A New Workflow to Purify Peptides

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Abstract

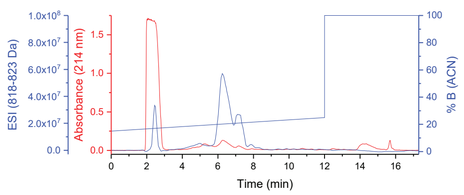
Peptides are increasingly used for medicinal compounds and for research; some examples include the antibiotic cyclosporin and GLP-1 agonists. Peptides are also used for agonist/antagonist assays, where a peptide is used as a model for larger compounds to evaluate their interaction with small molecules or other peptides for medicinal activity. Synthetic peptides are commonly purified using reverse phase columns. Some purifications require several tens of minutes using a gradient, limiting the peptide throughput.

A faster method to purify peptides makes use of an isocratic method using stacked injections. Although stacked injections are commonly used for chiral purifications, they also work well for the purification of achiral compounds including peptides. This technique is faster than traditional preparative gradients, saving time and solvent. Stacked injections increase throughput, eliminate time and solvent needed to re-equilibrate the column, and maximize the column capacity by using the elution time for the first sample injection to perform other injections. Scale-up simply means increasing the number of injections.

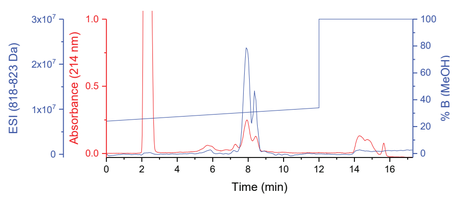
This poster explores how scouting runs are used to determine if a peptide mixture is suitable for a stacked injection, how to rapidly clean a peptide for stacked injections, and how to quickly determine a solvent system for stacked injections. The scouting runs are used to determine the isocratic method.

Background

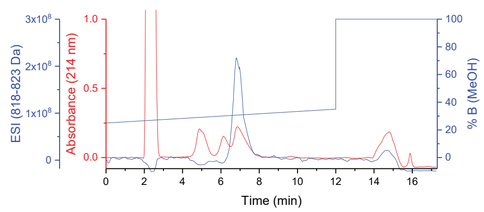
Peptides are commonly purified with a solvent system consisting of water/acetonitrile with 0.1% trifluoroacetic acid as a solvent modifier using long gradients. Using these methods, scaling up a particular peptide production requires larger columns. Another problem is loss of peptides due to incorrect buffering, where a peptide can elute as multiple peaks. Without a mass spectrometer, these multiple peaks seem to be impurities (top figure, below).



- *EGFRvii run with water and acetonitrile containing 0.1% TFA*
- *Mass spectrometer shows multiple peaks*
- *Using UV detection only, the largest peak is typically collected*
- *For preparative LC, sample concentration is generally high compared to TFA within a peak*



- *Strong solvent changed to methanol*
- *Water pH = 4.5 (50 mMol ammonium acetate)*
- *Mass spectrometer still shows multiple peaks*
- *EGFRvii pI = 5.5*

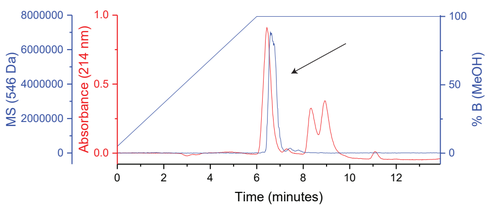


- *Water pH = 3.5 (50 mMol ammonium formate)*
- *Sample eluted as a single peak*

Scout Runs

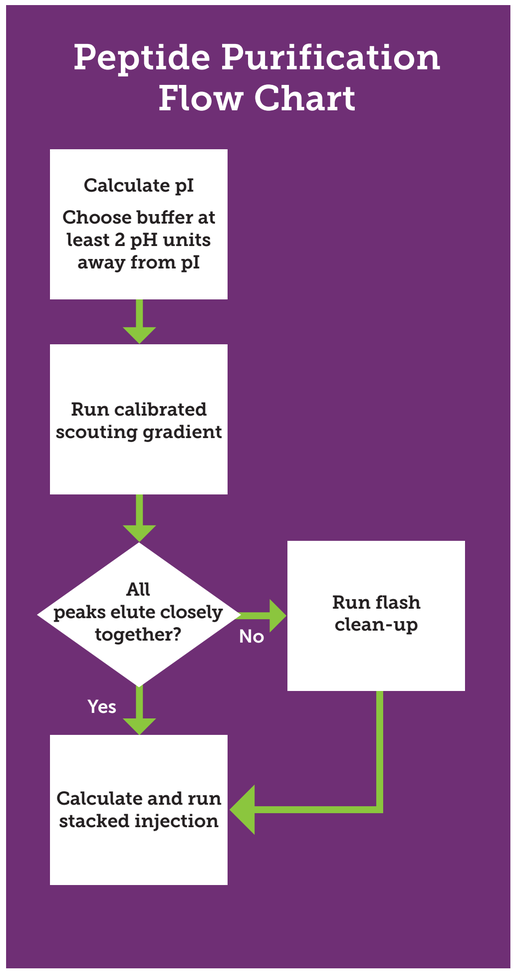
The scout run does several things:

