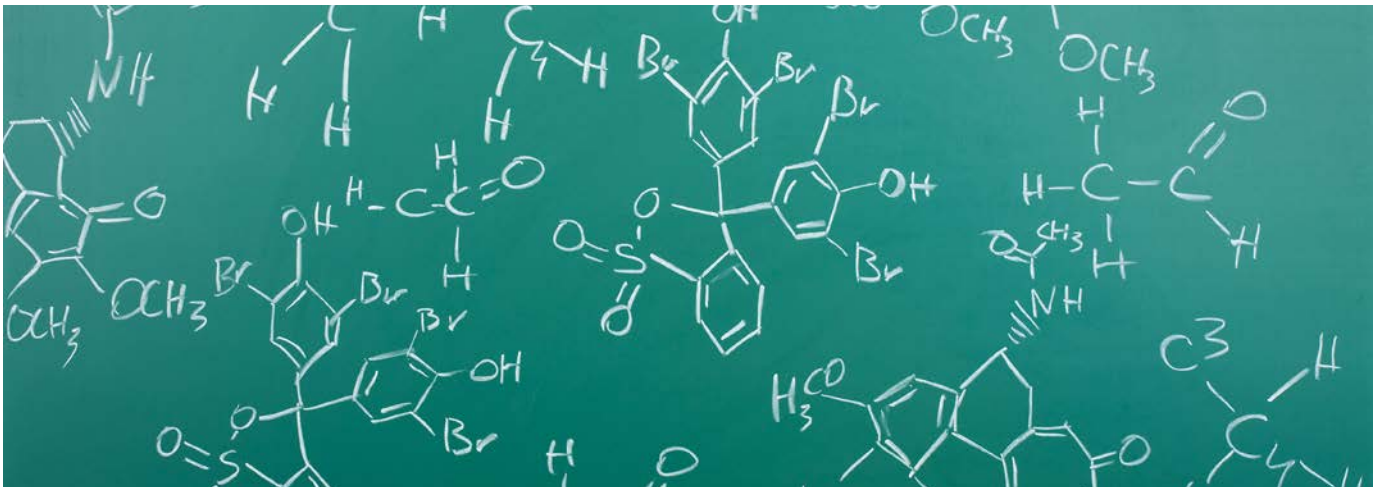
Agilent

Trusted Answers

Add a New Dimension to Your Research

Agilent 6560 Ion Mobility LC/Q-TOF



Reveal More Details Than Ever Before

Does your research involve characterizing small molecules or proteins, increasing metabolite coverage maps, or ensuring food safety? The Agilent 6560 Ion Mobility LC/Q-TOF will reveal details you've never been able to resolve before. Ion mobility mass spectrometry adds an orthogonal dimension of separation to your LC/MS analyses.

With exceptional selectivity and sensitivity, the 6560 Ion Mobility LC/Q-TOF can detect, identify, and characterize the components of your most complex samples.

With this sensitive LC/MS instrument, you will be able to:

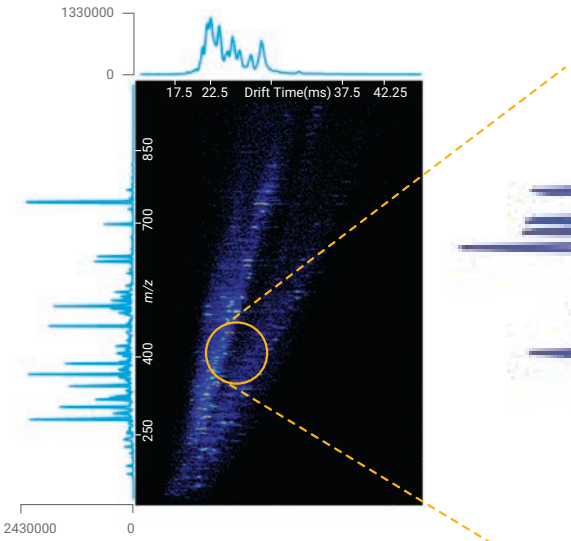
- Clearly separate molecules and isomers based on their size, shape, and charge
- Observe changes in the structure of proteins and other molecules by collision-induced unfolding (CIU)
- Calculate collision cross section (CCS) by first-principle measurements without standards
- Conserve the native state of molecules in the gas phase using the low-energy drift tube design
- Use multiplexing for substantially increased sensitivity and dynamic range
- Obtain ion mobility resolution of up to 250 independent of the acquisition

In fact, research scientists are already tackling these challenges using the 6560 high-resolution mass spectrometer.

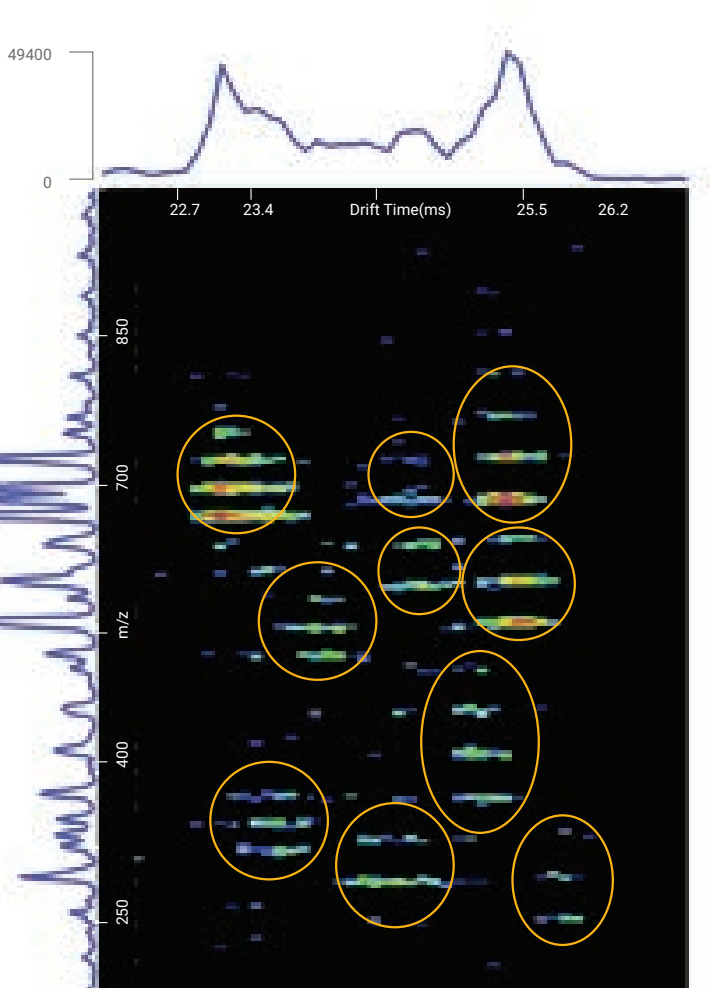
Solving the unsolved

Ion mobility reveals the full complexity of your sample

Biological matrices contain a very high number of compounds, which in many cases are overlapping. High resolution mass spectrometry can not always separate these, in particular over a broad range of abundances. Ion mobility adds another dimension and helps to separate otherwise unresolved ions.



The *m/z* versus drift plot shows the separation of tryptic peptides derived from a sample of mouse blood plasma spiked with 20 reference peptides. The sample was subjected to LC separation for 15 minutes before IM Q-TOF analysis.



The inset shows a zoomed-in region of the 3-D plot where 10 unique peptides were separated and identified.

