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MZ ANALYSENTECHNIK

General tips for preparative HPLC

1. General Information

The aim of preparative chromatography is to isolate, purify and collect substances in sufficient quantity and purity for subsequent use. Typical applications include the purification of peptides with reversed phase chromatography, the separation of enantiomers on chiral phases or preparative SFC applications in the pharmaceutical industry up to small research groups for natural products. The basic setup of a preparative HPLC-system is identical to that of an analytical HPLC with the addition of a fraction collector.

2. Objective

Preparative HPLC is mainly concerned with the parameters to be optimized: purity, throughput and yield. Since these are mutually dependent, it should be clear before purification which goal is to be primarily pursued. Maximum throughput can only be achieved at the expense of purity and yield. Maximum purity can sometimes only be achieved with long separation times and thus lower throughput. Maximum yields come at the expense of throughput and purity.

Ideally, as much substance as possible should be purified per injection in the best possible resolution and time. The peak shape of the target analyte is of secondary importance. In preparative HPLC, the primary concern is the resolution between the peak of interest and the peaks closest to it.

3. Pre-Testing and Up-Scaling

In order to save costs, it is recommended to first develop a suitable method for the respective substance mixture on an analytical column. To ensure smooth up-scaling, the following points should be considered:

- The analytical and preparative column should be packed with the same stationary phase.
- The particle size as well as the column length should ideally be identical for both columns.
- If the particle size of the preparative column differs, care should be taken to maintain the ratio of particle size to column length of the analytical column
- The mobile phase used should be identical for both separations.
- The samples should be dissolved in the same solvent for both separations and be present at the same concentration

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3.1 Adjustment of the flowrate F

For up-scaling, ensure that the linear flow velocities remain as constant as possible. The linear flow velocity is proportional to the cross-sectional area of the column and can be calculated for preparative columns using the formula below.

$$F_{\Pr ep} = F_{Analy} \cdot \left(\frac{d_{\Pr ep}}{d_{Analy}} \right)^2 \cdot \frac{p_{Analy}}{p_{\Pr ep}}$$

F_{Prep}	Flow rate of preparative system
F_{Analy}	Flow rate of analytical system
d_{Prep}	Internal diameter of preparative column
d_{Analy}	Internal diameter of analytical column
p_{Prep}	Particle size of preparative column
p_{Analy}	Particle size of analytical column

The following table contains the calculated volumetric flow rates (flow rates) at constant linear flow velocities for columns of different internal diameters but identical particle size.

Internal diameter of the column in mm	4.6	8.0	10	20	30	40	50
Flowrate in mL/ min	0.5	1.5	2.4	9.5	21	38	60
	1.0	3.0	4.7	19	43	76	120
	1.5	4.5	7.1	28	64	113	177
	2.0	6.0	9.5	38	85	150	236

3.2 Adjustment of loadability or injection volume v_{inj}

When a method is transferred to a preparative column or generally to a column with larger column volumes, the following equation helps to adjust the loadability or the injection volume v_{inj}. Also the loadability of the column is proportional to the cross-sectional area of the column.

$$v_{inj,\Pr ep} = v_{inj,Analy} \cdot \left(\frac{d_{\Pr ep}}{d_{Analy}} \right)^2 \cdot \frac{L_{\Pr ep}}{L_{Analy}}$$

$v_{inj,Prep}$	Injection volume of preparative system
$v_{inj,Analy}$	Injection volume of analytical system
d_{Prep}	Internal diameter of preparative column
d_{Analy}	Internal diameter of analytical column
L_{Prep}	Length of preparative column
L_{Analy}	Length of analytical column

3.3 Gradient transfer

In order to perform up-scaling as accurately as possible, the dwell volumes and the column dead volumes of both systems must not be neglected, especially for gradient separations. To compensate differences in the dwell volumes, short isocratic steps can be incorporated at the start of the gradient. The following equation should apply when gradients are to be transferred from one system to the other:

$$\frac{t_{D,Analy} + t_{I,Analy}}{t_{c,Analy}} = \frac{t_{D,\Pr ep} + t_{I,\Pr ep}}{t_{c,\Pr ep}}$$

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

3.4 Compensation of different dwell volumns of both systems

To compensate differences in the dwell volumes between the analytical and preparative systems, short isocratic steps can be incorporated at the start of the gradient. To determine this short isocratic step for the preparative system, it is necessary to know the dwell volumes and the column dead volumes for both systems.

$$t_{I,\Pr ep} = \left(\frac{t_{I,Analy} \cdot F_{Analy}}{v_{c,Analy}} + \frac{v_{D,Analy}}{v_{c,Analy}} - \frac{v_{D,\Pr ep}}{v_{c,\Pr ep}} \right) \cdot \frac{v_{c,\Pr ep}}{F_{\Pr ep}}$$

$t_{I,Prep}$	Initial hold of preparative system focused gradient
$t_{I,Analy}$	Initial hold of analytical system generic gradient
F_{Prep}	Actual flow in preparative system
F_{Analy}	Actual flow in analytical system
$v_{c,Prep}$	Column void volume of preparative system
$v_{D,Prep}$	Dwell volume of preparative System
$v_{c,Analy}$	Column void volume of analytical system
$v_{D,Analy}$	Dwell volume of analytical system

4. Loadability and overload effects

So-called overoad effects often occur during preparative separations. Especially when a large amount of pure analyte has to be recovered per time. Maximum loading studies should first be performed on the analytical column. There are two procedures for this:

1. A sample at high concentration is prepared. The injection amount is increased in defined steps.
2. Several samples of different concentrations are prepared. The injection volume remains constant.

