

## When Should I Use My UHPLC as a UHPLC?

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### 1.1 Introduction

Modern analytical LC systems are designed without exception as ultrahigh-performance liquid chromatography (UHPLC) systems. However, outside of pure research laboratories, a maximum of about 20–30% of the separations are performed under UHPLC conditions. By this, pressures above approximately 800 bar are meant. In which cases does it make sense, or is even necessary, to use the existing UHPLC system under truly UHPLC conditions? On the other hand, when should the UHPLC system perhaps be used as a fast, but “classical,” high-performance liquid chromatography (HPLC)? This chapter deals with exactly this point. To this end, the answers to two questions can help, both of which we will deal with. The first is “What do I really need?” Here it is necessary to define which characteristics of an HPLC method in exactly *this* situation are in the foreground, among others, for example, short retention times, a robust method, maximum resolution/peak capacity, and low detection limit. The second question is much simpler: “Why is the UHPLC more capable than the HPLC?” Afterward, we will discuss the key question: “How do I reasonably combine my requirements on the method and the potential of UHPLC – taking into consideration the real laboratory situation?”

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**Note** Familiarity with the theoretical background is assumed, and the principles of HPLC optimization are therefore only mentioned but not derived. For this, reference is made to the relevant literature (for example, [1–5]).

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## 1.2 What Do I Want to Achieve and What Is a UHPLC Capable of?

### 1.2.1 What Do I Want to Achieve?

One often wants more than only one attribute from an HPLC method, for example, “good” and “fast” separation. However, before deciding on the method design – which indeed includes the question of the necessity of UHPLC conditions – two points urgently have to be clarified. *Firstly*, what are the peculiarities of the method, and how is its environment? Here we are interested in, among other things, the following crucial features of the proposed analysis: matrix, time required for sample preparation and manual reintegration, experience of the user, changing or constant chromatographic conditions, research or routine laboratory, and so on. *Secondly*, what is the primary requirement for *this* method in the specific case? The main objective should be clearly identified, a second (or third?) regarded merely as a wish, for example: “In this case we need, for this reason the maximum possible sensitivity – if the method is also precise, that would be good too ... ” Four typical requirements for an HPLC method, which we will subsequently consider in more detail, are listed as follows:

- *Good separation*: this can mean, firstly, sufficient resolution – separation between two critical peaks or possibly between 2 and 3 relevant peak pairs. Or, secondly, sufficient peak capacity – separation of many (or all?) – possibly chemically similar components, see Section 1.3.1
- *Fast separation*: short retention times; this often goes hand in hand with a low solvent consumption, see Section 1.3.2
- *Sensitive measurement*: decrease in the detection limit, which means an improvement in the relative mass sensitivity, see Section 1.3.3
- *Robust conditions*: reliable methods, which lead to the avoidance of repeat measurements and minimization of equipment downtime, see Section 1.3.4.

### 1.2.2 What Is a UHPLC Capable of?

Put simply, a UHPLC system is an instrument that, first of all, compared to an HPLC system, has about 10 times lower dead volume (dispersion volume or “Extracolumn Volume”: the volume from the autosampler to the detector without a column) and also dwell/delay volume (the volume from the mixing valve/mixing chamber to the head of the column). The dead volume of a modern UHPLC system is nowadays about  $\leq 7\text{--}10\ \mu\text{l}$ , with the aid of special kits even about  $\leq 4\ \mu\text{l}$ , the dwell volumes are about  $100\text{--}200\ \mu\text{l}$  with low-pressure gradient (LPG), and about  $25\text{--}35\ \mu\text{l}$  with high-pressure gradient (HPG) systems.

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**Note** Nowadays, we talk less of “Extracolumn Volume” but rather of “Extracolumn Dispersion.” This takes into account the fact that the geometry of, for example, connections and mixing valves, and thus the flow profile, has more influence on the peak broadening than the absolute dead volume, see also Chapter 3. Secondly, a modern UHPLC system allows working pressures up to around 1500 bar.

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## 1.3 What Is Required from an HPLC Method?

### 1.3.1 Separate Well

First of all, we show briefly how the separation in HPLC can be improved in principle, and then we will have a closer look at the contribution UHPLC can make toward a better separation.

In chromatography, we distinguish with respect to the quality of a separation between two cases:

- 1) I am really only interested in one or a few components. It is therefore a question of – according to my individual criteria – sufficient separation between the component of interest and an “interfering” component – in other words, ultimately on the separation of two peaks. The focus can be on the critical pair (e.g., main and secondary components), possibly on two to three more peak pairs. The criterion here is the resolution, and when simplified, it describes the distance between the peaks at the baseline.

$$R_s = \frac{1}{4} \cdot \sqrt{N} \cdot \frac{k_2}{1 + k_2} \cdot \frac{\alpha - 1}{\alpha} \quad (1.1)$$

where  $R$  = resolution,  $N$  = plate number (fundamentally defined for isocratic conditions),  $\alpha$  = separation factor (formerly selectivity factor), and  $k$  = retention factor (formerly capacity factor  $k'$ ).

- 2) I want to or have to separate “all” existing peaks sufficiently well, that is, when possible with baseline separation. In this case, the peak capacity comes into play. This is the total number of peaks that I can separate in a certain time with a sufficiently good resolution (commonly  $R = 1$ ). The sum of all resolutions is often stated as a measure of the peak capacity. In the literature, one finds several formulas for the peak capacity, we consider here the two simplest:

$$n_c = \frac{t_{Rl} - t_{Re}}{w} \quad (1.2a)$$

or

$$n_c = \frac{t_G}{w} \quad (1.2b)$$

where  $n_c$  = peak capacity,  $t_{Rl}$  = retention time of the last peak,  $t_{Re}$  = retention time of the first peak,  $w$  = peak width, and  $t_G$  = gradient duration.

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**Note** About 70–80% of separations, nowadays, are gradient separations. Consequently, today's HPLC/UHPLC system is a high- or low-pressure gradient with DAD and/or MS/MS, and furthermore, aerosol detectors are becoming more common. The thoughts presented here apply in principle for both isocratic and gradient separations, but for the aforementioned reason, I will lay the focus a little more on gradient separations.

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Let us look first at the resolution.

Equation 1.1 shows that the resolution can be improved by increasing efficiency, selectivity, and retention. The requirement for the retention term is strong interactions, the optimum value lies around  $k \approx 3-5$ , and this means that the peaks of interest should elute by or after approximately three to five times the dead or mobile time. From Equation 1.1, it can be seen that the term for selectivity, and thus the separation factor  $\alpha$ , is by far the most sensitive function of the resolution:  $\alpha - 1/\alpha$ ! On the other hand, the plate number is under the root, a doubling of  $N$  improves the resolution by a factor of “only” 1.4. Two numerical examples illustrate this; for a detailed discussion, see [6]:

- 1) Assume that two peaks elute with an  $\alpha$ -value of 1.01. To achieve baseline separation of these two peaks, one would need about 160 000 plates. If the  $\alpha$ -value could be increased from 1.01 to 1.10, for the same resolution, just less than 2000 plates would be required. Even a seemingly small improvement in the  $\alpha$ -value from 1.01 to 1.05 means that instead of 160 000 plates, only about 6000 plates are necessary.
- 2) Further assume that we have a separation with the following values:  $k = 2$ ,  $\alpha = 1.05$ , and  $N = 9000$ . This results in a resolution of  $R = 0.76$ . This is not enough, and the resolution should be improved. To start with, the interactions can be increased, for example, through a more hydrophobic stationary phase or more water in the mobile phase. Assuming that the stronger interactions affect the two components equally, then the selectivity remains constant. The  $k$ -value increases from  $k = 2$  to, for example,  $k = 6$ , and the resolution increases to  $R = 0.97$ . Alternatively, one could use a column with 15 000 plates, and the resolution improves to  $R = 0.98$ . Both measures are therefore correct; however, they are not particularly effective when it comes to significantly improving the resolution. If the  $\alpha$ -value could be increased from 1.05 to 1.10, this would result in a resolution of  $R = 1.45$ . Let us finish the second example with the following observation: when two peaks are of different sizes (e.g., drug and impurity) and/or tailing is present, the resolution must be about  $R \geq 2$  if the error in integration is to remain below 1% [7]. In the present case, to improve the resolution to  $R = 2$ , there are two alternatives available: increase the  $\alpha$ -value from 1.10 just to 1.15 or double the plate number – at a constant  $\alpha$ -value of 1.10 – from 9000 to 18 000 plates. The last case would be possible with, for example, a 150 mm, 2.5  $\mu\text{m}$  column.

