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FEATURE ARTICLE

The 1000 bar and 24 hour limits of one-dimensional HPLC – graphical representations

Veronika R. Meyer

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Introduction

> *Past achievements suggest that columns providing efficiencies in excess of a million plates in less than 1 day are within the grasp of current technology.*
> Georges Guiochon [4]

A good understanding of chromatography can only be obtained by practical experience *and* a sound theoretical background. The latter can hardly be conveyed without mathematical equations. They allow us to play with the mutual dependencies of column dimensions, particle size or film thickness of the stationary phase, theoretical plate number, peak capacity, flow rate relationships, analysis time, and more [1]. Peak resolution is obtained by combination of plate number, retention factor, and chromatographic selectivity expressed as relative retention. The theoretical background, combined with some experimental data, allows the prediction of peak patterns and their optimization [2]. Thus, mathematics is indispensable for the superior use of the possibilities offered by the various chromatographic phase systems and the available hardware.

Mathematical relationships can be visualized, and by doing so the pleasure of insight can be increased. Out of the numerous functions and relationships this paper only deals with one question: what is possible in column liquid chromatography if today's instrumentation and column hardware allow working at a pressure of 1000 bar and if the user accepts an analysis time of one day? Is Guiochon's optimism (see his statement quoted above) justified? The answers are sought by construction of plots which reach these limits.

V. R. Meyer (✉)
EMPA St Gallen, Swiss Federal Laboratories
for Materials Testing and Research, Laboratory for Biomaterials,
Lerchenfeldstrasse 5,
9014 St Gallen, Switzerland
e-mail: veronika.meyer@empa.ch

The benefit of the optimum flow rate

If an analyst is willing to invest 1000 bar and to wait for one day it is obvious that the scenario yielding the maximum separation power should be selected from the various ones possible. It is a fact that any column performs best if it is run at its optimum flow velocity, i.e., at the lowest point of its van Deemter curve, resulting in the maximum possible number of theoretical plates [3–5]. If this condition is fulfilled the inevitable pressure limit will define the optimum length of a column which is packed with an optimum-diameter particular stationary phase. (Note that this paper does not deal with monolithic phases.) Deviations from the optimum flow velocity of the eluent result in poorer separation performance:

- If the flow rate (or, strictly speaking, the linear velocity) is lower than the respective van Deemter optimum, distinct band broadening will occur, resulting in reduced chromatographic resolution.
- If the flow rate is increased above the van Deemter optimum the same effects will result, although to a much lesser degree than in the case of too low a flow rate. Nevertheless, the pressure drop will increase and the plate number will decrease. Therefore the best utilization of pressure with regard to the resolution

power is obtained at the van Deemter optimum. If, with a certain particle diameter, the resulting pressure is lower than the maximum possible, a longer column can be used, giving the best separation power for a certain combination of particle size and pump model.

Therefore, and keeping in mind that commercial HPLC pumps come with an upper pressure limit, the Halász and Meyer plots shown in this paper represent columns used at their van Deemter optimum (whereas the Poppe plots do not do so directly).

Looking for theoretical plate numbers: the Halász plots

The plots published in 1982 by István Halász and Gerhard Görlitz [3] did not attract much attention, probably because the paper is written in German. The authors described the underlying mathematics in detail, so it is not outlined here again. It was necessary to define an empirical van Deemter equation, and the one selected was:

$$H = ad_p + b/u + cd_p^2 u \quad (1)$$

with H = height of a theoretical plate, d_p = particle diameter of the stationary phase, and u = linear velocity of the mobile phase. The constants a, b, and c depend on many parameters of a certain separation system. Halász and Görlitz selected:

a=1.5, b=6, c=1/16 for low-viscosity eluents (η between 0.4 and 0.6 mPa s);
a=2, b=3, c=1/8 for high-viscosity eluents (η between 0.7 and 1.5 mPa s).

They constructed nomograms for isocratic separations with the theoretical plate number N as the y-axis, the particle diameter as the x-axis, and three sets of inclined lines running through the graphs: one set for the column length L, one for the breakthrough time (or hold-up time) t_0, and a third for the resulting pressure drop Δp. Here the plots are re-drawn in order to avoid confusion with the symbols (the authors used n instead of the now common N for the plate number and δ instead of d_p for the particle diameter); in addition the 1000 bar line is highlighted and a horizontal line indicating the resulting plate number is drawn.

