A SYSTEMATIC APPROACH FOR PREVENTING THE LOSS OF HYDROPHOBIC PEPTIDES IN SAMPLE CONTAINERS

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INTRODUCTION

Successful protein and peptide quantitation using LC-MS requires continuous attention to detail in each step of the workflow, from sample prep 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. 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 blocking agents.

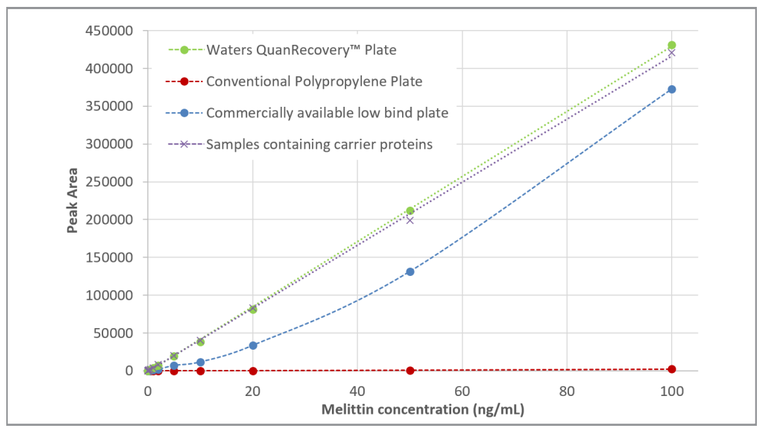


*Figure 1. Example calibration curves (50 pg/mL ~ 100 ng/mL) showing the non-linear relationship between the sample concentration and the peak area when there are analyte losses (**red** and **blue**). Compare with the straight calibration curves when the losses were avoided by adding 0.1% rat plasma (blocking agent/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.*

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

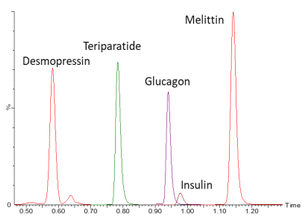
Separation: 0.5 mL/min, 15 to 45% B gradient in 1.2 min followed by 95% B wash for 0.5 min, and re-equilibration for 0.6 min

Peptide Sample Preparation

The solutions were prepared in groups with and without 0.1% rat plasma as carrier proteins: the peptide solutions containing rat plasma were used as recovery references. 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 sample storage conditions, such as storage time, 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 AND DISCUSSION

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

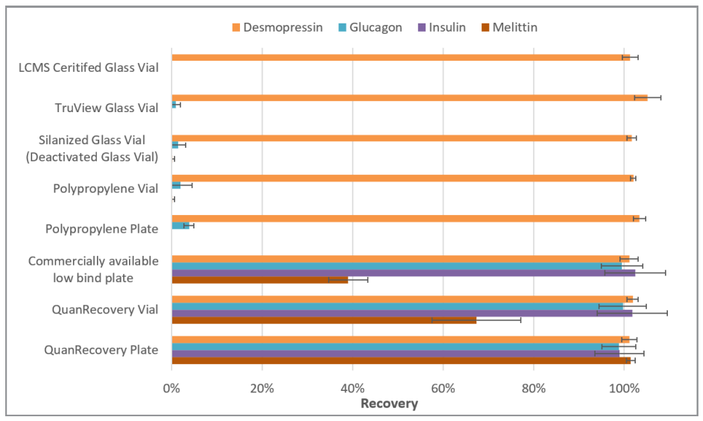


Figure 2. Average recoveries and standard deviations (n = 4) of four peptides (1 ng/mL per peptide) after 24 hours of storage at 4 °C. Peptide solutions were prepared in 80:20 water/acetonitrile + 0.2% TFA.

Analyte loss from NSB may be due to ionic interactions, hydrophobic interactions, and/or any other interactions. Mitigating NSB thus requires weakening all 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. Thus it is highly recommended to avoid glass containers for protein and peptide samples.

