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# Theory of peak capacity in gradient elution

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This paper is dedicated to the memory of Csaba Horváth.

**Abstract**

Peak capacity is the best measure of the performance of a gradient separation. In this paper, the theory of peak capacity for the standard operating conditions of reversed-phase and ion-exchange chromatography is outlined. The influence of the operating conditions on the peak capacity of a separation are discussed. Finally, bandspreading phenomena in gradient chromatography are analyzed.


*Keywords:* Peak capacity; Gradient elution; Theory of chromatography

## 1. Introduction

The earliest publications on the subject of peak capacity in gradient elution chromatography was a short note by C.G. Horváth and S.R. Lipsky in Analytical Chemistry in 1967 [1], following the creation and definition of the term by Giddings for isocratic chromatography [2]. The paper concludes: "It is apparent from the present considerations that the goal of fast chromatography can best be approached in liquid chromatography by employing gradient elution . . .". The present importance of gradient elution can be seen in the multitude of two-dimensional LC/LC separations that have moved to the forefront of today's separations technology [3–6]. In some cases, a two-dimensional separations scheme has also combined an isocratic separation in the first dimension with a gradient separation in the second dimension [7]. Also, the impact of gradient elution in simpler separation schemes can be seen in the work of Shen et al. who have achieved peak capacities in the order of 1000 in a single separation dimension [8]. The general importance of gradient separations in modern HPLC has been demonstrated in an article on comprehensive two-dimensional HPLC by Bushey and Jorgenson [9].

The majority of the discussions in the current paper focuses on reversed-phase chromatography. The foundations of our understanding of reversed-phase chromatography have been laid in the late 1970s by Horváth and coworkers [10,11]. Our knowledge has further improved over time (e.g. [12]) and still today progress is being made in resolving specific issues [13–15]. A subject that was studied by several workers has been the selectivity of different packings [16–19]. In recent times, new attempts have been made to understand and measure the selectivity of reversed-phase columns [20–22]. For methods development, gradient elution is commonly used as the first step [23], but in modern times, a gradient technique is often the final goal of the method development effort [24].

The general theories of gradient chromatography, were outlined in papers by Snyder [25] and Jandera and Churácek [26]. Later, Snyder and coworkers dealt specifically with the subject of gradients in reversed-phase chromatography [27,28]. Very readable reviews of the same theories can be found also in the newer references [23,29]. With today's knowledge, the elution patterns in gradient chromatography are predictable based on few preliminary experiments that embrace the experimental space (e.g. [30–32]).

While there is now a solid knowledge of the peak spacing phenomena in gradient elution, a complete measure of the quality of a separation requires the use of the peak widths as well. In this paper, we are advocating the use of peak capacity as such a measure in the case of a gradient separation, and we will develop the underlying theory. A very good definition of peak capacity has been provided by Giddings in his early work: "The peak capacity is the upper limit of resolvable components for a given technique under prescribed conditions"

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[2,33]. Grushka [34] elaborated on the factors that influence peak capacity, such as the influence of the plate number, the linear velocity or temperature. He also briefly discussed peak capacity under time normalization, as was done later in more detail by Neue and Mazzeo [35]. Peak capacity is a good measure of the quality of a separation, if other measures are absent. Stadalius et al. [36] used it for the assessment of the quality of a peptide separation, as was done in a general model for the HPLC separation of large molecules by Snyder and Stadalius [37]. Similarly, the concept was used for the separation of proteins by ion-exchange [38]. The concept was also used for the separation of complex mixtures [30], where an assessment of the total separation capability of the chromatographic setup is important. Peak capacity has occasionally also been used to assess the separation power of isocratic separations [39,40]. A special case was the assessment of the peak capacity in size-exclusion chromatography [41].

A complication for the theoretical assessment of peak capacity in gradient elution is the peak compression phenomenon postulated by Snyder and Saunders [42]. The idea of peak compression has also been adopted by other authors [43–45], but a proof of the existence of this phenomenon does not exist. Snyder's own research gave results that contradicted the existence of a peak compression phenomenon. First, Stadalius et al. [36,46] postulated an empirical factor $J$ that was compensating for the difference between the experimental results and the theoretical predictions based on a peak compression factor $G$. Hearn's results [47] demonstrated that for the peptides studied the measured peak width was much larger than the value predicted using the peak compression factor $G$. Then, Stuart et al. [48] confirmed their own earlier results and concluded that the product of the empirical factor $J$ and the gradient compression factor $G$ was rather constant and equal to 1.1.

