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Achieving Maximum Protein and Peptide Recovery, Sensitivity, and Reproducibility using QuanRecovery Vials and Plates

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WHAT IS NON-SPECIFIC BINDING AND WHY IS IT IMPORTANT IN QUANTITATIVE ASSAYS?

All successful quantitative LC-MS assays have something in common: high selectivity to accurately detect and identify analytes of interest, high sensitivity to precisely quantify the analytes at very low concentrations, and high reproducibility to ensure that the results can be trusted. While recent advancements in sample preparation methods and LC-MS technologies continue to push the envelope, the demand for greater selectivity, sensitivity, and reproducibility is growing at an even faster pace due to increasing sample complexity and decreasing detection limits. It has become almost impossible to develop a successful LC-MS assay method unless the whole workflow is scrutinized and optimized accordingly. One crucial yet often overlooked step that significantly influences assay sensitivity and reproducibility is sample storage before LC-MS analyses. It is surprisingly common that analytes stick to the surface of sample containers and are never recovered[1,2]. More alarmingly, these losses may not be recognized unless the data are carefully compared in a controlled manner. Such surface adsorption, and the consequent analyte loss, is referred to as non-specific binding (NSB) or non-specific adsorption (NSA).[3]

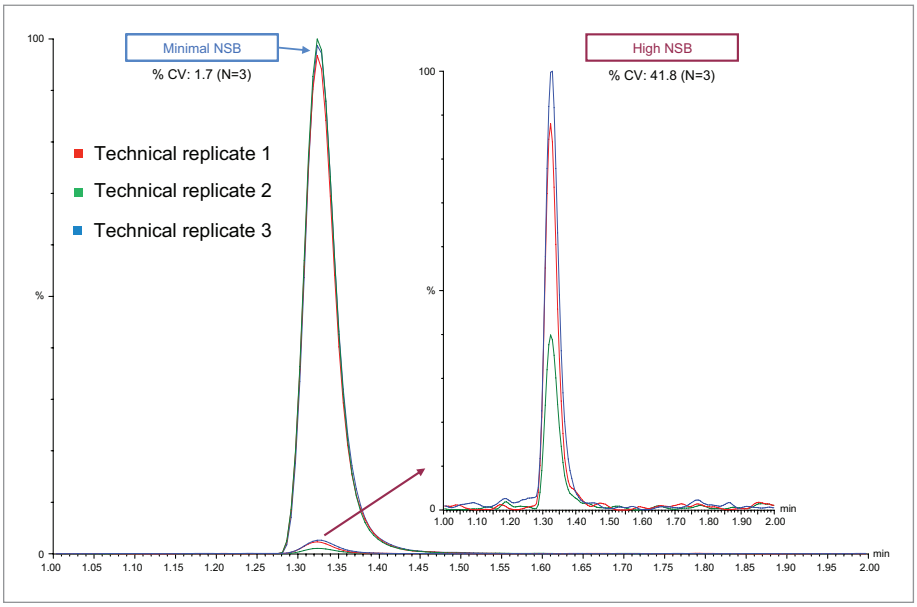

Figure 1. Comparison of leuprolide LC-MS/MS peaks (n = 3) with and without mitigating NSB. Note that the y-axis scales are not equal for the two panels: the scales are 1:50, also signifying the leuprolide losses if NSB is not properly mitigated.

The most obvious sign of NSB in a sample container is reduced peak area and consequent poor assay sensitivity. A less readily noticed but equally detrimental outcome of NSB is increased assay variability. Figure 1 shows example chromatograms of samples containing the peptide leuprolide (MW 1209.4) which were prepared with and without mitigating NSB. When there was NSB, the variation in triplicate leuprolide peak areas was as high as 41.8% CV. When leuprolide was not lost from NSB, the peaks were 50 times larger and the variability decreased significantly to only 1.7% CV. While it is a serious headache if found during method development, finding this problem at a later stage causes more troubles because this may require re-developing and re-validating the assay method. Failure to mitigate NSB early enough can thus lead to a significant waste of time and resources.

TYPICAL METHODS TO PREVENT SAMPLE LOSSES DUE TO NON-SPECIFIC BINDING

Non-specific binding happens at any surfaces that the analyte molecule has a chemical affinity to. And it is worth mentioning that sample containers are not the only place where NSB happens.[4] The two most common mechanisms for NSB are ionic interactions and attractions based on polarity. Once the interaction mechanism is identified, the NSB can be effectively mitigated by weakening the chemical affinity between the analyte molecule and the surface. Increasing the organic content in sample solutions or changing the solution pH are examples. This approach alone works well with relatively simple small molecules because their NSB is typically driven by a single attraction mechanism at a single functional site. In contrast, larger and more complex molecules, such as proteins and peptides, may form multiple heterogeneous affinity interactions with the surface to promote adsorption. Changing the composition of sample solutions may weaken one or two affinity mechanisms but may not affect other attractions. To make things worse, some folded proteins may change conformation upon binding to a surface,[5] forming additional affinity interactions and further strengthening the surface adhesion. It is more difficult to detach such deformed and adsorbed proteins, and thus it is most effective to prevent NSB before it happens.

Another effective strategy to mitigate NSB is coating the surface with something stickier ('blocking agent') so that the less sticky analytes of interest do not bind to the surface.[4,6] This is a popular approach to mitigate NSB of proteins and peptides because this technique interferes with various attraction mechanisms simultaneously by placing a blocking barrier between the surface and analyte molecules. Common blocking agents are large polymers, detergents such as TWEEN®-20 (Sigma-Aldrich) and Triton X-100, and carrier proteins such as serum albumin, casein, and plasma. While generally effective, polymers and detergents are detrimental to LC-MS analyses because they may change column selectivity and induce ion suppression. They can also be extremely difficult to remove from columns and LC-MS systems, shortening column life and requiring frequent system maintenance. A carrier protein is a more LC-MS friendly blocking agent, but it is not without any shortcomings. It adds unwanted complexity back into samples. This may complicate the LC-MS baseline of full scan or untargeted methods while it may indirectly affect targeted SRM (selected reaction monitoring) methods through ion suppression. Carrier proteins may also behave as mild surfactants in aqueous solutions to form froth, making it difficult to precisely pipet the solutions. Some biological samples contain endogenous components that may act as carrier proteins. Their concentrations may vary among samples from different origins (matrix, species, disease state, etc.) or samples treated with different upstream sample preparation methods. This variation introduces extra assay variability. There are some cases where a carrier protein is necessary, but it is far more desirable if one can prepare protein or peptide samples without one.

