Mechanisms of retention in HPLC

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Part 3

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3.1. Acid-base equilibria

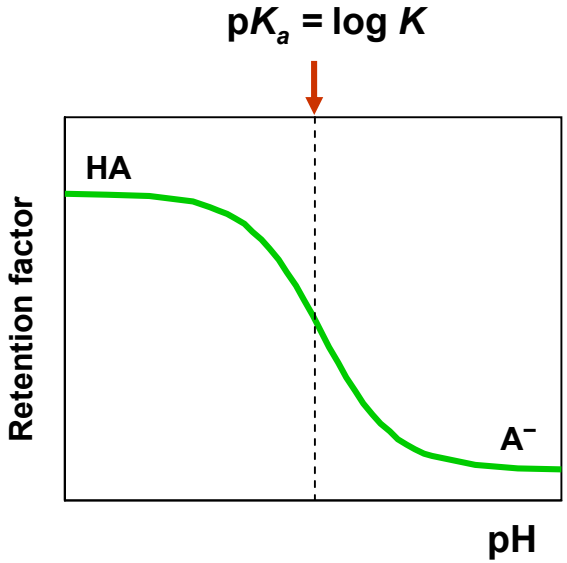
3.1.1. Changes in retention with pH

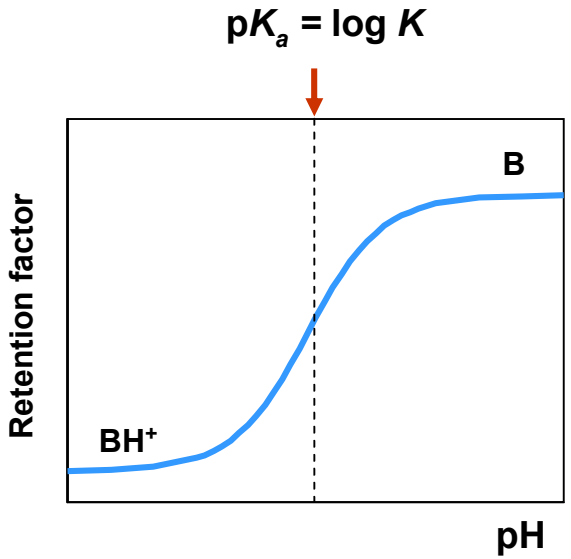
Sigmoidal dependence of RPLC retention *versus* mobile phase pH for weak acids and bases: pronounced drop around pH = pK_a in the direction of the ionic species

$$k = \frac{k_A + k_{HA} K h}{1 + K h}$$

$K = K_a^{-1}$: apparent protonation constant

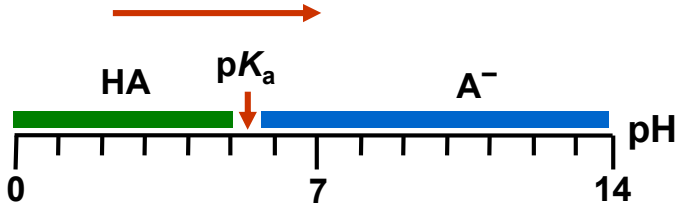
pH : log [H^+]





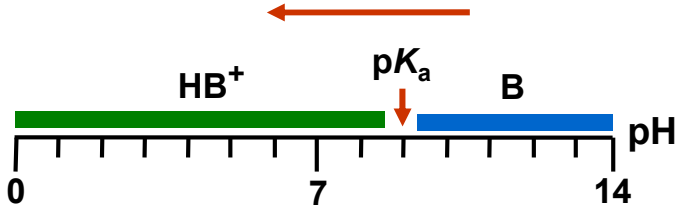
- **Neutral weak acids** lose a proton and become ionised when the pH increases

