Sample Clean-Up in the Fast Lane

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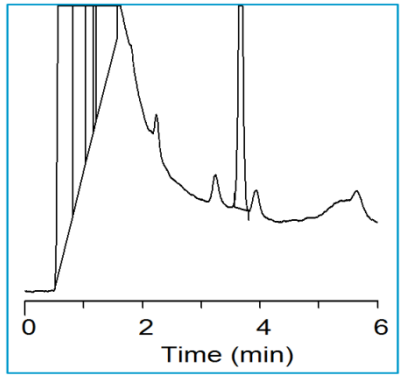
April 4, 2018

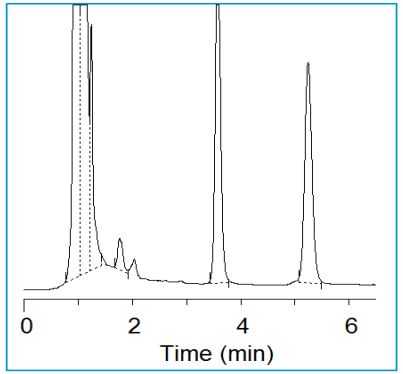
Why Perform Sample Clean-Up?

- To acquire desired sensitivity/selectivity
- To reduce contamination/carryover issues
- Use of sensitive and expensive instruments: *Protect your investment!!!*

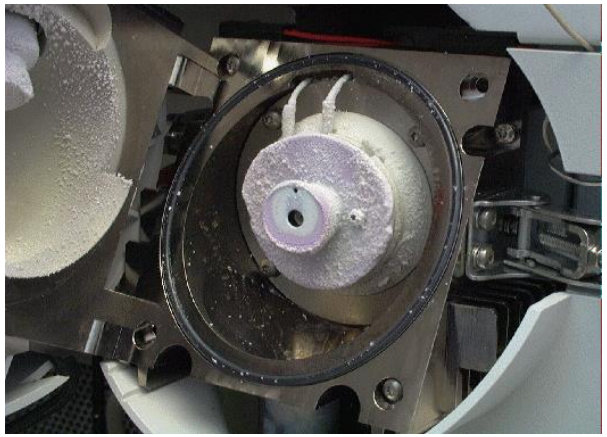
Pesticides in Avocado *without* sample clean-up



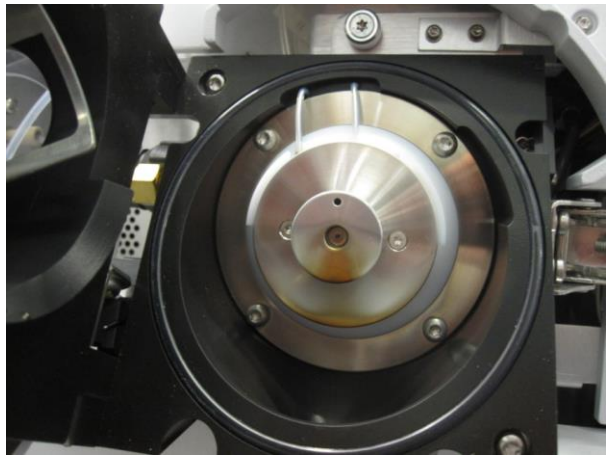
Pesticides in Avocado with sample clean-up



Instrument Contamination

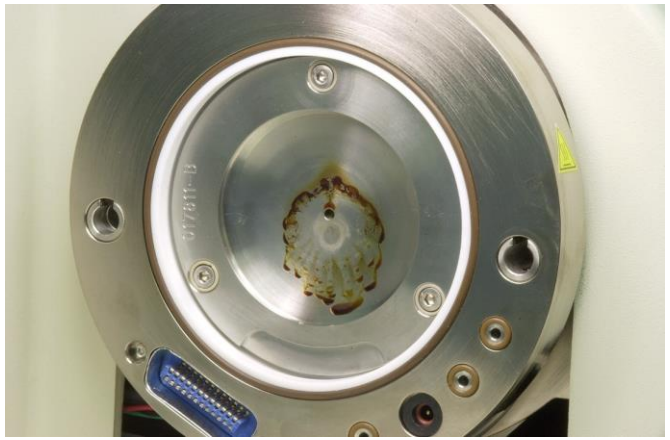


Salt build-up in LC-MS ion source from unextracted salts



ESI Ion Source contamination after 3000x Urine Dilute/Shoot Injections

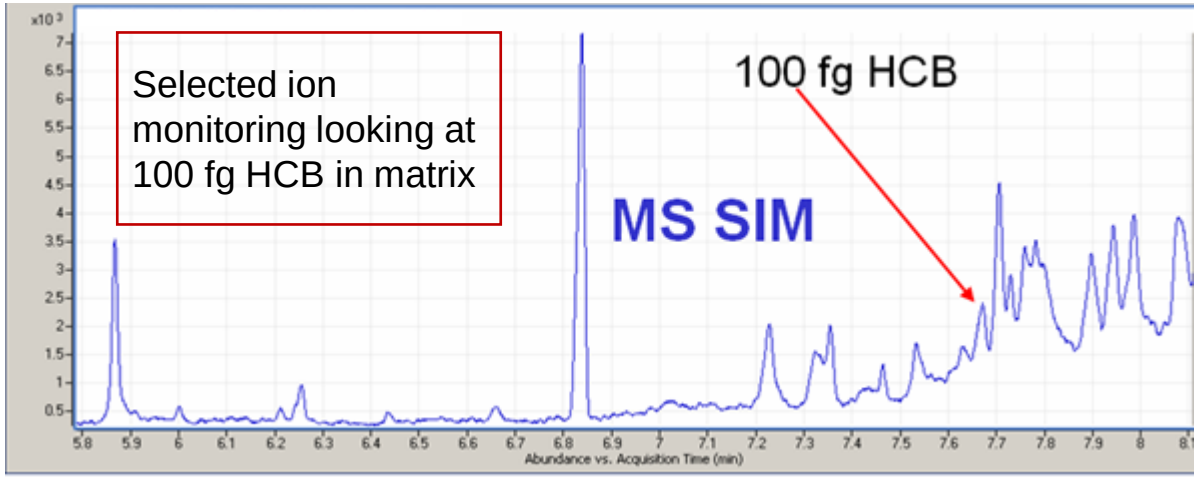
GC Inlet Seal

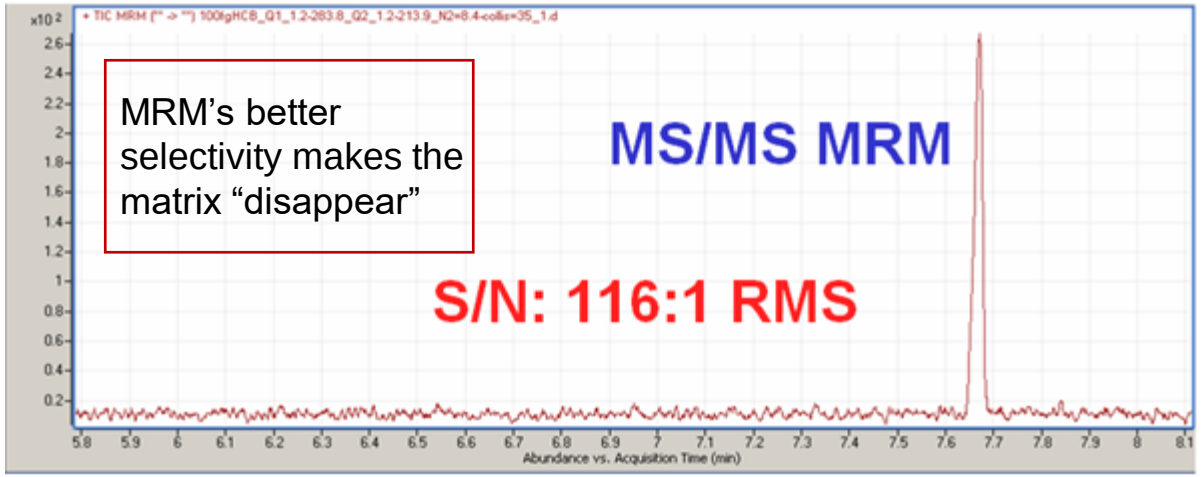


Curtain plate after injection of 25 samples with extractions from raisins without cleanup

GC Inlet Liner

Tandem Mass Spectrometry and "The Case of the Disappearing Matrix"





Finding the right sample clean-up for YOU!

Particulate Filtration

- Very fast!
- Only removes particulates

Protein Precipitation

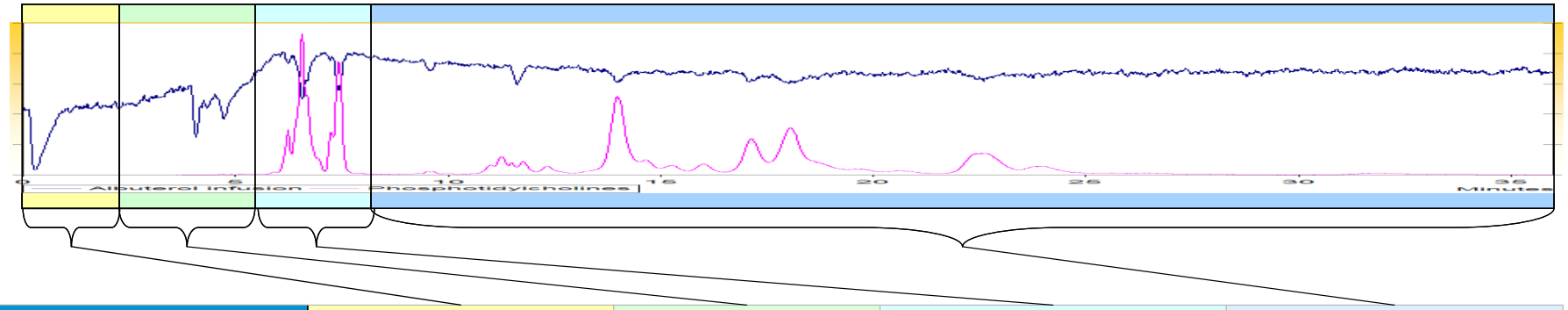
- Fast
- Removes ONLY proteins
- Extracts compounds from matrix

Solid Phase Extraction

- Provides very clean samples but time consuming
- Method development most likely required

Ion Suppression Regions by LC-MS/MS after protein crash

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

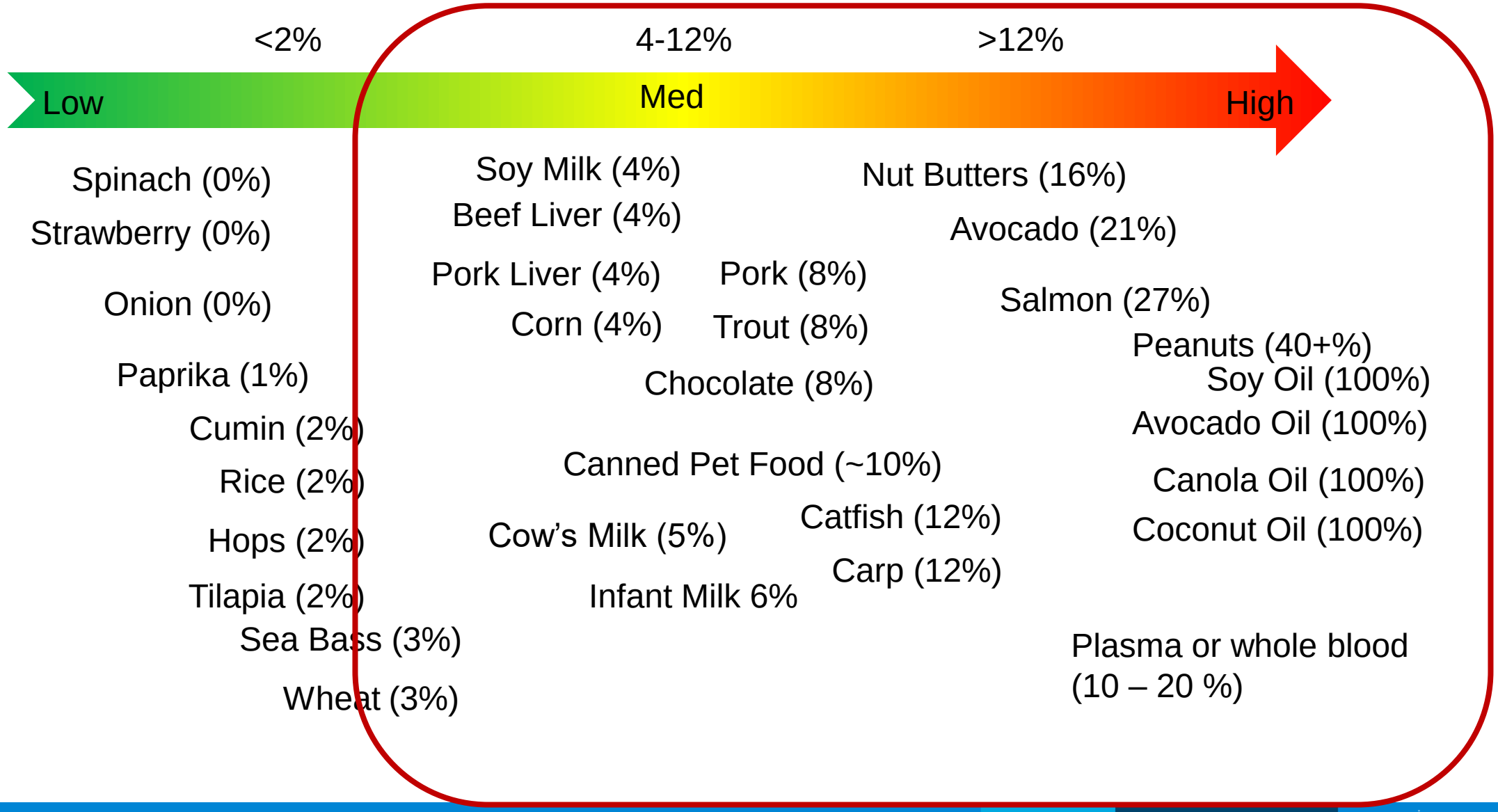
Protein precipitation is not sufficient to clean your sample!

