
We offer three kinds of service:
GOOD - CHEAP - FAST
You can pick any two
GOOD service CHEAP won't be FAST
GOOD service FAST won't be CHEAP
FAST service CHEAP won't be GOOD

Phases of the analytical service

- Preanalytical: sample collection, transport, storage and processing
- Analytical: peforming the assay
- Postanalytical: evaluating, validating, archiving and delivering results

The quality of the analytical service is directly and strongly related to finding and eliminating/optimally reducing errors in our processes

So what's the deal?

- Preanalytical error: 50-70% of all errors
- Analytical error: <15% of all errors
- Postanalytical error: 15-50% of all errors

The point of sample preparation is:

- To obtain a sample that can be assayed **efficiently** → depends on the analyte, the sample matrix, the analytical instrumentation and the range of available sample prep devices

- Protect the analytical equipment in order to maintain its performance and the long-term quality of analytical results

- Reduce exposure to certain preanalytical errors

Arsenal of sample preparation techniques:

- **physical:**
  - adsorption
  - centrifugation
  - dialysis
  - filtration
  - homogenization
  - incubation
  - mixing
  - sample concentration
  - sonication
- **chemical:**
  - digestion
  - dilution
  - distillation
  - extraction
  - homogenization
  - pH adjustment
  - protein precipitation
  - reactions (derivatization, oxidation/reduction etc.)
  - solvent exchange

Sample preparation for assaying small organic molecules

- Common approaches:
    - chemical derivatization
    - liquid-liquid extraction
    - pH adjustment prior to extraction or derivatization
    - phospholipid removal
    - protein precipitation
    - sample dilution
    - solid phase extraction
    - solvent exchange

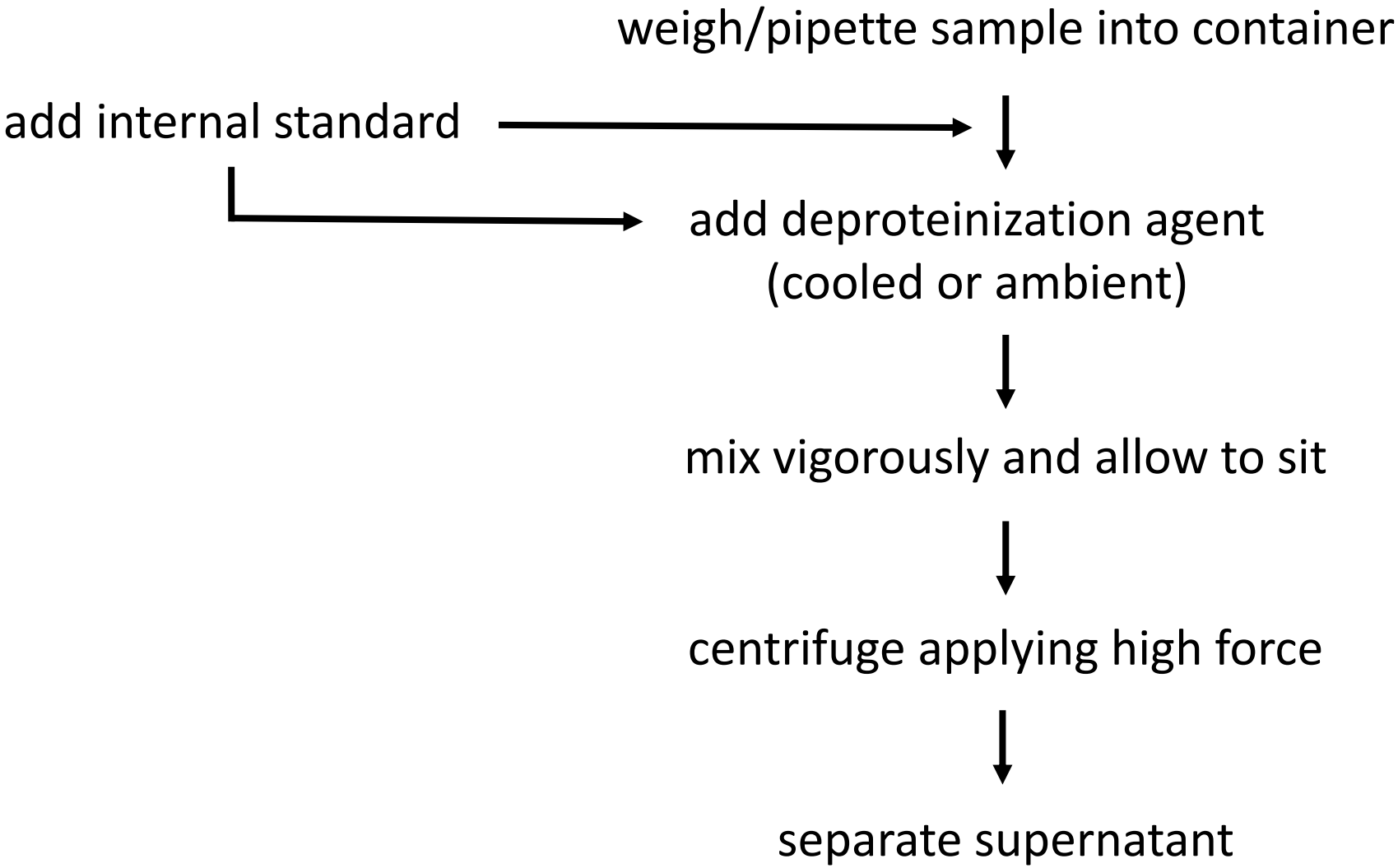
sample deproteinization

- aims:
  - to obtain a sample ready for assay
  - sample clean-up: protection of HPLC and MS, improvement of extraction recoveries, promotion of extraction procedure by reducing viscosity
  - recovery of protein-bound analyte fractions

- dilution of sample using solvents or solutions of inorganic salts

- commonly used deproteinizing agents:
  - organic solvents: acetonitrile, methanol, trifluoroacetic acid, trichloroacetic acid,
  - inorganic compounds: $HClO_4$, KOH, NaOH, $ZnSO_4$

deproteinization workflow



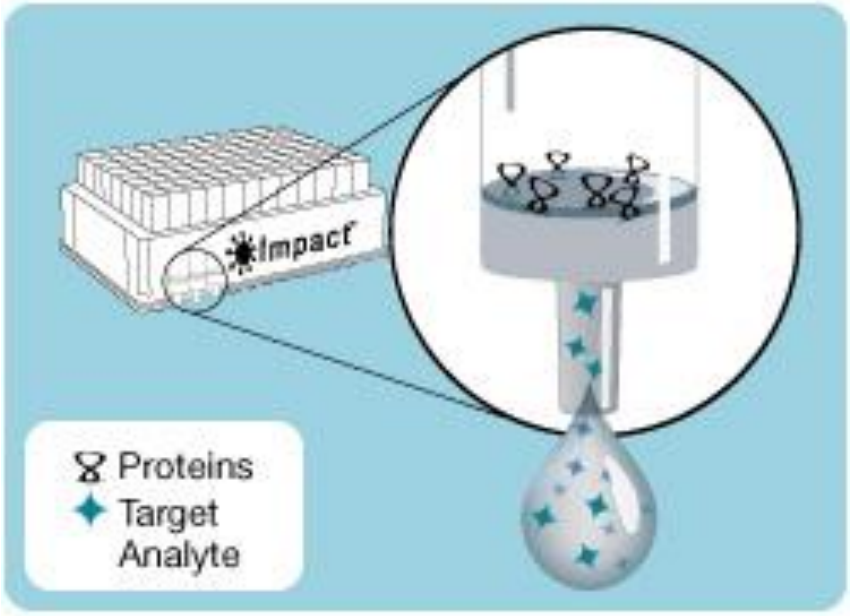
Factors in selecting the deproteinization agent

- type of sample
- type of analyte
- concentration range of analyte
- analytical aspects (instrumentation, chromatographic settings, injected volume)

Protein precipitation can be performed in a high-throughput format

Impact™

Finally an Easier Solution for Protein Precipitation



Liquid-liquid extraction

- extraction of analytes from aqueous matrix into organic

- advantages:
  - cheap
  - low matrix effect in MS analysis

- disadvantages:
  - laborious
  - difficult to increase throughput → simplified liquid extraction

