

Laboratory Chromatography Guide



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Publisher Büchi Labortechnik AG, CH-9230 Flawil, Switzerland
Cover NOSE Applied Intelligence AG, CH-8005 Zürich, Switzerland
Layout Atelier Güttinger AG, CH-9030 Abtwil, Switzerland

First edition
Printed in Switzerland
94175 0105

ISBN 3-033-00339-7

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“Laboratory Chromatography Guide” – A close look at preparative liquid chromatography

The present “Laboratory Chromatography Guide” is dedicated to preparative liquid chromatography, a common purification technique in most chemical or life science laboratories.

The performance of separations by chromatography is fairly well known in the scientific and industrial communities. Part 1, the “Flash Guide Basics”, gives consideration to this fact, proceeding swiftly through flash chromatography with an emphasis on speed, reliability and reproducibility of the separation.

But there are no rules without exceptions! As usual, problems appear with the most exciting and valuable compounds you want to purify. Therefore, you are personally challenged to understand and solve the purification task as fast as possible. The second part “Preparative Column Chromatography: Theory and Practice” helps you to overcome such drawbacks and leads you back to the shining path of your privileged profession: to understand and explore what modern science offers!

We at Büchi, as a leading supplier of high quality laboratory products and responsive services, wish you a lot of challenging and successful work!

Dr. Ernst Freydl
Büchi Labortechnik AG

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Abbreviations

TLC	Thin-layer chromatography
HPLC	High-performance liquid chromatography
$[C]_{\text{phase 1}}$	Concentration of the compound C in phase 1
GC	Gas chromatography
RPC	Reversed phase chromatography
S_i	Solvent strength
RI	Refractive index
S.I.	Symmetry index
F_m	Delivery rate
V_0	Dead volume
GLP	Good laboratory practice
MPLC	Medium pressure liquid chromatography
LC	Liquid chromatography
UV	Ultraviolet

Introduction

Chromatography has developed very rapidly over the past few years. It was a very long way from the first “capillary pictures” of Runge (1822–1850) through the early work of Tswett, the discoverer of Adsorption Chromatography (1903, separation of plant pigments) to modern HPLC from about 1967. Tswett had in fact adopted the name “Chromatography” for this separation technique (from the Greek *chromos* = colors, *graphein* = write).

However, the focal point of this enormous development was clearly in the area of analysis. In preparative chemistry, on the other hand, chromatographic separations are frequently carried out even today by a very simple method, i.e. with the aid of a simple glass column under hydrostatic pressure. The first publications on preparative chromatography under elevated pressure, so-called Flash Chromatography, only appeared towards the end of the seventies. This method too was subsequently further refined. This finally resulted in medium pressure liquid chromatography (called MPLC in the following), which is very efficient but nevertheless readily comprehensible and simple to carry out. At the same time, attempts were made to increase the size of the analytical HPLC systems and thus make them available also for preparative or at least semi-preparative work.

However, closer scrutiny reveals substantial differences between routine analysis and preparative separation. It is therefore essential for a preparative MPLC system to meet the specific requirements for such separations. The following factors must be noted in particular:

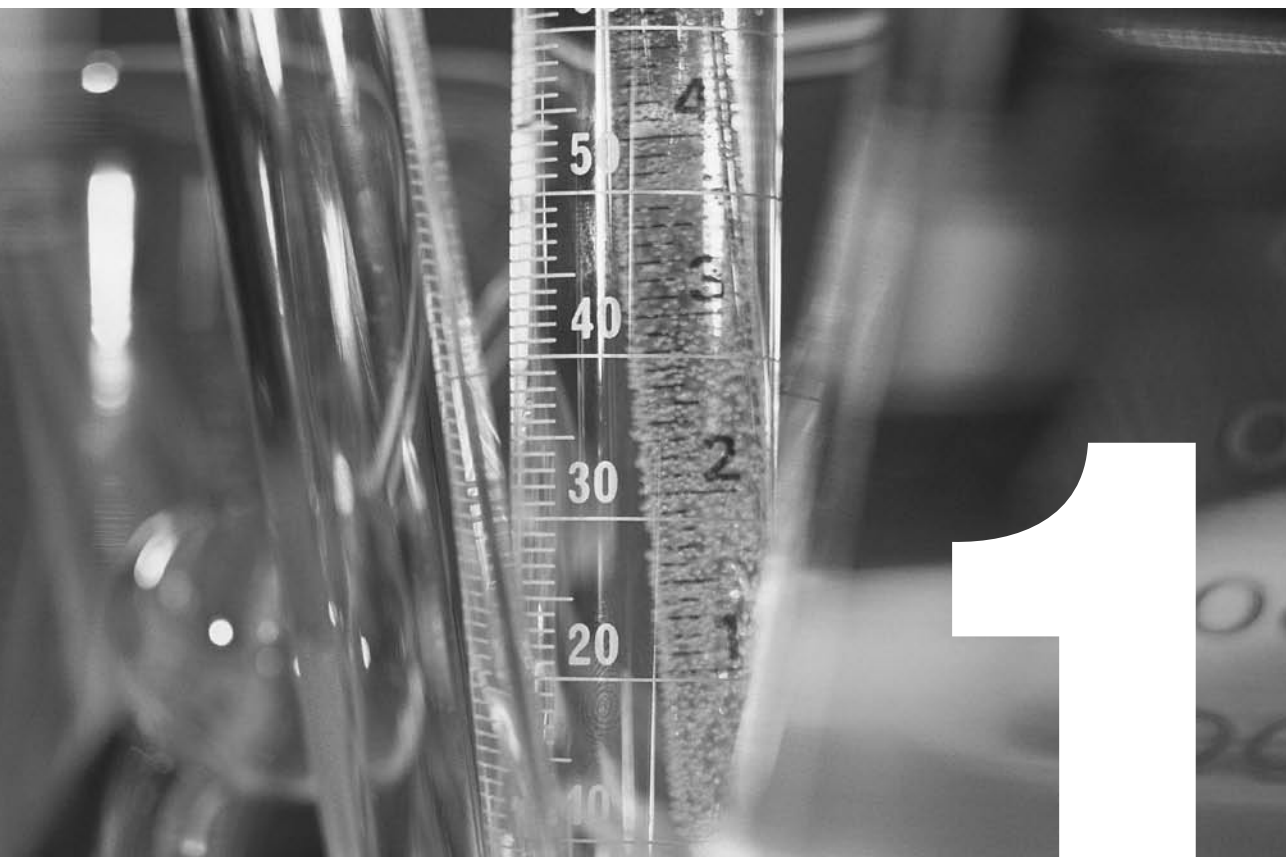
- Flexibility in the choice of column. The amount of substance and the required separating power differ for virtually every problem to be solved. Simple and economical adaptation to the particular separation problem must therefore be possible.
- High delivery of the pump. Large columns require large volume flows so that the desired linear flow rate can be achieved.
- Wide pressure range. The trend in preparative chromatography is clearly towards fine-grained adsorbents, which offer substantial resistance to flow.
- The apparatus must be simple to handle. In particular, filling and emptying of the columns as well as operation of the entire remaining system must be capable of being mastered immediately without a prolonged familiarization period. In the preparative laboratory, the liquid chromatography is in general not a specialized unit but rather a universal tool.

