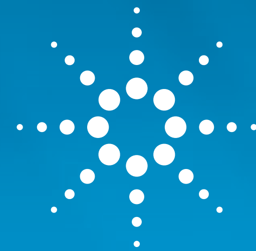

PRINCIPLES AND PRACTICAL ASPECTS OF PREPARATIVE LIQUID CHROMATOGRAPHY

Primer

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Helmut Schulenberg-Schell and Andreas Tei

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FOREWORD

As a synthetic chemist, biologist or engineer, it's your job to study the impact of particular chemical compounds. To achieve this goal you need to stay at the leading edge of your field of research. As a consequence, you often find yourself with less time for tasks that are not necessarily your core competences but nevertheless important for your workflows. Isolation and purification of chemical compounds are typical of these tasks.

In situations where the compound of interest is not available in pure form, you are challenged to purify it yourself. Possible scenarios include synthesis of the compound in multiple stages, using purification as an interim step, or isolation of the compound from a natural source when synthesis is too complex and tedious. Other scenarios that require you to turn to purification techniques include, for example, when flash chromatography did not yield the desired purity, or when crystallization did not work the way you expected.

Isolation of pure compounds was, in fact, the original purpose of liquid chromatography and as such drove the development of separation science during the last century – with close linkages to the discovery of natural sources and new synthetic pathways. The increasing need for high-value compounds deployed as pharmaceuticals, agrochemicals or nutraceuticals, has in turn justified the extra effort required for optimization of purification processes.

Today, preparative chromatography is no longer based on guesswork but is founded solidly on a set of well-documented rules to be followed for optimum results. Scouting for appropriate starting conditions, optimizing for speed, yield and purity are fundamental considerations. The desired sample throughput determines priorities: high yields for a few different samples justify optimization of yield, whereas dealing with large numbers of different samples at the milligram scale demands proper automation.

Now, it is all about getting started with preparative liquid chromatography without having to spend time delving deeply into the literature. Although a primer will never replace textbooks on preparative liquid chromatography to gain a full understanding of the theoretical background, this publication nevertheless bridges the gap between textbook literature and a typical system's user documentation that provides specific guidance on how to achieve optimum results.

Analytical liquid and gas chromatography are the techniques of choice for purity determination and indispensable tools for confirming the progress of purification processes. Preferably you should have made yourself familiar with these techniques prior to reading this Primer. This also includes the concepts of choosing the appropriate column chemistries as part of LC method development.

At this point we would like to give a word of caution to those who have already gained a high level of expertise in analytical liquid chromatography. In preparative chromatography there are additional rules and priorities as you try to optimize for speed, purity and yield. Hence, we hope this Primer proves to be worthwhile reading for everyone starting to care about efficient purification of compounds.

INTRODUCTION

In this Primer we give an introduction into the basic principles of preparative liquid chromatography, describe the components of a purification system, discuss strategies for collection of fractions and offer some practical solutions for common purification tasks.

We begin by redefining the difference between analytical and preparative liquid chromatography – not classically in terms of column dimensions or flow rates – but from the modern-day perspective of solutions for specific applications.

While this Primer gives a general overview of preparative liquid chromatography and its applications, we strongly recommend the Agilent Application Compendium entitled Solutions for Preparative HPLC (publication number 5989-5948EN) for further reading[1].

ABOUT THE AUTHORS

Helmut Schulenberg-Schell has a master's degree in chemistry and a Ph.D. in biochemistry from Münster University, Germany. Isolation of naturally occurring cyclopentenyl fatty acids and bacterial hopanoids, and purification of bovine lipid binding proteins introduced him to preparative chromatography early in his career. Later his interest focused on combining membrane separation techniques with biotechnology. Over the past 25 years he has worked in various positions in product and market development for Hewlett-Packard and Agilent Technologies to educate chemists, biologists and engineers about new technologies and products. Helmut currently holds the position of director of business development for liquid phase separations at Agilent Technologies.

Andreas Tei attained his master's degree in organic chemistry and his Ph.D. in natural sciences from Heidelberg University in Germany. During his Ph.D. studies he made his first experiences with the isolation of pharmaceutically active compounds from natural plant extracts by preparative chromatography. He started his industrial career with a scientific instrument manufacturer and held positions in service and support for LC/MS and GC/MS instrumentation. Later he worked for the major part of this time as an application chemist where he made intense experiences with onsite customer trainings for GC/LC-MS applications and mass-based purification. Automation of mass-based purification for medicinal chemistry laboratories has been one of the most exciting topics he has been involved with. This has gained importance as drug discovery teams are striving to become more efficient. He also obtained a global view on pharmaceutical workflows as a sales specialist and business development manager. During the next stage of his career Andreas was involved as product manager in the design of new modules and automation software for preparative chromatography systems. He is currently working as pharmaceutical segment manager for small molecules at Agilent Technologies.

Symbols	
A	absorption [AU]
c	concentration [mol/L]
d	path length [cm]
d_A	diameter of analytical column [mm]
d_P	diameter of preparative column [mm]
ϵ_λ	molar extinction coefficient
$f_{a,A}$	actual flow in analytical system [mL/min]
$f_{p,P}$	proposed flow in preparative system [mL/min]
k	retention factor
k_e	retention factor efficiency
L_A	length of analytical column [mm]
L_P	length of preparative column [mm]
N	number of theoretical plates
p_A	column particle size in analytical system [µm]
p_P	column particle size in preparative system [µm]
t_R	retention time [s]
$t_{D,A}$	dwell time of analytical system [s]
$t_{I,A}$	initial hold of analytical system generic gradient [s]
$t_{c,A}$	column pass time in analytical system [s]
$t_{D,P}$	dwell time of preparative system [s]
$t_{I,P}$	initial hold of preparative system gradient [s]
$t_{c,P}$	column pass time in preparative system [s]
$V_{D,A}$	dwell volume of analytical system [mL]
$V_{c,A}$	column void volume of analytical system [mL]
$V_{D,P}$	dwell volume of preparative system [mL]
$V_{inj,A}$	injection volume for analytical system [µL]
$V_{inj,P}$	injection volume for preparative system [µL]
w_h	peak width at half-height (in time units) [s]

Abbreviations	
DAC	dynamic axial compression
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EIC	Extracted ion chromatogram
id	inside diameter
IPA	isopropyl alcohol
SAC	static axial compression
TIC	Total ion chromatogram

1 INTRODUCING PREPARATIVE LIQUID CHROMATOGRAPHY

In this chapter we introduce preparative liquid chromatography (LC) by first making a clear distinction between preparative and analytical LC, and then discussing the diverse priorities that laboratories face when required to enrich or purify target compounds from mixtures.

1.1 Distinguishing between analytical and preparative liquid chromatography

Analytical liquid chromatography is a standard technique that needs to be embraced by any scientist or engineer interested in investigating mixtures of chemical compounds or biologically-derived molecules. A thorough qualitative or quantitative analysis of such mixtures can be achieved through chromatographic separation and selective detection of the mixtures' components.

In contrast, the need to enrich or purify target compounds from mixtures for further investigation or for commercial purposes is the key motivation to adopt and deploy preparative LC. For centuries multiple absorptive procedures have been developed to extract and enrich valuable substances. Towards the end of the 20th century the demand for compounds of highest purity in the food and pharmaceutical industries increased the pressure to advance preparative LC methodologies.

If we were to write a single statement that describes the distinction between liquid chromatography for analytical and preparative purposes, it would read like this:

"In preparative LC the separated compounds are collected in individual containers for further processing, whereas in analytical LC the laboriously separated compounds are simply diverted to waste or destroyed by a destructive detection technique!"

The classical approach of distinguishing between analytical and preparative chromatography in terms of column dimensions or flows rates is no longer appropriate.

Analyzing our generic description gives us an impression of just how common the use of preparative LC is. Completely independent of flow rates, preparative LC is deployed for collecting tiny protein fractions at flows of nano or microliters per minute as well as at high flow rates in industrial-scale purification of proteins.

In this Primer we focus on preparative LC chromatography as a simple yet sophisticated technique to separate and extract one or more target compounds from a mixture. A sample of the mixture is driven batch-wise through a tube containing absorptive layers of stationary phase. This process separates the mixture into its constituent components. Subsequently the target compounds are collected from the eluent stream.

1.2 Setting priorities for compound purification

When only limited amounts of raw material are available such as in the fractionation of complex natural product mixtures, preparative LC at lowest flow rates in the nanoliter or microliter range is deployed – possibly enabling novel discoveries in life sciences.

In contrast, high flow rates of multiple liters per minute are common in manufacturing processes for highly valuable compounds. Exact scale-up procedures and tightly-controlled, manual collection of fractions by experienced process engineers with a sound understanding of chromatography yield several kilograms of pure product – with a potential market value of millions of dollars.

Synthetic chemists working in pharmaceutical drug discovery or agrochemical research laboratories are focused constantly on the compromise between sample throughput, yield and purity. The injected amounts of crude sample are typically in the range of 100 to 500 mg. Key pharmaceutical laboratories are often purifying between 50 and 100 different samples on each system every day. High levels of system automation allow even non-expert chromatographers to purify their precious samples in self-service purification labs. To ensure every chemist can purify samples quickly and securely, and to be able to continue with synthetic work, the systems must be highly robust. With large numbers of different samples it is virtually impossible to individually optimize the purification parameters unless the processes can be automated[2–6].

In process development, chemists and engineers are focused mainly on pilot-scale purification in the range of multiple grams to kilograms of intermediates, fine chemicals or biological compounds. When it is required to purify large quantities of the same compound repetitively, it is worthwhile to tune the process thoroughly. As a consequence experienced chromatographers carefully elaborate scale-up processes

for each compound. Optimized gradients and often manually-controlled fraction collection are common practice. The purified compounds are usually very precious and although the number of purified samples per system per day is low, the value of the product could be huge. Hence, an efficient purification process is mandatory to sustain a profitable business model.

When separating complex samples such as metabolites in a biological matrix, the chromatographic resolution has the highest priority. For these challenges column sizes of 4.6 by 150 mm with 3 to 5-micron particles or even sub-2-micron particles are required, together with chromatographic conditions close or even identical to those used for typical analytical separations. Slow gradients combined with low-carryover autosamplers and fraction collectors are used to guarantee highest purity and recovery of the separated compounds. Typically, the concentrations in the crude sample are low. Hence, the compounds have to be enriched from large volumes of dilute sample (for example, urine) or recovered with maximum yield from small amounts of biological tissue.

KEY ASPECTS OF COLUMN SELECTION

2

Preparative liquid chromatography begins with an analytical separation. First, we need to confirm the presence of the target compound in the sample. Then, we must assess whether the estimated amount of the target compound we can recover from the sample justifies the subsequent purification effort. In this chapter we take a closer look at the separation column, offering decision criteria for column selection based on purification requirements.

2.1 Choosing a separation column

The amount of pure substance we need to recover within a given time determines the dimensions of the separation column and, in turn, these dimensions dictate the capacity of the purification system. For example, if an analytical column is large enough to deliver the required amount of pure substance, all we need to do is add a fraction collector to the analytical system.

The number of different samples per day or week dictates the levels of automation and capacity that the purification system needs to achieve. A high number of different samples every day creates a preference for a generic methodology for all samples with minimum time for method optimization, whereby automated sample introduction is desirable.

Purification of large amounts of a single compound justifies optimization of yield and purity, particularly when the same target compound needs to be purified on a regular basis. In this scenario we could even consider selection of a less expensive methodology. If large amounts of pure compounds are seldom required, repetitive injections of smaller amounts can be a feasible solution.

Figure 2.1 shows an overview of recommended flow rates and sample amounts for a range of column sizes typically deployed in preparative LC. The given purification ranges correspond to the amounts of crude product and are independent of purity or yield.

	Analytical		Semi-preparative		Preparative		Pilot		
Purification range [mg]	1–15	7–70	30–300	64–640	180–1800	400–4000	700–7000	600–16000	2800–28000
Column inside diameter [mm]									
4.6 mm	0.8–2.0 mL/min								
9.4 mm (0.5 inch)		4–10 mL/min							
21.2 mm (1 inch)			18–42 mL/min						
30 mm				34–85 mL/min					
50 mm (2 inch)					94–236 mL/min				
75 mm (3 inch)						212–931 mL/min			
100 mm (4 inch)							378–945 mL/min		
150 mm (6 inch)								800–2100 mL/min	
200 mm (8 inch)									1100–3375 mL/min

Figure 2.1 Overview of recommended flow rates (in mL/min) and sample amounts (in mg) for typical column sizes.

Typically, the amount of crude sample can vary from 0.1 to 1.0 % based on the weight of the sorbent. Recommended values are based on reversed-phase sorbent with a density of 0.6 g/mL and for a column or bed length of 150 mm. A 21.2 by 150 mm column contains about 32 g of sorbent.

Let us consider this example of a typical scientist's requirement;
"I would like to purify 100 mg of crude material per injection. Which column dimensions and what flow rate do you recommend?"

The recommendations in Figure 2.1 suggest that this demand can be met using a 21.2-mm id column with particle sizes between 5 and 10 microns, which deliver good results for the majority of purification tasks. The available column lengths from 50 to 250 mm need to be matched with the requested injection amounts. A simple rule to select a suitable length of a 21.2-mm id column is: 50 mm for 50 mg; 250 mm for 250 mg.

If separation becomes too difficult, the amount of injected crude material needs to be decreased or the amount of stationary phase needs to be increased. When decreasing the injected amount, always first reduce the injection volume, then the concentration. Increasing the amount of stationary phase can be realized by increasing the column diameter or the column length, or both.

In terms of flow rate a useful rule of thumb is to take a 21.2 by 100 mm column and scale up to 21 mL/min from a typical analytical flow rate of 1 mL/min for a 4.6-mm id analytical column, which reflects the well-described scale-up equations in the literature. When it is required to obtain shorter gradient times or to increase the daily throughput, the flow rate can be increased up to 42 mL/min, typically without significant loss in purity or recovery.

2.2 Choosing a large-scale preparative column

Large sorbent beds with inside diameters above 30 mm and lengths of 50 mm or more have an increasing tendency to settle continuously over time as a result of changing chromatographic conditions such as pressure, flow, temperature or other eluent properties that the beds are exposed to. Transport is another root cause of settling, if the columns are not handled with due care and attention.

During the column packing process axial compression is used to force the sorbent particles into a tightly packed bed and thereby maintain bed stability. There are two different types of axial compression used in column packing technology; static axial compression and dynamic axial compression. In static axial compression (SAC) the column bed is compressed and the plunger is held in a static position by a locking mechanism. In contrast, dynamic axial compression (DAC) keeps the sorbent bed under constant compression throughout deployment of the column.

In general, sorbents with spherical particles in the size range of 5 to 10 microns can withstand substantial compression forces and are used for large-scale purification applications. For these types of sorbents, preparative LC columns such as Agilent Load & Lock columns can be deployed in both SAC and DAC modes, see Figure 2.2.

Figure 2.2 Agilent Load & Lock columns are available with inside diameters of 1, 2 and 3 inches. A packing station provides for easy handling and facilitates both SAC and DAC modes.

In contrast, we recommend to use static compression for particle sizes of 10 microns or larger, or where the sorbent could be easily damaged by dynamic compression. For example, 300-Å particles break easily in so-called fines during hydraulic cycling that is typical for dynamic compression. Further examples would be irregularly shaped particles or sensitive gels for biological applications.

SAC/DAC columns are also an option when the required chemistries are available as bulk material. Such chemistries are often recyclable when contamination is affecting peak shape or when the sorbent bed has been damaged. The column can be unpacked, and the sorbent can be cleaned and then repacked. Packing Agilent Load & Lock column is easy to learn and with a little experience you can achieve more than 30,000 plates per meter.

If the separation efficiency in terms of resolution begins to deteriorate in an SAC packed column, for example, as a result of bed wearing, Agilent Load & Lock columns can simply be recompressed. This is done by placing the column on the packing station, recompressing the column, and then relocking the holding mechanism.

2.2.1 Choosing a compression system

SAC and DAC columns require a compression system for packing and unpacking operations. Ideally, a single system serves as an onsite packing station for the three laboratory-scale column sizes of 1, 2 and 3-inch inside diameters. The packing station should comprise a double-acting hydraulic cylinder, which is controlled by an air-driven, constant-pressure hydraulic pump. It should facilitate both static and dynamic axial compression. A source of compressed air at about 6 bar (90 psi) would be required to drive the hydraulic pump.

The hydraulic components – including pump, reservoir, cylinders, control panel and column attachment fixtures – should all be mounted on a mobile unit. Typical axial compression columns deploy a single hydraulic cylinder mounted vertically on the same axis as the column. However, mounting two cylinders on either side of the column in parallel with the column axis is a more advantageous configuration, facilitating the use of smaller diameter cylinders and resulting in a lower overall height when in the retracted position. The mobility of the module and the reduced height configuration are important considerations when using and handling such large equipment within a laboratory environment where floor space and door sizes are limiting factors.

2.2.2 Packing SAC/DAC columns

Agilent Load & Lock columns can be packed using different methods, depending on the physical length of the column bed or the quantity of sorbent desired. The two methods used to pack high performance sorbents in this type of column are the rapid-pack method and the aspiration method.

The most common method is the rapid-pack or slurry method that utilizes 60 % or less of the available column bed length. In this method, no reservoir is required. The slurry is introduced into the column, the end cap attached and the slurry solvent removed by hydraulic compression. When the compression pressure is reached, the compression piston is locked in place. This packing method requires a few minutes, is residue free and utilizes the entire aliquot of sorbent.

The aspiration or slow method typically utilizes the entire available bed length, which could be up to 90 % of the column volume. As a consequence, the method requires the addition of a plastic reservoir at the top of the column to accommodate the larger volume of packing slurry. The slurry is introduced in one aliquot and then the solvent is removed by vacuum aspiration from the bottom. After the bed dries, any excess resin is cut off the top of the column, the cap is attached and the bed is compressed. When the compression pressure has been reached, the compression piston is locked in place. The time required to pack a column using this method is dependent on the column length, type of sorbent, type of slurry solvent and available vacuum. Times can range from as little as 30 minutes to several hours. The ratio of hydraulic pressure to the mechanical pressure on the bed is given in Table 2.1.

	Column inside diameter		
	1 inch (27 mm)	2 inch (50 mm)	3 inch (75 mm)
Mechanical pressure [psi]	1,000	1,000	1,000
Hydraulic pressure [psi]	400	1,500	3,000
Ratio hydraulic/mechanical	1:2.5	1.5:1	3:1

Table 2.1 Ratios of hydraulic and mechanical pressures on the sorbent bed (C18 sorbent with a packed bed density of 0.59 g/mL).

3 COMPONENTS OF A PREPARATIVE LC SYSTEM

An analytical LC system can be adapted easily for purification by the simple addition of a fraction collector. As such, both analytical and preparative LC systems have essentially the same flow path from solvent delivery through to detection. The solvent delivery system generates the eluent mixture from solvents contained in reservoirs. For high flow rates the solvent supply means a substantial investment. For safety reasons the main solvent storage is typically located outside of the laboratory. A suitably sized intermediate storage device ensures proper supply to the pumps in the laboratory. The tubing to the autosampler, switching valves, columns, detectors and finally on to the fraction collector has to be optimized. If the tubing dimensions are large, additional dispersion occurs but backpressure is kept to a minimum. In contrast, if the tubing dimensions are small, the opposite is likely to happen. Each component contributes to the total performance of the purification system.

3.1 Solvent delivery

3.1.1 Low-pressure mixing of solvent gradients

In this method of solvent delivery the eluent composition is controlled by a proportioning valve and mixed at the low pressure side in a mixing chamber before being pressurized in the pump's cylinders. For flow rates up to 10 mL/min the solvents must be degassed using vacuum degassing. For higher flow rates helium purging is often deployed. However, it is difficult to feed the pumps directly by pipelines from larger tanks when using helium purging. In this case commercially-available external vacuum degassing equipment can be used. The advantages of low-pressure mixing compared to high-pressure mixing are lower investment costs, the ability to generate quaternary gradients, or the flexibility to add modifiers directly in the eluent flow. The major disadvantages of semipreparative low-pressure mixing systems are the larger dwell volume and degassing issues, which both lead to poorer gradient performance.

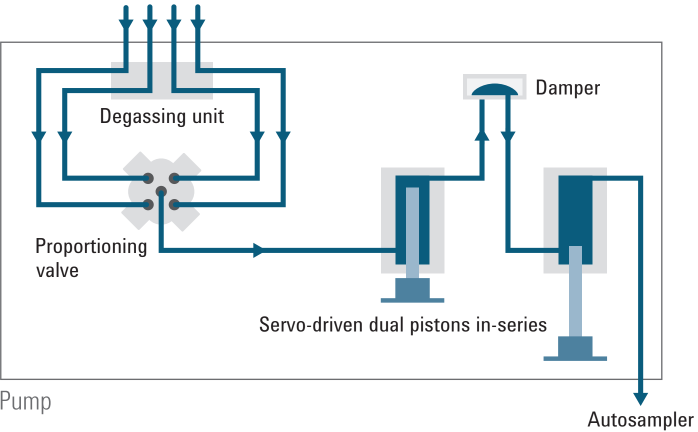


Figure 3.1 Schematic of a solvent delivery system for low-pressure mixing of gradients, showing degassing unit, proportioning valve and pump cylinders.