Partitioning between immiscible liquid phases at equilibrium

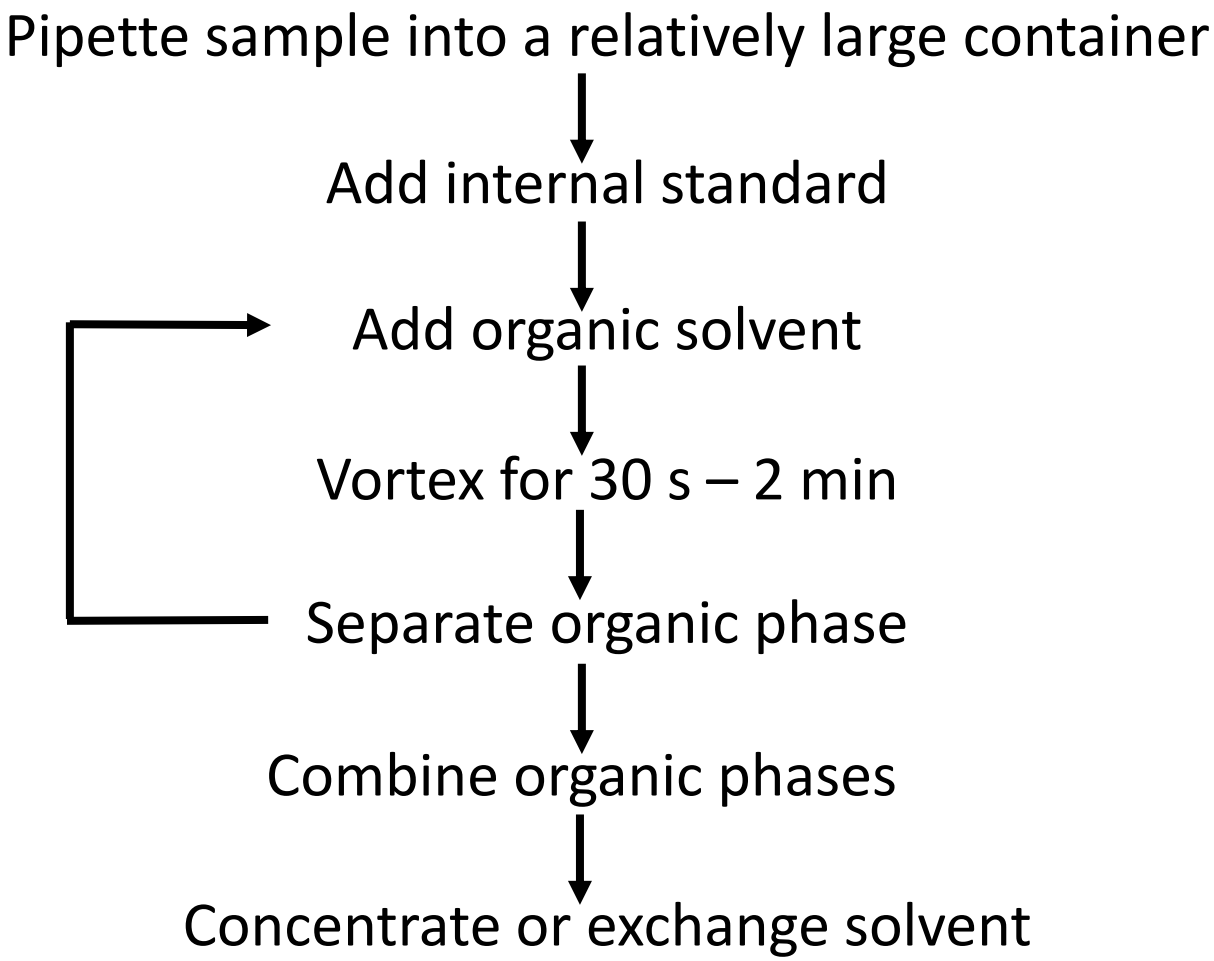
- Non-ionizable compounds: partition coefficient (log P)

$$logP = log\frac{c_{octanol}}{c_{water}}$$

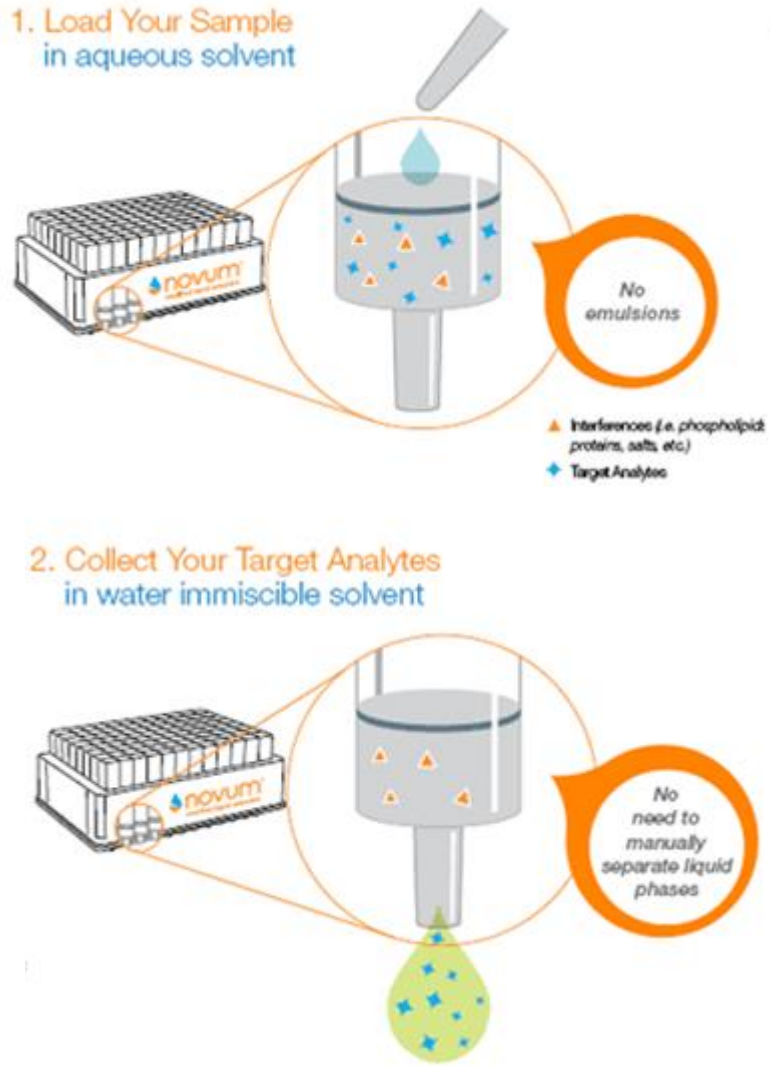
- Ionizable compounds: distribution coefficient (log D)

$$logD = log\frac{c_{octanol,ionized} + c_{octanol,unionized}}{c_{water,ionized} + c_{water,unionized}}$$

Liquid-liquid extraction workflow



Simplified liquid extraction



Add internal standard to aliquoted sample

↓

Dilute sample using appropriate buffer
(render analytes in non-ionized form)

