Flash Chromatography, a Fast and Efficient Technique for Purification of Peptides:

Improved Separation Performance Using Biotage® SNAP Bio Wide Pore media Cartridges – Part 1

Introduction

Despite the improvements in automated solid phase peptide synthesis, such as the use of elevated temperatures, whereby peptides of greater complexity and purity can be synthesized routinely, the purification step is one of the main bottlenecks in the peptide synthesis workflow. Preparative RP-HPLC is normally the method of choice but is limited by small loading amounts, long separation times, poor recoveries and high costs. In addition, crude synthetic peptides contain impurities with retention characteristics very similar to the target peptide which can present additional purification challenges. Although there are a number of examples in the literature,[1] flash chromatography or medium pressure liquid chromatography (MPLC) is almost never considered as a technique for purification of synthetic peptides, as it is not perceived to be suitable for this application. However, with recent advances in flash using 20–25 micron spherical particles, making flash chromatography an efficient technique for synthetic peptide purification.

Biotage® SNAP Bio flash cartridges were developed with a small particle size (20 µm) and large pore size (300 Å) to provide increased resolution and effective separation of complex peptide mixtures. Flash chromatography can be used either as the sole purification method or as a front end clean-up prior to a final RP-HPLC step.

In part 1 of this application note we show a variety of examples demonstrating how flash chromatography can be used as the sole purification method for purification of crude synthetic peptides to speed up your workflow.

Experimental

Materials

All materials were obtained from commercial suppliers; Sigma-Aldrich (acetonitrile, formic acid, sodium hydrogen carbonate and methanol). Milli-Q (Millipore) water was used for LC-MS analysis. Life Technologies (human insulin), Amersham Pharmacia Biotech (ribonuclease A and ovalbumin).

Synthesis

General Solid-Phase Synthesis of Peptides

All peptides were prepared by Fmoc solid-phase peptide synthesis on a Biotage® Initiator+ Alstra™ fully automated microwave peptide synthesizer using standard methods.

Peptides were cleaved from the resin with TFA-H_2O-TES (95:3:2) for 5 min. and then for 2 h and precipitated with cold diethyl ether.

Analysis of the peptides and proteins were performed by ULC-MS on a QTOF Impact HD, RSLC Dionex Ultimate 3000 (Thermo) using a Kinetex 2.6 µm EVO 100 Å C18 column (50 × 2.1 mm, Phenomenex). Analysis of some peptides and proteins used an Aeris 3.6 µm Widepore C4 column (50 × 2.1 mm, Phenomenex) with a flow rate of 0.5 mL/min. The following solvent system was used: solvent A, water containing 0.1% formic acid; solvent B, acetonitrile containing 0.1% formic acid. The column was eluted using a linear gradient from 5%–100% of solvent B.

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Flash chromatography was performed in reversed-phase mode on an Isolera™ flash purification system equipped with either Biotage® SNAP KP-C18-HS 12g, SNAP KP-C18-HS 30g, SNAP Ultra C18 30g, SNAP C18 300 Å 10g, SNAP C18 300 Å 25g, or SNAP C4 300 Å 25 g cartridges respectively.

Results & Discussion

Ac-DWLKAFYDKVAEKLKEAF-NH_2 ('18A')

The '18A' peptide (MW 2242.58 Da), which forms nanodiscs when dissolved with phospholipids, was synthesized with a crude purity of 60% measured by analytical LC-MS (Figure 1).

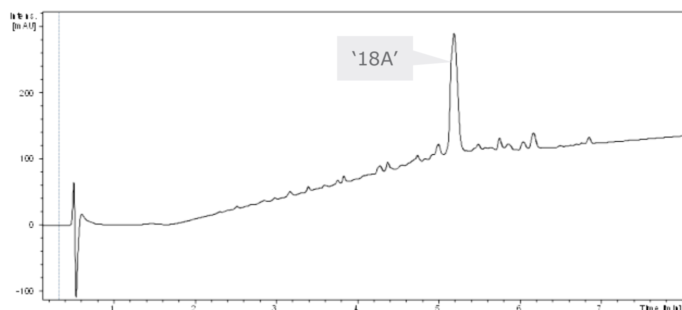


Figure 1. LC-MS graph of the crude '18 A' peptide.