3.1.2 High-pressure mixing of solvent gradients

An alternative method is to deploy dedicated pumps for each solvent channel, which deliver the respective solvent at the programmed flow rate and composition to achieve the desired gradient. Mixing occurs at the high-pressure side, whereby the mixing process can be a limiting factor when striving for highest chromatographic performance. Frits or stainless-steel balls are commonly used in passive mixers whereas rotating stirrers are used in active mixers. When mixing at high pressure, solvent degassing is not usually necessary as long as the eluent remains pressurized at a minimum of about 3 bar (40 psi) until it has passed the flow cell of the detector. Backpressure caused by the flow cell outlet or additional backpressure regulators prevent degassing during detection.

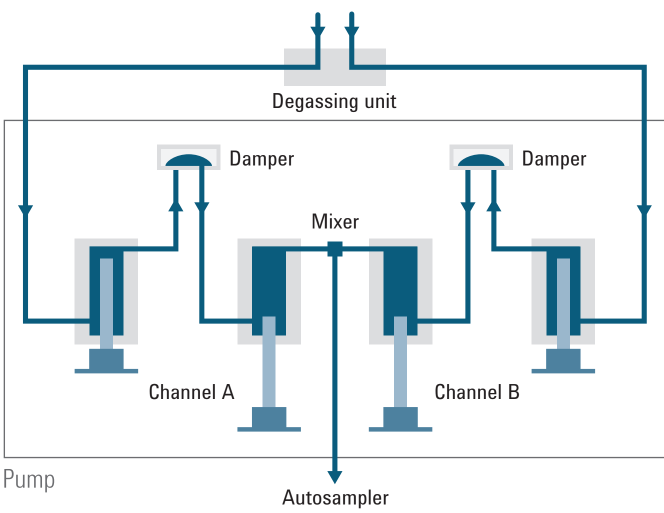


Figure 3.2 Schematic of a solvent delivery system for high-pressure mixing of gradients, showing the two solvent channels for generation of binary elution gradients.

3.2 Sample introduction

In preparative LC the number of samples and the amount of sample to be injected varies significantly. As a consequence we need to consider manual injection techniques as well as automatic liquid samplers – known as autosamplers – and injection pumps as possible techniques for transfer of the sample to the flow path. Autosamplers are available with two different design approaches; flow-through-needle and fixed-loop.

3.2.1 Flow-through-needle design of autosampler

The flow-through-needle design easily handles a variety of injection volumes and there is usually no loss of sample when filling the sample loop to the maximum. However, a disadvantage of this design is the larger dwell volume resulting from the size of the sample loop and metering device. This is a particular drawback when switching between analytical and preparative injection modes. Nevertheless, flow-through-needle technology facilitates easier workflows and eliminates sample losses caused by improper injection steps. Figure 3.3 shows how the eluent flows through the needle and onto the column.

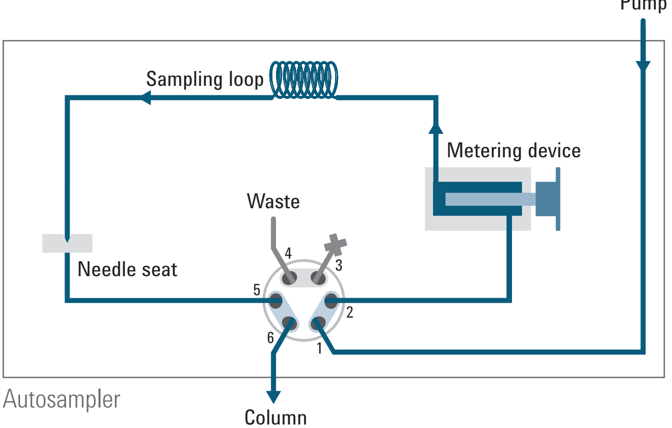


Figure 3.3 When the autosampler is in flush mode, the eluent from the pump flows through the switching valve, metering device, sampling loop, injection needle and needle seat, and again through the valve to the column.

For injection of larger sample volumes up to several milliliters, the capacity of the seat capillary between the needle seat and switching valve can be increased. This extra volume is used as a buffer to hold multiple aliquots drawn from a sample vessel and this procedure is appropriately termed the multi-draw approach. Increasing the seat capillary volume does necessarily increase the dwell volume. However, switching to bypass mode alleviates this dilemma as explained in later sections.

At the start of an injection cycle the switching valve moves to the bypass mode, diverting the eluent from the pump directly to the column. This takes the metering device, sampling loop and injection needle out of the flow path in preparation for sampling, see Figure 3.4.

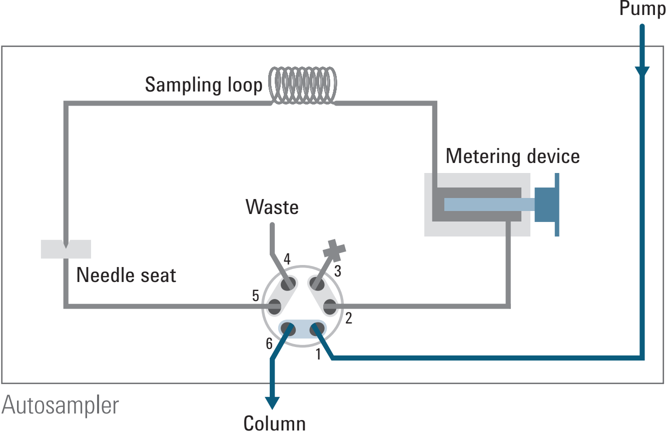


Figure 3.4 When the autosampler is in bypass mode, the eluent flow from the pump is diverted directly to the column, leaving the metering device and injection needle free to begin sampling.

Switched out of the flow path, the injection needle is raised, a sample vessel such as a vial placed underneath, and the needle lowered into the sample. Withdrawing the plunger of the metering device pulls sample through the needle into the sampling loop, see Figure 3.5.

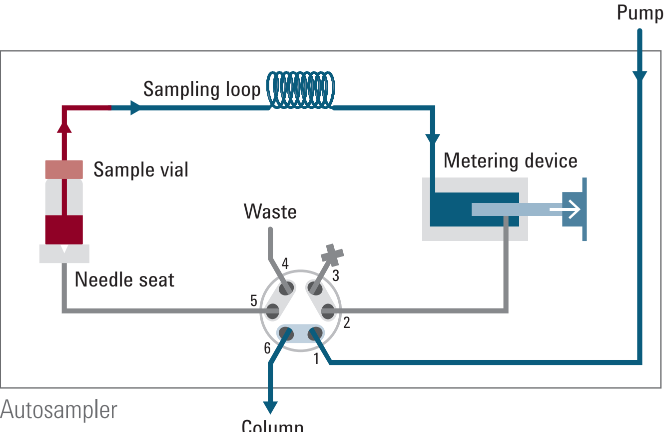


Figure 3.5 Drawing sample through the needle and into the sampling loop.

When the required amount of sample has been transferred to the sampling loop, the needle is raised out the sample vessel, the vessel moved away, and the needle lowered onto the needle seat. The switching valve now

returns to flush mode, sweeping the sample out of the loop, through the needle and onto the column, see Figure 3.6.

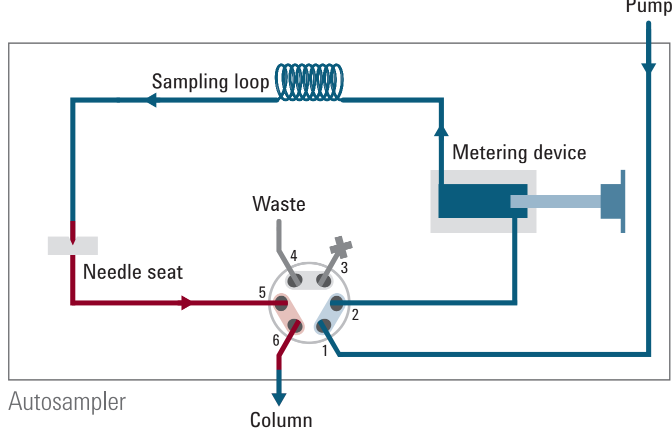


Figure 3.6 Switching back to flush mode sweeps the sample onto the column.

To reduce the dwell volume, we recommend returning to the bypass mode as soon as the sample has been transferred to the column. This can be accomplished using a programming step, whereby it is important to calculate exactly the amount of time required to transfer the sample to avoid trapping a portion of the sample volume in the valve or capillaries, see Figure 3.7.

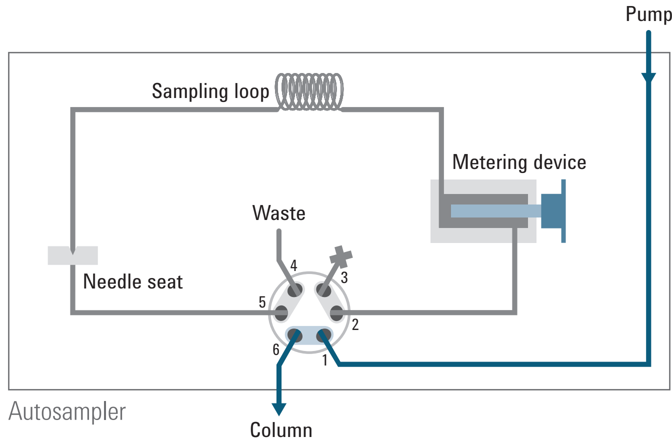


Figure 3.7 Switching to bypass mode after injection to reduce dwell volume.

3.2.2 Fixed-loop design of autosampler

When working with a system where – in alternating mode – analytical-scale and preparative scale injection volumes are required, the size of the sample loop of a flow-through-needle design of autosampler will contribute significantly to the dwell volume. Systems with large dwell volumes will not perform well when using analytical flow rates. For these applications the fixed-loop design of autosampler with two sample loops of different sizes can eliminate this dilemma. A switching valve is used to create completely separate analytical and preparative flow paths. Nevertheless, there are several aspects we need to consider to be able to work successfully with this fixed-loop design of autosampler with dual loops.

Figure 3.8 shows the dual-loop concept. In this setup, the upper sample loop has a larger volume suitable for preparative work at higher flow rates. In contrast, the lower injection loop has a smaller volume and used for analytical scouting at lower flow rates. Separate needle seats facilitate individual filling of the loops with sample. We describe the injection cycle in more detail in later sections.

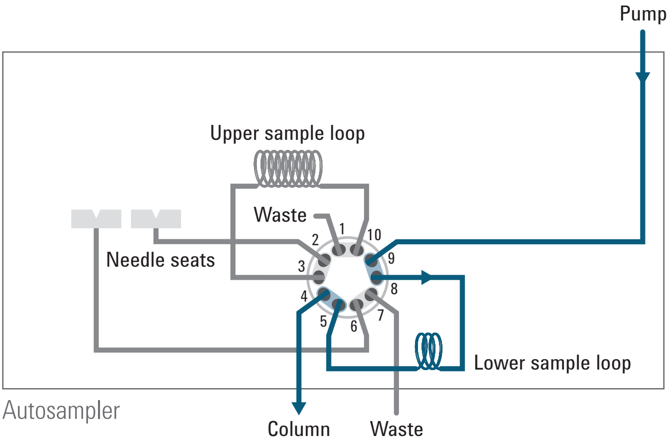


Figure 3.8 Schematic of a dual-loop autosampler, showing the flow directed through the lower, small-volume sample loop for analytical application work.

Because the loop is a part of the capillary system, the dwell volume increases with increasing loop size. As a consequence, we recommend keeping the dwell volume as small as possible, balancing it against the flow rates that will be used for chromatographic separation. As a rule of thumb, the ratio of total system void volume to applied flow rate should be equal to or less than 2:1 to obtain reasonable chromatographic performance.

When deploying fixed-loop autosamplers, the sample loops can be partially filled or overfilled, see Figure 3.9 and Figure 3.10.

Figure 3.9 Partial fill of sample loop.

Figure 3.10 Overfill of sample loop.

Partial filling of the sample loop is the most common approach, whereby we recommend not exceeding 65 to 70 % of the loop's capacity. Attempting to inject volumes approaching the capacity of the loop can lead to loss of sample caused by the sample flowing out of the other end of the loop. You can check this easily by using a colored sample.

Overfilling or full-loop injection is required for accurate, quantitative work and we do not recommended this technique for purification work as a large portion of a precious sample will be lost. For accurate analytical-scale injections we recommend an overfill factor between 2.3 and 5 to purge the loop completely with sample.

As mentioned earlier in this section, the dual-loop design of autosampler is well suited to meet the requirements of both analytical and preparative LC where vastly differing sample volumes need to be injected. The dual-loop design minimizes the total dwell volume of the injection system and facilitates analytical scouting with optimum separation efficiency prior to sample purification.

Figure 3.11 shows a dual-loop autosampler in flush mode, whereby the flow is directed through the upper, larger volume sample loop for preparative application work. For analytical applications, the flow path through the smaller loop is used as shown earlier in Figure 3.8.

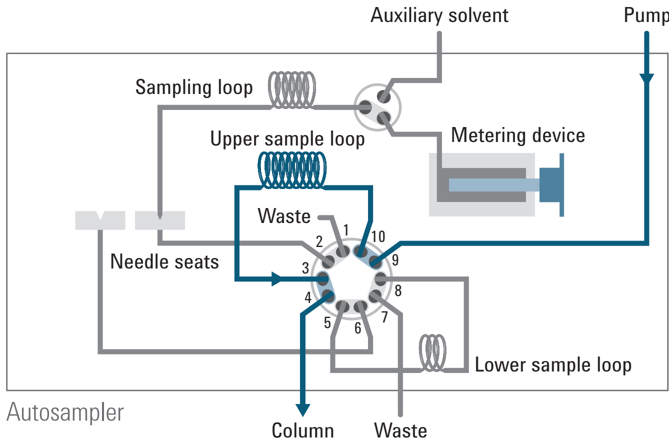


Figure 3.11 Dual-loop autosampler in flush mode, showing the flow directed through the preparative sample loop.

The injection cycle starts by switching the valve into the bypass mode, directing the eluent from the pump through the analytical sample loop. This takes the preparative sample loop out of the flow path in preparation for sampling, see Figure 3.12. To make sure the analytical column is equilibrated correctly we recommend purging the analytical sample loop with the eluent that is to be used at the start of the next run.

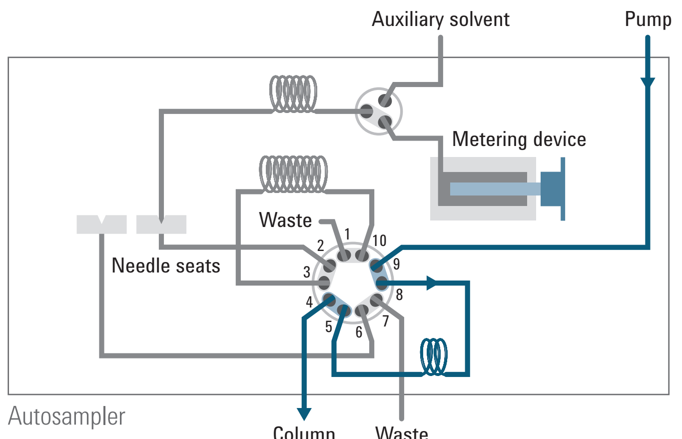


Figure 3.12 Dual-loop autosampler in bypass mode, showing the flow directed through the second sample loop, in this example a loop for analytical injection volumes.

The injector needle is raised, a sample vessel such as a vial placed underneath, and the needle lowered into the sample. Withdrawing the plunger of the metering device pulls sample through the needle into the sampling loop, see Figure 3.13.

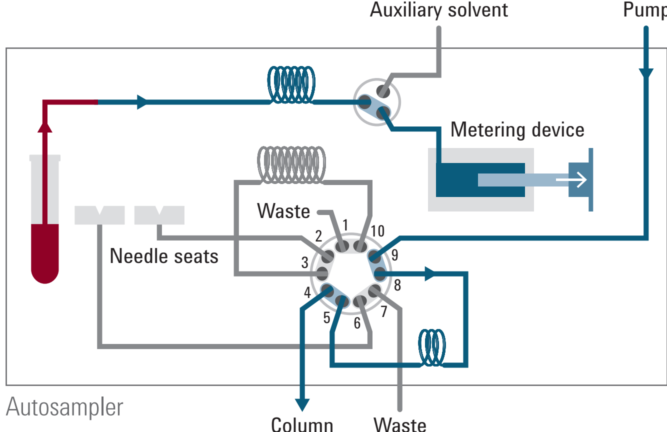


Figure 3.13 Drawing sample through the needle and into the sampling loop.

When sufficient sample has been drawn into the sampling loop, the needle is raised out the sample vessel, the vessel moved away, and the needle lowered onto the needle seat. The metering device now drives the sample out of the sampling loop, through the needle and needle seat and into the preparative sample loop, see Figure 3.14.

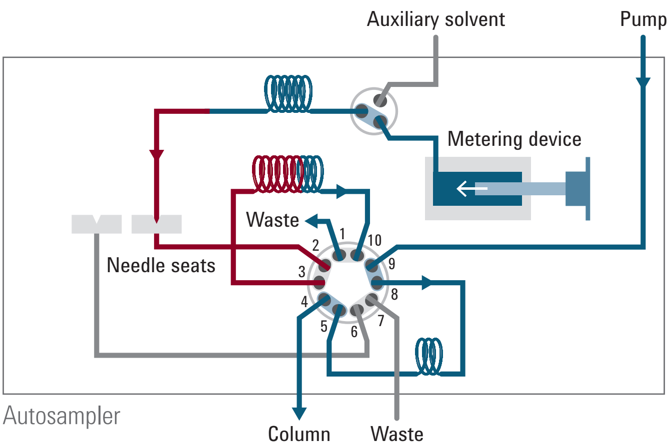


Figure 3.14 Driving the sample into the preparative sample loop.

Moving the switching valve back to flush mode sweeps the sample out of the preparative sample loop and onto the column, see Figure 3.15.

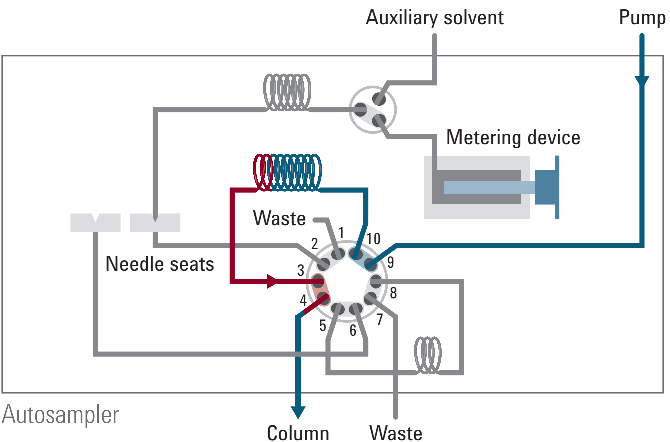


Figure 3.15 Switching the valve back to flush mode sweeps the sample out of the loop and onto the column.

When deploying a dual-loop autosampler, it is essential to have the possibility to flush the sampling loop and the needle seat capillary with a well-degassed auxiliary solvent, and also to wash the needle's outside surfaces for at least 10 seconds. This eliminates sample carryover between injections and prevents air bubbles building up in the loop. The auxiliary solvent is drawn into the metering device by switching the auxiliary solvent valve and withdrawing the plunger. The valve is then switched again and the plunger of the metering device drives the solvent through the sampling loop and needle seat capillary, see Figure 3.16.

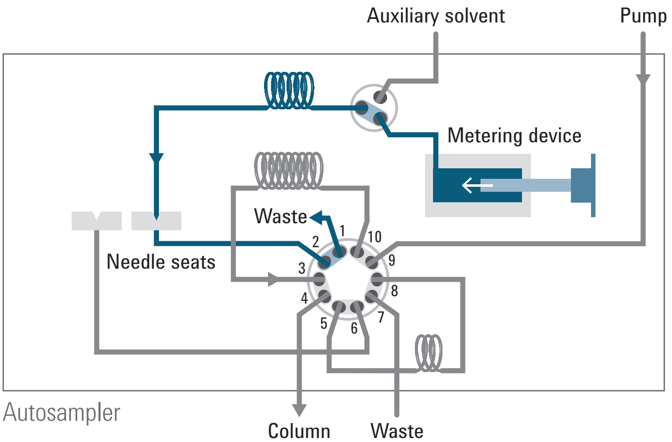


Figure 3.16 Flushing the buffer loop and the needle seat capillary eliminates carryover.

3.2.3 Special injection techniques

Compound solubility, sample preparation and storage conditions often demand the use of other solvents than are required as eluents for best chromatographic performance. In these situations special techniques for sample injection have been developed.

3.2.3.1 Sample sandwiching

This injection technique can be applied when samples tend to precipitate on contact with the mobile phase. Plugging of injector needles and capillaries can be possibly avoided. By programming the autosampler, the sample can be embedded or sandwiched between two plugs of an appropriate solvent that avoids precipitation in the sample loop, see Figure 3.17. DMSO or solvents immiscible with water can be used. For analytical injections the volume of injected DMSO has to be as low as possible. We recommend using two 5-µL plugs. As a rule of thumb for preparative injection volumes, we recommend using a total plug volume of about 10 % of the total injection volume. Good results can be achieved using dichloromethane as a sandwich solvent.

