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Graphical Method for Choosing Optimized Conditions Given a Pump Pressure and a Particle Diameter in Liquid Chromatography

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Abstract

The general limitations on liquid chromatographic performance in isocratic and gradient elution are now well understood. Many workers have contributed to this understanding and to developing graphical methods, or plots, to illustrate the capabilities of chromatographic systems over a wide range of values of operational parameters. These have been invaluable in getting a picture, in broad strokes, about the value of changing an operational parameter or the value of one separations approach over another. Here we present a plotting approach more appropriate for determining how to use chromatography most efficiently in one's own laboratory. The axes are linear: column length vertical and mobile phase velocity horizontal. In this coordinate system, straight lines with intercept zero correspond to different values of t_0 . Hyperbolas correspond to values of pressure as the product of length and velocity is proportional to pressure. For a given relationship between theoretical plate height and velocity (e.g., van Deemter), the number of theoretical plates as a function of column length and mobile phase velocity is a surface (z direction) to the x and y of velocity and length. By representing the surface as contours, a two dimensional plot results. Any point along a constant pressure hyperbola represents the best one can do given the particle diameter, solute diffusion coefficient and temperature. The user can quickly see how to use the pressure for speed or for more theoretical plates. Sets of such plots allow for comparisons among particle diameters or temperatures. Analogous plots of peak capacity for gradient elution are equally revealing. The plots lead instantly to understanding liquid chromatographic optimization at a practical level. They neatly illustrate the value (or not) of changing pump pressure, particle diameter, or temperature, for fast or slow separations in either isocratic or gradient elution. They are illustrated with a focus on maximizing plate count with a given analysis time (isocratic), the effect of volume overload (isocratic), and separations of a limited number of peptides with a peak capacity coming from statistical peak overlap theory (gradient).

TOC image

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Associated Content

The Supporting Information is available free of charge on the ACS Publications website. Original versions of the historical plots used for chromatographic optimization; equations used for chromatographic optimization; surface plot analogs of contour plots; statistical peak overlap theory calculations.

The MATLAB programs for creating each type of plot are available at stephen-weber-chemistry.squarespace.com (tab: "USEFUL LINKS") or by emailing the corresponding author.

Introduction

It takes time to get information about a sample from liquid chromatography. How much depends on the nature of the question and the sample. It also depends upon several parameters related to the liquid chromatographic (LC) system itself. The complexity of chromatographic instruments and the significant number of parameters that control them has spawned many efforts to systematize procedures for optimizing the performance of packed-bed LC which are cited below. Graphical depictions of chromatographic performance as a function of parameters and variables can be useful because they contain a lot of information, however the sheer number of parameters in modern LC confounds efforts to create simple graphics in a two-dimensional format that are easily interpreted. In this work we briefly review previously developed optimization diagrams and present a new graphical approach to determine optimized LC parameters for practitioners wishing to maximize isocratic plate count (N) or peak capacity in gradient elution (n_c) in a given analysis time when using a particular particle diameter and pump pressure.

Most famously, van Deemter et al. developed a plot that continues to guide virtually all reasoning about chromatographic optimization¹. The van Deemter plot arose from their application of the mass balance approach^{2,3} to solving for a chromatographic band width as a function of mobile phase velocity. Based on earlier work by Klinkenberg and Sjenitzer⁴, van Deemter et al. put meat on the bones of the theoretical plate concept of Martin and Synge⁵. What leaps out of the van Deemter plot is the presence of a minimum in theoretical plate height, the change in zone variance (in length units) with a change in zone position as it travels down the column. But, until recently, working at a velocity corresponding to that minimum made for chromatographic run times that were uncomfortably long. Thus, "Ultimately all questions about optimization are related to a compromise between efficiency and time"⁶. Key parameters in realizing a particular chromatographic figure of merit are the available pressure and the particle diameter. The former limits the column length's magnitude, L , and the degree to which particle diameter, d_p , can be decreased. The latter, as well as partly governing the pressure required is also a significant parameter in the van

Deemter equation which bounds separating power. These limitations and opportunities have been described in a number of key papers, and interesting plots, in the decades of the '60s – '80s^{7–13} some of which we return to below.

