

FACTORS THAT INFLUENCE THE RECOVERY OF HYDROPHOBIC PEPTIDES DURING LC-MS SAMPLE HANDLING

Moon Chul Jung, Kim Haynes, Markus Wanninger, Waters Corporation, 34 Maple Street, Milford, MA 01757

INTRODUCTION

Successful protein and peptide quantitation using LC-MS requires continuous attention to detail in each step of the workflow, from sample preparation to chromatographic separation and mass spectrometry detection. One frequently overlooked fact is that the analytes can be lost before LC-MS injections, especially in the sample container, such as a collection plate or a vial. Some proteins and peptides in solution tend to stick to the surface of the container, and may be permanently lost. This non-specific binding (NSB) can lead to inaccurate quantitative results, and thus can limit the LC-MS assay capability. Using a blocking agent has been the most common workaround to mitigate NSB problems, but it may not be fully compatible with the downstream LC-MS conditions. In this study, we review multiple factors that affect the recoveries of peptides and propose a systematic approach to prevent the losses without using a blocking agent.

METHODS

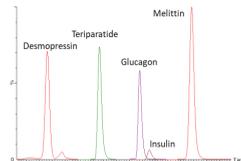
LC-MS Setup

LC: ACQUITY UPLC I-Class with a Fixed Loop injector
 MS: Xevo TQ-S with a Universal Source
 Column: CORTECS C₁₈+ Column, 90 Å, 1.6 µm, 2.1 x 50 mm at 55 °C
 Injection: 10 µL full loop injections
 Mobile phase A: 0.1% formic acid in water
 Mobile phase B: 0.1% formic acid in acetonitrile
 Flow rate: 0.5 mL/min
 Gradient: 15 to 45% B in 1.2 min followed by 95% B wash for 0.5 min, and re-equilibration for 0.6 min
 Total cycle time: 2.8 min