$$HA \leftrightarrows A^- + H^+$$



- **Aminic bases** accept a proton when pH decreases

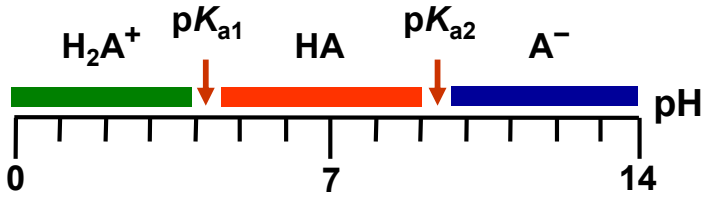
$$HB^+ \leftrightarrows B + H^+$$

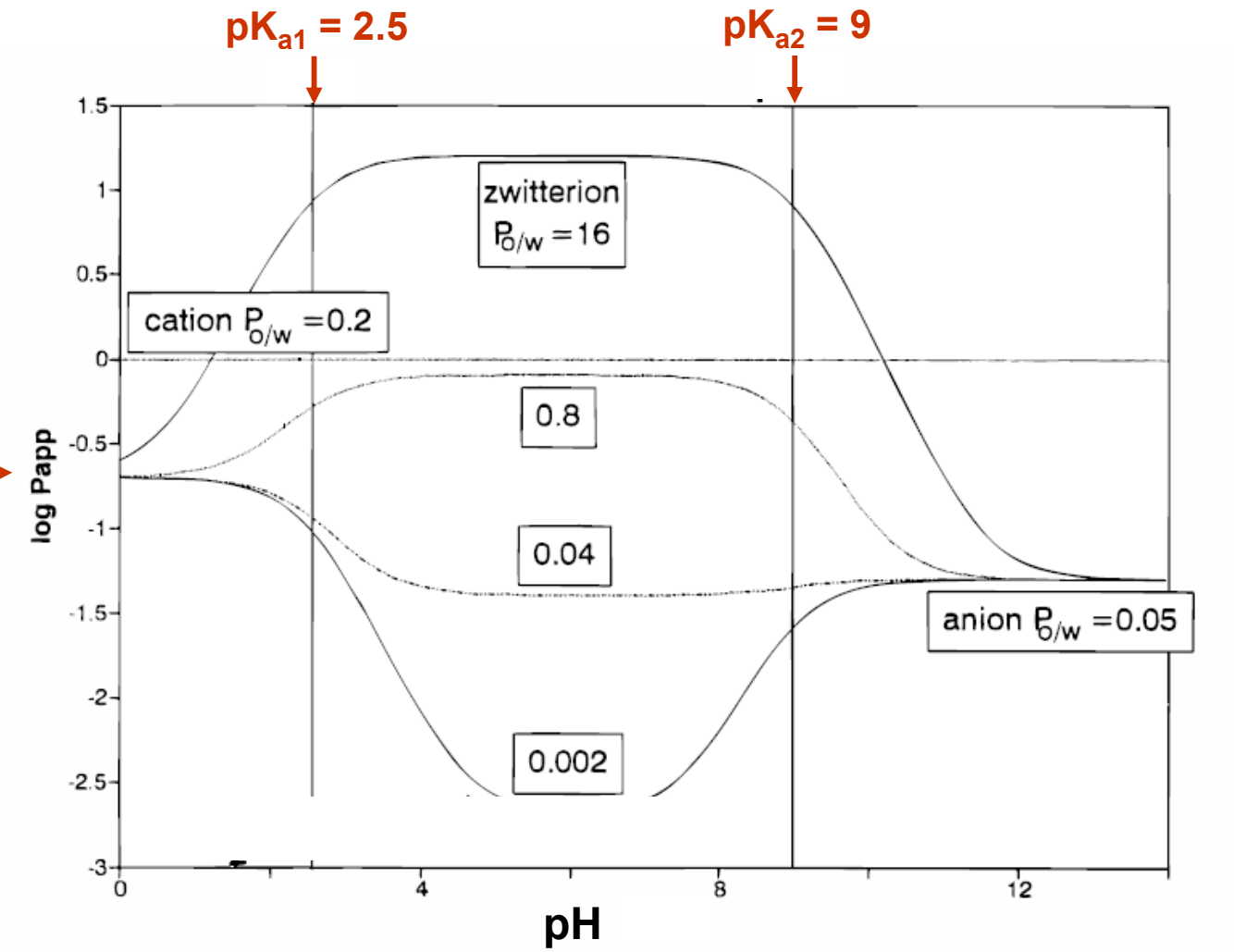


- For **polyprotic compounds,** the *k*-pH curve depends on the charge of the different acid-base species

$$H_2A^+ \leftrightarrows HA + H^+$$

$$HA \leftrightarrows A^- + H^+$$





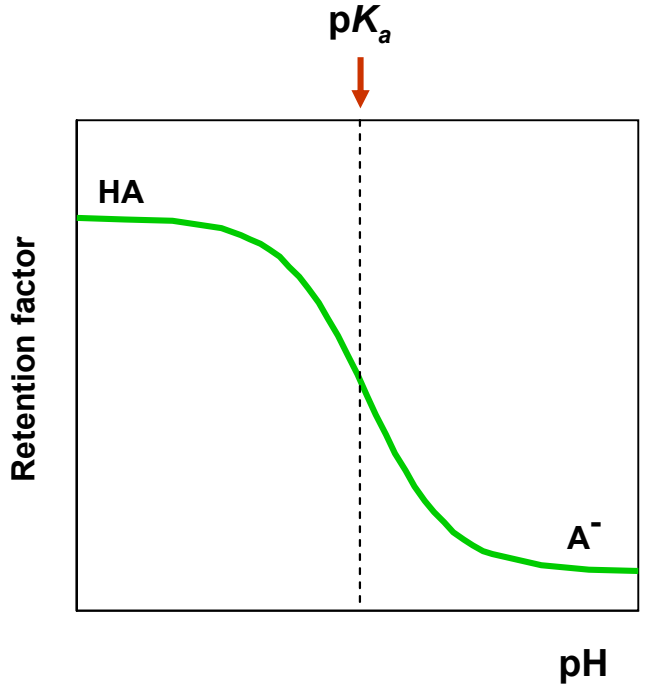
$H_2A^+ \leftrightarrows HA^{+/-} + H^+$

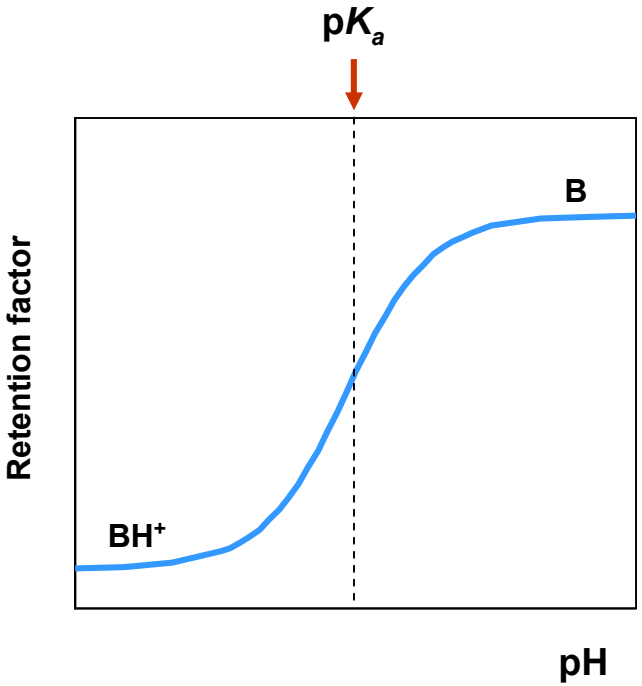
$HA^{+/-} \leftrightarrows A^- + H^+$

$$P_{app} = \frac{P^{+/-} + P^{+}\frac{[H^+]}{K_{a1}} + P^{-}\frac{K_{a2}}{[H^+]}}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}}$$

Retention drops

- The drop height depends on the difference in polarity between the acid-base species.
- Small variations in the mobile phase pH at values close to the pK_a result in significant changes in retention and selectivity. Therefore, the pH in this region needs to be tightly controlled to achieve robust procedures.



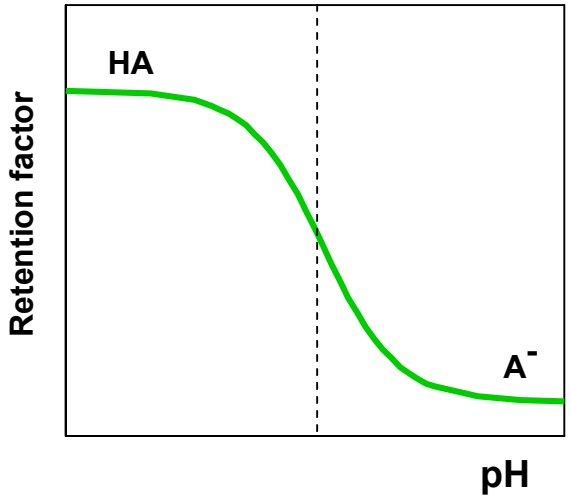


Acidic pH

- **Robust methods are achieved in regions scarcely affected by changes in pH.**

Weak acids: region of predominance of the neutral species (acidic pH)
(ion suppression chromatography)

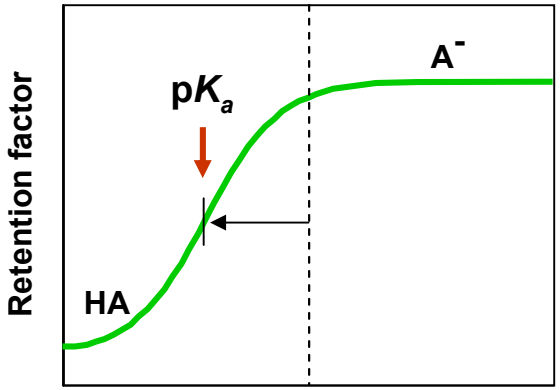
Basic compounds: acidic pH is also used to protonate (deactivate) silanols on the stationary phase.

