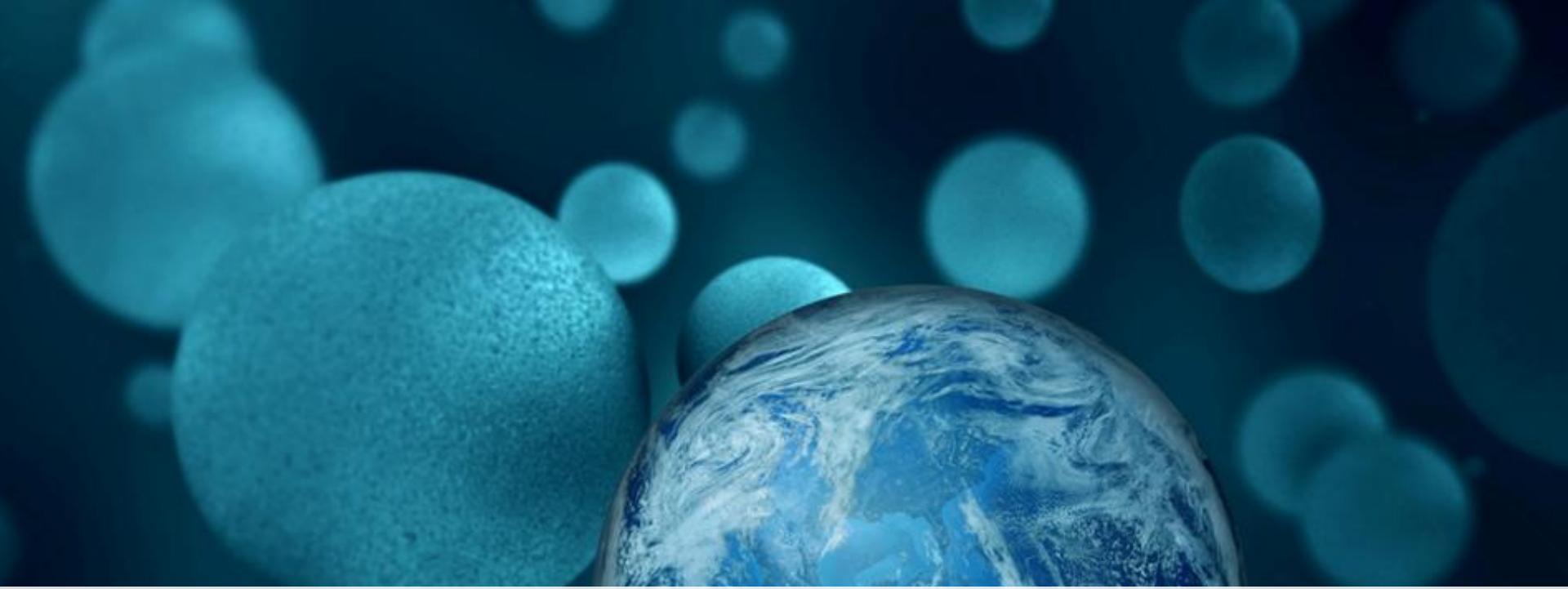

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HPLC Winter Webinars Part 2: Sample Preparation for HPLC

Jon Bardsley, Application Chemist
Thermo Fisher Scientific, Runcorn/UK

What am I Going to Talk About?

- **What do we mean by sample preparation?**
- **Why perform sample preparation?**
- **What are the options?**
- **A closer look at some types of sample preparation products**

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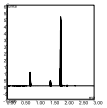
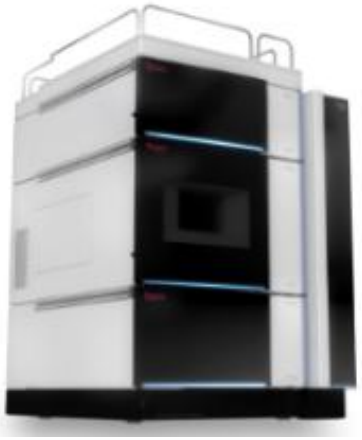
What do We Mean by Sample Preparation?

In short, any manipulation of your sample prior to further analysis.





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What do We Mean by Sample Preparation?

In short, any manipulation of your sample prior to further analysis.

There are many techniques used, each with their own benefits. Examples of widely used techniques are;

- Dilution
- Centrifugation
- Filtration
- Precipitation
- Liquid extraction
- Supported Liquid Extraction (SLE)
- Solid Phase Extraction (SPE)

- Immunoaffinity capture
- Protein digestion
- Derivatization
- Size exclusion
- Solvent switching
- Homogenising
- Accelerated Solvent Extraction (ASE)

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Why Perform Sample Preparation?

- **Compatibility to further analysis**
- **Simplify complex samples**
- **Remove interferences from the matrix**
- **Concentrate or dilute the sample**
- **Speed of analysis**
- **System Robustness**

Filtration

Sample filtration is a fast and cost effective way of removing particulates from your sample.

Wide variety of membrane material available.
(Cellulose, Glass microfiber, nylon, PES, PTFE, PVDF....)

Chemical compatibility between the filter membrane and your sample is key!

- Solvent resistance
- Binding of analyte to membrane
- Binding of analyte to housing

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Compatibility to Further Analysis

- Solid or semi-solid sample required for liquid chromatography
- Sample composition incompatible for chromatography (immiscible liquids)
- Sample contains non-volatile buffer for MS analysis or damaging reagents

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Compatibility to Further Analysis

Solid samples such as food or soil samples require extraction before analysis (LC, GC..).

Typically this is performed by homogenising the sample followed by solvent extraction.

This is simple but time consuming and can result in a very complex extract.

As well as extracting your compounds of interest, you may extract lots of matrix components that will interfere with your downstream analysis.

This extract may be analysed as it is, but its a good idea to further clean the extract with another technique such as SPE or QuEChERS

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Simplify Complex Samples

(Or remove the analyte from the interferences)

Isolating a group of compounds for analysis based on a common property (such as an ionic species) simplifies the sample and separation prior to detection. This greatly improves accuracy and precision, along with increased system robustness!

Solid phase extraction (SPE)

Liquid / Liquid extraction

Other properties can be used, such as hydrophobicity, to separate out the sample components.

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Reduce Interferences from the Matrix

(Or remove the interference from the analyte)

Rather than target the analytes, this approach targets and removes the interferences present in the matrix. Typically this is done by pass-though SPE, or dispersive SPE.

μElution SPE

QuEChERS

This approach is very common in the analysis of solid samples, particularly in pesticide analysis from food stuff.

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QuEChERS

Quick
Easy
Cheap
Effective
Rugged
Safe

QuEChERS is a multi-step, manual process of extracting analytes from solid samples such as food or soil.

1. Samples are homogenized and solvent extracted, usually by acetonitrile. Salts, acids, and buffers may be added to enhance extraction efficiency.

2. The extract cleaned further, normally by addition $MgSO_4$ to remove excess water, and dispersive SPE (dSPE) material to remove matrix components such as pigments and lipids.

3. Sample extract can be pH adjusted or solvent-exchanged for analysis.

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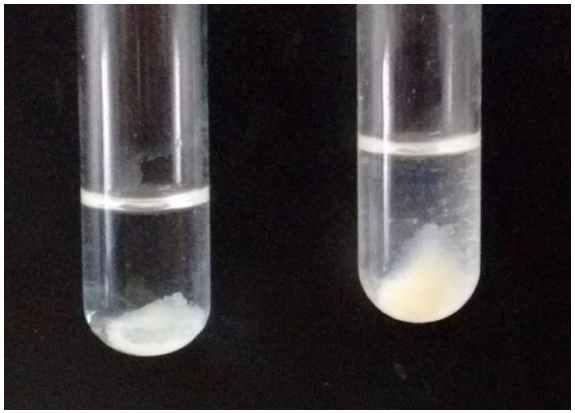
Protein Precipitation (PPT)

Protein precipitation is a popular method for the analysis of biological samples, especially in small molecule analysis.