QUANRECOVERY VIALS AND PLATES: A NEW OPTION TO MITIGATE NON-SPECIFIC BINDING

As mentioned earlier, it is not trivial to mitigate protein and peptide NSB by changing the composition of sample solutions alone. The effort, however, can be greatly assisted by using a container that has an inert surface. One example is using a deactivated (or silanized) glass container for a moderately basic analyte, instead of using a glass container that has a high surface silanol activity. For more strongly basic analytes, a polypropylene container with a pure hydrocarbon surface would work better than deactivated glass containers. Some proteins and peptides bind to glass surfaces via ionic attraction between their basic surface groups and the silanol groups on the surface of the glass. Such NSB is more common with proteins and peptides, especially when they are large and complex. This is because some complex proteins and peptides have multiple basic surface groups, even though their overall pI is lower than 7. It is therefore highly recommended to use polypropylene containers for protein and peptide samples to prevent ionic NSB. However, the hydrocarbon-rich surface of polypropylene is more likely to induce hydrophobicity-based attraction, promoting NSB for hydrophobic molecules. To address this challenge, Waters™ introduced QuanRecovery™ Vials and Plates with MaxPeak™ High Performance Surfaces (HPS) (Figure 2) for protein and peptide applications. QuanRecovery Vials and Plates are made with high-purity polypropylene and are designed to suppress hydrophobic NSB. QuanRecovery Vials and Plates are enabled by MaxPeak High Performance Surfaces Technologies. MaxPeak HPS are new and innovative technologies designed to increase analyte recovery, sensitivity, and reproducibility by minimizing analyte-surface interactions that can lead to sample losses. This white paper will review how hydrophobic NSB can be mitigated without using carrier proteins by optimizing the sample matrix and experimental parameters while using QuanRecovery Vials and Plates.

HOW WE MEASURED PEPTIDE RECOVERIES

Peptide solution standards, in the concentration range of 50 pg/mL to 100 ng/mL, were prepared in various sample matrices and stored in several commercially available sample containers prior to LC-MS analyses. To accurately determine the recovery of challenging peptides, solutions containing carrier proteins were used as recovery reference solutions: the solutions were prepared in groups, with and without 0.1% rat plasma, and the peptide recovery was calculated by comparing the peptide peak area from the solution that did not contain the blocking agent to the reference peak area. Peptides in each sample were separated using a 2.1 x 50 mm CORTECS™ UPLC C_{18+} Column (p/n: 186007114) on an ACQUITY™ UPLC™ I-Class PLUS System with a water/acetonitrile linear gradient, each with 0.1% formic acid, and detected using a Xevo™ TQ-S tandem quadrupole MS system in the Selective Reaction Monitoring (SRM) mode. To understand the role of the container's surface properties on peptide recovery, we used Waters polypropylene vials and 96-well plates, Waters QuanRecovery Vials and Plates, Waters TruView™ glass vials, and commercially available low bind plates. Other experimental conditions, such as composition of the peptide sample and peptide concentration, were varied to clearly highlight how these experimental factors affected peptide recoveries.

Figure 2. QuanRecovery Vials and Plates with MaxPeak HPS.

GLASS OR PLASTIC? WHICH TO CHOOSE

We showed why it is important to control NSB as early as possible, preferably during method development to avoid wasting time and resources in the future. The first step to control NSB of the target analyte is choosing the right container. While it is quite common that sample containers are selected without much consideration, the properties of sample containers, especially the material of construction and surface properties, are the most crucial factors that influence analyte NSB.

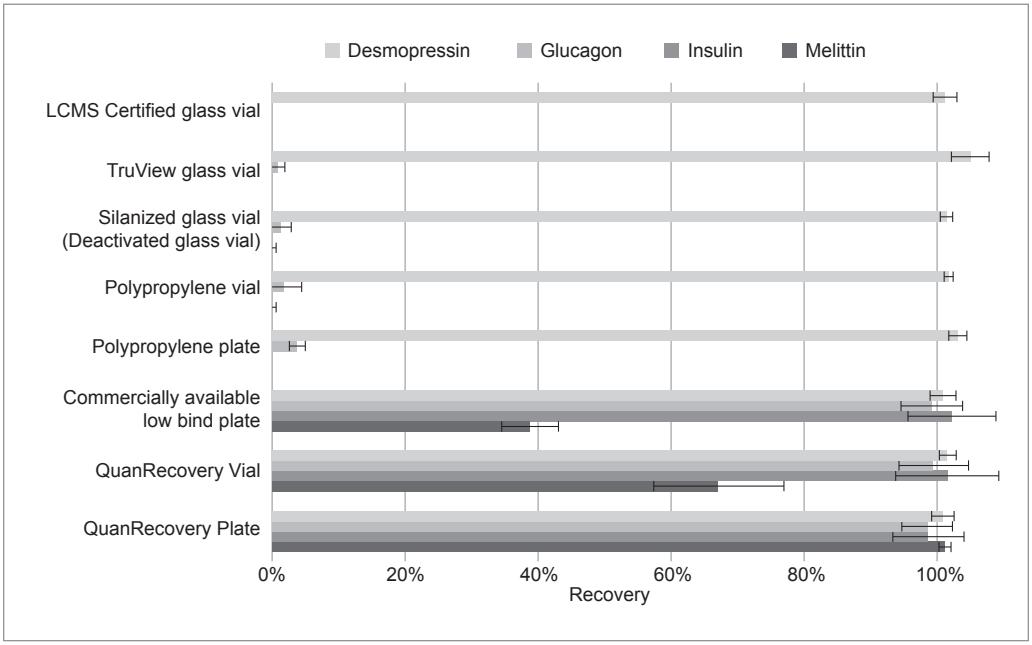

Figure 3. Average recovery (n = 4) of four peptides (1 ng/mL per peptide) after 24 hours of storage at 4 °C. The error bars show the standard deviations. Peptide solutions were prepared in 80:20 water-acetonitrile which was acidified with 0.2% trifluoroacetic acid (TFA). The more hydrophobic peptides are marked with darker grey.

Figure 3 compares the recovery of four peptides prepared in a typical LC-MS diluent and stored in various sample containers. The recoveries of peptides correlated well with their relative hydrophobicity, estimated by their calculated HPLC index numbers or by their relative retention in reversed-phase separations. The least hydrophobic peptide, desmopressin (MW 1069, HPLC index 16.8), was completely recovered from all sample containers, including glass containers and polypropylene containers. More hydrophobic peptides, glucagon (MW 3482, HPLC index 86), bovine insulin (MW 5734, HPLC index >120), and melittin (MW 2846, HPLC index 124.4), were less recovered, or more lost, in general. All glass containers, regardless of the surface treatment, and polypropylene containers showed little or no recovery for the three hydrophobic peptides. Even TruView Vials showed loss of hydrophobic peptides. This is, however, not a surprising result because TruView Vials were designed to reduce polar adsorption. As such, they can still be vulnerable to hydrophobic adsorption. In contrast, containers designed to reduce hydrophobic adsorption, such as QuanRecovery products and a commercially available low bind plate, showed good recoveries. With glucagon and insulin, there were no recovery differences between QuanRecovery products and the other low bind plate, but the difference was more obvious with the most hydrophobic peptide, melittin. It is thus confirmed that the loss of peptides in the polypropylene containers depends on their relative hydrophobicity, and we may further mitigate the losses by regulating the strength of the hydrophobic interactions.

