Herzlich Willkommen zum

Agilent Tipps & Tricks Seminar: Probenvorbereitung

Agilent Technologies

Teilnehmerzertifikat & Feedback Form

Teilnehmerzertifikat

Erhältlich am Ende des Seminars

Feedback Form

Ihre Meinung ist uns wichtig! Bitte lassen Sie uns wissen, ob Ihnen das Seminar zugesagt hat, ob wir etwas verbessern können und ob Sie weitere Wünsche haben.

Bitte füllen Sie den Feedback Bogen aus. Als Dankeschön erhalten Sie einen USB Stick mit wertvollen Unterlagen

Die **Vorträge** senden wir Ihnen nach der Veranstaltung in einem separaten eMail zum Download zu.

Agenda Pharma / Biolog. Flüssigkeiten nachmittags

Title	Speaker	
Session I – Optimale Ergebnisse mit Probenvorbereitung *Wie lässt sich durch Probenaufreinigung der Betriebsablauf im Labor positiv beeinflussen?*		30 min
Session II, Teil A – Applikative Lösungen , Methodenentwicklung & Fehlersuche		30 min
Kaffeepause		15 min
Session II, Teil B – Applikative Lösungen , Methodenentwicklung & Fehlersuche		30 min
Session III – Die beste analytische Säule für die aufgereinigte Probe		30 min
Session IV – Zu guter Letzt: Die Analysengeräte		30 min

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Optimale Ergebnisse mit Probenvor-bereitung

Great Results Start with Sample Preparation

Dr. Ulrike Jegle

Agilent Technologies

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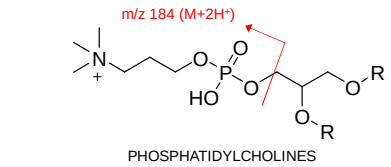
Tips and Tricks

Throughout this presentation will be a series of tips and tricks to help improve your results and productivity.

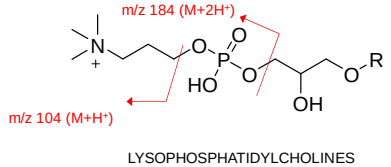
Watch out for the Orange Boxes!

BUT please let us if there is a TIP or TRICK that you need in your lab

TIP: Monitoring Phospholipid Removal



PHOSPHATIDYLCHOLINES



LYSOPHOSPHATIDYLCHOLINES

- Monitoring the 184 → 184 m/z trace in MS/MS gives a measure of the total phospholipids and lyso-phosphlipids in a sample
- Measures the ionic head group common to all classes of phospholipids

"We are now monitoring the 184→184 transition in all of our bioanalytical method development studies"

DMPK Group, AstraZeneca, UK

Sample Prep Terminology

Analyte(s): Molecule(s) of interest

Matrix: The sample (urine, blood, saliva, tissue)

Interferences: Entities inside the sample which will inhibit analysis of desired analyte

IS: Internal standard

LLE: Liquid-liquid extraction

SLE: Supported-liquid extraction

SPE: Solid phase extraction

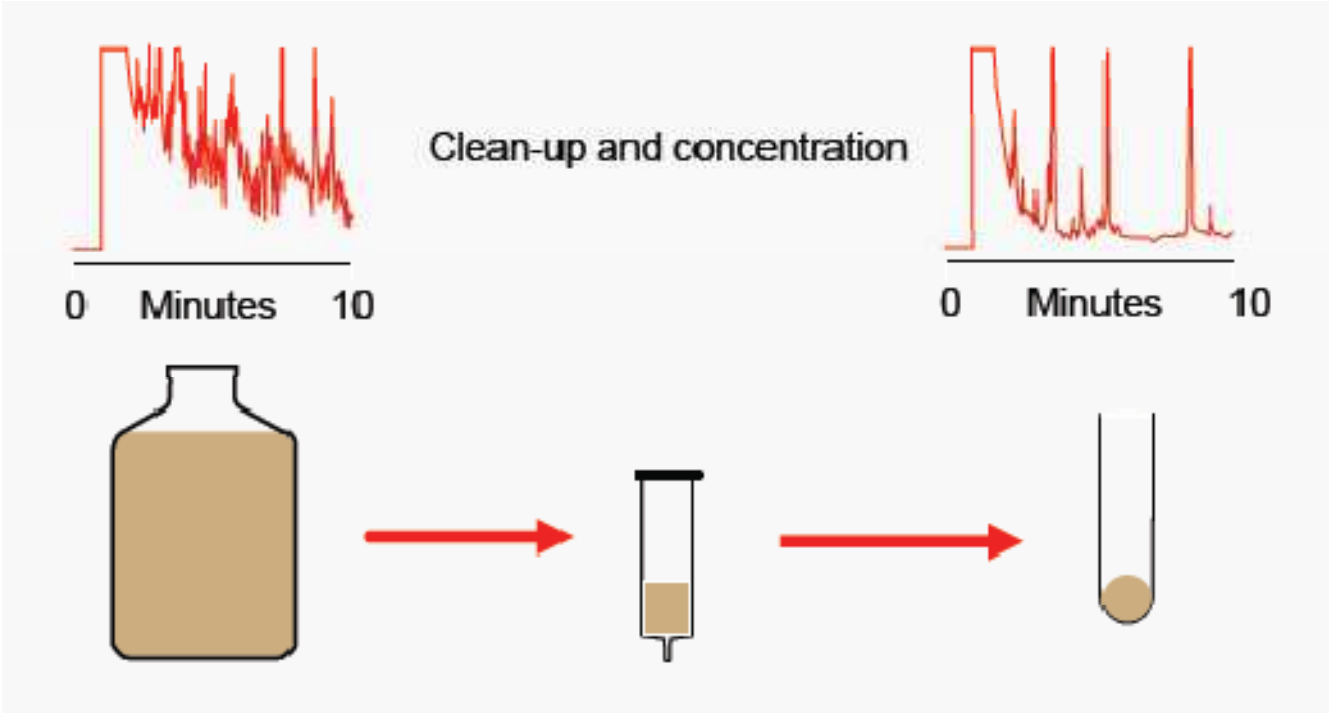
LC (MS): Liquid chromatography (mass spectroscopy)

GC (MS): Gas chromatography (mass spectroscopy)

LOQ: Limit of Quantification **LLOQ**: Lower limit of quantification

RSD: Relative standard deviation

Why Do Sample Preparation?



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

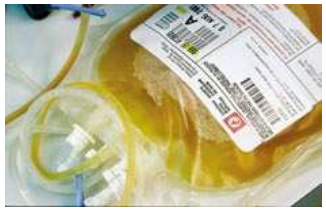
Effects of Endogenous Interferences

- Poor Chromatography
- Mechanical issues (particulates, blockages)
- LC Column Lifetime Issues
- Instrument Downtime
- Carry Over
- Increase in sample run time/ cost
- Ion Suppression
- Overall loss in sensitivity

Let’s take a look at some common matrices

Sample Types

Plasma



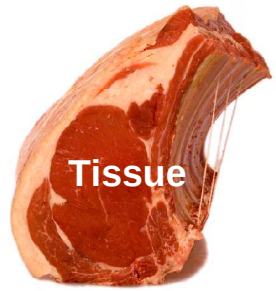
Water (92%)
Serum albumin (7%)
Blood-clotting factors
Immunoglobulin (antibodies)
lipoprotein particles
Other proteins
Various electrolytes
(Na+, Cl-, Ca++, K+ etc)

Whole Blood

Water (90% by volume)
Serum albumins
Red Cells (erythrocytes)
White cells, Platelets
Lipids
Proteins/peptides
Hormones
Salts + mineral ions
Carbohydrates
(Prescription) drugs

Tissue

Water
Fats, Lipids, Proteins
Cells
Collagen
Myosin + actin
Myoglobin
Carbohydrate
Salts + electrolytes



Saliva

Water
Mucus
Salts & Electrolytes
Enzymes & Proteins
Bacteria
Broken down foodstuffs

When saliva is sampled it is often added to a preservative solution. This contains surfactants, PEG, Tween and other salts and buffers.

Urine

Water (95% by volume)
Urea (deamination product)
Creatinine
Salts & Uric Acid
Pigments
Carbohydrates
Enzymes
Mucins (glycosylated proteins)

In some urine sample analysis, an enzyme called Beta-glucuronidase needs to be used. This can cause issues with analyte detection

Dilution

Sample dilution (with an internal standard added) aka “dilute and shoot” is a commonly adopted simple approach to sample analysis

Advantages

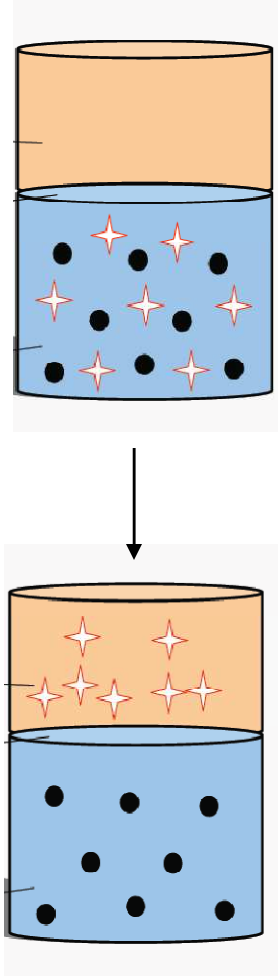
- Fast and easy

Limitations

- Interferences are not removed
- Analyte concentration is reduced
- Instrument and Column contamination
- Ion Suppression

Liquid-Liquid Extraction (LLE)

Technique which uses two immiscible solvents to partition analytes of different polarities



Advantages

- Low Cost
- Effective for Inorganic/Salt removal

Limitations

- No concentration of analyte achieved, sample will need to be reconstituted prior to analysis
- Uses large volumes of solvents (often chlorinated)
- No selectivity for organic soluble analytes
- Matrix specific (e.g. Urine vs Tissue)
- Difficult to automate

Supported-Liquid Extraction (SLE)

Technique which uses a highly wettable inorganic sorbent (diatomaceous earth).

Advantages

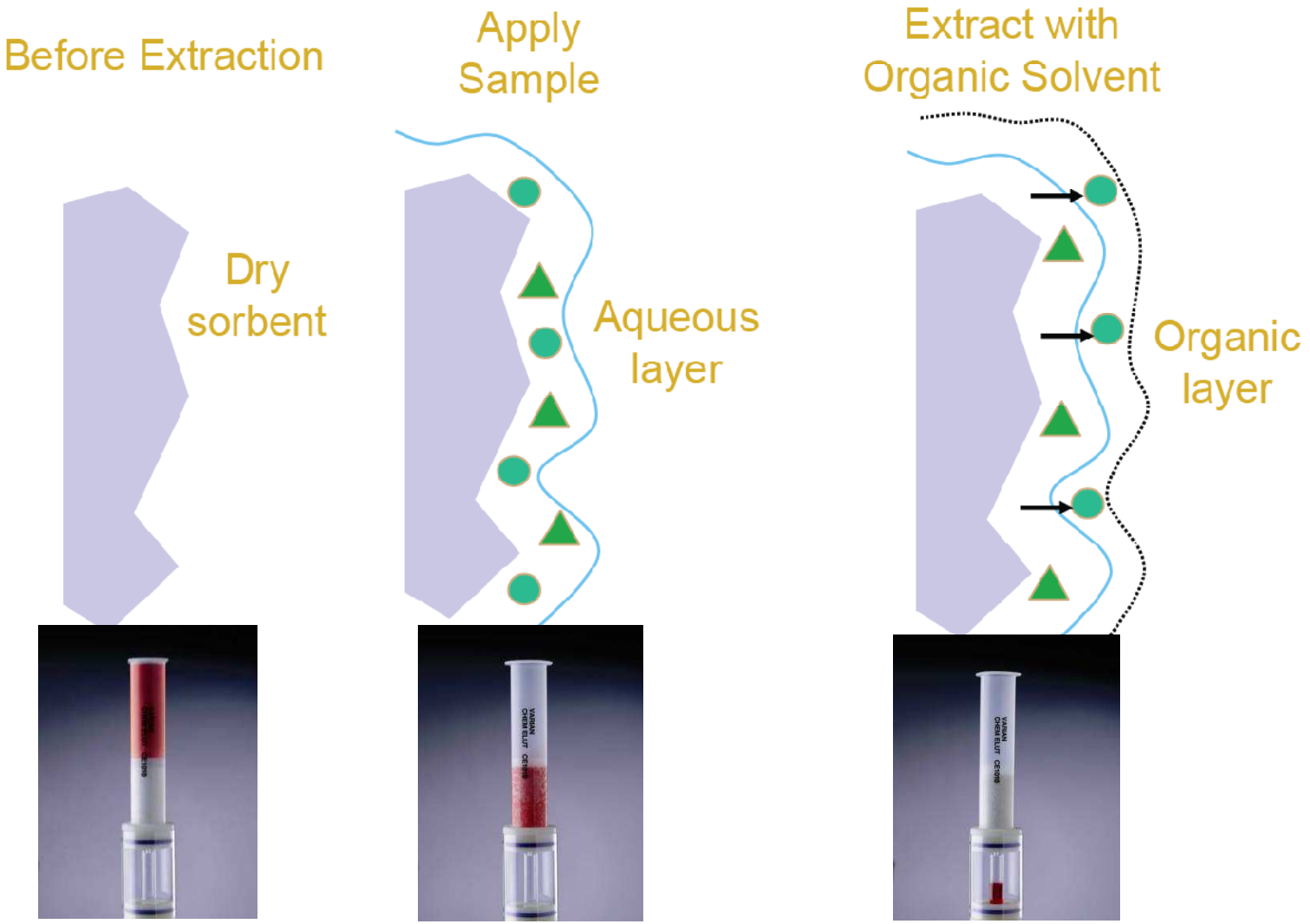
- Low Cost
- Effective for Inorganic/Salt removal

Limitations

- No concentration of analyte achieved, sample will need to be reconstituted prior to analysis
- Uses large volumes of solvents (often chlorinated)
- No selectivity for organic soluble analytes
- Matrix specific (e.g. Urine vs Tissue)
- Can be automated

Chem Elut Produkte

SLE Mechanism



Matrix Solid Phase Dispersion (MSPD)

Mechanical grinding/homogenisation of a functional sorbent with a solid sample followed by elution from the sorbent