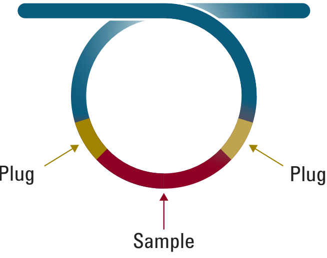


Figure 3.17 Profile of a sandwich injection using plugs of appropriate solvent either side of the sample to avoid precipitation in the sample loop or connection capillaries.

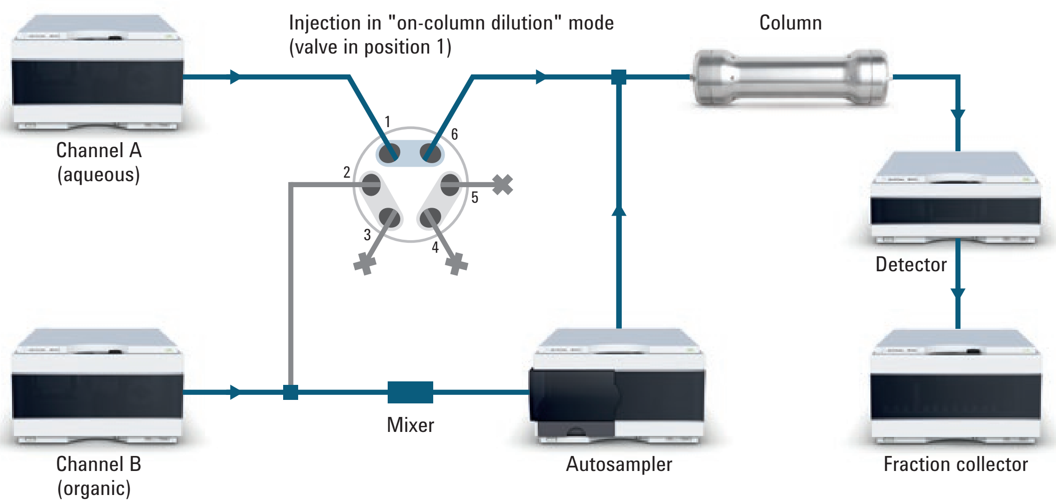
3.2.3.2 Injecting large volumes of strong organic solvents

Most compounds originating from organic synthesis work are well soluble in DMSO or DMF. These solvents offer strong solubility but exhibit high elution strength and may distort the chromatographic separation. This occurs especially when the compounds to be separated have relatively high polarity and hence low retention.

Column dimensions (id x length in mm)	2.1 x 150	4.6 x 150	9.4 x 150	21 x 150	30 x 150	50 x 150
Recommended injection volume [µL]	2	10	100	500	1000	2000
Maximum injection volume [µL]	5	20	200	1000	2000	3000

Table 3.1 Recommended injection volumes of strong solvents. When exceeding the maximum injection volumes the chromatographic performance will be strongly affected. Parts of the sample may elute with or as part of the initial solvent peak.

If the required volume needs to exceed the maximum injection volume for the given column size, we recommended to inject the sample in pure organic solvent and then dilute with the aqueous solvent directly before the column (known as on-column dilution injection mode). To inject in this way, a simple T-piece is added in the flow path immediately before the column. To inject the sample in pure organic solvent, channel B is used to deliver a flow rate of 5 to 10 % of the total flow. A complementary percentage of water (90 to 95 % of the total flow) is delivered by channel A and added through the T-piece. Two T-pieces and a switching valve facilitate injection of large volumes of strong organic solvents without causing precipitation of the sample, see Figure 3.18.



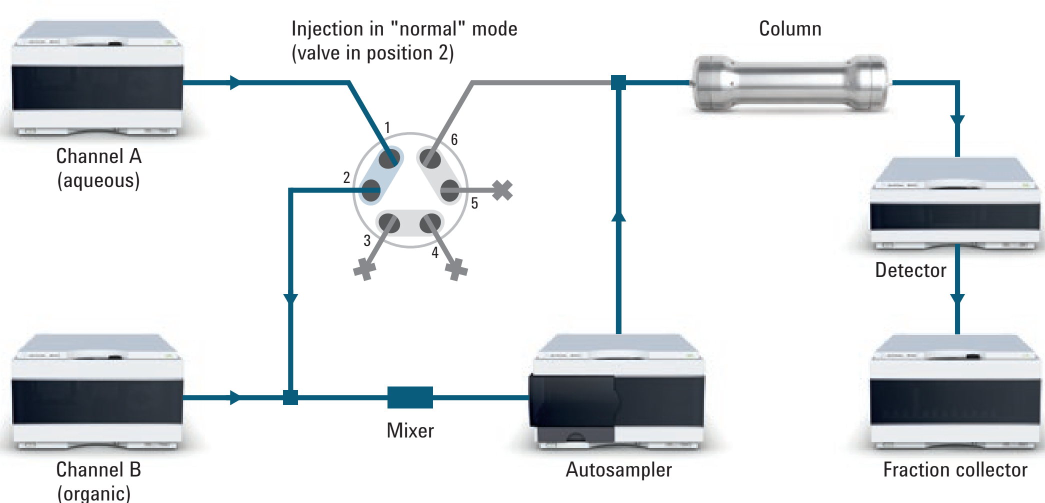


Figure 3.18 Schematics of a system configuration for different modes of sample injection. With the valve in position 1, the sample is injected with pure organic solvent delivered by the channel B pump. With the valve in position 2, solvents A and B are mixed before passing the injector. This mode can be defined as the "normal" injection mode.

When deploying a configuration such as shown in Figure 3.18, it is important to keep the distance between the mixing point – the T-piece – and the column as short as possible to avoid precipitation of the sample.

After injection we recommend keeping the eluent composition constant – on isocratic hold – until the sample has been transferred to the column. A further isocratic-hold step with low %B is added to flush out the organic solvent. The gradient can now be ramped up to elute the compounds from the column. This approach also reduces pressure shocks after injection of large volumes of highly viscous sample solutions. The loading process is extremely smooth, extending column lifetime while increasing column load as well as chromatographic resolution.

However, nonpolar compounds often exhibit poor peak shape when using the described injection mode. With a configuration as shown in Figure 3.18 it is possible to switch between the two different injection modes. Sandwich injections also deliver good results especially when the compounds are highly nonpolar and likely to precipitate easily.

Figure 3.19 shows the chromatogram after a standard injection of a high sample volume with strong eluents. Sample loss occurred as indicated by the badly distorted peak shape. In contrast, using the alternative on-column dilution injection mode resulted in no sample loss with the polar compounds focused on the column, see Figure 3.20.

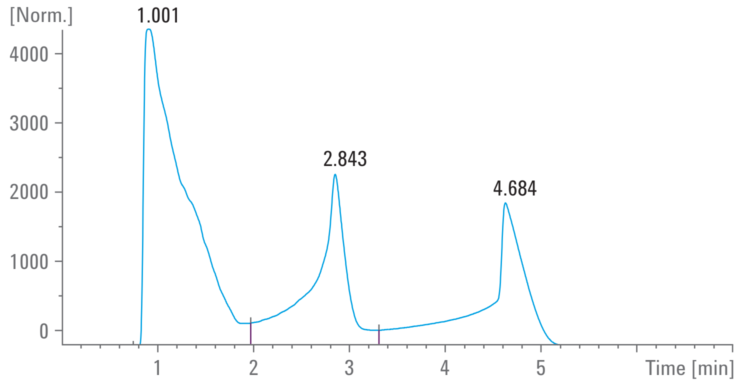


Figure 3.19 High-volume injection with strong eluents in standard injection mode. The chromatogram shows strongly distorted peak shapes of the compounds at 2.84 and 4.68 minutes. The solvent peak at 1.00 minute contains sample breakthrough – as a consequence sample is lost.

Sample: 50 mg acetamidophen and 50 mg caffeine in 5000 µL DMSO
Column: Agilent ZORBAX SB C18, 21.1 x 150 mm, 5 µm
Applied gradient profile for standard injection mode:

Time	Flow	%A	%B
0	37	93	7
0.6	37	93	7
6.0	37	78	22
6.1	37	2	98
9.0	37	2	98
9.1	37	93	7
14	37	93	7

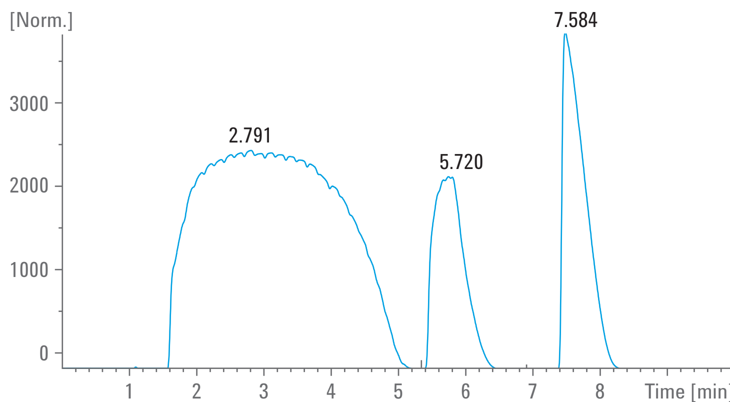


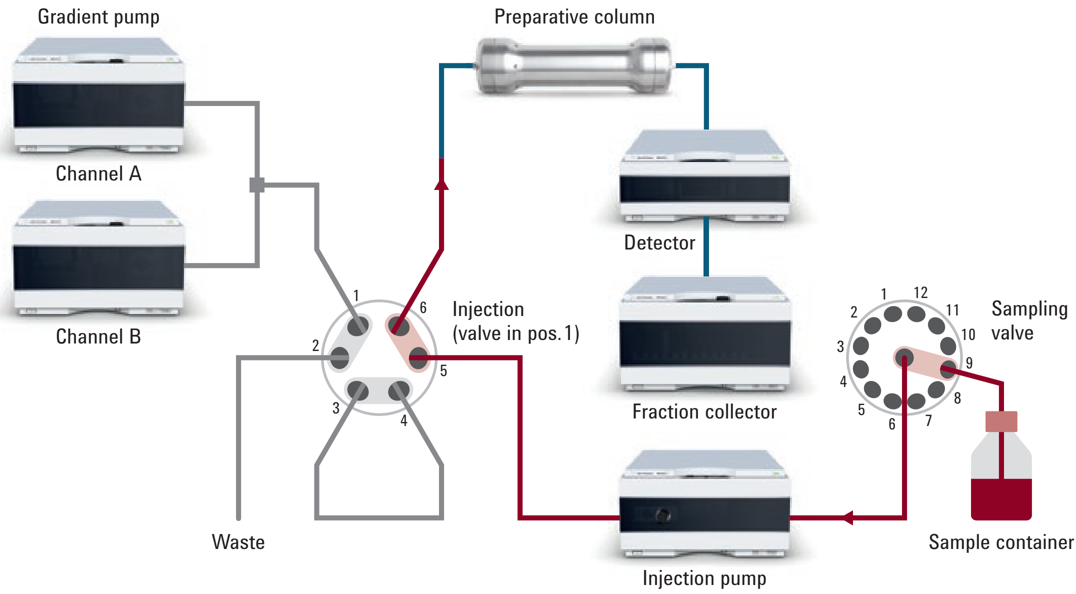
Figure 3.20 High-volume injection with strong eluents using the alternative injection mode. A broad solvent peak is obtained unless the DMSO has been washed out of the column. The two compounds at 5.72 and 7.58 minutes have been retained on-column and eluted as baseline separated peaks. No sample loss has been observed.

Sample: 50 mg acetamidophen and 50 mg caffeine in 5000 µL DMSO
Column: Agilent ZORBAX SB C18, 21.1 x 150 mm, 5 µm
Applied gradient profile for sandwich injection mode – a longer isocratic holding step is needed to transfer the sample to the column prior to ramping up the gradient:

Time	Flow	%A	%B
0	37	93	7
4.0	37	93	7
10.0	37	78	22
10.1	37	2	98
13.0	37	2	98
13.1	37	93	7
16.0	37	93	7

3.2.3.3 Injecting large sample volumes using an injection pump

For separation of highly diluted samples in aqueous solvents, large injection volumes that exceed the typical loop sizes of autosamplers are often required. In these situations an injection pump can be used to load the sample onto the column. Injection pumps can also be used for applications such as enantiomer separation. Here, the same sample solution must be loaded periodically and an injection pump can achieve this much faster than an autosampler. After sample transfer to the column, thoroughly purge the injection pump, the sampling and injection valves, and all connection capillaries to avoid sample carryover. Figure 3.21 shows a typical system configuration for sample introduction using an injection pump.



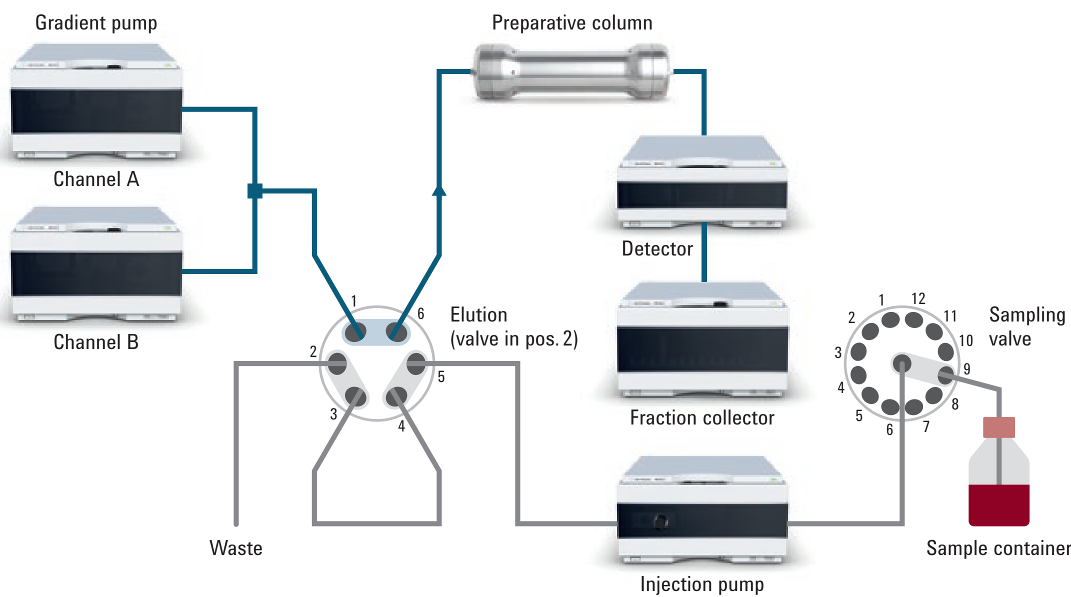


Figure 3.21 Schematics of a system configuration for sample introduction using an injection pump. With the valve in position 1, the injection pump draws the sample from the container and delivers to the column. For elution, the valve is switched to position 2 so that channels A and B combine, generating the required eluent composition for compound separation.

3.3 Flow splitting

When using a destructive detector in preparative LC, a flow splitter is necessary to divert the majority of the eluent to the collection device. Flow splitters can also be deployed to reduce the flow rate to within the analytical range when detectors' flow cells are not compatible with high flow rates. Combined with a make-up flow a splitting approach also secures the major part of the purified compound while the additional solvent prevents the detector flow cell from reaching saturation through high compound concentration. Different designs are applied to serve fundamentally similar purposes.

3.3.1 T-piece flow splitting

A simple, inexpensive approach to flow splitting is to use a T-piece, see Figure 3.22. Single T-piece splitters are used at low flow rates in combination with analytical columns, for example, to connect the LC system to a destructive detector such as one based on mass or evaporative light scattering. This approach secures the majority of the eluent that contains the target compound.

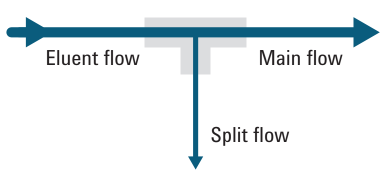


Figure 3.22 T-piece splitter – the split ratio is regulated by the backpressure resulting from the dimensions of the outlet capillaries.

The split ratio is dependent on the ratio of the backpressures at the outlets of the main stream and the split stream and must to be adjusted experimentally to meet the needs of the application. Using capillaries with different lengths and inside diameters alters the backpressure ratio and consequently changes the split ratio.

3.3.2 Double T-piece flow splitting

When using typical semi-preparative columns, the concentrations of the compounds as well as the buffers are often too high for direct introduction with a single T-splitter into a mass-selective detector. In these situations, the split flow needs to be diluted with a make-up solvent, see Figure 3.23. Make-up solvents support electrospray ionization and guarantee stable and fast compound transport from the splitting point to the detector. Mass-selective, evaporative light scattering and refractive index detectors all require make-up solvents for preparative applications.

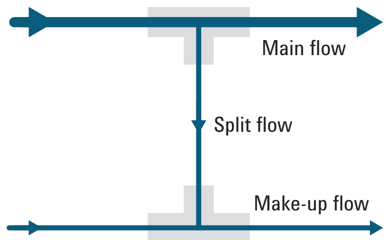


Figure 3.23 Double T-piece splitter–often used when a make-up flow is required for special detection techniques. The split ratio is regulated by the backpressure.

In a double T-splitter the split ratios are dependent on multiple parameters, whereby the major dependence is on the pressure difference between the main flow and the make-up flow. The pressure difference is typically set at 6 bar (90 psi) based on experiment using different restrictors for different flow rates. At this pressure splitter also works in gradient mode, in which the pressure of the main flow changes according to the viscosity of the solvent composition. The split ratios of commercially available splitters are preset by using different diameters and lengths of the split flow capillary. Some splitters have additional needle valves for further regulation of the pressure ratios. The split ratios obtained are only estimated values and not true actual values. Nevertheless, using these T-splitters provides for true splitting in real time without losing any information.

3.3.3 Active flow splitting

Active flow splitters are devices – typically valves – that transfer mechanically a small aliquot from the main stream to the split stream, see Figure 3.24.

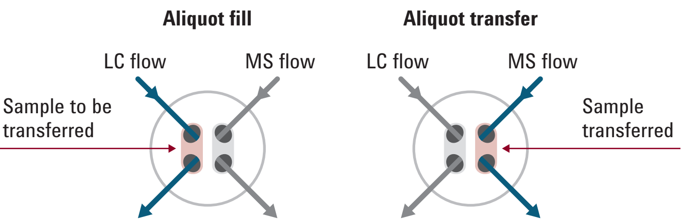


Figure 3.24 Schematics of an active flow splitter, showing the fill and transfer positions of the valve.

The split ratio depends on the switching frequency of the valve, the volume of the grove and the applied LC flow rate, see Equation 3.1.

$$\text{Split ratio} = \frac{\text{LC flow rate [µL/min] / Valve groove volume [µL]}}{\text{Switching frequency [Hz] x 60}}$$

Equation 3.1 Calculating the split ratio of an active flow splitter.

A choice of different groove volumes in the nanoliter range can be made through software to adapt for the flow rate.

Typically, a switching frequency of 1 Hz delivers good results. The make-up flow rate is an important factor affecting the signal strength and the delay time between the splitting point and the detector. Some mass-selective detectors may show only limited sensitivity at high flow rates while others can cope with flow rates above 1 mL/min.

The lifetime of rotor seals in the splitter can be extended by activating the splitting process only when fraction collection is required. The splitter can be deactivated and the flow diverted to waste during column purging and equilibration, and during the injection cycle, resulting in longer preventive maintenance intervals.

3.4 Detection

The most common type of detection used in liquid chromatography is based on absorbance of ultraviolet (UV) and visible light. Detection at a fixed wavelength is the most economic approach. For analytical scouting, detectors with photodiode arrays – known as diode array detectors (DAD) – are a safer choice because the spectral information acquired can be used to confirm compound identity, determine peak purity, and select the best wavelength for detection in subsequent purification processes. Figure 3.25 shows the optical system of a diode array detector.

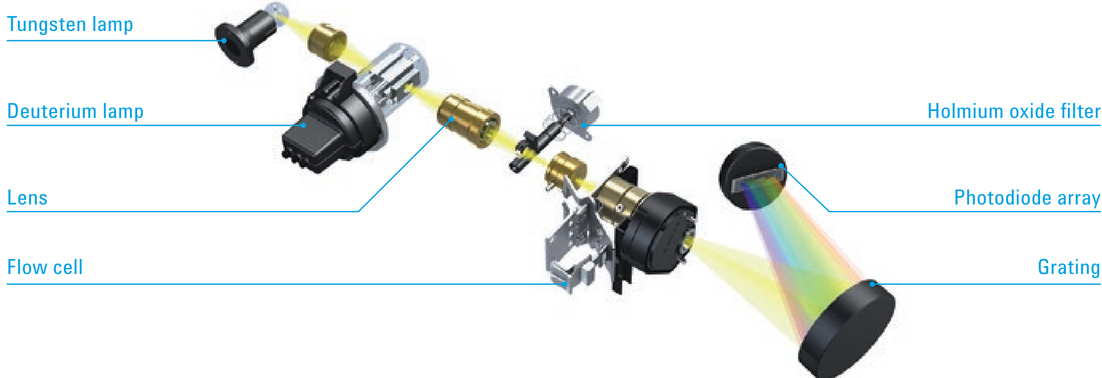
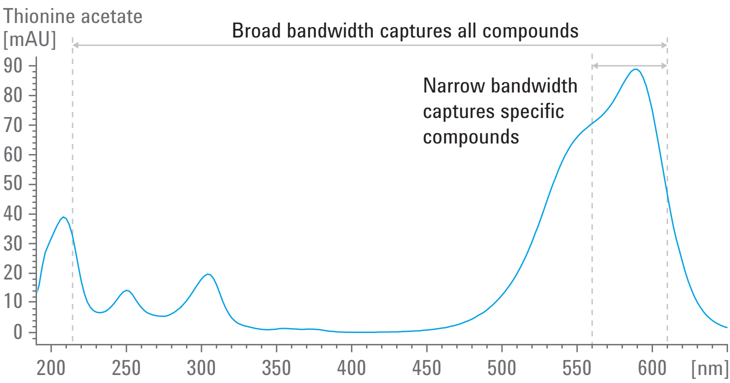


Figure 3.25 Optical system of a diode array detector.