This peptide is challenging to purify because of its low solubility which as a consequence normally requires multiple injections when purified by prep RP-HPLC.[2]

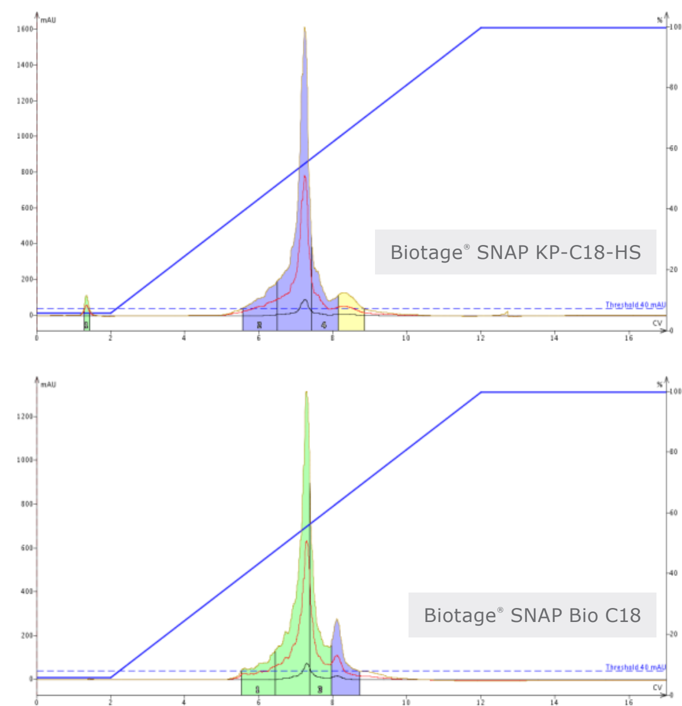
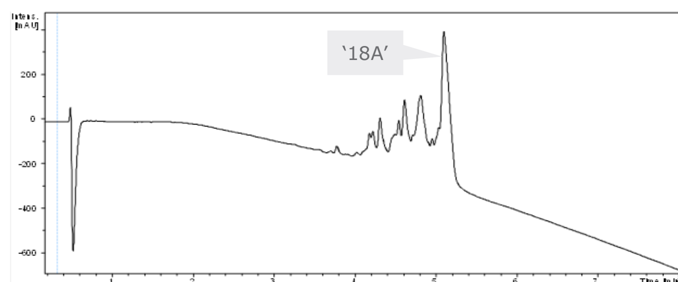


Figure 2. Chromatograms from the purification of '18 A' peptide using Biotage® SNAP KP-C18-HS and Biotage® SNAP Bio C18 flash cartridges.

In this example we compare the purification of '18A' on a 50 µm irregular silica C18 cartridge (Biotage® SNAP KP-C18-HS) with a 20 µm spherical silica Biotage® SNAP Bio C18 300 Å cartridge. Aqueous acetonitrile solution (including 0.1% formic acid) was used as mobile phase with gradient elution.

200 mg of crude '18A' peptide was dissolved in 10 mL of 20% aq. acetonitrile. 5 mL samples of this crude peptide solution were purified on both types of flash cartridges and the chromatograms are shown in Figure 2.

A 5 mL injection containing 100 mg crude sample (loading capacity of 0.8%) was purified on a Biotage® SNAP KP-C18-HS cartridge, and the target peptide '18A' was collected in two fractions with purities of 60% and 88% (based on HPLC analysis) and after lyophilization the dry weight of the fractions were found to be 15 mg and 33 mg respectively (Figure 3).



