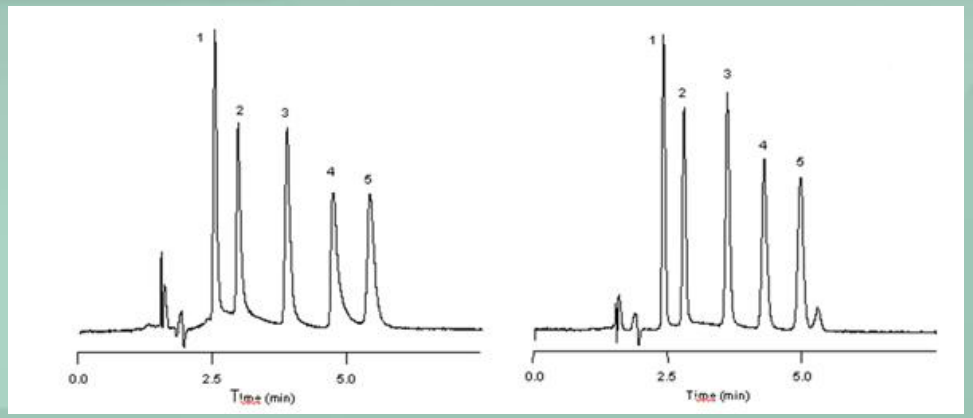

BASIC PRINICPALS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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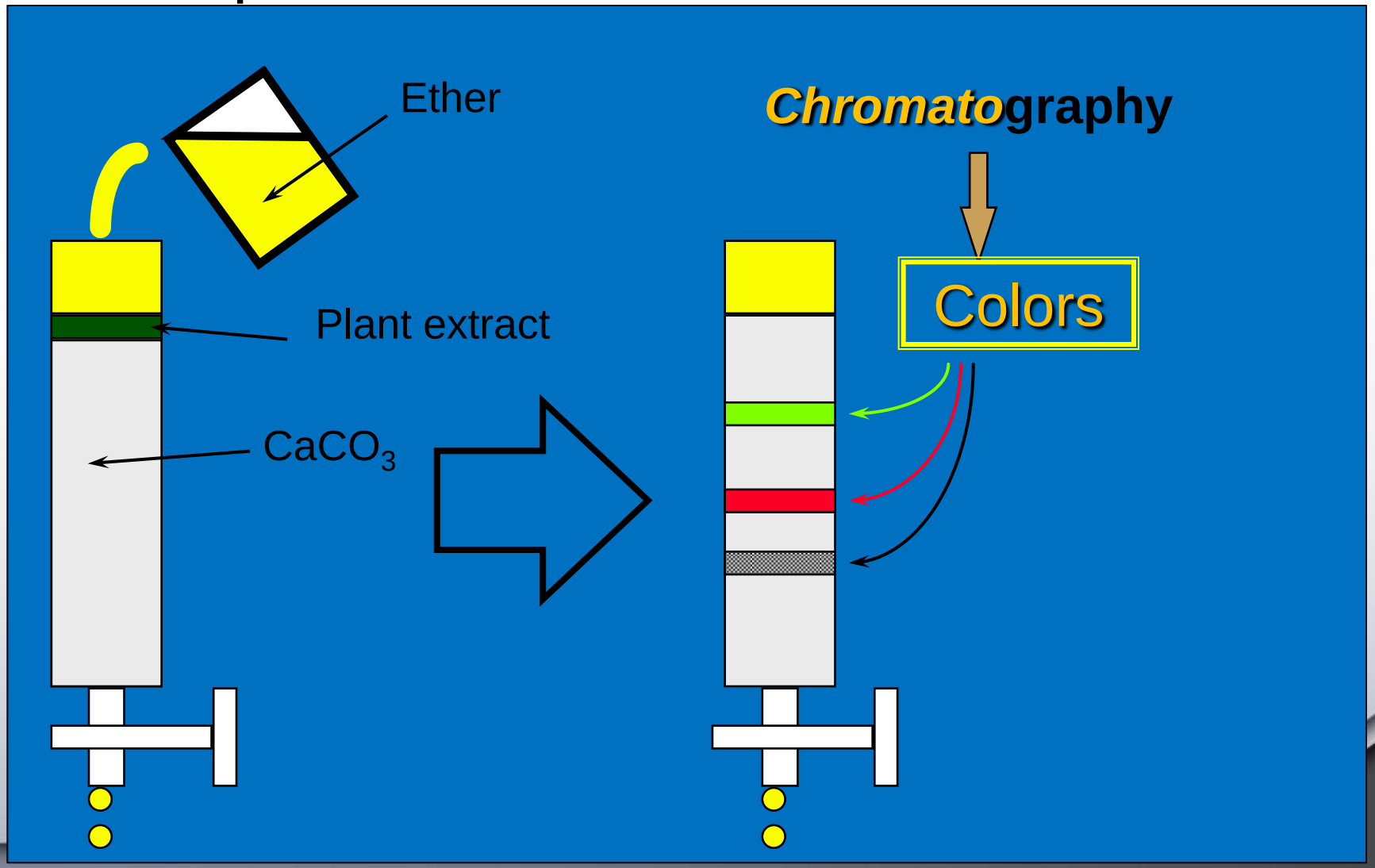
What is liquid chromatography?

- **Liquid chromatography (LC) is an analytical technique based on the separation of molecules due to differences in their structure and/or composition.**
- **Liquid chromatography was defined in the early 1900s by Mikhail S. Tswett.**
  - **Separation of compounds (leaf pigments) extracted from plants using a solvent, in a column packed with particles.**

Tswett's Experiment



Chromatographic methods are applied for:

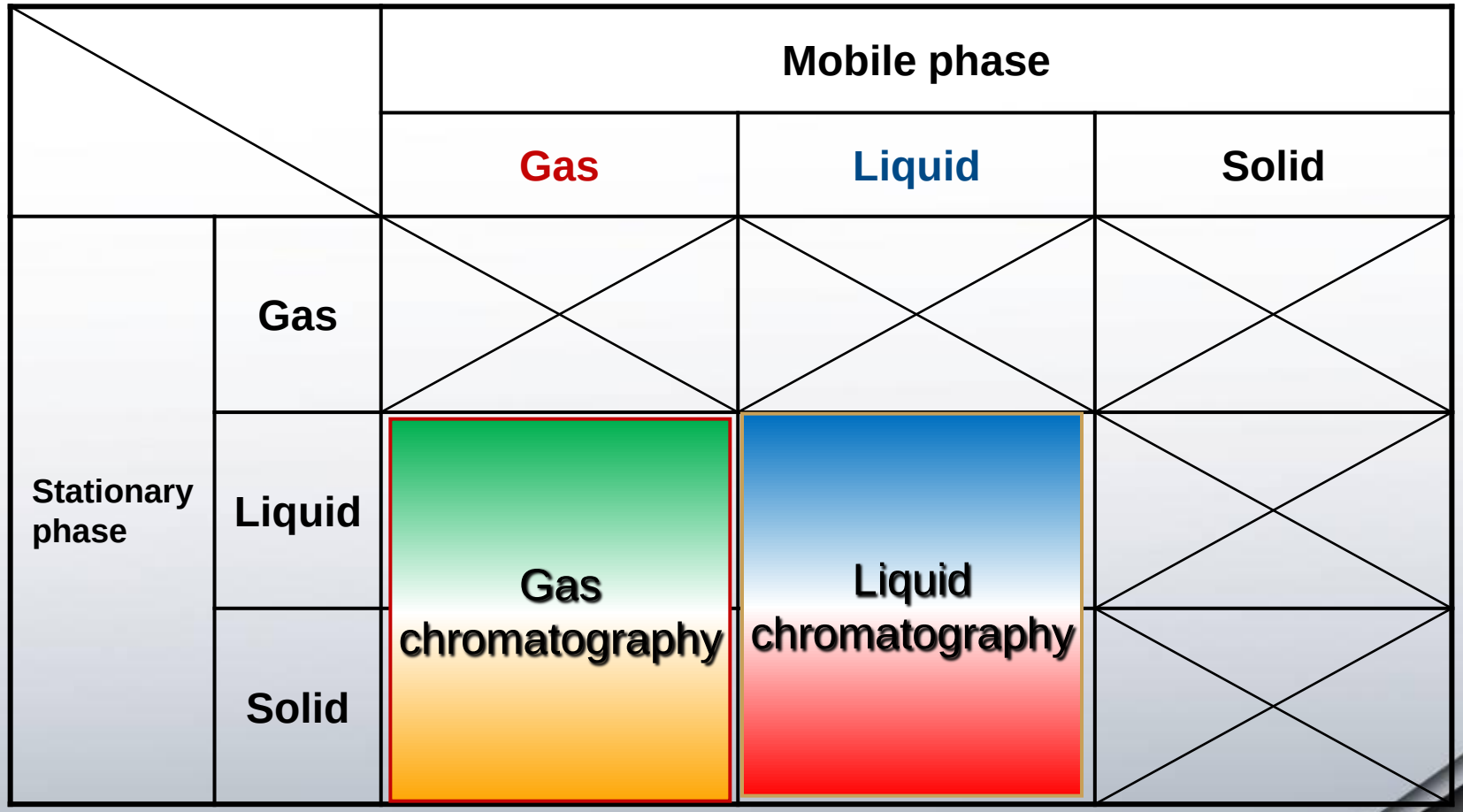
- **SEPARATION OF COMPOUNDS in a mixture**
  - Identification and determination