↓

Load sample, wait

↓

Elute

↓

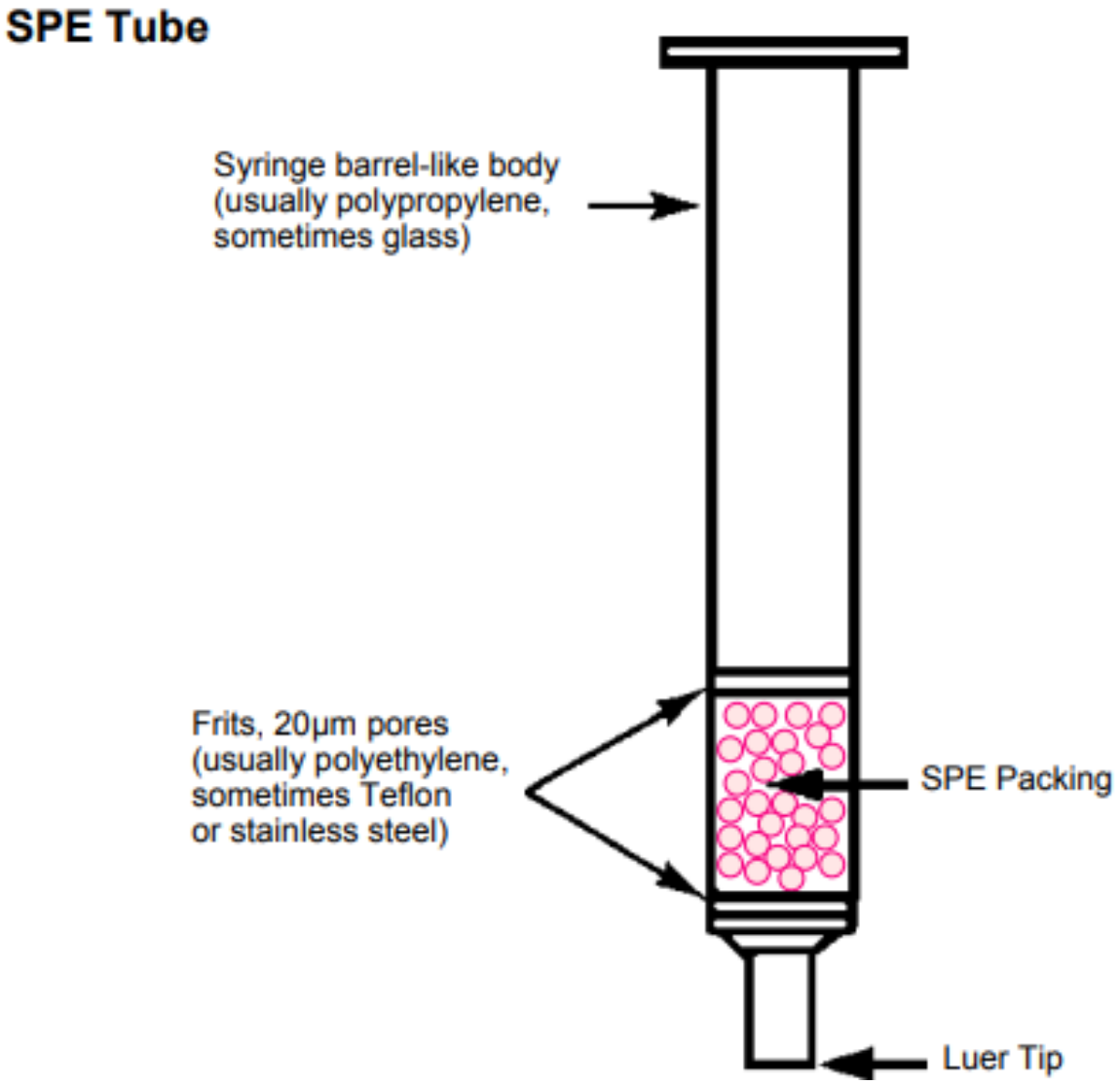
Concentrate or exchange solvent

Analysis of vitamin D metabolites in plasma

- take 0.5 mL plasma
- add 10 µL IS solution (2.5 µg/mL $^{2}H_{6}$-25-hydroxycholecalciferol in methanol)
- add 0.5 mL water
- extract 2 times using 1 mL ethyl acetate
- combine organic phases
- evaporate to dryness
- reconstitute using 0.5 mL methanol
- concentrate to 0.15 mL
- add 0.15 mL water

Karvaly G et al. Clin Chem Lab Med 2017;55:385.

Solid phase extraction



Bulletin 910: Guide to solid phase extraction. Sigma Aldrich, 1998.

The principles of solid phase extraction are very similar, but not analogous to liquid chromatography

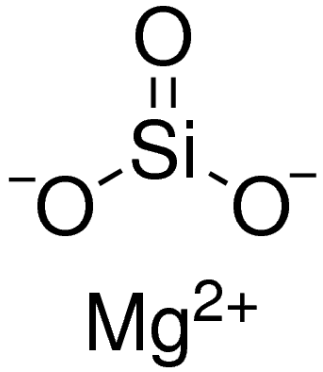
- several HPLC phases are used
- particle diameter is much larger (app. 30-60 µm)
- extraction tubes are tightly packed

Sorbents employed in solid phase extraction

- normal phase:
  - alumina-based
  - polymer-based
  - silica-based

- reversed phase:
  - DVB (styrene-divinylbenzene)
  - polymer-based
  - silica-based

- ion exchange:
    - polymer-based



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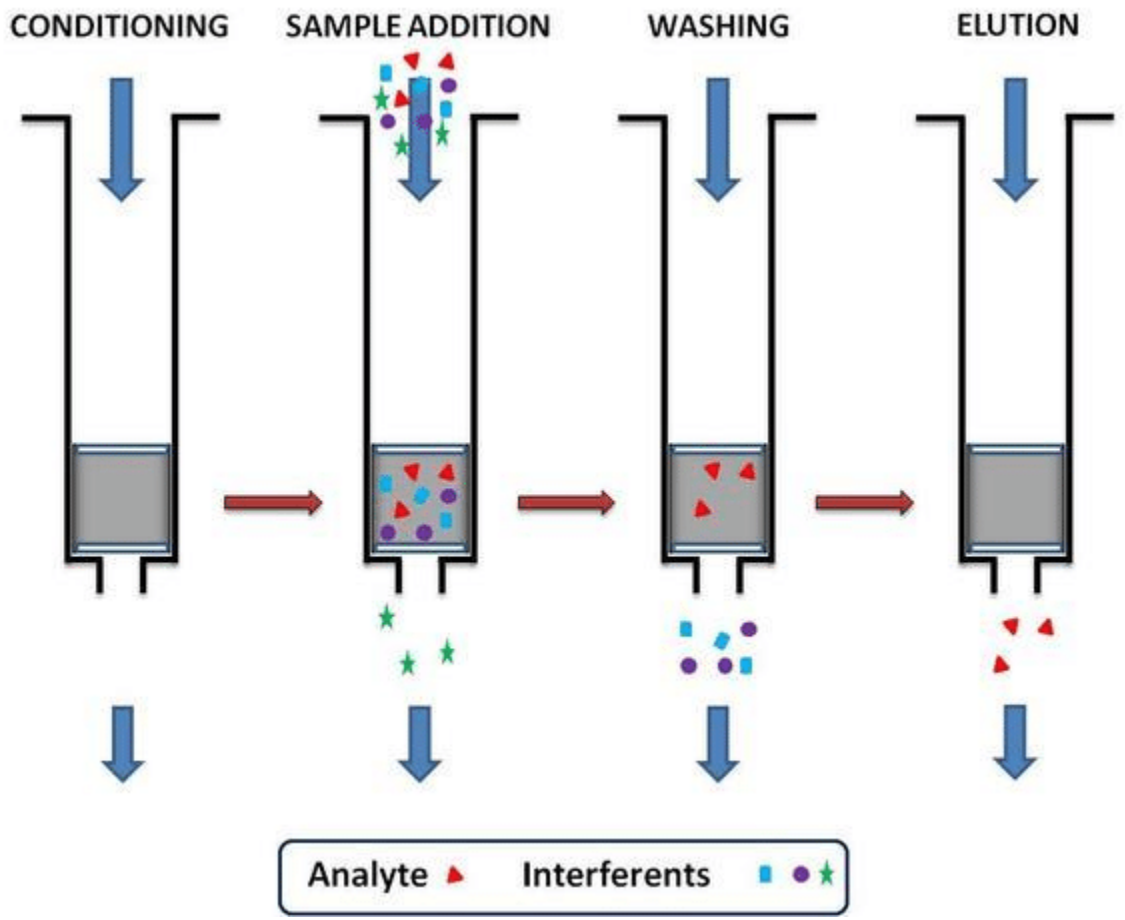
Steps in developing an SPE method

- select a sorbent phase you expect to be suitable
- develop initial protocol: use analyte solution first
- compare to alternatives
- optimize variables using spiked matrix (solvents, pH, volumes, steps)
- check for interferences („ghosts”)
- check for robustness
- validate method

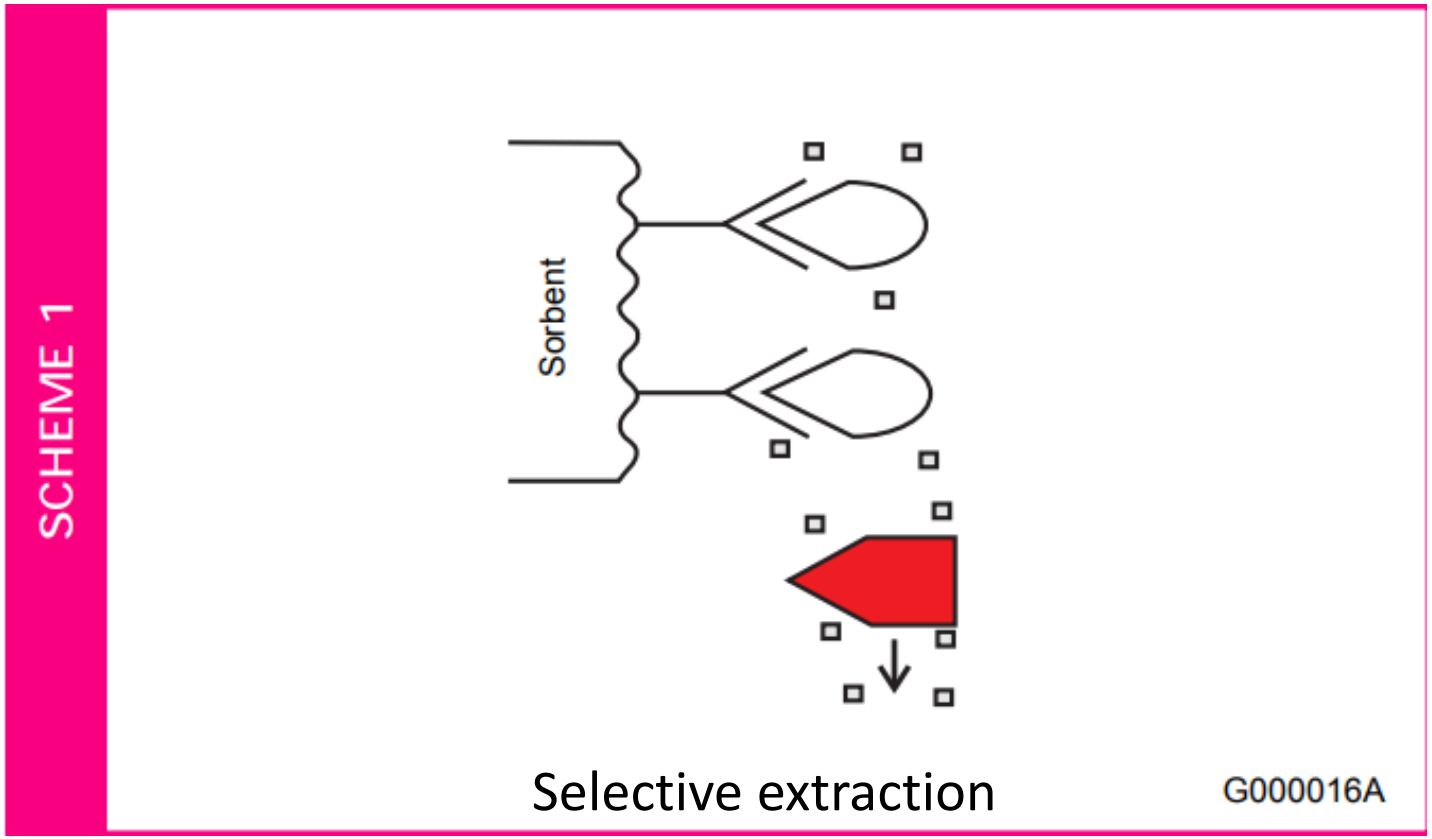
Analyte recovery is important, but is only one variable among many!

