

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

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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 μ m
Column temp.:	40 °C
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Injection volume:	5.0 μ L 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 <i>m/z</i>

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.

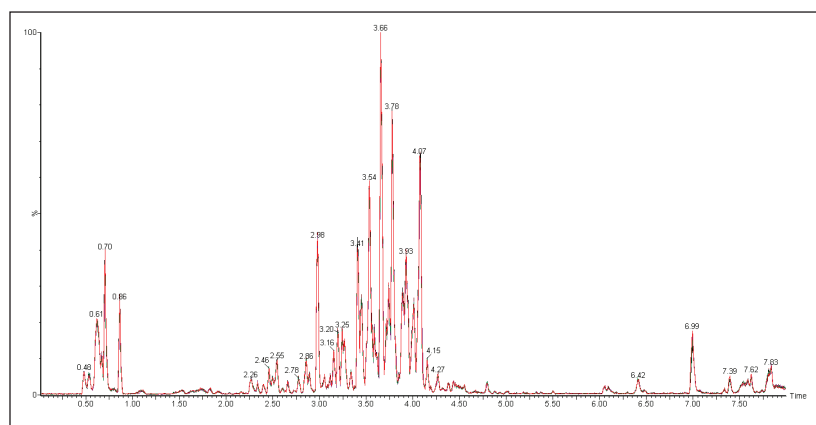


Figure 1. Overlay BPI chromatograms of extracted *Ficus* sample (six injections).

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.

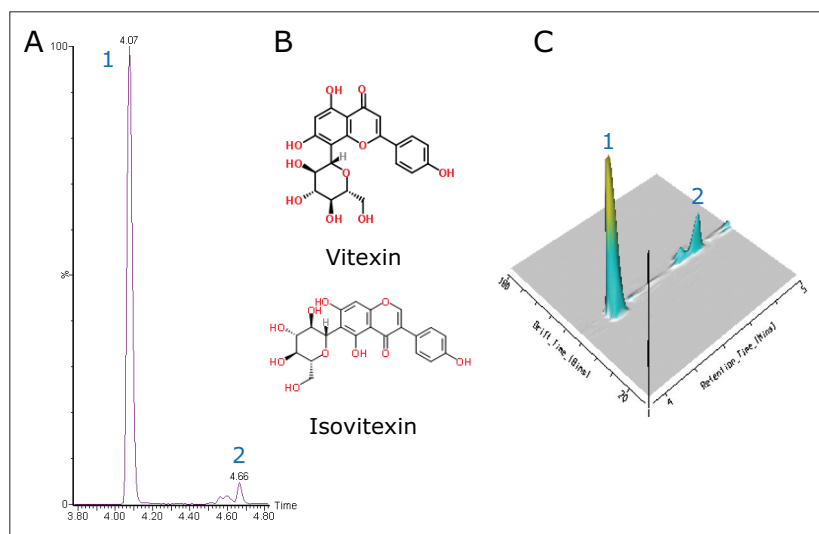


Figure 2. UPLC/SYNAPT G2 HDMS of *Ficus* sp. extract. 2A. XIC of *Ficus* sp. extract at 431.0978 m/z . 2B. Molecular structure of vitexin and isovitexin. 2C. 3D illustration of *Ficus* sp. extract from 4 to 5 min. The 3D plot shows the components were separated by chromatographic retention time. Vitexin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

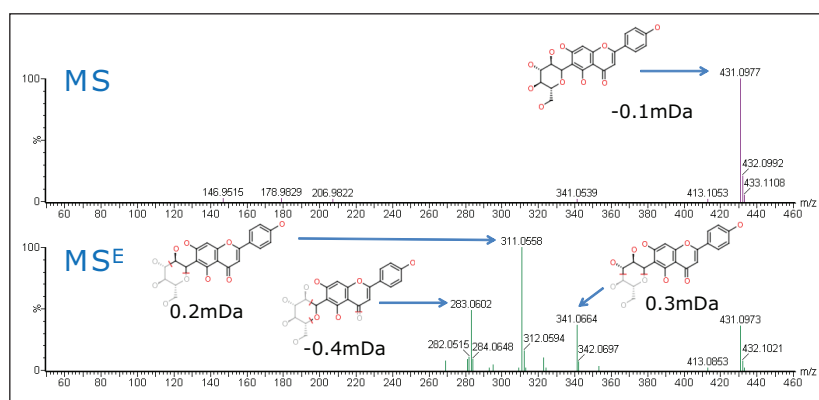


Figure 3. MS and MS^E spectra of isovitexin (with mass error) at 4.66 min.

