Measurement of optimal flow rate in gradient elution liquid chromatography

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Abstract

Method development in gradient LC relies upon the selection of a solvent time program and a mobile phase flow rate. The flow rate, optimal for gradient separation cannot be inherently predicted by the isocratic value optimal for a given analyte, and rather should be identified independently to ensure the highest separation performance of gradient analysis. The optimal flow rate (F_{opt}) is defined herein as the solvent volumetric flow rate (F) maximizing the separation (Δs) of a predetermined peak-pair or the separation capacity (s_c) of the entire LC analysis. The theoretical background and the experimental technique of measurement of F_{opt} in gradient elution analysis were considered and experimentally demonstrated. The technique of measuring F_{opt} is based on translatable changes of F where the product Ft_M was the same for all values of F. The F_{opt} was found as F corresponding to the maximum in Δs or in s_c.

1. Introduction

The relation of a solvent volumetric *flow rate* (F) to its optimum (F_{opt}) is an important factor of LC column operation. It is assumed throughout this report that F and the column *temperature* (T) do **not change** during the analysis, and that the time (t) is measured **since the sample introduction** (and, therefore, the effect of dwell time is eliminated).

It might be desirable to operate a column at $F > F_{opt}$. This, together with accompanying increase in the column *length* (L) makes it possible to reduce the *analysis time* (t_{anal}) required for a predetermined *separation performance* (*separation* of a critical peak pair, *peak capacity*, etc.) [1] (assuming that this approach is not prevented by the factors like maximum pressure available from the equipmet and/or accetable for the column, column overheating, etc.). On the other hand, increasing F far above F_{opt} together with increasing L (to maintain a required separation performance) might result in practically insignificant shortening of t_{anal} while significantly increasing the solvent consumption. Hence, there is a compromise for practically reasonable ratio F/F_{opt}. For example, Scott and Hazeldean suggested [1] that the mobile phase *optimal practical velocity* in open-tubular columns (OTC) corresponds to $F/F_{opt} = 2$. The practically justified F/F_{opt} ratio in packed columns can be larger than 2. These considerations indicate that it is important to know F_{opt} in a given analysis in order to choose a reasonable value of F.

Typically, F_{opt} is understood as the one that causes the minimum (H_{min}) in *plate height* [2] (*apparent plate height* [3]):

$$H = L / N \tag{1}$$

where, in *isocratic* analysis with no extra-column peak broadening [4, 5],

$$N = (t_R / \sigma)^2 \quad \text{(isocratic)} \tag{2}$$

is the *plate number* [2] (*number of theoretical plates* [6]) for a peak with *retention time* (t_R) and *width* (*standard deviation*) σ. Generally, N and, therefore, H can be different for different solutes. Frequently, however, the difference is practically insignificant and N can be considered as the column parameter the same for all solutes.

The relations are more complex in gradient LC (see the Appendix). H, and therefore, H_{min} and F_{opt} for a given solute in a given column, depend on the solute retention factor which, in turn, depends on the solvent *composition* (the volumetric fraction, ϕ, of the stronger component) that changes during gradient analysis and can be different at different locations along the column. Additionally, to calculate H at the time (t_R) of elution of the solute of interest, one needs to know the solute distribution between the stationary phase and the solvent at t_R [7, 8]. As shown in the Appendix, this distribution cannot be measured directly. In view of these complications, one might choose to measure F_{opt} in isocratic analysis and treat it as F_{opt} in gradient analysis. However, there is no guarantee that the optimal F in isocratic analysis is also optimal in gradient analysis.

The coordinate-dependent ϕ within a column in gradient LC analysis is controlled by the time-programming of the solvent composition (ϕ_i) at the column inlet. Generally, the program can consist of a sequence of several *ramps* [9] and *holds*. The inlet composition (ϕ_i) changes during the ramps and remains fixed during the *holds*. The choice of the column, solvent, and its programming can be time consuming. Because F_{opt} might depend on the column, the solvent and its programming details (the initial and the final ϕ_i, the *rates* of changing ϕ_i during the ramps, the holds, etc.), it is desirable to optimize F after the solvent programming has been developed. This, together with the complications in practical measurement of H_{min} in gradient LC calls for a second look at the minimization of H as the flow optimization goal. After all, the goal of chromatographic analysis is the separation of solutes in a

sample and the minimization of H is a way of achieving that goal, but not the goal itself. The goal of the flow optimization in chromatography, including gradient LC, might be achieving the best separation of a target peak-pair in the sample, or maximizing the number of *resolved* (quantifiably and identifiably separated) peaks, or improvement of other metrics of separation performance. This approach was used in temperature-programmed GC [10] and in comparison of specific columns in gradient LC [11]. General theoretical and practical aspects of these techniques in gradient LC are the topic of this report. The **bold face** type highlights the assumptions in this study. The experimental results are compiled in the Supplementary material.

2. Theory

2.1 Method translation

The change in the solvent composition (ϕ) within a column in gradient LC is implemented by time *programming* of solvent composition (ϕ_i) at the column inlet. A program (Figure 1) can consist of one or several *mixing ramps* [9] of *durations* Δt_{G1}, Δt_{G2}, …, etc., and *holds* of *durations* Δt_{h1}, Δt_{h2}, …, etc. between the ramps. During a hold, ϕ_i remains fixed, while, during a ramp, ϕ_i changes from that in proceeding hold to the one in the subsequent hold. The derivative $R_\phi = d\phi_i/dt$ is the *mixing rate* [9] (also referred to as *gradient slope* [12-14], *time steepness of the gradient* [11, 15], *time-based gradient steepness* [16], or *temporal gradient steepness* [17]). A ramp can be either *linear* (Figure 1) with R_ϕ remaining fixed during the ramp or *non-linear* (*curved*) otherwise. The mixing rate of a linear ramp can be found as $R_\phi = \Delta\phi_i/\Delta t_G$ where Δt_G and $\Delta\phi_i$ are, respectively, the ramp duration and the solvent composition increment during the ramp.