This booklet aims to provide both non-specialists and specialists with short and basic as well as with more detailed explanations of the different procedure steps encountered during a liquid chromatography separation.

The first part, "Quick Guide", is a short, practice-oriented overview of liquid chromatography (LC) for quick reference searches and the second part provides a broader and deeper description of the process, under both practical and theoretical considerations.

Flash Guide

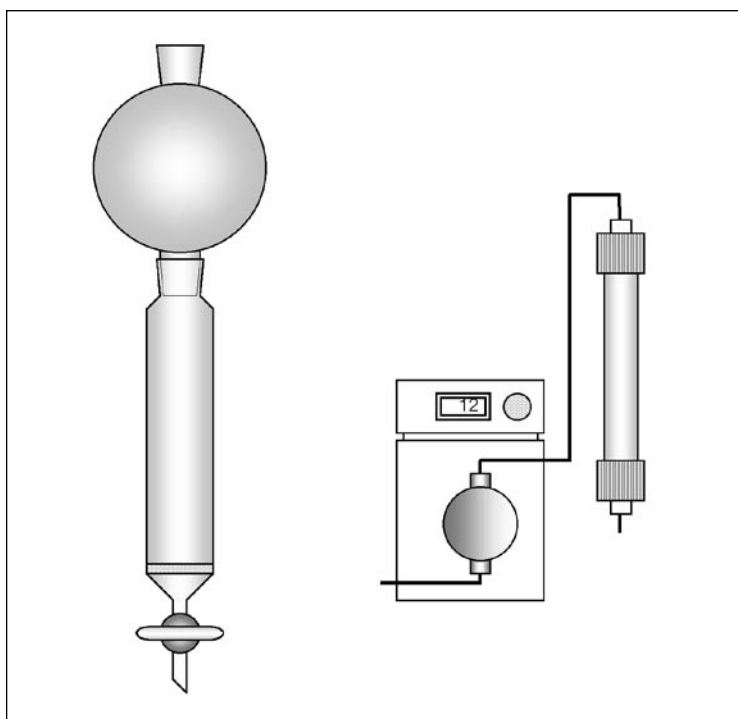
Basics



1 Introduction

Chromatography is a standard method used in preparative laboratories to isolate and purify substances. In the early days of chromatography simple glass columns were chiefly used, operated by means of the hydrostatic pressure of the solvent acting as an eluent. In a publication in 1978 Clark W. Still explored the possibility of accelerating the separation process in simple glass columns, which was until then the commonly used method, and thereby considerably increasing the efficiency of the technique. The results were convincing and the foundations of modern flash chromatography were laid. It triumphantly established itself in laboratories as an indispensable purification method in preparative chemistry. Flash chromatography has since undergone constant development, and has been adapted to meet present day expectations in terms of equipment and convenience.

*Figure 1:
From the simple glass
column to modern flash
chromatography.*



Modern flash chromatography systems are popular nowadays because they are simple to handle, flexible and can be universally employed. The first part of this brochure aims to give simple, accessible advice, which should ideally instantly lead to effective laboratory elutions.

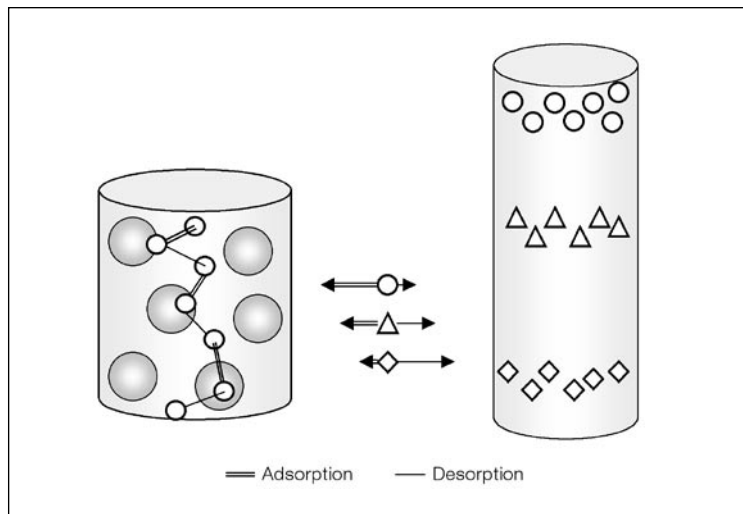
The following abbreviations are used in the first part:

TLC	Thin-layer chromatography
RP	Reversed phase, modified silica gels
NP	Normal phase polar silica gel phases
UV	Ultraviolet
S_i	Solvent strength (substitutes polarity)
% A	% solvent with low solvent strength
% B	% solvent with high solvent strength
R _f	Retention factor (from thin-layer chromatograms)
CV	Column volumes
ΔCV	Difference in column volumes
R _{f1}	Retention factor of first substance (substance which spreads onto the TLC plate the quickest. The index increases according to the time the substance takes to spread).

2 Principle of chromatography

Chromatographic separation is based on a balanced state among the components to be separated, an adsorbent agent in the column (= stationary phase) and a solvent flowing through it (mobile phase). When a component settles on the stationary phase this is defined as adsorption, while detachment by the mobile phase is defined as desorption. A high adsorption capacity between the components of interest and the stationary phase means that there is a high retention of these components and that there is a considerable delay in elution from the column. The separation of a mixture into its individual components is only possible if the individual components in a combination of stationary and mobile phases have different adsorption/desorption properties.

