Agilent InfinityLab

PRINCIPLES AND PRACTICAL ASPECTS OF PREPARATIVE LIQUID CHROMATOGRAPHY

A Primer

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Trusted Answers

Principles and practical aspects of preparative liquid chromatography

A primer

Content

1 Foreword

As a synthetic chemist, biologist or engineer, it's your job to study the impact of chemical compounds. To achieve this goal, you need to stay at the leading edge of your field of research. Consequently, 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.

2 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.

3 About the Authors

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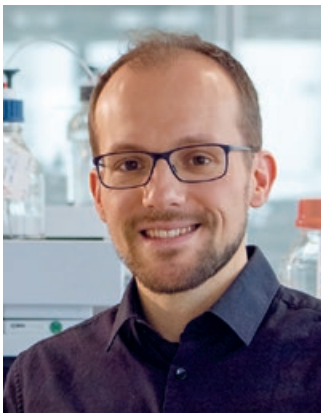
Helmut Schulenberg-Schell has a master's degree in chemistry and a Ph.D. in biochemistry from Münster University, Germany. Isolation of naturally occurring cyclopentenyl fatty acids and bacterial hopanoids, and purification of bovine lipid binding proteins introduced him to preparative chromatography 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.

Dr. Andreas Tei

Andreas Tei attained his master's degree in organic chemistry and his Ph.D. in natural sciences from Heidelberg University in Germany. He started his industrial career with one of the market-leading scientific instrument manufacturers and held various positions in technical support, application support, sales and marketing for LC/MS and GC/MS systems. In his years as an application scientist he was involved with the automation of mass-based purification for medicinal chemistry laboratories. In 2011, Andreas joined Agilent as product manager for preparative chromatography systems. In this role he was responsible for the design of new modules and automation software. He went on to become a marketing segment manager for pharma small molecules and now manages the customer success portfolio at Agilent.



Dr. Florian Rieck

Florian Rieck holds a master's degree in food chemistry and a Ph.D. in analytical chemistry from Karlsruhe Institute of Technology (KIT) in Germany. Detecting and characterizing new potentially bioactive plant metabolites during his Ph.D. studies, he gained first experience with preparative liquid chromatography. Florian joined Agilent Technologies in 2015, where he started as an applications chemist for liquid chromatography in Waldbronn, Germany. Working initially on general LC applications projects, Florian soon focused on preparative liquid chromatography and mass-based purification applications.

Ronald Guilliet

Ronald Guilliet received his education in analytical chemistry from the University of Applied Sciences in Vlissingen, The Netherlands. He started his career with a scientific instrument and consumables manufacturer and held positions in production and marketing for HPLC columns. Later, his interest focused on large-scale (process) purification. He gained a global view on pharmaceutical large-scale workflows as business development manager and sales specialist with a strong focus on India and Asia where he successfully offered total solutions. Ronald joined Agilent in 2010 as product manager for preparative chromatography and supercritical fluid chromatography. At this stage of his career, he is currently responsible for the design of the Agilent InfinityLab LC Purification portfolio.

4 Symbols and Abbreviations

Symbols

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

Principles and Practical Aspects of Preparative Liquid Chromatography

5 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.

5.1 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 nanoliters 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 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.

5.2 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 nonexpert chromatographers to purify their precious samples in self-service purification labs.

To ensure that 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 impossible to individually optimize the purification parameters unless the processes can be automated [1–4].

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. Consequently, 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 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 × 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 must be enriched from large volumes of dilute sample (for example, urine) or recovered with maximum yield from small amounts of biological tissue.

6 Key Aspects of Column Selection

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.

6.1 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 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	
Purification range [mg]		1–15	7–70	30–300	64–640	180–1800	400–4000
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

Figure 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 × 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 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 required 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 × 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.

6.2 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.

Figure 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 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.

In contrast, we recommend using 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.

6.2.1 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.

6.2.2 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. Consequently, 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 depends 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 1.

Table 1. Ratios of hydraulic and mechanical pressures on the sorbent bed (C18 sorbent with a packed bed density of 0.59 g/mL).

	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

7 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 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 to the fraction collector must 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.

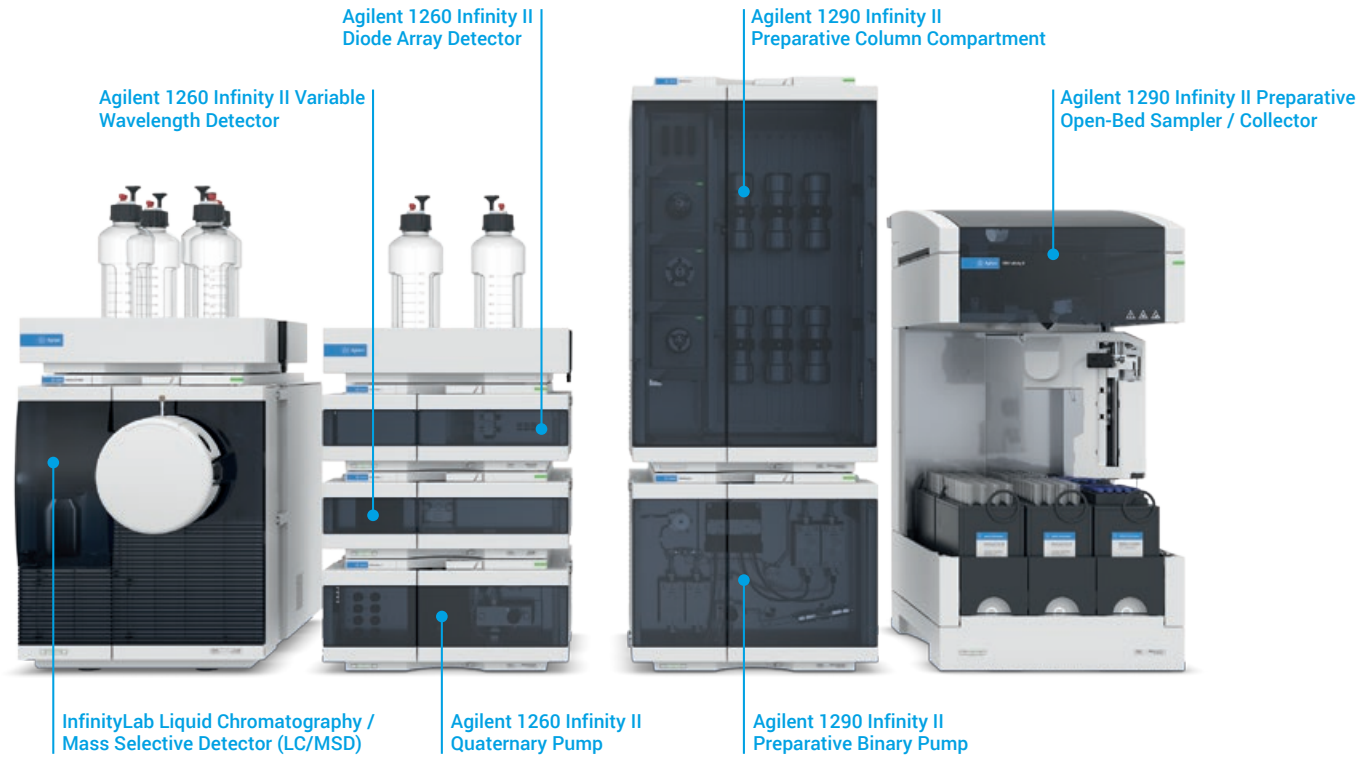


Figure 3. Agilent 1290 Infinity II Autoscale Preparative LC/MSD system.

7.1 Solvent delivery

7.1.1 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 to 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 4. Agilent 1290 Infinity II Preparative Binary Pump.

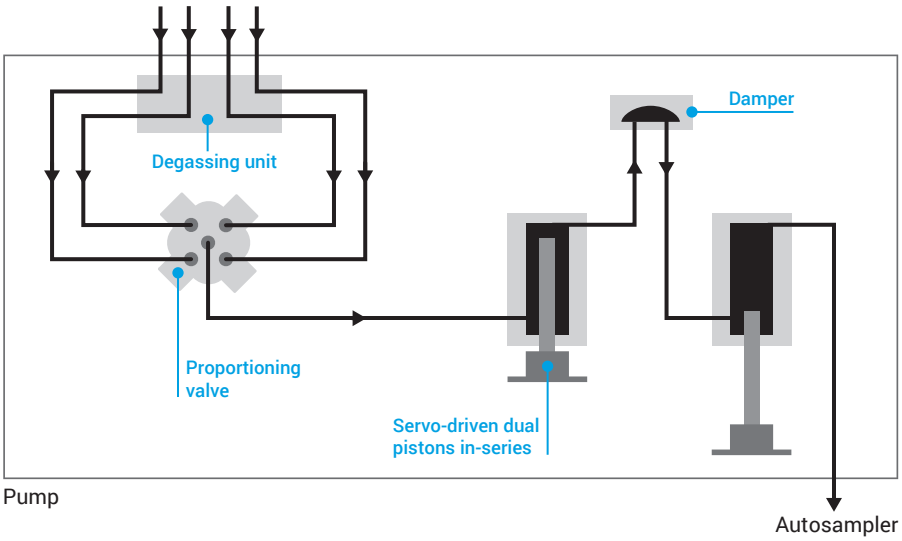


Figure 5. Schematic of a solvent delivery system for low-pressure mixing of gradients, showing degassing unit, proportioning valve, and pump cylinders.

7.1.2 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 usually not necessary if 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.

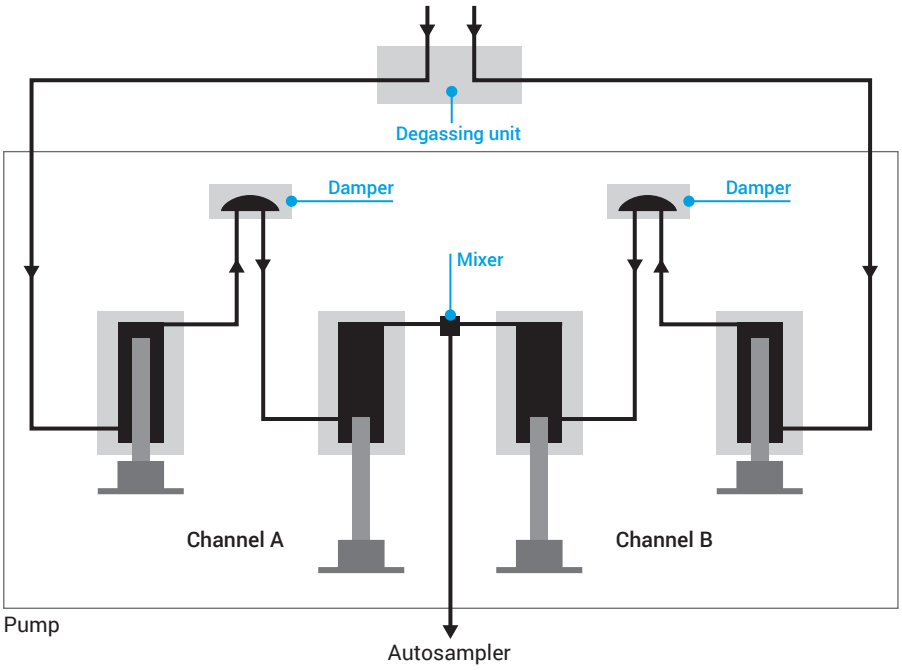


Figure 6. Schematic of a solvent delivery system for high-pressure mixing of gradients, showing the two solvent channels for generation of binary elution gradients.

7.2
Sample introduction

In preparative LC, the number of samples and the amount of sample to be injected varies significantly. Consequently, 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.

7.2.1 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 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 8 shows how the eluent flows through the needle and onto 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 multidraw approach. Increasing the seat capillary volume does necessarily increase the dwell volume. However, switching to bypass mode alleviates this dilemma as explained below.

Figure 7. Agilent 1290 Infinity II Preparative Open-Bed Sampler/ Collector.

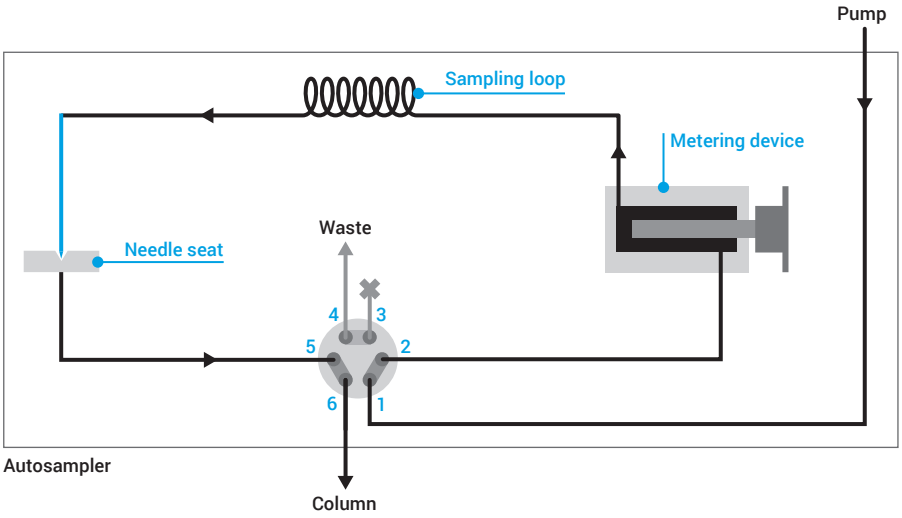


Figure 8. When the autosampler is in mainpass, the eluent from the pump flows through the switching valve, metering device, sampling loop, injection needle and needle seat, and again through the valve to the column.

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 9.

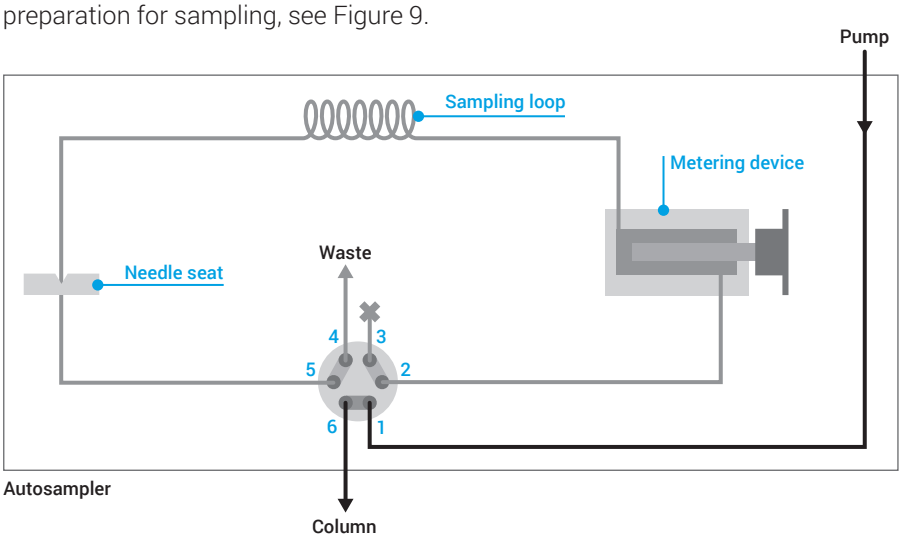


Figure 9. When the autosampler is in bypass mode, the eluent flow from the pump is diverted directly to the column, leaving the metering device and injection needle free to begin sampling.

Switched out of the flow path, the injection needle is raised out of the needle seat and lowered into the sample vessel. Withdrawing the plunger of the metering device pulls sample through the needle into the sampling loop, see Figure 10.

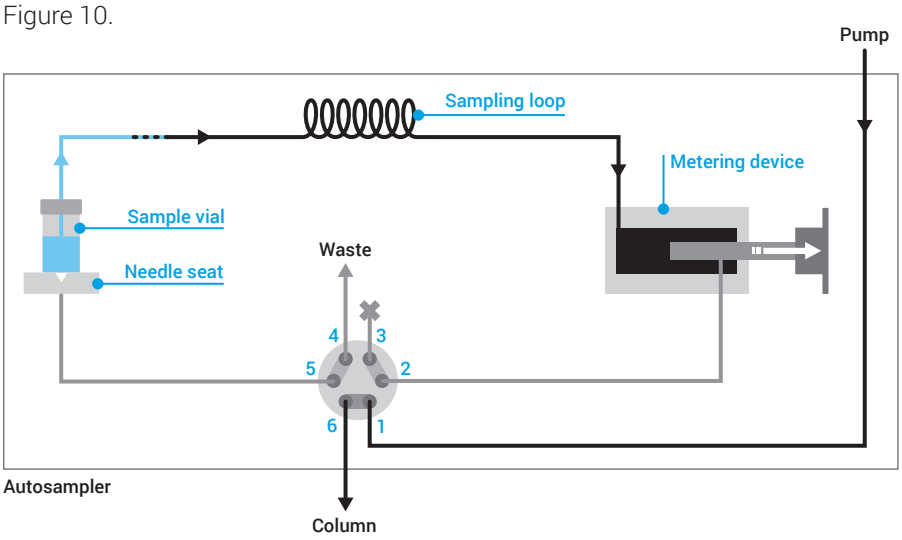


Figure 10. Drawing sample through the needle and into the sampling loop.