SPE workflow



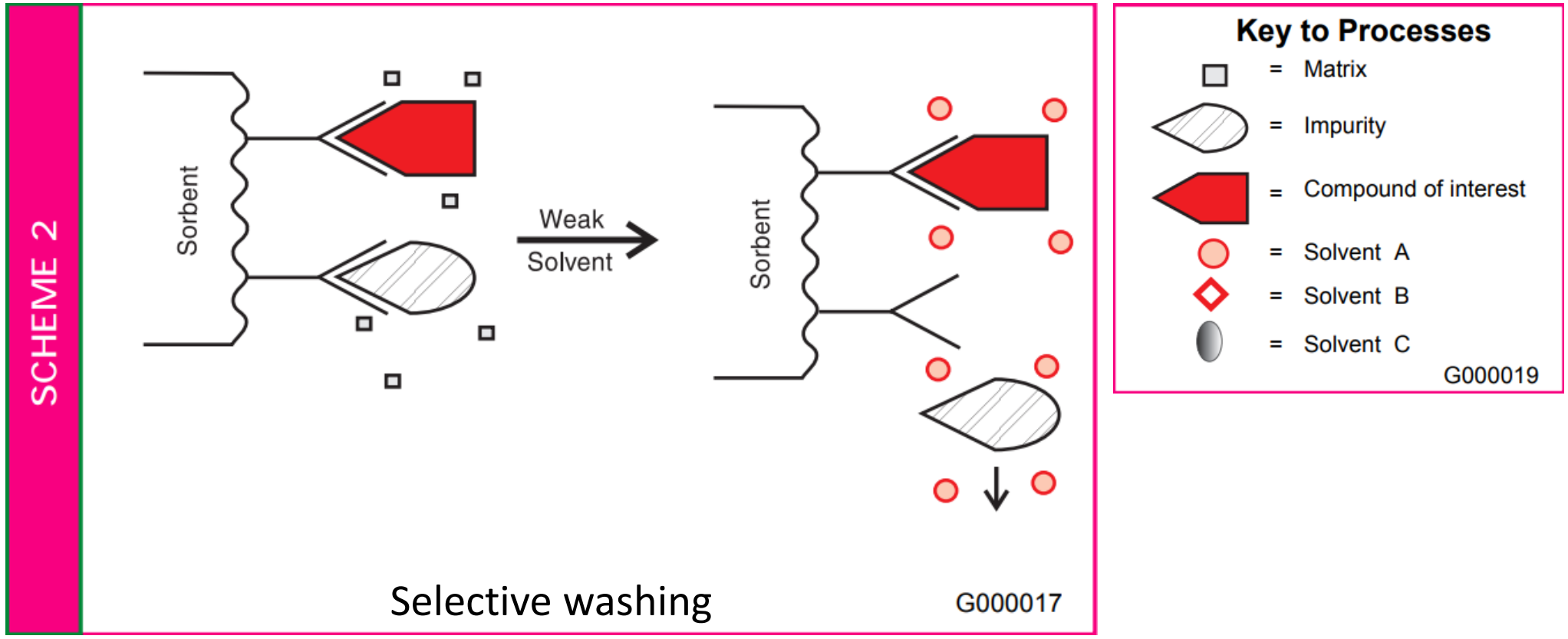
Bulletin 910: Guide to solid phase extraction. Sigma Aldrich, 1998.

Uses of SPE



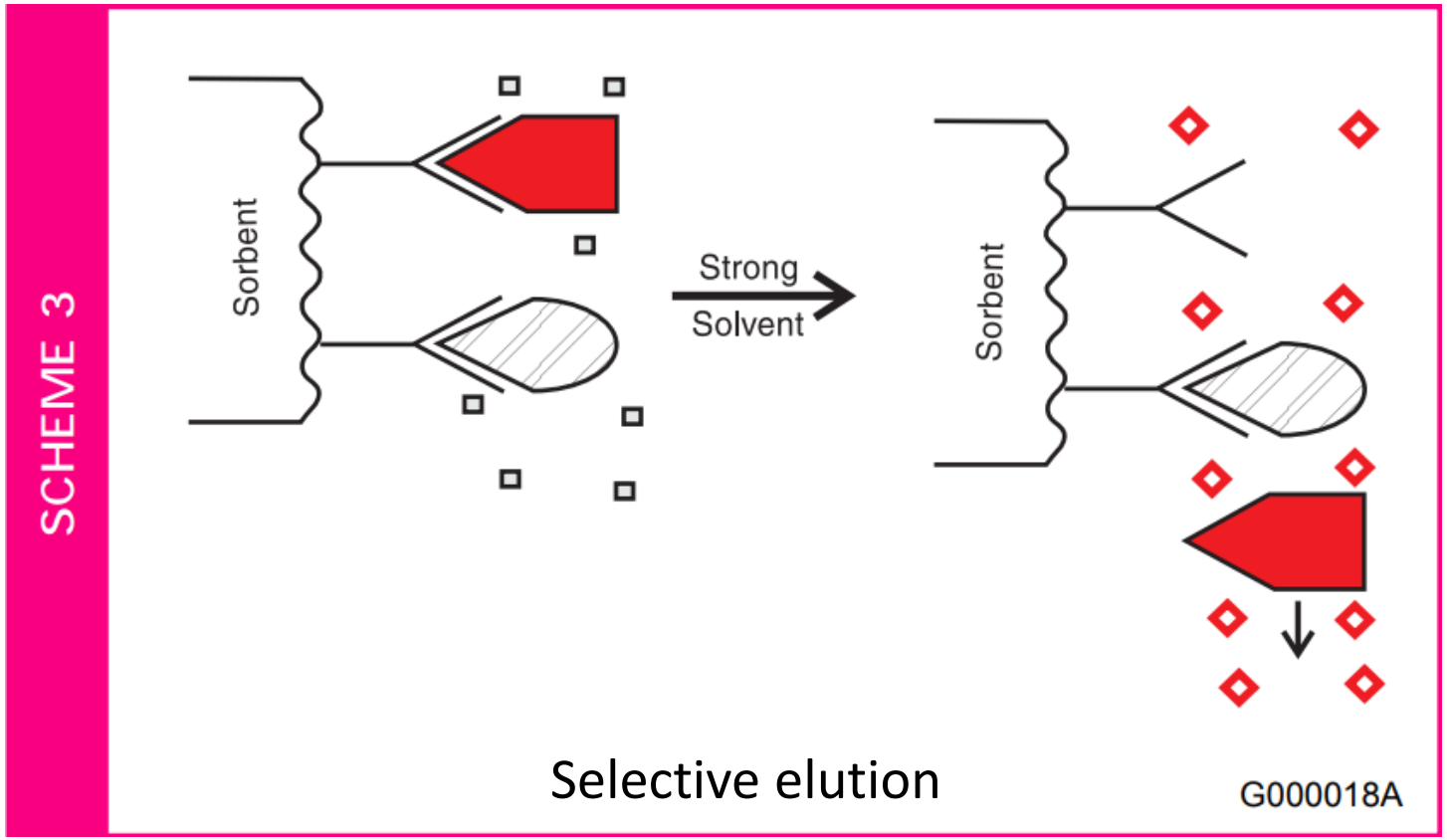
Bulletin 910: Guide to solid phase extraction. Sigma Aldrich, 1998.

