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Application Note

Deep Characterization of Aspartic Acid Isomerism Using High-Resolution Cyclic™ Ion Mobility and Electron-Capture Dissociation

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Application Brief

This is an Application Brief and does not contain a detailed Experimental section.

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Abstract

Aspartic acid (Asp) isomerization in therapeutic proteins can impact safety and efficacy, making accurate characterization essential. Conventional peptide mapping often fails to fully resolve isomeric species such as L-Asp, D-Asp, L-isoAsp, and D-isoAsp due to their subtle structural differences and identical elemental composition. In this study, the combined power of high-resolution cyclic ion mobility spectrometry (IMS) and electron capture dissociation (ECD) for deep characterization of Asp and isoAsp variants in monoclonal antibody peptides is demonstrated. Using the SELECT SERIES™ Cyclic IMS Mass Spectrometer with an integrated ECD cell, complete separation of all four isomeric forms in a synthetic peptide mixture and in spiked digest samples was achieved, where chromatographic resolution alone was insufficient. Furthermore, ECD fragmentation

provided definitive identification of isoAsp residues through characteristic product ions, enabling confident differentiation between Asp and isoAsp species. This workflow offers unprecedented clarity in the analysis of aspartic acid isomerism, supporting robust biotherapeutic characterization.

Benefits

- Achieve complete separation – High-resolution cyclic ion mobility fully resolves Asp and isoAsp isomers that co-elute during conventional LC analysis.
- Accelerate workflows – Rapidly distinguish isomeric peptides using multipass Cyclic IMS, reducing reliance on extended LC gradients.
- Lower development costs – Confidently identify Asp and isoAsp variants with ECD fragmentation, minimizing the need for synthesizing reference peptides.

Introduction

Isomeric amino acid residues have an effect on protein function, sometimes significant enough to contribute to diseases such as Alzheimer's disease.[1] In the realm of biopharmaceutical drug products, these isomerized protein species can have undesired effects on safety or efficacy and therefore must be investigated and characterized. The introduction of structural isomers and stereoisomers can occur during cell culture, downstream processing, or while in storage.[1,2] Asp, specifically, can be present in a protein in four forms: L-Asp, D-Asp, L-isoAsp, or D-isoAsp. The most common form is L-Asp, which can isomerize or racemize into the other three forms through a succinimide intermediate (Figure 1).[1] This modification is particularly challenging to characterize as it is a relatively small spatial change that is isomeric and therefore cannot be distinguished by typical characterization methods such as mass spectrometry. Various methods for the investigation of Asp and isoAsp are being employed in the industry today. The most common is peptide mapping, in which the protein is enzymatically digested and the resulting peptides are separated chromatographically in order to quantify isomer species by either UV or ion peak areas.[3]