When the required amount of sample has been transferred to the sampling loop, the needle is raised out the sample vessel, and lowered onto the needle seat. The switching valve now returns to mainpass, sweeping the sample out of the loop, through the needle and onto the column, see Figure 11.

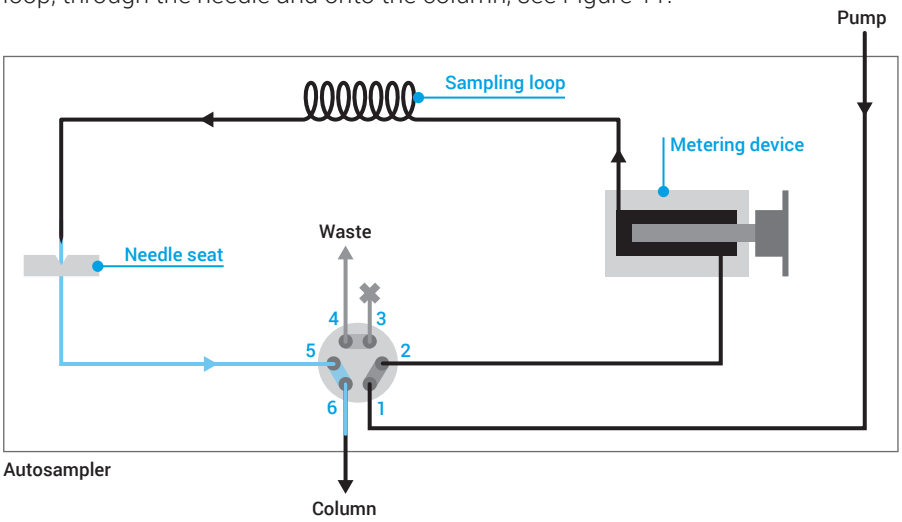


Figure 11. Switching back to mainpass sweeps the sample onto the column.

To reduce the dwell volume, we recommend returning to the bypass mode as soon as the sample has been transferred to the column. Some instruments offer injector programs or a method option to switch to bypass after a certain flush-out time; others do not have these capabilities. In either case, 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.

7.2.2 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 separate analytical and preparative flow paths.

Most instruments of this type are designed in a way that the preparative flow path serves as the bypass when the analytical loop is filled, and vice versa. Some instruments, however, offer special valve designs with dedicated bypass positions for both flow paths. This latter design offers more flexibility when the analytical and preparative flow paths are to be optimized with respect to loop volume and id – the risk of overpressure when switching from mainpass to bypass in preparative mode during high flows is eliminated.

When deploying fixed-loop autosamplers, the sample loops can be partially filled or overfilled, see Figure 12 and Figure 13. Partial filling of the sample loop is the most common approach. Due to Taylor dispersion, the interface between auxiliary solvent and sample will not be flat but parabolic. Therefore, we recommend not to exceed 60 % of the nominal loop volume. 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 using a colored sample.

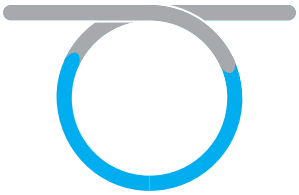


Figure 12. Partial fill of sample loop.

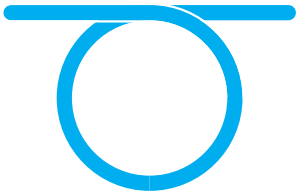


Figure 13. Overfill of sample loop.

One way to minimize Taylor dispersion is to use air plugs between the auxiliary solvent and the sample. The air-liquid interface within a capillary will be flat, which will extend the usable volume of a preparative sample loop to more than 75 % of its nominal volume.

Overfilling or full-loop injection is required for accurate, quantitative work and we do not recommend this technique for purification work as a large portion of a precious sample will be lost.

Because the loop is a part of the capillary system, the dwell volume increases with increasing loop size. Therefore, we recommend keeping the loop 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.

An autosampler with separate analytical and preparative flow paths is well suited to meet the requirements of both analytical and preparative LC where vastly differing sample volumes need to be injected. The design with two loops minimizes the total dwell volume of the injection system and facilitates analytical scouting with optimum separation efficiency prior to sample purification.

Fixed-loop autosamplers are designed in one of two ways to draw the sample and transport it into the loop, which are called push-to-fill and pull-to-fill principle. As the flow path between needle tip and metering device is not part of the high-pressure flow path, both principles require an auxiliary solvent to fill the injection flow path.

Figure 14 shows the push-to-fill principle. For a preparative-scale injection, the valve is switched to preparative bypass, which moves the analytical loop into the high-pressure flow path. The needle is lowered into the sample vial. The metering device pulls sample through the needle into the sampling loop.

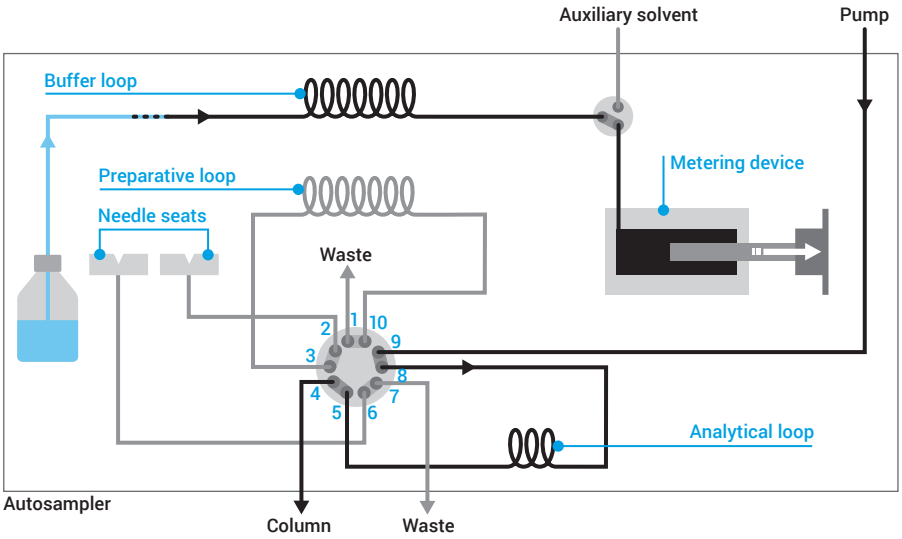


Figure 14. Drawing sample in a fixed-loop autosampler with push-to-fill principle.

Once the sample has been drawn into the sampling loop, the needle is raised out the sample vial, the vial 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 15.

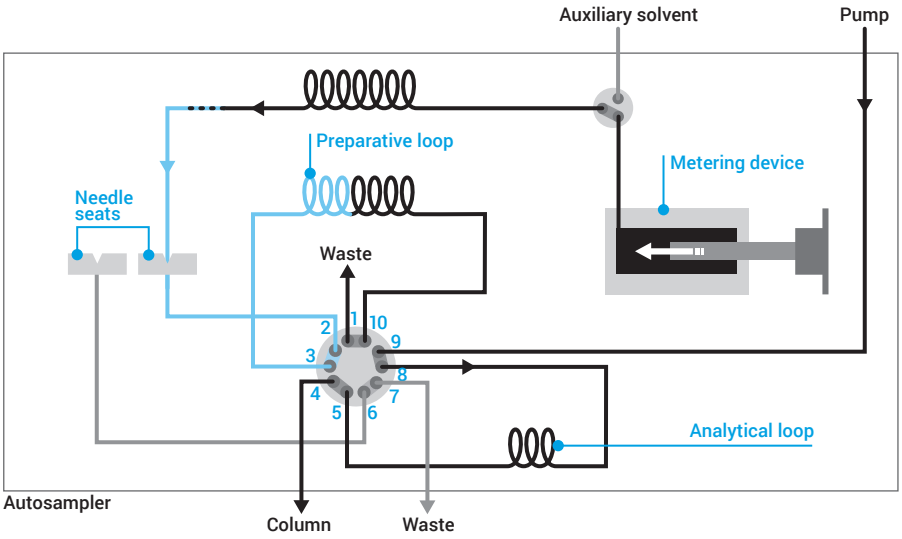


Figure 15. Driving the sample into the preparative sample loop.

Moving the switching valve back to preparative mainpass sweeps the sample out of the preparative loop and onto the column, see Figure 16.

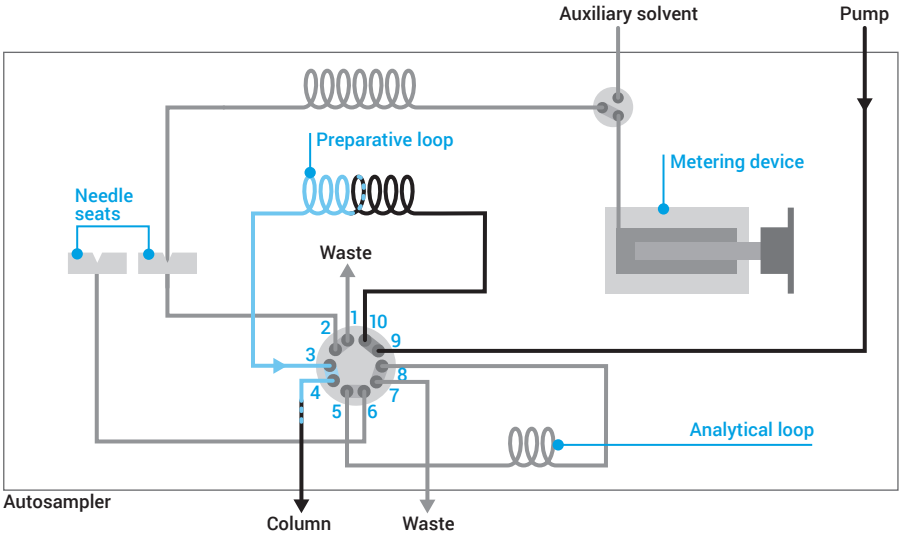


Figure 16. Switching the valve back to preparative mainpass sweeps the sample out of the loop and onto the column.

From the schematics, it becomes evident why this principle is called push-to-fill: the metering device first draws the sample into the sampling loop, then pushes it through the needle seat into the sample loop. A drawback of this two-step process is that each of the two flow paths requires a dedicated needle seat. The pull-to-fill principle, on the other hand, does not have this disadvantage.

Compared with push-to-fill instruments, fixed-loop autosamplers working by pull-to-fill principle share the concept of a sampling path that is separated from the high-pressure flow path. The needle and the metering device, however, are not directly connected but only linked through the injection valve, see Figure 17.

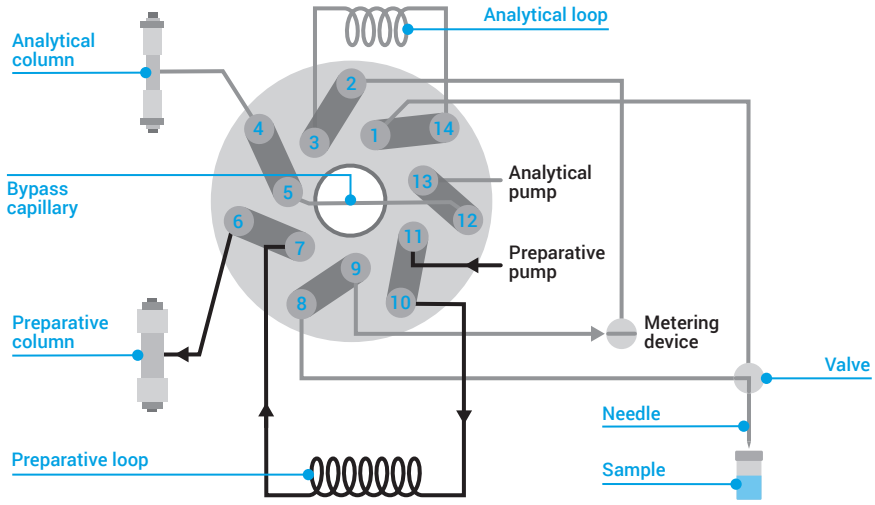


Figure 17. Pull-to-fill autosampler in preparative mainpass position. The eluent flows from the pump through the loop to the column. The needle and metering device, however, are not part of the flow path.

In a pull-to-fill sampling process, the valve switches to bypass, the needle moves into the sample vial, and the metering device draws the sample through the needle and the valve into the preparative loop, see Figure 18.

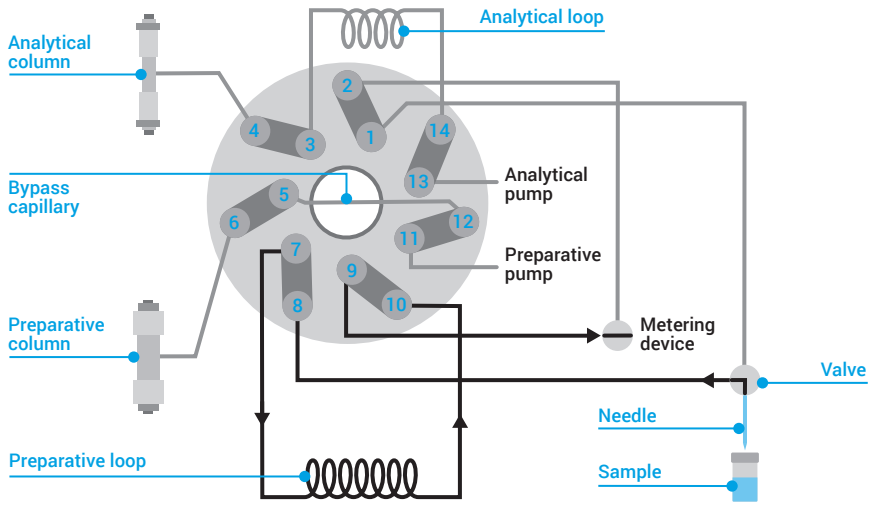


Figure 18. Drawing a sample in preparative bypass position of a pull-to-fill autosampler.

Once the sample has been drawn, the needle moves out of the sample vial and the metering device continues drawing until the content of the needle, needle tubing, and valve groove has been transported into the loop. The valve switches to mainpass, moving the preparative loop into the high-pressure flow path, where the sample is transported to the column, see Figure 19.

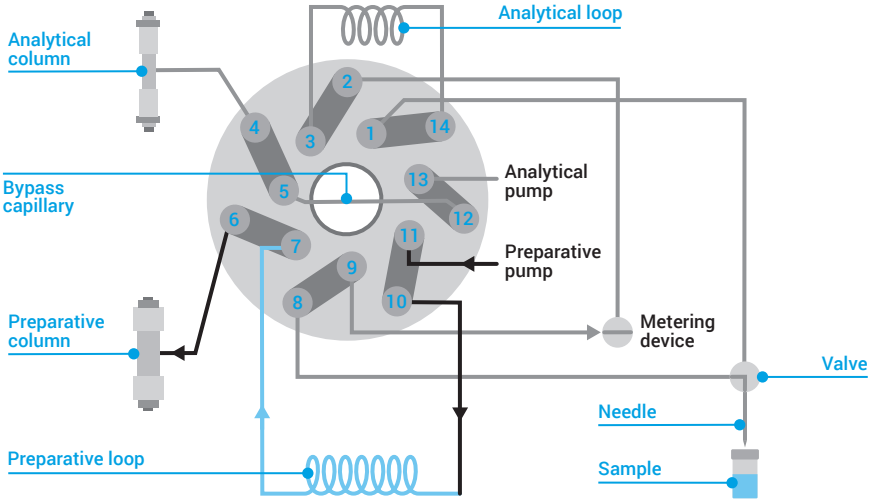


Figure 19. Switching the valve back to preparative mainpass position sweeps the sample onto the preparative column.

When working with fixed-loop autosamplers, it is essential to have the possibility to flush the sampling loop and the needle tubing with a well-degassed auxiliary solvent, and also to wash the needle's outside surfaces for at least 10 seconds. This procedure eliminates sample carryover between injections and prevents air bubbles building up in the loop.

In preparative chromatography, washing the inside of the needle and tubing is essential to maximize sample recovery. Concentrated samples, and nonpolar samples in particular, tend to stick to the inside wall of the tubing. Pure solvent drawn after the sample and transported into the sample loop will collect sample residue and increase recovery, see Figure 20. Some autosamplers offer dedicated method settings to incorporate these postsample or sample positioning plugs. Another type of solvent plug is used to inject highly concentrated samples in strong solvents. Read about these special injection techniques in the next section.

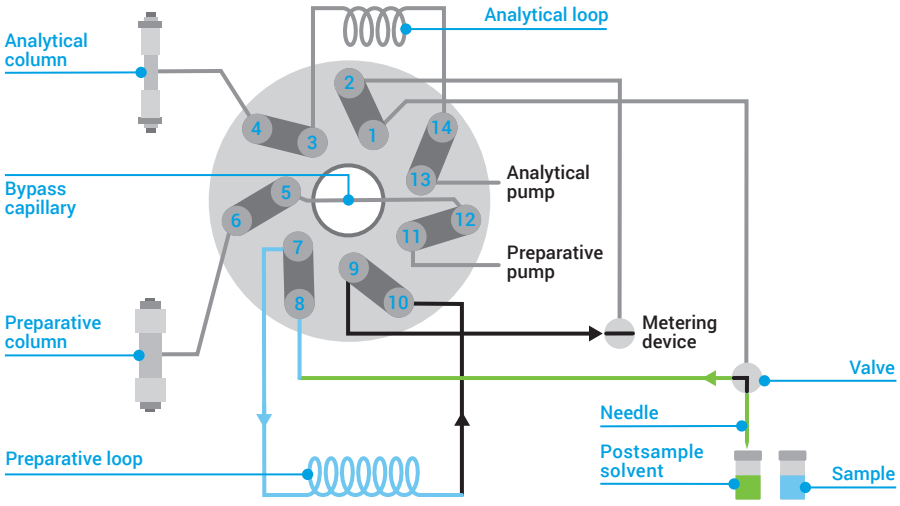


Figure 20. Postsample plug of pure solvent is drawn after the sample to transport sticky residue into the sample loop.

