Sample Preparation for Improved LC Bioseparations

Melissa Goodlad
Columns and Supplies Technical Support
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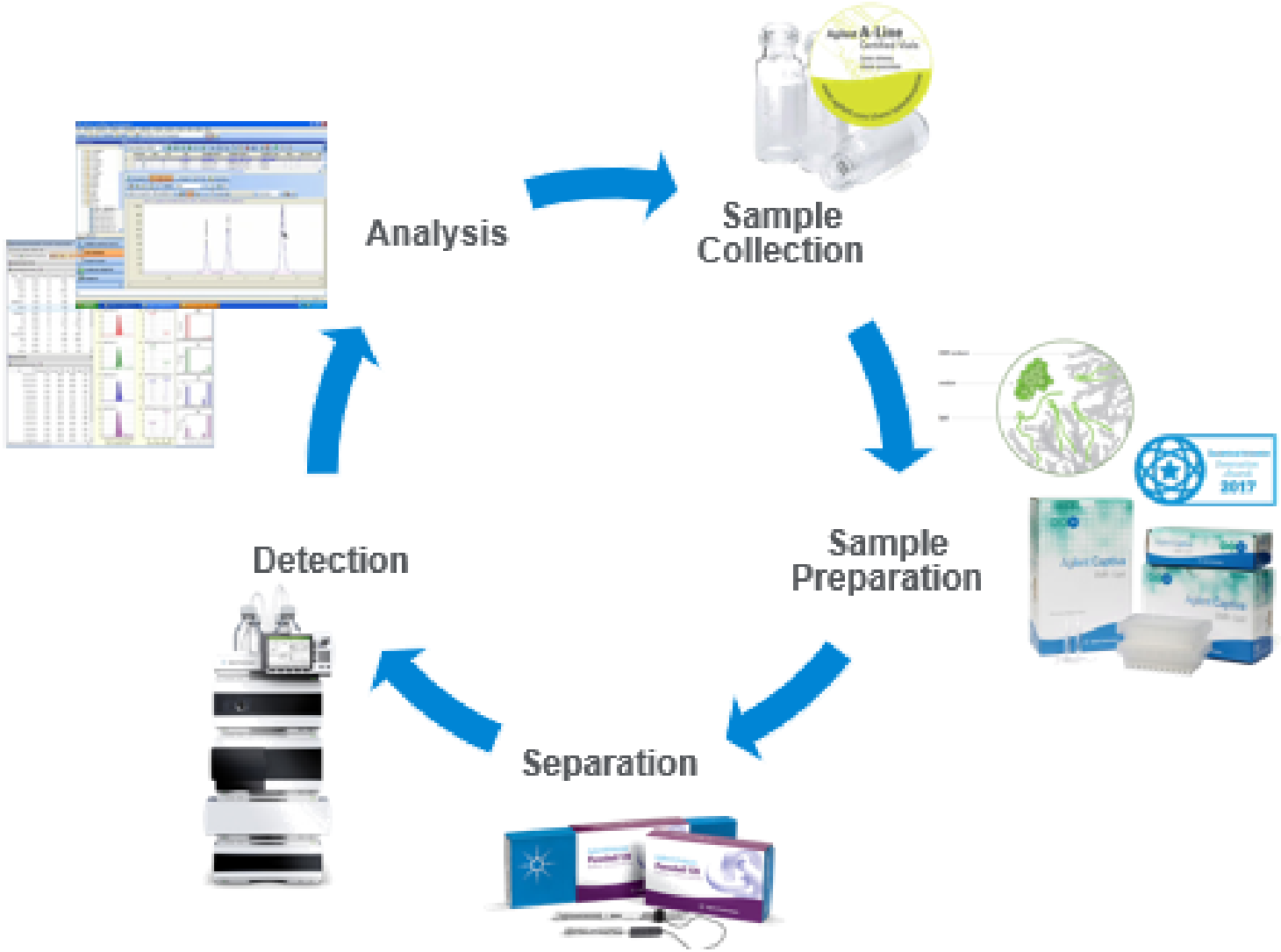
Agilent
Trusted Answers

Sample Preparation for Improved LC Bioseparations

Agenda

- **Why Is Sample Preparation Necessary?**
- **Background**
  - Analyte properties
  - Matrix considerations
  - Common techniques
- **Sample preparation techniques for:**
  - Lipids
  - Peptides
  - Proteins
  - Oligonucleotides



Sample Preparation Fundamentals for Chromatography (agilent.com)

Sample Preparation for Improved LC Bioseparations

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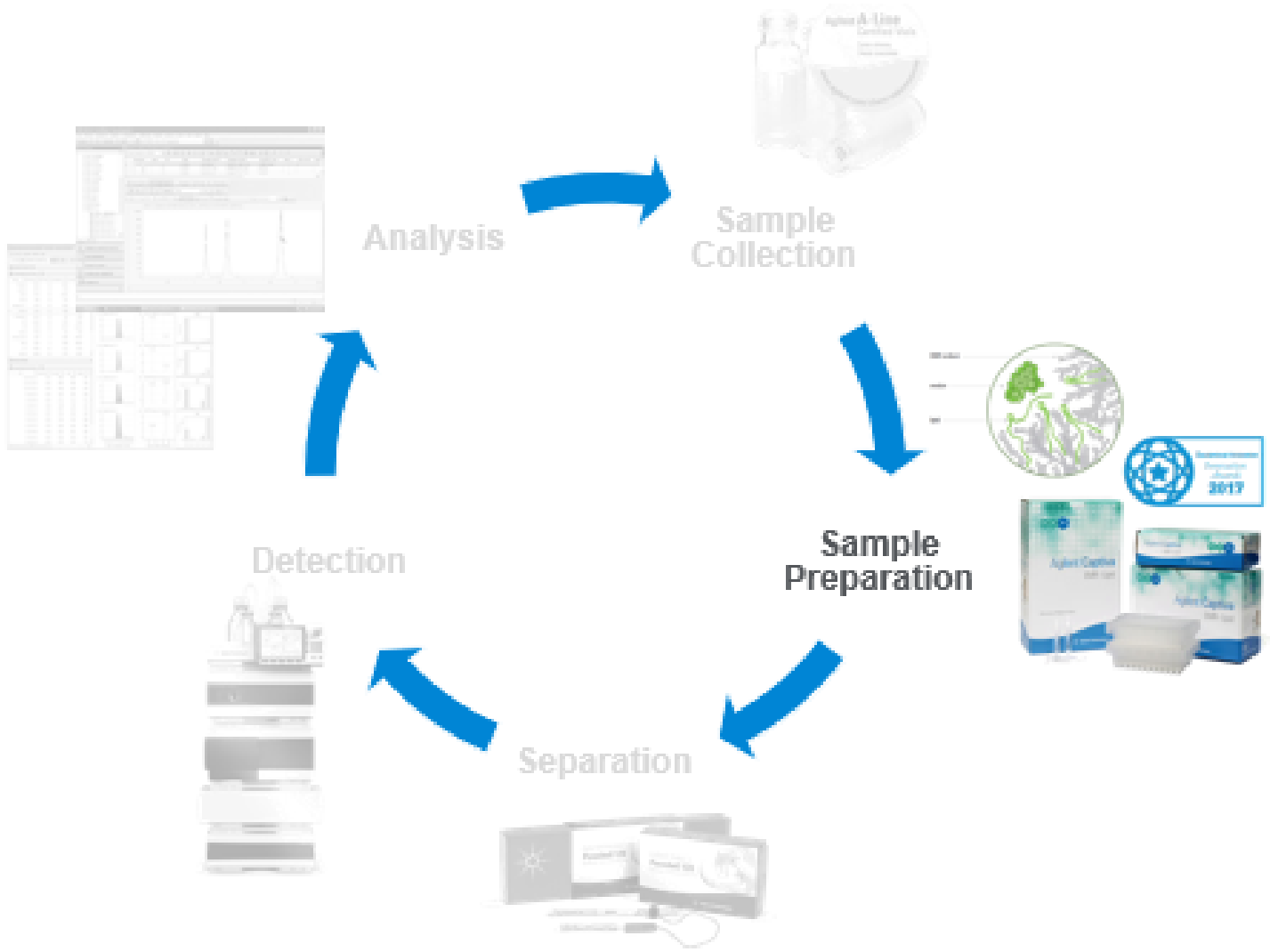


Sample Preparation Fundamentals for Chromatography (agilent.com)

Sample Preparation for Improved LC Bioseparations

Overview

Speakers

Golnar Javadi
Application Engineer
Agilent Technologies, Inc.

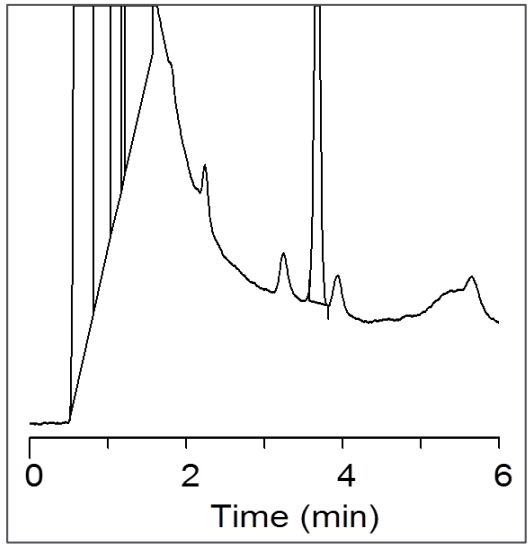
[Tips for Developing Successful Solid Phase Extraction Methods (on24.com)]

Why Is Sample Preparation Necessary?

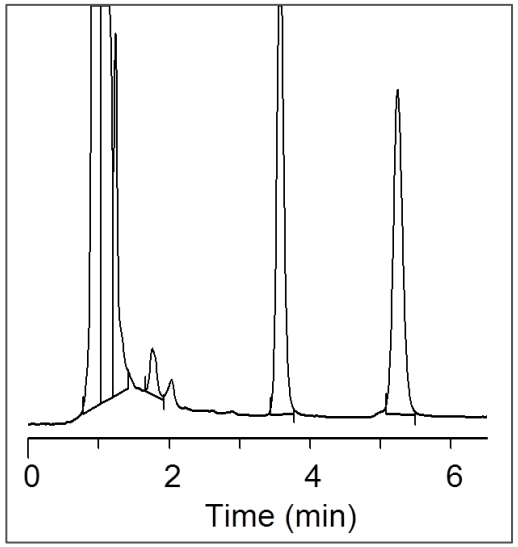
Why Is Sample Preparation Necessary?

Improve data quality

Improve sensitivity

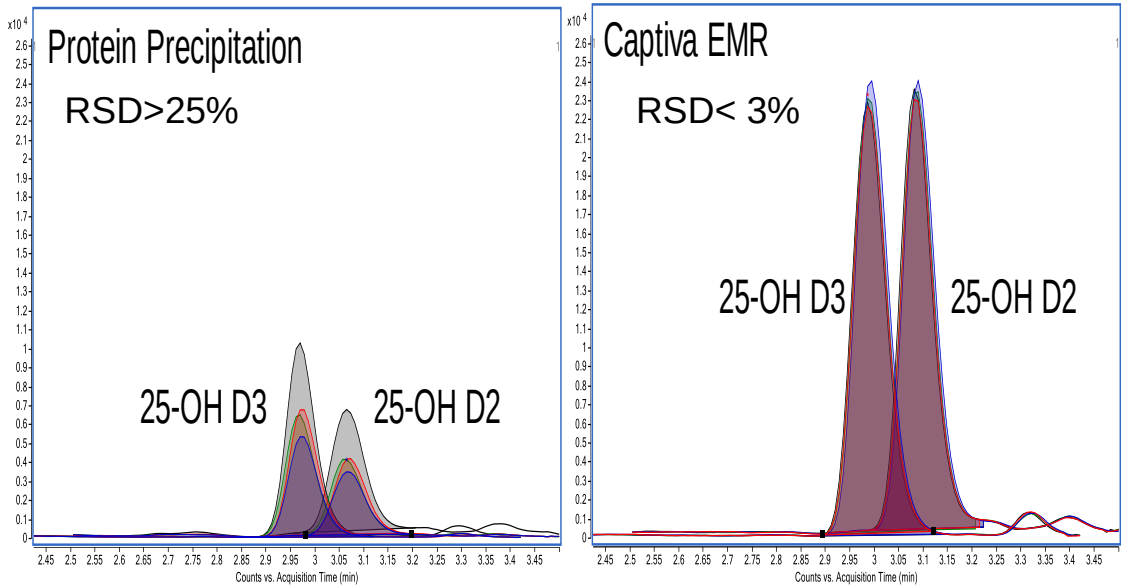


Without sample prep



With sample prep

Improve reproducibility



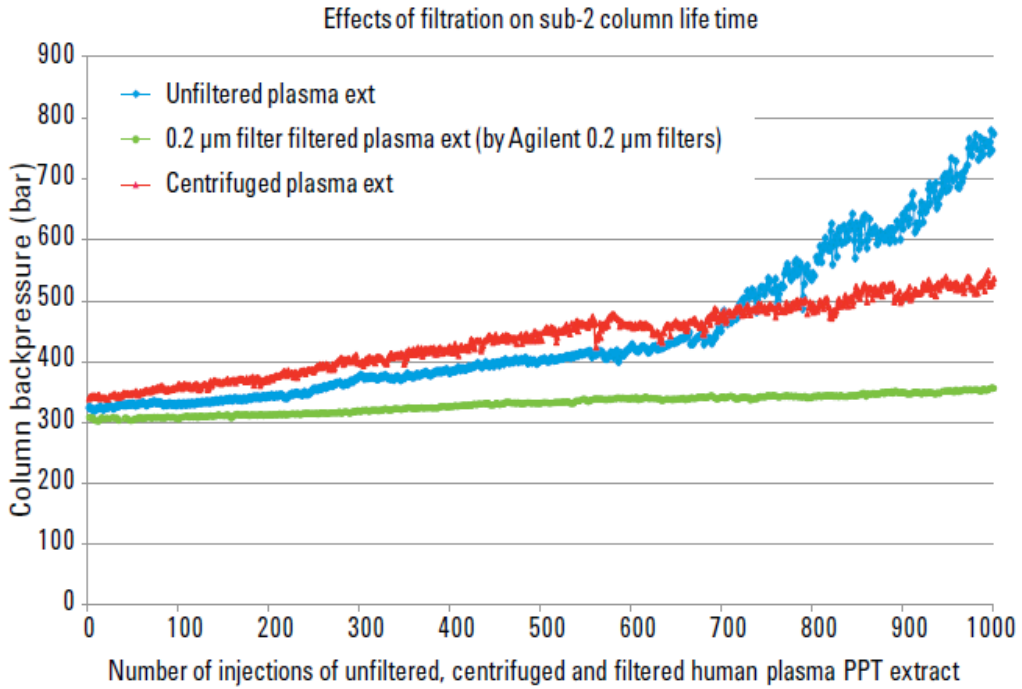
MRM overlay (n=4) of 10 25-OH D2 and 25-OH D3 at 50 ng/mL with protein precipitation and Agilent Captiva EMR-Lipid

5991-7956EN.pdf (agilent.com)

Why Is Sample Preparation Necessary?

