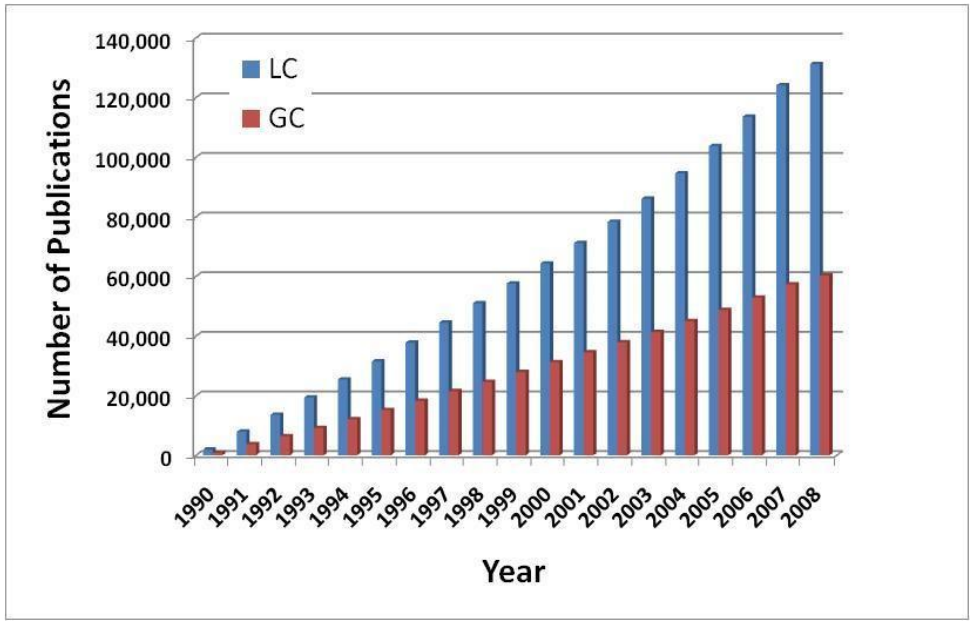
Liquid Chromatography

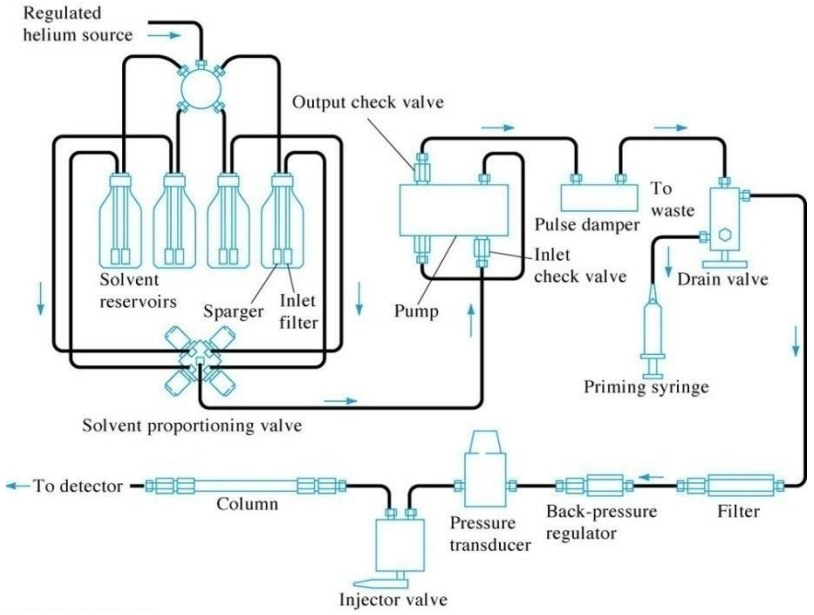
A.) Introduction:

Liquid Chromatography (LC) is a chromatographic technique in which the mobile phase is a liquid.

LC is a much older technique than GC, but was overshadowed by the rapid development of GC in the 1950's and 1960's.

LC is currently the dominate type of chromatography and is even replacing GC in its more traditional applications.





Advantages of LC compared to GC:

1.) LC can be applied to the separation of any compound that is soluble in a liquid phase.

, LC more useful in the separation of biological compounds, synthetic or natural polymers, and inorganic compounds

2.) Liquid mobile phase allows LC to be used at lower temperatures than required by GC

LC better suited than GC for separating compounds that may be thermally labile

3.) Retention of solutes in LC depend on their interaction with both the mobile phase and stationary phase.

, GC retention based on volatility and interaction with stationary phase

, LC is more flexible in optimizing separations → change either stationary or mobile phase

4.) Most LC detectors are non-destructive

, most GC detectors are destructive

, LC is better suited for preparative or process-scale separations

Disadvantage of LC compared to GC:

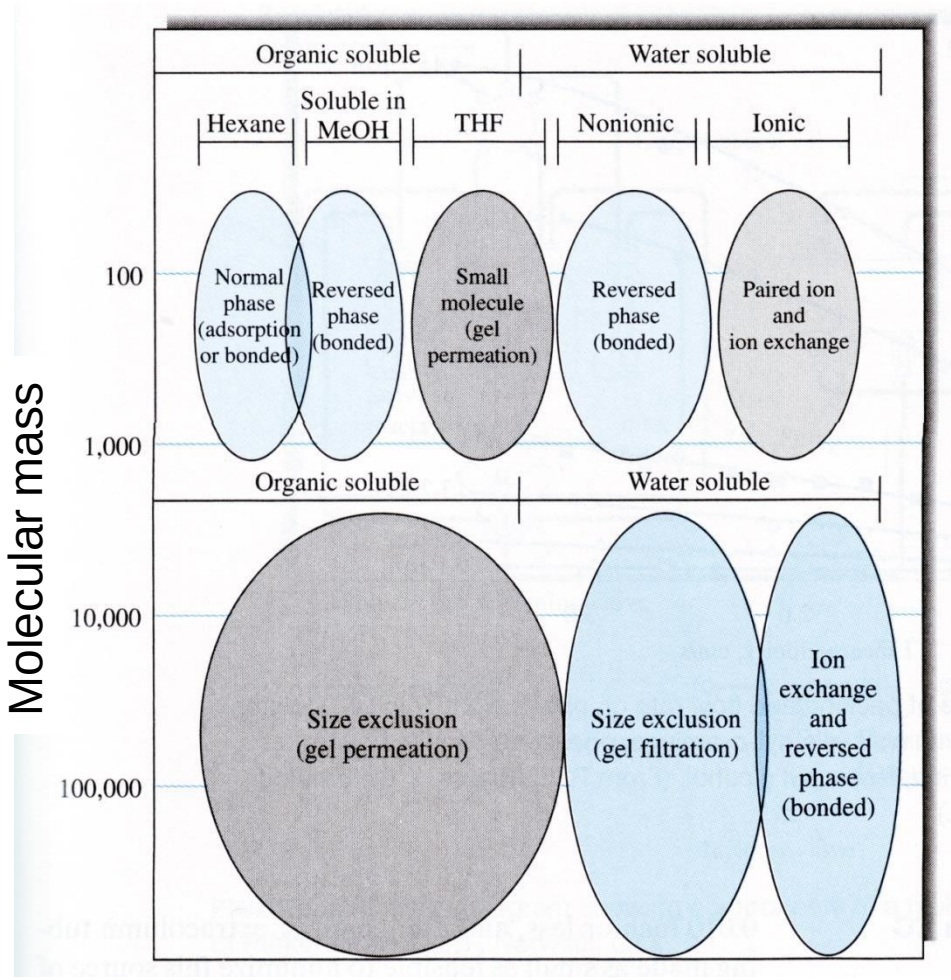
1.) LC is subject to greater peak or band-broadening.

, much larger diffusion coefficients of solutes in gases vs. liquids

B.) Low- and High-performance Liquid Chromatography:

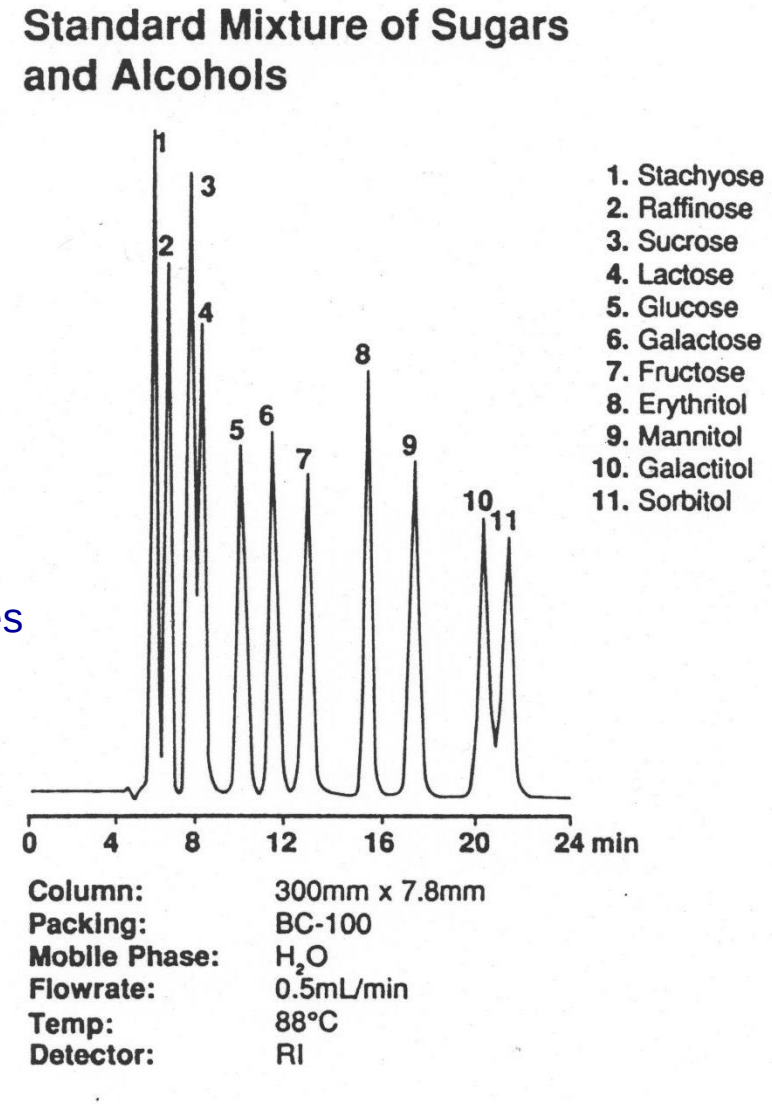
Many types of liquid chromatography are available, based on different stationary phase and mobile phase combinations.

