What you don't see…

…CAN hurt you

Sample Prep for Today's Analytical World

Tina Chambers
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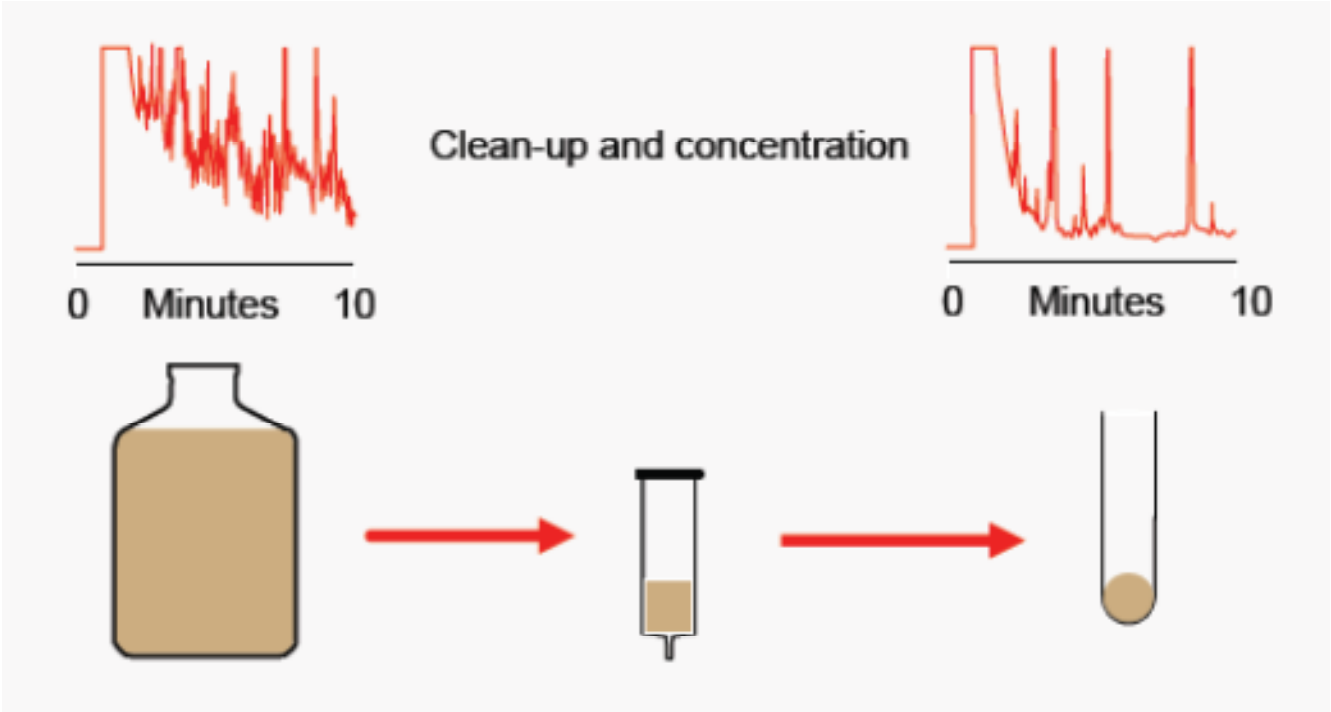
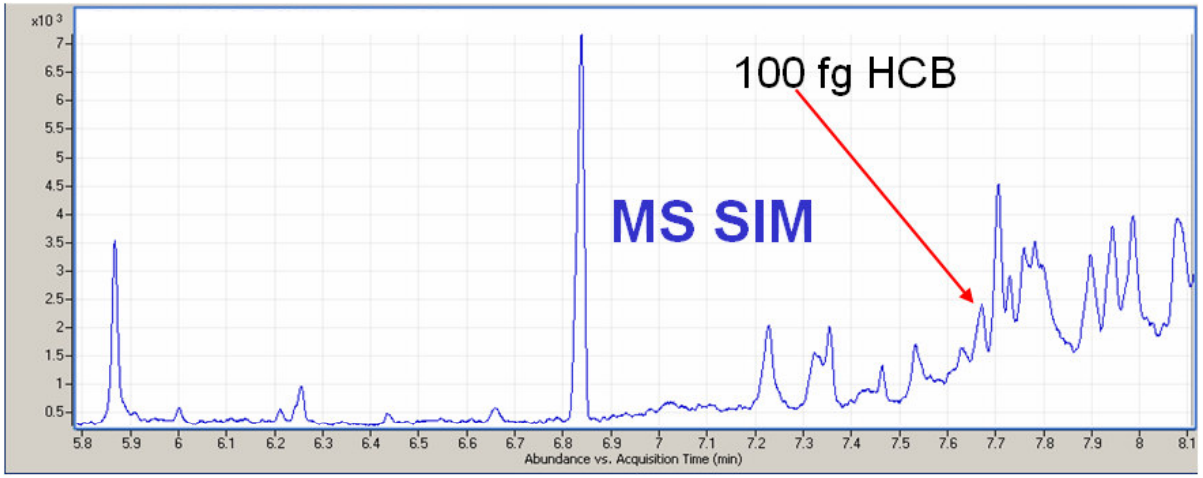
Application Engineers, Sample Prep

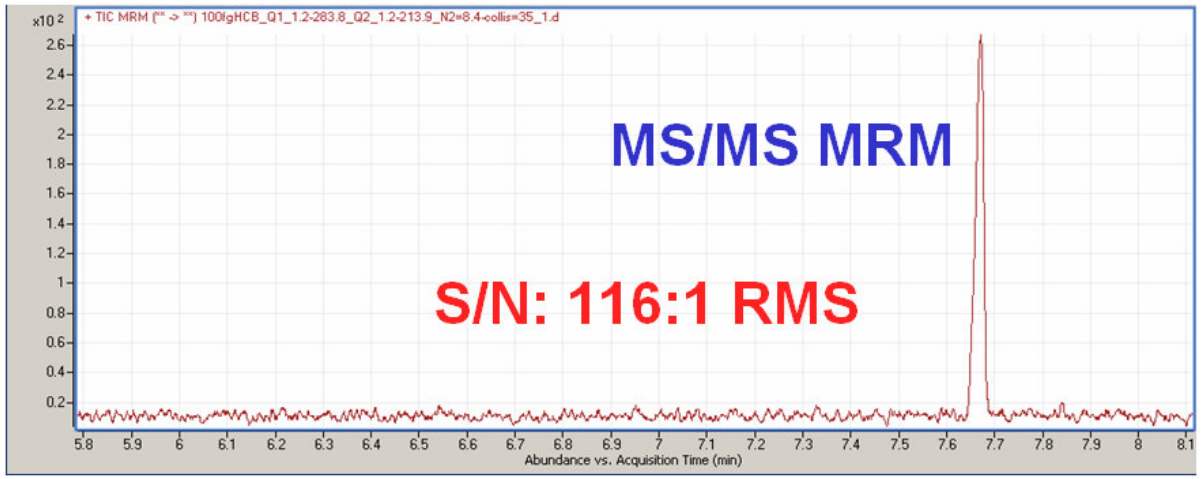
Traditional Reasons for Sample Prep



- Removal of interferences which would otherwise affect detection of analyte
- Concentration of an analyte to detectable levels
- Solvent switching into an analytically more compatible solvent

Today's World





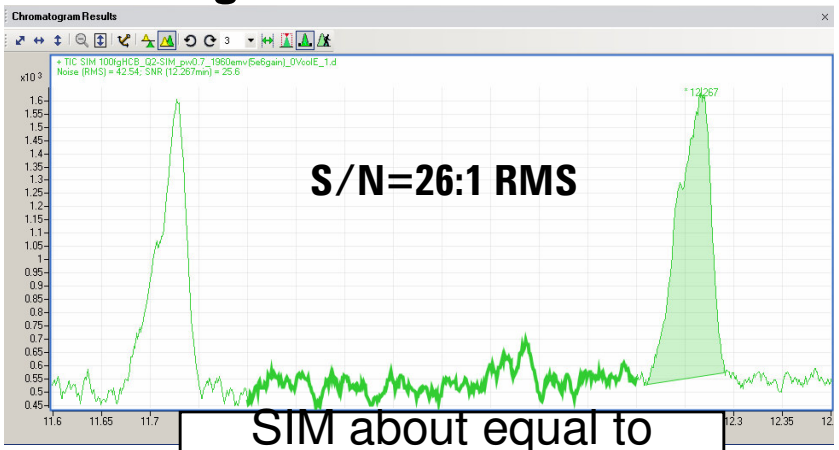
A chromatographer's dream: single peak on flat baseline

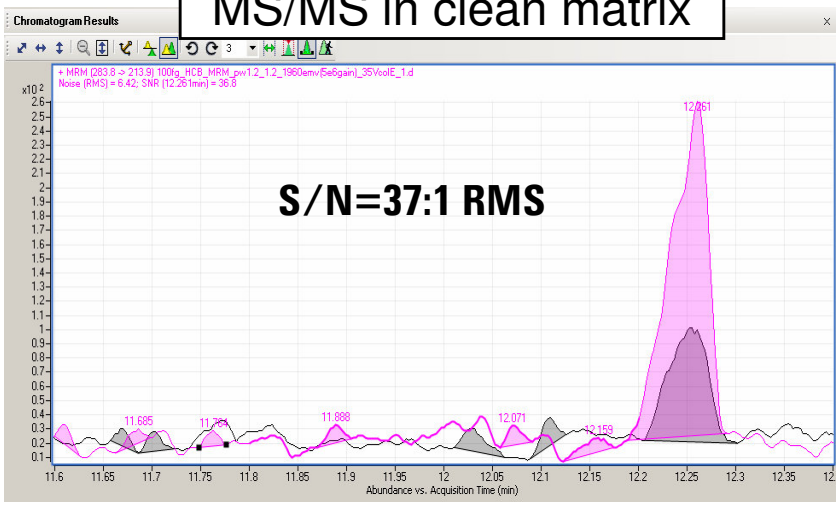
Some samples are very conducive to reduced Sample Prep…

