Estación Biológica Doñana CSIC

February 22-25, 2022

4th STABLE ISOTOPE COURSE IN ECOLOGY AND ENVIRONMENTAL SCIENCES

Daniel Carrizo, Laboratorio de biomarcadores e isótopos estables en ambientes extremos (CSIC-INTA), Madrid.

February 23, 2022

Sample preparation in CSIA (sampling, preservation and storage, processing, analytical set-up, derivatization reactions, etc.)

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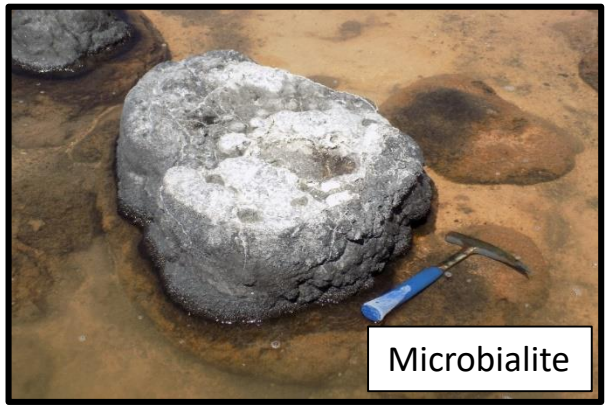
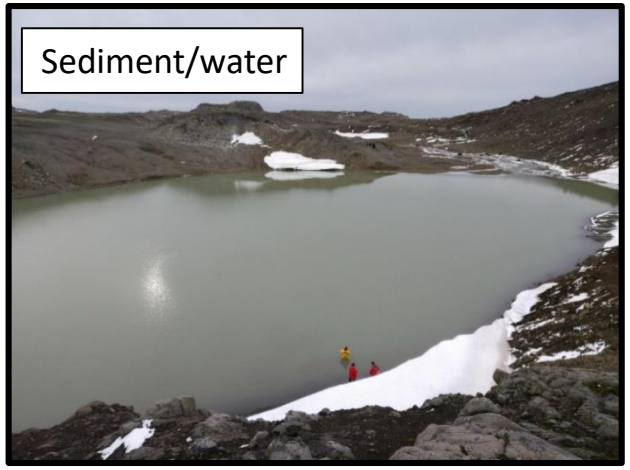
EXCELENCIA MARÍA DE MAEZTU
CENTRO DE ASTROBIOLOGÍA

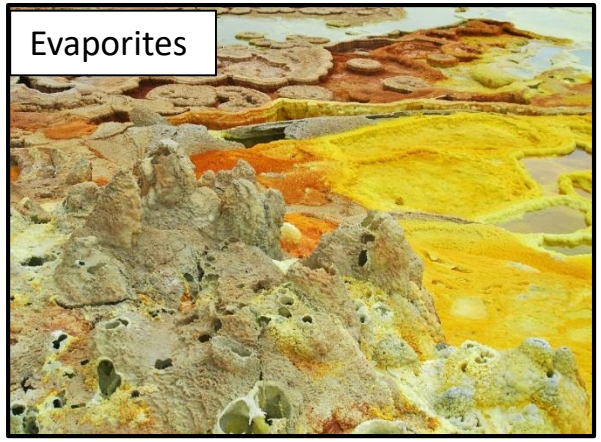
Sampling

- What we want to study?
- Type of samples (biological material, rocks, sediments, water….)
- Sample clean protocol (gloves, metal tools and glass material…)
- Sample packing and transport …

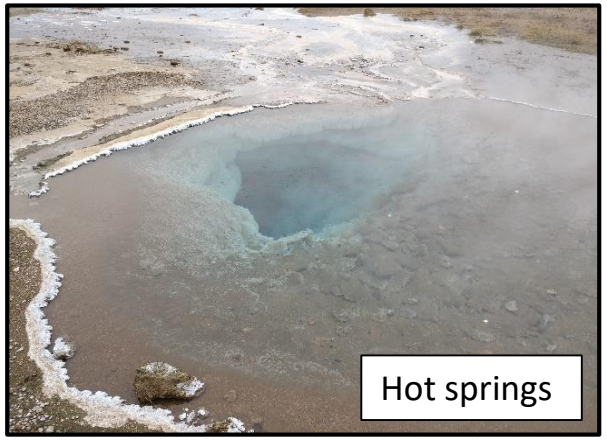
Samples…..


Microbialite

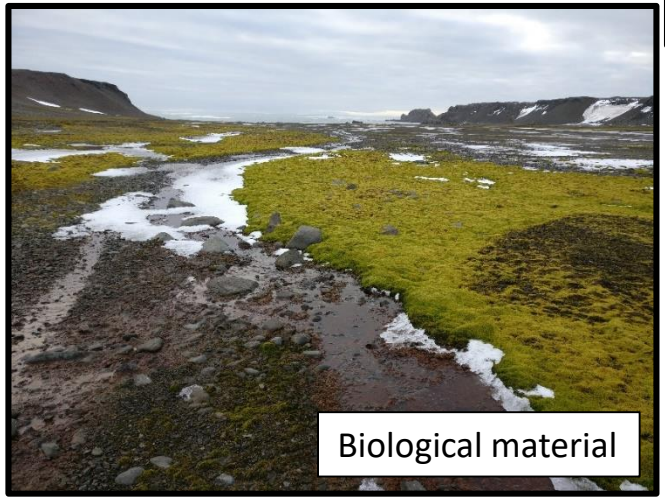

Sediment/water


Evaporites


Ice core


Hot springs


Salt crusts


Biological material


Geyser


Mud pots


Microbial mats

Sample processing

SAMPLE

"BIOLOGICAL"

"GEOLOGICAL"

- "Soft"
- High OM content
- Freeze dry (sub-sample)
- Mortar pestle (homogenization)
- Low amounts needed (1-10 g)

- "Hard"
- Low OM content
- Need to use a rock cutter
- Mortar and pestle grinder
- Higher amounts needed (50-300 g)

Sample

The idea is to have the best sample

Contamination

1. During "sample processing"

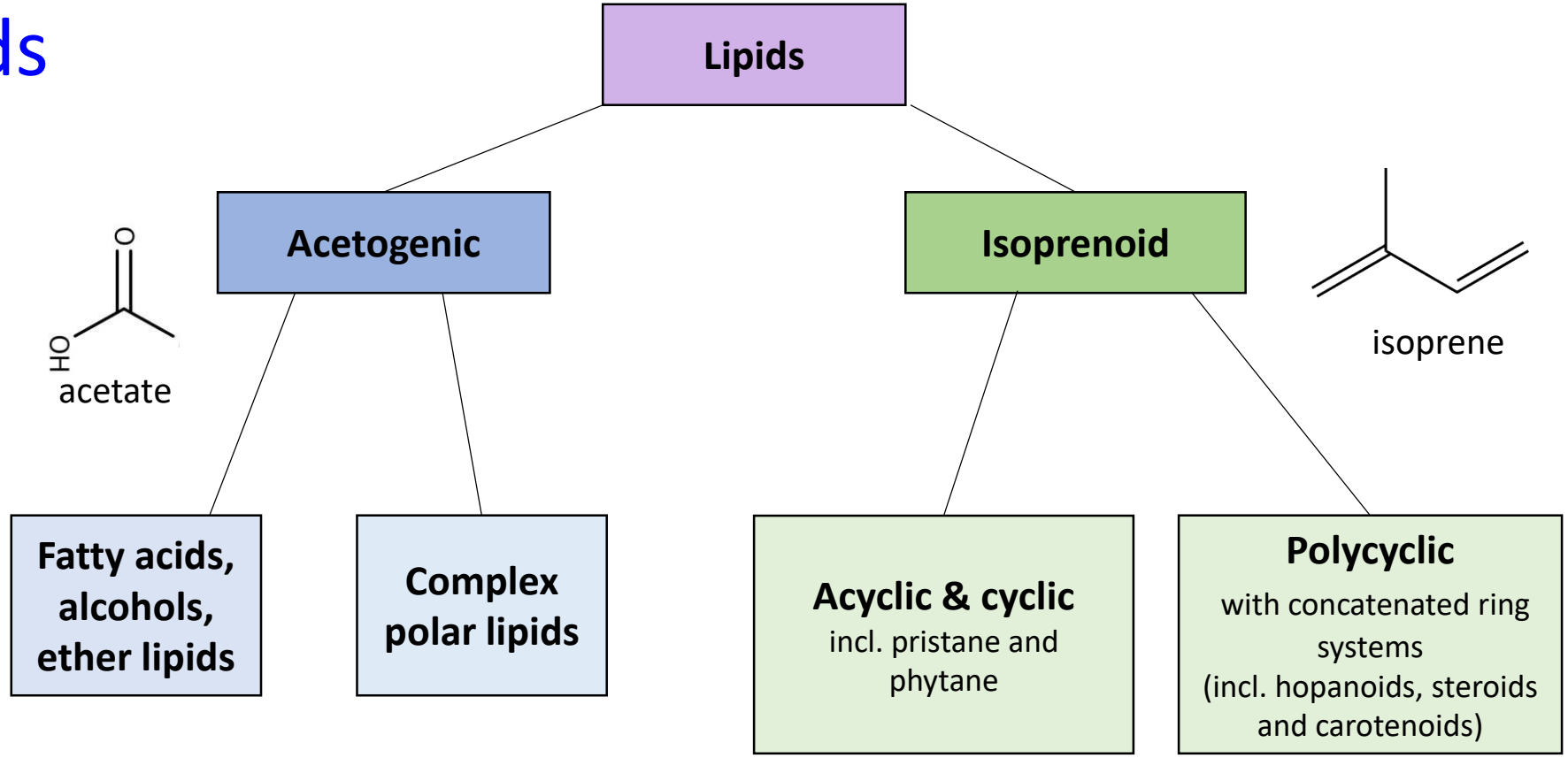
- Cut external surfaces
- Organic solvent clean (ultrasonic bath)
- All materials should be previously clean (miliQ water, solvents)

2. During "analytical protocol"

- Glassware:
  need to be clean (furnaced)
  adsorbents (silica, alumina, sodium sulfate), glass wool, etc
- Need to be clean (solvent extracted/furnaced)
- Plasticisers (everywere…)
- Cross-contamination
- People!

- Contamination may be more abundant than target compounds
- Co-elution with target compounds (impact isotopic ratios!)
- Some times the contaminant may be the same as our target compound!

Lipids



Lipids are organic compounds containing mainly C and H with minor O, N, P and S.