HOW THE SAMPLE MATRIX AFFECTS PEPTIDE RECOVERY

Organic solvent content in the sample matrix

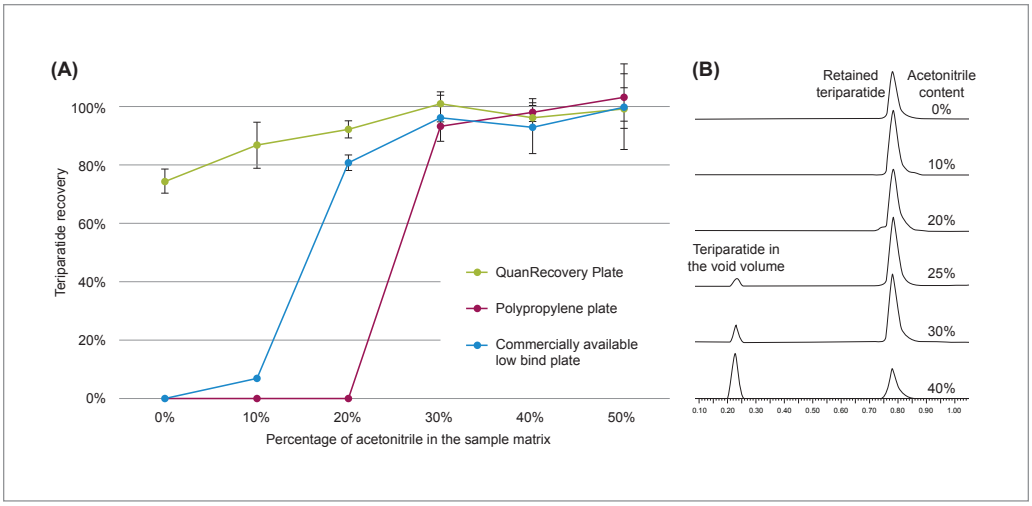

Figure 4. (A) Average recovery (n = 4) of 1 ng/mL teriparatide after 24 hours of storage at 4 °C. The error bars show the standard deviations. The peptide solutions were prepared in various water/acetonitrile mixtures and acidified with 0.2% TFA. After 24 hours of storage, the samples were diluted with appropriate water/acetonitrile mixtures to adjust the acetonitrile content to 20% before injection. The teriparatide peak quantitation, and thus the recovery calculation, was not compromised by poor retention. (B) Example chromatograms of teriparatide. Teriparatide samples were prepared in sample matrices with varied water/acetonitrile ratio and were injected without dilution.

We saw from the previous section that the peptide losses on polypropylene containers are driven by hydrophobic interactions between the analyte and the surface. While we can simply choose a container such as a QuanRecovery Vial or Plate to reduce the hydrophobic interactions, the hydrophobicity of the target analytes may not be changed easily unless the sample matrix is changed. The most effective method to regulate the strength of hydrophobic interactions between the analytes and the surface is to introduce a more organic-rich sample solution. This is in fact analogous to solid-liquid partitioning where the analytes are preferentially adsorbed onto the hydrophobic stationary phase until an organic (less hydrophilic) liquid phase is introduced, or gradient reversed-phase chromatography where the analytes are retained on a hydrophobic stationary phase until the mobile-phase strength is increased, i.e., the fraction of the organic mobile phase is increased. Figure 4A shows the same phenomenon for the recovery of teriparatide, another hydrophobic peptide (MW 4118, HPLC index 90.4), in sample matrices with varied acetonitrile content. As expected, recoveries improve as the sample matrix contains more acetonitrile, while the minimum acetonitrile concentration that led to full recovery was not the same for different sample containers. To achieve a teriparatide recovery greater than 90%, one must prepare the solution with more than 30% acetonitrile if using a polypropylene plate, and more than 25% acetonitrile if using a commercially available low bind plate. Using a QuanRecovery Plate, the sample solution can be made with as little as 10% acetonitrile.

It may appear that this difference between the sample containers is trivial, as teriparatide solutions prepared with 30% or more acetonitrile could be stored in any of the three containers without the risk of analyte loss. This is an acceptable solution only if sample storage is the last step in your analysis workflow. Otherwise, requirements from the downstream workflow should be considered as well. Especially in LC-MS, analytes in samples prepared in highly organic injection solutions may not retain well on the chromatographic column. Figure 4B shows the disrupted retention of teriparatide as the acetonitrile concentration in the sample matrix was increased. Teriparatide breakthrough peaks were observed in the void volume when the acetonitrile content in the sample matrix was equal to or greater than 25%, and thus it was necessary to prepare teriparatide samples with less than 25% acetonitrile to achieve good chromatography. As we noted from Figure 4A, we can expect to lose teriparatide if the samples were stored in either a polypropylene plate or a commercially available low bind plate. Only the samples prepared in a QuanRecovery Plate could achieve the maximum recovery without impacting the downstream LC-MS analysis.