- Allows calculation of an efficient isocratic run for the peptide (if a calibrated scouting gradient is used)
- Allows evaluation of impurities
- Provides an initial assessment of the interaction of the solvent, column, and sample
- Suggests an injection solvent—compounds with a calculated gradient of 30% organic solvent or less are usually soluble in water



- *Water pH = 3.5 (50 mMol ammonium formate)*
- *Late eluting impurities suggest pre-purification needed to improve preparative HPLC efficiency*
- *Reversed-phase flash chromatography is a fast way to remove well-resolved impurities quickly*

Peptide Purification Flow Chart



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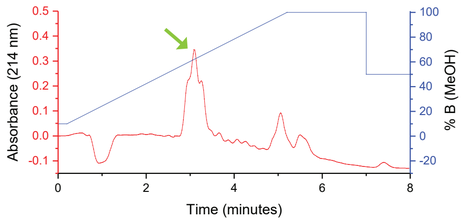
SCAN ME

[1] Silver, J. Overview of Analytical-to-Preparative Liquid Chromatography Method Development. *ACS Comb Sci* **2019**, *21* (9), 609-613. DOI: 10.1021/acscombsci.8b00187

Cleanup

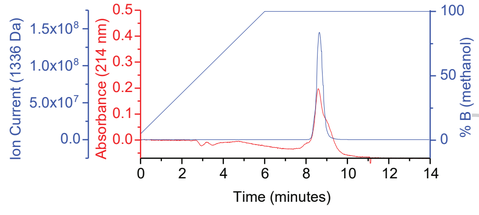
- A cleanup run removes early and late eluting peaks that extend run time
- An extra step that may save time
- Little method development needed—run a standard gradient



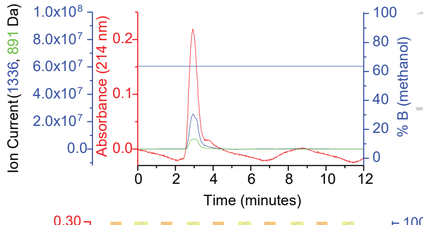
- *Water pH = 3.5 (50 mMol ammonium formate)/methanol gradient*
- *15.5 g RediSep Gold® C18 flash column*
- *50 mg EGFRvii peptide loaded*
- *Peptide collected, methanol evaporated using an Ecodyst evaporator*
- *Peptide used for stacked injection from aqueous solution*
- *Be mindful of the container used for injection.* ***Peptides may adsorb on plastic vials!***

Final Purification

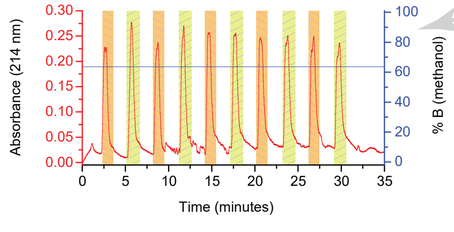
- This run used a different peptide
- Stacked injection
- Isocratic method calculated scouting gradient using the Time-on-Target algorithm[1] (built into the ACCQ*Prep*® system)
- Peptide peak can be shaved to remove closely eluting impurities



Scouting run shows impure peptide as shoulders around the peak detected with the Purlon mass spectrometer. An isocratic method was calculated from the scouting run using PeakTrak software and a 10 x 150 mm RediSep Prep C18 column. The calculated method used 63.6 % methanol in 50 mMol ammonium carbonate at pH 7.0.



Stacked injection used peak collection to determine the collection time windows. The peptide was collected only within the time windows. Only two prior injections (the scout and the isocratic run above) were needed to generate the stacked injection below. The calculated gradient suggested a mixture of methanol and water would be a good injection mixture. DMSO was avoided, as the DMSO peak would increase the stacked injection cycle time.



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