Many graphical illustrations of chromatographic performance have been designed to explore the effect of key parameters like particle diameter, pressure, and temperature. The plots that result provide information at three limits: What is the effect of the chosen parameter on the maximum number of theoretical plates? On the highest speed? On the position of the minimum height equivalent to a theoretical plate, H ? Giddings's used a so-called kinetic plot to demonstrate the limiting performance of an LC system over long and short timescales in comparison to GC (Figure S1)⁸. The clear conclusion from the plot was that LC will provide more theoretical plates at long times, but GC will be capable of more theoretical plates with higher speed at short times. The long time limit of the plot is equivalent to about 30 years. While the plot clearly makes its point related to the comparison of GC and LC, it does not provide easily accessible information for practitioners about how to achieve the best performance at reasonable times. Similarly, Knox and Saleem⁹ compared LC and GC over a wide range of conditions specifying those required to operate at or near the minimum H while using some or all of the available pressure. Again data are plotted (Figure S2) over many orders of magnitude focusing attention at the limits of chromatographic performance. Flow rates near the maximum hypothetical value of v correspond approximately to filling an Olympic sized swimming pool every 30 minutes with mobile phase passing through a 2.1 mm ID column. Clearly, the point of these early plots is to illustrate broadly the best approach to attain a large number of theoretical plates, or achieve a certain effective number of theoretical plates in a given time.

Poppe reinvigorated the discussion of separation power in a paper comparing various formats for liquid-phase separations including pressure-based chromatography¹⁴. This work led to another graphical approach to chromatographic optimization that came to be known as the Poppe plot which expresses the plate time (t_0/N) as a function of N (Figure S3). More recently, the Desmet group demonstrated the information content in a family of kinetic plots^{15–24} (Figure S4) developed to expand on the separation impedance concept developed by Knox²⁵. These plots speak more directly to practical figures of merit over a very wide range of conditions for different chromatographic supports. The Carr group expanded on that of Poppe to generate a plot for gradient peak capacity production²⁶. The same group also clarified the algebraic relationships among the interacting parameters in liquid chromatography⁶. The latter work is quite helpful as it clearly distinguishes two important cases: global optimum (maximum plates per time given a certain pressure and mobile phase viscosity) which they called the three-parameter problem, and the two-parameter optimization when one additional parameter, usually d_p , is fixed. Although not as crisply articulated, it is noteworthy that Knox and Saleem described the same thing in 1969⁹. There is no question that plots have great value for understanding in broad strokes how to think about chromatographic optimization.

Both Guiochon¹³ and Halasz and Görlitz²⁷ created nomograms for estimating laboratory conditions (Figures S5 and S6). While they do cover a rather wide range of conditions they are also useful for determining actual experimental conditions. For example, in Figure S5,

we illustrate how to find conditions to do a one-minute separation ($k' = 3$, mobile phase viscosity = 4×10^{-4} Pa s) with 600 bar pressure: the particle size should be 1 μm . Similarly, the Halasz and Görlitz plot can be used to deduce similar information. For a $k' = 3$ and a one-minute separation, t_0 is 15 s. The intersection of that value and $P = 600$ bar is shown approximately as a dot on Figure S6. This guides us to a particle size of about 1.5 μm . Chen and Horvath arrived at similar conclusions for separations of small and large molecules using reasoning analogous to that of Guiochon²⁸. Later Meyer extended the Halasz and Görlitz nomogram concept to maximize isocratic and gradient peak capacity^{29,30}.

In the process of working on fast neurotransmitter separations, we have realized a need for a plot that illustrates these same concepts, but that also fulfills three other criteria: (1) it is accessible to a non-expert, (2) it is focused on practical parameter ranges over a few powers of 2, not several orders of magnitude, reflecting reality in the lab, and (3) provides experimental parameters of column length and velocity immediately. Here, we explain the plot and demonstrate its value in determining conditions to achieve certain objectives from applications taken from our laboratory under isocratic and gradient elution conditions.

Theory

Information needed to construct the plot

Estimates, of the plate height/velocity and pressure/velocity relationships are required to construct optimization graphics. Thus, step one involves selection of the desired solute, mobile phase composition and column temperature. The latter two parameters play a critical role in determining chromatographic performance due to their influence on eluent viscosity and analyte diffusivity. Step two comprises a flow study to measure the kinetic and permeability properties of the column. We used A , B , and C values in the reduced van Deemter equation shown in Eq. 1 from Zhang et al.³¹ for columns packed with 1.7 μm fully porous particles. Conditions used in the calculation were 25 °C, with a 96:4 water/acetonitrile mobile phase. (Other chromatographic conditions are provided below.)

$$h = \frac{H}{d_p} = A + \frac{BD_m}{u_e d_p} + \frac{Cu_e d_p}{D_m} \quad \text{Eq. 1}$$

where h is the reduced plate height, H is the dimensioned plate height, d_p is the particle size, D_m is the solute diffusion coefficient in the mobile phase, u_e is the interstitial linear velocity and A , B , and C are the reduced dimensionless van Deemter coefficients.

