# Principles in preparative HPLC

A Primer

Agilent Technologies

# Principles in preparative HPLC

Udo Huber
Ronald E. Majors

# Principles in preparative HPLC

Since the early days of synthetic chemistry, the generation of compounds has consisted of two steps: The synthesis of the compound and its purification. In addition to the traditional purification techniques, like crystallization, extraction and distillation, the first instruments for preparative column chromatography were developed in the 1950's and 60's. They usually consisted of a column and an eluent reservoir set up above the column. The sample was applied manually to the column head and the column was then connected to the eluent reservoir. Since the system did not include a pump, the flow was achieved through the hydrostatic pressure of the eluent. To increase throughput and separation power the first preparative HPLC systems were developed in the 1970's. Due to the better packing material of the columns with smaller particle size, high-pressure pumps were used to generate the flow[1].

When Merrifield developed the solid phase synthesis for peptides in 1963, he avoided purification of the crude reaction mixture after each synthesis step by attaching the C-terminal amino acid to an insoluble polymeric support resin. Each amino acid of the peptide sequence was then attached to this immobilized intermediate using the reagents and protective groups of the traditional peptide synthesis. The advantages were that the reaction compounds could be applied in high concentration, which usually leads to a good conversion rate, and that the purification step was a simple filtering procedure washing away everything that was not coupled to the resin[2].

This principle was taken over by the pharmaceutical industry to synthesize large numbers of compounds in a combinatorial way to feed the previously developed high-throughput screening assays. But despite the solid-phase approach it turned out that the target compounds removed from the resin beads were still not pure enough for use in the assays directly. To avoid creating a bottleneck in the drug discovery process, systems for high-throughput purification of the target compounds were required.

Traditional methods, like distillation or extraction, lack the high level of automation that is required to keep pace with the chemists high-throughput synthesis. The only method that fulfills the requirements for automated and easy-to-use purification of large numbers of compounds is preparative HPLC.

The first systems were put together by the users using parts and modules from different suppliers and were operated by self-written software. Today several completely automated purification systems from different vendors are available on the market.

Whereas analytical HPLC became a standard tool in the pharmaceutical industry there is still a lot of momentum and ongoing development in the field of preparative HPLC. For example, trends are going towards high-throughput purification systems to purify hundreds of compounds per day or towards walk-up systems where a system administrator controls the system and users can independently purify their samples.

In this Primer we will give an introduction into the basic principles of preparative HPLC, describe the components of a purification system, talk about fraction collection strategies and offer some application solutions for common tasks and problems in preparative HPLC. While this Primer gives a general overview on preparative HPLC, we recommend the Purification Solutions Guide[3] and the Purification Application Compendium[4] from Agilent Technologies for further reading.

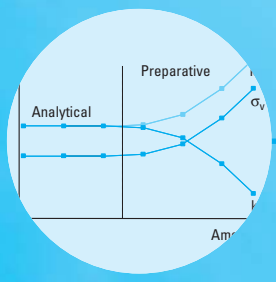

**Udo Huber**
**Ronald E. Majors**

*Udo Huber has a Ph.D. in organic chemistry from the University Karlsruhe/Germany and worked as postdoctoral fellow on natural products at the University of Hawai'i, Manoa. He is currently senior application chemist at Agilent Technologies, Life Sciences and Chemical Analysis, Waldbronn, Germany.*

*Ronald E. Majors, Ph.D. has worked with the company for nearly 15 years with focus in the areas of sample preparation and column technology. He is currently a master R&D applications chemist at Agilent Technologies, Life Sciences and Chemical Analysis, Wilmington, Delaware, USA.*

i

# Table of content




# Chapter 1

## Introduction into preparative HPLC

1

# Introduction into preparative HPLC

## 1. What does preparative HPLC mean?

The term preparative HPLC is usually associated with large columns and high flow rates. However, it is not the size of the instrumentation or the amount of mobile phase pumped through the system that determines a preparative HPLC experiment, but rather the objective of the separation. The objective of an analytical HPLC run is the qualitative and quantitative determination of a compound. For a preparative HPLC run it is the isolation and purification of a valuable product (table 1). Since preparative HPLC is a rather expensive technique, compared to traditional purification methods such as distillation, crystallization or extraction, it had been used only for rare or expensive products. With increasing demand for production of highly pure compounds in varying amounts for activity, toxicology and pharmaceutical screenings the field of operation for preparative HPLC is changing.

| Analytical HPLC | Preparative HPLC |
|---|---|
| Sample goes from detector into waste | Sample goes from detector into fraction collector |
| **Goal:** Quantification and/or identification of compounds | **Goal:** Isolation and/or purification of compounds |

**Table 1**
**Definition of analytical and preparative HPLC**

## 2. Working areas of preparative HPLC

*The preparative HPLC scale is determined by the amount of compound to be purified!*

Preparative HPLC is used for the isolation and purification of valuable products in the chemical and pharmaceutical industry as well as in biotechnology and biochemistry. Depending on the working area the amount of compound to isolate or purify differs dramatically. It starts in the µg range for isolation of enzymes in biotechnology. At this scale we talk about micro purification. For identification and structure elucidation of unknown compounds in synthesis or natural product chemistry it is necessary to obtain pure compounds in amounts ranging from one to a few milligrams. Larger amounts, in gram quantity, are necessary for standards, reference compounds and compounds for toxicological and pharmacological testing. Industrial scale or production scale preparative HPLC, that is, kg quantities of compound, is often done nowadays for valuable pharmaceutical products. The working areas for preparative HPLC are summarized in table 2.

| Compound amount | Working area |
|---|---|
| **µg** | • Isolation of enzymes |
| **mg** | • Biological and biochemical testing<br>• Structure elucidation and characterization of<br>- Side products from production<br>- Metabolites from biological matrix<br>- Natural products |
| **g** | • Reference compounds (Analytical standards)<br>• Compounds for toxicological screenings<br>- Main compound in high purity<br>- Isolation of side products |
| **kg** | Industrial scale, active compounds, drugs |

**Table 2**
**Working areas of preparative HPLC**

## 3. Method development and scale-up calculations

In analytical chromatography the sample amounts applied to the column are typically in the µg range but can be lower. The mass ratio of compound to stationary phase on the column is less than 1:100000. The applied sample volume is also usually much smaller than the column volume (< 1:100). Under these conditions good separations with sharp and symmetrical peaks can be achieved. The biggest difference in preparative HPLC is the much higher amount of sample applied to the stationary phase. The impact on the chromatography, the methods of injecting larger amounts of sample and the scale up of an analytical method are described in the next chapters.

### 3.1. Adsorption isotherm

The goal of analytical HPLC is the quantitative and/or qualitative determination of a compound. Important chromatographic parameters to achieve reliable and accurate results are resolution, peak width and peak symmetry. If more and more sample amount is applied to the column, the peak height and peak area increases but the peak symmetry and the capacity factor remain unchanged as shown in figure 1.

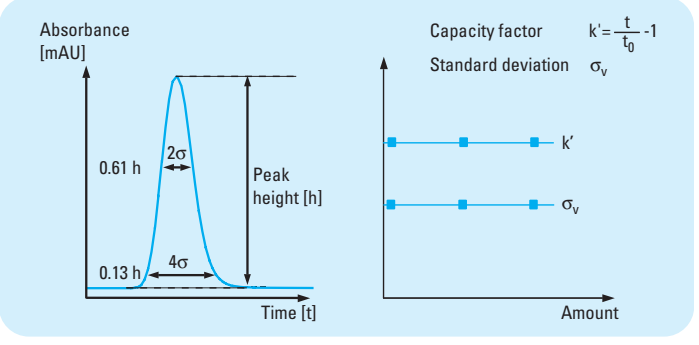



**Figure 1**
**Peak shape for analytical HPLC**

In analytical HPLC the optimal peak shape resembles a *Gaussian* curve. The peak's standard deviation $\sigma_V$ describes its symmetry and how well it resembles a *Gaussian* curve. The capacity factor (k') is the retention time (t) relative to the retention time of a non-retained compound ($t_0$). If more than a certain amount of sample is injected onto the column the adsorption isotherm becomes non-linear. This means the peak becomes un-symmetrical, shows strong tailing and the capacity factor decreases as shown in figure 2. In preparative HPLC this effect is called concentration overloading. In some cases, depending on the compound, the capacity factor increases with increasing overloading, which leads to a strongly fronting peak. Since the adsorption isotherm is dependant on the compounds the chromatographic system column loadability has to be determined for each preparative HPLC experiment.

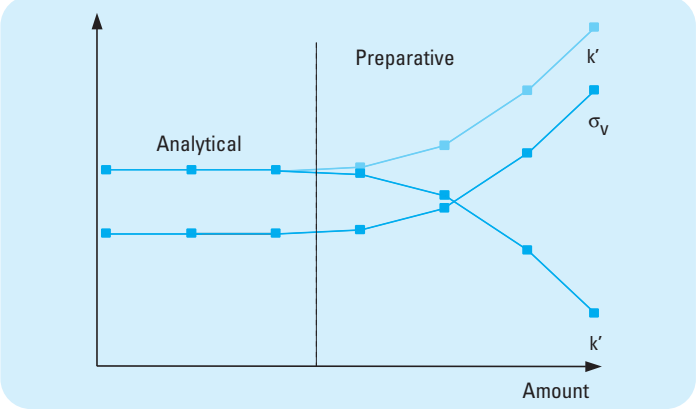



**Figure 2**
**Capacity factor and peak standard deviation for preparative HPLC**

### 3.2. Column loading and overloading

For purification of large sample amounts two methods are possible: Scale-up of the analytical system or column over-loading. Scale-up of the analytical system would mean

*What is concentration and volume overloading?*

using larger column diameter, higher flow rate and increasing the sample volume with the column length and sample concentration remaining constant. The peaks would then remain sharp and symmetrical. The disadvantage of this method is that large columns and high solvent volumes are required to separate rather small amounts of compound, hence, the method would not be economical. Therefore, column overloading, that is, increasing the applied sample amount under the same analytical conditions, is usually the method of choice. Using column overloading allows to separate samples in the milligram range even on analytical columns. For larger amounts of sample an additional scale-up of the system is necessary. Column overloading can be done in two ways – concentration or volume overloading. In concentration overloading the concentration of the sample is increased but the sample volume injected remains the same. The capacity factor, k', decreases and the peak shape changes from a *Gaussian* curve to a triangle as shown in figure 3. Concentration overloading is only possible when the sample compound has good solubility in the mobile phase.

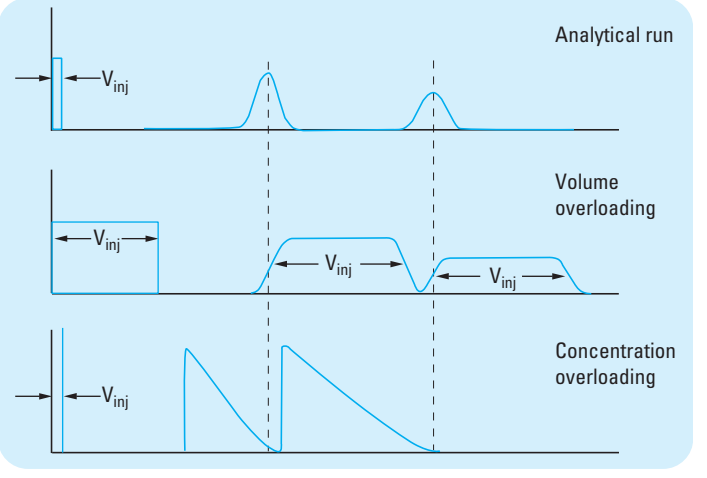



**Figure 3**
**Peak shapes of volume and concentration overloading**

If the compound has poor solubility, concentration overloading cannot be used and more sample volume must be injected. This technique is called volume overloading. Beyond a certain injection volume the peak height does not increase and the peaks become broader and rectangular. In preparative HPLC concentration overloading is favored over volume overloading because the sample amount, which can be separated, is higher. Since the solubility of compounds is usually a limiting factor both overloading techniques are used in combination. Table 3 gives an overview of the overloading techniques.

| Concentration overloading | Volume overloading |
|---|---|
| • Determined by solubility of compound in mobile phase | • Determined by injection volume |
| • "Preparative" area of adsorption isotherm | • "Analytical" area of adsorption isotherm |
| • Throughput determined by selectivity | • Throughput determined by column diameter |
| • Particle size of stationary phase of low influence | • Small particle size required |

**Table 3**
**Overview of concentration and volume overloading**

### 3.3. Method scale-up

Both concentration and volume overloading lead to decreasing resolution of the compounds. Since a certain resolution is required for the separation of the compounds it is important to optimize the resolution (figure 4) when developing the analytical method. As selectivity and overloading potential are dependent on each other, improving the selectivity increases the amount of sample that can be separated in one run. Therefore, the optimization and scale-up of an analytical to a preparative method is done in three steps:

1. *Optimization of the analytical method regarding resolution*
2. *Column overloading on the analytical column*
3. *Scale-up to the preparative column*

$$R_S=(\alpha-1)\left(\frac{k_1'}{1+k_1'}\right)N^{0.5}$$

$R_S$ = Resolution
$\alpha$ = Relative selectivity
$k'$ = Capacity coefficients
$N$ = Number of theoretical plates

**Figure 4**
**Equation for chromatographic resolution**

### 3.4. Scale up calculations

The two parameters that must be scaled up when going from a column with smaller i.d. to a column with larger i.d. are the flow rate and the sample amount applied to the column. To scale up the flow rate the upper equation in

**Analytical column** → **Preparative column**

$$\frac{\dot{V}_1}{\dot{V}_2}=\frac{r_1^2}{r_2^2}$$

Flow: 0.6 mL/min → Flow: ~ 30 mL/min

Volume: 15 µL/injection → Volume: 750 µL/injection

$$\frac{x_1}{\pi \times r_1^2}=\frac{x_2}{\pi \times r_2^2}\times\frac{1}{C_L}$$

$x_1$ = max. volume column 1 = 15 µL
$r_1$ = radius column 1 = 1.5 mm
$x_2$ = max. volume column 2 = ?
$r_2$ = radius column 2 = 10.6 mm
$C_L$ = ratio lengths of columns = 1

**Figure 5**
**Equations for scale up calculations**

figure 5 is used, in the shown example the flow of 0.6 mL/min on a 3 mm i.d. column is scaled up to a 21.2 mm i.d. column. For scaling up the amount of compound loaded onto the column the lower equation is used, it makes no difference if it is used to scale up the concentration or the injection volume. The factor $C_L$ equals 1 if two columns of the same length are used. After the scale-up calculations and the first preparative run, it is not uncommon to further optimize the parameters to achieve the best separation results[5].

## 4. Objectives of preparative HPLC

The three important parameters used to judge the result of a preparative run are purity of the product, yield and throughput. Since the parameters are dependent on each other it is not possible to optimize a preparative HPLC method with respect to all three parameters (figure 6).