Separate unresolved analytes with sensitivity that shows

What sets the 6560 Ion Mobility LC/Q-TOF apart?

In short, this instrument combines ion mobility with liquid chromatography and mass spectrometry. Erin Baker, a leading ion mobility scientist and ASMS Bieman medalist, collaborated with Agilent on this system. The results are impressive to say the least.

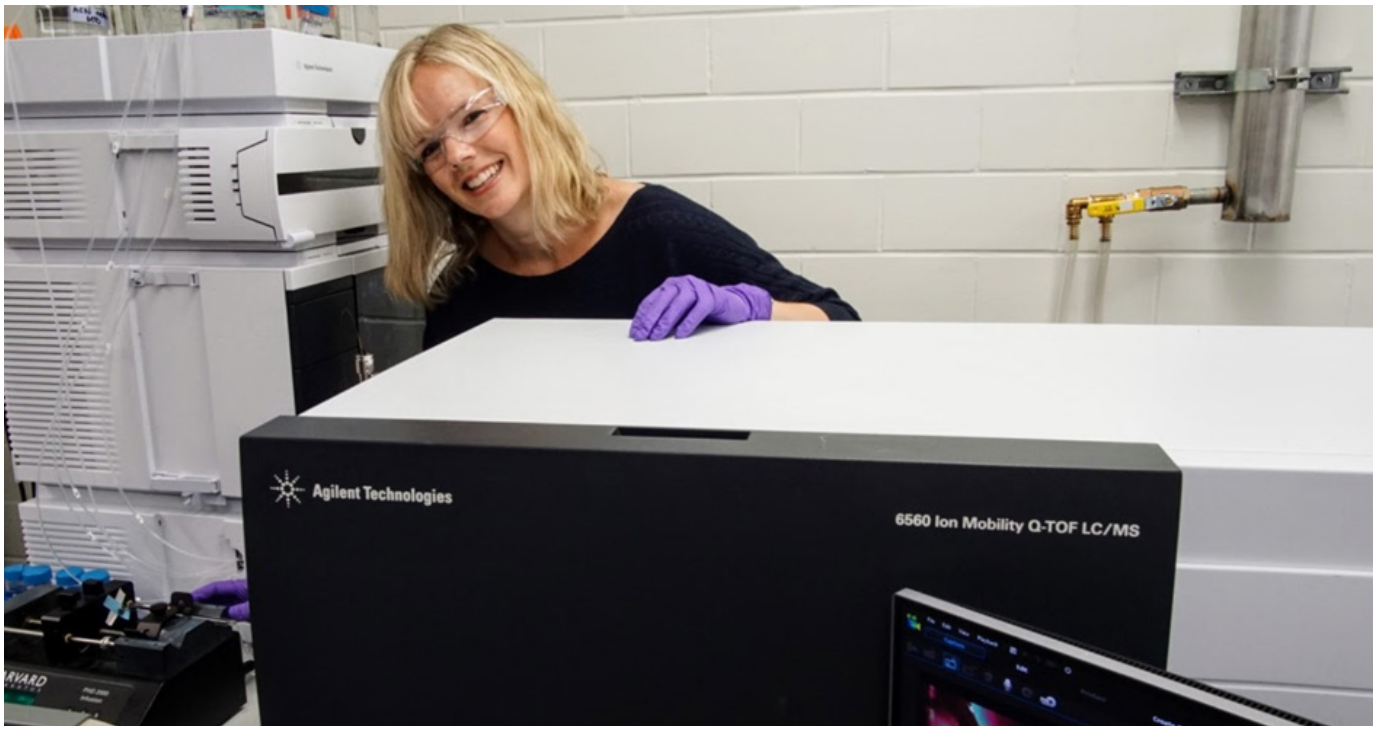
"A lot of times it's the things you don't know that end up being the most important," Dr. Baker says. "This system can give you information about everything in your sample."

If one molecule has the same mass-to-charge ratio as another and is present at a very low concentration, it can be nearly impossible to detect with other techniques.

If you're looking for low-level peptides—which researchers like Dr. Baker call the needle in the haystack—that sensitivity becomes important. In a number of instances, the shape or confirmation of a protein is the crucial clue in clinical research.

A mass spectrometer can only tell you which protein is there, but not the shape. "With ion mobility you can tell if the protein is all tangled up, or it's more extended, or it's malformed in some way," says Dr. Baker. "That's vital information."

With this new instrument from Agilent, you can get a lot of detailed information very quickly.



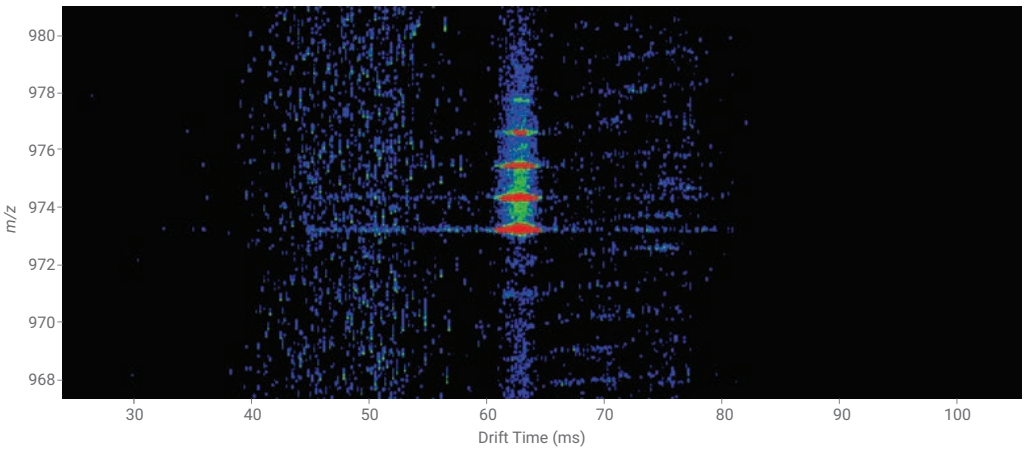
"This system can give you information about everything in your sample. With ion mobility, we're able to detect molecules at really low concentration levels," Dr. Baker says. "Where we used to be looking at ng/mL, we are now looking at high pg/mL."

— **Erin Baker,** Associate Professor
University of North Carolina at Chapel Hill

Precision opens new opportunities

"We have a tool here that gives us some opportunities to address questions that perhaps we've never even imagined before."

That's how Dr. Alfred Yergey, Scientist Emeritus with the National Institutes of Health in Bethesda, Maryland, assesses the Agilent 6560 Ion Mobility Q-TOF LC/MS system.



Positive spectrum of cyclodextrin, a compound at the time of measurement with no known CCS value or standards.

An instrument to believe in: Confidence and ease of use

The 6560 Ion Mobility LC/QTOF is the first commercial instrument that enables researchers to address truly fundamental questions about structure by first principle measurements. This allows for a deeper understanding in function and the workings of complex biological systems with real confidence and ease.

Much of Dr. Yergey's enthusiasm stems from the accuracy of the system. "When you calculate a collision cross section with this device, what you get is a believable number," he says.