It requires pressure to force eluent through a chromatographic column. Eq. 2 shows a semiempirical equation derived from Darcy's law which relates column parameters to pump pressure, P , under conditions typically experienced when fluids are passed through packed beds.

$$P = \Phi \eta \frac{u_e L}{d_p^2} \quad \text{Eq. 2}$$

The plots require the dimensionless flow resistance parameter, Φ , from Eq. 2 which can be determined from the slope of pressure vs velocity data. Here, we have used Φ and column porosity values, e_c and e_{tot} , reported by Zhang et al.³¹ in our calculations.

Numerical values used in subsequent optimization calculations are provided in Table 1.

Results and discussion

Introduction to the plot

Figure 1 shows an example of a plot generated from the values in Table 1 and equations 1, 2, and the well-known relationships among average velocity, interstitial velocity and porosity, and among average velocity, length, and time. The vertical axis represents column length (cm); the horizontal axis interstitial velocity, u_e , (cm/s). Dashed lines indicate constant values for t_0 , from 1 to 80 s with particular values indicated adjacent to each line. Colored contour lines denote constant values of N . Contours begin with $N = 1000$ and increase in 1000 plate divisions until $N = 10000$, where spacing increases to 5000 plates per division up to a maximum of 30000 plates. At any point on the plot, a value of u_e can be chosen which (with data from Table 1) provides a plate height. Then a value of L can be chosen which provides a value of N . The values of u_e and L immediately dictate t_0 . For example, near the top of the plot, we see that 20000 theoretical plates can be generated (purple line) with a t_0 of 20 s by using approximately a 15 cm long column and a u_e of 1.0 cm/s. Of course, not all lengths and velocities are accessible because the available pumping pressure is a limitation. Black hyperbolas are constant pressure isobars for 600 and 1000 bar. Conditions below and to the left of these hyperbolas are achievable combinations of L and u_e for systems with 600 and 1000 bar limits. Thus, we see that the 20000 theoretical plate/20 s t_0 point is not accessible even with a 1000 bar system (at 25 °C). Optimum conditions occur along an isobar. While one can operate below the isobar, performance is better when the operating point is on the isobar. Values of L , u_e , t_0 , and N along the isobar are referred to by Carr et al.⁶ with asterisks, e.g., L^* etc. To summarize, for a given d_p and T (and other particular attributes of the system as in Table 1), each point on this plot represents values of L , u_e , t_0 , and N . One cannot operate to the right of an isobar. Operating to the left is permissible. Operating on the isobar is best. Points along any isobar correspond to the results of Carr et al.'s "two-parameter" optimization⁶ for a given P_{max} .

Figure 1 presents a significant amount of useful information regarding optimization concepts and chromatographic performance. First, ignore for the time being the need for a particular pressure and focus on the contour lines for N . The constant plate contour lines reflect the shape of the van Deemter curve. This can be understood by following a horizontal, constant L , trajectory across the plot. Consider $L = 9$ cm. For the lowest interstitial velocity, N is low. As velocity increases, N increases over 10000 then reaches a maximum of about 20000 where the contour line reaches a minimum near $u_e = 0.33$ cm/s and t_0 about 40 s. The

minimum in the contour line for N represents the shortest column at which that number of theoretical plates can be generated. As velocity is increased further, N decreases. This is consistent with the fact that $N = L/H$ (Eq. 1 and L is constant). As a vertical trajectory is taken at constant velocity, N increases steadily, again in agreement with $N = L/H$ (and H is constant). The B-term region of the van Deemter curve corresponds to the left side of Figure 1 where linear velocity is below the optimum of 0.33 cm/s. This is the long L , long t_0 section of the plot. The C-term dominated portion of Figure 1 is in the lower right corner and corresponds to short columns and analysis times.

Now, let us consider the case where we will use the maximum pressure of 600 bar. Under what conditions can we obtain 20000 theoretical plates as we did for $L = 9$ cm, $u_e = 0.33$ cm/s, $t_0 \sim 40$ s at the van Deemter optimum velocity? The 20000 theoretical plate contour crosses the 600 isobar at the point designated by the letter *A*. Operating at 600 bar maintains the plate count but decreases t_0 to about 35 s.

There is one place where the isobar crosses a constant N curve at its minimum indicated by the letter *B*: It is at the van Deemter optimum velocity with column length such that system pressure equals $P_{\max}$. The conditions for point *B* are: $P_{\max} = 600$ bar, $u_e = 0.33$ cm/s, $L = 13.4$ cm, $t_0 = 63.7$ s and $N = 30000$. Conditions represent the maximum N for $t_0 = 63.7$ s for the 600 bar system with 1.7 μm particles at 25 °C. This is the *only point* on the isobar that is at the van Deemter optimum velocity. This point is frequently referred to as the Knox-Saleem limit^{6,36,37}.