- each type may be further characterized based on its overall efficiency or *performance*



High-performance liquid chromatography (HPLC)

– LC methods that use small, uniform, rigid support material
  , particles < 40 μm in diameter
  , usually 3-10 μm in practice
– good system efficiencies and small plate heights
– such systems have the following characteristics:
  , narrow peaks
  , low limits of detection
  , short separation times
  , columns can only tolerate high operating pressures and faster flow-rates

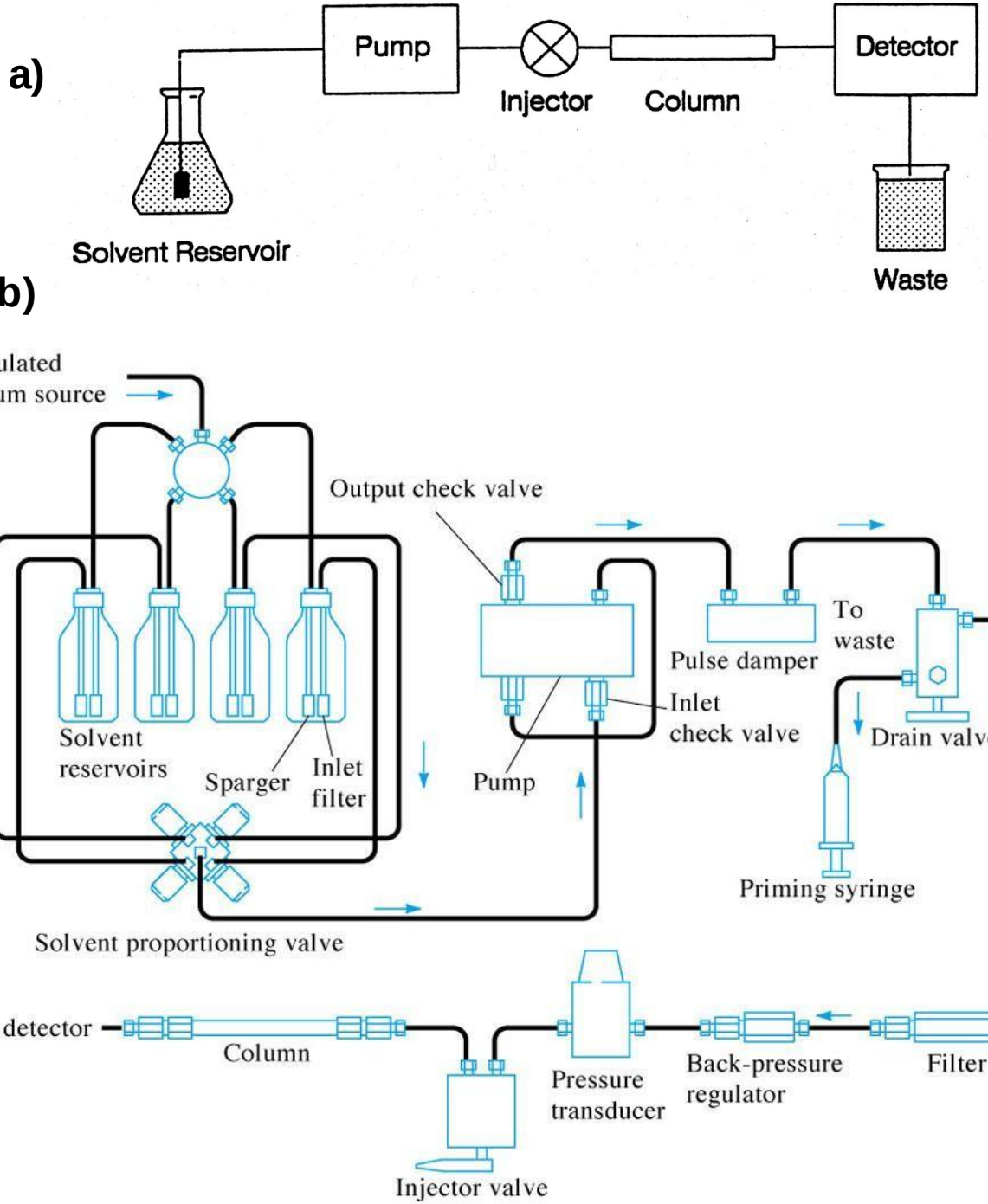


A typical HPLC system:

- Higher operating pressures need for mobile phase delivery requires special pumps and other system components

- Sample applied using closed system (*i.e.*, injection valve)

- detection uses a flow through detector



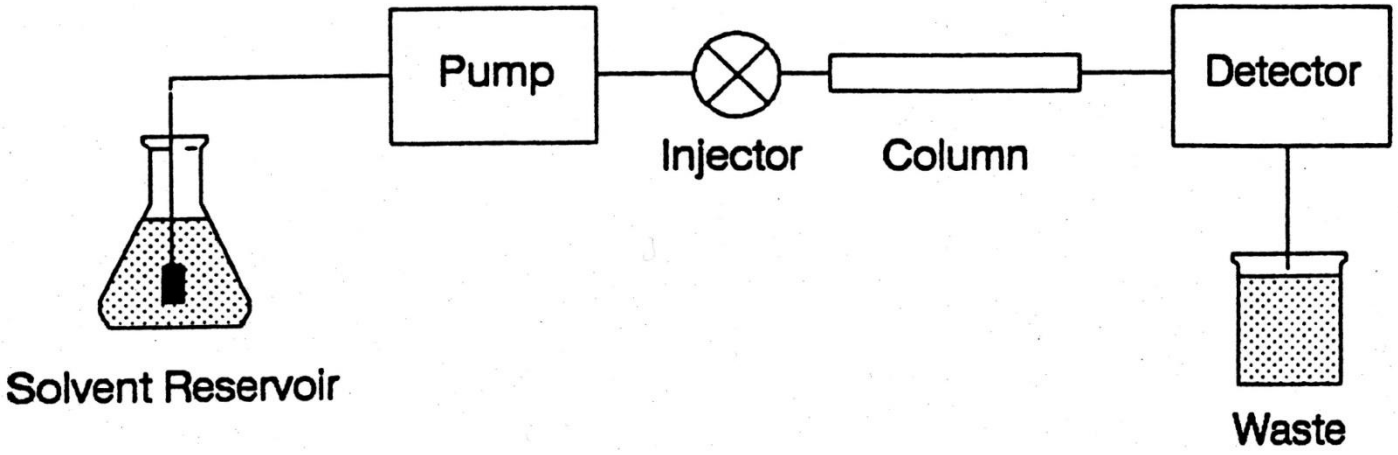
High-performance liquid chromatography

advantages:

– fast analysis time
– ease of automation
– good limits of detection
– preferred choice for analytical applications
– popular for purification work

disadvantages:

– greater expense
– lower sample capacities



C.) Elution:

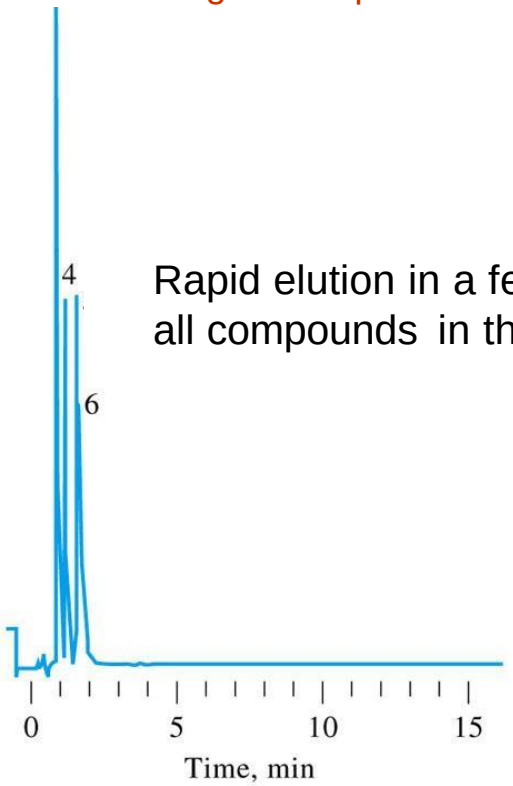
Retention and elution of solutes in LC depends on the interactions of solutes with <u>both</u> the mobile and stationary phases.

- to describe how well solutes are retained on a column with different solvents, the terms *weak mobile phase* and *strong mobile phase* are used.

<u>Strong mobile phase</u>: a solvent that quickly elutes solutes from the column (*i.e.*, small k')

This occurs if the mobile phase is very similar to the stationary phase in its intermolecular interactions with the solutes

- polar solvent would be a strong mobile phase for a column containing a polar stationary phase



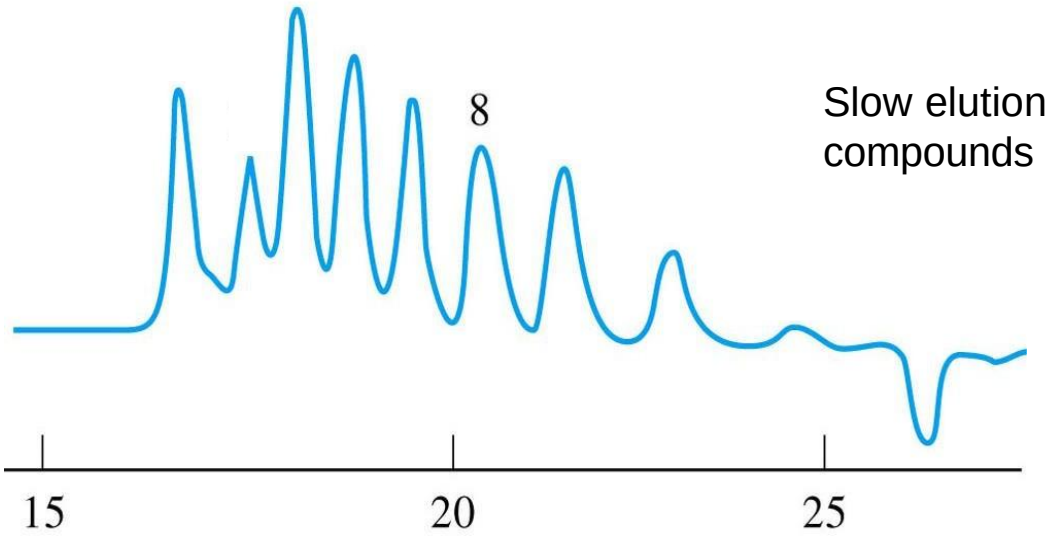
Rapid elution in a few minutes for all compounds in the mixture

C.) Elution:

Weak mobile phase: a solvent that slowly elutes solutes from the column (*i.e.*, high solute retention or large k')

This occurs if the mobile phase is very different from the stationary phase in its intermolecular interactions with the solutes

- a non-polar solvent would be a weak mobile phase for a column containing a polar stationary phase



Slow elution (~ 20 minutes) for all compounds in the mixture

Note: whether a solvent is a weak or strong mobile phase depends on the stationary phase being used. Hexane is a weak mobile phase on a polar stationary phase, but a strong mobile phase on a non-polar stationary phase.

Similar to GC, solutes can be eluted from a column by using either a constant column conditions or gradient elution

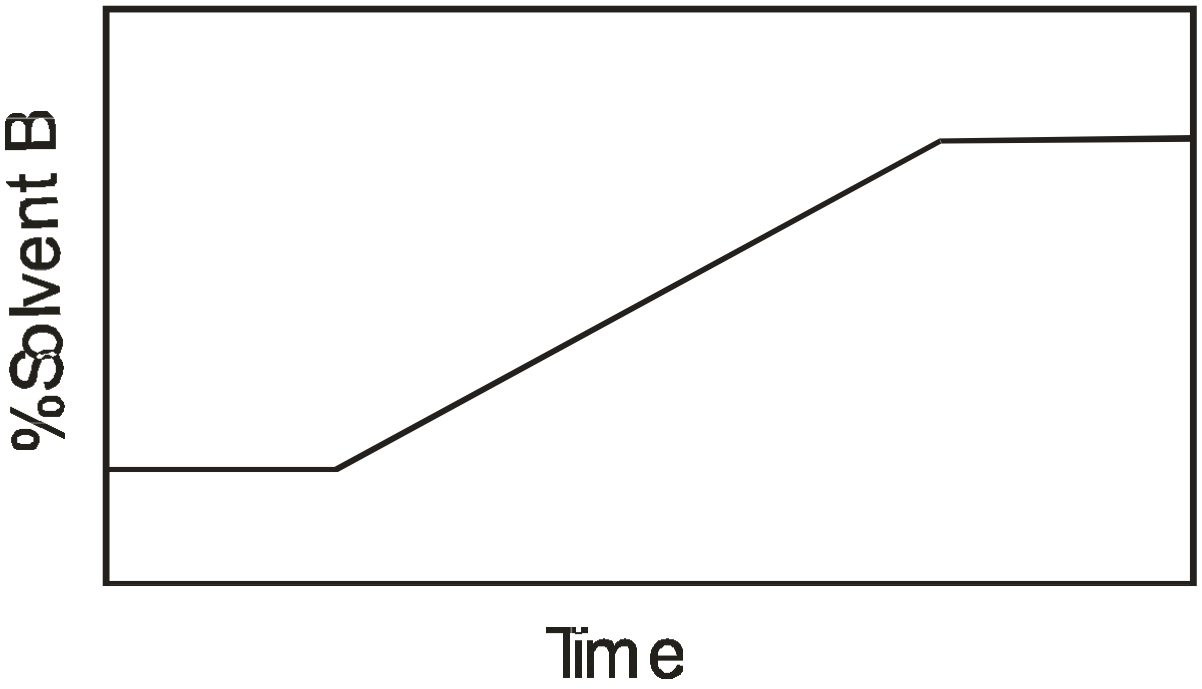
Gradient elution: changing composition of mobile phase with time → *solvent programming*

, going from a weak mobile phase to a strong one.

