



## ADD A NEW DIMENSION TO YOUR RESEARCH WITH THE AGILENT 6560 ION MOBILITY Q-TOF LC/MS

The Measure of Confidence

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### Applications Highlights

The Agilent 6560 Ion Mobility Q-TOF LC/MS system is the first commercially available uniform field ion mobility system. When coupled with the Agilent Infinity II UHPLC system, it provides a new dimension of separation power combining the selectivity of liquid chromatography, ion mobility, and mass spectrometry techniques.

Laboratories can accelerate research and gain greater confidence in compound identification with the additional dimension of mobility separation and collision cross section, as well as the structural information provided by ion mobility measurements. This instrument simultaneously provides high sensitivity and accurate collision cross section measurements. This document provides an overview of the technology and examples of real-world applications to demonstrate its capabilities.

### What is Ion Mobility?

#### Principles of ion mobility separation

In a classical uniform field drift tube, the electric field within the drift cell moves ions through the device while the drag force acts against the electrical force that moves the ions, due to the collisions of these ions with the stationary buffer gas molecules. The drag force experienced by the ions depends on their collision cross sections (a function of size and shape), electrical charge, and mass. Multiple charged ions move through the buffer gas more effectively than single charged ions, because they experience a greater force due to the electric field. Ions with larger cross sections are slowed more easily by collisions with the buffer gas in the drift tube. The drag force resulting from collisions of ions with buffer gas molecules acts against their acceleration. Thus, an equilibrium state is quickly reached, and the ions start moving with constant velocity ( $V$ ), proportional to the applied electric field ( $E$ ). The proportionality constant ( $K$ ) is the gas phase mobility of an ion. This process can be expressed in the equation:  $V = KE$ .



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Mobility is a function of an ion's interaction with the buffer gas, its mass, and its electrical charge. Furthermore, mobility depends on the gas temperature and the mass of the buffer gas molecules.

$$K_0 = \frac{L}{t_d E} \frac{P}{760} \frac{273.2}{T}$$

- $L$  is the length of the drift cell
- $t_d$  is the corrected drift time
- $E$  is the electric field across the drift cell
- $P$  is the pressure of the drift cell
- $T$  is the temperature of the buffer gas

## Why Ion Mobility?

### Achieve greater analytical detail for complex samples

The 6560 system was developed with the collaboration of scientists from a number of academic institutions and government laboratories. In multiple studies, the instrument has demonstrated the ability to reveal significantly greater analytical detail for complex samples, compared to high resolution mass spectrometry technology alone.

Researchers have reported that while high resolution mass spectrometry has become the analytical cornerstone for proteomics, metabolomics, and other research applications requiring the analysis of highly complex samples, there has also been significant interest in the use of ultra-fast orthogonal techniques to provide added dimensions of separation.

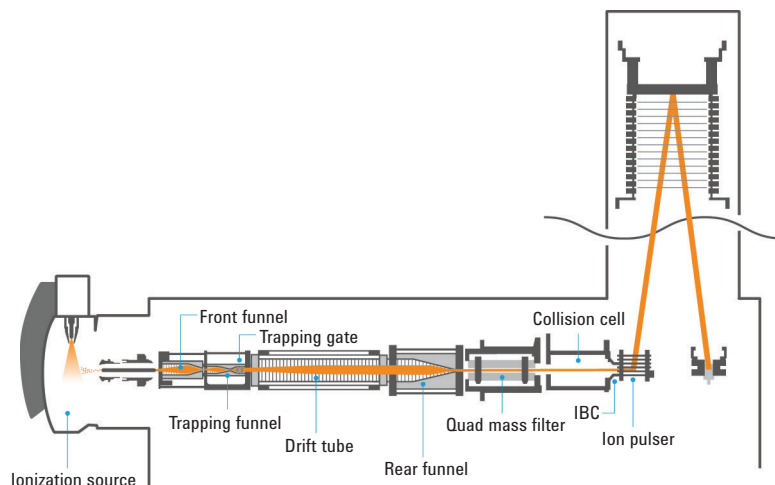


Figure 1. Schematic of an Agilent uniform field drift tube coupled to a quadrupole time-of-flight mass spectrometer using a hexapole ion guide. The Q-TOF MS has a mass resolution of over 40,000, and five orders of magnitude dynamic range in Q-TOF mode of operation.

The Agilent 6560 ion mobility system can provide researchers with greater analytical detail for the following challenges:

#### Resolving structural isomers

- Probe the molecular structure and conformation of peptides and proteins using high-resolution ion mobility separation.
- Directly determine molecular size (from collision cross sections) without reference standards or calibration tables.

#### Increasing peak capacity

- Effectively resolve individual components in complex mixtures with the combined power of UHPLC, ion mobility, and mass spectrometry.
- Obtain optimal ion mobility separation with double-grid trapping technology.

#### Finding and confirming minor components

- Readily detect low femtogram analytes in complex matrices using electrodynamic funnel technology.
- Confidently identify compounds using All Ions MS/MS.

#### Preserving protein conformations

- Easily study gas phase peptide and protein structures.
- Effectively minimize ion heating effects to maintain molecular conformations.

## Agilent Ion Mobility: higher quality MS/MS spectra at trace levels

The Agilent 6560 Ion Mobility Q-TOF LC/MS system enables direct collision cross section (CCS or  $\Omega$ ) measurements without calibration standards. It operates with uniform low field conditions, allowing the drift time information for ions to be used to determine collision cross section measurements. With the Agilent exclusive iFunnel technology, this instrument dramatically increases the ion sampling into the mass spectrometer, and results in higher quality MS/MS spectra at trace levels. For more details on this technology, read Agilent Technical Overview 5991-3244EN.

## Realize significant gains in ion mobility performance

The 6560 delivers an optimized uniform drift field mobility cell and interface to a high resolution Q-TOF instrument, providing a significant gain in ion mobility performance. The use of ion funnel technology pioneered by Agilent for both triple quadrupole and Q-TOF instruments over the past 3 years has been incorporated into the IM-QTOF system. This has resulted in combined ion mobility separation and mass resolution with high sensitivity.

Applications in this document demonstrate that the instrument delivers:

- Greater separation of lipids and glyco-peptides
- More accurate collision cross section measurements, enabling more confident characterization of structural conformations and isomeric compounds
- Greater numbers of trace level peptides in complex matrices
- Preservation of structural fidelity of metallo-proteins in solutions

To maximize the analytical utility of this system, Agilent has also developed software tools for the visualization of ion mobility data. Agilent MassHunter Software is designed to allow researchers to interrogate mobility/mass domain data, and easily determine collisional cross section values with high precision and accuracy. Agilent also provides advanced browsing capability and feature finding tools to take advantage of the mobility data.

On the following pages, we'll share applications from collaborators using the Agilent 6560 for a variety of analyses. The examples are grouped into four categories, as discussed, to demonstrate the key capabilities of the system:

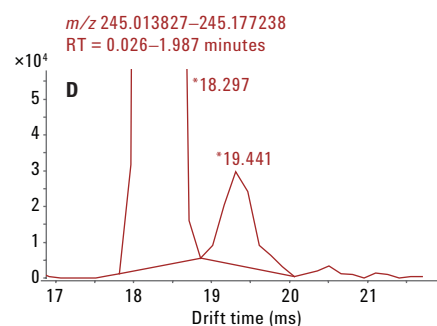
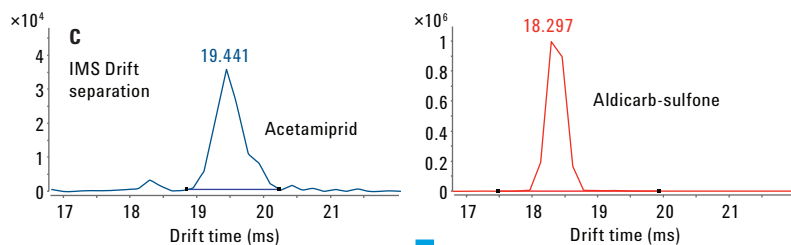
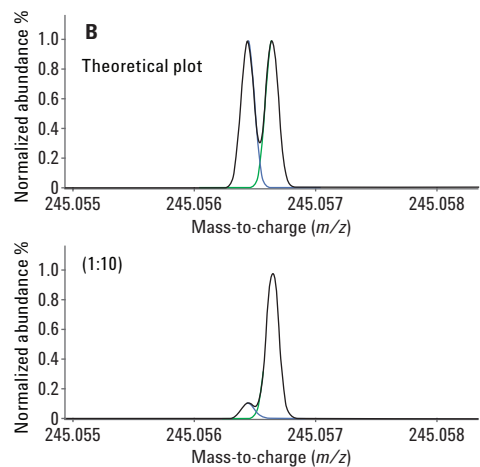
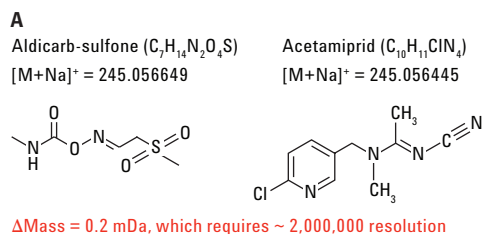
1. Resolving structural isomers
2. Increased peak capacity and specificity
3. Find and confirm minor components
4. Preserve protein conformations