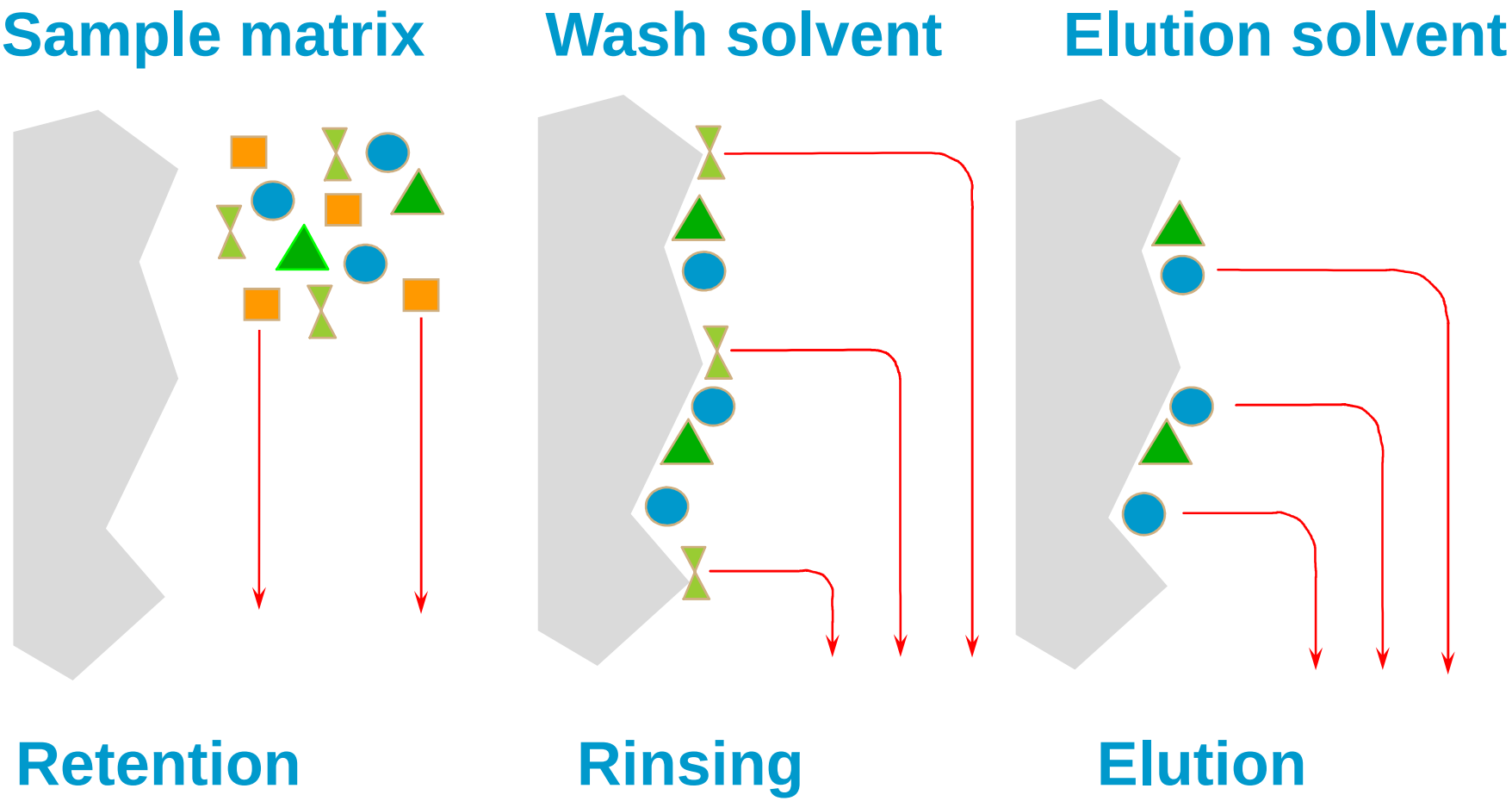
Advantages

- Excellent for tissue/solid sample
- Different functional sorbents can be used

Limitations

- Interferences often co-extracted
- Can use large volumes of solvents
- Labour intensive
- Difficult to automate
- Matrix specific (e.g. Solids,Tissue, Post mortem bloods)

SPE – Solid Phase Extraction
The Retention/Elution Process



Typical Approaches to Plasma

Dilute and Shoot: Dilution & addition of ITSD

LLE or SLE: Agitation, partition or Tube

Precipitation: Centrifugation, Filter Plate

Solid Phase Extraction: Tube or Plate

	Dilution	LLE	Precipitation (Captiva)	SPE (Plexa)
Proteins	No	Partial	Yes	Yes
Lipids	No	No	No	Yes
Salts	No	Yes	No	Yes

Protein Precipitation

Protein Precipitation (PP) is a technique primarily aimed at the removal of proteins (albumins) from plasma samples.

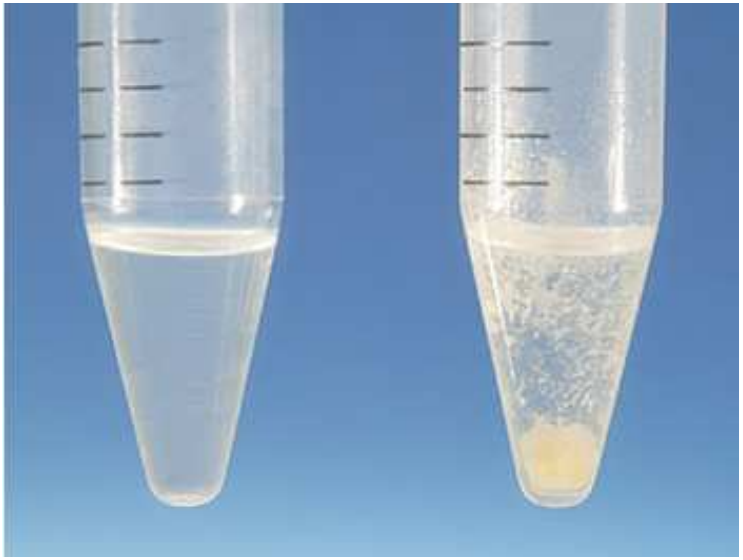
This is achieved by crashing protein via the addition of organic solvent. The resulting precipitate can be removed by centrifugation or filtration.

Advantages

- Effective removal of protein
- High throughput and automation friendly
- Generic methods across plasma types
- Flexibility

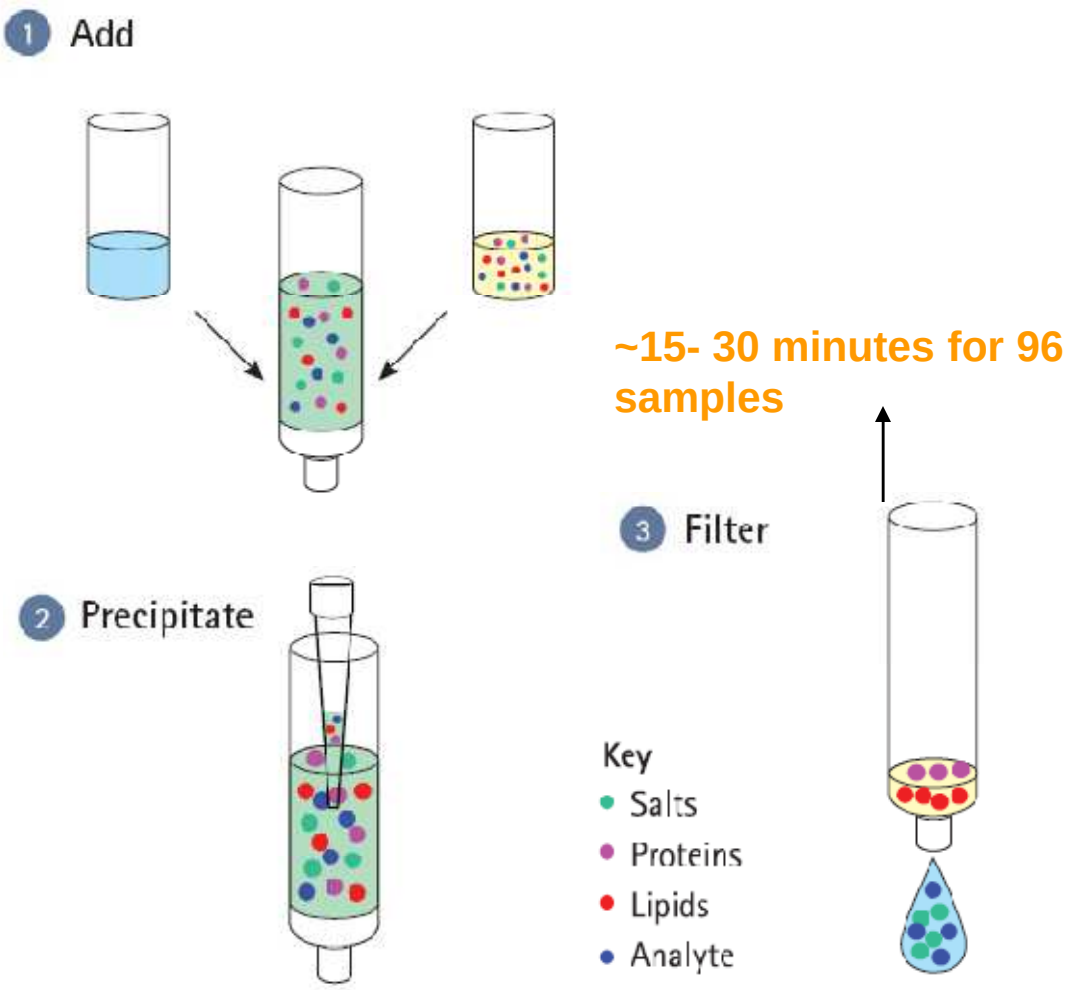
Limitations

- Low selectivity for analytes
- Salts and Lipids not removed
- Low levels of peptides/small proteins
- Column and Instrument contamination



Sample Preparation Workflow: Protein Precipitation

- **Fractionate** Plasma from whole blood (centrifuge)
- **Dilute** plasma 3:1 with MeOH or ACN.
- **Agitate** the sample
  - Aspiration
  - Vortexing
  - Shaking
  - Stirring
- **Filter** under vacuum
- **Inject** sample into LCMS



~15- 30 minutes for 96 samples

Solid Phase Extraction

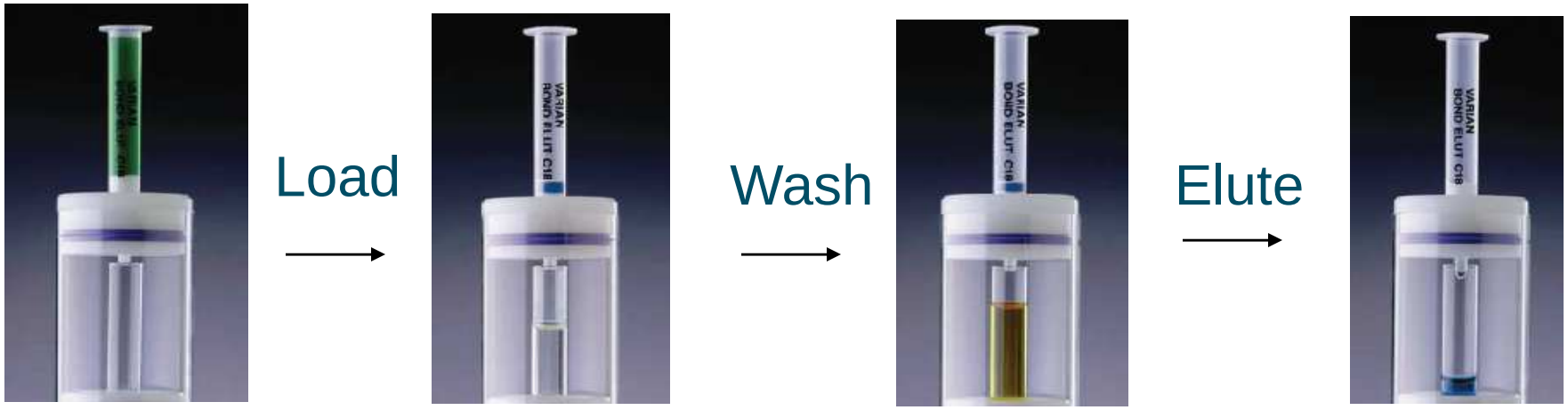
- **Solid Phase Extraction is the most commonly used sample preparation technique.**
- **The use of an immobilized sorbent, pre-packed into a device.**

Advantages

- Best interference removal
- Gain concentration effect
- Extract whole suites of drugs
- Flexibility and Automation

Limitations

- Requires method development
- Difficult to separate analytes with similar properties/structure!



Sample Preparation Workflow: SPE

- **Fractionate** Plasma from whole blood (centrifuge)
- **Precondition** tube
- **Dilute** sample with appropriate buffer (add IS)
- **Apply** the sample
- **Wash** steps
- **Elution** (pH modified)
- **Filter** under vacuum
- **Inject** sample into LCMS

Direct Injection or
Dry-down /reconstitute

	Plexa (30 mg, 1mL)
Sample	100 µL human plasma
Pretreatment	Dilute 1:3 w/ 2% NH_4OH (bases) 2% Phosphoric (acids)
Conditioning	1. 500 µL MeOH 2. 500 µL H_2O
Washes	5 % MeOH in water
Elution	2 x 250 µL 50:50 ACN/MeOH (or 100% MeOH)

Silica and Polymer Based SPE
A Plethora of Options

Bond Elut AccuCAT
Bond Elut Alumina (AL-A)
Bond Elut Alumina (AL-B)
Bond Elut Alumina (AL-N)
Bond Elut NH_2
Empore Anion-SR
Bond Elut Atrazine
BioBond Elut
Bond Elut C1
Bond Elut C2
Bond Elut C8
Elut C18
Elut C18 EWP
Bond Elut C18 INT
Bond Elut C18 OH
Bond Elut CBA

Bond Elut Certify
Bond Elut Certify II
Bond Elut CH
Bond Elut DEA
Bond Elut Diol (2OH)
Bond Elut ENV
Bond Elut FL
Bond Elut NH2
Bond Elut Plexa, PCX, PAX
PAXBond Elut PBA
Bond Elut PCB
Bond Elut PH
Bond Elut PPL
Bond Elut PRS
Bond Elut PSA
Bond Elut SAX
Bond Elut SCX
Bond Elut SI

40 + Phases and over 2000 Applications

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Bond Elut SPE

Non-polar

Plexa, C18, C8, C2, C1
C18 variations in carbon load and endcapping
EnvirElut
CH – cyclohexyl
CN-E – endcapped cyano
PH – phenyl
ENV, LMS, PPL, Focus, Nexus
Plexa

Polar

Plexa PAX
PSA - primary and secondary amine
NH2 - aminopropyl
DEA - diethylaminopropyl
Diol
Si - silica

Cation Exchange

Plexa PCX
SCX – benzenesulfonic acid
PRS – propylsulfonic acid
CBA – carboxylic acid

Reversible Covalent

PBA – phenylboronic acid

Specialty Phases

AccuCAT
Atrazine
Etc..

Anion Exchange

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

Mixed mode IEX/NP

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

Alumina – aluminum oxide
Florisil – magnesium-silica
Carbon
Carbon/NH2

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Formats

The most comprehensive range of formats in the industry

Tubes

1mL to 60mL Straight Barrel (50mg –10g)

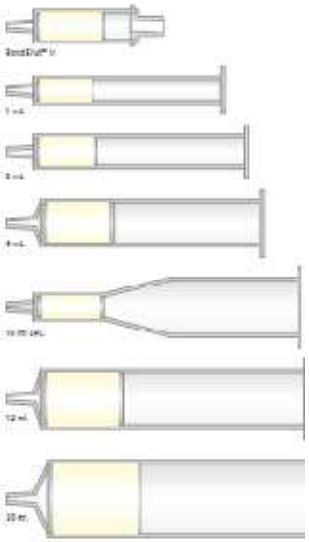
Bond Elut Jr (500mg –1g)

LRC (Large Reservoir Capacity)

(100-500mg)

Mega Bond Elut

12-150mL (2g-70g)



Multi-Array

1mL, 2mL 96 Well

Automation

Hamilton

TomTEC

Gilson ASPEC

Gerstel

Spark Holland Prospekt

SPE Manifolds

96 Well

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

VacElut 12, 20 and SPS 24

vacuum manifold for SPE syringe barrels.

Basic Accessories

Bond Elut Adaptors

- Connect cartridges in series
- Fit syringe for positive pressure

Luer stopcocks

- Stop flow on all Luer tip style cartridges

Frits, Reservoirs, Fritted reservoirs

SPE Methods? Where Do I Start?

Consider your analyte?

- Do the analytes dissolve into the matrix
- Does the analyte appear to be polar or non polar
- Do the analytes contain any ionic groups
- Are the compounds unstable in acid or base
- What is the method of analysis ?
- What is the concentration of the analyte in the sample

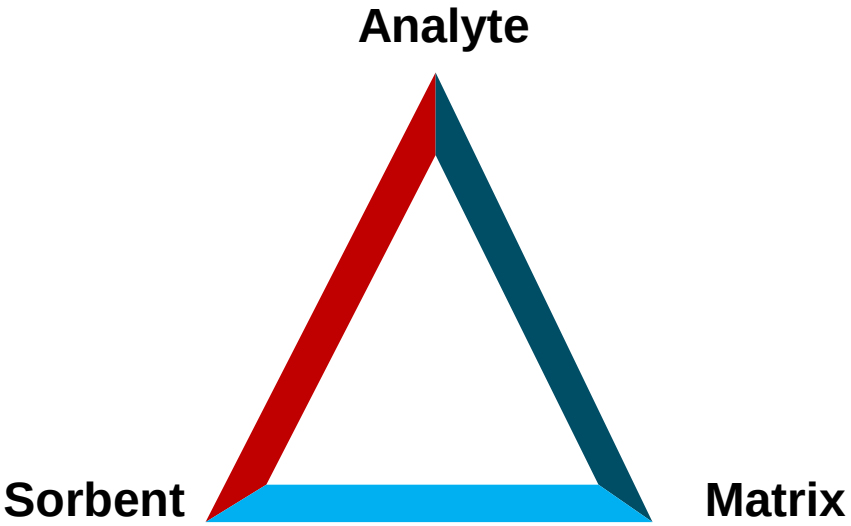
Consider your matrix?