The bulk proteins in the sample are precipitated and removed, typically by addition of organic solvent, and the supernatant analysed.



Protein precipitation

Protein precipitation plate

This process is cheap, but manual and slow.
It can be automated by use of a PPT filter plate.

Extract is NOT very clean and can still contain many matrix interferences.

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Liquid / Liquid Extraction (L/L or SLE)

Liquid / Liquid extraction is a technique for extraction of samples based partitioning of compounds between a polar and non-polar solvent.
Excellent for clean up of biological samples and removes more matrix components than protein precipitation

Liquid / Liquid extraction

Very manual and time consuming;
Can be automated with SLE cartridges or plates

Supported liquid extraction (SLE)

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Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) is a targeted extraction technique for isolation of a compound(s) from complex matrices e.g. biological samples.

Separation is achieved by the affinity of the compound(s) of interest for the active components of the stationary phase.

This allows removal of the compound(s) of interest from the matrix for subsequent analysis.

Typical formats are SPE tubes or 96 well plates.

Silica beads or polymeric material is bonded with specific functional groups to create the stationary phase.

Many different chemistries are available such as reverse phase, ion-exchange, HILIC.....

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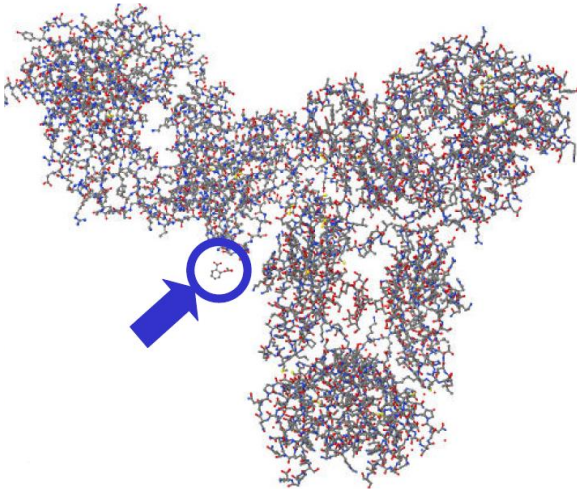
Solid Phase Extraction (SPE)

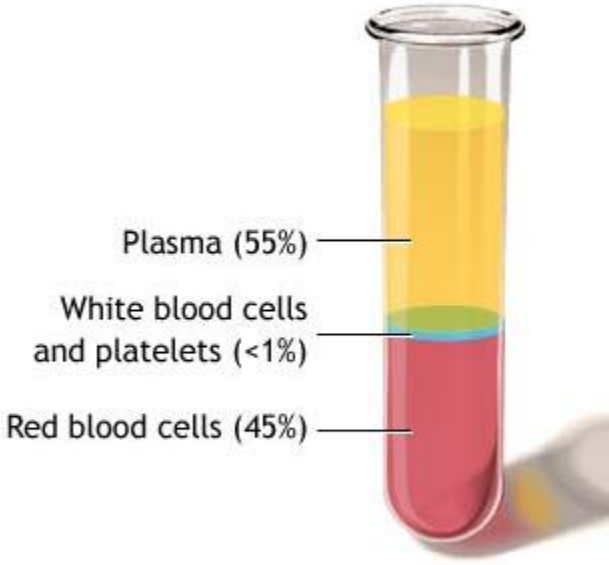


Isolation of specific compounds from very complex samples, such as a biological sample like plasma, can be challenging.

Even within a animal species, there can be huge variance sample-to-sample and so methods need to be very specific.

Poor extraction methods can cause serious problems with chromatography, detection and robustness of the instruments.

SPE can provide very clean extracts which prevents such issues.



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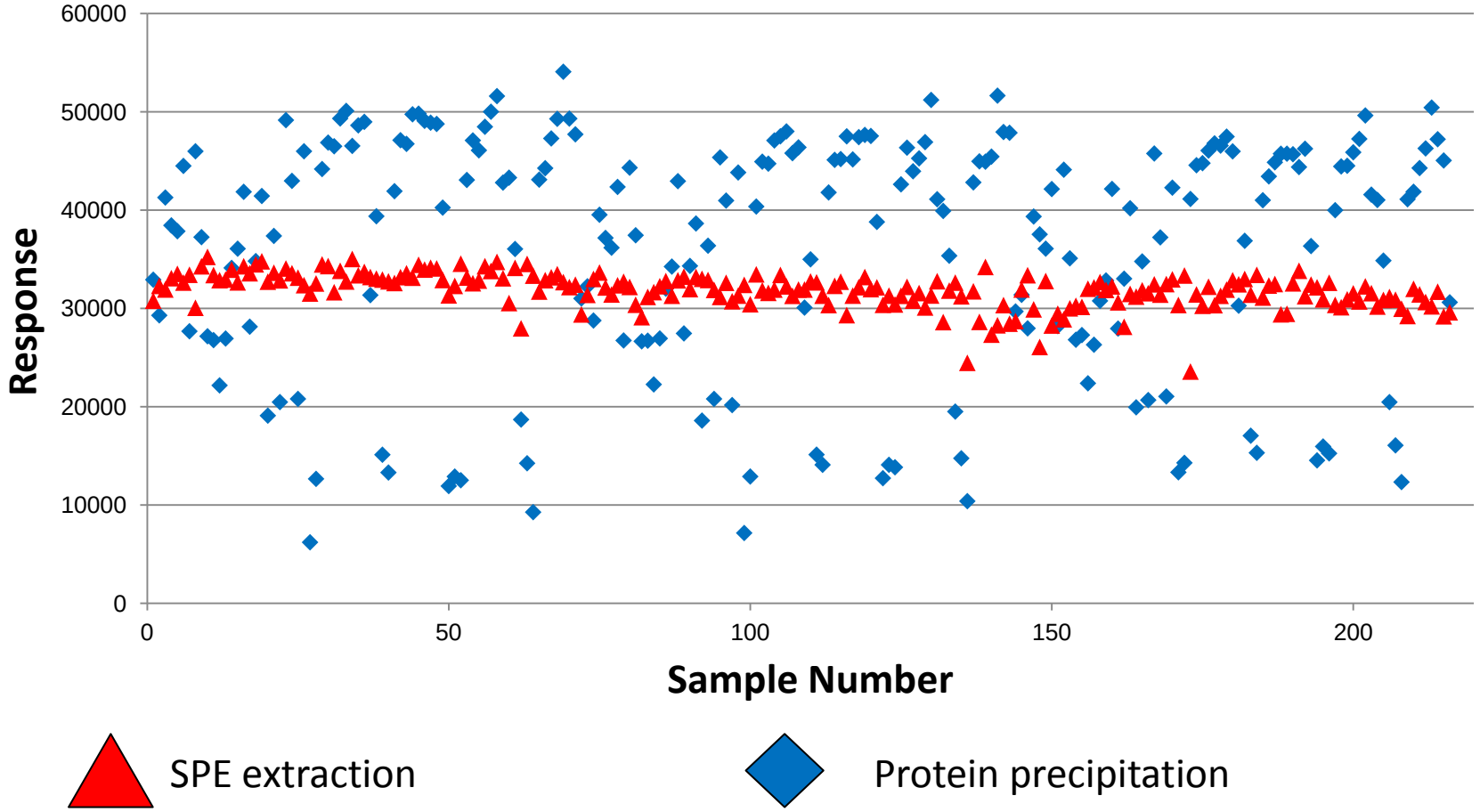
Reproducibility

Poor sample extraction can lead to variation in results. Here we show repeat injections of an SPE extract against an PPT extract.



