Chapter 1
Aspects of Gradient Optimization

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1.1 Introduction

Gradients are versatile and therefore find wide application. For example, gradients are just as essential in method development of unknown samples as for quantification at trace levels. The theoretical background of gradient elution is quite complex, because what happens in the column during gradient elution, compared to isocratic separations, is affected by more factors; these sometimes act in opposite directions or are multiplicative.

Herein, we will focus on selected aspects of the optimization of gradient separations in RP chromatography in deliberately simple form. Other important aspects of the gradient such as theory, equipment, and troubleshooting are left to other sources [1–4]. First, we briefly describe the action of a gradient in the column, then using some basic formulas we discuss the characteristics/features of the gradient. On the basis of this, possibilities for optimization of the following objectives will be shown: low detection limit, high peak capacity, sufficient resolution, and the shortest possible retention times. Finally, there is a summary with some basic rules and recommendations.

1.2 Special Features of the Gradient

In HPLC, interactions of different strengths between the analytes on the one hand and eluent components and the stationary phase on the other usually occur during separation. In the case of isocratic separations there is a predetermined, constant eluent composition, consequently during chromatography an interaction of constant strength takes place between the eluent molecules and the phase material.

What happens now in a gradient run? During gradient separations the strength of the mobile phase increases, consequently its interaction with the stationary phase also increases during the gradient run. The rule in RP chromatography is: the more organic, nonpolar/hydrophobic the eluent becomes during the separation (more % B, ACN or MeOH), the stronger its interaction with the organic, nonpolar surface of an RP material becomes – it is indeed "like with like," that

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means the nonpolar ACN or MeOH molecules naturally "like" for example nonpolar C18 alkyl groups.

In the course of a gradient, because of the ever increasing concentration of ACN/MeOH molecules, the substance molecules become subject to increasingly strong competition in their interactions with the C18 alkyl groups. Because of this, the substance molecules are increasingly forced to leave the stationary phase faster, go into the mobile phase earlier and thus elute earlier compared to isocratic separations. With 100% MeOH or ACN at the end of the gradient even the very hydrophobic components of the sample elute, maybe even persistent organic contaminants that may have accumulated on the surface of the stationary phase – as a side effect the column is flushed at the same time.

Focusing on the peak form, with gradients we have two opposing trends. On the one hand, the later the peaks elute, the more the substance zone is subject to dispersion processes in the column and thus band broadening initially increases – analogous to isocratic separations. On the other hand, the acceleration of the migrating substance zone increases to the same extent, since the elution strength of the eluent permanently increases from the beginning to the end. As a result, these effects compensate each other and with a gradient we usually have narrow peaks. Note that with a gradient the concentration of the elution band constantly increases leading to lower band broadening in comparison with isocratic separations, consequently resulting in low detection limits.

This is true both for the front and for the end part of the chromatogram, in the ideal case the peak width remains constant. For this reason, in conjunction with the gradient speaking of a "plate number" is not allowed. The plate number, a measure of band broadening, is defined only for isocratic conditions. The phenomenon described here means, among other things, that in practice a reduction in packing quality and a suboptimal hardware (system dead space), which with isocratic separations leads to broad peaks, is not as noticeable with gradient separations. Even with "poor" equipment and "poor" columns chromatograms from a gradient elution look good, especially if the gradient is steep and ACN is used as the organic content of the eluent – a welcome fact for sample chromatograms in manufacturer's brochures …

Positive from the user perspective is, that simple gradient separations using 20–50 mm columns on conventional equipment generally prove to be no problem, at least as far as the peak shape is concerned. Also the advantage of smaller particle sizes, for example 2 or 3 µm particles compared to 5 µm particles, is less relevant in many applications. In the case of a difficult matrix, 3.5–5 µm material should therefore initially be considered. Unless one has to separate a large number of very similar analytes – then of course the separating efficiency of $\leq$ 2 µm particles also becomes relevant for gradients. In this context, it is also pointed out that as the eluent permanently becomes stronger (= nonpolar), the migrating substance molecules at the end of a peak, i.e., at the trailing edge, move faster than those at the beginning of the peak as the later eluting molecules of the substance band are always pushed "forward" faster. This fact, known as "peak compression," has the effect that in gradient separations tailing is rarely observed.

Peak symmetry is about 10% better compared to an isocratic run with equivalent eluent composition (H.-J. Kuss, personal communication).

1.3 Some Chromatographic Definitions and Formulas

Let us now consider some chromatographic definitions which are known from theory – which, by the way, was developed originally for GC and much later for isocratic LC separations. The derivation of the formulas used below is omitted, they are only used to elaborate the consequences for practical optimization. For a more detailed discussion, see references [2–4] and in particular [1].

The resolution R is, in simplified form, the distance between two peaks on the baseline. The retention factor k (formerly the capacity factor k') is the ratio of the time a component spends in/on the stationary phase and in the mobile phase, that is the quotient of the net retention time t'_R (time spent in the stationary phase) and the flow-through or dead or mobile time t_0 and t_m (time spent in the mobile phase). It thus represents a measure of the strength of the interactions of *these* components on *this* column under *these* conditions: $k = t'_R/t_0$. However, the retention factor is not constant for a gradient. Very high at the beginning (with 100 or 95% water/buffer the substances literally "stick" to the beginning of the stationary phase), it becomes less during the separation and at the end of the gradient is very small. With 90 or 100% MeOH or ACN, the substance molecules hardly have a chance to stay on the stationary phase, because the competition for the "attraction" of the C18 group has now become huge. Put simply, with a gradient from 100% water/buffer to 100% MeOH/ACN, the k value at the beginning is virtually infinite – in some references numbers between 3500–4000 are given – and at the end almost zero. Since the k-value changes during gradient elution, a k^* value (or $\overline{k}$) was introduced to take account of this particular feature [1]: this is the k-value of a component when it is just in the middle of the column.

Although the need for such a term to describe the gradient may be questioned, the k^* value is used here because it has advantages for our deliberations. And that the interactions, and therefore a measure for them, a retention size, is important for optimization considerations, is clear – however such a term may be defined.

The separation factor α is the quotient of the retention factors of two components that one wishes to separate, k_1 and k_2, and describes the ability of the chromatographic system to separate these two components. In the literature, different formulas are used for R and k^*. However, they are quite similar and ultimately lead, when the focus is on the practice, to similar numerical values and thus to similar propositions.

Here is an example: in Eq. (1.1) for the second term (the selectivity term), in addition to $(\alpha - 1)$ the terms $\ln \alpha$ or $\alpha - 1/\alpha$ are also to be found in the literature. Assuming a α-value of 1.05, the following numerical values for the selectivity term are found: 0.048, 0.049, and 0.050. However, these different numbers affect the value of the resolution only in the second decimal place.

Five simple equations are given below. They are sufficient to draw conclusions for practical optimization.

$$R = \frac{\sqrt{N}}{4} \cdot (\alpha - 1) \cdot \frac{k}{1+k} \tag{1.1}$$

$$k^* = \frac{t_G \cdot F}{\Delta\%\,B \cdot V_m \cdot S} \tag{1.2}$$

$$\alpha = \frac{k_2}{k_1} \tag{1.3}$$

$$k = f\left(\frac{V_D}{V_m}\right) \tag{1.4}$$

$$n_c = \frac{t_{Rl} - t_{Re}}{w} \quad \text{or} \quad n_c = \frac{t_G}{w} \tag{1.5}$$

With:

R	Resolution;
N	Number of theoretical plates, defined fundamentally for isocratic conditions;
α	Separation factor (formerly selectivity factor);
k	Actual (measured) retention factor of a component;
k^*	Retention factor of a component in the middle of the column;
t_G	Gradient duration;
F	Flow;
Δ %B	Difference between the start and end concentration of the organic component in the mobile phase;
V_m	Hold-up volume of the column (also referred to as flow-through or mobile volume, which is the volume of the mobile phase in the column). This corresponds to the geometric volume of the column minus the skeletal volume of the stationary phase and is sometimes referred to as the "effective volume" of the column. Simplified, V_m may be set equal to the volume of the column);
S	Constant (resulting from the structure of the analyte and the chromatographic conditions);
V_D	Dwell or delay volume (volume between the mixing chamber and the column);
n_c	Peak capacity;
t_{Rl}	Retention time of the last peak;
t_{Re}	Retention time of the first peak;
w	Peak width.

The product of $t_G \times F$ is called the gradient volume.

Comments on Eqs. (1.2)–(1.4), or: Isocratic vs. Gradient Separations

In accordance with Eq. (1.2), a change in the flow rate and/or the column dimensions results in a change of the k^* value. Note that during a gradient this influence fundamentally affects the retention factor – regardless of whether the

middle, k^*, or the actually measured, k, is considered. Furthermore, according to Eq. (1.4), the dwell volume as well as the column volume influence the k^*/k value. A change in these factors can now fall out differently for different analytes. Since $\alpha = k_2/k_1$ is true, then consequently the selectivity can also change!

For example, after finding a suitable stationary phase during method development a user then employs a longer column with the identical phase material and is surprised that the selectivity/elution order changes. Or during method transfer: the instruments used are virtually identical, the volumes of the mixing chambers are identical, nevertheless there could possibly be very small differences in the dwell volume present caused for example merely by differences in the loop volumes in the autosamplers (see also Chapter 2). In this case too, not only the well-known changes such as retention time, peak shape, and resolution should be expected, but also a possible change in the selectivity or even the elution order as well, because – see Eq.(1.4) – V_D remains constant but V_m changes, this is especially noticeable with very short/thin columns.

The problems described here are noticed more often when the sample contains so-called irregular components, see below. Conversely, in isocratic systems a larger dead volume (= volume between the autosampler and the detector – without the column) leads "only" to broader peaks and thus to a deterioration of the resolution. Analogously, with isocratic separations a longer column affects the retention time, peak shape, and resolution. Unlike gradient separations, the length of the column can change neither the selectivity nor the elution order!

And in this context one last example: – assume a component elutes in isocratic mode at a flow rate of 1 ml/min and with a given eluent composition in 10 min. When the flow is increased to 2 ml/min, the component elutes after 5 min, because it "goes along with" the new speed, the time it spends in the column is reduced by a factor of 2. Suppose now that a component in the gradient mode leaves the stationary phase when the eluent contains 40% B, from then on it travels at the speed of the eluent – as usual. Let us further assume that the gradient reaches this 40% B after 10 min, the substance elutes after 12 min. That means, the substance stays on the stationary phase for 10 min, and migrates through the column at a flow of 1 ml/min in 2 min. With an increase of the flow to 2 ml/min, the substance still stays on the stationary phase for 10 min – the modified flow rate with which the eluent molecules now travel does not affect the interactions. The time spent in the mobile phase decreases, of course, by a factor of 2 (from 2 to 1 min), the component now elutes after 11 min. This means that a higher flow brings the molecules forwards faster, what's most important however has already been achieved by the elution strength. Thus, with gradient elution, the elution strength with which a component leaves the stationary phase is much more important for the retention time than the flow.

In isocratic separations the flow is the driving force, in gradient separations, it is the slope, in end effect % B/ml. Thus, an increase in the flow by a factor of 2 – under otherwise constant conditions – leads to a decrease in the retention time of only about 5–15%, depending on the gradient slope. These considerations regarding retention time apply correspondingly to the column length. Under otherwise constant conditions, a shorter column results – in contrast to isocratic separations – in just a marginally shorter gradient duration (example

chromatograms are shown further below). And finally, as both the k^* and k_e values ($k_e = k$-value at the moment of elution from the column) as well as the peak widths for the early and the late peaks in the ideal case remain constant or are very similar, the following applies: in contrast to isocratic separations, the resolution between the early peaks of the chromatogram is not in principle worse than that between the later peaks. The simple formula "early peaks, poor resolution" does not apply with gradients – in gradient separation the resolution is a value that can behave very "individually."

Note:
In isocratic separations, physical parameters such as flow, column dimensions, and dead volumes affect the retention time and also, through the peak shape, the resolution; these parameters do not intervene in the interactions ("chemistry"). With gradient separations however, further to this the selectivity and elution order can also change; furthermore these changes may be different at the beginning of the gradient than at the end.

1.4 Detection Limit, Peak Capacity, Resolution – Possibilities for Gradient Optimization

1.4.1 Detection Limit

The three main objectives of optimization are: low detection limit, "good" separation and, last but not least, fast separation. We start with the detection limit. Perhaps this task is the easiest – at least as far as the implementation of possible measures is concerned. Here applies, with the aim of a low detection limit, – analogous to isocratic separations:

- Optimal wavelength but also optimal settings – very important especially for small, early peaks, for example: small time constant ("Rise Time," "Filter Time," "Response Time," "Dwell Time"), large data recording rate ("sample rate," "sampling time"), large bandwidth, large gap ("slit"), and "appropriate" reference wavelength with the DAD (see also Chapter 2).
- Small dead volume: short and especially thin capillaries, small cell volume – but with the longest possible path in the case of a UV detector (see also Section 1.2).
- One should ensure a good peak/noise ratio, for example: measure more sensitively (the peak/noise ratio will become more favorable), optimal state of detector parts (e.g., no deposit in the UV cell, no "blind" mirror), no corroded circuit boards, no deposits on the MS interface, clean electrode surface in an electrochemical detector, etc. When necessary, the electronic noise of AD converters and other interfaces should be reduced by using electronic dampers.
- One should ensure a concentration/focusing of the substance zone at the column head ("On Column Concentration"). The sample solution should be weaker – i.e., more polar – than the starting eluent, dilute the sample solution with water or add a neutral salt or buffer.

- Make use of miniaturization: use shorter and above all thinner columns – be careful of possible overloading with the main peak/the matrix – use smaller particles/Fused Core materials.
- Increase the temperature (important!).

And now, very briefly with a gradient:

1. Small gradient volume.
2. a) In the case of very early, chemically similar peaks, start with "a lot" of water/buffer to achieve an "On Column Concentration."
   b) With simple separations and not too polar components, start with 50–60% B.
3. Use a short, steep gradient with higher final concentration of % B. The steeper the gradient, the sooner the eluent brings the components to the column outlet and the lower the volume of mobile phase needed will be.

