentrations of organic modifier in the gradient

t_G : total gradient time (time at which the gradient program reaches the final composition)

Analytical integration of the linear models

$$t_0 = \int_0^{t_g - t_0} \frac{dt_c}{k(\varphi(t_c))} \qquad (6.13)$$

$$k = k_w\, e^{-S\varphi} = k_w\, e^{-S(\varphi_0 + m t_c)} = k_w\, e^{-S\varphi_0}\, e^{-Sm t_c} = k_0\, e^{-Sm t_c} \qquad (6.16)$$

dwell time term

$$t_0 = \int_0^{t_d} \frac{dt_c}{k_0} + \int_{t_d}^{t_g - t_0} \frac{dt_c}{k_0\, e^{-Sm(t_c - t_d)}} = \frac{t_d}{k_0} + \frac{1}{Sm\,k_0}\left[e^{Sm(t_g - t_0 - t_d)} - 1\right] \qquad (6.17)$$

gradient time

$$t_g = \frac{\ln\left[1 + Sm(k_0 t_0 - t_d)\right]}{Sm} + t_0 + t_d = \frac{t_0}{b}\log(1 + 2.3\, k_0\, b) + t_0 + t_d \qquad (6.18)$$

$$b = S\, m\, t_0$$

retention factor

$$k_g = \frac{\ln\left[1 + Sm(k_0 t_0 - t_d)\right]}{Sm\, t_0} + \frac{t_d}{t_0} = \frac{\ln\left[1 + Sm t_0 (k_0 - f)\right]}{Sm\, t_0} + f \qquad (6.19)$$

t_d : dwell time

k_0 : retention factor for the initial gradient conditions

Jandera's model

The analytical integration can be applied to other equations, as that proposed by Jandera in NPLC, also useful for RPLC.

$$t_0 = \int_0^{t_g - t_0} \frac{dt_c}{k(\varphi(t_c))} \qquad (6.13)$$

$$\varphi = \varphi_0 + m t_c \qquad (6.15)$$

$$\frac{1}{k} = [a + b\varphi]^n \qquad (6.20)$$

$$t_0 = \int_0^{t_d} \frac{dt_c}{k_0} + \int_{t_d}^{t_g - t_0} \frac{dt_c}{[a + b(\varphi_0 + m(t_c - t_d))]^{-n}} = \frac{t_d}{k_0} + \frac{[a_0 + bm(t_g - t_0 - t_d)]^{n+1}}{(n+1)bm} - \frac{a_0^{n+1}}{(n+1)bm} \qquad (6.21)$$

$$t_g = \frac{\left[\left(t_0 - \frac{t_d}{k_0}\right)(n+1)bm + a_0^{n+1}\right]^{1/(n+1)} - a_0}{bm} + t_0 + t_d \qquad (6.22)$$

a, *b*, *n* : fitting parameters

$$a_0 = a + b\varphi_0 \qquad (6.23)$$

6.3.2. Non-integrable retention models

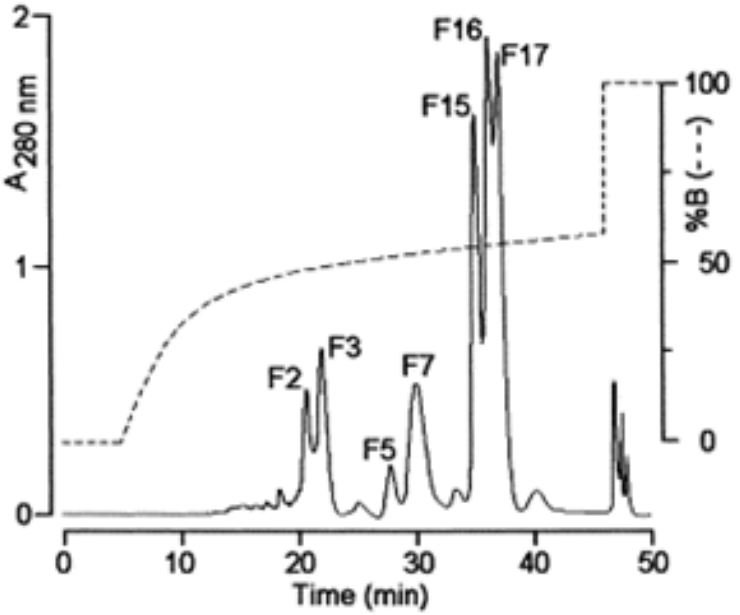
The linear retention model describes the retention accurately only in narrow concentration ranges of organic modifier. For wider ranges, a quadratic term should be added to make a proper description of the retention.