Figure 2 shows that hydrophobic peptides are completely lost in all glass containers, regardless of the surface treatment on the glass surface. Polypropylene vials and plates showed almost no recovery for hydrophobic peptides. In contrast, containers designed to reduce hydrophobic adsorption showed good recoveries, while their recovery values are different. The most hydrophobic peptide, Melittin, shows the greater 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

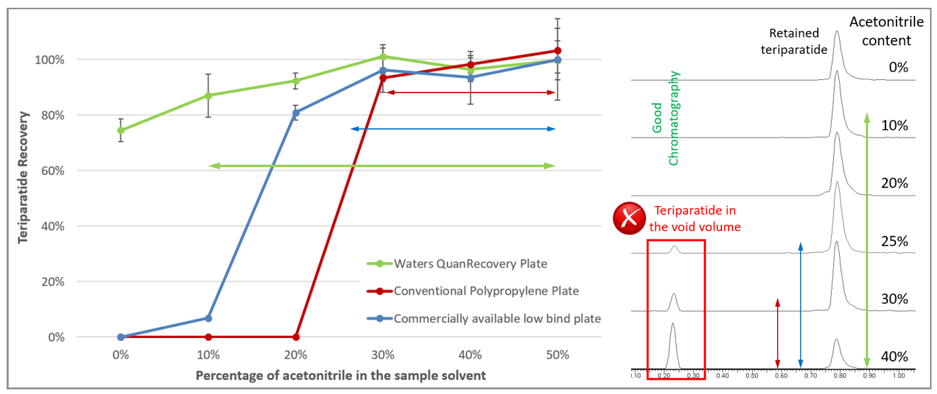
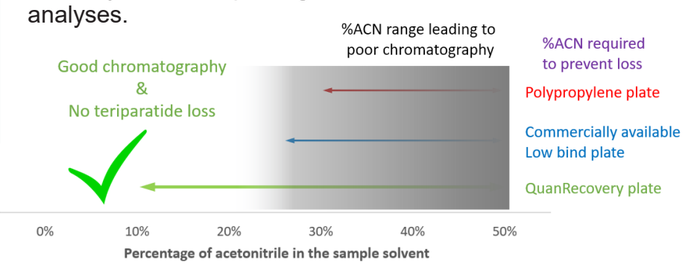


Figure 3. Average recoveries and standard deviations (n = 4) of 1 ng/mL teriparatide after storing the sample for 24 hours at 4 °C. The right panel shows example chromatograms of teriparatide in sample solvents with varied water/acetonitrile ratio.

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 (Figure 3). In this example, 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

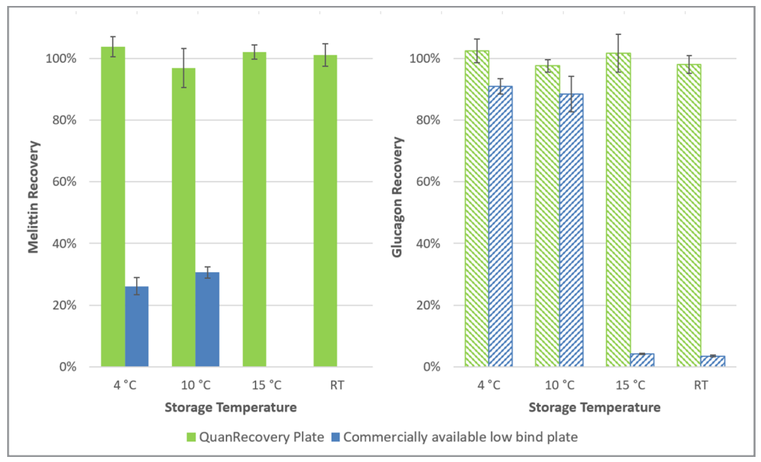


Figure 4. Average recoveries and standard deviations (n = 4) of 1 ng/mL melittin (solid bars) and glucagon (checked bars) after 47 hours of storage at different temperatures. The peptide solutions were prepared in an 80:20 water/acetonitrile mixture and acidified with 0.2% TFA.

Protein and peptide NSB is affected by many other factors, such as concentration, storage time, sample handling, and storage temperature. Figure 4 shows the effect of storage temperature on peptide recovery, demonstrated using melittin and glucagon. Both peptides showed reduced recovery when they were stored in commercially available low bind plates. The recovery further decreased if they were stored at a temperature higher than ~10°C. The same samples stored in the QuanRecovery plates showed excellent peptide recoveries, even at room temperature (~25 °C). Using an inert container thus provides a wide range of options without impacting protein and peptide recovery.

CONCLUSIONS

- **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 sample losses in the containers.**
  1. **Choose an appropriate container.**
  2. **Select a compatible sample solvent.**
  3. **Select an optimal sample storage condition.**

References

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