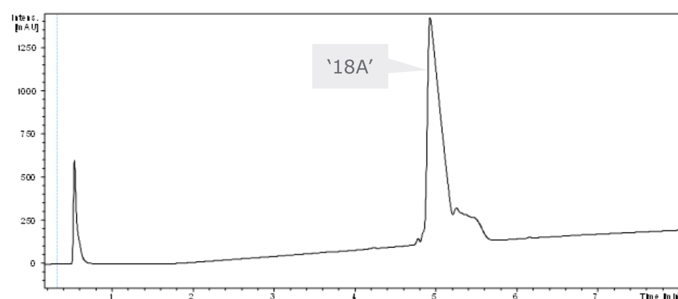


Figure 3. HPLC chromatogram of '18A' peptide fractions after flash purification using a Biotage® SNAP KP-C18-HS cartridge.

A 5 mL injection containing 100 mg crude sample (loading capacity of 1%) was purified on a Biotage® SNAP Bio C18 300 Å cartridge, and the target peptide '18A' was collected in one main fraction which was >98% purity (based on HPLC analysis, Figure 4) with a recovery yield of 66% (40 mg).

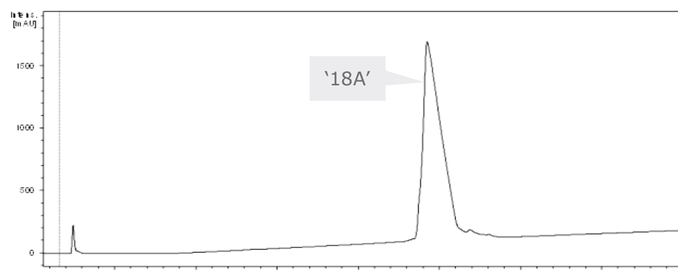


Figure 4. HPLC chromatogram of flash purified '18A' peptide after flash purification using a Biotage® SNAP Bio C18 300 Å cartridge.

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Chromatography Method

Cartridge	Biotage® SNAP KP-C18-HS 12 G	Biotage® SNAP Bio C18 300 Å 10 G
Flow Rate	12 mL/min	12 mL/min
Solvent A	H_2O (0.1% formic acid)	H_2O (0.1% formic acid)
Solvent B	CH_3CN (0.1% formic acid)	CH_3CN (0.1% formic acid)
Equilibration	5% B, 3 CV	5% B, 3 CV
Gradient	5% B, 2 CV 5–100% B, 10 CV 100% B, 5 CV	5% B, 2 CV 5–100% B, 10 CV 100% B, 5 CV

The Biotage® SNAP Bio C18 300 Å flash cartridge with wide pore media, showed increased resolution with better separation and better recovery of purified peptide in comparison with the standard Biotage® SNAP KP-C18-HS flash cartridge.

H-IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2 (PYY 3-36)

The gut hormone PYY 3-36 is a 34-amino acid peptide involved in appetite regulation.

The crude peptide obtained from the synthesis included the desired peptide and a high concentration of residual protecting groups (Figure 5).

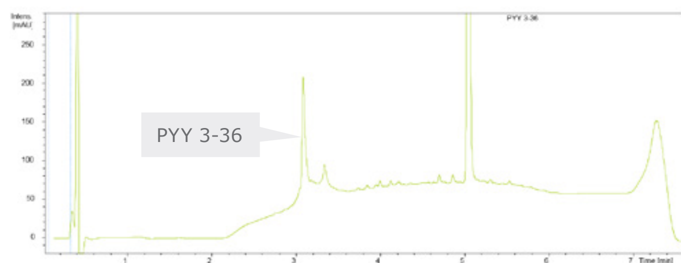

Figure 5. HPLC chromatogram of crude PYY 3-36 peptide, crude purity 65%.

500 mg of crude peptide (crude purity of 65%) was then dissolved in 15 mL of 50% aq. acetonitrile. This crude peptide is difficult to get in solution and forms a slurry. Nevertheless it could still be injected on to a disposable flash cartridge. A 5 mL sample was injected on to a Biotage® SNAP Ultra C18 30 g flash cartridge using aqueous acetonitrile solution (including 0.1% formic acid) as mobile phase with gradient elution (Figure 6).