$$\ln k = \ln k_w - S\varphi$$

$$\ln k = \ln k_w - a\varphi + b\varphi^2$$

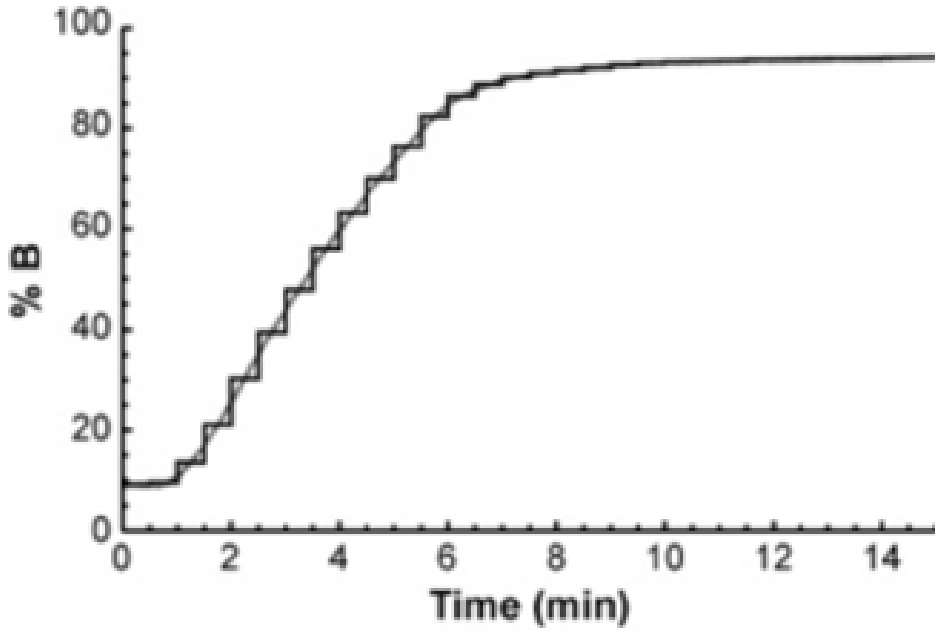
$$t_0 = \int_0^{t_g - t_0} \frac{dt_c}{k(\varphi(t_c))}$$

- A drawback of the quadratic model is that the analytical integration is not possible, or at least not straightforward.
- Independently of the retention model, the analytical integration cannot be applied when the change in the concentration of organic modifier with time is non-linear (non-linear gradients).



Solution for non-linear gradients

Non-linear gradients can be satisfactorily solved by approximating them as summations of several linear segments.



Numerical integration

Gradient retention times are calculated by splitting the integral in small isocratic steps.

$$t_0 = \int_0^{t_g - t_0} \frac{dt}{k(t)} = \int_0^{t_1} \frac{dt}{k(t)} + \int_{t_1}^{t_2} \frac{dt}{k(t)} + \ldots + \int_{t_{i-1}}^{t_i} \frac{dt}{k(t)} + \int_{t_i}^{t_{i+1}} \frac{dt}{k(t)} \qquad (6.25)$$

$$t_0 \approx \frac{t_1}{k_{0,1}} + \frac{t_2 - t_1}{k_{1,2}} + \ldots + \frac{t_i - t_{i-1}}{k_{i-1,i}} + \frac{t_{i+1} - t_i}{k_{i,i+1}} \qquad (6.26)$$