- **QUALITATIVE ANALYSIS (retention time, UV-Vis spectra, MS spectra)**

- **QUANTITAVIE ANALYSIS (peak area or peak height)**

- Separation is performed between two phases, **mobile** and **stationary**.
- Compounds which are longer retained at the stationary phase will elute later, compared to those which are distributed into the mobile phase.

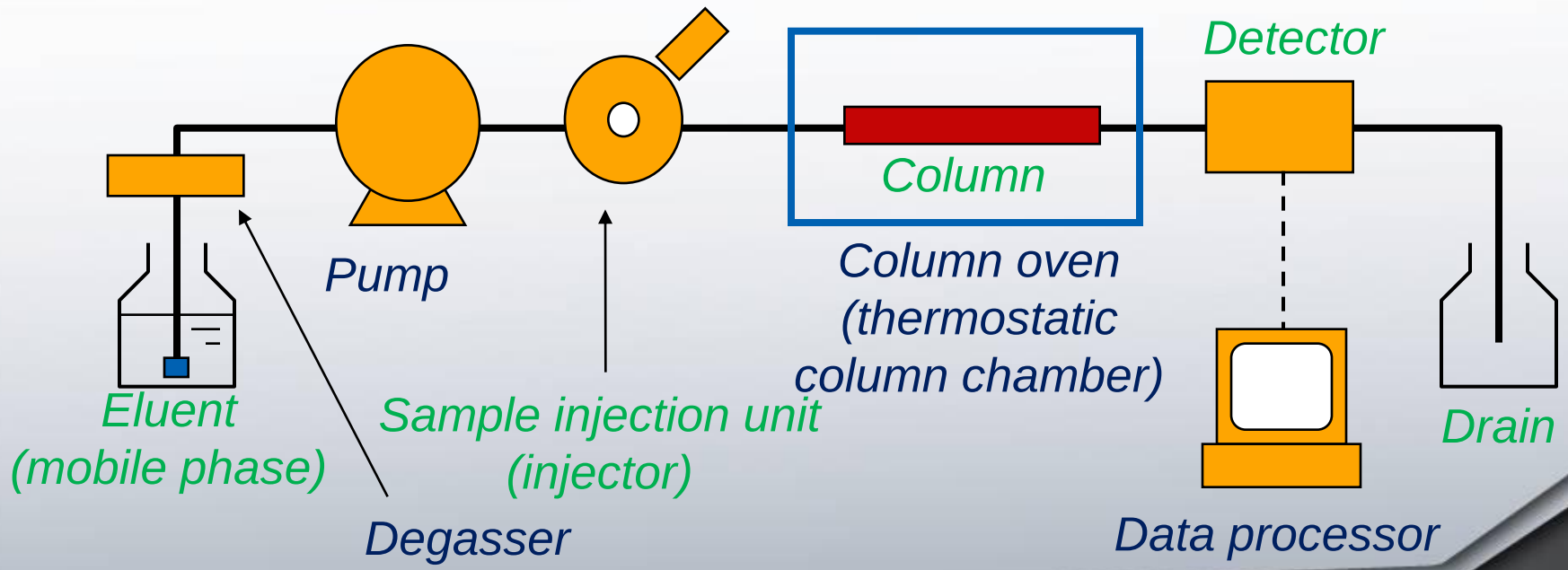
Chromatography Types



High performance liquid chromatography (HPLC) system

- HPLC is a form of liquid chromatography used to separate compounds that are dissolved in solution.

- HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, detector and data processor.



HPLC instruments

HPLC columns

- The column is the "**core**" of any chromatographic system
- One of the most important components where the separation of the sample components is achieved
- Columns are commercially available in different lengths, bore sizes and packing materials.

➢The most widely used packing materials for HPLC separations are **silica-based.**

➢The most popular material is octadecyl-silica (ODS-silica), which contains **C18 coating**

- materials with C1, C2, C4, C6, C8 and C22 coatings

➢The column life can be prolonged with proper maintenance:

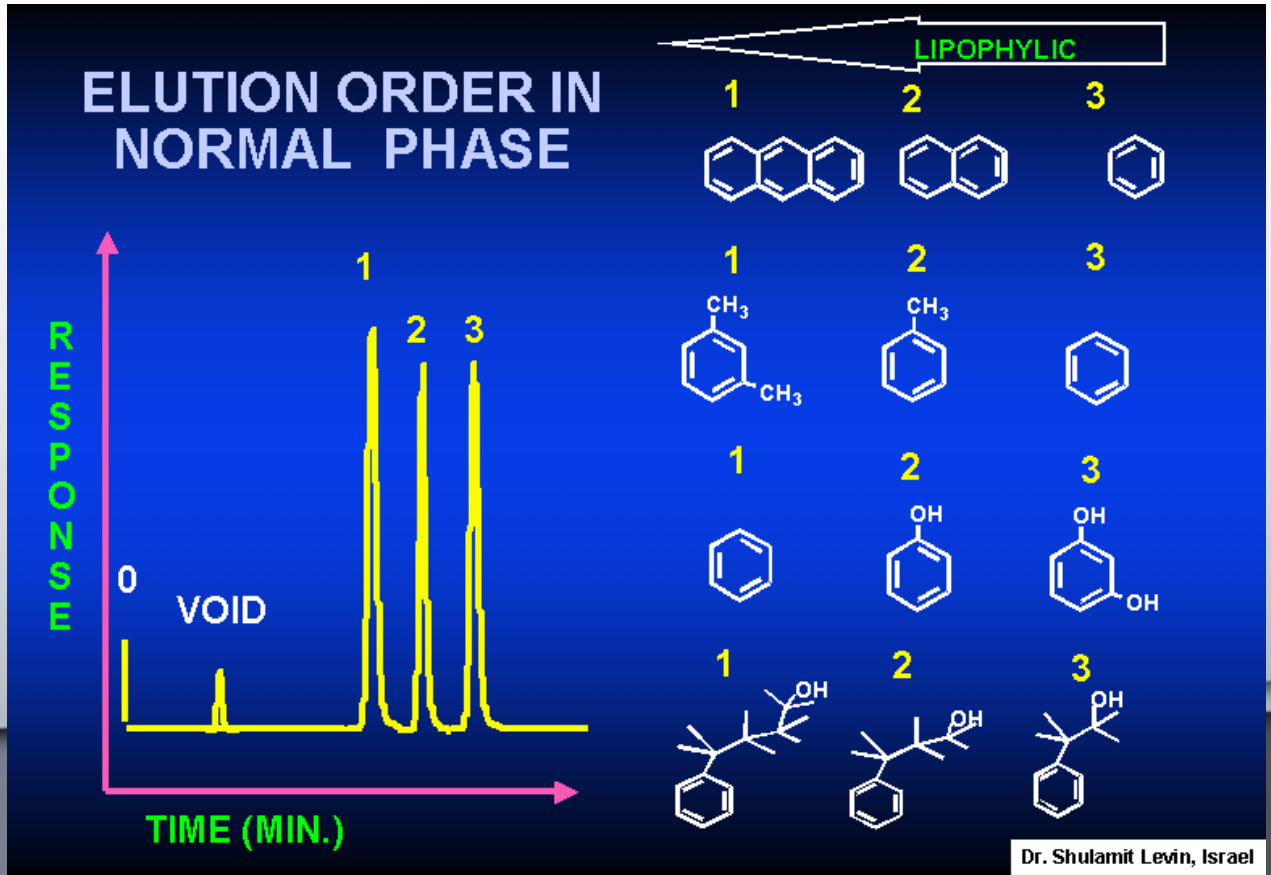
- flushing a column with mobile phase of high elution strength following sample runs is essential.
- When a column is not in use, it should be capped to prevent it from drying out.
- Particulate samples need to be filtered and when possible a guard column should be utilized.