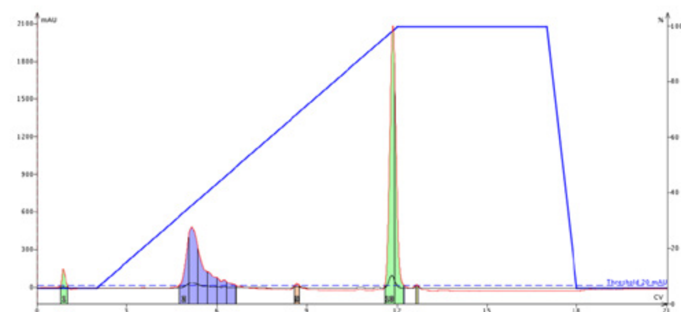

Figure 6. Chromatogram of PYY (3-36) peptide purified using a Biotage® SNAP Ultra C18 30 g flash cartridge.

The pure peptide was obtained in fractions 2 and 3, which were combined and freeze dried to give 15 mg of pure peptide with >95% purity.

The purification was repeated on a Biotage® SNAP Bio C18 300 Å flash cartridge and again a 5 mL crude peptide sample was injected on to the cartridge and the purification was performed with the same gradient (Figure 7). Pure peptide was found in fractions 3–6 which were combined and freeze dried to give 20 mg of pure peptide with >95% purity (Figure 8).

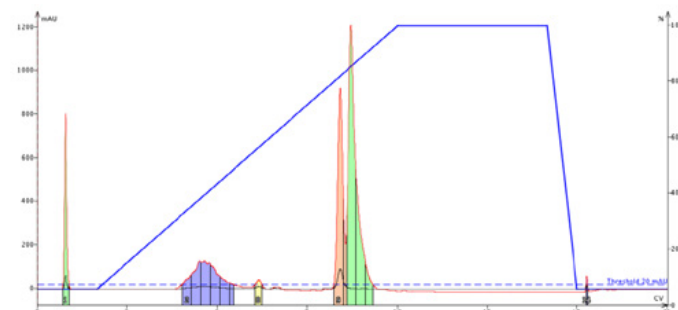

Figure 7. Chromatogram of PYY (3-36) peptide purified using a Biotage® SNAP Bio C18 flash cartridge.

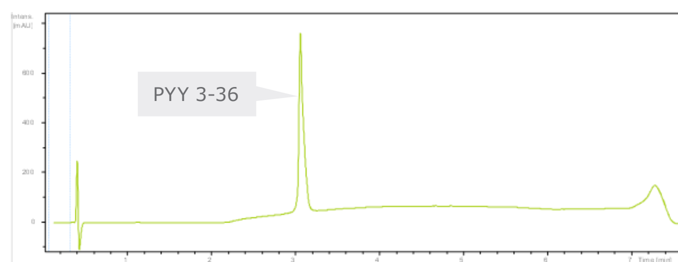

Figure 8. HPLC chromatogram of flash purified PYY (3-36) peptide, >95% purity.

Despite the peptide solubility issues which can hinder separation, a large amount of peptide could be processed in a single injection. It would normally take 3–4 purification runs on prep RP-HPLC to obtain this quantity of pure peptide due to the low sample loading capacity. The larger recovery obtained from the wide pore media cartridge (Biotage® SNAP Bio) is a result of its improved resolution and retentive properties providing more fractions containing pure peptide compared with the Biotage® SNAP Ultra C18 cartridge.

Chromatography Method

Cartridge	Biotage® SNAP Ultra C18 30 G	Biotage® SNAP Bio C18 300 Å 25 G
Flow Rate	45 mL/min	45 mL/min
Solvent A	H_2O (0.1% formic acid)	H_2O (0.1% formic acid)
Solvent B	CH_3CN (0.1% formic acid)	CH_3CN (0.1% formic acid)
Equilibration	5% B, 3 CV	5% B, 3 CV
Gradient	5% B, 2 CV 5–100% B, 10 CV 100% B, 5 CV 100–5% B, 1 CV 5% B, 3CV	5% B, 2 CV 5–100% B, 10 CV 100% B, 5 CV 100–5% B, 1 CV 5% B, 3CV

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33-mer and 36-mer

In the next example two long peptides (sequence information not disclosed) which are normally difficult to purify by prep RP-HPLC were purified using flash chromatography on a Biotage® SNAP Bio C18 300 Å 10 g flash cartridge. The synthesis afforded peptides with crude purities of 38% and 40% respectively and were able to be purified by flash giving purities >90% of the target peptides as judged by HPLC analysis (Figure 9).