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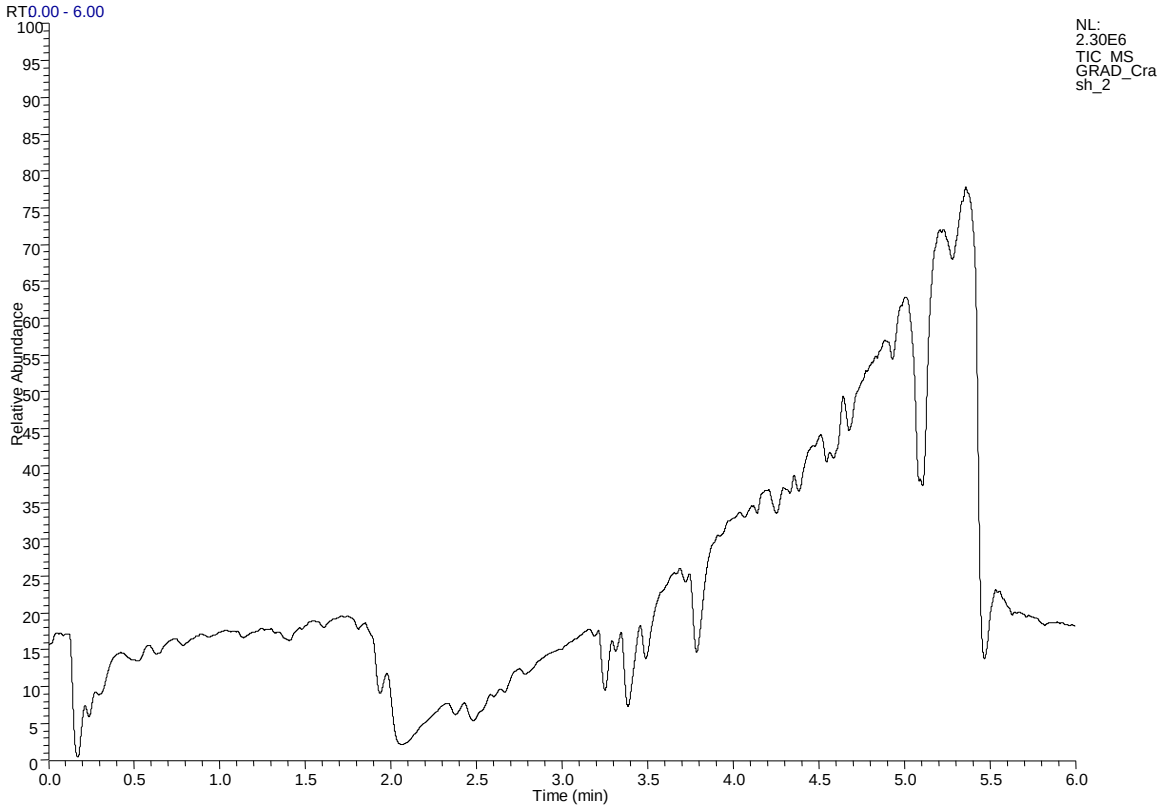
Reproducibility – Matrix Effects

Matrix effects can be varied but generally consist of;

- Loss of system robustness
- Chromatographic interference
- Ion-suppression in mass spectrometry



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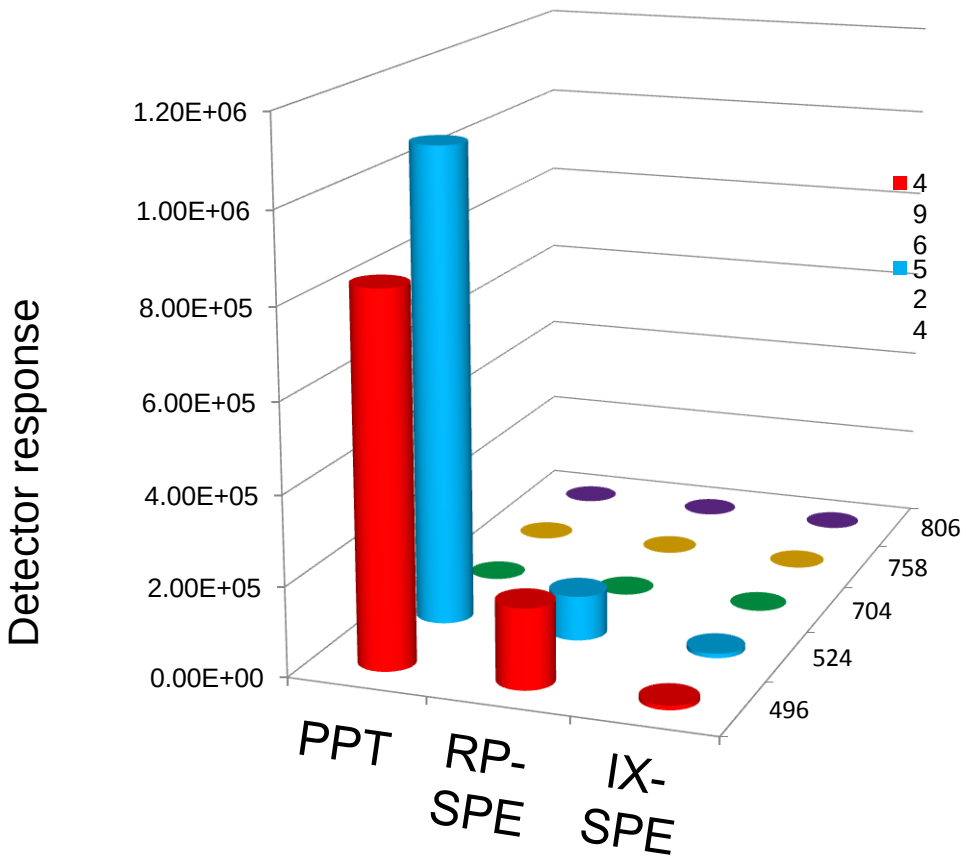
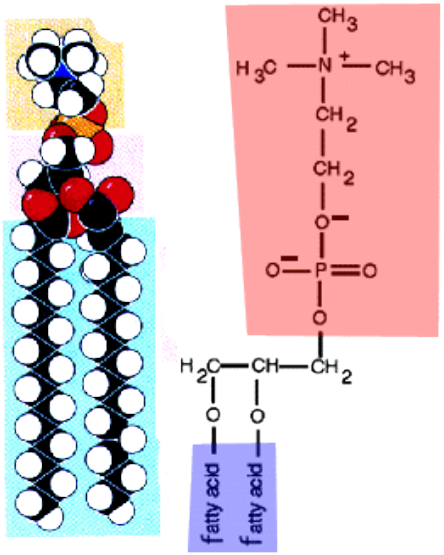
Reproducibility – Matrix Effects



A well know cause of ion-suppression are Phospholipids.

Here we compare PL removal using
PPT, RP-SPE, and IX-SPE



Far better clean up is observed using SPE than PPT.
Ion-exchange SPE also cleaner than RP-SPE.

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Reproducibility – Packing Effects

HDPE frits

+

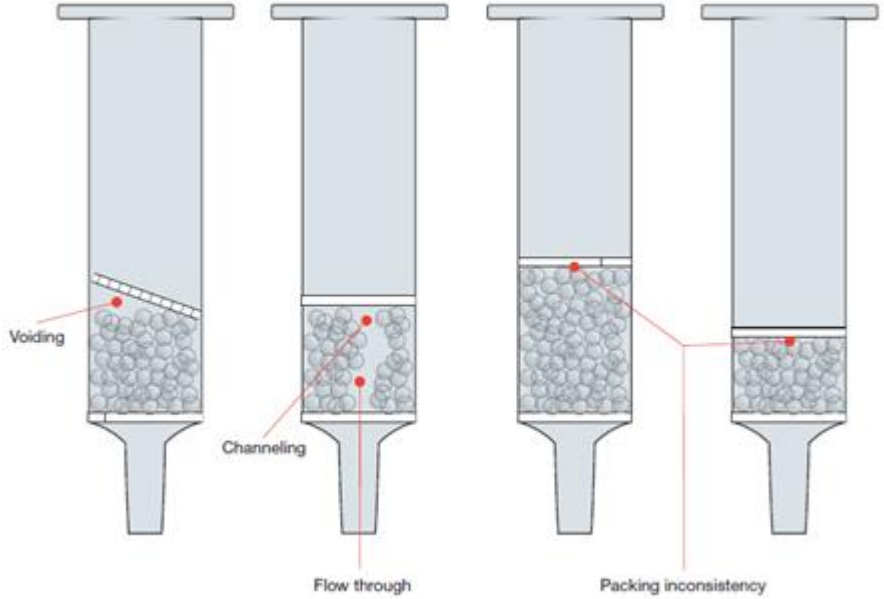
Polymeric sorbent material

+

Polypropylene cartridge/plate

=

Frits keep the sorbent material in place in the cartridge, they perform no other function.



