Application Note

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Optimizing Analysis and Purification of a Synthetic Peptide Using PLRP-S Columns

Reliable columns and media with the scale and pore size for successful purification of synthetic peptides

Authors

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Abstract

Reversed-phase ion-pair chromatography has become increasingly useful for the analysis and purification of synthetic peptides using mobile phases containing trifluoroacetic acid (TFA) as an ion-pair reagent. Scaling up from analytical to preparative high performance liquid chromatography (HPLC) can be costly, time consuming, and often difficult to perform due to differences in chemistries, pH conditions, particle sizes, and column length. This application note describes the analysis of a synthetic peptide and how to scale-up methods using an Agilent PLRP-S analytical HPLC column. The gradient and loading information are applied directly to a larger preparative column packed with an identical material.

Introduction

Peptide therapeutics are gaining popularity because of developments in biotechnology and bioengineering, including cancer diagnosis and treatment, antibiotic drug development, and new vaccines. Most peptide drugs are produced using solid-phase peptide synthesis (SPPS). The synthesis is performed on a polymeric support or resin, which can easily be filtered from reactions. The synthetic route includes multiple deprotection, activation, and coupling steps. The final peptide sequence is separated from the resin using a cleavage cocktail containing scavengers and other components resulting in the final crude product that is ready for purification.

Crude peptides synthesized by solid-phase peptide synthesis are analyzed by HPLC using reversed-phase columns with gradient elution using aqueous acetonitrile (typically containing 0.1% trifluoroacetic acid (TFA) as the ion-pair reagent). Liquid chromatography/mass spectrometry (LC/MS) based peptide analysis is normally used to confirm the structure of the target molecule. However, TFA is not ideal for LC/MS since it causes ion suppression, creating a weaker MS signal. The preferred ion-pair reagent for LC/MS methods is formic acid (FA), a weaker acid than TFA.

This work uses human glucagon-like peptide-1 (GLP-1) 7-36 amide, a single polypeptide chain containing 30 amino acids, which has a molecular mass of 3,297.7 Daltons (Da) (Figure 1).

This application note describes the ability to perform direct scale up from analytical PLRP-S 4.6 × 250 mm, 8 µm columns to larger scale preparative PLRP-S 21.2 × 250 mm, 8 µm columns. Two pore sizes appropriate for the separation of peptides, 100 and 300 Å, were investigated. Agilent PLRP-S is a rigid macroporous styrene divinylbenzene (PS-DVB) HPLC stationary phase with outstanding chemical and physical stability. PLRP-S HPLC media is inherently hydrophobic and does not require a bonded alkyl chain such as C8 or C18 to confer hydrophobicity. The characterization of the final product was confirmed using an Agilent 6545XT AdvanceBio liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF) with an orthogonal AdvanceBio Peptide Mapping column, 2.1 × 100 mm, 2.7 µm.

Sample preparation

Glucagon-like peptide GLP-17-36 amide was synthesized by CS Bio (Menlo Park, CA 94025, USA). The solid supports for the synthesis were provided by Agilent Technologies. The synthesis was achieved using standard side chain protection strategy and coupling conditions (fluorenylmethoxycarbonyl (Fmoc) chemistry).

Analytical equipment

An Agilent 1290 Infinity II LC system was composed of the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117C) with a 10-mm InfinityLab Max-Light Cartridge Cell (G7117-60020)

Preparative equipment

An Agilent 1290 Infinity II preparative LC system was composed of the following modules:

- Agilent 1290 Infinity II preparative binary pump (G7161B)
- Agilent 1260 Infinity II fraction collector (G7157A)
- Agilent 1290 Infinity II preparative column compartment (G7163B)
- Agilent 1260 Infinity II diode array detector (G7165A)

LC/MS equipment

Agilent 1290 Infinity II LC system coupled to the 6545XT AdvanceBio LC/Q-TOF (G6549AA)

Software and data processing

- Agilent OpenLab software suite, version 2.6
- OpenLab ChemStation CDS, version C01.09
- Agilent MassHunter data workstation acquisition, version B10.00
- Agilent MassHunter BioConfirm software, version 10.00

H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2

Figure 1. The amino acid sequence of synthetic GLP-1 (7-36) amide.

