Science Together

KNAUER

# Good HPLC practice guide

HPLC

**Support:**

**International Support:**

Contact your local KNAUER partner for support: www.knauer.net/en/Support/Distributors-worldwide

**Support in Germany**

(Austria & Switzerland on case-to-case basis):

Phone: +49 30 809727-111
(workdays 9-17h CET)

Fax : +49 30 8015010
Email: support@knauer.net

**Publisher:**

KNAUER Wissenschaftliche Geräte GmbH
Hegauer Weg 38
14163 Berlin

Germany

Phone: +49 30 809727-0
Fax: +49 30 8015010
Internet: www.knauer.net
E-Mail: info@knauer.net

**Version information:**

Document number: V6887
Version number: 1.0
Release date: 2021/03/01

# 1. Mobile Phase

### What quality should my used solvents have?

All used solvents should be of the highest quality. HPLC grade or MS grade solvents are suitable because they do not contain impurities that might lead to contamination peaks. Impurities in the solvents can lead to ghost peaks and pressure spikes in your system. Aquaeous solvents should be deionized and purified.

### How do I prepare buffers?

All prepared buffer solutions should be clear, homogeneous, and free from non-dissolved particles. They should be prepared freshly at the day of use. Adjusted pH values should be checked to avoid an effect on chromatography. If buffer solutions should be stored, keep in mind that they have limited lifetime.

### Do I need to filter the mobile phases?

Mobile phases should be filtrated if necessary. They can be filtered through a 0.45 µm filter. This removes any particulate matter that may cause blockages. Dependent on the used mobile phase the filtzer should be chosen regarding the solvent properties, e.g. hydrophilic or hydrophobic. Filtering HPLC solvents will benefit both your chromatography and HPLC system. Pump plungers, seals and check valves will perform better, and lifetimes will be maximized.

### How and why should the mobile phases be degassed?

Before pumping the freshly prepared mobile phase through the HPLC system, it should be thoroughly degassed to remove all dissolved gasses. Offline degassing can be performed via:

- Bubbling with helium
- Sonication
- Vacuum filtration

Additionally, modern HPLC pumps have an integrated module for online degassing. If the mobile phase is not or incomplete degassed, air bubbles can form in the system resulting in problems like for example system instability or spurious baseline peaks.

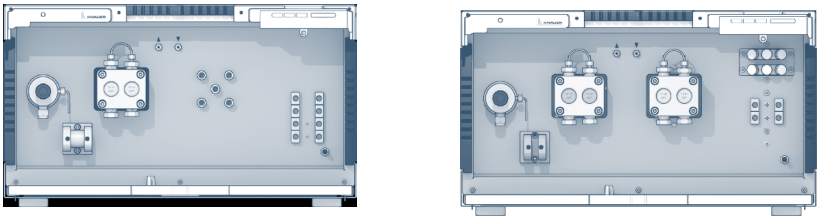

**Fig. 1:** AZURA P 6.1L LPG with degasser (left), AZURA P 6.1L HPG with degasser (right)

### Are there limitations relating to mobile phase properties?

Do not use highly basic or acidic solvents unless your HPLC system and applied column are able to withstand these properties. Seals and other wetted parts can be damaged by extreme pH conditions. Highly aqueous mobile phase can only be used with certain columns. A high-water content in the mobile phase can promote bacterial growth. The specifications for the applicable pH range should be provided with the additional column and device information. For additional information on pH robustness of your columns and wetted parts please contact your vendor or visit the KNAUER website at www.knauer.net.

**Note:** Always flush columns and the components of HPLC systems with organic solvent before storage.

### How to change solvents?

Before performing any solvent change, make sure the actual used solvent in the system is compatible with the new solvent. If the miscibility is not given, an additional flushing procedure with an appropriate solubilizer is necessary. Removal of the column from the system before flushing is recommended.

### Are all solvents miscible with each other?

Not all solvents are miscible. If you are unsure about the miscibility, then try mixing in a vessel before using the HPLC instrument. The below shown miscibility chart is also a helpful tool.
(white = miscible, black = immiscible)

**Example:** When running the system under reversed phase conditions using a mixture of acetonitrile/water or methanol/water, it is mandatory to flush the system with a solutizer when switching to normal phase conditions. Possible solutizer could be isopropanol. If aqueous buffers were used, the system needs to be flushed with pure water to get rid of possible salt decontaminations and to prevent precipitation of the buffer salts when changing back to organic solvents.