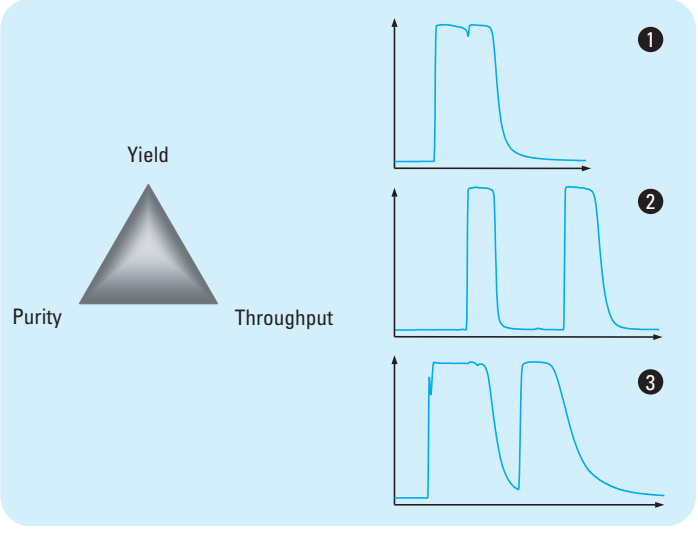



**Figure 6**
**Results of a preparative HPLC run**

1

*A purification run can never be optimized with respect to the three parameters: purity, recovery and throughput!*

Chromatogram 1 shows a preparative HPLC run capable of very high throughput but the separation of the two compounds is poor. It might be possible to obtain some fractions with high purity for each compound but the recovery, that is, the yield is rather low. In chromatogram 2 the peaks are well separated, therefore, it is possible to get both compounds in high purity and yield but the throughput is very low. Chromatogram 3 would be an optimized preparative HPLC run with a compromise to all three parameters. The peaks are almost baseline-separated, which leads to high purity and yield and throughput is as high as possible. The most important parameter for which the separation has to be optimized depends on the application. If, for example, a compound has to be isolated for activity or toxicity testing, it is necessary to obtain this compound in high purity. Throughput and the yield are less important. If a synthesis intermediate has to be purified the purity is not of highest importance as long as it is sufficient for the next synthesis step. However, throughput is an issue here because it is usually necessary to complete the entire synthesis as fast as possible. The yield is also important because the compound is valuable and the loss of compound should be minimized.

# Chapter 2

## The role of the column in preparative HPLC

2

# The role of the column in preparative HPLC

## 1. Introduction

It has often been stated (or perhaps overstated) that the column is the heart of the liquid chromatograph. Choice of the wrong column and mobile phase conditions for the sample at hand can trivialize all of the advantages of expensive, sophisticated instrumentation and data systems in a laboratory. In preparative chromatography, this statement is also true. Since one is often working with a sample that may have limited solubility in the injection solvent and/or mobile phase and injections of large volumes of sample for increased throughput are frequently used, an unsuitable column/mobile phase combination can be even more disastrous with possible sample precipitation playing havoc on expensive wide bore prep columns and instrumentation. The role of the column is pivotal in developing a rugged, reproducible preparative HPLC method. In this chapter, we will explore the columns used in preparative HPLC – how to select the appropriate mode, mobile phase system and operating conditions. The assumption is that the reader has a familiarity with analytical HPLC method development and separation optimization.

Analytical separations often assume Langmuir-like isotherms and that the resolution equation (chapter 1, figure 4) is strictly obeyed. In preparative HPLC, where columns are frequently, and sometimes heavily, overloaded, the actual isotherms and the commonly accepted relationships no longer apply quantitatively. For example, in analytical HPLC, a commonly accepted definition of column capacity is that the injected sample mass causes a 10% decline in column efficiency. In preparative chromatography, the amount of sample injected may exceed this value by an order of magnitude so rarely is column capacity given an absolute value. Instead, overload

in preparative HPLC, is defined as that loading which no longer permits the isolation of product at the desired purity or recovery levels[6]. Column capacity must take into account other molecules in the sample including the matrix. Remember, when we discuss capacity we mean the sample capacity and not the capacity factor, which is a measure of analyte retention. The goal of a preparative purification is the maximum production of purified product per injection.

*Overloading and scale-up experiments must be done on an analytical column with the same packing material as the preparative column!*

As was discussed in chapter 1, scale-up to preparative-scale HPLC from analytical liquid HPLC data is often time-consuming and wasteful of materials unless an optimized scale-up strategy is employed. A recommended method development strategy was to develop and optimize the initial separation on an analytical size column, overload the column while maintaining adequate separation of components of interest, then scale-up accordingly to a preparative column of appropriate dimensions based on the amount of compound needed to purify, using guidelines previously outlined. Thus, the choice of analytical column is often dictated by the availability of preparative columns containing the same column packing material, either in prepacked columns or in bulk media that can be packed by oneself. Then, using the equations provided in chapter 1 (section 3.4), the scale-up should be linear with perhaps only minor "tweaking" required to finalize the preparative method. It is highly recommended that one ensures that both analytical and preparative columns from the same line of packing material be readily available prior to beginning the preparative method development and optimization process.


## 2. Choosing the appropriate mode and stationary phase in preparative chromatography

In preparative scale-up, in theory the same separation modes that were used in analytical-scale chromatography may be employed. However, due to cost and availability of high performance preparative packing materials, the cost of mobile phase and mobile phase additives, the high throughput requirements and the need to recover isolated fractions in a high purity state, users may limit themselves to the more popular modes of adsorption and reversed-phase chromatography. Size exclusion- and ion-exchange chromatography are sometimes employed for initial preparative scale up but are usually followed by a secondary techniques.

For decades, adsorption chromatography on silica gel or other liquid-solid media (e.g. alumina, kieselguhr) was the main technique for purification of a variety of synthetic organic mixtures as well as other sample types. However, the overwhelming popularity of reversed-phase chromatography (RPC) in the analytical world has shifted the emphasis to this mode of operation for preparative HPLC. Also, many analytical HPLC users are not familiar with the principles of adsorption chromatography whereas they are more comfortable with RPC; thus, they tend to gravitate to it when facing a preparative need.

One consideration in preparative chromatography that is usually of lower importance in analytical chromatography is the capacity of the packing material. Since throughput (i.e. the amount of material purified per unit time) is a primary criterion in preparative HPLC, columns with higher capacity can handle more material per injection. For adsorption chromatography, the surface area of an adsorbent dictates the capacity. A higher surface area sorbent will allow larger mass injections than a low surface area sorbent. In RPC, in addition to solubility of the analyte, the bonded phase coverage in RPC determines the sample

capacity. Although one may think that the capacity of the RPC media is dictated by the alkyl chain length of the bonded phase (e.g. C18 vs. C4), it is more influenced by the bonded phase coverage.

*What determines the capacity of a preparative HPLC column?*

Surface coverage is often expressed as micromoles/$m^2$. For a typical silica gel packing, there are roughly 8-micromoles/$m^2$ of surface silanols available for bonding. Since in adsorption chromatography, the silanol group is responsible for analyte retention, the larger the surface area the more silanols are present and the greater the retention. In RPC, the mechanism is hydrophobic interaction between the alkyl and aryl groups on the analyte with the bonded phase. For a typical monomeric C18 bonded phase, due to steric reasons, the surface phase coverage is usually in the range of 2.5-3-micromoles/$m^2$. Due to its smaller footprint on the surface, a monomeric C8 phase may have a slightly larger coverage and a C4 even more. So, the coverage of a shorter chain alkyl phase may actually exceed that of a C18 bonded phase. Thus, the amount of available carbon for the hydrophobic interaction with the analyte may actually provide a better measure of surface coverage than the alkyl chain length. Most manufacturers provide the level of carbon coverage for their particular reversed-phase packings.

Resolution is seen as the most important factor in analytical chromatography and is equally important in preparative chromatography. However, since columns are frequently overloaded in preparative HPLC and peaks are broadened, selectivity is often a very important factor in successfully using preparative HPLC. If the selectivity between two sample components that are to be isolated is high, then one may overload the column to a much greater extent than if the selectivity is low. Thus, choice of stationary phase may be critical to provide the best selectivity for

the components of interest. Table 4 provides some rough guidelines on sample capacity for ZORBAX RPC columns as a function of α (selectivity). The actual sample capacity for the actual sample components may have to be determined by trial-and-error measurement.

| Column ID | α < 1.2 | α > 1.5 |
|---|---|---|
| 4.6 mm | 2-3 mg | 20-30 mg |
| 9.4 mm | 10-20 mg | 100-200 mg |
| 21.2 mm | 50-200 mg | 500-2000 mg |

**Table 4**
**Guideline for capacity of preparative HPLC columns**

### 3. Particle size and column dimensions

*What are the advantages and disadvantages of smaller particle sizes in preparative HPLC?*

Particle size is an important parameter for analytical HPLC. Generally the smaller particle size allows greater efficiency and permits the use of shorter columns to increase separation speed. In preparative chromatography, the particle size is important but since column may be used in an overloaded state, the smaller and more expensive particles of 1.8- and 3.5-µm average diameters that are used in analytical columns are generally not used in larger scale preparative columns. If a sample is very complex with poor resolution and selectivity among compounds of interest and overloading is sometimes difficult, then 5-µm particles are frequently employed. For well-resolved samples, larger particles of 7- and 10-µm can be used. Since pressure drop is inversely proportional to the particle diameter squared, larger particles also give lower pressure drop that allow higher flow rates which increases the throughput of preparative columns.

Column dimensions are dictated by the amount of material per injection that one desires to inject. The amount of sample that can be injected increases with column internal diameter and length so using the equations supplied in chapter 1, one can calculate the column diameter that fits

sample size required. Typically, 4.6-mm i.d. is for small-scale preparative HPLC, 7.8-mm i.d. columns are for semi-preparative HPLC, and 21.2-mm i.d. columns are for larger scale preparative HPLC. Columns of even larger diameters of 30-mm and 50-mm are available for even higher levels of scale-up. Beyond these diameters may require larger scale preparative and process instruments capable of extremely high flow rates (hundreds of milliliters per minute) and thus have a higher degree of solvent usage.

### 4. Choice of mobile phase

Often a direct transfer of the chromatographic conditions may be achieved during scale up. It should be decided during the analytical method development on the solvent system that will be employed. Factors influencing solvent choice(s) are:

- stationary phase and mobile phase conditions with optimum selectivity for compounds of interest;
- spectroscopic characteristics of mobile phase solvent(s) (i.e. UV transparency, fluorescence properties, mass spectroscopic compatibility);
- volatility for easy removal from isolated fraction(s);
- viscosity for low column back pressure;
- purity for low levels of non-volatile contaminants;
- good solubility properties for maximum sample loads;
- cost of solvents employed.

For the latter property, figure 7 gives a general idea of the cost of high purity solvents needed for preparative fractionation.[7]

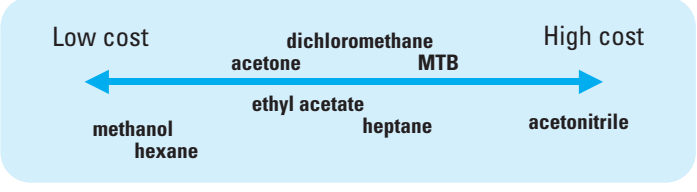



**Figure 7**
**Relative costs of organic solvents used in preparative HPLC**

*In preparative HPLC preferably volatile buffers should be used!*

Not surprisingly, solvent systems in normal phase chromatography often fulfill these criteria but nevertheless RPC still commands the most attention. In analytical RPC, non-volatile buffer salts are frequently used to ensure proper pH and prevent tailing and poor peak shape. In the development of the analytical separation destined for preparative scale up, it is recommended that a volatile buffer or mobile phase additive such as ammonium formate or ammonia being used for the initial method since removal will be much easier in the final stages of the method and, if mass spectrometry is used for confirmation, such a system will be more compatible. A list of recommended volatile buffers is included in table 5.

| Volatile buffer salts | |
|---|---|
| Trifluoroacetate | xx –1.5 |
| Ammonium formate | 3.0 – 5.0 |
| Pyridinium formate | 3.0 – 5.0 |
| Ammonium acetate | 3.8 – 5.8 |
| Ammonium carbonate | 5.5 – 7.5 |
| | 9.3 – 11.3 |
| Ammonium hydroxide | 8.3 – 10.3 |

**Table 5**
**Common volatile buffers used in HPLC**

## 5. Example of principles of scale up from analytical to preparative chromatography

The principle of scaling up from an analytical chromatography column to a preparative column will be illustrated for the separation of two xanthines, caffeine and theophylline. These compounds can be easily separated using reversed-phase chromatography as depicted in figure 8 that shows increasing amounts (from 0.025-µg each to 500-µg each) of the two xanthines on a 3.0-mm i.d. by 150-mm ZORBAX StableBond C18 analytical column. The separation was performed isocratically at a flow rate of 0.6-mL/min using a water-acetonitrile mobile phase system without any additives being present. Since a standard 10-mm path length UV flow cell was used at a wavelength of 270-nm, the detector electronics saturated at the higher sample

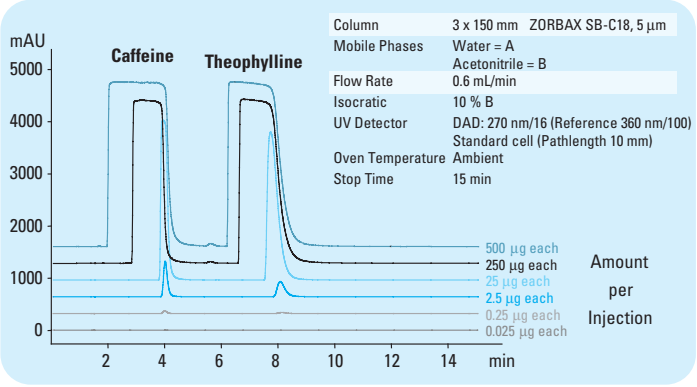



**Figure 8**
**Overloading experiments on the analytical column**

amounts resulting in the expected flat-top peaks. Since these two compounds displayed very good separation selectivity, the two chromatographic peaks were well resolved, even at the highest loading. Such good selectivity predicted good overloading in the next stage of scale up. Using the calculation formula from chapter 1 and depicted in figure 9, we next determined the maximum mass that could be loaded on our preparative column which contained the same packing material but with column dimensions of 21.2-mm i.d. by 150-mm length, the same length as our

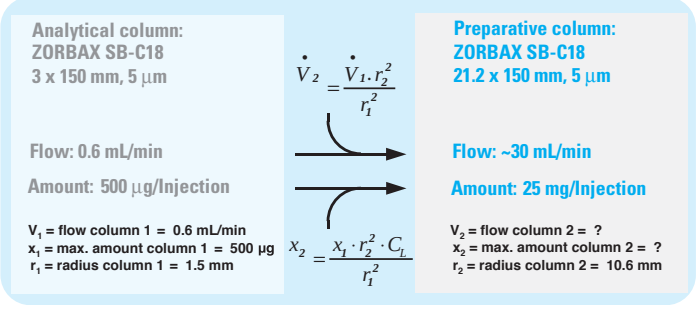



**Figure 9**
**Scale-up calculations – actual example**

analytical column. Figure 10 shows the preparative chromatogram on the larger column this time using a short pathlength preparative flow cell with the UV detector. This shorter path length was needed to prevent early detector electronics saturation and allowed us to observe the eluted xanthines, even at the 25-mg injected level. Note that the flow rate was adjusted to 25-mL/min instead of the 30-mL/min that was calculated. The separation of the two xanthines was just to baseline meaning that both compounds could be collected at high purity.