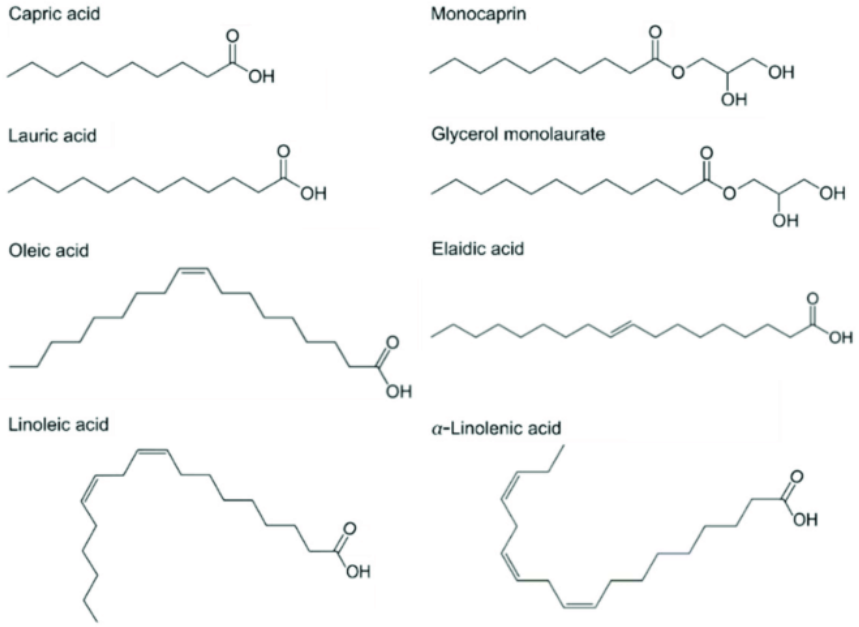
Lipids are made by living organisms and are insoluble in water, and soluble in non-polar solvents such as DCM.

Lipids comprise a wide array of chemical structures and functions.

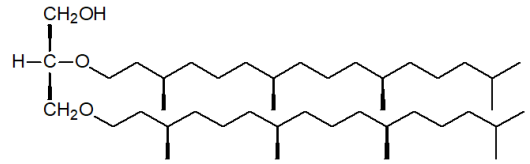
Acetogenic Lipids

Alkanols

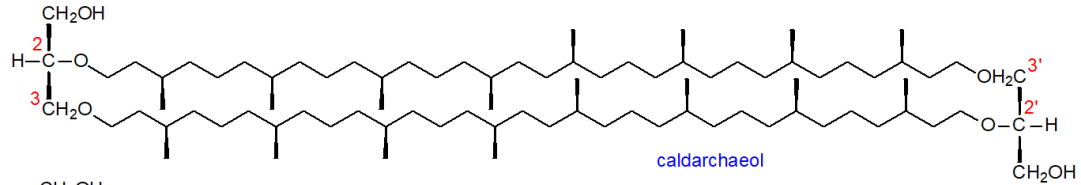
Fatty acids



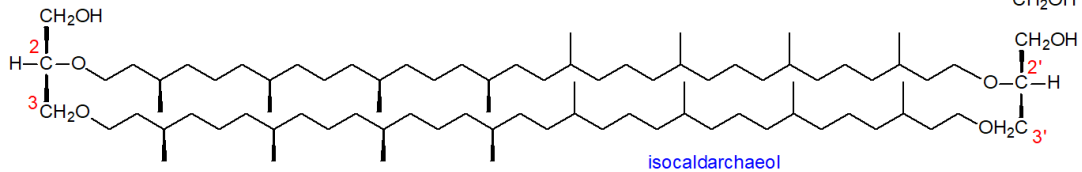
Ether lipids



2,3-diphytanyl-*O*-*sn*-glycerol (archaeol)

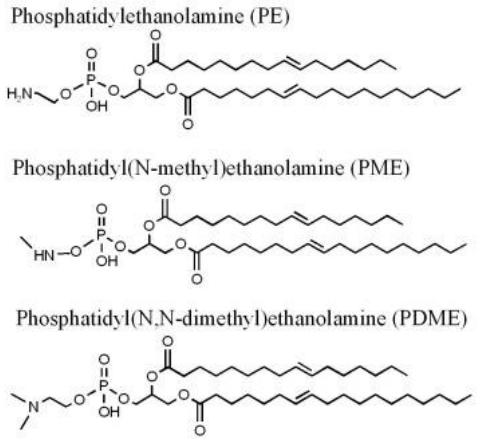


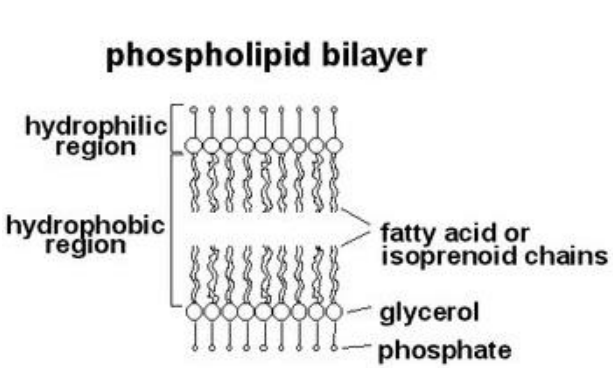
caldarchaeol

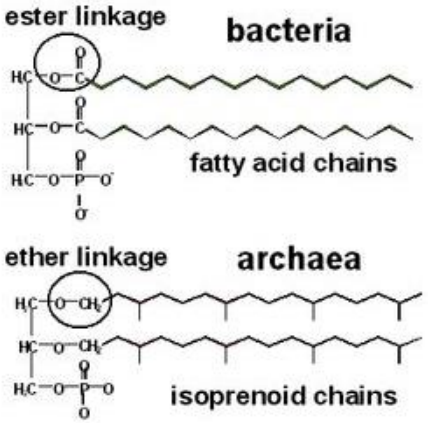


isocaldarchaeol

Complex polar lipids



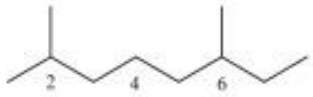




Isoprenoid Lipids

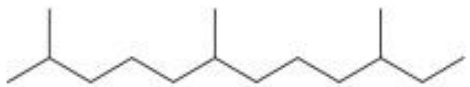
Acyclic

Monoterpane (C_{10})



2,6-Dimethyloctane

Sesquiterpane (C_{15})



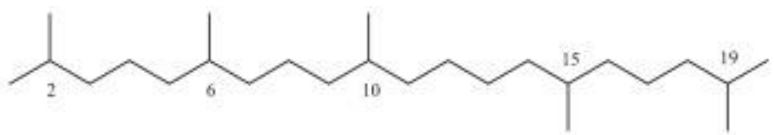
Farnesane (2,6,10-trimethyldodecane)

Diterpane (C_{20})

Pristane
(C_{19}: 2,6,10,14-tetramethylpentadecane)

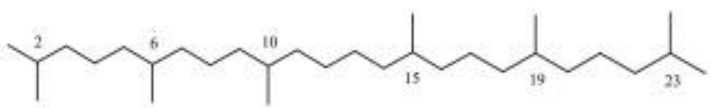
Norpristane
(C_{18}: 2,6,10-trimethylpentadecane)

Sesterterpane (C_{25})



2,6,10,15,19-Pentamethyleicosane

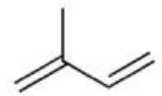
Triterpane (C_{30})



Squalane

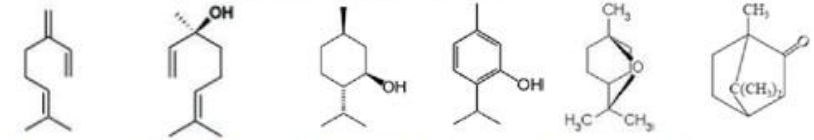
Cyclic and polycyclic

Hemiterpenoids



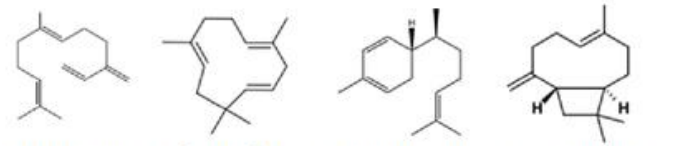
Isoprene

Monoterpenoids



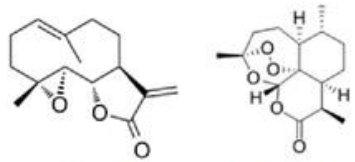
Myrcene Linalool Menthol Thymol Eucalyptol Camphor

Sesquiterpenoids



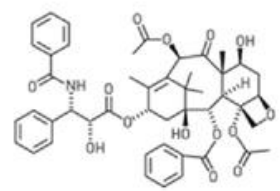
β-franesene α-humulene Zingiberene Caryophyllene