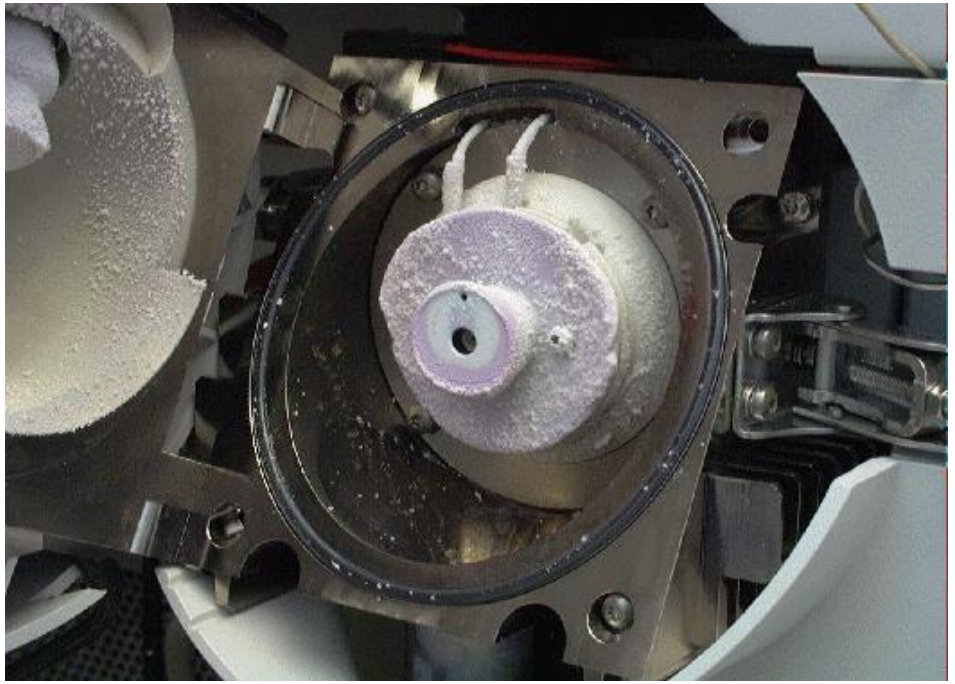
Improve throughput

Replacement of parts



Effect of filtration on a sub 2-micron column lifetime

System downtime

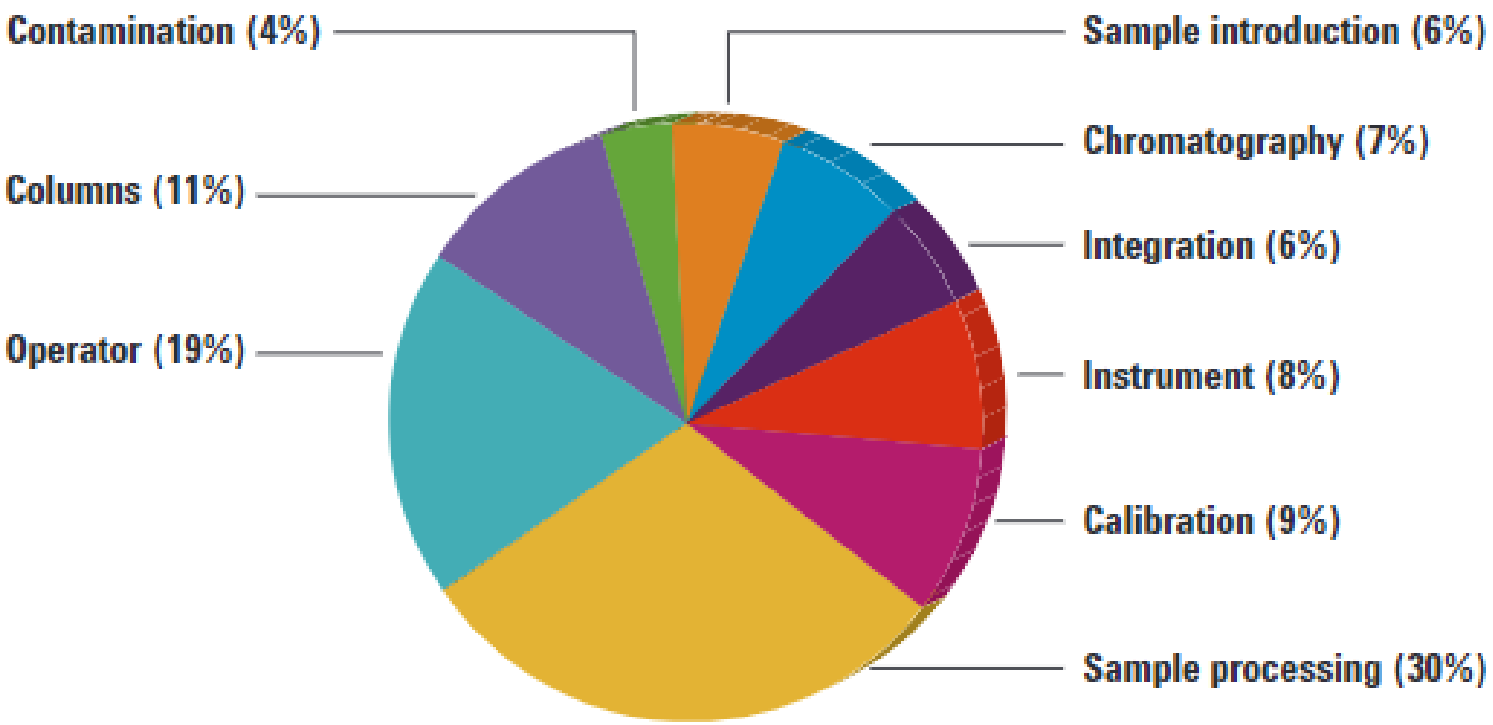


Salt build-up in LC-MS ion source from unextracted salts

Why Is Sample Preparation Necessary?

Figure 1.3

Sources of Error Generated During Chromatographic Analysis

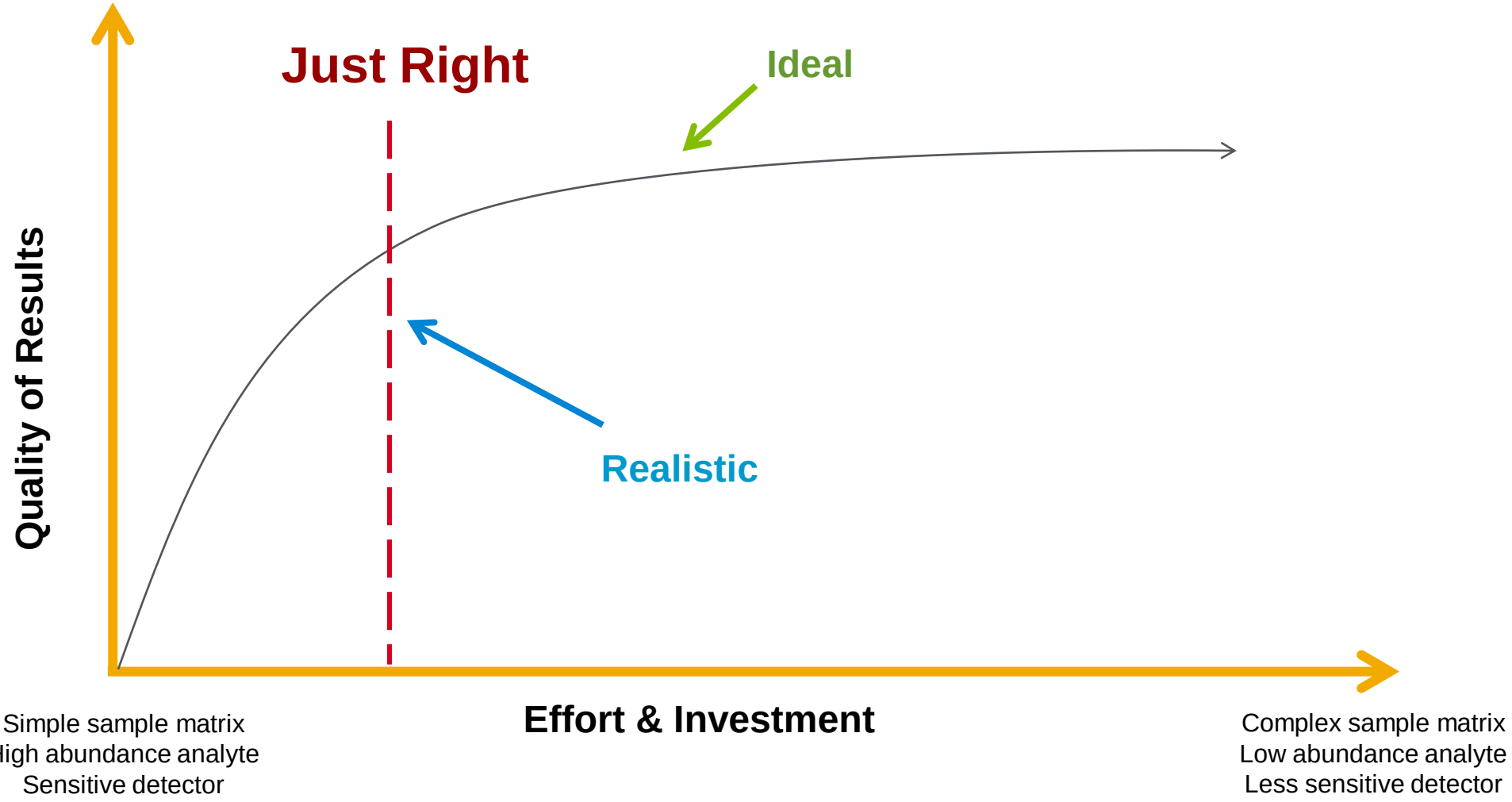


Data taken from Agilent Technologies survey

[Sample Preparation Fundamentals for Chromatography (agilent.com)]

Why Is Sample Preparation Necessary?

"Just right" sample prep



Sample Preparation for Biomolecules

Making Decisions about Sample Preparation

What should I know about my analyte?

- Is the analyte nonpolar or polar?
- Does the analyte contain any ionic groups?
- How large or small is the analyte?
- Is the analyte unstable in acid or base?
- What it the logP and pKa of the analyte?
- What is the approximate concentration of the analyte in sample?
- Any likelihood of the analyte undergoing nonspecific binding to glassware or plastic?
- What is the detection limit of the analytical system for the analyte

Making Decisions About Sample Preparation

What should I know about my sample matrix?

- What is the sample matrix?
- What sample pre-treatment steps may be required?
- What are the key functional groups on your matrix, interferences and analyte of interest?
- What key interferences are endogenous to the sample?
- What level of interference removal is required for analysis?

Salts, residual buffers, nucleotides, metabolites, phospholipids, ionic detergents, nucleic acids, polysaccharides, lipids, phenolic compounds, insoluble material

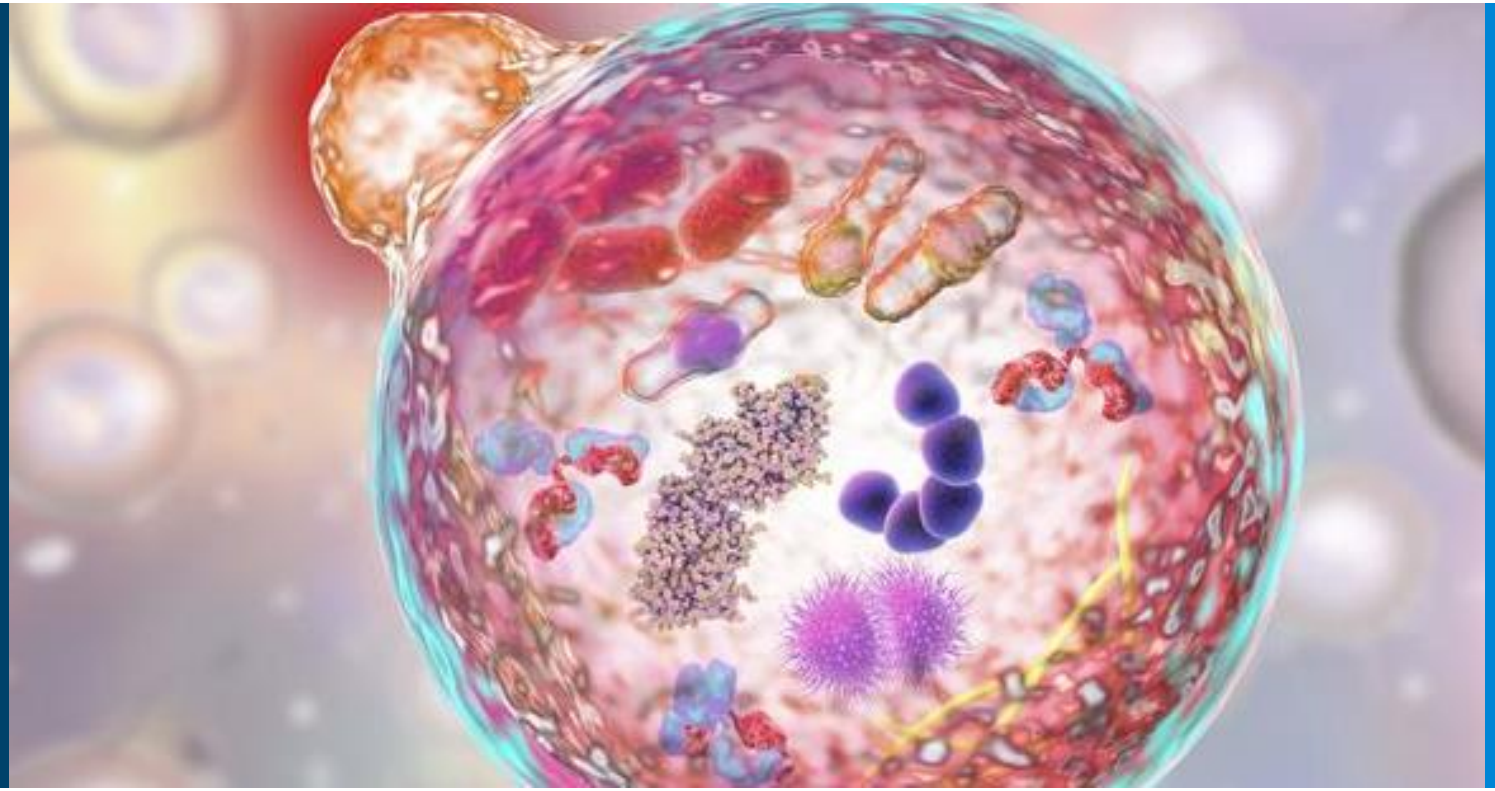
Making Decisions About Sample Preparation

Less Selective → More Selective

Technique	Strength	Weakness
Dilute & Shoot	Fast, cheap, simple	All matrix components still present Negative impact on LOD, column and instrument lifetime
Filtration	Fast, cheap, simple Less likely to clog column	All matrix components still present Negative impact on LOD, column and instrument lifetime
Protein Precipitation (PPT)	Simplicity, easy to combine with other methods	Only removes proteins from the matrix, multiple steps and transfers, low throughput
Liquid Liquid Extraction (LLE)	Simple operation, relatively efficient purification	Requires method development for analyte, difficult
Solid Phase Extraction (SPE)	Highly selective and efficient purification, automatable, scalable	Requires method development for analyte

Sample Preparation by Analyte Type

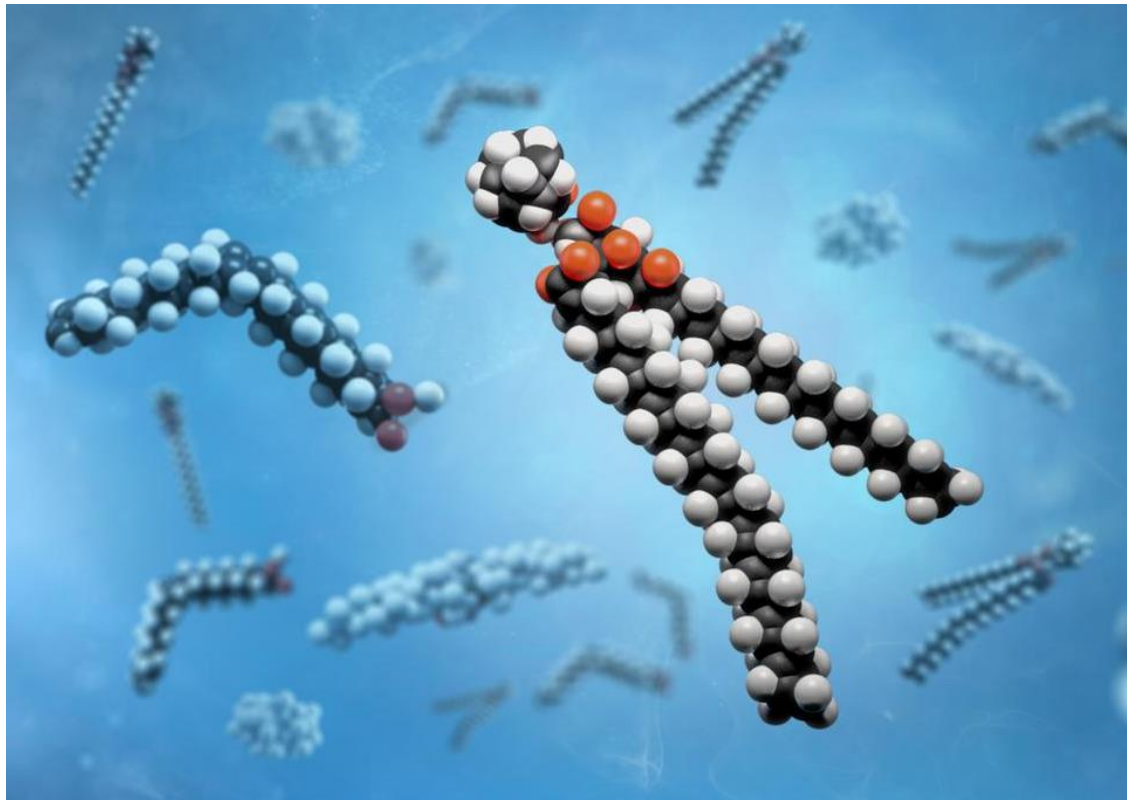
Lipids, Proteins, Peptides and Oligonucleotides



Lipids

Properties

- Includes 8 different classes of bio-molecules
  - Fatty acyls, glycerolipids, phospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, polyketides
- LC separations can be the most time-consuming aspect of bioanalysis
- **Challenge:** Wide diversity in structure, polarity and low water solubility



Lipids

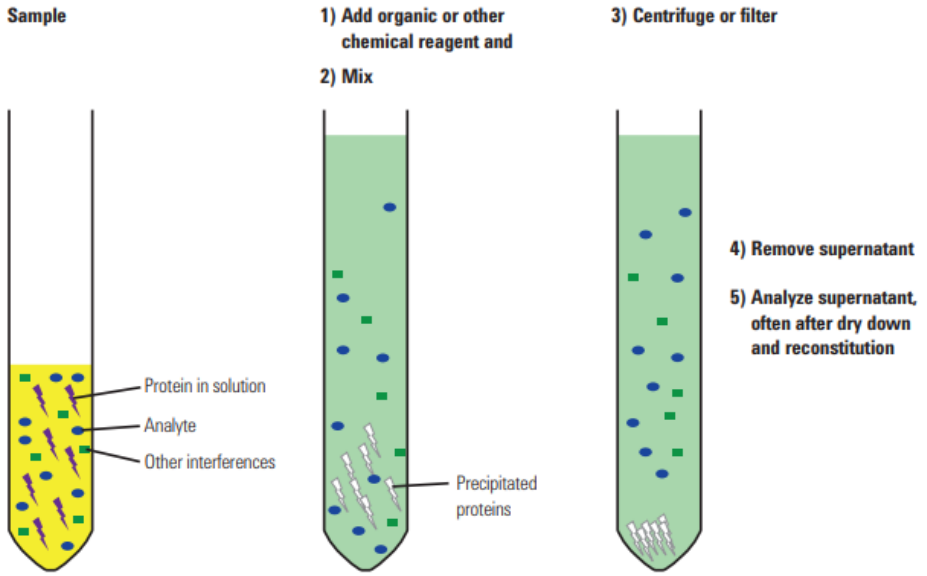
Protein Precipitation (PPT)

The solubility of proteins in aqueous buffers depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein's surface. Charged and polar residues increase the solubility of the proteins.