Food!

Oh where can these lipids be???

Biologicals!

Matrices and Approximate Total Lipid Content



Impact of Lipids on workflow

- **Inability to meet detection criteria**
  - Longer method development time
  - Method troubleshooting
  - Variability depending on matrix
  - QC/LOQ issues/reruns
  - **Indeterminate time**
- **Data variability**
  - High RSDs
  - Data accuracy
  - Variability depending on matrix
  - **Reruns, data analysis time**

- **Mass Spec Cleanliness**
  - Cleaning the source
  - Cleaning/replacing capillary
  - Liner Lifetime/Inlet issues
  - Establishing vacuum levels
  - Retuning
  - Any additional troubleshooting
  - **4 hours - > 1day**
- **Lipid Build Up on Column**
  - Column longevity / GC column cutting
  - Back pressure
  - Column flushing / equilibration
  - **Approximately 2 hours**

Current Sample Clean-Up Techniques

Method	How Lipids are removed	Weakness
Dilute and Shoot	No lipid removal, only dilution	No lipid removal
Protein Precipitation	PPT followed by centrifugation	Insufficient lipid removal
	PPT followed by filtration with or without sorbent	Insufficient lipid removal; low analyte recovery
QuEChERS	**PSA/C18 sorbent (dSPE)**	**Not selective; insufficient lipid removal; analyte loss**
	Zr-containing sorbent	**Low total lipid capacity; analyte loss**
	Freeze sample	Time needed; loss of analyte
SPE/SLE	**Load and elute**	**Time needed; solvent usage; extensive method development**
SEC/GPC	Chromatographic separation	Uses copious amounts of solvent and time; capital expense

Agilent's Answer to the Lipid Problem…

Captiva EMR-Lipid

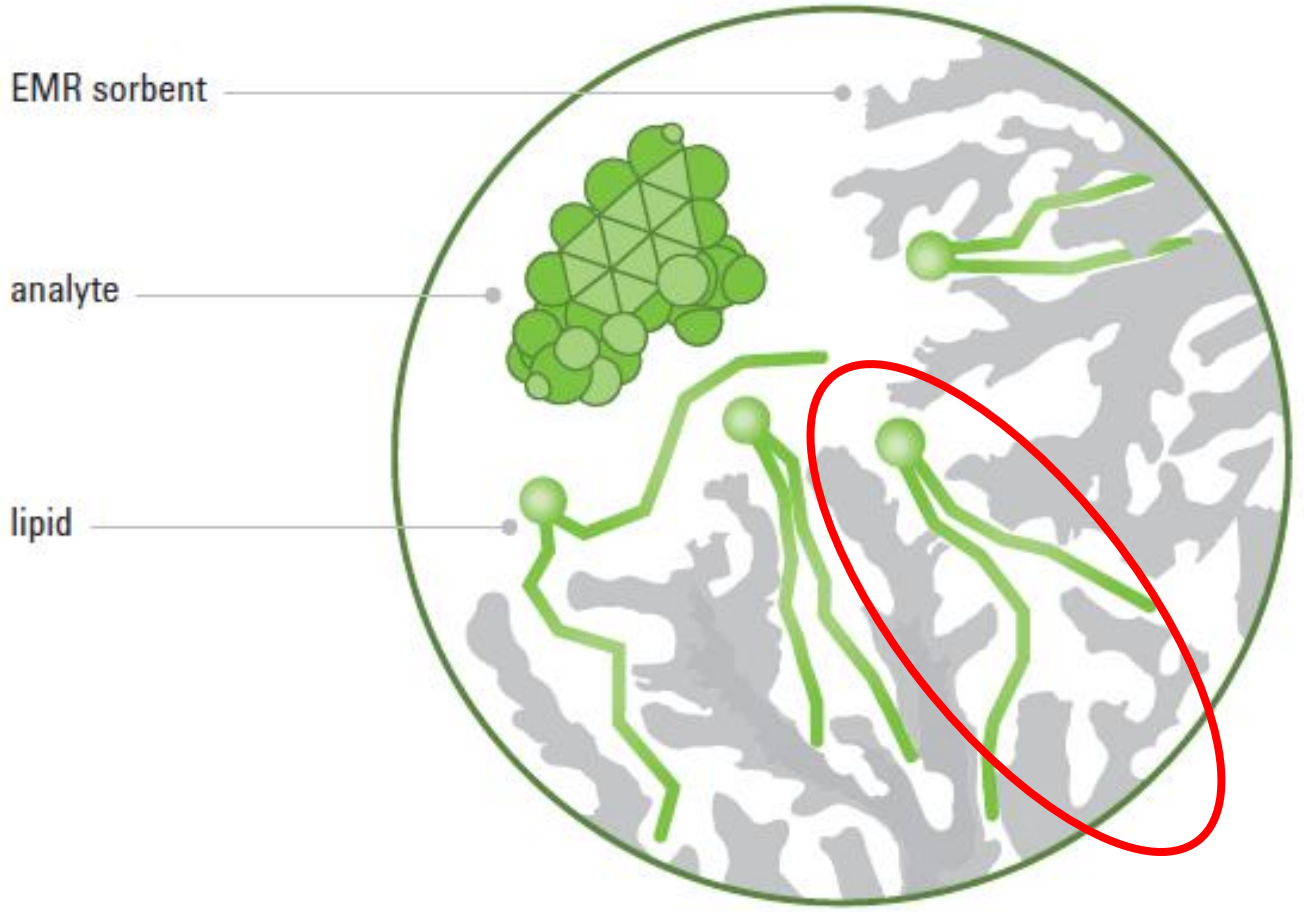
The Analytical Scientist Innovation Awards 2017

- One of Agilent's newest products with a 2 in 1 benefit of removing proteins and lipids
- Simple pass-through format
- Solvent-retention frit in 1 mL cartridge/96-well plate format for in well protein precipitation (*in situ*)
  - Unique cartridge/well construction minimizes clogging – and **ensures protein and lipid removal** (no cloudy samples)
- 3 and 6 mL cartridge format for larger samples
  - Do not contain solvent retain frit which allow for gravity flow
  - Protein precipitation performed offline (QUECHERS, etc.)
- Unique cartridge/well construction minimizes clogging – and **ensures protein and lipid removal** (no cloudy samples)
- High analyte recoveries
- Effective use will reduce ion suppression, increase analyte sensitivity, and detection, and extend the lifetime of your analytical column

What is it??

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

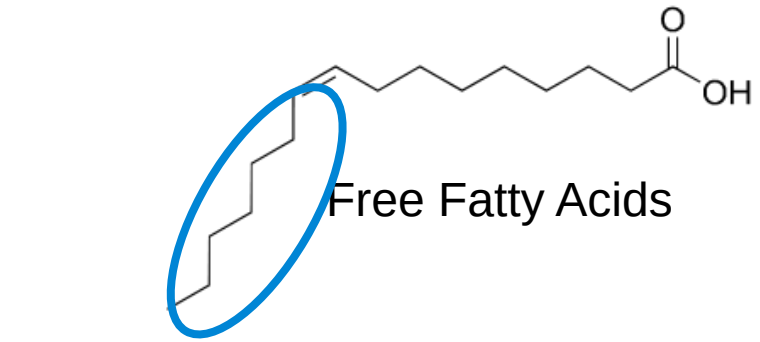


… and what does it do?

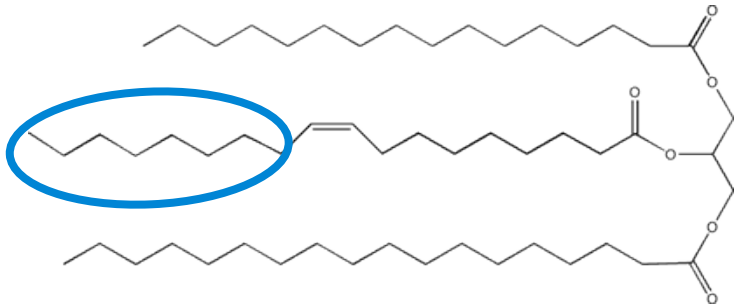
EMR-Lipid sorbent removes Lipids

What are Lipids?

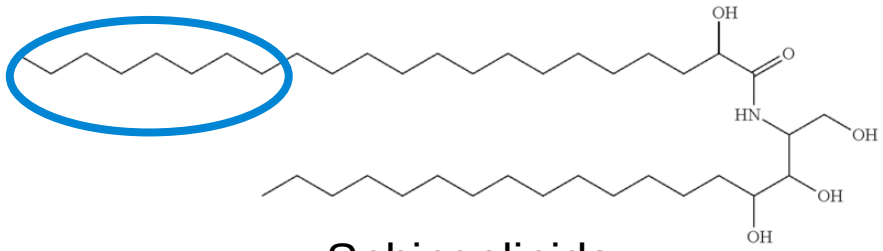
A class of naturally occurring hydrocarbon containing compounds commonly known as fats and oils



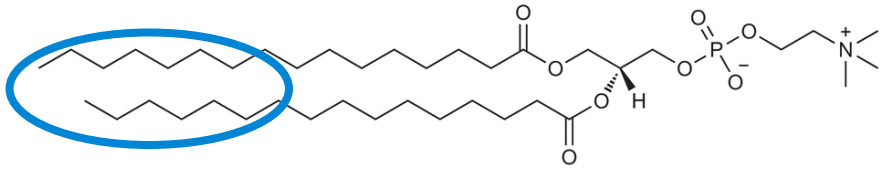
Free Fatty Acids



Triglycerides



Sphingolipids



Phospholipids

EMR-Lipid = Finger Trap

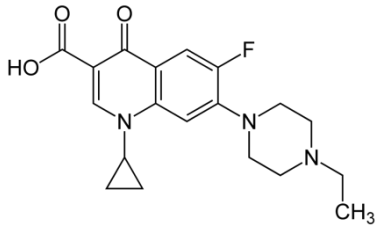
Finger = carbon chain of lipids

What Does EMR-Lipid *NOT* Interact With?

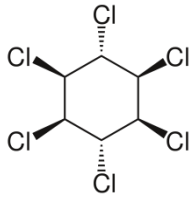
EMR-Lipid does NOT remove analytes of interest

Exceptions?