Method translation in chromatography [18] is valid for linear and non-linear mixing ramps in the same or in different columns. However, as the ramps in practical gradient LC analyses are typically linear, and as only the linear ramps in the same column were used in experiments for this report, only the **linear mixing ramps** in **the same column** are assumed from now on.

According to the method translation theory [18],

- the mixing rates, R_ϕ, and the hold durations (Δt_h) in the solvent strength programs are not meaningful in themselves, but only in relation to the analysis *void time* (t_M)

two gradient analyses (A and B) utilizing the same column and the same solvents are *mutually translatable*, i.e. are *translations* of each other if (Figure 1):

- both start at the same *initial* ϕ_i (ϕ_{init}) and end at the same *end* ϕ_i (ϕ_{end})
- each hold in A has its counterpart in B and vice-versa – both maintaining the same ϕ_i during the times, $\Delta t_{h,A}$ and $\Delta t_{h,B}$, relating to each other as $\Delta t_{h,B}/t_{M,B} = \Delta t_{h,A}/t_{M,A}$ where $t_{M,A}$ and $t_{M,B}$ are the void times in analyses A and B, respectively
- each ramp in A has its counterpart in B and vice-versa – their durations, $\Delta t_{G,A}$ and $\Delta t_{G,B}$, relate to each other as $\Delta t_{G,B}/t_{M,B} = \Delta t_{G,A}/t_{M,A}$

In short, the solvent strength programs in mutually translatable analyses are the *rescaling* of each other in time domain so that the duration of a ramp or a hold in analysis B is in the same proportion to the void time in analysis B as the duration of its counterpart in A to the void time in A.

If analyses A and B are mutual translations of each other, then the ratio $G_s = t_{R,A}/t_{R,B}$ of the retention times ($t_{R,A}$ and $t_{R,B}$) for a solute in analyses A and B is the same for all solutes [18]. This justifies the interpretation of G_s as the *speed gain* in B relative to A [19]. As a consequence, a solute *elution solvent composition* (ϕ_R) – the outlet solvent composition at the solute retention time (t_R) – is the same in all

analyses that are translations of each other. As a consequence of that, the solute *elution retention factor* (k_R) – the ratio of its amounts in the stationary phase and in the solvent at the time t_R – is the same in all analyses that are translations of each other. Only the **translatable changes** in F are considered below.

In analysis with a single linear *mixing ramp* starting from $\phi_i = \phi_{init}$ at $t = 0$, the ϕ_R of a solute with retention time t_R can be found as:

$$\phi_R = \phi_{init} + R_\phi (t_R - t_M) \tag{3}$$

2.2 Optimal flow rate

As shown in the Appendix, it is unknown how to measure the plate number (N) or the plate height (H) in gradient LC analysis and, therefore, how to find the solvent optimal flow typically considered as the one minimizing H and maximizing N. Instead, the optimal flow rate (F_{opt}) in this report is the solvent volumetric flow rate (F) that maximizes a column separation performance.

2.2.1 Separation measure

The separation performance of a column in LC analysis can be expressed as the *resolution* (R_s) [2, 20] of two neighboring peaks (e.g., the resolution of a *critical* peak-pair in the sample), or as the *peak capacity* (n_c) [21] of the entire analysis. Both metrics have shortcomings. Introduced by Giddings [21] as the quantity "which approximates the maximum number of peaks to be separated on a given column", n_c has since been inconsistently defined [22] and its use for practical evaluation of the number of peaks that a chromatographic analysis can resolve (identifiably and quantifiably separate) requires additional explanations. The shortcomings of R_s were discussed in several studies [8, 22, 23]. Originally introduced [20] "only … for time being", R_s has two widely known definitions [2, 24] that

are compatible with each other only for Gaussian peaks and may not make sense otherwise. Among other shortcomings of R_s that it is *not an additive* metric [22] – if A, B and C are consecutive peaks then, unless all three are Gaussian and have equal widths, $R_{s,AC} \neq R_{s,AB} + R_{s,BC}$ where $R_{s,AC}$, $R_{s,AB}$, $R_{s,BC}$ are the resolutions of, respectively, peaks A and C, A and B, B and C. As a result, and because the definitions of R_s and n_c are based on different underlying principles, the sum, $R_{s1} + R_{s4} + R_{s4} + \ldots$, of the resolutions of all neighboring peaks in a chromatogram starting from unretained peak and ending at the last peak of interest might be substantially different from n_c [23]. For example, the resolution of the unretained and the last peak in isocratic analysis cannot exceed $\sqrt{N}/2$ $(R_s \leq \sqrt{N}/2)$ [23] no matter how long the analysis lasts, while there is no limit to increasing n_c of the isocratic analysis with increasing its analysis time.

In this report, a single metric – the *separation measure* (Δs) [22, 23] – is used for representing the separation of peak pairs and of the number of peaks that can be resolved by a column. The *standard deviation* (σ) is the only peak *width* measure in this report, unless contrary is explicitly stated. This makes all results of the study suitable for any peak shape.

The separation measure (Δs) *of a* Δt-wide *time-interval* (t_A, t_B) *between time markers* t_A *and* t_B *in the separation space of a chromatogram is the number of adjacent σ-slots (σ-wide segments) in* (t_A, t_B)

If t_A *and* t_B *are retention times of peaks A and B then* Δs *is the separation of peaks A and B. Otherwise,* Δs *is the separation capacity of the interval* (t_A, t_B)

Both, the peak separation and the peak capacity of an arbitrary interval in the separation space of a chromatogram are transparent additive metrics defined from a single perspective and representing a

158 *system* of mutually compatible metrics based on the same principle of the number of σ-slots in an
159 interval in the separation space of a chromatogram.

