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A Systematic Approach for Improving the Recovery of Hydrophobic Peptides during LC-MS Analyses

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Proteins and peptides can be quite sticky!

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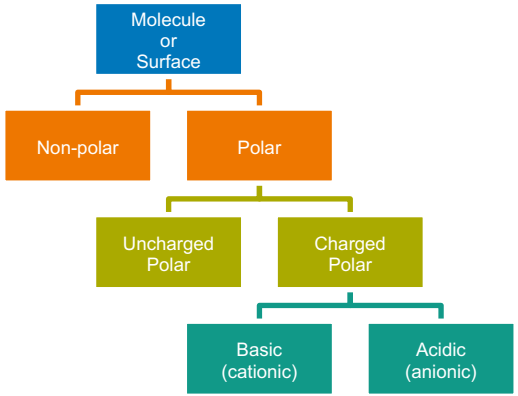
- Non-Specific Binding (NSB) or Non-Specific Adsorption (NSA)
  - Biomolecules tend to adhere to ***any*** exposed surfaces.
  - ***Any*** chemical interaction can be the source of NSB, *but most dominantly…*
    - Polarity-based interactions, *e.g., hydrophobic attraction*
    - Ionic interactions, *e.g., coulombic attraction*



- NSB of biomolecules is more difficult to deal with compared to NSB of small molecules
  - Biomolecules are larger and more complex than small molecules.
    - There may be ***multiple binding interactions*** between biomolecules.
  - Proteins may be ***cooperatively deformed*** during the adsorption process.
    - And may be permanently lost.

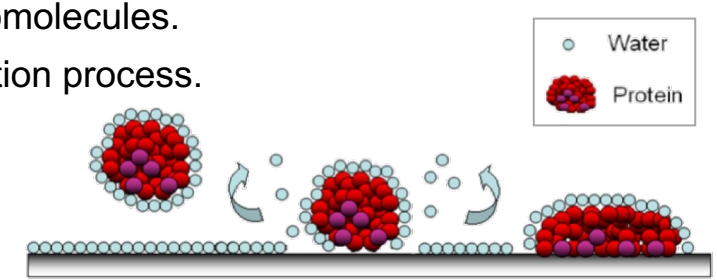


Figure from F. Poncin *et al.*, *J. Func. Biomater.* **3**(3), 528, 2012

How does Non-Specific Binding (NSB) affect analyses?

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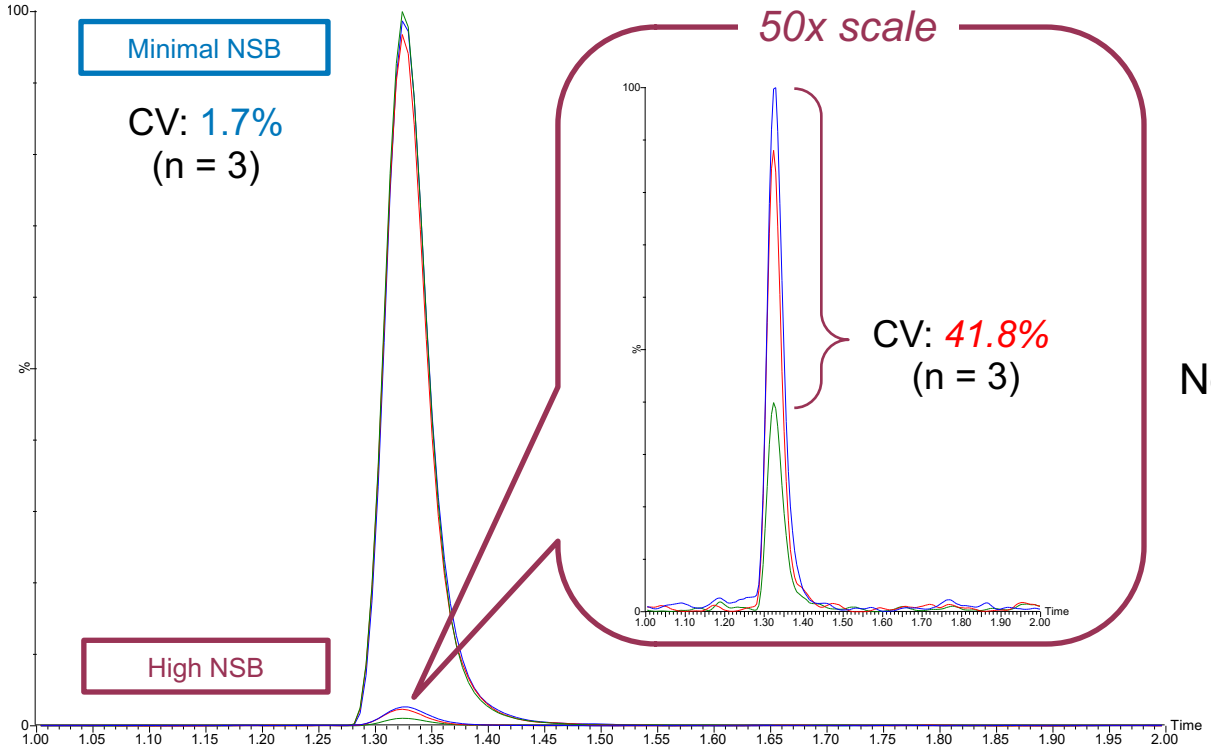
Leuprolide (MW 1209.4) hormone antagonist peptide



Non-specific binding can lead to:

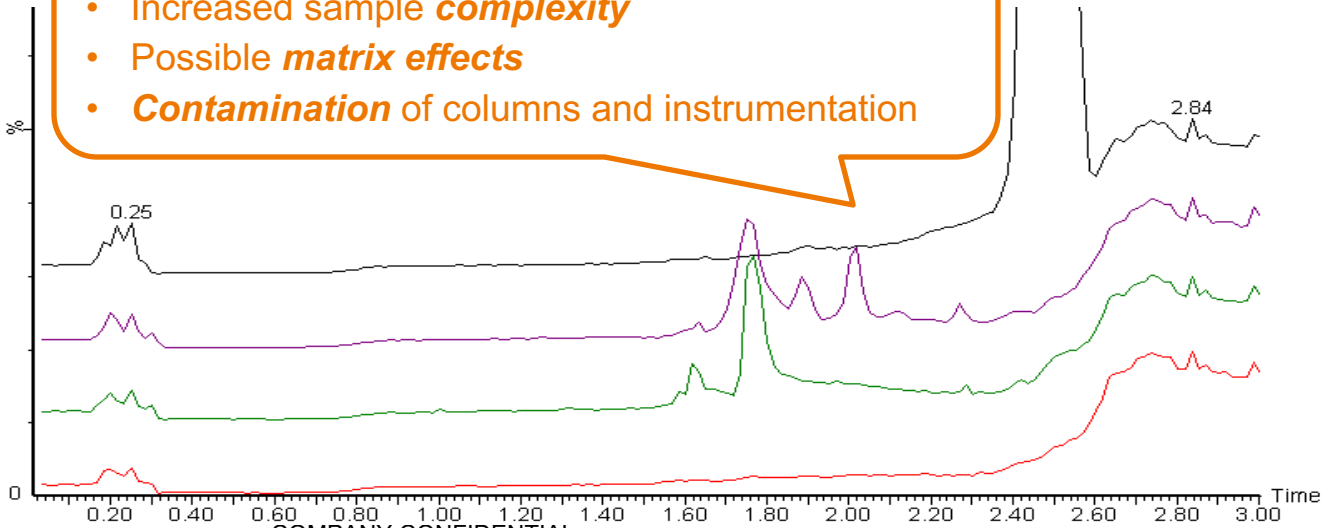
- Low sensitivity
- High variability
- Unreliable analytical results

Blocking agent: an alternative strategy to mask NSB

- Detergents (surfactants), such as Tween-20 or Triton X-100
- Large polymeric molecules, such as polyethylene glycol (PEG)
- Carrier proteins, such as bovine serum albumin, casein, or rat plasma

May be effective in reducing NSB, but may induce…

- Increased sample ***complexity***
- Possible ***matrix effects***
- ***Contamination*** of columns and instrumentation



Froth formation during pipetting leads to measurement errors

0.002% PEG

0.1% rat plasma

100 µg/mL BSA

No blocking agent

A ***dilemma*** in the peptide bioanalysis workflow

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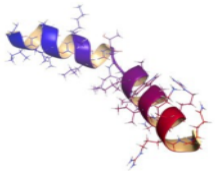

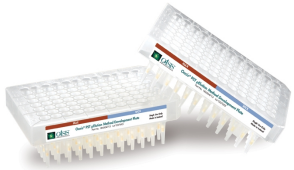

*Do we want to add **blocking agents** to the clean samples?*

Sample

+ matrix

Sample Preparation

Sensitivity ↑
Specificity ↑
Recovery ↑
Matrix Effects ↓

Cleaned-up samples

*Can we **risk** the analyte loss?*

LC-MS/MS Analysis

Waters introduced QuanRecovery Plates and Vials

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QuanRecovery™ WITH MAXPEAK™ HPS

Use it, don't lose it!

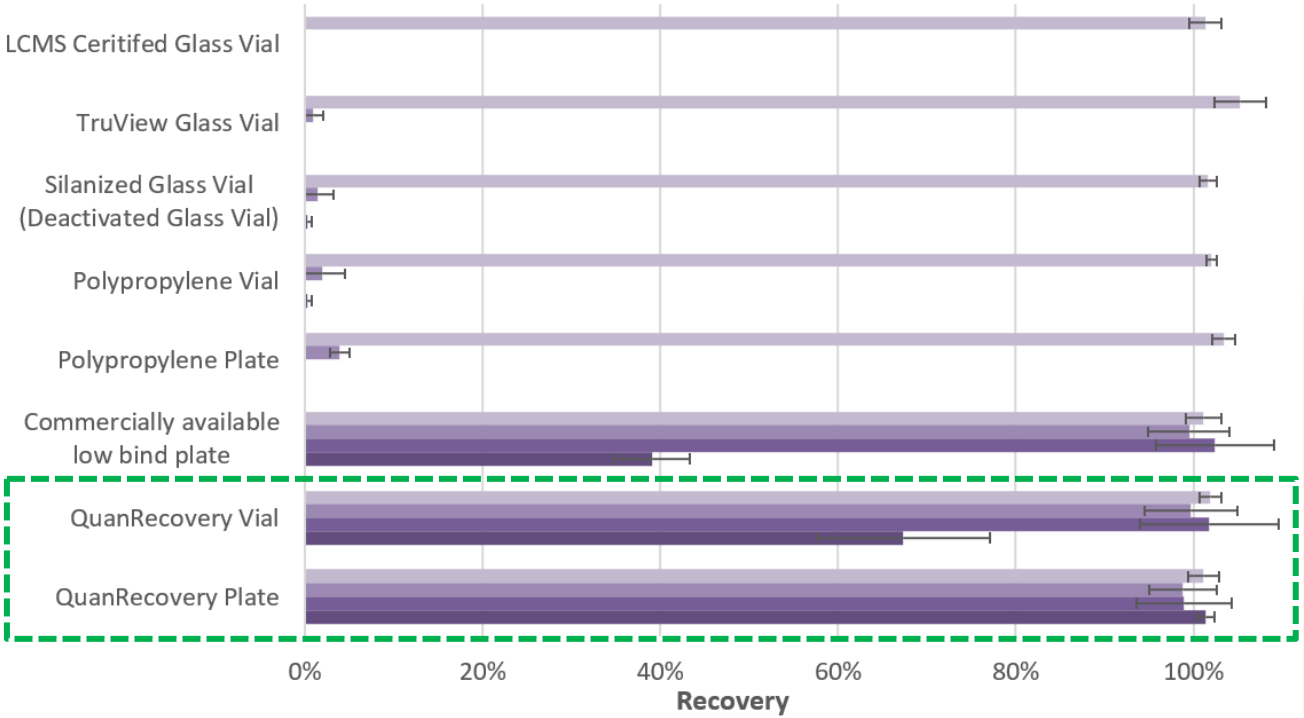
Clean polypropylene sample containers for LC/MS applications: **no silanol activities**

