Bioanalytical Sample Preparation and Method Development for Therapeutic and Endogenous Peptides

OASIS®
SAMPLE EXTRACTION PRODUCTS

Includes guidelines for the following:

✓ **Typical Challenges and Key Considerations in Peptide Bioanalysis**

✓ **Format**

✓ **Sorbents**

✓ **Sample Preparation Method: Therapeutic and Endogenous Peptides**

✓ **Sample Preparation Method: Tryptic Peptides from Protein Digestions**

✓ **Column Choices for Peptide Quantification**

✓ **Laboratory Terms and Information**

✓ **Calculating Recovery**

✓ **Calculating Matrix Effects**

✓ **Experimental Set-Up**

✓ **Phospholipid Monitoring**

✓ **Troubleshooting**

✓ **Sample Pre-Treatment**

✓ **The Peptide and Protein Bioanalysis Boot Camp**

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Typical Challenges and Key Considerations in Peptide Bioanalysis

1. **Protein binding:** Protein binding must be disrupted to separate peptides from sample matrix components, such as proteins. Failure to disrupt protein binding will cause the protein bound peptides to pass through the SPE sorbent, unretained due to size exclusion mechanisms. We suggest diluting plasma 1:1 with either a 4% solution of H_3PO_4 or a 5% solution of NH_4OH as a starting point. More aggressive pre-treatments may be needed, such as a 1:1 protein precipitation or denaturation with guanidine HCL, urea, or sodium dodecyl sulfate (SDS).

2. **Non-specific binding (NSB):** Peptides are notoriously sticky and tend to bind (adsorb) to glass and other surfaces. Non-specific binding is defined as analyte losses that occur as a result of binding to any surface to which the analyte is exposed.

   Strategies to reduce NSB include:

   a. Avoid using glass for sample preparation or storage. Polypropylene and/or low bind materials are recommended.

   b. Use of additives such as organic solvents, detergents, and carrier proteins. Use of organic solvents helps to promote solubility. Addition of detergents like SDS and guanidine help to denature a peptide/protein and help promote solubility. The addition of carrier proteins (5% rat plasma or 40 μg/mL solution of BSA) to neat standards solutions or biological matrices containing low protein concentrations, such as CSF, saturates the binding sites of surfaces a peptide may come in contact with and prevents loss of the peptide of interest.

   c. Avoid evaporation or dry down, as these can also lead to NSB.

   d. Preparation of high concentration stock solutions and subsequent serial dilution.

3. **Solubility:** Maintaining solubility throughout sample preparation and analysis is critical, starting from initial solubilization from powder, through sample extraction and LC-MS analysis. Since peptides are quite different from small molecules, we recommend limiting organic concentrations to no more than 75% and using modifiers to promote solubility. Modifier concentrations can be much higher than what is typically used with small molecules, ranging from 1% to 10% acid or base, such as TFA, FA, AA, or NH_4OH. Ensuring or maintaining solubility is especially important in the SPE elution step and in chromatographic separations. Poor solubility can lead to poor recoveries as well as column fouling and carryover.

4. **Specificity:** To achieve highly sensitive and robust bioanalytical peptide assays, high specificity is required. While protein precipitation is a very common and effective sample preparation strategy for quantification of small molecules from serum/plasma (effectively disrupting protein binding, affording high recovery, and minimizing matrix interferences), it is often detrimental for large molecule (peptide/protein) quantification. Due to their size and nature, protein precipitation, using high ratios of organic solvent, often results in peptide loss due to undesired precipitation of the peptide itself. PPT also lacks in specificity, resulting in issues with matrix effects. Additionally, PPT often requires sample evaporation/concentration to achieve high sensitivity. Mixed-mode solid-phase extraction (ion-exchange combined with reversed-phase) provides an orthogonal sample clean-up with high specificity to selectively retain the target peptides while removing extra matrix interferences.

5. **Low recovery:** This is the most common symptom of failure to successfully disrupt protein binding, avoid non specific binding or ensure peptide solubility (see points 1, 2, and 3 on this card).

Selecting the Correct SPE Format

SPE Format

The 96-well plate µElution™ format was specifically chosen for the extraction of peptides from biological matrices for several reasons. The use of the µElution format allows one to load up to 375 µL of sample (prior to dilution) and elute in as little as 25 µL, providing the possibility of up to a 15X concentration factor (often necessary to reach required detection limits) without evaporation and reconstitution. The ability to elute in small volumes eliminates potential peptide losses during evaporation due to adsorption to the walls of collection plates, chemical instability or lack of resolubilization upon reconstitution after evaporation. In addition, an entire 96-well plate can be processed manually in under 30 minutes, or less than 20 seconds per sample, significantly increasing throughput. The Oasis™ µElution Peptide Separation Technology Method Development Plate contains 48-wells of each of the two Oasis Sorbents, WCX, and MAX, described in the "Sample Preparation Method: Therapeutic and Endogenous Peptide" section.

Oasis µElution Plate Technology

- Patented µElution plate design.
- Ideal for SPE clean-up and analyte enrichment of sample volumes ranging from 10 µL to 375 µL.
- No evaporation and reconstitution necessary due to elution volumes as low as 25 µL.
- Up to 15X concentration without evaporation, often necessary to reach LOD's with peptides.
- Helps minimize analyte loss.
- Elimination of dry down step helps minimize sample loss due to problems resolubilizing after dry down or thermally unstable peptides.
- Residual volume using a 96-well 1 mL Collection Plate, <15 µL residual volume.
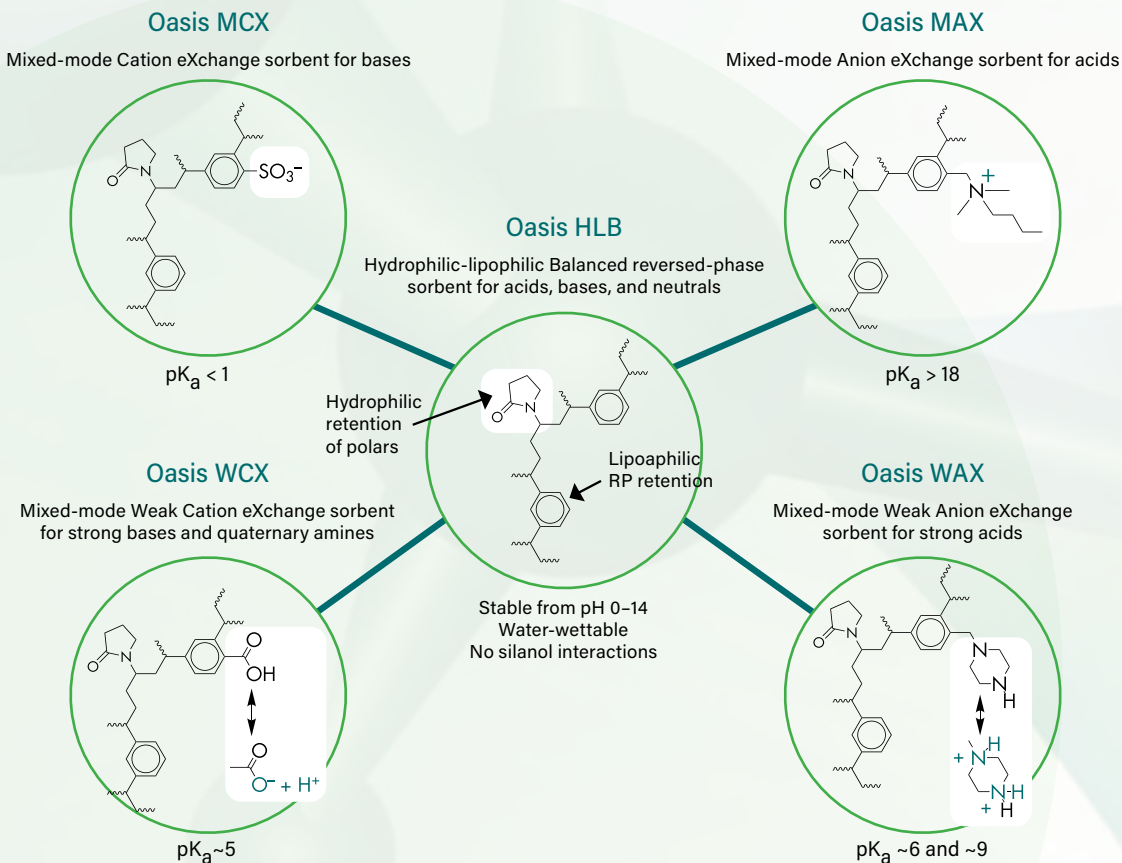- Compatible with most liquid-handling robotic systems for automated, reliable high-throughput SPE (HT-SPE).

