SUMMER SCHOOL ON:

DEVELOPMENT AND PRE-CLINICAL

EVALUATION OF RADIOPHARMACEUTICALS

Analytical control and purification of radiopharmaceuticals

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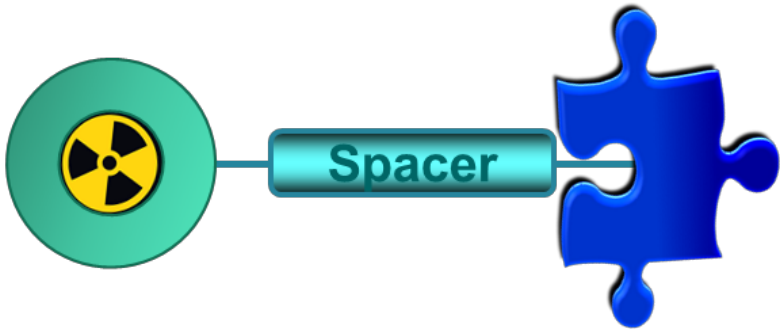
RADIOPHARMACEUTICAL

***Ph. Eur.*: Any medicinal product which, when ready for use, contains a radionuclide included for a medicinal purpose.**

Comply with all Quality, Safety and Efficacy requirements applied to medicines



Radiometal coordinated with a bifunctional chelator

Target specific vector

Radiopharmaceuticals: Quality Control

Controls on the product and its components to check that they comply with all the specifications that have been previously established for each Radiopharmaceutical

- **Radioactive concentration**
- **Radionuclide purity**
- **Radiochemical purity (RCP): Labelling Efficiency**
- **Chemical purity: Related with the chemical species in the formulation regardless of the presence of radioactivity**
- **Sterility**
- **Apyrogenicity**
- **pH**

Responsibility of the approved manufacturer and supplier

Radiochemical Purity (RCP)

RCP may be defined as "the proportion of the total radioactivity in the radiopharmaceutical which is present as the desired radiochemical species"

Determines the biodistribution of the Radiopharmaceutical

$$\text{RCP}(\%) = \frac{\text{Radioactivity component}}{\text{Total Radioactivity}} \times 100$$

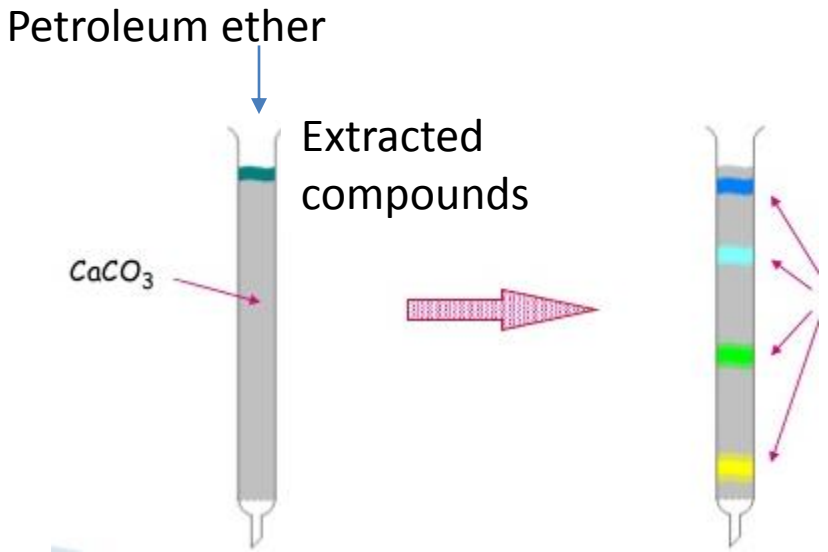
Measurement of RCP requires a method to separate and accurately detect all the different radiochemical species that can be present in the radiopharmaceutical preparation

Chromatographic Techniques and Electrophoresis

Origin of Chromatographic Methods

by the Russian botanist, Mikhail S. Tswett (1903)

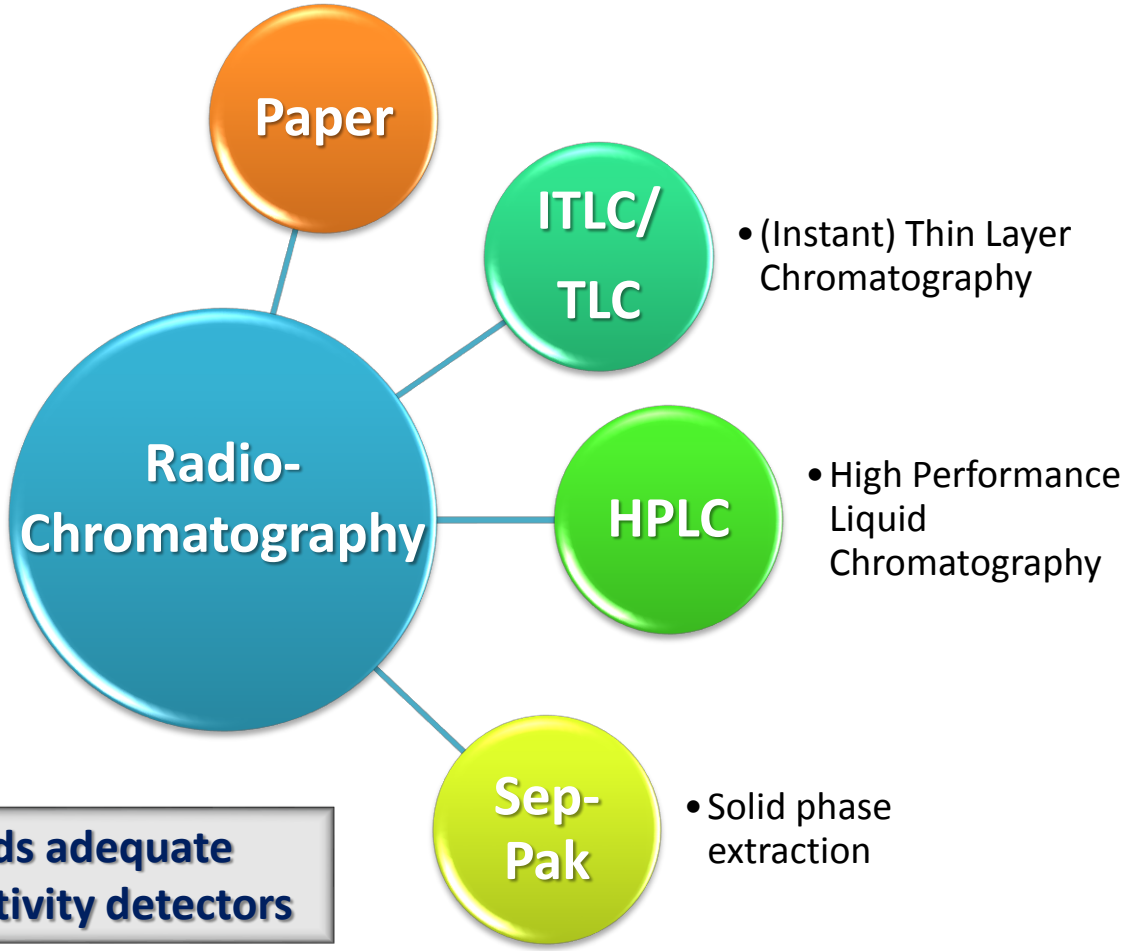
Compounds [leaf pigments], extracted from plants were separated using a solvent in a column



Tswett's Experiment

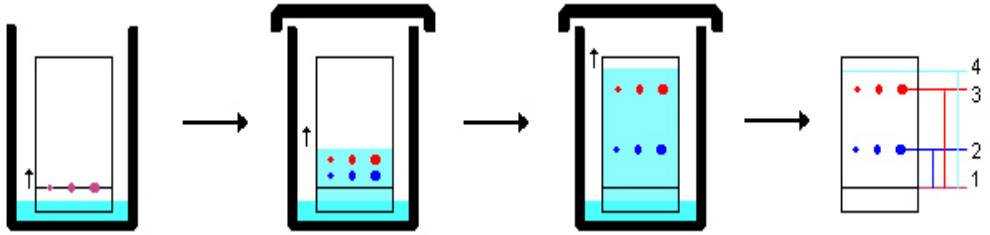
Tswett coined the name **chromatography** [from the Greek words **chroma**, meaning **color**, and **graph**, meaning **writing**— literally, color writing] to describe his colorful experiment

Analytical Control of Radiopharmaceuticals (RCP)



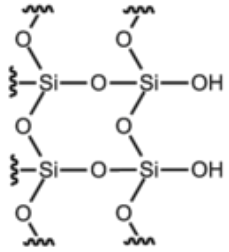
Demands adequate radioactivity detectors

TLC or ITLC-SG

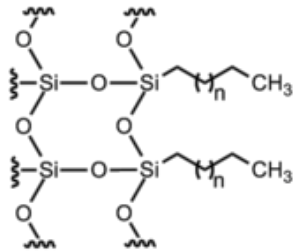


Detection and quantification ??

- **Stationary phase**: thin layer of an adsorbent (silica gel, reversed phase silica gel, alumina,…) coated in a support;
- **Mobile phase** is a solvent/ mixture of solvents
- The compounds are separated based on their interaction with the **stationary phase** and the **mobile phase**
- R_f: For a given solvent and stationary phase, each compound will have a characteristic **retention factor** (Rf) that can be used to identify it



Normal phase silica



Reversed phase silica

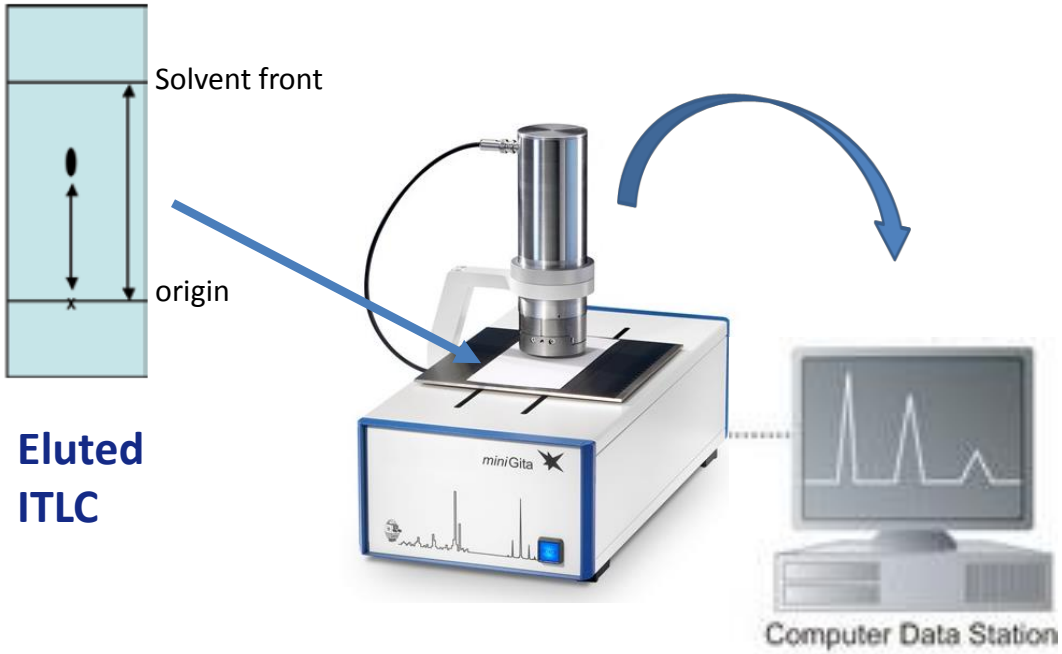
$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}$$

RADIO-TLC or ITLC-SG

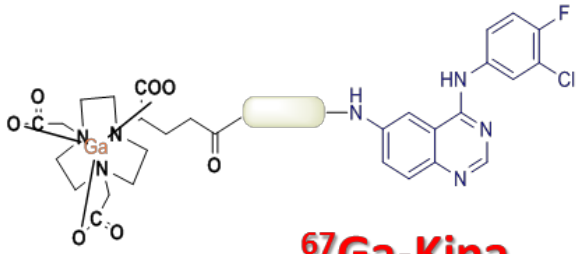
Detection and quantification ??

Equipment used for quality control should be regularly calibrated

- Cut and count in the dose calibrator
- Gamma counters
- Dedicated scanners

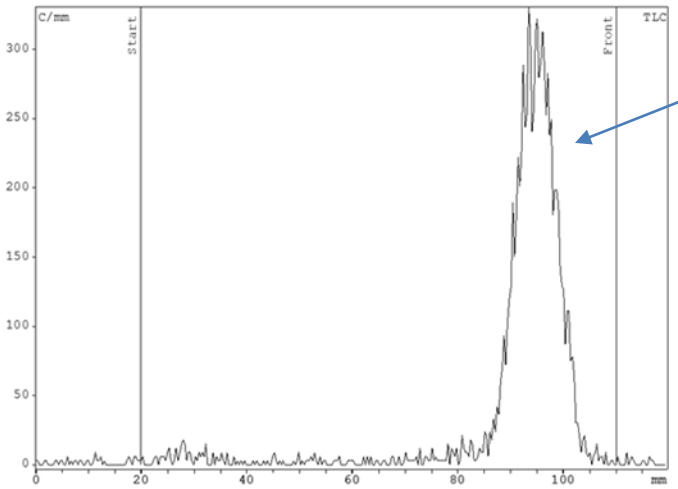


Radio-TLC scanner for rapid and accurate determination of radiochemical purity



^{67}Ga-Kina

^{67}Ga-colloid
Rf = 0

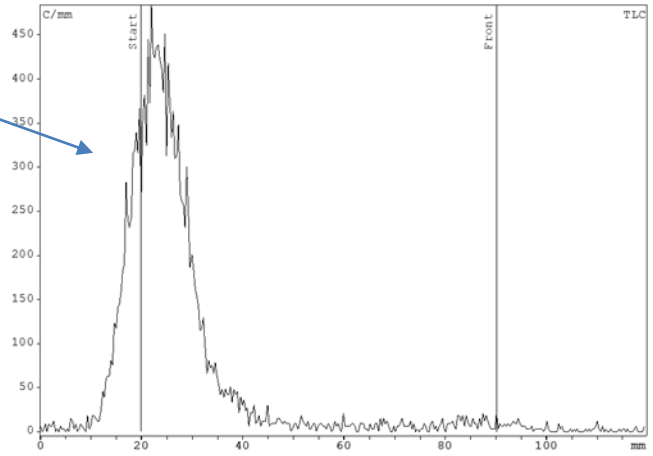


^{67}Ga-Kina

ITLC-SG

RCP >95%

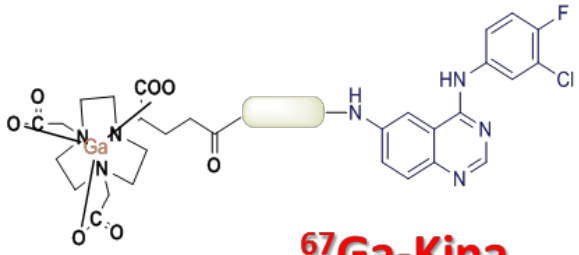
^{67}Ga-Kina



"free" ^{67}Ga
Rf = 1;

ITLC-SG

RCP
>95%



^{67}Ga-Kina

However, we only confirmed the absence of “free” radiometal and radiocolloidal species

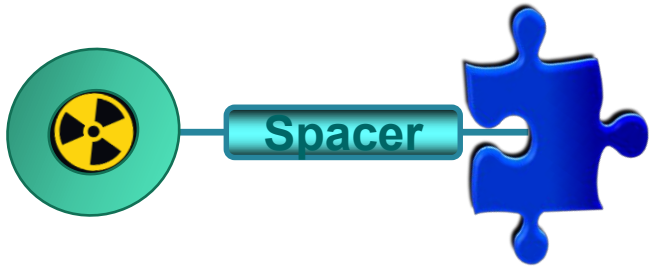
Is the detected radioactivity in the proposed chemical structure?

