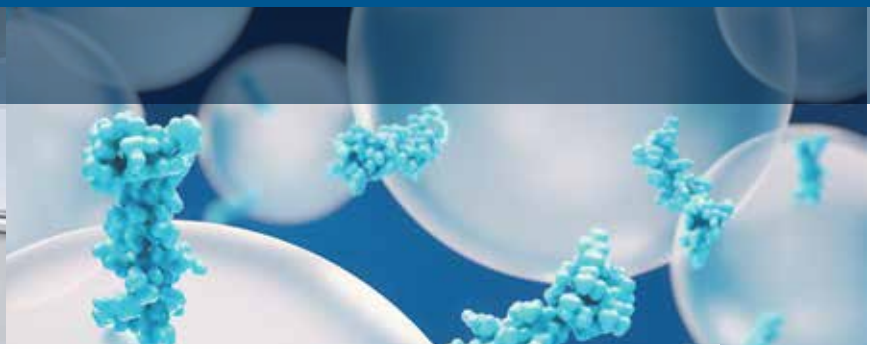
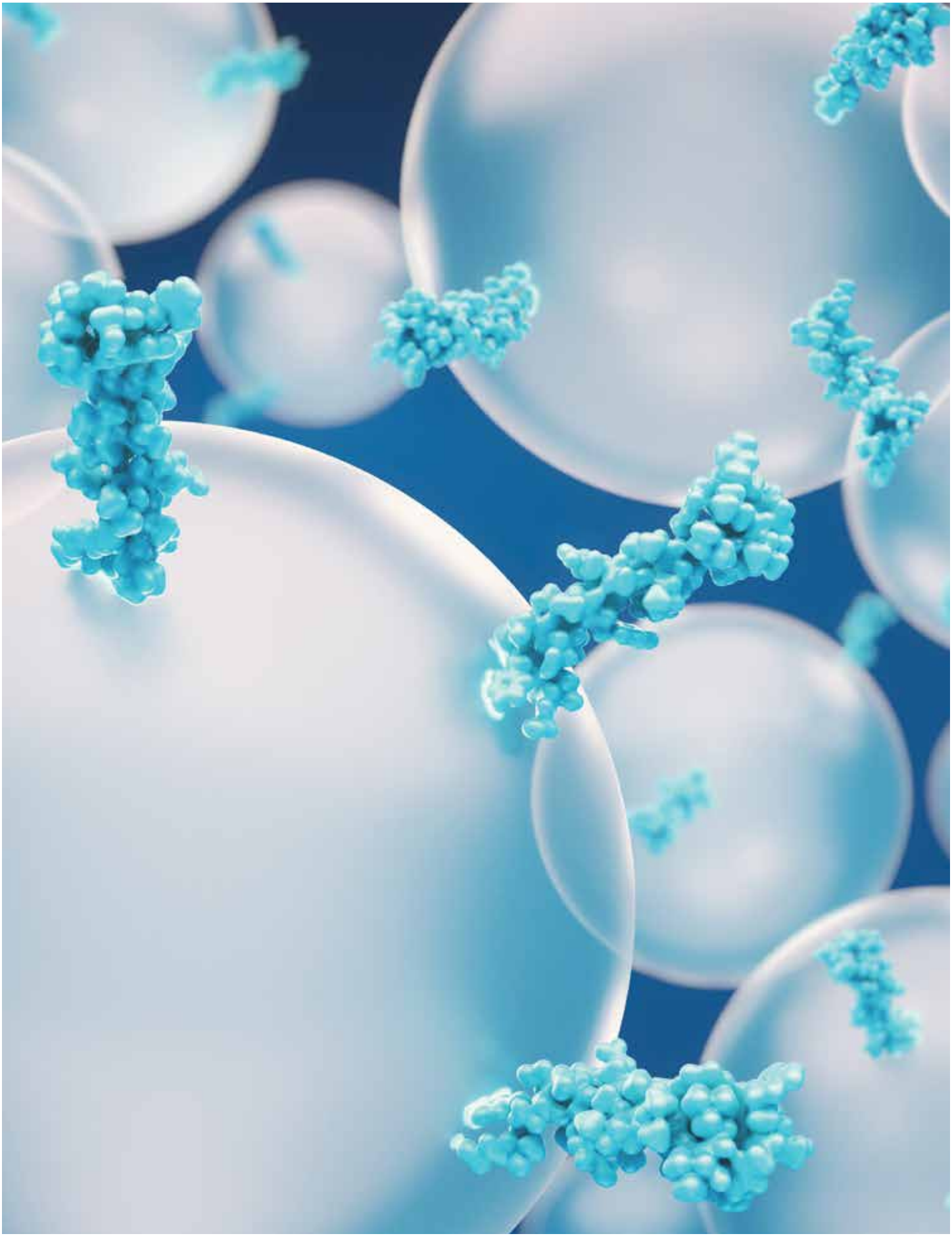
Agilent