7.2.3 Special injection techniques

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

Sample sandwiching

The sandwich injection technique can be applied when samples tend to precipitate on contact with the starting composition of the mobile phase. Clogging of injector needles, valves, and capillaries can be possibly avoided. By programming the autosampler, the sample can be embedded ("sandwiched") between two plugs of an appropriate solvent that avoids precipitation in the sample loop, see Figure 21. DMSO or solvents immiscible with water can be used. For analytical injections, to maintain chromatographic performance, the volume of injected DMSO should be kept as low as possible. We recommend using two plugs of 5 µL. As a rule of thumb for preparative injection volumes, we recommend using a total plug volume of about 10 % of the total injection volume.

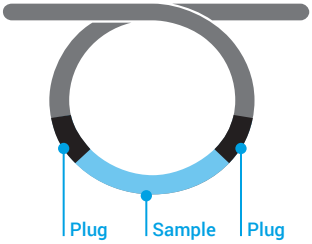


Figure 21. Profile of a sandwich injection using plugs of appropriate solvent either side of the sample to avoid precipitation in the sample loop or connection capillaries.

Injecting large volumes of strong organic solvents

Most compounds originating from organic syntheses 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.

If the required volume needs to exceed the maximum injection volume for the given column size (Table 2), we recommend organic-phase injection: connect the injector with the organic pump channel, inject the sample in pure organic solvent, and dilute it with the aqueous solvent directly before the column.

Table 2. Recommended injection volumes of strong solvents. When exceeding the maximum injection volumes, the chromatographic performance will be affected. Parts of the sample may elute with or as part of the initial solvent peak.

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

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 at least 5 % of the total flow. The complementary percentage of water is delivered by channel A and added through the T-piece, see Figure 22.

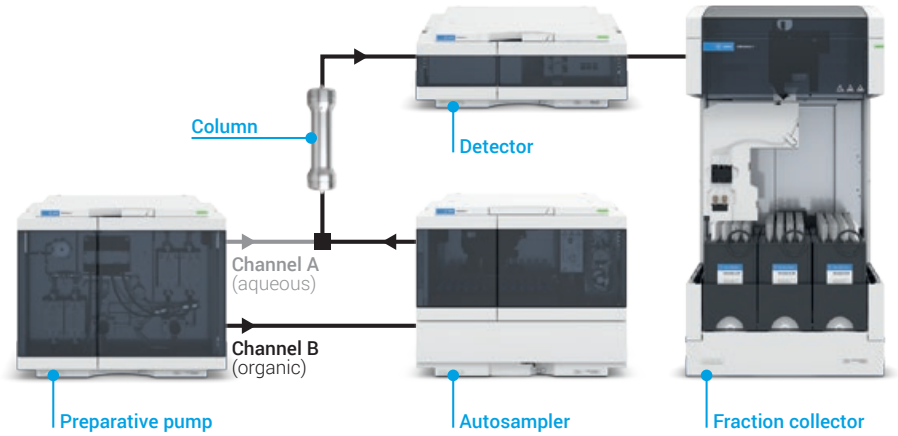
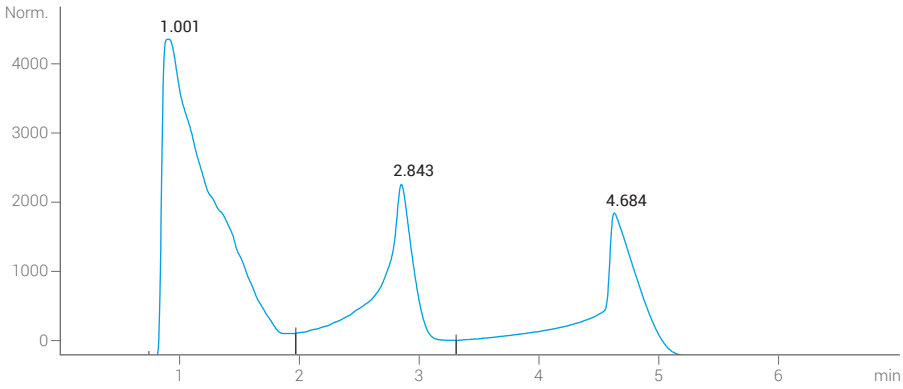


Figure 22. Schematics of a system configuration for organic phase injection.

When deploying a configuration as shown in Figure 22, 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 and the sample solvent flushed out of the column. 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 smooth, extending column lifetime while increasing column load as well as chromatographic resolution.

Figure 23 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 organic phase injection mode resulted in no sample loss with the polar compounds focused on the column, see Figure 24.

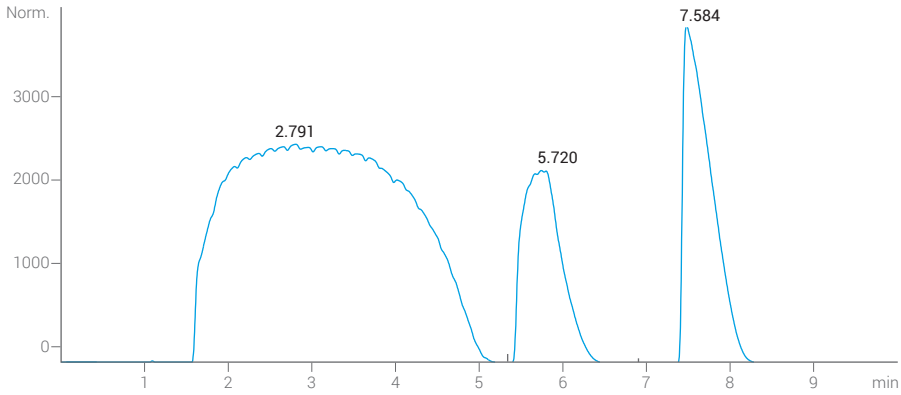


Sample: 50 mg acetaminophen and 50 mg caffeine in 5000 µL DMSO
Column: Agilent ZORBAX SB C18, 21.1 × 150 mm, 5 µm

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 23. High-volume injection with strong eluents in standard injection mode. The chromatogram shows 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 acetaminophen and 50 mg caffeine in 5000 µL DMSO
Column: Agilent ZORBAX SB C18, 21.1 × 150 mm, 5 µm

Applied gradient profile for organic phase 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

Figure 24. High-volume injection with strong eluents using organic phase injection. A broad solvent peak is observed until 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 was observed.

Injecting large sample volumes using an injection pump

Separations of highly diluted samples in aqueous solvents often require large injection volumes that exceed the typical loop size of autosamplers. In these situations, an injection pump can be used to load the sample onto the column. To avoid sample carryover, it is important to flush the injection pump, the valves, and all capillary connections after the sample has been transferred to the column.

Injection pumps can also be used for applications such as enantiomer separation. Here, the same sample solution must be loaded periodically, which is realized by a valve switching between inject and elute positions. Figure 25 shows a typical system configuration for alternating sample introduction and elution.

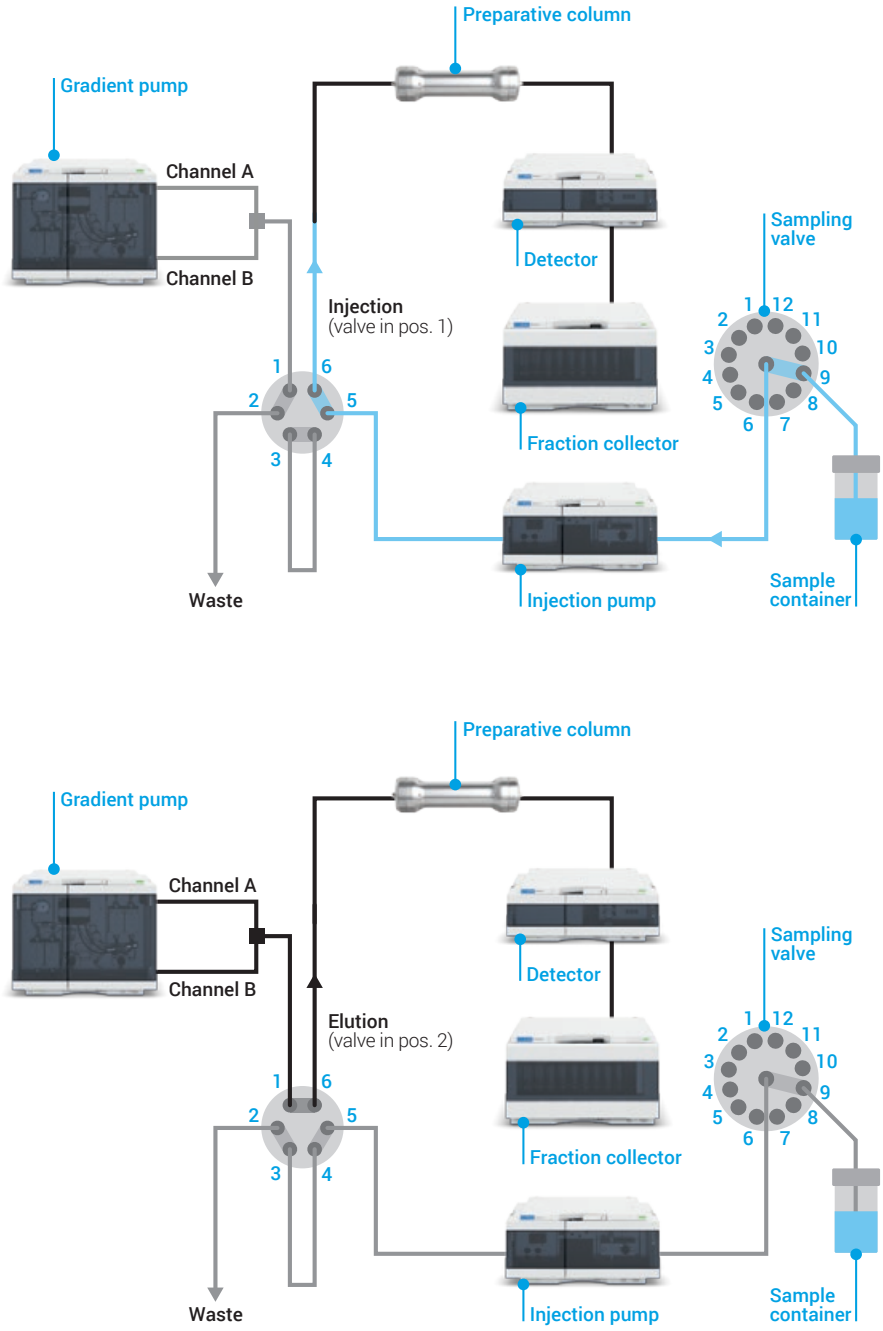


Figure 25. Schematics of a system configuration for alternating sample introduction and elution using an injection pump and a valve. With the valve in position 1, the injection pump draws the sample from the container and delivers to the column. For elution, the valve is switched to position 2 so that the gradient pump flow is directed to the column.

Some preparative binary pumps have built-in solvent selection valves that enable switching between sample introduction and elution within a single module. One of the aqueous channels is then used for the eluent, the other one for the diluted sample, see Figure 26. When this kind of pump is used for sample introduction, switching from injection to elution automatically flushes the critical parts of the flow path: the valve outlet, the pump heads, and the connections between valve, pump heads, and column.

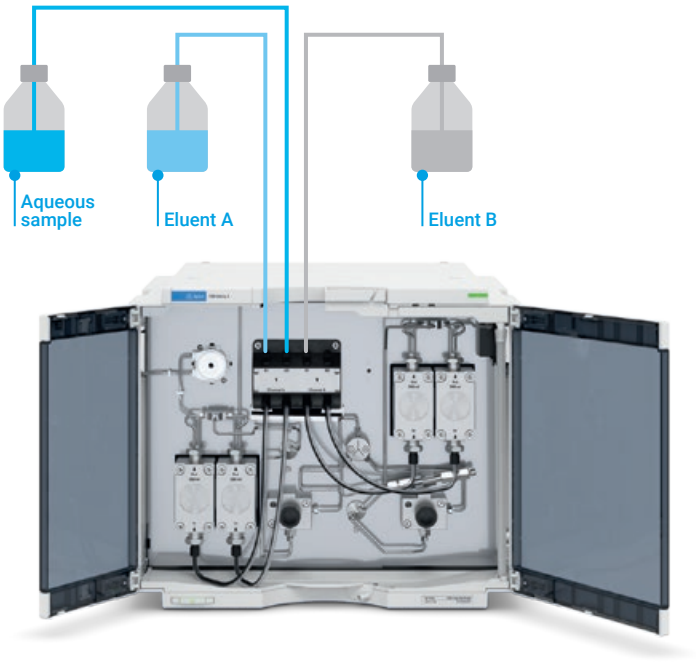


Figure 26. Schematics of alternating injection and elution enabled by the internal solvent selection valve of an Agilent 1290 Infinity II Preparative Binary Pump.[5]

7.3 Flow splitting

When using a destructive detector in preparative LC, a flow splitter is necessary to divert most of the eluent to the fraction collector. Flow splitters can also be deployed to reduce the flow rate to within analytical range when detector flow cells are not compatible with high flow rates. A splitting approach combined with a make-up flow diverts the major part of the flow to the fraction collector, while the split flow can be further diluted according to the detector range. Different designs are applied to serve fundamentally similar purposes.

7.3.1 T-piece flow splitting

A simple, inexpensive approach to flow splitting is to use a T-piece, see Figure 27. 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 a mass-selective or evaporative light scattering detector. This approach secures most of the eluent that contains the target compound.

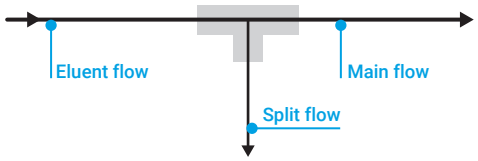


Figure 27. T-piece splitter – the split ratio is regulated by the backpressure resulting from the dimensions of the outlet capillaries.

The split ratio depends on the ratio of the backpressures at the outlets of the main stream and the split stream and must 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.

7.3.2 Double T-piece flow splitting

In preparative-scale chromatography, the concentrations of the compounds as well as the buffers are often too high for direct introduction into a mass selective detector with a single T-splitter. In these situations, the split flow needs to be diluted with a make-up solvent, see Figure 28. 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.

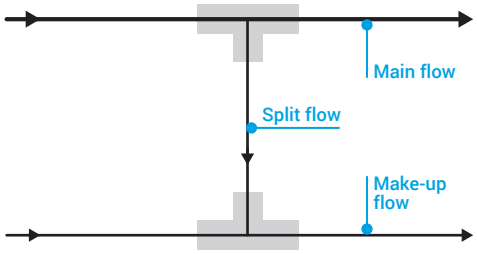


Figure 28. Double T-piece splitter – often used when a make-up flow is required for special detection techniques. The split ratio is regulated by the backpressure.

In a double T-splitter. the split ratio depends on multiple parameters, whereby the major dependence is on the pressure difference between the main flow and the make-up flow. In gradient elution, the viscosity of the mobile phase will change with the composition and thereby affect the split ratio. A customized double T-splitter needs to be carefully set up and adjusted experimentally for the flow rate used. The pressure difference is typically set at 6 bar (90 psi) based on experiments using different restrictors for different flow rates. 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 using different diameters and lengths of the split flow capillary. Some splitters have additional needle valves for further regulation of the pressure ratios. However, the split ratios obtained with these are only estimations and not true actual values. Nevertheless, using these T-splitters ensures true splitting in real time, without losing any information.

7.3.3 Active flow splitting

Active flow splitters such as the Agilent 1290 Infinity II MS Flow Modulator (Figure 29) are devices – typically valves – that transfer mechanically a small aliquot from the main flow to the split flow, see Figure 30.

Figure 29. Agilent 1290 Infinity II MS Flow Modulator

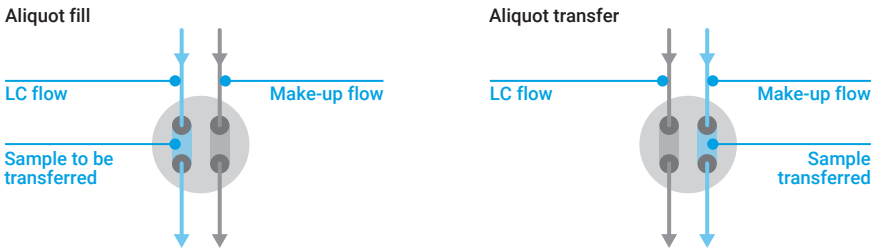


Figure 30. Schematics of an active flow splitter, showing the fill and transfer positions of the valve.