Columns

- **Analytical columns:** Agilent PLRP-S 100 Å, 4.6 × 250 mm, 8 µm (part number PL1512-5800); Agilent PLRP-S 300 Å, 4.6 × 250 mm, 8 µm (part number PL1512-5801)
- **Preparative columns:** Agilent PLRP-S 100 Å, 21.2 × 250 mm, 8 µm; Agilent PLRP-S 300 Å, 21.2 × 250 mm, 8 µm (custom dimension)
- **LC/MS column:** AdvanceBio Peptide Mapping 2.1 × 100 mm, 2.7 µm (part number 655750-902)

Solid support

- AmphiSpheres 40 RAM 0.4 mmol/g 75 to 150 µm (part number PL3867-4764)
- PL-Rink Resin (1% DVB) 0.3 mmol/g 75 to 150 µm (part number PL1467-4749)

Reagents and chemicals

All reagents were HPLC grade or higher.

Method conditions

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II Analytical LC System	
Parameter	**Value**
Column	Agilent PLRP-S, 4.6 × 250 mm, 8 µm
Thermostat	4 °C
Solvent A	0.1% TFA in water
Solvent B	0.1% TFA in acetonitrile
Gradient	Gradient 1: Time (min) %B 0 to 2 35 2 to 22 35 to 50 22 to 24 50 to 90 24 to 28 90 28 to 30 90 to 35 30 to 36 35 Gradient 2: Time (min) %B 0 to 2 35 2 to 22 35 to 65 22 to 24 65 to 90 24 to 28 90 28 to 30 90 to 35 30 to 36 35
Column Temperature	25 °C
Flow Rate	1.0 mL/min
Injection Volume	5.0 µL
Agilent 1290 Infinity II Preparative LC System	
Column	Agilent PLRP-S, 21.2 × 250 mm, 8 µm
Thermostat	4 °C
Solvent A	0.1% TFA in water
Solvent B	0.1% TFA in acetonitrile
Gradient	Time (min) %B 0 to 2 35 2 to 22 35 to 50 22 to 24 50 to 90 24 to 28 90 28 to 30 90 to 35 30 to 45 35
Column Temperature	Ambient
Flow Rate	21.2 mL/min
Injection Volume	100 µL
Fraction Collection	2.5 mL fractions; time based

Table 2. LC/MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Parameter	**Value**
Source	Dual AJS
Polarity	Positive
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Skimmer	65 V
Acquisition Mode	2.5 Hz
Mass Range	100 to 2,100 *m/z*
Acquisition Rate	5 spectra/s
Agilent 1290 Infinity II LC System	
Column	AdvanceBio Peptide Mapping, 2.1 × 100 mm, 2.7 µm
Thermostat	4 °C
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	Time (min) %B 0 to 2 3 2 to 23 3 to 47 23 to 25 47 to 50 25 to 26 50 to 97 26 to 27 97 to 3 27 to 30 3* *isocratic (postrun)
Column Temperature	55 °C
Flow Rate	0.3 mL/min
Injection Volume	20 µL

Results and discussion

Two resins were used for the synthesis of the target GLP-1 7-36 amide peptide. The first, AmphiSpheres 40 RAM 0.4 mmol/g 75 to 150 µm, contains a polyethylene glycol chain to improve the performance of the resin for difficult peptides. The second, PL-Rink resin (1% DVB) 0.3 mmol/g 75 to 150 µm is lower loading and suitable for the synthesis of longer peptide chains.

The synthesis was performed under identical conditions and resulted in two crude peptides: peptide 1A (from the AmphiSpheres resin) and peptide 1B (from the PL-Rink resin).

Purification of peptides usually requires a pore size of 100 or 300 Å. This pore size maximizes the loading capacity while minimizing restricted access or exclusion of larger molecules and retaining the desired mass transfer to achieve best separation.

Analytical chromatography of the crude peptides is necessary as a starting point to confirm the presence of the desired molecule and understand the elution characteristics. Initially the elution profile of the two samples was unknown, so different gradient methods were screened (Figures 2A, 2B, 3A, and 3B), and the best (35 to 50 %B) was chosen for the preparative runs. For actual method conditions, see Table 1.