Diode array detectors deliver full spectra in the UV and visible ranges. The sampling frequency for all wavelengths can be as fast as 240 Hz without loss in sensitivity. For most preparative applications a data rate of 20 Hz delivers good results. If the compounds of interest have completely different spectra, setting a broad bandwidth on the diode array detector facilitates monitoring of the entire UV absorption as a single chromatogram. For example, dyes with different UV spectra, as shown in Figure 3.26, can be analyzed by setting the detection wavelength to 420 nm with a bandwidth of 400 nm. In this example, by applying a continuous band from 220 to 620 nm the compounds with different absorption maxima can be detected and displayed in a single chromatogram, whereas specific wavelengths are used with smaller bandwidths to display the compounds selectively.



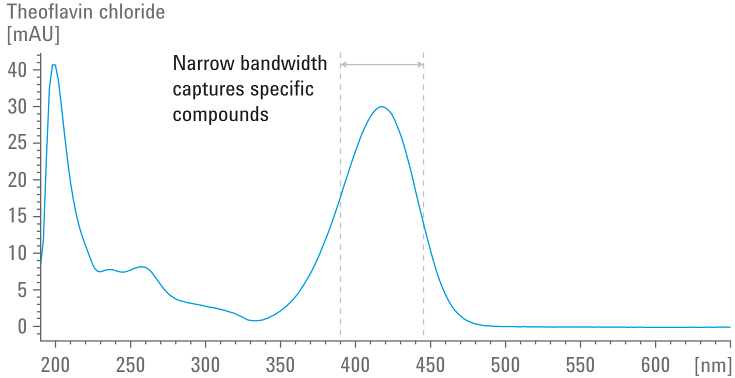


Figure 3.26 Two compounds with different UV spectra. Data acquisition using a wide bandwidth facilitates monitoring these different compounds in a single chromatogram. Using a small bandwidth instead monitors selectively only one of the compounds.

3.4.1 Matching concentration range with dynamic range

Variations in sample concentration can be dramatic and place significant demands on the optical design of any detector. Impurities should be visible during analytical scouting – even when they have low absorption coefficients. In contrast, target compounds can have extremely high concentration when working at high purity and with high column loading.

According to the Lambert-Beer law, see Equation 3.2, the most important parameter that can be used to match the concentration range is the path length of the flow cell.

$$A = \epsilon_\lambda c d$$

Equation 3.2 *The Lambert-Beer law.*

A	absorption
ϵ_λ	molar extinction coefficient
c	concentration [mol/L]
d	path length [cm]

For analytical work, typically a path length of 10 mm is used. However, during analytical scouting the sample concentrations are generally much higher than in analytical work. In these situations, a flow cell with a path length of 1 to 3 mm is a good starting point. Semi-preparative work on 21-mm id columns can be realized with a path length of 0.3 mm. For larger sample amounts applied to larger column diameters with higher flow rates, we recommend a path length of 0.06 mm.

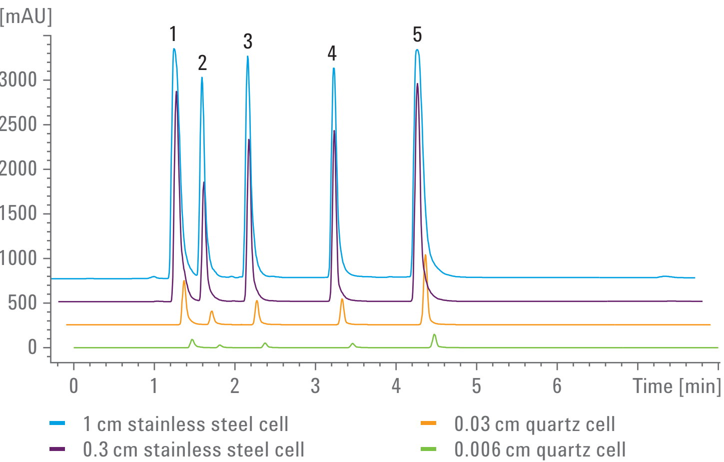


Figure 3.27 Flow cells with shorter path lengths reduce peak areas.
1 Caffeine, **2** Primidone, **3** Phenacetine, **4** Mandelic acid benzylester, **5** Biphenyl

An additional approach to increase the dynamic range is to measure the UV absorbance simultaneously with two light paths of different lengths in one or two detectors. At low concentrations the signal from the longer light path is taken. When the signal reaches the saturation level of the measuring diode, the system uses the signal from the shorter light path. This combination can extend the dynamic range of detection by at least one order of magnitude. Figure 3.28 shows a cut-away schematic of a dual-path flow cell.

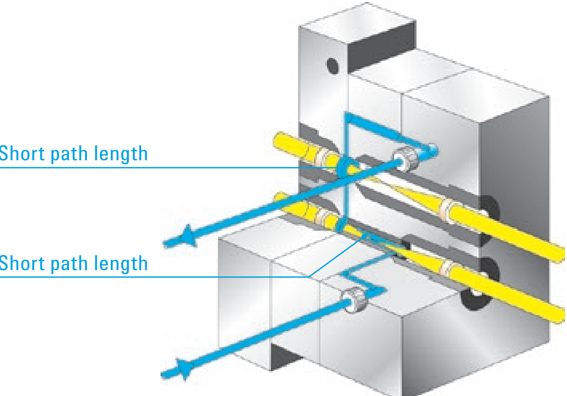


Figure 3.28 Schematic of a dual-path flow cell.

3.5 Fraction collection

The addition of a fraction collector with triggering of eluent collection into respective collection vessel constitutes the difference between an analytical and a preparative LC system – regardless of flow rate or pump capacity. During manual control of fraction collection the operator decides on the appropriate timing of when to start and stop collection. Time-based collection ensures collection of all relevant fractions throughout the entire run. Typically, this approach often means a higher workload because a large number of fractions are collected and need to be analyzed and processed. Further, fractions are not pure when different peaks are collected in the same vial. Triggering fraction collection based on selective detection helps to reduce the number of fractions to process and thereby increases the efficiency of the laboratory.

In a multi-user environment it is desirable to configure multiple fraction collectors in one system where each fraction collector can be allocated to an individual user. The fraction collectors are connected together by a multipurpose valve and form a software-controlled cluster. When the bed of the first fraction collector exceeds its capacity the multipurpose valve diverts the flow stream automatically to the next available fraction collector, if desired.

3.5.1 Collecting fractions manually

If samples are of particular value or exhibit unknown behavior, individual operator control is often the preferred approach. A graphical user interface with an online signal plot allows the operator to control fractionation by simple mouse-click.

3.5.2 Collecting fractions based on time

A simple mode of collection is time-sliced fraction collection. In this mode, fractions are taken periodically, see Figure 3.29. This collection mode is often applied when purifying complex mixtures such as natural compounds from plant extracts.

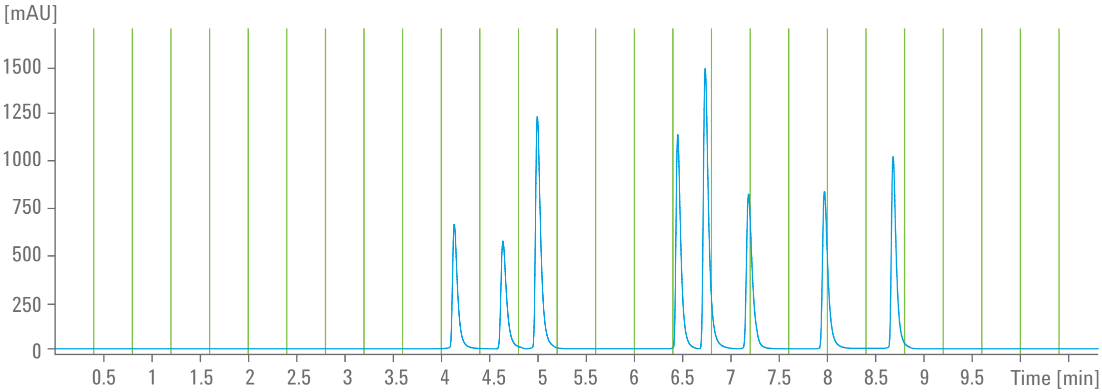


Figure 3.29 Fraction preview for time-based fraction collection. A new fraction is taken every 0.40 minutes.

3.5.3 Collecting fractions based on UV detection

When using peak-based fraction collection, compounds are collected according to slope or threshold settings, or by a combination of both, from a UV or additional detector such as an evaporative light scattering (ELS) detector or similar. When the signal exceeds the threshold the fraction collector starts to collect. To avoid false-positive triggering when the baseline rises during the gradient slope, a second parameter – called slope recognition – can be combined with the threshold trigger.

Collection parameters can be set before purifying the actual sample by loading a previously acquired preparative chromatogram into a fraction preview tool, This chromatogram should contain standard compounds at concentrations similar to those used later. The chromatogram is displayed and the parameters for fraction collection can be set interactively, see Figure 3.30.

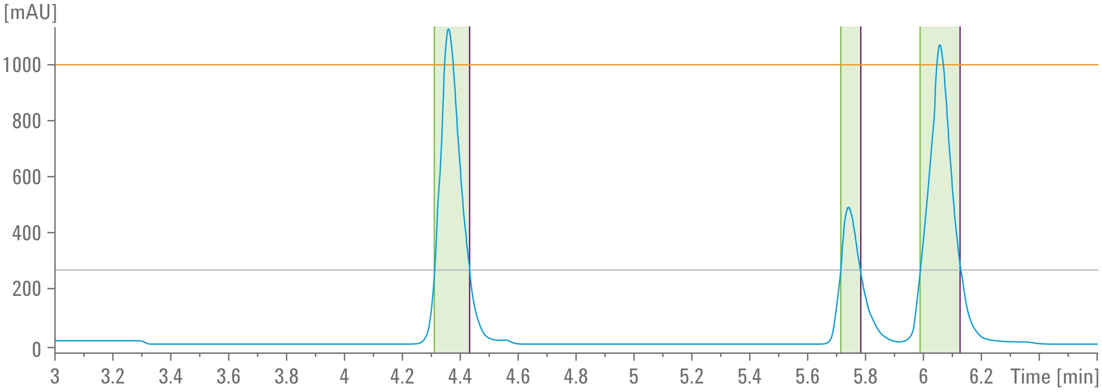


Figure 3.30 Fraction preview tool – setting trigger threshold and slope parameters interactively.

3.5.3.1 Setting the threshold level

If the signal intensity rises above the threshold level, fraction collection begins. When the signal falls below the threshold level, fraction collection stops, see Figure 3.31.

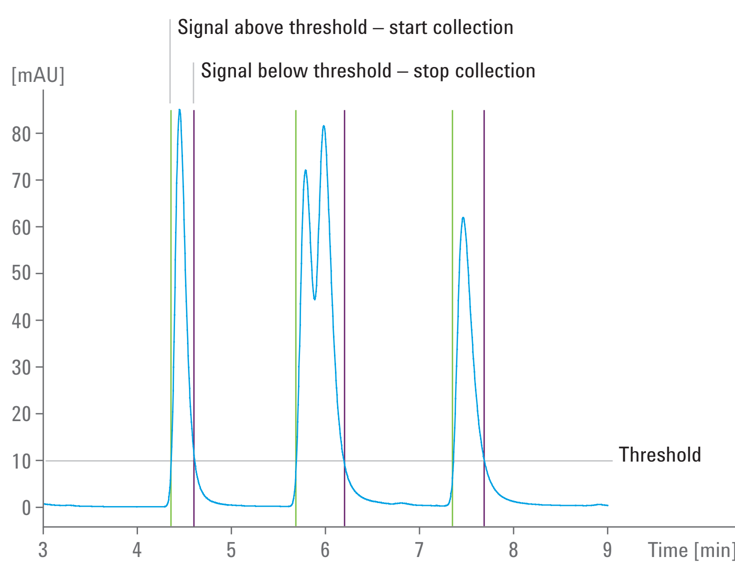


Figure 3.31 Fraction collection triggered by threshold setting.

3.5.3.2 Setting slope parameters

The slope of a chromatographic trace can be calculated as the first derivative. This is an appropriate parameter for indication of an eluting peak. The obtained signal rises from the baseline until the first inflection point of the peak is reached. Moving along the curve of the peak the first derivative becomes negative. After the second inflection point of the peak the first derivative reaches its start value, see Figure 3.32.

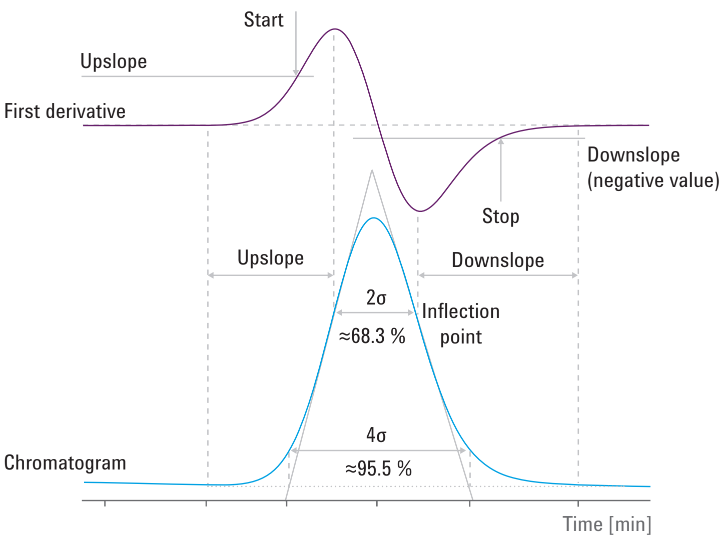


Figure 3.32 Slope recognition using the first derivative of the chromatographic trace.

Upslope and downslope settings for fraction collection avoid false-positive triggering when the monitored baseline rises slowly (baseline drift) due to the solvent gradient. Further, these settings help to separate non-baseline-resolved chromatographic peaks.

3.5.3.3 Setting an upper threshold limit

With high sample loading or high absorption coefficients, the detector electronics become saturated and create flat-top peaks potentially with signal fluctuations. This behavior creates the impression that these fluctuations could be additional peaks and cause the fraction collector upslope and downslope algorithms to trigger collection of additional fractions. An upper threshold limit prevents the detector from such triggering unless the volume exceeds the capacity of the fraction collection container. Typically, the upper threshold limit is set to approximately 90 % of the maximum signal strength before saturation occurs. If the detector

signal is permanently reaching the saturation limit, we recommended decreasing the length of the light path with a shorter flow cell to enable peak-based fraction collection. Injecting smaller sample amounts is another option.

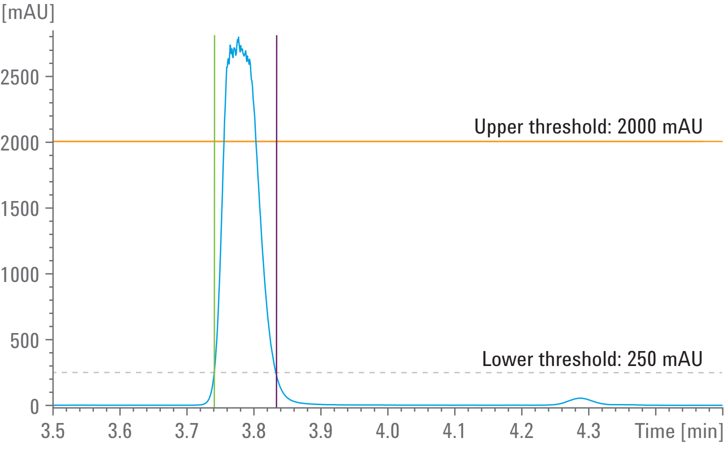


Figure 3.33 An upper threshold limit prevents fraction triggering caused by artificial signal fluctuations when detector saturation occurs.

3.5.4 Collecting fractions based on evaporative light scattering detection[7]

Absorption of UV or visible light by the target compounds is a prerequisite for detection using a diode array or other UV detector. If the target compounds do not contain any chromophore structures, evaporative light scattering (ELS) detection is an alternative solution. ELS detection has proven to be very useful as a complementary technique to UV absorption, particularly when dealing with unknown mixtures such as natural product extracts, peptides, lipids, oils or other aliphatic compounds. Even with mass spectrometry, ELS detection can be successfully deployed when ionization of target compounds is suppressed or just difficult to achieve.

Light scattering occurs when eluents are evaporated and solutes have ideally formed particles or droplets with residual solvent in a drying gas stream. Diverting the majority of sample for collection, while meeting the dynamic range of the ELS detector, is achieved by splitting the column effluent between the fraction collector and the ELS detector, see Section 3.3 "Flow splitting".

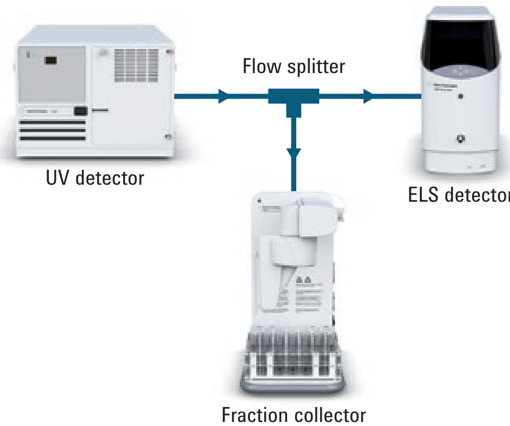


Figure 3.34 Schematic of a simple T-splitter when using a complementary detection technique.

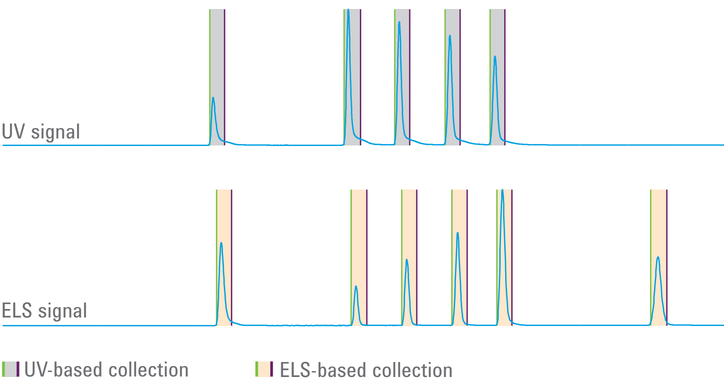


Figure 3.35 UV and ELSD-based fraction collection. Collection was triggered on both peak detectors. The gray areas represent the fractions collected by the UV detector, and the orange areasrepresent the fractions collected by the ELS detector. Green and red tick marks represent the start and end of collection.

3.5.5 Fraction delay

Signals from a UV detector can be processed to decide whether the eluent is diverted to collection vessels or to waste. Processing of the detector signal occurs while the potential fraction travels through tubing between the outlet of the detector flow cell and the diverter valve in the fraction collector. The void volume of this tubing has to be large enough to retain the peak of interest before the valve is switched to the desired position. The time required depends on the response time of the detector, the flow rate, and the void volume between the detector and the fraction collector. Figure 3.36 shows the functional parts of a fraction collector.

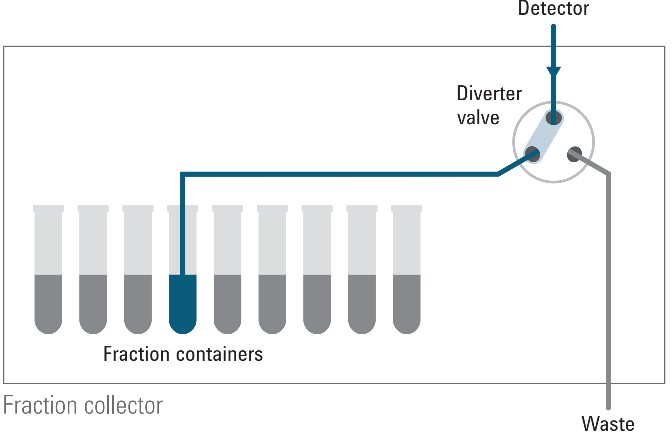


Figure 3.36 Schematics of a fraction collector, showing the basic functional parts.

3.5.5.1 Fraction delay sensor

To determine the exact delay time of a peak between the detector flow cell and the diverter valve, Agilent fraction collectors can be equipped with a fraction delay sensor. During a calibration process this device measures the time required for the peak to travel from the detector to the delay sensor, which is located adjacent to the diverter valve. The measured time difference is transformed into a delay volume using the applied flow rate feedback from the solvent delivery system. The value of the delay volume is saved in the firmware of the fraction collector for future calculations of delay time when different flow rates are used. Delay time calibration is not repeated as long as the tubing remains the same.

