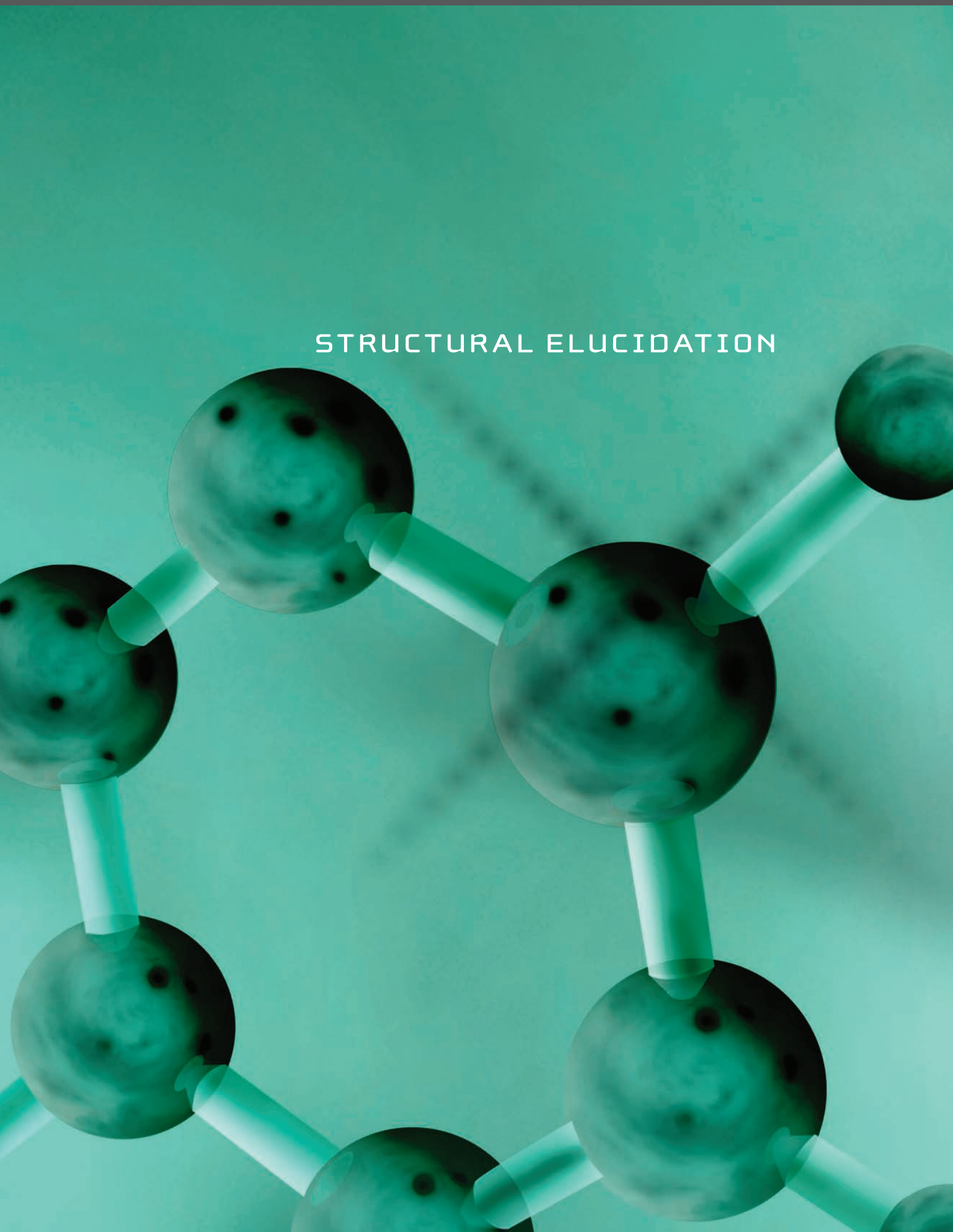
STRUCTURAL ELUCIDATION



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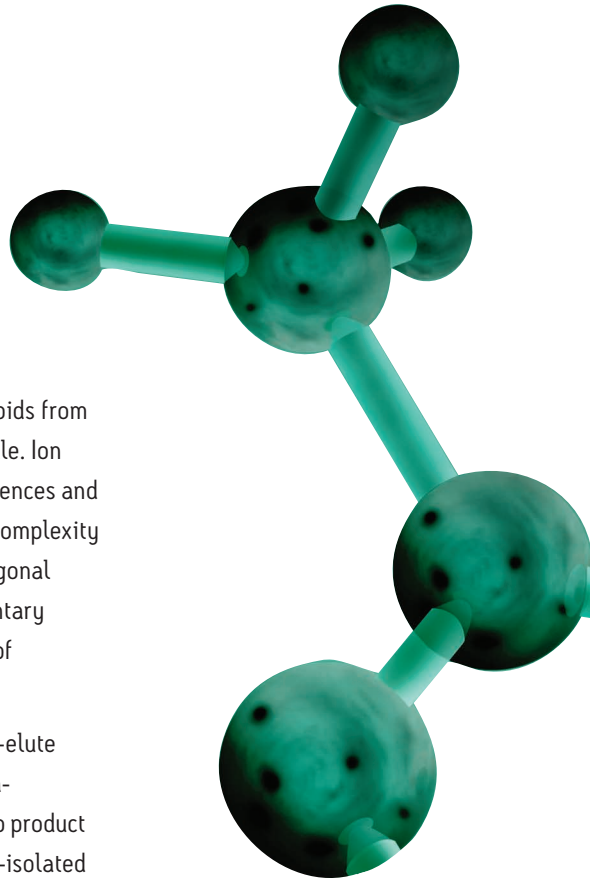
STRUCTURAL ELUCIDATION



There are significant challenges when detecting and characterizing metabolites and lipids from biological matrices due to the large number of potential interferences on the mass scale. Ion Mobility-MS can provide an opportunity to better detect ions in the absence of interferences and subsequently deliver cleaner, more reliable spectral tandem MS data. Because of the complexity of the biological metabolome and lipidome, the addition of mobility times as an orthogonal measurement to mass, retention times and MS-MS fragmentation provides complementary information regarding the analyte, which adds further specificity to the identification of metabolites and lipids.

Thousands of metabolites and lipids exist in biological samples, many of which can co-elute at the same retention time or appear in similar regions of the *m/z* scale. Applying data-independent (DIA) or data dependant acquisition (DDA) to these samples would lead to product ion spectra, containing a mixture of fragments deriving from multiple co-eluting or co-isolated precursors, which may complicate the interpretation of spectra. To aid the identification of complex mixtures of analytes, DIA fragmentation of precursor ions can be performed following the ion mobility separation. The incorporation of ion mobility separation of co-eluting precursor ions before fragmentation produces cleaner, product-ion spectra, which improves identification and reduces false-positive assignments. In the Waters' SYNAPT® Mass Spectrometer, such a mode of operation is referred to as HDMS^E (High Definition® MS^E).

The elucidation of the chemical structure of complex lipids and metabolites often requires multiple fragmentation techniques and/or methods. Time-aligned parallel (TAP) fragmentation is a particular mode of acquisition typical of the Waters' SYNAPT, which contains an ion-mobility separation cell between two collision cells (C1, C2). In this mode of operation, the precursor ion of interest is selected with the quadrupole mass filter and subjected to fragmentation in the first collision cell, C1. Next, the packet of fragment ions produced are subjected to ion mobility separation followed by secondary fragmentation in the second collision cell, C2 (pseudo-MS3). This process results in a driftogram, showing the different drift regions for each of the first-generation fragment ions aligned with the second generation fragment ions. This mode of acquisition has been used for fine, structural characterization of complex structures in a single analytical step.

- Unique gas phase separation of isobaric species by ion mobility (HDMS®)
- CID and ETD fragmentation capabilities
- Confirmation of metabolite/lipid identity by CID fragmentation in conjunction with ion mobility separation (HDMS^E, TAP)

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Traditional Herbal Medicine Structural Elucidation using SYNAPT HDMS with Time-Aligned Parallel (TAP) Fragmentation

Kate Yu, Jose Castro-Perez, and John Shockcor,
Waters Corporation, Milford, MA, USA

INTRODUCTION

The challenges in analyzing Traditional Herbal Medicine (THM) or Traditional Chinese Medicine (TCM) samples arise from the complexity of the matrix as well as variability from sample to sample. We have reported a generic intelligent work flow[1-2] that allows fast sample analysis while obtaining maximum information by effectively utilizing UPLC®/HDMS™ analysis in time-of-flight (TOF) mode with a variety of advanced profiling software tools (MetaboLynx™ and MarkerLynx™ Application Managers).

In this application note, we demonstrate a strategy that provides an effective means of confirming known components and elucidating structures of unknown components.

Ion mobility mass spectrometry (IMS) allows the separation of ionic species as they drift through a gas phase under the influence of an electric field. The rate of an ion's drift depends on the mass of the ion, the charge state of the ion, as well as the average collisional cross-section of the ion. With IMS, it is possible to separate ions with the same nominal mass if they have different charge states or different collisional cross-sections.