Supposing we desire a t_0 of 20 s with a 600 bar system pressure limit? From the plot, we can expect that 15000 theoretical plates are generated using a 7.5 cm long column at a linear velocity of 0.57 cm/s. The plot is also useful for a rapid assessment of the consequences of using a particular column length, e.g., 5, 10, or 15 cm.

Further, the plot helps to see whether increasing pressure can aid the chromatographer who is interested in speed, for example, $t_0 < 5$ s. A system with a 600 bar limit generates 2000 plates for a $t_0 = 1.8$ s (Figure 1 points *C*). Increasing $P_{\max}$ to 1000 bar has a negligible impact on speed for the 2000 plate separation, decreasing t_0 only 0.1 s. Clearly increasing system pressure alone is not a viable solution to increase speed under these conditions. Graphically, this information is evident as follows: constant t_0 lines are practically parallel to the constant plate contours. Traveling along a constant t_0 line is achieved by changing pressure while changing L and u_e proportionally. On the other hand, if system pressure is increased with a commensurate decrease in particle size both plate count and separation speed are improved⁶. It is fair to say that, while this plot tells the reader that increasing pressure alone when working on high-speed separations will have minimal effect on separation quality, it does not explain why. Probably the algebraic representation as expressed in Carr et al.⁶ (particularly the discussion centered around eqs. 14 – 17) is the best way to understand why this is the case.

Application of the plot to several problems

With this orientation to the plot, we now demonstrate the efficacy of Figure 1 based on our laboratory's applications. We have an interest in neurochemistry, specifically the study of

monoamine neurotransmitters serotonin (5-HT) and dopamine (DA) *in vivo* by microdialysis coupled online to LC. Changes in 5-HT and DA concentration are measured in response to various chemical and physical stimuli *in vivo* using online LC. The speed of the separation currently limits the time resolution. Better time resolution means we can extract more information.

In our first demonstration of the graphic in Figure 1 we require the quantitative determination of 5-HT by LC coupled online to microdialysis with high time resolution. Thus, high speed conditions are required, while the separation must also generate a predetermined number of plates to ensure accurate quantitation. This is a classic two-parameter optimization problem. Temperature, particle diameter, and pressure are the major elements of chromatographic optimization. Thus, we use the plot to assess the role of elevated column temperature and different particle sizes to improve the speed of our neurotransmitter analyses. Our application yields small sample volumes (10 – 500 nL) with low concentrations, thus quantitation is a challenge. Selection of the best column diameter represents a compromise between sample dilution (diameter too large) and volume overload (diameter too small). In a second demonstration we have incorporated the influence of volume overload into the neurotransmitter optimization procedure. Finally, we expand the scope of the plot from purely isocratic elution using the theoretical plate concept to gradient elution to increase the peak capacity production for the analysis of neuropeptide mixtures.

Maximizing isocratic plate count for fixed analysis time—For accurate quantitation neurotransmitter separations require a few thousand theoretical plates – here we specify 5000 theoretical plates. We focus on 5-HT, results are similar for DA. Chromatography uses “ion-pairing” reversed phase LC, with a small portion of acetonitrile in the mobile phase, 96:4 aqueous/acetonitrile, to achieve $k' \sim 10^{33}$. In Figure 2A, the red point is at 5000 theoretical plates and 600 bar. It has been calculated for 5-HT using 1.7 μm fully porous particles and a column temperature of 25 °C (see Table 1 for conditions). Under the 5000 plate requirement we see from the light yellow contour, 5000 plates are generated with a t_0 of about 5 s for the 600 bar limit system. 5-HT retention time is 55 s and time resolution is one minute. These results are similar to our previous *in vivo* work with 1-minute temporal resolution separations of 5-HT and DA^{38,39}. Now the question becomes how can we adjust chromatographic parameters to achieve the necessary 5000 plates in less time to improve temporal resolution. Briefly, we again consider what benefit there is to increasing pressure alone as shown by the blue arrows. Blue arrows illustrate the effects. The column's length could be increased to provide more plates, but the chromatographic time increases. The velocity could be increased, decreasing t_0 significantly, but with a concomitant decrease in plates. One could adjust length and velocity to keep N constant, but only a very small increase in speed would be gained. While it cannot be stated that increasing pressure does not help for a $t_0 = 5$ s separation, it helps very little to increase speed without sacrificing efficiency. Fortunately, there is a simple solution to help increase speed.