Figure 1 shows the Halász plot for typical reversed-phase separations using a mobile phase with viscosity 1.2 mPa s, a value selected by the authors probably because it is somewhere in the middle of the viscosity range which can occur with such analyses (pure acetonitrile has 0.4 mPa s at 25 °C, mixtures of water and methanol have a maximum of 1.6 mPa s). It is obvious that the plate number obtained at 1000 bar (or at any given pressure) increases with increasing breakthrough time: a long t_0

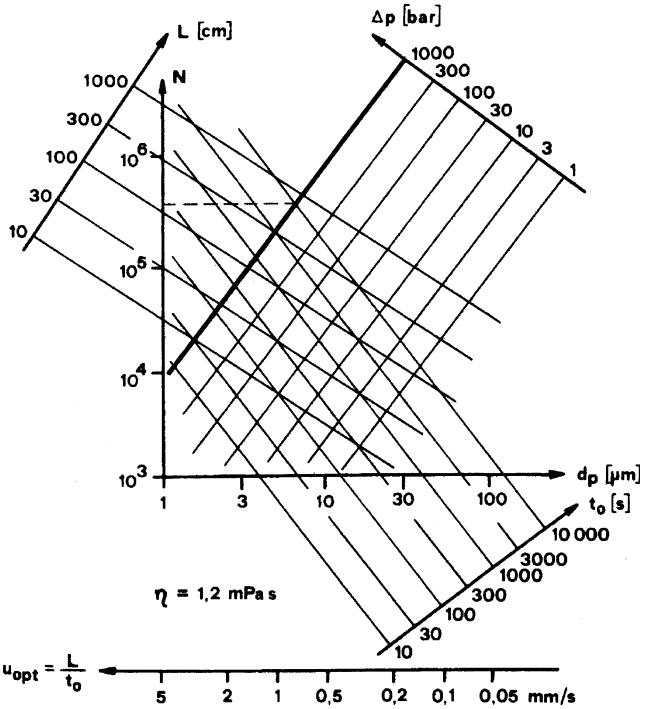

Fig. 1 Halász plot for an isocratic reversed-phase HPLC system, run at the van Deemter minimum with a mobile phase of viscosity η= 1.2 mPa s. Any two variables can be selected, then the other three are fixed and can be read off. Re-drawn and slightly modified after Ref. [3]

opens the door to high resolving power. The longest t_0 in the plot is 10,000 s or 2.8 h. With 1000 bar a plate number of 350,000 is obtained, using a column of 7.5 m length, packed with a stationary phase of 6.6-µm particles and run at its optimum flow rate. (These values can easily be calculated by using the "ready-made" equations outlined in Ref. [6].) However, if a maximum analysis time of 24 h is allowed we only reach a maximum retention factor k_{max} of:

$$k_{max} = \frac{t_{r,max} - t_0}{t_0} = \frac{24 - 2.8}{2.8} = 7.6 \quad (2)$$

Therefore the maximum peak capacity, n [7], i.e., the hypothetical number of peaks consecutively eluted with resolution 1.0, is:

$$n = 1 + \frac{\sqrt{N}}{4} \ln(1 + k_{max}) = 1 + \frac{\sqrt{3.5 \cdot 10^5}}{4} \ln 8.6 = 320 \quad (3)$$

The other Halász plot in the 1982 paper shows the more favourable situation with normal-phase separations; Fig. 2 is the re-drawn representation of it. The mobile phase is not aqueous, therefore the usual viscosities are markedly lower. The authors selected a viscosity of 0.44 mPa s, typical for dichloromethane, tetrahydrofuran, or ethyl acetate at 20 °C (mixtures of these solvents with hexane would result in

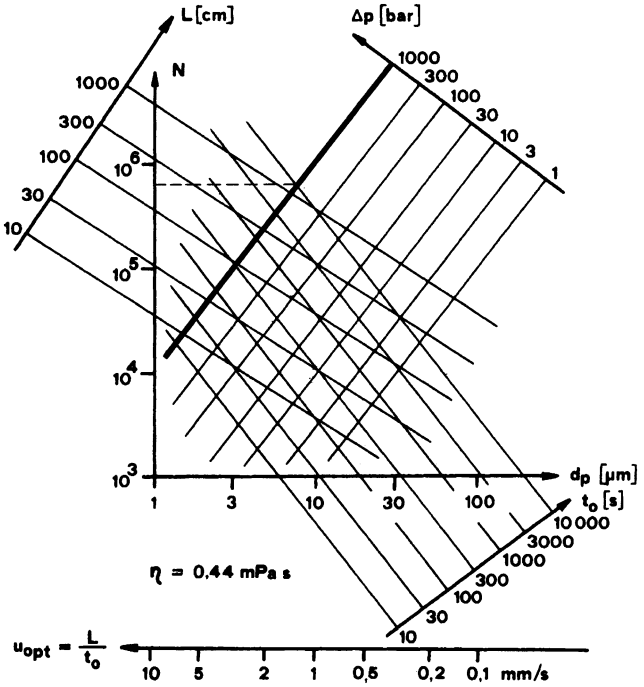

Fig. 2 Halász plot for an isocratic normal-phase HPLC system, run at the van Deemter minimum with a mobile phase of viscosity η=0.44. Re-drawn and slightly modified after Ref. [3]