Figure 2:
Adsorption und
Desorption, schematic
illustration of the
chromatographic
separation process.



3 Choice of the appropriate stationary phase

Chromatographic separation can be carried out on both polar and apolar stationary phases, and suitable sorbents are available from various manufacturers.

“Standard” chromatography requires the use of polar stationary phases such as silica gel and nonpolar solvents. The individual components are delayed as a result of a reaction between the polar function component groups and the polar groups of the sorbent. Low polarity substances are eluted first, followed by components of increasing size.

In “reversed phase” chromatography, however, the stationary phase is nonpolar and elution is by means of polar solvents. These stationary phases are produced by modifying silica gel with nonpolar groups such as C-18 or similar substances. Substances are eluted in order of decreasing polarity from reversed phase columns, i.e. the substance with the highest polarity appears first. Reversed phase materials are considerably more expensive than standard stationary phases, and this is one of the reasons why standard stationary phases are primarily used in flash chromatography. If the substance classes to be separated allow, modified stationary phases can nonetheless be used without restrictions or problems.

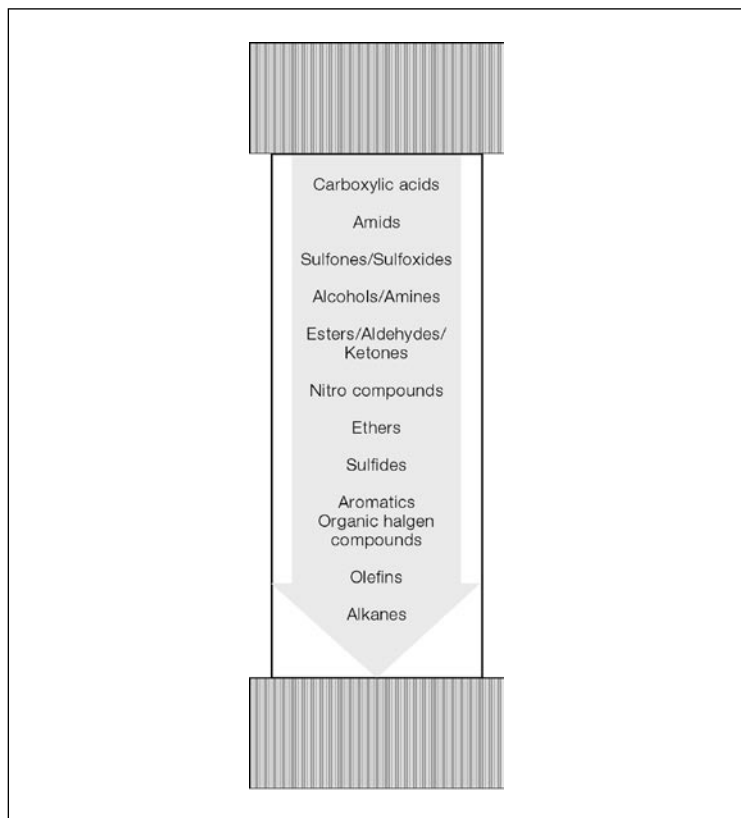


Figure 3:
Elution sequence for
normal silica gel.

4 Evaluation of the chromatographic system by thin-layer chromatography (TLC)

As mentioned earlier, most elutions in flash chromatography use normal silica gel, or modified silica gel in special cases or for highly polar substances. In all these cases it is advisable to carry out a thorough TLC pre-elution so that, with a minimum investment of time and material, promising elution conditions can be found, which can then be applied to the cartridge. The following applies:

1. Define stationary phase
2. Find mobile phase with best selectivity
3. Set solvent strength

Ideally the sorbents on the TLC plate and in the cartridge should be identical (type and pore size) so as to successfully apply TLC conditions to the cartridge!

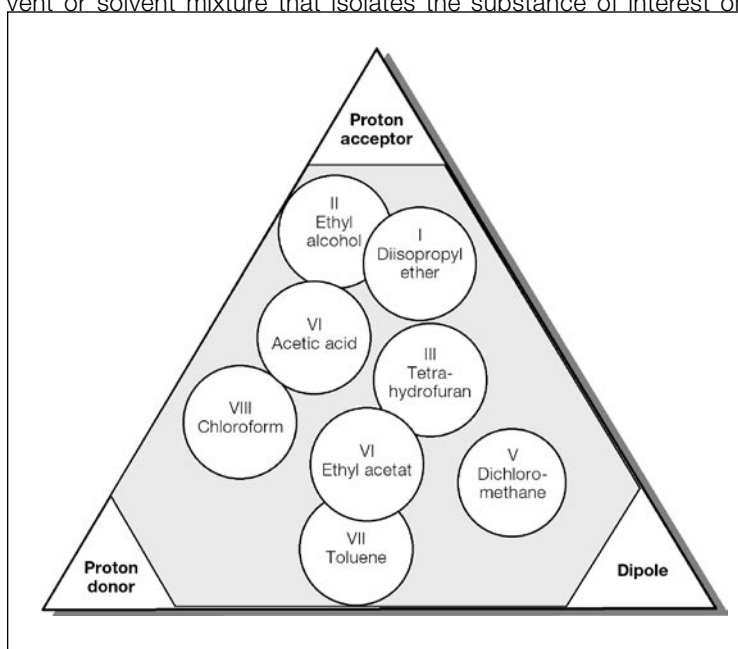
4.1 Evaluation of the stationary phase

The laboratory's experience with TLC tests, with which most laboratories are familiar, can help to you make the right choice. If TLC plates with normal silica gel are used for the tests, column separation can also be carried out using normal silica gel. If the results of this prove unsatisfactory, it is then advisable to switch to RP plates.

4.2 Selectivity of the solvent

Once the stationary phase has been established the mobile phase with the most suitable selectivity needs to be found, i.e. the solvent or solvent mixture that isolates the substance of interest on

Figure 4:
Selectivity triangle
with various selectivity
groups.



the TLC plate with the greatest possible distance to the adjacent components.

In general every solvent has its own defined selective properties; some tend to be similar to each other, while others can differ greatly. L.R. Snyder and J.J. Kirkland investigated and compared the properties resulting from various solvents and grouped solvents with similar effects together into what are known as **Selectivity Groups**.