Trusted Answers

Analysis and Purification of Synthetic Peptides by Liquid Chromatography

Consumables workflow ordering guide





Synthetic Peptide Therapeutics

Synthetic peptide therapeutics have become increasingly important in drug development research because of their increased efficacy, specificity, and lower toxicity. Peptides play a critical role in a wide variety of biological pathways, from pathway signaling, to enzyme regulation and cell communication. Synthetic peptides are designed to replicate the function of naturally occurring peptides or proteins in the body that play important roles in these various biological functions. They can also be engineered to target new functions and can be further modified structurally to increase stability and bioavailability to improve the overall therapeutic efficacy.

Techniques for purification of peptides

Peptides are short amino acid polymers ranging in sequence length from 2 to 50 amino acids. Many synthetic peptides are produced using solid-phase peptide synthesis (SPPS), which involves the step wise addition of amino acids to a peptide chain that is anchored to a polymeric solid support resin. The carboxyl group on one amino acid reacts with the amino group of another to form a peptide bond. The process of creating synthetic peptides through SPPS includes multiple deprotection, activation, and coupling steps, followed by the cleavage of the final sequence from the solid support. The crude final product contains impurities that can affect the safety and efficacy of the drug, and thus must be separated, characterized, and monitored by liquid chromatography.

Reversed-phase high-performance liquid chromatography (HPLC) coupled with UV detection is an established approach for this separation. Trifluoroacetic acid (TFA) is a common mobile phase additive and ion-pair reagent used for this analysis, creating a low pH environment that protonates the carboxyl groups of the amino acid sequence reducing secondary interactions and promoting better chromatographic separation and peak shapes, which is critical when looking to scale up your analytical methods for preparative purification. TFA can also form ion-pairs with positively charged functional groups in the peptide sequence, increasing the peptides overall hydrophobic interaction with the non-polar reversed-phase chromatographic media. For confirmation and characterization of the purified fractions with mass spectrometry (MS) detection, TFA, however can inhibit ionization efficiency and cause signal suppression. An alternative LC/MS method using formic acid is ideal for final product characterization.

Agilent reversed-phase columns for peptide purification

Agilent offers a variety of reversed-phase columns and media designed to simplify your synthetic peptide analytical workflows. Agilent PLRP-S reversed-phase columns contain rigid polystyrene/divinylbenzene (PS/DVB) particles that are available in a range of pore and particle sizes making it an ideal particle chemistry for analytical peptide separations that can be easily scaled up to preparative purification. The polymeric particle is inherently hydrophobic and free from surface silanols and trace metals found in traditional silica based particle supports that can cause unnecessary secondary interactions with charged functional groups in peptide sequences, leading to band broadening and peak tailing.