160 Being an additive metric, the *separation capacity of an analysis* (s_c) can be found as

161

162 $$s_c = \Delta s_1 + \Delta s_2 + \Delta s_3 + \ldots + \Delta s_{last} \quad (4)$$

163

164 where Δs_1, Δs_2, Δs_3, … are the separations of consecutive peak-pairs from unretained peak to the last
165 peak of interest in the analysis. If peaks A and B with retention times t_A and t_B have the same σ then
166 their separation is $\Delta s = \Delta t / \sigma$ where $\Delta t = t_B - t_A$. Otherwise [22, 23]:

167

168 $$\Delta s = \int_{t_A}^{t_B} \mathrm{d}s = \int_{t_A}^{t_B} \frac{\mathrm{d}t}{\sigma(t)} \quad (5)$$

169

170 where $\sigma(t)$ is a known dependence of σ on t. If $\sigma(t)$ changes linearly from σ_A to σ_B (like it does in, e.g.,
171 isocratic LC and isothermal GC) then Eq. (5) yields [22]:

172

173 $$\Delta s = \frac{\Delta t}{\sigma_B - \sigma_A} \ln\left(\frac{\sigma_B}{\sigma_A}\right) \quad (6)$$

174

175 If a wider of two peaks (A and B) is no more than 50% wider than the narrower one then their

separation can be approximated with less than 1.5% error [22] as[1]:

$$\Delta s \approx \Delta t / \bar{\sigma}\,, \quad \bar{\sigma} = (\sigma_A + \sigma_B)/2 \tag{7}$$

2.2.2 Optimal flow rate and velocity

Definition. Optimal flow rate (F_{opt}) *is the one that maximizes the separation* (Δs) *of two predetermined solutes in the chromatographic analysis.*

The two *predetermined solutes* in this definition can be represents by two neighboring or not neighboring peaks the chromatogram. In the case when these are the unretained peak and the last peak of interest, Δs is the separation capacity (s_c) of the analysis and F_{opt} is the flow rate maximizing s_c.

If the void time (t_M) and its optimum ($t_{M,opt}$) are measured along with the measurement of F and F_{opt} (by having unretained solute in the sample) then velocities $u_M = L/t_M$ and $u_{M,opt} = L/t_{M,opt}$ of transporting the solvent molecules from the inlet to the outlet are, respectively, the solvent *transport velocity* [25], (briefly, *velocity*, also known as the *linear velocity* [26-29], the *migration linear velocity* [30], the *unretained peak velocity* [31], the *chromatographic velocity* [29]) and its optimum. The $u_{M,opt}$ can also be found from the measurement of the longes t_M ($t_{M,max}$) when F is the smallest in the experiments (F_{min}), and the shortest t_M ($t_{M,min}$) when F is the largest in the experiments (F_{max}). Once

[1] In this case, $R_s \approx \Delta s/4$ although, as mentioned earlier, so found R_s might not make sense for non-Gaussian peaks, while Eqs. (5), (6) and (7) for Δs are suitable for any peak shape.

193 F_{opt} is found, $u_{M,opt}$, can be found from linear interpolation:

194

195 $$u_{M,opt} = u_{M,min} + \frac{F_{opt} - F_{min}}{F_{max} - F_{min}}(u_{M,max} - u_{M,min}) \quad (8)$$

196

197 where $u_{M,min} = L / t_{M,max}$, $u_{M,max} = L / t_{M,min}$.

198 Typically, the values of F and, therefore, F_{opt} are readily available while measurement of u_M and
199 $u_{M,opt}$ requires the presence of unretained solute in the sample in, at least, two analyses – at F_{min} and
200 F_{max}. On the other hand, F_{opt} is less universal than $u_{M,opt}$. In the case of, e.g., particulate columns, the
201 former depends on the particle size (d_p) and on the *internal diameter* (d) of the column tubing, while
202 $u_{M,opt}$ is essentially independent of d. If F_{opt} and $u_{M,opt}$ for column A are known, then their counterparts
203 for column B having the same structure but possibly different d and d_p can be found as:

204 $$u_{opt,B} = \frac{d_{p,A} u_{opt,A}}{d_{p,B}}, \quad F_{opt,B} = \frac{d_{p,A}}{d_{p,B}} \frac{d_B^2}{d_A^2} F_{opt,A} \quad (9)$$

205 ## 3. Experimental

206 All experiments were made using Vanquish Horizon UHPLC (Thermo Fisher Scientific, Germering,
207 Germany), a C18 core-shell column (Thermo Fisher Scientific, Bellefonte, PA), and ACN in water
208 solvent (both HiPerSolv HPLC grade from VWR, Radnor, PA). Column parameters are listed in Table
209 1. A mixture of uracil, acetophenone, butyrophenone, and valerophenone referenced below as solutes
210 0, 1, 2 and 3, respectively, was analyzed in single-ramp gradient analyses with no initial holds. Several
211 combinations of flow rates and gradient times were used, Table 2. All analyses were designed to be
212 translations of each other with the volume-fraction (ϕ) of ACN changing from 10% to 100% and the
213 gradient solvent volumes, $V_G = Ft_G$, being the same in all analyses.

In addition to the gradient analyses, the plate heights of solutes 2 and 3 were measured in isocratic analyses at all flow rates in Table 2 and outlet solvent composition existed at the elution time of solutes 2 and 3, respectively, in gradient analyses.

Each analysis was repeated 3 times with essentially identical results. The experimental results for run #2 are provided in the Supplementary material (Table S.2 - S.4). They served as the basis for the following discussion.

4. Results

4.1 Gradient analyses

The gradient volumes (V_G) were essentially the same in all analyses (Table 2). As all analyses started at the same solvent composition (ϕ_{init}) and ended at the same ϕ_{end}, they were essentially the translations of each other. As a result, a given solute eluted at essentially the same outlet solvent composition (ϕ_R) in all analyses (Table 3). Two gradient elution chromatograms are shown in Figure 2.

Depending on the flow rate (F), there was substantial (up to 3-fold) difference in the widths of peak 0 and its neighbor peak 1, Table 4. In view of that, Eq. (6) was used for calculation of separations (Δs) of these peaks. The peak width difference in other two neighbors (1-2 and 2-3 pairs) was lower than 10% (column σ-ratios in Table S.2. of the Supplementary material) and, therefore, Eq. (7) could have been used for Δs calculations of these pairs. However, for the sake of their uniformity, Eq. (6) was used in all calculations of Δs. The separation capacity (s_c) of the entire analysis was calculated as the sum, Eq. (4), of the separations of all neighboring peak-pairs. The results are illustrated in Figure 3. The solvent optimal flow rates corresponding to the maxima in the curves of Figure 3 are listed in Table 5 along with optimal velocities calculated from Eq. (8).