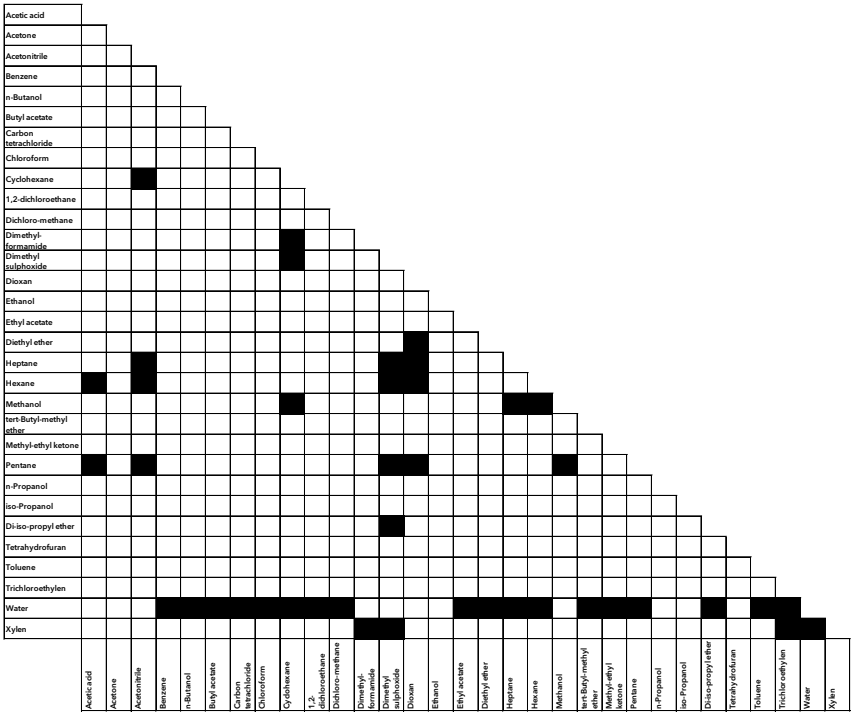


**Fig. 2:** Miscibility chart

### How does the eluting strength of my solvent influence the separation?

The eluting strength is classified with the eluotropic series. In this series the solvents are arranged in order of their relative abilities to effect elution or respectively the order of solvents runs in the order of their polarity.

If you have a mixture of acetonitrile/water 50:50 (v/v) and want to switch to methanol, you need about 20 % more methanol to get the same eluting strength. You also must consider the possible interaction between the different molecules or functional groups of your sample, mobile phase, and stationary phase.

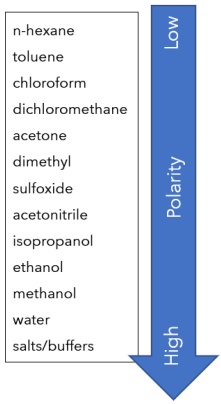


**Fig. 3:** High/Low polarity

## 2. Detection

### Is every substance detectable with a UV detector?

Not every compound can be detected with an UV detector. If you are not sure if your analyte can be detected with UV, the attached list of chromophore systems will help you.

| Chromophore | $\lambda_{max}$ [nm] | Chromophore | $\lambda_{max}$ [nm] |
|---|---|---|---|
| Ether | 185 | Thioketone | 205 |
| Thioether | 194/215 | Ester | 205 |
| Amine | 195 | Aldehyde | 210 |
| Thiol | 195 | Carboxyl | 200 - 210 |
| Disulfide | 194/255 | Sulfoxide | 210 |
| Bromide | 208 | Nitro | 210 |
| Iodide | 260 | Nitrite | 220 - 230 |

| Chromophore | $\lambda_{max}$ [nm] | Chromophore | $\lambda_{max}$ [nm] |
|---|---|---|---|
| Nitrile | 160 | Azo | 285 - 400 |
| Acetylide | 175 - 180 | Nitroso | 302 |
| Sulfone | 180 | Benzene | 184/202/255 |
| Oxime | 190 | Biphenyl | 246 |
| Azido | 190 | Naphthalin | 220/275/312 |
| Ethylene | 190 | Anthracene | 252/375 |
| Ketone | 195/270 - 285 | Pyridine | 174/195/251 |

### What is the UV cutoff and why do I need to know it?

Every solvent has its specific absorbance cutoff wavelength. Below this wavelength the solvent itself absorbs the light. When choosing a solvent be aware of its cutoff and where your desired analytes will absorb. If the wavelengths are close, choose a different solvent. The list below displays the UV cutoff wavelengths of common solvents.

| Solvent | λ Cutoff [nm] | Solvent | λ Cutoff [nm] |
|---|---|---|---|
| Acetic acid | 260 | Heptane | 197 |
| Acetone | 330 | Hexane | 210 |
| Acetonitrile | 190 | Methanol | 210 |
| Chloroform | 245 | 2-Propanol | 210 |
| Cyclohexane | 210 | Tetrahydrofuran | 220 |
| Dimethyl sulfoxide | 265 | Toluene | 286 |
| Ethanol | 210 | Water | 191 |
| Ethyl acetate | 255 | --- | --- |

# 3. Sample preparation

### Why should you perform sample preparation?

Sample preparation helps to remove interferences caused by sample matrices. It can simplify complex samples. It can make your sample compatible for further analysis and furthermore, it can dilute or concentrate samples. Altogether, a good sample preparation helps to keep your column clean and makes it last longer.
There are different techniques that can be used for preparation such as: filtration, dilution, centrifugation, precipitation, various extraction methods and many more.

