Notes in HPLC

JP Da Silva

jpsilva@ualg.pt

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Outline

HPLC system

The mobile phase

Pumps and injector

Stationary phases and column

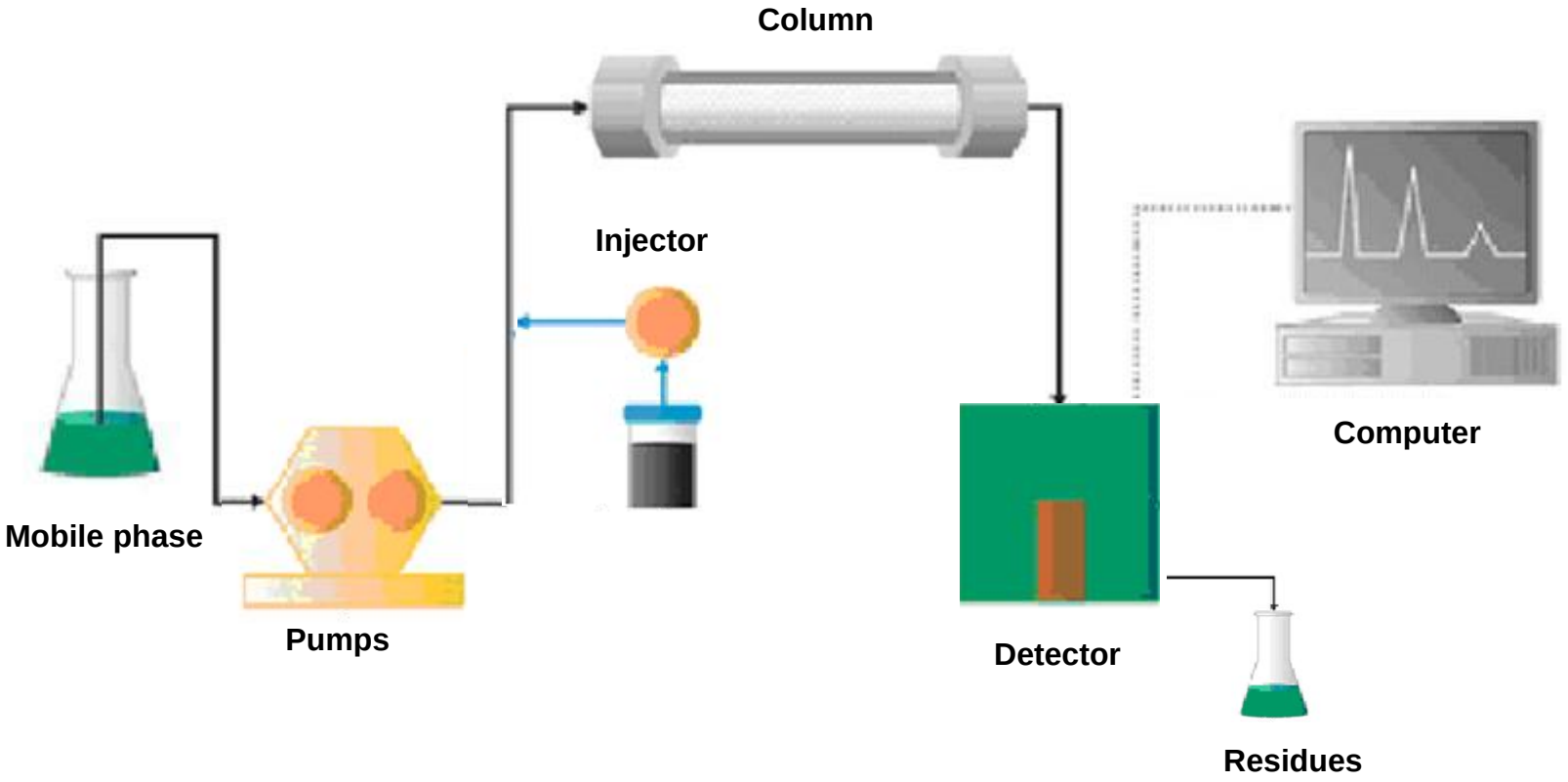
UV and Vis detectors

Examples

General LC system

Six basic units

A mobile phase supply system **(1)**, a pump and programmer **(2)**, a column **(3)**, a sample valve **(4)**, a detector **(5)** and finally a means of presenting and processing the results **(6)**.



LC @ Ualg/CCMAR

1986

2008

2019

A - Pump + degasser

B - Autosampler

C – Column compartment

D – Diode Array Detector

The mobile phase

Solvents

Water
Acetonitrile
Methanol
Ethyl acetate
Hexane
Chloroform
Methylene chloride
Ethyl ether
1- or 2-Propanol
……

Other chemicals

Buffers, ionizing agents…

Solvents should be:

1 - LC grade, isocratic or gradient

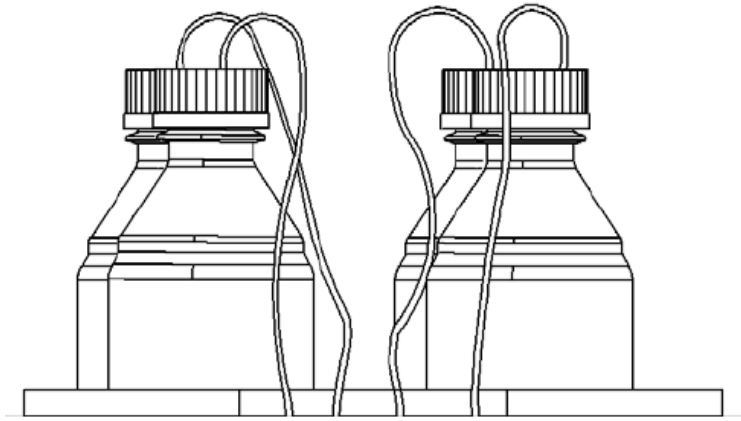
2 - Filtered

Removes **Particles** and **Algae**

3 - Degassed

The Mobile Phase Supply System

The mobile phase supply system consists of a number of reservoirs (200 ml to 1,000 ml in capacity), usually glass flasks, with an exit port open to air.



Why degassing?

- **Unstable flow**
- **Baseline noise**
- **Sample degradation**
- **Fluorescence quenching**

How to degas the Mobile Phase

Sonicate

Apply partial vacuum while agitating

Helium bubbling

In-line degasser

Pumps and Elution Types

Isocratic – where the eluent is at a fixed composition.

Gradient – where the eluent composition and strength are changing.

The gradient programmer

Two basic types of solvent programmer:

a) **Mixing at high pressure**

b) **Pre mix at low pressure**

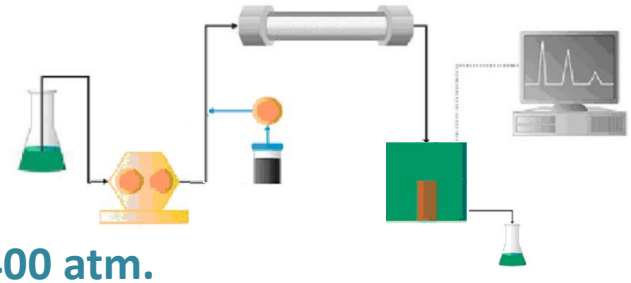
Pump care

Flush with water after running a buffer.
(note there are special procedures when using reverse phase columns)

Replace seals in a timely manner.

Do not allow solids in the mobile phase.

Injector



Especial valve because is able to sustain pressures up to 400 atm.