In this paper, I will first derive the equations that describe the peak capacity in gradient chromatography both for reversed-phase chromatography and ion-exchange chromatography. This is followed by a demonstration of how the peak capacity changes with a change in the gradient operation. In spite of the complexity of the underlying equation, a simple pattern describes the evolution of the peak capacity with gradient retention in reversed-phase chromatography. In ion-exchange chromatography, the dominating factor of the evolution of peak capacity is the ratio of the analyte charge to the charge of the eluting ion. Finally, I will briefly discuss the phenomenon of peak compression in reversed-phase chromatography from a new point of view.

## 2. Derivation of the theory and discussion of the results

### 2.1. Definition of the peak capacity

The peak capacity $P_c$ is defined as number of peaks that can be separated within a retention window. With other words, it is the retention time measured in peak width units. The peak width is commonly defined as four times the value of the standard deviation $\sigma$ of a peak. If the peak width is changing with the retention time $t_r$, the peak capacity needs to be expressed in the following integral form:

$$P_c = 1 + \int_{t_0}^{t_r} \frac{1}{4\sigma} dt \quad (1)$$

$t_0$ is the retention time of the unretained peak. In all the following discussion, we will assume that the gradient starts at the column inlet immediately after the injection.

The peak width of every peak is a function of the retention factor $k_e$ at the point of elution and the column plate count $N$:

$$\sigma = \frac{t_0}{\sqrt{N}}(k_e + 1) \quad (2)$$

With this, we obtain for the peak capacity in the gradient:

$$P_c = 1 + \frac{\sqrt{N}}{4} \int_{t_0}^{t_r} \frac{1}{t_0} \frac{1}{k_e + 1} dt \quad (3)$$

Of course, the implicit assumption in this formula is that the plate count is not a function of the gradient and is uniform for all analytes. In reality, this is not the case, since the plate count depends on the diffusion coefficient of the analyte, which in turn depends on its molecular weight and the viscosity of the solvent. In order to proceed beyond this, one needs to know more about the relationship of the diffusion coefficient with the elution pattern of the analytes. Such a pattern can be found for example in the case of a separation of oligomers [49], where diffusion decreases as retention increases. For the general case to be discussed here, we simply suppose a uniform sample, for which an average plate count can be assumed.

The retention factor at the point of elution (and thus the peak width) is a function of the gradient retention factor $k_g$, which is defined as usually:

$$k_g = \frac{t_r}{t_0} - 1 \quad (4)$$

This allows us to express the peak capacity as a function of the gradient retention factor:

$$P_c = 1 + \frac{\sqrt{N}}{4} \int_0^{k_g} \frac{1}{k_e(k_g) + 1} dk_g \quad (5)$$

In order to assess the peak capacity for different gradients, we need to express the retention factor at the point of elution as a function of the gradient retention factor for every gradient technique that we wish to consider. This can be accomplished for the gradient techniques for which solutions to the gradient equation are known.

### 2.2. Solution to the general gradient equation

In the following, we will derive the peak capacity equation in detail for reversed-phase chromatography and ion-exchange chromatography. In order to do this, we start with

the fundamental definition of the retention factor under gradient conditions:

$$k = \frac{dt_s}{dt_m} \tag{6}$$

Since the retention factor is variable, we have defined it in the differential form. $t_s$ is the residence time in the stationary phase, and $t_m$, the time spent in the mobile phase. This equation can be rewritten:

$$\frac{dt_s}{k(t_s)} = dt_m \tag{7}$$

and integrated:

$$\int_0^{t_r - t_0} \frac{dt_s}{k(t_s)} = \int_0^{t_0} dt_m \tag{8}$$

The right-hand side is nothing but the residence time of an unretained peak:

$$\int_0^{t_r - t_0} \frac{dt_s}{k(t_s)} = t_0 \tag{9}$$

A solution to this equation will yield the gradient retention factor for the gradient considered. In some cases, the change in retention with time can be expressed as follows:

$$k(t) = k_0 f(t) \tag{10}$$

In this case, the change in retention with time is exclusively a function of the dependence of the retention with the solvent composition combined with the change in solvent composition with time.

Substituting this expression into the gradient equation above, we obtain:

$$\int_0^{t_r'} \frac{1}{k_0} \frac{dt}{f(t)} = t_0 \tag{11}$$

or:

$$k_0 = \frac{\int_0^{t_r'} (f(t))^{-1} dt}{t_0} \tag{12}$$

With this, the retention factor at the point of elution is

$$k_e = \frac{f(t_r')}{t_0} \int_0^{k_g} \frac{dt}{f(t)} \tag{13}$$

And the peak capacity becomes:

$$P_c = 1 + \frac{\sqrt{N}}{4} \int_0^{k_g} \frac{1}{(f(t_r')/t_0) \int_0^{k_g} (f(t))^{-1} dt + 1} dk_g \tag{14}$$

This is the general equation for the peak capacity under gradient conditions. For some conditions, such as reversed-phase chromatography or ion-exchange under specific conditions, the equation can be integrated.