Possible production and shipping issues

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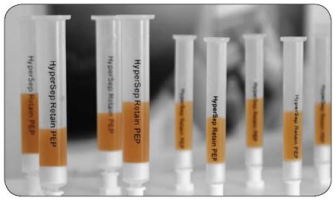
Reproducibility – Packing Effects

HDPE powder
+

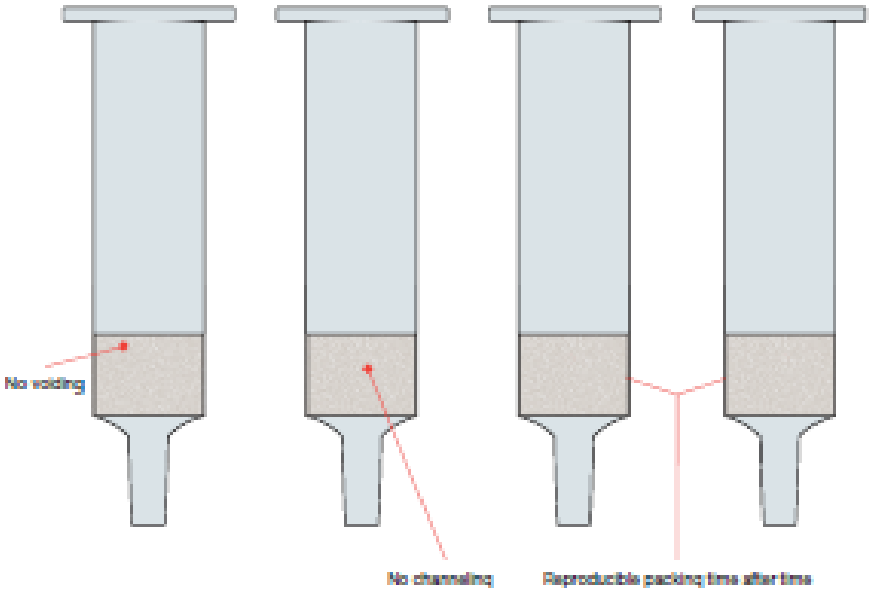
Polymeric material
+



Polypropylene cartridge/plate

=

Thermo Scientific™ SOLA ™ cartridge/plate is a solid single unit with integrated frit material



- Reproducible production
- Designed to eliminate packing quality issues
- Designed to eliminate shipping problems

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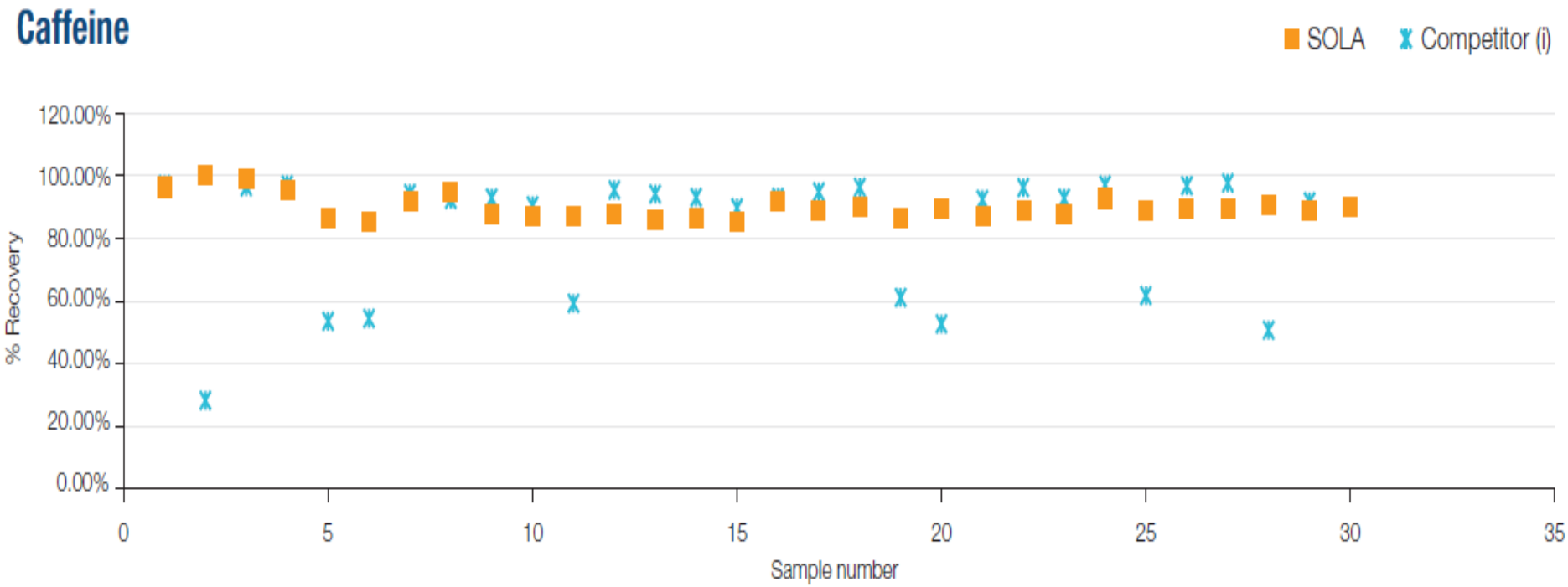
Reproducibility – Packing Effects



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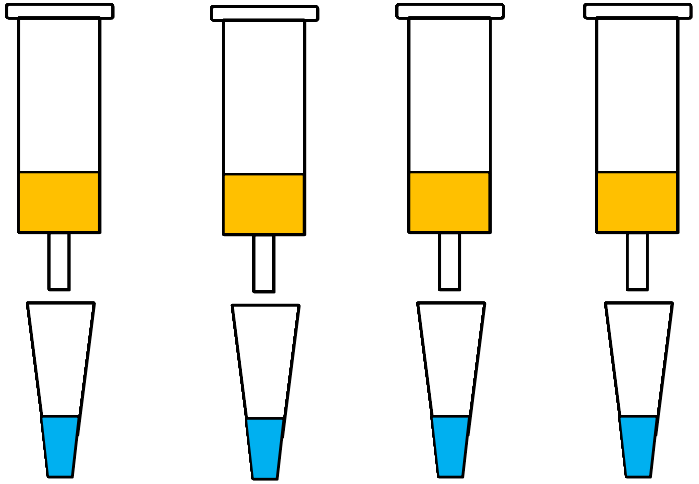
SPE Method Optimization

Typical SPE methods involve

1. Sorbent conditioning
2. Sample Loading
3. Washing
4. Elution of your analyte(s)



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SPE Method Optimization

	Sample	Dilute sample 1:1 w/ acid	
	HRP	**SCX *and* WAX**	**SAX *and* WCX**
Equilibrate	Methanol	Methanol	Methanol
Condition	Water	Water	Water
Sample load	Diluted sample	Diluted sample	Diluted sample
Wash	n% Methanol	2% Formic acid	5% Ammonia
Wash	-	Methanol	Methanol
Elute	n% Methanol	5% Ammonia in Methanol	2% Formic acid in Methanol
	Final extract	**Dilute or dry down and reconstitute**	

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	Final extract	**Dilute or dry down and reconstitute**	

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SPE Method Optimization

Elution profile experiment

- Load sample
- Wash with incremental strengths of solvent
- Capture all the steps and analyse
- Plot a chart to determine method conditions