The strategy reported here comprises of an initial sample screening using the Waters® ACQUITY UPLC® System with the SYNAPT™ HDMS System in full-scan IMS mode. Once a compound or class of compounds are identified, a targeted fraction collection is performed in analytical scale and the fraction collected can be infused into the mass spectrometer at a nano-scale flow rate so that structural elucidation can be performed for the compound of interest. The nano-scale flow rate allows infusion to be carried out over an extended time period, making it possible to conduct multiple MS/MS experiments, including time-aligned parallel (TAP) fragmentation experiments.

TAP, which is CID-IMS-CID, allows users to take the advantage of the Triwave(tm) configuration on the SYNAPT HDMS (Figure 1). This configuration allows pre-IMS T-Wave™ and post-IMS T-Wave to operate as two separate collision cells.

The fragment ions produced in the Trap T-Wave (pre-IMS) can be separated based on their charge states and sizes as they move through the IMS cell. Ions separated by drift time can be fragmented further in the Transfer T-Wave (post-IMS). As a result, the fragment ions generated in the Transfer T-Wave are drift time-aligned with their respective precursor ions, resulting in Time Aligned Parallel (TAP) fragmentation patterns. When these fragmentation results are combined with tools such as MassFragment™ Software, structural elucidation for small molecule is simplified.

Here, a Chinese Ginseng extract was analyzed by utilizing the ACQUITY UPLC/SYNAPT HDMS systems operating in IMS mode. Fraction collection was performed using a TriVersa NanoMate (Advion). The fractions collected were directly infused into the SYNAPT HDMS for analysis, providing more in-depth information about the compounds of interest. The example analyte discussed in this application note is the Ginsenoside Rb1.

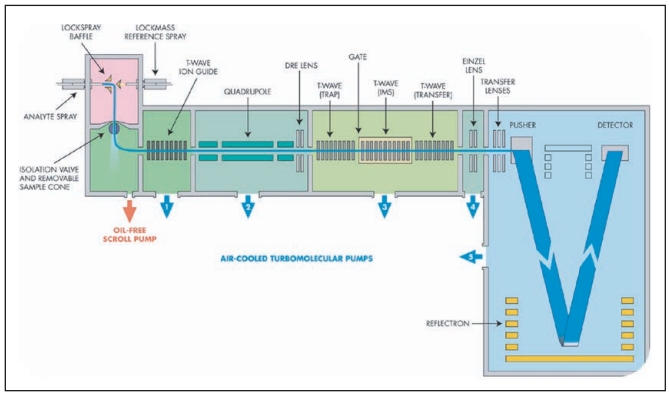

Figure 1. Schematic of the SYNAPT HDMS System's Triwave design.

EXPERIMENTAL

A Chinese Ginseng extract was used for this work. The sample was filtered prior to injection. A chip-based nano-electrospray device (TriVersa NanoMate, Advion) was used as the mass spectrometer interface.

LC conditions

LC system:	Waters ACQUITY UPLC System		
Column:	ACQUITY UPLC HSS T3 Column 2.1 x 100 mm, 1.7 µm, 65 °C		
Flow rate:	600 µL/min		
Mobile phase A:	Water + 0.1% Formic Acid		
Mobile phase B:	MeOH		
Gradient:	Time	Composition	Curve
	0 min.	95%A	
	10 min.	30%A	Curve 6
	17 min.	0%A	Curve 6
	20 min.	95%A	Curve 1

Fraction collection (FC) conditions

FC system:	Advion TriVersa NanoMate
Flow split:	300 nL/min flow to the MS and the rest of the flow to the waste or the collection plate when triggered
Collection plate:	96-well plate
Collection time:	7 sec per well
Trigger:	Time-based

MS conditions

MS system:	Waters SYNAPT HDMS System
Ionization mode:	ESI Negative
Capillary voltage:	3000 V
Cone voltage:	35 V
Desolvation temp:	450 °C
Desolvation gas:	800 L/Hr
Source temp:	120 °C
Acquisition range:	50 to 1500 *m/z*
Collision gas:	Argon
IMS carrier gas:	Helium
He gas flow:	80 L/min

RESULTS

For THM studies, it is typically desirable to have the compound of interest physically separated from the raw extract so that it can be analyzed in detail and to enhance structural elucidation. Even though preparative-scale chromatography is a common practice for fraction collection in the THM field, it is often desirable to determine a component's structure prior to rigorous isolation of a pure compound in preparative scale.

In this work, we have connected the ACQUITY UPLC/SYNAPT HDMS systems with a TriVersa NanoMate[3-4] such that fraction collection can be performed in the analytical scale. For this sample analysis, we have set the NanoMate for the collection of the major peak at *m/z* 1107, which corresponds to the Ginsenoside Rb1.

The NanoMate utilizes a chip-based nano-electrospray as the LC/MS interface.[3] This allows samples to be analyzed in the nL/min flow range. As a result, fractions collected can be analyzed by direct infusion. The low flow rate allows small volume of sample to be infused for a considerable amount of time (about 30 to 40 minutes) such that compounds at low concentration levels can be analyzed utilizing various full-scan MS, and MS/MS acquisition modes including TAP (Figure 2).

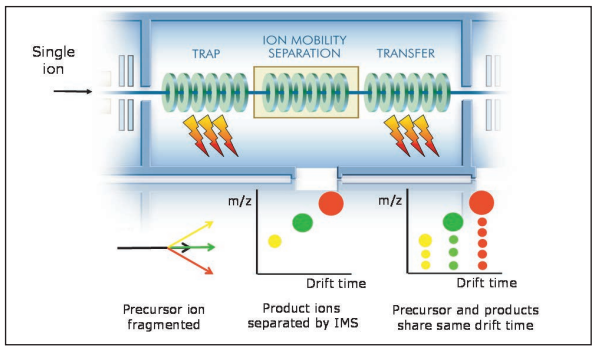

Figure 2. TAP fragmentation for m/z 1077 by direct infusion of fraction collected for the 10.02 minute peak.

The chemical structure and the possible major fragment ions for the Ginsenoside Rb1 are shown in Figure 3. The fragmentation is described left to right. As Ginsenoside Rb1 losses the sugar moiety in sequence, it generates fragment ions corresponding to *m/z* 945, *m/z* 783, and *m/z* 621. The *m/z* 459 is from the core ring structure of Rb1.

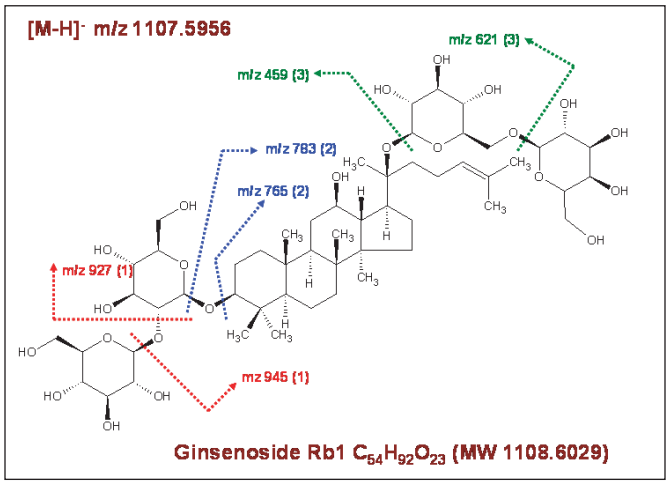

Figure 3. Chemical structure for Ginsenoside Rb1.

To demonstrate the TAP behavior, Figure 4 shows a DriftScope™ Plot comparison from two separate experiments. In Figure 4a, fragmentation was conducted in the T-Wave Trap region only. The fragment ions with a different numbers of sugar moieties migrated through the drift tube at different rates.

Drift time 1 in Figure 4a shows mainly the deprotonated molecule and the major fragment ion at *m/z* 945, which is the loss of one sugar moiety. Major ions in drift time 2 are *m/z* 945 and *m/z* 783 (loss of two sugar moieties). And drift time 3 mainly contains fragment ions generated from the sugars and from the fragmentation of core structures rings.

Figure 4b shows the TAP fragmentation data. At each drift time region, the precursor ions or first-generation ions were further fragmented, producing second-generation fragment ions. These ions are drift time aligned with the first-generation product ions and obtained in parallel. This produces a fragmentation tree that allows the user to account for the source of the second-generation fragments within the proposed structure. The true advantage of this experiment is that the entire second-generation fragment ions can be generated on the fly, i.e., in parallel with the generation of the first-generation product ions.

Typically a single ion-mobility experiment is carried out every 10 milliseconds. For nanoflow-scale infusion, it is possible to average many spectra across the infusion experiment to obtain a good signal-to-noise. Moreover, this approach allows the user to conduct multiple stages of fragmentation for compounds of interest that may exist in extremely low levels.

Figure 5 shows a combined TAP spectrum of the three regions that correlates to Figure 4b. Figure 6 shows the individual MS spectrum for each region (1, 2, and 3) with a few of the proposed structures shown therein. This provides valuable information for the study of the fragmentation mechanisms. For example, it should be noted that the fragment ion at *m/z* 323, which consists two sugars, is observed in drift times 1 and 2, but not 3. This indicates that the precursor ions for region 3 do not have the di-sugar side chain.

