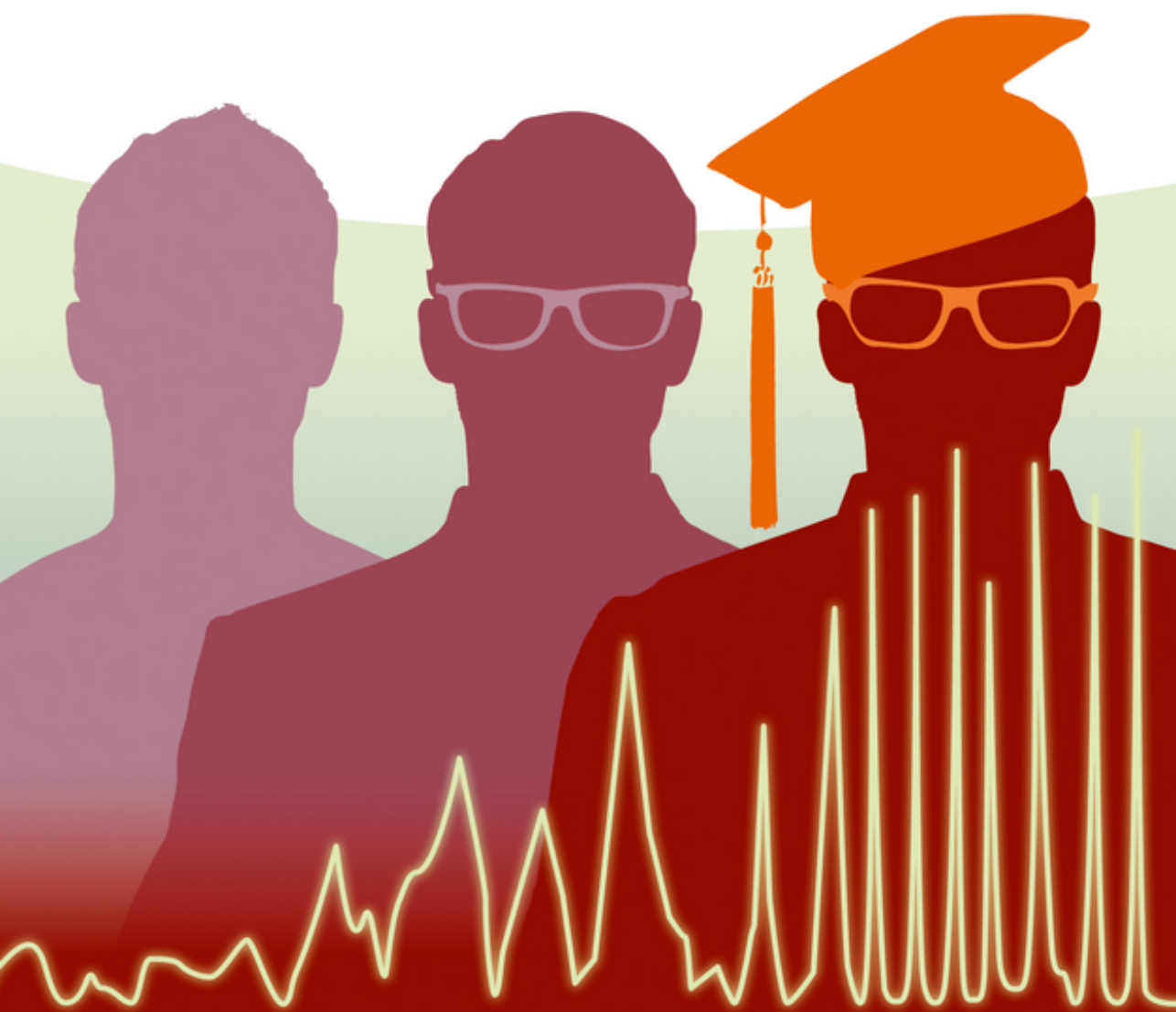


Edited by Stavros Kromidas

The HPLC Expert

Possibilities and Limitations of Modern
High Performance Liquid Chromatography



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The structure of “The HPLC-Expert”

This book contains the following chapters:

Chapter 1 (LC/MS coupling) is dedicated to the most important coupling technique of the modern HPLC. In the first part of the chapter, Oliver Schmitz overviews the *state of the art of LC/MS coupling* and opposes different modes. In the second part, Markus Martin shows *Pitfalls of LC/MS coupling* and provides precise and specific hints on how LC/MS coupling can successfully be established in a daily routine. LC/MS coupling is often linked to life science and environmental analysis. Alban Muller and Andreas Hofmann show a *concrete example of LC/MS coupling in ion chromatography* as an unfamiliar application.

In Chapter 2, Frank Steiner, Stefan Lamotte, and Stavros Kromidas go in detail *into optimization strategies for RP-HPLC* and discuss, on the basis of selected examples, which parameters seem promising in which case.

Chapter 3 is devoted to the gradient elution. Stavros Kromidas, Frank Steiner, and Stefan Lamotte discuss about *aspects of gradient optimization* in a dense form in the first part and offer simple “to-do” rules. In the second part, Hans-Joachim Kuss shows that *predictions of gradients runs* with excel can be very unerring and that the often used linear model represents a simplified approximation.

Chapter 4 is about the *comparison and choice of modern HPLC columns*; Stefan Lamotte, Stavros Kromidas, and Frank Steiner give an overview of different columns and come forward with proposals for pragmatic tests for columns as well as column portfolios, depending on the separation problem.

In Chapter 5, Juergen Maier-Rosenkranz introduces *separation techniques in the biochromatography*, illustrates their characteristics compared with RP-HPLC, and describes the advantages and disadvantages of the individual modes.

Evaluation programs have several strengths, extents, and opportunities. In Chapter 6, Amo Simon shows as a neutral insider advantages and disadvantages of the most known software on the market: *modern HPLC-Software programs – characteristics, comparison, outlook*.

During integration of peaks, which are not separated by base line, there might amount enormous and often undetected mistakes. Mike Hillebrand presents in Chapter 7 *prospects of the “right” integration nowadays*. At the same time, he introduces among other things two software tools, which allow to determine

objectively the deviation from desired value as well as the identification of the "true" peak area.

Chapter 8 is a question of *HPLC in the regulated field*. In the first part, Stefan Schmitz shows opportunities and gives a great many of hints in terms of *intelligent documentation*. Iris Retzko and Stefan Schmitz also give *many hints for a successful FDA inspection* in the second part. Especially, psychology and some simple tricks act a crucial part.

To gather information in an intelligent way is not only for secret services of prime importance. *Efficient information collecting in the era of web 2.0 at the example of HPLC* is the topic of Torsten Beyer in Chapter 9. Some links are presented, which might be useful to find specific information and the quality of these sources is also examined.

MS coupling has difficulties with isobar compounds; furthermore, there are some interesting molecules that are not UV active and finally refraction index detectors cannot be used in case of gradient elution. In Chapter 10, *trends of detection techniques*, Stefan Lamotte is giving a short overview of aerosol detectors und presents advantages as well as disadvantages.

The reader is not obliged to read the book linear. Every chapter represents a self-contained module, so jumping in between chapters is always possible. In this way, the character of the book gives justice to meet the requirements of a reference work. The reader may benefit thereof. At the end: some of the readers might want to use the EXCEL-Makro of Hans-Joachim Kuss for predicting gradient runs. Also the software tools of Mike Hillebrand to estimate integration errors might have drawn the interest of the reader. After all, Torsten Beyer's collection of useful links might be worth one's weight in gold and save unnecessary search. We want to give you the opportunity to use these tools online. WILEY-VCH makes the following link available: <http://www.wiley-vch.de/textbooks/>, where you can find the original-makro of Hans-Joachim Kuss for prediction of gradient runs, a demo version of the two integration tools of Mike Hillebrand as well as a list of links from Thorsten Beyer. We hope this offer obtains approval.

Preface

The HPLC-user fortunately can find nowadays many and good textbooks for the HPLC-methodology. Also applied literature, for example, for the pharmaceuticals or for techniques such as UHPLC or gradient elution is available.

In this book, we cover different topics in the field of modern HPLC. The purpose is to demonstrate current developments and dwell on techniques which recently found their way to the HPLC-laboratory or will do in near future.

At the same time, we offer knowledge in condensed form. In 10 chapters experts address the skilled user and the laboratory head with practical attitudes, who are searching for profound (background-)knowledge and new insights.

Our purpose is on the one hand to point out for the reader unknown mistakes and on the other hand to offer him latest tips, which are hard to get in this condensed form. I hope this choice of topics meets the audience with approval.

My acknowledgments belong to the colleagues who placed their experience and knowledge at the disposal. Special thanks go to WILEY-VCH and especial Reinhold Weber for the extraordinary good cooperation.

Blieskastel, February 2016

Stavros Kromidas

1

LC/MS Coupling

1.1

State of the Art in LC/MS

Oliver Schmitz

1.1.1

Introduction

The dramatically increased demands on the qualitative and quantitative analysis of more complex samples are a huge challenge for modern instrumental analysis. For complex organic samples (e.g., body fluids, natural products, or environmental samples), only chromatographic or electrophoretic separations followed by mass spectrometric detection meet these requirements. However, at certain moments, a tendency can be observed in which a complex sample preparation and pre-separation is replaced by high-resolution mass spectrometer with atmospheric pressure (AP) ion sources. However, numerous ion – molecule reactions in the ion source – especially in complex samples due to incomplete separation – are possible because the ionization in typical AP ion sources is nonspecific [1]. Thus, this approach often leads to ion suppression and artifact formation in the ion source, particularly in electrospray ionization (ESI) [2].

Nevertheless, sources such as atmospheric-pressure solids-analysis probe (ASAP), direct analysis in real time (DART), and desorption electrospray ionization (DESI) can often be successfully used. In ASAP, a hot nitrogen flow from an ESI or AP chemical ionization (APCI) source is used as a source of energy for evaporation, and the only change to an APCI source is the installation of an insertion option to place the sample in the hot gas stream within the ion source [3]. This ion source allows a rapid analysis of volatile and semi-volatile compounds, and, for example, was used to analyze biological tissue [3], polymer additives [3], fungi and cells [4], and steroids [3, 5]. ASAP has much in common with DART [6] and DESI [7]. The DART ion source produces a gas stream containing long-lived electronically excited atoms that can interact with the sample and thus desorption and subsequent ionization of the sample by Penning ionization [8] or proton transfer from protonated water clusters [6] is realized. The DART source is used for the direct analysis of solid and liquid samples.

A great advantage of this source is the possibility to analyze compounds on surfaces such as illegal substances on dollar bills or fungicides on wheat [9]. Unlike ASAP and DART, the great advantage of DESI is that the volatility of the analyte is not a prerequisite for a successful analysis (same as in the classic ESI). DESI is most sensitive for polar and basic compounds and less sensitive for analytes with a low polarity [10]. These useful ion sources have a common drawback. All or almost all substances in the sample are present at the same time in the gas phase during the ionization in the ion source. The analysis of complex samples can, therefore, lead to ion suppression and artifact formation in the AP ion source due to ion-molecule reactions on the way to the mass spectrometry (MS) inlet. For this reason, some ASAP applications are described in the literature with increasing temperature of the nitrogen gas [5, 11, 12]. DART analyses with different helium temperatures [13] or with a helium temperature gradient [14] have been described in order to achieve a partial separation of the sample due to the different vapor pressures of the analyte. Related with DART and ASAP, the direct-inlet sample APCI (DIP APCI) from Scientific Instruments Manufacturer GmbH (SIM) was described 2012, which uses a temperature-push rod for direct intake of solid and liquid samples with subsequent chemical ionization at AP [15]. Figure 1.1 shows a DIP-APCI analysis of a saffron sample (solid, spice) without sample preparation with the saffron-specific biomarkers isophorone and safranal. As a detector, an Agilent Technologies 6538 UHD Accurate-Mass Q-TOF was used. In the upper part of the figure, the total ion chromatogram (TIC) of the total analysis and in the lower part the mass spectrum at the time of 2.7 min are shown. The analysis was started at 40 °C and the sample was heated at 1°s⁻¹ to a final temperature of 400 °C.

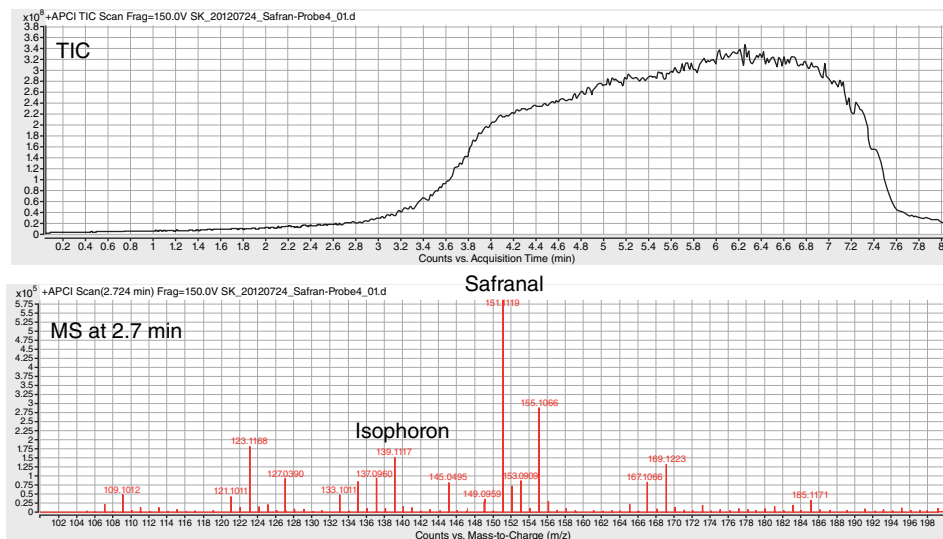


Figure 1.1 Analysis of saffron using DIP-APCI with high-resolution QTOF-MS.

These ion sources may be useful and time-saving but for the quantitative and qualitative analysis of complex samples a chromatographic or electrophoretic pre-separation makes sense. In addition to the reduction of matrix effects, the comparison of the retention times allows also an analysis of isomers.

1.1.2

Ionization Methods at Atmospheric Pressure

In the last 10 years, several new ionization methods for AP mass spectrometers were developed. Some of these are only available in some working groups. Therefore, only four commercially available ion sources are presented in detail here. The most common atmospheric pressure ionization (API) is ESI, followed by APCI and atmospheric pressure photo ionization (APPI). A significantly lower significance shows the atmospheric pressure laser ionization (APLI). However, this ion source is well suited for the analysis of aromatic compounds, and, for example, the gold standard for polyaromatic hydrocarbon (PAH) analysis. This ranking reflects more or less the chemical properties of the analytes, which are determined with API MS:

Most analytes from the pharmaceutical and life sciences are polar or even ionic, and thus efficiently ionized by ESI (Figure 1.2). However, there is also a considerable interest in API techniques for efficient ionization of less or nonpolar compounds. For the ionization of such substances, ESI is less suitable.

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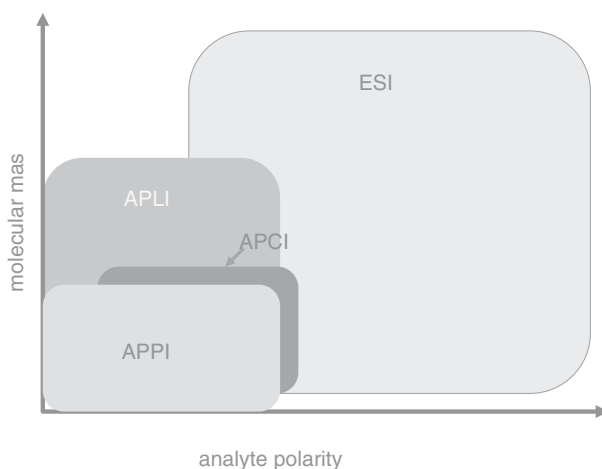


Figure 1.2 Polarity range of analytes for ionization with various API techniques. Note: the extended mass range of APLI against APPI and APCI results from the ionization of nonpolar aromatic analytes in an electrospray.

1.1.2.1 Overview about API Methods

Ionization methods that operate at AP, such as the APCI and the ESI, have greatly expanded the scope of mass spectrometry [16–19]. These API techniques allow an easy coupling of chromatographic separation systems, such as liquid chromatography (LC), to a mass spectrometer.

A fundamental difference exists between APCI and ESI ionization mechanisms. In APCI, ionization of the analyte takes place in the gas phase after evaporation of the solvent. In ESI, the ionization takes place already in the liquid phase. In ESI process, protonated or deprotonated molecular ions are usually formed from highly polar analytes. Fragmentation is rarely observed. However, for the ionization of less polar substances, APCI is preferably used. APCI is based on the reaction of analytes with primary ions, which are generated by corona discharge. But the ionization of nonpolar analytes is very low with both techniques.

For these classes of substances, other methods have been developed, such as the coupling of ESI with an electrochemical cell [20–31], the “coordination ion-spray” [31–46], or the “dissociative electron-capture ionization” [37–41]. The APPI or the dopant-assisted (DA) APPI presented by Syage *et al.* [42, 43] and Robb *et al.* [44, 45], respectively, are relatively new methods for photoionization (PI) of nonpolar substances by means of vacuum ultraviolet (VUV) radiation. Both techniques are based on photoionization, which is also used in ion mobility mass spectrometry [46–49] and in the photoionization detector (PID) [50–52].

1.1.2.2 ESI

In the past, one of the main problems of mass spectrometric analysis of proteins or other macromolecules was that their mass was outside the mass range of most mass spectrometers. For the analysis of larger molecules, such as proteins, a hydrolysis and the analysis of the resulting peptide mixture had to be carried out. With ESI, it is now possible to ionize large biomolecules without prior hydrolysis and analyze them by using MS.

Based on previous works from Zeleny [53], and Wilson and Taylor [54, 55], Dole and co-workers produced high molecular weight polystyrene ions in the gas phase from a benzene/acetone mixture of the polymer by electrospray [56]. This ionization method was finally established through the work of Yamashita and Fenn [57] and rewarded in 2002 with the Nobel Prize for Chemistry.

The whole process of ion formation in ESI can be subdivided into three sections:

- formation of charged droplets
- reduction of the droplet
- formation of gaseous ions.

To generate positive ions, a voltage of 2–3 kV between the narrow capillary tip (10^{-4} m outer diameter) and the MS input (counter electrode) is applied. In the exiting eluate from the capillary, a charge separation occurs. Cations are enriched at the surface of the liquid and moved to the counter electrode. Anions migrate to the positively charged capillary, where they are discharged or oxidized. The accumulation of positive charge on the liquid surface is the cause of the formation of

a liquid cone, as the cations are drawn to the negative pole, the cathode. This so-called Taylor cone resulted from the electric field and the surface tension of the solution. At certain distance from the capillary, there is a growing destabilization and a stable spray of drops with an excess of positive charges will be emitted.

The size of the droplets formed depends on the

- flow rate of the mobile phase and the auxiliary gas
- surface tension
- viscosity
- applied voltage
- concentration of the electrolyte.

These drops loose solvent molecules by evaporation, and at the Raleigh limit (electrostatic repulsion of the surface charges > surface tension) much smaller droplets (so-called microdroplets) are emitted. This occurs due to elastic surface vibrations of the drops, which lead to the formation of Taylor cone-like structures.

At the end of such protuberances, small droplets are formed, which have significantly smaller mass/charge ratio than the “mother drop” (Figure 1.3). Because of the unequal decomposition the ratio of surface charge to the number of paired ions in the droplet increases dramatically per cycle of droplet formation and evaporation up to the Raleigh limit in comparison with the “mother drops.” Thus, only highly charged microdroplets are responsible for the successful formation of ions. For the ESI process, the formation of multiply charged ions for large analyte molecules is characteristic. Therefore, a series of ion signals for, for example, peptides and proteins can be observed, which differ from each other by one charge (usually an addition of a proton in positive mode or subtraction of a proton in negative mode).

For the formation of the gaseous analyte, two mechanisms are discussed. The charged residue mechanism (CRM) proposed by Cole [58], Kobarle and Peschke [59], and the ion evaporation mechanism (IEM) postulated by Thomason and Iribarne [60]. In CRM, the droplets are reduced as long as only one analyte in the microdroplets is present, then one or more charges are added to the analyte. In IEM, the droplets are reduced to a so-called critical radius ($r < 10$ nm)

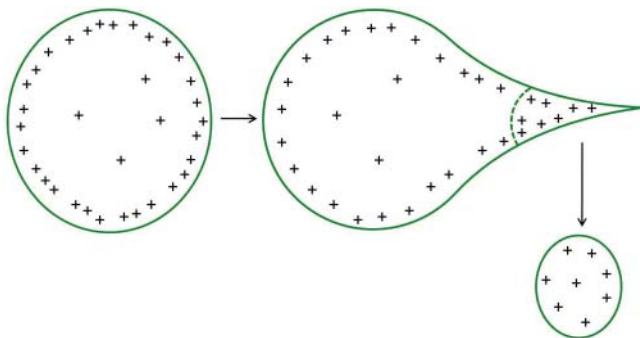


Figure 1.3 Reduction of the droplet size.

and then charged analyte ions are emitted from these drops [61]. It is essential for the process that enough charge carriers are provided in the eluate. This can be realized by the addition of, for example, ammonium formate to the eluent or eluate. Without this addition, ESI is also possible with an eluate of acetonitrile/water (but not with MeOH/water), but a more stable and more reproducible electrospray with a higher ion yield is only formed by adding charge carriers before or after high-performance liquid chromatography (HPLC) separation.

1.1.2.3 APCI

This ionization method was developed by Horning in 1974 [62]. The eluate is introduced through an evaporator (400–600 °C) into the ion source. Despite the high temperature of the evaporator, a decomposition of the sample is only rarely observed, because the energy is used for the evaporation of the solvent, and the sample is normally not heated above 80–100 °C [63]. In the exit area of the gas flow (eluate and analyte), a metal needle (Corona) is mounted to which a high voltage (HV) is applied. When the solvent molecules reach the field of high voltage, a reaction plasma is formed on the principle of chemical ionization. If the energy difference between the analyte and reactant ions is large enough, the analytes are ionized, for example, by proton transfer or adduct formation in the gas phase.

For the emission of electrons in APCI, a corona discharge is used instead of the filament in GC-MS (CI) because of the rapid fusion of the filament at AP. In APCI, with nitrogen as sheath and nebulizer gas and atmospheric water vapor (also in 5.0 nitrogen sufficient quantity of water is available), $N_2^{+\cdot}$ and $N_4^{+\cdot}$ ions are primarily formed by electron ionization. These ions collide with the vaporized solvent molecules and form secondary reactant gas ions, such as H_3O^+ and $(H_2O)_nH^+$ (Figure 1.4).

The most common secondary cluster ion is $(H_2O)_2H^+$, together with significant amounts of $(H_2O)_3H^+$ and H_3O^+ . These charged water clusters collide with the analyte molecules, resulting in the formation of analyte ions:



The high collision frequency results in a high ionization efficiency of the analytes and adduct ions with little fragmentation. In the negative mode, the electrons that are emitted during the corona discharge form – together with large amounts of N_2 and the presence of water molecules – OH^- ions. Due to the fact that the gas phase acidity of H_2O is very low, OH^- ions in the gas phase form by proton transfer reaction with the analyte H_2O and $[MH]^-$ (M = analyte) [63]. The problem with

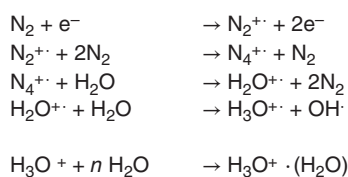


Figure 1.4 Reaction mechanism in APCI.

APCI is the simultaneous formation of different adduct ions. Depending on eluent composition and matrix components, it is possible that Na^+ and NH_4^+ adducts can occur besides protonated analyte molecules, making the data evaluation more difficult.

1.1.2.4 APPI

APPI is suitable for the ionization of nonpolar analytes, in which the photoionization of molecule M leads to the formation of a radical cation $M^{\bullet+}$. If the ionization potentials (IPs) of all other matrix elements are greater than the photon energy, then the ionization process is specific for the analyte. However, in the APPI, different processes can very strongly influence the detection of $M^{\bullet+}$:

- 1) In the presence of solvent molecules and/or other existing components in large excess, ion-molecule reactions can proceed.
- 2) VUV photons are efficiently absorbed from the gas phase matrix.

Thus, for example, in the presence of acetonitrile (often used mobile phase in HPLC), mainly $[M + H]^+$ is formed even though the IP of acetonitrile is more than 2.2 eV higher as the photon energy [64]. In general, in the case of polar compounds, which are dissolved in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, the formation of $[M + H]^+$ is usually observed, while nonpolar compounds such as naphthalene usually form $M^{\bullet+}$ [65]. A detailed mechanism for the formation of $[M + H]^+$ was proposed by Klee *et al.* [66]. In APPI, the ion yield is reduced due to the limited VUV photon flux, and the interactions with solvent molecules. Therefore, the DA-APPI was introduced as a new ionization method from Bruins and employees [65].

The total number of ions, which are formed by the VUV radiation, is significantly increased by the addition of a directly ionizing component (dopant). If the dopant is selected such that the resulting dopant ions have a relatively high recombination energy or low proton affinity, then the dopant ion can ionize the analytes by charge exchange or proton transfer. In addition to acetone and toluene also anisole was found to be a very effective dopant in APPI [67]. By adding dopants the sensitivity can be increased, but the possible adduct formations often lead to significantly more complicated APPI mass spectra [44, 65, 67]. Recent studies suggest that the direct proton transfer from the initially formed dopant ions plays only a very minor role, and the ionization process is dominated by a very complex, thermodynamically controlled cluster chemistry.

1.1.2.5 APLI

APLI was developed in 2005 [68]. It is a soft ionization method with easy-to-interpret spectra for nonpolar aromatic substances and only minor tendency for fragmentation of the analytes. APLI is based on the resonance-enhanced multiphoton ionization (REMPI), however, at AP. The REMPI method allows the sensitive and selective ionization of numerous compounds. Here, for example, the following approach is used:





Reactions a and b represent a classical ($m + n$) REMPI process, where $n = m = 1$ is often very beneficial for the ionization of PAH. Because the absorption bands of PAHs are relatively broad at room temperature, and PAHs have high molecular absorption coefficient in the near ultraviolet and a relatively long lifetime of the S1 and S2 states a fixed-frequency laser, for example, the 248 nm line of a KrF excimer laser, can be used. Under these conditions, an almost selective ionization of aromatic hydrocarbons can be achieved.

A great advantage of APLI in comparison to APPI is that neither oxygen nor nitrogen and the solvents typically used in the HPLC (e.g., water, methanol, acetonitrile) have appreciable absorption cross-sections in the used wavelength range. An attenuation of the photon density within the ion source, that is, a significant coupling of electronic energy into the matrix, as observed in the APPI, does not take place in APLI. The APLI is very sensitive in the determination of PAHs and, therefore, represents a valuable alternative to APCI and APPI, but APLI is not only restricted to the analysis of such simple aromatic compounds. Also, more complex oligomeric or polymeric structures and organometallic compounds can be analyzed [69]. It is also possible to analyze nonaromatic compounds after derivatization of their functional group with so-called ionization markers, in analogy to fluorescence derivatization [70]. With this technique, you can benefit from the selectivity of the ionization (only aromatic systems) and the outstanding sensitivity of the method. In addition, a parallel ionization of sample components with ESI or APCI together with APLI was realized [71, 72] to analyze polar (ESI) or nonaromatic medium polar (APCI) compounds together with aromatic (APLI) compounds.

1.1.2.6 Determination of Ion Suppression

In many mass spectrometric analysis of complex samples, the ion suppression leads to a more difficult quantitative determination and often time-consuming sample preparation is required. It should, therefore, be studied more in advance whether there is a signal-reducing influence of the matrix.

For the investigation of ion suppression, the sample solution (without analyte) is injected in the HPLC and a solution with the analyte (stable-isotopic labeled analyte, if no sample solution without analyte is available) is mixed behind the separation column via a T-piece to the eluate, and the mass trace of the analyte (or stable-isotopic labeled analyte) is analyzed during the total analysis time. After the column, the separated matrix ingredients are mixed with the analyte in the T-piece and are transported into the ion source. The change in intensity of the analyte mass trace before and after the injection of the matrix provides information about a possibly occurring ion suppression.

Figure 1.5 shows the determination of ion suppression of a PAH analysis in urine with APCI-quadrupole-time of flight (QTOF). During the analysis time between 80 and 400 s, the mass trace is considerably diminished and reaches the normal

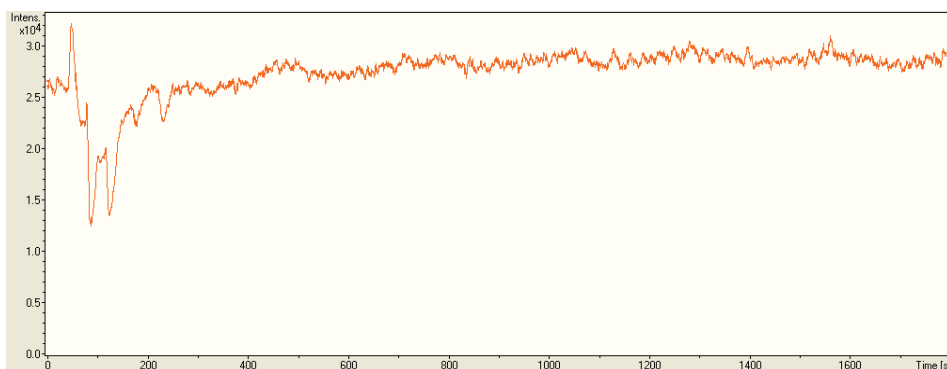


Figure 1.5 Ion suppression in APCI-MS of PAH in urine.

level after about 450 s. This means that between 80 and 400 s disturbing matrix components of urine left the column, which leads to ion suppression.

1.1.2.7 Best Ionization for Each Question

On the basis of Figure 1.2, the method can roughly estimate which allows the most effective ionization for the analyte of interest. Depending on the polarity of the analyte, the ionization should be done with ESI (polar analytes), APCI (moderately polar analytes), APPI (nonpolar analytes), or with APLI (aromatics). However, the matrix plays an important role in making this decision. For complex samples, an ion suppression with ESI is more likely and more pronounced than for the other ionization methods discussed here. The ion beam line also plays an important role in the inlet region of the mass spectrometer. ESI ion sources with a Z-spray inlet often show less ion suppression than normal ESI ion sources. Also, the eluate flow must be adapted to the ion source. For example, slightly higher fluxes than with ESI sources can often be used in APCI sources. Although MS manufacturers promise other flow rates, so it is with regard to spray stability, reproducibility and ion suppression useful to operate ESI sources with fluxes below 300 $\mu\text{l}/\text{min}$ and APCI, APPI, and APLI sources with fluxes below 500 $\mu\text{l}/\text{min}$. Of course, due to the application even larger flows can be used, but often problems such as ion suppression or spray instability are observed.

1.1.3

Mass Analyzer

The most frequent mass spectrometers, which are routinely coupled to the LC, are as follows:

- Quadrupole
- TripleQuad
- IonTrap
- oaTOF
- Orbitrap.

With regard to sensitivity and ratio of price and performance (including maintenance), a quadrupole MS is a very good purchase. With single ion mode (SIM), a very good sensitivity can be achieved and a fast quadrupole (from about 25 to 50 Hz) allows the coupling with a fast ultra-high-performance liquid chromatography (UHPLC) separation.

Based on quadrupole MS, a further development represents the triple quadrupole mass spectrometer, which plays an increasingly important role, especially in the target analysis in complex samples. The sample preparation is minimized, a preliminary separation is often omitted and the potentials of the first and third quadrupole are adjusted so that only a certain mass is allowed to pass these quadrupoles. In the first quadrupole, the ion of the target analyte and in the third quadrupole a characteristic ion fragment, which is induced by collisions with argon in the second quadrupole, is passed through. Due to the analysis of the fragment ion, the chemical noise (matrix) is greatly reduced and triple quadrupole mass spectrometer are one of the most sensitive and selective mass spectrometers. Detection limits in zeptomoles area (amount of substance on the separation column) have been realized for some analytes.

Similar to a quadrupole, an ion trap is constructed. However, the ions are collected in the trap, and then, either a mass scan or single to multiple fragmentation of the target analyte can be performed. Modern ion-trap MS systems are characterized by a very good linearity and sensitivity and a fast data acquisition (e.g., 20 Hz) and thus can even be coupled with UHPLC. They are particularly suitable for structure determination of biomolecules (carbohydrates, peptides, etc.).

For more than 20 years the use of time-of-flight (TOF) mass spectrometer is increasing, which is related to the orthogonal ion beam guiding in the device. The orthogonal ion beam has made it possible to couple even continuous ion sources, such as ESI and APCI, without loss of resolution to a TOF-MS. Recently, the resolution was steadily improved through the introduction of repeller electrodes, ion funnels, more powerful electronics, and so on, so that now several manufacturers offer TOF-MS systems with resolutions between 40 and 50 000 while realizing data acquisition rates of 20 Hz or more. Thus, these devices are ideally suitable for the coupling of fast separation techniques such as UHPLC and can also provide assistance in the identification of unknown sample components due to the high resolution and mass accuracy (<1 ppm).

One of the latest mass analyzer is the linear-trap quadrupole (LTQ) Orbitrap mass spectrometer. In this, the commercial LTQ is coupled with an ion trap, developed by Makarov [73, 74]. Due to the resolving power (between 70 000 and 800 000) and the high mass accuracy (2–5 ppm), Orbitrap mass analyzers, for example, can be used for the identification of peptides in protein analysis or for metabolomic studies. In addition, the selectivity of MS/MS experiments can be greatly improved. However, the coupling is not useful with UHPLC for rapid chromatographic pre-separation, as the data acquisition rate is too low for a reproducible integration of the narrow signals produced with UHPLC.

In addition to some other mass spectrometers, FTICRMS devices are also used. The latter, in addition to very high acquisition and operating costs (e.g., helium), has the disadvantage of low data acquisition rate (same problem as with the Orbitrap), so the coupling with a fast analysis, such as UHPLC, cannot be realized. However, they are unbeaten in resolution and an extremely useful tool in metabolomic research.

1.1.4

Future Developments

The trend in mass spectrometry is currently clearly toward higher resolution and faster data acquisition.

Probably, in future, resolution of about 100 000 and data rates of 20–40 Hz can be achieved with TOF-MS. With Orbitrap-MS, it is assumed that resolutions of more than 800 000 will be possible by more precise production of the cell and electronic devices. This would make it possible to reduce the scanning speed and then to realize the coupling with UHPLC also with good mass resolution.

By connecting an ion mobility spectrometry (IMS) in front of a QTOF-MS, another dimension of separation is realized. Unseparated isobaric compounds, which have the same m/z value, can be separated after ionization by the structure-dependent drift time through the IMS. The combination of IMS with QTOF is also a powerful tool for nontarget analysis in complex samples, due to the fact that the chemical noise is drastically reduced by IMS.

Another focus in future developments will be the optimization of ion sources with respect to ion generation and ion transport at different flows, which are used in nano- and micro-HPLC, LCxLC, and SFC to increase the sensitivity.

1.1.5

What Should You Look for When Buying a Mass Spectrometer?

In addition to the available budget in my opinion, the following points play a central role in making buying decisions:

- a target analysis or a comprehensive analysis of the sample are carried out
- needed sensitivity
- software
- sample throughput
- MS analysis with or without pre-separation process.

If only target analyses is planned (e.g., analysis of known impurities in a product or pesticide analysis), a quadrupole or triple quadrupole-MS would be the best choice. With these devices a very sensitive analysis will be guaranteed, and also a quick pre-separation (e.g., UHPLC) is now possible for many devices.

If nontarget analysis should be realized, high-resolution mass spectrometer such as QTOF or Orbitrap would facilitate the analysis considerably. Even if a high sample throughput is still necessary, the QTOF would get precedence over the slow Orbitrap in high-resolution mode. However, regarding the resolution Orbitrap, in comparison with QTOF, is the more powerful system. The sensitivity of a QTOF is about a factor 10 lower than that of a triple quad, but detection limits in the lower parts per billion range are quite possible.

Perhaps, due to a high number of samples, no pre-separation will be done. But then, it should be ensured that suitable so-called ambient desorption ionization techniques such as DESI, DART, ASAP, and DIP-APCI can be coupled to the MS.

Finally, there are large differences in the respective MS software. Here, the user should provide an overview of the strengths and weaknesses of the software.

In addition to the price of the system, operating costs should also be considered. Besides a high nitrogen consumption, the mass spectrometer should be serviced annually. Just the maintenance leads depending on the effort and manufacturer to an annual cost of €5–20 000.

1.2

Technical Aspects and Pitfalls of LC/MS Hyphenation

Markus M. Martin

For almost two decades, the coupling of liquid chromatography (LC) and mass spectrometry (MS) has left the stage of breadboard lab designs and is commercialized with manifold off-the-shelf products. Frankly, the first systems on the market required a strong expertise and highly skilled users and thus were exclusively applied in highly specialized research laboratories; however, due to intensive research and development work, the robustness and ease of use of LC/MS systems have improved so much over the years that LC/MS techniques are established meanwhile even in many routine applications. Considering how different the two worlds of a separation in the liquid phase via LC and in the gas phase via MS are, this is truly a remarkable fact. Both liquid chromatographs and mass spectrometers have meanwhile achieved a high degree of technical perfection that allows even the less experienced users to create reliable results in a fairly short learning time; nevertheless, the list of potential error sources in the LC/MS hyphenation is still long these days. It starts with the selection of an unsuitable Instrumentation and does not yet end with the wrong interpretation of experimental results. Some errors are specific for instruments, methods, or applications – think, for instance, of the countless variants of matrix effects in the field of food analysis; their individual discussion is beyond the scope of this section. Other aspects are more of a general or fundamental nature – this is what is discussed in this chapter.

1.2.1

Instrumental Considerations**1.2.1.1 Does Your Mass Spectrometer Fit Your Purpose?**

It is a long-stressed platitude that the right tool makes all the difference: anyone who has ever tried to fix an inch hex bolt with a metric wrench will confirm this from personal experience. Well, what applies to screwdrivers is in fact not different to high-tech analysis equipment in your lab, and it is particularly correct for mass spectrometry. Currently, five different mass analyzer principles are established in the market for LC/MS applications:

- Quadrupole (Q)
- Ion trap (IT)
- Time of flight (TOF)
- Orbitrap
- Ion cyclotron resonance (ICR).

Nearly all commercial LC/MS instrumentations rely on (at least) one of those five mass selectors; more sophisticated devices may either vary slightly in their technical design (e.g., 3D or Paul trap, quadrupole ion trap (*QIT*), vs. linear ion trap (*LIT*)), or come as hybrid instruments combining two or more of these analyzer types (e.g., Triple Quadrupole, *QQQ*, *QQ-TOF*, Ion trap-Orbitrap, *LIT*-Orbitrap, or even Tribbrids merging 3 different analyzers into one device). Each of those solutions has its strengths and weaknesses, which make it more appropriate for certain applications than for others. The previous chapter of this textbook gives a comprehensive overview on the technological state of the art; for additional information refer also to [75, 76].

But whatever field of application you are looking for – nearly every analytical challenge requiring mass spectrometric detection can be reduced to either one of the two aspects:

- selective detection of previously known analytes with highest sensitivity for quantitation, *or*
- identification and structure elucidation of unknown compounds.

Combining these tasks with the technical potential of UHPLC, which enables ultra-high separation performance and/or high speed of analysis, will then result in a very attractive technology for the fast *and* comprehensive screening of complex samples with low sample preparation efforts (*dilute-and-shoot*) and high throughput. However, the capabilities of a mass spectrometer need to keep pace with the increasing requirements dictated by higher sample complexity and shorter analysis times. Of course, you can apply a given mass analyzer type also to analytical questions where it would not be your spontaneous first choice. For instance, nothing speaks against the use of a single-quad mass spectrometer to quantify targeted analytes in a fairly simple sample of low complexity. Most single-quads achieve very low limits of quantitation (LoQ) when run in the *single-ion monitoring (SIM)* mode; and as long as you can be sure that only one

analyte species exists with your given target mass, this – rather low-tech – mass spec type can deliver reliable results. Or, to stress another extreme: in case of the measuring time not being a limiting factor, on principle you could also (mis)use an Fourier transform ion cyclotron resonance (FTICR) mass spectrometer for a super-sensitive routine screening analysis, although this would be a decadent waste of money given the immense investments you would need to make. However, you will get the highest confidence in your result if you apply the most suited mass spectrometer to a given analytical problem. Let us briefly discuss now the pros and cons of the various mass spectrometer types for our two core analytical tasks mentioned earlier, either the quantitation of known analytes as specific and sensitive as possible (*Targeted Screening*), or the fishing in the troubled water of samples where you do not have a clue about what compounds to expect (*Screening for Unknowns*).

For **Targeted Screening**, with a clear focus on quantitation of previously known target analytes, all those MS types are preferred that combine two mass analyzers with a collision cell in-between, allowing for *collision-induced dissociation (CID)* by *tandem-MS in space*. From all potential MS/MS operation modes offered by these instrument types, Targeted Screening is most frequently run in the *Selected Reaction Monitoring (SRM, also called Multiple Reaction Monitoring, MRM)* mode. This operation mode requires that you have a good understanding of how your target analyte dissociates into characteristic and ideally specific fragments after exciting it to vibrations by collision with an inert gas in a collision cell. For the collision gas, the heavier argon is typically preferred over the light nitrogen for a higher kinetic impact. You will operate the two mass analyzers as ion filters then; the first one in front of the reagent cell eliminates all unwanted ions so that only the ions with an m/z value of your target analyte, the *precursor ions*, enter the collision cell. The second mass filter behind the cell then is set to the m/z value(s) of the expected *fragment ions*. This SRM operation mode features two main advantages: the combination of precursor ion with as many characteristic fragment ions as possible substantially increases the detection specificity, and it ensures tremendously low limits of detection (LoD) and quantitation. Running the MS in SRM mode not only filters out all unwanted interfering ions, thus virtually eliminating baseline noise; it also reserves the full MS duty cycle exclusively for the detection of the target analyte ions, allowing you to detect a much higher amount of your target ions than in a full scan mode. Up to now, triple quadrupole mass spectrometers (QqQ) are the uncrowned leaders in the Targeted Screening domain, being superior to Qq-TOF or other instrument types with respect to sensitivity, ease of use, result robustness, and profitability. Particularly, ion-trap mass spectrometers, which basically offer the inherent advantage of *tandem MS and MSⁿ in time* for even more specific fragmentation experiments, are not ideal for quantitation purposes due to their limited linear detection range (refer also to the *space charge* phenomenon in Section 1.2.3.5). In addition, ion traps will completely fail for all MS/MS operation modes that require a scan process as the first step in a row of MS experiments due to their operation principle. If you need to perform a “true” *precursor ion scan* or a

constant neutral loss scan for your analysis (and not merely a software data set reconstructing these scan modes out of a set of sequential MSⁿ experiments), then the use of a *tandem MS in space* machine such as a triple quad device is imperative. It should be noted that depending on the molecular mass of your target analytes, the preferred MS/MS instrument type may slightly vary. For small molecules of typically less than 1000–1200 Da, a triple quad machine clearly rules out other MS types due to its benefits in robustness, sensitivity, and investment costs. However, the comparably low upper *m/z* limit of QqQ's is of slight disadvantage; hence, Qq-TOF and Orbitrap instruments are more in favor for large and macromolecules.

The other focus for LC/MS applications is the **Screening for Unknowns**, where you primarily need to learn about unknown sample constituents as much as you can with a very low experimental effort – ideally within one single LC/MS injection. The most relevant information you would need to gather comprises

- *the elemental composition* – which can be derived from high resolution/accurate mass (HR/AM) measurements
- *molecular substructures* – to be determined by MS/MS or MSⁿ experiments
- *the signal intensity ratio of the isotopes*, the so-called isotope pattern, which backs up the elemental composition calculation based on HR/AM results.

As already discussed in Section 1.1.1, only TOF, Orbitrap, and FTICR mass analyzers allow for reliable HR/AM measurements with a sufficient mass accuracy of less than 5 ppm and resolving power. Coupling these analyzers with a quadrupole and a collision cell upfront enables you to additionally measure CID fragment spectra revealing details on molecular substructures, functional groups, and so on, thus supporting structure elucidation. Data acquisition speed and resolving power *R* behave strictly opposite within these three MS types: as of today, TOF devices are by far the fastest mass spectrometers on the market (max. scan rate of up to 200 Hz), followed by Orbitraps (up to 18 Hz) and FTICR (1 Hz or less); In contrast, FTICRs lead in terms of resolving power (*R* up to 10 000 000), followed by Orbitraps (*R* up to 500 000) and TOFs (*R* up to 80 000).

- *TOF devices* offer exciting scan speeds, high mass accuracy, and resolving power at a good price per performance; however, they tend to be very prone even to minor variations of the environmental conditions. As with all materials, also the flight tube of a TOF-MS expands with higher temperature. An elongation (or shrinking) of the flight tube even only on the micrometer scale will substantially affect the accuracy of the mass determination (to be precise: the mass/charge determination) and the resolving power. For a stable and rugged experimental result, you will need a powerful and precise air conditioning in your MS lab (be also aware of sun glare shining on the mass spectrometer through the windows!) as well as a regular mass calibration, for example, on a 1 h frequency or even more often, to compensate for any drifts. As a drifting mass axis calibration can easily occur already on the timescale of one LC separation, the highest confidence in your mass accuracy can only be guaranteed by an internal

mass calibration where known mass calibration compounds are permanently co-infused into the MS during the LC run. Some TOF devices offer a continuous calibrant infusion as a *lock spray* into the ion source using a revolving aperture that alternately passes either the LC effluent or the calibrant solution into the mass analyzer. As an alternative, the calibrant solution can also be added to the LC effluent in front of the ion source by a simple tee piece setup. For a correct data analysis, every measured m/z value taken from the LC/MS data set is then referenced against the m/z values of the known calibrants. This may sound a bit clumsy, but it is the only way for TOF devices to fully reach their maximum specified mass accuracy.

- *Orbitrap devices*, in contrast, are much more rugged against changes in the ambient conditions due to their inherently different design and operation principle. For routine applications, a mass calibration once a week is typically sufficient (depending on the application and lab conditions). Next to the higher analysis ruggedness, they are significantly superior to TOF devices in terms of mass resolution and at least par with respect to mass accuracy – in fact they are the only mass analyzers that come even close to the accuracy of FTICR but with much less challenging claims for technical infrastructure, as they are true benchtop instruments today.
- *FTICR instruments* are very, very sensitive, being capable to detect even down to 10 individual molecules within their detection cell; and they are unbeaten yet in terms of mass resolving power and mass accuracy. However, the very low data rate, the limited linear detection range, their bulky size, and last but not least the massive total costs of operation (think not only of the device alone but also of the demanding infrastructure for the superconductive magnet) will make this mass spectrometer type a highly dedicated expert system also in the foreseeable future, asking for a high level of user expertise and by that not having a real chance to establish themselves in routine applications.
- *Ion traps* (being the only MS type together with FTICR) featuring tandem MS in time and by that MSⁿ experiments with $n \geq 2$ are the most versatile instruments for substructure elucidation by gas phase fragmentation reactions. Due to their limited mass accuracy of typically greater than 10 ppm and only moderate resolving power, they are not really suitable for HR/AM analyses. Their preferred field of application is, therefore, the elucidation of analyte structures for compound classes with only a limited variability of building blocks, such as the analysis of peptides, proteins, and nucleic acids.

A rather special position in the MS world is held by the fairly simple single quadrupole mass spectrometers. With their low mass accuracy (>100 ppm) and quite poor resolving power (R about 1000 for $m/z = 1000$), they are neither good for structure elucidation/screening for unknowns nor for a specific targeted screening. Their strengths are robustness and a low price, and their mass results can at least support peak assignment during method development and serve as a negative confirmation on the absence of a compound of interest within the limit

Table 1.1 Suitability and purpose of various mass spectrometer types.

	Structure elucidation			Simple quantitation	Targeted screening
	Elemental composition	Determination of substructures	Screening for unknowns		
Q	–	–	–	+	o
QqQ	–	o	o	+	+
QIT	–	+	o	–	o
LIT	–	+	o	o	o
QTRAP	–	+	o	+	+
TOF	+	–	–	o	o
Qq-TOF	+	+	+	o	+
Orbitrap	+	o	o	+	o
Q-Orbitrap	+	+	+	+	+
LIT-Orbitrap	+	+	+	o	+
FTICR	+	+	–	o	–

+ = well-suited; o = moderately suitable; – = inappropriate.

of detection. Therefore, they are frequently used as screening detectors with samples of low complexity, for instance, in the open-access process control analysis of combinatorial reactions. Due to their limited mass spectrometric performance, many users do not even perceive single quads as true mass spectrometers but much more as mass-selective detector (MSD), a concept that is meanwhile widely adopted by the marketing activities of various single quad manufacturers.

Table 1.1 gives a rough overview on the suitability of most common mass spectrometer types of today in combination with UHPLC for various application scenarios. In addition to the earlier discussed Targeted Screening and Screening of Unknowns, more generalized aspects of structure elucidation and quantitative amount determination are listed as well. It should be mentioned that this table has of course to live with a certain generalization. All major instrument manufacturers may offer individual, highly specialized flavors of the one or the other type of mass analyzer, which exceeds the general limitations predicted by this list, but from a general perspective, this categorization applies very well to the different mass spectrometer capabilities and applicability.

1.2.1.2 (U)HPLC and Mass Spectrometry

UHPLC has meanwhile been widely accepted and established in the last years, for both LC standalone and LC/MS workflows. UHPLC is highly attractive to mass spectrometry detection due to either the gathering of the same analytical information as a conventional HPLC separation in much shorter time or, thanks to a significantly improved chromatographic resolution, collecting much more information on your sample in a given time. A shot-run method, specially designed for very fast analyses, enables high-throughput screening (HTS) and improves both workload and payback period of a mass spectrometer.

A high-resolution separation, however, that avoids co-elution of analytes reduces competing ionization and ion suppression in the MS ion source, resulting in higher sensitivity and better spectra quality (cf. also Section 1.2.3.3). But it is exactly this UHPLC potential of high speed and efficiency that requires a thorough optimization of your instrumentation to ensure that the high separation performance of your UHPLC column is translated lossless into a perfect LC/MS chromatogram.

Speed in LC/MS Analysis I: Struggling with the Gradient Delay HTS is one focus area for LC/MS applications, for instance, in drug research and development in the pharmaceutical industry. Analysis times of less than 2–5 min for samples of modest complexity enable the fast and reliable processing even of large sample pipelines in an uninterrupted 24/7 routine operation, which makes this approach highly attractive, for example, for combinatorial synthesis monitoring or drug metabolism and pharmacokinetics studies (DMPK). With such short analysis cycles, the gradient delay volume (GDV) of a UHPLC system becomes a critical factor for the overall sample throughput. The GDV is defined as the sum of all volume contributions from the point of gradient formation to the column head. Hence, the GDV has a major impact on the appearance of a chromatographic gradient separation; it is the reason for any gradient separation to begin with an isocratic hold-up step, which takes as long as a change in the mobile phase composition needs to reach the column head and to interfere with the separation process. LC/MS applications in particular ask for separation columns with small inner diameters (from 2.1 mm I.D. columns for analytical scale separations down to several dozens or hundreds of microns in nano- and cap-LC applications), which come along with downscaled flow rates of less than 1 ml/min, with typical values between 50 and 500 $\mu\text{l}/\text{min}$. A small GDV is of high advantage here: the fastest gradient program is useless if a GDV of 500 μl in combination with a flow rate of 500 $\mu\text{l}/\text{min}$ makes the changed eluent composition arrive at the UHPLC column head with a delay of one full minute. And please do not get blinded by smart marketing messages of the instrument vendors, which in most cases only specify the mixer size of the (U)HPLC pump: of course, the mixer volume is part of the GDV, but the total GDV amount will be much more than that; it includes the sample loop and other fluidic parts of the autosampler as well as all connecting tubing or, for instance, the whole pump head fluidics in case you are using a low-pressure gradient (LPG) pump. Therefore, a small mixing volume only pays off if it provides sufficient mixing efficiency together with the entire rest of the (U)HPLC system also matching the fluidic requirements for fast LC.

Pump type and mixing volume: To some degree, all modern (U)HPLC pumps allow you to realize an overall GDV of 250 μl or less – getting much below 100 μl of GDV, however, is still a major challenge. Due to their operation principle, *high-pressure gradient (HPG) pumps* have an inherent advantage with respect to GDV

compared with *LPG pumps*; this makes a HPG pump the preferred one particularly for LC/MS applications. Using an LPG limits your LC method speed-up capabilities for LC and LC/MS applications significantly; this can only be overcome by reducing all potential GDV contributions, for example, by installing a smaller eluent mixer. But, wouldn't the mixing efficiency suffer from such a mixer change? Well, it depends on which perturbation effect would be affected, the *radial mixing* (i.e., along the cross section of your fluidics) or the *longitudinal or axial mixing* (along the flow direction) of your mobile phase. Radial mixing is regularly achieved by complex shifts and changes in the liquid stream, for instance, induced by a mixing helix or by branched channel structures on a chip-design mixer. Radial mixing is most required by HPG pumps due to their operation principle, and fortunate enough this needs only a small mixing volume. Axial mixing in contrast is most effectively achieved by larger mixing volumes. Unfortunately, it is the LPG pump operation principle that asks mostly for axial mixing. As a consequence, reducing the volume of your mixing device will have much less of an impact on the performance of an HPG than of an LPG. In addition, baseline stability, drift, and noise, suffers less from an axial inhomogeneity of the mobile phase in MS detection than in UV detection. All these are good arguments that a small mixing volume combined with an HPG pump is much less of a problem for LC/MS applications than it is for standalone (U)HPLC.

The GDV discussion is a particularly difficult one for pumps still having membrane-based pulsation dampeners. In this case, the GDV also depends on the system pressure, and by that on the separation flow rate [77]. While all major manufacturers of modern UHPLC pumps nowadays have established electronic control mechanisms in their high-end and most middle-class instruments that allow a virtually ripple-free flow delivery without dampeners, some older pumps or simpler entry-level models still have to rely on mechanical dampening. It would not be appropriate in general to use such pump types together with MS detection.

But what to do if you cannot further reduce the GDV of your system but still want to profit from a very short and steep, a *ballistic* gradient separation? Well, a workaround can be a delayed sample injection. It sounds simple: your autosampler does not inject the sample simultaneously with the pump starting the gradient program, but the sample is introduced with a certain time delay, which equals the GDV to be saved at the programmed flow rate. This operation principle is especially applicable if you need to transfer a separation method coming from a system with a lower GDV than yours, as it allows you to reduce the effective isocratic hold-up the sample goes through after injection. But do not be deceived – this is beneficial if you look on one single chromatogram, as it reduces the overall data acquisition time for this run: data recording still starts with the time of injection, not with the start of the pump program. However, it is the total run time for this separation, your cycle time, which still remains the same. Thus, delayed injection is a nice workaround for method transfer, but it will not help you to increase your sample throughput.

Sample Injection: Another important contributor to the GDV is the autosampler, which offers a lot of optimization potential. Users can typically choose between different sample loop sizes (read: GDV contributions); the default sample loop and system tubing are typically selected in a way that they universally cover nearly every injection volume from low μl to up to 100 μl volumes and more. This requires sample loops of significantly more than 100 μl internal volume. Most UHPLC-MS separations, however, run on 2.1 mm I.D. columns (or less, see also Section 1.2.2.1) and work with much less than 10 μl injection volume to avoid volume and/or mass overloading of the stationary phase. Cutting your sample loop size from nominal 100 μl to less than 30 μl will also reduce the GDV contribution of the autosampler accordingly. Autosamplers with the injection needle being part of the sample loop (*split-loop* principle, also *Flow-through-needle* principle) can additionally benefit from a small-sized needle seat capillary. If your system comes with a motorized high-pressure syringe as part of the sample loop (a *metering device*, as realized, e.g., by Agilent Technologies and Thermo Scientific), this will also contribute to the system GDV. The Vanquish UHPLC systems from Thermo Scientific offer users to modify the GDV setting by a variable metering device piston positioning, which allows a flexible adaptation of the autosampler GDV contribution to your LC separation – a feature that is particularly beneficial for method transfer. And last but not least, many instrument control software offer a bypass mode for autosamplers, which optionally turns the injection valve back to the “load” position after injection. This eliminates the sample loop contribution to the GDV for the rest of the run – a quite significant amount for all split-loop autosamplers. A drawback of this feature is that it cuts a certain volume segment out of a running gradient program, which may have adverse effects on the separation.

System Tubing: A factor that is frequently rather overrated than underrated (in contrast to the topics previously discussed) is the GDV contribution of the system tubing between the pump and the LC column. Particularly, with a bottom-up installation of a modular (U)HPLC system, the connection capillaries can potentially even be slightly longer than in a conventional top-down setup (if, e.g., the degasser is not integrated in the pump module, as exemplified in Figure 1.7b). But no worries – even if a connection line of 0.18 mm I.D. has a length of 19.7"/500 mm, this tube will have “only” 15 μl in volume, which is typically much less than 10% of the total system GDV. You might think that these 15 μl , however, could potentially harm much more as a contribution to band broadening by extra-column volumes (ECVs). Well, this depends on where this tubing is placed. Whenever a larger bore capillary is installed in front of the autosampler, the sample does not encounter it anyway, and peak broadening is no issue at all. And even with wider capillaries positioned between sample loop and column head – the overwhelming majority of LC/MS separations are run in gradient mode. Due to the sample refocusing effect of the gradient program that enriches the analytes on the column head by a huge initial retention at the very low starting solvent strength, the impact of band broadening volumes in

front of the LC column is massively reduced. Hence, any capillary tubing affects noticeably neither the gradient delay nor the band broadening. This statement, however, is only valid for analytical scale LC separations – things look different for capillary and nano LC applications.

Case 1 Take-Home Messages

- Minimize the GDV of your LC system – it will help to remarkably reduce analysis time. HPG pumps are of inherent advantage here.
- GDV means more than the pump mixer. Assess all volumes from the gradient formation point to the column head for minimum GDV, but without sacrificing mixing performance.
- Contributions of connection tubing to the GDV can typically be neglected in case of analytical scale LC separations.

Speed in LC/MS Analysis II: The Total Cycle Time or How Fast Can I Be? As already discussed earlier for the *delayed injection*, it is not the speed of your LC separation alone, typically in gradient mode, which matters for the total cycle time. Various other actions add up to it here, including every step of liquid handling such as sample aspiration, needle wash cycles, or column re-equilibration at the end of your separation.

The first bottleneck for speeding up the total cycle time is already the preparation of the sample injection, as this is fairly time-consuming and depends also on various instrumental properties. Fast state-of-the-art autosamplers can realize injection cycle times of less than 10–30 s. This impressive speed, however, can be achieved only with very high sample draw speed and without any external needle wash steps. So, there is a price to be paid: too fast a sample aspiration negatively affects the injection precision, especially with viscous samples or low-boiling sample solvents, while not cleaning the exterior of the injector needle will lead to enhanced carryover effects. Many UHPLC control software offer a “prepare next injection” feature, which already initiates drawing a new sample into the bypassed sample loop while the previous LC separation run is about to be finished, for example, during the column re-equilibration step at the gradient program end. This partial parallelization of injection preparation and chromatographic separation indeed leads to a shortened total cycle time; in real life, however, it is barely possible to achieve a precise and an ultra-low carryover injection in less than 30 s. Besides, this interlacing of injection and analysis steps always requires the sample loop being switched off the fluidic path by turning the injection valve from the *Inject* back to the *Load* position at a certain time in your LC separation. With modern split-loop samplers where the sample loop is a permanent part of the fluidic path, thus ensuring low sample carryover due to a continuous loop rinse by the mobile phase, it is essential that this switch-back takes place only when the sample loop is filled with your initial mobile phase composition. Otherwise, this

will cut a certain volume segment out of your gradient profile, thus actively interfering with the chromatographic process running in your LC column. Hence, an ideal point in time to trigger all kinds of bypassing actions would be during the column re-equilibration step at the gradient end.

The time needed to fill the sample loop can be optimized both by the piston speed of the injection device (typically a glass syringe or a high-pressure piston) and by the injection volume. Thanks to the small injection volumes in UHPLC of less than 1–5 μl , a moderate piston draw speed of 250 nl/s still ensures a rapid though reproducible sample dosage even for viscous samples or highly volatile sample solvents. But liquid handling is more than only drawing and injecting dissolved samples. Cleaning internal parts of the sampler fluidics that are in touch with the sample but not continuously flushed by the mobile phase can also become a time-critical step. Some instrument hardware designs use an injection syringe for these internal rinsing steps, and so this cleaning takes the longer the smaller this syringe volume is. Washing a sampler tubing of, for instance, 40 μl with only the fourfold volume of 160 μl wash liquid can take a considerable amount of time, and a 100 μl syringe finishes this cleaning obviously four times faster than a 25 μl syringe. With very unfavorable settings, so in a system with large tubing volume, small cleaning piston volume, and very fast LC separations of less than 2 min of run time, cleaning the autosampler fluidics can even take longer than the entire analytical separation.

But it is not only before or at the beginning of your separation where you have the potential for cycle time optimization. There is also one time-burner at the end of your chromatography, and it can be a substantial one: it is the column re-equilibration. When running a gradient separation, it is imperative to recondition the stationary phase back to the initial mobile phase composition of the gradient once the solvent strength gradient has reached its final level. This is the only way to ensure the mobile and stationary phase being in an equilibrium state, which is a prerequisite for stable retention times. As a rule of thumb, it is recommended to flush the separation column with at least the fivefold column void volume V_{m} of mobile phase for a stable equilibrium state. With challenging analysis conditions, the required equilibration volume can easily go up to 8–10 $\times$ of the void volume; This is frequently the case either at low initial organic solvent amounts of less than 5% or with analytes strongly affected already by minor deviations from the equilibration state – typically observed for analytes with retention factors of $k < 1$ or for pH-sensitive separations. A short example shall illustrate the time impact here. We will calculate the column void volume V_{M} from the geometrical column volume V_{C} using Eq. 1.1:

$$V_{\text{M}} = \varepsilon_{\text{t}} \cdot V_{\text{C}} \quad (1.1)$$

with ε_{t} = total porosity, r = column radius, L = column length, and $V_{\text{C}} = \pi r^2 \cdot L$

Table 1.2 Recommended re-equilibration volume for high-throughput and high-resolution columns under typical MS-compatible conditions.

Column dimension I.D. $\times$ L (mm)	V_M (μ l)	Required re-equilibration volume (rounded; μ l)	Flushing time for 5–8 V_M at 0.5 ml/min
2.1 $\times$ 50	113	570–900	1.1–1.8 min
2.1 $\times$ 250	563	2800–4500	5.6–9.0 min

Table 1.2 lists two different use cases for comparison, a fairly short UHPLC column for HTS and a long column for a high-resolution analysis, both columns operated at 500 μ l/min, which is a good average for a sub-3 μ m packing material and still being MS-compatible. You will immediately see that even the HTS column of 2.1 $\times$ 50 mm and a typical total porosity of $\epsilon_t = 0.65$ needs a re-equilibration time of 1.1–1.8 min. The five times longer high-resolution column consequently will require the fivefold of reconditioning time, ending up at between 5.5 and 9 min. With any regular UHPLC system, this amount of time adds to each and every single injection, no matter how fast the gradient separation itself will be. Hence, a total cycle time of less than 2 min is barely achievable. The only way out of this dilemma would be a second separation column of identical properties to the original one, which could be equilibrated in parallel to a running analysis using a second pump and a suitable switching valve. Once the analysis on one column has finished, the next injection is then done alternatingly on the other column, with the previous column being equilibrated simultaneously (Figure 1.6).

Finally, concluding these considerations on side procedures of (U)HPLC separations will leave us with one quite sobering finding. Although some side actions of a separation can be parallelized to a running separation, a fast separation method alone is by far no guarantee for a high sample throughput and short cycle times. Typically, the most time-burning process is the column equilibration, which in most cases can only be shortened at the expense of reproducibility. If we do not consider injection interlacing steps, then any high-throughput UHPLC analysis is on average extended by 0.5 min for preparing the sample injection and by 1.5 min for column reconditioning, with longer times required easily, depending on sample type, potential wash cycles, and column dimensions. A considerably fast LC method of 2 min then quickly takes double the time, and even an injection preparation parallel to the final phase of a running analysis does not help substantially to shorten the total cycle time. A minimum cycle time of 4–5 min is, therefore, hard to beat, even with the fastest separation programs on the most advanced UHPLC equipment.

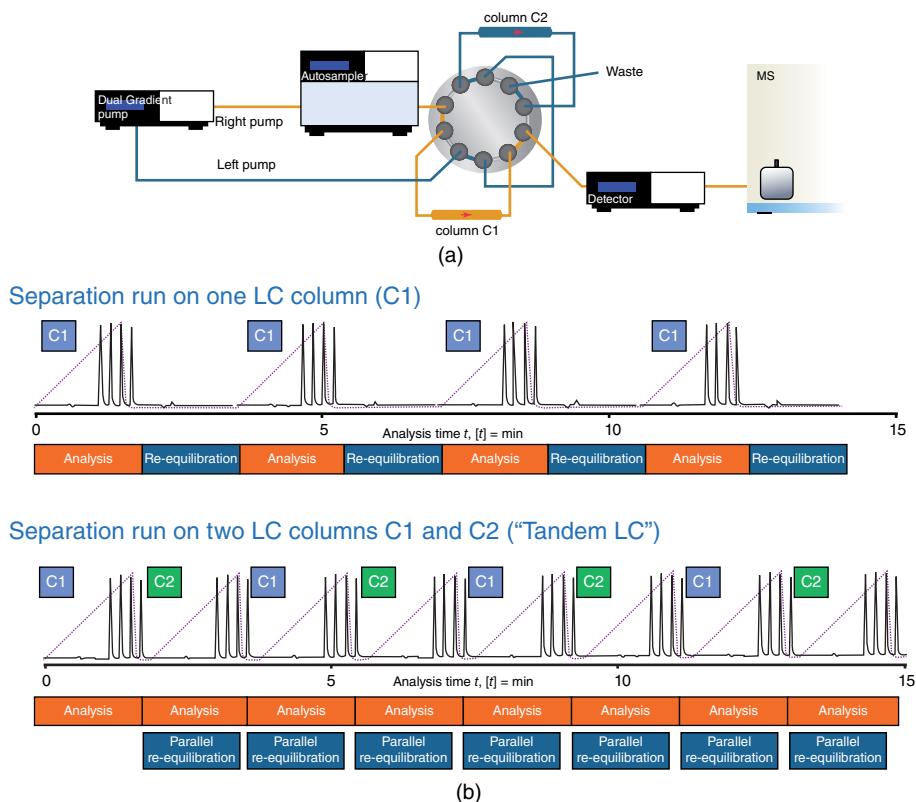


Figure 1.6 Reduction of re-equilibration time and throughput enhancement by using a second separation column and alternating sample injection (tandem LC); (a) flow scheme, (b) injection interlacing.

Case 2 Take-Home Messages

- A rapid separation means much more than an ultra-short (ballistic) gradient program.
- Fast autosamplers help to shorten the delay time prior to the sample injection. Preparing the next injection while the current analysis is still ongoing can help to additionally reduce the cycle time.
- Column re-equilibration is a time-burner, which, however, is hard to avoid, as a thorough column equilibration is mandatory for robust separation results.

Extra-Column Volumes As with UHPLC standalone installations, also LC/MS hyphenated systems are significantly prone to ECV contributions. It is the same rule of thumb that applies here: the maximum ECV between sample introduction and point of detection should not exceed 10–15% of the peak volume of an eluting sample zone. A quick calculation illustrates the situation: a compound

eluting in a 10 s wide peak (baseline width – a level easily undercut by superfast UHPLC separations, which can provide less than sub-2 s baseline widths) at a flow rate of 500 $\mu\text{L}/\text{min}$ has a peak volume of 83 μL . This translates in a tolerable ECV of only 8–10 μL . This is even more a challenge in LC/MS, as here the bridge between the UHPLC outlet and the mass spectrometer inlet contributes to the ECV. Thus, this bridging tube ideally would have the smallest volume you can think of, just to minimize unwanted band broadening effects. We can achieve this quite easily by using a capillary of a very small I.D., which should also be as short as possible. However, this capillary cannot be infinitely short – due to the physical dimensions and the geometric arrangement of UHPLC and MS instruments there always will be a certain minimum distance that you will need to bridge. Simultaneously, slim capillaries always generate high system backpressures – just remember Hagen–Poiseuille's law (Eq. 1.2), which describes the capillary pressure as being inversely proportional to the fourth power of the capillary radius:

$$F = \frac{V}{t} = \frac{\Delta p \cdot \pi \cdot r^4}{8 \cdot \eta \cdot L} \quad (1.2)$$

with F = flow rate, V = volume, t = time, Δp = pressure difference, r = capillary radius, η = fluid viscosity, and L = capillary length.

Considering this, we can deduce three general recommendations:

1) *Install your (U)HPLC system in a smart way*

Reducing the pathway length between LC and MS starts already with setting up your UHPLC instrumentation. The conventional LC setup typically follows a top-down path of your mobile phase (Figure 1.7a): with the solvent bottles on top, the stack sequentially contains the degasser, the pump, the autosampler, the column thermostat, and finally the detector(s) downstream. Most of all commercial mass

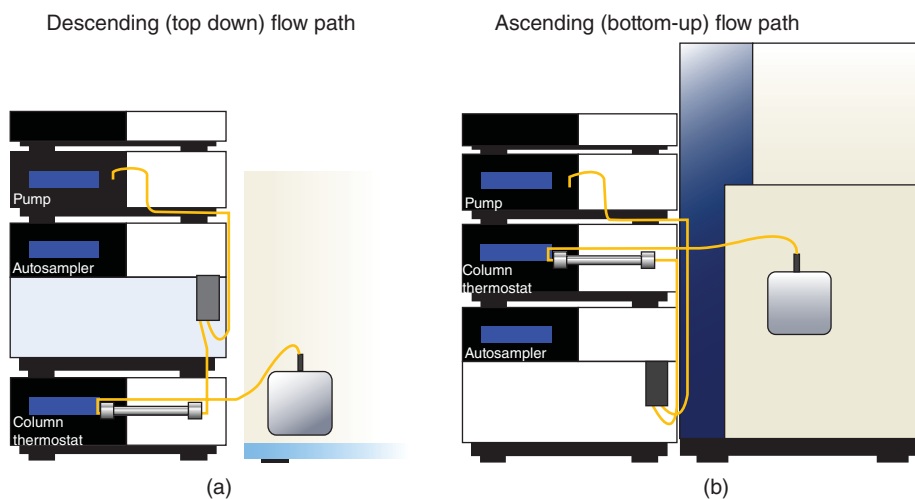


Figure 1.7 Top-down (a) and bottom-up (b) flow path for minimizing the connection tubing length between LC column outlet and MS inlet (ion source).

spectrometers, however, have the ion source inlet placed in a given height above benchtop level, typically between 12 and 25" (300–600 mm). Hence, a bottom-up installation of the UHPLC flow path would be more appropriate – ideally with the pump and degasser at the bottom, then upwards followed by the autosampler, the column thermostat, and – if required – inline detectors (Figure 1.7b). Most modern compact instruments are already designed like that by default. Modular LC systems can be individually configured by the user, allowing for a reduction of the LC-MS connection capillary length by up to 8–12" (200–300 mm) in a bottom-up setup compared with a conventional top-down installation. This, for instance, translates into a 2.4 µl void volume saving when using a 100 µm I.D. connection tubing. In some cases, the bottom-up setup may come along with a slightly longer tubing in front of the LC column; however, as already discussed in section “Speed in LC/MS Analysis I: Struggling with the Gradient Delay”, this does not noticeably impact the separation quality in case of a gradient separation.

2) *Keep your connection tubing slim and short*

Reducing the internal tubing volume always goes in line with short lengths and small I.D. Thus, the connection tubing between your LC outlet (either the column or, if present, an additional detector, e.g., a DAD) and your MS inlet (the ion source) should have the smallest inner diameter possible, which does not eat up too much of your (U)HPLC system pressure capabilities, and which does not compromise the pressure stability of any part of the flow path prior to the connection line (e.g., UV detection flow cells). As an example, take a connection line of 0.13 mm I.D., having a length of 30" (750 mm): running this capillary at 25 °C and a flow rate of 500 µl/min would generate a backpressure of moderate 11 bar (160 psi) for a mobile phase with a viscosity of 1.2×10^{-4} Pa·s (which is slightly more than the viscosity maximum of water/acetonitrile mixtures at ambient temperature). However, this capillary would contribute 10 µl of ECV *behind* your LC column, where it is particularly critical. Converting this tubing to 0.10 mm I.D. reduces the ECV contribution to 5.9 µl, but it comes along with a rise in pressure to 31 bar (450 psi); A 0.075 mm I.D. capillary reduces the void volume contribution further down to 3.3 µl, but at the cost of a considerably high backpressure of 97 bar (1410 psi). For further illustration, Table 1.3 summarizes some model calculations for typical LC/MS application conditions. As we can see, a significant speed-up of LC separations at flow rates beyond 1 ml/min is barely possible (leaving out the question if this was useful with respect to the MS detection sensitivity at such high flow rates).

In case you have an additional detector in front of your mass spectrometer, for example, a UV detector, you also have to take care of the detector flow cell pressure limit. Depending on the design principle, the maximum pressure limit of commercial UV flow cells can vary between 870 and 4350 psi (60 and 300 bar). Please be aware that it is not only the MS connection tubing that generates an additional pressure load to your UV flow cell; many mass spectrometers use internal switching valves to introduce calibrant solutions into the MS ion source, which block the flow path completely for a fraction of seconds when they are actuated, thus

Table 1.3 Volumes and backpressure of a 30''/750 mm capillary with different I.D. in the maximum viscosity of water/acetonitrile and water/methanol mixtures.

	Volume	0.13 mm I.D. 10.0 μ l	0.10 mm I.D. 5.9 μ l	0.075 mm I.D. 3.3 μ l
Water/acetonitrile 91/9 v/v, η = 1.06 cp @ 25 °C	Pressure at F = 0.5 ml/min	9.5 bar/138 psi	27 bar/392 psi	85 bar/1233 psi
	Pressure at F = 1.0 ml/min	19 bar/276 psi	54 bar/783 psi	171 bar/2480 psi
Water/methanol 40/60 v/v, η = 1.56 cp @ 25 °C	Pressure at F = 0.5 ml/min	14 bar/203 psi	40 bar/580 psi	125 bar/1813 psi
	Pressure at F = 1.0 ml/min	28 bar/406 psi	80 bar/1160 psi	251 bar/3640 psi

generating a very short but also very high pressure peak to any technical part in the flow path in front of it. In case you have a more fragile detector cell, you may want to consider either splitting your LC column effluent by a tee piece, or bypassing the MS switching valve and connecting your LC system with the ion source sprayer directly. The former reduces peak efficiencies (by the band-spreading tee piece connection) and sensitivity (only the split fraction of your effluent runs into UV and MS detectors), the latter even tends to improve your peak efficiency in the MS chromatogram, as switching valves in general are plate count killers due to their large bore and groove sizes; however, bridging the internal MS switching valve prevents you from automated recalibration of your mass spectrometer in a sequence run. How critical this is depends on the mass spec type; as already discussed in Section 1.2.1.1, some mass spectrometers need a frequent, if not permanent calibrant infusion, while others do not.

Just as a concluding note – although the tubing I.D. now may be seen as problematic due to its huge impact on the system pressure, it is by far the better optimization parameter to reduce volume contributions. The I.D. goes with the volume by the second power while the length contributes only linearly to it. For comparison reasons, let us consider again our 30'' (750 mm) long capillary of 0.13 mm I.D. (a quite common example for connecting LC with MS), which has an internal volume of approximately 10 μ l. To reduce this to the half, you would need to cut the tube down to half the length, so 12.8'' (375 mm) – which would be too short to make your LC/MS connection. Changing to an I.D. of only 0.10 mm (–23%) brings you down to a volume of 5.9 μ l, which comes close to the reduction by factor 2, but preserves your original capillary length so that you still have a good chance to be in line with your instrument arrangement. Alternatively, going down to a 0.10 mm I.D. capillary would enable you to make the capillary $(13/10)^2 = 1.7\times$ longer (i.e., 43.4''/1275 mm) but still keeping the same internal volume of 10 μ l. We see clearly now: for extra-column and GDV matters, capillary I.D. rules over length.

3) *Take care of your fitting and tubing connection quality*

It is not only the hold-up volume of your tubing that matters. Also, the quality of your fitting system has a large impact on the quality of your LC/MS chromatogram – a factor that is typically underrated in everyday lab life. It is common use still today to connect a UHPLC system with a mass spectrometer by cutting a poly(ether ether ketone) (PEEK) tube of an appropriate length from the bulk and installing it using PEEK fingertight fittings. However, due to improper cutting quality, nonrectangular tubing ends and careless fitting, this introduces a measurable but pointless and avoidable void volume that can be a real efficiency killer. Various dead-volume reducing fitting systems are nowadays commercialized; however, most of them are specially designed and tailored to a respective (U)HPLC equipment and thus not universally applicable. Only four universal UHPLC fitting systems are currently available on the market, not all of them covering true UHPLC pressure loads. These are the Viper™ fingertight fitting technology from Thermo Scientific [78] (maximum pressure of up to 22 000 psi/1500 bar), the A-Line™ fitting design from Agilent Technologies [79] (maximum pressure of up to 18 850 psi/1300 bar), Sure-Fit™ from MicroSolv Technology Corporation [80] (now IDEX; maximum pressure of up to 6000 psi/413 bar), and MarvelX™, also from IDEX (up to 19,000 psi/1310 bar) [81]. Figure 1.8 impressively illustrates how using such virtually zero-dead volume connections provide a remarkable gain in plate numbers and resolution. The upper chromatogram (Figure 1.8a) was generated using a standard PEEK capillary of 0.13 mm I.D., cut from the bulk by a standard tube cutter and installed between LC column outlet and MS ion source inlet by regular PEEK fingertight fittings. The lower chromatogram (Figure 1.8b) was run under exactly the same conditions, the only difference being a Viper stainless steel (SST) capillary of identical size and dimension of the PEEK one between column and ion source. The significant rise in chromatographic resolution of 47% from 1.72 to 2.53 clearly lines out how much separation power is wasted in most LC/MS installations simply due to the use of improper tubing and fitting quality.

Case 3 Take-Home Messages

- Keep your eyes on the shortest distance possible between LC outlet and MS inlet already while setting up your UPHPLC system.
- Do not worry about the ECV in front of the LC separation column – it can typically be ignored in gradient elution mode thanks to a sample refocusing effect.
- Focus on the ECV behind the LC column instead: shorter and slimmer tubing always pays off. However, take care of the backpressure generated by very thin capillaries – they eat up pressure reserves of your UHPLC system and could potentially kill your UV flow cell.
- Get rid of any uncontrolled ECV contributions due to improper tubing cuts and connections by using state-of-the-art zero-dead-volume fitting systems.

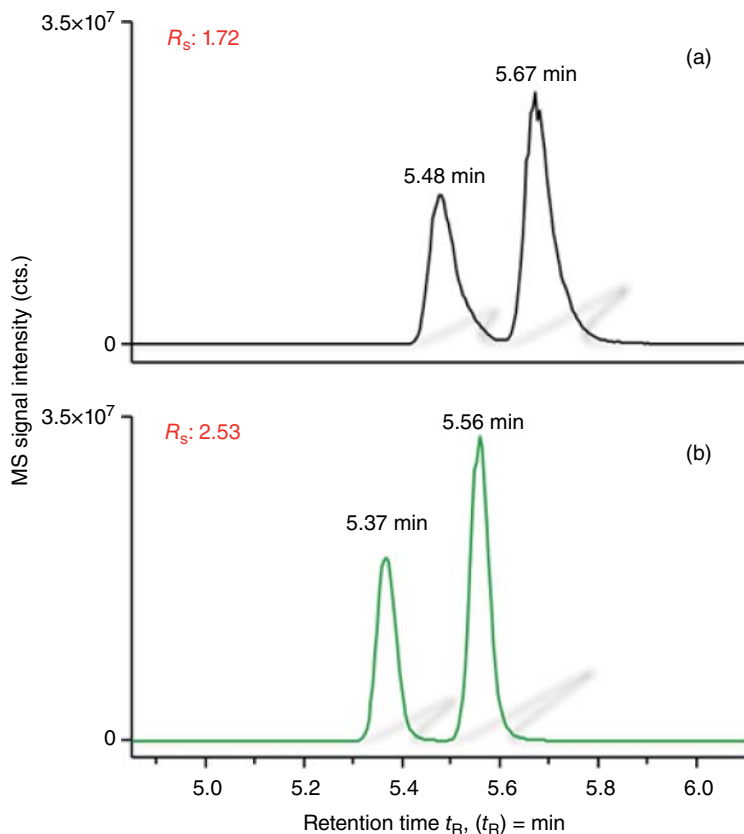


Figure 1.8 LC/MS chromatogram of two isomers, $m/z = 240.10$; (a) PEEK bulk capillary behind the column (0.13 mm I.D.), PEEK fingertight fittings; (b) SST capillary with virtually zero-dead volume connection behind the column (Viper™ fingertight fitting technology, 0.13 mm I.D.).

Data Rates and Cycle Times of Modern Mass Spectrometers It is common sense that an accurate quantitative result can only be generated from the best possible calculation of the *peak area* for your chromatographic peak (and that of reference compounds of course); quantitation based on peak areas beats the peak height determination approach by far with respect to error deviation. Hereby, the higher the number of data points that scan the elution profile of your analyte, the less the error in peak area calculation and in deviation between the experimentally determined and the ideal, theoretical peak profile will be. To ensure an acceptably well-recorded data set, the measured chromatographic peak should at least be described by 25–30 data points. For classical LC detectors, this is no real challenge, as spectroscopic detectors (UV absorption, fluorescence) today provide data acquisition rates of up to 250 Hz, which is more than enough to cope even with ultrafast UHPLC separations and peak widths in the 1-s range.

Mass spectrometers, however, are by far not able to keep pace with the speed performance of state-of-the-art UV detectors. Moreover, data acquisition rates and duty cycles of mass spectrometers behave opposite to the data quality: in most cases, high data rates and short duty cycles come along with poor mass accuracy and reduced resolving power. Especially if the instrument needs to perform complex MS/MS or MSⁿ experiments, the duty cycles for the individual fragmentation experiments will take so much time that a high chromatographic data acquisition rate simply cannot be realized anymore: the mass spectrometer will then be blind for a new package of the continuously infused ions as long as it processes the experimental steps of the previous set of ions. It is then up to you as the user, to find the ideal balance between the requirements for high-quality *LC/MS chromatograms*, that is, a high data rate for best describing the concentration distribution of an eluting sample zone, and high-resolution *mass spectra* for high-confidence compound confirmation. The exact data acquisition rate of a mass spectrometer hereby depends on many different parameters: instrumental criteria such as the mass analyzer type, the technical features and properties of your particular instrument such as electronics design, processor speed, and so on, and on experimental conditions such as the MS experiment type (whether it is in full scan mode, in SIM or SRM mode, precursor ion scan, etc.), the data acquisition range, or type and number of subsequent fragmentation steps (MSⁿ, data-dependent or data-independent MS/MS acquisition etc.). Today, TOF mass spectrometers represent the fastest mass analyzers with specified data acquisition rates of up to nominal 200 Hz for MS and 100 Hz for MS/MS runs [75]. This speed sounds very impressive, but it should be mentioned that this high speed does not allow to simultaneously achieve highest resolving power and spectrum quality. For comparison, triple quadrupole mass spectrometers that are the most widely used MS types for routine quantitation offer typical data acquisition rates of 5–15 Hz. This can already be challenged by a well-optimized conventional HPLC separation – to meet the requirements of ultrafast UHPLC separations with ballistic gradients, this will definitely be too slow. For a more detailed discussion on the selection of appropriate data rates, refer to Chapter 2.

Complementary Information by Additional Detectors or Mass Spectrometry Will Not Save the World It is a well-known saying that mass spectrometry is one of the most powerful analytical tools the world has ever seen. Without a shadow of a doubt, the sheer amount, the detail degree, and the accuracy of analytical information provided by mass spectrometers is very impressive; however, they cannot solve the impossible, and performing miracles beyond common sense is also not their business. Here is a small collection of the most widespread hypes and (partially) wrong assessments on mass spectrometers:

A Mass Spec Is a Universal Detector: This is a frequently quoted claim, which, however, is not getting right by frequent repetition. The advocates of this phrase typically compare MS with UV detection, referring to the fact that spectroscopic detection could only measure analytes having suitable chromophores that

interact with electromagnetic waves of a certain energy (represented by the wavelength). This is perfectly right, but unfortunately it is only an indicator for the selectivity of UV absorbance detection, but not for the pretended universality of MS detection. Indeed, it ignores the fact that also the detectability in a mass spectrometer is analyte-dependent, because it relates to the ionizability of your compound of interest; this, however, is not only related to the amount of ionizing energy present in the ion source, but also to analyte-specific properties. Molecules that do not have a considerable gas phase proton affinity will lead to a very poor ion yield in ESI or APCI mode, resulting in only a low amount of detectable molecular ions. Hence, every analyte species has its own individual mass spectrometric response factor, which indeed might be too low for a proper MS detection, depending on the selected ionization principle. Simply said: ESI and APCI are selective toward molecules with a certain gas phase acidity or basicity. Mass spectrometers are highly flexible in their wide application range and can easily be adapted to analyte requirements by a simple change of the ionization mode – but they are far away from being a universal detector.

It should be mentioned here that in many conversations and also in some literature there is no clear distinction between a *universal* and a *uniform* detection. The latter one describes the requirement of providing a homogeneous, identical response factor for all analytes of interest, independent from their molecular properties. This not necessarily has to come along with universal detection, but in real life it is an extended feature of (virtually) universal detectors. However, if a mass spectrometer does not detect universally by definition, there is even less of an argument for a mass spec being a uniform detector, due to the different ionizability of the analytes. This implies that for all quantitation experiments, the mass spectrometer must be calibrated for each individual analyte – which comes along with a significant experimental effort. True universal detectors in contrast (or technologies that come close to this ideal), such as charged aerosol detectors (CAD) or evaporative light scattering detectors (ELSD), can massively reduce (for exact quantitation) or even virtually eliminate the calibration efforts (for semiquantitative results and/or with constant matrix content).

There's No Detector Which Is More Sensitive than a Mass Spec.: This phrase touches the same misapprehension as the previous one. Sensitivity and the LoD and LoQ in mass spectrometry are not by default superior to any other detector. Under favorable conditions, like high ion formation yield and good ion transmission through the mass analyzer to the mass detector, mass spectrometers are indeed very powerful, allowing LoQs down to a femto- or even attomol level. However, in case of poorly ionizable analytes, an inappropriate ionization principle and/or perhaps not the most sensitive MS instrument design, there may be other detection principles that are clearly in favor, for instance electrochemical or fluorescence detection.

Identify All Your Analytes with 100% Certainty Using a Mass Spec.: Also this claim cannot be confirmed without limitations. Whatever you could do with your molecular ions in a modern mass spectrometer, such as performing sophisticated

gas phase experiments – at the end of the day, a mass spectrometer is “merely” a highly accurate kind of balance to determine molecular masses. However, identical molecular masses by far do not equal identical molecular structures and appearances. Even the most accurate molecular mass determination will fail in distinguishing isobaric compounds entering the mass spectrometer at the same time, without further discrimination. Any kind of isobaric compounds – *E/Z* isomers, diastereomers, enantiomers, and so on – cannot be differentiated by an MS experiment in the first way, as they all have the same molecular mass. As soon as isobaric species show a different fragmentation behavior, you will have a certain chance to separately identify them based on their fragmentation pattern in an MS/MS spectrum; however, this is unfortunately not a given in many cases.

All these aspects clearly show that other, more classical detection principles are by far not obsolete only because you have a mass spectrometer in your lab. Especially, when it comes to the structure elucidation of small molecules, spectroscopy (UV absorption, fluorescence) or electrochemistry provides valuable and complementary information helping to interpret your MS results (with NMR being the gold-standard of course, but LC-NMR hyphenation is by far more complex and less widespread than most other detection principles). One example to showcase the issue with isobaric compounds: many isomers differ significantly in their UV absorption spectrum; and due to the unique capability of reversed-phase (RP) chromatography to provide a structural recognition mechanism, they will also show different retention times. This is illustrated in Figure 1.9, which shows a reaction control analysis by LC-UV-MS to monitor the progress and yield for an *N*-aryl coupling reaction; unfortunately, the educt is not pure but is contaminated with a certain amount of the competing isomer – which could potentially lead also to an *E/Z* mix of product molecules. The coupling reaction indeed is stereoselective as expected (only one product isomer can be detected); however, based on the LC/MS chromatograms alone, there is no way to correctly assign the two educt peaks to the related *E* and *Z* isomer – the extracted ion chromatogram (EIC) for the educt mass just shows two peaks belonging to the two educt species, without any further indication of which one is which. However, as the *E* and *Z* educt isomers show significantly different UV spectra (not shown) and RP chromatography provides you with an excellent separation of the two species, a correct peak assignment based on the diode array detection spectra can be achieved seamlessly.

Whenever you plan to combine a mass spectrometer with a second detector, it is this additional detection principle that tells you how to technically realize the hyphenation. Do not forget – the mass spectrometer always eliminates your analyte while measuring and detecting it, so it must be the last detector in your instrument arrangement. If you want to add a nondestructive detector to your system – all spectroscopic detectors are of that kind – you can simply connect this in line with your LC column upfront and the mass spectrometer behind. One thing to take care of is the additional volume of the detector flow cell, which in most cases also adds a measurable contribution to band broadening. Using UV

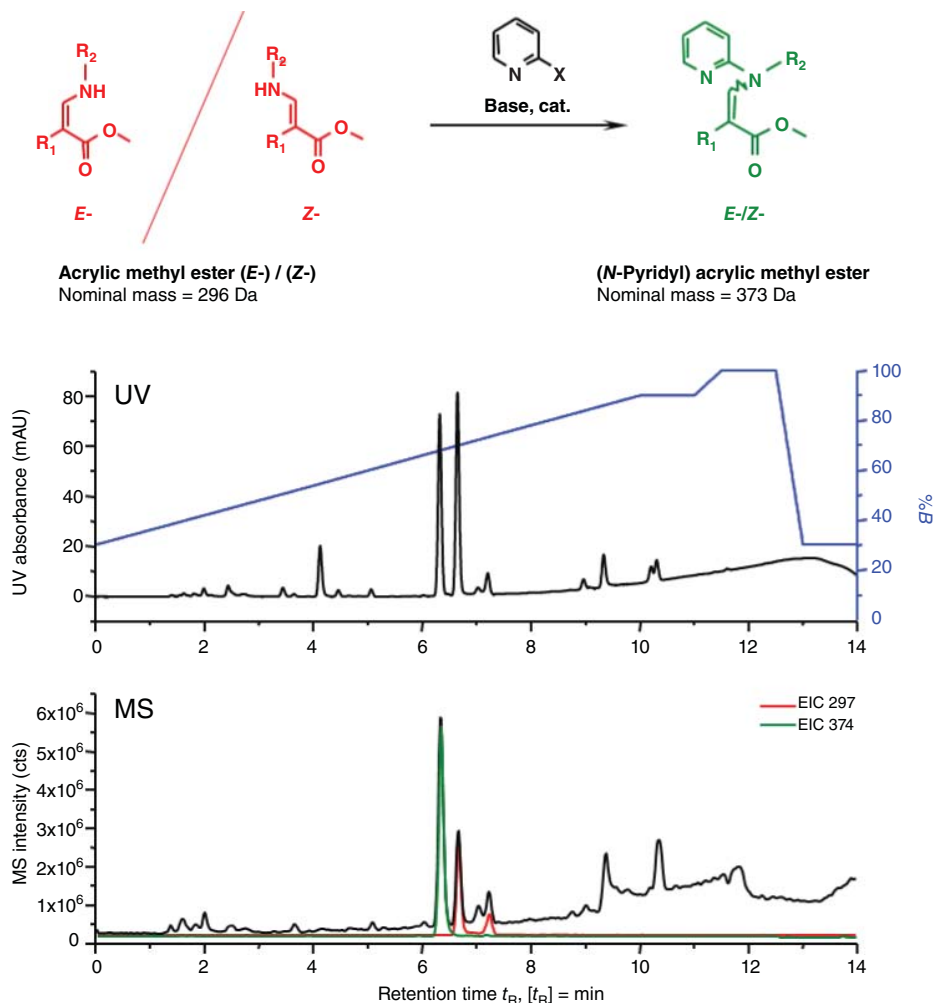


Figure 1.9 Distinction of isobaric compounds by RP chromatography and UV detection for a reaction control analysis (*N*-arylation of an *E/Z* acrylic ester mixture).

detectors, you would thus ideally look for a flow cell with a very small internal volume at maximum light path length. Typical UV flow cells of conventional design are commercially available with volumes down to 2–5 μL . A very popular topic these days are flow cell designs featuring a capillary-based flow cuvette, glass-fiber optics, and internal total reflection, enabling very long light paths of 10 up to 60 mm, combined with very low internal volumes, which allow for remarkably low levels of detection and a superb sensitivity. Unfortunately, these flow cell designs have two inherent drawbacks: next to their high price, the term *high sensitivity* also applies to their lower mechanical ruggedness. To some degree, all these

high-sensitivity cells have a pressure limitation below 1450 psi/100 bar, which is considerably lower than for conventional UV flow cells. As long as the UV detector is the last in your row, this does not really matter, but in combination with a mass spectrometer (or, for instance, a fraction collector) it does. Another alternative in this case would be specifically optimized capillary flow cells. They are made from fused-silica capillaries and can be used and handled like any other conventional flow cell; they combine excellent pressure resistance (up to 4350 psi/300 bar) with impressing low internal volumes of less than 50 nL. However, these cell types are highly limited in their linear range, which does not recommend them for quantitative analyses, but makes them the ideal UV monitor cell with no measurable band-broadening impact on a classical UHPLC separation on 2.1 mm I.D. columns.

By contrast, destructive detection principles like nebulizer-based detectors (CAD, ELSD, etc.) must be connected parallel to the mass spectrometer using a tee piece. This may sound clumsy, but it is not totally disadvantageous. Depending on the LC method settings, a post-column split may be a good idea anyway – it would allow you to run your LC separation with the best-suited linear velocity (in the van Deemter minimum of your column or beyond) and still to reduce the flow rate entering the MS ion source. A potential drawback of the split flow approach would be the addition of another ECV, potentially affecting your chromatographic efficiency and resolution. But in turn you would end up with a very versatile and powerful analysis tool: combining a uniform detection principle such as CAD with mass spectrometry accelerates screening experiments massively. One single chromatographic run in such a setup will give you a very good (semi)quantitative result from one of the most unspecific detectors commercially available, while a parallel-running HR/AM mass spectrometer gives you excellent qualitative data for (virtually) unambiguous compound identification. A very interesting extension in this context can be offered by electrochemical detectors (ECD). By making use of the redox activity of analytes to generate a detector signal, they will alter the analyte species; a MS in series behind will thus no longer detect the molecular ion mass of the initial species, but of the oxidized or reduced one. If you needed the unchanged mass of the original analyte, you would then add the ECD in a parallel split. By plugging the ECD in line with the mass spectrometer, however, you will get the highly interesting option to enhance the detection sensitivity of your MS by electrochemically converting your analytes into oxidized or reduced species that may show a much better signal response than the original molecule. You can even use this approach for bioanalytical applications, for instance, by electrochemically mimicking certain metabolism processes and to investigate them immediately, on the fly so to speak, in the mass spectrometer.

Case 4 Take-Home Messages

- Mass spectrometers are not universal detectors.
- Mass spectrometers detect highly selectively, depending on their operation settings, but not specifically.
- For the unambiguous compound structure confirmation, you will always need (at least) one additional structure elucidation method (e.g., NMR spectroscopy) next to your MS(/MS) findings.
- In all cases where molecules cannot be distinguished based on their molecular mass within the experimental error (isobaric compounds), additional and complementary detection principles are imperative. The related detector modules can be added either serially or in parallel by splitting the LC column effluent, depending on the detection principle.

1.2.2

When LC Methods and MS Conditions Meet Each Other

When LC meets mass spectrometry, two very different worlds with highly contrary physical requirements need to come together. While an HPLC separation works against ambient pressure at the outlet end, a mass spectrometer always asks for a high-quality vacuum to operate. The interface between LC and MS, the ion source, must therefore handle multiple tasks simultaneously: transferring the dissolved analytes into the gas phase, separating the analytes from the residual mobile phase (typically done by gas phase transfer), controlled ionization of the analyte molecules, and a focused analyte ion transfer into the evacuated mass analyzer. None of these jobs is a simple one – just take the mobile phase removal: as we know, the molar volume of a gaseous compound is 22.4 l under normal and 24.5 l under standard conditions. Hence, water at a flow rate of 1 ml/min, equaling 1/18 mol/min, forms 1.2 l of vapor every minute, which needs to be completely removed from the analytes and drained out of the ion source. Therefore, the LC separation needs to meet certain limiting requirements to ensure a smooth signal generation in the mass spectrometer.

1.2.2.1 Flow Rate and Principle of Ion Formation

Let me open this section with a short remark on the term *sensitivity*. In the first instance, “sensitivity” is defined as the slope of the response function that describes the signal change depending on a change in analyte amount or concentration. The slope, also known as response factor, always has a physical

dimension (signal unity per concentration measure), which makes it impossible to directly compare response factors of different detection principles. However, the sensitivity is always linked to the ratio of signal intensity to baseline noise – a dimensionless measure, which is also used to determine LoD and LoQ (and which allows a comparison between different detection methods). The following discussion covers both of these interpretations.