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SPE Method Optimization

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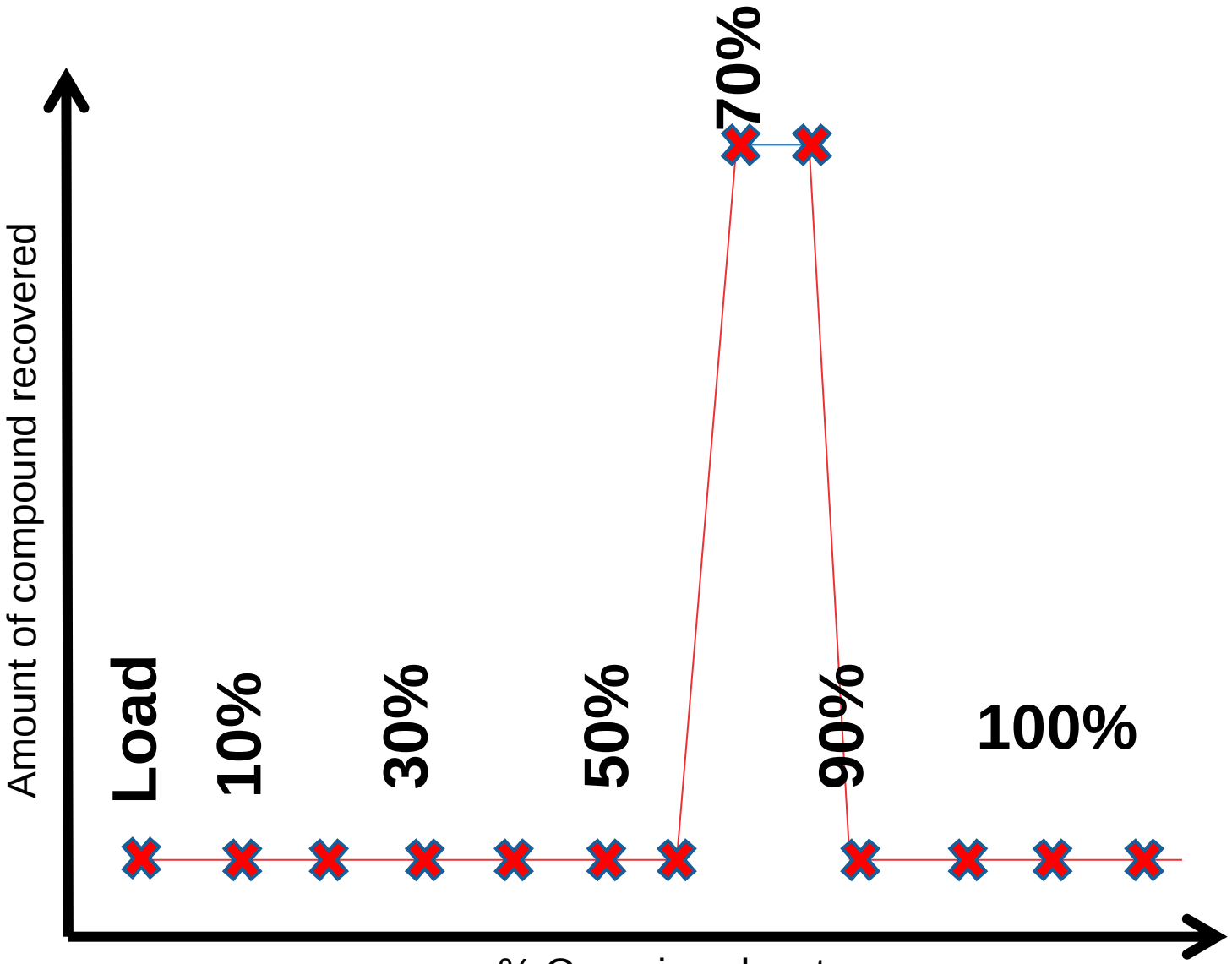
SPE Method Optimization



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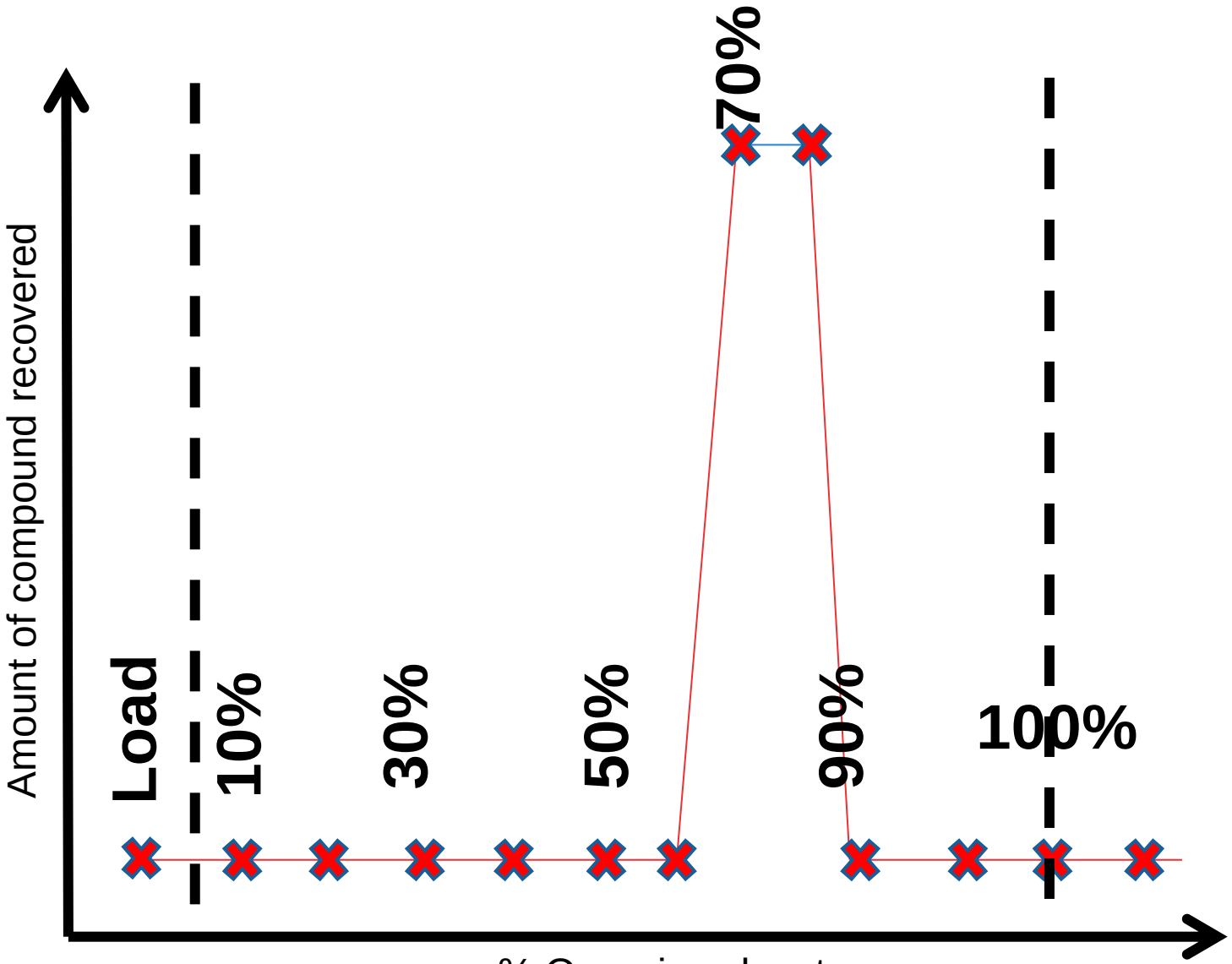