96-well Extraction Plates

- Innovative, award-winning two-stage well design.
- High throughput and high recovery.
- Available with 5 mg, 10 mg, 30 mg, and 60 mg of sorbent per well.
- Compatible with most liquid-handling robotic systems for automated, reliable high-throughput SPE (HT-SPE).

Syringe-Barrel Cartridges

- Ultra-clean syringe barrel and frits.
- Available with cartridge sizes ranging from 1 cc/10 mg up to 35 cc/6 g.
- Flangeless syringe-barrel cartridges available in 1 cc, 3 cc, and 6 cc configurations.

The Oasis SPE Family of Sorbents

As a unique, water-wettable polymeric sorbent, Oasis products can be used without the conditioning and equilibration steps required by other polymeric and silica-based sorbents. Historically, those steps were required to obtain retention of analytes by reversed-phase SPE. The water-wettable nature of Oasis allows direct loading of aqueous samples without sacrificing recovery.

Oasis MCX
Mixed-mode Cation eXchange sorbent for bases

—SO$_3^-$

pK$_a$ < 1

Oasis MAX
Mixed-mode Anion eXchange sorbent for acids

pK$_a$ > 18

Oasis HLB
Hydrophilic-lipophilic Balanced reversed-phase sorbent for acids, bases, and neutrals

Hydrophilic retention of polars

Lipoaphilic RP retention

Stable from pH 0–14
Water-wettable
No silanol interactions

Oasis WCX
Mixed-mode Weak Cation eXchange sorbent for strong bases and quaternary amines

OH

O$^-$ + H$^+$

pK$_a$ ~5

Oasis WAX
Mixed-mode Weak Anion eXchange sorbent for strong acids

pK$_a$ ~6 and ~9

Oasis PRiME HLB* makes solid-phase extraction easy to implement into routine laboratory use by providing generic, simple methods that remove 95% of common matrix interferences such as phospholipids, fats, salts, and proteins.

Oasis HLB is the backbone of all Oasis Sorbents. It is a multi-purpose reversed-phase sorbent that provides high capacity for a wide range of compounds.

Analyte specificity and sensitivity can be increased by using a **Mixed-Mode Oasis** Sorbent, which includes both reversed-phase and ion-exchange functionality for orthogonal sample preparation.

Oasis PRiME MCX can be used with a simple, 3 or 4 step protocol to selectively retain, concentrate, and elute compounds with basic characteristics while removing phospholipids and proteins.

*Oasis PRiME HLB is a proprietary, patent pending sorbent.

Sample Preparation Method: Therapeutic and Endogenous Peptides

Available in the Oasis Peptide Separations Technology μElution Plate for easy method development.
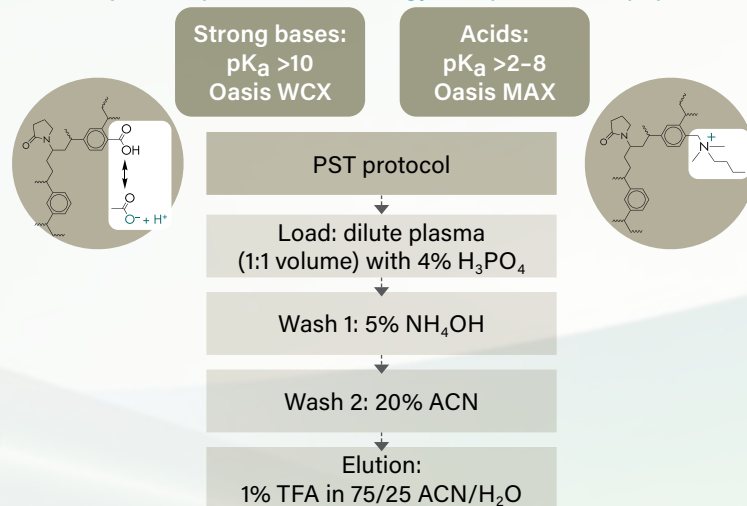
Oasis Sorbents for Solid-phase Extraction, Clean-up, and Concentration

The increasing number of peptide and protein based therapeutics brings new challenges to the typical bioanalytical laboratory. Sensitive, selective, and robust bioanalytical methods must be developed for these types of compounds. Because of their size and charge state distribution, sensitivity by mass spectrometry may be lower for biomolecules than typical small molecules, necessitating sample concentration and other means of increasing overall method sensitivity.

The Oasis family of solid-phase extraction (SPE) products is designed to simplify and improve sample preparation by combining the right sorbent chemistry, device format, and methodology. The zwitterionic nature of peptides makes it hard to predict which sorbent will work best. In our own testing, most peptides eluted best with WCX and MAX.

✓ Single protocol specifically for peptides which eliminates peptide precipitation and facilitates full peptide solubility.

✓ Increase selectivity with a 20% acetonitrile wash, removing polar interferences but without eluting polar peptides.

✓ Hydrophobic interferences retained on the sorbent, reducing matrix effects.

✓ Peptide solubility maintained through the use of 75% organic in the elution step, sufficient for even the most hydrophobic peptides.

✓ Excellent recovery and peak shape with TFA compared to formic acid in the final elution for peptides and improved recovery for acidic peptides.