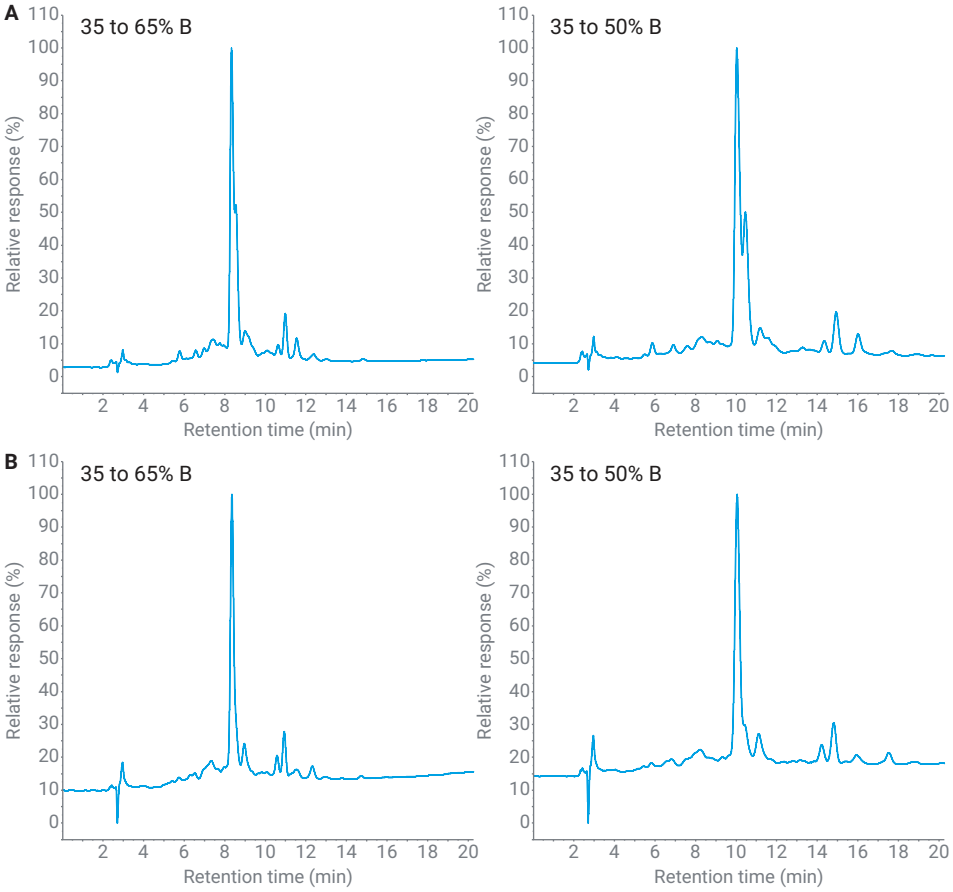

Figure 2. (A) Gradient optimization of peptide 1A on an Agilent PLRP-S 100 Å column. (B) Gradient optimization of peptide 1B on an Agilent PLRP-S 100 Å column.