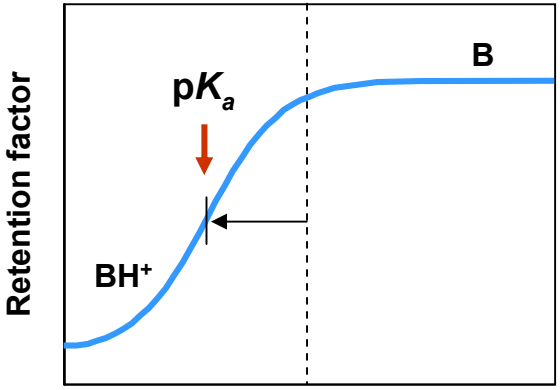


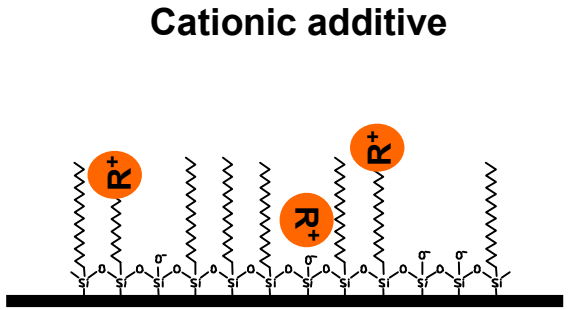
Separations at very low pH are not always feasible, due to column instability and long analysis times for some solutes.

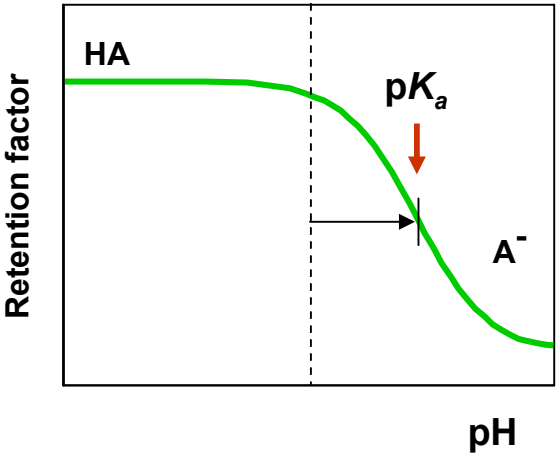
Addition of IIC reagents

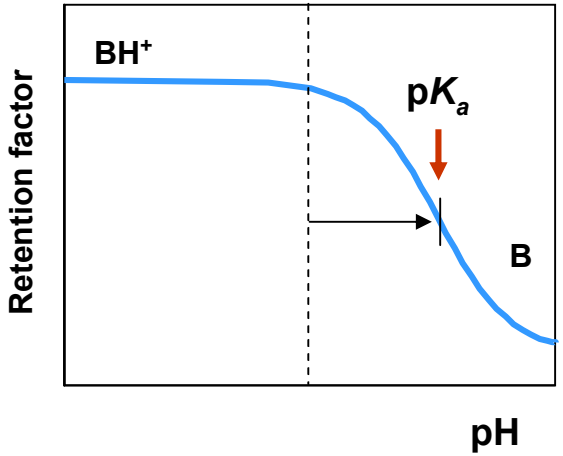
The pK_a value is shifted and the curve trend may be changed by interaction of the ionic species of the acid-base pair with an adsorbed ion on the stationary phase, which depends on the charge of both ions.

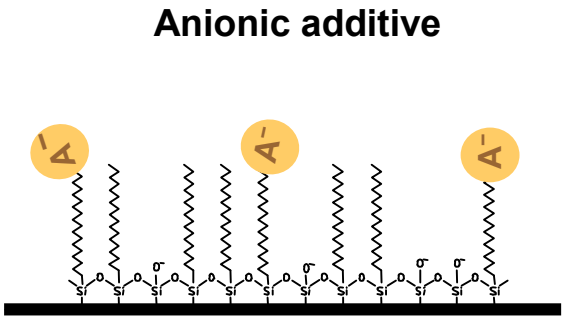












3.1.2. Buffers and measurement of pH

The pH range for conventional columns in RPLC is 2.5/3.5-7.0/7.5, but for some special columns, it can be extended to 2-12. For ionisable compounds, the addition of an appropriate buffer is needed to fix the pH and achieve reproducible retention.

- **Common buffers correspond to the acid-base systems:**

  tris(hydroxymethyl)aminomethane (Tris)

  phosphoric **citric**

  phthalic **acetic**

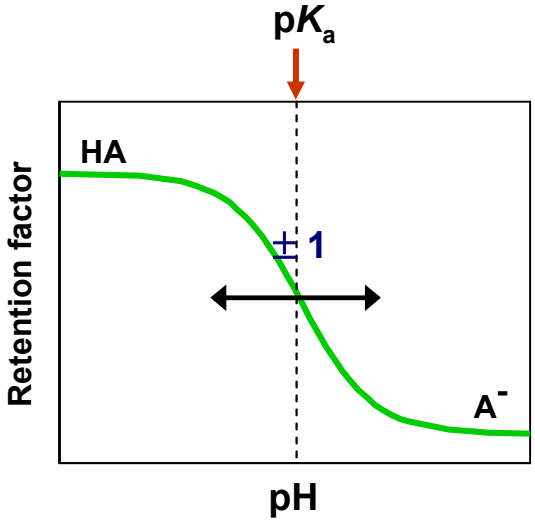
  formic **ammonium**

- **The buffering capacity occurs in the range**

  $pH = pK_{a,buffer} \pm 1$

- **Only volatile buffers are compatible with ELS and MS detection.**

- **Care should be taken with buffers that facilitate algae formation !!!**



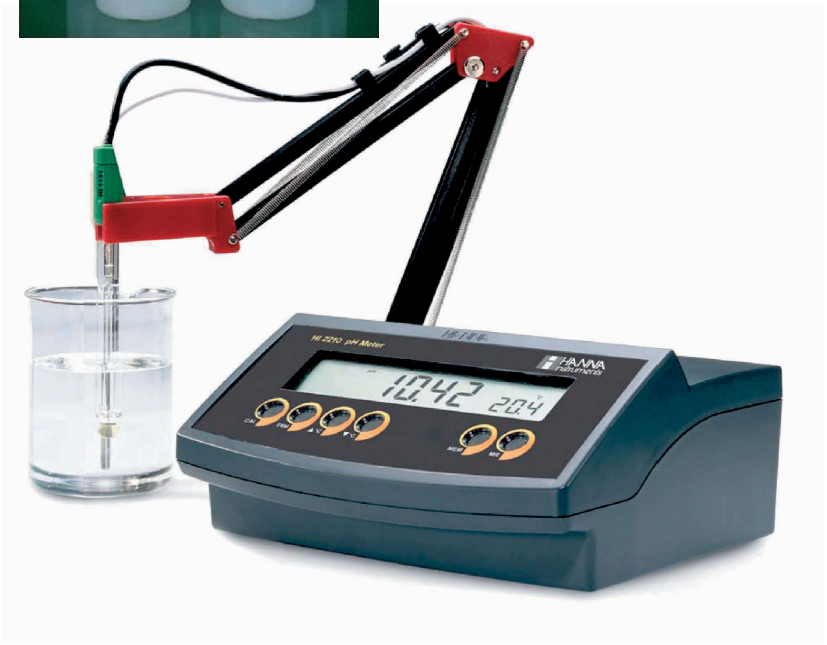
The pH should be measured in the hydro-organic mixture, better than in the aqueous buffer.