### 2.3. Peak capacity in reversed-phase chromatography

In reversed-phase chromatography, the retention factor is an exponential function of the solvent composition. If we change the solvent composition linearly with time, the local retention factor changes with time as follows:

$$k = k_0 \, e^{-B\Delta c \cdot t / t_g} \tag{15}$$

$\Delta c$ is the difference in solvent composition between the beginning and the end of the gradient. $B$ is the slope of the relationship between the natural logarithm of the retention factor and the organic solvent concentration. $B$ depends on the analyte, but is for the most part a function of the molecular weight of the analyte. $t_g$ is the gradient run time.

The gradient equation thus becomes:

$$\frac{1}{k_0} \int_0^{t_r - t_0} e^{B\Delta c \cdot t / t_g} dt = t_0 \tag{16}$$

Integration yields:

$$\frac{1}{k_0} \frac{t_g}{B\Delta c} (e^{B\Delta c (t_r - t_0)/t_g} - 1) = t_0 \tag{17}$$

which can be rearranged to yield:

$$k_g = \frac{t_r - t_0}{t_0} = \frac{1}{B\Delta c} \frac{t_g}{t_0} \ln \left( k_0 B \Delta c \frac{t_0}{t_g} + 1 \right) \tag{18}$$

If we define the gradient slope as follows:

$$G = B\Delta c \frac{t_0}{t_g} \tag{19}$$

the retention factor under gradient conditions is:

$$k_g = \frac{1}{G} \ln(G k_0 + 1) \tag{20}$$

The retention factor at the beginning of the gradient can be derived from the last equation:

$$k_0 = \frac{1}{G} (e^{G k_g} - 1) \tag{21}$$

The retention factor at the point of elution $k_e$ can be obtained as:

$$k_e = \frac{k_0}{G k_0 + 1} \tag{22}$$

If the retention factor at the beginning of the gradient is large, then Eq. (22) simplifies to:

$$k_e = \frac{1}{G} \tag{22a}$$

This implies that the solvent composition at the point of elution of a compound remains constant, if the compound is well retained at the beginning of the gradient and if the gradient slope does not change. In addition, for compounds that are well retained at the beginning of the gradient, the retention factor at the point of elution is uniform, if the slope $B$ does not vary much, as is often the case for analytes of similar molecular weight. This also means that the peak width is rather constant, in agreement with practical experience (see also the discussion of Eq. (29) below).

Combining Eqs. (21) and (22) allows us to express the retention factor at the point of elution as a function of the gradient retention factor:

$$k_e = \frac{e^{Gk_g} - 1}{G\, e^{Gk_g}} \tag{23}$$

We will put this expression for the retention factor at the point of elution into the equation for the peak capacity:

$$P_c = 1 + \frac{\sqrt{N}}{4} \int_0^{k_g} \frac{1}{(e^{Gk_g} - 1)/(G\, e^{Gk_g}) + 1} dk_g \tag{24}$$

With rearrangement, we obtain:

$$P_c = 1 + \frac{\sqrt{N}}{4} \int_0^{k_g} \frac{e^{Gk_g}}{(e^{Gk_g} - 1)/G + e^{Gk_g}} dk_g \tag{25}$$

Integration yields the following expression for the peak capacity under reversed-phase conditions:

$$P_c = 1 + \frac{\sqrt{N}}{4} \frac{1}{G+1} \ln\left(\frac{G+1}{G} e^{Gk_g} - \frac{1}{G}\right) \tag{26}$$

If we want to consider the entire peak capacity from the beginning to the end of the gradient, we need to express the gradient retention factor as a function of the solvent composition at the end of the gradient. With the definition of the gradient slope $G$ in Eq. (19), the factor $Gk_g$ required in the last equation is nothing but the following:

$$Gk_g = B\Delta c \tag{27}$$

Thus, we can study the influence of the gradient slope $G$ for a fixed elution window $\Delta c$ and a type or molecular weight range of compounds characterized by a typical slope $B$ of the relation of the logarithmic retention factor with solvent composition with the following equation:

$$P_c = 1 + \frac{\sqrt{N}}{4} \frac{1}{G+1} \ln\left(\frac{G+1}{G} e^{B\Delta c} - \frac{1}{G}\right) \tag{28}$$