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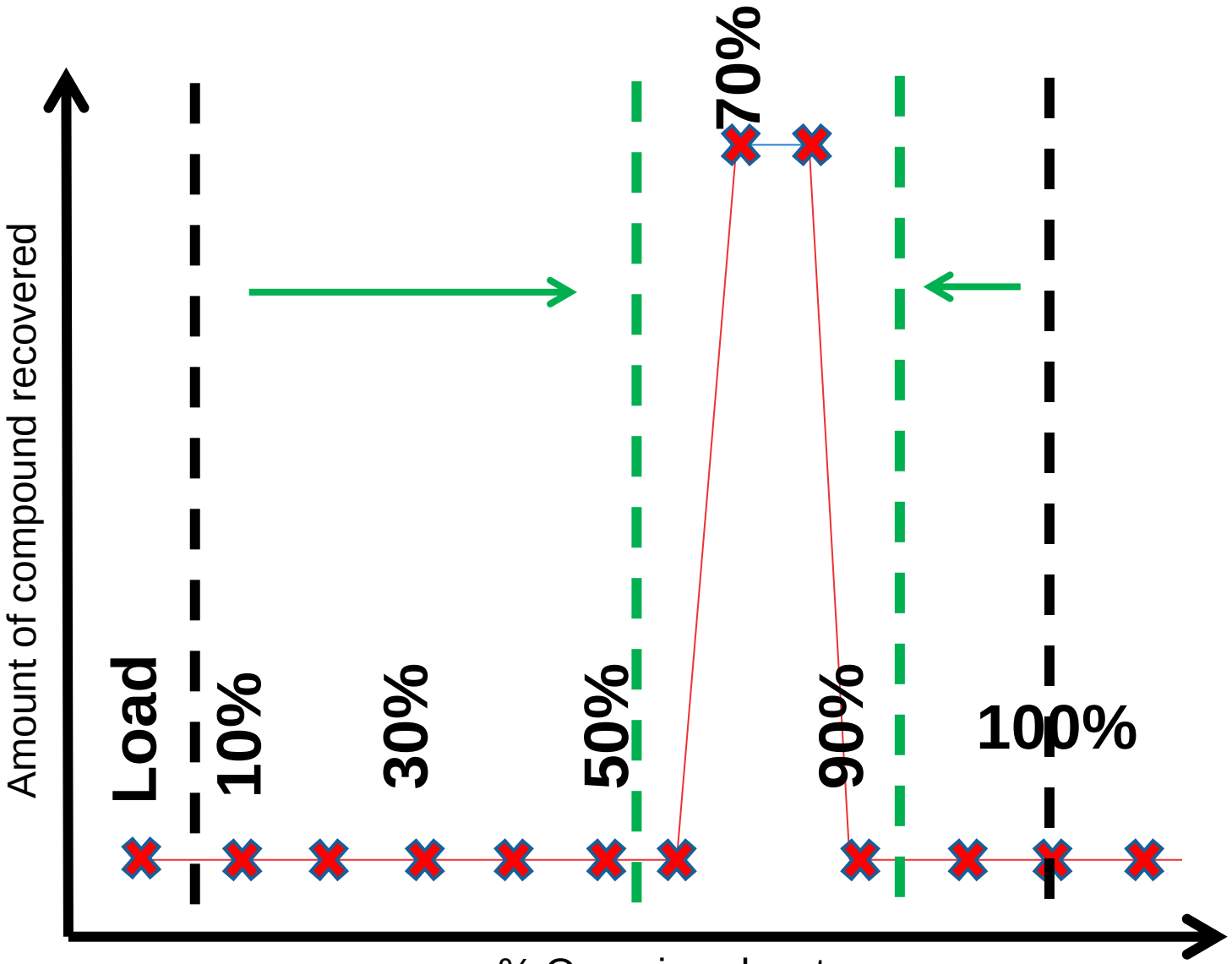

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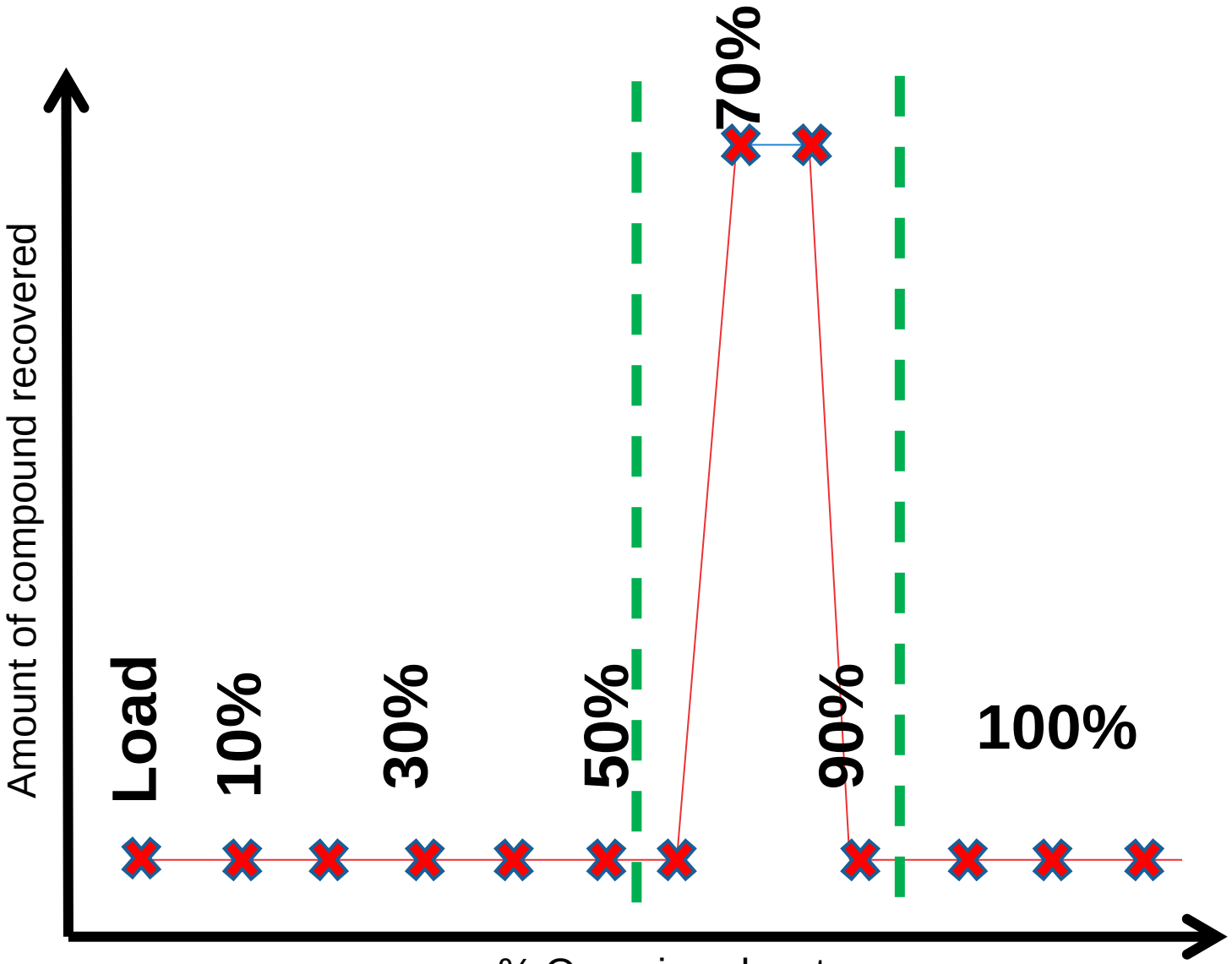
SPE Method Optimisation



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	Final extract	**Dilute or dry down and reconstitute**	

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SPE Method Optimization

- Equilibration steps not always needed
- Strength of counter-ion appropriate
- Organic solvent used
- Organic solvent composition
- Volume of elution solvent

	Sample	Dilute sample 1:1 w/ acid	
	HRP	SCX *and* WAX	SAX *and* WCX
Equilibrate	Methanol	Methanol	Methanol
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Elute	n% methanol	5% Ammonia in Methanol	2% Formic acid in Methanol
	Final extract	Dilute or dry down and reconstitute	

Smaller elution volumes allow for more concentrated sample, and remove the need for post-extraction steps.

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SPE Method Optimization

Concentration of you sample is often required in order to analyse for very low levels of compounds.

Evaporation (and reconstitution) of the sample, or an extract of the sample, is a popular method for achieving this.