Single MS: SIM 283.8

MS/MS: 283.8:213.9

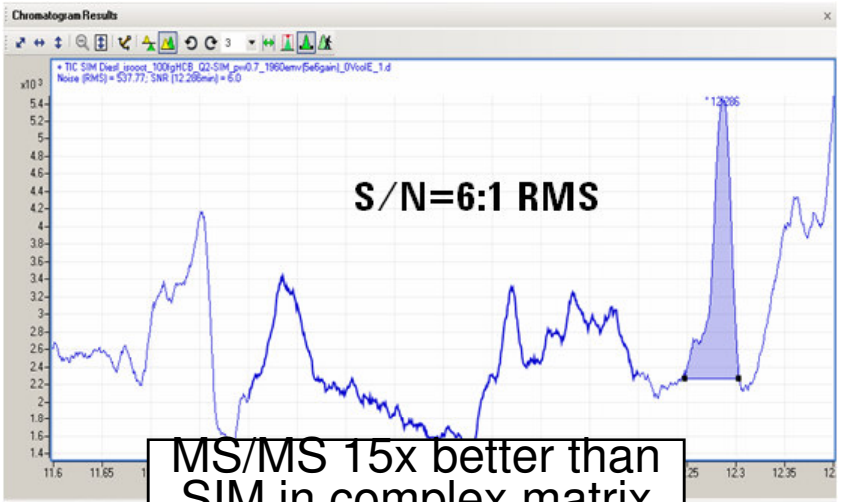
100 fg HCB in Clean Matrix

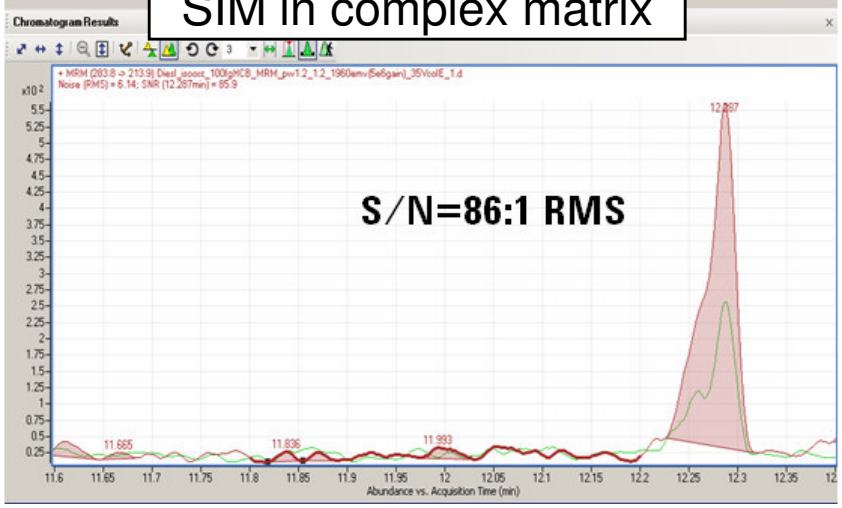




SIM about equal to MS/MS in clean matrix

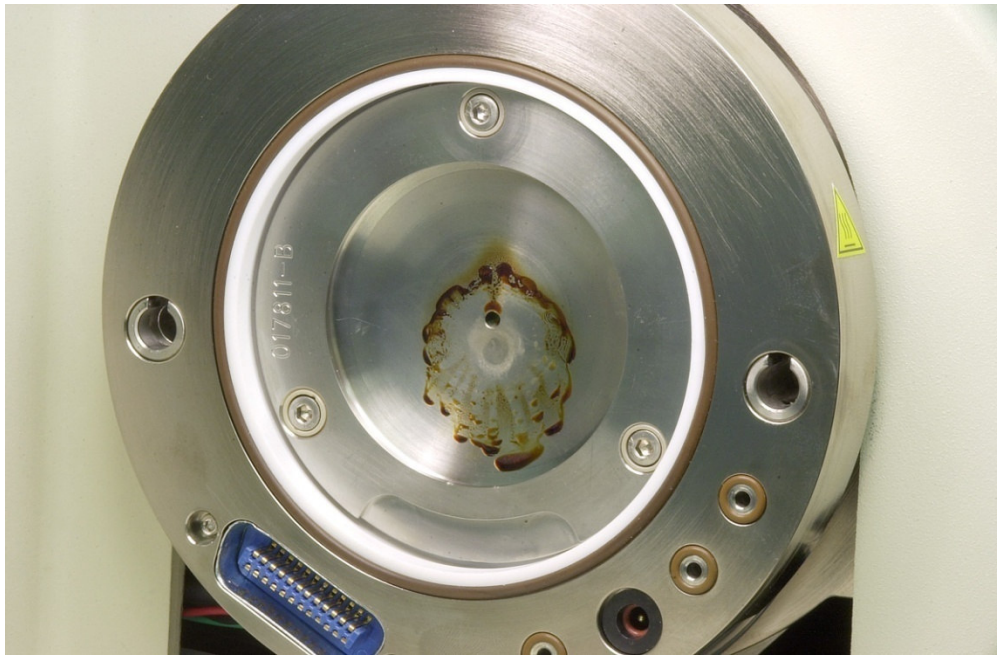
300 fg HCB in Diesel





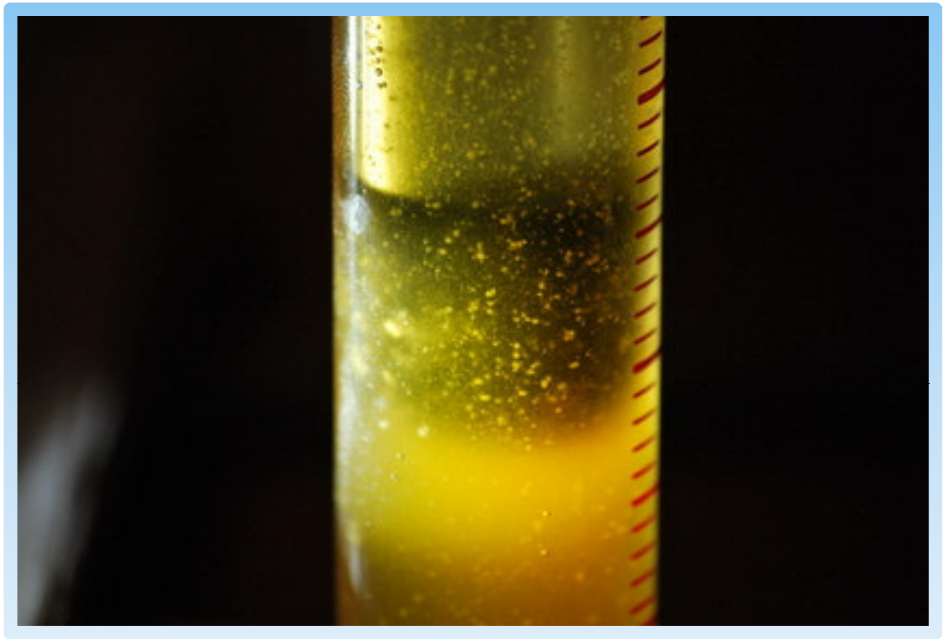
MS/MS 15x better than SIM in complex matrix

…others not so much



Curtain plate after injection of 25 samples with extracts from raisins without cleanup

Common Contaminant #1: Particulates



Why Filter?

Benefits of Filtration

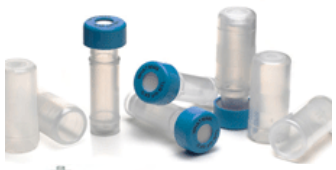
Optimal Instrument Performance	Less System Downtime	Extend Column Lifetime	Greater Sample Integrity
			

Achieve lower detection limits

Agilent recommends filtering prior to chromatographic analysis to remove particulates from the sample

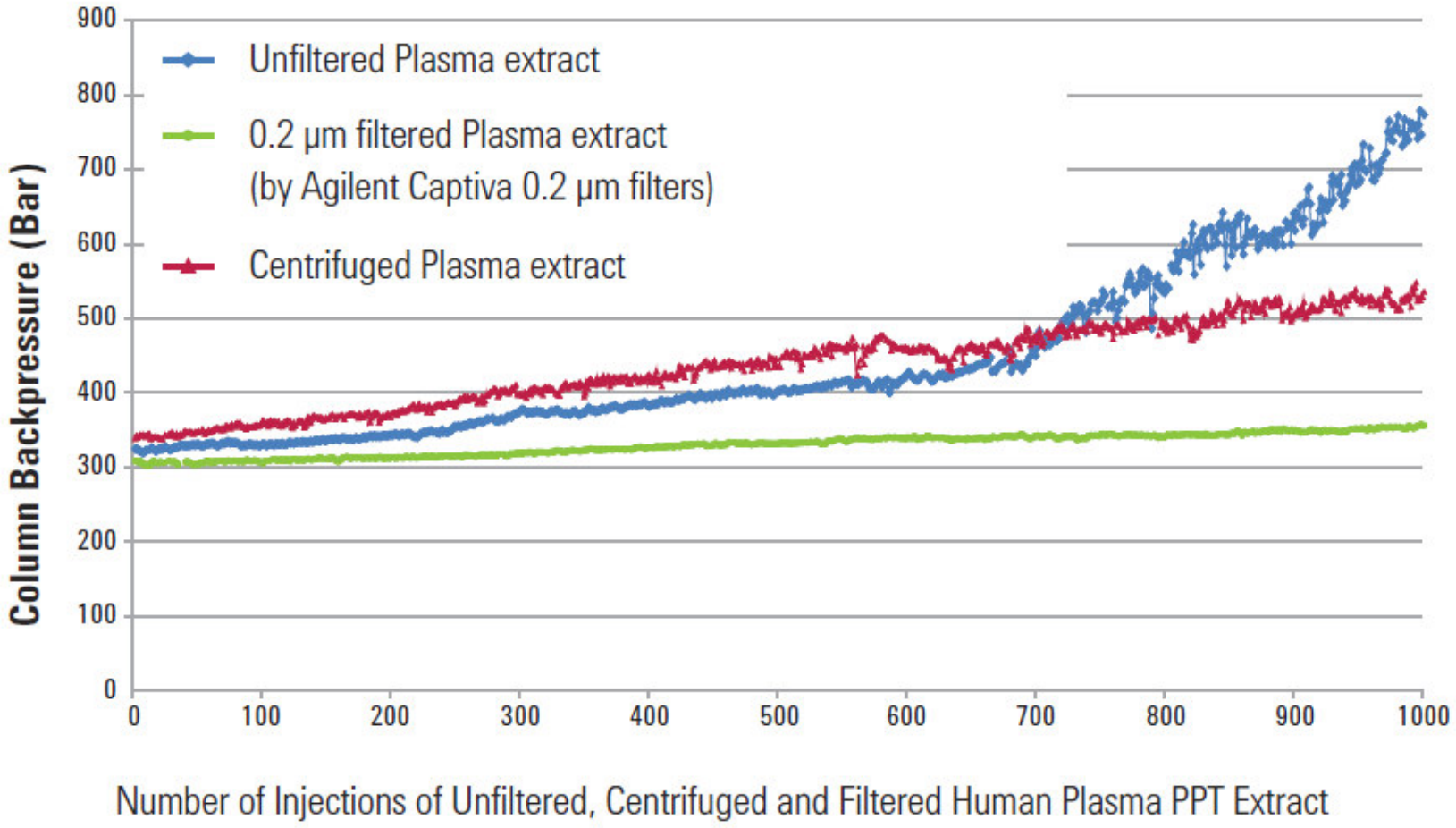
Why Filter the Sample?

Extreme Performance Requires Better Sample “Hygiene”



- Prevents blocking of capillaries, frits, and the column inlet (especially important for UHPLC)
- Results in less wear and tear on the critical moving parts of injection valves
- Results in less downtime of the instrument for repairs
- Produces improved analytical results by removing potentially interfering contamination
- “Functionalized” filtration can improve results over mechanical filtration by removing chemical interferences in addition to particulates

Syringe Filter Benefits: Improved sub-2 micron LC Column Lifetime – With Human Plasma Extract