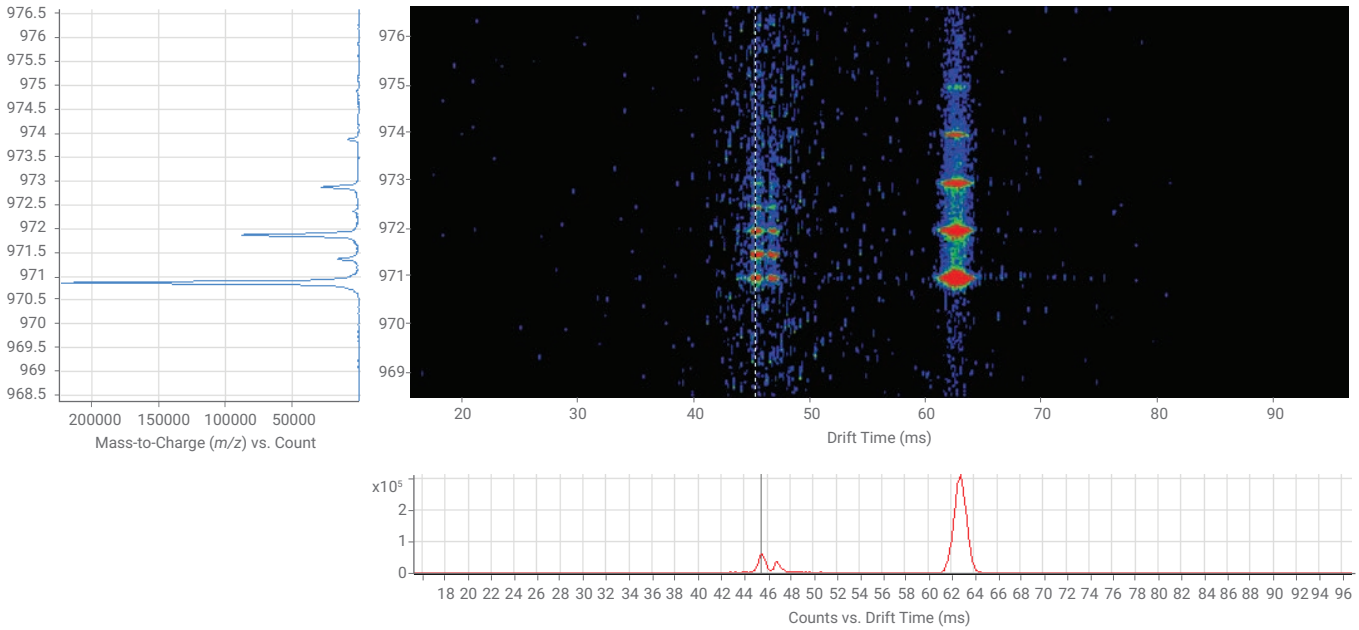
With other commercial systems, results must be calibrated in comparison to previously established values for similar compounds found in scientific literature. That's a big drawback if you're dealing with molecules that don't yet have an established value.

"The results that you get with this tool can be justified on the basis of first principles," Dr. Yergey observes. "The device behaves exactly the way you would expect it to behave in light of the long history of gas phase ion chemistry."

Here's what one analyst had to say about the precision of the 6560 Ion Mobility LC/Q-TOF.

"It's a tool that opens up one's ability to imagine different kinds of experiments. It's basically a way of interrogating gas phase ion chemistry in a fashion that one really couldn't imagine before the existence of this device."

— **Dr. Alfred Yergey,** Scientist Emeritus,
National Institutes of Health
Bethesda, Maryland



Both positive ion and negative ion nitrogen drift data of the same compound (cyclodextrin) in protonated and deprotonated form. CCS calculations in positive and negative mode showed CCS values within 2% accuracy. A) One well resolved drift peak in positive mode. B) Two different peaks (singly charged monomer as well as doubly charged dimer) in negative mode. Lower intensity peak contains two different conformers, possibly indicating that the dimers exist as two conformers.

Seeking the unknown: A pan-omic discovery tool

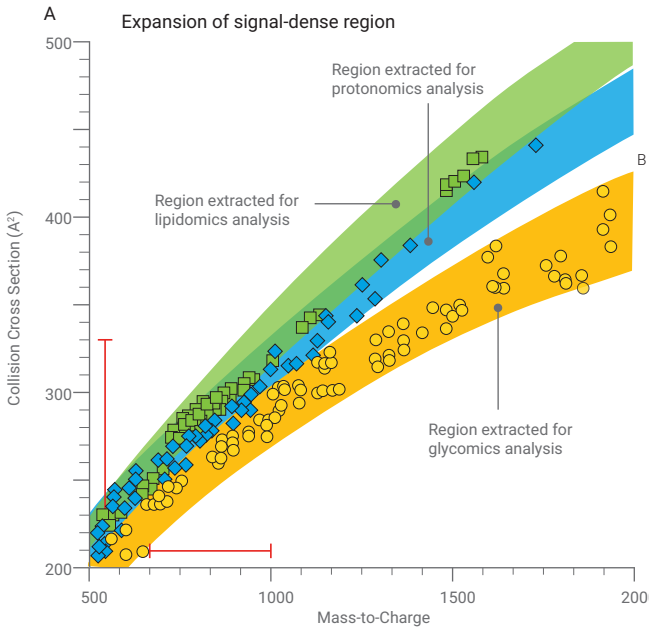
The Agilent 6560 Ion Mobility LC/Q-TOF represents an incredible advance for biologists trying to understand how genes, proteins, and metabolites interact as a whole system.

Just ask Dr. John McLean of Vanderbilt University in Nashville, Tennessee. He heads the Laboratory for Structural Mass Spectrometry, which performs experiments for biologists, immunologists, pathologists, and other scientists.

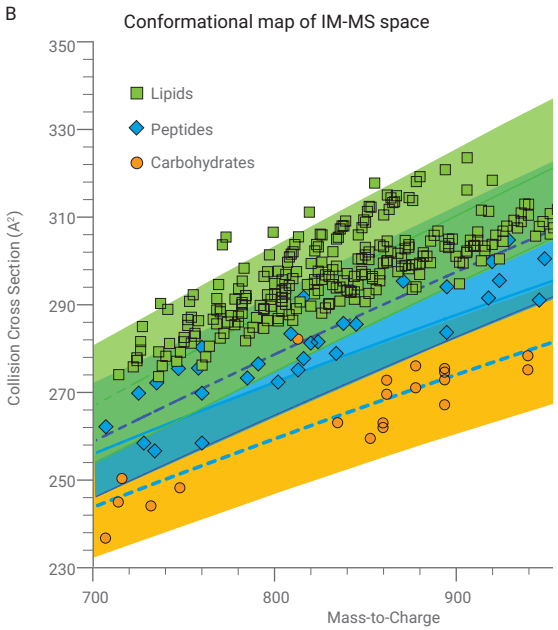
"The real problem with systems biology is you have to do millions of experiments to be able to discern small things, small networks in biology," says Dr. McLean. "In proteomics, you will be waiting for hours to detect the changes in protein expression levels. Metabolomics, on the other hand, provides a rapid reflection of biological responses that can serve as an effective indicator of biological state. If you're trying to understand disease states, for example, you have to be able to look at the molecules that are being expressed together under those conditions."

And, that's where the 6560 Ion Mobility LC/Q-TOF really shines.