Manual injection

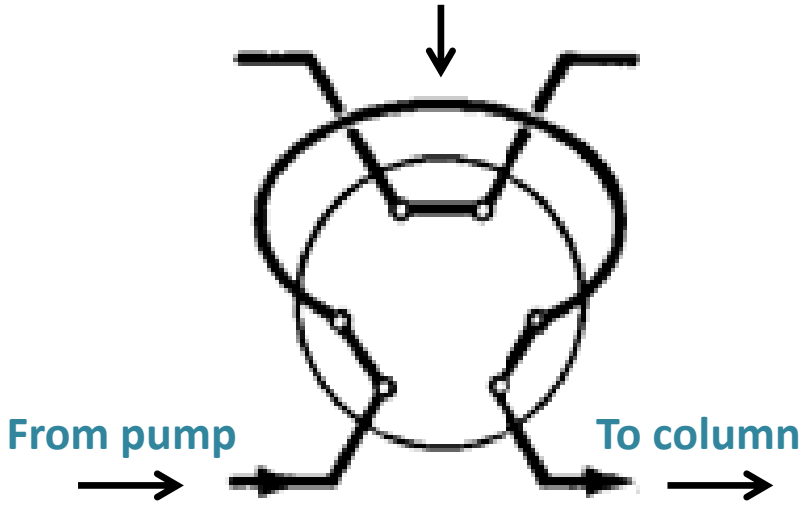
LOAD position

1 - Never use a pointed or bevel tip needle.

2 - Rinse after the use of buffer solutions.

3 – Avoid particles by filtering samples before injection.

loop INJECT position

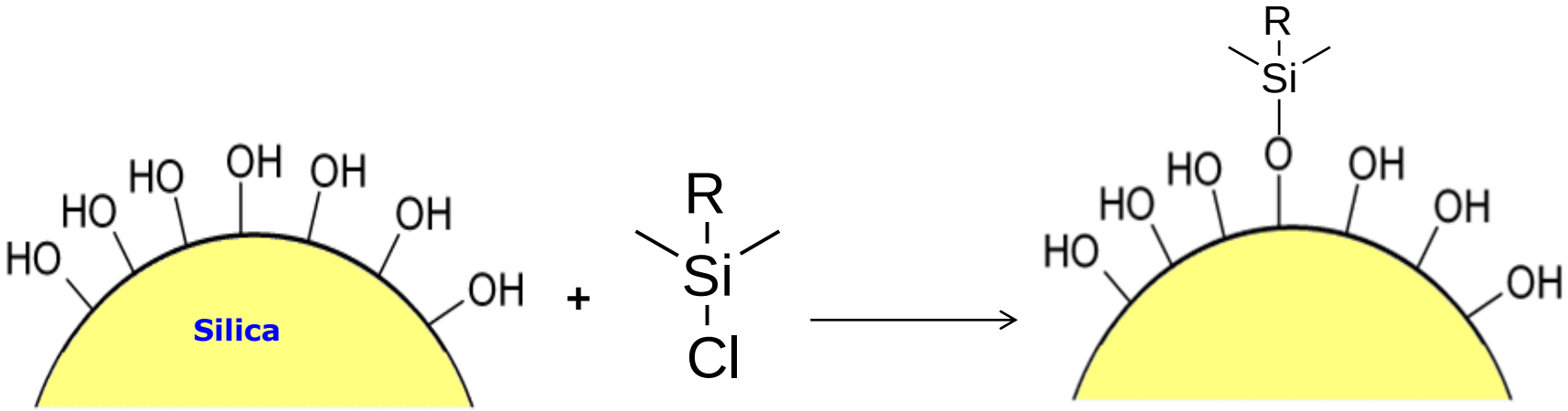


The stationary phase

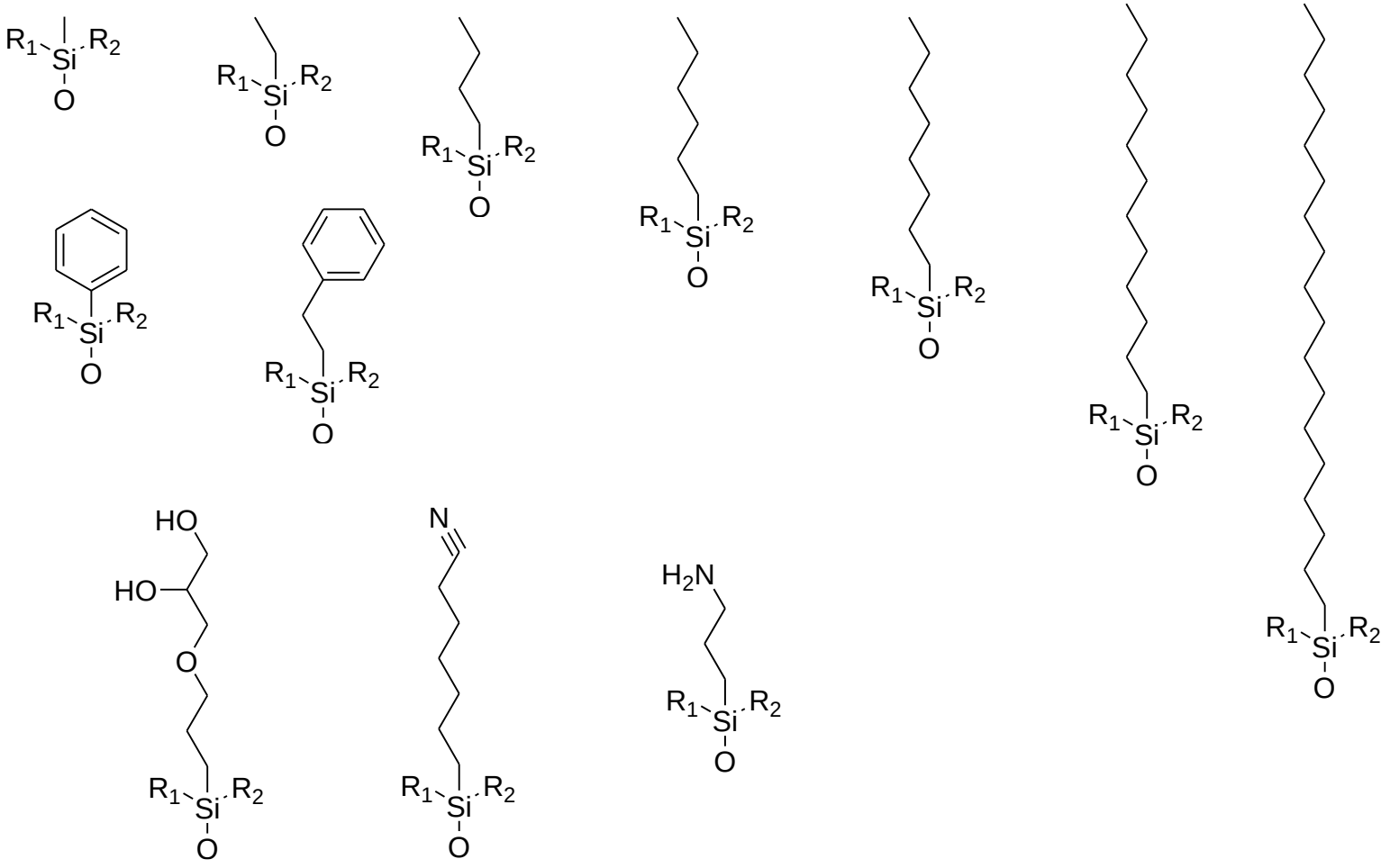
1 - The **traditional stationary phase** used in LC is **silica gel**, which separates solutes largely on the basis of polarity.

2 - The **bonded phases** were introduced to provide a material that would separate solutes by dispersive interactions and also to provide some intermediate polarity stationary phases. The bonded phases are **also based on silica gel**.