$$k_{i,i+1} = \frac{k(t_i) + k(t_{i+1})}{2} \qquad (6.27)$$

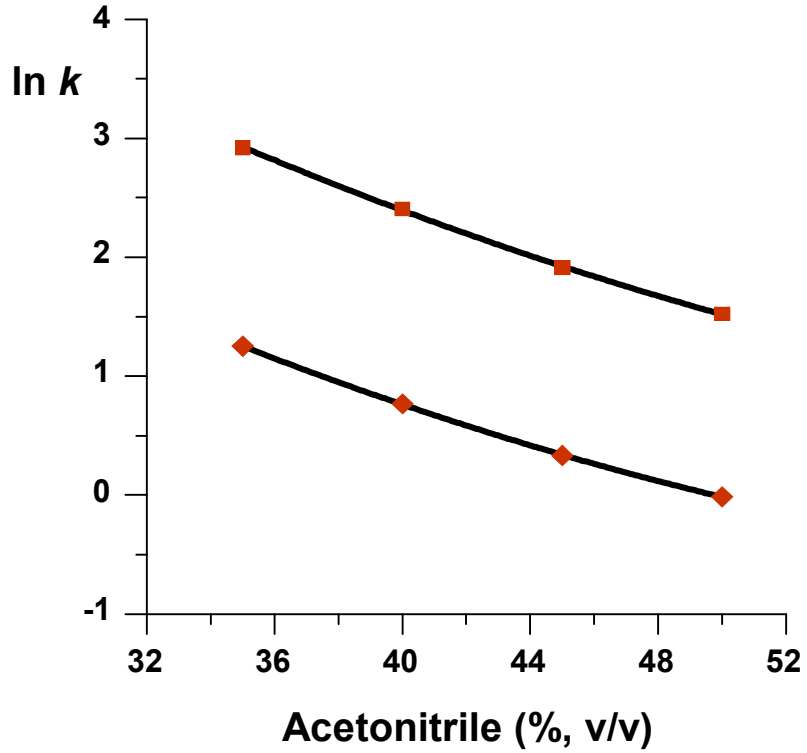
- $k(t)$ is assumed to be constant inside each step.
- The precision in t_g can be increased to any desired level, just narrowing the time intervals $(t_{i+1} - t_i)$.
- The higher the precision, the longer the computation time.

to consider !!

- Limitation associated with the pumping system: gradients are generated by the instrument by approximating changes in composition in small steps. Therefore, investing more effort in increasing the precision is useless.

In any case, the numerical integration is suitable. It is a fully flexible universal approach, and therefore, it is preferred by some analysts. However, its computation is somewhat slower and requires programming skills. In contrast, the analytical integration (when possible) is rapid, and the mathematical expressions are relatively simple, being relatively easy to be applied by any analyst.

Another alternative is possible !!!



$$\log k = c_0 + c_1 \varphi + c_2 \varphi^2$$

$$\log k = c_0 + c_1 \varphi$$

Retention in RPLC using linear gradients can be predicted assuming that the non-linear retention behaviour is not far from the linear behaviour. Therefore, it is possible to describe the retention based on the integral equation obtained for the linear model.

Alternative approach: assumptions

- The retention behaviour under gradient elution is formally similar to that for the linear model.
- Since *S* is the minus slope of the logarithmic linear model, for the non-linear model it is the minus derivative of the quadratic function at the initial gradient condition:

$$\ln k = \ln k_w - S\varphi$$

linear model

$$\ln k = \ln k_w - a\varphi + b\varphi^2$$

quadratic model

$$S = -\frac{\ln k - \ln k_w}{\varphi}$$

$$S = -\left(\frac{d \ln k}{d\varphi}\right)_0 = (a - 2b\varphi_0) \qquad (6.28)$$

linear model

$$k_g = \frac{\ln[1 + S m t_0 (k_0 - f)]}{S m t_0} + f \qquad (6.198)$$

← linear solvent strength model

quadratic model

$$k_g = \frac{\ln\left[1 - \left(\frac{d \ln k}{d\varphi}\right)_0 m t_0 (k_0 - f)\right]}{-\left(\frac{d \ln k}{d\varphi}\right)_0 m t_0} + f = \frac{\ln[1 + (a - 2b\varphi_0) m t_0 (k_0 - f)]}{(a - 2b\varphi_0) m t_0} + f \qquad (6.29)$$