Pan-omics mapping of empirical data

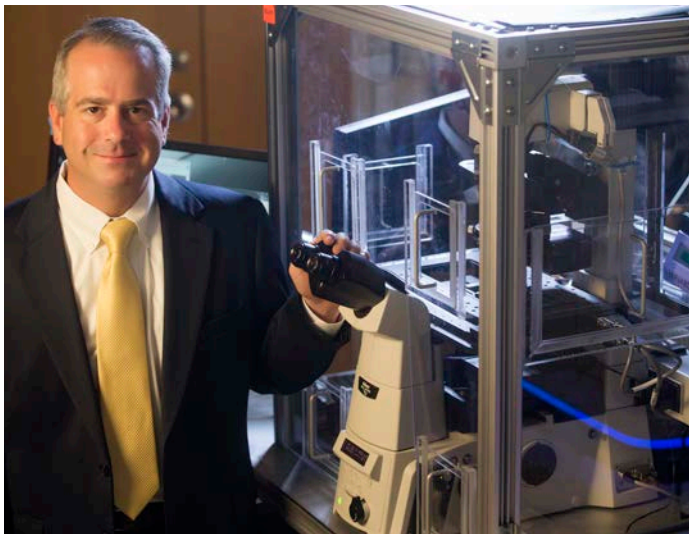


A) A sample containing lipids, peptides, and carbohydrates was infused directly into the instrument, then resolved using 2-D IM Q-TOF analysis by collision cross section and *m/z*. Conformational space maps allow complex mixtures to be separated based on different biomolecular classes with little front-end sample workup.*



B) A zoomed-in region with a high signal density. Distinguishing individual ion signals by the *m/z* measurement alone is challenging. Yet, the combined structural separation of IM Q-TOF allows the data to be delineated into chemical class-specific regions.*

** May, J.C., Goodwin, C.R., Lareau, N.M., Leaptrot, K.L., Morris, C.B., Kurulugama, R.T., Mordehai, A., Klein, C., Barry, W., Darland, E., Overney, G., Imatani, K., Stafford, G.C., Fjeldsted, J.C., McLean, J.A. Anal Chem 2014 (Feb 18, 2014; 2107-2116). Conformational Ordering of Biomolecules in the Gas-Phase: Nitrogen Collision Cross-Sections Measured on a Prototype High Resolution Drift Tube Ion Mobility-Mass Spectrometer.*



"We're breaking the paradigm of proteomics studies or genomics studies—any of the individual omics—and instead taking what we like to think is a truly untargeted, unbiased survey of the molecular inventory," he says, "using ion mobility coupled with mass spectrometry."

— **John A. McLean,** Stevenson Professor of Chemistry Vanderbilt University, Nashville

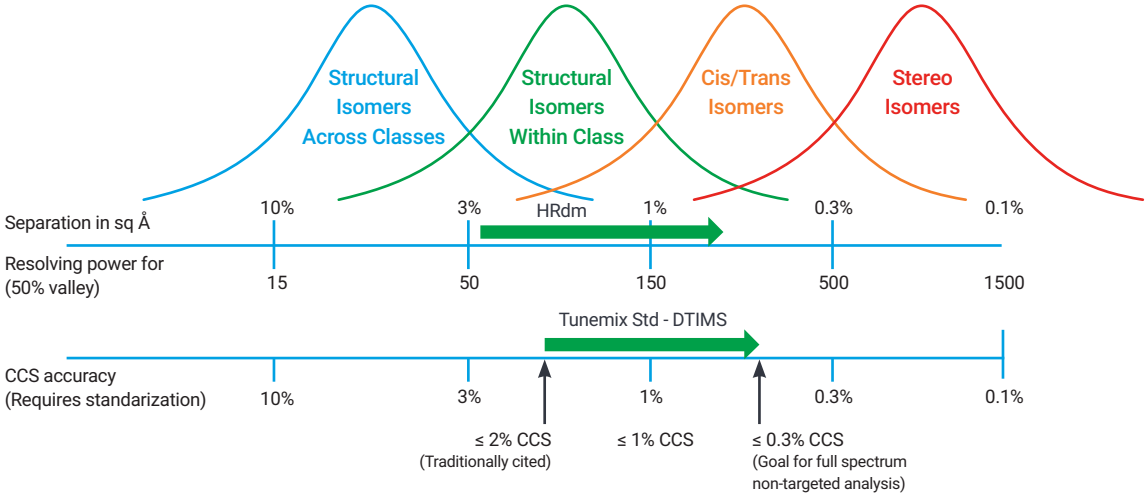
Dr. McLean and his team at Vanderbilt have a deep appreciation of how these technologies work together. They have been building their own systems for years, where resolving power is a key metric for the separation of different molecular classes.

"In our experience, a resolving power of greater than 20 is necessary to begin to resolve out chemical classes in conformation space, but with the Agilent platform, in some cases we've achieved 80," he says. "This allows us to resolve out the fine structure in molecular class distributions for even higher confidence assignments within a molecular class—for example, distinguishing molecular subclasses such as sphingolipids from glycerophospholipids."

Structural isomers across classes can be separated with traditional drift tube resolving powers of up to 50–60. In order to separate structural isomers within a class, higher IM resolving powers of 150–200 are needed, which can be obtained with high resolution demultiplexing. Separating cis/trans isomers and stereoisomers require even higher IM resolving powers. CCS accuracy, which has been demonstrated on drift tube ion mobility systems, becomes even more important when resolving closely eluting isomers as often these species have CCS values that fall within 1–3% of each other. For full spectrum, untargeted workflows <0.3% CCS accuracy is desired. The enhanced sensitivity and resolution helps uncover more compounds in complex mixtures.

Dr. McLean notes that other researchers are often surprised by the pan-omic nature of the technology. "This is going to make a big splash in biology," he says.

Examining the CCS scale



Different types of isomers require increasing IM resolving power. The same applies to identification, where CCS accuracy is of critical importance.

Characterizing biotherapeutics

Approaching the impossible—expedited, reliable biopharmaceutical development

The drug discovery process has been continuously refined over the years. However, the basic approach remains largely unchanged despite many technological advancements. So, some are asking, "Is it time to fundamentally modify the way we approach new pharmaceutical development?"

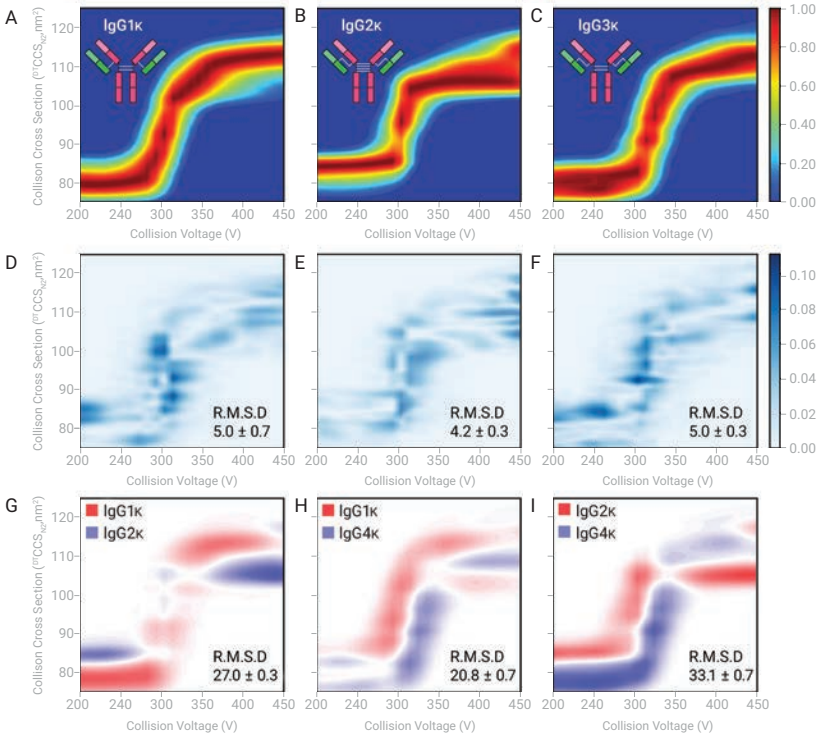
A team at the University of Michigan, led by Brandon Ruotolo, professor of chemistry, believes it is, and they think they have the right tool for the job: the new Agilent 6560C Ion Mobility LC/Q-TOF. Collision-induced unfolding, or CIU, is a rapid way to evaluate the stability of a protein therapeutic or target. Typically, protein-based therapeutics should be as stable as possible to retain efficacy and safety. A major benefit of CIU is that it allows access to stability information without needing to generate large amounts of purified protein. With CIU, stability can be assessed within minutes, and it can be completed orders of magnitude faster than with conventional technology. Thousands of protein-based drug candidates could easily be screened, providing information-rich data, which could improve and expedite the pharmaceutical pipeline.