For confirmation analysis where MS characterization is needed, Agilent AdvanceBio Peptide Mapping and AdvanceBio Peptide Plus columns with the high resolution 2.7µm Agilent Poroshell particle support, are ideally suited for LC/MS separations where formic acid is used as an alternative mobile phase additive for TFA. The AdvanceBio Peptide Mapping column utilizes a C18 chemistry that has been especially designed to ensure suitability and robustness with peptide separations. For more challenging peptide separations, where secondary interactions with surface silanols cause peak broadening and tailing, the AdvanceBio Peptide Plus column chemistry was designed with an alternative C18 selectivity by incorporating a charged surface modification that helps reduce those unwanted secondary interactions for improved peak shape and resolution.

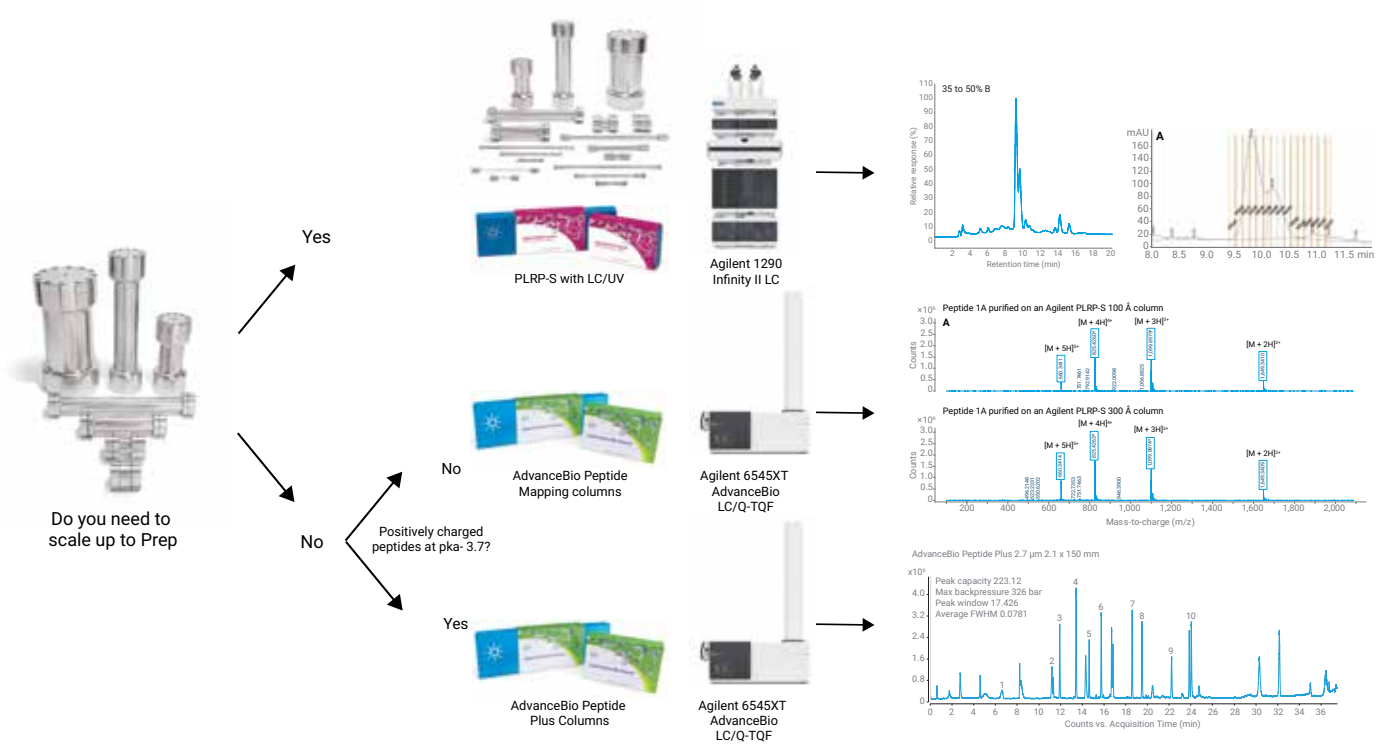


Figure 1. Choosing an analytical workflow for synthetic peptides depends on the scale of separation and need to characterize the sample with MS detection.