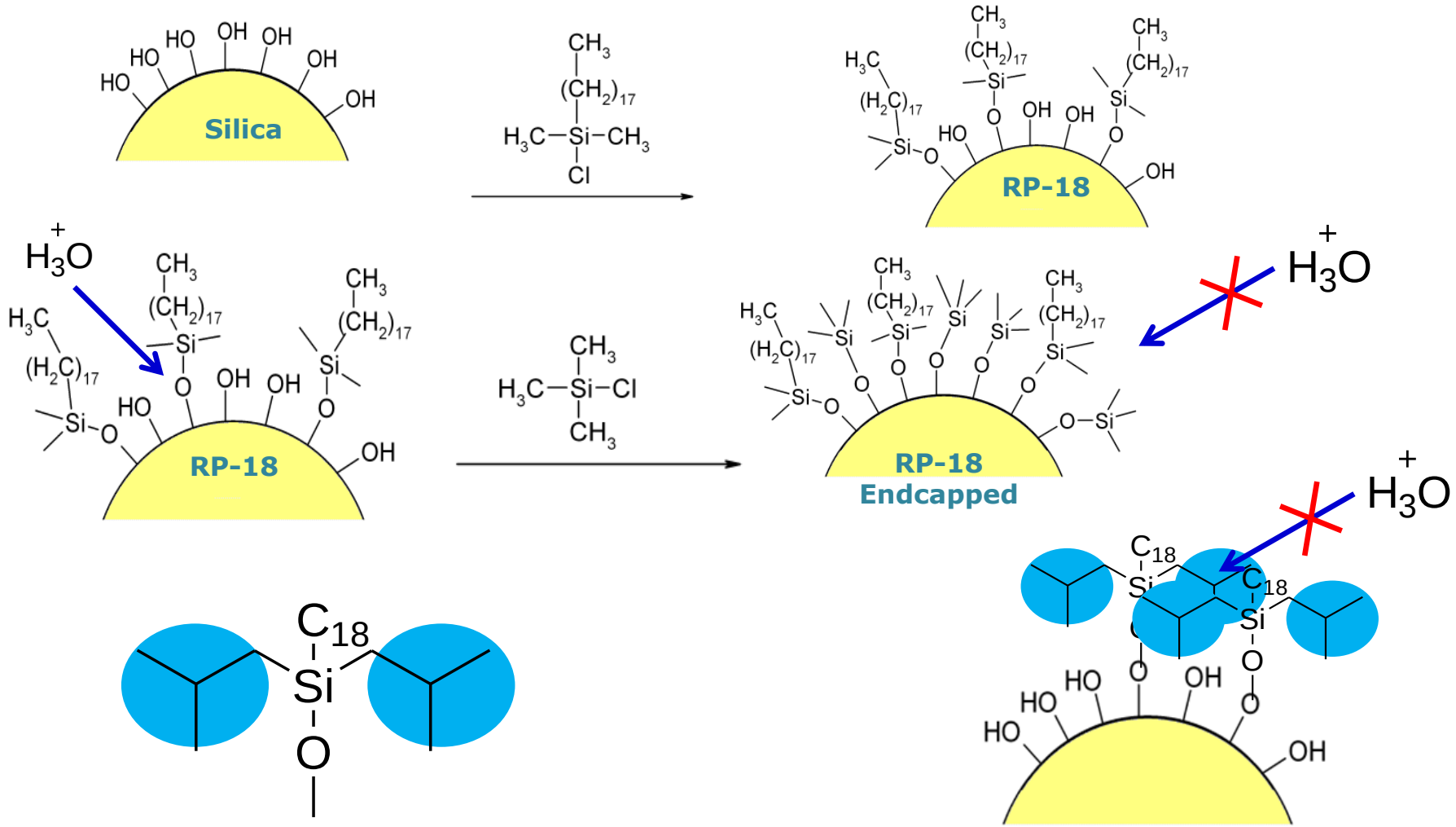
3 - More recently, ***polymeric* stationary phases** were also introduced to provide materials that are insoluble in water and **stable at extreme pH values**.



Bonded phases

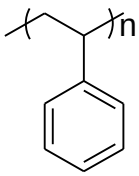


Protection from hydrolysis



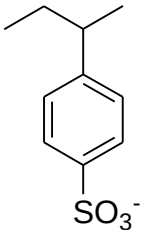
Macroporous polymers

1 – More popular **co-polymerization of polystyrene and divinylbenzene**.



2 - Underivatized, they are an alternative to the **C8 and C18 reverse phase** columns based on silica

3 - In the case of the **ion exchange materials**, inorganic groups of appropriate charge were chemically attached (*e.g.*, by sulfonation).

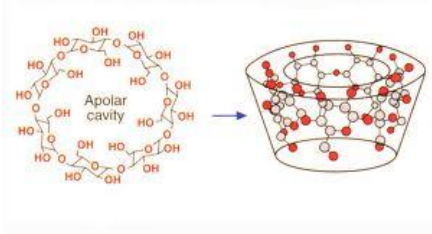


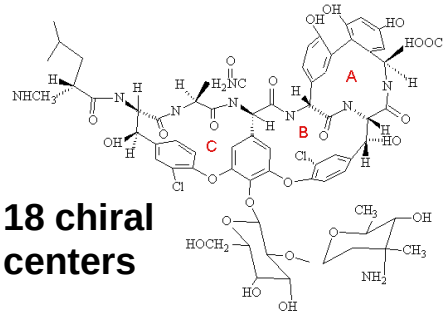
4 - S**tability under extremes of pH**, their use for peptide and proteins at both high and low pH has been well established.

Chiral stationary phases

5 – Peptides and cyclodextrin based materials.

Beta-cyclodextrin has 35 stereo centers.





18 chiral centers

Column care

The **mobile phase** must be chosen **to complement the stationary phase so that the selected interactions are concentrated in the stationary phase**.

Guard column: Protects the analytical column from particles and other interferences

After the use of **buffer solutions**:

1 - Do not flush with 100% of water as your first step.

2 - Substitute water for the buffer but leave the remaining proportions the same. Run through about 5 column volumes.

3 - Wash through 10 column volumes of a strong organic solvent, example – Methanol.

4 - If you plan to store the column, read the directions.

UV detectors - General

1 - Respond to those substances that absorb light in the range **180 to 350 nm.**