CIU experiments continued, and Ruotolo described early data as protein structural "fingerprinting." He said, "Early on, most data could not be tied to specific structural features. We just knew that the protein increased in size due to internal temperature changes throughout the experiment." He continued, "However, now we can discern some structural information."

The 6560C has proven itself to be a powerful platform to look at different proteins and protein complexes across a wide range of masses and structural states. The CIU assay is very sensitive to differentiate biosimilars and proteins based on disulfide bonding, unfolding, and changes in covalent bonding. It can differentiate where disulfide bonds are located within different IgG subclasses with high levels of confidence. This will be helpful for pharmaceutical development, as IgGs are used as scaffolding to build biotherapeutic proteins.

"We record patterns in structural changes as a function of energy applied. Then, compare with another sample that has been through cellular stress or bound to a ligand, and we see how data changes between the two" he explained. "CIU data analysis often involves a lot of references between samples. Data for standard proteins must be highly reproducible to draw conclusions from unknowns with confidence, and the 6560C has excelled here."

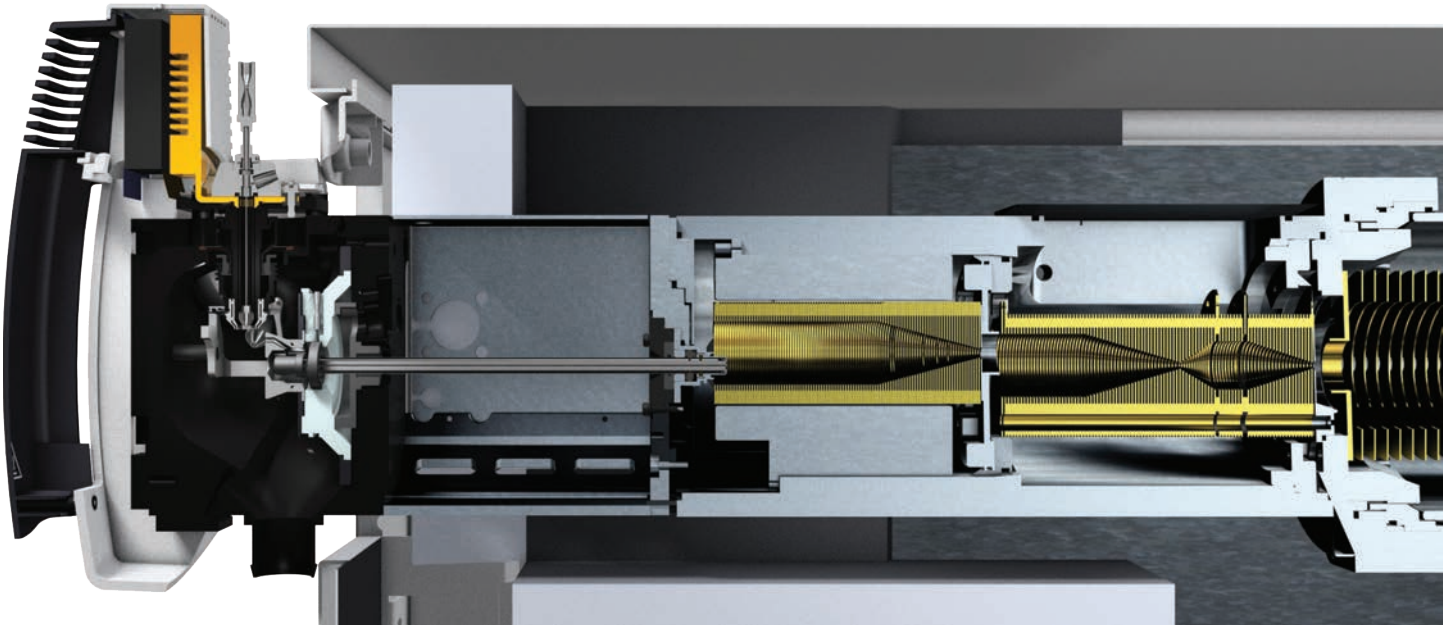
— **Brandon Ruotolo,** Professor of Chemistry
University of Michigan



J CIU Fingerprint R.M.S.D. (%)

	IgG1k	IgG2k	IgG4k
IgG1k	5.0±0.7	27.0 ± 0.3	20.8 ± 0.7
IgG2k		4.2 ± 0.3	33.1 ± 0.5
IgG4k			5.0 ± 0.3

Results from CIU experiments. Figures A–C: CIU curve for IgG1 kappa, IgG2 kappa and IgG3 kappa. Figures D–F: CIU curve average Root Square Mean Deviation (RMSD) value for multiple replicate runs of IgG1–3 kappa. Figures G–I: Comparison CIU curve average RMSD value for IgG1 kappa versus IgG2–4 kappa, with the image showing the comparison plots for the summed IgG's kappa.



Built on breakthough technology

Why all the excitement about a separation technique that has been around for more than 100 years? Because only now is it realizing its true potential, thanks to a number of recent innovations.

The emergence of modern ion funnel technology coupled with uniform field drift tubes was pioneered by Dr. Richard Smith, Pacific Northwest National Laboratory (PNNL). It has enabled more than 50-fold sensitivity gains for ion mobility coupled with high-resolution mass spectrometry.

Now, the 6560 Ion Mobility LC/Q-TOF takes this technology further than ever with an exclusive ion funnel design. Its dual funnel assembly includes a front funnel for sample enrichment, a trapping ion funnel, a drift tube, and a focusing rear funnel. All are designed to improve ion transmission from the source to the Q-TOF high-resolution mass analyzer.

Uniform field ion mobility designs have existed for years. However, they suffered from high ion losses (> 99.9%) without the use of electrodynamic funnels.

The Agilent drift funnel design preserves ions along each segment of the optics pathway. How? Through careful ion focusing in every segment of the electrodynamic ion funnel. This design results in only a two-fold loss of ion signal, compared to standalone high-resolution Q-TOF LC/MS instruments.

What's more, Agilent iFunnel technology provides robustness unmatched by other dual-funnel designs by combining true orthogonal electrospray orientation with Agilent JetStream ionization. This design minimizes the transmission of uncharged species and ion clusters, which reduces background noise.

"Ion funnel technology could possibly be the most significant MS development since the introduction of the API. It delivers a fundamental sensitivity and detection-limit breakthrough—resulting in performance far exceeding the capabilities of conventional mass spectrometers."