lower viscosities). With 1000 bar and again a breakthrough time of 10,000 s or 2.8 h a theoretical plate number of 640,000 is obtained. The column has a length of 13 m and a packing of 7.5-µm particles. As before, the k_{max} within 24 h is 7.6, resulting in a peak capacity of 430.

It is easier to realize a million theoretical plates with a normal-phase system than with an aqueous separation mode such as reversed-phase, due to the lower viscosity of the former. Is this possible within one day? The required configuration can be extrapolated from Fig. 2 or calculated by use of equations outlined in Ref. [6]. The breakthrough time is 24,500 s or 6.8 h. The monstrous column should be 26 m long with a 10-µm packing. Within 24 h a k_{max} of 2.5 is possible, giving a peak capacity of 314 with Eq. 3. The huge effort is not really worthwhile, because the more modest 13-m column discussed above yields a higher peak capacity.

Guiochon [4] presents an interesting equation which defines the optimum particle diameter (Eq. (38) in his paper):

$$d_{p,opt} = \sqrt{\frac{\varepsilon \eta D_m \nu_{opt} h_{min} N}{K \Delta P}} \quad (4)$$

with:

ε total porosity of the column, for porous particles around 0.7 (dimensionless)

η viscosity of the mobile phase, here 0.44 mPa s

D_m diffusion coefficient of the analyte in the mobile phase, for small molecules in normal-phase solvents approx. 4×10^{-9} m^{2}s^{-1} (in reversed-phase solvents approx. 1×10^{-9} m^{2}s^{-1})

ν_{opt} optimum linear flow velocity of the mobile phase, usually 3 (dimensionless) [8]

h_{min} minimum reduced plate height, often 3 (dimensionless) [8]

N number of theoretical plates, here 10^6 (dimensionless)

K column permeability, 10^{-3} (dimensionless); this is the Kozeny–Carman factor [9]

Δp pressure drop, here 10^3 bar or 10^8 Pa

Equation 4 is, in fact, based on an old but important paper by Knox and Saleem [10], however, these authors used a nomenclature which is no longer in use.

By this calculation we obtain the same value as from the Halász plot, namely $d_{p,opt}$=10 µm for the desired conditions of 10^6 theoretical plates, realized with 10^3 bar.

With the more viscous reversed-phase system a million theoretical plates with 1000 bar can be obtained under the following conditions: a t_0 of 81,000 s or 22.5 h, a column of 36 m length, and a particle diameter of 11 µm. The retention factor at 24 h is only 0.07, which means that no useful peak capacity can be developed within an elution time window of 1.5 h; n is a mere 17. The conditions would be more favourable if an eluent with high acetonitrile content would be used; pure acetonitrile has a viscosity of 0.37 mPa s at 20 °C, i.e. even less than the 0.44 mPa s used in the normal-phase Halász plot, giving better performance than a dichloromethane–silica separation system.

The nomograms of Figs. 1 and 2 do not show the ultimate limits of separation power. They are constructed on the basis of a reduced plate height at the van Deemter optimum of h=3; i.e. three particle diameters of the stationary phase are equivalent to the height of a theoretical plate. The best currently obtained values are h=2, resulting in higher plate numbers per given column length. On the other hand, many "everyday separations" show poorer performance, broader peaks, and higher values of h.

The Halász plots make clear that small particles are needed for fast separations but that they are not useful for highest plate numbers if pressure is limited. This point is also mentioned by Guiochon [4]. Equation 4 above shows that $d_{p,opt}$ is proportional to $\sqrt{N}$ or, inverted, that N is proportional to $d^2_{p,opt}$. In addition, the pressure drop per unit length decreases with increasing particle diameter, enabling the use of longer columns.

For mutual consistency with the Halász plots, the following Meyer and Poppe plots were calculated by using the total porosities ε as proposed by Halász and Görlitz: ε= 0.70 for chemically derivatized porous silica (e.g. reversed

phases) and $\varepsilon = 0.82$ for underivatized silica (normal phases). Other numeric parameters, for example the van Deemter constants or the viscosities, were also taken from the Halász–Görlitz paper.