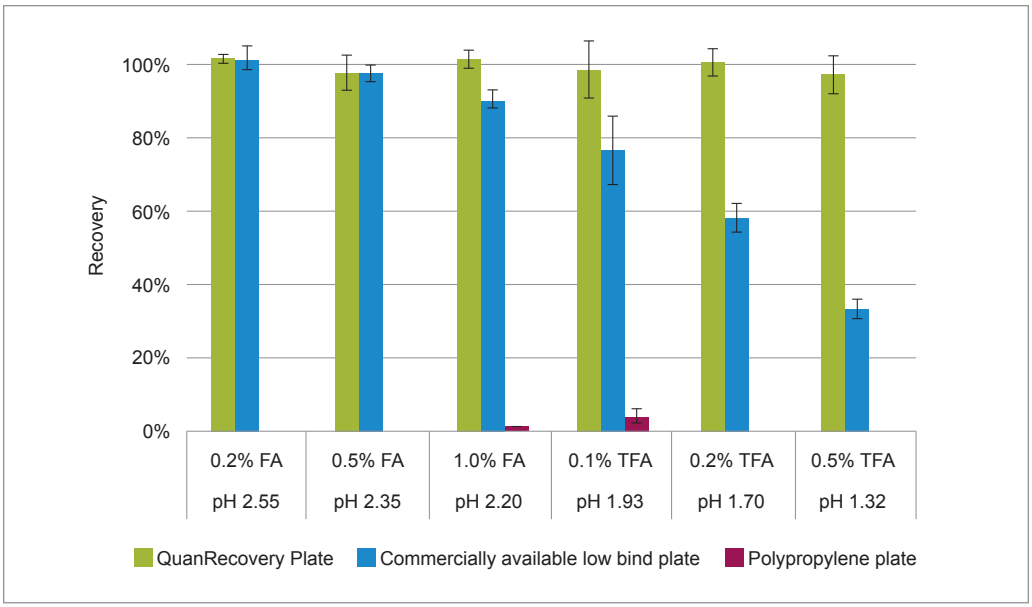

Figure 5. Average recovery (n = 4) of 1 ng/mL melittin after 75 hours of storage at 10 °C. The error bars show the standard deviations. The peptide solutions were prepared in an 80:20 water/acetonitrile mixture and acidified with formic acid (FA) or trifluoroacetic acid (TFA) while varying the volume (v/v). The pH of the solutions were experimentally measured.

The acidic/basic additives and the pH of the sample matrix

In addition to the organic solvent content in the sample matrix, the choice of the additive or the pH in the sample matrix affects the recovery of peptides. Figure 5 shows the recovery of melittin after 75 hours of storage in sample matrices prepared with various acidic additives. Using a weak acid such as formic acid at a low concentration helped increase the melittin recovery, but the increase was not as drastic as changing the acetonitrile concentration in the sample matrix. Melittin was never recovered from the polypropylene plate by changing the additive only, whereas it was completely recovered from QuanRecovery Plates regardless of the additive type and concentration. When using a plate that may have an intermediate surface binding activity, such as the other low bind plate, it is highly advised to monitor the recovery using various additives in different concentrations during method development as highlighted by the recovery changes shown in Figure 5. It should be noted that the choice of the acidic additive also has an influence on the peak shape in the downstream LC-MS analyses[7], although the effect was not as drastic as the additives in the mobile phase. Formic acid in the sample, being a volatile additive, provided a stronger MS signal but led to poor chromatographic peak shapes compared to trifluoroacetic acid.

The choice of the sample additives is not limited to acids. Sometimes it is preferred to prepare peptide samples in basic conditions to increase solubility or to achieve better downstream chromatography. Changing pH is also a versatile tool for controlling the analyte chemical properties to promote or prevent both ionic and hydrophobic interactions. QuanRecovery Vials and Plates can be used for storing basic sample solutions as well as acidic solutions. Figure 6 shows the recovery of two hydrophobic peptides, teriparatide, and enfuvirtide (MW 4492, HPLC index 155.9), in basic sample solutions (pH 11.5) containing 2% ammonium hydroxide. The two hydrophobic peptides were completely recovered from the QuanRecovery Plate while they were almost totally lost when the sample was stored in a polypropylene plate.

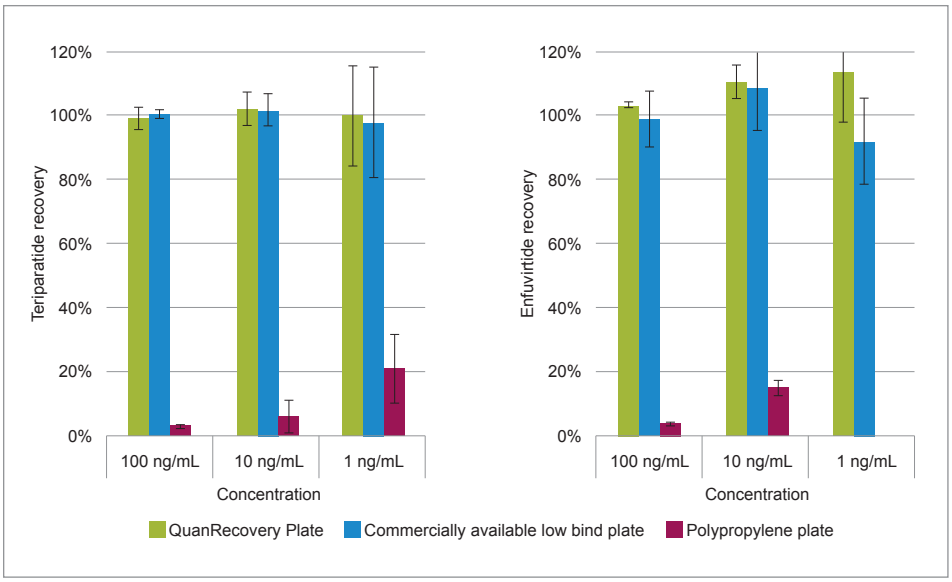

Figure 6. Average recovery (n = 3) of teriparatide and enfuvirtide at various sample concentrations after 24 hours of storage at 10 °C. The error bars show the standard deviations. The peptide solutions were prepared in an 80:20 water/acetonitrile mixture with 2% ammonium hydroxide. The pH of the solutions was 11.5 from the direct measurement. Note the increased measurement variability at lower sample concentrations (greater error bars), which also affected the accuracy of recovery from the polypropylene plate.

Figure 7 shows another example of using QuanRecovery products in extreme pH conditions. Occasionally sample plates and vials are exposed not just to moderately acidic or basic solutions but to extremely caustic solutions which can damage the surface and modify its properties. To investigate the effect of extreme acids or bases on the surfaces, sample containers were exposed to 1 M nitric acid or 1 M sodium hydroxide (pH 0 and 14, respectively) for 24 hours prior to measuring peptide recoveries. Both QuanRecovery Vials and Plates showed no change in glucagon recoveries after the exposure to either strong acid or base while the other low bind plate showed a decreased recovery after exposing it to 1 M nitric acid.

The pH of the sample matrix and the choice of additives are variables that influence peptide recoveries, but the selections may be limited according to the experimental conditions, for example, upstream and/or downstream workflows and analyte stability. Using QuanRecovery Vials and Plates, which offer maximum recoveries over a wide range of experimental conditions, makes it easier to select an optimal sample matrix condition that is compatible with the rest of the workflow while maintaining the analyte stability and recovery, in comparison with a sample container that may be used for only limited conditions.