– **Dr. Richard Smith,** Inventor of the ion funnel

Take advantage of all three dimensions of separation with one system

The 6560 Ion Mobility LC/Q-TOF combines the power of the 1200 Infinity Series HPLC, ion mobility, and a high-resolution, accurate-mass Q-TOF LC/MS system. So, you can easily expand your scientific research capabilities.

VacShield

– Allows vent-free capillary removal.

CIU

– Collision-induced unfolding for structural characterization.

Multiplexing

– Higher dynamic range and sensitivity, dramatically increased IM resolution with post processing algorithms.

Resolve structural isomers

– Effortlessly 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.

Increase peak capacity

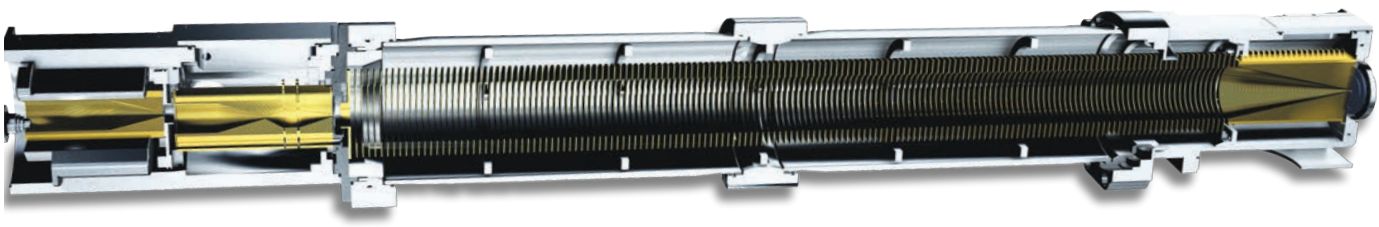
– Resolve individual components in complex mixtures with the combined power of UHPLC, ion mobility, and mass spectrometry.
– Obtain optimal ion mobility separation with double-grid trapping technology.
– Multiplexing to increase sensitivity, dynamic range, and resolution via high-resolution demultiplexing.

Find and confirm minor components

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

Preserve protein conformations

– Easily study gas phase peptide and protein structures.
– Maintain molecular conformations by minimizing ion heating effects.



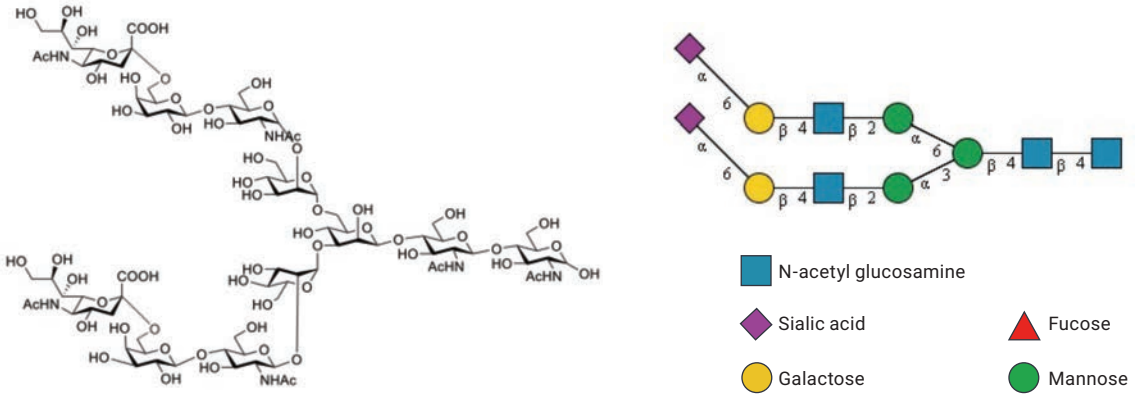
Reach new heights in peak capacity

Combining the orthogonal separation techniques of liquid chromatography, mass measurement, and ion mobility tremendously enhances overall peak capacity. So, you can more effectively characterize diverse molecules.

For in-depth analysis of complex samples, complete separation of multiple compounds may not be possible with liquid chromatography alone. Even subsequent high-resolution mass analysis may be insufficient to separate and identify isobaric compounds. The 6560 Ion Mobility LC/Q-TOF adds gas phase ion mobility separation—dramatically increasing the peak capacity of your analysis. That means you can resolve and detect a greater number of compounds and components than ever before.

Exact structure determination of complex glycans

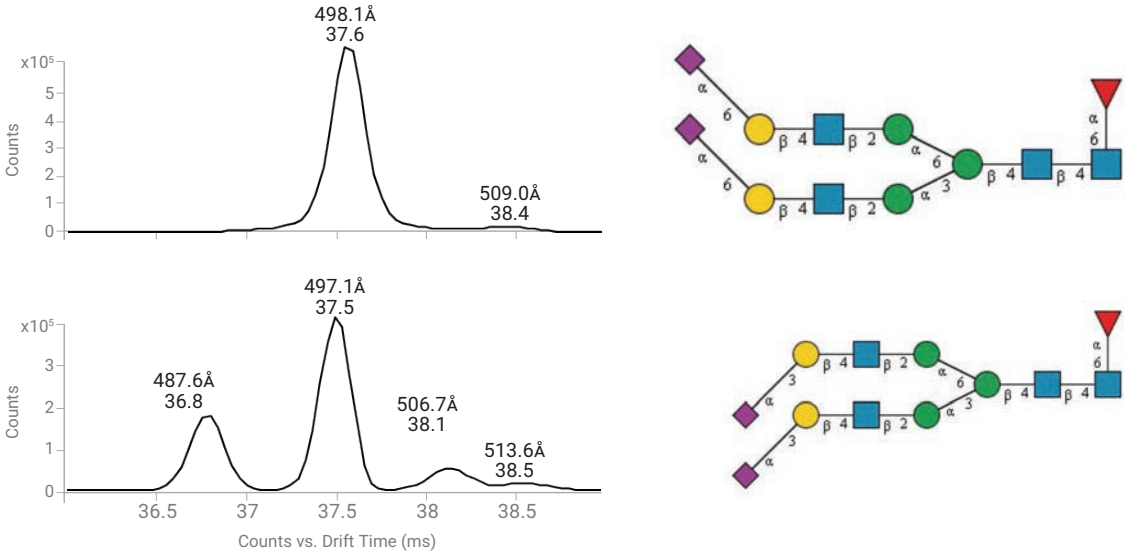
MS analysis of glycans is gaining considerable interest. These biomolecules have been implicated in biological and disease processes, can serve as biomarkers, are critical components of biologicals, and impact infant nutrition. Due to their structural complexity, identifying exact glycan structures by TOF-MS is challenging. Combining TOF-MS and DTIMS with multiplexing and HR-demultiplexing offers a faster, easier alternative.



