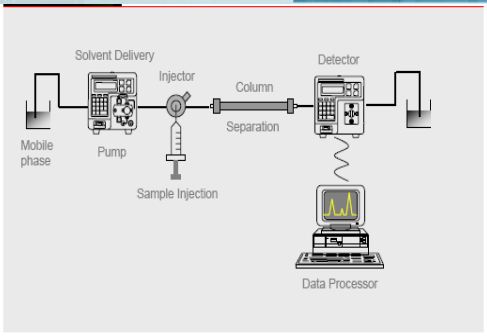
Determination L-ascorbic acid in some tropical fruits by High performance liquid chromatography.

By RIBE Team
HCMC - July 2012

HPLC Basic instrument

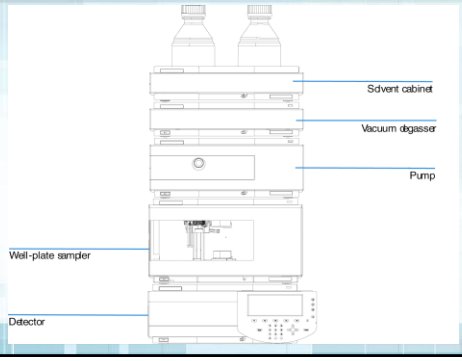


History:

- Discovered by Russian botanist Mikhail Tswett at the turn of the century. He separated various plant pigments by passing solutions through a glass column containing calcium carbonate.

- Chroma meaning "color" + graphein meaning "to write"

Typical HPLC Stack (Agilent 1100)



Chromatography Principle

- In chromatography process, separations are based on differences in migration rates among the sample components because they have differences interactive forces with stationary phase.

	Gas chromatography	Liquid chromatography
Mobile phase	Carrier gas: N2, He, Ar…	Polar and non-polar solvent such as: MeOH, acetonitril, water…
Stationary phase	Solid: film layer in capillary column	Solid or liquid covering particles in packed column
Analyzed objects	Samples easily evaporate (<300°C) or durable with heat.	Samples easily degrade by heat or has molecule mass > 3000 u.

Liquid Chromatography (HPLC)

High Pressure Chromatography
High Performance Chromatography

Suitable for separation of "small" organic compounds, but also proteins and peptides. Especially those with UV-Vis absorption or fluorescence.

Typical applications

- Vitamins
- Drugs
- Steroids
- Organic acids
- Pesticides
- Mycotoxins
- Flavours
- Carbohydrates

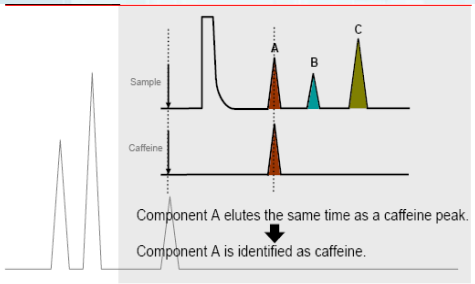
Advantages

- ✓ Speed and improved resolution
- ✓ Reusable columns
- ✓ Sample recovery
- ✓ Low temperature separation
- ✓ No need for derivatization

Vegetable, Fruit and Products Concerned (96/23/EC)

Identification

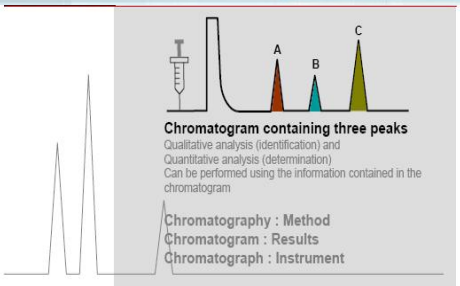
What is component A?



Component A elutes the same time as a caffeine peak.

Component A is identified as caffeine.