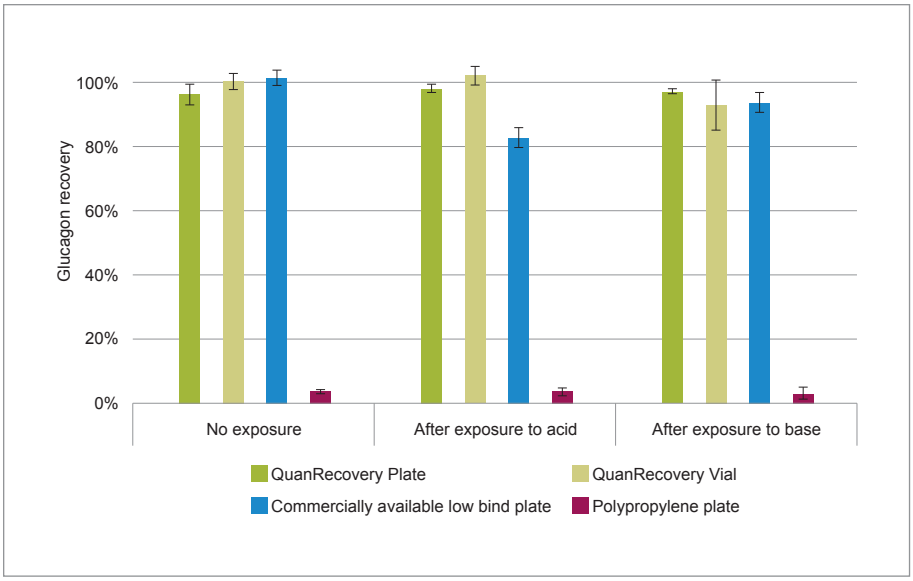

Figure 7. Average recovery (n = 3) of 1 ng/mL glucagon after 20 hours of storage at 4 °C, in sample containers previously exposed to a strong acid or base. The error bars show the standard deviations. Tested sample containers were first filled with 1 M nitric acid or 1 M sodium hydroxide solutions (pH 0 and 14, respectively) and sealed with appropriate caps to prevent evaporation. After 24 hours, the containers were emptied and thoroughly rinsed with DI water three times while aspirating the water up and down during the rinses to facilitate mass transfer in the containers. Recoveries of 1 ng/mL glucagon were measured after 20 hours of storage at 4 °C using the vials and plates that were exposed to the acid and base and then rinsed, as well as using reference vials and plates that were not exposed to the acid or base (no exposure). The glucagon solutions were prepared in an 80:20 water/acetonitrile mixture with 0.2% TFA.

THE EFFECT OF PEPTIDE CONCENTRATION ON RECOVERY

The peptide concentration is often not a variable that scientists can change. It is rather the information that scientists seek to determine as the result of an assay. This task is rather straightforward if there is no peptide loss. Unfortunately, the peptide concentration also affects the recovery and consequently makes it difficult to determine the correct concentration. In concentrations relevant to LC-MS analyses, the amount of peptide lost on the surface is more strongly dependent on the surface area and the adsorption kinetics than the peptide concentration in the solution. In a well-controlled sample set where all other factors are kept constant except the peptide concentration, the peptide recovery appears to be greater when the concentration is higher. This is because a similar mass of peptide is lost regardless of the peptide concentration, and thus the relative loss is greater in lower concentrations. This explains why analyte losses are more apparent in dilute sample solutions but not easily noticed in more concentrated samples. This non-linearity is particularly problematic when constructing a calibration curve, where the occurrence of peptide loss is easily identified from the shape of the curve.[8] Figure 8 shows the calibration curves for melittin using various sample containers. To illustrate the ideal calibration curve that is not affected by NSB, one sample set was prepared with 0.1% rat plasma as carrier proteins. Linear calibration curves were obtained when no peptide loss occurred on the sample container, such as with rat plasma or when the solutions were prepared in a QuanRecovery Plate. Using a polypropylene plate, the peptide loss was so severe that the calibration curve could not be constructed at all. Even when the peptide loss was not so severe, as shown in Figure 8 with the curve using the other low bind plate, it was impossible to construct a linear calibration curve. This example clearly shows why using sample containers that have maximum analyte recovery is crucial in quantitative analyses.

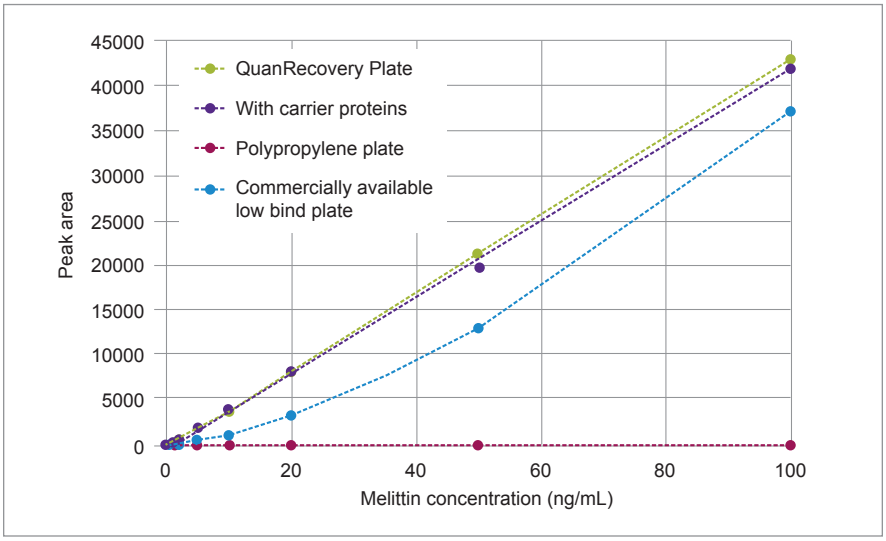

Figure 8. Calibration curves for melittin in the concentration range of 50 pg/mL to 100 ng/mL. The calibration standards were prepared with 80:20 water/acetonitrile + 0.2% TFA in each sample container by serial dilution. To obtain the 'true' calibration curve without suffering from the analyte loss, one set of calibration standards was prepared with 0.1% rat plasma as carrier proteins. All other standards was prepared without carrier proteins.

STORAGE TIME AND TEMPERATURE: FACTORS THAT AFFECT THE SPEED OF LOSS

While it is tempting to regard non-specific binding as a fast process that happens immediately upon contact, this is rarely the case. Figure 9 shows the recoveries of 1 ng/mL melittin and teriparatide stored in various sample containers, monitored over 51 hours. From previous examples, we know that melittin and teriparatide stored in a polypropylene plate would be completely lost in 24 hours. If we follow their recoveries over time, the recoveries were about 20% after 1 hour and dropped to almost zero after 8 hours. Peptides stored in other containers followed a similar pattern, where their recoveries are gradually decreased over time but at different rates depending on the peptides and containers. In general, peptides stored in a QuanRecovery Plate were much better recovered than peptides in other containers. Both melittin and teriparatide showed increased loss over time but followed different trends. Most notable from the data using the commercially available low bind plate, a significant amount of melittin was lost within the first hour while the losses during the following hours were not as quick. On the other hand, teriparatide showed a consistent rate of loss over time. A similar but much more subtle pattern could be noted from the data using the QuanRecovery Plate, where the recovery of teriparatide decreased over time while melittin recovery changed little. We speculate that this is due to the difference in adsorption kinetics for melittin and teriparatide. Regardless of the difference in kinetics, QuanRecovery Plates offered better recovery than other containers even after extended storage.