4.2 Isocratic analyses

Plate heights (H) and *phase retention factors* [32] (k) of solutes 2 (butyrophenone) and 3 (valerophenone) were measured under isocratic solvent compositions close to their respective elution averages (Table 3) in gradient analyses. The H vs. F plots are shown in Figure 4. The solvent optimal flow rates (F_{opt}) and velocities ($u_{M,opt}$) for each solute are listed in Table 6 along with the solvent compositions (ϕ) in respective isocratic analyses and the solute retention factors (k).

5. Discussion

The goal of this study was not to optimize a particular analysis, but to justify and demonstrate a technique of measurement of the solvent optimal flow rate (F_{opt}) and related parameters in gradient analyses. The lowest separation (Δs) of two peaks (peaks 2-3) at suboptimal F (0.25 mL/min) was larger than 50 (Figure 3c) – more than 50 σ-slots between the peak retention times. This is larger than probably might ever be necessary in practice (Figure 2a). Here, however, the impacts of suboptimal operations are discussed as if the separation of the critical peak-pair and the separation capacity (s_c) of the analysis were barely adequate, and the column optimization was practically important. The discussion is based on the assumption the flow rate (F) optimization in gradient analysis is performed after the solvent composition program has been developed at some F chosen more or less arbitrarily.

Suppose that solutes 2 and 3 were a critical pair. F_{opt} maximizing their gradient separation (Δs) is 1.5 mL/min (Table 5), while F_{opt} minimizing the plate height (H) of one of the solutes in the pair (solute 3) in isocratic analysis is 1.10 mL (Table 6). In other words, F_{opt} for a member of a peak-pair in isocratic analysis was almost 30% lower than F_{opt} for the separation of the pair in gradient analysis.

If the purpose of measuring F_{opt} is to operate the analysis at the highest Δs for a *given column* then 30% error in measuring F_{opt} can have barely noticeable effect.

Example 1. In a packed column with A-term of van Deemter equation being approximately half of H_{min} [33], 30% departure of F from F_{opt} results in 3% larger H and 1.5% lower Δs compared to their respective optimal levels.

However, to reduce the analysis time for a required separation performance, a method developer might choose not to operate the column at F_{opt}, but to operate it at $F > F_{opt}$ (together with using longer column if necessary) [1] with a predetermined ratio $X_F = F/F_{opt}$. According to van Deemter equation, when X_F is large, H is almost proportional to X_F, and, as Δs and s_c are inversely proportional to $\sqrt{H}$, the departure of Δs from Δs_{max} or s_c from $s_{c,max}$ might be proportional to $1/\sqrt{X_F}$, causing a substantial reduction in Δs or s_c compared to their targets. Furthermore, the difference between F_{opt} in actual gradient analyses and in its counterpart found from isocratic measurements might be larger than the 30% observed in our limited set of experiments. Among the reasons for that could be (a) greater than in our experiments dependence of F_{opt} on the solvent composition, (b) ϕ_R calculated from Eq. (3) for a solute and used as ϕ in isocratic analysis of the solute only approximately represents ϕ actually experienced by the solute during its migration in the gradient analysis, (c) gradient distortion [34] making Eq. (3) unsuitable for calculation of ϕ_R and further increasing the difference between ϕ in isocratic analysis of the solute and ϕ actually experienced by it in the gradient analysis. These and other factors together with substantial difference between F_{opt} in isocratic and gradient analyses found in our experiments suggest that the direct measurement F_{opt} in gradient analysis considered herein might lead to its substantially better optimization.

6. Conclusions

Optimal flow rate (F_{opt}) is defined herein as the solvent flow rate (F) maximizing the separation of a predetermined peak-pair or the separation capacity of the entire LC analysis. The theoretical

background and the experimental technique of measurement of F_{opt} in gradient elution analysis were considered and experimentally demonstrated. Alternatively, F_{opt} for a peak-pair in gradient analysis or for the entire gradient analysis can be considered as F minimizing the plate height of a peak in isocratic analysis. General considerations and experimental results show that F_{opt} found under isocratic conditions can be substantially different from that actually optimal for gradient analysis and can result in its substantial under-performance. The experimental results are compiled in the Supplementary material.

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8. Appendix

8.1 Measurement of plate number in gradient elution LC

It follows from Eq. (1) (main text) that F_{opt} minimizing the plate height (H) in a given column can be found as F corresponding to the maximum in the column plate number (N) which, in gradient analysis can be defined as [7, 8]:

The plate number (N) for a solute in gradient LC analysis is the one in isocratic analysis operated under conditions existing in the gradient analysis at the solute retention time

297 To extend Eq. (2) for N in isocratic analysis, one can notice that the retention time (t_R) in isocratic

298 analysis can be expressed as:

299

300 $$t_R = (1+k)t_M \qquad (A.1)$$

301

302 where k is the solute *phase retention factor* [23, 32] (briefly, *retention factor*) – the ratio of the solute

303 amount in the stationary phase to that in the mobile phase. Substitution of Eq. (A.1) in Eq. (2) yields

304 $N = (1+k)^2 t_M^2 / \sigma^2$. A form of this equation valid for isocratic and gradient LC can be expressed as [7,

305 8]:

306

307 $$N = (1+k_R)^2 t_M^2 / \sigma_{iso}^2 \qquad (A.2)$$

308

309 where k_R is k and σ_{iso} is σ – both in isocratic analysis under conditions existing at t_R in gradient

310 analysis[2].

311 Eq. (A.2) shows that three parameters (k_R, t_M and σ_{iso}) should be known in order to compute N for a

312 solute in gradient LC analysis.

313 The effect of F on t_M can be expressed as:

314

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[2] Sometimes k_R of a solute in gradient analysis is defined as $k_R = t_R / t_M - 1$. This quantity can be substantially different from k_R in this report when gradient analysis is considered. Essentially, k_R in this report is the ratio of the solute amount in the stationary phase to that in the mobile phase at the time (t_R) of the solute elution (see general definition of k in comments to Eq. (A.1)).