Agilent Captiva Filtration Products: Setting a New Standard in Filtration

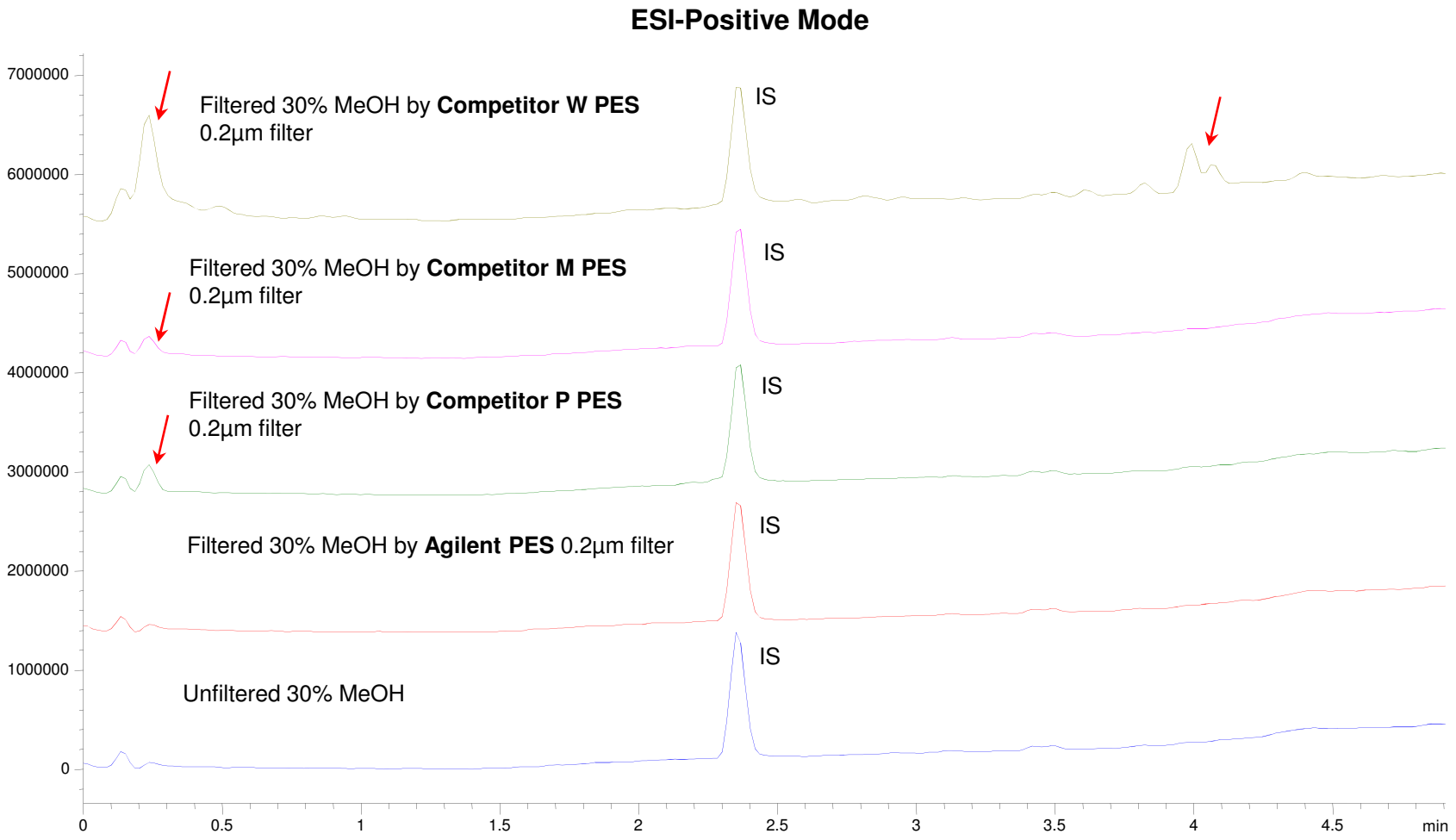
Captiva Premium Syringe Filters

- Certified to be free of UV detectable extractables on HPLC
  - PES & Glass Fiber also certified for LC/MS
- Color-coded boxes for easy identification
- Comprehensive portfolio to meet your application needs

Premium Syringe Filters						
Membrane	Diameter / Pore size					
	4 mm		15 mm		25 mm (* 28 mm)	
	0.2 µm	0.45 µm	0.2 µm	0.45 µm	0.2 µm	0.45 µm
PTFE	♦	♦	♦	♦	♦	♦
Nylon			♦	♦	♦	♦
PES	♦	♦	♦	♦	♦	♦
Regenerated Cellulose	♦	♦	♦	♦	♦	♦
Cellulose Acetate					♦	♦
Glass Microfiber			♦		♦*	
Depth filters: Glass / PTFE			♦	♦	♦	♦*
Depth filters: Glass / Nylon			♦	♦	♦	♦

Cellulose Acetate | Nylon | Glass Fiber/ PTFE | Glass Fiber | PES | PTFE | Regenerated Cellulose | Glass Fiber/ Nylon

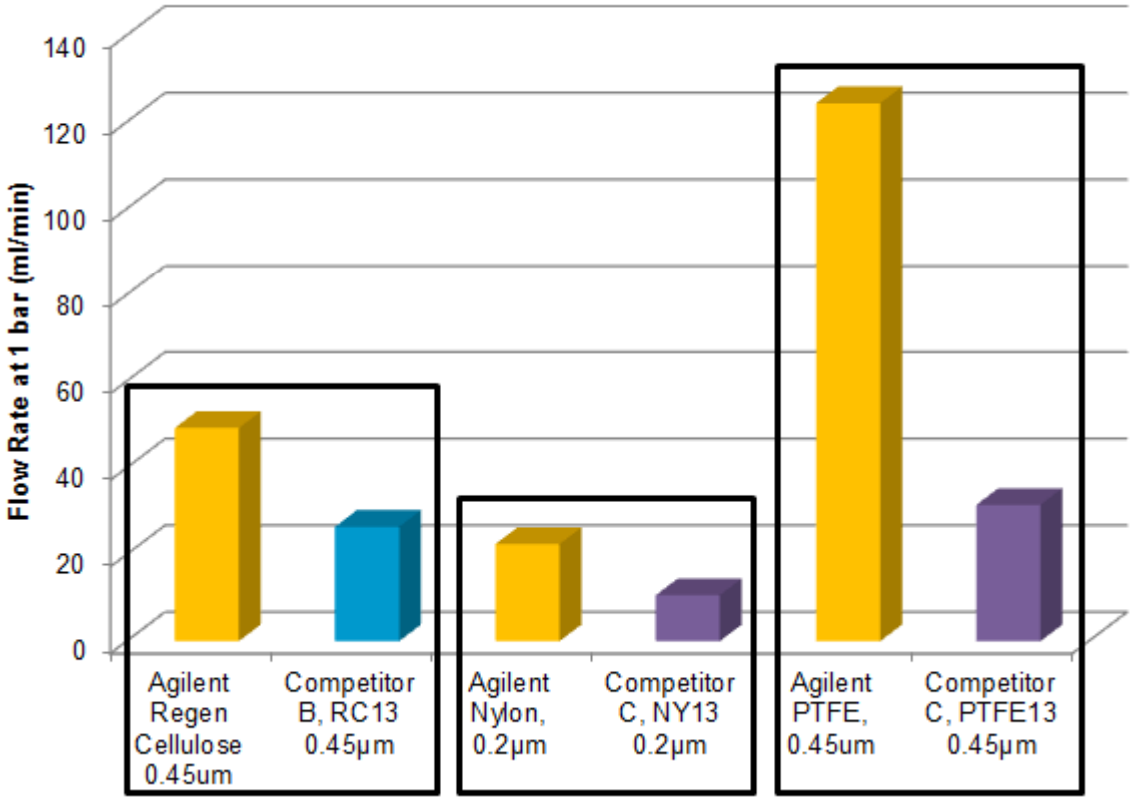
Cleanliness of Agilent Captiva PES Filters



Agilent Technologies

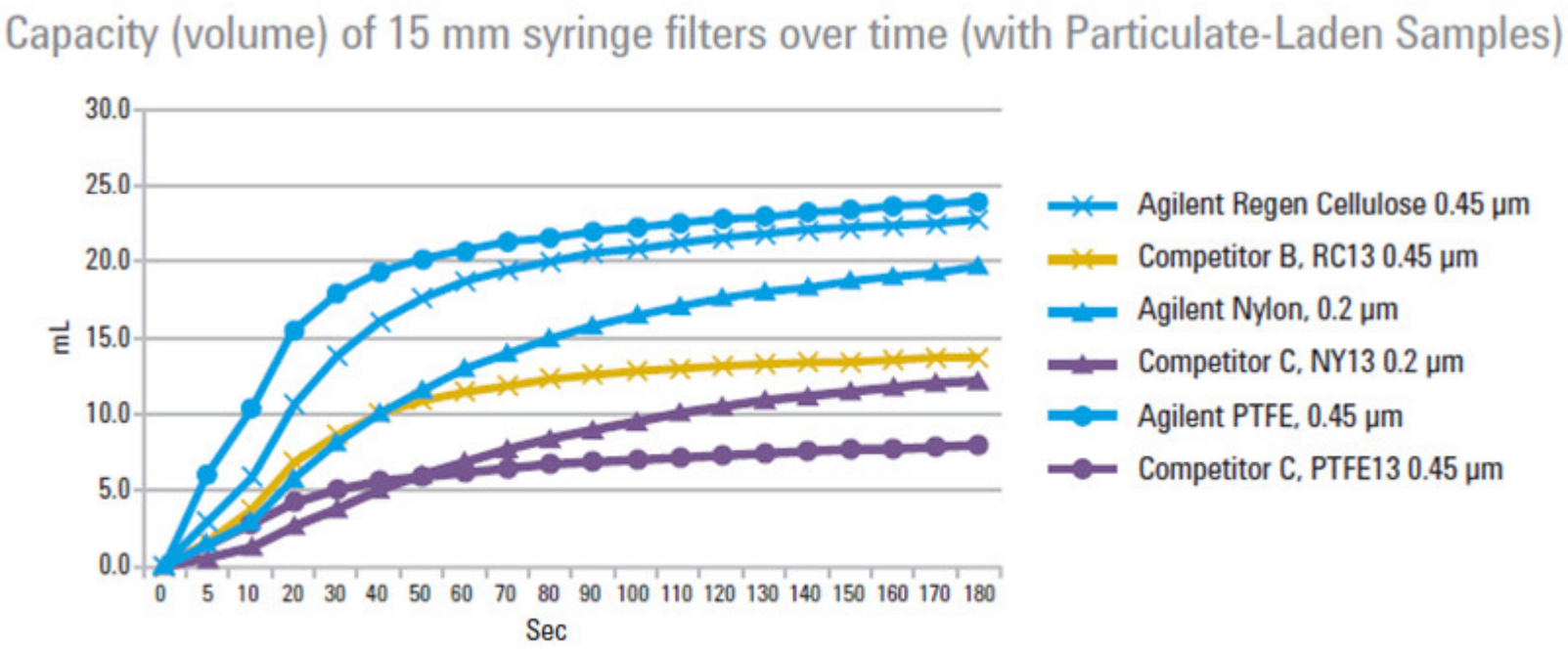
Captiva Premium Syringe Filters: Fastest Flow Rates

Agilent **RC**, **Nylon** and **PTFE** filters compared to competitors



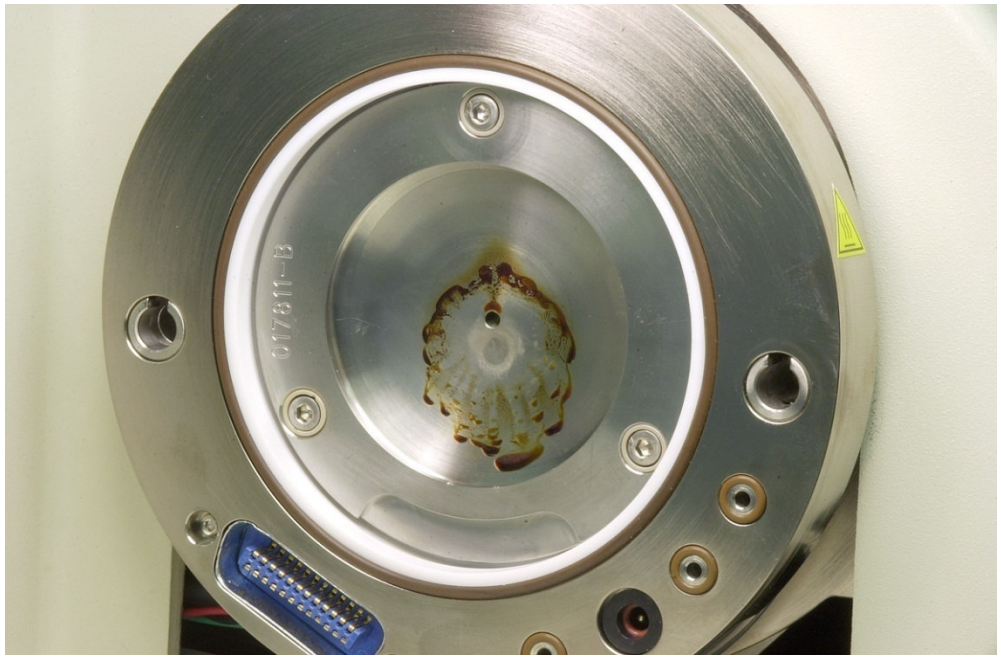
Captiva Premium Syringe Filters: High Loading Capacity

- ✓ Due to nature of membrane, there is less clogging by particulates
- ✓ Assures more sample will be filtered



…Now back to our source.

Will filtration solve everything?