?

Identification / Characterization
Demands Higher Resolution

HPLC

Development of radiopharmaceuticlas



Different Radioprobes

Small molecules/peptides
(lipophilicity, charge...)

Macromolecules

Antibodies, etc

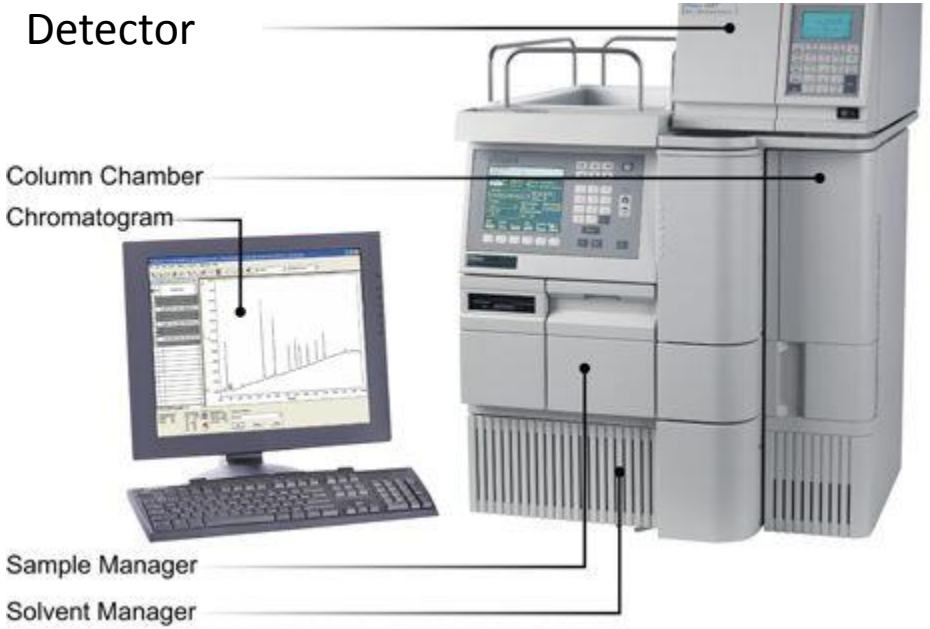
Nanoparticles

Determines the Type of Chromatography to be used in their analysis

HPLC

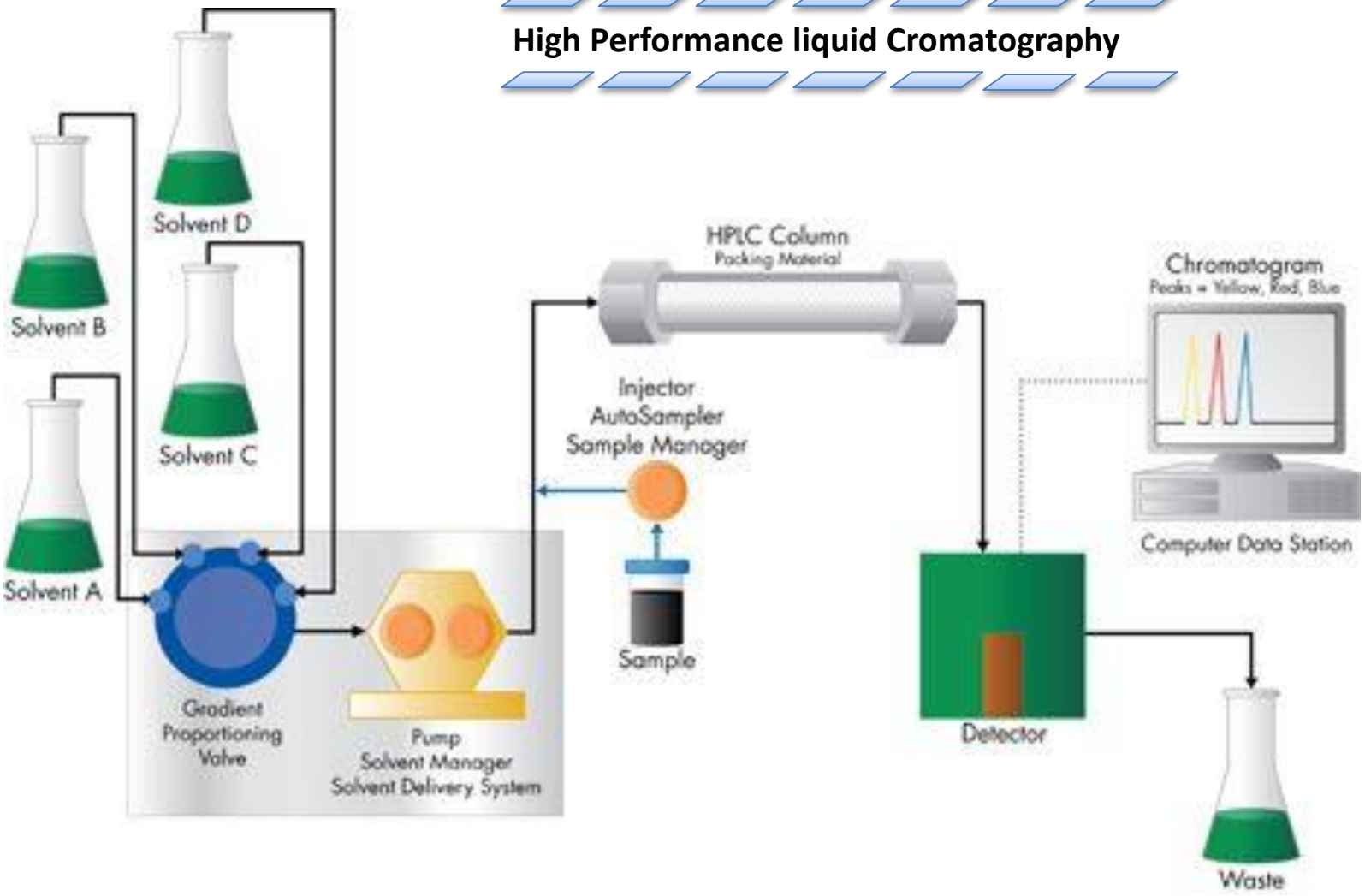
High Performance liquid Cromatography

Radioactivity Detector



Radio-HPLC

High Performance liquid Cromatography

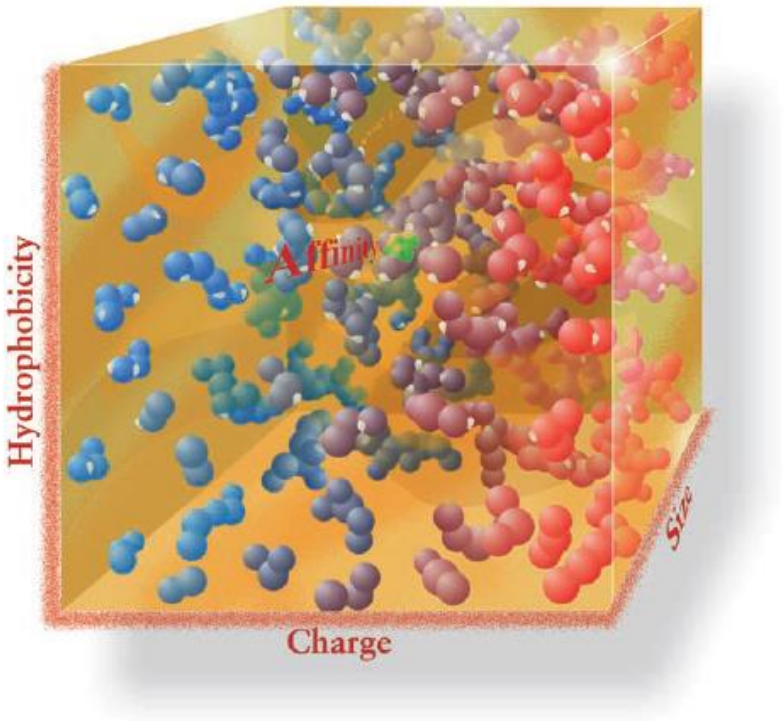


DETECTORS

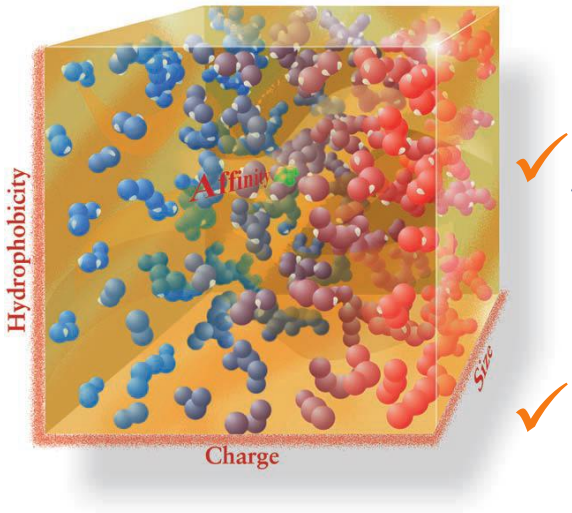
- UV/VIS Absorption Detectors
- Refractive Index (RI)
- Evaporative Light Scattering Detector (ELSD)
- The Fluorescence Detector
- Electrochemical Detectors (ECDs)
- Conductivity Detector
- MS
- **Radioactivity**

The type of detector(s) depends on the characteristics of the sample to be analysed

Types of HPLC Chromatography



Types of HPLC Chromatography



✓ Size Exclusion Chromatography (SEC)

Separates proteins according to differences in molecular size.

✓ Hydrophobic Interaction Chromatography (HIC)

Separation of biomolecules based on differences in their surface hydrophobicity.

✓ Affinity Chromatography (AC)

Reversible interaction between a protein (or group of proteins) and a specific ligand attached to a chromatographic matrix.

✓ Ion Exchange Chromatography (IEC)

IEC separates proteins based on differences in their net surface charge.

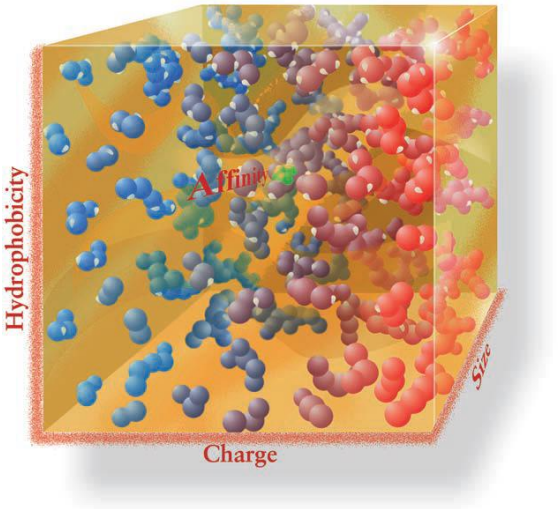
✓ Reversed Phase Chromatography (RPC)

RPC separates molecules based on the reversible interaction between the molecule and the hydrophobic surface of a chromatographic medium.

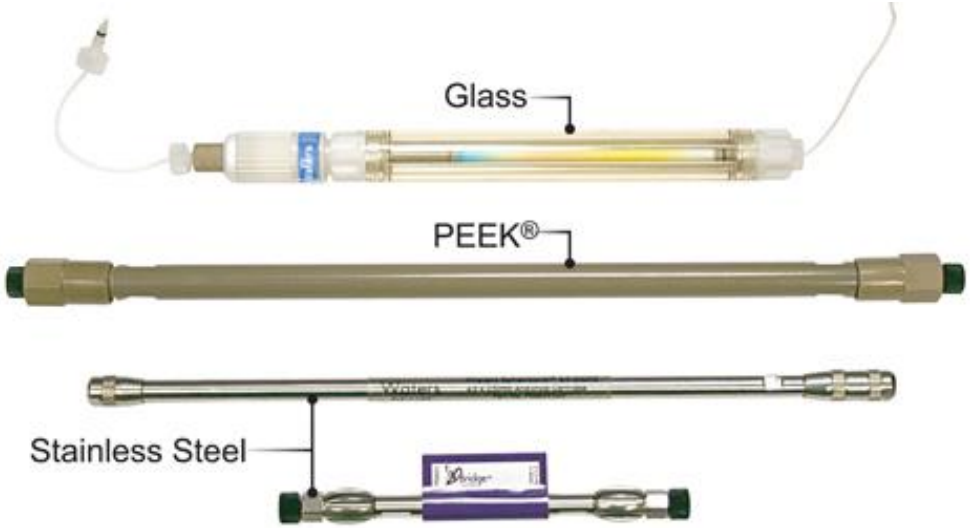
Types of HPLC Chromatography

✓ Reversed Phase Chromatography (RPC)

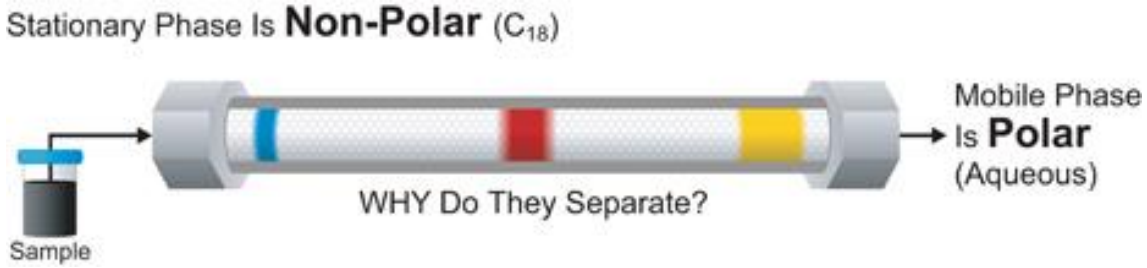
RPC separates molecules based on the reversible interaction between the molecule and the hydrophobic surface of a chromatographic medium.