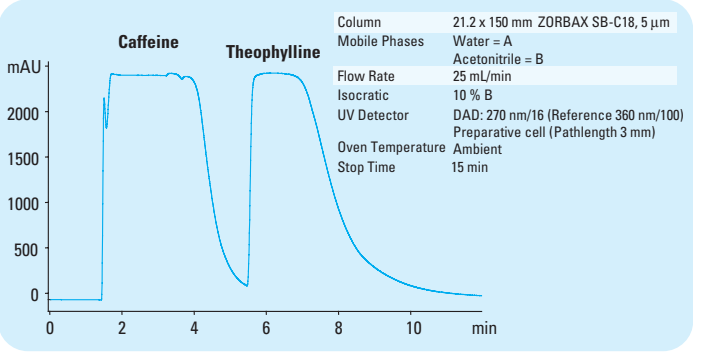



**Figure 10**
**Preparative separation resulting from scale-up calculations**

## 6. Successful use of preparative chromatography

Many of the factors in successful analytical HPLC are prevalent in preparative HPLC also, but some are even more of a factor. Since samples in preparation applications often are crude mixtures, impurities may accumulate at the head of the column and if not removed can cause peak shape and retention time change. Sometimes accumulated impurities do not affect retention but may change the column pressure so one must watch for increased column pressure. It is a good idea to occasionally flush the column with increasingly stronger solvents to remove bound impurities[8].

*For best performance the column should be flushed occasionally with strong solvents to remove bound impurities!*

Buildup of material in a packed column occurs most frequently when the injection solvent is weaker than the mobile phase and is especially noticeable when isocratic elution is used. The stronger solvent strength used in gradient elution tends to help with removal of strongly held impurities. Silica gel adsorbent tends to hold onto more polar analytes, especially basic compounds, while reversed-phase packings tend to favor more hydrophobic impurities.

Knowing the history of the preparative column is of utmost importance. Since impurities from previous samples can show up unexpectedly when new preparative separation conditions are developed, it is advisable to start with a fresh column. If this is not feasible, a solvent washing procedure for the column is suggested. Of course, if the cost of the solvents required is more than packing or column replacement, then it makes sense to either clean the packing or replace the column. For self-packed columns, externally cleaning the loose packing may be easier than when in the packed column. Often the first few centimeters of a column suffer the most contamination and removing this material and replacing it with fresh packing can be performed relatively easy.

## 7. Conclusions

Further reading on the "in's and out's" of preparative chromatography columns and proper usage can be found in reference books and recent reviews devoted to the principles and applications[6,9-13]. Modern preparative instruments coupled to high efficiency and high throughput columns have made the purification job of impure substances much easier. Rugged preparative columns that can withstand many injections are getting to be the "norm" and further work in special preparative phases such as monoliths and high capacity sorbents will continue.


2

# Chapter 3

## The purification system

3

# The purification system

## 1. How does a fraction collector work?

As mentioned in chapter 1 the only difference between analytical and preparative HPLC is what happens to the sample after it has left the detector. Whereas the sample travels directly into the waste receptacle in analytical HPLC it goes to a fraction collector in preparative HPLC. Based on certain triggering decisions (see chapter 4) the fraction collector diverts the flow either to waste or, the desired part of the injected sample, to a fraction container via the fraction collection needle. This is achieved using a diverter valve that can be switched, for example, by time programming or based on a detector signal. A schematic drawing of a fraction collector is shown in figure 11.

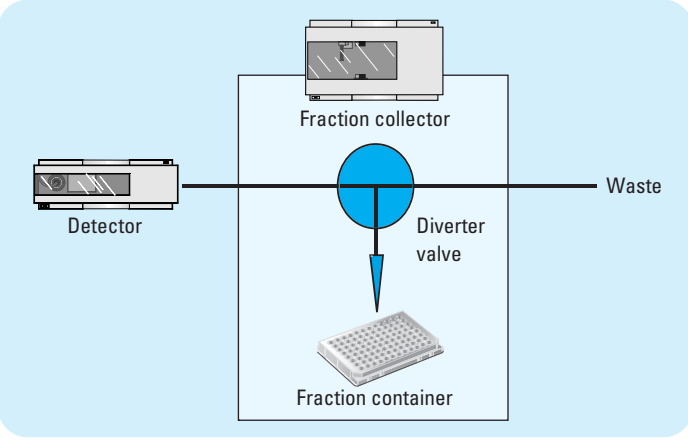



**Figure 11**
**Schematics of a fraction collector**

Fraction collectors are commercially available in different sizes and designs: While some can be used from very low to high flow rates, Agilent Technologies offers dedicated fraction collectors for three flow rate ranges. The micro

fraction collector is designed for flow rates below 100 µL/min, the analytical scale fraction collector is designed for flow rates below 10 mL/min and the preparative scale fraction collector is designed for flow rates up to 100 mL/min. Some instruments combine the autosampler and the fraction collector on a single platform either with a single needle and valve for injection and fraction collection or with two devices, one for injection and the other one for fraction collection. For the collection of fractions several vials, test tubes or well-plates are commercially available; most fraction collectors can handle all those fraction containers. A special fraction container is the Agilent funnel tray (figure 12). Rather then collecting directly into the fraction container the needle goes into an injection-port-like funnel, to which a piece of tubing is attached. This tubing can be placed into any fraction container, for example into round-bottom flasks that can directly be used on a rota-vapor after fraction collection is completed. The funnel tray allows the collection of virtually unlimited fraction volume.

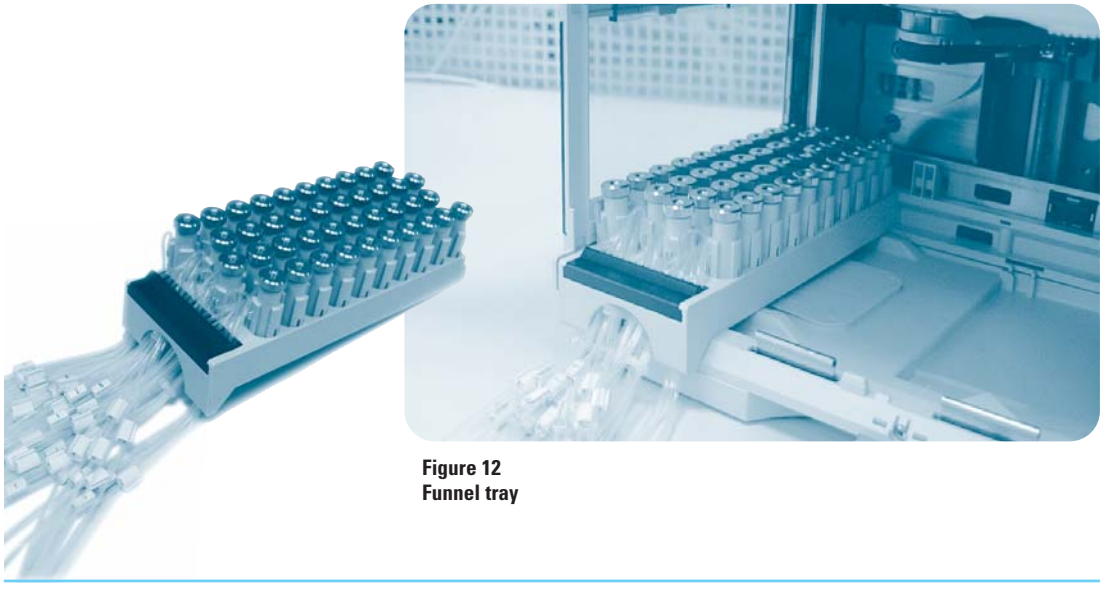


**Figure 12**
**Funnel tray**

## 2. What is the fraction delay time?

*To obtain the highest purity and recovery it is important to apply the accurate fraction delay times correctly!*

The system setup shown in figure 11 – a detector in front of the diverter valve – would be the typical set up for a system designed for peak-based fraction collection. As soon as the detector detects a peak that meets the triggering criteria (see chapter 4), the diverter valve has to be switched from the waste to the collect position. However at the precise time the detector finds a peak start ($t_0$, figure 13) the detected compound is in the detector cell and not at the diverter valve, therefore it would be too early to switch the valve to the collect position. The valve switching has to be delayed until the compound has moved from the detector cell to the inlet of the diverter valve. This time is called the delay time ($t_{D1}$) and must be determined beforehand in what is called the delay calibration procedure. The same delay time must be used to switch the valve back to the waste position when the detector finds the end of a peak ($t_E$). For best recovery results the additional delay time $t_{D2}$ should also be included to make sure the end of the peak has not only reached the diverter valve but also the end of the collection needle.

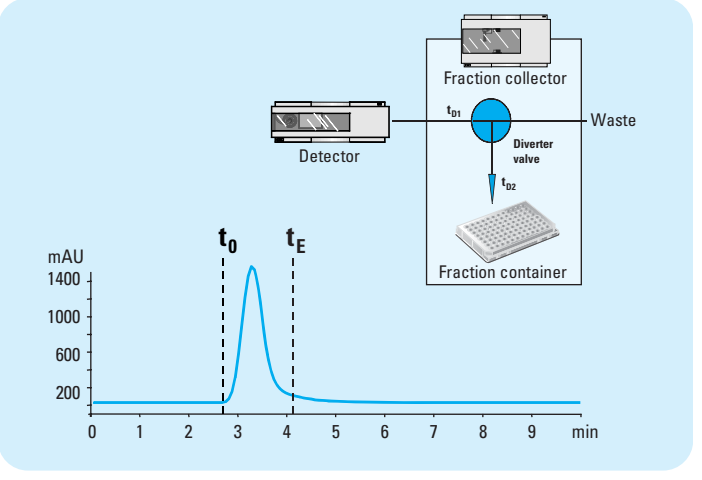



**Figure 13**
**Peak start and peak end time**

Applying the delay times in the following way gives the best fraction collection results regarding purity and recovery:

Start of fraction collection → when start of peak arrives at diverter valve
Start of fraction collection: $t_0 + t_{D1}$

End of fraction collection → when end of peak arrives at needle tip
End of fraction collection: $t_E + t_{D1} + t_{D2}$

In a system used for peak-based fraction collection, as shown in figure 11, the delay time depends on the flow rate. If the delay time for a given flow rate is measured, the delay volume $V_{D1}$ between detector and fraction collector can be calculated. The delay time for any flow rate can be calculated after identifying $V_{D1}$ avoiding the necessity to recalibrate the system.

## 3. The delay calibration procedure

The delay calibration procedure is used to determine the delay time between detector and fraction collector. In this chapter two traditional methods for delay calibration and the advanced method using the Agilent Technologies fraction delay sensor are described. Also two additional delays, the so-called detector delay and the system delay will be explained[14].

### 3.1. Traditional delay calibration procedures

The traditional delay calibration procedure is a tedious and error-prone process. One possibility to determine delay time is to inject a concentrated dye on the system and to monitor the detector signal in the online display of the control software. As soon as the peak can be seen in the online display a timer is started and stopped again when the dye can be seen coming out of the fraction

collection needle. This delay time, which is the sum of the delay times $t_{D1}$ and $t_{D2}$, is then entered into the software. If the software does only allow the input of a delay time, the calibration procedure must be repeated for any flow rate.

A more accurate but time-consuming procedure is to start with an estimated or calculated delay volume or delay time. A sample with known sample amount is injected and a fraction containing the compound is collected. The amount of compound in the fraction is determined, for example by analytical HPLC, and then the delay volume is altered. The experiment is repeated and the recovery is compared to the first result. This procedure is repeated until the delay time or delay volume that leads to a maximum recovery is found.

### 3.2. The fraction delay sensor

With the Agilent 1200 Series purification system delay calibration is done using the fraction delay sensor (FDS)[15], which is a simple, small detector built into the fraction collector (figure 14).

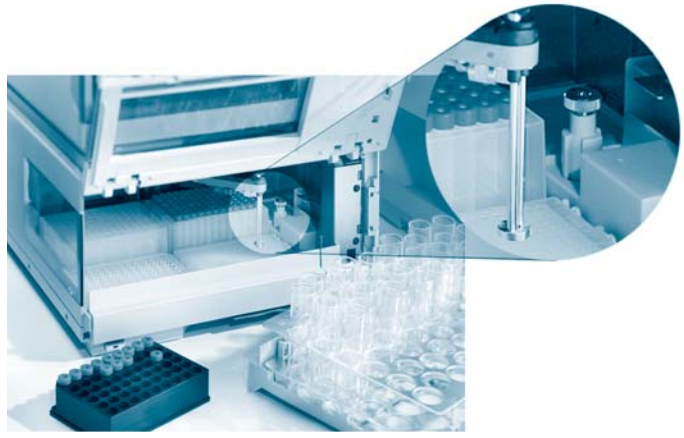


**Figure 14**
**Agilent fraction delay sensor**

*The fraction delay sensor makes the accurate measurement of the delay time a simple and automated task!*

In the completely automated process to determine the delay volume $V_{D1}$ of the system a dye is injected and the signal of the dye from the detector and from the FDS is recorded (figure 15). The overall system delay volume is calculated by means of the time difference of the signals $t_D$ and the flow rate used for the experiment. To determine the required delay volume $V_{D1}$, the known volumes, $V_{D2}$, which is the volume between the diverter valve and the fraction collection needle tip, and $V_{D3}$, the volume of the FDS, are subtracted from the overall delay volume. Once the delay volume $V_{D1}$ is identified, the software automatically calculates the delay time $t_{D1}$ for any other flow rate. The re-calibration of the system is not necessary unless any hardware changes, like changing the capillaries or the detector flow cell, are done.