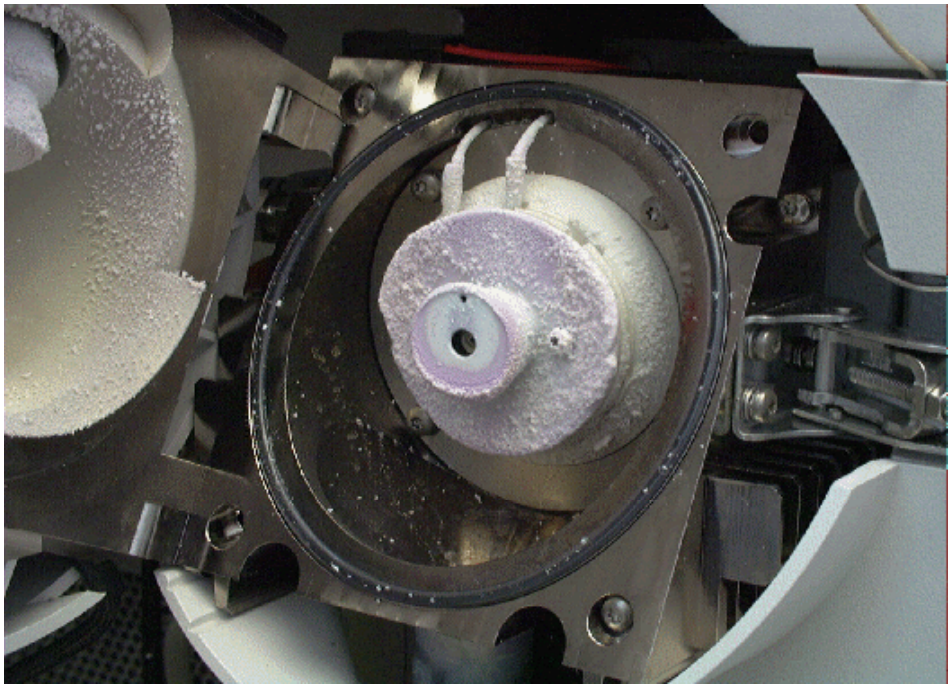


Curtain plate after injection of 25 samples with extracts from raisins without cleanup

Common Contaminant #2: Salts and Minerals

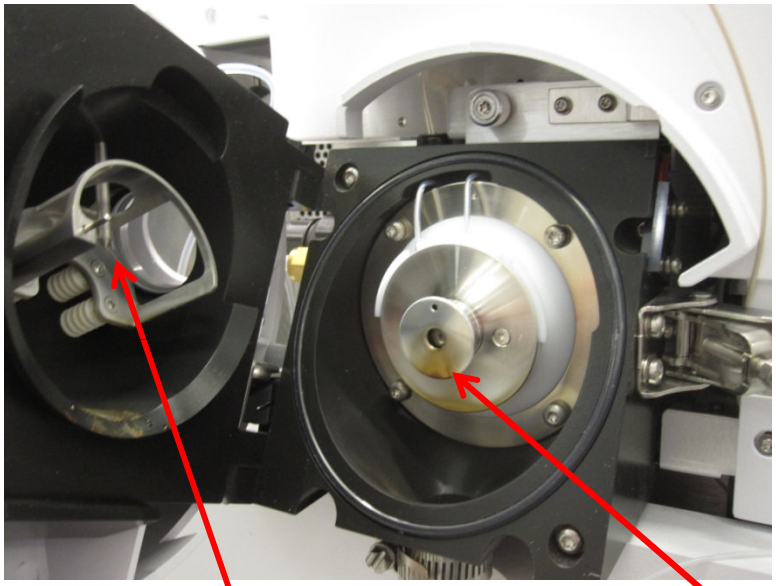
- Non-Volatile: will form residues in injection liner of ANY GC system
- Very water soluble: will carry through to detector in ANY LC system
- Ubiquitous in water (4g/L-30g/L) and body fluids (40-220 meq/L/day in urine)
- In LC/MS systems, ion suppression and precipitation in ion source/loss of sensitivity
- Easily eliminated by liquid-liquid extraction (conventional or solid-supported), but very polar compounds are difficult to extract

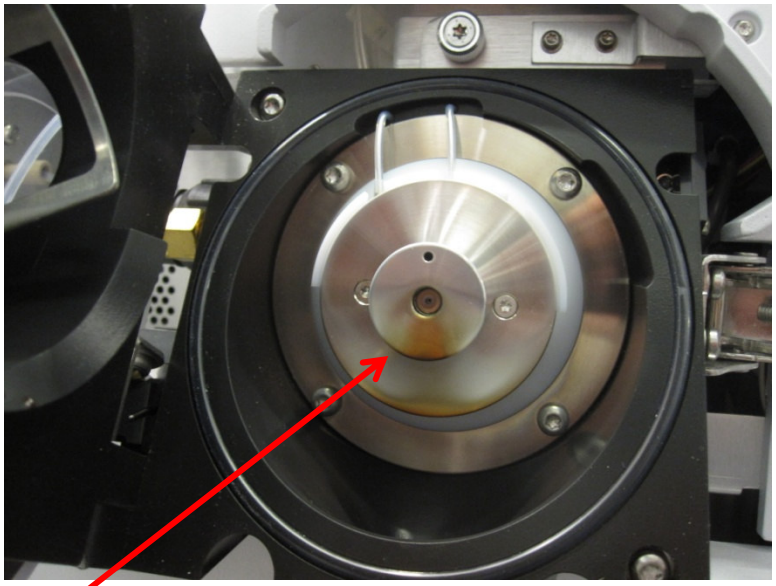
Salt Precipitation in Ion Source



Easily cleaned with MeOH, but downtime/run interruptions still exist

Orthogonal ESI Ion Source Condition after 3000x Urine Dilute/shoot Injections





Spray Shield/MS Inlet/Capillary

Nebulizer/Sprayer

6410 QQQ Sensitivity Results

Dilute/Shoot (1/10 dilution) versus SPE Sample Preparation

Opiates/Opioids

Compound	D/Shoot LLOQ (ng/ml)	SPE LLOQ (ng/ml)	ULOQ (ng/ml)
6-monoacetyl morphine	10	<1	1000
buprenorphine	10	1	1000
codeine	25	<1	1000
dihydrododeine	25	<1	1000
EDDP	10	<1	1000
fentanyl	1	<1	1000
heroin	10	<1	1000
hydrocodone	10	<1	1000
hydromorphone	5	<1	1000
meperidine	5	<1	1000
methadone	10	<1	1000
morphine	5	<1	1000
naloxone	5	<1	1000
naltrexone	10	<1	1000
N-desmethyltramadol	10	1	1000
norbuprenorphine	25	3	1000
norfentanyl	1	<1	1000
normeperidine	5	<1	1000
norpropoxyphene	5	<1	1000
o-desmethyltramadol	5	<1	1000
oxycodone	10	<1	1000
oxymorphone	5	<1	1000
propoxyphene	5	<1	1000
tapentadol	5	<1	1000
tramadol	1	<1	1000
trazodone	1	<1	1000

Sedatives/hypnotics

Compound	D/Shoot LLOQ (ng/ml)	SPE LLOQ (ng/ml)	ULOQ (ng/ml)
2-OH-ethylflurazepam	200	5	1000
7-aminoclonazepam	10	<1	1000
7-aminoflunitrazepam	5	<1	1000
alpha-OH-midazolam	10	<1	1000
alprazolam	10	<1	1000
a-OH-alprazolam	20	<1	1000
a-OH-triazolam	50	<1	1000
chlordiazepoxide	10	<1	1000
clonazepam	25 to 50	<1	1000
desalkylflurazepam	20	1	1000
diazepam	10	<1	1000
flunitrazepam	10	1	500
flurazepam	5	1	1000
lorazepam	50	20	1000
midazolam	10	<1	1000
nitrazepam	25	5	1000
nordiazepam	25	<1	1000
oxazepam	50	25	1000
temazepam	25	<1	1000
triazolam	5	<1	1000
zolpidem	5	<1	1000

Sample Prep Solutions

- Liquid/Liquid or SSLE for non-polar compounds
- SPE for very complex matrices or large sample volumes
- QuEChERS for polar compounds (with or without dispersive SPE)

- Let's look at these in turn-

Sample Preparation – Supported LLE (SLE)

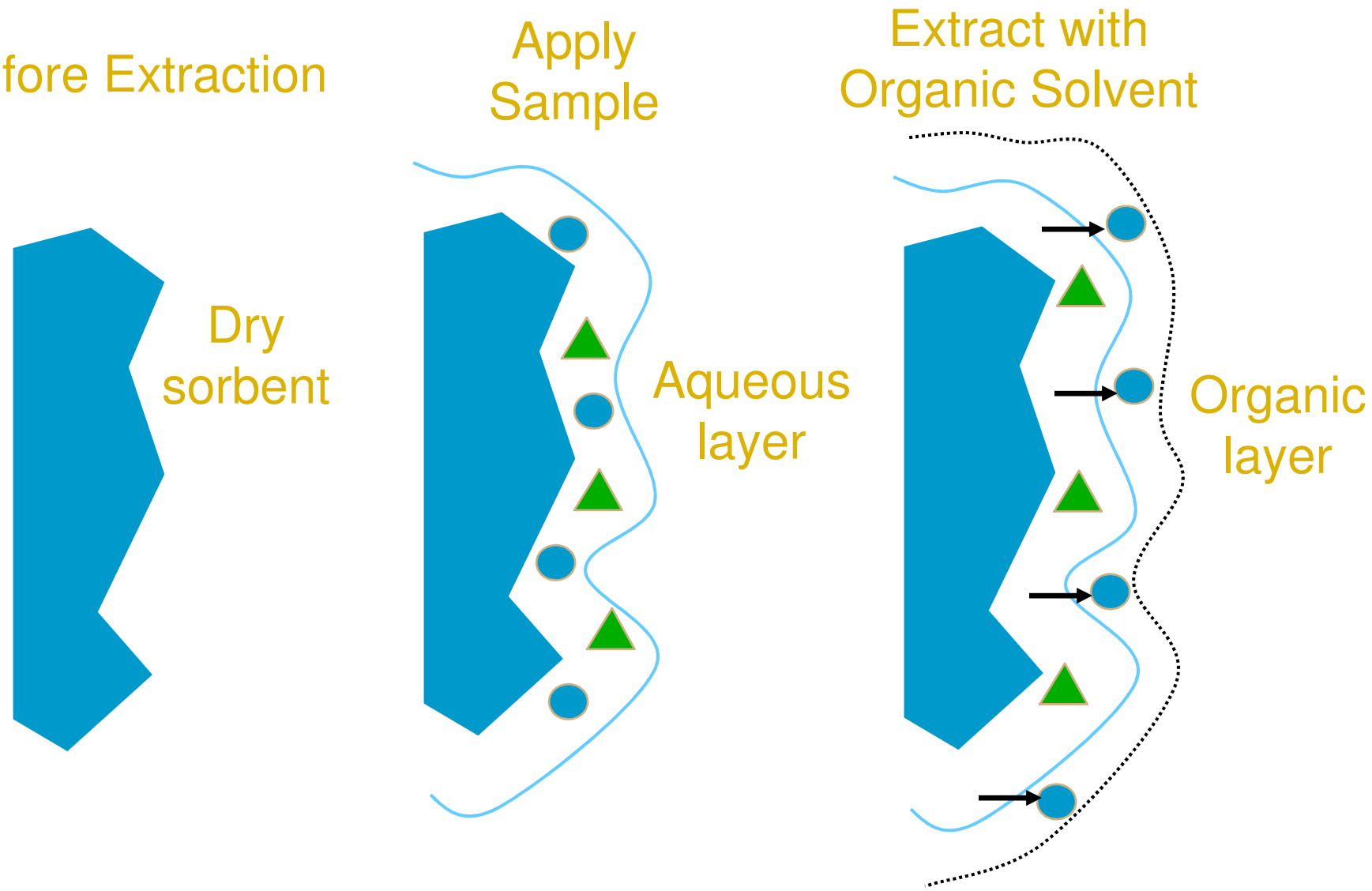
Hydromatrix™ - diatomaceous earth sorbent

- Composed of fossilized diatoms
- Purified at high temperatures
- High surface area for water adsorption
- Very polar surface