- Common operational methodology in RPLC is measurement of pH in water before the addition of the organic solvent. Of course, the reference buffers are also prepared in water ($_{w}^{w}$pH scale).
- This procedure has the advantage of reducing the number of measurements, since the pH value will be the same for all mobile phases prepared with the same buffered solution, independently of the amount of organic modifier.
- However, it does not take into account the influence of the organic modifier on pH.
- Predictions can differ strongly from the expectancies!!!

$_{w}^{w}$pH

The pH should be measured in the hydro-organic mixture, better than in the aqueous buffer !!!

- The electrode system ideally should be calibrated with standard buffers prepared using the same solvent composition as the mobile phase, which gives rise to the so-called $^{s}_{s}pH$ scale.
- These standards are not usually commercially available and need careful maintenance. A solution is to measure the pH in the hydro-organic mixture and calibrate the electrode system with aqueous buffers, giving rise to the $^{s}_{w}pH$ scale.
- Fortunately, both scales can be easily converted to each other.



$^{s}_{w}pH$ ⟷ $^{s}_{s}pH$

pH correction

$$ {}^{s}_{s}\mathrm{pH} = {}^{s}_{w}\mathrm{pH} - \delta(\varphi) \qquad (3.1) $$

δ : **correcting term** depends on the **solvent nature and concentration**

Example: For 0–60% acetonitrile and pH 3–7

$$ \delta(\varphi) = -0.348\varphi^{2} - 2.796\varphi^{4} \qquad (3.2) $$

Transfer of log *K* values between both scales is similar:

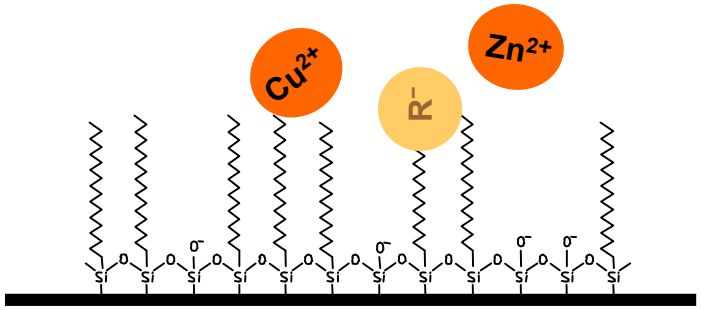
$$ \log {}^{s}_{s}K = \log {}^{s}_{w}K - \delta(\varphi) \qquad (3.3) $$

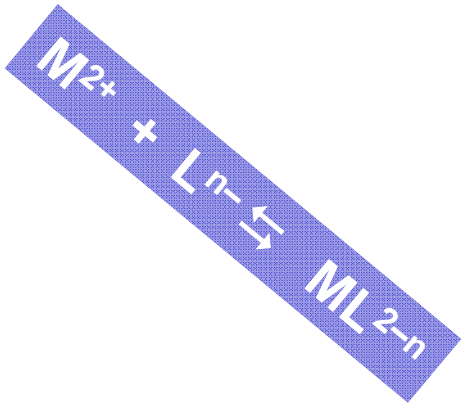
- The **column temperature** should be controlled, since it affects strongly the **ionisation degree of analytes and buffers.**
- The **ionic strength** of the reference buffers and mobile phases should agree to avoid changes in **liquid junction potentials.**

3.2. Metal complexation

3.2.1. Determination of metal ions

RPLC is a good alternative to spectroscopic methods and ion exchange chromatography for metal analysis. It allows the simultaneous determination of several metals, interference removal, coupling with different detectors, and high sensitivity.





Alternatives

- **The direct IIC separation of transition-metal ions is difficult, since the behaviour of hydrated metal ions is not different enough.**
- **The required selectivity should be achieved using a number of side-reactions:**

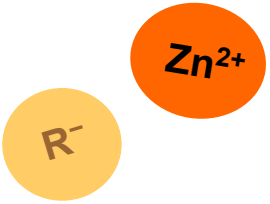
  complexation

  ion-pairing

  dynamic ion exchange

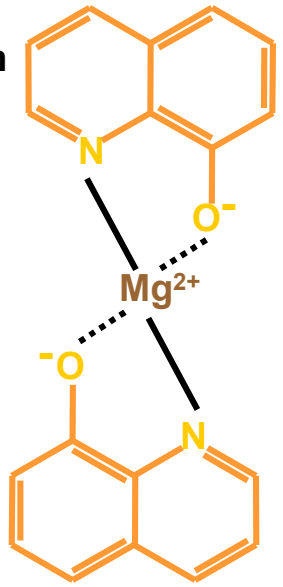
  association with a micelle in the mobile phase

in addition to acid-base equilibria !!!



The separation of chelates using **metallochromic ligands** with **highly absorbing chromophores** avoids the need of post-column derivatisation, with **sub-μg/mL-level detection limits.** Higher selectivity and sensitivity can be achieved with **fluorimetric complexing reagents.**

- **Neutral complexes** ➡ Hydro-organic mixtures without additives
- **Anionic complexes** ➡ **IIC mode** with alkylammonium salts with or without a competing anion in the mobile phase
  ➡ **IIC mode** with cationic surfactants as cetyltrimethylammonium bromide or cetylpyridinium chloride below or above the CMC