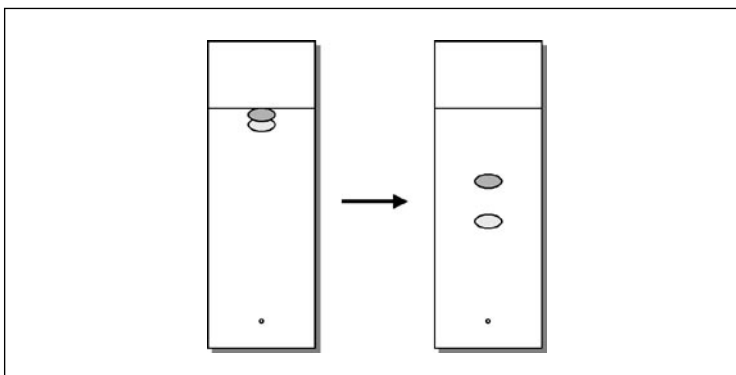
The selectivity groups allow us to focus our search. There is little point in comparing different solvents from the same selectivity group, as they all have the same properties. What we have to do is compare solvents from the various selectivity groups, as this is the only way to see the difference immediately. The most important solvents for our separation are compiled in the following table. This only shows solvents that are suitable for separation with UV detection, and do not make detection impossible as a result of high energy absorption.

Solvent	Group	Strength S_i	UV limit
n-Hexane	–	0.1	200
Cyclohexane	–	0.2	210
Diisopropyl ether	I	2.4	220
Diethyl ether	I	2.8	220
Ethanol	II	4.3	200
Methanol	II	5.1	200
Tetrahydrofuran	III	4.0	220
Acetic acid	IV	6.0	
Dichloromethane	V	3.1	250
Ethyl acetate	VI	4.4	260
Aceton	VI	5.1	330
Acetonitrile	VI	5.8	210
Toluene	VII	2.4	290
Xylene	VII	2.5	290
Chloroform	VIII	4.1	250

Table 1:
The most common solvents with selectivity group allocation and solvent strength S_i .

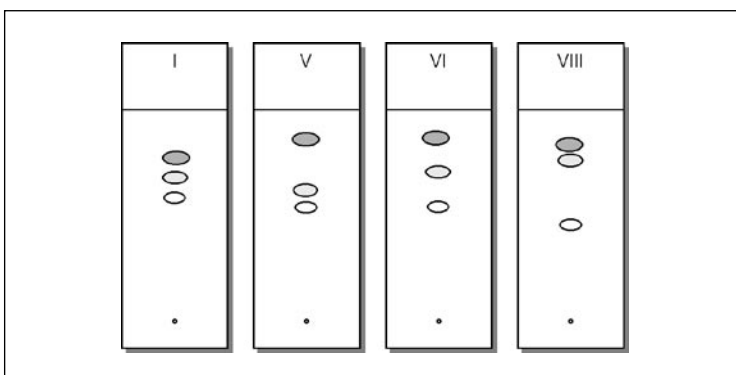
Depending on the polarity of the components to be separated, the entire mixture can flow onto the TLC plate with the solvent front; the solvent is too strong and the TLC separation cannot be assessed in this form. In these cases the solvent strength is reduced by diluting the solvent with hexane, for instance, and the TLC separation is then repeated.

Figure 5:
Reducing solvent strength so that the selectivity can be assessed in the first place. The TLC on the left was developed in dichloromethane, and the TLC on the right in hexane/dichloromethane 3:1.



Adding hexane reduces the solvent strength, but does not affect the selectivity!

Figure 6:
Evaluation of the optimal selectivity. In this example this is clearly in the system of selectivity group VI, where the individual components have been separated most effectively. If there is only interest in component 1 (top mark), the choice is V.



Ideal = the other directly adjacent substances are separated as well as possible from the substance of interest.

4.3 Solvent strength

Every solvent has its own characteristic strength (which used to be known as its polarity). The higher the figure, the stronger the solvent and the quicker substances are transported through the chromatographic system. Rapid transport through the column does however mean that there is less interaction between the stationary and the mobile phase, and that the separation is therefore not as effective. It is thus very important to have the correct solvent strength so as to achieve optimum separation results.

Solvent	Group	Strength
n-Hexane	–	0.1
Cyclohexane	–	0.2
Diisopropyl ether	I	2.4
Diethyl ether	I	2.8
Ethanol	II	4.3
Methanol	II	5.1
Tetrahydrofuran	III	4.0
Acetic acid	IV	6.0
Dichloromethane	V	3.1
Ethyl acetate	VI	4.4
Aceton	VI	5.1
Acetonitrile	VI	5.8
Toluene	VII	2.4
Xylene	VII	2.5
Chloroform	VIII	4.1

Table 2:
Strengths of the most
common solvents.

Using this table, solvents with different selectivity and usually with different strengths can be set at identical solvent strengths and thereby directly compared by mixing them with unselective solvents such as hexane. The diagram in figure 8 shows the mixing ratios for the most common solvents.

$$S_i = \frac{\% \text{ solvent A}}{100} + \frac{\% \text{ solvent B}}{100} \quad \text{B = Solvent with higher polarity}$$

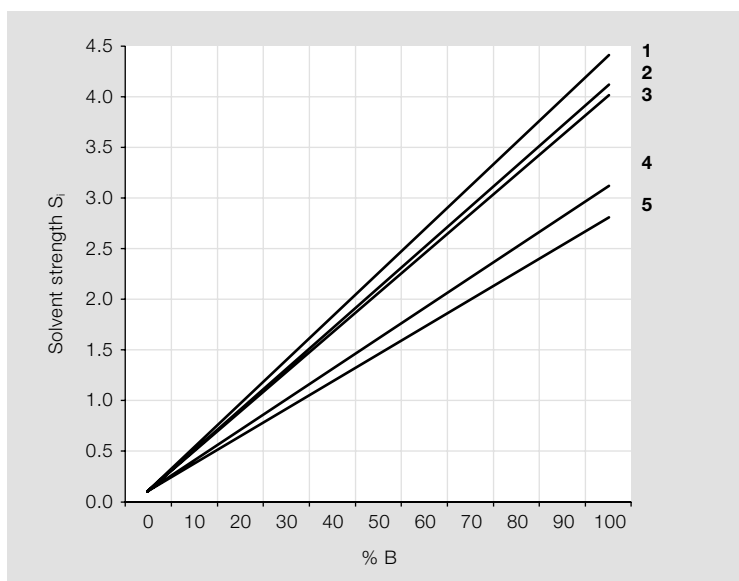
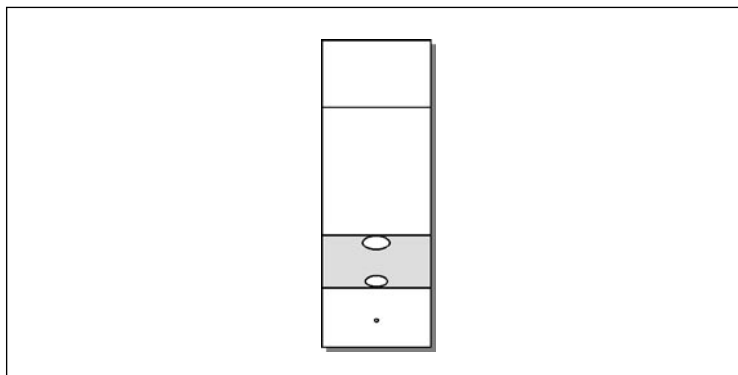


