Application Note

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# Analysis of a Synthetic Peptide and Its Impurities

## Using mass spectrometry compatible mobile phases with an Agilent AdvanceBio Peptide Plus column


**Authors**

Andrew Coffey and
Veronica Qin
Agilent Technologies, Inc.



## Abstract

Conventionally, chromatographic peptide separation with UV detection is performed using C18 reversed-phase HPLC columns and mobile phases containing trifluoroacetic acid (TFA) as ion pair reagent. However, although TFA provides improved resolution, it can suppress the mass spectrometry (MS) signal. Formic acid (FA) is a preferred ion pair reagent for MS detection but can result in suboptimal separation with many traditional C18 columns. This application note presents the use of the Agilent AdvanceBio Peptide Plus column to separate synthetic peptide impurities with MS-compatible FA as a mobile phase modifier.

## Introduction

Most peptide drugs are manufactured using solid-phase peptide synthesis. Synthetic peptide-related impurities can come from raw materials, manufacturing processes, or be generated by degradation during manufacturing or storage.[1] Traditionally, peptide separation is achieved using reversed phase columns with trifluoroacetic acid (TFA) as a mobile phase modifier and UV as a detector. However, TFA is not ideal for mass spectrometry (MS) since it can suppress the MS signal.

To identify the impurity peaks in an LC/MS method, formic acid (FA) is a preferred mobile phase modifier but results in suboptimal separation with traditional C18 columns.

TFA (pKa ~0.23) can lower the pH to protonate residual (incompletely alkylated or endcapped) silanol sites on the stationary phase surface, leaving no negative charge to interact with positively charged peptides facilitating good peak shape. In addition, TFA anions form an ion pair with positively charged peptides, increasing their hydrophobicity and increasing their retention time. By contrast, FA (pKa ~3.77) is a weaker acid than TFA and cannot lower the pH enough to protonate all the silanol sites so the interaction between the silanols and peptides is not masked completely. This often leads to broader peaks, increased tailing, and overall lower resolution and peak capacity compared with using TFA as a modifier.

The Agilent AdvanceBio Peptide Plus stationary phase possesses a hybrid, positively charged surface, and can provide better peak shape and separation with FA as a modifier than traditional C18 columns. This note describes an LC method to separate synthetic peptide impurities using FA as a mobile phase modifier that can be run with either UV or MS detection, therefore making method transfer between LC/UV and LC/MS easier. Both LC/MS and LC/MS/MS are used to positively identify some of the impurities found in the sample, synthetic bivalirudin, Figure 1.

Bivalirudin is a 20 amino acid synthetic peptide that reversibly inhibits thrombin.

Quality control of the synthetic peptide requires the identification and determination of impurities. The amino acid sequence of bivalirudin (FPRPGGGGNGDFEEIPEEYL) has a monoisotopic mass of 2178.9858 Da.

Therefore, using LC/MS can accurately determine the mass of the peptide, but by also using MS/MS analysis it is possible to confirm the sequence through the predicted fragmentation pattern, as shown in Table 1.

## Experimental

### Reagents and chemicals

All reagents were HPLC grade or higher.

### Sample preparation

Aged synthetic peptide bivalirudin trifluoroacetate hydrate was purchased from Selleckchem and reconstituted with 0.1 % FA in water to 1 mg/mL.