2 - Most popular and widespread LC detectors

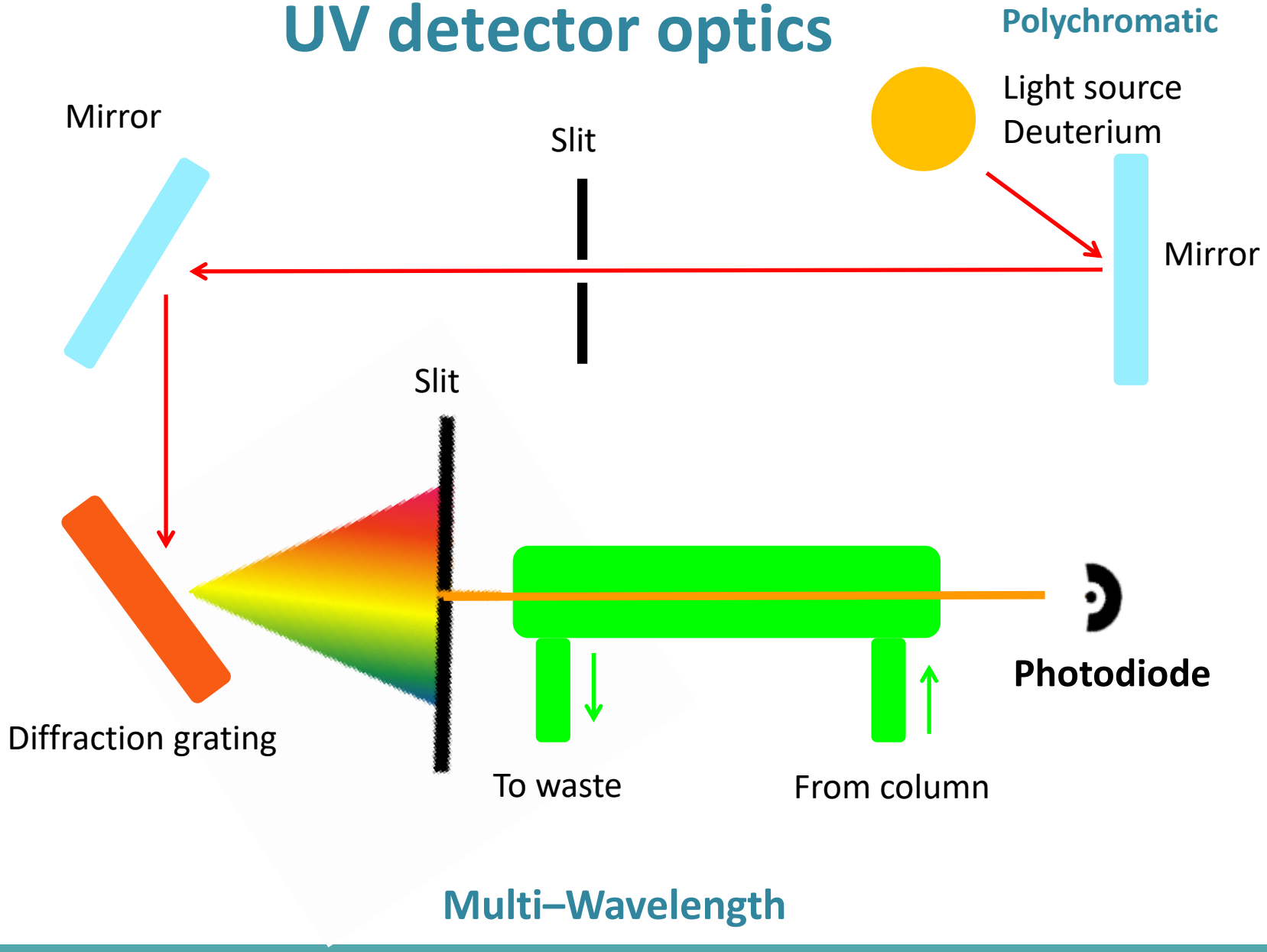
3 - Many (but not all) substances absorb light in this wavelength range.

4 - These include compounds having **one or more double bonds** (p electrons**) unshared** (unbounded**) electrons**, *e.g.* all **olefins**, **all aromatics** and compounds, for example, containing **>C=O, >C=S, –N=N– groups**.

5 - The sensitivity for a given compound will be directly proportional to its extinction coefficient

$$A = \varepsilon \left[A\right] l$$

UV detector optics



Multi–Wavelength

Cut-off points of solvents

The lower wavelength range of a UV detector is limited by the UV **cut-off** of the mobile phase.

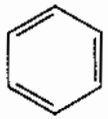
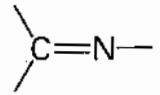
The **UV cut off** is the wavelength where the absorbance is **1.0** in a cell **of 10 mm** length, with air as the reference.

Lower Limit	Solvent
180–195 nm	Sulfuric acid (96%) Water Acetonitrile
200–210 nm	Cyclopentane n-Hexane Glycerol 2,2,4-Trimethylpentane Methanol
210–220 nm	n-Butyl alcohol Isopropyl alcohol Cyclohexane Ethyl ether
245–260 nm	Chloroform Ethyl acetate Methyl formate
265–275 nm	Carbon tetrachloride Dimethyl sulfoxide Dimethyl formamide Acetic acid
280–290 nm	Benzene Toluene m-Xylene
Above 300 nm	Pyridine Acetone Carbon disulfide

UV absorption of chromophores

TABLE 10-1

Electronic Absorption Data for Isolate Chromophores*

Chromophore	*Example*	*Solvent*	λ_{max} *(nm)*†	ε *(liter mol^{-1} cm^{-1})*
C=C	1-Hexene	Heptane	180	12,500
—C≡C—	1-Butyne	Vapor	172	4,500
	Benzene	Water	254	205
			203.5	7,400
	Toluene	Water	261	225
			206.5	7,000
C=O	Acetaldehyde	Vapor	298	12.5
			182	10,000
	Acetone	Cyclohexane	275	22
			190	1,000
	Camphor	Hexane	295	14
—COOH	Acetic acid	Ethanol	204	41
—COCl	Acetyl chloride	Heptane	240	34
—COOR	Ethyl acetate	Water	204	60
—$CONH_2$	Acetamide	Methanol	205	160
—NO_2	Nitromethane	Hexane	279	15.8
			202	4,400
=$\overset{+}{N}$=$\overset{-}{N}$	Diazomethane	Diethyl ether	417	7
—N=N—	*trans*-Azomethane	Water	343	25
	C_2H_5CH—NC_4H_9	Isooctane	238	200

* From J.B Lambert, H.F. Shurvell, L. Verbit, R.G. Cooks, and G H. Stout. *Organic Structural Analysis,* Macmillan Publishing. New York 1976

† Chromophores often have more than one absorption band

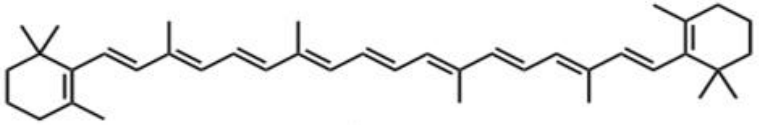
Effect of conjugation on UV maximum

Effect of Extended Conjugation in Alkenes on Position of Maximum Absorption*

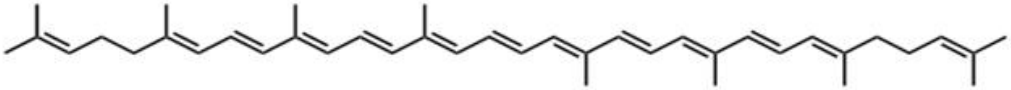
n in $H(CH{=}CH)_nH$	λ_{max} (nm)	ε_{max} (liter $mol^{-1}cm^{-1}$)	Color
1	162	10,000	Colorless
2	217	21,000	Colorless
3	258	35,000	Colorless
4	296	52,000	Colorless
5	335	118,000	Pale yellow
8	415	210,000	Orange
11	470	185,000	Red
15	547†	150,000	Violet

