Sample preparation

Overcoming nonspecific binding in liquid chromatography: enhancing assay sensitivity, accuracy, and reproducibility in peptide/protein workflows

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Abstract

Purpose: Investigating the impact of nonspecific binding (NSB) on the performance of LC-MS methods by evaluating influencing factors of NSB. Research ultimately seeks to improve method validation by enhancing the reliability and reproducibility of bioanalytical assays to support more effective biopharmaceuticals.

Methods: The workflow uses a Thermo Scientific™ Vanquish™ Horizon LC system in the reversed phase (RP) analysis with UV detection and implementing Thermo Scientific™ Hypersil GOLD™ C18 or Thermo Scientific™ MAbPac™ RP Phenyl columns.

Results: Five NSB influencing factors—vial fill volume, storage time in vial, autosampler temperature, organic modifier in diluent, and vial material—were analyzed for their effects on the recovery of four distinct proteins. The data revealed differential impacts of these factors on protein recovery, highlighting the variability in how each factor influences NSB in different material vials. Vial fill volume showed no significant influence and autosampler temperature effect varied based on protein tested. Organic additive greatly improved recoveries for majority of proteins and sample storage in autosampler over time showed influence on some proteins.

Introduction

The use of advanced high-throughput LC–MS methods are required in the evolving landscape of drug discovery and the development of complex biotherapeutics such as peptides and proteins. As the industry strives to improve drug efficacy and analyte concentrations decrease, these methods require more selective and sensitive techniques to meet stringent method validation standards. A critical challenge in this context is the phenomenon of nonspecific binding (NSB).

NSB is commonly overlooked and can lead to poor, nonlinear, or non-reproducible analyte recoveries and negatively impact the overall method robustness. Analytes, from sample preparation until entering an analytical instrument, are prone to adsorb onto various surfaces including sample handling equipment, the LC instrument and analytical column. While pharmaceutical guidelines require consistent recoveries, stability, accuracy, and precision in analytical methods, they do not extend the investigation of NSB to sample handling equipment. This oversight can critically influence the method's robustness (sensitivity, precision, accuracy). Furthermore, the lack of consideration for cross-validation between different materials can result in potential inaccuracies and inter-laboratory imprecision as there is insufficient information on analyte adsorption properties.

The analyte loss may occur due to multiple complex interactions such as electrostatic, hydrogen, or hydrophobic/hydrophilic bonding with adsorption surfaces, including sample containers, pipette tips, and vials. Differences in sample nature and vial materials, such as glass or plastic (polypropylene), can lead to significant levels of analyte loss during analysis due to NSB. Since samples are periodically stored in vials, this study focuses on the impact of vial material, sample environment and instrumental method conditions. By investigating various biotherapeutics each exhibiting different adhesion mechanisms, a better understanding how to mitigate analyte loss is achieved.

Materials and methods

In total four protein samples were used for this study:

- Insulin (MW = 5808 Da, Gibco™, P/N 12585014)
- Glucagon (MW = 3483 Da, Bio-Techne, P/N 6927)
- Semaglutide (MW = 4114 Da, AdipoGen™, P/N AG-CP3-0040)
- NISTmAb (MW = 148 kDa, NIST, P/N 8671)

Vials/inserts of different surface chemistry were used:

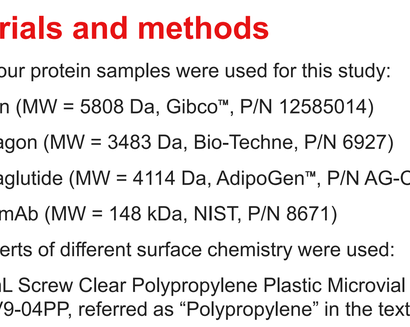
- 0.3 mL Screw Clear Polypropylene Plastic Microvial (P/N 6ESV9-04PP, referred as "Polypropylene" in the text)
- 0.3 mL GOLD-Grade Borosilicate Glass Insert (P/N 6PME03C1SPG, referred as "Unmodified glass" in the text)
- 0.3 mL GOLD-Grade Clear Glass Silanized Insert (P/N 6PME03C1SSP, referred as "Silanized glass" in the text)

Sample Preparation

Samples were prepared from stock solutions by diluting it to two different concentrations with 0.1% FA in water. One is high concentration reference for recoveries determination at 100 µg/mL for all proteins. Second is trace concentration test sample (4 µg/mL for Insulin, Glucagon and Semaglutide, 2 µg/mL for NISTmAb). These values were based on recovery versus concentrations curves – high enough concentration for reference to consider adsorption effects negligible and trace concentrations where significant recovery drop is observed.