H-D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH

**Figure 1.** The amino acid sequence of synthetic bivalirudin.

**Table 1.** The predicted MS/MS fragmentation pattern for bivalirudin.

| Seq | No. | b | y | No. (+1) |
|---|---|---|---|---|
| F | 1 | 148.076 | 2179.993 | 20 |
| P | 2 | 245.129 | 2032.925 | 19 |
| R | 3 | 401.230 | 1935.872 | 18 |
| P | 4 | 498.282 | 1779.771 | 17 |
| G | 5 | 555.304 | 1682.718 | 16 |
| G | 6 | 612.325 | 1625.697 | 15 |
| G | 7 | 669.347 | 1568.675 | 14 |
| G | 8 | 726.368 | 1511.654 | 13 |
| N | 9 | 840.411 | 1454.632 | 12 |
| G | 10 | 897.433 | 1340.589 | 11 |
| D | 11 | 1012.460 | 1283.568 | 10 |
| F | 12 | 1159.528 | 1168.541 | 9 |
| E | 13 | 1288.571 | 1021.472 | 8 |
| E | 14 | 1417.613 | 892.430 | 7 |
| I | 15 | 1530.697 | 763.387 | 6 |
| P | 16 | 1627.750 | 650.303 | 5 |
| E | 17 | 1756.793 | 553.250 | 4 |
| E | 18 | 1885.835 | 424.208 | 3 |
| Y | 19 | 2048.899 | 295.165 | 2 |
| L | 20 | 2161.983 | 132.102 | 1 |

## Instrumentation

For HPLC experiments, an Agilent 1290 Infinity LC was used comprising:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)
- Agilent 1260 Infinity II diode array detector (DAD) (G7115A)

For LC/MS experiments, the same 1290 Infinity LC configuration was used with an Agilent 6545XT AdvanceBio LC/Q-TOF detector.

## Data processing

LC/UV data was processed using Agilent OpenLab 2.2 CDS. LC/MS data was processed using Agilent MassHunter BioConfirm B.08.00 software. MS/MS spectra were used to confirm the identities of the synthetic peptides and their impurities.

## Method conditions

| HPLC Conditions | |
|---|---|
| Column | Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm (p/n 695775-949) |
| Mobile Phase | A) 0.1 % formic acid in water<br>B) 0.1 % formic acid in acetonitrile |
| Gradient | 0 min: 17% B<br>2 min: 17% B<br>22 min: 37% B<br>24 min: 95% B<br>26 min: 95% B<br>26.1 min: 17% B |
| Post Time | 5 min |
| Flow Rate | 0.4 mL/min |
| Column Temperature | 60 °C |
| Injection Volume | 5 µL (UV); 1 µL (MS) |

| Parameter | Value |
|---|---|
| Instrument | Agilent 6545XT AdvanceBio LC/Q-TOF |
| Source | Dual Agilent Jet Stream |
| Gas Temperature | 350 °C |
| Drying Gas Flow | 10 L/min |
| Nebulizer Gas | 30 psi |
| Sheath Gas Temperature | 275 °C |
| Sheath Gas Flow | 12 L/min |
| Capillary Voltage | 4,000 V |
| Nozzle Voltage | 0 V |
| Fragmentor | 125 V |
| Skimmer | 65 V |
| Oct 1 RF Vpp | 750 V |
| Mass Range | *m/z* 100 to 1,700 (MS);<br>*m/z* 50 to 1,700 (MS/MS) |
| MS Scan Rate | 8 spectra/s |
| MS/MS Scan Rate | 3 spectra/s |
| Acquisition Mode | Positive, extended dynamic range (2 GHz) |
| Collision Energy | 3.6 × (*m/z*)/100 – 4.8 |

## Results and discussion

Figure 2 shows the separation profile of an aged bivalirudin peptide sample using FA as a mobile phase modifier with UV detection. LC/MS/MS is used to identify several major impurity peaks in the profile, as shown in Table 2, with very low mass error.

Common impurities include deletion sequences (where an individual amino acid is missing), the presence of incompletely removed protecting groups or modifications of the peptide during removal of the protecting groups, loss of water and, in this particular peptide sequence, Asn is prone to deamidation, which could occur during manufacture or upon storage.

A total of five peaks were selected to illustrate the techniques used for identification using a combination of LC/MS and LC/MS/MS.