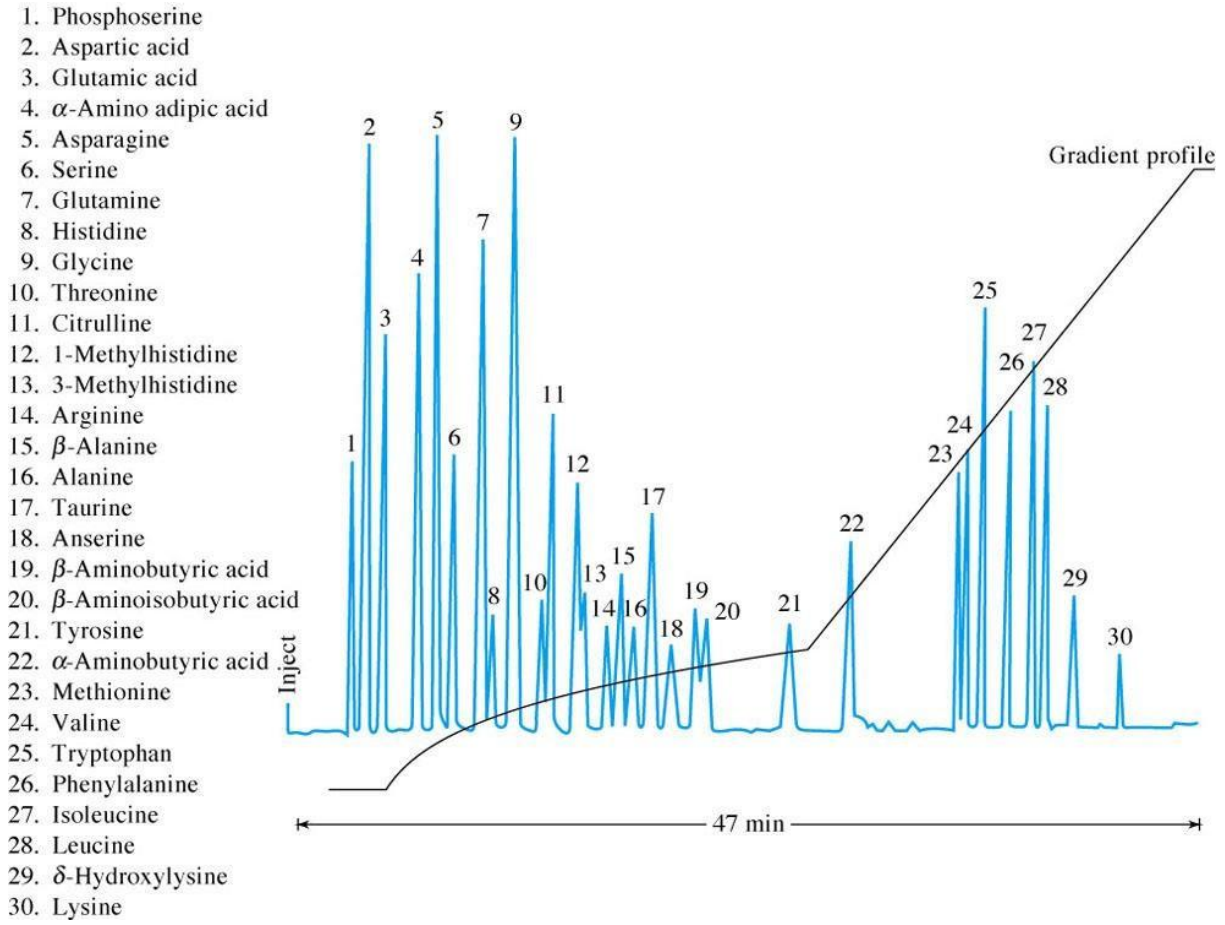
, weak mobile phase → *solvent A*

, strong mobile phase → *solvent B*

, solvent change can be stepwise, linear or non-linear



Gradient elution of mixture of 30 amino-acids

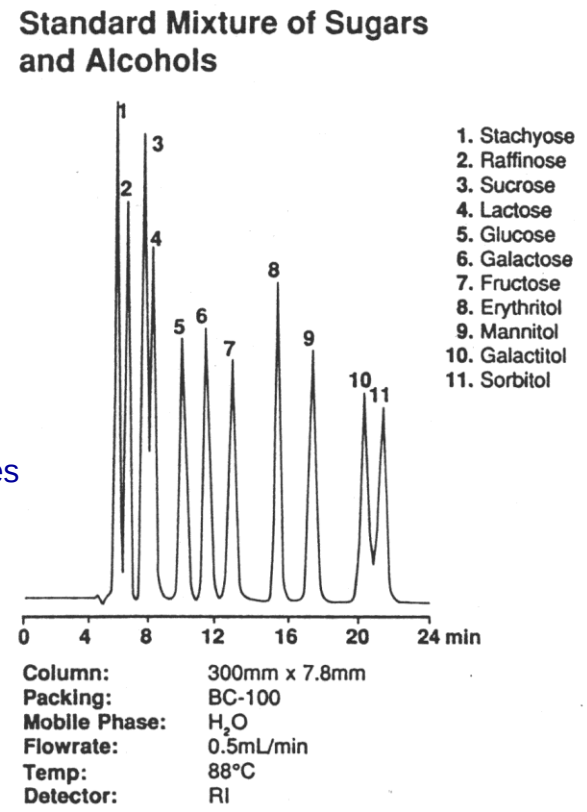


In choosing a mobile phase for LC, several factors need to be considered

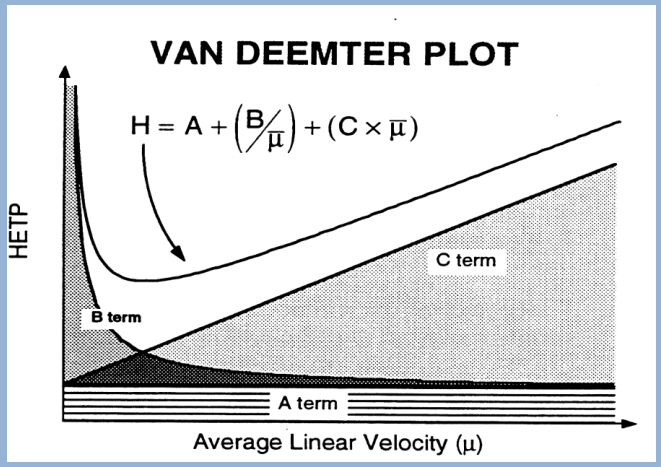
– type of stationary phase used
, determines what will be a strong or weak mobile phase
– solubility of the solutes
– viscosity of the mobile phase
– type of detector used and solvent's background signal
– purity of the solvents
– miscibility of the solvents (for gradient elution)

High-performance liquid chromatography (HPLC)

– LC methods that use small, uniform, rigid support material
  , particles < 40 μm in diameter
  , usually 3-10 μm in practice
– good system efficiencies and small plate heights
– such systems have the following characteristics:
  , narrow peaks
  , low limits of detection
  , short separation times
  , columns can only tolerate high operating pressures and faster flow-rates



Van Deemter equation: relates flow-rate or linear velocity to H:



$H = A + B/\mu + C\mu$ where:

μ = linear velocity (flow-rate x V_m/L) H = total plate height of the column
A = constant representing eddy diffusion & mobile phase mass transfer
B = constant representing longitudinal diffusion
C = constant representing stagnant mobile phase & stationary phase mass transfer

Plate height (H) may be used to relate these kinetic processes to band broadening to a parameter of the chromatographic system (e.g., flow rate). This relationship is used to predict the resulting effect of varying this parameter on the overall efficiency of the chromatographic system.

Number of theoretical plates(N) $(N) = 5.54\ (t_R/W_h)^2$ *peak width (W_h)*

$H = L/N$

μ optimum

Optimum linear velocity (μ_{opt}) - where H has a minimum value and the point of maximum column efficiency:
μ_{opt} is easy to achieve for gas chromatography, but is usually too small for liquid chromatography requiring flow-rates higher than optimal to separate compounds

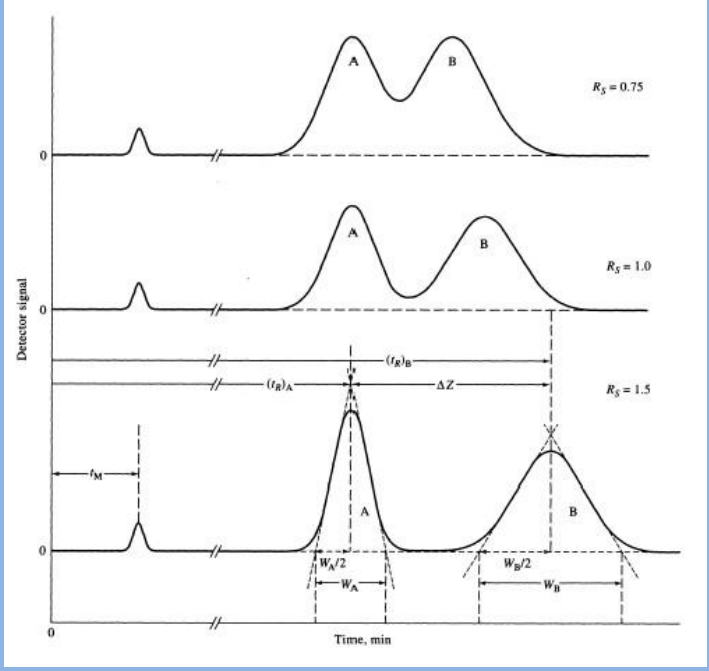
resolution (R_S) – resolution between two peaks is a second measure of how well two peaks are separated:

$$R_S = \frac{t_{r2} - t_{r1}}{(W_{b2} + W_{b1})/2}$$

where:

t_{r1}, W_{b1} = retention time and baseline width for the first eluting peak

t_{r2}, W_{b2} = retention time and baseline width for the second eluting peak



R_s over 1.5 represents *baseline resolution*, or complete separation of two neighboring solutes → ideal case.

R_s over 1.0 considered adequate for most separations.