Alternative approach: polarity model

$$\ln k = \ln k_w - S\varphi$$

linear model

$$\ln k = \ln k_0 - a\frac{2.068\varphi}{1+1.341\varphi} \qquad (6.30)$$

polarity model

$$S = -\frac{\ln k - \ln k_w}{\varphi}$$

$$S = -\left(\frac{d\ln k}{d\varphi}\right)_0 = \frac{2.068a}{(1+1.341\varphi_0)^2}$$

linear model

$$k_g = \frac{\ln[1+Sm t_0(k_0-f)]}{Sm t_0} + f \qquad (6.19)$$

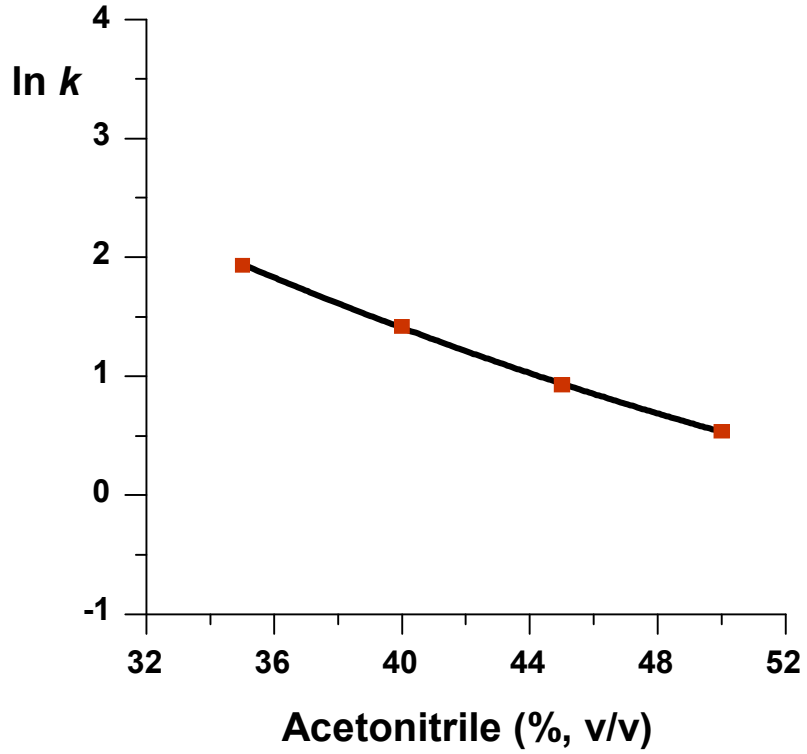
polarity model

$$k_g = \frac{\ln\left[1+\frac{2.068a}{(1+1.341\varphi_0)^2}mt_0(k_0-f)\right]}{\frac{2.068a}{(1+1.341\varphi_0)^2}mt_0} + f \qquad (6.31)$$

$$k_0 = e^{\ln k_w - a\frac{2.068\varphi_0}{1+1.341\varphi_0}} \qquad (6.32)$$

6.4. Representation of retention factors: isocratic elution

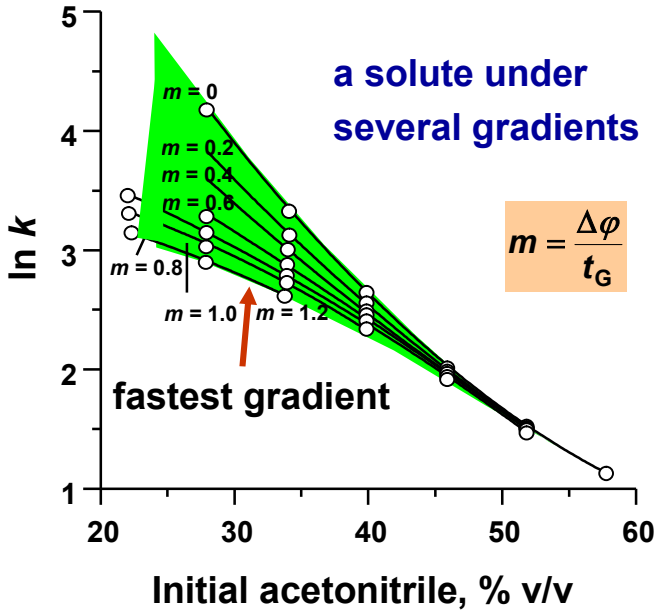
The plot of log *k* as a function of the organic solvent content is conventionally used to illustrate isocratic elution.