By these means the peaks elute early, they are narrow and high. Note that the steeper the gradient, the more similar the peak widths and the more similar – and smaller – the band broadening. However, a steeper gradient often means – depending on the wavelength with a UV detector and the solvents and eluent additives used – a noticeable drift, which can be counterproductive. Here it is necessary to find a reasonable compromise between the initial % B, wavelength, and steepness. Conversely, the steepness of the gradient is rarely a serious problem in LC-MS coupling, with aerosol detectors a steep gradient is even beneficial for the detection limit. Finally, in certain cases a concave or convex gradient profile may be thought about, when the detection limit of certain peaks specifically in the rear or the front section of the chromatogram is to be improved.

1.4.2 Peak Capacity and Resolution

Usually peak capacity is defined as the number of peaks per unit time, see Eq. (1.5). The peak capacity as a separation criterion proves to be important when very similar components have to be separated, such as components of a homologous series, for example oligomers; in this case one expects equidistant spacing of the peaks. With similar components, we can hardly expect different interactions and thus a good selectivity. The situation is similar in the case of complex mixtures and/or a difficult matrix. Again, realistically selectivity does not bring us any further. A separation in this case will be possible, if the many (similar?) components can be eluted as narrow peaks distributed throughout the chromatogram.

Conversely, the more the components differ, the more the peak capacity recedes into the background and the "chemistry" moves into the foreground, because here we would at least have the chance to induce different interactions and thereby to very effectively improve the selectivity and subsequently the resolution. Simply put, this means that with similar components and resulting lower selectivity, the (important) selectivity term in Eq. (1.1) recedes into the background and the resolution required can only be achieved by increasing the efficiency terms – assuming reasonably strong interactions.

How can the peak capacity now be increased? The three main factors are: the largest possible gradient volume, gradients as steep as possible (keeping the gradient volume constant!), and peaks as narrow as possible. These in turn initially mean the following: a long column, a long gradient plus high flow, higher end % B. The more efficient the column (high plate number, which means a long column filled with small particles), the longer the gradient should be to achieve good peak capacity. The shorter the column, the less important the gradient duration becomes; it is not worthwhile running long gradients with short columns. Note that a long gradient alone has little effect; a gradient twice as long leads to an increase in the peak capacity of only about 20%, because the components elute at lower elution strength, the peaks become broader – and of course the peak width is determined by the % B with which the component elutes. Note also that the benefit of a longer gradient becomes less the larger the ratio gradient volume to column volume, V_G/V_m, becomes. In practice, it is hardly worth going above a factor of 30.

When UHPLC is available, it is additionally recommended to use a long column filled with sub 2 µm particles – one could also possibly think of core shell or monolith columns, see Chapter 5 – at a high flow, run a steep gradient and additionally increase the temperature. The following trick, with many similar components, often leads to good resolution *and* narrow peaks: start with high % B and run a relatively flat gradient. For further information regarding peak capacity, see Chapter 2.

Resolution

Let us now turn to the objective of "good" resolution, which is by far the most difficult case. Let's make the following clear: good resolution means, simply put, a large distance between two peaks at the peak base. However, it may be that not only one or two critical pair(s) exist, but rather complete regions in the chromatogram which are critical. The focus during optimization then lies on a good resolution in this region of the chromatogram. If I have to separate "everything" well (critically question whether this is really necessary), then the sum of all the resolutions will become the most significant separation criterion and we are back to the peak capacity again, see further above. From Eq. (1.1) can be seen that the resolution is influenced by an efficiency, a selectivity, and a retention term. An improvement of the resolution is achieved by increasing the values of these three terms: that means the largest possible number of plates, a good selectivity – interactions of different strength between the components of interest and the stationary phase – as well as strong interactions in general.

Number of Theoretical Plates – Efficiency Term

A good efficiency, meaning a high number of plates, is generally desirable. However, in the case of a gradient for "normal" separation this is of secondary importance. Firstly, the plate number is under the root and even an increase in the number of plates by as much as a factor of two (for example, from a conventional plate number of 10 000 to an – in UHPLC in practice achievable – plate number of 20 000) leads to an increase in resolution of $\sqrt{2}$, a factor of only 1.4 – and

this under isocratic conditions! And secondly, especially with a gradient the plate number plays a subordinate role: as explained above, because of the permanently increasing elution strength the peaks are always narrow, the plate number is large and in the ideal case constant.

Is a higher separating power nevertheless required? In this case, this should be aimed for by the use of smaller particles rather than a longer column, unless you're dealing with a very difficult matrix and/or the robustness is in the forefront and not the time. A brief explanation is allowed at this point. For both isocratic and gradient separations the following applies: Chromatography is a dilution process, a broadening of the substance zone always takes place, the diffusion of the molecules plays a part in this. This contribution is, given a similar molecular weight of the analytes, practically constant, moreover with a gradient very small due to the reasons shown above.

Furthermore, in the case of slow kinetics another – sometimes substantial – contribution to peak broadening must be reckoned with. Because of, for example, additional ionic interactions – but also complex formation or displacement of equilibria due to an inadvertent pH gradient during the run – wide/tailing peaks can also be obtained with gradient runs. Regarding peak broadening due to slow kinetics, see below.

Separation Capability – Selectivity Term

In practice, we have to deal with α-values between about 1.02 and 1.1, in the case of substantially differing components perhaps of 1.2. Selectivity is by far the most sensitive function for the resolution, even a minimal improvement in the α-value leads to a dramatic increase in resolution, for details and numerical examples see Chapter 3. In that Chapter, and also in Chapter 5 (RP columns), it is reported in detail how the selectivity can be influenced. Let's go back to the gradient: $\alpha = k_2/k_1$, i.e., all factors that influence the retention factor (Eq. (1.2)) can also influence the selectivity – and in any case the resolution. We will come back to this further below.

Strength of the Interactions – Retention Term

The value of this term asymptotically approaches the number 1. For instance, with a k^* value of 5 the retention term has a value of 0.84 and with a k^* value of 20 a value of 0.95.

Let us look now in more detail at Eq. (1.2): many an interesting conclusion for routine use can be derived from this. In Section 1.6 some example chromatograms are shown which illustrate these findings.

$$k^* = \frac{t_G \cdot F}{\Delta\%\,B \cdot V_m \cdot S}$$

As with any fraction, here the k^* value increases when the numerator increases or the denominator decreases. What does this mean in concrete terms?

- The k^* value, and as a result the resolution, increase when the gradient volume increases. An increase in the gradient volume through the flow (constant gradient duration, increase in the flow rate) has the charm that while the re-

tention time remains constant, the resolution can improve. Conversely, am I satisfied with the resolution? In this case, it is necessary to check that, for example, with a gradient shorter by a factor of 2 and a flow higher by a factor of 2, I am achieving about the same resolution – but in the half of the time (constant gradient volume). The context discussed here also leads to the following statement: the shorter the gradient, the higher the flow should be, otherwise one does not reach a sufficiently large gradient volume, which may be necessary for the separation. Disadvantages of an increase in flow could possibly be the following:
Regarding quantification: with concentration-sensitive detectors (DAD, FLD, etc.), decrease of the peak area. Because of the increase in flow the components elute at a lower elution strength, the peak volume increases, thereby the dilution too, the concentration at peak maximum decreases.
Regarding resolution: peak broadening, and as a result a reduction in the resolution, is possible with larger particles such as 5 µm and/or methanol/water, because of the viscosity. With multiple mechanisms as in the case of EPG or mixed-mode phases, HILIC, charged molecules, isomers etc., peak broadening is probable. In both cases one is working in the C-term of the Van Deemter equation, the slow kinetics have already caused broad peaks, which even in the gradient mode could be difficult to cope with.
The disadvantage repeatedly mentioned in this context, namely the shortening of the life of the column due to the increased pressure at higher flow rates, is often overestimated. Firstly, the silica-based columns are more robust than sometimes feared, secondly, as a rule the time saved through higher flow rates is out of all proportion compared to the price of a column and especially thirdly, what should count is not the absolute lifetime of the column, but rather the number of injections per unit of time – and that remains (theoretically) constant. For example, in the half of the time (column lifetime reduced by a factor of 2 due to the pressure being increased by a factor of 2), I have performed the same number of injections.
Finally, the legitimate question: "All well and good, should I now opt for a longer gradient or for a higher flow"? The simplified answer is: is the ultimate resoluton in the foreground and does the run time play a secondary role? In addition to this, does one have a small difference in % B? When "yes," then in this case you should increase the gradient duration. Is the separation problem not that difficult? Then you should increase the flow. The possible minimal decrease in resolution is likely to have little impact, however you are finished quicker. Think in this context of the following analogy: with a gradient, the gradient duration corresponds to the aqueous fraction with an isocratic separation. In both cases, an increase leads to small, broad peaks, longer retention times, and better overall resolution (sum of the resolution between all peaks).

- Δ% B and V_m should be small, which means first of all a flat gradient as well as a short column. Even if one would intuitively always use a longer column to improve the resolution, examples with proteins, but also with smaller molecules, which are not shown here, show that in fact a shorter column can lead to a possible improvement of the resolution. This is because, according to Eq. (1.2), a longer column leads to a decrease of k^*; conversely, a longer column means

a higher plate number. As mentioned above, the plate number plays a subordinate role in a gradient, the advantage of a per se higher plate number can only partially cancel out the first-mentioned disadvantage. Note that with a gradient, the column volume is less significant compared to the gradient volume. Numerous measurements have shown that for up to about 20–25 peaks a 125 mm column is usually long enough – for a common problem even a 50 mm column is often completely sufficient.

Let us summarize once again the influence, discussed above, of the numerator and the denominator on the k^* value in Eq. (1.2) in the following statements – certainly not to be generalized for all cases:

- With short gradients and large Δ% B (from approximately 60% B, often the case with generic overview-gradients), initially a higher flow would be recommended, that means the gradient volume necessary should be achieved by means of the flow and not the gradient duration.
- The flatter the gradient (smaller Δ% B), the smaller is the advantage of an increased flow. The gradient becomes more "isocratic," in the case of complex mixtures the increase in the peak widths can lead to a decrease in resolution. When it has already been decided in favor of a flat gradient and on top of this, for example through secondary equilibria, the kinetics are also slow, then in *this* case it is not to be recommended to needlessly provoke an increase in band broadening by increasing the flow rate.
- Likewise, the flatter the gradient, the less important is the gradient duration: a smaller Δ% B in the denominator results in a sufficiently large k^* value, a large gradient volume – simply by means of a long gradient – is not all that necessary.
- The larger Δ% B is, the smaller the k^* value will be – leading to a decrease in the resolution, which may be counteracted by increasing the flow. Regarding this point please note the following aspect: when the flow is increased, the retention time of the peaks, and also that of the dead time, t_0, decreases. But the quotient t_{R1}/t_0 increases, which means, the first peak can be separated better from the front.
- A similar observation: when a long column is used (as with Δ% B, V_m would increase in the denominator), the flow should be increased. Apart from the above-mentioned cases, for example slow kinetics, etc., the advantage of an increase in gradient volume through the flow outweighs the disadvantage of the decrease in the plate number. Since the column volume should be small (V_m in the denominator!), the shortest possible columns should be used, especially with gradient separations, see the examples below.
- When steep gradients are run, it is also counterproductive to use a long column: the k^*-value of the components tends quickly towards 0, the length of the column will not be used, the result is a rather small but nevertheless unnecessary band broadening.

Rapid separations are often sought. A simple way in practice is to increase the flow rate, in any case rather than using a shorter column, which – when actually present – must first be installed and flushed, etc. Therefore, it is allowed here again to think finally about an increase in the flow. The flow should be increased

in the following cases: large difference in % B, ≤ 3 µm particles, long column, long gradient, fast kinetics. This measure should be critically questioned in the following cases: in particular LC-MS modes (see Section 1.2), at trace levels with DAD/FLD detection, multiple interactions and as a result slow kinetics – the use of ≥ 5 µm particles for analytical separations is likely to be the exception nowadays. By increasing the flow rate the gradient volume increases, and thereby the resolution can also at first increase. But the later eluting molecules are in the faster eluent for a longer time compared to the early eluting molecules. A result of this fact, in combination with slow kinetics, may be that an increase in the flow rate results in better resolution in the front area of the chromatogram, the resolution at the end, however, becomes worse. We have observed this with EPG- and mixed-mode phases as well as in experiments at low temperatures.

1.5 Gradient "Myths"

With the routine use of gradient separations in the laboratory, practices have become normal and ideas fixed that are not always appropriate or do not always correspond to reality. In the following, some of these myths are mentioned and briefly commented on. Relevant examples are provided below.

- *"A good overview-gradient ('generic gradient') for a new method is, for example, from 5–10% B to 90–100% B"*
  No, certainly not always. In several cases we have found that both the resolution within certain areas of the chromatogram and the peak capacity in general improve if one starts with about 30–40% B. A specific literature search on recent publications has confirmed these findings. The significant elution strength of such an initial mixture obviously leads to an early differentiation of the sample components: their potentially different properties and thus their different degrees of interaction with the phase material become noticeable with approximately 30–40% B, while with approximately 95% water/buffer almost all components "sit" at the column head, making a separation of relatively early peaks more difficult.
- *"For samples with a large number of components one should run a long gradient"*
  The improvement in the separation with a long gradient has its limits. With a shorter gradient the peaks elute earlier, but the peak width is narrower. Long gradients often lead to a significantly better resolution only for the late-eluting peaks.
- *"Use more % B at the start, so that the whole separation becomes faster"*
  Of course this is correct, however the resolution may also change.
- *"An isocratic step at the beginning improves the resolution"*
  This can but need not be, see further below. Bear in mind that during the isocratic stage the peak does migrate a little, the gradient thus acts quasi in a "shorter" column.
- *"A flat gradient improves the resolution"*
  Again, this can but need not be. In fact a flatter gradient does increase the average resolution, the sum of all resolutions increases. In certain areas in the

chromatogram however it can increase or decrease. What really happens in the particular case can only be predicted in advance with (at least) two runs.

- *"Mixing valves with small volumes require a small dwell volume – which is fine as long as the quality of the mixture is guaranteed"*
  Even if it is slowly becoming boring, this can but need not be. Bear in mind that a different dwell volume may result in a change in the chromatogram. For example, a change in the order of elution, the selectivity, the peak shape or the resolution may occur. And change means precisely "change," which can be positive or negative depending on the case.
- *"In the case of a complex mixture use a long column, a long gradient, and a slow flow"*
  In the case of irregular components, this statement requires, at the very least, a big question mark, see below and also the comments further above.