It is already the selected ionization principle that tells you about the maximum LC flow rate you should confront your mass spectrometer with. ESI, which is applied in about 82% of all published online-LC/MS hyphenations (the rest is shared between APCI with 16% and APPI and others with 2%; LC-MALDI-MS in contrast is a classical offline hyphenation example) [75], enables the use of 50–300 $\mu\text{l}/\text{min}$ flow rates at best sensitivity if assisted by a pneumatic nebulizer gas [81]. To some degree, all commercial ESI interfaces (excluding nanospray sources) can be operated at much higher flow rates of up to 1 ml/min and more. While being a concentration-sensitive process, ESI by theory is barely affected by the flow rate; the peak height should not change significantly with the LC flow rate. Indeed, there are many literature examples demonstrating that the sensitivity of ESI methods suffers only if the excess of mobile phase cannot be removed effectively anymore. This, however, can happen at LC flow rates beyond 1 ml/min, depending on the ESI interface design and the efficiency of the source heating or the supporting nebulizer [82]. In reality, experimental conditions such as composition and mixing change of the mobile phase over time can lower the sensitivity already at flow rates higher than 300–500 $\mu\text{l}/\text{min}$. It is hard to predict the extent of this reduction as there is no mathematical model or a rule of thumb for this; so it is highly recommendable to monitor the signal intensity and the signal/noise ratio for your target analytes at different flow rates by a flow injection analysis (FIA). Depending on the solvent removal capacity of the ESI interface, the maximum sensitivity might be reached at flow rates that are lower than the van Deemter minimum of the LC column packing material. Then, it is a case-by-case decision where to set the priority, on highest detection sensitivity or best chromatographic efficiency.

APCI tolerates much higher flow rates than ESI, which lies in the nature of the process – it simply needs a minimum amount of solvent vapor to create the reagent gas that is responsible for the analyte ionization. APCI is a mass-sensitive process [83], which benefits from higher flow rates because more analyte molecules per time enter the APCI interface, thus leading to increased peak heights at higher flow rates (the exception proves the rule [84]). The operation range of APCI starts at 150–200 $\mu\text{l}/\text{min}$ and ends at maximum 2 ml/min, with a sensitivity loss being observed also here at very high flow rates, depending on the interface design and the evaporation capacity. Similar to ESI, monitoring the sensitivity in dependency of the flow rate should be a no-brainer to determine the ideal LC/MS flow rate during the MS method development. Table 1.4 summarizes the usable and the most effective working ranges of ESI and APCI.

Hence, APCI seems to be a very suitable interface principle, particularly for fast UHPLC-MS separations. It should also be mentioned that APCI is less prone to matrix effects in many applications, thus having the tendency of being more

Table 1.4 Applicable and ideal working ranges for selected ionization processes.

	Applicable working range	Ideal working range
Nano-ESI source (without nebulizer gas)	<5 μ l/min	20–800 nl/min
Standard ESI source (with nebulizing support)	0.01–1.5 ml/min	0.05–0.3 ml/min
APCI source	0.2–2 ml/min	0.3–1 ml/min

robust and accurate than ESI (refer also to section “Gas Phase Adducts”). However, the user is not free in his or her choice, as the analyte properties dictate which ionization principle has to be applied. Due to the analyte polarity, ESI is the default choice in most cases. However, a dedicated LC column hardware can comply very well with the low flow rates ideal for ESI: columns of 2.1 or 1 mm inner diameter allow operating even UHPLC stationary phase materials with average particle diameters below 2 μ m at optimum linear velocities, as this translates in still very low volume flows in the microliter per minute range at those small column I.D. If the sensitivity loss with ESI at ideal chromatographic linear velocity was still too high, a post-column split of the LC effluent could be a good way out of this dilemma. Such a split is easily realized by a tee piece and two restriction capillaries – their dimensions will determine the split ratio between the primary flow to the MS and the bypass to the waste. As an extension, the split bypass does not mandatorily have to go to the waste: it can also be used for a second, ideally mass-sensitive detector. Just think of the combination of a mass spectrometer and a nebulizer-based detector such as ELSD or CAD, characterized by a virtually uniform, analyte-independent response, which would give you a very powerful tool for the identification and parallel (semi)quantitation even of unknown compounds. Nevertheless, such a split always bears also the risk for band-broadening void volumes, so great care must be taken while selecting the different pieces and assembling the split construction.

1.2.2.2 Mobile Phase Composition

MS compatibility of the mobile phase means that *all* ingredients of the eluent have to be volatile. To some degree, all solvents used in RP chromatography comply with this rule; water, which is the chromatography liquid with the highest evaporation enthalpy, is very well compatible with ESI and APCI processes – even more, a minimum amount of water is vital for an acceptable ionization yield. Organic solvents enhance the spray drying not only due to their higher vapor pressure but also by reducing the surface tension of the solvent droplets in the electrospray, which facilitates the evaporation of residual solvent molecules. As a practical consequence, higher organic content leads to a better spray stability and increased signal/noise ratio – which can also be observed in any RP gradient run; in ESI, the signal intensity typically goes linearly with the increase in organic content in the

mobile phase at up to 80% [81]. The trend behavior can significantly differ between pure solvents and solvent mixtures – for a more detailed discussion refer to the literature [85].

Volatility, however, must also be a given for all kinds of additives to the mobile phase. All modifiers forming nonvolatile salts or precipitates lead to a massively enhanced suppression of ion formation in the ion source (*ion suppression*) and to a rapid and tough contamination of the ion source with salt crusts. Next to the frequent cleaning efforts, this leads inevitably to a pronounced sensitivity loss in your MS chromatogram, although many major instrument vendors claim the opposite. As a consequence, most of the classical buffering agents, acids, bases, and additives well-known in LC standalone such as phosphate or borate buffers, and more generally sodium salts have to be avoided in combination with LC/MS. Instead, various (semi-)volatile organic acids, bases and their respective ammonium salts are viable alternatives. For acidic pH, formic acid (FA), acetic acid, and trifluoroacetic acid (TFA) are most popular, while aqueous ammonia solutions or alkyl amines, for example, triethyl amine, cover the alkaline range. If buffer capacity is needed, ammonium salts such as ammonium formate, acetate, or bicarbonate are of first choice. Also, oxidizing agents are not appropriate for LC/MS eluents: it is known that chloride ions not only promote ion suppression, but the electrospray can oxidize them into chlorine, which chemically modifies your analyte and over time also attacks your ion source hardware such as the spray needle [86]. Volatile ionic detergents, however, are also not a good choice, as they can deposit on the surfaces of the ion optics and the mass analyzer when entering the mass spectrometer; this can lead to electrical discharges and instrument malfunctions over time.

A topic frequently and controversially discussed in literature should briefly be mentioned here, which is the pros and cons of the use of FA and TFA as modifiers in LC/MS applications. These moderate organic acids generate a pH of 2–3, depending on the concentration, while still being MS-compatible as they do not form any nonvolatile precipitates in the ion source. Simultaneously, their anions – formate and triflate – are also ion-pairing reagents and thus actively controlling the retention mechanism of many analytes. Hence, cationic analytes potentially show a higher retention on an RP stationary phase. In many cases, also an improved peak shape with lower asymmetry can be observed. However, this effect, which is much appreciated in liquid chromatography, has its drawback in the MS ion source, as it compromises or even suppresses the formation of ions in the gas phase – a classical conflict of interests for the LC/MS analyst. TFA hereby is a much more effective ion-pairing reagent than FA. This leads to a better chromatographic elution behavior with enhanced retention and reduced peak asymmetry, but it also comes at the price of a noticeably worse signal/noise ratio in the MS chromatogram due to stronger suppression of ion formation in the ion source. When analyzing small molecules, the retention-enhancing effect of TFA is not much pronounced, but you clearly will observe ion suppression. Therefore, FA is more appropriate for the LC/MS analysis of small molecules. A classical tradeoff is the analysis of larger biomolecules, such as peptides or proteins,

which need to be detected in very low concentrations. For best sensitivity, FA would also here represent the modifier of choice, but TFA can significantly improve the chromatographic performance. For bioanalytical questions, which frequently are realized including a trap column in an online enrichment setup, it is, therefore, recommendable to use TFA in the trap column flow path, but change to FA in the analytical separation; in some cases, the addition of TFA also in the analytical flow path may be inevitable, though. Figure 1.10 illustrates nicely the retention-enhancing effect of TFA.

1.2.3

Quality of Your Mass Spectra and LC/MS Chromatograms

The previously discussed considerations cover very fundamental aspects of an LC/MS method setup; they are discussed typically once, at the beginning of your work, as they deal with generic questions such as “which instrument should I use” or “how should my LC/MS method look like.” Once decided, these things do not change significantly across the lifetime of a method. But there are many minor

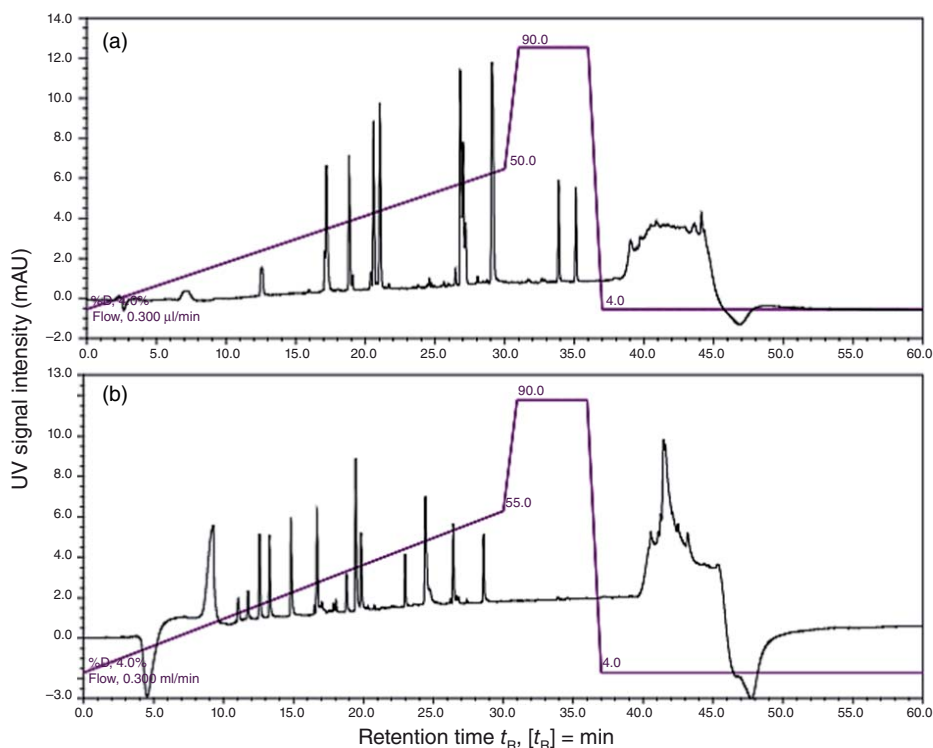


Figure 1.10 Separation of a cytochrome C digest by adding 0.05% TFA (a) or 0.1% FA (b) under identical chromatographic conditions.

or major deviations from the routine happening in the lab every day. Surprises and pitfalls are many, from wrong mass assignments or unknown mass signals, noisy MS spectra to a totally empty mass signal and sheer baseline noise. This final section should help to showcase very common error symptoms of LC/MS lab life and to point out ways how to tackle them.

1.2.3.1 No Signal at All

The most striking problem while running a sample analysis is described very quickly: after thoroughly developing a robust and sensitive LC/MS analysis method, you start an injection and you see-nothing. Obviously, the root cause for this “nothing” can be either the LC or the MS part of your hyphenated LC/MS system, with the worst case being that both chromatography and mass spectrometry do not run as expected. The hard truth is that in most of these cases, the instrument hardware is operating properly – none of the diagnostic tools for the devices report any error for the electric and electronic instrument subassemblies. Most of the time, it is an inadequate ion formation that is responsible for weak or no MS signals, so the system struggles somewhere with the ion source, the ion transfer to the vacuum section of the MS, or with the sample introduction. Technical defects behind the ion source, be it in the ion optics, the mass analyzer, or the mass detector, are observed much less frequently.

To pin down the error source, it is very helpful to check first the signal intensity and the noise level of the baseline both in the LC/MS chromatogram and in the mass spectra via the online view of the instrument control software. A very low signal intensity of only several hundreds of ion counts and a noise pattern that looks much more like an erratic flaring of signal spikes than a continuous base level signal are strong indicators that virtually no ions at all are reaching the mass analyzer or the mass detector. The core reason for this is typically a quite rough mechanical or electrical defect either in the LC or the ion source sprayer assembly. To further troubleshoot, the LC part of your system, a UV detector is of invaluable help – no other detection principle is even par with UV with respect to ruggedness. Nowadays, dedicated UV monitor flow cells of only a few dozens of nanoliters internal volume allow even for a permanent monitoring of your LC/MS separation all the time as they do not contribute measurably to band broadening and thus reduced peak efficiency. This allows for a rapid and doubtless verification of your chromatographic separation running properly. Having confirmed that chromatography separates your analytes and transports them toward the outlet of the LC/MS connection capillary accordingly, there must be something wrong within the MS ion source. For instance, a deformed or even broken spray capillary or needle prevents effectively the LC effluent from entering the MS ion source; hence, no stable nebulizing spray can be created. Very popular here is a spray needle tip that is cracked, resulting from a short touch on the lab bench or during a careless insertion into the source assembly. ESI and APCI ion sources that apply the HV for the electrospray or ion formation to the spray needle (such as Sciex and Thermo Scientific devices) instead of the MS vacuum inlet

(Bruker Daltonics, Agilent Technologies) can additionally suffer from a damage in the electrical wiring or plug connections due to improper handling.

With a chromatography and spray unit assembly being intact and a baseline noise that indicates a reasonably stable spray being formed, obviously your system generates ions properly, but for whatever reason a too little amount of ions truly reaches the mass analyzer. The ion current measured along the ion transfer capillary in the evacuated part of your mass spectrometer is a good indicator for an acceptably high ion transfer yield. In the case of this being very low, your first step of action should be a thorough clean-up of the ion source including the ion transfer capillary. Analyte or dirt deposits on the various surfaces, which cannot even be seen by your eyes, can suppress ion formation and need to be removed by cleaning. It is highly recommendable to check the state of the spray needle, particularly the tip, with a magnifier or a suitable microscope. If the needle tip is bent or cracked, you will need to replace it. Depending on the sprayer assembly design and thus MS vendor, also the alignment and positioning of the spray needle in the sprayer assembly should be checked; a re-adjustment of the needle alignment can be very helpful, as the extent of the tip protrusion out of the sprayer assembly can change the detection sensitivity by several orders of magnitude.

1.2.3.2 Inappropriate Ion Source Settings and their Impact on the Chromatogram

The baseline quality of our LC/MS chromatogram is a good indicator if the processes within your ion source are running seamlessly; same for the base signals in every individual mass spectrum. Typical error patterns can mostly be divided into two categories: increased baseline noise and poor baseline stability. Both phenomena suggest that something is going wrong with the selective creation of analyte ions or the continuous removal of residual solvent, for instance due to spray instability. An increased baseline noise is mostly a clear sign for dirt into the MS ion source, as it is the result of too many different ions being created over a wide m/z range and entering the mass spectrometer simultaneously. The reasons for this so-called *chemical noise* can be manifold. The first thing to check would be the cleanliness of the ion source and when it has been cleaned up most recently. Any residues on the spray needle tip, on the internal surfaces of the ion source assembly, on the orifice and metal plates of the vacuum inlet, on the ion transfer capillary or the first stages of ion lenses are very good candidates for increased baseline noise. Very obvious sources for these chemical contaminations are not only sample or matrix components but also wanted or unwanted parts of your mobile phase, and the dry gas or nebulizer gas of the ion source. The LC solvents used to compose your mobile phase should, therefore, always be of LC/MS purity grade (labeled "LC-MS grade", "ULC/MS", etc., depending on the supplier). The very popular "gradient grade" purity solvents, however, are merely optimized for a minimal amount of UV-absorbing impurities and, therefore, are typically not pure enough for LC/MS applications.

The gas used to dry and/or to nebulize the LC effluent – typically nitrogen – can also introduce contaminations into the ion source and thus reduce

your signal quality. The gas purity level should be at least 99.0% or better 99.5% and higher. Depending on the nitrogen origin, the types of contaminants can vary. Many mass spectrometers have a very high gas consumption of up to 10–25 l/min, especially when combined with analytical-scale LC flow rates of several hundreds of microliters per minutes. This locks out the nitrogen supply from gas cylinders – although this ensures a very high gas purity, the average gas consumption of a mass spectrometer would typically empty these cylinders every 1–2 days, which is not very economic and not convenient either. Higher amounts of nitrogen can only reasonably be provided by nitrogen generators or a nitrogen supply line in your lab based on liquid nitrogen evaporation. The latter typically ensures the highest gas purity grade. Nitrogen generators, in contrast, are fed with pressurized ambient air and then the oxygen removed by a membrane separator. The pressurized air, however, is provided by a compressor and, therefore, frequently contains residual oil mist and other hydrocarbons diffusing out of the compressor hardware. These contaminants must be effectively eliminated from the nitrogen stream by gas filters based on activated carbon filter assemblies or other adsorbents. If you miss such a filter or the filter is fully loaded and needs to be replaced, you will observe a significant rise in the baseline noise, too.

Next to these “real” contaminations originating from unintended chemicals, gas phase aggregates or *clusters* built from residual solvent molecules and charge carriers can be formed under nonoptimized ion source conditions. These clusters can in summary be heavy enough to create signals within the m/z detection range of your MS experiment and thus permanently contribute to the baseline noise. A thorough optimization of the ion source parameter settings can effectively suppress this cluster formation. Figure 1.11 illustrates this by depicting the MS signal intensity of a target analyte (Astemizol, m/z 459.3) introduced by FIA under varying dry gas temperatures. In Figure 1.11a, we see that the individual analyte signal intensity in the EIC remains nearly constant with rising dry gas temperature, so the analyte ion yield does not really change with higher temperatures. Figure 1.11b reveals, however, that the overall noise level in the TIC significantly varies with the drying temperature, which is a clear sign for a more efficient gas phase aggregate destruction (*declustering*).

Finally, an increased baseline noise can also be caused by too high of an electrospray voltage (called ESI voltage in the following). In extreme cases, you can even see this with the naked eye as a pale blue glow discharge on the ESI needle tip. This glow discharge is facilitated by too many charged species in your mobile phase, for instance, by using a highly concentrated buffer salt, acid, or base; next to a disrupting ion beam in your MS source, this can even lead to a voltage flashover in parts of your ion optics. If possible, you should then reduce the ESI voltage, the buffer concentration in your eluent, or both.

Baseline stability issues, in particular spikes or spontaneous drops, typically indicate an improper nebulizing or an unstable electrospray. A poor nebulizer gas flow or pressure rate as well as a nonideal ESI voltage then lead to the formation of larger liquid droplets or clusters in the ion source; these droplets tearing down from the ESI needle will lead to negative drops in your baseline, while a

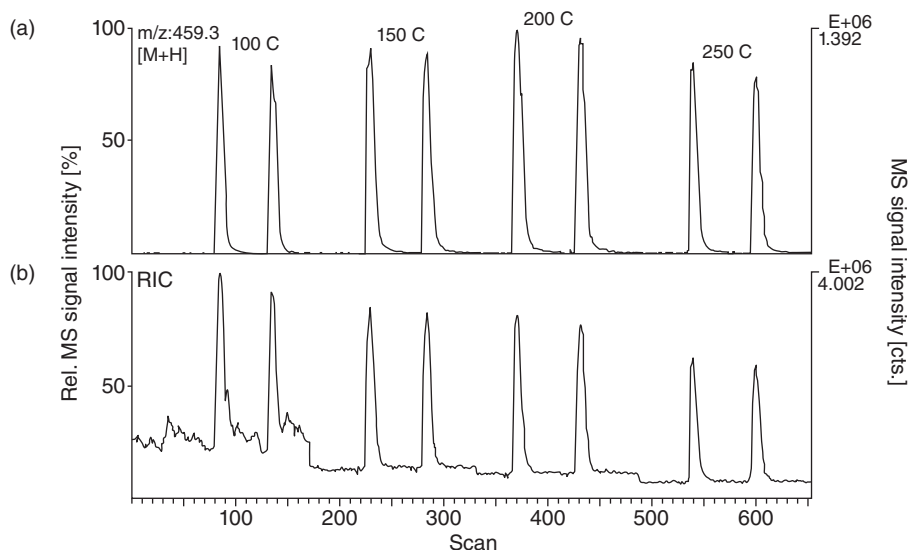


Figure 1.11 Influence of the dry gas temperature on MS signal quality using FIA of Astemizol in 10 mM aqueous ammonium acetate/methanol 20/80 (v/v) at 50 μ l/min

on a Triple-Quad instrument; (a) extracted ion chromatogram of Astemizol ($[M+H]^+$), m/z 459.3; (b) reconstructed total ion chromatogram.

droplet burst releases many ions from this droplet simultaneously and thus generates characteristic positive spikes.

1.2.3.3 Ion Suppression

Whenever possible, the LC/MS method development is done with high-purity reference compounds. Real samples, however, can host significant amounts of impurities and matrix components; those can lead to a substantial signal intensity reduction for your analytes at the same identical concentration as in your purified method development standard solutions. This is a very frequent problem in matrix-rich sample analyses such as food, blood and plasma, or cell tissue. The origin of this deviation in signal intensity is the co-elution of the target analyte with other compounds that interfere with each other during the ionization process and thus affect the ion yield for your analyte of interest. Depending on the mechanisms behind, either a signal enhancement or a signal reduction could be observed, the latter being called *ion suppression*, which is already discussed in Section 1.1. The reasons for a signal-enhancing or signal-reducing effect are manifold. An in-depth discussion would exceed the scope of this section, so I refer to the literature instead [87]. Signal suppression can be observed both with ESI and APCI interfaces, for positive as well as negative polarity. However, there are many examples indicating that APCI is less prone to ion suppression than ESI, and negative polarity seems to be less affected than positive. We already learned about how to qualitatively determine the signal enhancement or suppression effect

using a *post-column infusion* setup (see Section 1.1). A quantitative assessment of matrix effects requires a comparative analysis of a spiked, matrix-free sample and a matrix-containing sample. A comparison of peak areas from both experiments will tell you then about the extent of a signal change by matrix effects. It is explicitly this signal intensity change caused by matrix effects that passionately pleads for a high-quality LC sample separation as an essential requirement for a robust and reliable quantitation with mass spectrometry. Infusing an unknown and nondiscriminated bulk solution of your sample into a mass spectrometer will always have the potential for many disturbing interactions of the analytes with each other during the ion formation process; as a result, you would barely be able to assess whether you truly see every analyte in your sample, not to speak of a reliable analyte quantitation.

1.2.3.4 Unknown Mass Signals in the Mass Spectrum

Many LC/MS users are permanently facing the challenge that they cannot plausibly assign all signals in a mass spectrum to a given target analyte. Either the measured mass signals differ from what is expected, or the spectrum shows more m/z values than predicted. In the following, we discuss a selection of root causes being responsible for this mismatch between experiment and expectation. However, it would be beyond the scope of this book to talk comprehensively about virtually all aspects of observing unknown mass signals; therefore, I refer also to MS-specific literature for further reading [76, 88, 89].

Gas Phase Adducts The most frequent reason by far for unknown mass spectrum peaks is the creation of adducts between the analyte molecular ion and other low-molecular-weight ions and/or neutral chemical entities in the gas phase. Hereby, the type and extent of adduct formation varies substantially with the ionization principle, the ion source parameter settings, the analyte properties, and the quality of the mobile phase. However, gas phase adduct formation or *clustering* is not disadvantageous by default; it is even imperative for the conversion of a neutral molecular species into a charged one with ESI and APCI, as adding a proton to (in positive polarity mode) or subtracting a proton from (in negative polarity) an analyte of the molecular mass M converts it into the charged state of $[M + H]^+$ and $[M - H]^-$, respectively. Consequently, the measured m/z ratio differs from the theoretical one of the neutral species by the amount of one proton mass. But next to this fundamental prerequisite for the mass detection, many other charged adduct species can be observed in reality. The virtually ubiquitous sodium and potassium cations leaching from the glass surfaces of the solvent bottles, for instance, frequently lead to the respective adducts $[M + Na]^+$ and $[M + K]^+$. The longer the shelf life of your solvents, the more these sodium and potassium clusters are even in favor compared with the proton adduct – shifts in the adduct ratio between proton and alkali metal-based ionic species can, therefore, even be used as a rough estimate of the solvent age in your LC/MS system. With the sodium or potassium adduct becoming the most prominent m/z signal in your spectrum over time, it is high time to

prepare a fresh lot of mobile phase for your UHPLC separation. Due to the well-known chemical similarity between the alkali metal ions and the ammonium ion, the use of ammonium salts as buffering agents in LC/MS applications will lead to the analog creation of the ammonium adduct $[M + \text{NH}_4]^+$ instead. Next to those species, also higher aggregates involving three or more – mostly neutral – molecules can be observed; depending on the solvent evaporation efficiency, excessive solvent molecules can then cluster with the analyte and the charge carrier, resulting, for example, in $[M + \text{H}_2\text{O} + \text{H}]^+$ or $[M + \text{CH}_3\text{CN} + \text{H}]^+$. Even clusters of multiple analyte molecules sharing one proton or sodium cation as $[2M + \text{H}]^+$ or $[2M + \text{Na}]^+$ can often be detected. All these adducts reveal very characteristic mass differences in comparison to the simple proton adduct. Table 1.5 lists the most common gas phase adducts with their respective nominal mass difference to the singly protonated ($[M + \text{H}]^+$) or deprotonated ($[M - \text{H}]^-$) reference. More in-depth information on that matter can also be found in the literature [90].

In general, the trend to form those adducts can be controlled quite effectively via the ion source parameters. An appropriate setting for the drying conditions of the source (such as dry gas temperature, nebulizer gas pressure) allows the declustering of higher aggregates into less complex ones. Also, APCI is typically less prone to higher aggregate formation with, for example, alkali metals, as the charge transfer to the analyte molecule happens after the evaporation, that is, entirely in the gas phase; In ESI, in contrast, the charge transfer takes place parallel to the gas phase transfer, so while the analyte still partially is in the liquid phase. For the same reason, APCI is in many cases also less affected by matrix effects, depending of course on the application. As the cluster formation is also influenced by type

Table 1.5 Common gas phase adducts at positive (left) and negative (right) polarity.

Positive polarity		Negative polarity	
Gas phase adduct	Nominal mass difference (ΔDa)	Gas phase adduct	Nominal mass difference (ΔDa)
$[M + \text{NH}_4]^+$	+17	$[M - \text{H} + \text{H}_2\text{O}]^-$	+18
$[M + \text{H}_2\text{O} + \text{H}]^+$	+18	$[M - \text{H} + \text{CH}_3\text{OH}]^-$	+32
$[M + \text{Na}]^+$	+22	$[M + \text{Cl}]^-$	+36
$[M + \text{CH}_3\text{OH} + \text{H}]^+$	+32	$[M - \text{H} + \text{CH}_3\text{CN}]^+$	+41
$[M + \text{K}]^+$	+38	$[M + \text{HCOO}]^-$	+46
$[M + \text{CH}_3\text{CN} + \text{H}]^+$	+41	$[M + \text{CH}_3\text{COO}]^-$	+60
$[M + \text{H}_2\text{O} + \text{CH}_3\text{OH} + \text{H}]^+$	+50	$[M + \text{Br}]^-$	+80
$[M + \text{CH}_3\text{CN} + \text{Na}]^+$	+63	$[M + \text{HSO}_4]^-$	+98
$[2M + \text{H}]^+$	–	$[M + \text{H}_2\text{PO}_4]^-$	+98
$[2M + \text{Na}]^+$	–	$[M + \text{CF}_3\text{COO}]^-$	+114
$[2M + \text{K}]^+$	–	$[2M - \text{H}]^-$	–

Mass differences refer to the difference between $[M + \text{H}]^+$ (left) or $[M - \text{H}]^-$ (right), respectively, and the related gas phase adduct.

and amount of mobile phase, the signal intensity of different gas phase species can change with the UHPLC flow rate.

In-Source Collision-Induced Dissociation Gas phase fragmentation reactions are a fundamental element of mass spectrometric experiments. Imagine an analyte that is excited to vibrations in a well-controlled manner: this molecule will then selectively break into pieces at the weakest bonds, thus creating a characteristic fragment spectrum. As long as this fragmentation reaction can be stimulated reproducibly, these fragmentation patterns provide you with a molecular fingerprint, which tremendously helps to identify a chemical compound. And even if you cannot afford to run your entire LC/MS experiment in full scan mode with parallel MS/MS fragmentation because you need highest sensitivity, the detection of as many known characteristic fragments as possible in SRM mode increases the level of confidence for your compound confirmation substantially. However, ESI and APCI are rather mild ionization principles transferring the analyte molecules into the gas phase as a whole, so fully intact and nonshattered – which is very different from EI (also earlier known as *electron impact ionization*) in GC/MS. The benefit is that you will be able to determine the molecular mass of the intact molecule, but you lose the chance to learn more about the structural properties and chemical nature of your analyte by fragmentation patterns. Sophisticated tandem-MS techniques, however, enable you to stimulate well-controlled decomposition conditions in the collision cell of a tandem mass spectrometer. The user typically knows about the most characteristic fragments of the target analyte, so not too many surprises are to be expected then. Many years of extensive research meanwhile allowed unveiling a huge set of decomposition reactions and their follow-ups in the gas phase; a very informative and comprehensive tutorial by Holcapek *et al.* is a valuable starting point for your own interpretation of small molecule fragmentations in API mass spectrometers [90].

Next to these intended fragmentation reactions in a tandem MS, the user will always have the chance – or the risk – to shatter the analyte in an uncontrolled way, typically stimulated by unfavorable ion source or ion transfer conditions. As long as the analytes have not entered the final high vacuum section of the mass spectrometer, that is, while they still are in the ion source or the transfer section of the ion optics that come along with a staged pressure reduction, these ions will have to remain intact in an environment where their mean free path is only in the range of a few micrometers ($\sim 50\ \mu\text{m}$ at 1 mbar of ambient pressure) and not several dozens of inches ($\sim 20''/500\ \text{mm}$ for a vacuum of 10^{-4} mbar). A collision with excessive ambient gas molecules is very likely there, and the higher the collision impulse, the more you will see an unwanted fragmentation taking place already in the entrance area of the ion source. You can master this process to some extent by a smart selection of acceleration voltages in your ion optics. High voltages, to be applied, for example, along the ion transfer capillary or to the skimmer electrodes, strongly accelerate the ions while traveling through the ion optics and induce more effective collisions with residual gas molecules, thus resulting in more fragment signals in the mass spectrum (also called *nozzle-skimmer dissociation*). In case

of the MS method development, it is a useful approach to change the parameter settings for the ion source and the transfer section stepwise while monitoring the signal change and the spectrum quality accordingly to avoid excessive analyte fragmentation. However, you can also make use of this principle to artificially stimulate ion fragmentation and to learn more about unknown compounds even with rather simple and cost-effective instruments such as single-quad mass spectrometers.

A very comprehensive table on the generation of fragments out of various functional groups can be found in the literature [90]. We briefly discuss the behavior of alcohols, aldehydes, and carbonic acids as representative examples for frequently occurring fragmentation reactions. These compound classes have a strong heteropolarity of the carbon–oxygen bond in common due to the high electronegativity of the oxygen atom. One immediate result of this is the neutral loss of water; the loss of carbon oxides is another one, depending on the chemical composition of the analyte. Once protonated in positive mode, alcohols preferably split off water (R-OH_2^+ from R-OH), thus generating an R^+ fragment (equaling $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$), which is lighter than the expected ion of the intact molecular ion by nominal 18 Da. Aldehydes lose carbon oxide (CO), ending up in a fragment ion $[\text{M} + \text{H} - \text{CO}]^+$. Aliphatic carbonic acids typically lose the thermodynamically very stable carbon dioxide after protonation, while aromatic carbonic acids under the same conditions preferably “only” split off water, leaving us with an acylium cation for detection.

Contaminants Eluting from the Instrumentation Nevertheless, there will still be many situations where a good knowledge of gas phase fragmentation reactions and chemical expertise will not help to explain the existence of prominent mass signals in your MS spectrum: for instance, the sheer amount of mass signals being so high that they cannot be deduced by fragmentation reactions, or mass interferences not only showing up as one individual signal but as a series with distinctive patterns. The root cause here can be very trivial – contaminants are eluting from your chromatography or the mass spectrometer hardware. If your MS was thoroughly cleaned recently, then the (U)HPLC system and any fluidics connected with it would be blamed for that, and the potential contamination sources are numerous:

- *Bleeding* of a separation column being either old or unsuitable for MS detection results in an increased elution of the stationary phase bonding, which is split off the carrier material surface and leads to an increased noise in the LC/MS chromatogram.
- *Plasticizers* are ubiquitous in almost all modern plastic materials, from sample vials over solvent lines, piston seals to filter frits and many other pieces. Very common plasticizers are phthalates which can rapidly be identified based on their characteristic masses (m/z 279, 391, 413, 429, 454, and many more). Also lubricants and separating agents such as Erucamide (m/z 338, 360) can frequently be observed.

- *Polyethers* such as *polyethylene glycol* (PEG) and *polypropylene glycols* (PPG) are another frequent contamination type, which can be identified quickly due to their characteristic MS spectrum pattern. Also they are nearly ubiquitous in plastic materials, but they can also be introduced into the MS by working sloppily with disposable gloves or skin care products. These compounds never show up with only one single mass signal but always come with a very characteristic polymer distribution pattern [91]. The mass distance of the monomeric units are $\Delta m/z = 44$ for PEGs and 58 for PEGs, which immediately reveals the chemical nature of these contaminants.
- *Polysiloxanes (silicones)* are core ingredients of many modern high-performance oils and vacuum grease. Oil vapor traces from the rough vacuum pump leaking into the mass spectrometer produce characteristic signals of, for example, m/z 371, 445, or 519. In such a case, the ion optics, such as focusing multipoles or ion funnels, should be checked for cleanliness. A continuous stream of oil mist entering the MS can even lead to a razor-thin oil film coating the metal surfaces of the ion guides or, in some cases, on the mass analyzer over weeks and months. This results not only in contaminant signals in your mass spectrum but also in a measurable sensitivity loss, which makes a thorough and extensive cleaning mandatory in the end. But how would it happen anyway that oil mist from the rough pump(s) could enter the MS interior in a way that also comes along with ion generation? Well, the most obvious reason for this is a nonideal installation of the various exhaust hoses of your MS. For convenience, the exhaust tubes of the vacuum pump(s) and of the ion source drainage are frequently tied together into the same lab exhaust ventilation nozzle. But by doing so, you allow the vacuum pump exhaust to diffuse backward into the MS ion source and further down into the mass spectrometer. Installing the pump exhaust and the ion source draining tube into different connectors of the lab ventilation with a distance of 1.5–3' (0.5–1 m) in-between is a very simple and effective solution to that issue.
- *Metal ions* can be a great origin for the generation of larger gas phase adducts with your target analyte molecules; alternatively, metal ions can also react with parts of your sample and thus inhibit the detection of compounds. We already discussed the formation of alkali metal adducts in section "Gas Phase Adducts"; A SST LC fluidics or massive hardware defects in a biocompatible UHPLC system, for example, a damaged injection valve, can result in a propagated release of iron ions and thus in iron/analyte clusters in the mass spectrum; these clusters reveal themselves very quickly due to their multicharge state and the isotope pattern of iron, which significantly deviates from those of the usual elements in organic matter such as carbon, hydrogen, nitrogen, and oxygen. Biochemical applications are particularly prone to issues created by heavy metals in the mobile phase. A lot of biological compounds tend to form either precipitates or nonvolatile aggregates with iron, or alternatively to irreversibly adsorb on iron surfaces. A frequently described phenomenon is the "vanishing," so the nondetectability of phosphorylated peptides and

proteins in a separation system with an SST fluidics. To avoid this, bioanalytical applications are preferably run on instrumentation with an iron-free fluidics, which can be made of titanium, biocompatible metal alloys like MP35N, or PEEK, with the latter being not very pressure-resistant (tubing typically up to 5–6000 psi/350–400 bar) and thus not being suitable for UHPLC applications.

- *Dissolved residual gases* in the mobile phase, however, typically result in an unstable spray and/or interfering spikes in the LC/MS chromatogram and the mass spectra rather than modifying the analyte mass signals.

The aspects discussed here can only represent a selection of some very popular phenomena, but this list is far away from being a fully comprehensive compilation of known interference signals [88]. Already some years ago, Keller *et al.* published an excellent and highly detailed, tabulated collection of all literature-known MS contaminants known at this point [92]. Most MS vendors discuss this topic very openly as well and compiled various collaterals on MS contamination sources [89, 93], and last but not least the internet offers various public data search engines on that matter. One example to be mentioned is the *MaConDa* (*Mass spectrometry Contaminant Database*) database maintained by the University of Birmingham [91], which allows to search for more than 300 contaminants based on accurate mass, compound class, and mass spectrometer type (operation principle and manufacturer), featuring also many literature references.

1.2.3.5 Instrumental Reasons for the Misinterpretation of Mass Spectra

Finally, we investigate some selected instrumental reasons that can lead to the misinterpretation of mass spectra. As discussed earlier, all MS types have their strengths and weaknesses that affect also the quality of your analytical result. Let us discuss these in the following on three scenarios:

False Mass Assignment Depending on Ionization Principle As already discussed in section “Gas Phase Adducts”, the selected ion creation principle determines which m/z value is shown in your mass spectrum for an unknown analyte species. The most frequently used ionization process by charge transfer, that is, proton association or distraction (used in ESI and APCI), does not result in the molecular mass of the intact molecule being measured, but in an m/z value differing by one proton mass (or multiple proton masses at a respectively corrected fraction of the intact molecule mass for multicharged molecular ions). Other processes such as APPI or EI (hardly used in LC/MS), which can create ions also by transferring electrons instead of protons lead to a measured m/z value, which deviates from the theoretical value of the intact molecule only by the much smaller mass amount of an electron. With ESI, APCI, and APPI, the formation of adducts with alkali metals and/or residual solvent molecules can lead to misinterpreting a numerical m/z value as representing an $[M + H]^+$ ion species, which in fact would be, for instance, an $[M + H_2O + Na]^+$ ion instead. A thorough look on your mass spectrum can be very helpful here, as many

adduct species coexist with others, with characteristic m/z differences between the various ion aggregates. So if you find a new m/z value, which you may take for an $[M + H]^+$ ion, just check for further signals with m/z differences to the first one of, say, $[“M + H” + 22]^+$, $[“M + H” + 38]^+$, or $[“M + H” + 41]^+$ – with these being a sodium, potassium, and ACN/proton adduct, respectively; this series can additionally confirm (or disprove) your originally assumed mass assignment.

False Interpretation Due to Poor Mass Resolving Power Another reason leading to a mass signal misinterpretation is a poor or inappropriate mass resolution. Imagine two different analyte species having only very small differences in their m/z values and arriving in the mass analyzer simultaneously; a low-resolving mass spectrometer will not be able to sufficiently discriminate the two different masses. The resulting mass spectrum will thus show only the envelope curve for the two different mass patterns, and the peak maxima of this envelope function do not necessarily have to be identical to the mass signal maxima of the mass spectrum of each individual compound. Also, low-resolution mass spectrometers will not be able to resolve higher charge states; we know that the m/z distance between the isotope pattern signals of a compound equals the $1/n$ th fraction of the charge state n , which means at the same time that for analyte ions with three or more charges the isotope pattern cannot be resolved appropriately with low-res mass specs. In summary, co-eluting contaminants or impurities could not be identified as such, or mass signals are erroneously assigned to the wrong compounds. It depends – next to your budget of course – on the sample complexity and the quality of your separation how good the resolving power of your mass spectrometer must be at minimum. The better your chromatography, the more unambiguous the interpretation of your mass spectrum will be in the end. As a general recommendation, a mass spectrometer for the determination of accurate masses should have a resolving power R of 10 000 – 15 000 at least; the scientific literature typically defines R of 10 000 as the minimum for high-resolution and R of 100 000 for ultra-high-resolution mass spectrometry [75].

False Mass Determination Due to Inappropriate or Unstable Experimental Conditions

It is an obvious fact: a mass determination is always achieved by comparing your instrumental MS data for the analyte with the mass signals of known calibration standards; thus the quality and the long-term stability of the mass calibration are critical for a reliable mass measurement. Expired or contaminated reference standards with partially degraded ingredients should of course not be used for calibration anymore. An undefined number of mass signals during the mass calibration process compromises the correct mass assignment and impairs or even blocks software tools such as autotune algorithms. Once successfully calibrated, the quality of the mass axis calibration needs to be verified regularly. As discussed earlier, TOF instruments are particularly prone to drifts in the mass axis calibration even on short-term periods of (much) less than 1 h. Hence, an internal mass calibration by a continuous calibrant infusion is essential for a

reliable mass determination. Mass spectrometers of the ion trap design (QIT, LIT, FTICR, Orbitrap) are additionally influenced by the spatial density of the ions stored in the electromagnetic field cage of the ion trap. The circulating ion packages in the trap act as so-called *space charges*, which induce additional electrical fields, shielding and thus locally distorting the external electromagnetic trapping field. Higher charge densities, which means high amount of analyte ions in the trap cell and/or multiply-charged ion species, translate then into a pronounced shift of the resonance conditions for your circulating ion packages, which impairs mass accuracy and mass resolution significantly. An overfilled ion trap will then give you shifted and thus falsified mass signals. In contrast, the fewer ions you trap, the more you will lose sensitivity. The ideal filling degree for an ion trap is dynamically calculated in real time by modern MS control software – feature names here are *ICC*, *AGC*, or others, depending on the MS vendor. Very concentrated samples, however, cannot always fully be intercepted by the control algorithms and thus will still lead to a short-time overfilling of the ion trap. Due to their design principle with a stretched longitudinal cell construction, LIT typically suffer less from space charge effects than circular traps (QIT).

1.2.4

Conclusion

For almost two decades, the coupling of LC and mass spectrometry (MS) has been successfully commercialized now. With the first instrument generation being true divas requiring in-depth expert knowledge, nowadays this technology has reached a fairly mature development state, which substantially lowered the entry barrier to this technique; this results in many robust LC/MS solutions being established in the market, and LC/MS is more and more penetrating the field of routine applications, as users do not have to adopt a high amount of expertise to create quick and reliable results. Within these 20 years of growth and evolution, not only new MS technologies such as the Orbitrap have seen the light of day but also LC has made a big step ahead by moving from HPLC to UHPLC with much higher separation efficiencies and shorter run times. So from a bird's perspective, LC/MS has evolved to a very powerful analytical tool, which is surprisingly easy to use given the high complexity of the technologies involved. But nevertheless, mass spectrometry is not the analytical all-purpose weapon as it is advertised in some cases, and it will not be for a long time. Key to the highest analytical benefit of an LC/MS installation is the thorough mutual physicochemical optimization of the LC and the MS world; this chapter hopefully is one contribution to better understand the technical needs and to avoid the most general pitfalls. As the scientific progress is moving forward, several new technological territories will be entered in the future for sure, be it for even higher speed and resolution, for instrument miniaturization, or for enhanced usability by new and powerful software tools. However, one thing is for sure: it is not only the liquid phase separation that

benefits from the mighty analytical information creation by mass spectrometry but also MS technologies will massively fall short on their potential without a thoroughly optimized chromatography upfront. For the foreseeable future, both concepts, chromatography and mass spectrometry, will continue to depend on each other.

1.2.5

Abbreviations

AP(C)I	atmospheric pressure (chemical) ionization
CAD	charged aerosol detection
CID	collision-induced dissociation
ECD	electrochemical detection
EI	electron ionization (also: electron impact ionization [obs.])
EIC	extracted ion chromatogram
ESI	electrospray ionization
ELSD	evaporative light scattering detector
FA	formic acid
FIA	flow injection analysis
FT	Fourier transformation
GDV	gradient delay volume
HPG	high-pressure gradient pump
HR/AM	high-resolution/accurate mass
HTS	high-throughput screening
HV	high voltage
ICR	ion cyclotron resonance
I.D.	inner diameter
LIT	linear ion trap
LPG	low-pressure gradient pump
MRM	Multiple Reaction Monitoring
MSD	mass selective detector
PEEK	poly(ether ether ketone)
PEG	poly(ethylene glycol)
PPG	poly(propylene glycol)
QIT	quadrupole ion trap
SIM	single-ion monitoring
SRM	Selected Reaction Monitoring
SST	stainless steel
TFA	trifluoroacetic acid
TOF	time of flight

1.3

LC Coupled to MS – A User Report

Alban Muller and Andreas Hofmann

In comparison to coupling LC to UV, coupling LC to mass spectrometry (MS) requires important adaptations. Water–methanol or water–acetonitrile gradients are often applied in RP chromatography. Only volatile buffers should be used in order to prevent contamination of the MS instrument by nonvolatile salts. For example, FA or acetic acid can be used for acidic conditions and ammonia can be used for alkaline conditions. If samples contain nonvolatile salts, the LC flow can be directed to waste at the beginning of the analysis to prevent contamination of the MS instrument. A two-dimensional LC system with an enrichment column is another option to analyze samples containing nonvolatile salts. Matrix effects can reduce signal-to-noise ratios of analytes in complex biological samples. Adding heavy isotope-labeled standards to samples allows to take losses during extraction and matrix effects into account. ^2H , ^{13}C , and ^{15}N are often used to isotopically label standards. In contrast to ^2H -labeled standards that can show small retention time shifts, ^{13}C - and ^{15}N -labeled standards elute at the same time as the analytes.

The most suitable LC system can be selected based on the required sensitivity and the desired analysis time. The MS signal intensity is proportional to the concentration of the analyte. Nano LC systems are mainly used to achieve highest sensitivity. High sensitivity is especially critical for the analysis of low abundant endogenous molecules, for example, in proteomics experiments. In proteomics experiments, chromatographic columns have often an inner diameter of only 75 μm or lower. The low solvent flow rate (e.g., 250 nl/min) in nano-LC/MS methods can lead to an analysis time of 1 h and more, but allows the most sensitive approach to detect endogenous molecules. Higher LC flow rates (400–600 $\mu\text{l}/\text{min}$) are usually applied for the detection and quantification of analytes with higher concentrations. Chromatography columns with a 1 or 2.1 mm inner diameter are often used for U(H)PLC applications that allow to complete an analysis in only few minutes.

The method of ionization is chosen based on analyte properties and the LC flow rate. ESI is suitable for a very broad spectrum of analytes and LC flow rates. Hydrophilic as well as hydrophobic substances can be well analyzed with ESI. APCI and APPI are mainly applied for the analysis of very hydrophobic substances and require high LC flow rates. In general, the ion source temperature and the gas flow rates need to be increased with increasing solvent flow rates. Recommended parameters for different LC flow rates can be found in the manual of the ion source. These recommended parameters often represent a good starting point for your own optimization. In addition to parameters that depend on the solvent flow rate, analyte-dependent parameters of the ion source need to be optimized. Analyte-dependent parameters can be optimized by direct infusion of a pure analyte solution with a syringe pump, in order to adjust all parameters for a maximal signal-to-noise ratio. Combining the flow of the syringe pump via a T-piece with the LC flow allows to simulate conditions close to the final

analysis conditions. Relatively high concentrations of analyte have to be used for direct infusion, which can lead to background signals of the analyte. Different instrument parameters can also directly be varied during the LC/MS analysis, due to the short analysis time of U(H)PLC systems and the fast scan speed of modern MS instruments. A lower concentration of analyte can be used for the variation of parameters during the LC/MS analysis compared with direct infusion. Many steps of the optimization can automatically be done by the instrument software or platform-independent software. However, automatically determined parameters should always be checked for plausibility. Orifices of MS instruments have been enlarged over the last years in order to increase ion transmission. Not only more ions but also more neutral particles enter the MS instrument through the enlarged orifice; therefore, strong roughing and turbo pumps are necessary to maintain the required vacuum. Furthermore, the geometry of the ion optics were changed to efficiently separate ions from neutral particles, for example, by using the StepWave™, iFunnel, or electrodynamic ion funnel technology. The improved ion transmission of modern MS instruments results in higher signal-to-noise ratios, more robust methods, and facilitated optimization of instrument parameters.

Mass accuracy, mass resolution, scan speed, sensitivity, and many other parameters of MS instruments can differ greatly. Some applications may only be feasible with one specific type of MS instrument. Basic MS instruments, such as single quadrupole, ion trap, or TOF instruments, can be used to analyze samples with low complexity. Hybrid MS instruments, combining two mass analyzers, are often used to analyze complex biological samples. Hybrid MS instruments with high mass accuracy and mass resolution, for example, Q-TOF, TOF-TOF, ion trap-Orbitrap, or Q-Orbitrap instruments, allow the identification of unknown substances. Triple quadrupole MS instruments with high scan speed and excellent sensitivity are often applied for quantitative analyses. A triple quadrupole instrument consists of three quadrupoles arranged one after the other (Figure 1.12a). The first quadrupole is used as a mass filter for the ionized, intact analyte. The ionized, intact analyte is also called parent ion. The parent ion is then fragmented in the second quadrupole by collision-induced dissociation. Subsequently, the third quadrupole filters for a specific fragment of the parent ion, also called daughter ion. Assays on triple quadrupole instruments are very sensitive and selective due to the double filtering in the first and third quadrupole.

For example, isomers of the hydroxy-eicosatetraenoic acid are difficult to separate chromatographically (Figure 1.12b), and a simple mass analysis shows overlapping peaks for the parent ions (Figure 1.12c). However, a triple quadrupole instrument enables to measure specific daughter ions of the two isomers and the daughter ion traces show no interferences between the two isomers [94].

Ion-pairing reagents, such as TFA for acidic conditions or triethylamine for alkaline conditions, can be used for the separation of hydrophilic analytes by RP chromatography. In general, ion-pairing reagents often lead to a reduced signal-to-noise ratio and to high background signals when the polarity is switched.

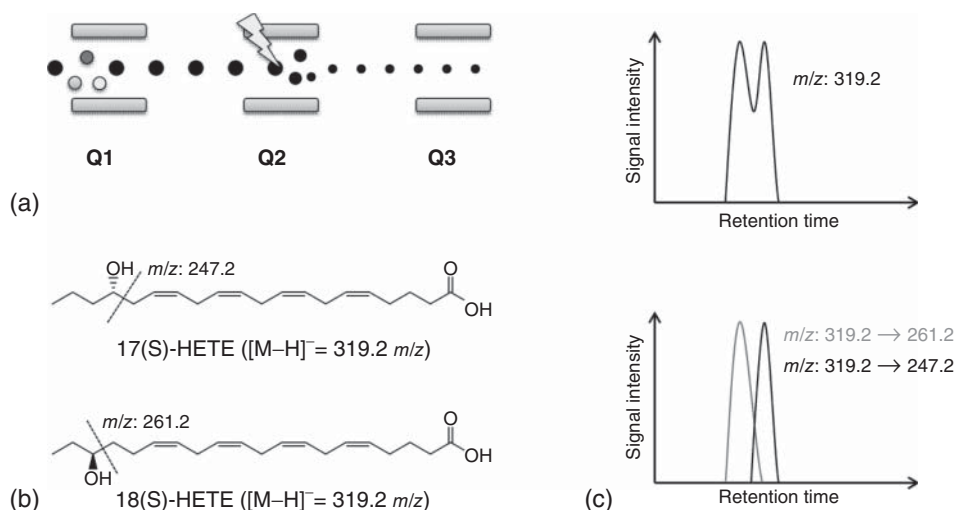


Figure 1.12 (a) Schematic representation of the quadrupoles of a triple quadrupole instrument. Only two quadrupole rods of the four quadrupole rods are shown for each quadrupole. The first quadrupole (Q1) filters for the precursor ion, the precursor ion is

fragmented in Q2 by collision-induced dissociation and Q3 filters for a specific daughter ion. (b) Isomers of hydroxy-eicosatetraenoic acid (HETE). (c) Schematic representation of ion traces by measuring just the precursor ions or daughter ions of HETE isomers.

Ion chromatography (IC) is a reliable alternative to separate very hydrophilic, charged molecules, which cannot be readily analyzed by RP chromatography. Analytes are separated based on their charge and size by IC. In contrast to silica-based RP columns, the stationary phases of IC columns are polymer-based. Therefore, IC columns are very stable under alkaline conditions. Potassium hydroxide is often used for anion exchange chromatography, and the eluting strength is directly proportional to the potassium hydroxide concentration. Methanesulfonic acid is often used for cation exchange chromatography.

An example of an anion exchange IC coupled to a triple quadrupole MS is described in the following section. A potassium hydroxide gradient is produced by the eluent generator and analytes are separated on the IC column. Thereafter, potassium ions are exchanged by hydronium ions in the electrochemical suppressor. Usually, IC is coupled to a conductivity detector, which determines the conductivity of the solution. A conductivity detector is relatively insensitive and not well suited for biological applications, for example, in the field of metabolomics. Furthermore, the conductivity is not selective so that analytes with the same retention time cannot be distinguished. In contrast, a triple quadrupole instrument enables a very sensitive and selective detection of different analytes. Without electrochemical suppressor, potassium ions would lead to a strong suppression of analyte signals and contamination of the MS instrument. The electrochemical suppressor generates an aqueous solution with a low salt concentration of a few microsiemens. The efficiency to generate negative ions

can be increased by adding an organic solvent, such as methanol, via a T-piece to the aqueous solution. A Dionex ICS-3000 IC system, an ESI ion source and an AB Sciex QTrap 5500 MS instrument was used for the analysis described here. The triple quadrupole instrument allows a very sensitive and selective detection of analytes via MS/MS experiments.

1.3.1

Conditions of the Ion Chromatography

Eluent generator	EGC III KOH
Enrichment column	Ion Pac AG20 2 × 50 mm
Analytical column	Ion Pac AS20 2 × 250 mm
Column temperature	35 °C
IC pump	Isocratic, 250 µl/min
Suppressor electric current	62 mA
Loop volume	2 µl
Methanol flow rate	50 µl/min

1.3.2

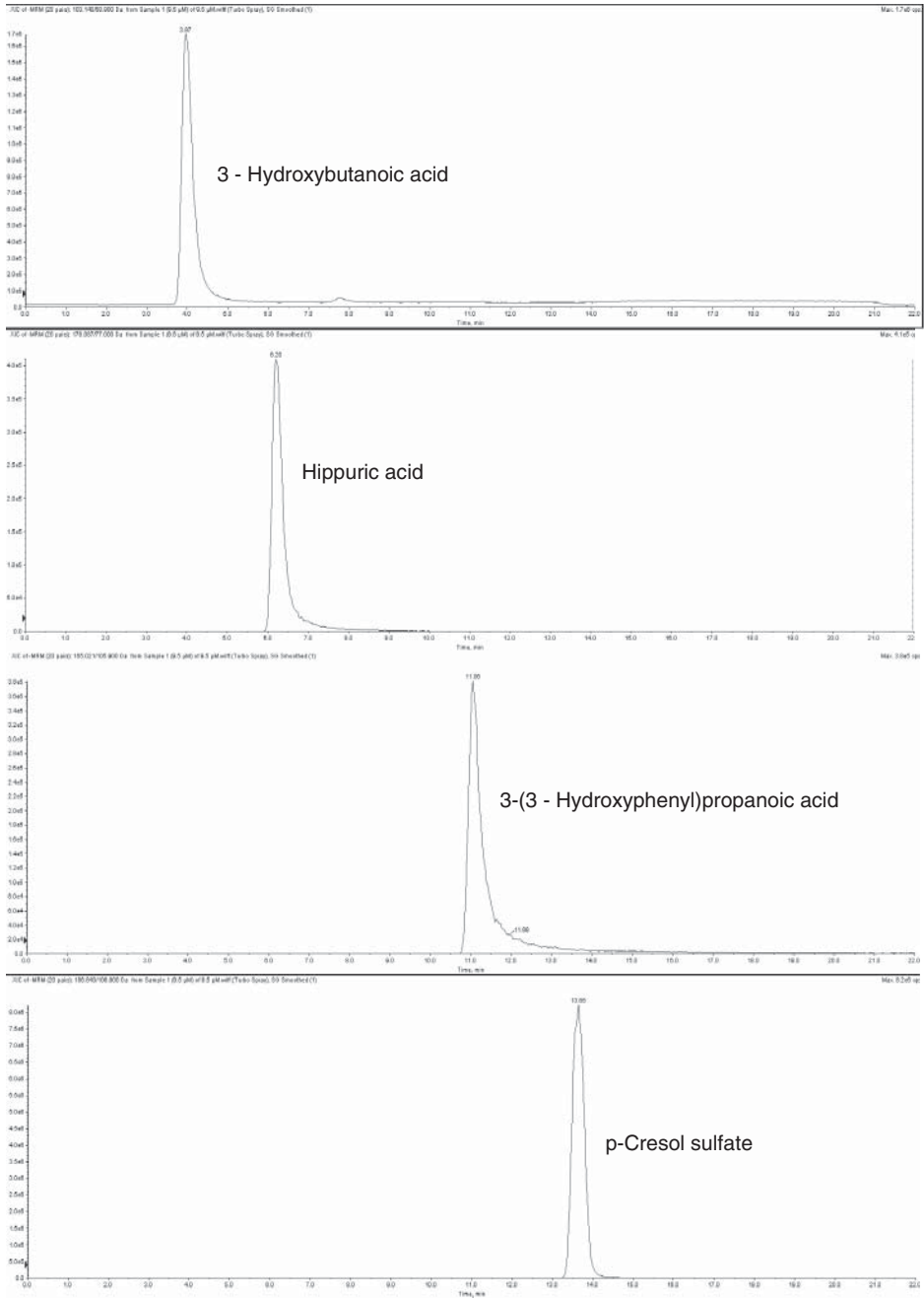
Gradient Generator

Time (min)	[OH ⁻] (mM)
0	10
7.5	45
17.5	48
18.5	100
22.5	100
22.6	10
25	10

1.3.3

Transitions

Analyte	Q1 mass (Da)	Q3 mass (Da)
3-Hydroxybutanoic acid	103.1	58.9
Hippuric acid	178.1	134.1
3-(3-Hydroxyphenyl)propanoic acid	165.0	105.9
<i>p</i> -Cresol sulfate	186.8	106.9



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2

Optimization Strategies in RP-HPLC

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2.1

Introduction

Optimization of devices and processes is very common in today's efficiency-oriented world with everyone striving to do things faster but with no loss of quality. It is, therefore, important to define the best approach in which optimization can be achieved and how the related quality can also be measured. There are numerous parameters in a technique such as HPLC of either independent of each other or that have a related nature to other parameters. Optimization goals can vary and mostly depend on specific needs of analytical laboratories. The most important optimization criteria in HPLC will be elucidated and discussed in this introductory section.

It is important to note that the majority of rules in this chapter on optimization are valid beyond reversed phase (RP) separation mechanisms, though most of the given examples are based on RP-HPLC and the same applies for the concepts on selectivity optimization. In spite of a rapid development of mixed-mode phase-based and HILIC separations, RP continues to be the most widespread separation mode in modern HPLC and UHPLC.

2.1.1

Speed of Analysis

Speed is the most common HPLC optimization criterion. A method is considered optimized if it allows the separation of all relevant components with sufficient resolution in the shortest time possible. Such speed increase only translates into an economical benefit for the laboratory if it actually enables productivity increase of the respective lab. Labs with particularly high sample load are typical beneficiaries of this, but so are all labs that directly profit from knowing analytical results within hours or even minutes after receiving the samples in order to react on it. Productivity increase often requires optimization of the entire lab workflow which will include more than the analytical separation process. It is rather common that samples do not arrive in a ready to inject format and thus require sample preparation

steps. If the time to perform these steps cannot be optimized to a similar extent, they will become the bottleneck in the workflow. Consequently, faster separations may only be of very limited benefit for the overall productivity.

In-process analysis depends very often on fast analysis, no matter on where this occurs – either research or production processes, continuous or batch processes. A reduction in the analysis time always results in a higher information density, which normally translates into an economical benefit downstream. It is an interesting notion the speed potential of modern UHPLC finally enabled the use of liquid chromatography for current in-process analysis, but is yet very rarely found there.

2.1.2

Peak Resolution

The resolution between neighboring peaks is another common optimization goal. There are two approaches to influence resolution in chromatography:

- increasing column efficiency
- improving the selectivity, by changing the relative retention times of the respective components.

Optimizing the resolution enhances the accuracy and precision of quantitative analysis and can also improve method robustness. It can even reduce the amount of sample preparation provided that the optimized method can handle a direct injection of samples with more complex matrices. Improved separation efficiency also translates into a higher number of components that the method is able to separate, the so-called theoretical peak capacity. Economical benefit may result from simplified workflows with less sample preparation and improved data quality to account for more accurate analyses.

2.1.3

Limit of Detection and Limit of Quantification

The limit of detection (LOD) is the smallest amount of a specific analyte that is visible with given significance. It is an important parameter for trace analysis. A distinction between the lowest concentration of the analyte in the sample (relative value) and the smallest absolute amount that can be detected with the respective method is important. This also has to take the type of detector into account that can either be concentration dependent or amount/mass dependent in its signal generation. The latter type of detectors is in general destructive in that it is not possible to recover the sample. With concentration-dependent detectors, such as the common UV absorbance detector, the concentration detection limit can only be improved on the detector itself, while the absolute detection limit depends both on the detector and on the column dimension and separation efficiency. For truly mass proportional detectors, the column dimension does

not matter as long as the flow rate does not affect the sensitivity of the detector. Zone-sharpening effects in gradient elution help to improve both limits on all types of detectors. It can be concluded from these considerations that limits of detection are in no way a simple matter in liquid chromatography. The smallest concentration or mass of analyte that can be quantified with a given accuracy is the limit of quantification (LOQ). All considerations for the LOD apply, respectively. Optimization of these parameters can also save sample preparation steps (such as preconcentration) and improve quality of trace analysis results. Reducing sample preparation will speed up the total analysis workflow and can also eliminate sources of error, in particular if these steps were manually done in the original method.

2.1.4

Costs of Analysis

In terms of direct costs, these are primarily defined by the required consumables, for example, columns, solvents, chemicals, disposable filters, and cartridges, but also include the often significant costs for their disposal. Other cost types are maintenance and service, instrument amortization (or leasing) costs, and last but not least labor costs. For a fair comparison, the total costs per successfully analyzed sample must be calculated. This is achieved by dividing the sum of the above-mentioned cost types per period (e.g., month or quarter) by the number of analyzed samples in that period. If an instrument is used for more than one method, however, it is obviously more difficult to determine the cost of analysis for one specific method. Cost reduction by solvent savings is a very effective way and also reduces the environmental footprint. It can be achieved through reduction of the column diameter and also through shortening of column length combined with increasing stationary phase efficiency. The related savings can be counteracted by the cost of columns. Modern volume-minimized and efficiency-optimized UHPLC columns are mostly more expensive than conventional LC columns, but do not necessarily allow more analyses over a column lifetime. Even if the column cost per injection increases, this must be considered against the extra savings associated with faster analysis and higher productivity.

It should not be overlooked that highly optimized UHPLC separation methods may require more thorough sample preparation than a method with a more forgiving classical column. The extra sample preparation can result in a solvent mismatch effect if the analyte gets dissolved in a very strong solvent (e.g., DMSO in RP-HPLC), which has to be corrected by solvent removal and reconstitution of the sample. It is also possible that additional filtration or dilution steps are needed with UHPLC. In some cases, and depending on how this dilution and solvent transfer is executed, this will result in additional solvent consumption and increased environmental footprint. If consumable and disposal costs in total are higher after such an optimization from conventional LC to UHPLC, the productivity increase and data quality improvement should still clearly justify the change.

2.2

LC Fundamentals

This section describes the fundamental relationships that are required to understand the rationale behind optimization of LC separations and to make predictions based on a theoretical understanding. For experienced users, it is an opportunity to add to their knowledge, as well as being a timely revision lesson on some of the more fundamental aspects. Beginners should refer to a text book that addresses HPLC knowledge from the scratch [1], since the understanding of the chromatographic process and the functional parts of the HPLC instrument is a premise.

Readers that tend to shy away from mathematics will possibly not initially enjoy this chapter, however and with time they will certainly recognize how formula and particularly rules of thumb derived thereof can be very beneficial for a systematic approach to optimization as opposed to trial-and-error experiments.

2.2.1

Peak Resolution

The *resolution* R_s of two substance zones recorded as a chromatogram is defined by the quotient of the peak apex distance (expressed by the difference in retention times) and the average width at the peak base w_b . It is calculated by 2.1 as follows:

$$R_s = \frac{t_{R2} - t_{R1}}{(w_1 + w_2)/2} \quad (2.1)$$

Resolution optimization is not equivalent to maximizing resolution, but is about finding the most appropriate resolution that is just sufficient for an error-free and robust peak integration to account for accurate quantitative results. The sketch of asymmetrical peak shapes shown in Figure 2.1b is more representative for a real-world chromatogram than peaks that follow a Gaussian distribution. Nevertheless, the Gaussian peak shape is the foundation for the calculation of plate numbers and plate height equivalents in chromatography. If two peaks exhibit ideal Gaussian shape and are of similar height, a resolution of $R_s = 1.5$, equivalent to a 6σ distance of the peaks (six times the standard deviation of the concentration distribution in each zone) is considered sufficient for quantitative analysis. Under these conditions, only 2% of the peak area overlaps with the respective adjacent peak. As real world chromatograms mostly exhibit less symmetrical peaks that can be of significantly different height, a minimum resolution of 1.8 is recommended for a robust method, and some work instructions even define an $R_s = 2.0$ requirement. It should also be noted that most USP methods suggest $R_s = 2.0$ as the minimum resolution. Statistics can prove though that any resolution increase beyond $R_s = 2.0$ will not further improve the accuracy of peak integration, but may negatively affect analysis times.

It is very helpful for any effective separation optimization in chromatography to know how the *resolution* R_s of two neighboring peaks depends on the three fundamental parameters. These are the *plate number* N , the *retention factor* k , and the *separation factor* α . Let us make sure we understand these parameters well.

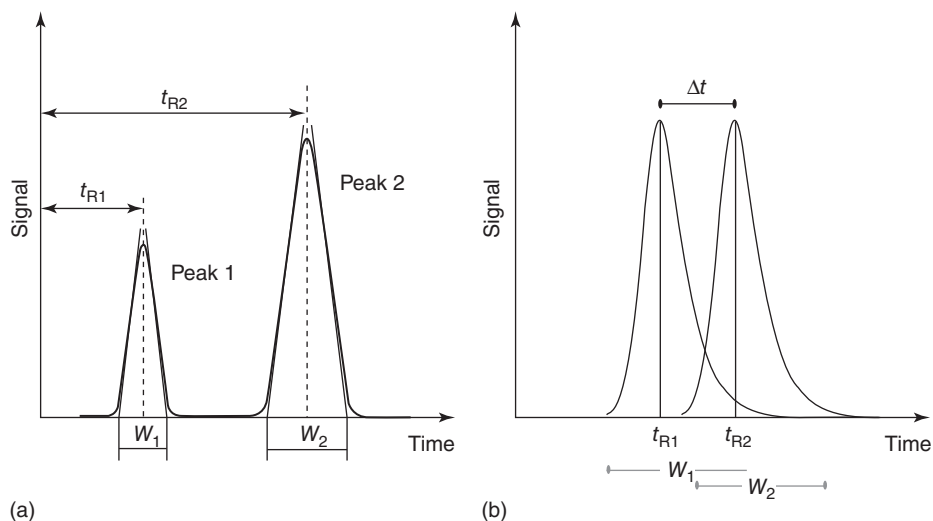


Figure 2.1 Schematic representation of ideally symmetrical (a) and real world asymmetrical (b) peak pairs and how the resolution is calculated.

The *plate number* N is a compound-specific measure (it, therefore, applies to each individual peak) for the separation efficiency of a column under clearly defined mobile phase and temperature conditions. It will change over the column lifetime and can also be influenced by the HPLC instrument. The plate number increases proportional to the column length L , provided all other conditions remain constant (except for the column pressure). It also increases at constant column length when the stationary phase particle size, particle architecture, or bonding chemistry is optimized in way that accounts for less band dispersion. The respective columns exhibit increased separation efficiency per unit column length. Once the plate number of a column or method is increased, one can separate more analytes or separate analytes with better resolution under otherwise constant conditions. The formula to calculate the plate number from the peak parameters retention time t_R and peak width at base w_b or peak width at half height w_h is as follows:

$$N = 16 \cdot \left(\frac{t_R}{w_b} \right)^2 = 5.54 \cdot \left(\frac{t_R}{w_h} \right)^2 \quad (2.2)$$

The *retention factor* k was defined as a retention parameter that is in contrast to the retention time t_R independent of column dimensions and the mobile phase flow rate F . It is characteristic for the given analyte under given stationary and mobile phase conditions as well as temperature. The retention factor can be derived from the peak retention time t_R and the column hold-up time t_M (elution time of a nonretained compound, the so-called inert marker) and equals the ratio of the so-called net retention time t_R' (difference of retention time and hold-up time) and the hold-up time:

$$k = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M} \quad (2.3)$$

Of highest importance for the separation of two compounds is their relative retention. This is commonly calculated for two neighboring peaks and is defined as the *separation factor* α , often referred to as selectivity α . The separation factor by definition is never negative, so the two considered peaks have to be assigned accordingly. The higher the separation factor α , the easier two compounds can be separated under otherwise constant conditions. If the goal is to characterize the method considering the relative retention of a compound pair, the parameter α is named the selectivity of the method for the respective substances. The selectivity of LC methods is the most important parameter when it comes to peak resolution optimization. It equals the ratio of the retention factors (or net retention times) of the two peaks and can thus be easily calculated as follows:

$$\alpha = \frac{t'_{R2}}{t'_{R1}} = \frac{k_2}{k_1} \quad (2.4)$$

Equation 2.5 shows how the peak resolution depends on these three parameters and can be viewed as the most important equation in chromatography. It considers the combination of the factors that drive the separation (retention and selectivity) and the counter-productive but inevitable band dispersion. The band dispersion in relation to the path length of the separation column is expressed as the column plate number N , where a high plate number accounts for low dispersion on a given column length.

$$R_S = \frac{1}{4} \cdot \sqrt{N_2} \cdot \frac{k_2}{1 + k_2} \cdot \frac{\alpha - 1}{\alpha} \quad (2.5)$$

The next section discusses how the variation of the three parameters affects the resulting resolution of adjacent peaks. This is expressed in the three overlaid curves in Figure 2.2. The respective ranges of the parameters (x -axis) as well as the constant value of the respective remaining two parameters is deliberately chosen to highlight the importance of each parameter appropriately and to reflect typical and realistic values of α , k , and N .

Thus, the constant values for each parameter are as follows:

- Separation factor = 1.05
- Retention factor = 5
- Efficiency = 5000.

The intersection of the three curves allows the constant values of all the parameters to be deduced.

The scale of the individual axes is chosen to accommodate the fullest range that could be anticipated for each of these parameters. It should be mentioned though that gradient elution is much more recommendable to separate complex mixtures rather than distributing them up to a retention factor of 25 in an isocratic run. Plate numbers up to 25 000 per column are demanding to achieve in conventional LC but readily available in modern UHPLC. The continuously strong slope of the

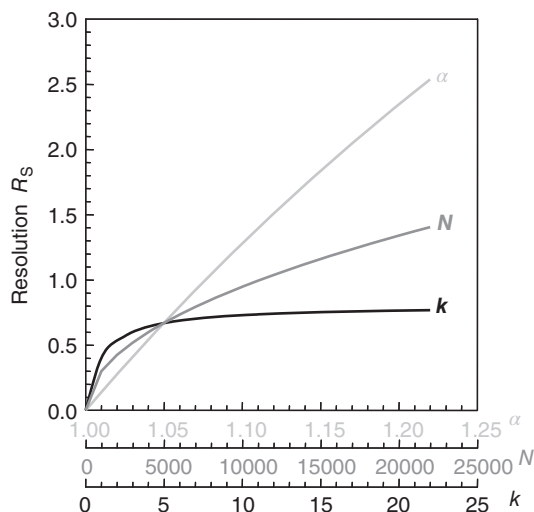


Figure 2.2 Curves to demonstrate the individual influence of the three parameters selectivity α , plate number N , and retention factor k on peak resolution. The constant value of the respective two remaining parameters can be read from the intersection point.

curve for the selectivity α over the relevant range up to 1.25 shows the importance and dynamics of this parameter. It should be mentioned that the selectivity curves also saturates above values of 4, but such high separation factors between two adjacent peaks are rare in practice and completely useless for productive chromatographic separations.

The retention factor can be well controlled in liquid chromatography. Beyond the stationary phase, it strongly depends on the composition of the mobile phase and the temperature. As a general rule, it should never be smaller unity ($k = 1$), as the range from 0 to 1 in real samples is often affected by matrix components. Moreover, the resulting resolution drops markedly below $k = 1$, and as can be seen in Figure 2.2 it already reaches 50% of its maximal contribution at this level. At $k = 2$, it rises to 67% and above $k = 5$ the increases of resolution is only marginal and can no longer justify the longer analysis time that comes with it.

The contribution of the plate number, N , on R_s is a square root function, resulting in continuously decreasing slope without saturation. Doubling the plate number always increases the resolution by factor 1.4.

An important fact becomes very obvious from Figure 2.2: The most effective parameter for resolution optimization in LC is selectivity. This consideration becomes even more important in relation to gas chromatography (GC). Based on the much stronger intermolecular forces in liquid phases, it is easier to generate differences in retention and a multitude of parameters based on mobile phase composition enable control this. In GC, there is a completely different situation as temperature is the only parameter to influence retention once the stationary phase is defined. To compensate for smaller selectivities GC column

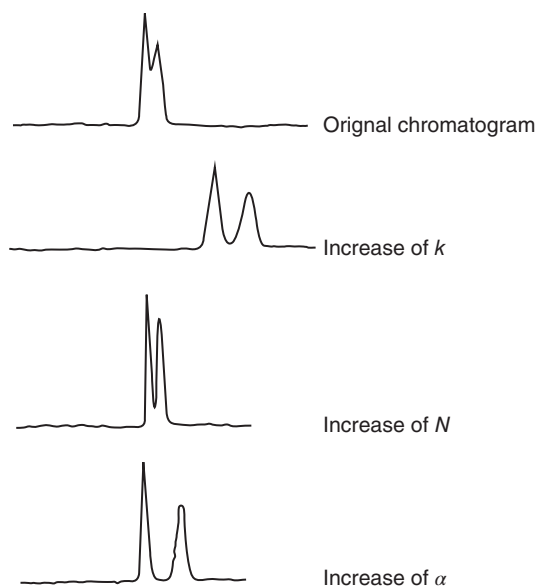


Figure 2.3 Influence of a change in k , N , and α on the chromatogram for a critical peak pair.

can achieve impressive plate numbers. Even in conventional capillary GC with 250 μm diameter columns, plate numbers of 200 000 are readily accessible, thanks to column lengths of up to 100 m. This is different in conventional HPLC where it is even challenging to exceed plate numbers of 10 000 in methods with equivalent analysis times. Modern UHPLC with coupled column chains can do this up to plate numbers of 100 000. This is once again easily outperformed by modern GC with 100 μm diameter thin-layer columns run at temperatures above 300 °C. Those can easily generate 1 000 000 theoretical plates.

The conclusion is straightforward, but of paramount importance: GC separates primarily through plates, LC primarily through selectivity. This finding points to the importance of understanding the basics of selectivity optimization very well. It implies both an excellent knowledge of the multitude of stationary phases as well as the influences of mobile phase composition.

The influence of the three variables k , N , and α on the look of a critical peak pair in a chromatogram is illustrated in Figure 2.3. To close this section, we summarize the importance of these parameters, weigh them against each other, and discuss what to do when.

- 1) The selectivity α is the most important parameter of the three and should always be addressed first to optimize resolution. It describes the difference in interaction of two analytes with a stationary phase under given eluting conditions (mobile phase and temperature). A certain difference in the interaction is a fundamental prerequisite for a chromatographic separation. It can

- be influenced by many parameters, but there are hardly any rules for a systematic optimization with guaranteed success. Users mostly optimize selectivity based on experience gained through previous trial and error approaches; however, software-assisted optimization workflows for this purpose are increasing in popularity. The latter are sometimes applied in a systematic design of experiment (DoE) approach to account for quality by design (QbD).
- 2) If the user fails to find conditions to improve selectivity for critical pairs, the increase of plate number N provides the best alternative strategy for optimizing resolution. The advantage of this approach is a clear prediction of the required boost in N to achieve a given resolution under constant selectivity, so the optimization can be made very systematically. The prerequisite is, however, the availability of a column with better efficiency (normally through a smaller particle size) but equivalent selectivity. The most straightforward way would be to increase the column length, but this also increases analysis time. In any case, will the optimization of N imply the investment for another column?
 - 3) The last choice to optimize R_s is the (nonselective) increase of interaction of both analytes. This is mostly the application of a mobile phase with weaker elution strength that directly leads to increase of the retention factors k_1 and k_2 . This ranking is based on the assumption that the retention factor for the first eluting peak is larger than 1 in the original method with insufficient resolution. The increase in retention is always accompanied by an increase in analysis time and above $k = 3$ it is only of marginal impact on resolution.

It is important to note that all the above-mentioned rules and calculations are strictly limited to isocratic methods. In gradient elution, the parameters k , α , and N do not follow the given definitions, and the slope and shape of the gradient is of critical importance for peak shape and resolution. The fundamental considerations still apply, but modified parameters need to be taken into account for the mathematical description (also see Chapter 3 on gradient elution).

2.2.2

Optimization of Efficiency (The Kinetic Approach)

This section teaches us that the so-called kinetic optimization is the key strategy to achieve a given resolution in a shorter time. It can be expressed in a quantitative way as the ratio of theoretical plates per unit time. It is, therefore, crucial to understand on the variables that affect the plate number, N . We first define the height equivalent to one theoretical plate (also called plate height), H , which is characteristic for a stationary phase, eluting condition, and analyte combination, but independent of the column length L . The plate height, H , can not only be easily calculated from plate number, N , and column length, L , but can also be determined directly from peak width and retention time:

$$H = \frac{L}{N} = \frac{L}{16} \cdot \left(\frac{w_b}{t_R} \right)^2 = \frac{L}{5.54} \left(\frac{w_h}{t_R} \right) \quad (2.6)$$

The plate height observed in modern UHPLC is in the single-digit micrometer range, and it corresponds to the length increment of one theoretical plate on the column in the direction of the mobile phase flow. The plate height is a good measure for band dispersion. Kinetic optimization is thus related to minimizing plate heights. The flow rate, F , is an important parameter in this optimization. In the early days of HPLC, this was mostly neglected, but the advent of UHPLC has emphasized its importance over the last decade. Increasing F directly increases the speed of analysis. If it was only about increasing F deliberately to make separations faster, things would be easy; however, this is not the case in reality. To understand the related implications, we will not consider the flow rate, but rather the linear velocity, u . This parameter is not so common for users, mainly because it cannot be set directly at the instrument. As it is independent of the column diameter and stationary phase properties, it is the more suitable measure for a straightforward discussion of kinetic optimization. The linear velocity, u , is the speed at which the liquid mobile phase travels along the column and is mostly given in millimeter per second. To calculate u from the set flow rate given in milliliter per minute, the following equation applies:

$$u = \frac{66.67 \cdot F}{d_C^2 \cdot \pi \cdot \epsilon_T} \quad (2.7)$$

The internal diameter d_C is inserted in millimeter, and ϵ_T is the total porosity of the interior of the separation column. It is a dimensionless ratio between the volumes occupied by the mobile and stationary phase inside the column and for modern columns packed with spherical, porous stationary phases has a value around 0.7. Another way to calculate u is based on the hold-up time simply by the quotient L/t_M . This is intuitive as t_M is the time that the nonretained analyte needs to travel the length L of the column.

The important relationship between the band dispersion (expressed as plate height H) and the linear velocity u is shown in Figure 2.4. It can be described by an equation that is mostly referred to as the van Deemter equation. The original van Deemter equation [2] was developed for GC with packed columns, but in this very general form it is valid for LC and GC to calculate H/u curves:

$$H = A + \frac{B}{u} + C \cdot u = 2\lambda dp + \frac{2\gamma D_m}{u} + \frac{\omega dp^2}{D_m} \cdot u \quad (2.8)$$

We will not need to discuss the factors λ , γ , and ω to a greater extent as they do not vary significantly, cannot be influenced by the user, and are not necessary for understanding the fundamental relationships. It is important to understand the meaning of the diffusion coefficient (or diffusivity) D_m and the stationary phase particle diameter dp . The diffusion coefficient describes the speed of diffusion of the analyte in the mobile phase (in this simplified approach both inside and outside the stationary phase pores). It depends on the molecular properties of the analyte, as well as on the temperature-dependent viscosity of the mobile phase. While the particle diameter is fixed with a given column, the diffusion coefficient can be altered without the need to change the column. It can change with the

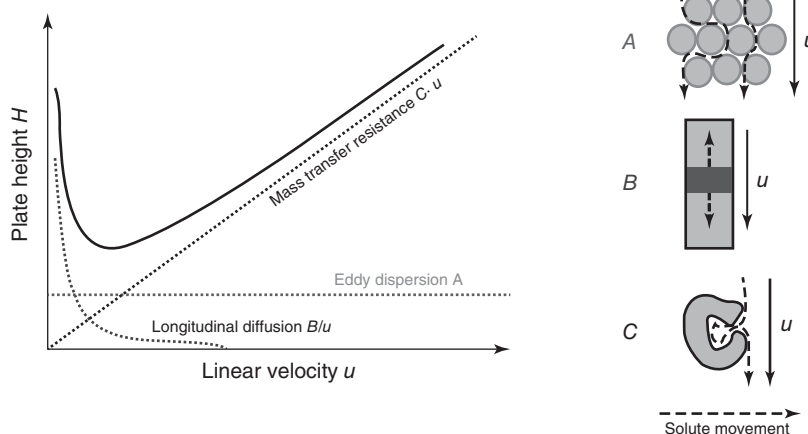


Figure 2.4 The three terms of the van Deemter equation expressed in individual curves and the fluidic explanation in related sketches.

mobile phase composition and the column temperature, because both parameters influence the mobile phase viscosity. There are more advanced versions of the van Deemter equation that distinguish between the diffusion inside and outside the stationary phase, but for the considerations made in this chapter, the simplified version as shown in 2.8 is sufficient.

The equation shows that the first term, A , is independent of the linear velocity u and grows proportional to the particle diameter dp . The term C grows proportional to the linear velocity u and even grows with the square of the particle diameter dp . The remaining term B is also independent of the particle diameter but is inversely proportional to the linear velocity; hence it decreases with increasing speed of the separation. The physicochemical background of the three terms of the equation is elucidated in Figure 2.4 through the sketches next to the three individual curves of the terms A , B , and C .

2.2.2.1 The Term Describing the Eddy Dispersion (A-Term)

The eddy dispersion term relates to the band broadening not only based on the different distances that analytes have to migrate around the particles, but also due to following a countercurrent movement in the eddy streams between the particles. It is often considered independent of the linear velocity and we also treat it like this, though there are other equations that consider a dependence on u with a power smaller than unity. The A -term grows with increasing particle diameter as the amount of length differences grow with that. It also depends on the packing technique and surface properties and is small when the particle size distribution is very narrow. All these effects are represented in the factor λ that is nevertheless close to unity.

In a nutshell: The A-term is small (good for small band dispersion) if the particle size is small, the particle size distribution is narrow and the packing structure is

homogeneous. All these conditions can only be controlled by the column manufacturer.

2.2.2.2 The Term Describing the Longitudinal Diffusion of Analyte Molecules (B-Term)

The *B*-term represents the band broadening due to the diffusion of the molecules in and against the direction of the mobile phase flow. This can be imagined quite easily as the broadening of a zone in the column after stopping the flow. In this scenario, the band would spread over time as the concentration of the analyte gradually equilibrates throughout the column. It is obviously a function of the time the analyte spends in the column; however, this is dominated by the time the analyte spends in the mobile phase as there is a much slower diffusion in the immobilized state on the surface of the stationary phase. The longitudinal diffusion is, therefore, inversely proportional to the linear velocity and thus disappears with very fast separations that do not leave the analyte sufficient time for this (diffusion in liquid phases is generally rather slow).

2.2.2.3 The Term Describing the Hindrance of Analyte Mass Transfer (C-Term)

To understand the hindrance of the transfer of analytes between the mobile phase and especially the porous stationary phase, it is important to note that there is practically no flow of the mobile phase through the pores of the packing material. This is because of the far higher viscous friction in the much smaller channels that exist in the pores compared with the channels between particles. In general, the difference in size is about 1000. The mobile phase in the pores is considered stagnant and not actively moving. This implies that the analyte molecule cannot be actively transferred into the pores by the pressure-driven flow of the mobile phase. At the same time, the stationary phase inside the pores is of paramount importance for the chromatographic retention, as by far the majority of stationary phase is situated there, and only a negligible portion is outside the pores at the outer particle surface. The pores are filled with mobile phase and there is continuous exchange of this liquid, but not by a mechanical-driven transport from the pressure gradient. The driving force for the exchange is diffusion, which is very dependent on the concentration gradient between the channel and the pore. This diffusion transfers the analyte molecules into the pores and hence onto the stationary phase. The diffusion is, of course, bi-directional. It first occurs toward the stationary phase at a distinct position in column where the moving analyte zone arrives, and as soon as the zone is moving further ahead, the concentration gradient is inverted and the direction of diffusion is oriented away from the stationary phase and out of the pores. This diffusion can be considered approximately radial in the column, hence orthogonal to the mobile phase movement and it is crucial that this mass transfer can keep pace with the speed of the mobile phase passing through the column. The more this diffusion lags behind the mobile phase flow, the further the analyte zone in the mobile phase will move ahead of the respective zone in the stationary phase and the more the overall zone is broadened. This is a very important effect in chromatography and is the reason for increased band dispersion (increase of plate height, *H*) when speeding up chromatographic separations by increasing the flow rate, *F*.

At this point, the stationary phase particle diameter is extremely important for the kinetic optimization of separations. A smaller particle diameter reduces the distance for the necessary radial diffusion of analyte molecules on the one hand, but increases the geometrical radial concentration gradient that drives the diffusion. Both effects are synergistic for an efficient analyte transport and this is the physicochemical foundation for the decrease of the C -term with the squared particle diameter (dp^2). This will be used effectively in the speed optimization strategy.

An important note to the user: both the B -term and the C -term depend on diffusion of the analyte in the mobile, as can be seen by the diffusion coefficient, D_m , represented in both terms. While the B -term decreases with the linear velocity, the C -term increases with it and this leads to a minimum in the resulting van Deemter curve where the separation is most efficient. If the mobile phase composition or column temperature is changed, the diffusion coefficient will change as well, resulting in a change to the linear velocity of the mobile phase required for the van Deemter minimum. In other words, the optimum linear velocity will change and has to be determined once again after changing eluting conditions.

Reducing the separation time is always about optimizing the C -term, since the B -term disappears at linear velocities, and hence reduced analysis times, due to increased flow rates. The following rules for the C -term have to be considered:

- The smaller the molecule, the faster it can diffuse and the greater the separation can be sped up.
- From a purely kinetic point of view, acetonitrile is always better than methanol as organic modifier for fast separations with good resolution due to its lower viscosity.
- About 50% (volume %) of methanol in water is typically the worst scenario for fast and efficient separations.
- Temperature increase always helps for a kinetic optimization of separations (it does, however, require prerequisites regarding the column thermostating and stationary phase temperature stability).

Nevertheless, there are more factors to be considered for reducing the analysis time:

- the pressure limit and sometimes also the maximum flow rate of the LC instrument at the required pressure
- efficiency loss due to thermal effects that are related to viscous heating (see Sections 2.3 and 3.2.2)
- retention mechanisms with slow kinetics or mixed retention mechanisms such as the combination of reversed phase and ion exchange. This is a complex matter and must be studied in more specialized literature [3].

2.2.3

The Influence of the Column Dimension

The dimensions of a separation column are defined by the column length L and the internal diameter d_c . Both determine the column volume V_C , the length having a

linear correlation with the volume, and the internal diameter having a square correlation to the column volume. At a given mobile and stationary phase, the solvent consumption is strictly proportional to the column volume V_C . In particular, the internal diameter of the column is a parameter that can help to save solvent and reduce the environmental footprint of a method. Reducing d_C from 4.6 to 2.1 mm reduces the solvent consumption by more than 80% through a reduction of the flow rate by the same factor. Reduction of the column length from 250 to 125 mm in a method optimization reduces the solvent consumption by 50%. The flow rate remains constant in this case, but the analysis time is reduced approximately to half (gradient methods require additional consideration of gradient time and system dwell volume). While the actual separation time is reduced to half, but it must be considered that the solvent consumption during the injection cycle still remains constant.

Beyond the solvent consumption, the volume of the column also has a strong influence on the dilution of the analyte in the column. All chromatographic processes imply a dilution of the sample zone as a prerequisite from thermodynamic considerations, because the overall entropy in a system cannot be reduced as a result of the separation. The entropy gain is eventually achieved by that dilution. With all other factors constant, the volume in which the analyte is dissolved when eluted from the column defines the dilution factor. This so-called peak volume is strictly proportional to the column volume. Therefore, more sample mass must be injected with increasing column volume in order to keep the concentration in the detector constant. This is critical for the limits of detection and quantification with concentration-dependent detectors such as a UV detector. This rule also implies a reduction of the column diameter is beneficial, if only a small amount of sample is available for the analysis. It must be noted though that any reduction in the column diameter increases the demands on the instrument. The instrument must be able to handle smaller flow rates and injection volumes accurately. In addition, all system volumes have to be reduced proportionally to the column volume reduction in order to keep the contribution of extra column band broadening to the same relative amount. If extra column band broadening in the instrument becomes dominant, it will destroy the chromatographic separation delivered by the column.

For method transfer, the following rule is of great practical relevance: with any change in column diameters at constant stationary and mobile phase and temperature, the linear velocity u must always be kept constant through a respective adaptation of the flow rate F according to Eq. 2.7. A reduction of the column diameter from 4.6 to 2.1 mm implies a reduction in the flow rate to 0.2 ml/min (exactly 0.208 ml/min) if the original flow rate was 1 ml/min. This reduces the solvent consumption and the environmental footprint, but also improves the compatibility of the method with electrospray ionization mass spectrometry (ESI-MS). This is discussed in more detail in Sections 1.1 and 1.2 on LC/MS coupling.

Another important consideration is the dependence of the radial temperature distribution in a column as a function of the internal diameter. A smaller diameter can also be advantageous in this regard. This can both relate to a proper preheating

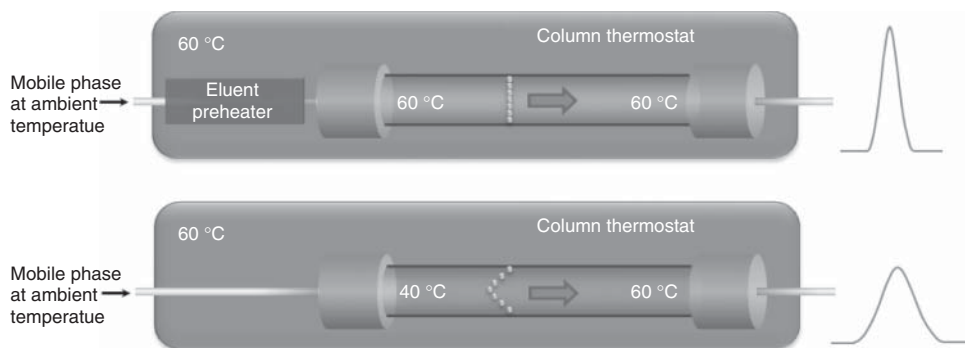


Figure 2.5 Band broadening effect of radial temperature inhomogeneity as a result of insufficient mobile phase preheating. The spheres represent the analyte zone.

of the mobile phase especially for methods at higher temperatures, as well as for the effects related to the dissipation of frictional heat in the column, especially in UHPLC. A homogeneous radial temperature distribution is a crucial prerequisite for optimal chromatographic performance. Any deviation from a constant radial temperature profile leads to peak distortion. This is because a temperature increase results in increased linear velocity under a given pressure gradient due to lower viscosity and generally also in reduced analyte retention. Both effects lead to a faster flow of the analyte in the hotter part of the column. The effect is called a thermal mismatch and can reduce plate numbers in 4.6 mm columns to less than half the original efficiency. The smaller the column diameter the less pronounced is this effect.

Figure 2.5 illustrates the effect of a radial temperature gradient. The given scenario is the insufficient mobile phase preheating due to absence of a precolumn heat exchanger. This leads to lower temperature in the column center, at least in the first part of the column and consequently slower movement of the analyte molecules there. Overall, this will significantly broaden the peak when compared with proper prethermostating, as can be seen in the sketch.

If an increased temperature in the center of the column is induced through frictional heating in UHPLC, the analyte molecules will move faster in the column center, but the negative impact on the peak shape is similar. This is another reason, why UHPLC columns normally have reduced internal diameters, as this helps to minimize thermal mismatch effects.

This consideration on frictional heat effects is only applicable in this simple form, if the thermostating of the column follows the so-called isothermal concept, in other words the thermostat attempts to keep the column at a defined temperature by removing the frictional heat. The larger the column diameter the more difficult it is to complete heat dissipation. Alternative to the removal of frictional heat is the adiabatic column thermostating [4] where in the ideal case the column would be thermally insulated and all frictional heat remains in the column. Thermostats without a fan for heat circulation come closer to this adiabatic mode

of operation and are relatively common in UHPLC instruments. They can reduce the thermal mismatch from frictional heating significantly, but at the same time they allow the mobile phase to be heated as it passes from the column inlet to the column outlet. As a consequence, the column temperature will be longitudinally inhomogeneous and any increase in flow rate will further increase this effect. The consequence will be an alteration of retention as a function of the flow rate and the user will no longer be able to control the effective temperature inside the column.

2.3

Methodology of Optimization

Section 2.1 covered the important HPLC fundamentals together with some rather basic concepts for the chromatography practitioner. This section on systematic methodology provides detailed knowledge to effectively and systematically translate this into practice. The core goals for optimization as introduced in Section 2.1 are considered. Separation speed and resolution can both be a target of kinetic optimization, and it is not always reasonable to consider them strictly independent. The outline thus predominantly follows the individual criteria or parameters, for example, the temperature and its multiple influences. Two of the core goals are addressed in dedicated sections though. These are the systematic speed optimization at constant resolution (Section 2.3.4.1) and the limits of detection and quantification (Section 2.3.7). The closing Section 2.3.8 provides a summary of all the rules and formula that have been discussed in all forgoing sections. We initially start with a brief discussion on how to optimize selectivity in LC.

2.3.1

How to Optimize Selectivity

We discussed in Section 2.2.1 the parameters that influence peak resolution in chromatography and learned about the importance of the selectivity α . Before going into further detail, we need to distinguish between fundamental method development and the optimization of existing methods. The goal of method development is to generate a functioning method, fit for an analytical purpose. Optimization takes a functioning method as basis and improves it for defined criteria as discussed in Section 2.1. Successful method development thus implies that a defined peak resolution was achieved, and this again implies respective selectivity has already achieved. A prerequisite for any chromatographic optimization is the investigation of the parameters of the existing method regarding selectivity, retention factors, and plate numbers. Based on the related findings, the further strategic optimization can be defined, for example, is increasing the selectivity an appropriate action (and usually it is). An important part of method development is the proper selection of the column and a good understanding of selectivity optimization through changes of the eluting conditions on this specific column. These aspects are discussed in more detail in Chapter 4 on modern HPLC columns.

2.3.1.1 The Role of Selectivity in Practical Method Optimization

A poor separation method is mostly caused by poor peak resolution or an uneven distribution of the peaks across the chromatogram. The latter issue is always caused by the selectivity, with the cause of the former also very often by this; however, there are also other parameters that can affect poor peak resolution. To obtain clarity on this and to express the characteristics of a method quantitatively, the user should first calculate the parameters plate number, N , selectivity, α , of selected peak pairs and retention factor, k , in particular for the first and the last peaks in the chromatogram. The related formulas for this calculation have been introduced previously in Section 2.2.1. Special emphasis should always be given to peak pairs, where the resolution does not meet the discussed requirements.

We now study an example, where the resolution of a peak pair in the original method of a reversed phase separation is only $R_S = 0.76$. The parameter analysis yields the results shown in Table 2.1. A calculated plate number of $N = 9000$ meets the expectations for a 150 mm long column packed with 5 μm particles well, and the second peak exhibits a very reasonable retention factor of $k_2 = 2.0$. The poor separation factor $\alpha = 1.05$ between the two peaks is the obvious reason for the imperfect resolution and hence the method lacks selectivity to separate these two compounds. One could now reduce the organic content of the mobile phase by 10% and expect an increase in retention of factor 2. This would increase the analysis time by 67% (it is proportional to $1 + k$), but the resolution would only increase to $R = 0.98$ and still be insufficient. Further extrapolation shows that no increase of retention without a change in plate number or selectivity could bring the resolution in the above-mentioned example $R_S = 1.1$.

Alternatively, the plate number could be increased to $N = 15\,000$ by changing to a column length of $L = 250$ mm packed with the identical stationary phase and keeping the eluting conditions constant. Coupled with the effort and investment of organizing such a column, this would also increase the analysis time (which is proportional to the column length L) by 67%, but once again lead to insufficient resolution of $R_S = 0.97$. Could we, however, increase the selectivity from 1.05 to 1.10 and keep the retention of the second peak in the order of $k_2 = 2$, the

Table 2.1 Example case study for resolution optimization by changing the individual parameters that control selectivity.