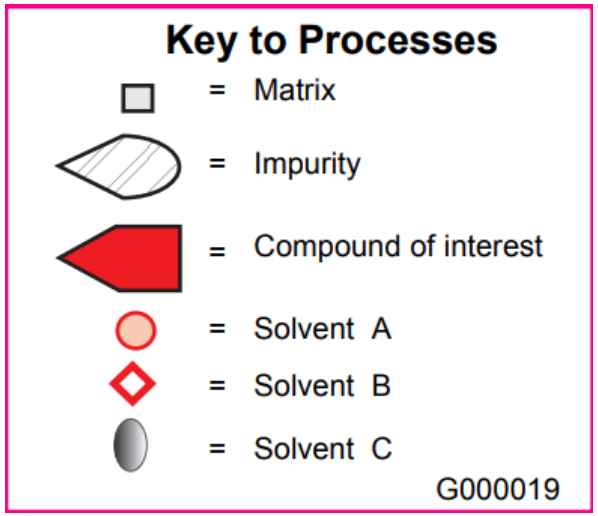
Uses of SPE



Bulletin 910: Guide to solid phase extraction. Sigma Aldrich, 1998.

Uses of SPE





Bulletin 910: Guide to solid phase extraction. Sigma Aldrich, 1998.

Mechanisms of retention on SPE phases

Reversed Phase
(polar liquid phase, nonpolar modified solid phase)

Hydrophobic interactions

- nonpolar-nonpolar interactions
- van der Waals or dispersion forces

Normal Phase
(nonpolar liquid phase, polar modified solid phase)

Hydrophilic interactions

- polar-polar interactions
- hydrogen bonding
- pi-pi interactions
- dipole-dipole interactions
- dipole-induced dipole interactions

Ion Exchange

Electrostatic attraction of charged group on compound to a charged group on the sorbent's surface

Adsorption
(interactions of compounds with unmodified materials)

Hydrophobic and hydrophilic interactions may apply
Depends on which solid phase is used

Bulletin 910: Guide to solid phase extraction. Sigma Aldrich, 1998.

Suitable target analytes

- inorganic ions (SAX, SCX)
- acidic and basic substances (NH_2, WAX, WCX)
- neutral small molecules (reversed phase)
- large biomolecules (proteins, nucleic acids)

Sample Characteristics Determine Your SPE Procedure

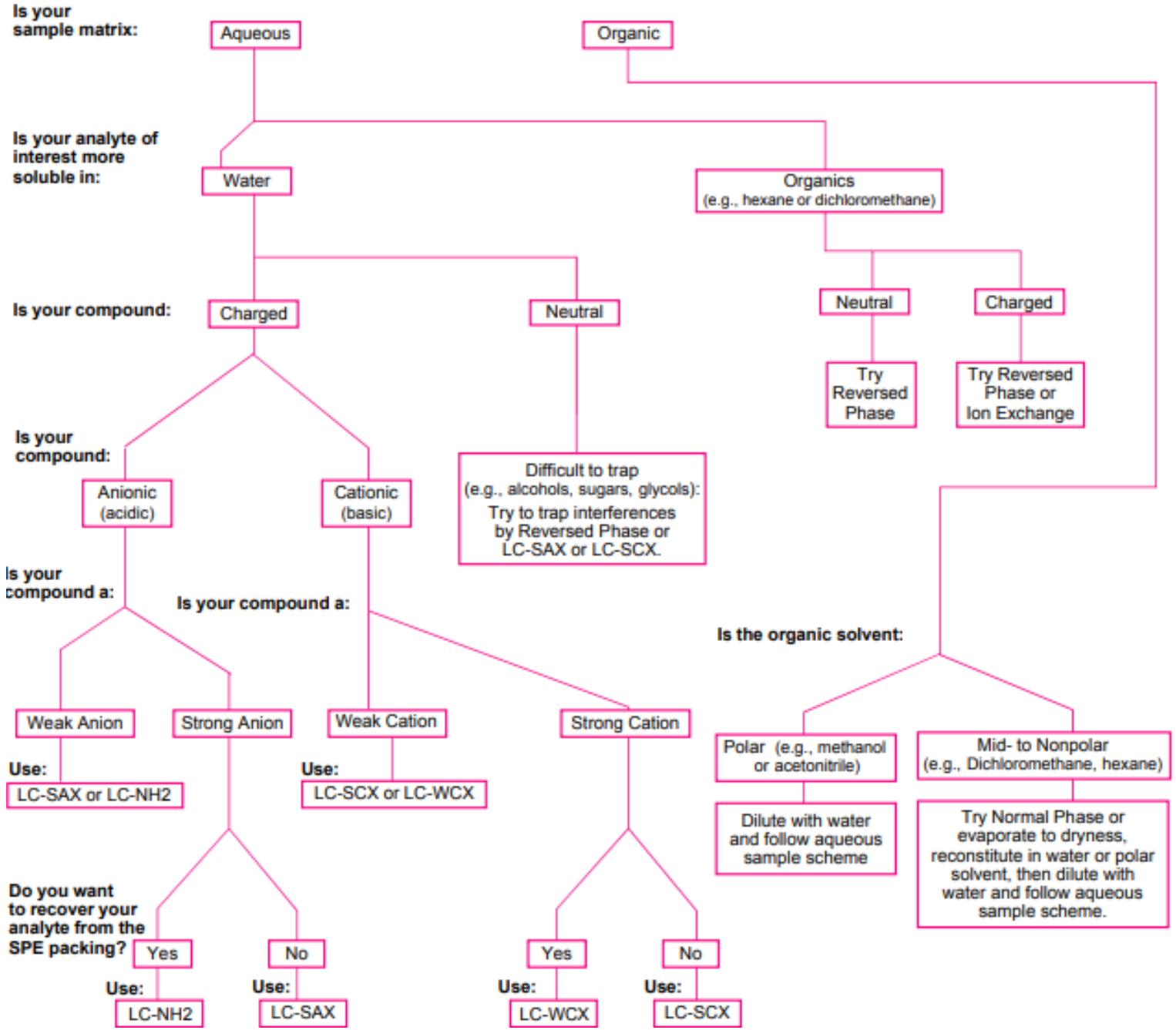


Table A. Characteristics of Solvents Commonly Used in SPE

Polarity			Solvent	Miscible in Water?
Nonpolar	Strong Reversed Phase	Weak Normal Phase	Hexane	No
			Isooctane	No
			Carbon tetrachloride	No
			Chloroform	No
			Methylene chloride (dichloromethane)	No
			Tetrahydrofuran	Yes
			Diethyl ether	No
			Ethyl acetate	Poorly
			Acetone	Yes
			Acetonitrile	Yes
			Isopropanol	Yes
	Weak Reversed Phase	Strong Normal Phase	Methanol	Yes
			Water	Yes
Polar			Acetic acid	Yes

SPE formats

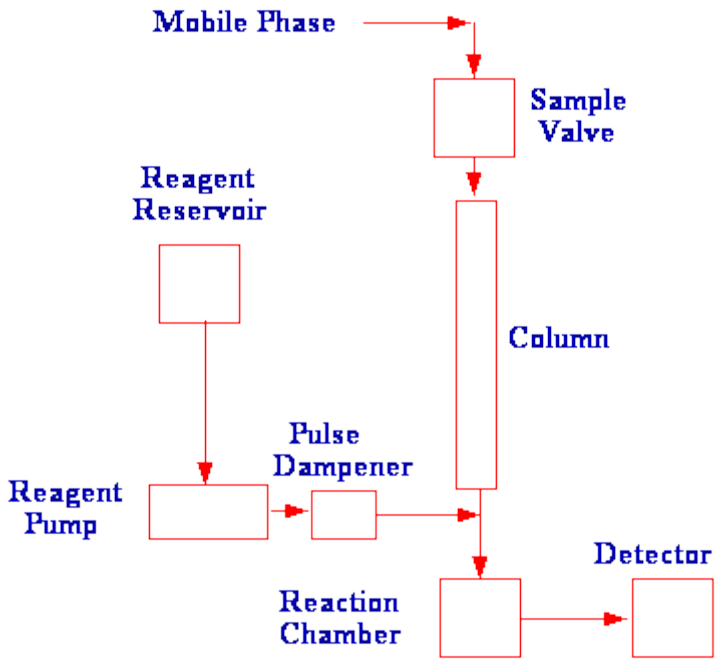
- standalone tubes
- tabless tubes
- 96-well plates

Derivatization in liquid chromatography and mass spectrometry

- Aims:
    - increase analyte stability
    - increase solubility
    - improve chromatographic properties
    - increase detection sensitivity
    - increase selectivity
    - reduce matrix effect
    - allow chiral separation in achiral systems

Derivatization in liquid chromatography and mass spectrometry

- pre-column derivatization: part of the preanalytical workup
- post-column derivatization: part of the analytical assay