- Is the matrix polar or non polar: (olive oil or urine?)
- is the matrix high in organic or ionic content? (will dilution of the sample be necessary)
- What is the sample size?
- Does the sample require pH adjustment?
- Is an internal standard required?
- Is filtration or centrifugation necessary upfront?

The SPE Triangle

Consider LC Theory

- The same physical processes in liquid chromatography also apply in SPE
- Think of SPE as a digital LC process
  - Analyte is **ON** (retention)
  - Analyte is **OFF** (elution)
- We are not looking at separation
- Normal or Reverse phase?
- Ionic modifier?

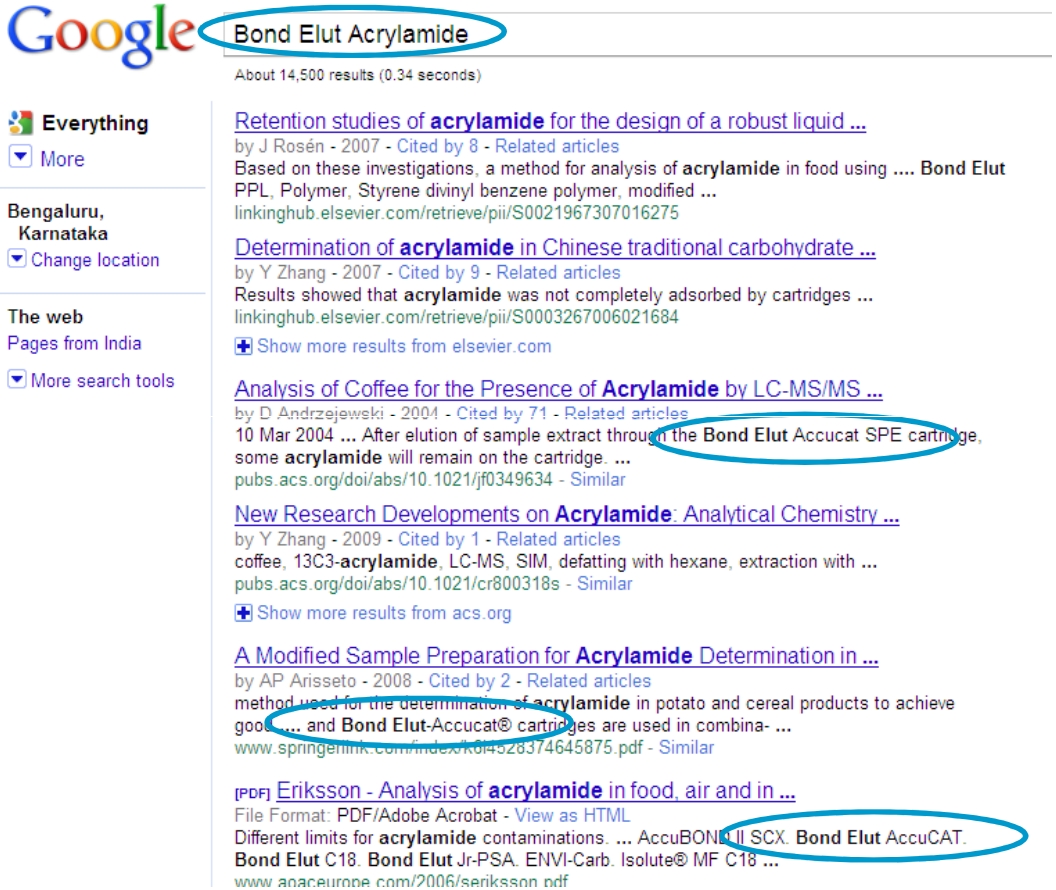


TRICK: Use Google!

- **Bond Elut SPE has more published methods than any other SPE platform**
- 1000s of articles
- Official methods
- 31 years of heritage

BOND ELUT + (Analyte)

Don't re-invent the wheel!



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How do Sample Prep Options Compare?

Solid Phase Extraction (Bond Elut)

- Highest level of clean up
- Potential for the most selectivity

Protein precipitation (Captiva, Captiva NDLipids)

- Works for most analytes with no or very little method development
- Using Captiva can improve throughput and ease-of-use
- Captiva ND Lipids removes lipids also

Liquid/Supported Liquid Extraction (Chem Elut)

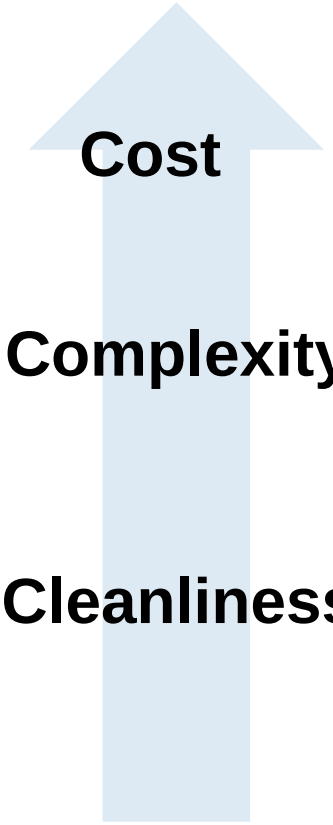
- Reasonably clean, very inexpensive
- Can be labor intensive, especially for larger sample sizes.

Filtration

- Mechanical removal of particulates only

Dilute and Shoot

- No clean up, no interference removal



Fragen???

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Teil A – Applikative Lösungen , Methodenent-wicklung

Ion Suppression

Polymer based SPE Sorbents

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Polymeric SPE - Plexa: Technology and Applications

Advanced Polymeric SPE

- Non polar (Bond Elut Plexa)
- Ion Exchange applications (Bond Elut Plexa PCX and PAX)

Plasma based applications

- Ion suppression studies
- Multi suite drug extraction

Alternative Matrices

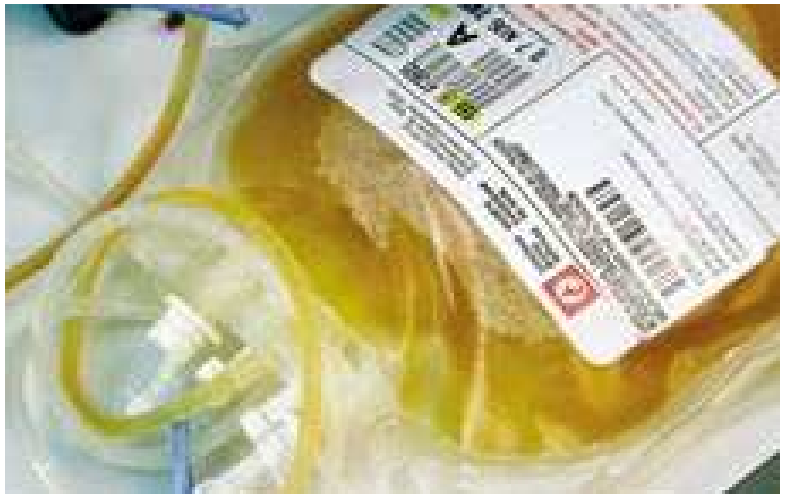
- Saliva (Multi-suite DoA analysis)
- Urine (Therapeutic and DoA multi-suite testing)

Plasma

Composition

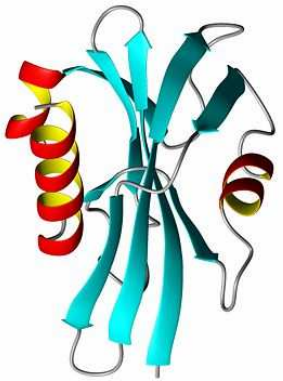
- Water (92%)
- Serum albumin (7%)
- Blood-clotting factors
- Immunoglobulin (antibodies)
- lipoprotein particles
- Other proteins
- Various electrolytes

  (Na, Cl, Ca, K etc)



Ion Suppression

Ion Suppression is a deleterious phenomenon that can be experienced during LCMS analysis.



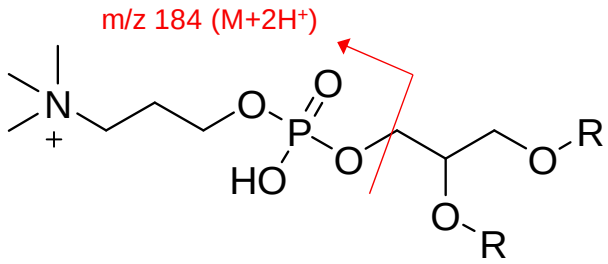
Causes

Endogenous matrix interferences

- Proteins and Peptides
- Lipids (phospho- and lysophospholipids)
- Salts
- Other hydrophobic species
- Dosing vehicles/preservatives (Tween, PEG)

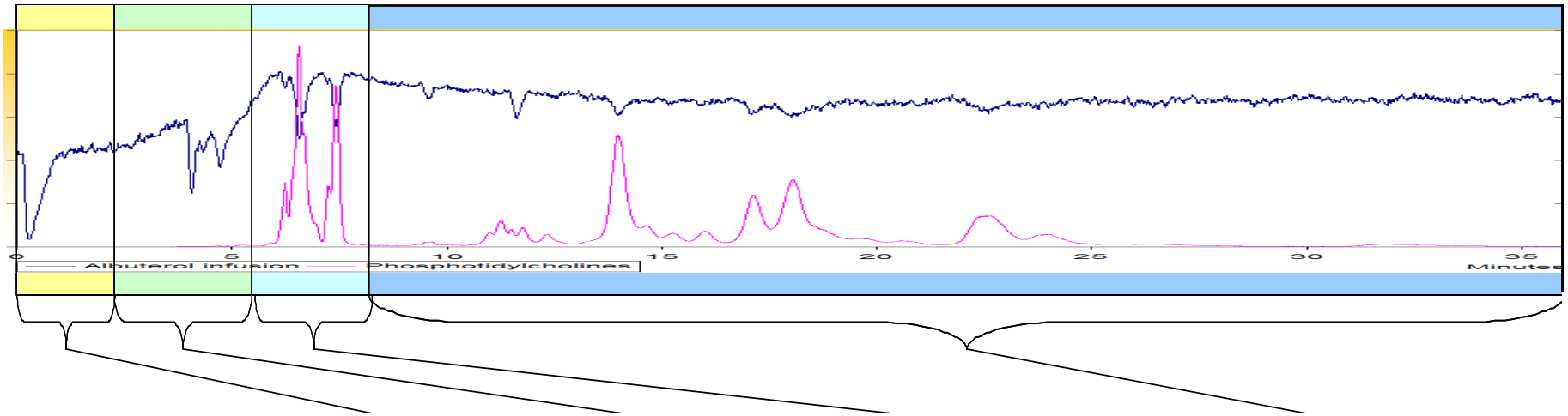
Effect on Data

- Loss of Ion signal will inhibit quantification of analyte
- Loss of sensitivity, precision and accuracy



PHOSPHATIDYLCHOLINES

Ion Suppression Regions



Interference	Salt/ Polar Ionics	Proteins & Peptides	Lyso-phosphatidylcholines	Lipids & Hydrophobics
Typical Elution Conditions (C18)	At Void with <20% Organic	40-70% Organic (10 Column Volumes)	70-90% Organic (10 Column Volumes)	>90% Organic (10 Column Volumes)
Short Term effect	Ion Suppression	Ion Suppression	Ion Suppression	Retained on LC Column
Long Term Effect	Unknown	Column Soiling	Decreased Sensitivity	Decreased Sensitivity
Likely Long Term Causes	Ion Source Contamination	Ion Source Contamination	Ion Source Contamination Column Build-up	Ion Source Contamination Column Build-up

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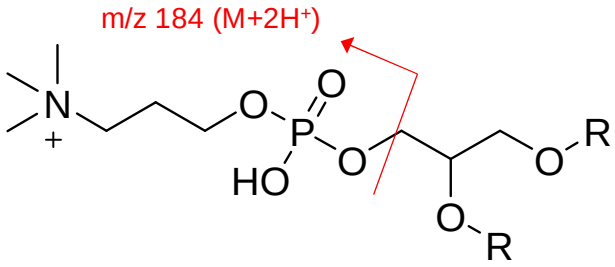
TIP: Monitoring Phospholipid Removal



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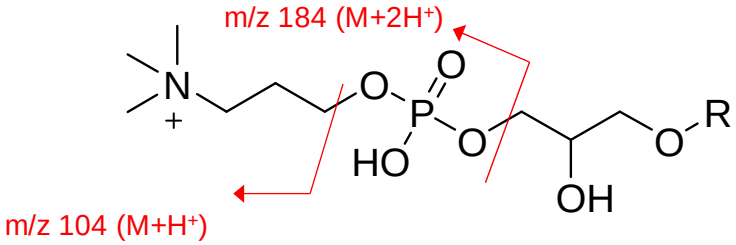

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- Monitoring the 184 → 184 m/z trace in MS/MS gives a measure of the total phospholipids and lyso-phospholipids in a sample
- Measures the ionic head group common to all classes of phospholipids
- Install a new LC column first !

"We are now monitoring the 184→184 transition in all of our bioanalytical method development studies"

DMPK Group, AstraZeneca, UK

Typical Approaches to Plasma

Dilute and Shoot: Dilution & addition of ITSD

LLE: Partitioning between immiscible solvents

Precipitation: Centrifugation, Filter Plate – Unretained Method

Solid Phase Extraction: Tube or Plate – Retained Method

	Dilution	LLE	Precipitation (Captiva)	SPE (Plexa)
Proteins	No	Partial	Yes	Yes
Lipids	No	No	No	Yes
Salts	No	Yes	No	Yes

Polymeric SPE : Advantages and Benefits

Some advantages that are common to all polymeric SPE compared to silica SPE are:

- Stability in a wide pH range – useful for all method options
- High capacity/loadability – smaller SPE bed masses minimize solvent usage and create greater througput
- Loading of 10-12% of bed Capacity (Interferences count!)
- Resistance to drying after conditioning – creates greater error-free ease of use and throughput for end users

Polymeric SPE : Advantage to Pharma/biofluids

The adoption of polymeric SPE for bio-fluid analysis is the industry norm:

- Simple and robust methodologies.
- It is resource heavy to method develop, unknown or novel compounds – offers different interaction possibilities.
- Multi-suite analysis large number of internal standards need extra capacity- offers different interaction possibilities.
- Very cost effective.
- Long shelf life (non-hygroscopic, stable material)

The Bond Elut Plexa Family

Bond Elut Plexa

Non-polar polymer with a hydroxylated surface functionality is the best choice for a wide range of acidic, neutral and basic analytes.