Looking for peak capacities: the Meyer plots

For the question discussed here, namely "1000 bar within 24 hours", the Halász plots are not really ideal. First, the maximum breakthrough time shown by them is 10,000 s, resulting in a maximum retention factor of 7.6 although the graphs could be expanded towards longer breakthrough times. But a second fact is more important: the plots do not show the best conditions for obtaining a high peak capacity. Analysts want peak resolving power within a certain time range, and this objective is better represented with the peak capacity than with the plate number. In addition, the fact that, in the normal-phase example, the million plates/26 m column mentioned above yields a lower peak capacity within 24 h than the 13 m column with 640,000 plates is not intuitively obvious.

Therefore I developed the original Halász plots further [6]:

- The *x*-axes of the graphs shown in Ref. [6] cover a retention factor range of $k = 0$–20, presented with a maximum retention time of one hour. Therefore t_0 is 60/(20 + 1) min = 2.8 min. A k_{max} of 20 is somehow the upper limit which makes sense in isocratic elutions because the last peaks are broad and no longer high.
- The *y*-axis shows the peak capacity instead of the theoretical plate number.

In addition to the Halász and Görlitz publication a plot for reversed-phase gradient separations was also developed. Reference [6] explains the necessary conditions for the plots in detail.

Here, some peak capacity plots illustrating the 1000 bar, 24 h question are presented. The retention or gradient time was expanded to one day and in the isocratic plots k_{max} is again 20; therefore the breakthrough time is 24/(20 + 1) h = 1.15 h or 1 h 9 min.

Figure 3 presents the situation in reversed-phase systems. The peak capacity increases with increasing analysis time (this is trivial) and reaches $n = 355$ after 24 h if a pressure of 1000 bar can be applied. The column is 3.8 m long and is packed with a 5.4-µm phase, giving 235,000 theoretical plates at the van Deemter optimum with $h = 3$. The slightly more favourable situation, compared with Fig. 1, for which a maximum peak capacity of 320 was calculated, comes from the fact that this separation runs to a higher k_{max}. But, as mentioned above, the separation time or the retention factor cannot be expanded to just any possible value because the peaks soon become flat and the signal-to-noise ratio decreases.

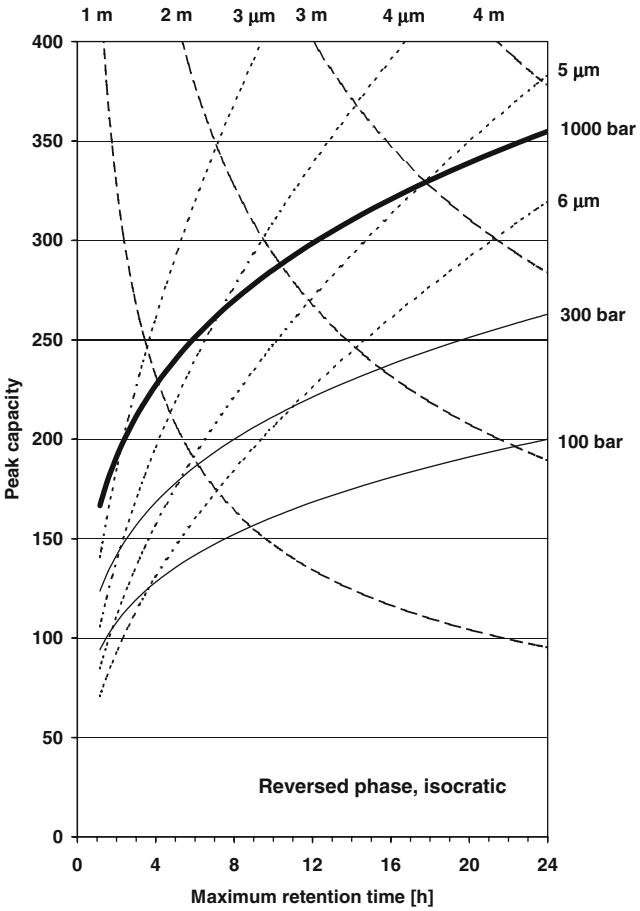

Fig. 3 Peak capacity plot for the same isocratic reversed-phase conditions as in Fig. 1, however with a k_{max} of 20, thus t_0 is 1.15 h. On any point of a pressure line (*solid*) the resulting peak capacity and the necessary column length (*dashed lines*) and packing diameter (*dotted lines*) can be read off

Again, the normal-phase system yields higher peak capacities than the reversed-phase one, namely 475 within 24 h (Fig. 4). This performance is obtained with a 6.6-m column and 6.2-µm stationary phase at 1000 bar, giving 355,000 theoretical plates.