Chem Elut™ - pre-assembled cartridges with Hydrom

Combilut ™- 96-well plate filled with Hydromatrix

The SLE Process



The Chem Elut Method

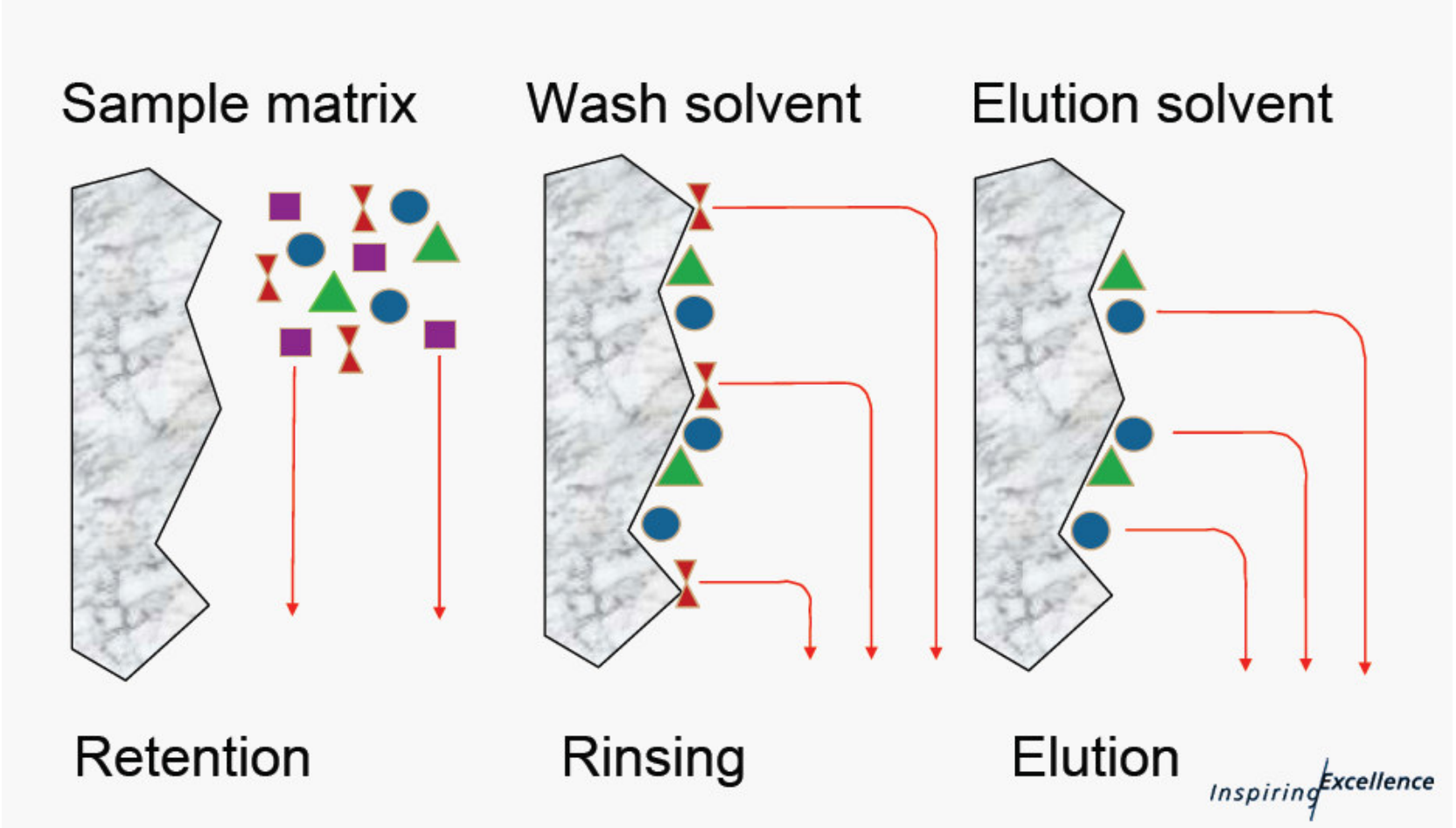
Aqueous sample being applied

Solid support adsorbs water onto high surface area particles

Organic extraction solvent

Agilent Technologies

The SPE mechanism

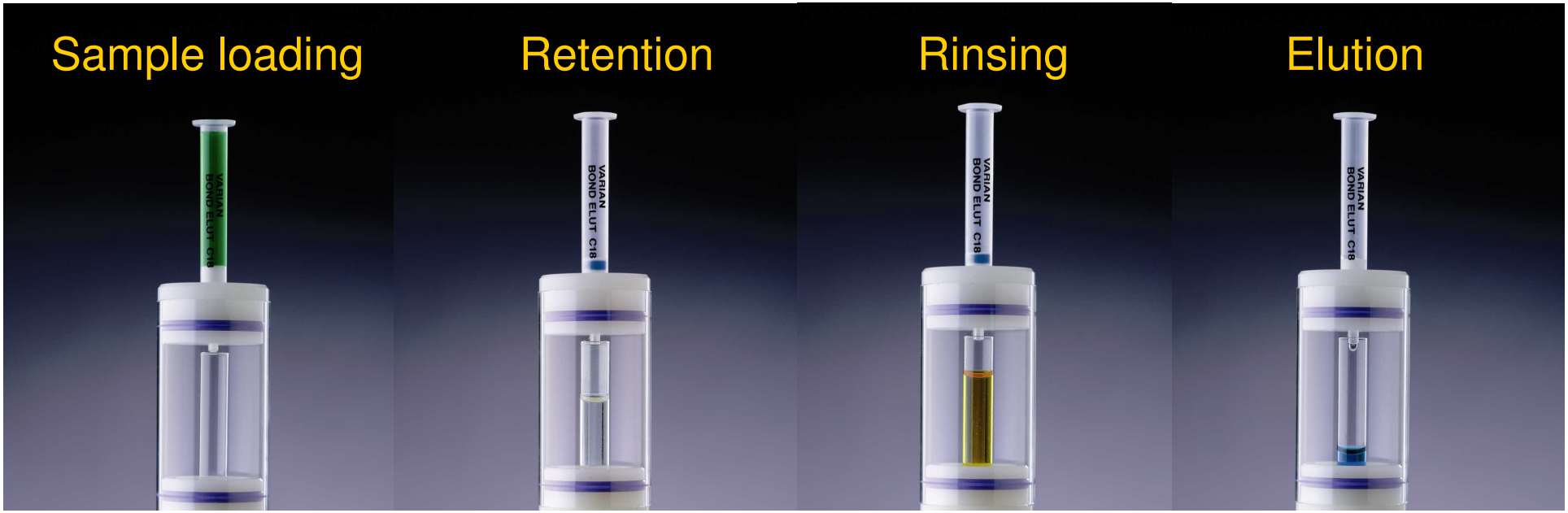


The Bond Elut Method

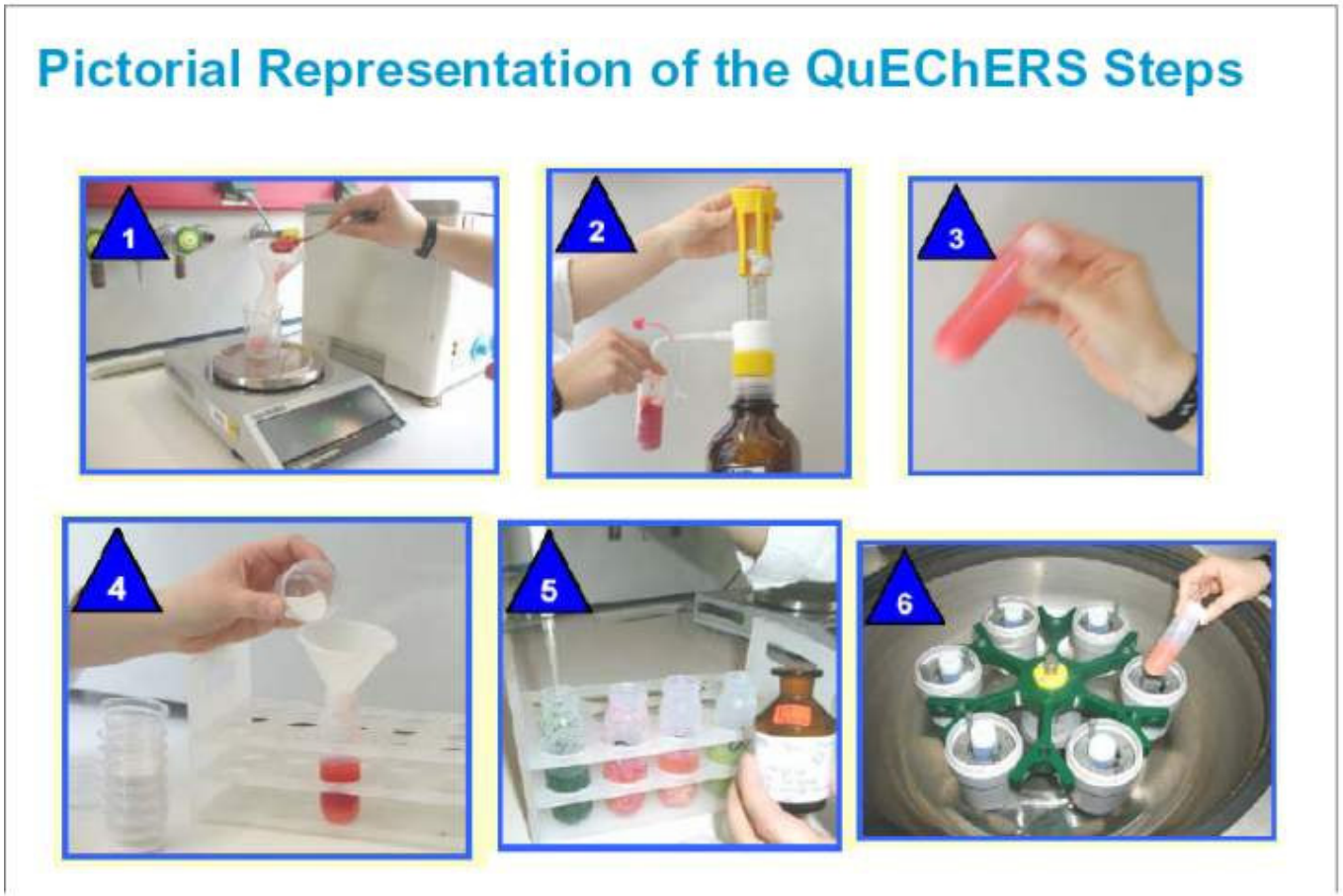
Green = Blue and Yellow

Blue is more non polar than yellow

Blue is retained

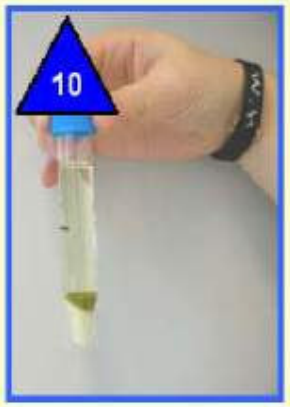


QuEChERS
First step extraction



1) Weigh sample, add water if needed, spike
2) Add 10ml ACN
3) Vortex
4) Add salt packet
5) Shake 1 minute
6) Centrifuge at 4,000 rpm for 5 minutes

Second Step – Dispersive SPE



7) Choose d-SPE kit based on matrix characteristics

8) Transfer 1-8ml alliquot, vortex 1 minute

9) Centrifuge

11) Analyze by GC/MS or LC/MS

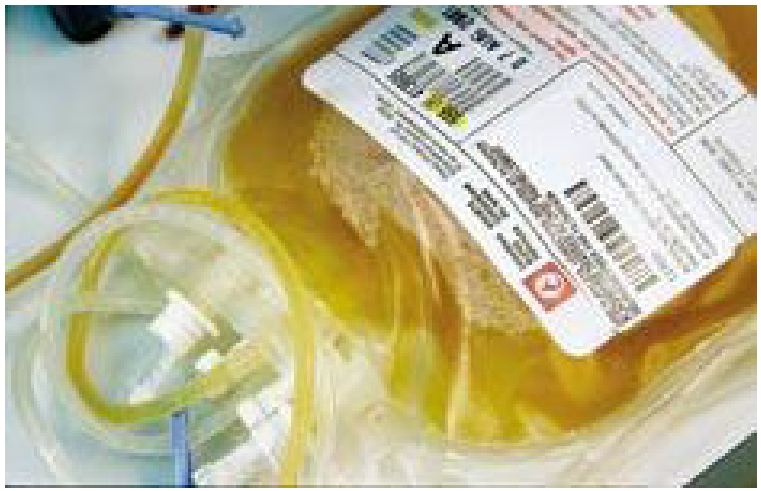
Common Contaminant #3: Proteins and Lipids