No. AA	Crude Purity	Quantity injected	Sample Loading Capacity	Purity after Flash Chromatography	Recovery	Yield
33	38%	73 mg	0.73%	94%	16 mg	58%
36	40%	40 mg	0.4%	94%	11 mg	69%

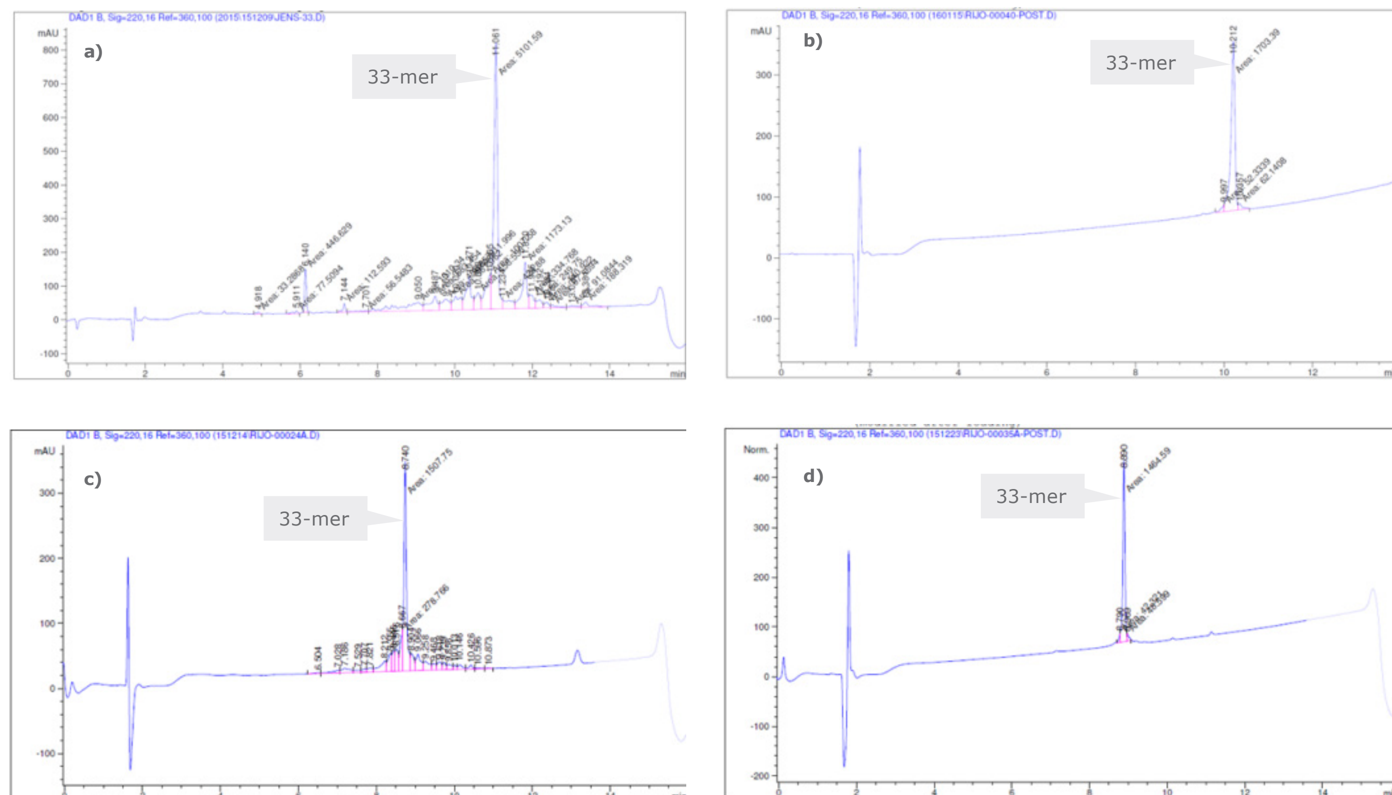

Figure 9. a) HPLC chromatogram of crude 33-mer peptide, crude purity 38%; b) HPLC chromatogram of flash purified 33-mer using a Biotage® SNAP Bio 300 Å cartridge, purity 94%; c) HPLC chromatogram of crude 36-mer peptide, crude purity 40%; d) HPLC chromatogram of flash purified 36-mer using a Biotage® SNAP Bio 300 Å cartridge, purity 94%.

Human Insulin

The retentive properties of the wide pore media cartridges were evaluated with human insulin protein (~6 kDa). A sample of human insulin was injected on to three different flash cartridges to evaluate their retentive properties.