As a rule of thumb, for a baseline separation, one could remember the following:

- If, in a real sample, the  $\alpha$ -value of the critical pair is about 1.05, then for a baseline separation, about 20 000 plates would be necessary.
- If the  $\alpha$ -value is approximately 1.02, you would need about 100 000 plates.
- With an  $\alpha$ -value of 1.01, you will hardly be successful without 2D chromatography (see Chapter 6).

These numerical examples are valid for both isocratic and gradient separations.

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**Note** The plate number is defined for isocratic separations. There are several formulas, of which the simplest is

$$N = \frac{L}{H} = \frac{16 \cdot t_R^2}{w^2} \quad (1.3)$$

where  $N$  = plate number,  $L$  = length of the column,  $H$  = height of one theoretical plate,  $t_R$  = retention time, and  $w$  = width of the peak at its base.

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Let us look first at the question “what significance does the plate number have in isocratic and gradient separations?” When a peak elutes later in an isocratic run, it becomes wider, the ratio  $t_R/w$ , however, remains constant and therefore also the plate number. Note that the plate number for a component in the isocratic mode is – at least theoretically – a constant. This means it is independent of the retention time as long as alterations in this are due to a change in the stationary or mobile phases and/or temperature – but not in the flow! Once again, the peaks elute later or earlier and thus are wider or narrower – the ratio retention time/peak width remains constant and therefore also the plate number. Here, it is assumed that the mechanism of the interaction with the stationary phase remains constant.

How are the relationships in gradient mode? In the literature, it is often stated that, strictly speaking, the plate number can only be determined for isocratic separations. In connection with gradients, the terminology “separation efficiency” is often used. Nevertheless, also with gradients, there is no reason in principle against talking about a “plate number”  $N_{Gr}$  – at least as an idea.

Consider Equation 1.3 and assume that, due to any measure, a peak elutes in a gradient method later. In this case, the “plate number”  $N_{Gr}$  increases, because  $t_R$  increases but  $w$  remains constant. When simplified, the following applies: isocratic: ratio  $t_R/w$  constant and plate number constant; gradient:  $w$  constant and the “plate number”  $N_{Gr}$  increases.

What significance does this have for the separation? In isocratic separations, I can push the critical pair into the optimal retention area and, due to the optimal selectivity then existing, achieve the maximum resolution – but only for *this* critical pair, it is possible that other peak pairs may be less well separated. In a gradient run, because the peaks move closer together, the result is lower selectivity, but as we have seen that the plate number is higher, the peaks are narrow. As described earlier, the selectivity influences the resolution considerably more than does the plate number, and therefore, for this pair, we have better resolution under isocratic conditions as with a gradient run. Unless there is a particular case where the kinetics of desorption of one or more components from the stationary

phase are very slow, e.g. high enthalpy of adsorption, multiple mechanisms, and large molecules. Here the advantage of a higher plate number with a gradient predominates, and under gradient conditions, we have better resolution. With a gradient, compared to isocratic separations, additional possibilities for altering the selectivity in the “front” and “rear” areas of the chromatogram are available.

In comparison to isocratic separations, increased “plate number”  $N_{Gr}$  is the reason for an improved average resolution (often reported as the sum of the resolutions), which ultimately means a better peak capacity; see the following discussion. From a practical point of view, this means that, especially for gradient separations, the high plate number of a column is not as important as often suggested in the brochures of column suppliers. Example: “Our new column XYZ has 450 000 plates/m.” Common chromatographic conditions for such runs are acetonitrile/water, low flow, simple, neutral aromatics, and 1  $\mu$ l injection volume. Users can now so understand the specification “450 000 plates/m” to mean that during the use of the column, this plate number (with a 100 mm column, about 40 000–45 000 plates) would actually be available. Note, however, that the plate number is affected not only by the quality of the packing and the particle size. Far more than 10–15 factors play a role, such as eluent composition and temperature (viscosity), the dead volume of the system (more precisely, the dispersion of the substance bands), particle size distribution, retention time, flow, injection volume and sample concentration, constitution and pH of the sample solution, chemical structure and diffusion coefficient of the analyte, and, last but not least, the parameter settings affect the appearance of the peaks.

For example, broad, tailing peaks indicate a slow kinetic (e.g., additional ionic interactions, large molecules) or a significant dead volume in *this* system with *this* column – despite a “good” plate number. In conclusion, note the following: for improvement of the resolution, an increase in selectivity principally “brings” the most, an increase in plate number is secondary, the van Deemter H/u curves are much overrated by the marketing of the manufacturers.

How can I improve the selectivity? A change of pH, the addition of modifiers, and the use of alternative stationary phases are important factors and independent of the hardware. Let us look now at UHPLC. What can it actually accomplish? Of the two advantages of UHPLC – the small dead/delay volume and the ability to work at higher pressures – the second advantage can be used here. In the following cases, the efforts to improve the selectivity are accompanied by an increase in pressure, without doubt, a situation for which a UHPLC system is designed.

- *Methanol as organic solvent*: this often results in better selectivity than with acetonitrile by the separation of polar molecules.
- *Lower temperature*: by the separation of certain substances (enantiomers,  $\alpha$ - $\beta$ -/double-bond isomers), an improvement in selectivity is often seen at lower temperatures.
- *Pressure*: at pressures above around 600–700 bar, the polarizability of certain molecules (e.g., prednisone/prednisolone, conformational isomers, tocopherols, etc.) changes. The selectivity also changes (improvement?), and in combination with certain stationary phases ( $C_{30}$ , “Mixed Mode Phases” and

other “shape selectivity phases”), interesting possibilities arise. For example, immediately after the column, a restrictor with a minimal volume could be added. However, the robustness is to be seen critically by small pressure fluctuations in this region.

- *Flow*: increase in the flow leads to an increase in the gradient volume (gradient volume = gradient duration  $\times$  flow).

You can, of course, change two factors simultaneously and make the best use of the possibility of UHPLC to work at higher pressures. For example, with gradient runs, lower the temperature to 10 °C and at the same time increase the flow. In one case, we have increased the pressure to 1000 bar, lowered the temperature to 15 °C, and, at the same time, increased the gradient volume, once by means of the gradient duration (in this case, necessary due to the slow kinetics) and once by means of the flow. The number of peaks, which then appeared, had increased, compared with the original validated method, by about 30%.

Now we come to peak capacity.

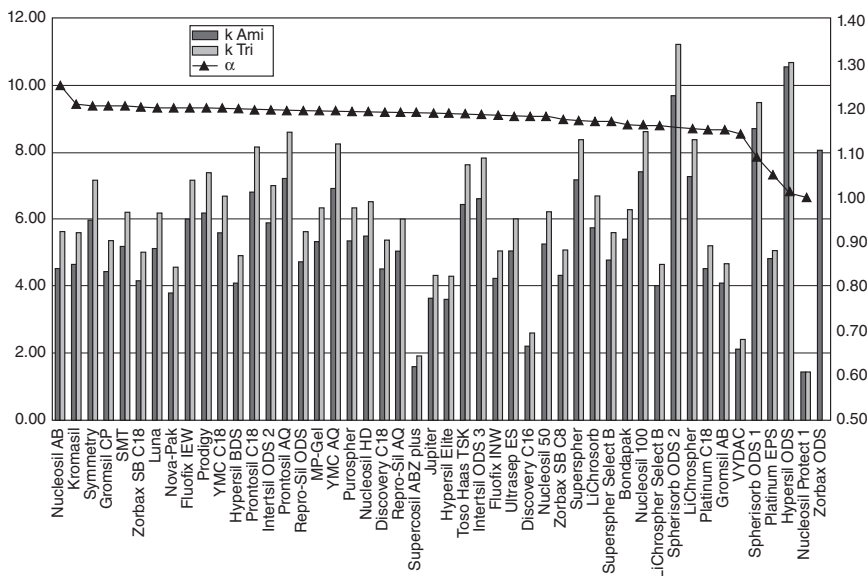
There are cases in which an improvement in selectivity is hardly possible, for example:

- A large number of possibly even similar components, in addition, may be in a complex matrix.
- When hydrophobic interactions dominate, these are not particularly specific, and there is hardly any noticeably different selectivity. When, for example, basic compounds are neutralized by pH, they are present as neutral molecules, and the interactions with the stationary phase are hydrophobic in nature and thus rather unspecific. In such cases, in the course of optimization experiments and when using different stationary phases, interactions of varying strength do occur, resulting in differing retention times and  $k$ -values, but the selectivity is often comparable, see Figure 1.1: differing  $k$ -values (see bars), but very similar  $\alpha$ -values (see lines) are found.