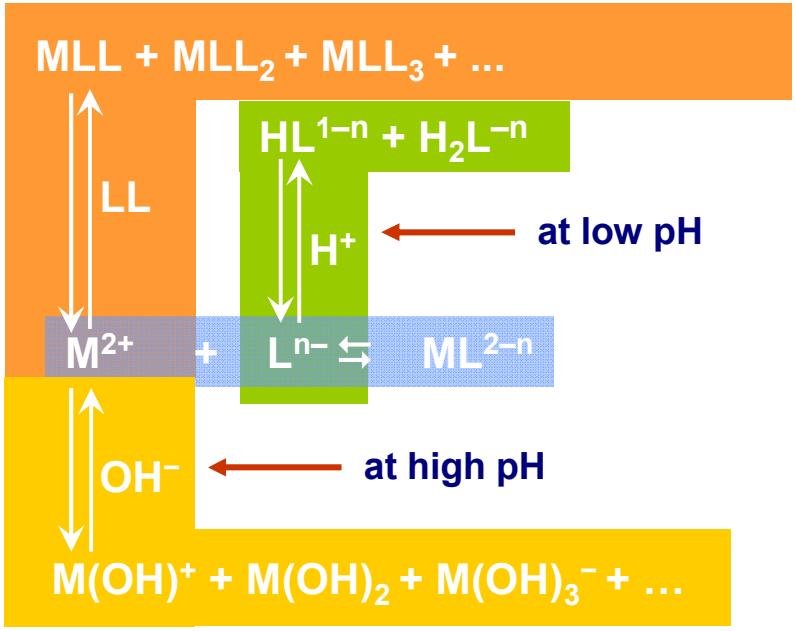


- **Binary complexes** are usually formed, with a few examples of **ternary complexes** that enhance the selectivity and sensitivity.
- **Chelating reagents** used in **spectrophotometric methods:**

8-hydroxyquinoline	4-(2-pyridylazo)-resorcinol (PAR)	1,10-phenanthroline
dithiocarbamates	azo dyes, etc.	

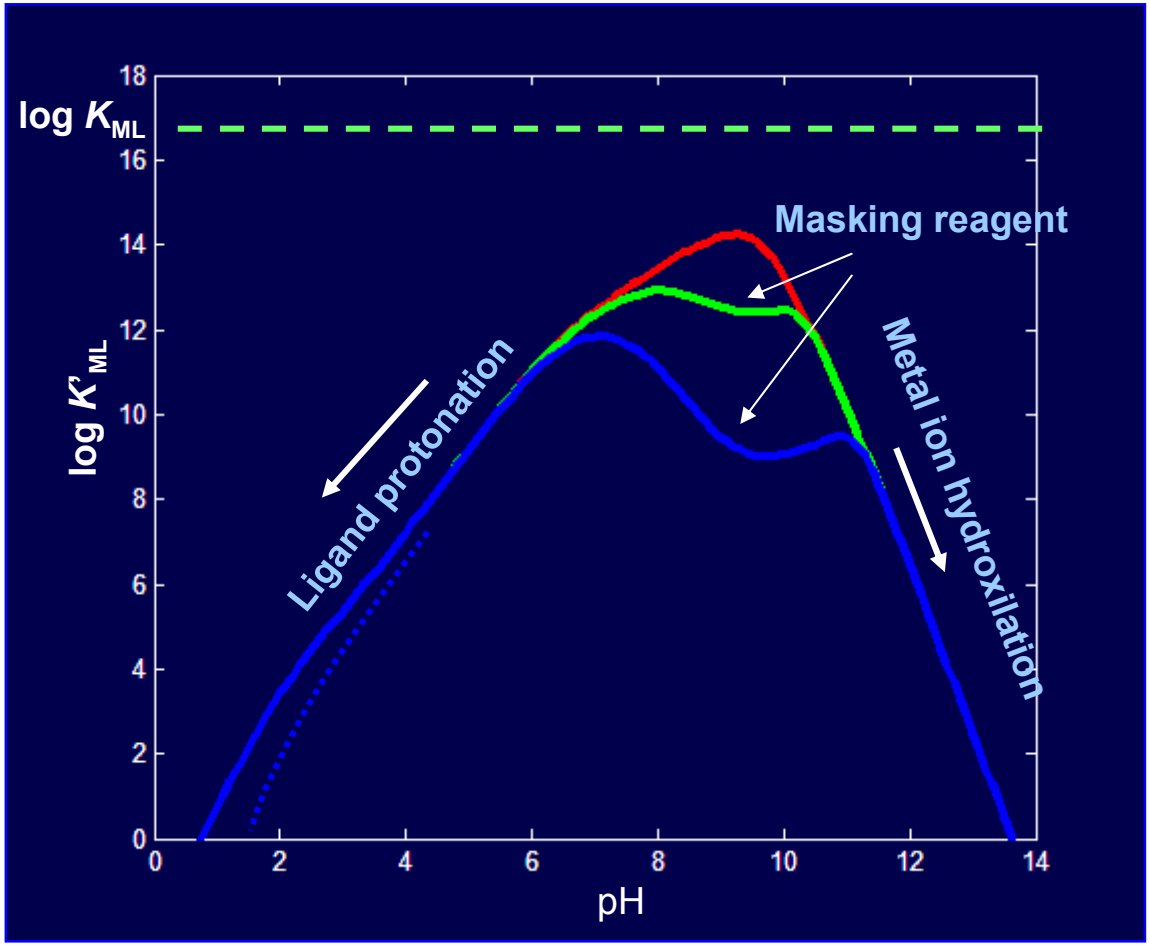
Side reactions

In some cases, selectivity is improved by adding a second ligand to mask the metallic interferences (eliminate the corresponding peak). The integrity of metal chelates is susceptible to pH, since side reactions are expected at low pH with the ligands (protonation) and at high pH with metal ions (hydroxylation).



Poor water solubility of some chelates requires a mobile phase with a high proportion of organic solvent or a surfactant !!!