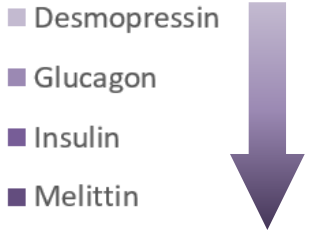
Hydrophilic surface modification: no coating or extra chemicals on the surface

300 µL injection vial

700 µL 96-well plate

What is the impact of the sample container on recovery?





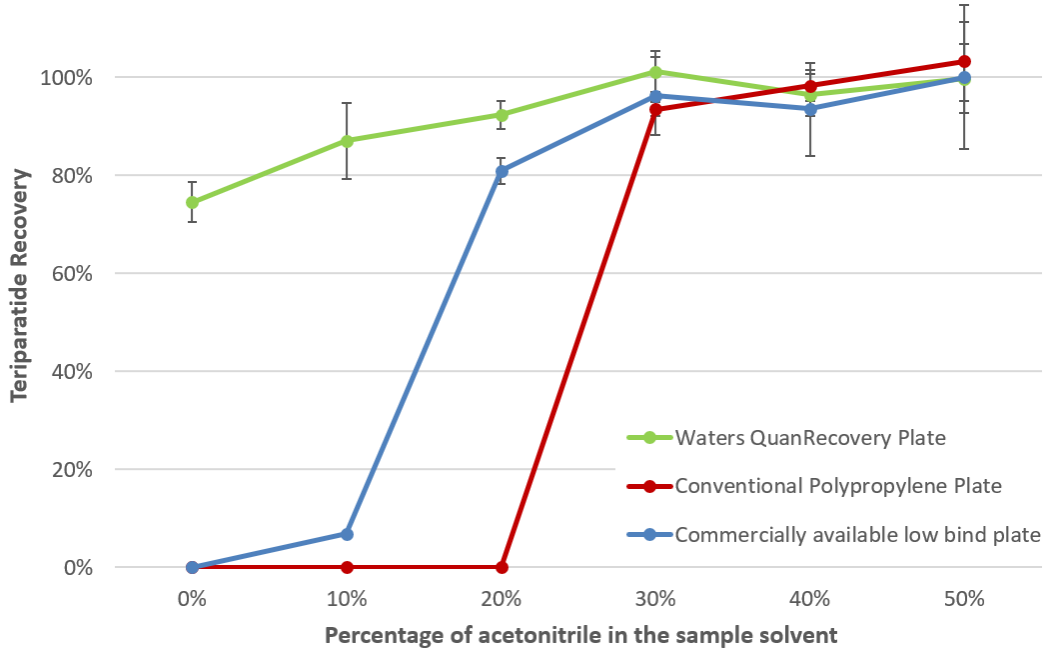
Increasing hydrophobicity

i.e., more challenging peptides

- Recovery of hydrophobic peptides is a good indicator for the NSB losses.
- Not all containers are suitable for peptides.

Average recovery (n=4) of four peptides (1 ng/mL each) after 24 hours storage at 4 ℃.
Sample prepared in 80:20 water/ACN + 0.2% TFA.

How does the sample matrix composition affect recovery?



Average recovery of 1 ng/mL teriparatide (n=4) after 24 hours of storage at 4 ºC. Samples prepared in water/ACN mixtures of various ratios, all acidified with 0.2% TFA.

QuanRecovery showed greater recoveries in low organic sample matrices.

…but is this a significant benefit?

How does the sample matrix composition affect recovery?