- Low volatility in any GC systems, contaminate inlet liner, gold seal, front end of column
- Limited solubility of proteins in high organic reverse phase mobile phases in HPLC systems
- Both lipids and proteins coat column in any HPLC system: decrease/alteration of chromatographic separation
- Commonly encountered in human and animal blood and tissue samples, including mother's milk
- These will typically NOT show up as discreet contamination peaks

GC System Components Contamination with Biological Samples

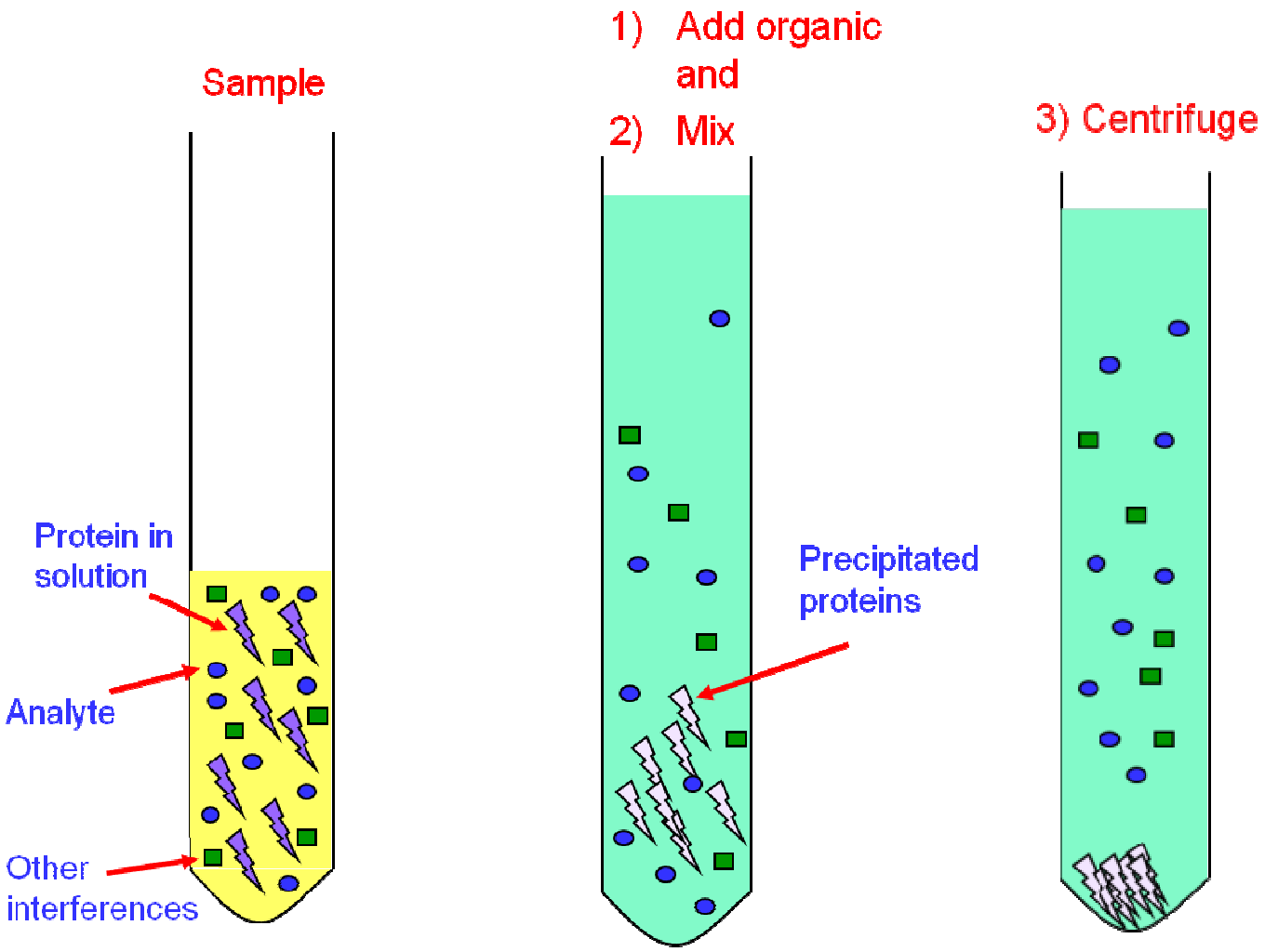
Sample Prep Solutions

- Protein Precipitation with or without Lipid Removal
- SPE
- QuEChERS for Fatty and Waxy Samples



Plasma contains high levels of proteins and lipids

Manual Protein Precipitation



4) Remove supernatant

5) Analyze supernatant, often after dry down and resuspension

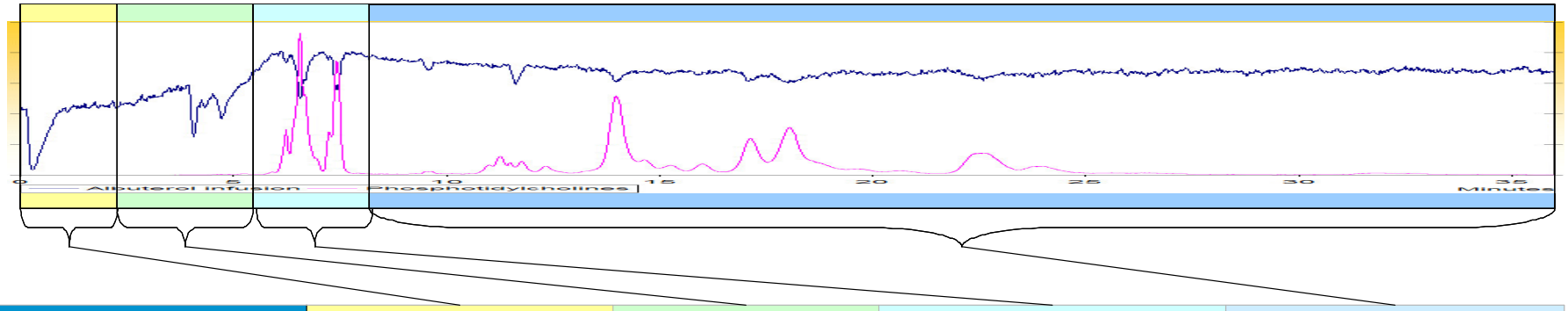
Faster or High Throughput with Filtration Plates

Captiva and Captiva ND are mechanical filters only

- Five times faster than manual (5 min vs. 26 min)
- No transfer steps
- Easy method transfer
- Filtration from 0.2um to 20um pore size

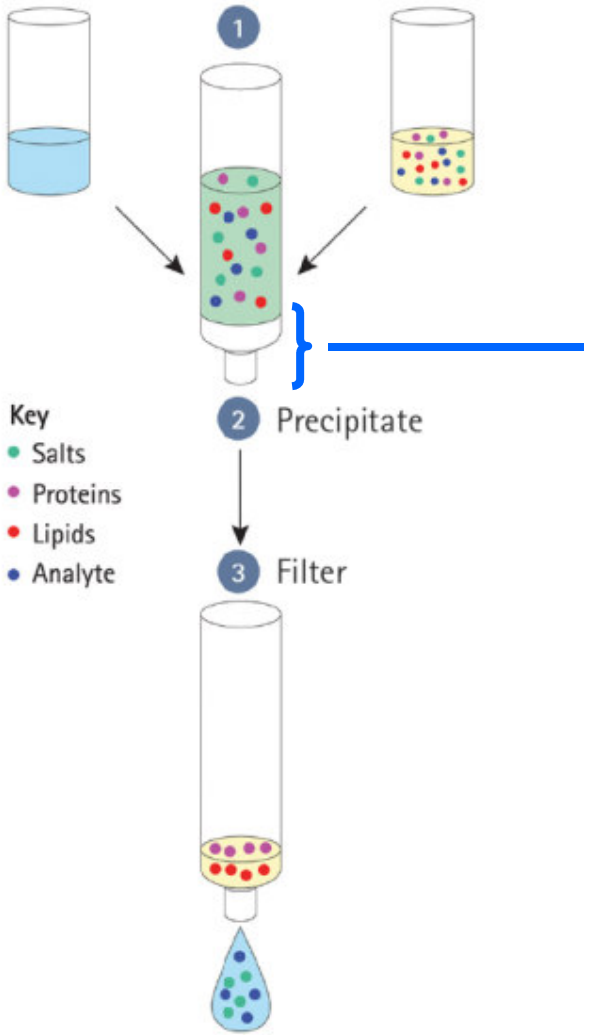
Ion Suppression Regions

Protein precipitation sample
PCI with procainamide



Interference type	Salt/Polar ionics	Proteins/ Peptides	Lyso-phosphatidylcholines	Lipids and other hydrophobics
Typical Elution Conditions (C18 column)	At or near void with < 20% organic	10's of column volumes at 40% - 70% organic	10's of column volumes at 70% - 90% organic	10's to 100's of column volumes at > 90% organic
Short term effect (single injection)	Significant ion-suppression	Significant ion-suppression	Significant ion-suppression	Some ion suppression, however, usually retained on LC column)
Long term effect (multiple injections)	Unknown	Unknown	Decreased sensitivity, Increased variability	Decreased sensitivity, Increased variability
Likely long term causes	Ion source contamination	Ion source contamination	Ion source contamination, Some column build-up	Ion source contamination, Column build-up

Captiva™ ND*Lipids* Lipid removal filtration plate



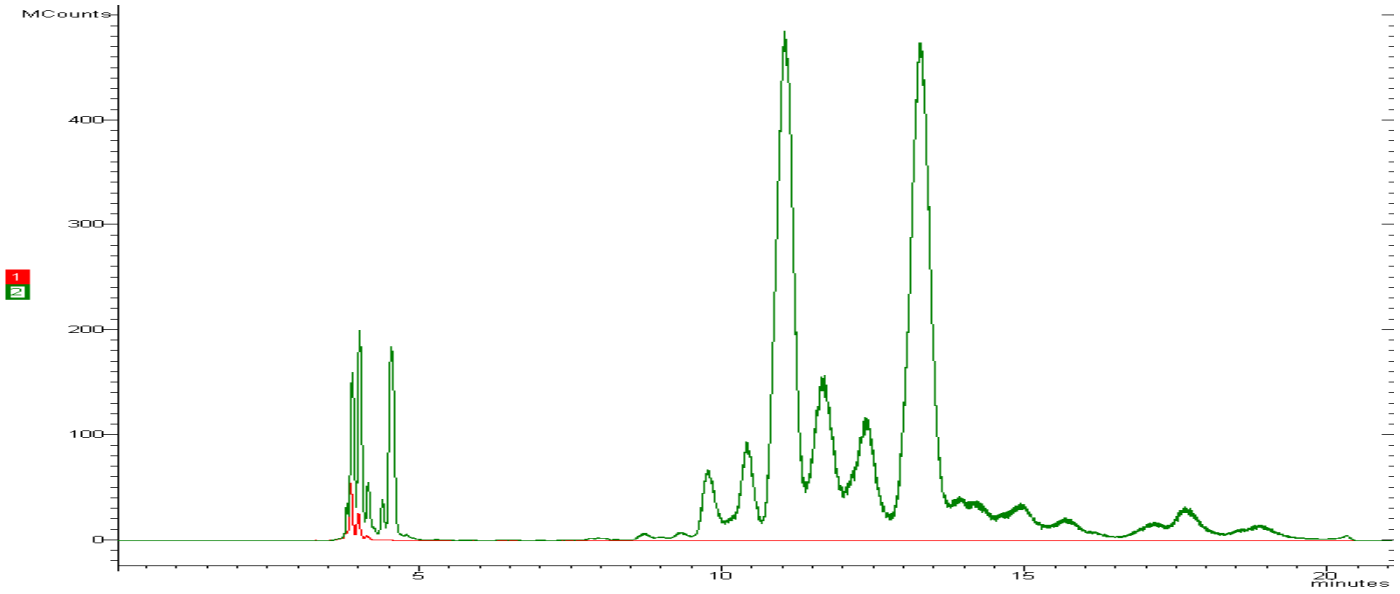
Features:

- Captiva particulate filter → removes protein interferences
- Proprietary Lipid Stripping Media → removes lipids
- Non-Drip Membrane → ease of use

Lipid removal

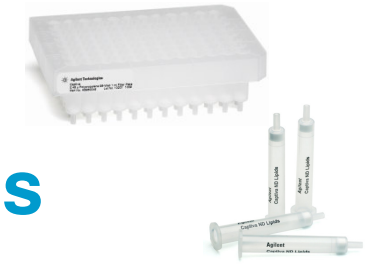
Green = ppt only

Red = lipid-stripped ppt with CaptivaND*Lipids*



MS Transition 184 →184

Sample Preparation Time Comparison
PPT (centrifugation) vs. Captiva ND Lipids



Captiva ND Lipids	Time (min)
Add 0.6 mL of MeOH and 0.2 mL of plasma sample to Captiva ND 96-well plate.	5
Mix each well with a pipette 5 times and apply vacuum for filtration.	
Directly transfer injection plate for analysis.	0
Total time required for sample preparation	5

This time comparison is based on the preparation of 96 samples.