Derivatization for LC-MS/MS assays

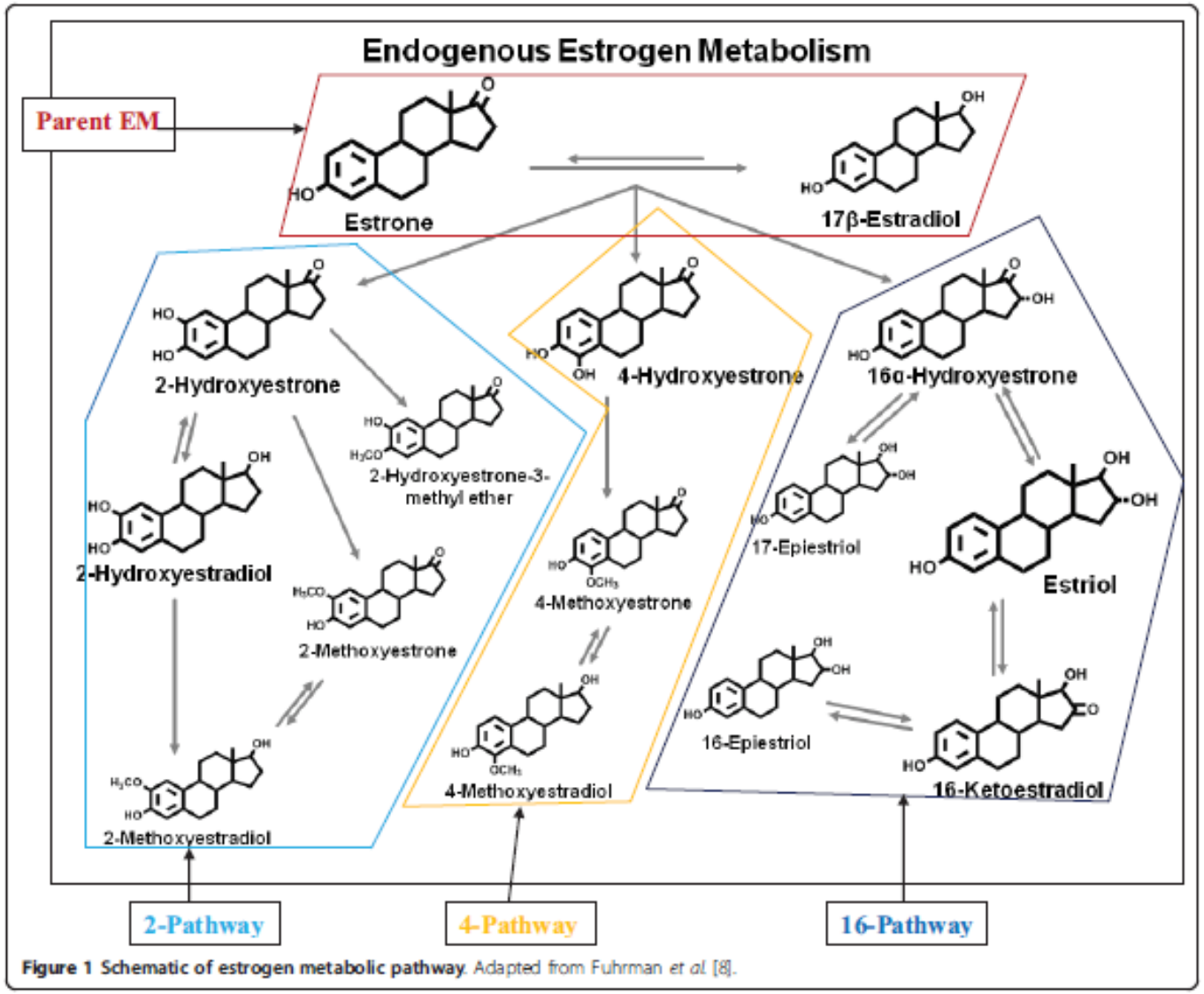
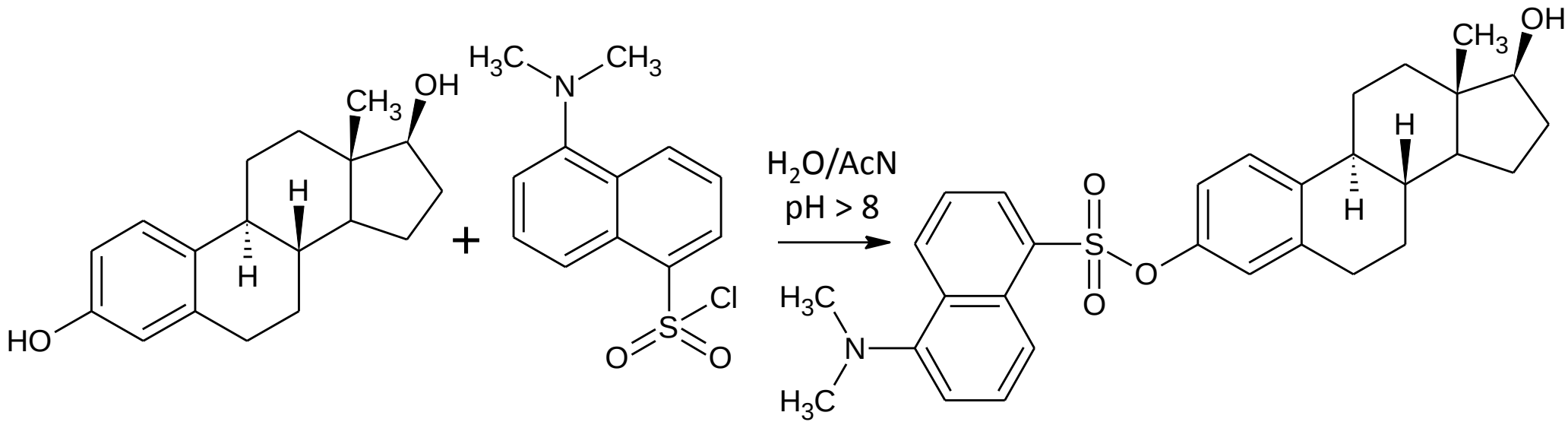
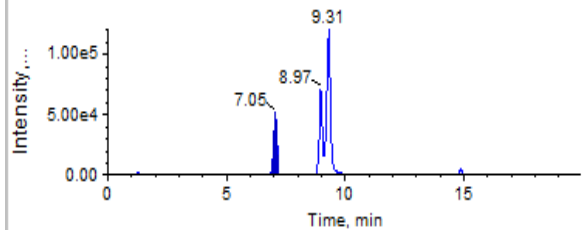


Figure 1 Schematic of estrogen metabolic pathway. Adapted from Fuhrman *et al.* [8].

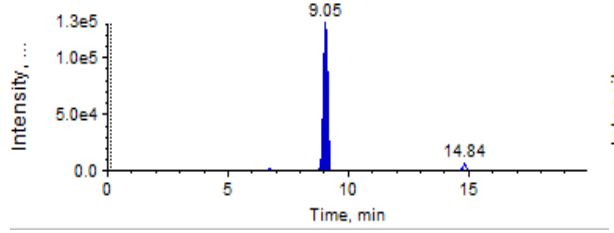


estradiol dansyl chloride

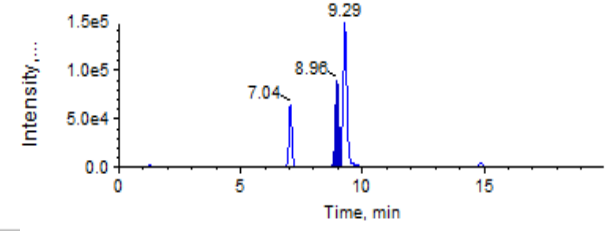
Emix-2 - estriol (Unknown) 522.000/171.000 Da - sample 10 of 12 from E..
Area: 4.47e+005 counts Height: 5.14e+004 cps RT: 7.05 min



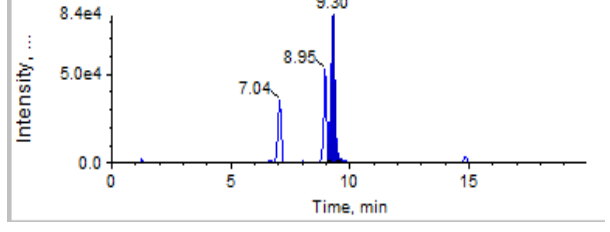
Emix-2 - 16OHE1 (Unknown) 520.500/171.000 Da - sample 10 of 1...
Area: 1.44e+006 counts Height: 1.31e+005 cps RT: 9.05 min



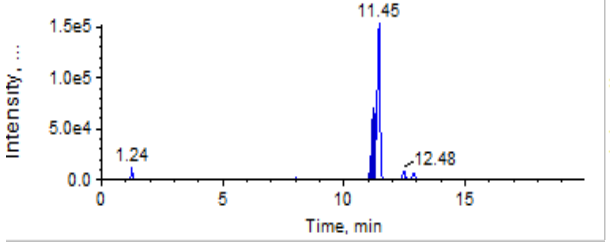
Emix-2 - 16epiE3 (Unknown) 522.100/171.100 Da - sample 10 of 12 from
Area: 8.28e+005 counts Height: 8.90e+004 cps RT: 8.96 min