Sesquiterpenoid lactones



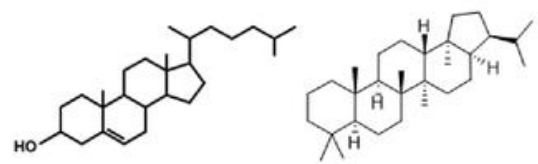
Parthenolide Artemisinin

Diterpenoids



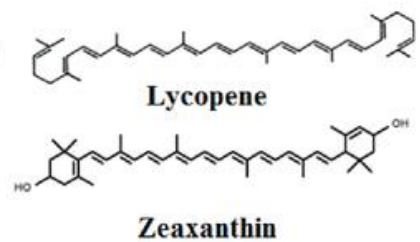
Taxol

Triterpenoids



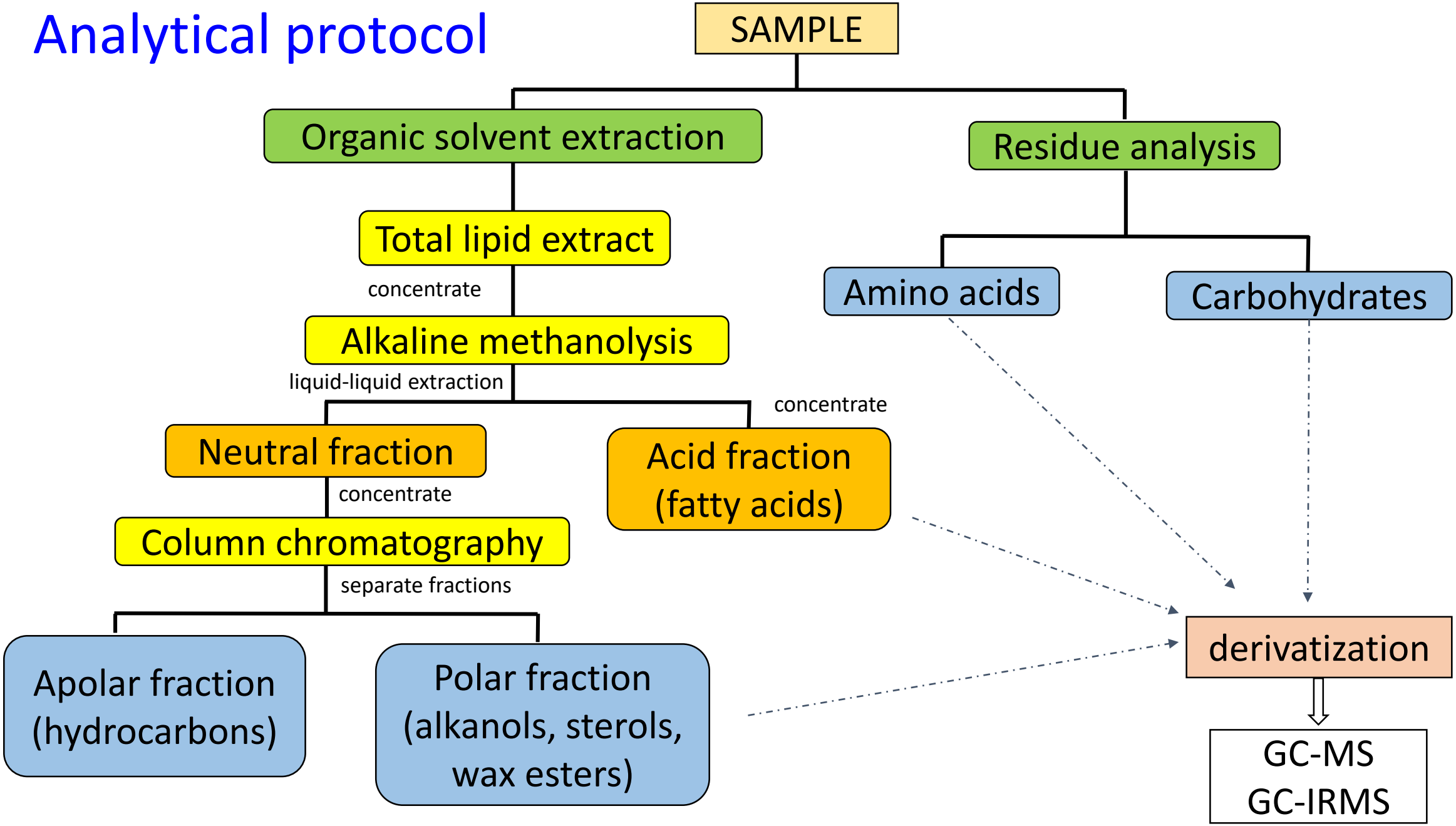
Cholesterol Hopane

Tetraterpenoids



Lycopene Zeaxanthin

Analytical protocol



Analytical protocol

Organic Solvent Extraction

Depending on the sample "size"

Ultrasonic bath

"Small size"
1-10 g
(high OM content)
Low solvent volume

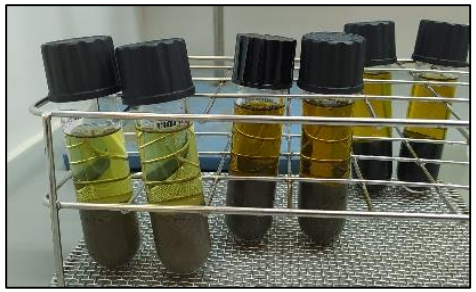
Soxhlet

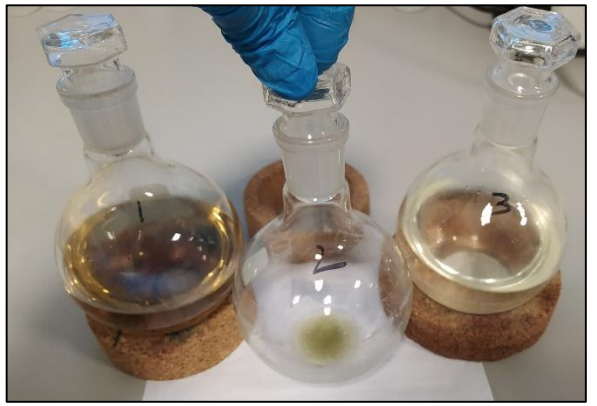
"Big size"
50-200 g
(low OM content)
High solvent volume

- Mixture of organic solvents (wide variation of combinations, polar/non-polar)

Other lipid extraction techniques:

Microwave-assited extraction (MAE)
Accelerated solvent extraction (ASE)





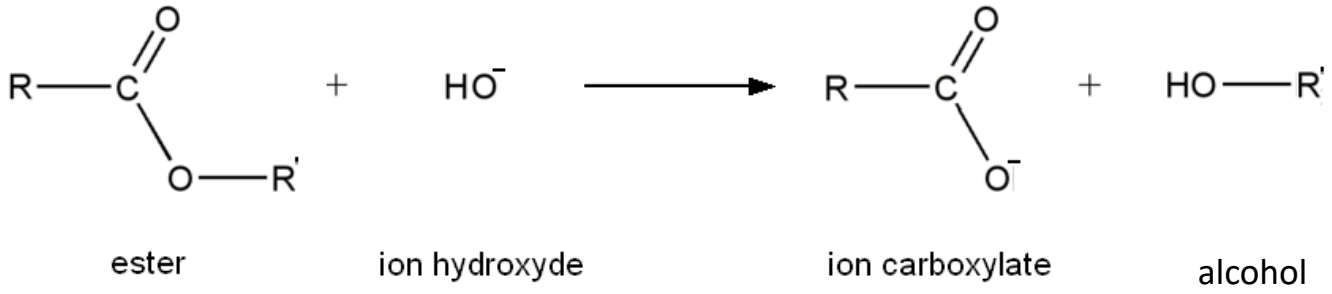
Analytical protocol

Concentration (big volumen)

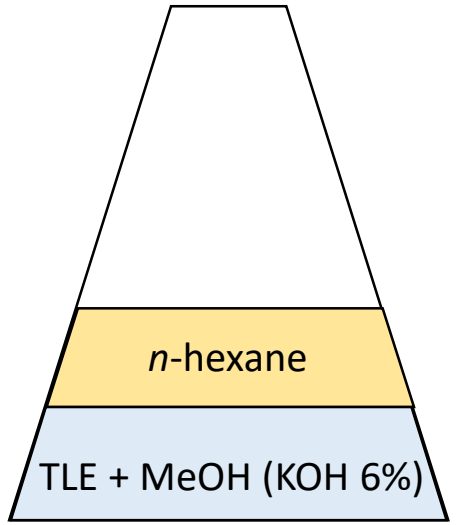
- Rotoevaporation
- Vaccum, hot water bath (25-35ºC) and cold serpentine (7ºC or less)
- The idea is evaporate the organic solvent
- With minimal loss of target compounds

Analytical protocol

Alkaline methanolysis

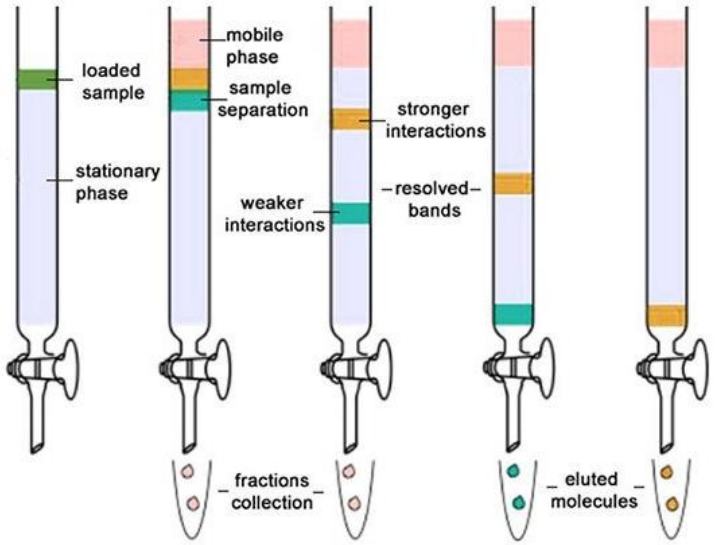


- Saponification (convert a more complex lipid into an alcohol and a fatty acid)
- Load the TLE (1-2 ml) onto 35 ml of MeOH (6% KOH)
- Leave react overnight at room temperature
- Liquid extraction with *n*-hexane (3 times x 30 ml): 1st extraction (Neutral fraction : apolar/polar compounds)
- Add HCL until pH 2, extract with *n*-hexane (3 times x 30 ml): 2nd extraction (Acid fraction)



Analytical protocol

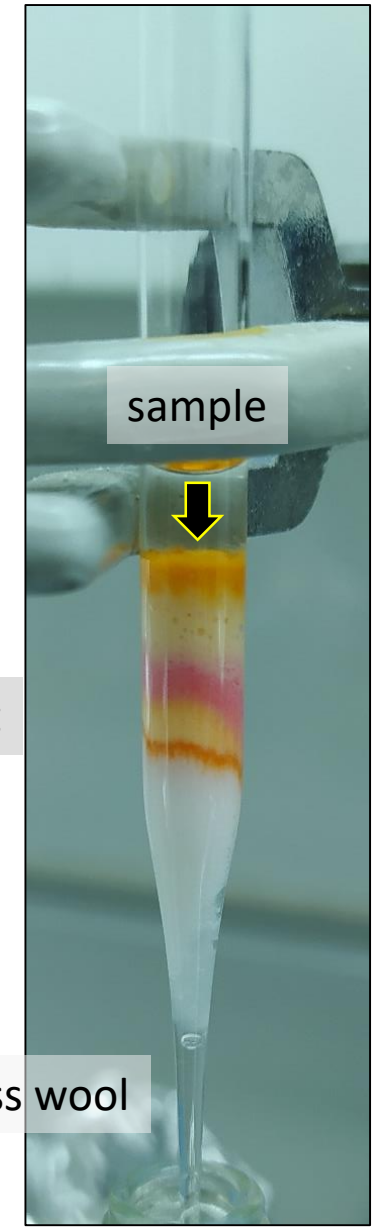
Column chromatography



- Load ”minimal” amounts of sample extract
- Adsorbents (Al_2O_3, SiO_2), glass wool and glass material should be clean (furnace, solvent clean)
- Solvents added in sequence:

From low polarity (hexane/DCM) for hydrocarbons

to high polarity (MeOH) for alcohols, sterols, etc.



