

# Separation of Deamidated Peptides with an Agilent AdvanceBio Peptide Plus Column

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## Abstract

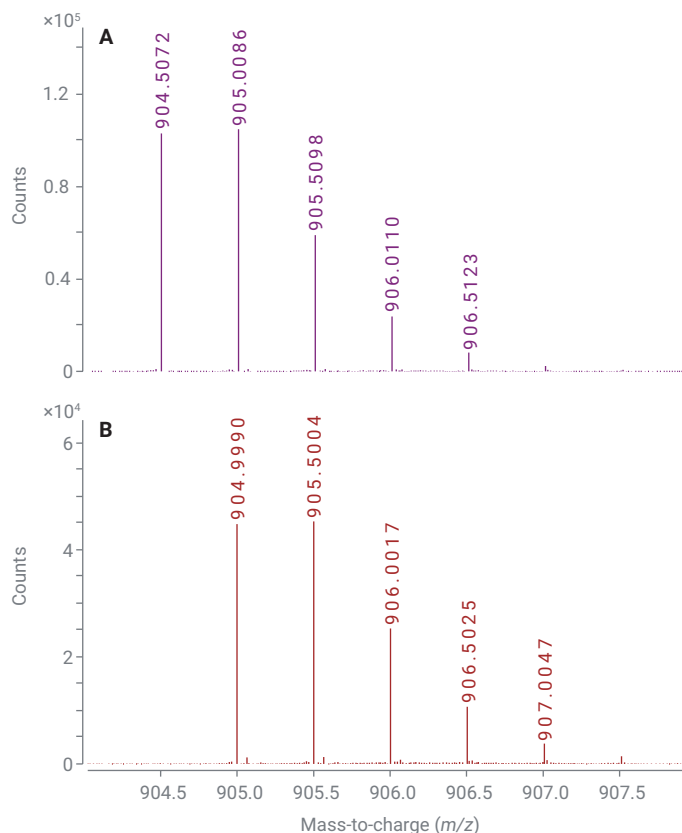
Deamidations of glutamine and asparagine are amongst the most common degradations affecting proteins. However, analysis of deamidation by mass spectrometry is challenging due to the small mass shift of less than 1 Da versus the unmodified form. Site-specific deamidation is often determined by protease digestion followed by LC/MS analysis, but even this approach can fail when the unmodified and deamidated forms are not chromatographically resolved. Fortunately, a charged surface C18 column dramatically improves the resolution of deamidated peptides from their unmodified variants. Furthermore, mobile phase optimization can provide additional control over the resolution of these analytes.

## Introduction

Analysis of protein deamidation is important for multiple stages of biopharmaceutical drug development and production. From an analytical perspective, deamidation eliminates an amide group and introduces a new carboxylic acid group, potentially enabling analysis by charge-based methods such as IEX and isoelectric focusing.<sup>1</sup> However, these techniques do not always resolve deamidated products and cannot confirm deamidation at a given site within the protein. Therefore, many analysts perform a protease digest of the sample and analyze the resultant peptides by reversed-phase LC/MS. Deamidation of asparagines or glutamines is identified by a mass increment of 0.9840 Da versus the unmodified form of the peptide. In some cases, peptides containing these degraded sites are well-separated, and relative quantification can be performed based on the relative signal intensity of the different forms.<sup>2</sup> However, deamidated peptides sometimes coelute with their nondeamidated forms since conversion of asparagine/glutamine to their corresponding carboxylic acids does not result in a large change in hydrophobicity at low pH.<sup>3</sup> Such coelutions result in an overlap of the deamidated peptide signal with the highly abundant <sup>13</sup>C isotopes of the unmodified form, as illustrated in Figure 1. This can impact the quantitation of deamidation, and in some cases, may even prevent detection of the deamidated variant.