HPLC Columns

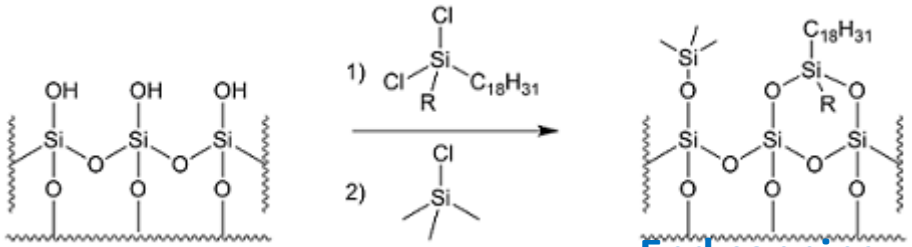


Reversed-Phase HPLC



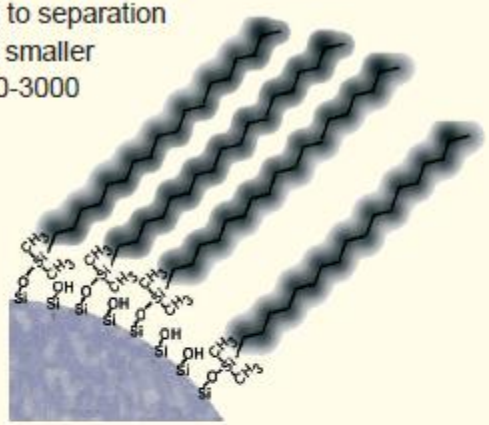
RP-HPLC separates molecules according to differences in their **hydrophobicity**.
Essential tool in the separation and analysis of lipophilic small molecules and peptides because of its **resolution**, **versatility**, **sensitive detection** and its ability to work together with techniques such as mass spectrometry.

RP- HPLC

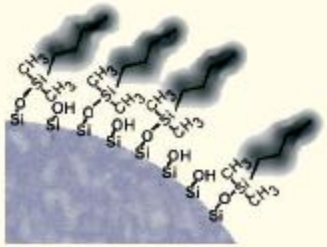


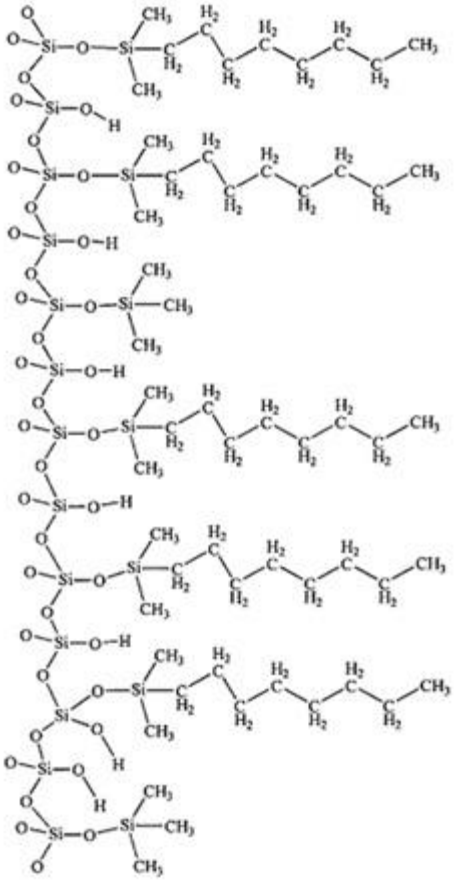
End capping

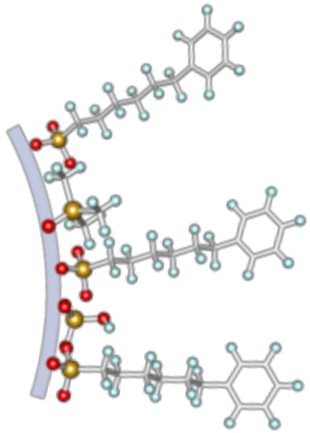
A. C18 hydrophobic phase is best suited to separation of peptides smaller than ~ 2000-3000 daltons



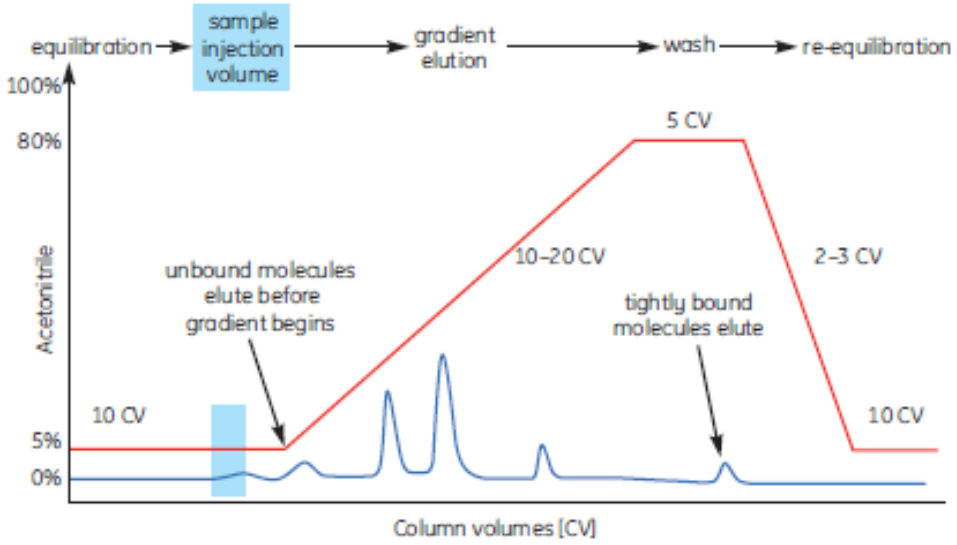
B. C4 hydrophobic phase is best suited to separation of peptides greater than ~ 3000 daltons and proteins







RP-HPLC: Gradient Elution



Sample Preparation: Samples **must be free from particulate matter** and, when possible, dissolved in the starting mobile phase.
Sample can be: **Centrifuged** and/or **Filtrated** with 0.22 or 0.45 µm filtres.

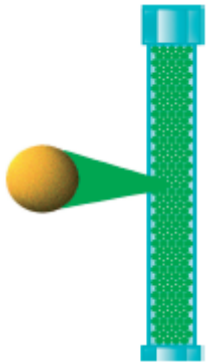
If sample is insoluble try different solutions:
10-30% acetic acid, formic acid**, DMSO** (dimethyl sulphoxide), **TFA** or acetonitrile.

Note that a very hydrophobic molecule/peptide dissolved in DMSO may precipitate or bind irreversibly to an RPC matrix. Test first with aliquots of sample.

RP-HPLC: Column Characteristics

Peptides are separated by interacting with the hydrophobic surface of particles packed in columns

Particles



The particles in the column are usually made of **silica**:

- ✓ Physically robust,
- ✓ Stable under most solvent conditions (except at pH > 7),
- ✓ **Spherical particles** of various **sizes** and **pores**.

Silica purity: Demands on high performance RP silicas

- ➢ **Synthetic, high purity silica gel**
- ➢ **Totally spherical particle with outstanding surface geometry**
- ➢ **Ultra low metal content**
- ➢ **Remarkable pressure stability**
- ➢ **Prolonged column lifetime**
- ➢ **high batch-to-batch reproducibility**

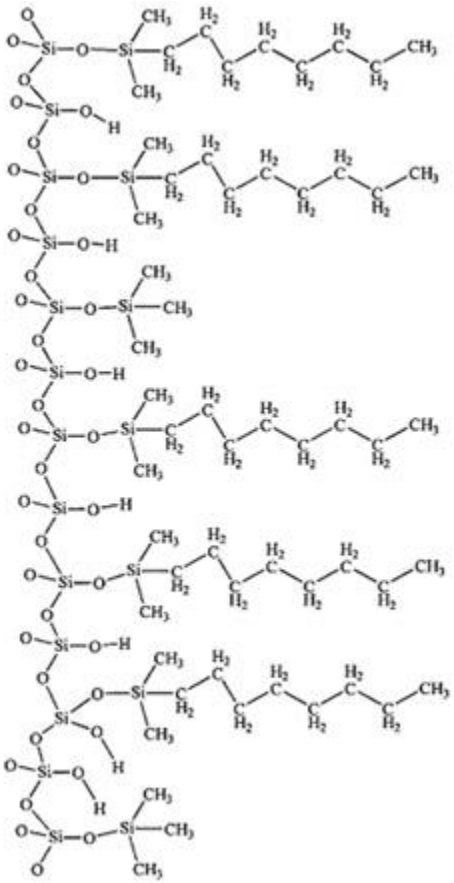
Al, Fe, Na and Ca < 5 ppm

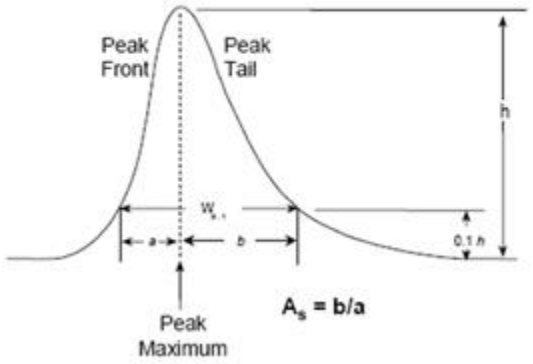
Ti and Zr < 1 ppm

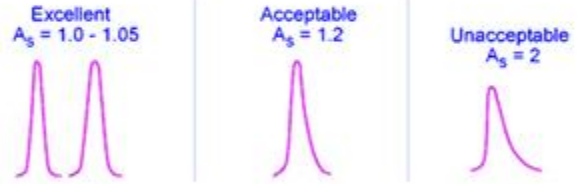
As < 0.5 ppm

Hg < 0.05 ppm

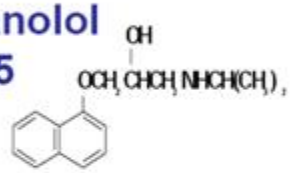
Asymmetry





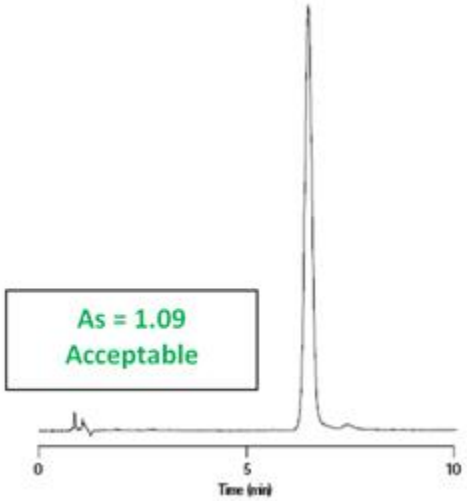


Propranolol
pKa 9.5



As = 2.9
Unacceptable

As = 1.09
Acceptable



Mobile Phase

Organic Modifier: The purpose of the organic solvent is to desorb peptide molecules from the adsorbent hydrophobic surface. Slowly raising the concentration of organic solvent (gradient) until the polypeptides/peptide of interest desorb and elute.

✓ **Acetonitrile:** The organic solvent most commonly used
- ❑ Is volatile and easily removed from the sample.
- ❑ Has **low viscosity** and thus **low back pressure**.
- ❑ Is quite transparent to low wavelength UV light.
- ❑ Has a long history of successful separations.

✓ **Isopropanol:** Isopropanol plays a particular role in polypeptide chromatography
- ❑ The major disadvantage of isopropanol is the **high viscosity** (consequently high back **pressure**)
- ❑ Isopropanol is useful **to improve recovery** of some polypeptides, particularly very hydrophobic proteins.
- ❑ added to acetonitrile (1 – 5%) to enhance recovery of hydrophobic proteins.