# Resolve Structural Isomers

## Application examples

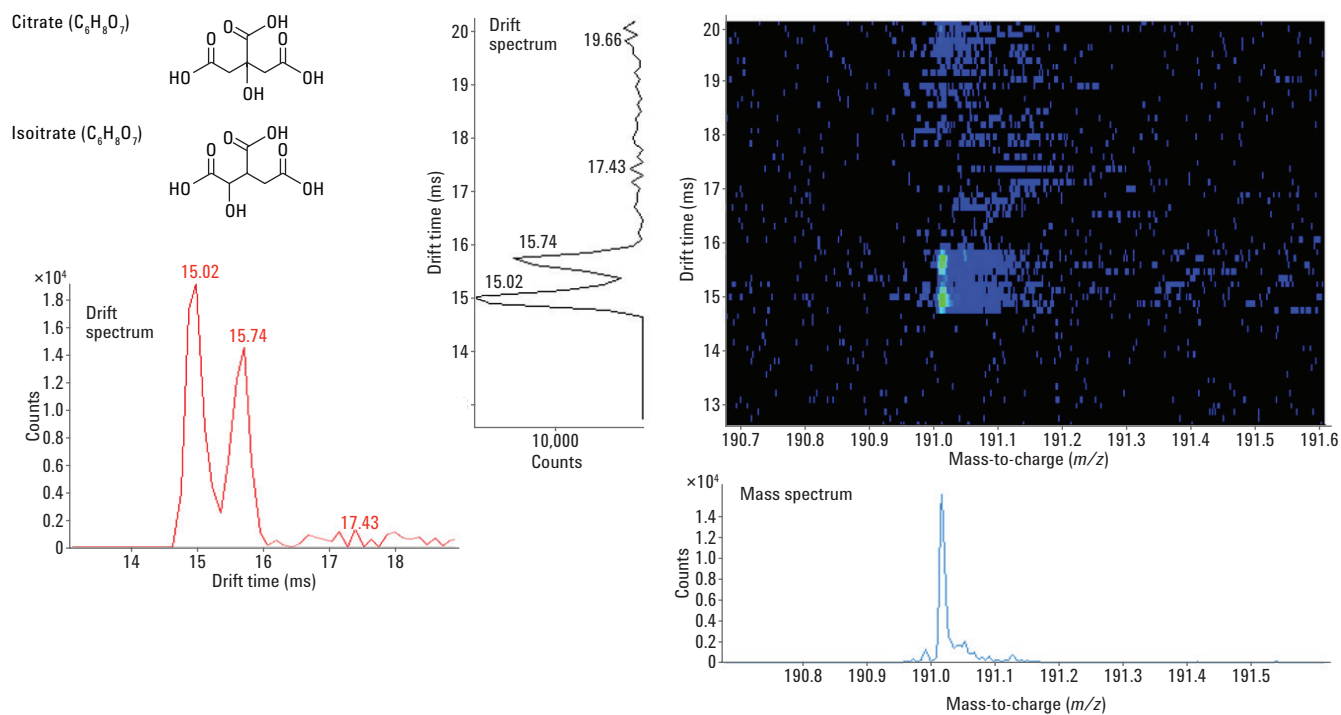
### Separation of isobaric pesticides



This example shows the combined separation power of UHPLC, ion mobility, and mass resolution. Two pesticides, differing in mass by less than 0.2 millidaltons, require overall separation power of approximately 2,000,000x to resolve them. B) shows the theoretical plot of the two compounds. C) and D) show clear IMS drift separation of the two compounds (blue and red), which are separated by 1.144 milliseconds in drift time. Even at a concentration difference of 10:1 between the two compounds, the drift resolution is sufficient to separate the two isobaric compounds without the use of UHPLC.

## Resolve Structural Isomers Examples

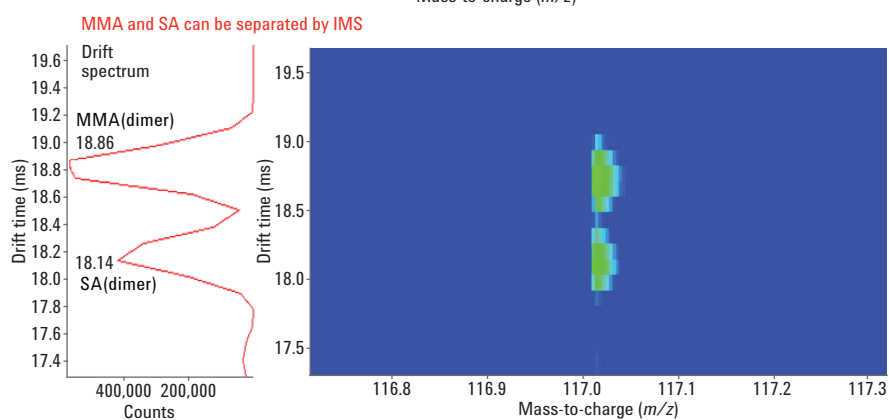
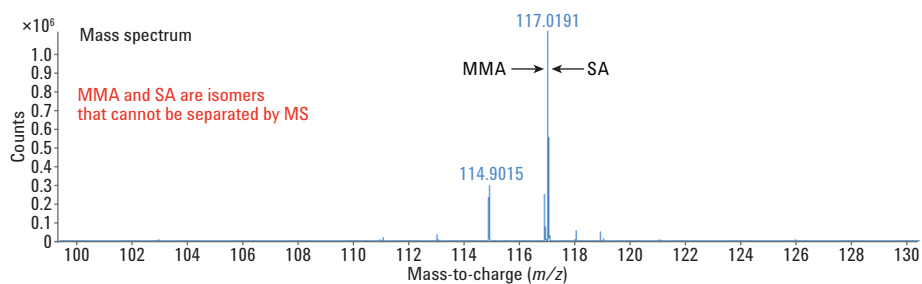
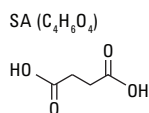
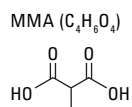
### Separation of isomers: citrate and isocitrate



Citrate and isocitrate are isomers that are very similar in physical properties and structure. This presents challenges in their bio-analytical characterization. The Agilent 6560 Ion Mobility Q-TOF LC/MS shows clear resolution between these isomers.

## Resolve Structural Isomers Examples

### Separation of methylmalonic acid (MMA) and succinic acid (SA)

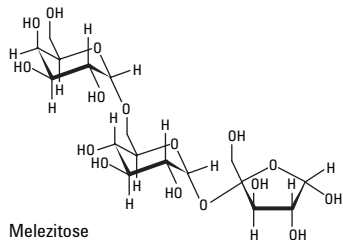


This is another example showing the ion mobility separation of the isomers of MMA, which is the vitamin B12 deficiency marker. In human plasma, its isomer (SA) is approximately 20 to 100x higher in concentration than MMA. These isomers are very difficult to separate by HPLC due to their polar properties. Our data demonstrate that the 6560 can resolve these isomers clearly, and the result can be used for accurate quantitation.

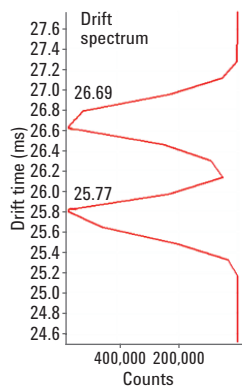
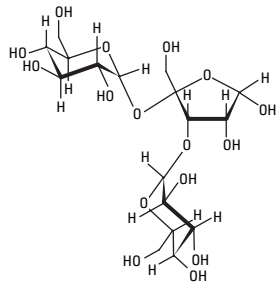
## Resolve Structural Isomers Examples

Resolving structural sugar isomers  $C_{18}H_{32}O_{16}$

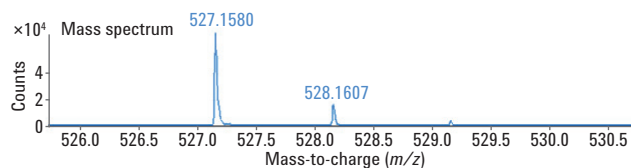
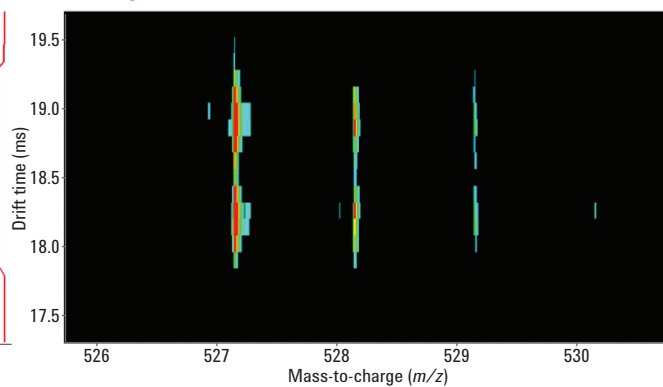
Raffinose



Melezitose



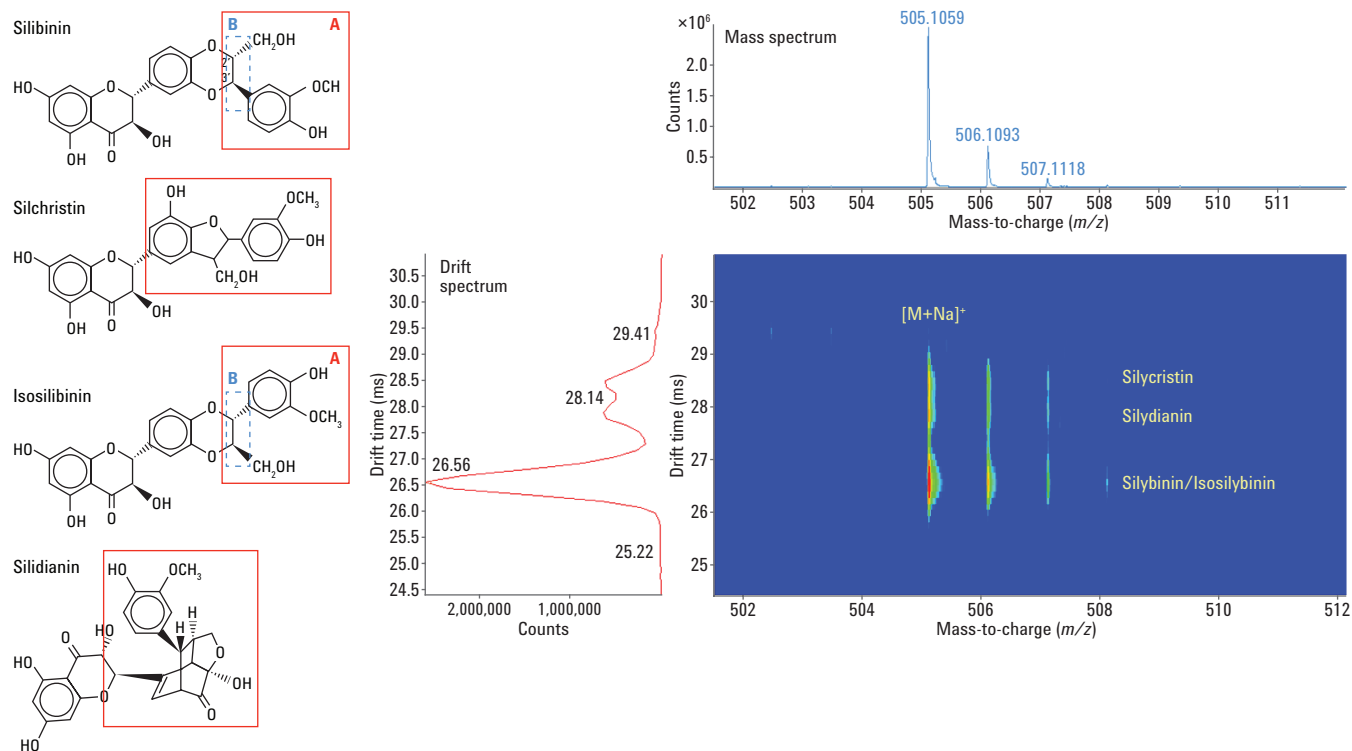
Resolving two isobaric tri-saccharides



This example demonstrates how ion mobility can be used to resolve two different isobaric tri-saccharides with the same exact mass. These isomeric structural differences cannot be resolved using an traditional premium high resolution mass spectrometry system.