Comparison to Protein Precipitation Antidepressants

Method Used:

Sample preparation with Captiva ND*Lipids* filtration plates:

1. Add 600 µl methanol (with 0.1% formic acid) into the well of the Captiva ND*Lipids* filtration plate.
2. Add 200 µl rat plasma (spiked, 1 – 128ng/mL) into each well, and process 6 replicates for each concentration, mix.
3. Process the plate by vacuum (15” Hg) for 4 min, and collect filtrate.

Sample preparation with standard protein precipitation:

1. Add 200 µl rat plasma (spiked, 1 – 128ng/mL) into 1.5 ml vial, 6 replicates for each concentration.
2. Add 600 µl methanol (0.1% formic acid) into the vial, vortex.
3. Centrifuge for 10 min at 14,000 rpm.
4. Take 600 µl aliquots of the supernatant from each vial, transfer to a 96-well sample plate.

Application note 01736

Absolute Recoveries; Sensitivity Enhancement Over Protein Precipitation

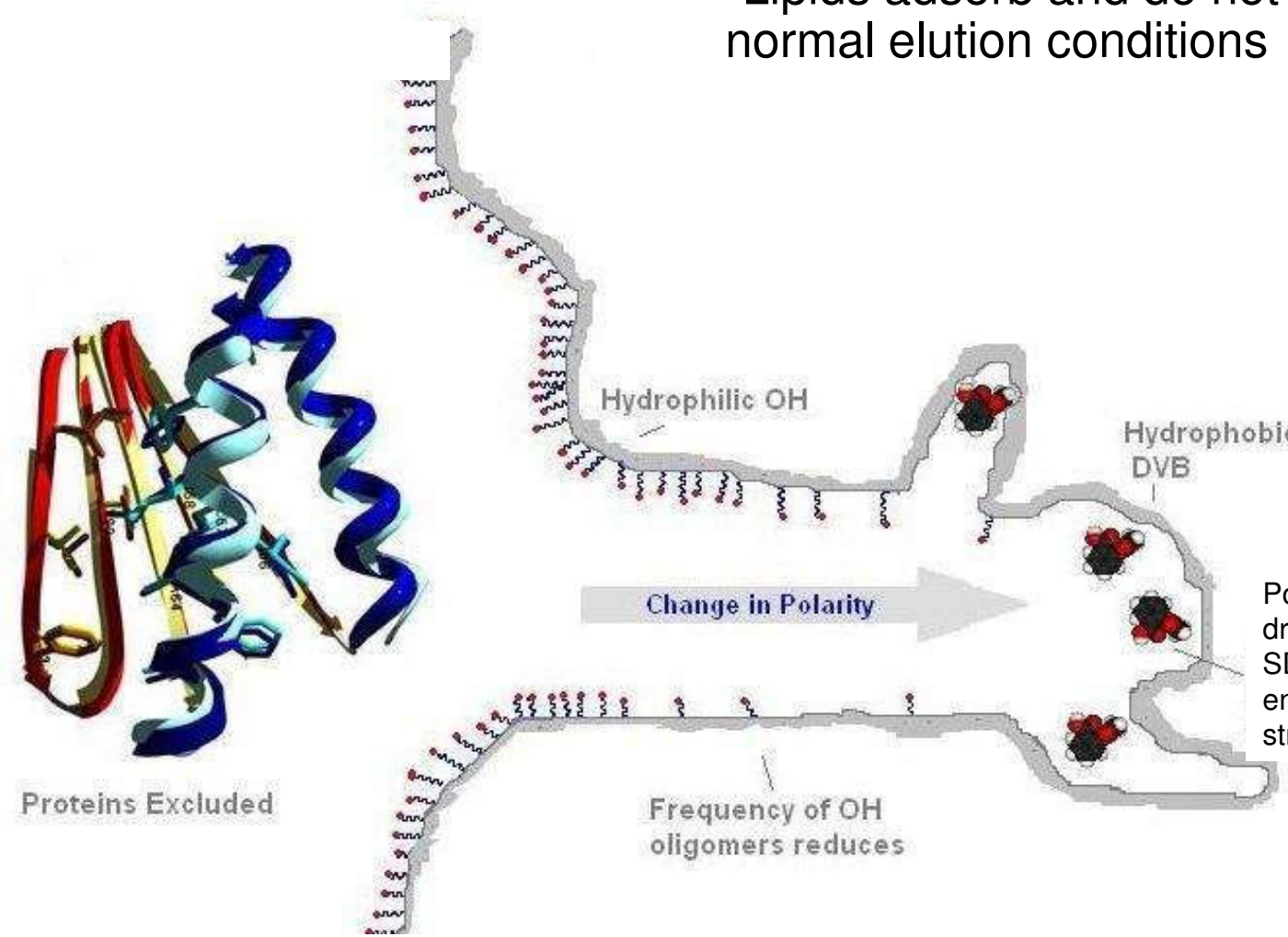
Recovery of antidepressant drugs from rat plasma

Compound	Extraction Method	Avg Rec%
Tranylcypromine Log P: 1.4	CaptivaND*Lipids*	**67.1**
	Protein Precipitation	**29.1**
Nomifensine Log P: 2.9	CaptivaND*Lipids*	**77.4**
	Protein Precipitation	**38.1**
Amoxapine Log P: 3.4	CaptivaND*Lipids*	**77.8**
	Protein Precipitation	**38.8**
Maprotiline Log P: 5.1	CaptivaND*Lipids*	**82.8**
	Protein Precipitation	**71.1**
Nefazodone Log P: 4.7	CaptivaND*Lipids*	**75.0**
	Protein Precipitation	**63.1**

Greatest enhancement is seen with lower logP compounds (<5) although enhanced sample cleanliness is observed with all

Protein and Lipid removal using the SPE approach

- Big proteins will be excluded
- Lipids adsorb and do not elute under normal elution conditions



Polar and non polar drugs bind in the SDVB hydrophobic end of the pore structure

Protein and Lipid Removal using QuEChERS

- Proteins precipitate during extraction step due to 50% ACN content
- dSPE kits for fatty/waxy samples available, contains C18 which adsorbs lipids
- Very fatty matrices (e.g. cooking oil) require addition of hexane in extraction step, see acrylamide application note for further details

Common Contaminant #4: Pigments and Dyes

- Various polarities and chemical structures, therefore various effects
- Anthocyanins (reds/blues) commonly found in berries, grapes, wine scavenged by PSA
- Chlorophyll (green) commonly found in foliage, vegetables, hay and carotenoids, scavenged by GCB
- SPE methods available for large number of chemical dyes
- dSPE (low capacity) or scavenging cartridges (high capacity)

Common Contaminant #4: Carbohydrates and polysaccharides

-Found in produce and beverages

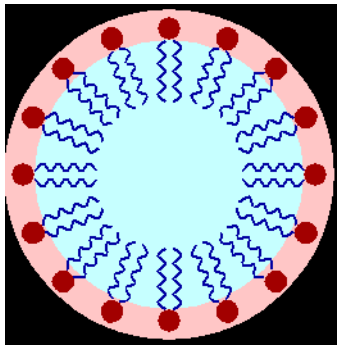
-Very polar and often of high molecular weight (ex: starch)

-Viscous in organic solvents

-Best bet is with non-polar SPE, just like protein removal

-QUECHERS are a good option

Common Contaminant #6: Surfactants



-Can be polar, non-polar and ionic simultaneously.

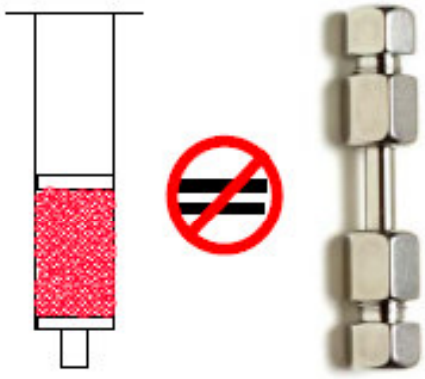
-Often in high concentrations

-Can cause non specific binding on SPE cartridges

-Use ion-exchange SPE for ionic surfactants

-Use polar SPE sorbents like diol for non-ionic surfactants

What is SPE?



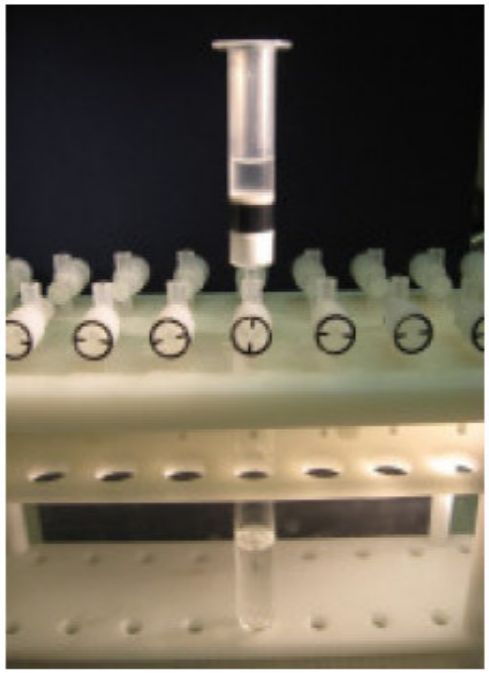
- Can be thought of as digital chromatography – compounds either bind or flow through

- Has a wide choice of sorbents with selectivities similar to sorbents used in HPLC, but it is not HPLC

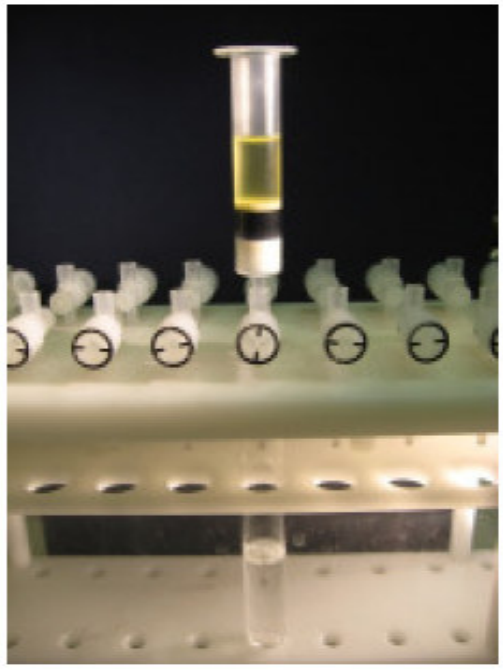
- Many samples can be analyzed in parallel. Manifolds of 10 and 20 ports are readily available.