A few comments might be necessary with respect to the magnitude of the common values of the gradient slope $G$ and the slope $B$ of the relationship of the logarithm of the retention factor and the solvent composition. For small molecules, the value of $B$ is about 10 (if the solvent concentration is expressed as the volume fraction). Typical values for $G$ for small molecules are 0.1–0.3. The largest value obtained with very steep gradients is around 3, the smallest value for very flat gradients is around 0.03. Typical values of $G$ for peptides are over two times larger. This stems from the fact that a typical value of $B$ is about 40 for a small peptide, but the gradient is typically executed over a narrower span of organic solvent.

The function in $G$ and $B\Delta c$ of Eq. (28) is shown in Fig. 1. To obtain the peak capacity from this function, the value on the vertical axis needs to be multiplied by one fourth of the square root of the plate count, e.g. 25 for a plate count of 10 000. The gradient steepness function $G$ covers the entire range of interest from very flat gradients ($G = 0.01$) to very steep gradients ($G = 8$). The axis that describes $B\Delta c$ ranges

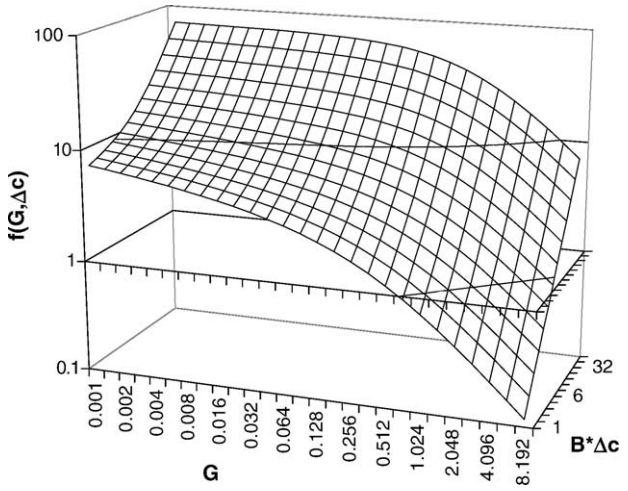


Fig. 1. Display of the function in $G$ in Eq. (28). The function is proportional to the peak capacity.

from 1 to 64. The smallest value exemplifies a gradient with small molecules over a range of 10% organic. The largest value is a representation of a gradient over the full solvent composition range with peptides as samples. All axes are logarithmic. For a steep gradient over a limited solvent range, the function is very low, under 1. For a flatter gradient with a gradient steepness of around 0.1, most of the gradient performance has been reached already, especially for gradients covering a larger range in solvent composition.

In Fig. 2, the increase in the peak capacity $P$ is shown with an increase in the gradient duration for a few selected gradients. The column plate count was assumed to be 10 000. The lines shown apply to (from bottom to top) to gradients covering a span of 20%, 30%, 40% and 100% organic. For the nearly parallel lines that cover a narrower gradient span, the peak capacity increases by only about 50% if the gradient duration is increased by a factor of 10. For the gradient covering the entire solvent range, the increase in peak capacity is initially about a factor of 2 with a 10-fold increase in the gradient duration, but then the gain decreases for still slower

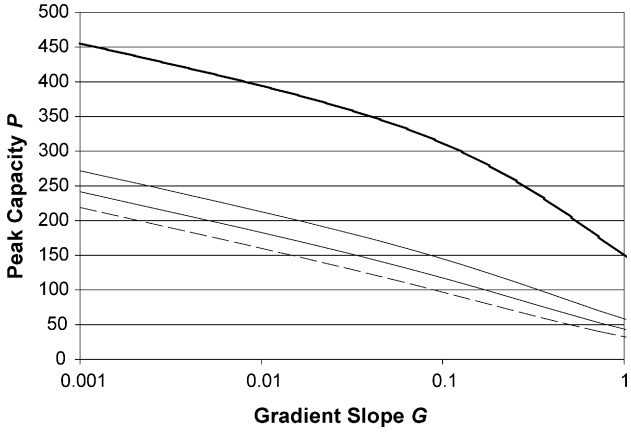


Fig. 2. Peak capacity as a function of the gradient steepness $G$: from bottom to top: 20%, 30% 40%, 100% organic.

gradients as well. This rather limited increase in separation power with a significant increase in analysis time constrains the manipulation of a separation via a simple expansion of the gradient run time.