## Resolve Structural Isomers Examples

### IM Q-TOF Analysis on the silymarin family

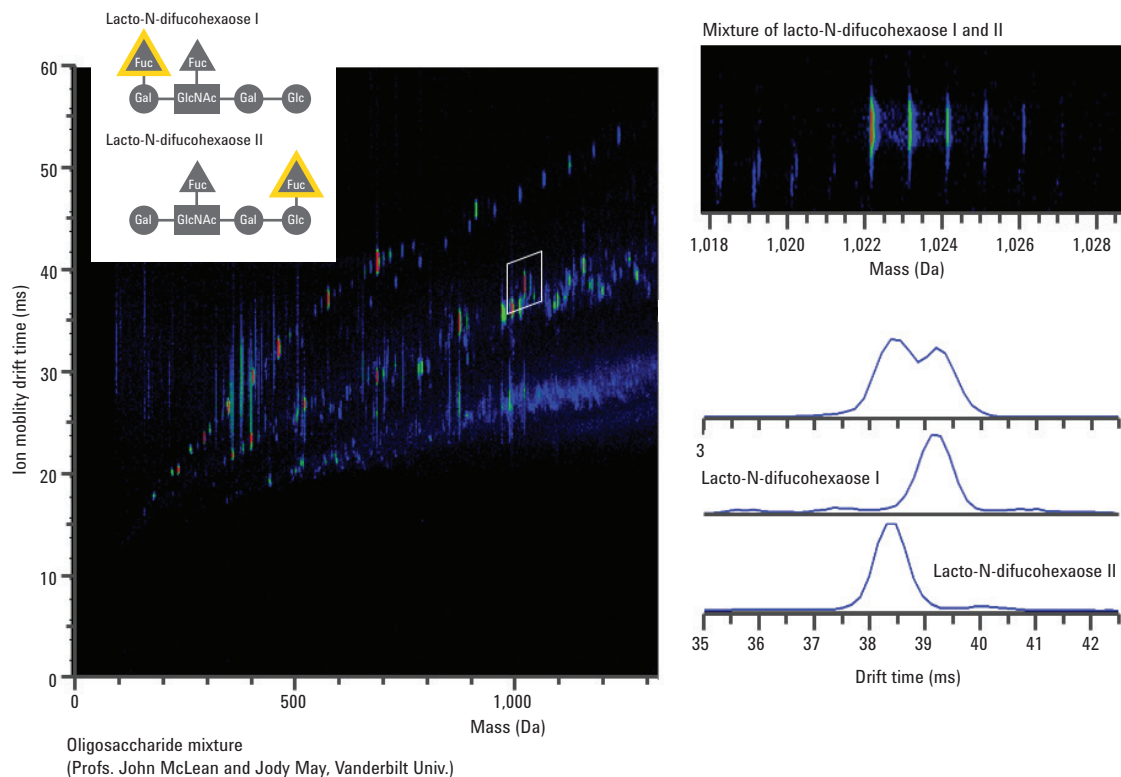


Silymarin, the active extract from plants, contains a mixture of flavonolignans consisting of silibinin, isosilibinin, silicristin, silidianin, and others. The most active compound in silymarin is silibinin. It has been extensively used in patients with liver disease. Ion mobility can be used to characterize these silymarin isomers. In this study, various isomers of silymarin are separated, and each of their unique collision cross-section values are determined (data not shown).



## Resolve Structural Isomers Examples

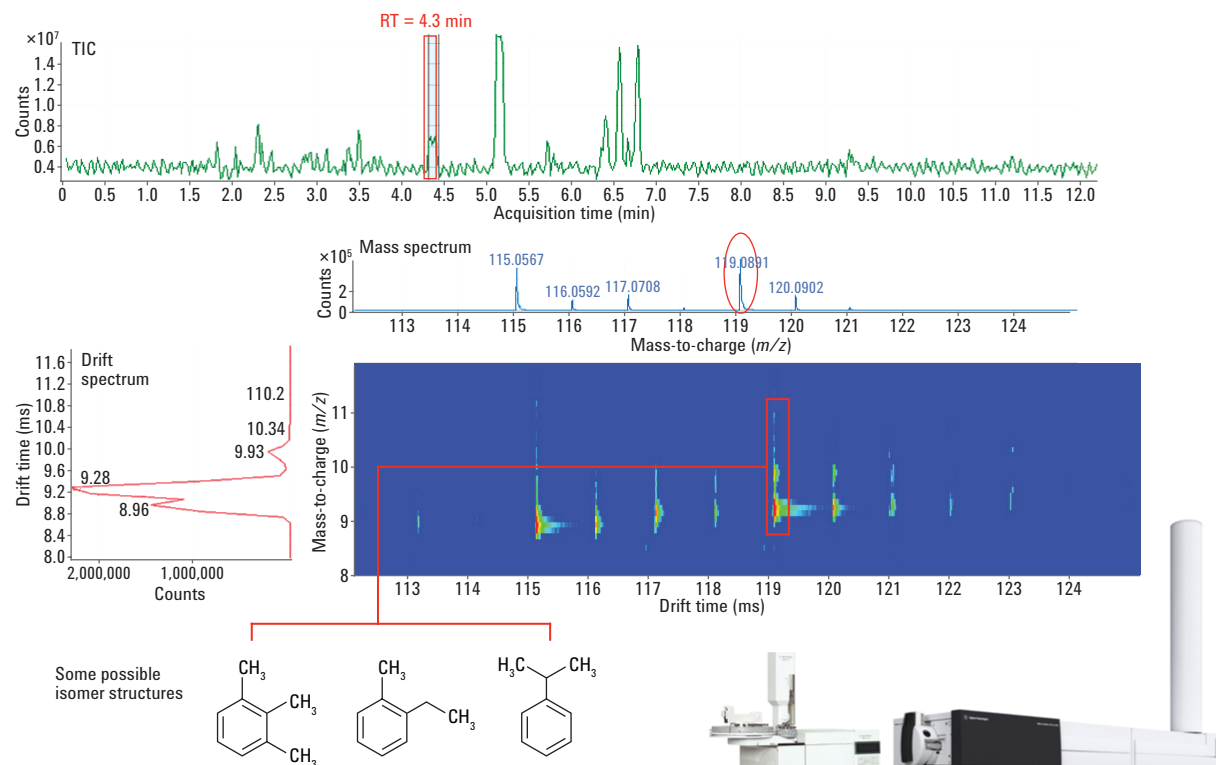
### Carbohydrates analysis by IM-MS



A mixture of lacto-N-difucohexaose I/II isomers, which differ by the location of the fucose group, is discovered in a complex sample mixture using the 6560 IM-Q-TOF. Upon individually analyzing the standards, the observation of two species of human milk oligosaccharide isomers is confirmed.

## Resolve Structural Isomers Examples

### GC-APCI/IMS-Q-TOF analysis of ASTM compound mixture

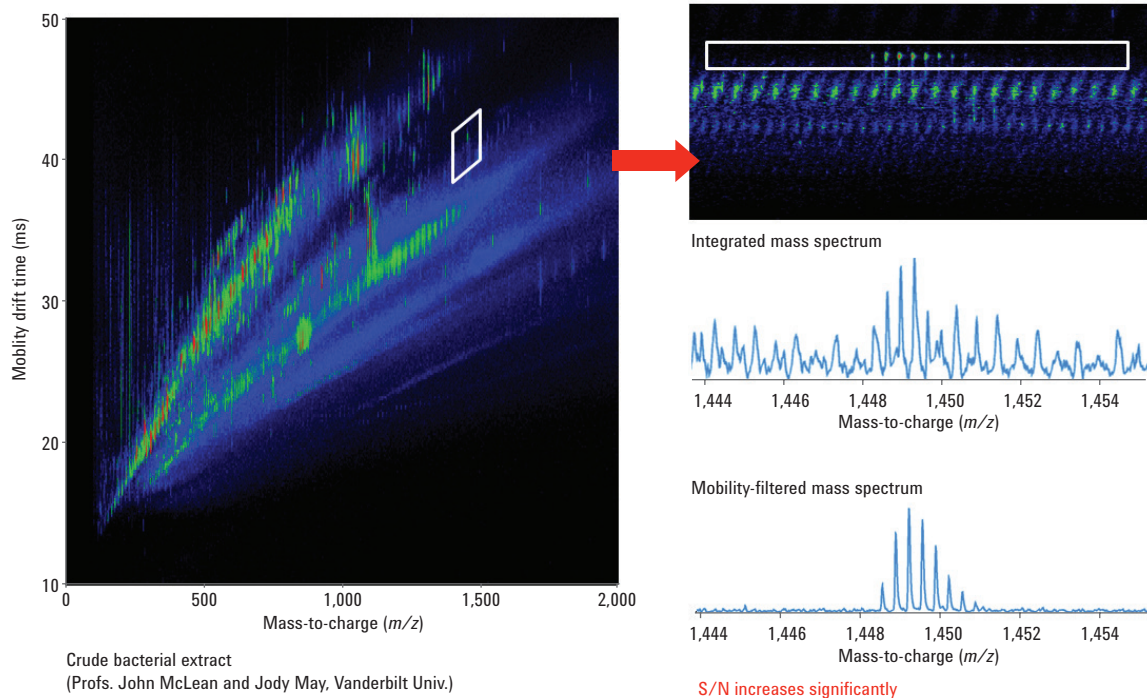


In this study, we use the GC-APCI interface coupled with ion mobility to analyze the ASTM standard compound mixture. For example, at the GC peak of 4.3 minutes, more than three separated compounds with many associated isomers in the mass range of 110 to 125 Da were found. Therefore, the Agilent 6560 provides higher peak capacity to successfully resolve the different isomers.