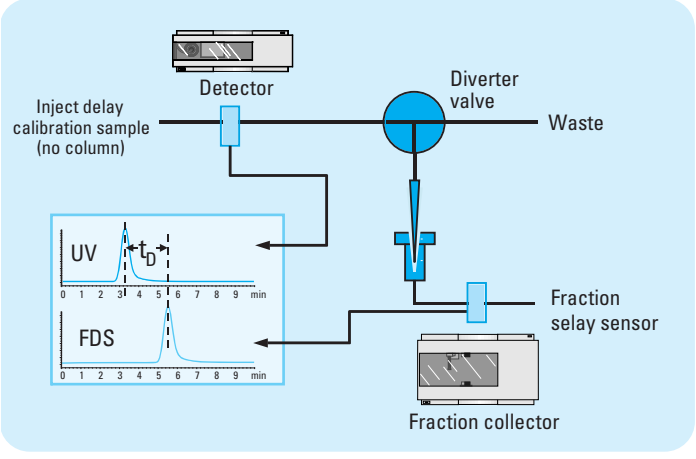



**Figure 15**
**Delay calibration process**

### 3.3. The detector delay

When recording a signal from a detector, for example an UV detector, a smooth signal as shown in figure 16b is expected. However the signal the detector actually measures looks, due to electronic noise, more like the signal shown in figure 16a.

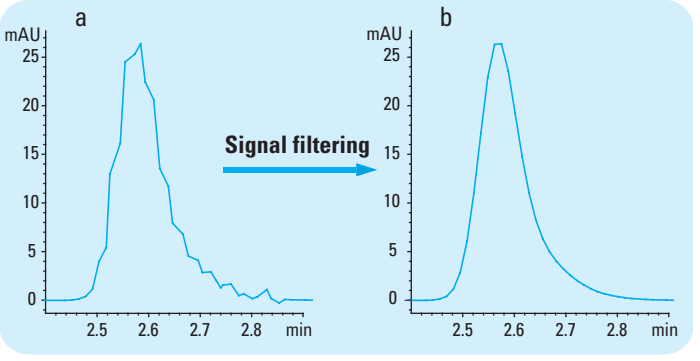



**Figure 16**
**a) Rough signal b) Filtered signal**

*How does signal filtering in the detector affect the fraction delay timing?*

To smoothen the signal mathematical filtering is applied to the rough signal by the control software. Therefore several data points are measured by the detector then averaged using a smoothing algorithm and finally displayed, for example in the online display of the software. However the signal filtering does not only smoothen the signal it also delays it for a certain time. Since the data point drawn in the software is the result of a calculation over several measured data points the online display is always a little bit behind the actual measurement point in the detector (figure 17). This is called the detector delay. The length of the detector delay depends on how much filtering is applied, it can range from below 1 up to more than 10 seconds. Given that in peak-based fraction collection the triggering of the fraction collector has to be done on the filtered signal, the rough signal would be too noisy and would lead to many unwanted fractions (see chapter 4), the detector delay has to be taken into account when

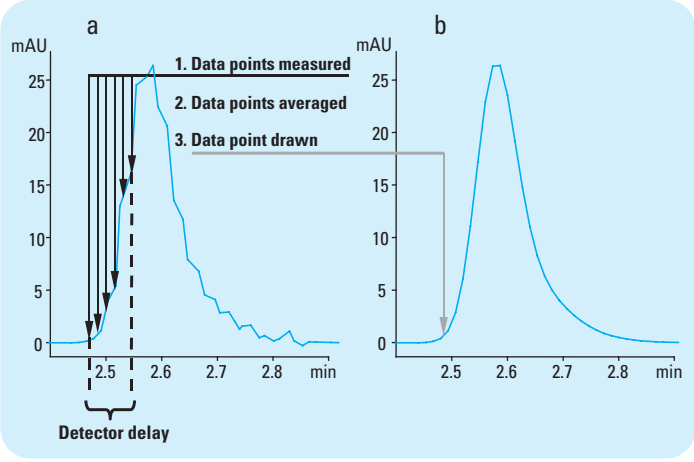



**Figure 17**
**Detector delay due to signal filtering a) Rough signal b) Filtered signal**

measuring the delay time. The easiest way to do this is to perform the delay calibration using the same filtering settings as in the actual purification run. With the Agilent purification system the time adjustment for different filtering settings is done automatically by the fraction collector software.

### 3.4. The system delay

Particularly in larger purification laboratories computers are usually not directly connected to the instruments they control but all equipment is connected via a Local Area Network (LAN). When the purification system detects a peak that meets the triggering criteria it sends this information to the computer. The computer applies the delay time and then sends the signal to switch the diverter valve of the fraction collector. Taking into account that the information from the detector to the computer and the trigger signal from the computer to the fraction collector are both sent via the LAN several factors can influence the fraction delay timing. If any other data, for example a large print job, is sent over the same LAN the data traffic

will be delayed, which means the overall delay time will be incorrect. Additionally, if the operating software installed on the computer controls the fraction collection process and applies the delay time then high CPU usage will have an impact on the overall delay timing process, for example when other software programs are running simultaneously. Therefore the following points should be considered:

- If the purification system is connected to the computer via LAN this network should not be used for anything else that could cause heavy network traffic. The instruments could also be connected directly to the computers.
- If the computer software controls the purification process no other programs should be running on the computer at the same time.

*Heavy LAN network traffic or CPU usage can influence the timing of fraction triggering!*

To avoid the problems mentioned above, i.e. influence of LAN traffic and CPU usage on the delay timing, the Agilent purification system works in a different way. When a run is started the method is downloaded completely from the computer to the modules. The modules communicate with each other via the Controller Area Network (CAN), a direct connection between the modules. If, for example the detector triggers a peak it sends this information to the fraction collector that reads the actual flow rate from the pump, calculates the delay time and triggers the diverter valve. With this system-integrated intelligence all decisions are made within the system, the computer is only the interface to the user to set up the method and display the results, which means that high LAN traffic or CPU usage do not have any influence on the fraction delay timing[6].

### 4. Configuration and delay calibration of a system for mass-based fraction collection

Configuration and fraction delay timing of a system that does not contain a mass selective detector (MSD) for fraction collection is quite simple. The complete flow coming from the column goes through the UV detector cell and from there to the fraction collector. When the delay volume between the two modules is determined the delay time

can be calculated for any given flow rate. As the MSD does not tolerate flow rates above several milliliters and because it is a destructive detector a system for mass-based fraction collection must be set up differently.

## 4.1. Configuration

Given that the MSD is a destructive detector the flow coming from the column must be split into the main flow going to the fraction collector and into the split flow going to the MSD for fraction triggering. This is achieved using a device called the flow splitter (figure 18).

*Why is a flow splitter required in a mass-based fraction collection system?*

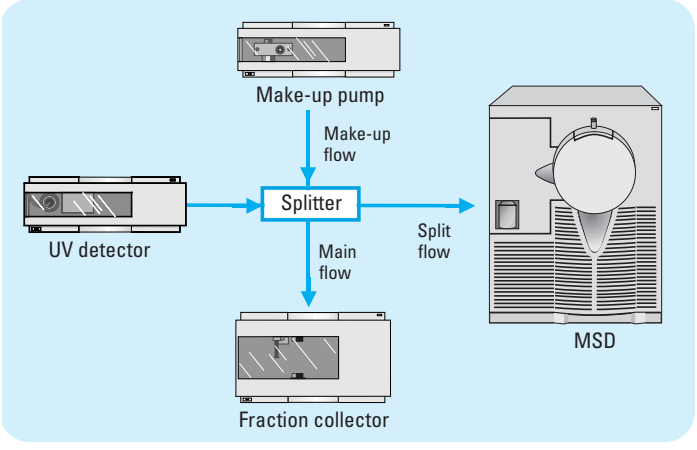



**Figure 18**
**Configuration of a mass-based purification system**

Since the MSD triggers the switching of the diverter valve in the fraction collector, the portion of the compound in the split flow must arrive at the MSD earlier than the portion in the main flow at the fraction collector. This time difference between the MSD and the fraction collector is the fraction delay time. Since the flow is usually split by a factor of 1000 – 20000 the flow going towards the MSD must be sped up using a make-up pump (figure 18). Further factors for using a make-up pump are:

- Increase and stabilize flow for better nebulization
- Dilute sample to good range for MS (< 500 ng/s)
- Provide optimum ionization conditions for MS, for example add acid to make-up flow

## 4.2. Flow splitter considerations

The traditional flow splitter is a passive splitter achieving the split using restrictive tubing (figure 19).

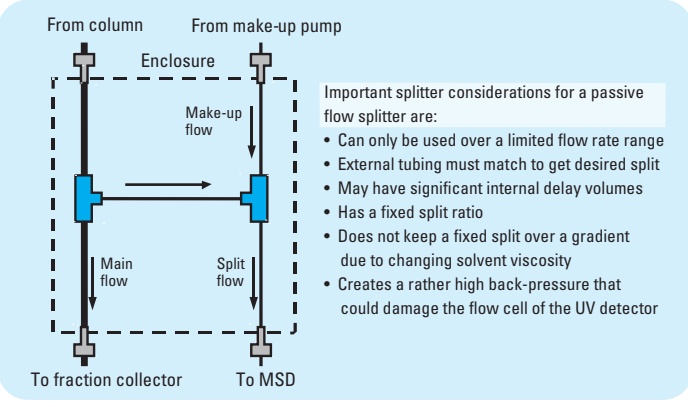



**Figure 19**
**Passive splitter design**

A different approach is the active splitter. It has two completely separated flow paths and a rapid switching valve that transfers the compound from the main flow into the make-up flow. The operating principle is shown in figure 20.

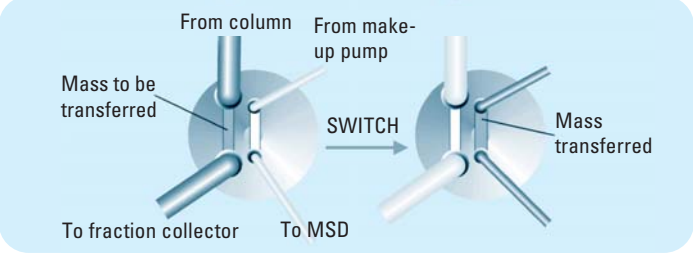



**Figure 20**
**Active splitter design**

The biggest advantage of the active splitter is the possibility to select different split ratios by changing the switching frequency of the valve. Other aspects of the active splitter are:

- Constant, accurate split ratios unaffected by viscosity, temperature, and tubing length
- Minimal post-column volumes, minimizes dispersion
- Fraction delay time independent of split ratio
- Two independent flow paths allow the usage of different modifiers in the main and make up flow, for example trifluoracetic acid for good chromatography in the main flow and formic acid to avoid ion suppression in the make-up flow
- Rotor seals must be exchanged after about 1.5 million movements, approximately every 4 – 6 months
- Minimal back-pressure, no danger to damage the UV detector cell

### 4.3. Delay time calibration for a mass-based fraction collection system

For a mass-based fraction collection system the delay time is the time between the compound arriving in the MSD and in the fraction collector. For a peak-based fraction collection system the delay time can be calculated from the delay volume and the flow rate of the pump. For a mass-based fraction collection system this cannot be done because the delay time depends on the flow rates of two pumps: The main pump and the make-up pump. What happens if one of those flow rates is changed, is shown in the figures 21a and 21b.

Figure 21a illustrates what happens to the delay time if the flow of the make-up pump is increased. The flow to the MSD is increased and therefore the compound arrives there earlier. Since the flow to the fraction collector is unchanged the overall delay time also increases.

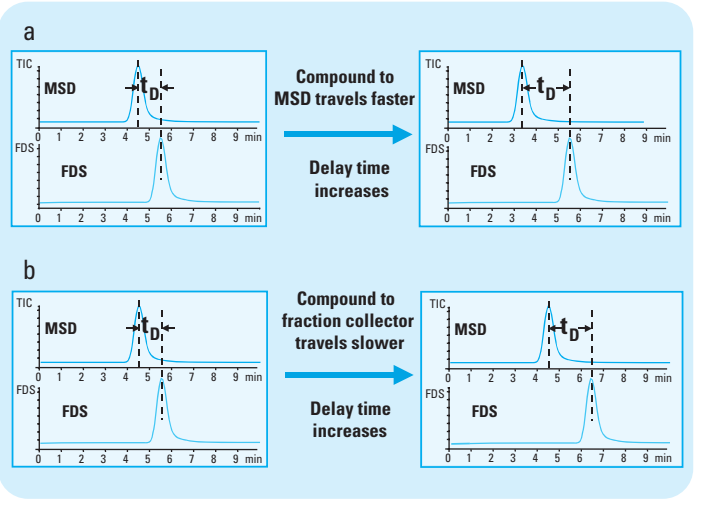



**Figure 21**
**a) Increased make-up flow**
**b) Decreased main flow**

*Since the delay time for a mass-based fraction collection system depends on the flow rates of the main pump and the make-up pump it is a method parameter!*

Figure 21b illustrates what happens when the flow of the main pump is decreased. While the flow rate to the MSD remains the same the compound arrives at the fraction collector later. This means that the delay time increases. The delay volume for a peak-based fraction collection system depends only on the tubing used between the detector and fraction collector and is therefore a configuration parameter. Consequently the system must only be re-calibrated when the configuration is changed. For a mass-based fraction collection system the delay time depends on the flow rates of the two pumps. Given that the flow rates of the pumps are method parameters the delay time is also a method parameter. This means the system must be re-calibrated whenever one of the flow rates, main flow rate or make-up flow rate, is altered.

## 5. System optimization for highest recovery

An often overlooked parameter in preparative HPLC is the dispersion, which is the peak broadening when a compound moves through a capillary. When a peak is detected in the detector it has to move through a capillary of a certain length until it reaches the fraction collector, which leads to changing peak shape due to dispersion. Fraction triggering is done based on the peak shape in the detector. Therefore, the recovery and purity of the compound in the collected fraction is not always as anticipated[16].

### 5.1. Influence of the dispersion on the recovery

Figure 22 shows a peak in the detector and the changing peak shape when the peak moves through capillaries with increasing length. While the peak area measured at the fraction collector remains the same the peak height decreases and the peak width increases.

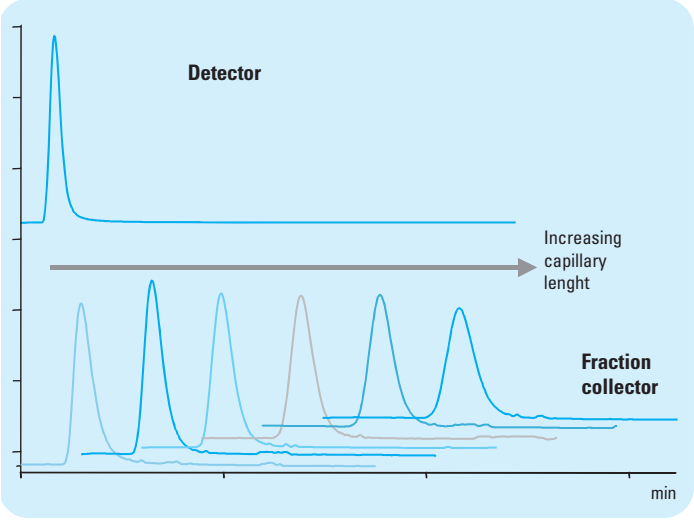



**Figure 22**
**Changing peak shape due to dispersion**

Figure 23 shows the influence of the dispersion on the recovery for a collected fraction. A fraction will be collected for a given threshold as indicated by the box in the detector signal as the triggering is based upon a signal from the detector. If this box is transferred to the peak at the fraction collector it can be noticed that a part of the compound will be not be collected. Everything that is framed by the grey boxes will be lost. As the peak gets broader and broader with increasing capillary length the collected part of the peak, and therefore the recovery, decreases.