The column lengths calculated so far are huge compared with those for the 10 cm or 25 cm lengths which are common today. Long columns used in isocratic mode are not the solution for the highest plate numbers or peak capacities (maybe with the exception of preparative separations with the objective of isolating one or a few pure compounds) but gradient separations are needed. Therefore, a one-hour gradient plot was developed in Ref. [6]. Here it is expanded to one day (Fig. 5). The underlying theory was developed by Uwe Neue [11]. For the plot a wide %B range was selected, in fact the widest one which makes sense, namely 0.9 representing the amount of B

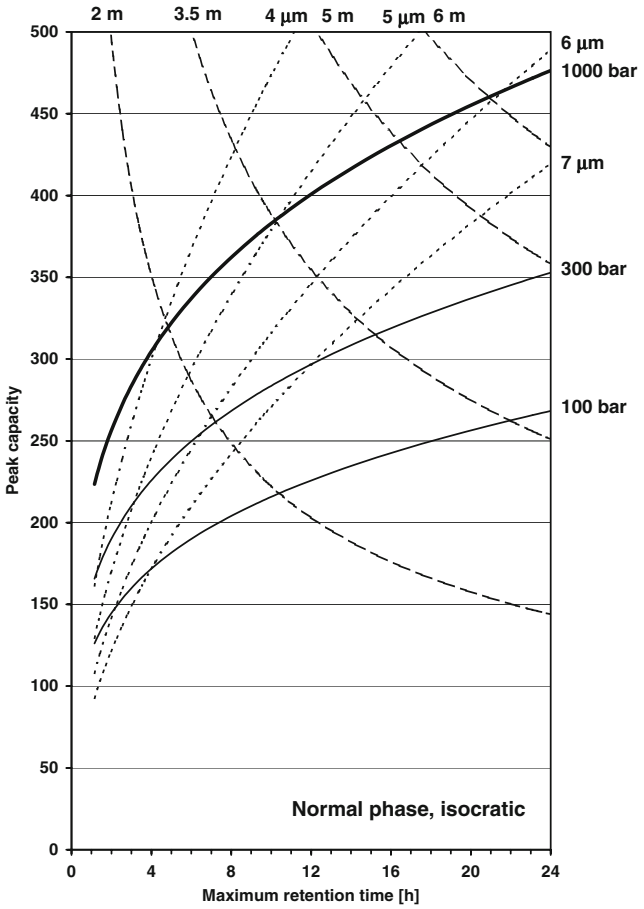

Fig. 4 Peak capacity plot for the same isocratic normal-phase conditions as in Fig. 2, however with a k_{max} of 20

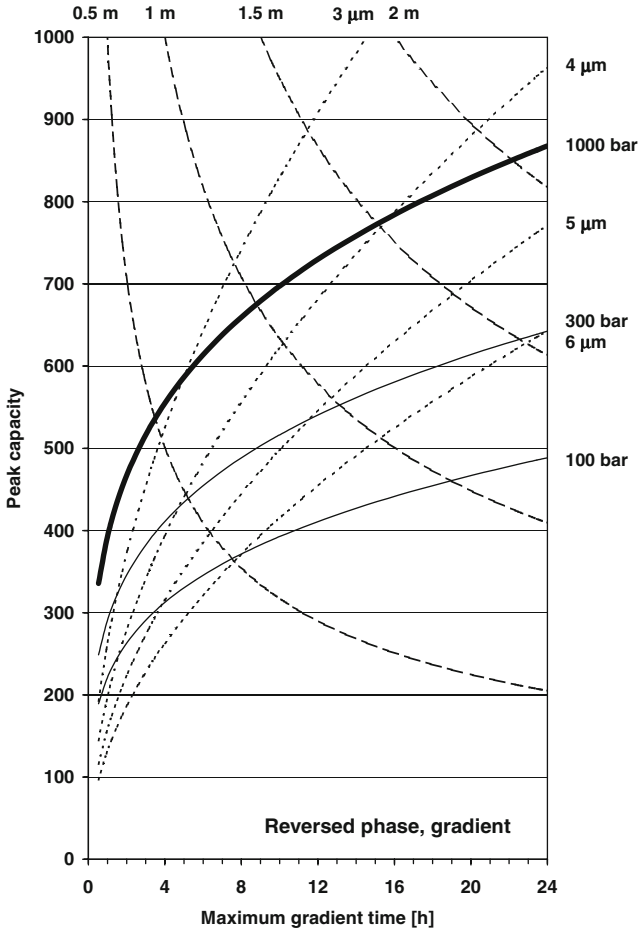

Fig. 5 Peak capacity plot for reversed-phase systems run with a gradient using an eluent composition change of 0.9 (for example 10–90% B). The flow rate is always at the van Deemter optimum and t_0 is 32 min

solvent changing from 10 to 100% (because a start at 0% B is not recommended because most reversed phases do not perform well under totally aqueous conditions). With Neue's values it turned out that the gradient run time of the plot is 45 t_0 [6], i.e. t_0 is 0.53 h or 32 min for a 24-h separation.