Analytical protocol

Concentration (small volume)

- Evaporation (gentle nitrogen flow)
- Tubes can be inside a heater (25 ºC)
- Dryness with minimal target compound loss
- Avoid high N flow and high temperature

Analytical protocol

Vials and inserts

- Evaporate under N and redisolved in *n*-hexane
- Redisolved volume (depends...)
- Vials and inserts should be clean (furnace overnight)
- Different vials and inserts

Sample storage

“Raw sample or ready to extract ”

- Bulk sample maintain at -20ºC
- After clean and before extraction:

  Use clean glass tube/flask to store at -20ºC

“sample extracts”

- TLE (can be store at 4ºC)
- Sample solvent organic fractions (can be store at 4ºC)

“sample vials”

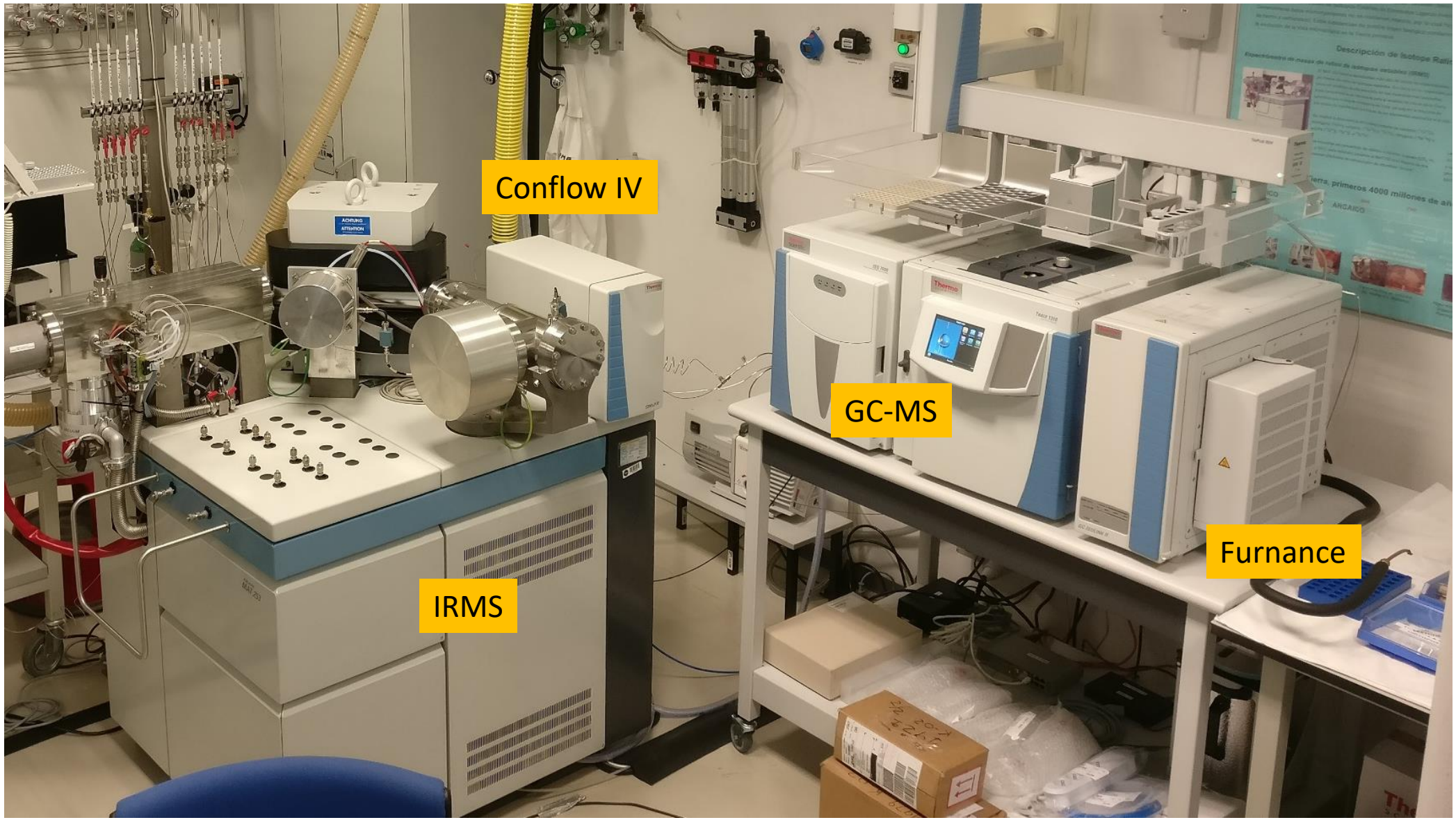
- Vials need to be store at -20ºC
- After inyection:

  “hydrocarbon” vials can be store at -20ºC

  Other fractions vials (FAMEs or alcohols/sterols) can be re-inyected within a week or less at -20ºC

- Always change tap

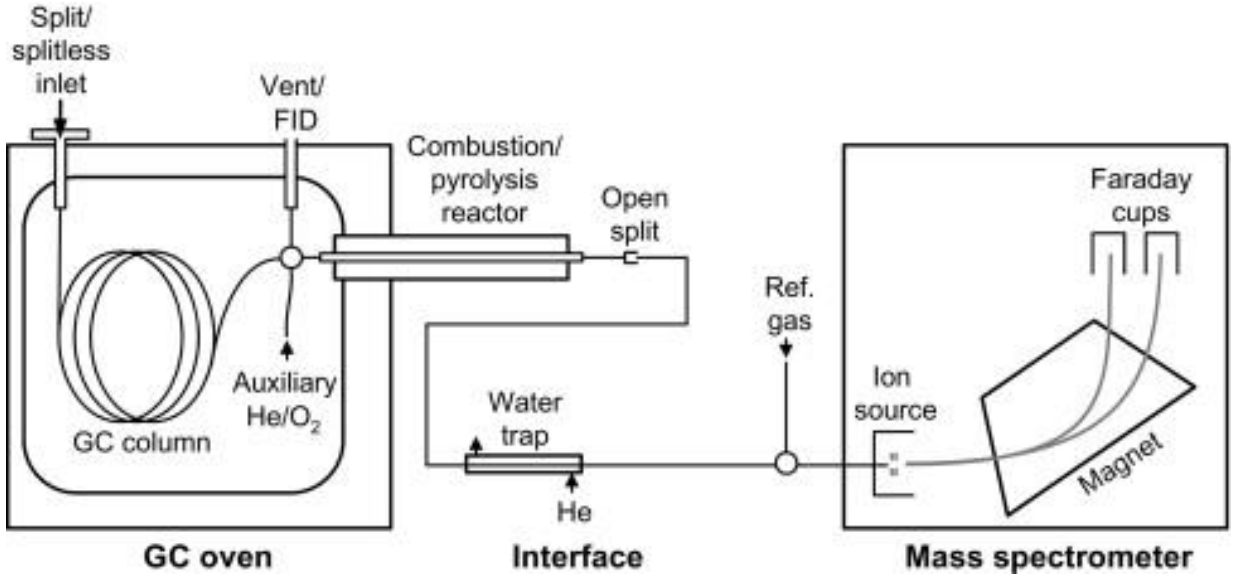
GC-IRMS system

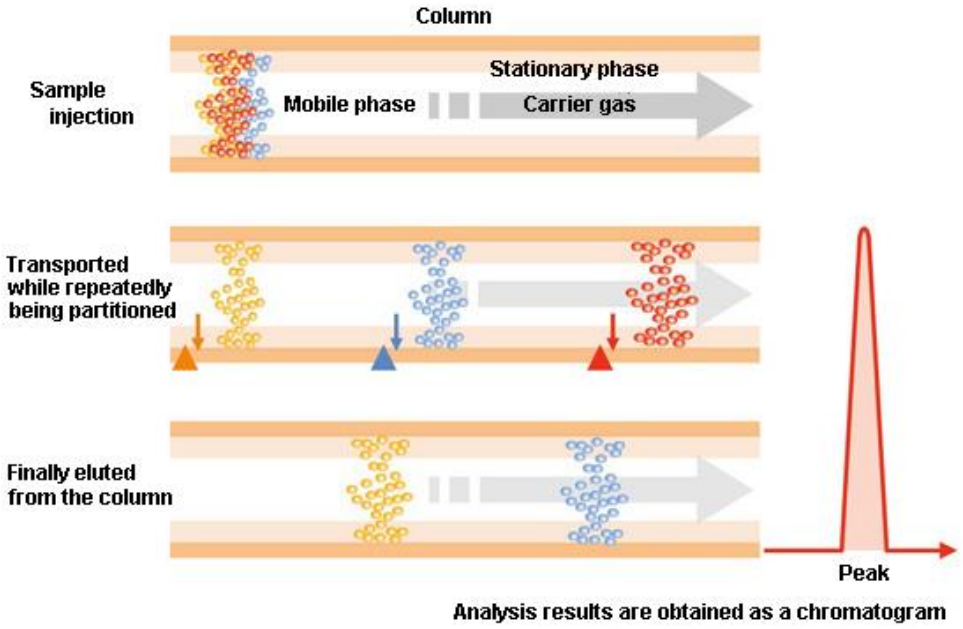


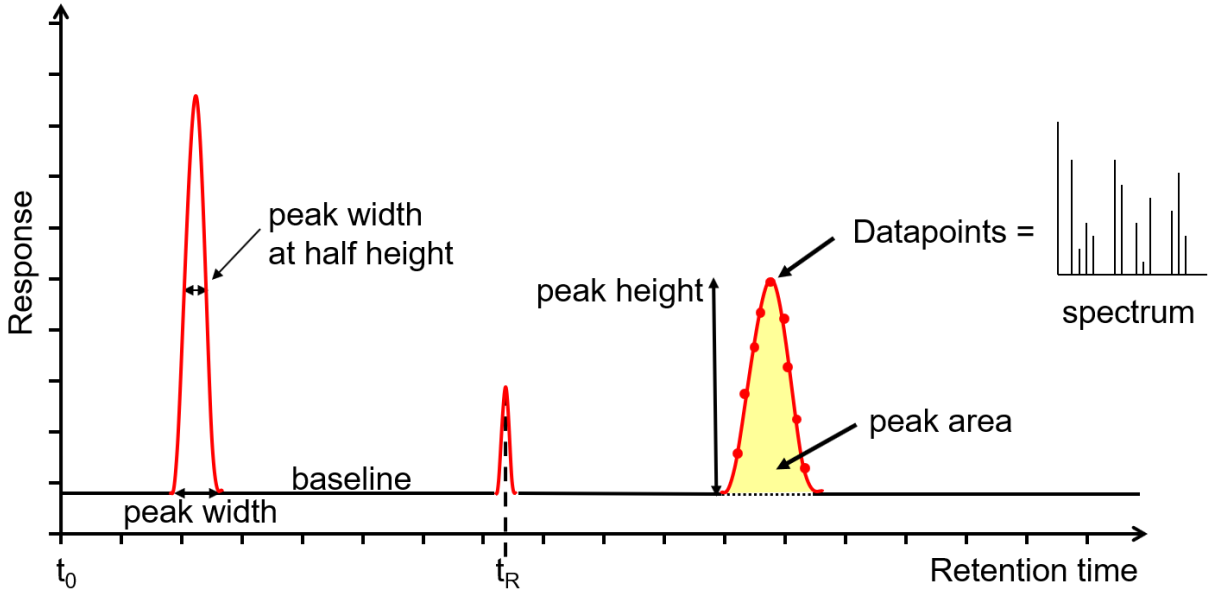
GC-IRMS system

KEY:

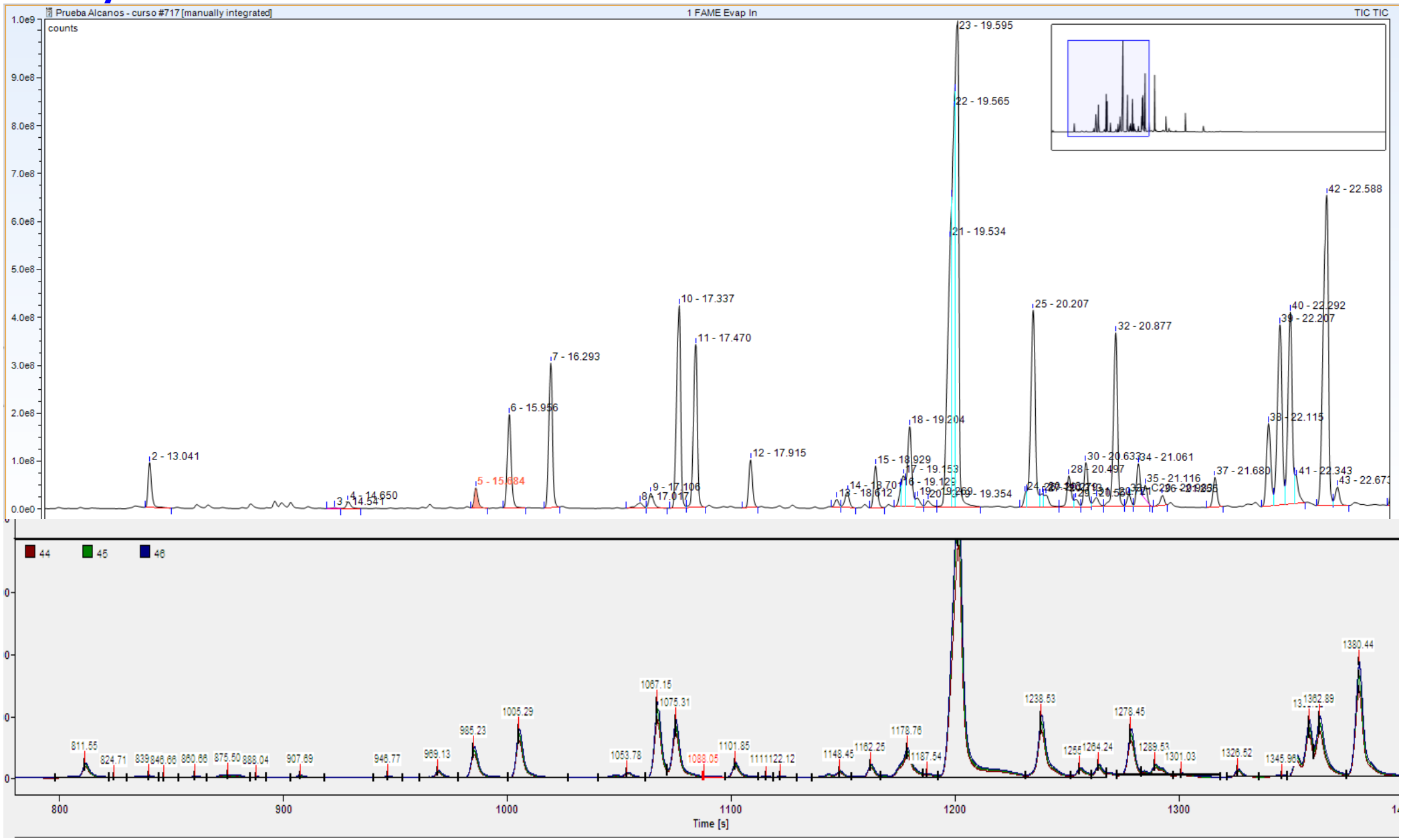
- good peak shape and chromatographic separation, low base line and low column bleed means good IRMS isotopic data!!





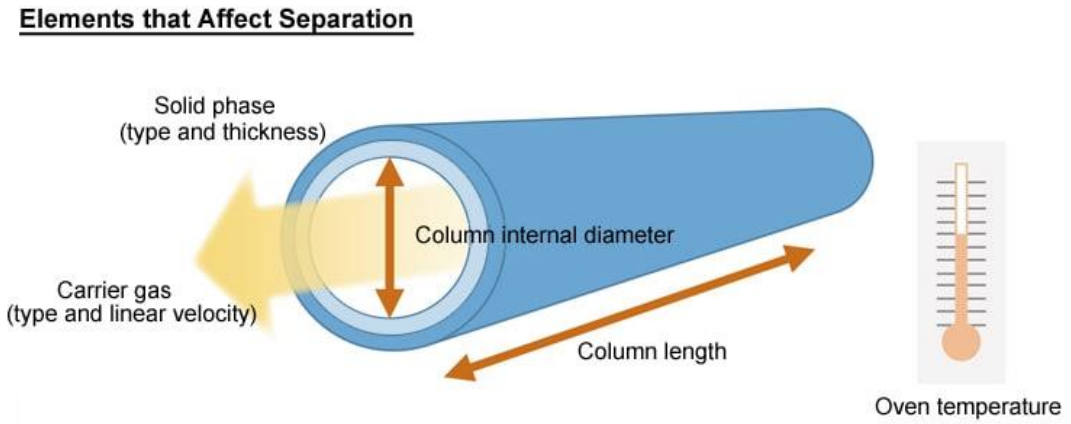


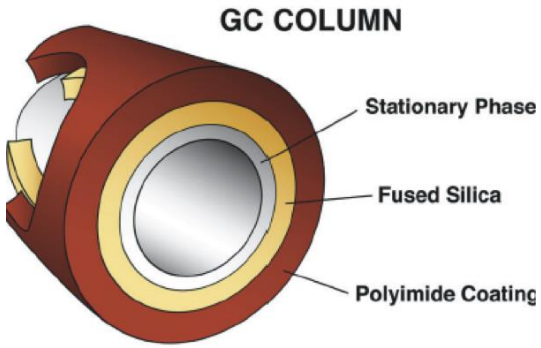
GC-IRMS system

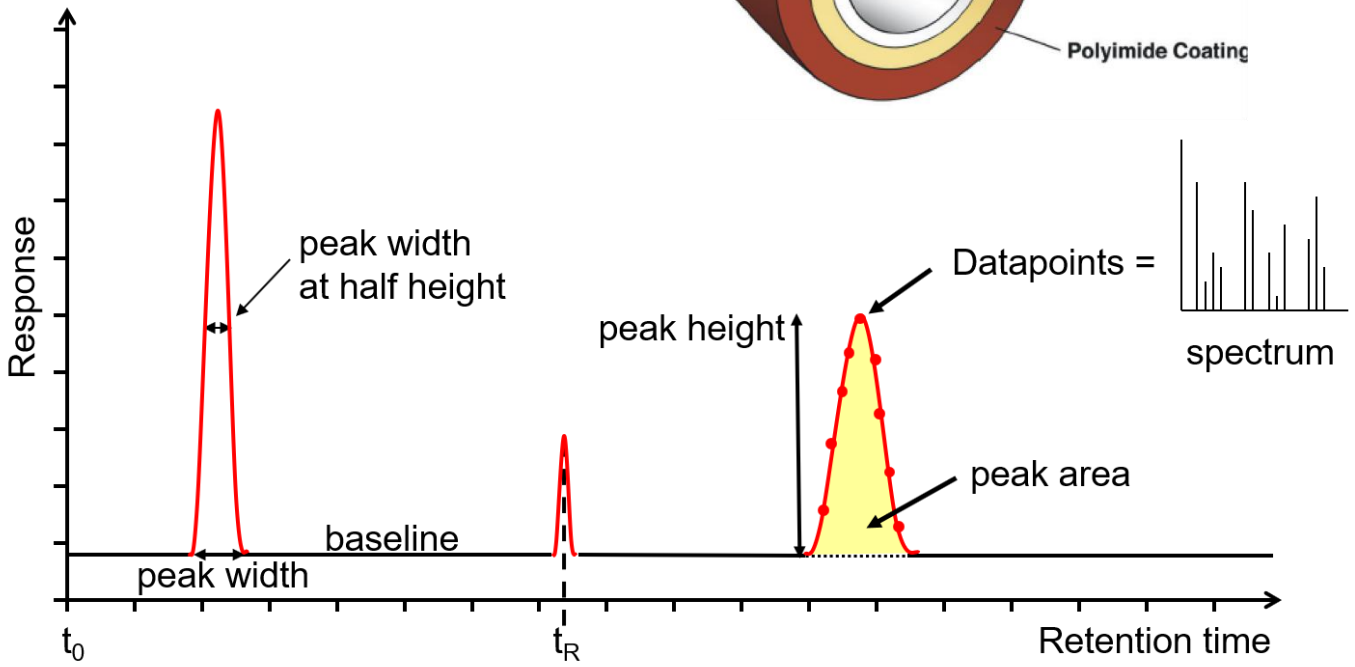


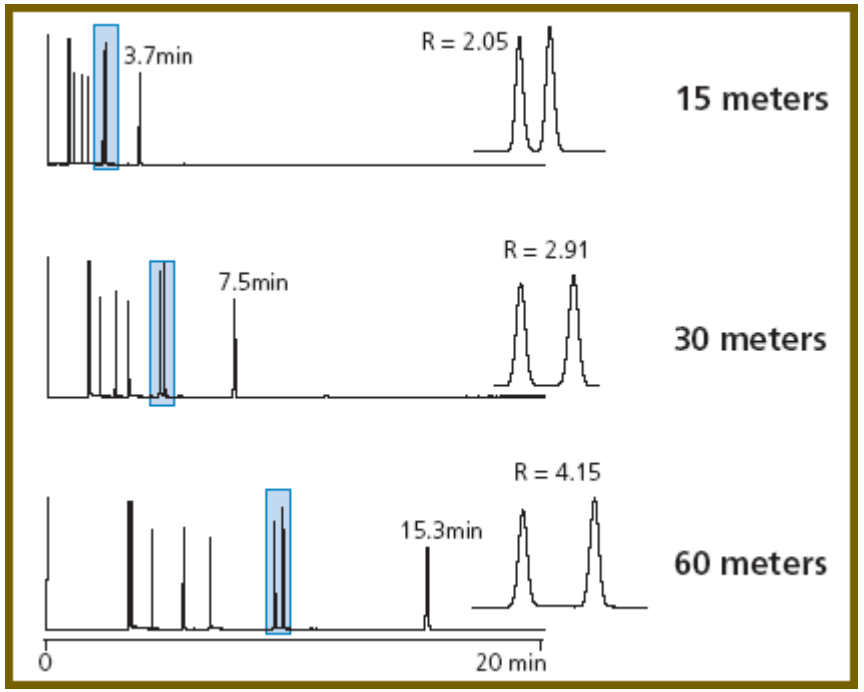
GC Columns

- From low to high polarity
- Depending on the type of compounds
- Wide variety of applications
- Ramp temperature (50ºC to 310ºC)