Bond Elut Plexa PCX

based on Plexa, with a strong cation exchange functionality, specifically designed for effective results for a wide range of pKa and logP basic compounds from plasma

Available SOON

Bond Elut Plexa PAX

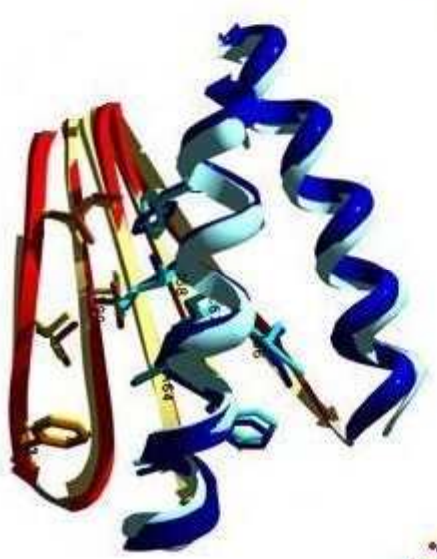
based on Plexa, with a strong anion exchange functionality, specifically designed for effective results for a wide range of pKa and logP acidic compounds from plasma

Bond Elut Plexa - Aufbau

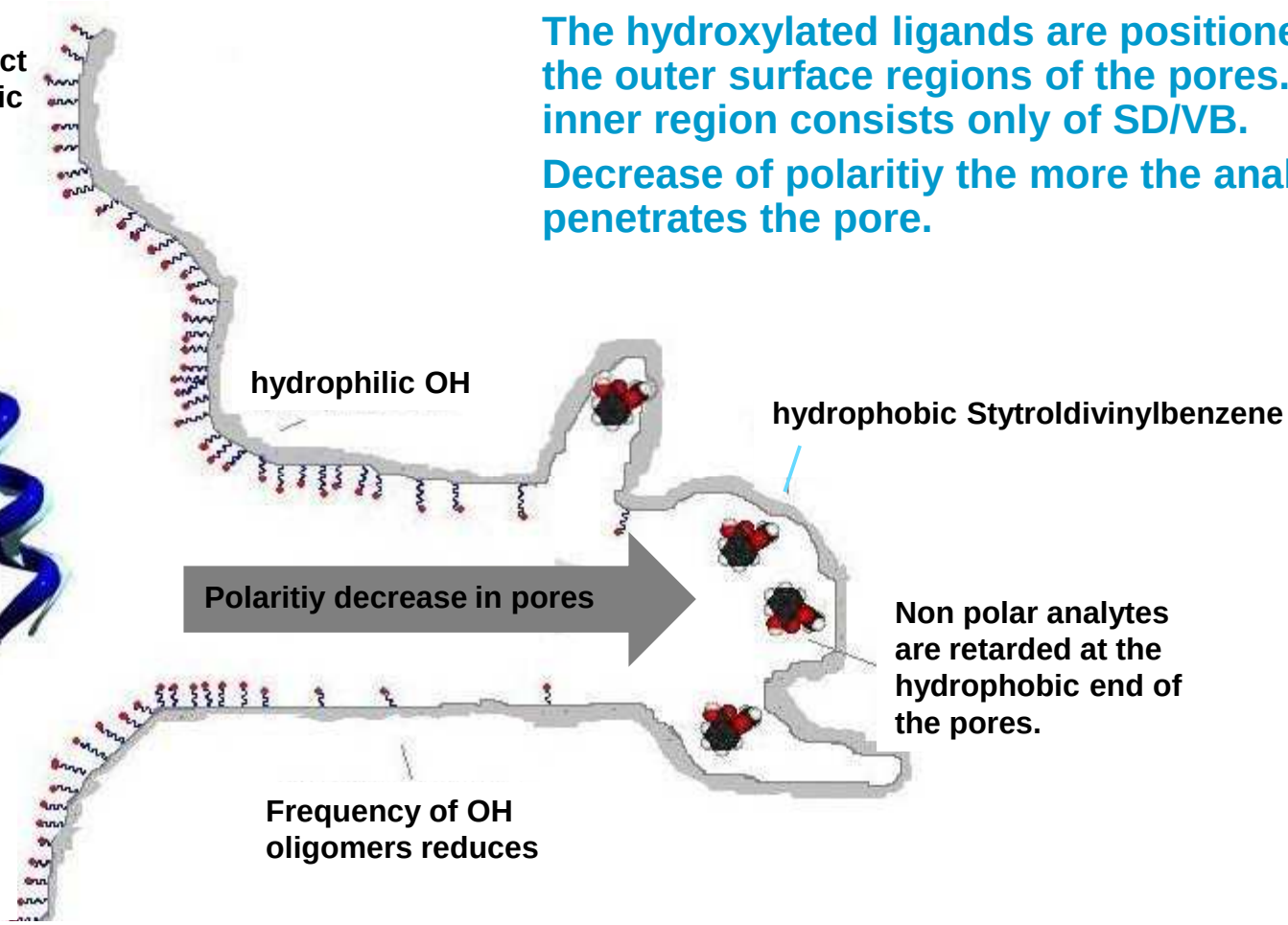
Polare analytes interact at the outer hydrophilic surface areas

The hydroxylated ligands are positioned at the outer surface regions of the pores. The inner region consists only of SD/VB.

Decrease of polaritiy the more the analyte penetrates the pore.



z. B. Proteins and Huminc Acids are excluded – in generell: macro molecules

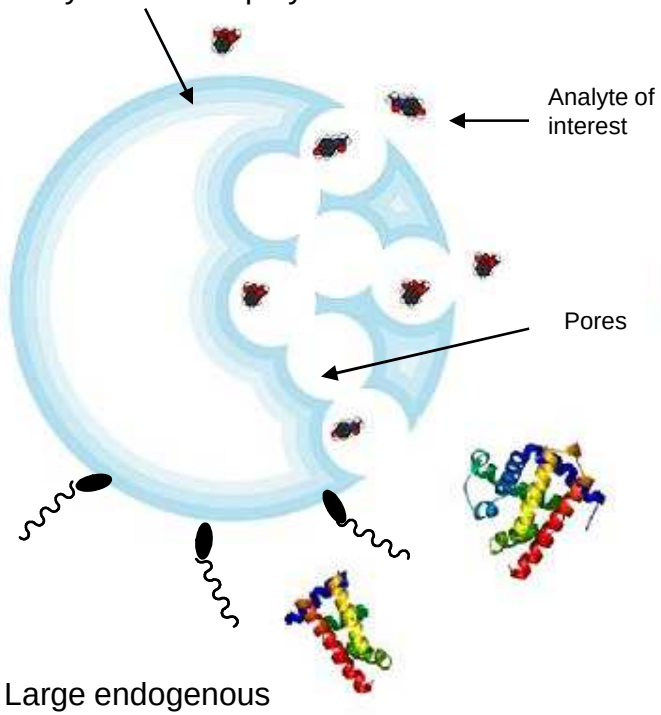


Amid-functionality, which could cause interferences with the matrix are not present at all.

How Bond Elut Plexa works

Apply Sample

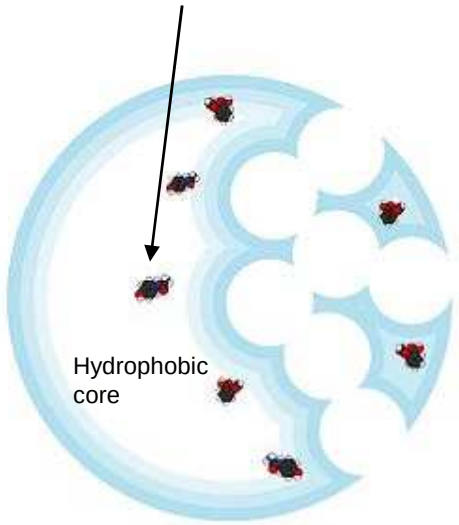
Water Rich Hydrophilic component allows excellent phase transfer of analytes into the polymer core



Large endogenous proteins do not bind to the surface of the polymer and cannot access pore structure.

Washing

Analytes remain tightly bound in the hydrophobic core.



Interferences (lipids and proteins) washed away without leaching into the analytes of interest

Elution

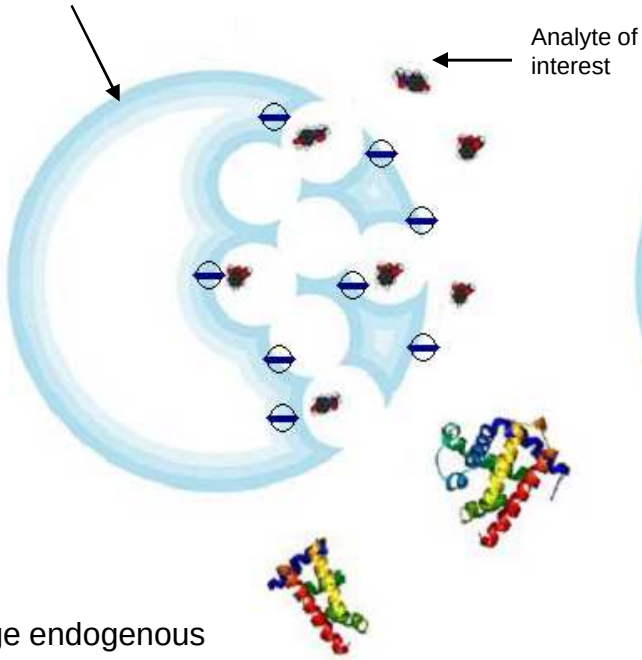
Specially engineered pore structure allows excellent mass transfer out of polymer

Clean extract with high recovery

How Bond Elut Plexa PCX works

Apply Sample

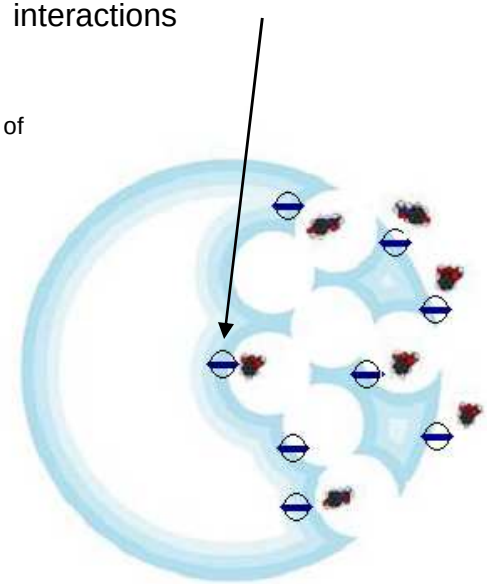
Water-rich Hydrophilic component allows excellent phase transfer of analytes to the cation exchange sites within the polymer.



Large endogenous proteins cannot access pore structure.

Washing

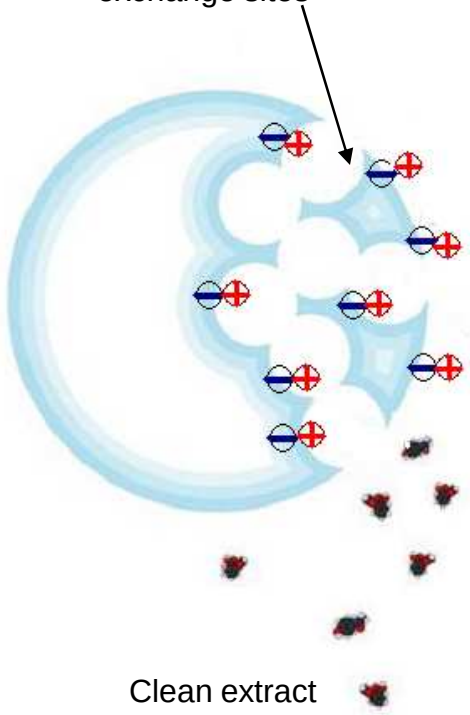
Analytes remain tightly bound to the polymer through strong ionic interactions



Interferences can be washed away without the leaching of basic analytes

Elution

Change in pH allows the elution of analytes from the strong cation exchange sites



Clean extract with high recovery

Plexa Recoveries Versus Other Polymer

Analyte	Plexa	Other Polymer	pKa	LogP
Albuterol	97.9	115.4	5.9	1.3
Atenolol	97.0	94.0	4.2	4.2
Loratadine	71.0	49.0	5.7	1.5
Metoprolol	92.0	74.0	5.7	1.5
Naltrexone	85.7	13.0	4.9	5.2
Pravastatin	85.0	59.0	4.9	5.2
Propranolol	55.0	35.0	4.9	5.2
Zolpidem	93.0	96.8	9.9	3.4

Plexa gives equivalent or better absolute recoveries than other polymer for a wide range of pKa and logP compounds

Conditions: Absolute Recovery from human plasma
Basic load conditions, n=6, 200 ng/ml

Absolute vs. Relative Recovery

Absolute Recoveries (AR)

Plasma + ISTD + drug

Extract

Inject

Compared to

Mobile Phase + ISTD + drug. No extraction, no loss of recoveries, no matrix effects

Relative Recoveries (RR)

Plasma + ISTD + drug

Extract

Inject

Data is then compared to a graph of extracted drug controls.

Drugs are the same and should extract at the same efficiencies.

If the standard curves were extracted at 80% and the unknowns at 80% the relative recoveries will show to be at about 100%!!

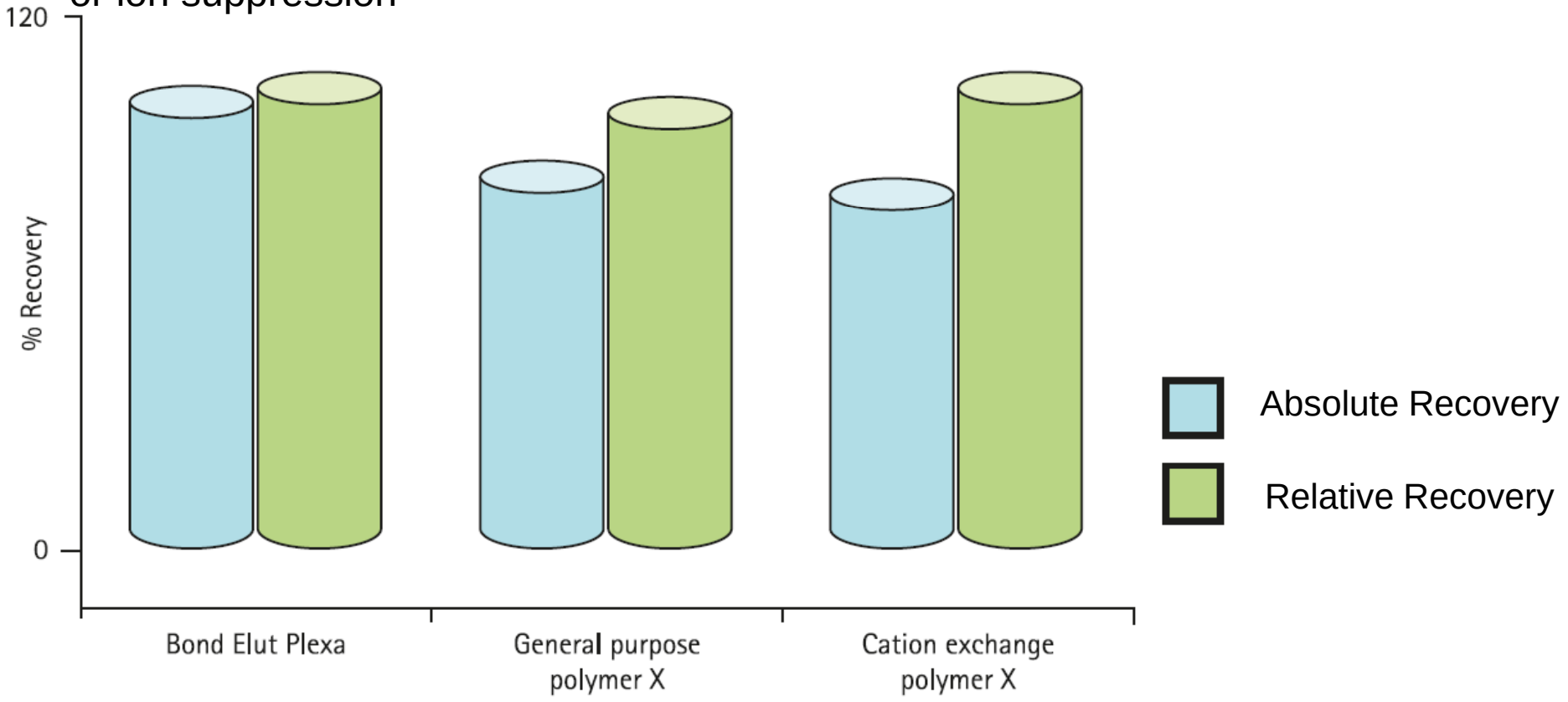
Relative recoveries (via calibration curves) can mask the affects of ion suppression, whereas absolute recoveries do not "cancel" these affects.