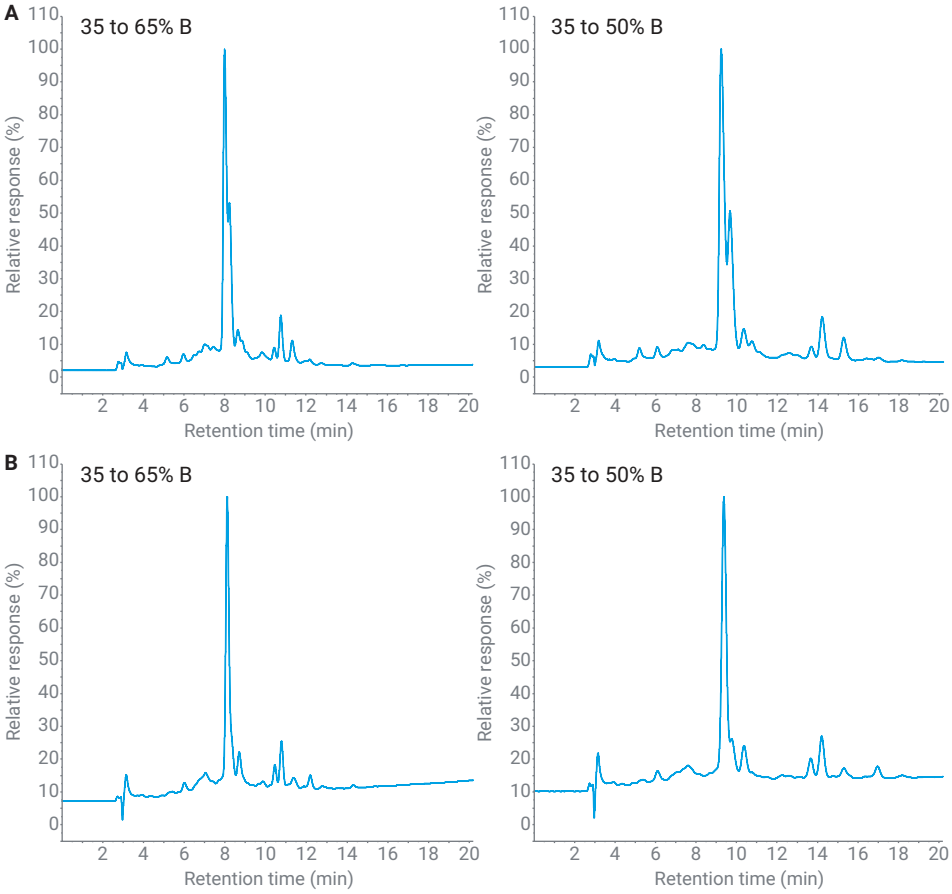

Figure 3. (A) Gradient optimization of peptide 1A on an Agilent PLRP-S 300 Å column. (B) Gradient optimization of peptide 1B on an Agilent PLRP-S 300 Å column.

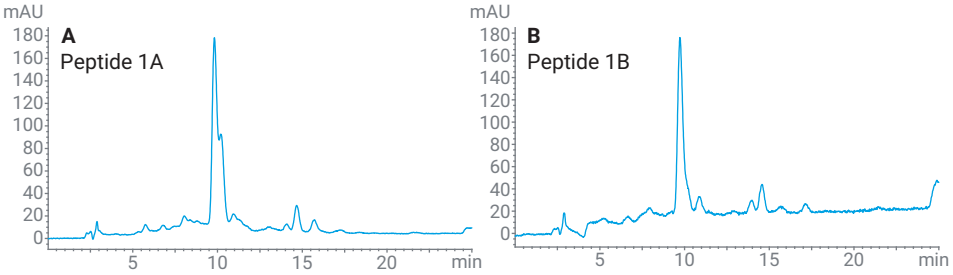

Figure 4. Preparative chromatograms of peptide 1A (A), and peptide 1B (B) on an Agilent PLRP-S 100 Å column.

It is evident that peptide 1B (prepared using PL-Rink resin 0.3 mmol/g) resulted in higher crude purity than AmphiSpheres 40 RAM in this example (Table 3).

Table 3. Crude peptide purity.

	Peptide 1A	Peptide 1B
PLRP-S 100 Å	33.15%	43.19%
PLRP-S 300 Å	41.23%	46.53%

The preparative scale separation was performed by injecting 100 µL of crude peptide at a concentration of 1 mg/mL dissolved in mobile phase A (containing water with 0.1% TFA). A total amount of 1 mg was purified on both PLRP-S 100 Å and PLRP-S 300 Å columns with a 21.2 mm id scale up from 4.6 to 21.2 mm id column dimensions (Figures 4 and 6).

The fraction collector was set to collect the full-length product (FLP) using fixed 2.5 mL volume fractions over the time period that the main peak eluted. The product and any closely eluting impurities could easily be identified by reanalyzing the appropriate fractions on the analytical columns (Figures 5 and 7).

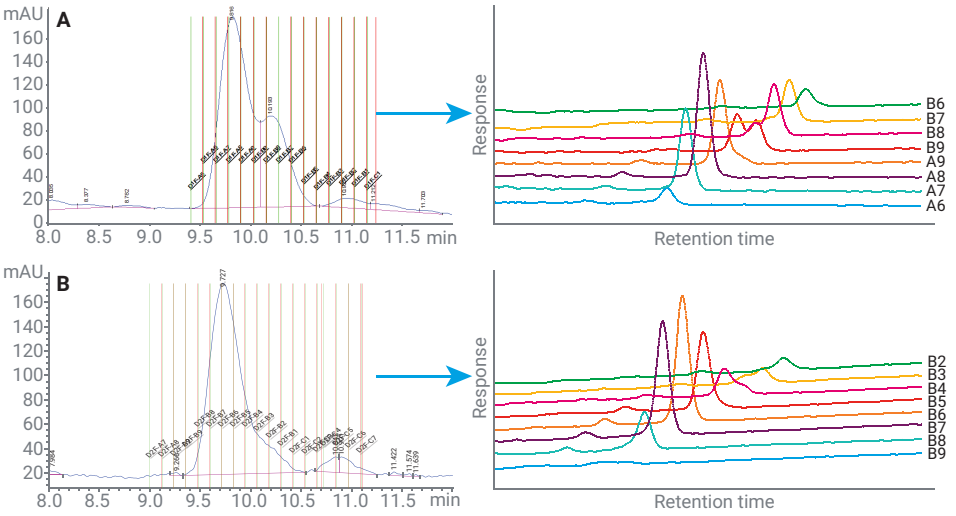

Figure 5. (A) Peptide 1A on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right). (B) Peptide 1B on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right).