Peptide Sample Preparation

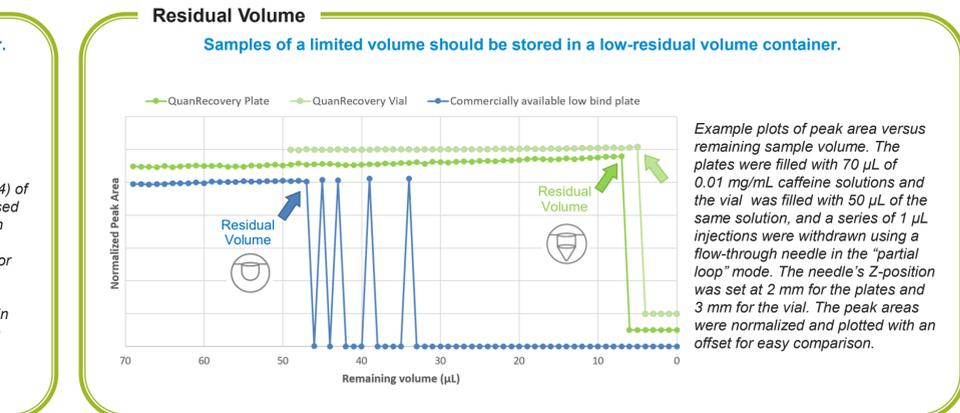
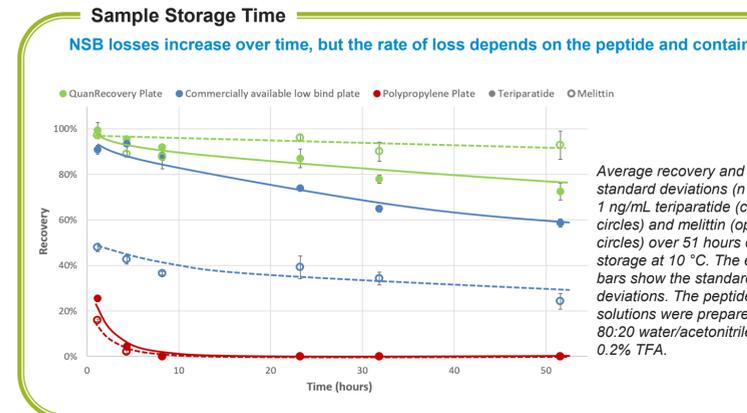
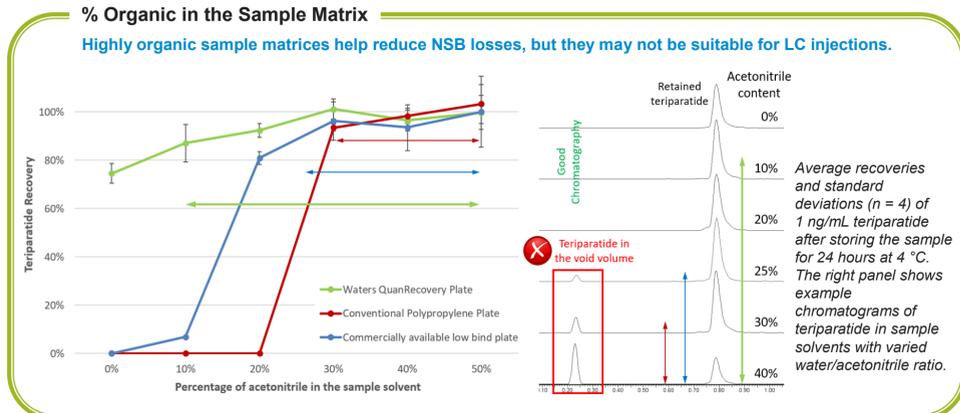
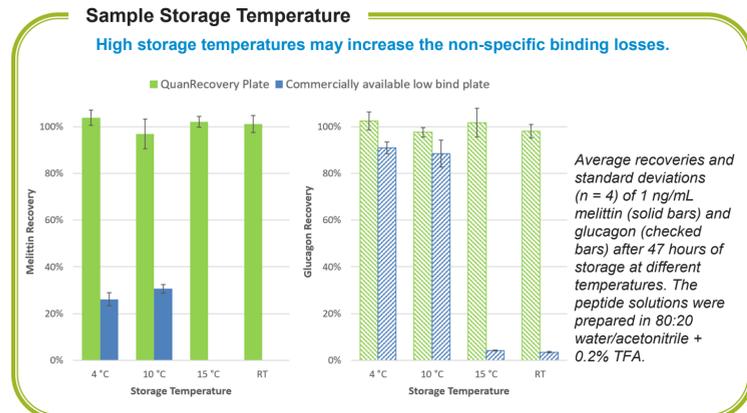
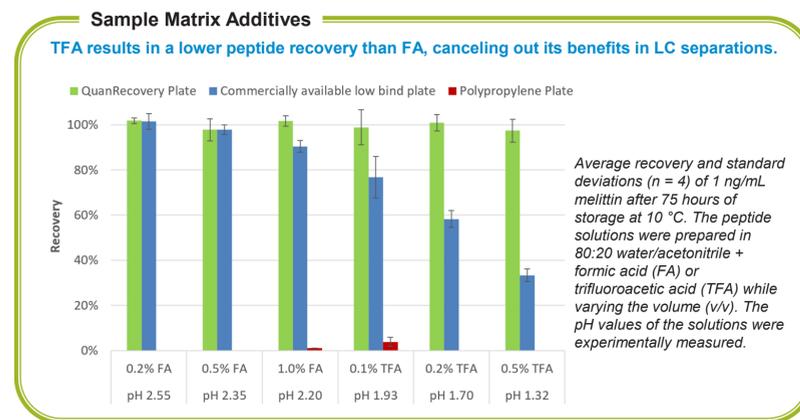
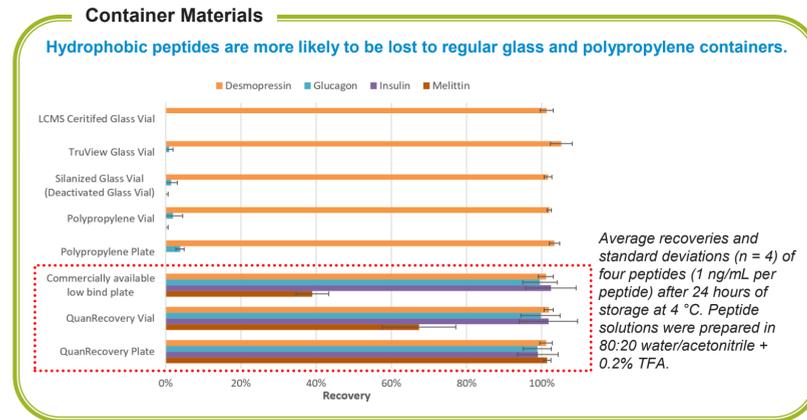
For all peptide recovery measurements, the peptide samples were prepared in two groups. One group was prepared in neat water-acetonitrile solution with an acidic or basic modifier. The other group, which served as references, was prepared in the same water-acetonitrile solution plus 0.1% rat plasma as carrier proteins. The peptide recovery was calculated by comparing the peptide peak area from the solution that did not contain rat plasma to the reference peak area.

Other conditions, such as sample storage temperature and duration, composition of the peptide solution, peptide concentration, etc. were varied to clearly highlight how these experimental factors affected peptide recoveries.



	MW	pI	HPLC index
Desmopressin	1069	8.6	16.8
Teriparatide	4118	9.1	90.4
Glucagon	3482	7.3	86
Insulin	5734	5.3	>120
Melittin	2846	12.1	124.4

RESULTS



DISCUSSION

Simple experimental factors, such as temperature, storage time, sample matrix composition, all influenced the peptide recovery. Because these factors are dependent on each other, finding the optimal condition for maximum recovery by trial-and-error could be quite challenging. Here we propose a systematic guideline to optimize the storage conditions.