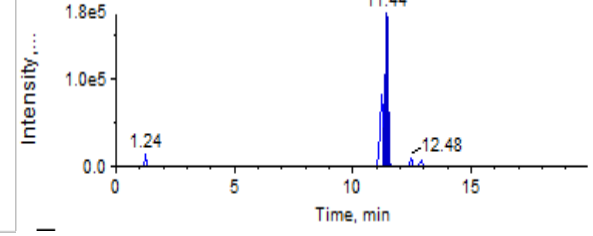
Emix-2 - 17epiE3 (Unknown) 521.900/170.900 Da - sample 10 of 12 from
Area: 8.13e+005 counts Height: 8.26e+004 cps RT: 9.30 min



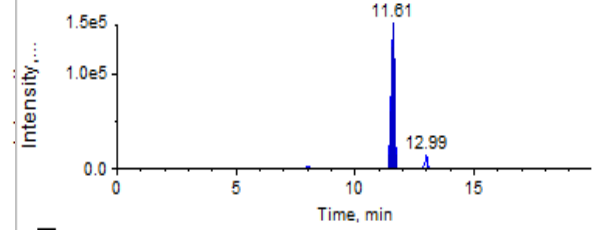
Emix-2 - 2OMeE2 (Unknown) 536.500/171.000 Da - sample 10 of 12 from
Area: 6.43e+005 counts Height: 7.05e+004 cps RT: 11.2 min



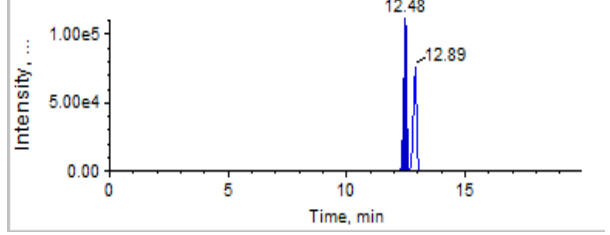
Emix-2 - 4OMeE2 (Unknown) 536.400/171.100 Da - sample 10 of 12 from.
Area: 1.51e+006 counts Height: 1.75e+005 cps RT: 11.4 min



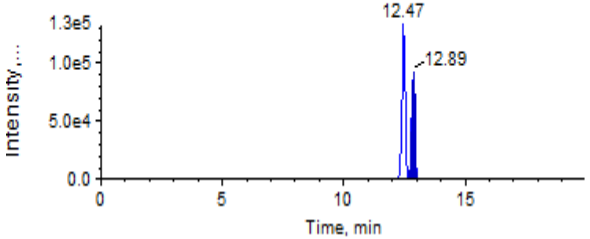
Emix-2 - estradiol (Unknown) 506.500/171.000 Da - sample 10 of 12 from
Area: 1.32e+006 counts Height: 1.52e+005 cps RT: 11.6 min



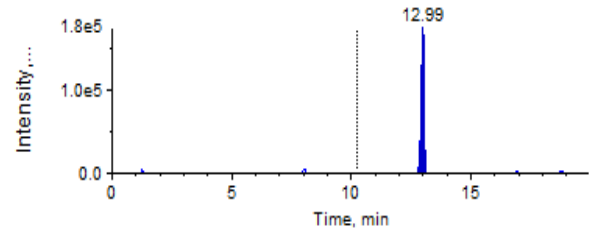
Emix-2 - 2OMeE1 (Unknown) 534.500/171.000 Da - sample 10 of 12 from.
Area: 8.99e+005 counts Height: 1.11e+005 cps RT: 12.5 min



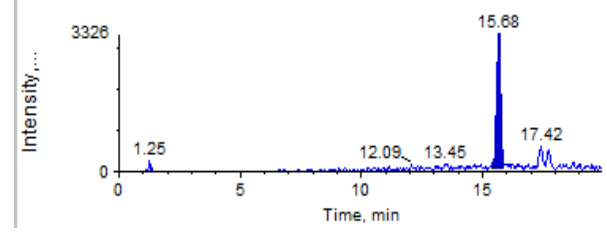
Emix-2 - 4OMeE1 (Unknown) 534.400/171.100 Da - sample 10 of 12 from
Area: 7.85e+005 counts Height: 9.21e+004 cps RT: 12.9 min



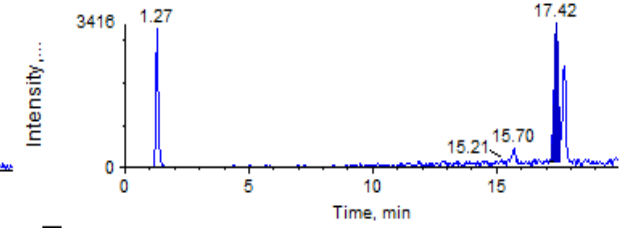
Emix-2 - estrone (Unknown) 504.500/171.000 Da - sample 10 of 12 from ..
Area: 1.45e+006 counts Height: 1.76e+005 cps RT: 13.0 min



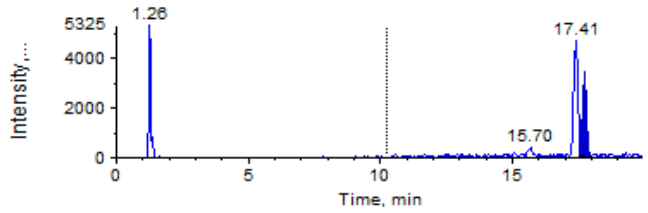
Emix-2 - 2OHE2 (Unknown) 756.000/171.000 Da - sample 10 of 12 from
Area: 3.45e+004 counts Height: 3.23e+003 cps RT: 15.7 min

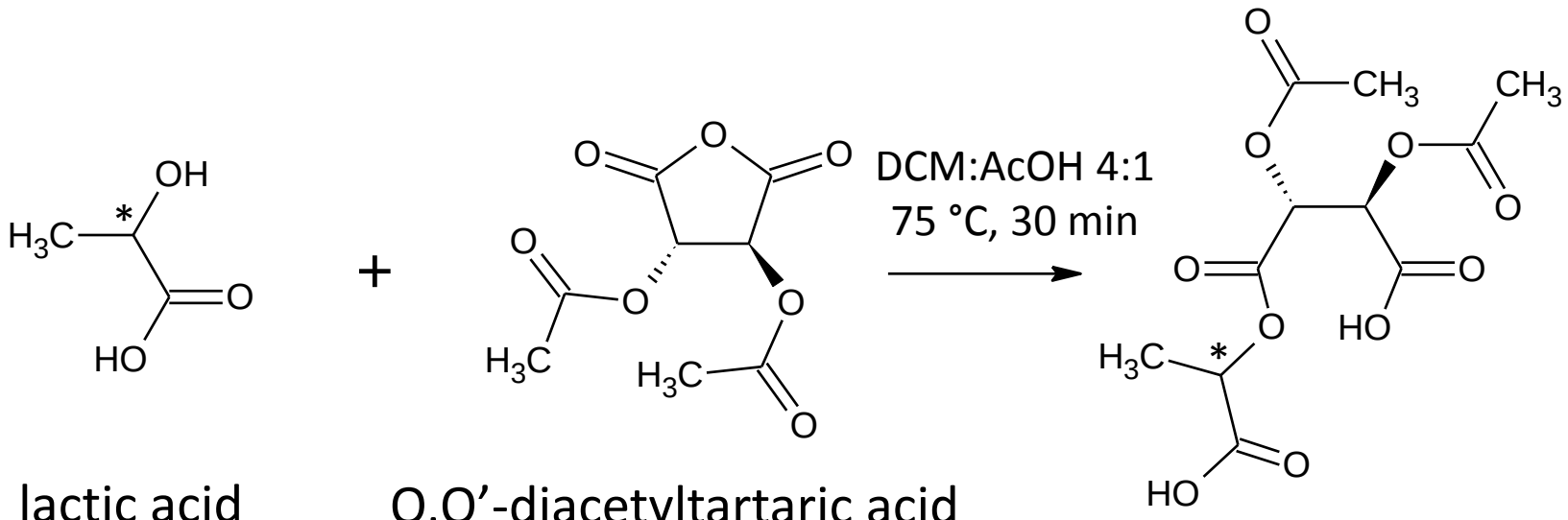


Emix-2 - 2OHE1 (Unknown) 754.000/171.000 Da - sample 10 of 12 from E
Area: 3.42e+004 counts Height: 3.26e+003 cps RT: 17.4 min