This application note demonstrates that a charged surface C18 column greatly enhances selectivity for deamidated variants of peptides versus their unmodified forms when compared to a traditional C18 column. This increases confidence in the ability to detect and quantify deamidation at the peptide level.

Based on Agilent superficially porous Poroshell technology, Agilent AdvanceBio Peptide Plus columns feature a hybrid, endcapped C18 stationary phase on a 120 Å pore size, 2.7 µm particle modified to have a positively charged surface. This provides alternative selectivity compared to traditional C18 columns



**Figure 1.** Mass spectrum of VVSVLTVLHQDWLNGK (A) and a deamidated variant of that peptide (B), showing the overlap between mass spectra.

## Experimental

### Materials

The mAb sample was expressed and purified from chinese hamster ovary cells. The sample was digested by trypsin, adjusted to a pH of approximately 11 using reagent-grade ammonium hydroxide (Sigma-Aldrich), and incubated for 4 hours at 60 °C to accelerate deamidation. LC/MS-grade formic acid (part number 533002) and acetonitrile (part number 900667) were also purchased from Sigma-Aldrich.

### Instrumentation

#### LC system

An Agilent 1290 Infinity II LC system with the following configuration was used:

- Agilent 1290 Infinity II binary pump (G4220A)
- Agilent 1290 Infinity II autosampler (G4226A)
- Agilent 1290 Infinity II thermostatted column compartment (G1316C)

#### MS system

Agilent 6546 LC/Q-TOF

#### Data processing

LC/MS data were processed by Agilent MassHunter BioConfirm software (version 10.0 SP1) and MassHunter Qualitative Analysis software (version 10.0).

## Results and discussion

Five peptides in the mAb digest showing partial deamidation were identified, shown in Table 1. These peptides were used to investigate how the choice of column and mobile phase affects the separation of the unmodified peptide from its deamidated variants.

### LC/MS conditions

Parameter	Agilent 1290 Infinity II LC
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, (p/n 695775-949) Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, (p/n 653750-902)
Column Temperature	60 °C
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow Rate	0.4 mL/min
Gradient	Time (min) % B 0 3 2 3 40 40 50.5 100 53 3
Post Time	7 minutes
Injection Volume	3 µL

Parameter	Agilent 6546 Q-TOF
Source	Agilent Jet Stream
Gas Temperature	323 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 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	<i>m/z</i> 300 to 1,700
MS Scan Rate (spectra/s)	5
Acquisition Mode	Positive mode, extended dynamic range (2 GHz) Centroid data format

Table 1.

Peptide	Sequence (Nondeamidated Form)	<i>m/z</i> of [M+2H] <sup>2+</sup>
A	NQVSLTCLVK	581.8103
B	FNWYVDGVEVHNAK	839.4047
C	VVSVLTVLHQDWLNGK	904.5071
D	NTAYLQMNSLR	655.8300
E	GLEWVGYPDPSNGETTYNQK	1136.0323