	N	k_2	α	R_S
Original method	9 000	2	1.05	0.76
Increase of k_2	9 000	4	1.05	0.98
Increase of N	15 000	2	1.05	0.97
Increase of α	9 000	2	1.10	1.45
	9 000	2	1.15	2.06
Combined improvements	15 000	2	1.10	1.87
	18 000	2	1.10	2.05
	15 000	2.5	1.10	2.00

resolution would almost double to $R_S = 1.45$ without a significant impact on analysis time. This resolution would still fail to meet the requirement of $R_S \approx 2$ for a robust routine method. A further increase of the selectivity to $\alpha = 1.15$ would, however, perfectly meet this resolution requirement, as can be seen in Table 2.1.

This discussion has demonstrated the importance of analyzing the parameters of a separation thoroughly and it showed the tremendous effect of improving the selectivity, α . The challenge with this finding is that unlike for the plate number, N , and the retention factor, k , there is no general recipe on how to improve the selectivity for a distinct peak pair. Moreover, it is virtually impossible in practice to change the selectivity without affecting the plate number or retention time (as was assumed in the given simulated example mentioned earlier). Achieving the described increase from $\alpha = 1.05$ to 1.15 is very difficult just by empirically changing the mobile phase conditions, temperature, or swapping to another RP column. Therefore, it often helps in practice to strive for a simultaneous optimization of N , k , and α , to finally obtain the required resolution. This is also demonstrated in Table 2.1. If the selectivity cannot be increased above $\alpha = 1.10$, we can first discuss increasing the plate number with the column length. A value of $N = 15\,000$ from $L = 250$ mm would yield $R_S = 1.87$, which is still below the required value. Increasing to $N = 18\,000$ would generate $R_S = 2.05$, but requires a column length of $L = 300$ mm. Finally, the combination of $\alpha = 1.10$, $N = 15\,000$, and $k_2 = 2.5$ paves the way to the target of $R_S = 2$. Comparing this method to the original poorly resolving method, it can be seen that the analysis time has almost doubled, resulting in a doubling of the consumption of mobile phase. Reducing the retention of the first peak in the critical peak pair sufficiently to obtain $\alpha = 1.15$ without changing anything else, would have resulted in a $R_S = 2$ without any additional analysis time, without any additional eluent consumption, and without any increased pressure due to a longer column.

It cannot be stressed enough that selectivity is the key in liquid chromatography, but there is no simple rule available how to improve it. The following section gives us some insights into selectivity control and should help the user to address this challenge more systematically.

2.3.1.2 How to Control Selectivity in HPLC?

To address this essential question, the triangle of interactions in liquid chromatography has to be initially investigated. While the mobile phase in GC has only the function to transport the analyte through the column without undergoing any interactions, the situation in LC is far more complex. The interactions between analyte molecules and the stationary phase (same as in GC) have to be considered, as well as the interactions between the mobile phase molecules and the stationary phase. In a simple model, we can assume that mobile phase molecules and analyte molecules compete for spaces on the stationary phase. This competition is expressed in thermodynamic equilibria and thus also controlled by the temperature. Last but not least, there is also the interaction between analyte molecules and mobile phase molecules to be taken into consideration.

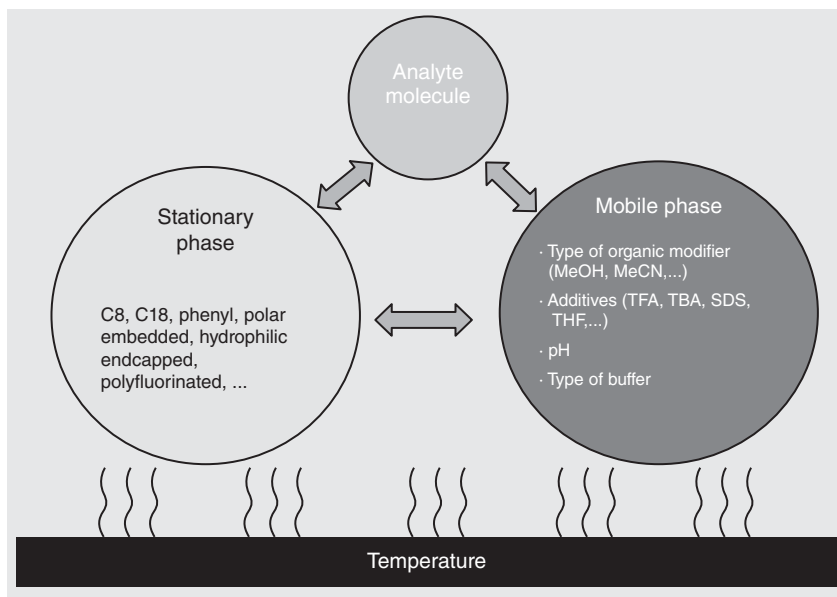


Figure 2.6 The temperature dependent triangle of interaction in liquid chromatography and some options to vary stationary and mobile phase in RP chromatography.

Figure 2.6 summarizes the complex situation that exists between the different interactions that can exist in liquid chromatography. It is necessary to discuss the individual variables further to ensure that the user can make predictions on required changes to achieve targeted selectivities. However, Figure 2.6 does provide a good overview that can also be considered a check list for options. There are some rules of thumb to predict retention changes based on modifying temperature, mobile phase composition, or stationary phase chemistry, and most of them are summarized in Table 2.10 in Section 2.3.8.2. Unfortunately, there is no predictive formula that is required for determining selectivity, which would allow for simple rules of thumb to be available to aid the chromatographer. Even current levels of knowledge on physicochemical interactions in LC and all the listed physicochemical properties of compounds does not allow for a theoretical prediction of compound retention or selectivity. Practical improvement is thus based on empirical approaches that can nevertheless be executed in a very systematic way by DoE strategies. It is a hot topic to achieve method QbD and can be further studied in the respective literature [5].

Experienced HPLC users with a reasonable chemical knowledge will have individual strategies for selectivity improvement. These are commonly based on assessment of the relative changes of molecular interactions between analytes and the surface of the stationary phase, as well as analytes and mobile phase components. All possible physicochemical interactions such as London dispersion forces, dipole interaction, hydrogen bonds, coulomb interaction, π -electron interactions, or complex formation are present in LC. The order given relates

to the use by separation scientists and not by the strength of the respective interactions. The following very basic rules are helpful to make some predictions:

- increasing the interaction between analyte molecules and stationary phase surface increases retention
- increasing the molecular interaction between mobile phase components and stationary phase surface decreases retention
- increasing the interaction between analyte molecules and mobile phase components decreases retention.

It is also important to understand, that retention in RP-LC is never solely based on a pure hydrophobic or dispersion interaction, but always influenced by secondary retention mechanisms. These secondary mechanisms can be both of wanted or of an unwanted nature. A good understanding of these relationships is extremely helpful for selectivity optimization and the fundamentals are discussed in more detail in Chapter 4 on modern HPLC columns.

Some basic rules are as follows:

- Retention of neutral molecules is almost not affected by buffers and mobile phase pH. Optimization options are limited to stationary phase properties and mobile phase solvent composition.
- Mobile phase pH is a very effective optimization parameter for analyte molecules that exhibit a pH-dependent net charge, such as acids and bases. Beyond the pH, the type of buffer and the column temperature is of great relevance for selectivity of such analytes. The use of ion-pair-forming additives is also often helpful.
- RP-HPLC notoriously struggles when it comes to separation of positional isomers, while it is extremely potent to separate alkyl homologs. If the position of a polar group in the molecule distinguishes two analytes, it is helpful to use polar modified RP materials or mobile phase additives that adsorb to the stationary phase and induce polar interactions.
- The protic organic modifier *methanol* is superior for selectivity tuning if acidic analytes (proton donors) are separated on stationary phases with proton acceptor capabilities (N-containing functional groups).

Before we discuss the influence of pH, additives, and temperature in more detail, we need to understand another important factor for optimization of peak resolution. Peak resolution is affected by the difference in retention time between peaks and the peak shape. We have discussed the intrinsic effect of band dispersion in chromatography, which leads to a symmetrical broadening of peaks. This peak width is described in a standardized way by the plate number. In practical LC, however, perfectly symmetric peaks are rather the exception, and it is an important criterion of method optimization to remove root causes for peak distortion. Every increase in peak asymmetry negatively affects resolution (under otherwise constant conditions). It must be emphasized in this context that Eq. 2.1 in Section 2.2.1 is only valid for Gaussian peaks with perfect symmetry, while any peak distortion will lead to smaller effective resolution. There are cases where the

removal of asymmetry is of greater impact than the increase of selectivity. There are many reasons for peak asymmetry and are beyond the focus of this textbook. Most of the considerations above are also effective to influence peak asymmetry. Peak asymmetry is often related to the influence of secondary interactions (those of unwanted nature) and altering the experimental conditions normally changes the extent of those effects.

Selectivity Control by Mobile Phase pH and Column Temperature These two parameters are of major importance for selectivity control in HPLC, especially with acidic and/or basic analytes. The charge state of analyte molecules influences both their hydrophobic (dispersion type) interaction, as well as possible ionic secondary interactions (e.g., with dissociated residual silanol group on silica-based stationary phases). The relatively wide range of so-called mixed-mode phases make very effective use of ionic interactions, but specialty phases are outside the scope of this chapter (refer to Chapter 4 for more information).

For silica-based reversed-phase materials without any special modifications, the following rules apply:

- The more an acid is protonated and the more a base is deprotonated, the stronger is the hydrophobic retention, because neutral molecule exhibit stronger dispersion interactions than charged molecules. This is schematically represented in Figure 2.7.
- Amphoteric substances show the highest retention at a pH that equals their isoelectric point (pI).
- At neutral pH, basic analytes can experience ionic interaction with dissociated residual silanol groups and thus additional retention. At $pH < 3$ some stationary phases show ion exclusion effects with basic analytes that can even lead to elution before t_M .

The situation described in the third bullet is often accompanied by peak asymmetry. More advice on this can be found in Chapter 4.

The curves shown in Figure 2.7 demonstrate the sensitivity that acidic and basic compounds have with respect to the pH, with neutral compounds being

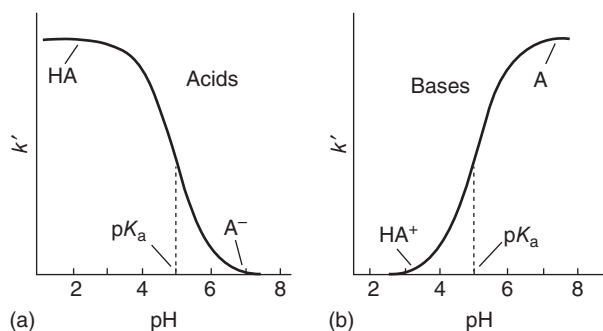


Figure 2.7 RP Retention of acids and bases as a function of the mobile phase pH. Reproduced from Snyder *et al.* [3] with permission of John Wiley & Sons.

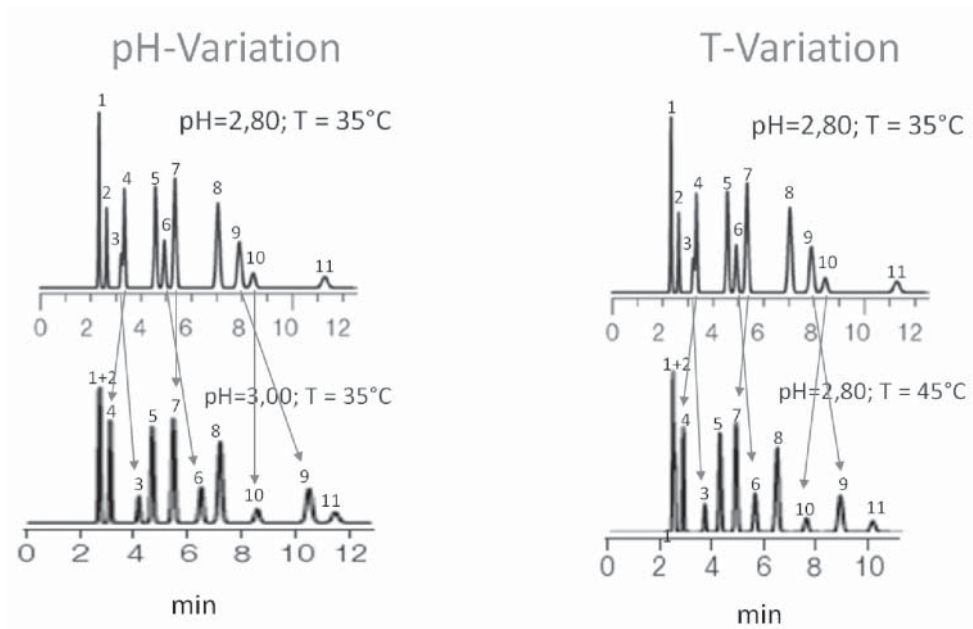


Figure 2.8 Similar influence of pH and temperature change on the selectivity of a separation of acids and bases. Column: silica based C18, mobile phase: phosphate buffer/ACN 50/50 v/v, peaks: (1) 2-phenylpyridine, (2) ketoprofen, (3)

4-*n*-pentylaniline, (4) diflunisal, (5) 4-*n*-butylbenzoic acid, (6) 4-*n*-hexylanilin, (7) diclophenac acid, (8) 4-*n*-pentylbenzoic acid, (9) 4-*n*-heptylaniline, (10) mefenamic acid, (11) 4-*n*-hexylbenzoic acid. From Dolan [7].

unaffected by alterations in the pH. This is the foundation of a very effective individual change of retention as a function of pH and thus a change in selectivity.

Thermodynamics teaches us that the state of equilibrium depends on the temperature, and this also applies to the dissociation of acids or protonated bases. In other words, their pK_a values are temperature dependent. Therefore, the temperature also affects the absolute position of the curves shown in Figure 2.7, this is without considering the direct influence on the effective pH or the pH^* in a mixed aqueous–organic medium that temperature has. The physicochemical fundamentals on this can be studied elsewhere [6], but are not necessary to understand the relationships discussed here. Figure 2.8 demonstrates the very dramatic effect that both temperature and pH can have on selectivity. This figure shows chromatograms of a mixture of weakly basic and weakly acidic compounds that elute close to each other with crossover of acids and bases. As the pH affects both the charge state of the analytes as well as that of the silica-based stationary phase, we do not discuss the root causes of individual retention changes in any further detail. It is only important to note that the change from pH = 2.8 to 3.0 at constant temperature of 35 °C has almost the same influence as the change of temperature from 35 to 45 °C at constant pH. This example was carefully picked by the

author Dolan [7] and is certainly a special case, but it impressively demonstrates the rules described above. It is not feasible to draw any general conclusions for any quantitative predictions from this example, but it does provide a qualitative rule regarding the effect that temperature and pH can have on chromatographic retention. Both the increase of pH and the temperature enforce the dissociation of acids. This makes bases more neutral and acids more charged and thus changes retention in opposite directions between the two groups.

If selectivity tuning of certain separations is possible by changing pH or changing temperature, the question arises what is the preferred way. It is obviously more convenient to change the temperature. This can be easily set at the instrument and does not require further mobile phase preparation work. Moreover, there is limited flexibility for pH changes with a given buffer type, especially if the buffer capacity has to be maintained. These considerations clearly promote the temperature as the first choice, and this optimization experiment can easily be automated. In contrast, it requires much more effort and a special instrumental setup for pH optimization.

One warning should be given though. The changes in pH and temperature have a tremendous influence on selectivity, due to the operation of the separation in the steep part of the curves in Figure 2.7. This very effective change in selectivity by minor modifications in pH and temperature implies that the methods may not be overly robust with respect to these two parameters. This should not prevent the user from making use of these effects, but it is always recommended to search for a pH and/or temperature where none of the analyte compounds is in the steep range of the curve, which occurs when the pK_a is relatively close to the effective pH. This should be verified and if such conditions are not among those which provide the favorable selectivity, the method must be questioned. Robustness is a very important quality criterion of any routine analysis method.

Selectivity Control by Mobile Phase Additives It is first necessary to clarify what is an additive or answer the question if a buffer compound qualifies as additive or not. There is no general definition for this in place but it will be assumed that those compounds that directly influence the triangle of interaction are considered as additives (see Figure 2.6) by introducing additional intramolecular forces that would not exist in their absence. As the primary role of a buffer is to stabilize the mobile phase pH, buffers normally would not belong in this category. This can be challenged though as there is hardly any buffer type, that does not interact with either the stationary phase or the analyte directly, beyond its buffering role. If this was not the case, it would not matter if a distinct pH is either adjusted by phosphate or by citrate. Every experienced HPLC user knows that the type of buffer employed can affect the separation. While the phosphate buffer undergoes strong interactions with silica-based stationary phases, citrate is a complexing agent that can interact specifically with certain analytes. Moreover, this change could not easily be executed at constant ionic strength without significantly affecting the buffer capacity. These considerations are equally important as almost every buffer sustainably modifies a stationary phase. For this reason, care has to be taken when

using nonbuffered mobile phases as the stationary phase will behave very different dependent on whether the column was new or has been used extensively in another buffer system.

The true mobile phase additives can be subdivided into the following categories:

- ion-pair-forming additives
- additives that induce a polar modification of the RP material
- silanol group masking additives
- complex-forming additives
- chaotropic additives.

Ion-pair-forming additives are the most common representatives of this list, even though their importance decreased with the introduction of modern high-purity silica-based RP materials. In the early days of RP chromatography, it was quite difficult to analyze acids or bases with reasonable peak shape and retention without the addition of ion-pair reagents to form additives. Essentially, all cationic surfactants (e.g., tetrabutylammonium chloride or the respective hydrogen sulfate) are ion-pair formers for anionic compounds or dissociated acids. Anionic surfactants (e.g., sodium dodecylsulfate) are ion-pair reagents for cations or protonated bases. There are several retention mechanisms in discussion which range from a true ion pair formed in the organic-rich layer that solvates the alkyl chains of the RP material to a dynamic ion exchange mechanism after hydrophobic immobilization of the surfactant molecule to the stationary phase surface. It is not of practical relevance what mechanism really applies and will not be further discussed here. Of relevance is, however, that the surfactant concentration does not exceed the critical micellar concentration, as this would induce other more complicate mechanisms and would not necessarily improve the separation. It is also important to note that surfactants can never be completely removed again from an RP material, so the respective column should not be used for other any other methods subsequent to its use with a surfactant. Another reason why most ion-pair reagents are not so prominent anymore is their poor compatibility with LC/MS due to their low volatility. Special notice should be given to trifluoroacetic acid (TFA). This is a strongly acidic modifier on the one hand, but the trifluoro acetate also acts as an ion-pair reagent for cationic analytes. This is used to strongly retain very polar basic or amphoteric compounds in RP chromatography. It is a very common way to achieve effective peptide separations with good peak shape and good retention of the very polar peptides. At the same time, TFA causes many baseline issues in UV detection due its self-absorbance and can cause significant ion suppression issues with ESI-MS detection.

All neutral substances with surfactant properties can be considered polar-modifying additives. They can be retained on the RP material with their nonpolar part of the molecule while the polar end can induces respective secondary interactions with polar analytes and also enhance the solvation (wetting) of the stationary phase at very low organic content of the mobile phase. A common practice was not to use typical neutral surfactants but simply tetrahydrofurane

(THF) up to a maximum content of 5% in the mobile phase. This is adsorbed to the stationary phase and induces polar interactions as well as hydrogen bond acceptor behavior. This modification can be very effective for the separation of positional isomers in RP-HPLC. Due to its strong UV absorbance up to a wavelength of 240 nm, THF cannot be used for gradient elution though. It is also very aggressive to most plastic surfaces of the standard HPLC instrumentation. The introduction of RP materials that contain polar groups means that THF is fortunately rarely needed in modern HPLC anymore.

Another type of additives are those that are masking silanol groups and these tend to be strong bases, for example, *n*-butylamine. Even at contents below 1% it can almost completely suppress the interaction of basic analytes with residual silanols. Although they enable the generation of symmetrical peaks obtained with strong bases on classical RP materials, they are not overly popular due to their bad smell and the aggressive behavior toward the silica backbone of the stationary phase. Another issue is that these compounds are highly retentive and subsequently can hardly be removed from a column. Modern RP materials on ultrapure silica with very effective endcapping no longer require such masking agents and even for extremely strong bases as analytes, the phases with polar groups embedded in the alkyl chain can also be very effective at maintaining a good peak shape.

Complexing additives play a dominant role in the chromatographic analysis of permanent cations, so-called ion chromatography. They can very specifically control the charge state of certain ions and thus the ion exchange interaction with the stationary phase. In RP chromatography, they are mainly used for indirect effects, for example, to mask residual cations in the mobile phase. Some analytes undergo such strong complexing interactions with alkali or alkaline earth cations that even trace amounts in the mobile phase can affect the retention and peak shape. The addition of strong complexing agents such as ethylenediaminetetraacetic acid (EDTA) can effectively mask such cations in the mobile phase. A practical example is the addition of less than 0.1% EDTA to the mobile phase for the analysis of isohumulones with good peak shapes [8]. Isohumulones are a very important ingredient in beer that contributes to the bitter taste.

Chaotropic additives are used to break intermolecular hydrogen bonds in macromolecules and thus strongly influence their chromatographic behavior. They can change the tertiary structure of proteins in solution, and have a similar denaturing effect to acetonitrile or methanol. This property can very effectively be used in dedicated separation systems but their relevance is yet very limited and restricted to specific applications. More information can be found in dedicated literature [9].

2.3.2

The Role of Temperature in HPLC

We have seen in the previous sections that retention in chromatography follows the rules of equilibrium (or reversible) thermodynamics. This is the foundation for the very important effect of temperature on retention and sometimes also

on selectivity. The theoretical background on how effectively a separation can be achieved in the shortest time is described by the kinetics of chromatography. This is mainly about the influence of the temperature on molecular diffusion as described by the van Deemter theory (Section 2.2.2). Increasing the temperature speeds up diffusion, which is a prerequisite for good peak efficiency at elevated linear velocity of the mobile phase. This makes temperature in general an important variable in HPLC optimization, which is unfortunately often neglected, but sometimes also overrated. We discuss this in the following sections in greater detail.

2.3.2.1 Retention and Selectivity Control via Temperature: Possibilities and Limitations

The general influence of temperature on chromatographic retention can be explained as follows. In order to be retained on the stationary phase, molecules need to transform from a state of very little order while floating in the mobile phase to a much more ordered immobilized state in the stationary phase. This is a process that implies a reduction in entropy. In order to make retention still an energetically favorable process, thermodynamics defines that it must release heat. Thus, retention in chromatography is typically an exothermic process. If the temperature is increased, exothermic processes escape from this constraint by shifting the equilibrium to the original side. This implies that temperature increase is accompanied by a shift to the desorbed state of the molecules and thus a lower retention. There are some rare exemptions to this rule (as always), but those are based on secondary equilibria that overrule the effect described earlier.

We first review a separation that shows several interesting temperature effects. It is indeed a column test developed by Neue [10] that is also presented in Chapter 4 and generally referred to as the Waters Test. The original protocol defines a column temperature of 23 °C, which already points to a certain relevance of proper column thermostating. Therefore, the temperature dependence of this separation will be investigated. The respective chromatograms of the separations recorded at 21, 30, and 40 °C are overlaid in Figure 2.6. Next to the observation of a general decrease in retention with increasing temperature, there is a very obvious change in selectivity. This shift of relative retention leads to a very special chromatogram at 30 °C. On the stationary phase applied in this example, we observed a complete co-elution of both the peak pair of butylparabene (3) and dipropylphthalate (4), as well as acenaphthene (6) and amitriptyline (7). Operating at this temperature would lead to the false assumption that only five components rather than seven are present in the mixture. The obvious conclusion from this observation is that there is a change in the elution order with two peak pairs within the investigated temperature range. Moreover, the peak of butylparabene (3) comes very close to that of propranolol (2) at the highest temperature. From a method optimization perspective, one can conclude that the separation at 40 °C is the best of the three. All peaks are sufficiently resolved, but they are more evenly distributed across the chromatogram than at 21 °C, and the separation is 5 min (or 20%) faster than at the lowest temperature. Based on this observation, two interesting questions arise. How can these changes in selectivity be explained and how can temperature optimization be performed in a truly systematic way? To find answers to both these

questions, it is necessary to take a closer look into the molecular structure of the test compounds and to investigate the relationship of retention on temperature in a quantitative way. The latter scenario will initially be discussed.

Retention in chromatography is controlled by thermodynamic equilibria. The partition of the analyte between the mobile and the stationary phase is in control of the retention factor. This partition can be described by the laws of reversible thermodynamics. Therefore, we also borrow the thermodynamic description of the temperature dependence of equilibria. This is the so-called van't Hoff equation, which is the quantitative expression of the Le Chatelier principle. According to this, the temperature dependence of the retention factor k can be described by 2.9, with R being the general gas constant, ΔH^0 the molar enthalpy (heat tone) related to the transition of the analyte from mobile to stationary phase, ΔS^0 the molar entropy change for this transition, and β the so-called phase ratio of the packed stationary phase in the column.

$$\ln k = \frac{-\Delta H^0}{R} \cdot 1/T + \frac{\Delta S^0}{R} + \ln \beta \quad (2.9)$$

While this formula may appear slightly daunting at the first glance, it simply expresses that there is a linear relationship between the natural logarithm of the retention factor and the inverse absolute temperature (measured in K). This simple relationship is very powerful for temperature-based selectivity optimization and is the origin of the van't Hoff plot depicted in Figure 2.10. The plot contains the retention data at 25 and 35 °C in addition to the data from the three chromatograms in Figure 2.9.

A further interpretation of the respective retention behavior can be related to the molecular structures and the resulting retention mechanisms. Naphthalene (5) and acenaphthene (6) are pure hydrocarbons without any functional groups and can only interact with the stationary phase through London dispersion forces (hydrophobic retention). The heat of transition to the retained state is negative (exothermal process), and this explains the positive slope of the curve if the negative prefix for ΔH^0 in 2.9 is considered. With all other compounds present in the test sample, we can assume secondary interactions due to their functional groups. Dipropylphthalate (4) is a potent hydrogen bonding acceptor and will interact with nondissociated residual silanol functions. Butylparabene (3) can also be a hydrogen acceptor due to its phenolic hydroxide group, but this group is simultaneously a strong hydrogen bond donor. The embedded amide group on the stationary phase becomes very pertinent at this point as it can provide the complementary acceptor function. Another characteristic of this type of stationary phase (ultrapure silica, polar embedded, endcapped) is a very low silanol activity, as residual (non-endcapped) silanols interact with the sterically closer amide group, rather than with the analyte molecules and are thus shielded. Therefore, the most intense of the possible interactions discussed earlier will be between the parabene as a donor and the amide group embedded in the stationary phase bonding. Hence, the retention mechanism between butylparaben and dipropylphthalate will be markedly different. This can explain the difference in the transition enthalpy between both

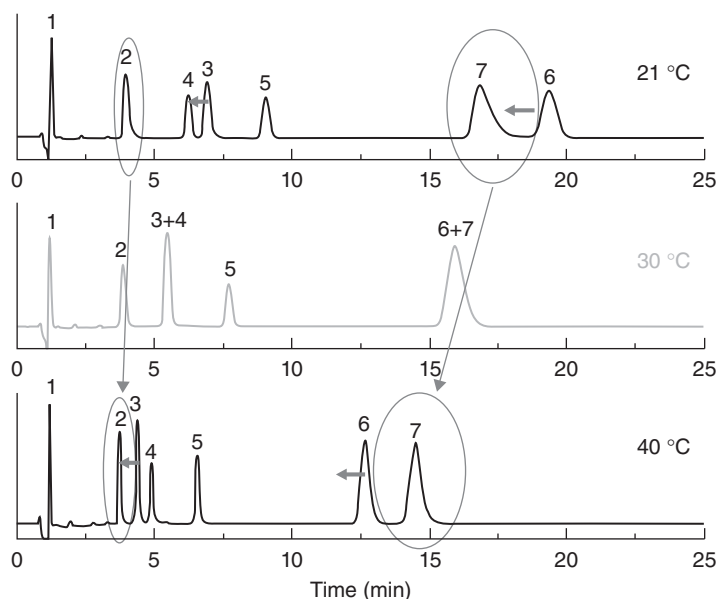


Figure 2.9 Temperature dependence of the Waters column test (according to Uwe Neue). Column: ProntoSIL C18 ACE EPS, 125 mm × 4 mm (Bischoff); Eluent: phosphate buffer 20 mM pH = 7/MeOH 35/65 v/v; peaks: (1) uracil, (2) propranolol, (3) butylparaben, (4) dipropylphthalate, (5) naphthalene, (6) acenaphthene, (7) amitriptyline.

analytes, which is expressed in the different slopes of the van't Hoff plot and eventually leading to the change in elution order.

Propranolol (2) and amitriptyline (7) exhibit secondary amine function and are thus strong bases that will be almost completely protonated at pH = 7. The silanol groups, albeit present with a relatively small activity, will be highly dissociated at this pH. As a consequence, there will be a cation exchange interaction possible between the protonated bases and the silanols. The related Coulombic interaction is energetically very strong and likely to dominate the hydrophobic interaction of these basic compounds, which is anyway small due to their charged status (see Figure 2.7). This implies a strong influence of the temperature dependence of the cation exchange mechanism and secondary equilibria come into play. It can be concluded that a temperature rise is accompanied by increased silanol group dissociation and even leads to a slight increase in retention, which contradicts the rule of the entropy–enthalpy balance. We have learned in Section 2.3.1.2 that temperature increase induces stronger dissociation of acids and this effect increases the cation exchange capacity of the stationary phase due to increased presence of dissociated silanol groups. Eventually, the increasing temperature increases the cation exchange-based retention (slightly negative slope in the van't Hoff plot). This effect is stronger than the temperature effect on the very weak hydrophobic retention of the two strong bases. The opposite slopes of the curves of strong base

amitriptyline (7) and the neutral compound acenaphthene (6) lead to the change of elution order as the temperature is changed.

It is important to note the difference between the example of the Waters–Neue test and the example that was discussed in Section 2.3.1.2. In the previous discussion, the pH was closer to the pK_a of the respective substances whose retention was significantly changed by small variations of the two parameters. The strong bases in the Waters–Neue test have pK_a values that are about four pH-units higher than the applied pH = 7. In this scenario, the conditions are completely different from those observed when there was a very strong dependency on the dissociation and the pH (see Figure 2.7).

The following rules of thumb apply for retention and selectivity optimization:

- An increase in temperature by 1 °C decreases the retention factor of substances undergoing regular hydrophobic retention by 2–3%. From this rule and considering the exponential relationship 2.9, a decrease of 22–35% corresponds to a T decrease of 10 °C and 50–80% to 20 °C, respectively.
- The stronger the retention mechanisms differences between analytes, the more the temperature can be used for selectivity optimization. This is particularly effective for the separation between acids and bases versus neutral substances, even if functional groups in neutral molecules differ significantly.
- The linear relationship between $\ln k$ and $1/T$ allows with a small number of experiments to create a plot similar to Figure 2.10 to easily determine the optimal temperature for best overall selectivity. The temperature range for the experiments should cover at least 20 °C.

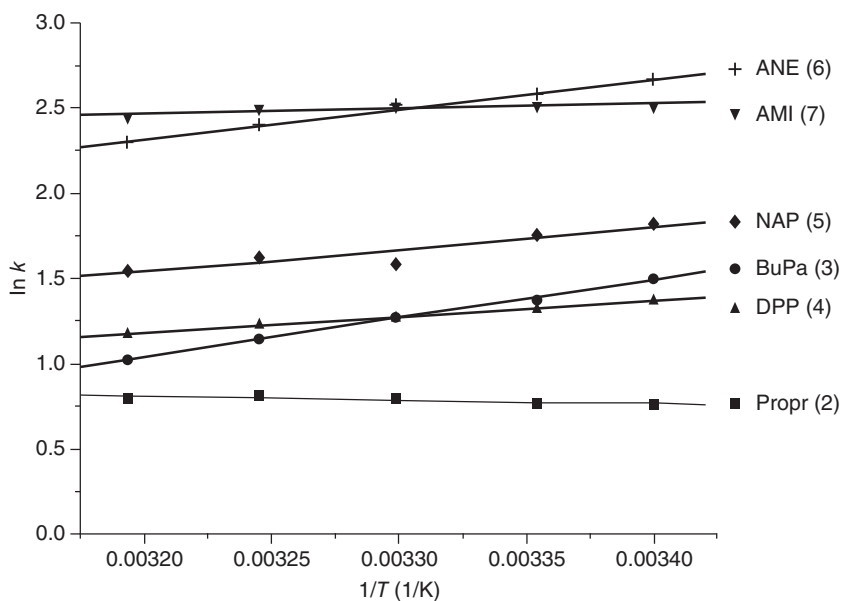


Figure 2.10 Van't Hoff Plot to show the temperature dependence (21–40 °C) for the probe compounds in the Waters column test, chromatographic conditions as in Figure 2.9.

From the second bullet, it can be concluded where the limitations of the temperature concept are. In cases where the retention mechanism between two substances does not differ, there is no way to influence selectivity by temperature alteration. This is usually the case if compounds do not differ in the type or number of functional groups, but, for example, only in the length of alkyl chains. This example of a homologous series will be investigated in the following section.

2.3.2.2 Separation Acceleration through Temperature Increase

Prior to the discussion on the influence of a wide range of temperature variation on the separation of a homologous series, another aspect will be elucidated with regard to the chromatograms shown in Figure 2.9. There is an additional effect next to the changes in retention. This becomes most obvious if we look at the changing peak shape of amitriptylin (7). The relatively broad and clearly asymmetric peak at 21 °C develops into a much sharper and more symmetrical peak when the temperature is increased. To express this more quantitatively, the asymmetry (determined at 5% peak height) decreases from 2.62 to 1.41 and the plate number increases by 50% from 3000 to 4500. A similar effect can be seen with propranolol (2), but due to the smaller peak width at lower retention, it is just less obvious, but the change in quantitative peak parameters is actually similar. Looking at the peak of acenaphthene (6), this sharpening effect at higher temperature is much less pronounced than with propranolol and amitriptyline. The acenaphthene substance band is even at the lowest temperature much more symmetrical, but shows an increase by only 17% from 5400 to 6300. Amitriptyline and propranolol present the characteristic peak shapes of strongly basic compounds. Those show energetically strong cation exchange interaction with silanol groups. The silanol groups are very small in numbers on such ultrapure silica-based endcapped materials and thus easily overloaded by mass, resulting in a mixed adsorption isotherm. Moreover, there are different kinetics between the ion exchange and the hydrophobic retention mechanisms. Both effects are root causes for the asymmetric peak shape. Increase of temperature induces silanol dissociation and increases the cation exchange capacity, thus reducing the overloading effect. At the same time it speeds up the exchange kinetics and reduces the observed retention time differences between the two mechanisms there. Overall the positive effect of a 40 °C column temperature on this example is tremendous relative to 20 °C, while at 30 °C the separation is impossible due to co-elution effects. Even in the absence of special and strongly basic compounds, there is an important kinetic influence of temperature, which is also of importance for increasing separation speed. In the next example, the systematic influence of temperature on peak shape over a wider temperature range will be studied. A significant influence of the temperature on column back pressure will also be observed.

In Figure 2.11, the separation of seven alkylphenones on a polymer-based stationary phase over the temperature range from 30 to 100 °C is shown. Three effects can be seen from this figure:

- 1) Retention drops to less than 50% going from the lowest to the highest temperature.

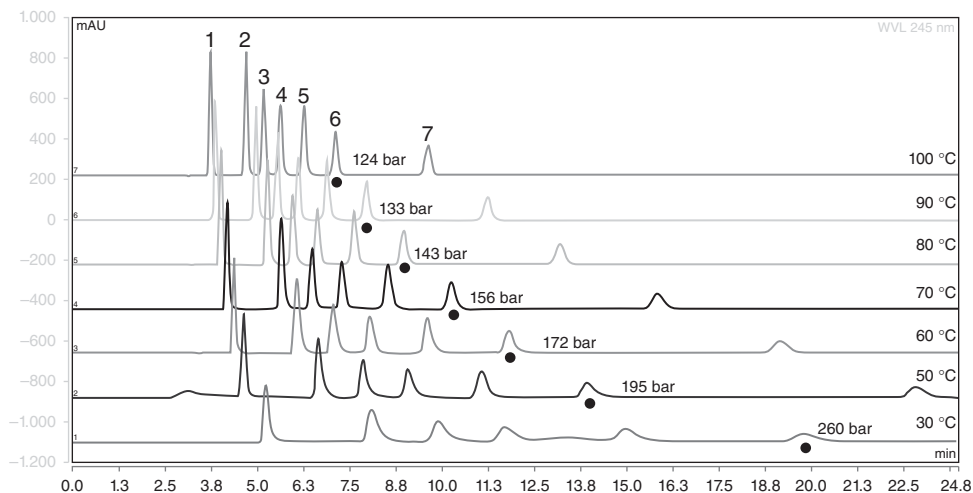


Figure 2.11 Separation of alkylphenones on a C18-modified polyvinyl alcohol (PVA) phase over a wide range of temperature. Column: Shodex® ET-RP1 4D 5 μ m, 250 mm $\times$ 3 mm; eluent: H₂O/MeOH/25/75

v/v; flow: 0.5 ml/min; peaks: (1) acetophenone, (2) propiophenone, (3) butyrophenone, (4) pentanophenone, (5) hexanophenone, (6) heptanophenone, (7) octanophenone.

- 2) The column head pressure drops to less than 50% of the original value going from the lowest to the highest temperature.
- 3) The peaks appear much sharper at the elevated temperature, which translates into better resolution in spite of the markedly lower retention.

At first glance, the selectivity appears to be unaffected by temperature. To validate this, a van't Hoff plot was generated, which can be seen in Figure 2.12.

The plot clearly demonstrates that all the lines are almost parallel. This means that temperature has a marginal influence on selectivity, if at all. This is typical behavior for the separation of a homologous series in RP chromatography because the retention mechanism is uniform across all analytes. It can be concluded, therefore, that the temperature will not always help to aid optimization of selectivity in LC.

The true motivation for these experiments was the application of temperature to accelerate the separation. Very obviously, this goal was clearly achieved by the strong decrease in retention, but a similar effect would have been possible by increasing the methanol content in the mobile phase to greater than 85%. However, there is another important comment to be made. In Section 2.2.1, with the help of Eq. 2.5, it was noted that a strong decrease in retention should be accompanied by a loss in resolution, at least for the early eluting peaks. As the resolution in Figure 2.11 obviously did not suffer, another parameter must have changed as well. It has been shown that the selectivity remained constant, so the only option left is an increase in plate number. The calculated plate numbers as a function of the temperature are plotted for the peak of heptanophenone (6) in Figure 2.13.

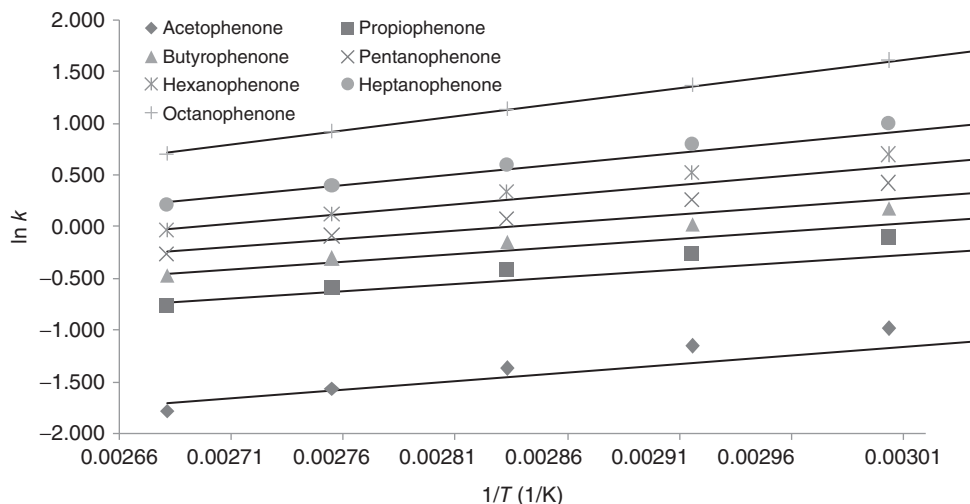


Figure 2.12 Van't Hoff Plot to show the temperature dependence of the retention of seven alkylphenones from 30 to 100 °C, chromatographic conditions as in Figure 2.11.

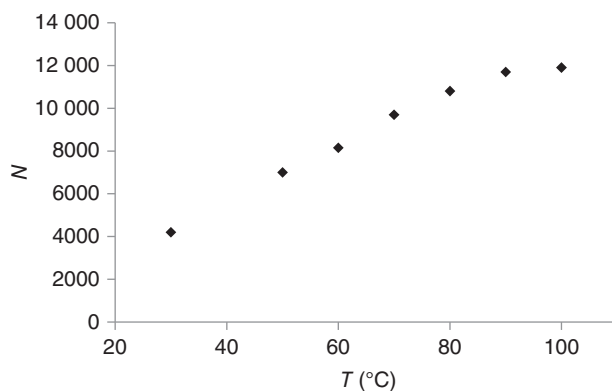


Figure 2.13 Temperature dependence of the plate number of heptanophenone (marked with dot in Figure 2.11) on C18 modified PVA phase (chromatographic conditions as in Figure 2.11).

It can be clearly seen that there is a steady increase in efficiency over a wide temperature range with an asymptote being reached at temperatures above 80 °C. How can we explain this systematic increase in efficiency and how can we understand that it suddenly loses traction at higher temperature? Before diving deeper into this discussion, it is necessary to assess the absolute level of theoretical plates in this example. With only 4200 plates under conditions slightly above room temperature this must be rated a very poor efficiency for a 250 mm long column packed with 5 μ m material. It takes temperatures above 80 °C to jump over the 10 000 plate mark and make the column perform reasonably. The poor efficiency at lower temperature is a typical phenomenon on polymeric stationary phases as

they do not possess pore structures for unhindered and fast diffusion even for small molecule analytes. This leads to slower mass transport than silica-based columns of a similar particle size. So it must be assumed that a significantly higher slope of the C -term in the van Deemter equation 2.8 is obtained. It has previously been derived from this equation that the C -term is controlled by the diffusion coefficient. So the next logical step would be to discuss how the diffusion coefficient depends on temperature.

Equation 2.10, which is a simplified form of the Wilke–Chang equation [11], shows the relationship between temperature and diffusion. In this equation, Ψ is a constant that depends both on the solvent and the analyte molecule. For those who are interested in the quantitative relationship, the diffusion coefficient is inversely proportional the molar volume to the power of 0.6, so approximately to the square route of molecular mass (depending on detailed molecular structure, in particular for macromolecules). In this example, neither the solvent nor analyte is altered, and thus it can be directly concluded how the temperature influences the diffusion 2.10. It shows the linear increase of D_m with increasing temperature, but at the same time we have to consider the decrease in viscosity, which is also a function of temperature, thus increasing the diffusion coefficient even more.

$$D_m(T) = \Psi \cdot \frac{T}{\eta(T)} \quad (2.10)$$

The dependence of the viscosity is unfortunately not linear but rather exponential and the curve is strongly dependent on the molecular properties of the solvent. The experimentally determined dependence over the span from 25 to 200 °C is shown in Figure 2.14 for three typical RP-HPLC solvents and one mixture of water and acetonitrile. Water shows the strongest deviation from a linear relationship and between 25 and 50 °C with the viscosity dropping by 40% relative to its original value, but with the subsequent increase to 75 °C only further 20% decrease in the

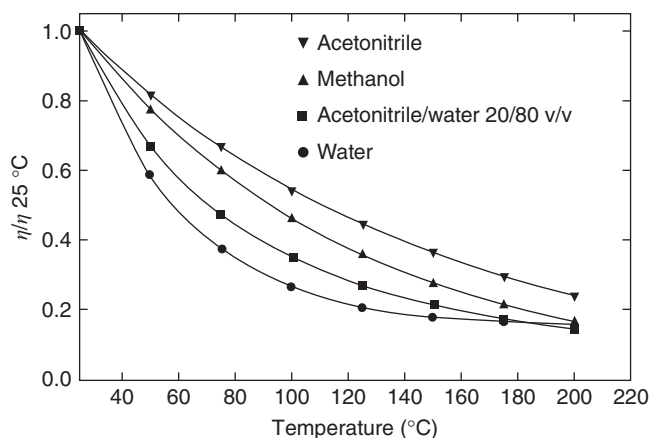


Figure 2.14 Dependence of the (dynamic) viscosity η on temperature for three representative HPLC solvents and one acetonitrile–water mixture. Curves show the progression as relative values of η related to the viscosity at 25 °C.

viscosity is observed and by less than further 15% as the temperature is increased to 100 °C. Above 125 °C, there is minimal effect in increasing the temperature. The two organic solvents acetonitrile and methanol show less curved but overall much weaker temperature dependency than water. Adding high water content to acetonitrile brings the curve closer to that of water, but still significantly different with an interesting observation occurring at extreme temperatures close to 200 °C where the response curve crosses that obtained with 20% acetonitrile content.

It can be concluded from these curves and the considerations related to Eq. 2.10 that the viscosity effect in a water–acetonitrile 20/80 v/v mixture increases the diffusion coefficient by a factor of 3.5 when going from 30 to 100 °C. In addition to this phenomenon is a direct temperature effect on diffusion due to the higher kinetic energy of the solvent molecules. It can be seen that the relationship is linear but it is important to consider the absolute temperature, which only increases from 303 to 373 K (please note that temperature is always expressed in degree celsius, unless the context requires K). This factor of 1.23 can almost be neglected relative to the factor 3.5 resulting from the viscosity. Nevertheless, it can be expected to observe more than a factor of 4 faster mass transport in this scenario. The example shown in Figure 2.11 uses a water–methanol 25/75 v/v mobile phase, which will create a viscosity curve relatively similar to that of methanol. It can be assumed that there is a viscosity decrease by more than a factor of 2, which combined with the direct temperature effect (1.23) represents a 2.5-fold increase in D_m over the given temperature range. The van Deemter equation predicts that the optimal linear velocity (minimum of the curve) linearly increases with D_m . At the same time, the slope of the C -term linearly decreases with D_m . Both of these observations are favorable for fast chromatography. The optimal resolution is obtained at higher analysis speeds and there is only a much smaller efficiency loss when increasing the linear velocity into the C -term. We have not recorded temperature-dependent van Deemter curves for the application in Figure 2.11. Experimental van Deemter curves for pentylbenzene in a relatively viscous eluent running on a silica-based reversed phase are shown in Figure 2.15 for three different temperatures (25, 40, and 60 °C). The faster diffusion reduces the C -term in this viscous eluent by a factor of almost 3 over the 35 °C temperature range and with this factor the optimal linear velocity increases from 0.7 to 2 mm/s. By the mathematical derivation of Eq. 2.8 for u , it is possible to determine that the minimum of the curve is proportional to D_m/dp giving an important consideration for optimization the speed of analysis. With organic-rich eluents the minimum of the van Deemter curve moves approximately linearly with temperature increases, with the minimum observed when the stationary phase particle is decreased to moving to the right (see Eq. 2.10 and Figure 2.14).

This knowledge, allows the plate number curve in Figure 2.13 to be discussed with a clearer understanding of what is happening. It is a reasonable conclusion that increases in the diffusion coefficient will not just linearly grow up to 80 °C and then stop as the temperature is further increased. Such strict proportionality between D_m and N could only be expected in a certain range of the C -term regime. The van Deemter curve for heptanophenone on the polyvinyl alcohol

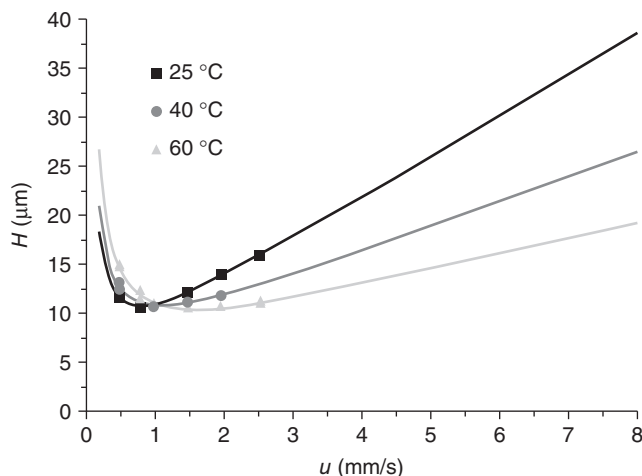


Figure 2.15 Van Deemter curves recorded at three different temperatures, column: Pron-toSIL KromaPlus C18 5 μm , 250 mm $\times$ 4.6 mm, eluent: EtOH/H₂O 80:20 (v/v), analyte: pentylbenzene.

(PVA) stationary phase is not known, but it is possible to calculate from the flow rate of 0.5 ml/min that the linear velocity is approximately 1.5 mm/s. For efficient silica-based 5 μm stationary phases running with a viscous (water–methanol) eluent at room temperature, this should be close to the van Deemter minimum for a small molecule. We already discussed that polymer-based stationary phases often suffer from slower mass transport due to their pore structure. The strong efficiency increase with increasing temperature leads to the conclusion that $u = 1.5$ mm/s is already located far in the C -term, resulting in slow mass transport on the PVA material. If the conditions are chosen such that the dispersion processes are dominated by the C -term and the temperature is increased, the main effect that comes into play is the decreasing slope of the C -term resulting in efficiency increase. At the same time, the minimum of the curve moves to the right, which can be considered as rolling down the C -term toward the curve minimum as a consequence. As soon as the curve minimum is approached, the efficiency increase will slow down and on increasing the temperature, will result in the B -term becoming the dominant dispersion process, leading to an efficiency drop with further temperature rises. This explains why the slope of the curve in Figure 2.13 flattens at 90 °C, and it can be concluded that it takes approximately 100 °C to be at optimal efficiency with $u = 1.5$ ml/min on the PVA material. So the PVA needs this high temperature to enable chromatography with reasonable speed, but fortunately this phase is stable at 100 °C, whereas most silica-based phases would fail, even in the absence of buffers.

This discussion reveals a risk involved with temperature increase: for a method that is efficiency optimized at ambient temperature, but is used at increased temperature, the efficiency will drop because the B -term of the van Deemter curve comes into play if the linear velocity is increased accordingly.

The relationship between temperature and viscosity has been explicitly discussed, and it was also briefly mentioned how the pressure change can be used as an indicator for the viscosity change. Now it is the time to treat this in quantitative ways, as pressure is always an important limiting factor in kinetic optimization of HPLC. Equation 2.11 puts all pressure determining variables in context. Pressure increases linearly with column length L and linear velocity u and is inversely proportional to squared particle diameter (dp^2). Pressure increases with the viscosity, which is a nonlinear function of temperature according to the curves shown in Figure 2.14. For organic-rich eluents, the viscosity drops between 30 and 80 °C approximately by 10% per 10 °C temperature increase. Hence, the column head pressure under organic mobile phase content above 60% is at 80 °C only about half that at ambient temperature.

$$\Delta p \sim \frac{\eta \cdot L \cdot u}{dp^2} \quad (2.11)$$

Decreasing the retention time is the first step in the speed-up of the separation. The related decrease in viscosity lowers the column pressure and allows for an increase in linear velocity. It was mentioned above that this is actually necessary, to prevent longitudinal diffusion, B -term, processes becoming dominant and hence losing separation efficiency. In the example of the alkylphenol separation, it can be seen the potential reduction in analysis times related to a temperature increase from 30 to 80 °C with the following aspects:

- 1) The retention and thus analysis time reduces by a factor of 3, without a significant change in resolution.
- 2) The column pressure at the same linear velocity is reduced by a factor of 2.
- 3) Doubling the flow rate at the highest temperature, in order to generate the original back pressure (260 bar), would allow for a sixfold reduction in analysis time.

This speed-up potential appears very appealing, and it raises the question why temperature is not used to a much greater extent for expediting LC analyses. Wouldn't it be a great alternative to the use of sub-2 μm particles that mostly require UHPLC instrumentation to be able to perform at higher pressures? Three important considerations have to be made to assess this, which will highlight the negative aspects to this approach:

- 1) The preservation of the resolution is not guaranteed due to potential differences in selectivity. In the given example, the experimental parameters are chosen such that the dispersion processes are dominated by the C -term, with a strong dependency between flow and the C -term, due to the slow mass transfer of the polymer material. Starting at the curve minimum, it would not be possible to increase the chromatographic efficiency by increasing the temperature, not even with proper adaptation of u . In such a case, there would be a high risk of losing resolution due to the lower retention (see Eq. 2.5 and Figure 2.2), and thus the faster method would possibly no longer meet the requirements.

- 2) It has been discussed in this section that temperature changes can strongly change selectivity. It was shown in Figure 2.9 that these effects can even cause co-elution of compounds that are separated at a different temperature. Homologs are a case where the retention mechanism in RP-HPLC is as uniform as can be, which virtually excludes an influence of temperature on selectivity. Most analytical challenges, however, are more complex than this.
- 3) The endurance of stationary phases is always a strong function of temperature though it depends on other operational parameters, too (see Chapter 4 for more details). Kinetics of disintegration of the base material or the surface modification (bonding) simply follows the rules of chemical kinetics and so column deterioration is exponentially accelerated with temperature. Excellent stability for use at temperatures of 100 °C and above can only be attributed to stationary phases based on organic polymers, porous graphite, and ceramics such as titania or zirconia (nonbonded). A few highly efficient silica-based materials exhibit impressive stability at temperatures of 100 °C or more thanks to hybrid-silica and/or special bondings that protect the silica material. Nevertheless, the vast majority of silica-based RP-materials do not provide acceptable endurance at temperatures above 60 °C under common eluent compositions.

The three considerations above clearly show that the example of alkylphenones on a polymer-based stationary phase show the limitations of this particular application. Nevertheless, the use of temperature to speed up separation is, in spite of the discussed limitations, always worth a consideration. If method selectivity and the instrument capabilities allow for this, there are few reasons not to operate at temperatures of about 50 °C to allow for the advantages of reduced analysis times to be observed. To speed up a method by using elevated temperature, three important considerations must be made:

- 1) The new method must still provide sufficient retention and resolution, so the rules of $k > 1$ for the first eluting peak and $R_s > 2$ for critical pairs should still apply.
- 2) For the modification of isocratic methods, the flow rate should be accelerated in order to obtain the same column pressure as at the lower temperature. As in the relevant temperature range, the diffusion coefficient changes approximately with the same factor as the viscosity. Simultaneously, it allows for full exploitation in the reduction in analysis times.
- 3) There are two more considerations for gradient methods. In order to respect the gradient volume concept (see Chapter 3 and Section 2.3.4.1), the gradient time must be shortened by the same factor as the flow rate is increased. The higher temperature will normally cause elution of the last eluting peak at an earlier position in the gradient. To exploit the full speed-up potential, the end of the gradient should be cut in order to place the last eluting peak back at the end of the gradient window.

With the information given in this section, separation scientists should feel forearmed to make proper use of temperature for both selectivity and speed

optimization. The mobile phase composition in LC also has a similar effect to the temperature. It influences both the mobile phase viscosity, with all respective consequences and it also influences retention and potentially selectivity. This will be addressed in the following section along with a perspective to what has already been discussed on temperature.

2.3.3

The Value of Mobile Phase Composition versus Temperature in the Strive for Optimization in HPLC

The effect of eluent composition on the mobile phase viscosity was briefly addressed in the previous sections and Figure 2.14 clearly demonstrates this. It was mentioned that there are no simple linear relationships between viscosity and mixing ratio of solvents. The nonlinear effects are always related to the amount of heteromolecular interactions in the mixtures, which is a complex field handled by thermodynamics of mixing. It is outside the scope of this textbook to treat it in a fundamental physicochemical way, but we discuss it empirically and point to all effects that are relevant for the LC user. It is a well-known fact that mixing of water and alcohols is an exothermic process, so the mixture heats up when methanol is poured into a water containing flask. This occurs because the intermolecular forces between methanol and water molecules are even stronger than those between the molecules in the pure phases. This releases energy, facilitating the mixing process, but it also leads to a stronger association of molecules in the mixed phase than in the pure phases. One consequence is the pronounced viscosity maximum that is observed in water–alcohol systems.

The experimentally determined dynamic viscosity η is shown in Figure 2.16 for RP-HPLC relevant eluent mixtures. This also includes the quite uncommon mixtures of water with alcohols other than methanol. All values are measured at 20 °C, while the relatively complicated temperature dependence was discussed in the previous Section 2.3.2.2. It is important to note that the mixing ratio is given as mass-%. The advantage of mass-% is the absence of temperature influences due to the different changes in solvent density with temperature. It is, therefore, a much more precise practice to prepare mixtures for mobile phases gravimetrically than volumetrically. The downside is that programming of mobile phase mixtures with gradient instruments only allows control of volume-%. This has important relevance for the quantitative conclusions from Figure 2.16. Due to the lower density of the organic solvents (methanol and acetonitrile <0.8 g/ml, while water has 1.0 g/ml at ambient temperature), the maxima in the plot would move to the right when transferring to volume-%. The shift is not linear, but increases with increasing organic content. While it changes the maximum for water–acetonitrile by less than 3% the water–methanol maximum is altered by almost 10% to the right and thus located at close to 50% methanol in volume.

While the curves in Figure 2.16 represent the situation at 20 °C, we can draw similar conclusions for the entire temperature range from 10 to 50 °C. The amplitudes of the curves are normally reduced with increasing temperature but the

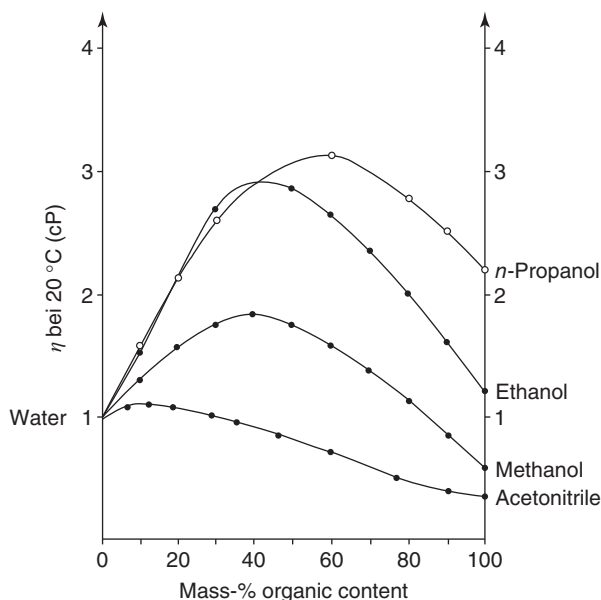


Figure 2.16 Dynamic viscosity of mixtures of water with acetonitrile and different alcohols at 20 °C. Please note that the x-axis is in mass-% rather than the more common volume-%.

maxima do not shift significantly in this temperature range. The following conclusions are helpful in practice:

- With water–acetonitrile, it is observed that a viscosity maximum at 15% v/v, which is about 15% higher than the viscosity of pure water. The viscosity changes only slightly between 0% and 30% v/v, but drops stronger at higher content and yields 40% of the viscosity of water in pure acetonitrile.
- With water–methanol, we observe a steeper increase striving toward the maximum at 50% v/v, which is about 80% higher than the viscosity of pure water. The viscosity drops very strongly right of the maximum and reaches 60% of the viscosity of water in pure methanol.
- Mixtures of water with longer chain alcohols exhibit pronounced viscosity maxima which is in the case of *n*-propanol more than a factor of 3 higher than the viscosity of pure water. Even pure *n*-propanol and ethanol show higher viscosity than water.
- The fact that water–methanol shows a 60% higher viscosity maximum makes it much less attractive than water–acetonitrile from a purely kinetic standpoint. The extremely high maxima of the longer chain alcohols virtually exclude them from standard application use (let alone the tax issue with ethanol). Nevertheless, the positive impact of the different solvation properties of the alcohol modifiers should never be underestimated for selectivity and peak shape optimization.

We have seen in Section 2.3.2.2 with Eq. 2.11 that the column pressure is proportional to the eluent viscosity. Hence, the pressure with a 50% v/v methanol eluent would be almost twice as high as with acetonitrile at the same composition. The fact that the isoeluotropic composition of water–methanol is approximately 5% higher, albeit helpful does not make a great difference here. Users of modern UHPLC instruments that provide up to almost four times the pressure capability of a conventional HPLC instrument may be inclined to just ignore this pressure difference of factor 2. This should not be taken too easy though and to understand this we need to look back into the Eqs. 2.8 and 2.10. We can derive that the molecular diffusivity depends on solvent viscosity. We have learned in the context of temperature effects that solvent viscosity matters for the shape of the van Deemter curve, both with respect to the position of the minimum and the steepness of the C -term. So we can directly conclude that the viscosity changes related to the eluent composition will change diffusion coefficients to a similar extent and have thus similar influences on the van Deemter curves. The van Deemter curves for the same analyte and the same stationary phase used in Figure 2.15 are shown for different mobile phase compositions but constant temperature in Figure 2.17. Eluent composition was adapted respectively for isoeluotropic conditions to allow for a fair comparison. The viscosity of the water–methanol eluent is approximately twofold higher, while that of the water–ethanol eluent is more than fourfold higher than with water–acetonitrile. We do not discuss the van Deemter curves in a quantitative manner, as the scatter of the experimental

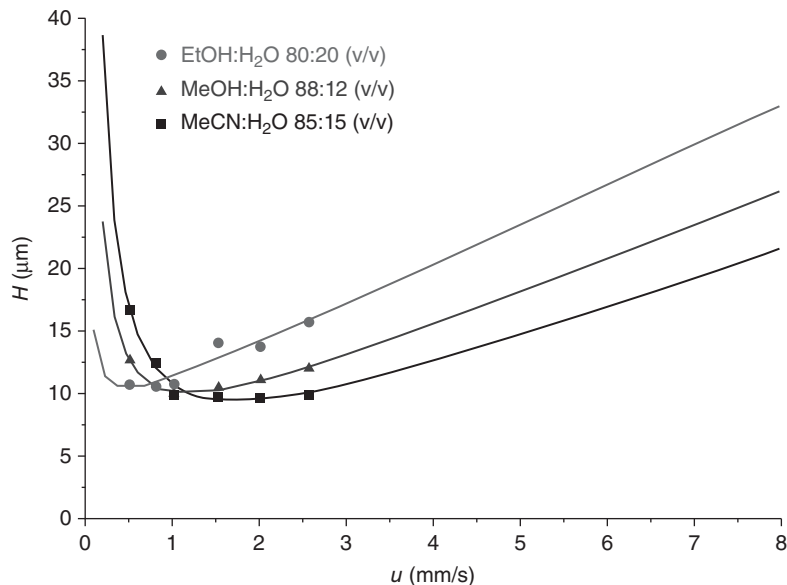


Figure 2.17 Van Deemter curves recorded at three different isoeluotropic eluent compositions that differ markedly in viscosity, column: ProntoSIL KromaPlus C18 5 μm , 250 mm $\times$ 4.6 mm, temperature: 25 $^{\circ}\text{C}$, analyte: pentylbenzene.

data is relatively high, in particular for the water–ethanol system. Nevertheless, one can clearly see a massive impact of the solvent composition on the mass transfer (C -term), which is much higher (slower mass transfer) in the high viscosity compositions. Comparing the curves for acetonitrile and methanol, the rule of shifting the position of the curve minimum by the same factor as the diffusion coefficient (thus the same factor as viscosity and pressure) perfectly applies. The optimum linear velocity moves by factor 2 from 1 to 2 ml/min when changing from methanol to acetonitrile. This proves that there is more than the pressure limit of the instrument to take into account for the speed-up potential of methods based on different eluents.

$$\Delta p \sim \frac{\eta \cdot L \cdot u}{dp^2} \quad D_m(T) = \Psi \cdot \frac{T}{\eta(T)} \quad u_{\min} \sim \frac{D_m}{dp} \quad (2.12)$$

To express these relationships quantitatively, we have summarized all relevant equations introduced before as 2.12. These dependencies lead to the following statements:

- Doubling the viscosity η doubles the column pressure Δp and reduces the molecular diffusivity D_m by half.
- To stick to the optimal linear velocity $u_{\min}$ under this slower diffusivity, the flow rate F (and thus u) must be reduced by half as well.
- Having reduced the flow rate, the original column pressure will be observed, but simultaneously the analysis time has doubled.

Based on this we can draw two important conclusions:

- 1) With a given column type, temperature, and analyte, the minimum of the van Deemter curve will always be at the same pressure level, no matter what the mobile phase composition is, but the flow rate will have to be optimized individually.
- 2) As the mobile phase viscosity and the corresponding molecular diffusivity changes during a gradient run, the constant flow mode is not the best option for efficiency optimization in gradient elution.

The second point leads to the conclusion that a pressure-constant mode in HPLC, with the resulting flow gradients would be the optimal approach to gradient elution. These flow gradients could go over a wide range of up to threefold changes when running from the maximum viscosity composition all the way to the pure organic component (see viscosity changes in Figure 2.16). This pressure-constant mode would also improve the column lifetime, which is reduced with the continuous pressure changes during gradient analysis. At the same time, the control of the gradient formation under constant pressure is a challenge, as the gradient slope would need continuous adaptation with the changing flow rate in order to respect the gradient volume principle (Chapter 3). This purely technical challenge can be solved through intelligent instrument control though. However, there is another important consequence of the constant pressure mode, as it removes the strict relationship between elution volumes and

retention times that only exists in the flow-constant mode. This has implications for the chromatographic behavior as the column ages. When the column permeability goes down over the lifetime of the column (it accounts for the resulting back pressure at constant flow), the retention times will change as a consequence, because the instrument control reduces the flow to keep the pressure constant. The only way to present chromatograms when operating in the pressure-constant mode consistently would be plotting the detector signal over the elution volume rather than over the retention time. This will not easily find acceptance in the user community and is the most obvious reason why the pressure-constant mode for gradient elution has not yet gained ground.

The change of solvent composition as opposed to temperature change is an important consideration to make in method optimization. It is important to oversee the kinetic consequences of both approaches beyond retention and selectivity when it comes to decisions. A practical challenge may be the decision whether to increase temperature or content of the organic mobile phase when the goal is a reduction of retention. To discuss this more quantitatively, we will learn another important rule of thumb:

Horvath rule for retention control in RP-HPLC: increasing the organic content in the eluent by 1% has approximately the same effect as increasing the column temperature by 5 °C.

This rule can be derived from the facts that

- 1% increase in methanol or acetonitrile decrease retention by 5–8%
- an increase in temperature of 1 °C decreases retention by 1–2%.

It is very obvious from these relatively wide ranges that the Horvath rule can only give rough guidance, but it is still very helpful for practical method development. It is crucial to note that both organic content and temperature change retention exponentially based on the fundamentals of equilibrium thermodynamics. Together with the applicable ranges for temperature and solvent composition, this allows for some important general considerations:

- The accessible range of temperature variation on common LC columns (using a column thermostat that allows working at subambient temperature) is approximately 10–50 °C. In this span, the retention factor can be changed by not much more than factor 2.
- Mobile phase composition on every RP column can be changed between 5% and 100% and allows changes in retention by a factor of 5000–10 000. It is thus so much more effective than temperature that both can hardly be compared.
- To reduce the retention by factor 2 can either be achieved by increasing the temperature by 30–40 °C or increasing the organic content by approximately 10%.
- A temperature increase of 30 °C will improve the speed-potential of a method with more than 90% water in the eluent by 50–80%. With more than 90% methanol or acetonitrile content this potential will only be 20–40%.
- The increase of 10% acetonitrile in the mobile phase will not remarkably influence the speed-potential of the method as long as the initial content is below

25%. If the initial content is above 30% the speed-potential will increase by approximately 10%.

- Thorough consideration is very relevant in methods using methanol. With more than 55% initial methanol content the speed-potential at 10% higher methanol increases by approximately 20%. If the initial methanol content is below 40%, however, the speed-potential is *decreased* by approximately 15% because of the strongly increasing viscosity in this range.

There is no generally applicable rule whether mobile phase composition or temperature is the better way to reduce retention from a kinetic point of view. The above-mentioned rules can give some guidance, but in practical method optimization it is more important how the selectivity of the method is affected by either organic content or temperature change and how much the higher temperature affects column endurance with the given application. The picture is clearer if the original method uses high water content (e.g., more than 80%). In this case, temperature is normally the better choice, if no other consideration speaks against it. If the organic modifier is even methanol and the original method has more than 60% water in the mobile phase, there is a clear incentive to employ a temperature increase. The increase of 10% methanol-content will *reduce* the analysis speed by 10–20% which is not very appealing. This competes against more than 50% speed improvement related to 30 °C temperature increase. If column stability and selectivity allow for it, it is very clear what the better choice for water-rich methanol eluents is: temperature increase without a shadow of a doubt.

So we have seen how complex the influence of temperature in HPLC can be, but we have learned so many helpful rules that the user should really apply temperature effectively in method optimization. Especially the combination of temperature and mobile phase composition may be an excellent way to optimize methods. Temperature should always be considered as an important method variable. If a method uses temperatures below 40 °C, the reason for this should always be challenged. There are three important conclusions for this chapter that should take away all myths and misconceptions about the use of temperature in method speed-up.

- 1) The fact that temperature increase reduces retention has nothing in common with temperature-based kinetic improvement or speed-up of separations. The related reduction of retention may even be a risk for the method robustness or resolution. Retention in LC can be much more effectively reduced with mobile phase changes, and it is a purely thermodynamic consideration that has no place in kinetic optimization.
- 2) Kinetic improvement is solely about the time needed to generate a certain number of theoretical plates. Plates per time depend on molecular diffusivity, which can directly be influenced by temperature, mainly through reduction of eluent viscosity. From this, we can conclude a *maximum practical speed-up potential by temperature by factor of 2*, as this is the accessible range to influence the diffusion coefficient in practice.

- 3) The common assumption that temperature increase can generally replace pressure increase for method speed-up is simply wrong. We have learned that a proper method transfer to a higher temperature implies working at the same pressure as with the original method. If a given column type requires for a given application a certain pressure to work in the van Deemter minimum, but the instrument is not capable to support this, it will not help to just increase the temperature. While the lower viscosity will allow increasing the linear velocity at the given pressure, the minimum of the van Deemter curve will move by the same factor to right. *There is no way to escape the B-term that is not accompanied by pressure increase.*

2.3.4

Accelerating Separations through Efficiency Improvement of Stationary Phases

We have learned in the previous section that the often neglected increase in temperature has a fundamental potential for reducing analysis times, but we also identified the limitations. Using equivalent plate number as a comparator, the use of temperature cannot realistically reduce analysis times by more than a factor of 2. This section addresses the much more common approach to reducing analysis times by improvements in the column performance. The theoretical foundation will primarily be the dependence of the van Deemter curve shapes on the stationary phase particle diameter dp . Later, we also briefly discuss the potential of superficially porous and monolithic stationary phase materials.

2.3.4.1 Systematic Speed-Up by Optimization of Particle Diameter and Column Length

In Sections 2.3.2.2 and 2.3.3, the influence of the diffusion coefficient on the shape of van Deemter curves was explicitly discussed. This discussion is progressed to determine the influence of the particle diameter and how it translates into practical value for the user. It was seen in Section 2.2.2 that the particle diameter dp has an effect on the A -term and the C -term, with the C -term being related to dp^2 . This points to an even stronger influence on efficiency than that of the diffusion coefficient, D_m . To discuss this, the response of the van Deemter curves to changes in dp will initially be simulated. This will require a simple transformation of Eq. 2.8 into a generic form with dp as the only variable. A value of 1 is assigned to the constants λ and γ . The mass transfer factor ω will be assigned the value of 0.15 and D_m will be approximated with $0.003 \text{ mm}^2/\text{s}$ ($3 \cdot 10^{-5} \text{ cm}^2/\text{s}$), which represents a typical small molecule of 200–300 Da diffusing at ambient temperature in a medium with viscosity of 1 cP. We have discussed that D_m can be decreased by a factor of 2 by raising the temperature but this is not relevant for the relative comparison of different particle diameters. After inserting the above-mentioned values for chromatography of a small molecule in aqueous-rich mobile phases at ambient temperature into Eq. 2.8, the generic form of a van Deemter equation is obtained, as shown in the last term in 2.13. This equation was published by Halász in 1975 [12], and it certainly contains empirical elements. The formula is set up very conveniently, so that the user can insert the linear velocity u in millimeter

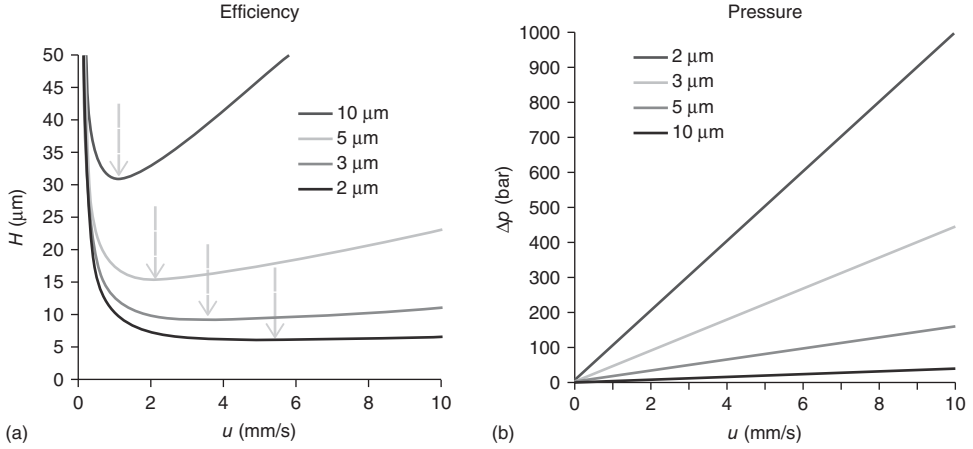


Figure 2.18 (a) Simulated van Deemter curves calculated with Eq. 2.13 for different particle diameters and (b) simulated column pressure curves for a viscosity of 1 cP and a column length of 150 mm.

per second and the particle diameter dp in micrometer, in order to obtain the theoretical plate height H in micrometer.

$$H = 2\lambda dp + \frac{2\gamma D_m}{u} + \frac{\omega dp^2}{D_m} \cdot u = 2dp + \frac{6}{u} + \frac{dp^2}{20} \cdot u \quad (2.13)$$

This formula allows the calculation of the van Deemter curves shown in Figure 2.18a. We can draw the following conclusions from the comparison of the curves for different dp :

- 1) The plate height H at the minimum of the curve decreases systematically with decreasing particle diameter dp . This allows improving separation efficiency by reducing dp at constant column length.
- 2) The optimal linear velocity u_{opt} (x -value of the curve minimum) systematically shifts to higher linear velocity with decreasing dp . This automatically speeds up the separation when the linear velocity is correctly adapted to a decreased particle diameter.
- 3) The slope of the C -terms is drastically reduced when dp is decreased. This facilitates further speed-up beyond u_{opt} on small particles without significant loss in efficiency.

Before discussing what implications the optimal use of smaller particles has for the column pressure, it is necessary to understand the above-mentioned findings in a more quantitative way. From the derivative of 2.13 to u , we can calculate the position of the curve minimum as a function of dp . This is done in 2.14 and yields an easy approximate relationship to estimate the optimal linear velocity as function of dp .

$$u_{\text{opt}} = \sqrt{\frac{B}{C}} = \sqrt{\frac{120}{dp^2}} \approx \frac{10}{dp} \quad (2.14)$$

From 2.14, it can be concluded that the optimal linear velocity for the use of a 5 μm stationary phase is in the order of 2 mm/s. This is only an approximation as this value also depends on the molecular diffusivity D_m of the analyte. It has been seen in the previous sections that D_m , even for a given analyte, can change by a factor of 2 to 3 when the temperature or mobile phase conditions are varied and the value for u_{opt} will change proportionally. This is the reason why $\sqrt{120}$ has been approximated to 10 without losing any relevant accuracy. The most important information in 2.14 is not the absolute value for u_{opt} as a function of dp , it is rather the fact that u_{opt} is strictly proportional to $1/dp$. With that knowledge, it is possible to state that the linear velocity must be exactly doubled when changing from 10 to 5 μm particles in order to maintain the optimal linear velocity.

The next important quantitative conclusion can be derived by inserting the value for u_{opt} , namely $10/dp$ into 2.13. This is done in 2.15 and allows to estimate the theoretical plate height in the minimum of the curve as a function of dp :

$$H_{\min} = 2dp + \frac{6 \cdot dp}{10} + \frac{10 \cdot dp}{20} \approx 3 \cdot dp \quad (2.15)$$

It is once again of use to derive the fact that the plate height in the curve minimum is strictly proportional to dp . This leads to the important conclusion that the change from 10 to 5 μm with properly adapting the linear velocity and keeping the column length constant will exactly double the column efficiency expressed in the plate number N .

There is also a practical use in the absolute value for $H_{\min}$ that can be calculated with 2.15. With that equation, it is possible to estimate that the plate height in the curve minimum must approximately equal three times the particle size of the stationary phase. For a well-packed modern column, we can expect even smaller values for λ than unity. This would lead to the conclusion that a measured $H_{\min}$ of $3 \cdot dp$ is a worst-case scenario for a modern column. From modern HPLC practice, one can tell that $H_{\min} > 4 \cdot dp$ is a clear indicator that either something is wrong with the column, or the instrument is not appropriate to operate the column (too high band dispersion in the system). The following quantitative conclusion can be drawn from 2.13, which allows for Figure 2.15a to be interpreted in a truly quantitative way:

- 1) To halve the particle diameter doubles to optimum linear velocity.
- 2) To halve the particle diameter doubles the plate number at the optimum linear velocity.
- 3) To halve the particle diameter reduces the slope of the C -term by factor 4.

All these findings clearly prove the paramount advantage of smaller particles: they allow better separations in shorter analysis time, but there is a price to pay for this huge practical benefit. This can be determined from Eq. 2.11 in Section 2.3.2.2 and has been expressed graphically in Figure 2.15b. Under otherwise identical conditions, the column pressure Δp increases with $1/dp^2$. If a given column packed with 10 μm particles creates a pressure of 40 bar, then a column of the same length, operated under equivalent conditions, but packed with 2 μm particles will

generate 25 times this pressure, consequently 1000 bar. In addition to that effect of dp on the column permeability, we must also consider the need to adapt u to maintain the minimum of the van Deemter curve with the 2 μm column. It has been seen that it is necessary to increase u by the same factor as dp is reduced, in this case a factor of 5. This would further increase the column pressure to 5000 bar and directly raises the question, how is it feasible to work in practice with such small particles at the optimum linear velocity. There must be something that has not been considered that needs to be incorporated into the discussion.

For the discussion of a method transfer to a column with a smaller particle size, a simple isocratic separation of uracil and five phenylalkanes will be considered. This separation will be optimized in two steps by changing the column and the linear velocity twice until a significant speed optimization as shown in Figure 2.18 is achieved. The original separation was based on very traditional parameters, as the column was a 10 μm material packed in a 250 mm column length and operated at a flow rate of 1 ml/min. The peak resolution was larger than required, so there was no need to improve it. It can be seen that there is a systematic reduction in analysis times by reducing the particle size and adapting the other parameters accordingly. This process requires that the equivalently bonded stationary phase material is available with other particle sizes packed in the respective column lengths. The two simple rules to consider are as follows:

- 1) The column length L must be reduced by the same factor as the particle size dp is decreased, so we keep the ratio L/dp constant. According to the fundamental rules in chromatography presented in Section 2.2 this will keep the plate number N also constant, as long as the column with smaller particles is still operated in the van Deemter minimum.
- 2) The linear velocity u must be increased by the same factor as dp is reduced in order to stick to the van Deemter minimum u_{opt} . If the column diameter is not varied, we will achieve this by increasing the flow rate F to the same factor as dp is decreased.

Following these two simple rules, it is possible to first reduce L from 250 to 125 mm, as dp was reduced from 10 to 5 μm and simultaneously doubling F from 1 to 2 ml/min. With these two changes, a fourfold increase in the analysis speed was obtained, since a column of half the length, and with twice the linear velocity is being used. It is possible to go one step further by reducing dp to 3 μm and L accordingly to 75 mm ($L/dp = \text{const}$ rule). Once again F needs to be corrected by the same factor to 3.33 ml/min and another almost threefold speed increase is observed. Obviously, this optimization could have been achieved in just one step going from the 10 μm particle column to a 3 μm particle and obtaining directly an 11-fold (3.33^2) separation speed-up.

How does this parameter change then affect the column pressure Δp . Δp increases inversely proportional to dp^2 ; however, L and F , which affect the pressure, have also been simultaneously altered. To halve dp at otherwise constant conditions would result in a fourfold increase in Δp . The simultaneous halving of L would result in a pressure increase at constant F of only twofold. However, as F

is doubled to maintain the van Deemter minimum, the effective overall pressure increase by systematically transferring a method to halve the original particle size is fourfold. In general, we find the following important relationship for analysis speed and column pressure:

$$(1/t_{\text{analysis}}) \sim \Delta p \quad (2.16)$$

The pressure will always increase by the same factor as the analysis speed. We can see this proven in the recorded pressure levels shown in Figure 2.19. The speed was overall increased 11-fold and so the pressure increased 11-fold from 16 bar (rounded) to 175 bar. It is worth noting that this speed-up experiment was carried out on a conventional HPLC instrument with nonoptimized fluidics. This can be concluded from the low back pressure of 16 bar with the original method. A modern UHPLC would generate at $F = 1 \text{ ml/min}$ with the given eluent viscosity a pressure of 30–50 bar in the instrument alone without mounting a column. So the pressure resistance of a UHPLC instrument is two to three times higher than that of a $250 \text{ mm} \times 4.6 \text{ mm}$ column packed with $10 \mu\text{m}$ particles. It can also be seen from the zoom into the chromatogram recorded on the short $3 \mu\text{m}$ column that the early eluting peaks show a pronounced tailing. This is a consequence of severe dispersion effects in the conventional HPLC instrument that is not appropriate to operate a $75 \text{ mm} \times 4.6 \text{ mm}$ column packed with $3 \mu\text{m}$ particles.

With the next example shown in Figure 2.20, the speed-up of a gradient separation performed on modern UHPLC-type columns and instrumentation is discussed. The attempted use of a narrow bore (2.1 mm) column packed with $2.2 \mu\text{m}$ particles requires an instrument with significantly reduced extra-column volume. This also includes the adaptation of the flow cell volume and the requirement of faster data handling by the detector electronics (which was already a prerequisite in the example in Figure 2.19). Moreover, the instrument must be able to handle precise injection of $2 \mu\text{l}$ sample volume and withstand a column pressure of almost 600 bar.

As well as the two rules discussed for the speed optimization of isocratic methods, it is necessary to include another rule to allow for the correct optimization of gradient programs. In order to make sure that the shorter and more efficient column is not overloaded by volume and to achieve the same peak height in a concentration proportional detector, it is necessary to include another rule in both isocratic and gradient speed-up. If the column diameter is not changed during the method change, these additional rules are as follows:

- 1) The gradient time t_G must be shortened by the product of the flow increase factor times the factor at which we reduced the column length. Thus, the following proportionality must be considered $t_G \sim F \cdot L$ which implies $t_G \sim 1/dp^2$, respectively.
- 2) The injection volume V_{inj} must be reduced by the product of column reduction factor times the square root of the particle size reduction factor ($V_{\text{inj}} \sim \sqrt{dp \cdot L}$).

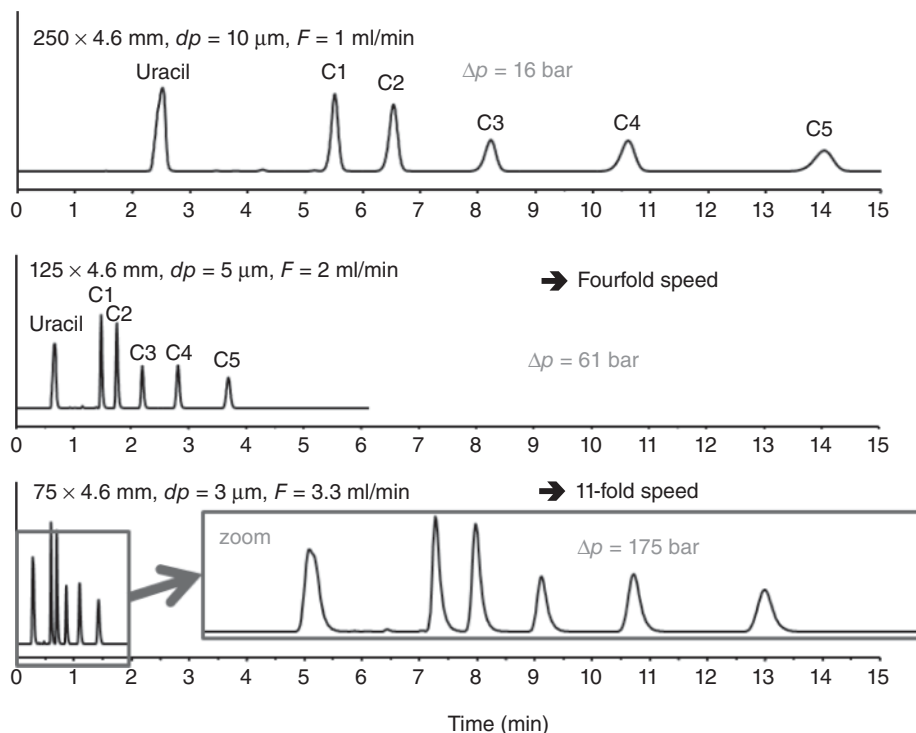


Figure 2.19 Example for a systematic method speed-up by optimization of particle diameter dp and column length L . Sample: uracil und phenylalkanes C_1 – C_5 ; station-

ary phase: Prontosil 120-C18-AQ (Bischoff), mobile phase: 80/20 (v/v) ACN/ H_2O , temperature: 20°C ; $L/dp = 25$. All other conditions as shown in the figure.

For the example presented in Figure 2.20, it is necessary to shorten the t_G by a factor of 2.8 for the first optimization step and by a factor of 6.2 for both steps in total. It can be seen that the duration of the gradient time also corresponds to the reduction in analysis time. If t_G is not altered in a gradient method and only the two rules for isocratic optimization are considered, the analysis time will not be markedly reduced and the chromatogram will look different.

For the injection volume, V_{inj} , it is necessary to consider the changes in L and dp according to the above-mentioned rule. This implies a reduction factor of 2 for the first step and a volume reduction of 4 overall. In this case, all parameter changes have been rounded (L , F , t_G , and V_{inj}) and for the second optimization step it is assumed $dp = 2 \mu\text{m}$ instead of $2.2 \mu\text{m}$. This was done to facilitate the following calculation. It can be shown in practice that this minor change does not matter for the look of the chromatogram; however, the speed-up factor was 20% higher than it would have been with the correct calculation. The reason is that resolution in gradient methods is less influenced by the exact particle diameter than is the case in isocratic methods.

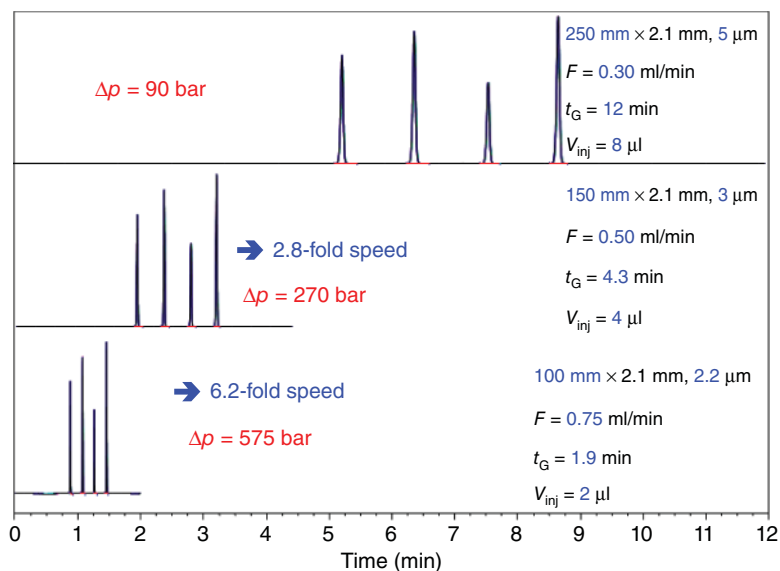


Figure 2.20 Example for a systematic speed-up of a gradient separation. Sample: parabens C_1 – C_4 ; stationary phase: Acclaim 120-C18 (Thermo Scientific, Sunnyvale, USA),

gradient: from 60/40 to 20/80 (v/v) H_2O/ACN with the respective t_G ; temperature: $40^\circ C$; $L/dp = 50$. All other conditions as shown in the figure.

For the discussion on column pressure change, it is necessary to take into account the pressure in a gradient method changes during the run. All pressure values given Figure 2.20 apply to the initial phase of the gradient where the viscosity is at the highest level. Looking at the figures, we could prove with this second example the rule from 2.16 valid. This rule implies an important conclusion for the speed potential of UHPLC in both gradient and isocratic methods:

In a fair comparison based on equivalent resolution and by exploiting both the full pressure capability of a 400 bar conventional HPLC system as well as that of a 1200 bar UHPLC system, the analysis speed will be increased by not more than factor 3.

This finding may take away some of the magic of UHPLC, but in any case it initiates the question what is behind the often reported speed-up factors of 10 and more when going from HPLC to UHPLC. The two major reasons are that the HPLC is not used to the full pressure capabilities (which is often hampered by system volume and detector influences as well), but mostly the fact that the resolution is reduced during the method speed-up (no proper method transfer). This is illustrated in the example given in Figure 2.20.

The fastest separation in Figure 2.20 is taken as a starting point for method optimization. The average resolution of $R_s = 7$ is much higher than required, which allows the column length to be shortened, to 50 mm half of the original column length, L . The results obtained from the shorter column are shown in Figure 2.21.

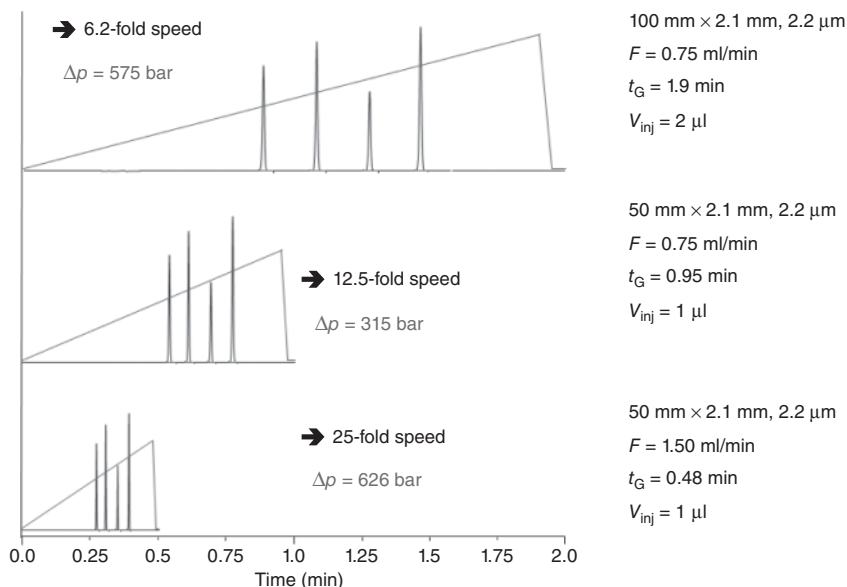


Figure 2.21 Further speed increase of the separation shown in Figure 2.20. A reduced resolution was tolerated here, as L/dp was reduced from 50 to 25.

The gradient time is reduced by half, following the rule mentioned previously (0.85 min instead of 1.9 min). The injection volume is also reduced to half of the original value (1 μ l instead of 2 μ l). Comparing the upper two chromatograms in Figure 2.21, it can be observed that the pressure was reduced to the half, although the separation is twice as fast. This means that there is a drop in the resolution, which goes with the square root of the column length, in this case the resolution is decreased from 7 to 5, still much more than is required. The low pressure encourages a doubling in F and accordingly halving t_G . The resulting chromatogram can be seen at the bottom of Figure 2.21. Since the 2.2 μ m particles exhibit a flat C -term, the resolution is still close to the value of 5. Comparing this chromatogram with the original separation (upper chromatogram in Figure 2.20), it can be seen that a sevenfold pressure increase (from 90 to 630 bar) gave us a 25-fold increase in analysis speed. The run time was reduced from 12 min to half a minute. Nevertheless, this is an unfair comparison and the result of a noncorrect method speed-up, as we reduced the resolution relative to the original chromatogram. In fact, most of these impressive speed-up factors often shown in marketing literature profit from the fact that the resolution in the original separation was way higher than needed. During the “optimization,” the method is not only accelerated, but resolution is sacrificed by working on shorter columns, which gives rise to the much higher speed increase than pressure increase. This example also demonstrates that a 12-fold speed increase with an allowed drop in resolution is easily achievable within the pressure range of a conventional HPLC instrument (middle chromatogram in Figure 2.21 running at only 315 bar). The essence of correct method acceleration in

LC is to increase speed without changing resolution by running smaller particles in shorter columns at higher linear velocity. Considering all the rules, the speed and the pressure are increased as a function of the squared particle diameter (dp^2). Going from a 150 mm column packed with 5 μm particles to a 50 mm column with 1.7 μm and correctly adapting F by factor 3, would result in a ninefold speed increase and ninefold pressure increase. The three times shorter column length also allows a decrease in the solvent consumption by a factor of 3. If the column diameter is also reduced, from 4.6 to 2.1 mm, another fivefold solvent saving is achieved. Hence, the overall solvent saving is by a factor of 15 and thus less than 7% of the original mobile solvents are consumed.

All the rules and formula required for such a speed-up of a gradient method even with reduction of column diameter are summarized in the tables in Section 2.3.8.2. There are also web-based calculators that do all the calculations automatically and the Thermo Scientific Chromeleon Chromatography Data System even has such a calculator embedded in the method editor.

We have shown in Section 2.3.2.2 that analysis times can be reduced just based on a temperature increase, which provides three clear advantages:

- 1) The need of a column with equivalent selectivity but smaller particle size and shorter length as a possible road block is removed.
- 2) Effort and cost to acquire such a column prior to starting the experiment is removed.
- 3) The instrument does not have to support higher pressures.

These advantages are opposed by three important disadvantages of temperature increase:

- 1) Temperature increase only moves the minimum of the van Deemter curve to the right and flattens the C -term, but does not reduce the plate height at u_{opt} . Hence, it does not enable to shorten the column length without reducing resolution and will thus only allow moderate speed-up factors up to 3, but never a factor of 10.
- 2) The temperature change may result in selectivity change and will result in reduced retention. Therefore, the accelerated separation does not necessarily fulfill the resolution requirements.
- 3) The increased temperature, in particular, with water-rich eluents at elevated pH can deteriorate silica based columns relatively fast. So it often would eventually be cheaper to invest into a UHPLC column and run that at temperatures not higher than 40 °C.

A moderate temperature increase can provide a synergistic accompaniment to reduction in particle size to allow speed-up with transfer to a UHPLC-column. The combination of both (starting from a method that runs at ambient temperature) can yield speed-up factors of 20 instead of 10 with just reducing dp and L by a factor of 3.

2.3.4.2 Monoliths and Solid Core versus Fully Porous Phase Materials

It has been shown in the last section how small particles in shorter columns allow systematic method speed-up. It has also been shown that the operating pressure increases with the same factor as the reduction in analysis times. While this appears to be a fair deal, having an understanding of the root cause may enable the identification of even more favorable approaches to reducing analysis times. Smaller particles provide lower dispersion due to the shorter pathways to circumvent them and to diffuse into their internal pore structure. When packed into a dense structure they generate smaller flow-through channels. With these narrower channels, it is comparable to drinking through narrow straws as opposed to wide straws. It takes more suction effort to consume the same amount of beverage over time. The narrow channels generate a higher viscous friction and the same applies in the packing of LC columns. Thus finding an approach to keep the more efficient smaller particles at a larger distance from each other and consequently increase the channel widths would have some benefits. This would combine the incentive of small particles with that of large particles. Another obvious consideration is the search for larger particles with improved efficiency thanks to a different structure. Other alternative concepts are treated in more depth in Chapter 4 with its focus on columns. These are columns based on monolithic stationary phase structure as well as those packed from superficially porous particles that will be referred to as solid core materials for simplification.

Both concepts serve the demand of an improved efficiency to pressure ratio. While monolithic columns in spite of their continuous improvement over time still lag behind on efficiency [13], we find that the solid core materials outperform classical fully porous ones [14] of comparable particle size. Both concepts have two disadvantages though:

- 1) The choice of columns with different bondings and different base materials is still much smaller than that of totally porous particle packed ones. In terms of monolithic columns, this applies even more to the number of different manufacturers.
- 2) The accessible stationary phase surface is smaller in relation to the column volumes, which results in a lower loading capacity and smaller retention factors compared with fully porous materials with an equivalent diameter of diffusion pores.

These two disadvantages are less pronounced with solid core particles than with silica-based monolithic phases. Moreover, the retention behavior of every monolithic column can be considered one of a kind given the manufacturing process for the bonding. There are three more considerations with monolithic columns:

- 1) The structure-inherent higher total porosity (relatively wide channels between the smaller size structure of stationary phase skeleton) leads to a higher V_M at given column size and thus to a higher solvent consumption. The higher permeability encourages increasing the flow rates and this consumes additional solvent during the injection process and the termination of automated analysis runs (delays from the data system).

- 2) With concentration proportional detectors, the absolute peak area decreases with the same factor as the flow rate increases. This leads to smaller peak areas and since proper peak integration may always be a challenge, it can negatively affect quantification accuracy.
- 3) Even with the relatively powerful newer generation of silica-based monoliths, it is a fact that they still lag behind the efficiency (expressed as plate height H) of modern sub-2 μm particulate stationary phases, while the recent small diameter solid-core particles outperform even the most efficient totally porous particle phases.

With respect to a discussion of the practical value of alternative stationary phase architectures, we, therefore, restrict this section to the solid core approach.