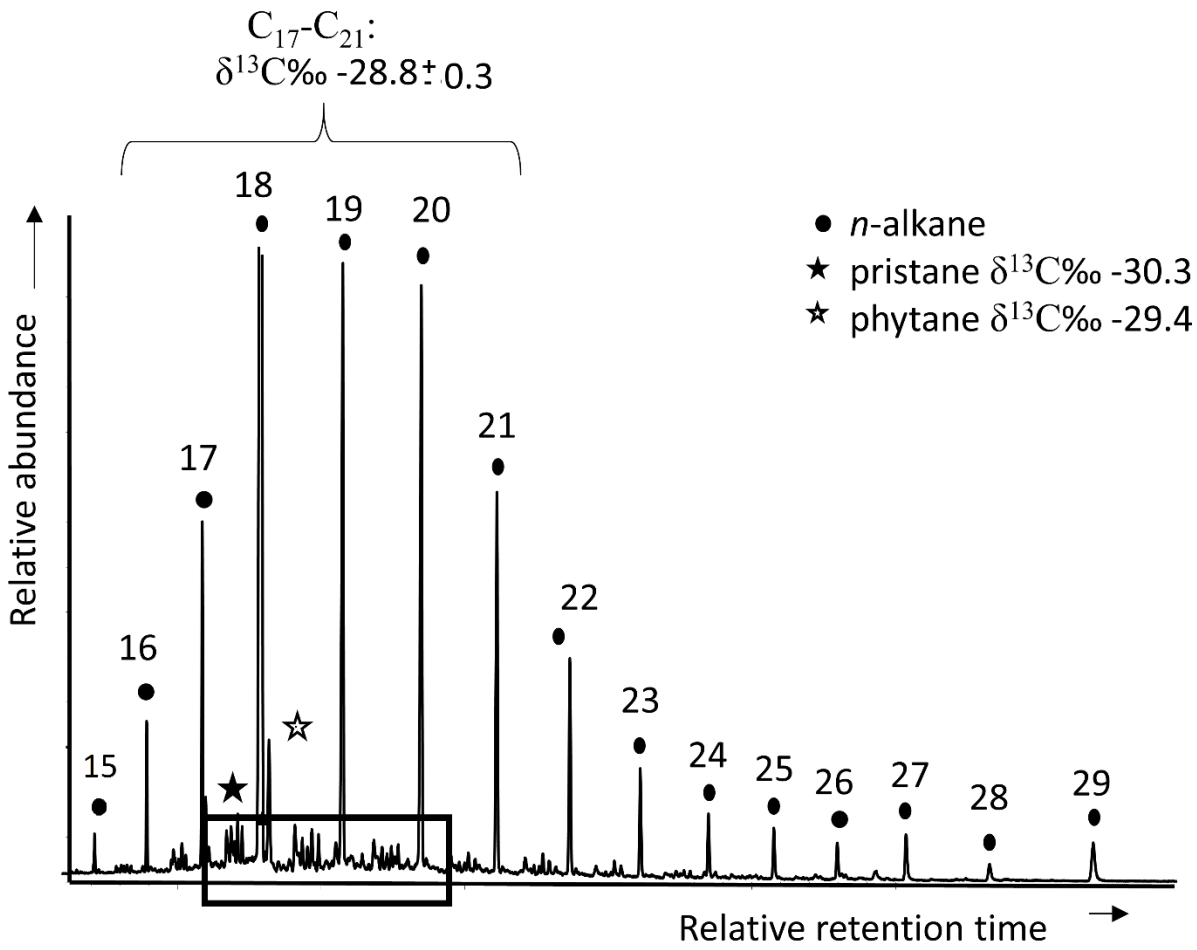
Chromatography

- Apolar (hydrocarbons) fraction
- Polar (alcohols, sterols, etc.) fraction (needs derivatization)
- Acid fraction (needs derivatization)

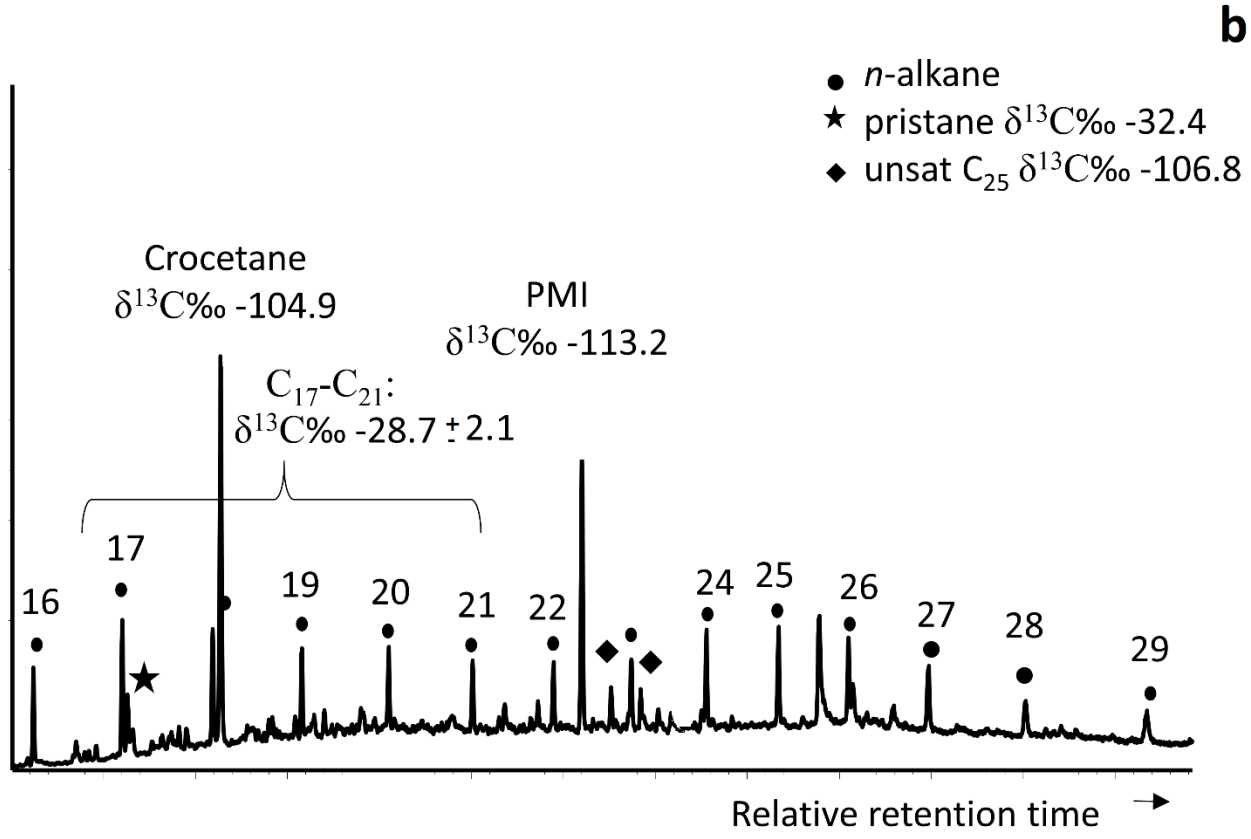
hydrocarbons

✓ NO functional groups

✓ NO derivatization needed



Deep sea Chimney (Cádiz)



Deep sea Chimney (USA)

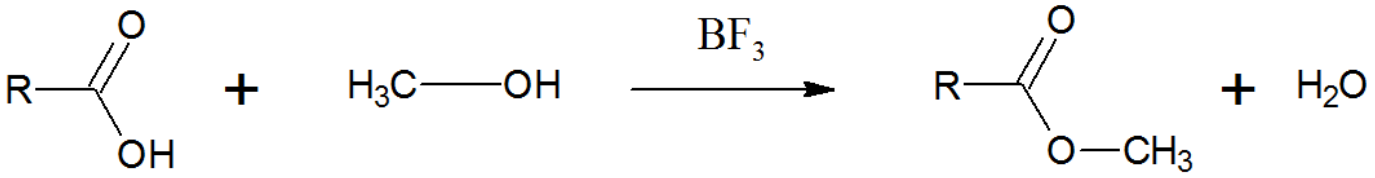
Derivatisation requirements

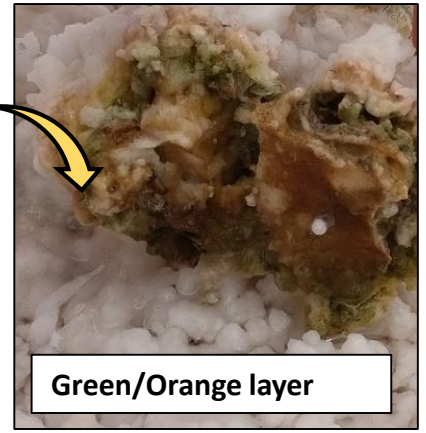
- Kinetic Isotope Effect
- Minimal addition of C/H as possible
- Fast and easy reaction
- Good separation of target compounds
- No by-products interference
- Stable end-products

Derivatisation

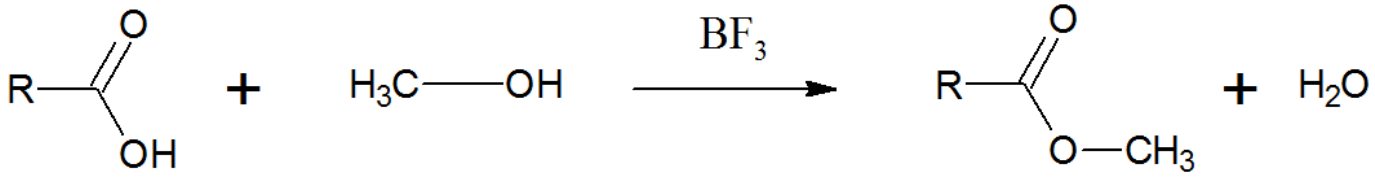
- Needed for non-volatile compounds, due functional groups
- The aim is to “blocked” the functional groups by apolar groups
- Common functional groups:
  - Carboxylic acids
  - Hydroxy
  - Amino
- Some derivatization reactions:
  - Esterification
  - Silylation
  - Acetylation
- Which one choose?