Step 1. Choose an appropriate container for the analytes of interest

Analyte loss from NSB may be due to ionic interactions, hydrophobic interactions, and/or any other interactions. Mitigating NSB requires weakening all possible interactions between the analyte and the surface.

Analytes with basic groups → Avoid glass containers

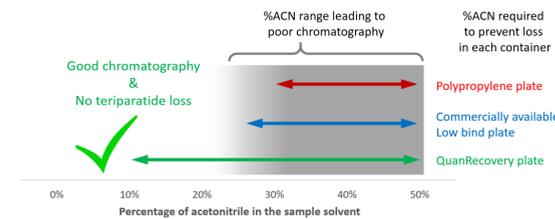
Note that many proteins and peptides have basic surface groups even though their overall pI is neutral or acidic. Therefore, it is highly recommended to avoid glass containers for protein and peptide samples.

Hydrophobic peptides, such as glucagon, insulin, melittin, were completely lost in all glass containers and polypropylene containers [See [Container Materials](#)]. In contrast, containers designed to reduce hydrophobic adsorption showed good recoveries, while their recovery values are different. The most hydrophobic peptide, Melittin, shows the greatest difference in recovery among containers.

Hydrophobic analytes → Use specially designed containers

Step 2. Select a sample solvent that reduces analyte loss while being compatible with the overall workflow

Protein and peptide NSB in a polypropylene container is driven by hydrophobic interactions, which can be modulated by changing the acetonitrile content in the sample solvent. However, this approach may not be compatible with downstream LC-MS analyses because proteins and peptides may not retain on the column [See [% Organic in the Sample Matrix](#)]. Different acidic modifiers also affected the recovery and the peak shape [See [Sample Matrix Additives](#)]. Only samples prepared in QuanRecovery plates could achieve maximum teriparatide recovery without impacting the downstream LC-MS analyses.



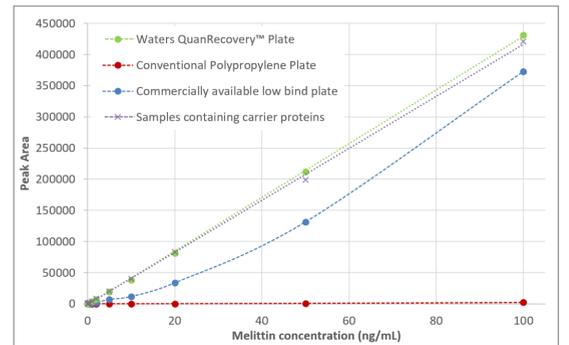
Step 3. Select an optimal condition for sample storage before LC-MS analysis

Protein and peptide NSB is affected by many other factors, such as concentration, storage time, sample handling, and storage temperature. When all other conditions were the same, samples stored in QuanRecovery Vials or Plates consistently showed a better recovery than samples stored in other containers. This superior NSB protection provides a wide range of options without impacting protein and peptide recovery.

Another factor to consider is the residual volume [See [Residual Volume](#)]. A considerable fraction of the sample may not be available for injection if the sample container has a high residual volume. Samples of limited volume should not be stored in such containers.

CONCLUSIONS

Mitigating the non-specific binding losses of challenging peptides is crucial for successful quantitative analysis. If the loss is not mitigated, the assay may suffer from low sensitivity, poor reproducibility, and unreliable results. The following example shows how non-specific binding losses may distort the calibration curve to make a peptide quantification assay unreliable.



Example calibration curves (50 pg/mL ~ 100 ng/mL) showing the non-linear relationship between the sample concentration and the peak area when there are analyte losses (red and blue). Compare with the straight calibration curves when the losses were avoided by adding 0.1% rat plasma (blocking agent or carrier protein) to the sample solutions (purple, $r^2 = 0.998$) or by preparing the sample in a QuanRecovery plate without using carrier proteins (green, $r^2 = 0.997$). All samples were prepared in 80:20 water/acetonitrile + 0.2% TFA, and stored at 4 °C for 24 hours before analyses.

- Proteins and peptides may adsorb to sample containers while waiting for LC-MS injections, and may not be recovered.
- Such losses are detrimental to the assay because they negatively affect recovery, sensitivity, and reproducibility.
- Follow these steps to optimize the sample storage conditions to prevent losses in the containers.
 - Choose an appropriate container.
 - Select a compatible sample solvent.
 - Select an optimal sample storage condition.

References

- M.C. Jung, Achieving Maximum Protein and Peptide Recovery, Sensitivity, and Reproducibility using QuanRecovery Vials and Plates, *Waters White Paper*, 72006543EN (2019)
- Bobaly, B., Sipko, E. & Fekete, J. Challenges in liquid chromatographic characterization of proteins, *J Chromatogr B Analyt Technol Biomed Life Sci* **1032**, 3-22 (2016).
- Rabe, M., Verdes, D. & Seeger, S. Understanding protein adsorption phenomena at solid surfaces, *Adv Colloid Interface Sci* **162**, 87-106 (2011)