A precipitating agent decreases the solubility by lowering the dielectric constant of the solution which increases the electrostatic interaction between protein molecules.

Figure 16.3

Protein Precipitation



Chemical Reagents for Protein Precipitation from Plasma

Type of Reagent	Typical Reagents	Comments
Organic solvent	Acetonitrile, methanol, ethanol	Acetonitrile is the most popular; irreversibly denatures three-dimensional structure of proteins
Acids	Trichloroacetic acid, perchloric acid, tungstic acid	Irreversibly denatures three-dimensional structure of proteins
Metal ions	Zinc hydroxide, copper sulfate-sodium tungstate	
Salts	Ammonium sulfate	Salting out effect; denaturization potentially reversible by dialyzing out the excess salt

[Sample Preparation Fundamentals for Chromatography (agilent.com)]

Lipids

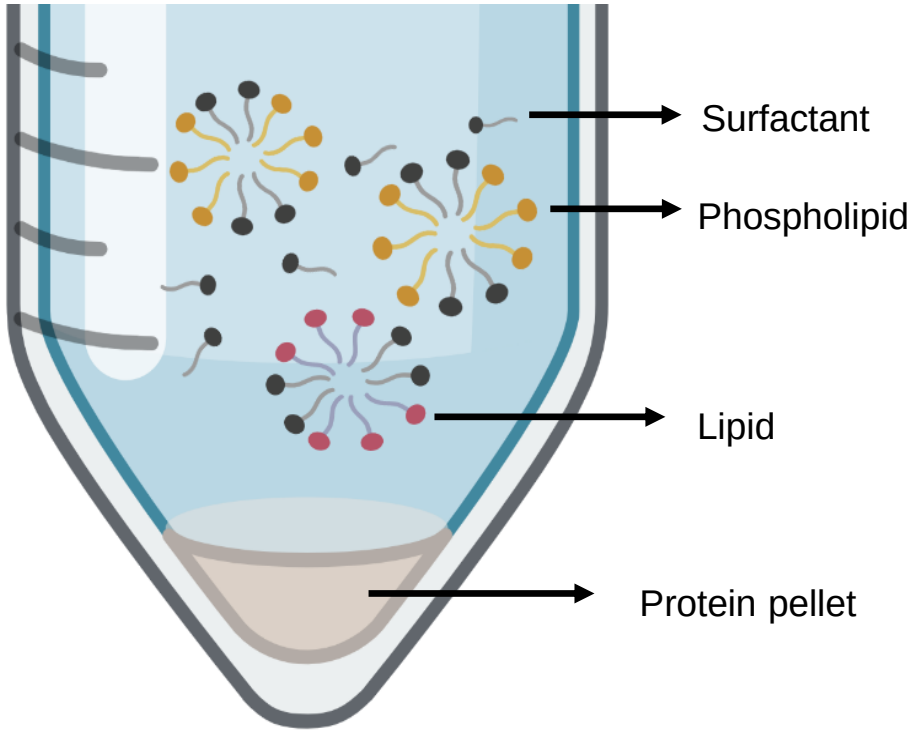
Protein Precipitation (PPT)

Strength:

- Quick and simple removal of proteins
- Many published methods

Challenge:

- Only removes proteins from the sample
- All other matrix components will remain



Lipids

Liquid Liquid Extraction (LLE)

- Lipid LLE methods use non-polar solvents like chloroform, MTBE or butanol.

Strength

- Relatively simple

Challenge

- Variability of yield and purity
- Difficult to automate

	Folch[1]	Bligh-Dyer[2]	Matyash[3]	BUME[3]
Organic:MeOH:Water	8:4:3	2:2:1.8	10:3:2.5	1:1 (single phase)
Sample (mL)	0.1	0.1	0.1	0.1
Methanol (mL)	0.53	.68	0.38	0.45
Organic Solvent (mL)	1.06	.68	1.29	0.45
Water (mL)	0.4	0.62	0.32	NA
Organic Solvent:	Chloroform	Chloroform	MTBE	Butanol/MeOH 5mM Am.Acetate

1. Folch, J., et al., J Biol Chem 226, 497-509 (1957).
2. Bligh, E. G. & Dyer, W. J.. Can J Biochem Physiol 37, 911-917, doi:10.1139/o59-099 (1959).
3. Matyash, V., et al., J Lipid Res 49, 1137-1146, doi:10.1194/jlr.D700041-JLR200 (2008).
4. Alshehry, Z. H. et al., Metabolites 5, 389-403, doi:10.3390/metabo5020389 (2015).

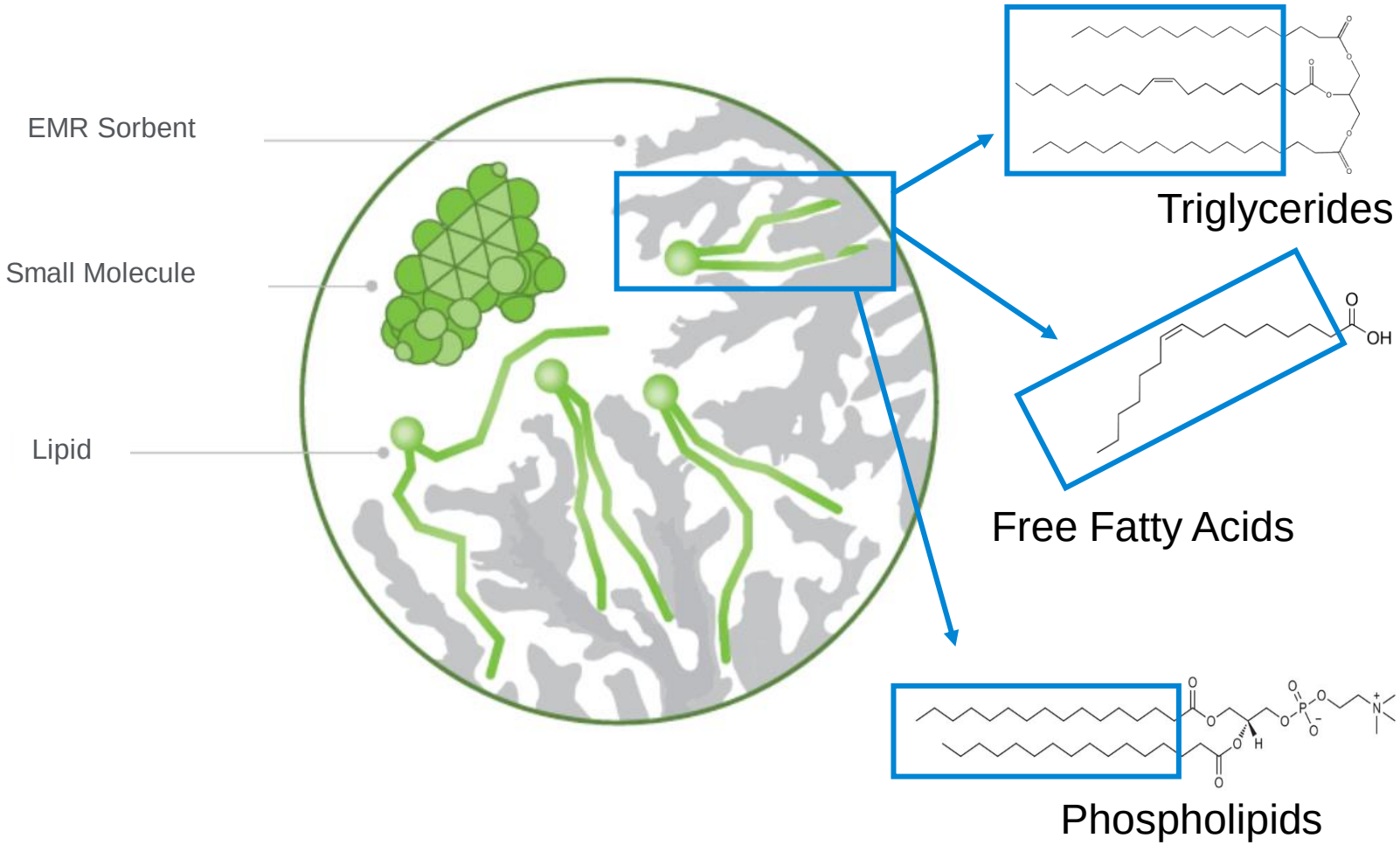
Lipids

Solid Phase Extraction (SPE)

Retention of lipids with Bond Elut Lipid Extraction is based on a combination of size exclusion and hydrophobic interaction mechanisms. Proteins precipitated from sample are retained by a filtration process.

Strength

- Higher selectivity than PPT or LLE
- Consistency of results
- Automatable and scalable



Lipids

SPE: Bond Elut Lipid Extraction

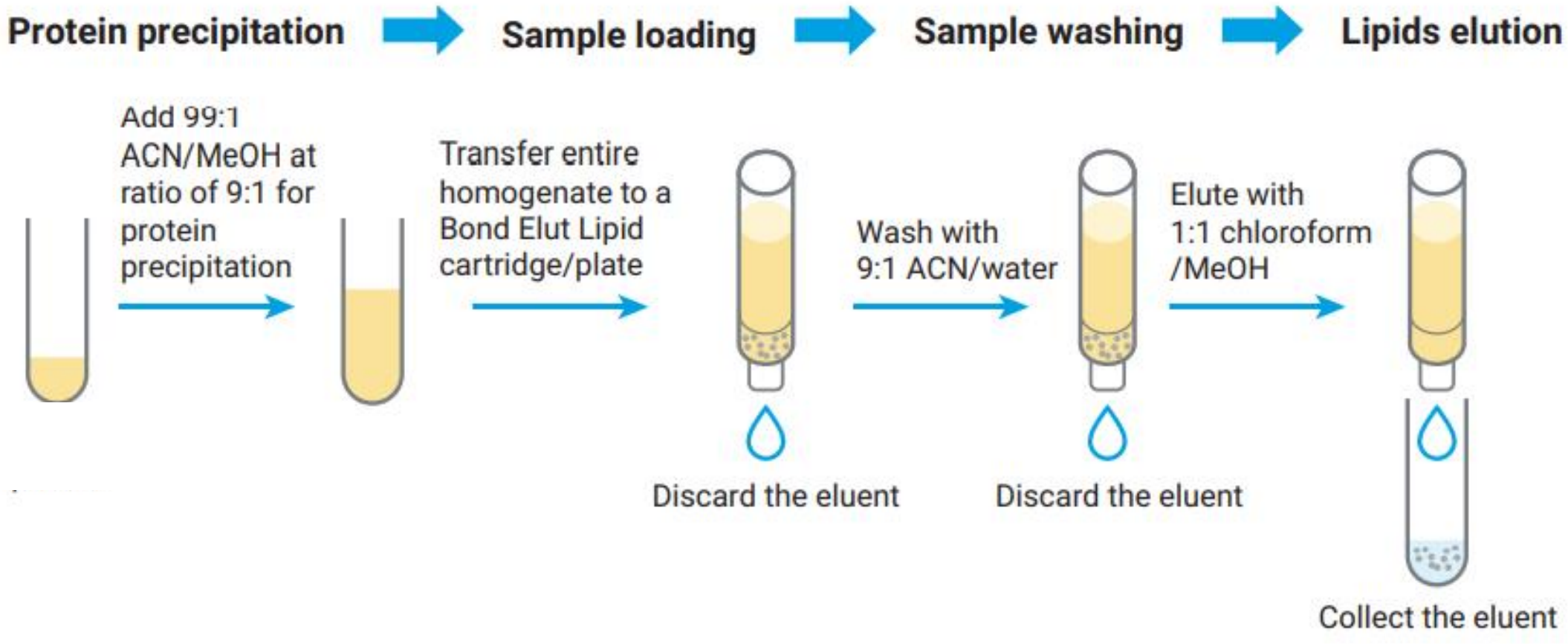


Figure 2. Sample preparation workflow for lipidomic study using Agilent Bond Elut Lipid Extraction 1 mL cartridge

Lipids

SPE: Bond Elut Lipid Extraction

Bond Elut Lipid Extraction shows higher reproducibility compared to the LLE techniques

	Average Peak Area RSD (All Identified Features)				
	Bond Elut Lipid Extraction	**LLE Folch**	**LLE Bligh-Dyer**	**LLE Maytash**	**LLE BUME**
LC/MS Replicates	6.4%	6.3%	5.8%	7.1%	6.0%
Extraction Replicates	9.4%	12.2%	22.6%	11.2%	19.8%

Bond Elut Lipid Extraction Method Simplifies and Accelerates Sample Processing

	Bond Elut Lipid Extraction	Liquid-Liquid Extraction
Coverage	+++	+++
Selectivity [1]	+++	+++
Time (batch of 1–48 Samples)[2]	30 minutes	60–90 minutes
Ease of Use	++	-
Reproducibility	<10% RSD	10–20% RSD
Ease of Automation	+++	-

1. Selective isolation of lipids in complex matrix
2. Not including 1–2 hours N_2 drying time.

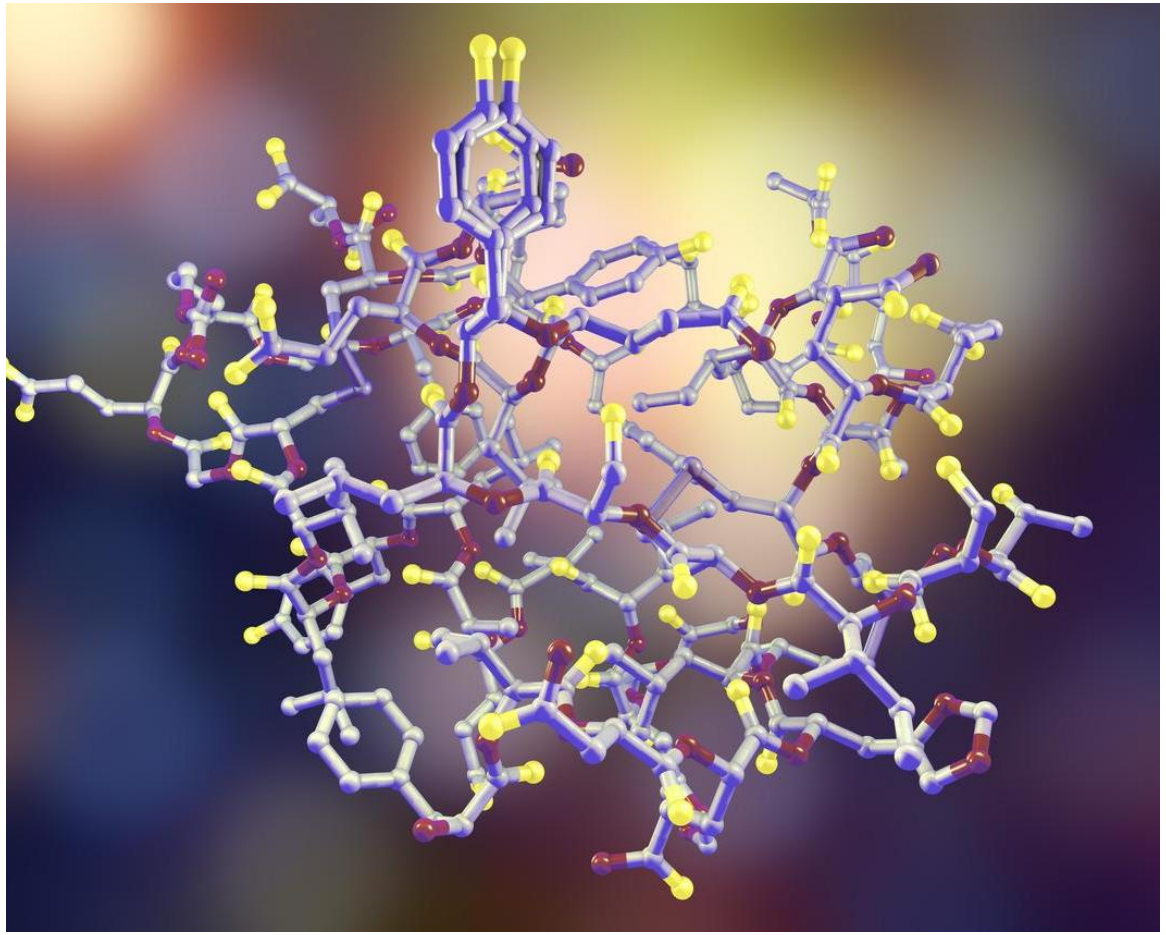
Peptides

Properties

Peptides are typically considered to be a single chain of less than 50 amino acids, or to have molecular weights less than 10 kDa. Physicochemical properties depend on amino acid makeup of the chain

Challenges:

- Separating peptides from abundant proteins
- Phospholipids may build up on LC column if not removed
- Matrix effects can decrease sensitivity making it hard to detect low abundant peptides



Peptides

Protein Precipitation (PPT)

Peptides do not precipitate as well as larger proteins so PPT can be used to remove proteins

Strengths:

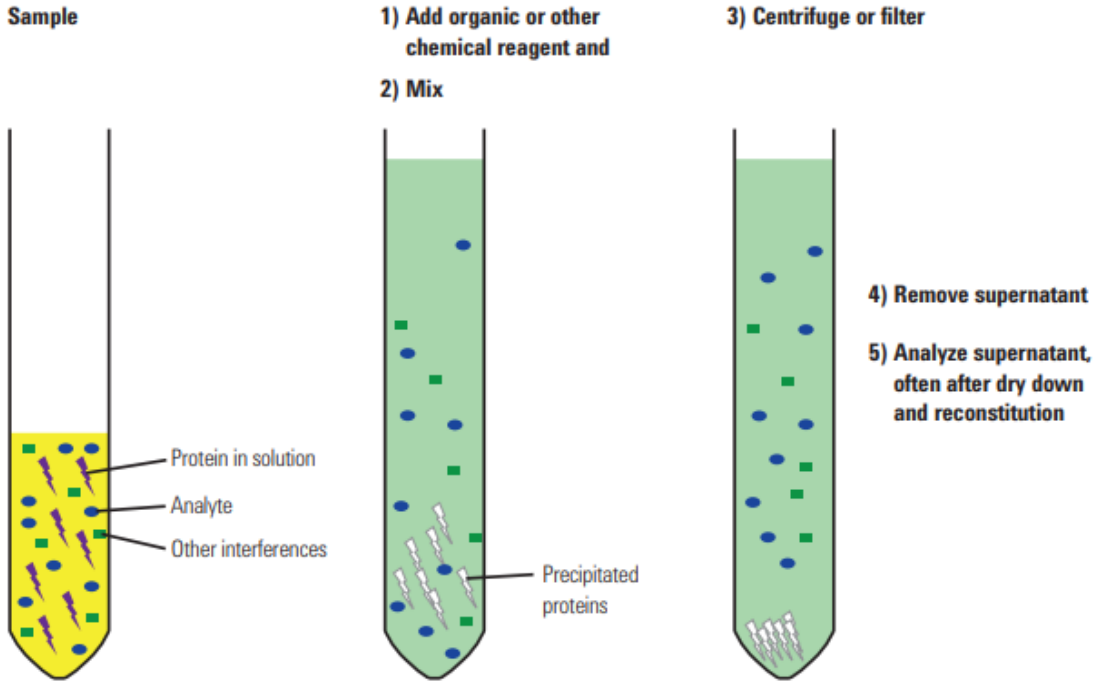
- Fast and simple

Weaknesses:

- Does not remove phospholipids or salts
- Larger peptides could coprecipitate with proteins

Figure 16.3

Protein Precipitation



Sample Preparation Fundamentals for Chromatography (agilent.com)

Peptides

Protein Precipitation (PPT)

Captiva ND Lipids

Pairing PPT with a filtration product such Captiva ND Lipids can further reduce prep time and remove remaining lipids from matrix

Captiva ND Lipids 96-well filtration starter kit, A59640002SK

Centrifugation Protein Precipitation (PPT)	Time (min)	Captiva ND Lipids*	Time (min)
Add 0.2 mL of spiked plasma sample and 0.6 mL of ACN + 0.1% formic acid to centrifugation tubes or an empty 96-well plate.	5	Add 0.2 mL of spiked plasma sample and 0.6 mL of ACN + 0.1% formic acid to Captiva ND 96-well plate.	5
Centrifuge at 10,000 RPM for 10 min.	11	Mix each well with a pipette 5 times and apply vacuum for filtration.	
Transfer supernatant to 2 mL injection vials (if tubes were used) or a new empty 96-well plate for analysis (if plate format was used).	10	Directly transfer injection plate for analysis.	0
Total time required for sample preparation	**26**	Total time required for sample preparation	**5**

* Based on the automation using Tomtec or other machine.

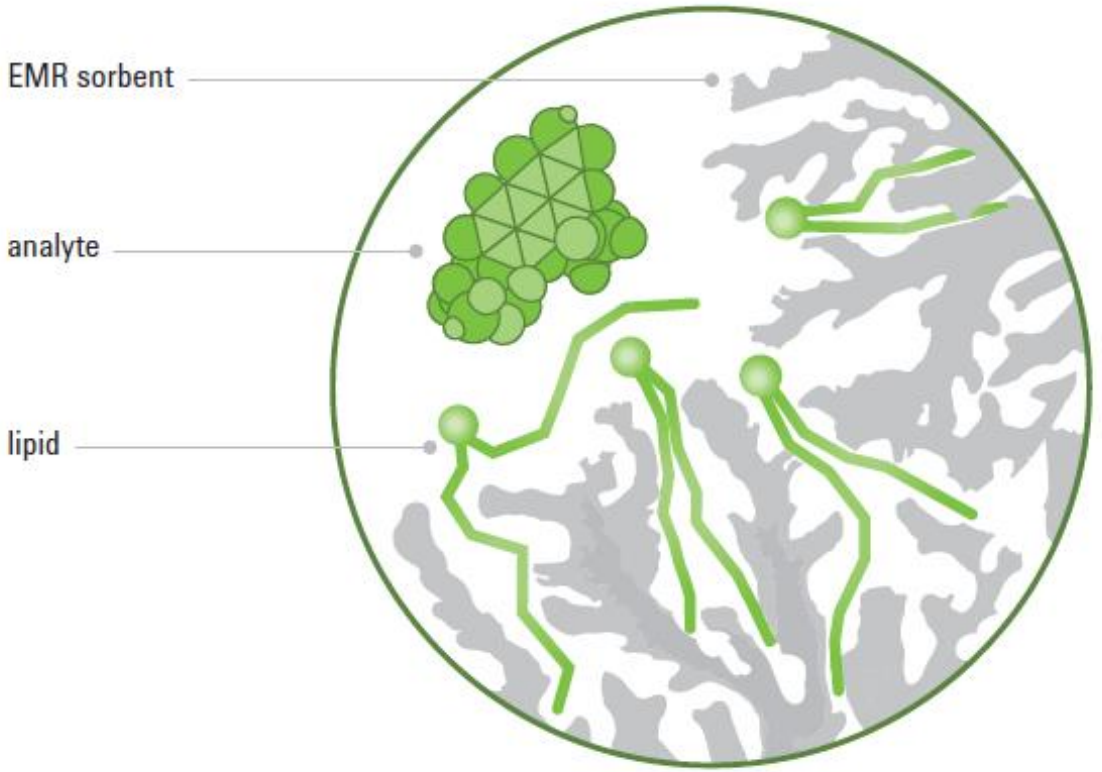
Peptides

Protein Precipitation (PPT)

Captiva EMR-Lipid

EMR-Lipid sorbent technology effectively traps lipids through two mechanisms:

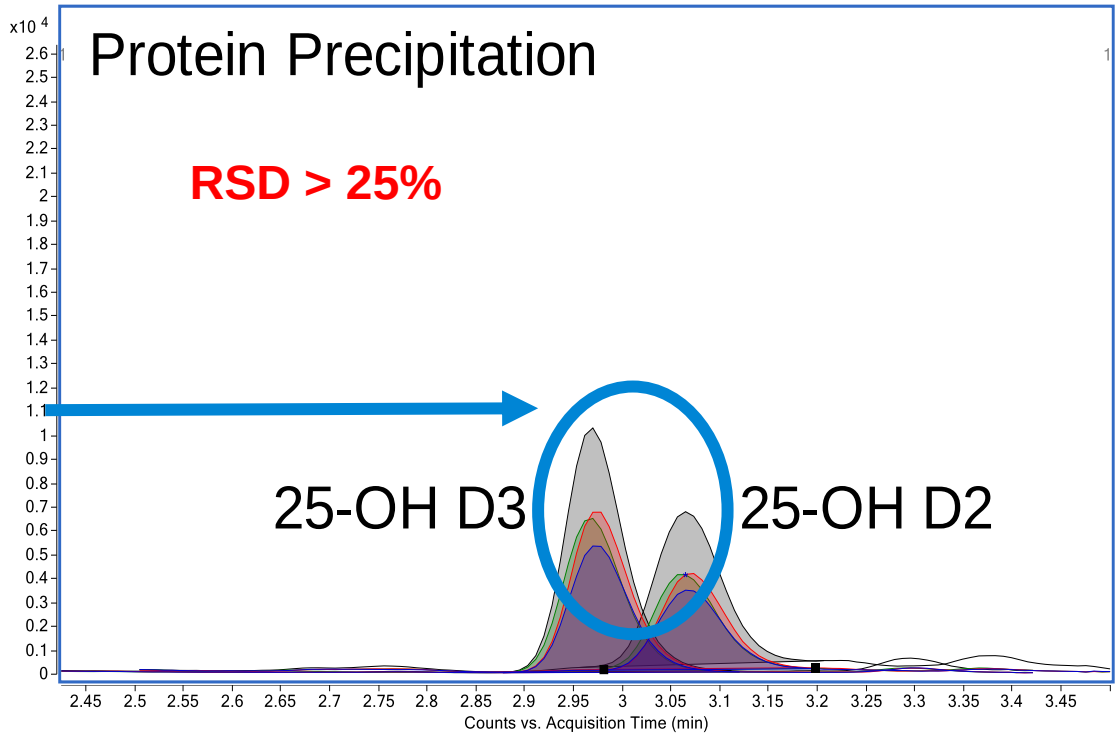
- **Size exclusion –** Unbranched hydrocarbon chains (lipids) enter the sorbent; bulky analytes do not
- **Sorbent chemistry** – Lipid chains that enter the sorbent are trapped by hydrophobic interactions

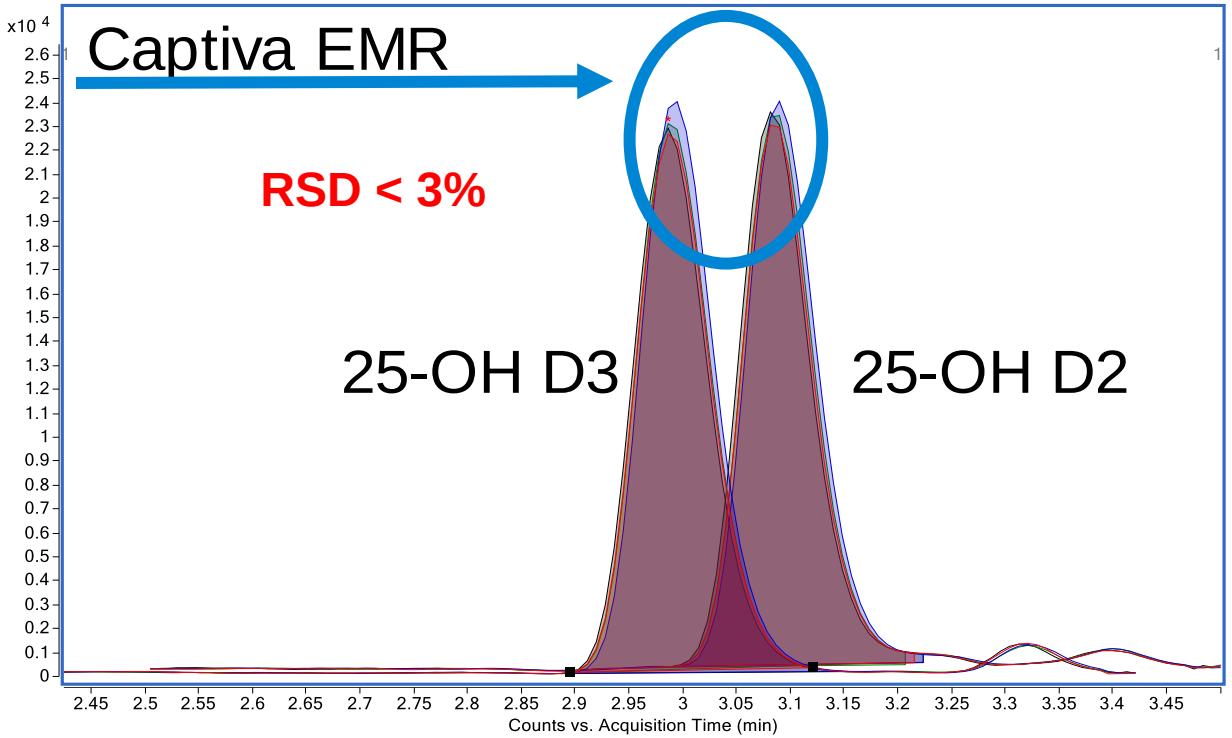


Peptides

Protein Precipitation (PPT)

Captiva EMR-Lipid





Peptides

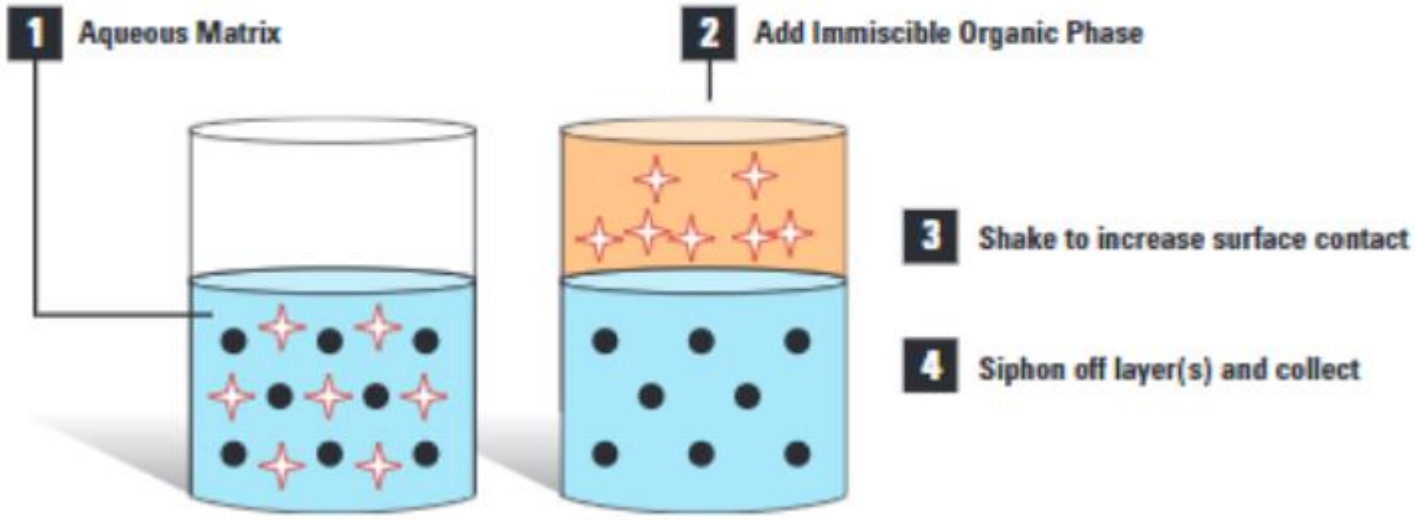
Liquid Liquid Extraction (LLE)

LLE can be used to extract hydrophobic peptides

- Strengths: Simple method, cleaner results than PPT, can be automated
- Weaknesses: Limited to hydrophobic peptides, low recovery of analytes, will not remove lipids from matrix

Figure 7.1

Typical Liquid-Liquid Extraction



Peptides

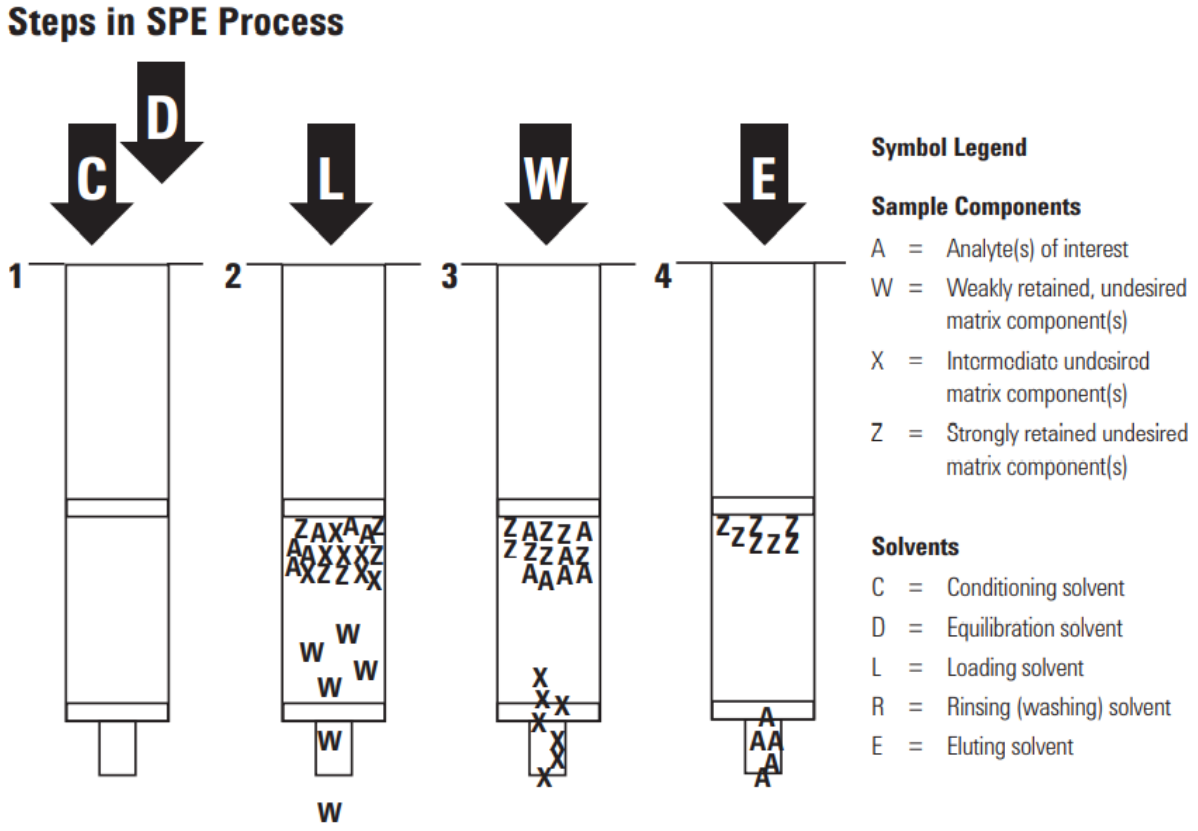
Salting-Out Assisted Liquid Liquid Extraction (SALLE)