A thousand bar, or any other pressure shown in Fig. 5, is the maximum which occurs during the separation; because the viscosity of the eluent changes during a gradient separation the pressure will also change. The maximum peak capacity obtained now is 870 with a 2.1-m column and a 4.4-µm stationary phase.

A peak capacity of 870 may seem high. For complex mixtures of analytes, however, this resolving power is still somewhat disappointing. For separations which are not especially optimized, the probability P' of resolving all components of a sample is given by [12]:

$$P' = \left(1 - \frac{m-1}{n-1}\right)^{m-2} \quad (5)$$

with

m number of analytes present in the sample
n peak capacity

For a sample with 30 components, a peak capacity of 870 gives P'=0.39, i.e. the chance that all the compounds can be resolved is not higher than 40%. This approach is statistical in nature; therefore, the circumstances of a certain separation can be more or less favourable than indicated with Eq. 5. But it shows that a simple linear gradient even on a column longer than 2 m gives no guarantee at all of a successful separation. The way out of this problem lies in clever coupling with mass spectrometry or in comprehensive two-dimensional chromatography [13].

Figures 3, 4, 5 clearly show the immanent drawback of one-dimensional separations: the peak capacity increases only moderately with time. In all nine cases - three separation systems run at three different pressures each - the peak capacity after one hour is already 45% of the number one gets

after 24 h. Note that this fact is also true for the gradient approach. Why wait for one day if the capacity can be increased in a shorter time even with a rather simple off-line, non-comprehensive two-dimensional approach?

Looking for comparison: the Poppe plots

In 1997, Hans Poppe proposed a special type of plot which can be used to compare the properties of different stationary phases when used at a certain pressure [14]. Such a plot does not pay special attention to the optimum flow rate but it shows the performance of hypothetical columns of such a length that a certain, selected pressure is needed to use them. The subject is not straightforward to understand, and Poppe gave only a cryptic description on how to generate the plots. Nevertheless, they became astonishingly popular, and other plots of similar "kinetic" types were proposed. Detailed descriptions of the various possibilities and how to draw kinetic plots can be found in papers by Desmet et al. [15], Fountain et al. [16], and Neue [17].

The classical Poppe plot shows t_0/N, the residence time of the eluent in a theoretical plate, as a function of N. Both axes are logarithmic, and the resulting curves look parabola-like with the opening towards the upper left corner of the plot. Each curve is valid for a certain particle diameter and a certain pressure (plus a certain van Deemter function, eluent viscosity, column permeability, and column packing porosity) under isocratic conditions. Any point of a curve represents a defined column length and linear flow velocity which together, in combination with the other variables, yield the desired or available pressure. The curves have two asymptotes [4]:

- The vertical one marks the highest possible plate number which could be obtained with the given stationary phase if the column length reaches indefinite length and the flow velocity approaches 0.
- The horizontal (leftwards) one marks the system operating at indefinitely high velocity and a column length approaching 0.

The curves do not show the column length at a certain point or its corresponding flow rate. If an envelope is laid under a set of isobars, a straight line with slope +1 is the result. It touches the curves at their individual van Deemter optima [18]. Breakthrough times can be represented as diagonal lines with slope −1 (because log t_0/N is just a function of log N); therefore it is possible to show some of them in a plot, and the position where a breakthrough line crosses a Poppe curve marks the breakthrough time of the respective hypothetical column.

Poppe plots allow the comparison of different types of column packings, i.e. particle sizes or particulate vs. monolithic stationary phases. They show, e.g., if a certain plate number can be achieved with a given stationary phase but the means to reach it are hidden.

The plots of Figs. 6 and 7 were obtained by spreadsheet calculation as follows:

- The range of each curve was limited by the reduced velocity: ν was varied between 1 (too slow, unfavourable, upper right end of a curve) and 18 (far away from the optimum velocity which is at approx. ν=3, lower left end of a curve). The reduced velocity is a dimensionless characterization of the eluent velocity and is defined as:

$$\nu = \left(u d_\mathrm{p}\right)/D_\mathrm{m} \tag{6}$$

i.e. it is linked to the diffusion coefficient of an analyte and the particle diameter.