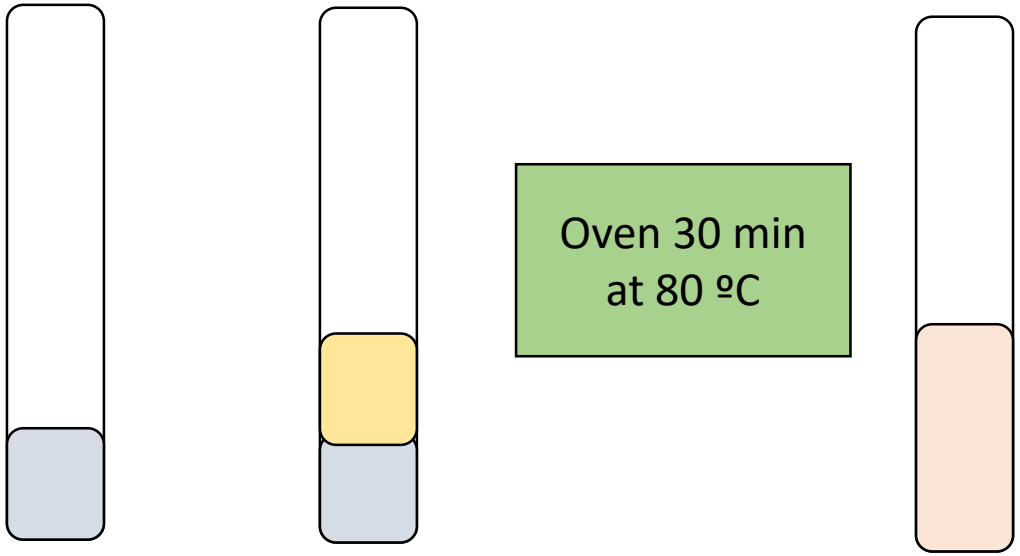
Fatty acids (esterification with boron trifluoride -BF_3)





Fatty acids (esterification with boron trifluoride -BF_3)



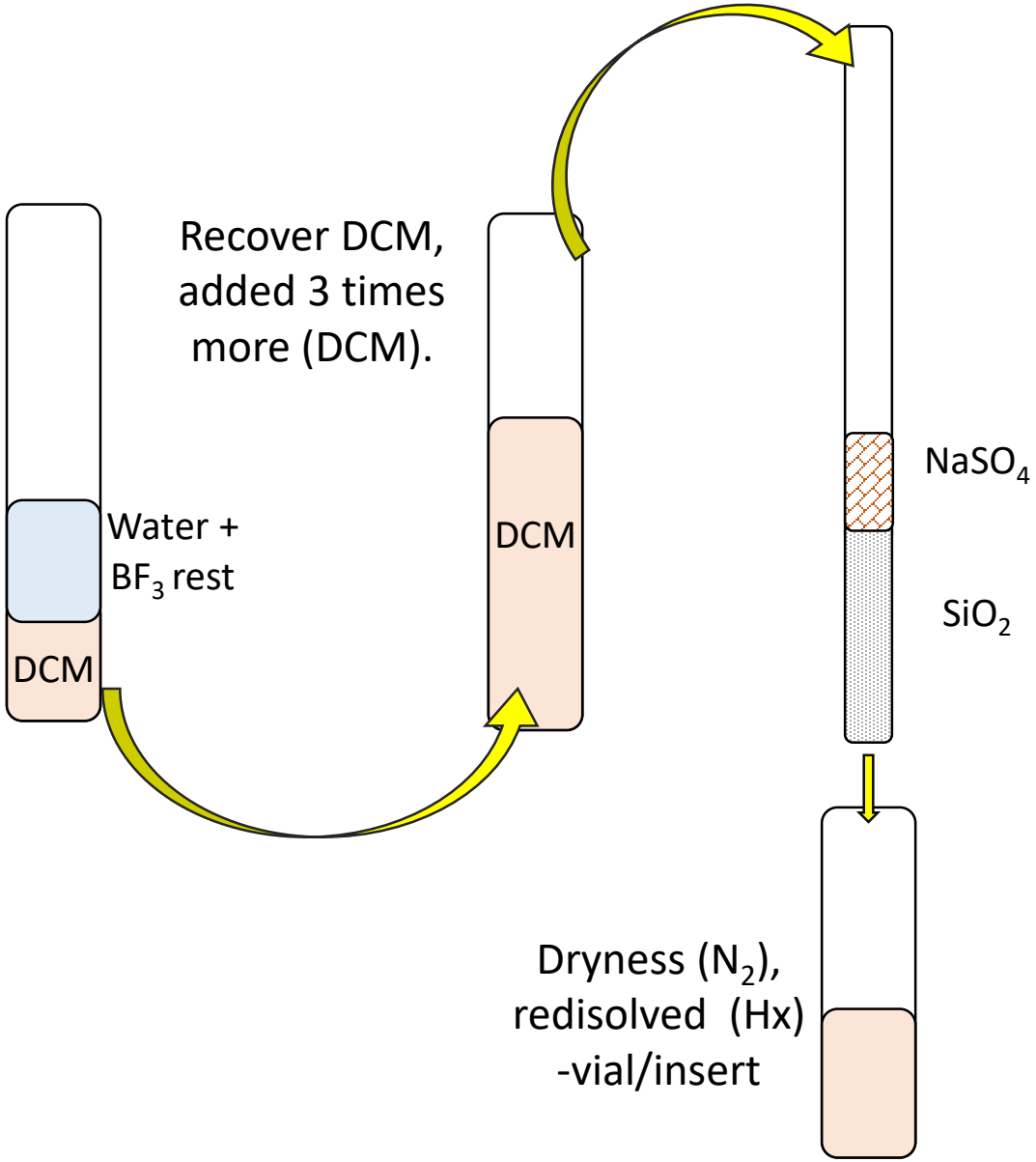


acid fraction: dryness and redisolved in 1 ml DCM

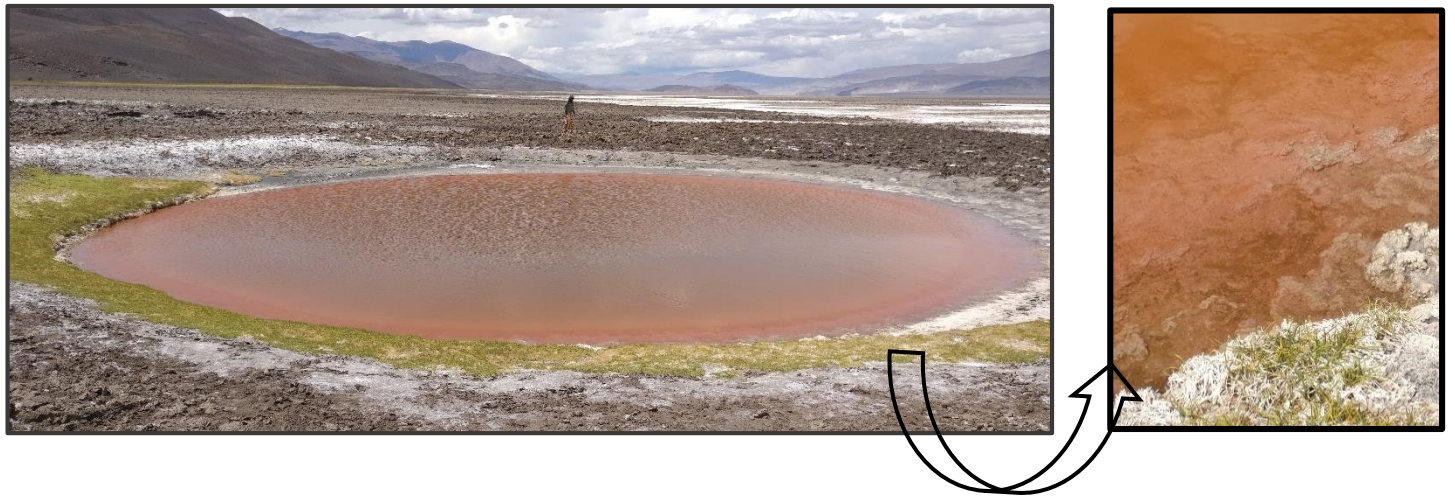
Add 1 ml of BF_3 (mix)

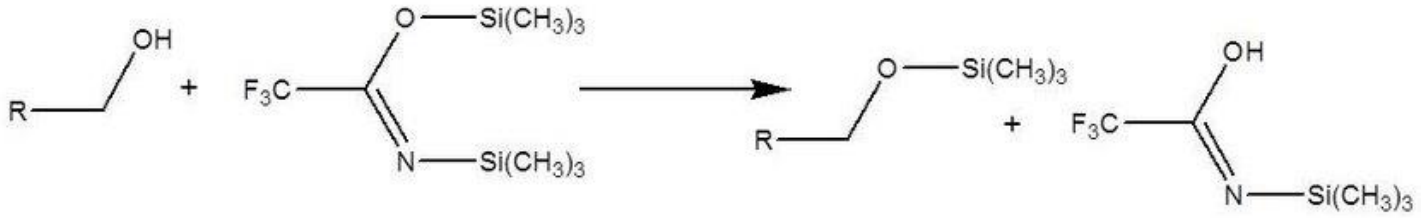
Oven 30 min at 80 ºC

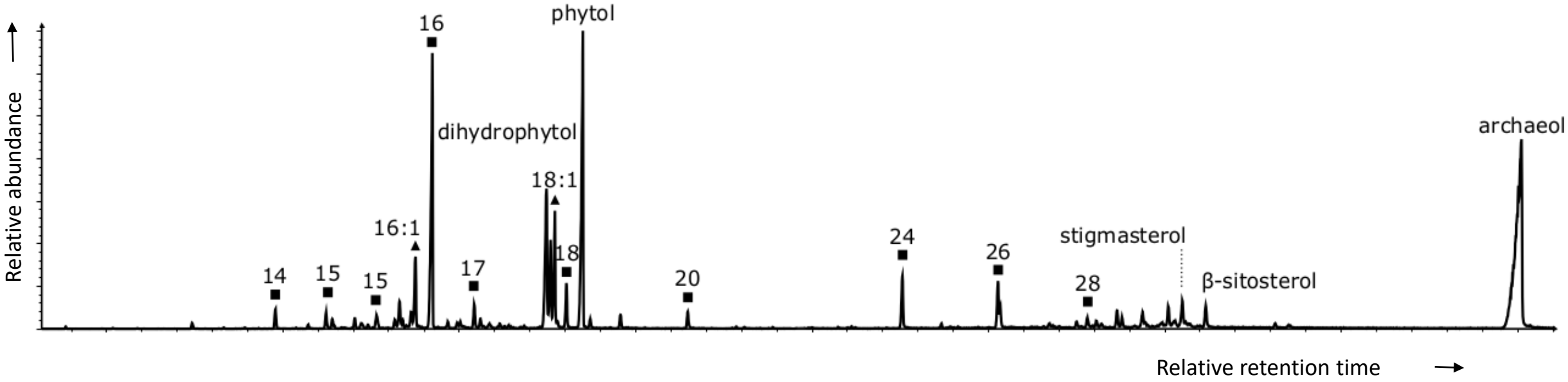
Add 1 ml of Milli-Q water (mix)