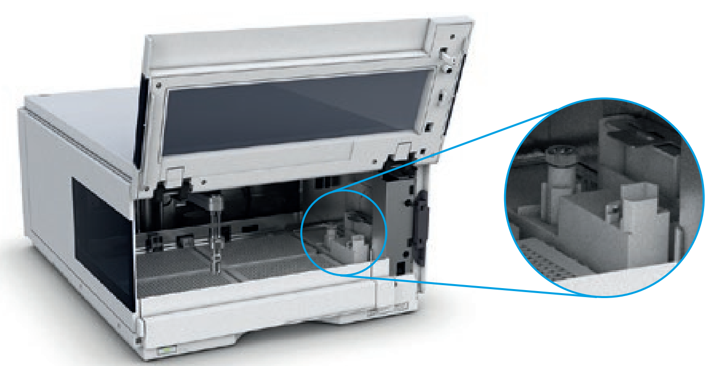


Figure 3.37 Location of fraction delay sensor in Agilent 1260 Infinity preparative-scale fraction collector.

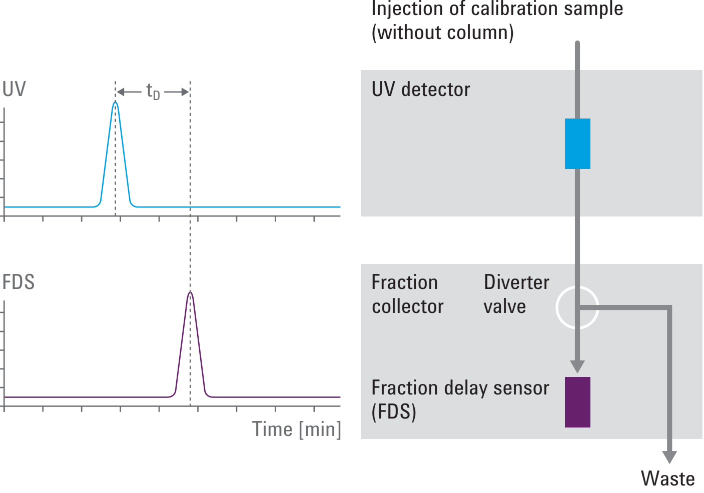


Figure 3.38 Time difference between detector and fraction delay sensor. The delay volume is calculated automatically by software during the calibration process.

3.5.6 Collecting fractions based on mass-selective detection

Mass-based fraction triggering increases the efficiency of the purification workflow significantly and is the method of choice when the number of samples per day and the number of collected fractions can no longer be handled. The number of collected fractions is reduced dramatically when using mass-selective triggering. The unequivocal characterization of the collected compounds is done instantaneous. In other words, there is no need to take aliquots of all the collected fractions and submit them to a separate LC/MS system for identification.

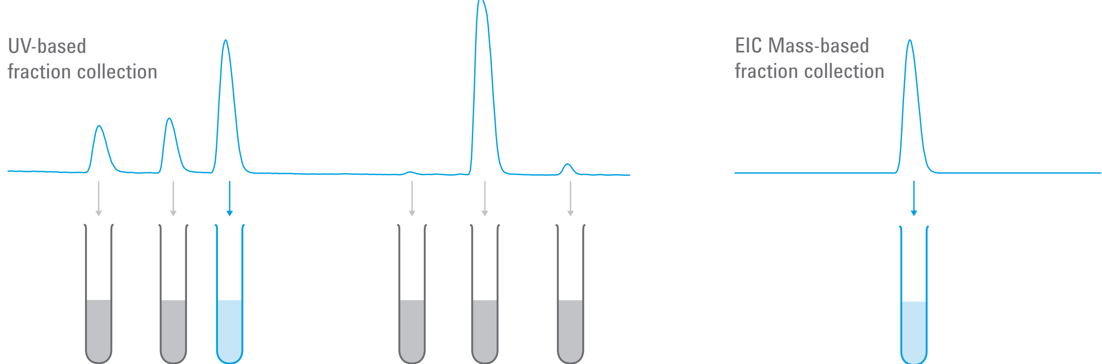


Figure 3.39 Mass-based purification is a selective method and reduces the number of collected fractions compared to UV-based collection.

It is common practice to combine the signals of the non-selective UV and selective mass detector to trigger fraction collection. The highest purity is ensured by applying Boolean AND logic. The UV signal as the principal signal is better resolved than the mass signal. Therefore the purity of the collected fractions will increase when the better-resolved signal is used to control fraction triggering. The selectivity factor is added by the mass signal.

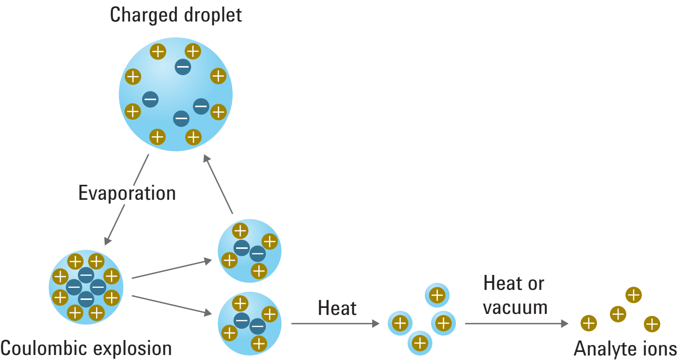


Figure 3.40 The electrospray Ionization process. After evaporation of the solvent the charge density inside the droplet is increased until free ions are ejected into the gas phase. The ionization process is affected by the volume of eluate which has to be vaporized and the concentration of buffers or matrix compounds within the droplets which suppress the ionization process.

The mass of the target compound can be obtained from analytical LC/MS results or predicted through synthesis planning. This enables triggering of fraction collection with a mass-selective (MS) detector with unmatched productivity. Unless multiple isomers occur and elute at different times this approach results in a very limited number of fractions, if not just a single fraction. Analysis of fraction purity and pooling requires much less effort. However, ionization of the target compound has to be assured under the chromatographic conditions to make the target detectable with a mass-selective detector. The usage of mono-isotopic (not average) masses is crucial to be successful. The software should allow entering the total sum formula and the adduct information. The sum of the target mass and the adduct ion equals the trigger ion by which the MS detector triggers fraction collection. When a combination of UV or ELS detectors is used with the MS detector, the delay time has to be considered for synchronization of the detector signals. In this case, the fraction delay sensor measures the different delay volumes and ensures maximum purity and recovery.

Figure 3.41 shows the time difference between UV and MS detectors, and fraction delay sensor. The delay time for MS triggering is calculated by a software tool during the calibration process. A small aliquot from the main flow is diverted by the splitter and diluted with the make-up solvent. If the make-up flow is more than 0.5 mL/min, another T-splitter before the MS inlet may be required to keep the flow in the ion source close to 0.5 mL/min as higher flow rates will decrease detector sensitivity. The ionization process is dependent on the make-up solvents used. Usually, a mix of 95 % acetonitrile, 4.9 % water and 0.1 % formic acid, or alternatively a mix of 75 % methanol, 20 % acetonitrile, 4.9 % water and 0.1 % formic acid can be used for APCI ionization.

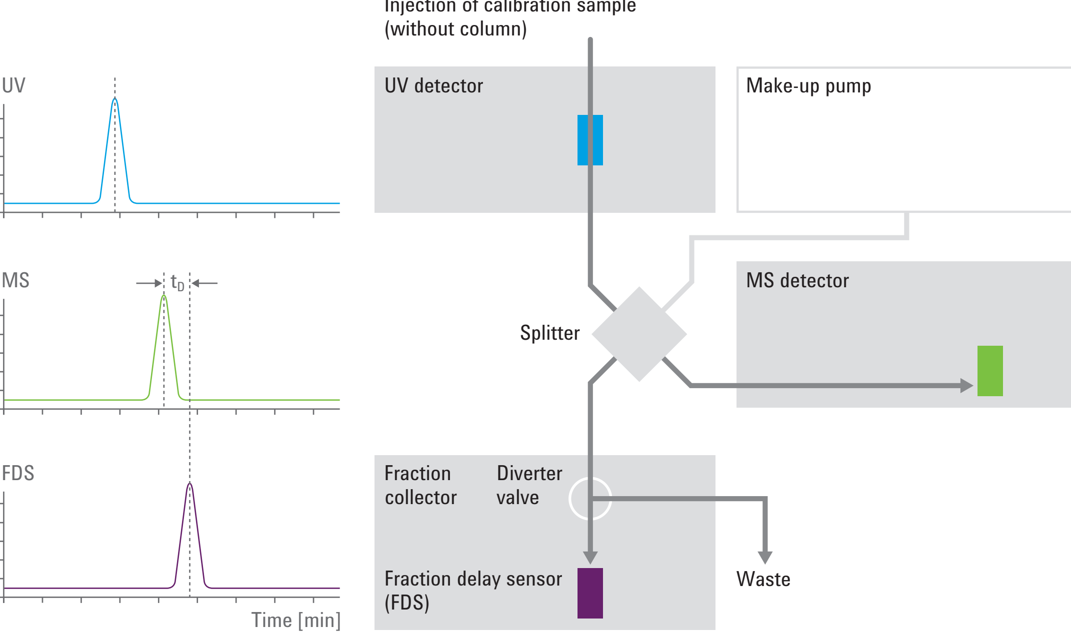


Figure 3.41 Time difference between UV and MS detectors, and fraction delay sensor.

It is characteristic of the molecule itself whether negative or positive charged ions are generated. If the compound has a basic character, protonation of the compound is likely. Hence, generation of positive ions is preferred. If the compound has some acidic groups, deprotonation is more likely. In this case the generation of negative ions is preferred. Further, the strength of the buffers used can have an influence on the ionization mode. When using low concentrations of formic acid or acetic acid, both ionization modes work. Very strong modifiers such as

trifluoroacetic acid (TFA) suppress negative ionization but also distort positive ionization because of ion-pair building with the compound itself.

Ion formation in solvents such as water and methanol is very efficient. The less polar the solvents are, the more inefficient the ionization process becomes. Particularly normal-phase solvents, for example, hexane or ethyl acetate, do not support compound ionization in electrospray mode. A postcolumn make-up solvent can address this situation. For example, mixtures of isopropanol and methanol (1:1) with 0.1 % formic acid as modifier, or dichloromethane and methanol (1:1) with 0.1% formic acid as modifier, are miscible with various nonpolar solvents and create predefined ions in solution.

Unfortunately it is still a common practice to use only the positive ionization mode in LC/MS, although there is a high risk of not detecting a significant number of compounds due to their low proton affinity.

To achieve the most efficient ionization process we recommend ionizing alternately in positive and in negative mode (in the absence of trifluoroacetic acid as a buffer). In this case, complementary and more reliable information from the sample is received, as shown in Figure 3.42.

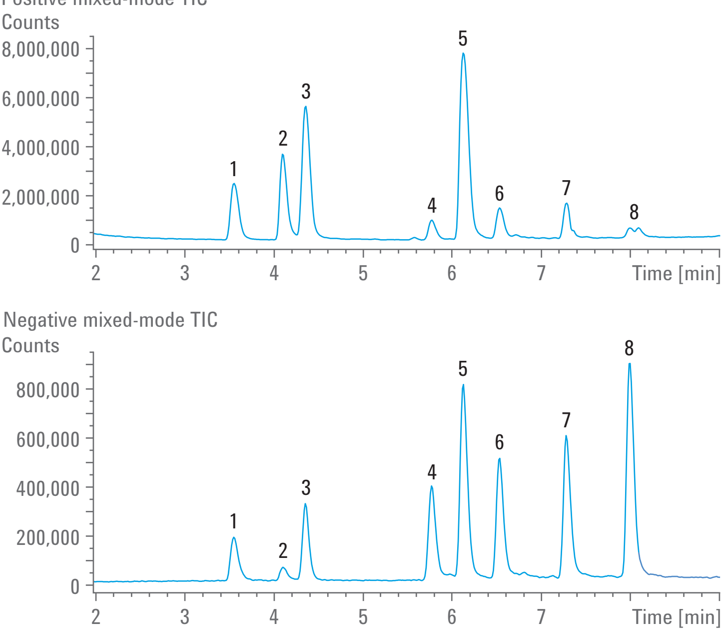


Figure 3.42 Dual ionization mode delivers complementary and reliable information.
1 Acetamidophen, **2** Caffeine, **3** Sulfamerazine, **4** Methylparabene, **5** Sulfadimethoxine, **6** Ethylparabene, **7** Propylparabene, **8** Benzylparabene

When acquiring in scan mode a total ion current (TIC) chromatogram is obtained. The TIC chromatogram is not a mass-selective signal specific for a target compound. In Figure 3.43, the third peak represents the target compound, sulfamerazine (M = 264).

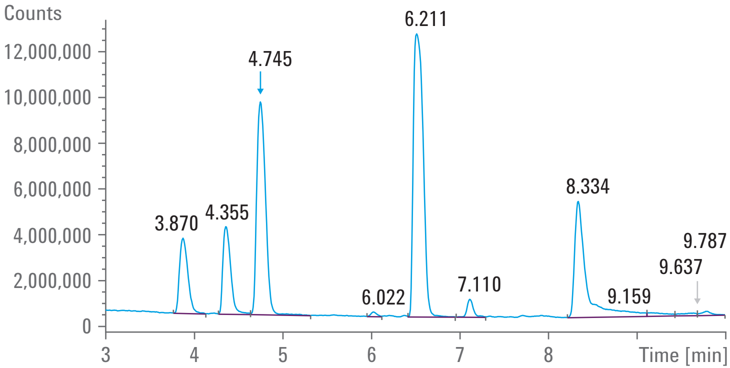


Figure 3.43 TIC chromatogram, no specific compound information is obtained.

When the molecular mass for sulfamerazine is entered, an extracted ion chromatogram (EIC) is generated in real-time in the background. The data system adds the selected adduct masses (in most cases a proton) and monitors the EIC at m/z = 265. To trigger the fraction collector, the observed signal strength has to exceed the threshold level of the selected trigger, see Figure 3.44.

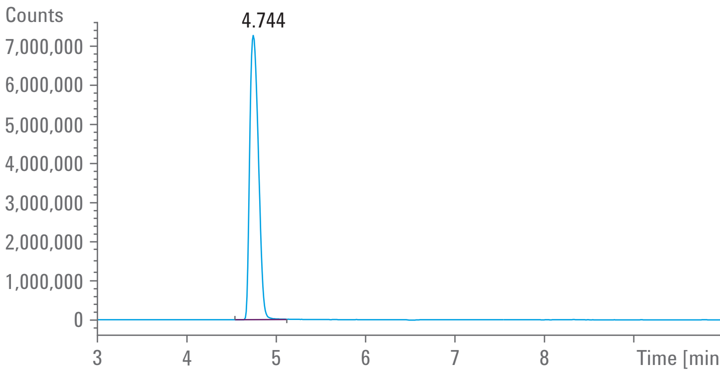


Figure 3.44 Selective extracted ion chromatogram for the target compound at m/z = 265.

3.5.7 Collecting fractions using multiple detector configurations

When multiple detectors are configured in a purification system, fraction triggering has to be set up properly and several ways of decision making are possible. A universal interface box can pick up signals from a variety of detectors. Fractions can be collected when either one or more detectors recognize an eluting peak. Boolean AND/OR logic is available for this step.

3.6 Recovery collection

The objective of any purification task is to collect all compounds of interest based on identification by retention time, UV signal intensity at a specific wavelength, or abundance of target mass. There can be several possible reasons for the compounds of interest not being collected:

- Improper fraction collection settings, for example, target mass, or lower and upper threshold
- Lack of ionization in the mass-selective detector
- Improper selection of methods such as in a walk-up environment
- Mechanical failure or software error

To avoid complete loss of a sample, more and more purification systems are equipped with sample recovery locations or dedicated recovery fraction collectors connected to the waste line of the primary fraction collector, see Figure 3.45. For each sample there should preferably be a separate container available to collect what is expected to be waste. The recovery position together with the sample information must be reported. This ensures you are able to return the entire sample back to whoever submitted the sample for purification.

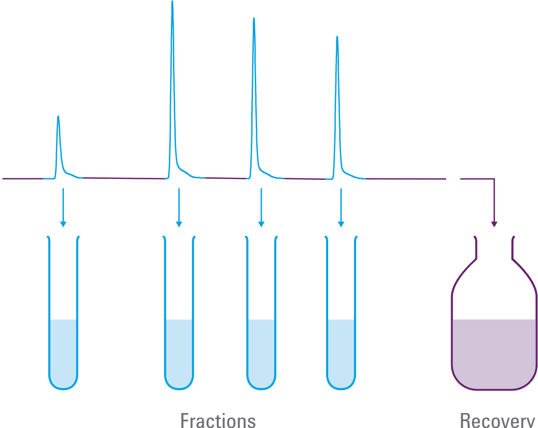


Figure 3.45 Recovery collection – the volume of solvent not collected by the main fraction collector is captured by the recovery collector.

3.7 System considerations

Purification systems can be scaled to accommodate a variety of column sizes and flow rates. Scaling is desirable when the flexibility to apply a variety of sample sizes is of high priority. However, it has a significant impact on the tubing dimensions and thus the internal volume of the system. The resulting dispersion in the system impacts the chromatographic performance and hence the purity and recovery of the target compound. When scaling up from the dimensions of analytical conditions the system dwell volume needs to be taken into consideration.

Increasing the injector loop size is a simple way to facilitate injection of larger volumes and thereby increase the amount of sample that can be purified per run. Larger inside diameter capillaries help to deal with the backpressure caused by higher flow rate. These would appear to be logical steps to adapt an analytical LC system for preparative work. However, these measures can severely impact the separation efficiency of your application.

3.7.1 System dwell volume and column void volume[8]

The system void volume comprises the volume contributed by the flow path and the column void volume. The dwell volume has been defined as the void volume from the mixing point of two eluents to the column head. Dwell volume and column void volume can be measured as described in Section 3.7.1.4 "Determination of dwell volumes and column void volumes", Section 5.2 "Determining the system dwell volume" and Section 5.3 "Determining the column void volume".

Figure 3.46 shows the impact of system dwell volume on chromatographic performance. The resolution of early eluting compounds can be low on systems with low dwell volumes, especially when large sample volumes are injected when the gradient starts immediately after injection. An isocratic holding step helps to improve the separation in the first part of the chromatogram.

The resolution of late eluting compounds is often poor on systems with larger dwell volumes. The cycle time to elute all compounds is larger at the same flow rate. Poor chromatographic results can only be improved by reducing the dwell volume.

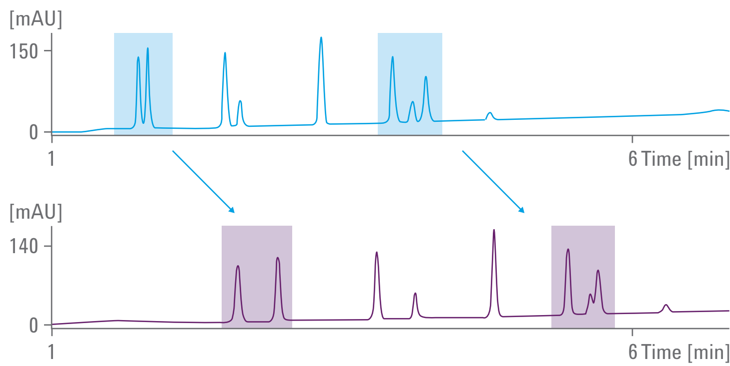


Figure 3.46 The effects of system dwell volume on separation efficiency. The upper chromatogram was obtained using a system with low dwell volume. In contrast, the lower chromatogram was obtained on a system with large dwell volume.

3.7.1.1 What constitutes optimized system setup?

A preparative LC system with appropriate dimensions can be used for both analytical scouting runs on 4.6 mm-id columns as well as for purification on a 50 mm id preparative column. In this case, the system dwell volume must be as small as possible to obtain good results when working in gradient mode.

As a general rule – system optimization is achieved when the ratio between dwell volume and column void volume is equal or less than one. Further, the ratio of the total system void volume to the applied flow rate should be equal to or less than 2:1 to obtain reasonable chromatographic performance.

Larger ratios extend the length of the chromatogram and will reduce chromatographic performance as shown in Figure 3.46. The volume of capillaries, mixer and injection loop have a strong impact on the dwell volume. Hence, it is important to keep loop sizes as small as possible or use two different loops and flow paths. Further, the correct capillary diameter for the applied flow rate must be used to reduce systems void volumes and as a consequence the peak dispersion.

3.7.1.2 What is the impact of capillary length and inside diameter?

The inside diameters of the capillaries have to be synchronized with the flow rate, see Table 3.2.

For most semi-preparative applications using a flow range between 10 and 100 mL/min, standard 1/16-inch capillaries of 0.02 or 0.03-inch id can be used.

- Standard 1/16-inch capillaries with an inside diameter of 0.04 inch (1 mm) can be used for flow rates up to 200 mL/min. This is sufficient even for 50-mm id columns.
- 1/8-inch capillaries can be used when systems are used only at flow rates above 100 mL/min and can even be used up to 500 mL/min.
- 1/4-inch capillaries can be used when systems are used at flow rates above 200 mL/min up to flow rates of several liters per minute.