Results obtained by HPLC



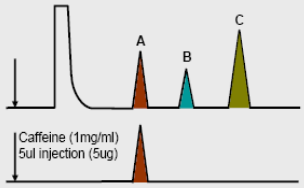
Chromatogram containing three peaks

Qualitative analysis (identification) and
Quantitative analysis (determination)
Can be performed using the information contained in the chromatogram

Chromatography : Method
Chromatogram : Results
Chromatograph : Instrument

Determination

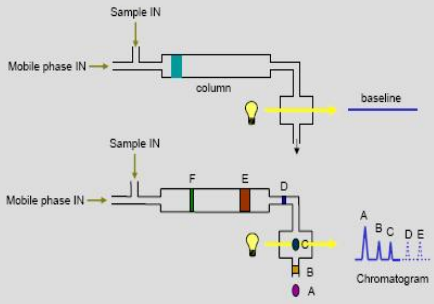
What is the concentration of component A?



Peak area (or height) is proportional to the concentration (or amount) of the component.

The concentration of component A(caffeine) is determined by comparing the peak area with that of the standard caffeine peak.

Chromatogram



In HPLC

- Stationary Phase:

Typical surface coatings:

Normal phase (-Si-OH, $-NH_2$)
Reverse phase (C_8, C_{18}, Phenyl)
Anion exchange ($-NH_4^+$)
Cation exchange ($-COO^-$)

- Typical Solvents

- hexane, methylene chloride, chloroform, methanol, acetonitrile (normal phase)
- methanol, acetonitrile, water (reverse phase)

HPLC - Column

Stainless steel tubing with low volume end fittings.

Length 3 - 30 cm, becoming shorter

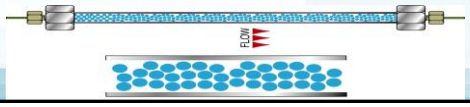
Inner diameter usually 3 – 4.6 mm

Packing material

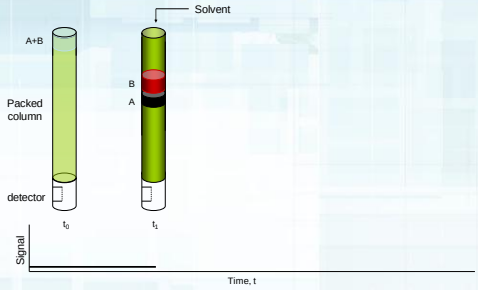
- Microparticular 3, 5 or 10 µ
- Irregular or spherical shape
- Different types, silica based

Guard column

- Short column of same packing material before separation column
- Adsorb impurities and particles
- Can be replaced



Separation



HPLC - Detectors

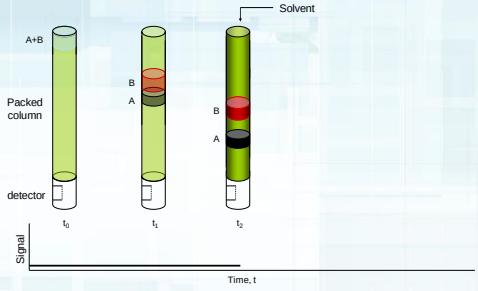
Table 25-2

Comparison of commercial HPLC detectors

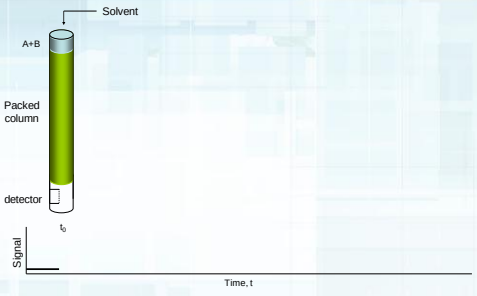
Detector	Approximate limit of detection[a] (ng)	Useful with gradient?
Ultraviolet	0.1–1	Yes
Refractive index	100–1 000	No
Evaporative light-scattering	0.1–1	Yes
Electrochemical	0.01–1	No
Fluorescence	0.001–0.01	Yes
Conductivity	0.5–1	No
Mass spectrometry	0.1–1	Yes
Fourier transform infrared	1 000	Yes

a. Most detection limits from F. W. Yeung and R. E. Synovec, *Anal. Chem.* **1986**, *58*, 1237A.