Oasis Peptide Separation Technology SPE protocol for peptides

Strong bases: pK_a >10 Oasis WCX	Acids: pK_a >2–8 Oasis MAX

PST protocol

Load: dilute plasma (1:1 volume) with 4% H_3PO_4

Wash 1: 5% NH_4OH

Wash 2: 20% ACN

Elution: 1% TFA in 75/25 ACN/H_2O

Solutions needed

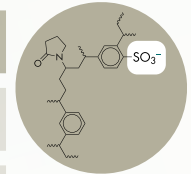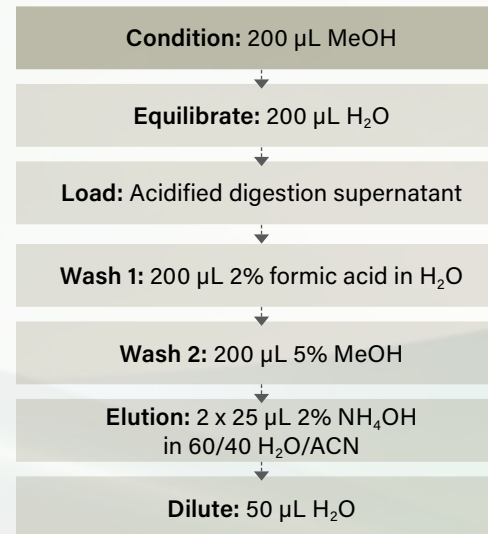|	
Pretreatment:	4% phosphoric acid in water, by volume
Wash 1:	5% NH_4OH in water, by volume
Wash 2:	20% ACN in water, by volume
Elution:	75/25% acetonitrile/water containing 1% trifluoroacetic acid, by volume

ProteinWorks µElution SPE Kit for Protein Digest Purification

Tryptic peptide digest clean-up presents unique but equally important challenges. Tryptic peptides contain basic side chains resulting from the arginine and lysine residues. The Oasis MCX Sorbent features a strong cation-exchange functionality, perfect for interacting with the residual positive charge on the lysine and arginine side chains of the peptide fragments.

The ProteinWorks™ µElution SPE Clean-up protocol is a simple, logical approach for extraction of tryptic peptides resulting from the digestion of proteins from plasma/serum biological matrices. This protocol allows the extraction of tryptic peptides; yielding high SPE recoveries while removing matrix components and digest reagents that may interfere with analysis.

✓ Remove interfering buffer salts and digest reagents.

✓ Recover unique and generic signature tryptic peptides with high efficiency using a single SPE method.

✓ Minimize sample loss with µElution format.

✓ Concentrate the sample up to 15x.

✓ Retention of very polar tryptic peptides.

Oasis MCX µElution Protocol

**Condition:** 200 µL MeOH

↓

Equilibrate: 200 µL H_2O

↓

Load: Acidified digestion supernatant

↓

Wash 1: 200 µL 2% formic acid in H_2O

↓

Wash 2: 200 µL 5% MeOH

↓

Elution: 2 x 25 µL 2% NH_4OH in 60/40 H_2O/ACN

↓

Dilute: 50 µL H_2O

Solutions Needed

Condition:	Methanol
Equilibration:	Water
Pretreatment:	4% phosphoric acid in water, by volume*
Wash 1:	2% formic acid in water, by volume
Wash 2:	5% methanol in water, by volume
Elution:	60/40% water/acetonitrile containing 2% ammonium hydroxide, by volume

*For protein digested supernatant resulting from use of the Waters ProteinWorks eXpress Digest and Direct Digest Kit, pre-treatment with an acidified aqueous solution is not necessary.

Column Choices for Peptide Quantification
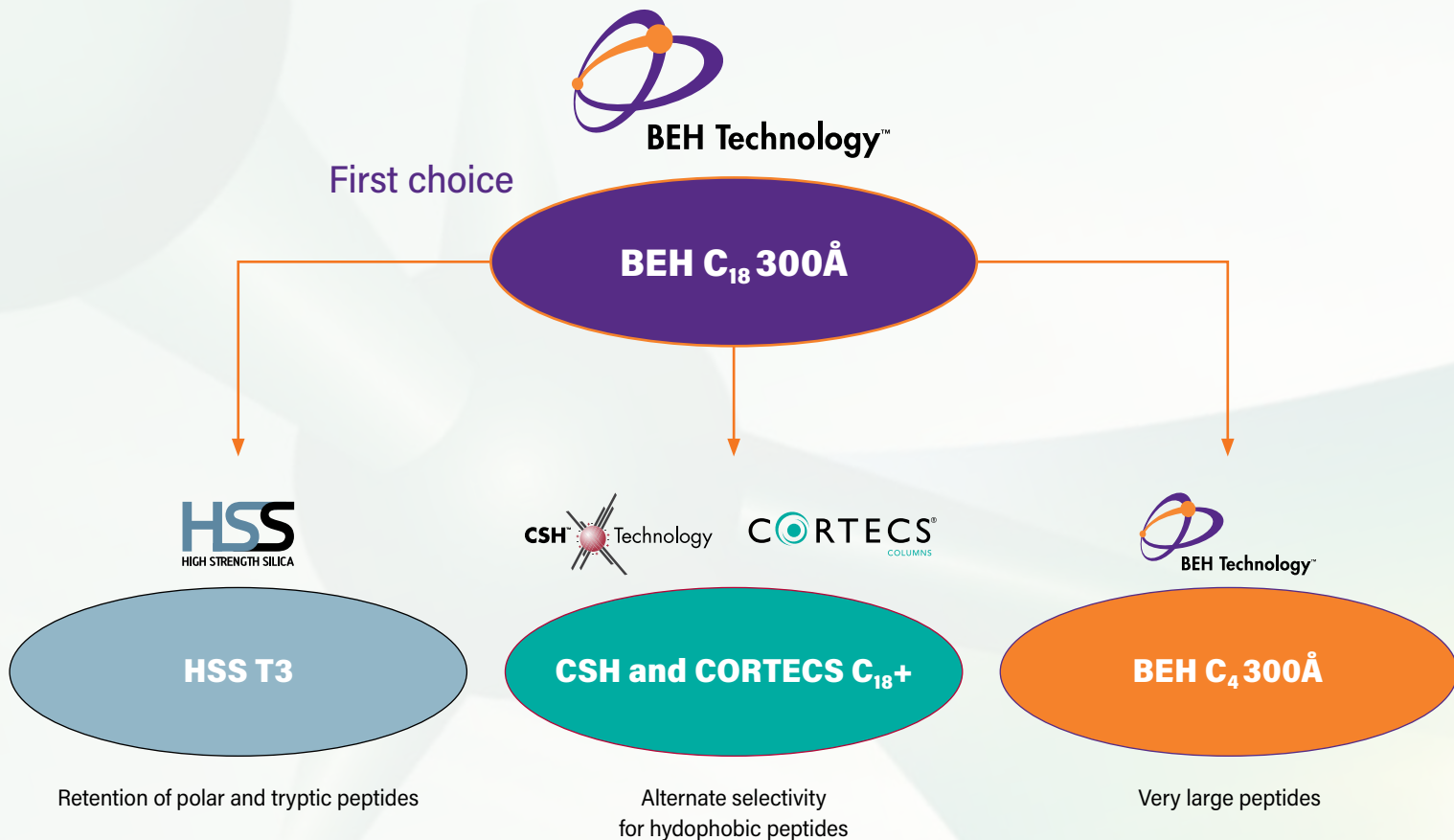
Tryptic Digest Supernatant Loading Volumes

Recommended maximum digest loading volumes for the ProteinWorks µElution SPE Clean-up Kit

Oasis MCX 96-well µElution Plate recommended maximum digest loading volumes

Starting plasma/serum volume*	SPE loading volume (µL)	
	Direct digest**	Post-generic affinity digestion†
15 µL	110–200	Total digest supernatant
25 µL	70–140	Total digest supernatant
35 µL	50–100	Total digest supernatant
50 µL	35–70	170–200
70 µL	25–50	120–200

* Starting volume of plasma added for protein digestion or affinity purification using the ProteinWorks eXpress Digest Kit and protocols, with a final digestion volume of 200 µL.