Tips for Optimal Chromatographic Separation and Detection

Sample preparation

- Agilent StratoSpheres product line of high-performance and high-quality resins[1] represent a wide variety of polymer supports for the development and manufacture of synthetic peptides through solid support peptide synthesis pathways.
- Compatible with Boc and Fmoc chemistries, StratoSpheres SPPS resins provide superior synthesis performance for higher purity peptides.
- Solid support resins allow for simplified cleanup of process related impurities and side products, simplifying the complexity of the crude sample.

Chromatographic Separation – UV Detection

- The choice of UV detection in your workflow allows for the use of TFA as a mobile phase additive. With a pKa ~ 0.23, TFA will lower the pH to protonate carboxyl functional groups on your peptide and residual silanols of the silica surface, to reduce unwanted secondary interactions that lead to peak broadening and tailing.
- The PLRP-S column portfolio offers flexibility of choice of particle and pore size. Evaluate different pore sizes (100Å or 300Å) depending on the sequence length of your synthetic peptide.
- Start your analytical method optimization[2] with an initial generic scouting gradient from 5% MPB to 95% MPB to understand the elution profile of your target product and what impurities are still in your crude sample.
- A more focused gradient can be developed to shorten your overall analysis time and optimize the resolution between the full-length target product and any closely related impurities.
- Once an optimized separation method has been developed at the analytical scale, your method can be easily scaled up to larger inner diameter columns to allow for purification of larger sample sizes.
- It is critical to optimize your gradient on an analytical column dimension before scaling up to prep, as resolution will dictate the purity and sample size that can be purified at the prep scale

Chromatographic Separation – MS Detection

- The choice of MS detection in your workflow limits the ability to use TFA as a mobile phase additive. TFA will affect the ionization efficiency of your sample and cause signal suppression which can inhibit your ability to detect and characterize potential low abundance impurities in your sample. Formic Acid is a more compatible acidic modifier for MS detection, but with a higher pKa (~ 3.7), it can affect how your sample interacts with the stationary phase compared to TFA.
- AdvanceBio Peptide Mapping and AdvanceBio Peptide Plus column chemistries[3-4] on the 2.7 µm Poroshell particle support allow for high-resolution separations.
- Start your analytical method optimization with an initial generic scouting gradient from 5% MPB to 95% MPB to understand the elution profile of your target product and what impurities are still in your crude sample.
- If the peptide has a high degree of positively charged functional groups in its sequence and the separation on the AdvanceBio Peptide Mapping column shows peak broadening and tailing, this may be due to interactions from the sample with residual surface silanols. Evaluate the AdvanceBio Peptide Plus column.
- After deciding on a column chemistry, a more focused gradient can be developed to shorten your overall analysis time and optimize the resolution between the full-length target product and any closely related impurities.

Mass spectrometry

- Do not use TFA or phosphate-containing buffers with MS detection!
- Divert the LC stream to waste outside of the retention time(s) of interest, especially during a high organic rinse at the end of the method and, if possible, as the void volume elutes.
- Use HPLC or higher-grade solvents.
- Establish a regular cleaning routine for the MS source.