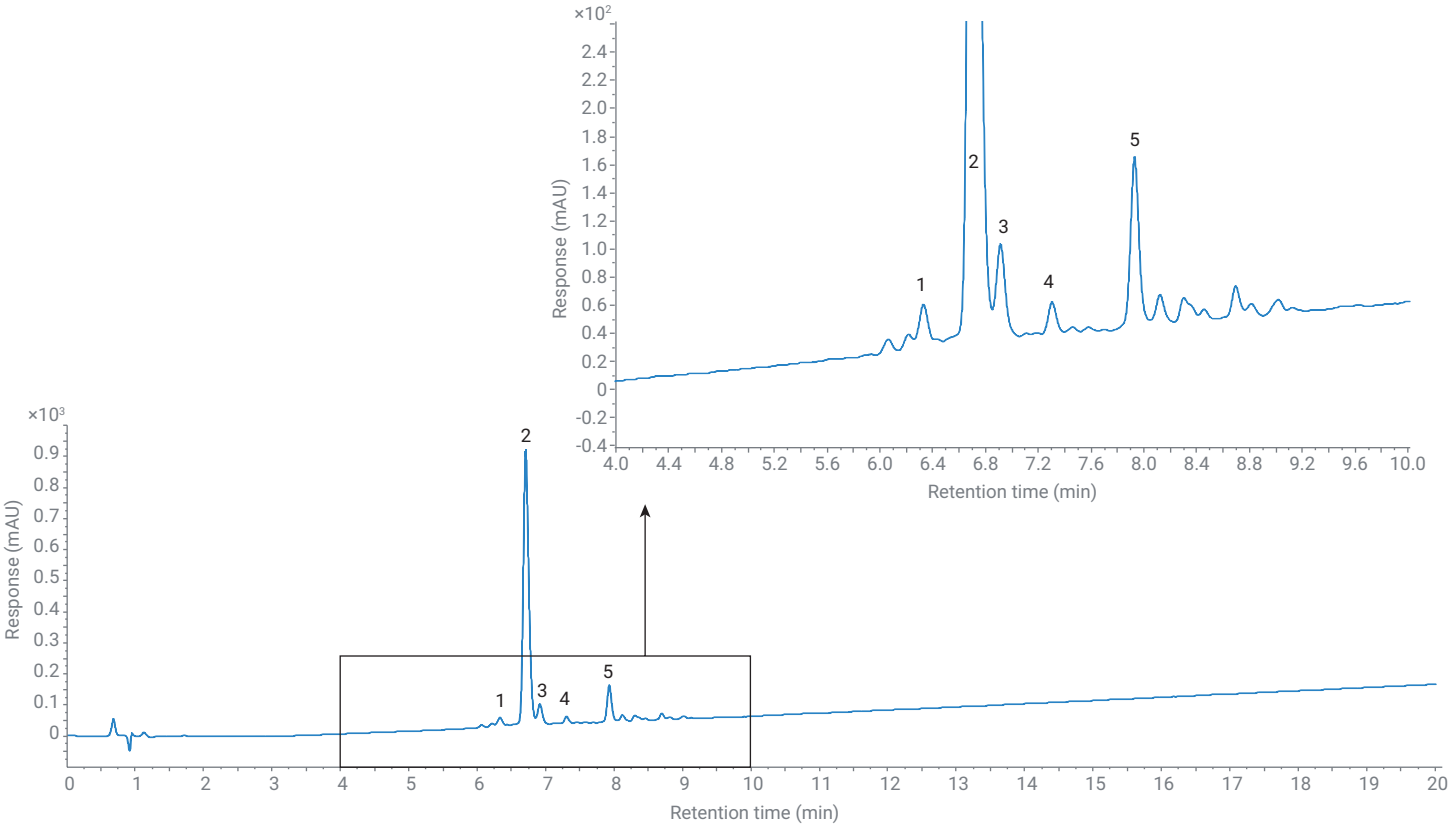


**Figure 2.** LC/UV chromatogram of synthetic bivalirudin. A zoomed baseline region of synthetic bivalirudin is shown.

**Table 2.** Peak identification of aged bivalirudin peptide and major impurities.

| Peak | Mass (Da) | Peak ID | Target mass (Da) | Mass error (ppm) |
|---|---|---|---|---|
| 1 | 2,049.9467 | Deletion of Glu | 2,049.9432 | 1.71 |
| 2 | 2,178.9894 | Product | 2,178.9858 | 1.65 |
| 3 | 2,121.9663 | Deletion of Gly | 2,121.9644 | 0.90 |
| 4 | 2,160.9764 | Loss of $H_2O$ | 2,160.9705 | 2.73 |
| 5 | 2,179.9742 | Deamidation | 2,179.9698 | 2.02 |