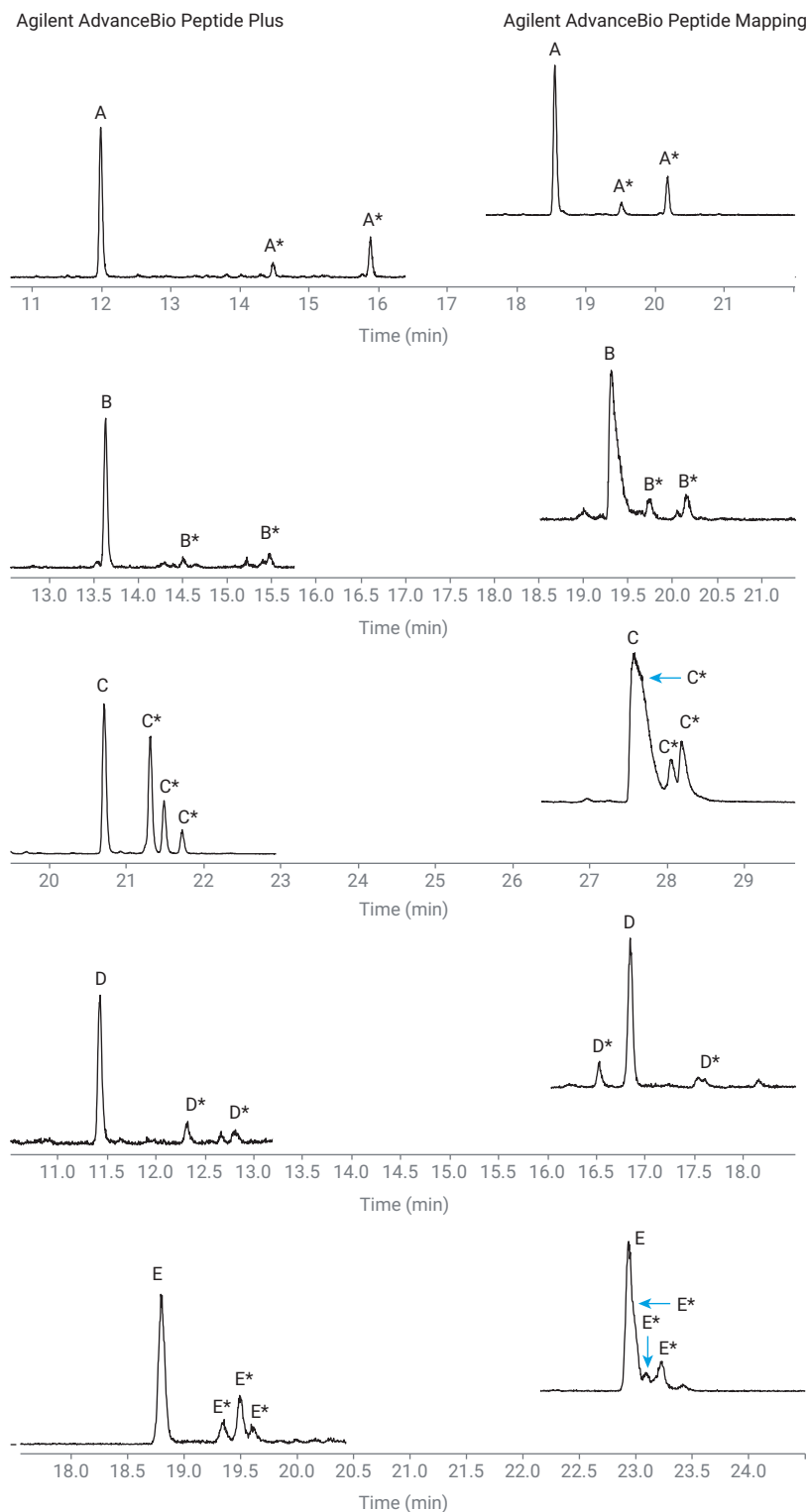
## Column type

Figure 2 shows the separation of peptides and their deamidated variants on two different C18 columns in a typical LC/MS analysis method using a formic acid-modified water/acetonitrile gradient.

The AdvanceBio Peptide Mapping column uses an endcapped C18 silica based on 2.7  $\mu\text{m}$  superficially porous particles with a 120  $\text{\AA}$  pore size. On this column, deamidated variants typically eluted slightly later than the unmodified form. At least two deamidated variants are detected in each case, likely representing conversion of asparagine into aspartate and isoaspartate. However, in the case of peptide D, one deamidated variant elutes before the unmodified form, while for peptides C and E, a deamidated variant coelutes with the unmodified form. These findings demonstrate that a standard C18 column will not resolve deamidated variants from their unmodified forms in a significant minority of cases.

Meanwhile, the AdvanceBio Peptide Plus column incorporates a positively charged surface on the same type of particle with similar C18 functionalization and endcapping. On this column, all deamidated variants were well resolved from the unmodified form. Furthermore, all deamidated variants of all five peptides eluted later than their modified forms.