Another important factor that influences the kinetics of non-specific binding is temperature. In kinetic theory, temperature influences many reactions by promoting molecular movement and/or supplying energy. Figure 10 shows the effect of sample storage temperature on the recovery of melittin and glucagon after 47 hours. It is remarkable that highly hydrophobic melittin can be completely recovered from QuanRecovery Plates stored at various temperatures including room temperature (~25 °C). It is however not recommended to store samples at room temperature because of other concerns such as sample degradation. Melittin stored in commercially available low bind plates was mostly lost. About 25% was recovered if stored at or below 10 °C while complete loss was observed if stored above 10 °C. To confirm whether the abrupt transition in recovery at approximately 15 °C was true, the recovery of glucagon, a less hydrophobic peptide, was examined. While the recoveries were in general greater than those for melittin, the same abrupt transition in recovery was observed. Because the same peptides were completely recovered from the QuanRecovery Plate at temperatures over 15 °C, we can conclude that the losses observed from the other low bind plate are due to NSB rather than peptide degradation. This result is another example demonstrating that peptide adsorption on QuanRecovery Vials and Plates is a slower and less favorable process compared to similar adsorption on other containers, making it more compelling to choose QuanRecovery Vials and Plates for working with sticky analytes.

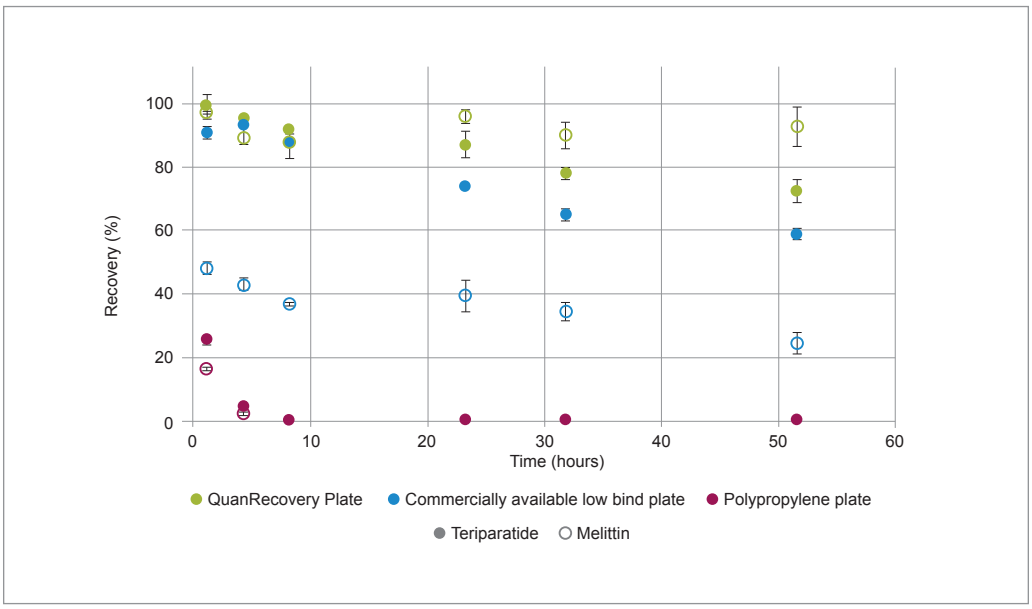

Figure 9. Average recovery (n = 4) of 1 ng/mL teriparatide (closed circles) and melittin (open circles) over 51 hours of storage at 10 °C. The error bars show the standard deviations. The peptide solutions were prepared in an 80:20 water/acetonitrile mixture and acidified with 0.2% TFA.

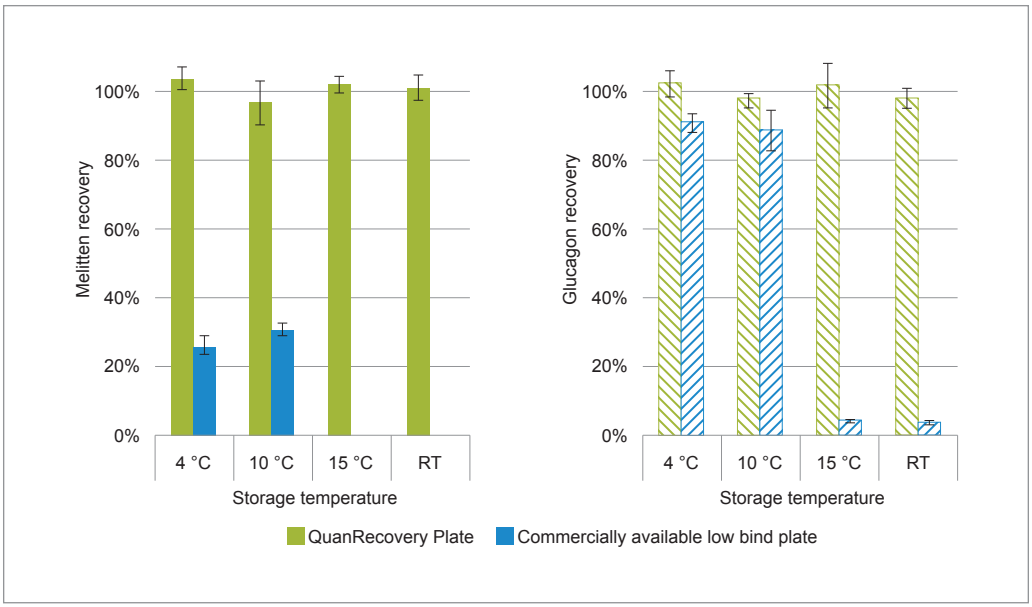

Figure 10. Average recovery (n = 4) of 1 ng/mL melittin (solid bars) and glucagon (checked bars) after 47 hours of storage at different temperatures. The error bars show the standard deviations. The peptide solutions were prepared in an 80:20 water/acetonitrile mixture and acidified with 0.2% TFA. Room temperature (RT) was approximately 25 °C.