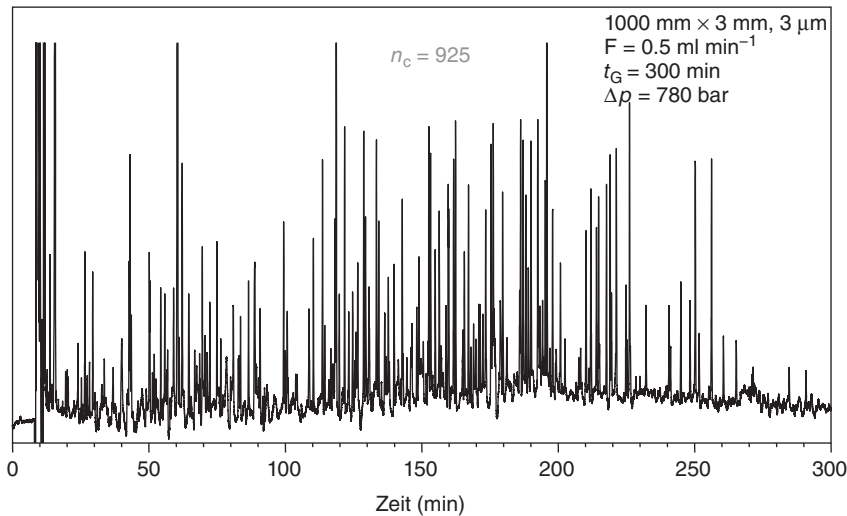
In such cases, a noticeable improvement of the selectivity is hardly feasible. Even if it were possible to improve the selectivity at one specific point in the chromatogram, it could become worse elsewhere. In a case such as this, the peak capacity comes into focus: peaks as narrow as possible (i.e., maximum achievable “plate number”  $N_{Gr}$ /separation efficiency), ideally evenly distributed over the entire chromatogram, see, for example, Figure 1.2 (taken from [6]). Here, a separation with a (theoretical) peak capacity of 925 peaks on four 250 mm columns connected in series is shown.

Before we look at how the UHPLC can profitably be used, let us note with reference to Equations 1.2a and 1.2b how in principle the peak capacity can be increased:

- 1) I need a long gradient or rather a large difference in retention time between the first and the last peak. This requires a large gradient volume, possibly also a long column.
- 2) I need a small peak width; in other words, I aim for narrow peaks. I can achieve this through a steep gradient, a high start and also end % B, small particles, low viscosity, and high temperature.



**Figure 1.1** Retention (bar) and separation factors (line) of tricyclic antidepressants in acidic acetonitrile/phosphate buffer on differing RP phases; for details, see text. (From “HPLC richtig optimiert,” Figure 7, S. 176.)



**Figure 1.2** High-resolution 1D-UHPLC separation of a tryptic digestion of five proteins. A chain of four 250 mm columns was constructed using dead volume couplings based on Viper fittings (Thermo Scientific). Stationary phase: Acclaim 120 C18 (Thermo Scientific), temperature: 30 °C. Theoretical peak capacity calculated from the peak width of individual well-resolved peaks. (From “HPLC-Experte,” Figure 3.25, S. 164.)



As known, the UHPLC gives us small dead volumes and allows high pressures. With reference to the last named advantage, the following would be possible: a long column or several columns connected in series plus perhaps small particles. Note that when columns are connected in series, the negative influence of the dead volume ("Extra Column Effects") is minimized. From experience, this becomes quite apparent in the case of very small column volumes in spite of the most modern UHPLC design, see further discussion and Chapters 3 and 4. Because with gradient separations, the particle size is not so crucial, one possibility would be as follows:

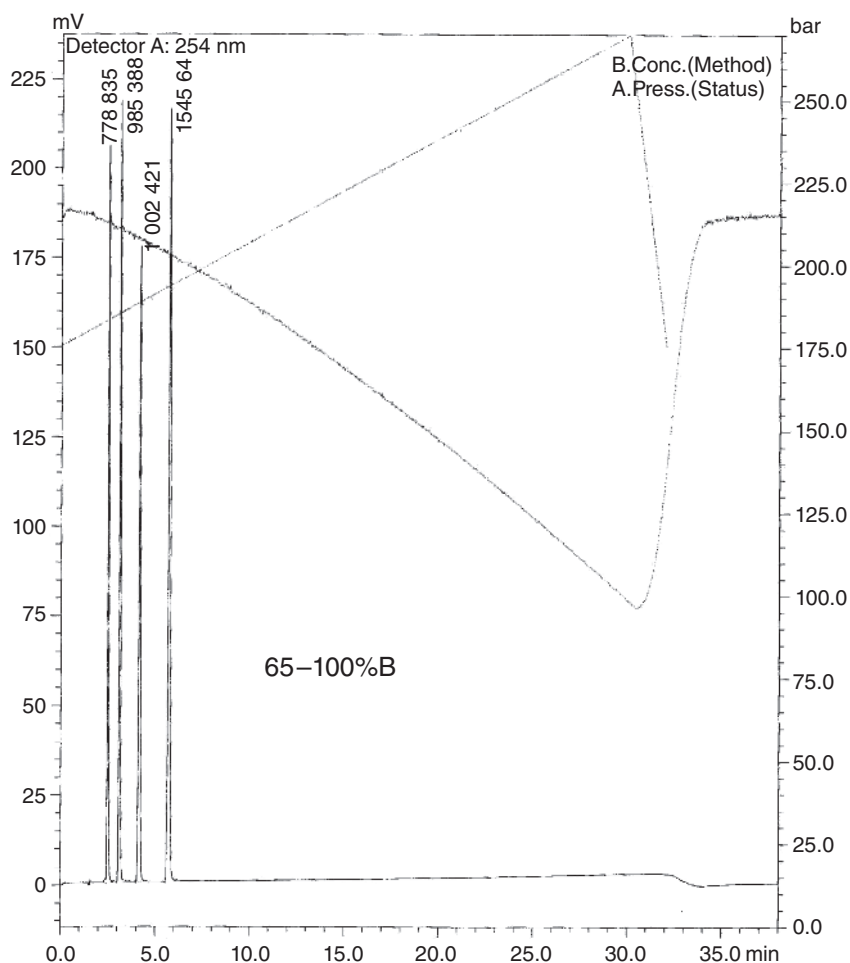
Three 150 mm  $\times$  3 mm columns, 2.5–3.5  $\mu$ m particles, in series plus 40–50 °C.

A survey of the literature showed that separations with higher peak capacity under UHPLC conditions are published more and more frequently. To start with, the following examples seem to be realizable for a "Real-Life" laboratory.

2  $\times$  150 mm, 1.9  $\mu$ m, 1200 bar at 45 °C: 480 peaks in 40 min (12 peaks/min)  
 3  $\times$  150 mm, 2.6  $\mu$ m fused core, 1200 bar at 45 °C: 600 peaks in 50 min (12 peaks/min)  
 4  $\times$  250 mm, 3.0  $\mu$ m, 1200 bar at 30 °C: 1000 peaks in 300 min (3–4 peaks/min).