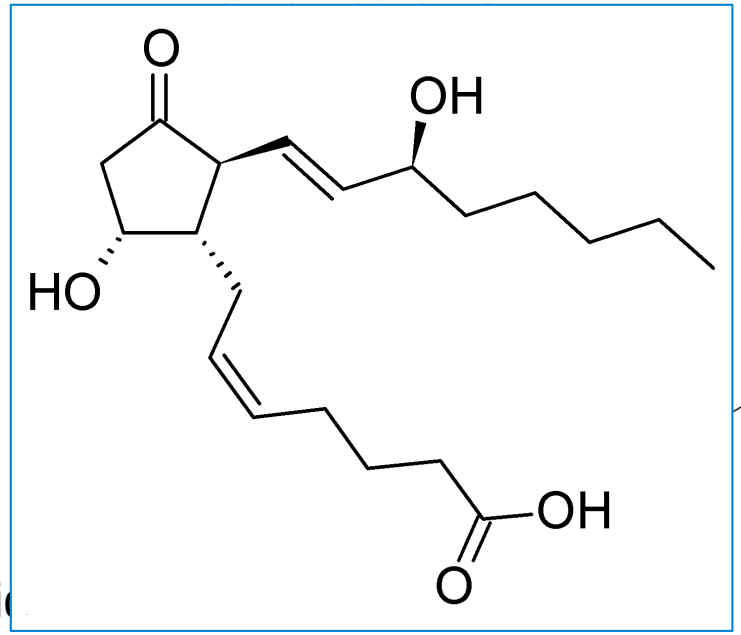
Compounds containing unbranched carbon chains (e.g. prostaglandins)

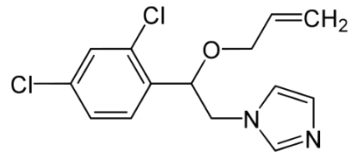


Fluoroquinolones

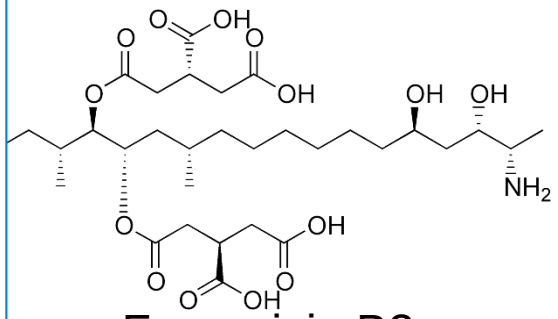


Organochlorine Pesticic





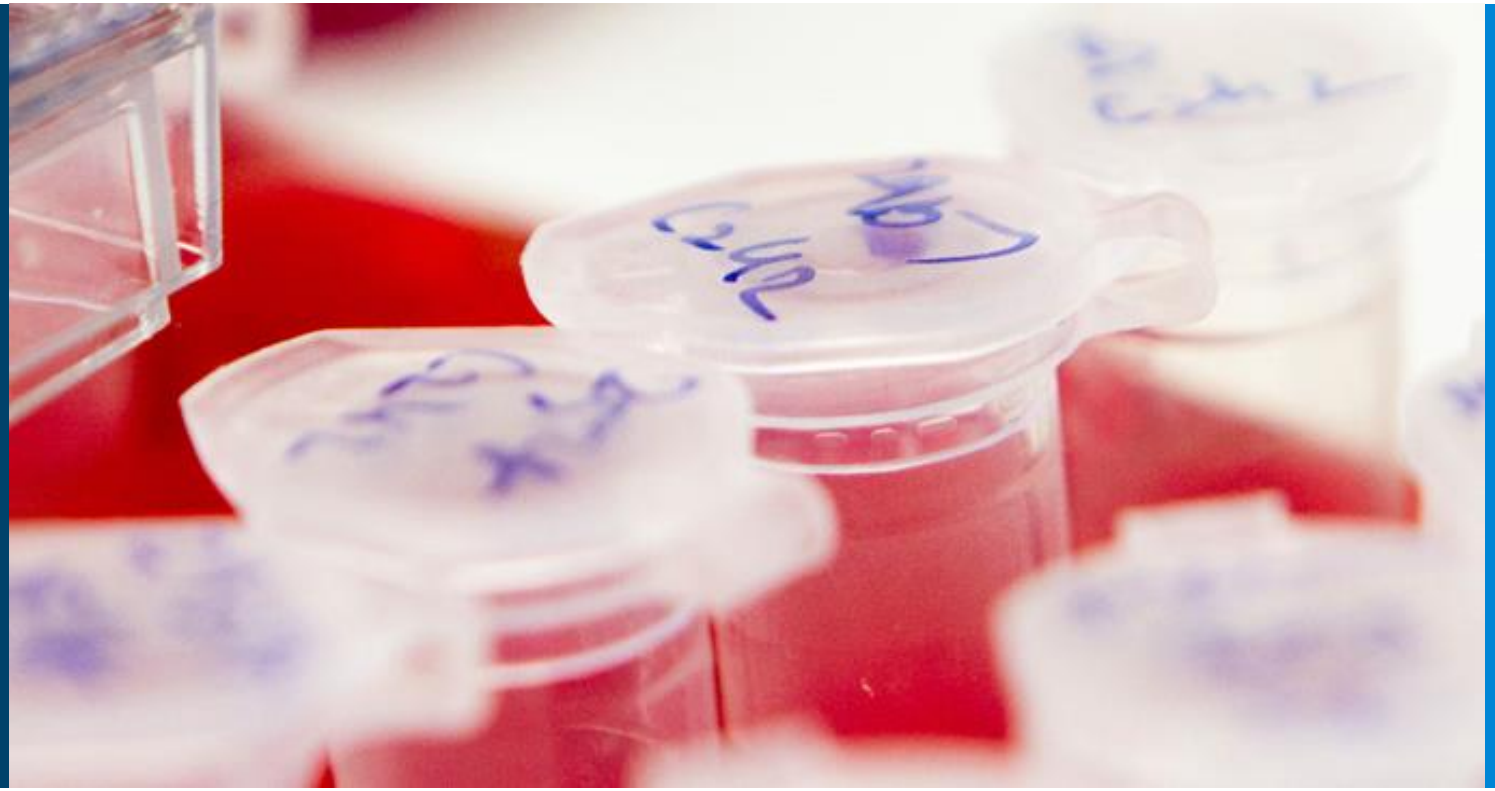
Imidazole pesticides



Fumonisin B2

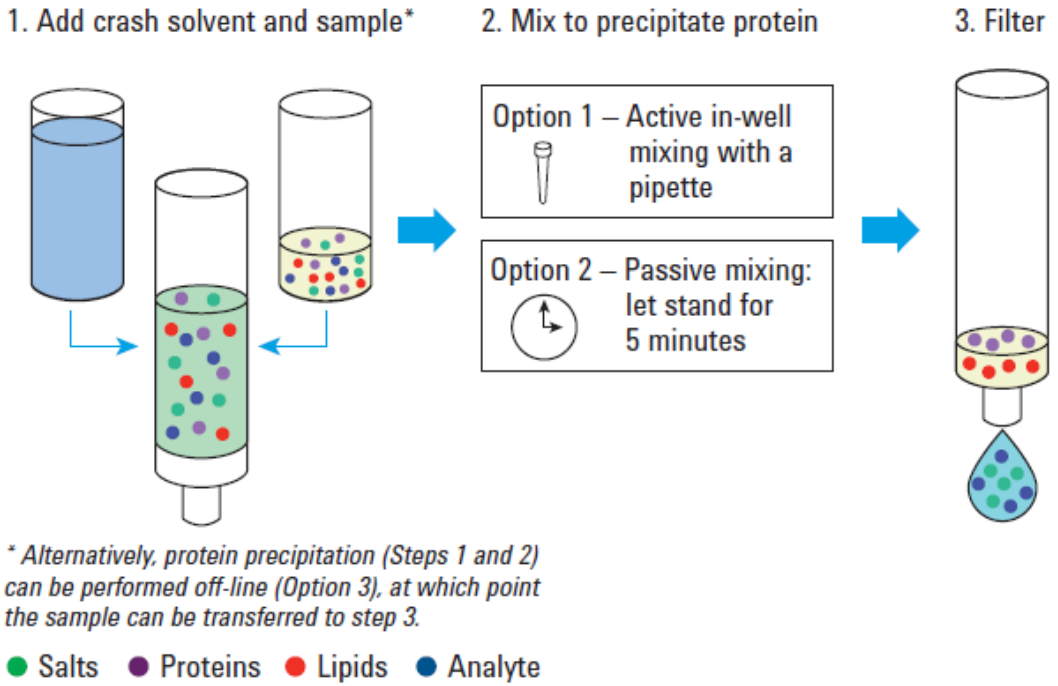
Biological Applications

96-Well Plates and 1 mL cartridges



Captiva EMR-Lipid General Protocol: Biologicals using 1 mL Cartridge

Operating Instructions for Agilent Captiva EMR-Lipid 1 mL Cartridge



Crash solvent or sample can be applied first (order does not matter)

Sample and crashing solvent should contain 20% water

Vacuum, positive pressure, or centrifugation can be used

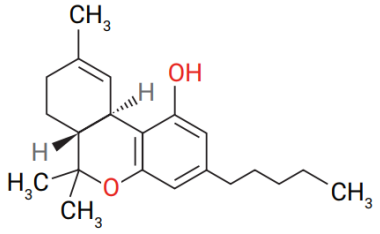
1 drop every 3-5 seconds

Extra elution step of 80:20 ACN:H_2O can improve recovery

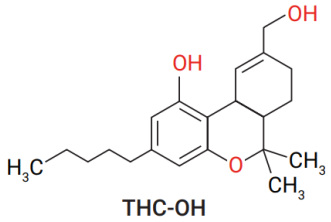
With biological applications, the sample is often highly aqueous already (plasma) so would have to add organic

Captiva EMR-Lipid Method Guide 96 well-plate and 1 mL cartridge formats

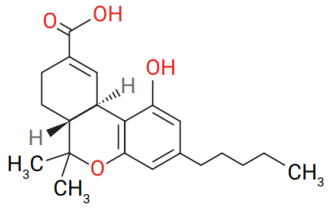
Efficient Quantitative Analysis of THC and Metabolites in Human Plasma Using Agilent Captiva EMR-Lipid and LC-MS/MS (5991-8636EN)



THC



THC-OH



THC-COOH

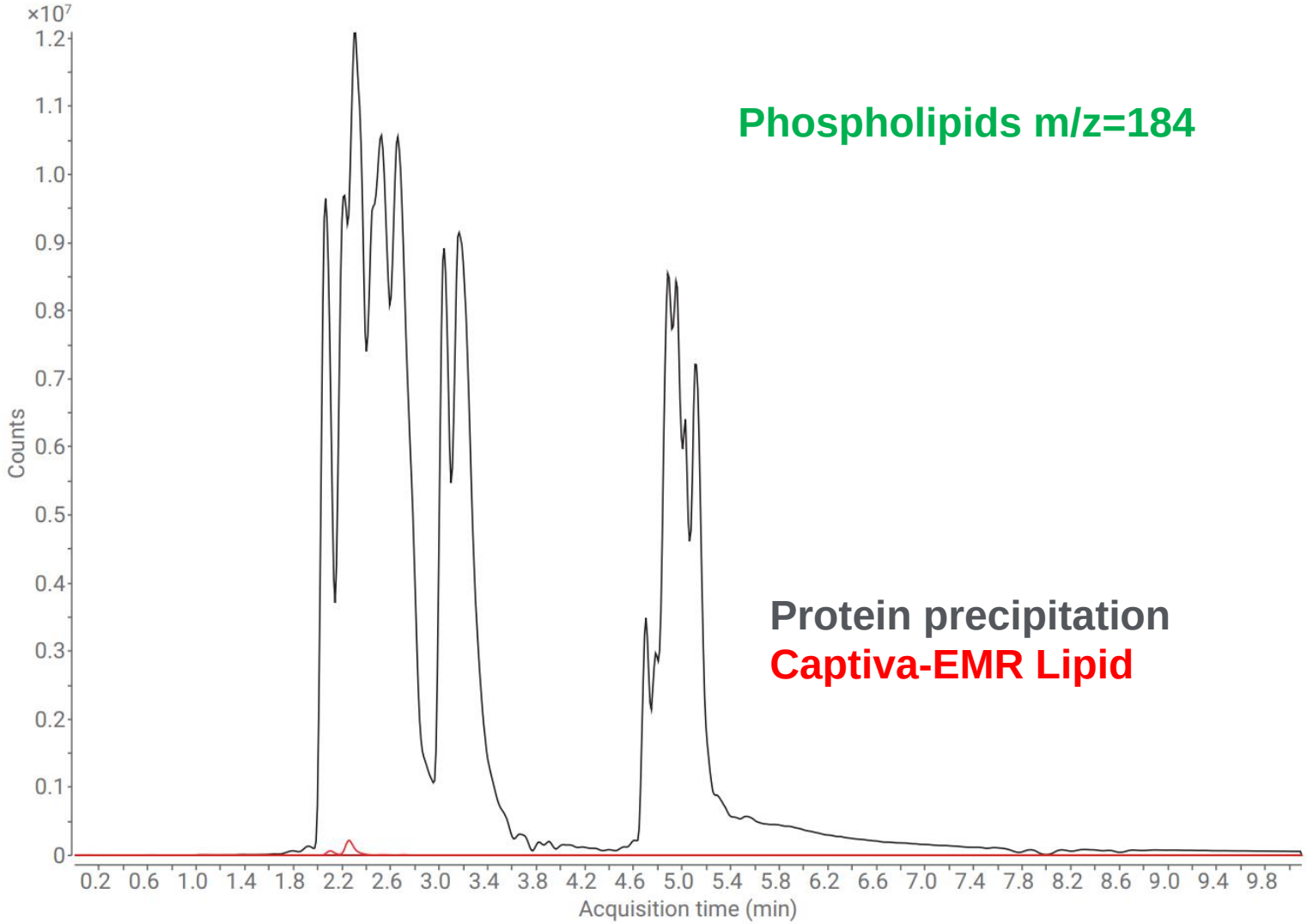
Sample Preparation Procedure

1. Add 500 µL of ACN (1 % FA) to an Agilent Captiva EMR—Lipid 1 mL cartridge.
2. Add 100 µL of human plasma.
3. Thoroughly mix, in-well.
4. Pull a vacuum of 1.5–3 psi.
5. Add 200 µL of 1:4 H_2O:ACN.
6. Pull the vacuum until the entire volume has passed through the cartridge, then increase the pressure to 11–13 psi to pull the remaining solvent through.
7. Evaporate under N_2 at 45 °C, then reconstitute in 100 µL MeOH (0.1 % FA).
8. Inject 5 µL + 10 µL of water for dilution directly into the LC system.