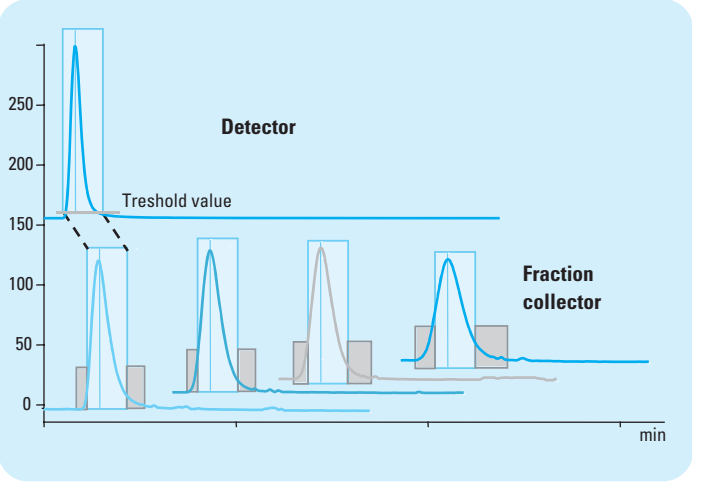



**Figure 23**
**Decreasing recovery with increasing delay volume**

*Unnecessarily long capillary connections between the detector and fraction collector will lead to lower recovery!*

Therefore, when setting up the purification system, the capillary connections between the detector and the fraction collector should be optimized, ideally kept as short as possible. Although the effect of recovery loss due to dispersion is extremely important for purification at low flow rates, it can still be seen on systems with flow rates of 25 to 35 mL/min.

## 5.2. Influence of the dispersion on the purity

Figure 24 shows the influence of dispersion on a sample that contains two peaks. As both peaks broaden with increasing capillary length between detector and fraction collector the compounds start to remix while they move to the fraction collector.

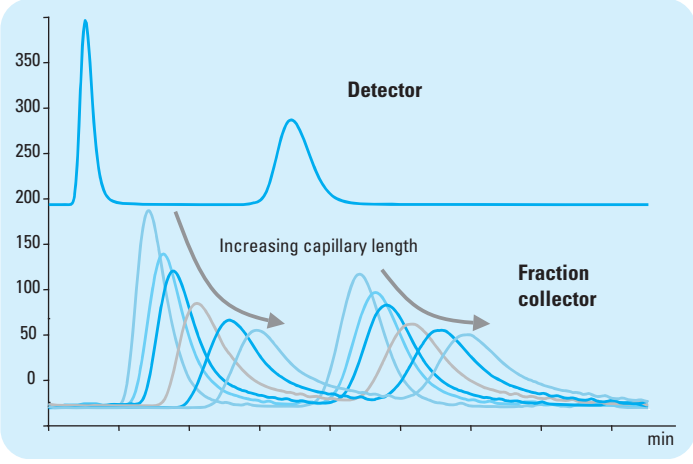



**Figure 24**
**Remixing of peaks due to dispersion**

To reaffirm, the recovery for each compound will decrease with increasing capillary length as the triggering is done based on the peak width in the detector. However due to the partial re-mixing of the compounds the purity of each collected fraction also will be lower than expected. The resolution is a parameter to measure how well two peaks are separated. Figure 24 shows the resolution of the two peaks for different added delay volumes. As the detector is positioned before the added delay volume, the resolution of the peaks in the detector does not change. After measuring the resolution at the fraction collector with the standard capillary set up (added delay volume = 0 µL) delay volume was added using a 0.25 mm i.d. and a 0.8 mm i.d. capillary, respectively.

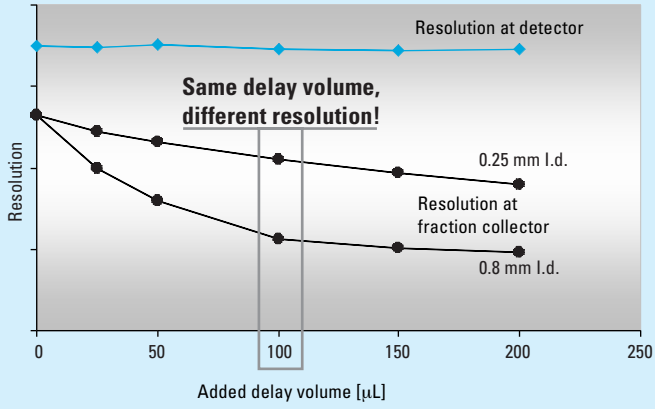



**Figure 25**
**Influence of capillary length and i.d. on the resolution**

*Capillary connections with inappropriate inner diameters will either lead to high back pressure or to low recovery and purity!*

Notice that the resolution of the two peaks decreases with increasing delay volume added with a capillary of a certain inner diameter. Interestingly, the resolution is poorer if the same delay volume is added using a capillary with a larger inner diameter, which can be seen in figure 25 (window): When adding 100 µL delay volume with a capillary with an inner diameter of 0.8 mm the resolution is much poorer then when adding the same delay volume with a capillary with an inner diameter of only 0.25 mm. This can easily be explained with the *Aris-Taylor* equation shown in figure 26. Band broadening $\sigma^2$ is directly proportional to flow rate and capillary length but it is proportional to the fourth power of the capillary radius.

$$\sigma^2 = \frac{\pi \times r^4 \times F \times L}{24 \times D_m}$$

**Figure 26**
***Aris-Taylor* equation**

Therefore it is important to always use capillaries with an inner diameter according to the flow rates required for the application. An inappropriate inner diameter leads either to high back pressure if the capillary is too narrow or to poor fraction collection performance if it is too wide.

To summarize the effects described in 5.1. and 5.2.:

- To minimize dispersion and maximize recovery it is important to keep the capillary connections between the detector and the fraction collector as short as possible.
- If delay volume must be added for any reason it is important to use capillaries with appropriate inner diameter because wide capillaries lead to band broadening and therefore to lower recovery and purity.

## 6. Safety issues

When working in preparative HPLC the volumes of dangerous organic solvents like acetonitrile or methanol are much higher than in analytical HPLC. Therefore special care must be taken to avoid solvent spills if, for example, a leak occurs in the system. The fractions also contain a large amount of organic solvent that will evaporate into the laboratory if the fractions are not immediately dried down after collection. Therefore the purification system must be equipped with safety features to prevent endangering the operator and the laboratory environment.

### 6.1. Leak handling

Contrary to, for example, capillary HPLC detecting leaks in preparative HPLC is usually easy. Due to the high solvent volumes used in preparative HPLC it is not recommended to run a system unattended if no precautions were taken in case a leak occurs. However the disadvantage of an unrecognized leak during a sequence of purification runs is not only the amount of spilled solvent but also the loss of valuable samples. If the system does not recognize the leak it keeps injecting samples, which not

*How does the leak sensor prevent the loss of valuable samples?*

only means that the spilled solvent presents a health risk to the operator and a threat to the laboratory environment but also that the valuable samples injected after the occurrence of the leak are lost. Therefore a purification system should be equipped with a leak sensor, which shuts down the system pumps and stops the injection of the pending samples. In the Agilent 1200 Series purification system each module is equipped with a leak sensor (figure 27) that stops the system as soon as a few drops of liquid are detected. A drainage system makes sure that wherever the leak occurs the spilled solvent is always guided to the leak sensor of a module.

**Figure 27**
**Leak sensor**

### 6.2. Solvent vapor

At the end of a sequence of purification runs the collected fractions can contain several liters of organic solvent. This solvent will evaporate from the fraction container into the laboratory air, where it can easily reach the maximum concentration allowed for a working environment. Therefore, as a minimum the fraction collector of the purification system must be placed under a fume hood. A new approach was taken in the Agilent 1200 Series

purification system: Due to the closed design of the fraction collector the solvent vapor cannot evaporate out of the fraction collector. A small fan at the rear of the fraction collector (figure 28) removes the solvent vapor from the fraction collector interior and guides it through a tube into a fume hood, for example.

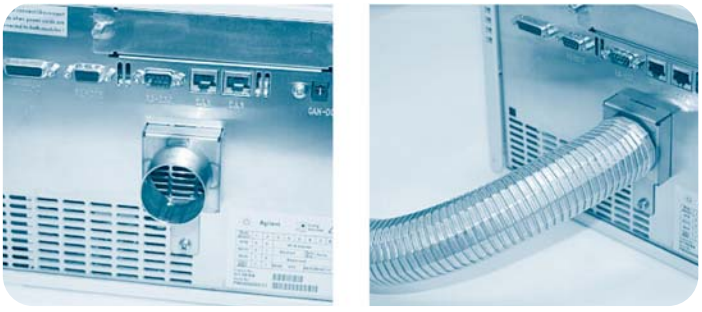


**Figure 28**
**Forced fume extraction**

# Chapter 4

## Fraction collection strategies

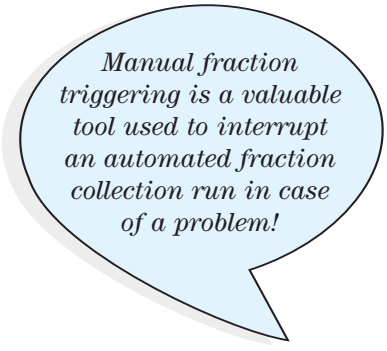
4

# Fraction collection strategies

In chapter 3 the hardware configuration of the fraction collector was described and how the diverter valve of the fraction collector must be switched from the waste to the collect position after taking the delay time into account. In this chapter the different mechanisms that trigger the valve switching are described as well as the parameters that are used to decide whether a fraction must be collected or not. The following trigger mechanisms are explained:

- *Manual fraction collection*
  The switching of the diverter valve is triggered manually by pressing a button either on the instrument or in the control software
- *Time-based fraction collection*
  Collection of certain time intervals from a purification run
- *Peak-based fraction collection*
  Collection based on a detector signal
- *Mass-based fraction collection*
  Collection if a user-selected target mass is found by an MSD

## 1. Manual fraction collection




Manual fraction collection means that the user triggers the switching of the diverter valve manually, usually based on a signal plot on the instrument display or in the software. Using manual fraction collection offers the highest flexibility because only the desired parts of the purification run are collected. The drawback is certainly the lack of automation and the rather low throughput that can be achieved with this method. Therefore manual fraction collection is usually only used for:

- Low-throughput applications
- Very valuable samples
- As a safety feature to manually interrupt an automated purification run.

Two aspects are important for accurate manual fraction collection: First the delay time must also be applied to the triggering decision. The time when a peak is seen in the signal plot is not the time the compound arrives at the diverter valve. Therefore the system must wait for the delay time after the operator had pressed the button to trigger the fraction. Furthermore the signal plot must be a real-time plot, which means there must be no delay between the measurement in the detector and the displayed signal.

With the Agilent 1200 Series purification system manual fraction collection can be done using the handheld controller, also to manually interrupt a purification run.

### 2. Peak-based fraction collection

Peak-based fraction collection is an automated way of collecting fractions based on a detector signal, very often from a UV detector. The parameters used to decide whether a peak in the chromatogram should trigger the fraction collector are usually threshold and/or slope.

The easiest approach for peak-based fraction collection is to trigger on threshold only: As soon as the signal exceeds a predefined limit a fraction start is triggered; when the signal falls back below the specified threshold collection is terminated.

Figure 29 shows the result of peak-based fraction collection based on threshold only. Although the first and the last peak were collected accurately the two peaks in the middle were collected as a single fraction despite the compounds being separated on the column to a certain extent. The only way to collect them in separated fractions would be to increase the threshold, but that would lead to the loss of some compound at the beginning and the end of all peaks. Furthermore, for a generic method that has to work for compounds with different response factors, the

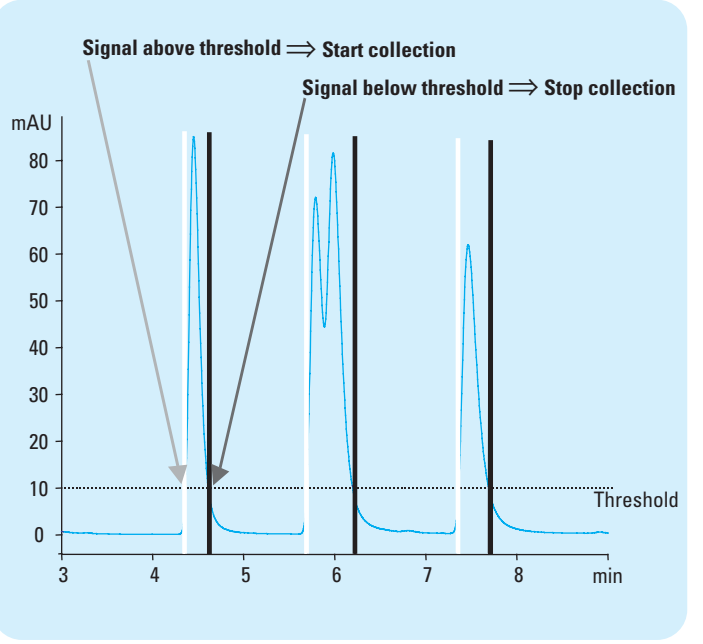



**Figure 29**
**Peak-based fraction collection on threshold**

threshold must not be set too high because otherwise some peaks could be missed completely.