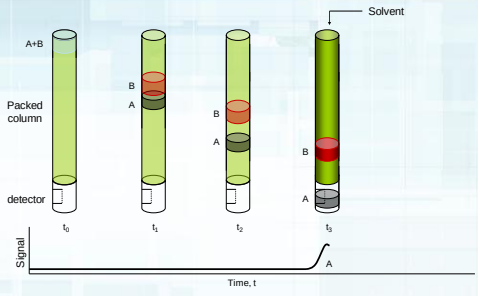
Separation



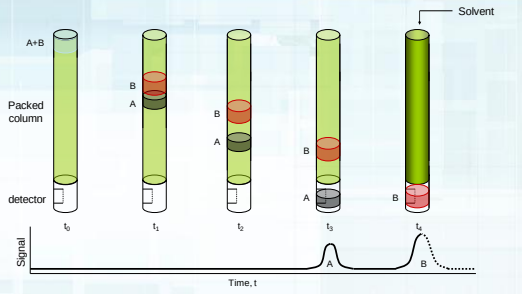
Separation



Separation



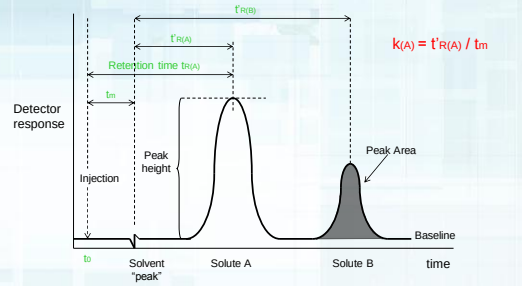
Separation



Preparation of ascorbic acid standard curve

- Stock solution: dissolve 10 mg of ascorbic acid in 10 ml of extracting solution. Carry to 10 ml using dark volumetric flask.
- Prepare ascorbic acid standard solutions of ascorbic acid with concentrations of 5,10, 20, 30, 40 mg/l.
  - Mix 2.5 ml stock solution with extracting solvent, fill up to 50 ml in volumetric flask-→ solutions of ascorbic acid with concentrations of 50 mg/l.
  - Mix 1 ml stock solution with extracting solvent, fill up to 25 ml in volumetric flask-→ solutions of ascorbic acid with concentrations of 40 mg/l.
  - Mix 1.5 ml stock solution with extracting solvent, fill up to 50 ml in volumetric flask-→ solutions of ascorbic acid with concentrations of 30 mg/l.

Factors Affecting Chromatography



Preparation of ascorbic acid standard curve

- Mix 0.5 ml stock solution with extracting solvent, fill up to 25 ml in volumetric flask-→ solutions of ascorbic acid with concentrations of 20 mg/l.
- Mix 0.5 ml stock solution with extracting solvent, fill up to 50 ml in volumetric flask-→ solutions of ascorbic acid with concentrations of 10 mg/l.
- Mix 1 ml solution of ascorbic acid with concentrations of 50 mg/l with extracting solvent, fill up to 10 ml in volumetric flask→ solutions of ascorbic acid with concentrations of 5 mg/l.