When column temperature is increased the mobile phase viscosity decreases significantly, e.g., $\eta_{75\text{ }^\circ\text{C}} = 0.4\text{ cP}$, $\eta_{25\text{ }^\circ\text{C}} = 0.9\text{ cP}$. This viscosity decrease has the same mechanical effect as increasing the system pressure limit: for the same packed bed, the mobile phase velocity and thus separation time (all else constant) are improved resulting in an increase in speed. At

the same time, there is an increase in 5-HT diffusion coefficient, $D_{m,75\text{ }^{\circ}\text{C}} = 1.4 \times 10^{-5} \text{ cm}^2/\text{s}$, $D_{m,25\text{ }^{\circ}\text{C}} = 0.54 \times 10^{-5} \text{ cm}^2/\text{s}$, which is an increase of a factor of 2.6. In high-speed separations where the van Deemter C-term is important we note that the C-term is proportional to the parameter d_p^2/D_m . Increasing temperature has an effect that is similar to decreasing particle diameter.

Let us return to the 5-HT problem. Increasing the column temperature to 75 °C while maintaining $N \approx 5000$ should increase speed. As Figure 2A and B show, this is indeed the case. Now, for $P_{\text{max}} = 600 \text{ bar}$, $N = 5000$ is achieved with $t_0 = 1.9 \text{ s}$, $L = 3.5 \text{ cm}$, $u_e = 2.81 \text{ cm/s}$ (red point, Figure 2B). This corresponds to a retention time of 21 s for 5-HT and an analysis time of 25 s. Increasing column temperature would permit 30 s time resolution in the microdialysis experiment. It is interesting to compare the effect of decreasing particle diameter, Figure 2C shows the same plot as Figure 2A with $d_p = 1.0 \text{ }\mu\text{m}$, $T = 25\text{ }^{\circ}\text{C}$. The line for 5000 plates and 600 bar cross at about $L = 1.5 \text{ cm}$ and $u_e = 1.0 \text{ cm/s}$ with $t_0 = 2.3 \text{ s}$. The decrease in particle size has a similar effect on the separation quality as raising the temperature, although the speed is 20% poorer. Increasing the available pressure to 1000 bar (red dot, Figure 2C) provides virtually equivalent performance to the 75 °C, 600 bar, 1.7 μm particle, case (Figure 2B). Finally, we can see from the red arrow in Figure 2D that increasing the temperature from the conditions in Figure 2C more than halves t_0 to an impressive 0.8 s with $L = 1.7 \text{ cm}$, $u_e = 3.4 \text{ cm/s}$ and $N = 5000$ (location of point not shown).

Finally, and briefly, we have been discussing the creation of non-standard length columns. This is not a problem for capillary columns packed in-house, but these plots readily provide useful information about the performance of columns of fixed length at various conditions.

Volume overload and its impact on optimization—Basal 5-HT concentration *in vivo* in the rat striatum is about 1 nM or less, sample volume is 500–1000 nL, consequently accurate quantitation for online neurotransmitter monitoring requires detection limits sufficient to determine quantitatively $\sim 1 \text{ fmol}$ or less. It is important to minimize the volume of the chromatographic zone that contains that femtomole to achieve the highest possible signal from a concentration sensitive detector. Thus, to avoid significant dilution, capillary columns are used. Column volume is typically 200–500 nL. Under these conditions injection volume often is equivalent to, or may exceed the column fluid or dead volume. Large volume injections can result in a phenomenon known as volume overload. Volume overload occurs when the injection volume contributes significantly to the observed band spreading, decreasing the apparent efficiency of the separation. It is worth noting that this phenomenon was explored by van Deemter et al. in their seminal 1956 paper¹.

The combination of on-column- and volume overload-induced band spreading leads to an observed column efficiency, N_a , shown in Eq. 3:

$$N_a = \frac{L}{H_a} = \frac{L}{H + \left(\sigma_{inj}^2 / L \right)} \quad \text{Eq. 3}$$

where L is column length, H_a is the apparent plate height, H is the non-overload plate height (calculated as above), and σ_{inj}^2 is the injection variance. The injection variance is a function of the injection volume, column volume and solute retention factor in the sample solvent⁴⁰. Figure 3 shows the plot with contours of N_a , rather than the column's intrinsic plate count, N . The apparent plate count has been calculated for fixed 500 nL injections into 100 (A), 150 (B) and 250 (C) μm ID columns while the remaining parameters are as in Figure 2B. Holding the injection volume constant reflects the situation in online analysis (e.g., microdialysis) in which the fixed time resolution corresponds to a particular, fixed volume of sample from a constant flow rate stream. The solute retention factor in the sample solvent was 10. What is most obvious from Figure 3A is the significant loss in observed column efficiency due to the 500 nL injection. Under the same conditions optimized for speed in Figure 2B, indicated by the red dot, $N = 5000$, $t_0 = 1.9$ s, $L = 3.5$ cm, $u_e = 2.81$ cm/s, plate count has decreased from 5000 to 1430.