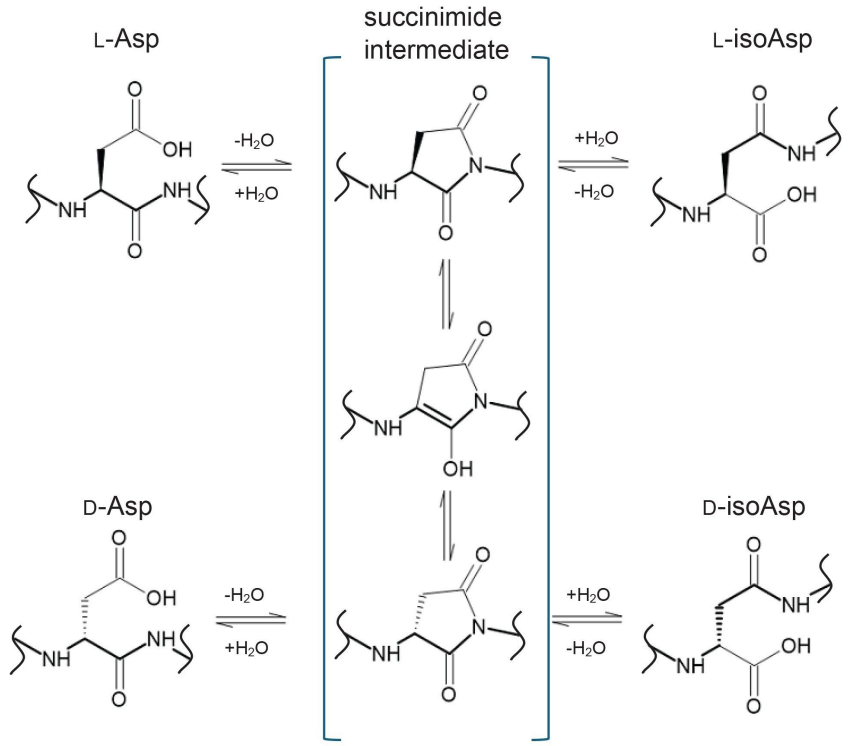

Figure 1. Isomerization scheme for an L-Asp-containing peptide. Isomerization between Asp and isoAsp forms proceeds through a succinimide intermediate. Isomerization between D and L forms (racemization) proceeds through the enol form of the succinimide. Bonds in bold highlight the peptide backbone.

Cyclic ion mobility mass spectrometry is highly powerful in the separation of isomeric species, including small molecules,[4] natural products,[5] lipids,[6] peptides,[7] and oligonucleotides.[8] By separating on the basis of molecular shape in a high-resolution ion mobility device, Cyclic IMS provides a means to distinguish ions with identical elemental formulae but distinct arrangements of atoms in space.

Collision-induced dissociation is by far the most widely used fragmentation technique in tandem MS studies, providing high efficiency fragmentation with broad applicability in the structural elucidation of both small and large molecules. However, some types of isomerism are out of reach of CID, which leads to the need for alternative techniques. ECD is an emerging tool capable of identifying peptide isomers directly from tandem MS spectra, depending on the type of isomerism.[9,10] It is well established that ECD can distinguish some isomeric

amino acids in leucine- and isoleucine-containing peptides, as well as Asp- and isoAsp-containing peptides, by virtue of characteristic product ions. In the latter case, for an isomerization at position n in a peptide of length m, characteristic ions $c_{(n-1)}$+57 Da and $z_{(m-n-1)}$-57 Da can be observed. For further information on comprehensive biotherapeutic characterization using ECD, there is an existing Waters application note.[11]

In this application brief, the depth of characterization achieved using the SELECT SERIES Cyclic IMS Mass Spectrometer equipped with the Waters ECD cell option is demonstrated. The power of high-resolution cyclic ion mobility enables full separation of the four isomer mix of a mAb tryptic peptide with L-Asp, L-isoAsp, D-Asp and D-isoAsp forms, where only partial chromatographic separation is achieved. Furthermore, ECD fragmentation clearly distinguishes the Asp isomers from their isoAsp counterparts, providing unprecedented clarity in the analysis.

Results and Discussion

During routine peptide mapping of the mAb Trastuzumab, two major isomeric peaks for the T12 peptide from the heavy chain were detected. In cases such as this, the major peak is the native peptide, and the minor peak is putatively identified as the isoAsp variant (Figure 2). These two variants are well separated in retention time, leading to facile relative quantification.