Column types

- ***Normal phase***
- ***Reverse phase***
- ***Size exclusion***
- ***Ion exchange***

Normal phase

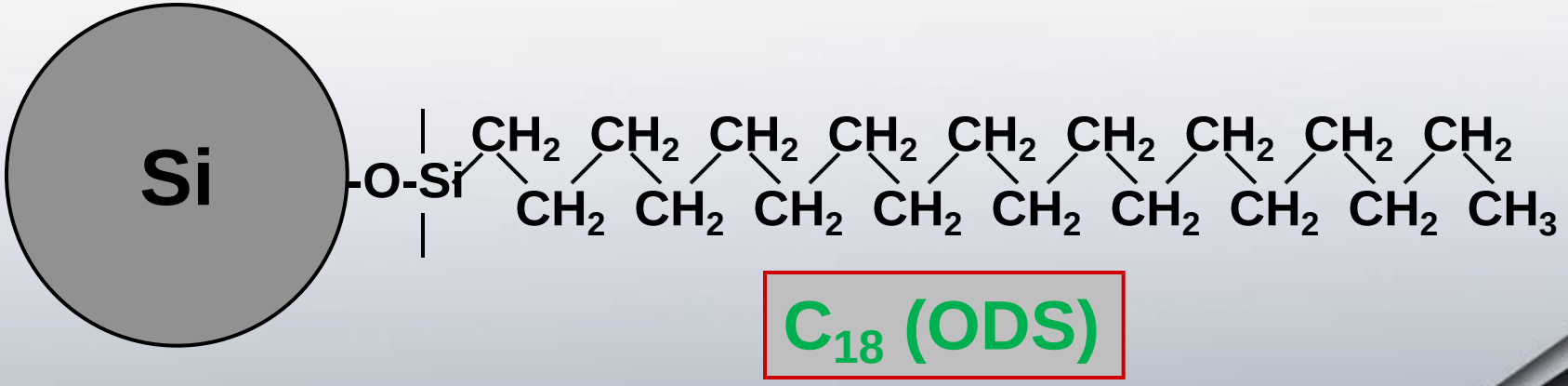
- Stationary phase: High polarity
  - Silica or organic moieties with cyano and amino functional groups
- Mobile phase: Low polarity
  - Hexan or heptan



Reverse phase

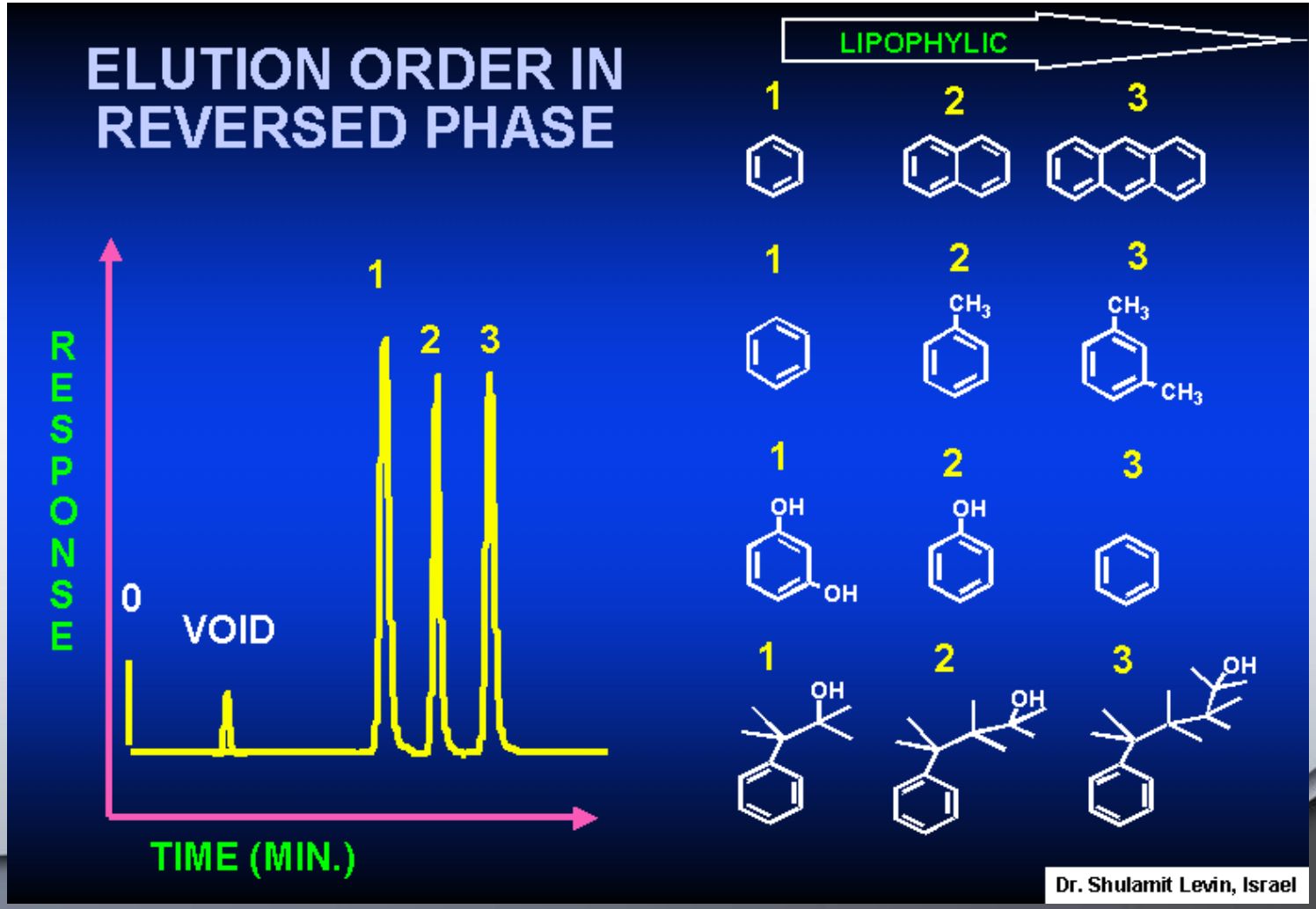
- Stationary phase: Low polarity
  - Octadecyl group-bonded silical gel (ODS)
- Mobile phase: High polarity
  - Water, methanol, acetonitrile
  - Salt or acid is sometimes added.