**Based on a total protein content of 75 mg/mL in whole plasma/serum.

† Based on a total protein content 15 mg/mL post-generic affinity purified plasma (Protein A/G) and assuming all affinity captured sample is used for digestion.

Laboratory Terms and Information

1. Converting g/mL to mol/mL

Examples:

Peptide molecular weight
1000 = 1 μg/nmol or 1 ng/pmol

Peptide molecular weight
3000 = 3 μg/nmol or 3 ng/pmol

pI = isoelectric point

pH at which the molecule contains no net electrical charge or where the positive and negative charges are equal.

At pH below pI = net positive charge

At pH above pI = net negative charge

HPLC index

Provides a sense of relative hydrophobicities of peptides.

Hydrophobic peptides (HPLC index ≥ 30)

Polar peptides (HPLC index ≤ 30)

2. Preparing a 1 molar (1 M) solution

- Molar (M) solutions are based on the number of moles of chemical in 1 liter of solution
- Determine the molecular weight of each atom in the chemical formula

  $NaOH$ = 1 x Na (22.99), 1 x O (15.999), 1 x H (1.008)

  $NaOH$ = 39.997

- 1 M NaOH consists of 39.997 g in 1 L of distilled water
- What if 100 mL of 0.1 M of NaOH is required?

  Grams of chemical =
  (molarity of solution in mole/litre) x
  (MW of chemical in g/mole) x (mL of solution) ÷ (1000 mL/L)

  Grams of NaOH = 0.1 x 39.997 x 100 ÷ 1000

  100 mL of a 0.1 M NaOH consists of 0.39997 g of NaOH

3. Preparing a weight/volume percentage (w/v%) solution

- The following calculation is used to calculate w/v% solutions

  w/v(%) =
  weight of the solute ÷ volume of the solution x 100

- What is the w/v(%) of an 250 mL aqueous sodium chloride (NaCl) solution containing 8 g of sodium chloride

  w/v(%) = 8 g ÷ 250 mL x 100

- 250 mL aqueous sodium chloride solution containing 8 g of sodium chloride is 3.2% (w/v%)
- To determine how much chemical to add to make a w/v% solution

  grams of chemical = volume of solution ÷ 100 x w/v%

- What weight of NaCl is required to make 250 mL of a 3.2% w/v% solution

  grams of NaCl = 250 ÷ 100 x 3.2

  A 3.2% in 250 mL w/v% solution of NaCl consists of 8 g of NaCl

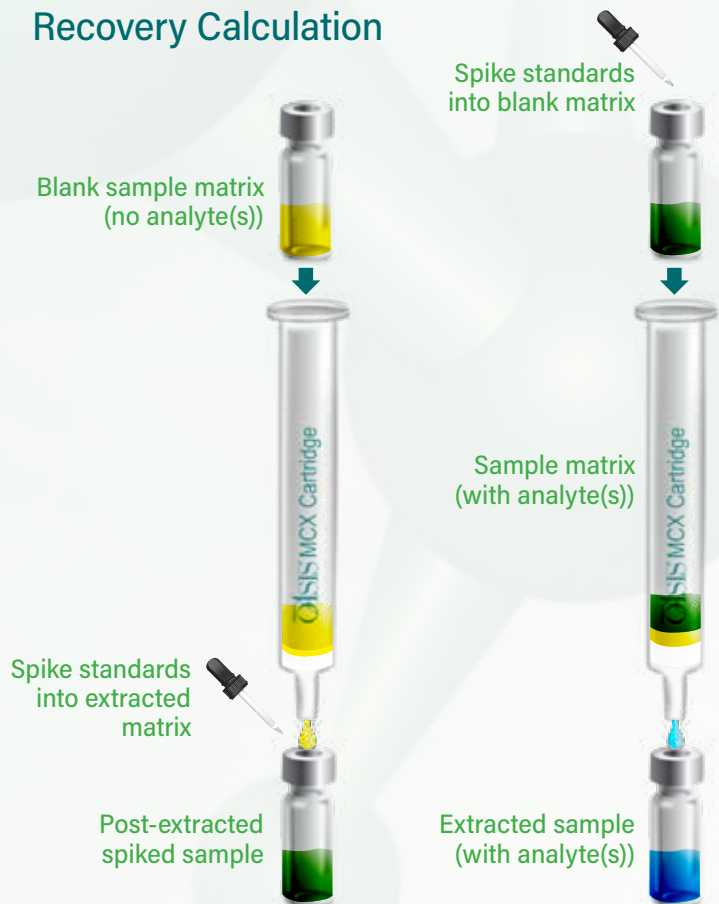## 4. Reagent/Sample dilution calculation

$$C_1 * V_1 = C_2 * V_2$$

- Where: C_1 = Initial concentration, C_2 = Final concentration, V_1 = Initial volume, V_2 = Final volume
- For example, to prepare 5 mL of a plasma solution containing 50 ng/mL of a target analyte from a 50 ng/mL stock solution.

  C_1 = Initial concentration = 10 μg/mL = 10,000 ng/mL

  C_2 = Final concentration = 50 ng/mL

  V_1 = Initial volume = unknown

  V_2 = Final volume = 5 mL = 5,000 μL

  $$C_1 * V_1 = C_2 * V_2$$

  $$V_1 = (C_2 * V_2)/C_1$$

  $$V_1 = (50\ ng/mL * 5,000\ μL)/(10,000\ ng/mL)$$

  V_1 = 25 μL

Calculating Recovery

To determine the success of the SPE method, there are two key parameters that must be evaluated. These are **recovery** and **matrix effects**. **Recovery** will determine how successfully the SPE method has isolated your compound(s) of interest. Matrix effects will determine if you have removed matrix components that may interfere with your ability to accurately and consistently quantify your compound(s).