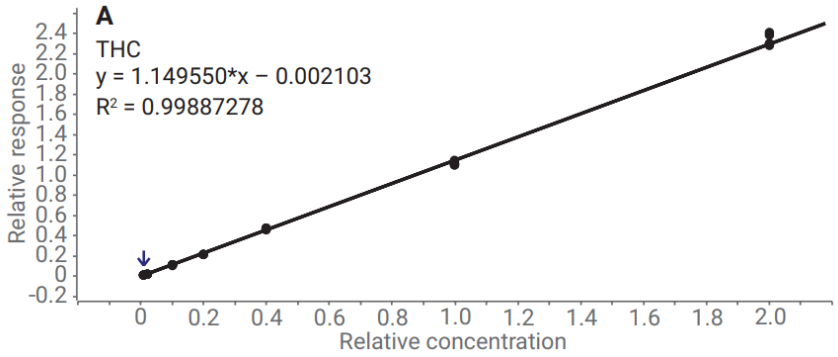
Products Used

- Agilent ZORBAX Rapid Resolution High Definition (RRHD) Bonus RP **(857768-901)**
- Agilent Captiva EMR-Lipid 1 mL cartridge **(5190-1002)**
- Agilent Vac Elut SPS 24 Vacuum Manifold with collection rack for 12 X 75 mm test tubes **(12234041)**

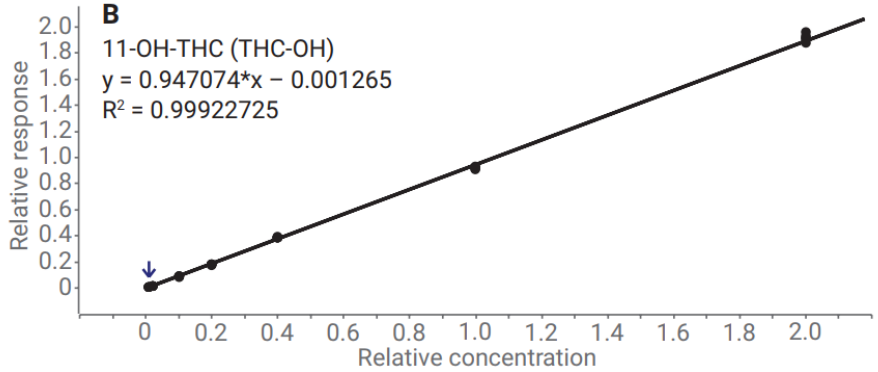
Effective Phospholipid Removal

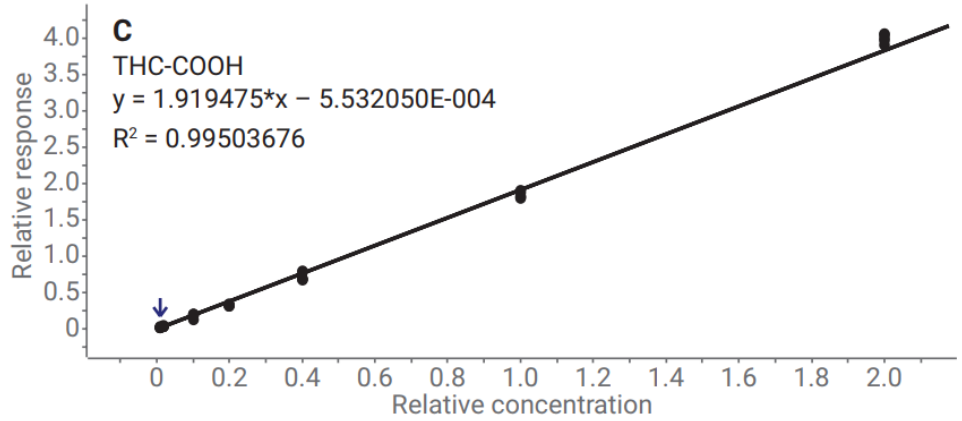


Great Recovery and Linearity using Captiva EMR Lipid



Compound	1 ng/mL		10 ng/mL		50 ng/mL	
	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
THC-OH	100	7.6	106	1.4	101	1.4
THC	107	1.2	105	3.2	104	3.2
THC-COOH	97	5.6	107	4.2	103	4.2

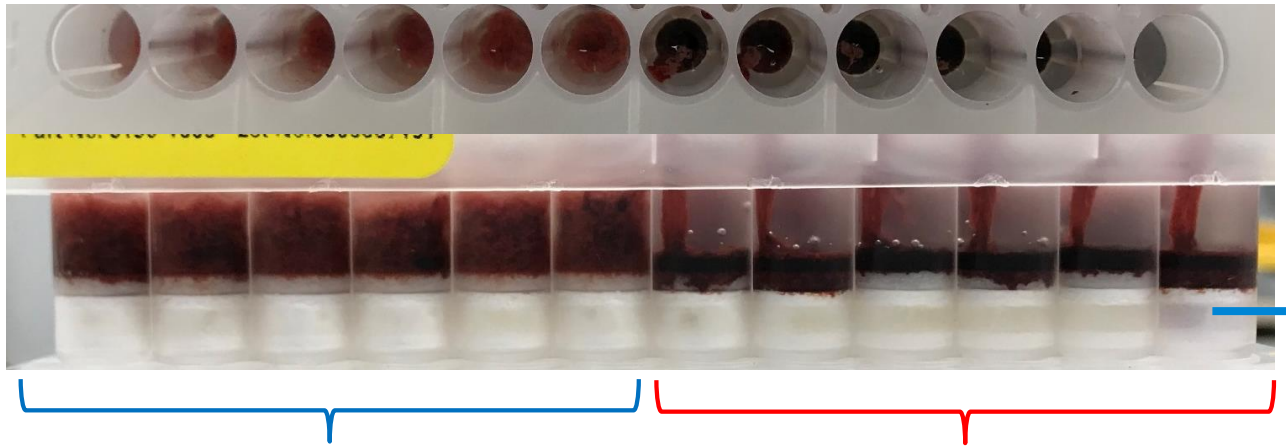




Captiva EMR-Lipid General Protocol: Biologicals using 96 Well-Plate

Operating Instructions for Agilent Captiva EMR-Lipid 96-Well Plate

Benefits of Adding Crashing Solvent to Sample for 96 Well Plate Captiva EMR-Lipid



Whole blood settled at the bottom, not being protein precipitated.

Adding crashing solvent into whole blood

Adding whole blood into crashing solvent

Sample First, then Solvent regardless of matrix

Benefits of Adding Solvent into Bio-fluids

- Provides a more complete/instant protein precipitation
  - Precipitation is homogenous without the need for mixing
- Elution is easier and consistent well-to-well
  - No clogging
- Sample can be added first to EMR-Lipid plate directly followed by addition of internal standard
  - No need for transfer and no extra collection plate needed
- More straightforward workflow with one less step transfer
  - Less collection plates, pipette tips needed = cost savings!
  - No need for active mixing with pipette tips reduces risk for cross contamination in 96-well plates

Multi-Residue Analysis of Abuse Drugs in Whole Blood Using In-well Protein Precipitation Followed with Captiva EMR-Lipid Cleanup by LC/MS/MS

Target Analytes

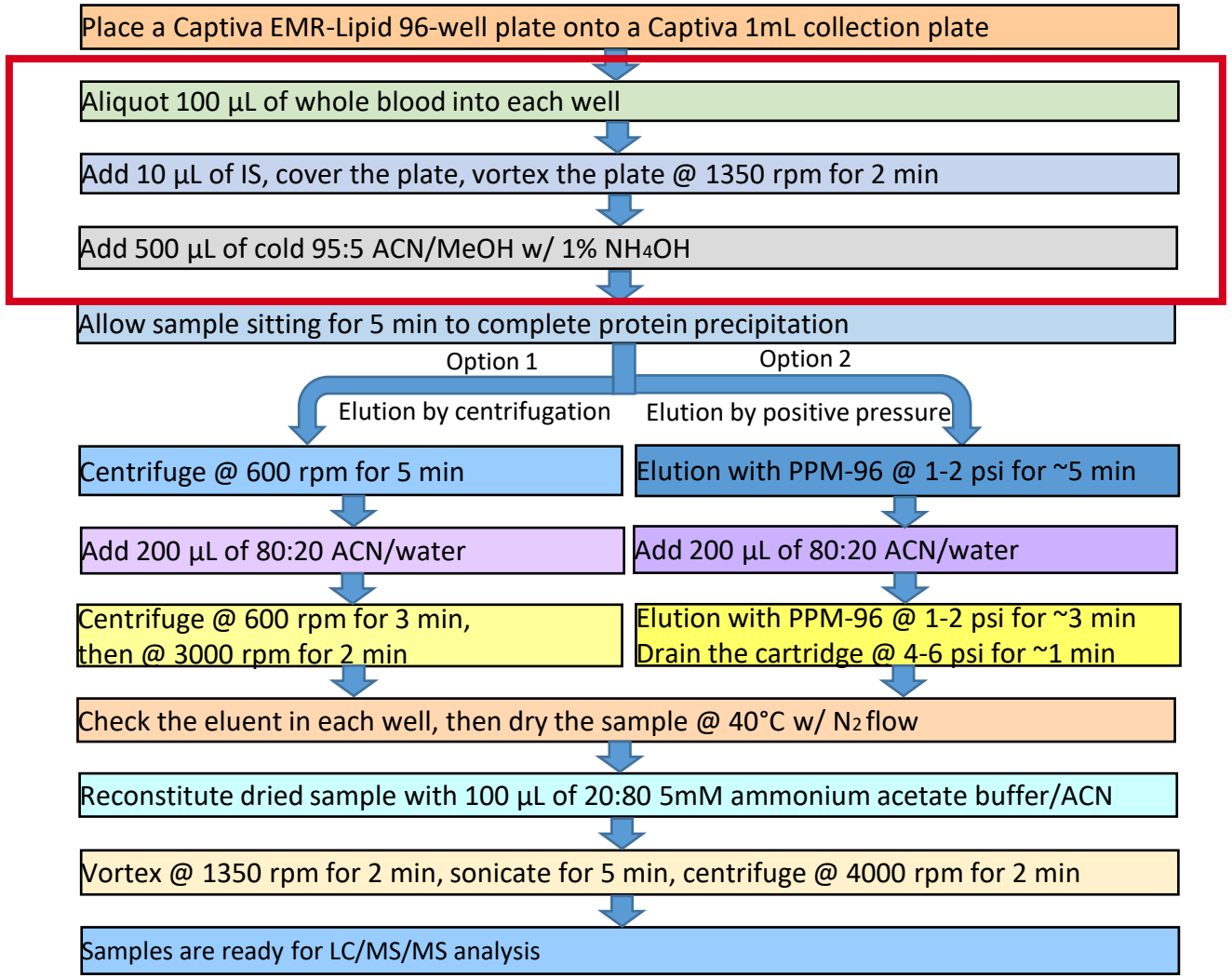
Representative and popular abuse drug compounds were selected from different classes.