- Typical stationary phases are nonpolar hydrocarbons (such as C18, C8, etc.) and the solvents are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water.



C_{18} (ODS)

Reverse phase



Normal Phase/Reversed Phase

Type	Stationary phase	Mobile phase
Normal phase	**High polarity (hydrophilic)**	**Low polarity (hydrophobic)**
Reversed phase	**Low polarity (hydrophobic)**	**High polarity (hydrophilic)**

- **The polarities of stationary phase and mobile phase have to be different!**

Elution

➢ Isocratic

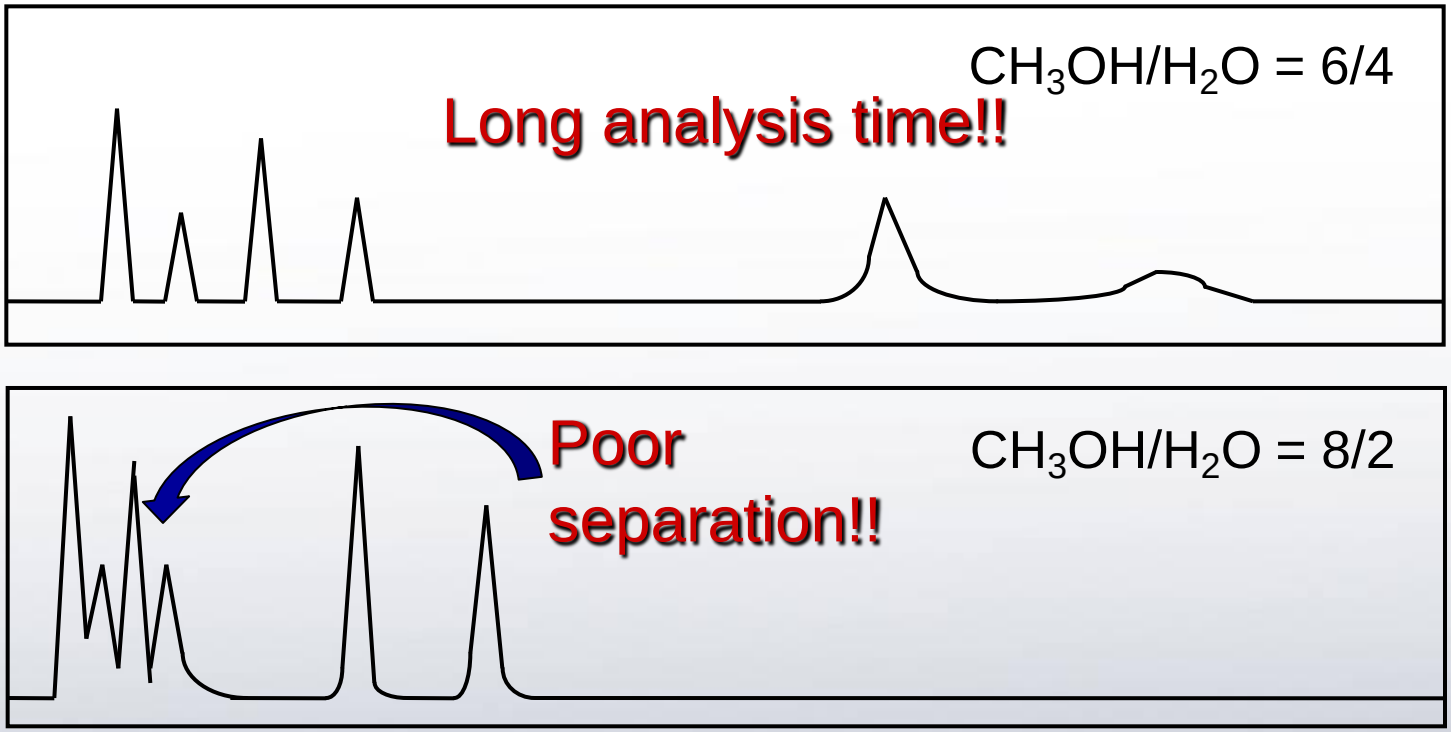
- Constant eluent composition, same eluent: for example 50 % methanol

➢ Gradient

– Varying eluent composition

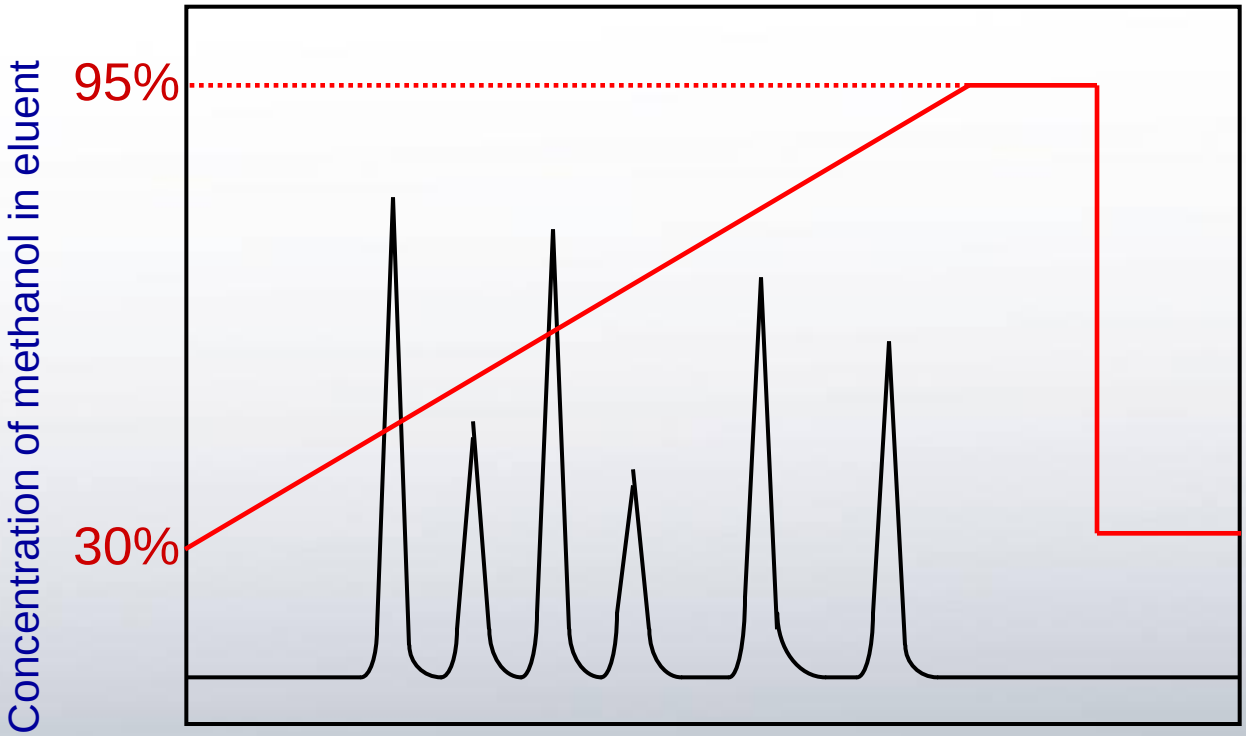
- HPGE (High Pressure Gradient): High gradient accuracy, complex system configuration (multiple pumps required)
- LPGE (Low Pressure Gradient): Simple system configuration, degasser required