The major component of the LC/UV chromatogram, Peak 2, gave an MS spectrum shown in Figure 3. This corresponds to $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ *m/z* which, when deconvoluted, gives the mass of 2178.9894 corresponding to the full-length peptide sequence of bivalirudin, FPRPGGGGNGDFEEIPEEYL.

A similar approach is used to identify the earlier eluting impurity, Peak 1. In this case, a similar MS spectrum is obtained (Figure 4A), however, the mass of the impurity is 2049.9467 following deconvolution. The mass difference is −129 Da indicative of the loss of glutamic acid. By closer inspection of the LC/MS/MS spectrum, it is possible to identify the position of the missing Glu residue (Figure 4B).

The BioConfirm software has identified the $b_{15}$ and $y_4$ fragments for FPRPGGGGNGDFEEIPEYL, a 19 amino acid sequence, indicating that the sequence is missing a glutamic acid at position 17 or 18.

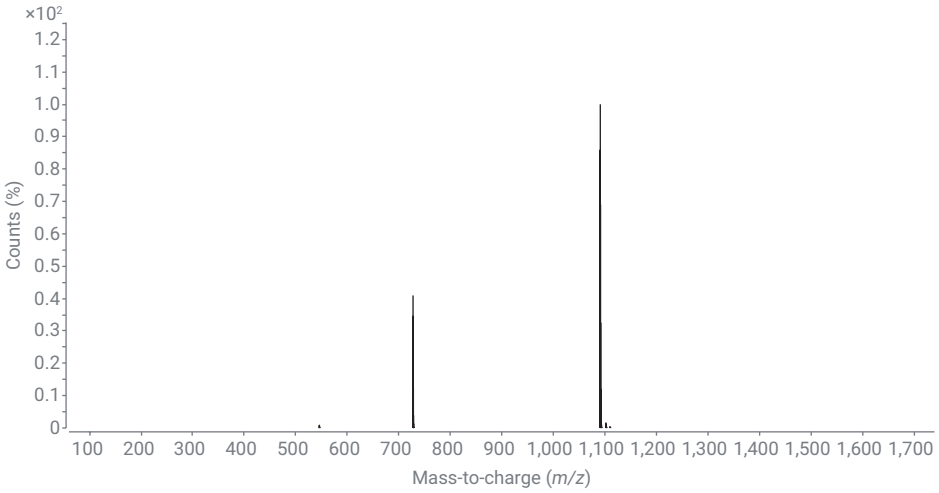


**Figure 3.** MS spectrum of the main product (peak 2)

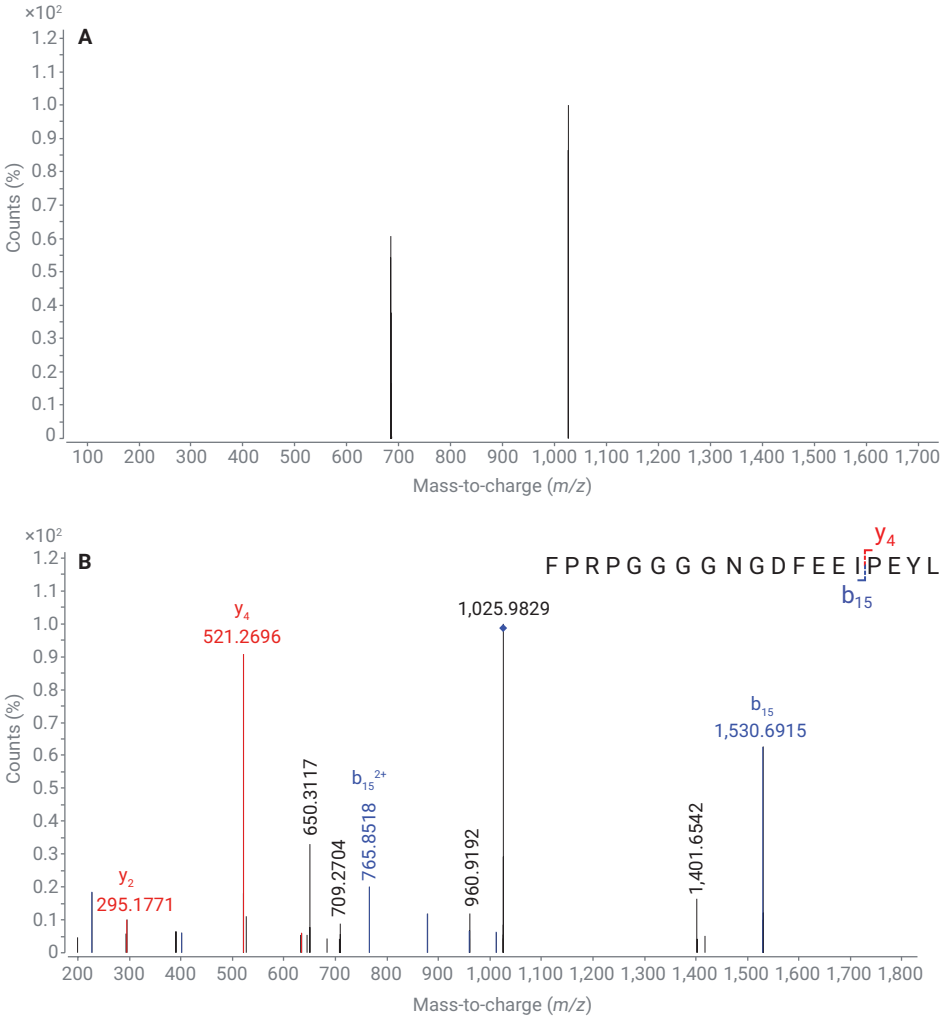


**Figure 4.** (A) MS spectrum of impurity, peak 1. (B) MS/MS spectrum of impurity, peak 1.

Analysis of impurity peak 3 gives a mass difference of −57 Da, indicative of a missing glycine (Figure 5). Impurity peak 4, meanwhile has a mass difference of 18 indicative of dehydration through loss of $H_2O$ (MS spectrum not shown).

Finally, analysis of impurity peak 5 gives a mass difference of +1 Da, indicative of deamidation (Figure 6A). A closer look at the MS/MS data for this impurity reveals the software has identified that Asn at position 9 (N) has been converted to Asp (D) through deamidation.