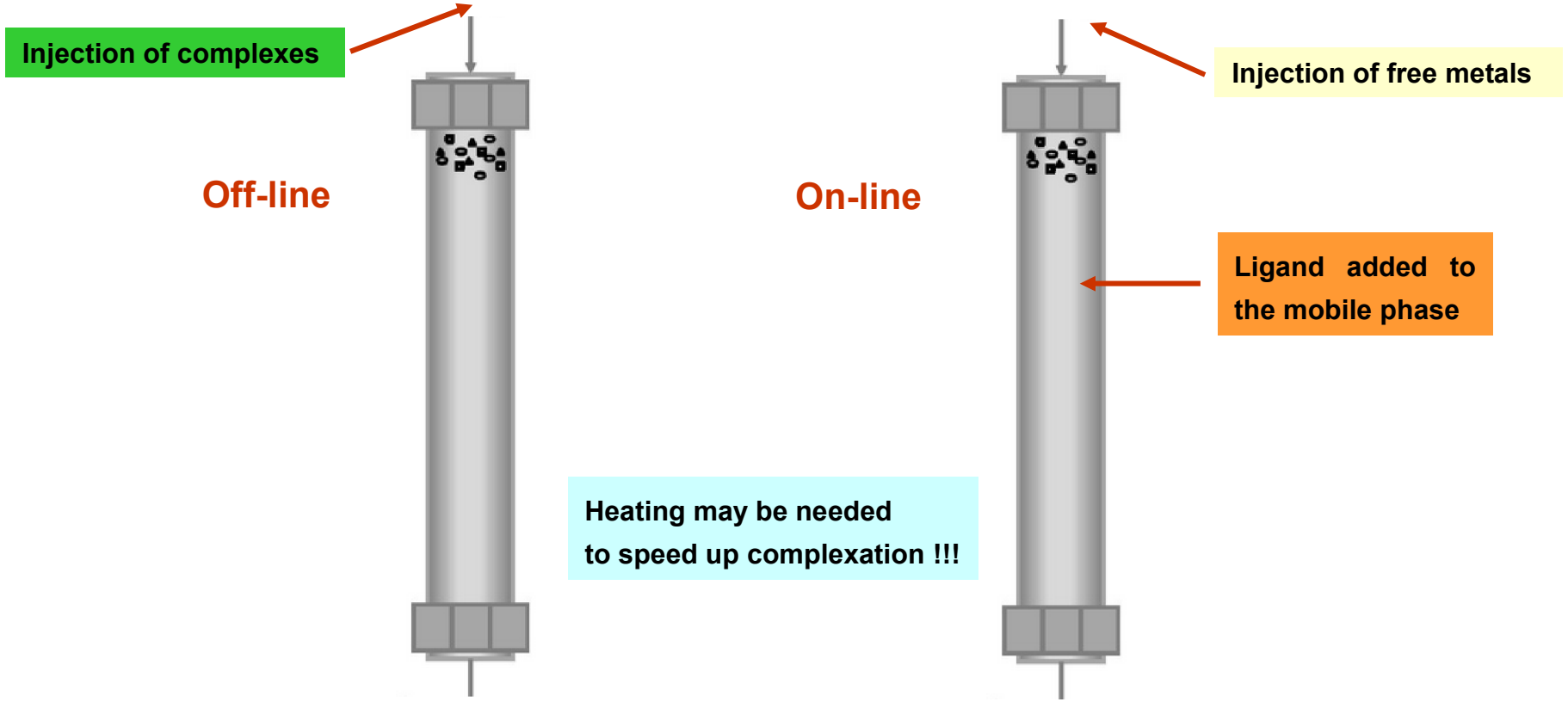
Effect of pH



The narrow pH range of conventional columns may be unsuitable for complex formation.

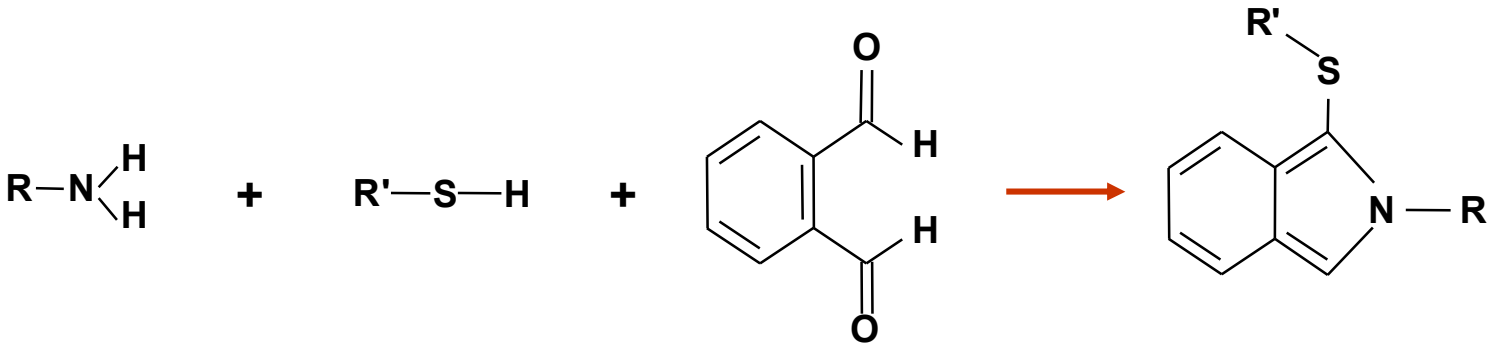
3.2.2. Off-line and on-line modes

There are two main operational modes in metal complexation RPLC: Off-line (pre-column) formation of the complexes with subsequent separation, and on-line formation with a ligand added to the mobile phase.



Derivatisation reactions

These operational modes are also used in the analysis of organic compounds by derivatisation reactions to modify their retention and allow detection.

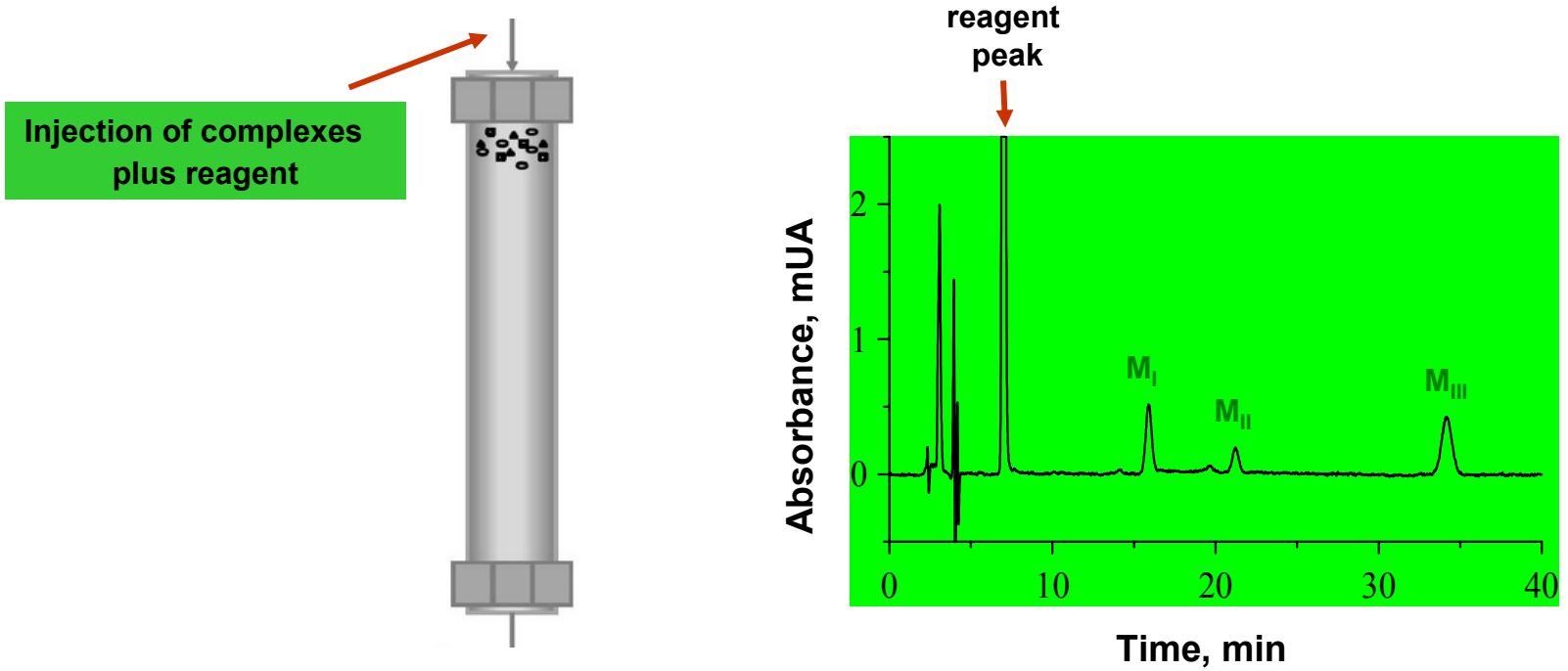


Amino acid Thiol *o*-Phthalaldehyde (OPA) Isoindole

Analysis of amino acids by isoindole formation

Off-line approach: excess reagent

Chelates can be completely separated from the excess of reagent added at the off-line chelation step. This allows detecting only the chelate signals with no background.