Analyte	Chemical class	Analyte	Chemical class
Codeine	Opioid	Amphetamine	Monoamine alkaloid
Oxycodone		MDA	
Hydrocodone		Methamphetamine	
Heroin		MDMA	
Meperidine		Phentermine	
Methadone		MDEA	
Proadifen	--	Nitrazepam	Benzodiazepine
Trazodone	Triazolopyridine	Oxazepam	
PCP	Arylcyclohexylamine	Lorazepam	
Verapamil	Phenylalkylamine	Alprazolam	
Strychnine	Alkaloid	Temazepam	
Cocaine		Diazepam	

Products Used

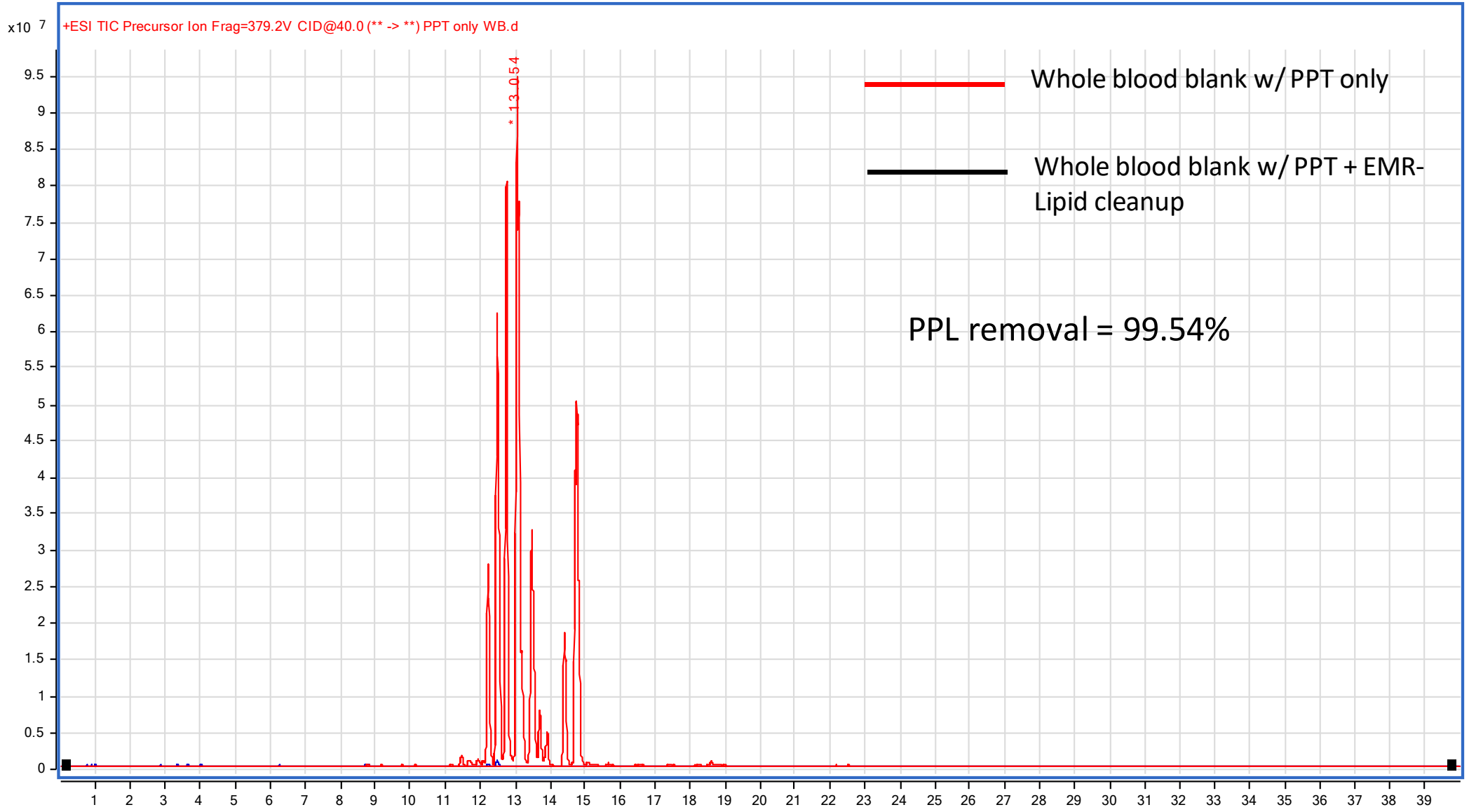
- Agilent Infinity Lab Poroshell 120 EC-C18 **(695775-906)**
- Agilent Captiva EMR-Lipid 96-well plate (**5190-1000); (5190-1001** [5/pk])
- Captiva Collection plate **(A696001000)**
- Agilent Captiva 96-well plate cover 10/pk **(A8961007)**
- Agilent Positive Pressure Manifold **(5191-4116)**

Full Procedure of 96-Well Plates from Start to Finish

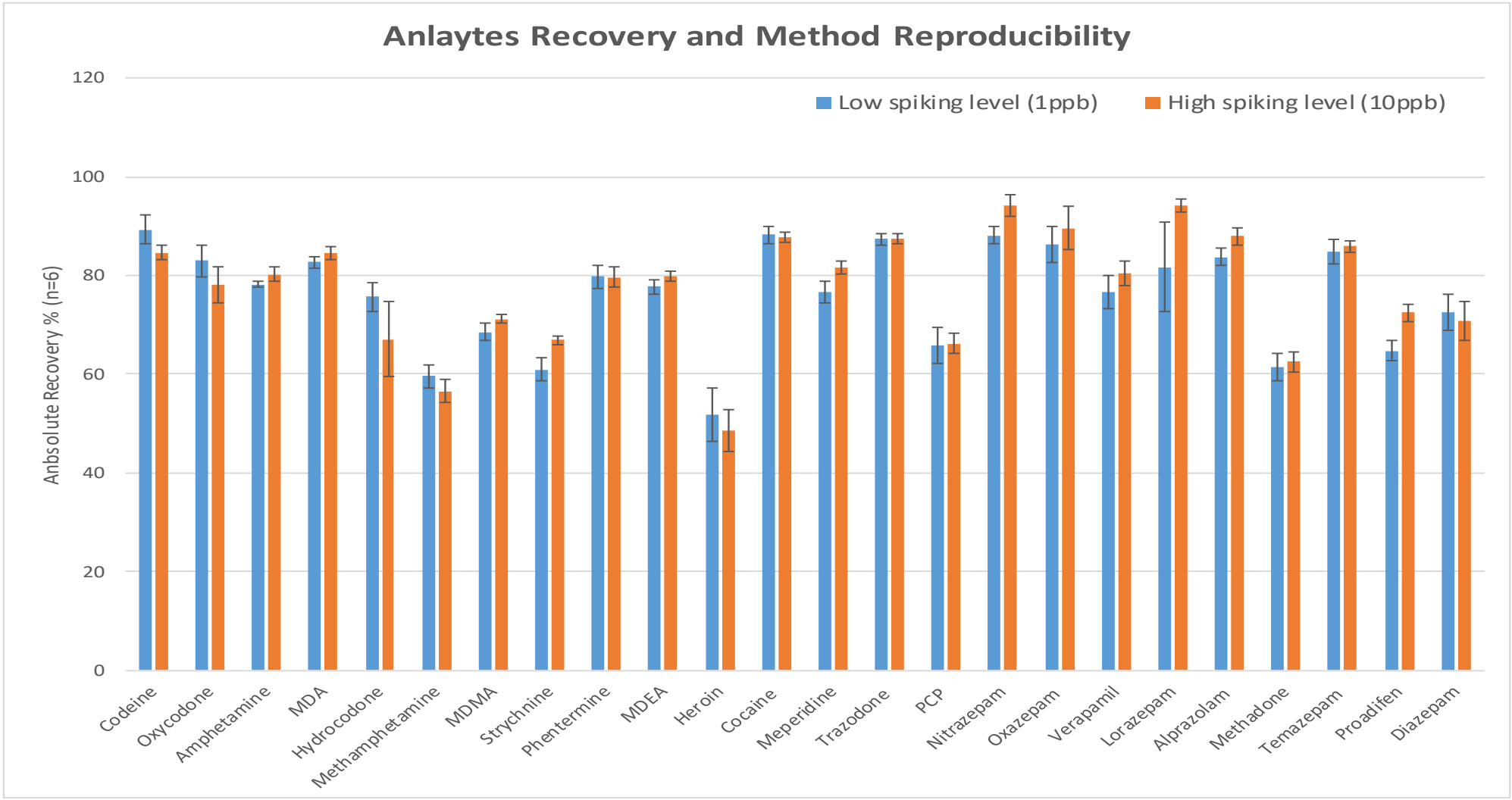


Sample first, solvent second

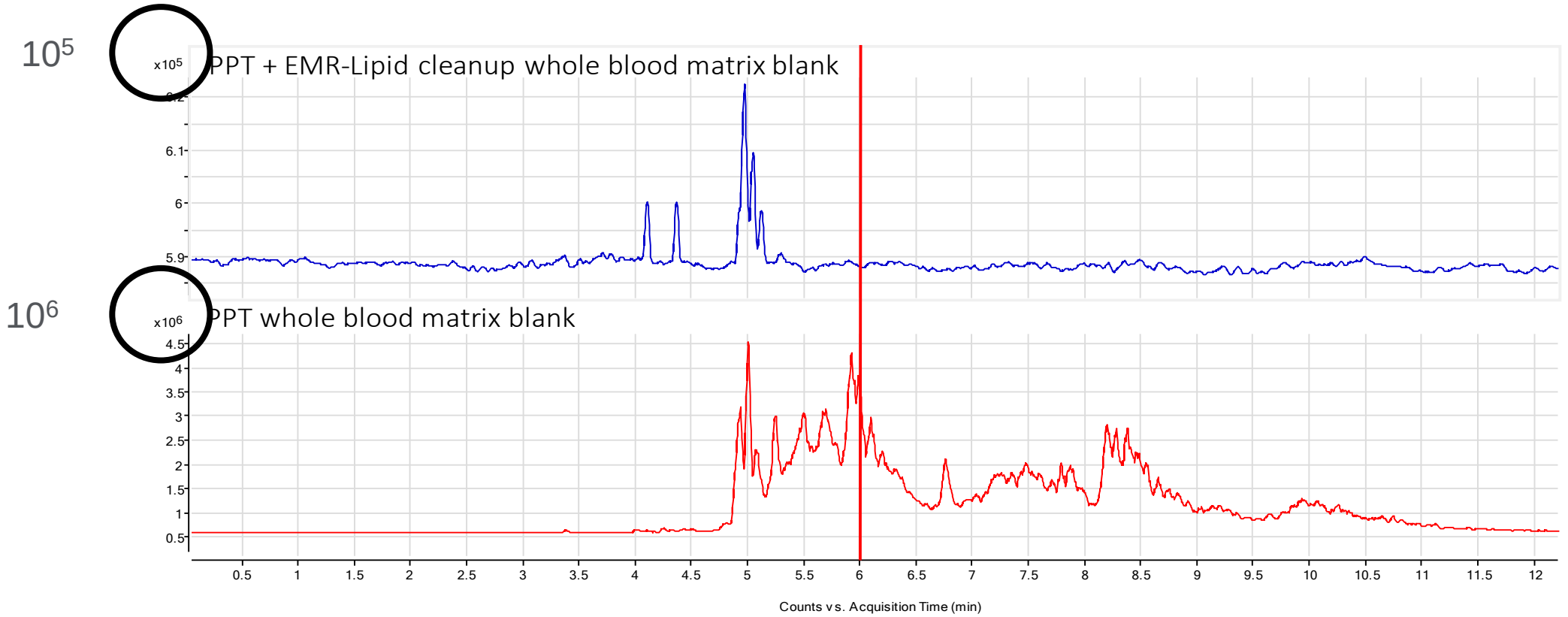
Effective Phospholipids Removal



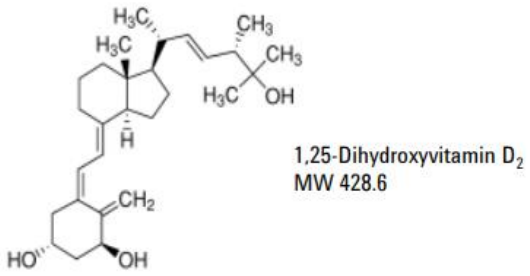
Acceptable Analyte Recovery and Superior Reproducibility