## Increased Peak Capacity/Specificity

### Application examples

#### Ion mobility provides greater specificity

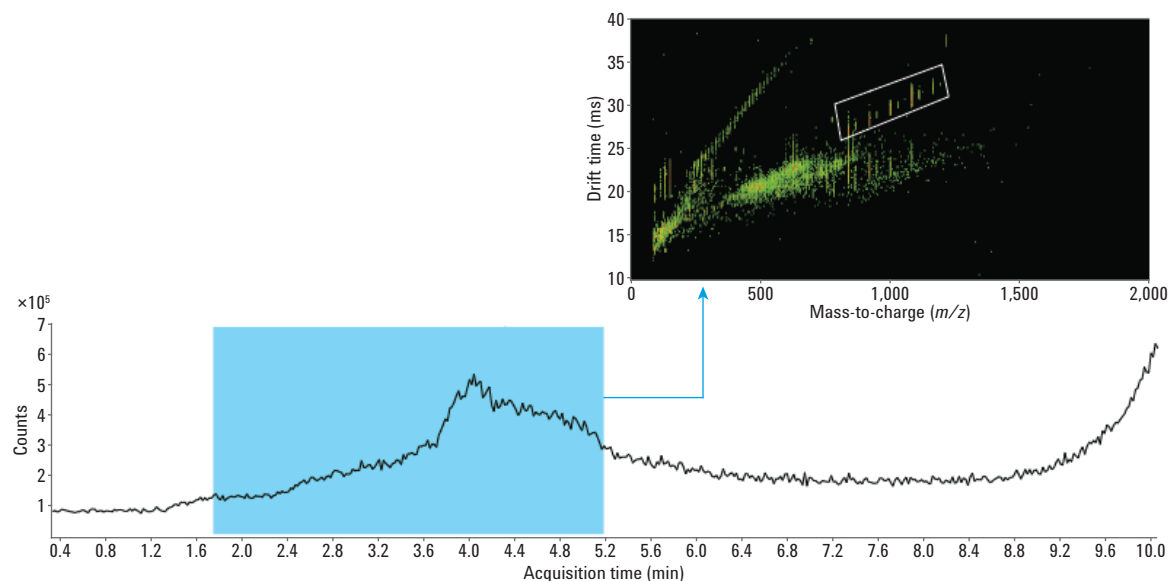


Another value of ion mobility is to effectively clean up background chemical noise from a crude bacterial extract. This mobility heat map shows hundreds of components in the sample with overlapping compounds at nearly every  $m/z$  value. In the highlighted polygon region of the heat map, see the integrated mass spectrum that has too many ions to provide confident compound identification. The bottom graphic shows the mobility-filtered mass spectrum, which eliminates many of the overlapping chemical background ions. This enables fast and confident compound identification.

## Increased Peak Capacity/Specificity Examples

### Ion mobility provides greater specificity

RNAseB Native glycans analysis Enable the extraction of ion series of interest - a group of glycans from matrix for further study. (Prof. Cathy Costello, Boston University)

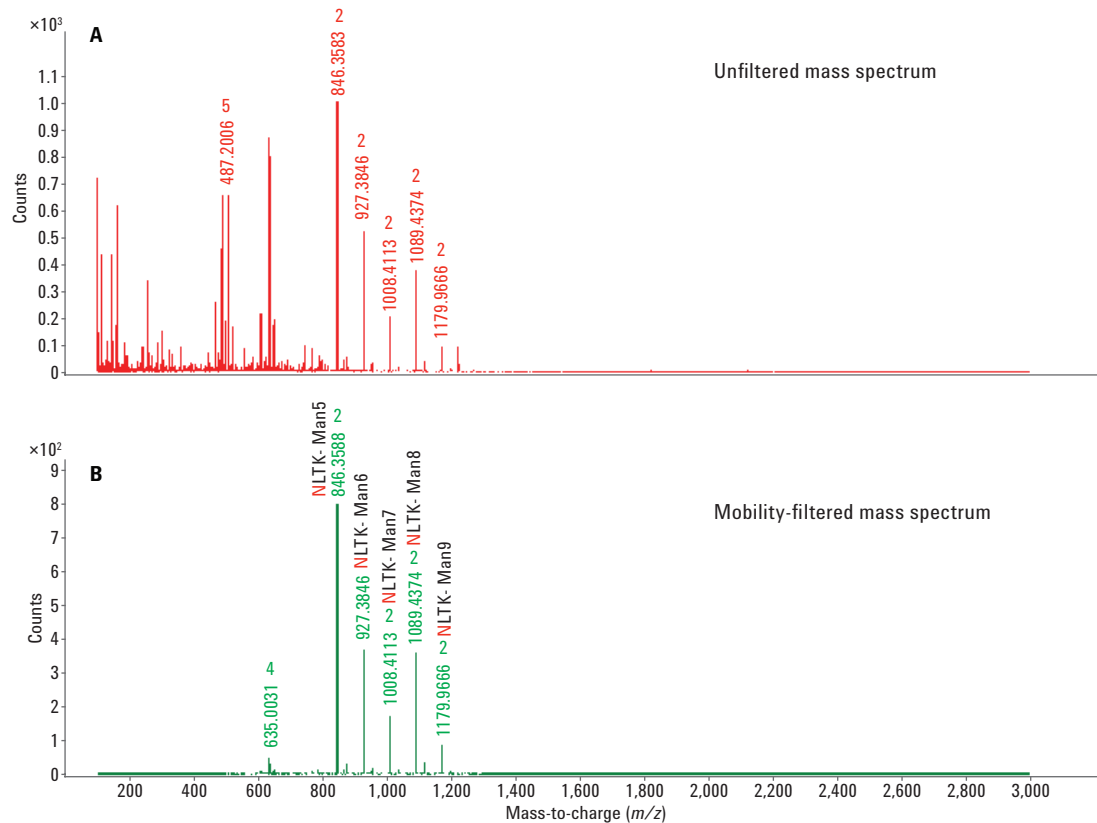


In this example, Professor Cathy Costello at Boston University uses an Agilent 6560 Ion Mobility Q-TOF LC/MS system to selectively isolate a group of RNaseB glycopeptides from the background matrix. A selected region from an LC chromatogram (blue) is displayed in the IMS-MS heat map (insert). One region of distinct mass differences (white box), as shown by the trend line, is clearly distinguishable as a trendline with increasing number of monosaccharides in the glycopeptide compositions. The signal from these glycopeptide ions can be selected in the mobility trace and separated from other ions present in the spectrum, for further study.

## Increased Peak Capacity/Specificity Examples

### Ion mobility simplifies complex spectra

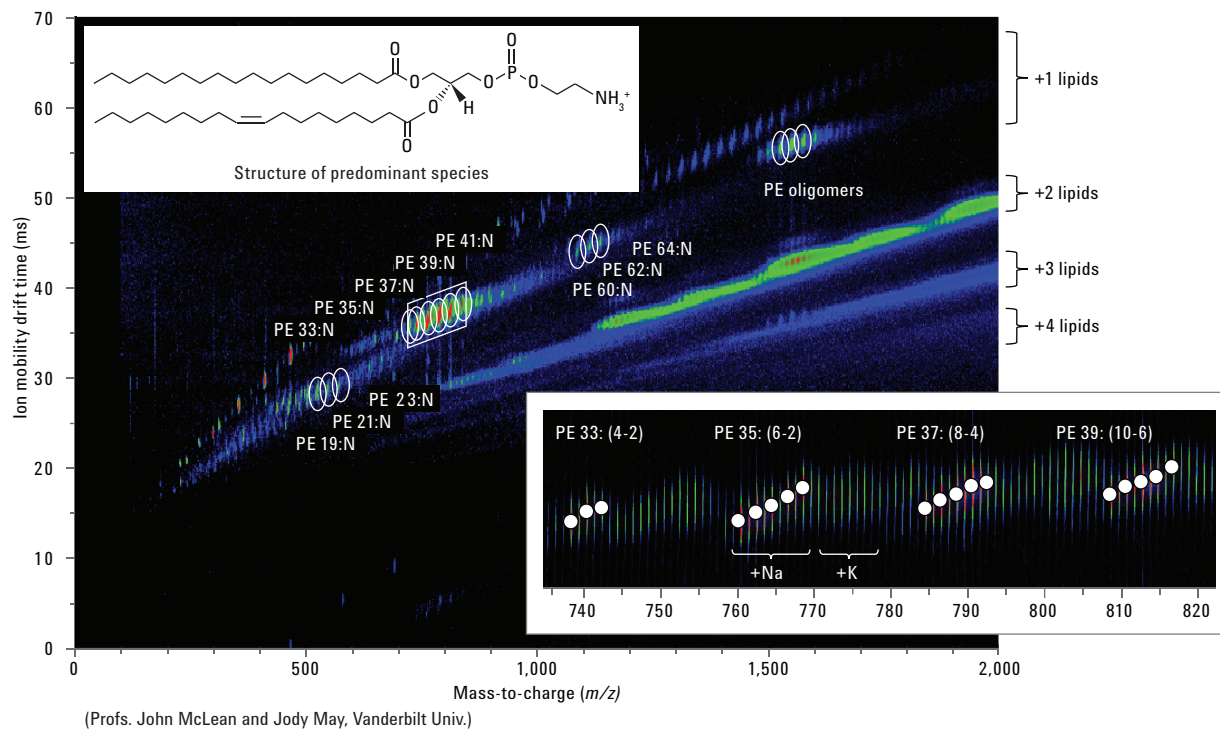
RNAseB glycopeptides NLTK- Man5 through 9



In this example, ion mobility is used to simplify complex glycopeptide spectra. A) RNAseB glycopeptides on a custom HILIC-C18 HPLC-chip on the 6560 shows all of the combined ions corresponding to compounds eluting in the selected retention time window. B) RNAseB glycopeptides on HILIC-C18 chip on the 6560 with IMS separation shows a simplified mobility spectrum for the different glycoforms of a single glycopeptide. The peaks with a distinct delta of 162 correspond to increasing number of mannose units in the high-mannose N-linked glycopeptide NLTK, as labeled.