THE EFFECT OF SAMPLE VOLUME ON RECOVERY

In typical chemical reactions, the difference in volume is seldom an important factor that influences the kinetics. For surface adsorption on a sample container, however, sample volume does influence NSB because the exposed surface area is changed. For most sample containers, the surface-to-volume ratio increases as sample volume decreases, and consequently more NSB is observed. This is particularly undesirable in challenging LC-MS analyses where samples are limited in volume and analyte concentrations are low. Figure 11 shows the effect of sample volume on peptide recoveries. Teriparatide stored in a commercially available low bind plate showed decreased recovery and increased variability as the sample volume is decreased. On the other hand, teriparatide stored in a QuanRecovery Plate was completely recovered regardless of the sample volume, without showing increased variability at low concentrations.

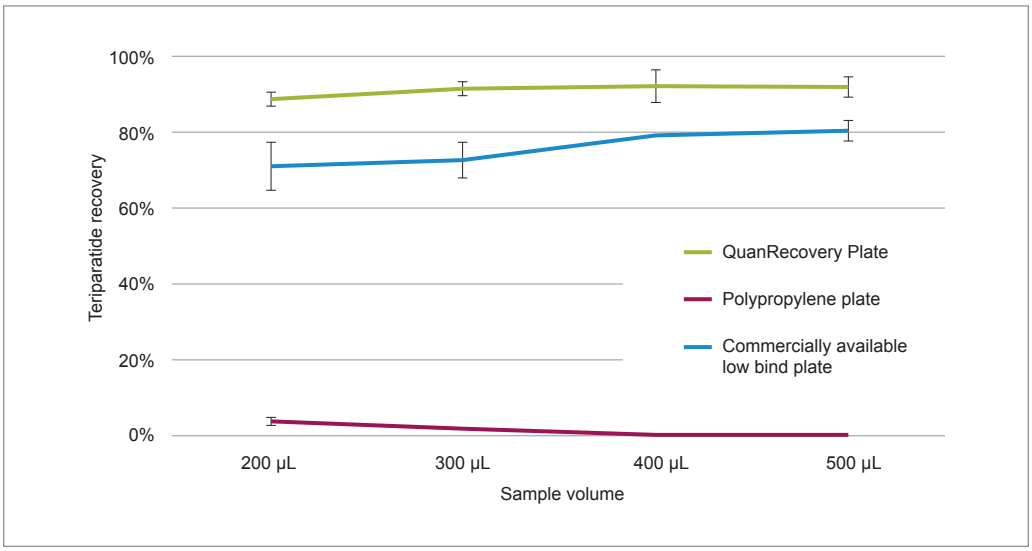

Figure 11. Average recovery (n = 4) of 1 ng/mL teriparatide after 24 hours of storage at 4 °C. The error bars show the standard deviations. The peptide solutions were prepared in an 80:20 water/acetonitrile mixture and acidified with 0.2% TFA.

SEALING OPTIONS FOR PLATES AND THEIR CONTRIBUTIONS TO RECOVERY

A cap or a sealing mat is an essential, complementary element for a sample container to prevent contamination, evaporation, and accidental splashing. Just as vials have various caps, there are a few sealing options for plates that are also compatible with LC-MS injectors. Generally flat in shape, some sealing caps have embossed structures that match the shape and size of the wells so that the caps are held on top of plates by friction. Other caps are flat films with an adhesive side to attach the film to the plate. Regardless of the shape and sealing mechanism, it is recommended that the caps should not be in direct contact with sample solutions to prevent potential contamination and sample loss. Because sample loss is a particularly important consideration when choosing a low bind product such as a QuanRecovery Plate, we tested several sealing caps to compare peptide losses on the caps. QuanRecovery Plates were filled with peptide solutions and four different caps were used to seal the wells. The four caps tested were: 1) a polypropylene cap mat (p/n: 186002483), 2) a PTFE/silicone cap mat (p/n: 186006332), 3) adhesive tape seal (p/n: 186006336), and 4) a strip plug cap. Three plates were identically prepared, and one plate was left undisturbed; the second plate was agitated by vortex mixing and centrifuged to bring down the solution into the wells; and the third plate was agitated by vortex mixing only. After these three QuanRecovery Plates were stored for 24 hours, the peptide recoveries were measured (Figure 12). If the solutions were left undisturbed, the sealing caps did not contribute to peptide losses. When the plates were agitated by vortex mixing, and thus solutions in the well were in contact with the seals, measurable losses were observed. The losses also depended on the hydrophobicity of the peptide: melittin in general was affected more than less hydrophobic teriparatide. Centrifugation after vortex mixing did not prevent the peptide losses once the solutions touched the caps. Some caps showed less peptide losses than others, possibly due to their difference in hydrophobicity and wettability by the tested solutions. In a few test cases, the adhesive tapes showed small leaks around the wells after agitation. It is thus strongly recommended to avoid agitating plates with caps on, and to test the impact if it is unavoidable in your workflow.

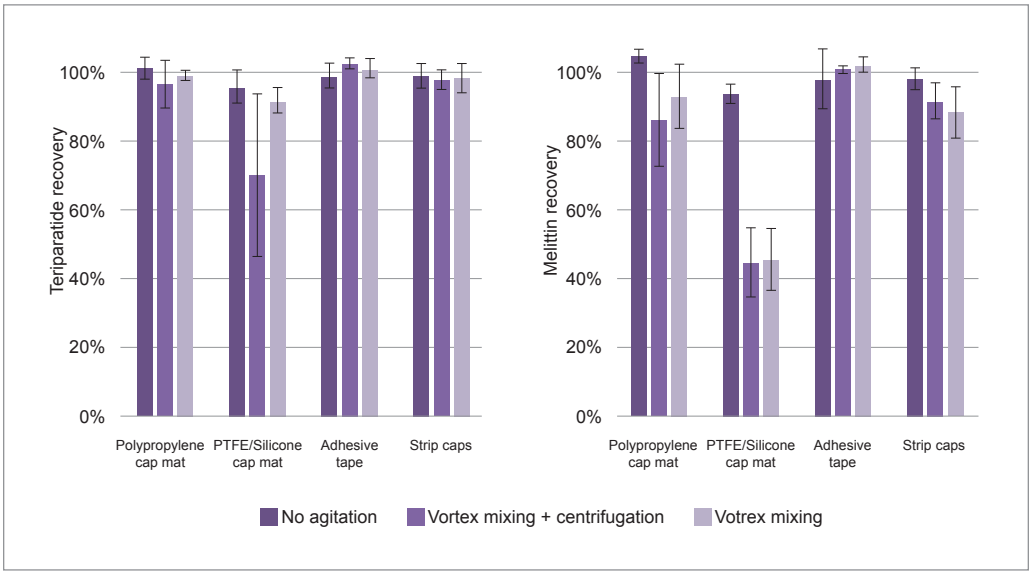

Figure 12. Average recovery (n = 4) of 1 ng/mL teriparatide and melittin in QuanRecovery Plates capped with various sealing options and physical agitations. The plates were stored at 10 °C for 22 hours before LC-MS analyses. The error bars show the standard deviations. The peptide solutions were prepared in an 80:20 water/acetonitrile mixture and acidified with 0.2% TFA. Tested caps were: 1) a polypropylene cap mat, 2) a PTFE/silicone cap mat, 3) adhesive tape seal, and 4) a strip plug cap. After capping the plates, one plate was left undisturbed; the second plate was agitated by vortex mixing for 5 min and centrifuged at 4000 rpm for 1 min to bring down the solution to the wells, and the third plate was agitated by vortex mixing for 5 minutes only.