Off-line approach: complex stability

- Only thermodynamically or kinetically stable chelates will reach the detector cell, since each chelate migrates completely apart from the ligand resulting in a very steep decrease in ligand concentration in the nearby of the chelate band.
- Weak complexes will dissociate in the analytical column, through solvolysis or ligand-exchange reactions.
- Slow formation of the complexes is not necessarily detrimental for the analysis. The column can work not only as a conventional separation device, but also as a powerful kinetic discriminator for chelates:

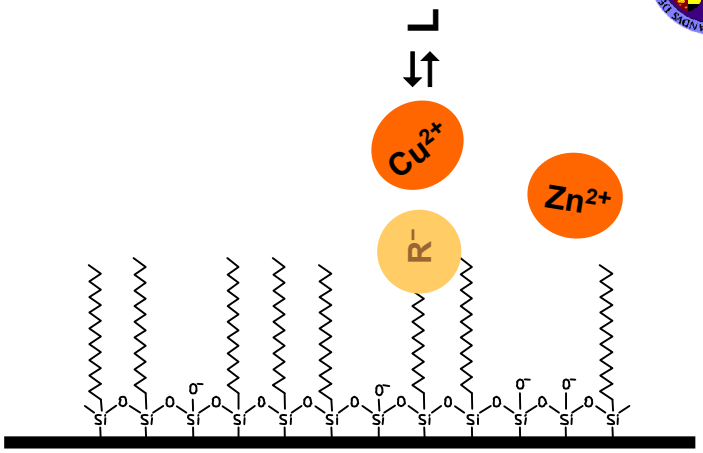
kinetic differentiation chromatography

The synergic interactions of four origins of unique selectivity are combined:

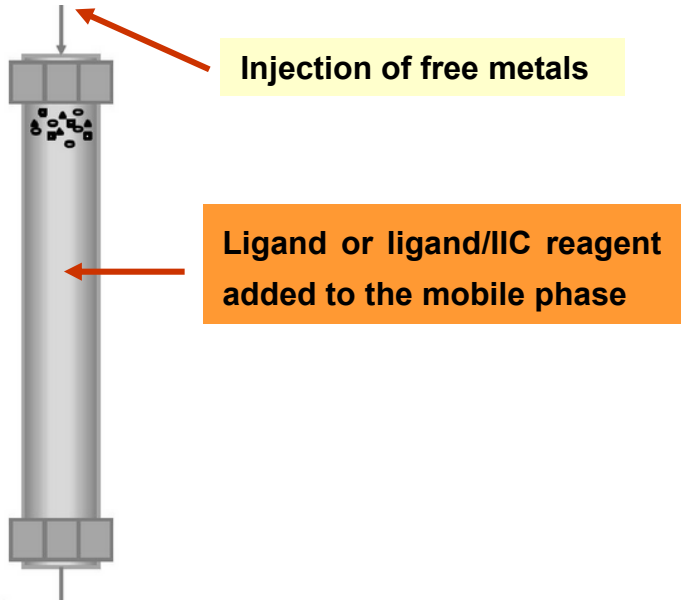
pre-column chelation
chromatographic separation
dissociation kinetics
spectral selectivity

On-line approach:

Dynamic chelating chromatography



- The simplest operational mode: free metal ions are injected and complexed inside the column.
- Combination of complexation and ion exchange reactions: the strengths and rates of the reactions of the metal with the ligand and the IIC counterion added to the mobile phase give rise to the observed selectivity.



The selectivity and sensitivity of the analysis in the off-line mode can be enhanced by combining off-line complexation with solvent extraction. However, many chelates used to determine metal ions by spectrophotometry using solvent extraction are not sufficiently strong, and dissociate in the RPLC column.

Combination of off-line and on-line modes

- A strong chelating reagent can be idoneous for extraction of the metal ions in a sample, but not at all for an RPLC separation, due to:

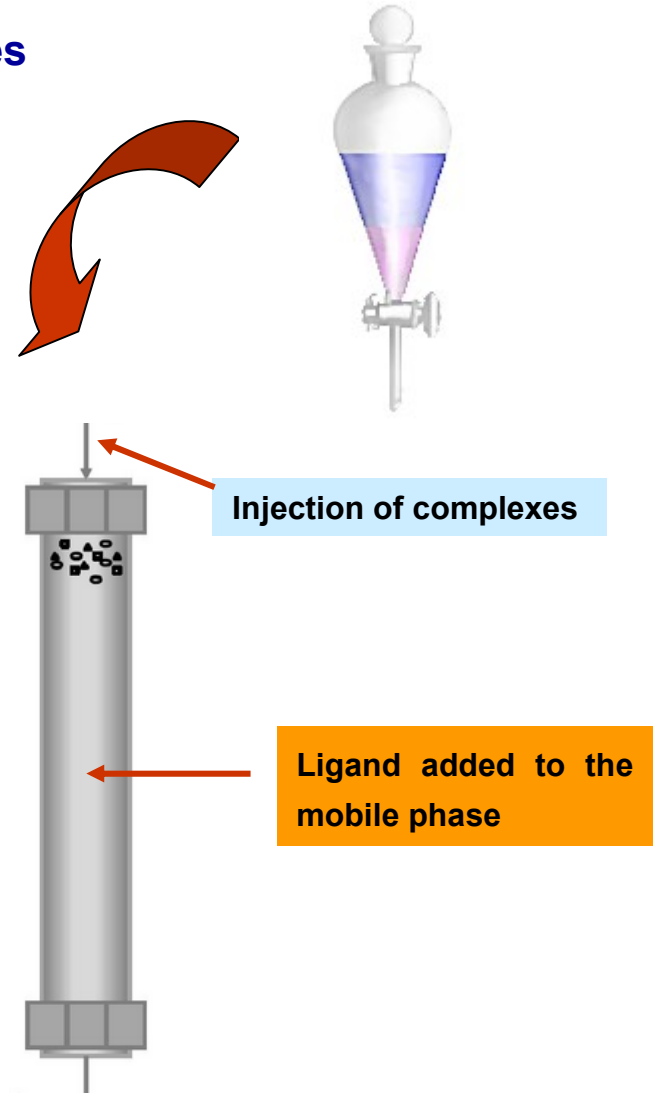
  lack of selectivity

  instability of the complexes
  at the column experimental conditions

  undetectability

- A solution is the combination of the off- and on-line modes.