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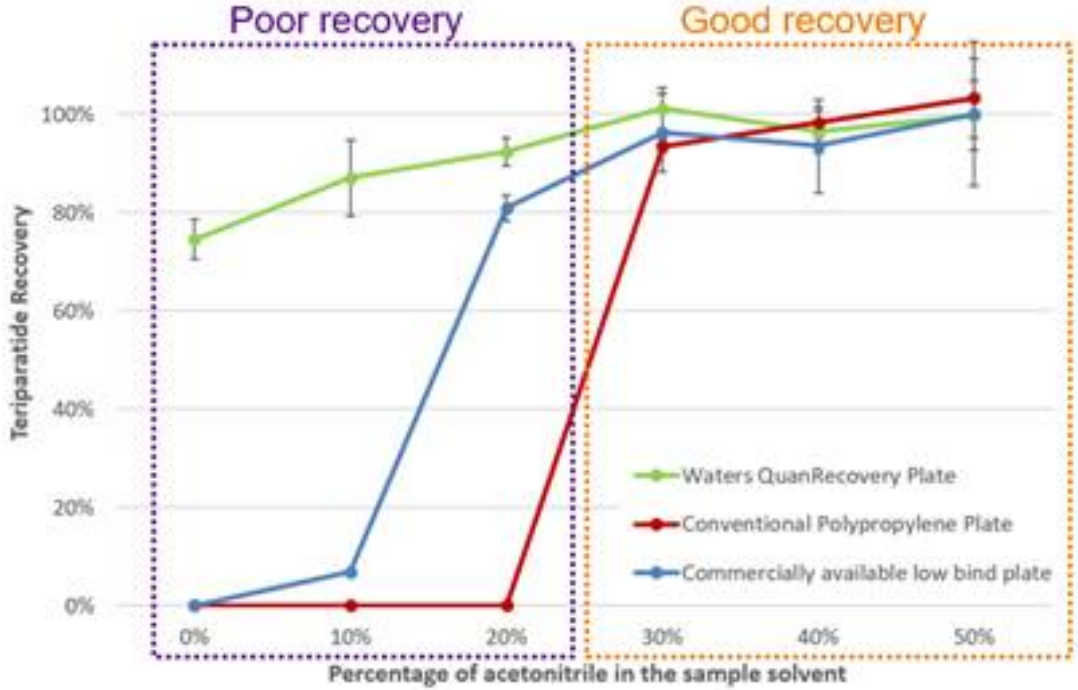
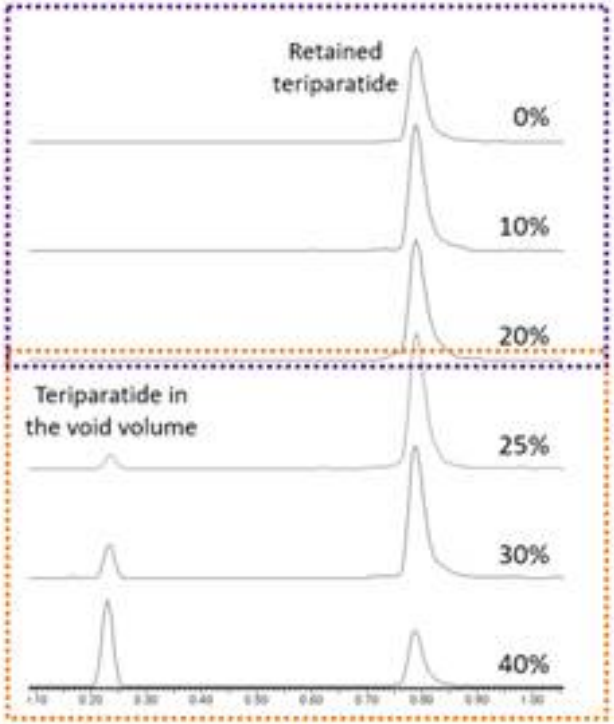


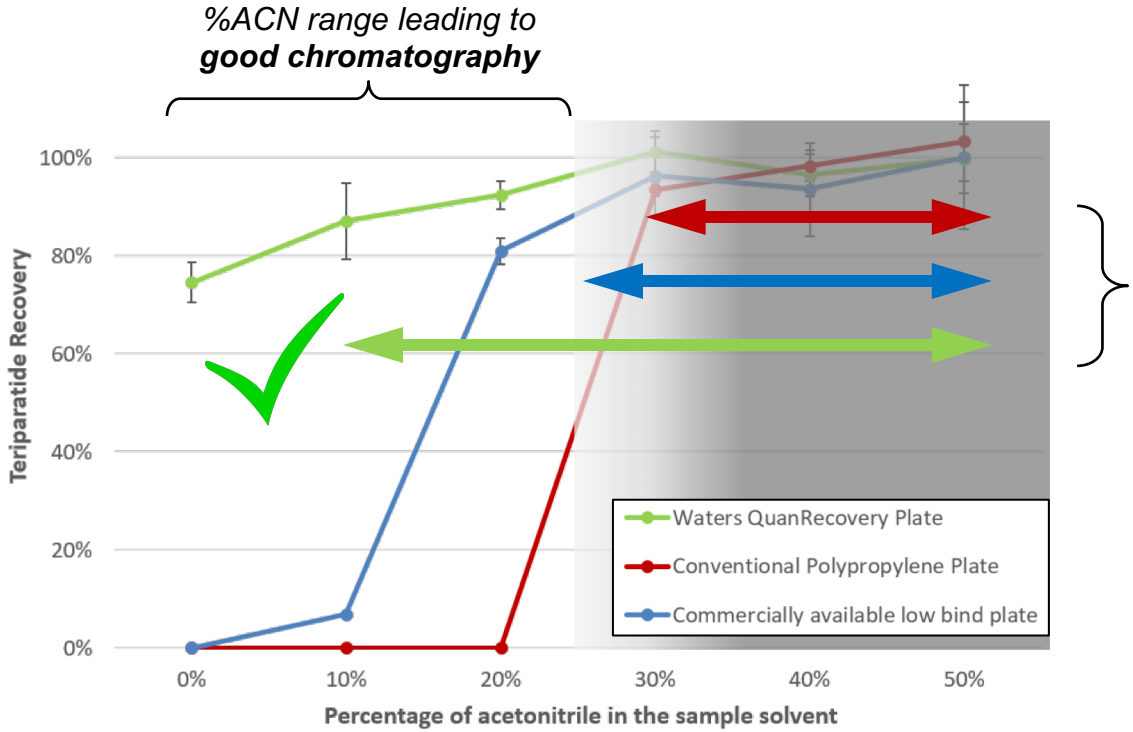
Average recovery of 1 ng/mL teriparatide (n=4) after 24 hours of storage at 4 °C. Samples prepared in water/ACN mixtures of various ratios, all acidified with 0.2% TFA.