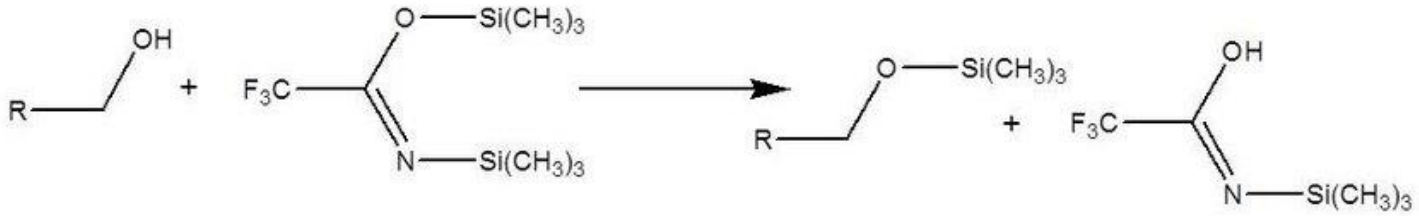
Alcohols (sylilation with BSTFA)

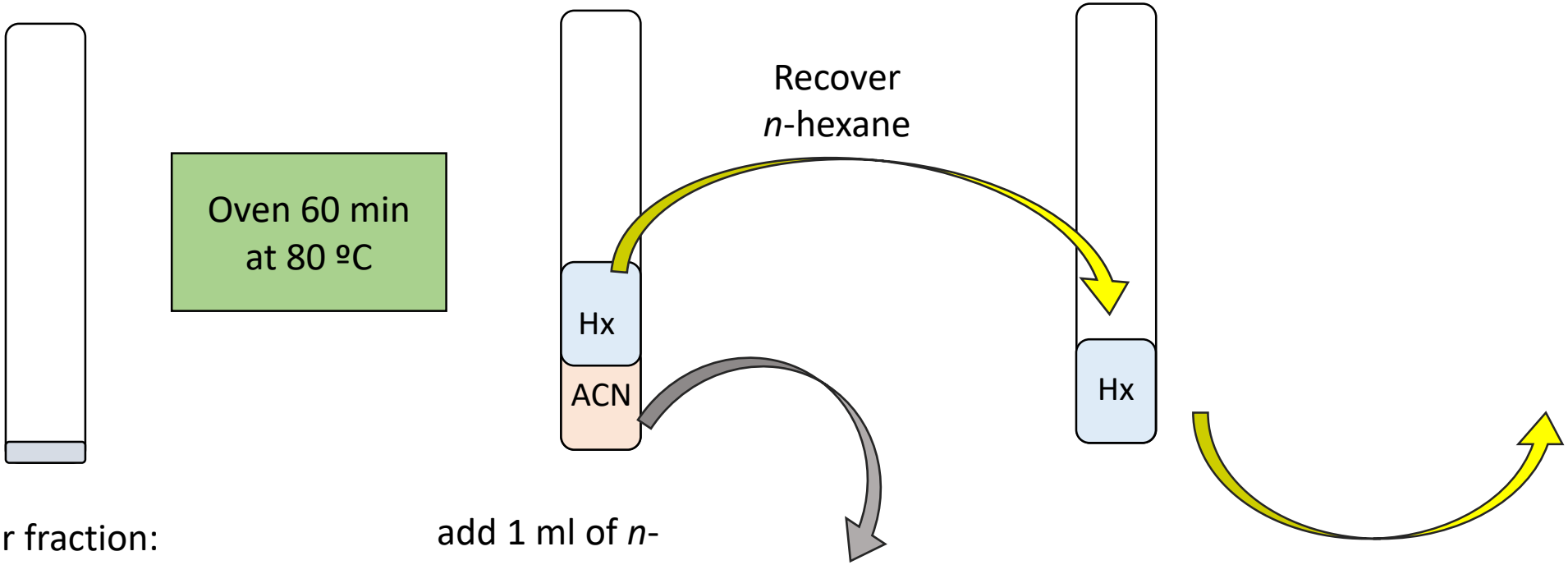






Alcohols (sylilation with BSTFA)





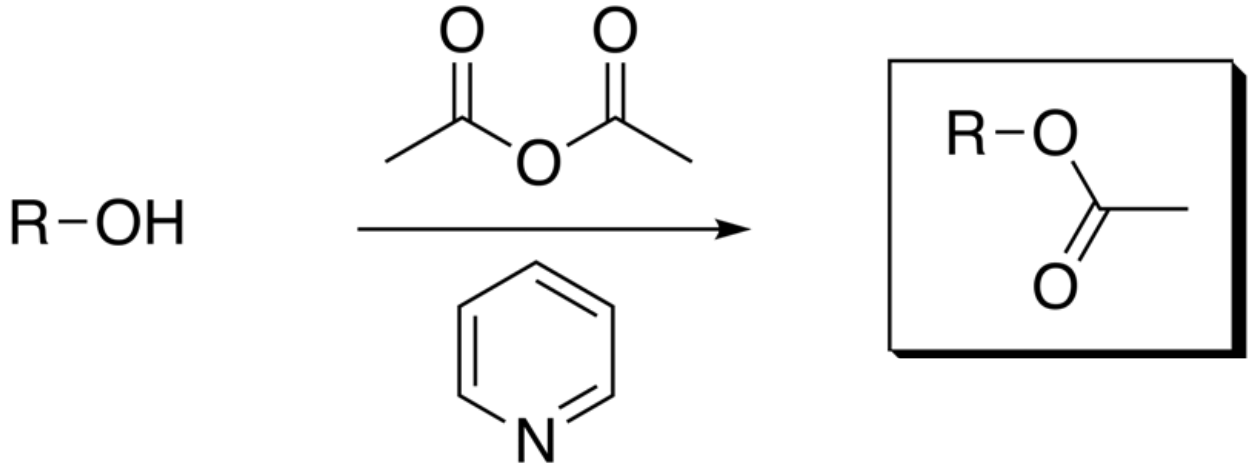
polar fraction:
Dryness, add
100 µL BSTFA

add 1 ml of *n*-
hexane and
1 ml ACN
(mix)

Recover &
throw ACN,
added 3 times
more (ACN).

Disadvantage: products are stable but only for a relative short time

Acetylation

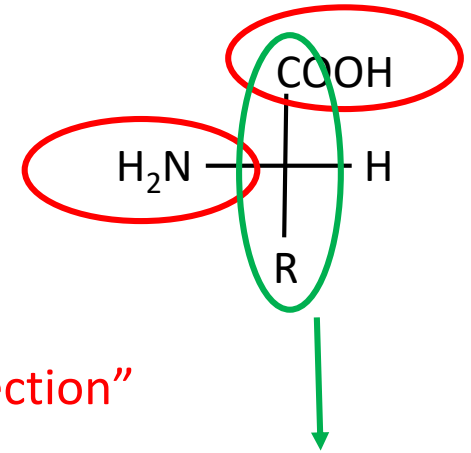


- For a wide variety of compounds (alcohols, amides, thiols, etc)

  A catalyzer is used for the reaction: e.g., pyridine or 4-(dimethylamino)pyridine (4-DMAP)

- Drawbacks: harsh reaction environment, poor yields of the desired product, the formation of side products, and longer reaction time

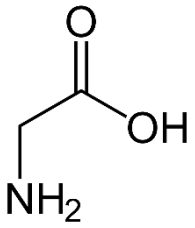
Amino acids



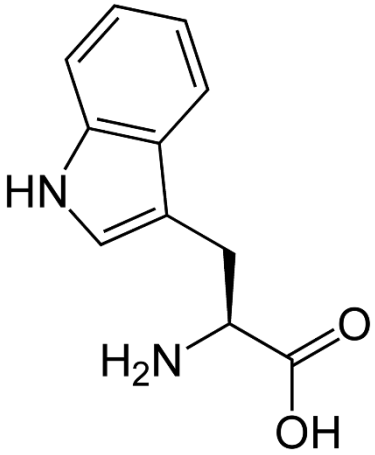
Main problems:

- Two different “active” groups which needs “protection”
- Small number of “own” carbon atoms

2 (Glycine) to 11 (Tryptophan)

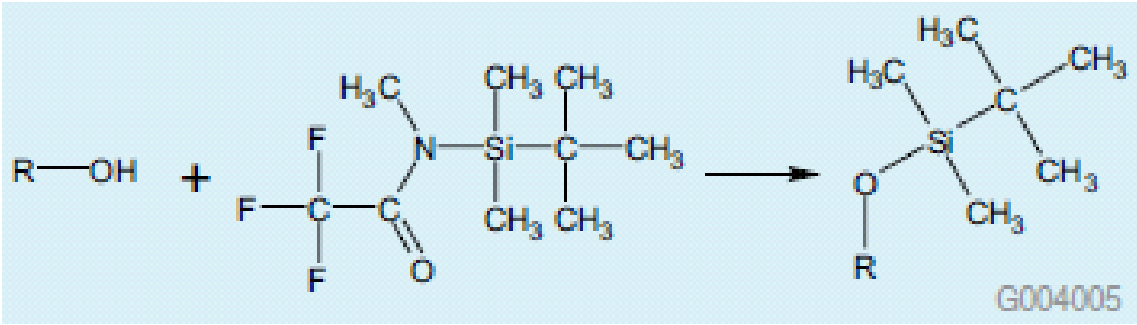


Glycine



Triptophan

Amino acids



Derivatization with MTBSTFA:

- N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide
- forms tert-butyl dimethylsilyl (TBDMS) derivatives

Drawbacks:

- Relative large number of C atoms added (5 at least)
- A reaction with an isotope effect, however can be corrected

Correction for derivatising groups

- With no kinetic isotope effect, e.g. methylation of fatty acids with BF_3.
- Using a simple mass balance equation:

$$RCO_2H \xrightarrow{BF_3/MeOH} RCO_2Me$$

$$n_{cd}\delta^{13}C_{fd} = n_c\delta^{13}C_c + n_d\delta^{13}C_d$$

Where:

n is the number of atoms of carbon/hydrogen
c refers to the target compound
d refers to the derivative group
fd refers to the derivatized fatty acid


THANK YOU !