Let us look at the method acceleration of a herbicide analysis that starts from a conventional method on a 250 mm column with totally porous 5 μm particles (similar to the example in Figure 2.20). In contrast to using a 2 μm totally porous material in the last stage of the example in Figure 2.20, a 2.6 μm solid core stationary phase with a similar selectivity will be used. If the 2.6 μm material had an efficiency which corresponded to its particle size and strictly following the rules from Section 2.3.4.1, the new column length would need to be 130 mm in order to achieve the same plate number. Since such a column length was not available and instead two options were available, namely a 150 and 100 mm length column. In general, solid core particles provide better efficiency compared with their fully porous counterparts, and so a 100 mm solid core column was chosen to progress the method acceleration. With the assumption that the efficiency of this 2.6 μm solid core material is 2.5-fold better than that of the original 5 μm material, it is also necessary to accelerate the linear velocity by factor 2.5 to maintain the van Deemter minimum. To reduce the amount of solvent consumption the column diameter was reduced from 4.6 to 2.1 mm and due to the fivefold reduced cross section resulted in a reduction in the flow rate of fivefold to achieve equivalent linear velocity. Thus, for the method transfer initially the linear velocity should be increased by a factor of 2.5 to account for the efficiency alterations, and then the linear velocity is divided by a factor of 5 to adapt to the smaller column diameter. This results in a total flow rate conversion factor of $1/2$, resulting in the flow rate being reduced from 1 to 0.5 ml/min. Since the method applies gradient elution, it was also necessary to alter t_G . This must consider the reduction of the column length and the increase of the linear velocity, so the factor is $2.5^2 = 6.3$. The reduction of t_G directly translates into the speed-up factor of the separation. The analysis time is eventually reduced by a factor of 6.3 from 16 to 2.6 min. After finally adapting the injection volume and the detector settings, the method transfer is complete with the resulting chromatogram shown in Figure 2.22. A more thorough comparison between the two chromatograms will reveal that the ratio in retention time does not exactly follow the factor 6.3 for all peaks, but is for most peaks even smaller than 6. So the peaks do not exactly elute at the same gradient composition as this is a consequence of the not perfectly identical stationary phase chemistry and the change in the total porosity ε_T of the column, a fundamental challenge when changing between totally porous and solid core materials.

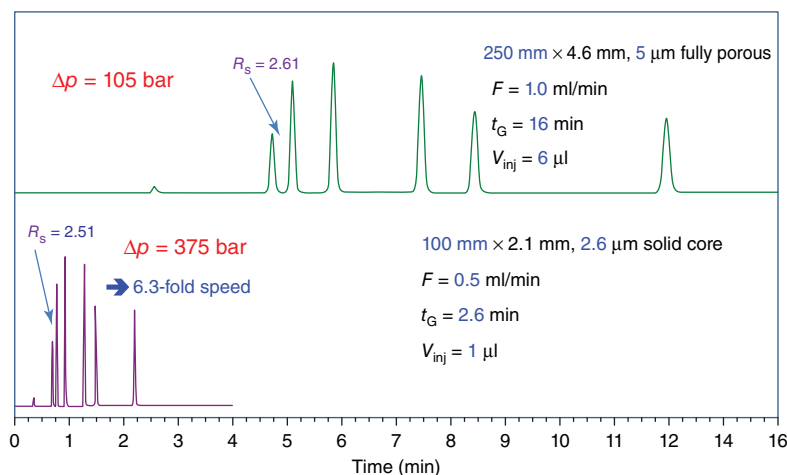


Figure 2.22 Speed-up of a gradient separation with transfer from the fully porous stationary phase Hypersil Gold (Thermo Scientific, Runcorn, UK) to the solid core material Accucore RP-MS (Thermo Scientific, Runcorn, UK). Gradient from 65/35 to

40/60 (v/v) $\text{H}_2\text{O}/\text{ACN}$ in the respective t_G ; temperature: 30°C ; peaks in order of elution: tebuthiuron, metoxuron, monuron, chlorotoluron, diuron, linuron. All other conditions as shown in the figure.

To assess the quality of the new method, the resolution for the critical pair was analyzed. In this case, the critical pair are the first two peaks: Tebuthiuron and Metoxuron. A drop in resolution from 2.62 to 2.52 was observed during method optimization; however, the new method still meets or even exceeds the regulatory requirements for resolution. It must be acknowledged that the chromatogram pattern and peak resolution could not be exactly transferred; however, a very similar separation was achieved in only 16% of the analysis time and with 90% solvent savings.

The most interesting aspect of this method speed-up example is the change in column pressure though. In the original method, a pressure of 105 bar was observed at the start of the gradient. Following the rule shown in Eq. 2.16, it would be expected to observe a 6.3-fold pressure increase associated with a respective speed increase. Actually, it can be seen in Figure 2.22 that the pressure increased by less than a factor of 3.6—375 bar. Relative to the method transfer to a $2.2\text{ }\mu\text{m}$ totally porous material with the same reduction in column length, more than 40% pressure increase has been saved. This proves the outstanding potential of these sub- $3\text{ }\mu\text{m}$ solid core materials. They allow for a sixfold speed increase accompanied by a roughly threefold pressure increase, but without relevant loss in resolution.

Before closing this section, it is appropriate to give a short insight into the overall market perspective of superficially porous or solid core materials. While the related fundamental concept is as old as HPLC (Horvath reported in 1967 the use of surface etched glass beads for the first published HPLC separation [15]), their broad-based commercialization is relatively new and evolved predominantly over

the past 10 years. In fact, the efficiencies associated with earlier versions were not very good and so there was not much uptake of the technology. Meanwhile, there are many different materials of this type available from a multitude of vendors under product brands such as Accucore (Thermo Scientific), Ascentis Express (Sigma Aldrich), Cortecs (Waters), Halo (Advanced Material Technologies), Kinetix (Phenomenex), Poroshell (Agilent), Raptor (Restek), or Titan (Sigma Aldrich) just to list the most important brands in alphabetical order. While the range of selectivities still lags somewhat behind that offered with totally porous materials, there is now a decent choice of solid core-based different stationary phases. It is also noteworthy that several solid core materials also exist nowadays in a sub-2 μm format (Accucore Vanquish 1.5 μm , Cortecs 1.6 μm , Kinetix 1.3 and 1.7 μm , Titan 1.9 μm). With these smaller particle diameter phases, the domain of solid core particle-based columns now surpasses the totally porous particle-based column on efficiency [16]. There is a trend observed that the efficiency advantage of solid core materials over fully porous ones of equivalent size decreases with the particle diameter [17]. There is another potential advantage associated with these particles though, and this is their generally increased mechanical stability over totally porous materials. Specifically, the use of particle diameter much lower than 2 μm when packed into columns of 100 mm or greater in length generates an extreme pressures to achieve the van Deemter minimum. While the recent UHPLC instruments can go far beyond 1000 bar (currently up to 1500 bar), it is a challenge to manufacture stationary phases that provide acceptable endurance under such conditions. Here, the sub-2 μm solid core materials may be the format of choice, but this is still an ongoing debate. Still under investigation is also the fundamental understanding of the performance advantage of solid core materials [14] and with that all the related quantitative predictions as a function of particle size that would be very helpful to design parameter calculators for method speed using solid core materials. We can expect more to come in this field.

2.3.5

Optimizing Resolution by Particle Size and/or Column Length

We have seen with the speed-optimization in Section 2.3.4 that the particle size dp is the predominant parameter to influence the plate number, N , being the primary descriptor for efficiency to enable kinetic improvement of HPLC methods. Temperature can also change kinetic parameters in HPLC, but to a smaller extent than particle size and it simultaneously changes thermodynamic parameters such as retention and can sometimes even alter the selectivity. Kinetic optimization is mostly about increasing speed of analysis by providing a method that generates the same N in a shorter time. We now see how an increase of N through column parameters can improve resolution and what considerations have to be made.

Understanding the influence of L , dp , and u on N and Δp gained from Section 2.3.4.1, it becomes apparent that there are three different ways to increase the plate number N together with the related implications:

- 1) Increase of the column length L which leads to equivalent increase in N . As the linear velocity u must be kept constant. Hence, the analysis time increases with the same factor as L .
- 2) Reducing the particle size dp , which leads to increase in N by the same factor. At constant u an increase in Δp by the squared change of dp would be observed. As we need to adapt u to the smaller Δp in order to stick to $H_{\min}$, we further increase the pressure, but also reduce the analysis time relative to the original method.
- 3) A well-matched combination of reduction in dp and increase in L . In fact, it is this the only way to increase N without changing the analysis time, as the increased L can be compensated by the increased u (provided availability of the required combination of L and dp).

The three options are discussed using example generated through numerical simulations which generate the chromatograms presented in Figure 2.20. This figure depicts a chromatogram with a peak triplet with a poor resolution of 0.94 for both adjacent bands.

In Section 2.2.1, Eq. 2.5 that relates the resolution to the square root of the plate number ($R_s \sim \sqrt{N}$) was discussed. This rule is instrumental for the prediction of the resolution gain in the examples shown in Figure 2.23c and d. They all have in common that the ratio of L/dp was increased from 20 to 50 relative to the original method. So the resolution will increase by factor $\sqrt{2.5} = 1.58$ from $R_s = 0.94$ to 1.5. In Figure 2.23b, this was achieved simply by increasing L from 100 to 250 mm. This results in a relatively moderate pressure increase from 40 to 100 bar (the change in pressure being directly proportional to the change in column length, L). Simultaneously the analysis time increased 2.5-fold and the related 1.58-fold dilution (peak volume grows with $\sqrt{L}$) makes the peaks broader and lower in height. The overall result is not very appealing, although we could achieve the target resolution of 1.5. The chromatogram shown in Figure 2.23 appears much more appealing. Here, L is kept constant and dp was reduced 2.5-fold from 5 to 2 μm accompanied by the required 2.5-fold flow rate increase from 1.5 to 3.75 ml/min. As a result of these changes, the target resolution was achieved in a fraction of the original analysis time, but the price for this is a 15.6-fold pressure increase from 40 to 625 bar. As already mentioned, the simultaneous and matched change of L and dp is the only way to achieve a resolution increase without changing the analysis time. This is shown in Figure 2.23d, which is obtained from the simulation using the practically best possible scenario. Here, L was increased 1.5-fold from 100 to 150 mm, accompanied by a 1.67-fold decrease in dp from 5 to 3 μm . Hence, the increase in L approximately compensates for the required increase in u . The analysis time is only increased by 10% here, but at the expense of a 6.9-fold pressure increase from 40 to 275 bar (however, still in the range of a conventional HPLC instrument).

Next, the quantitative relationship between resolution and pressure will be reviewed but allowing for a fair comparison. Very simplistically, to achieve a twofold resolution increase it is necessary to increase L by factor 4. In order to perform the analysis in the same time, it is necessary to also increase F (ignoring

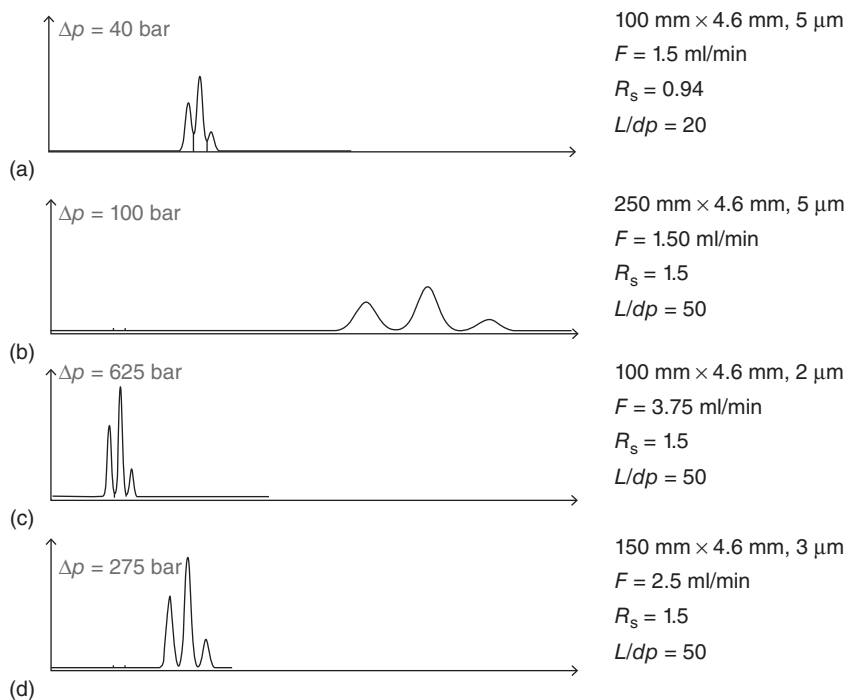


Figure 2.23 Simulated chromatograms of an original poorly resolving method ($R_s = 0.94$) showing three different approaches to increase the plate number by factor 2.5 and thus the resolution by factor 1.67. Note that the analysis time and column pressure dif-

fers significantly. The virtual mode of the Chromeleon (Thermo Fisher Scientific, Germany, Germany) UCI-50 A/D interface control was used for the chromatogram simulation.

efficiency drop from a C -term effect) by factor 4. This modification results overall in a 16-fold pressure increase for kinetically doubling the resolution and with the conclusion being that Δp increases with R_s to the power of 4, as expressed in Eq. 2.17. Even when L , dp , and u are optimized to maintain the van Deemter minimum, Eq. 2.17 remains valid for resolution improvement at constant analysis time.

$$\Delta R_s \sim \Delta p^4 \quad (2.17)$$

This finding is relatively sobering, but pressure capabilities of modern UHPLC instruments do allow the user a greater leeway in resolution due to the extra pressure capacity. It is obvious that the use of sub-2 μm materials will provide even higher potential for resolution increase. Several constructed scenarios are compared in the next section (listed in Table 2.2) to demonstrate how this may appear in practice. u always has to be increased by the same factor as dp decreases to maintain the optimum efficiency, which induces tremendous pressure increases. At constant column length the pressure increases with the third power of the

Table 2.2 Example for the attempt to improve resolution through efficiency increase when transferring from HPLC to UHPLC. All numbers are theoretically modeled on the basis of the artificial method #1 (*method #2 is not feasible in practice).

Method	dp (μm)	L_{column} (mm)	F (ml/min)	N (plates)	t_{analysis} (min)	R_s	Δp (bar)
M1	5 _{FullyPorous}	100	0.25	6 000	10	1.0	100
M2*	1.7 _{FullyPorous}	100	0.75	18 000	3.3	1.7	2 700
M3	1.7 _{FullyPorous}	50	0.625	9 000	2	1.2	1 125
M4	2.5 _{SolidCore}	250	0.625	~45 000	10	2.5	1 000
M5	2.5 _{SolidCore}	150	0.625	~30 000	6.7	2.0	670

particle size reduction, while N and the analysis speed increase linearly. Some example figures of obtained during such an optimization are shown as methods M1 and M2 in Table 2.2. Starting with method M1 running on a typical conventional column generating a back pressure of 100 bar (for the sake of simplicity), an analysis time of 10 min and a resolution of $R_s = 1.0$, due to the poorly resolved critical peak pair. From method M2, it can be seen that maintaining a constant L , as the particle size is changed from a 5 to 1.7 μm yields threefold reduction in the analysis time, 1.7-fold resolution boost ($R_s \sim \sqrt{N}$) but at the price of 27-fold pressure increase. As the 2700 bar are in no way feasible on commercial instrumentation, the only solution is to shorten L and slightly the decrease flow rate (M3). While this brings the pressure into a commercially viable operational range (~ 1100 bar), the related plate number increase only translates into $R_s = 1.2$, still insufficient for practical requirements.

However, an alternative approach can be considered using superficially porous particles (dp of 2.5 μm for simplicity) run at 2.5-fold higher u (or F) and the reasonable assumption that a similar efficiency increase as with changing to 1.7 μm totally porous particles occurs. As optimizing the analysis time is not the goal, the column length is increased to 250 mm by the same factor as the eluent velocity (this was practically impossible with the 1.7 μm material) was increased. The calculated result is shown as M4 and yields $R_s = 2.5$ (more than required) with the same analysis time as method M1 and still at a practically very feasible Δp of 1000 bar. Moving on to M5 finally yields the perfect resolution ($R_s = 2.0$) combined with a 33% reduction in analysis time but still very much in the pressure comfort zone of modern UHPLC instruments (670 bar). These observations allow for three principal conclusions:

- 1) It is much more effective to increase selectivity for resolution improvement in LC, as was already concluded in Section 2.3.1.1.
- 2) If an increase in resolution and not a reduction in analysis time is the goal, then the focus should be on a moderate reduction in dp and use the required increase of u to run a longer column at similar analysis time.
- 3) As superficially porous particles with diameters between 2.5 and 3 μm can provide similar efficiencies to fully porous sub-2 μm ones, they may offer best potential for kinetic resolution boost (provided the selectivity fits).

2.3.6

High-Resolution 1D-LC and 2D-LC to Fully Exploit the Potential

The term high-resolution LC is not generally defined. It has been shown that the kinetic resolution potential of a chromatographic method correlates with the square root of the achievable plate number for the given analytes. However, there may be different approaches on how to realize this resolution potential. When faced with the challenge of separating two compounds when no method with $\alpha > 1.05$ is available, it is necessary to have $N > 20\,000$ to obtain the required R_S . If selectivity is as low as $\alpha = 1.02$, then $N \approx 100\,000$ will be needed to gain an adequate separation. An example for an extreme scenario could be the chromatographic separation of isotopes, where even $\alpha \approx 1.01$ is difficult to achieve. Addressing such a challenge with several hundred thousand plates could be a justification for ultra-high-resolution chromatography.

Much more common in the lab than those cases of two compounds that are extremely difficult to separate is the challenge of analyzing very complex mixtures. In such a complex mixture, it will be impossible to tune individual selectivities. These will be situations where a higher selectivity at a distinct part of the chromatogram comes at the expense of another part of the chromatogram. This is an inherent issue if a high number of substances must be separated from a complex sample, because it is generally assumed that all parts of the separation space have an equal number of components. Any chromatographic method can be assessed for a theoretical number of compounds it can separate in the best case. This is done by the so-called theoretical peak capacity n_C and considers perfectly equidistant peaks at a resolution of 1.0 for all adjacent bands. It is the maximum number of compounds that can be separated by the respective method under these prerequisites. The theoretical peak capacity n_C can be calculated based on chromatography fundamentals that already have been introduced and is in general proportional to $\sqrt{N}$.

The peak capacity in isocratic separations $n_{C,i}$ is a function of the plate number N and the retention factor of the last eluting peak k_i , calculated according to 2.18:

$$n_{C,i} = 1 + \frac{1}{4} \cdot \sqrt{N} \cdot \ln(1 + k_i) \quad (2.18)$$

The calculation of peak capacity in gradient elution $n_{C,G}$ is much more complicated and we do not derive the exact mathematical expression here. The relevance of peak capacity in gradient is much higher though, since significantly better values can be achieved thanks to the fact that peak width does not increase significantly with retention increase, as is the case in isocratic separations. For a good estimation of the peak capacity, Eq. 2.19 can be used:

$$n_{C,G} \approx 1 + \frac{1}{4} \cdot \sqrt{N} \cdot \frac{10}{1 + 10 \frac{t_M}{t_G}} \quad (2.19)$$

It becomes obvious that the relative gradient volume, the ratio of t_M to t_G , adopts a similar role as t_i in isocratic separations. From Eq. 2.18, it can be concluded that the maximum achievable peak capacity in a reasonable analysis time using

isocratic elution is close to the square root of the plate number, N . This level is reached at $k_i = 50$, while doubling the analysis time to $k_i = 100$ will only increase $n_{C,i}$ by another 17%. This is much more favorable in gradient elution, where with a $t_G/t_M = 50$ an $n_{C,G} \approx 2 \cdot \sqrt{N}$ is achievable. Gradient elution also permits a peak capacity corresponding to $\sqrt{N}$ at $t_G/t_M = 7$ to be achieved, which is seven times as fast at generating the same peak capacity as that obtained isocratically. Finally, it can be concluded from Eq. 2.19 that even in very shallow gradients a peak capacity of $2.5 \cdot \sqrt{N}$ cannot be practically exceeded. Finally, it can be concluded that about 160 000 theoretical plates are required to achieve a peak capacity of 1000 in an extremely shallow gradient ($t_G/t_M > 1000$). 160 000 plates are quite a challenge even for UHPLC and the resulting analysis time from $t_G/t_M > 1000$ would not be very appealing. Hence, even $N = 250\,000$ is required to achieve $n_C = 1000$ with a reasonable gradient slope of $t_G/t_M = 50$.

As a take home message for estimation of peak capacity, we should remember the following:

- 1) The theoretical peak capacity is proportional to the square root of the plate number of the column in use.
- 2) In a $t_G/t_M = 50$ gradient method, it is necessary to have 2500 plates to achieve $n_C = 100$; 16 000 plates to achieve $n_C = 250$, and 250 000 plates to achieve $n_C = 1000$.

2.3.6.1 The 2D-LC Approach to Increase Peak Capacities Beyond These Limits

A 150 mm long column packed with sub-2 μm particles will, even in the best case, generate no plate number larger than 40 000. Does this then mean that even modern UHPLC cannot support peak capacities larger than 400? This is clearly not the case but nevertheless it is important to investigate approaches to advance LC toward such high peak capacities. A very effective way to boost peak capacity further is to add another dimension of separation, in other words to advance from 1D-LC to 2D-LC. In the ideal case, the peak capacities of the two individual dimensions multiply for the total peak capacity of a 2D-LC method, following Eq. 2.20.

$$n_{C,2D-LC} = {}^1n_C \cdot {}^2n_C \quad (2.20)$$

This would imply that by simply combining two methods with $n_C = 100$, a 2D-LC method with $n_C = 10\,000$ could be achieved. The practical implementation of 2D-LC, however, is not a separation in true two-dimensional space, as is seen in gel electrophoresis or from a 2-step orthogonal development on a thin-layer chromatography plate. 2D-LC in practice is based on cutting fractions from the separation in the first dimension and injecting them subsequently into a second dimension. This can be done in a manual or automated way. In the automated implementation, one distinguished between the online and the offline variant. The online variant is characterized by parking the fraction in a loop, from which it is directly switched to a second dimension. In a standard type setup, this implies a perfect time match between cutting fractions from the first dimension and analyzing them in the second. This challenge can be partially overcome by using a

storage loop array for a higher number of fractions from the first dimension. In the offline mode, the fractions are collected in vials and from them a certain aliquot is re-injected into the second dimension. Specialized autosamplers that can both fractionate and reinject allow this fully automated without using an extra module. The application of such a setup for proteomics workflows is described in a paper from Eeltink *et al.* [18], which also presents peak capacity in comparison to 1D-UHPLC methods. The instrumental prerequisites and also the commercially available solutions can fill a decent monograph, which is completely outside the scope of this chapter. Users who are interested in further details may refer to more specialized textbooks [19].

What should be covered though are the prerequisites for the validity of Eq. 2.20. It is necessary to fulfill three important criteria to take full advantage of the peak capacity from both dimensions:

- 1) The two combined methods must exhibit completely independent separation criteria, or in other words they must have orthogonal selectivities.
- 2) The complete 2D retention space must be occupied, which means that the peaks must spread from the lowest to the highest retention (in practice from the start to the end of the gradient) in both dimensions.
- 3) The number of fractions taken from the first dimension must reflect its separation performance adequately. The statistically required minimum oversampling equals two fractions per theoretical peak volume 4σ according to the Murphy–Schure–Fowley rule. This implies a number of fractions that is twice as high as the peak capacity of the first dimension.

An example for two automated offline 2D-LC method will now be reviewed and a discussion had on how well the three rules are respected in these examples. After that the challenges associated with translating theoretical peak capacity into practical analytical value is discussed.

In Figure 2.24, a 2D retention map, as the chromatography software Chromeleon™ (Thermo Scientific, Germering, Germany) displays it, is shown. The separation was done in the automated offline mode hence the fractions cut from the first dimension were collected in well plates and subsequently automatically injected into the second dimension by the specialized fraction collection autosampler. In the 2D retention map report view, the chromatograms from the second dimension are vertically arranged from bottom to top and positioned at the retention times on the *x*-axis that correspond to the time point when the respective fraction collection was finished. To create the closed spots from each peak, the software mirrors the chromatograms vertically and fills the resulting spots with color with an intensity proportional to the peak height. This creates a similar picture as the protein scientists are familiar viewing with 2D gel electrophoresis.

The 2D-separations will be analyzed and discussed further. The separation shown in Figure 2.24a combines a salt gradient-based SCX separation in the first dimension with a reversed-phase separation running at pH = 1.9 in a TFA-based water–acetonitrile gradient in the second dimension. The separation

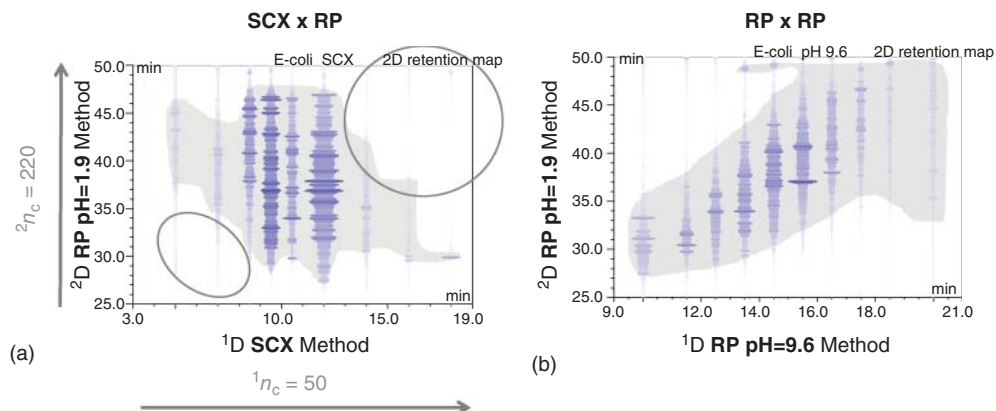


Figure 2.24 Automated off-line 2D-LC separation of an *Escherichia coli* tryptic digest. Ten fractions of 1 min were collected from first dimension. First dimension run on 300 μ m id, 150 mm length capillary columns at 6 μ l/min flow, second dimension run on 75 μ m id, 150 mm length nano columns at 300 μ l/min flow. SCX column was a PolySUL-FOETHYL Aspartamide 5 μ m (PolyLC Inc.) run

in a phosphate buffer at pH=3 with salt gradient up to 0.6 M NaCl in 15 min. All RP columns were Acclaim PA2 5 μ m (Thermo Scientific). First dimension RP was run at pH=9.6 with triethylamine and gradient to 50% acetonitrile in 15 min. Second dimension RP (both methods) was run at pH=1.9 with TFA and gradient to 50% acetonitrile in 30 min.

in Figure 2.24b combines a reversed-phase separation running at pH=9.6 in a triethylamine modified water–acetonitrile gradient with the equivalent pH=1.9 RP separation from method (a) in the second dimension. Comparing the patterns, it can be seen that the peaks appear more or less statistically distributed in the SCX $\times$ RP method, as shown in Figure 2.24a. If the retention criteria of both dimensions would be correlated, we would see the peaks arranged close to a diagonal line originating from the origin and with a gradient of 1. Looking at the RP $\times$ RP method shown in Figure 2.24b it can be seen that such a diagonal pattern is present. This suggests that orthogonality is better fulfilled with the SCX $\times$ RP method than with the RP $\times$ RP, which is not surprising. The first prerequisite appears well fulfilled with SCX $\times$ RP method shown in Figure 2.24a. The next question addresses the coverage of the retention space in this method. The two ovals in Figure 2.24a demonstrate that there are spaces at bottom left, and even more pronounced at top right where hardly any peaks are located. From this, it must be concluded that the second prerequisite is only partially fulfilled. Based on the individual peak capacities (shown on the arrows in Figure 2.24a) and applying Eq. 2.20, it would be expected a total peak capacity of $^1n_c = 50$ times $^1n_c = 220$ resulting in 11 000 to be observed. The fact that the retention space is not fully covered will reduce this number. The third prerequisite, namely the proper oversampling from the first dimension will now be discussed in detail. For the SCX method a peak capacity of $^1n_c = 50$ was calculated, so following the Murphy–Schure–Fowley rule it is necessary to transfer a minimum of 100 fractions. This would imply cutting a fraction every 6 s and running subsequently

100 separations with 30 min gradients. Taking the column re-equilibration of another 6 min into account, this would result in a total analysis time of 3600 min (60 h). Such conditions appear to be not very practical and mainly for this reason only one fraction was collected every minute resulting in a total of 10 fractions from the first dimension. This is by far insufficient to secure the theoretical peak capacity from the SCX separation. Following the Murphy–Schure–Fowley rule, it can only be assumed, in a best-case scenario, an effective peak capacity equal to half the number of fractions, namely 5. So the maximum total peak capacity considering $^2n_C = 220$ from the second dimension would be 1100. This is only one-tenth of the $n_{C,\text{total}} = 11\,000$ value that we calculated based on fully exploiting the first dimension and the complete retention space. The retention space coverage will be discussed later; however, initially important conclusion from the data and facts presented above can be made:

The theoretical peak capacity of the first dimension in a 2D-LC method (no matter if performed online or offline) is relatively meaningless in practice.

To discuss this further, a scenario where equivalent peak capacity of $n_C = 100$ in both dimensions that would yield $n_{C,\text{total}} = 10\,000$ and under ideal conditions is discussed. Ideal conditions would imply transferring 200 fractions from the first dimension, as has already been shown the Murphy–Schure–Fowley dictates the number of fractions is $2 \times {}^1n_C$. It will now be assumed that challenge to provide a UHPLC method in the second dimension that can provide $n_C = 100$ with total cycle time (injection, separation, column re-equilibration in case of full gradients) of 4 min can be solved. Even if this is achieved, the total analysis time to run the 200 fractions would sum up to 800 min (200×4 min), thus more than 13 h. While it is obvious that the second dimension has to be fast, it is still much more beneficial to increase the peak capacity there, but reduce the number fraction from the first dimension. Especially with very steep gradients, where the cycle time is very much influenced by column re-equilibration. Radically optimizing the second dimension for peak capacity per unit time can bring peak capacities in the range of $n_C = 200$ in 6 min, when using a 150 mm column packed with a material that generates $N = 50\,000$. This is an extreme challenge, but feasible today. At pressures above 1200 bar and with use of increased temperature a $V_G/V_M = 15$ is possible with such a column, which enables $n_C = 200$ in 6 min. It is, therefore, possible to generate $n_{C,\text{total}} = 10\,000$ with ${}^1n_C = 50$ from the first dimension and this would need 600 min (100×6 min) total analysis time. This is still 10 h, but a clear productivity advantage over the approach with equivalent n_C in both dimensions.

The practical implementation of faster 2D-LC methods builds on reducing the number of fractions from the first dimension to a reasonable level of 30–50 (yielding maximum effective peak capacity ${}^1n_C \leq 25$), and to further reduce the analysis time in the second dimension by using, for example, very effective 30 mm solid core columns or 50 mm second-generation silica monolith columns [20]. These methods allow analysis times of less than 2 min per second dimension run, but only yield peak capacities below 100. Depending on how the first dimension is implemented, the related 2D-LC methods can be run in a total analysis time of not more than 1 h, but would hardly exceed a total peak capacity

of 1000. If 50 fractions are run into a fast ${}^2n_C = 200$ method, the total peak capacity achievable in 5–6 h would be 4000, which can be considered a practical limit for 2D-LC runs in acceptable analysis times. There are several different approaches to combining the gradients in the two dimensions and there is a wide performance range that is spanned by the different ways to execute the second dimension, which must also be matched to the molecular size of the analytes. An excellent overview on these possibilities is presented in a recent review article [21].

If the first dimension of 2D-LC method is radically under sampled, as is relatively common in offline 2D-LC methods, a consideration that differs markedly from the commonly accepted way of assessing appropriate sampling rates can be made. The separation in Figure 2.24a is discussed to exemplify this point. The given SCX method generates peaks with a $\sigma_t = 3$ s, so one fraction of 1 min corresponds to $20 \times \sigma_t$. The probability that the maximum of a randomly located peak zone in this volume fraction is less than $1 \times \sigma$ away from the fraction start or end is less than 5% and this suggests that it is unlikely that a peak significantly smears over more than one fraction. At such a degree of undersampling in the first dimension, it could be justified to consider the number of fractions equivalent to the effective peak capacity contribution from the first dimension. This approach would claim in no respect that the separation from the first dimension is appropriately reflected in the graphical representation of the 2D chromatogram. It only claims that the probability of a substance zone to significantly spread over two fractions is relatively low. This consideration is also the foundation for the validity of the 2D retention maps shown in Figure 2.24 to graphically represent 2D-LC separations. They display the zones from two different fractions as separated nonidentical compounds. At the given level of undersampling there is a sufficiently high probability that this assumption is valid.

The discussion on how peak capacity can translate into practical value for the analyst will now be reviewed. The examples shown in Figure 2.24 will again serve for that. The peak capacity contribution from the first dimension is now considered to be equivalent to the number of fractions, so we assume ${}^1n_C = 10$. Even more important is the fact that both the SCX method shown in Figure 2.24a and the alkaline RP method shown in Figure 2.24b will contribute the same peak capacity of ${}^1n_C = 10$, no matter whether their individual theoretical efficiencies differ. They are both limited by the same number of fractions and assuming they are sufficiently undersampled, considerate is reasonable to assume ${}^1n_C = n_{\text{fractions}}$. As the second dimension is identical in both methods, the outcome would be that both methods should provide a total peak capacity of $10 \times 220 = 2200$. The second dimension nano-LC was run into an ion trap mass spectrometer, which allows identification of the respective peptides and construct the original proteins with the help of appropriate software tools. The details on this are outside the scope of this chapter, but typical workflows are common to proteomics experts. It is assumed that for the given discussion that the number of identified proteins is a good measure for the analytical value of both methods.

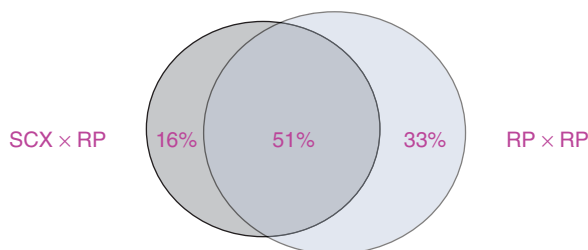


Figure 2.25 Venn diagram to compare both 2D-LC methods presented in Figure 2.24 with respect to identified proteins. The percentages are calculated relative to the total number of identified proteins by combination of both methods, but not relative to the number of proteins in *E. coli*.

The result of the method comparison regarding identified proteins is graphically presented in a Venn diagram shown in Figure 2.25. The RP $\times$ RP helped to find 84% of the total number of identified proteins, while the SCX $\times$ RP method only enabled 67% to be identified. It is also noteworthy that 16% of all identified proteins could solely be found with the apparently less powerful SCX-RP method. This shows that both methods were complementary to a certain extent. We now want to characterize the two methods from Figure 2.24 in more detail. We already discussed that the peak pattern in Figure 2.24a speaks for a more orthogonal method than the one in Figure 2.24b. Yet, was the RP $\times$ RP method (b) more successful for identification of *Escherichia coli* proteins. The next step is to determine the retention space coverage achieved in both separations. There are several methods published for its calculation [21] where the binning method according to Gilar [22] is a quite popular one. A very simplistic approach is applied, similar to the binning method that accounts for the consideration of the 10 fractions from the first dimension as 10 discrete spaces. The determination of retention space coverage was simply done by measuring the length portion of signals above a certain threshold in each of the 10 individual traces. To achieve the total coverage for the entire method the average of these 10% was calculated. How this percentage space coverage can be envisioned is illustrated by the gray transparent shapes in Figure 2.24 that surround the occupied spaces. The quantitative results yielded a surface coverage of 58% for the RP $\times$ RP method, but only 47% for the SCX $\times$ RP method. If these coverage levels with the theoretical peak capacity of 2200 are multiplied for both methods, it can be concluded that it is possible to have a practically effective peak capacity of 1280 for the RP $\times$ RP method and an effective peak capacity of 1040 for the SCX $\times$ RP method. So the effective n_C of method (b) is 24% higher than that of method (a). Comparing the percentages of identified proteins, namely 84% versus 67% it can be seen that the analytical success measured like this is 25% higher with method (b) than with method (a). Hence, it may be concluded that the retention space coverage correction is a meaningful way to determine effectively 2D-LC peak capacities that appear to translate systematically into analytical value.

2.3.6.2 Boosting Peak Capacity in 1D-LC Further and How This Translates into

Analytical Value

After the quite sobering conclusions from the previous section that it is difficult to generate really high peak capacities in 2D-LC within reasonable analysis times, approaches to increasing the peak capacity in a single LC-dimension beyond $n_C = 500$ (representing an approximate value to achieve on a 150 mm long 1.7 μm column in gradient mode) will be considered. Peak capacities can be increased by increasing plate numbers and plate numbers can be increased by extending column lengths. If longer columns packed with respective stationary phases are not available, there is another approach that can be used to increase L without changing the stationary phase. If a coupling device that generates no measurable additional band broadening is available, this can be used to simply couple two or more columns in a similar fashion to a daisy chain. This also allows a thermostatted separation path length of several times the maximum length that would generally fit into a column compartment. As has previously been discussed, extending L significantly will also lead to a pressure increase. So the question is whether these column lengths can still be operated in the van Deemter minimum using commercially available. This question will not be discussed in greater detail in this chapter, but examples of using extreme separation path lengths by coupling LC columns will be presented.

Initially, a 1000 bar instrument that can just handle methods above 800–900 bar for routine use, which means that it is not possible to run very long sub-2 μm columns. The first example to be reviewed will be based on a column chain of four 250 mm columns packed with 3 μm stationary phases, with $L = 1000$ mm and $N \sim 130\,000$, which was experimentally determined for this arrangement [23]. It can be calculated, with the help of Eq. 2.19, that with a gradient method, theoretical peak capacities of 800–900 could be achieved based on such a plate number. The resulting chromatogram obtained with such a daisy chain of four 250 mm columns packed with 3 μm material is shown in Figure 2.26. From the averaged base widths of isolated peaks across the entire gradient a theoretical peak capacity of $n_C = 925$ could be determined. This fully confirms the expectation based on calculations from the plate number. The analysis time is significant though, as the gradient volume required for this resolution ($V_G/V_M = 35$) results in a total analysis time of 5 h.

In the RP $\times$ RP separation shown in Figure 2.24, with proper consideration of the retention space coverage, an effective peak capacity of 1200 in an analysis time of 10 h could be achieved, which corresponds to a peak production rate of 2 peaks/min. The 1D-UHPLC chromatogram shown in Figure 2.26 with complete use of the retention space provides a 50% higher productivity of 3 peaks/min. Moreover, the much higher instrumental effort to execute the 2D-LC in Figure 2.24 must also be considered.

Similar to the attempt of significantly increasing the peak capacity per unit time in 2D-LC with a respectively productive UHPLC method in the second dimension, boosting the effectiveness of 1D-UHPLC in order to separate such complex samples faster, can also be considered. Knowing that today UHPLC instruments

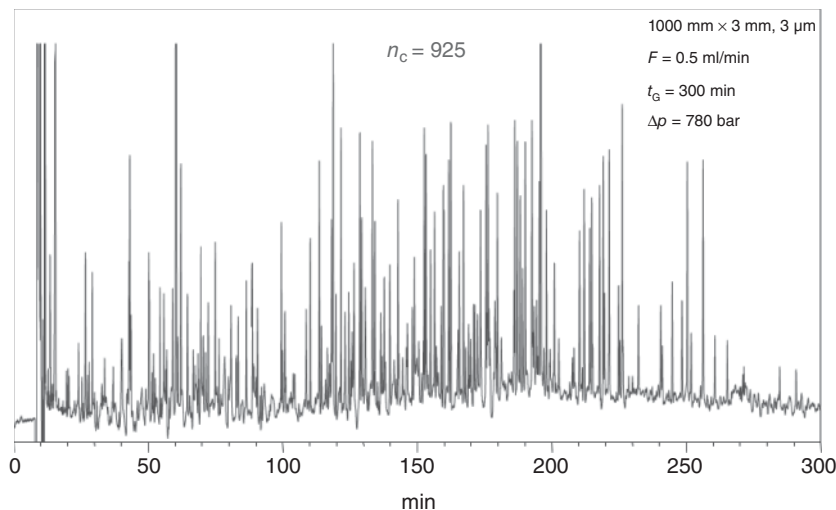


Figure 2.26 High resolution 1D-UHPLC separation of a tryptic digest from a mixture of five proteins. A daisy chain of four 250 mm columns was coupled with zero dead volume Viper couplers (Thermo Scientific, Ger-

mering, Germany). Stationary phase: Acclaim 120 C18, 3 μm (Thermo Scientific, Sunnyvale, USA), temperature: 30 $^{\circ}\text{C}$, $t_{\text{G}} = 300$ min, n_{C} calculated from peak width of well resolved peptides.

can easily support 1200 bar methods, it is possible to increase the flow rate in the method in Figure 2.25 to 0.75 ml/min (would correspond to 1200 bar) and shorten the gradient time respectively to 200 min ($t_{\text{G}}/t_{\text{M}} = 35$, as before). The van Deemter characteristic of the material would easily allow for the resulting linear velocity and if everything scales according to the theory, the resulting productivity rate would be 5 peaks/min. Nevertheless the experiment was not executed as the pressure of 1200 bar would have resulted in the Acclaim columns being operated far outside of their pressure specification range.

It is very obvious that the elevated pressure capabilities of recent high end UHPLC instruments offer opportunities to achieve extremely high peak capacities in very reasonable analysis time. The question is what the right stationary phase format would be to use with these instruments. One conclusion that can be drawn with the help of kinetic plots is that higher pressure capabilities allow the use of smaller particle sizes for extremely complex analyses. This is because these stationary phases can then be very effectively operated in longer columns or column chains. An interesting particle size for such an attempt may be the 2.2 μm format, which is in the middle between 3 μm and sub-2 μm phases with respect to pressure permeability. At the same time, it can provide significantly higher efficiencies than 3 μm material. The approach of generating high peak capacities very fast on 2.2 μm phases will be elucidated with the help of a natural compound analysis example, more specifically a Traditional Chinese Medicine (TCM) sample.

Similar to most natural products, TCM samples are normally very complex and can contain hundreds of compounds that are relevant for a screening assay to characterize them. Figure 2.27 demonstrates how the use of $2.2\ \mu\text{m}$ particles combined with extended column lengths can facilitate such challenging analyses. The original method on a 250 mm column with gradient duration of 18 min yielded a peak capacity of $n_C = 590$ and created a pressure of 915 bar at the start of the gradient. To fully exploit the 1500 bar pressure capability of the Vanquish UHPLC system, the column length was extended to 400 mm and the gradient time, respectively, to 29 min. It was hoped that higher peak capacities could be generated by better exploiting the pressure capabilities of the Vanquish UHPLC instrument. The resulting pressure at the gradient start now increased to 1360 bar, but an impressive peak capacity of $n_C = 720$ could be determined for this method. While $n_C = 720$ may be considered not too impressive relative to the $n_C = 925$ shown with the method in Figure 2.26, it is worthwhile to note that the analysis time was reduced 10-fold from 300 to 30 min. If the production rate is calculated, 24 peaks/min is achieved which is definitely a big improvement from all the methods discussed earlier, regardless of whether 1D-LC or 2D-LC.

The observed increase in peak capacity of 22% matched the theoretical expectation of a peak capacity increase with the square root of the column length extension under respective gradient adaptation ($\sqrt{(L_2/L_1)} = \sqrt{(40/25)} = 1.26 \approx n_{C2}/n_{C1} = 720/590 = 1.22$). A zoom into a particular part of the chromatogram shows how the improved method provides better resolution (Figure 2.27). It can be seen how peak 1 is split into two peaks and how some other peaks are getting sharper and resolution increases, while peaks 2 and 3 actually lose resolution. It is important to be bale to characterize the overall benefit of the method with increased peak capacity and to identify an approach, which can be used to quantify the benefit in a screening method where peaks are not identified. Table 2.3 shows some figures of merit to compare both methods. Under defined integration parameters, the overall number of peaks, as well the number of peak pairs with a certain

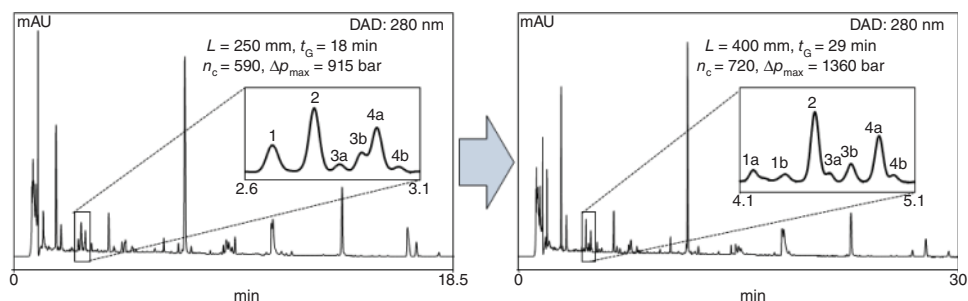


Figure 2.27 Comparison of two generic exploratory methods for traditional Chinese medicine (TCM) run on a Vanquish UHPLC system (Thermo Scientific, Germering, Germany) with two different column lengths as specified. Column id: 2.1 mm, $L = 400$ mm setup:

coupling 150 and 250 mm columns with Viper coupling device (Thermo Scientific, Germering, Germany), gradient : from 0% to 100% acetonitrile, flow rate: 670 $\mu\text{L}/\text{min}$, column temperature: 45 °C.

Table 2.3 Comparison between methods (a) and (b) from Figure 2.27 for number of identified peaks and minimum level of resolution between all adjacent peak pairs as quality criteria to assess the value of different peak capacities.

Criterion	$n_C = 590$ method (a)	$n_C = 720$ method (b)
Total number of identified peaks	125	206
Number of peaks with $R_S > 0.8$	118	187
Number of peaks with $R_S > 1.0$	114	179
Number of peaks with $R_S > 1.2$	108	168
Number of peaks with $R_S > 1.5$	101	163

resolution is depicted. The number of identified peaks was more than 60% higher on the 400 mm column setup. Even more relevant are the statistics on the peak pair resolution. At all resolution levels the improved method provided more than 50% additional peak pairs to meet this criterion. At the relevant $R_S = 1.5$ level, the gain was even more than 50%. At least for this TCM example, the result is a clear demonstration of the practical value of n_C to improve methods for complex samples.

The following conclusions from the data presented here and from the findings reported in more recent literature on highly optimized 2D-LC can be made:

- 1) With proper consideration of orthogonality, surface fractional coverage and oversampling of the first dimension, the maximum achievable practical peak capacities in 2D-LC are in the ballpark of 3000–4000.
- 2) Even with speed optimized UHPLC methods in the second dimension, such methods take at least 4 h to run, which corresponds to a peak production rate of 10–15 peaks/min. Highly speed optimized 2D-LC methods for moderate peak capacities of up to 1800 can nowadays be run in approximately 100 min, which corresponds to peak production rates of up to 18 peaks/min.
- 3) Using UHPLC pressure capabilities of 1400 bar allows the user to operate 3 μ m stationary phases in column chains up to 150 mm in length with $V_G/V_M = 35$ at reasonable analysis times of 4 h as well. This results in very impressive peak capacities for 1D-LC that can exceed $n_C = 1000$, but only moderate peak production rates of 4–5 peaks/min.
- 4) With 1D-UHPLC pressures of 1400 bar it is possible to achieve the highest productivity for complex sample analysis when using $dp \approx 2.2 \mu$ m in 400–500 mm column lengths. Such methods can achieve peak capacities of 700–800 in only 30–40 min, which corresponds to amazing productivity rates of 20–25 peaks/min.
- 5) If peak capacities significantly higher than 1000 are required, 2D-LC is the only option. For peak capacities of 500–700, 1D-UHPLC can provide excellent production rates and the effort of 2D-LC is difficult to justify, unless a specific selectivity combination from the two dimensions proves indispensable.

2.3.7

Optimization of Limits of Detection and Quantification

In the introductory Section 2.1.3, it was discussed that an important aspect of optimization can be to improve a method for its applicability in trace analysis. The nature of the mode of detection is very relevant in this case; whether the applied detector is concentration proportional like the very common UV detector or mass proportional like nebulizer-based detectors, for example, evaporative light scattering detector (ELSD) or charged aerosol detector (CAD). This textbook contains dedicated chapters on nebulizer-based or aerosol detectors (Chapter 10 on trends in detection), as well as for the coupling of LC with mass spectrometry (Chapter 1). Here, the focus is on concentration proportional detectors; UV detectors (VWD, DAD), fluorescence detectors (FLD), electrochemical detectors (ECD), and refractive index (RI) detectors.

With concentration proportional detectors in particular, the difference between the concentration-related and the absolute (mass- or amount-related) LOD and LOQ must be identified. This was already briefly discussed in Section 2.1.3, with the following sections diving deeper into the details together with mathematical calculations. This will guide the practitioner to the optimization parameters and should also take away some common misconceptions. The typical acronyms LOD and LOQ will be used.

One of the simplest ways to determine both LOD and LOQ is based on the ratio of the peak height to the noise in the chromatogram baseline, mostly called the signal-to-noise ratio (S/N). It is common practice to define the LOD at $S/N > 3$. For the LOQ, the respective rule is $S/N > 10$. The LOD (and LOQ) of a method is thus low if the method shows a steep slope of the calibration curve combined with a low baseline noise. The slope of the calibration curve expresses the change of detector signal as a function of change in analyte concentration (or mass). This slope is defined as the so-called sensitivity of a method, which is often confused with the LOD or trace detection performance. Both are related, but they are not identical and measured in different units. Since LOD and LOQ are strictly proportional, we only discuss LOD in this section.

Both the sensitivity of a method and the LOD can be defined for the analyte concentration or the absolute analyte mass (the amount injected onto the column). The concentration-related LOD c_{LOD} can be calculated as follows:

$$c_{\text{LOD}} = \frac{3 \cdot n_b}{h_p / c} \quad (2.21)$$

This calculation requires the baseline noise n_b as well as the quotient of the peak height h_p and the analyte concentration c . The latter equals the slope of the calibration curve for determination of analyte concentration in the sample by peak height. The absolute (mass related) LOD m_{LOD} is calculated similarly:

$$m_{\text{LOD}} = \frac{3 \cdot n_b}{h_p / m_{\text{inj}}} \quad (2.22)$$

Here, the quotient h_p / m_{inj} equals the slope of the calibration curve for determination of analyte mass in the sample by peak height. It is much more straightforward to define m_{LOD} for a method than to define c_{LOD} , due to the impact of injection volume size. This is shown with more detailed discussion and formula deviation in the following sections where the absolute detection limit m_{LOD} is initially discussed.

2.3.7.1 Absolute Detection Limit (Related to Analyte Mass on Column)

The discussion is restricted in the section to UV absorbance detection. For this, we can use Beer's law and calculate the peak height h_p as follows:

$$h_p = \frac{c_{\text{max}} \cdot \epsilon \cdot d}{M_w} \quad (2.23)$$

In this formula, c_{max} is analyte concentration (mass per volume) at the maximum of the analyte band in the detector, ϵ the molar absorptivity (see Beer's law), d the optical path length in the detector flow cell, and M_w the molecular mass of the analyte. It is possible to calculate the concentration at the peak summit in the detector as follows:

$$c_{\text{max}} = \frac{m_{\text{inj}} \cdot \sqrt{N}}{\sqrt{2\pi} \cdot V_M \cdot (1 + k)} \quad (2.24)$$

In this, we use the fundamental parameters of the kinetic and thermodynamic theory of chromatography plate number N , column hold-up volume V_M , and retention factor k . They all determine the peak volume, which is crucial for the concentration at the peak summit. Substituting Eq. 2.24 into 2.23, solving for h_p / m_{inj} and eventually substituting this into 2.22 yields for the following equation to determine the absolute LOD:

$$m_{\text{LOD}} = \frac{3 \cdot n_b}{\epsilon \cdot d / M_w} \cdot \sqrt{2\pi} \cdot \frac{V_M}{\sqrt{N}} (k + 1) \quad (2.25)$$

This equation only applies for isocratic HPLC separations, as the parameters N and k are not defined as such in gradient HPLC. This formula will be used to first calculate some representative values of m_{LOD} in conventional HPLC and then for equivalent UHPLC scenarios. For both scenarios, the calculations are made for two different molar absorptivities ϵ and two retention factors k . The lower value of ϵ represents an analyte with relatively poor UV detectability (e.g., acetic acid at 200 nm), the higher value reflects excellent UV detectability (e.g., chrysene at 266 nm). The two retention factors 2 and 5 represent the typical working range observed in isocratic HPLC separations.

For the conventional column, a 250 mm × 4.6 mm dimension packed with 5 μm at a total porosity of $\epsilon_T = 0.75$ is used. This would typically result in $V_M = 3.1$ ml and $N = 16\,700$ in the van Deemter minimum. A noise level of the conventional

Table 2.4 m_{LOD} for an analyte of 300 Da in *conventional HPLC* using a 250 mm $\times$ 4.6 mm column packed with 5 μm particles on a UV detector with 10 mm light path and 50 μAU baseline noise at different k and significantly different ϵ .

Retention factor	$\epsilon = 100 \text{ l/mol}\cdot\text{cm}$	$\epsilon = 100\,000 \text{ l/mol}\cdot\text{cm}$
$k = 2$	$m_{\text{LOD}} \approx 80 \text{ ng}$	$m_{\text{LOD}} \approx 80 \text{ pg}$
$k = 5$	$m_{\text{LOD}} \approx 160 \text{ ng}$	$m_{\text{LOD}} \approx 160 \text{ pg}$

detector of $5 \cdot 10^{-5} \text{ AU}$ (50 μAU) is assumed, which is a realistic value for n_b for a conventional HPLC detector. For the UHPLC scenario, a column with the dimension 100×2.1 and densely packed with 1.7 μm particles at a total porosity of $\epsilon_T = 0.65$ is used. This column will only yield a slightly higher efficiency of $N = 20\,000$, but a significantly lower hold-up volume $V_M = 0.225 \text{ ml}$. It is assumed that for a powerful modern light pipe detector optimized for such a column format that $n_b = 10 \mu\text{AU}$. Although very ambitious for a baseline in real-life applications, this is possible after thorough optimization. For the sake of simplicity it will be assumed that a light path of 10 mm in both detectors exists. This is a reasonable assumption, as typical modern light pipe flow cells with extended flow path (e.g., 60 mm) are not compatible with such a column format due to mismatch between peak volume and flow cell volume. The molecular mass of the analyte should be $M_W = 300 \text{ g/mol}$ for both scenarios.

Equation 2.25 is used to calculate the m_{LOD} values for the four representative conventional HPLC scenarios, with results shown in Table 2.4. It can be seen that the absolute LODs in conventional HPLC are in the order of 100 ng for analytes with very poor absorptivity and 100 pg for analytes with excellent absorptivity. For typical aromatic compounds such as caffeine, a molar absorptivity of $\epsilon = 10^4 \text{ l/mol}\cdot\text{cm}$ is a reasonable estimate. LODs for those compounds in conventional HPLC would vary between 10 and 25 ng depending on the retention factor.

It is now interesting to see how much these absolute detection limits can be decreased when transferring to a modern UHPLC method. The results of the respective calculations based on the given assumptions on the column format and detector are represented in Table 2.5. It can be seen that the absolute LODs can be improved by a factor of approximately 80, suggesting that a factor of 100 is a good estimate in general. Now the LODs for the compound with very poor ϵ are in the single-digit nanogram range, for the analyte with excellent ϵ they are in the single-digit picogram range. The major contribution for these lower LODs stems from the increase of efficiency and column volume miniaturization.

The factor $V_M/\sqrt{N}$ is 10- to 20-fold smaller in UHPLC than in conventional HPLC. Based on the assumption made previously on the detector performance, the residual factor 5 came from the reduction of detector noise. If the conventional method would be run on a top performing modern UHPLC light pipe detector, the absolute LOD would only be factor 10–20 higher than that of the UHPLC method on the same instrument.

Table 2.5 m_{LOD} for an analyte of 300 Da in modern UHPLC using a 100 mm × 2.1 mm column packed with 1.7 μm particles on a UV detector with 10 mm light path and 10 μAU baseline noise at different k and significantly different ϵ .

Retention factor	$\epsilon = 100 \text{ l/mol}\cdot\text{cm}$	$\epsilon = 100\,000 \text{ l/mol}\cdot\text{cm}$
$k = 2$	$m_{\text{LOD}} \approx 1 \text{ ng}$	$m_{\text{LOD}} \approx 1 \text{ pg}$
$k = 5$	$m_{\text{LOD}} \approx 2 \text{ ng}$	$m_{\text{LOD}} \approx 2 \text{ pg}$

2.3.7.2 Concentration Detection Limit

It was shown in Section 2.3.7.1 that the absolute detection limit is a strict function of the volume and efficiency of the separation column, as well as the response factor and baseline noise of the detector. To calculate the LOD in concentration units (c_{LOD}), it was shown in Eq. 2.24 that the injected analyte mass m_{inj} must be taken into account. At a given analyte concentration in the sample, and in this case this relates to c_{LOD} , m_{inj} can be varied, by changing the injection volume V_{inj} . Thus, the concentration LOD can be lowered by just injecting a larger volume on the column. Of course this is only valid as long as the increase of V_{inj} does not significantly affect the resulting plate number N . At a certain V_{inj} , however, once the column has become overloaded with the increasing sample volume, N starts to drop remarkably. It is possible to derive that the percentage of the V_{inj} relative to the resulting peak volume V_{peak} from a given column actually equals the percentage drop in N . So if V_{inj} adopts 10% of the V_{peak} , a column that could provide 10 000 plates would only give 9000 plates. Tolerating a loss of 10% in N would result in approximately 5% loss in resolution R_s . While this is mostly tolerated, it will be assumed as a general rule that only 5% loss in N resulting from the injection variance is tolerable for optimal practical conditions. This would correspond to approximately 2.5% loss in R_s . The loss factor is defined as θ , and based on that it is possible to formulate a general equation for calculating V_{inj} from the column parameters:

$$V_{\text{inj}} = \theta \cdot V_{\text{peak}} = \frac{\theta \cdot 4 \cdot V_M \cdot (1 + k)}{\sqrt{N}} \quad (2.26)$$

With Eq. 2.26 both the theoretical peak volumes V_{peak} for representative conventional HPLC and UHPLC column formats as well as V_{inj} at a given value of θ (Table 2.6) can be calculated. The resulting injection volumes may appear relatively low, but they should be considered for making best use of the respective column efficiency. If a loss in N of 10% can be tolerated, one can inject twice this volume.

To derive a formula for the calculation of c_{LOD} , it is necessary to divide Eq. 2.26 by 2.25 and to obtain 2.27, which includes an approximate calculation for c_{LOD} based on $\theta = 0.05$:

$$c_{\text{LOD}} = \frac{3 \cdot n_0}{\theta \cdot 4 \cdot \epsilon \cdot d / M_W} \cdot \sqrt{2\pi} \approx \frac{40 \cdot n_0}{\epsilon \cdot d / M_W} \quad (2.27)$$

Table 2.6 Peak volume and injection volume levels for representative column formats.

dp (μm)	5	3	2.2	1.7
L (mm)	250	150	100	50
d_C (mm)	4.6	3.0	2.1	2.1
V_{peak} (at $k=2$) (μl)	300	75	25	15
V_{inj} (at $\theta=0.05$) (μl)	15	4	1.25	0.75

The calculations assume $H=3 \cdot dp$ for all columns, $\epsilon_T=0.75$ for 5 and 3 μm columns and $\epsilon_T=0.7$ for 2.2 and 1.7 μm columns.

One approximation is made in deriving this, namely that N does not change at the V_{inj} related to $\theta=0.05$, which appears to be fair given the considerations above. With the help of Eq. 2.27, c_{LOD} can be calculated for a molecule with a M_W of 300 Da and a ϵ of 10 000 l/mol·cm is in the range of 20 $\mu\text{g/l}$ with a modern light pipe detector and 100 $\mu\text{g/l}$ on a more conventional detector. Interpreting Eq. 2.27 further and comparing it to Eq. 2.25 the following very important conclusions for trace analysis can be made:

- 1) The concentration related LOD (c_{LOD}) is *independent* of the column volume and column efficiency as long the injection volume V_{inj} is properly adapted to the respective column. Hence, c_{LOD} cannot be improved by the column parameters, as long as sufficient sample is available to apply the required V_{inj} for optimum column load.
- 2) Provided sufficient sample, c_{LOD} for a given analyte at the optimal detection wavelength can only be influenced by the detection path length d and the baseline noise n_b with the given method and detector settings. Modern light pipe detectors with more than 50 mm light path and low noise levels enable a 10–20-fold lower detection limit than conventional detectors, but normally tolerate no peak volumes smaller than 100 μl without loss in resolution.
- 3) A reduction in column dimension and an increase in column efficiency can *solely* improve the absolute detection limit m_{LOD} . This can be very relevant if the sample size is limited though and it was the key incentive to develop nano- and capillary-HPLC for certain application fields like proteomics.

2.3.8

Practical Guide for Optimization

2.3.8.1 General Optimization Workflow and Important Considerations and Precautions

Before a method can be optimized, there must be a respective method available as a starting point. It was not the goal of this chapter to provide guidance for fundamental method development from the scratch, but to develop an existing method. Prior to starting method optimization, the user must become clear on the possible deficiencies of the current method and identify its relevant optimization potentials in terms of parameters like, for example, resolution, speed, or detection limits. Once a deficiency is identified, the user must also analyze the method for the root cause for these deficiencies. If a method shows insufficient resolution for a

critical peak pair, the next step is to analyze retention factors, selectivity and plate numbers as well as peak asymmetries of the respective peaks to draw the proper conclusions. This analysis will quickly show if the required minimum retention of $k = 1$ is fulfilled (or if the peaks actually elute during the gradient), if no peak asymmetry is higher than 1.5 and if the plate numbers meet those of the column test protocol (at comparable retention factors). Poor peak shapes should be addressed by verifying the state of the column with a standard test, too low retention may be addressed by decreasing the solvent strength of the mobile phase or lowering the starting composition of a gradient. However, it is often the selectivity that is the root cause of insufficient resolution. This has to be empirically optimized starting from a change in the eluting conditions. This can be either a change of the organic modifier under more or less isoeutropic conditions, or potentially slight temperature or pH-variations. Another option is the change to a different column with ideally orthogonal selectivity. If such a column is available, it is a quick test, if it has to be purchased, one should start with variation of eluting conditions. Several rules for a systematic procedure have been developed with valuable hints on the related decision criteria discussed in detail. A greater understanding of these rules is given in Chapters 3 and 4 of this text book.

In subsequent optimization steps the analysis time may be reduced. The first approach to reducing the analysis time should be a stepwise increase in the flow rate, possibly coupled with a temperature increase. In gradient methods, the gradient time must be reduced by the same factor as the flow rate is increased. Loss of resolution or if a lower resolution does not meet the method requirements can be quickly seen as the flow is increased or if there is a matched increase of the flow and temperature (considering constant pressure). If such problems are encountered, the next approach to reducing the analysis time should be to change to a shorter column with smaller particles respecting the $L/dp = \text{const}$ rule. If the flow rate and gradient time (if applicable) are adapted according to the rules, this will be a guaranteed success, provided the selectivity of the new column does not significantly deviate. If the original method provides relatively high resolution for the critical pair ($R_S > 2.0$), it will tolerate deviations in selectivity as well as flow and temperature increases, or even a reduction of L/dp much more readily.

It is always highly recommended to verify the robustness of the optimized method before implementing it in routine. If no elution parameter other than flow rate was changed and the column is still well in the specified operating pressure range, a thorough evaluation of peak resolution should be sufficient. If the temperature was increased beyond 40 °C, the new method should be verified for column stability by injecting 100–200 samples with no relevant change being observed in retention or efficiency. If mobile phase composition, pH or temperature were varied, robustness of the new method must be proven with single parameter variation of the variable that was altered.

2.3.8.2 Overview on Valuable Rules and Formula

This section will give a brief summary on all the relevant rules and formula that apply in method optimization. They help to make estimations and give guidance

to avoid critical failures. Wherever applicable, it also provides simplified approximations for calculations. In Table 2.7 the relevant information on instrumental considerations can be found. The reader should also consider Section 1.2 in this context, as it contains very useful information that can be readily applied beyond the application of instruments in LC/MS. The following tables lead to method related optimization criteria. This will address the separation column (Table 2.8), the analysis speed (Table 2.9), the retention in RP chromatography (Table 2.10), the resolution (Table 2.11), and also the peak capacity (Table 2.12). The formula related to LOD have been developed and discussed in Section 2.3.7 and will not be repeated here.

The following rules and formula cannot be complete, but they provide a concise summary of all relevant information in this chapter and they can be used for a repetition as well as a general reference work.

2.4

Outlook

It has been shown in this chapter how modern UHPLC with pressures up to 1500 bar and optimized use of particle sizes slightly above $2\mu\text{m}$ can achieve theoretical peak capacities of almost 1000 in less than 1 h. It is also possible to perform relatively demanding separations in about 1 min on sub- $2\mu\text{m}$, ideally using solid core based short columns. Selectivity continues to be the most important parameter in LC when it comes to resolution optimization. This is the incentive for column manufacturers to continuously develop materials with new selectivities for relevant and challenging applications. At the same time there is a trend toward solving as many applications as possible on a small number of different columns, ideally only one. This is facilitated through the high plate numbers that UHPLC columns can generate in relatively short times. Since selectivity is still indispensable there is also desire for phases that allow for significant changes of selectivities by altering the elution conditions. Mixed mode or multi mode phases have a high potential for such a tuning via pH, ion strength and organic modifier, while the temperature is not yet widely used as an additional parameter. Alternatively, there is still significant improvement potential for the kinetic performance of such phases. Most of them do not exist in particle sizes below $3\mu\text{m}$, they are often not compatible with higher pressures, and peak symmetries are sometimes not comparable to the quality obtained with modern C18 phases of equivalent particle size. There are two fundamentally oppositional trends observed. One is the increasing number of columns customized for certain applications, for example, distinct biopharma characterization workflows, the other trend is striving for generic methods to solve as many analytical problems as possible without any method development on just one column. While the new generic approaches are driven by the kinetic potential of modern UHPLC often in combination with fast and powerful mass spectrometry, specialty phases for certain applications hardly take advantage of the kinetic capabilities of UHPLC and there is still discussion to determine if there is a need for these types of phases.

Table 2.7 Instrument related volumes and detector settings.

Parameter	Formula	Approximations/ rules	Comments
Gradient delay volume (Gradient dwell volume)	$GDV_{\text{system}} = GDV_{\text{pump}} + GDV_{\text{sampler}} + V_{\text{capillary(pump-sampler)}} + V_{\text{capillary(sampler-column)}}$	$GDV_{\text{HPG}} \approx V_{\text{Mixer}}$ $GDV_{\text{sampler}} \approx V_{\text{loop}} + 5 \mu\text{l}$ $V_{\text{cap(100 } \mu\text{m)}} \approx 0.08 \mu\text{l/cm}$ $V_{\text{cap(130 } \mu\text{m)}} \approx 0.1 \mu\text{l/cm}$ $V_{\text{cap(180 } \mu\text{m)}} \approx 0.3 \mu\text{l/cm}$ $V_{\text{cap(250 } \mu\text{m)}} \approx 0.5 \mu\text{l/cm}$	HPG: High pressure gradient pump (binary gradient pump) LGP: Low pressure gradient pump (quaternary gradient pump) LPG: Consider the GDV specified by the manufacturer or better determine it yourself (Dolan-Test)
		Rules: <i>Ideal:</i> $GDV_{\text{system}} \leq V_{\text{M}}/2$ <i>Fair:</i> $GDV_{\text{system}} \approx V_{\text{M}}$ <i>Maximum tolerable:</i> $GDV_{\text{system}} \approx 2 \cdot V_{\text{M}}$ V_{M} = column hold-up volume (siehe "column")	

Table 2.7 (continued)

Parameter	Formula	Approximations/ rules	Comments
Volumes of connection tubings	No exact formula	Rules: <i>Ideal:</i> • $V_{\text{tubings}} \leq V_{\text{peak}}/5$ <i>Maximum tolerable:</i> • $V_{\text{tubings}} \leq V_{\text{peak}}$ See above for estimation of tubings volumes for different capillary IDs V_{peak} = Peak volume (see “column”)	• Keep all connections as short as possible • Keep tubing ID as low as possible, in particular behind the column • Instruments with maximum 600 bar pressure limit: Capillary IDs $\geq 130 \mu\text{m}$ to avoid over pressure
Detector flow cell volume	No exact formula	Rules: <i>Ideal:</i> • $V_{\text{cell}} < V_{\text{peak}}/10$ <i>Maximum tolerable:</i> • $V_{\text{cell}} \approx V_{\text{peak}}/3$ See “column” für V_{peak}	• V_{cell} is the light irradiated volume in the flow cell • Challenge manufacturer specifications as not all list the irradiated volume • For band dispersion consider also the volume of heat exchanger in front of the flow cell

(continued overleaf)

Table 2.7 (continued)

Parameter	Formula	Approximations/ rules	Comments
Data collection rate in detector (f_{det})	$f_{\text{det}} \geq \frac{25}{\sqrt{\frac{16 \cdot H}{L}}} \cdot 1/t_R$	Approximation: $f_{\text{det}} \approx \frac{6 \cdot \sqrt{N}}{t_R}$ with N from column test protocol	• Formula applies for 25 data points at the peak base w_b ($4\sigma_t$) • Corresponds to 30–40 data points within the integration limits • Is valid for isocratic analysis and should consider t_R of most early eluting peak • For gradient methods replace t_R by t_M
Time constant of detector (t_C)	$t_C \leq 0.1 \cdot \sqrt{\frac{16 \cdot H}{L}} \cdot t_R$	<i>Alternatively: insert H in exact formula as $3 \cdot dp$</i> Approximation: $t_C \approx \frac{t_R}{2 \cdot \sqrt{N}}$ with N from column test protocol	• The inverse data rate is also a good estimation for t_C • If the detector settings require “rise time” order “response time” instead of “time constant” apply twice the value • For gradient methods replace t_R by t_M

Table 2.8 Columns, particle sizes and their operational conditions.

Parameter	Formula	Approximations/ rules	Comments
Column hold-up volume V_M	$V_M = L \cdot \frac{d_C^2}{4} \cdot \pi \cdot \epsilon_T$	Approximation: $V_M \approx \frac{L \cdot d_C \cdot d_C}{2}$	• Insert L and d_C in mm to obtain V_M in microliter • Approximation uses $\epsilon_T = 0.63$, a good approximation for UHPLC columns with totally porous particles (conventional columns will have up to 25% higher V_M, solid core columns up to 25% lower)
Theoretical peak volume V_{peak}	$V_{\text{peak}} = \sqrt{H \cdot L \cdot d_C^2} \cdot \pi \cdot \epsilon_T \cdot (1 + k)$	Approximation: $V_{\text{peak}} \approx \frac{10 \cdot V_M}{\sqrt{N}}$	• V_{peak} is defined as the peak volume at the peak base w_b (4σ), the volume within the integration limits is 20–50% higher • V_p can also be determined from the chromatogram by calculating $w_b \cdot F$ • If $V_{\text{peak real}} \gg V_{\text{peak theoret.}}$ the correct detector setting must be checked
Calculate V_M with approximation above <i>Alternatively:</i> insert H in exact formula as $3 \cdot dp$			

(continued overleaf)

Table 2.8 (continued)

Parameter	Formula	Approximations/ rules	Comments
Optimum flow rate F	No general exact formula (depends on analyte, mobile phase, and temperature)	Approximation: $F_{\text{opt}} \approx \frac{d_C \cdot d_C}{3 \cdot dp}$	- Exact determination always requires measuring a van Deemter curve - Approximation valid for totally porous material - For solid core materials assume 50% higher value (20% for sub-2 μm)
Linear velocity u (conversion from flow rate)	$u = \frac{4 \cdot F}{d_C^2 \cdot \pi \cdot \epsilon_T}$	Insert d_C in millimeter and dp in micrometer, F results in milliliter per minutes Approximation: $u \approx \frac{30 \cdot F}{d_C \cdot d_C}$	- For a totally porous material u_{opt} for small molecules is approximately $10/dp$ - For a sub-3 μm solid core material u_{opt} for small molecules is approximately $15/dp$
		Insert F in milliliter per minutes and d_C in millimeter to obtain u in millimeter per second	

Table 2.9 Method speed-up.

Parameter	Formula	Approximations/ rules	Comments
Analysis time t_R in isocratic runs	$t_R = \frac{L}{u} \cdot (1 + k)$	Approximation: $t_R = \frac{L \cdot dp}{100}$	This equation applies when the flow rate is near the theoretical H/u minimum and for retention factors up to $k = 5$
Analysis time t_R in gradient runs	$t_R = \frac{L}{u} \cdot \frac{V_G}{V_M}$	Insert L in mm and dp in μm to obtain t_R in min Approximation: For very steep gradients with $V_G \approx 6 \cdot V_M$ the same approximation as for isocratic runs can be used	The formula does not consider the time for column re-equilibration but the pure separation time. Cycle times in practice will be 20–50% longer
Method speed-up in isocratic separations	$\frac{L}{dp} = \text{const.}$ $u \cdot dp = \text{const.}$ $t_R \sim dp^2$	Rules: Lower dp and lower L proportionally and increase u (at constant d_C also F) by the same factor	The related reduction in V_{peak} and w_b may require changes at instrument volumes and detector settings
Method speed-up in gradient separations	$\frac{L}{dp} = \text{const.}$ $u \cdot dp = \text{const.}$ $\frac{F \cdot t_G}{V_M} = \frac{V_G}{V_M} = \text{const.}$ $t_R \sim dp^2$	Rules: Respect the same rules as in isocratic applications and additionally keep the ratio of V_G/V_M constant	The related reduction in V_{peak} and w_b may require changes at instrument volumes and detector settings
Required pressure increase for speed-up	$\frac{1}{t_R} \sim \Delta p$	Rule: Pressure increases at the same factor as speed	At constant R_S and applying columns with totally porous particles

Table 2.10 Retention dependencies in RP chromatography.

Parameter	Formula	Approximations/ rules	Comments
Retention dependency on stationary phase	$k = \frac{c_S}{c_M} \cdot \frac{\%C \cdot \rho}{\epsilon_T}$	Rule: Retention increases with carbon load %C of stationary phase	• Applies at constant packing density and porosity • If V_M/V_C matches, requirement is fulfilled
Retention dependency on organic content in mobile phase	$\ln k = \ln k_{\text{aqueous}} - B \cdot \%_{\text{organ.}}$	The plot of $\ln k$ versus % organic content is linear and allows retention prediction	• Perfect linearity is not always achieved across the full range • Approximations are only rules of thumb, exact behavior can markedly vary with analyte and stationary phase
Retention dependency on temperature	$\ln k = \frac{-\Delta H^0}{R} \cdot 1/T + \frac{\Delta S^0}{R} + \ln \beta$	Approximations: k decreases by 3-9% with 1% increase in organic content k halves with 10% increase in organic content The plot of $\ln k$ versus $1/T$ is linear and allows retention prediction	• Perfect linearity is not always achieved across the full range • The approximation applies primarily for neutral analytes
Relationship between change of mobile phase composition and temperature change	No exact formula	Approximation: k decreases by 2–3% with 1 °C increase in temperature Rule: 1% increase in organic content corresponds approximately to 5 °C temperature increase	• Known as Horvath rule • The rule applies primarily for neutral analytes

Table 2.11 Peak resolution and dependencies on fundamental method parameters.

Parameter	Formula	Approximations/ rules	Comments
Dependency of resolution R_S on selectivity α	$R_S \sim \frac{\alpha-1}{\alpha}$	Rules: • R_S increases by 80% when increasing α from 1.05 to 1.1 • R_S increases by factor 3.7 when increasing α from 1.1 to 1.5 • R_S increases by 52% when increasing α from 1.5 to 2.0	• α is the most important parameter to control R_S in LC • The influence is very pronounced at $\alpha < 1.2$ • It does not provide practical value to work at $\alpha > 1.5$
Dependency of resolution R_S on plate number N	$R_S \sim \sqrt{N}$	Rules: Doubling N increase R_S by factor 1.4 To double R_S a fourfold increase in N is required	• Increase in N may help if it is difficult to increase α • Very high N is needed to separate very complex samples
Dependency of resolution R_S on retention k	$R_S \sim \frac{k}{1+k}$	Rules: • R_S increases by 34% when increasing k from 1 to 2 • R_S increases only by 12% when increasing k from 2 to 3 and another 11% when increasing k from 3 to 5	• k can be very effectively controlled by eluent composition • Increase in k also increases analysis time • k should never be smaller than 1

Table 2.12 Peak capacity in 1D-LC and 2D-LC.

Parameter	Formula	Approximations/ rules	Comments
Peak capacity n_C in isocratic separations	$n_C = 1 + \frac{1}{4} \cdot \sqrt{N} \cdot \ln(1 + k_i)$ $k_i = \text{retention factor of latest elution peak}$	Approximation: $n_C \approx \sqrt{N}$	• n_C is valid for equidistant peaks at $R_S = 1.0$ • The approximation is achieved at $k_i = 50$ • At $k_i = 10$ already 60% of this value is accessible • $k_i = 100$ allows only 17% increase over $k_i = 50$
Peak capacity n_C in gradient separations	$n_C = 1 + \frac{1}{4} \cdot \sqrt{N} \cdot \frac{\frac{\Delta c \cdot B}{1 + \Delta c \cdot B} \cdot \frac{V_M}{V_G}}$	Approximation: $n_C \approx 2 \cdot \sqrt{N}$	• Approximation is only valid for normal elution behavior, $\Delta c > 70\%$ and $V_G/V_M \geq 40$ • With only $V_G/V_M = 10$ gradients can achieve the same n_C as isocratic runs with $k_i = 50$, but almost fivefold faster
Peak capacity n_C in 2D-LC	$\Delta c: \text{change in } \%_{\text{organ}} \cdot \text{over entire gradient}$ $B: \text{slope in } \ln k = \ln k_{\text{wässr.}} - B \cdot \%_{\text{organ.}}$ $n_C = {}^1n_C \cdot {}^2n_C$ $V_{\text{fraction}} \leq {}^1V_{\text{peak}}/2$	Rules: n_C in 2D-LC equals the product of n_C in the first dimension times that of the second dimension Fraction volumes cut from the first dimension must not be larger than half the V_{peak} from the first column	• Product rule only applies if the retention criteria in both dimensions are orthogonal • Highly optimized 2D-LC can achieve four to sixfold higher n_C in the same analysis as 1D-LC

The further enhancement of the kinetic performance of UHPLC is certainly a very relevant aspect in future developments. Column manufacturers would be capable to produce sub-1 μm particles, but it is still more than questionable if such formats will ever gain ground in routine analysis. A 0.7 μm solid core material efficiently packed in a 20 mm long column could principally generate 20 000 theoretical plates. Such a stationary phase would require 15–20 mm/s to achieve the van Deemter minimum for small molecules and this would imply a t_{M} as low as 1 s. This could enable to run even demanding separations in not more than 10–20 s. It is worth to note that such a phase run in a 2.1 mm I.D. column would require flow rates of 2 ml/min and would generate pressures higher than 2000 bar at that flow. While the trend to further increase the maximum pressure of UHPLC instruments without sacrificing on flow rates is currently continuing, but there is still some way before a high flow 2500 bar technology in commercialized instruments becomes a reality. Beyond the pressure and flow requirements, the biggest challenge will be the extra column peak dispersion when using such demanding columns. The best instruments available today could possibly not generate more than 5000 plates at $k=2$ with such a column and the instrumental challenges are not easy to overcome. It seems thus much more likely that the current trend of using 1.5–1.8 μm solid core materials will continue and columns that attempt to achieve a maximum number of possible plate numbers will not be shorter than 50 mm, which will not allow running them far into the *C*-term. Regarding the maximum pressure it is fair to say that current instrumentation can support routine analysis at 1200–1300 bar. In spite of the trend to further increase of pressure capabilities in a very competitive high end UHPLC market, some critical reflections on the related practical value are certainly worthwhile. Broekhoven and Desmet have recently published interesting modeling to support this discussion [24]. Their predictions showed that the possible gain by moving from 1200 to 2400-bar instruments would at most lead to a 40% increase in efficiency and only 20% in resolution or peak capacity, which is not overly impressive. At the same time they point out the almost insurmountable instrumental challenges with respect to extra column effects in real world instruments.

Another interesting question addresses the future trend regarding column diameters. Today's UHPLC is predominantly run on 2.1 mm column, while 1 mm are continuously gaining importance. Next to the reduced environmental footprint through solvent savings, thermal effects are also decreasing with lowering column diameters. This counts both with respect to effective and accurate solvent preheating, as well as dissipating viscous heating that occurs quite intensely at elevated pressure. It is also of interest in this respect, whether the quasi adiabatic still air thermostating concept will become generally accepted in LC instrumentation thanks to its efficiency advantage under viscous heating. The question is though how it will be dealt with the fact that the true column temperature under frictional heating cannot be controlled and is not known when using a still air thermostat. Considering the total of advantages related to environmental impact, required size of samples, better handling of thermal effects with even enabling temperature

gradients and last but not least good compatibility with ESI-MS, there may be a trend toward a broader based application of capillary LC with diameters of 200–500 μm . The intrinsic challenge to be solved are once again the instrumental challenges with extra column effects, but also with the gradient delay volume when flow rates are down to 5–20 μl . This trend could then abet further development of highly efficient silica based monoliths in capillary formats. Imagine that column manufacturers would succeed in producing 300 μm I.D. monoliths that generate plate heights of 2–3 μm in real world separations with permeabilities equivalent to 3 μm particles, but being available in lengths of 1000 mm or more, stable to work at extreme pressure of, for example, 1500 bar, and able to be coiled into standard size column compartments without breaking and efficiency losses. This could be a true game changer in LC column technology. Such columns operated at pressures of 1200–1500 bar in combined elution and temperature gradients would enable to easily generate GC-like plate numbers in not more than 30 min. They could certainly be the vehicle to help liquid chromatography to take the magic hurdle of one million theoretical plates in very reasonable analysis times.

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3

The Gradient in RP-Chromatography*

3.1

Aspects of Gradient Optimization

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3.1.1

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 with isocratic separations, is affected by more factors; these sometimes act in opposite directions or multiplicative.

In this chapter, we focus on selected aspects of the optimization of gradient separations in reversed-phase (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. Based on 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. In the subsequent Section 3.2, Hans-Joachim Kuss deals with the prediction of gradients.

3.1.2

Special Features of the Gradient

In high-performance liquid chromatography (HPLC), interactions of different strengths between the analytes on the one hand and eluent components and the stationary phase on the other hand usually occur during separation. In the case of isocratic separations, there is a predetermined, constant eluent composition,

*Translated from German by Steve Ross.

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 means the nonpolar ACN or MeOH molecules naturally “like,” for example, nonpolar C18 alkyl groups.

In the course of a gradient, due to 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 with 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 – analog 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 become 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 it is not allowed to speak of a “plate number.” The plate number, a measure of the 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 (dead volume or extra column volume), 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 with 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 $\leq 2 \mu\text{m}$ 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, that is, at the trailing edge, move faster than those at the beginning of the peak, the later eluting molecules of the substance band are always pushed “forward” faster. This fact, known as *peak suppression*, has the effect that in gradient separations tailing is rarely observed. Peak symmetry is about 10% better compared with an isocratic run with equivalent eluent composition (Hans-Joachim Kuss, personal communication).

3.1.3

Some Chromatographic Definitions and Formulas

Let us now consider some chromatographic definitions, which are known from the theory – which, by the way, was developed originally for the 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 Refs. [2–4] and in particular [1].

The resolution R is, simplified, 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 and 4000 are given – and at the end almost zero. Since the k -value changes during gradient elution, a k^* value (or $\bar{k}$) 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. (3.1), 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 an 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 for the resolution only in the second decimal place.

Five simple equations are given in the following. They are sufficient to draw conclusions for practical optimization. A more detailed discussion follows in Section 3.2.

$$R = \frac{\sqrt{N}}{4} \cdot (\alpha - 1) \cdot \frac{k}{1 + k} \quad (3.1)$$

$$k^* = \frac{t_G \cdot F}{\Delta\%B \cdot V_m \cdot S} \quad (3.2)$$

$$\alpha = \frac{k_2}{k_1} \quad (3.3)$$

$$k = f\left(\frac{V_D}{V_m}\right) \quad (3.4)$$

$$n_c = \frac{t_{RI} - t_{Re}}{w} \quad \text{or} \quad n_c = \frac{t_G}{w} \quad (3.5)$$

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,

$\Delta\%B$ = 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_{RI} = 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 to Eqs. (3.2)–(3.4), or: isocratic versus gradient separations.

In accordance with Eq. (3.2), 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. (3.4), 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 Section 1.2). 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. (3.4) – 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, discussed later. On the other hand, 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. Analog, 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 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 is 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 later. 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 as at the end.

3.1.4

Detection Limit, Peak Capacity, Resolution: Possibilities for Gradient Optimization

3.1.4.1 Detection Limit

The three main objectives of optimization are low detection limit, “good” separation and, not in the 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 3).
- 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. 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 – that is, more polar – as 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. The steepness of the gradient, on the other hand, 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.

3.1.4.2 Peak Capacity and Resolution

Usually, peak capacity is defined as the number of peaks per unit time, see Eq. (3.5). 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 here we do not come any further via selectivity. A separation in this case will be possible, when the many (similar?) components can be eluted distributed as narrow peaks throughout the chromatogram.

Conversely, the more the components differ, the more the peak capacity recedes into the background and the “chemistry” comes into the foreground, because here we would have at least the chance to induce different interactions and thereby to improve very effectively the selectivity and following this the resolution. Simplified, this means that with similar components and resulting lower selectivity, the (important) selectivity term in Eq. (3.1) recedes into the background, 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, gradient 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, that 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 worthwhile to go above a factor of 30.

When UHPLC is available, it is additionally recommended to use a long column filled with sub 2 μm particles – possibly think also of core shell or monolith columns, see Chapter 5 – at a high flow, run a steep gradient and additionally increase the temperature. A trick that, 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 3.

Resolution Now let us turn to the objective of “good” resolution. This is by far the most difficult case. Make the following clear – good resolution means,

simplified, 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 earlier sections. From Eq. (3.1) 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. First, the plate number is under the root, an increase in the number of plates by as much as a factor of 2 (e.g., from a conventional plate number of 10 000 to an – in the UHPLC in practice achievable – plate number of 20 000) leads to an increase in resolution of $\sqrt{2}$, a factor of only 1.4 – this under isocratic conditions! And second, especially with a gradient the “plate number” plays a subordinate role: as explained earlier, due to the permanently increasing elution strength the peaks are always narrow, the “plate number” is large and in the ideal case constant.

Is, nevertheless, a higher separating power 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 foreground and not the time. At this point, a brief explanation is allowed, a detailed discussion is to be found in Chapter 3. 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 discussed earlier.

Furthermore, in the case of slow kinetics it must be reckoned with another – sometimes substantial – contribution to peak broadening. Due to, 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. There, as also in Chapter 5 (RP columns), is reported in detail how the selectivity can be influenced. Let us go

back to the gradient: $\alpha = k_2/k_1$, that is, all factors that influence the retention factor (Eq. (3.2)) can also influence the selectivity – and in any case the resolution. We discuss this in the later sections.

Strength of the Interactions: Retention Term The value of this term approaches asymptotically 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. (3.2): many an interesting conclusion for the routine can be derived from this. In Section 3.1.6, 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 great charm that while the retention time remains constant, the resolution can improve. On the other hand, 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. Due to 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, due to 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. First, the silica-based columns are more robust than sometimes feared, second, as a rule the time saved through higher flow rates is out of all proportion compared with the price of a column and especially third, 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 resolution 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 so difficult? Here 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, better overall resolution (sum of the resolution between all peaks).

- $\Delta\%B$ 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. (3.2), a longer column leads to a decrease of k^* , on the other hand, a longer column means a higher plate number. As mentioned earlier, 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 with 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 earlier, of the numerator and the denominator on the k^* value in Eq. (3.2) in the following statements – certainly not to be generalized for all cases:

- With short gradients and large $\Delta\%B$ (from $\sim 60\%B$, 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 $\Delta\%B$), 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 $\Delta\%B$ in the denominator results in a sufficiently large k^* value, a large gradient volume – simply by means of a long gradient – is not so necessary.

- The larger $\Delta\%B$ 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, that means, the first peak can be separated better from the front.
- A similar observation: when a long column is used (as with $\Delta\%B$, V_m would increase in the denominator), the flow should be increased. Apart from the above-mentioned cases, for example, slow kinetics, and so on, 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 examples discussed later.
- When steep gradients are run, it is also counterproductive to use a long column: the k^* -value of the components tends quickly toward 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, and so on. 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 $\%B$, $\leq 3\ \mu\text{m}$ 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 $\geq 5\ \mu\text{m}$ 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 with 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.

3.1.5

Gradient “Myths”

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

- *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, see Section 3.2.

- *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," that 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, behind this statement belongs, at the very least, a fat question mark, see later and also the comments mentioned earlier.

3.1.6

Examples for the Optimization of Gradient Runs: Sufficient Resolution in an Adequate Time**3.1.6.1 About Irregular Components**

Before we start to talk about examples of individual optimization parameters, here an important hint; ignoring this fact in the routine 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, and so on. 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. For example, the $\ln k/\%B$ -plot of SunFire is not similar to the image of any of the other columns which we have recorded, see Figure 3.6 in Section 3.2. The curvatures of the curves can also differ greatly. The result can be: co-elution, deterioration “here,” improvement “there.”

3.1.6.2 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 alkyl-benzenes 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 there. But first, again the following remark: 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 its part ...

We have discussed the “plate number” earlier, it will no longer play a role here. We will concern ourselves rather with interactions, with the selectivity and the retention term in Eq. (3.1); the objective is sufficient resolution. We have treated the influence of the important parameters solvent (ACN against MeOH), pH and temperature on the 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 Refs. [5, 6].

Rather, we focus on the gradient specifics, see Eq. (3.2): 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. Furthermore, the effective volume of the column, in practice this means the length of the column. In addition, 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.

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 3.1.

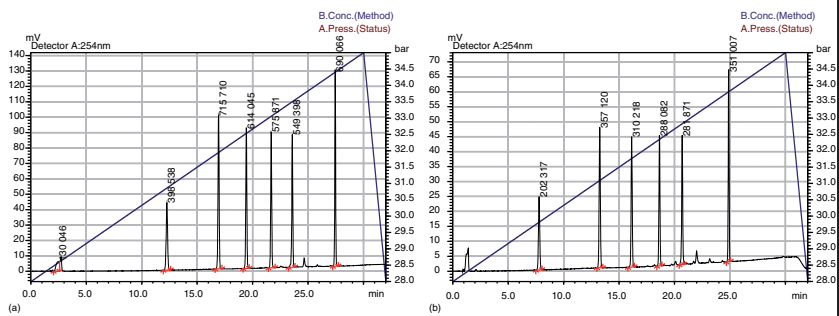


Figure 3.1 Influence of the flow rate, XBridge Shield 150×4.6 mm, 5 μ m. (a) 0–100%B, 1 ml/min, $t_G = 30$ min, (b) 0–100%B, 2 ml/min, $t_G = 30$ min.

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 3.2.

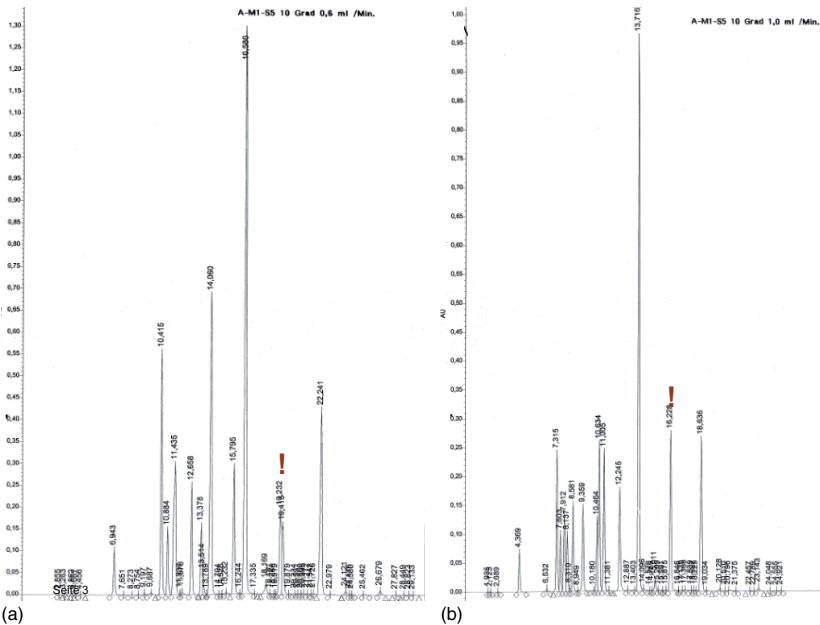


Figure 3.2 Influence of the flow rate, (a) 0.6 ml/min, 10 °C, (b) 1 ml/min, 10 °C.

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 the half of the time, see Figure 3.3. Often the resolution becomes a little worse, in the case of fast kinetics however the loss of resolution is rather limited.

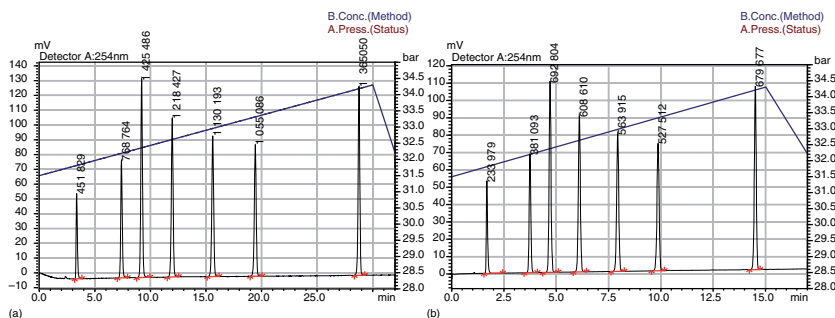


Figure 3.3 Influence of the flow rate, XBridge Shield, 150×4.6 mm, $5 \mu\text{m}$, (a) 50–90%B, 0.5 ml/min , $t_G = 30 \text{ min}$, (b) 50–90%B, 1 ml/min , $t_G = 15 \text{ min}$.

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 3.4.

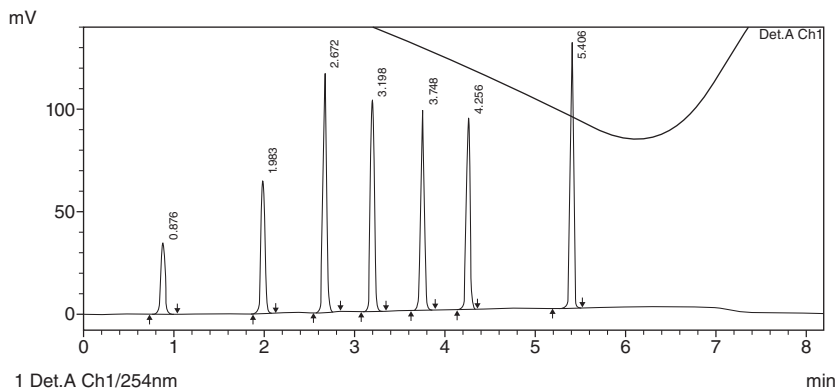


Figure 3.4 The gradient duration required, Zorbax SB C8, 150×4.6 mm, $5 \mu\text{m}$, 40–90%B, $t_G = 5 \text{ min}$, 2 ml/min .

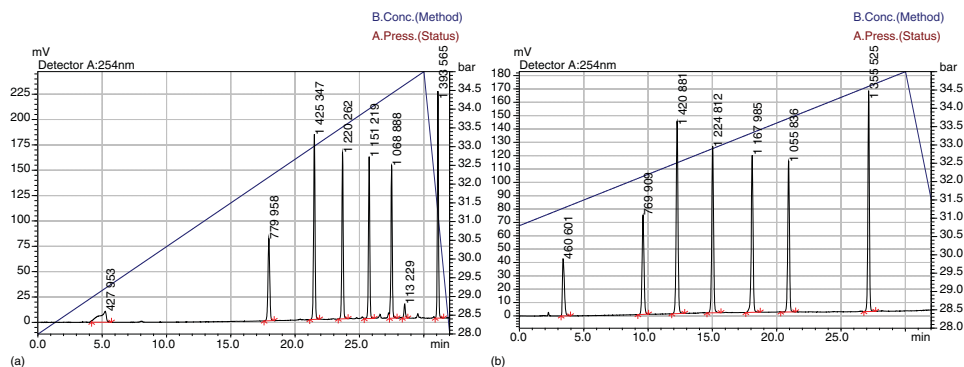


Figure 3.5 Effect of initial %B, XBridge Shield, 150×4.6 mm, $5 \mu\text{m}$, (a) 0–100%B, 0.5 ml/min, $t_G = 30$ min, (b) 40–100%B, 0.5 ml/min, $t_G = 30$ min.

Initial and Final %B, Slope As already mentioned earlier, the common practice in method development for an unknown sample, to start with a gradient of, for example, 5–100%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, are narrower and higher, see Figure 3.5. 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 3.6.

Note:

The initial %B is relatively unimportant for the elution of the last peaks, the same with an isocratic step at the beginning. To put it simply, 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, a flat gradient often leads to an improvement in resolution only in the later part of the chromatogram. Consider Figure 3.7, where the following two gradients are shown, 40–100%B and 50–90%B. With almost the same gradient duration is, with 50–90%B, the peak height in the first half of the chromatogram large, the resolution small, with 40–100%B exactly the opposite. 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.