For example, insulin recovery versus concentration linearity curve was generated by diluting 4 mg/mL of stock solution to 200, 100, 50, 20, 10, 4, 2 and 1 µg/mL concentrations (Figure 1). Recoveries were based on considering 200 µg/mL as a 100% point.

Figure 1. Insulin recovery versus concentration curve in polypropylene (diluent – 0.1% FA in water, autosampler temperature - 5ºC, vial fill volume – 1/3 of total volume).



Design of Experiment Setup

To evaluate adhesion effects, the experimental matrix with different factors and variables was generated (Table 1). Selected conditions are applicable to test with relatively simple preparation and setup – accounting to only instrumental methods modification, vial material change and addition of one organic solvent (acetonitrile).

Table 1. Design of experiment

Factor	Variable A	Variable B	Variable C
Vial fill volume	1/3 of total vial volume	2/3 of total vial volume	-
Vial storage time in autosampler	6 h	12 h	24 h
Autosampler temperature	5ºC	25ºC	-
Organic diluent content	0% ACN	20% ACN	-
Vial material	Unmodified glass	Silanized glass	Polypropylene

Test Method(s)

Table 2 shows the chromatographic conditions of the RP-HPLC methods for both small proteins and mAb.

Table 2. Chromatographic conditions

Parameter	Value
Column	Hypersil GOLD C18 100 x 2.1 mm; 1.9 µm (P/N 25002-102130) for Insulin, Glucagon and Semaglutide. MAbPac RP Phenyl (P/N 088647) 100 x 2.1 mm, 4 µm for NISTmAb.
Solvent A	0.1% FA in water
Solvent B	0.1% FA in ACN
Gradient (Insulin and Glucagon): Equilibrate for 4 min with 20% B, then ramp to 45% B in 4 min, clean with 95% B for 2 min.	
Gradient (Semaglutide): Equilibrate for 4 min with 35% B, then ramp to 65% B in 4 min, clean with 95% B for 2 min.	
Gradient (NISTmAb): Equilibrate for 3 min with 18% B, then ramp to 40.5% B in 4 min, clean with 60% B for 0.5 min.	
Flow rate	0.8 mL/min (0.5 mL/min for NISTmAb)
Column temperature	50ºC (80ºC for NISTmAb) with active preheater at 50ºC (80ºC for NISTmAb) , still air mode, post column cooler at 40ºC
Needle wash solution	75/25 isopropanol/water (v/v) + 0.1% FA
Needle wash mode	Both (before and after)
Injection volume	10 µL
Detector settings	Detection wavelength: 280 nm

Data Analysis

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.3.2 was used for data acquisition and processing. Errors were calculated by measuring standard deviation of 3 consecutive injections.