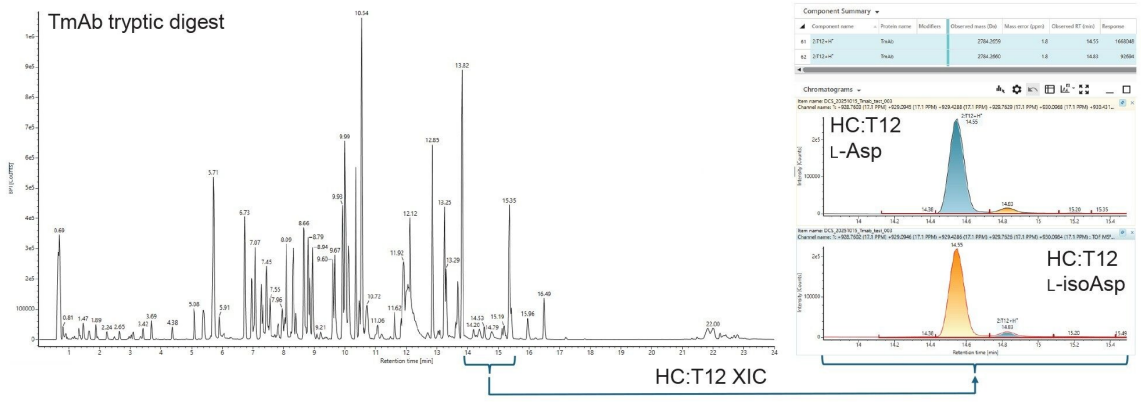

Figure 2. LC-MS of a tryptic digest of trastuzumab. Left: BPI chromatogram, Right: Extracted ion chromatograms of the L-Asp and L-isoAsp forms of the T12 peptide of the heavy chain. L-isoAsp is quantified as 5% of HC:T12.

Given the potential of the native L-Asp-containing peptides to racemize to the D-Asp form, the ability of the peptide mapping assay to fully separate a mixture of peptides populating the L-Asp, L-isoAsp, D-Asp and D-isoAsp variants was assessed. For this, synthetic forms of the HC:T12 peptide with sequence WGGD GFYAMDYWGQGTLVTVSSASTK were purchased, with all Asp isomeric residues at position 4. Performing LC-MS of the mixture of the four synthetic HC:T12 peptides yielded a chromatogram with four peaks, three of which are partially resolved (Figure 3a). Resolution of co-eluting components is made more challenging still when there are minor components in the mix. This can be seen in the chromatogram in Figure 3b, where the synthetic peptides were each spiked at a 5% level into the trastuzumab tryptic digest sample. This chromatogram shows that the species at 14.6 and 14.7 minutes are considerably less simple to distinguish from the L-Asp peak at 14.56 minutes.