### How to weigh a sample?

Substances tagged according to Globally Harmonized System of Classification, Labelling and Packaging of Chemicals (GHS) should only be weighed under an extractor hood. It is always important to keep the weighing place clean to avoid contaminations. Other colleagues should be informed about special exposures, for example when you are working with CMR (carcinogenic, mutagenic, reproductive toxic) substances.

### Which solvent is the best for dissolving my sample?

Ideally the sample should be dissolved in the solvent that correspond to your analysis starting conditions.

**Example:** If your gradient starts at a solvent composition of 10 % acetonitrile, your sample should be dissolved in the same composition if possible to prevent disturbing peaks. If this is not possible, try to solve the sample in the most similar solvent composition, to minimize any potentially disturbance.

### How to proceed if the solubility of the sample is unknown?

If you know the ingredients/composition of your sample, you should look up their chemical and physical properties. Often information about solubility are mentioned.  If you do not know the formulation of your sample, you can perform a solubility study.

### How to perform a solubility study?

If the solubility of your sample/compound is unknown, it is necessary to dissolve a constant amount of sample in different solvents and document the behaviour. You should consider polar as well as non polar solvents commonly used for HPLC analysis.

Determination of whether a chemical has dissolved is based on visual observation. A chemical has dissolved if the solution is clear and shows no signs of precipitation.

# 4. Lab safety

### Do I need safety glasses?

In most laboratories wearing safety glasses is mandatory. The requirements for personal eye protection equipment is described in DIN EN 166. In special cases the use of a goggle or even a complete face protection shield can be necessary.

### Do I need safety gloves?

For every exposure with chemicals, safety gloves should be worn. Dependent on the chemical properties of the used substance, the appropriate glove must be chosen. Therefore, most vendors of safety gloves provide tables with permeability times for certain substances. Furthermore, the gloves are classified in scale numbers/permeation levels from 1 to 6. These are fixed in DIN EN 374.

**Example:** Nitrile gloves (thickness ~ 0.14 mm) are suitable for working with phosphoric acid (permeation level 6, breakthrough time > 480 min) but only provides limited protection for ethanol (permeation level 1, breakthrough time 20 min) and no protection for methanol (permeation level 0, breakthrough time 7 min). The appropriate glove for your application should at least provide protection at permeation level 2 (breakthrough time > 30 min).

### When do I need to change gloves?

Some gloves are for single use only and should be thrown away directly after using them or rather when a contact with the chemical has happened. Other gloves are for multiple use and need to be cleaned after usage. Nevertheless, they should be replaced within a predefined period. This depends on how often they have been used.

### Can I wear the gloves all the time?

No! Gloves are only for the direct contact with chemicals and must not be worn when opening doors or using the computer keypad, for example. This might cause contaminations and possible exposition of used chemicals to other colleagues.

## How do I know which protective equipment I need to wear?

Most often before using chemicals, a safety instruction for the handling and information about potential risks is carried out by qualified staff. If not, all required information can be found in the material safety data sheet, short MSDS.

Risks to Human Health and the Environment

**Danger**

GHS02 GHS07

- H225 - Liquid and vapour highly flammable.
- H302 - Harmful if swallowed
- H312 - Harmful in contact with skin.
- H319 - Causes serious eye irritation.
- H332 - Harmful if inhaled.

**Water hazard class 3 (highly hazardous to water)**

Safeguards and Rules of Conduct

- P210 - Keep away from heat/sparks/open flames/hot surfaces – No smoking
- P233 - Keep container tightly closed.
- P240 - Ground/bond container and receiving equipment.
- P280 - Wear protective gloves/protective clothing/eye protection/face protection.
- P403 - Store in a well ventilated place.

**01. Technical**

- Keep container in a cool, well-ventilated place.
- When damping and fogging, work in a fume cupboard and keep the sash closed.
- Keep away from ignition sources, as it is a flammable liquid with a flash point lower than 45°C. Only work in a fume cupboard with explosion-proof fan.