Sample method and chromatogram for each method

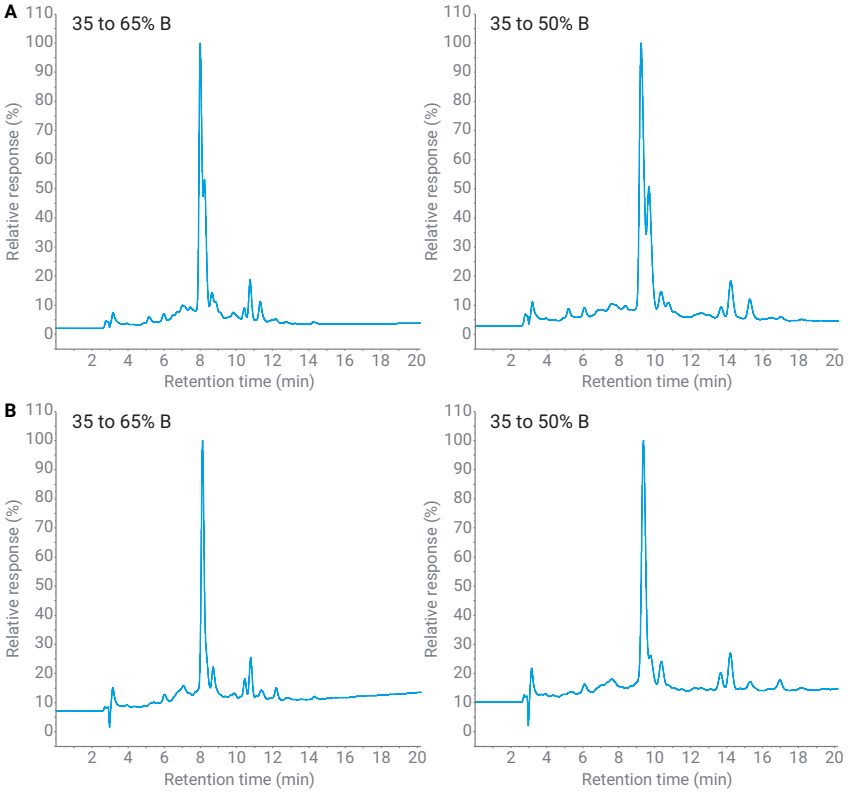


Figure 2. [2](A) Gradient optimization of peptide 1A on an Agilent PLRP-S 300 Å column.
(B) Gradient optimization of peptide 1B on an Agilent PLRP-S 300 Å column.

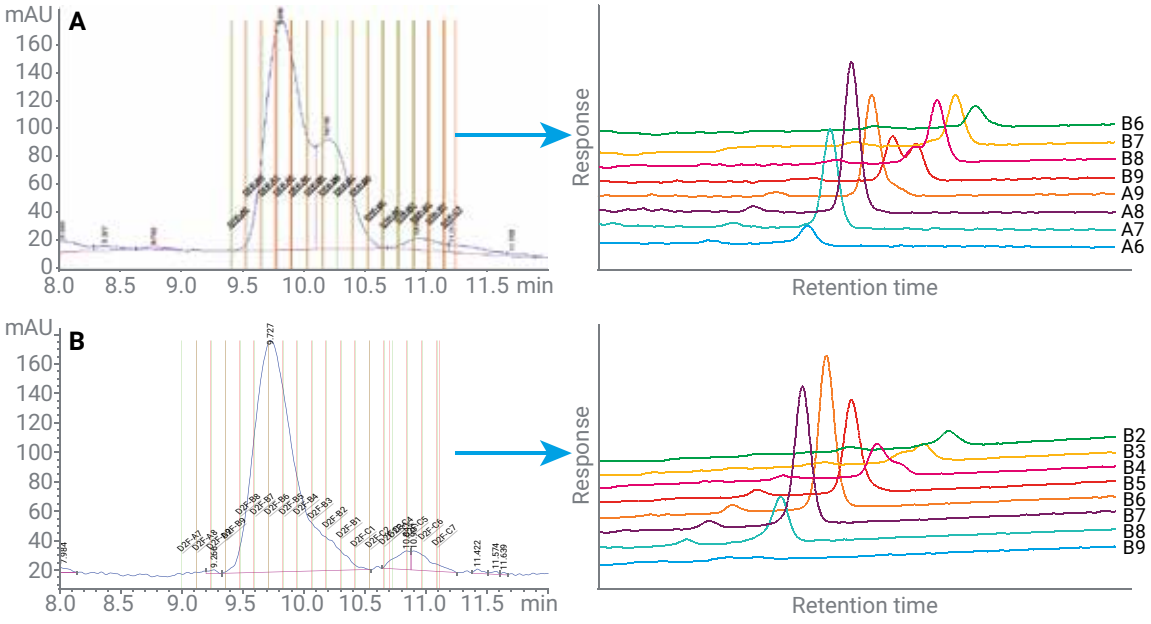


Figure 3. [2] (A) Peptide 1A on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right).
(B) Peptide 1B on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right).