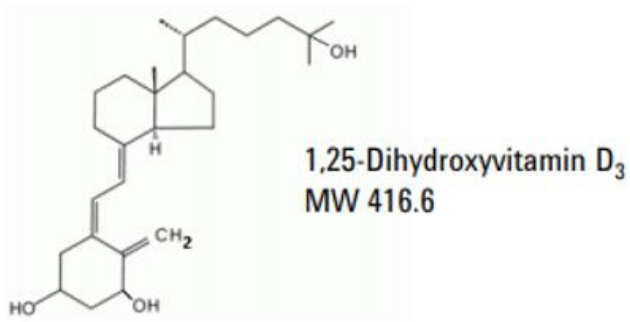
Reduced Column Contamination Allows Shorter Cycle Time



Vitamin D Metabolite Analysis in Biological Samples (5991-7956EN)



1,25-Dihydroxyvitamin D_2
MW 428.6



1,25-Dihydroxyvitamin D_3
MW 416.6

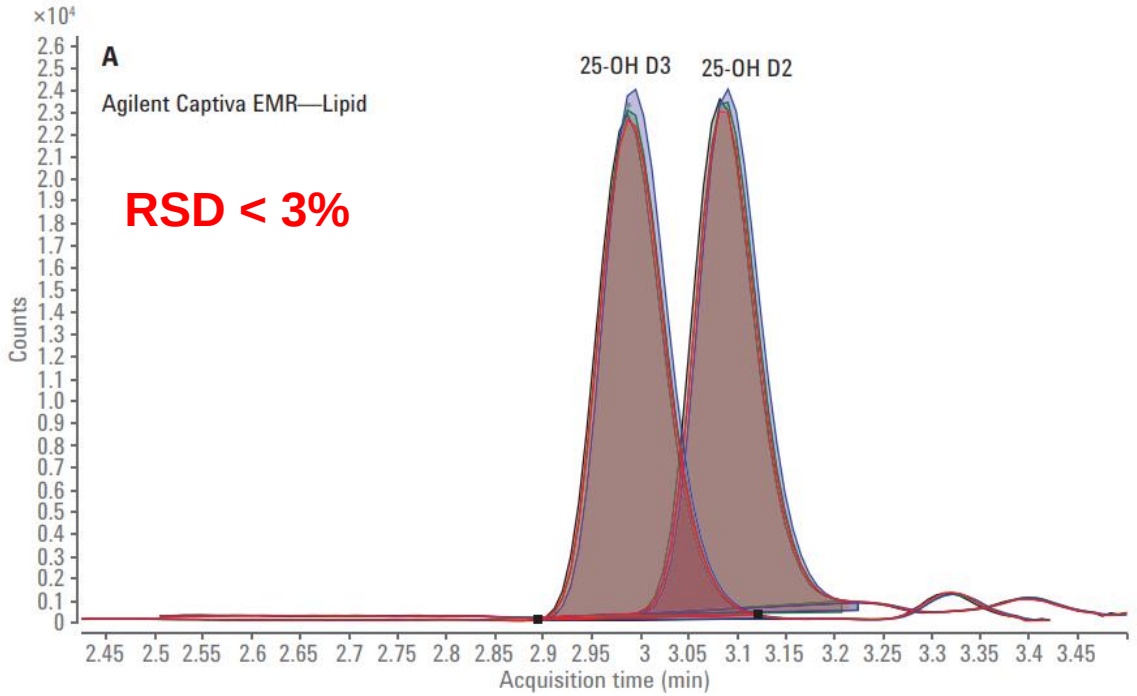
	25-OH D2		25-OH D3	
	Absolute recovery (%)	%RSD	Absolute recovery (%)	%RSD
10 ng/mL	106.4	4.8	106.3	10.2
20 ng/mL	88.8	9.5	94.2	9.2
30 ng/mL	90.8	5.0	94.7	6.3
250 ng/mL	96.9	14.6	96.6	4.2
500 ng/mL	97.1	11.5	100.1	9.5
750 ng/mL	96.9	6.4	100.3	5.0

Part Numbers

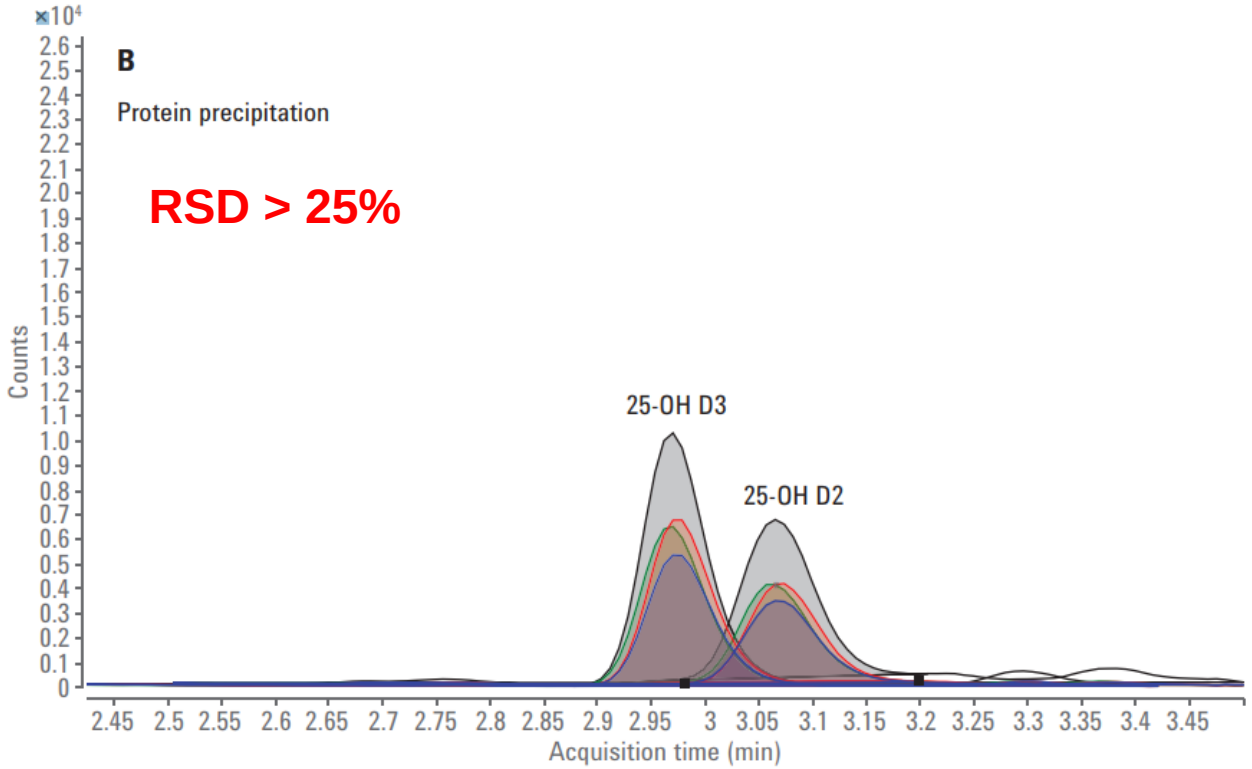
- Agilent Infinity Lab Poroshell 120 EC-C18 **(699775-902)**
- Agilent InfinityLab Poroshell 120 EC-C18 guard column **(821725-911)**
- Agilent Captiva EMR-Lipid 96-well plate **(5190-1000); (5190-1001** [5/pk])
- Captiva 96-deep well collection plate, 1 mL **(A696001000)**
- Agilent Captiva 96-well plate cover 10/pk **(A8961007)**

Protein Precipitation vs. Captiva EMR-Lipid RSD and Peak Area

Captiva EMR-Lipid



Protein Precipitation



Lipids cause reproducibility problems resulting in high RSD values

Using Captiva EMR-Lipid → low RSD values and higher peak areas

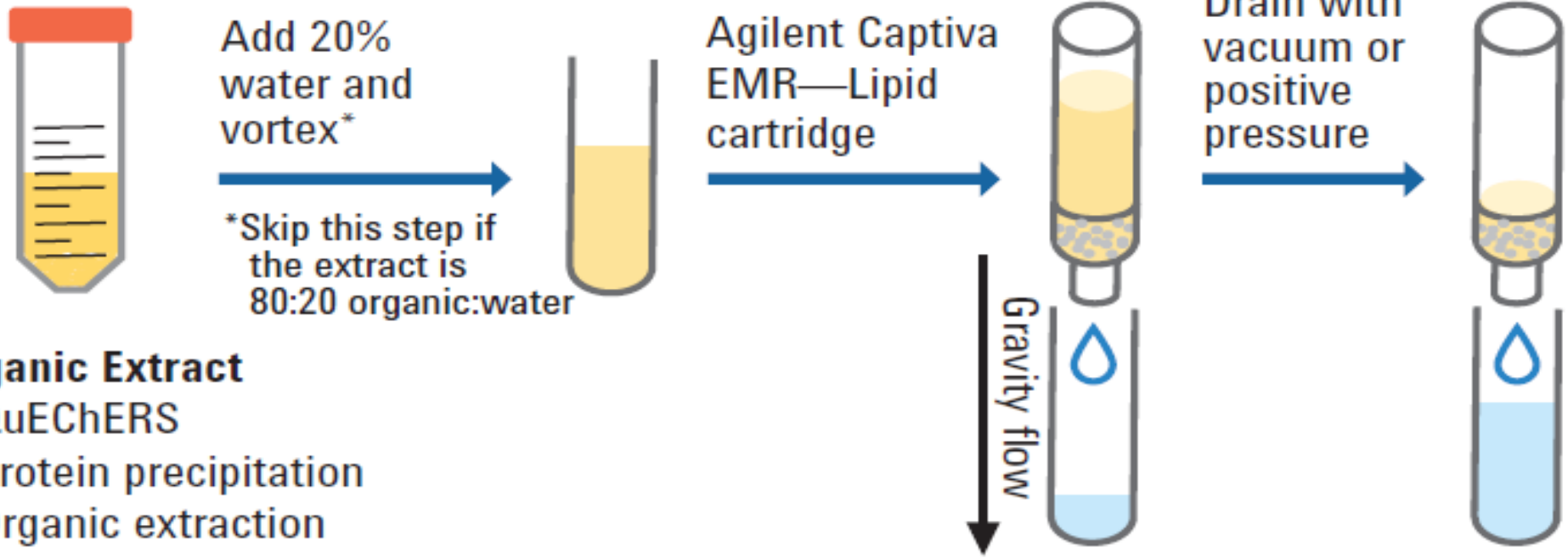
Higher peak area due to less ion suppression → can lead to lower detection limits

FOOD APPLICATIONS

3 and 6 mL Captiva EMR-Lipid Cartridges

Let's Take a look at the Captiva EMR-Lipid Procedure: Food and Food Products

Operating instructions and tips for Agilent Captiva EMR—Lipid 3 and 6 mL Cartridge Products



Organic Extract

- QuEChERS
- Protein precipitation
- Organic extraction

ACN preferred

Captiva 3 and 6 mL Cartridge Features

- No retention frit in 3/6 mL cartridges-slow sample flow achieved by gravity
  - Followed by vacuum or positive pressure to recover all sample
  - Simple pass through/clean-up
- Contain larger sorbent amounts for larger food samples
- Food applications: extracting in 100% organic
  - Need to add water to achieve 20% water before applying
- Additional elution with 80:20 ACN:H_2O can help improve recoveries
- Captiva EMR Lipid 3/6 mL Cartridge Method Guide

Five Meat Matrices

	Beef	Pork	Bovine liver	Bovine kidney	Chicken liver
Matrix major components					
Fat	2-30%	1-47%	5-7%	6-7%	6-7%
Proteins	17-22%	11-23%	29%	17%	26%
Water	55-75%	41-75%	59%	75%	75%

Multiclass Multiresidue Veterinary Drug Analysis in Beef Using Agilent Captiva EMR-Lipid Cleanup and LC/MS/MS (5991-8598EN)