A likely explanation for the difference in behavior between the two columns starts with the observation that the positively charged C18 phase is less retentive for peptides in general versus the standard C18 phase. This reduced retention may result from ionic interactions with peptides since they generally carry a positive charge in the presence of 0.1% formic acid.<sup>4</sup> This retention-reducing effect is stronger for highly basic peptides, and becomes less significant on peptides with greater numbers of acidic amino acids. Since deamidation



**Figure 2.** Separation of peptides and their deamidated variants (indicated by \*) on the Agilent AdvanceBio Peptide Mapping column (elevated and eluting later) and the Agilent AdvanceBio Peptide Plus column (eluting earlier) under the same conditions with 0.1% formic acid mobile phase modifier.

introduces an additional acidic group, deamidated peptide variants are less basic than their unmodified forms and therefore show greater retention on the charged column.

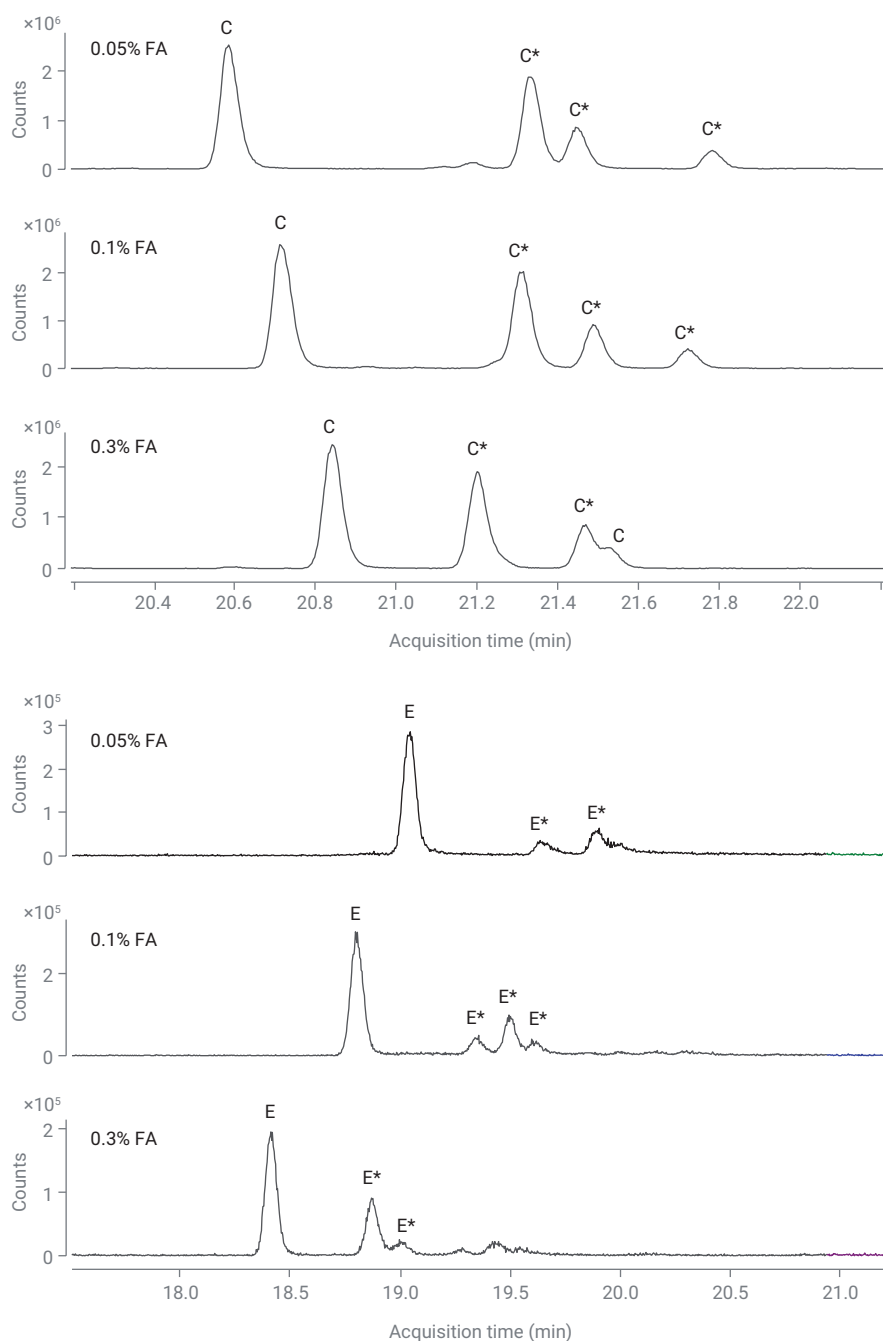
The enhanced selectivity for deamidated peptide variants versus their unmodified forms greatly reduces the chance of a coelution between these analytes on the charged column, thereby avoiding any challenges that would arise from their overlapping mass spectra.