Figure 7:
Setting the solvent
strength.
1 = Ethyl acetate
2 = Chloroform
3 = Tetrahydrofuran
4 = Dichloromethane
5 = Diisopropyl ether

solvent strength should be set so that the resulting substances are approximately 0.15–0.4.

Figure 8:
Optimum Rf range
to transfer the results
to the flash cartridge
are 0.15–0.4.



Why such low Rf values? The reason for this is evident if we look at the relationship between Rf values and column volume (CV).

An Rf value of 1 in the TLC means that the corresponding substance with the solvent front is flowing. The substance would also move with the solvent front in a flash cartridge and after 1 column volume would leave the cartridge. At an Rf value of 0.1 the flow distance is $\frac{1}{10}$ of the front distance – the substance would need 10 times longer to reach the front or in turn to reach the column exit, i.e. 10 column volumes. The substance would be held back for much longer and other components would therefore be separated. The following applies to the relationship between column volume and the Rf value:

- Column volume $CV = \frac{1}{Rf}$
- Rf value ranging from 0.15–0.4, corresponding to 2.5–6.6 column volume.

Table 3:
Correlation of Rf
values and column
volumes.

Rf value	Column volume CV	Rf value	Column volume CV	Rf value	Column volume CV
0.9	1.11	0.6	1.67	0.3	3.33
0.8	1.25	0.5	2.00	0.2	5.00
0.7	1.43	0.4	2.50	0.1	10.0

Summary

Optimize the TLC conditions by applying the following rules:

1. Use identical silica gels if at all possible (same type and pore size) for TLC plates and flash cartridges. Different silica gels behave differently.
2. Look for suitable selectivity. The ideal selectivity separates the components of interest well before adjacent components or impurities. The greater the difference, the more efficient the flash separation.
3. Optimize the solvent strength. Ideal solvent strengths display

Rf values ranging from 0.15–0.4 in TLC for the components of interest; the $\Delta CV > 1$.

Apply these conditions to the flash cartridge.

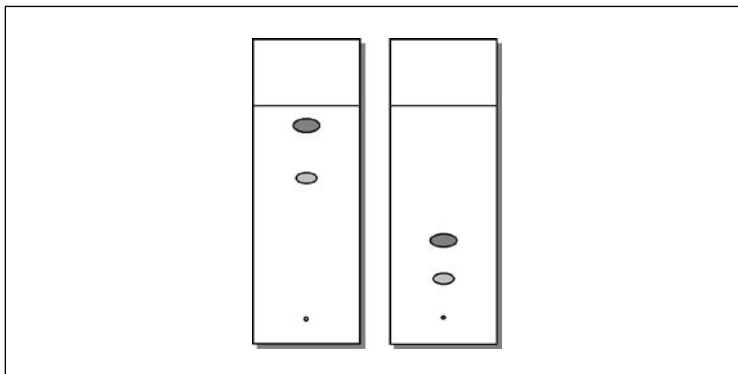


Figure 9

Example of pre-elution using TLC and transferring the results to a Büchi cartridge

Step 1: Selectivity

Substance	TLC 1			TLC 2			TLC 3		
	Rf	CV	ΔCV	Rf	CV	ΔCV	Rf	CV	ΔCV
1	0.54	1.8		0.56	1.8		0.61	1.6	
			0.5			0.6			0.3
2	0.43	2.3		0.42	2.4		0.45	1.9	
			0.6			1.4			1.0
3	0.34	2.9		0.27	3.8		0.35	2.9	
			3.3			8.7			2.3
4	0.16	6.2		0.08	12.5		0.19	5.2	

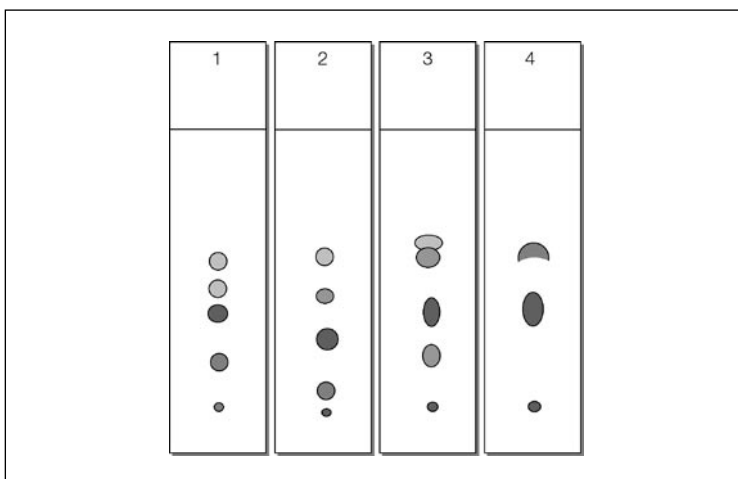


Figure 10: Evaluation of the mobile phases in terms of selectivity and assessment.

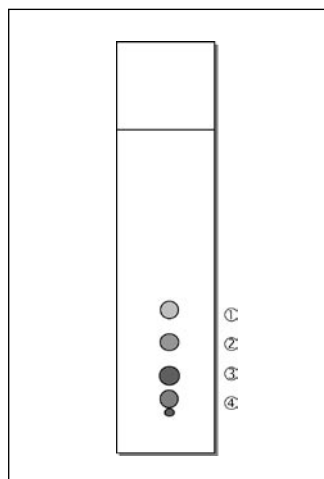
- 1 = ethyl acetate
- 2 = diisopropyl ether
- 3 = chloroform
- 4 = dichloromethane

TLC 2 clearly displays the best selectivity. TLC 4 was not evaluated.