Under some circumstances, e.g. for the separation of large molecules such as peptides or for rapid gradients that stretch over a large range of solvent composition such as the gradients used in combinatorial chemistry, Eq. (28) simplifies to:

$$P = 1 + \frac{\sqrt{N}}{4}\frac{B\Delta c}{G+1} \tag{29}$$

This form of the equation has been used in previous publications where such rapid gradients were discussed [35,50,51]. The simplified form of the equation results from the assumption that the peak width of all peaks in the chromatogram is essentially equal. This permits for example the assessment of the influence of extra-column effects on the peak capacity. However, for gradients over a limited range of solvent composition with small molecule analytes, the complete Eq. (28) should be used.

### 2.4. Peak capacity in ion-exchange chromatography

The theory of the gradient elution pattern in ion-exchange chromatography had already been elaborated in the late 1950s [52]. In pure ion exchange, the retention factor depends on the concentration of the salt solution $D$ and the ratio of the charge of the analyte to the charge of the competing ion in the mobile phase $n$.

$$k = \frac{K}{D^n} \tag{30}$$

The common way to execute an ion-exchange separation is to run a gradient with increasing salt solution (and constant pH). The concentration of the salt solution is programmed to increase with the gradient time $t$:

$$D(t) = D_f\frac{t}{t_g} \tag{31}$$

$D_f$ is the final salt concentration, and $t_g$ is the gradient run time. Thus, the retention factor changes as follows with time:

$$k\,(t) = \frac{K}{(D_f t/t_g)^n} \tag{32}$$

The equation for the retention time $t_r{}'$ under gradient conditions is:

$$\frac{1}{K}\int_0^{t_r'}\left(D_f\frac{t}{t_g}\right)^n \mathrm{d}t = t_0 \tag{33}$$

After integration, we obtain for $K$:

$$K = \frac{1}{n+1}\left(\frac{D_f}{t_g}\right)^n \frac{t_r{}'^{n+1}}{t_0} \tag{34}$$

The ratio $t_r{}'/t_0$ is the nothing but the gradient retention factor $k_g$. In the next step, we substitute Eq. (34) into Eq. (32), and

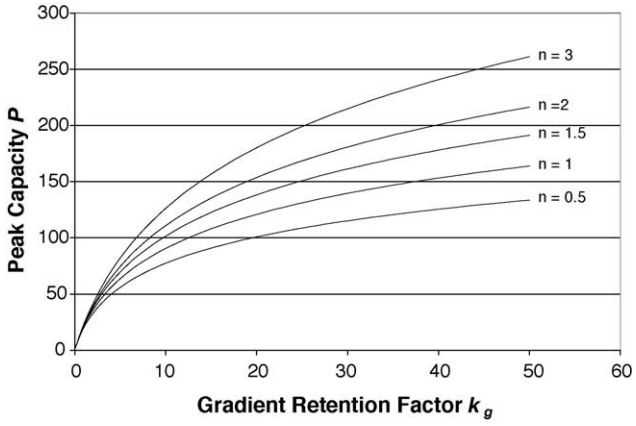


Fig. 3. Peak capacity $P$ as a function of the gradient retention factor $k_g$ for ion-exchange separations. The charge ratio for each line is marked on the graph.

the retention factor at the point of elution $k_e$ is:

$$k_e = k(t_r{}') = \frac{K}{(D_f t_r{}'/t_g)^n} = \frac{1}{n+1}\frac{t_r{}'}{t_0} = \frac{k_g}{n+1} \tag{35}$$

Now, in analogy to Eq. (3), the equation for the peak capacity is simply:

$$P_c = 1 + \frac{\sqrt{N}}{4}\int_0^{k_g}\frac{1}{k_g/(n+1)+1}\mathrm{d}k_g \tag{36}$$

The integration of Eq. (36) results in the explicit solution for the peak capacity in a salt gradient in ion-exchange chromatography:

$$P_c = 1 + \frac{\sqrt{N}}{4}(n+1)\ln\left(\frac{k_g}{n+1}+1\right) \tag{37}$$

An important aspect of this equation is the fact that the peak capacity depends on the charge of the ions to be separated, or—more specifically—on the ratio $n$ of the charge of the ions to be separated to the charge of the competing ion. The peak capacity is shown as a function of the gradient retention factor for values of $n$ from 0.5 to 3 in Fig. 3. For a particular gradient retention factor, the peak capacity increases with the value of $n$.