1.6 Examples for the Optimization of Gradient Runs: Sufficient Resolution in an Adequate Time

About Irregular Components

Before we begin to discuss examples of individual optimization parameters, here an important hint: ignoring this fact in routine work can lead to enormous headaches and frustration.

Substances are referred to as irregular when they differ chemically, especially when they show differing chromatographic behavior, – in other words when they are subject to interactions of significantly different strength with the stationary phase [1]. This can happen when, for example, the sample contains position/double-bond isomers, ionic/neutral, small/large, aliphatic/aromatic components, etc. With such samples, in the course of optimization a modified parameter may improve the resolution in the chromatogram "here," but make it worse "there," because one or more peaks may move faster or slower forwards or backwards and some hardly seem to react at all to the change.

Put differently, with regular components (chemically similar substances) a change in, for example, the column length, the flow, or the temperature leads only to a change of the k^*/k values. As a rule, the selectivity remains the same, an improvement or deterioration of the resolution results, which can be predicted very well in accordance with the equations and the rules derived from them. With irregular components, crossing points are obtained for the retention times, which, by the way, can also shift depending on the material. The result can be: coelution, deterioration "here," improvement "there."

Preliminary Remarks, General Conditions

Lately, to understand gradient elution we have carried out many gradient separations. Most of the measurements were performed by Hans-Joachim Kuss in Munich. With the aim of finding generally applicable rules, but also to verify the

theory of gradient elution in practice, we have chosen very different chromatographic conditions, below and in short form the most important:

- Instruments: first a UHPLC system, then a modern low-pressure gradient and finally and deliberately an old high-pressure gradient from the early 1990s with considerable dead – and dwell – volumes
- Columns: long (e.g., 150 × 4.6 mm, 5 µm), middle (e.g., 50 × 4 mm, 3.5 µm), and short (e.g., 20 × 2 mm, ≤ 2 µm) columns
  - Packing material: classical C18 columns (e.g., Symmetry C18, SunFire), monolith (e.g., Chromolith Performance and HR), hybrid materials (e.g., XBridge C18/Shield, Gemini-NX), Fused Core (e.g., Kinetex, Accucore, Ascentis Express), Mixed-Mode Phases (e.g., Primesep C, Obelisc N/R)
  - Mobile phase: various ACN/MeOH mixtures, some with modifier
  - Samples: mixtures with regular/irregular components, from fairly neutral alkylbenzenes over weakly polar/ionic components such as phenol to very strongly polar research substances.

It goes without saying that the presentation of the results including examples exceeds by far the scope intended here. Therefore the, in our opinion, most important results follow in condensed form further below. Some example chromatograms are intended to illustrate these findings. The fact that with UHPLC and Fast LC-systems narrow peaks and fast separations can be achieved is trivial. Here we only show examples obtained using the older system. Our intention is to show that with not very demanding separations diverse, even "difficult" gradients are quite possible with medium-quality equipment.

Even modern, short columns can be useful in that case. But first, the following remark once again: gradient elution is complex, please consider the following statements solely as recommendations, which, although compatible with the theory and repeatedly confirmed through cross-experiments, are on no account to be considered as a general "to-do" list. For example, a complex matrix can cause an unexpected result, even an unintentional pH gradient may contribute…

We have discussed the plate number further above, therefore it will no longer play a role here. We will concern ourselves rather with interactions, selectivity, and the retention term in Eq. (1.1); the objective is sufficient resolution. This book has treated the influence of the important parameters solvent (ACN against MeOH), pH, and temperature on selectivity in detail in Chapter 3 and partly in Chapter 5; reference is made here to these. Also, approaches for systematic pH-value, column, temperature, and eluent screening through the automation of experiments (AutoChromSword, DryLab, ACDLabs) are not considered here, see [5, 6].

Rather, we focus on gradient specifics, see Eq. (1.2): gradient duration and flow – that is, gradient volume – as well as start and end %B; from the latter and the gradient duration results the slope. Additionally, the effective volume of the column, which in practice this means the length of the column. Further, an isocratic step at the beginning – either deliberately included or due to the existing dwell volume. The constant S is derived from the substance structure and the chromatographic conditions and can thus be influenced only indirectly.

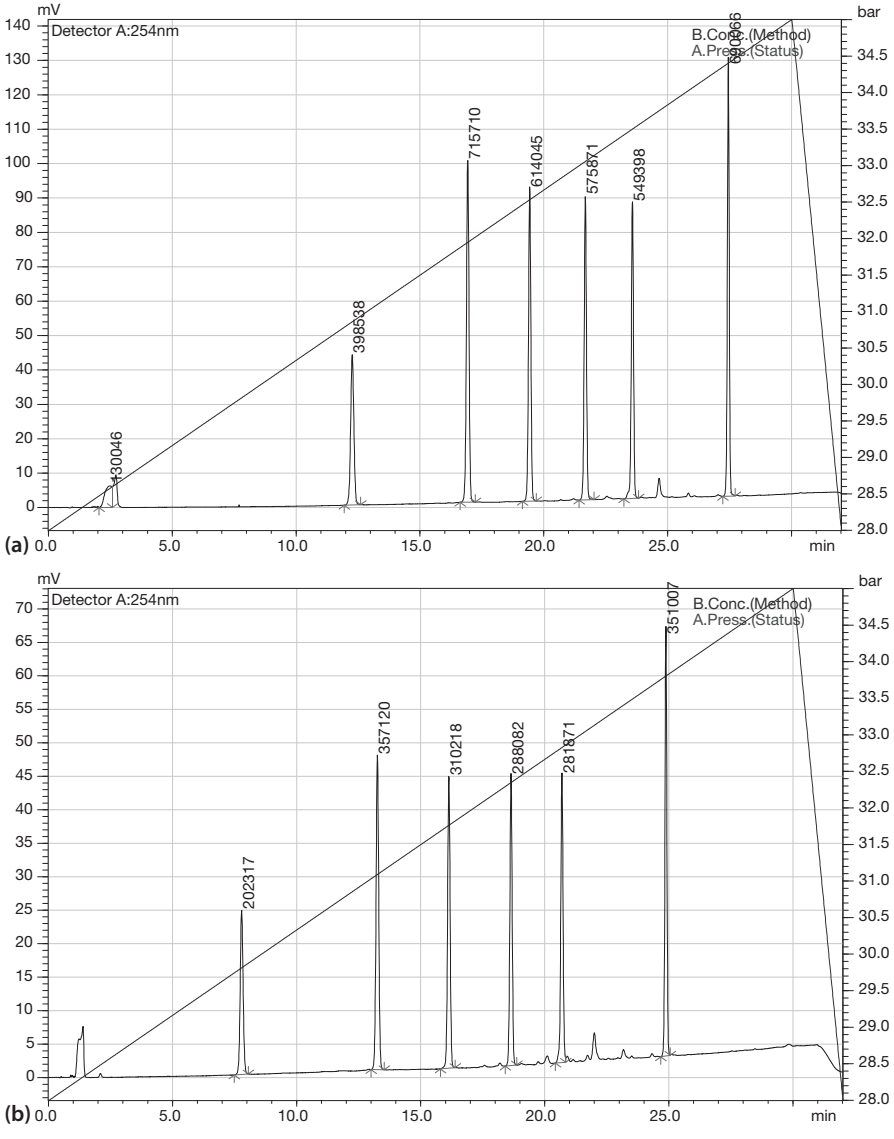


Figure 1.1 Influence of the flow rate. XBridge Shield 150 × 4.6 mm, 5 µm. (a) 0–100% B, 1 ml/min, t_G = 30 min, (b) 0–100% B, 2 ml/min, t_G = 30 min.

Gradient Duration and Flow

Case 1 The gradient duration remains constant and the flow is increased.

This always results in a decrease in the retention time. As far as the resolution is concerned, with regular components the separation remains about the same, see Figure 1.1.

Unfortunately, with irregular components a wide variety of cases are imaginable: the order of elution can be reversed; with the same elution order the resolution becomes better; the resolution is better only at the end; the resolution increases at the front of the chromatogram and decreases somewhat at the end, see Figure 1.2.

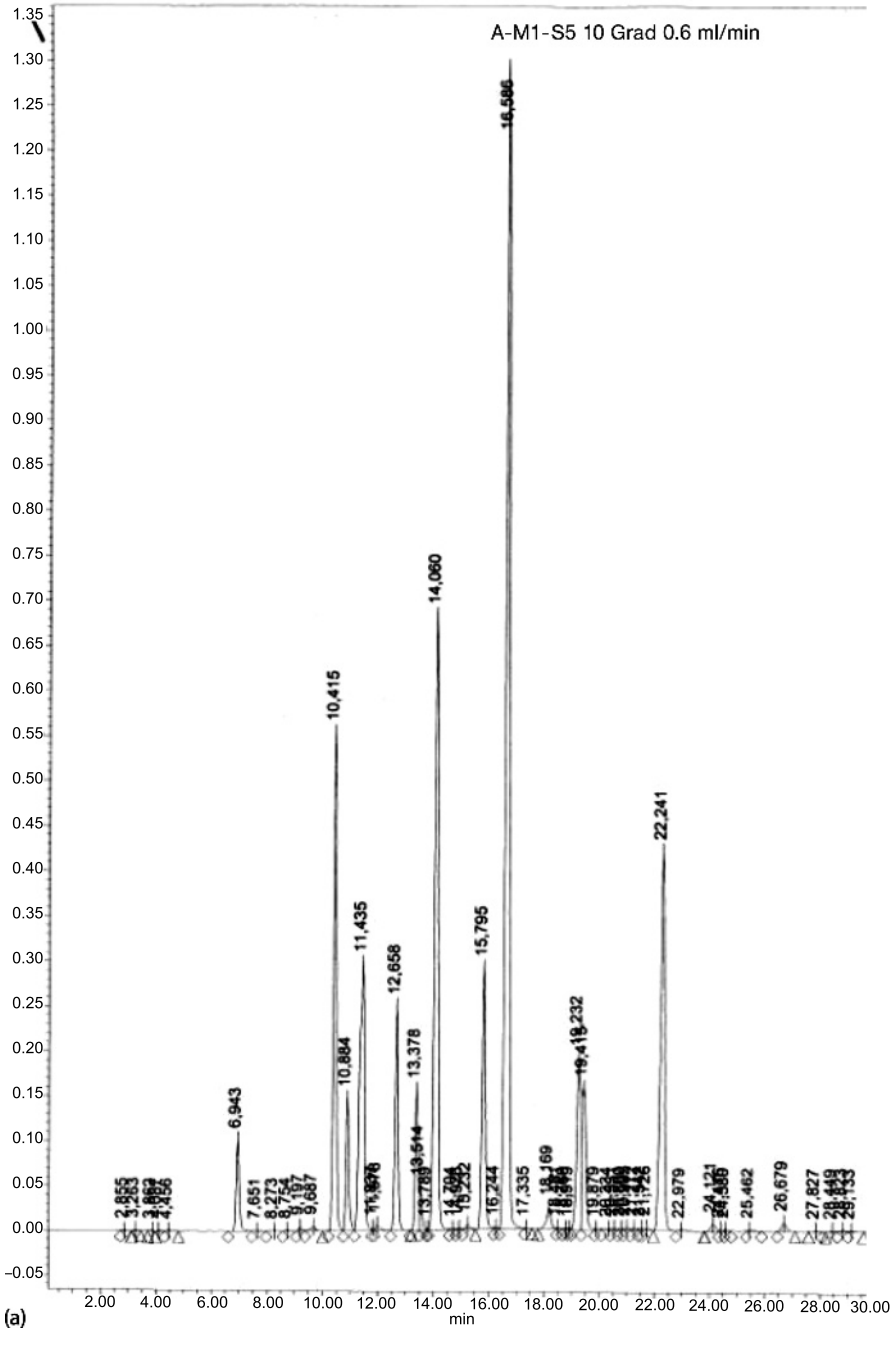
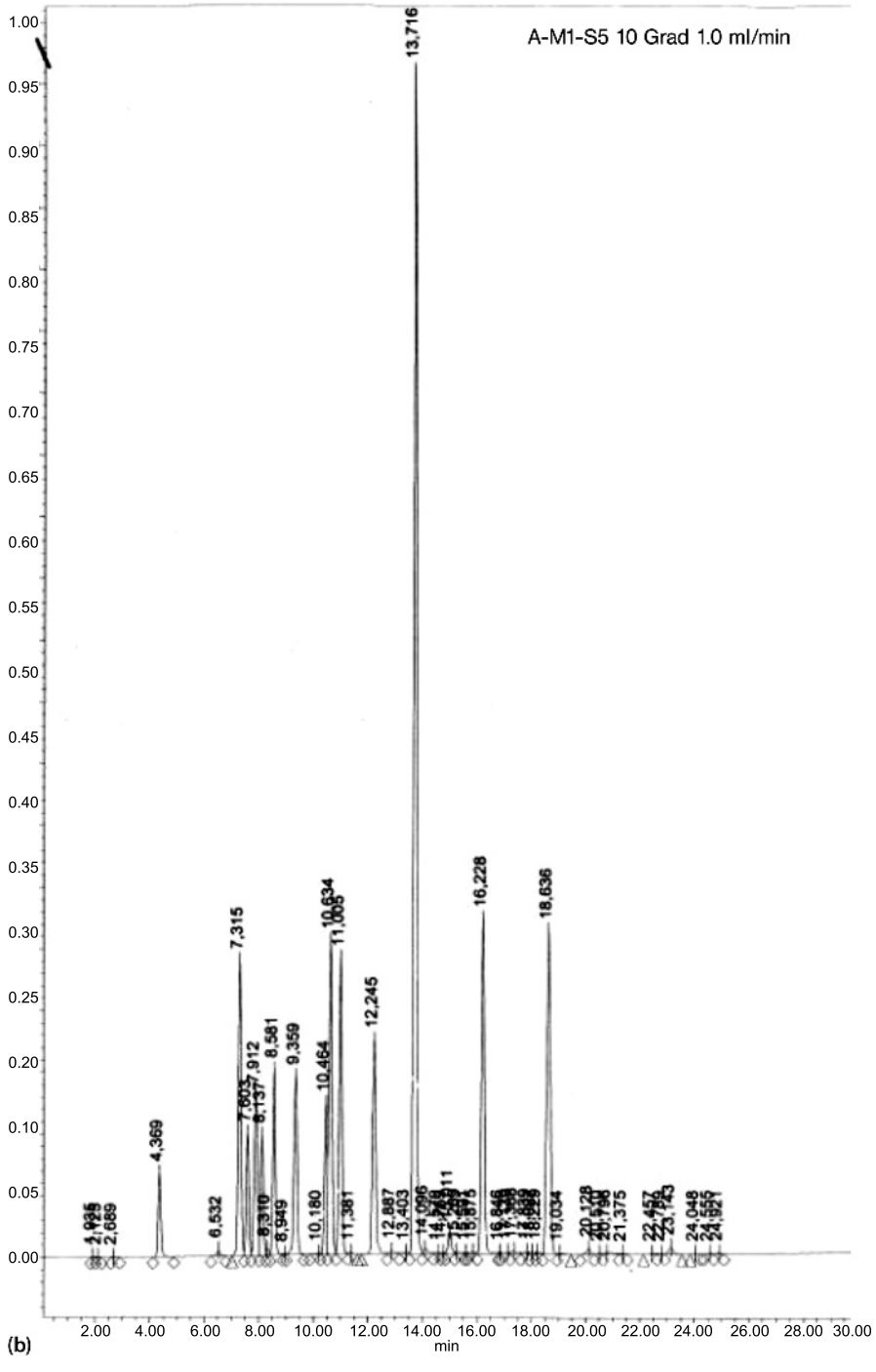


Figure 1.2 Influence of the flow rate. (a) 0.6 ml/min, 10 °C, (b) 1 ml/min, 10 °C.