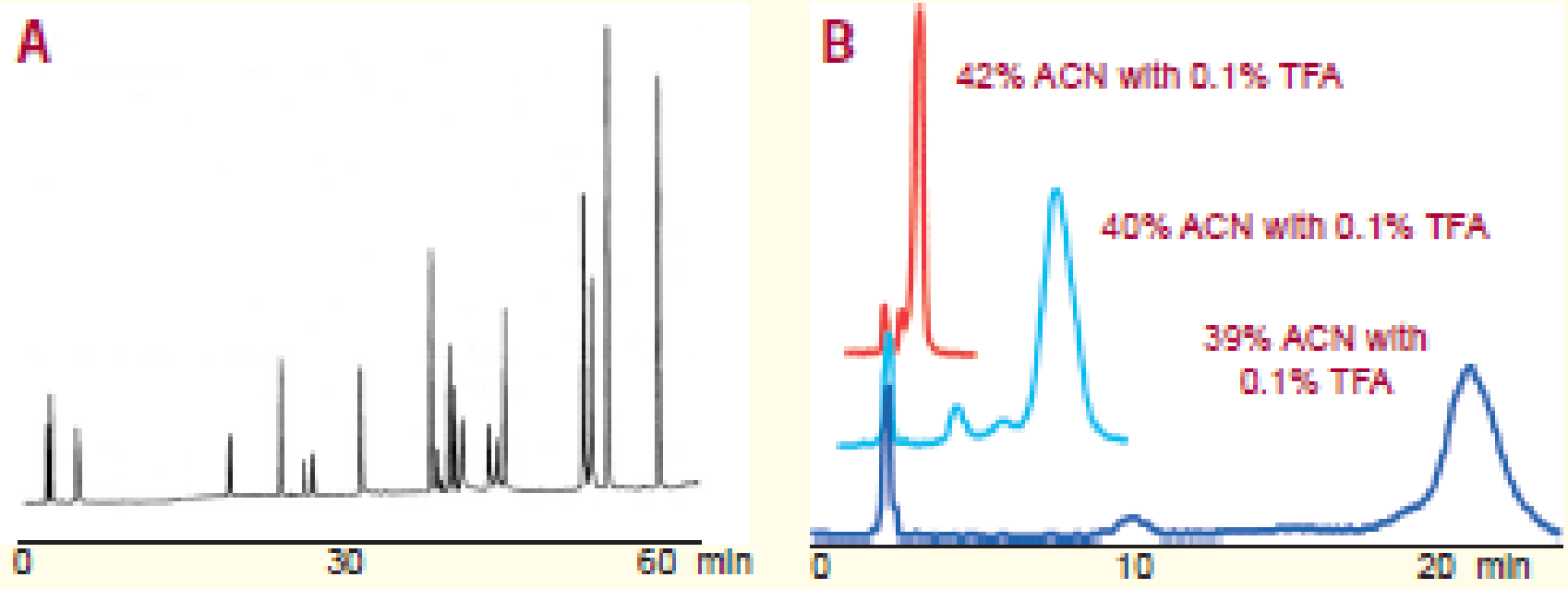
Other organic modifiers:

methanol or ethanol are seldom used except for very hydrophobic proteins. Ethanol is also used for large scale process purification of proteins because of its low toxicity (accepted by FDA) and low price.

Figure 6.

A. Peptides and proteins elute with sharp peaks during gradient elution.

B. With isocratic elution protein peaks, in this case lysozyme, are broad and small changes in organic solvent result in large changes in retention.



Column Characteristics: Pore Diameter

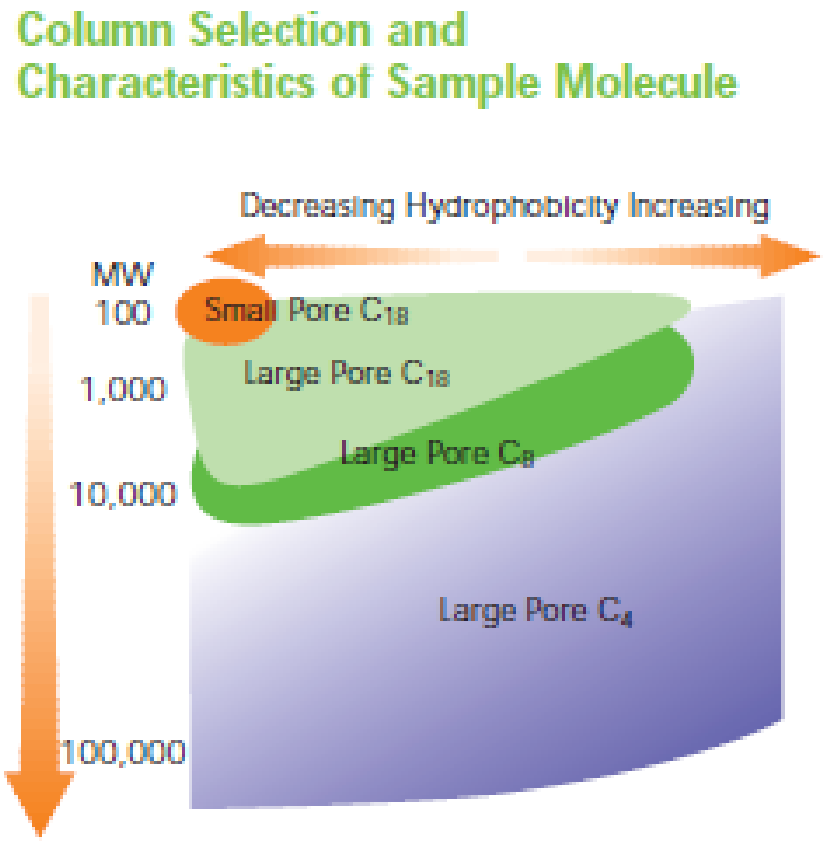
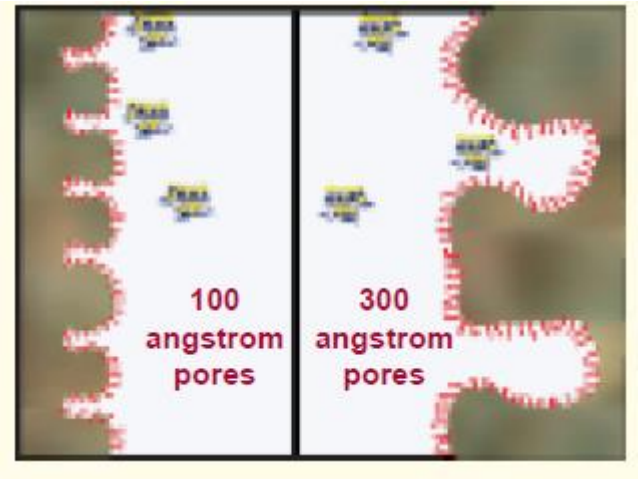


Figure 7. *This chart indicates the pore size and bonding recommended for various molecular weights and hydrophobicities.*



✓Small pore (~ 100 angstrom) particles (left) do not permit most proteins to enter the pores, limiting surface interaction.

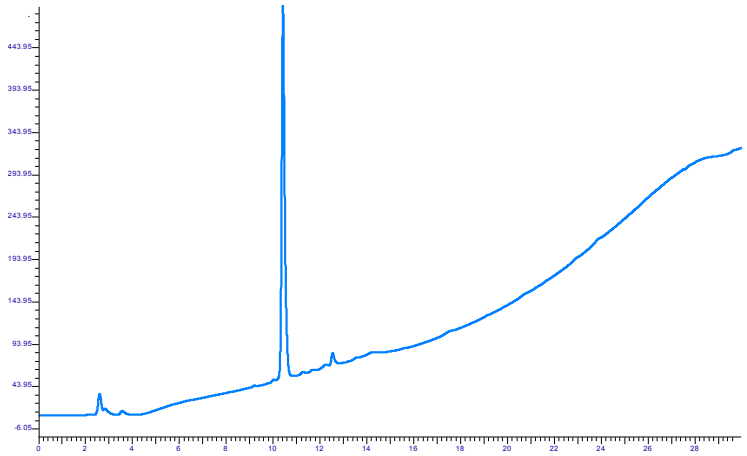
✓Particles with wide (~300 angstrom) pores (right) allow proteins to interact with the hydrophobic surface.

Ion-Pairing Reagents and Buffers

The ion-pairing reagent or buffer sets the eluent pH and interacts with the polypeptide/peptide to enhance the separation.

Trifluoroacetic acid (TFA):

- It is volatile and easily removed from collected fractions;
- It has little UV absorption at low wavelengths (210 - 220 nm);
- Proven reliability in RP-HPLC peptide separations.
- TFA is usually used at concentrations of **0.1%** but concentrations up to **0.5%** could be necessary for more hydrophobic peptides.
- New column developments allow the use of much lower TFA concentrations



To reduce baseline drift due to TFA
For example, use:
0.1% TFA in Solvent A;
0.085% TFA in Solvent B.

Ion-Pairing Reagents and Buffers

Alternative ion-pair reagents: sometimes used in protein/peptide separations

- Triethylamine phosphate (TEAP; 20-30 mM; pH 2 – 2.5); can also be used at higher pH changing selectivity.
- Heptafluorobutyric acid (HFBA);
- Formic acid (FA; 10-60%); Better for LC/MS.
- Acetate buffers.

Figure 18. The use of ion-pair reagents other than TFA may result in different selectivity

Conditions

Column: C18 wide pore, 4.6 x 250 mm

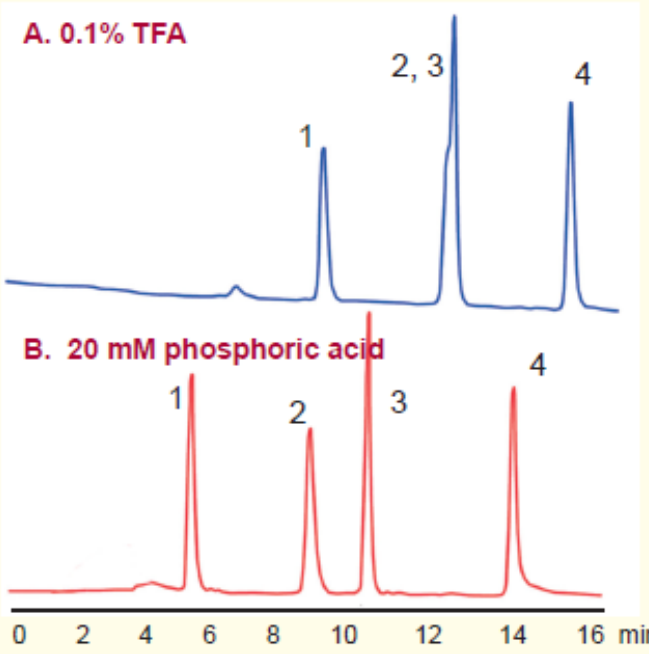
Eluent:

A. Gradient 4 - 40% ACN in 18 min with 0.1% TFA, pH 2

B. Gradient 4 - 40% ACN in 18 min with 20mM phosphoric acid, pH 2

Sample.

1. bradykinin
2. neurotensin
3. bombesin
4. eledoisin



Effect of pH

The Effect of pH on Peptide Separations

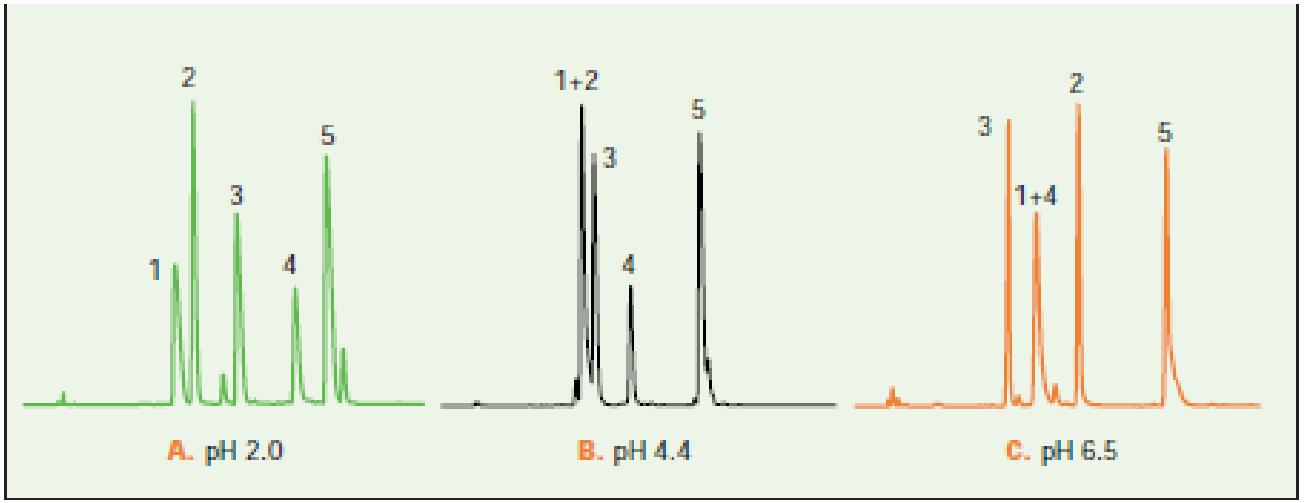
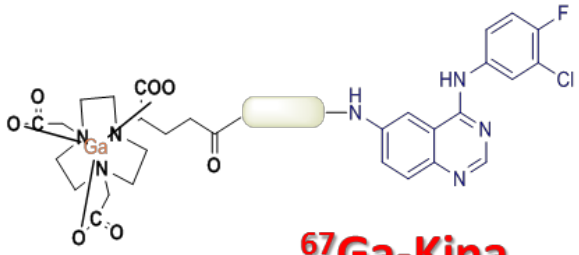


Figure 21. *Elution of five peptides at pH 2.0, 4.4 and 6.5 with phosphate as the buffer. Column: VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm). Eluent: 15–30% ACN in 30 min at 1.0 mL/min; plus A. 20 mM phosphate, pH 2.0 B. 20 mM phosphate, pH 4.4 C. 20 mM phosphate, pH 6.5 Peptides: 1. bradykinin 2. oxytocin 3. angiotensin II 4. neurotensin 5. angiotensin I.*

ITLC-SG

RCP
>95%



^{67}Ga-Kina

However, we only confirmed the absence of “free” radiometal and radiocolloidal species

Is the detected radioactivity in the proposed chemical structure?

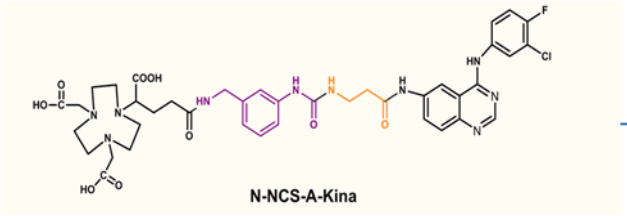
?