Recovery Calculation

Spike standards into blank matrix

Blank sample matrix (no analyte(s))

Sample matrix (with analyte(s))

Spike standards into extracted matrix

Post-extracted spiked sample

Extracted sample (with analyte(s))

Recovery of the extraction procedure (RE)* (or, SPE recovery)

$$\% \ RE = 100 \ x \ \frac{\text{Response Extracted sample (with analyte(s))}}{\text{Response Post-extracted spiked sample}}$$
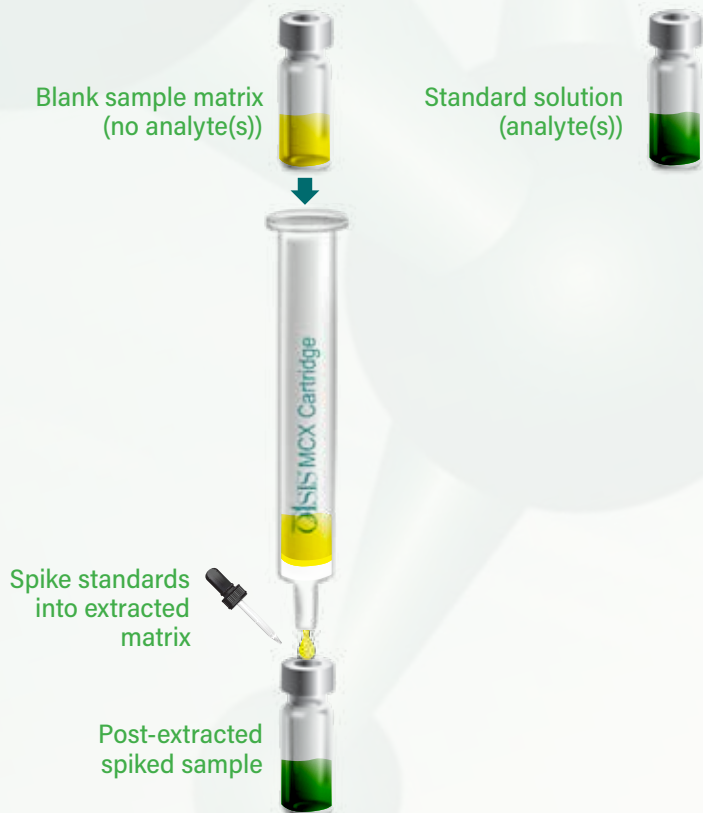
Both extracted samples should be in the same solution

*Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M. Anal. Chem. 2003, 75, 3019-3030.

Calculating Matrix Effects

To determine the success of the SPE method, there are two key parameters that must be evaluated. These are **recovery** and **matrix effects**. Recovery will determine how successfully the SPE method has isolated your compound(s) of interest. **Matrix effects** will determine if you have removed matrix components that may interfere with your ability to accurately and consistently quantify your compound(s).

Matrix Effects Calculation

Blank sample matrix
(no analyte(s))

Standard solution
(analyte(s))

Oasis MCX Cartridge

Spike standards
into extracted
matrix

Post-extracted
spiked sample

Matrix effects and matrix factor

$$\text{Matrix factor (MF)} = \frac{\text{Response}_{\text{Presence of matrix}}}{\text{Response}_{\text{Absence of matrix}}}$$

$$\text{\% Matrix effects (ME)} = \left(\left(\frac{\text{Response}_{\text{Post-extracted spiked sample}}}{\text{Response}_{\text{Solvent standard}}}\right) - 1\right) * 100$$

- Both samples should be in the same composition solution
- MF Value <1, negative % ME = suppression
- MF Value >1, positive % ME = enhancement

Calculating Recovery and Matrix Effects

Example of Recovery Sample Preparation

Post-extracted spiked sample

500 µL blank matrix

SPE – elute with 50 µL

Post-spike solvent – 50 µL of a 100 ng/mL = 5 ng (analyte)

50 µL extract

Extracted sample

500 µL matrix with 10 ng/mL analyte

500 µL of a 10 ng/mL = 5 ng

SPE – elute with 50 µL

Post-spike solvent – 50 µL (no analyte)

50 µL extract
5 ng analyte

- Both vials contain matrix components from 500 µL matrix, (eluted with the 50 µL elution solvent), 50 µL post-spike solvent, and theoretically an equivalent to 5 ng analyte in each solution.

Example of Matrix Effects Sample Preparation

Post-extracted spiked sample

500 µL blank matrix

SPE – elute with 50 µL

Post-spike solvent – 50 µL of a 100 ng/mL = 5 ng (analyte)

50 µL extract

Standard solution (analytes)

Post-spike solvent 50 µL of a 100 ng/mL

50 µL elution solvent

** Special consideration for preparing post-spiked solvent. Due to peptide solubility and non-specific binding considerations, we suggest adding 50 µL of stock solution containing carrier protein and organic (e.g., 20/80 ACN/water with 1% FA, containing 0.05% rat plasma).

- Both vials contain 5 ng analyte (50 µL elution solvent and 50 µL post-spike solvent).
- The post-spike sample also contains the components extracted from the sample matrix.

Experimental Set-Up

## Definitions for SPE Plate and Collection Plate Samples

96-Well plate template for recovery and matrix effects experiment

Will be used for recovery AND matrix effects calculations	Will be used for recovery calculations	Will be used for matrix effects calculations
Post-extracted spiked sample (PESS)	**Extracted sample (ES)**	**Standard solution (SS)**
Run your blank sample matrix through the SPE process then spike the standards directly into these wells at the end.	Spike your standards into the sample matrix before the SPE process and collect the final eluate in these wells.	Pipette the final elution solution used in the SPE protocol into these wells, then spike in the standards. No SPE performed into these wells.
4 replicates	4 replicates	4 replicates

Template for 96-well Plate Experiment to Determine Recovery and Matrix Effects
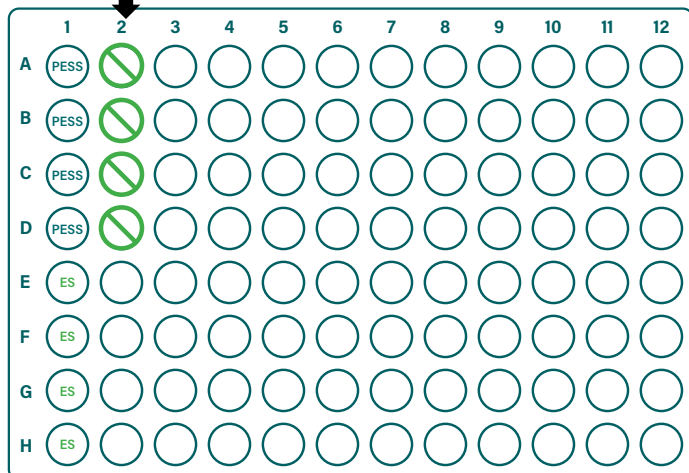
PESS = Post-extracted spiked sample, ES = Extracted sample
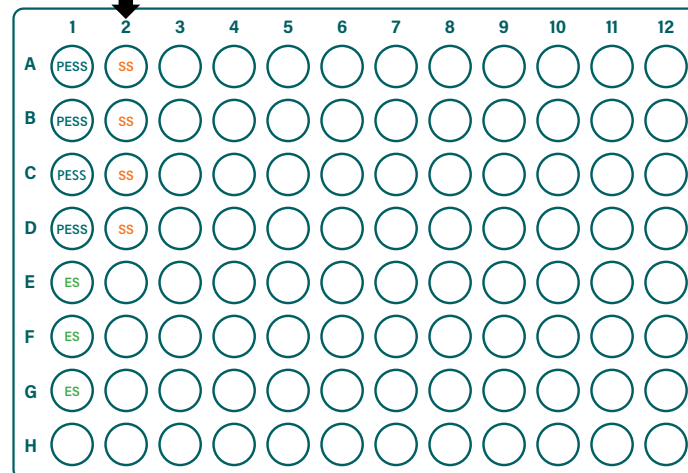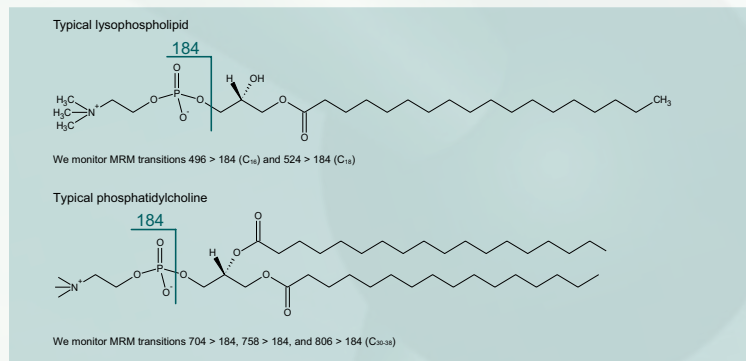
PESS = Post-extracted spiked sample, ES = Extracted sample, SS = Standard solution