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 co-elution, to an improvement of the

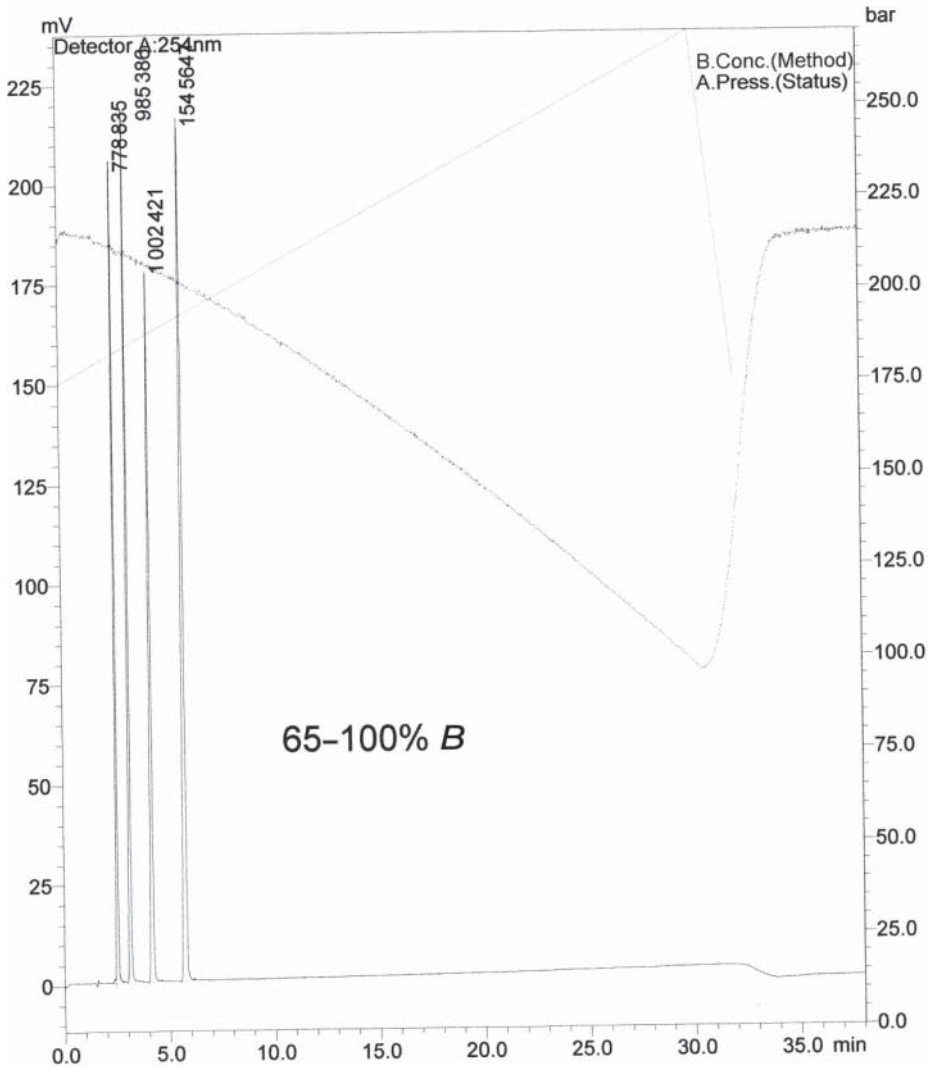


Figure 3.6 Regarding the starting conditions with a small number of peaks: Gemini NX, 50×4 mm, $3 \mu\text{m}$, 65–100%B.

resolution, see Figure 3.8: From 45–60% 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.

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

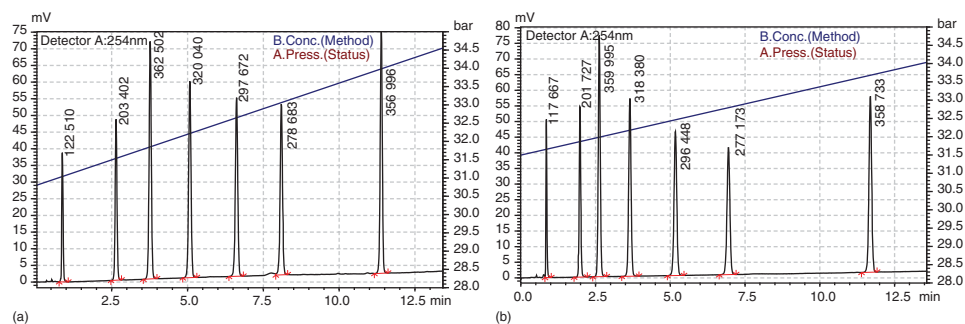


Figure 3.7 Effect of initial %B and slope, XBridge Shield, 150 × 4.6 mm, 5 μm, (a) 40–100%B, 2 ml/min, $t_G = 15$ min, (b) 50–90%B, 2 ml/min, $t_G = 15$ min.

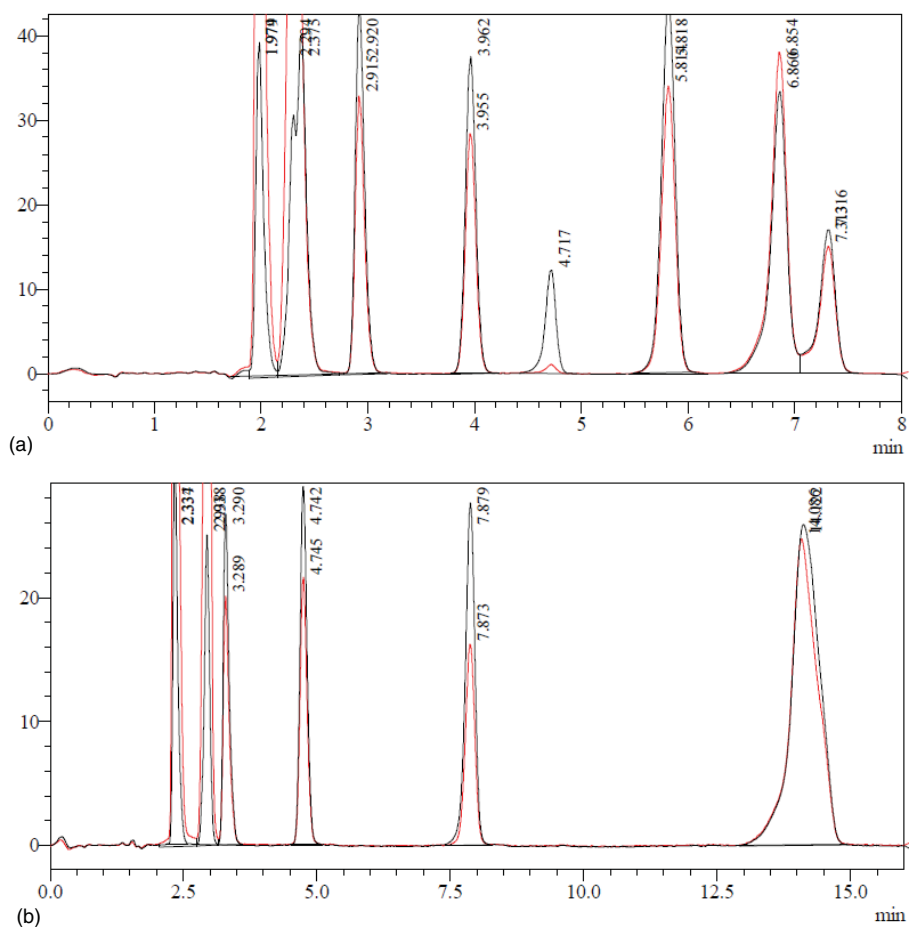


Figure 3.8 Influence of initial %B and slope, Symmetry C18 150 × 4.6, 5 μm initial %B: (c) 45%, (b) 60%, (c) 70%; final %B in all cases 100%.

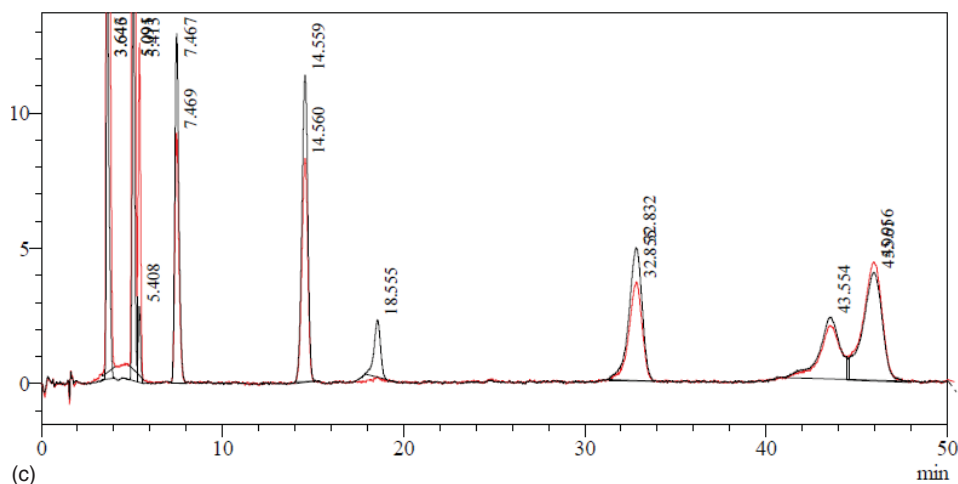


Figure 3.8 (Continued)

- Constant slope and modification of $\Delta\%B$ – that means parallel gradient profiles, similar chromatograms
- Constant $\Delta\%B$ and modification of the slope – that probably leads to different chromatograms

Initial and final %B and gradient duration – they determine the slope – or respectively column length and flow

As mentioned earlier, the two most important parameters for the resolution are $\%B/\text{min}$ (strictly speaking $\%B/\text{ml}$) and the gradient volume.

Consider the two following 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, $\%B/\text{min}$ remains more or less constant (about $\Delta 3\%B/\text{min}$), 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, $\%B/\text{min}$ 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.

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 3.9. Note the following: the gradient becomes flatter and longer, which gives a slope of $\Delta 16\%B/\text{min}$ through $\Delta 7\%B/\text{min}$ to $\Delta 3.3\%B/\text{min}$. 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 fourth peak, with changes in the order of elution and in the resolution (8 vs. 10 peaks).

Once again: in the case of not very difficult separations gradient duration and column length are not so important, see Figures 3.10 and 3.11. When one knows that only these seven peaks are to be expected, a 20 mm $\times$ 4.6 mm, 2 μ m column is sufficient, a 150 mm $\times$ 4.6 mm, 4 μ m column inevitably brings unnecessarily long

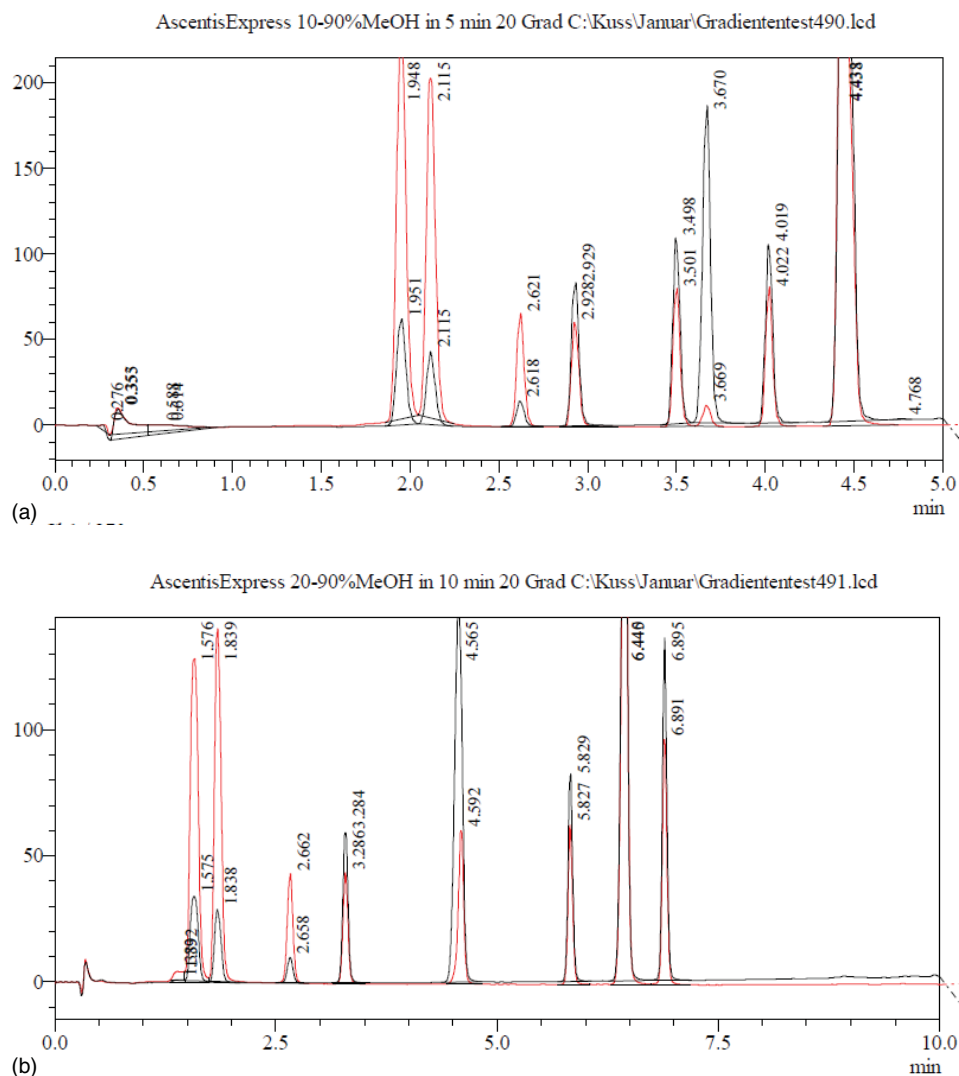


Figure 3.9 Effect of initial %B and gradient duration on elution order and resolution, AscentisExpress C18, 50 $\times$ 3 mm, 2.7 μ m, (c) 20–70%B, t_G = 15 min, (b) 20–90%B, t_G = 10 min, (a) 10–90%B, t_G = 5 min.

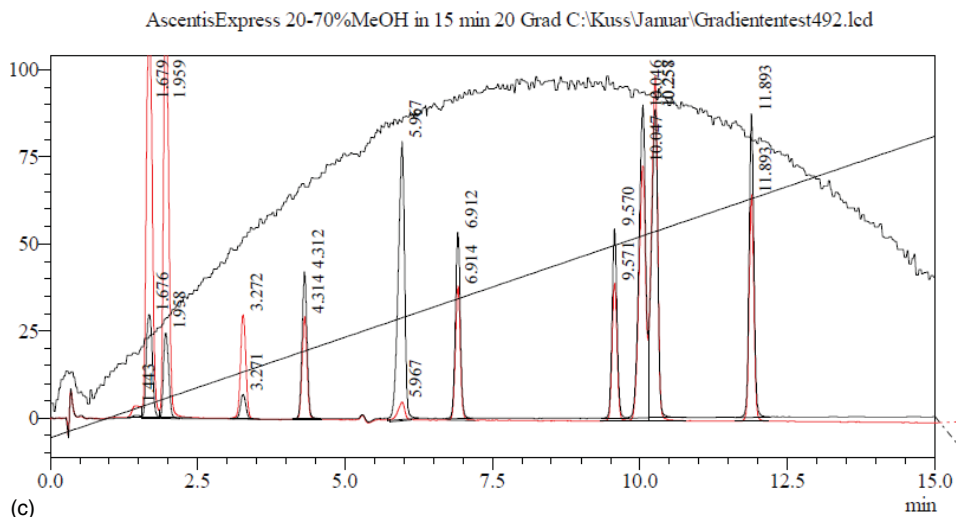


Figure 3.9 (Continued)

retention times. Figure 3.11 (column: 20 mm $\times$ 4 mm, 2 μ m), demonstrates what has already been described earlier. First, 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 with a 10 min gradient is equally sufficient (the drift in the lower image and the apparently broader peaks are no problem, the scaling is different).

Of course, with $\leq 2 \mu$ m particles and/or 20 mm columns, fused core materials, high throughput columns and Chromolith HR, we have noticed tailing and peak broadening due to the instrumental dead volume, 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 from the user as a disadvantage – and then it is no problem.

Now to conclude, some aphorisms regarding gradients as a compressed “take-home message,” many a repetition of the previous text is acceptable here.

3.1.7

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

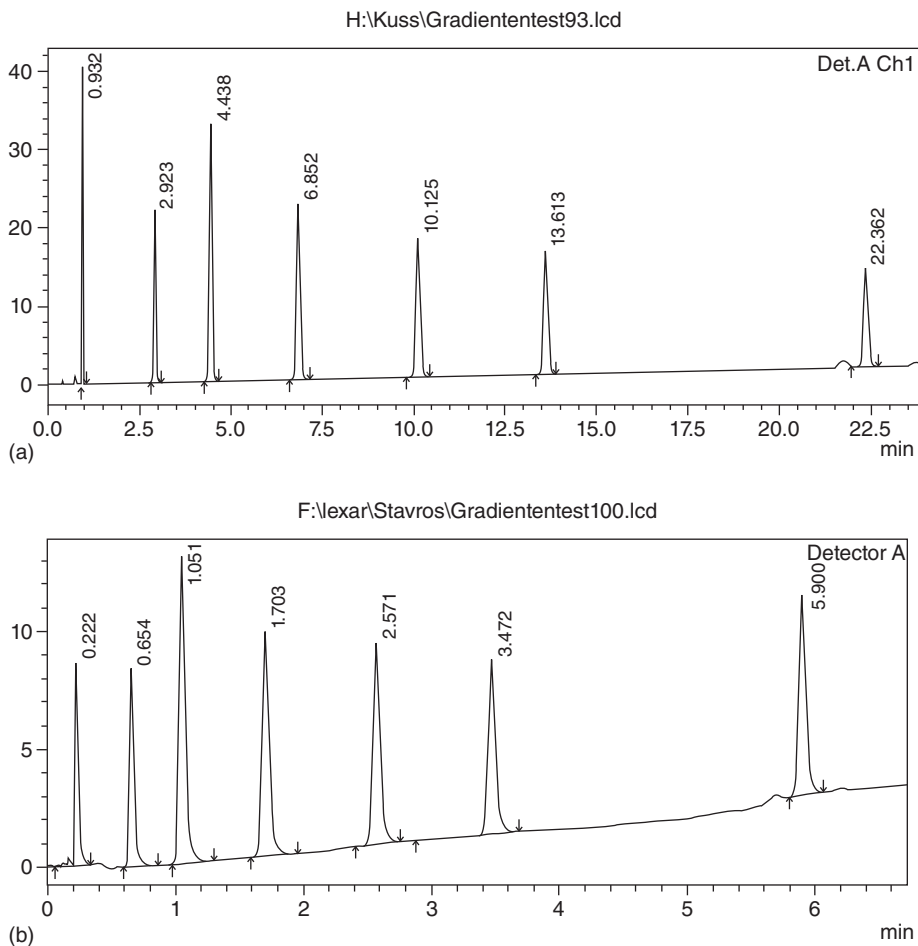


Figure 3.10 Influence of column length, (b) Synergi Fusion RP, 150 × 4.6 mm, 4 μm, (a) Synergi Fusion RP, 20 × 4.6 mm, 2 μm.

- Use a low flow with $\geq 5 \mu\text{m}$ particles, but also in the case of multiple interactions, that is, when slow kinetics are expected
- Try a high flow first when $\Delta\%B$ is $\geq 60\%$

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, $\Delta\%B$

- In general, $\Delta\%B$ 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 $\Delta%B$ and gradient duration and from these the slope

- With differing components, a $\Delta%B \geq 40$ 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 that the first peaks of interest elute after approximately two to three times the dead time – don't start unnecessarily with a high aqueous percentage. Choose the final %B so that all

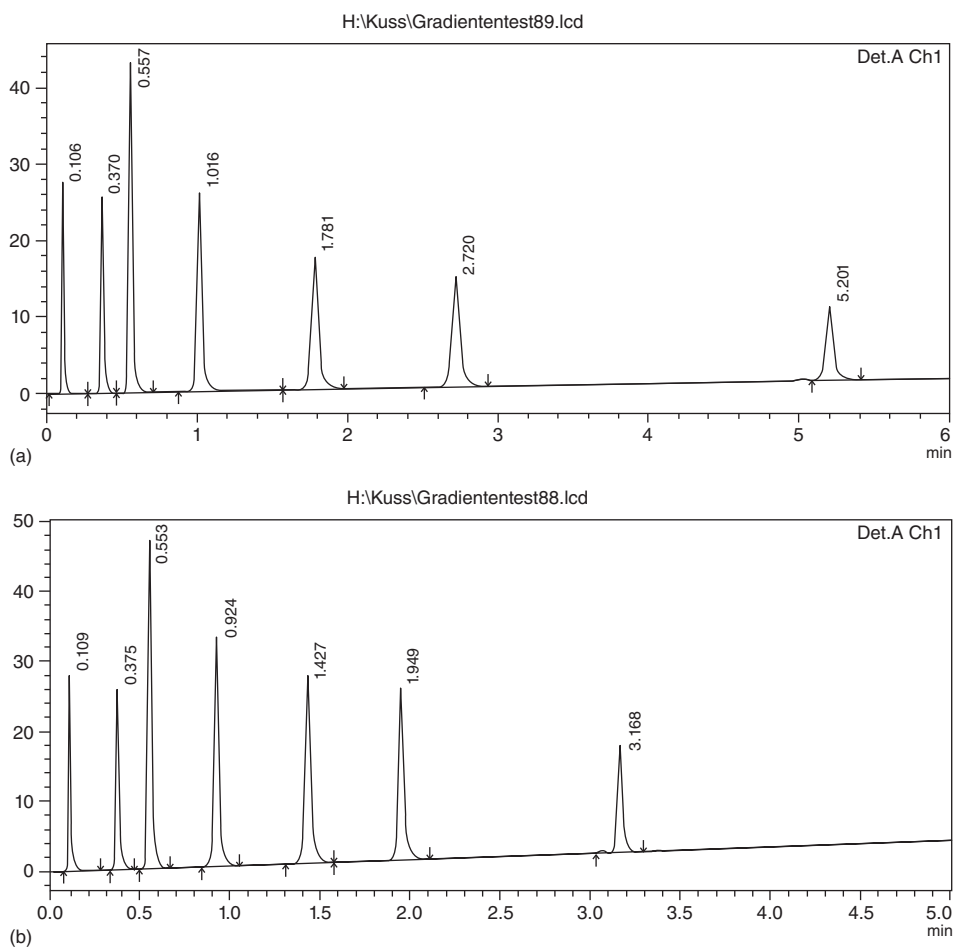


Figure 3.11 Influence of gradient duration, Synergi MAX RP 20 × 4 mm, 2 μ m, (a) gradient duration 2 min, (b) 5 min, (c) 10 min.

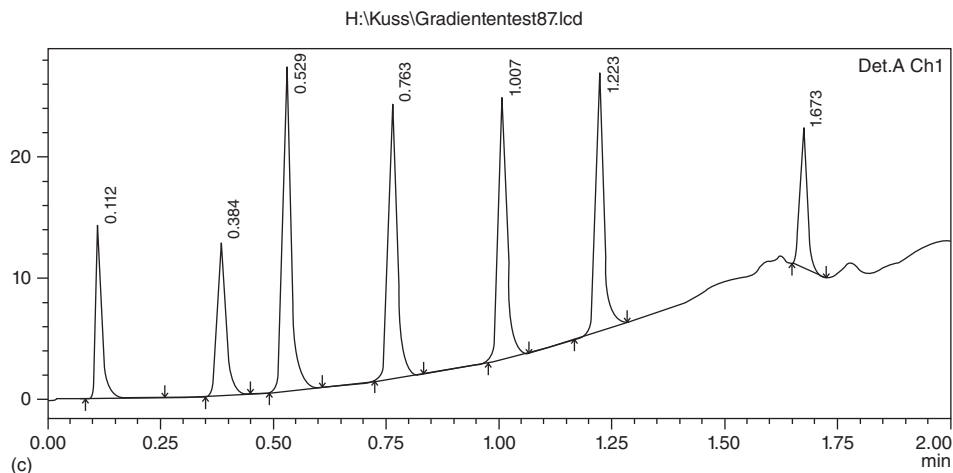


Figure 3.11 (Continued)

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 5–10 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* $\Delta\%B$): 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

- High peak capacity does not automatically mean good resolution overall
- The gradient compensates dead volume 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 Section 3.2.

Be courageous, when you have about ≤ 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 consequent, when you expect about ≥ 30 –40 peaks and have a UHPLC, then use a 150×3 or $2,1$ mm, $2\text{ }\mu\text{m}$ 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 $30\text{ }^{\circ}\text{C}$, 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 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 $5\text{ }\mu\text{m}$ particles, maybe also of a 4 mm or even 4.6 mm column.

3.2

Prediction of Gradients

Hans-Joachim Kuss

3.2.1

Linear Model: Prediction from Two Chromatograms

With only two chromatograms at appropriate isocratic conditions, the retention times for all other isocratic conditions can be predicted provisionally. Assume that we have a 15 cm SunFire column in the drawer as well as a test mixture of methyl-, ethyl-, propyl-, and butyl-paraben. Then we can start with, for example, 55% methanol and 45% water and obtain the chromatogram in Figure 3.12.

We can use our gradient system to make this eluent mixture. The horizontal line in all chromatograms shown in this chapter represents the set value of the methanol content, the rather uneven line the measured pressure. Both parameters should always be displayed as error indicators.

Now we have made an isocratic overview run and can see the four peaks with increasing retention times t_R in the above-mentioned order. Knowing the mobile time (formerly called dead time) of $t_0 = 0.75$ min (small peak, calculation see below) we can also calculate the retention factor k , which generally should be not

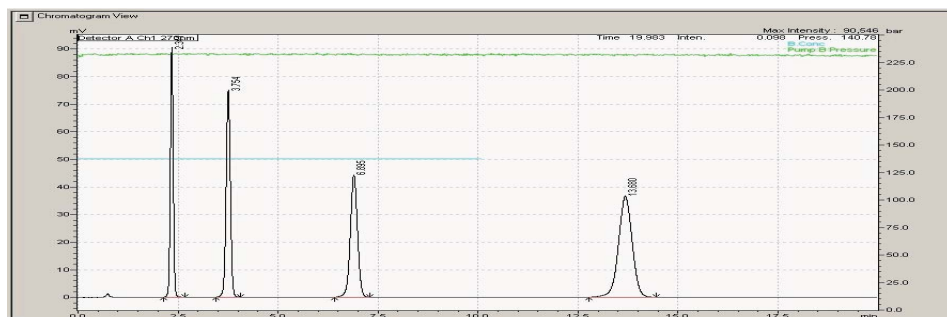


Figure 3.12 Parabens on a 150×4.6 mm SunFire 2 ml/min 55% MeOH/45% water at room temperature. The peak widths $w_{50\%}$ are 0.078; 0.115; 0.191; 0.365.

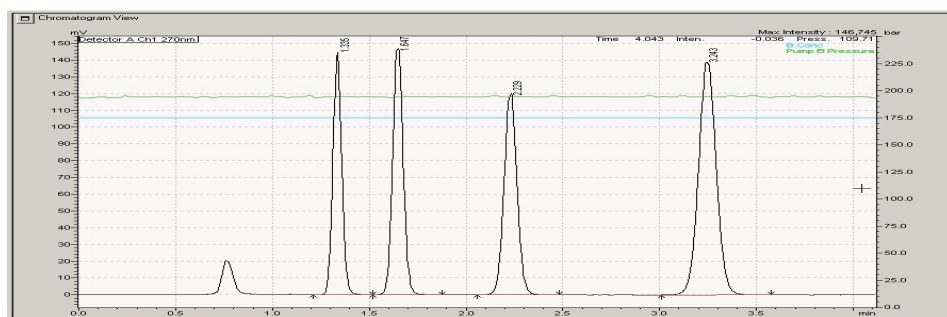


Figure 3.13 Parabens on a 150×4.6 mm SunFire 2 ml/min 70% MeOH/30% water at room temperature. The peak widths $w_{50\%}$ are 0.052; 0.058; 0.074; 0.098.

lower than 0.5. Methyl paraben has a k of 2.1. Therefore, a second measurement can be carried out at a higher methanol concentration, for example, at 70%B, as in Figure 3.13.

3.2.1.1 What Does the Retention Factor k Tell Us?

The *retention time* t_R is the primary variable in chromatography, from which we deduce whether an analyte is present or not. The retention times t_R are dependent on the column dimensions. When under the same conditions a column with three times the *length* L is used, then the retention times and t_0 increase by a factor of 3. A column with half the *internal diameter* d_c , that is, a quarter of the cross-section, reduces at the same flow rate the retention times and t_0 by a factor of 4.

To convert the retention times into the dimensionless *retention factors* k the *mobile time* t_0 is needed, which is calculated from the *mobile volume* V_0 and the *flow* F [7]. The mobile volume can be estimated from the internal volume of the column and a characteristic factor for the packing.

$$V_0 \approx 0,5d_c^2L \quad (3.6a)$$

$$t_0 = \frac{V_0}{F} \quad (3.6b)$$

When L and d_c are in centimeter, then V_0 is in milliliter. No separation can occur during t_0 , and so in the first step t_0 is subtracted from t_R . This gives the additional *hold-up time in the stationary phase* t_s . The relativization of t_s through the mobile time t_0 eliminates the influence of varying column dimensions.

$$k = \frac{t_R - t_0}{t_0} = \frac{t_s}{t_0} \quad (3.7)$$

The retention factor k depends on the interaction of the analytes with the respective column material but not on the column diameter d_c , the flow F , the column length L or the *particle diameter* d_p .

The retention factor k is a measure of the interaction

According to the Linear Solvent Strength Model [8], in RP HPLC k is logarithmically dependant on the *percentage of the organic fraction* %B, this means that a graph of $\ln(k)$ against %B results in a straight line for each substance under analysis with a large k_{00} for pure water and a characteristic $\ln(k)$ -slope S .

$$\ln(k) = \ln(k_{00}) - S\%B \quad (3.8)$$

The slope S is an individual, characteristic variable and dependant on the substance and the current interactions with the phase material. In the above-mentioned example (compare Figure 3.14), the four k -values are calculated and from them the $\ln(k)$ values. With the Excel functions “slope” and “intercept” the two characteristic straight line parameters are found. Then, for any other %B conditions the $\ln(k)$ and from them the k -values can be calculated and converted

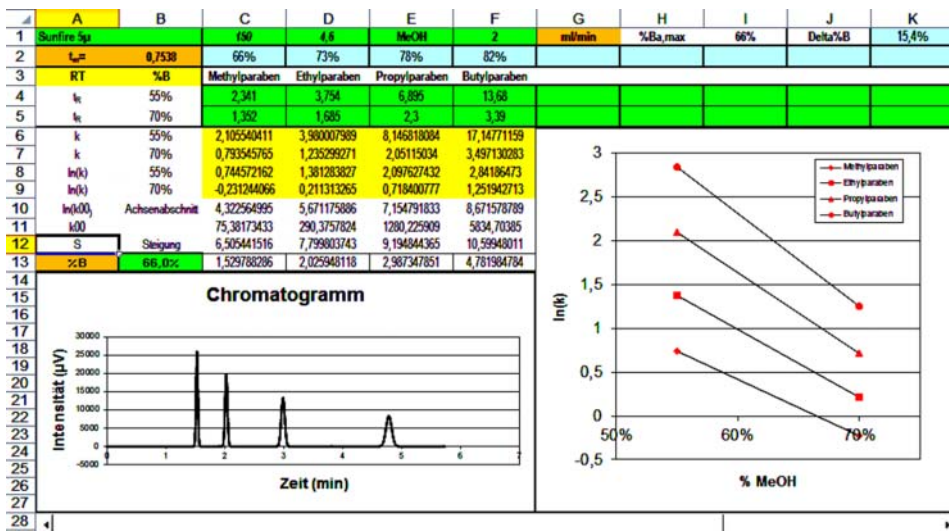


Figure 3.14 Detail from “2SIS SunFire MeOH.xls.” The dark grey shaded cells contain the input data. The bar in row 28 is a scroll bar that changes the value of %B in cell B13 and shows dynamically the chromatogram.

into retention times. With increasing chain length the parabens have much larger k_{00} -values, but also larger S values.

The usable *gradient range* $\Delta\%B = \%B_e - \%B_a$ (*final value minus initial value*) depends only on the conditions for the first and the last peak. We look for the leftmost and rightmost intersections of the straight line with the horizontal line for $\ln(k) = 0$, that is, $k = 1$. The $\%B$ value of the left intersection is the *maximum initial value of the organic modifier* $\%B_{a,\max}$ for both isocratic and gradient separations. Higher $\%B$ values as $\%B_{a,\max}$ lead to k -values less than 1, which should be avoided. Initial conditions of $\%B_a$ greater than $\%B_{a,\max}$ lead to the first peak being eluted too early and possibly unsatisfactorily separated. But lower initial conditions as $\%B_a$ can be set and thereby the separation modified. This variable is, therefore, in addition to t_g an optimization parameter. In Figure 3.3, the $\%B_{a,\max}$ in the $\ln(k)$ graph is shown as 66% MeOH. All four intersections can be calculated by dividing the intercept by the slope (see Figure 3.14, cells C2 to F2). In the cell I1, the minimum value of the intersections is requested and shown as 66%.

The right-hand intersection is a first guess of the $\%B$ -conditions by which the last peak must be eluted. Higher end values of $\%B_e$ do not alter the separation as the substances have already been eluted and no longer “see” the continued running of the gradient. Therefore, in the calculations $\%B_e$ is set to 100%. For the real measurement of chromatograms $\%B_e$ is reduced so far at constant gradient slope $\Delta\%B/t_g$ that the last peak remains “in the gradient.”

When the difference $\%B_e - \%B_a$ between the right and left intersections is larger than 40% [9], then to obtain all peaks within an acceptable analysis time a gradient must be used. In the above-mentioned example with $\%B_e - \%B_a = 15\%$ (see cell K1) no gradient is necessary. The shortest isocratic separation can be made with 66% MeOH. When a line is drawn vertically upwards at 66% MeOH, the chromatogram can almost be “seen” – the distance between the peaks increases, this is not very apparent on a logarithmic scale. At all $\%B$ -conditions visible here the lines are far apart, that is, the peaks have so much space between each other that they are very well separated, as shown by the chromatogram in Figure 3.14.

From a $\ln(k)$ versus $\%B$ graph the retention factors can be calculated for each (not measured) mixing ratio $\%B$ and thus for a given column dimension the retention times. With the retention times, the corresponding isocratic chromatogram can be visualized. The simulation of chromatograms from the report data is done with the Excel function “Normdist” [10].

The file “2SIS SunFire MeOH.xls” shows through movement of the scroll bar that with $\%B = 0\%$, that is, pure water, an analysis time of 3 days would be required and that between 90% and 100% the peaks coincide near t_0 . SIS stands for Single Isocratic Steps. In principle, the $\ln(k)$ versus $\%B$ -graph contains all the data required for the prediction of gradients, for this the integral over many isocratic conditions must be made. The “programming” of gradient prediction in Excel according to the linear model has been described in detail by Neue [11].

3.2.1.2 What Do the Two Retention Factors k_g and k_e Mean?

Imagine a car which, after a certain distance, passes through a light barrier at constant speed. Depending on the speed both the (retention) time t_R and the duration of the passage through the light barrier (= peak width w) are equally shorter or longer, so that t_R/w is constant. Remember that in chromatography t_R^2/w_b^2 is proportional to the *plate number* N . The *peak width* w_b can be determined asymptotically.

Then imagine a car moving at different speeds. The retention time depends on the integral of the speed, that is, from all speeds on the route. For the peak width only the speed when passing through the light barrier is relevant, no matter what has happened before.

In a gradient, both k_g (dependant on the course of the gradient) and k_e (dependant only on %B at the moment of exit from the column) must be calculated [11]. So on the face of it the retention factor k_g corresponds to the integral (simplified: the “average”) of the individual retention factors over the entire gradient under the selected conditions, while k_e is the retention factor at the moment of elution/exit from the column. As with the above-mentioned analogy, the *retention time* is calculated from k_g and the *peak width* from k_e . In isocratic separations, k_g and k_e coincide. The *gradient slope* G is explained below (Section 3.2).

$$k_g = \frac{1}{GS} \ln(k_0 GS + 1) \quad (3.9a)$$

$$k_e = \frac{k_0}{GS k_0 + 1} \quad (3.9b)$$

$$k_g > k_e \quad (3.9c)$$

The driving force in chromatography is the elution strength %B. In the chromatogram, in Figure 3.15 %B was changed from 55% to 70% methanol after 5 min. The first two peaks were eluted with only 55%, the last two peaks at first with 55% and then with 70%. The third and fourth peaks have a considerably longer

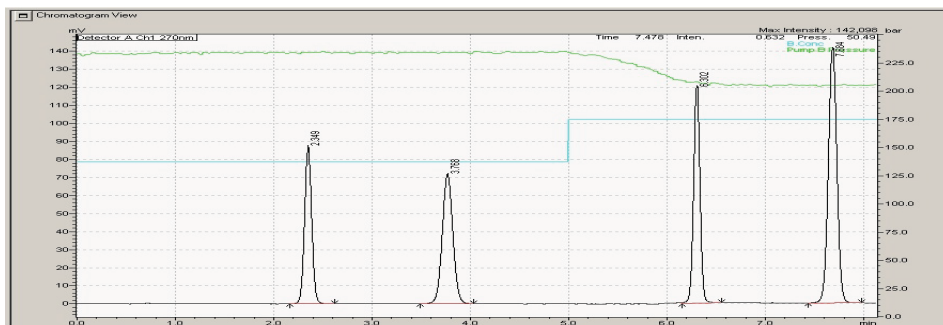


Figure 3.15 Parabens on a 150 × 4.6 mm SunFire 2 ml/min 55% MeOH for 5 min, then 70% MeOH at room temperature. The peak widths $w_{50\%}$ are 0.079; 0.116; 0.071; 0.093.

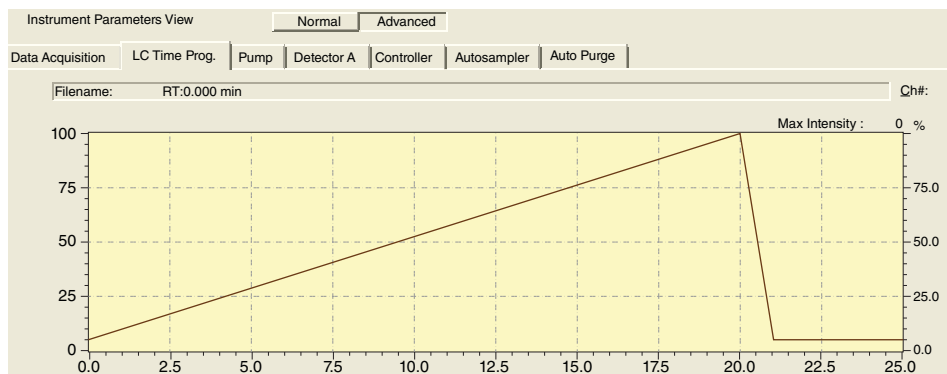


Figure 3.16 Gradient from 5% to 100%B in 20 min in the method file from Labsolutions (Shimadzu).

retention time than isocratically with 70%, but the peak width is identical to the 70% run.

3.2.1.3 How Does the Integration- and Control System See the Gradient?

With a simple linear gradient, the initial and end times of the gradient together with the two %B-values are entered into a table. The *Gradient time* t_g is calculated from the difference of the two times (Figure 3.16).

The instrumental gradient slope is obtained from the quotient:

$$\frac{\Delta \%B}{t_g}$$

The profile is presented graphically, including the return to the initial conditions after the gradient. After again reaching 5%B, 5–10 times t_0 have to be waited for equilibration of the column. When the column dimension (t_0) is not defined, then t_g cannot be rated as short or long.

When, for example, only the region of 20–80% MeOH is used, then to improve mixing it is useful to pre-mix the initial and final %B. The %B set instrumentally is then no longer identical with the real methanol content (also called Φ), but can be used directly just the same.

3.2.1.4 How Does the HPLC-Column See the Gradient?

As with the k -values, the relativization to the column dimensions is made by means of the relation of t_g to t_0 . This gives the *effective gradient slope* G . The quotient t_g/t_0 is a measure of how many times the mobile volume of the column flows through it during the gradient.

$$G = \frac{t_0 \Delta \%B}{t_g} = \frac{\Delta \%B V_0}{t_g F} \quad (3.10)$$

During a gradient, the analytes are moved by two driving forces. On the one hand at constant speed, dependant on the flow, on the other hand with increasing speed,

due to the gradient slope. Both effects complement each other multiplicatively. When I double the flow, then I have to halve the gradient slope if I want to get the same gradient and chromatogram, resulting in the same separation in half the analysis time.

Two columns 25 cm 5μ and 15 cm 3μ with the same RP-material also give the same separation when $t_g/t_0 = V_g/V_0$ remains constant. This means that at the same flow, t_g and parallel to this t_0 of the shorter column must be 5/3 lower in order to obtain exactly the same k -values for all analytes. The analysis time is reduced accordingly. Because the expected number of plates in both columns is about 10 000, the peak widths relative to the retention times are the same.

We have three instrumental variables: $\%B_a$, F , and t_g/t_0 . I can reduce the maximum initial concentration $\%B_a$ from the maximum value $\%B_{a,max}$ down to 0% (only with water compatible columns, otherwise 5% or 10%), usually I can increase the flow only by a factor of 2 or 3, so that t_g/t_0 remains as the main variable, which I can alter in the region of about 3–60.

3.2.1.5 How Do the Substances to Be Analyzed See the Gradient?

The individual analytes “experience” the gradient differently. The initial $\%B_a$ is the same, but each substance “sees” the gradient only until it leaves the column in the eluent with the $\%B$ current at this time. The effective gradient slope G is equal for all substances, but is modified by the varying influence of the organic modifier $\%B$ on the interactions, which is measured by the $\ln(k)$ -Slope S .

$$GS = \frac{\Delta \%B t_0 S}{t_g} \quad (3.11)$$

This is the *intrinsic gradient slope* (GS) individually effective on the analytes. The instrumental gradient slope G acts on a column of defined dimensions, and in conjunction with the interaction of each analyte with the column material used the substances are eluted. I have to work with the intrinsic GS , but I am free to change the eluent or to use a column with preferably other characteristics in order to change the interaction and thus the retention.

3.2.1.6 Interpretation of the $\ln(k)$ to $\%B$ Graph

This graph is the focal point for the gradient. From it can be estimated how difficult it will be to optimize a separation. The more substances that have to be analyzed, the more difficult the assignment of the peaks will be.

When in Figure 3.17 at a methanol content of, for example, 40% you move slowly vertically upwards, you pass through the isocratic chromatogram again. The distance between the straight lines corresponds to the distance between the peaks. The critical peak pair chlorobenzene/toluene can immediately be seen. With a gradient you go from $\%B_a$ diagonally up toward the right to $\%B_e$. However the gradient is also, and strongly, dependent on the gradient duration t_g .

The intersections indicate conditions $\%B$ where two peaks directly coincide and where a change in the peak order occurs, that is, a point to be avoided. If there are many intersections, which of course becomes more likely with an increasing

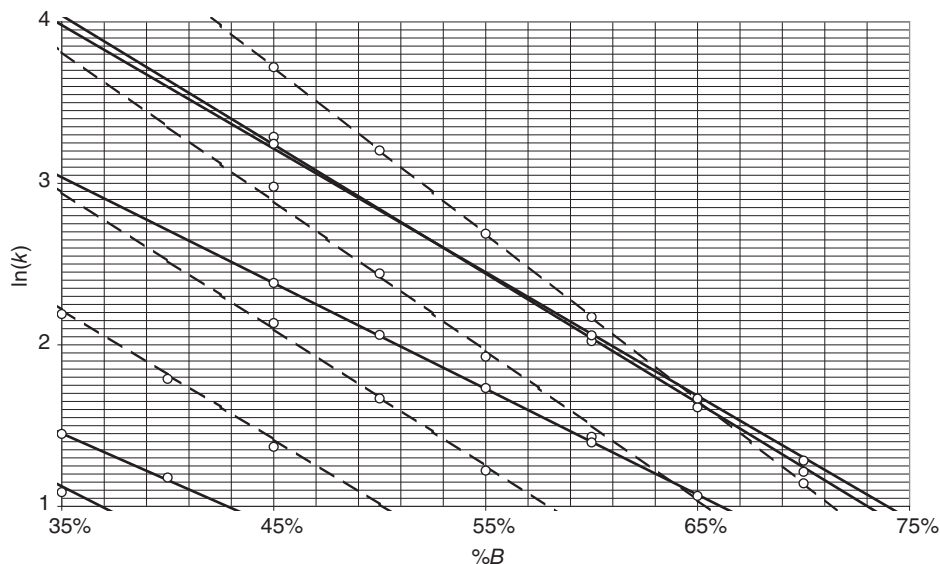


Figure 3.17 $\ln(k)$ versus $\%B$ graph on a 150×4.6 SunFire 5μ at 30°C . The four dashed lines are the four parabens in Figures 3.1–3.153.4. The solid lines from

bottom to top are phenol, benzaldehyde and benzene, followed by the two closely adjacent straight lines corresponding to toluene and chlorobenzene.

number of analytes, then the separation problem is difficult. When the intersections are widely distributed relative to $\%B$, the separation problem becomes even more difficult as several $\%B$ must then be avoided.

The probability of obtaining intersections is large with chemically different analytes, as the interactions with the stationary phase will differ. The more substances to be separated, then the sooner intersections become inevitable. Especially complicated are adjacent lines, that is, analytes with similar retention times and similar slopes. When the lines directly overlap then the separation problem cannot be solved, even with a gradient.

The separation factor α is the distance between the straight lines

The separation factor α is the quotient of the k -values of two neighboring peaks. The larger value (following peak) is always divided by the smaller value (preceding peak). A chromatogram with several peaks, all having $\alpha = 1.05$, means that for all peak pairs the second peak has a k -value about 5% higher than the respective first peak

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_m}{t_{R1} - t_m} = \frac{t_{S2}}{t_{S1}} \quad (3.12)$$

The separation factor α (formerly: selectivity factor) is the quotient of the two hold-up times in the stationary phase, that is, the relative interaction strength of a peak pair. Instead of α the value $\alpha - 1$ in percent can be used, that is, instead of $\alpha = 1.05$ then $\alpha - 1 = 5\%$, which is much easier to visualize.

With a glance at Figure 3.17 it can be seen that the separation problem has become difficult due to the five substances present in addition to the parabens. Phenol and benzaldehyde (bottom left) separate well over the entire %B range. In Figure 3.6, four intersections can be seen and a further intersection for ethyl paraben and benzene at about 30% can be suspected.

A $\ln(k)$ -axis is basically also a linear α -axis, because an equal interval in the logarithm of two values means the same quotient: $\alpha = k_2/k_1$. The logarithm of 1.05 (the α value necessary for a resolution greater than 1 with 10 000 theoretical plates) is 0.05. This means that when the $\ln(k)$ axis has a 0.05 grid, then the condition where $\ln(\alpha) > 0.05$ is reached can easily be seen. The interval of the lines must be at least one vertical box.

If the polarity of the analytes differs so much that they can be separated isocratically only in an unacceptably long analysis time, then a gradient is essential. If for each peak pair no differing isocratic conditions in which adequate separation results exist, then a gradient cannot be successful either. This can be seen by exactly overlying lines in the $\ln(k)$ versus %B-graph. This is a rare but possible case.

The main problem immediately recognizable, the separation of the nine substances on a SunFire column, is due to the very similar manner of interaction of toluene and chlorobenzene, which coincide by 55% methanol. Only at %B = 35% or at B = 75% are the lines separated by $\ln(k) = 0.05$ (one box vertically). At 75% the front peaks have almost no retention. Therefore, in the isocratic mode this separation problem can only be solved with a methanol content of 35% or lower. Then, however, the last peak butyl paraben has a $\ln(k)$ -value of 4.75, that is, $k = 115.6$, and a retention time of 175 min. So here there is no acceptable isocratic chromatogram.

With the calculation of k_g all chromatograms with a simple linear gradient can be predicted. As an initial value $\%B_{a,\max}$ is chosen as the first step and $\%B_e$ set to 100%. In contrast to real chromatography, no time is lost when the gradient runs further unnecessarily. Then t_g/t_0 is varied in the range of 3–60 in increments of one, and all values for k_g are calculated. Excel then calculates the retention times, which can be sorted by the function Small (); see Figure 3.18.

3.2.1.7 The Instrumental Gradient Delay (Dwell Time)

The gradient at the column outlet lags behind the start of the gradient through the system controller by the gradient delay time (*Dwell Time*) t_d . During this time, the analytes move with the initial gradient conditions $\%B_a$ isocratically. The instrument-specific time t_d must be taken into account when transferring gradient separations from one instrument to another. Regarding this problem in the UHPLC and with LC/MS-gradient separations, see Section 1.2.

The dwell time is best measured under realistic conditions, for example, with %B = 0–100% in 15 min. Instead of the column a capillary with preferably small internal diameter is used, in order to build up a realistic back pressure while increasing the volume as little as possible. Eluent A is water, eluent B water with a few parts per thousand of acetone. When the UV absorbance is set to zero, eluent B should have a maximum absorption of slightly less than 1 AU. The gradient is

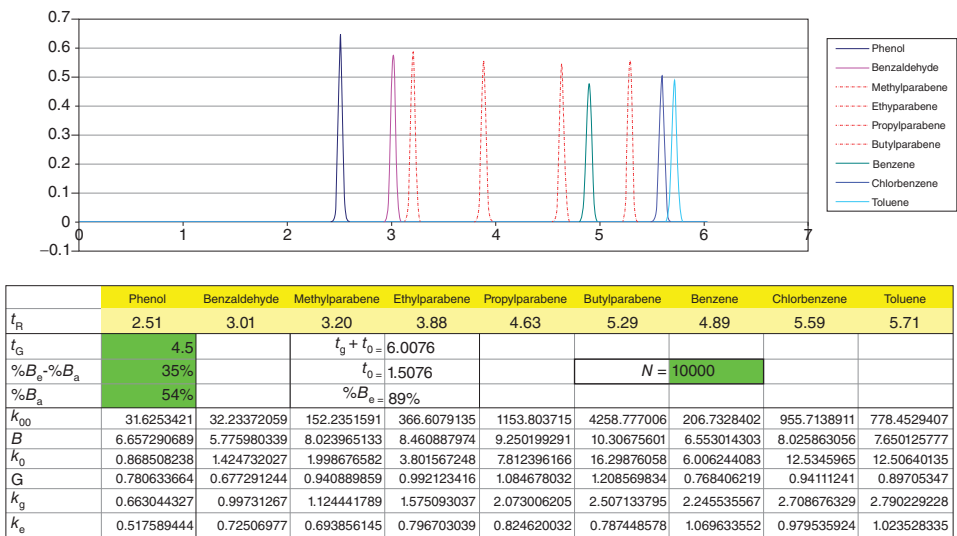


Figure 3.18 Shows the calculation and the predicted chromatogram with a satisfactory separation using the linear data of Figure 3.17 (listed in the lower part of Table 3.2).

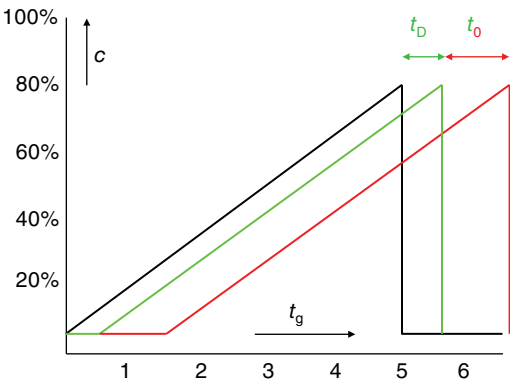


Figure 3.19 At the end of the column, the analytes exit delayed by the dwell time t_d and the mobile time t_0 .

run and the time $t_{50\%}$ determined, which is when half the maximum absorption is reached. To obtain the dwell time, half the gradient time is subtracted from $t_{50\%}$ (Figure 3.19).

The dwell time t_d is a (hopefully) short isocratic separation preceding the gradient. Therefore, t_d must be taken into account in the equations. Before the eluent reaches the column outlet, additionally t_0 elapses (see Figure 3.17). Only when the analyte has left the column can it no longer be affected by the continued running of the gradient. In Figure 3.15, the third peak, which comes about 1.3 min after the eluent change, is clearly eluted in 70% MeOH. Also the base line offset and the pressure profile indicate that $t_d + t_0$ is about 1 min. At a flow of 2 ml/min

$t_0 \approx 0.75$ min and $t_d \approx 0.25$ min, and so the dwell volume can be estimated at about 0.5 ml. A more accurate measurement as described here results in a dwell volume of 0.325 ml.

During t_d , the analytes move with different speeds and with an individual *retention factor* k at the initial conditions % B_a through the column:

$$\frac{t_d}{t_R} = \frac{t_d}{t_0(k_0 + 1)} \quad (3.13)$$

Substances with long retention times are relatively unaffected, on the other hand substances with short retention times and small retention factors k_0 are strongly affected.

The mobile time is the sum of an *isocratic fraction* $t_{0,d}$ and a *gradient fraction* $t_{0,g}$. (Note: The mobile or dead time remains of course overall unchanged, because whether 100% water or 100% acetonitrile is used is irrelevant for the elution of a true inert component, which is influenced only by the column dimensions and the flow. In reality, there is a slight influence:

$$t_0 = t_{0,d} + t_{0,g} \quad (3.14a)$$

and

$$\frac{t_d}{t_R} = \frac{t_{0,d}}{t_0} \quad (3.14b)$$

Summarized and solved for $t_{0,g}$, the result is

$$t_{0,g} = t_0 - \frac{t_d}{k_0 + 1} \quad (3.15)$$

With decreasing k_0 the substances have moved further into the column. Therefore, $t_{0,g}$ is different for each substance and only the remaining part of the column is available for the gradient. Of course, the predetermined instrumental gradient slope does not change, but the effective gradient slope does, as the column available for the gradient has become “shorter.”

The various molecules all move into the column during the same time t_d . But as they are retained to varying extents under the initial gradient conditions % B_a they move different distances, the “early” substances a long way, the “late” substances not so far.

The retention factor k_0 is different for each substance and for each (initial) eluent mix % B_a . This means that during t_d each substance moves a different distance into the column, and this is dependent on % B_a . The “remaining column” available for the gradient is, therefore, different every time, which manifests itself in differing $t_{0,g}$. Of course, the shorter the column used then the shorter the “remaining column” is (see Eq. (3.12): t_d is constant, but t_0 is shorter).

Many HPLC manufacturers now offer the possibility of delayed injection, in which the injection is delayed by t_d after the start of the gradient. With a software modification this should also be possible with older systems. It is not so much a matter of the additional computing effort, rather the noncomparability of gradients on different systems with differing t_d (Table 3.1).

Table 3.1 Contains in the upper part the retention times of the eight gradient chromatograms with an instrumental gradient slope of 5% MeOH/min.

Retention times using a 100*3 Chromolith-column ($t_0 = 0.4275$ min, 1 ml/min) gradient extension at constant gradient slope (5%B/min)											
%B	tG	Phenol	Benzaldehyde	Methylparabene	Benzene	Ethylparabene	Propylparabene	Toluene	Chlorobenzene	Butylparabene	
45-70	5	0.964	1.158	1.397	2.498	1.997	3.02	3.867	3.867	4.191	
40-70	6	1.089	1.366	1.864	3.062	2.68	3.86	4.684	4.684	5.147	
35-70	7	1.229	1.533	2.316	3.696	3.474	4.769	5.543	5.543	6.123	
30-70	8	1.435	1.773	2.769	4.325	4.305	5.695	6.412	6.431	7.1	
25-70	9	1.714	2.066	3.397	5.022	5.183	6.653	7.259	7.344	8.095	
20-70	10	2.075	2.413	4.134	5.695	6.112	7.628	8.168	8.274	9.093	
15-70	11	2.545	2.822	4.956	6.371	7.069	8.614	9.092	9.217	10.092	
10-70	12	3.138	3.305	5.844	7.062	8.041	9.613	10.03	10.171	11.089	
Difference	6-5	0.125	0.208	0.467	0.564	0.683	0.84	0.817	0.817	0.956	
Difference	7-6	0.14	0.167	0.452	0.634	0.794	0.909	0.859	0.859	0.976	
Difference	8-7	0.206	0.24	0.453	0.629	0.831	0.926	0.869	0.888	0.977	
Difference	9-8	0.279	0.293	0.628	0.697	0.878	0.958	0.847	0.913	0.995	
Difference	10-9	0.361	0.347	0.737	0.673	0.929	0.975	0.909	0.93	0.998	
Difference	11-10	0.47	0.409	0.822	0.676	0.957	0.986	0.924	0.943	0.999	
Difference	12-11	0.593	0.483	0.888	0.691	0.972	0.999	0.938	0.954	0.997	

The lower part of the table shows the seven differences from the second to the first chromatogram, and so on (e.g., 0.125 min = 1.089-0.964), that is, the additional retention caused by the extension of the gradient.

3.2.1.8 Extension of the Gradient Downwards by Constant Slope

The earlier gradient start from $%B_a = 45\%$ down to 10% in steps of five causes a significant change in the separation. The two peaks for ethyl paraben and benzene even move past each other. Interesting is that the last peak is shifted toward the end only by almost a minute (differences between 0.97 and 1 are shaded gray), this means that butyl paraben in contrast to all earlier peaks remains “stuck” at the front of the column. The peak width of butyl paraben remains constant, although the retention times increase. The elimination concentration does not change either.

The smaller the differences are, the further the substances move due to the respective “gradient extension.” Only when $\ln(k) \approx 5$, that is, $k \approx 150$ do the substances “freeze,” they do not move any further, and without forward movement no separation is possible. In contrast to propyl paraben, toluene and chlorobenzene do show a certain degree of mobility despite higher retention times, even in the 12-min chromatogram. This is explained by the higher $\ln(k)$ -slope of the parabens, through which the $\ln(k)$ limit is reached earlier.

It should be noted that by reducing the gradient initial value $%B_a$ more changes are to be expected in the front part of the chromatogram as in the rear section. The upshot of the gradient extension and the dwell time is: when $%B_a$ is reduced from its maximum value $%B_{0,\max}$ down to zero, then the farther forward that the peaks come in the chromatogram, the greater is the change in the separation. This can lead to a better separation, but also a change for the worse. In the rear section, only a slightly modified separation is to be expected.

3.2.2

Curvilinear Model: More than Two Input Chromatograms

3.2.2.1 The $\ln(k)$ -Straight Lines Are Often not Straight at All

When measurements are made at 5–10 different $%B$ values, then the $\ln(k)$ values often show a curvature, which is less with methanol and stronger with acetonitrile and tetrahydrofurane. In the equation, the curvature is superimposed over the straight line and a curvilinear course results. As a result, a two-point linear fit can give slightly varying intercepts and slopes, depending on which two data points have been measured. Furthermore, due to opposite curvature two curves can in reality drift further apart than can be shown with the straight lines. Finally, the opposite curvature can also lead to two intersections, which two straight lines cannot have.

Schoenmakers [12] writes in the summary: “Firstly, the relationship between $\ln k$ and ϕ is generally nonlinear, in accordance with previous statements” and further below: “Consequently $k(\phi)$ should correctly be expressed as $\ln k = A\phi^2 + B\phi + C$. Snyder and co-workers, conceding this point, nevertheless argue that the linear relationship provides an adequate approximation for practical gradient elution.” In other words, Schoenmakers proposes describing the curvature with a superimposed parabola.

With today's HPLC instruments running automatically overnight, then unlike previously it makes little difference whether only 2 instead of 10 chromatograms are run. The real effort is in the sample preparation, the programming of the sample sequence and possibly the preparation of the eluents (Table 3.2).

The data of Table 3.2, whose graphs are shown in Figures 3.17 and 3.20, were taken during one night. This input mask can be used just as well for only two measurements. Chromatograms with a difference of less than 5%B will hardly be needed. The shaded cells in the first three rows have to be filled in, as they are repeatedly required further below.

When comparing Figures 3.20 (curved) and 3.17 (linear) with the identical measurement points, it can be seen that the linear fit to all data points sometimes clearly misses these. Not all data points are shown in the figures, in order to show the section enlarged. In Figure 3.20, two additional intersections can be seen between 35% and 40%. The quite significant deviations from reality in Figure 3.17 lead even with isocratic predictions to an error of far more than 1%, although methanol causes only slight deviations from linearity. These errors are inevitably contributory factors in the gradient prediction – if this is based on the linear model.

3.2.2.2 The $\ln(k)$ to %B Fit According to Neue

The correction of the $\ln(k)$ to %B curves can also be described with a hyperbolic function [13], which has the advantage that it can be simply integrated. This is taken into account by a *curvature factor* a . The quadratic function proposed by Schoenmakers [12] sometimes shows a (physically implausible) upturn of $\ln(k)$ in the upper %B-region, which however usually lies in the region where the substances have already been eluted.

$$\ln(k) = \ln(k_{00}) + 2 \ln(1 + a\%B) - \frac{S\%B}{1 + a\%B} \quad (3.16)$$

The gradient parameters are named differently depending on the author and abbreviated differently. In contrast to [14], %B is used here for the organic fraction instead of c , the $\ln(k)$ -slope named S instead of B and the effective gradient slope G instead of S .

At the lower end of Table 3.4 the characteristic values %B-slope S and intercept $\ln(k_{00})$ for the linear model are calculated directly and the curvature factor set to zero. The three values for the curvilinear model according to Eq. (3.16) are determined iteratively using the Excel Solver and copied into the table.

The equations for k_g and k_e are frighteningly long [14]. Repetitive calculations are summarized in this chapter in the three auxiliary variables H , I , and J , which are built successively.

$$H = 1 + a\%B_a \quad (3.17a)$$

$$I = k_{00} e^{-S\%B_a/H} \quad (3.17b)$$

Table 3.2 The input cells are in the upper part in the range of 0% to 100% MeOH.

Sunfire 5 μ 30° 150 4.6		MeOH								1 (ml/min) $t_m = 1.508$
Retention time		Phenol	Benzaldehyde	Methylparabene	Ethylparabene	Propylparabene	Butylparabene	Benzene	Chlorobenzene	Toluene
0% MeOH										
5% MeOH										
10% MeOH										
15 MeOH										
20% MeOH										
25% MeOH		10.872	12.844	34.342						
30% MeOH		7.996	10.127	22.683						
35% MeOH		5.983	7.925	14.984						
40% MeOH		4.773	6.403	10.527						
45% MeOH		3.849	5.144	6.403	14.258	31.156	72.159	17.852	41.891	40.179
50% MeOH		3.217	4.213	5.475	9.497	18.828	39.91	13.361		
55% MeOH		2.773	3.527	4.214	6.621	11.858	22.92	10.03		
60% MeOH			3.017	3.392	4.839	7.807	13.668	7.592	12.906	13.317
65% MeOH				2.852	3.751	5.493	8.7	5.879	9.076	9.489
70% MeOH					3.048	4.087	5.885	4.664	6.584	6.945
75% MeOH						3.22	4.24	3.771	4.908	5.226
80% MeOH						2.673	3.256	3.161	3.841	4.082
85% MeOH							2.646	2.709	3.101	3.283
90% MeOH									2.609	2.741
95% MeOH										
100 MeOH										
Curvature factor a		0	0	0	0	0	0	0	0	0
%B-slope linear		6.657	5.776	8.024	8.461	9.250	10.31	6.553	7.995	7.650
ln(k_{00}) linear		3.454	3.473	5.025	5.904	7.051	8.357	5.331	6.837	6.657
Curvature factor a		0.465	-0.091	0.403	0.698	0.781	0.737	0.052	0.203	0.130
%B-slope curved		10.44	5.164	12.116	18.505	22.659	24.56	7.107	10.827	9.338
ln(k_{00}) curved		3.943	3.386	5.648	7.927	9.925	11.576	5.47	7.585	7.102

In this example, the empty white cells represent double peaks, where the retention time cannot be clearly assigned and, therefore, are better omitted, as otherwise they increase the inaccuracy. The lower part shows the linear fit and shaded the fit according to Neue.

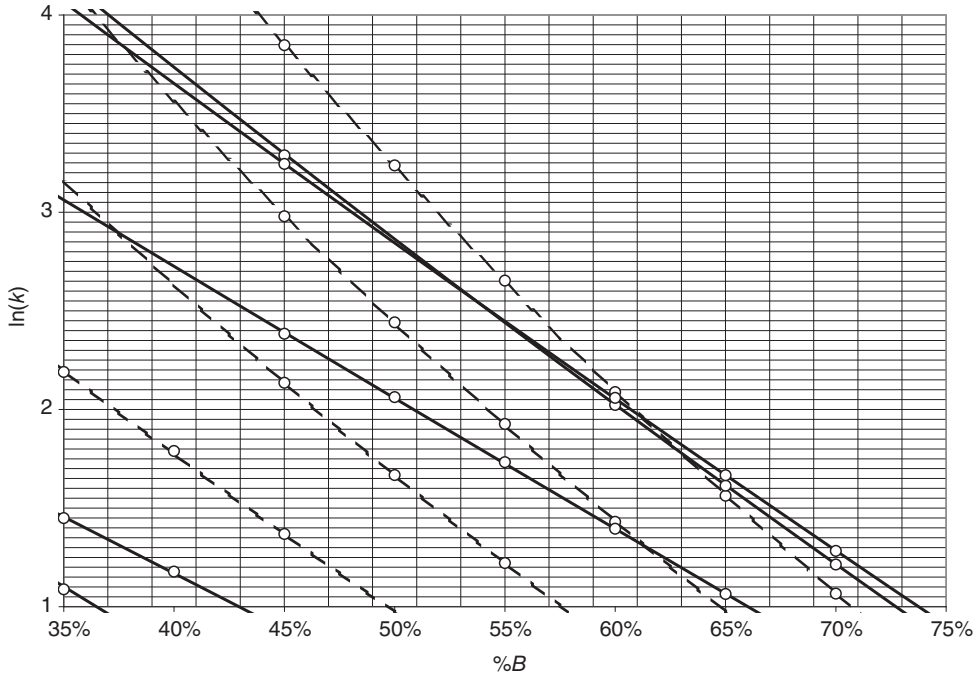


Figure 3.20 Is identical to Figure 3.17; however, the fitted lines are curvilinear.

$$J = \ln(GSI/1) \quad (3.17c)$$

Then k_0 , k_g , and k_e are calculated:

$$k_0 = k_{00} H^2 e^{-S\%B_s/H} \quad (3.18a)$$

$$k_g = \frac{1}{GS} \frac{I^2 J}{1 - (a/S)HJ} \quad (3.18b)$$

$$k_e = \frac{I}{J} \left\{ \frac{H}{1 - (a/S)HI} \right\}^2 \quad (3.18c)$$

and from this t_R with the fractions $t_{R,grad}$, $t_{R,d}$ (the isocratic prerun) and σ as a function of the square root of the plate number N .

$$t_R = t_{0,g}(k_g + 1) + t_{0,d}(k_0 + 1) \quad (3.19a)$$

$$\sigma = \frac{t_0(k_e + 1)}{\sqrt{N}} \quad (3.19b)$$

The resolution is calculated from

$$R_s = \frac{t_{R,n} - t_{R,n-1}}{2\sigma_n + 2\sigma_{n-1}} \quad (3.20)$$

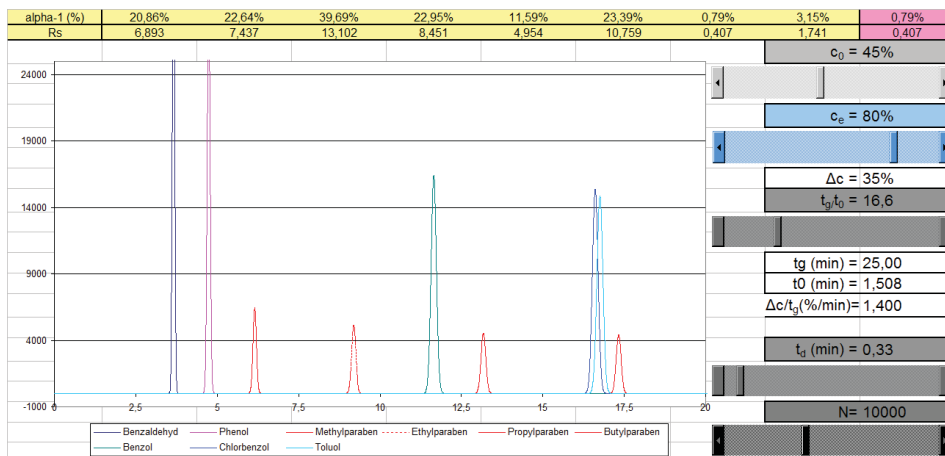


Figure 3.21 Cutout from the file “SunFire MeOH 30Grad.” The peaks are shown individually colored in the file for easier identification of overlaps. The four parabens are

the four small peaks. The order of the larger peaks is phenol, benzaldehyde, benzene, chlorobenzene, and toluene.

3.2.2.3 Predictions with Excel

These calculations are entered in the file “GS SunFire MeOH 30Grad.xls” and shown graphically in a chromatogram (Figure 3.21). With the scroll bar the parameters $\%B_a$, $\%B_e$, t_g/t_0 , t_d , and N can be varied continuously.

This file can be used universally. If the linear fit is preferred or only two measurement points are available, then the respective k_{00} and S -values can be copied into the file and a set to zero. Zero can also be entered for the dwell time. It would easily be possible to combine the input tables with the gradient simulation. Only for the curved lines is it necessary to adjust k_{00} , S and the curvature factor a (offline) by use of the Solver. However, in all cases it is recommended to check and interpret the $\ln(k)$ to $\%B$ graphs, as they are the key to the gradient calculations. Then, the checked data from the EIS-File (“Individual Isocratic Steps”) are copied into the “gradient simulation” GS-File.

With the Excel table in Figure 3.21 a simple linear gradient is predictable. With the scroll bar the dwell time t_d and the initial and final conditions $\%B_a$ and $\%B_e$ are set and the relative gradient time t_g/t_0 selected. Then, the conditions can be run through continuously and the changes in the chromatogram observed. Figure 3.21 shows the prediction with the same conditions as the real chromatogram (Figure 3.22). The agreement is very good.

The fit according to Neue does not give a result visually significantly different than the result from the linear model. The above-mentioned separation between the penultimate (double peak) and the last peak is, however, overestimated by the linear model, as can be seen in Table 3.3. The deviations according to Neue are less than 1%. The deviations resulting with the linear model are not only higher, but also have from peak to peak opposite signs (Table 3.3).

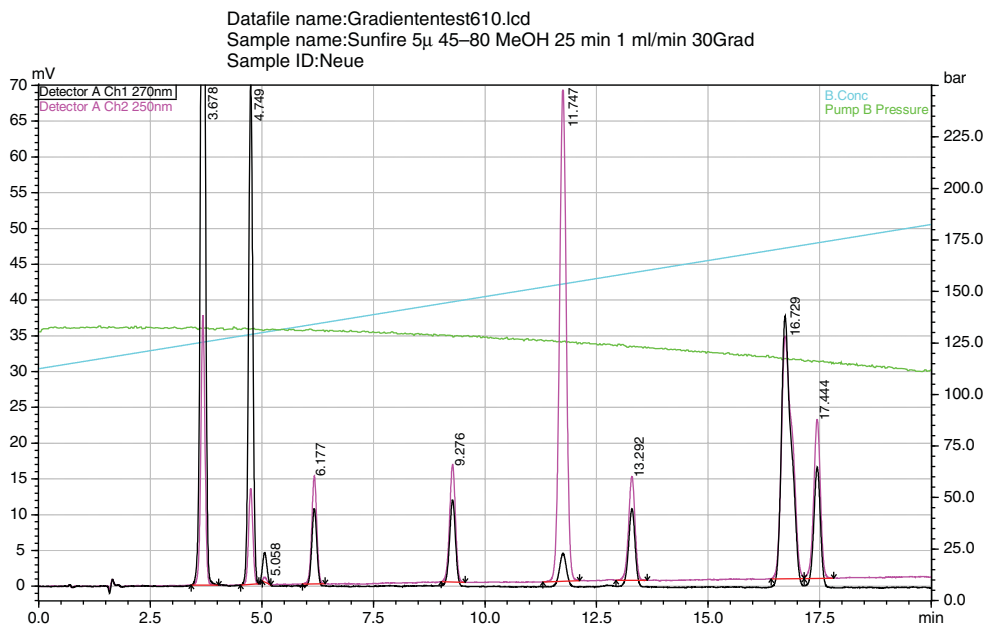


Figure 3.22 Measurement of nine substances on a SunFire column and with a gradient of 45–80% methanol in 25 min with a flow of 1 ml/min at 30°C.

Table 3.3 Retention times of the prediction according to Neue, according to the linear model and measured values, as well as percentage deviations from the measured values.

t_R (min)	Neue	Linear	Measured	Dev. Neue (%)	Dev. Linear
Phenol	3.667	3.698	3.678	–0.30	0.54
Benzaldehyde	4.748	4.731	4.749	–0.02	–0.38
Methylparabene	6.170	6.329	6.177	–0.11	2.46
Ethylparabene	9.223	9.269	9.276	–0.57	–0.08
Propylparabene	13.216	13.391	13.292	–0.57	0.74
Butylparabene	17.375	17.748	17.444	–0.40	1.74
Benzene	11.680	11.679	11.747	–0.57	–0.58

The nonseparated substances toluene and chlorobenzene were omitted.

With the linear model, an absolute satisfactory prediction often results. But how does one know when it is not satisfactory? It is recommended to use 5–8 measurements to exactly determine the curvature of the lines. More accurate basic data lead to more accurate predictions.

3.2.2.4 The Interaction Is Temperature Dependant

The logarithmic retention factors are, in compliance with thermodynamics, inversely proportional to the temperature (°K). $\ln(k)$ against $1/T$ is extrapolated

to absolute zero, $\ln(k_{0,T})$ is found and from this and the slope D the value of $\ln(k_{00})$ at the desired temperature $T(^{\circ}\text{K})$ is calculated. Similarly, the $\ln(k)$ -slope S with its temperature dependence can be calculated.

$$\ln(k_{00}) = \ln(k_{0,T}) + \frac{D}{T} \quad (3.21a)$$

$$S = \left(1 + \frac{D}{T}\right) S_T \quad (3.21b)$$

The temperature, in HPLC usually between 10 and 60 $^{\circ}\text{C}$, is an effective additional optimization parameter. The corresponding Excel table (Table 3.4 shows the table header) was developed by U.D. Neue. By entering the desired temperature in cell B3, k_{00} and S are adjusted according to the above-mentioned equations.

3.2.2.5 Optimization Parameters

The general separation problem is to separate all the peaks present sufficiently well from each other, that is, all peak pairs must have a minimum separation. This requirement is quantified in the standard operating procedures (SOPs) by a *resolution* R_s , which represents a minimum value. R_s is calculated from the difference in the retention times of a peak pair divided by the average of the two peak widths measured asymptotically.

A value for R_s under 1 corresponds to an unacceptable separation, between 1 and 2 (baseline separation) the separation improves continuously. For peaks of about the same height, the separation does not become better at $R_s > 2$. Therefore, R_s is not a suitable optimization parameter for iteration programs such as the Solver, $1/R_s$ or $1/R_s^2$ is better. The *Peak Separation Index (PSI)* would be even better, but this is not given by the integration systems. This percentage depth of the valley between two peaks runs only from 0% (shoulder peak) to 100% (baseline separation), based on the height of the smaller peak. With the PSI no improvement to baseline separation is possible, while the resolution (and also the separation factor α) continue upwards, without leading to a chromatographically meaningful improvement.

3.2.2.6 Commercial Optimization Programs

The two commercial programs DryLab and ChromSword are mentioned here as examples. The Excel tables for prediction of a single linear gradient previously shown cannot replace these commercial programs in their perfection. The Excel tables are intended as an introduction to gradient prediction and should lead to a better understanding of gradients. With concrete data, however, and all the means of prediction mentioned here, "What happens when" questions can be answered, which with real measurements is very time consuming (Figure 3.23).

For evaluation of the predicted chromatograms DryLab uses a resolution map, which with measurements at, for example, two columns temperatures or differing pH is three dimensional. Gradients are shown on a graphic interface, where t_g , $\%B_a$ and $\%B_e$ can be changed with the mouse. The gradient editor allows entry

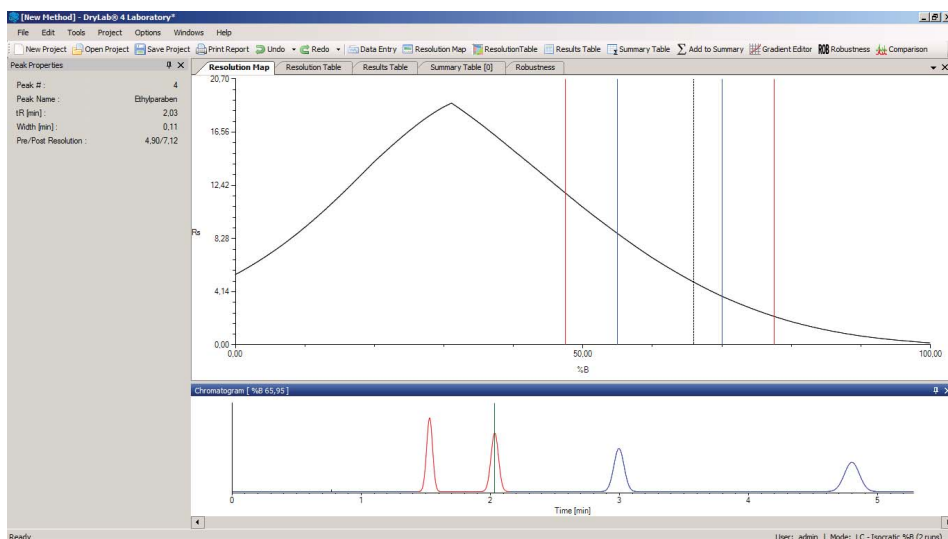


Figure 3.23 After entering the retention time in Figures 3.1 and 3.2, with DryLab the Resolution Map and the predicted isocratic chromatogram at 65.95% MeOH can be seen. The predicted retention times agree very well with those of Figure 3.3.

of 10 gradient points at which the instrumental gradient slope can be changed (Figure 3.24).

ChromSword includes an optimization algorithm. It can, however, also be used as a prediction program:

The data of up to 20 chromatograms can be entered and a linear, quadratic, or cubic function fitted to the $\ln(k)$ values. Temperature and pH are optional variables. The up to 10 nodes – that is, points at which the gradient slope changes – can be moved using the mouse or numerically and the predicted chromatogram displayed. The “Optimize” function allows the automatic optimization of the gradient.

3.2.2.7 How Accurate Must the Prediction of k_g and k_e Be?

Assume a separation whose critical peak pair has an α of just 1.05. Also assume an uncertainty in the retention factor of 1%, which works so that the value of the front peak is correct and that of the rear peak 1% too high. Then instead of 1.05 we would find an α of 1.06. This is a 20% increase in $\alpha - 1$ and R_s will be predicted 20% too high. With several critical peak pairs this error can be too large. Ideally the error in the prediction of k_g should not be more than 1%. A similar deviation in R_s of 20% would only occur after a change in the plate number N from 10 000 to 14 400. The uncertainty in N is usually negligible. Therefore, the prediction of k_e can be much less precise than for k_g .

The retention makes the separation, but without plate numbers it won't work

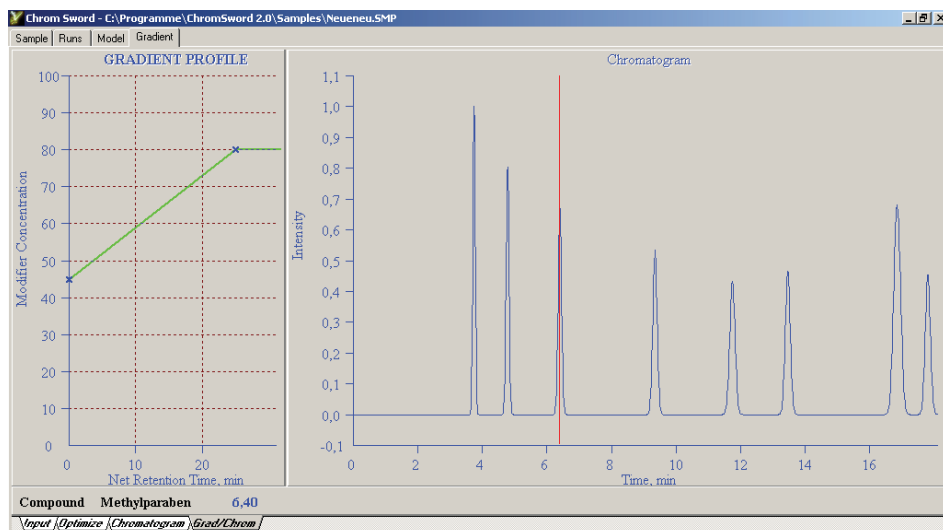


Figure 3.24 ChromSword prediction for a gradient from 45 to 80% methanol in 25 min based on the linear model. With one click the quadratic function is selected and the

last two peaks come closer together, as in the real measurement shown in Figure 3.22. The input data are those from Table 3.4.

3.2.3

How to Act Systematically?

- 1) First decide whether and if yes then which buffer is useful as eluent A. Measure for an overview two (or more) isocratic chromatograms with different organic fraction %B, in which for each substance at least twice a distinct retention time can be assigned, that is, only a slight fusion of the peaks is allowed.
- 2) Calculate the k -values and draw a $\ln(k)$ to %B graph – this can also be done with semi-logarithmic graph paper, three or four decades. The more intersections that can be seen, the more complicated is the separation problem. From this graph, all isocratic chromatograms with any desired %B can be read, a calculation is more accurate.
- 3) The leftmost %B value for $\ln(k) = 0$ (i.e., $k = 1$) is $\%B_{a,max}$, the maximum value for an isocratic method and the highest possible initial value for a gradient. The rightmost %B value for $\ln(k) = 0$ gives a preliminary estimate of $\%B_e$. Is $\%B_e - \%B_a$ above 30–40%? Look at the isocratic prediction at $\%B_{a,max}$ and lower, by which the k -value of the last peak should not be over 20. Decide whether a gradient is necessary or useful.
- 4) Very acute angles at the intersections of the $\ln(k)$ curves mean a poor separation at all isocratic and gradient conditions. Several acute angles are very unfavorable.

- 5) The greater the difference in the %*B*-values of the various intersections, the more difficult they are to “avoid.” Even if one wants to optimize a gradient without the help of computers, the information referred to here should not be disregarded.
- 6) Now turn on (reluctantly) the computer and decide if the linear model seems to be sufficient. Otherwise run 3–5 additional isocratic chromatograms, as far as possible for all substances in the range from $k = 1$ to 150. Sometimes the sample has to be split into “early” and “late” substances. With the information from Point 2 an automatic analysis sequence from high to low organic content can be run overnight, as the retention time of the last peak and thus the analysis time is known. Use the Excel Solver to modify the coefficients k_{00} , *S* and the curvature factor *a*.
- 7) Start the gradient prediction with the % $B_{a,max}$ analog to Point 3, set % B_e to 100% and t_g to $3t_0$. Increase t_g to about $60t_0$. When this gives a good chromatogram, then reduce % B_e simultaneously with t_g , whereby $(\%B_e - \%B_a)/t_g$ (and the separation) remain the same.
- 8) When a good chromatogram (especially in the front part) cannot be obtained, reduce % B_a and repeat Point 7 (if necessary several times).
- 9) When a good chromatogram still cannot be obtained, make additional measurements at a different temperature and vary the temperature in the prediction.
- 10) When even that was unsuccessful, use methanol instead of acetonitrile, or vice versa.
- 11) After that the only thing left is to try one (or more) other columns preferably with different characteristics. When choosing the column, it is sufficient to consider Point 2. With more input chromatograms, the reliability of the gradient prediction is improved.
- 12) Check the prediction with a real measurement. With problems: check the assignment of the peaks again. And everyone has already experienced this: when a computer gives erratic results, the error is very often due to the user.

3.2.4

List of Abbreviations

% <i>B</i>	percentage flow of the pump <i>B</i> , often identical with Φ
% B_a	initial value of % <i>B</i> with gradients
% $B_{a,max}$	maximum value of % B_a for isocratic and gradient methods
% B_e	final value of % <i>B</i> with gradients
<i>a</i>	curvature factor
<i>D</i>	slope of the temperature dependence
d_p	particle diameter (μ)
<i>F</i>	flow (ml/min)

G	effective gradient slope
GS	intrinsic gradient slope
k	retention factor
k_0	retention factor at the initial condition % B_a
$k_{0,T}$	retention factor for the temperature correction
k_{00}	retention factor for pure water
k_e	retention factor in gradients for calculation of the peak width
k_g	retention factor in gradients for calculation of the retention time
L	column length (cm)
N	plate number
PSI	peak separation index
R_s	resolution
S	$\ln(k)$ to % B slope
S_T	$\ln(k)$ to % B slope for the temperature correction
t_0	mobile time
$t_{0,g}$	gradient fraction of the mobile time (min)
$t_{0,d}$	isocratic fraction of the mobile time (min)
t_d	gradient delay time “dwell time”
t_g	gradient time (min)
t_s	hold-up time in the stationary phase (min)
V_0	mobile volume (ml)
w_b	peak width measured asymptotically
α	separation factor
$\Delta \%B$	gradient region
Φ	organic fraction in the eluent, often identical with % B

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4

Comparison and Selection of Modern HPLC Columns

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4.1

Supports

From the beginning, a wide range of different materials have been used as stationary phases in liquid chromatography. Their selection depends primarily on three key properties: chemical stability, mechanical stability, and specific surface area. The chemical stability is important for achieving the greatest possible compatibility with the mobile phase used, and on this depends also the possibility of bringing the analytes to be determined into solution.

The second equally important point is the mechanical stability. Since HPLC in the form UHPLC can take place at pressures up to and above about 130–150 MPa (1300–1500 bar, bar is used throughout the chapter), the materials of the stationary phase are potentially subject to high mechanical loads. As a consequence, only certain supports come into question.

Finally, for liquid chromatography, it is important to use base materials having a high specific surface area. This is necessary to guarantee a certain phase relation between the mobile and stationary phases and thus ensure an appropriate loadability of the stationary phase. Such a high specific surface area can only be achieved with a porous support. The porosity in turn has an influence on the mechanical stability of the stationary phase.

Thus, a variety of materials can be ruled out, and the selection is limited to metal oxides such as SiO_2 , Al_2O_3 , TiO_2 , and ZrO_2 , as well as cross-linked polymers based on polystyrene-divinylbenzene and acrylates, and also carbon.

Other important points in the selection of the support are its production cost and the possibility of a chemically simple, flexible, and cost-effective surface modification.

For these reasons, silica has asserted itself as a support over all other materials and is now by far the most commonly used base material for HPLC. Nevertheless, there are some weaknesses in the use of silica, so that in some applications quite different supports are used, as discussed later in detail.

4.1.1

Why Silica Gel?

In addition to its mechanical stability, silica has the particular advantage of a low-cost and easy synthesis and a simple, inexpensive, and flexible means of surface modification. Furthermore, in contrast to, for example, polymeric materials, it is possible to synthesize silica with a specific and closely distributed pore size. This is an important point for optimization of the separation kinetics, thereby achieving narrow elution bands. Detailed information about the preparation of silica, its characteristics, and chemistry can be found in the book by Isler [1]. In actual fact, chromatography is not the main use of silica, much more common is its use as a pigment, as filler in the cosmetic and plastic industries, and as an adsorbent and support for catalysts (keyword: zeolites).

For practical HPLC, interesting are the different ideas – you could almost say philosophies – which hide behind the characteristics of the various silicas and their planned purpose and application. Here, it is helpful to look at the Wheeler equation:

$$\frac{4 \times 10^4 \times V_p}{S_{\text{BET}}} = D[A] \quad (4.1)$$

where V_p is the pore volume in ml/g, S_{BET} is the surface area in m^2/g , and D is the pore size in Å.

From this, by way of example, the following values for silica with a pore size of 100 Å (10 nm), typically used in the HPLC, can be calculated:

Pore 100 Å = 10 nm

V_p (ml/g)	0.1	0.3	0.7	1.0	1.5	2.0
S_{BET} (m^2/g)	40	120	280	400	600	800

And for a surface area of 300 m^2/g typically used in the HPLC, the following are the possibilities to achieve this:

Specific surface area 300 m^2/g

V_p (ml/g)	0.3	0.5	0.8	1.0	1.5	2.0
D (Å)	40	70	100	130	200	270

But what does this mean in practice? A small pore volume results in faster diffusion, leading to narrower elution bands (diffusion is less hindered) and thus higher plate numbers in direct comparison to larger pore volumes. However, when the

pore volume is reduced the pore sizes should not at the same time become too small, in order to prevent size-exclusion effects on the analytes. The chromatographically active specific surface area of the stationary phase is always the most important parameter of the resulting support and, therefore, always the focus of optimization during the development of new materials.

A high specific surface area of the stationary phase is desirable as this allows stronger interaction of the analytes with the stationary phase, often leading to higher resolution of difficult-to-separate analytes and also counteracting problems with loadability, which manifest themselves in asymmetric and broad elution bands.

Moreover, it should also be noted that an increase in pore size also leads to reduced mechanical stability, since the structure of the support becomes thinner. This is particularly important with materials for UHPLC because there the pressure can reach 1500 bar and the columns are packed at even higher pressures.

Depending on the desired application, different chromatographic supports have, therefore, been developed. With silica, pore volumes between 0.3 and 1.5 ml/g, pore sizes between 60 and 300 Å, and surface areas between 80 and 500 m²/g are found. Later, examples are given showing when for which analytes which parameters are made use of.

4.2

Stationary Phases for the HPLC: The Historical Development

Parallel to the development of silica with respect to purity, stability, and kinetics, the development of the surface modification must also be considered.

As HPLC began, irregular silicas prepared by polymerization of water glass were used as supports. As a result, depending on how the pH of the solutions was adjusted and also the origin of the water glass used, the metal content and the pH of the resulting silicas differed considerably. The acidity of the silica in turn has a decisive influence on its reactivity, and thus for modified silicas on their key properties: hydrophobicity, hydrophobic selectivity, silanophilic activity, shape selectivity, and polar selectivity.

Irregular silicas were largely replaced in the late 1970s and early 1980s by spherical silicas. The advantage of these supports was primarily their higher packing density and lower back pressure compared with irregular materials. A direct result of this is increased mechanical stability and thus a longer lifetime of the column. Nevertheless, these silicas still had a high and also varying metal content, which lead to fluctuations in the properties of the resulting stationary phase. Stable retention times and uniform separation properties, however, are essential for the use of HPLC in the chemical and pharmaceutical industry.

It still took a few years until in the mid-1980s the first high-purity silicas based on the polymerization of tetraethoxysilane (TES) were introduced. The metal content (mainly Fe and Al) of this support is in the parts per million range. As a result, it was now possible to comply with the requirements of HPLC quality

control procedures and produce very reproducible separation media. These so-called third generation silicas or Type B silicas (as they are often called in the English-speaking countries) now dominate the market for silica.

Nevertheless, the history of supports for HPLC does not stop at this point, and there are several further developments aimed at either a kinetic optimization of the materials (see Chapter 3), an extension of the usable pH range, or the pressure stability of the silicas. In the course of kinetic optimization of the materials, essentially the diffusion paths of the analytes interacting with the stationary phase are shortened. This is done primarily by reducing the particle diameter of the supports.

While at the beginning of the 1990s it was still common to use supports having a particle diameter of 5 μm , by the mid-1990s it was quite normal to use 3 μm materials from time to time. In the mid-2000s – with the introduction of UHPLC – sub-2 μm supports came into common use. An important trend in the 2010s now aims at the use of materials with nonporous silica cores covered by a porous shell (subsequently referred to as solid core material).

As a result, due to the shorter diffusion paths the separation efficiency of a 1.7 μm material can be reached at the back pressure of a 3 μm material. These materials achieve this by the use of a well-known concept developed since the 1970s. Even the earliest activities with HPLC used so-called porous layer beads [2, 3]. Admittedly, at that time these materials with particle sizes of about 50 μm were used as an improvement to the conventional irregular materials (fraction 40–63 μm). The disadvantage was the significantly lower loadability (you only had a 1 μm thick porous shell around a 50 μm thick nonporous glass core, which could not be compensated for by the advantage of the significantly higher separation efficiency).

With the newer materials, the ratio of core to shell is much better, so that the issue of loadability plays only a minor role. The first solid core materials with particle sizes between 2 and 3 μm clearly aimed at providing an improved alternative to porous sub-2 μm materials.

In comparison to fully porous sub-2 μm materials, the user has the choice between either lower back pressure with the same separation efficiency or significantly higher separation efficiency at the same back pressure, as he is able to use longer columns. Since the elution bands of solid core materials analog to fully porous sub-2 μm materials are very narrow, then also with these materials – despite statements to the contrary in glossy brochures – it is not possible to use conventional HPLC systems. In order to use the maximum performance of these columns, it is imperative to optimize the band broadening outside the separation column and to use small-volume flow cells and higher data acquisition rates. Recently, solid core materials with larger particle sizes for use in the HPLC have been developed. These have particle sizes between 4 and 5 μm , are filled in classical HPLC column dimensions, and also show significantly higher separation efficiencies as fully porous materials of the same particle size.

At this point, it should be mentioned that due to the nonporous core, columns filled with solid core materials intrinsically have a lower total porosity and hence a higher packing density. In practice, this means that when comparing a fully porous and a solid core column of the same column dimensions, which are being used at the same flow (specifically: volume flow), the linear flow rate in the solid core column is higher, and thus a 20–30% higher back pressure is observed compared with the corresponding fully porous material. If the characteristics of the columns are to be compared in a scientifically correct manner, then measurements must be made at comparable linear flow rates, that is, the solid core column must be operated at lower flow rates. The linear flow rate is very easy to measure, the column length is divided by the hold-up or dead time t_M . The hold-up time can be determined from the chromatogram; it is the time at which the disturbance of the base line, caused by the injection of the sample, often occurs. The solid core columns packed with 4–5 μm material can be used with conventional HPLC equipment.

In addition to these larger solid core particles, smaller sub-2 μm solid core particles with particle sizes of 1.7 and 1.3 μm are commercially available. However, their theoretical potential with respect to separation efficiency and speed of analysis, which certainly exists, cannot be used in practice. This is because no HPLC systems are available with the necessary small volumes (of both the capillaries and detector cells) and which also have the data acquisition rate required to detect and record the separations achieved on these columns without loss. Today's modern UHPLC systems are already quite well optimized, but their performance is by far not good enough for these applications; the manufacturers face further challenges here. As with all materials, it is highly recommended to check the batch-to-batch reproducibility especially of solid core phases.

Parallel to the development of particulate materials, there is the possibility of using monolithic columns. This technique, developing since the late 1990s, uses the idea of an inverse silica structure with the same goal: acceleration of the kinetics. The biggest advantage of this technique is the very low column back pressure, the separation efficiencies are quite comparable to columns filled with sub-2 μm supports and the separations are fast [4]. The biggest advantage of monolithic columns is the higher total porosity of the material compared with porous silica columns. This leads not only to a much lower back pressure, but also to a comparatively higher column volume. This is exactly the opposite as with the solid core materials. In practice, this means that monolithic columns show a much larger tolerance toward band broadening outside the column and thus high separation efficiencies can be achieved and also measured with conventional HPLC equipment without considerable efforts for optimization.

Another advantage is the improved tolerance of these materials toward highly contaminated samples. These columns do not become contaminated as easily as similar particulate materials. The disadvantages, however, should also be mentioned. The columns are limited to a pressure of 200 bar as the monolithic silica rods are seated in a PEEK hull. Furthermore, only a few different selective stationary phases are available. Although recently a few additional bondings such as Phenyl-Hexyl and Cyanopropyl have been announced, only two companies so far

offer these promising columns based on silica. At the moment, however, monolithic columns are the most robust and elegant way to achieve fast separations with sufficient separation efficiency (effectively 20 000 plates over a separation distance of 100 mm).

4.3

pH Stability and Restrictions in the Use of Silica

Silicas are the ideal support for stationary phases in the HPLC, as they are mechanically stable, can be produced easily and inexpensively and are chemically relatively inert. As anhydrides of silicic acid, however, they dissolve in alkaline medium. This drawback of silica was taken into account in the history of HPLC and counteracted using different approaches. The first step was the development of high-purity silica. While the supports of the first generation had a high acidity, due to a high metal content, the materials of the second generation were significantly purer and thus more pH stable at high pH values. Another approach toward achieving higher pH stability was the coating of the silica with metal oxides such as Al_2O_3 , TiO_2 , or ZrO_2 , which reduce the dissolution of the silica at high pH values [5]. A further possibility is a high surface coverage of the silica-based reversed phases. With reversed phases having a high surface coverage the dissolution of the silica at high pH values is impeded as the hydrophobic surface is not easily accessible for the polar bases. Therefore, the higher the surface coverage of the support and the larger the base used, the more stable is the stationary phase. Other approaches to obtaining increased pH stability are polymeric coatings around the silica and the introduction of hybrid materials – these are composites of TES and bridged organosilanes.