Identification / Characterization
Demands Higher Resolution

HPLC

Identification/ Characterization

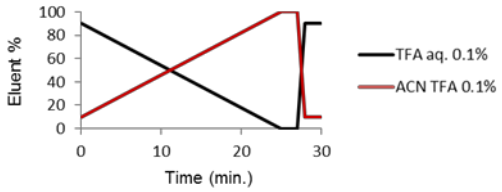
Ga Nitrate
pH 5.5, rt, 3 h
[L] = 1.0 x 10^{-5} M

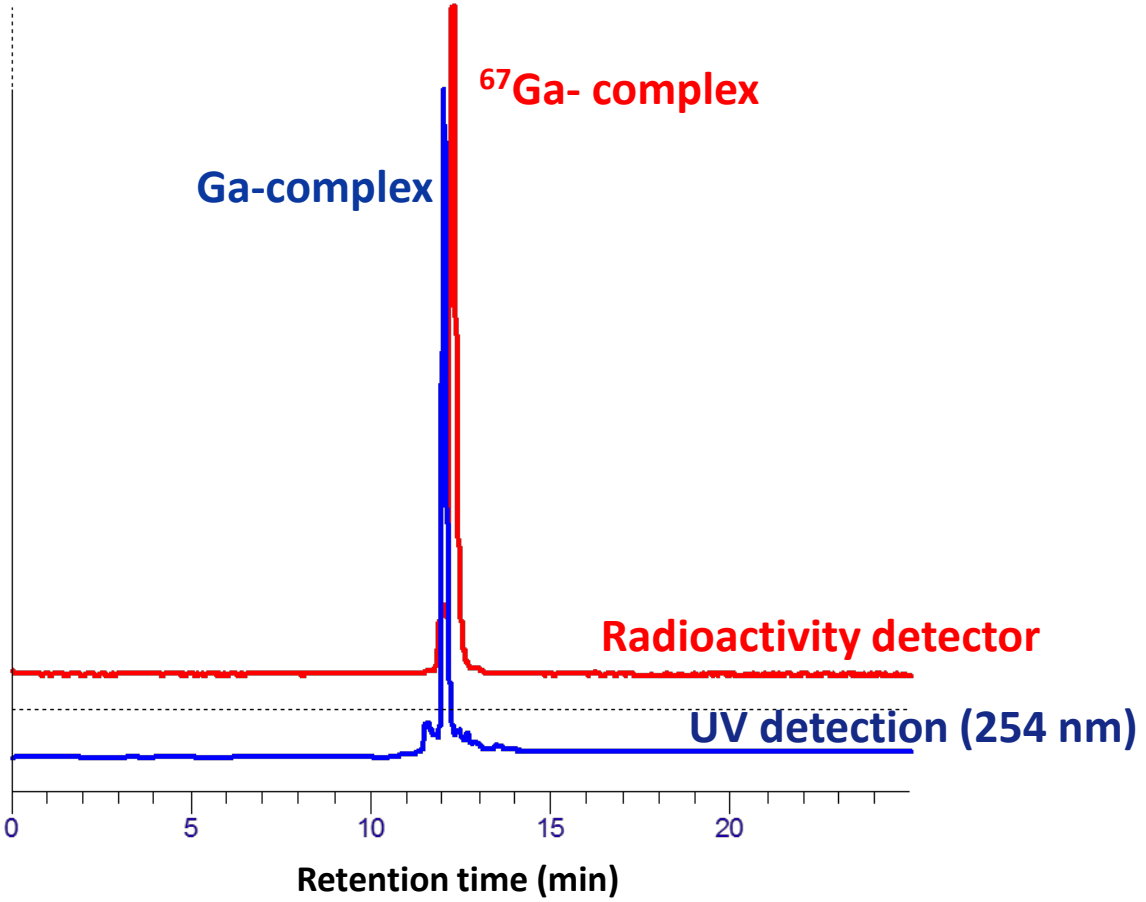


$^{67}GaCl_3$
pH 5.5, rt, 20 min
[L] = 1.0 x 10^{-5} M

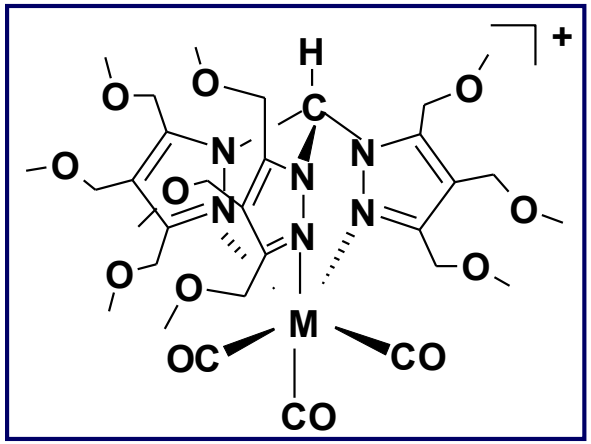
The inactive Ga complex was caracterized by ESI-MS and NMR

Nucleosil C18 column



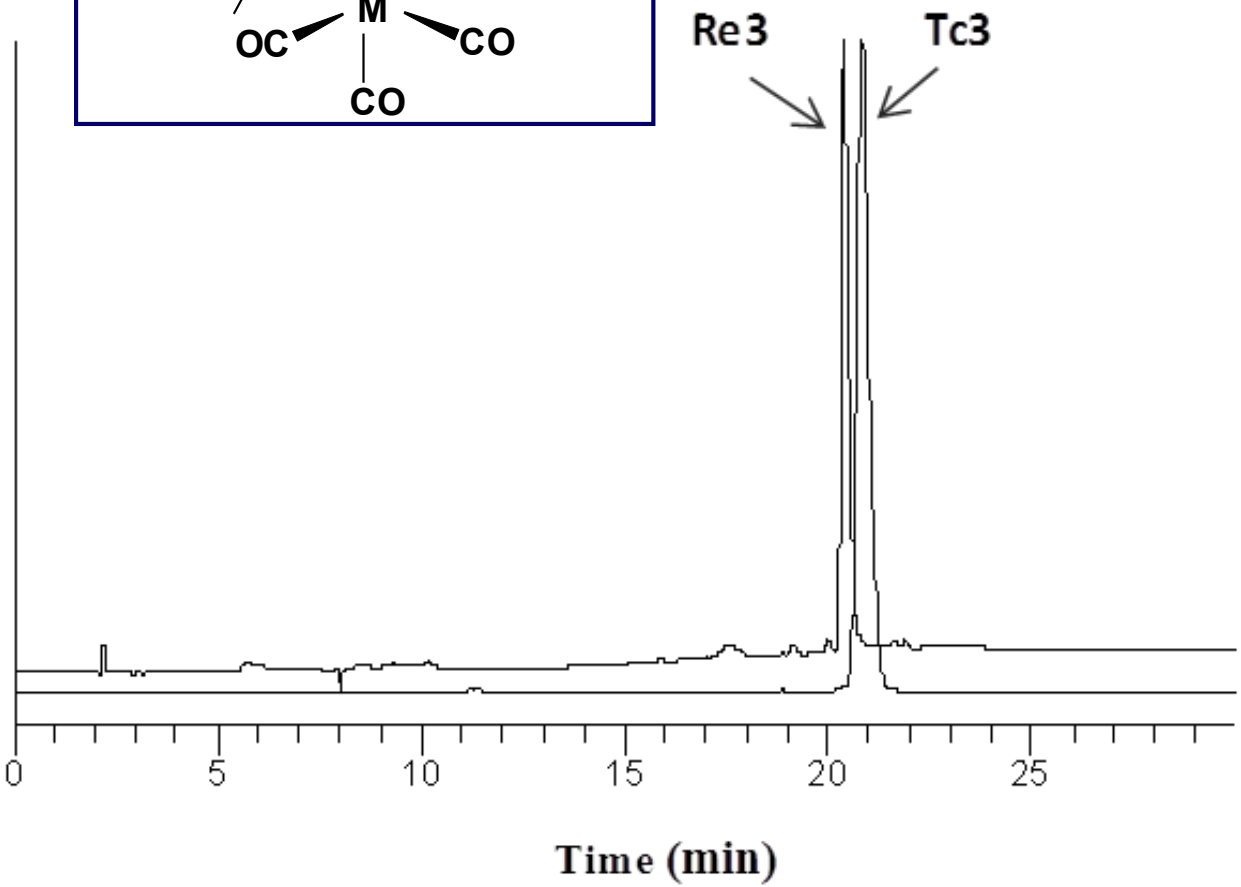


Identification/ Characterization



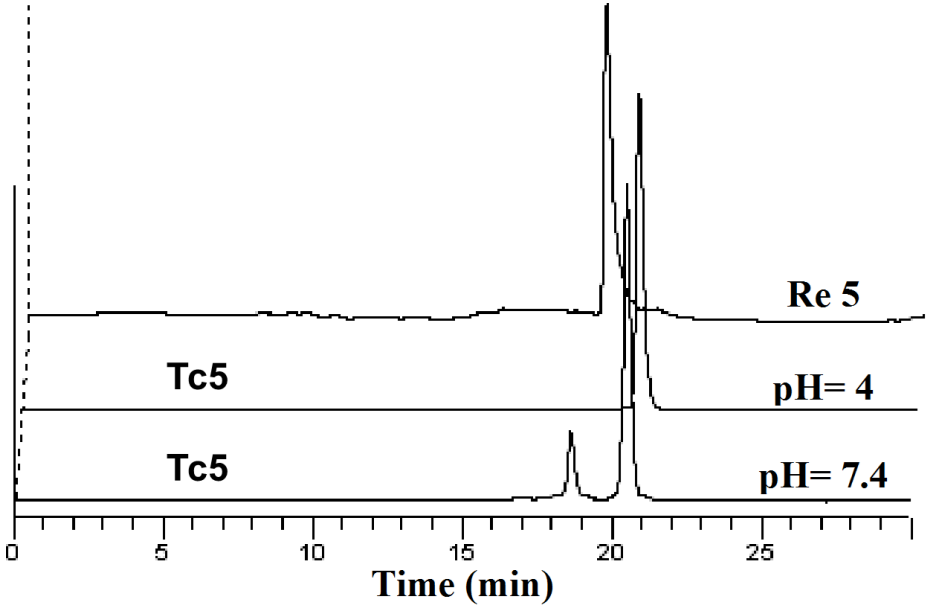
^{99m}Tc
HPLC

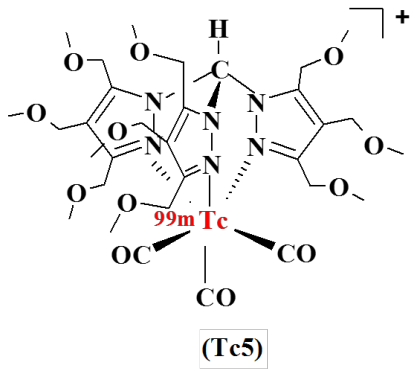
Re
HPLC
^{1}H/^{13}C NMR
ESI-MS
IR



Identification/ Characterization

Optimization of radiolabeling conditions



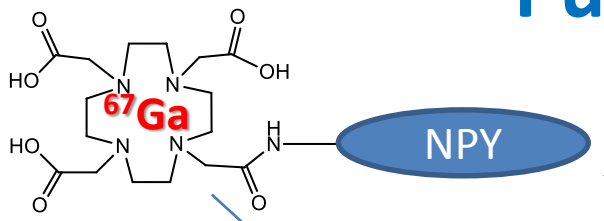


HPLC chromatograms of complexes Tc5 (prepared at pH=4 and pH=7; radiometric detection) and Re5 (UV detection)

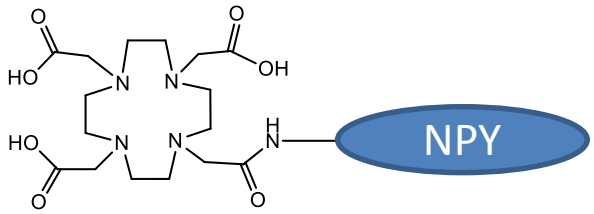
RH-HPLC / ITLC

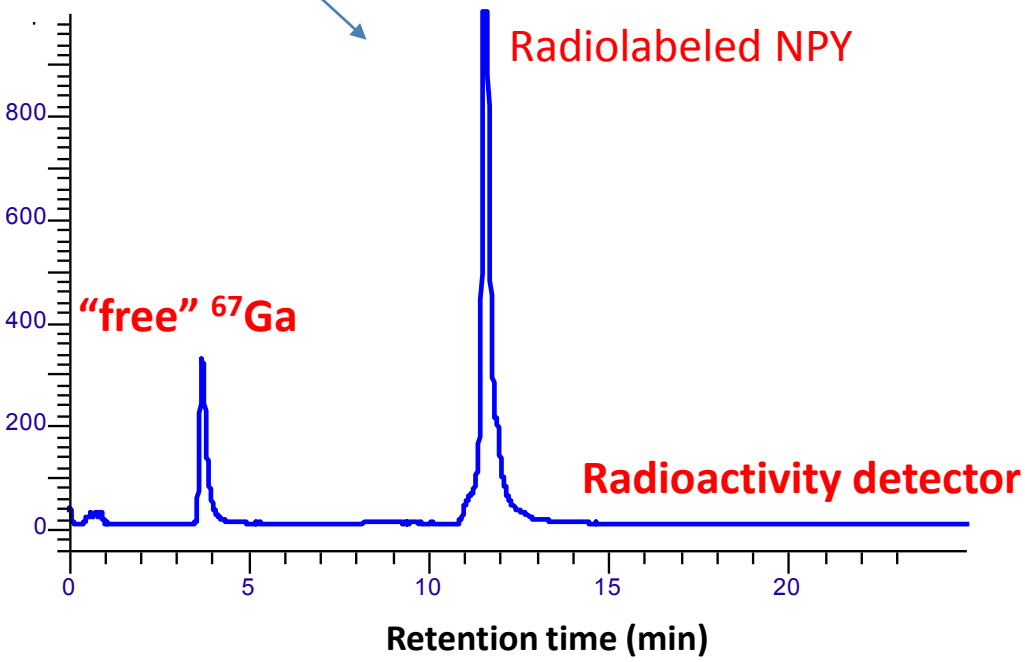
- In the RP-HPLC the colloidal species and aggregates are retained in the top of the column. They are not detected!
- Influence in the biodistribution: uptake in liver, lung.
- The colloidal species should be evaluated by **planar chromatography: ITLC or paper**

Purification



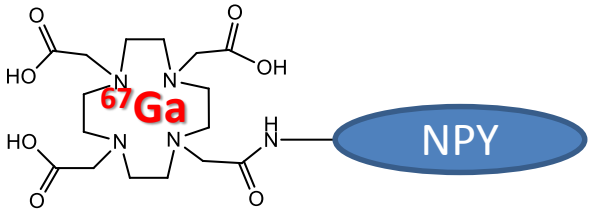
$^{67}GaCl_3$
pH 5.5, 95ºC, 30 min
[L] = 1.0 x 10^{-5} M