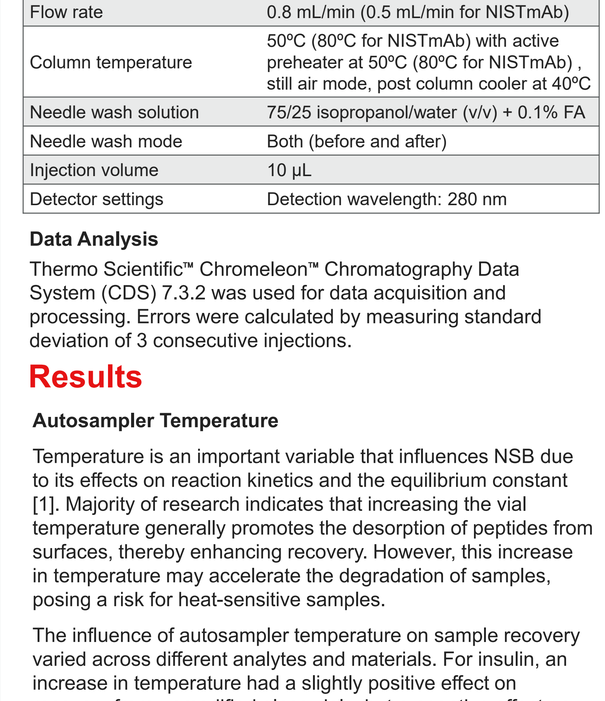
Results

Autosampler Temperature

Temperature is an important variable that influences NSB due to its effects on reaction kinetics and the equilibrium constant [1]. Majority of research indicates that increasing the vial temperature generally promotes the desorption of peptides from surfaces, thereby enhancing recovery. However, this increase in temperature may accelerate the degradation of samples, posing a risk for heat-sensitive samples.

The influence of autosampler temperature on sample recovery varied across different analytes and materials. For insulin, an increase in temperature had a slightly positive effect on recovery from unmodified glass vials, but a negative effect on recovery from silanized glass vials. In contrast, glucagon exhibited the most pronounced recovery increase across all materials tested. Semaglutide demonstrated only minor recovery improvements with polypropylene and silanized glass vials. NISTmAb did not exhibit any significant response to temperature variations (Figure 2).

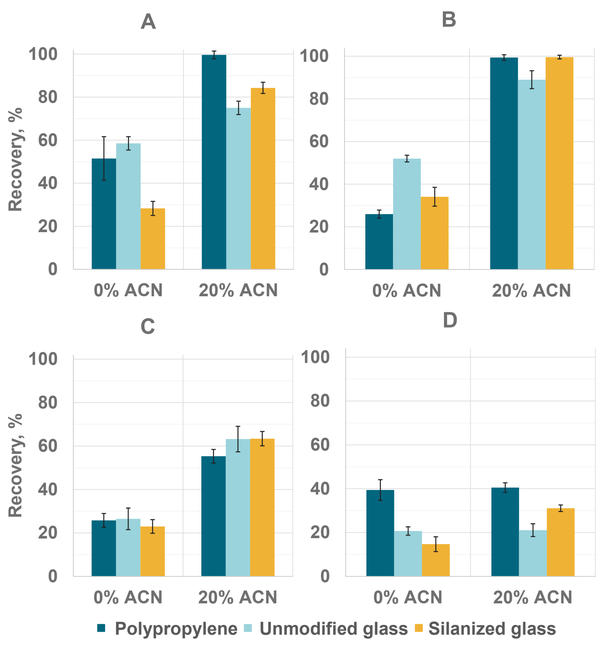
Figure 2. Autosampler temperature influence on recovery. A – insulin, B – glucagon, C – semaglutide, D – NISTmAb.



Organic Diluent Content

As previously described, NSB of an analyte to a surface strongly depends on the equilibrium constant between solvent and surface and therefore is relevant to investigate the impact of the solvent additives e.g., acetonitrile. These additives can enhance the solubility of hydrophobic peptides and reduce sample affinity to the vial surface by decreasing hydrophobic interactions. However, the concentration of organic additives must be carefully controlled to avoid "salting-out" effects, which can decrease solubility and lead to sample precipitation, particularly in samples with high salt concentrations. To mitigate these effects, only limited amounts of organics were added into design of experiment.

Figure 3. Organic diluent content influence on recovery. A – insulin, B – glucagon, C – semaglutide, D – NISTmAb.



Among the variables tested, the presence of organic additive had the most significant impact on the majority of protein samples, with some instances achieving up to 100% recovery. The influence of the solvent was particularly pronounced in more hydrophobic vial materials, such as polypropylene and silanized glass, as observed in the cases of insulin and glucagon. This suggests a strong suppression of protein hydrophobic interactions with the surface. However, larger protein samples, such as NISTmAb, exhibited minimal to no response to the addition of organics, highlighting the need for caution when applying this approach universally (Figure 3).