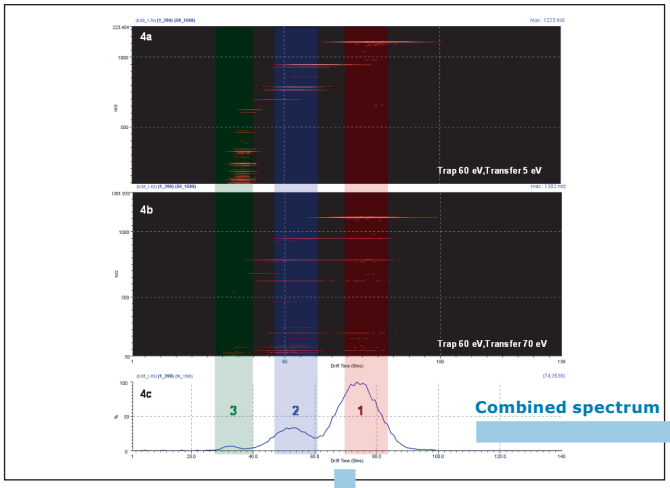

Figure 4. Fragmentation results obtained for Ginsenoside Rb1 using different fragmentation strategies.

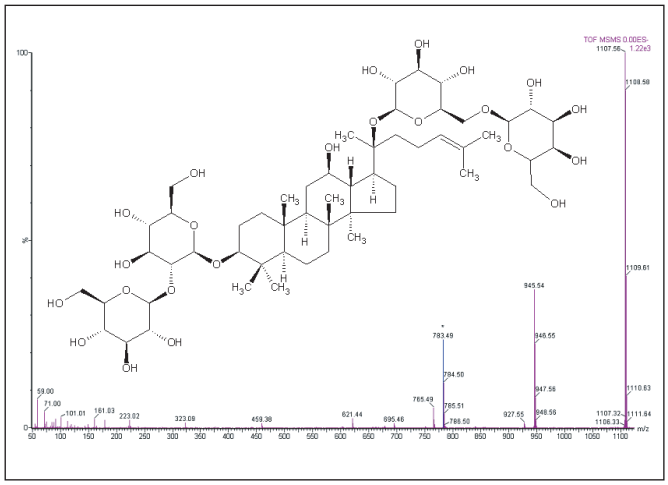

Figure 5. The MS spectrum of Ginsenoside Rb1 by combining the three driftogram peaks from 4c.

Spectrum for each peak

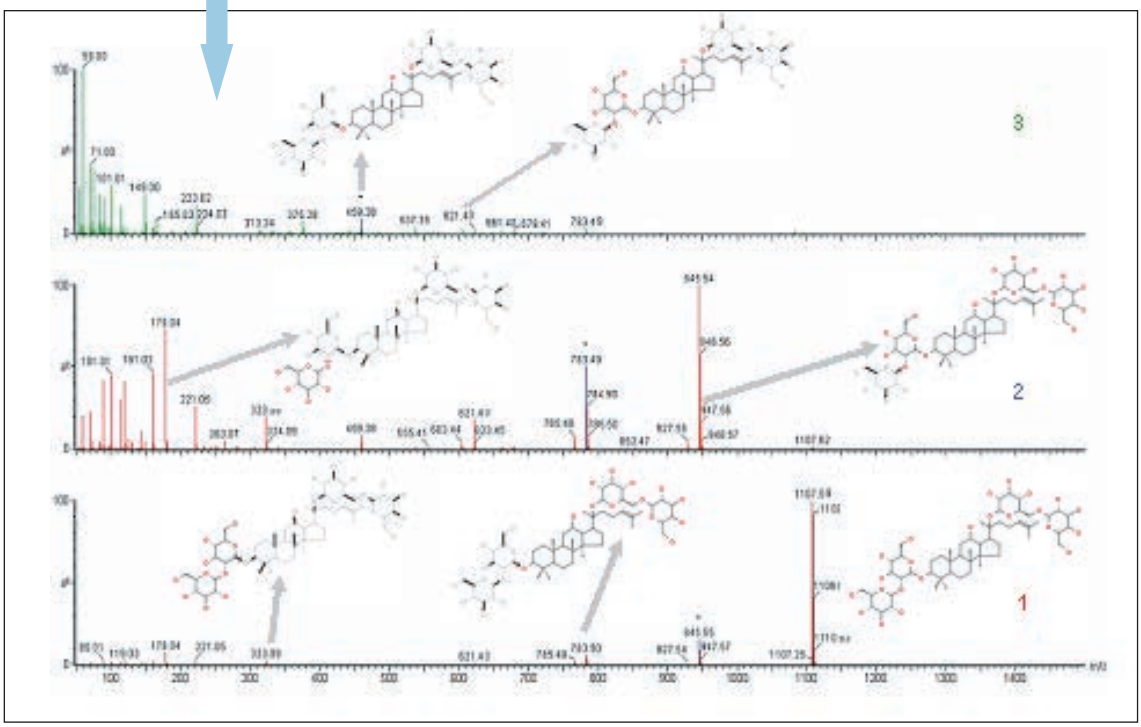

Figure 6. The individual spectrum for each region corresponds to driftogram peak 1, 2, and 3.

CONCLUSION

UPLC/HDMS and analytical-scale fraction collection combined with TAP fragmentation is complementary to the UPLC/TOF MS workflow. As a result, Traditional Herbal Medicine (THM) samples can be analyzed with high resolution, high sensitivity and fast turnaround time.

This technique enhances the user's ability to perform structural elucidation for individual components from a complex matrix. TAP fragmentation, used in combination with the MassFragment structure elucidation software tool, provides a fast and accurate approach to solving complex elucidation problems.

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THE BENEFITS OF GAS-PHASE COLLISION CROSS-SECTION (CCS) MEASUREMENTS IN HIGH-RESOLUTION, ACCURATE-MASS UPLC/MS ANALYSES

The rotationally-averaged collision cross-section (CCS) represents the effective area for the interaction between an individual ion and the neutral gas through which it is travelling. CCS is an important distinguishing characteristic of an ion in the gas phase, being related to its chemical structure and three-dimensional conformation. CCS affects the mobility of an ion as it moves through a neutral gas under the influence of an electric field and ions may be separated accordingly using ion mobility spectrometry (IMS).[1] CCS values may be measured experimentally using IMS. CCS values may also be estimated computationally if the 3D structure of the molecule is known.[2]

Figure 1 illustrates the separation of ionic species achieved by IMS. In this example, two ions of equal mass and charge, but different three-dimensional conformation, will travel through an IMS device at rates dependent on their mobilities and emerge at different times (drift times). The ion with a more compact three-dimensional conformation has a shorter drift time through the IMS device because its smaller CCS value gives it a higher mobility than its neighbour with a more open three-dimensional conformation.

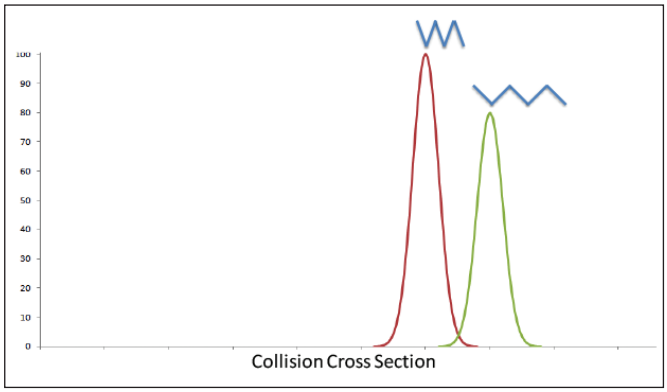

Figure 1. The CCS value of an ion may be determined using its drift time through an IMS device.

By calibrating the IMS device using ions of known CCS, the drift times measured during subsequent analyses of unknown species may be converted directly to CCS values.

The Waters® SYNAPT® G2-S*i* HDMS™ Mass Spectrometer incorporates high-efficiency T-Wave™ ion mobility separations and enables CCS values to be measured routinely as an integral part of high-resolution, accurate-mass LC/MS experiments.[3] This allows CCS to be used alongside the traditional molecular identifiers of precursor ion accurate mass, fragment ion accurate masses, isotope pattern, and chromatographic retention time as a confirmation of compound identity or as an indicator of compound structure.

CCS FOR MAXIMIZING ANALYTICAL PEAK CAPACITY AND SELECTIVITY

The selectivity of an analytical method depends on the ability of the analytical system to resolve sample constituents from each other. Often, a sample may contain many thousands of individual components.

Chromatography and mass spectrometry are techniques typically used to resolve sample constituents. The resolving power of modern UltraPerformance LC® (UPLC®) instruments and high resolution mass spectrometers, when combined, deliver very high levels of analytical selectivity but many analyses either cannot make full use of chromatographic separations (MALDI[4] or direct analysis techniques such as DART, DESI, and ASAP[5]) or demand levels of selectivity that cannot be provided even by the combination of state of the art LC/MS systems.