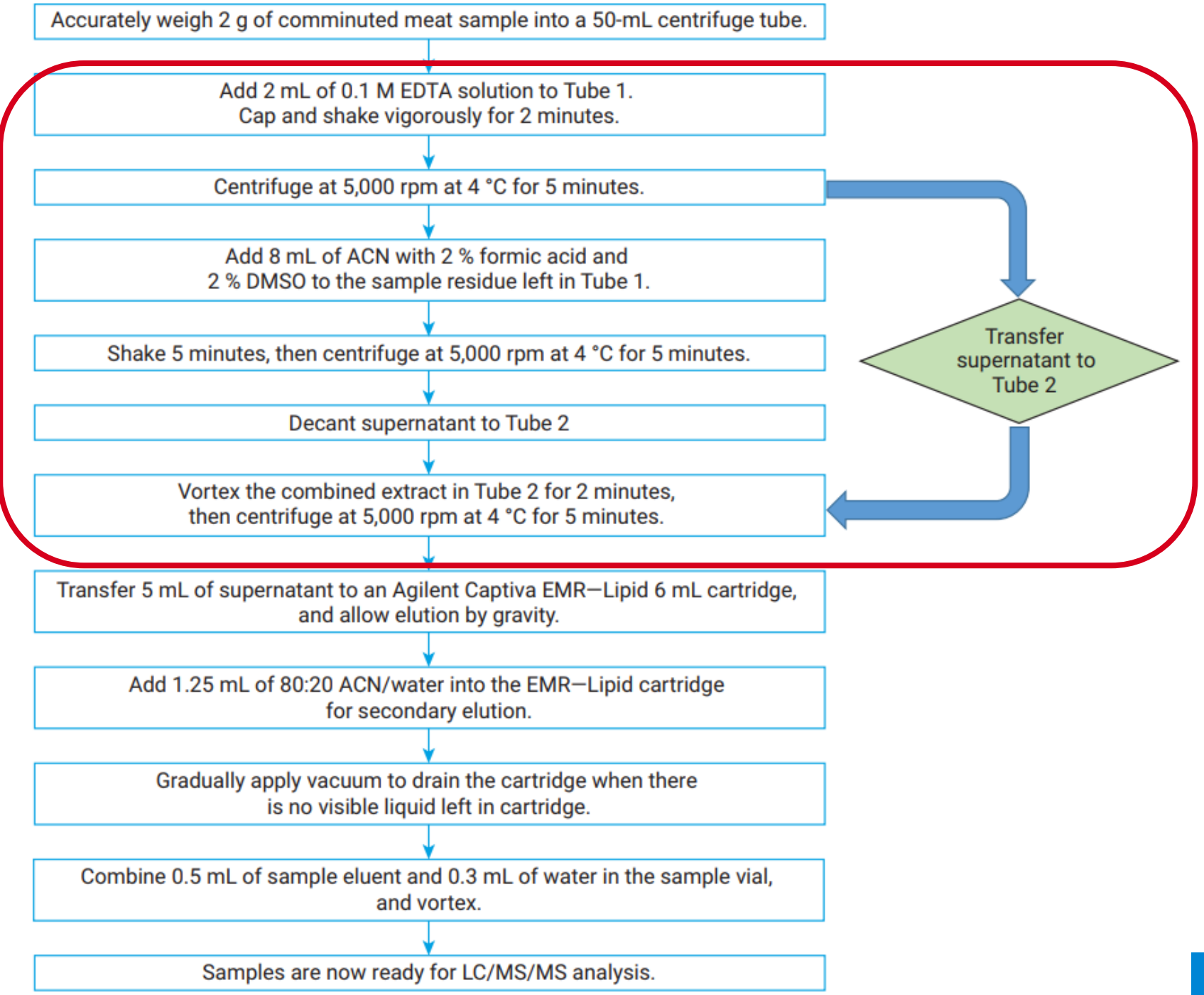
Representative Vet Drug	Drug Class	Representative Vet Drug	Drug Class	Representative Vet Drug	Drug Class
Amoxicillin	β-Lactam	Prednisone	Corticosteroid	Gamithromycin	Macrolide
Nafcillin		Oxyphenylbutazone	NSAID	Tylosin	
Ampicillin		2-Thiouracil	Thyreostat	Oxytetracline	Tetracycline
Penicillin V		Metronidazole-OH	Nitroimidazole	Doxycycline	
Cloxacillin		Fenbendazole	Anthelmintic	Tetracycline	
Oxacillin		Lavamisole		Minocycline	
Difloxacin		Morantel		Demeclocycline	
Sulfamethizole	Sulfonamide	Bithionol	Flukicide	Chlortetracycline	
Sulfamethoxypridazine		Clorsulon		Acepromazine	Tranquilizer
Ciprofloxacin	Fluoroquinolone	Niclosamide		Chlorpromazine	
Norfloxacin		Florfenicol	Phenicol	Ketoprofen	
Danofloxacin		Chloramphenicol		Cefazolin	Cephalosporin
Ractopamine	β-Agonist	Lincomycin	Lincosamide	Melengesterol acetate	other

Representative drugs were selected based on

- Popular and challenge classes: 17 different classes
- Polar to non-polar compounds: Log P range of -1.5 to 5.51;
- Acidic, neutral to basic compounds: pKa range of 2.3 to >12.

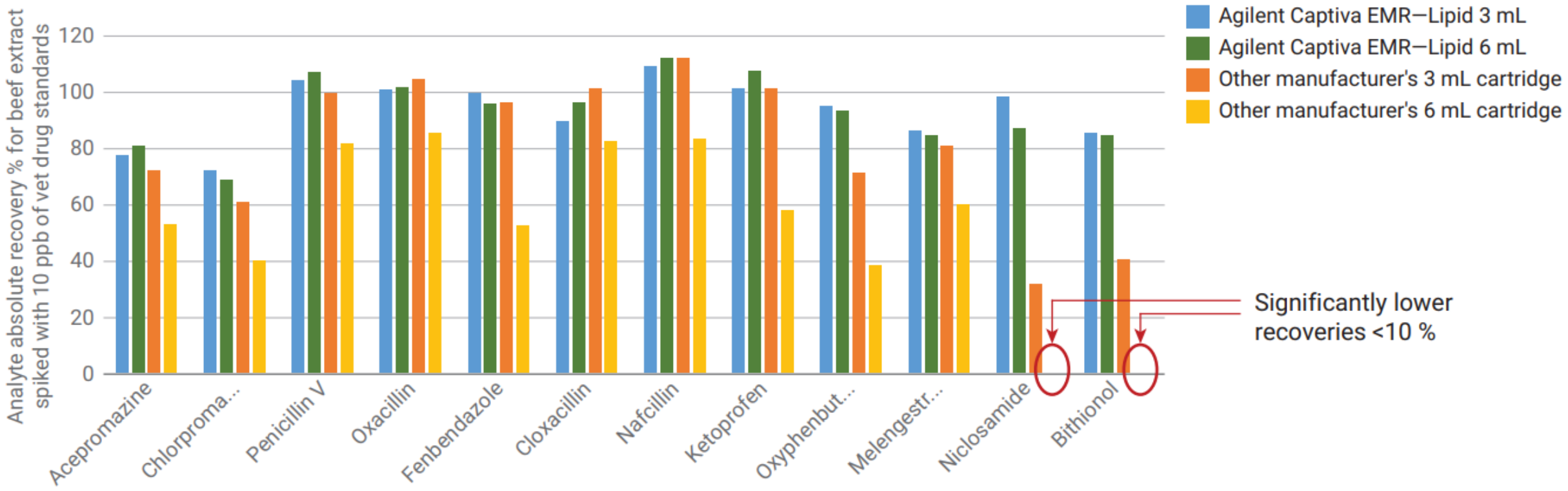
Part Numbers

- Agilent Infinity Lab Poroshell 120 EC-C18 **(693775-902)**
- Agilent InfinityLab Poroshell 120 EC-C18 guard column **(821725-911)**
- Agilent Captiva EMR-Lipid 3 mL/300 mg **(5190-1003)** and 6mL/600 mg **(5190-1004)**
- Agilent Vac Elut SPS 24 Vacuum Manifold for 16X100 mm test tubes **(12234004)**



Two-step extraction improves hydrophilic and hydrophobic analyte extraction. Improves protein precipitation efficiency. EDTA addition to prevent sensitive analytes loss due to chelation and binding with matrix

Analyte Recovery Comparison for Vet Drugs in Beef



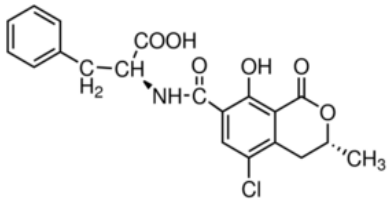
Competitor lipid removal products in most case uses a hydrophobic retention mechanism which leads to lower recoveries especially for hydrophobic compounds

Penicillin V log P = 1.88

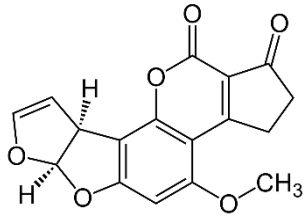
Bithionol log P = 5.51

Multiclass Mycotoxin Analysis in Cheese Using Agilent Captiva EMR-Lipid Cleanup and LC/MS/MS (5991-8694EN)

Compound	Log P
Aflatoxin M1	0.93
Aflatoxin G2	1.36
Aflatoxin G1	1.37
Aflatoxin B2	1.57
Aflatoxin B1	1.58
Fumonisin B1	-0.67
Ochratoxin B	4.01
Mycophenolic acid	3.53
Fumonisin B3	4.10
Zearalenone	3.83
Fumonisin B2	4.39
Ochratoxin A	4.31
Sterigmatocystin	1.62



Ochratoxin A
$C_{20}H_{18}ClNO_6$
403.81 g/mol
LogP ~ 4.31



Aflatoxin B1
$C_{17}H_{12}O_6$
312.28 g/mol
LogP ~ 1.6

Matrix major components	Blue Cheese	Parmesan Cheese
		
Fat	27%	28%
Proteins	21%	42%
Water	50%	26%

Part Numbers

- Agilent Infinity Lab Poroshell 120 EC-C18 **(693775-902)**
- Agilent InfinityLab Poroshell 120 EC-C18 guard column **(821725-911)**
- Agilent Captiva EMR-Lipid 6 mL/600 mg cartridge **(5190-1004)** and 3 mL/300 mg (**5190-1003)**
- Agilent Vac Elut SPS 24 Vacuum Manifold with collection rack for 16X00 mm test tubes **(12234004)**

Sample Preparation – Blue and Parmesan Cheese

QuEChERS Extraction

- Weigh 2 g sample into 50 mL centrifuge tube
- Add 10 mL water and soak for 5 min.
- Add 10 mL acetonitrile w/ 2% formic acid, then QuEChERS salts (MgSO4, NaCl).
- Mix on GenoGrinder for 10 min then centrifuge at 5000 rpm for 5 min.

Captiva EMR-Lipid Pass-through Cleanup

•Transfer 8 mL upper ACN extract into 15 mL tube and mix with 2 mL water (20% water by volume).

•Load 2.5 mL extract onto 3 mL Captiva-EMR tube (300 mg).

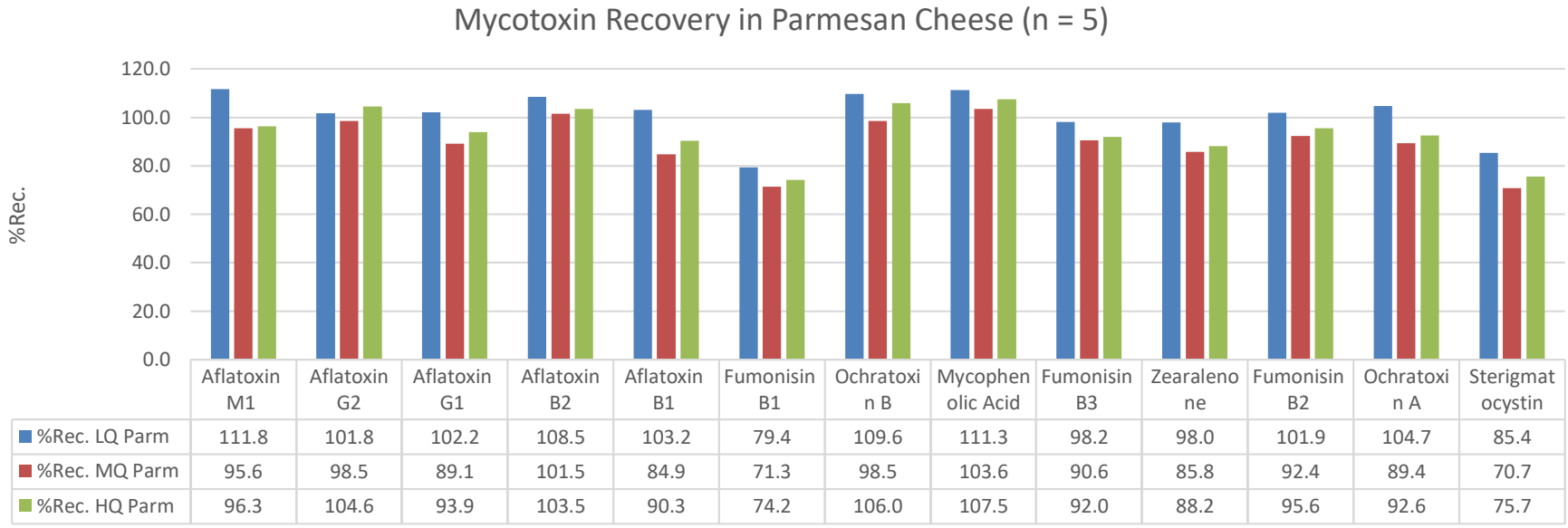
•Allow to elute with gravity over 10 min. then ramp up to ~10 in.Hg to dry Captiva bed.