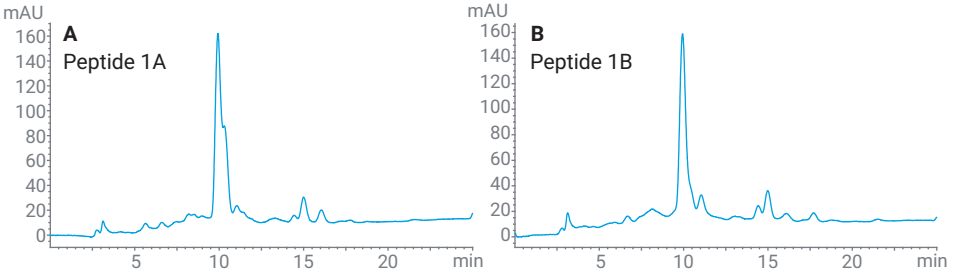

Figure 6. Preparative chromatograms of peptide 1A (A), and peptide 1B (B) on an Agilent PLRP-S 300 Å column.

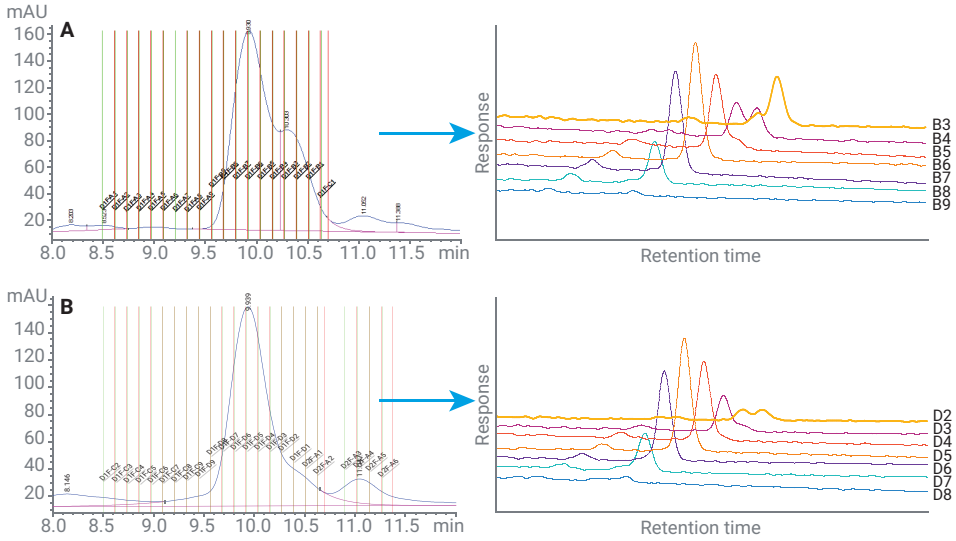

Figure 7. (A) Peptide 1A on an Agilent PLRP-S 300 Å column showing fraction reanalysis (right). (B) Peptide 1B on an Agilent PLRP-S 300 Å column showing fraction reanalysis (right).

Overall purity level is calculated from the peak area percentage of each fraction (Table 4).

Table 4. Summary of purity and yield from combination of fractions.

Peptide 1A	Area% (Purity)	Overall Yield%
PLRP-S 100 Å (Fractions A6-B9)	89.28	85.59
PLRP-S 300 Å (Fractions B8-B5)	90.26	73.02
Peptide 1B	**Area% (Purity)**	**Overall Yield%**
PLRP-S 100 Å (Fractions B8-B4)	97.81	92.69
PLRP-S 300 Å (Fractions D7-D3)	90.55	90.93

LC/MS analysis of the main component purified was performed on an AdvanceBio Peptide Mapping column to confirm its identity.