In such cases T-Wave ion mobility separation provides an additional dimension of resolution that works orthogonally to both chromatography and mass spectrometry to multiply the peak capacity and selectivity of an analytical method.[6] By incorporating CCS based separations into an analytical method it is possible to distinguish analyte from matrix interferences or even resolve structural isomers[7], conformers, and protomers.[8] For MALDI Imaging experiments, where samples may be complex and chromatographic separation is not possible, CCS based separation provides the selectivity required to accurately and confidently determine the spatial distribution of important molecular species.[9]

CCS FOR CONFIRMING COMPOUND IDENTITY

Because CCS measurements are undertaken in the gas phase, remotely from the ion source, CCS values are unaffected by sample matrix and are consistent between instruments and across a range of experimental conditions. The precision of CCS measurements obtained with T-Wave IMS can be used in combination with other molecular identifiers to increase the confidence of compound identifications.

As an example, Figure 2 shows the average measured CCS values from a range of pesticides analysed in a variety of different sample extracts, compared with the values measured in solvent standards. The results show that CCS may be measured within 2% of the expected value regardless of the nature and complexity of the analytical sample.

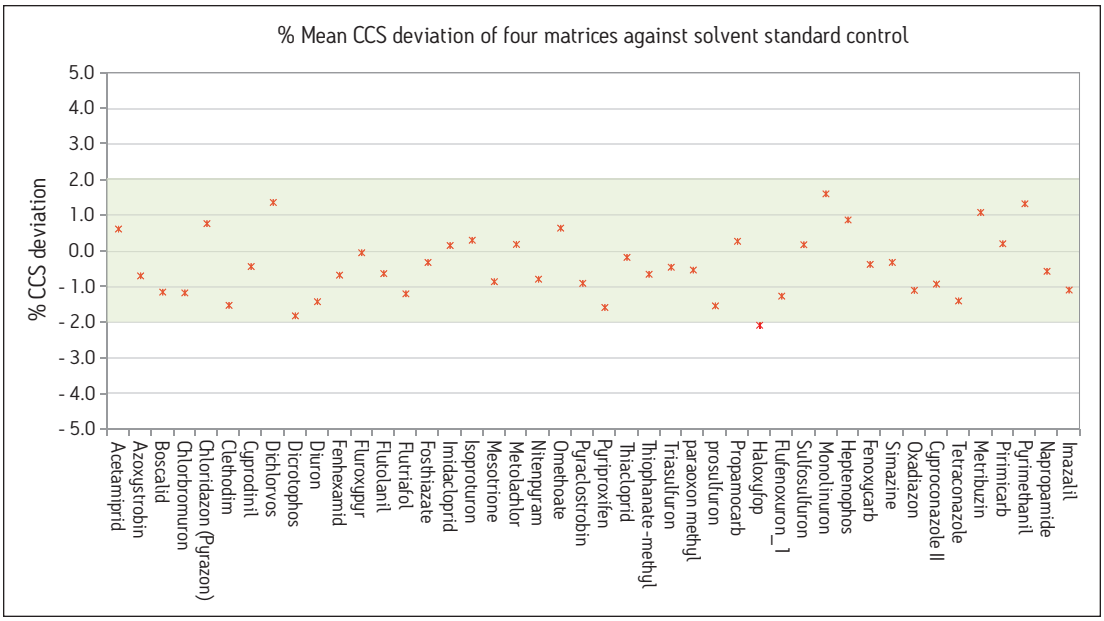

Figure 2. Average measured CCS values for a wide range of pesticide compound classes over a range of sample matrices deviate by less than 2% from CCS values measured using solvent standards.

Large numbers of pesticides can be screened for in high-resolution, accurate-mass LC/MS experiments but often, components in the sample matrix give rise to signals that can obscure the signal from a residue present in the sample (false negative detection) or can be mistaken for pesticide residues not present in the sample (false positive detections). The number of false negative and false positive detections can be minimised by choosing appropriate mass accuracy and retention time tolerance windows and by filtering the results based on the presence of expected fragment, adduct or isotope confirmatory ions. The CCS values of pesticides may be used as an additional, orthogonal means of filtering the data to significantly reduce the proportion of false positive and false negative detections.

A spiked extract of mandarin fruit, produced for a European Union Reference Laboratories (EURL) proficiency test, was analysed in a blind study using a Waters SYNAPT mass spectrometer. The data were searched against a library of approximately 480 pesticide compounds. Approximately 50,000 sample components were observed and filtering these components according to chromatographic retention time, the presence of expected fragment ions and a 5 ppm mass tolerance gave 9 identifications, with 1 false negative and 2 false positive results. Increasing the mass tolerance to 10 ppm gave 10 identifications with 2 false positive results. Applying a CCS tolerance of ± 2%, as a further filter to the results reduced the number of identifications to 8 as shown in Figure 3. The 8 identifications obtained using CCS as an extra, orthogonal data filter were all correct and, in this particular case, the false positive and false negative identifications were removed.

	Without CCS	Without CCS	With CCS
m/z tolerance (+/-)	5 ppm	10 ppm	10 ppm
rt tolerance (+/-)	2.5%	2.5%	2.5%
CCS tolerance (+/-)	–	–	2%
Correct IDs	7	8	8
False Negatives	1	0	0
False Positives	2	2	0

Figure 3. EURL proficiency test sample results: Pesticide screening without using CCS shows a number of false positive and false negative identifications. For this study, using CCS information removes false positive and false negative identifications.

The use of CCS as an additional, orthogonal means of filtering the data effectively allows a more balanced set of tolerance criteria to be applied to compound screening experiments, which results in more confident compound identifications.

CCS AS AN INDICATOR OF MOLECULAR CONFORMATION

Theoretical CCS values determined by molecular modelling may be compared directly with experimentally derived CCS values obtained by T-Wave ion mobility separations, in order to yield useful information on the structures and shapes of large proteins/protein complexes, peptides, organometallic complexes and small

molecules; information that can provide unique insights into important biological and chemical processes.

The use of T-Wave IMS has enhanced knowledge in diverse areas of scientific research, from mapping the size distribution in oil and petroleum samples[10] to revealing the molecular architecture of multi protein complexes[11,12] and to rapidly determining sites of biotransformation in drug metabolism studies.[13,14]

These types of studies are able to provide useful scientific information far more rapidly and efficiently than by more traditional techniques. Often, using CCS to probe molecular structure can yielded scientific advances not possible by other means.

CONCLUSION

The ability to separate ions by T-Wave IMS and measure their CCS values as an integral part of high-resolution, accurate-mass LC/MS experiments allows significant increases in the peak capacity and resolution of analytical methods. It enables the separation of isomers, conformers and protomers that cannot be separated by other means. It also provides a unique, orthogonal identifier for target analytes in screening experiments and can be used to probe the structures and conformations of diverse types of molecule. All these experiments can be carried out on the Waters SYNAPT G2-S*i* HDMS Mass Spectrometer, enabling significant increases in the performance of analytical methods and delivering unique capabilities not available on conventional mass spectrometers.

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SYNAPT G2 High-Defintion Mass Spectrometry: Separation and COLLISION CROSS-SECTION Determination of Leucine and Isoleucine by Travelling Wave Ion Mobility Mass Spectrometry

Iain Campuzano, Kevin Giles, Kieran Neeson, and Keith Richardson
Waters Corporation, Manchester, UK

INTRODUCTION

Here we demonstrate the use of travelling wave ion mobility mass spectrometry with the SYNAPT™ G2 High Definition Mass Spectrometry™ (HDMS™) platform to separate the two amino acid structural isomers isoleucine and leucine, which differ in a collision cross-section (CCS; (Ω)) by less than 3 Å^2.

Distinguishing between isoleucine and leucine has only previously been demonstrated on instruments such as magnetic sectors or MALDI Tof-Tof mass spectrometers that are capable of performing high energy Collision Induced Dissociation (CID).

Following separation of these isomers, the T-Wave™ collision cross-section values were automatically generated with DriftScope™ Informatics (v2.1). This new capability allows one to automatically generate a T-Wave ion mobility calibration function, and derive CCS values for such compounds.

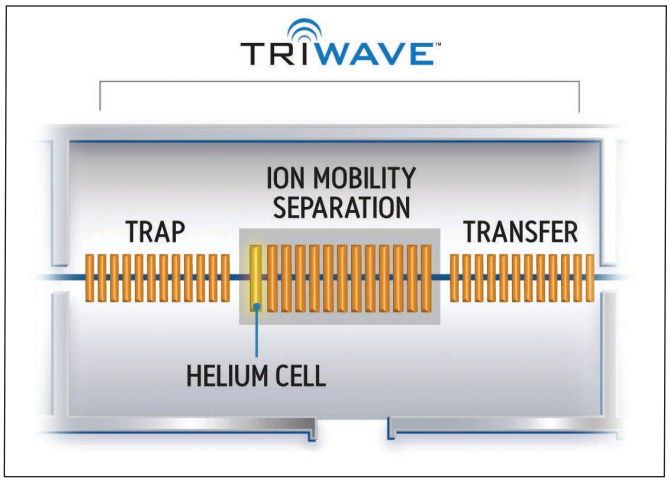

Figure 1. Schematic of the second-generation Triwave™ Technology of SYNAPT G2. The enhanced IM resolution is achieved through both the increased length and pressure of the IMS T-Wave region.