Minor differences in absolute vs. relative recoveries is indicative of extract cleanliness or superior extraction characteristics.

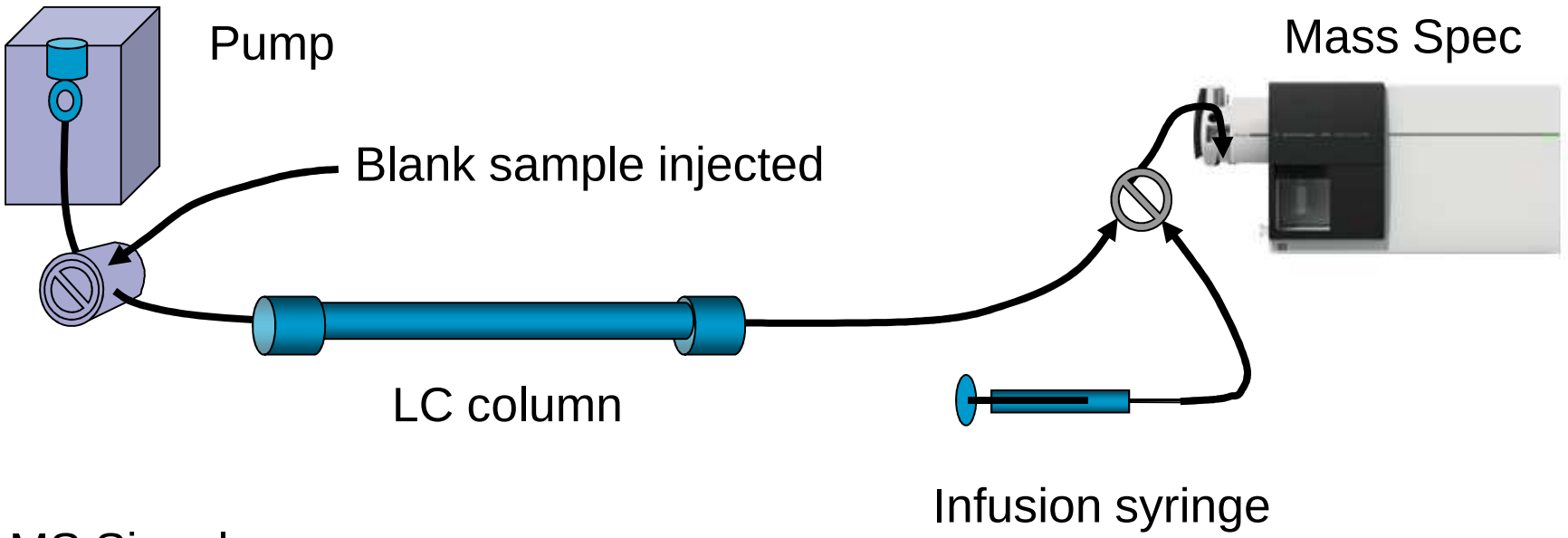
Absolute vs. Relative Recovery

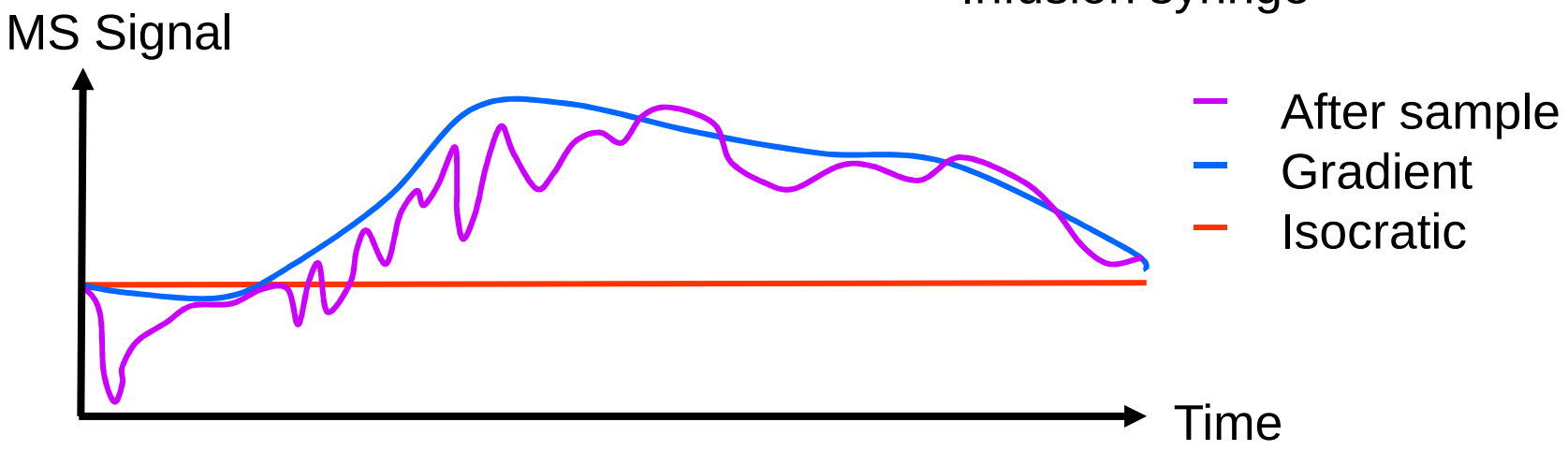
Small differences between AR and RR indicate high extraction efficiency and low signal loss due to ion suppression.

Larger differences indicate sensitivity problems arising from low extraction efficiency or ion suppression

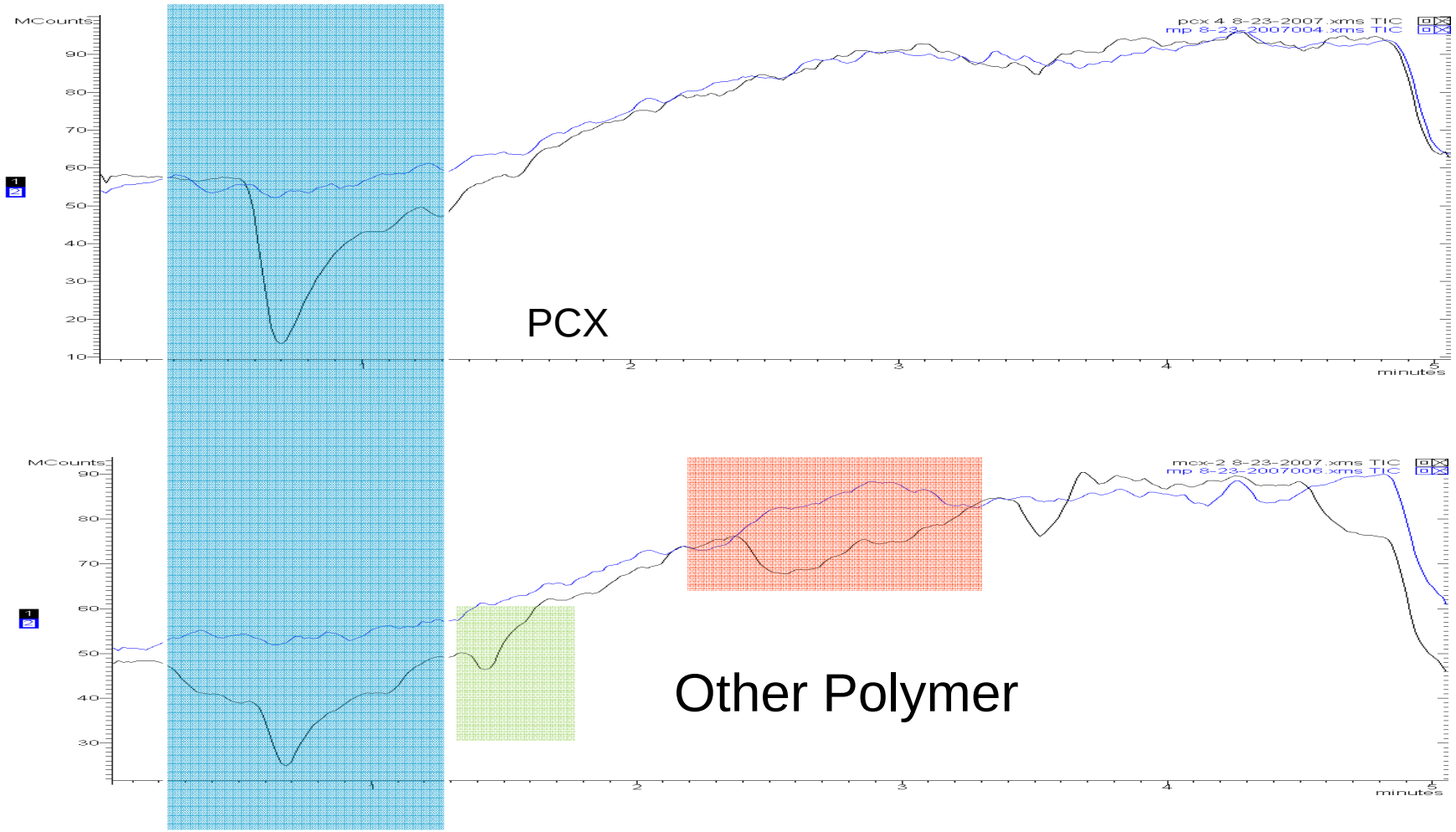


Post Column Infusion Experiments





Improved Precision - Reduced Ion Suppression



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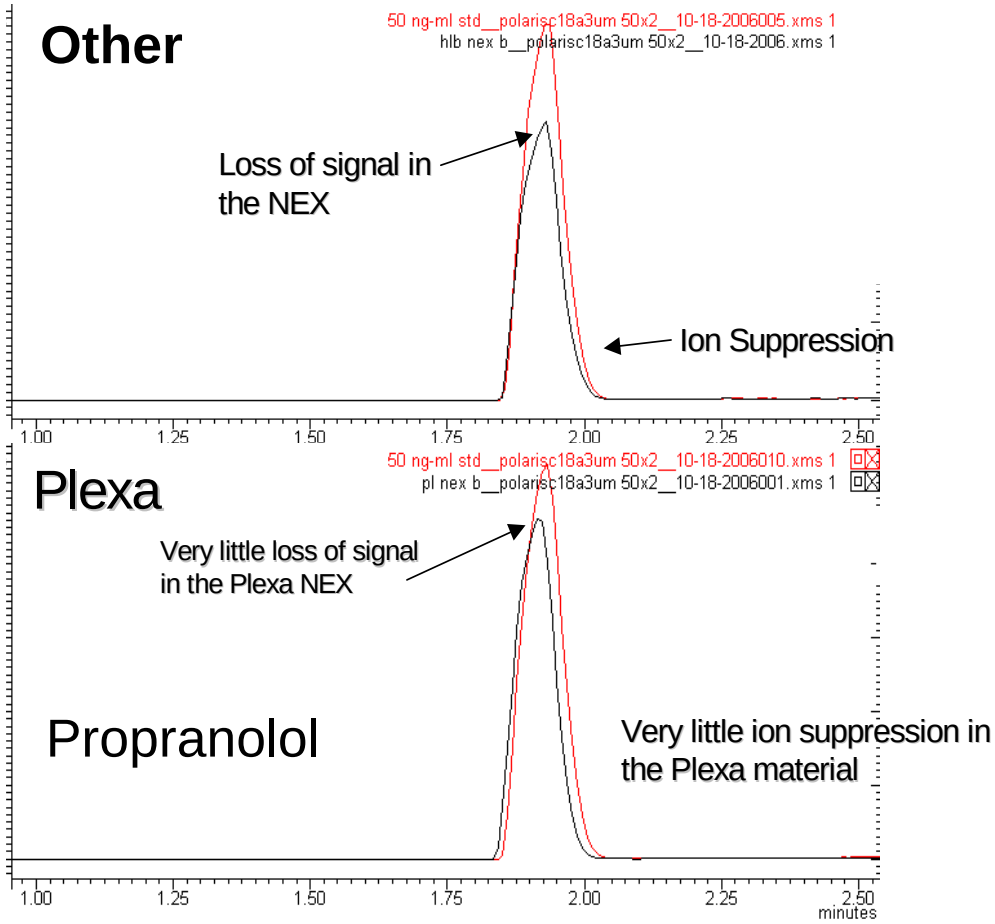
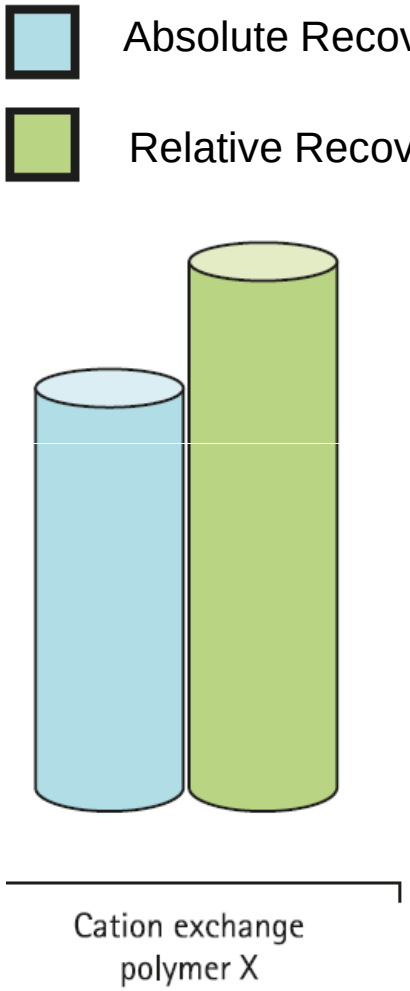
Typical PCI Experiment



Ion Suppression is a very real and concerning phenomenon in the Pharmaceutical Industry

- The loss of quantification of an analyte can create serious issues for the analytical chemist.
- The FDA recently published guidelines on how to determine the effects of ion suppression for low abundance metabolites in DMPK studies (FDA MIST regulations)
- Over 120 publications since 2005 which look at ion suppression in detail

TIP: Evaluating Ion Suppression



- **Simple Route:**

  Compare absolute and relative recoveries (if the difference is >10% after n = 3 then you have a significant matrix effect.