- With the given particle diameter and diffusion coefficient it is possible to calculate u.
- The height of a theoretical plate H was obtained with Eq. 1 and the variables given above.
- Two equations describing the column length can be used to calculate N; the second one is derived from the Kozeny–Carman equation [9]:

$$L = NH = N\left(a \cdot d_\mathrm{p} + b/u + c \cdot d_\mathrm{p}^2 \cdot u\right) \tag{7a}$$

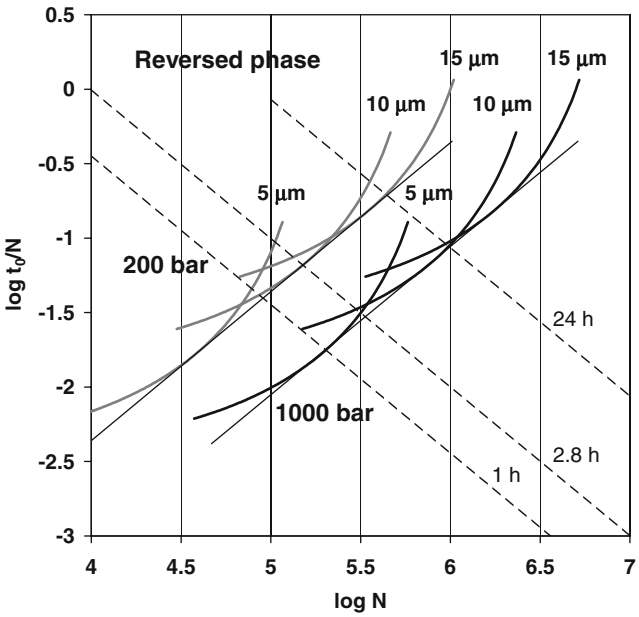

Fig. 6 Poppe plot for isocratic reversed-phase systems. The black lines are valid for 1000 bar, the grey ones for 200 bar. The diagonal envelopes under the curves represent the positions of the van Deemter optima. Three breakthrough time lines are also shown

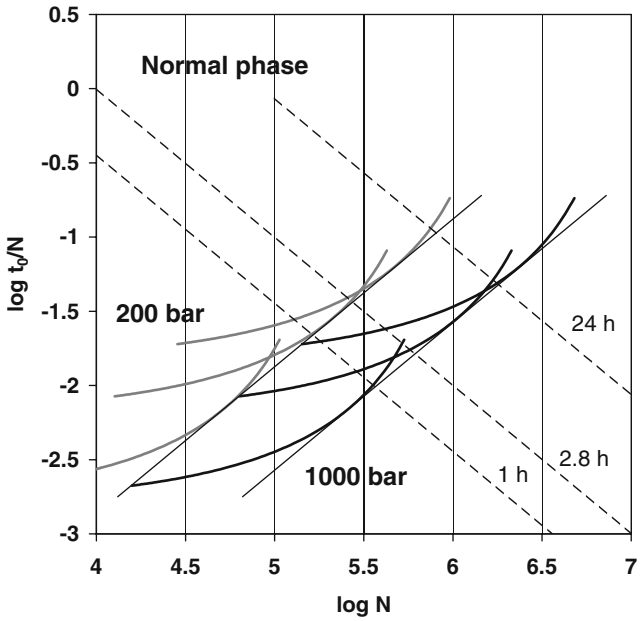

Fig. 7 Poppe plot for isocratic normal-phase systems. The representation is identical with that in Fig. 6 with particle sizes of 5, 10, and 15 μm. Both axes have the same scale as in Fig. 6 to show clearly that normal-phase separations are faster (t_0 is smaller) than reversed-phase ones

$$L = \frac{\Delta p \cdot d_p^2}{1000 \cdot \varepsilon \cdot \eta \cdot u} \tag{7b}$$

$$N = \frac{\Delta p \cdot d_p^2}{1000 \cdot \varepsilon \cdot \eta \cdot u\left(a d_p + b/u + c d_p^2 u\right)} \tag{8}$$

- t_0 is calculated from Eq. 7b and the relationship $t_0 = L/u$:

$$t_0 = \frac{\Delta p \cdot d_p^2}{1000 \cdot \varepsilon \cdot \eta \cdot u^2} \tag{9}$$

- t_0/N, the y-axis of the plots, is then a simple division.

Figure 6 is for reversed-phase systems and Fig. 7 is for normal-phase ones. Particle diameters of 5, 10, and 15 μm were selected, and 1000 bar (black lines) and 200 bar (grey lines, for comparison) as pressures. Breakthrough times of 1 h, 2.8 h (which gives a maximum retention factor of 7.6 within 24 h as discussed above), and 24 h (giving no eluted chromatogram within one day) are also shown. It must be stressed that the axes present logarithmic data; therefore the difference of the system performances shown by the individual curves are much larger than it may seem at first glance.