- In isocratic mode



(Column: ODS type)

- In gradient mode



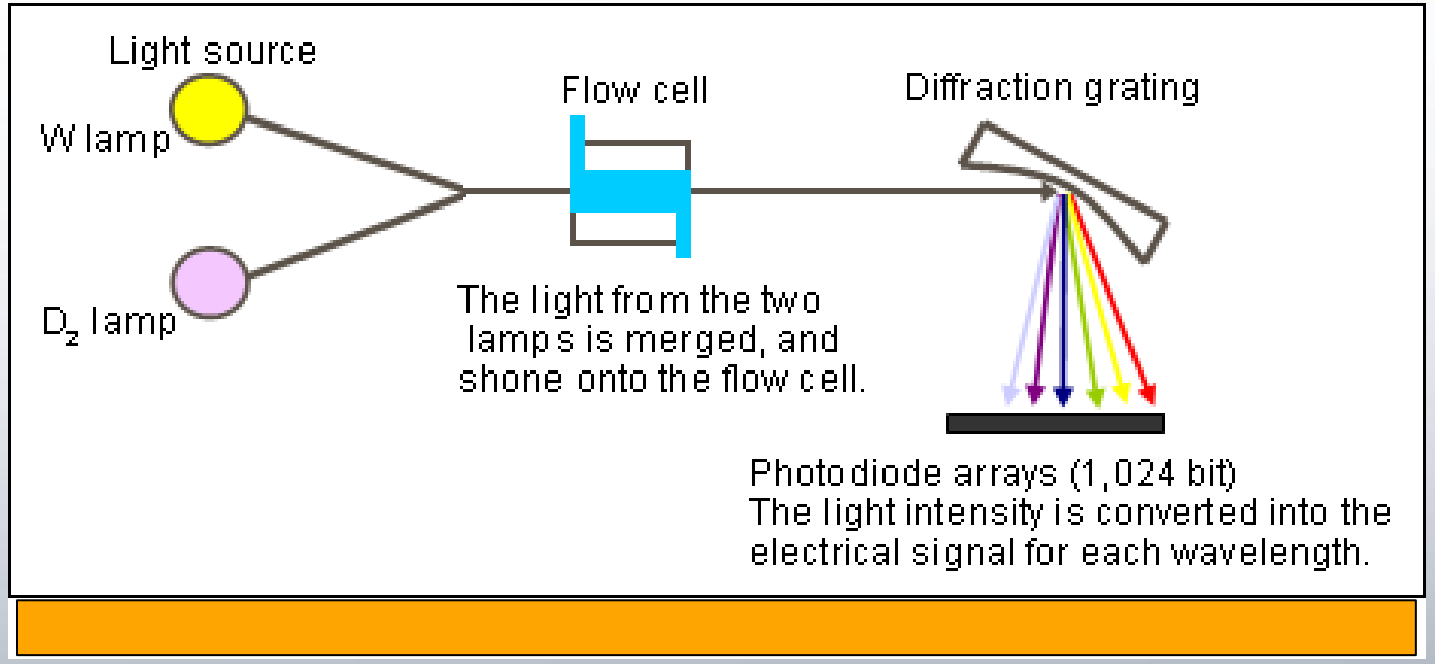
Detector requirements

- **Sensitivity**
  - The detector must have the appropriate level of sensitivity.
- **Selectivity**
  - The detector must be able to detect the target substance without, if possible, detecting other substances.
- **Adaptability** to separation conditions
- **Operability**, etc.

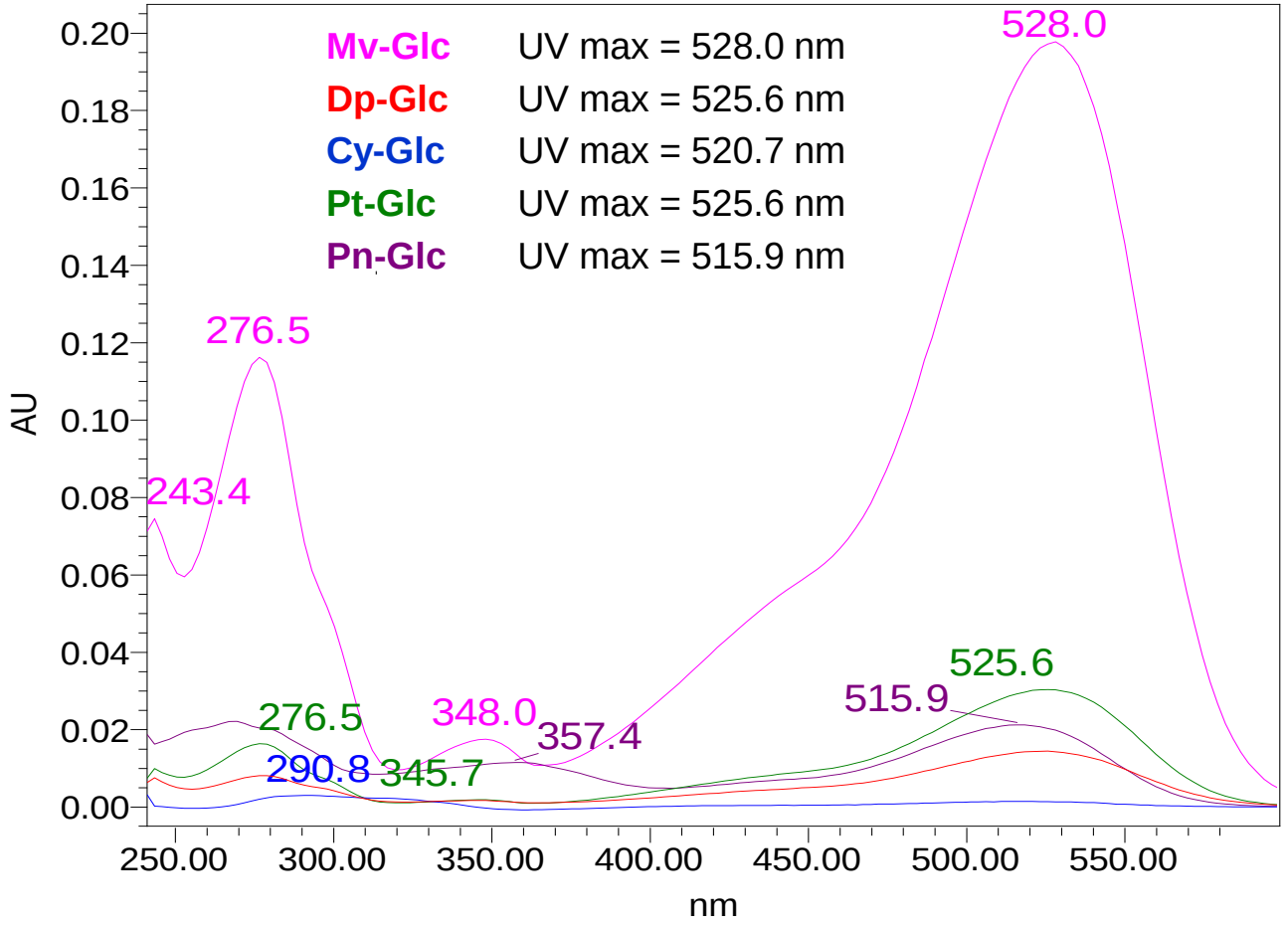
Types of Detectors

- ✓ UV-Vis absorbance detector
- ✓ Photodiode array-type UV-VIS absorbance detector (DAD)
- ✓ Fluorescence detector
- ✓ Refractive index detector
- ✓ Electrical conductivity detector
- ✓ Electrochemical detector
- ✓ Mass spectrometer