315 $$t_M = V_M / F \quad \text{(A.3)}$$

316

317 where V_M is the column *void volute* [35] – a column parameter that can be measured as the solvent
318 volume consumed between injection and elution of an *unretained solute*.

319 The peak width (σ_{iso}) in isocratic analysis under conditions existing at t_R in gradient analysis can be
320 different from the actual peak width (σ) at t_R in gradient analysis. Several factors can cause the
321 difference. One is the solute *zone compression* due to negative gradients in the solute velocities [36,
322 37]. The difference can also be caused by the fact that the solvent composition (ϕ) at the location of a
323 migrating solute changes during the analysis. This changes the solute's k. As a result, the solute's H
324 changes during its migration. This affects the width of the eluting solute zone and the width (σ) of the
325 corresponding peak. The difference between σ_{iso} and actual σ in gradient analysis can be expressed via
326 the *peak formation factor* [8]:

327

328 $$G_p = \sigma / \sigma_{iso} \quad \text{(A.4)}$$

329

330 Substitution of Eqs. (A.3) and (A.4) in Eq. (A.2) yields:

331

332 $$N = \frac{(1 + k_R)^2 V_M^2 G_p^2}{\sigma^2 F^2} \quad \text{(A.5)}$$

333

334 Among parameters in the right hand side of this equation, two (F and σ) can be measured in each
335 particular case. Furthermore, V_M is a column parameter. It can be measured or calculated once for each
336 specific column, it does not depend on F, and is treated herein as a known quantity. Due to the
337 changing mobile phase, and thus changing conditions of solute migration in gradient analysis, it is

unclear how to measure the remaining two parameters (k_R and G_p) of a given analyte under these transient conditions. As a result, it is unknown how to find N and, therefore, N_{max}, and eventually F_{opt} defined as F at $N = N_{max}$. Fortunately, this is not practically necessary for uptimizing F in gradient analysis. In the main text of this report, F_{opt} is defined as F resulting in the highest separation of a predetermined peak pair (rather than in the lowes H of the nighest N for a predetermined peak).

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Figures

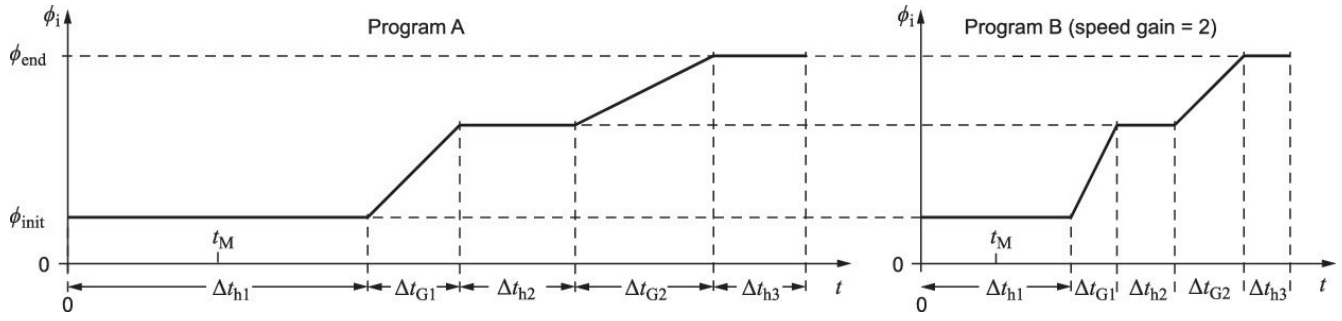


Figure 1. Program A of changing of inlet solvent composition (ϕ_i) in time (t) and its translation (Program B) with the speed gain of 2.

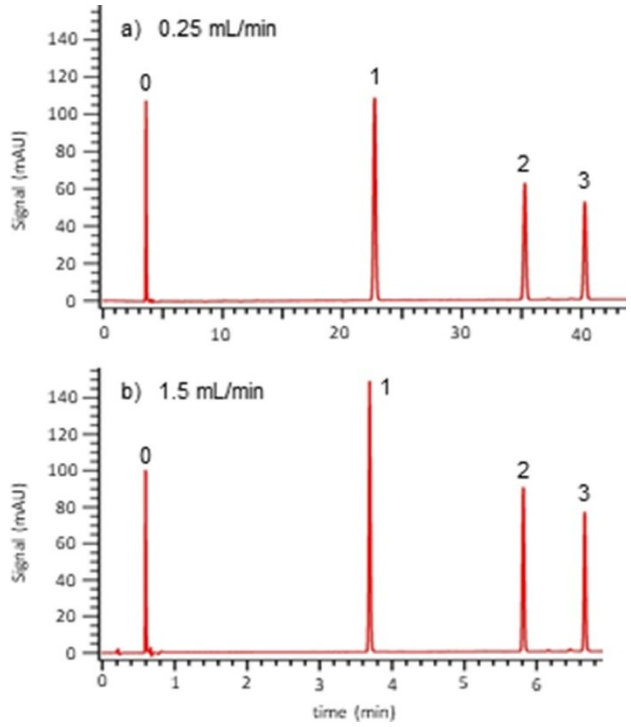
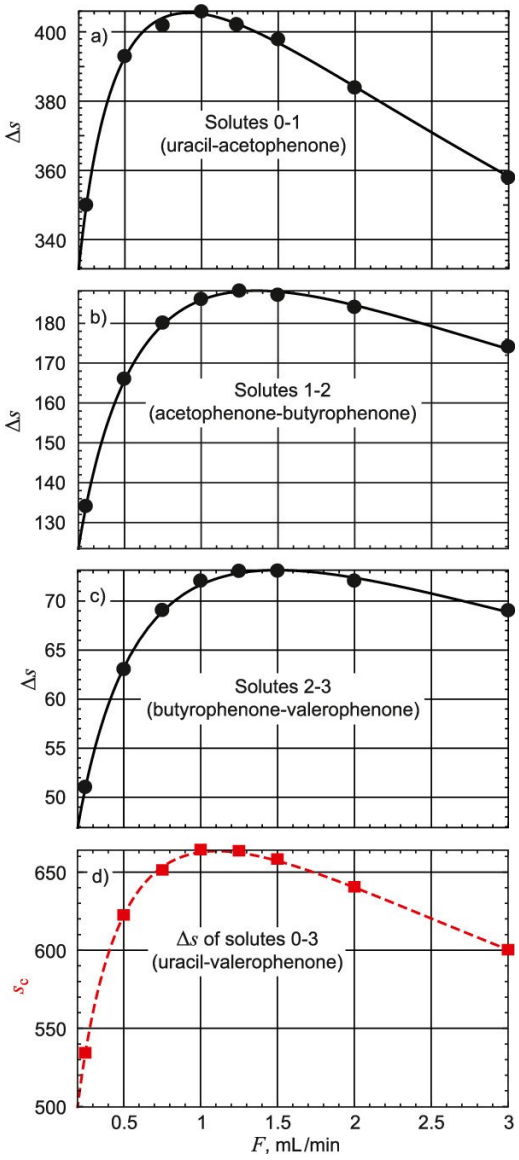