Step 2: Solvent strength

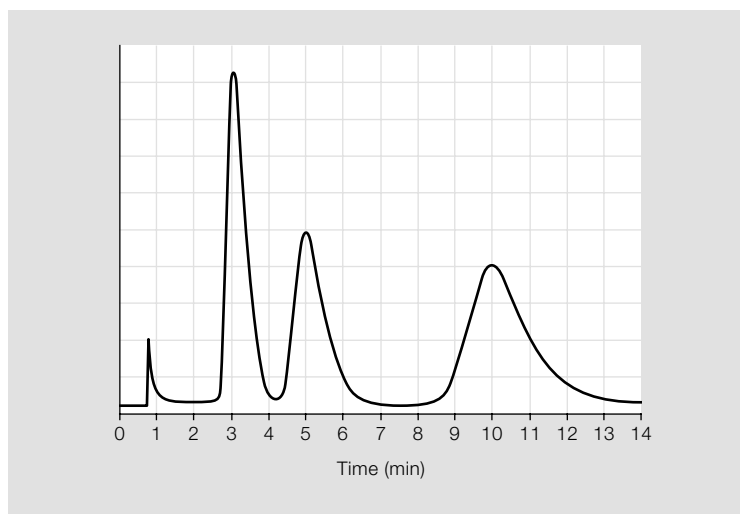
Figure 11:
Setting the solvent strength. The ratio of hexane/diisopropyl ether = 3:1. Components 2 and 3 are of interest.

Substance	TLC		
	R _f	CV	ΔCV
1	0.37	2.7	
			1.3
2	0.25	4.0	
			4.4
3	0.12	8.4	
			16.8
4	0.04	25.2	



Step 3: Applying result to the cartridge

Figure 12:
Applying the conditions to a Büchi flash cartridge 12x150 mm. Eluent = hexane/diisopropyl ether 3:1, flow rate 14 ml/min, detection = UV 254 nm.



5 Injection/Column loading

Injecting the sample is usually a simple procedure in analytical chromatography. The quantities to be injected are low and solubility is hardly an issue.

In preparative separations, on the other hand, the columns are overloaded and the injection of the sample is of primary importance.

When loading the column the mixture to be separated should be applied to the column bed in as compact a form as possible, i.e. in a narrow horizontal band. Preparative separations are usually in larger quantities, i.e. grams.

For a long time the general rule for preparative separations was that a column can be loaded with an approximately 1% mixture, in terms of the silica gel level. The use of modern flash systems and optimizing the mobile phase (R_f 0.04–0.4, $CV > 1$) means that nowadays the load can be increased to up to 10% – separation is faster and more cost effective – more efficient all round!

ΔV_s	Approximate possible load in g*			
	Cartridge 12x75 mm	Cartridge 12x150 mm	Cartridge 40x75 mm	Cartridge 40x150 mm
1	0.15	0.3	1.2	2
2	0.3	0.6	2.5	5
6	0.6	1.2	5	10

Table 4:
Approximate values
for loading at
 $R_f = 0.15-0.4$.

* Values are given as a guide and depend on the silica gel used and the percentile sample composition

Example of preparative separation at high load

Optimizing the conditions on the TLC plate silica gel 60:
Hexane/diisopropyl ether 95:5 ($CV > 1$, $\Delta CV > 1$).

Substance	R_f	CV	ΔCV
1	0.95	1.05	
			1.1
2	0.48	2.1	
			2.2
3	0.23	4.3	

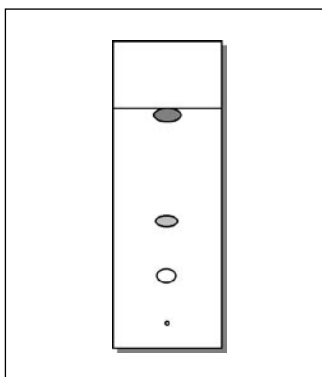


Figure 13:
Optimized conditions
on TLC.

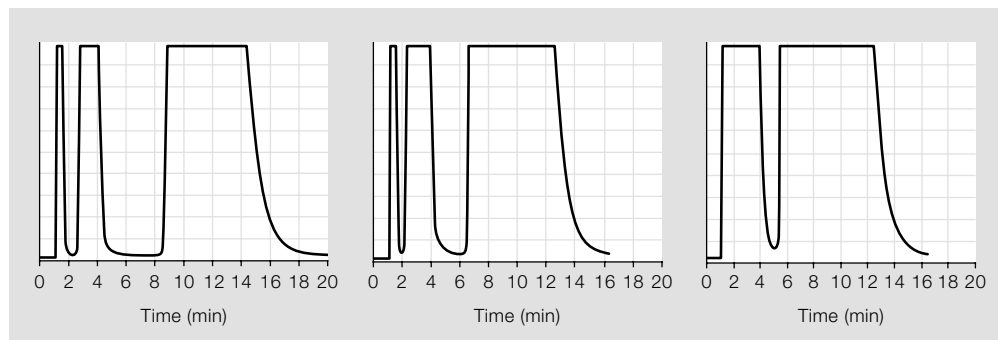


Figure 14:
The impact of increasing the load on the separation. Cartridge 12x150 mm. Silica gel 60, 40–63 μm , eluent hexane/diisopropyl ether 95:5, 14 ml/min, load (from left to right) 300 mg, 600 mg and 1200 mg. Injection volume 1 ml.

The volume must be low so that the sample can be compactly applied to the column bed. If the volume is too high, the band is considerably widened and the separation is less efficient.