The split ratio depends on the switching frequency of the valve, the volume of the valve groove and the applied LC flow rate, see Equation 1. Some instrument manufacturer's software calculates the available split ratios based on the flow rate, which facilitates choosing the correct frequency and groove size.

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

Equation 1. Calculating the split ratio of an active flow splitter.

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 starting the splitting process only when fraction collection is required. The splitter can be deactivated during column purging and equilibration, and during the injection cycle, resulting in longer preventive maintenance intervals.

7.4 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), see Figure 31 – are a safer choice because the spectral information acquired can be used to confirm compound identity, determine peak purity, and select the wavelength best suited for detection in subsequent purification processes. Figure 32 shows the optical system of a diode array detector.

Figure 31. Agilent 1260 Infinity II DAD-WR.

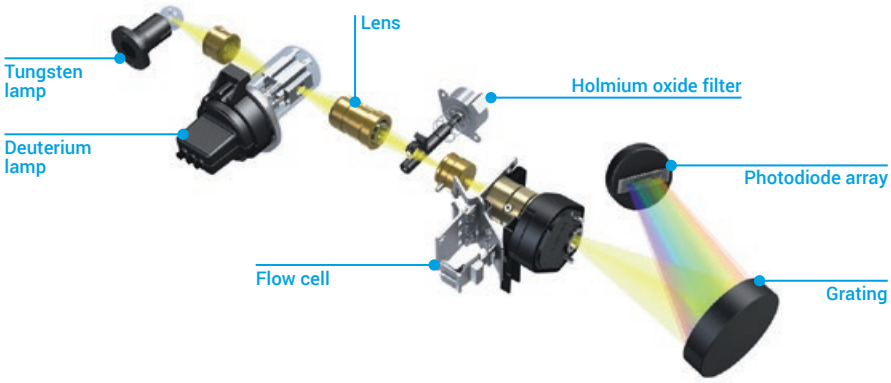


Figure 32. Schematic of optical system of a diode array detector.

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 33, 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.

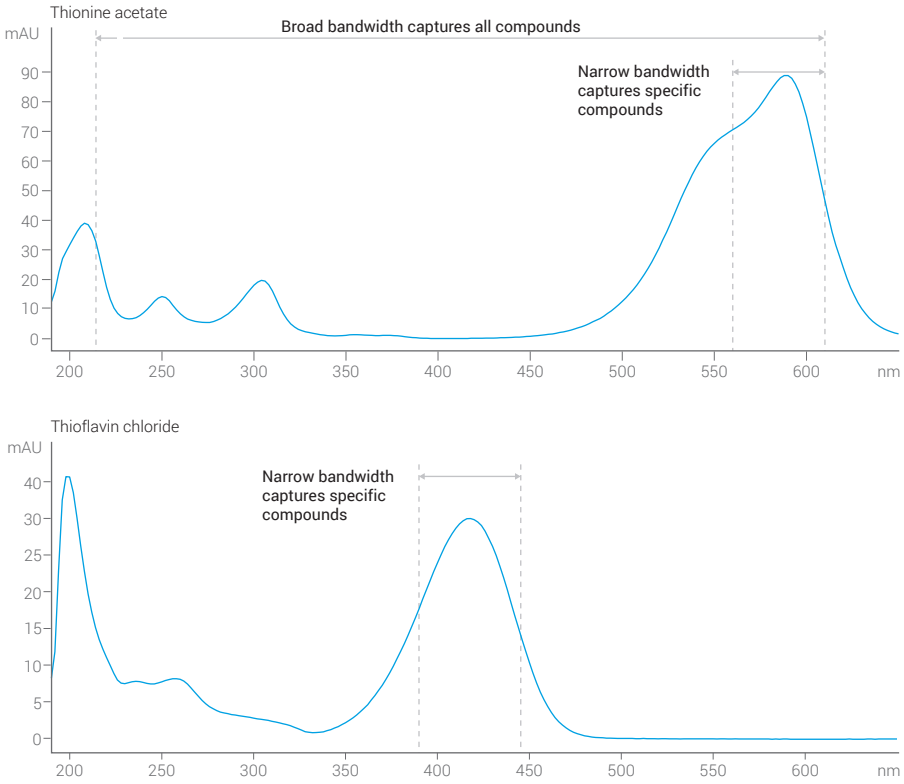


Figure 33. Two compounds with different UV spectra. Data acquisition using a wide bandwidth facilitates monitoring these different compounds in a single chromatogram. Using a small bandwidth instead monitors selectively only one of the compounds.

7.4.1 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 high concentration when working at high purity and with high column loading.

According to the Lambert-Beer law, see Equation 2, the most important parameter that can be used to match the concentration range is the path length of the flow cell.

$$A = \varepsilon_\lambda cd$$

Equation 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 for purification 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. Semipreparative 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.

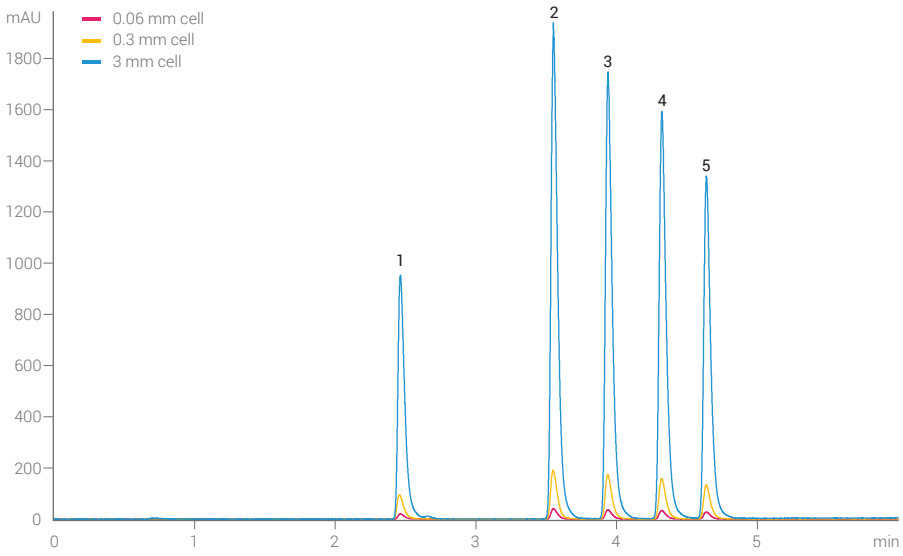


Figure 34. Flow cells with shorter path lengths reduce peak areas. 1 Caffeine, 2 Methylparaben, 3 Ethylparaben, 4 Propylparaben, 5 Benzylparaben.

7.5
Fraction collection

The difference between an analytical and a preparative LC system – regardless of flow rate or pump capacity – is determined solely by the absence or presence of a fraction collector. 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 a defined time window. 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 detector signals helps reduce the number of fractions to process and thereby increases the efficiency of the laboratory.

7.5.1 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 enables the operator to control fractionation by a simple click.

Figure 35. Agilent 1290 Infinity II Preparative Open-Bed Fraction Collector.

7.5.2 Collecting fractions based on time

A simple mode of collection is time-based fraction collection with fixed time or volume slices. In this mode, fractions are collected periodically, see Figure 36. This collection mode is often applied when purifying complex mixtures such as natural compounds from plant extracts.

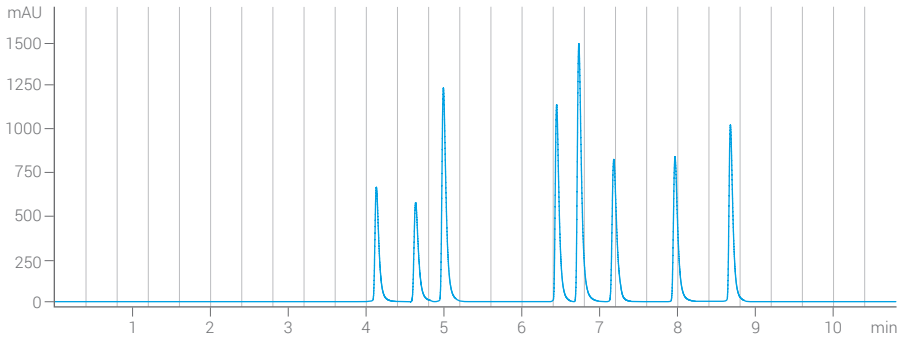


Figure 36. Time-based fraction collection: a new fraction is taken every 0.40 minutes.

7.5.3 Collecting fractions based on UV detection

When using peak-based fraction collection, compounds are collected based on a detector signal, for example, from a UV or evaporative light scattering detector. Two parameters, signal threshold and signal slope, can be used separately or combined to trigger fraction collection. The following sections explain when and how to use these parameters.

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 37.

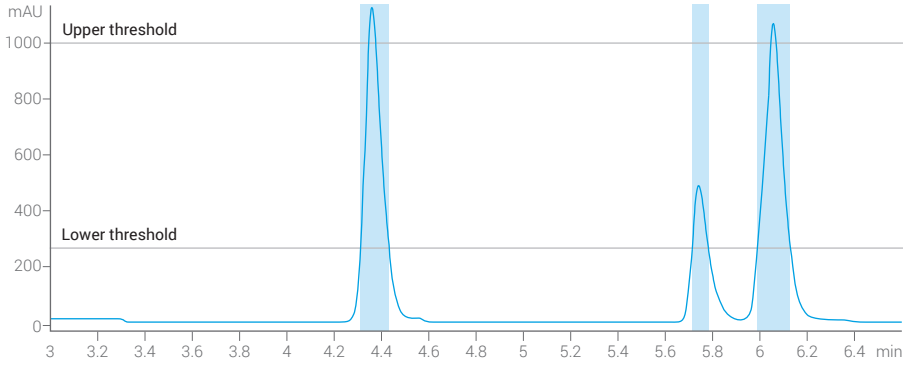


Figure 37. Fraction preview tool – setting trigger threshold and slope parameters interactively.

Setting the threshold level

If the signal intensity rises above the defined threshold level, fraction collection begins. When the signal falls below the threshold level, fraction collection stops, see Figure 38.

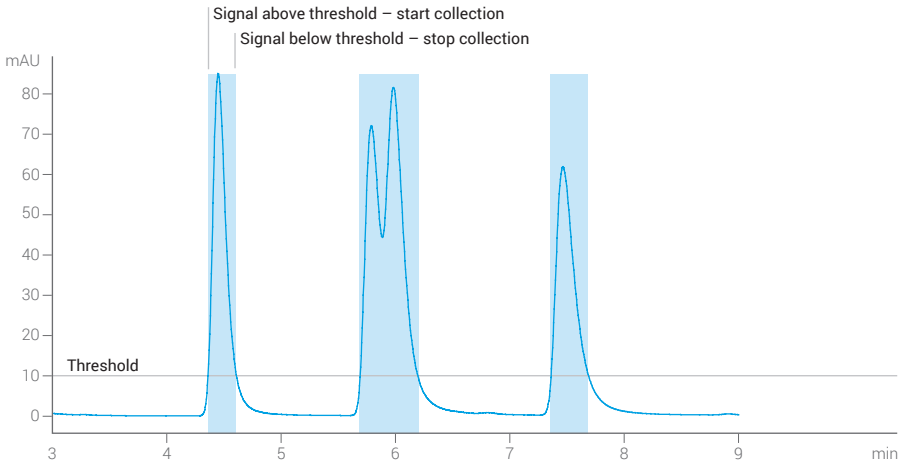


Figure 38. Fraction collection triggered by threshold setting.

Setting slope parameters

The slope of a chromatographic trace can be calculated as its first derivative and is an appropriate parameter to indicate an eluting peak. At the baseline, the signal slope is zero. When a peak elutes, the first derivative rises until the first inflection point of the peak is reached. Moving along the curve of the peak to the apex and the second inflection point, the first derivative declines and becomes negative. After the second inflection point of the peak the first derivative reaches its start value, see Figure 39.

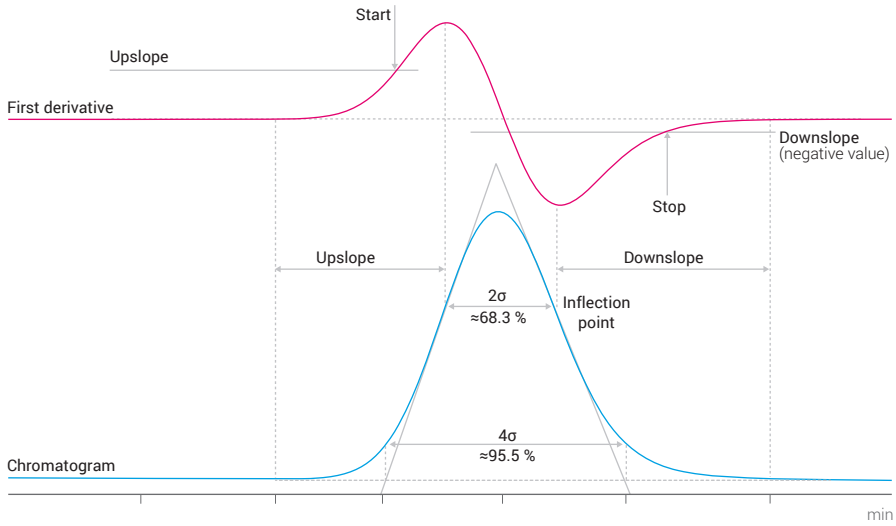


Figure 39. Slope recognition using the first derivative of the chromatographic trace.

Upslope and downslope settings for fraction collection avoid false-positive triggering by threshold when the monitored baseline rises slowly (baseline drift) due to the solvent gradient. Further, these settings can help to separate poorly resolved chromatographic peaks: the falling and rising slope will trigger a new fraction even if the signal does not drop below the defined threshold, see Figure 40.

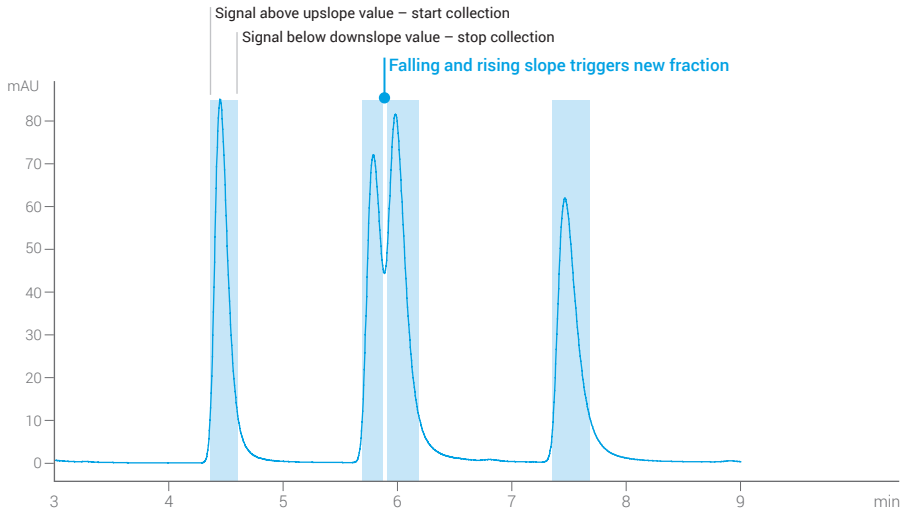


Figure 40. Fraction collection triggered by slope setting.

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: slope changes above the upper threshold are ignored by the fraction collector, see Figure 41. 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 recommend 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.

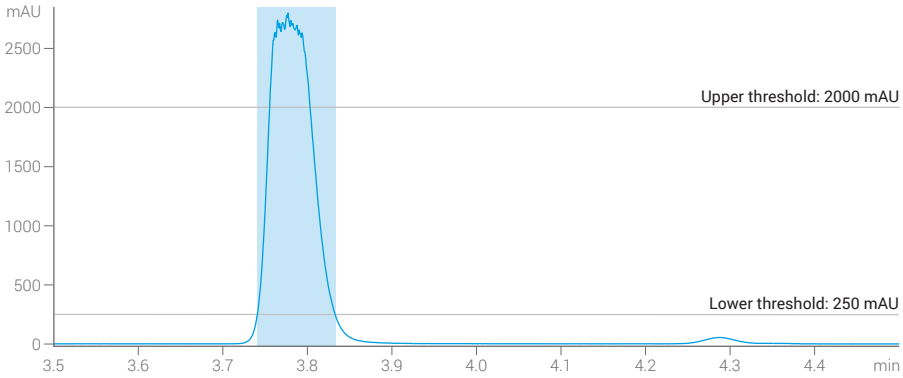


Figure 41. An upper threshold limit prevents fraction triggering caused by artificial signal fluctuations when detector saturation occurs.

7.5.4 Collecting fractions based on evaporative light scattering detection[6]

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, see Figure 42. ELS detection has proven to be 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.