To collect peaks that are not baseline-separated a second parameter, the peak slope, is required. The slope is the first derivative of the chromatogram, for a simple *Gaussian*-shaped peak the slope is displayed in figure 30[17]. As shown in figure 30 the slope reaches its maximum at the first inflexion point of the peak, falls back to zero at the peak apex and reaches its minimum at the second inflexion point of the peak. For peak-based fraction collection based on the peak slope this means a peak start can only be triggered before the first inflexion point by increasing

*The slope is the first derivative of the chromatogram!*

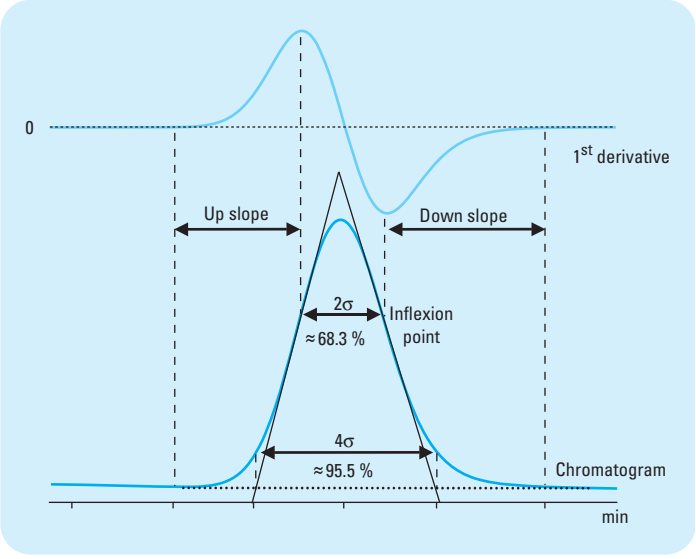



**Figure 30**
**Chromatogram and slope of a *Gaussian* peak**

the slope value. If a slope value higher than the value at the inflexion point is used the peak will not be collected at all. Since the same is valid for the second inflexion point it is impossible to trigger the start and/or stop of a fraction between the inflexion points of a peak by triggering on the peak slope only! While the triggering of the peak start using the slope value is simple – as soon as the specified slope is exceeded a start is triggered – the termination of the fraction collection based on the slope value is more difficult. Since the slope value at the peak apex equals zero and falls then to a minimum the Agilent software uses the following algorithm to trigger a peak stop: one slope parameter, the up-slope, is used to trigger the peak start; a second parameter, the down-slope is used to trigger the peak stop. Applying the down-slope entered by the user

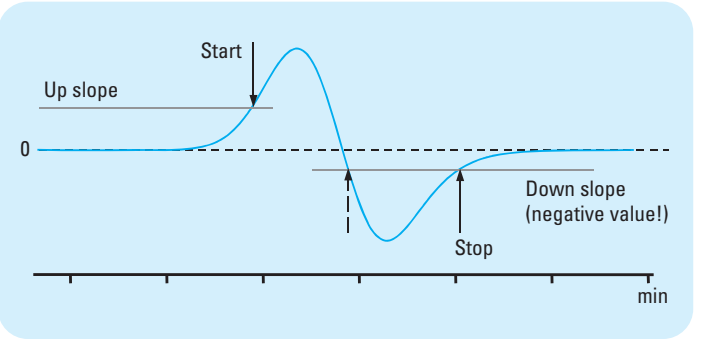



**Figure 31**
**Triggering on up- and down-slope with the Agilent software**

the software takes the negative value and then triggers a stop when the slope first falls below and then rises back above this value (figure 31). A problem can arise when triggering on slope and the signal goes into the saturation of the detector. The tops of the peaks are only flat at first sight, they are quite noisy due to electronic noise (figure 32) when zoomed in. Since the slope for such a peak has several

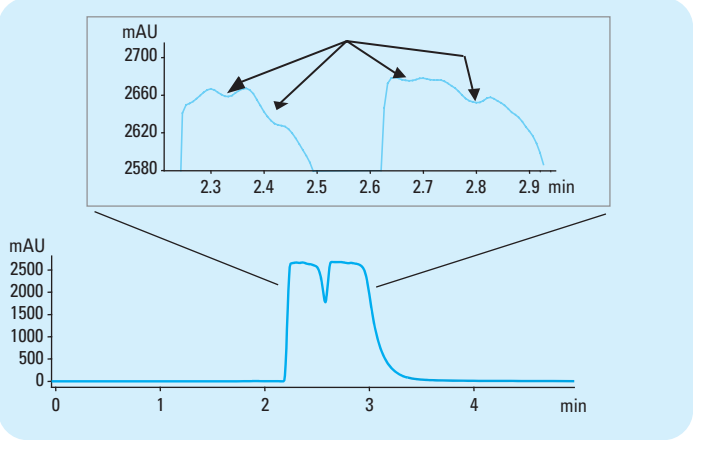



**Figure 32**
**Electronic noise in peaks in detector saturation**

maximums, zeros and minimums several fractions would be triggered using the slope parameters.

*How is the upper threshold used to avoid the collection of unwanted fractions if peaks go into detector saturation?*

To avoid triggering of those unwanted fractions a further parameter, which is called upper threshold in the Agilent ChemStation software, can be used. When the signal rises above the upper threshold start and stop triggers from the slope are ignored. To make sure that real valleys between two peaks are not ignored the upper threshold should be set near the saturation limit of the UV detector.

As shown in figure 29 two non-baseline separated peaks cannot be collected in separate fractions using a reasonable threshold. When collecting only on slope the two peaks could be separated but also unwanted fractions could be triggered due to noise in the baseline. Therefore a combination of the parameters threshold and slope are usually used for fraction collection. How those parameters are combined logically is described below, the result of the collection is shown in figure 33.

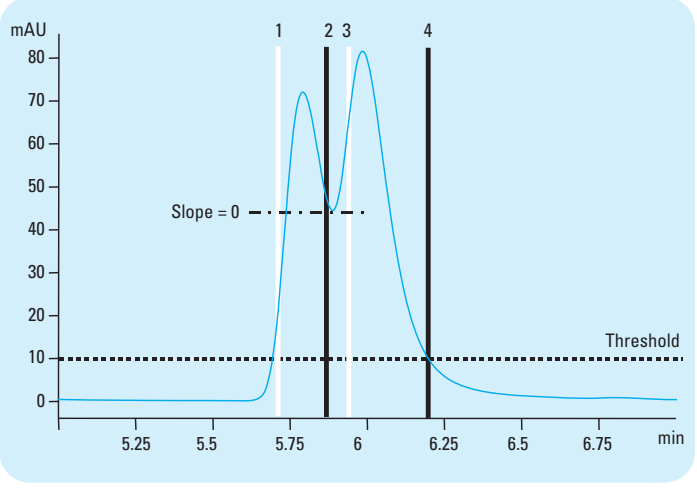



**Figure 33**
**Collection of non-baseline separated peaks**

A peak start, indicated by line 1 in figure 33, is triggered when both criteria are met: The signal must rise above the threshold and the slope must rise above the specified up-slope value. To trigger a peak stop only one of the stop criteria must be met: Either the signal falls below the threshold or the down-slope criterion is triggered as previously described. Since the slope at the valley between the peaks equals zero, a peak stop as indicated by line 2 in figure 33, is triggered before the valley. The start of the second fraction is triggered shortly after the valley because the threshold is still exceeded and the slope rises again above the specified up-slope value (line 3). The end of the second fraction is triggered as indicated with line 4 in figure 33 because the signal falls below the threshold setting though the down-slope criteria is still not met.

Depending on the application, it may be necessary to have the system set-up to be triggered not only by the signal from a UV detector but also by a signal from any other detector, for example, a evaporative light scattering detector (ELSD). These detectors are not necessarily from the same manufacturer as the purification system but nevertheless there should be a possibility to trigger a peak start or stop upon a signal from these detectors. The Agilent purification solution provides the universal interface box (UIB), which can be used to trigger the system by a signal from any detector that can be connected via an analog output to the UIB.

### 3. Mass-based fraction collection

In peak-based fraction collection all compounds are collected if their peaks meet the triggering criteria. Only the compound with the desired mass is selectively collected when doing mass-based fraction collection. Therefore the number of collected fractions is much lower than in peak-based fraction collection, many times it is only one fraction per sample. For successful mass-based fraction collection

*For mass-based fraction collection it is crucial to use the monoisotopic mass of a compound as the target mass!*

two requirements must be met: The molecular mass of the compound of interest must be known and the compound must ionize to make it detectable by the MSD.

The sample specific target mass must be entered into the software for a successful mass-based purification run. It is absolutely necessary to enter the monoisotopic mass of the compound and not the average mass, although some software allows entering the totals formula also. As the target mass is a sample specific parameter the expected adduct depends on the mobile phases used in the main flow and the make-up flow and is therefore method specific. If, for example, a mobile phase with low pH is used in the make-up flow the $[M+1]^+$ can probably be seen in the MSD. If the make-up mobile phase contains sodium the $[M+23]^+$ adduct is expected. The sum of the target mass and the adduct ion equals the trigger ion by which the MSD triggers the fraction collector (figure 34).

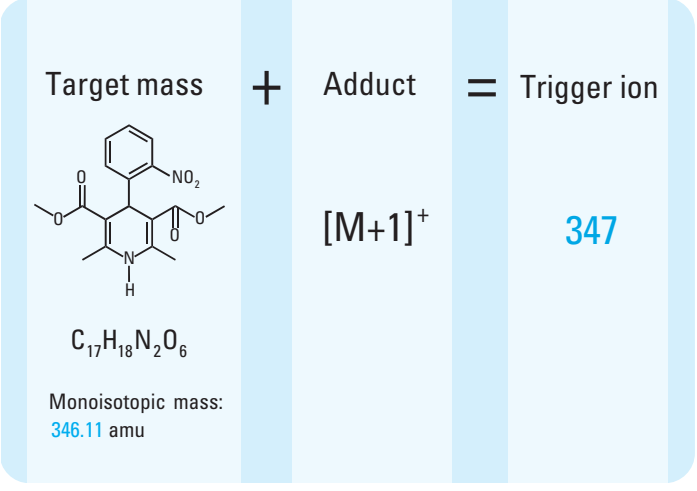



**Figure 34**
**Example calculation of the trigger ion**

Based on the trigger ion the MSD calculates the extracted ion chromatogram (EIC) from the total ion chromatogram (TIC) and applies the triggering parameters threshold and slope to it. When the criteria are met the MSD triggers the fraction collector to collect a fraction (figure 35).

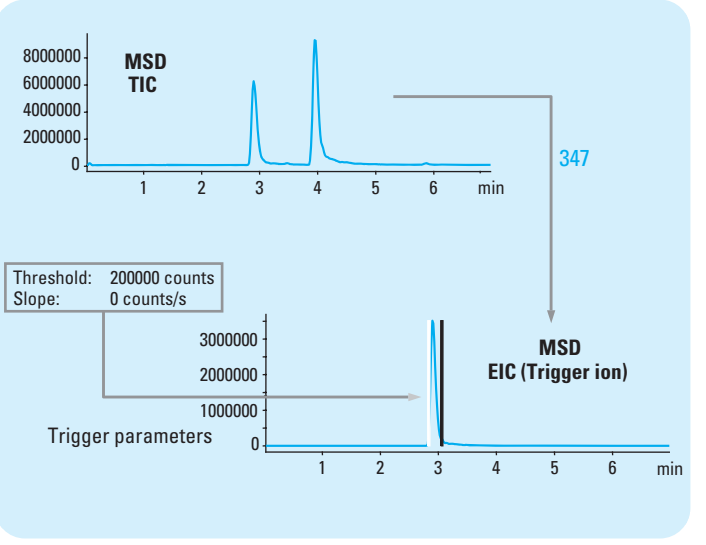



**Figure 35**
**EIC of the trigger ion**

Entering the correct target mass for the sample is obviously the critical part in mass-based fraction collection. If the incorrect mass is entered no fraction will be collected and the target compound will go to waste. To avoid the complete loss of the sample more and more mass-based purification systems are equipped with sample specific recovery locations (see chapter 5, 5. Recovery collection), in which all of the sample is collected that was not identified as a fraction.

## 4. Combining mass-based and peak-based fraction collection

In analytical HPLC one advantage of the MSD is its higher sensitivity compared to a UV detector, for example. In preparative HPLC sensitivity is usually not a problem; the higher sensitivity of the MSD can even be a disadvantage. Even when using a high split ratio (see chapter 3) the signal of the MSD is usually much broader than the signal of the UV detector in the flow path between the column and the splitter. This peak broadening is due to the overloading of the MSD, especially of the spray chamber and the dielectric capillary. Therefore it can happen that closely eluting peaks are not well collected by the purification system because the target ion "tails" into the next peak. An example is shown in figure 36.

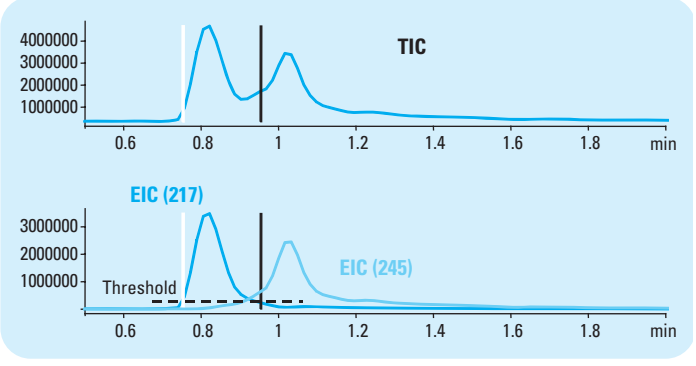



**Figure 36**
**Tailing of trigger ion into next peak**

When looking at the TIC it can be seen that the fraction – indicated by the white and black line – should have been terminated earlier for best purity results. However, the EIC of the trigger ion (217) shows that the fraction was collected correctly providing the EIC of the trigger ion was above the specified threshold.

A better fraction collection result can be achieved if the target mass of the closely eluting compound is also

entered into the software. As both trigger ions (217 and 245) are collected, the first fraction will be terminated as soon as the second trigger ion (245) becomes dominant over the first trigger ion (217) in the mass spectrum. Although the fraction collection results achieved with this technique are satisfactory, the problem is that the target mass of the partly co-eluting impurity must be known before starting the purification run. This is certainly not the case for all the impurities in the sample. Therefore an analytical run must be done before the preparative run to determine the target masses of possible impurities. This alogorithm works fine as long as the elution order and resolution in the preparative run are comparable to the analytical run. If the elution order or the resolution changes it can disturb the fraction collection process leading to lower recovery and purity.

A better solution to this problem is the combination of the MSD signal with the signal of a UV detector placed in the flow path between the column and the splitter. This detector will display the actual peak shape of the compounds coming from the column and going into the fraction collector if the dispersion effect is minimized as described in chapter 3.

*For best purity results the MSD signal should be combined with the UV detector signal using a logical AND combination!*

Figure 37 shows the result of the fraction collection of the same sample as shown in figure 36 but with a logical AND combination of the UV and the MS detector signals. That means a fraction is only collected if both detectors, the UV and the MS detector, see a signal that meets the triggering criteria taking the different delay times of both detectors into account. In the example shown in figure 37 the triggering in the MSD was done the same way as described for figure 36, in the UV detector the triggering was done on slope only. The fraction was therefore terminated early

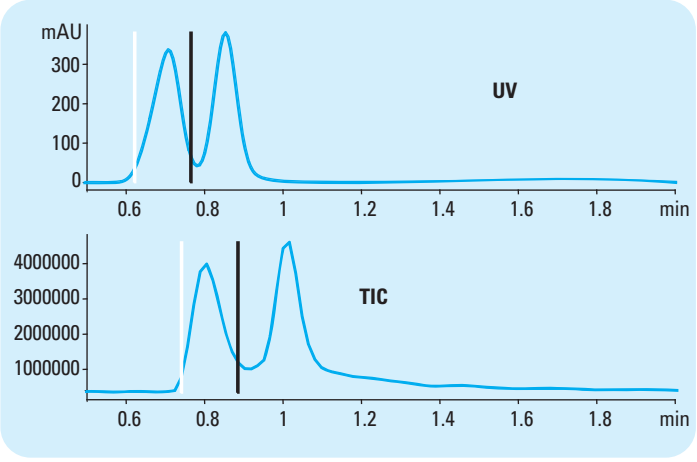



**Figure 37**
**Fraction collection on UV and MSD signal using a logical AND combination**

enough because the down-slope criterion in the UV detector was met while the trigger ion was still present.