If time is not a significantly limiting factor and the matrix not extremely difficult (polymers, foods, fermented cultures), with UHPLC, about 600–1000 peaks can theoretically be separated, see also Chapter 12. For such cases, in the mid-term long columns with 2.1 mm internal diameter and 1.5–2.6  $\mu$ m, fused-core material could represent one of the most interesting possibilities. Under optimal conditions and with the most modern UHPLC hardware, the target is "100/100": 100 peaks/100 s. To date, separations with a theoretical peak capacity of 730 peaks in 30 min or 530 peaks in 13 min have been reported. The higher the peak capacity – made possible through an optimal combination of UHPLC system and column – the less necessary a good selectivity becomes, the improvement of which in any case is not exactly a trivial task, especially when pressed for time.

Now let us look at everyday use. In a real chromatogram – except perhaps with a sample containing only homologs – the peaks are rarely evenly distributed. Especially when the peaks also have to be quantified – that is, a resolution of 1.5 or at least 1 is necessary – in practice, only a much smaller capacity can be achieved. According to statistical calculations from Giddings, with a theoretical peak capacity of 1000, 184 peaks could be separated. Taking into consideration a difficult matrix and/or possibly suboptimal equipment, a good rule of thumb is considered to be about 1/10 of the theoretical peak capacity, for the last given example in reality, 100 peaks. Put simply, for really demanding problems (multicomponent samples and/or a complex matrix), the best method is 2D chromatography with orthogonal separation mechanisms, the next best is the modern UHPLC, which nevertheless can provide one-dimensionally a theoretical peak capacity of around 1000.



**Figure 1.3** Separation of polystyrene; a good peak capacity is obtained starting with 55% B and using a flat gradient. (Source: Waters.)

Here too, you could try to use simultaneously as many parameters as possible, which can contribute to a good peak capacity. The following variations correspond to an “optimal” combination.

A long column (or multiple columns in series), 2–3  $\mu\text{m}$  particles, a high flow, 40–50  $^{\circ}\text{C}$ , acetonitrile as the organic solvent, gradient starting at about 40% B. With ionic components, one could try to achieve good peak symmetry by altering the pH. Depending on the mechanism, a steep gradient, occasionally also a flat gradient, can be beneficial, see Figure 1.3.

Column length and gradient duration have one thing in common: both have less influence on the peak capacity than is generally believed. For example, gradients longer than 20–25 min only make sense in the case of very complex mixtures. With respect to column length and gradient duration, note the following simplified rules of thumb for an optimal peak capacity:

- 50 mm  $\leq$  5 min
- 100 mm  $\approx$  10–20 min
- 150 mm  $\geq$  20 min.

In this context, the aforementioned statements should be recalled. Firstly, increasing the plate number is not really the most effective way to improve the resolution. Secondly, gradient separations produce narrow peaks anyway, and a high “plate number”  $N_{Gr}$  is relevant only in difficult cases – when a high peak capacity is needed. Furthermore, a decrease in the particle size by a factor of 2 led (by a fourfold increase in pressure) to an improvement in resolution by a factor of 1.4. An increase in column length by a factor of 2 (the pressure increased only by a factor of 2, the analysis time was longer) also led to an improvement in resolution by a factor of 1.4. If I continue to increase the column length (or use multiple columns), up to a given/critical pressure, I achieve significantly more plates. Thus, if the ultimate aim is “maximum plate number” (question this critically!), then I should connect several columns in series, filled with 2.5–3.5  $\mu$ m particles – in this case, I would have to accept the long retention time. In other words, I should use the pressure allowed by UHPLC for long columns rather than for small particles; see also comments in Chapter 12. Some numerical examples follow to illustrate this:

- A typical UHPLC column, 100 mm  $\times$  2.1 mm, 1.7  $\mu$ m, and at a pressure of about 1000 bar has approximately 20–25 000 plates. If a pressure of about 1000 bar should be regarded as a limit for prolonged routine use, one soon sees the limits of small particles as a source of maximum efficiency – despite UHPLC. Thus, it becomes clear that the UHPLC, under normal conditions, cannot provide the resolution that would be necessary for difficult separations
- Four 250 mm  $\times$  4.6 mm, 5  $\mu$ m columns in series have approximately 100 000 plates – at a pressure of about 600 bar (interesting: additionally set the temperature to 80 °C; result: excellent peak capacity at moderate pressure).

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**Note** For separations under “Ultrahigh-Resolution Separation” conditions (plate number >150 000 plates), the temperature should not be increased – this would have a negative effect on the B-term of the van Deemter equation, and the result would be increased diffusion.

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- One 250 mm  $\times$  2.1 mm, 1.9  $\mu$ m column provides approximately 55 000 plates – at a pressure of about 960 bar. This plate number should be about the maximum, which can nowadays be achieved with a column under real conditions.

In summary, the conclusion regarding column length, number of plates, and pressure is as follows.

Doubling the column length leads to twice the pressure and an improvement in resolution/peak capacity by a factor of 1.4. Half of large particles lead to fourfold pressure and also to an improvement in the resolution/peak capacity by a factor of 1.4. From this it follows that at a given pressure, the plate number can be increased

more effectively with a longer column (or columns in series) than by using smaller particles. Once again, for gradient separations, both longer column(s) and small particles do not necessarily have really essential advantages – with the following exceptions: very many, very similar components (long column), very slow kinetics, and low mass sensitivity (small particles).

In this context, the experience that is still valid even after 50 years of HPLC is recalled: a 250 mm × 4.6 mm column can be filled more easily and better than a short and especially a thin column. Another advantage of the long column is the long lifetime. Disadvantages are long runs and high solvent consumption – both not to be underestimated in the long term.

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**Note**

- 1) As we have seen earlier, neither good packing quality nor small particles guarantee a good peak shape, and among other factors, the dead volume can play an important role: with particles  $\leq 1.7 \mu\text{m}$ , the plate number and also the peak symmetry often increase with later eluting peaks. This shows that the dead volume of today's UHPLC equipment is too large to fully exploit the efficiency of these particles. A further indication of this fact is that the resolution of early eluting peaks on  $5 \mu\text{m}$  columns is often better than on  $\leq 2 \mu\text{m}$  columns.
  - 2) As just stated, in gradient separations, neither the length of the column nor the gradient duration or the particle size is of crucial importance – rather the gradient volume, initial and end % B, and the slope. Especially for gradient separations, “real” UHPLC conditions are only needed for very challenging separation problems (e.g., complex mixture, high peak capacity required) and/or in cases where several parameters need to be altered simultaneously, thereby increasing the pressure. If, for example, in the case of polar components, both selectivity and peak capacity are to be improved, one could proceed as follows: long column (or several columns in series), 2–3  $\mu\text{m}$  particles, high flow, 40–50 °C, plus methanol, and as an alternative to increasing temperature, try a run at 10–15 °C. Especially with such experiments, carried out in a short time, the UHPLC can show its strengths.
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**1.3.2 Separate Fast**

First of all: if the selectivity very good, then one could use a 3 mm,  $5 \mu\text{m}$  column with relatively high flow – in any case, one achieves this way a faster separation than under UHPLC conditions. Note that due to the good selectivity, the low plate number resulting from the high flow is of no great importance. The strength par excellence of UHPLC lies in the following situation: when the column being used has almost the optimal selectivity, then a sufficiently good resolution in a short time is obtained. In other words, under UHPLC conditions, we get the best plate number/time ratio – that means the lowest retention time at a given efficiency and also low solvent consumption. A decrease in column length and, at the same time, a decrease in particle size gives us an “identical” separation in a shorter time at a lower solvent consumption.

Requirements for an “identical” separation are equally well-packed columns, no noticeable decrease in the plate number due to the wider size distribution for particles  $\leq 1.7\ \mu\text{m}$ , and no deterioration in peak shape (tailing) due to dead volume, especially with the early eluting peaks. Put differently, by keeping the column length constant and using smaller particles, I can achieve, through an increase in the plate number, better resolution in the same retention time. Here I could even at the same time reduce the retention time, because according to the  $H/u$  curve, with the now smaller particles, I can increase the flow without appreciable loss of efficiency. Note, however, that with concentration-sensitive detectors, the area decreases.