(b)

Figure 1.2 Continued.

Case 2 The gradient duration is halved and the flow increased by a factor of 2. In this case, the gradient volume remains constant, ideally the same chromatogram (same resolution) is obtained, however, in half of the time, see Figure 1.3. Often the resolution becomes a little worse, in the case of fast kinetics however the loss of resolution is rather limited.
The gradient duration is not as important as generally assumed. For 6–8 peaks a gradient longer than about 5–7 min is rarely necessary, see Figure 1.4.

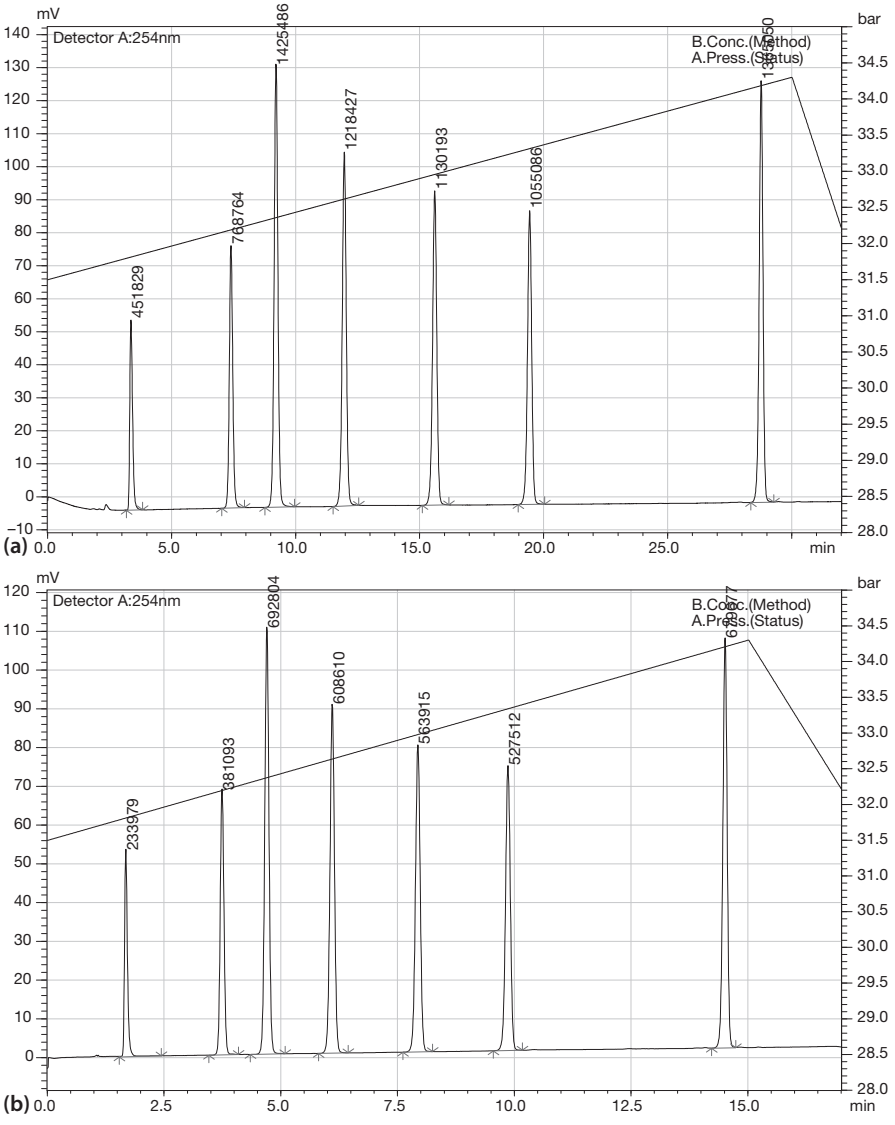


Figure 1.3 Influence of the flow rate. XBridge Shield, 150 × 4.6 mm, 5 µm. (a) 50–90% B, 0.5 ml/min, t_G = 30 min, (b) 50–90% B, 1 ml/min, t_G = 15 min.

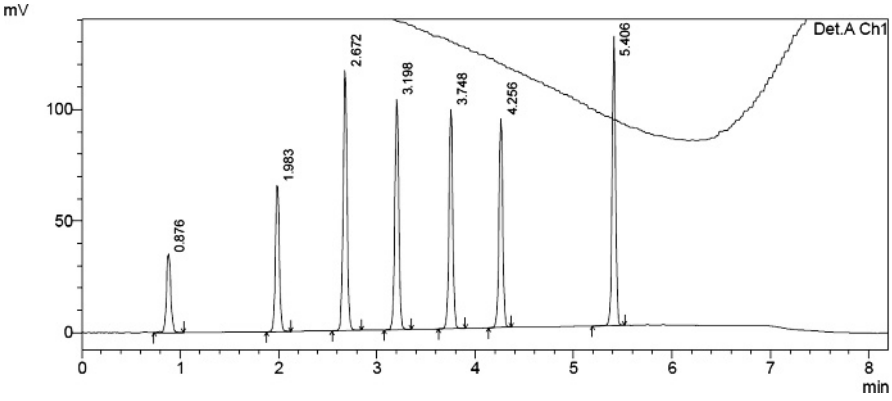


Figure 1.4 The gradient duration required. Zorbax SB C8, 150 × 4.6 mm, 5 µm, 40–90% B, t_G = 5 min, 2 ml/min.

Initial and Final % B, Slope

As already mentioned above, common practice in method development for an unknown sample, i.e., starting with a gradient of for example 5% to 100% B, rarely proves to be wise: often the first peaks of interest start to elute relatively late. Another disadvantage is that impurities from the water appear as small interfering peaks ("ghost peaks"). A start at about 40% B often has the following advantages: the peaks are distributed evenly over the whole chromatogram, small interfering peaks seldom emerge, the peaks elute earlier and are narrower and higher, see Figure 1.5. In our view, a start with 0–5% B only makes sense if strongly polar components are to be separated on a C18 column, when peaks directly at the dead time are expected. The situation is similar with an isocratic step at the start: only in the case of early peaks and a gradient start with a high aqueous portion is an improvement in the separation to be expected.

If the number of peaks is known, with the aim of improving the detection limit one can easily start with an even higher % B, see Figure 1.6.

Note

The initial % B is relatively unimportant for the elution of the last peaks, the same with an isocratic step at the beginning. Simply put, what happens at the "front" hardly concerns the later peaks. Conversely, the final % B hardly affects what happens in the first third of the chromatogram, this means: initial % B is important for the "front," final % B for the "end." Further, a steep gradient lowers the detection limit, while a flat gradient often leads to an improvement in resolution only in the later part of the chromatogram. Consider Figure 1.7, where the following two gradients are shown, 40 to 100% B and 50 to 90% B. With almost the same gradient duration, with 50–90% B, the peak height in the first half of the chromatogram is large, the resolution small, while with 40–100% B the opposite is seen. Depending on the choice of initial % B and slope, one can selectively influence both resolution and detection limit in the front and rear areas of the chromatogram.

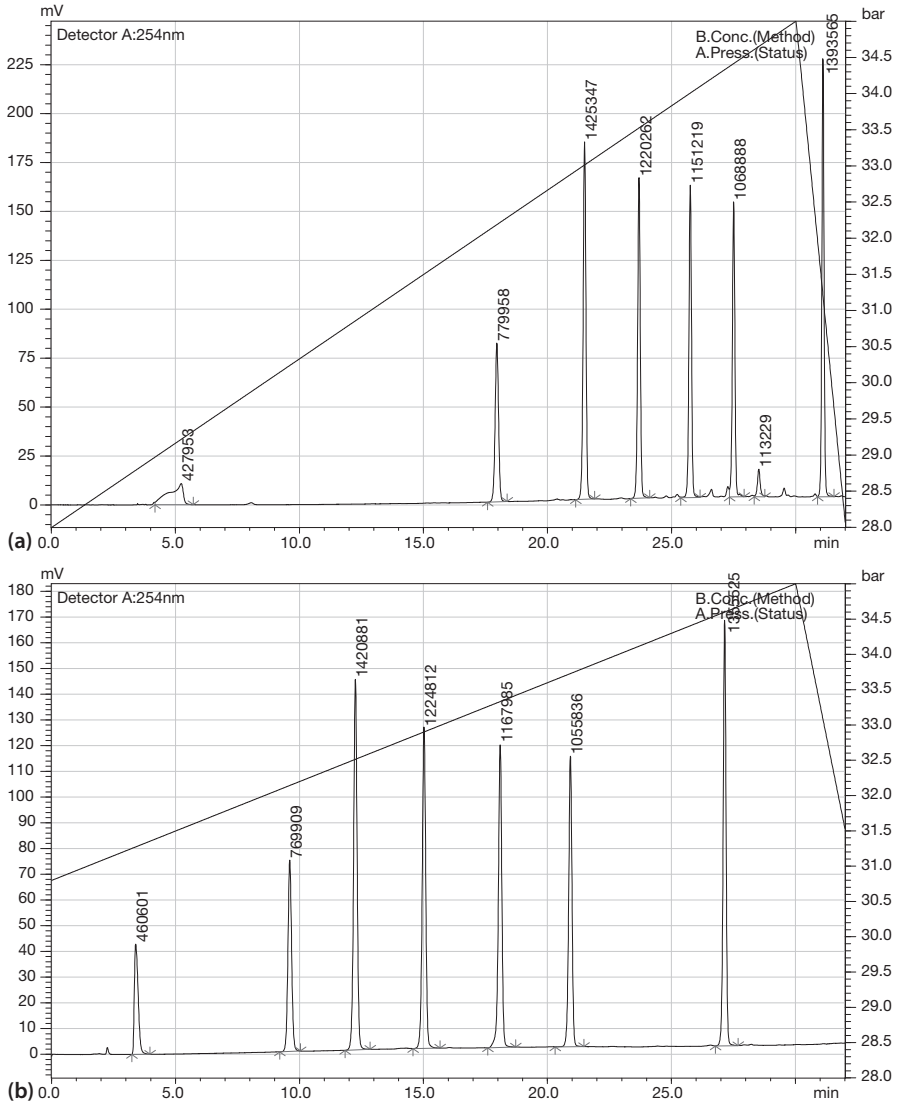


Figure 1.5 Effect of initial % B. XBridge Shield, 150 × 4.6 mm, 5 µm. (a) 0–100% B, 0.5 ml/min, t_G = 30 min, (b) 40–100% B, 0.5 ml/min, t_G = 30 min.

Note that in the case of irregular components, an extension of the gradient duration and/or modification of the initial or end % B – and thus the slope – can lead to a reversal of the order of elution, to coelution, and improvements in the resolution, see Figure 1.8: From 45% through 60% to 70% B as the starting conditions, the separation at the front becomes increasingly worse, at the end, however, from "reasonable" and nine peaks, through "bad" and six Peaks to "really good" and nine peaks.

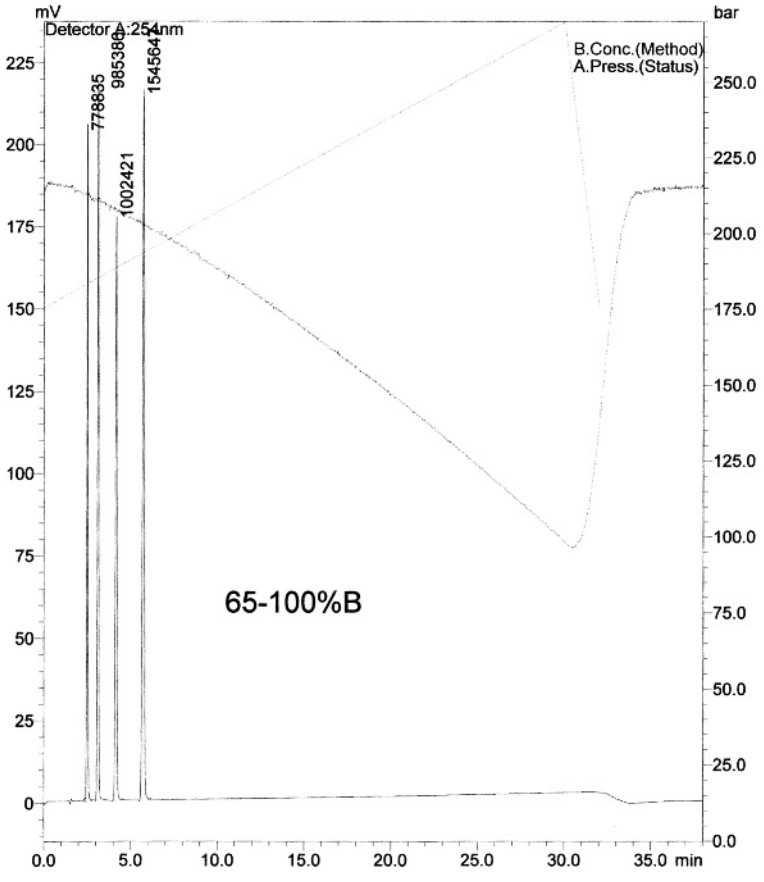


Figure 1.6 Regarding the starting conditions with a small number of peaks. Gemini NX, 50 × 4 mm, 3 µm, 65–100% B.