Color	Inside diameter [inch]	[mm]	Recommend flow [mL/min]	Void volume [µL/cm]	Calculated backpressure [bar] per meter at maximum recommended flow*
Black	0.004	0.102	0.1 – 0.8	0.081	103
Red	0.005	0.127	0.2 – 1.5	0.127	72
Yellow	0.007	0.178	0.8 – 3.0	0.249	37
Blue	0.01	0.254	2.5 – 10	0.507	30
Orange	0.02	0.508	7.5 – 100	2.027	19
Green	0.03	0.762	20 – 150	4.56	6
Tan	0.04	1.016	30 – 200	8.11	2

Table 3.2 Recommended inside diameters of PEEK capillaries for different flow rates (*backpressure measured using methanol/water 1:1 as solvent).

3.7.1.3 Column void volume

With preparative columns the column void volume contributes significantly to the total void volume of the system. The column void volume comprises the volume inside the column housing that is not occupied by the packing material, see Figure 3.47. Except for the inner column size the porosity of the particles has to be accounted for. Agilent ZORBAX SB C18 columns have a porosity factor of 0.53, see Table 3.3.

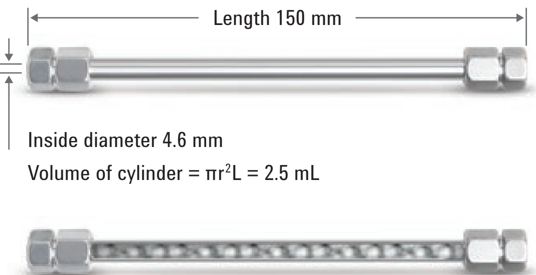


Figure 3.47 Mathematical determination of column void volume.

Diameter [mm]	Length [mm]	Particle size [µm]	Porosity	Void volume [µL]
2.1	50	5.0	0.53	0.092
3.0	50	5.0	0.53	0.187
4.6	50	1.8	0.53	0.440
4.6	50	3.5	0.53	0.440
4.6	50	5.0	0.53	0.440
4.6	100	5.0	0.53	0.881
4.6	150	5.0	0.53	1.321
9.4	50	5.0	0.53	1.839
21.2	50	5.0	0.53	9.354
21.2	100	5.0	0.53	18.708
21.2	150	5.0	0.53	28.063

Table 3.3 Column void volume for different Agilent ZORBAX SB C18 columns. The porosity of ZORBAX SB C18 material is 0.53.

3.7.1.4 Determination of dwell volumes and column void volumes

The dwell volume and even the column void volume of a system can be measured in different ways. In this section we describe a method using a linear gradient from 5 to 95 % of solvent B during a 10-minute run. In this determination, Solvent A is organic solvent (acetonitrile) and solvent B is also organic solvent (acetonitrile) but spiked with 1 % acetone as a tracer that is measured at 270 nm by UV detection. The column is replaced by a zero dead volume connector during the first step to measure the system's dwell volume. The absorption curve (red) is plotted against the gradient curve (blue). The time difference between the two curves at 50 % multiplied by the applied flow rate determines the dwell volume of the system. The column is then remounted and a second absorption curve (green) acquired, which determines the total void volume of the system. The column must be well equilibrated – flushed with at least ten times the combined column and system volume – to avoid retention of the tracer. The column void volume is given by the difference between the dwell volume and the total system volume, see Figure 3.48.

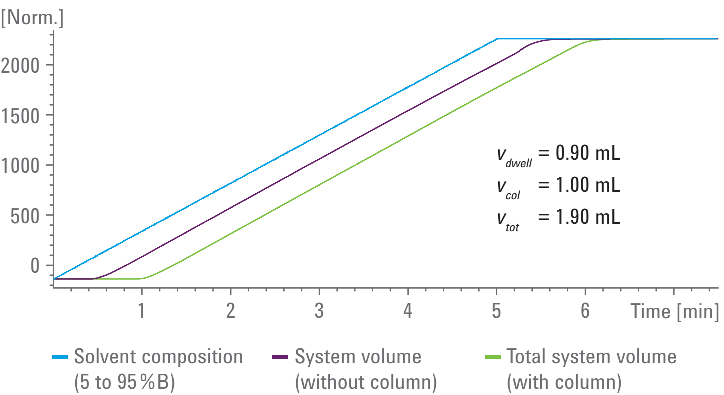


Figure 3.48 Dwell volume and column void volume determination by linear gradients.

3.7.1.5 Equilibrating and purging the column

Sufficient column equilibration time is required to obtain reproducible retention times. We recommend an equilibration phase of three column void volumes and an additional two dwell volumes after each column purge phase. For a proper purge at least two column void volumes are required.

3.7.2 System communication

Agilent LC systems use a controller area network (CAN). Peak trigger assessment processes and the simultaneous monitoring of different signal traces, especially when data intensive devices such as diode array detectors and mass selective detectors are involved, create a heavy load on workstations. Activities such as virus scans or software update/ download can interfere with fraction collection processes. As Agilent's CAN communication operates independently from the Windows-based software, it is a direct, rapid and robust communication line between the different system modules, which guarantees maximum of robustness. Furthermore, purification systems with CAN communication synchronize the delay time between the UV detector and the fraction collector, facilitating deployment of different flow rates.

Figure 3.49 CAN communication lines – fast direct communication between the modules is independent from the host software.

3.7.3 Safety concepts

When performing preparative LC the volumes of organic solvents used are much larger and hence the potential hazards are much greater when compared to analytical-scale work. Special care must be taken to avoid solvent spills when, for example, a leak occurs. Further, solvent vapors derived from numerous fractions containing volatile organic solvents can lead to harmful incidents in the laboratory. Purification systems with proper safety features can prevent such situations that endanger both laboratory personnel and equipment. Loss of samples can be an additional consequence of leaks in the system.

Leak detectors in each module can stop the solvent delivery and prevent the system from proceeding with the next sample, see Figure 3.50. A drainage system should be available to route the effluent to waste collection containers. Solvent vapors can be removed from the collection area by an extraction fan and directed to an exhaust line, for example, a fume hood, see Figure 3.51.

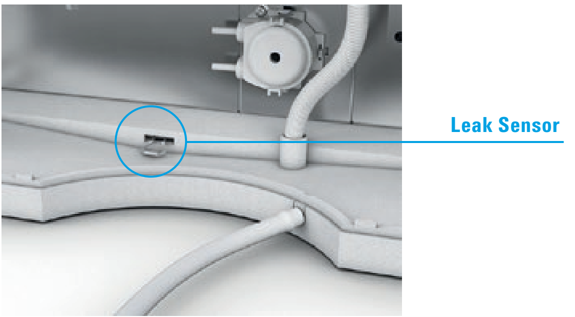


Figure 3.50 Leak sensor and leak management, showing drainage system to lead leaking solvent to waste containers.

Figure 3.51 Adapter on rear of fraction collector for forced-fume extraction.

4 STRATEGIES FOR SCALE-UP [9-11]

In this chapter we describe scale-up from a 4.6 by 150 mm analytical column to a 21.2 by 150 mm preparative column, starting with a generic elution gradient. A correct scale-up process between two different column geometries can only be achieved when the same chemistries, pH conditions, particle sizes and column lengths are used.

4.1 Analytical chromatography as a starting point for scale-up

Analytical chromatography is required as a first step to confirm the presence of the target compound and if it could be separated from the other compounds under the selected chromatographic conditions. Typically, a gradient profile from 2 to 98 % of the organic content is best suited to accommodate for a variety of compounds. An additional isocratic step directly after injection makes sure early eluting compounds are retained – especially when the sample has been dissolved in a solvent with high elution strength for better solubility, for example, DMSO. The UV signal or the TIC trace of the mass-selective detector can be used to monitor this step.

Generic gradient profiles of 10 %B/min can be used for column lengths of 150 mm. To be as efficient as possible the slope can be increased up to 30 %B/min for a column length of 50 mm. In contrast, the slope must be decreased to 6 %B/min for a column length of 250 mm. Using an appropriate flow rate for the selected column diameters, resolution can be increased by varying the steepness of the gradient slope.

Experiments performed on our system showed that a flow rate of 1.5 mL/min delivers highest resolution and number of theoretical plates using a 4.6 by 150 mm, 5 µm column. This flow rate is the starting point for all further scale-up processes.

Switching to a preparative system requires changes to flow rates, additional isocratic holding steps, modified gradient slopes, and adjusted injection volumes and run times, whereby the preparative gradient profile should look similar to the analytical gradient profile.

Figure 4.1 shows the gradient profile of an analytical scouting run. Figure 4.2 shows the gradient profiles after transferring the method from a system with a small dwell volume to a system with a larger dwell volume and larger column inside diameter. The isocratic holding step has been extended to compensate for the differences in dwell

volume of both systems. The linear method transfer from an analytical to the preparative system was accomplished using the formulas in Section 4.2 "Formulas for linear scale-up from analytical to preparative columns".

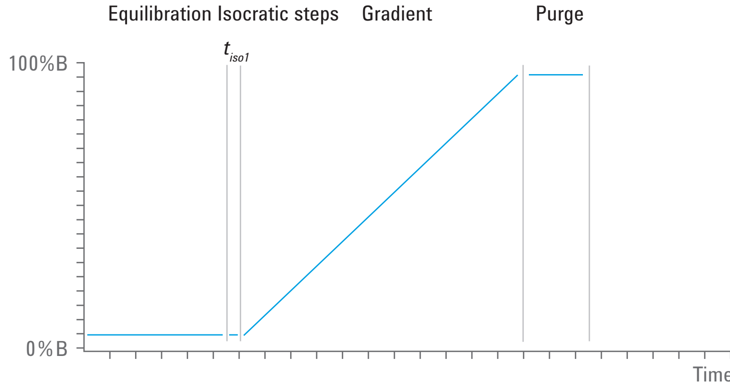


Figure 4.1 Gradient profile for an analytical scouting run using a 4.6-mm id column, comprising injection, optional isocratic hold, gradient slope and purge phases.

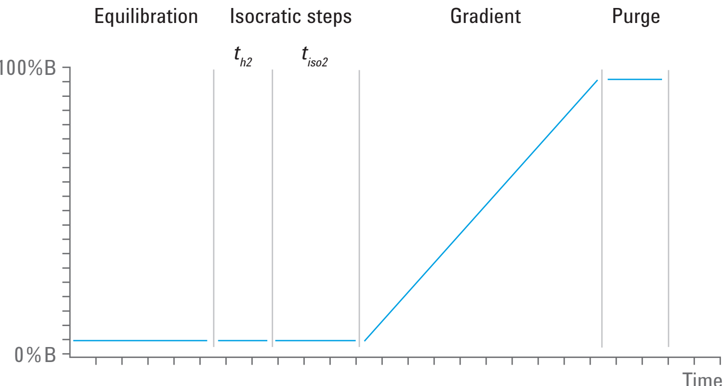


Figure 4.2 Gradient profile after scale-up to a 21.2-mm id column, comprising an extended isocratic step to compensate for the applied flow rate with respect to the difference in dwell volumes between the analytical and preparative system.

4.2 Formulas for linear scale-up from analytical to preparative columns

The diameters and particle sizes of both analytical and preparative columns need to be considered for scale-up of flow rates. If the particle size remains constant, a flow of 1.5 mL/min on a 4.6-mm id column results in a flow of 31.86 mL/min on a 21.2-mm id column, see Equation 4.1.

$$f_{p,P} = f_{a,A} \frac{d_P^2}{d_A^2} \frac{p_A}{p_P}$$

Equation 4.1 Calculation of flow rate for analytical to preparative scale-up.

d_A	Diameter of analytical column
d_P	Diameter of preparative column
$f_{a,A}$	Actual flow in analytical system
$f_{p,P}$	Proposed flow in preparative system
p_A	Column particle size in analytical system
p_P	Column particle size in preparative system

Initial isocratic holding steps compensate for differences in dwell volume between the analytical and preparative systems. Further, gradients can be transferred between different systems when the conditions according to Equation 4.2 are fulfilled.

$$\frac{t_{D,A} + t_{I,A}}{t_{c,A}} = \frac{t_{D,P} + t_{I,P}}{t_{c,P}}$$

Equation 4.2 Gradient transfer conditions.

$t_{D,A}$	Dwell time of analytical system
$t_{I,A}$	Initial hold of analytical system generic gradient
$t_{c,A}$	Column pass time in analytical system
$t_{D,P}$	Dwell time of preparative system
$t_{I,P}$	Initial hold of preparative system gradient
$t_{c,P}$	Column pass time in preparative system

The initial holding phase of the preparative gradient is determined based on the one-time determination of the system dwell volume and column void volume, see Equation 4.3.

$$t_{I,P} = \left(\frac{t_{I,A} \; f_{a,A}}{V_{c,A}} + \frac{V_{D,A}}{V_{c,A}} - \frac{V_{D,P}}{V_{c,P}} \right) \frac{V_{c,P}}{f_{a,P}}$$

Equation 4.3 Calculating the initial isocratic hold step.

$f_{a,A}$	Actual flow in analytical system
$f_{a,P}$	Actual flow in preparative system
$t_{I,A}$	Initial hold of analytical system generic gradient
$t_{I,P}$	Initial hold of preparative system focused gradient
$v_{D,A}$	Dwell volume of analytical system
$v_{c,A}$	Column void volume of analytical system
$v_{D,P}$	Dwell volume of preparative system

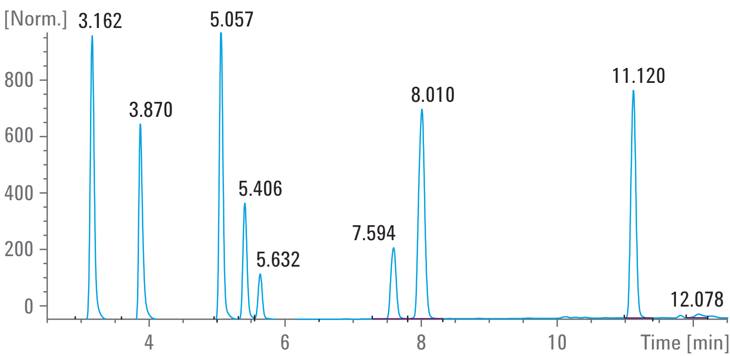
When transferring a method to a column containing a larger amount of stationary phase, a simple equation can be used to calculate the column load or injection volume, see Equation 4.4.

$$v_{inj,P} = v_{inj,A} \; \frac{d_P^2}{d_A^2} \; \frac{L_P}{L_A}$$

Equation 4.4 Scale-up calculation for column load.

d_A	Diameter of analytical column
d_P	Diameter of preparative column
L_A	Length of analytical column
L_P	Length of preparative column
$v_{inj,P}$	Injection volume for analytical system
$v_{inj,A}$	Injection volume for preparative system

Figure 4.3 shows the results of a method transfer after applying the equations in this section.



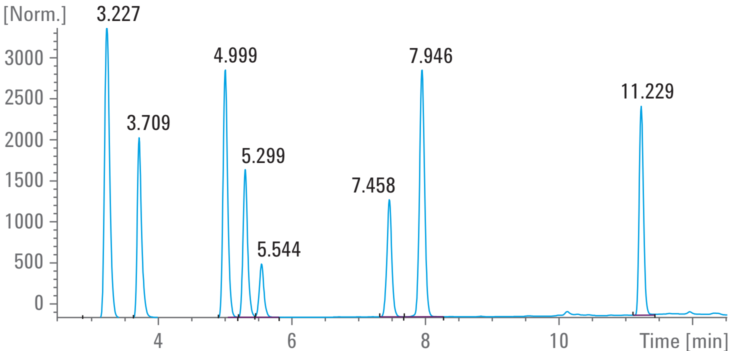


Figure 4.3 Results after linear scale-up from a 4.6 x 150 mm, 5 µm analytical column to a 21.2 x 150 mm, 5 µm preparative column. Retention times on both columns are very similar.
Upper chromatogram: Preparative run with 500-µL injection and 31.8-mL/min flow
Lower chromatogram: Analytical run with 5-µL injection and 1.5-mL/min flow

Methods can be transferred from 4.6-mm id to 21.2-mm id columns after the determination of the dwell volumes by using the scale-up equations. Retention times of all compounds on both chromatograms are very similar.

4.3 Increasing efficiency through focused gradients[12–15]

To obtain maximum efficiency the column load needs to be maximized and the runtime needs to be minimized. However, increasing column load decreases resolution because peaks become broader. Additional separation efficiency for the target compound is desirable to enable high loading, sufficient resolution and hence pure fractions with maximum recovery.

4.3.1 The concept of focused gradients

The resolution between groups of adjacent peaks can be increased by using a shallow gradient profile focused on a target peak. In this section we show different focused gradient profiles and how to generate them.

Dissolving all compounds in a sample – despite different polarity and high concentrations – is the key for robustness of the methodology. The starting conditions in a focused gradient profile are derived from the polarity of the target compounds. At the same time, retention has to be reached using as low a concentration of the organic solvent B as possible. For polar compounds good results are obtained by starting at a low percentage of solvent B. Even the polar compounds are then retained at the column head. After the isocratic hold, a steep gradient step can be used to ramp up to the starting point of the focused gradient for each zone. The applied gradient profile is shallow to achieve optimum separation efficiency for the target compound and close eluting impurities.

For non-polar compounds the solubility is higher at larger percentages of solvent B. The risk of precipitation and plugging of capillaries during injection can be reduced when the starting conditions are close or even the same as the initial conditions of the applied gradient profile for this elution zone. After sample transfer to the column has succeeded a shallow gradient profile will be applied around the elution zone of the compounds of interest. After elution of these compounds the column will be purged immediately. All other compounds are purged out. The process is optimized to reduce the run times.

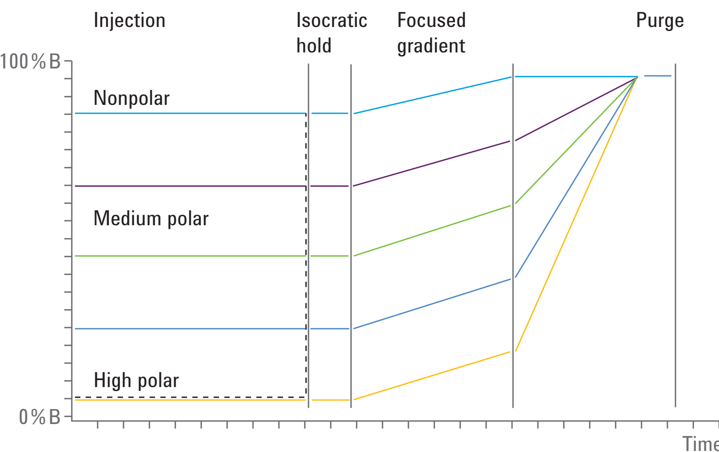


Figure 4.4 Focused gradient profiles for target compounds with different polarities. The dashed line represents an alternative step-gradient profile.

4.3.2 Developing focused gradients

The process of developing a focused gradient starts with an analytical scouting run using a linear gradient profile from 2 to 98 % organic solvent. The analytical scouting run gives us the retention time of the target compound. The exact gradient conditions at elution of the individual target compound are calculated from the results of the scouting run when the dwell volume and the column dead volume of the system have been determined beforehand. The offset time between the programmed solvent and the actual solvent composition at the column head is calculated by dividing the combined void volumes by the flow rate. The virtual elution point, which reflects the actual solvent composition when the peak has been detected, is calculated by determination of the gradient composition after subtracting the offset time from the retention time.

After calculation of the virtual elution point for the target compound, a new shallow or focused gradient slope has to be applied. Typically, good results are obtained when the focused gradient step starts 15 percentage points below the virtual elution point and can ramp up to 5 points above the calculated elution point. Or, in more general terms, the new gradient step reaches the virtual elution point of the target compound at about 75 % of the gradient's length. The slope is varied primarily based on the column length as listed below for a scouting flow rate of 1.5 mL/min:

- 250-mm column = 10 minutes; slope = 2.0 %B/min
- 150-mm column = 6 minutes; slope = 3.33 %B/min
- 100-mm column = 4 minutes; slope = 5.0 %B/min
- 50-mm column = 2 minutes; slope = 10.0 %B/min

With this concept the earliest gradient start for early eluting compounds would be 2 % organic content. In this case, the lowest matching elution point is 17 %B. If compounds are eluting much earlier, we recommend finding different chromatographic conditions (solvent composition, pH, chemistry) that lead to later elution of the target compound.

4.3.3 Simplified approach to creating focused gradients

Figure 4.5 shows a simplified concept of focused gradients applied to a sample. The programmed gradient (green) leads to the true gradient (orange) caused by the system delay volume and column void volume. In this example, the chromatogram has been sliced into six time windows or zones – with start and end zones plus four zones each with an increase of 20 %B. For example, a focused gradient for zone 2 starts at 5 %B and increases to 25 %B with a reduced slope. For a column length of 150 mm the gradient step takes six minutes. For other column lengths, use the slope duration as described in Section 4.3.2 "Developing focused gradients".