180 mg of insulin was dissolved in 10% aq. acetonitrile. (In order to get the insulin in solution the pH was first raised to 8.5 with $NaHCO_3$ and once in solution, the pH was lowered to 6.5 with formic acid and the volume was adjusted to 15 mL). 5 mL of this solution containing 60 mg of insulin was injected on to each of the three flash cartridges and the chromatography assessed, and all collected fractions were freeze dried to provide the dry weight data required for recovery calculations.

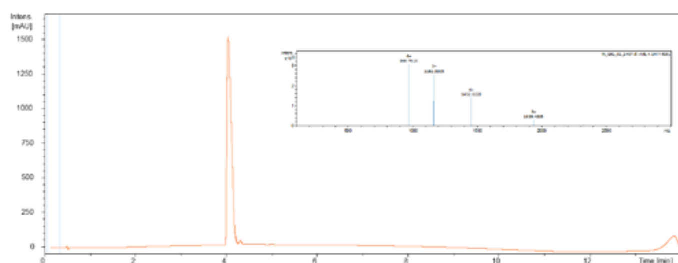

Figure 10. Analytical HPLC of human insulin.

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The chromatograms show the comparison of peptide retention and peak shape observed on the different cartridges (Figure 11).

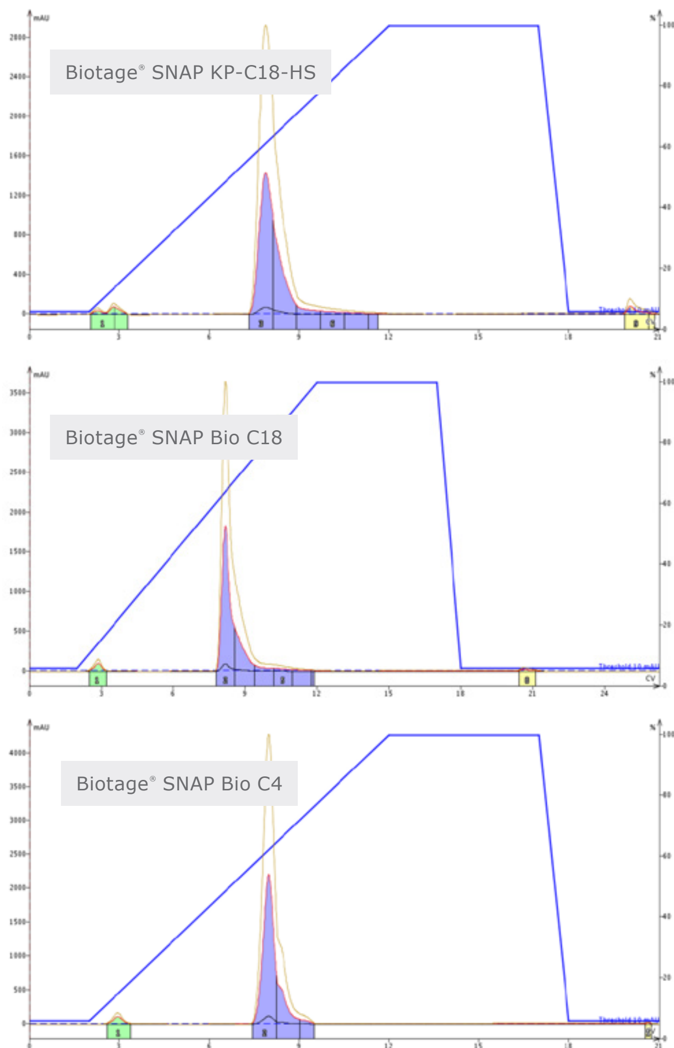

Figure 11. Chromatograms of flash purification of human insulin protein using three different Biotage flash cartridges.