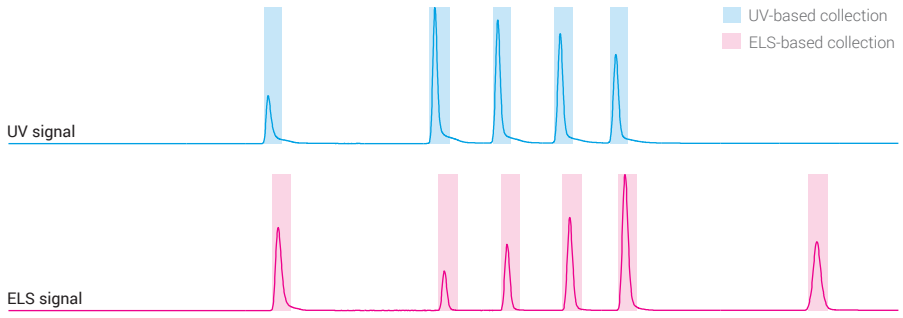


Figure 42. UV and ELS-based fraction collection. Collection was triggered on both peak detectors. The blue areas represent the fractions collected by the UV detector, and the red areas represent the fractions collected by the ELS detector.Without fraction collection triggered by the complementary ELS detection, the last compound would have been undetected and lost.

Light scattering occurs when eluents are evaporated, and solutes have ideally formed particles or droplets with residual solvent in a drying gas stream.
The dynamic range of the ELS detector must be met by appropriate flow splitting between the fraction collector and the ELS detector, see section 7.3 “Flow splitting”. Figure 43 shows the relevant flow path of a system with UV and ELS detector.

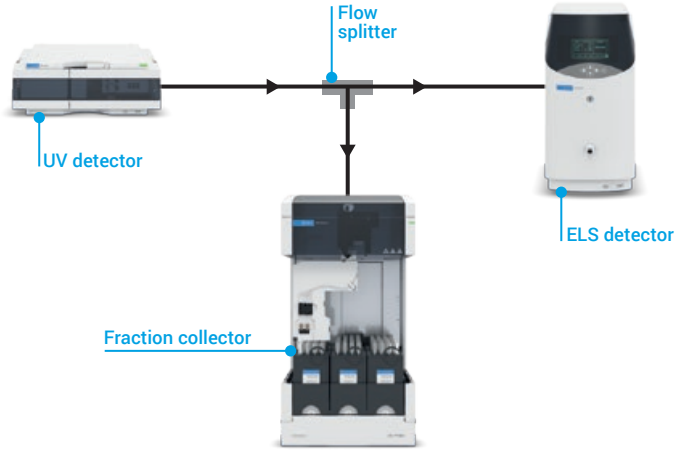


Figure 43. Schematic of a simple T-splitter when using a complementary detection technique.

7.5.5 Fraction delay

With peak-based fraction collection activated, the system needs to process the detector signals to decide whether the eluent is diverted to collection vessels or to waste, see Figure 44.

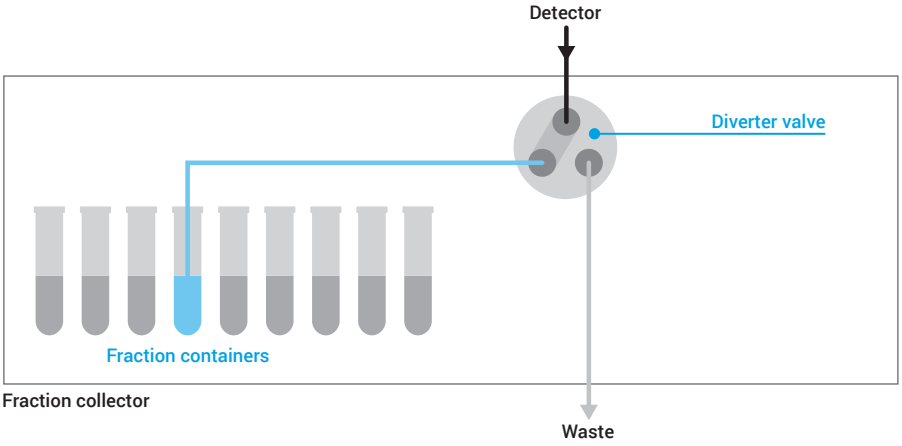


Figure 44. Schematics of a fraction collector, showing the basic functional parts.

During processing, the potential fraction travels with the main flow through tubing between the detector flow cell outlet and the diverter valve in the fraction collector. The volume of this tubing must be large enough to retain the peak of interest until the system has decided whether to switch to a collection vessel. The time required for this decision depends on the response time of the detector. In most UV-based systems, the delay volume between detector and fraction collector is larger than signal processing requires.

Knitted delay coils

In systems with a mass selective detector, additional delay volume might be required. To increase the fraction delay volume, it is not sufficient to simply increase the capillary volume between detector outlet and fraction collector inlet. Due to Taylor dispersion, the peak shape will drastically deteriorate in straight capillaries with laminar flow. As a consequence, the fraction start and end times communicated from the detector will not represent the actual peak start and end times in the fraction collector – a significant part of the fraction will be lost.

To prevent Taylor dispersion caused by the parabolic flow profile in the capillary, the flow must be turbulent. This can be achieved using knitted delay coils: the narrow coils cause the flow to become turbulent, which reduces peak dispersion to a minimum, see Figure 45.

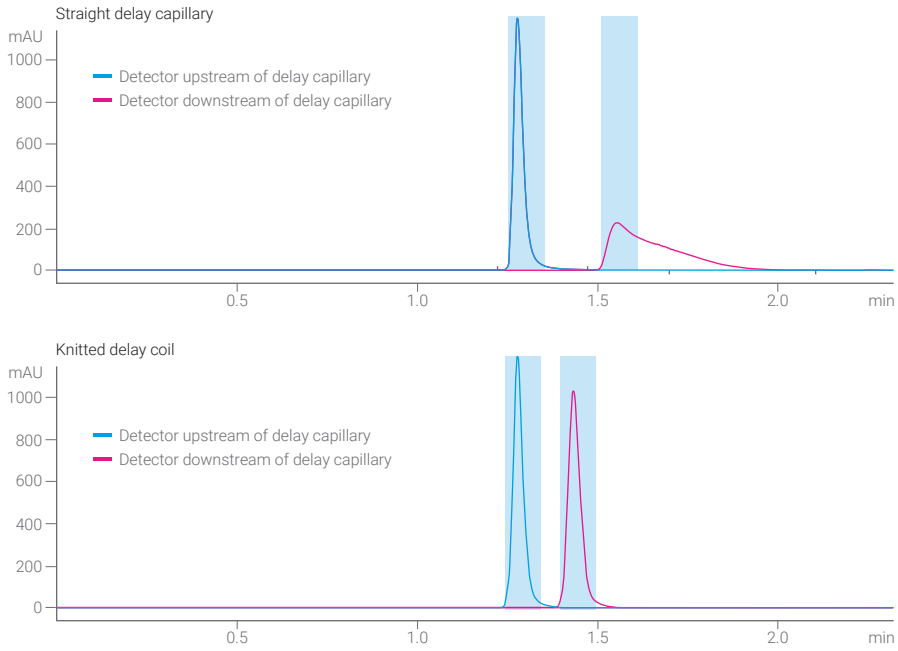


Figure 45. Comparison of a straight versus a knitted delay capillary. The blue signal represents a compound as detected by the UV detector, which is upstream of the delay capillary. The red signal traces were recorded by a second UV detector located downstream of the delay capillary, representing the same compound entering the fraction collector. The fraction trigger window defined by the first UV detector is highlighted in blue and copied to the second detector signal. Due to peak dispersion, sample loss would be >50% in case of a straight capillary.

Fraction delay sensor

The volume between detector outlet and fraction collector diverter valve must be known to the system to time the exact delay between detection and fraction collection. In most systems, this volume must be either calculated based on the capillary length or determined experimentally by visual inspection of a colored compound. Due to production tolerances and manual interaction, respectively, both methods are not accurate.

To determine the exact delay time of a peak between the detector flow cell and the diverter valve, Agilent fraction collectors are equipped with a fraction delay sensor, see Figure 46. 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.

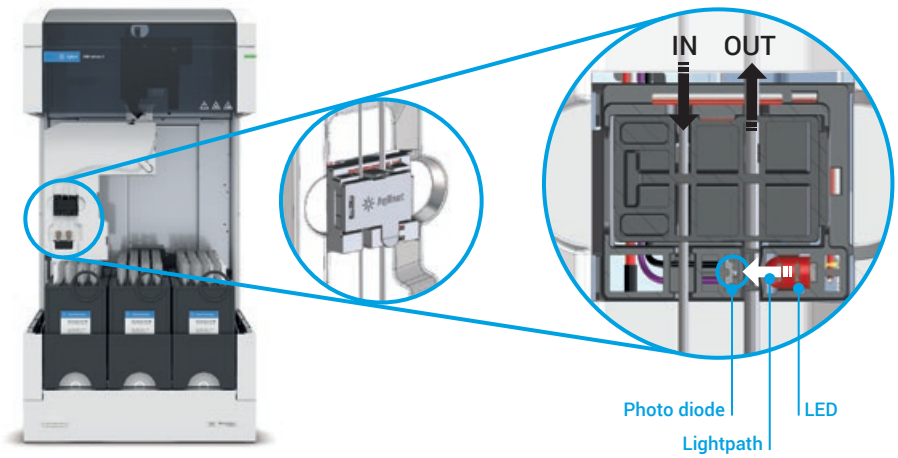


Figure 46. Location of fraction delay sensor in Agilent 1290 Infinity II Preparative Open-Bed Fraction Collector.

The measured time difference is transformed into a delay volume using the applied flow rate feedback from the pump, see Figure 47. 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 need not be repeated as long as the tubing remains the same.

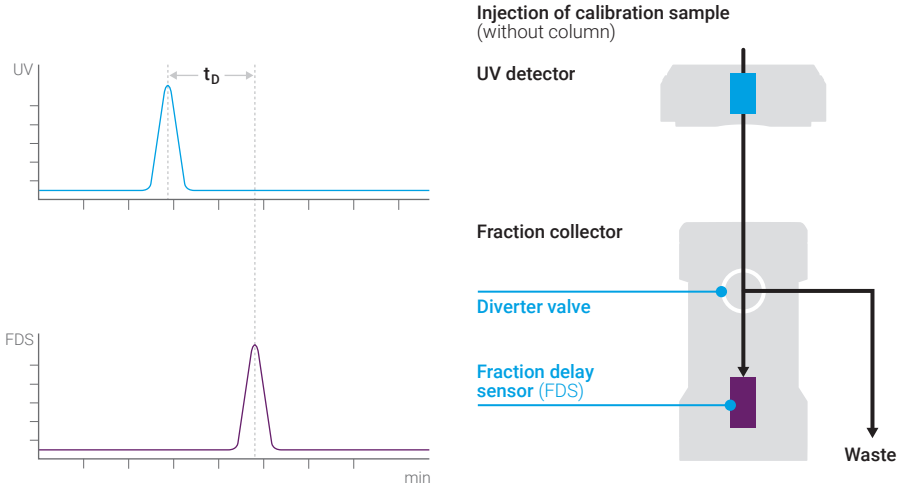


Figure 47. Time difference between detector and fraction delay sensor. The delay volume is calculated automatically by software during the calibration process.

7.5.6 Collecting fractions based on mass selective detection

Due to its specificity and selectivity, mass-based fraction triggering increases the efficiency of the purification workflow significantly. The number of collected fractions is reduced dramatically when using mass-selective triggering, making it the method of choice when the number of samples per day can no longer be handled by unspecific UV-based collection. The unequivocal characterization of the collected compounds is done instantaneously – there is no need to take aliquots of each collected fraction and submit them to a separate LC/MSD system for identification.

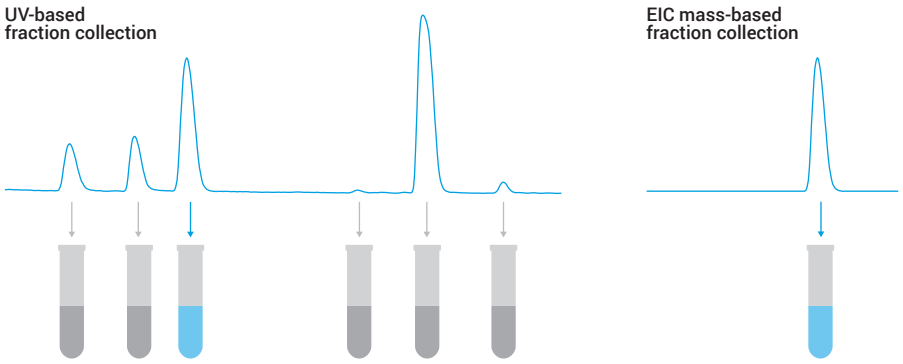


Figure 48. Mass-based purification is a selective method and reduces the number of collected fractions compared to UV-based collection.

It is common practice to combine the signals of the nonselective UV detector and the selective mass detector to trigger fraction collection. The highest purity is ensured by applying Boolean AND logic: a fraction will only be collected when both signals detect the compound. The UV signal as the principal signal is better resolved than the mass signal. When the better-resolved signal is used to control fraction triggering, the purity of the collected fractions will increase. The selectivity factor is added by the mass selective detector signal.

The mass of the target compound can be obtained from analytical LC/MSD results or predicted through synthesis planning. This enables triggering of fraction collection with a mass selective detector (MSD) with unmatched productivity. Unless multiple isomers occur and elute at different times this approach results in a 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 (Figure 49) has to be assured under the chromatographic conditions to make the target detectable with a mass selective detector. The use of monoisotopic (not average) masses is crucial for successful triggering. The software should allow for 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 MSD triggers fraction collection. When a combination of UV or ELS detectors is used with the MSD, the delay time of each has to be considered for synchronization of the detector signals. The fraction delay sensor in Agilent fraction collectors facilitates the measurement of these different delay volumes and ensures maximum purity and recovery.

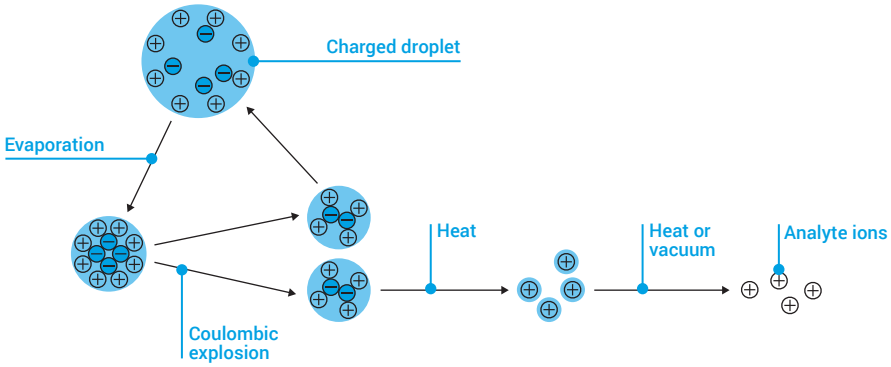


Figure 49. The electrospray Ionization process. After evaporation of the solvent the charge density inside the droplet is increased until free ions are ejected into the gas phase. The ionization process is affected by the volume of eluate that has to be vaporized and the concentration of buffers or matrix compounds within the droplets, which suppress the ionization process.

Figure 50 shows the time difference between UV and mass-selective detectors, and the fraction delay sensor. The delay time for MSD triggering is calculated by software 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 MSD 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 depends on the make-up solvent used. A mixture of 70 % methanol or acetonitrile, 29.9 % water, and 0.1 % formic acid is a good starting point for electrospray ionization.

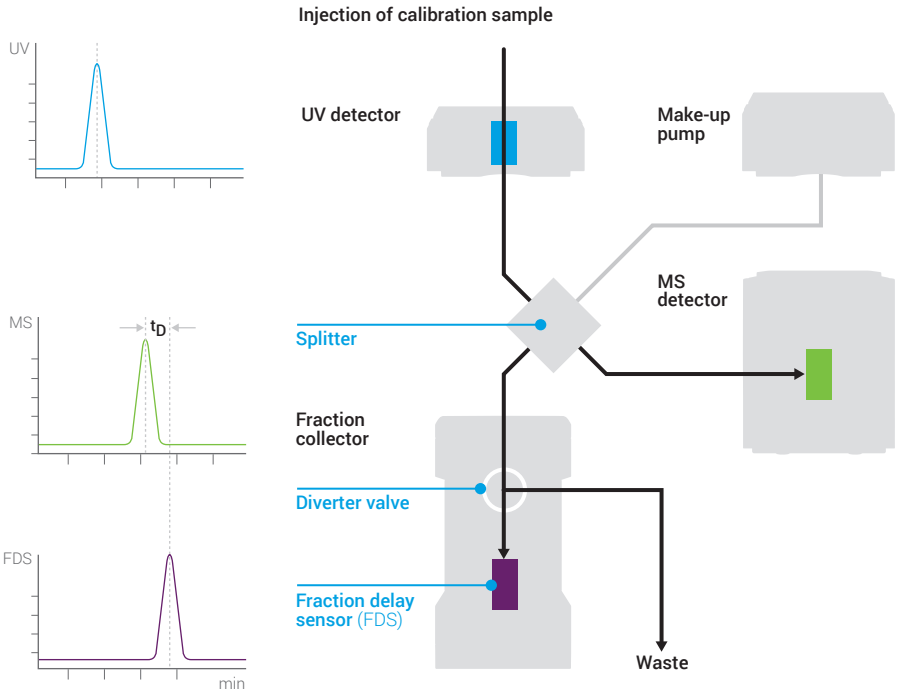


Figure 50. Time difference between UV and MSD detectors, and fraction delay sensor.

It is characteristic of the molecule itself whether negatively or positively 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. Strong modifiers such as trifluoroacetic acid (TFA) suppress negative ionization but also distort positive ionization because of ion-pair formation with the analyte itself.