All these approaches lead to a slight improvement in pH stability by about one pH unit, but it is falsely suggested to the user that he can always use these columns at pH values greater than 9. This is incorrect! Even as a hybrid material and coated with polymer, silica is still the anhydride of silicic acid and dissolves at pH values greater than 7. The rate of dissolution can be reduced by means of a high surface coverage of the stationary phase, a coating or an organosilane hybrid. Furthermore, with mobile phases containing a high proportion of aprotic organic solvents (e.g., acetonitrile), the dissociation of the bases and of the silanol groups of the silica is suppressed, which in practice leads to much lower pH values. (Careful: up to a pH of about 8, the pH increases after the addition of methanol/acetonitrile [6].) If a stability study of a silica-based HPLC column that reaches 10 000 column volumes at pH 12 without loss of efficiency is found in the glossy brochure of a column manufacturer, then this is only possible if the organic solvent content is at least 70%, the base very bulky (e.g., triethylamine or pyrrolidine) and the column was used at room temperature. If the same column was used at, for example, 60 °C with a small base (ammonia or hydroxide) in mobile phases containing less than 10% organic solvent, the service life would be less than 500 column volumes. So, care should be taken here!

Must chromatography be performed routinely at a pH above 8, then it may make more sense to use alternative materials based on polymers, other metal oxides such as TiO_2 , ZrO_2 , or pure carbon.

There is also the possibility of using a saturation (or conditioning) column. This is a tried and tested method of increasing the lifetime of silica-based columns at high mobile phase pH without using a different support, which could possibly bring selectivity problems. A saturation column is a short column, for example, a guard column, filled with coarse-grained silica. Its particles are slowly dissolved by the basic mobile phase, which in turn becomes saturated with the dissolved silica, thus sparing the column. Admittedly, it is also possible just to use a column filled with large particle silica. The saturation column is installed between the pump and injector, note that this has an effect on the dwell volume of the system.

Why does chromatography at high pH make sense at all? The answer is simple: at a basic pH, basic analytes are present in the unprotonated form. Thus, the hydrophobic interactions between the stationary phase and the analyte are stronger. The ionic interactions are weaker, and as a result the elution bands of the basic analytes are narrower and more symmetrical than when chromatographed at acidic or neutral pH. Furthermore, since at high pH the basic analytes are no longer ionic, they are more hydrophobic and thus more strongly retained on reversed phases, which is often very advantageous as it enables movement of the basic components into a different elution window. Moreover, bases in an uncharged state show a significantly higher loadability on reversed phases [7].

Frequently, however, the basic compounds in the uncharged state are significantly less stable than in the charged state. This is a well-known observation often noticed in stability studies. In chromatography, sometimes artifacts are observed, which indicate the decomposition of the products, a fact often kept quiet. Therefore, caution should be exercised when working at high pH. Nevertheless, the variation of the pH is, alongside the selection of the stationary phase, the most important optimization parameter for the separation of polar or ionic/ionizable components.

4.4

The Key Properties of Reversed Phases

The properties that essentially influence the selectivity of the reversed phase are hydrophobicity, hydrophobic selectivity, silanophilic activity, shape selectivity, polar selectivity, and metal content. These properties are different for each different support and, therefore, essentially determine the separation.

4.4.1

The Hydrophobicity of Reversed Phases

The hydrophobicity of the reversed phase indicates how strongly an analyte interacting primarily via hydrophobic interactions with the stationary phase is

retained. In short, the stronger the retention power of the stationary phase, the higher the hydrophobicity. The hydrophobicity of a column is determined by the surface of the support, its carbon content and the surface coverage of the support. Phases that have the same hydrophobicity can produce quite different separations (even for homologous series!). Therefore, the hydrophobic selectivity should also be taken into account, which is more important for the separation than the hydrophobicity itself [8].

4.4.2

The Hydrophobic Selectivity

The hydrophobic selectivity is the methylene group selectivity of the stationary phase, that is, the selectivity of two compounds differing by a methylene group, which are retained predominantly due to their hydrophobicity. This selectivity is to be used, for example, in the case of neutral, hydrophobic analytes for the comparison of columns and assessment of their similarity, and has a much higher significance than the hydrophobicity treated in Section 4.4.1, and later sections.

4.4.3

The Silanophilic Activity

Silica has about $9 \mu\text{mol}/\text{m}^2$ silanol groups on its surface [1]. When silica is treated with C18-silane, about $3 \mu\text{mol}/\text{m}^2$ of these silanol groups can be made to react, leaving about $6 \mu\text{mol}/\text{m}^2$. Further reactions with shorter chained silanes (usually trimethylsilane functionalized silanes) – also known as endcapping – leads to a conversion of about a further $3 \mu\text{mol}/\text{m}^2$ of silanol groups, so that in the final reversed phase about $3 \mu\text{mol}/\text{m}^2$ of silanol groups remain. These are not convertible but can certainly interact with analytes affine to silanol groups, and so contribute to the selectivity of the corresponding reversed phase.

This is the so-called *silanophilic activity* of the reversed phase. The compounds affine to silanol-groups are usually bases that interact with the acidic silanol groups via ionic interactions. These interactions are energetically much stronger than the usual hydrophobic interactions of reversed phases, which usually lead to wider elution bands for basic analytes on classical reversed phases.

Therefore, the aim of the manufacturer in the development of new reversed phases is primarily the minimization of the silanophilic activity. This is also the most critical parameter for the reproducible production of reversed phases. The silanophilic activity of a reversed phase is determined by the following parameters: manufacturing process of the silica, surface coverage of the stationary phase, and type of silane used (reactive and functional groups of the silane).

At this point, however, it should be noted that it is not always desirable to use stationary phases with low silanophilic activity. For example, when no basic compounds are present in the sample, then silanol groups may well make a positive contribution to the selectivity of the stationary phase. Thus, hydrogen bonds are

often observed between silanol groups and carbonyl groups, without which some separations would not be possible.

4.4.4

Shape Selectivity (Molecular Shape Recognition)

Reversed phases also have the ability to differentiate between the spatial configurations of analytes in solution, that is, whether analytes are present in a planar or bent form, as is often the case with, for example, *cis*-, *trans*-isomers. This property is called shape selectivity. The parameters of the stationary phase primarily responsible for the strength of the shape selectivity of the reversed phase are surface coverage of the reversed phase, chain length of the reversed phase, additional polar interactions, and pore size of the silica. The following applies:

- The higher the surface coverage, the higher the shape selectivity.
- The longer the alkyl chain, the higher the shape selectivity.
- The wider the pores, the higher the shape selectivity.
- The more additional polar interactions, the higher the shape selectivity.
- The lower the temperature and the higher the back pressure of the column, the higher the shape selectivity.

Why Is This So? The answer is clear. The shape selectivity of a stationary phase depends crucially on the increased possibility for interactions between the planar analyte and the stationary phase and the simultaneous discrimination against interaction of analytes present in the bent form. If the surface coverage is high, then only the more planar molecule together with all its interaction sites can penetrate completely into the pores of the stationary phase and consequently be more strongly retained than the bent molecule.

Analog is the situation when the stationary phase contains longer alkyl chains. Here, the alkyl chains are attracted more strongly to each other, and the more flexible (longer) they are, the smaller the resulting gaps into which only the planar molecules can penetrate. The same is true if the temperature is lower and the pressure drop higher. The dependence on the pore size is due to the fact that more than 99% of the chromatographic surface is within the silica particles. If the pores are too small, it is simply impossible to achieve the surface coverage required for sufficient shape selectivity. Therefore, most of the shape selective reversed phases have pore sizes larger than 150 Å (15 nm). Here again, of course, exceptions confirm the rule.

4.4.5

The Polar Selectivity

Polar selectivity is generally understood to refer to the differing retention of polar analytes relative to neutral analytes in comparison to classical reversed

phases. On polar selective stationary phases, acids are more strongly and bases slightly weaker retained in comparison to classical reversed phases. The polar selectivity is achieved by means of polar groups (amide, carbamate, urea, diol, etc.), which are applied to the surface of the silica and which can undergo additional interactions with the polar analyte. Polar selective reversed phases are the first alternative if classical reversed phases do not function, but more on this later.

4.4.6

The Metal Content

The metal content is dependent on the preparation of the silica; the content is higher or lower depending on the starting material and equipment used. The first generations of silica were prepared from water glass. In these silicas, the metal content is relatively high, especially aluminum, and iron are to be found. But why is it so serious if the silica contains metal impurities? Strictly speaking this should only cause problems with just a few analytes that are able to interact with these metals (e.g., complex builders or some proteins). These should undergo additional interactions with the stationary phase, consequently resulting in broad unsymmetrical elution bands. However, the metals also influence the acidity of the silica and thus the silanophilic activity (see Section 4.4.3) and, therefore, the reactivity of the silanol groups with the silanes during the surface modification.

Even that would not be so bad, if the metal content of the original silica remains constant. However, this is not the case. Thus, the batch-to-batch reproducibility of stationary phases based on non-metal-free silica (the English-speaking literature often addresses Type A silica) is much worse than that of metal-free silica (so-called Type B silica). But here too, some separations require additional metal interactions and in certain cases it is quite possible that the separation will only work on Type A silica. So even today some users still swear on “their” LiChrospher, Select B or Spherisorb ODS 1.

4.5

Characterization and Classification of Reversed Phases

Based on the above-mentioned six key properties of reversed phases, the stationary phases can be characterized. A wide variety of literature exists on this subject [9–15]. Of course, the synthesized stationary phases can be subjected to a full physicochemical examination (nitrogen adsorption measurements to determine the specific surface area, the pore volume and the pore size, CHN analysis to determine the surface coverage of the stationary phase, particle size measurements, etc.). However, all these characterizations are not really to the point, because in the end only the chromatographic separation counts. As a result, chromatographic tests for the characterization and classification of reversed phases have established themselves, from which a representative few, without any claim to being exhaustive, are presented here (Figures 4.1–4.3).

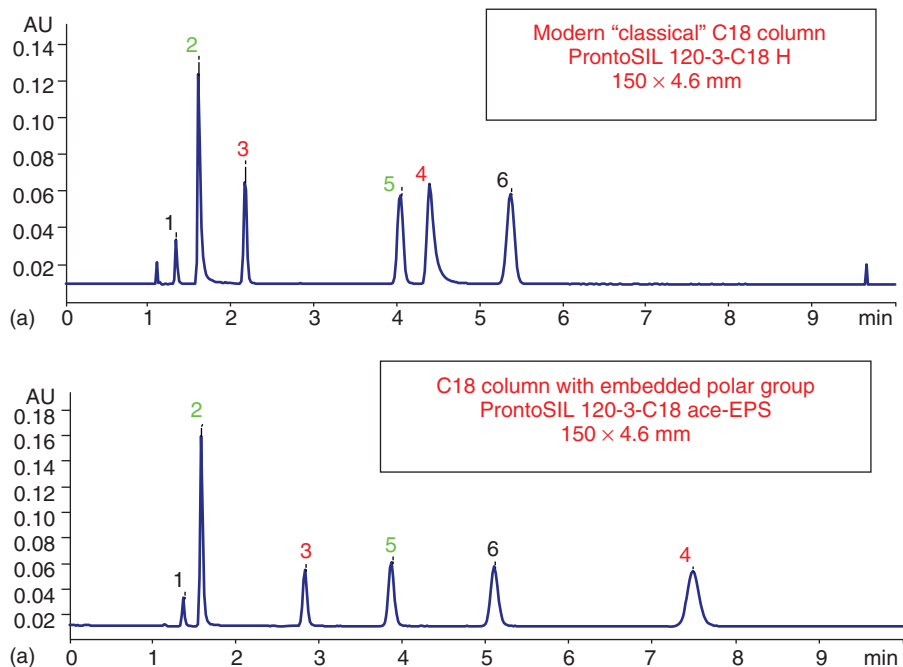


Figure 4.1 Test of polar selectivity: (a,b) neutral (black), acidic (dark gray), and basic (light gray) analytes.

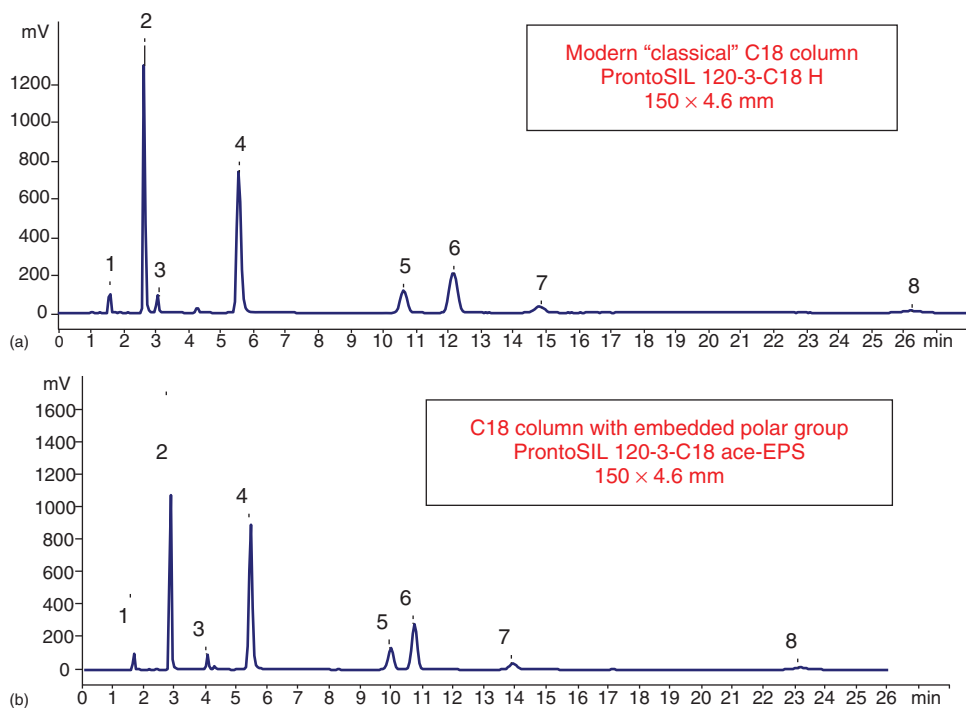


Figure 4.2 Engelhardt test: (a,b) comparison between classical reversed phase and reversed phase with bedded polar group.

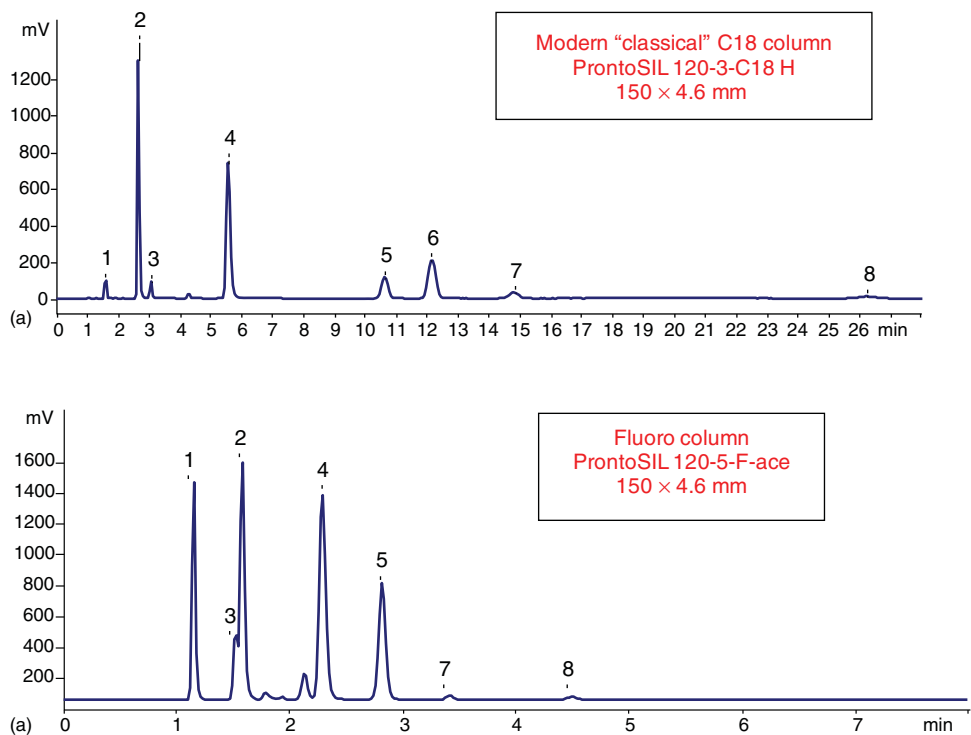


Figure 4.3 Neue test: comparison between reversed phase with bedded polar group and fluoro phase.

Test	Polar selectivity	
Mobile phase	ACN/50 mM Phosphate buffer pH = 3, 65/35 (v/v)	
Temperature	40 °C	
Flow	1.0 ml/min (column ID 4.0 mm)	
Detection	UV @ 254 nm	
Analytes	Analyte	Characteristic
Peak no. 1	Uracil	Dead time marker
Peak no. 2	Pyridine	Silanophilic activity
Peak no. 3	Phenol	Polar selectivity
Peak no. 4	4-Butylbenzoic acid	Polar selectivity
Peak no. 5	<i>N,N</i> -Dimethylaniline	Silanophilic activity
Peak no. 6	Toluene	Hydrophobicity

Test	Engelhardt test	
Mobile phase	MeOH/Wasser 49/51 (w/w), respectively, (55/45 (v/v))	
Temperature	40 °C	
Flow	1.0 ml/min (column ID 4.0 mm)	
Detection	UV @ 254 nm	
Analytes	Analyte	Characteristic
Peak no. 1	Uracil	Dead time marker
Peak no. 2	Aniline	Silanophilic activity
Peak no. 3	Phenol	Polar selectivity
Peak n o. 4	4-Ethylaniline	Silanophilic activity
Peak no. 5	<i>N,N</i> -Dimethylaniline	Silanophilic activity
Peak no. 6	Ethyl benzoate	Chain length selectivity
Peak no. 7	Toluene	Hydrophobicity/hydrophobic selectivity
Peak no. 8	Ethylbenzene	Hydrophobicity/hydrophobic selectivity

Test	Neue test	
Mobile phase	MeOH/20 mM Phosphate buffer pH = 7, 59.4/40.6 (w/w) (65/35 (v/v))	
Temperature	23 °C	
Flow	1.0 ml/min ((bei ID 4.0mm Säulen) column ID 4.0mm)	
Detection	UV @ 210 nm	
Analytes	Analyte	Characteristic
Peak no. 1	Uracil	Dead time marker
Peak no. 2	Propranolol	Silanophilic activity
Peak no. 3	Butylparabene	Polar selectivity
Peak no. 4	Dipropylphthalate	Polar selectivity
Peak no. 5	Naphthalene	Hydrophobicity
Peak no. 6	Amitriptyline	Silanophilic activity
Peak no. 7	Acenaphthene	Hydrophobicity

It should also be mentioned that the characteristics of the columns change with use, depending on the buffer, additive, and also the sample matrix. In practice, this has several consequences:

- During method development and optimization, HPLC columns “see” several mobile phases of differing pH and containing different buffers, as well as additives. If you have reached the end of a method optimization, then it is best to

characterize the column using a chromatographic test. This allows you to identify any subsequent variations and quickly find alternative solutions. We discuss this topic in more detail later under the heading of column databases.

- During HPLC method validation, it is imperative to test the finished optimized method on several (usually three) new columns from different production batches in order to see whether the separation still functions. If this is the case, you can be hopeful that future batches will work. Sometimes, it is necessary, however, to precondition the columns by means of a defined conditioning program before they meet the requirements of the separation. This is the reason why the verification of the selectivity is so important.

4.5.1

The Significance of Retention and Selectivity Factors in Column Tests

4.5.1.1 Preliminary Remark

The hydrophobicity of a stationary phase is responsible for the retention of the analytes. This is mainly determined by the specific surface area and the carbon content, discussed earlier. But as far as the selectivity is concerned, in reversed-phase (RP)-chromatography hydrophobic interactions usually play a much less important role than is generally believed, ionic/polar interactions, and steric aspects – if they come into play – are more dominant factors. Only by means of these influencing factors can analytes be differentiated. This is also the reason why a correlation between retention and selectivity factors is rather seldom observed, discussed later. Such a correlation may at the best be expected from a uniform RP mechanism in which van der Waals interactions predominate, which is seldom the case (homologous series or simple pairs of analytes such as toluene/ethyl benzene). This is because the molecules to be analyzed mostly have groups that are capable of ionic/polar interactions. Even with hydrophobic aromatic compounds, we are dealing with induced dipoles; in addition, the polarity may increase through resonance.

Otherwise, “similar” molecules also differ in their spatial dimensions; their steric properties influence their diffusion behavior in the pores of the phase surface. Examples here are the polarizability of molecules due to double bonds, or their rigidity/flexibility depending on the location of substituents on the aromatic ring.

In conclusion: in an RP-system hydrophobic interactions are primarily responsible for the retention, in the case of ionic analytes naturally also ion exchange when this is possible. On the other hand, polar/ionic interactions are decisive for the selectivity. These are primarily ion exchange as well as hydrogen bonding and dipole-dipole interactions, possibly even complex forming/chelation.

4.5.1.2 Criteria for the Comparison of Columns

In the literature, the hydrophobicity is often used as a criterion for comparison of stationary phases. For this purpose, $\ln k$ values (k = retention factor) of typical RP analytes on different stationary phases are compared, on “similar” hydrophobic phases the analyte will be equally strongly retained, leading to similar $\ln k$ values. This practice should be critically questioned. Without going into details about

the suitability of the (usually simple) analytes used, the fundamental question of whether, if at all, retention factors represent a suitable criterion for comparison should be dealt with.

From a practical perspective, the retention behavior (a measure of the hydrophobicity) is only of fringe interest. The reason is that in comparative tests the user is more interested in finding columns that show similarly good separation for certain analytes rather than in finding columns on which the analytes elute after a similar retention time. And a measure of the separating efficiency happens to be the selectivity (separation factor, α). Similarly, strong interactions – and thus similar retention factors – do not at all mean that the respective phases also show similar selectivity.

This has been repeatedly confirmed in several studies; see, for example, Figure 4.4. Here, the retention (bars) and separation factors (lines) of tricyclic antidepressants in acidic acetonitrile/phosphate buffer are shown. In the case of quite different retention factors depending on the hydrophobic character of the phases (differently strong interactions), apart from a few exceptions very similar separation factors result. Although the analytes are retained for different lengths of time on the stationary phases, they show a similar selectivity behavior. This is observed especially with neutral or – by means of the pH-neutralized molecules. In this case, mainly hydrophobic interactions between the analytes and the surface of the material dominate. These are not especially specific; the individual differences of the phases with respect to selectivity do not come into effect.

Figure 4.5 illustrates this phenomenon for the separation of ketones, polar but nonionizable analytes. Moreover, a strong interaction does not automatically mean good selectivity – see also Figure 4.6, in which, in principle, this phenomenon is presented slightly differently. There, retention factors on various columns are plotted against the corresponding separation factors. The fact is that the peaks elute early or late on the different columns (according to “Hydrophobicity” these are phases with different properties) but the selectivity of these columns is very similar – all α values lie between about $\alpha = 1.1$ – 1.2 . For a separation technique such as HPLC, selectivity should be the most important or most meaningful criterion when it comes to assessing the similarity of RP phases.

To verify this assumption, several chemometric analyses (with strongly differing separation conditions and analytes) were performed and extensive data collected. Figure 4.7 shows an example of these studies. The farther apart the variables (here retention and separation factors) are located around the origin on the y -axis, the greater their influence on the differentiation of objects, here columns. As can easily be seen, the α -values are suitable for this. If nevertheless k -values must be used, then at the most “problem” substances are suitable. For example, polar analytes in unbuffered eluent (e.g., k Cos, caffeine) or strong acids in buffered eluents (k Phs, phthalic acid, and k Tes, terephthalic acid) because the differences between the phases only become visible when polar (ionic) interactions are possible. This, however, is not the case when only hydrophobic interactions are expected; the issue of “column comparison” and thus “column selection” is then not so critical, any “good” column can be used and for logical reasons optimization is more easily

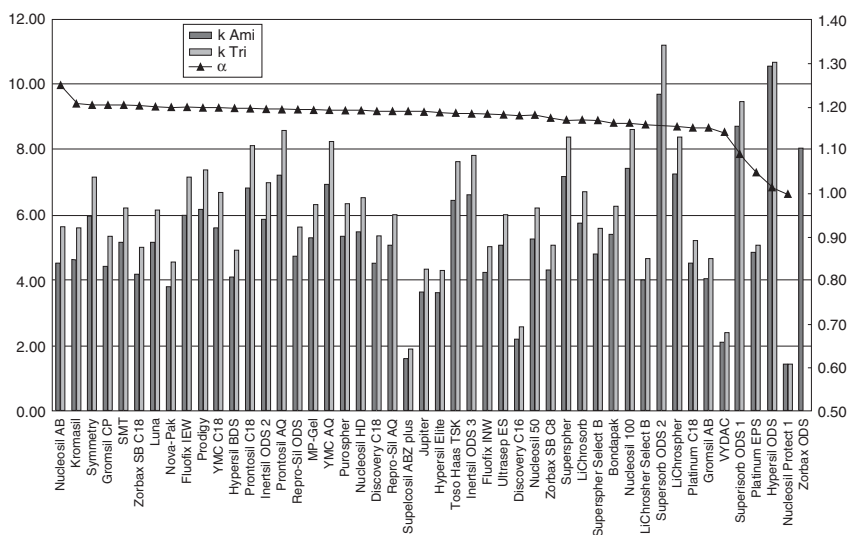


Figure 4.4 Characterization of different RP stationary phases. Reproduced from Kromidas [6], with permission of Wiley.

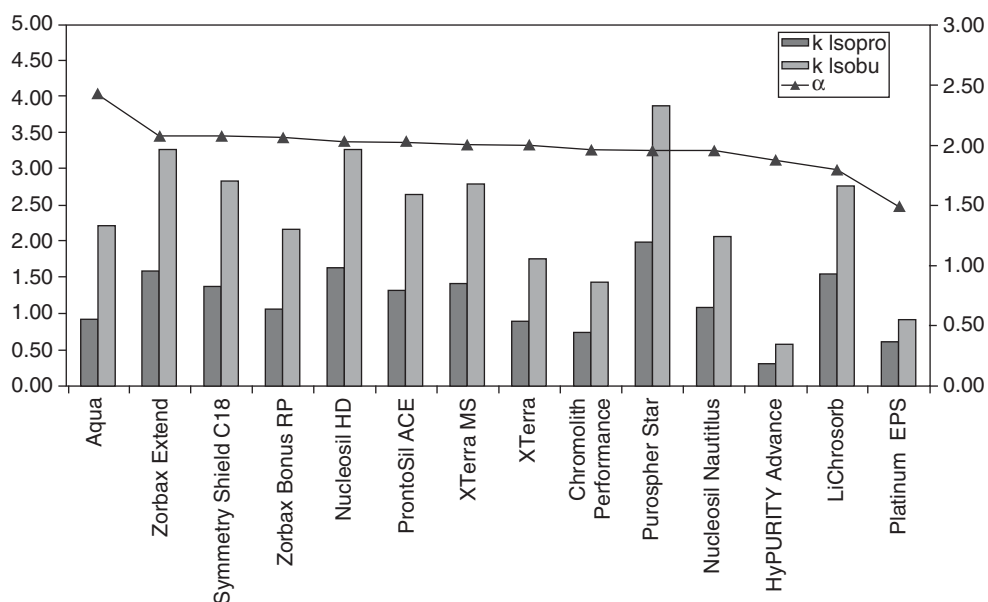


Figure 4.5 Hydrophobicity and Methylene group selectivity. Reproduced from Kromidas [6], with permission of John Wiley & Sons.

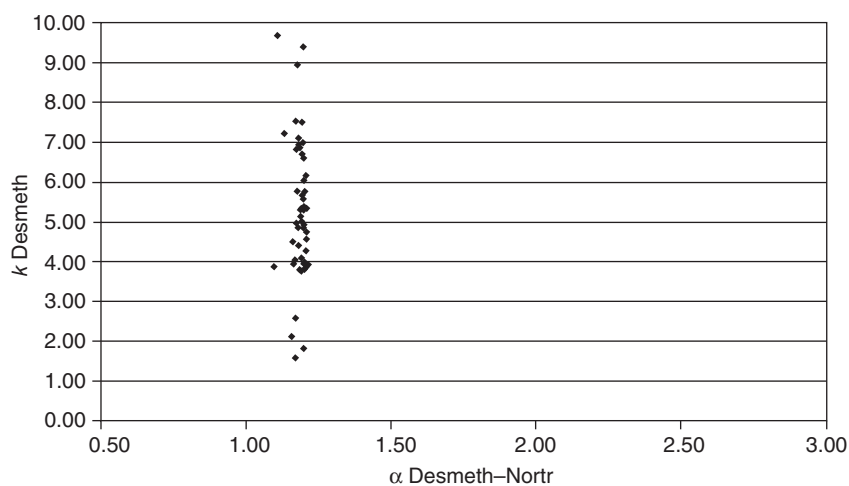


Figure 4.6 On the bandwidth of retention and separation factors by the separation of metabolites of tricyclic anti-depressants in acetonitrile/acidic phosphate buffer. Reproduced from Kromidas [6] with permission of John Wiley & Sons.

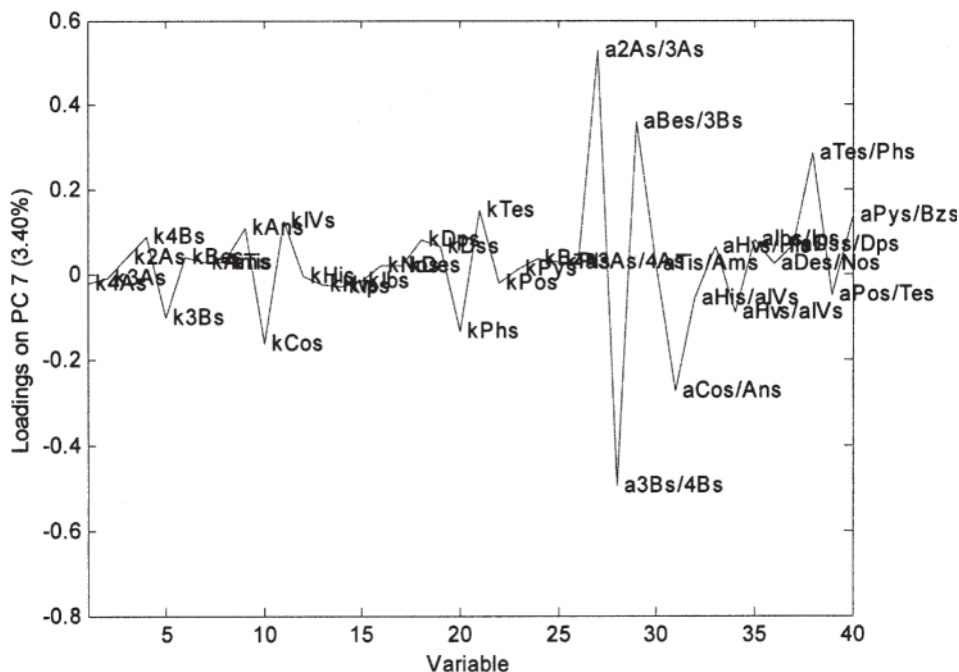


Figure 4.7 On the suitability of k - and α -values for a differentiation of stationary phases; for comments, see text. Reproduced from Kromidas [6] with permission of John Wiley & Sons.

done with the eluent. Nevertheless, having the optimal column that can be used in combination with an easy mobile phase makes chromatography in general more robust and better transferable from one lab to another. Therefore, finding the optimal column for our separation task should be our first predominant focus during method development.

4.5.2

Column Comparison, Comparison Criteria: Similarity of Selectivities

A comprehensive review of diversified tests and visualization tools for the comparison of columns is available in Ref. [8]. In the following, we present three tests that appear to us to be quite informative. For the reasons set out earlier, the selectivity (separation factor) is used as the classification criterion for comparison of columns.

1) Selectivity plots

Here, separation factors, determined by means of separations on supposedly similar phases under defined chromatographic conditions, are plotted against each other. The more similar the phases, the closer to the resulting diagonal the individual values should lie. In a series of experiments, 68 different pairs of analytes were separated under deliberately varying chromatographic conditions (nine different eluents) and the resulting α -values plotted against

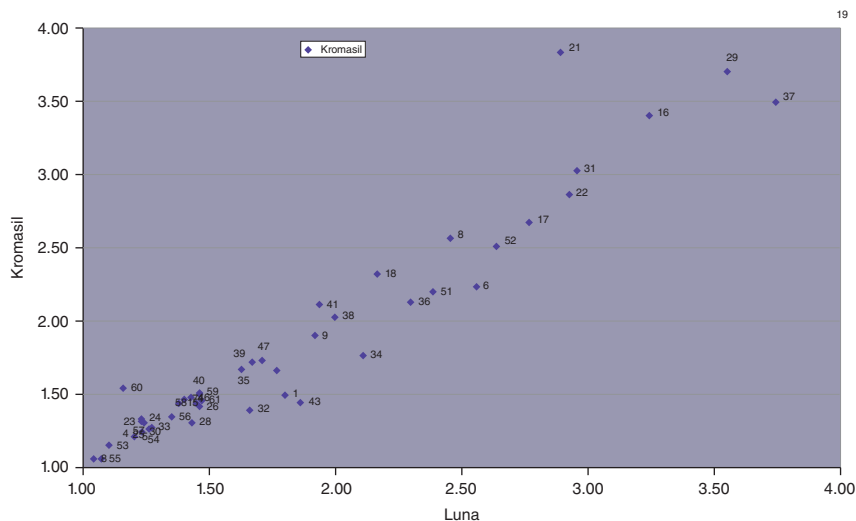


Figure 4.8 Plot of the separation factors of analyte pairs that differ as much as possible, on two columns; for explanation see text.

each other. Due to the large number of values and the different experimental conditions, quite substantiated assertions can be made regarding the similarity of the phases. An example of such a plot is shown in Figure 4.8, in which two C18 columns, Kromasil and LUNA (2), are compared.

From Figure 4.8, first, it can be seen that both columns have a similar character. This is to be expected, as both columns are hydrophobic endcapped C18 phases with a classical high surface coverage and based on high-purity silica. What is striking is the outlier “21.” The α -value corresponds to the separation of 2,2’-/4,4’-bipyridine after the column has been in use for 4 weeks. A large α -value is an indication of the presence of heavy metal ions in the silica matrix, as 2,2’-bipyridine builds complexes, whereas 4,4’ does not. Apparently, during use of the column more metal ions accumulate on the silica surface of Kromasil, and the tendency to complex formation increases. As both silicas are originally ultrapure, the metal stems either from the frit on the column inlet or from the eluent and is enriched in the column since silica-based columns are always cation exchangers. Otherwise, this batch of silica from Kromasil contains traces of metal ions that are missing from LUNA. Furthermore, with LUNA, the slightly higher hydrophobic character is apparent; see separation “37,” the separation of 3-hydroxy/4-hydroxybenzoic acid, two weak acids. In fact, LUNA does have a slightly higher degree of coverage compared with Kromasil; the phase has a slightly greater hydrophobic selectivity.

With such plots, it can be determined whether two supposedly similar columns do indeed have a similar selectivity performance for *this* analyte. Also, with this method the batch-to-batch reproducibility of a material can be tested, or whether during routine use *this* sample matrix and/or

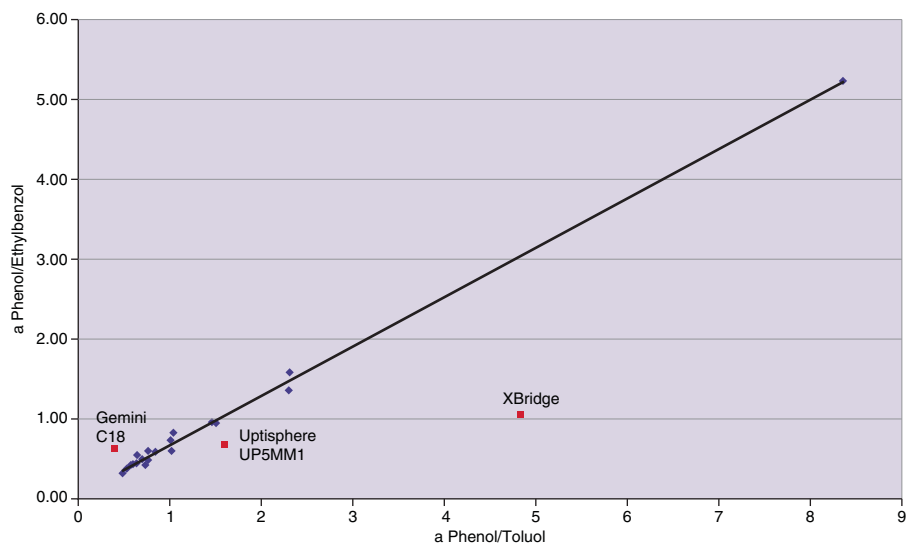


Figure 4.9 Plot of the separation factors of two characteristic analyte pairs on several columns; for explanation see text.

these chromatographic conditions have caused (irreversible) changes to the character of a certain column.

- 2) Plot of the separation factors of two very similar analyte pairs, obtained on the columns to be compared, for example, phenol/ethyl benzene and phenol/toluene.

Since these molecules are typical, simple RP analytes and are also very similar (a difference of only one methylene group between toluene and ethylbenzene), separation on different phases with similar selectivity factors should be possible. Only phases with “really” different characters should be “out of line” here. The three outliers in Figure 4.9 do in fact represent exceptions: Gemini and XBridge are hybrid materials, and Uptisphere UP 5MM1 is the only phase with a phenyl and a cyclopentenyl group as end groups. With this test, it can, for example, be tested which column(s) show an entirely different selectivity for which analyte. From the results, suitable candidates for orthogonal tests can also be determined.

- 3) Plot of the separation factors of two analyte pairs of interest on different columns

Analyte pairs that reveal the similarity of the phases with respect to selectivity for the current issues should be chosen. Assume that in one case the separation of both simple neutral molecules as well as aromatics is relevant; see Figure 4.10. Ethylbenzene/fluorenone is a measure for the hydrophobic selectivity and chrysene/perylene for the aromatic selectivity. XTerra MS and AQUA as quite hydrophobic phases show good hydrophobic selectivity,

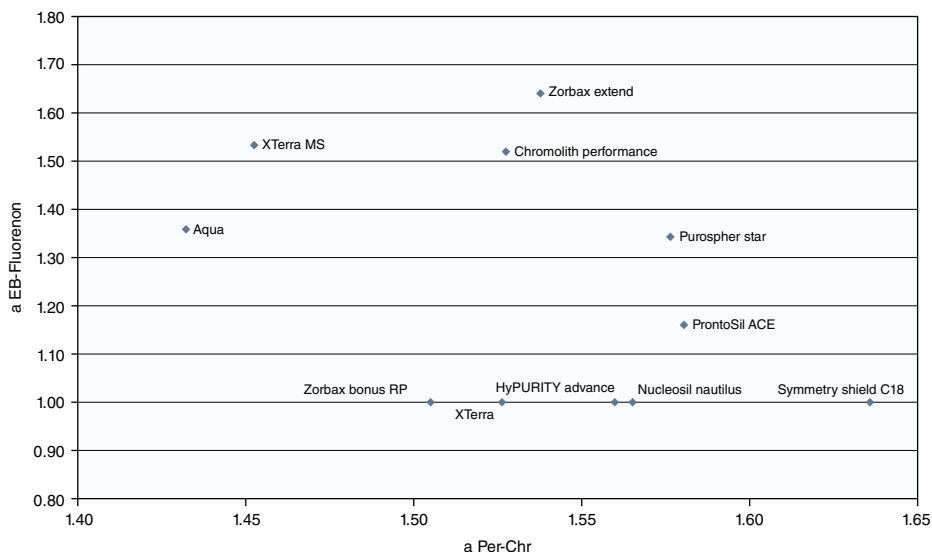


Figure 4.10 Two simple tests for the characterization of RP-phases.

whereas Symmetry Shield and Nautilus as polar phases exhibit good aromatic selectivity. If one wants to separate both small, neutral, and large, polycyclic aromatics, then Zorbax Extend, Chromolith, and Purospher Star would be a good choice – the position of a column in the upper right corner of the α/α -diagram signifies a good selectivity for both analyte pairs. With this test, the similarity of the selection of columns used for the substances to be analyzed can be determined.

4.5.3

Two Simple Tests for the Characterization of RP Phases

The tests just described undoubtedly provide useful and accurate information; however, they represent an effort that is justifiable probably only in the least laboratories. A few years ago, using results from several studies, we developed two simple tests that have proven to be effective: isocratic runs, no buffer, retention time less than 10 min – and at the same time yielding interesting information. They are briefly outlined in the following:

Test 1

Eluent: 80/20 methanol/water (v/v, add MeOH first)

Flow: 1 ml/min

Temperature: 35 °C

Detection: UV, 254 nm

Injection of ethylbenzene and fluorenone, dissolved in eluent

Test 2

Eluent: 40/60 methanol/water (v/v, add MeOH first)

Remaining, as described in Test 1

Injection of phenol and benzylamine dissolved in eluent (freshly prepared solutions)

Results and comments

4.5.3.1 Test 1

If a column shows a separation factor greater than 1.4 then it is a phase with high hydrophobic selectivity, a separation factor greater than 1.6 indicates a phase with a very high hydrophobic selectivity. Phases with high hydrophobic selectivity are suitable for the separation of small monocyclic aromatics, for homologs with a difference in the alkyl chain, for aldehydes, ketones, esters, and for neutral “unproblematic” analytes such as weak organic bases and weak organic acids. Columns that only show small separation factors in this test are suitable for the separation of smaller polar impurities and twisted structures, as well as carotenoids, steroids, double bond isomers, polycyclic aromatics, and analytes present in ionic form such as strong acids or in general any analytes having polar character, such as phospholipids and dyes.

4.5.3.2 Test 2

The later benzylamine elutes compared with phenol, the stronger the polar character of the phase (more precisely: it has groups that are able to undergo ionic interactions). An elution close to phenol or co-elution indicates a hydrophobic phase, elution even before phenol a very well coated, very hydrophobic RP phase or a stationary phase where interactions with silanol groups are suppressed by polar functional groups such as amide or other nitrogen-containing groups. If such a phase shows the desired selectivity for the current issue, then serious problems with this column are hardly to be expected in routine use, it offers good symmetrical peak shape, reproducible retention times, and long life.

4.6**Procedure for Practical Method Development**

The selection of the column is of crucial importance and, therefore, has the utmost priority in any method development. In this chapter, a guide is given to the strategy by which it should be possible to rapidly find the most appropriate column. But, before we come to the list, first some important rules – these are often ignored but, when complied with, lead sooner to success.

4.6.1**The Interaction between Mobile and Stationary Phase**

The separation results from the combination of mobile and stationary phases (and of course temperature). Some combinations are not practicable and, therefore,

either cannot be considered at all, or only (almost in despair) when all other combinations do not work.

The following is the most important basic principle here:

For the chromatography of basic compounds on nonpolar stationary phases (classical reversed phase), use preferably aprotic solvents (e.g., acetonitrile) as the mobile phase and on polar stationary phases (reversed phase with embedded polar groups) preferably protic solvents (e.g., methanol).

4.6.1.1 Why Is This?

Protic solvents solvate polar functional groups and thereby make them more exposed. The only polar functional groups that can be found on a conventional nonpolar reversed phase are the silanol groups. If these are made more prominent by solvation, then the undesired ionic interactions with basic analytes come more to the fore and the elution bands become broad and asymmetric. If, however, an aprotic solvent such as acetonitrile is used with these columns, then the lone pair electrons of acetonitrile can form hydrogen bonds with the silanol groups, and these will be shielded from the basic analytes. The elution bands are much narrower and more symmetrical.

With polar reversed phases, the effect is exactly the opposite. Here, the polar groups (usually nitrogen-containing functional groups) become exposed by the use of protic solvents and thereby interact with the silanol groups. This has the advantage that then the basic analyte cannot interact with these silanol groups. As a result, when protic solvents are used bases elute from polar reversed phases with symmetrical elution bands.

When acidic analytes are being separated, for maximum selectivity the strongest possible interactions of the analytes with the silanol groups of the stationary phase are required. Consequently, in this case it makes sense with classical, nonpolar stationary phases to use protic solvents such as methanol as the mobile phase. With polar stationary phases the polar interactions should be suppressed as much as possible, and so here aprotic solvents such as acetonitrile should be used as the mobile phase.

When both acids and bases are present in a sample, then mobile phases suitable for the bases are normally used, as broad and asymmetric elution bands generally have a more serious effect on the analytical problem as a slightly inferior selectivity. In such cases, as a compromise mixtures of protic and aprotic solvents (methanol and acetonitrile) are sometimes used.

Furthermore, in the reversed-phase HPLC stationary phases whose surface has been modified with phenyl or pentafluorophenyl groups are also used. These functional groups can only develop their maximum selectivity when they are solvated. Therefore, with these columns it is imperative to use a protic solvent as mobile phase. The use of acetonitrile is ruled out, since its lone-pair electrons interact with the stationary phase and thus the interaction of the phenyl groups with the analyte is at least suppressed, if not completely blocked.

When these few rules are followed, then success comes rapidly.

4.6.2

Which Columns Should Be Used, and How Do I Use Them?

First of all, we need to distinguish between whether all the analytes to be determined and all the analytical issues are known, or if there is the possibility that unknown impurities are present.

When our analytes and our analytical goal are known, then we can select the column directed toward this aim. If, for example, we want to separate organic acids differing primarily in the chain length of the aliphatic rest, that is, in their hydrophobic selectivity, then it is best to try a classical endcapped, hydrophobic reversed phase, because this provides the methylene selectivity necessary for this problem.

If, on the other hand, we want to separate aromatic carboxylic acids, which differ in the position of the functional groups on the aromatic ring, then to start with a classical reversed phase would not promise much success. Here, the analytes differ mainly in their π -electron density on the aromatic ring and such analytes can be separated most effectively via π - π interactions. Thus, in this case the use of a stationary phase with phenyl groups would be the first choice. As mentioned in Chapter 3, in HPLC the separation and thus the resolution are primarily achieved through the selectivity, and this in turn depends strongly among other things on use of the correct column.

So if we have information on the analytes to be separated, then we can use this for clever selection of the column to be used.

If we have to clarify the heterogeneity of an unknown sample, the sample must be examined on various columns with orthogonal selectivity to ensure that a possible co-elution is not overlooked. Selection of these columns is best orientated on the key characteristics of the columns, which were considered in Section 4.4.

Therefore, a modern C18 or C8 column with a good efficient endcapping should be tried first. In the most cases this will be successful. Two typical examples here would be Chromolith High Resolution RP 18e from Merck and YMC Pro C8. The Chromolith column has the advantage of high separation efficiency at relatively low back pressure and at the same time shorter column length. Due to their chromatographic performance, C8 columns are to be preferred to C18 columns as they usually have a higher surface coverage and, therefore, fewer unwanted secondary interactions between bases and the stationary phase are possible. However, if strong methylene group selectivity is required (i.e., two large molecules that differ in just one methylene group need to be separated) a C18 is always superior compared with a C8 stationary phase. If somewhat stronger bases are expected in our sample, a particulate C8 column is preferable to the monolithic C18 column. The retention on a monolithic C8 column would be too weak and it is, therefore, not really suitable for general use, in special cases, however, it is definitely an alternative.

If the separation does not work on a classical reversed phase, then the type of stationary phase must be radically changed. A change to a classical packing from a different manufacturer is only successful in isolated cases. The column with which

one has the most success after the classical packing is a reversed phase with an embedded polar group. As typical representatives, ProntoSIL C18 ace-EPS from Bischoff or Symmetry Shield RP 18 from Waters could be named here. These show similar hydrophobic retention to classical reversed phases (most reversed phases with bonded polar groups are weakly retentive) and have a fairly high polar selectivity.

If even this column does not give the desired separation, the next choice would be a phenyl column. Examples of this column type are XSelect CSH Phenyl-Hexyl from Waters or Zorbax Phenyl from Agilent Technologies. Many manufacturers use hexyl as a spacer for the phenyl group. With increasing chain length the strength of the $\pi-\pi$ interactions increases [16]. Recently, Restek introduced a biphenyl-bonded stationary phase, which provides slightly different selectivity compared with traditional phenyl columns.

And if even this column does not work, then a classical reversed phase with high metal content and without endcapping or a modern non-endcapped C30 column with high shape selectivity would be the column of choice. Recommendations from this category are LiChrospher RP 18 from Merck or ProntoSIL 200 C30 from Bischoff. At this point, a practical tip to shape selectivity: the shape selectivity is strongly temperature dependent; the lower the column temperature, the higher the shape selectivity. Therefore, “shape”-selective separations should best be done at low temperatures. The same effect as lowering the temperature can be observed by increasing the back pressure of the column. This can be achieved either by using smaller particle diameters or a restriction capillary after the column. This effect is observed at back pressures of 500 bar and higher.

A further option would be the use of a cyano (CN) column. A typical classical CN column would be Nucleosil CN from Macherey Nagel or LUNA CN from Phenomenex. These columns separate via dipole interactions and thus preferentially compounds that differ in their dipole moment. Due to their low hydrophobic selectivity, however, with these columns, it often comes to co-elution of compounds having similar dipole moments and only slightly differing hydrophobicity. Nevertheless, these columns offer an interesting alternative for group separations, which then show their strengths in combination with 2D-LC or phase-optimized liquid chromatography (POPLC).

Very hydrophobic analytes often interact very strongly with the stationary phase and are sometimes not eluted from conventional reversed phases. Here, the use of a very short chain alkyl phase modified only with trimethylsilane groups helps. A well-proven column here is Zorbax TMS from Agilent Technologies. Other manufacturers also offer this kind of column and call them C1, RP2, or SAS.

During selection of these columns, it may happen that on some columns two components are separated, but other components that are separated on other columns suddenly coincide. In such cases, POPLC can be used [17, 18]. The principle is shown in Figure 4.11. A set of segmented columns is contained in a column kit. This set represents the columns of differing selectivity described earlier. From an initial measurement on the individual columns, the retention time of each

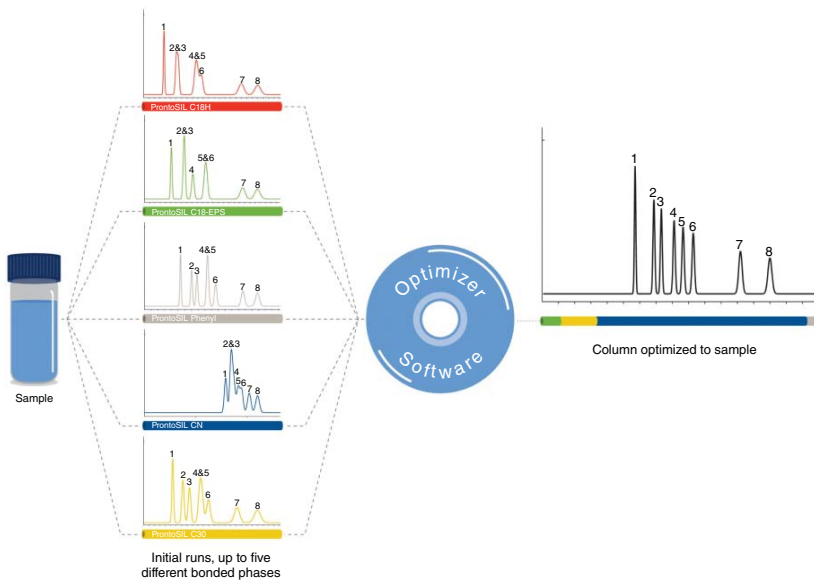


Figure 4.11 POPLC scheme.

compound is determined and by means of a software program the ideal composition of the separation column determined. Through objective-based coupling POPLC can deliver the “best” column composition for the current separation. However, when the number of analytes to be separated exceeds about 20, then usually POPLC optimization does not give a solution either. Here, only high peak capacity (i.e., 2-D HPLC) or a high plate number can help.

4.6.3

What to Do, When the Analytes Are Very Polar and Are not Retained on the above-Mentioned Columns?

Polar analytes are very often a major challenge to reversed-phase chromatography. This is because even with very polar (completely aqueous) mobile phases only little or no retention can be achieved. For these cases, there are certain strategies that are discussed in the following.

4.6.3.1 AQ Columns, Polar RP Columns, and Ion-Pair Chromatography

If polar analytes are to be separated with reversed-phase HPLC, then very polar mobile phases must be used. An increase in the water content of the mobile phase leads to an increase in retention. With classical reversed phases, this is the case (at least for pressures under 200 bar) only when the proportion of organic solvent is above 5%. Below this, the retention suddenly collapses, so classical C18 columns cannot be used with 100% aqueous eluents. This is due to the high surface tension of water. More than 99% of the specific surface area of the stationary phase is located in the pores. If the proportion of organic solvent in the mobile phase falls below 5%, a “drop” forms at the entrance to the pores, which prevents the movement of analytes into and out of the pores. Only when the pressure rises above 200 bar is this again possible [7].

As a result, stationary phases have been developed that allow the reversed-phase mode to be used in purely aqueous media. These include so-called AQ columns, which often have AQ in their name or are called aqua or hydro. Sometimes, their names are only associated with water, such as Atlantis. In principle, the water compatibility can be achieved in three ways:

- a) Lower surface coverage. For example, Platinum EPS from Alltech or Atlantis T3 from Waters. These columns are classical C18 columns but have a significantly lower surface coverage than conventional C18 columns. They often have a conventional endcapping (Platinum EPS does not) and also exhibit the selectivity of conventional columns. Due to the low surface coverage, however, a better accessibility of the surface is achieved; residual polar silanol groups can be “seen” and solvated and thereby the pore accessibility is maintained.
- b) Polar endcapping, for example, Aquasil C18 from Thermo or ReproSIL C18 AQ from Dr Maisch. With these stationary phases, endcapping is achieved with a small silane containing a polar group instead of conventional endcapping with trimethylsilane groups. This ensures the wetting of the surface and, therefore, the accessibility of the pores.

- c) Reversed phases with an embedded polar group, for example, Ascentis RP Amide from Sigma Aldrich or XBridge Shield RP18 from Waters. These stationary phases have a polar group (amide, carbamate, or urea) incorporated into the alkyl chain. The endcapping of these materials is conventional (trimethylsilane groups). With these columns the polar group built into the alkyl chain ensures the surface wetting.

Finally, other highly polar phases can be thought of such as various fluorinated phases, Zorbax Bonus RP, or Synergi POLAR RP.

An unusual but repeatedly successful measure is a typical RP eluent (e.g., buffer/ACN) plus silica. This is the “aqueous normal phase” chromatography known from the 1980s (in the gradient mode nowadays known as hydrophobic interaction liquid chromatography (HILIC), discussed later).

If the analytes to be separated are so polar that even 100% aqueous mobile phases are not sufficient to retain them on the above-mentioned columns, then at least for ionizable analytes ion-pair chromatography helps. In ion-pair chromatography, reagents of opposite charge to the sample and which contain a polar group and a nonpolar residue are added to the mobile phase. These additional anionic or cationic surfactants then form together with the polar cationic or anionic analytes a so-called ion pair, which is then outwardly electrically neutral and can be retained on reversed phases. Other conceptions assume that the surface of the stationary phase becomes coated with the corresponding ion-pair reagent and so an ion exchanger is present, on which it is then possible to separate the oppositely charged analyte ions. The truth probably lies between these two models. Ion-pair chromatography is a very elegant means to chromatograph ionic analytes in reversed phase mode; however, in practice some points have to be considered.

- a) Equilibration of an ion-pair reagent with the stationary phase takes a long time, the column should be equilibrated overnight at a low flow. AQ columns are the most suitable for use in ion-pair chromatography as they allow good access to the complete chromatographic surface and are normally equilibrated twice as fast as conventional C18 columns (this is also true for the customary use of these columns in nonpolar applications).
- b) When using an ion-pair reagent, care must be taken to ensure that at all times both the analyte and the ion-pair reagent are present in dissociated form. Therefore, appropriate buffering of the eluent is necessary.
- c) Ion-pair reagents are also always surfactants and so difficult to obtain commercially in ultrapure form. As a result, the ion-pair reagents often contain impurities, which in the case of gradient elution are initially enriched at the head of the column, elute with increasing solvent strength, and then appear in the chromatogram as so-called “ghost peaks.” This is not serious when no co-elution with the analyte to be measured occurs. However, it is necessary to locate and know the ghost peaks in the chromatogram. Therefore, a blind injection () must be made before the actual chromatographic run and also the equilibrium time between the individual chromatographic runs must remain

- constant, as otherwise after prolonged equilibration the intensity of the “ghost peaks” changes.
- d) From experience, for the separation of anions the purest ion-pair reagent is tetrabutylammonium hydrogen sulfate (TBAHSO_4), for the separation of cations sodium dodecyl sulfate (SDS). Typical concentrations used in the eluent are 10 mM ion-pair reagent and 20 mM buffer. However, it is always worth varying the ion-pair concentration during method development to examine the influence on peak shape and selectivity and, therefore, robustness of the method.
 - e) As ion-pair reagents are not volatile, mass spectrometric and aerosol detectors cannot be used. It is, however, possible by means of suppressors before the detector to free the eluate from the ion-pair reagents; these detectors can then be used [19].

Another elegant way to avoid ion-pair chromatography is the use of so-called “mixed-mode” columns.

4.6.3.2 Mixed-Mode Columns

Mixed-mode columns contain reversed as well as various other functional groups, for example, often groups capable of forming complexes or ion-exchange groups. Because here the interaction-capable groups are bonded covalently to the stationary phase, these materials provide an elegant way of retaining ionizable polar analytes without the addition of ion-pair reagents. When using mixed-mode columns, volatile buffers can be added to the mobile phase, allowing the use of aerosol and mass spectrometric detectors. There are some column manufacturers who have specialized in mixed-mode columns. Frequently used mixed-mode columns are the Primesep columns from SIELC. These are obtainable as strong cation exchanger (Primesep 100), weak cation exchanger (Primesep 200), strong anion exchanger (Primesep B), and weak anion exchanger (Primesep B2), so that the corresponding column for each analytical application is available. A column with a complex-capable group on the alkyl residue would be Primesep C. Further examples of mixed-mode columns are Acclaim Mixed-Mode HILIC 1, Scherzo SM-C18, and Obelisc R or N.

Other columns with multiple ion-exchange groups and an additional heteroatom such as sulfur appear to us to be too special and are not further considered here. The special practical charm of these columns lies in the fact that by increasing the acetonitrile content during the gradient not only the solvent strength for the RP retention mechanism is increased, but at the same time the protolysis equilibrium of the ion-exchangeable groups is suppressed, resulting in the elution of ionic analytes.

In fact, every silica-based reversed phase represents a mixed-mode column, because besides the alkyl chains it has silanol groups, which are groups capable of weak cation exchange. Some C18 materials of the first generation, such as Partisil ODS from Whatman or Spherisorb ODS1 from Waters, have only a very low carbon content (<5%) and a high number of free silanol groups. With these

columns, strongly basic compounds can be separated and elute with symmetrical peak shapes. This is because in these stationary phases the silanol groups are present in such a large number that they are not overloaded and, therefore, symmetrical elution bands result.

In the earlier sections, we have discussed the options available to retain ionizable polar analytes. In the following, we show possibilities to retain and chromatograph neutral and ionizable analytes together.

4.6.3.3 Ion-Exchange Columns/Ligand-Exchange Chromatography

These materials are polymer-based stationary phases, more exactly exhaustively sulfonated polystyrene-divinylbenzene copolymers. The retention mechanism of these separations is very complex and is often founded on the possibility for the analytes to undergo interactions similar to ligand complexes with the cation of the stationary phase [20]. However, the separations often work very well especially for polar analytes containing many hydroxyl groups, such as sugar. The mobile phase used in this case is slightly acidic water. These columns are available not only in the acidic H^+ form but also containing various metals (Ca^{2+} and Pb^{2+}), resulting in varying selectivities. For the separation of sugars, primarily the metal-containing columns are used, while for the separation of organic acids the use of the H^+ form is preferred. These columns are available from many manufacturers. As a proven column, Shodex K-801 is mentioned. These stationary phases generally have small pores. To accelerate the mass transfer, it is recommended to run the columns at elevated temperatures such as $60-80^\circ C$.

4.6.3.4 HILIC (Hydrophilic Interaction Liquid Chromatography)

In the case of HILIC, polar stationary phases are used with mobile phases consisting of at least one polar and one nonpolar solvent. Usually, these are acetonitrile/water mixtures with a high proportion of acetonitrile. HILIC is, therefore, an aqueous normal phase chromatography [21, 22]. As shown in Figure 4.12, the more polar part of the mobile phase accumulates on the polar stationary phase. The more polar analytes prefer to stay close to the polar surface and are, therefore, more strongly retained.

In practice, the use of HILIC is completely different from reversed-phase chromatography and so users accustomed to working with RP chromatography

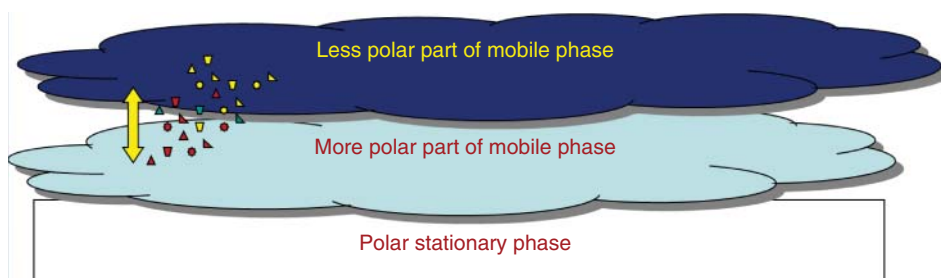


Figure 4.12 HILIC scheme.

often founder when working with HILIC. If the following points are observed, then success comes sooner:

- a) Equilibration takes much longer with HILIC as with RP separations, usually twice as long. While in the reversed-phase chromatography 5–10 column volumes are sufficient for equilibration of the column after a gradient run, with HILIC 10–20 column volumes are required.
- b) The gradient in RP chromatography usually covers almost the entire range of polarity from water to acetonitrile. With HILIC, the gradient is run only from 95% acetonitrile merely to a maximum of 60% (usually even only 70%) acetonitrile. As with reversed-phase chromatography, the gradient volume is 5–15 column volumes.
- c) The loadability of HILIC columns is often significantly less than that of RP columns. This must also be taken into account.
- d) The buffer and above all the buffer concentration have a stronger influence on the separation than in the reversed-phase chromatography. It has a direct influence on the thickness of the layer of the more polar mobile phase (“pseudo-stationary phase”) near the surface. Here too, robust conditions must be ensured.
- e) Dissolve the sample always in the eluent in which the chromatography starts. If this is not done no retention for the analytes will be obtained. This is the most prominent mistake HILIC rookies make.

Despite these limitations, HILIC provides an elegant way to retain and separate polar analytes.

With HILIC, various polar stationary phases with differing selectivity are used. Basically, it must be distinguished between three different selective types: weak anion exchanger (silica modified with aminopropyl groups) and amide columns, weak cation exchanger (usually unmodified (bare) silica) and neutral supports (diol or zwitterionic stationary phases (ZIC-HILIC)). With ionizable compounds, in addition to the distribution equilibrium between the mobile phase (“pseudo-stationary phase”) near to the polar surface and the less polar mobile phase, ionic interactions can also occur, resulting in differing separation characteristics on the different stationary phases.

In the case of electrically neutral stationary phases the zwitterionic phases, on the surface of which a betaine is bonded, have the advantage over the diol phases that with the same mobile phase the retention of the stationary phase is much higher. This is because due to the ionic groups the more polar mobile phase near the surface can form a thicker layer and, therefore, the retention of the polar analytes is increased. The selectivity of zwitterionic stationary phases is similar to that of diol phases.

For this reason, in practice, it is recommended to check the selectivity of neutral, anionic, and cationic polar stationary phases.

4.6.3.5 Porous Carbon

Porous carbon as a stationary phase was developed and studied in the early 1980s by John Knox and co-workers [23]. Commercially, these columns are obtainable

from Thermo under the trade name Hypercarb. Alternatively, a zirconium dioxide material coated with carbon and having similar selectivity is available from Zirchrom. The advantage of the graphite material is that certain polar organic molecules can undergo especially strong interactions with the carbon and thus exhibit a very high retention. The main reason for this is that analytes with lone pair electrons, present, for example, in amino, hydroxyl or carbonyl groups, can interact with the electron cloud of the conductive graphite. The high selectivity for the separation of isomers containing π -electrons is based on this. Since these π -electrons are more or less heavily exposed in the analyte, more or less strong interactions with the stationary phase occur resulting in higher selectivity.

Even highly polar analytes such as underivatized amino acids, oligosaccharides, or nucleobases can be retained on these columns.

A further advantage is the high temperature and pH resistance of the carbon materials.

The disadvantage of the carbon columns compared with silica is a poorer pore size distribution, which in practice results in a slightly asymmetric peak form, with tailing in the lower 10% of the peak height.

4.7

Column Screening

In the course of a new method development, a suitable column should be found as quickly as possible. The most efficient and quickest approach here is a column screening, in which the above-described, preferably orthogonal stationary phases are tested in a screening and the number of components, which can be separated determined. The stationary phase that provides the best separation (largest number of peaks) can then be used for fine-tuning.

For fast and effective column screening, a column switch and up to six different columns in the column oven are usually used. Preferably short columns (length 50–100 mm) with inner diameters of 3.0–4.6 mm are recommended for use here. Smaller inside diameters would be unsuitable for screening, since the ratio of column volume to volume of the apparatus would be less than a factor of 10. When the volume of the apparatus is more than 10% of the column volume then significant broadening of the peaks outside of the column occurs, the practical consequence of which is wider peaks, which in effect cancel out the separation that took place in the column.

During column screening, the complete range of mobile phase polarity should be covered. With classical reversed phases, this means a gradient from 10–20% to 95% acetonitrile (with regard to gradients, see also Chapter 3). With polar stationary phases methanol instead of acetonitrile must be used, or a mixture of the two. Furthermore, differing pH values must also be covered, so that any pH-dependent changes in selectivity can be detected. Consequently, the screening should cover pH 2, pH 7 and, if basic components are expected then also pH 9. At this point, it is emphasized once again that when working above pH 7 caution is necessary

(see Section 4.3, pH-stability). During screening, the linear velocity of the flow through the columns should be about 3 mm/s (for a 4.6 mm ID column this corresponds to approximately 1.5 ml/min, with a 3 mm ID column about 0.7 ml/min). The gradient slope should be about 10 column volumes. This means that with the 50 mm column, the gradient passes through in about 4 min and with the 100 mm column in about 8 min. With this experimental design, it is possible to run a column and eluent screening overnight.

The effective approach to method development is summarized once more in Figures 4.13–4.16:

Finally, we present some examples of suitable column assortments depending on the issue or the possible preference for a manufacturer. The columns mentioned are certainly not to be understood as recommendations, but rather they represent examples for a specific intention. Naturally alternative columns, which can be found with the help of databases (discussed later) can also be considered.

Explanation: depending on the case either the type of stationary phase (e.g., “biphenyl” or “PFP”) or the name of a specific column can be found in the two tables. In the first case, depending on availability and supplier preferences you can choose which bi(di)phenyl or pentafluorophenyl column you want to use. In the second case, the name of the column is mentioned as a typical representative of this characteristic type of material.

Table 4.1 Examples (!) of column assortments for special cases and/or conditions.

One company (e.g., Waters) variety	Strong bases	Polar analytes	Strongly polar analytes	Mixed mode	One company + polar analytes
X-Bridge C18	Gemini/NX	Synergi FUSION RP	Synergi POLAR RP	Acclaim Trinity P1	Discovery HS polyethylene glycol (PEG)
CSH PFP	YMC-Triart C18	“AQ”	Kinetex HILIC	Scherzo SM-C18	Ascentis Express Amide
HSS PFP	Pathfinder XQ	Biphenyl	ZIC-HILIC	Obelisc R (Obelisc N)	Ascentis Express F5
Atlantis HILIC	XSelect CSH (XBridge)	Phenyl-Hexyl	Zorbax SB CN	Acclaim Mixed-Mode HILIC	Ascentis ES Cyano
Symmetry Shield	Kromasil Eternity	Zorbax SB C8	Ascentis HS F5	Nucleodur Sphinx	Ascentis Express HILIC
SunFire	Zorbax Extend	Hypersil GOLD	Platinum EPS	RP-WCX	Ascentis Phenyl

Our opinion: Also think about, for example, Hypersil ODS, LiChrospher Select B 60, Superspher or Cosmosil NAP; additionally, for example, Jupiter, Zorbax SB 300 as well as Nucleosil 50 – then orthogonality is given for all cases.

Comments on Table 4.1

Column 1: Suppose you want to use columns from Waters, because Waters is your main column supplier. In this fictitious example, you have a completely unknown sample and for the overnight run using the column switch you would like to test quite different columns, so you need a variety of Waters columns.

Column 2: Suppose you suspect strong bases in your sample and you would like to test columns that are stable under alkaline conditions.

Columns 3 and 4 are self-explanatory.

Column 5: These columns are suitable for the case where you expect substances of completely differing character in your sample.

Column 6: The Situation: You want to use columns from Sigma Aldrich and you know you probably have polar analytes, so you need the entire range of polar phases from Sigma Aldrich.

After finding the “best” column overnight, in accordance with our comments another run would make sense: better to be safe than sorry, and an orthogonal test in the case of unknown samples provides parallel to a DAD-LC/MS–MS coupling more security regarding peak homogeneity.

Table 4.2 Examples (!) of column assortments for special cases and/or conditions.

Shape ^{a)} selectivity new and others	Shape ^{a)} selectivity ... old Säulen	Low pH-value	2D- separation	Mixed mode in general	Orthogonality of column types
ACE 3 C18	Novapak	Zorbax SB C18	RP-HILIC	RP-WAX	C18 Nr. 1: (Atlantis d)
PFP im HILIC Modus	Nucleosil 50	XSelect CSH	RP-SEC	RP-WCX	C18 Nr. 2: (InterSustain)
ASCENTS Express Amide	Sphersisorb ODS 1	YMC Triart	IEC-SEC	RP-HILIC	PEG (Poly ethylene glycol)
Biphenyl Cosmosil	Jupiter	Gemini/NX	Achiral–Chiral	HILIC–WAX	PFP
Cholesterol	Zorbax SB 300	Hypercarb	PFP-RP/PFP- NP	HILIC–WCX	Biphenyl
Ultra II Aromax	Symmetry 300	Lichrospher	Mixed-Mode RP-NP (SiO ₂ !)	Zorbax ODS	Primesep C

a) In such cases, remember the “3”: low flow, methanol as organic solvent, low temperature.
Superficially porous material Cortecs, Accucore, PoroShell, Kinetex EVO, Coresep.

Comments on Table 4.2

Columns 1–3 are self-explanatory

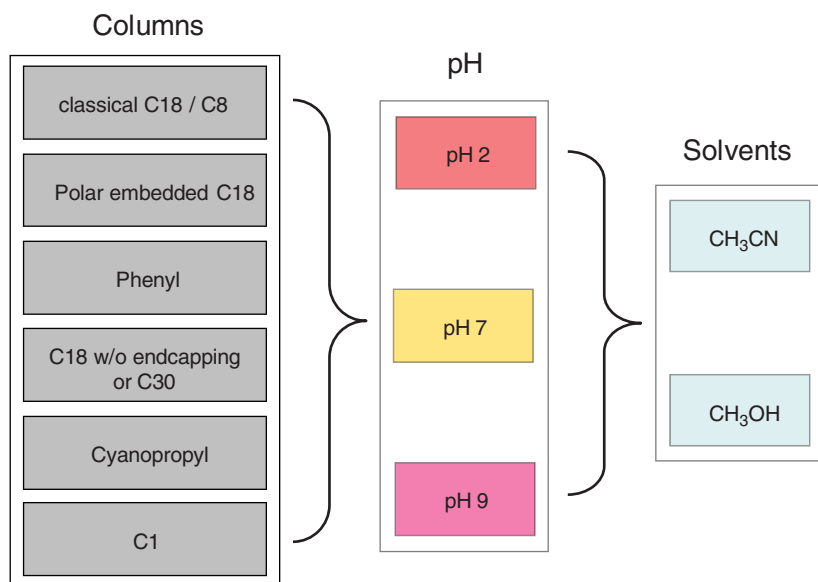


Figure 4.13 Procedure with classical reversed-phase chromatography.

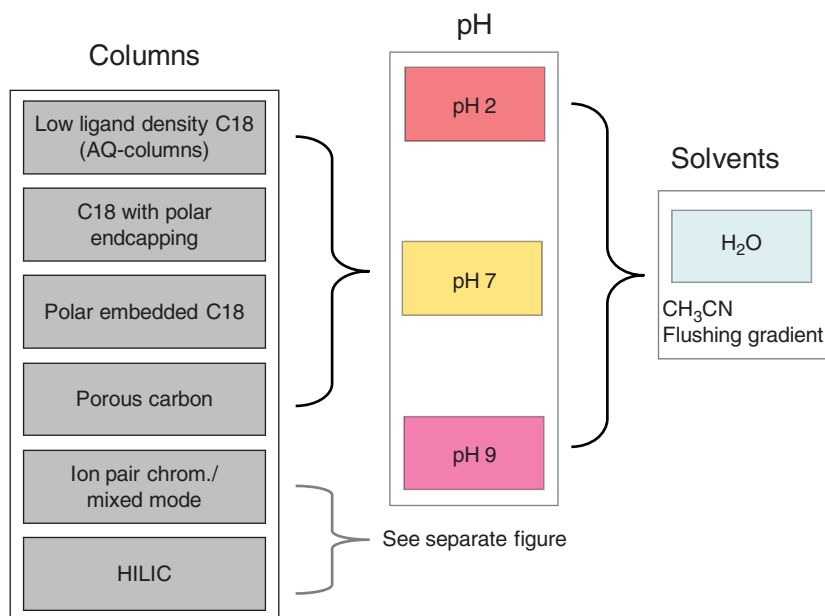


Figure 4.14 Procedure with polar analytes.

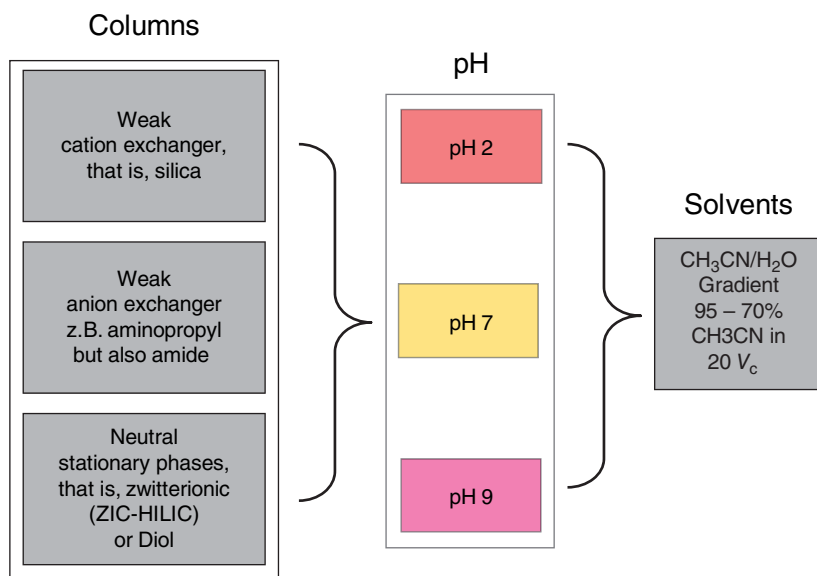


Figure 4.15 Procedure with HILIC.

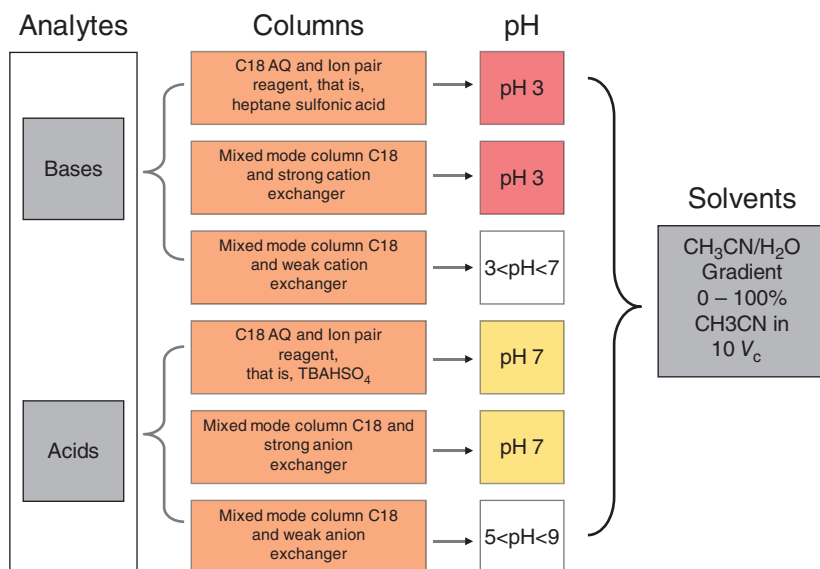


Figure 4.16 Procedure with ion-pair chromatography or by the use of mixed-mode columns.

Column 4: With 2D experiments (see also Chapter 3) orthogonal selectivities are essential. For the given column types, the selection of a suitable mobile phase should not cause any major problems.

Column 5: In addition to examples of modern types of columns with various combinations of functionality, we have also added Zorbax ODS. As, for example, LiChrospher, this old classic represents a stationary phase, which allows almost “all” types of interactions that may be important for a good selectivity, ranging from hydrophobic interactions (about 17% C) through ion exchange, hydrogen bonding, dipole interactions, and so on to complex formation. The material has among others free, geminal and vicinal silanol groups, siloxane bonds, and as contaminants metal ions.

Finally, column 6 contains examples of materials that have quite different characters; we have also deliberately included two completely different C18 columns.

To the commentary: The more similar the molecules are chemically (also similar polarity) the more important is the use of methanol and chromatography at low temperatures. Furthermore, higher flows are not allowed due to the slow kinetics.

Recently, several column manufacturers offer selectivity kits for certain purposes, for example, Waters offers a family of differently selective columns called XSelect CSH which is aimed for use with LC/MS compatible mobile phases such as formic acid.

4.8

Column Databases

Often you have a good column, but for some reason it is not available or its production has been stopped or new production batches of the columns show a completely different selectivity and the separation suddenly no longer works. You may also have to prove the correctness of the first method using a preferably orthogonal HPLC method, or co-elution occurs in a method development. We have all experienced this during the laboratory routine. The question here is: How do I find the most similar or most dissimilar column to my current one?

As so often nowadays, the answer is in the Internet. Under <http://apps.usp.org/app/USPNF/columnsDB.html> two column databases can be found. The databases contain data from hundreds of HPLC columns, characterized by means of chromatographic tests such as those described in Section 4.5 [24–27]. The first database is based on the USP test from the National Institute of Standards and Technology (NIST) for the characterization of reversed phases. Unfortunately, this test is not very precise.

The second database, the PQRI database, contains data for about 750 columns. The data for each column were determined by means of five chromatographic tests carried out at different pH values, so that with this database a very good comparison of stationary phase selectivity, also as a function of the mobile phase

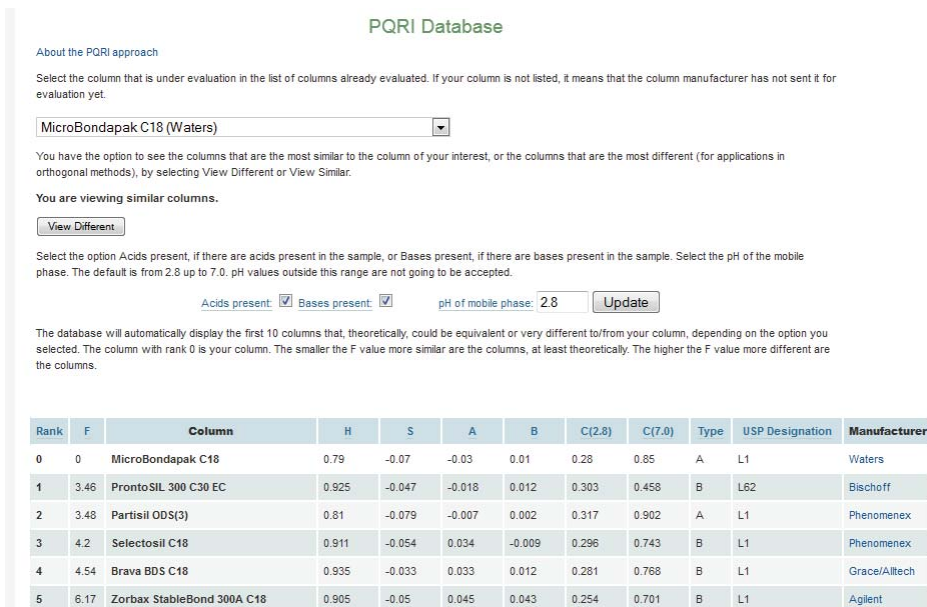


Figure 4.17 Screenshot of the PQRI-database.

pH, is possible. The different column parameters are weighted according to the pH of the mobile phase and whether acidic or basic analytes are present in the sample. When, for example, the sample contains no basic analytes, then it would be unnecessary to consider the ion exchange capacity of the two columns when calculating the fitting factor.

When two columns are compared a fitting factor is obtained, which is a direct measure of how well the columns match. For any method, the most similar and also the most dissimilar column to the chosen column can easily be identified, as can be seen in the figure 4.17. For the sake of completeness, a further database, based on the work of Tanaka, Euerby, and Peterson, is mentioned (ACD Labs Column Selection Database):

<http://www.acdlabs.com/products/adh/chrom/chromproc/index.php#colsel>.

These databases are a great help in finding your way through the jungle of HPLC columns.

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5

Introduction to Biochromatography

Jürgen Maier-Rosenkranz

5.1

Introduction

During the recent years, biochromatography has increasingly gained in importance, particularly in the pharmaceutical industry for the development, analysis, and production of active pharmaceutical ingredients, the so-called APIs, with a significantly increased number of biomolecules.

Together with this increased interest in biochromatography, its application and definition have also expanded. From a historical perspective, biochromatography developed largely independent from the traditional HPLC, mostly used for smaller molecules (<1000 Da). In most cases, the equipment, columns and packing materials used were provided by other manufacturers. Traditionally, biochromatography was mostly carried out on polymer-based materials with larger particles in the low- or medium-pressure range. The use of highly concentrated salt solutions as mobile phases also made it difficult to apply these methods for classic HPLC systems. Today, both areas have approached and benefit from each other, though there is still great potential for development in the comprehensive networking of these two areas.

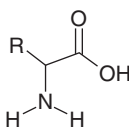
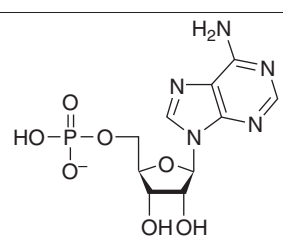
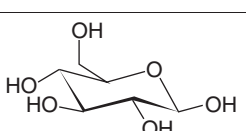
Conceptually, biochromatography can be seen as the separation (analysis and purification) of biomolecules.

Basically, the term *biomolecule* is broader than usual in chromatographic applications nowadays:

Biomolecules are molecules of organic substances that exist in life-forms. They mainly consist of carbon and hydrogen, which form chemical compounds with oxygen, nitrogen, phosphorus, or sulfur. Other elements can be a component of biomolecules too, though they appear less often. (Source: Wikipedia December 31, 2009)

According to this definition, biomolecules comprise a very wide-ranging group of compounds, which also includes substances such as lipids, phospholipids, glycolipids, sterols, vitamins, hormones, or neurotransmitters. Referring to

Table 5.1 Biomolecules - monomers.

α -aminoacid	Nucleotide	Monosaccharide
		

biochromatography, this grouping is, however, often reduced to the separation and purification of peptides and proteins.

In chromatographic reality, it is, however, more comprehensive and suitable to extend the definition of biomolecules to include these three groups of substances and their oligomers and polymers (Table 5.1).

At first sight, sugars play a minor part in this context; they are, however, very important in the glycosylation of peptides or proteins, for example, the glycopeptide vancomycin.

Table 5.2 lists the 20 proteinogenic amino acids.

Table 5.2 List of the 20 proteinogenic amino acids.

Amino acid	Three-letter	One-letter	Side-chain polarity	Groups	pI value
Alanine	Ala	A	Nonpolar		6.0
Arginine	Arg	R	Basic polar	NHR 3×	10.8
Asparagine	Asn	N	Polar	NH ₂ –CO	5.4
Aspartic acid	Asp	D	Acidic polar	COOH	2.8
Cysteine	Cys	C	Polar	SH	5.1
Glutamic acid	Glu	E	Acidic polar	COOH	3.2
Glutamine	Gln	Q	Polar	NH ₂ –CO	5.7
Glycine	Gly	G	Nonpolar		6.0
Histidine	His	H	Basic polar	Imidazole	7.6
Isoleucine	Ile	I	Aliphatic		6.0
Leucine	Leu	L	Aliphatic		6.0
Lysine	Lys	K	Basic polar	NH ₂	9.7
Methionine	Met	M	Nonpolar	SCH ₃	5.7
Phenylalanine	Phe	F	Aromatic	C ₆ H ₆	5.5
Proline	Pro	P	Nonpolar		6.5
Serine	Ser	S	Polar	OH	5.7
Threonine	Thr	T	Polar	OH	5.9
Tryptophan	Trp	W	Aromatic	Aminoindole	5.9
Tyrosine	Tyr	Y	Aromatic	OH–C ₆ H ₆	5.7
Valine	Val	V	Nonpolar		6.0

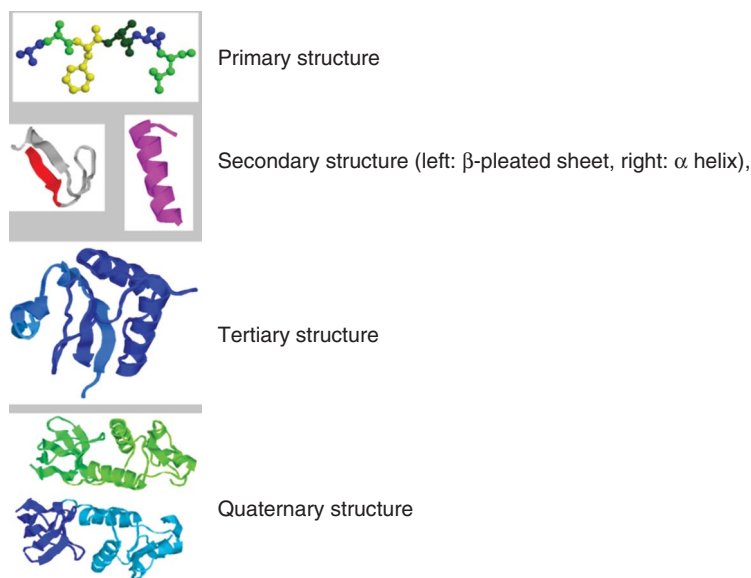


Figure 5.1 Levels of protein structure Source: Wikipedia 2012.

In addition to these primary differences, there are also bigger structural differences as the molecules get bigger.

Table 5.2 and Figure 5.1 show the differences between the various amino acids, peptides and proteins, which can be used for chromatographic separation:

Separation according to	Method
Shape/size	Size-exclusion chromatography (SEC)
Loading	Ion-exchange chromatography (IEC)
Polarity, solubility	Reversed phase; HIC, HILIC
Biological activity	Affinity chromatography (AC)

These six different chromatographic techniques will be explained in detail in the later sections.

Beforehand, however, the stationary phases will be explained, because the base material and its structure and parameters are identical for all chromatographic techniques. The focus is here biochromatographic application and detailed information to RP materials are in Chapter 4.

5.2

Overview of the Stationary Phases

Today, there are a variety of different stationary phases on the market, and it is often difficult to purposefully select an appropriate phase.

This section describes the criteria for the evaluation of stationary phases and their respective influence on separation efficiency and loading.

5.2.1

Base Materials

First of all, the stationary phases differ by the base material used. Ideally, a base material is chemically stable against acids, bases and solvents, has good mechanical stability (pressure resistance), no change in volume when the mobile phase changes and no competing interactions with regard to the chromatographical principle used. Unfortunately, not all of these requirements can be realized with the respective materials. Table 5.3 lists the most common base materials and their corresponding classifications.

In addition to these four main materials, there are some others which are, however, not very common: hydroxylapatite, zirconium dioxide, titanium dioxide.

5.2.2

Characterization of Stationary Phases

A stationary phase is comprehensively described with the following parameters: particle form and size, pore size, surface area, bonding density, purity and functional group. In this context, it is strongly recommended to study the manufacturers information (technical data sheet) and evaluate it according to the separation to be realized.

Table 5.3 Overview different base materials for Stationary phases.

		Silica gels	PS/DVB polystyrenes	Acrylates	Dextrans
Mechanical stability	Pressure resistance	++	+	0	–
Mechanical stability	Swelling/shrinking	++	0	–	–
Chemical stability	Solvent	++	+	0	–
Chemical stability	pH stability – acid	++/≥ 1	++/≥ 1	+/≥ 2	+/≥ 4
Chemical stability	pH stability – alkaline	–/≤ 8	++/≤ 14	++/≤ 13	++/≤ 13
Purity		++ metals (Fe, Al, etc.)	+ Synthetic by-products	+ Synthetic by-products	+ Synthetic by-products

++, applies completely; +, applies partly; 0, applies in a limited way; –, applies poorly.

5.2.2.1 Particle Form

There are three different particle forms: irregular, spherical and spheroidal. Irregular particles are produced by milling larger particles. This is done with some silica gels. Previously, this was the primary manufacturing method and, therefore, these silica gels are regarded as out-of-date. Today, however, good irregular silica gels can be produced, which show very good parameters and, therefore, can clearly be packed better and more stably than the corresponding old materials.

Today, spherical materials are the technical state of the art. Mostly, they can be packed easily and give a good performance.

Spheroidal forms are less common.

5.2.2.2 Particle Size

The particle size, d_p , is the mean particle diameter and is normally indicated in micro meter. The particle size defines two important values:

Plate number	$N \approx 1/2 * L/d_p$
Pressure	$P \sim 1/d_p^2$

If the number of plates is increased by using smaller particles then, as a consequence, the pressure increases by square at the same rate.

In this context, the particle size distribution is especially important. An increased share of small particles, especially, increases the back pressure. When there are more bigger particles, the number of plates decreases. An additional problem of a broad particle size distribution is that the material is more difficult to pack.

If pressure-sensitive soft gels are used and the amount of small particles, so-called fines, is too high, this of course directly influences the field of application (flow rate, column length). In the case of analytical separations, this often does not matter much, because pre-packed columns are mostly used. In the case of preparative purifications, however, a column with a low number of plates and an increased back pressure negatively influences the loading, purity, run time, and lifetime.

To evaluate the particle size distribution, besides d_{50} (= median particle diameter) the values for d_{90} and d_{10} are normally also indicated.

In Figure 5.2, $d_{50} = 11.2 \mu\text{m}$; $d_{90} = 14.4 \mu\text{m}$, $d_{10} = 8.7 \mu\text{m}$.

This means that 90% of the volumes of all particles are smaller than $14.4 \mu\text{m}$ and that 10% of the volumes of all particles are smaller than $8.7 \mu\text{m}$. For an easier evaluation and better comparability, mostly d_{90}/d_{10} are defined. The value for d_{90}/d_{10} should be as small as possible: suitable values are less than 2. The example in Figure 5.2 shows a $d_{90}/d_{10} = 1.66$, which is good.

The advantage of the smaller particles to show a better resolution because of the higher plate number is used directly for better analyses. Today, materials from

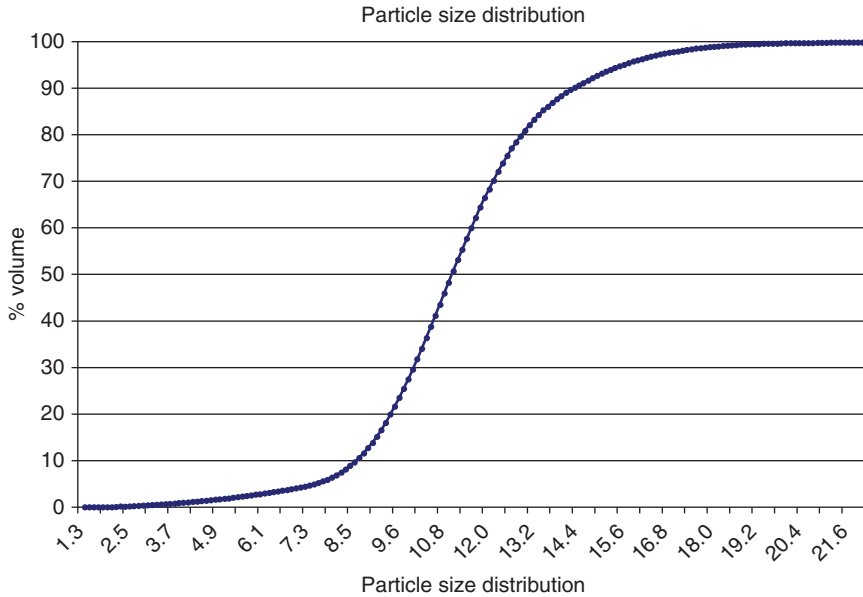


Figure 5.2 Particle size distribution measured by laser diffraction.

1.5 to 5 μm are typical, or up to 10 μm in the case of materials with low pressure stability. In the case of preparative separations and high loadings, the advantage of smaller particles is less important, as can be seen in Figure 5.3.

When smaller particles are used, the peak broadening caused by loading is clearly higher than with bigger ones. The selection of the particle size depends, of course, on the given separation and is normally a compromise between resolution, loadability, and the possible pressure and flow rate ranges. In the

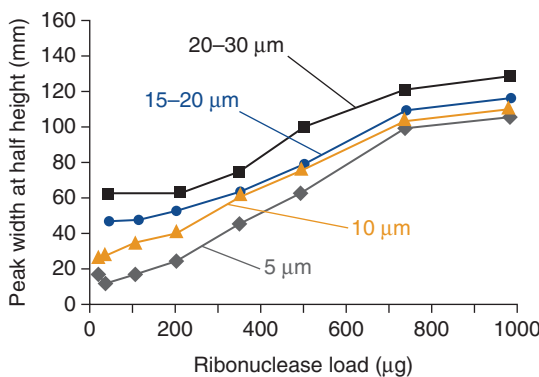


Figure 5.3 Protein loading capacity of RP-HPLC materials of different particle sizes. Source: *Protein and Peptide Analysis and Purification*, Vydac Reversed Phase Handbook, 5th edition, W.R. Grace, 2013.

preparative scale, 5 μm is only used if highest performance is needed, such as for the purification of synthesized peptide obtained from a synthesizer with impurities having only small differences to the target peptide. The consequence is that the loading is limited.

5.2.2.3 Pore Size and Surface

Biomolecules exist in a very broad size range. Therefore, it is very important to select the right pore size. Traditionally, the pore size is indicated in angstrom, $10 \text{ \AA} = 1 \text{ nm}$. Phases with smaller pores have a bigger surface and thus more possibilities for interaction. They can also have a higher loading:

60 $\text{\AA}$	$\sim 500 \text{ m}^2/\text{g}$
120 $\text{\AA}$	$\sim 300 \text{ m}^2/\text{g}$
200 $\text{\AA}$	$\sim 200 \text{ m}^2/\text{g}$
300 $\text{\AA}$	$\sim 120 \text{ m}^2/\text{g}$
1000 $\text{\AA}$	$\sim 30 \text{ m}^2/\text{g}$

Depending on the molecule weight the following pore sizes are suitable:

60 $\text{\AA}$	<1 000 MW
120 $\text{\AA}$	<10 000 MW
200 $\text{\AA}$	<50 000 MW
300 $\text{\AA}$	<100 000 MW
500 $\text{\AA}$	<250 000 MW
1 000 $\text{\AA}$	>250 000 MW

These values are only approximate, because the pore availability depends on the sample molecules topology and the pores themselves. The indication of the pore diameter always refers to the unbonded base material. Depending on the bonding, the available space in the pore is reduced.

It is important for chromatography to have a very tight pore size distribution to guarantee that the given surface is also available chromatographically. In the case of a broad pore size distribution, most of the surface is in the small pores, which are not available to bigger molecules. It is, therefore, possible that stationary phases with nominally bigger pores and thus a smaller surface nonetheless have more capacity, because the surface in the bigger pores is better available due to better accessibility.

The pore size and distribution are defined by nitrogen adsorption (BET) or mercury porosimetry. In chromatographic terms, inverse size-exclusion chromatography (SEC) is particularly suitable, because only those pores are measured that are chromatographically available.

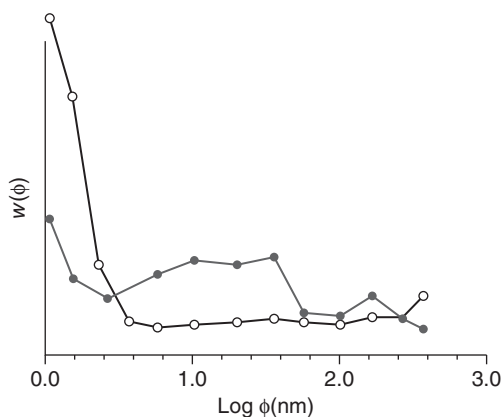


Figure 5.4 Inverse size-exclusion chromatography from two different polystyrene phases. Source: J. Maier-Rosenkranz, PhD thesis, Tübingen, 1992.

In Figure 5.4, two polystyrene phases are compared. W shows the respective share of the different pore diameters (Φ) in the total pore volume. One aspect typical of polymer materials is the high proportion of microporosity and a pore size distribution, which tends to be broad. In this example, the pore size distribution is particularly broad; especially for the phase indicated with $\bullet$ —a very high microporosity can be detected, and the proportion of bigger pores is evenly distributed over all sizes. Therefore, for bigger molecules only a very limited share of the surface is available. For RPC or IEC, a phase of this type would be unsuitable; for SEC, however, it would cover a broad molecular range with a good selectivity. The second phase, indicated with $\circ$ —, shows much less microporosity and a high proportion of pores with $\log \Phi$ from 0.8 to 1.8, which corresponds to a pore diameter of 60–600 Å.