## Increased Peak Capacity/Specificity Examples

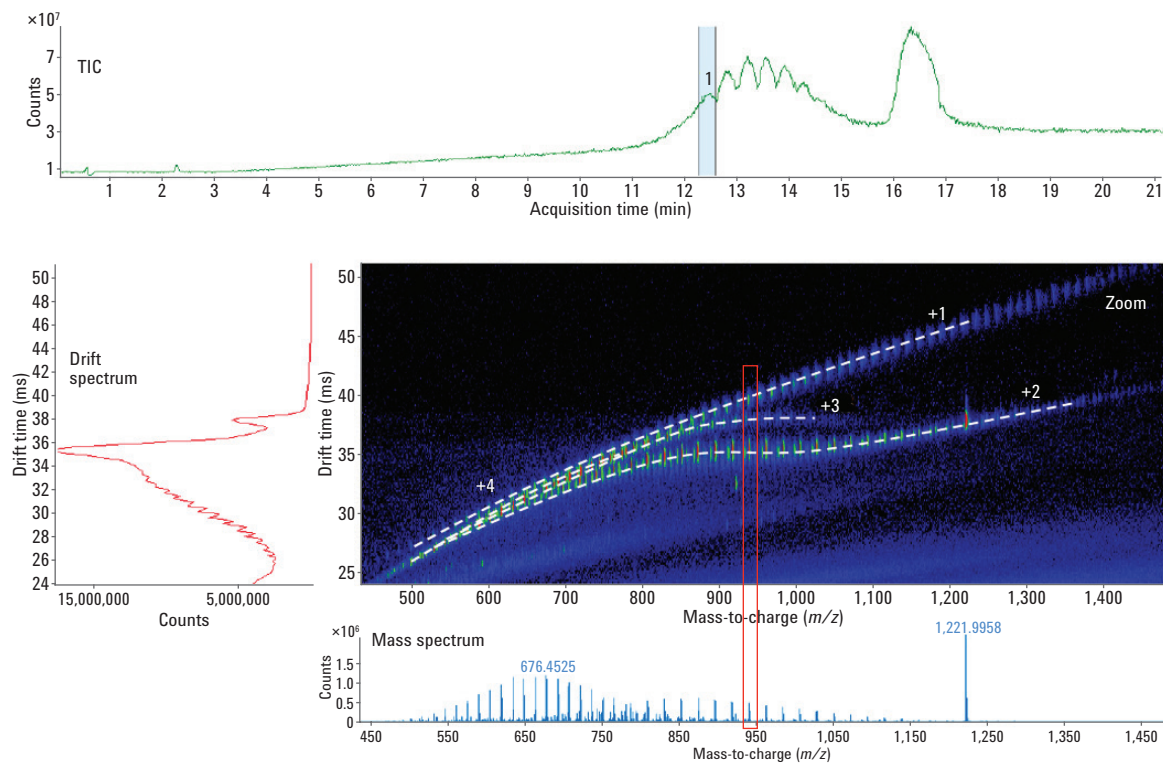
### Lipid analysis: Mixture of L- $\alpha$ -phosphatidylethanolamine (PE) lipids



In this example, a class of lipids, the phosphatidylethanolamines, is separated by ion mobility. Professor John McLean's group at Vanderbilt University quickly identifies over 200 different lipids and oligomers that fall on a specific trend line. Another interesting discovery is made while evaluating the data within the main trend of the lipids; they observe a secondary trend consisting of differing degrees of unsaturation.

## Increased Peak Capacity/Specificity Examples

### Ion mobility of polymeric ink dispersants

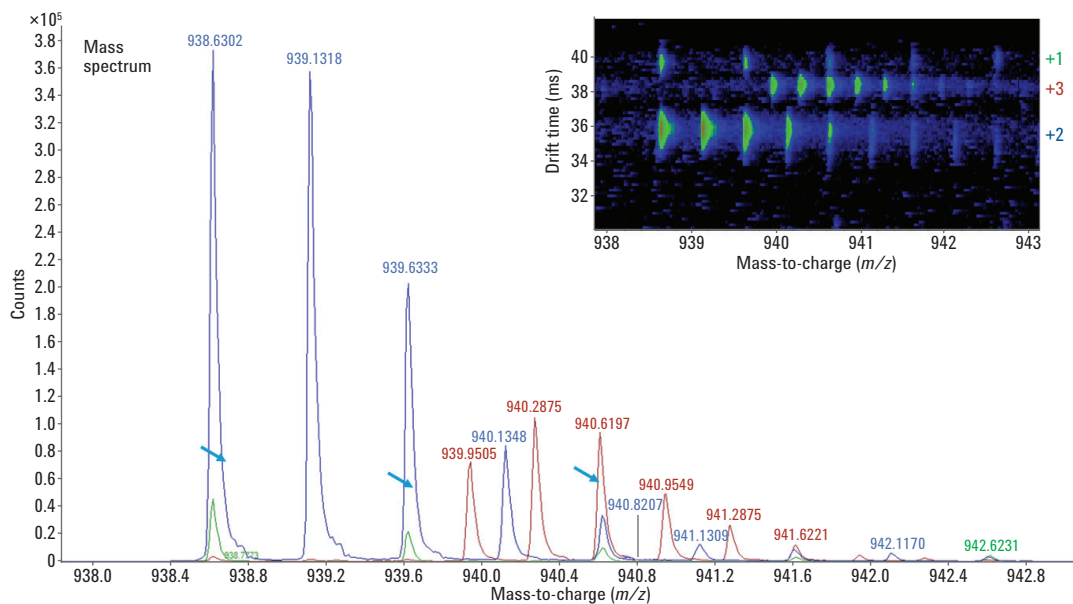


In this example, ion mobility is used to identify different hydrocarbon ion series in polymeric ink dispersants. The trend-lines illustrating the various charge-state hydrocarbon ion series are clearly resolved. In addition, the ion mobility specificity can significantly reduce the background matrix effect.

## Increased Peak Capacity/Specificity Examples

### Ion mobility of polymeric ink dispersants – hydrocarbon molecules

Resolving overlapping charge-state isotopes by ion mobility

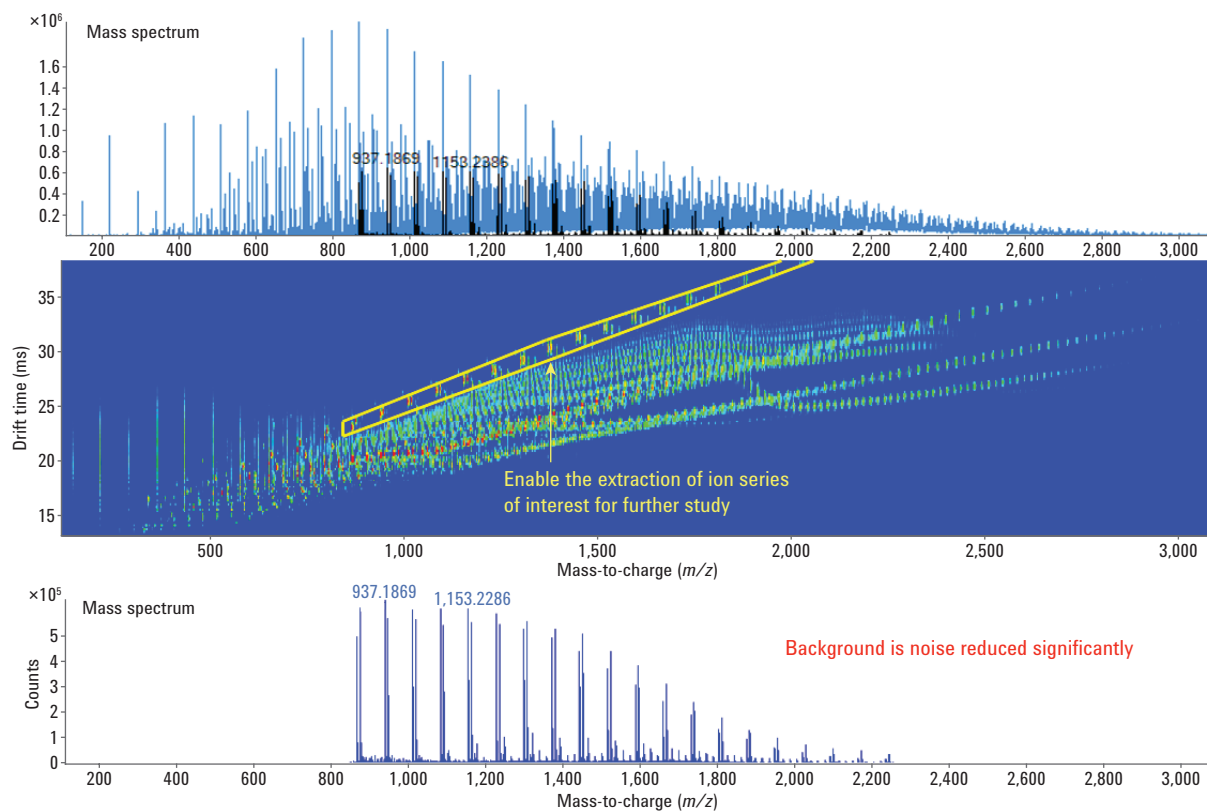


In this example, ion mobility is used to resolve overlapping charge-state isotopes that were unresolved by mass resolution only. This information is used to compare and confirm the quality level of the various ink products.