Post Treatment

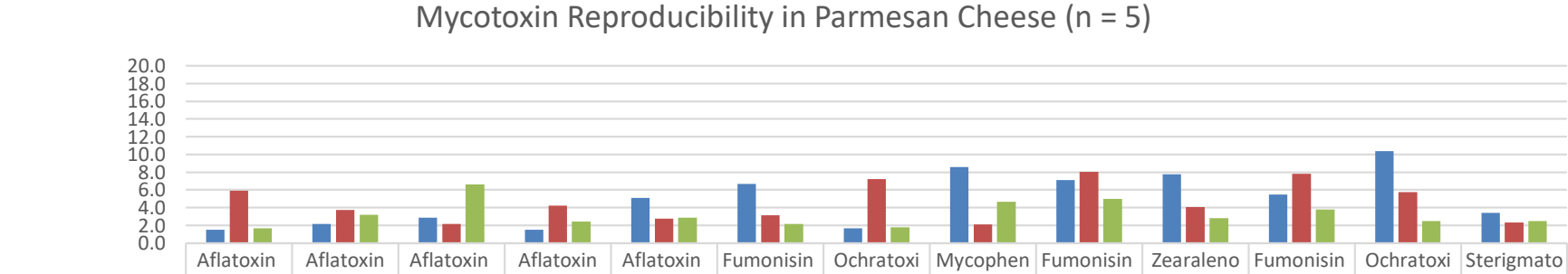
- Transfer 1.25 mL into a glass collection tube and evaporate to dryness on Turbo Vap.
- Reconstitute with 0.200 mL, 85:15 buffer*:ACN.

* 5 mM NH4 formate + 0.1% Formic acid

Validation Results – Parmesan Cheese



	Aflatoxin M1	Aflatoxin G2	Aflatoxin G1	Aflatoxin B2	Aflatoxin B1	Fumonisin B1	Ochratoxin B	Mycophenolic Acid	Fumonisin B3	Zearalenone	Fumonisin B2	Ochratoxin A	Sterigmatocystin
%Rec. LQ Parm	111.8	101.8	102.2	108.5	103.2	79.4	109.6	111.3	98.2	98.0	101.9	104.7	85.4
%Rec. MQ Parm	95.6	98.5	89.1	101.5	84.9	71.3	98.5	103.6	90.6	85.8	92.4	89.4	70.7
%Rec. HQ Parm	96.3	104.6	93.9	103.5	90.3	74.2	106.0	107.5	92.0	88.2	95.6	92.6	75.7



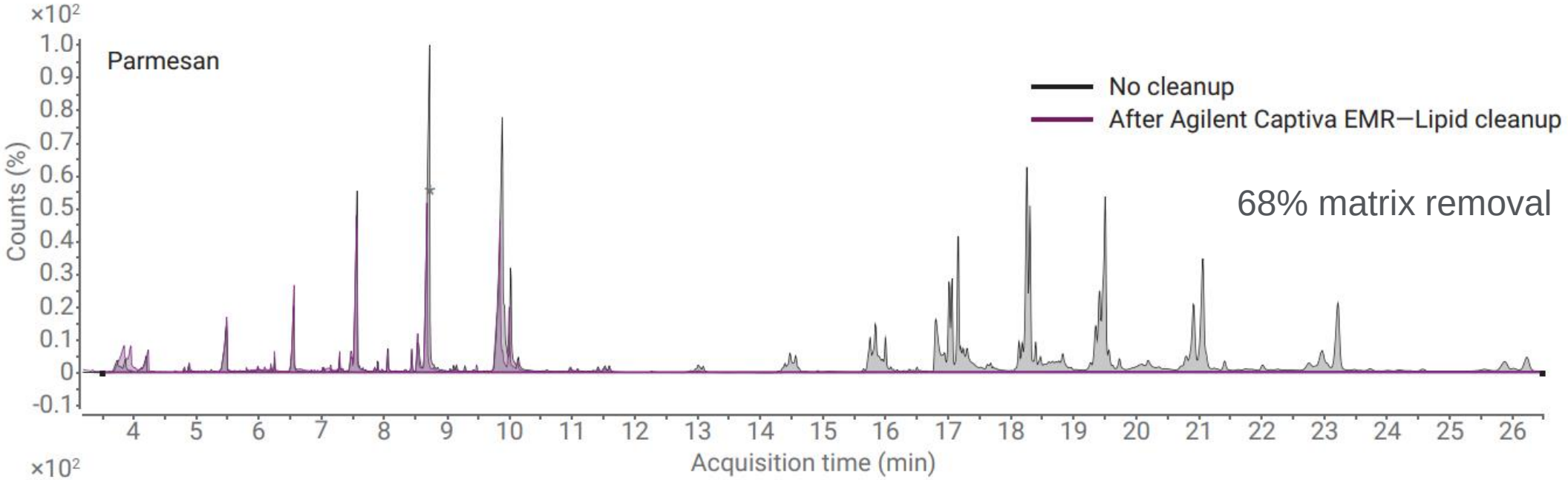
	Aflatoxin M1	Aflatoxin G2	Aflatoxin G1	Aflatoxin B2	Aflatoxin B1	Fumonisin B1	Ochratoxin B	Mycophenolic Acid	Fumonisin B3	Zearalenone	Fumonisin B2	Ochratoxin A	Sterigmatocystin
%RSD LQ Parm	1.5	2.2	2.8	1.5	5.1	6.7	1.7	8.6	7.1	7.8	5.5	10.4	3.4
%RSD MQ Parm	5.9	3.8	2.2	4.2	2.7	3.2	7.2	2.1	8.1	4.0	7.8	5.7	2.3
%RSD HQ Parm	1.7	3.2	6.6	2.4	2.9	2.2	1.8	4.6	5.0	2.8	3.8	2.5	2.5

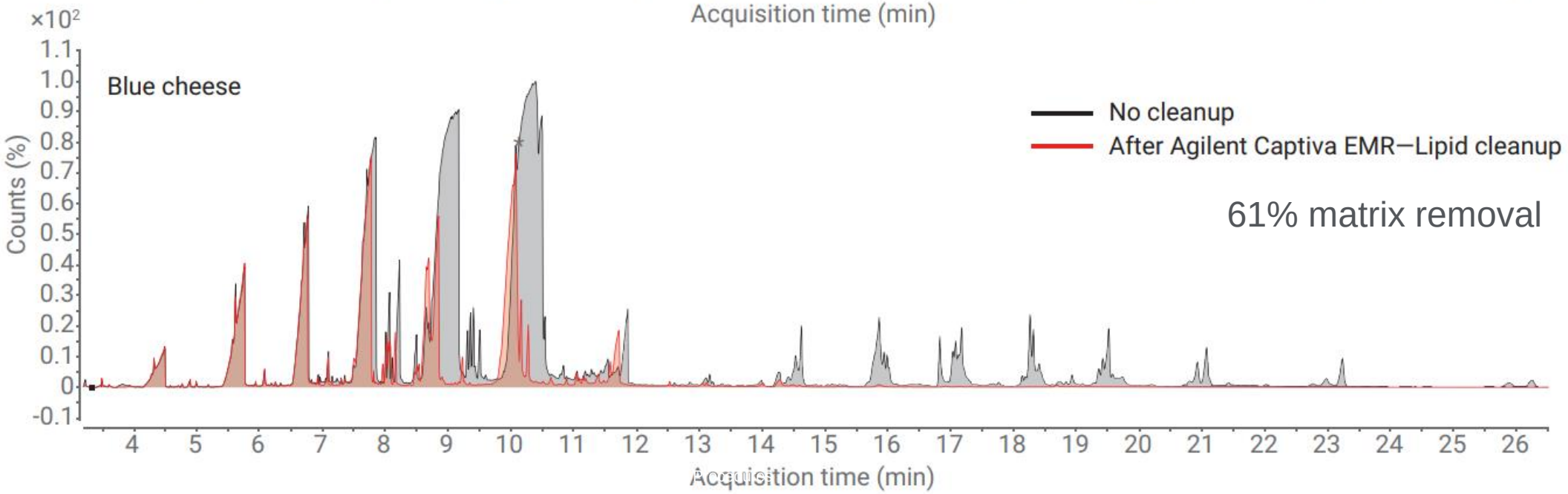
* Internal Standard = $^{13}C_{17}$ Aflatoxin B1

Quantitation result – competitive comparison

Recovery and precision comparison of Captiva EMR-Lipid and Competitor A pass-through cleanups (Parmesan cheese, 5 ng/mL, n = 4).				
	Captiva EMR-Lipid		Competitor A	
	%Rec.	%RSD	%Rec.	%RSD
Aflatoxin M1	96.1	3.6	93.5	4.4
Aflatoxin G2	100.9	0.5	89.5	4.4
Aflatoxin G1	102.4	1.6	86.1	4.8
Aflatoxin B2	100.8	3.2	84.2	4.7
Aflatoxin B1	98.4	4.0	85.3	5.5
Fumonisin B1	96.6	3.4	77.3	3.8
Ochratoxin B	104.9	6.4	76.7	7.5
Mycophenolic Acid	90.8	7.2	79.3	7.0
Fumonisin B3	103.1	11.6	76.8	11.5
Zearalenone	96.1	3.1	46.7	7.5
Fumonisin B2	85.0	6.9	85.1	9.6
Ochratoxin A	95.1	10.9	66.4	11.7
Sterigmatocystin	99.6	4.1	50.1	10.3

Matrix Removal evaluated by GC-MS full scan





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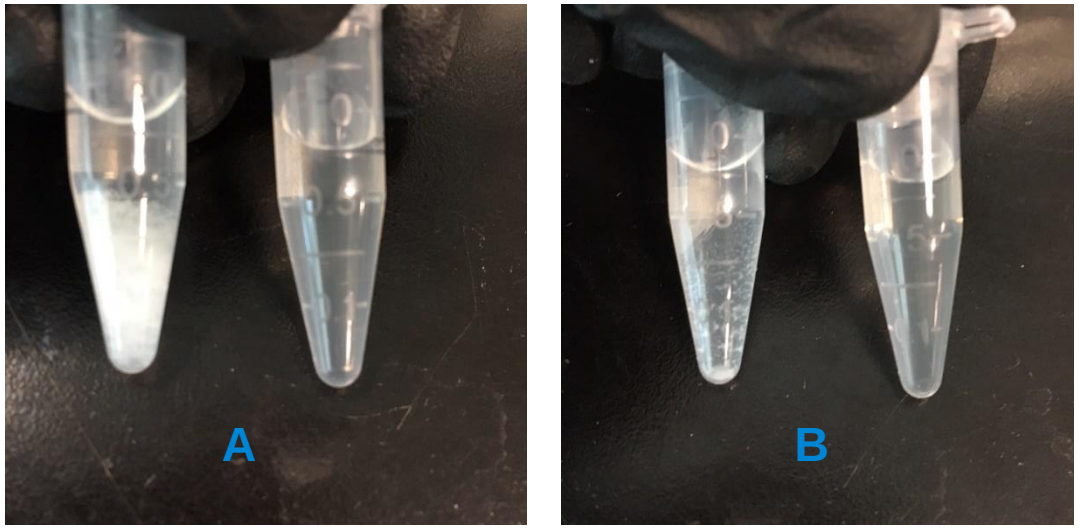
Lipid Freeze-out Comparison



blue cheese (A)
parmesan cheese (B)

A qualitative comparison placed untreated cheese samples and Captiva EMR-Lipid treated samples in a freezer at 0°C for 1 h and recorded precipitated lipid observations . As shown, untreated blue cheese contains a large amount of precipitated fats while parmesan shows a small amount clinging to the plastic vial. Captiva EMR-Lipid treated samples contained no observable fats after lipid freeze-out.

Conclusion

- Captiva EMR-Lipid is a simple and FAST pass through sorbent that removes classes of lipids from sample matrix
- Captiva EMR-Lipid does not interact with functional groups on compounds allowing to analyze for a wide variety of compound classes
- Wide variety of formats
  - 96-well plate and 1 mL cartridges for biological applications
  - 3 and 6 mL cartridges for food applications
- Provides better data and better analytical method robustness

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