Cartridge	Size	Sample Loading Capacity	Recovery	Yield
Biotage® SNAP KP-C18-HS	30 g	0.2%	42 mg	70%
Biotage® SNAP Bio C18	25 g	0.24%	47 mg	78%
Biotage® SNAP Bio C4	25 g	0.24%	55 mg	92%

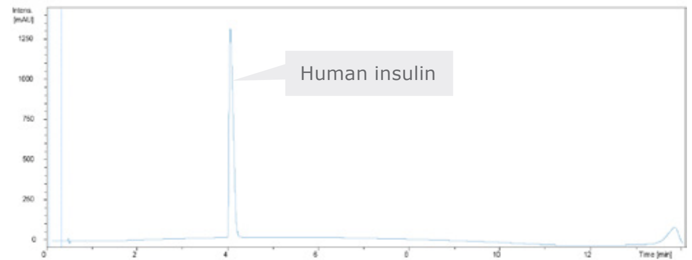

Figure 12. Representative example of analytical HPLC of a freeze-dried fraction.

The chromatograms showed that the protein sample was able to be retained on all cartridges. The Biotage® SNAP Bio C4 flash cartridge showed the least amount of tailing and superior resolution and recovery.

Chromatography Method

Flow Rate	45 mL/min
Solvent A	H_2O (0.1% formic acid)
Solvent B	CH_3CN (0.1% formic acid)
Equilibration	5% B, 3 CV
Gradient	5% B, 2 CV
	5–100% B, 10 CV
	100% B, 5 CV
	100–5% B, 1 CV
	5% B, 3CV

Protein Test Mix

The C4 cartridge was evaluated further. In this example, a test mix sample containing three different proteins (in PBS buffer with additional 6M guanidine hydrochloride solution). The proteins were human insulin (~6 kDa), ribonuclease A (~13,7 kDa) and ovalbumin (~34 kDa). A 2 mL sample of the solution was injected onto a Biotage® SNAP Bio C4 300 Å 10 g flash cartridge. The sample contained around 1.5 mg of each protein (Figure 13–17).

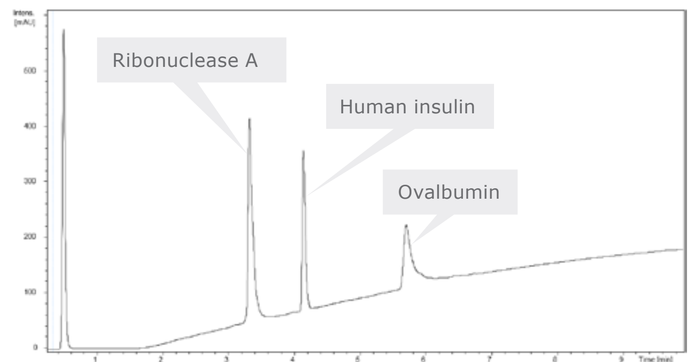

Figure 13. Analytical HPLC of the three protein test mix sample.

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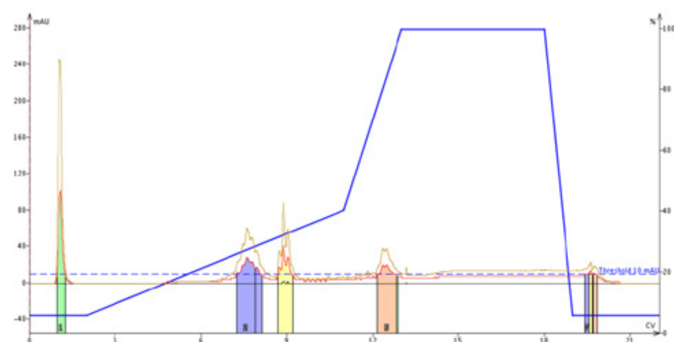

Figure 14. Chromatogram from Isolera™ shows the protein mixture was adequately resolved by flash chromatography using the Biotage® SNAP Bio C4 300 Å 25g flash cartridge.