## Increased Peak Capacity/Specificity Examples

### Ion mobility of diesel (hydrocarbons) sample

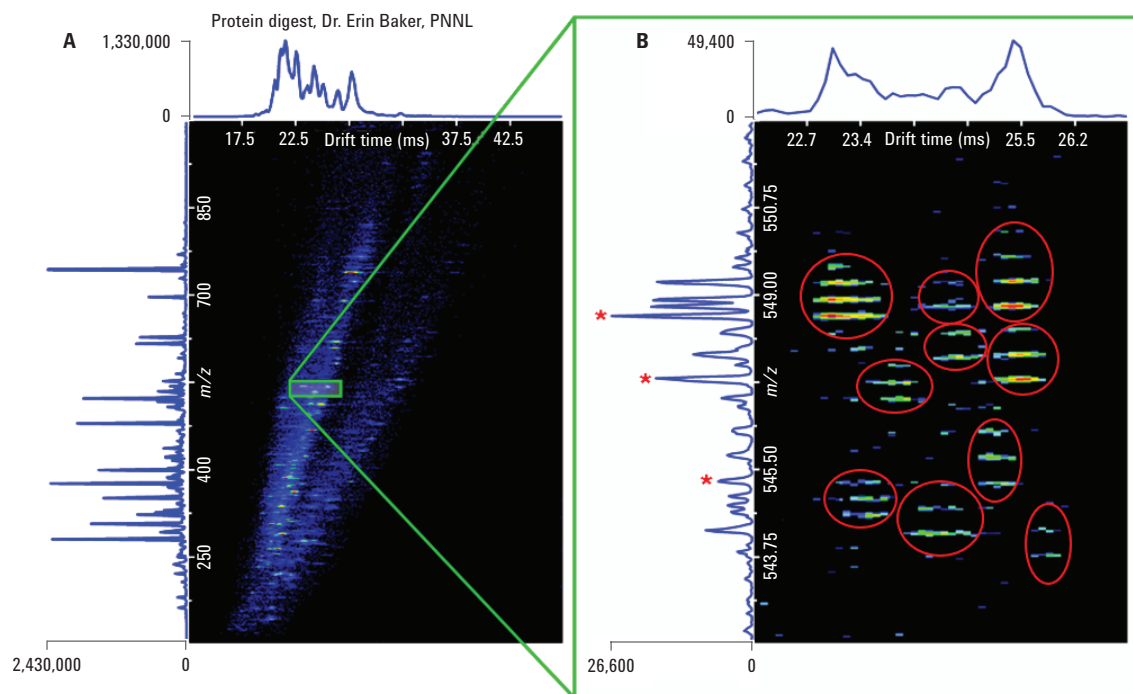


In this example, ion mobility is used to identify different hydrocarbon ion series in a diesel sample. The middle panel shows how ion mobility enables the extraction of ion series of interest for further study. The lower panel shows how ion mobility significantly reduces the background noise.

## Find and Confirm Minor Components

### Application examples

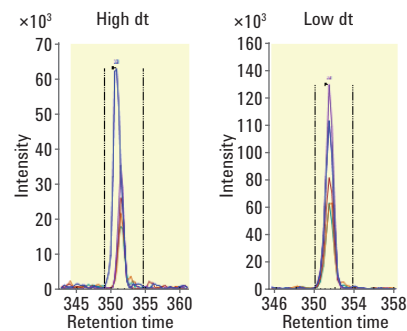
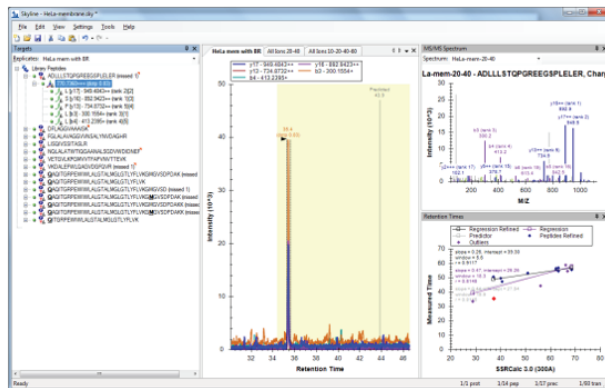
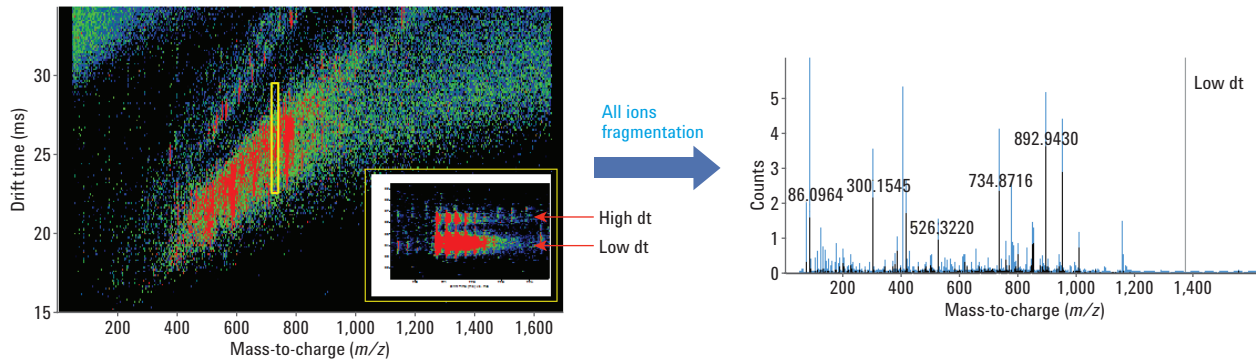
#### Ion mobility provides greater detection for proteomics



In this example, ion mobility is used to enhance the identification of tryptic peptides in mouse (A) and human blood plasma, which is useful for analyzing disease states. The inset graphic (B) shows a zoomed-in region of the 3-D plot where 10 peptides were identified easily with IMS in 0.5 seconds of the 15-minute LC run (red circles). By comparison, the same sample was run with a 100-minute LC gradient, using a high-resolution MS instrument, which yields only three identifications at the same region (indicated by red asterisks). In summary, the 6560 detects > 3x the peptides > 5x faster.

# Find and Confirm Minor Components Examples

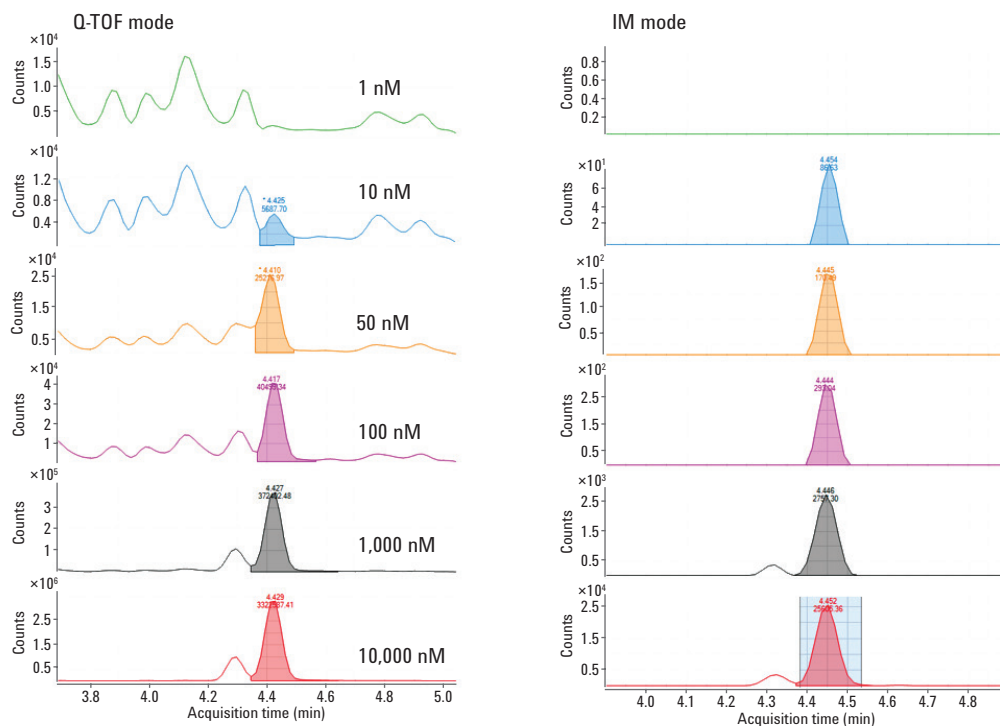
## IMS-MS for discovery proteomics: transmembrane spanning peptides of HeLa digest



In this sample, we are able to identify multiple potential isoforms of transmembrane-spanning peptides using ion mobility. Using Agilent All Ions MS/MS and an MS/MS library from our Spectrum Mill proteomics software, we use Skyline software from the MacCoss Group at the University of Washington to match some transmembrane-spanning peptides with known helical structures. In this way, we are able to determine the relative quantitation ratio between an helical form (condensed, lower dt) and a denatured form (higher dt).

## Find and Confirm Minor Components Examples

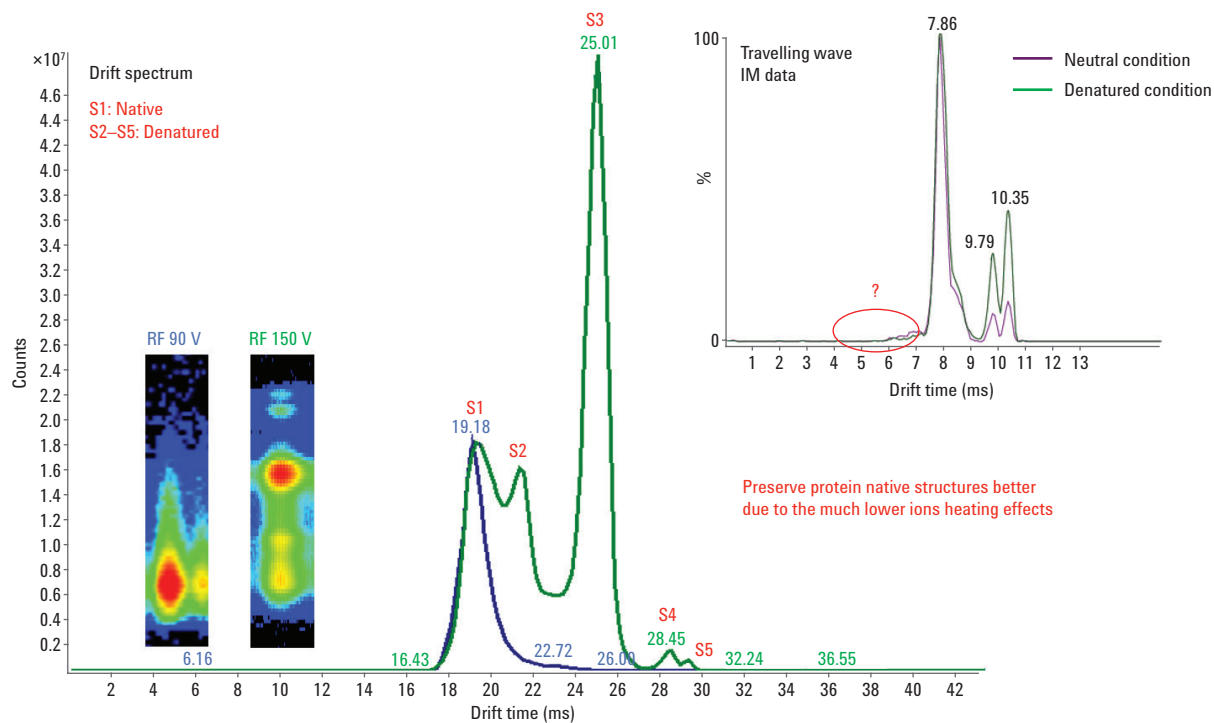
### Sensitivity: detection limit of spiked compound in urine



This example shows a comparison of detection limits between the Q-TOF and the IM Q-TOF modes of the system for a spiked compound (biological marker) in urine. Although very similar limit of detection (LOD) sensitivity is observed between these data acquisition modes, superior signal-to-noise (S/N) data using the IM-Q-TOF mode are obtained. Improvement in quantitative results are achieved due to the much lower background noise level.