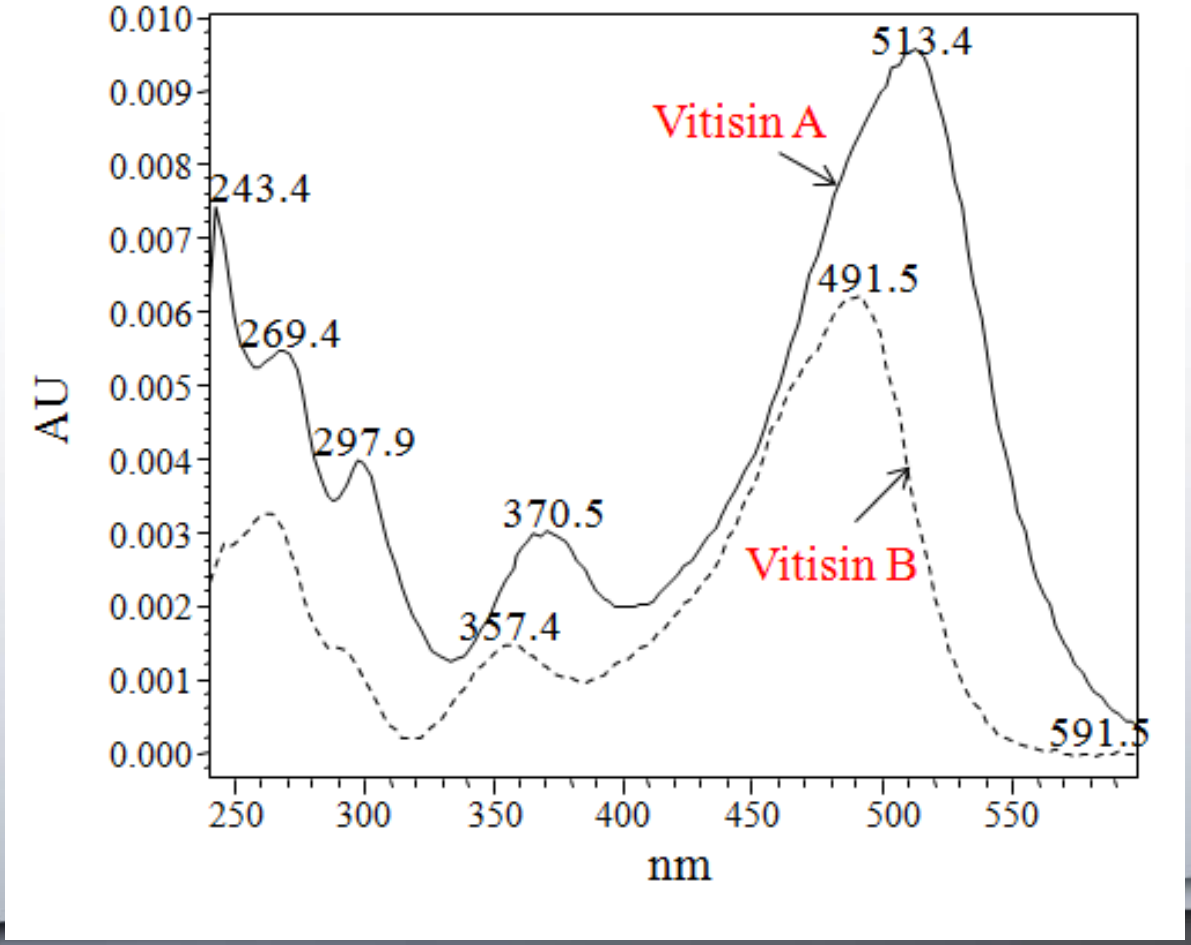
-**UV-Vis** detector has **only one** sample-side light-receiving section
-**DAD** has **multiple** (1024 for L-2455/2455U) photodiode arrays to obtain information over a wide range of wavelengths at one time



UV-Vis spectra of anthocyanin monoglucosides



UV-Vis spectra of vitisin A and vitisin B



Fluorescence detector

- **The most sensitive among the existing modern HPLC detectors.**
- Typically, fluorescence sensitivity is 10 -1000 times higher than that of the UV detectors
- Fluorescence detectors are very specific and selective among the others optical detectors.

- Roughly about 15% of all compounds have a natural fluorescence - derivatization is necessary

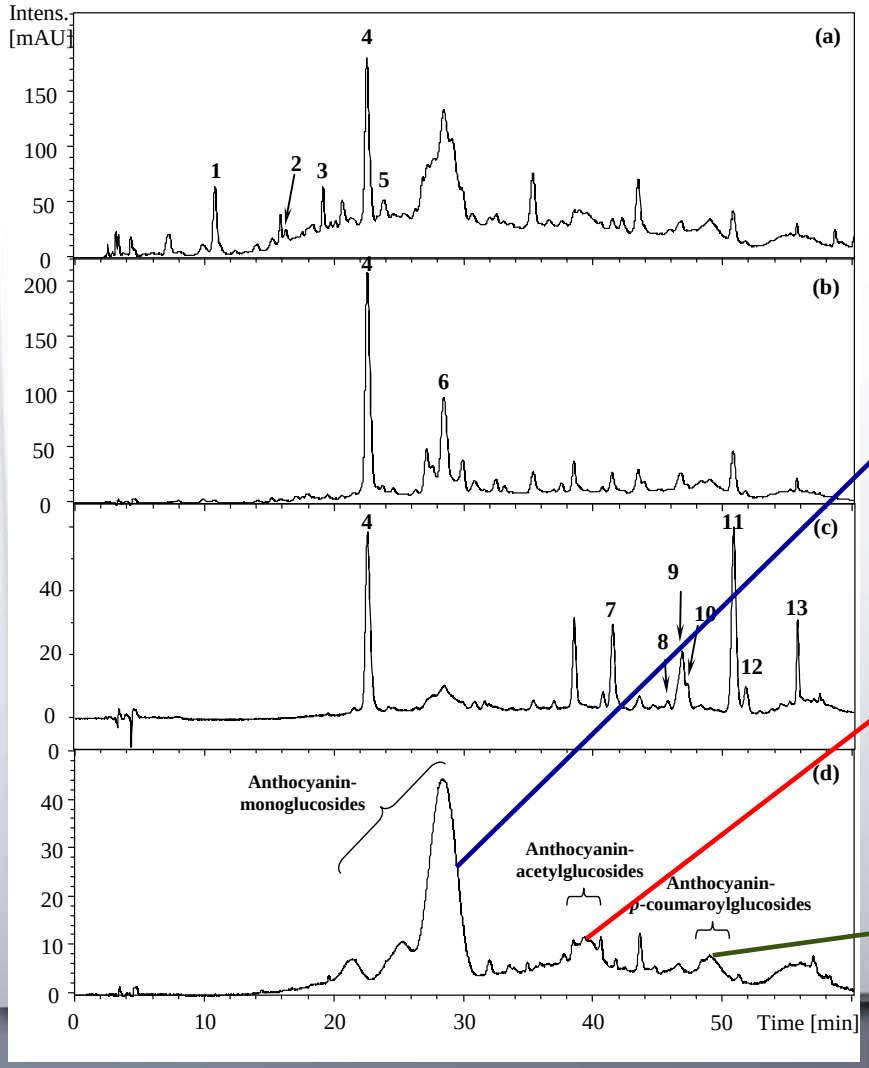
Refractive index detector

- Measures the refractive index of an analyte relative to the solvent
- They can detect anything with a refractive index different from the solvent, but they have **low sensitivity**
- Very sensitive to slight changesd of the mobile phase, not compatible for gradient elution