D.) Types of Liquid Chromatography:

Techniques in LC are classified according to the method of solute separation

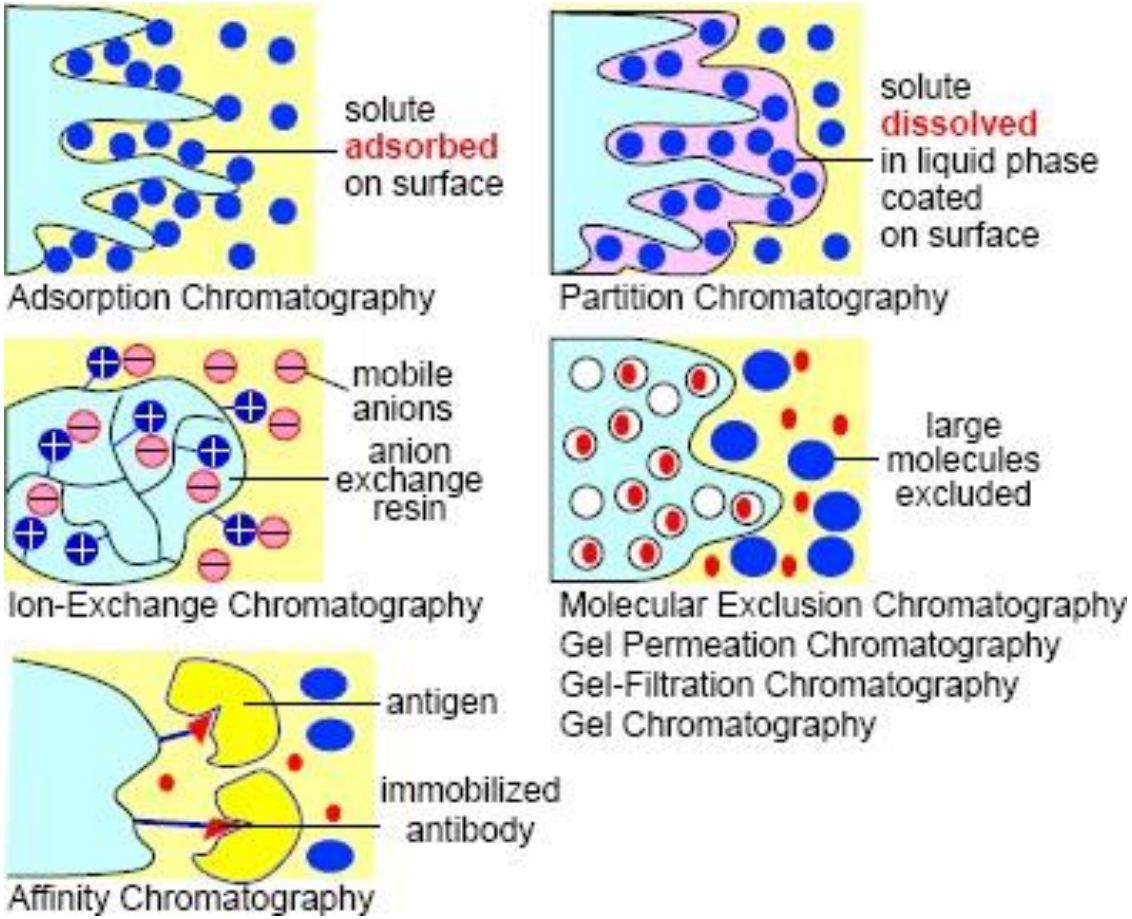
, Adsorption chromatography

, Partition chromatography

,Ion-exchange chromatography

, Affinity chromatography

, Size-exclusion chromatography



E.) LC Detectors:

Common types of LC Detectors

, Refractive Index Detector, Conductivity Detector

UV/Vis Absorbance Detector, Electrochemical Detector

, Fluorescence Detector

As in GC, the choice of detector will depend on the analyte and how the LC method is being used (*i.e.*, analytical or preparative scale)

Detector	Selectivity	Sensitivity	Notes
Refractive Index	**Poor**	**Poor**	Any component that differs in refractive index from the eluate can be detected, despite its low sensitivity. Cannot be used to perform gradient analysis.
UV/Vis	**Moderate**	**Good**	A wide variety of substances can be detected that absorb light from 190 to 900 nm. Sensitivity depends strongly on the component.
Fluorescence	**Good**	**Excellent**	Components emitting fluorescence can be detected selectively with high sensitivity. This is often used for pre-column and post-column derivatization.
Conductivity	**Moderate**	**Good**	Ionized components are detected. This detector is used mainly for ion chromatography.
Electrochemical	**Good**	**Excellent**	Electric currents are detected that are generated by electric oxidation-reduction reactions. Electrically active components are detected with high sensitivity.

2.) UV/Vis Absorbance Detector

Measures the ability of solutes to absorb light at a particular wavelength(s) in the ultraviolet (UV) or visible (Vis) wavelength range.

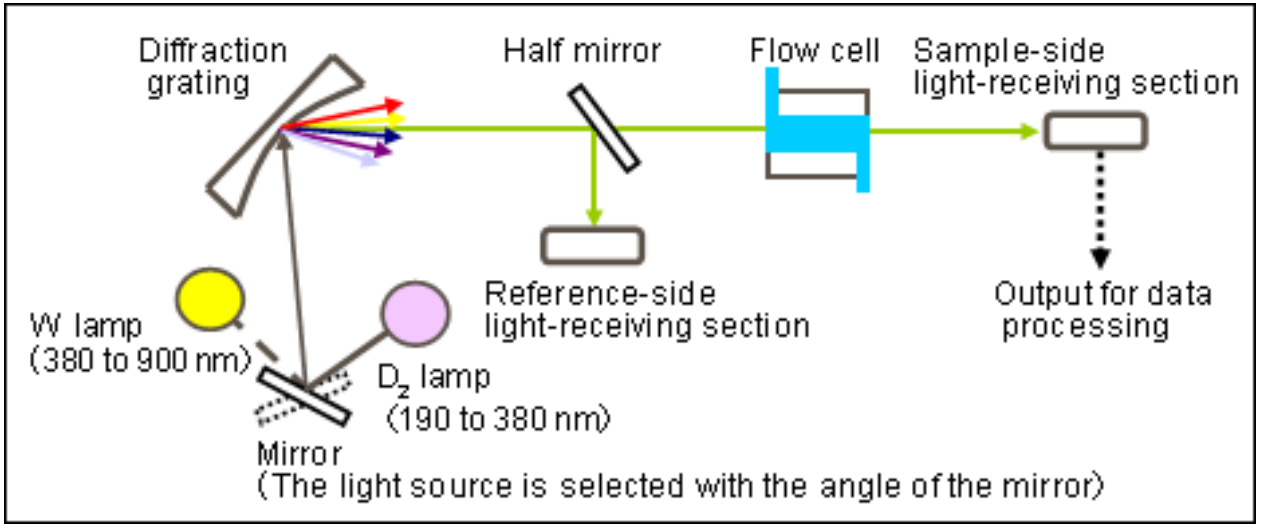
, most common type of LC detector

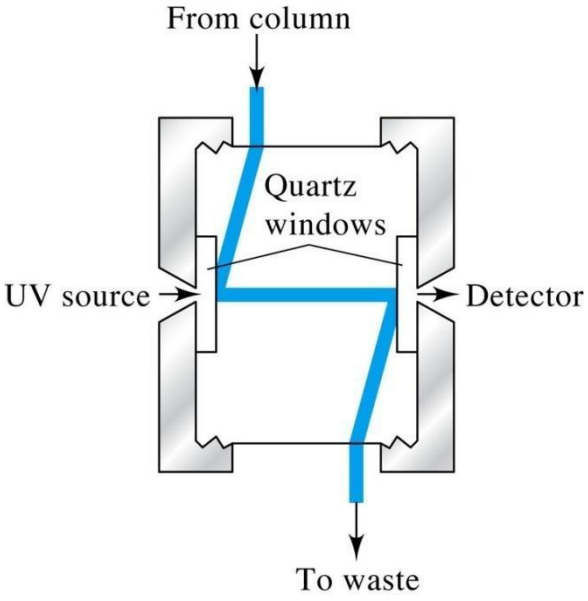
Three Common types of UV/Vis Absorbance Detectors

, Fixed wavelength detectors

, Variable wavelength detectors

, Photodiode array detectors





2.) UV/Vis Absorbance Detector

Fixed Wavelength Detector absorbance of only one given wavelength is monitored by the system at all times (usually 254 nm)

, simplest and cheapest of the UV/Vis detectors

, limited in flexibility

, limited in types of compounds that can be monitored

Variable Wavelength Detector a single wavelength is monitored at any given time, but any wavelength in a wide spectral range can be selected

, wavelengths vary from 190-900 nm.

, more expensive, requires more advanced optics

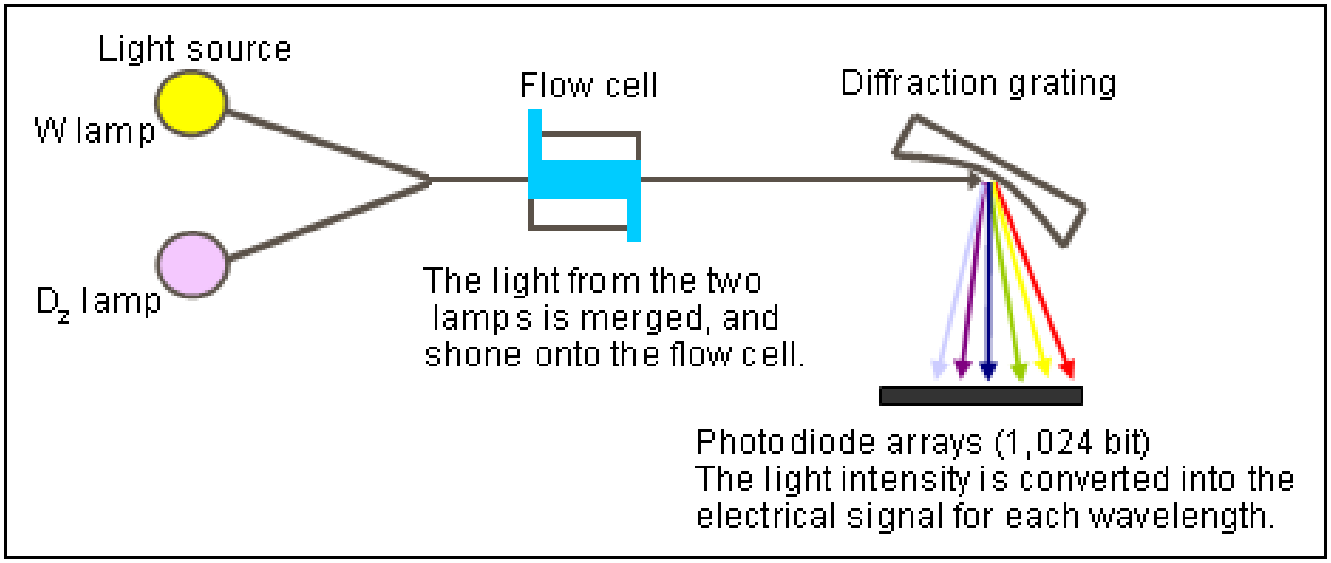
, more versatile, used for a wider range of compounds

Photo Diode Array Detector operates by *simultaneously* monitoring absorbance of solutes at several different wavelengths.

, uses a series or an array of several detector cells within the instrument, with each responding to changes in absorbance at different wavelengths.

, entire spectrum of a compound can be taken in a minimum amount of time

, useful in detecting the presence of poorly resolved peaks or peak contaminants

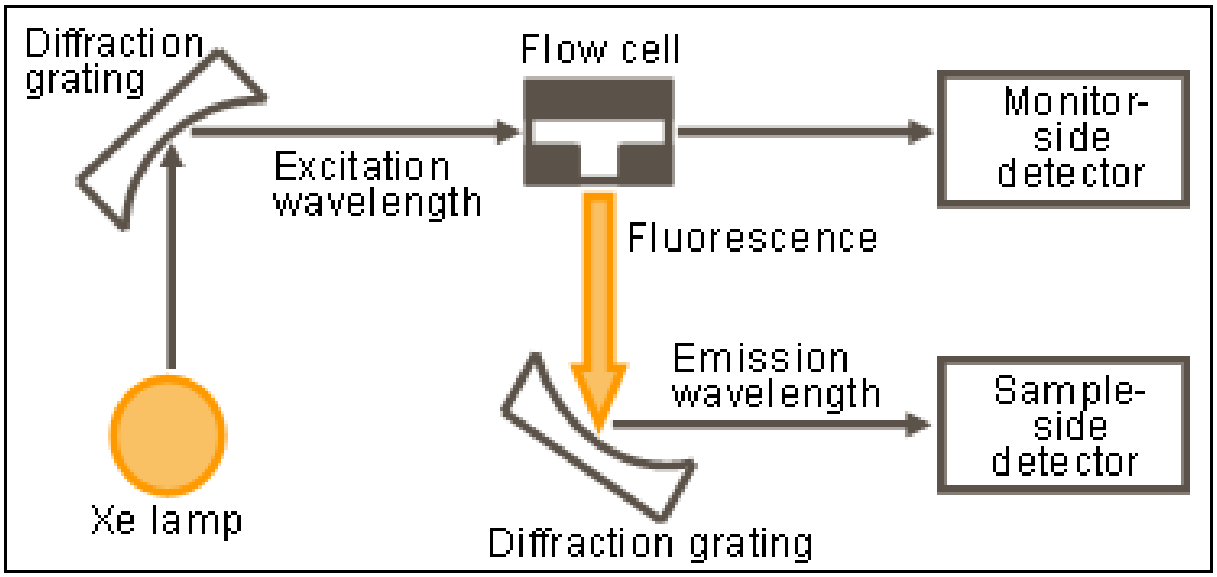


Applications:

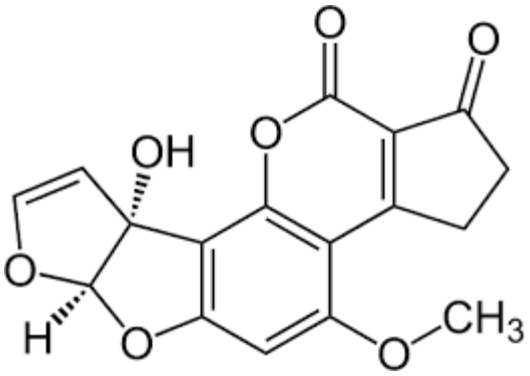
- UV/Vis absorbance detectors can be used to detect any compound that absorbs at the wavelength being monitored
- Common wavelengths:
  - , 254 nm for unsaturated organic compounds
  - , 260 nm for nucleic acids
  - , 280 or 215 nm for proteins or peptides
- Absorbance detectors can be used with gradient elution
  - , wavelength being monitored is above the cutoff range of the solvents being used in the mobile phase
- limits of detection for fixed and variable UV/Vis absorbance detectors are ~ 10^{-8} M
- limits of detection for photodiode array detectors are ~ 10^{-7} M

3.) Fluorescence Detector

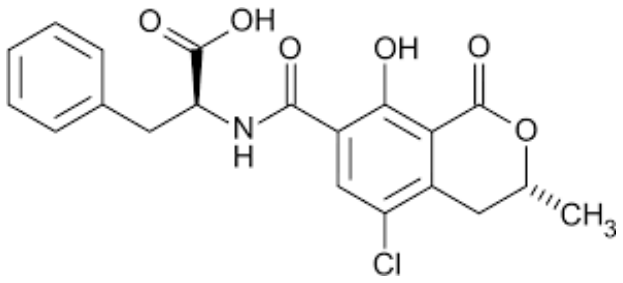
A selective LC detector that measures the ability of eluting solutes to fluoresce at a given set of excitation and emission wavelengths



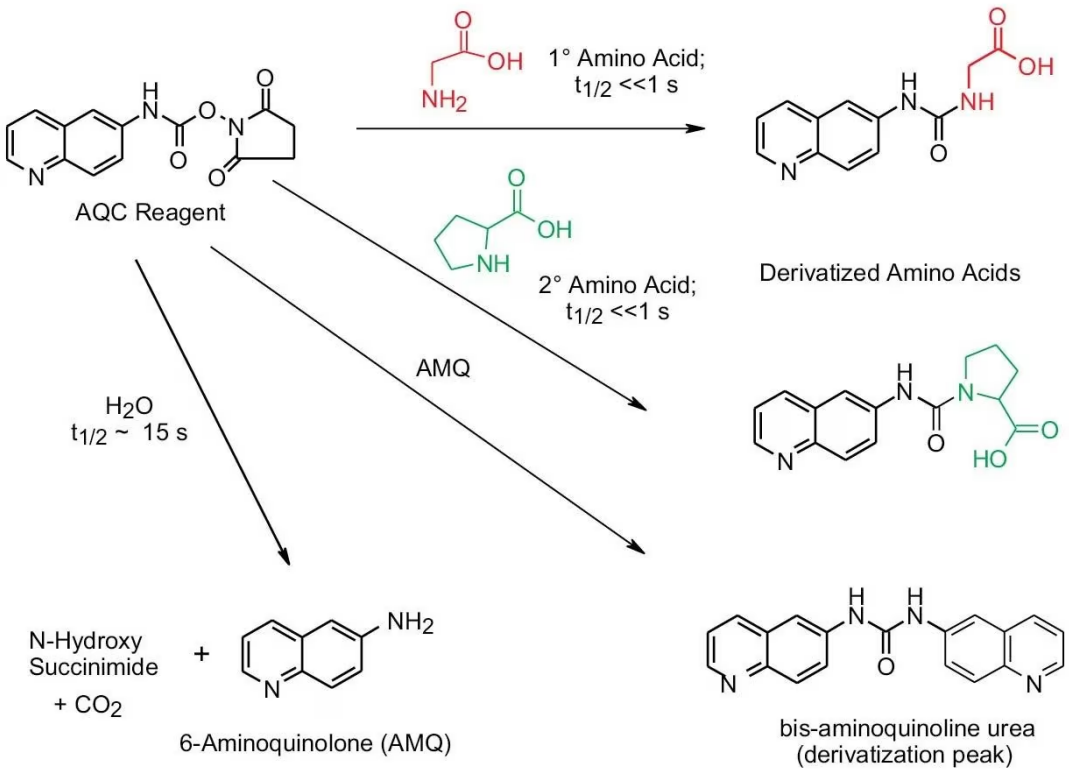
Application in food; Mycotoxins (aflatoxins, ochratoxin A etc…), derivatised compounds (e.g. aminoacids)



Aflatoxin M1



Ochratoxin A


AQC Reagent
1° Amino Acid;
$t_{1/2}$ <<1 s
2° Amino Acid;
$t_{1/2}$ <<1 s
Derivatized Amino Acids
AMQ
H_2O
$t_{1/2}$ ~ 15 s
N-Hydroxy
Succinimide
+ CO_2
+
6-Aminoquinolone (AMQ)
bis-aminoquinoline urea
(derivatization peak)

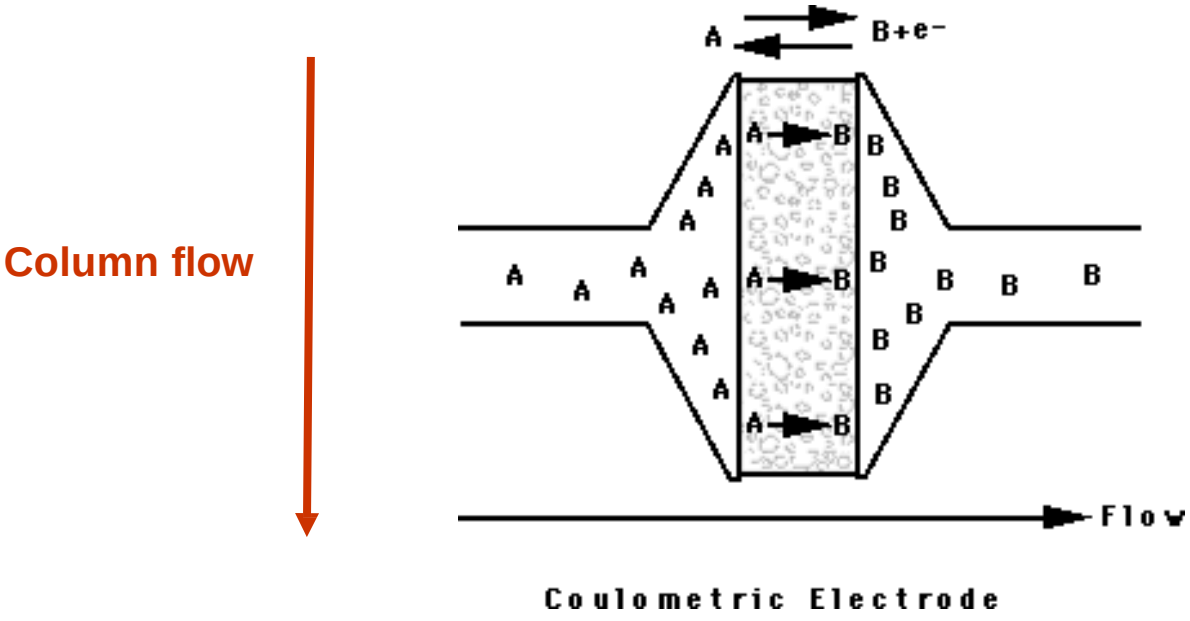
5.) Electrochemical Detector

Used to monitor any compound in the mobile phase that can undergo an oxidation or reduction

, electrochemical detection in liquid chromatography is sometimes referred to as LC/EC

, generally includes two or more electrodes which monitor the current that is produced by the oxidation or reduction of eluting compounds at a fixed potential

, generally electrical output is an electron flow generated by a reaction that takes place at the surface of the electrodes.



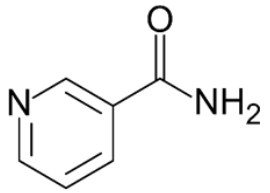
Application in food; sugars, any compound that can be reduced/oxidised (not polyphenols!)

Vitamin B3

- Niacin, also known as vitamin B3, is one of the essential human nutrients. Vitamin B3 comprises nicotinic acid, nicotinamide, and numerous enzymatic forms.
- Nicotinamide is a precursor of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), two redox coenzymes primarily involved in the production of energy from dietary proteins, carbohydrates, and fats. They play a key role in several biochemical paths like Krebs' cycle or glycolysis.
- In meat the niacin content is mainly present in the form of nicotinamide: while in living tissues nicotinamide is a component of the coenzymes NAD and NADP, in meat it is found also in the free form because of postmortem hydrolysis of NAD.
- Chicken meat is a really good source of vitamin B3. In particular white meat (breast e.g.) contains a higher amount of niacinamide with the respect of dark meat cuts (thigh and drumstick e.g.).

Determination of vitamin B3 (niacinamide) in poultry meat samples

	t 0gg – 3gg - 7gg 1 5gg		
	MAP	SKIN	STRETCH
Drumstick	8-12* conf	8-12* conf	8-12* conf
Breast	8-12* conf	8-12* conf	8-12* conf
Thigh	8-12* conf	8-12* conf	8-12* conf



Different cuts

Breast

Thigh

Drumstick

Extraction procedure

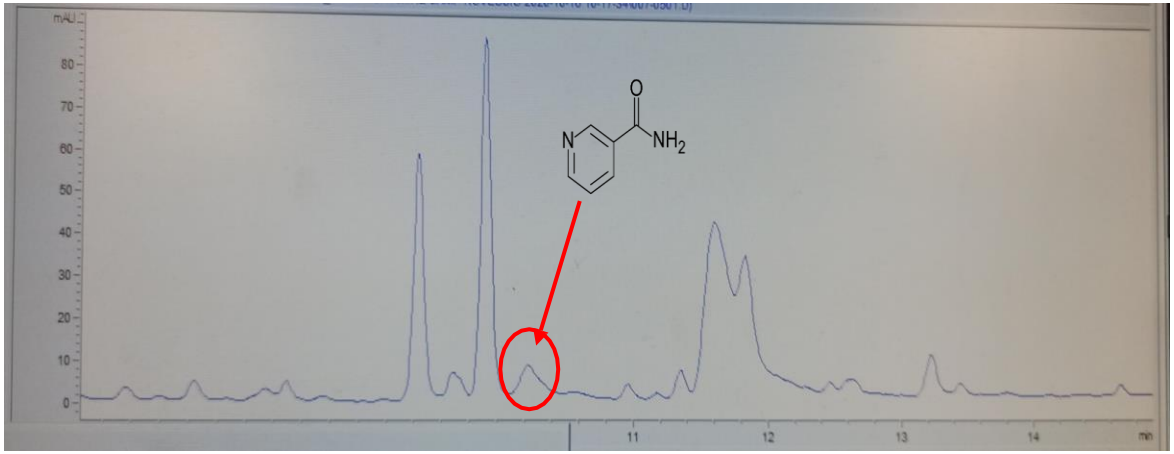
- 0.5 g of samples dissolved in 4.5 mL of HCl 0.1 M
- Homogenization with ultra turrax (1 min)
- Heat treatment (100 °C, 1 h)
- pH correction to 4-4.5, using solution of Sodium Acetate 2.5 M
- Addition of 500 uL of taka-diastase (hydrolysis of phosphorylated forms) 10 % (w/v) solution
- Incubation at 48 °C, 3 h
- Centrifugation at 10000 g, 10 min, 4°C
- Recovery of the supernatant, taken up to volume to 5 mL
- 500 uL of sample in HPLC vial

Ultra turrax homogenizer

Chromatographic method

- Instrument: HPLC Perkin Elmer sieres 200 equipped with autosampler, pump and Uv-vis detector.
- Column:C18 Luna Omega Polar 3 um 4.6x150.
- Mobile Phases
  - A: ammonium acetate 0.01 M; methanol 0.01 %
  - B: methanol
- Gradient: from 100 % to 30 % in 30 min
- Wavelength: 260 nm