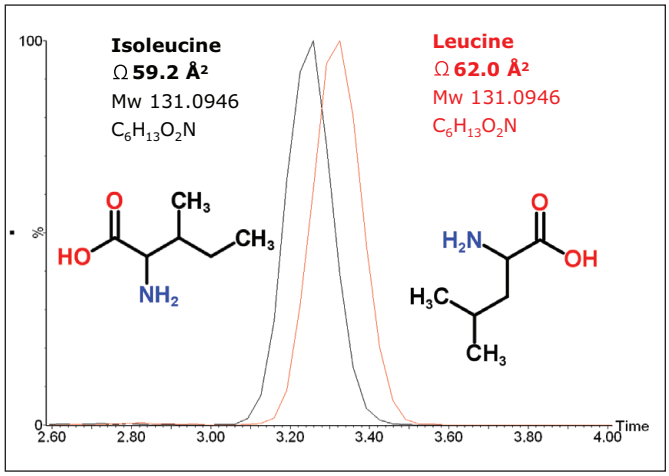

Figure 2. T-Wave ion mobility drift time (msec) chromatograms for isoleucine and leucine and their respective T-Wave derived collision cross-sections (Ω).

EXPERIMENTAL

SYNAPT G2 is an innovative hybrid quadrupole IMS oa-Tof mass spectrometer incorporating second-generation Triwave Technology (Figure 1), which provides significantly enhanced ion mobility resolution (over 40 (Ω/ΔΩ)). The increased pressures of the drift gas (e.g. N_2) and overall length of the IMS T-Wave provide an ion mobility resolution increase of up to a factor of 4 compared to traditional Triwave Technology, while maintaining high transmission efficiency via the novel Helium-filled entry cell. The T-Wave ion mobility calibration was carried out using previously determined collision cross-section values for polyglycine (from www.indiana.edu/~clemmer).

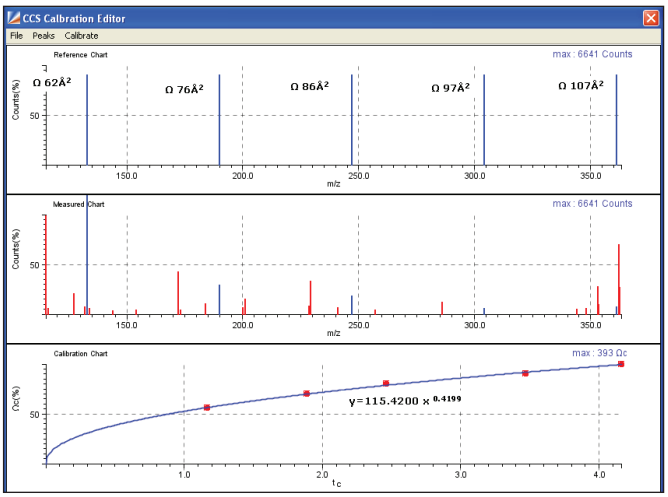

Figure 3. DriftScope Software's (v2.1) automated T-Wave ion mobility collision cross-section calibration editor. Data displayed are polyglycine.

An automated T-Wave ion mobility calibration can be carried out using DriftScope Software (v2.1) (Figure 3). The top panel shows the ion mobility calibration compound (polyglycine) with annotated collision cross-sections. The second panel shows a polyglycine mass spectra with automated peak selection. The third panel shows a charge and reduced-mass corrected collision cross-section vs. drift time plot, fitted with a power relationship, which can be used to derive CCS values for unknown compounds.

CONCLUSIONS

It is possible to distinguish between the structural isomers of leucine and isoleucine by travelling wave ion mobility MS where the absolute collision cross-section measurements of structural isomers differ by less than 3 Å^2.

DriftScope Informatics enable routine collision cross-section determination of such compounds with the use of an automated T-Wave ion mobility calibration editor.

SYNAPT G2 with HDMS now enables such structural studies to be performed with enhanced specificity (enhanced IM resolution (over 40 (Ω/ΔΩ)) and speed (DriftScope Informatics).

Reference

1. The travelling wave device described here is similar to that described by Kirchner in US Patent 5,206,506 (1993).

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Flavonoids Identification in Complex Plant Extracts using Ion Mobility TOF MS and MS^E

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Waters Pacific Pte Ltd., Singapore

APPLICATION BENEFITS

- Screening of flavonoids in complex plant extract (*Ficus* sp.) using ACQUITY UPLC®/SYNAPT® G2 HDMS.™
- Identification of isomers using ion mobility and MS^E acquisition functionality.
- HDMS^E provides another dimension of orthogonal separation, delivering unprecedented peak capacity for increased confidence in isomers identification of complex mixtures.

WATERS SOLUTIONS

ACQUITY UPLC System

SYNAPT G2 High Definition Mass Spectrometry™ (HDMS)

DriftScope™

MS^E Data Viewer

ACQUITY UPLC HSS (High Strength Silica) Technology

KEY WORDS

Flavonoids, isomers, ion mobility, TOF, HDMS

INTRODUCTION

Flavonoids are a remarkable group of plant metabolites that ubiquitously exist in natural products that have been considered as an active ingredient of many medicinal plants.[1] Generally, the backbone of flavonoids consists of two phenyl rings and a heterocyclic ring, but they are often conjugated to a carbohydrate moiety with individual differences arising from various chemical processes, such as hydroxylation, methoxylation, glycosylation, and acylation.[2]

Plants containing flavonoids have been used for thousands of years in traditional Eastern medicine. In recent years, plant flavonoids have been shown to be of vital significance to humans. They have been linked as active contributors of health benefits, including its antioxidant properties in beverages such tea and wine, and in foods such as fruits and vegetables.

Waters® SYNAPT G2 High Definition Mass Spectrometry (HDMS), a combination of high-efficiency ion mobility separation (IMS) and time-of-flight (TOF) mass spectrometry, has been used to effectively separate and identify natural product structural isomers.[3] The rapid orthogonal gas separation technique in the IMS T-Wave™ allows another dimension of separation via their mass and shape without compromising MS data quality or sensitivity.

MS^E is an acquisition technique that provides a simple, unbiased, and parallel route to deliver exact mass, low energy precursor (MS) and high energy fragment ion (MS^E) information from every detectable component, without the need for multiple injections.

This application note describes the analysis of *Ficus* sp. extract using Waters ACQUITY UPLC System combined with the SYNAPT G2 HDMS System with IMS and MS^E functionality to provide chromatographic and isobaric separation for a more comprehensive structural characterization of flavonoids.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY HSS T3 2.1 x 100 mm, 1.8 µm
Column temp.:	40 °C
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Injection volume:	5.0 µL PLNO
Total run time:	10.0 min

MS conditions

MS System:	SYNAPT G2 HDMS
Ionization:	ESI negative
Capillary voltage:	1.7 kV
Sampling cone:	30 V
Extraction cone:	4.0 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas:	1000 L/hr
Cone gas:	50 L/hr
Trap CE:	4 V
Transfer CE:	0 V
Trap/transfer gas:	Ar
IMS gas:	N_2 (~3.4 mbar)
IMS T-Wave speed:	650 m/sec
IMS T-Wave height:	40 V
Mass range:	50 to 1200 *m/z*

Sample preparation

Plant samples (*Ficus* sp.) were extracted in 50% methanol/water solution. The extract was then centrifuged and the supernatant was collected for further analysis.

Mobile phase gradient is detailed in Table 1.

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.4	99	1	–
1.0	0.4	95	5	6
6.5	0.4	50	50	6
7.5	0.4	5	95	6
8.0	0.4	99	1	6
10.0	0.4	99	1	6

Table 1. ACQUITY UPLC System mobile phase gradient.

RESULTS AND DISCUSSION

In this profiling study, the base peak ion chromatograms of the extracted *Ficus* sp. samples showed a high degree of complexity, with numerous co-eluting components and also the presence of isomers. The advantages of ACQUITY UPLC not only produces highly reproducible chromatograms between injections, as shown in Figure 1, but also high throughput with a rapid analysis time of 10 mins. When coupled together with IMS and MS^E functionality of the SYNAPT G2 HDMS, another dimension for the separation of isomers/isobaric compounds is attained. With this system, comprehensive structural information can be acquired without compromising sensitivity and analysis time.

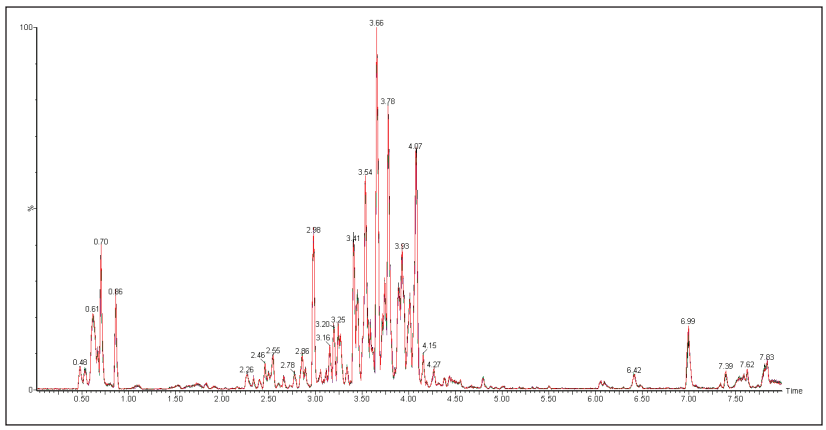

Figure 1. Overlay BPI chromatograms of extracted Ficus sample (six injections).