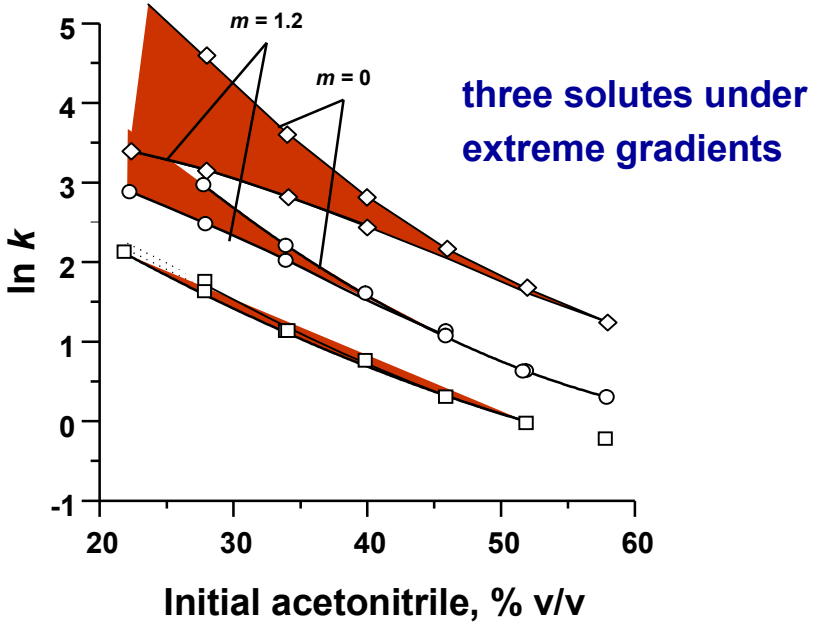


6.4. Representation of retention factors: triangular elution plots

The plot of log *k* for different gradients versus initial organic solvent content allows a convenient visualization of gradient elution.

- The diagram is similar to the conventional diagram in isocratic elution.
- Upper line: isocratic elution. Other lines: different gradient slopes (*m*).
- The lines delimit all possible retention factors and define a triangular region.
- The effect of the gradient is larger for the most retained solutes, and practically null for those weakly retained.