- Methods are simple to perform, but the process can be time-consuming, however SPE can be readily automated

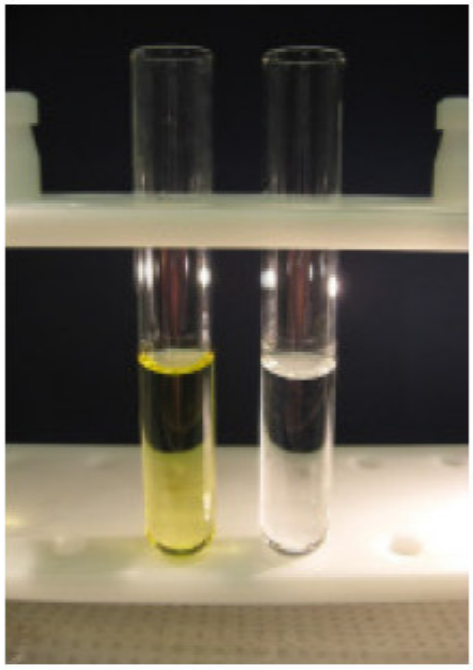
- SPE methods give high selectivity, recovery and reproducibility



Conditioning



Clean-up



Before and after clean-up

SPE Manifolds

96 Well

CaptiVac Collar: For use with Bond Elut 96 1mL and Captiva filtration plates

VacElut 12, 20 and SPS 24

Vacuum manifolds for SPE barrels.

SPE Modes—"Digital Chromatography"

Analyte Adsorption (Bind-Elute)

Analyte(s) retained ($K_D >> 1$)

Matrix unretained ($K_D \sim 0$)
and/or strongly retained ($K_D >> 1$)

Preconcentration factor

Cleaner extracts

Load at 1-3 drops/sec (recovery ∝ 1/flow)

Capacity issues may be more important

Matrix Adsorption (Interference Removal)

Analyte(s) unretained ($K_D \sim 0$)

Matrix retained ($K_D >> 1$)

No preconcentration advantage

Eluates may not be as clean

Sample loading often gravity fed

Used less often than analyte adsorption

Solid Phase Extraction: Most efficient clean-up for complex matrices

Four Step of SPE:

•**Conditioning**: Preparation of the sorbent prior to sample addition

• **Retention**: Analytes of interest and other interferences adsorb onto the surface of the sorbent during sample addition (over 40 chemistries available!)

• **Washing**: Elimination of undesired interferences

• **Elution**: Selective desorption and collection of desired analytes from the sorbent

Retention Mechanisms

Polar

matrix is organic (ie organic phase from a liquid/liquid extraction)

analyte is water soluble

wash solvents are non-polar (hexane, methyl t-butyl ether etc)

elution solvents are polar (water, methanol, acetonitrile etc)

Non-polar

matrix is aqueous (foods, biological fluids)

analyte is organic soluble

wash solvents are aqueous

elution solvents are organic

Mixed mode

matrix is aqueous (foods, biological fluids)

analyte can be polar, hydrophillic, or hydrophobic

wash solvents are aqueous and organic

elution solvents are organic

sorbents are either mixed silica (such as C8/SCX) or polymer

Retention Mechanisms

Cation exchange

matrix is aqueous (foods, biological fluids)

analyte is basic (cationic)

wash solvents are aqueous

elution solvents are :

- high ionic strength,
- pH is increased above the pKa of the target compound,
- competition with a cation (such as Na^+) with greater affinity for the sulfonic acid

Anion exchange

matrix is aqueous (foods, biological fluids)

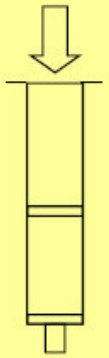
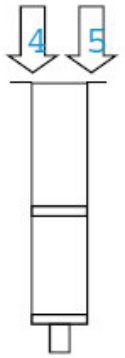
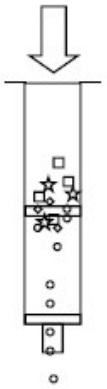
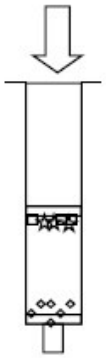
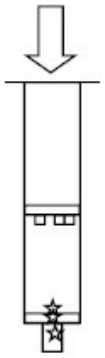
analyte is acidic (anionic)

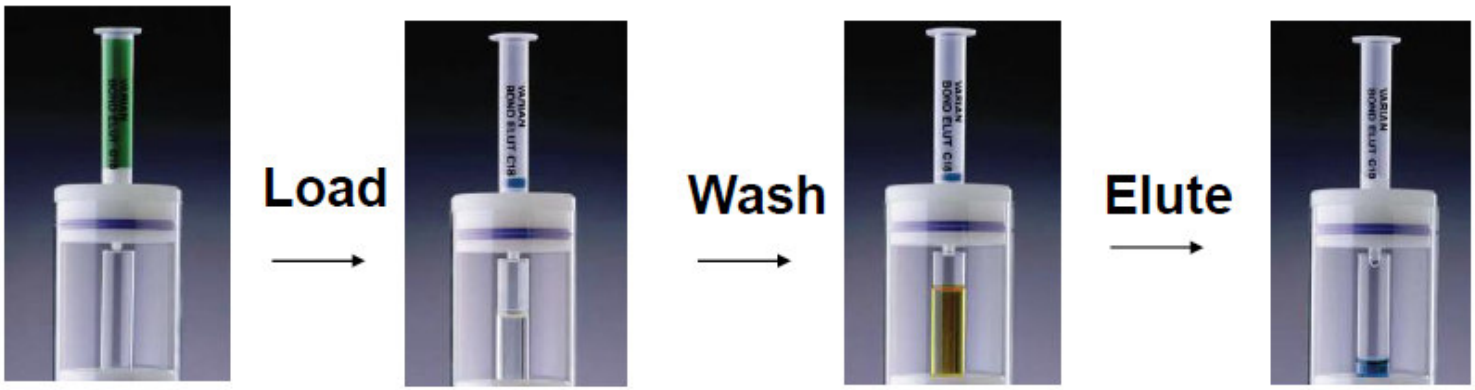
wash solvents are aqueous

elution solvents are :

- high ionic strength,
- pH is increased below the pKa of the target compound ,
- competition with an anion (such as SO_3^-) with greater affinity for the positively charge amine

Fundamental Steps for "Bind-Elute" SPE

Prewash*	Precondition	Load	Wash	Elute
Remove contaminants that could elute with analyte	Prepare cartridge to accept sample	Load sample and rinse reservoir(s)	Wash with solvent that won't elute analyte	Elute analyte in smallest volume possible
				
If elution solvent will be stronger than precond. solvent	1. MeOH or ACN 2. Weak solvent (water, buffer)	Weakly retained matrix compds elute	Analyte and other matrix compds retained	Elute analyte leaving highly retained compds



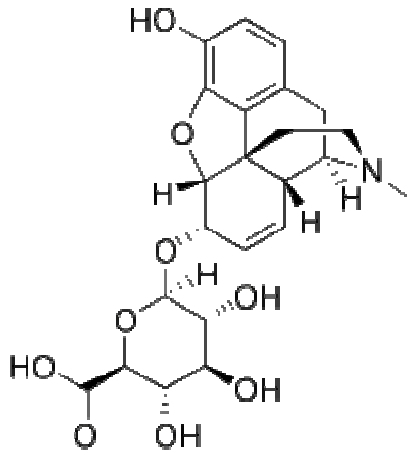
Sample Pre-treatment or prewash

Pre-extraction compound preparation

- Dilution
  - Reduce organic content before RP SPE
  - Reduce salt concentration before IEX SPE
  - Facilitate flow
  - Plasma is typically diluted 2:1 or 3:1
- Addition of internal standard
  - Deuterated or compound similar to analyte of interest
- Filtering, settling or centrifugation
  - Remove particulates to facilitate flow and reduce chance of clogging
- Buffering
  - Establish necessary pH
    - Neutralize analytes for RP SPE
    - Charge analytes for IEX SPE

Hydrolysis

For some clinical or drugs of abuse methods, many drugs, during metabolism, will take a glucuronide group to make the compound more polar allowing urinary excretion. Morphine is an example: morphine-3-glucuronide

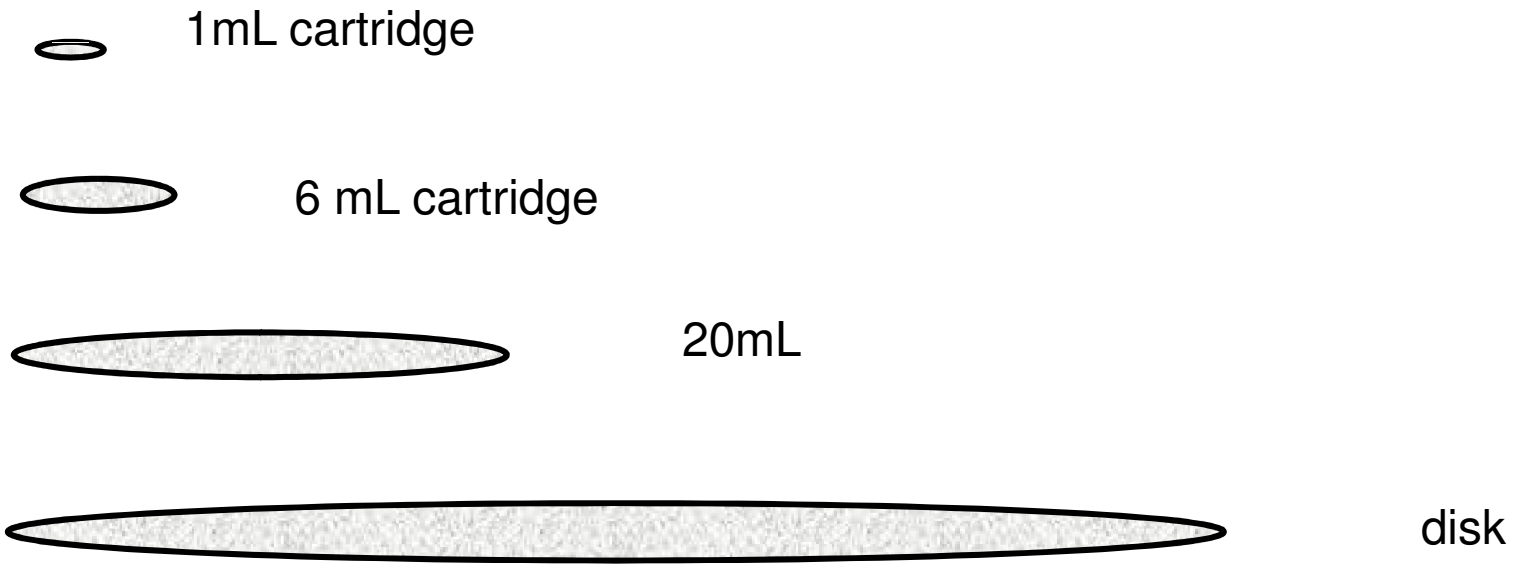


Many GC applications will require

chemical or enzymatic hydrolysis.

Large Sample Volumes

If sample has particulates (ground water, etc) sample needs to be filtered for cartridge applications. The smaller the bed mass diameter, the faster the SPE format will clog.



Protein Precipitation

- Used mostly for serum, plasma, and blood
- Precipitates cell wall and other proteins, which are then filtered out or centrifuged down
- Proteins are the single largest interference in biological samples
- Can also free protein-bound analytes
- Acetonitrile, methanol, and acetone are most commonly used.
- 3:1 to 10:1 ratio solvent:sample

Whole Blood

When ACN precipitation is not effective:

- Isotonic solutions with or without sonication
  - Dilution with water causes cells to burst from taking in water
  - Sonication facilitates the process
  - Pro: Does not add acids or salts
- See Certify Manual for other approaches
  - Acids
  - Zinc sulfates
  - others

Note – unlike many matrices, some compounds can be retained in the cell wall of the blood. Spiking whole blood samples, while still the best experimental approach doesn't simulate the difference between free and bound analytes.

Solid samples

- typically need to disrupt cell walls and/or separate bound analytes from the matrix
- Soil
  - Tumbling in a solvent is often used
  - Polar solvents (water, MeOH, acetone) used for polar and mid-polar compounds
  - Nonpolar solvents are used for more non-polar extractions
- Biological tissues and food
  - MSPD
  - Quechers
  - Homogenization with a solvent
  - freezing followed by milling (food)
    - esp when enzymes that can alter accurate analyte quantitation are present.

Non-aqueous samples

Oil samples are often diluted in hexane or other nonpolar solvents. Depending on the interferences either polar extraction techniques (diol, NH2, etc) can be used

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Agilent Bond Elut Sample Prep Solutions

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Thank You!