It is also noteworthy to see the reflection of Eq. 3 in Figure 3A. The injection variance term is not velocity dependent. As a result, for short columns where the injection variance term is largest for a fixed injection volume and column diameter the N_a contours are flat at sufficiently high velocities.

Apparent column efficiency when injecting 500 nL onto the 100 μm ID column does not yield the required 5000 plates for accurate quantitation. There are several solutions available to regain column efficiency: decrease injection volume, increase column length, increase column diameter, improve focusing. Reducing injection volume increases efficiency by reducing volume overload effects, but a smaller volume injection decreases detector signal as the 5-HT average concentration in the eluting peak will have decreased. This is not a desirable solution. Increasing column length adds plates, improving the separation, but also adds time to the separation. The purple dot in Figure 3A shows this, for $N_a = 5000$, $L = 6.9$ cm and $t_0 = 7.2$ s. Improving sample focusing (increase k' in the sample solvent) is the best solution to maintain efficiency with large injection volumes, but is not always simple to implement. Thus we focus on the role of column diameter. Previously we have shown this parameter to be critical to the optimization for speed and sensitivity³³. Figure 3B and 3C highlight this importance. As in Figure 3A purple dots in 3B and 3C show the conditions required to achieve $N_a = 5000$ in the shortest time (operating at $P_{\text{max}} = 600$ bar). Open dashed circles in each panel represent the conditions for the target 1.9 s t_0 and 30 s temporal resolution microdialysis experiments. For the 150 μm ID column in Figure 3B 5000 plates are generated using $L = 4.4$ cm, $t_0 = 3.0$ s and $u_e = 2.24$ s. Increasing diameter to 250 μm results in conditions in which $L = 3.6$ cm, $t_0 = 2.0$ s and $u_e = 2.75$ cm/s, virtually identical to the parameters found without volume overload.

Peak capacity optimization in gradient elution separations—Thus far we have optimized performance of isocratic separations using a series of graphics based on Figure 1. Under gradient conditions, peak capacity is the most relevant metric to assess the performance of a separation⁴¹. In this section we have reformatted Figure 1 to optimize separations of neuropeptides by maximizing peak capacity at a given gradient time. Neuropeptides are an important class of signaling molecules in the brain, regulating

processes involved with pain and learning⁴². We have a longstanding interest in the fate of neuropeptides in the brain, specifically their hydrolysis by ectopeptidases^{43,44}. To understand the kinetics of neuropeptide hydrolysis, samples must be analyzed using solvent gradient elution to efficiently separate moderately hydrophobic peptides from their often very hydrophilic hydrolysis products. We illustrate this with an example, the quantitative analysis of leucine enkephalin (YGGFL) and its primary hydrolysis product in tissue, GGFL. Inclusion of sampling and LC internal standards yields a relatively simple mixture composed of four solutes accessible with isocratic elution⁴⁴. However, much more biochemical information could be obtained by simultaneously determining other products such as YG, YGG, GFL, and FL. Gradient elution would be the best approach. Furthermore, other neuropeptides and their products span an even greater range of hydrophobicity which requires a gradient method for analysis.

Using an approach similar to that for creating Figure 1 we calculated values for peak capacity, n_c , using Neue's formulation⁴¹:

$$n_c = 1 + \frac{1}{4} N(t_0, L, u_e)^{1/2} \frac{1}{G+1} \ln \left[\frac{G+1}{G} e^{S\Delta\phi} - \frac{1}{G} \right] \quad \text{Eq. 4}$$

where N is the column efficiency, a function of t_0 , L , and u_e , S is the slope of the log of solute retention factor vs eluent composition from Snyder's linear solvent strength theory⁴⁰, ϕ is the range in eluent composition and $G = S \cdot \phi t_0 / t_G$, t_G is the gradient time.