Good chromatography



Poor chromatography

How does the sample matrix composition affect recovery?

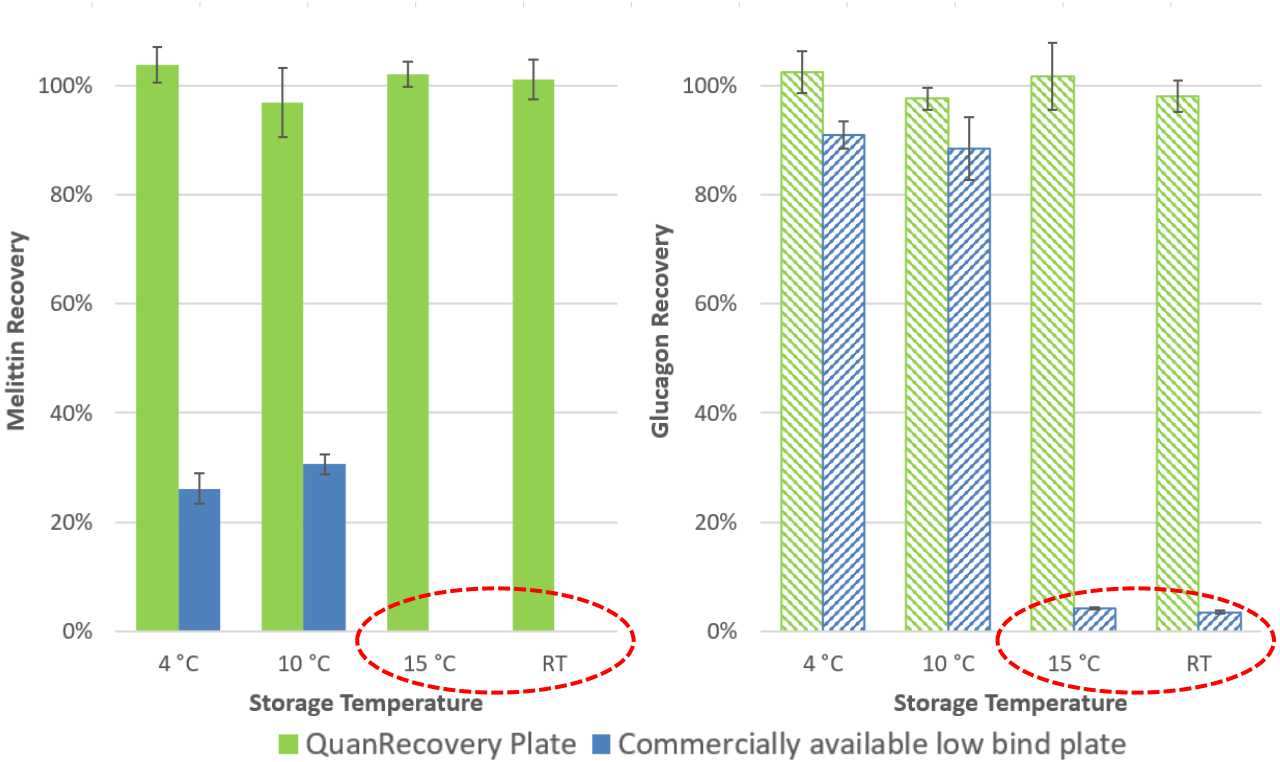


%ACN range leading to ***good recovery (>90%)*** *in each container*

Highly organic sample matrices help reduce NSB losses, but they may not be suitable for LC injections.

Average recovery of 1 ng/mL teriparatide (n=4) after 24 hours of storage at 4 ºC. Samples prepared in water/ACN mixtures of various ratios, all acidified with 0.2% TFA.