Determination L-ascorbic acid (AA)

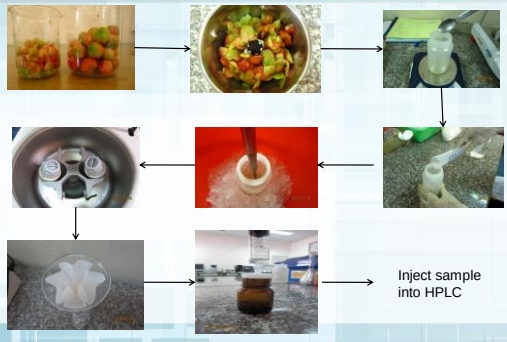
(reference to Agilent application)

- AA is a water soluble organic compound that participates in many biological processes.
- In samples, vitamin C concentration is total content of ascorbic acid and dehydroascorbic acid; dehydroascorbic acid did not account for more than 10% of total vitamin C in any of the analysed fruits as has been described by Lee and Kader (2000).
- In our lab, we extract AA in matrices by using acid solvent which has pH 2.1 (titrate by H_2SO_4) and quantify its concentration by using HPLC with RP-column and UV detector.

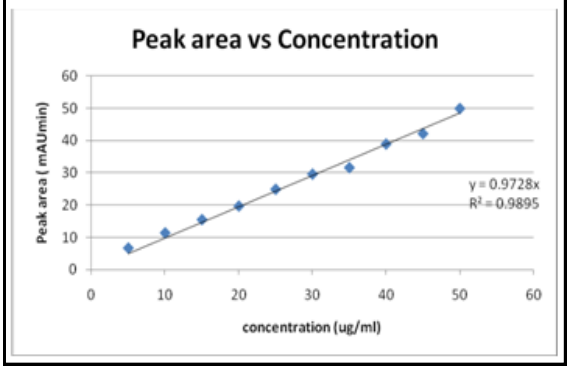
Procedure of determination AA

- Weigh 2-5 g sample into centrifugal bottle.
- Add 50 ml acidic solvent pH 2.1 (extracting solvent).
- Mix thoroughly to obtain homogenous slurry by using Ultra-Turax in 3 minutes.
- Centrifuge at 4000 rpm, 4°C in 20 minutes.
- Give extract in 100 volumetric glass flask.
- Wash centrifugal bottle with 10 ml acidic solvent pH 2.1
- Combine washing solvent in volumetric glass flask and add acidic solvent up to 100 ml.
- Filter extract through 0.2 µm membrane.
- Inject 20 µl filtrate into HPLC system.

Practical steps:



Standard curve



Analytical conditions

- Agilent 1100
- Column: Agilent 10 cm x 4.6 cm x 3 µm
- Flow: isocratic elution 0.5 ml/minute.
- UV Detector: 254 nm.
- Mobile phase: 100 % acidic solvent pH 2.1

Calculation:

$$\text{Vitamin C conc.} = \frac{Cm \times V\ (ml)}{m\ (g \text{ or } ml)} \quad (mg/Kg \text{ or } mg/L)$$

Cm: Vitamin C conc. is calculated base on standard curve.
Vm: 100 ml (the final volume of extract).
m: weight of sample (g).

Reference

- M.A. Romero R., M.L. Vazquez O., J. Lopez H., J. Simal L.Determination of vitamin C and organic acids in various fruits by HPLC (1992). *Journal of Chromatographic Science*, 30(11):433-437
- HPLC for food analysis (Agilent's application)

Example HPLC Separation

Chromatographic conditions for UV detection

The HPLC method presented here was used to analysis vitamins in a vitamin drink.

Sample preparation	filtration
Column	100 x 4 mm Hypersil BDS, 3 µm
Mobile phase	A= water with pH = 2.1 (H_2SO_4) = 99 % B = ACN 1 %
Gradient	at 3.5 min 1 % B at 11 min 25 % B at 19 min 90 % B
Post time	6 min
Flow rate	0.5 ml/min
Column compartment	30 °C
Injection volume	2–5 µl
Detector	UV-DAD detection wavelength 220/30 nm, reference wavelength 400/100 nm

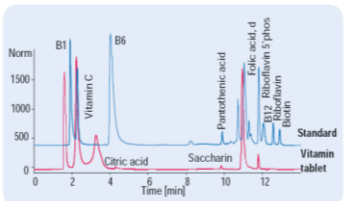


Figure 32
Analysis of water-soluble vitamins in a vitamin tablet