- The addition of salts can induce the separation of water-miscible organic solvents from water to form a two-phase system. The addition of salt can decrease the solubility of polar nonionic peptides in the aqueous phase.
- Strengths: Simple method, cleaner results than PPT, can be automated
- Weaknesses: Method development, will not remove lipids from matrix

Peptides

Solid Phase Extraction (SPE)

- The mechanism of SPE is similar to LC in that it is based on the affinity between solutes and the sorbent material.
- Strengths: Much more selective than PPT or LLE, can remove phospholipids and salts, wide variety of sorbents available, able to concentrate analyte, scalable, and automatable
- Weaknesses: Method development, more expensive than PPT or LLE



[Solid Phase Extraction (SPE) - Cartridges, Well Plates, Sorbents | Agilent]

Peptides

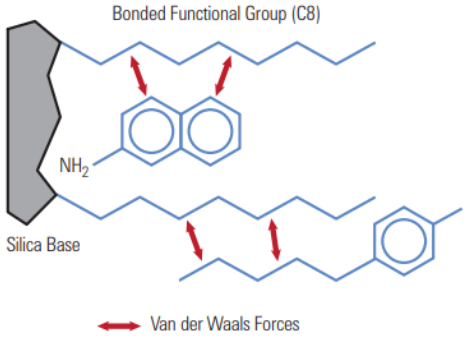
Solid Phase Extraction (SPE)

The best analyte retention is obtained when the polarity of the analyte is most similar to the polarity of the phase

SPE type	Usage
Reverse-phase (C18)	Peptides
Polar phase	Glycopeptides Peptides
Cation Exchange	Basic peptides
Phenylboronic acid	Glycopeptides

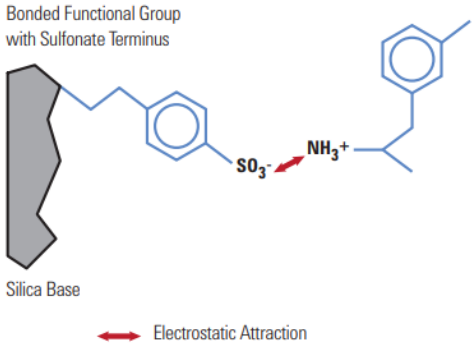
Non-Polar Interactions

Figure 9.10A



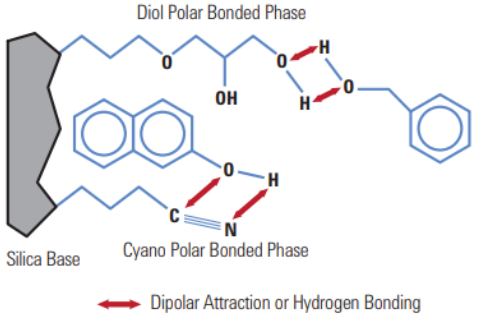
Ion Exchange Interactions

Figure 9.10C



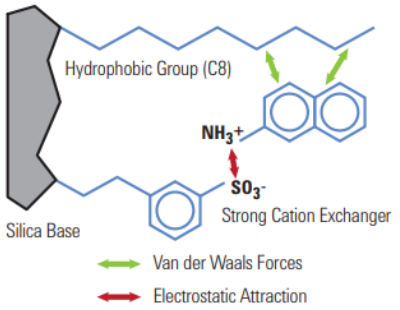
Polar Interactions

Figure 9.10B



Mixed Mode SPE

Figure 9.10D



[Solid Phase Extraction (SPE) - Cartridges, Well Plates, Sorbents | Agilent]

Peptides

Solid Phase Extraction (SPE)

Nonpolar

C18, C8, C2, C1

C18 variations in carbon load and endcapping
EnvirElut
CH – cyclohexyl
CN-E – endcapped cyanopropyl
PH – phenyl
Plexa, PPL, ENV, LMS, Focus, Nexus

Polar

PSA - primary and secondary amine
NH2 - aminopropyl
DEA - diethylaminopropyl
Diol - diol
SI - silica
CN - nonendcapped cyanopropyl

Cation Exchange

SCX – benzenesulfonic acid
PRS – propylsulfonic acid
CBA – carboxylic acid
Nexus WCX

Anion Exchange

SAX – quaternary amine
PSA – primary and secondary amine
NH2 – aminopropyl
DEA – diethylaminopropyl

Covalent

PBA – phenylboronic acid

Silica/Polymeric

Mixed Mode IEX/Nonpolar

Certify – SCX/C8
Certify II – SAX/C8
Plexa PCX
Plexa PAX
PFAS WAX

Specialty Phases

Lipid Extraction
AccuCAT
Atrazine
Mycotoxin

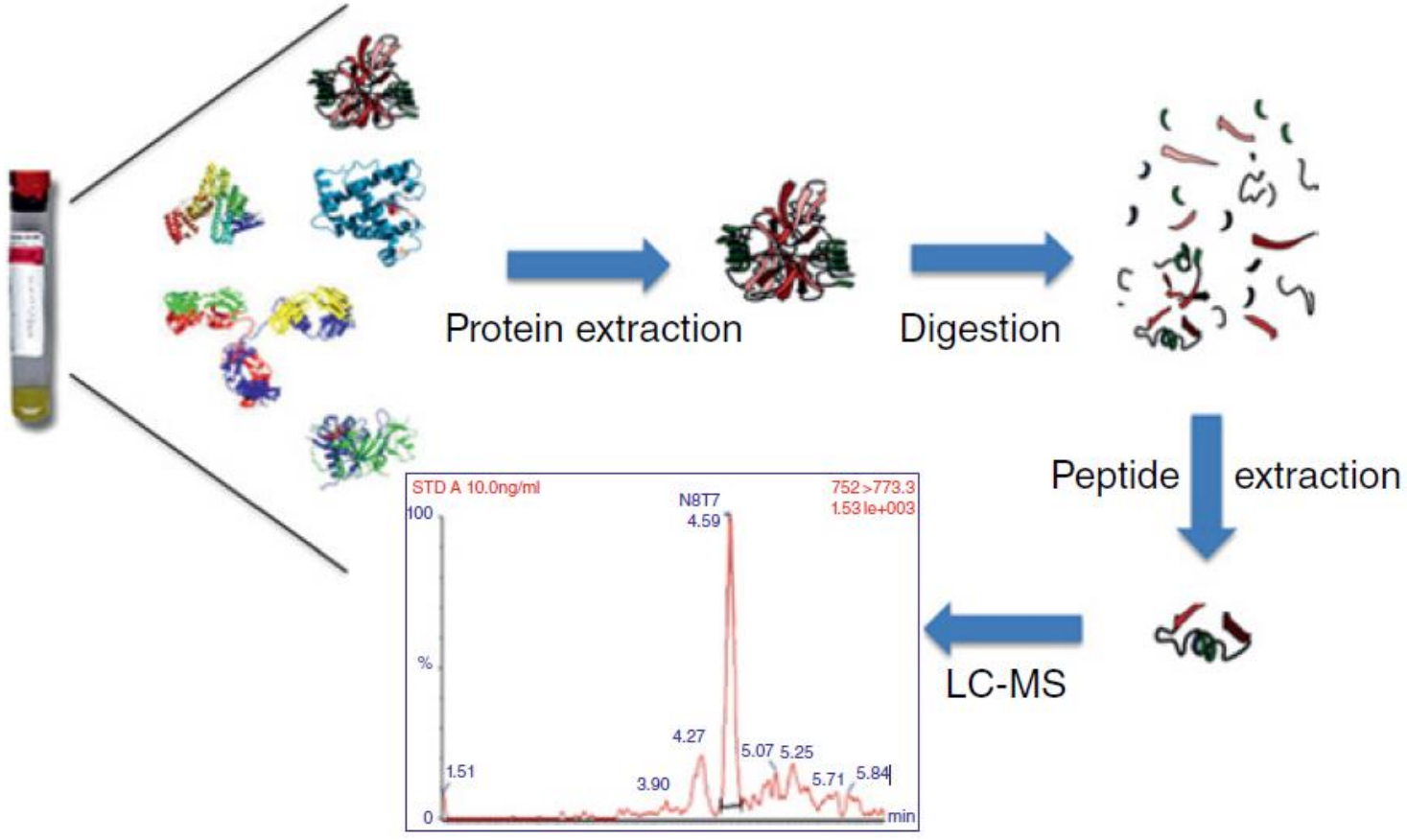
Alumina – aluminum oxide
Florisil – magnesium-silica
Carbon S – synthetic carbon
Carbon
Carbon/NH_2

Solid Phase Extraction (SPE) - Cartridges, Well Plates, Sorbents | Agilent

Peptides

Digested proteins

Digested proteins produce peptides that are 500-2000 Da in size. C18 Spin columns and pipette tips are widely used to remove salts and ion-suppressing interferences before MS analysis.



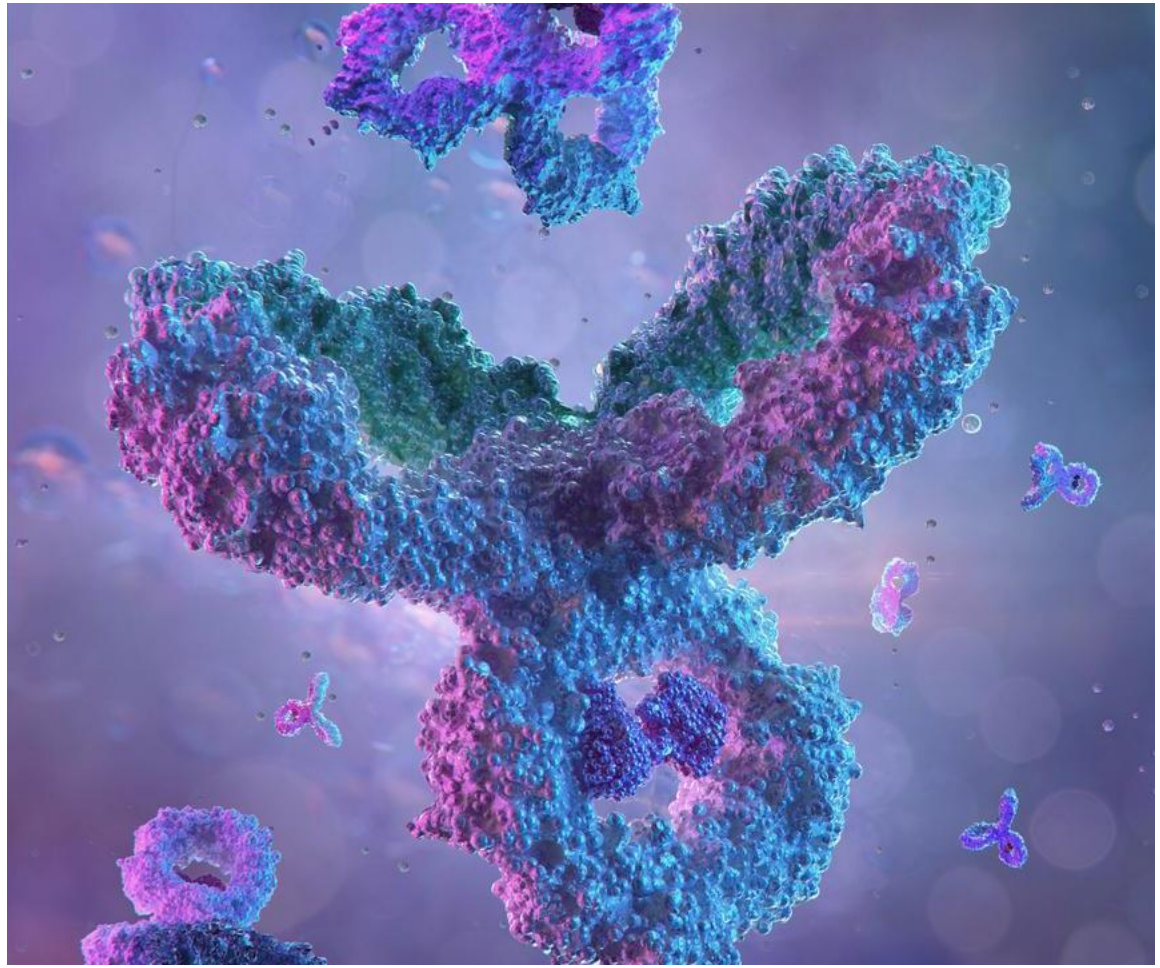
[OMIX Tips | Agilent]

[Peptide Cleanup C18 Spin Tubes | Agilent]

Proteins

Properties

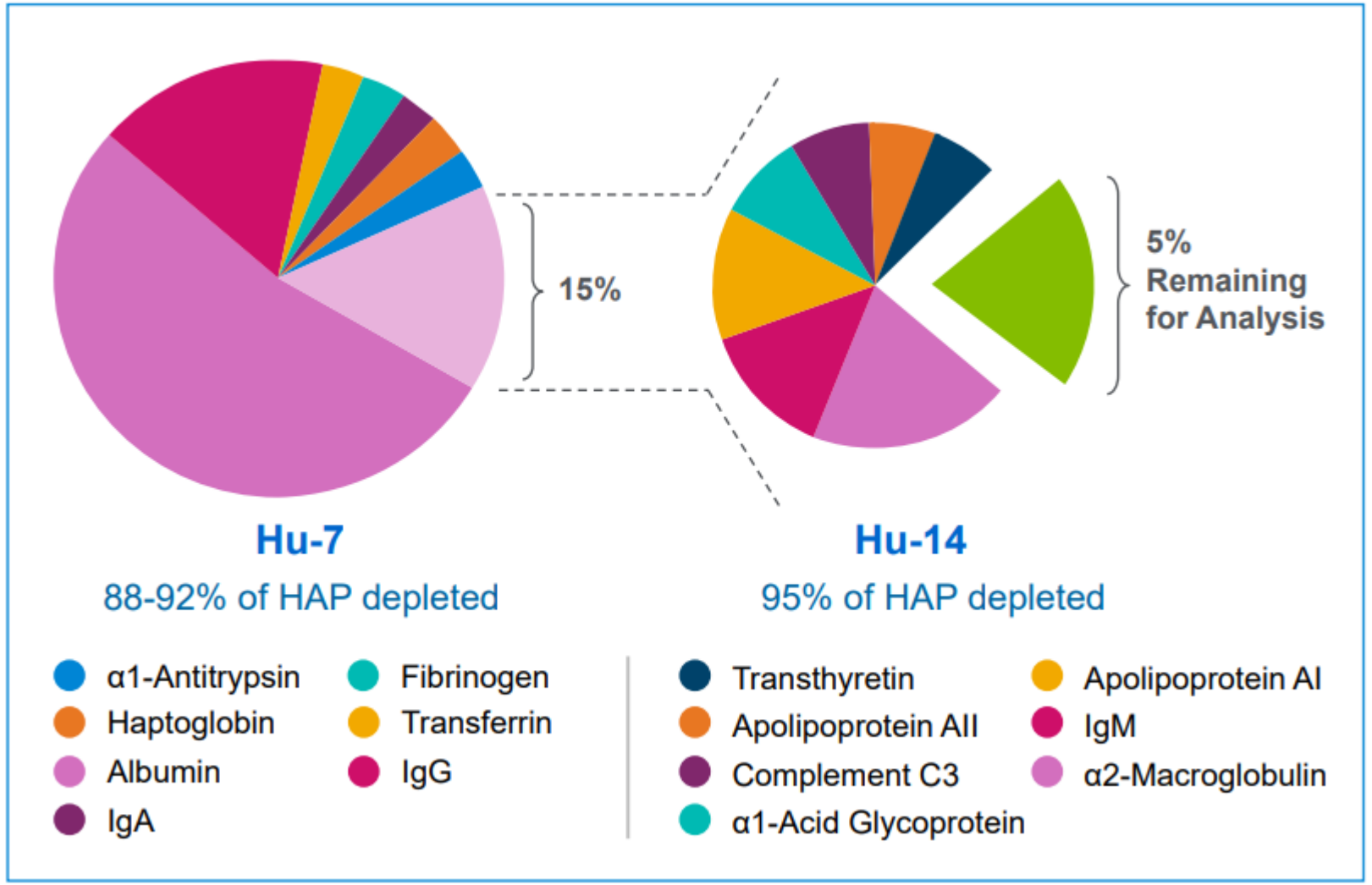
- Proteins are large (MW > 10 kDa) complex molecules made of hundreds or thousands of amino acids with unique 3D structures
- Challenges:
  - Complexity and diversity of proteins in samples needs to be reduced to provide analytical results
  - Analyte typically needs to be quantified among matrix proteins that will have similar physicochemical properties at much higher concentrations
  - No protein amplification method