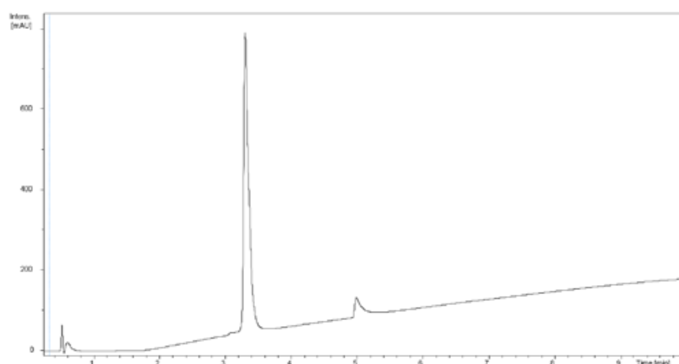

Figure 15. Ribonuclease A, fraction purity 94%.

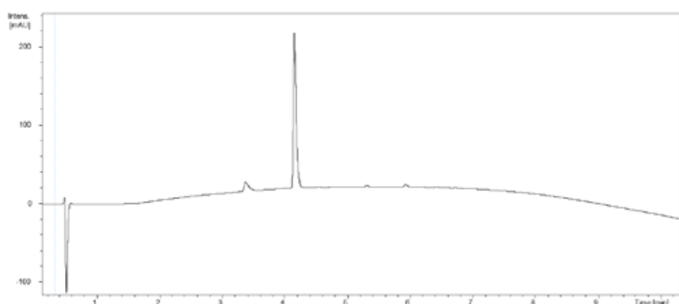

Figure 16. Human insulin, fraction purity 93%.

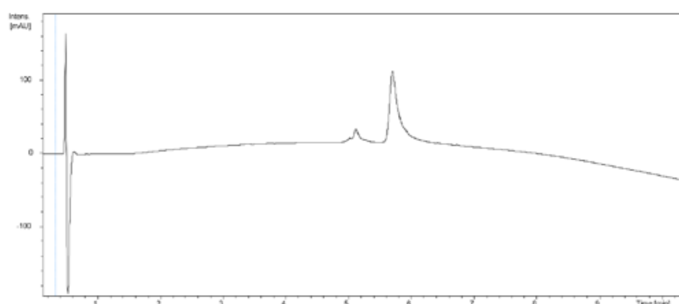

Figure 17. Ovalbumin, purity 99%. Fractions consist of unfolded ovalbumin (peak 1, 15%) and folded ovalbumin (peak 2, 84%).

Chromatography method

Cartridge	Biotage® SNAP Bio C4 300 Å 25g
Flow Rate	45 mL/min
Solvent A	H_2O (0.1% formic acid)
Solvent B	CH_3CN (0.1% formic acid)
Equilibration	5% B, 3 CV
Gradient	5% B, 2 CV 5–80% B, 7 CV 80–100%, 3 CV 100% B, 5 CV 100–5% B, 1 CV 5% B, 3CV

Conclusion

The results demonstrate that flash chromatography is a fast and efficient technique to dramatically clean-up synthetic peptides and larger molecules and can be used as the sole method of purification for some crude peptide samples. Flash chromatography enables an increase in sample loading capacity compared to prep RP-HPLC which results in a greater peptide quantity to be processed in a single injection.

The Biotage® SNAP Bio 300 Å flash cartridges with wide pore media, provided increased resolution with better separation and better recovery when purifying crude peptide mixtures in comparison to standard C18 flash cartridges.

References

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2. Biotage® application note AN103: High Recovery and Yield Of Amphipathic Peptide '18A'.

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