## Preserve Protein Conformations Examples

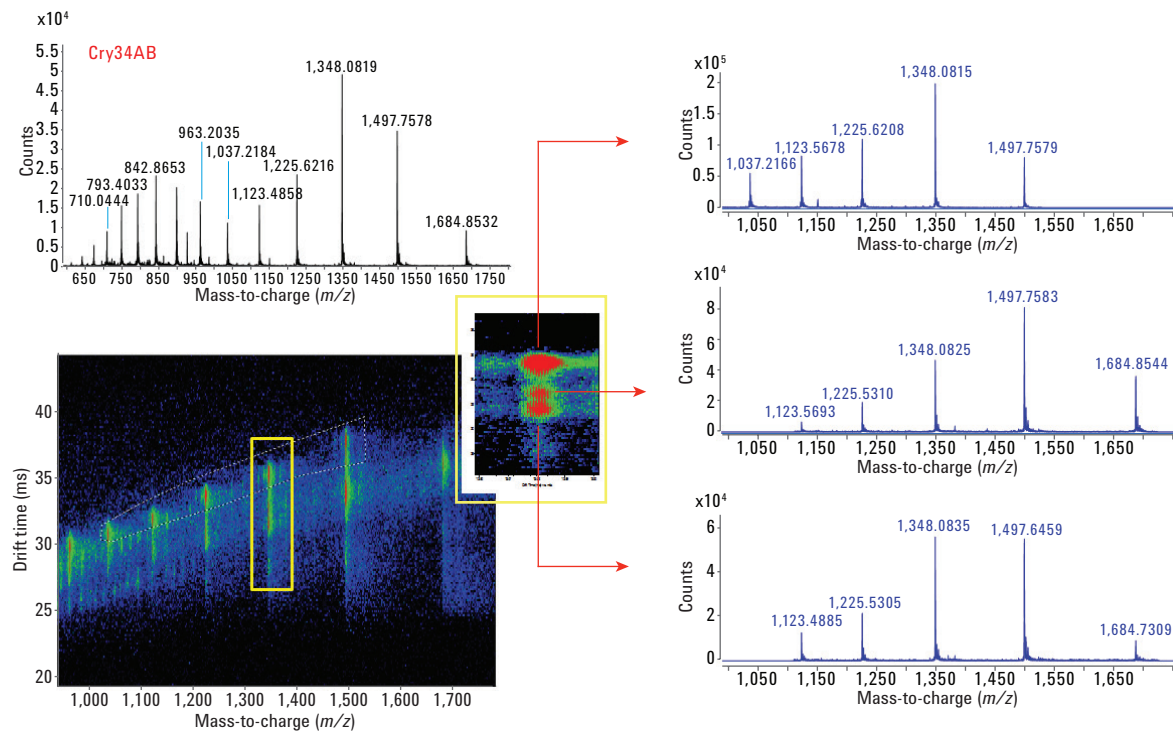
### IM Comparison on Cytochrom C (+8): (uniform drift tube versus travelling wave)



The minimal ion heating effect from the Agilent 6560 Ion Mobility Q-TOF LC/MS system is critical for maintaining a native protein conformation. By altering the trap RF voltages, we can change the protein conformation from its native state (S1) to various degrees of denatured states (S2–S5). By comparison with an alternative travelling wave IM system, the Agilent drift tube IM system requires a much lower energy, minimizing the ion heating effect that causes the denaturation of the protein molecules.

## Preserve Protein Conformations Examples

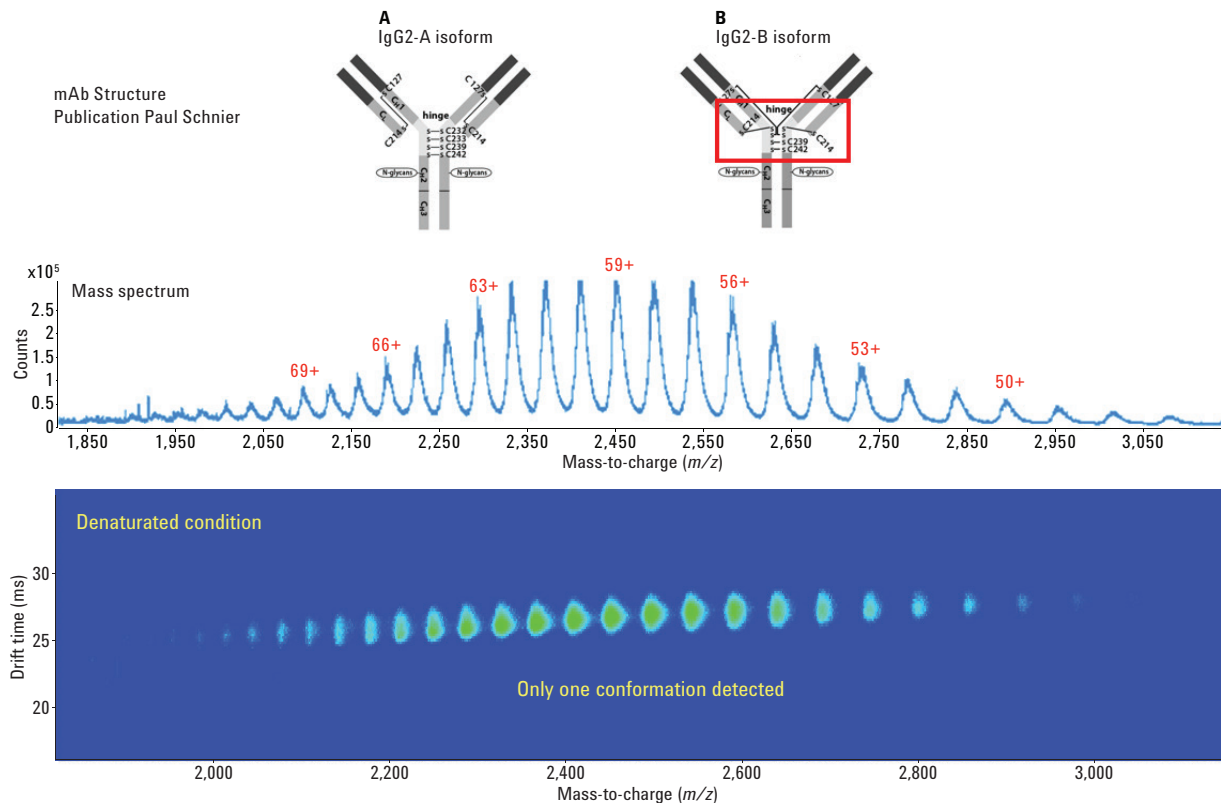
### Ion mobility Q-TOF on protein isoform analysis



A protein sample (CRY34AB) under native and denaturing conditions is analyzed using ion mobility. Using just the IM information, it appears that there are possibly three isoforms, which are clearly separated. The IM results also confirm that the different isoforms generate different charge envelopes, indicating various protein folding structures, consistent with time-consuming X-ray crystallography.

# Preserve Protein Conformations Examples

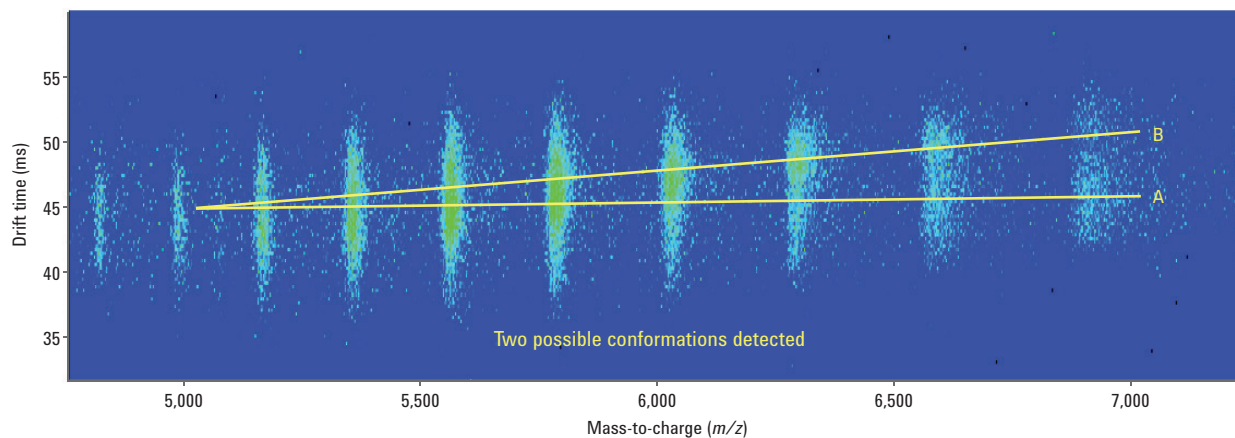
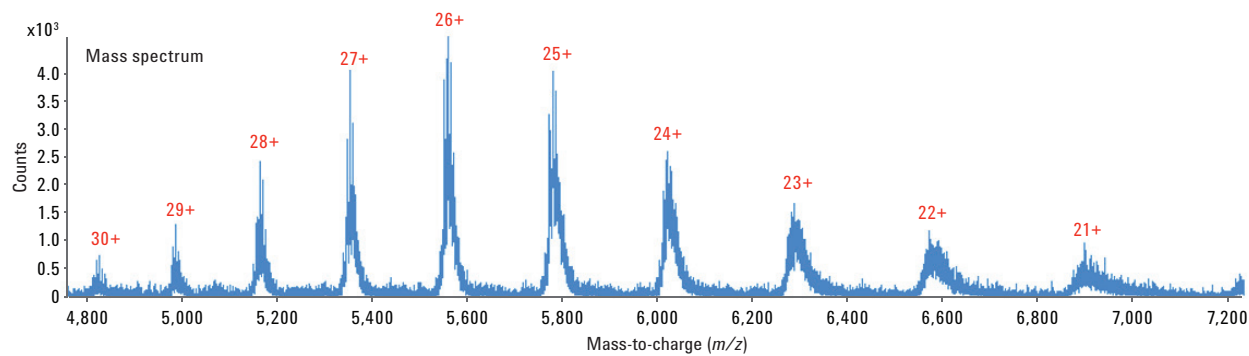
## Resolving isoforms of IgG-2



The IgG-2 molecule has two different conformations (isoforms A and B) under native conditions. However, normal LC/MS conditions, with a high content of organic solvent and 0.1% formic acid, will destroy the native structure. Only one denatured protein conformation can then be detected.