The first, simplified rules for the start:

- Use a reasonable starting % B and initially vary only the slope as needed – this could already lead to success
- Constant slope and modification of Δ% B, – which means parallel gradient profiles, similar chromatograms
- Constant Δ% B and modification of the slope – which probably leads to different chromatograms.

Initial and Final % B and Gradient Duration – they Determine the Slope – or Respectively Column Length and Flow

As mentioned above, the two most important parameters for the resolution are % B/min (strictly speaking % B/ml) and the gradient volume.

Consider the following two cases:

1. In three gradient runs, from 10 to 100% B in 30 min, from 10 to 50% B in 13 min, and from 10 to 40% B in 10 min, % B/min remains more or less con-

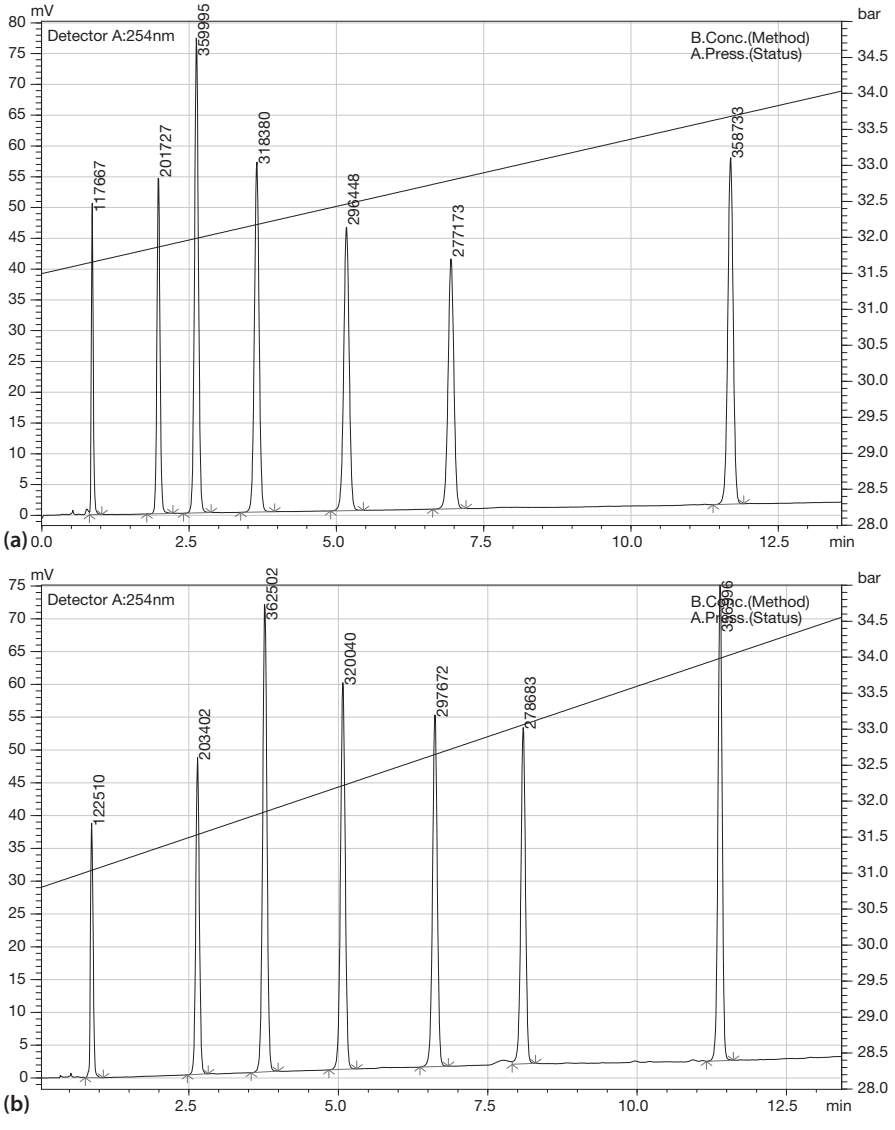


Figure 1.7 Effect of initial % B and slope. XBridge Shield, 150 × 4.6 mm, 5 µm. (a) 40–100% B, 2 ml/min, t_G = 15 min, (b) 50–90% B, 2 ml/min, t_G = 15 min.

stant (about Δ3% B/min), the result is the same resolution but with different gradient durations.

2. In three gradient runs, all from 10 to 90% B in 20 min, the last peak elutes at 0.5 ml/min in 16 min, at 1 ml/min in 8 min and at 2 ml/min in 4 min. Here too, % B/min remains constant, the result is the same resolution (the gradient volume remains constant at 8 ml), the retention time of the last peak however becomes increasingly lower.

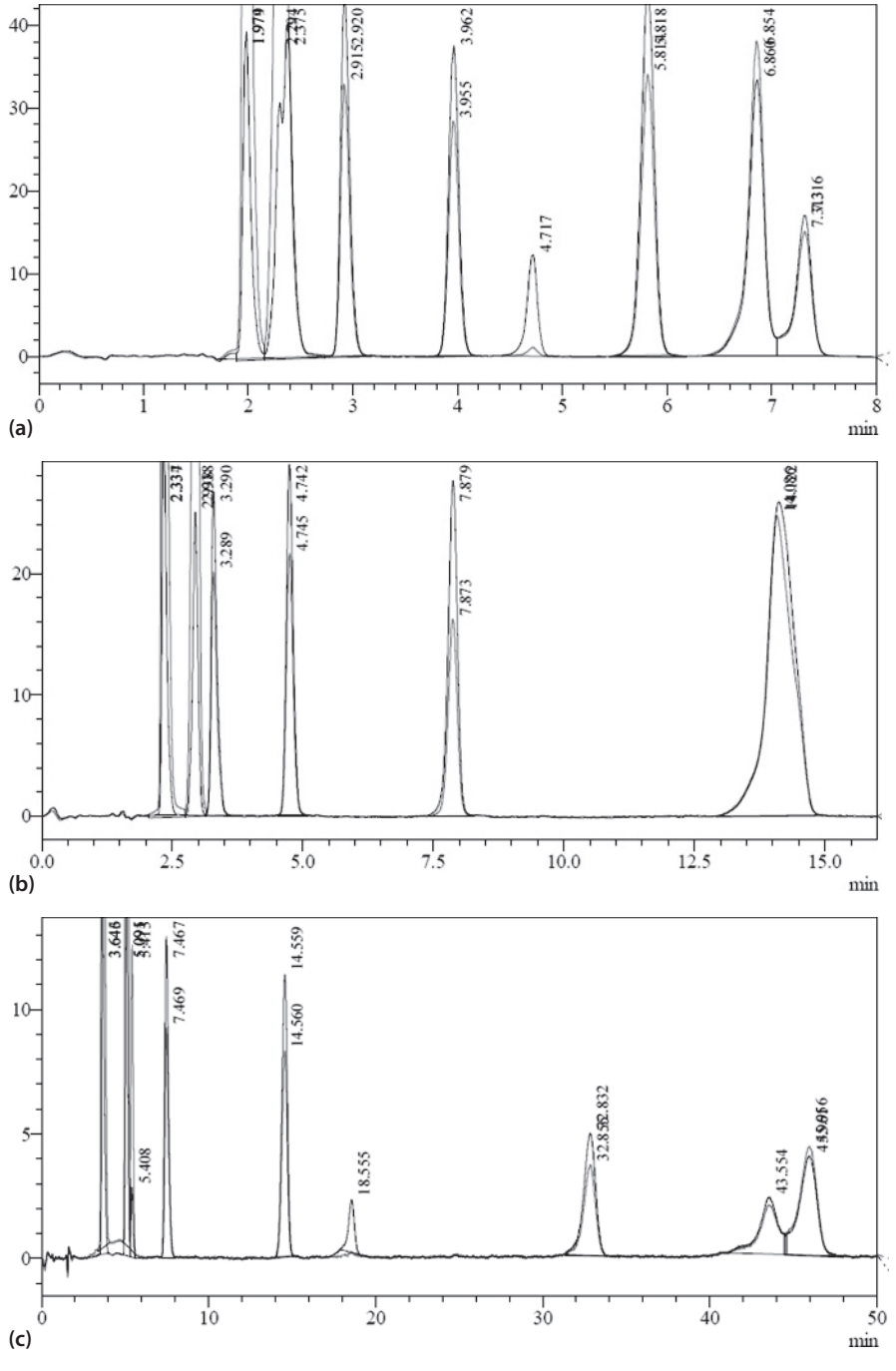


Figure 1.8 Influence of initial % B and slope. Symmetry C18 150 × 4.6 mm, 5 µm. Initial % B: (a) 45%, (b) 60%, (c) 70%; final % B in all cases 100%.

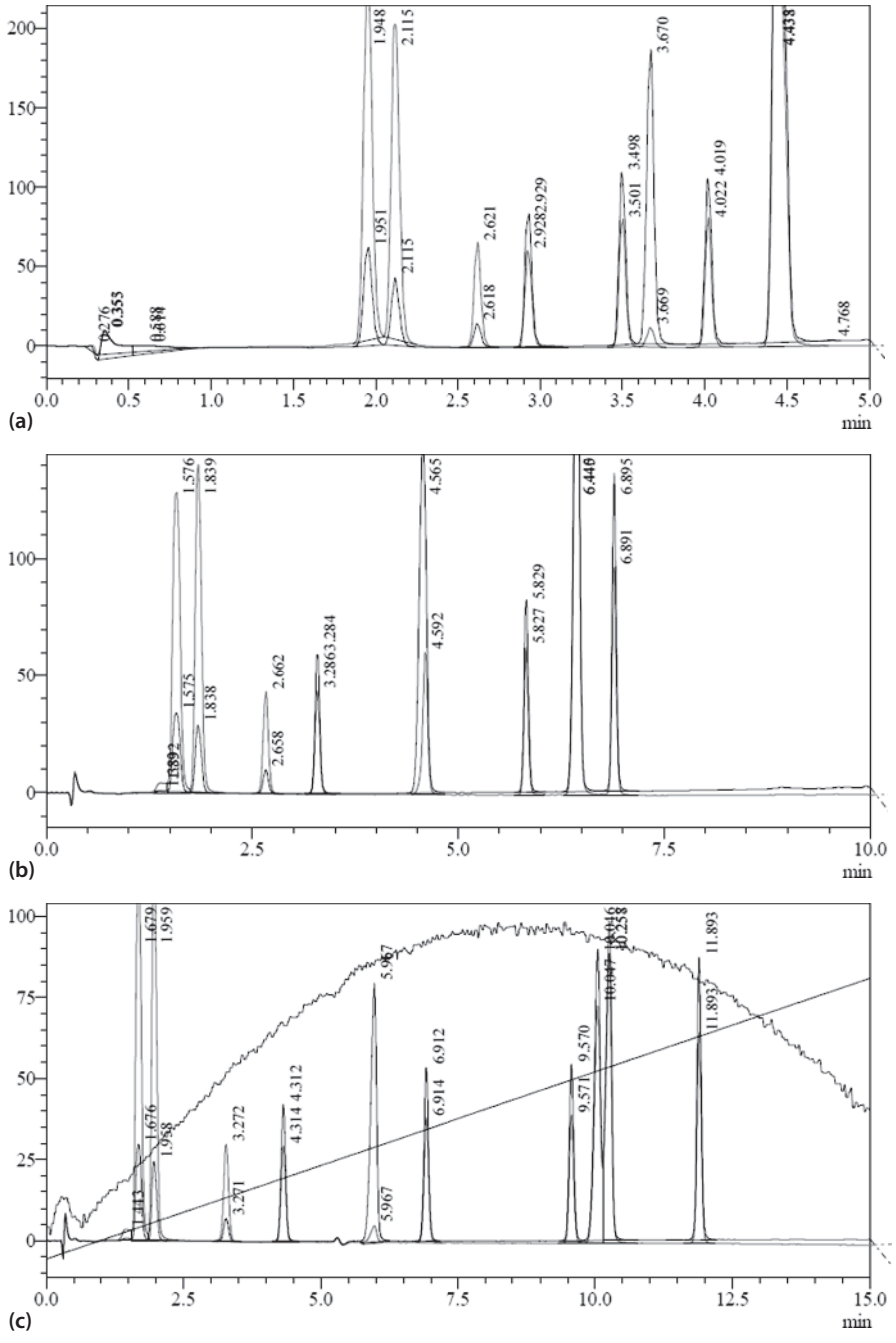


Figure 1.9 Effect of initial % B and gradient duration on elution order and resolution. Ascentis Express C18, 50 × 3 mm, 2.7 µm. (a) 10–90% B, t_G = 5 min. (b) 20–90% B, t_G = 10 min. (c) 20–70% B, t_G = 15 min.

Following a change in the slope due to a change in the initial % B and/or the gradient duration, with irregular analytes one should expect both a change in the elution order as well as an improvement or deterioration of the resolution, see Figure 1.9. Note the following: the gradient becomes flatter and longer, which gives a slope of Δ16% B/min through Δ7% B/min to Δ3.3% B/min. What do you notice? In spite of this large difference, the separation of the first four peaks is only slightly affected. The chromatogram varies significantly only after the 4th peak, with changes in the order of elution and in the resolution (eight vs. ten peaks).