Can have issues with

- Loss of volatile analytes
- Loss of analytes due to NSB
- Increase in workflow (time and money)

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SPE Method Optimization

Concentration of you sample is often required in order to analyse for very low levels of compounds.

Evaporation (and reconstitution) of the sample, or an extract of the sample, is a popular method for achieving this.

Applying a large sample to an extraction media, such as SPE, and using a smaller volume to recover the sample, can both clean and concentrate the sample.

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SPE Method Optimization

SOLA SPE cartridges/well plates
- 10mg SPE
- Excellent reproducibility
- Robust polymeric material
- Multiple chemistries

SOLAμ SPE well plates
- 2mg SPE
- Excellent reproducibility
- Robust polymeric material
- Multiple chemistries
- Elution volumes as low at **25 µL**

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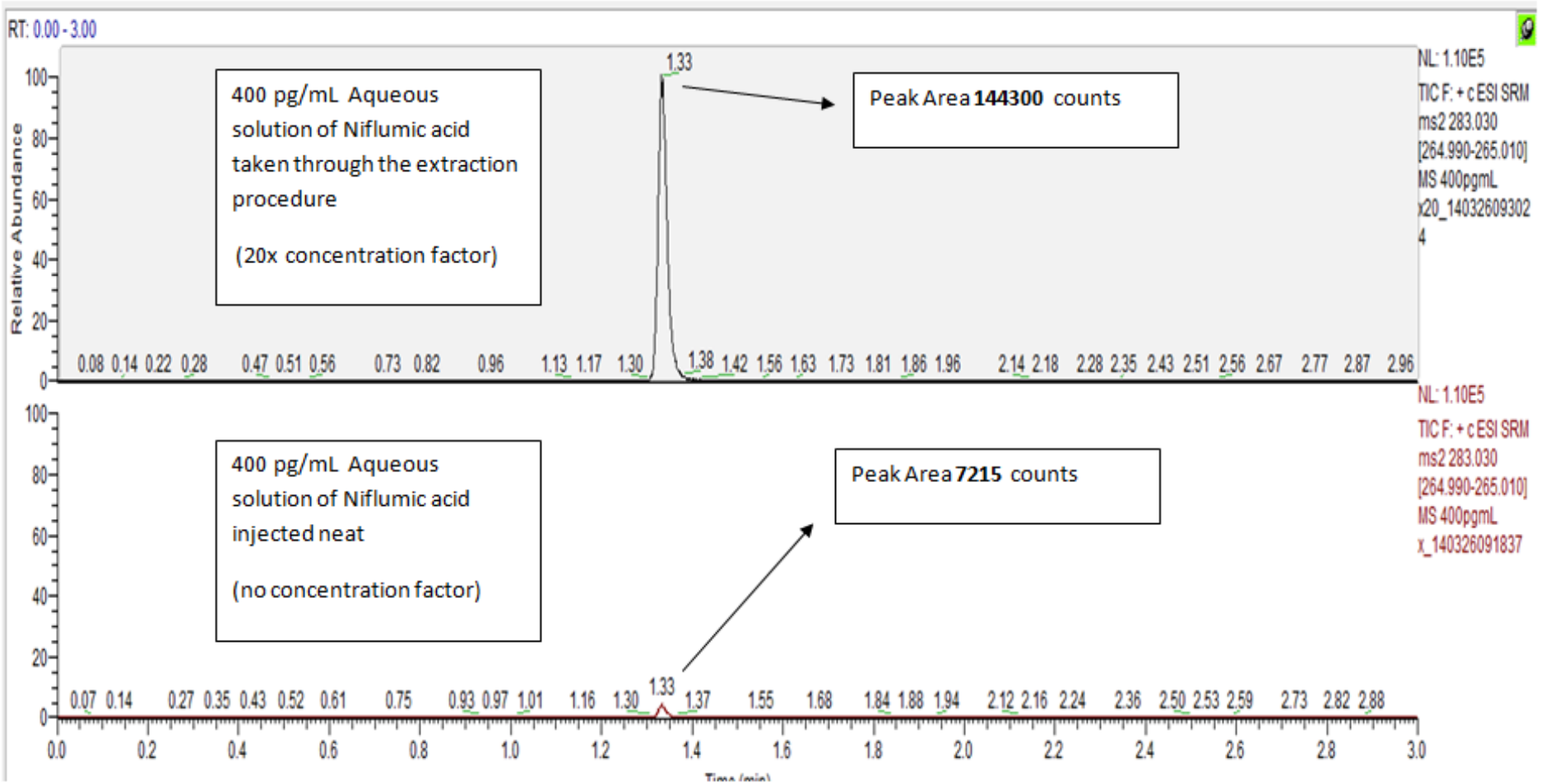
SPE Method Optimization



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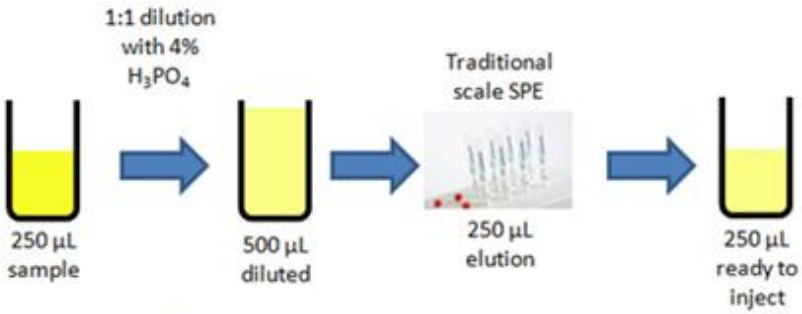
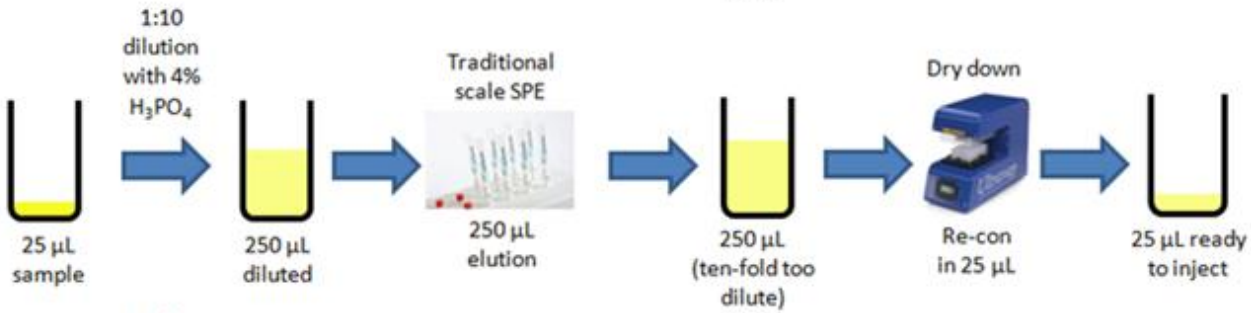
SPE Method Optimization

A



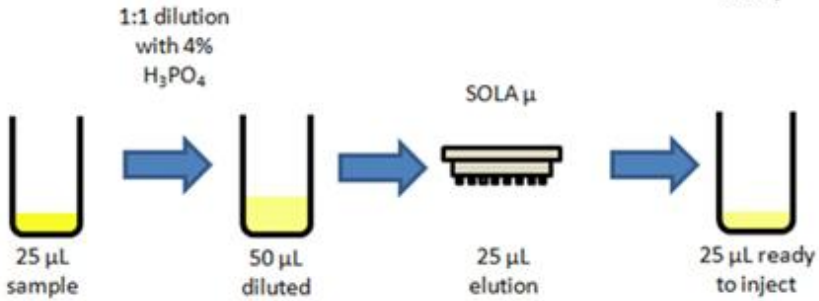
Problem; large sample volume requires reduction

B



Option 1; introduce additional steps with associated issues

C



Option 2; Scale volumes directly with no other method changes

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So Many Options....?

With such a big range of sample preparation products on the market how do I chose which is right for my analysis?