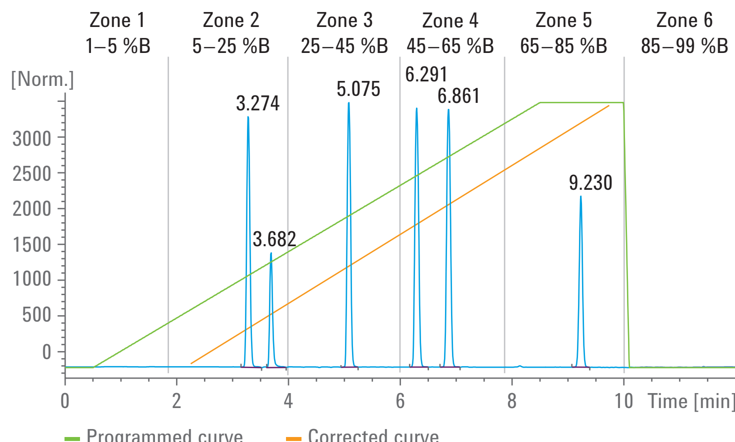


Figure 4.5 Slicing of a generic gradient into six time windows. A focused gradient step is defined for each part of the chromatogram.

Figure 4.6 shows the results after applying a focused gradient profile. In this example the target compound elutes in zone 2 during the generic scouting run. A focused gradient profile from 5 to 25 %B has been applied. The compound which elutes within the gradient slope shows a significant improved resolution. Compounds which are not of interest are purged out. A larger sample amount can be loaded to increase the efficiency of the process. Please be aware that the simplified approach with a reduced number of focused gradients will deliver less chromatographic performance than the more accurate procedure based on the calculation of the virtual elution point from each target compound described in Section 4.3.2 “Developing focused gradients”.

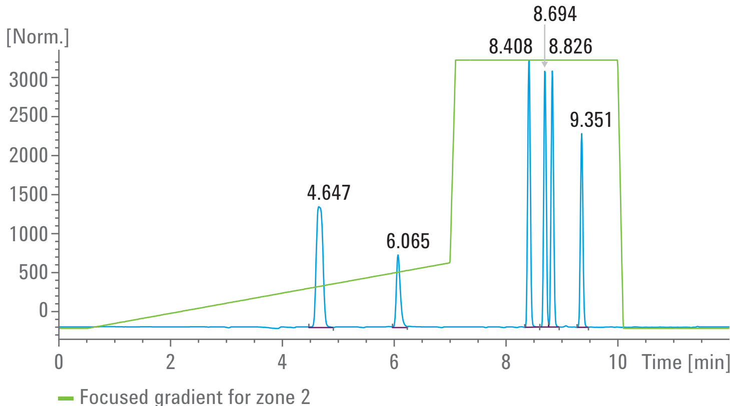


Figure 4.6 Application of focused gradient for target compounds eluting in zone 2.
Column: ZORBAX SB C18, 4.6 x 150 mm, 5 µm; **Flow:** 1.5 mL/min;
Gradient: 5 to 25 % ACN in 6 min

4.4 Describing the entire scale-up workflow[16]

The most critical step in the purification workflow is the scale-up process from analytical column dimensions to semi-preparative or preparative column dimensions. Scale-up can be done quickly using a generic gradient profile when the development of a focused gradient is perceived as an additional burden and too many samples are in the queue. However, if maximum performance is a must then a focused gradient is the best choice. Automated systems can provide the gradient calculations within a few clicks when the number of samples and users justifies the investment. The manual scale-up workflow can be executed following the scheme described here. It can be summarized in four different steps as shown in Figure 4.7. The spreadsheet-based scale-up calculator from the University of Geneva helps to address the calculations.
It is available as a free download at:
http://www.unige.ch/sciences/pharm/fanal/lcap/telechargement-en.htm (accessed November 1, 2014)

Step 1	Step 2	Step 3	Step 4
Determination of correct analytical scouting conditions such as pH, mobile phase and chemistry	Application of focused gradient to optimize resolution and column load	Determination of maximum load on analytical column (loading study)	Scale-up for preparative injection and fraction collection

Figure 4.7 Four-step, scale-up process.

A fundamental question during scale-up is the solubility of the sample in the used solvents. Strong solvents such as DMSO or DMF dissolve most compounds while obtaining high concentrated solutions. However, injecting large volumes of solutions from polar compounds can have a detrimental effect on chromatographic resolution. It is useful to have an idea which solvents fit best for dissolving the compounds, see Section 3.2.3.2, "Injecting large volumes of strong organic solvents."

4.4.1 Step 1 – Determining the correct analytical scouting conditions

To obtain first chromatographic information from the sample we recommend injecting a typical analytical volume of 1 to 5 µL on a 2.1, 3.0 or 4.6 by 50 mm UHPLC or HPLC column. A retention factor, k, of the target compound between 2 and 9 should be achieved. If the retention factor is too low, the compounds are not retained. If the retention factor is too high, peak broadening is likely to be observed as the compounds are eluted during the isocratic purge phase of the gradient profile.

Basic compounds will be protonated when using acidic modifiers. If the concentration of the modifier is abundant enough to protonate all injected molecules, symmetric peak shapes can be expected. When using too low modifier concentrations not all molecules of the injected sample are protonated. This combination often causes peak splitting and poor peak shapes. Adding some acid when dissolving the sample can reduce this phenomenon.

When poor retention or poor resolution under low pH conditions is observed, it is worthwhile analyzing the sample at high pH using 0.5 % ammonia as modifier in combination with a column chemistry that is resistant to high pH conditions, for example, ZORBAX Extend C18. Note that ammonia is suitable for electrospray ionization in mass-based purification workflows.

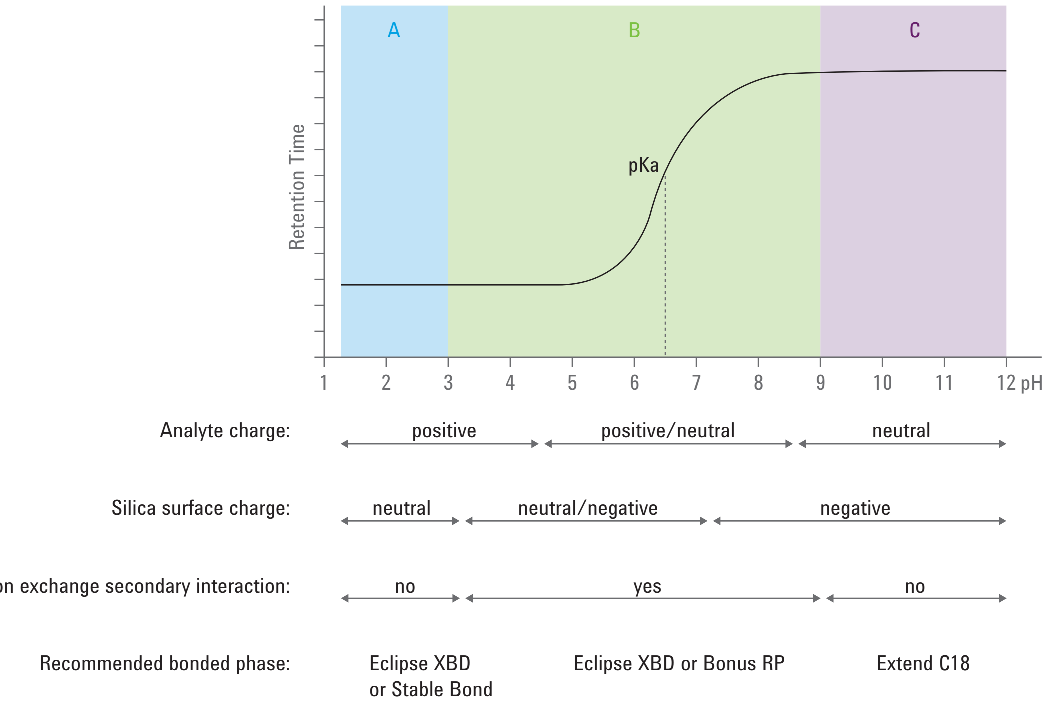


Figure 4.8 Recommended chemistries for different pH ranges. ZORBAX SB C18 columns and ZORBAX Extend C18 columns facilitate scale-up from 4.6 mm to 21.2-mm id at low pH and high pH conditions.

4.4.2 Step 2 – Applying a focused gradient

As described in Chapter 3 "Components of a Preparative LC System", focused gradients are useful for sample amounts that exceed the purity, loadability or recovery achieved under the conditions of the generic gradient. When the number of samples increases, a semi-automatic – or preferably – a fully automatic approach to gradient development is required. In the latter case, software can automatically prepare the preparative method based on the scouting run and the dimensions of the preparative system and column.

4.4.3 Step 3 – Determining the maximum column load

Column load is a critical factor for the success. A well-elaborated focused gradient profile on an analytical column can cause poor separation in preparative chromatography when the column is used beyond its Langmuir absorption isotherm in overload mode. The most significant effect on peak shape and resolution is caused from volume overload when injecting too much strong solvent such as DMSO. Figure 4.9 shows the results of a loading study, in which column load is increased until the limit of resolution is reached.

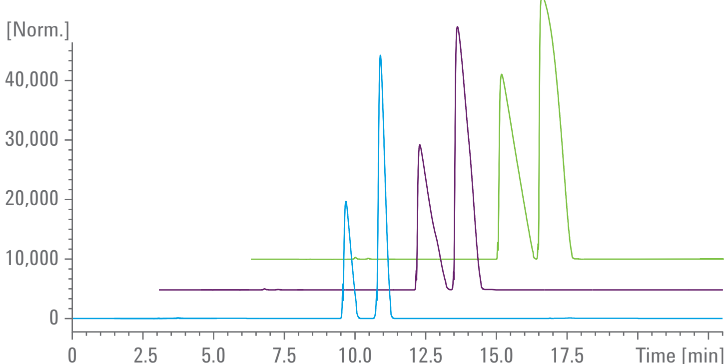


Figure 4.9 Results of loading study – the column load is increased until the limit of resolution are reached.
Column: Agilent Scalar, 4.6 x 250 mm, 10 µm
Flow: 1.5 mL/min
Injection volumes: 5 to 50 µL
Gradient:

Time	Flow	%B
0	1.5	2
0.01	1.5	2
16.01	1.5	98
19.0	1.5	98
19.5	1.5	2
25.0	1.5	2

By applying a focused gradient on the analytical column, the injection volume is increased in 5-µL steps to determine the limits of column load. With 40 µL (18.7 mg) the separation of the two compounds is still obtained with a resolution, R_S, of 1.11.

The final injection volume for the purification step on the 50 mm id column is calculated by using the column scale-up equation (see equations in Section 4.2 "Formulas for linear scale-up from analytical to preparative columns"). The same resolution on the 50-mm id column is obtained after injecting 4500 µL or 2.11 g of mixture.

4.4.4 Step 4 – Scaling-up for preparative injection and fraction collection

Figure 4.10 shows the chromatogram after the scale-up calculation. By applying the formulas from Chapter 4 "Strategies for Scale-Up", the preparative chromatogram is exactly predictable. Both compounds are baseline separated and have been collected with a purity of greater than 95 %.

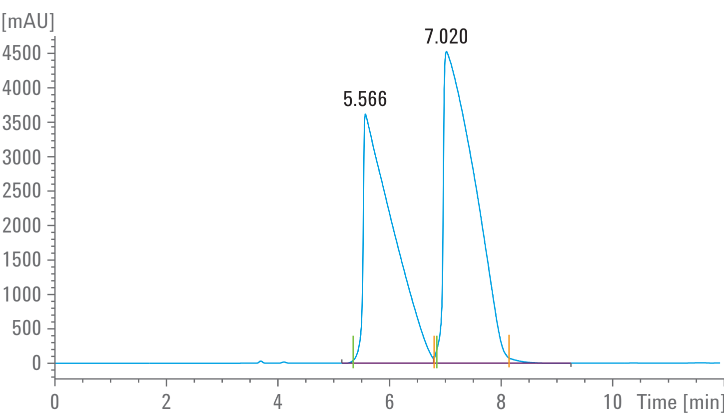


Figure 4.10 Preparative-scale chromatogram. The tick marks indicated the collected fractions. Based on the results from the loading study on the analytical 4.6-mm id column, the method has been scaled-up matching the flow rate, gradient profile and injection volume for a 50-mm id column. This chromatogram shows the separation of the two target compounds after 4500 µL have been injected.

5 PRACTICAL GUIDELINES AND DETAILED PROCEDURES FOR PREPARATIVE LC

This chapter gives you practical guidelines for preparative LC, including procedures for column packing, dwell volume determination, column equilibration and column loading.

5.1 Packing procedure for SAC/DAC columns

SAC/DAC column media can be prepared and packed using the following step-by-step procedure.

1. Calculate and weigh the appropriate amount of dry material based on the required column volume, column id and length to be packed. Most media are supplied as dry powder ready for use.
2. Calculate the volume of the column:

$$V_{col} = \left(\frac{id}{2}\right)^2 \pi L$$

3. Calculate the amount of media required:

$$M = V_{col} \, x \, \rho$$

$$(\rho \sim 0.668 \text{ g/mL})$$

4. Calculate the density of the media (M):

$$\rho = \left(\frac{g_M}{V}\right)$$

5. Disperse the material in packing solvent in the ratio 1 g of dry media to 2 mL of packing solvent. Packing solvent is usually isopropanol (IPA). To ensure the media fully disperses and is free of lumps, the packing slurry should be shaken not stirred, or bottled-rolled for approximately five minutes. As with all HPLC media, do not use a magnetic stirring bar as this grinds the particles and produces fines. It is essential that the slurry be well mixed. Since there are significant differences in the characteristics of stationary phases (for example, particle size, shape, nature of functional groups, and so on), you should consult the resin manufacturer to determine the optimal slurry solvent and compression pressure. As you gain experience in column packing with a given resin, the packing conditions can be adjusted to optimize results. The packing slurry is now ready for use.

6. Take the homogenous, free-flowing slurry and pour quickly into the assembled column in one continuous action.

7. Complete the assembly of the column and operate the packing station according to the instructions supplied. Mechanical pressure of approximately 1000 psi is recommended for reversed-phase media with 100-Å pore size. Hence a hydraulic pressure of 1500 psi is required. Make sure that the hydraulic pressure has been set using the correct ratio for the combination of column id and packing station being used.

8. Once column packing is complete, the flow of packing solvent has ceased and the pump has stopped, allow the column to stand/equilibrate for 10 minutes. The column is ready to be transferred to the chromatographic eluent. If required, the column plunger can be locked in the compressed position so that the column can be operated in SAC mode.

9. The packed column is now ready for use. It can be used while still assembled on the packing station or it can be undocked for use in a purification facility.

The typical column efficiency for 10-micron reversed-phase media in a bench-top Load & Lock column is 30,000 plates/meter.

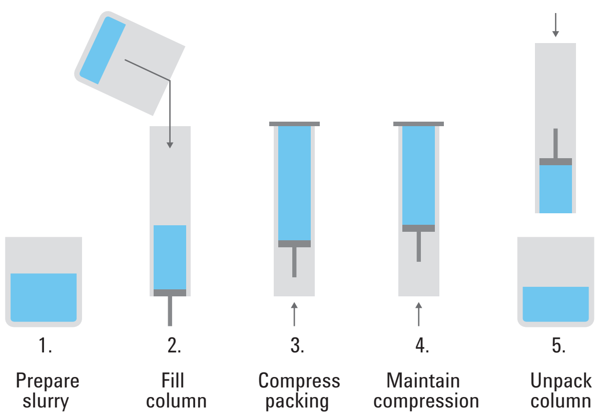


Figure 5.1 Basic packing and unpacking procedure for DAC/SAC columns.

Inside diameter [mm]	Length [mm]	Volume [mL]	Amount of sorbent [g] (silica-based ρ ≅ 0.67 g/mL)	Amount of sorbent [g] (polymer-based ρ ≅ 0.33 g/mL)
25	250	122.7	82	41
	450	220.8	148	73
50	250	490.8	329	162
	450	883.4	592	292
75	250	1104.2	740	364
	450	1987.6	1332	656
100	250	1963.1	1315	648
	480	3769.2	2525	1244
150	250	4417.0	2959	1458
	480	8480.7	5682	2799
200	250	7852.5	5261	2591
	480	15076.8	10101	4975

Table 5.1 Required resin aliquot sizes when packing DAC/SAC columns.
For polymer-based columns, please consider the much lower pressure limits.

5.1.1 Determining the chromatographic plate number

After the column has been packed, a column efficiency test is required. The column is flushed with an 80:20 mix of acetonitrile and water, and equilibrated at an appropriate flow rate with respect to the column dimensions. To equilibrate the column, we recommended flushing with at least four column void volumes and two dwell volumes. In this example a flow rate of 100 mL/min in isocratic mode is used and 500 µL of a solution

of 1 % toluene in acetonitrile is injected. The theoretical plate number can be calculated using Equation 5.1. The number of theoretical plates should exceed 30,000 per meter.

$$N = 5.545 \left(\frac{t_R}{w_h} \right)^2$$

Equation 5.1 Calculation of theoretical plates.

N	Number of theoretical plates
t_R	Retention time
w_h	Peak width at half-height (in time units)

The column can be kept in the packing station under pressure using the DAC mode, or it can be removed from the packing station using the SAC mode. When using polymer media it is important to stay within the pressure limits of the media or it will become unusable. In this case the SAC mode is required.

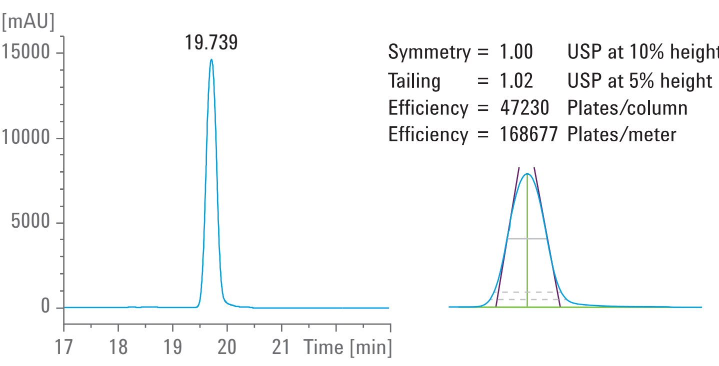


Figure 5.2 Injection of 1 % toluene in acetonitrile to determine the chromatographic plate number.

5.1.2 Unpacking the column

Before unpacking the column, we recommended flushing the column with at least four column volumes using IPA as solvent. The column is mounted into the packing stand and the top lid removed. Now the bed can be removed by using the hydraulic piston. Strongly contaminated column material can be removed while the rest of the stationary phase can be washed out with IPA and dried in a rotary evaporator. Special caution is needed as inhalation of the particles can be harmful.

5.2 Determining the system dwell volume

Two different methods for determination of the system dwell volume are available. For systems capable of delivering highly accurate analytical flow rates such as 1 mL/min, a method with a linear gradient profile delivers more accurate results than a step method. The method can also be used to measure the column void volume.

5.2.1 Determining the dwell volume of analytical systems

Use the following procedure to determine the dwell volume of analytical systems capable of delivering accurate flow rates.

1. Prepare solvent A: 100 % water
2. Prepare solvent B: 99 % acetonitrile with 1 % acetone as tracer
3. Prime the system with the solvents A and B.
4. Set the detection wavelength to 263 nm.
5. Replace the column by a low dead volume restriction (maintaining a backpressure of 50 bar).
6. Run a linear gradient from 0 to 10 minutes with 5 to 95 %B at a flow rate of 1 mL/min.

Note that when using preparative sample loops with volumes larger than 1 mL, the flow rate needs to be increased to finish the dwell volume determination in a reasonable time. A good compromise is to keep the flow rate equal to the loop size.

7. Determine the difference in time (t_{dwell}) between the programmed and actual elution time of the gradient at 50 % of the composition.
8. v_{dwell} can be calculated from $v_{dwell} = t_{dwell} \times f$

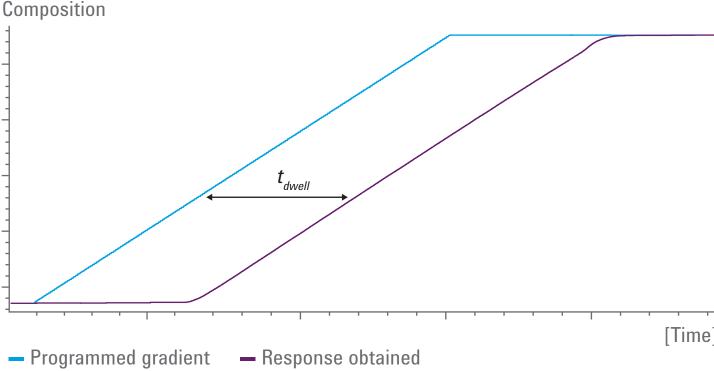


Figure 5.3 System dwell volume determination for analytical systems.

5.2.2 Determining the dwell volume of preparative systems

Use the following step gradient method to determine the dwell volume of a system with preparative pumps.

1. Prepare solvent A: 100 % water
2. Prepare solvent B: 99 % acetonitrile and 1 % acetone
3. Replace the column by a restriction capillary 0.005 inch id (0.12 mm id) x 750 mm.
4. Set the detection wavelength to 263 nm.
5. Run a step gradient at 4 minutes from 10 to 90 %B at a flow rate of 2 mL/min.

Note that when using sample loops with volumes larger than 5 mL, the flow rate needs to be increased. A good compromise is to keep the flow rate equal to the loop size.