Looking at the EIC's of the two masses 217 and 245 (figure 38) shows that the collection of the fraction was stopped before contaminating it with the partly co-eluting compound.

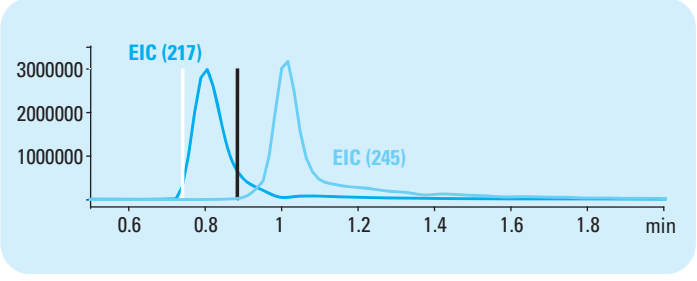
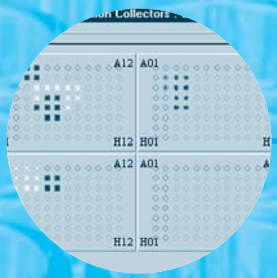



**Figure 38**
**EIC of trigger masses of target compound and impurity**

The combination of the UV and the MSD signal using a logical AND has several advantages over the combination of the target mass with the masses of impurities[18]. It is not necessary to specify the target masses of the possibly co-eluting impurities as triggering on the UV signal is not selective, which means it has no influence on the fraction collection if the elution order of the compounds in the preparative run changes compared to the analytical run. It is also not necessary to identify the target masses of the possibly co-eluting impurities in an analytical run before the preparative run is started.




# Chapter 5

## Application solutions

# 5 Application solutions

In this chapter solutions for several typical applications and possible problems in preparative HPLC are described:

- Purification in medicinal or high-throughput chemistry
- Purification in natural product chemistry
- Purification of byproducts for impurity analysis
- Column overloading
  - Injection of high-concentration samples
  - Injection of high-volume samples
- Recovery collection
- Automated fraction re-analysis
- Walk-up system

While more purification applications are described in detail in the Agilent Purification Application Compendium[4] this section gives a general overview of the applications mentioned above.

### 1. Purification in medicinal or high-throughput chemistry

Today most compounds in drug discovery are synthesized by medicinal or high-throughput chemistry groups. In high-throughput chemistry the chances to find an active compound are increased by synthesizing large numbers of compounds while medicinal chemistry takes a closer look at the drug target. As a result, fewer but more specific compounds are synthesized by the chemists. Regardless if high or low numbers of compounds are synthesized all compounds must be purified before they can be released for activity testing. Mass-based fraction collection on the calculated molecular mass is the purification method of choice as the structures of the synthesized compounds are known but the available sample amount is limited[19-21]. Due to the high number of compounds in high-throughput chemistry very often preparative HPLC is not done by the chemists themselves but by a purification group. The challenge for this group is not only the purification

process itself but also to create a seamless workflow of sample submission, purification and re-assembling of the purified compounds. In medicinal chemistry the purification process is often done within a group of chemists because the number of compounds to be purified is much lower than in high-throughput chemistry. As the chemists are not necessarily purification experts, the purification system is very often set up as a walk-up system (see 7. Walk-up system).

Requirements for a purification system for medicinal or high throughput chemistry:

- Mass-based fraction collection
- High-throughput capability
- Walk-up capability
- Safety features, like leak sensor, for unattended operation
- Robustness for 24 hour operation
- Data import, for example from Microsoft® Excel® or from a database

## 2. Purification in natural product chemistry

The traditional task of natural product chemistry is the isolation of active compounds from active crude natural product extracts. As the structure of the active compound is usually not known, it is not possible to calculate the molecular mass and perform mass-based fraction collection, therefore time- or peak-based fraction collection are the methods of choice[22]. As the crude extract is a very complex mixture the purification process usually consists of several consecutive purification and activity testing steps until the active compound is available in pure form for structure elucidation. Currently natural product extracts are also used to generate compound libraries with high diversity. Basically as many compounds as possible

are isolated from a crude extract, again by consecutive purification steps using time- and peak-based fraction collection.

Requirements for a purification system for natural product chemistry:

- Time- and peak-based fraction collection
- Robustness for difficult samples (particles or cells in the samples)
- Safety features, like leak sensor, for unattended operation

### 3. Purification for impurity analysis

At later stages in the drug discovery and development process it is not only important to isolate the compound of interest in pure form but also possible impurities, down to 0.1 % of the main compound. These impurities must be isolated in sufficient amounts as reference standards for the later production process[23]. Whenever an unexpected compound is observed during production, it can be compared to the previously isolated reference standards. The challenge is to separate the minor impurities from the main compound and to get several milligrams in pure form. As frequently enough starting material is available, the purification run can be optimized to make sure the impurities are well separated from the main peak. Therefore, it is not necessary to perform mass-based fraction collection, particularly because the main compound will heavily overload the MSD. The optimized purification run must be repeated several times to isolate enough material because the column loadability is determined by the amount of the main compound. After the consecutive time or peak-based purification runs, the fractions containing the desired impurity must be pooled together. Depending on the control software for the purification system this can also be done automatically during the fraction collection process.

Requirements for a purification system for impurity analysis:

- Time- and peak-based fraction collection
- Precision for repetitive runs
- Capability to pool fractions automatically
- Fraction containers for large volumes
- Safety features, like leak sensor, for unattended operation

## 4. Column overloading

To increase the throughput on a purification system the preparative column has to be overloaded as described in chapter 1 and chapter 2. As mentioned there, overloading can be done as concentration or volume overloading: In concentration overloading the sample volume is kept as small as possible but the sample concentration is increased. In volume overloading the concentration is kept constant and the injection volume is increased. Both methods have their own restrictions, possible problems and solutions for those problems as described in the following sections.

### 4.1. Injection of high concentration samples

When concentration overloading has to be performed, the limiting factor for the amount of sample that can be loaded onto the column is the solubility of the compound in the sample solvent and the mobile phase. When doing gradient runs with a mobile phase with a high water content at the beginning of the gradient, it is usually impossible to use the same mobile phase composition as sample solvent because the sample solubility in it is rather low. Therefore strong sample solvents, like DMSO or DMF, are used. Although the sample solubility is usually good in those solvents a problem arises right after the injection: As soon as the sample starts to mix with the mobile phase of the gradient starting composition the solubility is

instantaneously decreased and the sample starts to crystallize (figure 39). The flow path of the system is blocked as this happens most of the time in the injector valve. Then the system must be taken apart and the valve has to be cleaned, recovering as much of the sample as possible.

*How can high-concentration samples be injected without precipitation?*

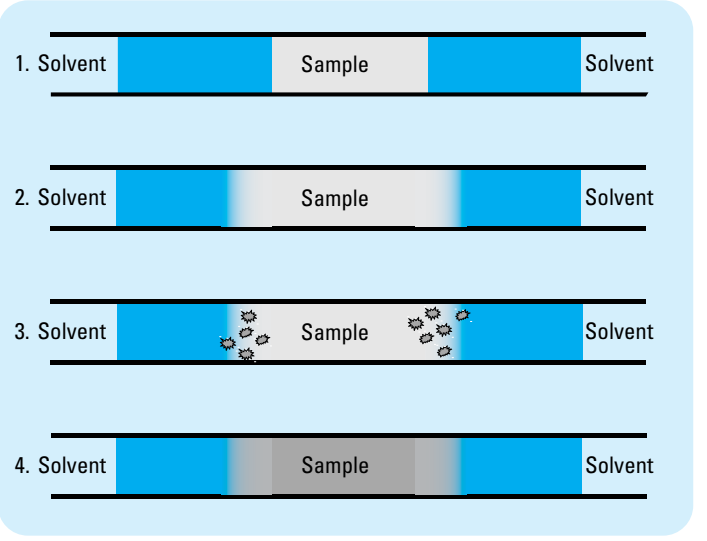



**Figure 39**
**Crystallization of sample during injection cycle**

There are basically two possible approaches to avoid this problem, the organic-phase injection and the sandwich injection.

### 4.1.1 Organic-phase injection

In this approach the autosampler is set up in the flow path of the pump delivering the organic mobile phase prior to the mixing point with the aqueous mobile phase (figure 40)[24] For seamless operation of the system a simple T-piece, positioned as close as possible to the column, must be used instead of a mixer for the mobile phases. As the

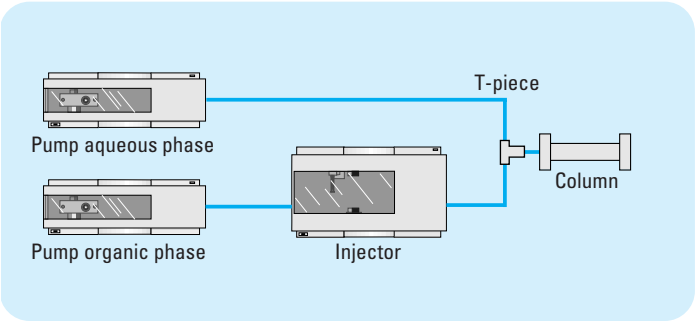



**Figure 40**
**System configuration for organic-phase injection**

mixing performance of the T-piece is much lower than that of a passive mixer, for example, the chromatographic performance may suffer from this disadvantage.

Another shortcoming of this approach is that the sample is transferred to the mixer only by the flow coming from the pump delivering the organic phase, which is rather low at the start of the gradient. While running at a flow rate of 20 mL/min, for example, the flow rate of the pump delivering the organic phase is only 2 mL/min when using a composition of 10 % organic mobile phase as gradient starting conditions. Depending on the delay volume of the pump, the injector and the capillary connections, the gradient starting conditions must be held until the compound reaches the T-piece.

Although this approach prevents sample crystallization in the injector valve very often the blocking problem is just moved to another part of the system: When the sample in the organic mobile phase starts to mix with the aqueous mobile phase in the T-piece the crystallization occurs there. This quite often leads to the blockage of the T-piece but even if the sample crystals are washed to the column

the compound will not be adsorbed on the column material. The crystals will be held back by the frit at the column head, which means the increasing amount of organic mobile phase during the gradient run must dissolve the crystals first before the compound is adsorbed on the column material. As a result no real chromatography is done but a mixture of sample dissolution and chromatography. The overall chromatographic results is rather poor.

### 4.1.2. Sandwich injection

A much better approach to transfer the sample onto the column is by means of sandwich injection. The idea is to protect the sample with a plug of pure sample solvent before and after the sample (figure 41)[25].

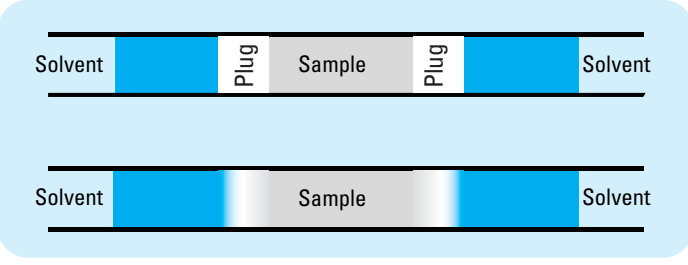



**Figure 41**
**Sandwich injection**

Using an injector program, for example, a plug of pure sample solvent is positioned before and after the actual sample and the complete sandwich is injected onto the system. When the sandwich comes in contact with the mobile phase, the sample starts to mix with the plug from one side and the mobile phase starts to mix with the plug from the other side. Since the plug solvent is the same as the sample solvent the sample will not precipitate on the sample side and because the sample concentration in the plug is zero no precipitation at the solvent side will occur.

It is important to keep the volume of each plug at about 5 -10 % of the sample volume as strong solvents, like DMSO or DMF, can lead to peak broadening or even to the column break-through of the sample. Using two plugs of that size no significant peak broadening or column break-through will be observed.

### 4.2. Injection of high volume samples

*For the injection of sample volumes of several milliliters up to several liters an injection pump is used!*

The limiting factors for volume overloading are usually the maximum injection volume of the autosampler and the volume of the sample container. When volumes of several milliliters up to several liters have to be injected an autosampler is no longer the optimal device, also because the number of samples to be injected is then usually rather low. Therefore large sample volumes are often injected using a manual injector or an injection pump. Basically two approaches are possible using an injection pump to transfer the sample onto the column: Similar to an autosampler the pump can be used to fill a sample loop, which is then switched into the flow path. The advantage of this approach is that a low-pressure injection pump, like a peristaltic pump, for example, can be used. The disadvantage is that the low-concentrated sample in a large sample volume will lead to significant peak broadening in the purification run (figure 3, chapter 1). The second approach requires a high-pressure pump to load the sample directly onto the column. The advantage of the second approach is that the sample is pre-concentrated on the column head before the gradient starts, which leads to better chromatographic results.

An example for a system setup with an injection pump is shown in figure 42[26]. After the sample is applied to the column by the injection pump, the valve is switched and the gradient pump delivers the gradient. During the gradient run the injection pump and the valve must be washed and re-equilibrated prior to the next injection.

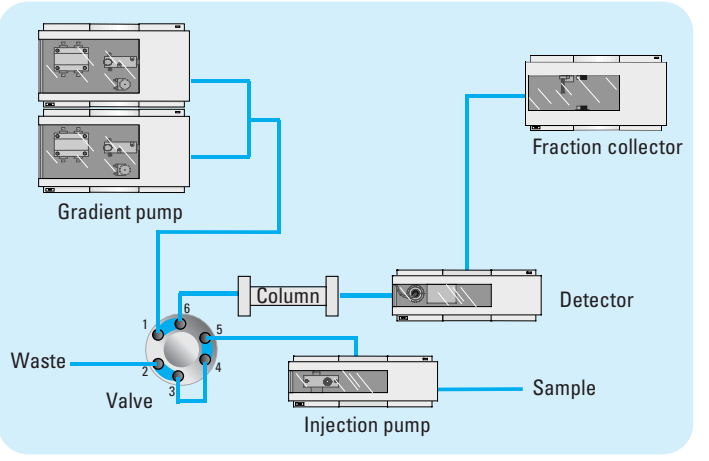



**Figure 42**
**System configuration using an injection pump**

## 5. Recovery collection

During a purification run all fractions of interest must be collected however, depending on the application, it is also necessary to collect everything else in a sample-specific location, the so-called recovery location (figure 43).