### 2.5. Peak width patterns as a function of the gradient

Even if the peak capacity in a particular gradient method cannot be calculated from scratch, it is still worth our while to see, how the retention factor at the point of elution changes with the gradient retention factor. The reason for this is the fact that the retention factor at the point of elution determines the peak width, as shown in Eq. (2). If a relationship can be found between the retention factor at the point of elution $k_e$ and the gradient retention factor $k_g$, the peak width can be calculated as a function of the gradient retention. For reversed-phase chromatography, such a dependence is found in Eq. (23), and in Eq. (35) for ion exchange.

For reversed-phase gradients, the following link between the peak width and the gradient retention factor can be established:

$$\sigma = \frac{t_0}{\sqrt{N}} \left( \frac{e^{Gk_g} - 1}{G\, e^{Gk_g}} + 1 \right) \quad (38)$$

For ion-exchange, we obtain:

$$\sigma = \frac{t_0}{\sqrt{N}} \left( \frac{k_g}{n+1} + 1 \right) \quad (39)$$

These two equations point to a fundamentally different relationship between reversed-phase gradients and ion-exchange gradients. In ion-exchange gradients, the peak width increases linearly with the gradient retention factor. This is not the case for reversed-phase chromatography, where the increase in peak width with gradient retention is much more moderate. This applies especially to the case of peptide separations, where the value of $Gk_g$ is much larger than 1, and the peak width becomes constant:

$$\sigma \approx \frac{t_0}{\sqrt{N}} \left( \frac{1}{G} + 1 \right) \quad (40)$$

This situation is commonly encountered in the case of peptide separations by reversed-phase chromatography. The cause of this is the fact that the dependence of retention on solvent composition is rather steep [53,54].

For the case of reversed-phase chromatography, the peak width pattern can be examined via the relationship in the parentheses of Eq. (38) (Fig. 4). The retention factor at the point of elution shown on the vertical axis is a measure of the peak width. The gradient retention factor forms the horizontal axis. For a "flat" gradient with $G = 0.1$ or less, as it can be executed with small molecules as samples, the peak width increases with increased retention. This is not dissimilar to isocratic chromatography, which is the limiting case for very "flat" gradients. On the other hand, for "steeper" gradients as they are common for peptide samples ($G = 1$), the retention factor at the point of elution and thus the peak width becomes quickly constant and independent of retention. Note also that the retention factor at the point of elution, and thus the peak width, is much narrower for the "steeper" gradient. As can be seen, the observed patterns of elution in reversed-phase chromatography depend strongly on the gradient steepness factor $G$.

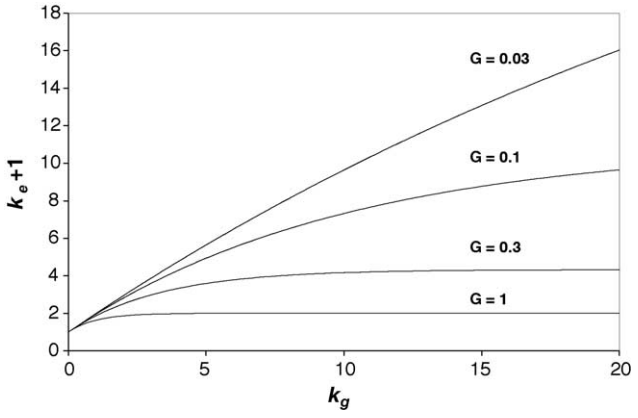


Fig. 4. The relationship between the parameter $k_e + 1$ and the gradient retention factor $k_g$ for various reversed-phase gradients with different gradient steepness functions $G$.

Eq. (39), which applies to ion-exchange chromatography, deserves an additional comment. The increase in peak width with retention decreases with an increased charge of the analyte. For highly charged analytes, the peak width will become rather constant and independent on retention. This is analogous to the situation in reversed-phase chromatography, where peptides exhibit a constant peak width throughout the gradient chromatogram. An example of such a situation is the anion-exchange chromatography of oligonucleotides [55].

### 2.6. Patterns of peak capacity with gradient execution

Up to now, the discussion has focussed exclusively on the influence of the gradient itself on the peak capacity, with a fixed column performance, implying a fixed linear velocity. However, a more interesting scenario can be found, if the velocity is change at a fixed gradient run time. Under these circumstances, the bandspreading phenomena inside the column as well as the execution of the gradient are changed simultaneously while maintaining a fixed solvent window for the elution of the analytes of interest. We have studied this situation in previous publications [35,50,51]. In general, higher resolution is achieved at higher flow rates, especially for rapid gradients from 0 to 100% organic, as they are common in the analysis of drugs in biological fluids with MS detection [56]. The same principle applies also to gradients that extend only over a narrower solvent composition range. An example is shown in Fig. 5. Plotted is the peak capacity as a function of the flow rate and the gradient duration for a gradient that stretches over a range of 30% organic. The column is a 50 mm × 2.1 mm column packed with 1.7 μm particles. For a very rapid gradient with a gradient duration of only 1 min, the best results are obtained at a flow rate around 1.2 mL/min. On the other hand, for a 60-min gradient the best conditions are around 0.25 mL/min. The more stretched-out gradient also achieves a higher peak capacity than the fast gradient, but if the analysis time is important, a well executed 1-min gradient can still achieve one third of the peak capacity of a 60-min gradient.