### Mobile phase

While all five peptides were well-resolved from their deamidated variants on the AdvanceBio Peptide Plus column in 0.1% formic acid, these separations are also greatly affected by mobile phase choice.

Figure 3 shows the separation of peptide C from its variants when the aqueous and organic mobile phases are modified with 0.05%, 0.1 or 0.3% formic acid. Dropping the concentration to 0.05% increased selectivity and resolution, while at 0.3% formic acid, resolution was somewhat reduced. A similar pattern is observed for peptide E.

While formic acid is often the favored mobile phase modifier for LC/MS peptide separations, trifluoroacetic acid (TFA) is sometimes used to improve peak shape, even though it has a detrimental effect on ESI-MS sensitivity.<sup>5</sup> TFA lowers mobile phase pH more than formic acid, and therefore suppresses the ionization of the carboxylic acids formed by deamidation. Furthermore, TFA is reported to reduce the impact of ionic interactions by acting as a stronger ion pair reagent than formic acid. These effects may impact the ability of the AdvanceBio Peptide Plus column to separate deamidated variants of peptides from their unmodified form.



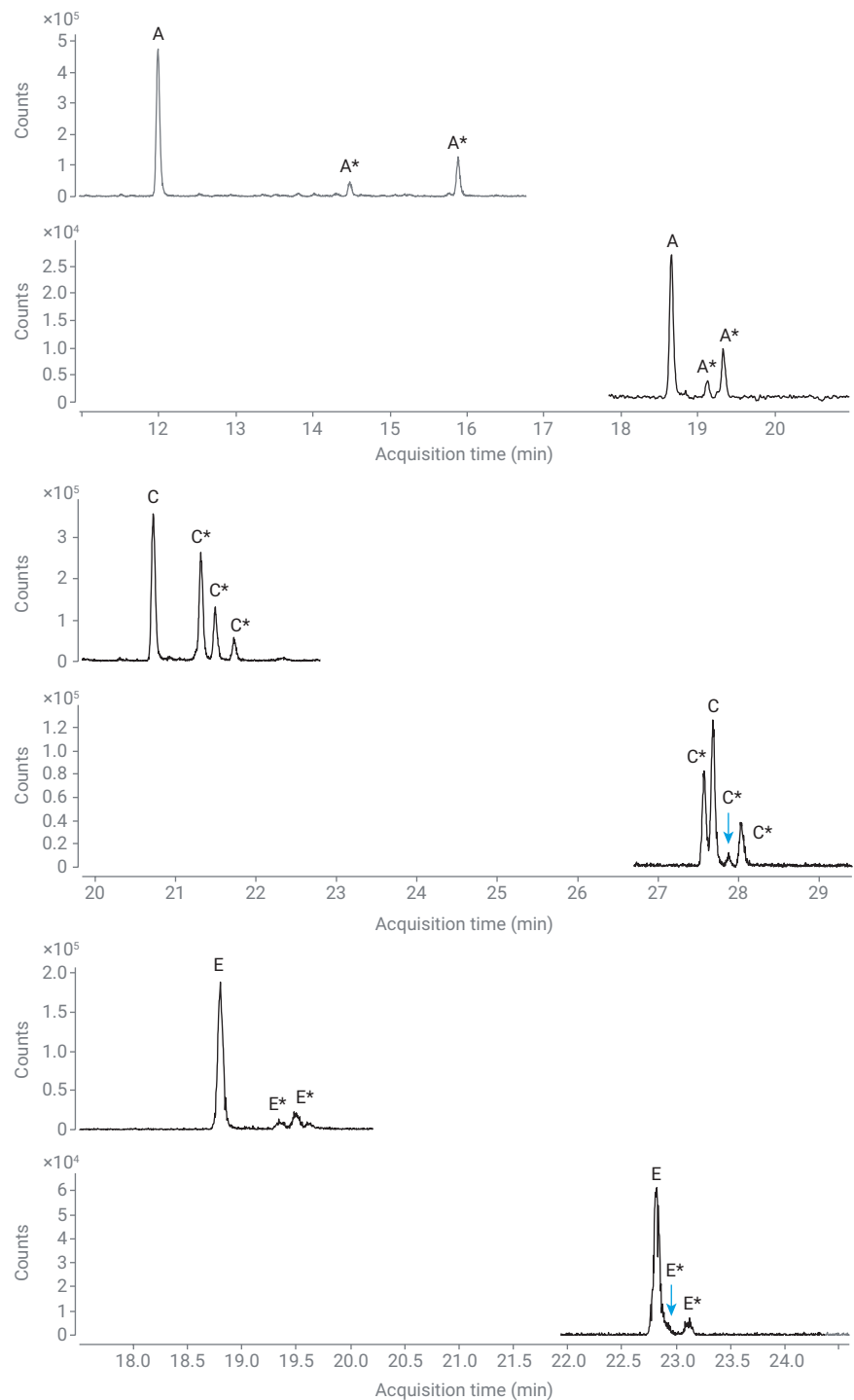
**Figure 3.** Separation of peptides on Agilent AdvanceBio Peptide Plus with increasing concentrations of formic acid mobile phase modifier. Selectivity for deamidated peptide variants over their native forms is maximized at lower concentrations.