Figure 2. Gradient elution chromatograms at (a) the lowest F in all experiments, and (b) F_{opt} for separation of solutes 2 and 3 (Table 5).



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442 Figure 3. Separations (Δs) of neighboring peak-pairs (panels a-c), and separation capacities (s_c) of the
443 analyses vs. flow rate (F). Each dot in panels (a-c) represents Δs calculated from the measurements
444 using Eq. (6). Each square in paned (d) represents s_c in Eq. (4). The lines are the least-square fits of the
445 curve $1/\sqrt{A+B/F+CF}$ to respective data, reflecting the proportionality of Δs and s_c to $1/\sqrt{H}$ with
446 H represented by the van Deemter model $H = A+B/F+CF$.

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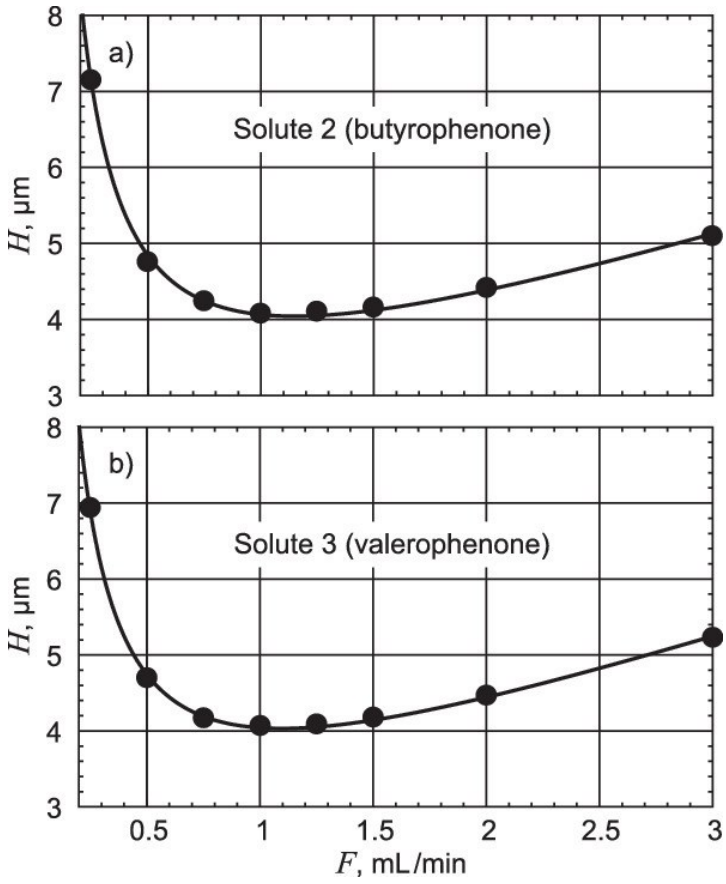

448

449 Figure 4. Plate heights for 2 solutes. The dots represent the measurement results. The lines are the

450 least-square fits of the curve $H = A + B/F + CF$ to respective data.

451

452

Tables

Table 1. Column parameters.

Parameter		Value
Column dimensions	$L\times d$	100 mm×4.6 mm
Temperature	T	25 ºC
Packing porosity	ϵ	0.41
Particle size	d_p	2.6 μm
Particle porosity	ϵ_p	0.25
Shell thickness		0.5 μm
Pore size		80Å

Table 2. Flow rates (F), gradient times (t_G) and volumes (V_G).

F, mL/min	0.25	0.5	0.75	1.0	1.25	1.5	2.0	3.0
t_G, min	64.71	32.208	21.402	16.056	12.852	10.728	8.1	5.436
$V_G = Ft_G$, mL	16.18	16.10	16.05	16.06	16.07	16.09	16.20	16.31

Table 3. Elution solvent compositions (ϕ_{R0}, ϕ_{R1}, ϕ_{R2}, ϕ_{R3}), %, of solutes 0 through 3, respectively, vs. flow rate (F, mL/min).

F	0.25	0.5	0.75	1.0	1.25	1.5	2.0	3.0
ϕ_{R0}	10	10	10	10	10	10	10	10
ϕ_{R1}	36.6	36.1	36.1	35.9	35.9	36	35.8	35.8
ϕ_{R2}	54.1	53.8	53.8	53.7	53.7	53.8	53.7	53.8
ϕ_{R3}	61.	60.8	60.8	60.8	60.8	60.9	60.8	61

Table 4. The widths (σ_0 and σ_1) of peaks 0 and 1 vs. flow rate (F, mL/min).

F	0.25	0.5	0.75	1.0	1.25	1.5	2.0	3.0
σ_0, s	1.8	0.87	0.6	0.45	0.37	0.31	0.25	0.18
σ_1, s	5.4	2.2	1.36	1.0	0.8	0.66	0.51	0.37
σ_1/σ_0	3.0	2.5	2.3	2.2	2.2	2.1	2.1	2.1

463 Table 5. Optimal solvent flow rates (F_{opt}) and velocities ($u_{M,opt}$) for separations of neighboring peak
464 pairs and for separation capacity (s_c) of the analysis (separation of peaks 0 and 3).