The monosaccharide building blocks of *N*-glycans can be linked in different sequences and linkages (left), yielding a large number of possible glycan structures, including many isomers. The representation of the molecular structure is commonly simplified by the use of symbols for the building blocks and annotation of linkages (right).[1]

High-resolution glycan conformer distribution fingerprinting

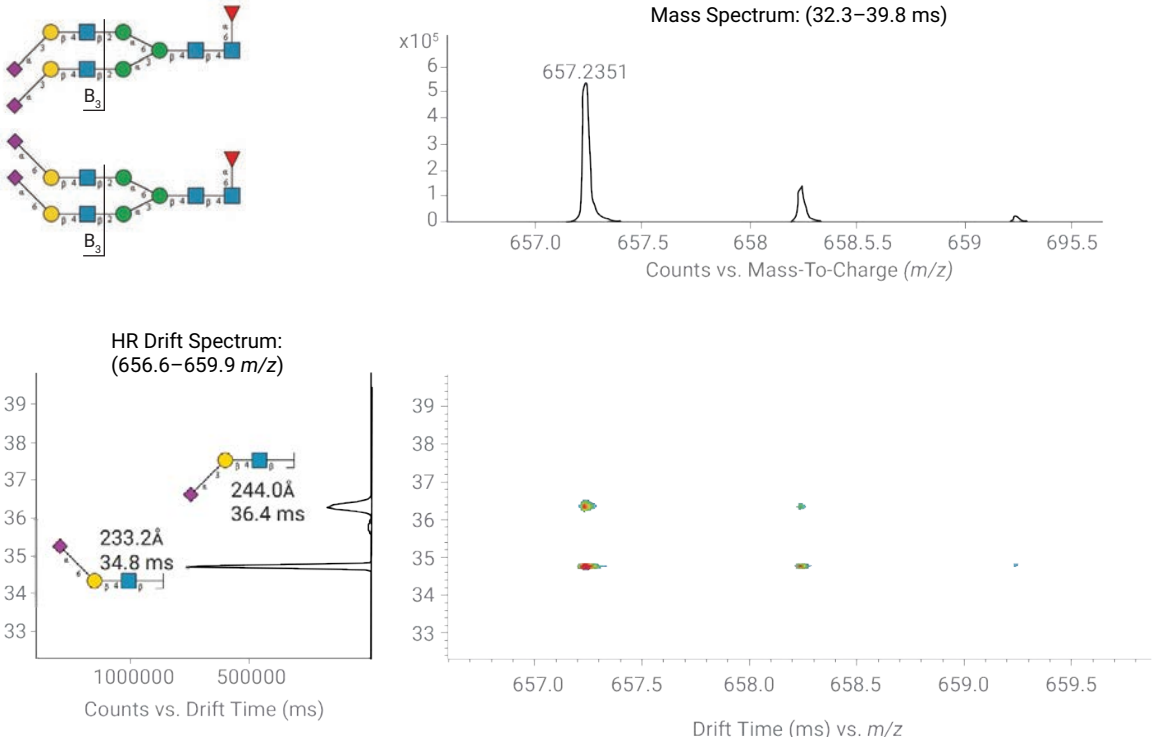
The flexibility between carbohydrate building blocks allows glycans to adopt several stable gas phase conformations, which can be resolved with DTIMS. These highly repeatable conformer distributions show unique fingerprints for each glycan.[2] A database with these fingerprints, together with accurate mass measurements and CCS values, can be used to identify the glycan structures without conducting multistage MS experiments.



Differentiation of isomeric *N*-glycans by their HR conformer distribution fingerprint and CCS values. The N-glycans were derivatized with 2-aminobenzoic acid at the reducing end and measured in negative ion mode as $[M-^{2}H]2-$ ions.

In-source activation and HR-DTIMS

DTIMS of glycan fragments, which are formed after in-source activation, offers the possibility to obtain detailed structural information, such as exact fucosyl positions[3] or sialic acid linkage type. By measuring CCS values of fragment ions and comparing these with reference data, isomeric fragment ions can be identified without the need for MS^3 experiments.



Separation of B_3 fragment ions (at 34.8 and 36.4 ms in the HR drift spectrum) obtained after in-source activation of two mixed isomeric *N*-glycans, differing only in sialic acid linkage (α2,3 or α2,6, see structures in inset figure). The *N*-glycans were derivatized with 4-amino-N-2-(diethylamino)ethyl-benzamide at the reducing end, in-source activated and measured in positive ion mode. The two B_3 fragment ions have identical *m/z* values ($[M+H]^+$ at *m/z* 657.24), but they can be separated by their different mobility in the drift tube, enabling sialic acid linkage differentiation by CCS values.

1. Damerell, D.; Ceroni, A.; Maass, K.; Ranzinger, R.; Dell, A.; Haslam, S. M., The GlycanBuilder and GlycoWorkbench glycoinformatics tools: updates and new developments. *Biol. Chem.* **2012**, 393 (11), 1357-62.

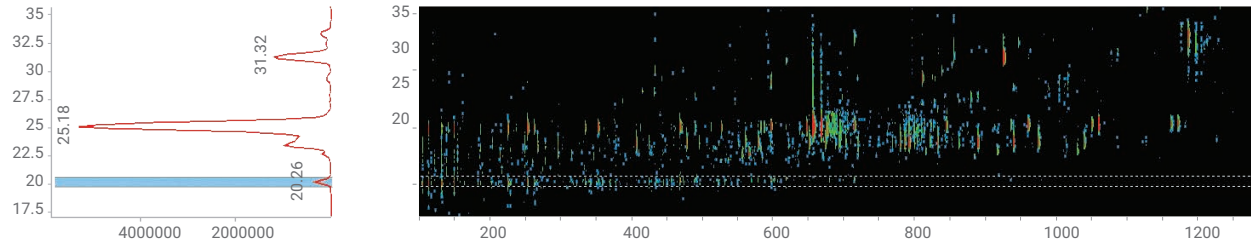
2. Sastre Torano, J.; Aizpurua-Olaizola, O.; Wei, N.; Li, T.; Unione, L.; Jimenez-Oses, G.; Corzana, F.; Somovilla, V. J.; Falcon-Perez, J. M.; Boons, G. J., Identification of Isomeric N-Glycans by Conformer Distribution Fingerprinting using Ion Mobility Mass Spectrometry. *Chem. Eur. J.* **2021**, 27 (6), 2149-2154.

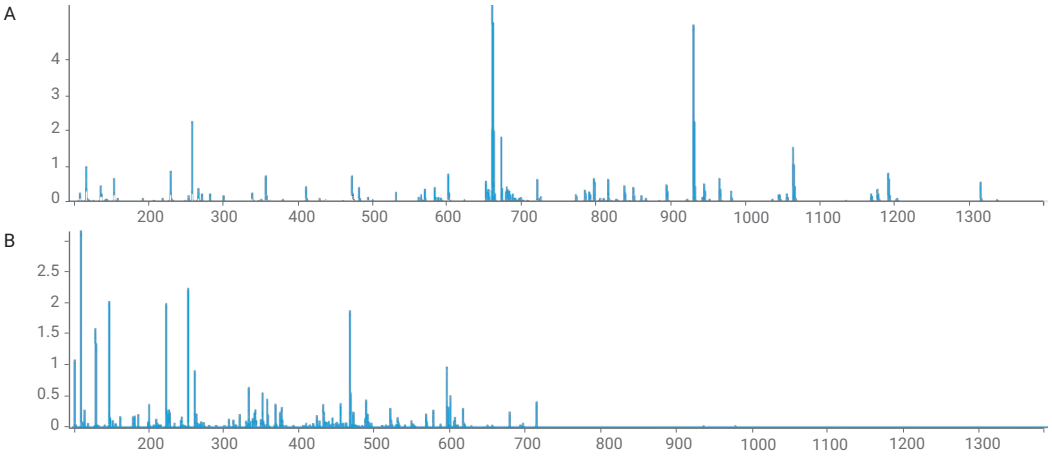
3. Sastre Torano, J.; Gagarinov, I. A.; Vos, G. M.; Broszeit, F.; Srivastava, A. D.; Palmer, M.; Langridge, J. I.; Aizpurua-Olaizola, O.; Somovilla, V. J.; Boons, G. J., Ion-mobility spectrometry can assign exact fucosyl positions in glycans and prevent misinterpretation of mass-spectrometry data after gas-phase rearrangement. *Angew. Chem., Int. Ed. Engl.* **2019**, 58 (49), 17616-17620.