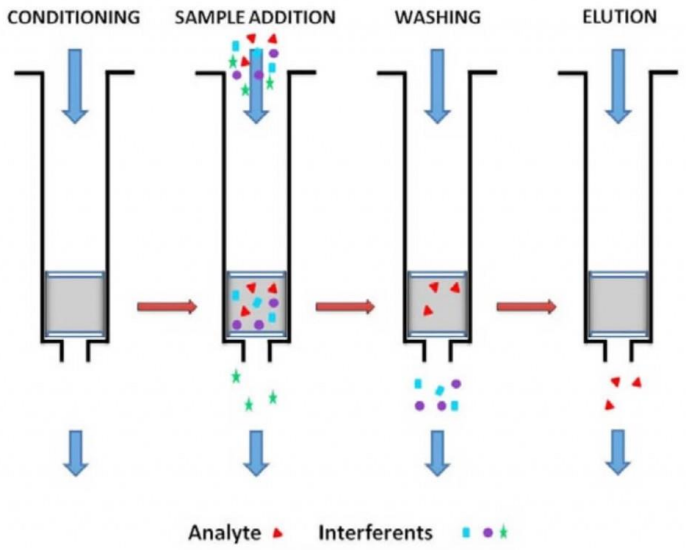




- **Purification to achieve adequate RCP**
- **How to purify?**
- **In this case we can try purification by SPE using classic C18 Sep-Pak cartridges**

Purification by SPE

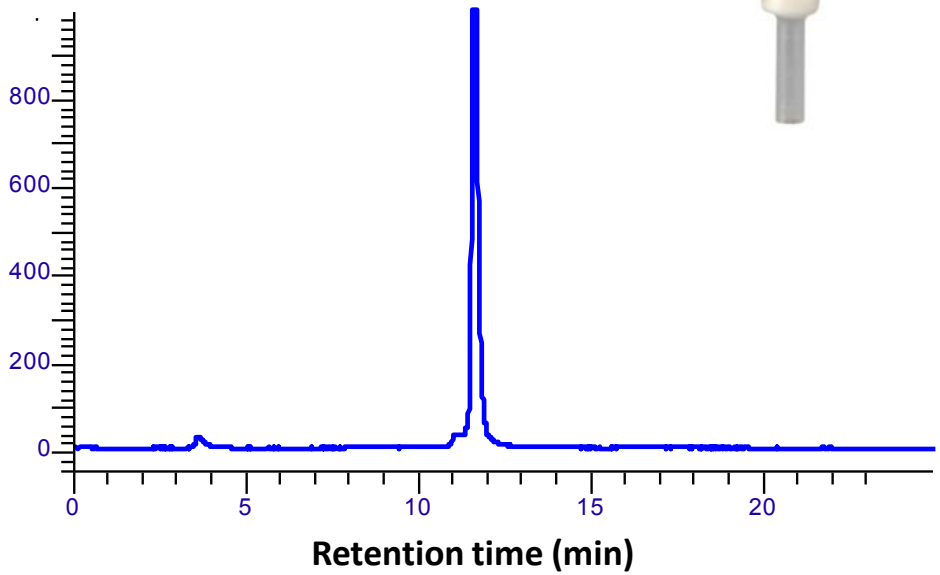




Schematic representation of SPE clean-up procedure

Sep-Pak®
Sample Extraction Products

After Purification



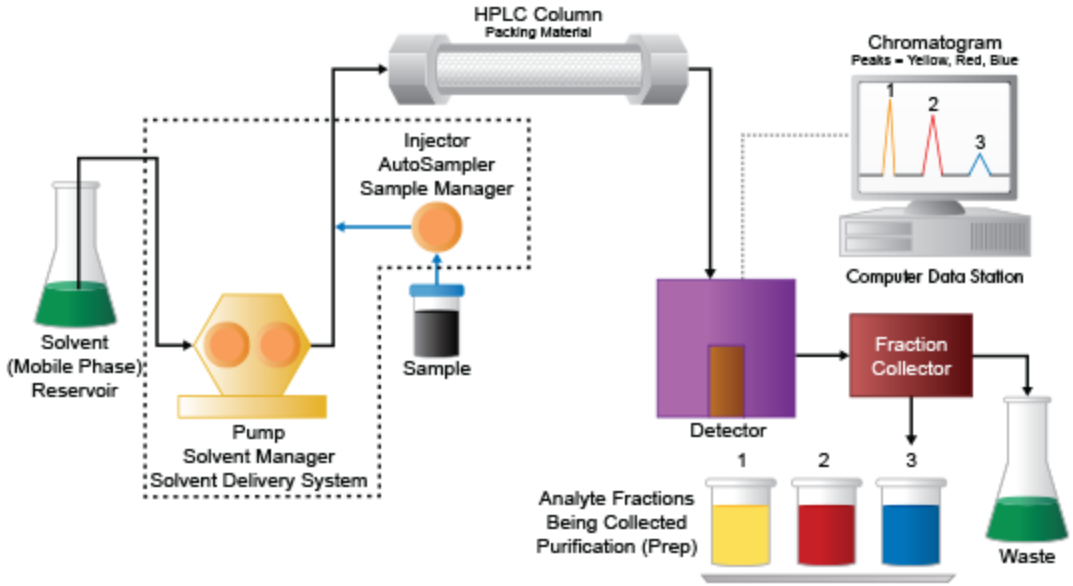
✓ **High radiochemical purity**

SPE could be used for desalting and buffer exchange

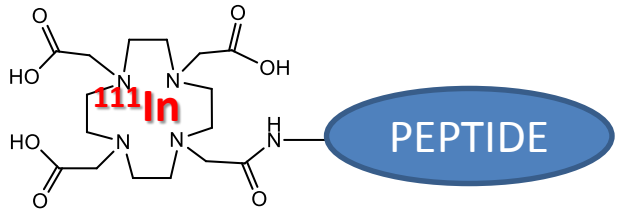
Plus Short	Plus Long	Plus Light	Classic Short	Classic Long	Vac 1 cc/50 mg	Vac 1 cc/100 mg	Vac RC/100 mg

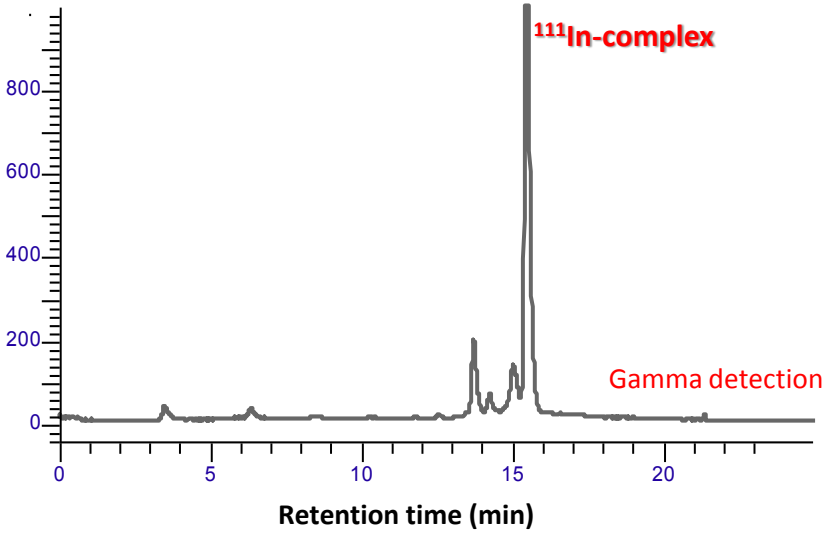
Vac 3 cc/200 mg	Vac 3 cc/500 mg	Vac RC/500 mg	Vac 6 cc/500 mg	Vac 6 cc/1 g	Vac 12 cc/2 g	Vac 20 cc/5 g	Vac 35 cc/10 g

PURIFICATION by RP-HPLC

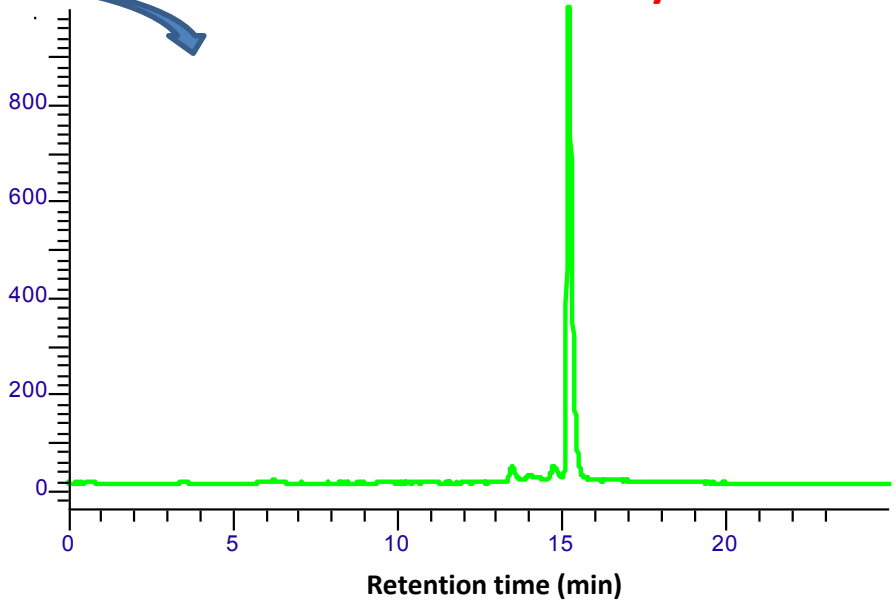


PURIFICATION by RP-HPLC





After Purification by HPLC

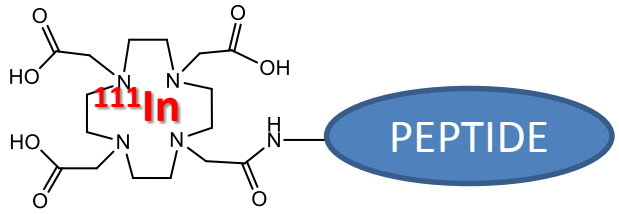


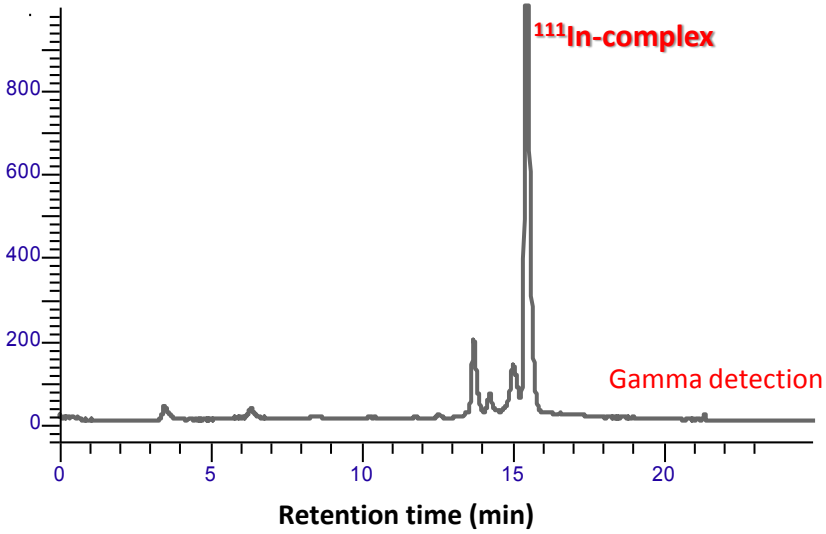
- **Purification to achieve adequate RCP**
- **How to purify? HPLC**

  Nucleosil C18 column

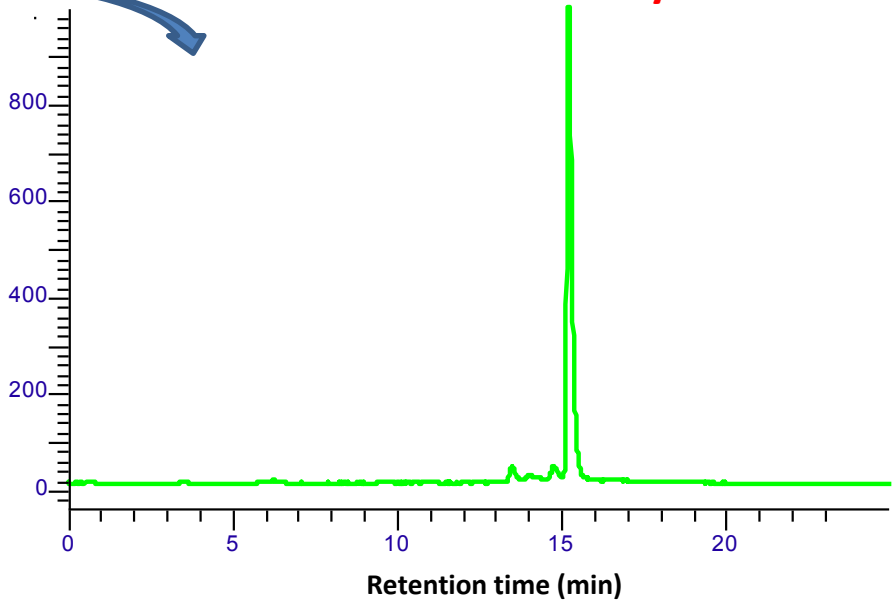
It is necessary evaluate the RCP after purification