## Preserve Protein Conformations Examples

### IM-Q-TOF Analysis of native IgG-2

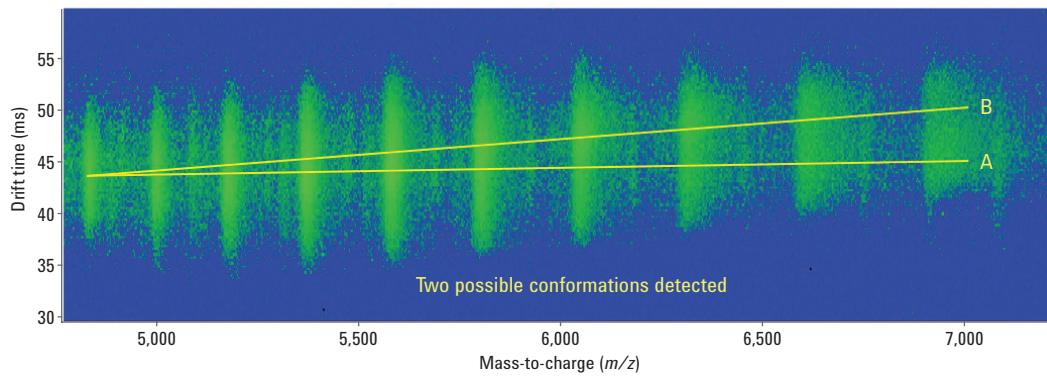
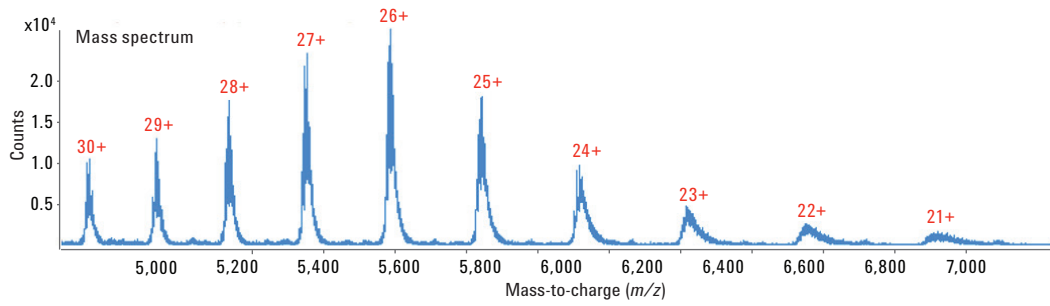


Under native conditions (100 mM ammonium acetate), the IgG-2 charge envelope will shift to higher  $m/z$  (5,000–7,000 range). Two protein conformations were clearly detected. Isoform A is the true native structure, and isoform B represents the IgG-2 with possible mismatch in disulfide bonds.



## Preserve Protein Conformations Examples

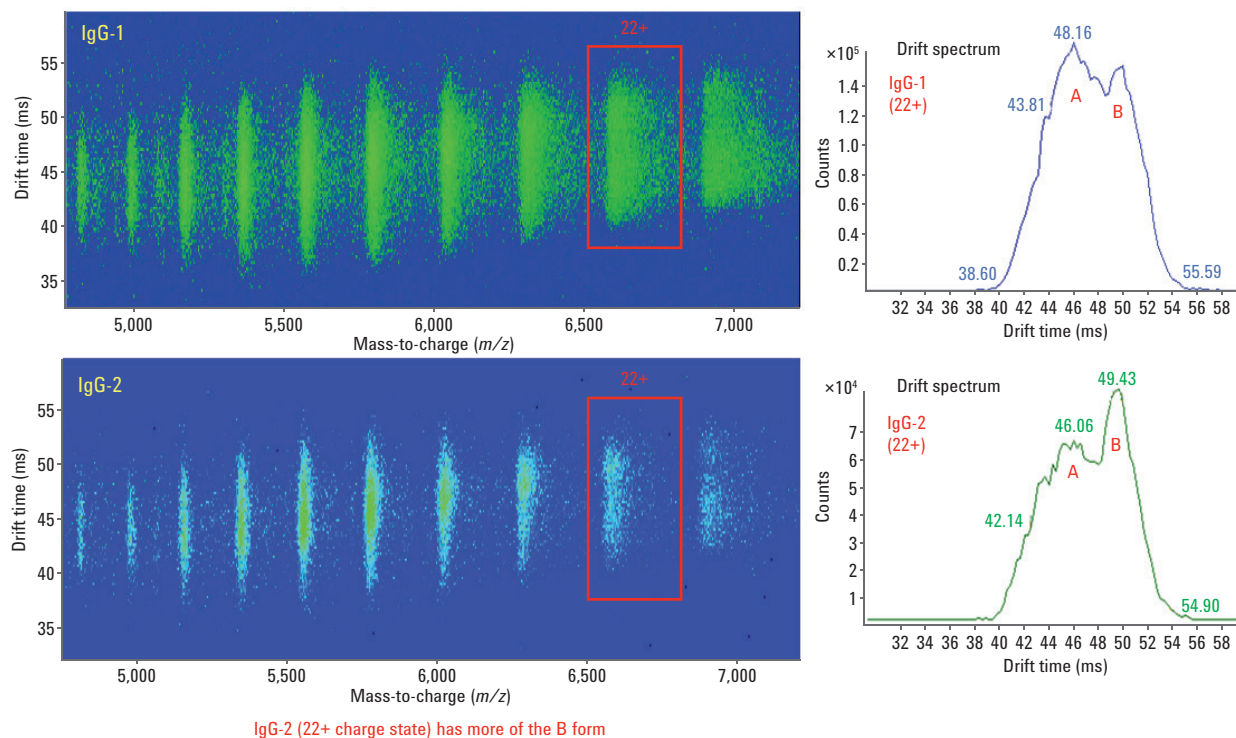
### IM-Q-TOF Analysis of native IgG-1



Two protein conformations are detected in the native IgG-1 sample using the 6560 system.

## Preserve Protein Conformations Examples

### IM-Q-TOF Comparison of IgG-1 and IgG-2



In this example, ion mobility is used to determine the relative amount of isoforms A and B in the IgG-1 and IgG-2 sample. In a side-by-side comparison, IgG-1 posts a slightly higher percentage of isoform A (native) than B at its 22+ charge state molecule. Conversely, a higher percentage of isoform B is detected in the IgG-2 sample.

## **Ion Mobility Adds A New Dimension to Your Research**

The Agilent 6560 Ion Mobility Q-TOF LC/MS is the first commercial instrument that enables researchers to address truly fundamental questions about structure, function, and the workings of complex biological systems with real confidence and ease. Combining the orthogonal separation techniques of liquid chromatography, mass measurement and ion mobility tremendously increases peak capacity, giving you the ability to more effectively characterize a variety of molecules.

The technology in the 6560 IM Q-TOF provide important benefits for analytical challenges:

- Added separation capability for isomeric compounds
- Increased peak capacity/specificity
- Added selectivity for very complex samples
- Reduced effect of chemical backgrounds
- Ability to distinguish different protein conformations

Simply put, you can now resolve and detect a greater number of compounds and components than ever before.

## **Acknowledgments**

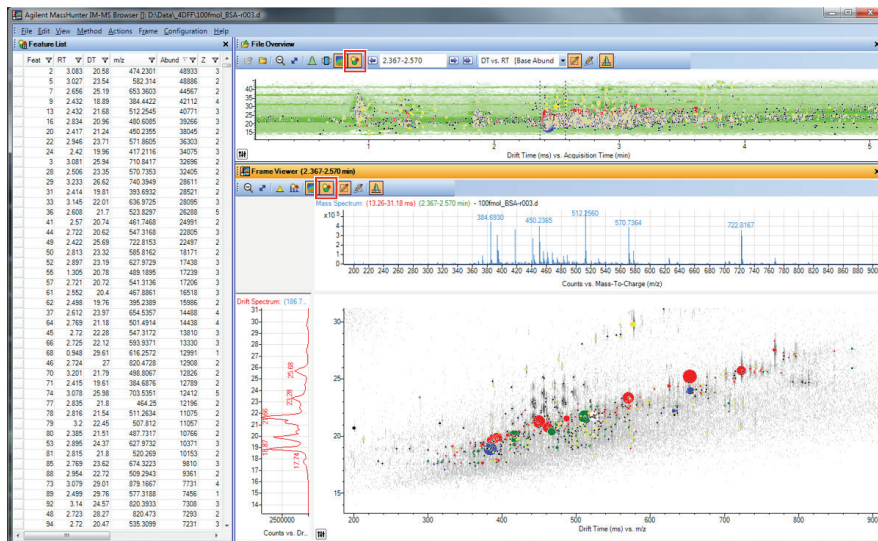
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- Single field CCS calculation
- Differential profiling using Mass Profiler, including statistical analysis and PCA plots

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