Proteins

Protein depletion

Protein depletion increases detectability of target proteins by removing high abundant proteins (HAP)

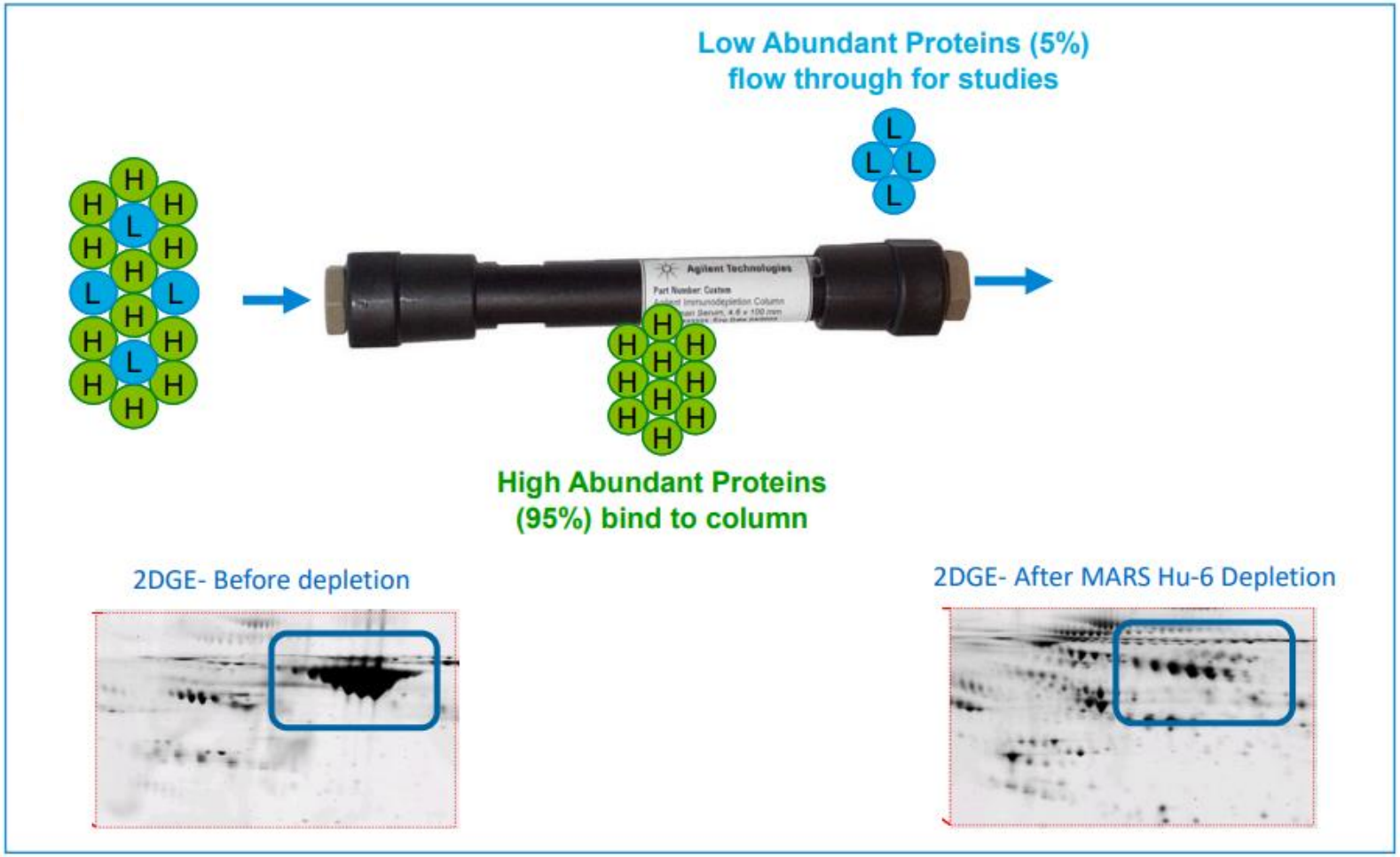


Proteins

Protein depletion

Multiple Affinity Removal System

- Robust chemistry enables long column lifetime and reusability
- Rapid, simultaneous removal of up to 14 proteins with one device
- Available in both standard LC column and spin cartridge format.

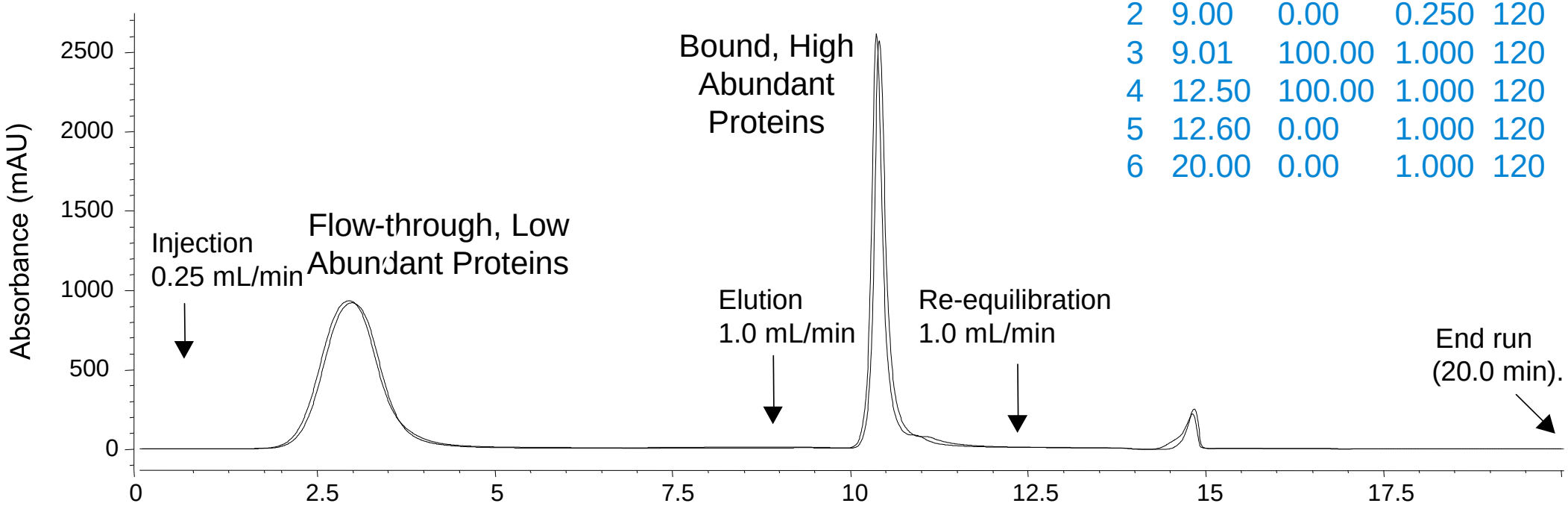


[Abundant Protein Depletion | Agilent]

Proteins

Protein depletion

Multiple Affinity Removal System



	Time (min)	%B	Flow rate	Max. pressure
1	0.00	0.00	0.250	120 bar
2	9.00	0.00	0.250	120
3	9.01	100.00	1.000	120
4	12.50	100.00	1.000	120
5	12.60	0.00	1.000	120
6	20.00	0.00	1.000	120

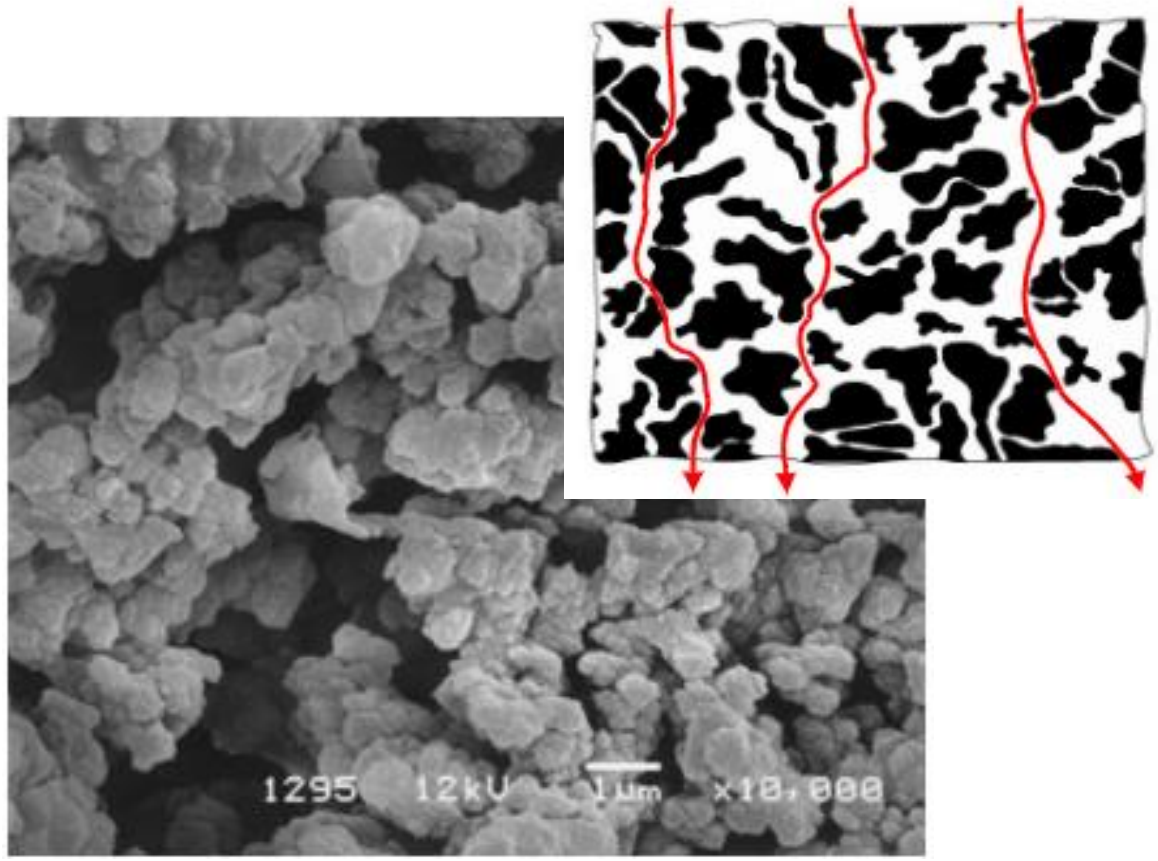
[Abundant Protein Depletion | Agilent]

Proteins

Solid Phase Extraction (SPE)

The best analyte retention is obtained when the polarity of the analyte is most similar to the polarity of the phase.

For larger proteins sorbents that are monolithic or large pore sizes will help with retention



SPE type	Usage
Reverse-phase (C4, C1)	Proteins
Polar phase (Amide)	Hydrophilic proteins
Cation Exchange	Basic proteins (pKa 6-10)

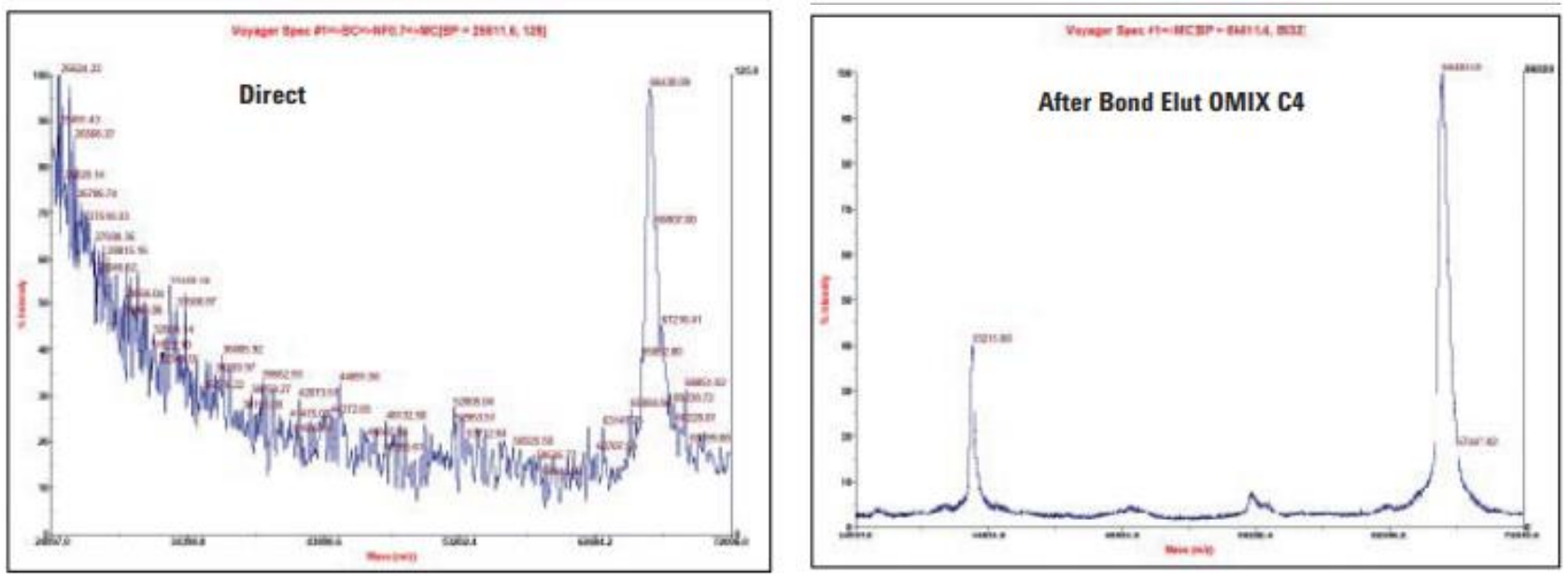
[Solid Phase Extraction (SPE) - Cartridges, Well Plates, Sorbents | Agilent]

Proteins

Solid Phase Extraction (SPE)

Bond Elut Omix C4

Proteins are more hydrophobic than peptides and bind too tightly to C18, making them difficult to elute and leading to low recovery. C4 is less hydrophobic and can be used to enrich picomolar and femtomolar concentrations of proteins while removing ionic interferences.



1 pmol bovine serum albumin in 2M guanidine analyzed by MALDI-TOF both before and after clean-up with Bond Elut Omix C4 10 µL tips

OMIX Tips | Agilent

5990-8885EN | Agilent

SPEC Disk SPE | Agilent

Proteins

Immunocapture using Magnetic Beads or resins

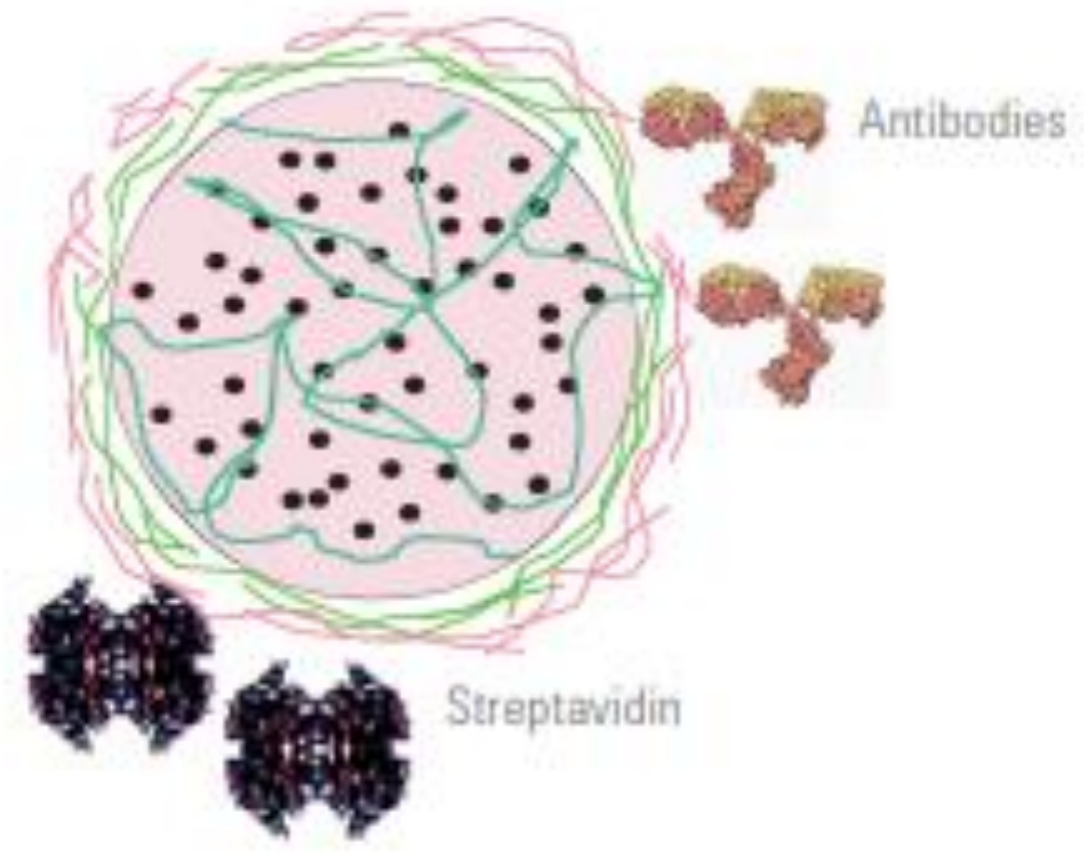
Immunocapture is the most rigorous sample cleanup technique. Magnetic beads or resins are coated with a protein that has a high binding affinity for the analyte, such as an antibody.