Figure 5.5a,b depicts the separation of paracelsin, a peptide (20-mer) with four sequence analogs, which differ only slightly, on an RP silica and a PS/DVB phase. The separation on the silica-based phase can be realized in about a quarter of the time used for the separation on the PS/DVB phase. In this case, this big difference can largely be attributed to the tighter pore size distribution of the silica-based phase.

5.2.2.4 Loading Density

The loading density is an important information for the selection of the stationary phase. Depending on the functional group, it is indicated differently.

In the case of reverse phases, it is indicated as %C or in $\mu\text{mol}/\text{m}^2$, and in the case of ion exchangers in $\mu\text{eq}/\text{ml}$ or $\mu\text{eq}/\text{g}$. In addition to this, there is the dynamic binding capacity, which indicates the capacity for a determined protein for a given flow rate. The dynamic binding capacity depends, of course, on the type and size of the sample molecules, the flow rate and the pore accessibility.

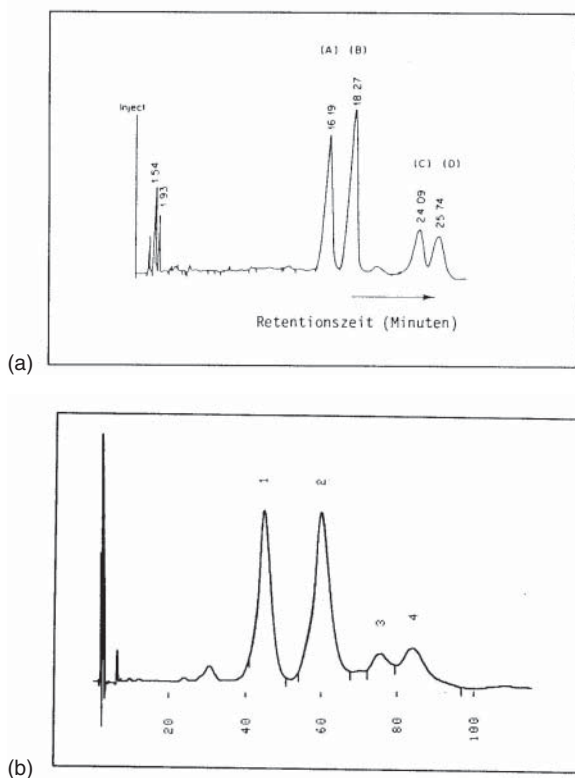


Figure 5.5 (a) Separation of paracelsin on a silica-based C8 phase. (b) Separation of paracelsin on a PS/DVB phase. Source: J. Maier-Rosenkranz, PhD thesis, Tübingen, 1992.

From a chromatographic point of view, it is the more important value, because it best reflects the real conditions. A high loading does not automatically mean a high capacity. If big molecules are adsorbed at the surface, accessibility to the neighboring groups is reduced, which means that these groups are not available. There are, therefore, also low-loaded stationary phases, because these can offer advantages over high-loaded ones.

5.2.2.5 Purity

Because biomolecules are multifunctional, they can of course interact with each other. Impurities in the base material can unintentionally interact with the sample, which can worsen the chromatography and even lead to a complete adsorption.

Normally, purity is specified as a percentage and should be greater than 99%.

Metals such as Al and Fe, which mainly exist in silica gels, should not exceed 10 ppm, or the surface coverage is so good that it is not important any more from the chromatographic point of view.

The main impurities are mostly by-products of polymerization. These can be both monomers or shorter polymer units and starters or emulsifiers.

5.2.2.6 Functional Group

The functional groups are described in the respective chapters on the chromatographic techniques.

5.3

Reversed-Phase Chromatography of Peptides and Proteins

RP chromatography is one of the standard methods for analyzing peptides and proteins. It stands out for a very good selectivity and performance.

One of the most common methods is the use of trifluoroacetic acid (TFA) in water against acetonitrile gradients. TFA has the advantages that it reduces the pH value, the carboxyl groups are protonized, and it forms an ion pair with the amino groups. Thus, the peptide or protein has no more free charges and, therefore, it is as hydrophobic as possible.

If none of the information that is important for chromatography is available, the starting point is normally a screening gradient of 5–95% 0.1% TFA against ACN 0.1% TFA. The following sections describe the different parameters for the development and adaptation of methods.

5.3.1

Retention Behavior of Peptides and Proteins

The retention of a small molecule is reduced when the ACN content increases. Roughly estimated: $+10\%B \sim 1/2 t_R$

The retention of a protein is suddenly reduced as soon as the critical point between adsorption and desorption is reached. This means that even changes of $\pm 1\%B$ decide if the protein has retention or if it passes through with t_0 . Figure 5.6 shows the various graphs from k to $\%B$. The bigger the peptide or protein the steeper these curves are, which means that it is more difficult to realize stable and reproducible retention times under isocratic conditions. Therefore, the gradient elution is preferred for peptides and proteins.

5.3.2

Gradient Design

As already described, the chromatography is normally started with a screening gradient between 5 and 95% 0.1% TFA to ACN 0.1% TFA. As a rule of thumb for the gradient duration, it takes 1 min/cm column length and, in the case of more complex samples, 2 min per column length. In a second step, the gradient is adapted such that the peaks are spread over the total time range. Usually, very good selectivities are achieved with this approach.

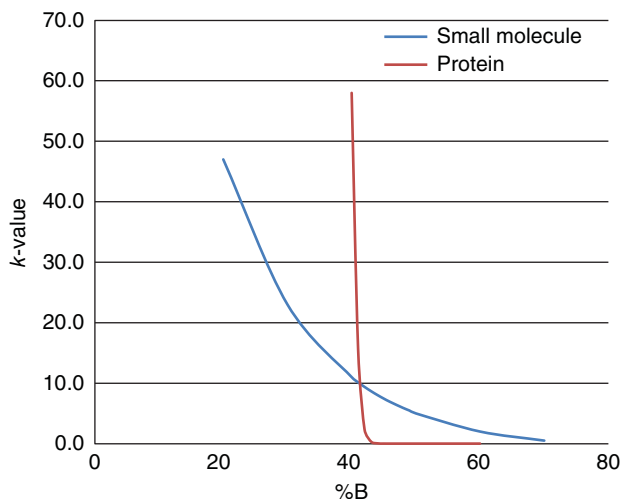


Figure 5.6 Influence of change %B on k .

The following gradient parameters influence separation selectivity:

Gradient slope: %/min

defined by the difference between the start and final concentrations as well as the time (gradient duration)

Gradient volume: ml

defined by flow rate and time

Reducing the gradient slope by increasing the gradient time is normally the fastest and easiest way to improve the resolution (Figure 5.7).

The second way to influence the resolution is to change the gradient volume. Traditionally, there are two ways to scale when changing to another column. In the case of linear upscaling/downscaling, the gradient duration is scaled at the same rate as the change in the column length:

column length halved = gradient times halved

In this case, the gradient volume is halved. The second way is to keep the gradient volume constant: time is adapted at the same rate as the column length changes and, at the same time, the flow rate is changed such that the same quantity of eluent is always flowing through the column:

column length halved = gradient times halved + flow rate doubled

Today, the constant gradient volume is mostly applied when changing from the traditional HPLC to UHPLC. If a column of 15 cm is changed to that of 5 cm, the gradient duration is reduced to 1/3 and the flow rate is tripled.

Both ways can lead to success. The result for method development is that, with a given column length and gradient duration, the variation of the flow rate is critically important. Because, as described in Section 5.3.1, different substances show

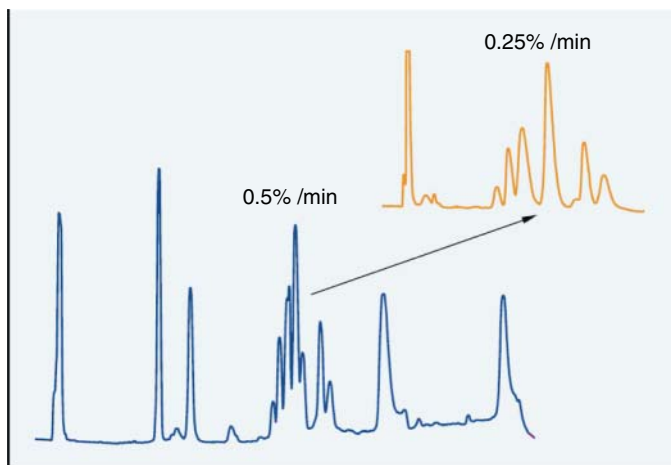


Figure 5.7 Influence of the gradient slope on resolution. Vydac 218TP C18, 4.6 × 150 mm, 10 μm; 25–50% ACN in 0.1% TFA ml/min. Source: *Protein and Peptide Analysis and Purification*, Vydac Reversed Phase Handbook, 5th edition, W.R. Grace, 2013.
Sample: Subunits of cytochrome c oxidase.

different behaviors when the organic modifier (%B) is increased, the k -values also show different behaviors when the flow rate is increase or reduced. This can also lead to a change in the peak sequence. Therefore, varying the flow rate by $\pm 50\%$ as a minimum is recommended. It must, however, be taken into consideration that, depending on the pore size, access to the pores is more difficult with high flow rates and big molecules and that, therefore, the Van Deemter curve strongly increases.

5.3.3

Organic Modifier

Acetonitrile is undoubtedly the most used organic modifier for analytic and preparative (laboratory scale) separations. The reasons for this are its good efficiency, low viscosity and low UV adsorption at wavelengths of approximately 210 nm.

Alcohols such as methanol, ethanol, and isopropyl alcohol are not as popular because of their high viscosity and the high back pressure resulting from it. Yet, these protic solvents strongly influence selectivity, especially of polar peptides or proteins on polar RP phases (C8, C4, CN, phenyl, diol). For the method development, it is, therefore, recommended to use and to vary mixtures of acetonitrile and alcohol as organic modifiers. This is often more efficient and faster than changing the stationary phase.

Ethanol and isopropyl alcohol are often used at a process scale, because these are cost-effective, and they can more easily be applied in the regulatory environment. It is also easier to keep up biological activity in comparison to acetonitrile,

which has a stronger denaturing effect. The use of isopropyl alcohol is, furthermore, favorable when hydrophobic peptides or proteins are separated, which can also increase recovery.

5.3.4

Ion Pair Reagent

TFA is the most common ion-pair reagent. The low pK_s value (0.8) protonizes the carboxyl groups and, therefore, retention is higher than with formic acid or acetic acid, with pK_s values of 3.75 or 4.76. The typical concentration is 0.1%. An increase in the ion-pair concentration results in the peptides and proteins tending to have a higher retention. Because this increase is different for the respective peptides or proteins, concentration is an important parameter for the method development.

With basic peptides and proteins, in particular, heptafluorobutyric acid (HFBA) can be used alternatively for TFA, which has the advantage of more sharper peaks. Another advantage of these two ion-pair reagents is their high UV transmittance at short wavelengths. With LC–MS applications TFA and HFBA are less often applied, because both cause a strong ion suppression. Good results are, however, achieved by combining ion-pair reagents with acids. To achieve sufficient capacity, formic acid is used in higher concentrations together with very low concentrations of HFBA or TFA to get the low pK_s value (see Figure 5.8).

Theoretically, other acids, such as phosphoric acid or hydrochloric acid, can also be used, because these can also positively influence selectivity. The reason for their rare use is, in the case of hydrochloric acid, its high potential for corrosion, and phosphates are not volatile, which is an exclusion criterion in the case of MS or preparative applications.

In the neutral pH range, ammonium formate, ammonium acetate, and triethylamine phosphate and in the alkaline range ammonia, trimethylamine, and triethylamine are used.

Columns that are to be used with ion-pair reagents should be reserved for this application only. Otherwise, there is a very high risk of a change in selectivity and associated with this, bad reproducibility.

5.3.5

Influence of the pH Value

Amino acids, as components of peptides and proteins, are zwitterions, that is, they have both positive ($-NHR_2^+$) and negative charges ($-COO^-$) depending on the solutions pH value. If the amino acid has the same number of positive and negative charges, namely at the pI value, it is neutral. Below the pI value, the peptides are cations and above it they are anions. The pI value of acidic amino acids is approximately 3, that of the polar, non-polar, and aromatic amino acids is 5–6.5 and that of the basic ones is greater than 7 (see Table 5.1).

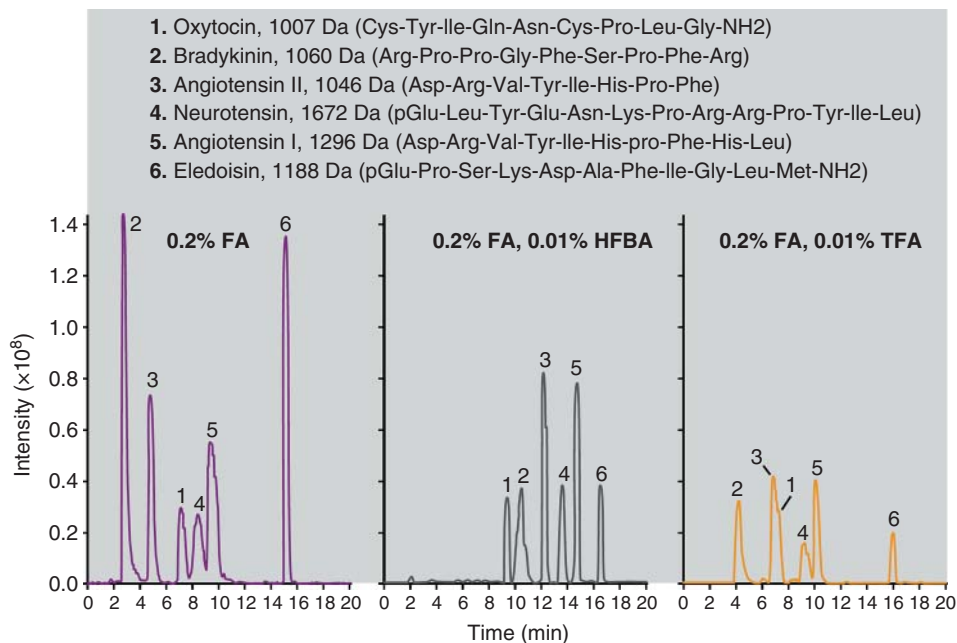


Figure 5.8 Effect of the combination of ion-pair reagents. LC–MS TIC of peptides. Column: Vydac® Everest® 238 EV, 300 Å, C18, 5 μ m 150 $\times$ 1 mm. Mobile phase: A: 95:5% water:ACN with ion-pair reagents. B: 20:80% water:ACN with ion pair reagents.

Flow: 50 μ l/min, gradient: 12.5–50% B in 30 min. Source: *Protein and Peptide Analysis and Purification*, Vydac Reversed Phase Handbook, 5th edition, W.R. Grace, 2013.

Depending on the peptide or protein structure, the number and distribution of the charges, and thus the molecules polarity, change with the pH value. This obviously has a direct influence on retention and selectivity (see Figure 5.9).

In the acid pH range, acid peptides have less charge and thus a higher retention; this is true for basic peptides in the basic range. During method development, the pH value is one of the dominant factors and if, in the acid pH range, the standard method with TFA or HFBA has no success, it is a good way to regulate the necessary selectivity via the pH value.

5.3.6

Pore Size

Section 5.2.2.3 already described the pore size influence in combination with stationary phases. Therefore, only the particularly RP-relevant points are specified here. Normally, stationary phases between 100 and 300 Å are used for peptides. The bigger pores, in particular, have the advantage of better accessibility. This is not only an advantage during chromatography, but also during the bonding. Due

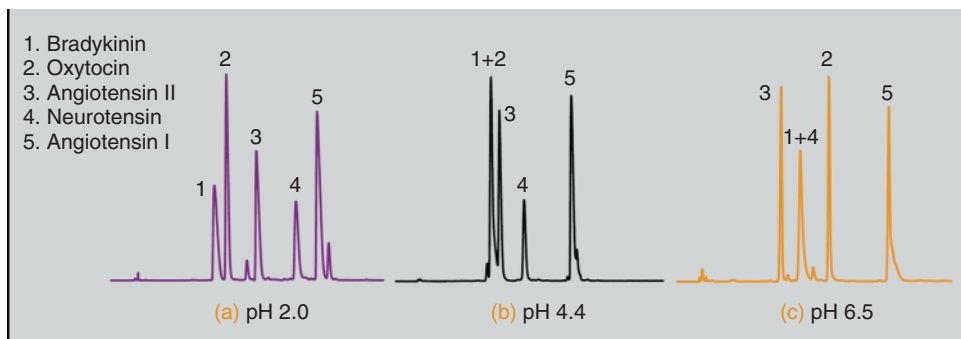


Figure 5.9 Separation of a peptide mixture at different pH values. Column Vydac® 218TP, 300 Å, C18, 4.6 × 250 mm, 5 μm. Mobile phase/gradient: 15–30% ACN in 20 mM phosphate, (a) pH 2.0, (b) pH 4.4, (c) pH 6.5. (1 ml/min). Sample: 1. bradykinin,

2. oxytocin, 3. angiotensin II, 4. neurotensin, 5. angiotensin I. Source: *Protein and Peptide Analysis and Purification*, Vydac Reversed Phase Handbook, 5th edition, W.R. Grace, 2013.

to the fact that alkyl silanes get better into the pores, materials with bigger pores often have a better surface covering and thus fewer unwanted interactions with the base material. In the analytic separation, this can, for example, be seen from the fact that the peak form is better or that low-concentration impurities can be seen and not adsorbed completely on the surface.

5.3.7

Bonding Chemistry

Usually, C18, C8, C4, phenyl and CN phases, or even polystyrenes are used for the RPC of peptides. The bonding used depends on the polarity, size, and higher structure of the peptides or proteins. Polar phases (C8, C4, phenyl, CN) tend to be used for polar compounds, because they have a higher selectivity in the polar range. C18 is usually used for lipophilic compounds, because it has the best solubility thanks to its high amount of ACN that is needed for elution. If the lipophilic peptide or protein sticks to the surface, polar phases are used too. In this case, it must be ensured that, when the amount of water increases, the peptide or protein is still soluble. In the case of bigger peptides or proteins, shorter chains (C4) are advantageous, because then the accessibility to the pores is increased.

5.4

IEC Chromatography of Peptides and Proteins

There are two types of ion-exchange chromatography (IEC): cation chromatography, that is, the separation of cations, which is used for proteins/peptides at a pH

value below their isoelectric point, and anion chromatography, that is, the separation of anions, which is used for proteins/peptides at a pH value higher than their isoelectric point or for oligonucleotides or polynucleotides.

The respective stationary phases are as follows:

Strong cation exchanger (SCX)	
S (methylsulfonate)	$-\text{CH}_2\text{SO}_3^-$
Sulfopropyl (SP)	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$
Weak cation exchanger (WCX)	
Carboxymethyl (CM)	$-\text{OCH}_2\text{COO}^-$
Strong anion exchanger (SAX)	
Quaternary ammonia (Q)	$-\text{NR}_3^+$
Weak anion exchanger (WAX)	
Diethylaminoethyl (DEAE)	$-\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_2\text{CH}_3)_2$
Diethylaminopropyl (ANX)	$-\text{CH}_2\text{CHOHCH}_2\text{N}^+\text{H}(\text{CH}_2\text{CH}_3)_2$
Quaternary aminoethyl (QAE)	$-\text{OCH}_2\text{CH}_2\text{N}^+(\text{C}_2\text{H}_5)_2\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$

In this relation, “strong” or “weak” does not mean the interaction strength, but the change in capacity of the ion exchanger depending on the pH value. Strong exchangers are available over a wide range with a constant capacity. As regards weak exchangers, the capacity changes in correlation with the pH value.

With IEC, the column is loaded at a low salt concentration and when the salt concentration increases, it elutes (see Figure 5.10).

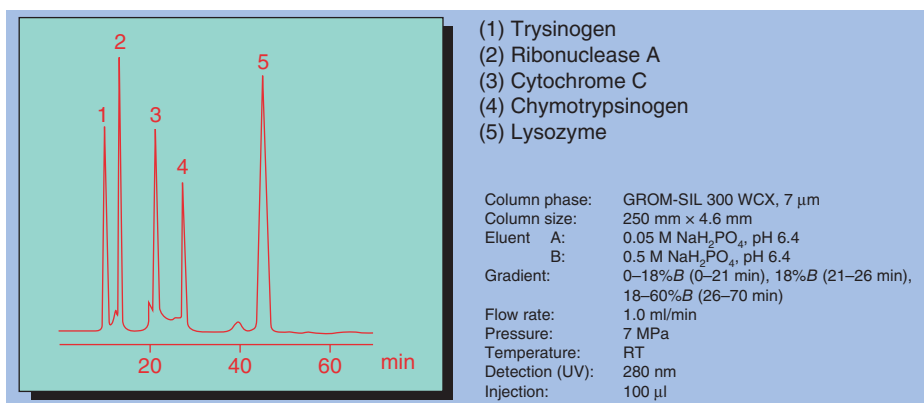


Figure 5.10 Example of ion-exchange chromatography. Source: Grom Analytik + HPLC GmbH, Application 09105; 2004.

5.4.1

IEC Parameters

5.4.1.1 Ionic Strength of the Sample

Proteins and peptides with a higher ionic strength have a higher retention. The ionic strength is, above all, defined by the number of charges.

5.4.1.2 Buffer Concentration of the Eluent

An increase in the buffer concentration results in shorter retention times. Usually, gradient separation is preferred. Resolution is usually set via the gradient slope. Flatter gradients increase resolution. In Figure 5.10, for example, an isocratic step is used to separate the cytochrome from the chymotrypsinogen.

5.4.1.3 pH Value

The pH value influences not only the samples charge conditions, which influence the selectivity strongly, but also those of the exchanger. At very low pH values, the acid group of cation exchangers is protonized and is thus not available for chromatography. If the pH value is higher, the retention of SCX and WCX increases, because more loadings are available on the ion exchanger.

The behavior of the anion exchanger is exactly the opposite. If the pH values are higher, SAX and WAX have a shorter retention.

Instead of a salt concentration gradient, a pH gradient can also be used. In this case, eluting conditions are if the pH value moves toward the samples pI value – there is no more retention at the pI value – or, as explained above, the charge number on the exchanger is reduced.

This variant can be applied for LC–MS, because this avoids large quantities of salt, which would significantly reduce the sensitivity of the analytic of peptides and proteins.

5.4.1.4 Organic Modifier

To improve solubility or to suppress unwanted interactions, it is possible to add an organic solvent, similar to RP chromatography. If ACN or alcohols are added, retention is reduced. For ESI-LC–MS, particularly, this is an advantage, because the mobile phase can be sprayed more easily with the organic solvent, which allows the salt concentration to be kept low. It can also be a good idea to use a gradient. The use of a gradient supports elution and selectivity.

5.4.1.5 Temperature

An increase in temperature usually improves kinetics, which causes the peaks to be narrower. The temperature also influences the pH value and the samples interactions. This also changes selectivity.

5.4.1.6 Flow Rate

Normally, the flow rate is slightly smaller than with RP-HPLC: 0.5–0.8 ml/min for a column of 4.6 mm. This corresponds to a linear flow rate between 4.3 and 6.9 cm/min. A good indication for a suitable flow rate is the dynamic capacity, which is usually specified in the technical data sheet. In the case of very big molecules, a low flow rate can be advantageous.

5.4.1.7 Pore Size

As already described in Section 5.2.2.3, the pore size selected defines the available surface.

5.4.1.8 Loading and Injection Volume

Because of the strong interactions, IEC can focus big injection volumes at the column head very well. It is also possible to highly load ion exchangers. Loading depends on many parameters (all the parameters described above), but it is particularly dependent on the size of the sample molecules and the static and dynamic capacity.

IEC is a suitable complement or alternative to RP chromatography. The elution and selectivity can be adjusted in very different ways. Figure 5.11 shows a

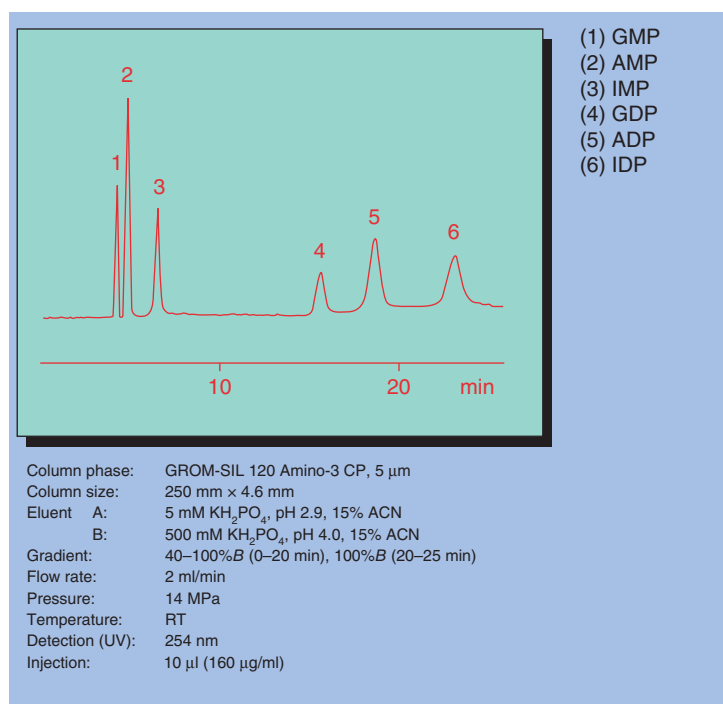


Figure 5.11 IEC of nucleotides using salt-, pH- and organic solvent-gradient Source: Grom Analytik + HPLC GmbH, Application 07127; 2004.

combination of salt concentration gradient, pH gradient and the addition of an organic modifier.

First, the monophosphates elute, then the diphosphates follow much later, because of the double charge.

IEC can even be used in LC–MS if the traditional salt gradient is replaced or reduced by changing the pH value and the concentration of the organic modifier. This broadens the field of application even further.

5.5

Size-Exclusion Chromatography of Peptides and Proteins

SEC is a special form of chromatography, which differs in the fact that there are (ideally) no interactions with the stationary phase.

The principle of separation is based on the different pore accessibilities of different molecule sizes. Small molecules can penetrate all pores and therefore, have a longer way, very big molecules are completely excluded and thus elute first (see Figure 5.12).

Separation is, therefore, based on the different available volumes. In the following, the volume types for a column of 300×8 mm are specified.

Column volume	15 ml	100%
Interstitial volume: V_i	~ 4 ml	$\sim 30\%$
Pore volume: V_p	~ 7 ml	$\sim 45\%$
Volume of the mobile phase (dead volume): V_0	~ 11 ml	$\sim 75\%$
Volume of the stationary phase	~ 4 ml	$\sim 25\%$
Volume available for separation $V_0 - V_i = V_p \sim 7$ ml		

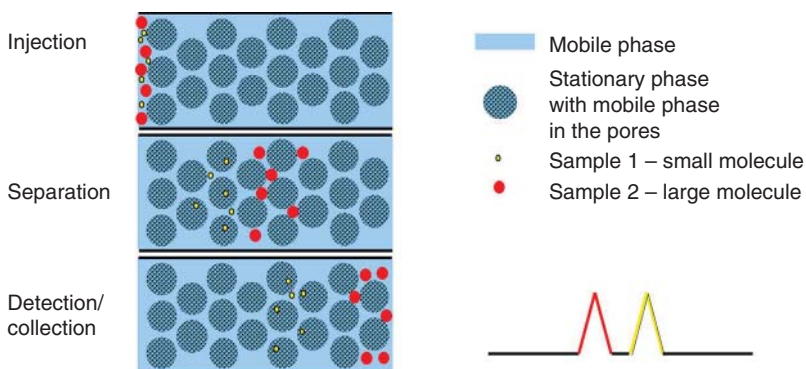


Figure 5.12 Principle of size-exclusion chromatography.

At a flow rate of 0.5 ml/min, the time up to the first peak (completely excluded) would be at approximately 8 min and for the last peak (completely penetrating, t_0) at approximately 22 min.

In the case of SEC, the chromatogram finishes – at t_0 – at the point when separation starts with other chromatography techniques.

At first sight, the mobile phase thus does not influence separation either, because, theoretically, there are no interactions. In reality, however, unintended interactions take place, which must be prevented with the mobile phase.

Ionic interactions	With a high salt concentration, pH value
RP interactions	With an organic modifier
Solubility	With an organic modifier, pH value

In Figure 5.13, a typical SEC separation can be seen. The phosphate buffer adjusts the pH value, the sodium chloride produces the high salt concentration, and the ACN acts as an organic modifier.

With this type of separation, the possible selectivity can also be assessed. Peptides or proteins, whose molecular weight differs by approximately a factor of 2,

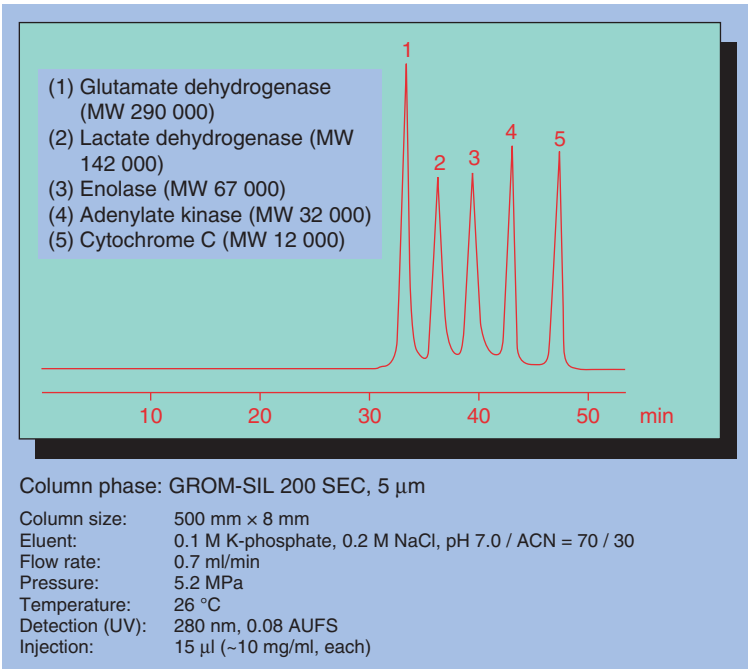


Figure 5.13 SEC separation. Source: Grom Analytik + HPLC GmbH, Application 09046b; 2004.

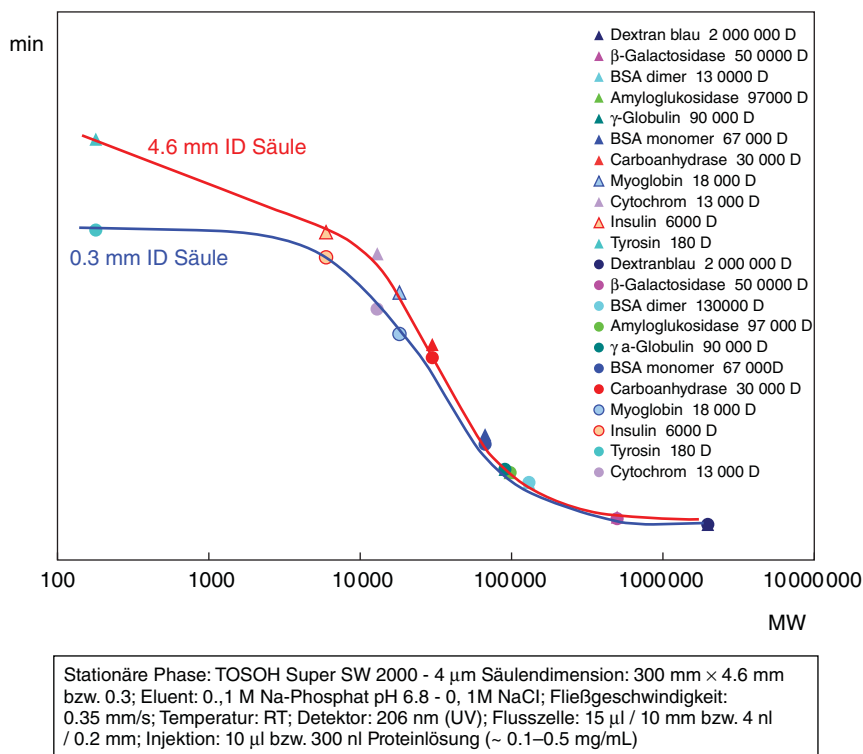


Figure 5.14 SEC curve. Source: Application Note No 005, Grom Analytik + HPLC GmbH, 2003.

can easily be separated. The retention time/molecular weight graph shows the range in which different molecular weight gives the change of the elution time. In Figure 5.14, this range goes from 6000 D (insulin) to 500 000 D (β -galactosidase). The field of application for the stationary phase depends on the pore size.

5.5.1

SEC Parameters

SECs performance is influenced by the following parameters:

5.5.1.1 Particle Size

The particles should be as small as possible to get short diffusion paths and narrow peaks. The disadvantage of small particles is the increased back pressure, which can cause problems depending on base materials such as acrylate or polystyrene.

5.5.1.2 Pore Size Distribution

A narrow pore size distribution causes a good separation for a small MW range of peptides and proteins. Broad pore size distribution allows separations over a

broad MW range with lower selectivity. This is usually balanced out with longer columns.

5.5.1.3 Pore Volume

The pore volume defines the possible run time/elution volume of the separation. The higher it is, the higher the peak capacity is and the chance of a good separation.

5.5.1.4 Flow Rate

Lower flow rates are showing higher plate numbers. Low flow rates allow particularly bigger molecules to diffuse better into the pores. In the case of high flow rates, these are, as a tendency, shifted toward lower elution volumes.

5.5.1.5 Temperature

A higher temperature reduces viscosity and, therefore, allows a better diffusion. Efficiency increases. Proteins, however, are temperature sensitive and, therefore, the temperature increase is limited.

5.5.1.6 Viscosity

A lower viscosity increases efficiency. Viscosity can be influenced by the type of salt, the salt concentration, and the organic modifier.

5.5.1.7 Loading and Injection Volume

Both of these should be as low as possible. As SEC has no retention, both parameters directly affect the peak broadening.

5.6

Further Types of Chromatography – Brief Descriptions

In addition to the types of chromatography described earlier in detail, three other types can be used for biochromatography.

5.6.1

Hydrophobic Interaction Chromatography

With hydrophobic interaction chromatography (HIC), hydrophobic proteins interact with slightly hydrophobic surfaces (propyl, ether, phenyl) at high salt concentrations. For elution, a salt gradient from high to low salt concentrations is used (see Figure 5.15).

5.6.2

Hydrophilic Interaction Chromatography

Hydrophilic interaction chromatography (HILIC) is particularly used for strongly polar compounds, that is, if these have a low retention at RP chromatography or

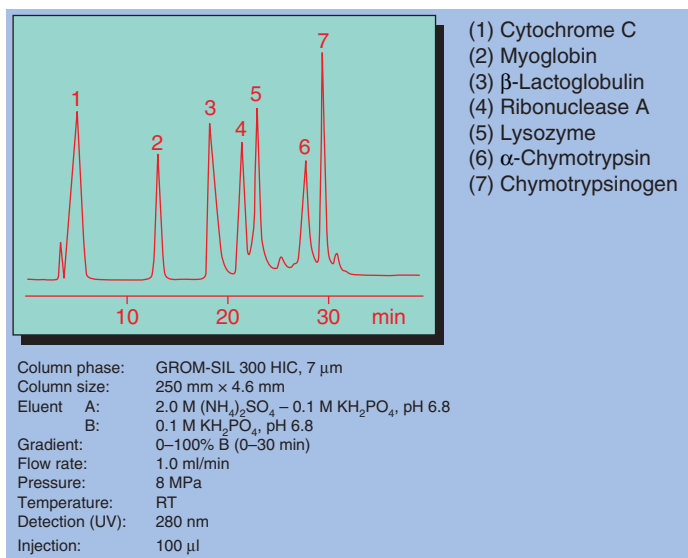


Figure 5.15 Example of an HIC protein separation. Source: Grom Analytik + HPLC GmbH, Application 09106; 2004.

if the peptides to be separated mainly differ in the polar groups. It is often applied for separating different degrees of glycosylation of a peptide.

The HILIC functional principle can be described as follows. On polar phases, polar molecules have retention when solvents that are more nonpolar than the stationary phase and that can be mixed with water are used. A layer of water develops on the phases surface. The polar sample spreads between this layer of water and the mobile phase.

Amino, diol, cyano, SAX, SCX, Si, and a series of special polar groups are used as stationary phases. Acetonitrile/water is usually used as a mobile phase, but methanol, ethanol, isopropyl alcohol, or a mixture of them are also common. If the amount of acetonitrile is increased, retention increases. If water is added, retention is reduced. The addition of acids, bases, and buffers is possible and is used for method development (Figure 5.16).

5.6.3

Affinity Chromatography (AC)

With affinity chromatography (AC), the biospecific interactions between, for example, a protein and the matching antibody are used to separate the protein or the antibody. This separation is based on a specific and reversible interaction between the molecules. A typical application is the purification of monoclonal antibodies. Under the given conditions, they bond to a stationary phase that is covered with the protein A. After a washing step, the conditions are changed (often change in the pH value) such that the antibody elutes.

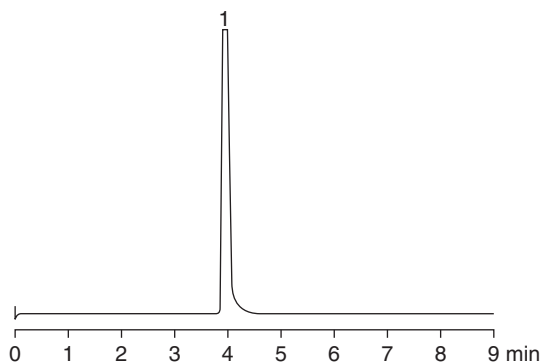


Figure 5.16 Chromatogram of bradykinin in the HILIC mode. Column: Alltima HP HILIC, 5 μ , 150 $\times$ 4.6 mm. Mobile phase: (70:30) acetonitrile: 10 mM ammonium acetate w/0.2% acetic acid; flow rate: 0.75 ml/min. Detector: ELSD. Source: Alltech Application Data Base, W.R. Grace; 2007.

AC is a highly selective separation method, which finds widespread application in research and production.

5.7

Summary

Both classical chromatographic areas, small molecules and biochromatography, comes more and more together today, especially with the goal to increase the performance of the separations. This development is speeded up through the increased number of biomolecules in the pharmaceutical development with the need of better HPLC for analysis and purification. This is also visible due to the fact that companies that are offering fused core materials, which have an increased market share, also offered quickly materials with a bigger pore size, which are also suitable for peptides. Also the high performance of the UHPLC systems and the increased peak capacity in combination with fast and high-resolution mass spectrometers will drive this progress.

6

Comparison of Modern Chromatographic Data Systems

Arno Simon

6.1

Introduction

The field of modern chromatography data systems (hereinafter CDS) now extends from the single-user solution for separate measuring systems to global network solutions in which laboratories from different countries and across continents work together. Hardly any other area in the analytical laboratory has changed so significantly as the laboratory data processing. The developments and the differences between these systems are presented here as well as the attempt is made to give an outlook on the next developments and trends.

The range of application for modern CDS runs today from single workplaces of separated analytical systems to global network solutions, where laboratories of different countries and continents collaborate in the same infrastructure. It can be assumed that no other area in the analytical laboratory has changed to such a significant extent as the management of laboratory data. This chapter presents the development and differences of these systems and tries to give a look-out to the next development steps and trends for the future.

6.2

The Forerunners for CDS

The triumphal procession of CDS started 30 years ago with the enhancements of the integrator. Based on contemporary computer platforms, such as digital VAX, a DEC professional or an HP1000, several manufacturers for HPLC and GC instruments have developed integration software programs for the control of their instruments and the evaluation of their chromatograms. All of them had a restricted scope of operation, compared with current standards, and were extremely cost-intensive. First, the IBM PC and the following clone-PCs were affordable for a conventional laboratory and have, therefore, been the cause for more and more manufacturer to invest into this sector of industry. With the

IBM PC entering the laboratories, the environment for the operational computer system has changed from UNIX-based central computers to MS-DOS and later on Windows-based office clients.

Over the years, the scope for software systems as a kind of an alternative integrator for the evaluation of peaks and quantification with limited tuning options has changed to complex solutions that were able to control many different instruments and to manage laboratory samples.

6.3

CDS Today

The first and perhaps most important difference of current CDS is how data storage is organized. Until 1992, when Waters has introduced the *Millennium*²⁰¹⁰ software, all established solutions were based on file-based data storage, as it was known for MS office programs. But *Waters Millennium*²⁰¹⁰ has used an integrated database for storing analytical data. While file-based systems save all information such as sample name in the data name of the data file and the instrument method including all settings in a method file, *Millennium*²⁰¹⁰ stores these data in a database. By this solution, new possibilities for the management of analytical data were given. Especially, method information has been saved again and again together with each new data file in order to provide traceability. With a database solution, all information and results are stored associated in a database having a link to the corresponding injections.

A database solution offers the possibility to easily search for any information demanded by the user. For example, the changes of retention times for a defined compound that should be considered over a longer period can be depicted in *Millennium* (a later on in *Empower*) by simple database query, where each first injection of a standard solution over the past 6 months is considered.

More and more manufacturers are following this idea and equip their CDS more or less halfhearted with a database. In many cases, only the administration of raw data files has been moved to a database, whereas the actual meta data (sample information, measuring data and results) are still stored as files.

Currently, all modern CDS have an integrated database. But file-based systems do still have advantages today.

6.4

Advantages and Disadvantages of File-Based CDS

File-based CDS store all information in single data files. This way, in most cases, a method file, which contains measuring and evaluation settings, and sequence file, defining the sample order of a measuring set, are stored together with the actual raw data files. These files are structured by a typical folder structure on the acquisition computer or on a network drive. For most users, this system gives the best

transparency of the software, as they are familiar with this structure, when having experience with Word or Excel. Anyway, this does not mean that the usability for this kind of CDS products does benefit from this approach!

The biggest problem of this technology is the missing possibility for administering several versions of a data file in the same data system. Actually, this means that, for example, a modified method replaces the previous version when saved under the same name. The only solution to keep previous versions is to rename the method or to store it several times in different directories. Both practices can become rather unclear and there is a risk of mixing them up.

With the increasing demands of laboratories in the regulated environment, further problems are faced: data accessibility needs to be controlled and traceability for data generation and modification needs to be given. But a Windows file system does not offer solutions to a sufficient extent. Indeed, access rights can be assigned to folders and files. However, the implementation is too little sophisticated, the structure is uncomfortable, and administration is difficult.

6.5

Advantages and Disadvantages of Database-Supported CDS

Database-supported systems do not know the above-mentioned limitations. The application itself is organizing the data and stores all significant information in a database. Raw data files themselves are not stored in the database. Especially, LC–MS and GC–MS couplings that are used more and more frequently in the laboratory produce an enormous amount of data volume. Therefore, raw data of modern systems are stored in file systems, as well. Anyway, the database is still assigning the names to these raw data files and takes care of their organization. Sample information such as sample name, measuring instrument and instrument method are saved together with all data file information (which often has a generic name, e.g., D04711) in the database, whereas the data file itself is stored separately.

The idea of separating database and data files offers the chance of scaling up a CDS installation from a single PC, on which database and file server are installed together, up to global solutions, where database server and file server are installed on different physical devices.

A further advantage of this technology is the simplified administration. Usually, data access is provided by the application itself and not by user definition. Sophisticated access rights on the data level are not necessary; the application performs the access control. However, the mentioned benefits of a database-supported CDS are paid with some restrictions. For example, data backup and archiving of file-based systems is rather simple. Complete directories can be moved or burned on a DVD. Data that are required again, can be read directly from the storage medium.

In contrast, the backup of a database or of single data is much more involved. A running database represents a kind of an open data file that cannot simply be copied on a backup medium. Backup conceptions are, therefore, often implemented by occasional “cold backups”, performed perhaps on a monthly base. For

those cold backups, the database needs to be stopped. In addition, daily “hot backups” are performed, where all modifications since the last backup are stored. In the case of a roll-back, first the latest “cold-backup” needs to be retrieved followed by all “hot-backups” generated after this. This can take a while.

Especially, for installations of a manageable size, the virtualization offers new chances.

On a virtualized host, the hardware of a CDS server is emulated. This virtualized CDS server runs as application on that host, what offers several advantages:

- Servers use the computing capacity only to a little extent. As the hardware of a virtualized host is used by several virtual servers, the hardware capacity can be utilized more efficiently.
- With increasing performance requirements for a CDS server, for example, by an increasing number of registered users, more storage or processor capacity can be assigned to the virtual machine without the need of adjusting the CDS installation.
- The possibility to retain the current status of the CDS server by a so-called “snapshot” permits to save the systems before the installation of patches and updates, what could be very comfortable for test systems.
- The backup of a running database server is more difficult due to the high number of open data. Therefore, the database needs to be stopped, before a backup can be performed. Operation of the CDS is suspended during this time. As a virtual server actually depicts a single data file, data backup programs are available on the market that can even save running databases. The operation of the CDS does not need to be suspended.

6.6

CDS in a Network Environment

More and more companies are integrating their CDS in the companies’ own network environment. Provided that the CDS supports network capability, many advantages are offered:

- Access to system and data is given from any PC in the network.
- Users can, for example, prepare and start their analyses in the laboratory, whereas the evaluation can take place in the quiet office room.
- Data are stored at a central location, which simplifies data backup.

A file-based network environment mostly consists of single workstations, which use a common storage for data files in the network. The whole software functionality is granted to every workplace.

In contrast, a database supported system has the database server as a central node in the network. Here, data acquisition clients are connected to for controlling analytical instruments and acquiring data, which then will transfer the data to the server. The user works at a client PC, where the evaluation part of the CDS software is installed.

On a data acquisition client, there is no other software installed except the CDS. As the user will never work actively on such a client and, for example, using the internet, there is no risk caused by malware or incompatibilities. The separation of instrument control and data acquisition on the one hand and the workplace clients on the other hand is an important factor for the stability of such systems. This way it is guaranteed that instrument control and data acquisition work reliably and no measuring data become lost, even if a problem occurs in the network or on the server.

Where the CDS is used for monitoring and operating production processes, the availability of the system is of high importance. Network interruptions or downtimes of a database server can potentially tie up the complete fabrication. The elaboration of an emergency concept is, therefore, of enormous significance. Although research laboratories can easily come to terms with an outage of, for example, 1 day, even a few hours downtime can be a big problem for in-process control laboratories. Therefore, conceptions that correspond to the individual requirements need to be established and tested. Often these concepts are not easy to develop, as the availability of a database server of a database-supported CDS is an essential precondition.

ThermoSCIENTIFIC (Dionex) is a CDS manufacturer that has presented an interesting solution on this with *Chromeleon 7*. The system copies the part of the central database that is relevant for an analytical instrument on the data acquisition client. In the case where the connection to the database gets lost, work can be continued with this local copy of the database. Once the database connection has been reestablished, the contents of the local copy and the central database will be synchronized.

6.7

Instrument Control

Originally, all CDS were control and evaluation systems of one instrument manufacturer. The aim was to provide a better and more convenient control of the in-house HPLC and GC instruments. As the solutions were always focused on the own instrument hardware, almost every manufacturer uses his own system to connect instruments to the computer hardware. Besides standardized formats such as IEEE488 Bus (*Waters & Agilent*), there can be found serial interfaces, later on often USB ports or proprietary connections. In correlation with standardized communication protocols, this can lead to incompatibilities, why some laboratories have decided to use several CDS each having its particular data system. In order to decrease the effort that arises from training laboratory staff and multiple installations, maintenances, and software validations, companies aim to decrease the number of CDS in their laboratories to a minimum. Having the focus on one CDS helps to define a unique workflow and ensures a consistent documentation of the processes.

For reaching this aim without being forced to replace a multiplicity of analytical instruments, the number of different instruments that can be controlled by a CDS has become a decisive aspect. No CDS manufacturer can get away with only being able to control his own instruments.

When surveying the currently available CDS products with regard to their capability of controlling different instruments, the following can be observed:

- Manufacturers, where the instruments have a big market share, have started lately to integrate external instruments. For them, the present instruments where the key to place their software in the laboratories.
- Manufacturer with a small market share must be able to control various instruments in order to get a chance of placing their software. Formerly, the control of the competitors' instruments has been realized by reading the data bus without the permission of the manufacturer. Today, the control codes are often exchanged between single suppliers.
- Manufacturers that do not have own chromatography instruments give licenses for their CDS to manufacturer of instruments without an own software; the CDS integration can be programmed by themselves. This kind of integration is in most cases very successful.

6.8

Documentation and Compliance

With the entrance of the information technology, the US Food and Drug Administration (FDA) has increased the demands for documentation. Especially, GxP areas in pharmaceutical industries, but also suppliers of the chemical industries are concerned. Information that formerly simply was filed in paper format is today a matter of strong obligations regarding traceability and secure data storage. This results in two basic requirements:

- All data and the ways of their generation need to be traceable.
- Electronic data must sometimes be archived for long periods during which their readability must be ensured.

A high degree of traceability has already been reached by optimized control options for chromatography instruments.

Besides the chromatogram, the instrument method is stored as well. In addition, the single work steps of the user, from the start of a measurement to the final result, must be stored in a traceable manner. In this aspect, database-supported CDS offer high benefits. Relations between single information can simply be depicted in a database, whereas file-based systems are charged with a redundant amount of information.

However, regarding the demand of archiving data, file-based systems offer one of the few advantages: As all data are kept in simple folder structures, those can

easily be transferred to corresponding longtime storage media and placed into stock.

From a database, where all information is linked to each other, single data cannot be removed readily and stored somewhere outside the database. For archiving, there is either a corresponding structure required or the CDS already offers special functionalities that make archiving possible.

6.9

Brief Overview of Current Systems

It seems to be impossible to provide an entire overview of all CDS that are currently on the market. Every now and then, some trade magazines are publishing overviews where the different functionalities are compared. But these comparisons do not really offer an objective view. Almost all systems have an audit trail function today, but the functional scope differs a lot between the single systems. Knowing that it is impossible to offer a real objective comparison, the most common systems should be presented from a subjective point of view. Besides some information about the CDS itself, the mentioned advantages and disadvantages reflect the ratings and perceptions of different users. Anyway, everybody is free to make his own appraisal.

6.9.1

Atlas

For many years, the standard CDS of *ThermoSCIENTIFIC* has been *Atlas*. With the acquisition of *Dionex*, the more modern and more powerful software, *Chromeleon* has become a product of *ThermoSCIENTIFIC*. Therefore, the decision was taken to replace *Atlas* by *Chromeleon* during the next years. Before this, the control of additional *ThermoSCIENTIFIC* instruments and the support of MS need to be implemented in *Chromeleon*. With *Chromeleon 7.2* that was announced for the middle of 2013, this implementation should be completed.

6.9.2

ChemStation

Agilent ChemStation is a well-proven and a popular software product. Over the years, the software has achieved a high degree of distribution, particularly at universities and public institutions. The software design of *ChemStation* has become out-of-date over the years. That's why *Agilent* has started a redesign, calling the software now *OpenLAB*.

Strengths: reasonably priced, easy to use.

Weaknesses: low performance, file-based software architecture.

6.9.3

Agilent OpenLAB CDS

In 2010, *Agilent* started launching *Agilent OpenLAB CDS*. *Agilent* talks about an evolution of its software instead of calling it a new CDS. Two versions are currently available on the market: the *ChemStation* and the *EZchrom* edition. Both of them use the same control panel module and the same report generator. All other software parts are adjusted to the customs of *ChemStation* and *EZchrom*, respectively.

This approach is less spectacular than the introduction of a new product. Anyway, a soft and less extensive changeover is possible.

Strengths: modern network technology, large integration into the *OpenLAB* family, for example, direct search in *OpenLAB ECM* from the CDS.

6.9.4

Chromeleon

Particularly, in Europe, *Chromeleon* could take root as a significant and great CDS. With version 7.0 introduced in 2010 and 7.1 released in the beginning of 2011, a fully new product has been developed. Strengths of the previous version 6.8 have been kept, and weaknesses have been eliminated forcefully.

Strengths: easy to use, support of the most common HPLC and GC instruments, database-supported architecture.

Weaknesses: MS support is not supported yet (planned with the introduction of version 7.2).

6.9.5

Empower

With *Empower* and its parent product *Millennium*²⁰¹⁰, *Waters* is the pioneer for database-supported CDS. With the current version *Empower3*, the final stage of advancements is supposed to be reached. Currently, *Waters* is developing a successor called *UNIFI* that follows a new conception and a redevelopment from the scratch: the combination of CDS, Electronic Lab Notebook (ELN), and Scientific Data Management System (SDMS).

Weaknesses: not easy to learn, support of controllable instruments could be extended. Software has reached the final stage of its life cycle.

6.9.6

EZchrom

Agilent has obtained the *EZchrom* software with the acquisition of *Scientific Software*. Over the years, *EZchrom* has become the core part of many other CDS. Just as *ChemStation*, the *EZchrom* software has been revised and is called *OpenLAB CDS EZchrom* Edition.

Strengths: a multiplicity of instruments can be controlled.

Weaknesses: low performance, file-based architecture.

6.9.7

Tabular Comparison of Empower and Chromeleon

Both *ThermoSCIENTIFIC Chromeleon* and *Waters Empower* cover the whole range of requirements of a modern CDS. Both are network capable (up to 1000 users and more) and can be run in a terminal server environment. Anyway, differences can be discovered going into detail, even though those are regarded and judged from a subjective point of view. In the following table, the existing differences are compared:

	Empower 2	Empower 3	Chromeleon 6.8	Chromeleon 7.x	OpenLAB CDS
Support of current OS	No (only Windows Vista and Windows 2003 Server)	Yes	Yes	Yes	Yes (32 bit and 64 bit)
Supported databases	Only Oracle		Various (Oracle, SQL, MS-Access)		Oracle and SQL (file- based as alternative)
Integrated outage protection	Limited (running data acquisitions are continued, evaluation and starting of new measurements is not possible)		Yes (in case of an outage the whole range of operations can be continued on an acquisition client)		Yes Operations on a client are possible Failover
Usability	Difficult (but easy access to functionalities of the database)		Easy adapted to the common use of Windows Explorer. Difficult for database queries		Easy
GUI	Traditional Windows Design with menu and toolbar			Current MS Office ribbon design	

	Empower 2	Empower 3	Chromeleon 6.8	Chromeleon 7.x	OpenLAB CDS
Support of MS detectors	Yes (some Waters MS detector)		No	Yes (Version 7.2)	Yes
Support of competitive instruments	Yes (limited to HPLC instruments of Shimadzu, Hitachi, Dionex and Agilent GC & HPLC Systems, and Beckman CE)		Yes (broad range of instruments from different manufacturers)		
Peak integration	Easy (graphical appliance, with ApexTrack almost automatic)		Normal (like all common systems)	Easy (graphical appliance, with Smartpeak Integration well supported)	Modern (data analysis)
Integrated automatization	Yes, but limited Injections as part of a Sample Set can be repeated or aborted driven by the calculated result		Yes, but limited conditions can be defined in methods – difficult to set	Ja by so-called eWorkflows processes can be depicted	Ja Smart Sequenz
Reporting	Reports can be designed individually, complex layouts are possible. Graphical editor is offered calculations that are embedded in the report are only possible to a restricted extent (table statistics)		Reports offer almost the same possibilities as MS Excel, Even complex calculations are possible		Intelligent report builder. (based on MS BIS)

	Empower 2	Empower 3	Chromeleon 6.8	Chromeleon 7.x	OpenLAB CDS
Customizing by own database fields	Any number of user-defined database fields can be created. Those can contain describing information (e.g., batch number), but also complex calculations (between peaks of the same chromatogram and between different sample lines)		Any number of additional database fields can be created. Those can contain describing information (e.g., batch number), calculations in user-defined database fields are not possible	Any number of additional database fields can be created. Those can contain describing information (e.g., batch number), calculations in user-defined database fields are also possible	Yes, alpha- numeric informa- tion only
Versioning of results	All method modifications are stored as a new method version, overwriting of data is not possible. Each result that was generated and stored can be retrieved at any time		Versioning of methods is not supported. Results are not stored, but created again each time from the scratch	All method changes are automatically stored as a new version. Data cannot be overwritten	Yes method versions and result sets

6.10

The CDS of Tomorrow

6.10.1

MS Integration

In the same way, as 20 years ago the diode-array detector became the default detector in the laboratories, MS detection has become rather popular. Even questions and answers concerning data technology were very similar: Unusual

data amounts need to be stored, operated, and backed up. At that time, the discussion was focused on megabyte ranges, whereas today giga- and terabytes need to be handled. As long as mass spectrometers are used as stand-alone installation in specific MS laboratories, the workplace is the most practical solution. Inexpensive NAS systems (network attached storage) are easily capable to store a data volume of 10 terabytes that can be transferred over a decided network to an acquisition or evaluation client. In the case where mass spectrometers today are integrated into a CDS, the infrastructure needs to be adapted to the postulated requirements. Unfortunately, strong and suitable solutions are not yet offered by any manufacturer.

6.10.2

Large Installation

More and more global companies extend their CDS to an installation across their locations over the world. The aim is to harmonize the processes by centralizing the data, in order to reduce costs and expenses for operating the system. A company-wide installation has many advantages, where different models can be considered:

With licenses organized by the *concurrent user model*, only active user accounts are counted, which are concurrently working with the CDS. By this model, the same licenses could first be used by a site in, for example, Japan and later on in the United States due to the time shift.

Using licenses according to the *named user model*, every single active account in the system is counted, no matter if this user has currently registered in the systems or not. Costs for licenses are saved by keeping unused licenses centrally. For example, each single site does not need to keep three "spare-licenses," but centrally five licenses are kept that can be offered to any site, if requested.

Centralization reduces costs for testing, validation, and documentation. Instead of having the effort for user management, data storage, and validation of user-defined calculations for many sites, this can be taken care of centrally only once being valid for all sites. The single laboratories connected to the system can simply access the already validated system and can refer to a centrally managed documentation. It is very obvious how expenses to run such a CDS can be reduced. As all data are stored at a central location and are not distributed at the single sites of a company, backup and archiving strategies as well as a conception for an outage of the system can be implemented much more easily.

However, company-wide installations still involve a big risk. A huge amount of data needs to be organized and managed. Although a database mostly can handle the high amount of data volume, the storage volume for data files is in most cases a problem. According to the selected security concept of the software, for example, copying of data into the companies' storage area network (SAN) could be misleadingly interpreted as data manipulation by the CDS. With *UNIFI Waters* has announced a new solution, where data files are kept in the database themselves. Administration of a large installation would be much easier by this approach.

A central system demands much more planning for administrating and performing updates. Especially, global installations only offer a few slots, where no user is working in the system, for example, performing a reboot of the server. For the installation of updates that need to be distributed on all servers and clients at the same time, several hours of even days are required, where the system is not available for the daily operation.

6.10.3

Easy and Intuitive Usability

For many years, CDS manufacturers have implemented new functionalities in the system that are now offered by a modern CDS and that cover almost all requirements for the operation in laboratory. Unfortunately, usability has suffered with the implementation of new functionalities. Most systems require a 5-day training course, in order to qualify the user for running the most important functions. For upcoming functional changes and enhancements that are implemented by a software update in the current CDS installation, new concepts are necessary in the future, in order to avoid a complicate operation and missing transparency.

With the release of *Chromeleon 7*, *ThermoSCIENTIFIC* has focused on usability aspects by reducing forcefully the number of required clicks for performing a special task and by offering a modern graphical user interface in the style of *MS Office*. *MS Office 2007* only displays the menu items that are required for the selected task.

Apple has demonstrated how the user acceptance of a product will increase by an elaborate usability. It will be interesting to see how manufacturer, especially *Waters*, is going to provide for this challenge with its new product *UNIFI*.

6.11

Special Extensions

Already today, different suppliers offer extensions and interfaces to their CDS. Those extensions increase the functional scope of the software or solve special problems or requirements. Some of these ideas are demonstrated in the following sections.

6.11.1

Support of Peak Integration

All CDS offer various possibilities for the integration of chromatograms. The aim of an integration is to determine the peak area as precise as possible. For this purpose, the integration needs to precisely follow the baseline. Where the user is unsure, all systems offer an overlay function by which, for example, the chromatogram can be compared with a blank injection. The blank depicts the best baseline course. Much more difficult to identify the “correct” integration for non-baseline separated peaks. Here, the CDS offer three different integration types:

- 1) Peaks can be separated by setting a drop line.
- 2) A peak can be skimmed from a mother peak by a tangential line (tangential skim).
- 3) A peak can be skimmed from a mother peak by an exponential curve (exponential skim).

Especially, the integration performed by a drop line and an integration performed by a skimming might provide very different peak area values. For example, when determining impurities and degradation products, the kind of integration will decide whether or not a product meets the specifications and still can be used. An assistance for the decision, which integration should be used, no CDS is offering today. Anyway, additional programs are available that can be connected to the software through a programmed interface. These extensions offer a mathematical well-founded rating and can support the decision in case of an audit.

6.11.2

Column Administration

Some suppliers have integrated the installed analytical column in their hardware systems for identification purposes. This can be performed by a “funk chip” that is connected to the column and can be recognized by the column compartment (*Agilent*) or by a clip that is connected to the column that is then fixed at the column compartment (*Waters*).

Here, the compatibility is the biggest problem again: Column information cannot be assigned to the corresponding injection in the CDS. If it would be possible to store the clear identification of a column for each injection automatically in the CDS, a column log book could be depicted without any additional effort.

6.11.3

Instrument Usage

In times of increasing competitive constraints, companies are more often deliberating whether the investment of a new chromatographic instrument is required or if the present instruments could be charged better to capacity. All information to support such a decision are actually stored in the CDS. Anyway, they cannot be exploited just like that.

The first step would be a summary of all run times of an instrument. But this only provides the actual measuring time. Times for equilibration and injections that are not stored in the CDS also need to be considered. All these times provide in summary the actual usage time of an instrument.

In addition, it needs to be considered that samples are measured as a series (sequence or sample set) in most cases. In order to judge the real usage, the times between two series needs to be considered, as well. In case of an idle running of perhaps 1 h no further samples could be measured during this time. If beyond that the column needs to be replaced and solvent need to be changed, even periods

of several hours could not be used for additional measurement. A functionality that provides information about the usage of an instrument and the detection of idle running would be very helpful, but is not part of any current CDS and can, therefore, only be implemented by a third party software program. Such a feature needs to have access on all data that have ever been generated by a system. For this purpose it is necessary to store all relevant data in separate database, a so-called data warehouse. Using the currently present information in the CDS, all data that already have been archived or deleted would be missing and the result of a query would not reflect the effective usage of an instrument.

6.11.4

Connection of Balances

Almost for every measurement that is performed by HPLC or GC systems, sample weightings need to be performed in advance. No matter if a sample weighting or if a solution for a reference solution is prepared, always measuring values from a balance are involved.

For a laboratory with a paperless workflow, these values are kept in the Laboratory Information and Management System (LIMS) and will be transferred to the CDS. Without this cross-linking, the manual input of weightings into the CDS would signify an enormous effort and the risk of transcription errors would exist. To avoid this, two approaches for the transfer of balance data to the CDS are thinkable:

First instrument driver of a balance could be connected directly to the CDS and transfer the values through this interface, for example, into the sample table of a CDS. However, although this seems to be a simple solution, there is a little pitfall: standard solutions are often prepared for the use of a defined period. The measuring value of a standard that was prepared on a Monday still needs to be available on a Tuesday. Does the expiry end after 3 days, this value must not be used on Friday. An administration of standard values, therefore, needs to be provided. Where solutions are available for later measurements until their expiry date.

If a weighting consists of several steps, the measuring value from the balance is not the value that should be used in the CDS. For example, 12 mg of substance A are weighed and 15 mg of substance B are added, the actual balance displays a value of 27 mg. Before transferring the values to the CDS, a difference between weighing A and weighing B needs to be calculated.

For this reason, a direct interface between the balance and the CDS is only productive for easy processes. The second solution should, therefore, be preferred:

Through an interface the CDS is connected to the balance software. Besides the mere data acquisition, current software products such as labX of Mettler-Toledo provide support for the measurement itself. Operation procedures (first weigh an empty measuring flask, then add about 15 mg of substance A, fill up with water) and further important information such as limits (15 mg of substance A in the range of 13–17 mg) can be transmitted to the user.

Instead of connecting a balance directly to a CDS, the integration of a balance software provides more functionality and a higher comfort.

6.12

Open Interfaces

Although CDS have been considered as an independent system so far, now a cross-linking becomes necessary with the increasing significance of the integration of various software systems. For example, a LIMS delivers the work orders to the laboratory, which then should be executed by the CDS. The obtained data then need to be evaluated somehow and retransferred to the LIMS. A cross-linking between LIMS, CDS, ELN, and SDMS is avoiding manual transfer error and provides potential for saving time and securing data that should not be misprized. Even the time-consuming review of transferred data is inapplicable. Unfortunately, there is today almost no harmonization of interfaces and data formats. Only the standards according to *AIA*, a definition of the *American Society for Testing and Materials (ASTM)*, offers a possibility for simple transfers of CDS data between the different systems. In the future, the current *ANDI/netCDF standard* is supposed to enable the exchange of 3D data, as they are generated by diode array detectors and mass spectrometers.

However, these formats only permit the transfer of mere measuring data (XY-data files) and eventually of some meta data, such as the sample name. Up to now, a real connection, where access is given to all information of the CDS including all measuring results, always needs to be customized as an individual solution. Even interfaces that are offered by some LIMS and CDS suppliers do not really offer an out-of-the box conception, but rather a tool box that allows the customer to create his own solutions. Here, new concepts are desirable that could simplify the cross-linking between different systems.

It is supposed that *Waters* offers a new approach with the *UNIFI* system. The basic idea is that all systems of a laboratory access the same data pool in the database. Here, the single components CDS, ELN, and SDMS can be connected to, which are required by the customer. By using a shared database all connection steps are obsolete, and the single system could cooperate seamlessly. The only question that has not been solved yet is how systems of other suppliers could dock on. In case *UNIFI* will be kept as a proprietary *Waters* system, this concept could not be considered as a comprehensive approach for the laboratory of tomorrow.

6.12.1

Instrument Integration

Today, not all instruments can be connected to every CDS in the laboratory. As a result, some laboratories need to purchase several CDS. To avoid this, suppliers for instruments and CDS should offer corresponding interfaces for the development of instrument drivers in the future. Up to now, the attempt has failed to harmonize

interface for instrument drivers, as in contrast to the supplier-independent integration of printer drivers in Windows, every supplier of analytical instruments uses his own protocol.

At least, *Agilent* has started with an attempt of harmonization offering the *Instrument Control Framework (ICF)*. A CDS supplier only needs to implement a driver for the ICF interface, *Agilent* cares for the connection of the driver to the instrument. By this, a better integration of the instrument to the CDS is given and drivers are available more quickly, as only *Agilent* needs to develop a new implementation. An agreement on a general standard for interfaces would also help the user in harmonizing and reducing test and validation procedures. User trainings would be simpler as the graphical interfaces for entering control settings would look similar for all systems.

6.13

The CDS in 20 Years

Which trends would find their way into the future of the CDS?

Comparing the systems of today with the standards of the 1980s, it can be assumed that the CDS of the 2030s would also look completely different.

One trend, which can already be suspected today, is the migration of the CDS from a local installation into a cloud. Due to the globalization, there are systems today where data are stored separated from their point of origin. For the user, the chromatogram can be found in the CDS – the actual storage location does not matter. In a next step, the application itself could be located in the cloud. The cloud is probably followed by mobile devices. Instead of the conventional PC, the user of the future will presumably walk with a tablet PC through the laboratory. On standing in front of an HPLC, the instrument would recognize the tablet PC by RFID technique and the CDS could transmit the current measurement, as well as all other entries such as, for example, audit trail.

Acknowledgment

Finally, I thank my colleague Ingo Green for his remarks and ideas, which were of great help for the preparation of this chapter.

7

Possibilities of Integration Today

Mike Hillebrand

Let us imagine a perfect chromatographic method. Every single component of a sample would be displayed as a fine single graph.

But (especially) in biotechnology or in the chemistry of natural products, this is not usual.

For example, if substances are generated by microorganisms, the by-products obtained are often very similar to the main product. And this will cause peaks with overlapping areas, which cannot be often separated completely.

7.1

Peak Overlay - Effect on the Chromatogram

If two peaks do overlap, this does not mean that one is hiding behind the other. Both signals are combined. So the signal from components A and B, and other possible components is assembled. The result is a sum curve or envelope.

Overlapping peaks always run the risk of making errors in area integration. As this is a systematic error, it usually remains undetected. Therefore, in the method development, baseline separation of the peaks is desirable. The larger the peak size ratio, the better the resolution needs to be. However, as explained in the introduction, even with enormous strain in method development, in some cases, a baseline separation is not possible.

If you have overlapping peaks, you have to be aware that you do not only have errors in area integration, but also in retention time and height. Both are affected by the peak overlay, this is shown in Figure 7.1. Both parameters are very important peak parameters, since they are used for quantitative and qualitative terms.

Figure 7.1 shows three overlapping peaks. The analyst would receive only the dark grey sum curve in his chromatogram. It consists of the combined signals of the three peaks (light grey). As you can see, each peak affects its neighbors, because the rise or fall of the respective neighbors, in turn, affects the cumulative

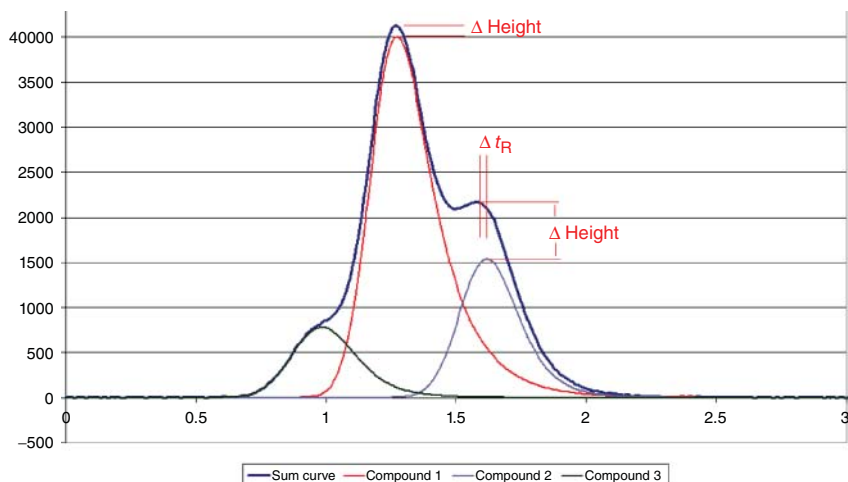


Figure 7.1 Effect of peak overlay on the determination of the peak height and retention time.

curve. So, the height and the retention time of the peaks, depending on the resolution varies can change within the cumulative curve.

7.2

Separation Techniques for Higher-Level Peaks

Having overlapping peaks in a chromatogram, the analyst has to solve the following question: “Which separation method is the best for separating these overlapping peaks?”

The vertical separation method (“Vertical skim,” “drop”), separation of the tangential (“skim Tangential”), the valley-to-valley separation method, the Gauss separation method (“Gaussian skim”), or the exponential removal (“Exponential skim”)?

Looking at the separation methods, you can see very quickly (just consider the vertical, the tangential, and the valley-to-valley separation method) that the separation must be faulty and full of “errors.”

Overlapping peaks and overlaid curves can only be determined in exceptional cases by separating with straight lines.

7.2.1

Lot Method

The vertical peak separation, which is called *Lot method*, separates peaks from each other by drawing a vertical line.

This is preferably (depending on the resolution or size) placed in the valley between the peaks (see Figure 7.2a).

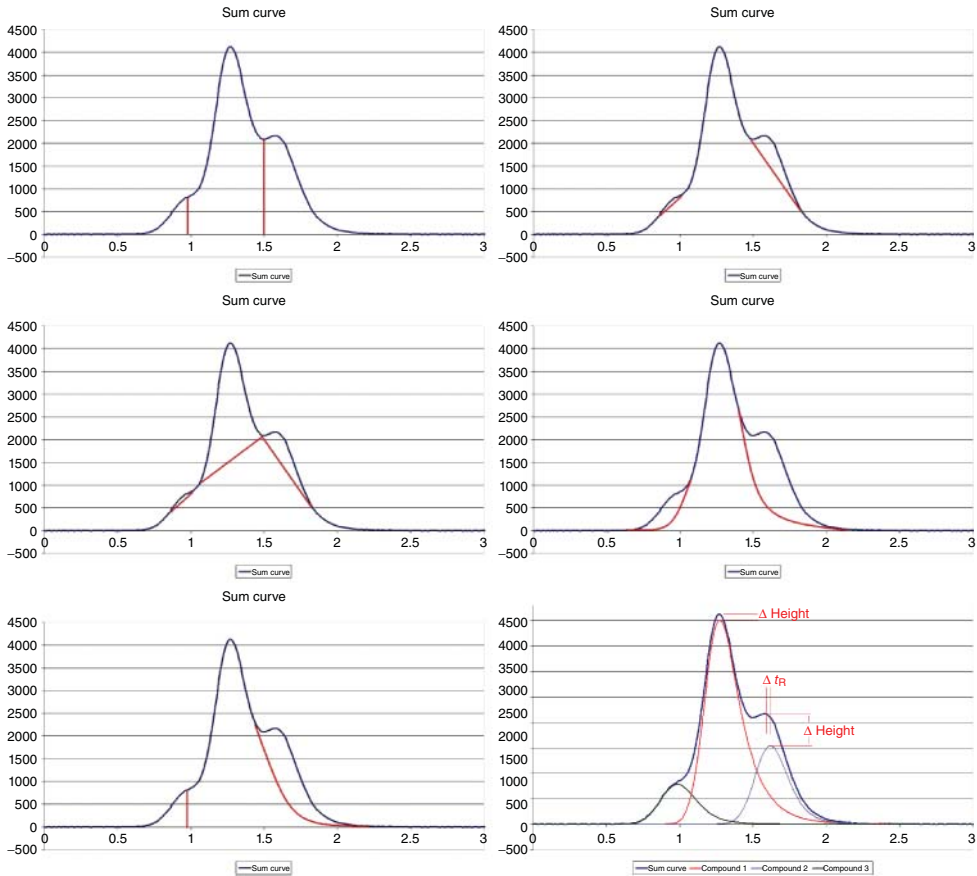


Figure 7.2 For available separation techniques for higher-level peaks and their comparison ((a) vertical separation, (b) tangential separation, (c) valley-to-valley separation, (d)

Gauss separation method, (e) exponential separation, (f) comparison, cumulative curve with single peak).

Mistakes/errors that may occur to the “Vertical skim” in terms of area analysis are described by V.R. Meyer and confirmed in several scientific work area rules.

7.2.2

Error by the Vertical Skim Overlapping Peaks (Area Rules to V.R. Meyer) [1]

- *Symmetric peaks*: the large peak is too large and the small is too small (regardless of elution) [1].
- *Peaks with tailing*: the first peak too small and the second too great (regardless of the area ratio) [1].
- The ratio of the relative error is inversely proportional to the ratio of the peak areas [1].

- Small peaks are more affected [1]. At an area ratio of 20 : 1, for example, the error for the small peak is 20 times greater than that for the large (relatively speaking).
- The errors grow with decreasing resolution and growing tailing. The stronger the peaks differ in their size, the greater is the relative error for the small peak and the lower for the great one [1].

In the automatic integration, the software used registers the lowest point between the peaks and places the lot. In the manual integration, the separation is made subjective, but should also take place at the lowest point between the peaks.

The area to the left and right of the separation are then assigned to the peaks.

After V.R. Meyer is generally true that the values from the lot method are too large and the values of the tangential separation method in general are too small for small peaks get on the flank of other peaks [1, 2].

7.2.3

Tangential and Valley-to-Valley Separation Method

The tangential separation replaces shoulder peaks by drawing a tangential line, which separates the shoulder tangent (see Figure 7.2b).

The valley-to-valley separation separates overlapping peaks by using the valley point. The base line is drawn from the valley point (see Figure 7.2c).

These two separation methods are only suitable, when the baseline runs accordingly and there are no alternatives available. Thus, the baseline must run within the peak cluster along the peak valleys. These separation methods apply only in exceptional cases.

7.2.4

Gaussian and Exponential Separation Method

The Gaussian (see Figure 7.2d) and the exponential separation (see Figure 7.2e) are calculated by an algorithm by the chromatography data system used. The existing peak top is extended Gaussian or exponential.

It is not calculated that the peak is lifted and moved from the neighboring peak. This will cause a large area of the observed peaks and – in turn – a small area of the neighboring peaks. The lower the resolution, the higher the error. Also, the error is higher if, the neighboring peaks become smaller.

7.3

Application of Separation Methods

The possible use of one or the other separation method is dependent on the chromatography data system that is used. Not all systems provide the full variety of methods. Which method is used, however, is often decided by the analyst and his “feeling.”

You can see very quickly that some separation methods do not provide exact values. The “blank” is a useful tool to determine the base curve in the first assessment.

The peak separation with any separation method currently available in the chromatography data systems is faulty (with some exceptional cases). Depending on the size difference of the peaks and the strength of the overlay, the mistakes increase. So you can, for example, within the area integration, reach the errors with enormous proportions.

These error values can be up in the order of about -80% (see, e.g., tangential and valley-to-valley separation methods in Figure 7.2) up to about $+200\%$ and be larger (e.g., in the application of vertical separation method of superimposed peaks with enormous differences in size and low resolution).

This means that, for example, an error value of -80% for a substance causes a value of 200 unit areas instead of the actually existing 1000 area units.

Especially, in areas of purity analysis in hazardous byproducts, this is unacceptable. Also think about how much expense and how many financial resources are invested in the analysis technology and the exact sample preparation.

In spite of all this, such errors are accepted, without being able to define the height of the errors made.

7.4

Chromatogramsimulation

The main problem is not necessarily the mistakes, but the very limited options of getting information about the error value. The analysts have no values that justify one or the other separation method even when knowing that the separation and the values given are faulty. Especially, in areas of federally inspected establishments, there should be a desire to clarify the audited inspector that one has dealt with this issue and the error values of the separation methods used for higher-level peaks were scientifically evaluated.

Making a simulation of the chromatogram can be a way to get a first look at the errors made.

For this purpose, you can simulate symmetrical peaks on the normal distribution function using Excel. Via the setting and adaptation of peak parameters, the simulation of the individual peak areas were adapted to the real chromatogram.

Instructions on how to create such a simulation can be found in the book “integrate chromatograms and properly evaluate” [3]. According to the instructions in Reference [3], the mistakes made with the separation methods can be well estimated by the created simulation. The creation of a simulation is relatively time consuming because you have to adjust the peak parameters frequently and empirically. Nevertheless, the creation of a simulation is a significant help, since one with the help of this, it is possible to get the area values of the real chromatogram.

A.W. Westerberg (1969) published, almost 40 years ago, the report of a chromatographic analysis with overlaid peaks [4]. In this work, Westerberg describes the disadvantages of vertical skimming with superimposed peaks and recommends a curve fitting, which is discussed in the following section.

7.5

Deconvolution

Deconvolution, based on overlaid peaks in the chromatographic process, is a curve fitting on theoretical mathematical models. This means that the cumulative curve of a chromatogram is broken down into individual summands, individual peaks of the components, with the aid of theoretical models (see Figure 7.2f).

From this report with overlaid peaks, a simulation is created using Excel (using the normal distribution function). This gives us a simulation, which is identical to the one published by A.W. Westerberg - at the same scale (see Figure 7.3).

The overlaid peaks of the simulation were separated with the vertical separation method for area integration. For comparison, the overlaid peaks of the simulation were separated with the recommended curve fitting by Mr Westerberg (deconvolution) using the Gauss curve model.

The values, from the report of A.W. Westerberg received and used as the target of the simulation area, were compared with the results of Vertical skim and the deconvolution. This comparison is shown in Table 7.1.

The thick black peaks in Table 7.1 are those within the simulation that are overlapping in great areas.

So peaks 6 and 10 are shoulder peaks with a very low resolution.

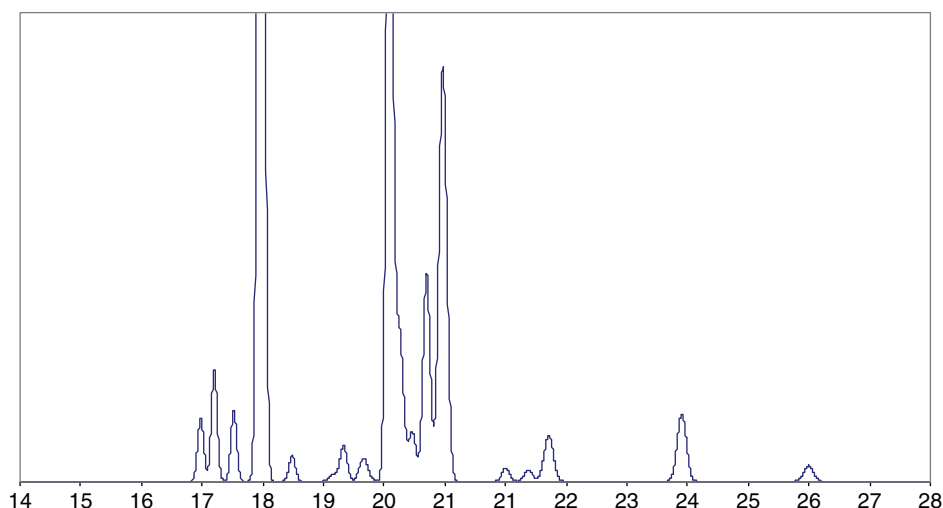


Figure 7.3 Simulation of the report of Westerberg [4] using MS-Excel.

Table 7.1 Comparison of area integration of Vertical skim and the deconvolution.

Peak	Area as it should be	Peak area		Peak area	
		Lot precipitation	Deviation from the area as it should be (%)	Deconvolution	Deviation from the area as it should be (%)
1	947.35	935.92	−1.21	947.41	0.01
2	1 544.05	1 555.42	0.74	1 544.09	0.00
3	1 015.7	1 015.32	−0.04	1 015.74	0.00
4	22 941.67	22 941.5	0.00	22 941.59	0.00
5	395.37	395.15	−0.05	395.17	−0.05
6	132.73	112.78	−15.03	132.78	0.04
7	621.22	642.32	3.40	621.15	−0.01
8	497.73	495.87	−0.38	497.57	−0.03
9	12 795.5	13 661.27	6.77	12 795.46	0.00
10	3 019.67	2 223.55	−26.36	3 019.82	0.01
11	881.4	781.5	−11.33	881.27	−0.01
12	3 583.83	3 555.65	−0.79	3 583.79	0.00
13	8 130.83	8 188.82	0.71	8 130.9	0.00
14	245.2	244.82	−0.16	245.29	0.04
15	236.33	237.45	0.47	236.38	0.02
16	1 040.27	1 038.87	−0.13	1 040.23	0.00
17	1 467.47	1 467.15	−0.02	1 467.42	0.00
18	367.75	367.42	−0.09	367.74	0.00

These are the interesting points of the chromatogram for the use and evaluation of separation methods and deconvolution.

The deviations from the target area, which are about 1%, were light gray and about 5% dark gray.

The deviation of the peaks in both methods is almost 0%. The existing deviation is partly due to the influence of the noise.

The Vertical skim supplies in the overlaid peak deviations up to 26% by peak 10.

Especially, the shoulder peaks have increased deviations and so is peak 6 No. 2 with 15%. Six of the nine overlaid peaks have deviations higher than 1%. Again, four of these six peaks have a difference of more than 5%. These enormous errors are just accepted while using vertical skim (Vertical skim). If the overlapping peaks additionally contain a tailing, the integration by vertical skim creates errors in the peak area of not less than 20–30% [3].

In the deconvolution using Gaussian peak model, all peaks have a deviation of less than 1%. These are even close to 0%, regardless of whether the peaks overlap.

Regarding overlapping peaks, deconvolution provides clearly less variation than the vertical skimming. Because the simulation, created through the normal distribution function, was used as the basis for the application of the deconvolution using Gaussian peak model; a very accurate area integration seems natural. In a real chromatogram, the accuracy depends on the symmetry of the individual

peaks using the Gaussian peak model. If peaks are symmetrical, a clear agreement with the Gaussian peak model can be seen.

A variety of chromatography data software, which also includes well-known names, still do not use deconvolution. In the example as well as in other studies [5] it has already been shown that the use of deconvolution results is a clear optimization for the separation of overlapped peaks. The area and height and retention time determinations are clearly more accurate. Even at the deconvolution the made error depends on the resolution. The lower the resolution, the larger the error.

Each separation method is ultimately based on mathematical methods. Thus, with their use, regardless of the method chosen, there are always deviations from the real values.

However, the deconvolution is the method that reflects reality most accurately. It is important to raise awareness about the mistakes made. The aim should be the separation method with the least deviations from the real values to use.

In any use of separation techniques, most accurate knowledge of the chromatogram, the components included, and the basis line course should be available. What is the resolution between the peaks? How is the baseline? Is there a baseline drift? What is my signal/noise ratio? What are the side component peaks relative to the maximum amplitude? Which symmetry does each individual peak have?

7.6

Evaluation of Separation Methods

To evaluate and optimize separation methods for overlapping peaks, there is a software tool called *hi|chromet*. With *hi|chromet*, the analyst has a tool for determining how well the chosen separation method in his chromatography data system really is it deals with symmetric as well as a symmetric peaks.

The aim of *hi|chromet* is to create a comprehensive evaluation report about the usability of different separation techniques for overlapping symmetrical and tailing peaks. This leads to the documented knowledge of how accurately the separation method used really is, what mistakes you make when using individual separation methods, and which peak parameters the individual peaks of the chromatogram really have.

The chromatogram and the determined integration results are imported and a simulation of the chromatogram is created. The simulation is adapted by means of visual shift. Deviation parameters determine the comparability with the original chromatogram. The report on the processed chromatogram provides an overview of the deviations of each separation method used. In addition, he receives a recommendation for which separation method he should apply for individual peaks overlapping, to obtain the least deviation.

The report refers not only to the peak area, but also the peak height, and retention time at peak overlays also, as shown in Figure 7.1, can have very large inaccuracies.

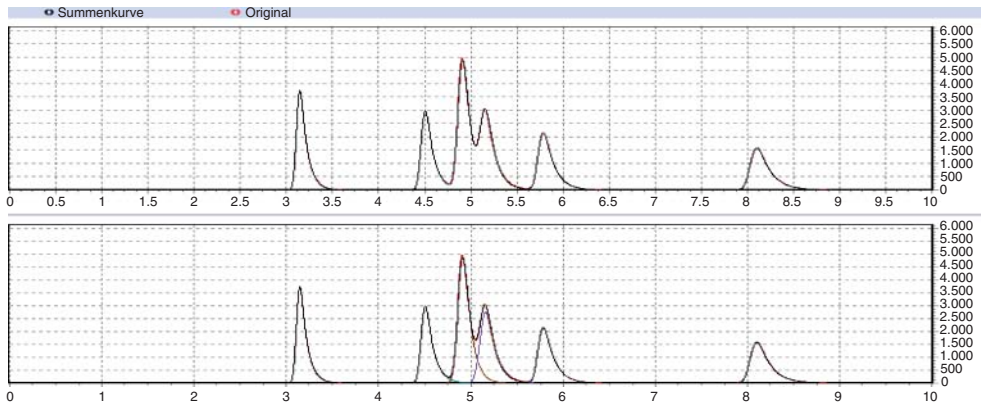


Figure 7.4 The user window of hi|chromet after completion of the Chromatogrammsimulation.

In the chromatogram of the following example, consisting of six separation peaks, peaks 3 and 4 overlap the most. Peaks 1, 5, and 6 elute baseline separated.

Now, the analyst is faced with the questions as to which of the available separation method he should use and which errors the values can have.

In order to give a decision aid on the basis of determined and thus known deviations, the chromatogram in hi|chromet was imported and a Chromatogramm simulation created. After completion, the correspondence with the imported chromatogram is shown in Figure 7.4. The variation coefficient amounts to 0.9994.

The tool hi|chromet compares the results of the chromatogram simulation with those of the respective separation methods after completing the report. Retention time, area, and height for each peak are listed individually. In Table 7.2, the results are shown as an example with reference to the surface.

In the report, the results of the surfaces are tabulated ("Area") from the individual separation methods with the corresponding deviations ("Deviation"). Again,

Table 7.2 Comparing the surfaces of the respective separation method with deviation.

Area	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Simulation	29 821	28 222	48 721	32 762	26 152	24 345
Vertical skim	29 879	27 435	45 438	36 703	26 158	24 343
Deviation (%)	0.19	-2.79	-6.74	12.03	0.02	-0.01
Exponential skim	29 879	27 065	54 118	26 354	24 401	24 343
Deviation (%)	0.19	-4.10	11.08	-19.56	-6.70	-0.01
Gaussian skim	29 879	27 380	52 143	29 539	25 302	24 343
Deviation (%)	0.19	-2.98	7.02	-9.85	-3.25	-0.01
Tangential skim	29 879	24 611	29 816	13 042	24 058	24 343
Deviation (%)	0.19	-12.79	-38.80	-60.19	-8.01	-0.01

peaks 2–4 with the lowest resolution separation methods for the assessment of the most interesting, as the other baseline separated peaks elute. For those, peaks consistently result in differences close to 0%. The respective slightest deviation per peak is marked in gray.

From the use of lot method (“vertical skimming”), resulting errors in the value are up to 12% (peak 4), the area of peaks 2 and 3, using the lot method, results in comparison to other separation methods to the lowest deviation. Peak 2 has here, for example, with the lot method an error value of –3% compared with the tangential separation method (“tangential skim”) with too small a surface area of –13%. Even more dramatic is the difference in peak 3 with an even lower resolution and a resulting clearer overlay; –7% here is the deviation of the lot method determination of the area. The exponential separation method (“Exponential skim”) provides 11% deviation, a very large surface. The largest deviation results here at peak 3 using the tangential separation method (“tangential skim”) with –39%.

The Gauss separation method (“Gaussian skim”) returns over the entire peak time almost identical error values as the lot method. The deviations in peaks 2 and 3 only in the first decimal larger, but at peak 4% to 2% lower. Twelve percentage of the lot method and –10% for the Gaussian separation method.

The obtained results are extremely helpful for the analyst, to make decisions about the use of one or another separation method. The Tangent and the Exponential separation method close due to their high deviations. In the narrow selection, the lot method as well as the Gaussian separation method occur. Both lead to approximately similar deviations, but the signs at peaks 3 and 4 are interchanged, that is, where the lot method leads to a larger area, the Gauss separation method leads to a lower area and vice versa. Depending on, for example, the primary substances to be determined, then one or the other separation method, the deviation can be selected, with the focus on certain peaks to minimize. What matters is that now there is the documented knowledge of the deviations of the individual separation methods.

Even when using a clearly erroneous method the knowledge of their error extent is available.

7.7

Practical Application of Deconvolution

As an alternative to the current situation that the use of the deconvolution is not included in any chromatography data system currently available in the market, recently a software tool became available, called hi|skim. With hi|skim, an imported chromatogram with overlaid peaks can be integrated, using mathematical models. This means, for the separation or analysis of overlaid peaks, deconvolution is averted. With hi|skim, the analyst receives a report in the usual way by integrating with the respective interesting peak parameters for further processing.

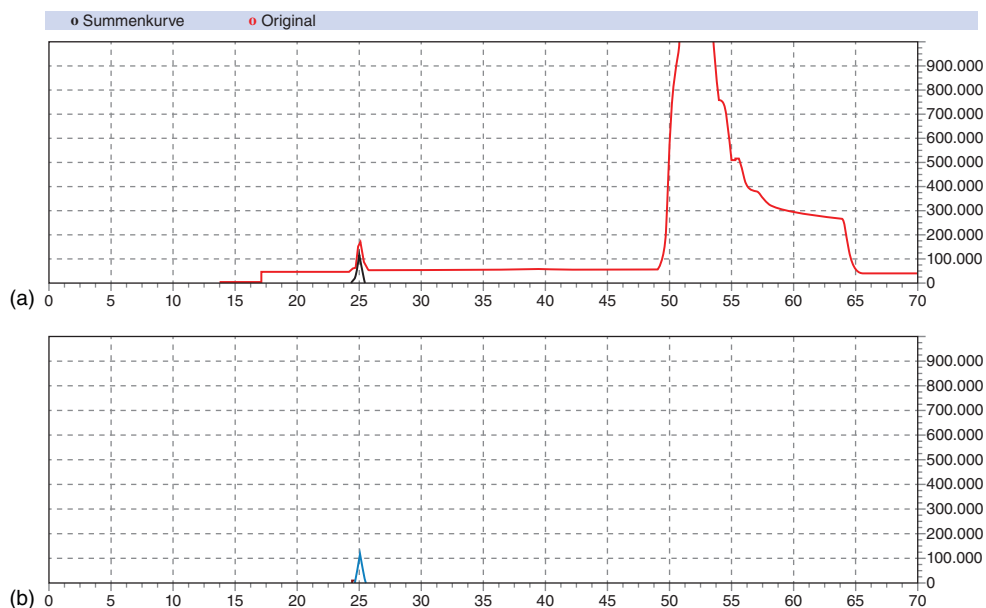


Figure 7.5 Chromatogram with forward simulation in hi|skim.

Figure 7.5 is a chromatogram (dark grey - in panel a) to determine two pharmaceutical substances (light gray – 6,7-dehydro-EE, and -9,11-dehydro-EE - in panel b), which was imported in hi|skim. The chromatograms used below were kindly provided by Dr Reinhardt, mibe GmbH medicines available. The deconvolution provides the individual peaks (panel b) and the resulting cumulative curve (black – in panel a).

The total chromatographic process takes about 70 min. The two-tailed substance peaks elute 23–27 min. Therefore, the evaluated range is limited to this time window (see Figure 7.6).

Because the peaks sit on a plateau (see original data - panel a (dark grey)) will be added a baseline curve (see Figure 7.7) as linear step gradient.

After the correction of the baseline curve, the curve fit is performed. The deviation parameters between the original and the sum curve from the deconvolution must be located within the required limits. After adjustment (Figure 7.8), for example, the coefficient of variation in this case is 0.9992.

The adjustment can be checked and corrected by means of zoom. When the process is finished, the respective peak parameters are reported.

The chromatograms of the individual peaks can be exported and re-imported to assist in the evaluation in a chromatography data system. Helpful for evaluating the chromatography data system (CDS) is an overlay of imported single peaks for the evaluation and application of separation methods.

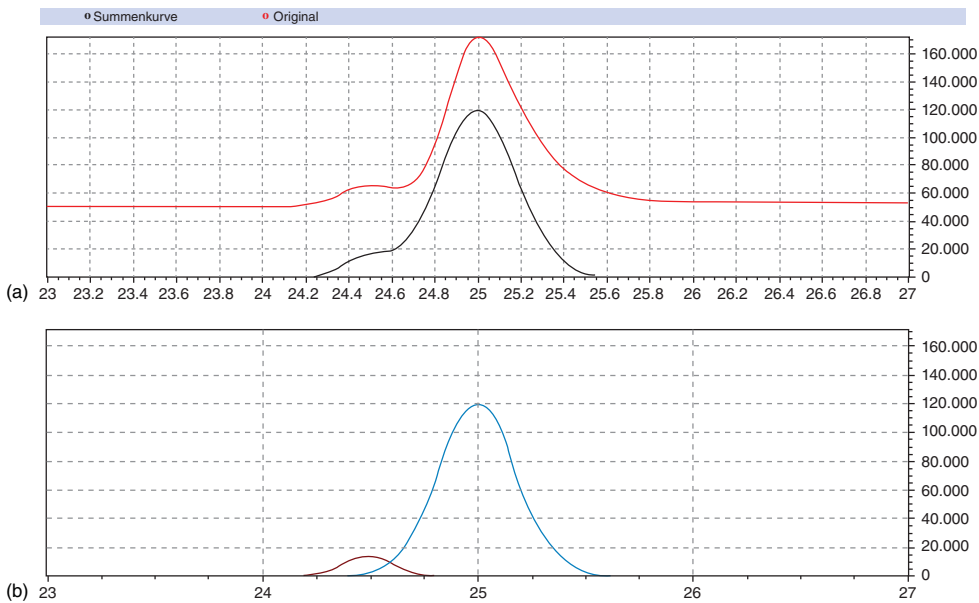


Figure 7.6 To-be-evaluated Chromatogrammbereich set to 23–27 min.

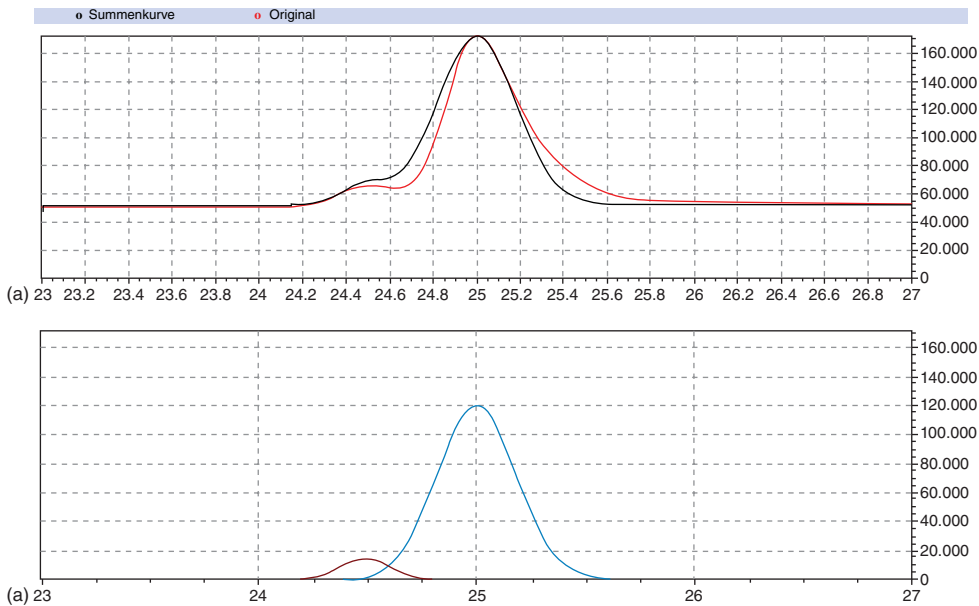


Figure 7.7 Added a step gradient of linear fits to the baselines development.