The sample that is to be separated can be brought into the separation system either as a solution or dry, adsorbed by silica gel. A classical solution sample injection requires that the sample can be sufficiently dissolved in the starting eluent. The injection volume should be no more than 10% of the column volume. The following injection volumes apply to the Büchi cartridges:

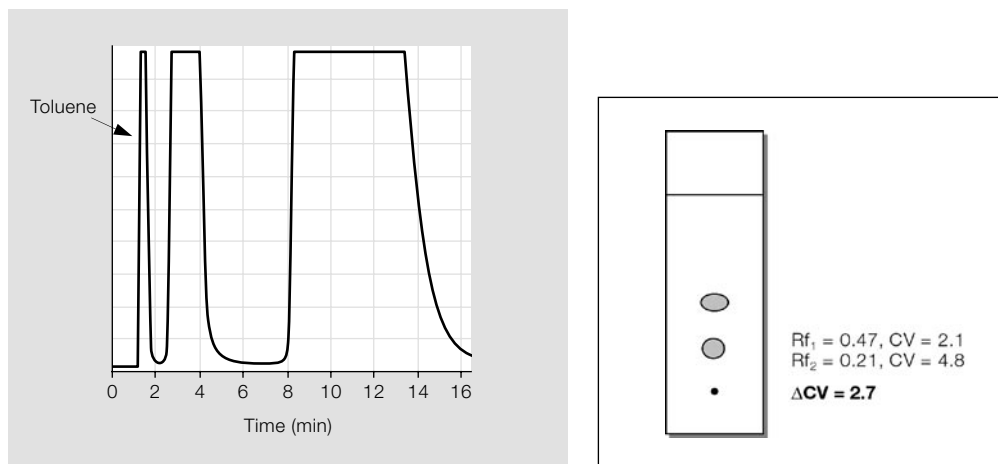
Table 5

Recommended max. injection volumes

Cartridge 12x75 mm	Cartridge 12x150 mm	Cartridge 40x75 mm	Cartridge 40x150 mm
1.5 ml	2 ml	10 ml	20 ml

If the sample cannot be sufficiently dissolved in the starting eluent, dry application can be carried out, where the sample is dissolved in any solvent and mixed with silica gel. The solvent is then distilled off using rotation. This dry silica gel is packed into a precolumn and this is then fitted in front of the separation column into the eluent flow. The components to be separated are then constantly eluted from the precolumn to the actual separation column. This procedure is also advisable if there are sticky or solid impurities in the sample which cannot easily be removed!

Another slightly unconventional injection method for samples in the form of solutions is not to dissolve the sample in the starting eluent but in a completely different solvent with excellent dissolving properties for the mixture. This “foreign” solvent is separated in the separation flow as an additional component. Retention times are usually in the range of the solvent front. If the components of interest are optimized to an R_f range of 0.15–0.4, separating the front is not a problem anyway.



This simple and unconventional injection procedure is often used to inject by-products that are not easily dissolved to the separation column. These substances are usually heavily adsorbed in the area where they initially enter the column and remain there. This is not, however, a problem if disposable cartridges are used, as the cartridge is changed anyway after the components of interest have been eluted.

Figure 15:
Liquid sample injection
in toluol, eluent = he-
xane/diisopropyl ether
9:1.

Special advice for injecting dissolved samples

In preparative chromatography, it is often not possible to spend the time pre-cleaning the samples and the mixtures are applied directly to the separation column with varying levels of accompanying substances. To ensure that the flash equipment operates smoothly it is therefore very important to **rinse the injection port clean after every injection, regardless of whether it is a quick stop valve or a device fitted with a tap system**. This is the only way to avoid problems such as sample contamination or leaking injection ports. The following should therefore be observed in the injection process:

1. Stop pump
2. Inject sample
3. Rinse injection port
4. Start pump

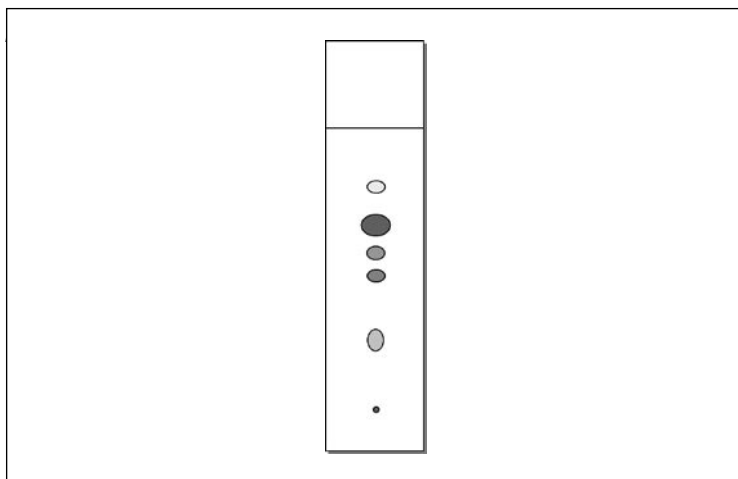
6 Gradient elution

The examples given are all separated isocratically, i.e. the mobile phase is identical throughout the entire separation process. In practice this is, however, often not possible, as the substances to be separated in adsorption often differ.

Substances that cannot be successfully eluted isocratically can be identified by pre-elution using TLC, and can be optimised accordingly. The following example explains the procedure:

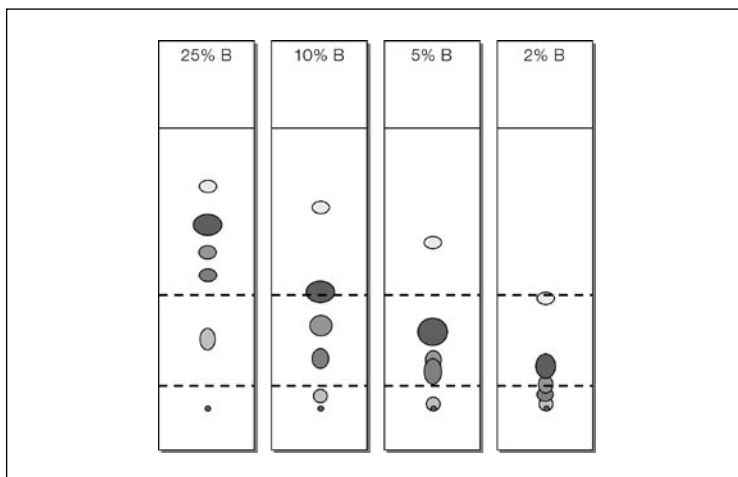
a) *Establishing the suitable selectivity (see 4.2).*

Figure 16:
Suitable selectivity
using ethyl acetate.



This is problematic – either the R_f values are so high that it is practically impossible to achieve separation with a preparative loading of the column, or the R_f values are so low that they can only be eluted from the column with a great deal of time and solvent.

Figure 17:
Different solvent
strengths, achieved
using hexane with
varying levels of ethyl
acetate.