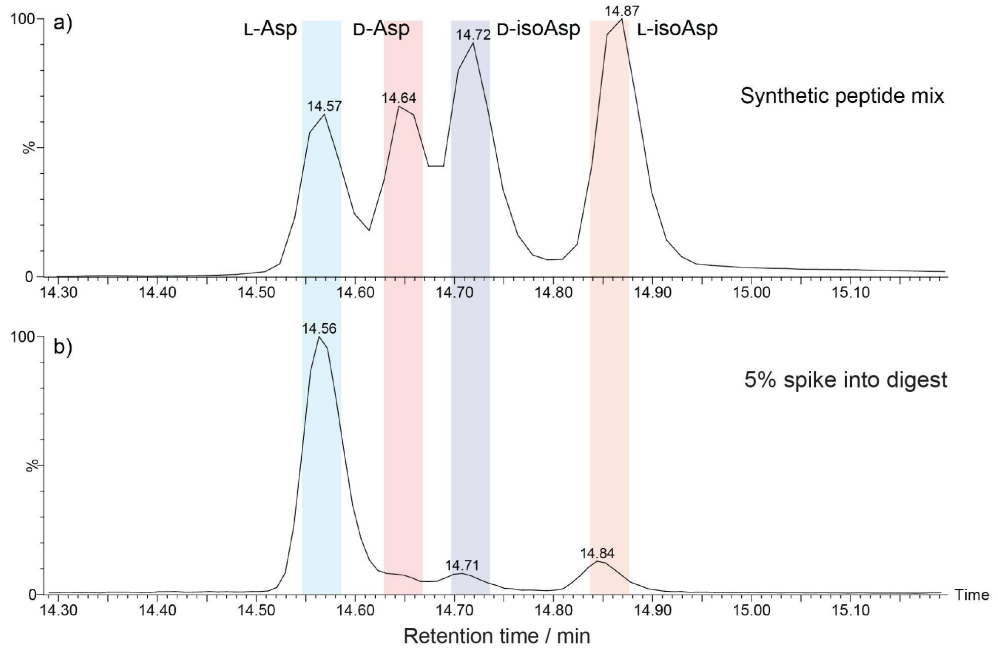

Figure 3. Separating aspartic acid isomers by LC-MS. a) Extracted ion chromatogram of the 4-way mixture of synthetic HC:T12 peptide isomers. The L-Asp, D-Asp and D-isoAsp peaks are partially co-eluting. b) The four peptides were spiked at a 5% level into the trastuzumab tryptic digest. The lower level isomers, particularly the D-Asp and D-isoAsp are more difficult to resolve with chromatography alone under these conditions.

To address this analytical challenge, these two samples were subjected to multipass cyclic ion mobility (10 passes in total). Figure 4 shows the resulting LC-Cyclic IMS-MS data for the 4+ charge state of the HC:T12 peptides at 696.85 *m/z*. For the synthetic peptide mixture (Figure 4a), complete resolution of the four species is observed at the arrival time versus retention time heat map, revealing unprecedented clarity in the separation. Furthermore, even in the spiked sample (Figure 4b), all four species are clearly separated. This high-resolution cyclic ion mobility separation in combination with LC-MS provides a means to confidently identify and accurately quantify these isomeric forms in biotherapeutic digests.