Peaks	0-1	1-2	2-3	0-3
F_{opt}, mL/min	0.91	1.36	1.5	1.11
$u_{M,opt}$, mm/s	1.69	2.51	2.76	2.04
Δs_{max}	406	188	73	663

465

466 Table 6. Solvent compositions (ϕ) in isocratic analyses of 2 solutes and the solute parameters.

Solute 2 (butyrophenone)				Solute 3 (valerophenone)			
ϕ	k	F_{opt}	$u_{M,opt}$	ϕ	k	F_{opt}	$u_{M,opt}$
%		mL/min	mm/s	%		mL/min	mm/s
53.8	2.6	1.15	0.666	60.9	2.7	1.10	0.635

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Supplementary material to Measurement of optimal flow rate in gradient elution liquid chromatography

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The purpose of this Supplemental material is to provide the experimental data and calculated parameters evaluated in the main text.

Table S.1. Nomenclature and Definitions

Symbol	Descriptions
A	peak area
F	solvent flow rate
Δs	peak separation, Eq. (6) of main text
s_c	separation capacity of analysis, Eq. (4) of main text
t_G	gradient time
t_M	void time (t_R of uracil)
t_R	retention time
V_G	gradient volume, $V_G = Ft_G$
$\bar{V}_G$	average V_G
w_A	peak area-over-height width, $w_A = A/Y_{max}$
w_b	peak base width
w_h	peak half-height width
Y_{max}	peak height
$\Delta\phi G$	ϕ increment during tG
$\Delta\phi_M$	void increment of ϕ (ϕ increment, $\Delta\phi_G t_M / t_G$, during t_M)
σ	peak standard deviation, $\sigma = w_A/\sqrt{2\pi}$ [1]
σ-ratio	σ/(σ of preceding peak)
ϕ	solvent composition (volume-fraction of stronger solvent)
ϕ_R	outlet ϕ at the time t_R
Isocratic analyses only	
H	plate height, Eq. (1), main text
N	plate number, Eq. (2), main text
k	retention factor, $k = t_R / t_M - 1$

In the tables below, non-shaded entries are the set-points (F, t_G) and parameters (t_R, w_b, w_h, A, Y_{max}) reported by the chromatographic system. The shaded entries were calculated from non-shaded ones using equations in the main text or the ones described in Table S.1, above.

Table S.2. Experimental results of gradient analyses.

F	t_G	t_R	w_h	w_b	A	Y_{max}	t_M	$\Delta\phi_M$	u_M	V_G	$\bar{V}_G$	ϕ_R	w_A	σ	σ-ratio	Δs	s_c
mL/min	min	min	min	min	mAu×min	mAu	min	%	mm/s	mL	mL	1	s	s	1	1	
Uracil																	
0.25	64.71	3.593	0.068	0.120	8.119	107.760	3.593	5.00	0.46	16.18	16.13	0.100	4.521	1.804			
0.50	32.208	1.790	0.033	0.060	4.003	110.410	1.790	5.00	0.93	16.10	16.13	0.100	2.175	0.868			
0.75	21.402	1.192	0.022	0.040	2.637	106.240	1.192	5.01	1.40	16.05	16.13	0.100	1.489	0.594			
1.00	16.056	0.894	0.017	0.030	1.972	105.770	0.894	5.01	1.86	16.06	16.13	0.100	1.119	0.446			
1.25	12.852	0.716	0.014	0.020	1.578	102.930	0.716	5.01	2.33	16.07	16.13	0.100	0.920	0.367			
1.50	10.728	0.598	0.012	0.020	1.311	100.130	0.598	5.02	2.79	16.09	16.13	0.100	0.786	0.313			
2.00	8.1	0.450	0.009	0.020	1.052	102.950	0.450	5.00	3.70	16.20	16.13	0.100	0.613	0.245			
3.00	5.436	0.302	0.007	0.01	0.7093	95.8	0.302	5.00	5.52	16.31	16.13	0.100	0.444	0.177			
Acetophenone																	
0.25	64.71	22.712	0.211	0.360	24.447	108.380	3.593	5.00	0.46	16.18	16.13	0.366	13.534	5.399	2.99	350	
0.50	32.208	11.143	0.085	0.150	12.363	135.100	1.79	5.00	0.93	16.10	16.13	0.361	5.491	2.190	2.52	393	
0.75	21.402	7.389	0.053	0.090	8.310	146.340	1.192	5.01	1.40	16.05	16.13	0.361	3.407	1.359	2.29	402	
1.00	16.056	5.517	0.038	0.070	6.203	149.610	0.894	5.01	1.86	16.06	16.13	0.359	2.488	0.992	2.22	406	
1.25	12.852	4.416	0.03	0.050	4.955	150.150	0.716	5.01	2.33	16.07	16.13	0.359	1.980	0.790	2.15	402	
1.50	10.728	3.692	0.026	0.040	4.116	148.380	0.598	5.02	2.79	16.09	16.13	0.360	1.664	0.664	2.12	398	
2.00	8.1	2.773	0.02	0.030	3.027	140.830	0.45	5.00	3.70	16.20	16.13	0.358	1.289	0.514	2.10	384	
3.00	5.436	1.863	0.014	0.02	2.0301	131.49	0.302	5.00	5.52	16.31	16.13	0.358	0.926	0.370	2.09	358	
Butyrophenone																	
0.25	64.71	35.271	0.23	0.390	15.331	62.490	3.593	5.00	0.46	16.18	16.13	0.541	14.720	5.873	1.09	134	
0.50	32.208	17.459	0.093	0.160	7.823	78.970	1.79	5.00	0.93	16.10	16.13	0.538	5.943	2.371	1.08	166	
0.75	21.402	11.601	0.056	0.100	5.257	86.770	1.192	5.01	1.40	16.05	16.13	0.538	3.635	1.450	1.07	180	
1.00	16.056	8.690	0.041	0.070	3.927	89.320	0.894	5.01	1.86	16.06	16.13	0.537	2.638	1.052	1.06	186	
1.25	12.852	6.959	0.032	0.050	3.149	90.530	0.716	5.01	2.33	16.07	16.13	0.537	2.087	0.833	1.05	188	
1.50	10.728	5.816	0.027	0.050	2.616	89.980	0.598	5.02	2.79	16.09	16.13	0.538	1.744	0.696	1.05	187	
2.00	8.1	4.381	0.04	0.040	1.443	64.510	0.45	5.00	3.70	16.20	16.13	0.537	1.342	0.535	1.04	184	
3.00	5.436	2.947	0.015	0.02	0.9617	60.81	0.302	5.00	5.52	16.31	16.13	0.538	0.949	0.379	1.02	174	
Valerophenone																	
0.25	64.71	40.288	0.235	0.400	13.058	52.240	3.593	5.00	0.46	16.18	16.13	0.610	14.998	5.983	1.02	51	600
0.50	32.208	19.976	0.094	0.160	6.699	66.850	1.79	5.00	0.93	16.10	16.13	0.608	6.013	2.399	1.01	63	640
0.75	21.402	13.284	0.057	0.100	4.497	73.280	1.192	5.01	1.40	16.05	16.13	0.608	3.682	1.469	1.01	69	658
1.00	16.056	9.955	0.041	0.070	3.363	75.580	0.894	5.01	1.86	16.06	16.13	0.608	2.670	1.065	1.01	72	663
1.25	12.852	7.974	0.033	0.060	2.686	76.520	0.716	5.01	2.33	16.07	16.13	0.608	2.106	0.840	1.01	73	664
1.50	10.728	6.664	0.027	0.050	2.233	76.330	0.598	5.02	2.79	16.09	16.13	0.609	1.755	0.700	1.01	73	651
2.00	8.1	5.023	0.021	0.040	1.140	50.790	0.45	5.00	3.70	16.20	16.13	0.608	1.346	0.537	1.00	72	622
3.00	5.436	3.381	0.015	0.03	0.7607	47.77	0.302	5.00	5.52	16.31	16.13	0.610	0.955	0.381	1.01	69	534