The flavone C-glycosides are an important subclass of the flavonoids family. Flavone C-glycosides are present in foodstuffs and nutraceuticals and they include orientin (luteolin-8-C-glucoside), isoorientin (luteolin-6-C-glucoside), vitexin (apigenin-8-C-glucoside), and isovitexin (apigenin-6-C-glucoside). They are also reported to exhibit anti-inflammatory and anti-nociceptive properties.[4,5]

Both vitexin and isovitexin have the same chemical formula of $C_{21}H_{20}O_{10}$ with an exact mass of *m/z* 431.0978 $[M-H]^-$. Using the above UPLC® method, the extracted ion chromatogram showed two peaks with a baseline chromatographic separation at 4.07 min and 4.66 min, as shown in Figure 2A. As both compounds are isobaric, the assignment of vitexin and isovitexin to these peaks (4.07 min and 4.66 min) are not possible with only high resolution spectra alone. The identities of these two peaks were further confirmed using MS^E and IMS.

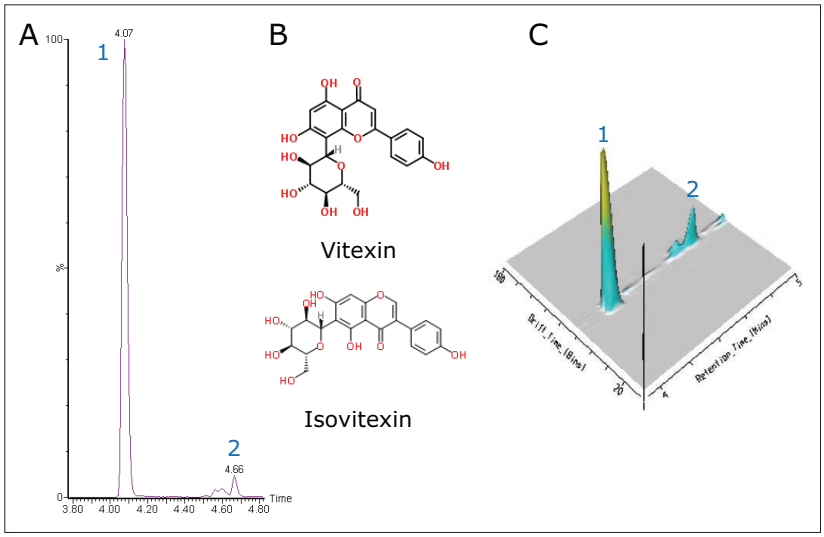

Figure 2. UPLC/SYNAPT G2 HDMS of Ficus sp. extract. 2A. XIC of Ficus sp. extract at 431.0978 m/z. 2B. Molecular structure of vitexin and isovitexin. 2C. 3D illustration of Ficus sp. extract from 4 to 5 min. The 3D plot shows the components were separated by chromatographic retention time. Vitexin and isovitexin are labeled as 1 and 2 respectively.

Baseline chromatographic separation of vitexin and isovitexin via retention time was achieved. However the fragmentation patterns observed in the MS^E spectra for both vitexin and isovitexin were identical. The predicted product ions of vitexin and isovitexin, were then cross-checked against the MS^E spectra of the *Ficus sp.* extract samples using MassFragment™ Software to provide added confidence. The MS and MS^E spectra of isovitexin are shown in Figure 3.

Using HDMS, both compounds were further separated via ion mobility based on their structural configuration and a 3D plot was generated, as shown in Figure 2C. From Figure 2C, it can be observed that vitexin and isovitexin have drift times of 81.78 bins (4.45 ms) and 83.44 bins (4.53 ms) respectively. Thus using retention times, MS^E and HDMS, the identity of vitexin and isovitexin can be determined.

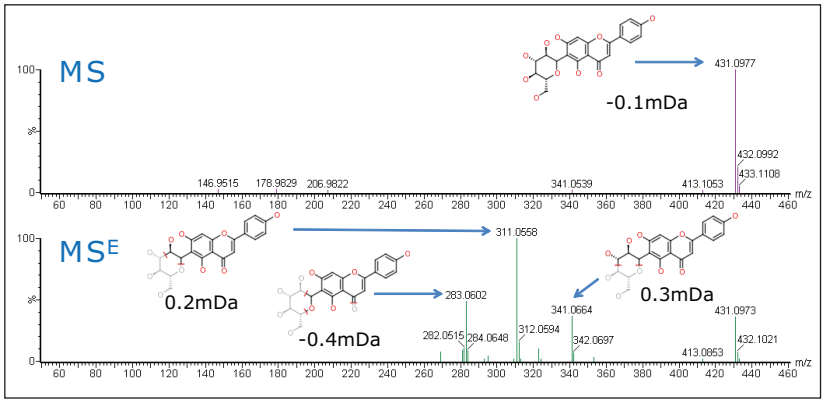

Figure 3. MS and MS^E spectra of isovitexin (with mass error) at 4.66 min.

Two additional important C-glycoside flavonoids are orientin and isoorientin. They have a chemical formula of $C_{21}H_{20}O_{11}$ with an exact mass of *m/z* 447.0927 $[M-H]^-$. The extracted ion chromatogram in Figure 4 shows two major peaks at 3.72 min and 3.83 min.

Baseline chromatographic separation of isoorientin and orientin via retention time was achieved. However upon further interrogation of these peaks using ion mobility and DriftScope Data Viewer, when a 3D plot was generated, shown in Figure 4C, it was observed that an unknown peak (*m/z* 635.1767) co-eluted with the orientin peak (marked with an asterisk in Figure 4C).

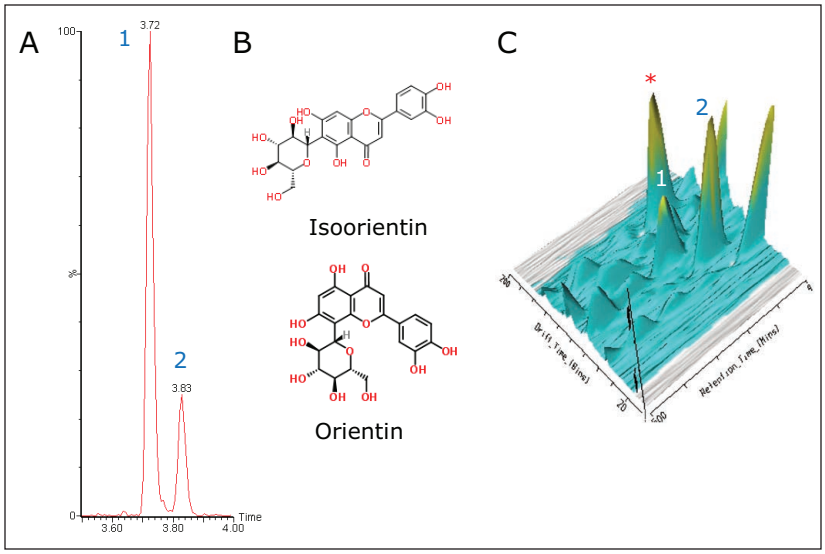

Figure 4: UPLC/SYNAPT G2 HDMS of Ficus sp. extract. 4A. XIC of Ficus sp. extract at 447.0927 m/z. 4B. Molecular structure of orientin and isoorientin. 4C. 3D illustration of Ficus sp. extract from 3.5 to 4.0 min. The 3D model shows the components were separated by chromatographic retention time and ion mobility drift time. Isoorientin and orientin are labeled as 1 and 2 respectively. An unknown co-eluting compound is marked with an asterisk.

The 2D DriftScope plot in Figure 5 illustrates the IMS separation of isobaric orientin and isoorientin (447.0927 *m/z*), showing two isomers with drift times of 76.83 bins (4.16 ms) and 84.33 bins (4.54 ms) respectively. Thus by estimating the cross-sectional structure of orientin and isoorientin , shown in Figure 4B, it can be proposed that the more compact orientin is the species with the drift time of 4.16 ms and the more extended structure of isoorientin has a longer drift time of 4.54 ms. Using the HDMS^E available on the SYNAPT G2 HDMS, the product ions of orientin and isoorientin were easily visualized by their drift times and mass-to-charge ratios, as shown in the insert in Figure 5.