### 2.7. Bandspreading and peak compression

The bandspreading of a peak depends to some degree on the retention factor. At low velocities combined with a high retention factor, stationary phase diffusion may be the dominant factor of peak broadening. At intermediate to low velocities, the bandspreading is dominated by packed bed non-uniformity terms, which do not depend on the retention factor. At high velocities, the dependence of the mass

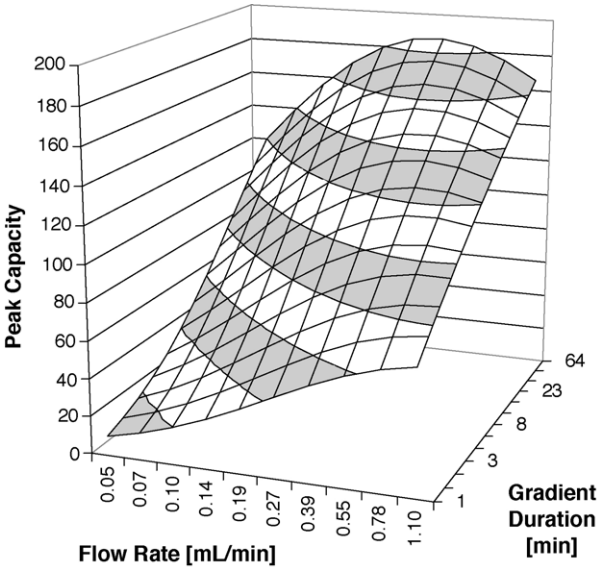


Fig. 5. Peak capacity as a function of flow rate and gradient duration for an analyte with a molecular weight of 250 on a 50 mm × 2.1 mm column packed with 1.7 μm particles in reversed-phase chromatography. Gradient span: 30%.

transfer on the retention factor is rather weak for retained peaks on fully porous packings [57].

Therefore, the height-equivalent to a theoretical plate $H$ is a function of the location $L$ inside the column [57]:

$$H = \frac{\mathrm{d}\sigma_L^2}{\mathrm{d}L} \tag{41}$$

and the total peak variance $\sigma_{L2}$ is, therefore:

$$\sigma_L^2 = \int_0^L H(L)\,\mathrm{d}L \tag{42}$$

The theoretical plate height $H$ depends on the diffusion coefficient $D_M$ and the retention factor $k$, and both in turn are a function of the solvent composition and, therefore, a function of the position $L$ of the band inside the column, which varies with the details of the gradient execution:

$$H(L) = f(D_M(L), k(L)) \tag{43}$$

While this complicates the theoretical assessment, there is no fundamental impediment to this measurement. A procedure for the measurement of the theoretical plate height in reversed-phase gradient chromatography has been proposed and demonstrated in [57].

We can use a modern version of the Van Deemter equation [58] to estimate the influence of the different contributions to the bandspreading in the column:

$$H = A + \frac{2}{u}(\gamma_M D_M(L) + \gamma_S D_S k(L)) + d_p^2 u \left( \frac{c_M}{D_M(L)} \frac{k^2(L)}{(k(L)+1)^2} + \frac{c_S}{D_S} \frac{k(L)}{(k(L)+1)^2} \right) \tag{44}$$

$A$ is a factor of the packed bed quality, the factors $\gamma_M$ and $\gamma_S$ are the obstruction factors in the mobile phase and the stationary phase, respectively, and $c_M$ and $c_S$ are the mass transfer coefficients for the mobile phase and the stationary phase. Both the diffusion term (the second term) and the mass transfer term (the third term) depend on the retention factor $k$ and the diffusion coefficient in the mobile phase $D_M$ and thus are variable during gradient elution. In Eqs. (43) and (44), this is expressed as the dependence on the position $L$ in the column. In addition, the stationary phase diffusion $D_S$ may also depend on the solvent composition, but the dependence may be much weaker, and we have, therefore, rather assumed an independence on the solvent composition.