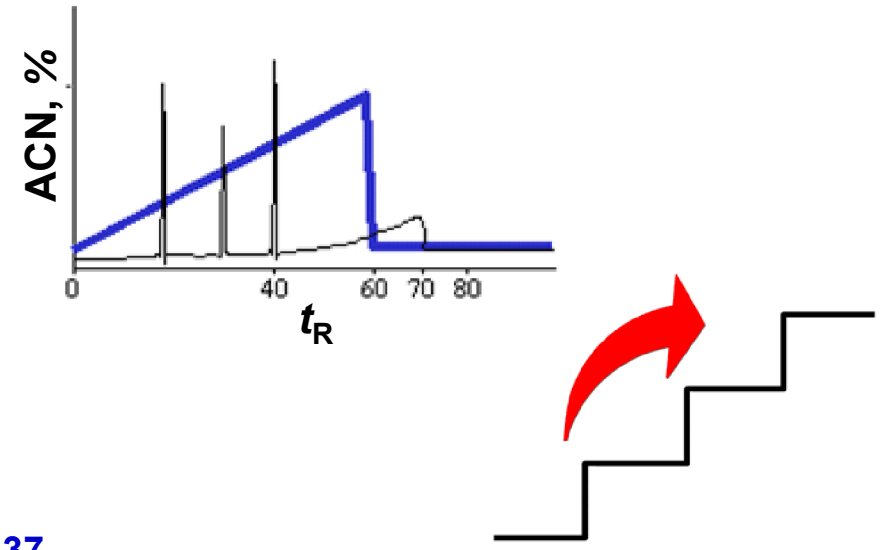


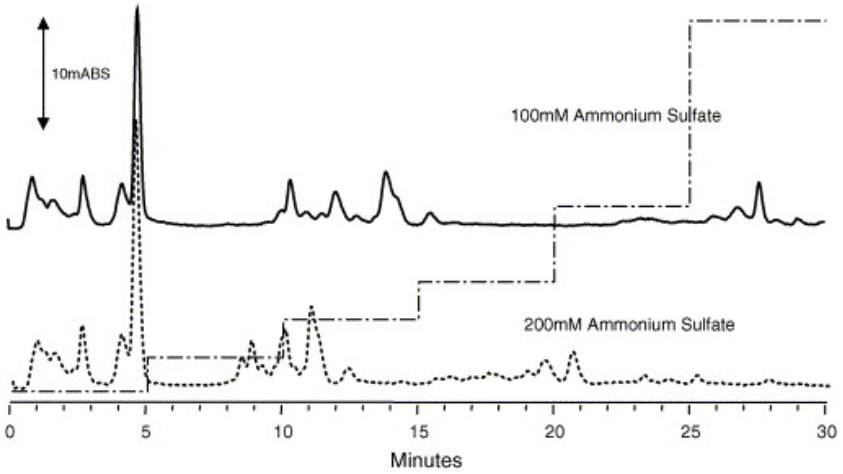


6.4. Multi-linear gradients

The number of gradient programs that can be implemented is virtually unlimited, and the success of the separation depends critically on the selected profile.

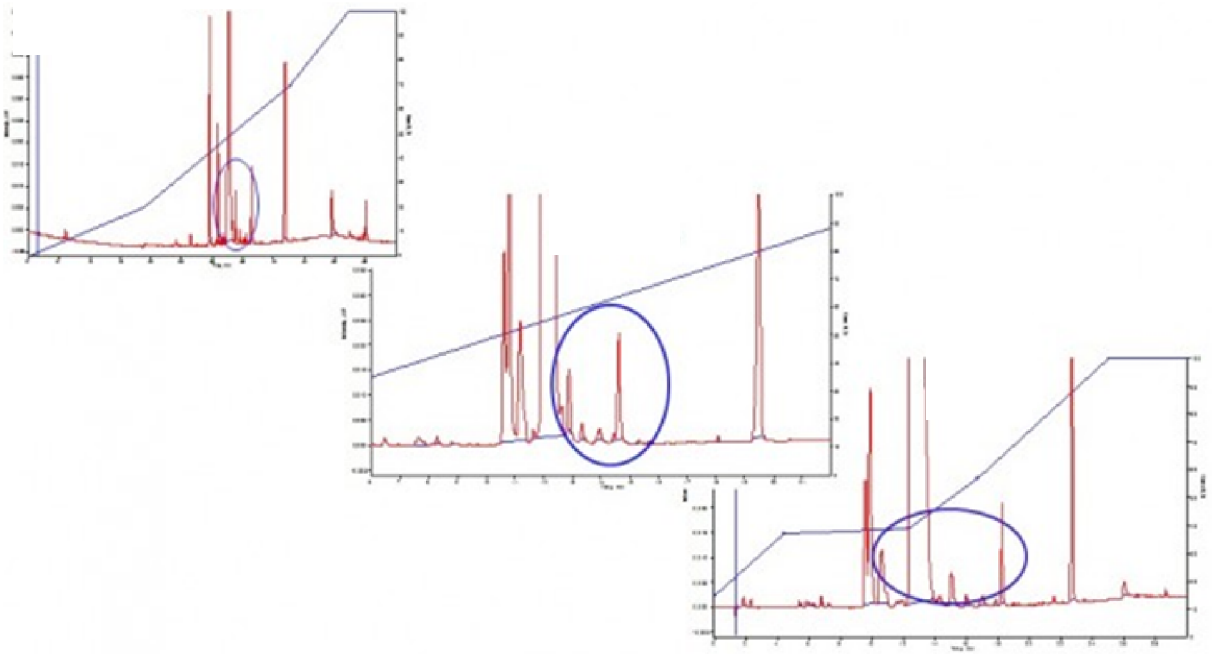
- **Gradients consist usually of simple linear increases of an experimental factor.**
- **Chromatographs generate gradients by performing consecutive small isocratic steps, usually at increasing modifier concentration.**
- **Gradients can also be approximated to a few large isocratic steps (stepwise or multi-isocratic gradients).**





Several segments of different slope, linear (multi-linear gradients) or curvilinear, can be also implemented.