PURIFICATION by RP-HPLC





After Purification by HPLC

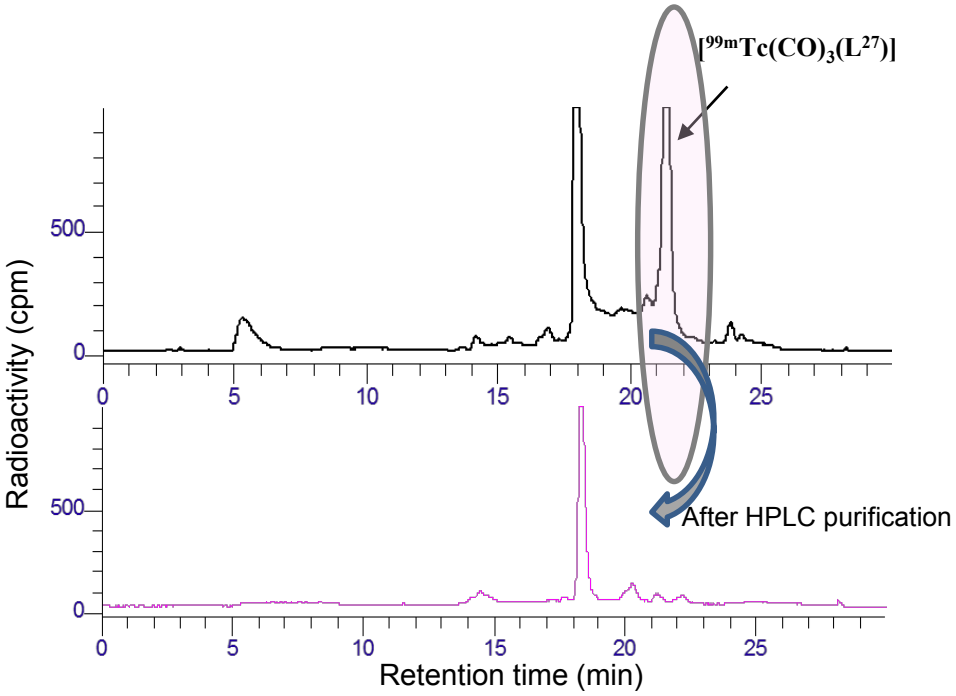


- **Purification to achieve adequate RCP**
- **How to purify? HPLC**

  Nucleosil C18 column

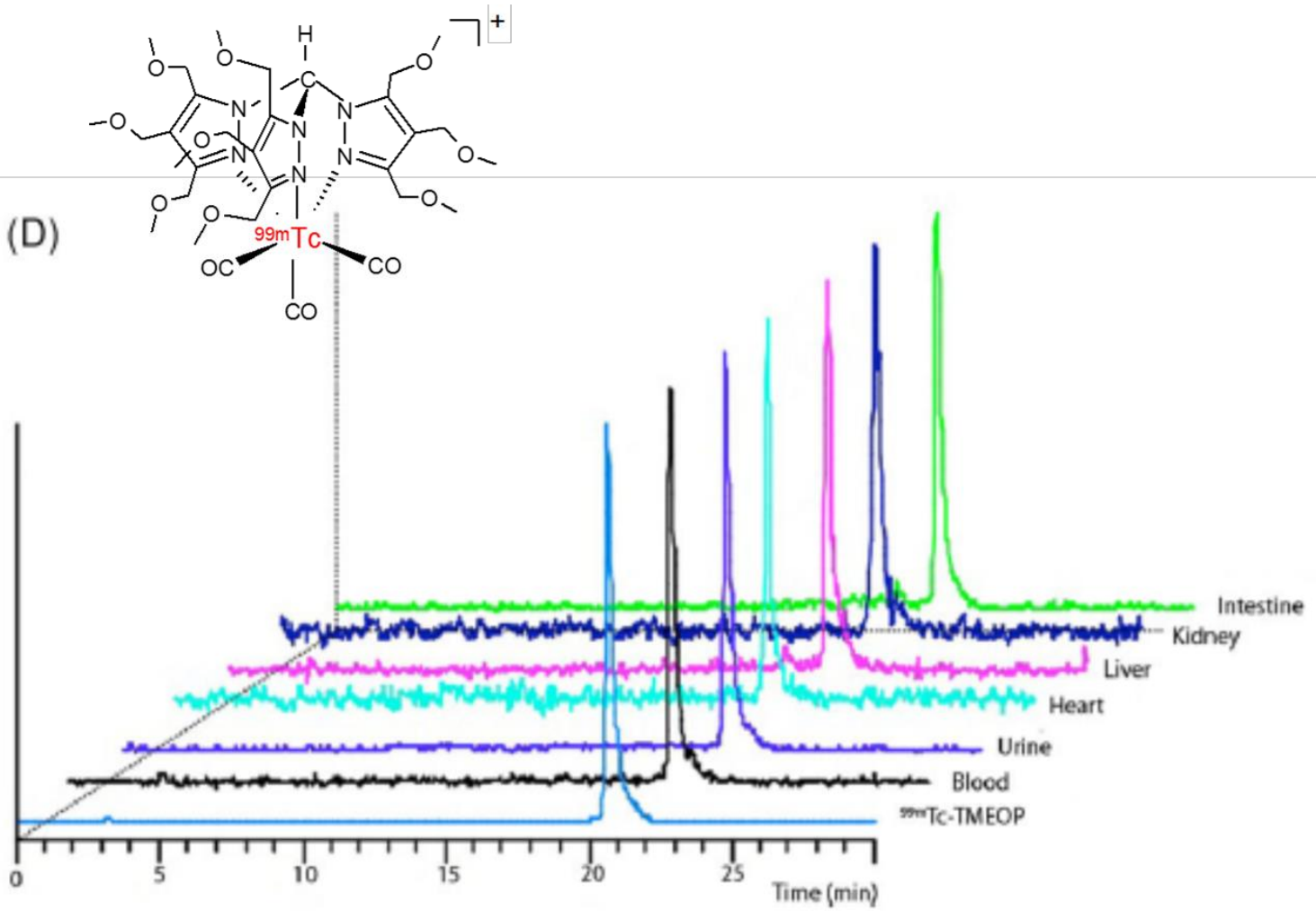
It is necessary evaluate the RCP after purification

PURIFICATION by RP-HPLC



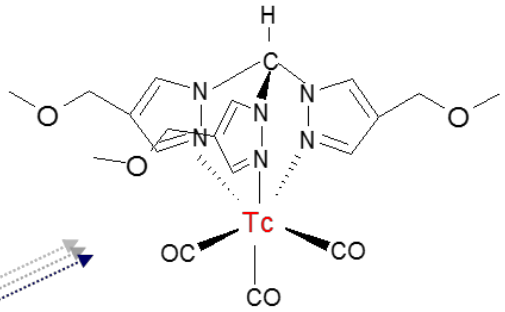
DECOMPOSITION DURING THE PURIFICATION PROCESS!

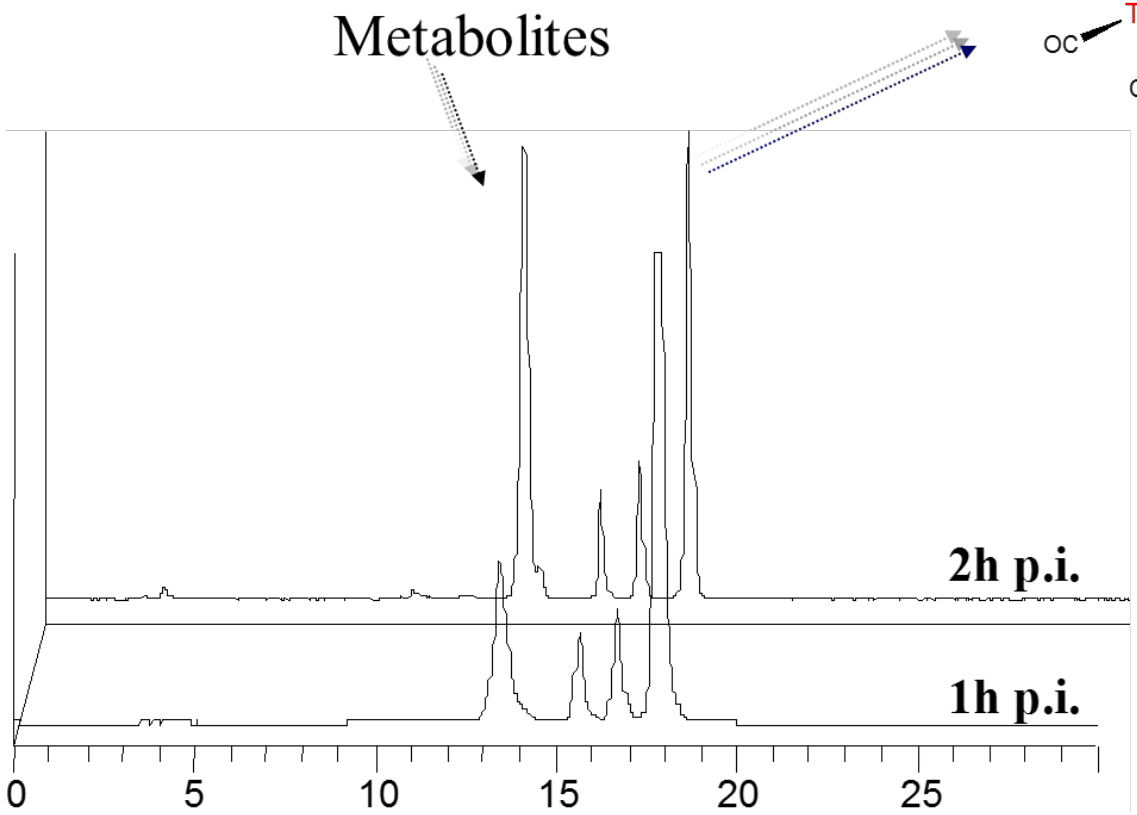
IN VIVO STABILITY: by RP-HPLC



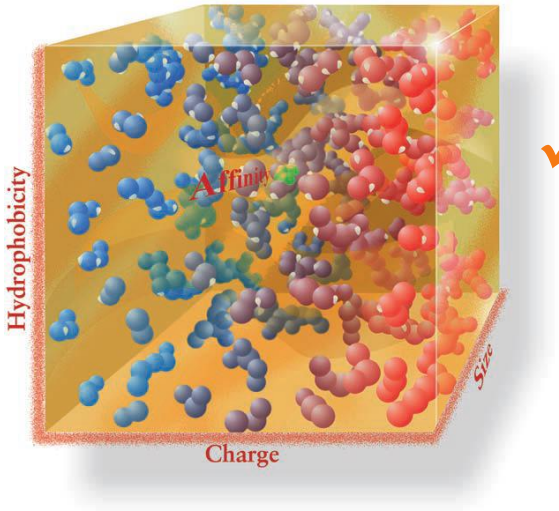
IN VIVO STABILITY: by RP-HPLC

Probable metabolic reactions: O-dealkylation followed by oxidation to carboxylic acids





A. Paulo et al. *J. Organomet. Chem.* **2014**, *760*, 138-148 A



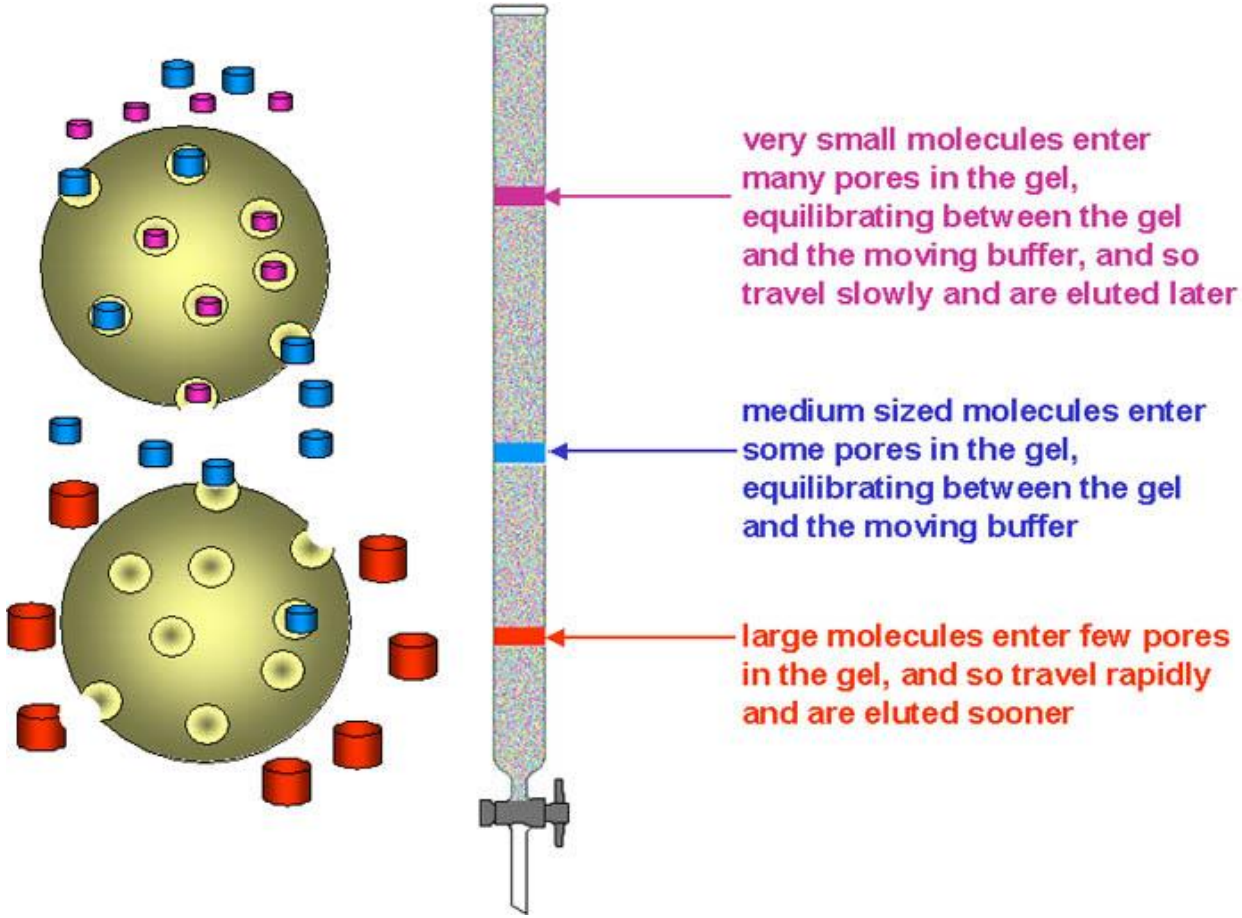
✓ Size Exclusion Chromatography (SEC)

Separates proteins, nanoparticles, polymers, etc, according to differences in molecular size.

Size-Exclusion Chromatography (SEC)

- ✓ Also named as gel filtration (GFC) and GPC.
- ✓ Separation is based on molecular size: larger molecules elute faster than smaller ones.
- ✓ Efficient separation method for macromolecules: proteins, polymers, mAb
- ✓ Not used for small molecules or small peptides.