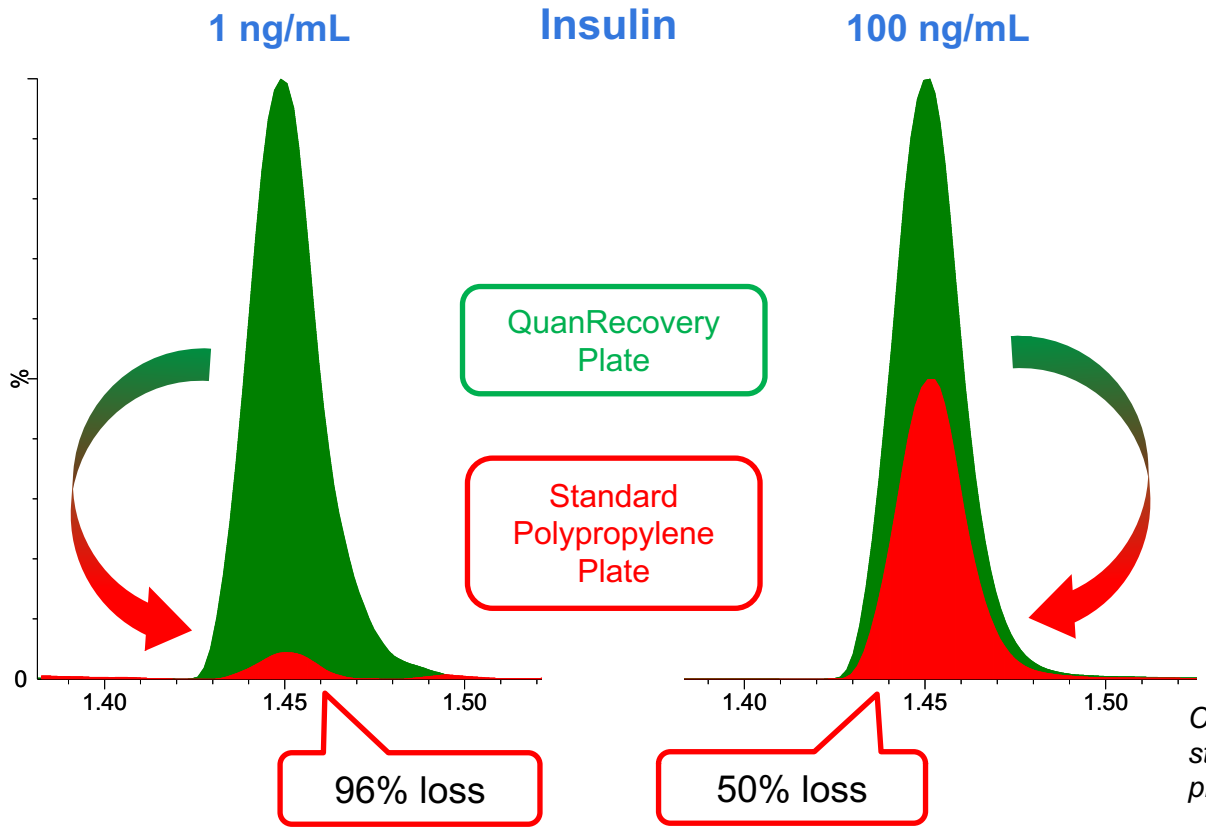
Temperature vs. peptide recovery



Non-refrigerated sample storage or the exposure to an elevated temperature during sample handling may increase NSB losses.

Average recovery of 1 ng/mL melittin and glucagon (n=4) after 47 hours of storage at various temperatures. Samples were prepared in 80:20 water/ACN + 0.2% TFA.

Non-specific binding losses at high and low concentrations



NSB losses are more apparent at low concentrations, but still cause challenges at high concentrations.

Chromatographic peaks of insulin after storage for 24 hours at 4 ºC. Samples were prepared in 80:20 water/ACN + 0.2% TFA.

Impact of non-specific binding on calibration curves

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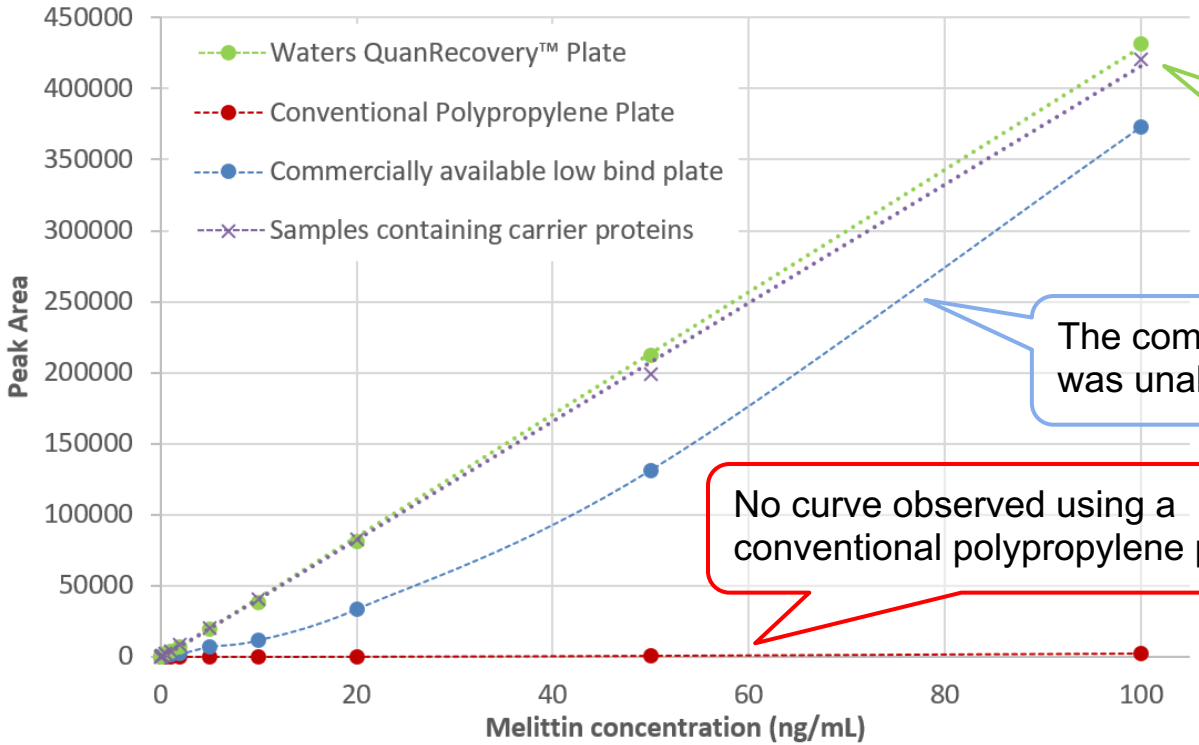

QuanRecovery Plate and the Carrier Protein Method both produced *linear* calibration curves.