- In multi-linear gradients, the modifier concentration is linearly increased, but the gradient has two or more segments of different slope.
- To speed up the elution, the gradient slopes should be always positive, but occasionally, one or more isocratic steps can be inserted, or the slope is decreased with respect to previous segments to get optimal separations.



Number of nodes

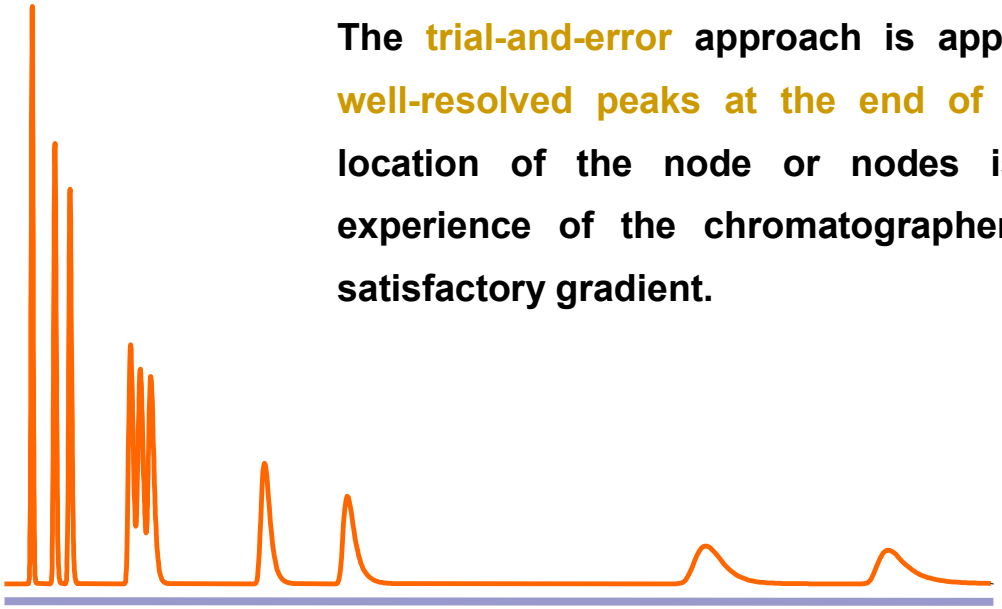
- The simplest multi-linear gradient approach consists of inserting, in a trial-and-error fashion, one or more nodes where the slope of the gradient is changed:

  one node will give rise to a two-segment gradient (bilinear gradient)

  two nodes will yield a three-segment gradient (trilinear gradient), etc.

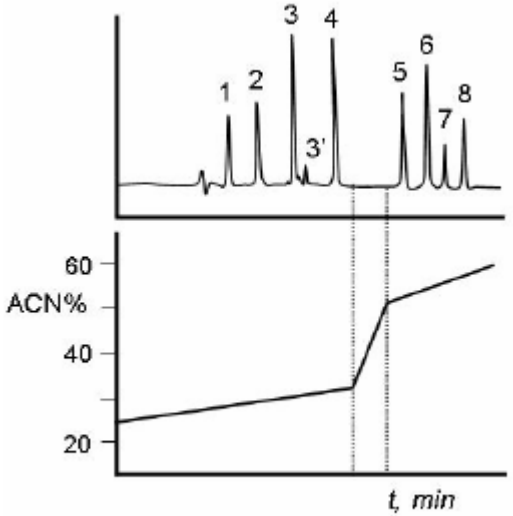
- The slope of the gradient can be particularised to each solute cluster in the eluted mixture.