Of course, an increase in flow only makes sense when by the interactions of the analyte with the stationary phase, fast kinetics and thus a small  $C$  term in the van Deemter equation result. To these relationships, the following remark is valid: they are neither new findings, nor is therefore a UHPLC necessarily required. Such improvements are – at least partially – up to a pressure of 400 bar with a classical HPLC system quite realizable. Only since the introduction of UHPLC technology in the mid-2000s does one risk working at higher pressures.

Conclusions for “fast separation” and UHPLC:

- 1) If the aim is “fast separation,” then UHPLC is outstandingly suited if, firstly, the separation problem is not very demanding ( $\leq 15\text{--}20$  peaks); secondly, constant, simple, robust chromatographic conditions dominate; and thirdly, there is an automated and constant sequence. As a typical application, the IPC (In-Process Control) could be named here.
- 2) The UHPLC is also suitable when in a short time I have to develop methods and/or identify trends or optimize existing methods by varying various parameters. The UHPLC allows many parameters to be tested rapidly, since short gradient runs using short columns with sufficiently good resolution/peak capacity are possible. The UHPLC is predestined for development departments, which, pressed for time, have to develop or optimize methods with varying parameters or also with the help of generic runs.

### 1.3.3 Improve Mass Sensitivity

This must be the simplest case, while my goal is clear – the quantification of small peaks. Equally clear is the contribution UHPLC can make with its characteristics. The following is relatively easy to implement:

- *Reduction of the internal diameter:* when one is not able to or allowed to inject more volume, a thinner column in combination with small particles leads to an improvement in the relative mass sensitivity.
- A shorter column with smaller particles leads to a smaller peak volume.

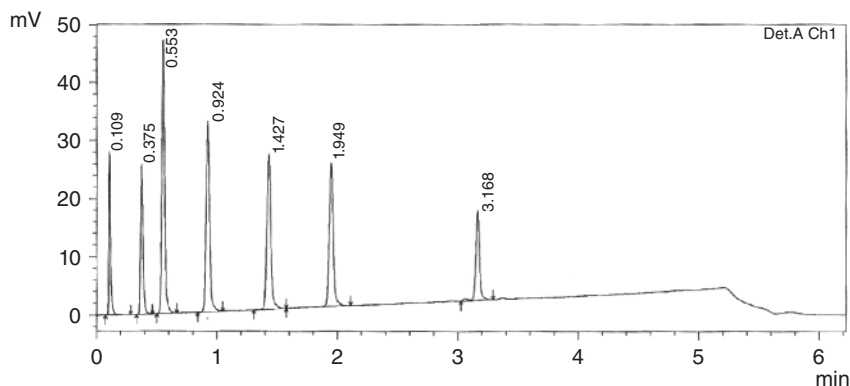
In this context, however, one should consider the following practical aspects.

A reduction of the column inner diameter from, for example, 2 to 1 mm did indeed lead – at a pressure increased by a factor of 4 – to an improvement in the relative mass sensitivity by a factor of about 20. However, to pack a 1 mm capillary well and reproducibly is not an easy task – both with porous and with – in particular! – fused-core materials. In addition, by the analysis of secondary components on thin columns or capillaries, there is the danger of the columns being overloaded by the main peak or the matrix. Finally, when one wants to advance into these regions, the dead volumes of even the most modern UHPLC systems need to be optimized. This means that when the maximum possible relative mass sensitivity does not necessarily have to be reached, under everyday conditions, a 2.1 mm, 1.5–1.7  $\mu\text{m}$  column would be well suitable, in matrix-free sample solutions and with optimal hardware perhaps 1.3  $\mu\text{m}$ . If the relative mass sensitivity has to be further improved, then the UHPLC reaches its limit, this is where nano or capillary LC comes into play.

Although the following points are not UHPLC-specific, for good sensitivity, they are important and are therefore briefly mentioned here:

- The sample solution should be weaker – in RP-HPLC more polar – than the initial eluent, which means dilute the sample solution with water; this leads to a concentration at the head of the column (“On-Column Concentration”)
- *With very early eluting, chemically similar peaks:* start the gradient with a high proportion of water/buffer and maybe use a short isocratic step; here too, a concentration is possible
- *For simple separations and with not too polar components:* start with 50–70% B, and run a steep gradient, see Figure 1.4

We conclude this section with the following tip: optimal parameters such as data recording rate (sample rate), and time constant/response time are important at high flows and in general with early eluting, narrow, small peaks. This is increasingly so under UHPLC conditions and even more so under UHPLC conditions and when striving for good mass sensitivity. For suitable numerical values, see Section 1.4.



**Figure 1.4** Initial gradient conditions with the focus on improvement in peak form; Column GeminiNX, 50 mm  $\times$  4 mm, 3  $\mu\text{m}$ , 65–100% B (acetonitrile/water).

**Note** In connection with the advantages of UHPLC, it is repeatedly claimed that the “sensitivity” in UHPLC is better in comparison to HPLC. But if here the sensitivity of detection is meant, then the opposite is the case! The explanation is that if one uses a conventional column and a DAD as a concentration-sensitive detector, a cell with a long path (Lambert–Beer “longs for” a long light path) can be used. The risk that the sensitivity could be neutralized by dead volumes hardly exists, as the column volume is large in comparison to these. Furthermore, as already known, the injection volume should be at the most 10% of the column volume, with low retention factors, band broadening makes itself noticeable from 1% injection volume. With a conventional column, using larger injection volumes is no problem. With the low-volume columns used in UHPLC, the injection volume should not exceed 1–2  $\mu\text{l}$ . For the same reason (small column volume), in the UHPLC, the “Extracolumn Volume” and consequently the detector cell volume must be small. Despite significant recent progress in cell design, with the cell volume of much less than 1  $\mu\text{l}$  required in the UHPLC, the light path in a detector cell cannot be significantly lengthened. For these reasons, detection sensitivity in UHPLC is intrinsically less sensitive than in HPLC. However, if from the outset, the injection volume is small or cannot/may not be increased, the UHPLC undoubtedly has an advantage: the mass sensitivity in an (optimized) UHPLC system due to the small peak volume is better by magnitudes than in the HPLC.

#### 1.3.4 Robust Separations in Routine Use

In a routine laboratory, the robustness of the method has top priority and the downtime should be reduced to a minimum. In the case of simple chromatographic methods, large numbers of samples, robust conditions, clear sample solutions, no/minimal sample preparation, automatic integration, and so on – see earlier discussion – UHPLC would no doubt come into question. In the following cases, the use of a UHPLC system should, however, be critically questioned:

- *Difficult matrix*: the sample preparation does not lead to homogeneous, clear sample solutions, and they contain possibly substances from the matrix (plant extracts, contaminated soils, coated tablets, ointments, polymers, biological matrices such as tissue, blood, etc.). Before we come to further critical points regarding an appropriate use of the UHPLC, here is an example of the unsuitability of UHPLC. Assume that the components of interest are only soluble in or extractable with acetonitrile, alcohols, or tetrahydrofuran, and the sample solution is then stronger (more organic) than the eluent/initial gradient. In particular areas – such as pharmaceuticals and environmental analysis – this is unfortunately unavoidable. A typical example would be the extraction/dissolving of active ingredients into ethanolic solutions (analysis of ointment). In this case, we have to deal with fronting, at the worst, even double peaks occur. Due to the small column volumes in the UHPLC (e.g., for a 5 mm  $\times$  2.1 mm column about 200  $\mu\text{l}$ ), the poor peak shape persists – in the UHPLC, the “lost” plate number remains permanently lost! Somewhat simplified, one can say: a matrix-contaminated sample and/or a strong solvent



let every UHPLC fail. With conventional columns (longer/thicker and thus with larger column volume), the problem usually occurs only with the early eluting peaks and injection volumes greater than about 15–20  $\mu\text{L}$ .