- **Comprehensive**:

  Run and isocratic or gradient (preferred) Post Column Infusion experiment

- **Extra View**:

  Monitor the 184→184 transition for lipids

Simple Plexa Methodology

Bond Elut Plexa

Bases/Neutrals

Condition:	500 µL MeOH 500 µL water
Apply sample:	100 µL plasma dil 1:3 w/ 2% NH_4OH
Wash:	500 µL 5% MeOH
Elute:	500 µL MeOH

Bond Elut Plexa PCX

Bases

Condition:	500 µL MeOH
	500 µL water
Apply sample:	100 µL plasma dil 1:3 w/ 2% phosphoric acid
Acidic wash:	500 µL 2% formic acid
Neutral wash:	500 µL MeOH:ACN (1:1 v/v)
Elute:	500 µL 5% NH_3 in MeOH:ACN (1:1)

Methods for 30 mg Plexa in 96 well plate

PAX Method

Bond Elut Plexa PAX

Acids

Condition:	500 µL MeOH 500 µL water
Apply sample:	100 µL plasma 1:3 w/ 2% NH4OH
Wash:	500 µL water 500 µL 5% MeOH
Elute:	500 µL MeOH w/ 5% Formic Acid

Methods for 30 mg Plexa in 96 well plate

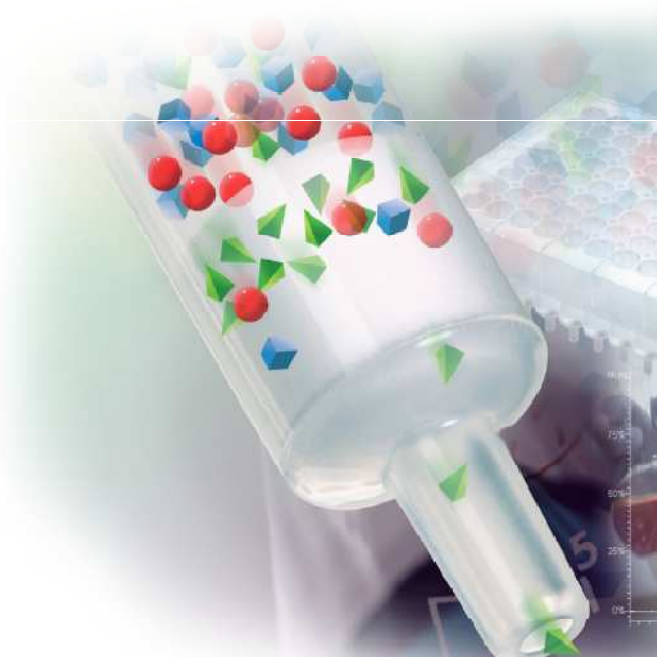
TRICK: Maximizing Elution Recovery

Elution Solvent Composition

- Most methods recommend 100% MeOH or 5% NH3 in MeOH elution (CX)
- **We recommend 50:50 MeOH/ACN**
- Combination of polar protic and dipolar solvent breaks more analyte interactions with SPE phase

Elution Volume

- Most methods recommend 1000uL of eluting solvent
- 2 x 500uL often gives higher recovery
- **For Plexa methods 2 x 250uL (30mg bed)**



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Multi-Suite SPE Method – Bond Elut Plexa PCX

Fractionation of **Acidic, Neutral, and Basic Drugs** from Plasma with Polymeric SPE cation exchange**,** **Bond Elut Plexa PCX** 10 mg

Acids:

Atorvastatin, Diclofenac, Furosemide, Pravastatin

Neutrals:

Cortisone, Cortisol

Bases:

Procainamide, Metoprolol, Paroxetine

Sample pre-treatment:

100 µL human plasma

Dilute 1:3 with 2% H_3PO_4

Condition 1. 500 µL CH_3OH

2. 500 µL DI H_2O

Load Plasma 1:3 with 2% H_3PO_4

Wash 1 500 µL 2% Formic acid

Elute 1 500 µL ACN:MeOH (1:1, v:v)

Acids, Neutrals

Elute 2 500 µL 5% NH_3 in ACN;MeOH

Bases

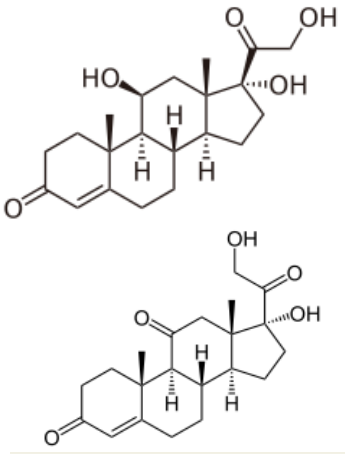
Recovery Data: Acids & Neutrals

Acids:

Analyte	% Rec 0.5 µg/mL	% RSD	% Rec 1µg/mL	% RSD
Diclofenac	101	4	101	5
Furosemide	99	3	96	2
Pravastatin	95	4	96	6
Atorvastatin	100	4	100	5

Neutrals:

Analyte	% Rec 0.5 µg/mL	%RSD	% Rec 1 µg/mL	% RSD
Cortisone	93	4	97	6
Cortisol	101	4	101	4



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Recovery Data: Bases

Bases:

Analyte	% Rec 0.5 µg/mL	% RSD	% Red 1 µg/mL	% RSD
Procainamide	100	5	98	3
Metoprolol	94	4	92	6
Paroxetine	94	5	99	4

Multi-Suite methods have excellent application opportunities

- Hospital or clinic based multi-therapeutic screening
- Drugs of Abuse confirmation
- Drug metabolism studies (where acid or neutral metabolites may need to be extracted using a different method)
- Save time, increase throughout and reduce no of SPE operations and sample injections

Urine

Composition

- Water (95% by volume)
- Urea (deamination product)
- Creatinine
- Salts & Uric Acid
- Pigments
- Carbohydrates
- Enzymes
- Mucins (glycosylated proteins)

In some urine sample analysis, analytes need to be hydrolysed from their glucuronide form using enzyme or base. This can affect extraction efficiency.

Plexa PCX Multi-Suite Urine Method: Bases

- **This generic method covers a broad range of basic drug classes**
- Amphetamines
- Opiates
- Benzodiazepines
- Ketamine/PCP
- Cocaine and Metabolites
- Antidepressants
- Synthetic Analgesics

- **Most multi-suite SPE devices have poor recovery with THC and THC-COOH**

  (next slide)

	Plexa PCX 96 well plate (30 mg, 1mL)
Sample	0.2 mL urine
Pretreatment	Dilute 0.6 mL 100 mM KH_2PO_4
Conditioning	1. 0.5mL MeOH 2. 0.5 mL H_2O
Washes	1. 0.5mL 50% MeOH in H_2O Dry 5 minutes
Elution	0.5 mL 50:50:5 EtAc:MeOH:NH_3

Plexa PCX: THC and THC-COOH

THC

Concentration	% Recovery	RSD (n=6)
1.0 ng/mL	102%	4%
50 ng/mL	107%	5%
1000 ng/mL	107%	3%

THC-COOH

Concentration	% Recovery	RSD (n=6)
1.0 ng/mL	101%	8%
50 ng/mL	97%	4%
1000 ng/mL	102%	4%

	Plexa PCX 96 well plate (30 mg, 1mL)
Sample	0.5 mL urine
Pretreatment	Dilute 0.5 mL 2% Formic Acid
Conditioning	1. 0.5mL MeOH 2. 0.5 mL H_2O
Washes	1. 0.5mL 2% Formic 2. 0.5mL 5% Aqueous MeOH Dry 1 minutes
Elution	0.5 mL MeOH

Saliva (Oral Fluid)

Composition

- Water
- Mucus
- Salts & Electrolytes
- Enzymes & Proteins
- Bacteria
- Broken down foodstuffs

When saliva is sampled it is often added to a preservative solution. This contains surfactants, PEG, Tween and other salts and buffers.

Trends in Oral Fluid Anlaysis

Trends

- Growing matrix for drugs of abuse testing
- Non intrusive test, can be performed in any location
- Low incidence /chance of sample adulteration/ spoiling
- Use of surfactants/preservatives in collection device
  - Stops drug binding to plastic and cellulose bud
  - Reduces sample degradation

Challenges

- Small sample volume
- Contains LCMS ion suppressing surfactants/ oligomers

Plexa PCX for Oral Fluid Analysis

Single Drug Suite

	Plexa PCX (30 mg 3mL)
Sample	0.5 mL oral fluid
Pre-treatment	Dilute 1:2 w/ 100mM phosphate buffer
Conditioning	1. 1 mL MeOH 2. 1 mL H_2O
Washes	1. 1 mL 100mM KH_2PO_4 2. 1mL 100% MeOH
Elution	1.0 mL 2% NH3 in EtAc

Multi Suite (Synergy Health)

	Plexa PCX (1mL 30mg)
Sample	600µl of buffered Saliva
Pre-treatment	500µl of 0.1M, pH6 phosphate buffer.
Conditioning	1. 1 mL MeOH 2. 1 mL H_2O
Washes	1. 1ml, 0.1M HCl 2. 1ml, 60:40 Methanol/0.1M HCl , then dry.
Elution	150µl, 50:50 (ACN:MeOH) 2x 150µl, 50:50:2 ACN/MeOH/ NH4OH

Recovery Data

Drug	% Recovery	%RSD
Morphine	82	15
Codeine	96	2.0
Dihydrocodeine	92	4.3
6-MAM	74	14
Amphetamine	75	5.5
Methamphetamine	93	2.8
MDA	78	8.1
MDMA	88	5.3
MDEA	97	3.3
Benzoylecgonine	95	2.8
Methadone	98	1.5
EDDP	31	7.0
Flunitrazepam	88	5.2
Aminoflunitrazepam	77	2.9
Aminonitrazepam	67	9.9
Diazepam	90	5.9
Nor-Diazepam	91	7.7
Temazepam	80	3.3

COMING SOON: Plexa PAX

Bond Elut Plexa PAX is specifically designed for effective results for a wide range of pKa and logP acidic compounds from plasma

- Made from the same base polymer technology
- Optimized loading of SAX groups ensures good retention/elution balance
- Robust, generic method, for the widest range of acidic analytes

PAX Applications

	Plexa PAX (10 mg)
Sample	100 µL human plasma
Pretreatment	Dilute 1:3 w/ 2% NH_4OH
Conditioning	1. 500 µL MeOH 2. 500 µL H_2O
Washes	1. 500 µL H_2O 2. 500 µL MeOH
Elution	500 µL 5% Formic Acid :MeOH

Compound	LogP	pKa
Atorvastatin	5.7	4.5
Diclofenac	4.2	4.2
Furosemide	1.5	4.7
Ketoprofen	3.2	5.2
Pravastatin	2.6	4.6

Mobile Phase –

A: 5mM Ammonium Formate

B: Methanol

Gradient –

t = 0 - 60% A : 40% B

t = 3:0 - 3:59 min 20% A : 80% B

t = 4:0 – 5:00 min 60% A : 40% B

Column –

Pursuit XRs Diphenyl 2.8 µm 50 x 2.0 mm

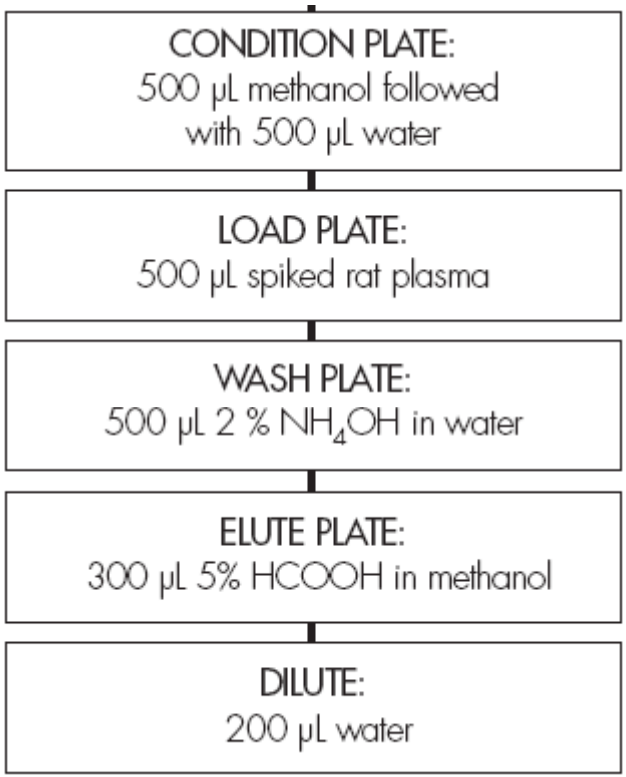
All samples evaporated and reconstituted in 100 µL of 80:20 5mM Ammonium Formate: MeOH

Method Comparison

	Plexa PAX (10 mg)
Sample	100 µL plasma
Pretreatment	**Dilute 1:3 w/ 2% NH_4OH**
Conditioning	1. 500 µL MeOH 2. 500 µL H_2O
Washes	1. 500 µL H_2O 2. 500 µL MeOH
Elution	2 x 250 µL 5% Formic Acid :MeOH

Base pre-treatment further activates AX groups, improving recovery

Other AX Plate



Recovery Data

- Absolute recoveries used against other AX product (10mg Plate)
- Other vendor's method had very poor recoveries, data shown is for Plexa method on other AX
- RSDs for AX methods typically high

Absolute Recoveries (100 ng/mL)				
Compound	**Bond Elut Plexa PAX**		**Other AX Polymer**	
	% Rec	RSD(n=6)	% Rec	RSD(n=6)
Atorvastatin	87	13	37	21
Diclofenac	67	16	42	22
Furosemide	99	11	40	26
Ketoprofen	71	18	49	31
Naproxen	93	15	50	20
Pravastatin	87	14	106	10

Summary

The Bond Elut Plexa family offers the opportunity to increase throughput and improve the quality of your data

- The hydroxylated surface minimizes binding of matrix interferences offering greater accuracy and precision
- Proven minimization of ion suppression in complex matrices
- Amenable to a broad range of biological matrices
- Ultra generic methodology for urine samples
- Multi-suite methods for saliva (inc. Tween exclusion)
- Simple methodologies allow rapid method development
- Three phases offer high recoveries for most compounds and matrices – simple choices for quick results

Kaffeepause

15 min

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Teil B – Applikative Lösungen , Methodenent-wicklung

Precipitation – Filtration

Troubleshooting

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Typical Approaches to Plasma

RECAP:

Dilute and Shoot: Dilution & addition of ITSD

LLE or SLE: Agitation, partition or Tube

Precipitation: Centrifugation, Filter Plate

Solid Phase Extraction: Tube or Plate

	Dilution	LLE	Precipitation (Captiva)	SPE (Plexa)
Proteins	No	Partial	Yes	Yes
Lipids	No	No	No	Yes
Salts	No	Yes	No	Yes

Typical Approaches to Plasma

What happens if plasma SPE methods are not attractive :

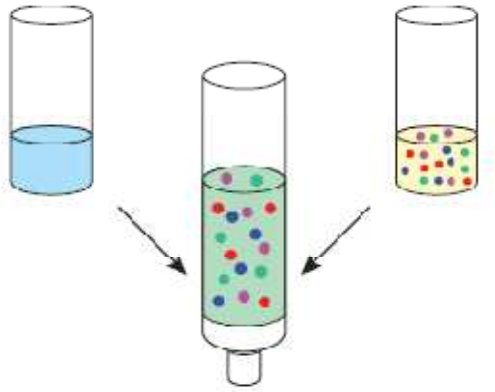
- Percieved cost?
- Workflow change?
- Complexity?
- Laziness?
- Sample throughput

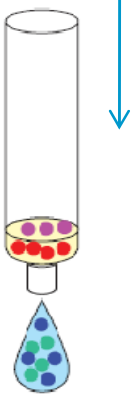
	Dilution	Precipitation (Captiva)	SPE (Plexa)
Proteins	No	Yes	Yes
Lipids	No	No	Yes
Salts	No	No	Yes

Captiva Filtration Products

They are Mechanical Filters

- Protein precipitated plasma (0.45µm)
- Fibrinogen from plasma storage (20µm)
- Tissue homogenates (0.45µm)
- Hepatocyte filtrate (liver cells) (0.45µm)
- General particulate filtration





How Captiva Improves Precipitation

- Centrifugation not necessary

  processing by vacuum or positive pressure

- Easier automation

  Captiva filter plates interface with most automation platforms (while the centrifugation method of ppt is nearly impossible to automate)

- Fewer transfer steps

  no pellet means dry down & reconstitution in single collection plate

- Cleaner, particulate-free samples

  - no pellet means direct trouble-free sampling from collection plate
  - filtration effectively removes more proteins from injection