The maximum loadability depends on many parameters and is individual for each separation problem. The maximum loadability for a preparative separation is usually determined empirically. So much is injected until the peaks of interest just start to touch each other or until the analyte can just be isolated in sufficient quantity and purity. If the peaks are not sufficiently resolved, there is a risk that the collected fractions will lose purity. Additional defined intermediate fractions are then obtained, which can subsequently be recycled if required. With simple separation and good selectivity, up to 10 mg per run can already be purified on a 250x4.6 mm column, for example. For difficult separations, the maximum loadability of such

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a column can also be only in the sub mg range. The better the resolution of the analytical separation, the more the preparative column can be loaded.

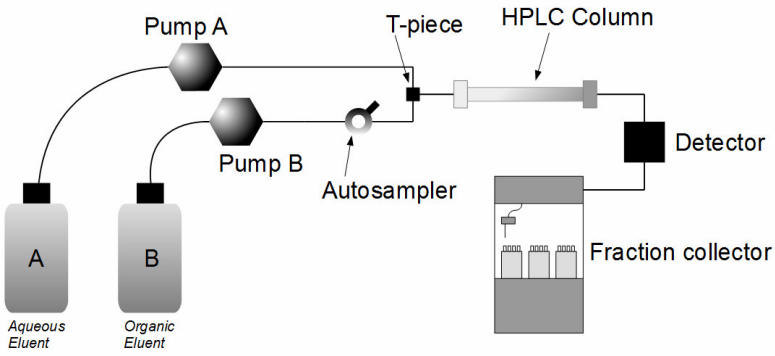
5. Injection with strong organic solvents

If the analytes can only be dissolved in strong organic solvents such as DMF, DMSO or NMP, a special injection technique can be used, sometimes referred to in the literature as "at-column dilution". Such strong organic solvents have a very high elution strength, which in many cases destroys the chromatographic separation when an ordinary injection is performed. The result is a strong peak broadening and a certain loss of substance. This occurs particularly when, in reversed-phase chromatography, the compounds to be separated have a relatively high polarity and are thus only weakly retarded. The following procedure has proven useful as a solution to this problem:

1. Connect the injector to the pump channel that supplies the organic eluent.
2. Inject the sample in pure organic solvent and dilute it with the aqueous eluent directly upstream of the column.

To do this, install a simple T-piece just before the entrance to the column. Note that the distance between the T-piece and the column should be kept as short as possible to minimize the risk of precipitation of the sample. During injection, channel B (organic eluent) should provide at least 5% of the total flow rate. The remaining portion of aqueous eluent (channel A) is mixed to it through the tee (see figure below).

Before the actual start of the gradient, a short defined isocratic run is then switched to flush the strong organic sample solvent from the column and concentrate the sample at the column head.



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6. Fraction collection

If the sample to be separated is still unknown, of special value, or if only a few runs are necessary, manual collection of fractions is often preferred. An online signal plot on the computer screen allows the analyst to control fractionation by simple clicks or to simply collect the fractions by hand into test tubes or vials according to detector flow rate. If large sample volumes are frequently purified preparatively by HPLC, fully automated instruments are often used to collect the individual fractions. Fraction collectors can be either time-programmed or signal-programmed. For fraction collection by time, either defined time windows are determined in which the eluate is collected or the sample is collected continuously and the time is determined for how long to collect into a test tube/vial. Another option is to collect fractions according to the detector signal, e.g. UV, ELSD or even MS. To trigger a sample collection, either a signal threshold and/or the signal slope can be used. Maximum purity and selectivity is achieved with a combined fraction collection of UV and MS signal. In addition, the number of collected fractions can be drastically reduced, which saves a lot of working time in the post-treatment of the collected samples.

7. Mobile Phase

To ensure that the separated components can be easily isolated in pure form, the entire mobile phase (including all buffer additives) must be of the highest purity and volatile. Volatile buffer additives include acetic acid, formic acid, trifluoroacetic acid, ammonia or triethylamine. Preparative separations should be run isocratically if possible, since gradients involve considerable additional work in many respects. Nevertheless, gradient separations are often used in preparative HPLC as well.

8. Detection

To avoid detector overload during preparative separations, insensitive detectors should be used for this purpose. Refractive index detectors or special UV detectors have proven successful for this purpose. ELSD or MS detectors in combination with a flow splitter are also sometimes used for preparative separations.

9. Column and system protection

To conserve the comparatively expensive preparative columns, only particle-free sample mixtures should be injected that contain only components that can also be eluted. It is advantageous if the sample is previously purified, e.g. by low-pressure column chromatography. To protect the main column from substances that are difficult or impossible to elute, it is advisable to use suitable precolumns and/or precolumn filters. Pre-columns protect the main column from impurities and substances that are difficult to elute. Pre-column filters retain small undissolved particles. Pre-columns and pre-column filters should be

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replaced at regular intervals, depending on the degree of contamination of the samples. Pre-columns (cartridges) are packed with the same stationary phase as the actual separation column. Sometimes materials with larger particles are packed into the precolumns to minimize cost and additional pressure rise. Manufacturers of preparative HPLC columns offer different types of precolumns. Please contact MZ-Analysentechnik GmbH to find out which types of precolumns are available for your preparative HPLC column.

For storage of preparative HPLC columns the solvents recommended by the manufacturer should be used. In general, the storage solvent should not contain more than 50% water to avoid the growth of bacteria, algae and fungi.