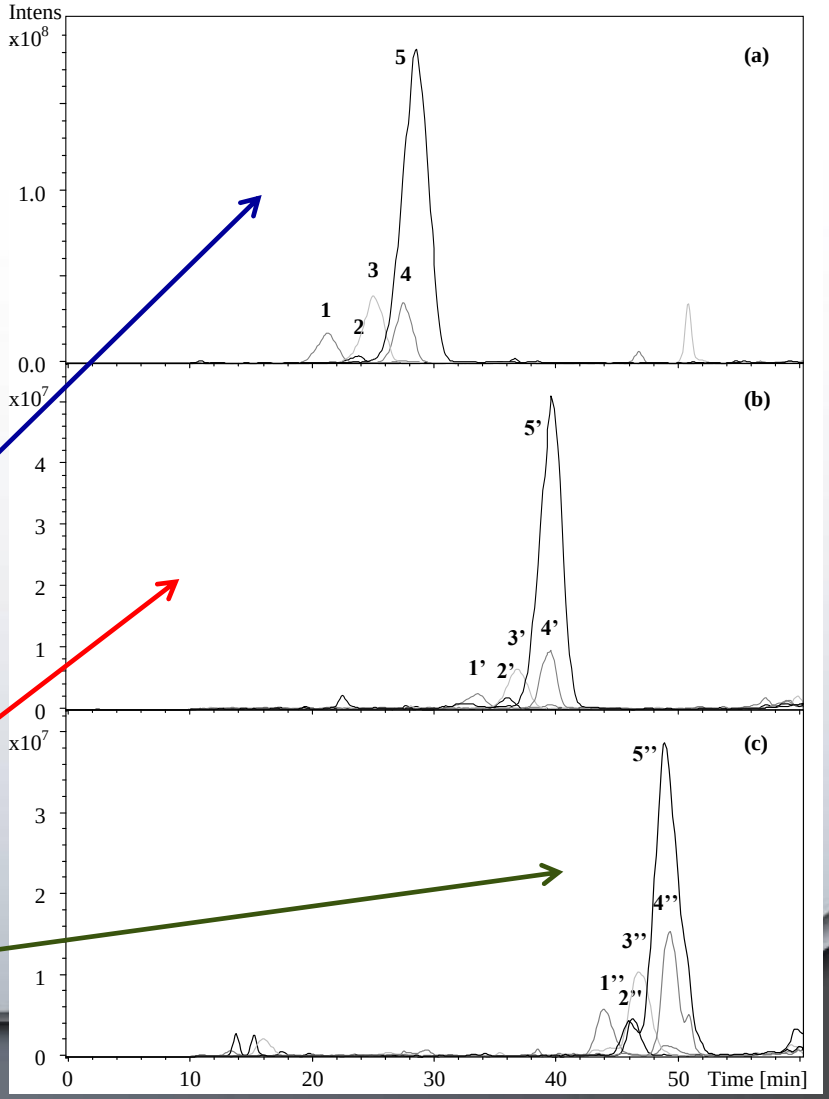
Mass spectrometer

- **Mass spectrometry (MS) is an analytical technique that ionizes chemical species and sorts the ions based on their mass to charge ratio.**
- Mass spectrum measures the masses within a sample.
- Mass spectrometry is used in many different fields and is applied to pure samples as well as complex mixtures.
- **Used for:**
  - **characterization of complex structures of compounds**
  - **detection of new compounds in different matrices**
  - **……**

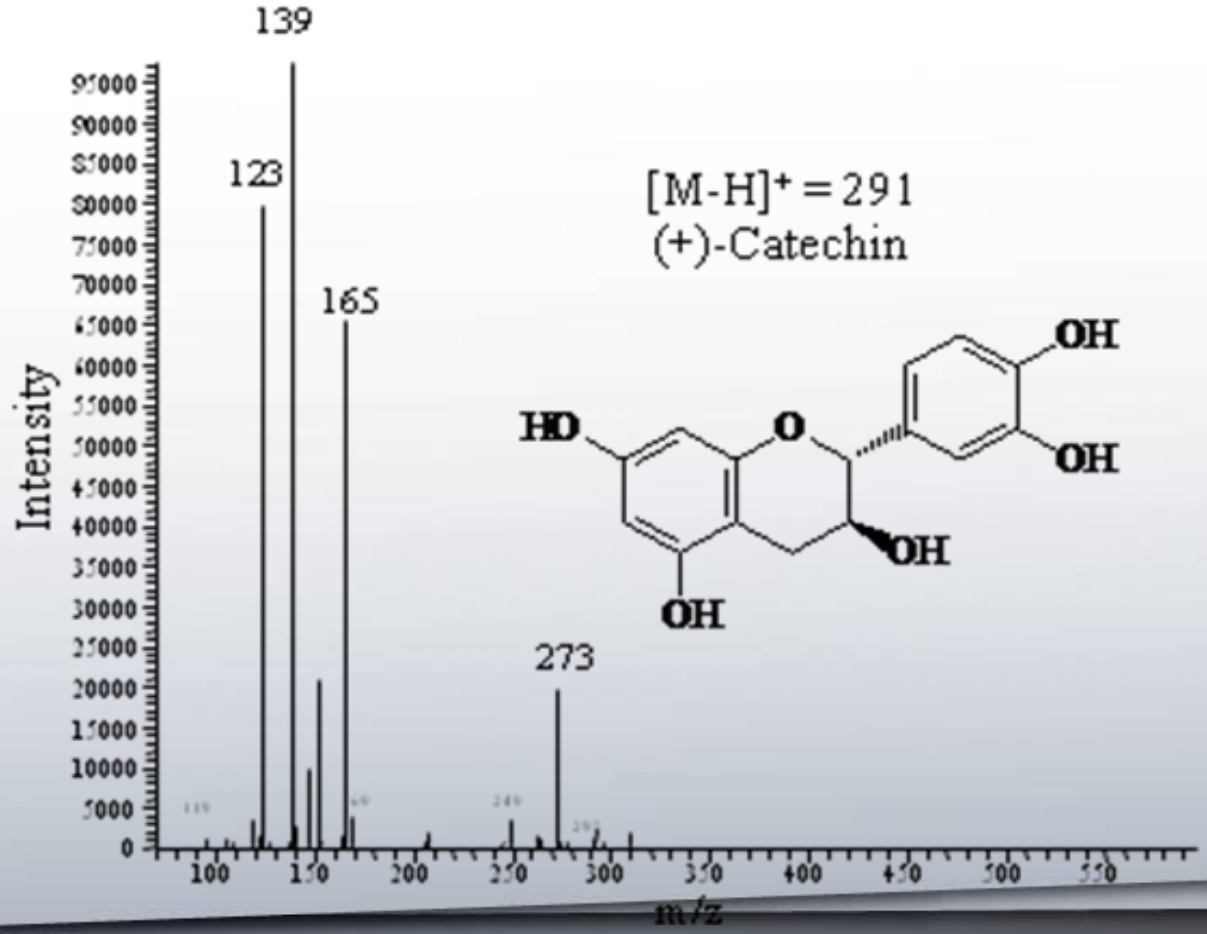
UV and visible chromatograms of polyphenols: (a) 280 nm, (b) 320 nm, (c) 360 nm, (d) 520 nm



Extracted ion chromatograms at different *m/z* values, which correspond to the M^+ signals of the anthocyanins