Figures 4A and 4B show peak capacity optimization plots similar to Figure 1 for the analysis of leucine enkephalin hydrolysis mixtures using short linear gradients. Axes remain L and u_e , dashed lines indicate constant t_0 , solid black lines show 600 and 1000 bar system limits. Colored contour lines in Figure 4 represent constant values of n_c calculated from Eq. 4. Contour spacing in Figures 4A and 4B are 12.5 peak capacity units. Conditions were: $S = 20$ (lower limit for small peptides)⁴⁵, $\phi = 0.5$, $t_G = 1$ and 3 minutes, $d_p = 1.7 \mu\text{m}$, $T = 25^\circ\text{C}$. For simplicity van Deemter coefficients matched those in Table 1 and plate height dependence on mobile phase composition was neglected. Column efficiency and pressure were calculated with mobile phase composition set to 0.2 acetonitrile/water. The diffusion coefficient for leucine enkephalin in water is $D_{m,25^\circ\text{C}} = 4.1 \times 10^{-6} \text{ cm}^2/\text{s}$ ³². The diffusion coefficient for leucine enkephalin under specific conditions of mobile phase composition and temperature was calculated using Eqs. SI1 and SI2.

What is obvious from Figures 4A and 4B is the difference in shape for the constant peak capacity contours for each gradient time and presence of a clear n_c maximum which is seemingly independent of column dimensions and system pressure. Unlike the isocratic efficiency plots (Figure 2) where characteristic van Deemter-curve shapes are visible under all conditions the peak capacity analogs provide a less consistent general shape, but one which is highly dependent on gradient time. At short gradient times it is particularly obvious that the contours have a shape that is different from the contours of N in the isocratic case. In both the isocratic and gradient plots, for a given length, there is an optimum velocity

(reminder: irrespective of pressure limitations). However, only in the gradient plots is there also an optimum length for a given velocity.

Now we return to the enkephalin separation. In Figure 4A, with 1000 bar pressure, the maximum value of n_c is 81 (purple dot). However, the contour becomes quite flat in this range of lengths and velocities for short t_G (See Figure SI8). Thus, lengths from about 3 – 6 cm with appropriate adjustments in velocity yield n_c greater than 75 for the one-minute gradient. Operating at a pressure of 600 bar, where the maximum value of n_c is 80 (red dot), involves no practical sacrifice. As with the isocratic separations, in this regime of speed there is little to be gained from increasing pressure alone.

If we relax the time requirement, Figure 4B shows similar general properties at $t_G = 3$ min but with increased maximum peak capacity: 133 at 1000 bar and 128 at 600 bar. When considering the two upper graphs with reasonably brisk separations, we note that the optimum t_0 hardly changes with changes in pressure. Further, the optimum t_G/t_0 is in the mid-teens.

As mentioned above, we might ask the question, “If we challenge tissue with a small number of neuropeptides, what is the best approach to separation and quantitation of all detectable components in the mixture following hydrolysis in the tissue?”. If we were to perfuse tissue with three neuropeptide substrates and expect 3 – 4 quantifiable products from each, the separation would focus on 9–12 peaks, a rather modest number. Even so, the statistical peak overlap theory⁴⁶ tells us that for a 90% likelihood of a clean separation of 12 components we would need a peak capacity of 228; for a 95% probability it would be 468. From Figure 4C we see that to achieve a peak capacity of 228 with 1.7 μm particles at 25 °C with a 1000 bar system limit (indicated by the red dot) we need to use a 20-minute linear gradient, a 5.9 cm column and set $u_e = 0.9$ cm/s yielding $t_0 = 9.1$ s. We should point out that n_c for the 20-minute gradient can be improved. Moving up and to the left along the 1000 bar pressure contour (to the purple point) leads to a maximum achievable peak capacity of 313. New conditions for the 20-minute gradient are: $L = 12.6$ cm, $u_e = 0.47$ cm/s and $t_0 = 40.8$ s. Contour spacing in Figure 4C and 4D is 25 peak capacity units.

In the interest of performing the minimum number of experiments in tissue a second question follows, “What is the maximum number of substrate peptides we can perfuse into tissue while still realizing enough peak capacity to achieve a satisfactory separation in a reasonable time?” We define a 60-minute gradient as a reasonable time. Figure 4D shows a maximum peak capacity of about 450 (pink contour) can be reached for the 1000 bar system using a 60-minute gradient. We should point out that further increase in column length does not yield appreciable increases in n_c , data not shown. Conditions to yield a maximum peak capacity of 460 (red point) are as follows: $L = 21.5$ cm, $u_e = 0.27$ cm/s, $t_0 = 121$ s, $T = 25$ °C. From statistical peak overlap theory and the same 90% likelihood of a clean separation we predict a peak capacity of about 450 could separate 24 peaks from 5–6 neuropeptide substrates.