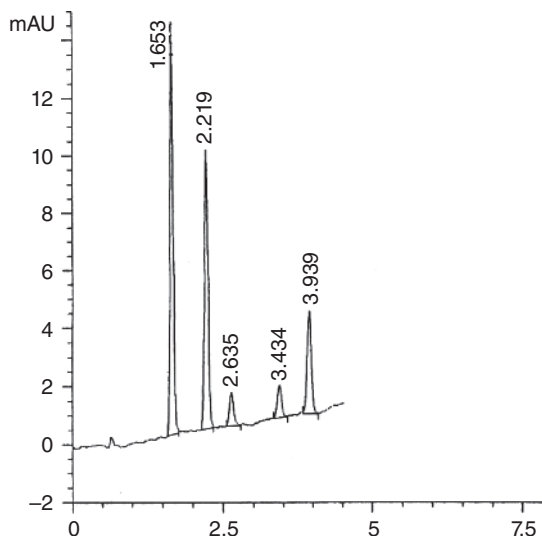
- Varying chromatographic conditions in routine use, poor robustness of the method(s).
- Sample preparation, manual integration, and other necessary steps such as documentation, and filing take much longer than the separation time.
- The number of samples is relatively limited.
- Frequent method transfers with several laboratories and anticipated differences in procedure, in know-how, and in the “culture.” Here are a few examples: a less experienced HPLC user simply takes a different capillary or cuts it off improperly or connects a PEEK fitting with a steel capillary or uses a column switch or a connector between column and detector, in order to fit columns from different suppliers. The UHPLC does not “forgive” such things, whereas the HPLC is more likely to.

Let us imagine a fictitious laboratory in the quality control of a pharmaceutical company or generic drug manufacturer, where tablets, capsules, or ointments are analyzed. Admittedly simplified, the situation often looks something like this: a user has possibly two HPLC systems to look after, and/or, on top of that, also has other jobs to do (among others, documentation). The methods are old: LiChrospher/Nucleosil 100/Hypersil ODS/Spherisorb ODS, and so on, phosphate buffer, possibly triethylamine or ion-pair reagents and so forth, things do not always run smoothly. Often, time is short, after a system failure – for whatever reason – the system must be requalified or at least, using system suitability tests and possibly repeat injections, the fault-free condition of the equipment must be verified. One already has an uncomfortable feeling before the impending method transfer, since from experience, something like that is seldom completed without complications.

When considering the possible introduction of a UHPLC, the objections could read something like “does it really help much when we reduce the retention time from 20 to 6 min, when the sample preparation takes half an hour and the frequently necessary manual integration just as long, not to mention screening the individual chromatograms? And what use is it, when on the one hand the sequence is already finished at 11 p.m. instead of 5 a.m. the next morning, and on the other hand the system more often shuts itself down? And as far as solvent consumption is concerned, in the laboratory we are talking about liters, in the production of hundreds of liters . . . . And anyway, we were unsatisfied with the life of the UHPLC columns during the test phase with the UHPLC.” One can certainly more or less understand these arguments – up to perhaps the last point: relevant is not the absolute column life, rather the number of injections per unit time or the number of column volumes run before the column becomes unusable. Based on the final criterion, and excluding matrix problems, and so on, no significant difference between HPLC and UHPLC columns can be seen. When a UHPLC is now to be used in this environment without changing



**Figure 1.5** Separation of seven components on a Synergi MAX RP 20 mm × 4 mm, 2 μm column in less than 2 min on a high-pressure gradient system from the beginning of the 1990s – the system had considerable dead volume.



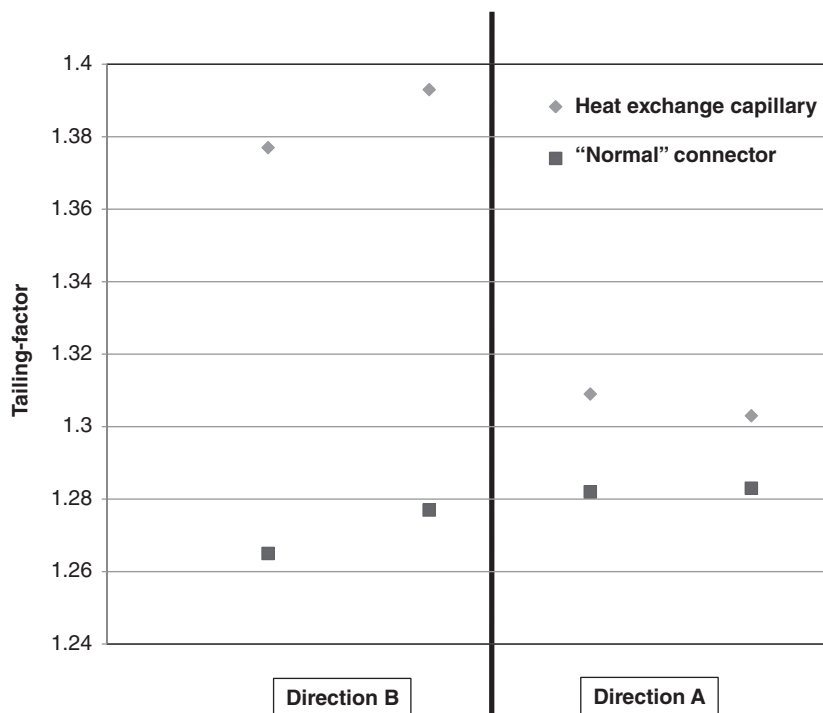
attitudes, working habits, and also the expectations on a UHPLC, then many annoying things can happen. Following are just three examples:

- A small salt crystal from the nonfiltered buffer or a part of the matrix can block the thin capillaries used in the UHPLC, resulting in leaks or, at the worst, the system shuts itself down.
- There is no UHPLC column available with similar characteristics to the column specified in the method.
- Method transfer: when SST criteria, for example, in addition to resolution, the symmetry factor, and relative retention time instead of relative retention [8] are required, then this is often quite difficult to achieve – in spite of the existing software tools for scaling method parameters to be found in the literature or available from the equipment manufacturer. The problems of method transfer from UHPLC to HPLC will not be discussed here in detail, reference is rather made to Chapter 4.

With any functioning, classical HPLC system, improvements are possible in any direction. Following are two examples that show that with older HPLC instruments, significant time savings can be achieved without noticeable loss of resolution and in compliance with the SST criteria (Figure 1.5).

## 1.4 The UHPLC in Routine Use – A Brief Report

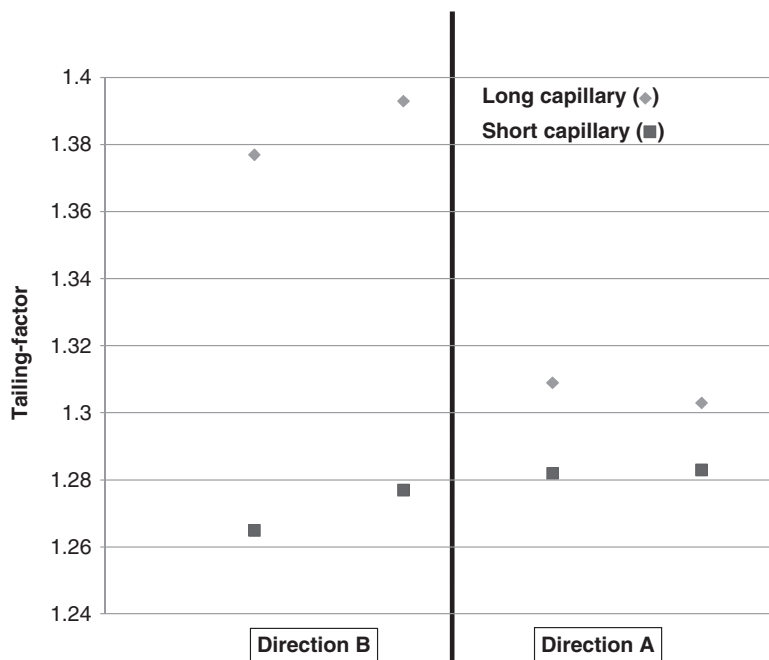
This section provides a brief, simplified description of the routine use of UHPLC. As mentioned at the beginning, since the mid-2000s, more and more UHPLC systems have come into use – with both satisfied and less satisfied users.