Both plots show clearly that "large" particles of the stationary phase are needed to obtain high plate numbers, as already mentioned above in the discussion of the Halász plots. With the reversed-phase system of Fig. 6 it is not possible to obtain a million theoretical plates with a 5-μm packing, even if a pressure of 1000 bar is available. The same seems to be true for the normal-phase system although it is more favourable, because of the lower backpressure and the higher diffusion coefficient. Because the upper end of every curve represents a reduced velocity of 1, this point and all possible ones which would follow towards the vertical asymptote are extremely unfavourable, because this is a region with much too slow a flow rate and broad peaks with regard to the van Deemter optimum.

One million theoretical plates can be obtained on both plots with either the 10-μm or 15-μm phase if 1000 bar can be applied. The conditions are noted in Table 1. (Note that these sets of conditions cannot be read off from Figs. 1 or 2 because they only show systems operated at their van Deemter minimum.) Again, the normal-phase systems allow faster separations which is represented by the fact that their curves lie lower in the plot than the reversed-phase curves–although breakthrough times of around 8 h are anything but "fast"!

Conclusions

So-called ultra-high-performance liquid chromatography (UHPLC) systems which allow pressures up to 1000 bar are on the market now although most published UHPLC separations are performed at lower pressures. They open the way to higher theoretical plate numbers and peak capacities than have been common so far. Nevertheless, the speed of liquid chromatography is governed by the

Table 1 Isocratic conditions for 10^6 theoretical plates if 1000 bar can be applied (Figs. 6 and 7)

System	Breakthrough time (h)	Reduced flow velocity	Column length (m)
Reversed phase, 10 μm	25.5	3.6	33
Reversed phase, 15 μm	27.5	7.8	51
Normal phase, 10 μm	8.4	2.4	29
Normal phase, 15 μm	9.8	5.0	47

rather poor, i.e. slow, diffusion coefficients of even small molecules in solvents. If high pressures are applied it is necessary to use rather coarse stationary phases, long columns, and long analysis times in order to reach the region of 10^5 to 10^6 theoretical plates. A critical overview on 40 years of efforts towards this objective was given by Guiochon [4]. With regard to high peak capacities it is absolutely indispensable to work with gradients [19, 20]. Even then a peak capacity of 1000 is not really satisfactory for mixtures of 100 compounds, because of the laws of statistical resolution probability. Possibilities of improving the situation are:

- Working at higher temperature shifts the van Deemter curve to the right because the diffusion coefficient of the analytes increases. Because most separations are performed at too high a flow velocity with regard to the van Deemter minimum, increasing the temperature (while holding the flow rate constant) usually results in higher plate numbers because the system is now closer to the optimum. In addition, the pressure drop is reduced. (However, note that the backpressure at the van Deemter minimum is not affected by temperature [21].) Sandra and Vanhoenacker obtained 200,000 theoretical plates or a peak capacity of 900 for analysis of tryptic digests by coupling eight columns of 25 cm length, packed with 5-μm reversed-phase material and operated at 60°C [22]. Their instrumentation allowed a maximum pressure of 600 bar but the column system was not exhausting this limit. Normal-phase systems are more advantageous than reversed-phase ones. High-temperature HPLC, especially the approach with superheated water [23], is most promising.
- Use of monolithic columns. Their backpressure is much lower than with traditional, packed columns [24]. However, so far it is not yet possible to produce long monoliths, therefore their number of theoretical plates is limited. The gateway to Guiochon's dream of a million plates within one day could be monolithic phases prepared in microbore tubes [4, 25].
- Performing demanding separations with comprehensive two-dimensional HPLC which is the silver bullet to obtain high peak capacity [11, 26].

Personally, I am not very optimistic that "a million plates in less than 1 day are within the grasp of current technology". There is a gap between the demands of all kinds of "fingerprint analysis" (in environmental and the various types of "omics" research) and the limits set by diffusion coefficients and pressure problems (including the limitations of material strength and the problem of heat transfer within a HPLC column [27]). Even if one million theoretical plates or a peak capacity of 1000 could be available in everyday work, the search for the highest performance would not come to an end. More and more peaks would be visible in complex samples, the mass spectrometer would still prove that there is peak overlap, and generations of researchers could devote their skills to finding better separation and identification techniques.

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Veronika R. Meyer is a chemist and works at EMPA, the Swiss Federal Laboratories for Materials Testing and Research in St Gallen. She is a book author in the field of HPLC and a lecturer at the University of Bern, Switzerland. Her scientific interests are analytical chemistry, especially liquid chromatography, quality assurance, and measurement uncertainty.