Synthetic peptide samples can often contain a high number of different molecular weights, impurities, missing amino acids in the sequence, loss of water, sometimes the protecting groups from the synthesis can still be attached to our target molecule in case of unsuccessful cleavage from the solid support. It is therefore critical that analytical methods for synthetic peptides cover a wide range of potential impurities. The major component from the purest fraction gave the expected $[M + 2H]^{2+}$ at 660.34, $[M + 3H]^{3+}$ at 825.42, $[M + 4H]^{4+}$ at 1,099.89, and $[M + 5H]^{5+}$ at 1,649.34. The results correspond to the full-length amino acids sequence of (GLP-1) 7-36 amide of 3,297.7 Da (Figure 8). For method conditions and instrument parameters, see Table 2.

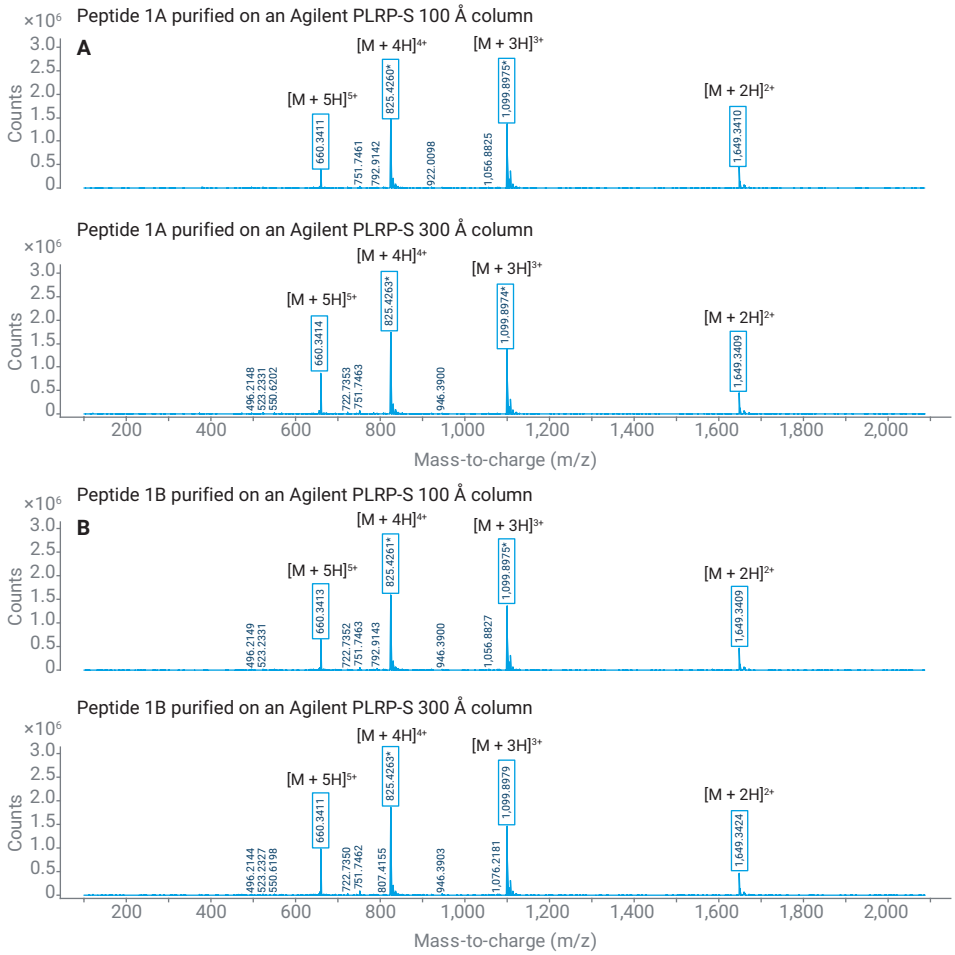

Figure 8. Mass spectral results of purified peptides analyzed by LC/MS on an Agilent AdvanceBio Peptide Mapping column (for method conditions, see Table 2).

Conclusion

This application note demonstrates that crude peptide purity may depend on synthesis conditions, including choice of solid phase resin. However, Agilent PLRP-S columns are an ideal choice for ion-pair reversed-phase purification of synthetic peptides. The smaller pore size 100 Å particles with higher available surface area can potentially offer higher purification capacity. Wider pore sizes can lead to better mass transfer and sharper peaks for larger species.

By coupling the Agilent PLRP-S preparative HPLC columns to the Agilent 1290 Infinity II preparative LC system, you can achieve efficient separations. Finally, for the LC/MS method, an Agilent AdvanceBio Peptide Mapping column was successfully used with formic acid as a mobile phase modifier to confirm the identity of the molecule.

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