The commercially available low bind plate was unable to produce a *linear* curve.

No curve observed using a conventional polypropylene plate.

The NSB losses skewed the calibration curves, and thus severely limited the assay dynamic ranges.

*The calibration curve for melittin **(0.05 – 100 ng/mL)** after standards were stored for 24 hours at 4 ºC. Standards were prepared in 80:20 water/ACN + 0.2% TFA.*

Another way of losing samples – residual volumes

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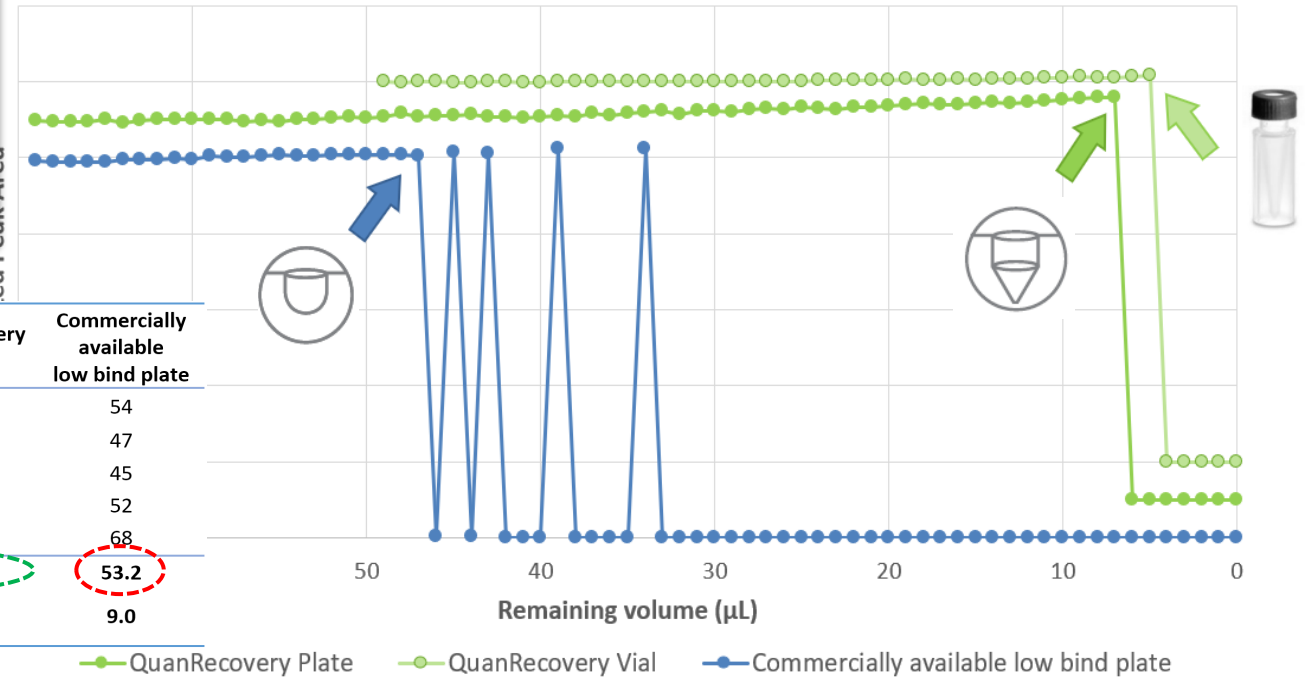
The container design influences the available sample volume for injections.

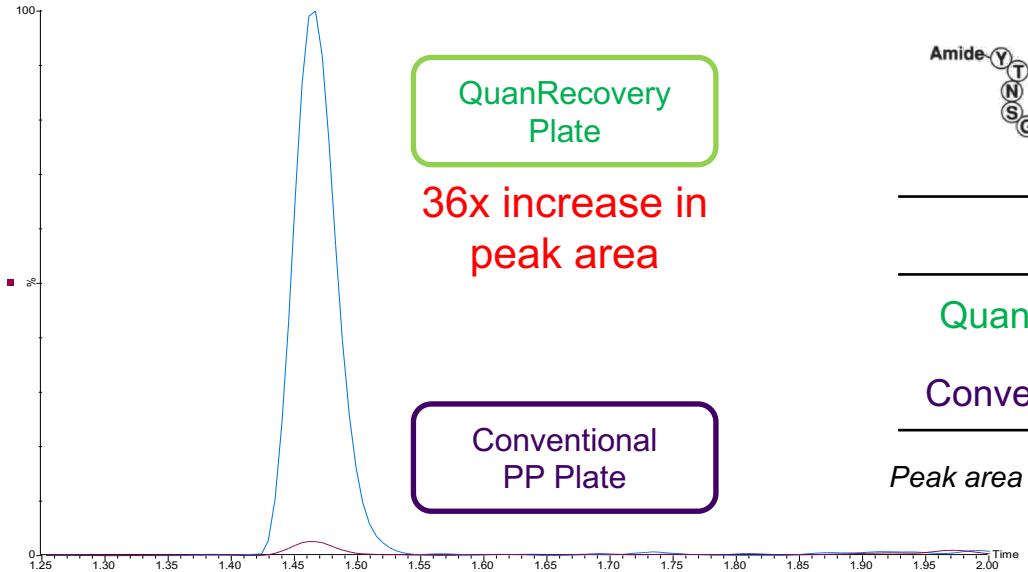
	QuanRecovery Plate	QuanRecovery Vial	Commercially available low bind plate
Top-Left	10	5	54
Top-Right	7	4	47
Bottom-Left	7	5	45
Bottom-Right	9	5	52
Center	9	6	68
Average	**8.3**	**5.0**	**53.2**
Standard deviation	**1.3**	**0.7**	**9.0**