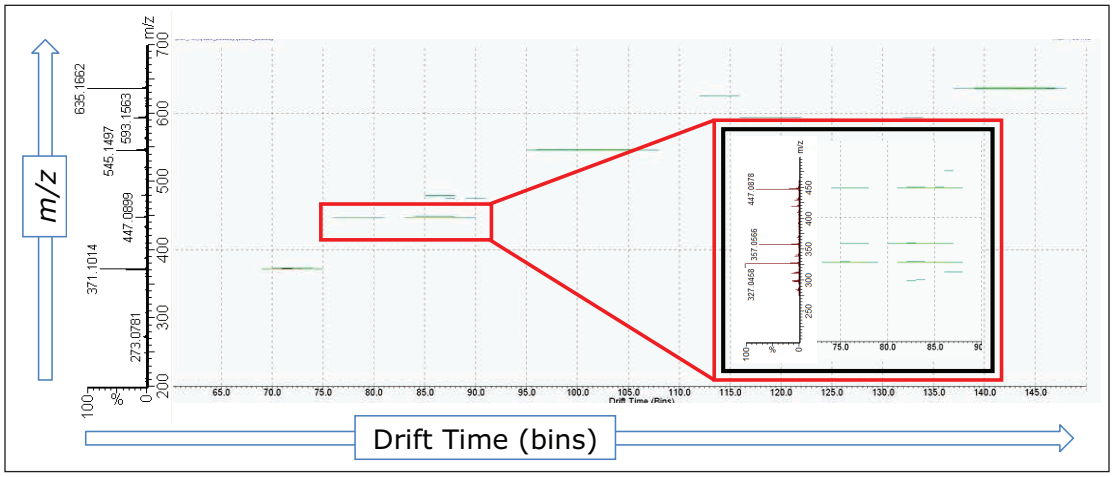

Figure 5. Visualization (drift time versus m/z) of compounds eluting between 3.7 and 3.9 min. Note the separation of orientin and isoorientin with drift times of 4.16 and 4.54 ms respectively. Inset: Product ions of orientin and isoorientin via post-IMS collision-induced dissociation.

Using the MS^E Data Viewer, with the selection of *m/z* 447.0927, the BPI chromatogram window showed two prominent peaks at 3.73 and 3.84 min, as shown in Figure 6A. However upon further data interrogation of the peak at 3.84 min, it was observed that there were several co-eluting compounds, which were of higher intensity than the peak of interest, as shown in Figure 6B. Thus due to the high complexity of the sample and the vast amount of product ions present in the MS^E spectra. It is impossible to accurately determine the fragmentation pattern of orientin, see Figures 6C and 6D.

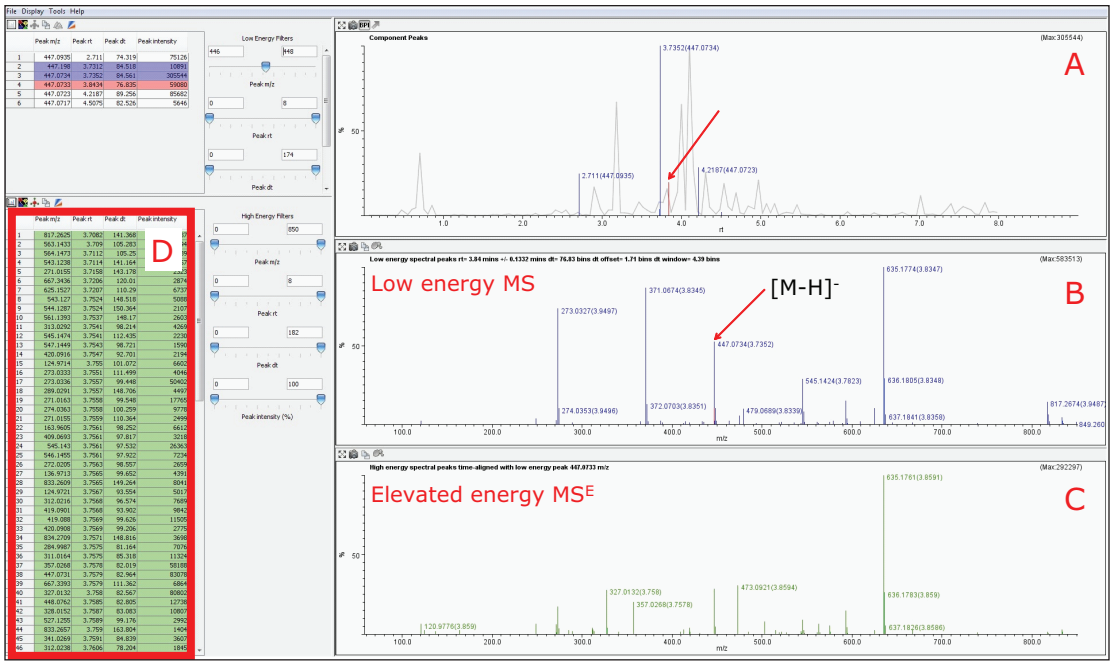

Figure 6. Orientin peak at 3.84 min using MS^E Data Viewer software. 6A. Chromatogram of peaks with m/z 447.0927. Orientin peak with retention time of 3.84 min was selected (highlighted in red with an arrow). 6B. Low energy mass spectrum (MS) of peak at 3.84 min. Molecular mass of orientin [M-H]$^-$ is indicated (arrow). 6C. High-energy mass spectrum (MS^E) of peak at 3.84 min. 6D. List of product ions present in the MS^E spectrum. HDMS function is not activated.

However, by activating the HDMS function of the MS^E Data Viewer, the co-eluting components in the same peak at 3.84 min could be mobility resolved. As shown in Figure 7B, the precursor ion of orientin has a drift time of 4.16 ms. With the HDMS functionality activated, the product ions were easily resolved and the list of product ions were also greatly reduced, as shown in Figures 7C and 7D, thus increasing the confidence level of identifying orientin. HDMS^E is an essential tool for separating compounds in complex mixtures containing numerous co-eluting compounds as it provides another dimension of orthogonal separation for increased confidence in isomer identification.

For isoorientin with a retention time at 3.73 min, a drift time of 4.54 ms with similar HDMS^E fragmentation pattern as orientin was observed, as shown in Figure 8.

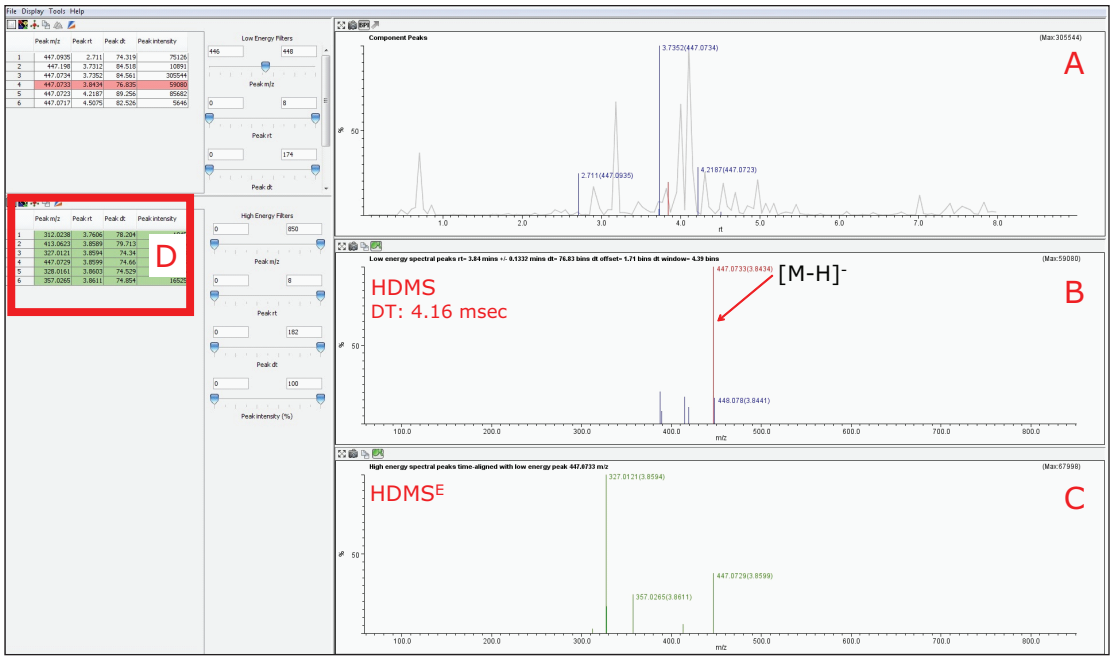

Figure 7. Orientin peak at 3.84 min with HDMS function activated. 7A. Chromatogram of peaks with 447.0927 m/z. 7B. Low energy mass spectrum (HDMS) with orientin peak selected (highlighted in red). 7C. High-energy mass spectrum (HDMS^E) of orientin peak. 7D. List of product ions present in the HDMS^E spectrum.

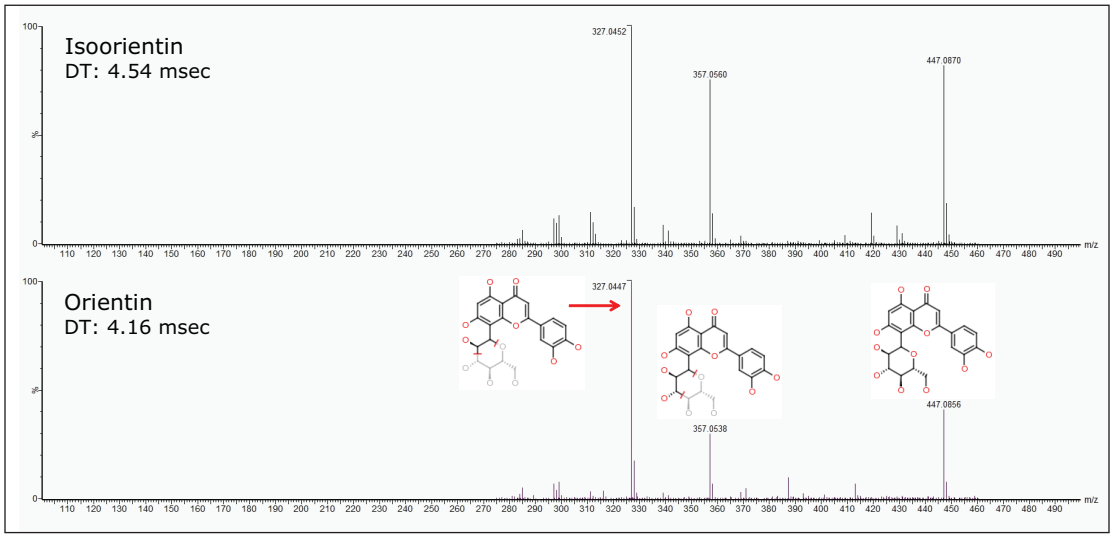

Figure 8. HDMS fragmentation pattern of orientin and isoorientin. Inset: Product ions of orientin generated using MassFragment Software.