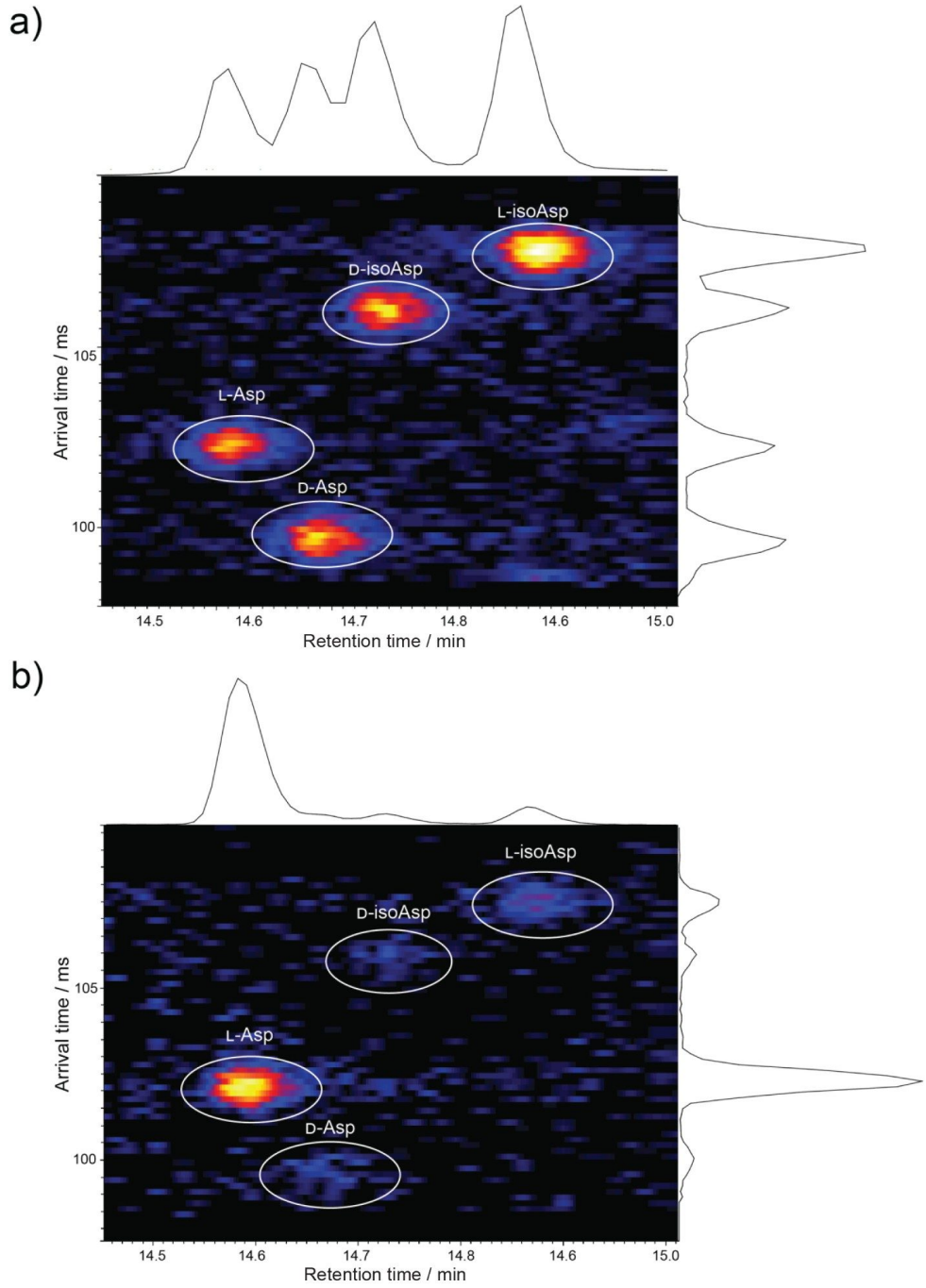

Figure 4. High resolution Cyclic IMS enables complete separation of all HC:T12 isomeric forms. a) The separation of the 4-way mixture by LC-Cyclic IMS. The partial separation in retention time is compensated for by the unmatched resolving power of the

multipass Cyclic IMS. The 2D heat map plot shows how the combination of LC and Cyclic IMS completely separate the four isomers. b) The same is true in the spiked digest. Even when present at minor levels, the isomers are clearly separated, aiding in detection, identification, and quantification.

Taking the work a step further, ECD fragmentation to differentiate the Asp and isoAsp forms in the mixture was used. Employing direct infusion electrospray ionization of the 4-way isomer mixture, 10 pass cyclic IMS separation was performed on the 4+ charge state, followed by ECD fragmentation. For these experiments, the ECD cell was positioned in the post-IMS configuration, meaning that ECD product ions are encoded with the arrival time of their corresponding precursor ions (Figure 5). For the HC:T12 peptide, the characteristic ions indicative of the presence of isoAsp are c_3+57 and z_{23}-57, with *m/z* values of 375.16 and 1206.06, respectively. Extracted ion mobilograms of these ions (Figure 5a, blue and red) revealed arrival times consistent with the two lower mobility species, confirming these to be L-isoAsp and D-isoAsp in agreement with the data above.