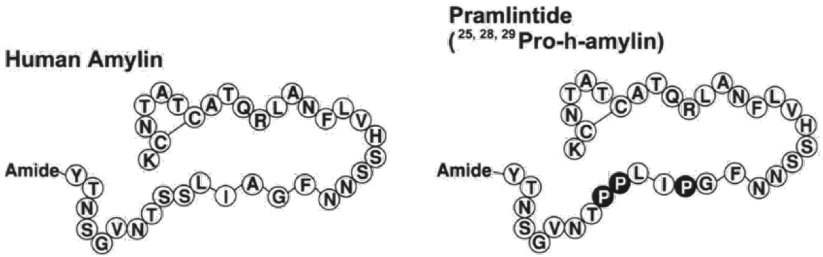


Peak areas from repeated injections of 1 µL sample from a single well/vial.

Does it really work? – Bioanalysis of synthetic peptides

- Pramlintide acetate (SYMLIN™) suffers from a high degree of non-specific binding.
  - It's a large peptide (MW 3949).
  - It's moderately hydrophobic (HPLC Index: 88.7).





	Peak Area	Recovery
QuanRecovery Plate	9278	99.0%
Conventional PP Plate	259	2.8%

Peak area and recovery of 10 ng/mL pramlintide stored in each plate.

Caitlin Dunning, look for her EBF poster for more details.

Does it really work? – mAb subunit analysis

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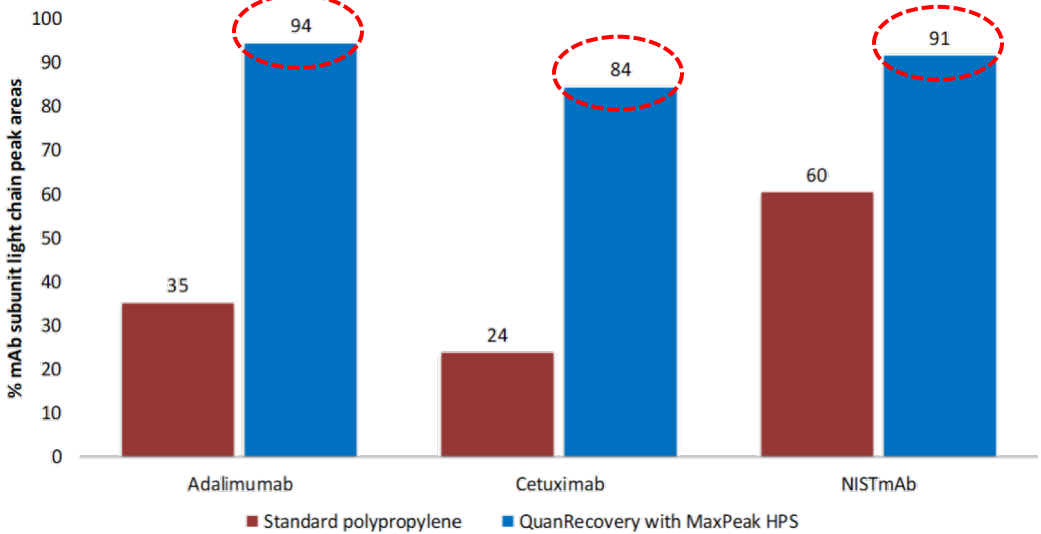
mAb	Precursor (m/z)	Fragment (m/z)	Fragment Identity
Adalimumab	1236.02		
Cetuximab	1236.81	1329.85	P119 – y96
NIST mAb	1288.84		

100 ng/mL mAb, after 24 hours of storage.



Summary

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- Proteins and peptides may adsorb to any surface, especially to sample containers, while waiting for LC-MS injections.
- Such losses are detrimental to the assay because they negatively affect recovery, sensitivity, and reproducibility.
- Optimizing experimental factors influence the severity of non-specific binding. Follow these steps to prevent the losses in the container.
  - Choose an appropriate container.
  - Select a compatible sample matrix.
  - Select an optimal sample storage condition.

Acknowledgements

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Waters EBF Team

EBF organizers

Thanks for attending!

To learn more about non-specific binding losses…

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- Posters at EBF
  - *Factors that influence the recovery of hydrophobic peptides during LC-MS sample handling*
  - *Development of a SPE LC-MS/MS Method for the Bioanalytical Quantification of Pramlintide from Serum*
- Waters QuanRecovery page

  http://www.waters.com/QuanRecovery
- Whitepaper on non-specific binding losses

  http://www.waters.com/waters/library.htm?lid=135018646

QuanRecovery™ WITH MAXPEAK™ HPS

Use it, don't lose it!

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