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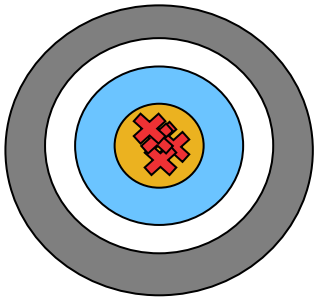
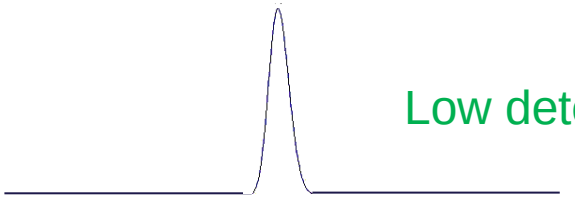


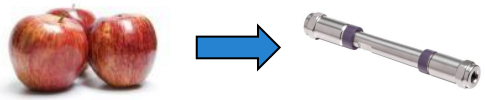
Assay performance

€ $ £ Cost



Low detection limits

System robustness



Compatibility to analysis

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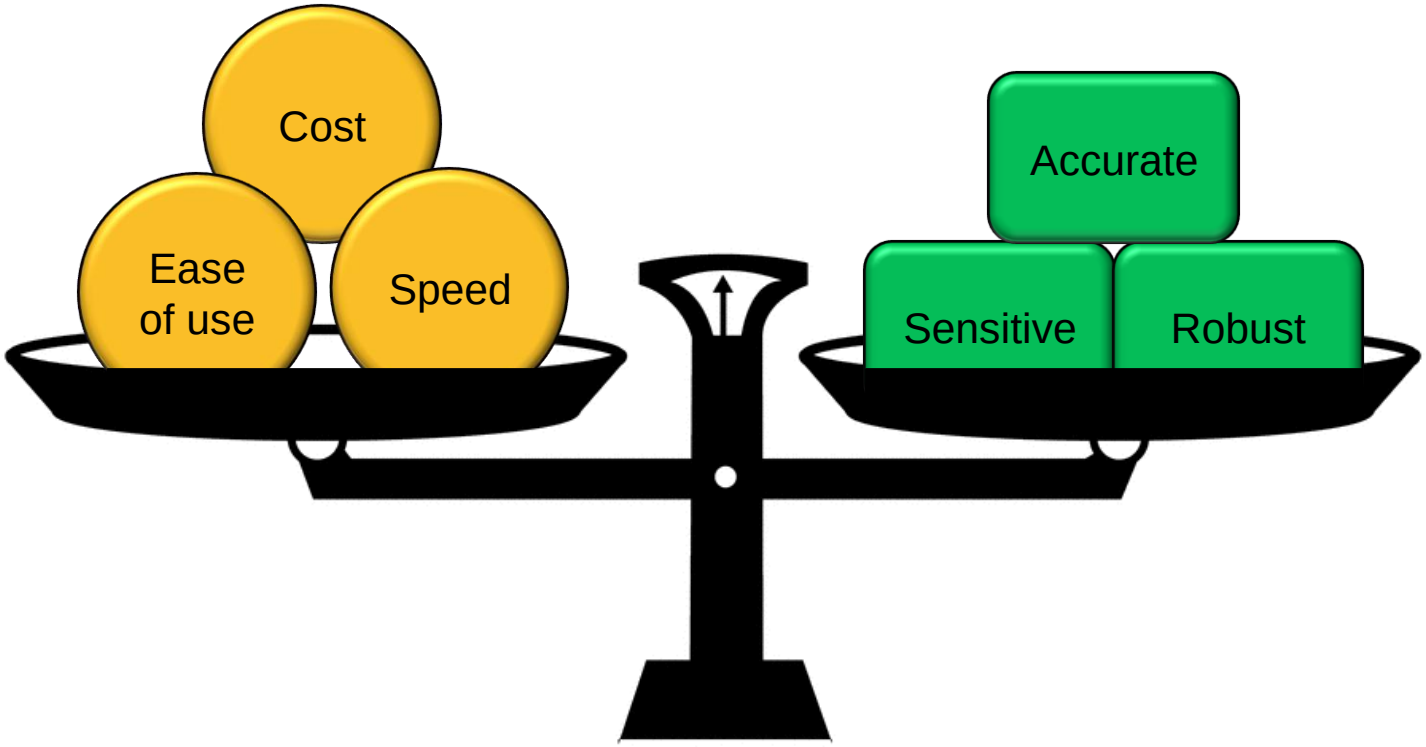

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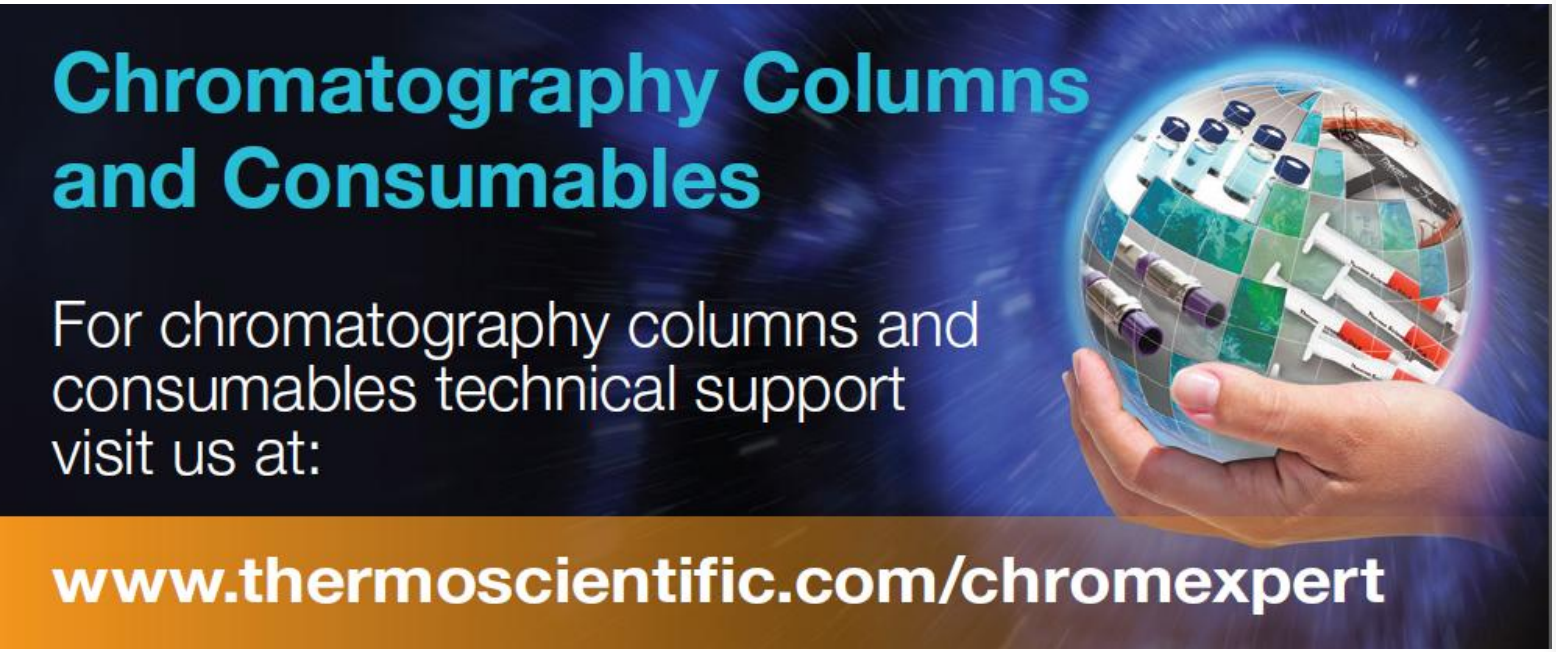
Thank you



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Any questions?

Do you have additional questions or do you want to talk to an expert from Thermo Fisher Scientific?

Please send an E-Mail to analyze.eu@thermofisher.com and we will get back to you.