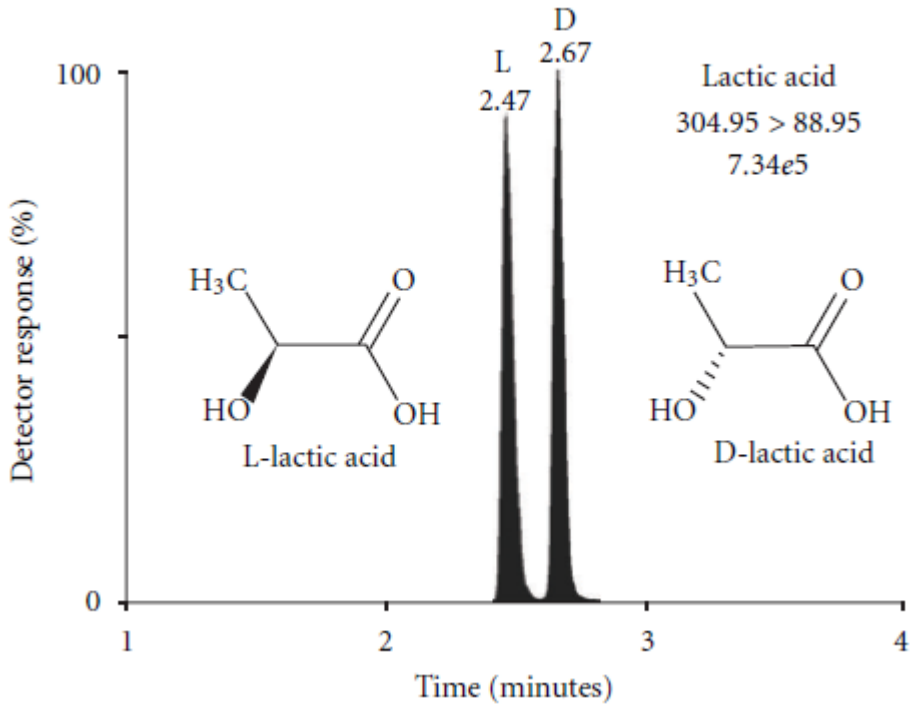
Emix-2 - 4OHE1 (Unknown) 754.100/171.100 Da - sample 10 of 12 from ..
Area: 3.41e+004 counts Height: 3.35e+003 cps RT: 17.7 min

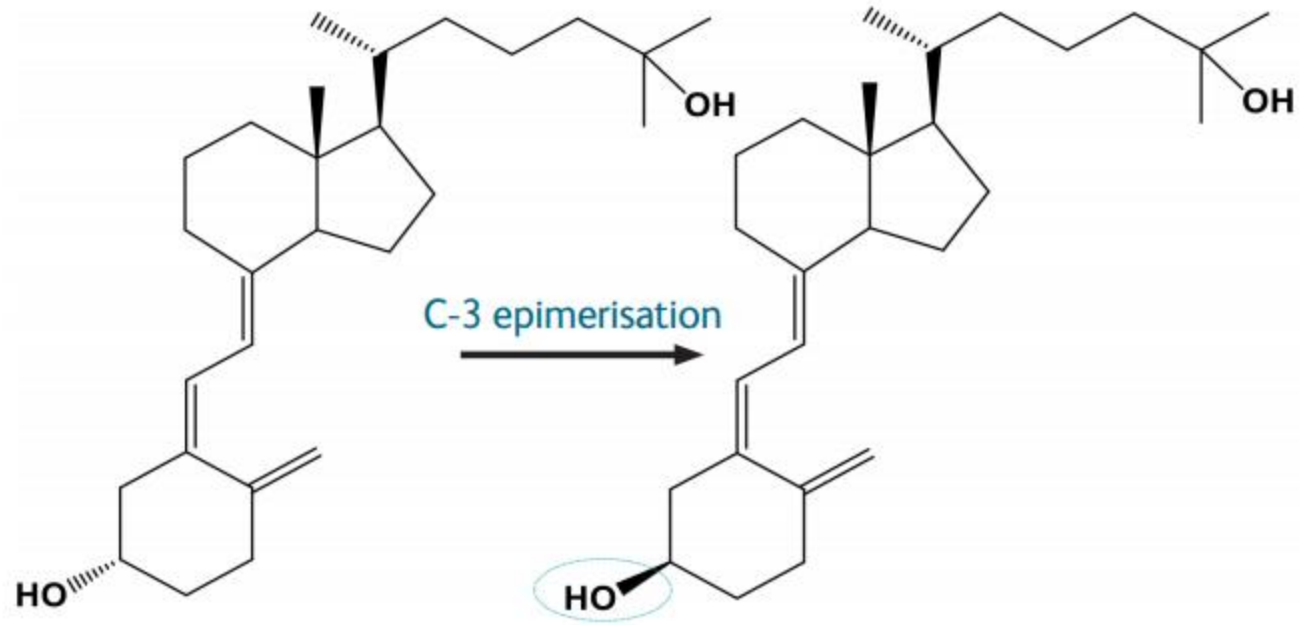




lactic acid

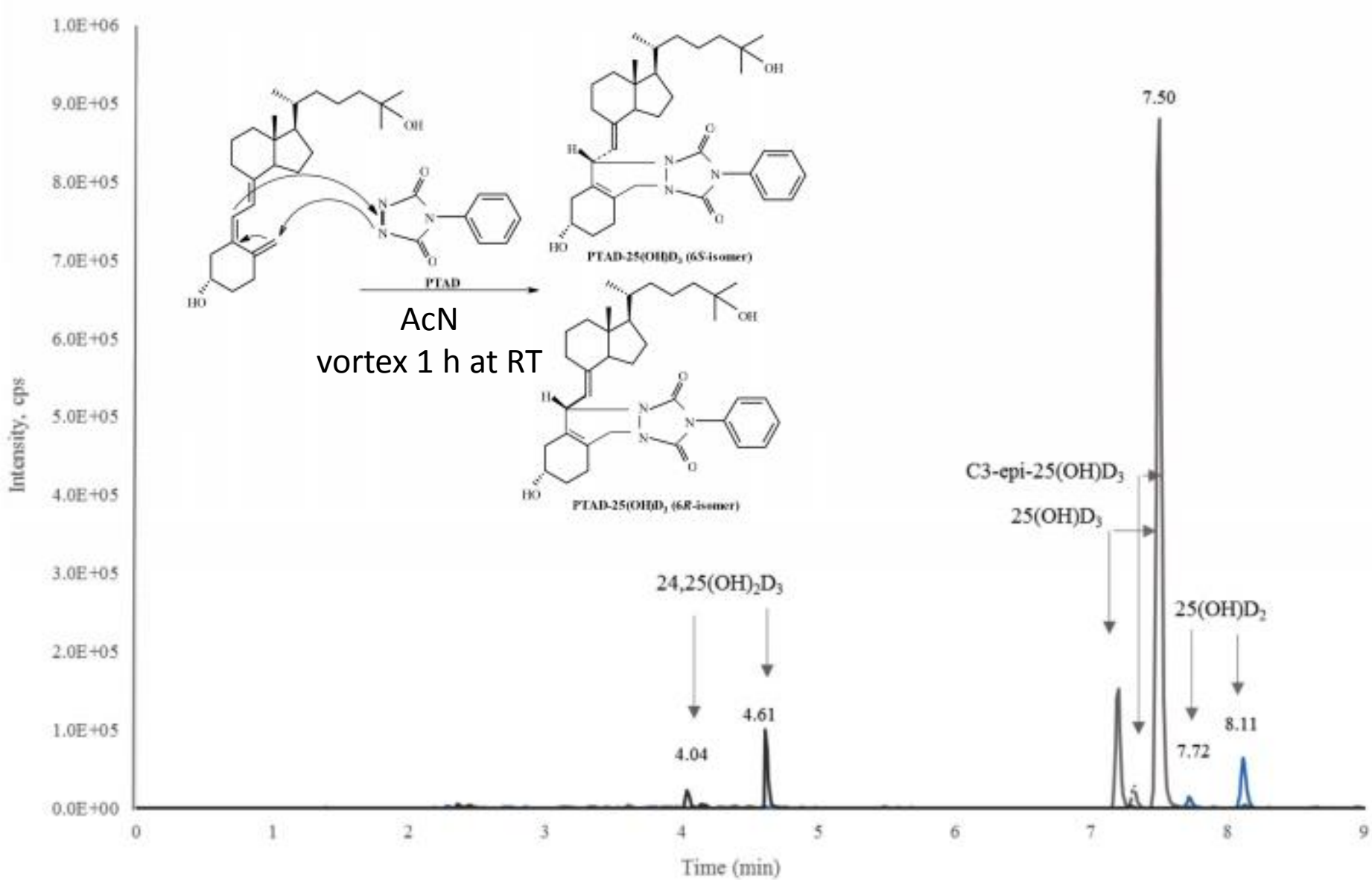
O,O’-diacetyltartaric acid





25-hydroxyvitamin D3

3-epi-25-hydroxyvitamin D3



PTAD: 4-phenyl-1,2,4-triazoline-3,5-dione

Higashi T et al. Anal Bioanal Chem 2008;391:229.

Review

DOI: 10.5582/ddt.2013.v7.1.9

Derivatization in liquid chromatography for mass spectrometric detection

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