HPLC system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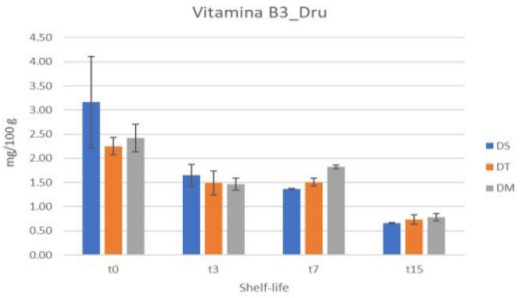
Vitamina B3_Dru
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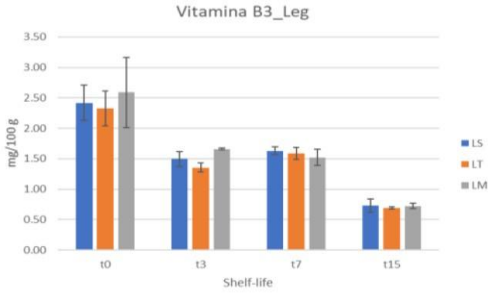
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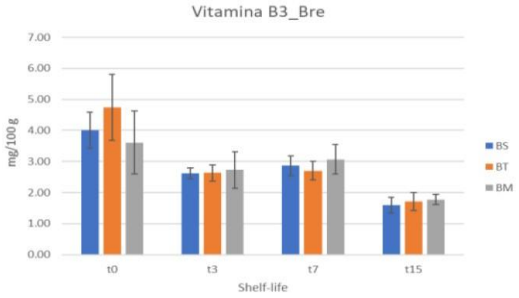
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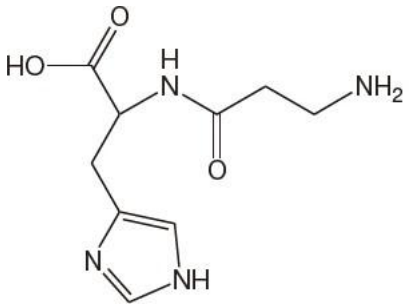
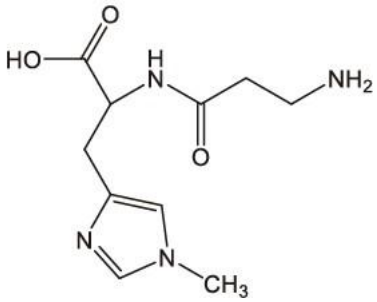
ANSERINE



CARNOSINE



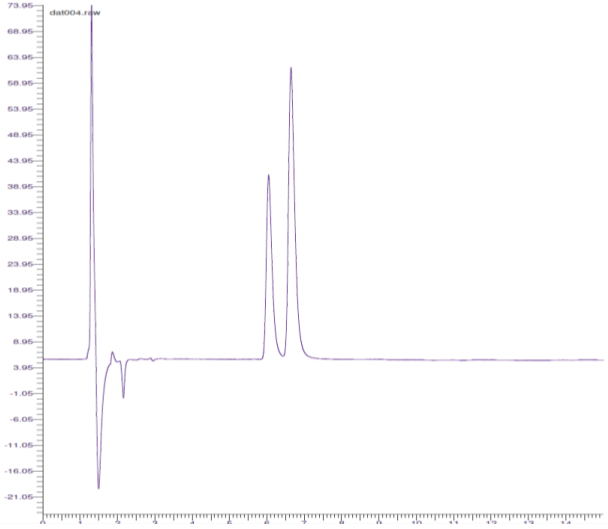
DIPEPTIDES PRESENT IN POULTRY MEAT IMPORTANT FOR

- ANTIOXIDANT ACTIVITY
- ANTI-AGING ACTIVITY
- BUFFER CAPACITY
- ANTI TUMORAL AND ANTIINFLAMMATORY ACTIVITY

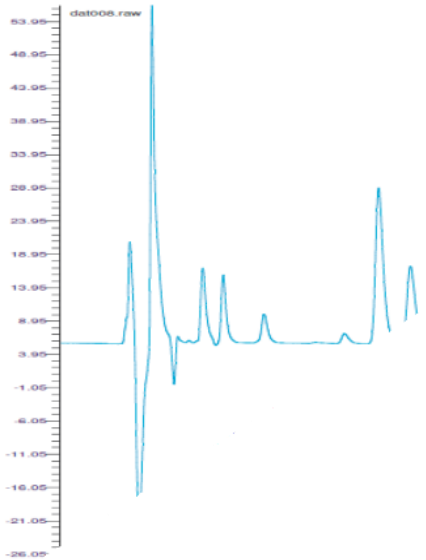
Sample	Anserine mg/100g	Carnosine mg/100g	C/A	Reference
Breast extract		71.8		Intarapichet and Maikhunthod 2005
Thigh extract		10.4		
Chicken- turkey burger 25-75	287.9	66.6	0.23	Gil-Agustì et al., 2008
20 wk breast	336.7	71.2	0.21	Kim et al., 2012
20 wk drumstick	116.5	39.5	0.34	
20 wk wing	259.4	38.4	0.15	
90 wk breast	291.5	55.3	0.19	
90 wk drumstick	69.3	14.2	0.20	
90 wk wing	226.4	35.9	0.16	
Breast	1170	700	0.60	Peiretti et al., 2011
Breast	1670	920	0.55	
Breast	1460	760	0.52	
Breast	710	255	0.36	Mori et al., 2015
Leg	220	106	0.48	
Fillet	608	157	0.26	
Breast (Conv)	92.6	63.16	0.68	Kim et al., 2020
Breast (Welfare)	117.54	65.30	0.56	

After elimination of fat and connective tissue 0.5±0,05 g treated with trichloroacetic acid (0.07%) and homogeneised. After centrifugation at 4000 rpm (15 min) 2 mL of supernantant then and centrifuged again (10000 rpm 15 min). Dilution 1:20 mobile phase 20 uL injected in HPLC.

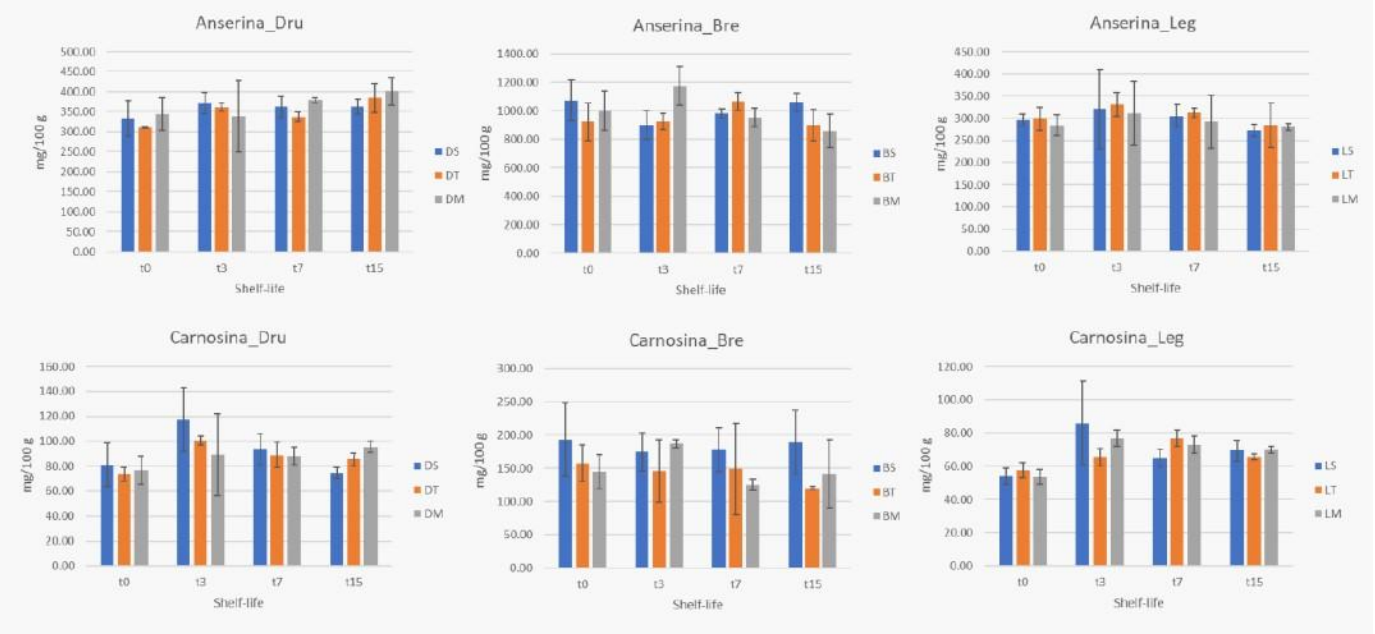
Column Agilent Zorbax Bonus RP 3.5µm, 4.6x150mm (densely packed diisopropyl-C14 groups). Mobile phase is 10 mM phosphate buffer in 5% acetonitrile and 5 mM octansulfonic acid **(octanesulfonate is an ion pairing reagent that is used in HPLC, notably in the analysis of small organic compounds. The anionic sulfonate counterion permits the separation and resolution of positively charged analytes).** 15 min isocratic at 1 mL/min flow rate, UV detector at 224 nm.



Standards



Sample



Changes are significant and different depending on the type of cut and packaging, may be possible markers of oxidation , to be investigated……

Application in food matrices:

Chemical characterization of Polyphenols in different extracts of :

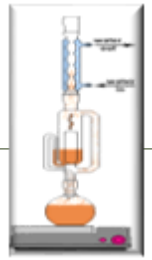
***Coriandrum sativum*L.**
(Apiaceae)

***Thymus vulgaris* L.**
(Lamiaceae)

***Cannabis sativa* L.**
(Cannabacee)

Extraction Techniques

(UAE)
Ultrasound Assisted Extraction



Soxhlet

(RSLDE)
Rapid Solid-Liquid Dynamic Extraction
(Naviglio extractor)

Maceration

Extractionparameters

Solvent: Ethanol
Extraction Time:

- 30 days for Maceration
- 20 minutes for Ultrasounds
- 2 hours and 6 hours for RSLDE and Soxhlet

The weight of the matrix subjected to extraction was calculated maintaining the same ratio for the different extraction techniques

HPLC-UV Analysis parameters

HPLC Perkin-Elmer series 200
Phase A (Inorganic): 1% acetic acid in water
Phase B (Organic): acetonitrile
Column:Kinetex C18 (dimensions: 250 x 4.6 mm, particle size: 5 µm, pore size: 110 Å)

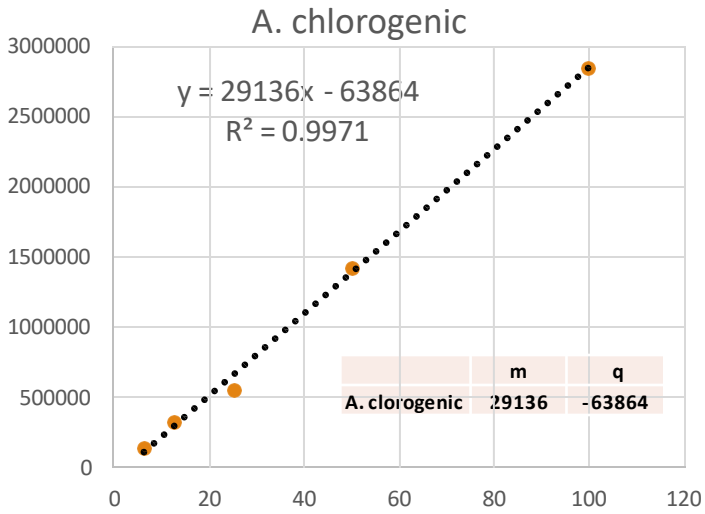
UV-Vis detector Perkin Elmer LC 240 set at 280 nm

