High Throughput Peptide Purification Strategies Using Silica -based 96-Well SPE Formats Prior to LC/MS Analysis

Elizabeth Denton[1], John Urh[1], Essra AboJasser[1], Yuan Li[1], Amit Mehrotra[2], Lee Williams[3] & Geoff Davies[3]

[1]Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte North Carolina 28269, USA
[2]Biotage AB, 751 03 Uppsala, Sweden
[3]Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Cardiff, CF82 7TS, UK

Introduction

Peptides have returned to the forefront of drug discovery efforts as unbiased screening technologies have improved and the rules defining passive cell permeability have been clarified. As a result, the demand for synthetic peptide libraries has increased significantly, particularly during secondary screening as lead compound programs progress. At these early discovery phases though, highly pure peptide samples may not be necessary when evaluating these compound libraries in assays. With this in mind, and given the improvements in automated peptide library synthesis strategies, a solid phase extraction methodology may be appropriate to parallelize peptide library cleanup and significantly reduce library purification time[1].

Herein we present data featuring the latest innovation from Biotage designed specifically to improve the parallel peptide synthesis workflow, with particular focus at the purification stage. The data will demonstrate that solid phase extraction can be a suitable replacement for traditional ether precipitation workflows without compromising peptide integrity, product yield, all the while potentially improving purity.

Experimental

Peptide Synthesis and Analysis

Peptides under investigation were synthesized automatically with a Biotage® Initiator+ Alstra™ peptide using Rink Amide resin, default methods, DIC/Oxyma as coupling reagents, and Fmoc-protected amino acids. Cyclic peptides were prepared with Fmoc-Cys(Acm)-OH building blocks, enabling on-resin orthongonal deprotection and concomitant oxidation.

Peptide cleavage occurred in an appropriate cleavage cocktail for 2 hours at room temperature. The cleavage cocktail was evaporated using the Biotage® V-10 Touch evaporation system and the resulting crude sample residue was dissolved for purification. Crude peptide samples were purified using a Biotage® Isolera™ Dalton 2000 equipped with a 25-gram Biotage Sfär Bio C18 column. Crude and purified peptides were analyzed for purity with an Agilent 1260 Infinity series HPLC.

Purified peptide samples were subjected to solid phase extraction (SPE) procedures using a Biotage® Extrahera™ sample preparation workstation equipped with Biotage® reversed phase media packed in 96-well plates. The sorbent plate was conditioned with 100% ACN, then equilibrated with 10% $ACN_{(aq)}$ prior to sample loading. After sample loading the sorbent was treated with 10% $ACN_{(aq)}$ to remove dissolution solvent, washed with 20% $ACN_{(aq)}$, then eluted with 70% $ACN_{(aq)}$, unless otherwise stated. All solvents utilized were modified with 0.1% TFA and solvent concentrations were selected based on calculated elution point using analytical HPLC.

Results and Discussion

Solid Phase Extraction Media Selection Impacts Results

Media utilized for traditional sample preparation methodologies are inherently designed to retain small molecules, like drugs of abuse, while excluding other biological matrix components like proteins, peptides, and lipids. This physical property however, could become a liability when applied to alternative chemical systems. The properties of three reversed phase media evaluated are shown in ***Table 1.***

Table 1. *Comparison of sorbents used for potential peptide cleanup.*

	PeptiRen C18	Wide-pore C18	"standard" SPE C18
Particle size (µm)	30	20	50
Pore size (Å)	100	300	60

To deploy a parallel workup protocol, one must be confident that *all* compounds treated simultaneously will elute from the stationary phase *predictably* and *completely.* Each of the three commercially available medias were compared using a purified, amphipathic 18 amino acid peptide loaded onto the sorbents and treated with 65% $ACN_{(aq)}$ and then 70% $ACN_{(aq)}$. ***Figure 1.***

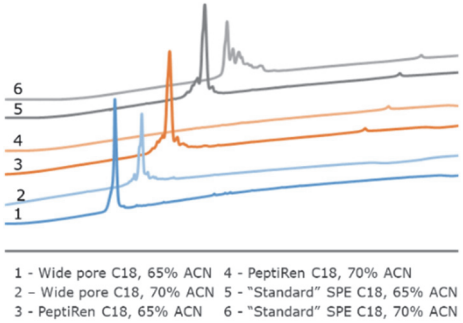

Figure 1. *Comparison of elution efficiency for three commercially available reversed-phase solid phase extraction medias.*

The peptide is expected to be present in the 65% $ACN_{(aq)}$ fraction based on its analytical HPLC elution profile, so any detectable peptide in the 70% $ACN_{(aq)}$ would have been lost. Only the PeptiRen C18 material allowed for both retention and complete elution of the peptide of interest. This observation has been replicated with a wide range of peptides (data not shown). Given the non-specific interaction leveraged in reversed phase chromatography, this technique can also be applied for non-linear peptide sequences, ultimately generalizing this strategy. Future efforts will focus on a variety of cyclic peptides, differing primarily in their cyclization strategies.