1,3-butadiene
λ_{max} = 217 nm

1,3,5-hexatriene
λ_{max} = 258 nm



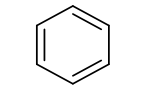
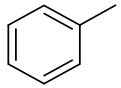
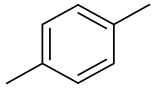
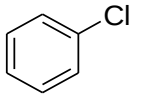
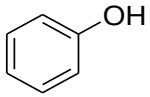
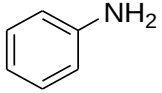
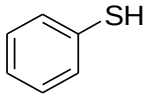
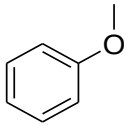
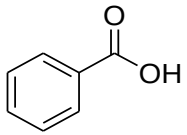
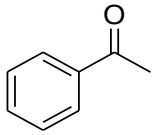
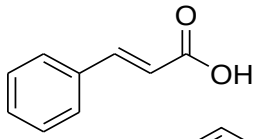
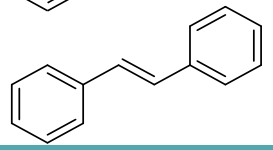
β-carotene
λ_{max} = 454 nm



lycopene
λ_{max} = 471 nm

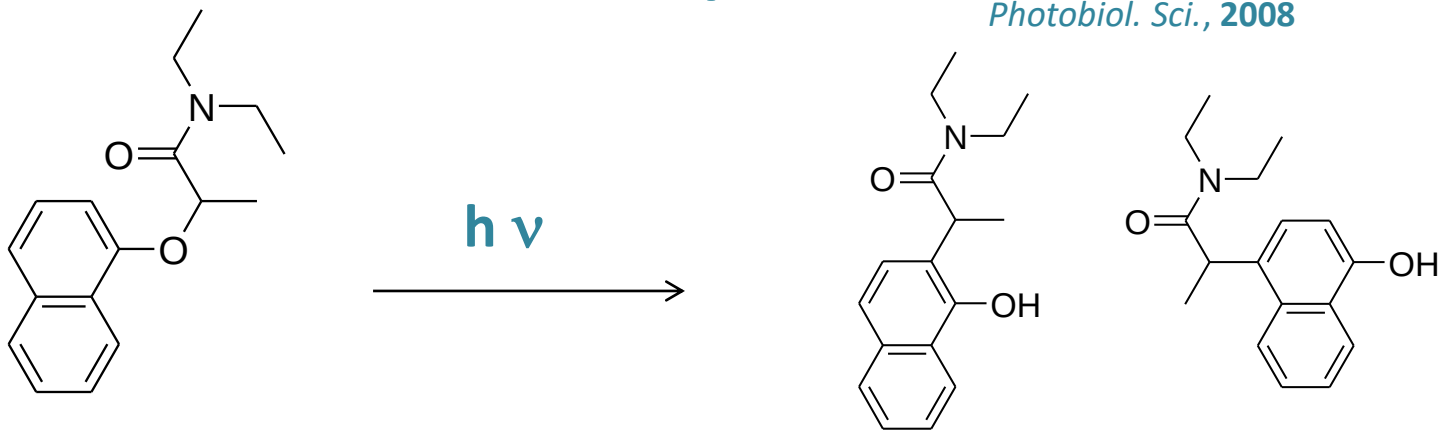
Absorption data for benzene derivatives

Compound	Solvent	λ_{max}	ε_{max}	λ_{max}	ε_{max}	λ_{max}	ε_{max}
Benzene	Hexane	184	68,000	204	8,800	254	250
Toluene	Hexane	189	55,000	208	7,900	262	260
p-Xylene	Ethanol			216	7,600	274	620
Chlorobenzene	Ethanol			210	7,500	257	170
Phenol	Water			211	6,200	270	1450
Aniline	Water			230	8,600	280	1400
Thiophenol	Hexane			236	10,000	269	700
Anisole	Water			217	6,400	269	1500
Benzoic acid	Water			230	10,000	270	800
Acetophenone	Hexane			238	13,000	276	800*
Cinnamic acid	Hexane	200	31,000	215	17,000	280	25,000
Stilbene	Etanol			225	24,000	274	10,000