**Figure 1.6** Tailing factor depending on the direction of the used capillary. For details, see text.

Satisfied users in routine laboratories are firstly those who, under UHPLC conditions, run chromatographically relatively simple analyses ( $\leq 20$ – $25$  components with quite different properties) under constant, stable conditions, or, secondly, those who operate their UHPLC system more or less as a fast HPLC, because in the past they have not – for whatever reasons – used the capabilities of their HPLC for rapid separations. The last group of UHPLC owners are not really UHPLC users in the true sense, but they are satisfied – so what? Both user groups are happy with the fast separations, the narrow peaks, and the reduced solvent consumption. Users in development laboratories are satisfied because in a short time they can perform many optimization experiments that does indeed make lot of fun!

Dissatisfied UHPLC users are primarily those who over the years were spoiled due to the relatively minor problems with their HPLC systems and are now faced with a UHPLC instrument with such problems in routine use: solutions and chemicals must be particularly pure or maybe even extra filtered, leaks in the autosampler are a nuisance, "good" columns suddenly show double peaks in the UHPLC, in the case of early eluting peaks, the parameters (sample rate, time constant, etc.) must be adapted, and so on. Even a capillary fitted the other way around can affect the peak symmetry, see Figures 1.6 and 1.7. In short: if, in a real environment, I do indeed want to use my UHPLC as a UHPLC, then I have to increase my care and effort compared to my previous HPLC habits – or I stay below about 600–650 bar and definitely have less problems in routine use.



**Figure 1.7** Influence of the direction of capillaries on the asymmetry factor; for details, see text.

**Note** In a highly regulated environment, absolute priority has, without doubt, the confirmation of the expected/desired results with minimization of trouble, repeated measurements, and system downtime. Time and solvent savings are desirable, in fact; however, these are second- or even third-ranking goals. The requirements for a routine method are therefore robustness, time, and only then cost savings. All this can be achieved to a large extent without UHPLC: the U.S. Pharmacopoeia allows a good many adaptations without having to revalidate the method. I could, for example, use a different  $C_{18}$ , a 70% shorter column, one 25% thinner, one filled with 50% smaller particles. Furthermore, according to the USP and also European Pharmacopoeias, it is permitted to alter the gradient, only the final % B must be maintained (for details, see [8–10]).

When, after an adjustment, the system suitability criteria – well thought out (!) – are still met, then a revalidation is not necessary. This topic is complex and alone could fill a book. We end this little excursion with the following personal appraisal: it is certainly not easy, but one could at least try to overcome one's own trepidation a little and make use of the flexibility allowed by the authorities and organizations. In the process I could maybe, in compliance with the requirements, make my method more robust or faster, thereby saving my employers money, sparing my nerves, doing something useful for the environment (solvent consumption) – and the adaptations are formally in order. If something similar to this is possible in such a highly regulated area as the pharmaceutical industry, then it could be implemented analogously even more so in other industries.

The message for a routine laboratory is, in my view, as follows: first, make full use of regulatory flexibility and the technical possibilities of your current HPLC system. Even this may be an important step that brings a noticeable improvement toward your aim. When you want to or are allowed to, with reasonable effort and small investment, you can achieve “UHPLC-like” separations on your HPLC system:

- Dilute the sample solution – concentration at the column head will be possibly resulting in an improvement in the peak shape, especially for the early eluting peaks.
- Use a smaller loop in the autosampler.
- Think of the parameters (Sample Rate, Time Constant/Response Time, Dwell Time, Bandwidth, Slit, see Section 1.5).
- Use a smaller UV cell in your DAD (2–4  $\mu\text{l}$  volume is OK, – optimal would, of course, be 0.25–1  $\mu\text{l}$ , length 60–85 mm).

When subsequently, the method works reliably for a while and you want to further improve it (does everyone involved really want this?), then of course you should, with an appropriate budget, now think of UHPLC.

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## 1.5 How Can the Potential of UHPLC Effectively Be Fully Exploited? (See Also Chapters 2, 3, and 9)

A look at the special features of UHPLC is also helpful in answering this question: small dead and delay volumes, high pressures possible. Furthermore, the current analytical problem should always be in the foreground. We can state the following: for not too difficult analytical problems, all commercial UHPLC systems are good enough – especially in gradient mode. The more demanding the separation problem is, the more important the parameter settings become and the more likely hardware optimization could be attained. To this end, the subsequent remarks should provide some clues. Using dead volume as an example, it will first be made clear that, depending on the current situation – which can only be assessed individually – the entire bandwidth is possible, from “nothing needs to be done” to “urgent need for action.”

### 1.5.1 Dead Volumes

The fact is that the dead volume of every UHPLC system on the market is much too large for a 50 mm  $\times$  2.1 mm,  $\leq 2 \mu\text{m}$  column or smaller. Depending on the manufacturer, the loss of efficiency is around about 20–40%, and this is openly admitted by most manufacturers. If you notice a conspicuous tailing in the early peaks under isocratic conditions, then you know that this is currently the case.

The question now is: does it bother me that, due to the dead volume, instead of, for example, the possible peak width of 4 s, it is now 6 s? Or not really, since I have a relatively simple separation problem and fortunately the required symmetry factor of 1.3 is not exceeded? With gradient separations, the dead volume (not the dwell volume!) is even less relevant, because here narrow peaks are almost

guaranteed. When, on the other hand, this loss in efficiency interferes with the separation of my early eluting peaks, then something must be done. In this case, optimization kits with particularly thin capillaries, dead-volume-“free” fittings, specially designed UV-cells, and so on, can help. These are now available from the manufacturers.

Conclusion: according to the situation, either “business as usual” or urgent action.

Numerical values for settings and chromatographic parameters as well as hardware requirements, which are necessary or useful for UHPLC separations:

- *Data recording rate (sample rate)*: usually 20–40 Hz, with runs of about  $\leq 1$ –2 min and about  $\leq 1$ –2 s peak width, more than 50 data points per peak are necessary to avoid loss of resolution.
- *Time constant*:  $\leq 50$  ms. In some software programs, the time constant is, however, coupled to the data recording rate.
- *Bandwidth and slit with a DAD*: for good detection sensitivity, 16 nm, for a good spectral resolution (e.g., for Peak Purity tests), 1–4 nm.
- *Injection*: injection cycles as short as possible. Here, injectors with fixed loops have an advantage, but the problem of carryover should not be lost sight of. Injection volume about 1–2  $\mu\text{l}$  (for a 50 mm  $\times$  2.1 mm column with a column volume of approximately 200  $\mu\text{l}$ ), in any case  $< 5 \mu\text{l}$ . As we have seen earlier, the 1% rule is valid – the injection volume must not exceed 1% of the column volume; otherwise, the volume overload is noticeable. Regarding the possibility of larger injection volumes, see Chapter 9.
- *Cell volume*: 1–2  $\mu\text{l}$ , 0.25  $\mu\text{l}$  is worth considering (regarding UHPLC/MS coupling, see Chapter 5).
- *Dead and delay volumes*: at the moment there exists here, so to speak, a sporting competition among the manufacturers, as to who can save how many microliters first. Note that 10  $\mu\text{l}$ , at the most 15  $\mu\text{l}$ , dead volume would be acceptable for a column with 100–300  $\mu\text{l}$  column volume. Here the 10% rule applies; the volume outside the column may not exceed 10% of the column volume; otherwise, the plate number falls considerably for details, see Chapters 2 and 3. As far as dwell volumes are concerned, low-pressure UHPLC instruments with a dwell volume of 100  $\mu\text{l}$  are indeed already available, but 150–200  $\mu\text{l}$  is certainly not bad. As is known, the dwell volume affects the separation; however, it is not possible to make a general statement about whether, as a matter of principle, a large or small dwell volume is good or bad for the separation.
- *In general*: at high pressures and at the same time high flow rates, heat is generated due to friction (“Frictional Heating”). This effect can significantly influence the separation, and one should bear this in mind (see Chapter 2.2). Furthermore, a preheater with a volume of 1–2  $\mu\text{l}$  (for example, a 300 mm, 100  $\mu\text{m}$  capillary prior to the column oven) is part of every modern UHPLC. Commercial UHPLC eluents are usually filtered through a 0.22  $\mu\text{m}$  filter. When, nevertheless, problems with blocked capillaries and frits occur, an additional filtering of the eluent and also of the sample solutions and other chemicals would be appropriate.