Trial-and-error



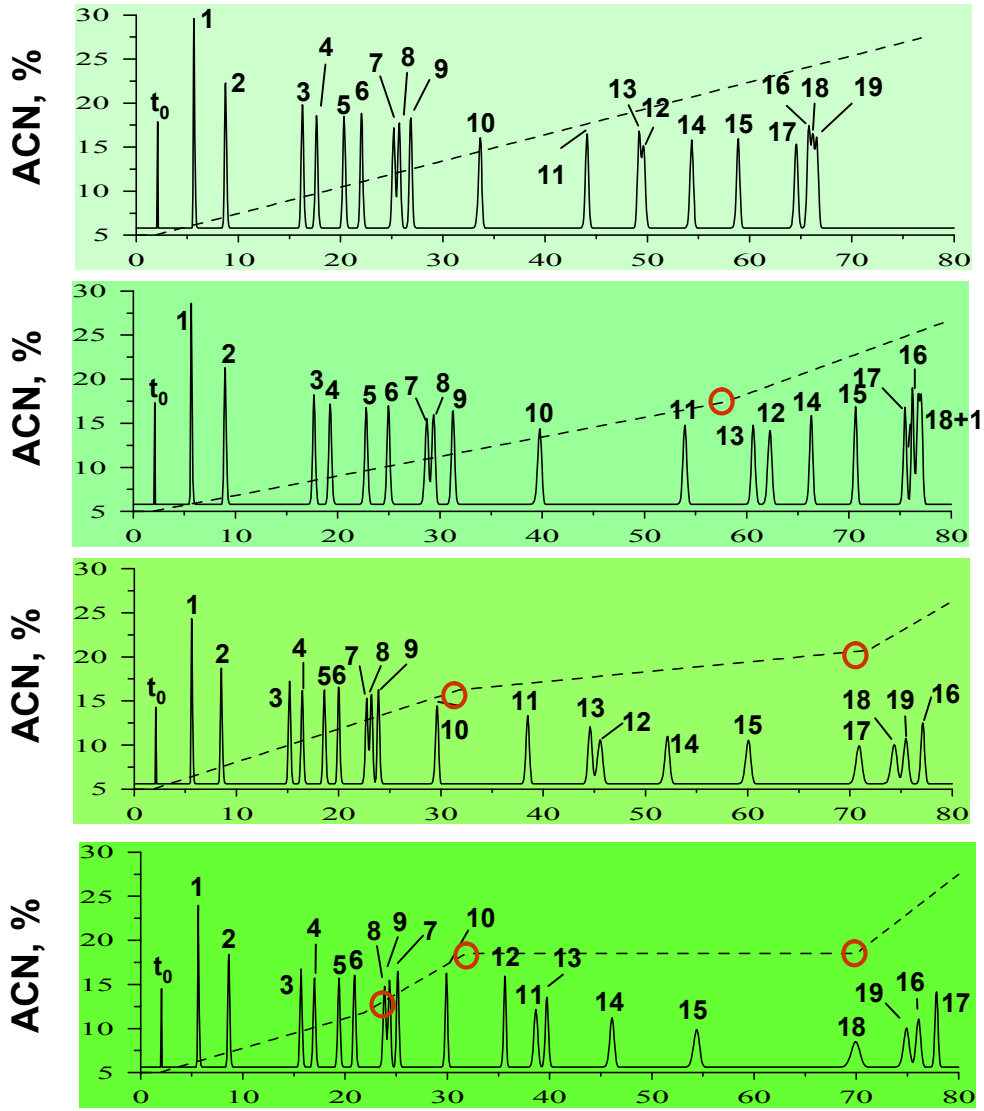
Retention time, min

The trial-and-error approach is appropriate for cases with well-resolved peaks at the end of the chromatogram: the location of the node or nodes is non-critical and the experience of the chromatographer is enough to find a satisfactory gradient.



Computer-assisted approach

- The selection of the location of the nodes and gradient slopes is optimal.
- To increase the possibilities of success, gradient interpretive optimisation can be adapted to the particularities of the sample by inserting a large number of nodes.
- There is no limit in the number of nodes considered in the optimisation, but when $n > 3$ the required computation time begins to constitute a limit that constraints the practical application of the algorithms.
- The architecture of the software can expedite the process.
- It is worth to check if the complexity of a gradient program deserves be increased to enhance resolution by inserting more linear segments, or on the contrary, no significant improvements are expected under more complex gradients.



***o*-Phthalaldehyde derivatives of proteic primary amino acids**