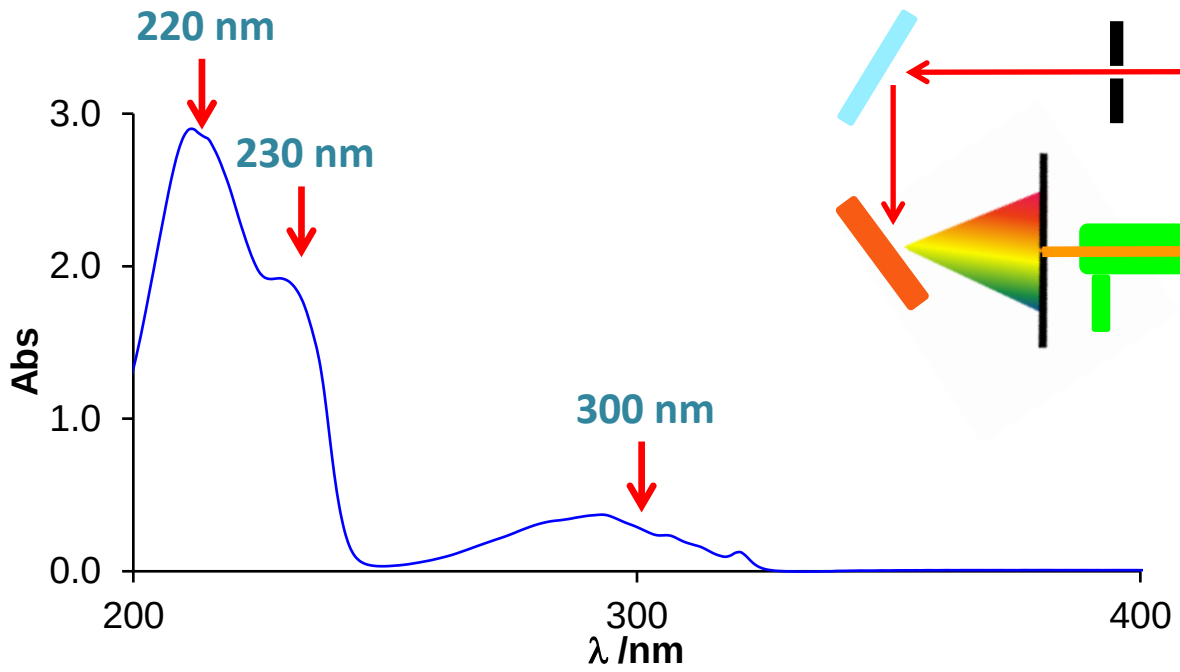
Example 1

Bastos et al., *Photochem. Photobiol. Sci.*, **2008**



Napropamide

UV dispersive detector



Bastos et al., *Photochem. Photobiol. Sci.*, **2008**

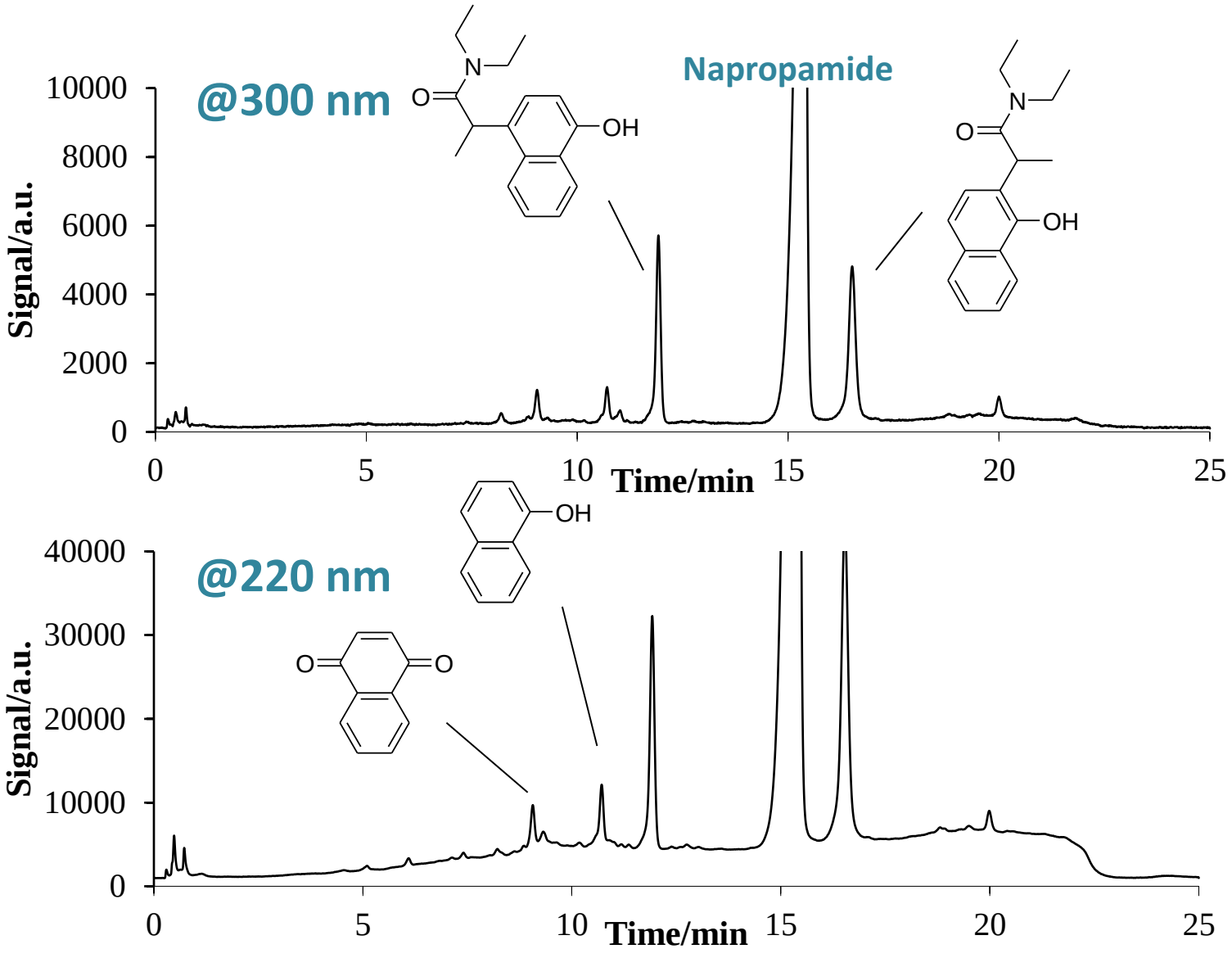
Time	Methanol	Water	Acetonitrile
0	15	85	0
8	15	50	35
14	15	25	60
19	15	25	60
20	15	85	0
24	15	85	0

Column: RP-18, 124 mm length, 5 μm dp,

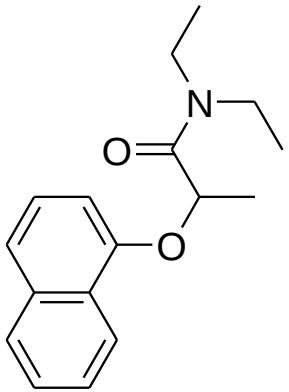
Flow: 1.5 mL/min

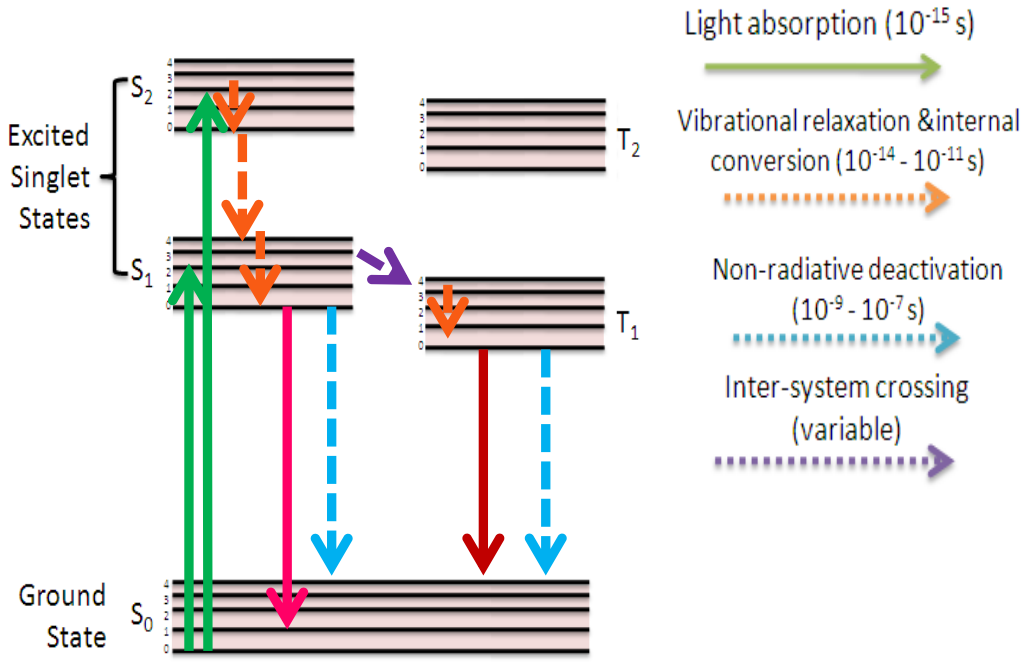
Inj Volume: 20 mL

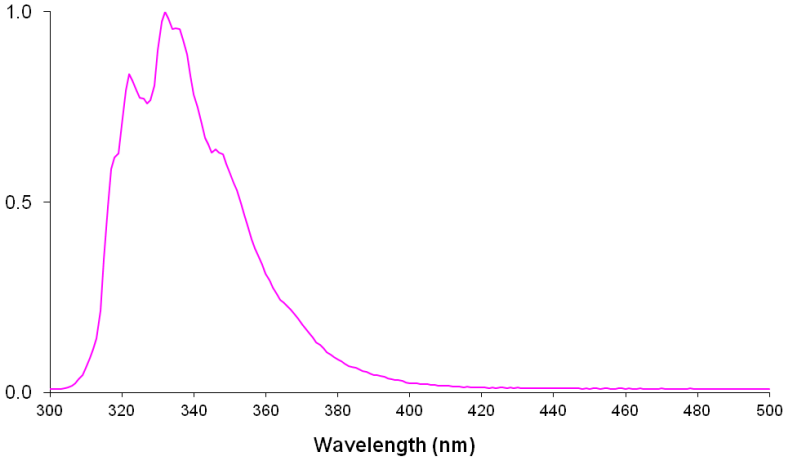
Bastos et al., *Photochem. Photobiol. Sci.*, **2008**