## 1.6 Summary and Outlook

Advantages of UHPLC:

- Increased flexibility in comparison to HPLC and increased capability for improving selectivity and resolution: methanol as the organic solvent, lowering of the temperature, increase in flow rate, and thereby increase of the gradient volume – also when using thin/long columns. The consequence of all these interesting possibilities is increased pressure.
- Peak capacity can be increased: connection of columns containing 2.5–3.5  $\mu\text{m}$  porous or 1.5–5  $\mu\text{m}$  fused-core material.
- For  $\leq 2 \mu\text{m}$  material, a very good plate number/retention time ratio and thereby sufficiently good resolution in a short time for less demanding separations, and as a result, increased throughput and reduced solvent consumption. The main practical advantage lies less in the rapid analysis of sequences – with autosamplers often possible overnight, rather in the fast control. I see much sooner if something is going wrong and can “rescue” my sequence before leaving work, instead of first noticing problems in the morning when everything is too late and I – also maybe the production – have already lost a lot of time again.
- The small dead volumes in combination with thin columns and small particles result in a small peak volume and thereby an improvement in the relative mass sensitivity/lowering of the detection limit.

As disadvantages, the following can be mentioned:

- Greater effort in sample preparation. In addition, matrix-contaminated samples and strong sample solvents present seemingly unsolvable problems. Especially in the last case, the small particles cannot compensate for the deterioration in the peak shape.
- The reproducibility under changing conditions is not always satisfactory.
- Above about 800 bar noticeable frictional heat, above approximately 600–700 bar, a change in selectivity is possible. The last two named points are one reason why the transfer of a method from HPLC to UHPLC and vice versa, it is not as simple as often presented.
- Difficulties in method transfer. Due to the small column volumes, small differences in dwell volumes with gradient systems cause considerably more problems than in HPLC.
- In general, more care is necessary in the overall handling.

As with almost every technique, depending on the case, typical characteristics of the technique prove to be advantageous as well as disadvantageous. We will finish this discussion with an example to support this. Consider the small column volume typical in UHPLC, the small column and dispersion volumes result in a small peak volume. This is the reason for the excellent mass sensitivity in the UHPLC. However, at the same time, the small column volume is also the reason for the strong fronting in the case of a strong sample solvent. Furthermore, especially in the early eluting peaks, this is also the reason for tailing – since the dispersion volume of UHPLC systems is still much too large for small column

volumes. Finally, the small column volume means that the injection volume cannot be increased, leading to the poor detection sensitivity of the UHPLC.

In the following, the practical possibilities of UHPLC for some typical situations are presented in a simplified way:

- 1) *Existing HPLC method, only small changes are allowed.* You can easily run the method with your classical HPLC column on a UHPLC system, only the injection volume and the gradient need to be adapted. This assumes that as a result of miniaturization, due to the thin capillaries, and so on, no blockage occurs (for details, see earlier discussion). The result is narrow peaks due to the smaller dead volume and thus probably better resolution at higher pressure.
- 2) *Existing HPLC method, column length, internal diameter, and particle size may be varied and the flow adjusted accordingly.* With the same stationary phase, you use a column shorter by a factor of 2 with particles also smaller by a factor of 2. Result: the “same” separation in half the time at a pressure higher by a factor of 2 and a detection limit lower by a factor of 2. An interesting side effect is solvent consumption reduced by a factor of 2. When instead of a 4 mm column, you use a 3 mm one (corresponding to a decrease by 25%, allowed according to USP and EP as this would be an adaptation and not a change), then this results in a solvent consumption reduced by about 45% (!), at a pressure increased by the same percentage.
- 3) *The resolution of the method is to be optimized, and you can change what you like.* As explained earlier, you can experiment with methanol as organic solvent, with lower temperatures and long columns/multiple columns. Result: all of this can, at higher pressure, lead to an improvement in selectivity (first two points) or an increase in the plate number/peak capacity (longer column/multiple columns).
- 4) *The method is fine so far, the resolution could still be a little better.* You may have little time to improve the selectivity by means of the mobile phase, pH, other stationary phases, modifiers, and so on, and just use a column with half as large particles. Result: at a pressure higher by a factor of 4, the plate number is increased by a factor of 2, thus improving the resolution by a factor of 1.4. This could possibly be sufficient and includes an interesting side effect: the detection limit is also reduced by a factor of 1.4.
- 5) *The resolution of the method is to be optimized. As before, you do not have much time to vary the chromatographic conditions.* You use a column longer by a factor of 2 with particles also smaller by a factor of 2. Result: with a retention time increased by a factor of 2, the resolution is also better by a factor of 2. The problem here is that the pressure is higher by a factor of 8. The original pressure was about 150 bar, it is now about 1200 bar, which a modern UHPLC system should be able to cope with even in routine use.
- 6) *The detection limit of the method is to be improved,* more precisely, the mass sensitivity, not the detection sensitivity, and that means you cannot/may not increase the injection volume. You use a column with a smaller internal diameter and smaller particles – if necessary, you also reduce the column length. Result: the relative mass sensitivity can be improved by a factor of 5–20, depending on the measures taken.

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**Note** The figures mentioned in the descriptions of example situations relate to isocratic separations. In gradient separations, other pressures can result depending on the gradient, and the retention times in cases 2 and 5 do not change by a factor of 2 but, depending on gradient-specific parameters, only by 10–30%, see also [11].

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### 1.6.1 Outlook

Modern UHPLC systems have more or less the following specifications: 80–100  $\mu\text{l}$  dwell volume with LPG or 25–35  $\mu\text{l}$  with HPG systems,  $\leq 3\text{--}5\text{ }\mu\text{l}$  dead volume, 1500 bar back pressure, flow up to 5  $\text{ml min}^{-1}$  possible. What can be expected in the future? The pressure on the manufacturers to keep bringing newer products onto the market will probably lead to further developments in the UHPLC hardware. It is quite possible that in the next generation of UHPLCs, the dead and dwell volumes will be further minimized. The focus here is clearly on the dwell volume as such and in particular on the geometry of the gradient mixing chamber, because the mixing quality should not be reduced as a result of miniaturization.

The second critical point is certainly the autosampler: fast injection cycles and prevention/minimization of carryover. The 1–2  $\mu\text{l}$  injection solution remains in the point of the (ceramic) needle until injection, and the loop is not used for the injection process. Instead, it serves as a “buffer” to provide flexibility in dwell volume with different systems. Furthermore, consider the problem of the enormous pressure surges to which the column is subject during injection, their minimization is a further challenge for autosampler design. Finally, pressures of about 2000 bar and flow rates of 1–3  $\text{ml min}^{-1}$  should be technically possible. The challenge here is less the pressure to be reached, rather material damage at these pressures.

What would be chromatographically possible on a system with such a design? With a future UHPLC system, 10–20  $\text{mm} \times 2.1\text{ mm}$  columns filled with 1–1.1  $\mu\text{m}$  porous or 0.7–0.8  $\mu\text{m}$  fused-core material could be used. Using the theoretically resulting 20–25 000 plates, challenging separations within a few seconds would be possible – assuming that the three modern detectors DAD, Corona, and MS/Ion mobilization can process the signals without loss of information (see also Chapters 4 and 8). Due to the following limiting factors, these column options will probably, however, not be put into practice: large particle size distribution with the small particles, considerable effort would be required to pack a 10–20 mm column with 0.7–1.0  $\mu\text{m}$  material efficiently and reproducibly, not to mention inhomogeneity in the packing at the column wall and the increased solubility of small particles. Other difficulties would be the – for such a column significant – dead volume of 2–3  $\mu\text{l}$  probably present and the enormous frictional heat, which would then be generated. The future user too will probably be willing to accept “long” runs of a few minutes when under robust conditions.

The more likely scenario for the UHPLC in the future: instead of striving toward extremely short retention times with extremely short columns and extremely small particles, its strengths will more likely be used for the use of long or coupled



columns with approximately 1.3–3  $\mu\text{m}$  material, the continuous improvement of peak capacity is likely to stand in the foreground. As far as the matrix of stationary phases is concerned, both porous and fused-core materials have their advantages and weaknesses. A coexistence will probably continue.

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