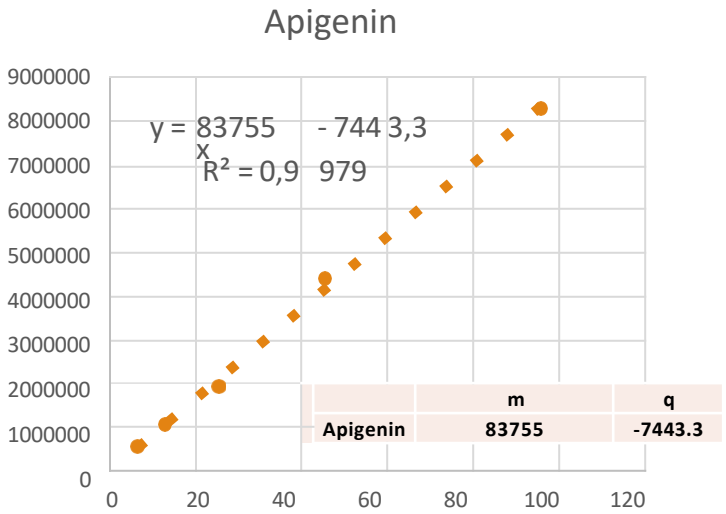
Polyphenols analyzed

The polyphenols chosen for the analysis are: gallic acid, p-OH benzoic acid, apigenin, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, rosmarinic at start. The working standard mixtures were prepared by appropriate dilution of the standards in methanol at concentration of 100 ppm

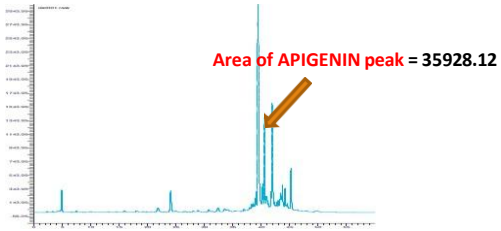
Quantitative Analysis

The quantification for each analyte was performed by means of a calibration line, starting from the mix of polyphenols at 100 ppm, a 6-point line (1-6.25-12.5-25-50-100 ppm)

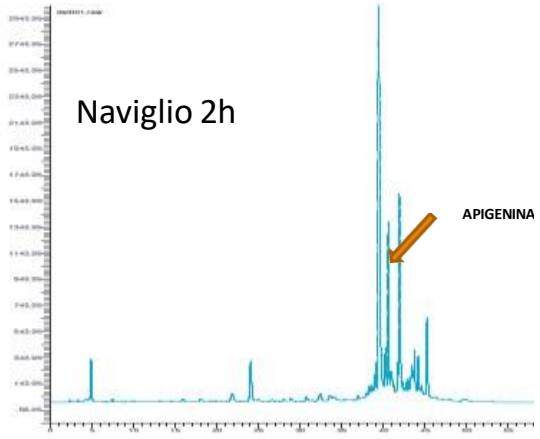


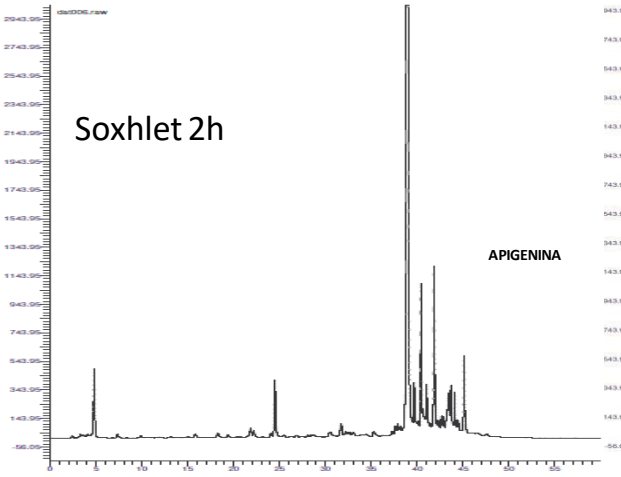


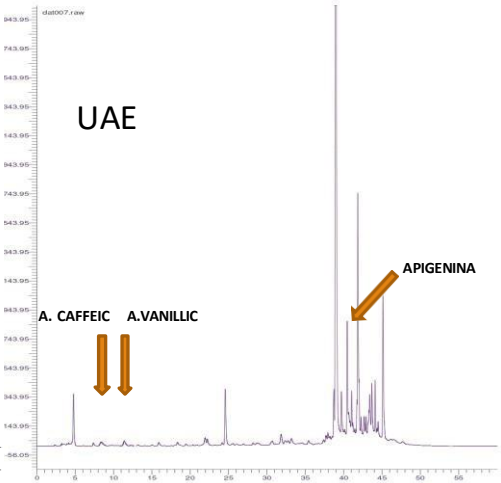
The concentration of the analyte present in the sample is calculated through the calibration line:
(Area of my peak - q) / m
The result are expressed in ppm .

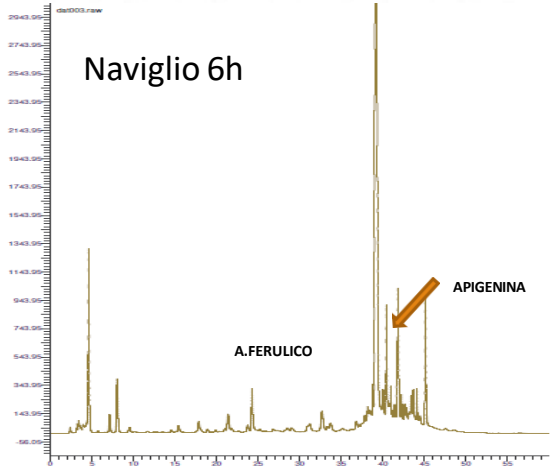


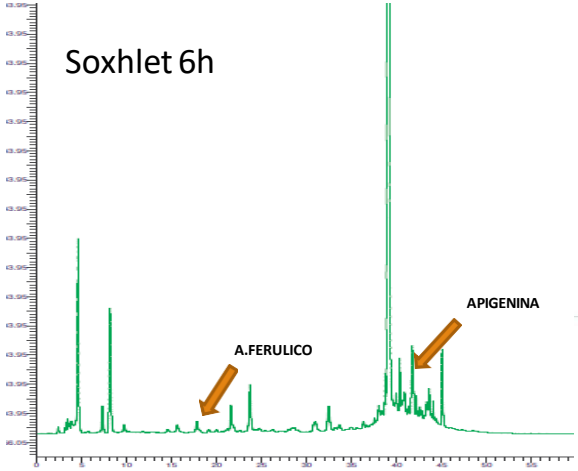
Chromatograms of Thymus extracts:

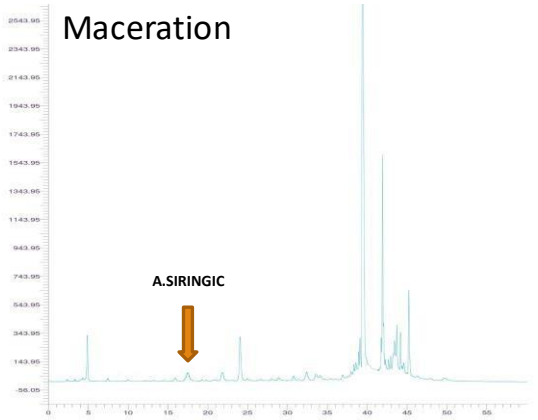






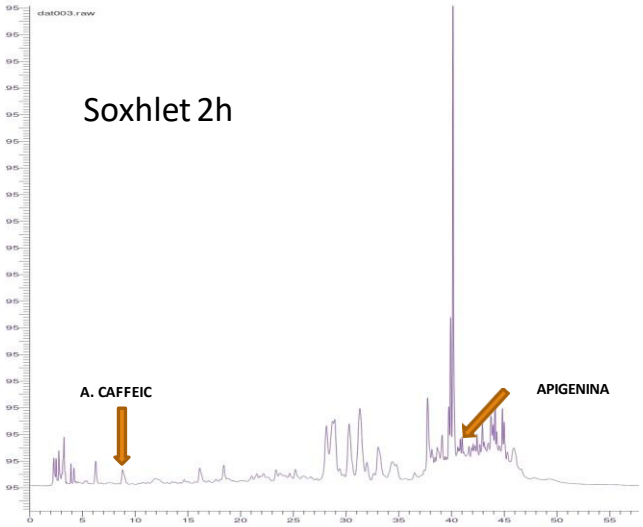


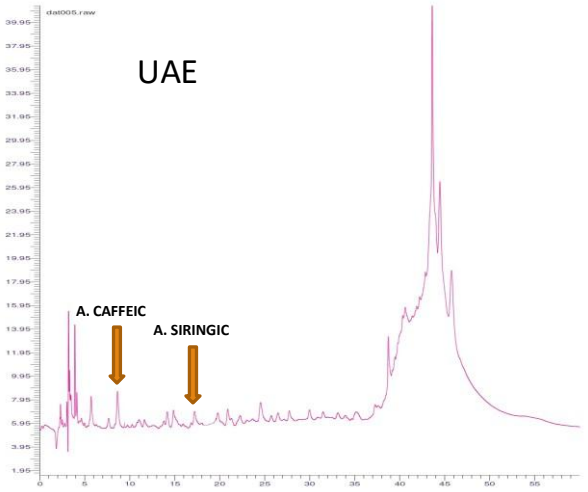


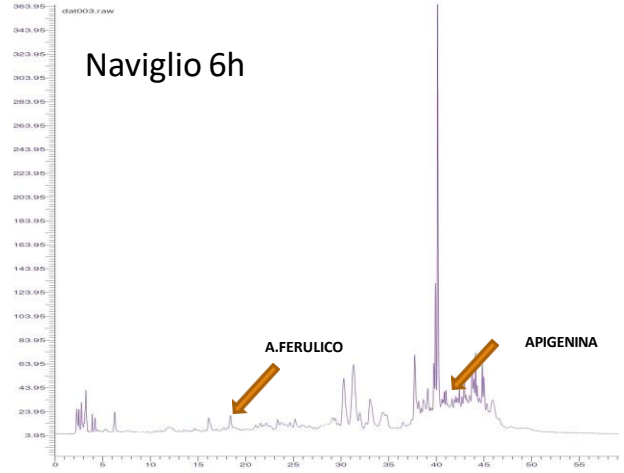


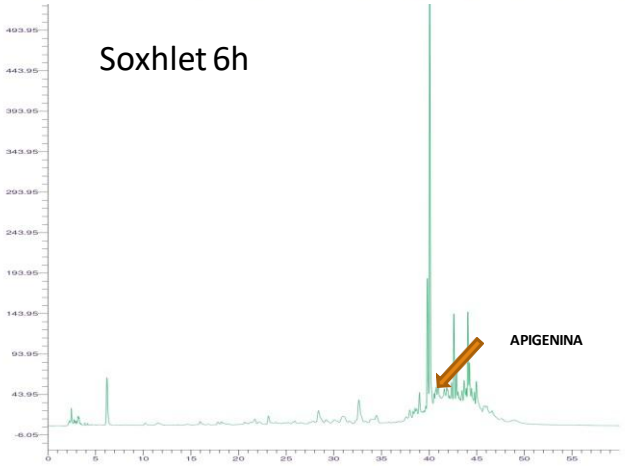
	Thyme					
	N 2 h	**N 6 h**	**S 2 h**	**S 6 h**	**UAE**	**M**
Gallic acid	**42.74±0.50 d**	**58.73±0.90 b**	678.67±0.40 a	48.78±0.32 c	-	40.23±0.89 e
p-OH-benzoic acid	**190.47±1.02 b**	**197.15±0.89 a**	-	148.68±0.98 c	55.04±0.95 d	17.97±1.02 e
Chlorogenic acid	**66.54±0.96 c**	**120.44±0.75 a**	30.07±0.89 d	97.21±0.98 b	-	-
Vanillic acid	**-**	**-**	-	-	376.27±0.56 a	-
Caffeic acid	**-**	**-**	-	-	145.92±0.85 a	-
Syringic acid	**-**	**-**	-	-	-	159.69±1.02 a
Ferulic acid	**-**	**139.24±0.96b**	-	516.02±0.84 a	-	-
Rosmarinic acid	**34201.41±1.05 b**	**33955.20±1.02 c**	51686.96±0.95 a	31549.93±1.25 d	15049.48±1.09 e	261.55±0.82 f
Luteolin	**2671.96±1.10 c**	**1554.86±1.05 f**	1931.34±0.95 d	1704.21±1.32 e	4143.43±0.65 b	2099.47±0.84 a
Apigenin	**6608.97±1.15 b**	**5309.40±1.03 c**	7618.77±0.98	2909.13±1.01 e	4416.70±0.87 d	-
Carvacrol	**3499.84±1.15 b**	**885.03±1.02 d**	2873.81±0.99 c	5595.41±1.05 a	295.41±1.05 f	220.49±0.98 e