6. Calculate the time difference (t_{dwell}) between the programmed gradient and the obtained signal curve at 50 %.
7. v_{dwell} can be calculated from $v_{dwell} = t_{dwell} \times f$

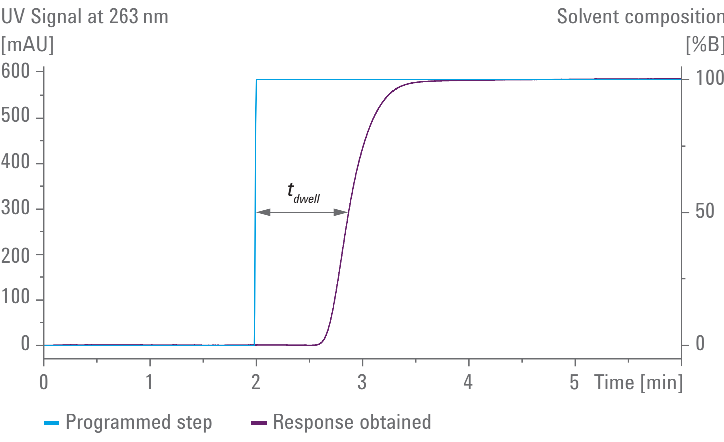


Figure 5.4 System dwell volume determination for preparative systems.

5.3 Determining the column void volume

Two procedures can be followed to determine the column void volume. The first is a simplified procedure that provides fast results. The alternative procedure is more advanced and yields more accurate results.

5.3.1 Simplified procedure for column void volume determination

1. Prepare solvent A: 100 % acetonitrile.
2. Prepare solvent B: 99 % acetonitrile with 1 % acetone as tracer.
3. Prime the system with the solvent A and B.
4. Set the detection wavelength to 263 nm.
5. Install the column.
6. Equilibrate the system with 95 %A and 5 %B until the baseline is stable.
7. Run a linear gradient from 0 to 10 minutes with 5 to 95 %B at a flow rate of 1 mL/min.

Please note that when the calculated total system volume (sample loop and column) is larger than 3 mL, we recommend increasing the flow rate to finish the dwell volume determination in a reasonable time. A good compromise is to keep the flow rate equal to the calculated system void volume.

8. Determine the difference in time (t_{dwell}) between the programmed and actual elution time of the gradient at 50 % of the composition.
9. v_{dwell} total can be calculated from v_{dwell} total = $t_{dwell\ total} \times f$
10. Replace the column by a low dead volume restriction (maintaining a backpressure of 50 bar).
11. Run a linear gradient from 0 to 10 minutes with 5 to 95 %B at a flow rate of 1 mL/min.
12. Determine the difference in time (t_{dwell}) between the programmed and actual elution time of the gradient at 50 % of the composition.
13. v_{dwell} can be calculated from $v_{dwell} = t_{dwell} \times f$
14. Subtract the system dwell volume, v_{dwell}, from $v_{dwell\ total}$ to calculate the column void volume.

5.3.2 Advanced procedure for column void volume determination

The following chemicals are required for both stand-alone analytical or preparative systems, or for a combined system:

- Solvent A: water (optionally with 0.1 % formic acid)
- Solvent B: acetonitrile (optionally with 0.1 % formic acid)
- Needle and purge wash solution (degas in ultrasonic bath): 80 % acetonitrile or other suitable solution

Thiourea dissolved in a mixture of acetonitrile and water (75:25) is required as a marker compound. Its concentration has to be correlated with the path

length – equivalent to sensitivity – of the UV cell used. Saturation must be avoided. For best column performance and lifetime, we recommended filtering the sample before use with a regenerated cellulose syringe filter (Agilent part number 5190-5108).

Characterization of the column volume described below is based on elution of a non-retained compound (marker), thiourea. In the first step a column is replaced by a zero volume connection and the marker is eluted using as low a flow as possible with reliable performance. A retention time in such a setup gives a dead volume of tubing between the points of injection and detection. In the second step the target column is installed and equilibrated with 75 % acetonitrile. The difference between retention volumes of the marker in the setup with and without the column gives the target column volume. For the best results measure column volumes on a standalone analytical LC system, if available. The following procedure describes the column volume characterization for a standalone analytical system. Characterization of column volume using thiourea was optimized for ZORBAX SB C18 columns. Another column type may require adjustment in solvent composition or even different marker compound.

1. Prepare a thiourea sample in one of the following concentrations, according to the path length of the detector's flow cell, and place the sample vial in the autosampler:
   - 3 mm flow cell: 0.5 mM thiourea in 75 % acetonitrile
   - 10 mm flow cell: 0.2 mM thiourea in 75 % acetonitrile
   - 60 mm flow cell: 0.03 mM thiourea in 75 % acetonitrile
2. Filter the sample before use with a regenerated cellulose syringe filter.
3. Replace the column by a zero dead volume connection.
4. Prepare solvents and wash solutions, and purge solvents lines:
   - Solvent A: water (optionally with 0.1% formic acid)
   - Solvent B: acetonitrile (optionally with 0.1% formic acid)
   - Needle wash solution: 80 % acetonitrile or other suitable solution
   - Purge solvent lines with new solvents
5. Setup the method:
   - Set stop time to no limit in all modules (infinite run time)
   - Set solvent B to 75 %
   - Clear timetable
   - Set injection volume to 1 µL
   - Set UV detection signal to 242 nm with 4 nm bandwidth, no reference

6. Display UV profile at 242 nm in online plot
7. Equilibrate the system with 75 %B using 1 mL/min for 2 minutes
8. Set flow to 0.2 mL/min (if necessary, use a restriction capillary of known volume to maintain backpressure above 15 bar)
9. Open the sample info dialog:
   - Enter the location of the sample vial
   - Enter a run name
   - Run the method
10. Stop the run after the marker peak has been recorded
11. Repeat the run twice (total 3 runs)
12. Install the target column
13. Equilibrate the column until pressure and UV absorbance are stable
14. Set a suitable flow in the range of 0.2 to 4 mL/min so that the marker elutes at 1 min or later.

    Since the expected column volume is about one half of a geometric column volume (that is, the cross-sectional area multiplied by the length) set the flow to be about one half of the geometric column volume in mL units. For example, a geometric volume of 4.6 x 50 mm column is (3.14 x 2.3 x 2.3 x 50) / 4000 = 0.83 mL, giving a flow (1/2 x 0.83) mL / 1 min ≈ 0.4 mL/min.
15. Adapt the injection volume to the applied flow (and column volume):
    - Flow 0.2 to 0.5 mL/min, inject 1 µL
    - Flow 0.5 to 1 mL/min, inject 2 µL
    - Flow 1 to 2 mL/min, inject 5 µL
    - Flow > 2 mL/min, inject 10 µL
16. Note the applied flow and injection volume.
17. Stop the run after the marker peak has been recorded.
18. Repeat and check for consistency.
19. Evaluate data.
    - Record the elution time of all peaks at the apex

- Calculate the elution volume without the column:
  - Calculate the average elution time of data without the column
  - Multiply it by the applied flow rate
  - Subtract the volume of the restriction capillary if used and one half of the injection volume
- Calculate the final column volume:
  - Calculate the average elution time with the column
  - Multiply it by the applied flow rate
  - Subtract the elution volume without the column and one half of the injection volume

5.4 Equilibrating the column and optimizing the flow rate

5.4.1 Equilibrating and purging the column

Sufficient column equilibration time is required to obtain reproducible retention times. We recommend an equilibration phase of four column void volumes and two dwell volumes after each column purge phase. At least three column void volumes are required for a proper column purge.

5.4.2 Optimizing the flow rate

What is the best flow rate for analytical scouting on a purification system?

A test mix was injected on a manual scale-up system with a 4.6 by 150 mm analytical column. The total system void volume (dwell and column void volumes) is approximately 3 mL. Using a flow rate of 1 mL/min and a generic gradient from 2 to 98 % organic solvent, a gradient slope of 10 %B/min was applied, see Figure 5.5. In two further experiments we increased the flow rate to 1.5 mL/min and 2 mL/min, see Figure 5.6 and Figure 5.7.

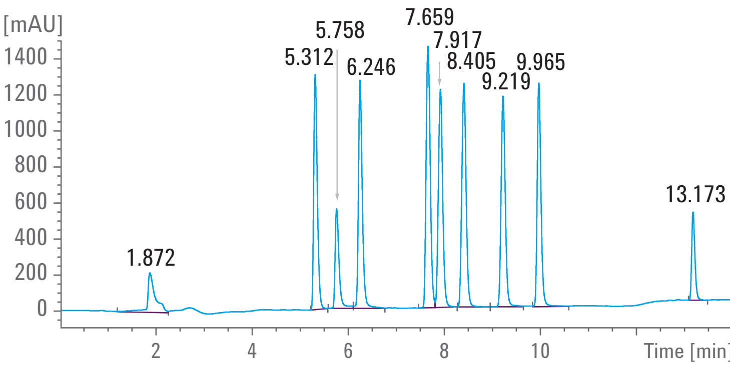


Figure 5.5 Chromatogram at 1 mL/min (4.6 x 150 mm, 5 µm column) for determination of optimum flow rate.

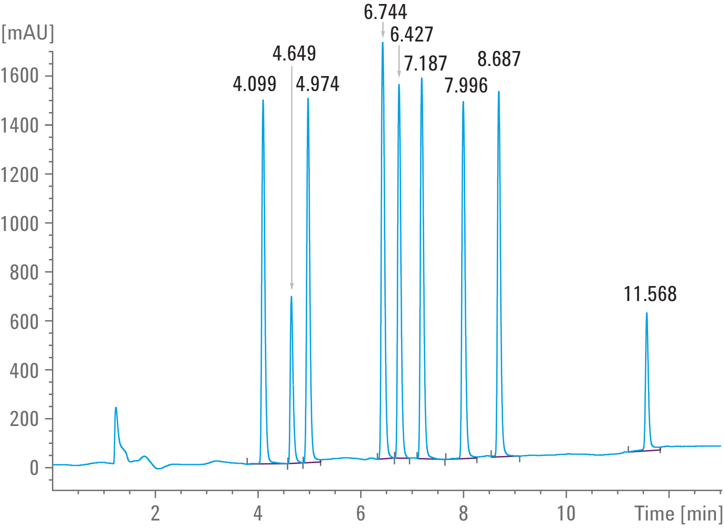


Figure 5.6 Chromatogram at 1.5 mL/min (4.6 x 150 mm, 5 µm column) for determination of optimum flow rate.

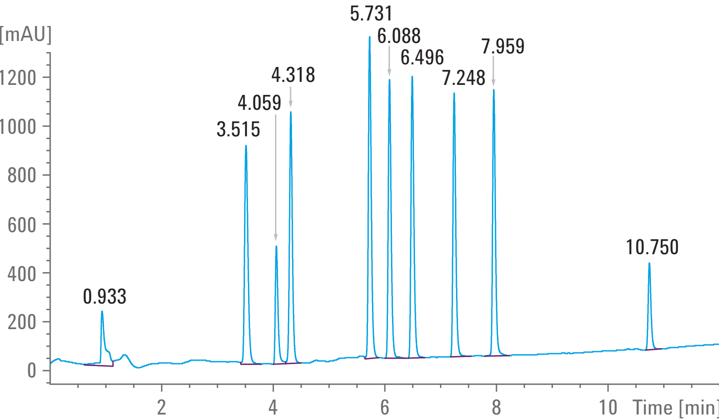


Figure 5.7 Chromatogram at 2.0 mL/min (4.6 x 150 mm, 5 µm column) for determination of optimum flow rate.

After the chromatograms were acquired the resolution between the peaks at 7.659 and 7.917 minutes in Figure 5.5 and the number theoretical number of plates was calculated. Table 5.2 summarizes all the results.

Flow [mL/min]	Resolution (preceeding peak)	Resolution (following peak)	Peak width	Number of plates
1.0	1.594	2.955	0.09708	36843
1.5	2.943	4.108	0.06208	65792
2.0	2.892	4.012	0.06292	64092

Table 5.2 Chromatographic resolution, peak width and theoretical number of plates. The flow rate of 1.5 mL/min is appropriate and delivers the best resolution on this system.

5.4.2.1 Comparing run time and solvent consumption

When comparing run time, solvent consumption and peak width, a flow rate of 1.5 mL/min gives best results, see Table 5.3. Increasing the flow rate from 1.5 to 2 mL/min reduces runtime by a further 10.7 % but increases the solvent consumption by 20.3 %, which is unfavorable. However, if the requirement sample throughput demands reduced runtime, the increased solvent costs must be accepted.

To obtain good results a simple rule is to use a ratio of system void volume to flow rate equal to one or less than two. Based on the results of this study all further scale-up calculations use a flow rate of 1.5 mL/min.

Flow [mL/min]	RT of target compound [min]	Solvent consumption [ml]	Time savings [%]	Increase in solvent consumption [%]	Peak width	Comment
1.0	7.917	7.917			0.09708	Reduced resolution
1.5	6.744	10.116	18.1	27.77	0.06208	Best resolution
2.0	6.088	12.170	10.7	20.3	0.06292	Increased solvent consumption

Table 5.3 Effects of different flow rates on resolution and run time – based on results obtained from system used for measurements.

5.5 Overloading the column

Demands in throughput increase when large quantities of pure compound are required. Multiple injections or an increased column load per injection are ways to address this challenge. Overloading the column can be done as concentration or volume overloading. In concentration overloading the volume is kept the same but the sample concentration is increased. In volume overloading the concentration is kept constant but the injection volume is increased. Unfortunately, samples can be problematic when solubility of impurities or target compound conflict with the requirements of chromatography. Various measures can be taken to deal with these challenges. In this section we look at how volume overloading affects peak shape and resolution.

5.5.1 Volume overload

Figure 5.8 shows the chromatogram obtained from a 4.6 by 150 mm column after a 10-µL injection of a sample dissolved in DMSO. All compounds are separated within an acceptable range of resolution and exhibit good peak shape. Figure 5.9 shows a 20-µL injection of the same sample. The peaks of two of the basic, nitrogen-containing compounds show strong fronting and are beginning to split. Figure 5.10 shows an injection of 30 µL of sample. Three peaks exhibit serious fronting and splitting effects. Strong fronting and poor peak shapes affect the triggering process especially when the slope recognition parameters are too sensitive.

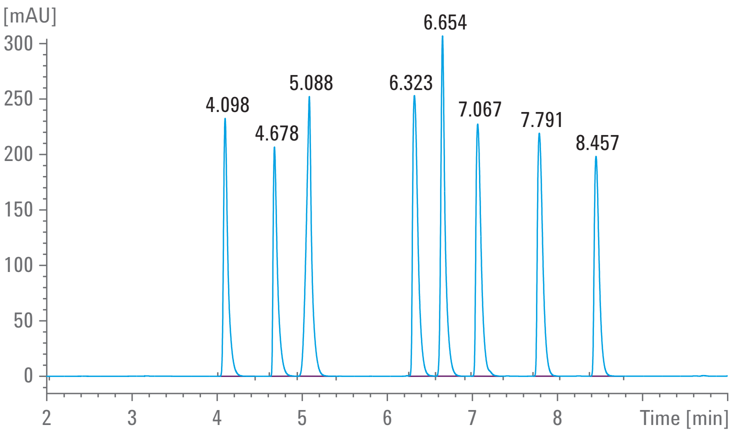


Figure 5.8 Injection of 10 µL of sample mix in DMSO on a 4.6 by 150 mm column. All components are separated and exhibit good peak shape.

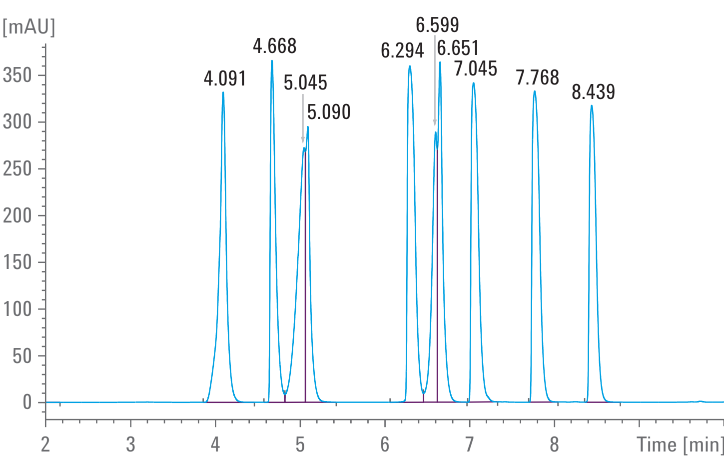


Figure 5.9 A 20-µL injection of the same sample shows strong fronting on two of the nitrogen-containing compounds as well as peak-splitting.

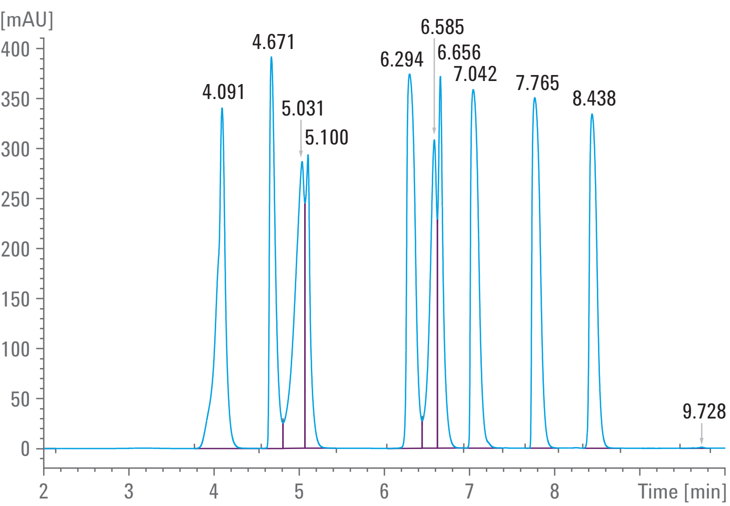


Figure 5.10 A 30 µl injection shows even more serious effects, as seen on the nitrogen-containing compounds 1, 3 and 5.

5.5.2 Mass loading of the column

Table 5.4 indicates the amount of stationary phase for different column dimensions and the recommended sample sizes by mass loading. A column load between 0.1 and 1 % of mass load with respect to the chromatographic task is frequently applied. The more difficult the chromatographic task, the less column load is required. For simple or mass-throughput separations, column loads of even 5 % weight of the stationary phase can be used.

Diameter [cm]	0.46	1.09	2.12	3.00	5.00
Length [cm]	15.00	15.00	15.00	15.00	15.00
Volume [mL]	2.49	42.55	52.97	106.07	294.64
Flow [mL/min]	1.50	25.59	31.86	63.80	177.22
Approximate sorbent required [g]	1.50	25.53	31.78	63.64	176.79
Very high efficiency 0.1 % load [g/run]	0.001	0.026	0.032	0.064	0.177
Load and efficiency 1 % load [g/run]	0.015	0.225	0.318	0.636	1.768
Throughput oriented 5 % load [g/run]	0.075	1.276	1.589	3.182	8.839
Run time based on 15 min run [h]	0.25	0.25	0.25	0.25	0.25
Throughput [g] 24 hours/day from 1 % load	1.4	24.5	30.5	61.1	169.7
Throughput [kg] 365 days/year, 24 hours/day	0.524	8.944	11.136	22.300	61.945

Table 5.4 Throughput calculations for Agilent Load & Lock columns.
Stationary phase density: 0.6 g/mL

With these calculations, the amount of sorbent for different column dimensions can be estimated when using a sorbent density of 0.6 g/mL. According to the task, a 30 by 150 mm column can be used from 64 to 640 mg of solid crude mix. As there are often solubility issues with the crude mix, the limits are often reached by the recommended injection volume. For these column dimensions we recommend to inject not more than 2000 µL of DMSO. The most limiting factor is the solubility of the compounds.

Table 5.5 shows recommend injection volumes of DMSO for different column diameters and length. All recommendations are based on experiences and lab experiments.

Column dimensions	Injection volume [µL]	Stationary phase [g] with density 0.6 g/mL	Column load [mg] 0.1 %	0.5 %	1.0 %
2.1 x 50 mm, C18	1.75				
4.6 x 150 mm, C18	25				
19 x 100 mm, C18	284	17	17	85	170
19 x 150 mm, C18	426	26	26	130	260
19 x 250 mm, C18	710	43	43	215	430
21.2 x 100 mm, C18	354	21	21	105	210
21.2 x 150 mm, C18	531	32	32	160	320
21.2 x 250 mm, C18	885	53	53	265	530
30 x 100 mm, C18	706	42	42	210	420
30 x 150 mm, C18	1063	64	64	320	640
30 x 250 mm, C18	1772	106	106	530	1060
50 x 100 mm, C18	1969	118	118	590	1180
50 x 150 mm, C18	2953	177	177	885	1770
50 x 250 mm, C18	4922	295	295	1475	2950

Table 5.5 Amounts of stationary phase for different column sizes and recommended sample load by mass.

If larger volumes need to be injected, we recommend using the on-column dilution method, see Section 3.2.3 "Special injection techniques". Another approach is to reduce the strength of the injected solvent, for example, by replacing DMSO by methanol, assuming the compounds are soluble. Alternatively, the compounds can first be dissolved in a small volume of DMSO and then diluted with methanol or other less-strong solvents.

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