Displacement Chromatography Effects Can Cause Highly Selective Sampling of Peptides During Solid Phase Extraction Cleanup

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INTRODUCTION

Small solid-phase extraction cartridges are frequently used to capture and process peptide samples, especially for desalting prior to analysis by mass spectrometry. Millipore Corp. pioneered this field with the ZipTip® product line. Other companies have since introduced small cartridges for similar applications. While evaluating one such alternative design, we observed an unexpectedly high degree of selectivity in peptide binding from complex mixtures. This poster explores the cause of the selectivity and how to avoid it if desired

MATERIALS AND METHODS

SPE Cartridges: NuTip® and TopTip® cartridges were products of Glygen Corp. (Columbia, MD). NuTip cartridges have the stationary phase embedded in the walls with an open channel in the middle, while TopTips are miniature packed beds with a fritless design; the slit at the bottom permits liquid to exit but not the packing material. The NuTips used here, for samples 10-200 µl, contained either C-18 coated silica (item# NT200C18) or graphitic carbon (item# NT200CAR) for reversed-phase chromatography (RPC). The TopTips used here (item# TT200HEA), also for samples 10-200 µl, were packed with PolyHYDROXYETHYL Aspartamide, a silica-based material used for hydrophilic interaction chromatography (HILIC)[1].

Peptide Samples: Lyophilized tryptic digests of transferrin and ovalbumin were kindly provided by Ron Orlando (Complex Carbohydrate Research Center/ Univ. of Georgia/ Athens, GA). The samples had been reduced and alkylated prior to digestion, which was performed with 1 M urea in an ammonium bicarbonate buffer. Samples were received in the form of a urea-rich gel.

HPLC Analysis: Peptides eluted from NuTips or TopTips were analyzed with a PolyHYDROXYETHYL Aspartamide column (PolyLC Inc., Columbia, MD) [item# 204HY0503; 200x4.6-mm; 5-µm, 300-Å]. Except as noted otherwise, the column was operated in the HILIC mode, with a gradient of 85-44% acetonitrile (ACN) in 15 mM triethylamine phosphate buffer, pH 3.0. Peptides elute in order of increasing polarity. This mode was selected because gradients start with a high level of organic solvent; samples eluted from the reversed-phase NuTip cartridges could be injected with no further processing. The HPLC system was the Essence, from Scientific Systems Inc. (State College, PA). Absorbance was monitored at 220 nm.

[1] A.J. Alpert, *J. Chromatogr. 499* (1990) 177-196.

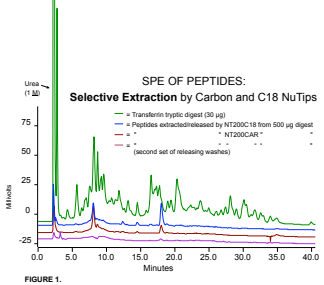


FIGURE 1.
Column: PolyHYDROXYETHYLA (item# 204HY0503), HILIC mode. Detection: A_{220}
Gradient: 40' linear, 85-32% ACN in 15 mM TEAP, pH 2.7

Figure 1. Selective Extraction by Carbon and C-18 NuTips.

500 µg of lyophilized tryptic digest of transferrin was dissolved in 250 µl water, resulting in a solution containing ~ 1-2 M urea.

First [top] Chromatogram: The complete digest. This profile is unremarkable. The offscale peak in the void volume is urea; the offscale peak immediately after it is DTT (left over from the reductive alkylation step).

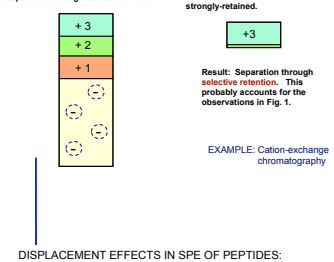
The 500-µg solution was aspirated/expelled 50x from a NuTip containing C-18-coated silica. This process was repeated with water (2x 20-µl volumes, aspirated 10x each) to wash out nonadsorbed peptides and urea. The adsorbed peptides were then eluted with 2x 10-µl washes (aspirated 10x each) of 15 mM TEAP, pH 2.7, containing 85% ACN. This process was repeated with a second NuTip and the eluates pooled for analysis.

Second Chromatogram: Pooled eluates from the C-18 NuTips. The binding capacity of each tip is apparently 2 µg peptide, judging from comparison with the first chromatogram. However, only a few peptides were retained by the NuTips.

Third Chromatogram: NuTips containing graphitic carbon had the same capacity as the C-18 tips and were selective in adsorbing the same few peptides from the complete digest, albeit in somewhat differing ratios.

Fourth Chromatogram: The used carbon NuTips from the initial experiment were eluted with a second round of 2x 10 µl releasing washes and the eluates again pooled for analysis. These contained < 10% of the peptide that the first releases did. Everything that could be released, was released. This suggests that the selectivity effects reflect selective adsorption of a small number of peptides rather than selective release of just a few.