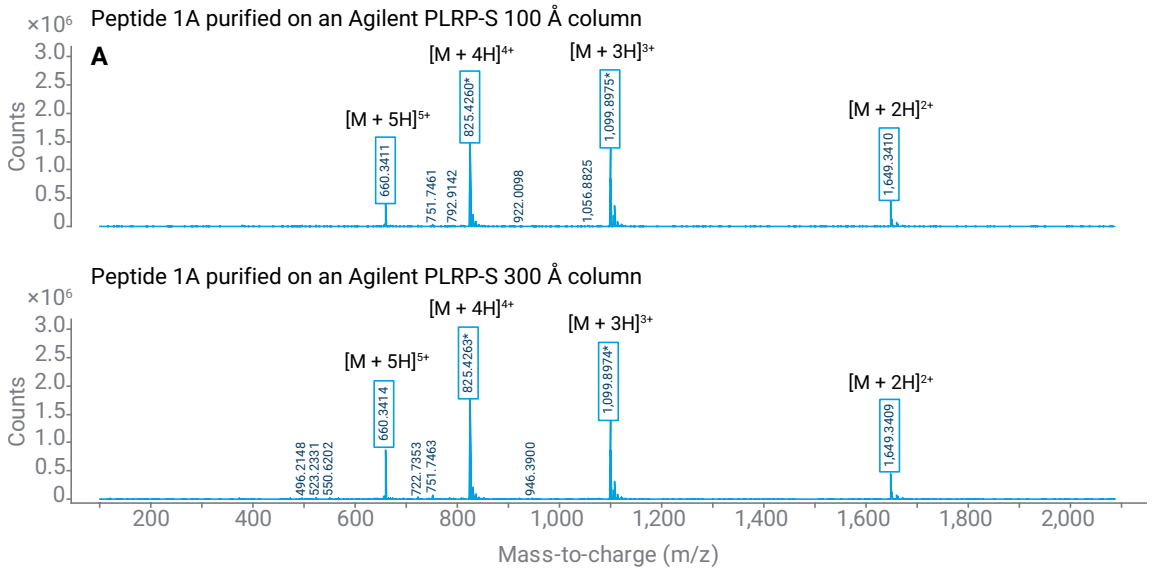


Figure 4. Mass spectral results of purified peptides analyzed by LC/MS on an Agilent AdvanceBio Peptide Mapping column (for method conditions see reference 2.)

References

1. Production-Scale Peptide Synthesis Using Agilent StratoSpheres Synthesis Support Resins, 5991-1485EN
2. Optimizing Analysis and Purification of a Synthetic Peptide Using PLRP-S Columns, 5994-6087EN
3. Analysis of a Synthetic Peptide and its Impurities, 5994-2760EN
4. Agilent AdvanceBio Peptide Plus 2.7 µm Column for Peptide Characterization, 5994-3508EN

Easy Selection and Ordering Information

To order items listed in the tables below, add items to your Favorite Products list by clicking on the MyList link in the header. Your list will remain under Favorite Products for your use with future orders. If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. If you do not have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled.

Individual items can also be ordered from the Agilent online store by clicking on the part number hyperlinks or through your regular sales and distributor channels.