Ion formation in solvents such as water and methanol are highly efficient. The less polar the solvents are, the less efficient 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 isopropyl alcohol 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 common practice to use only the positive ionization mode in LC/MSD, 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 modifier). In this case, complementary and more reliable information about the sample is received, as shown in Figure 51.

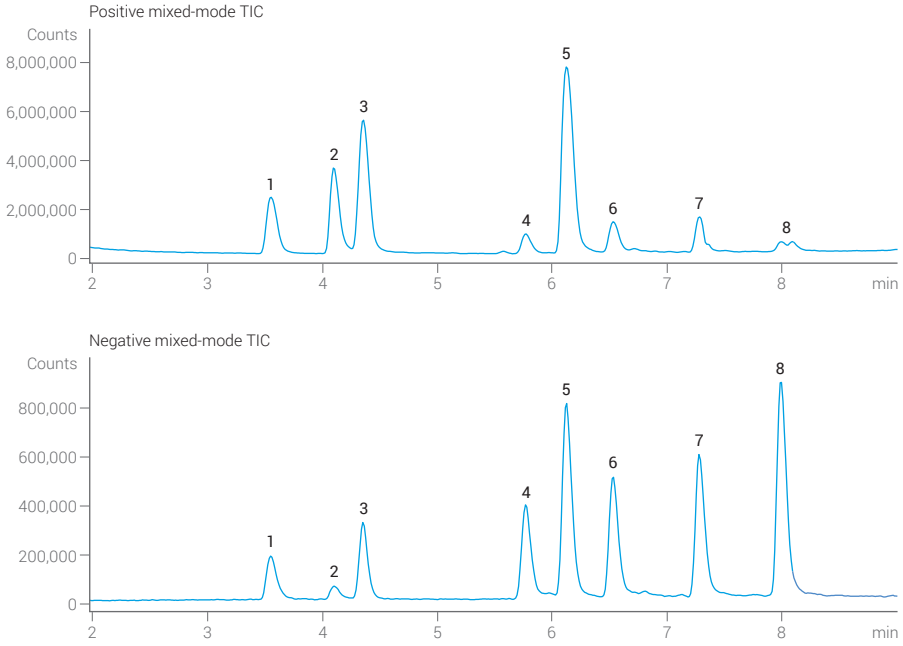


Figure 51. Dual ionization mode delivers complementary and reliable information. 1 Acetaminophen, 2 Caffeine, 3 Sulfamerazine, 4 Methylparaben, 5 Sulfadimethoxine, 6 Ethylparaben, 7 Propylparaben, 8 Benzylparaben.

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 52, the third peak represents the target compound, sulfamerazine (M = 264 g/mol).

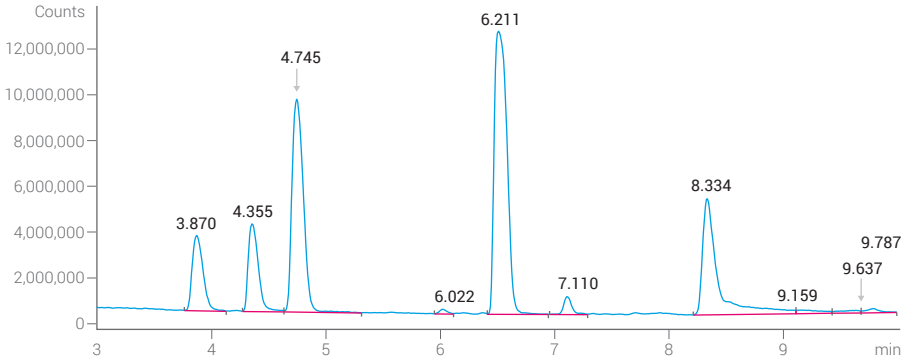


Figure 52. TIC chromatogram, no specific compound information is obtained.

When the molecular mass for sulfamerazine is entered, an extracted ion chromatogram (EIC) is generated in real time in the background, see Figure 53. 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 must exceed the threshold level of this signal.

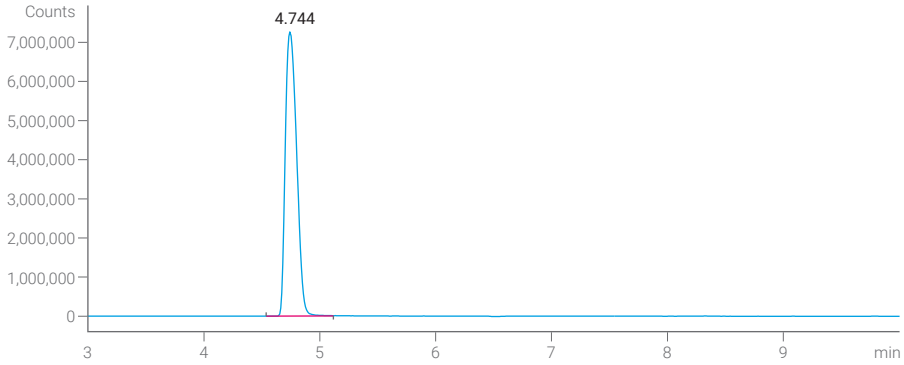


Figure 53. Selective extracted ion chromatogram for the target compound at m/z = 265.

7.5.7 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.

7.6 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, some 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 55. Preferably, there should be a separate location for each sample to collect what is expected to be waste. The recovery position must be reported together with the sample information. This allows to assign the recovery collection to the collected fractions and the submitter of the sample.

Figure 54. 1290 Infinity II Preparative Open-Bed Fraction Collector with 1260 Infinity II Preparative Valve-Based Recovery Collector.

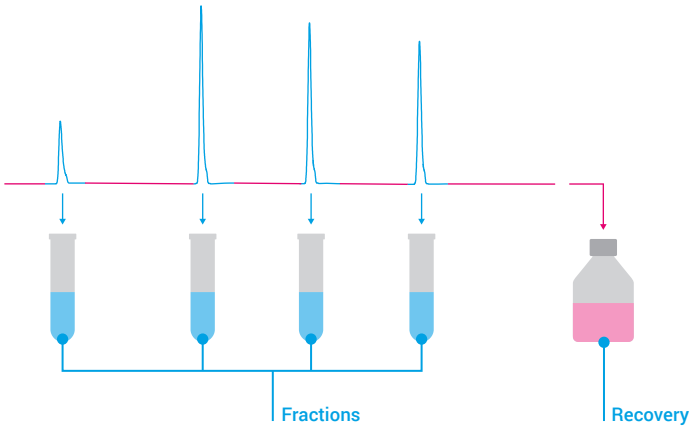


Figure 55. Recovery collection – the volume of solvent not collected by the main fraction collector is captured by the recovery collector.

7.7 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 variable sample sizes is of high priority. However, upscaling 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. Installing capillaries with a larger inside diameter helps to deal with the backpressure caused by higher flow rate. These two steps would appear to be logical to adapt an analytical LC system for preparative work. However, these measures can severely impact the separation efficiency of your application.

7.7.1 System dwell volume and column void volume[7]

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 9.2 “Determining the system dwell volume” and section 9.3 “Determining the column void volume”.

Figure 56 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, and 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. Chromatographic results with low resolution can only be improved by reducing the dwell volume.

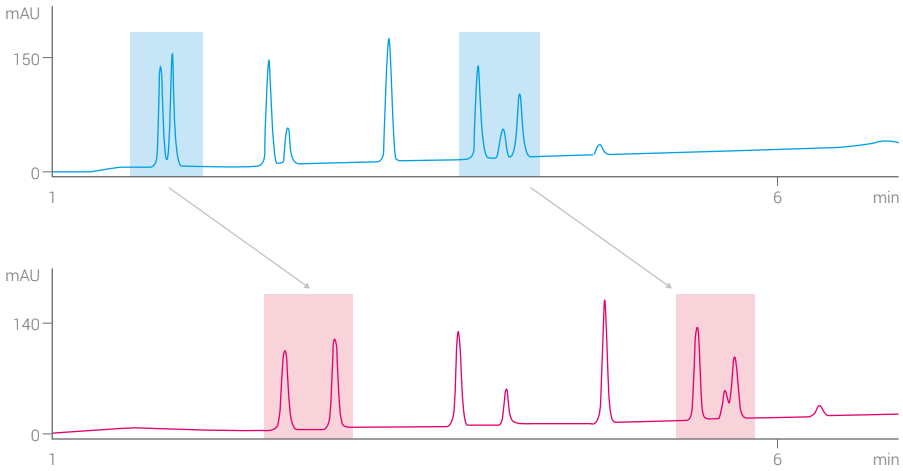


Figure 56. The effects of system dwell volume on separation efficiency. The upper chromatogram was obtained using a system with low dwell volume. In contrast, the lower chromatogram was obtained on a system with large dwell volume.

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 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 56. The volumes 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 for analytical- and preparative-scale work. Further, the correct capillary diameter for the applied flow rate must be used to reduce systems void volumes and minimize peak dispersion. Table 3 gives an overview of typical capillary inside diameters, their recommended flow range, and the resulting backpressure.

Table 3. Recommended inside diameters of stainless-steel capillaries for different flow rates (*backpressure calculated using methanol/water 1:1 as solvent).

Color	Inside Diameter [inch]	[mm]	Recommend Flow [mL/min]	Void Volume [µL/cm]	Calculated Backpressure [bar] per Meter at Maximum Recommended Flow*
Black	0.003	0.075	0.1–0.5	0.044	196
Red	0.005	0.127	0.2–1.5	0.127	72
Green	0.007	0.178	0.8–3.0	0.249	37
Gray	0.012	0.30	4–8	0.707	10
White	0.02	0.50	15–40	2.027	8
—	0.024	0.60	40–80	2.83	6
—	0.037	0.94	80–200	6.94	5

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 57. In addition to the volume of the inside of the column, the porosity of the particles has to be accounted for. Agilent ZORBAX SB-C18 columns have a porosity factor of 0.53, see Table 4.

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. Procedures on how to determine system dwell and column void volumes are described in sections 9.2 “Determining the system dwell volume” and 9.3 “Determining the column void volume”, respectively.

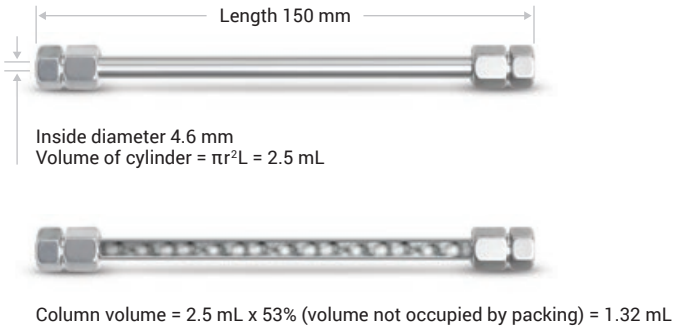


Figure 57. Mathematical determination of column void volume.

Table 4. Column void volume for different Agilent ZORBAX SB-C18 columns. The porosity of ZORBAX SB-C18 material is 0.53.

Inside 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

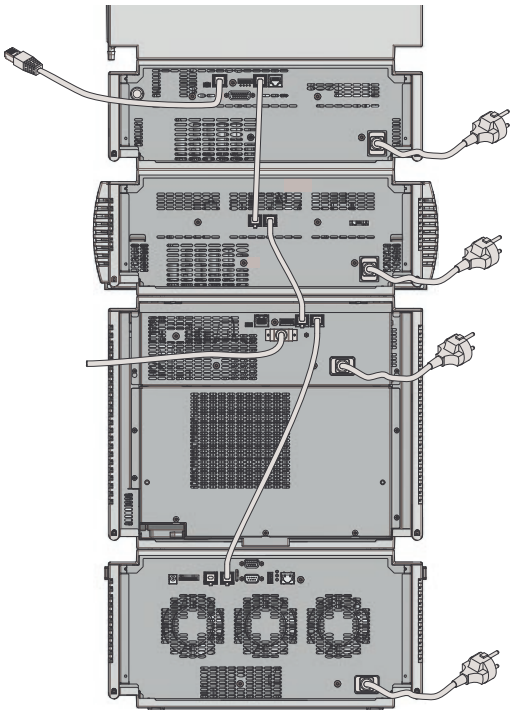


Figure 58. CAN communication lines – fast direct communication between the modules is independent of the host software.

7.7.2 System communication

Agilent LC systems use a controller area network (CAN), see Figure 58. Peak trigger assessment processes and the simultaneous monitoring of different signal traces create a heavy load on workstations, especially when data-intensive devices such as diode array detectors and mass selective detectors are involved. Activities such as virus scans or software update/download can interfere with fraction collection processes. Agilent CAN communication, however, operates independently of the Windows-based software, providing a direct, rapid, and robust communication line between the different system modules. Once a sample has been submitted, fraction collection will be successfully triggered even if the connection between PC and system is disrupted. Further, purification systems with CAN communication calculate the delay time between the UV detector and the fraction collector based on the actual flow rate transmitted by the pump.

7.7.3 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 59. 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 60.

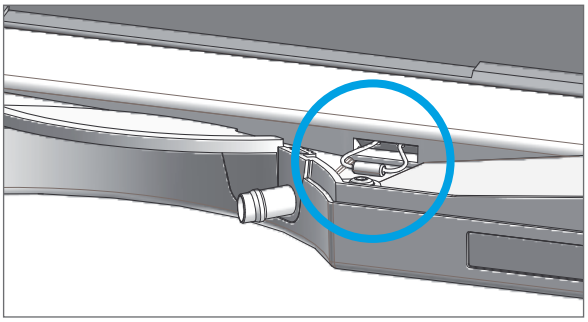


Figure 59. Leak sensor and leak management, showing drainage system to lead leaking solvent to waste containers.

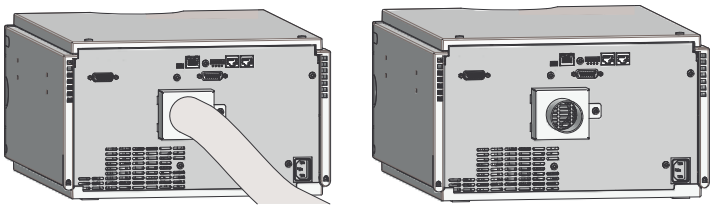


Figure 60. Adapter on rear of fraction collector for forced-fume extraction.

8 Strategies for Scale-up[8-10]

In this chapter, we describe scale-up from a 4.6 × 150 mm analytical column to a 21.2 × 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.

8.1 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 whether 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 × 150 mm, 5 µm column. This flow rate is the starting point for all further scale-up calculations.

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 61 shows the gradient profile of an analytical scouting run. Figure 62 shows the gradient profiles after transferring a method from an analytical system to a preparative system where the ratio of dwell volume to flow rate is smaller. Isocratic holding steps need to be applied to compensate the differences between both systems. The linear method transfers from an analytical to the preparative system were accomplished using the formulas in section 8.2 “Formulas for linear scale-up from analytical to preparative columns”.

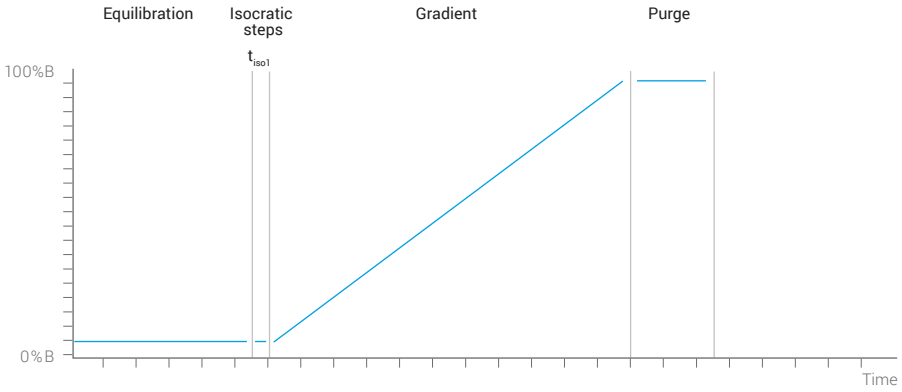


Figure 61. Gradient profile for an analytical scouting run using a 4.6 mm id column, comprising injection, optional isocratic hold, gradient slope and purge phases.

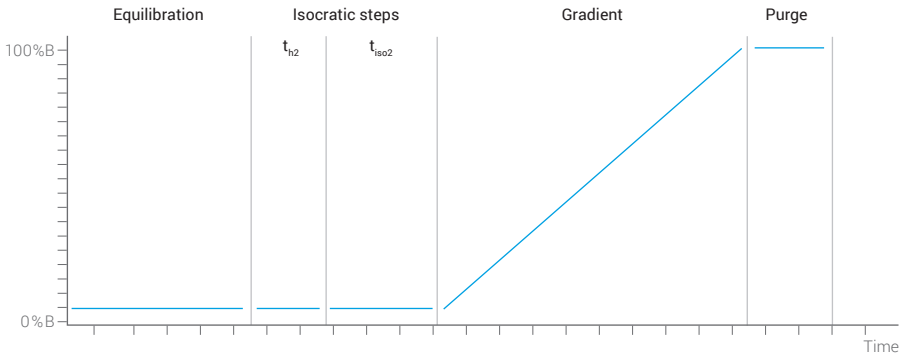


Figure 62. Gradient profile after scale-up to a 21.2 mm id column, comprising an extended isocratic step to compensate for the applied flow rate with respect to the difference in dwell volumes between the analytical and preparative system.