Strength

- Highest selectivity results in high sensitivity for analyte

Weakness

- Relies on the availability and quality of suitable affinity reagents

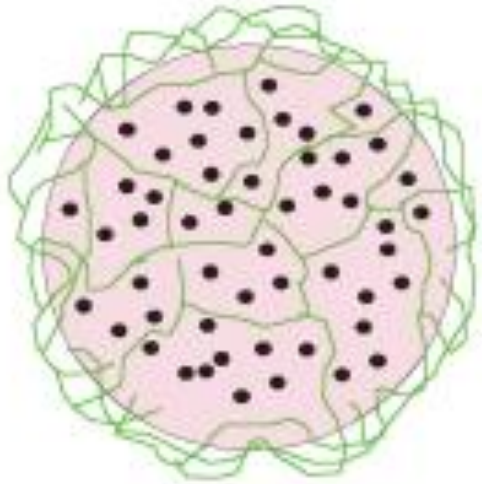


5991-1336EN.pdf (agilent.com)

Proteins

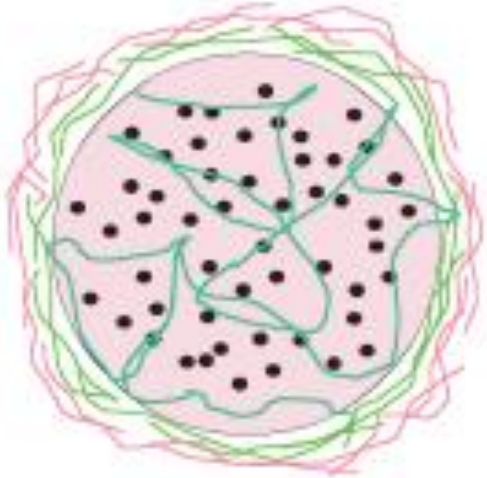
Immunocapture using Magnetic Beads or resins

Microcrystalline Fe distributed within the body of a precursor particle.



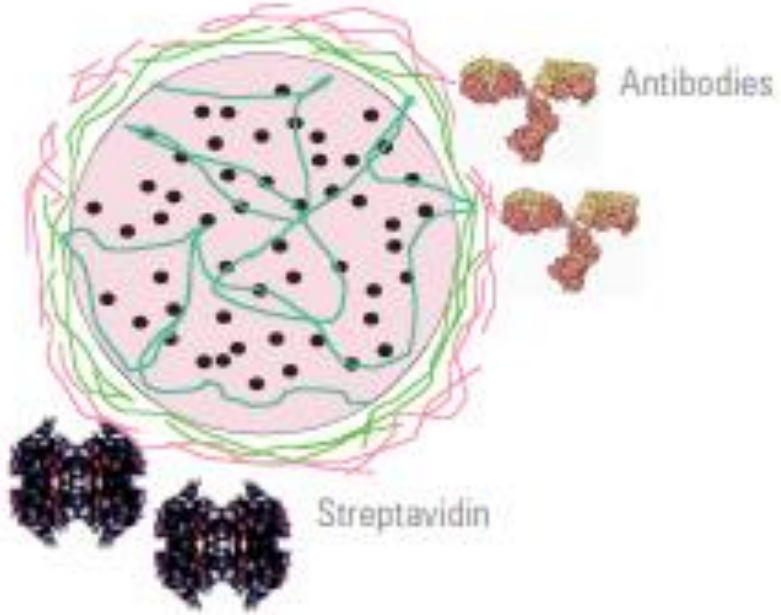
Polymer network provides a surface shell keeping the Fe hidden and acts as a base for surface chemistry addition.

Coating of polymer layers introduces specific properties for ligand binding, e.g. COOH.



Further processing controls other characteristics, e.g. reducing backgrounds or auto-fluorescence.

Ligand binding through standard chemistries.

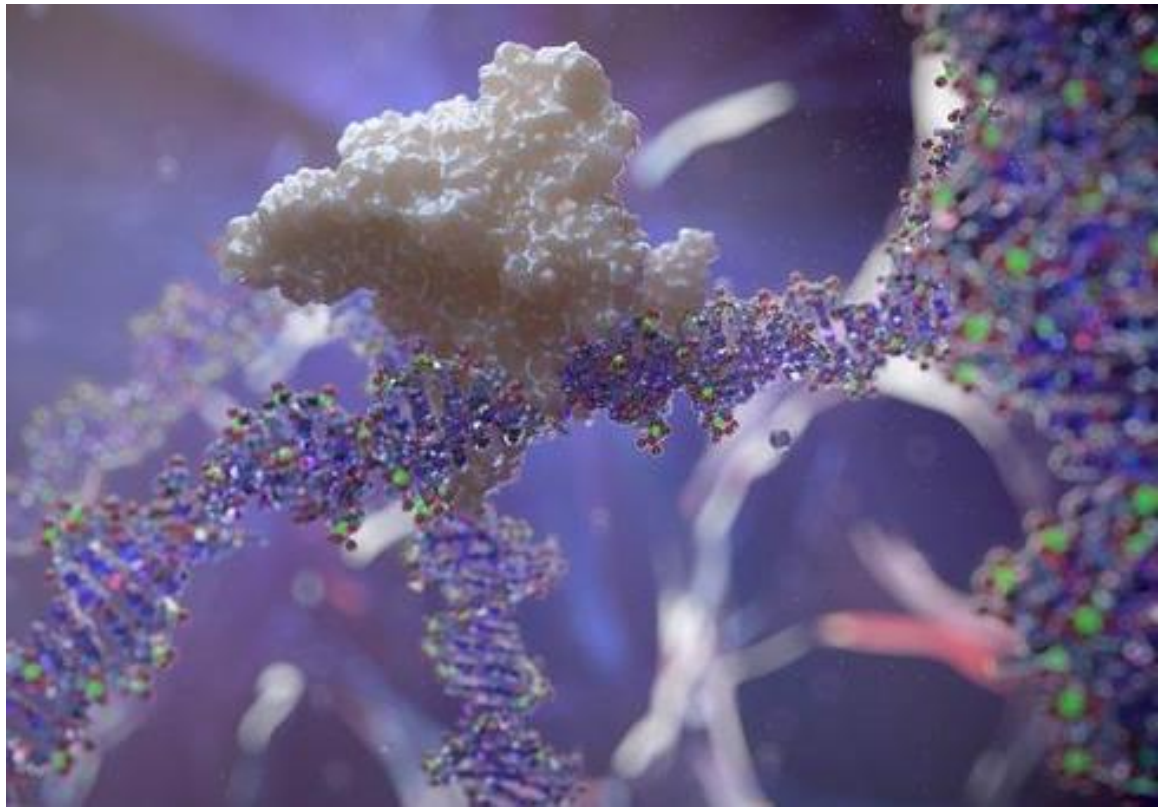


5991-1336EN.pdf (agilent.com)

Oligonucleotides

Properties

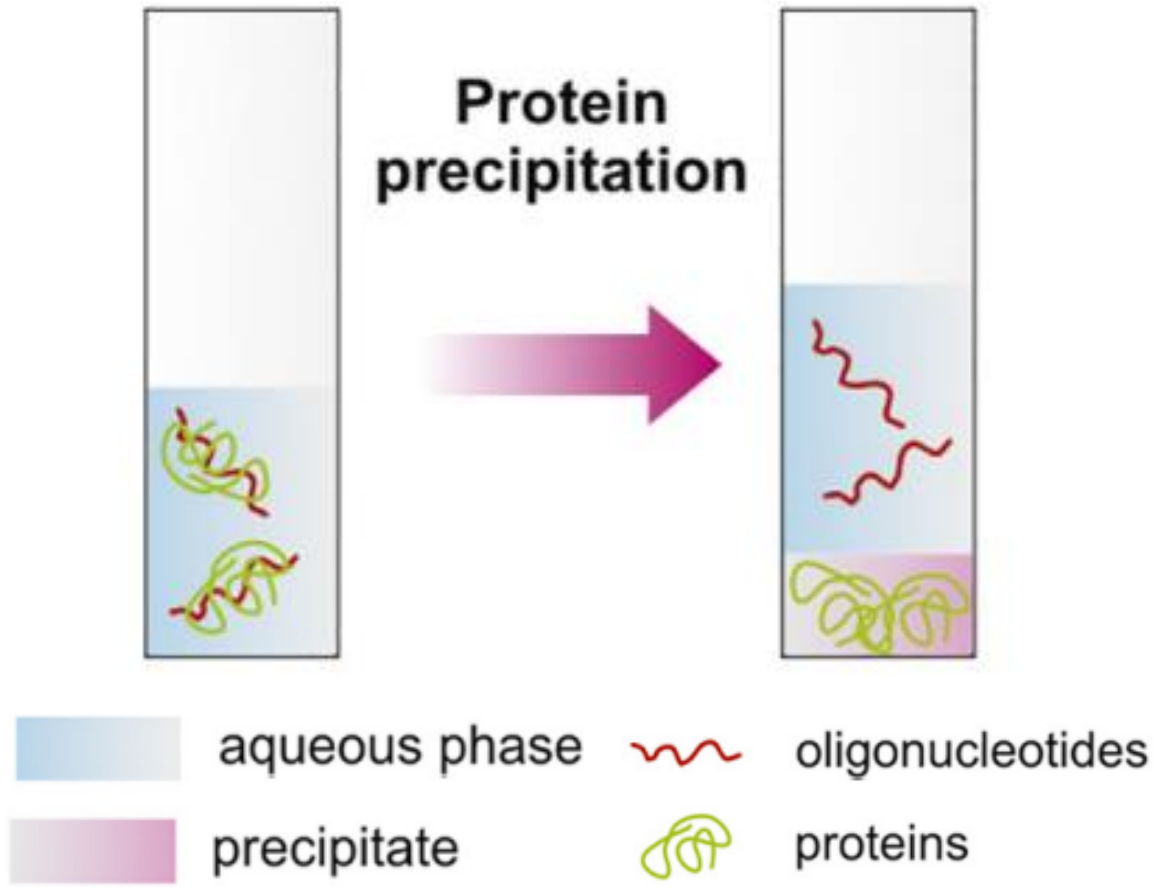
- Each nucleotide has 350 Da to size of oligonucleotide so even relatively small oligos are large for biomolecules
- Phosphate backbone creates substantial negative charge and increases water solubility
- Complex higher-order structures
- May be challenging to remove plasma proteins



Oligonucleotides

Protein Precipitation (PPT)

- Simplest method is PPT with ammonium acetate and methanol or acetonitrile
- Strengths: Produces relatively clean samples with a yield ~ 70% for oligonucleotides 10–30 bases in length
- Challenges: Poor recovery due to strong binding of oligos to precipitating proteins



Nuckowski, Ł., Kaczmarkiewicz, A., & Studzińska, S. (2018). Review on sample preparation methods for oligonucleotides analysis by liquid chromatography. *Journal of Chromatography B*, *1090*, 90–100. https://doi.org/10.1016/J.JCHROMB.2018.05.025

Oligonucleotides

Proteinase K digestion

- Removal of proteins by digesting them to amino acids by incubating samples with proteinase K
- **Strengths:** Limited number of transfer steps makes method more adaptable to automation, up to 90% recovery of small oligos, good at removing oligos from proteins
- **Challenges:** Does not separate phospholipids from sample, needs to be followed with addition purification steps, 3–12 hour incubation time

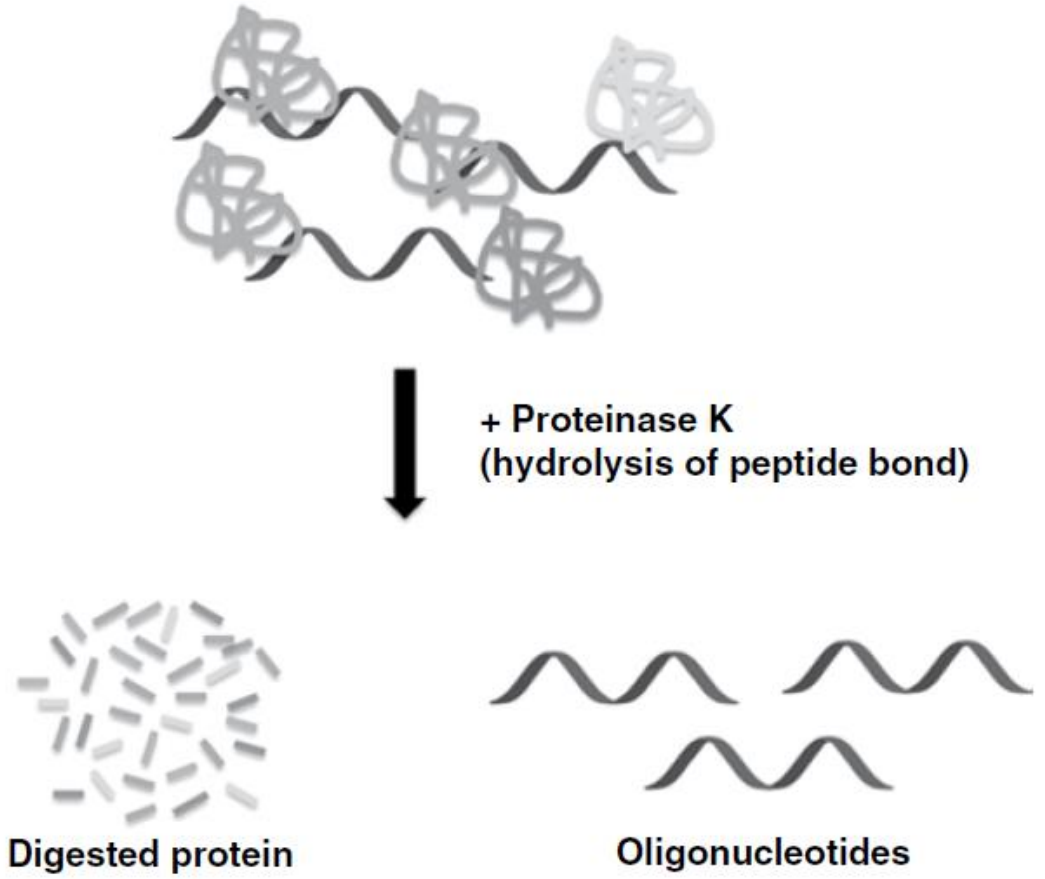


Figure 25.4 Oligonucleotide isolation by proteinase K digestion.

Bartlett, M. G., Kim, J., Basiri, B., & Li, N. (2019). *Sample Preparation in LC-MS Bioanalysis* (W. Li, W. Jian, & Y. Fu, Eds.). John Wiley & Sons.

Oligonucleotides

Solid Phase Extraction (SPE)

- SPE sorbent is typically a mix-mode or hydrophilic-lipophilic to take advantage of polar/charged oligo properties
- **Strengths:** Recovery in range of 60–90%, easily automated, scalable for high though-put
- **Challenges:** May need to be used in combination with LLE or ethanol precipitation for high purity

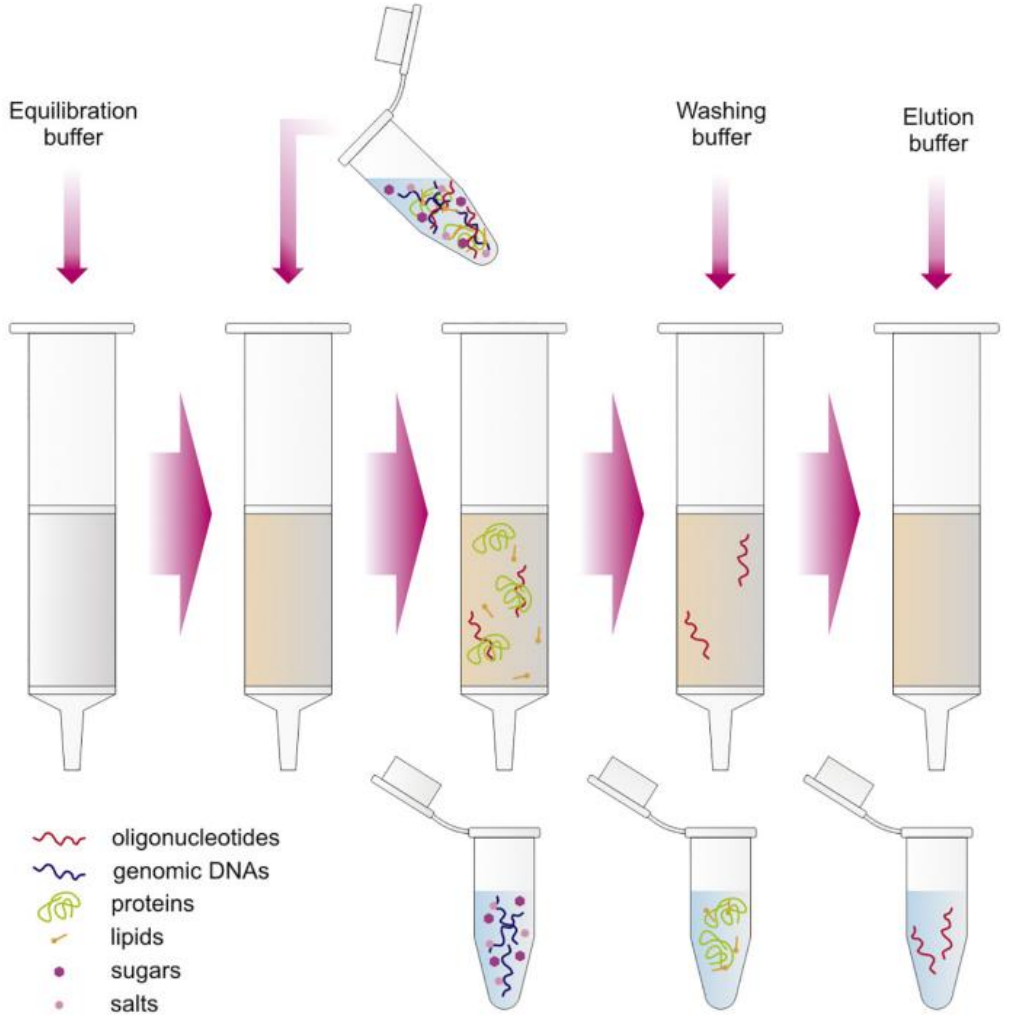


Fig. 3. Typical procedure for the solid phase extraction of oligonucleotides.