Conclusions

There are several published methods of plotting chromatographic performance vs. controllable conditions (variables) that precede the one(s) presented here. The underlying ideas and quantitative expressions of those ideas are the same in all of them. What distinguishes one plot from another is mostly its intended purpose. Plots of performance have tended to emphasize the big picture and traverse orders of magnitude of variable space. Our intention has been to emphasize what can be done in the lab over a restricted range of parameters. The Carr-Wang-Stoll “two parameter” optimization is easily portrayed in graphical format here. Note that the algebra involved in that optimization is not required; the plots embody the results naturally. In “three parameter” optimization (isocratic), the particle diameter is allowed to vary in a continuous fashion (rather than being limited to commercially available particles which come in discrete sizes). It turns out that using only commercially available particle diameters will yield a result that is acceptably close to the global optimum³⁷, as long as changes in diameter are made at the correct t_0 , thus a small set of the present plots at commercial particle diameters can provide a pragmatic three-parameter optimization. The application to gradient elution with a similar format to isocratic elution also turns out to be quite informative. This plotting approach is somewhat more limited with gradients because there are other parameters involved such as gradient time and $S \phi$. In addition, we have not accounted for gradient compression. However, it is still clear that some general properties become evident from observing the plots. And actual laboratory conditions can be estimated for a given gradient time and desired peak capacity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Plate count, t_0 , and P as a function of u_e and L (given the parameters in Table 1). A) Conditions required to generate $N = 20000$ in the shortest time, $t_0 = 35$ s, for a pump with a 600 bar pressure limit. B) Conditions that represent the maximum N for $t_0 = 63.7$ s for the 600 bar system. This point is often referred to as the Knox-Saleem limit where velocity is set to the van Deemter optimum and column length sufficient to operate the system at its pressure maximum. C) Points required to generate 2000 plates for the 600 and 1000 bar systems where the increase in system pressure has little influence on chromatographic speed.

Figure 2.

Influence of column temperature and particle size on isocratic separation performance. Red dots correspond to the values of L , u_e and N required to achieve 5000 plates in the shortest time with a maximum pressure of 600 bar for the 1.7 μm particles and 1000 bar for the 1.0 μm particles. Blue arrows in A present the series of options to improve performance when increase pressure from 600 to 1000 bar. A) Particle size: 1.7 μm , temperature: 25 $^{\circ}\text{C}$. B) Particle size: 1.7 μm , temperature: 75 $^{\circ}\text{C}$. C) Particle size: 1.0 μm , temperature: 25 $^{\circ}\text{C}$. D) Particle size: 1.0 μm , temperature: 75 $^{\circ}\text{C}$. All other conditions were identical to Figure 1. For clarity axes labels corresponding to linear velocity 0.01 cm/s and column length 0.1 cm have been omitted.

Figure 3.

Effect of volume overload and column diameter on apparent column efficiency optimization for 1.7 μm diameter particles. A) 100 μm ID column, B) 150 μm ID column, C) 250 μm ID column. The red dot in panel A corresponds to the values of L , u_c , and N required to generate 5000 plates in the shortest time for a 600 bar pressure maximum shown in Figure 2B. Open red circles in B and C represent the target conditions indicated by the red dot in A. Purple dots in each panel denote the new conditions required to achieve 5000 plates factoring in volume overload effects. Injection volume was 500 nL, retention factor in the sample solvent was 10, column temperature was 75 $^{\circ}\text{C}$. All other conditions were identical to Figure 1. For clarity axes labels corresponding to linear velocity 0.01 cm/s and column length 0.1 cm have been omitted.

Figure 4.

Plots for gradient elution peak capacity as a function of u_e , L and t_G for separations of neuropeptides. Gradient times were: A) 1 minute, B) 3 minutes, C) 20 minutes, D) 60 minutes. Particle size and column temperature were $1.7 \mu\text{m}$ and 25°C , respectively. See text and Table 1 for other chromatographic conditions. For clarity, axis labels corresponding to linear velocity 0.01 cm/s and column length 0.1 cm have been omitted.

Table 1

Parameters used in plots.

Parameter	Source	Value
$P_{\max}$ (bar)	Commercial	600, 1000
$D_{m,25\text{ }^{\circ}\text{C}}$ (cm^2/s)	Literature ³²	5.6×10^{-6}
$D_m(\phi, T)$	Literature ³³	See Eq. SI1
A	Literature ³¹	0.66
B		10.2
C		0.095
d_p (μm)		1.7
Φ		431
e_e		0.353
e_{tot}		0.537
T_{col} ($^{\circ}\text{C}$)	Experience	25
ϕ		0.04
$\eta(\phi, T)$ (cP)	Literature ^{34,35}	See Eq. SI2.