Once again: in the case of not very difficult separations gradient duration and column length are not that important, see Figures 1.10 and 1.11. When one knows that only these seven peaks are to be expected, a 20 × 4.6 mm, 2 µm column is sufficient, while a 150 × 4.6 mm, 4 µm column inevitably brings unnecessarily long retention times. Figure 1.11 (column: 20 × 4 mm, 2 µm), demonstrates what has already been described further above. Firstly, in spite of a small column volume and also a considerable dead volume of the apparatus the peaks look useful, the peak width remains constant. Secondly, for this small number of peaks a 2 cm column is sufficient. And thirdly, a 2 min gradient compared to a 10 min gradi-

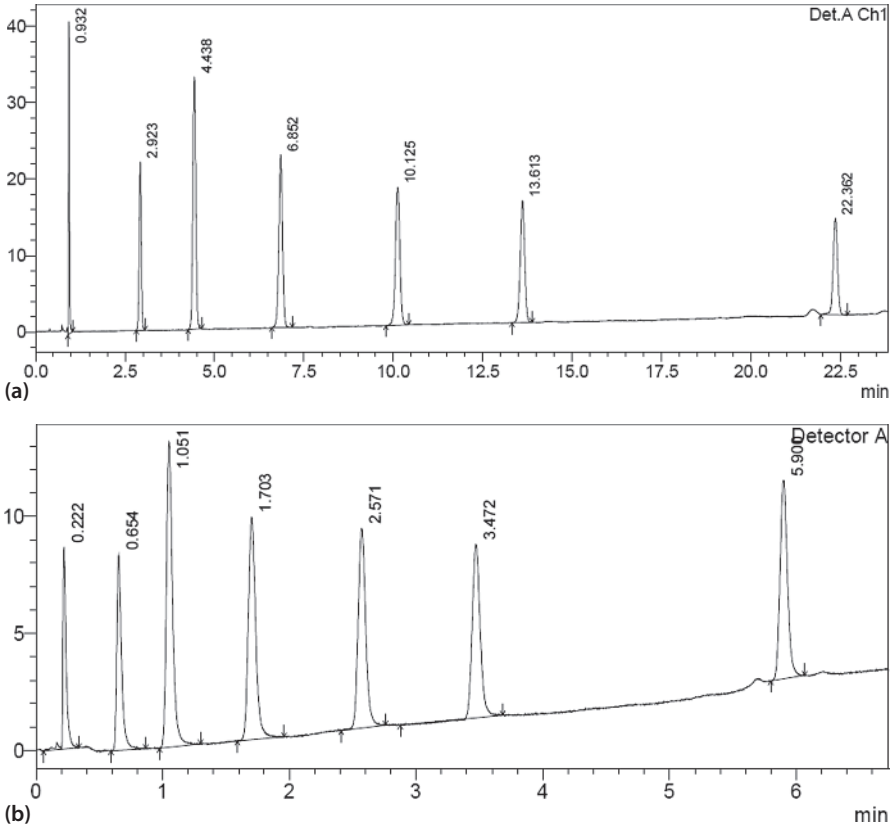


Figure 1.10 Influence of column length. (a) Synergi Fusion RP, 20 × 4.6 mm, 2 µm. (b) Synergi Fusion RP, 150 × 4.6 mm, 4 µm.

ent is equally sufficient (the drift in the lower image and the apparently broader peaks are no problem, the scaling is different).

Of course, with ≤ 2 µm particles and/or 20 mm columns, fused core materials, high-throughput columns, and Chromolith HR, we have noticed tailing and peak

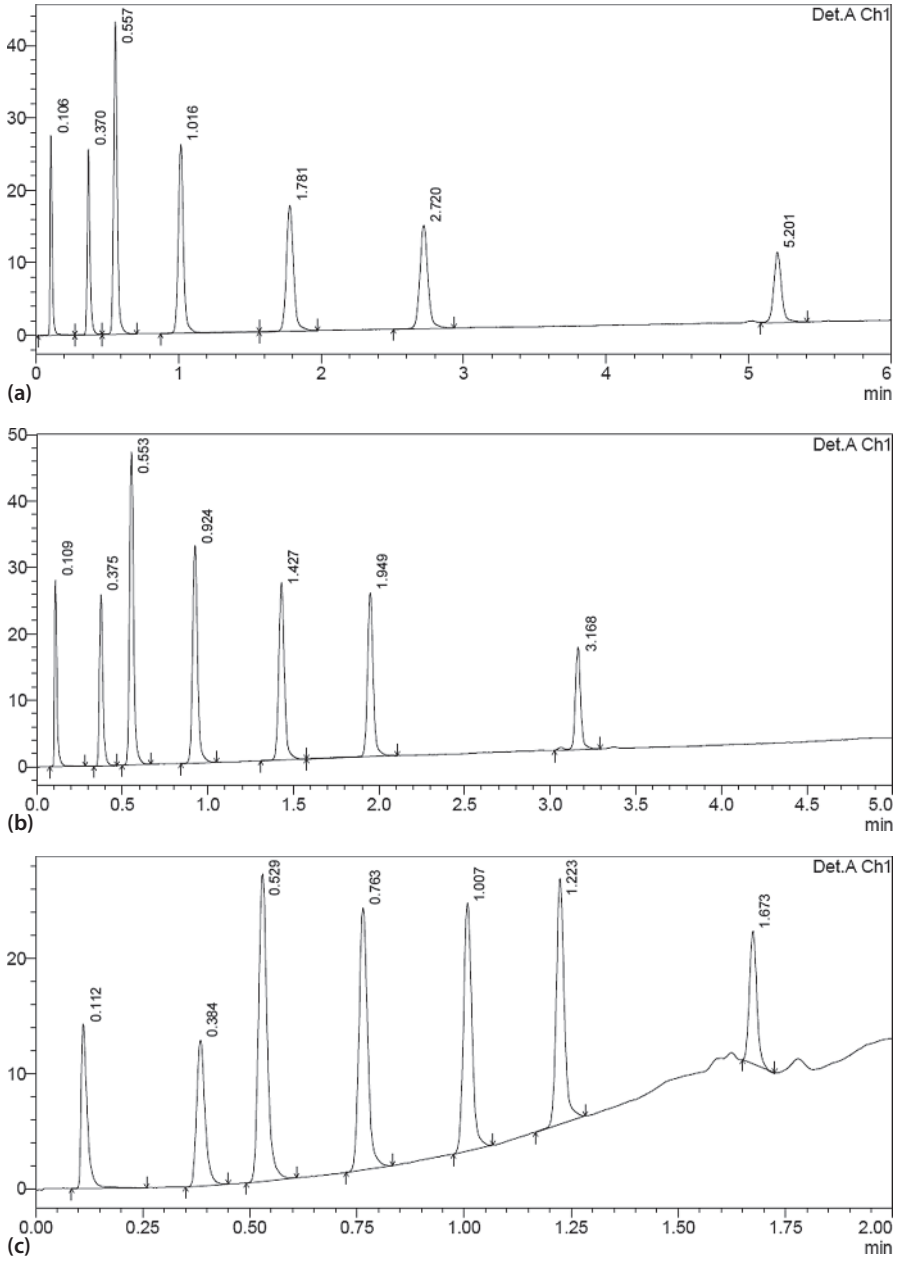


Figure 1.11 Influence of gradient duration, Synergi MAX RP 20 × 4 mm, 2 µm. (a) Gradient duration 10 min, (b) 5 min, (c) 2 min.

broadening due to the instrumental dead space, especially with the early peaks – even with modern equipment! But for simple separations we can live with the loss of 20–40% efficiency, because whether a peak has a peak width of 3 or 4 s will, due to the narrow peak form and a satisfactory separation, not usually be registered by the user as a disadvantage – and then it is no problem.

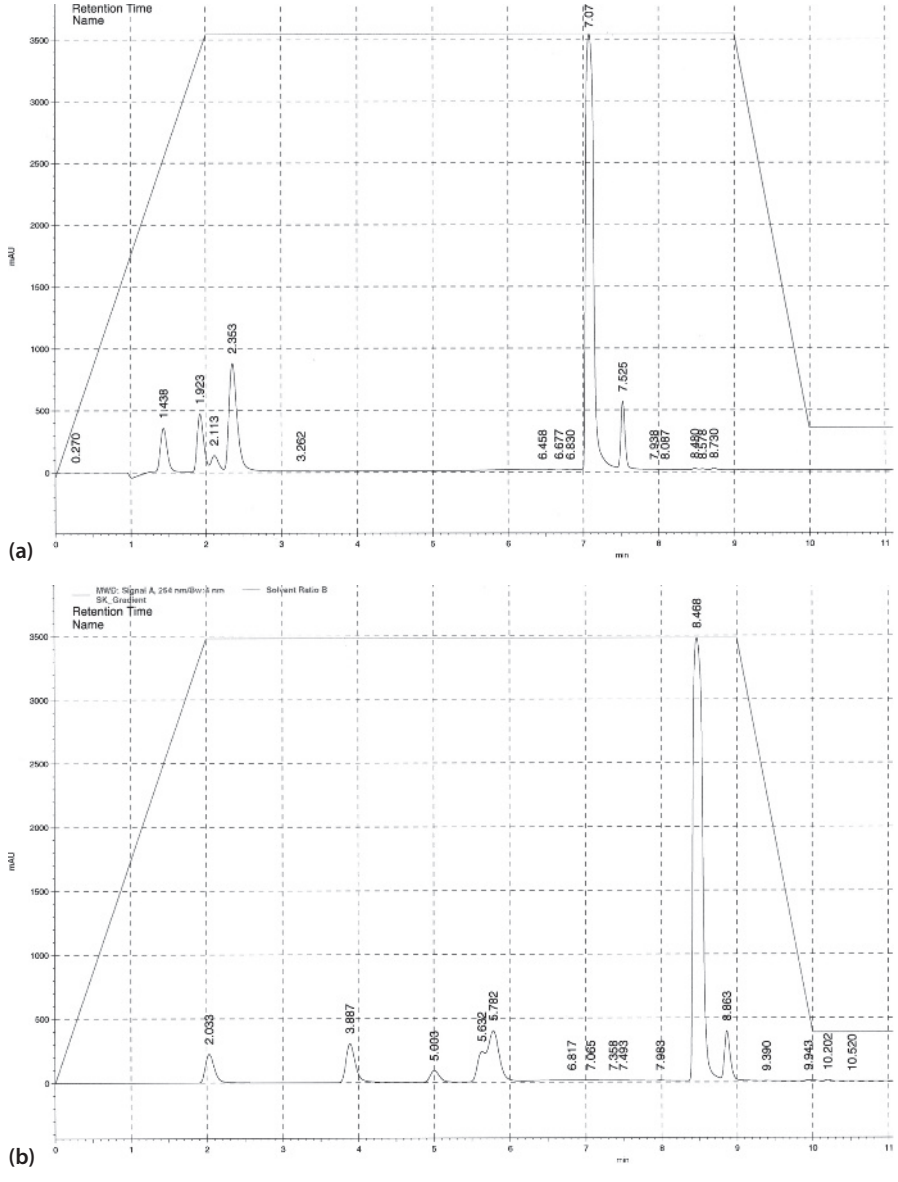


Figure 1.12 Gradient run at 35 °C (a) and 15 °C (b) on LUNA Omega PS. The largest selectivity difference is found for polar components, for details see the text.

Temperature

A decrease in the temperature often leads to an improvement in the separation, or at least to a change, and most often this change occurs in the front region of the chromatogram (elution of polar components). Conversely, the overall retention time changes relatively little in RP runs. In a series of experiments it could be further confirmed that temperature differences more strongly affect the separation if stationary phases with an additional polar character are used rather than hydrophobic materials. Two examples are given: in Figure 1.12a,b one sees separations at different temperatures on LUNA Omega PS, a C18 material with an additional positive charge on the surface. In Figures 1.13a,b, separations are shown on Primesep C, a mixed-mode material with a complex-capable group, where change of elution order is observed.

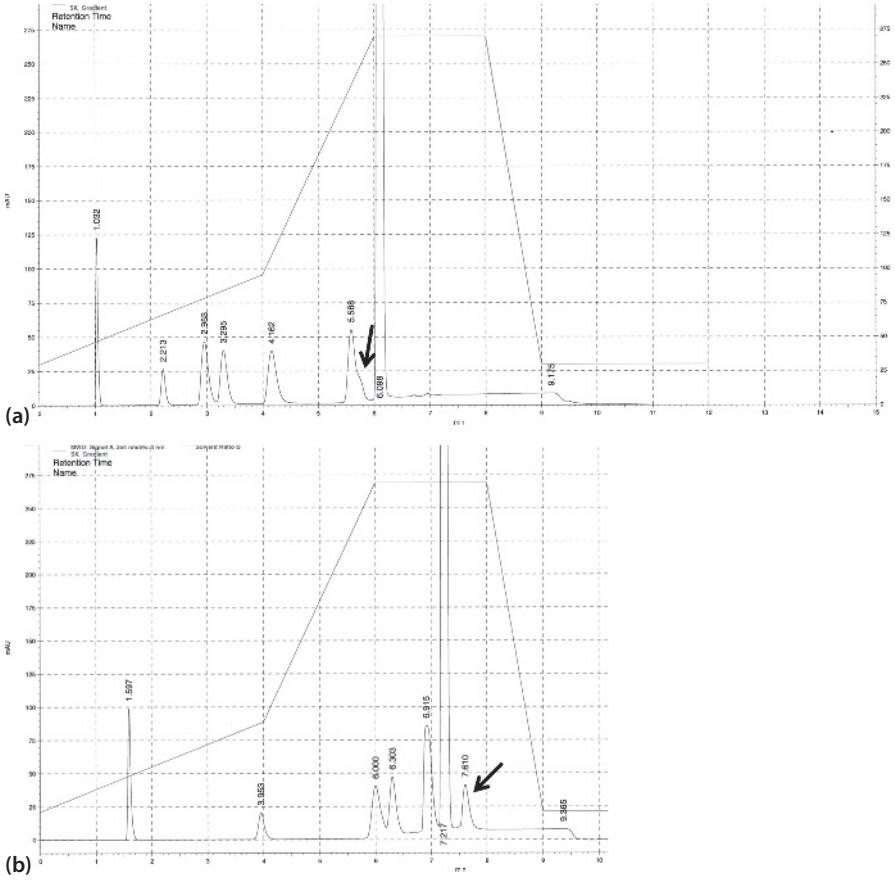


Figure 1.13 Gradient run at 35 °C (a) and 15 °C (b) on Primesep C. A change of elution order takes place on the mixed mode material, for details see the text.