Peptides are Recovered at High Percentages

Peptide libraries are typically synthesized on relatively small scale, demanding a high recovery percentage in order to have enough material to forward for evaluation. Once the optimal sorbent was selected, the amount of peptide one expects to be recovered from a solid phase extraction, ***Table 2***.

Table 2. *Sample recovery after subjecting a purified peptide at 0.5, 1, 2, and 3% loading levels (n=3) to solid phase extraction protocols. Across these loading ranges, an average of 87% of the loaded sample was recovered.*

Expected sample load (mg)	Actual sample load (mg)	Recovered sample (mg)	Percent recovery
2	2.3 ± 0.1	2.3 ± 0.1	100.2 ± 7.4%
5	4.05 ± 0.07	3.8 ± 0.3	88.7 ± 9.5%
10	8.7 ± 0.17	7.3 ± 0.4	78.8 ± 5.3%
15	12.8 ± 0.4	11.2 ± 0.6	81.6 ± 5.7%

These data, collected using a purified 10 amino acid representative peptide, suggest that high sample recoveries can be consistently achieved across a wide range of loading levels. Future work requires a similar analysis to be completed for a wider range of peptides varying size, structure, and other general physiochemical properties.

Eliminating Ether Precipitation from a Library Workflow

Adapting traditional peptide workflow steps to a plate-based peptide library can be more challenging than anticipated. Volume limitations in the well plate require a preliminary concentration step to ensure full miscibility of the ether with the cleavage cocktail. Removing the ether supernatant requires additional adaptation, all of which is a completely manual procedure.

Proof of concept experiments using SPE for crude peptide libraries indicated that residual protecting groups elute at grossly different chromatographic conditions than is observed for the majority of peptides, suggesting that PeptiRen SPE could replace ether precipitation in the traditional peptide library workflow, ***Figure 2.***

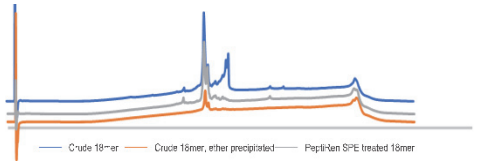

Figure 2. *Comparison of peptide purity for a crude peptide with no ether precipitation used (blue) and subjected to PeptiRen SPE protocols (grey) to the same crude peptide ether precipitated.*

These data clearly demonstrate that cleavage cocktail scavengers and residual protections groups (second large peak, blue trace) have distinctly different chromtagraphic behavior than other peptidic sample components. This peptide elute at approximately 68% $ACN_{(aq)}$ via C18-based analytical HPLC, and after a treatment with 70% $ACN_{(aq)}$ during PeptiRen SPE, can readily be separated from the non-peptidic sample components. This delineation point simplifies SPE clean up of peptide libraries, which likely contain peptides with grossly different chromatographic behavior. But more importantly, can be used as a suitable, less laborious, replacement for ether precipitation.

Conclusion

Herein we have shown data demonstrating that Biotage® PeptiRen SPE can be confidently deployed in a peptide library workflow, reducing manual steps and generally expediting delivery of the library for downstream evaluation, ***Figure 3***.

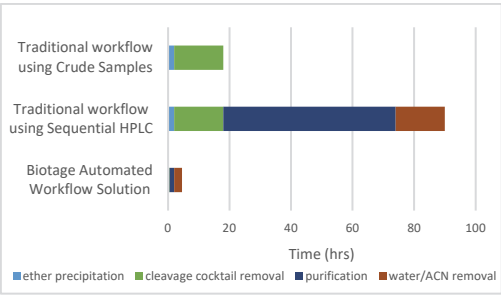

Figure 3. *Timeline outlining time investment required to workup a single 96 compound peptide library after the synthesis has completed. When coupled with a Biotage® Extrahera™ the PeptiRen-96 significantly reduces time and impact to deliver the library to downstream assays.*

Generation of synthetic peptide libraries requires a much different handling process than traditional, larger scale syntheses, even those done in parallel. Biotage® PeptiRen-96 well plates have been intentionally designed to disrupt the established peptide workup processes and overall workflow. Future work will focus on expanding the generality of this technique to include cyclic peptides and further evaluation of larger peptides as well.

Disclosure: The authors declare no competing financial interest. However, salaries of all authors are paid by Biotage.

[1] Bennett, R. et al Anal. Chim. A. **2020**, 1142, 10-18.

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