All ions, all the time: Quickly detect low-level compounds

All Ions fragmentation is available on all Agilent high-resolution LC/Q-TOF instruments. Combined with Agilent Personal Compound Databases and Libraries (PCDL), this technique gives you an unparalleled ability to confidently screen for compounds in complex mixtures.

Traditional data-dependent MS/MS experiments often miss low-abundance peaks, but with All Ions fragmentation experiments, all the ions from the source are directed to the collision cell for fragmentation. Data analysis software then uses the MS/MS spectra available in the PCDL to confidently extract compounds present in the sample. Agilent All Ions fragmentation is even more powerful when combined with ion mobility. That's because ion drift time provides an extra dimension of separation—further reducing sample complexity and enhancing the detection of low-level compounds. The benefits: less ambiguity in identifying compounds, and better detection limits for trace-level compounds.





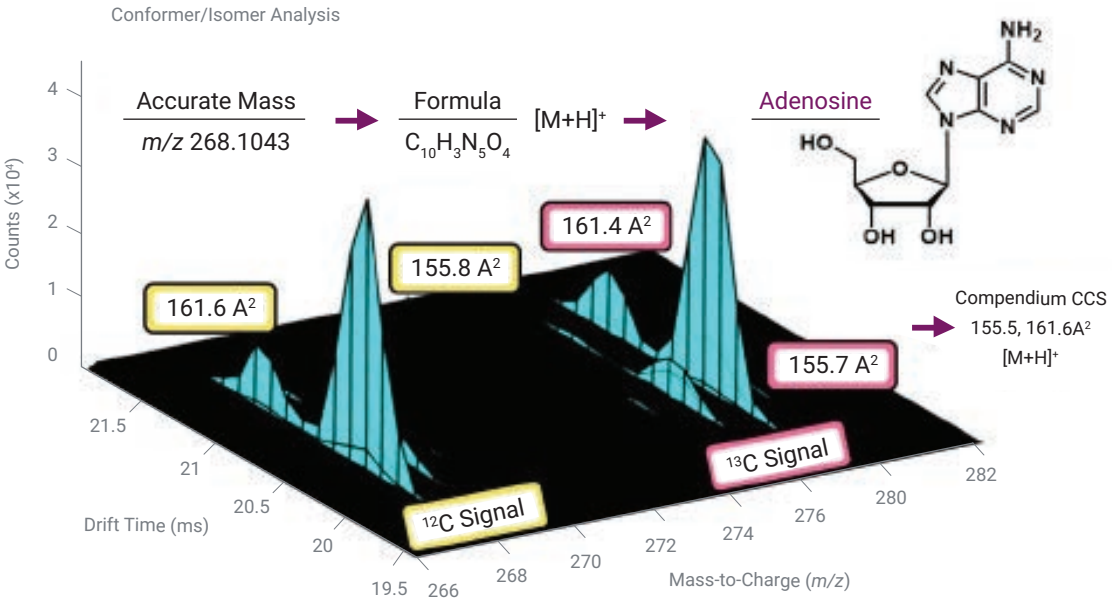
Agilent All Ions fragmentation was used to identify low-level peptides in a protein digest. The starred peak in the LC separation had six to seven peptide components, shown separated in the drift time heat map (inset). These components could not be detected and identified using just LC and MS.

A: Sum of fragment ion spectrum at 30 V across the starred peak. B: Drift separated fragment ion spectrum of the triply charged HLVDEPQNLIK peptide at 20.26 ms.

Untargeted metabolomics: Increased molecular annotation by CCS values

One of the greatest analytical challenges in untargeted metabolomics research is how scientists can increase confidence in molecular annotation of unknowns. Current methods utilize molecular descriptors, including accurate mass, isotopic pattern, and MS/MS spectra, to tentatively assign a molecular identity at a given level of analytical certainty. Utilizing ion mobility spectrometry (IMS) and collision cross section (CCS) values documented in the literature provides an additional form of molecular annotation that facilitates structural characterization of unknowns. The CCS values generated by the uniform field drift tube of the Agilent 6560 platform allow researchers to translate these molecular descriptors across laboratories with a high degree of precision. Here, we were able to annotate an unknown metabolite as adenosine based solely on accurate mass and CCS values calculated in an untargeted profiling experiment. The two conformers can likely be attributed to multiple sites of protonation on the purine nucleobase. The calculated CCS values at Erin Baker's lab at North Carolina State University showed agreement (less than 0.2% difference) with reference values published by the John McLean Lab at Vanderbilt University.

In addition, any heavy isotope-labeled metabolites (values in pink borders) have near identical CCS and conformer distributions compared to endogenous metabolites (values in yellow borders), which provides additional certainty in molecular assignment.



Data from Erin Baker, North Carolina State University.

Separate molecules by size and shape

Collision cross section (CCS) values—a measure of compound size and shape—are useful for characterizing polymers, proteins, peptides, lipids, biopharmaceuticals, and more. For these studies, CCS values often help distinguish between various forms of isomers or structurally similar molecules and complexes.

CCS values are derived from ion mobility measurements and can be directly calculated in the uniform field drift tube, thanks to the unique design used by Agilent.

With the Agilent 6560 Ion Mobility LC/Q-TOF, you can routinely achieve CCS measurements within <2% accuracy. Its uniform field drift tube provides excellent control of experimental parameters (pressure, temperature, and electric field), which the system maintains during mobility experiments.

Creating the CCS reference standard

The CCS reference standard using Agilent Tune Mix

Type Mix	CCS	% RSD	Tune Mix	CCS	%RSD
118	121.30 ± 0.20	0.17%	112	108.23 ± 0.20	0.19%
322	153.73 ± 0.23	0.15%	301	140.04 ± 0.29	0.21%
622	202.96 ± 0.27	0.14%	601	180.77 ± 0.21	0.12%
922	243.64 ± 0.30	0.12%	1033	255.34 ± 0.32	0.13%
1221	282.20 ± 0.47	0.17%	1333	284.76 ± 0.31	0.11%
1521	316.96 ± 0.60	0.19%	1633	319.03 ± 0.70	0.22%
1821	351.25 ± 0.62	0.18%	1933	352.55 ± 0.27	0.08%
2121	383.03 ± 0.64	0.17%	2233	380.74 ± 0.31	0.08%
2421	412.96 ± 0.58	0.14%	2533	412.99 ± 0.31	0.07%
2721	441.21 ± 0.59	0.13%	2833	432.62 ± 0.35	0.08%

The creation of the CCS reference standard based on Agilent Tune Mix was accomplished via an interlab study using a reference system and three other systems in additional laboratories. Four tuning modes on three different days in both positive and negative mode were used to acquire data in all laboratories. The corresponding RSD for CCS values obtained under repeatability conditions of measurement on the reference system was found to be less than 0.22% for all tune mix ions. This thorough evaluation was undertaken with a view to use these reference CCS values as calibrants for universal single-field measurements and are presented as reference values to support broad CCS standardization.

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Agilent MassHunter software, the IM-MS Browser and Mass Profiler, enables you to interrogate mobility and mass domain data, and easily determine collision cross-section values with precision.

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