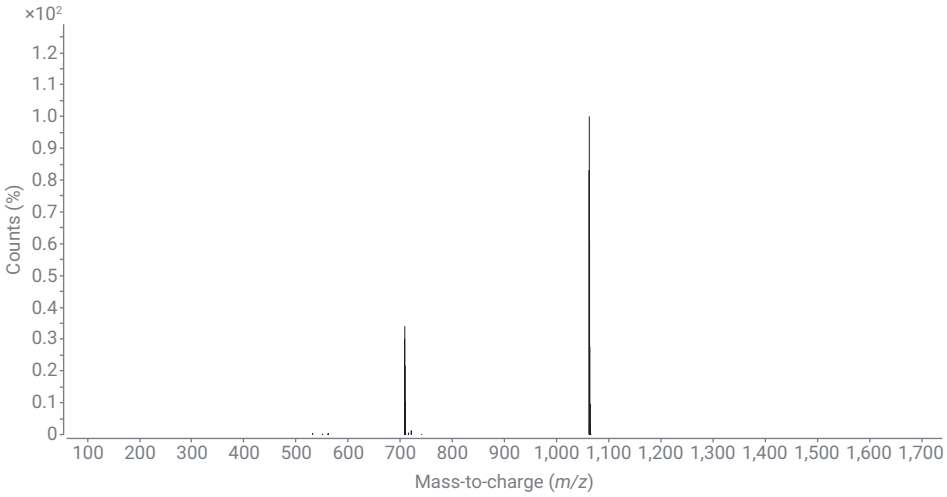


**Figure 5.** MS spectrum of impurity, peak 3

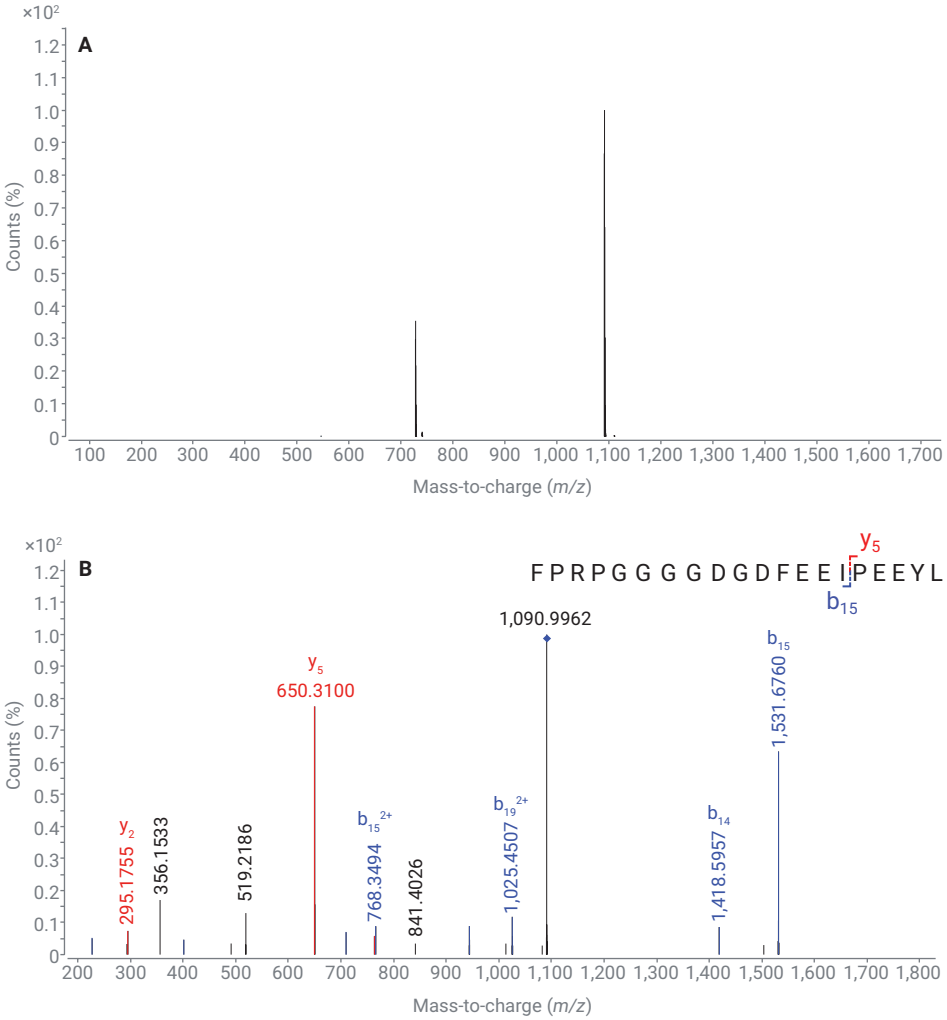


**Figure 6.** (A) MS spectrum of impurity, peak 5. (B) MS/MS spectrum of impurity, peak 5.

## Conclusion

In this study, an Agilent AdvanceBio Peptide Plus column was used with formic acid as a mobile phase modifier to analyze a synthetic peptide and its impurities. The method employed can be easily transferred between LC/UV and LC/MS.

## References


1. Eggen, I. *et al.* Control Strategies for Synthetic Therapeutic Peptide APIs Part III: Manufacturing Process Considerations. *Pharm. Technol.* **2014**, *38(5)*.

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