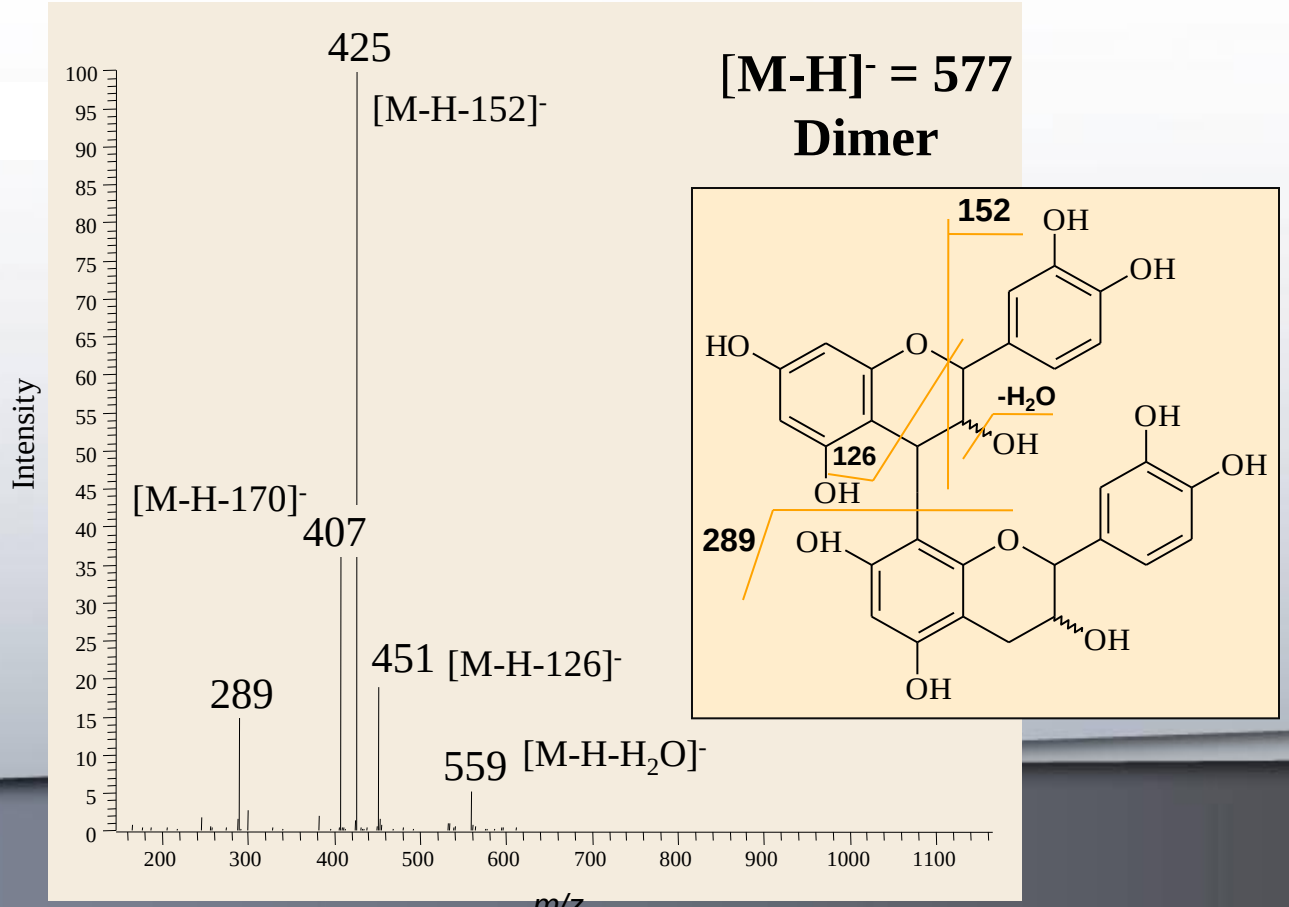


Mass spectrum of catechin (*m/z* 291) obtained under positive mode



Mass spectrum of procyanidin (*m/z* 577) obtained under negative mode

m/z 577 ⟶ 559, 451, 425, 289



Quantitative analysis

- Quantitation performed with peak area or height.
- Calibration curve created beforehand using a standard.
  - External standard method
  - Internal standard method
  - Standard addition method

External standard method

- The simplest method
- The accuracy of this method is dependent on the reproducibility of the injection volume.
- **Standard solutions of known concentrations of the compound of interest are prepared with one standard that is similar in concentration to the unknown.**
- A fixed amount of sample is injected.
- Peak height or area is then plotted versus the concentration for each compound. The plot should be linear and go through the origin.
- The concentration of the unknown is then determined according to the following formula:

$$\text{Conc.}_{\text{unknown}} = \left(\frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}} \right) \text{conc.}_{\text{known}}$$

Internal standard method

- The internal standard method tends to yield the most accurate and precise results
- **An equal amount of an internal standard, a component that is not present in the sample, is added to both the sample and standard solutions.**
- The internal standard selected should be chemically similar, to have similar retention time and derivatize similarly to the analyte, to be stable and does not interfere with any of the sample components.
- The internal standard should be added before any preparation of the sample so that extraction efficiency can be evaluated.
- Quantification is achieved by using ratios of peak height or area of the component to the internal standard.

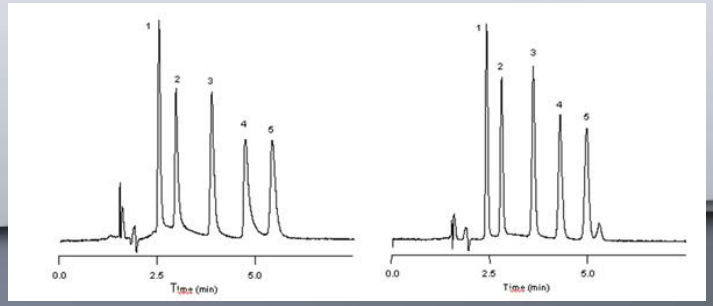
$$\text{Conc.}_{\text{unknown}} = \left(\frac{\text{Area}_{\text{Internal Std in known}}}{\text{Area}_{\text{Internal Std in unknown}}} \right) \times \left(\frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}} \right) \times \text{conc.}_{\text{known}}$$

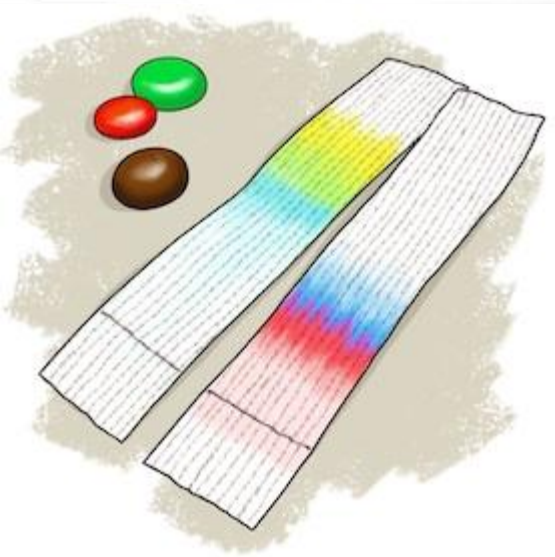
Fields in Which High Performance Liquid Chromatography Is Used

- Biogenic substances
  - Sugars, lipids, nucleic acids, amino acids, proteins, peptides, steroids, amines, etc.
- Medical products
  - Drugs, antibiotics, etc.
- Food products
  - Vitamins, food additives, sugars, organic acids, amino acids, polyphenols, biogenic amins
- Environmental samples
  - Inorganic ions
  - Hazardous organic substances, etc.
- Organic industrial products
  - Synthetic polymers, additives, surfactants, etc.

Conclusion

- HPLC offers high sensitive, accurate and fast analysis of various non-volatile compounds
- It is currently the most widely used method of quantitative analysis in the pharmaceutical industry, food and beverages analysis laboratories, environmental application etc.
- Connected to MS detectors is one of the most sophisticated techniques for quantification of very low concentrations of various compounds.





Thank you for your attention!