8.2 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 3.

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

Equation 3. 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 are fulfilled.

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

Equation 4. 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 5.

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

Equation 5. 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
$V_{c,P}$ Column void 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 6.

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

Equation 6. 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 63 shows the results of a method transfer after applying the equations in this section.

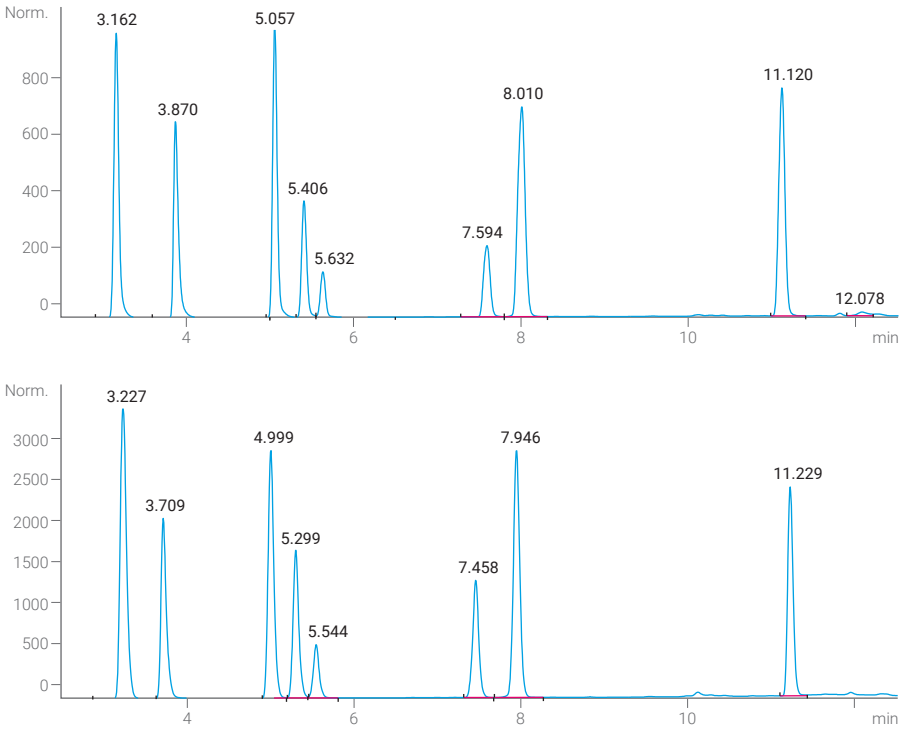


Figure 63. Results after linear scale-up from a 4.6 × 150 mm, 5 µm analytical column to a 21.2 × 150 mm, 5 µm preparative column. Retention times on both columns are similar. Upper chromatogram: Preparative run with 500 µL injection and 31.8 mL/min flow. Lower chromatogram: Analytical run with 5 µL injection and 1.5 mL/min flow.

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

8.3 Increasing efficiency through focused gradients[11–14]

To obtain maximum efficiency the column load needs to be maximized and the run time 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.

8.3.1 The concept of focused gradients

The resolution between groups of adjacent peaks can be increased 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 key for a robust separation method. The starting conditions in a focused gradient profile are therefore derived from the polarity of the target compound. At the same time, all compounds with higher polarity than the target compound need to be retained, which dictates as low a concentration of the organic solvent B as possible. Depending on the most polar compound in the sample, starting conditions of a focused gradient can vary, see Figure 64. After an initial isocratic hold, a steep gradient step can be used to ramp up to the starting point of the focused gradient for each zone. The focused gradient profile, however, is shallow to achieve optimum separation efficiency for the target compound and close eluting impurities.

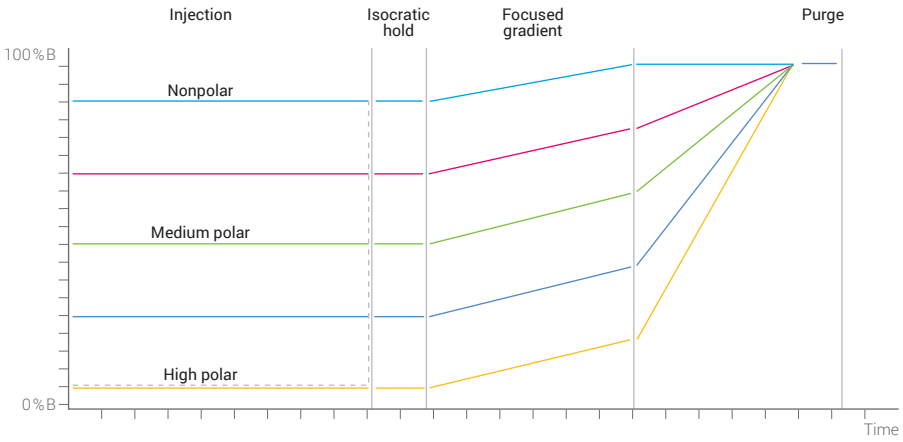


Figure 64. Focused gradient profiles for target compounds with different polarities. The dashed line represents an alternative step-gradient profile.

For nonpolar 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 to 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.

8.3.2 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 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 ramps up to five 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.

8.3.3 Simplified approach to creating focused gradients by Agilent Automated Purification Software

OpenLab CDS ChemStation and Automated Purification Software[16]

The Agilent OpenLab Chromatography Data System (CDS) facilitates complete control of purification processes. The software's interface simplifies method development as well as scale-up from analytical- to preparative-scale purification. Time, peak, and mass-based fraction collection – or any combination of these – are available and can be triggered by any detector signal. Intelligent real-time data processing facilitates instantaneous and precise fraction collection. Methods can be transferred from 4.6 to 21.2 mm ID columns after the determination of the dwell volumes using the scale-up equations. Retention times of all compounds on both chromatograms are similar.

Agilent Automated Purification Software (an add-on for OpenLab CDS ChemStation Edition) facilitates the automated transfer of purification methods from analytical to preparative scale. The add-on automates the transfer of data between process steps, streamlining your workflow. Algorithms calculate focused gradients on-the-fly for each target compound, ensuring highest purity of fractions collected during preparative-scale purification runs.

With a few clicks, you can select the desired combination of analytical and preparative columns, upload and process the analytical results, launch the purification run, and review the purification results. Full access to the entire functionality is available through the Expert mode, which also provides for configuration of preset methods for occasional users.

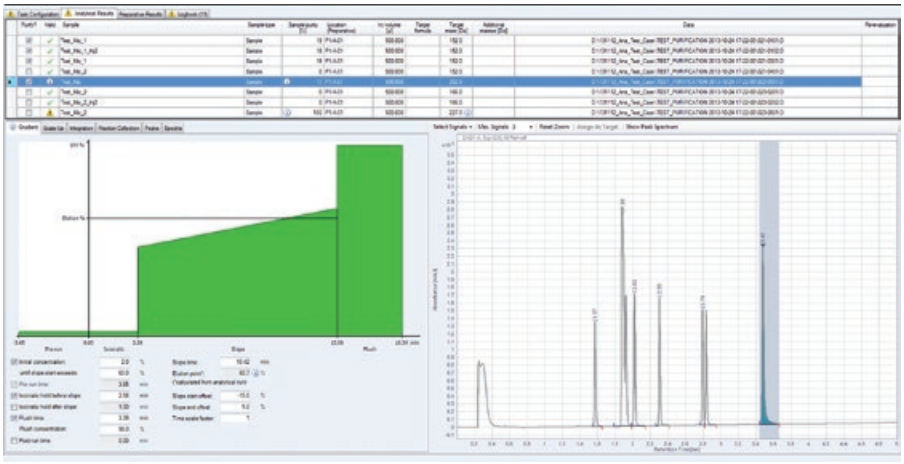


Figure 65. Calculated gradient profile of the detected target compound highlighted in the analytical chromatogram.

Automated Purification Software also supports a fully automated purification workflow. You can use *.txt or *.csv format for data import. The program facilitates the generation of sequence tables for analytical scouting. Furthermore, it provides automated target compound confirmation and calculates a focused gradient on-the-fly for the purification step, ensuring an optimized resolution and minimizing run time. With the Fractions Result Browser, the position of the collected fractions is quickly and clearly displayed. The purity of each collected fraction can be checked by reviewing UV and mass spectral data from the browser screen. A fraction selection tool permits the export of pure fractions to a liquid handling system, or the generation of a new sequence table for fraction reanalysis within a few clicks.

8.4 Describing the entire scale-up workflow[15]

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 66. The spreadsheet-based scale-up calculator from the University of Geneva helps to address the calculations.

It is available as a free download at:
https://ispso.unige.ch/labs/fanal/hplc_calculator:en (accessed April 1, 2019)

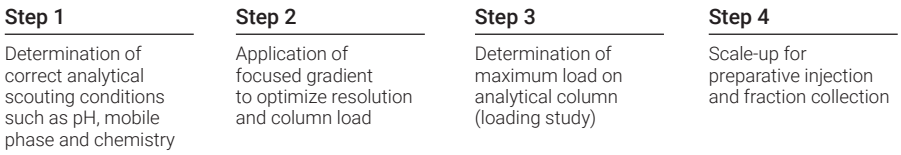


Figure 66. Four-step, scale-up process.

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 7.2.3 “Special Injection Techniques”.

8.4.1 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 × 50 mm UHPLC or HPLC column. The target compound should have a retention factor, k, between 2 and 9. 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 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.

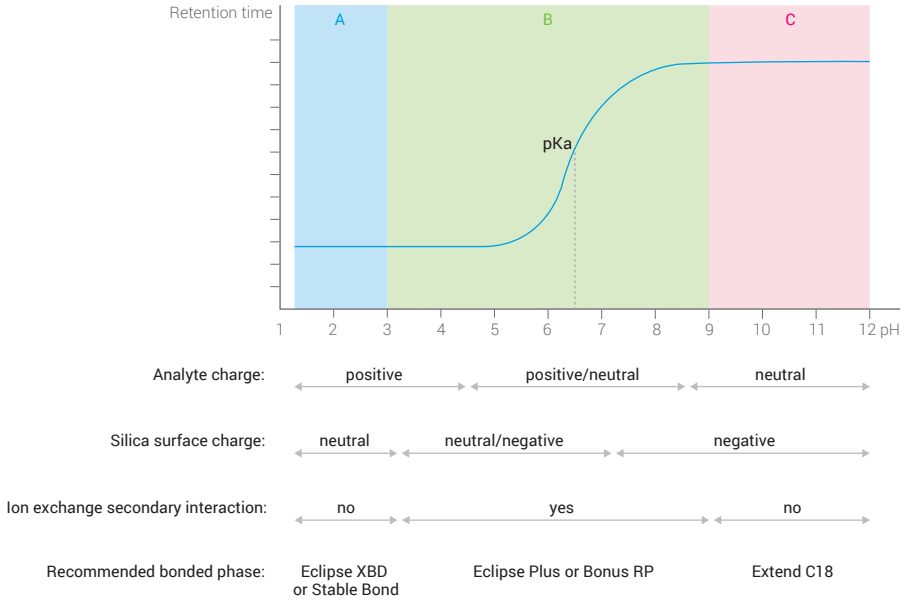


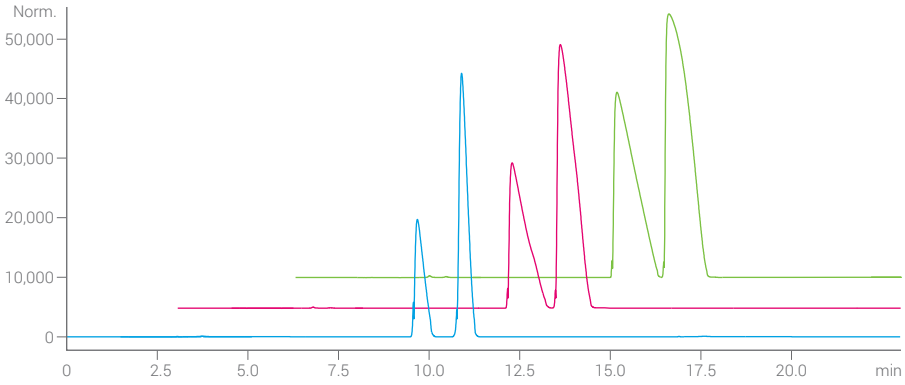
Figure 67. Recommended chemistries for different pH ranges. ZORBAX SB C18 columns and ZORBAX Extend C18 columns facilitate scale-up from 4.6 to 21.2 mm id at low pH and high pH conditions, respectively.

8.4.2 Step 2 – Applying a focused gradient

As described in Chapter 7 “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 calculate the preparative method based on the scouting run and the dimensions of the preparative system and column.

8.4.3 Step 3 – Determining the maximum column load

Column load is a critical factor for a successful purification. 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 by volume overload when injecting too much strong solvent such as DMSO. Figure 68 shows the results of a loading study, in which the column load is increased until the limit of resolution is reached.



Column: Agilent Prep-C18 Scalar, 4.6 × 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

Figure 68. Results of loading study – the column load is increased until the limit of resolution is reached.

By applying a focused gradient on the analytical column, the injection volume is increased in steps of 5 µL 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 using the column scale-up equation (see equations in section 8.2 "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.

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

Figure 69 shows the chromatogram after the scale-up calculation. By applying the formulas from section 8.2 "Formulas for linear scale-up from analytical to preparative columns", the preparative chromatogram is exactly predictable. Both compounds are baseline separated and have been collected with a purity of greater than 95 %.

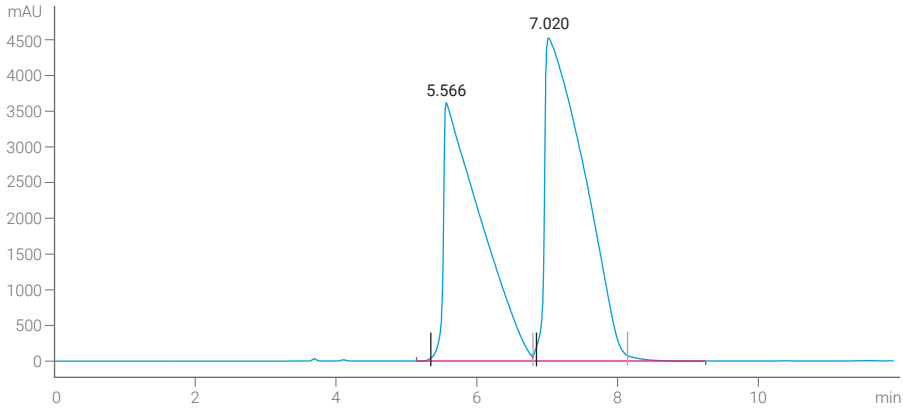


Figure 69. Preparative-scale chromatogram. The tick marks indicated the collected fractions. Based on the results from the loading study on the analytical 4.6 mm id column, the method has been scaled-up matching the flow rate, gradient profile, and injection volume for a 50 mm id column. This chromatogram shows the separation of the two target compounds after 4500 µL have been injected.

9 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.

9.1 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:

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

V_{col}	Volume of the column
id	inner diameter
π	3.14159265359
L_{bed}	packed bed length

3. Calculate the amount of media required:

$$m = V_{col} \times \rho$$

ρ	density
m	mass
V_{col}	Volume of the column

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

$$\rho = \frac{m}{V}$$

ρ	density
m	mass
V	Volume

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 that the media fully disperses and is free of lumps, the packing slurry should be shaken or bottle-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 silica media in a benchtop Load & Lock column is 30,000 plates/meter.

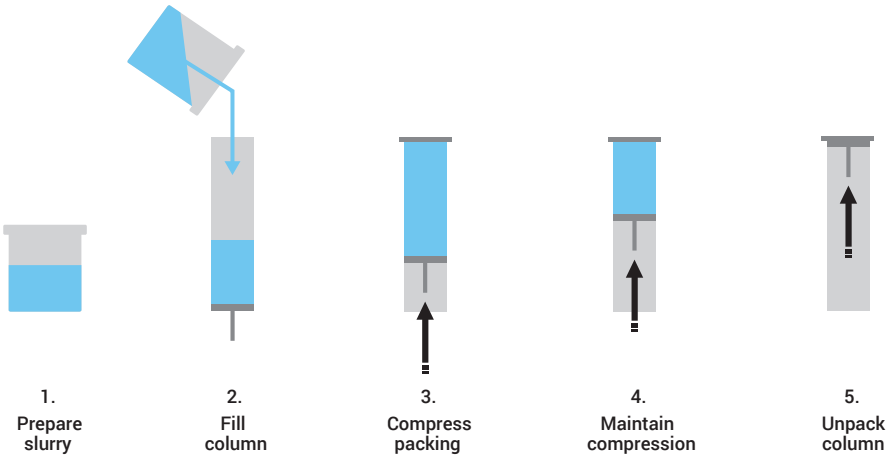


Figure 70. Basic packing and unpacking procedure for DAC/SAC columns.

Table 5. Required resin aliquot sizes when packing DAC/SAC columns. For polymer-based columns, please consider the much lower pressure limits.