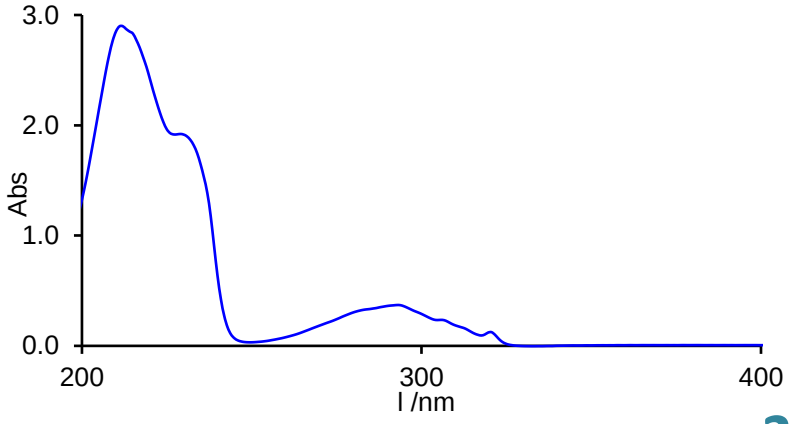


Fluorescence

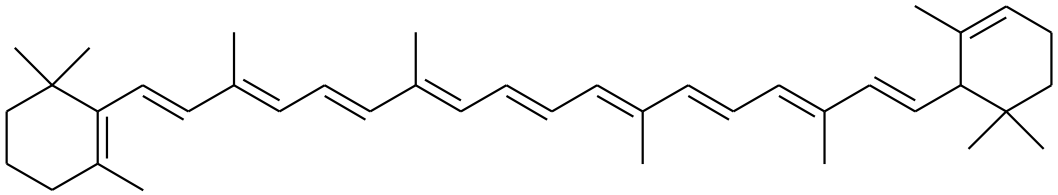




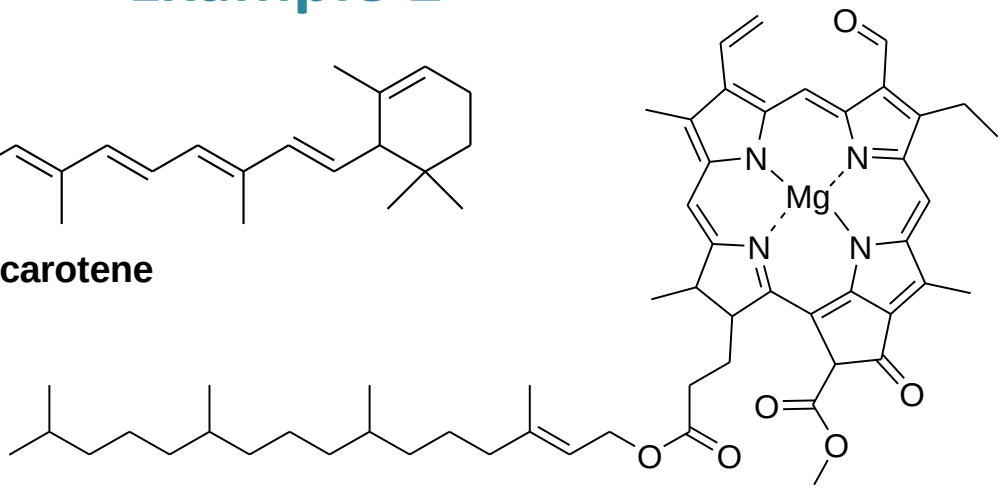




Example 2

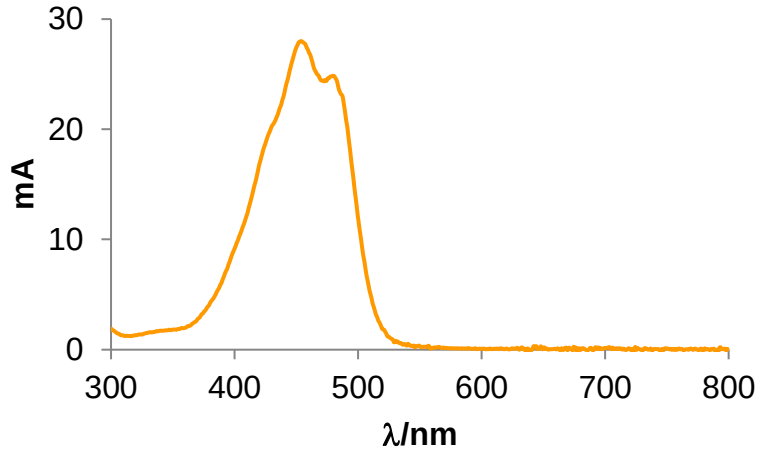


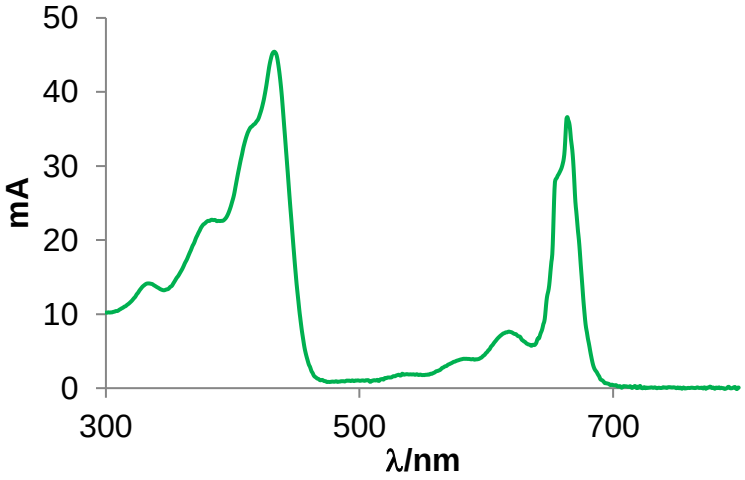
Alpha-carotene

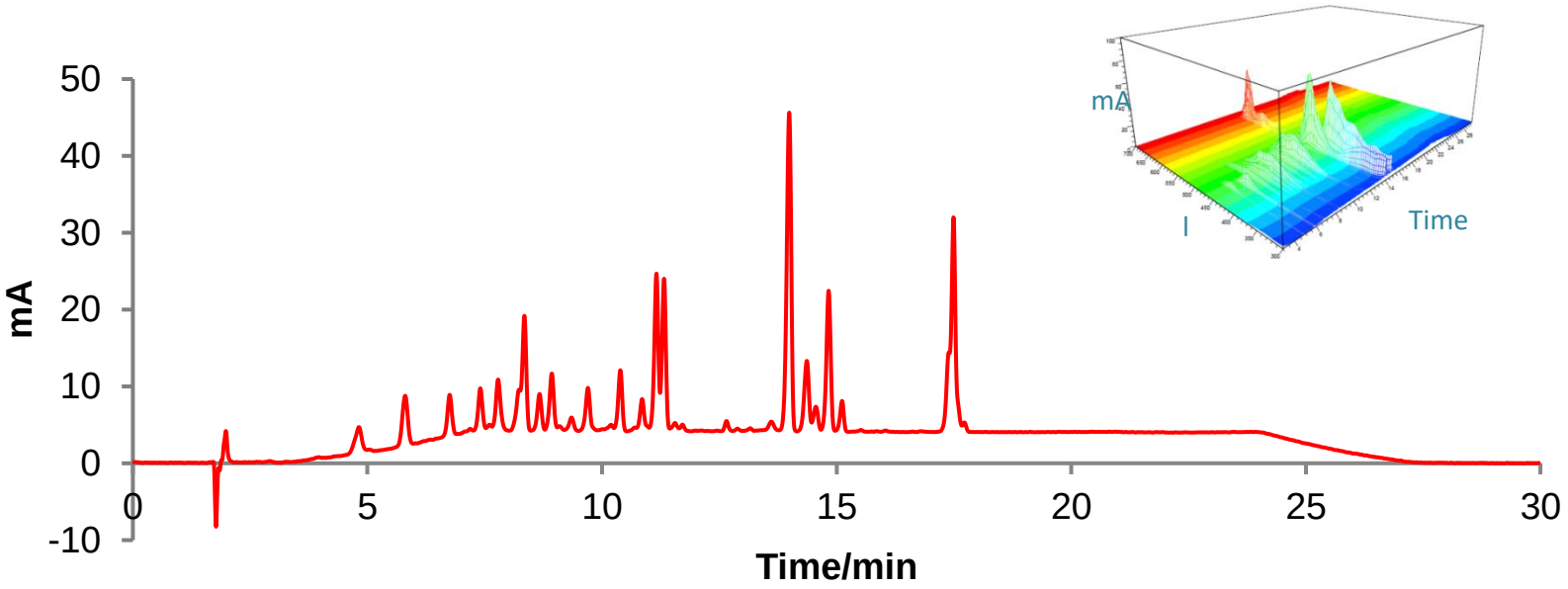


Chlorophyll b

Photodiode array detector







Time	Solv.B	Solv.C	Flow
0.00	0.0	0.0	1.000
4.00	100.0	0.0	1.000
18.00	20.0	80.0	1.000
21.00	100.0	0.0	1.000
24.00	0.0	0.0	1.000
29.00	0.0	0.0	1.000