MyList 1: Solid-phase supports for peptide synthesis

Description	Part Number
Solid-Phase Peptide Synthesis	
AmphiSpheres 40RAM, 0.4 mmol/g, 75-150 µm, 100 g	PL3867-4764
PL-Rink Resin (1% DVB) 0.3 mmol/g 75 to 150 µm, 100 g	PL1467-4749

MyList 2: HPLC columns for peptide purification

Description	Part Number
PLRP-S Columns	
PLRP-S 100 Å, 8 µm, 2.1 x 150 mm	PL1512-3800
PLRP-S 100 Å, 8 µm, 2.1 x 250 mm	PL1512-5800
PLRP-S 100 Å, 8 µm, 25 x 150 mm	PL1212-3800
PLRP-S 300 Å, 8 µm, 2.1 x 150 mm	PL1912-3801
PLRP-S 300 Å, 8 µm, 2.1 x 250 mm	PL1912-5801
PLRP-S 300 Å, 8 µm, 4.6 x 150 mm	PL1512-3801
PLRP-S 300 Å, 8 µm, 4.6 x 250 mm	PL1512-5801
PLRP-S 300 Å, 8 µm, 25 x 150 mm	PL1212-3801
PLRP-S 300 Å, 8 µm, 50 x 150 mm	PL1712-3801
AdvanceBio Peptide Mapping	
AdvanceBio Peptide Mapping 120 Å, 2.7 µm, 2.1 x 100 mm	655750-902
AdvanceBio Peptide Mapping 120 Å, 2.7 µm, 2.1 x 150 mm	653750-902
AdvanceBio Peptide Plus Columns	
AdvanceBio Peptide Plus 100 Å, 2.7 µm, 2.1 x 150 mm	695775-949

MyList 3: Supplies and solvents for HPLC purification of synthetic peptides

Description	Part Number
Ultra-Low Dispersion kits‡	
Ultra-low dispersion tubing kit for Agilent 1290 Infinity II	5067-5963
Ultra-low dispersion tubing kit for Agilent 1290 Infinity II Bio	5004-0007
Sample Containment supplies	
A-Line screw top vial, 2 mL, 12 x 32 mm (12 mm cap) amber, write-on spot, 100/pk	5190-9590
Screw cap, 12 mm, bonded, blue, PTFE/white silicone septa, 100/pk	5190-7021
Vial insert, 250 µL, 5.6 x 30 mm, deactivated glass with polymer feet, 100/pk	5181-8872
InfinityLab well-plate 96/0.5 mL, 30/pk	5043-9310
InfinityLab well-plate silicone closing mat for 96-well plates, 50/pk	5042-1389
Solvents & Additives	
InfinityLab Ultrapure LC/MS grade Water, 1 L	5191-4498
InfinityLab Ultrapure LC/MS grade MeOHL, 1 L	5191-4497
Formic acid, 99.5% purity, 5 mL	G2453-85060
Solvent Filtration Supplies*	
InfinityLab Solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, pore size 0.2 µm, 100/pk	5191-4341
Filter membrane, regenerated cellulose 47 mm, pore size 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168
Solvent Handling Supplies	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1L	9301-6524
InfinityLab solvent bottle, amber, 1L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2L	9301-6341
InfinityLab Stay Safe purging bottle	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap (charcoal filter 5043-1193 not included)	5043-1221
InfinityLab charcoal filter with time strip, 58 g (use with 5043-1221)	5043-1193

‡ If using a 1290 Infinity II Bio System, the Ultra-low dispersion tubing kit for Agilent 1290 Infinity II Bio is recommended.
* If using solvents other than those listed in this table, use the InfinityLab Solvent Filtration assembly prior to analysis.

Agilent CrossLab services

CrossLab is an Agilent capability that integrates services and consumables to support workflow success and important outcomes like improved productivity and operational efficiency. Through CrossLab, Agilent strives to provide insight in every interaction to help you achieve your goals. CrossLab offers method optimization, flexible service plans, and training for all skill levels. We have many other products and services to help you manage your instruments and your lab for best performance.

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