The diffusion term shows an increase in stationary phase diffusion with increasing residence in the column [59–61]. For slow, high-resolution gradients on modern, very small particles, stationary phase diffusion may be a more important factor than for larger particle sizes, where the longitudinal diffusion plays an insignificant role. On the other hand, smaller particles permit shorter run times for equal performance, and under these circumstances the influence of stationary phase diffusion is still small. With respect to the effect of the variable retention on the mobile-phase and stationary-phase mass transfer terms, the combination of both exhibits a maximum at intermediate retention [55]. The consequence of this is that the overall effect of the restriction to mass transfer is only small for practical small-molecule separations.

This treatment demonstrates that the bandspreading in gradient chromatography is a function of the details of the elution conditions. On the other hand, in realistic gradients under realistic flow rates, the variation of the HETP during elution is expected to be rather small. In most practical circumstances, the effective migration of an analyte will occur over a very similar range of solvent composition, and therefore, the bandspreading will be similar. In addition, the treatment shown here does not imply any band compression at all. The discussion above showed that all phenomena that contribute to bandspreading are essentially the same for an isocratic operation or a gradient operation. During the migration of a band inside the column, a band compression is therefore not expected. The only contribution from the changing solvent composition is an averaging effect over the contributions that one would have encountered in an isocratic operation, i.e. the dependence of some of the parameters of the Van Deemter equation on the position of the band inside the column discussed above. Of course, in gradient elution, the retention factor changes between the part of the peak that elutes early and the part of the peak that elutes late. On the other hand, the mere transformation of the peak from a band inside the column to a peak dissolved in mobile phase at the column outlet does not include any peak sharpening mechanism, except for under extreme circumstances not commonly encountered in the practice of gradient chromatography, i.e. very steep gradients or even step gradients. Consequently, if we accept the model of a separation of the bandspreading phenomena inside the column from the elution phenomena, the idea of

band compression needs to be dismissed. This is in agreement with the measured data in reference [48]. We have recently also executed computer models on peak compression under the condition of step gradients, using a random walk model in a porous packed bed. For any peaks that eluted unretained after the gradient step caught up with them, no peak compression was observed. On the other hand, for peaks that simply changed retention factor, some peak compression was observed, when the gradient step caught up with the peak close to the end of the column. Of course, computer experiments may contain some flaws in the thought process, as do pure theories. Therefore, carefully designed experiments are currently planned that will shed light on this subject and give a final answer to the old question of peak compression.

### 2.8. Measuring peak capacity

Since peak capacity is such a good tool to determine the quality of a gradient separation, a few words need to be said about how to measure it in a practical chromatogram. It is assumed that peaks occur over most of the gradient chromatogram. Then the peak capacity can be calculated from the peak widths $w$ in the chromatogram as follows:

$$P = 1 + \frac{t_g}{(1/n)\sum_1^n w} \tag{45}$$

$n$ is the number of peaks selected for the calculation. As above, $t_g$ is the gradient run time. Thus peak capacity is simply the gradient run time divided by the average peak width. In some forms of chromatography, for example peptide separations by reversed-phase chromatography, the peak width is rather uniform throughout the gradient chromatogram, and one can select any group of peaks for this calculation. In other cases, such as very flat gradients in reversed-phase chromatography or in the simplest cases of ion-exchange gradients, the peak width increases with retention. Under these circumstances, the peaks selected for the calculation need to be representative of the distribution of the peak width throughout the chromatogram, i.e. in the simplest case, a peak at the beginning and a peak at the end of the chromatogram need to be selected. If many peaks in the chromatogram are used for the calculation, the selection should not overemphasize peaks with low retention or high retention. We need to keep in mind that the prescribed calculation is an averaging process over the entire time scale of the gradient chromatogram. If this mandate is followed, the agreement between measured values and theoretical predictions is excellent, even if only a few representative peaks in the chromatogram are used for the calculation.

## 3. Conclusion

In this paper, I have advocated the use of peak capacity as a measure of performance in gradient separations. The theory that permits the prediction of the peak capacity for reversed-phase gradients and for ion-exchange gradients has been presented. Also, the combination of velocity-dependent bandspreading with the gradient elution profile can be derived to permit an optimal peak capacity at a given analysis time. I have also discussed bandspreading in gradient chromatography. Based on the thoughts presented here, the existence of significant peak compression phenomena in reversed-phase gradient chromatography is questionable. However, in addition to the theoretical discussion in this paper, rigorous experiments are planned to either dismiss or confirm the concept of peak compression.

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