**02. Organizational**

- Keep laboratory stocks as low as possible, keep it protected against the effects of flame and heat.
- Avoid splashing and overrun during filling and decanting.
- Keep away reactive substances or only add them in a controlled manner.
- Do not eat or drink in working areas.

**03. Personal**

- Use safety goggles or face protection according to DIN EN 166.
- Wear protective gloves, the following are suitable:
  - Mikroflex 93-260, Ansell
  - SHIELDskin CHEM NEO NITRIL 300
  - Ansell, Barrier
- Avoid contact with eyes and skin
- Before breaks and after work, take appropriate skin cleaning and skin protection measures.

**Fig. 4:** Exemplarily extract of safety instruction

# 5. Running the system

**Can I start the measurement immediately after switching on the system?**

After switching on the devices, they need time to warmup. Lamps of UV or DAD detectors need to be warmed to ensure a stable baseline. The flow cells of RI detectors also need a stable temperature. Additionally they need to be flushed with the solvent to avoid air bubbles and to adjust the zero glass. Furthermore, the HPLC column also needs time to equilibrate properly.

## 5.1 Checklist for starting up a system

- ☐ All required capillary connections, tubing and plugs are installed.
- ☐ Devices are switched on.
  **Attention:** consider detector warmup times!
- ☐ Mobile phase is ready.
- ☐ Refill necessary?
- ☐ Preparation of new mobile phase necessary?
- ☐ Degassing necessary?
- ☐ Filtration necessary?
- ☐ Back piston flushing is installed.
- ☐ Back piston flushing solvent is filled up and fresh.

  **Note:** Commonly used are: 50 % isopropanol or ethanol for RP, 100 % isopropanol for NP, 5 % isopropanol or ethanol for high salt buffers.
- ☐ The system is purged properly with mobile phase.
- **If not:** Open the purge valve and purge the mobile phase dependent on instructions of the used chromatography software.

- [ ] Close purge valve and flush the complete flow path/ capillaries with mobile phase at a moderate flow rate e.g. 1 ml/min.
  **Attention:** No column has been installed yet!
- [ ] No leaks are detected in the flow path.
- [ ] Autosampler washing bottle is installed.
- [ ] Autosampler washing solvent is filled up.

  **Note:** Washing solution should be equivalent to eluent starting composition.
- [ ] The autosampler syringe contains no air bubbles.
- [ ] Perform needle wash procedure to remove bubbles from the injector.
- [ ] Install column in the right direction.
  **Attention:** Consider recommended flow direction!
- [ ] Equilibrate column to your analysis starting conditions.

  **Note:** Typically, 10 to 20 column volumes are required when new column is installed.
- [ ] Ready for measurement.

## 5.2 Checklist for cleaning/shutting down a system

- [ ] ■ Measurements have been completed.
- [ ] ■ Clean/flush your column to remove residues of samples and sample matrices.
- [ ] ■ Clean/flush your column to remove buffer salts or modifiers .
- [ ] ■ Back piston flushing is installed.
- [ ] ■ Disconnect the column and install a coupling.
- [ ] ■ Empty the autosampler tray and dispose or store your sample vials.
- [ ] ■ Replace your mobile phase with isopropanol.

  **Note:** If buffers were used flush the system with water before switching to isopropanol.
- [ ] ■ Purge your pump with water or isopropanol.

  **Note:** Open the purge valve and purge dependent on instructions of the used chromatography software.
- [ ] ■ Close the purge valve and flush the complete flow path/ capillaries with water or isopropanol at a moderate flow rate, e.g. 1 ml/min.
  **Attention:** No column is installed!
- [ ] ■ Replace the autosampler washing solution with isopropanol.
- [ ] ■ Perform needle wash procedure.

  **Note:** 2-3 times
- [ ] ■ Perform advanced autosampler wash procedure to rinse the sample loop.

  **Note:** 2-3 times
- [ ] ■ Ready for non-usage.

# 6. References

www.knauer.net/en/Support/FAQ

www.knauer.net/en/Support/FAQ/Good_LC_practices

www.knauer.net/en/Support/FAQ/LC-Columns#Care

www.knauer.net/eluting_strength_influence

www.knauer.net/column_care_and_regeneration

www.knauer.net/column_care_hilic

www.knauer.net/column_care_and_silica_phases

www.knauer.net/column_care_eurospher_chiral_care

Science Together

KNAUER

Latest KNAUER instructions online:
www.knauer.net/library

KNAUER
Wissenschaftliche Geräte GmbH
Hegauer Weg 38
14163 Berlin

Phone: +49 30 809727-0
Fax: +49 30 8015010
E-Mail: info@knauer.net
www.knauer.net