Phospholipid Monitoring

You may wish to monitor the presence of phospholipids in your final sample to evaluate the degree of their removal during the SPE process. Phospholipid removal not only increases method robustness by reducing a common cause of matrix effects, it also increases instrument uptime and column lifetime. There are two common techniques used to monitor the presence of phospholipids. The first approach is to monitor 5 or more MRM transitions from individual phospholipids. The second approach is to monitor 1 MRM transition, the 184.4 fragment common to the polar head group of phosphatidylcholine containing phospholipids, the most abundant type. Either of these methods provides a good representation of the overall cleanliness of your sample.
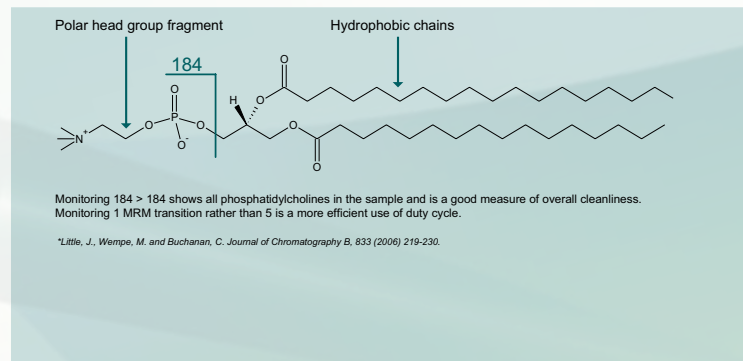
Phospholipid monitoring MS method 1:
Monitoring individual MRM transition

Typical lysophospholipid

We monitor MRM transitions 496 > 184 (C$_{16}$) and 524 > 184 (C$_{18}$)

Typical phosphatidylcholine

We monitor MRM transitions 704 > 184, 758 > 184, and 806 > 184 (C$_{30-38}$)

Phospholipid monitoring MS method 2:
Monitoring all phosphatidylcholines*

Polar head group fragment   Hydrophobic chains

Monitoring 184 > 184 shows all phosphatidylcholines in the sample and is a good measure of overall cleanliness. Monitoring 1 MRM transition rather than 5 is a more efficient use of duty cycle.

*Little, J., Wempe, M. and Buchanan, C. Journal of Chromatography B, 833 (2006) 219-230.

Mass Spectrometry conditions for phospholipid monitoring

Precursor ion (m/z)	Product ion (m/z)	Cone voltage	Collision energy
184.40**	184.40	90	3
496.40[†]	184.40	35	30
520.40	184.40	35	30
522.40	184.40	35	30
524.40*	184.40	35	30
704.40*	184.40	35	30

Precursor ion (m/z)	Product ion (m/z)	Cone voltage	Collision energy
758.40*	184.40	35	30
760.40	184.40	35	30
784.40	184.40	35	30
786.40	184.40	35	30
806.40*	184.40	35	30
808.40	184.40	35	30

** For individual MRM method
[†] Most frequently monitored

Troubleshooting Recovery

Problem: Exceeding capacity of the SPE sorbent, peptides pass through un-retained during load step.

Solution 1: Remove some matrix components prior to SPE, for example, using protein precipitation (1:1).

Solution 2: Reduce sample size/amount loaded onto sorbent.

Solution 3: Move to a larger sorbent mass, but use caution as dry down/evaporation steps can cause peptide losses.

Problem: Poor recovery of very polar peptides

Solution 1: Insufficient ion-exchange binding causes breakthrough of peptides on loading step. Most peptides retain by reversed-phase mechanism on load. Thus, some polar peptides may require modification of the pre-treatment step to change the ionization state of the compound, improving its initial retention.

Solution 2: Polar peptides can also be lost in the washes. For base labile analytes, consider substituting wash 1 for 10 mM phosphate buffer (pH7) or 10 mM ammonium acetate (pH~6) and/or decrease % organic of wash 2.

Problem: Peptide is not eluting from the SPE sorbent, but it is not detected in the load or wash steps. It is stuck on the sorbent. This is likely due to poor solubility.

Solution 1: Incorrect elution solvent or insufficient volume to ensure complete elution of peptides from the SPE sorbent.

Solution 2: Increase elution strength (no higher than 75% organic) or elution volume.

Solution 3: Reduce elution speed from SPE plate. Ensure slow, discreet droplets in elution.

Solution 4: The peptides have precipitated in organic concentration that is too high, reduce organic concentration to be less than 75%.

Solution 5: Add a higher concentration of modifier to promote solubility (more acid or base).

Problem: Recovery out of sample matrix is lower than recovery out of standard solution.

Solution 1: Failure to disrupt protein binding. Modify sample pre-treatment by adding 4% H_3PO_4 or 5% NH_4OH to plasma, or try doing a protein precipitation before SPE (1:1). In some cases, protein denaturation with detergents like SDS or guanidine HCl may be necessary.

Solution 2: Capacity of the SPE sorbent is insufficient for peptide retention due to competing matrix interferences saturating the binding sites of the sorbent. Either reduce sample mass/volume loaded onto the sorbent, or increase sorbent amount.

Important SPE Considerations

Flow rate

Low recovery can be caused by loading or eluting too fast (too much vacuum pressure/or too fast speed). Due to inherent size of peptides, resonance time (getting in and out of chromatographic pore) needs to be considered. Flow rates should not exceed 1.0 mL/min, then increase the vacuum to about 15–20" Hg to ensure that all solvent has passed through.