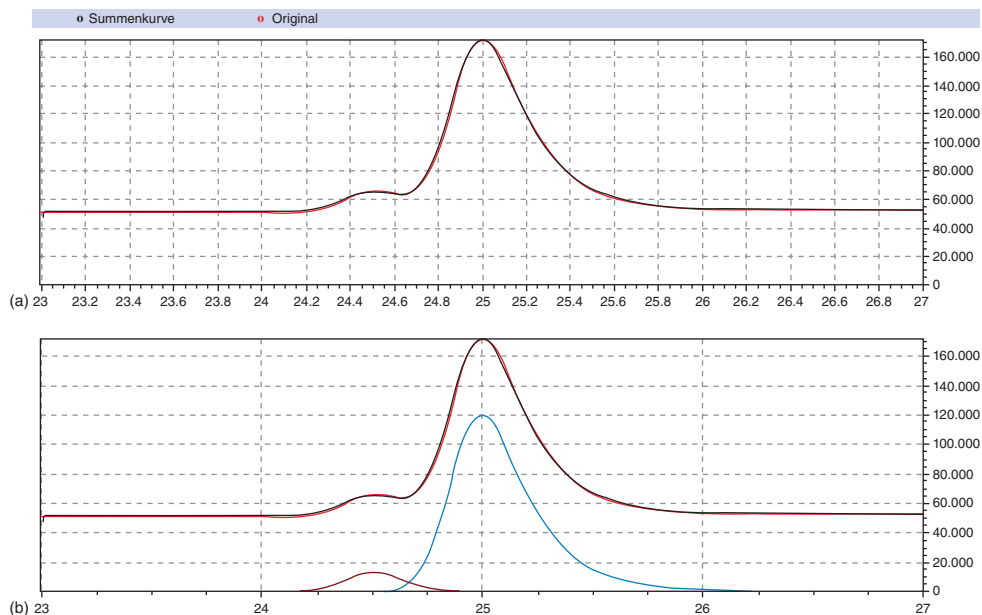


Figure 7.8 Complete the separation by means of deconvolution (variation coefficient: 0.9992).

Furthermore, the resulting peak parameters can be helpful for the optimization of a methods, for example, the peak symmetry of overlaid peaks is reality closer determined and not distorted on the separation methods.

With this chromatographic method, three analyses were performed with different concentrations of 6,7-dehydro-EE (see Figure 7.9) and imported, respectively, in *hi|chromet* to the deviations of the area values of the first peak (at 6,7-dehydro-EE) to define, using the soldering methods. The peak height ratios differ because only the concentration was varied to 6,7-dehydro-EE. Table 7.3 shows the results from *hi|chromet*.

The lot method leads by tailing peaks, as in the area rules described by Meyer [1], always to a loose in area for the first peak [(the 6,7-dehydro-EE-peak here)]. This is the case here. Reducing the concentration and thus the peak area or the peak height results in an increase of the deviation when using the method of Lot.

Thus, it can be documented and evaluated how the integration behaves when changing the chromatographic parameters. Our example is the change in the concentration. However, with this method, the influences of all other possible changes that have reference to the peak shape, resolution, heights, areas, and so on can be evaluated. Maybe – in one or the other situation – the use of another separation method is advisable or the results show that the resolution-sufficient precision, optimal or contrary, must be further optimized.

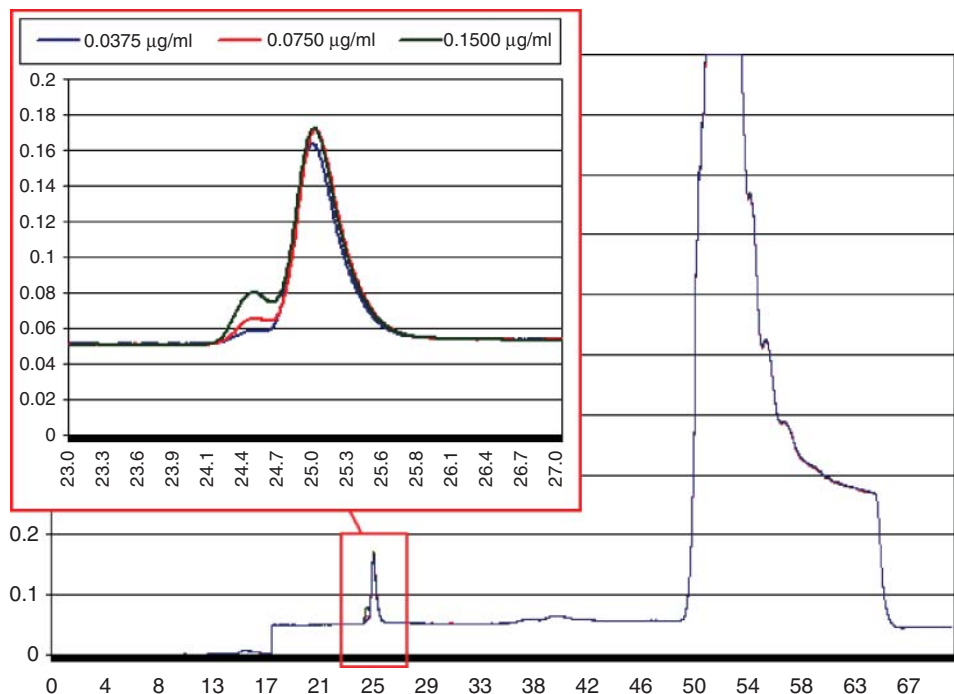


Figure 7.9 Overlay of the chromatograms with different concentrations of 6,7-dehydro-EE.

Table 7.3 Summary of results from hi|chromet to the deviation of soldering methods for the 6,7-dehydro-EE-peak.

Concentration of 6,7-dehydro-EE (µg/ml)	Peakhöhen	Fläche	Abweichung
0.0375	~1/17	107 312	-13.74
0.075	~1/9	245 837	-11.45
0.15	~1/4	538 855	-9.99

The currently available separation methods are, just like 40 years ago published [4] and illustrated in this chapter, not acceptable for an analytical method with such a high level of required accuracy, provided there is no additional information for their accuracy. Even today neither the available access via the deconvolution nor an assessment of the available separation methods within the chromatography data systems is available.

A first innovative move is made by the company beyonitics GmbH. For the two chromatography data systems Empower (Waters) and Chromeleon (Dionex), the company beyonitics GmbH is currently working on toolkits for hi|chromet and hi-skim. After completion, both will be available directly from

the chromatography data systems and can be used for evaluation. Thus, the direct evaluation of deconvolution and the assessment of separation methods in these two chromatography data systems are possible for the first time.

A demo version of hi|skim and hi|chromet can be ordered for free at www.hi-analysis.com.

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8

Smart Documentation Strategies

Stefan Schmitz

8.1

Introduction

Based on typical workflows and documents of an HPLC laboratory, fundamental procedures are presented, aiming at efficient laboratory documentation that meets the regulatory requirements e.g., data integrity.

The discussion about documentation in regulated laboratories in the chemical, pharmaceutical, biotechnological, food, and medical devices branches nearly takes up as much room as the processed technical topics. The increasing demands of regulatory requirements are often referred to as a burden by the operators and there are complaints that the documentation takes a growing proportion of the productive working time. In fact, the data sources, storage media, data formats, and related media breaks are more diverse than ever before and the classical definition that the raw data are exclusively archived on paper is on trial. The question of documentation is posed from multiple points of view, considering the ongoing technical possibilities, and should be answered in accordance with the currently existing organizational situation. The most common situation in regulated laboratories is a hybrid system, consisting of paper and electronic media (CDs, DVDs, magneto-optical disks, DLTs, hard drives on measuring computers, network drives on servers, or special NAS drives). The tried and true explanation “*Our raw data are paper and will be archived*” is due to this heterogeneous mix of various storage media, data formats, and elusive usage of electronic data, an ever-riskier line of defense in the course of official inspections.

This raises the question of a contemporary strategy on how to deal with documentation in the laboratory, in future. In addition to classical static documentation models, a process-oriented documentation life cycle is considered, comprising steps from creation (amendment), collection, organization, storage, distribution (publication) of documents for various target groups (assignment of access rights), and deletion of no longer relevant data.

Case 1 Box: Practical Tips on the Subject: Definition of Raw Data

- Define raw data as the first original recording on a storage medium.
- In this way you capture both the traditional paper records in laboratory notebooks and laboratory worksheets as well as the first recorded data in electronic form.
- Define the initially recorded electronic raw data as the “primary electronic raw data” and the derived data as the “secondary electronic raw data.”
- Example: The initially recorded proprietary chromatographic data (time axis in minutes vs. detector signal in mV) are your primary electronic data (source data), while the derived data, namely the integrated peaks, retention times, peak areas, peak heights, and the corresponding chromatograms are referred to as secondary electronic data, which are usually also stored in vendor-specific formats.
- This distinction makes sense because for the secondary electronic data a unified solution for long-term archiving is already available (see Box: PDF), while the standardization of the primary electronic data has not yet been achieved (see Section 8.2.3 *Network Common Data Form* (NetCDF) as an American Society for Testing and Materials (ASTM) standard) [1].
- It is very important for the recoverability of electronic data to be independent of vendor-specific formats, because only in this way long-term readability can be ensured.
- This is achieved by storing the data in the secondary electronic PDF/A-1 – format, a data format that is valid according to ISO 19005-1:2005 as long-term archivable (see Box: PDF).
- The nowadays often practiced alternative is to archive the derived data in paper form.
- An advantageous and profitable first step toward digital storage of processed data is to archive these data in PDF/A-1 format, instead of using paper format.
- For conservation and exchange of the underlying primary electronic data (source data), an international standard for chromatographic data has been developed, the ASTM E1947–98 (2009) [2]. This standard is based on the file extension *.cdf (derived from NetCDF) and contains typical header information such as instrument ID, column, operator, detector, and so on, as well as the x, y data, consisting of time and detector data.
- The implementation of this standard is not yet comprehensive across manufacturers and systems.

8.2

Objectives of Documentation

Documentation generally describes the process of creating documents, while documents represent physically existing information and data containers, which may exist either in paper form and/or electronic form.

Different perspectives on documentation allow a comprehensive overview of the functionality of the documentation in organizations.

The following aspects of documentation can be distinguished:

Perspective	What should be achieved?	How will the goal be reached?
1. Organization	What documents are needed by the organization to achieve its goals?	Definition of master documents, specification and verification documents for all regulated processes
2. Processes	What processes need to be documented for regulatory purposes?	Definition of regulated processes (SIPOC model) [3]
3. Communication	What is the workflow of information distribution?	Communication design, including definition of transfer documents (push or pull processes for transference of information)
4. Information	How is information available/readable/editable over longer periods?	Defining the types of data, for example, meta-data, raw data, definition of data media and formats, and transfer to long-term archivable formats (paper), various PDF formats (see Box: PDF) as well as generic formats of chromatographic raw data (NetCDF)
5. Knowledge storage	How can the information and knowledge stored in the data be made available and preserved in the organization?	Generation of indexed archives, databases, intranet portals, document management systems (DMSs), expert systems

8.2.1

Documentation from the Organizational Point of View

The document structure in regulated companies generally follows a hierarchical approach. Figure 8.1 shows three typical levels of documents and is considered the standard model.

The top level, shown in Figure 8.1, comprises the documents published and controlled by the management, quality management, and the departments, representing a general implementation strategy for official rules. These strategic documents, such as quality manuals or master plans, for instance, delineate the interpretations of the regulatory requirements by the organization. In regulated HPLC laboratories, for example, a validation master plan would be expected at this level that specifies how the validation of computerized systems is conducted.

On the middle level are the concrete specification documents for the operational level, which describe certain work processes and are also considered to be controlled documents, that is, they are subject to change control. Examples of documents on this level are, for example, standard operating procedures (SOPs), test instructions, manufacturing instructions, packaging instructions, specifications, calibration instructions, or maintenance instructions.

On the lowest level are the records that are partially filled by hand. This includes all documents from verification of the sampling, the printouts of the processed raw data up to the printouts of the final report of an analysis, a method development and validation, or for example a certificate of analysis (CoA).

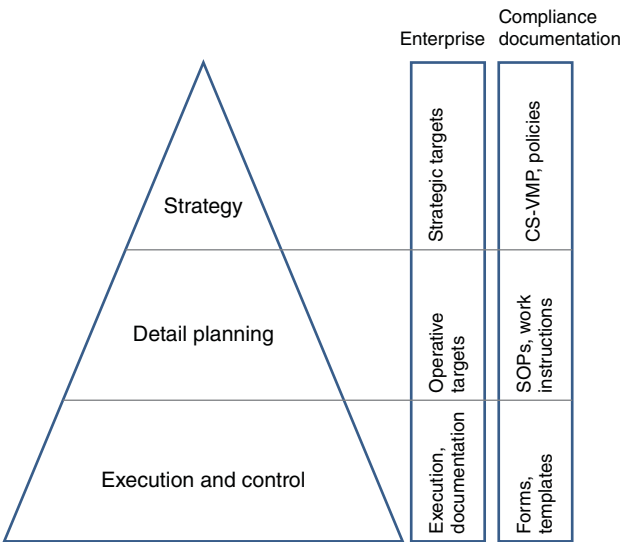


Figure 8.1 Hierarchical document structure in regulated organizations.

8.2.2

Documentation from the Process Point of View

Since documentation is a document creation process, it makes sense to describe the documentation in an organization by a process model. Such a process model that has been developed in the late 1980s as part of the total quality management (TQM) is the SIPOC model. The SIPOC model is independent of organizational or departmental boundaries and offers an overarching vision on the interactions involved in the processes. The acronym SIPOC stands for Supplier-Input-Process-Output-Customer and answers the following questions:

- Supplier: Who delivers?
- Input: What and how is it delivered?
- Process: What is done with it in the process?
- Output: What is the result of the process?
- Customer: Who gets the result in what form?

For the list of the process steps in which documents are created, it pays to consider the entire information chain and list it for instance in tabular form (see Table 8.1).

With this model, the complete information chain of a laboratory is made visible starting from the suppliers to the customers and valuable insights are provided for handling of documents that contain the essential data and information. For the proper treatment of the documents during creation, modification, distribution, and archiving, it is necessary to determine in which data formats they are available. Hence, it can be decided in what manner it should be possible to keep the data accessible, recoverable, and legible during the retention period.

Case 2 Box: Practical Tips for Identifying the Process Steps and the Resulting Data Formats

Work flow	Reference document	p ^{a)}	E ^{b)}	Data medium/format
Sample preparation	Test procedure	x		Paper
Sequence compilation	Sample set	x	x	Paper/SQL-DB-format
Instrument control	Instrument method	x	x	Paper/SQL-DB-format
Data acquisition	Method set	x	x	Paper/SQL-DB-format
Signal detection	Processing method	x	x	Paper/SQL-DB-format
System suitability test	Processing method	x	x	Paper/SQL-DB-format
Data analysis	Processing method	x	x	Paper/SQL-DB-format
Reporting	Report method	x	x	Paper/SQL-DB-format

a) Paper format.

b) Electronic format.

Table 8.1 Analytic process in an HPLC laboratory, according to the SIPOC-model.

Supplier	Input	Process	Output	Customer
Customer	Analysis work order	Sample reception	Labeled samples stored in the proper place, or database list of all samples received	Test laboratory
Sample reception	Storage and sample information	Sample assignment and supply	List of samples with assignment of the tests, storage, laboratories, or LIMS database	Test laboratory
Test laboratory	Test procedure	Sample preparation	Various raw data, such as weight, dilutions, mixing protocols of solutions, reference substances	Test laboratory
Test laboratory	Test procedure	Sequence compilation	Analysis sequence table with details about vial positions, sample name, standards, system suitability testing (SST) solutions, sample type, calibration information, sample volume, dilution factor, injection volume, method ID	Test laboratory
Test laboratory	Test procedure	Instrument control	Instrument method with information on flow rate, mobile phase composition, injection volume, column temperature (1 : 1 compilation of the testing instructions)	Test laboratory
Test laboratory	Test procedure	Data acquisition	Primary electronic raw data (source data) in the manufacturer-specific format (proprietary data)	Test laboratory
Test laboratory	Test procedure	Signal detection	Integration method (electronic and/or paper)	Test laboratory
Test laboratory	Test procedure	System suitability testing (SST)	System suitability test with evaluation protocol	Test laboratory
Test laboratory	Test procedure	Data analysis	Reporting templates	Test laboratory
Test laboratory	Reporting templates	Reporting	Printout or pdf files of evaluated chromatograms, peak lists, graphics, calibration data, result data	Test laboratory
Test laboratory	Specifications	Evaluation of results	Evaluated results, graphs in data bases/lists	Test laboratory
Test laboratory	Evaluated results, graphs in data bases/lists	Information to customer	Test report, certificate of analysis (CoA)	Customer

Table 8.2 Communication interfaces, according to the SIPOC model.

Interaction between ...	Documentation	Communication channels
Supplier and input	Internal creation of documents or database entries	Internal document creation and storage on a network drive or paper folders
Input and process	Transfer document, for example, sampling protocol or analysis order and so on, with handwritten entries, or entries in ERP and LIM systems	In the case of paper documents, the shipment of the sample and document is simultaneous, or alternatively send as an email attachment (scanned or real electronic document); in the case of databases (ERP), as a push/pull process data can be fetched [pull method] or be initiated actively [push method]
Process and output	Meta-data, raw data in different formats	Storage of data on network drives, storage of data in laboratory worksheets or lab journals, sending files as email, or sending-out links
Output and customer	Report (results), for example, certificate of analysis (CoA), stability report	Distribution by email (password-protected, encrypted, or unprotected), or providing on protected drive (VPN Internet)

The diversity of data formats described here led in the past to the situation that the users printed-out all the necessary information to achieve traceability and archived it in paper form during the legal retention period. An alternative would be to convert the print data into long-term archivable PDF/A-1 formats. The topic of the different formats of the source data is discussed in the following section.

8.2.3

Documentation from the Communication Point of View

If the SIPOC process map, as described in the earlier section is available, it is important to embed all communication partners in the overall process. Four connection points (interfaces) exist in this simple process image (Table 8.2).

The subtopic communication already reveals a major disadvantage of hybrid systems, that is, the integration of paper-based information and electronically available documents, as media breaks occur at the connection points (interfaces). In addition, long recovery times for documents arise, because of different system-related storage practices.

Case 3 Box: PDF – Formats for Long-Term Archiving (PDF/A-1, PDF/A-2, PDF/A-3) [4]

- In 2005, the ISO (International Standards Organization) published the PDF – PDF/A-1 standard (ISO 19005-1:2005) based on the PDF version 1.4, which could be produced with the then common ADOBE Acrobat™ 5.0 software.

- PDF/A-1 files facilitated the long-term readability and recoverability, regardless of tools and systems used for production, storage, and reproduction.
- Adobe™ 8, Microsoft Word 2007 and OpenOffice version 2.4 allow the storage in PDF/A-1 standard. In addition, there are numerous other commercial providers that support this long-term standard.
- All documents with character-, pixel-, and vector-graphics can be reproduced with the PDF/A-1 standard, because all the elements, such as fonts, are embedded in the file.
- The PDF/A-2 standard was released in 2011 (ISO 19005-2:2011) and is no longer based on an ADOBE PDF version, but on the standard ISO 32000-1 (PDF standard).
- PDF/A-2 has the advantages of JPEG 2000 compression for scanned documents or that collections of PDF/A-1 – documents (scanned documents) can be combined in a container PDF document, or even support of different levels (layers) in documents. Furthermore, the requirements for customer metadata (XMP) have been defined.
- In 2012, the standard PDF/A-3 (ISO 19005-3:2012) has been released, which allows to save the original source files, for example, XML, CSV, XLS, DOC, CDF, CAD, JPEGs, together with the long-term archiving enabled PDF/A format document in a container.
- Thus, PDF/A-3 has at least the advantage of storing the different file formats in one archive container, but this is not (yet) a guarantee for long-term readability of the embedded source files. This makes it possible, for example, to archive emails together with its annexes in different data formats in one container or chromatographic data with its source files in *.cdf format.

The use of standards, such as long-term archivable PDF/A-1, PDF/A-2, and PDF/A-3 in conjunction with standard protocols for chromatographic raw data (*.cdf derived from NetCDF), plays a crucial role in solving the above-mentioned challenges. The PDF standards, which were published by the ISO organization, allow the long-term archiving of all previously printable data in PDF/A-1 – format. The handwritten information available in paper form, for example, laboratory forms, or copies of lab notebook pages can be scanned as required and added to the processed data in compressed form (PDF/A-2). The PDF/A-3 standard, published in October 2012, allows the embedding of source files along with PDF files in a container, which can fill an important gap between the primary electronic (source data) and the secondary electronic data (processed data). The standardization of chromatographic source files has been pursued since 1988 for different motives over the last decades, by the chromatography instrument manufacturers (AIA, *Analytical Instrument Association*; ref. ASTM, ANDI). Software maintenance of the resulting and freely available program library NetCDF is taken over by Unidata, which is part of the American Association for Atmospheric Research (*University Corporation for Atmospheric Research* (UCAR), sponsored by the American “National Science Foundation”).

In 2009, ASTM Standard E1947–98 (2009) “*Standard Specification for Analytical Data Interchange Protocol for Chromatographic Data*” has been published by the ASTM. The specified objectives of this standard are

- 1) data transfer between instruments of different manufacturers;
- 2) data exchange with laboratory information management systems (LIMS);
- 3) connecting the data with document management systems (DMSs), for example, for regulatory submission purposes;
- 4) connecting the data with spreadsheet applications;
- 5) archiving of analytical data (with the possibility of subsequent processing).

The archiving standard PDF/A-3 and the unification of source standards for chromatographic data, together, generate a real future opportunity to ensure independence of manufacturers and systems for chromatographic data as report data, including all metadata, as well as their processing (reprocessability). With the realization of the above-mentioned options, the demands of some auditors are obsolete to keep older computers, software, and expertise in a validated environment, to possibly have access to older data.

8.2.4

Documentation from the Information Point of View

Information can be derived from data. The nature of the information obtained depends on the type of data being used. If it is meta-data (data about data), it can provide information that is necessary for the traceability of all processes. Is it result-related data, like raw data, for example, primary electronic raw data such as chromatography data files on the hard disk of the measurement computer, it can be associated with sample preparation data to calculate quantitative data, which are used in subsequent steps of the information generation process.

The following considerations apply to the verification documents at the lowest level of the document hierarchy shown in Section 8.2. The main objective of the verification documentation is that for all GxP-relevant processes traceable information is generated, accessible for the legally prescribed retention period. The purpose of the following meta-data model is to enable the complete traceability of the information and data, using the meta-data. From this information, no results can be derived, but they are an indispensable part of the raw data.

In batch-oriented quality management systems, such as good manufacturing practice (GMP), the batch number of an active substance or a product is the main index variable, where the entire information about sourcing of materials, manufacturing, analysis (control), packaging, and distribution is attached.

In study-based quality management systems, such as good laboratory practice (GLP) and good clinical practice (GCP), the study number is considered as the main index variable and any other information along the meta-data model can be derived from it. With the help of the meta-data model (see Box: Meta-Data Model), it can be checked easily if the information of the documented GxP-relevant process is complete.

Case 4 Box: Meta-Data Model	
1) WHO	• Operator
2) WHEN	• Date/time
3) WHAT	• Sample/material/batch
4) HOW	• Method/process
5) WITH WHAT	• Instrument/system/equipment
6) WHERE	• Filed path/location of data

A typical HPLC laboratory workflow is exemplified in Table 8.3, specifying which meta-information is usually communicated and must also be filed with the actual measurement data in order to ensure traceability.

Each machine printout or manually generated entry should be checked by the meta-data model to ensure traceability. Only when all six questions can be fully answered, the data are traceable in the sense of authority requirements.

In addition, GMP requirements in the pharmaceutical industry demand proving of *accuracy, completeness, and compliance* with internal and external requirements. This control process consists of complex manual steps, which in turn must be documented in the raw data.

8.2.5
Documentation from the Knowledge Storage Point of View

The traditional paper filing systems allow the preservation of knowledge in static form. If the data in paper form are archived, it is possible to directly reproduce the data in human-readable form, over a long period of time. The process of recovery of the data from the archive and the comparison with the current data, however, is complex and the electronic version inferior in several ways.

For real retrospective data analyses, such as stability tests, it is often necessary to compare data from different measurement points with each other. For this purpose, the use of electronically stored data is advantageous, because it is very easy to superimpose and compare chromatograms and search within the databases. However, electronic filing systems, for example, file or database formats, provide some challenges regarding the recoverability of the data during the regulatory retention period (see Section 8.2.4).

Forward-looking strategies for knowledge storage will focus on the availability of the electronic source data of the measured chromatograms (LC, UV, diode array, mass spectrometric data). Similar to the PDF-ISO standards for secondary electronic data, chromatographic information will be available as NetCDF-data

Table 8.3 Meta-data model of data traceability in an HPLC laboratory.

Type	Process step	Document Paper or electronic?	Meta-data 1) Who 2) When 3) What 4) How 5) With what 6) Where
	Logistic Sampling	Sampling protocol	1) Person drawing samples 2) Date of sampling 3) Sample ID, amount of sample 4) Sampling procedure 5) Sampling device 6) Filed path/location of sampling protocol
	Logistic Sample shipment	Shipment documentation	1) Sender, shipper 2) Date of shipment 3) Sample ID 4) Terms/conditions of transport 5) Shipment monitoring 6) Filed path/location of shipment information
1.	Sample receiving/transport/storage	Documentation of receipt	1) Receiver ID 2) Date of receipt 3) Sample ID, amount of sample 4) Storage conditions and storage location of samples 5) Equipment and room IDs of the storage locations 6) Filed path/location of receipt information
2.	Analysis job	Written order	1) Customer 2) Date of analysis order 3) Sample ID and definition of analysis 4) Assignment of methods 5) Assignment of measuring instruments (laboratory) 6) Filed path/location of written order
3.	Sample preparation	Test procedure/test protocol	1) Operator 2) Date of sample preparation 3) Sample ID (batch) 4) Number of test instruction 5) IDs of used instruments, standards, und solutions 6) Filed path/location of raw data on sample preparation
4.	Definition of acceptance limits for system suitability testing (SST)	SST specification for SST method	1) Operator 2) Date of specification 3) Type of SST parameters 4) Type of algorithms used for SST calculation 5) Software module for computing SSTs 6) Filed path/location of SST data

(continued overleaf)

Table 8.3 (Continued).

Type	Process step	Document Paper or electronic?	Meta-data	
			1) Who	2) When
			3) What	4) How
			5) With what	6) Where
5.	Sample assignment and provision	Sequence table	1) Operator	2) Date of sequence
			3) IDs of references and samples	4) Assignment of vial number, sample name, sample type, calibration data, sample amount, dilution factor, injection volume, method ID
			5) Instrument ID	6) Filed path/location of sequence table
6.	Instrument control	Method	1) Operator	2) Date of method generation
			3) Statement of instrument parameters: for example, flow rate, mobile phase composition, injection volume, column temperature, wavelength	4) Method ID ^{a)}
			5) Instrument ID	6) Filed path/location of method
7.	Data acquisition	Instrument method	1) Operator	2) Date of instrument method
			3) Statement of data acquisition parameters: for example, measuring time, data rate, channel, location, storage formats	4) Instrument method ID
			5) Instrument ID	6) Filed path/location of instrument method
8.	Signal detection	Integration method	1) Operator	2) Date of integration method
			3) Statement of signal detection parameters: integration window, start peak, end peak, area reject, threshold, type of integration method (Tangent Skim, Peak to Valley, and so on)	4) ID of integration method
			5) Instrument ID	6) Filed path/location of integration method

Table 8.3 (Continued).

Type	Process step	Document Paper or electronic?	Meta-data 1) Who 2) When 3) What 4) How 5) With what 6) Where
9.	Data analysis	Method of data analysis	1) Operator 2) Date of data analysis method 3) Type of data analysis, regarding calibration and calculation of results 4) ID of data analysis method 5) Instrument ID 6) Filed path/location of data analysis method
10.	Reporting	Reporting template	1) Operator 2) Date of reporting method 3) Qualitative and quantitative information, related to sample ID 4) Reporting template ID 5) Instrument ID 6) Filed path/location of reporting method
11.	Evaluation (comparison of the results with specification)	Certificate of analysis (CoA)	1) Operator 2) Date of result evaluation 3) Decision about in-specification or out-of-specification for sample (batches) 4) Comparison with specifications 5) ID of the used tools 6) Filed path/location of evaluated data/version
12.	Information to customer	Fax or pdf document	1) Operator 2) Date of result submission to customer 3) Contents of document 4) Path of submission, for example, email or fax 5) Device ID for result submission 6) Filed path/location of submitted document/archive

a) Ideally identical to the original name.

according to ASTM standard, really system-comprehensive, platform- and vendor-independent. The deletion of previously collected primary electronic data (source data) is not recommended here, because they will potentially be converted in the future via a converter program into long-term human readable formats.

8.2.6

Regulatory Requirements for Laboratory Documentation

The regulatory requirements aim at the controlled handling of documentation to achieve complete traceability of information over the regulated processes. The most widely used quality management systems in laboratories of pharmaceutical, biotech, medical device, and food (USA) industries are GMP, GLP, and ISO 17025 for accredited laboratories. The latter mentioned system is not a legal requirement, but an international standard, which is inspected by commercial certifiers.

For the batch-based quality system GMP, the following expectations of the authorities are relevant: The rules for documentation of the EU GMP Chapter 4

Table 8.4 Comparison of the requirements for documentation under GLP versus GMP regulation.

Requirement	Good laboratory practice (GLP)	Good manufacturing practice (GMP)
Legal regulations in USA, Europe (EU), Germany (D)	USA: 21 CFR 58 [5] EU: OECD "GLP" D: Chemicals Act (German: Chemical Law), section 6	USA: 21 CFR 210, 211 [6] pharmaceuticals/drugs USA: 21 CFR 110, 111 food and dietary supplements EU: EU GMP guidelines D: AMG (German Pharmaceuticals Act), AMWHV
Focus	Traceability of data	Safety, efficacy
Orientation	Study-based QM system	Batch-based QM system
Application in USA, EU, D	Nonclinical safety studies, for example, toxicology and PK studies (ADME), and analysis of body fluids from clinical trials	USA: Food, pharmaceuticals/drugs, medical devices, veterinary products, cosmetics EU, D: Pharmaceuticals/drugs
Legally responsible persons	Manager of the facility, Study director, Head of Quality Assurance, Archiving manager	USA: Quality assurance, management EU: Qualified Person (QP) D: Qualified Person (QP), Head of Production, Head of Quality control, Quality Management, Upper management
Worldwide inspections	FDA EMA Federal state authorities of Germany	FDA EMA Federal state authorities Germany (GMP inspectorates)
Expected retention periods	USA: depending on phase EU, D: 15 years CH: 10 years	USA: EU, D: at least 1 year after the expiration date, in practice at least 10 years

“Documentation” and “Computerized Systems” regulated in Annex 11 [7, 8]. For the United States, there is a special legal guidance that describes the expectations of the laboratory records, 21 CFR §211.194 “Laboratory Records”[9], and 21 CFR Part 11 “Electronic records, electronic signatures”[10].

In general, specification and verification documents can be distinguished, while in practice mixed forms of both document types exist. A typical specification document in the laboratory is a test specification, which contains all the requirements for carrying out the analysis starting with the mixing of solutions and reagents to the calculation of the analytical results. As a typical example of a verification document (record), a weighing record can be quoted. A mixed form of specification and verification documents is often found in test protocols that contain both requirements and relevant attestations [11].

In summary, the following requirements have to be fulfilled:

The document control has to describe all activities of creation, review, approval/authorization, distribution, modification and versioning, withdrawal, and archiving, and make it traceable for all quality review activities.

In practice, particularly records are subjected to a thorough review by a second person (four-eye principle), which proves the accuracy, completeness, and compliance with internal and external requirements. For electronic data, besides accuracy, integrity, availability, and human readability have to be safeguarded (Table 8.4).

8.3

The Life Cycle Model for Regulated Documents in Practice

The life cycle model of regulated documents officially starts with the planning of a document and ends with the withdrawal or deletion. Each individual document in the analysis process contributes to the traceability and quality of the result. Therefore, at least the following points need to be defined for each document (see Table 8.5).

Once created by the author, reviewed by quality management, and released/approved by management, specification documents, for example, SOPs are valid. They are distributed to the area of application (scope), relevant personnel are trained and the document can then be applied. After application, the related verification documents are collected, organized, and relevant parts thereof are published, for example, a CoA as an extract from a set of data, which in turn consists of a further set of raw data and meta-data.

Looking at this document life cycle from the perspective of a pure paper-driven laboratory, it can be seen that a significant amount of work is generated by the purely manual execution of these steps. For organizations that already operate an electronic document management system (DMS), there is the advantage that all steps can be conducted highly automated and with a minimum of effort, that is, media breaks can be avoided, consequently.

Table 8.5 Required definitions for documents.

No.	Subject	Example
1.	Data medium	Paper or electronic record
2.	Data formats, in the case of electronic records	PDF/A-1 PDF/A-2 PDF/A-3, and TIFF are considered to be long-term recoverable data formats, CDF for chromatographic data
3.	Author	Author of the document
4.	Review	Quality and technical review of the document
5.	Approval/release	Approval and release of the document
6.	Version control for changes	Versioning and change history of the document
7.	Review of the document	Reviewer checks for accuracy, completeness, and compliance with internal and external requirements
8.	Collection/structuring of documents	Aggregation of the data belonging to a batch analysis or a study
9.	Publication of documents	Distribution of verification documents of the laboratory, publishing the data in report form, for example, to customers, authorities, other clients
10.	Life cycle of documents	Definition of the intended duration of the availability of the document in human-readable form
11.	Storage/archiving	Definition of location and type of the archive. Ensuring long-term archiving and recoverability for the planned retention period

Even in organizations that operate a DMS, it is part of the reality that not all raw data are processed electronically, but usually only a small part of the data appears in electronic formats. Often, organizations begin to convert the essential specification documents such as SOPs into electronic formats, but this in turn leads to a hybrid situation, that is, the coexistence of paper and electronic documents.

In general, for regulated documents, document life cycle applies (see Figure 8.2):

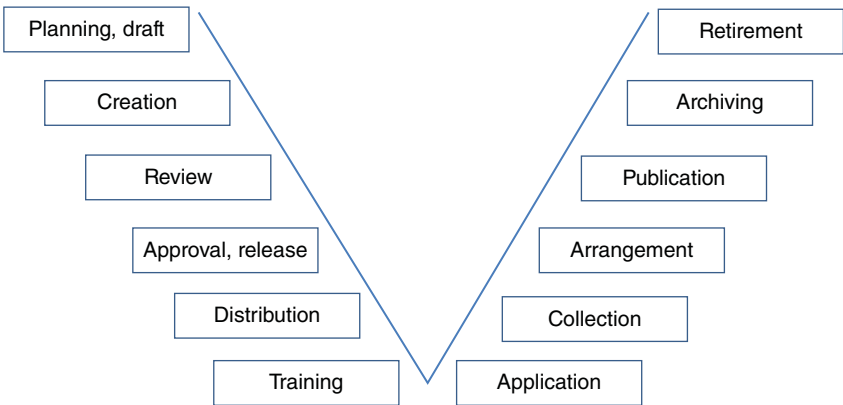


Figure 8.2 Document life cycle of regulated documents.

8.4

Dealing with Hybrid Systems Comprising Paper and Electronic Records

8.4.1

Advantages and Disadvantages of Paper Versus Electronic Documents

This section describes the steps that are required to define an appropriate strategy for dealing with mixed document systems (hybrid systems) of paper and electronic documents. Table 8.6 shows the advantages and disadvantages of using paper and electronic documents.

In hybrid systems, time-consuming media breaks often cannot be avoided. In some cases, electronic documents are printed, signed manually, scanned, and printed out completely with the signature page again. This is classic duplication of work, which could be avoided by using DMS.

Although for many years there are rumors about paperless offices and laboratories, it can be stated that more than ever paper is used for documentation purposes. But the road is mapped-out: similar to the field of photography where most images are incurred nowadays in electronic form, more electronic data will be generated and stored for regulatory purposes in the future. In principle, the same requirements apply to electronic documents as to paper documents; however, electronic documents have high demands for data security (see Table 8.6). In IT systems, access protection is ensured, for example, by user identity and password. Verification of the data integrity in IT systems can be performed very quickly and efficiently by data integrity checks using checksums (e.g., md5). For example, it is possible to detect data corruption due to tampering or disk errors.

Table 8.6 Overview of the dependencies of paper and electronic records.

Paper documents before archiving	Electronic documents before archiving
• Only one original • Cost of distribution by copies • Great effort for change process • Paper distribution slow and laborious • Long search times • Cost- and space-intensive intermediate storage	• High availability • Good integration • Searchability
Archived paper documents	Archived electronic documentation
• Access security • Immutability • Recoverability • Technological independence • Manual authentication	• Data security (physical and logic) • Data integrity and data medium aging • Recoverability • Electronic authentication

The recoverability and readability of electronic data depends on two factors:

- hardware for reading the media and
- software to ensure the readability of the data format.

On one hand, adequate reading devices must be available for this purpose, otherwise the data are worthless. On the other hand, suitable software must be able to make data formats readable.

If the primary electronic data (source data of the chromatograms) of a certain HPLC system exist in a vendor-specific (proprietary) format, sooner or later, the manufacturer will announce with a system update that the “old” data format is no longer supported.

In principle, three scenarios are possible when it comes to the consideration of the long-term archivability and readability of previously collected electronic information:

- a) Data are readable without converting (e.g., NetCDF as *.cdf files).
- b) Data can be restored only after conversion, which possibly goes along with a small loss.
- c) Data are no longer readable after changing the hardware/software by the supplier.

In case (a), the user would have no problem with the readability of the “old” data, because the new updated system (or a vendor-independent generic system) allows the recovery of data.

In case (b), the user has to conduct a data migration, according to a validated procedure.

In case (c), the data can no longer be restored in the chromatography data system (CDS) after an upgrade; this case can be compensated in several ways:

According to the FDA guideline “*Scope and Application*” published in 2003, it is possible to delete the underlying original data in proprietary data format, if content and meaning of the data are preserved and archived on a different platform (paper, vendor-independent electronic format, e.g., PDF/A-1).

... any copies of the required records should preserve their content and meaning. As long as predicate rule requirements are fully satisfied and the **content and meaning of the records are preserved and archived, you can delete the electronic version of the records**. In addition, paper and electronic record and signature components can coexist (i.e., a hybrid situation) as long as predicate rule requirements are met and the content and meaning of those records are preserved ... (citation from FDA directive “*Scope and Application*,” 2003) [12].

The PDF/A-1 format is according to ISO standard 19005 “*Document management – electronic file format for long-term preservation*” considered as long-term

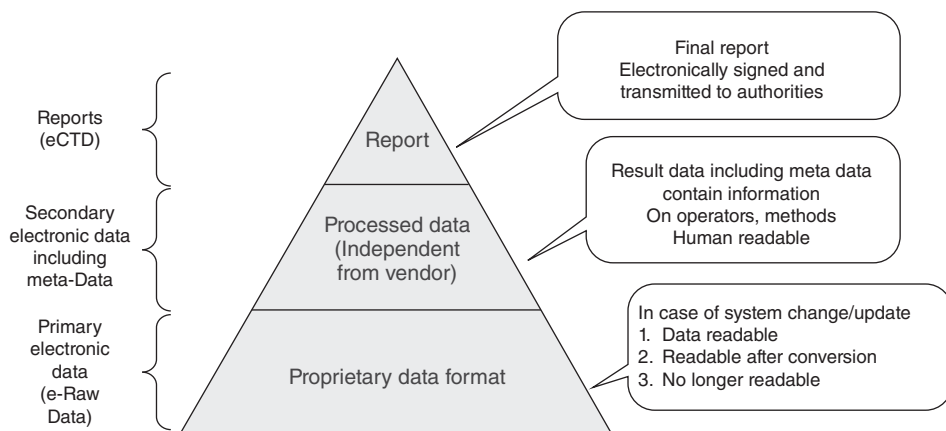


Figure 8.3 Data model of instrumental raw data, calculated result data, and report data.

archivable format and thus provides an elegant way to avoid paper documents and instead produce genuine electronic data that are independent of the manufacturer, for all applications that have been printed-out so far. Adobe™ has disclosed this format, so that this data format can actually be recovered in the future with any suitable pdf-reader (Figure 8.3).

8.4.2

Implementation Strategy

Based on the presented requirements and constraints, as well as the experiences gained in practice, different approaches to the challenges of the integrated documentation of paper and electronic records arise, depending on the technology level of an organization.

Three basic implementation strategies can be identified:

- a) organization with focus on paper-based documentation
- b) organization with “*isolated applications*,” using hybrid systems
- c) organization that is consistently aligned to integrated electronic solutions.

Strategy (a) represents the least sustainable model, because it consciously accepts the fact that the documents archived in paper form, already exist in an electronic format, and thus accepts media discontinuity, including high costs of production, distribution, modification, cancellation, redistribution, archiving, and not least their recovery.

Strategy (b) is found in organizations that aim on automation in some areas and are already working with partial solutions such as LIMS, with or without online instrument connections such as ... “*Enterprise Resource Planning*” (ERP),

DMS, and ELN “*Electronic Lab Notebook*.” These solutions must be operated in a validated environment, resulting in a very high burden during the design and implementation phases. In addition, a number of different electronic manufacturer-specific data formats are created, besides paper documents.

Strategy (c), that is, the consistent focus on purely electronic documentation has been realized only by a few companies. The benefits that result from this model outweigh the costs of implementation of such systems in a regulated environment. The consistent implementation of such systems includes the use of subsystems, as electronic signatures that comply with regulatory requirements. The digital signature must contain the following elements: the reason of the signature, date and time, time zone, and the full name of the signatory.

The biggest initial gain in the transition from paper-based to electronic documentation can be achieved by laboratories that take the decision to convert a paper-intensive process step into an electronic form. That means many companies opt for a gradual implementation in the form that the paper printouts of chromatograms are replaced by PDF/A-1 files. So step by step, the single documentation steps are converted from paper to electronic format and in the end will form an integrated solution that deserves the labeling “electronic documentation.”

8.5

Preview

In fact, regulatory authorities responsible for pharmaceuticals and medical devices more commonly request electronic registration documents, and it can be predicted that there will be no more traditional paper filings in the near future. First, for competitive reasons (time to market), and second due to time and cost aspects, because the electronic versions of registration documents allow the authorities representatives to copy and paste operations, search the documents, as well as analyze and integrate data by programs of the authorities.

It may be assumed for the future that owing to standardization of source data (NetCDF, *.cdf) and availability of source data in the PDF/A-3 format, the authorities will not only look at chromatograms but also reanalyze the data, such as detection or quantification limits to check or verify peak purity. In connection with audit trails used in the laboratories, such submitted chromatograms are clearly traceable and laboratories will be as transparent as never before.

The major disadvantages of “pure” paper solutions and hybrid systems demand for greater efforts toward comprehensive electronic solutions and it is a matter of time until separate systems such as LIMS, with online device connection ERP, DMS, and ELN will be integrated. In my personal opinion, as there is a PDF-reader for documents, there will be a CDF-reader for chromatographic data that makes chromatographic data independent of manufacturer, system, platform or age of the data, and allows chromatographic source data to be visible and processible.

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9

Tips for a Successful FDA Inspection

Stefan Schmitz and Iris Retzko

9.1

Introduction

The US health authority *Food and Drug Administration* carries out domestic and foreign inspections to investigate whether an organization works in accordance with the US law (*Code of Federal Regulations*, CFR) and international guidelines. For the production and control of classically and biotechnologically manufactured pharmaceuticals, the American laws *Current Good Manufacturing Practices in Manufacturing, Processing, Packaging or Holding of Drugs: General* and 21 CFR Part 211 *cGMP Current Good Manufacturing Practices For Finished Pharmaceuticals* constitute the essential legal framework for manufacturers and test laboratories.

For the handling of electronic records and the use of electronic signatures, the law 21 CFR Part 11 *Electronic Records, Electronic Signatures* applies. In addition, adherence to a number of guidelines is mandatory, concerning the development, manufacture, packaging, labeling, storage, logistics, and testing. In the course of the submission of a new product to the US FDA, all participating companies and contractors must be inspected for compliance with these laws and regulations. In this case, all companies involved in the filing, for example, sponsors, contractors, contract manufacturers (*Contract Manufacturing Organizations*, CMOs), or contract laboratories (*Contract Research Organizations*, CROs) have to pass a successful inspection, regardless of where in the world the service is provided.

The FDA inspectors, so-called *FDA Investigators* perform the inspection on the basis of internal FDA procedures, the *Compliance Program Guidance Manuals*. Inspections of finished medicinal products for example are carried out according to the CPGM 7356.002 *Drug Manufacturing Inspections* and inspections prior to approval follow the CPGM 7346.832 *pre-approval-inspections*. It falls to each inspector or team of inspectors, as well as the *District Office* in conjunction with the *Office of Regulatory Affairs* (ORA), which inspection approach is followed. This depends, among other things, on the education and qualifications, experience, the training received, and the relevant preference for certain core issues and cannot be predicted.

Usually, two inspectors perform a preapproval inspection (PAI), but it is very common in recent times that only one experienced person takes over the entire inspection, especially when follow-up inspections are conducted. Is there a team of two inspectors (female or male), one is usually responsible for the inspection of the quality systems, the other for the technical and scientific aspects.

Usually, the inspectors stay abroad for 3 weeks, and perform inspections at three to five different organizations. A primary source of information for foreign inspectors regarding inspection regulations is the inspection manual *Investigation Operations Manual 2014*. Another important basis for aspects of traveling is the *Guide to International Inspections and Travel, updated version 2009*.

Manufacturers and subcontractors (CROs, CMOs) that produce for the US market are obliged to register¹⁾ with the FDA and to disclose the activities related to production and testing. It is important for the inspection activities of the FDA that all companies are listed in the FDA database with accurate, complete and current information. Besides other information, the company name, company size, address, business activities, and the inspection status are recorded. Almost every FDA inspection includes the clarification of the registry and if it is not already done, it is required to start the registration process during the inspection. Other important functions of the FDA database are to inform politics and the public about the type and number of regulated companies and create budget plans for various compliance programs. A public database of the FDA for the representation of registered companies²⁾ is available to all interested parties. Here, the company name, the FEI number *Firm Establishment Identifier*, the DUNS number *Data Universal Numbering System*, address, and topicality of registration can be accessed.

As part of the preparation, it is important to clarify with the FDA, and/or licensing partners, customers, which products are focused by the planned inspection to enable concentrated preparatory activities on these product(s). In the case of an initial inspection, besides the inspection of the product-related issues, a general and coherent good manufacturing practice (GMP) inspection is carried out in the target organization. This may well mean that other areas that are regulated by the Act 21 CFR 211, are inspected, because the inspection focuses on all products or processes potentially destined for the American market.

9.2

Preparation with the Inspection Model

To support the preparation of an FDA audit in the laboratory, it seems reasonable to use a structured inspection model (see Figure 9.1), which categorizes the topics to be inspected, and it allows to distribute the work packages to several project members. All elements of this model have potential influence on the product and result quality and are, therefore, critical to the safety and efficacy of the product.

1) <http://www.fda.gov/ICECI/Inspections/FieldManagementDirectives/ucm096034.htm>.

2) <http://www.accessdata.fda.gov/scripts/cder/drls/default.cfm>.

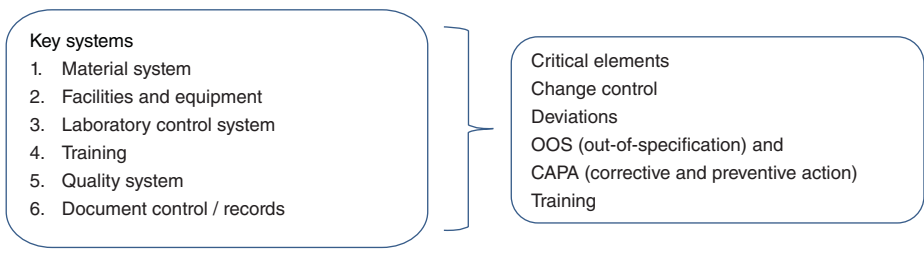


Figure 9.1 Inspection model for structuring the topics.

For understanding the key relationships and to explain complex issues, it is advantageous to create a historical overview of the projects and products that shows who have worked together at what time with which partners to develop, manufacture, store, process, or test the product. Sometimes, it is also necessary to de-archive older documents to be able to clarify historical facts. Sometimes, personnel, who were significantly involved in the project in the past, are no longer available, so that this historical analyses are of particular importance.

9.2.1

Materials, Reagents, and Reference Standards

This includes all incoming goods, materials, reagents, solutions, reference standards, and mobile and stationary phases that are relevant for the product of interest. The material flow, warehousing, containers used, labeling, testing, or the establishment of shelf-lives are typical questions on this group of themes. Furthermore, the concepts of identification, labeling, and traceability have to be made transparent, from delivery to usage or disposal. All software systems that have to do with the identification, registration, storage, redistribution, are subject to inspections that aim to prove the suitability of these systems (validation).

Case 1 Box: Practical Tips on the Subject: Materials, Reagents, Reference Standards
• Delivery notes of materials received shall be provided on request. • Goods receipts are clearly documented (paper and/or electronic version). • All reference substances are tested for validity, certificates are complete, and invalid substances are removed. • Material safety data sheets are available for all reference substances. • Mixed solutions, reagents, and mobile phases are clearly documented; all used test equipment such as balances, pipettes, refrigerators, and freezers are identified and calibrated. • Reagents, solvents, and mobile phases are controlled regarding expiration date and replaced if necessary, including laboratory water and other solvents.

- Samples, reagents, solvents, and mobile phases on laboratory benches and shelves are labeled clearly and in accordance with (legal) requirements.
- Samples and specimens in refrigerators and freezers, are checked, for example, for expiration date, clear labeling.
- Labels on solvent and reagent flasks are as specified, for example, not manually overwritten.
- Reference standards of the US Pharmacopoeia (USP) must be used, even if there are other alternatives.

9.2.2

Facilities and Equipment

For analytical instruments of a quality control laboratory, which are under GMP generally referred to as equipment, the evidence of qualified operation, qualification, and, for computer-based systems, a validation, have to be provided. The equipment, facilities, and systems must be described by appropriate system documentation and it is expected that in the vicinity of the system a system binder and logbook provide information on the qualification and/or the calibration status. In general, inspectors ask about the frequency of calibration intervals, the accompanying documentation, and the training of responsible personnel (system owner, equipment manager). If changes of the test equipment are carried out, this should be documented as part of the controlled change management (change control). It is useful to categorize the equipment according to chapter <1058> *Analytical Instrument Qualification* of the USP:

- A: Exempt equipment, such as magnetic stirrer, vortex mixer, calculators, LCD timer.
- B: Calibrated equipment, such as balances, pipettes, pH meters, refrigerators, and freezers.
- C: Computer-based systems, such as modular or compact HPLC instruments, spectrometers.

Case 2 Box: Practical Tips on the Subject: Facilities and Equipment

- Access controls for the building and all relevant subcompartments are enabled.
- Laboratory coat is worn; safety glasses are carried in the jacket pocket and worn where required.
- Visitor coat and glasses are kept ready for the inspectors.
- Hygiene requirements (e.g., cleanroom locks, disinfection) also apply for the inspectors.

- All equipment binders/logbooks are checked for good condition and maintained up to date.
- A validation master plan that contains concepts of qualification, re-qualification, risk assessment, validation, maintenance, periodic inspections, and system retirement is kept.
- All laboratory equipment is included in an inventory list that has at least the following attributes: device ID, location, person responsible for the equipment, purpose of use, reference to SOPs.
- Lab equipment is categorized according to USP <1058> into three classes (A, B, C).
- Qualification status of the relevant equipment is checked.
- Calibration and maintenance status (SOP) of the relevant equipment is checked, which also includes refrigerators, freezers, drying ovens, incubators, balances, dilutors, pipettes, and so on.
- For computerized systems, risk-based validations are available that cover access controls (data security), audit trails, viewing the data formats, recovery, handling in case of failure, backup and archiving, data integrity.
- Calibration documents of reference equipment are maintained.
- The person responsible for the equipment is able to present clear audit trail functions and records for a past time period on the screen.
- The screen-lock is active or a password-protected screensaver is set automatically after a defined time (e.g., company logo as screensaver).
- Equipment that is expired or no longer in-use is labeled as "Rejected" or qualified before the inspection.
- It should be possible to retrace and explain recent as well as former changes (Change Control).
- The persons responsible for equipment are trained for the presentation of important laboratory equipment (e.g., HPLC, balances, pipettes, pH meters, refrigerators).
- Wastepaper baskets are emptied to avoid discussions about raw data disposal.

The persons responsible for equipment have, in this part of the inspection, the important role of demonstrating to the inspectors that all significant aspects of GMP are under control. The persons responsible for equipment have to fulfill the following essential tasks:

- 1) Documented briefing and training by the supplier ("in-house expert")
- 2) Issue of an SOP for operation, function testing, calibration, maintenance and cleaning of the equipment
- 3) Issuing and maintenance of an equipment logbook to document relevant events during the life cycle
- 4) Responsibility for periodic functional tests and calibrations
- 5) In-house training and documentation of additional equipment users

- 6) Updating of qualification documentation after modifications and repairs
- 7) Definition and training of a representative
- 8) Closure of equipment in the case of a violation of acceptance limits
- 9) Ordering and stocking of important consumables and spare parts
- 10) Presentation of the equipment during audits and inspections.

In practice, it is important that the responsible persons can perform these topics using work instructions and that the operation is compliant with established procedures (SOPs). It is not unusual that FDA inspectors interview two persons about the same issue (investigative interview), and observe real laboratory operations on site.

9.2.3

Laboratory Controls

The testing of incoming goods, intermediate materials, packaging, in-process controls, and final release are tasks of the test facilities, such as the quality control laboratories. The tests shall be carried out by validated methods, especially in later phases or shortly before regulatory submission. The method validation should be planned, conducted, and documented in accordance with the guideline ICH Q2 (R1): *Validation of analytical procedures: Text and Methodology*. All relevant methods should be checked for their validation status, as well as their validity. Method development, validation, and transfer generally have a high priority in FDA inspections. It is advisable to list the method development, the resulting validated methods and versions to make a historical comparison of the respective valid specifications so that in case of doubt, it can be shown at any time, which method has been used in which validation status for testing against a specification. Further assistance can be provided by the FDA guidance *Analytical Procedures and Methods Validation* (2000), which gives valuable information on the expected type of documentation for analytical methods.

Transfers of analytical methods are also evaluated very critically, and analytical transfer plans with defined acceptance criteria are expected, as well as related transfer reports. On this topic, the USP <1224> *Transfer of Analytical Procedures* offers some guidance. For assistance on the confirmation of pharmacopoeia methods, the chapter USP <1226> *Verification of Compendial Methods* can be looked up.

Case 3 Box: Practical Tips on the Subject: Laboratory Controls

- The test specifications are saved in the instrument method of the measuring equipment and can easily be identified by an ID or a homonymous name.
- Instrument methods *must* be identical to paper-based version.
- The current test procedure is located near the workplace.

- All samples in the autosampler of the HPLC are listed exactly in the order of the analysis sequence and include system suitability tests (SSTs) and calibration samples.
- Samples that are no longer needed (elapsed) in the autosampler are removed.
- The validation documents for the relevant methods are available in written form, are signed, and comprise a validation plan and report.
- The corresponding raw data undergo a meticulous review, during preparation phase.
- Key elements of a validation should be documented by raw data, for example, robustness, with respect to pH of the mobile phase, the mobile phase composition, the shelf life of reference samples, and the column temperature.
- If the method is stability indicating, this should be demonstrated by stress-test data, measured during method-development.
- The essential data for complete traceability (who, when, what, how, what, where?) are documented on the printouts of the analytical results.
- Work instructions for all aspects of stability testing are available (e.g., planning, logistics, implementation, evaluation, qualification of stability storage cabinets, respective storage rooms).
- Written stability-testing protocols are available for all relevant tests. They are dated and signed.
- If manual integrations are performed in automated data analyses, they have to be conducted according to a current procedure. If only manual integrations will be applied, this has to be described as well, using illustrated and comprehensive examples.
- Ensure that all essential calculations are reviewed by a second person to ensure accuracy, completeness, and compliance with the applicable regulations, and that this procedure is regulated and documented.

9.2.4

Personnel

The training of staff is an important element of compliance with legal and self-imposed quality standards and is based on the education and qualification of a person, considering the dedicated job profile. It is expected that the company conducts training plans for employees who are subjected to regular target-performance comparisons, to continuously determine the training status. The trainings regarding the GMP requirements have to be carried out periodically and should be documented. A proof of the training success is also currently expected by the inspectors. For persons who are engaged for a long time in an important function, but do not have a training certificate for their activities, a document

should be created that attests these many years of experience as *training on the job* to provide a training record for the inspection. This training record should be signed by the person, line manager, and quality management.

Each employee should take the responsibility to update his/her training records and should know where the training records are kept, as part of the inspection preparation. A chronological list of all internal and external trainings and the respective training certificates should be available at a central location. For all key personnel, an updated job description should be available, describing the actual tasks correctly and completely. The individual job description should be known precisely by each employee, because inspectors often ask people during their company-walkthrough about their job profile or what they are doing currently.

Case 4 Box: Practical Tips on the Subject: Personnel

- A training plan for the employees is available and is updated.
- Each employee should review his/her training records, regarding, for example, vocational education and training, GMP training, and update as appropriate.
- Job descriptions for all key personnel are in an updated form and reflect the actual responsibilities of each employee.
- For groups of employees function-related *collective job descriptions* may be defined.
- Annual GMP refresher-trainings are conducted.
- Specific trainings are available for all testing activities (test procedures, equipment training).
- Trainings of employees who were absent for a longer time, for example, maternity leave, have to be refreshed upon come-back.
- Each employee catches up on his/her current job profile before the inspection and can also reflect it.

9.2.5

Quality Management

The Quality Management department is expected to carry out regular target-performance comparisons of specifications and requirements, document the evaluations, and provide management with information on the results. For all possible discrepancies from the desired state, for example, complaints, results out of specification (OOS), changes, deviations, and audit results (internal and external audits), the appendant information are collected centrally and processed by means of the *Corrective And Preventive Action* (CAPA) system. These monitoring and control functions are performed by the staff of quality management.

It is very likely that during the inspection historical events of the above-mentioned topics will be surveyed and it is expected that the involved persons have evaluated the processes with scientific expertise and appropriate risk assessments. A controlled, informed, and trained handling of these critical issues is mandatory. Compliance with set review-cycles for specification-documents will be checked randomly and should be considered in the preparation phase of the inspection.

Eventually, it should be paid attention to documentation in paper and electronic form, according to 21 CFR Part 11. Often a combination of paper and electronic records (hybrid solution) is used in companies and it has to be decided case by case which type of document is stored in which system. For important electronic systems, it has to be determined, for example, which data format is used, how the data retrieval over the retention period can be ensured (data format, data reliability, data integrity), and how the document has been authenticated.

For many inspectors, the assessment of the project or product documentation, in conjunction with the contractual agreements between sponsor and contractor, is an important element of compliance. Thus, the involved departments, particularly the purchasing department, research and development (R&D), quality control and, where appropriate, the production as well as critical suppliers should be involved in the review of these *quality and service agreements*. Agreements made earlier should be checked for up-to-dateness and specifications. Especially for critical suppliers, this review is of great importance, as it assures the validity and compliance with the agreements. Particular focus is set on the communication between the involved parties. The FDA is aware of the fact that there are communication problems in many cases between the parties, so that sufficient attention should be paid to the correspondence and/or telephone notes, because some important decisions in the project can be best documented by an e-mail, a written note, or the signed phone minutes of a conference call.

The different departments should verify the applicable parts of the registration dossiers and related documents and records for accuracy, completeness, and consistency. This ensures that there will be no surprises during the FDA inspection, which could lead to agitation and discussions.

Case 5 Box: Practical Tips on the Subject: Quality Management

- There is a list of all *standard operating procedures* (SOPs).
- The SOPs governing the activities of the QM department, are updated, valid and trained.
- All SOPs on the topics “change control” (Change Control), “deviations”, “results out of specification” (OOS), “corrective and preventive actions” (CAPA), as well as “training” are updated, valid and trained.
- Self-inspections are planned and conducted as scheduled.
- Supplier inspections are planned and conducted according to plan.

- Management reviews are carried out regularly and are documented.
- The job descriptions of all QM staff are available and updated.
- Lists of complaints, results OOS, deviations, and changes for the product that is in the inspection-focus are available, covering the past 24 months.
- For deviations from the past that have not been addressed satisfactorily, review-documents with conclusive explanations and consequences are available.

9.2.6

Documents and Records

Documents and records are the most important issue in FDA inspections, due to the irrefutable principle “What is not documented, is not done!” a fundamental differentiation is made between specification documents (e.g., test procedures) and verification documents (e.g., test protocols and laboratory records). More information is available in Chapter 8.

Case 6 Box: Practical Tips on the Subject: Documents and Records

- All standard operating procedures (SOPs), specifications, manuals, and instructions are available in the specified binder in the laboratory; check with table of contents, all documents up-to-date.
- Documents related to deviations, changes, OOS results, complaints, and CAPA are stored and managed centrally by the QM.
- All laboratory notebooks and worksheets are checked by a second employee with initials and date (accuracy, completeness, compliance), according to 21 CFR 211.194 (Lab Records).
- No self-adhesive labels (post-its) are used in the laboratory, even for private information.
- Blotting pads are blank, no calculations on calendar sheets.
- Wastepaper baskets are emptied; no raw data, chromatograms, or reports (even drafts) in the bins.
- No weighing printouts lying around on the balance printer or near by.
- All data storage media such as floppy disks, CDs, DVDs, memory cards are placed inside the drawers, because usually they are not labeled sufficiently.
- Binder labels should be uniform and appropriate to regulations (e.g., colors).
- Older versions of documents are removed from the laboratory.
- No handwritten entries in specification documents (SOPs or similar).
- Handwritten entries, for example, in verification documents only with indelible pens (DIN ISO 12757-2).
- No use of thermal paper and ink-jet printing for archival purposes.

- An archive is established and meets all the criteria of protection against fire, flood, and other catastrophic data loss.

9.3

Typical Course of an FDA Inspection

FDA inspections follow a typical course, which has developed in practice as the standard sequence (see Figure 9.2).

Once the inspectors have entered the audit room, they are greeted by the representative of the management. Then, the exchange of business cards and personal presentation follow. The other participants introduce themselves as well as the management representative and hand over their business cards. This usually happens before the inspectors have taken a seat. After the inspection, the business cards are a valuable source of information on names and functions of the contact persons who participated in the inspection. It is common that the inspectors identify themselves to members of management with their identity card “credentials” and thus begin the official part of the inspection.

Participants of the “opening meeting” are compiled from the persons in the audit room, back office, and other interested or maybe involved persons from the departments. The FDA inspectors are quite used to a larger number of participants in opening meetings. If the number of seats is limited, at least the persons directly involved in the audit room and the back office should take part in the opening

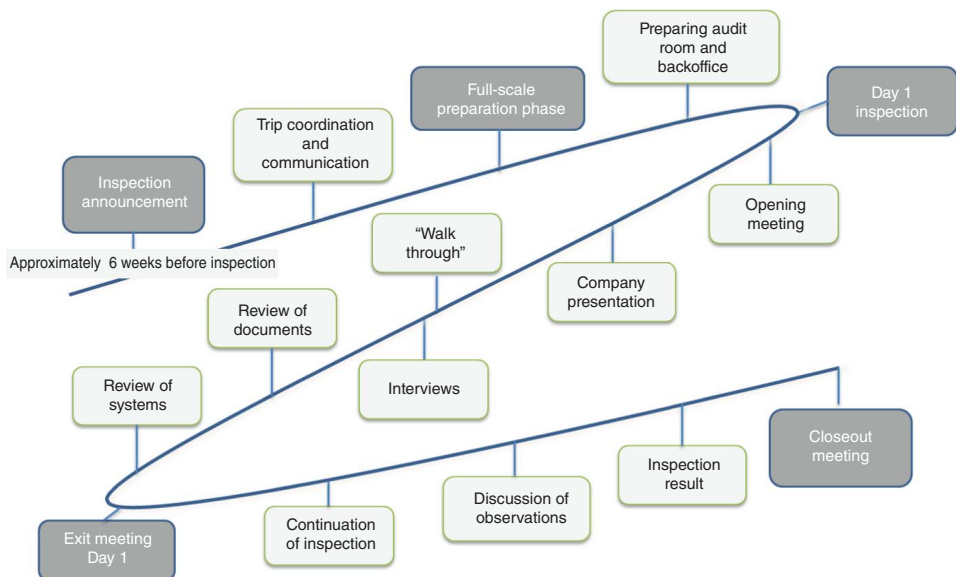


Figure 9.2 Typical course of an FDA inspection.

meeting. If the room size is sufficient 10–20 persons may participate. The opening meeting offers the opportunity for all participants to get a first impression of the inspectors. If the inspectors have taken a seat, the representative of the executive board should obtain permission from the inspectors to show a short company presentation, which is almost always accepted.

The company presentation for the opening meeting should be created and presented in English, and the inspectors should be provided with a printout so that they can take notes for future reference. Later, it facilitates writing of the report for the inspectors.

The contents of the company presentation are intended to familiarize the inspectors with the basic facts of the company in a short time and to give them a first impression of the organization. It is important that the presentation does not occupy too much time. Typical times are between 15 and 30 min for the entire company presentation. The presentation can be conducted by several persons, but it makes sense to limit the number of presenters on two or three. For example, the general part could be presented by a management representative, the history of the product, the associated contractors and processes by the product manager or head of Regulatory Affairs, and the part that deals with quality management by the head of Quality Management.

Case 7 Box: Company Presentation

- Overview of the company's name, affiliated group, headquarter, location(s)
- Overview of the economic indicators, number of employees, and so on
- Overview of the main products and markets, related to the US market
- Overview of the product focused by the current inspection, with information on history, processes, techniques, locations, subprocesses, key suppliers, or subcontractors, for example, sterilization process
- Organizational chart of the group, the location, the departments that are focused by the FDA, for example, QM, R&D, production, packaging, quality control laboratory
- Location plan; inspection relevant buildings should be marked
- Floor plans of essential facilities such as production, packaging, storage, quality control, laboratory
- Presentation of the audit core team (persons in the audit room are direct contact persons during the inspection)
- Presentation of the quality systems established on-site (e.g., ISO 17025, cGMP 21 CFR 211), with a short historical overview
- Information on audits of authorities (brief history and current inspection status, accreditations, and certifications)
- Information about any previous FDA inspection (on-site)
- Information on the integration of Quality Management into the value chain (opportunity to present the QM as an integral part of the company processes)

- Brief overview of the documentation systems of the company, for example, QM manual, process instruction (PI) sheets, test procedures and associated protocols, operating procedures (SOPs).

Immediately after the company presentation, the inspectors should be invited to ask questions. When all questions are answered, the lead inspector will introduce the team and reveal the contents of the inspection. This should bring no surprises, because already in the preparation phase of the inspection, it is of the utmost importance to strictly clarify the scope of the upcoming FDA inspection.

After agreement on the contents of the inspection, a little break to drink coffee, tea, or soft drinks can be used to give the participants who do not belong to the core team of the audit room, the opportunity to leave the room.

In most cases, the inspection begins with a company-walkthrough, which may include the usual areas such as incoming goods area, warehouse, product testing, quality control laboratories, manufacturing, packaging and storage, and logistics. It is difficult to predict which systems are studied in the first step, because the company-walkthrough serves to form a first overview of the company and it may well be that the tour will be continued later or on the following days.

9.4

During the Inspection

The “audit room” is the room, in which the inspectors and the core team stay during the inspection and where the documents and records are reviewed by the inspectors. This is also where the opening meeting and the “closeout meeting” take place. The management or management representatives must be present both at the beginning and at the end of the inspection.

The following requirements apply to the audit room:

- Room with good indoor climate (air conditioning in summer, heating in winter, natural light as possible and windows)
- Sufficiently large workspace on the tables, so that documents can be well spread
- Sufficient number of seats for the participants
- Ideally, for the opening meeting and the closeout meeting room for more participants should be provided.

Who should be in the audit room?

- Inspectors (typically, one to two persons)
- Management (only for welcome and farewell of inspectors)
- Head of quality management as the main contact person
- Representatives of the regulatory affairs department (optional)
- Note-taker and Moderator, usually in one person
- Interpreter(s), if this option is selected

- Two vacant seats for persons, who have to present results or documents (e.g., manager and employee).

Lists in the audit room:

- Audit room protocol (paper or better: intranet based, online connected with back office)
- List of participants (registration of all participants in order of occurrence after the beginning of the inspection, including name, position, topics discussed)
- Audit room tracking list (document for tracking the documents that come from the back office into the audit room, including comments on the status of the documents and whether the documents have been reviewed or copied and taken by the FDA inspectors).

The “back office” is the communication center in the background of an FDA inspection. Here, all information from the audit room about the questions or needs of the inspectors is gathered. These requests can be reported to the back office, either directly from the audit room or during the company-walkthrough. All documents, records, or samples requested by the inspectors shall be verified first in the back office and then transferred to the audit room. Similarly, the persons who have to present issues of their field of responsibility in the audit room will be prepared in the back office (briefing). These persons are kept ready and wait in the back office until they are called by the note-taker of the audit room to present their topic. These activities are to be documented by the note-taker in the back office tracking list.

The following requirements apply to the back office:

- Room situated within walking distance to the audit room (not more than 1–2 min foot-walk)
- Sufficiently large work surface on tables, so that documents can be well spread
- Sufficient number of seats for the team and persons waiting to be called to the audit room
- Space for the binders that are needed for the audit.

The technical equipment should enable network connections to access important documents (e.g., SOPs, process instructions, and manufacturing and test documents), records, journals, guidelines, or pharmacopoeias (e.g., USP). Internal roles and responsibilities as well as contact persons should be defined individually for each FDA inspection, as this may change due to the inspected issues. The back office administrates the “document tracking list,” which contains the status of all requested documents.

9.4.1

Behavior in Inspections

In general, the selection of persons who have direct contact with the FDA inspectors should consider that the following skills are well developed: balanced mood,

unexcited style of discussion, structured logical thinking and working, strong service orientation, stress resistance, general overview, and adaptability.

For the dress code, there is only one rule: You should feel comfortable in your clothes. Not too formal – not too casual, but comfortable and customary clothes that do not overly exceed the level of the inspectors' clothes. If you generally do not wear a neck-tie, you should not wear it in the inspection. Women should avoid shoes with very high heels and use decorative cosmetics, accessories and jewelry with restraint.

The inspectors are usually addressed by first-name, if he or she does not present and behave in a very formal way. This is common in the United States and, however, should not be interpreted as a chummy form of address (as it may imply for Europeans). It should be remembered that the inspection is a formal administrative act, similar to a tax audit or judicial investigation, despite the relaxed and casual appearance, or speech of the inspectors. Monitor the situation thoughtful and focused, and try to help the inspector to make his or her work as comfortable as possible. The general behavior should be characterized by friendliness and commitment, because people from the American culture are accustomed to a friendly and open environment. A natural smile or friendly gesture is the best guarantee that you will reach the “human touch” of the inspectors.

The form of address of your managers, colleagues, and subordinate employees should be retained, as it is customary in your company and in the individual relations: If you generally address by first-name, you should also do it in the inspection and the same when you generally use the more formal surname.

All inspection participants with direct FDA contact should be trained for the interaction with the inspectors, so that the chances of a successful communication increase. It has been proven in practice that it is helpful to observe the following points.

Case 8 Box: Practical Tips on the Subject: Behavior in Inspections

- Be friendly, relaxed and concentrate on the statements and questions of the inspectors.
- Make use of simultaneous interpreters, if you do not feel comfortable in English, because the inspection language is always English.
- All statements you make must be supported by a written evidence, for example, a test procedure or method, a standard operating procedure (SOP), or a technical test report.
- All of the statements you make must be honest and reflect the facts as completely as possible.
- Answer only the question with a precise response, without further embellishments or details that you plan for the future.
- If you do not understand the question, ask for repetition of the question.

- Be responsive to “small talk” offers of the inspectors, for example, on topics such as weather, culture, regional points of interest or culinary specialties, travel, history, countries, and customs, because that relaxes the atmosphere.

For the successful presentation of your position, it is important to be on a par with the inspectors. This means that you should neither present yourself technically more competent or superior, nor behave like you are in a weak position, or feel attacked and adopt a defensive position. If you do not succeed in presenting your compliance convincingly in some cases, remain calm and offer to procure additional data and facts from the back office, which can provide further clarification. If this additional information cannot be brought in a timely manner, ask (usually the note-taker or moderator) to shift the issue to a later point of time. Longer pauses and silence of the inspectors should not unsettle the core team, but are seen as a normal part of such audits.

9.4.2

Lab Walkthrough

As already mentioned, a laboratory-walkthrough is usually conducted immediately after the opening meeting. It may be that the inspectors plan the route with you, or join your proposed route. Eventually, it is irrelevant who proposes the route, because inspectors are trained to enter the essential premises and to conduct their investigations.

If there are two inspectors, it must be expected that the group splits, which should be considered as an option when planning the escort of the inspectors. Some inspectors inspect according to the material flow and track the full path of the testing procedure, but there are also some who take a seemingly arbitrary way and change from one topic to the next during their lab-walkthrough. The employees in the different areas of the company should continue to work normally, as if it were an ordinary working day, even if the FDA inspectors approach. Only on request, people should speak with the inspectors, otherwise continue to work routinely.

In general, as few persons as necessary shall accompany the inspectors on the company tour. In any case, the escort should include the head of quality management, a note-taker, a representative of each visited department, and a “stand-by person,” who may bring documents and records, if the employees of the department are currently occupied by the questions of the inspector.

9.4.3

The Inspection in the Audit Room (Front Office)

Most of the inspection time is usually spent in the audit room. The attendees shall deliver documents, records, procedures, and processes, on request

of the inspectors. While preparing the audit room a suitable place for the inspector(s) should be selected. It should be selected the best possible and comfortable place with sufficient lighting and no passage door behind the chair.

Documents are generally presented in the original form. In case the inspectors like to take along certain documents, they should be copied and stamped as *confidential information* so that the information cannot be disseminated. Under the FOI *Freedom Information Act* of the United States, information from FDA 483 forms and *warning letters* are accessible to the public via the Internet.

All records and documents that are related to regulated processes and products may be requested by the inspectors (see IOM, 2015, Chapter 5.6.2.3 – *Records*). However, the FDA exercises restraint with regard to the examination of internal audit reports or supplier audits. The FDA inspectors are entitled to demand and take samples, specimens and reference materials from the inspected company. The required samples shall be documented in a list and handed over to the inspector (see IOM, 2011, Chapter 5.10.4.3.17 – *Samples Collected*).

9.4.4

Dealing with Obviously Serious Observations

If there is a significant and serious observation, remain calm and friendly as it could be that additional data could clarify the situation. Therefore, ask for a postponement of the question until you can provide further data. However, you should not do this to fend off the question, but with the intention to support the inspector as best you can and help him or her with the investigation of the facts.

Use the gained time in the back office, involving all persons, who are able to contribute to the clarification and try to develop a conclusive answer. If you are unable to convince the inspector, please explain the circumstances on the basis of facts and mitigate the original observation by relativizing statements that are supported by facts. In practice, a three-step process for dealing with difficult observations has proved to be effective:

- 1) Agree to the obvious findings (honesty).
- 2) Provide additional information to clarify (relativization).
- 3) Describe procedures that will prevent such events in the future (CAPA or PACA, sometimes it depends on the order of corrective and preventive measures).

With the method described here, deficits can be dispelled, especially deficits found prior to the inspection by internal or third-party reviews and make them “harmless” for the FDA inspection. Experience reports that comprise a description of deficits, relativizing facts and appropriate CAPA are suitable tools to process the three-step method.

9.4.5

Documentation of Observations on Form FDA 483

If an observation is recorded on FDA Form 483, you have the opportunity to address and discuss this issue on each inspection day in the *exit meeting*. However, new data and additional information must be provided that was not available at the time, when the observation was made. Take advantage of the openness of the inspectors to discuss. Please keep in mind that for the inspectors the foreign environment and language are stressors and apply social and intercultural sensitivity in order to determine how far you can go in the discussion.

The opportunity of clarifying and possibly discussion of factual open questions or observations should be used in a short *exit meeting* on each inspection day by the core team in the audit room. All issues that can be solved on site should be addressed on site, because usually it is difficult to convince the FDA district office with written statements on complicated facts, after the inspection. Most investigators are very well trained and experienced, so that discussions with a technical background can be performed thoroughly. Again, the communication shall be very friendly and factual. It cannot be emphasized enough that the inspectors are trained to discuss the results of the FDA 483 observations and your proposed corrective actions (see IOM, 2014, Section 5.2.3), so you should use this opportunity during the inspection.

At the end of each day's "exit meeting," it should be asked what subjects are planned for the following day of the inspection, since the evening is still available to prepare essential things, such as translations of relevant texts into English, if desired, or to make graphical representations that can contribute to clarification and transparency. The agreement of a provisional agenda for the following day significantly facilitates the entry into the new inspection day and should be used as an opportunity.

In the final meeting of the FDA inspection, the *closeout meeting*, the presence of a representative of the management is mandatory. If there are any observations on Form FDA 483, the inspector is obliged to hand over this document to the management representative personally. At this meeting, there should be no surprises, as all observations have already been presented in the daily "exit meetings" on the previous days, and possibly were discussed. If no final agreement could be reached for an observation, the possibility of an *Annotation* to the FDA 483 observations can be used. If there are no FDA 483 observations on the form the meeting will be terminated quickly, while in the case of observations all violations will be read aloud. The observations are presented at this time without citation of the relevant legal text. The exact quote is created later by the *District Office* in the United States and is listed explicitly in the report of the inspector, the *EIR Establishment Inspection Report*.

The final report EIR is written in English (see IOM, 2014, Chapter 5.10.4) and usually follows 6–12 weeks after an inspection. The EIR contains

- printout of FACTS "Facility Establishment Inspection Record"
- acknowledgment statement ("endorsement," discussed later)

- a copy of the FDA Forms FDA 482, FDA 483, and FDA 484
- summarized report of the inspector
- copy of the inspection mandate, if available
- exhibits and finds that are described in the report
- checklists, for example, BSE Checklist
- the original checklists should be handed out with the EIR.

9.5

Post-Processing of the Inspection

The FDA Form 483 is used to log the observed GMP violations found by inspectors during the stay in the company (IOM, 2014, Chapter 5.2.3. seqq.) In principle, these observations are the result of subjective perceptions of the inspectors and can be based on misunderstandings, not fully clarified facts or faulty communication. However, there can be justified objectively measurable facts that are consistently seen by inspectors and inspected company.

Once an FDA Form 483 signed by the inspector is handed over to the management, only the written path can be taken to comment on the observations, to relativize, and to make suggestions for corrective and preventive measures. The expectation of the FDA is that 483 observations will be answered in writing within 15 working days.

If there are observations on the FDA Form 483, those persons involved in the inspection should be consulted that have detailed knowledge about how it came to this observation and may have already used the opportunity during the inspection to discuss with the inspector any responses or actions. In the case of experienced inspectors, these estimates can be very helpful for the design of a suitable response strategy.

The answers shall convince the FDA *district office*, from where the inspector was sent that the proposed action is likely to achieve compliance with applicable US laws. The answer should not only focus on short-term solutions, but also on a sustainable implementation of reasonable measures, because it is very likely that in a subsequent FDA inspection this topic will be revisited.

If the submitted answer does not convince the FDA and it is suspected that the proposed measures are not sufficient to solve the problem, the FDA will generate a *Warning Letter*, which is published on the website of the FDA and contains the observations and the assessments of the FDA. When even the written replies to this *Warning Letter* do not convince, legal measures, such as stopping a submission, import ban, confiscation can be taken by the FDA.

Further Readings

21 CFR 210 Current Good Manufacturing Practices in Manufacturing, Processing, Packing or Holding of Drugs: General,

<http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=210> (accessed 28 December 2015).

- 21 CFR 211 Current Good Manufacturing Practices For Finished Pharmaceuticals, <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=211> (accessed 28 December 2015).
- 21 CFR 11 Electronic Records, Electronic Signatures, <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=11> (accessed 28 December 2015).
- CPGM 7356.002 (2002) Drug Manufacturing Inspections, <http://www.fda.gov/downloads/ICECI/ComplianceManuals/ComplianceProgramManual/UCM125404.pdf>.
- CPGM 7346.832 (2010) Pre-approval-Inspections, <http://www.fda.gov/downloads/Drugs/DevelopmentApproval-Process/Manufacturing/Questionsand-AnswersonCurrentGoodManufacturing-PracticescGMPforDrugs/ucm071871.pdf>.
- FDA Regulatory Procedures Manual (RPM), Chapter 4 – Advisory Actions, Chapter 5 – Administrative Actions, Chapter 6 – Judicial Actions, Chapters 7–8 Recall and Emergency Procedures, Chapter 9 – Import Operations/Actions, <http://www.fda.gov/ICECI/ComplianceManuals/RegulatoryProceduresManual/> (accessed 28 December 2015).
- IOM FDA (2015) Investigation Operations Manual 2015, <http://www.fda.gov/iceci/inspections/iom/default.htm> (accessed 28 December 2015).
- FDA Foreign Inspections: Guide to International Inspections and Travel, updated version 27 April 2015, <http://www.fda.gov/ICECI/Inspections/ForeignInspections/ucm111443.htm>.
- FDA Compliance Policy Guide for the handling of internal quality records from self – inspections, CPG Sec. 130.300 *FDA Access to Results of Quality Assurance Program Audits and Inspections*, <http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm073841.htm> (accessed 28 December 2015).
- ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology, November 1996, <http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html> (accessed 28 December 2015).
- Analytical Procedures and Methods Validation, CMC documentation, <http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm386366.pdf>
- United States Pharmacopeia, USP <1224> Transfer of Analytical Procedures.
- United States Pharmacopeia, USP <1226> Verification of Compendial Methods.

10

HPLC – Link List

Torsten Beyer

Subsequently useful links in the field of HPLC and analytical chemistry in general as well are to be found in order to help users to find quick appropriate information.

10.1

Chemical Data

Wikipedia

<http://en.wikipedia.org>

TOXNET

<http://toxnet.nlm.nih.gov>

INCHEM

<http://www.inchem.org/>

SOLV-DB

<http://solvdb.ncms.org>

Osiris Property Explorer

<http://www.organic-chemistry.org/prog/peo>

ChemExper

<http://www.chemexper.com>

GESTIS-database on hazardous substances

<http://www.dguv.de/ifa/GESTIS/GESTIS-Stoffdatenbank/>

ASTM (with costs)

<http://www.astm.org/Standard/>

NIST Libraries of Peptide Tandem Mass Spectra

<http://peptide.nist.gov>

NIST Chemistry WebBook

<http://webbook.nist.gov/chemistry>

EU Pesticides database

<http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database>

Pesticides online

<http://www.pesticides-online.de>

Compendium of Pesticide Common Names

<http://www.alanwood.net/pesticides>
 Drugbank
<http://www.drugbank.ca>

10.2

Applications/Methods

10.2.1

Authorities and Institutions

EPA
<http://www.epa.gov/fem/methcollectns.htm>
 FDA
[http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/DIN/ISO/EN methods \(with costs\)](http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/DIN/ISO/EN%20methods%20(with%20costs))
<http://www.beuth.de/en/>
 OSHA (Occupational Safety & Health Administration)
<http://www.osha.gov/dts/sltc/methods>
 GESTIS – Analytical methods
<http://amcaw.ifa.dguv.de/>

10.2.2

Manufacturers of Analytical Instruments and Columns

Agilent Literature Library
<http://www.agilent.com/en-us/library/literature>
 Hamilton HPLC Application Index
<http://www.hamiltoncompany.com/support/hplc-application-index-search>
 Knauer Applications
<http://www.knauer.net/applications>
 Macherey Nagel Application Database
<http://www.mn-net.com/apps>
 Perkin Elmer Library Resources
<http://www.perkinelmer.de/tools/technicallibrarylandingpage.aspx>
 Phenomenex Application Database
<http://www.phenomenex.com/Application>
 Restek Searchable Chromatogram Library (LC)
<http://www.restek.com/chromatogram/search?s=type:LC>
 Shimadzu Literature
<http://search1.shimadzu.co.jp/search?site=EM0AJRZ6&design=2>
 Supelco HPLC Applications
<http://www.sigmaaldrich.com/analytical-chromatography/applications-search.html>
 Thermo Scientific (Dionex Application Notes)

<http://www.dionex.com/en-us/documents/application-notes-updates/lp-71593.html>
 Thermo Scientific (AppsLab Library) <https://appslab.thermoscientific.com/Waters>
<http://www.waters.com/waters/libraryList.htm>
 YMC Application Data Collection
<http://www.ymc.de/applications.html>

10.2.3

Journals and Web Portals

LCGC Featured Applications
<http://www.chromatographyonline.com/LCGC-Application-Notes>
 LCGC Online – Application Notebook Archive
<http://www.chromatographyonline.com/sourceissues?sourcelist=34>
 Separation Science
<http://www.sepscience.com/Techniques/LC/Applications>

10.3

Troubleshooting

Chromatography Forum
<http://www.chromforum.org>
 Google Group “sci.chem.analytical”
<https://groups.google.com/forum/#!forum/sci.chem.analytical>
 LCGC: LC Troubleshooting (John W. Dolan)
http://www.chromatographyonline.com/search/apachesolr_search/troubleshooting?filters=sm_pubAuthor%3A%22John%20W.%20Dolan%22
 Separation Science HPLC solutions (John W. Dolan)
<http://www.sepscience.com/Techniques/LC/Articles/214-/John-Dolan>
 Chromacademy HPLC Troubleshooting (registration necessary)
http://www.chromacademy.com/hplc_troubleshooting.html
 Chromedia LC Troubleshooting
<http://www.chromedia.org/chromedia?waxtrapp=lxsaqEsHqnOxmOIIecCxBwBdC&subNav=pmxgzFsHqnOxmOIIecCxBwBdCA>
 Agilent Troubleshooting Videos
<http://www.agilent.com/en-us/products/liquid-chromatography/lctroubleshootingvideos>
 Agilent HPLC Troubleshooting
<http://www.agilent.com/search/?Ntt=hplc%20troubleshooting&N=170>
 Knauer
http://www.knauer.net/fileadmin/user_upload/produkte/files/Dokumente/application_notes/vsp0003n_hplc_troubleshooting_guide.pdf

Macherey-Nagel

[http://www.mn-net.com/ChromatographyServices/Troubleshooting/](http://www.mn-net.com/ChromatographyServices/Troubleshooting/HPLCTroubleshooting/tabid/11836/language/en-US/Default.aspx)

[HPLCTroubleshooting/tabid/11836/language/en-US/Default.aspx](http://www.mn-net.com/ChromatographyServices/Troubleshooting/HPLCTroubleshooting/tabid/11836/language/en-US/Default.aspx)

Perkin-Elmer (Webcast HPLC Troubleshooting, registration required, free of charge)

[http://shop.perkinelmer.com/Account/FormBuilder.aspx?FormID=](http://shop.perkinelmer.com/Account/FormBuilder.aspx?FormID=HPLCWebCast2)

[HPLCWebCast2](http://shop.perkinelmer.com/Account/FormBuilder.aspx?FormID=HPLCWebCast2)

Phenomenex (HPLC Troubleshooting Guide, PDF)

[http://www.phenomenex.com/ViewDocument/?id=hplc+troubleshooting+](http://www.phenomenex.com/ViewDocument/?id=hplc+troubleshooting+guide)
[guide](http://www.phenomenex.com/ViewDocument/?id=hplc+troubleshooting+guide)

Restek (FAQ HPLC and UHPLC)

http://www.restek.com/Pages/faq_lc

Shimadzu

<http://www.shimadzu.com/an/hplc/support/faq> (tips for daily HPLC analysis)

http://www.shimadzu.com/an/lc_worldtalk (“LC World Talk”, registration required)

Supelco (HPLC Troubleshooting Guide)

[https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/](https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/Bulletin/4497.pdf)
[Bulletin/4497.pdf](https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/Bulletin/4497.pdf)

Waters HPLC Troubleshooting Guide (registration required)

<http://www.waters.com/waters/library.htm?cid=511436&lid=1528445>

10.4

Background Information and Theory

Chemgapedia

<http://www.chemgapedia.de/vsengine/en/>

Analytical Training Solutions (with costs)

<http://www.analyticaltraining-solutions.org/?pak=5575226378897885>

Chromacademy – HPLC-Training (limited free “Lite Membership,” full access with costs)

<http://www.chromacademy.com/hplc-training.html>

Chromedia LC Basics (mostly with costs)

<http://www.chromedia.org/chromedia?waxtrapp=yqegzCsHqnOxmOIIEcCbC>

Agilent LC Handbook (Guide to LC Columns and Method Development)

[http://www.chem.agilent.com/Library/primers/Public/LC-Handbook-](http://www.chem.agilent.com/Library/primers/Public/LC-Handbook-Complete-2.pdf)
[Complete-2.pdf](http://www.chem.agilent.com/Library/primers/Public/LC-Handbook-Complete-2.pdf)

UHPLC Simulation Sheet

<http://www.uplc.info>

Ionsource

<http://www.ionsource.com>

Waters Reversed Phase Column Selectivity Chart (also for columns of other manufacturers)

<http://www.waters.com/waters/selectivitychart>
 USP und PQRI Database
<http://apps.usp.org/app/USPNF/columns.html>
 Comparison Guide to C18 Reversed Phase HPLC Columns
http://www.crawfordscientific.com/downloads/pdf_new/Grace/grace_hplc_columns_introduction.pdf

10.5

Literature

10.5.1

Publishing Companies for Journals, Books, and Databases

American Chemical Society ACS
<http://pubs.acs.org>
 Elsevier
<http://www.sciencedirect.com>
 Royal Society of Chemistry RSC
<http://pubs.rsc.org>
 Springer
<http://www.springer.com>
 Wiley
<http://onlinelibrary.wiley.com>

10.5.2

Scientific Journals (Full Access with Costs)

Analyst (ISSN 0003-2654)
<http://pubs.rsc.org/en/journals/journalissues/an>
Analytica Chimica Acta (ISSN: 0003-2670)
<http://www.sciencedirect.com/science/journal/00032670>
Analytical and Bioanalytical Chemistry (ISSN 1618-2642)
<http://link.springer.com/journal/216>
Analytical Biochemistry (ISSN 0003-2697)
<http://www.sciencedirect.com/science/journal/00032697>
Analytical Chemistry (ISSN 1520-6882)
<http://pubs.acs.org/journal/ancham>
Analytical Letters (ISSN 1532-236X)
<http://www.tandfonline.com/toc/lanl20/current>
Analytical Methods (ISSN 1759-9660)
<http://pubs.rsc.org/en/journals/journalissues/ay>
Analytical Sciences (ISSN 0910-6340)
<http://www.jsac.or.jp/analsci>
Biomedical Chromatography (ISSN 1099-0801)
<http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291099-0801>

Chemosphere (ISSN: 0045-6535)
<http://www.sciencedirect.com/science/journal/00456535>
Chirality (ISSN 1520-636X)
<http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291520-636X>
Chromatographia (ISSN 1612-1112)
<http://link.springer.com/journal/10337>
Environmental Science and Technology (ISSN 0013-936X)
<http://pubs.acs.org/journal/esthag>
International Journal of Environmental Analytical Chemistry (ISSN 1029-0397)
<http://www.tandfonline.com/toc/geac20/current>
International Journal of Polymer Analysis & Characterization (ISSN 1023-666X)
<http://www.tandfonline.com/toc/gpac20/current>
Journal of Agricultural and Food Chemistry (ISSN 0021-8561)
<http://pubs.acs.org/journal/jafcau>
Journal of Analytical Toxicology (ISSN 1945-2403)
<http://jat.oxfordjournals.org>
Journal of AOAC International (ISSN 1944-7922)
<http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac>
Journal of Chromatographic Science (ISSN 1945-239X)
<http://chromsci.oxfordjournals.org>
Journal of Chromatography A (ISSN 0021-9673)
<http://www.sciencedirect.com/science/journal/00219673>
Journal of Chromatography B (ISSN 1570-0232)
<http://www.sciencedirect.com/science/journal/15700232>
Journal of Liquid Chromatography & Related Technologies (ISSN 1520-572X)
<http://www.tandfonline.com/toc/ljlc20/current>
Journal of Pharmaceutical and Biomedical Analysis (ISSN 0731-7085)
<http://www.sciencedirect.com/science/journal/07317085>
Journal of Separation Science (ISSN 1615-9314)
<http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291615-9314>
Phytochemical Analysis (ISSN 1099-1565)
<http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291099-1565>
Separation & Purification Reviews (ISSN 1542-2127)
<http://www.tandfonline.com/toc/lspr20/current>
Separation and Purification Technology (ISSN 1383-5866)
<http://www.sciencedirect.com/science/journal/13835866>
Solvent Extraction and Ion Exchange (ISSN 1532-2262)
<http://www.tandfonline.com/toc/lsei20/current>
Separation Science and Technology (ISSN 1520-5754)
<http://www.tandfonline.com/toc/lst20/current>
Talanta (ISSN 0039-9140)

<http://www.sciencedirect.com/science/journal/00399140>

Trends in Analytical Chemistry (ISSN 0165-9936)

<http://www.sciencedirect.com/science/journal/01659936>

10.5.3

OpenAccess Journals

Analytical Chemistry Insights (ISSN 1177-3901)

<http://www.la-press.com/analytical-chemistry-insights-journal-j1>

Camera Separatoria (ISSN 0860-2719)

http://dach.ich.uph.edu.pl/camera_separatoria.html

Chromatography Research International (ISSN 2090-3510)

<http://www.hindawi.com/journals/cr>

Eurasian Journal of Analytical Chemistry (ISSN 1306-3057)

<http://www.eurasianjournals.com/index.php/ejac>

International Journal of Analytical and Bioanalytical Chemistry (ISSN 2231-5012)

<http://urpjournals.com/journals.php?journalID=6>

International Journal of Analytical Chemistry (ISSN 1687-8760)

<http://www.hindawi.com/journals/ijac>

ISRN Analytical Chemistry (ISSN 2090-7311)

<http://www.isrn.com/journals/ac>

ISRN Chromatography (ISSN 2090-8636)

<http://www.hindawi.com/journals/isrn/contents/isrn.chromatography/>

Journal of Chromatography & Separation Techniques (ISSN 2157-7064)

<http://www.omicsonline.org/chromatography-separation-techniques.php>

Open Analytical Chemistry Journal (ISSN: 1874-0650)

<http://www.benthamopen.com/TOACJ/>

Pakistan Journal of Analytical and Environmental Chemistry (ISSN 1996-918X)

<http://www.ceacsu.edu.pk/Faculty/Journal.html>

10.5.4

Free Commercial Journals and Web Pages with Focus on Chromatography

LCGC

<http://www.chromatographyonline.com>

(LCGC North America, LCGC Europe, The Column, The Peak)

Chromatography Today (ISSN: 1752-8070)

<http://www.chromatographytoday.com>

Separation Science

<http://www.sepscience.com>

SeparationsNow

<http://www.separationsnow.com>

10.5.5

Literature Search Engines

Google Scholar <http://scholar.google.com>

Google Books <http://books.google.com>

10.6

Databases with Costs

10.6.1

STN Databases

- CA (Chemical Abstracts)
- ANABSTR (Analytical Abstracts database)
- KOSMET (The IFSCC Cosmetic & Perfume Science and Technology database)
- FSTA (Food Science Technology Abstracts)
- SPECINFO (Spectral database Information System)
- WATER (Water Resources Abstracts)

See: http://www.stn-international.de/sum_sheets.html

10.6.2

Data on Chemical Media

Reaxys

<http://www.reaxys.com>

Römp

<http://www.roempp.com>

10.6.3

Literature

Chromatography Abstracts (discontinued)

<http://www.rsc.org/publishing/currentawareness/chra>

10.7

Apps

Agilent LC Calculator (iOS)

<http://itunes.apple.com/us/app/lc-calculator/id355827606>

HPLC Troubleshooting App (iOS)

<http://kduoapps.com/app/hplc-app>

Sigma-Aldrich HPLC Calculator Android

<https://itunes.apple.com/us/app/hplc-calc/id533941172> (iOS) <https://play.google.com/store/apps/details?id=sial.andriod.calc> (Android)

Thermo HPLC Troubleshooting Guide (Android)
<https://play.google.com/store/apps/details?id=com.thermoscientific.troubleshootingguide>

10.8

Social Media

LinkedIn
<http://www.linkedin.com>
ResearchGate
<https://www.researchgate.net>

10.9

Twitter Pages (Examples)

PerkinElmer
<http://twitter.com/PerkinElmer>
Restek
<https://twitter.com/Restek>
Shimadzu Europa
<http://twitter.com/ShimadzuEurope>

10.10

Facebook Pages (Examples)

PerkinElmer
<http://www.facebook.com/PerkinElmer>
Shimadzu
<http://www.facebook.com/ShimadzuCorp>
Thermo Scientific
<http://www.facebook.com/thermoscientific>

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