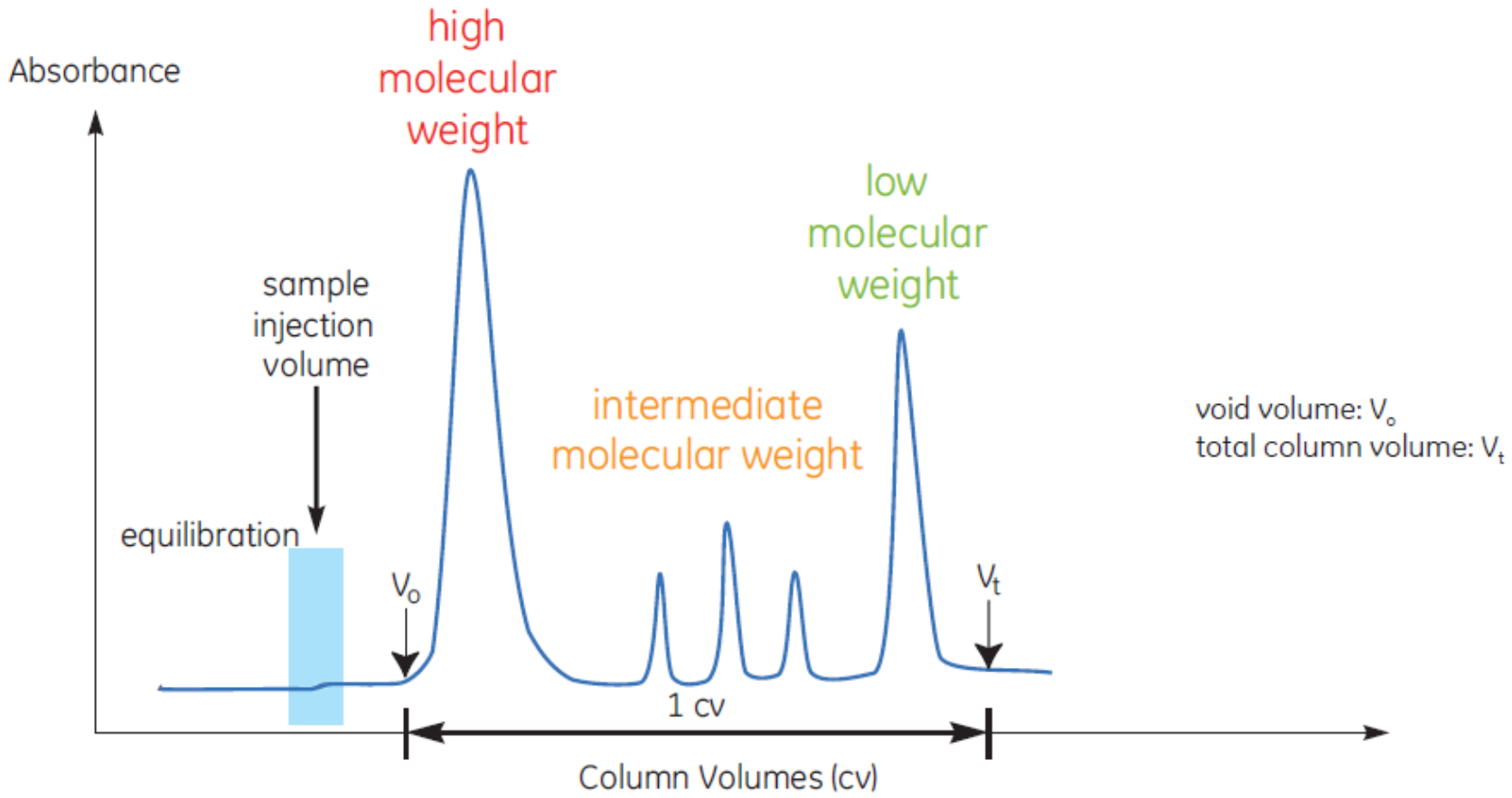
SEC separates molecules based on their size by filtration through a gel. The gel consists of spherical beads containing pores of a specific size distribution.



Desalting — A common use of SEC is for desalting proteins. The gel should have an exclusion limit significantly smaller than the molecule of interest.

Fractionation — Molecules of varying molecular weights are separated within the gel matrix. With this separation method, the molecules of interest should fall within the fractionation range of the gel.

Size-Exclusion Chromatography (SEC)



Sample volume and capacity: To achieve highest resolution, the sample volume must not exceed 5% of the total column volume.

Sample Preparation: Samples must be free from particulate matter. Viscous samples should be diluted.

Size-Exclusion Chromatography (SEC)

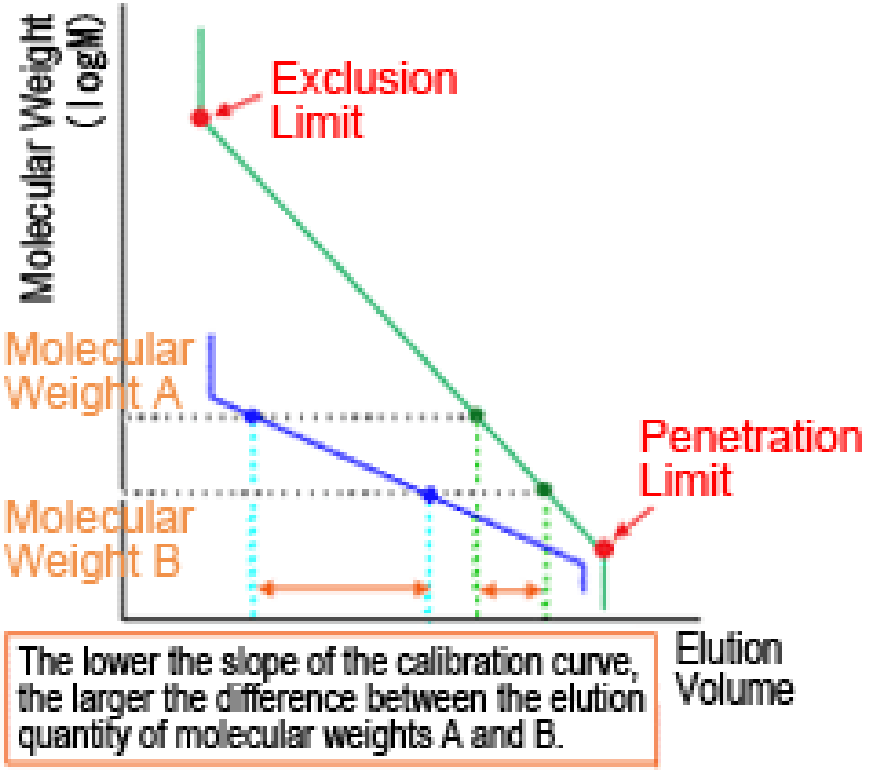
Column Dimensions — resolution increases with the column length, and as the column diameter increases, the capacity of the column increases due to the larger column or bed volume.

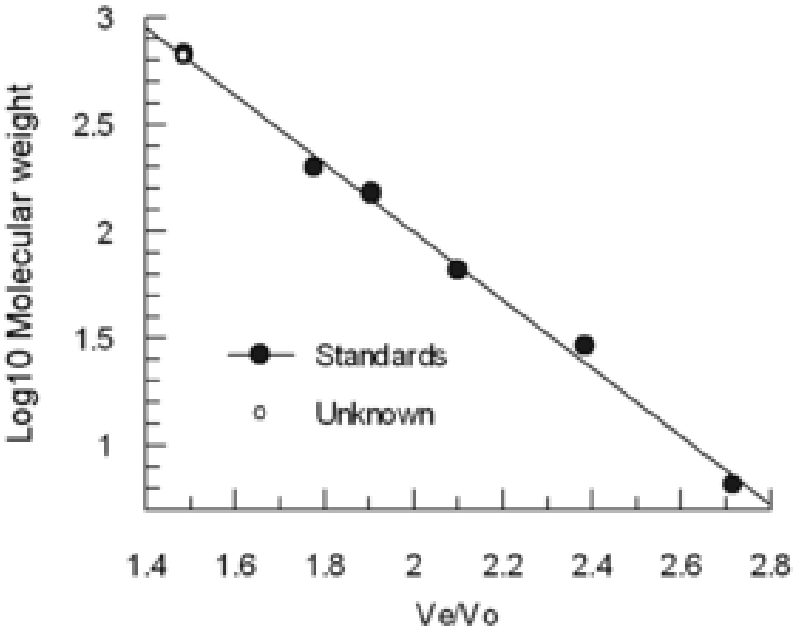
Flow Rate — moderate flowrates offer the highest resolution. Flowrates are specific to the type of media being used.

Column Packing — column packing is critical to resolution.

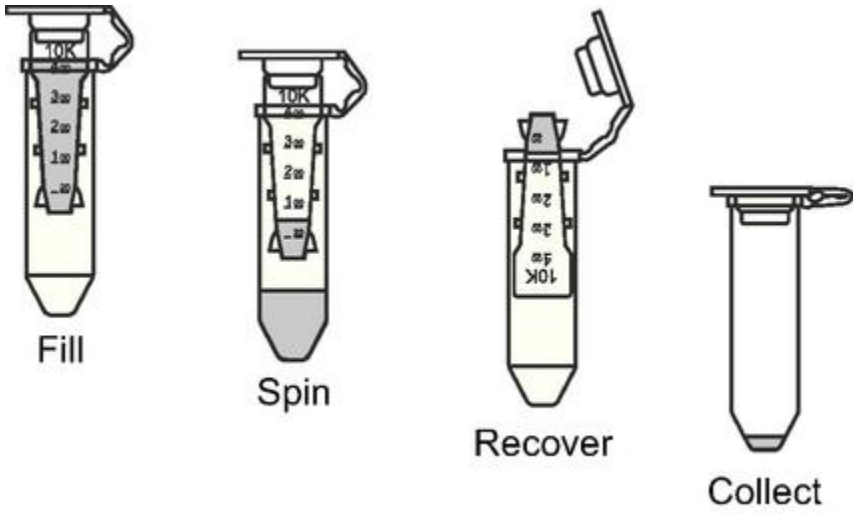
Particle and Pore Size — in general the smaller the particle size, the higher the resolution.

With proper column calibration using **MW standards, the molecular weights of unknown molecules** can be determined.





Ultra centrifugal filter Devices

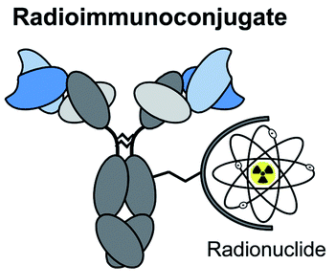


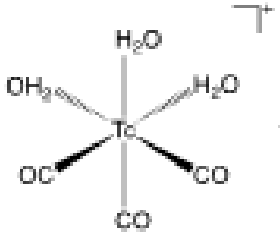
Available different Nominal Molecular Weight Limit

- 3k; 10k; 30k; 50k; 100k

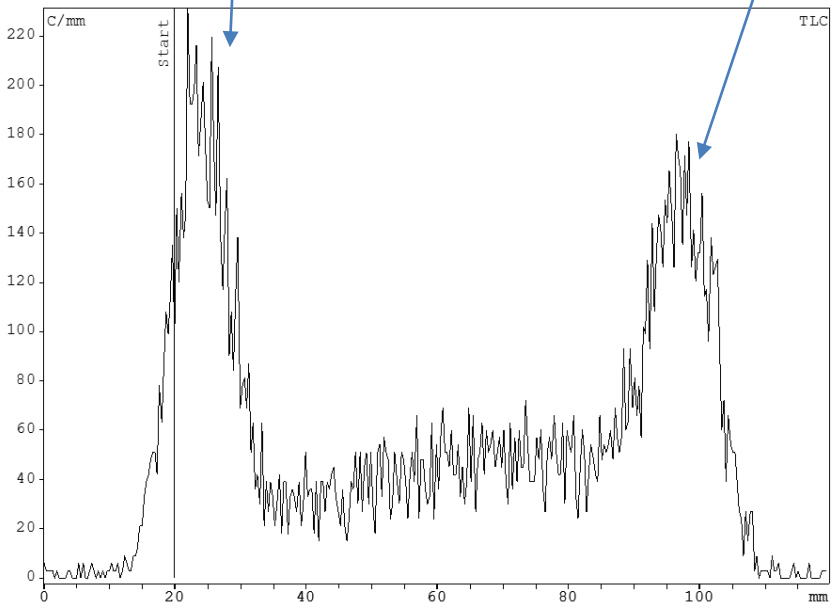
Applications

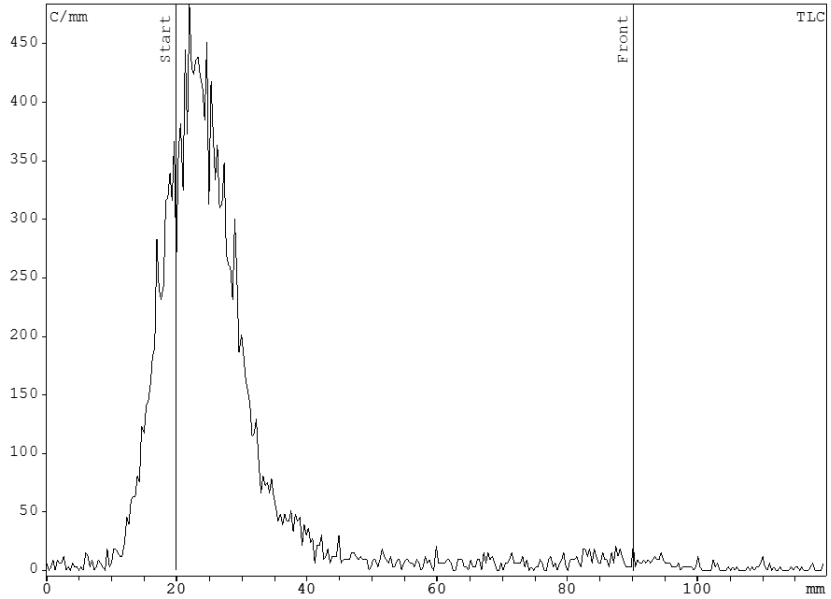
- Concentration of biological samples containig antigens, Ab, nucleic acids
- Purification of macromolecular components
- Desalting, buffer exchange





High RCP after purification





ITLC-SG
MeOH/HCl 6M (95/5)

Purification by ultrafiltration using Amicon Ultra; 100k