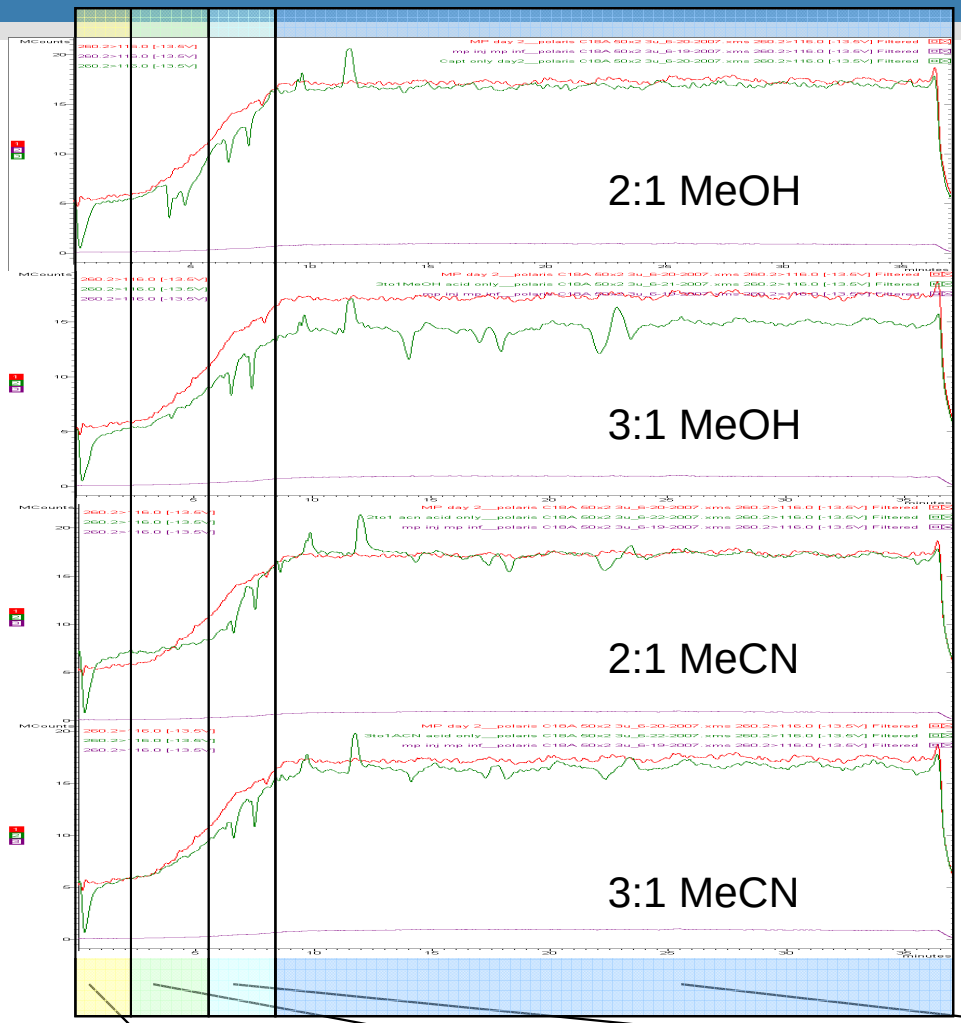
TIP: Method Development in Protein Precipitation

Method development in Protein Precipitation is not complicated

- **Acetonitrile** - good pellet “qualities” commonly used
- **Methanol** - fine particles , needs filtration
- Organic solvent ppt typically is a **3:1** or greater (as much as 10:1)

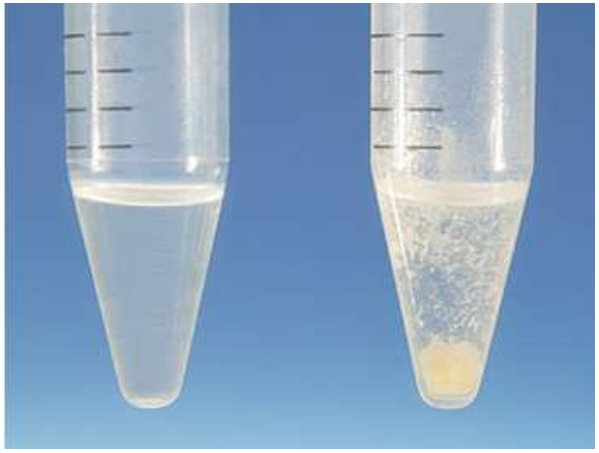
- **AVOID:**
- 10% aq. TCA - highly efficient, highly aqueous supernatant, destroys LC columns quickly
- metal salts - highly efficient, highly aqueous supernatant, not MS compatible

Post Column Infusion: Captiva



SALT/ POLAR IONICS	PROTEINS & PEPTIDES	LYSO-PHOSPHATIDYLCHOLINES	LIPIDS & HYDROPHOBICS

Propranolol
Post Column Infusion



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Advanced Precipitation Techniques

Is there a missing technology here?

- Is there a compromise between the ease of use of PP and the cleanliness of SPE?
- Salts are not a concern in reverse phase chromatography
- Lipid Removal PP?

	Precipitation (Captiva)	Lipid Removal Precipitation	SPE (Plexa)
Proteins	Yes	Yes	Yes
Lipids	No	Yes	Yes
Salts	No	No	Yes

Lipid Build-Up With a Ballistic Gradient

Sample: Protein Precipitated Porcine plasma (3:1 ACN precipitation)

Column: 40x4.0 Polar modified reverse phase

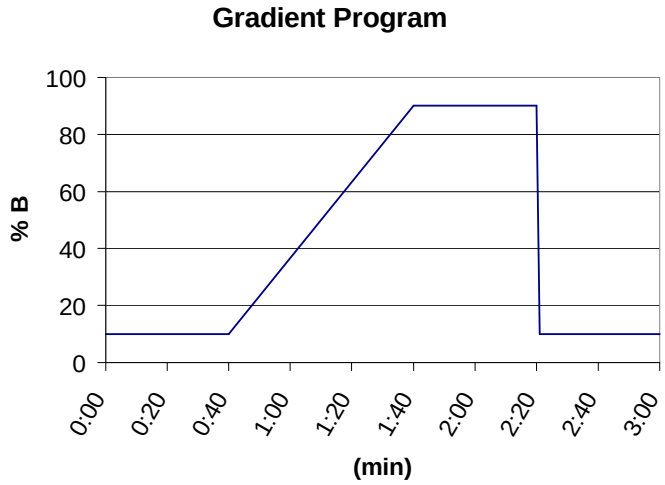
Solvents:

A: 0.1% Formic Acid

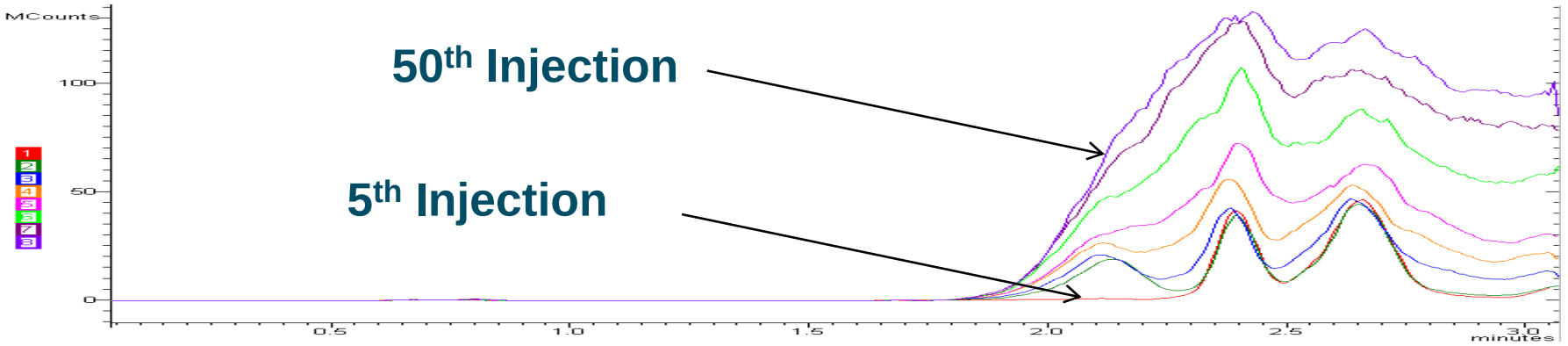
B: Acetonitrile

Gradient:

Time (min)	%B	Flow (µL/min)
0:00	10	500
0:40	10	500
1:40	90	500
2:20	90	500
2:21	10	500
3:00	10	500



Detection: 184→184 (nonselective Phospholipid head group channel)



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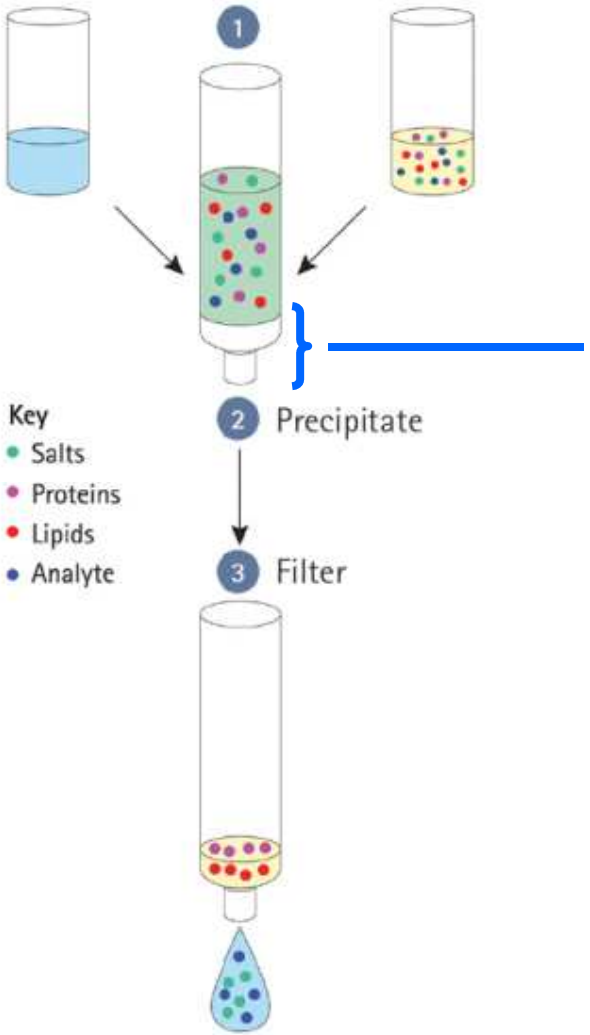
Lipid Removal Precipitation: Captiva ND*Lipids*

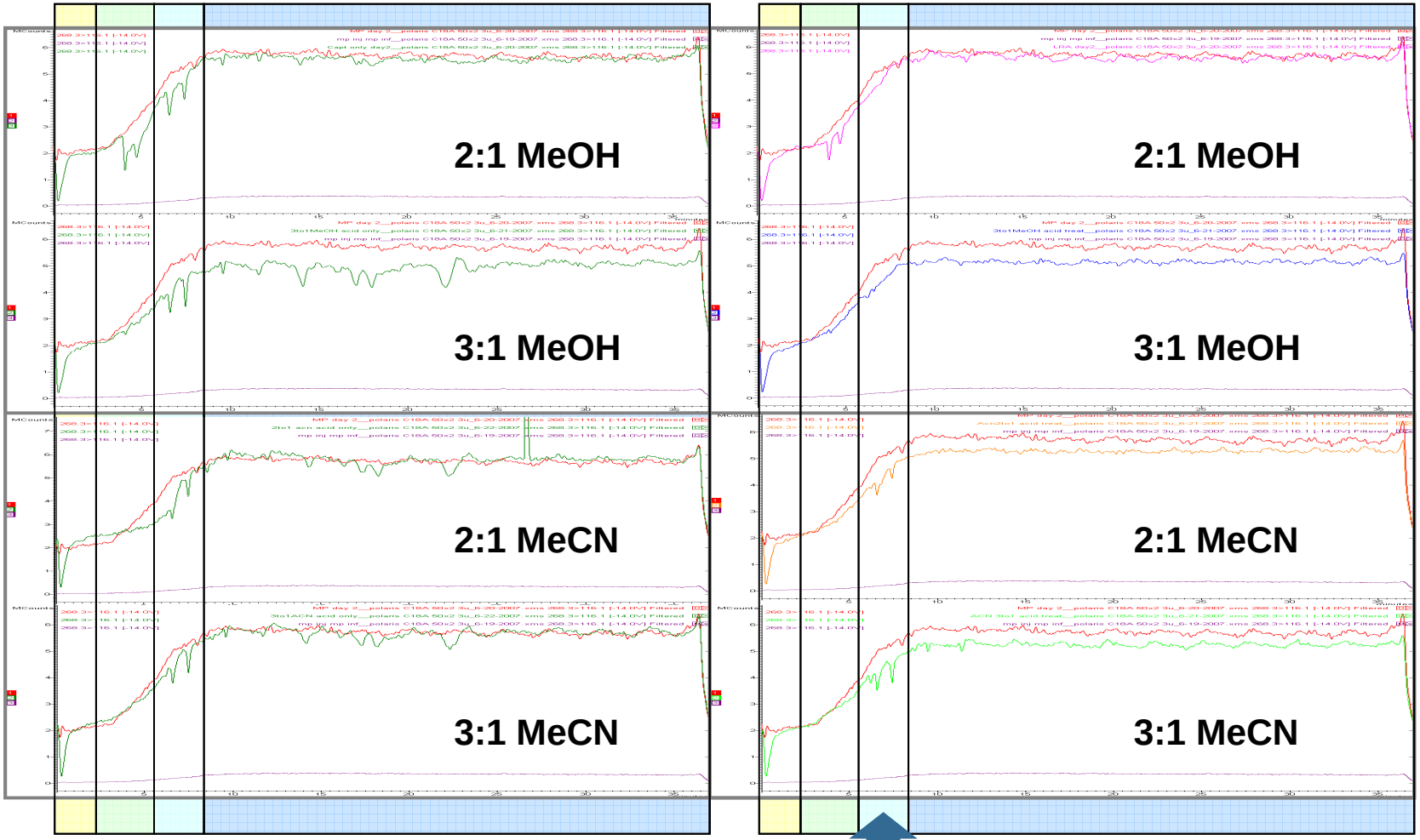


- Captiva particulate filter → removes protein interferences
- Proprietary Lipid Stripping Media → removes lipids
- Non-Drip Membrane → ease of use

Post Column Infusion Studies: Metroprolol

Protein Precipitation

Captiva ND*Lipids*



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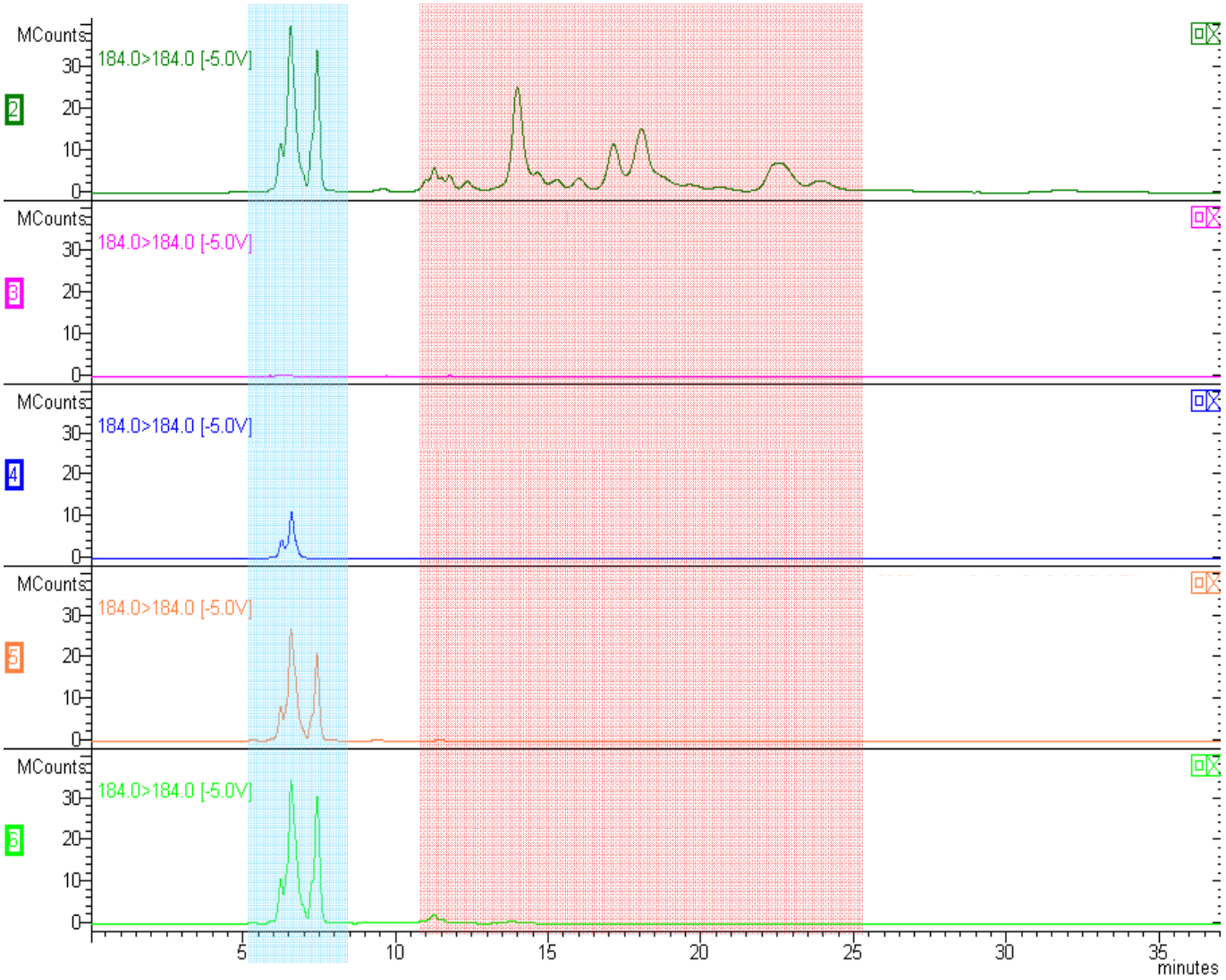
Lipid Removal