Vial Storage Time in Autosampler

Another factor analyzed in the study was storage time in the vials. Importance of this factor in the industry is largely associated with testing of large quantities of samples which results in extended run times of the analysis sequences. Furthermore, performing these tests may help understand more about the kinetics of NSB, as some may consider this as a fast process which occurs immediately upon contact, however, some research suggests that this process may not be instantaneous, leading to potential misinterpretations of data when using samples prepared in advance.

Figure 4. Insulin vial storage time in autosampler influence on recovery.

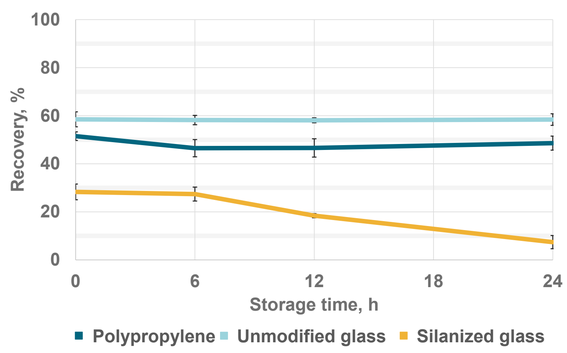
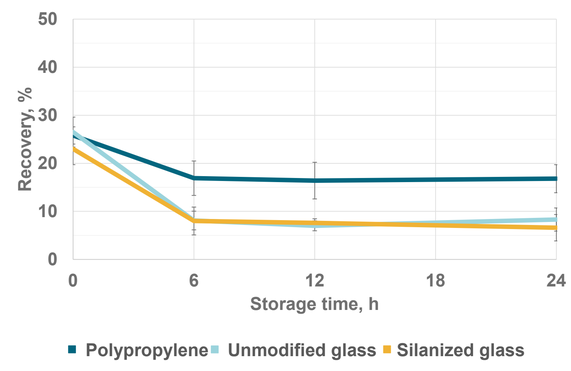


Figure 5. Semaglutide vial storage time in autosampler influence on recovery.



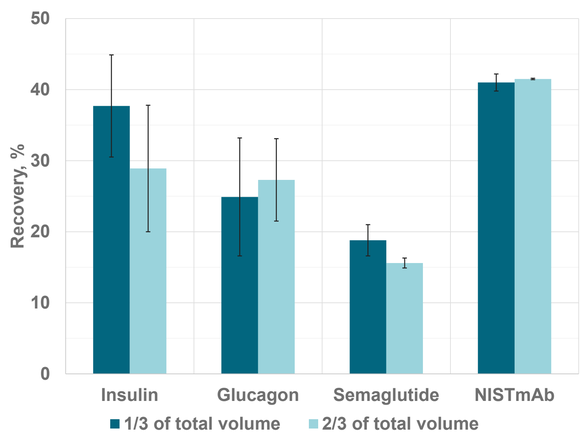
Data suggests that for vials with glucagon and NISTmAb this process is fast and occurs instantaneously. Some notable differences are decrease of recovery on silanized glass for insulin (Figure 4) and significant drop of recovery for all vials with semaglutide, particularly unmodified and silanized glass (Figure 5).

Vial Fill Volume

The rationale for evaluation of different vial fill volumes comes from the limited consideration of this variable in the industry, despite the changing exposed surface area to sample volume [2]. Generally, as sample volume decreases, the surface-to-volume ratio increases, potentially leading to higher NSB. This effect may be particularly significant when working with minimal sample volumes and trace concentrations. This could be especially relevant for high-recovery vial bottom shapes, which have the highest surface-to-volume ratio the lower the sample volume is.

However, experiments revealed no significant effects on protein samples, all within error ranges. Polypropylene example is presented for comparison (Figure 6).

Figure 6. Polypropylene vial fill volume influence on recovery



Conclusions

- Autosampler temperature influence varied for all tested protein samples, most evident positive impact was observed in glucagon.
- No significant influence for vial fill volumes for each tested protein samples. Results were within error.
- Presence of organic additive greatly improves recoveries for most proteins. NISTmAb did not show substantial influence.
- Over 24 hours of sample storage, no significant recoveries change were observed, the only exception being semaglutide with greatly reduced recoveries from 6th hour onwards.

Future research opportunities may involve more in-depth tested factors analysis, extension of tested vial materials, influencing factors and protein samples.

References

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