CONCLUSIONS

- The ACQUITY UPLC System combined with SYNAPT G2 HDMS is an effective system solution for rapid screening and identification of flavonoids in *Ficus* sp. extract.
- Plant extracts are complex matrices that contain many co-eluting compounds and isomers. HDMS provides an extra dimension of separation via ion mobility, which allows the separation of orientin from other co-eluting compounds. Together with the MS^E functionality, where both low energy (precursor) and high energy (product ion) data can be acquired within a single analysis, provides greater confidence in the identification of flavonoids in *Ficus* sp. extract.
- The co-eluting interference peak with orientin are easily visualized using 3D models and drift-time plots generated using DriftScope. While further spectra cleanup for accurate product ions fingerprint for qualitative analysis is achieved using MS^E Data Viewer.

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An Added Dimension for Metabolite ID Studies Using Ion Mobility Combined with MS^E

GOAL

To use HDMS^E data to generate cleaner, more precise data sets and to resolve isobaric species. When LC/MS and LC/MS/MS just isn't enough, using DriftScope™ Software allows you to interrogate your data in an extra dimension, RT, *m/z*, and now drift time (ion mobility separation).

HDMS^E (Ion Mobility Mass Spectrometry) provides researchers with added orthogonal separation and peak capacity to differentiate small changes in closely eluting isobaric metabolites.

BACKGROUND

When performing metabolite identification it is common to observe multiple biotransformations that give the same isobaric mass. These compounds are often nearly indistinguishable and can be very difficult to resolve by chromatography alone. Extremely high levels of matrix, as is often the case with *in vivo* studies compound the problem. Although re-optimized chromatography, improved instrument sensitivity and careful interpretation of data can lead to resolution of these species, they are time consuming steps and often require large data sets to be reacquired with modified conditions. Additional orthogonal separation such as ion mobility introduces selectivity that can often quickly resolve these differences and further improve spectral quality, leading to higher quality data sets and interpretations. This combination of Ion Mobility Separation (IMS) and MS^E creates High Definition Mass Spectrometry,™ HDMS^E, and gives the researcher another powerful tool to understand and probe their datasets. In this technology brief we will study the applicability of HDMS^E to complex datasets.

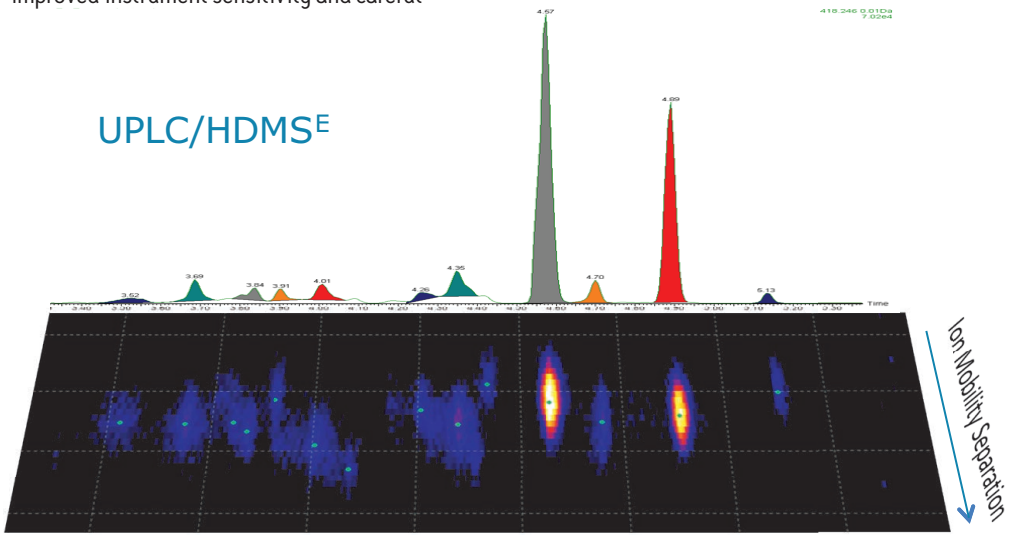

Figure 1. An additional three +32 Da metabolites of buspirone are clearly identified with the added dimension of separation generated by HDMS^E.

THE SOLUTION

Rat liver microsomes spiked with 10 µM buspirone were incubated for 0 and 20 min at 37 °C. Samples were quenched with one volume of cold acetonitrile + 0.1% formic acid and centrifuged. In order to evaluate the application of HDMS^E to *in vivo* metabolite identification studies, the above *in vitro* samples were diluted 10-fold with SD (Sprague Dawley) rat urine containing 0.1 % PEG400 by volume. Samples were analyzed using a Waters® SYNAPT® G2 coupled with an ACQUITY UPLC® System. Data acquisition was performed with HDMS^E in positive ion, sensitivity mode. 5 µL of sample were injected onto an ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 100 mm Column and run with a 20 min gradient using a flowrate of 0.7 mL/min. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Data was processed and visualized using DriftScope Software.

Figure 1 illustrates the added dimension of separation generated by HDMS,™ the additional peak capacity introduced by IMS clearly elucidates an additional three metabolites versus UPLC® alone.

Figure 2 shows a comparison between MS^E data for a dealkylation metabolite generated with and without IMS separation enabled. Precursor and fragment ions that co-elute perfectly with the compound of interest can be quickly resolved using IMS techniques alone. Dedicated software using patented MS^E and IMS peak peaking algorithms (Apex 4D) leads to clear resolution of all relevant peaks. The power of peak picking in four dimensions (RT, *m/z*, ion mobility, and intensity) allows for a thorough cleanup of background noise and artifact peaks.

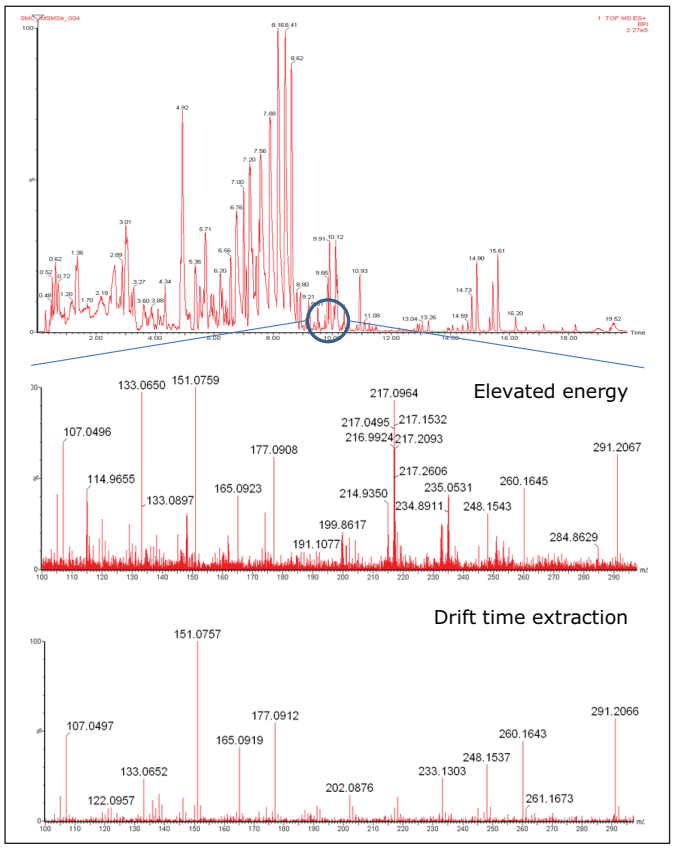

Figure 2. An illustration of the use of HDMS^E to remove background ions from a fragment ion spectrum in an in vivo sample.

SUMMARY

The advances presented in this technology brief facilitate the identification of metabolites, not only with better sensitivity and resolution, but through the unique properties of ion mobility. This allows the user to view data with less interference from matrix and other nominal mass interfering ions not separable through other methods.

Having an entirely unique mode of separation at your fingertips as an additional rich layer of information may mean the difference between an easy analysis and a costly, time-consuming revisiting of already worked out LC and MS methodology.

The benefits of UPLC coupled with SYNAPT G2 HDMS described in this technology brief are now available from Waters. As an additional weapon in your analytical toolkit, ion mobility separation can help you make insightful scientific discoveries and more keenly interpret and understand your experiments.

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