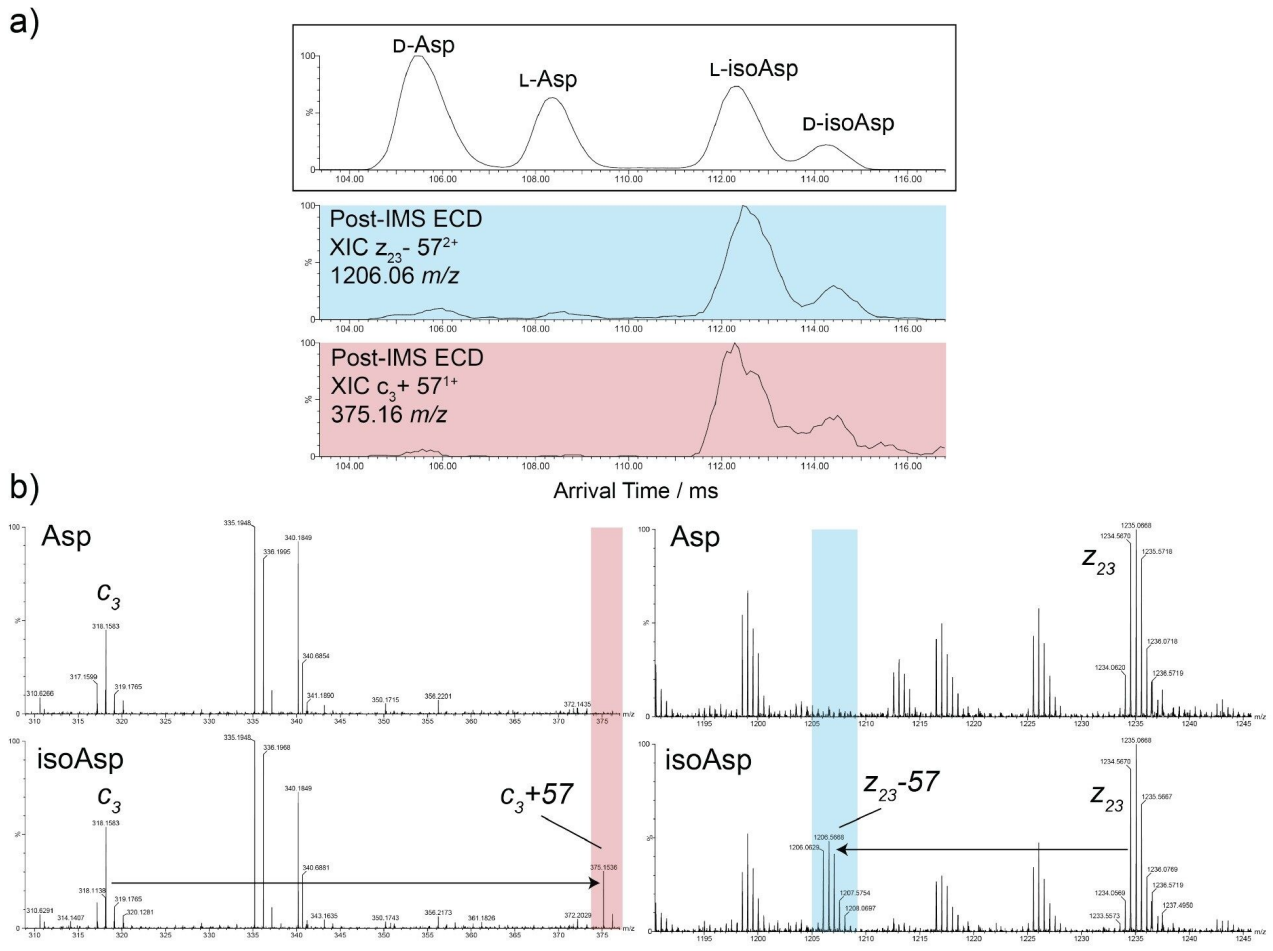

Figure 5. Cyclic ion mobility coupled with ECD. a) High resolution multipass Cyclic ion mobility of the 4-way mixture of synthetic Asp and isoAsp variants of HC:T12 of trastuzumab. A 10 pass separation of the 4+ charge state is shown. The extracted ion mobilograms of the z_{23}-57 at 1206.06 m/z and the c_3+57 at 375.16 m/z show arrival time distributions consistent with the two lower mobility features, showing these to be the isoAsp isomers. b) Mass spectra showing the characteristic ion signals for the z_{23}-57 and c_3+57 ions.

Conclusion

High-resolution cyclic IMS combined with ECD fragmentation delivers a powerful solution for resolving and

identifying aspartic acid isomers in complex biotherapeutic samples. Unlike conventional LC-MS peptide mapping, which provides only partial separation, multipass cyclic IMS achieves complete resolution of L-Asp, D-Asp, L-isoAsp, and D-isoAsp forms, even at low abundance. The integration of ECD further enhances specificity by generating diagnostic fragment ions that distinguish Asp from isoAsp residues. This approach reduces reliance on synthetic standards, accelerates analysis, and improves confidence in structural characterization. Overall, the workflow represents a significant advancement for monitoring critical quality attributes in monoclonal antibodies and other protein therapeutics.

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