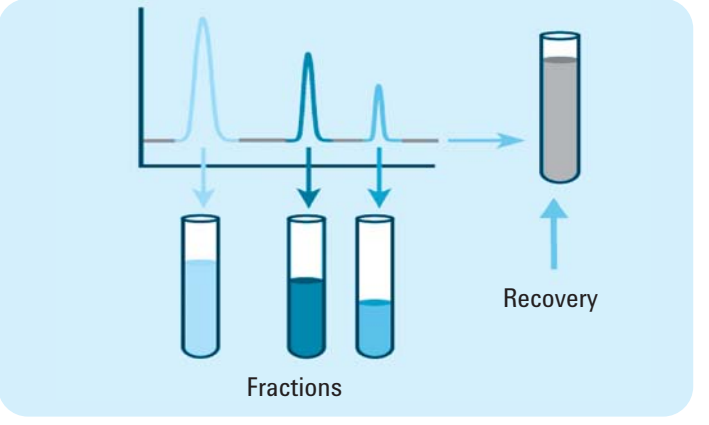



**Figure 43**
**Recovery collection**

*Recovery collection avoids the loss of valuable samples even if no fractions were collected!*

Recovery collection is used in a walk-up environment (see 7. Walk-up system), for very valuable samples and as a safety feature in case a peak or fraction of interest is not collected.

Possible reasons for non-collection are:

- User inputs wrong target mass or formula in mass-based fraction collection
- User selects inappropriate generic method
- Peaks fail to cross trigger threshold of generic methods
- Sample has poor ionization
- Mechanical or software failure

For recovery collection an additional fraction collector or a collection valve is configured into the system, which collects the recovery from the waste line of the fraction collector (figure 44). This ensures that a sample-specific recovery location is used for each sample. The recovery position together with the sample information must be reported to the user. Recovery collection certifies that the sample submitter gets the entire sample back[27].

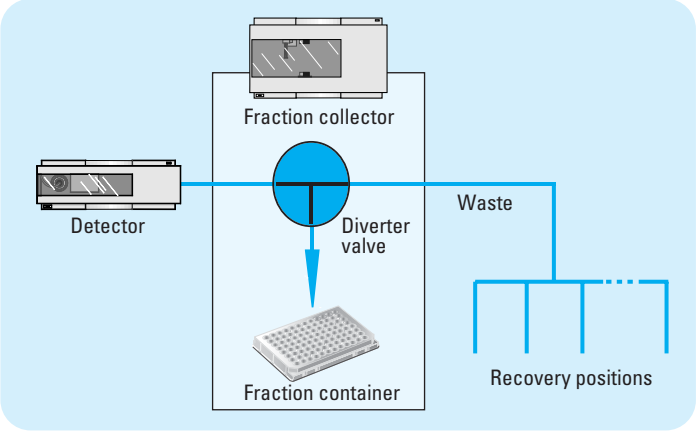



**Figure 44**
**System configuration for recovery collection**

## 6. Automated fraction re-analysis

The purification process of a compound usually consists of three steps as shown in figure 45: In the first step, the pre-preparative analysis, the identity, purity and target compound amount prior to the purification run is measured to decide whether it is worthwhile to purify the sample or not. After the purification run is completed, the fraction

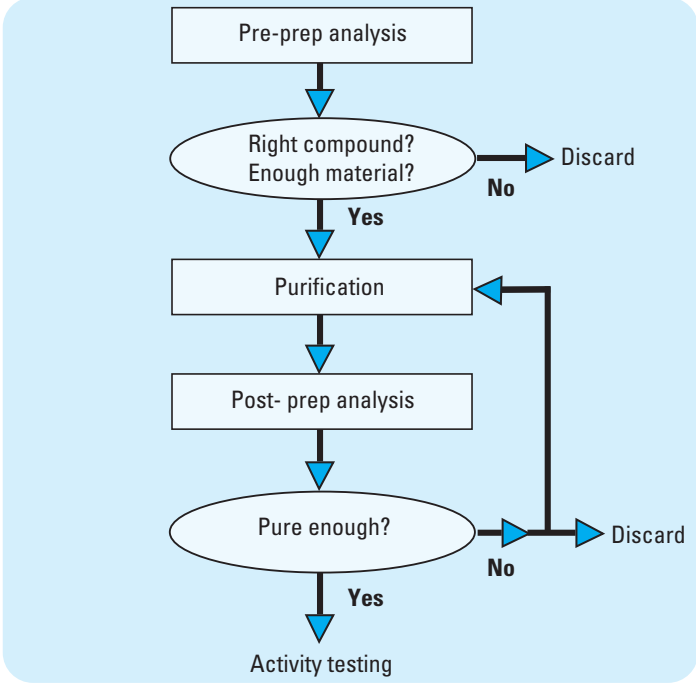



**Figure 45**
**Purification workflow**

containing the target compound must again be analyzed at a certain point in the workflow to assure that the right compound with sufficient purity is provided to the activity testing. Before the sample is sent to activity testing it is usually dried down, weighed, and re-dissolved to a specific concentration in an appropriate solvent, very often DMSO.

*Automated fraction re-analysis can lead to false purity results or even to the complete loss of an active compound!*

Basically there are two stages in this workflow where the purity analysis of the fraction containing the target compound could be done: Either directly after the fraction was collected or just before the compound goes to activity testing. While the first approach offers a high level of automation if the purification system offers the possibility to inject directly from the fraction containers onto an analytical system this process can lead to incorrect results.

Possible problems can be:

- Wrong purity results due to concentration gradients in the fraction
- Wrong purity results due to crystallization
- Unrecognized decomposition of the compound

When a fraction is collected the concentration of the compound in this fraction changes over the time period of collection. The concentration rises to a maximum at the peak apex and falls back to a low value at the end of the peak. This concentration gradient can be seen in the fraction right after the collection but does not disappear even after several hours as shown in figure 46.

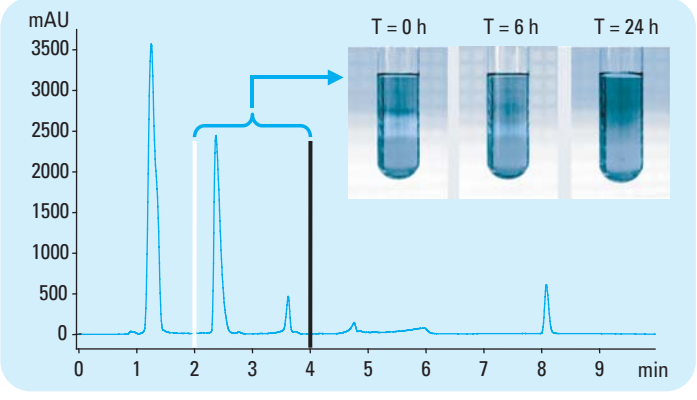



**Figure 46**
**Concentration gradient**

If a small volume is drawn out of a fraction container as shown in figure 46, the resulting analytical run would certainly not give a representative purity result of the complete fraction. The measured purity and concentration would be different depending on where in the vial the sample was drawn. The fraction must be mixed prior to the analytical run to make sure the drawn sample gives a representative result.

The purity measurement directly from the fraction container can also provide incorrect results if crystallization occurs. As the sample is usually submitted in a strong solvent like DMSO or DMF, in which it was highly soluble, the mobile phase consists of water and acetonitrile or methanol. It can be quite often seen that the sample starts to crystallize when the fraction remains in the fraction container over a certain time period. If a sample is now drawn from the liquid phase, the result of the analytical run provides the purity of the liquid phase and not of the complete sample in the fraction container.

The biggest problem of automated fraction collection is that a possibly active compound can be lost due to decomposition between the purity measurement and the activity test. If a purity analysis was done directly from the fraction container, the fraction is afterwards dried down, redissolved to a certain concentration and then a portion is taken for the activity test. If the target compound decomposes during the dry-down process, and this happens to 1 – 10 % of the compounds depending on the dry-down method, it will not be recognized that the decomposition product was given to the activity testing and not the target compound. As there is almost always a second analysis done on a sample that shows activity, such an analysis is not done on a sample that showed no activity.

Therefore the recommendation is to take a second portion from the sample sent for activity testing and do the analytical run and the purity analysis with this second portion on a dedicated, analytical system, which ensures that the result is absolutely representative. It also assures that the purification system is available for purification and is not tied up for routine analytical work[28].

### 7. Walk-up system

*A walk-up purification system can be used in a system administrator/system user environment!*

Depending on the organizational structure of the company but also, for example, on the company size, purification of the samples synthesized by the chemists is done by different people: Either every chemist purifies his samples on a purification system within the group of chemists or submits the samples to a group within or outside the company that does the purification as a service. Another possibility, which is called a walk-up approach, is that the purification system is operated and administered by a purification group but the chemists purify their samples themselves. In this case only the system administrator has complete control over the system and can set up methods, etc. The users have only limited access as specified by the system administrator and must, for example, login to the system using a password before they can use it. Then they are guided through the sample setup process, which usually consists of typing in some basic sample information, like operator name, sample name and target mass. The next step is to assign pre-defined methods to the samples, put the samples into the autosampler and receive notification, for example by e-mail, when the purification runs of their samples are finished. All this, the system administration as well as the interface for the users, is usually handled by dedicated software[29]. After the purification runs of a system user are completed, he is not only interested to get the fractions back but also to have a look at the data.

Therefore it is necessary to provide him not only a simple tool to operate the purification system but also one that allows fast and simple but also efficient data review, preferably at the user's office PC. Agilent not only provides a dedicated walk-up software (EasyAccess) but also has a tool for remote data review, the ChemStation Data Browser.

## References


1 Unger, K.K., "Handbuch der HPLC", *GIT Verlag, Darmstadt*, **1994**

2 Merrifield, R. B., *J. Am. Chem. Soc. 85, 2149*, **1963**

3 Huber, U., "Solutions for sample purification – Agilent 1100 Series purification systems", *Agilent Technologies Solutions Guide 5988-5315EN*, **2004**

4 Huber, U., "Solutions for preparative HPLC – Application Compendium", *Agilent Technologies Application Compendium 5989-5948EN*, **2006**

5 Nägele, E., Huber, U., "Method scale-up from analytical to preparative scale with the Agilent 1100 Series purification system PS", *Agilent Technologies Application Note 5988-6979EN*, **2002**

6 Bidlingmeyer, B.A., Editor, "Preparative Liquid Chromatography", *part of Journal of Chromatography Library, Volume 38, Elsevier Scientific Publishers, New York*, **1987**

7 Brandt, A., and Kueppers, S., "Practical Aspects of Preparative HPLC in Pharmaceutical Development and Production", *LC/GC North America, 20(1), 14-22*, **2002**

8 Majors, R.E., *LC/GC North America, 21(1), 19-26*, **2003**

9 Scott, R.P.W., "Preparative Chromatography", *Chrom-Ed Book Series, free on-line book at http://www.library4science.com/*

10 Grushka, E., Editor, "Preparative-scale Chromatography", *Marcel Dekker; ISBN: 0824780612*, **1988**

11 Rathore, A.S., Editor, "Scale-Up and Optimization in Preparative Chromatography: Principles and Biopharmaceutical Applications", *Chromatographic Science, Vol 88, Marcel Dekker, New York*, **2003**

12 Ganetsos, G., and Barker, P.E., Editors, "Preparative and Production Scale Chromatography", *Marcel Dekker, New York, ISBN: 0824787382*, **1992**

13 Snyder, L.R., Kirkland, J.J., and Glajch, J.L., "Practical HPLC Method Developments", *2nd Edition*, *Chapter 13 "Preparative HPLC Separation"*, *John Wiley and Sons*, *New York*, **1997**

14 Moritz, R., "Innovative fraction collection with the Agilent 1100 Series purification platform", *Agilent Technologies Application Note 5988-9250EN*, **2003**

15 Patent US6106710 A1

16 Huber, U., "Peak-based fraction collection with the Agilent 1100 Series purification system AS – Influence of delay volume on recovery", *Agilent Technologies Application Note 5988-5746EN*, **2002**

17 Huber, U., "Sophisticated peak-based fraction collection – working with up and down slope", *Agilent Technologies Application Note 5989-0511EN*, **2004**

18 Rosentreter, U., Huber, U., "Optimal Fraction Collection in Preparative LC-MS", *J. Comb. Chem*, **2004**

19 Moritz, R., Huber, U., "Mass-based fraction collection of compound libraries using the Agilent 1100 Series purification system", *Agilent Technologies Application Note 5988-7112EN*, **2002**

20 Rosentreter, U., Huber, U., "Purification of pharmaceutical drugs by mass-based fraction collection at higher flow rates", *Agilent Technologies Application Note 5988-7113EN*, **2002**

21 McConnell, D., Lubich, P., Egger, G., Huber, U., "Development of a compound purification strategy for a medicinal chemistry group", *Agilent Technologies Application Note 5989-0055EN*, **2003**

22 Nägele, E., Huber, U., "Isolation of formononetin and other phytoestrogens from red clover with the Agilent 1100 Series purification system", *Agilent Technologies Application Note 5988-5747EN*, **2002**

23 Moritz, R., Huber, U., “Isolation and purification of reaction by products using the Agilent 1100 Series purification system”, *Agilent Technologies Application Note 5988-5748EN*, **2002**

24 Blom, K. F., “Two-Pump at-Column-Dilution Configuration for Preparative Liquid Chromatography – Mass Spectroscopy”, *J. Comb. Chem. 4, 295-301*, **2002**

25 Leister, W., Strauss, K., Wisnoski, D., Zhao, Z., Lindsley, C., “Development of a Custom High-Throughput Preparative Liquid Chromatography/Mass Spectrometer Platform for the Preparative Purification and Analytical Analysis of Compound Libraries”, *J. Comb. Chem. 5, 322-329*, **2003**

26 Foster, G., O’Hanlon, R., Huber, U., “Injection of large samples volumes using the Agilent 1100 Series purification system with an injection pump”, *Agilent Technologies Application Note 5989-0029EN*, **2003**

27 Huber, U., Woodhouse, A., “Recovery collection with the Agilent 1100 Series purification system”, *Agilent Technologies Application Note 5988-9650EN*, **2003**

28 Huber, U., “Automated fraction re-analysis – does it really make sense?”, *Agilent Technologies Application Note 5988-8653EN*, **2003**

29 Duncan, W., McIntyre, D., “Increasing Productivity with LC/MS Easy Access Software”, *Agilent Technologies Application Note 5988-5525EN*, **2002**


Printed in Germany
April 2007

Publication Number 5989-6639EN

www.agilent.com/chem/purification

Agilent Technologies