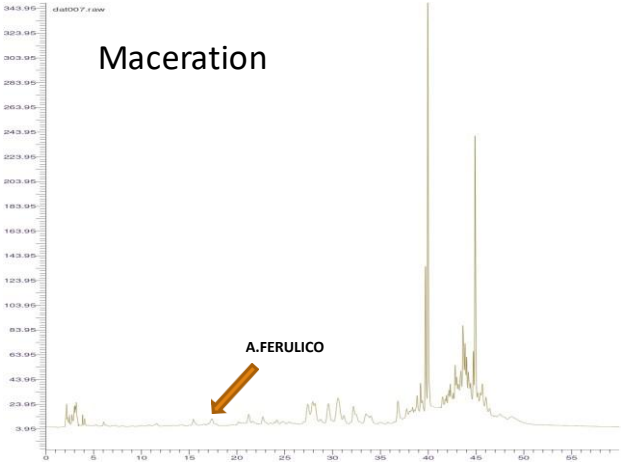
Chromatograms of Hemp extracts:





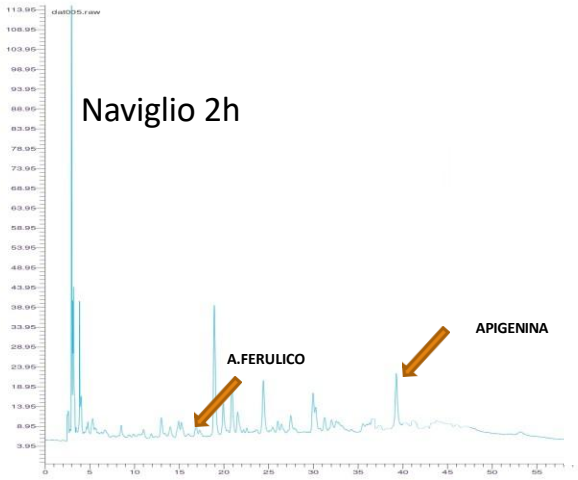


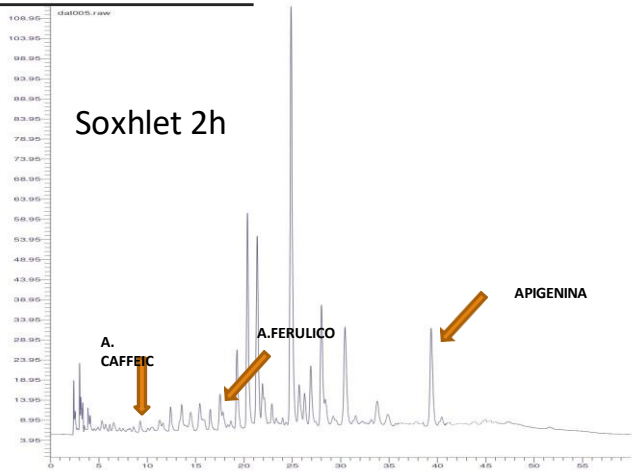


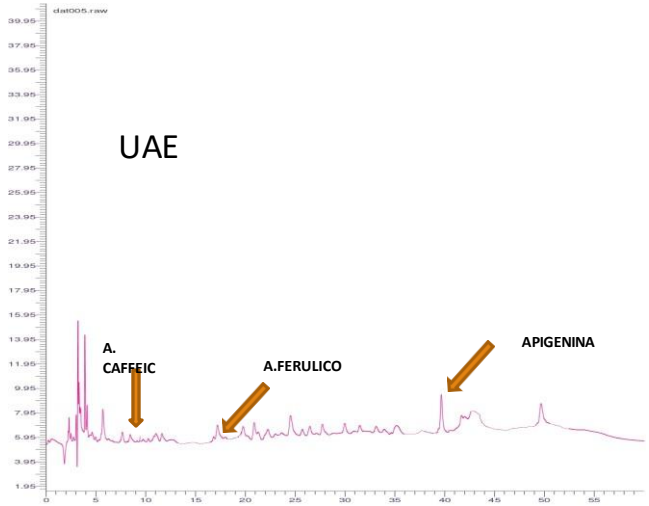


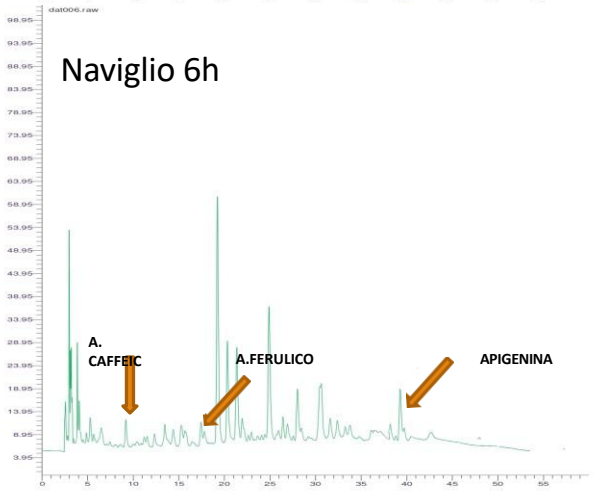
	Hemp					
	N 2 h	**N 6 h**	**S 2 h**	**S 6 h**	**UAE**	**M**
Gallic acid	-	**52.29±0.98 c**	118.07±0.32 b	408.92±0.63 a	35.10±0.98 d	36.02±0.65 d
p-OH-benzoic acid	-	**47.70±0.75 c**	95.18±0.95 a	36.42±0.35 d	-	52.41±0.96 b
Chlorogenic acid	-	**-**	-	-	-	-
Vanillic acid	-	**-**	-	-	-	-
Caffeic acid	-	**-**	36.98±0.48 b	-	81.81±0.91 a	-
Syringic acid	-	**-**	-	-	57.28±0.64 a	-
Ferulic acid	-	**247.77±0.64 a**	-	-	-	96.70±0.93 b
Rosmarinic acid	**259.56±0.97 c**	**27.09±0.85 f**	206.30±0.94 d	152.06±0.65 e	514.33±1.01 a	328.21±1.10 b
Luteolin	**1572.05±1.04 a**	**304.37±1.10 e**	502.83±0.95 d	753.01±0.84 c	1384.09±1.09 b	127.67±1.03 f
Apigenin	**72.99±1.02 a**	**51.43±0.48 c**	35.77±0.95 d	54.22±1.06 b	-	-
Carvacrol	-	-	-	-	-	-

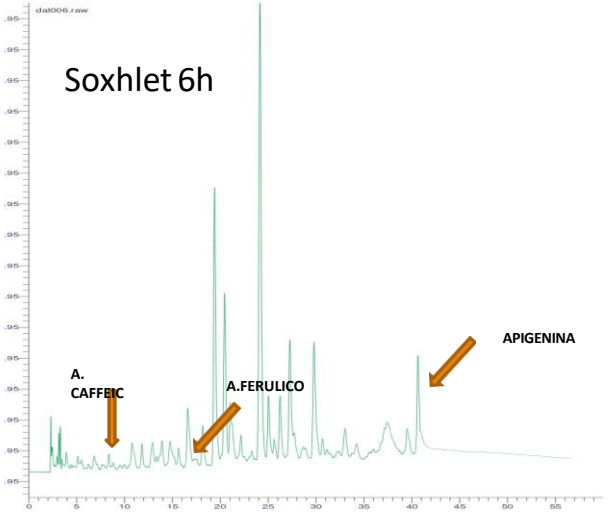
Chromatograms of Coriander extracts:

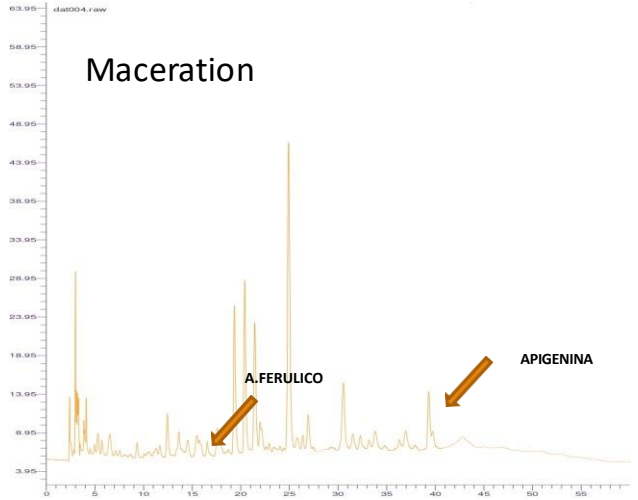












Coriander seeds						
	N 2 h	**N 6 h**	**S 2 h**	**S 6 h**	**UAE**	**M**
Gallic acid	**42.37±0.98 b**	**49.05±0.65 a**	22.45±0.35 d	22.73±0.36 d	-	31.02±0.39 c
p-OH-benzoic acid	**274.83±0.95 a**	**31.80±0.98 d**	-	89.22±0.84 b	-	46.61±0.91 c
Chlorogenic acid	**74.80±0.67 e**	**480.49±0.92 b**	149.47±0.41 c	490.56±0.35 a	146.90±0.83 d	27.82±0.94 f
Vanillic acid	**120.27±0.87 e**	**208.65±0.80 c**	440.90±1.25 a	273.23±1.65 b	205.46±0.96 d	-
Caffeic acid	**-**	**208.66±0.85 b**	440.17±0.75 a	54.21±0.65 c	27.88±0.35 d	-
Syringic acid	**-**	**-**	24.09±0.35 b	65.20±0.92 a	-	23.56±0.24 b
Ferulic acid	**188.92±0.95 b**	**78.81±0.85 c**	20.64±0.77 e	239.21±0.8 7a	22.75±0.64 d	23.37±0.81 d
Rosmarinic acid	**81.05±1.05a**	**82.13±0.97a**	34.78±0.91b	31.00±0.85c	21.97±0.98c	-
Luteolin	**324.50±0.94a**	**295.82±1.12b**	172.40±1.18c	86.90±0.98e	152.01±1.32d	-
Apigenin	**182.67±1.20a**	**182.04±1.15a**	30.09±1.06d	35.81±1.14b	101.07±1.23e	87.31±0.98c
Carvacrol	-	-	-	-	-	-

Palmieri, S., Pellegrini, M., Ricci, A., Compagnone, D., & Lo Sterzo, C. (2020). **Chemical Composition and Antioxidant Activity of Thyme, Hemp and Coriander Extracts: A Comparison Study of Maceration, Soxhlet, UAE and RSLDE Techniques.** *Foods*, *9*(9), 1221.

This polyphenols analysis is a part of a work, where these extracts have also been evaluated of volatile fraction and antioxidant activity by spectrophotometric assays *in vitro*. The aim of this work was to compare the extracts of two conventional, as maceration and Soxhlet technique, and two unconventional, the Ultrasound Assisted Extraction - UAE and the Rapid Solid-Liquid Dynamic Extraction - RSLDE performed by Naviglio Extractor®. Moreover, for the Soxhlet and RSLDE techniques, the effect of the extraction time was also investigated.

Our results, although not showing univocal trends because of the expected variability of the samples, have indicated the RSLDE technique as a very efficient method for recovering bioactive compounds from plant matrices, particularly to produce extracts retaining good antioxidant capacity. In comparison to the other techniques, has the advantages of reducing solvent and energy consumption.

Furthermore, given the limited effect of the increase in extraction time, the RSLDE technique performed by Naviglio Extractor® at 2 hours of extraction time can be considered an excellent standardized method to obtain extracts with good in vitro antioxidant activity and potential candidates as natural preservatives in food.