Inside Diameter [mm]	Length [mm]	Volume [mL]	Amount of Sorbent [g] (Silica-Based ρ = 0.60 g/mL)	Amount of Sorbent [g] (Polymer-Based ρ = 0.33 g/mL)
27	100	57.28	34.37	18.90
	250	143.20	85.92	47.25
50	100	196.43	117.86	64.82
	250	491.07	294.64	162.05
75	100	441.96	265.18	145.85
	250	1104.91	662.95	364.62

9.1.1 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 7. The number of theoretical plates should exceed 30,000 per meter.

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

Equation 7. 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.

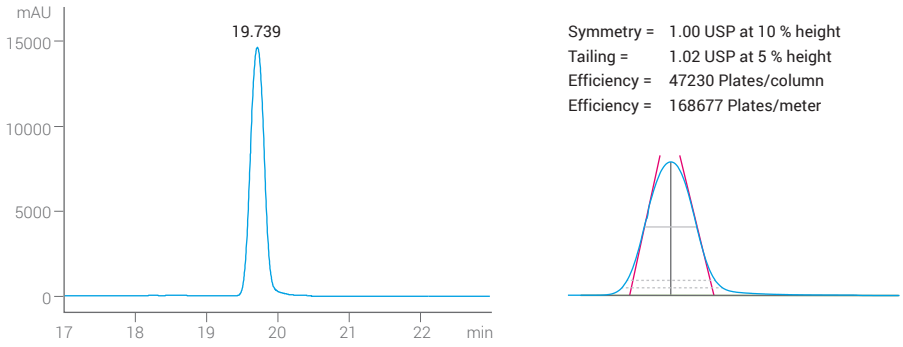


Figure 71. Injection of 1 % toluene in acetonitrile to determine the chromatographic plate number.

9.1.2 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. 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.

9.2 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.

9.2.1 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.

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 $v_{dwell} = t_{dwell} \times f$

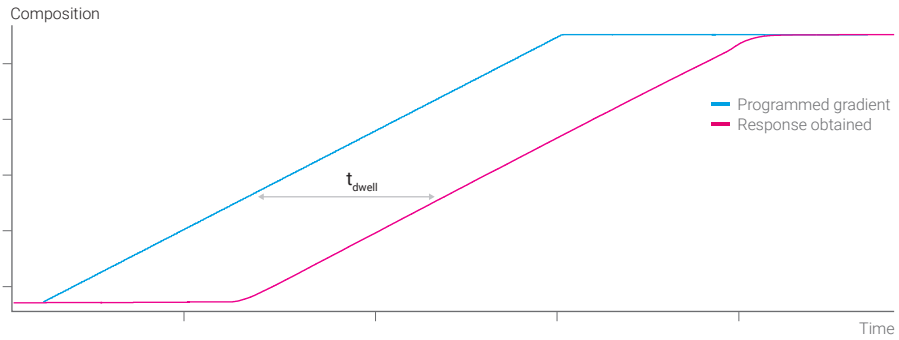


Figure 72. System dwell volume determination for analytical systems.

9.2.2 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" id (0.12 mm id) × 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 $v_{dwell} = t_{dwell} \times f$

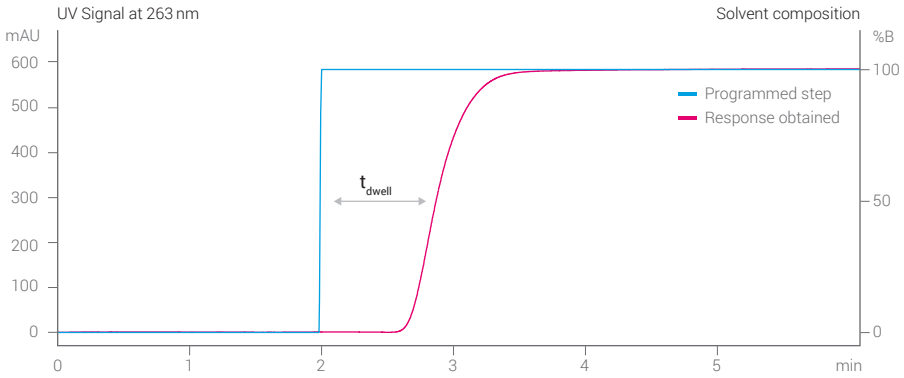


Figure 73. System dwell volume determination for preparative systems.

9.3 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.

9.3.1 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.

9.3.2 Advanced procedure for column void volume determination

The following chemicals are required for both standalone 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. Set up 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 screen:
   - 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 three 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 × 50 mm column is (3.14 × 2.3 × 2.3 × 50) / 1000 = 0.83 mL, giving a flow of (1/2 × 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

9.4 Equilibrating the column and optimizing the flow rate

9.4.1 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.

9.4.2 Optimizing the flow rate

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

The answer is not a simple number, but rather the ratio between the total system void volume and the flow rate. As a rule of thumb, good results can be achieved with a ratio of 2:1. This means that the flow rate can be significantly reduced when UHPLC systems with 2.1 mm ID columns are used for scouting. The larger the total system void volume (including column), the higher the required flow rate to achieve high-quality results. Agilent tested this rule experimentally on a combined analytical and preparative 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 74. In two further experiments we increased the flow rate to 1.5 and 2 mL/min, see Figure 75 and Figure 76.

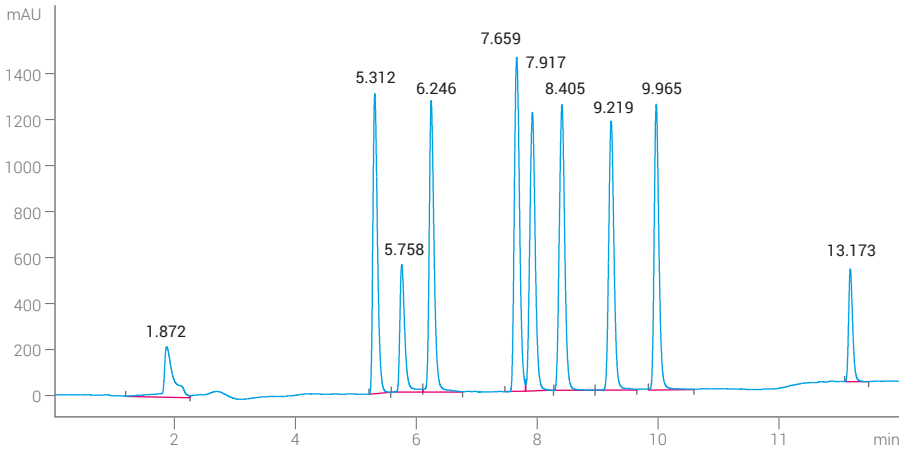


Figure 74. Chromatogram at 1 mL/min (4.6 × 150 mm, 5 µm column) for determination of optimum flow rate.

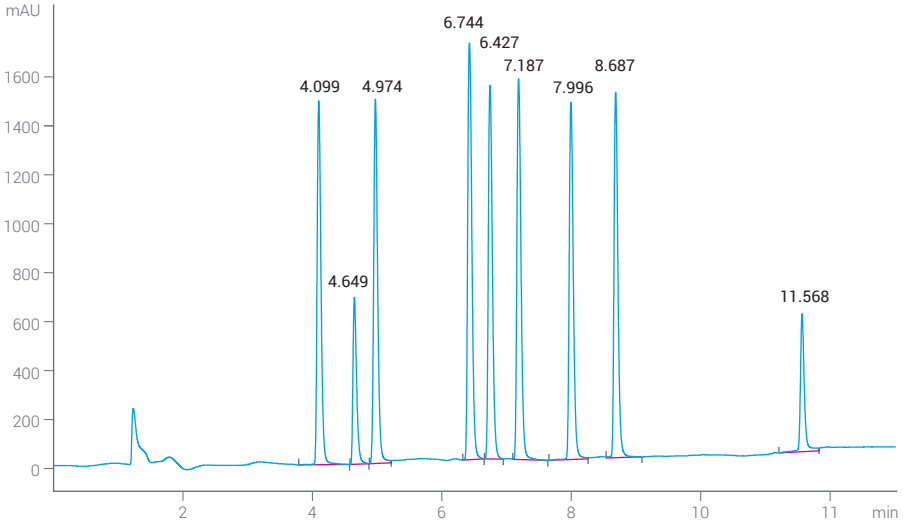


Figure 75. Chromatogram at 1.5 mL/min (4.6 × 150 mm, 5 µm column) for determination of optimum flow rate.

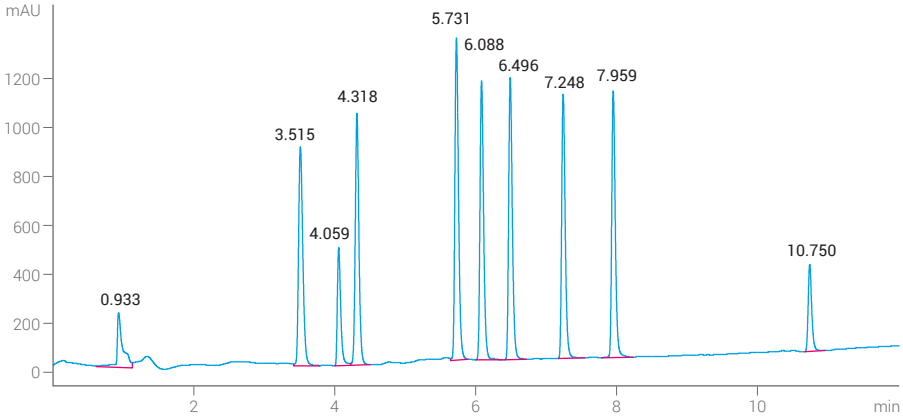


Figure 76. Chromatogram at 2.0 mL/min (4.6 × 150 mm, 5 µm column) for determination of optimum flow rate.

After the chromatograms were acquired, the resolution between the peaks at 7.659 and 7.917 minutes in Figure 74 and the number of theoretical plates were calculated. The highest plate number and resolution were achieved at a flow of 1.5 mL/min. Table 6 summarizes all the results.

Table 6. 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.

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

Comparing run time and solvent consumption

When comparing run time, solvent consumption and peak width in the above mentioned experiment, a flow rate of 1.5 mL/min gives best results, see Table 7. 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 required sample throughput demands reduced run time, the increased solvent costs must be accepted.

Table 7. Effects of different flow rates on resolution and run time – based on results obtained from system used for measurements.

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

9.5 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 the solubility of impurities or a target compound conflicts 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.

9.5.1 Volume overload

Figure 77 shows the chromatogram obtained from a 4.6 × 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.

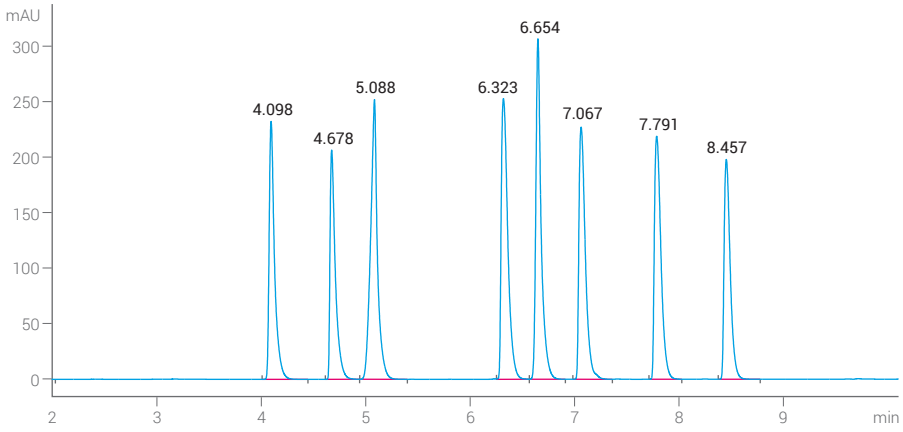


Figure 77. Injection of 10 µL of sample mix in DMSO on a 4.6 by 150 mm column. All components are separated and exhibit good peak shape.

Figure 78 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.

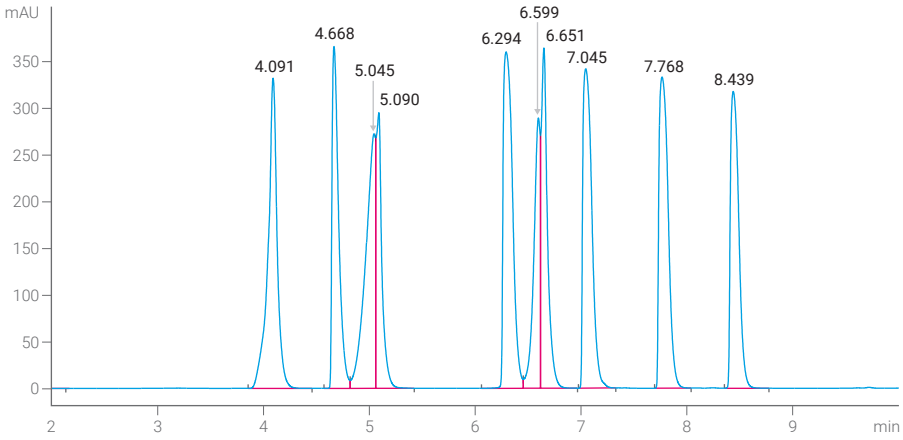


Figure 78. A 20 µL injection of the same sample shows strong fronting and peak splitting on two of the three basic compounds.

Figure 79 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.

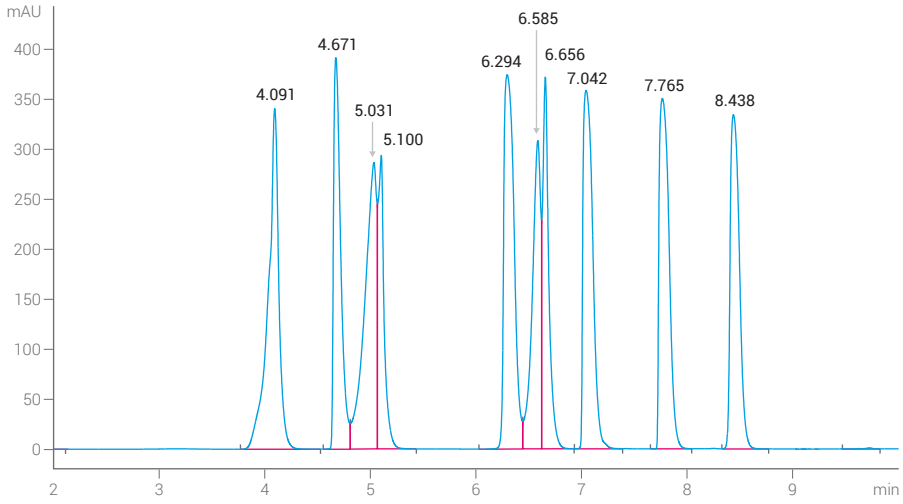


Figure 79. A 30 µL injection shows even more serious effects, as seen on the basic compounds eluting around 4 min, 5 min, and 6.5 min.

9.5.2 Mass loading of the column

Table 8 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 can be tolerated. For simple or high-throughput separations, on the other hand, column loads weighing up to 5 % as much as the stationary phase may be used.

Table 8. Throughput calculations for preparative LC columns. Stationary phase density: 0.6 g/mL

Diameter [mm]	4.6	10.0	21.2	30	50
Length [mm]	150	150	150	150	150
Volume [mL]	2.49	11.78	52.97	106.07	294.64
Flow [mL/min]	1.5	7.09	31.86	63.8	177.22
Approximate sorbent required [g]	1.5	7.07	31.78	63.64	176.79
Very high efficiency 0.1 % load [g/run]	0.001	0.007	0.032	0.064	0.177
Load and efficiency 1 % load [g/run]	0.015	0.071	0.318	0.636	1.768
Throughput oriented 5 % load [g/run]	0.075	0.353	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	6.8	30.5	61.1	169.7
Throughput [kg] 365 days/year, 24 hours/day	0.524	2.477	11.136	22.3	61.945

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 × 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 injecting not more than 2000 µL of DMSO. The most limiting factor is the solubility of the compounds.

Table 9 shows recommended injection volumes of DMSO for different column diameters and lengths. All recommendations are based on experience and lab experiments.

Table 9. Amounts of stationary phase for different column sizes and recommended sample load by mass.

Column dimensions	Injection volume [µL]	Stationary phase [g] with density 0.6 g/mL	Column load [mg] 0.1 %	Column load [mg] 0.5 %	Column load [mg] 1.0 %
2.1 × 50 mm, C18	1.75				
4.6 × 150 mm, C18	25				
10 × 100 mm, C18	79	5	5	24	47
10 × 150 mm, C18	118	7	7	35	71
10 × 250 mm, C18	197	12	12	59	118
21.2 × 100 mm, C18	354	21	21	105	210
21.2 × 150 mm, C18	531	32	32	160	320
21.2 × 250 mm, C18	885	53	53	265	530
30 × 100 mm, C18	706	42	42	210	420
30 × 150 mm, C18	1063	64	64	320	640
30 × 250 mm, C18	1772	106	106	530	1060
50 × 100 mm, C18	1969	118	118	590	1180
50 × 150 mm, C18	2953	177	177	885	1770
50 × 250 mm, C18	4922	295	295	1475	2950

If larger volumes need to be injected, we recommend using the organic-phase injection method, see section 7.2.3 “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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