As there is no basic common denominator for the conditions, the R_f values for highly adsorbent and less adsorbent components are optimised separately (both R_f values from 0.15–0.04).

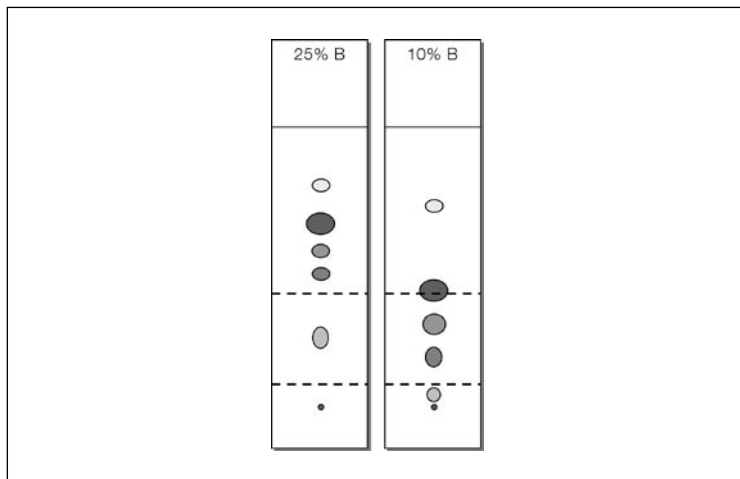


Figure 18: Selection the mobile phase for a step elution hexane with varying levels of ethyl acetate according to the indication on the TLC plate.

These are the conditions that apply when applying the separation to the column. The less adsorbent components are eluted with the weaker eluent. We then switch to the polar mobile phase (= higher level B). Depending on the equipment used, this switch can either be carried out gradually or in one step.

The following examples show how the separation can be affected by the choice of solvent strength. The level of ethyl acetate is entered in the chromatogram (% B).

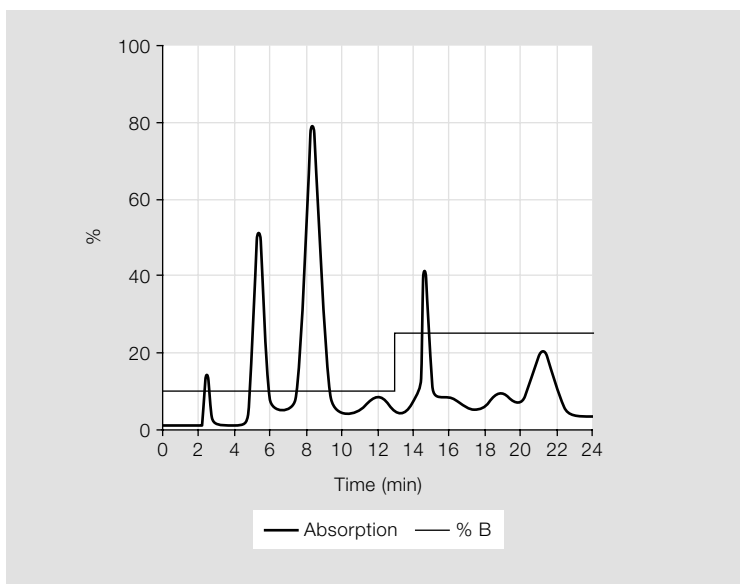


Figure 19: Separation using a step gradient 10% B/25% B.

Figure 20:
Separation with a
continuous gradient
10 \Rightarrow 20% B in 10 min,
20 \Rightarrow 45% B in 5 min,
then 45% B constant.

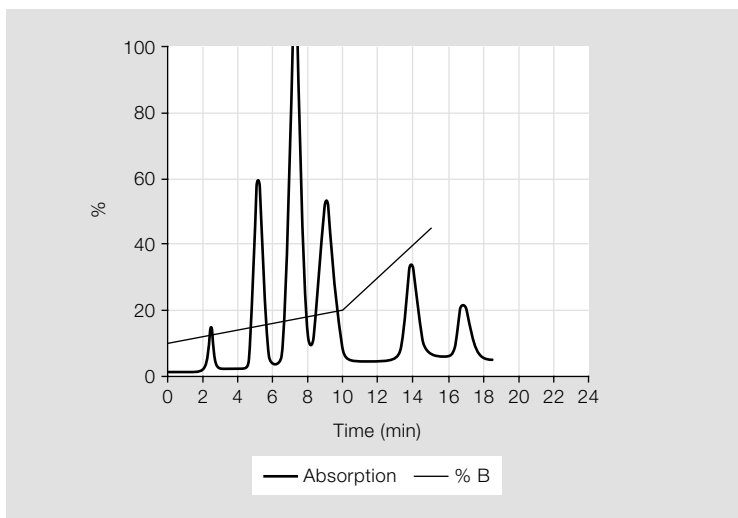
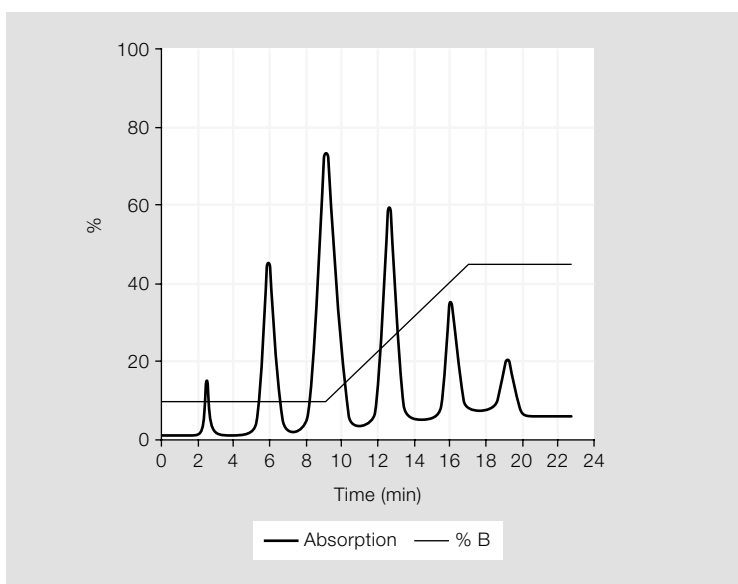


Figure 21:
Separation with
continuous gradient
10% B for 9 min,
10 \Rightarrow 45% B in 8 min,
then 45% B constant.



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ISBN 3-033-00339-7