Table S.3. Experimental results of isocratic analyses at ϕ = 53.8%.

F	t_R	A	Y_{max}	w_b	w_h	w_A	σ	N	H	k
ml/min	min	mAu×min	mAu	min	min	s	s	1	µm	1
Uracil										
3.00	0.268	0.644	111.13	0.01	0.005	0.348	0.139	13430	7.45	0
2.00	0.398	0.975	116.60	0.01	0.007	0.502	0.200	14237	7.02	0
1.50	0.529	1.313	120.37	0.02	0.010	0.654	0.261	14777	6.77	0
1.25	0.633	1.586	121.77	0.02	0.012	0.781	0.312	14848	6.73	0
1.00	0.790	1.988	123.98	0.02	0.014	0.962	0.384	15259	6.55	0
0.75	1.052	2.660	125.27	0.03	0.019	1.274	0.508	15427	6.48	0
0.50	1.577	3.987	127.71	0.05	0.028	1.873	0.747	16030	6.24	0
0.25	3.155	7.989	122.35	0.10	0.060	3.918	1.563	14671	6.82	0
Butyrophenone										
3.00	0.960	1.399	81.52	0.03	0.016	1.030	0.411	19661	5.09	2.58
2.00	1.426	2.119	89.30	0.04	0.022	1.424	0.568	22683	4.41	2.58
1.50	1.891	2.859	93.61	0.05	0.028	1.833	0.731	24083	4.15	2.57
1.25	2.263	3.448	94.97	0.06	0.034	2.178	0.869	24415	4.10	2.58
1.00	2.824	4.313	95.53	0.07	0.042	2.709	1.081	24578	4.07	2.57
0.75	3.762	5.769	94.05	0.10	0.057	3.680	1.468	23634	4.23	2.58
0.50	5.652	8.618	88.25	0.15	0.091	5.859	2.338	21047	4.75	2.58
0.25	11.343	17.225	71.69	0.38	0.225	14.416	5.751	14004	7.14	2.60
									Average:	2.58

Table S.4. Experimental results of isocratic analyses at ϕ = 60.9%.

F	t_R	A	Y_{max}	w_b	w_h	w_A	σ	N	H	k
ml/min	min	mAu×min	mAu	min	min	s	s	1	μm	1
Uracil										
0.25	3.152	8.859	135.21	0.10	0.060	3.931	1.568	14541	6.88	0
0.50	1.573	4.434	141.55	0.05	0.028	1.879	0.750	15848	6.31	0
0.75	1.050	2.957	139.79	0.03	0.019	1.269	0.506	15479	6.46	0
1.00	0.788	2.218	138.40	0.02	0.014	0.962	0.384	15188	6.58	0
1.25	0.632	1.769	136.81	0.02	0.012	0.776	0.310	15007	6.66	0
1.50	0.527	1.472	135.16	0.02	0.010	0.654	0.261	14704	6.80	0
2.00	0.397	1.091	129.85	0.01	0.008	0.504	0.201	14038	7.12	0
3.00	0.267	0.713	123.11	0.01	0.005	0.348	0.139	13339	7.50	0
Valerophenone										
0.25	11.523	14.384	59.83	0.38	0.225	14.425	5.755	14434	6.93	2.656
0.50	5.724	7.248	73.76	0.16	0.091	5.895	2.352	21323	4.69	2.639
0.75	3.822	4.832	78.16	0.10	0.057	3.709	1.480	24017	4.16	2.640
1.00	2.874	3.626	79.00	0.07	0.042	2.754	1.099	24640	4.06	2.647
1.25	2.308	2.900	78.44	0.06	0.034	2.218	0.885	24488	4.08	2.652
1.50	1.931	2.409	77.02	0.05	0.029	1.876	0.749	23958	4.17	2.664
2.00	1.458	1.790	73.36	0.04	0.022	1.464	0.584	22434	4.46	2.673
3.00	0.984	1.180	66.23	0.03	0.016	1.069	0.427	19152	5.22	2.685
									Average:	2.657

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