RESIDUAL VOLUMES OF PLATES AND VIALS: ANOTHER WAY TO LOSE SAMPLE

When selecting a sample container for LC-MS analyses, another factor that should be considered is the residual volume. It is quite common in challenging bioanalyses that the available sample volume is limited. The entire sample volume is not available for LC-MS injection because most LC autosamplers use a sample needle that accesses sample containers from above. This left-over volume is called residual volume, and it is determined by the shape of the container and the needle's vertical position (Z-position). It is therefore important to correctly set the needle position and to select a sample container that is designed to minimize the residual volume. Failure to do so would result in not just waste of precious sample but also inconsistent injections leading to poor assay reproducibility and quantitation errors.

We measured the residual volumes of several low bind sample containers. The plates were filled with 70 µL of sample in four corner wells and a center well, and a series of 1 µL injections were withdrawn. Similarly, five vials were filled with 50 µL samples and positioned at four corners and a central position on a 48-vial tray (ANSI-48 Vial 2 mL Holder). The needle Z-position was set to 2 mm for plates and 3 mm for vials. The residual volumes were defined by the remaining volumes when the first unsuccessful injection was made. Figure 13 shows examples of residual volume measurements for a QuanRecovery Plate, a QuanRecovery Vial, and a commercially available low bind plate. Table 1 summarizes the measured residual volumes from all plate wells and vials. The QuanRecovery Vials and Plates showed low average residual volumes (5 and 8 µL, respectively) while the other low bind plate showed a residual volume over 50 µL. Moreover, the standard deviations for residual volumes were much smaller with the QuanRecovery Vials and Plates than with the other low bind plate (about 1 vs 9 µL). This result, in addition to the greater recovery for peptides in small sample volumes as seen in the previous discussion (Figure 11), demonstrates that QuanRecovery Vials and Plates are ideal choices when analyzing samples with a limited volume.

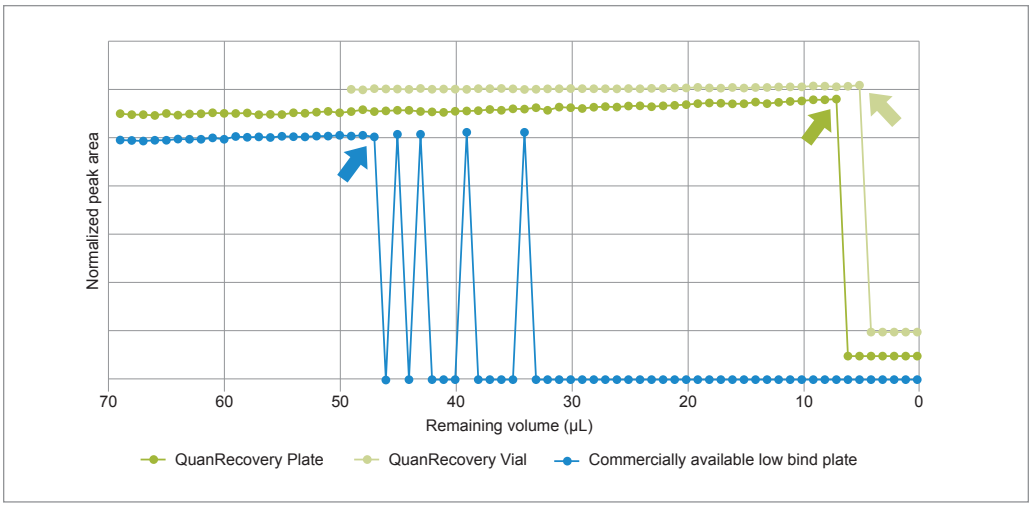

Figure 13. Example plots of peak area versus remaining sample volume, using a QuanRecovery Plate, a QuanRecovery Vial, and a commercially available low bind plate. The plates were filled with 70 µL of 0.01 mg/mL caffeine solutions and the vial was filled with 50 µL of the same solution, and a series of 1 µL injections were withdrawn using a flow-through needle in the "partial loop" mode. The needle's Z-position was set at 2 mm for the plates and 3 mm for the vial. The peak areas were normalized and plotted with an offset for easy comparison.

Table 1. Summary of measured residual volumes from each well and vial

	QuanRecovery Plate	QuanRecovery Vial	Commercially available low bind plate
Top-left	10	5	54
Top-right	7	4	47
Bottom-left	7	5	45
Bottom-right	9	5	52
Center	9	6	68
Average	**8.3**	**5.0**	**53.2**
Standard deviation	**1.3**	**0.7**	**9.0**

CONCLUSIONS

Quantitative LC-MS analyses for proteins and peptides are getting more challenging every day, requiring greater sensitivity and reproducibility from smaller amounts of samples. Analyte loss due to non-specific binding in sample containers is a significant problem in quantitation that is often not recognized early enough. Failure to mitigate this problem can lead to hours of wasted time during method development, or even worse, to suboptimal methods that are limited by poor analytical sensitivity and reproducibility. In this paper, we reviewed various factors that influence peptide losses. Understanding the mechanisms and the kinetics of the losses provided useful guidelines to mitigate this sticky problem by allowing us to consciously choose optimal experimental conditions. Some experimental factors, such as sample matrix, storage temperature, and sealing options may be readily varied within the workflow to maximize the recoveries. Other factors, such as storage time, sample volume, and peptide concentration may not be modified because the available options are dependent on other steps in the total workflow. While using containers that are designed to mitigate NSB cannot prevent all adsorption problems, it certainly allows more options than would be available if using other containers. We recommend selecting QuanRecovery Vials and Plates with MaxPeak High Performance Surfaces as the first step to achieve maximum protein and peptide recovery, sensitivity, and reproducibility in the most demanding bioanalysis applications and any other challenging assays for detecting difficult analytes at low concentrations.

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