FIGURE 2. Selectivity in Elution vs. Displacement Chromatography



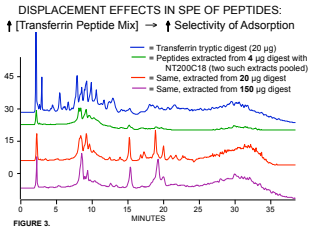


FIGURE 3.
Column: PolyHYDROXYETHYL A (item# 204HY0503), HILIC mode. Detection: A_{220}
Gradient: 40' linear, 85-44% ACN in 15 mM TEAP, pH 2.7

FIGURE 3. Adsorption Becomes More Selective as Sample Size Increases

First Chromatogram: Complete tryptic digest of transferrin

Second Chromatogram (peptide content of sample comparable to the binding capacity of the NuTip cartridge): The profile of the peptides bound/released is similar to that of the whole mixture .

Fourth Chromatogram (peptide content of sample greatly in excess of cartridge capacity): The most strongly adsorbed peptides are present in sufficient quantity to saturate the cartridge and displace all other peptides.

Third Chromatogram (intermediate peptide content): The most strongly-retained peptides are adsorbed in their entirety, but some capacity remains to adsorb others as well. The profile resembles that of the complete mixture with a few components bound/released to a much greater extent than the others.

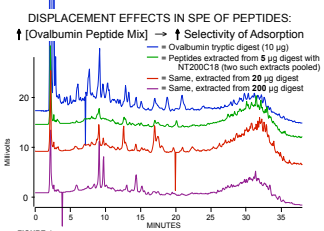


FIGURE 4.
Column: PolyHYDROXYETHYLA (item# 204HY0503), HILIC mode. Detection: A_{220}
Gradient: 40' linear, 85-44% ACN in 15 mM TEAP, pH 2.7

FIGURE 4. Displacement Effects with Ovalbumin Digest

The selectivity effects observed with the tryptic digest of transferrin are also observed with a digest of ovalbumin. As the amount of peptide in the sample increases, adsorption by a NuTip cartridge becomes more selective.

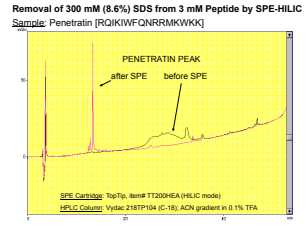


FIGURE 6. *(Data courtesy of Britt-Marie Olsson (Stockholm Univ.)*

FIGURE 6. Removal of SDS from a Synthetic Peptide with a TopTip.

SDS in the sample ruined its analysis by RPC. A HILIC TopTip successfully removed the SDS. The starting solvent, rich in ACN, broke the interaction between SDS and the peptide. The peptide was well-retained by the TopTip but SDS was not. The peptide was then eluted as in Fig. 5. The processed peptide eluted from an RPC column in a sharp peak.

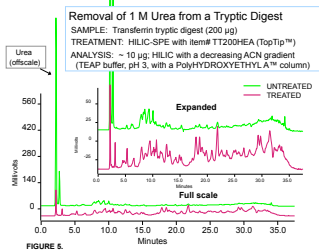


FIGURE 5.

FIGURE 5. Successful Removal of Urea from a Whole Digest with a TopTip.

TopTip cartridges have much higher capacity than do NuTip cartridges (~ 1 mg vs. 2 µg), and are used to capture and process a whole sample. In this case, 200 µg/100 µl of the tryptic digest of transferrin were mixed with 500 µl ACN and the resulting mixture was passed through the TopTip in several portions. The cartridge was flushed 3x 50 µl with 15 mM ammonium formate (pH 2.7) containing 85% ACN to eliminate urea and DTT. The peptides were then released with 4x 25-µl washes with 15 mM ammonium formate containing 10% ACN and the eluates were pooled.

Result: The bound/released peptides have a chromatographic profile similar to that of the starting mixture except that the level of urea is < 3% that in the starting mixture. Since the capacity of the cartridge was sufficient to adsorb all of the peptide, there was no selectivity due to displacement effects.

While these results were obtained with a HILIC TopTip cartridge, the same principle would apply to SPE cartridges operating in other modes such as RPC.

CONCLUSION

SPE cartridges such as NuTips can conveniently be used to desalt or otherwise process small amounts of a peptide sample. However, if the sample contains a number of different peptides in a quantity greatly exceeding the binding capacity of the cartridge, then the best-retained peptides can displace those less strongly adsorbed. The resulting adsorbed sample is not likely to be representative of the composition of the whole mixture. This problem can be avoided through the use of a SPE cartridge with a binding capacity exceeding that of the sample.

ACKNOWLEDGEMENTS

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ABSTRACT

Small solid-phase extraction cartridges are used for convenient cleanup of small amounts of peptide mixtures, especially for desalting prior to MS analysis. The assumption is that the peptides adsorbed are representative of the entire mixture. Our data indicate that this assumption is valid if the binding capacity of the cartridge is close to or exceeds the total amount of sample presented to it. If the amount of sample presented is greatly in excess of the cartridge's capacity, though, then peptides that bind with low affinity will be displaced by peptides that bind with high affinity. The consequence is that only two or three peptides may be sampled from a tryptic digest that contains 40-60 peptides. Examples are presented with tryptic digests of transferrin and ovalbumin that contain 1-2 M urea and a peptide sample containing 8.6% SDS, with cleanup via reversed-phase or hydrophilic interaction chromatography.