Dwell Volume

The dwell volume (volume from the point of mixing to the column head), as well as the type of mixing device and quality of the mixing itself are decisive factors in gradient separations. The relationships are discussed in detail in Chapter 2. The results of three experiments are briefly presented below. Here, we have used methanol/water gradients, the reason for that is the following: possible differences are often more visible with methanol as an organic solvent than with acetonitrile.

1. If the dwell volume of two apparatus is different, then it is necessary to count on "everything," a situation that does not facilitate the transfer of methods: identical separation, improvement/worsening of the separation, and also changes in retention time, peak shape, selectivity, and elution order. Also, the column material can eventually influence the result. We measured columns

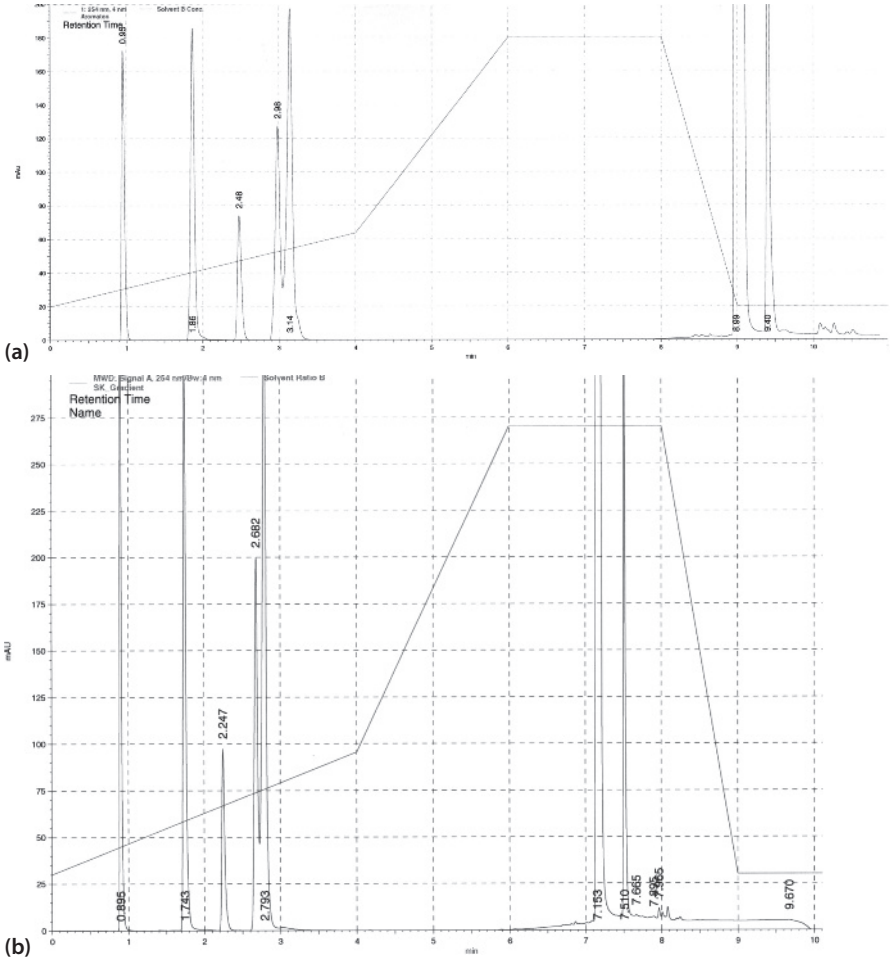


Figure 1.14 Gradient run on Cortecs C18 on two apparatus with different dwell volume; there is hardly any difference, for details see the text.

with different characteristics on a Shimadzu LC 20 (low-pressure gradient) and an Agilent 1200 (high-pressure gradient) system under identical chromatographic conditions. Here are some findings:

- See Figure 1.14a,b (Cortecs C18) and Figure 1.15a,b (Poroshell EC 120-C18), both are hydrophobic, end-capped core shell materials. For the Agilent unit (Figures 1.14b and 1.15b) with the smaller dwell volume, the peaks elute a little bit earlier, but the separation as such is barely noticeably different.

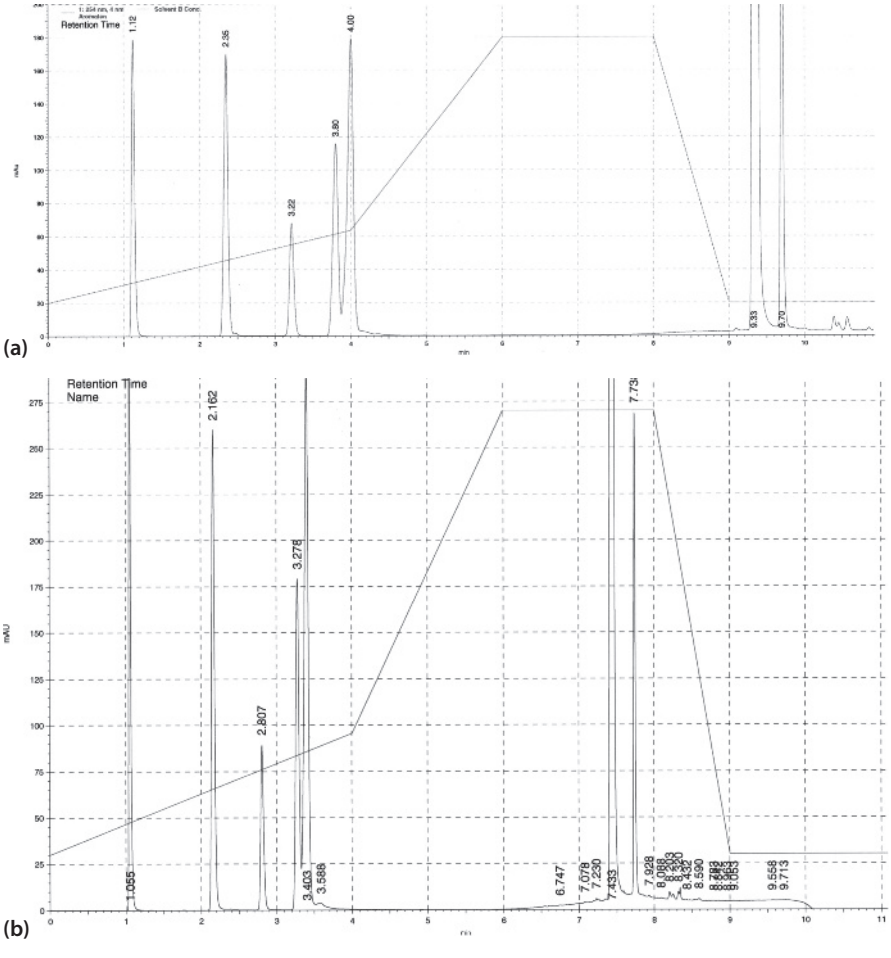


Figure 1.15 Gradient run on Poroshell EC 120-C18 on two apparatus with different dwell volume; there is hardly any difference, for details see the text.

- Figure 1.16a,b show chromatograms with Cortecs phenyl. At a phenyl phase, as a fairly polar stationary phase, RP interactions are weak. The larger dwell volume on the Shimadzu equipment (Figure 1.16a) not only leads to a longer retention time, the isocratic step at the beginning also "offers" the substance molecules a water-rich environment, the separation improves.

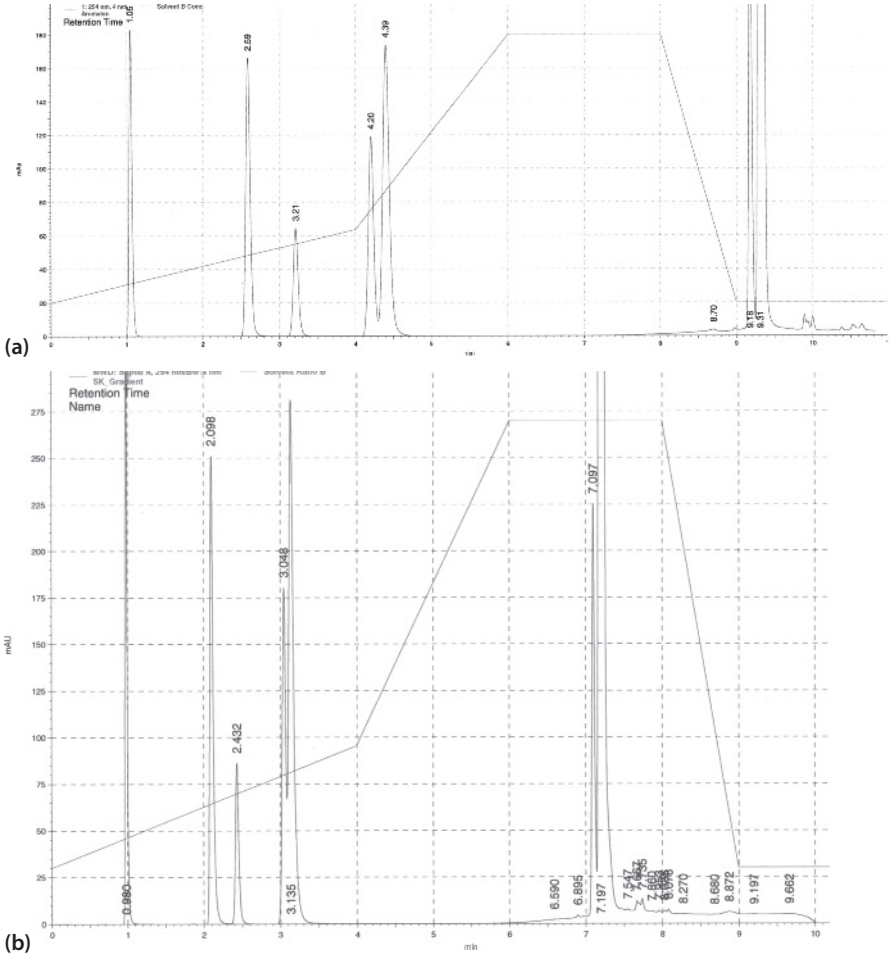


Figure 1.16 Gradient run on Cortecs Phenyl on two apparatus with different dwell volume. The unit with the larger dwell volume (a) shows a better separation.

- Compare Figure 1.17a,b, the column here is Atlantis T3. For the Agilent unit (Figure 1.17b), the early eluting peaks are slightly inferior and the late-eluting peaks slightly better separated.

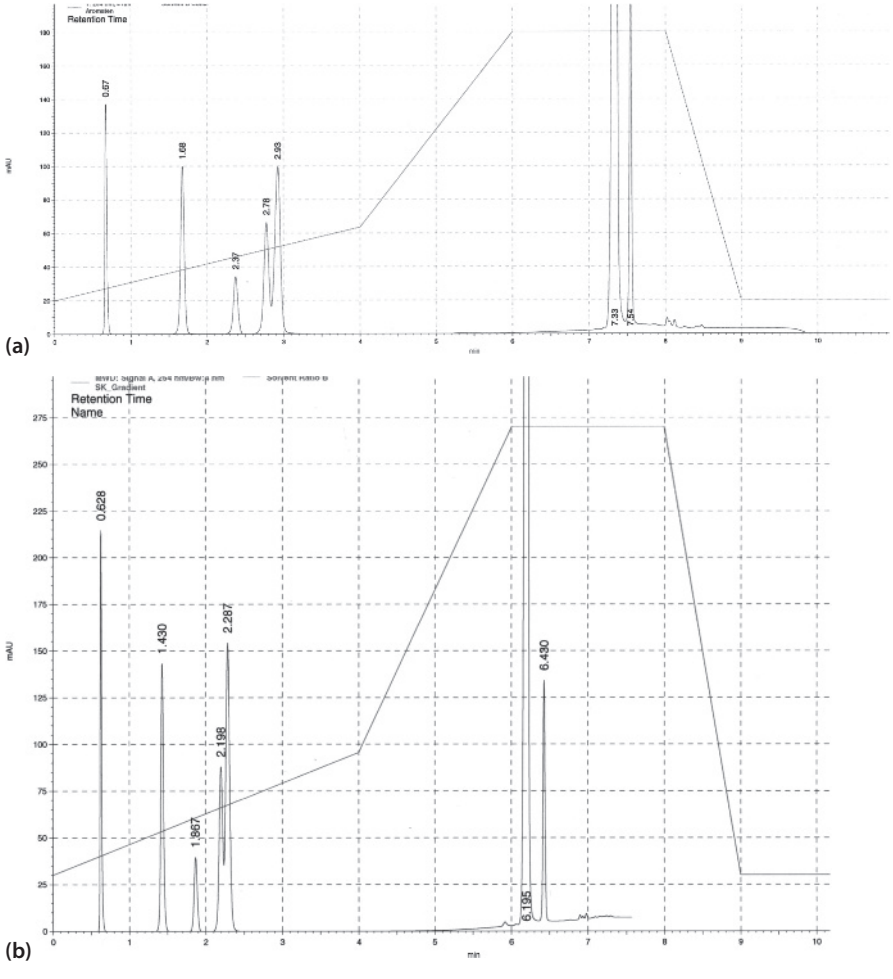


Figure 1.17 Gradient run on Atlantis T3 on two apparatus with different dwell volume. The smaller dwell volume (a) leads to a poorer separation of the early eluting peaks and to a better separation of the late-eluting peaks.

- For Primesep C, a mixed-mode material with a complex-capable group, we have a change of the elution order, see Figure 1.18a,b.