Figure 4 shows the effect of substituting 0.1% formic acid for 0.1% TFA. Under this condition, selectivity for deamidated variants over the unmodified form was significantly reduced. For peptide A, this simply resulted in less baseline between the unmodified form and the variants. However, in the case of peptide C, one of the deamidated variants eluted before the unmodified form. Meanwhile, resolution was lost between the unmodified form of peptide E and one of its deamidated variants.

Overall, the general pattern of deamidated peptides eluting later than the unmodified variant was preserved in the 0.1% TFA condition on AdvanceBio Peptide Plus. However, analysts should be aware that the chances of coelution when using TFA are much higher than when using formic acid.

## Conclusion

The AdvanceBio Peptide Plus column shows greater selectivity for deamidated peptide variants versus their unmodified form when compared to a standard C18 column. This dramatically improves confidence that deamidated forms of peptides can be detected and quantified either manually or by automated analysis software by preventing issues with overlapping mass spectra. Selectivity can be altered by increasing or decreasing the concentration of formic acid mobile phase modifier. These findings may be useful to anyone analyzing deamidation of proteins using LC/MS, as described in the application note *Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies*.<sup>2</sup>



**Figure 4.** Separation of peptides and their deamidated variants on Agilent AdvanceBio Peptide Plus with 0.1% formic acid mobile phase modifier compared to 0.1% TFA.

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