Nuckowski, Ł., Kaczmarkiewicz, A., & Studzińska, S. (2018). Review on sample preparation methods for oligonucleotides analysis by liquid chromatography. *Journal of Chromatography B*, *1090*, 90–100. https://doi.org/10.1016/J.JCHROMB.2018.05.025

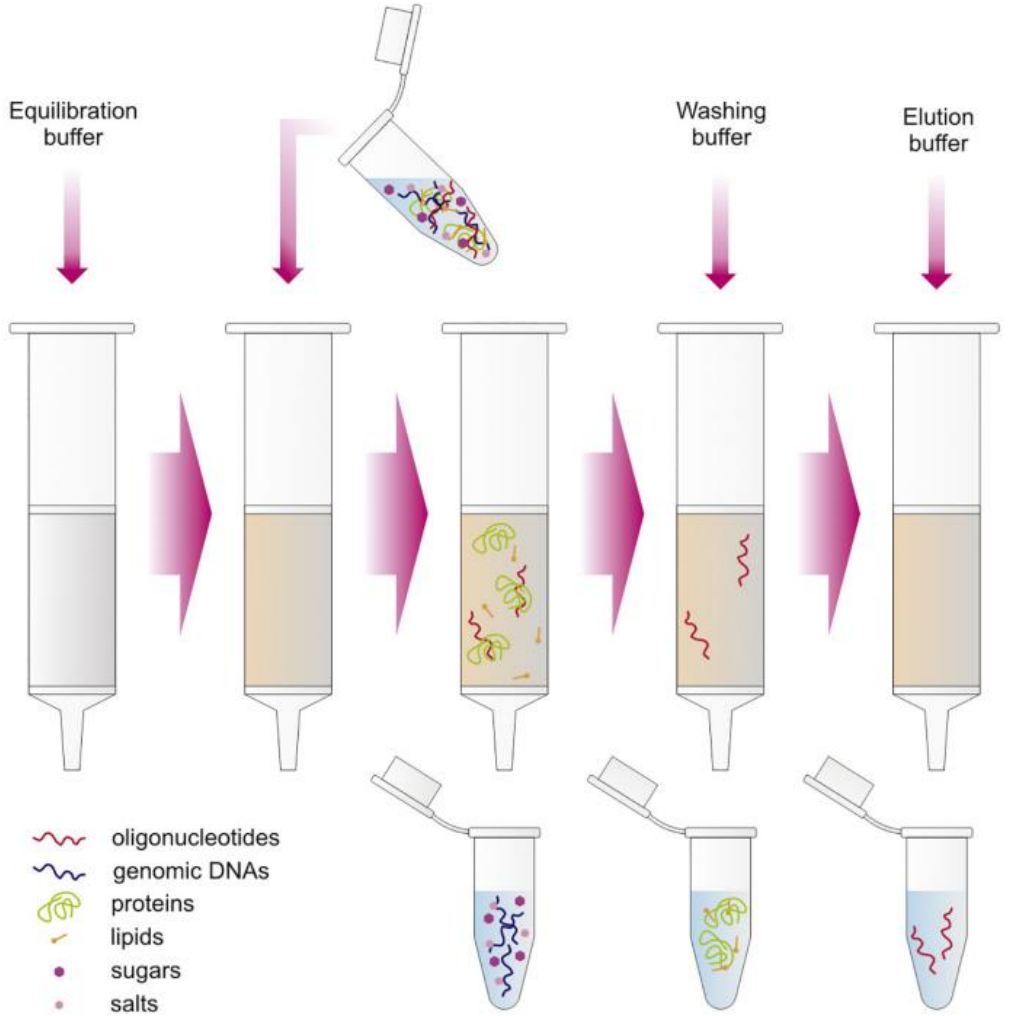
Oligonucleotides

Solid Phase Extraction (SPE)

Ion-Pairing SPE

Ion-pairing reagents:

- Tetraethylammonium bicarbonate (TEAB)
- Triethylammonium acetate (TEAA)
- Tetrabutyl ammonium hydrogen sulfate (TBAS)
- Trimethylamine/hexafluoroisopropanol (TEA/HFIP)



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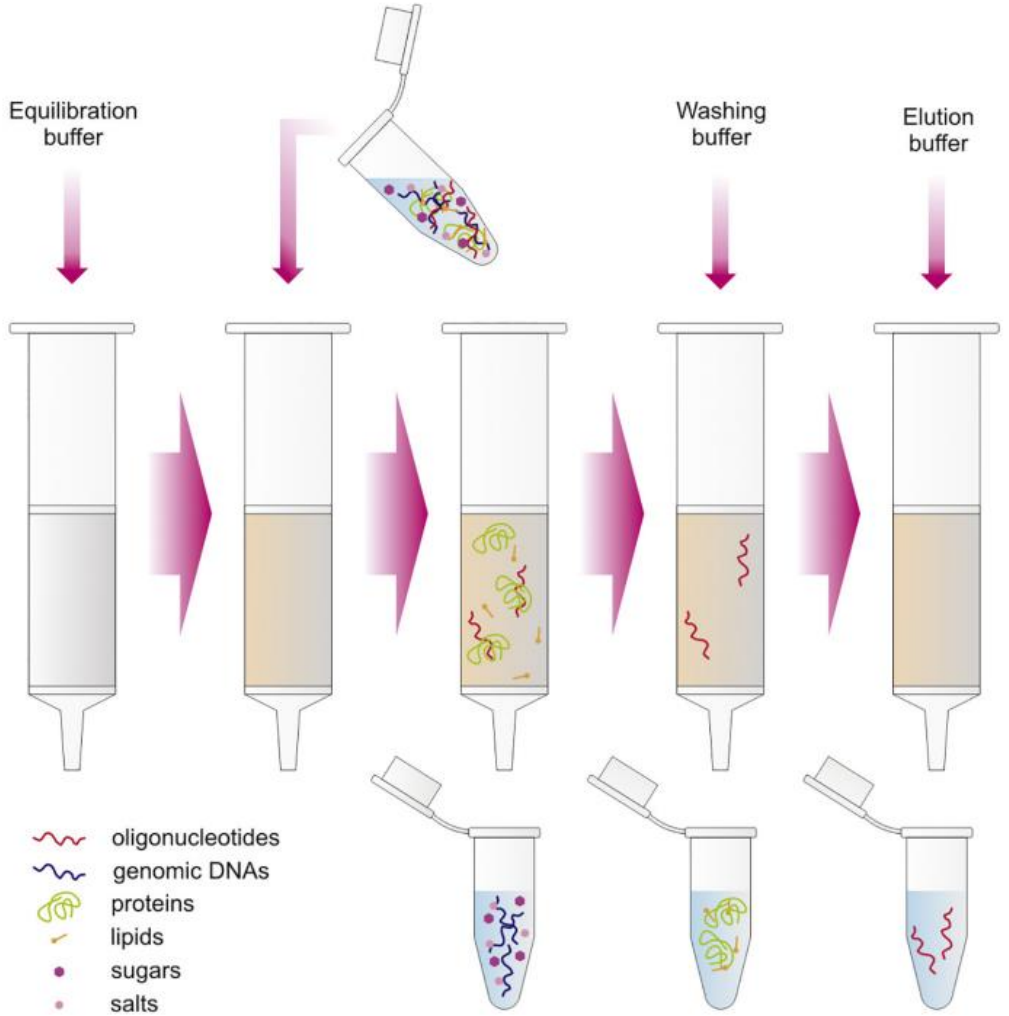
Oligonucleotides

Solid Phase Extraction (SPE)

Anion Exchange SPE

Strong anion exchange (SAX) has high yields, but uses involatile salts not compatible with MS

Weak anion exchange (WAX) has lower yield but better compatibility with MS



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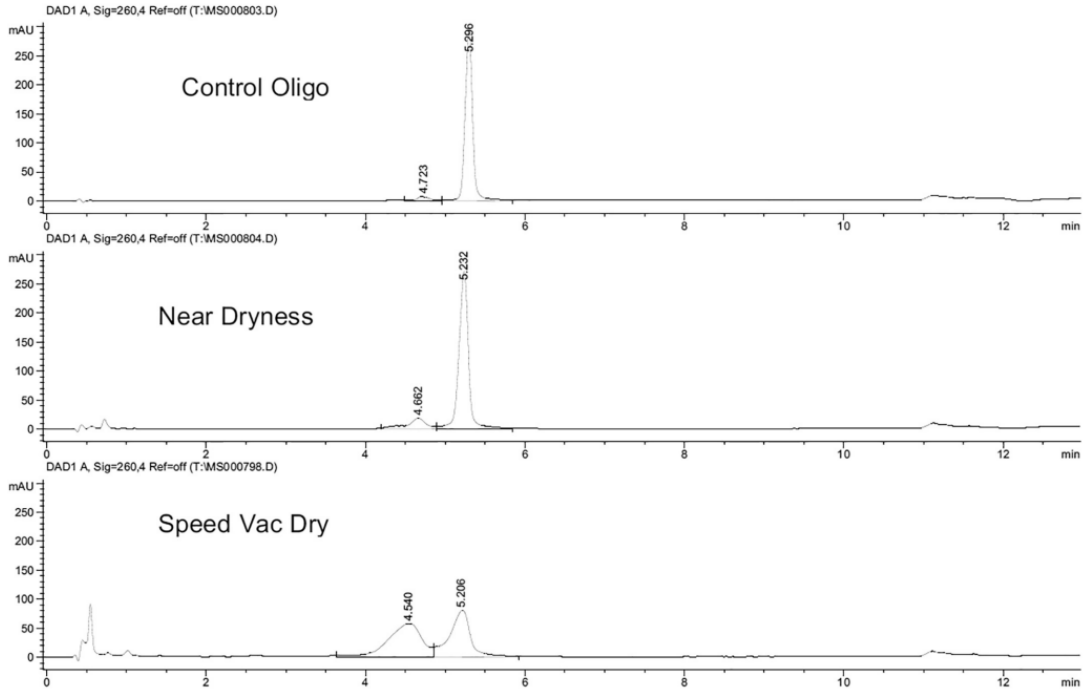
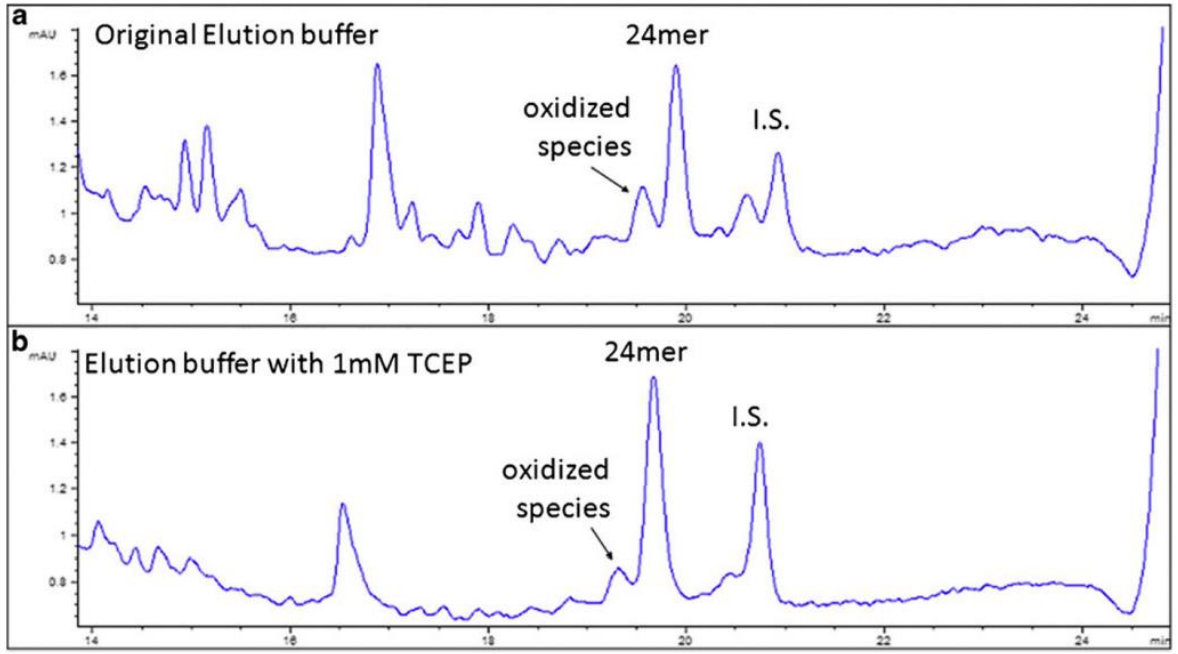
Oligonucleotides

Solid Phase Extraction (SPE)

! Important to know



Evaporating to dryness after SPE results in significant recovery loss



Addition of TCEP to elution buffers reduces oligo oxidation

Nuckowski, Ł., Kaczmarkiewicz, A., & Studzińska, S. (2018). Review on sample preparation methods for oligonucleotides analysis by liquid chromatography. *Journal of Chromatography B*, *1090*, 90–100. https://doi.org/10.1016/J.JCHROMB.2018.05.025

Oligonucleotides

Immunoaffinity and sequence capture

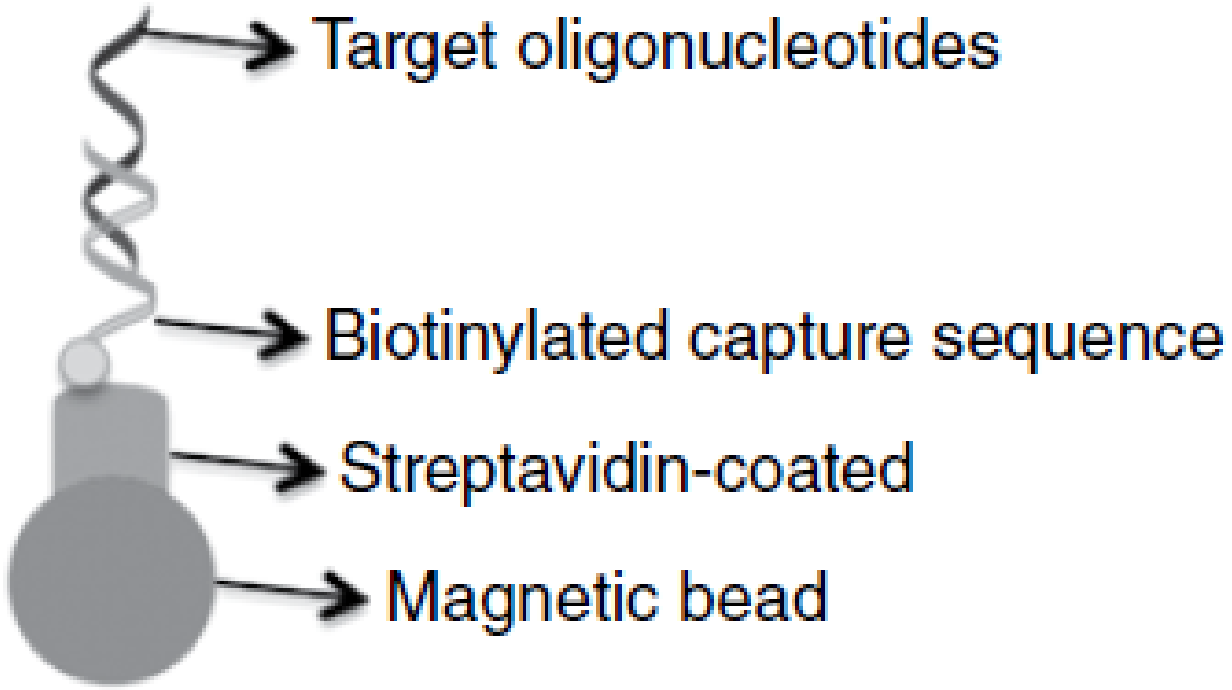
A magnetic bead can be functionalized with either a target protein or capture sequence

Strengths:

- Very high specificity for target oligos,
- High purity,
- High yield,
- Automatable

Challenges:

- Needs to have a conjugatable target
- Has not yet been used in large scale purifications



Bartlett, M. G., Kim, J., Basiri, B., & Li, N. (2019). *Sample Preparation in LC-MS Bioanalysis* (W. Li, W. Jian, & Y. Fu, Eds.). John Wiley & Sons.

Agilent Resources for Support

- Sample Preparation Fundamentals for Chromatography 3989-6969EN | Agilent
- Sample Preparation catalog 5994-0114EN | Agilent
- HPLC Advisor app HPLC Advisor app | Agilent
- Resource page http://www.agilent.com/chem/agilentresources
  - Quick reference guides, product catalogs
  - Online selection tools, “How-to” videos
  - Column user guides - User Guide | Agilent
  - Biocolumn user guides - Biocolumn User Guide | Agilent
- Tech support: http://www.agilent.com/chem/techsupport
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Thank you!