Sample pre-treatment

This step is essential to make sure that your analytes of interest are contained within a solution appropriate for your SPE protocol. For example, analytes in tissue or blood samples may need to be extracted into a separate solution prior to SPE. In addition, any drug-protein binding must be disrupted before SPE in order for the analytes of interest to be retained. This is often achieved by diluting the sample (i.e. plasma) 1:1 with a 4% H_3PO_4 (phosphoric acid) solution, to a final concentration of 2% H_3PO_4. In some cases, stronger disruptive action may be needed. Please see the Sample Pre-treatment section for additional suggestions.

Ionization states

When using the mixed-mode sorbents, it is important to think not only about the charge of your analyte of interest, but about the charge of the SPE sorbent as well. Strong ion-exchange sorbent will always be in a charged state. Weak ion-exchange sorbent can be charged or uncharged, depending on the pH of the solution flowing through the sorbent. It is important to understand the impact of these charge states on your sample. As a general rule, operate at least 2 full pH units away from the pI of the analytes and/or the pKa of the sorbent.

Sample Pre-Treatment

Plasma

The standard pre-treatment for plasma is a 1:1 dilution with 4% phosphoric acid. This dilutes the sample, decreasing viscosity and increasing the contact time with the sorbent. It also helps to disrupt protein binding. If the sample needs to be at a different pH for loading, try diluting 1:1 with 5% strong ammonia or with another appropriate buffer. If acid or base sample pre-treatment is not sufficient enough to disrupt protein binding, precipitation with an organic solvent may be necessary. Typical protein precipitation is not advised for peptides. A 1:1 protein precipitation is usually sufficient to ensure peptide recovery. Dilute the sample with aqueous such that the final concentration of organic is no more than 10–20%, or break-through during loading may occur.
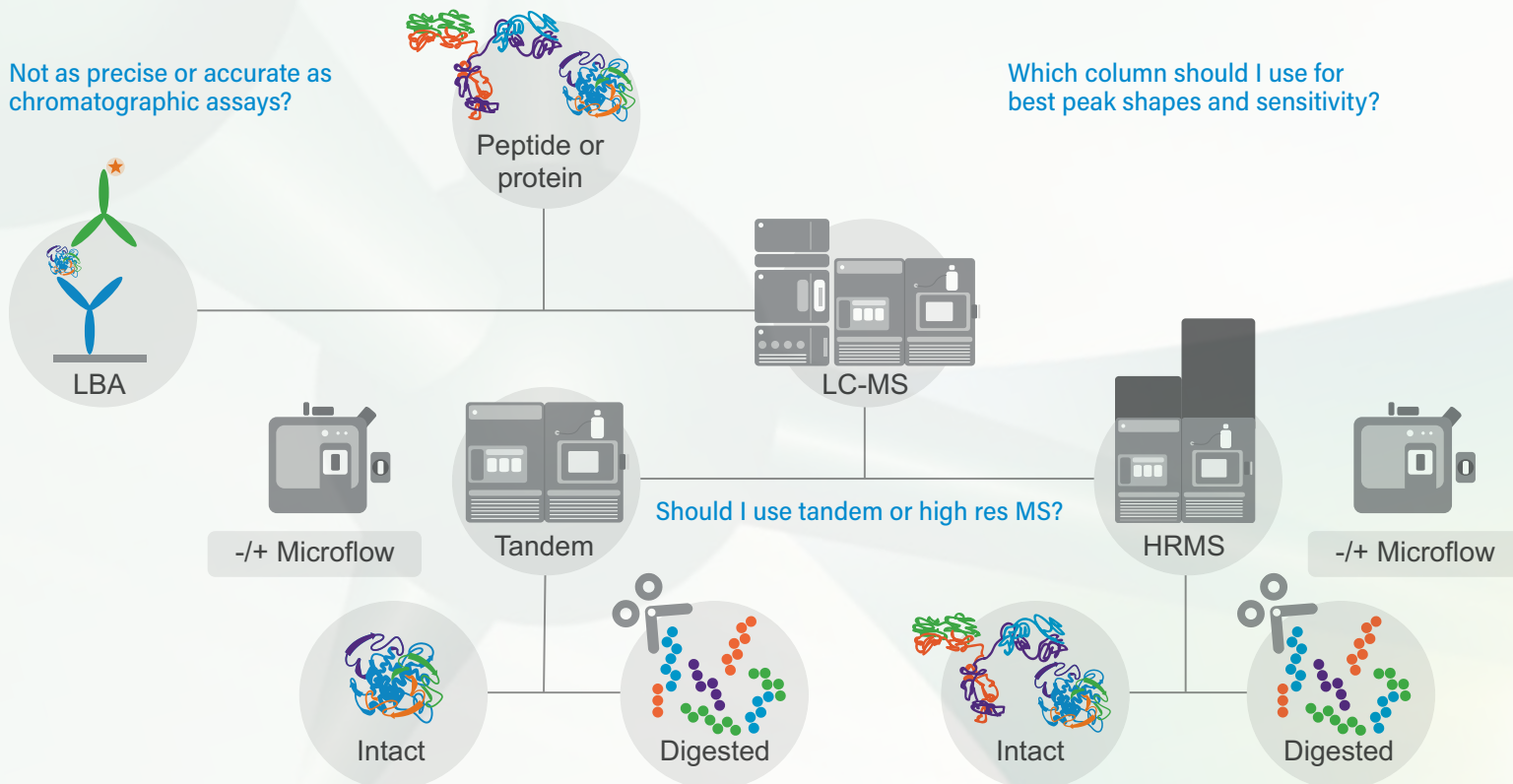
Whole blood

When preparing whole blood samples, the blood cells need to be lysed and the entire sample must be precipitated prior to SPE. For cell lysis, a 1:1 or 1:2 dilution with 0.1 M $ZnSO_4$ is usually sufficient. For example, 100 µL of whole blood can be treated with 50 or 100 µL of $ZnSO_4$. A solution of 0.1 M $ZnSO_4$ and 0.1 M ammonium acetate (NH_4OAc) can also be used. Following cell lysis, precipitate the sample with 1:1, organic solvent: sample.