Centrifuge PPT

2:1 MeOH

3:1 MeOH

2:1 MeCN

3:1 MeCN

TIP: Captiva ND Lipids Method

- Add 3:1 pH modified MeOH: plasma to the well
- Mix thoroughly
- Filter

- pH modification to charge the analyte(s)
- For most analytes, including polar basic small molecules 0.1% formic acid in MeOH is sufficient
  - For acidic analytes 5% ammonia can be used instead
- Acid concentration can be increased if necessary, but care needs to be taken to avoid sample gelation

- MeOH is the best solvent for phospholipid removal

Blood

Spotted blood samples:

- Add excised blot spot disk directly to Captiva ND*Lipids* well and use standard method

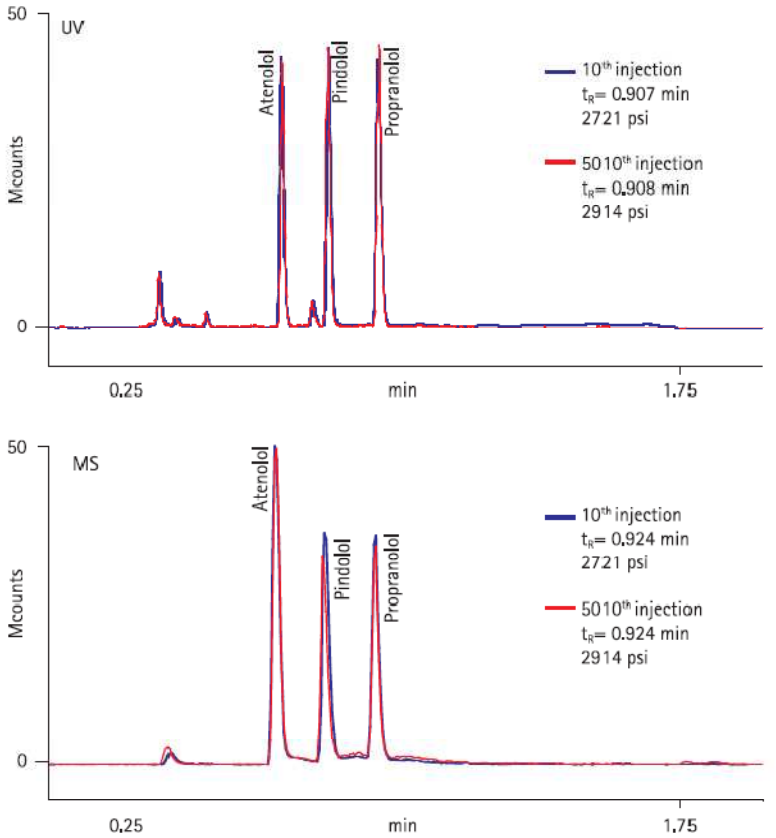
Whole blood:

1. Dilute whole blood sample 1:1 with 0.1% aqueous formic. Vortex and allow to sit for 1 hr.
2. Add MeOH with 0.1% formic acid : diluted blood sample to Captiva ND*Lipids* plate (3:1)
3. Seal the plate with DuoSeal cover and vortex for 1 minute.
4. Leave cover in place. Place plate on Captivac with collection plate in place. Apply mild vacuum.
5. Filtrate should be clear and may require dilution prior to LC/MS

Universality of CaptivaND*Lipids*

Analytes	LogP	Relative Response vs. Protein Precipitation Alone (%)
Zolpidem	3.32	109.6
Mianserin	3.52	167.4
Tranylcypromine	1.40	251.4
Nefazodone	4.70	144.9
Amoxapine	3.10	171.1
Maprotiline	5.10	159.0
Nomifensine	2.94	162.6
Warfarin	3.51	100.5
Sulindac	3.59	136.7
Loratidine	3.65	103.4
Loperamide	6.25	106.7
Vardenafil	6.01	106.5

Longevity study illustrating prolonged column lifetime when using Captiva ND*Lipids*



No significant changes in back pressure, retention time, and peak shape with Captiva ND*Lipids* after 10 and 5010 injections for LC/MS or LC/MS/MS bioanalysis (top = UV detection; bottom = MS detection).

Technique Summary

	Filtration (Captiva)	Filtration + (Captiva Lipids)	SPE
Proteins	Yes	Yes	Yes
Lipids	No	Yes	Yes
Salts	No	No	Yes

- Simple & easy to use
- Removes proteins
- Inexpensive

- Removes proteins & lipids - reduced ion suppression
- Simple & easy to use - 3 step process
- Low Cost

- More involved
- Removes proteins, lipids, salts etc
- Better for lipophilic drugs
- Reasonable

TIPS & TRICKS: Trouble Shooting

From our global helpdesk logs, we have identified the most common SPE issues encountered by our end users.

All of these issues can be linked to simple practical errors

1. **Low Recovery**
2. **Poor Flow**
3. **Loss of Analyte**
4. **Dirty Extracts**

TIP: Sorbent Conditioning

Sorbent conditioning is vital for good SPE performance

- An unconditioned sorbent bed can result in **Poor Flow** and **Poor Recovery**.
- Particularly for biological samples, where precipitation can occur on dry media
- Clogging and Channelling

Recommendations

Silica SPE	Polymeric SPE
>2 bed volumes of low viscosity organic solvent (e.g. MeOH)	>2 bed volumes of low viscosity organic solvent (e.g. MeOH)
Condition slowly under minimal vacuum <2” Hg	Wash with aqueous solution prior to sample load
Allow time for equilibration (30-60s)	More tolerant to accidental drying

TIP: Poor Flow - Vacuum Considerations

Vacuum control and experience comes with practice

- Flow rates should be kept on the lower side overall (2-5 mm Hg)
- 2-3 mL/min is optimal for load, wash and elution
- Ion Exchange as a primary or secondary interaction is especially affected by high flow rates (break through)
- Even C18 can show band broadening with high flow rates
- Employ variable vacuum or variable time (pulsing) depending on sample type and drying needs

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TIP: Poor Recovery - Capacity

Understanding the Capacity of your SPE Phase is Critical

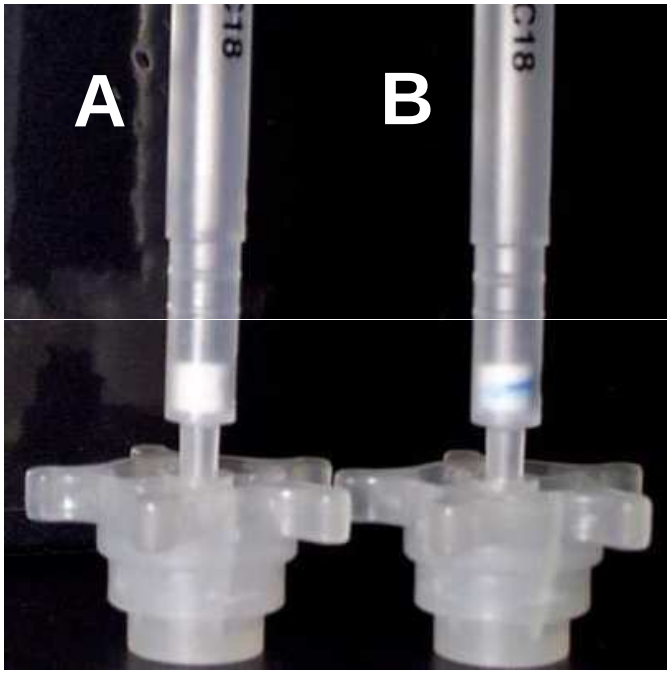
Sorbent Type	Capacity
Silica (Polar or Non-Polar)	1-4% of bed mass
Silica (Ion Exchange)	< 1.0 mmol/g
Polymeric	10 -12% of bed mass

- Assumes good analyte/sorbent interaction
- **Capacity does not distinguish between analyte and interference!**
- Do you know amounts of interferences present?
- Stacking two cartridges on top of each other will help determine if capacity is an issue

TIP: Poor Recovery - Elution

Elution volume optimization can be the simple to achieve

- Elution volumes can be minimized and recovery maximized if elution is done in aliquots.
- Four aliquots of 50uL give better results than 200uL in a single shot. 2 x 100uL is a more practical solution.
- Using mixtures of solvents can have a dramatic effect on recovery (50:50 MeOH/ACN)



A: 4 x 50 uL Aliquots
B: 1 x 200 uL Aliquot

TIP: Dirty Extracts

Dirty Extracts are common in un-refined SPE methods

- Phase may be too universal (e.g. C18)
- Extraction scheme is not specific enough
- Reduce concentration of Elution solvent
- Wash step is ineffective, increase organic concentration
- Ion Suppression - try alternative phase or change wash %
- Try polymeric or mixed mode SPE
- Pre-rinse cartridge with elution solvent

TRICK: If In Doubt … Mass Balance!

If you load ???ng of a sample onto a cartridge, tracking where it goes helps determine important method alterations

Method Development

1. Collect “effluent” that has passed through upon sample loading
2. Collection all wash steps
3. Collect all elution steps

Easy to Quantify if you have analyte still on the phase

If the matrix contains high levels of interference, mass balance can be difficult if analyte does not retain on the load step.

Sample Prep Summary

- In the ever demanding analytical chemistry environment, sample prep is becoming an essential component of good laboratory workflow
- The advanced sample prep techniques (SPE and Precipitation) offer clear ease of use and productivity advantages
- The quantification of endogenous materials and ion suppression is vital to getting the most from your sample
- Polymeric SPE and multi-suite methods is a key platform for biological fluids
- Agilent offers the broadest platform of chemistries in the widest range of formats in the industry
- **This was just a short seminar. Tell us if you have a challenge or application need in your laboratory… we probably have a solution already!**

Fragen???

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Vielen Dank!

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Agilent Sample Prep: Accuracy Starts Here

- Options for Your Needs
- Innovative Products Designed for Lab Efficiency
- Technical Support at Every Step
- World Class Manufacturing and Quality

Provide chromatographers with highly accurate, reproducible results and enable transfer of methods from lab-to-lab and around the world

Effect of Endogenous Interferences

- Poor Chromatography
- Mechanical issues (particulates, blockages)
- LC Column Lifetime Issues
- Carry Over
- Instrument Downtime
- Ion Suppression
- Overall loss in sensitivity
- Increase in sample run time/ cost

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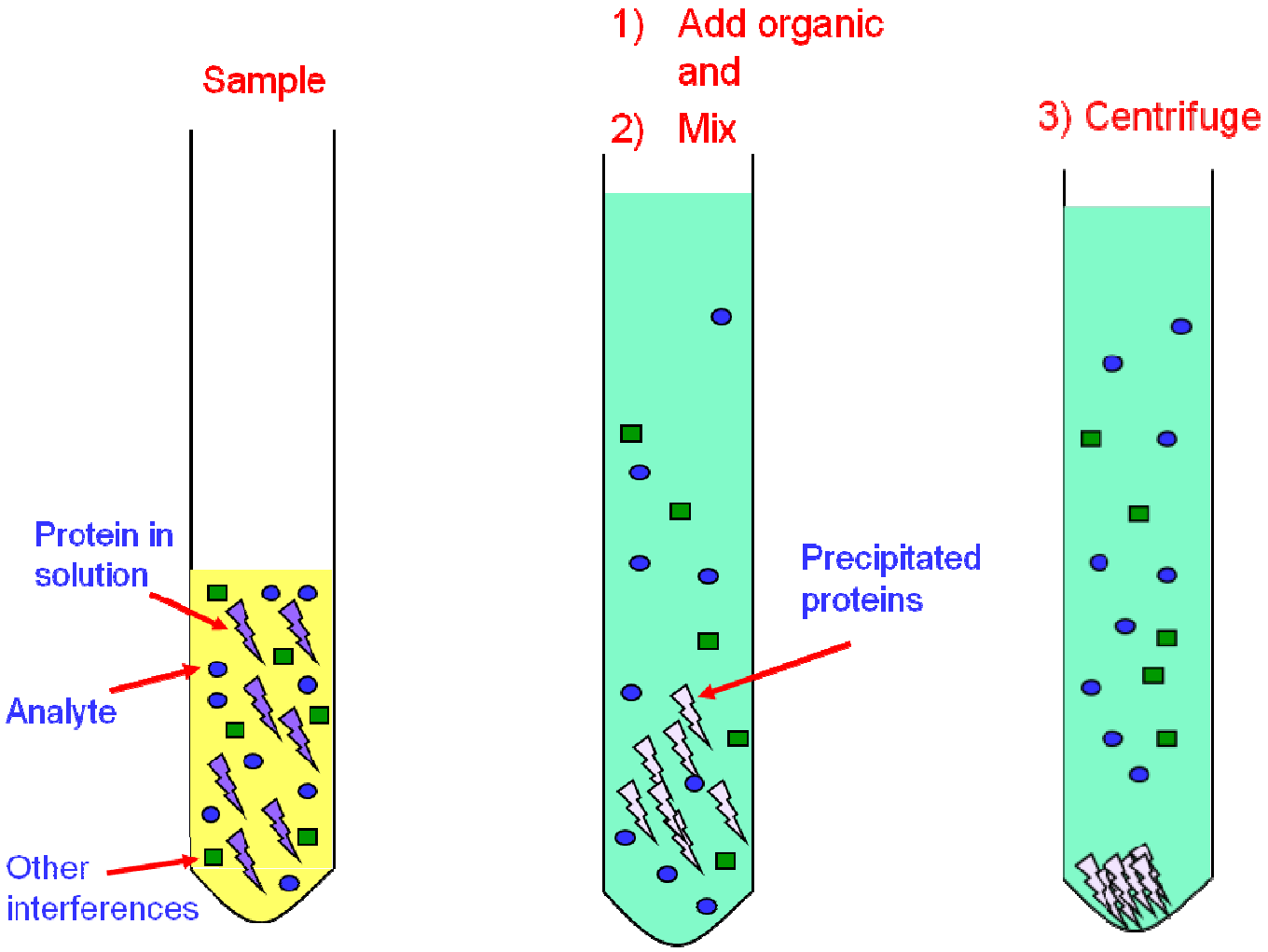
Protein Precipitation with Centrifugation



4) Remove supernatant

5) Analyze supernatant, often after dry down and resuspension

Where to go for more information

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