- The first ligand (used in the extraction step) can be also replaced with another added to the mobile phase in the so-called ligand-exchange approach.



Coating the stationary phase with a ligand

- Hydrophobic **metallochromic ligands,** such as xylenol orange or methyl thymol blue, have been used to **coat the RPLC stationary phase,** producing a chelating capacity to separate metal ions.

- **Two approaches** are possible:

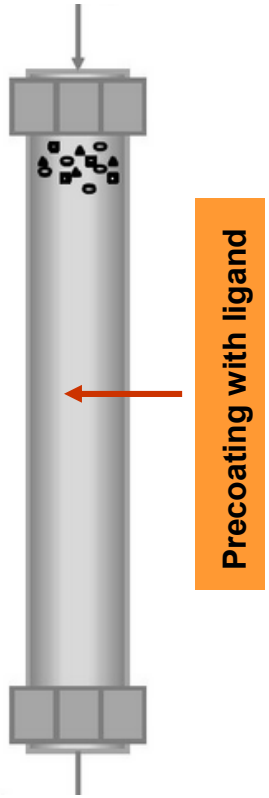
  - ➡ **pre-coating** the stationary phase with the ligand and elution with an inorganic salt
  - ➡ addition of the ligand **within the mobile phase** **(dynamic coating of the stationary phase)**

- **Dynamically coating of the stationary phase** allows:

  larger column capacity and stability

  improved separation efficiency and selectivity

  ability to exploit the ligand in the mobile phase for metal detection



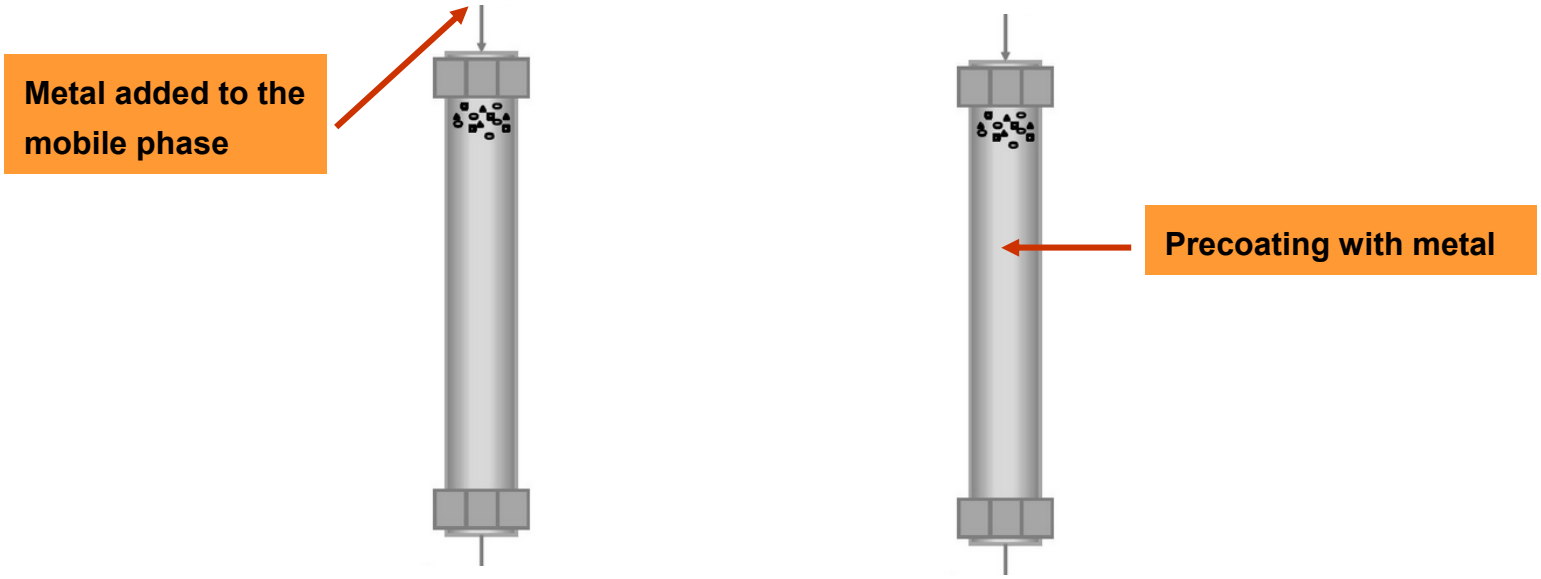
3.2.3. Determination of organic compounds by complexation

Metal cations can be also used to modulate the selectivity in the separation of organic compounds able to form complexes (that act as ligands).

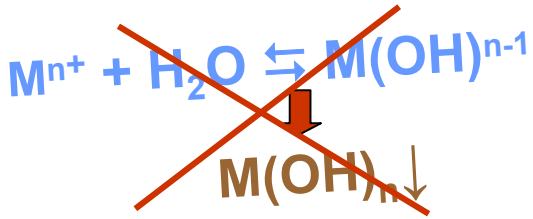
- There are two basic approaches:

  introduction of the metal ions into the mobile phase

  introduction of the metal ions into the stationary phase



- When metals ions are added as salts of non-complexing anions, such as nitrate or perchlorate, the mobile phase should be acidic to avoid metal hydrolysis. In this case, column performance is often poor in terms of selectivity and peak profile.



- The use of charged metal chelates (anionic or cationic) is a more versatile and simple approach, with enhanced performance against the use of conventional IIC reagents.

- When metal complexes are used, two mechanisms are possible:
  - ligand-exchange between the analysed organic compound and the ligands in the complexes
  - formation of ternary complexes (metal-ligand-organic compound)
- Both mechanisms involve:

  hydrophobic selectivity

  steric selectivity, related to the conformationally rigid structures of the chelates, which serve as templates.

$$ML + X \leftrightarrows MX + L$$

$$ML + X \leftrightarrows MXL$$

- The **metal choice** is a compromise between several factors:
  - ⇒ **ability** to form complexes with the analysed organic compound
  - ⇒ **solubility of the complex** in the hydro-organic mixture
  - ⇒ **detection of the complex**

- **Most common metals:** Cu^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}

- **Silver ion (argentation chromatography)** is used in a common method for the analysis of **lipids. Incorporation of Ag^{+} into the solid support is preferred,** since the addition of Ag^{+} in the mobile phase has the disadvantage of using a mobile phase troublesome to handle and is quite more expensive.