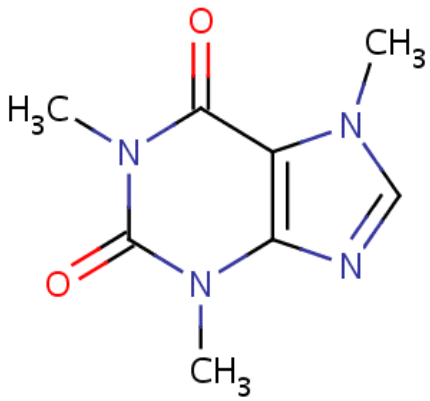
Column: RP-18, 4.6 mm ID, 150 mm length, 5 µm dp

Flow: 1.0 mL/min

Inj Volume: 50 mL

Example 3

Caffeine @ Diode array and MS detectors



An alkaloid from the xanthines group

Stimulating

Present in coffee, tea, cocoa, etc

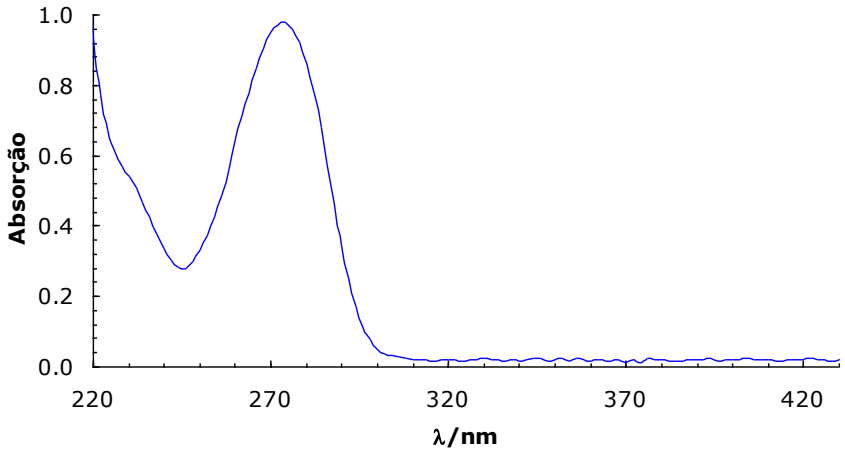
Natural pesticide

Column: **RP-18, 2.1 mm ID, 125 mm length, 5 μm dp**

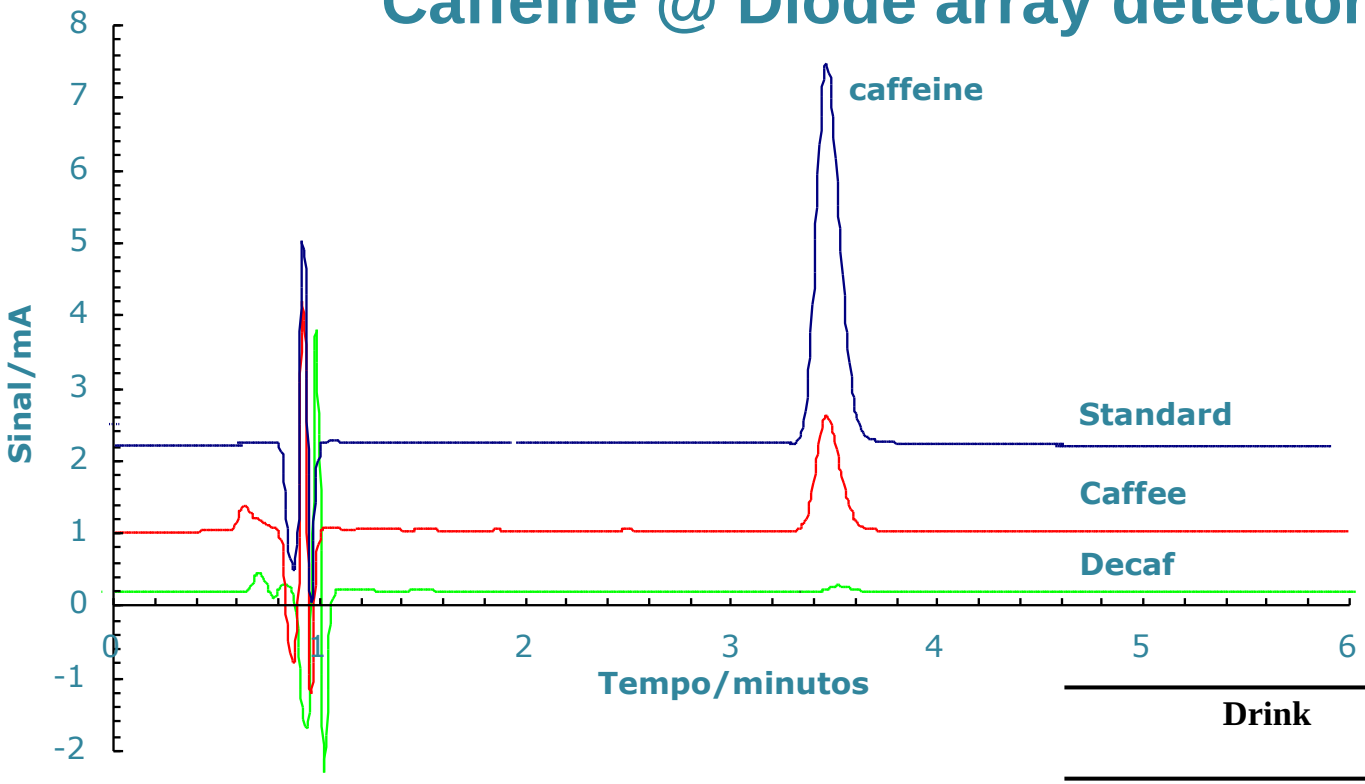
Eluent system: **Water-acetonitrile, 90:10, 0.1 % formic acid**

Flow: **0.4 mL/min**

Wavelength: **275 nm**



Caffeine @ Diode array detector



The concentration in the espresso was 2.9 g/L. In the decaf 0.01 g/L

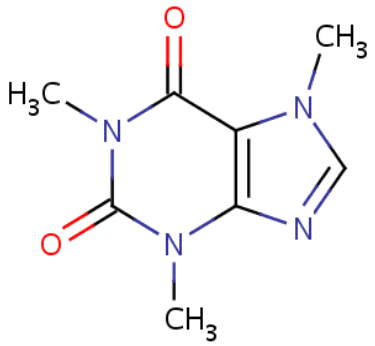
116 mg per espresso

0.4 mg per decaf

Drink	Caffeine, mg per 40 mL
Espresso	**53**
Decaf	**1**
Green tea	**7**
Black tea	**8**
Coca-Cola	**4**
Pepsi	**4**
Red Bull	**13**

Adapted from Hechman, M.A. et.al., Journal of Food Science, 75, R77, 2010

Caffeine @ MS



Nominal mass 194

Column: **RP-18, 2.1 mm ID, 125 mm length, 5 mm dp**

Mobile phase: **Water-acetonitrile, 90:10, 0.1 % formic acid**

Flow: **0.4 mL/min**

Wavelength: **275 nm**

MS

Ionization - Positive polarity

Mass range - 0 a 300