Urine

Urine is the most straightforward matrix to pre-treat. It should be diluted 1:1 with an appropriate aqueous solution. Water is usually sufficient for reversed-phase SPE, or if using mixed-mode ion-exchange sorbent, good choices include 4% phosphoric acid or 5% strong ammonia. It is important to make sure your analyte and/or sorbent are in the correct ionization state for loading onto the sorbent. If buffering to a specific pH is required, make sure to use a high enough molarity solution to overcome the natural buffering capacity of urine. Also, be aware of sorbent capacity when using ion-exchange sorbents for urine extractions.
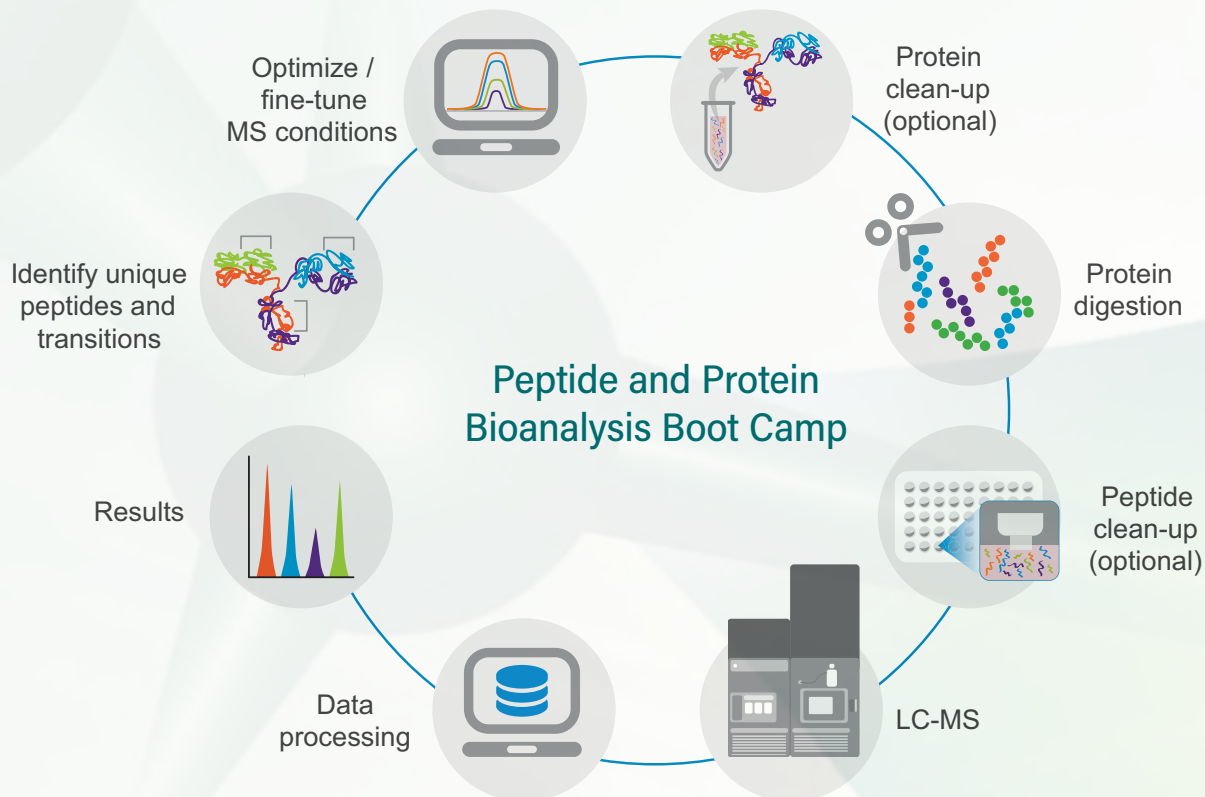
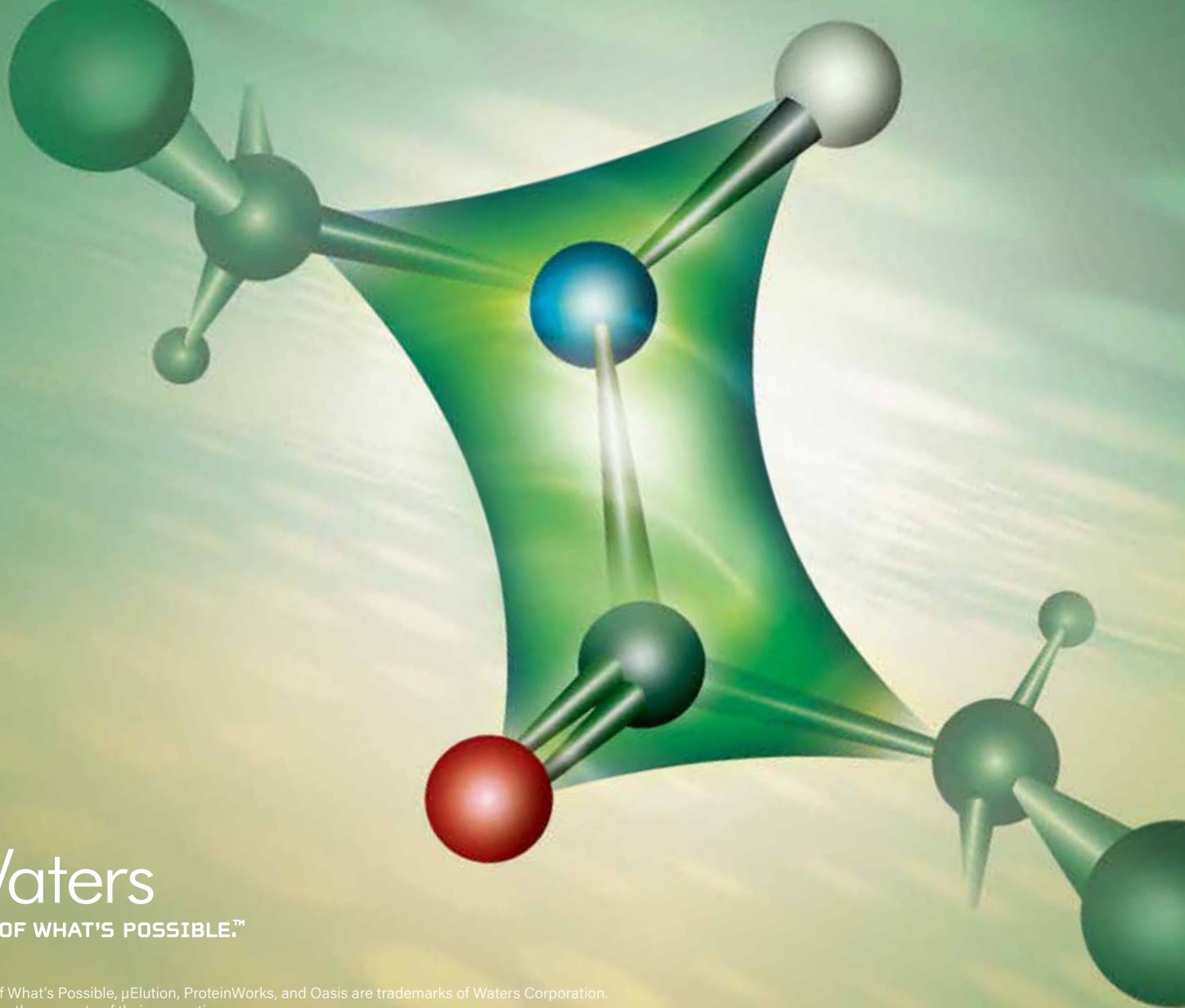# Peptide and Protein Bioanalysis Boot Camp

Where can I find the answers to these, and more questions?

Not as precise or accurate as chromatographic assays?

Peptide or protein

Which column should I use for best peak shapes and sensitivity?

LBA

LC-MS

-/+ Microflow

Tandem

Should I use tandem or high res MS?

HRMS

-/+ Microflow

Intact

Digested

Intact

Digested

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™