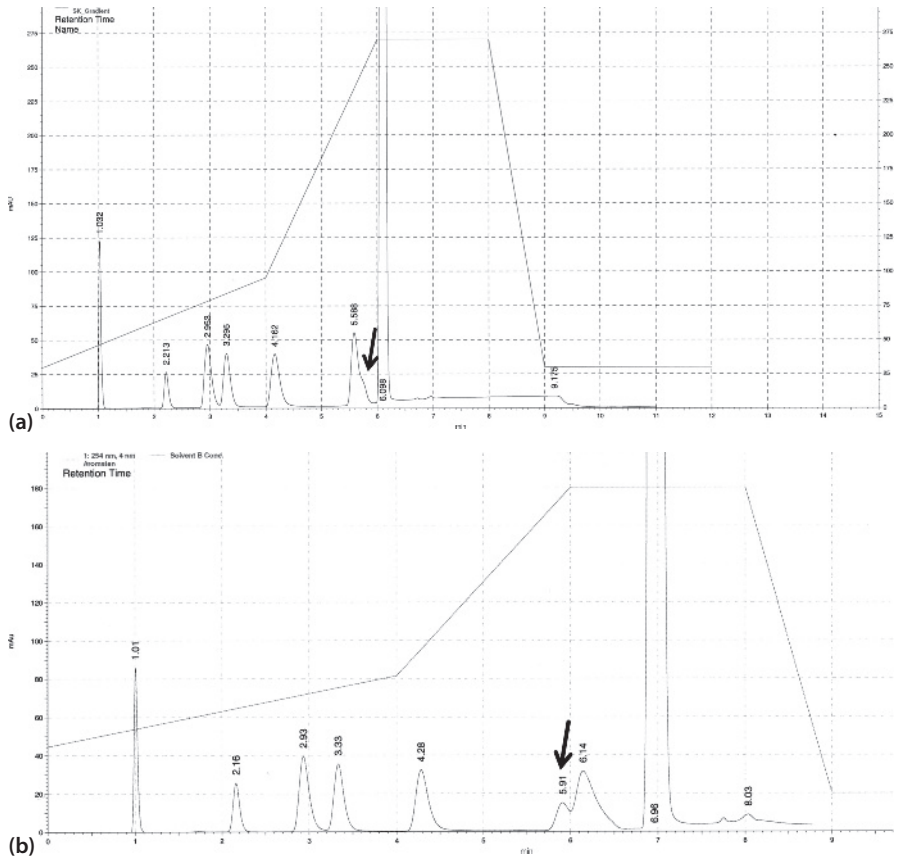


Figure 1.18 Gradient separation on Primesep C on two apparatus with different dwell volume. The larger dwell volume (b) not only leads to an increase in the retention time, but also to a change of the elution order.

2. In a further experiment, we replaced the usual mixing chamber with a volume of 1.7 ml in one unit (Shimadzu) with a mixing chamber with a volume of 2.6 ml. Here, too, several columns with different properties were tested. As mentioned above, a different dwell volume – here due to the volume of the mixing chamber – possibly leads to a different separation, which can also be different at different stationary phases: the components elute as expected, somewhat later on all columns due to the larger mixing chamber. However, there were quite big differences in terms of resolution depending on the column:
   - With Cortecs C18 and Poroshell EC 120, the larger mixing chamber gives a better separation of the early eluting peaks.
   - There was little difference with Atlantis T3.
   - With Cortecs-Phenyl, separation with the larger mixing chamber was better throughout the chromatogram.
   - With Primesep C the separation in the front part of the chromatogram remained the same, in the late part it improved.

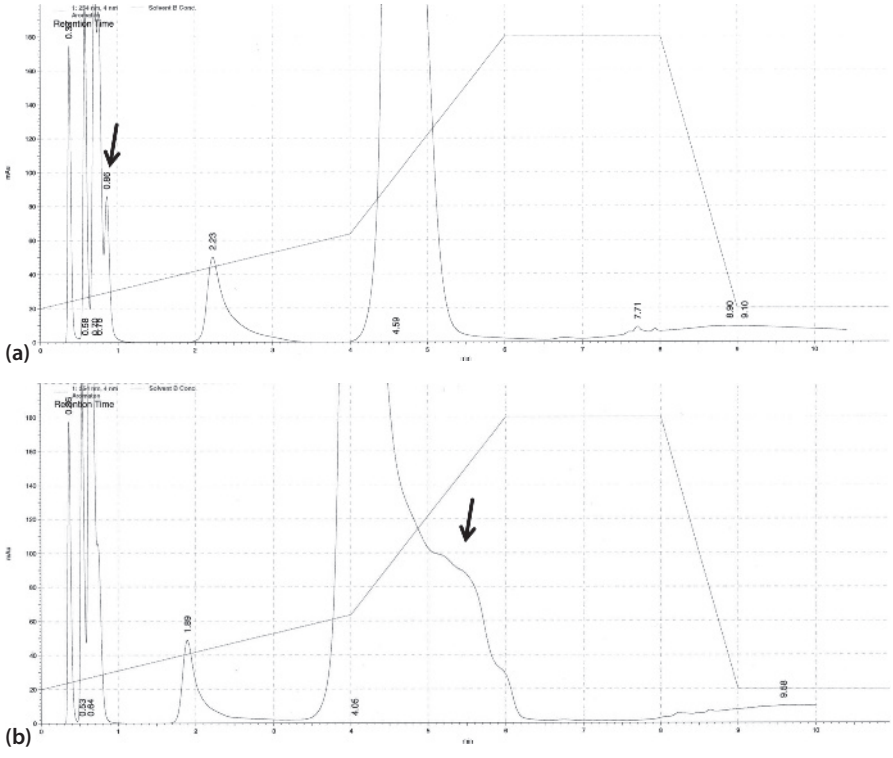


Figure 1.19 Different volumes of mixing chambers may have a different influence on the front area and on the back area of the chromatogram, for details see the text.

- With Obelisc R, a mixed-mode material, separation was better with the smaller mixing chamber in the first part of the chromatogram (Figure 1.19a), with the larger mixing chamber in the second part of the chromatogram (Figure 1.19b).

3. We checked the influence of the mixing device on the chromatogram from the Agilent 1200 high-pressure gradient system: the original Agilent mixer with a volume of 400 µl – which is located after the mixing chamber – was replaced with a LEE TCMA mixer with a volume of only 10 µl. Despite the small volume this mixer operates on the nozzle principle and thus has an excellent mixing efficiency. Hardly any difference could be detected, see Figure 1.20a,b (Poroshell EC 120): in a high-pressure gradient, the additional mixer will not inevitably have a noticeable impact if no extreme gradients or extremely small columns are used, see also Chapter 2.

Now to conclude, some aphorisms regarding gradients as a compressed "take-home message," many a repeat of previous text.

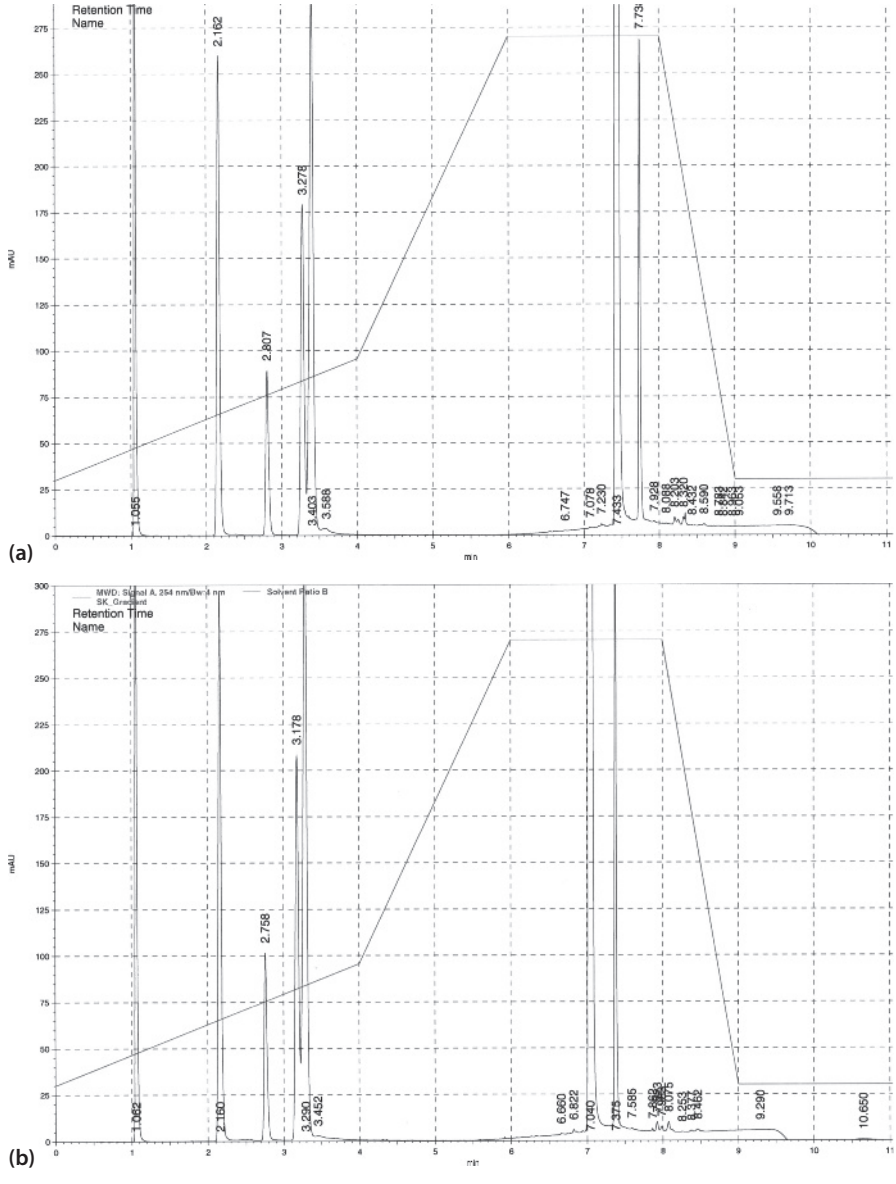


Figure 1.20 Mixers of different volumes: (a) 400 µl; (b) 10 µl, in a high-pressure gradient. There is no noticeable difference, for details see the text.

1.7 Gradient Aphorisms

Flow

- The resolution of later eluting peaks is more likely to be strongly negatively influenced by an increase in flow than that of earlier eluting peaks
- Increase in flow may change the selectivity/resolution
- Use a high flow with small particles and "simple" interactions
- Use a low flow with ≥ 5 µm particles, but also in the case of multiple interactions, that is when slow kinetics are expected
- Try a high flow first when Δ% B is ≥ 60%.

Gradient Duration

- The gradient duration is not as important as is generally believed, as a rule of thumb: gradient duration about 10–15 times the dead time
- If you use a long gradient, at least increase the flow.

The Initial and Final % B, Δ% B

- In general, Δ% B and gradient volume are the most important factors with respect to optimization, in practice – not always appropriately – the gradient duration is often changed instead of the flow. Consequently, essential optimization parameters are Δ% B and gradient duration and from these the slope.
- With differing components, a Δ% B ≥ 40 is necessary.
- The elution strength is more important than the flow, with the same gradient duration and twice the flow, the peaks elute only about 10% earlier.
- Start with, for example, 30–40% B, increase to 100% B and wait a few minutes to make sure that all the peaks elute during the gradient and not in the flushing phase. Then make the gradient flatter to test whether the resolution improves. Note as a general rule, the initial % B should so be chosen so that the first peaks of interest elute after approximately 2–3 times the dead time – don't unnecessarily begin with a high aqueous percentage. Choose the final % B so that all peaks which are to be separated actually elute – in no case choose a higher end % B, otherwise the gradient takes unnecessarily long and during this time nothing more happens. After elution of the last component, one should of course increase (fast, steep) the organic content to 100% B to flush the column. The flushing time depends on the matrix, the other organic impurities, and the additives in the eluent, and is usually 5 to 10 column volumes.

Length of the Column

- 20–30 mm, for about 5–8 peaks
- 50 mm, for about 8–12 peaks
- 100–125 mm, for about 20–25 peaks

Assumption: no additional peaks, for example from a difficult matrix

- Gradient volume (*and* Δ% B): much more important than the column volume
- Short column (and small particles)? Short gradient
- Long column? Longer gradient plus higher flow
- Short gradient? Length of the column rather unimportant.

Gradient Slope and …

- The steeper the gradient, the less the influence of the length of the column on the resolution
- The steeper the gradient, the less important the flow becomes
- Initial % B is more important for the first half of the gradient than the gradient slope
- The higher % B is at the start, the less of an advantage is, for example, a 30 min over a 20 min long gradient.

In General

- The shorter the column, the sooner the peaks will elute, they may even elute before they have "seen" the entire gradient; an isocratic step at the beginning affects the separation noticeably
- The later the peaks elute, the more important the gradient becomes, they "notice" the accelerating effect more than the early eluting peaks
- The early eluting peaks "see" less gradient like with a short column (that is, they undergo less acceleration), again the isocratic part of the run (the isocratic step) at the beginning is important
- High peak capacity does not automatically mean good resolution overall
- The gradient compensates dead volumes in the instrument and poor packing quality, a high plate number makes itself significantly noticeable only with really difficult separations
- The linear gradient model is sometimes insufficient, for example, often from about 80% B – here especially with acetonitrile – and in the case of additional ionic interactions, see Chapter 3.

Be courageous, when you have about $\leq$ 10 peaks and a relatively clean sample, then proceed as follows: short column, high flow, short, steep gradient starting at about 30–40% B.

Be consistent, when you expect about $\geq$ 30–40 peaks and have a UHPLC, then use a 150 × 3 mm, 2 µm column, run a steep gradient, and make sure that you have a gradient volume of about 25–30 ml. Subsequently run two gradients at two temperatures differing by about 30 °C, a long one with a low flow and a short one at a high flow – and keep an eye on the gradient volume. For 30–40 peaks, a gradient volume larger than about 30 ml for this column volume is not usually necessary. However, do you expect this number of peaks in a really difficult matrix, and over and above that are you aiming for a robust method? In this case think of 3.5 or even 5 µm particles, maybe also of a 4 or even 4.6 mm column.

References

1 Snyder, L.R. and Dolan, J.W. (2007). *High-Performance Gradient Elution*. John Wiley & Sons.
2 Wang, J., Ji, J., Aubry, A., Arnold, M., and Jemal, M. (2011). *Journal of Chromatography B* 879: 1917–1926.
3 Boswell, P.G., Schellenberg, J.R., Carr, P.W., Cohen, J.D., and Hegeman, A.D. (2011). *Journal of Chromatography A* 1218: 6742–6749.
4 Neue, U.D. and Kuss, H.-J. (2010). *Journal of Chromatography A* 1217: 3794.
5 Vogel, F. and Galushko, S. (2013). *International Labmate* 38: March.
6 Molnar, I. and Rieger, H.-J. (2013). *International Labmate* 38: April.