Two additional important C-glycoside flavonoids are orientin and isoorientin. They have a chemical formula of C₂₁H₂₀O₁₁ 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).

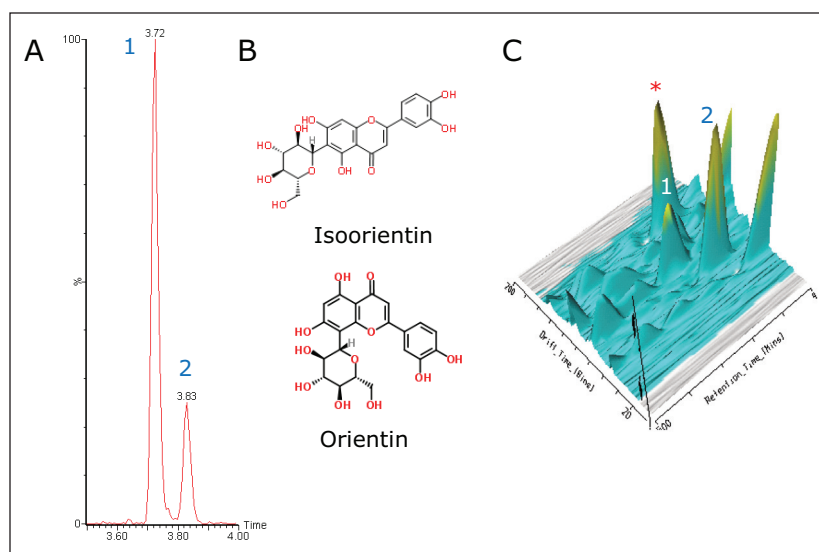


Figure 4: UPLC/SYNAPT G2 HDMS of *Ficus sp.* extract. 4A. XIC of *Ficus sp.* extract at 447.0927 m/z . 4B. Molecular structure of orientin and isoorientin. 4C. 3D illustration of *Ficus sp.* extract from 3.5 to 4.0 min. The 3D model shows the components were separated by chromatographic retention time 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.

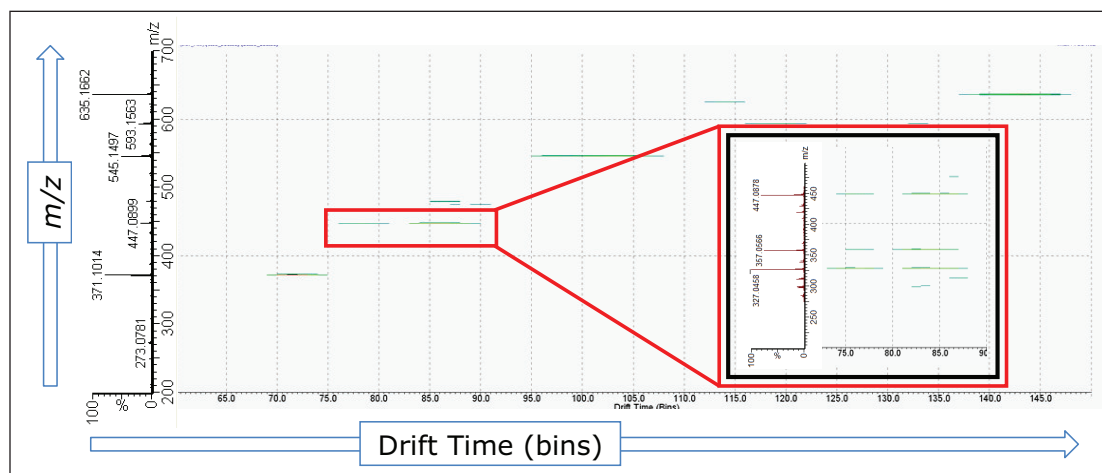


Figure 5. Visualization (drift time versus m/z) of compounds eluting between 3.7 and 3.9 min. Note the separation of orientin and isoorientin with drift times of 4.16 and 4.54 ms respectively. Inset: Product ions of orientin and isoorientin via post-IMS collision-induced dissociation.

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.

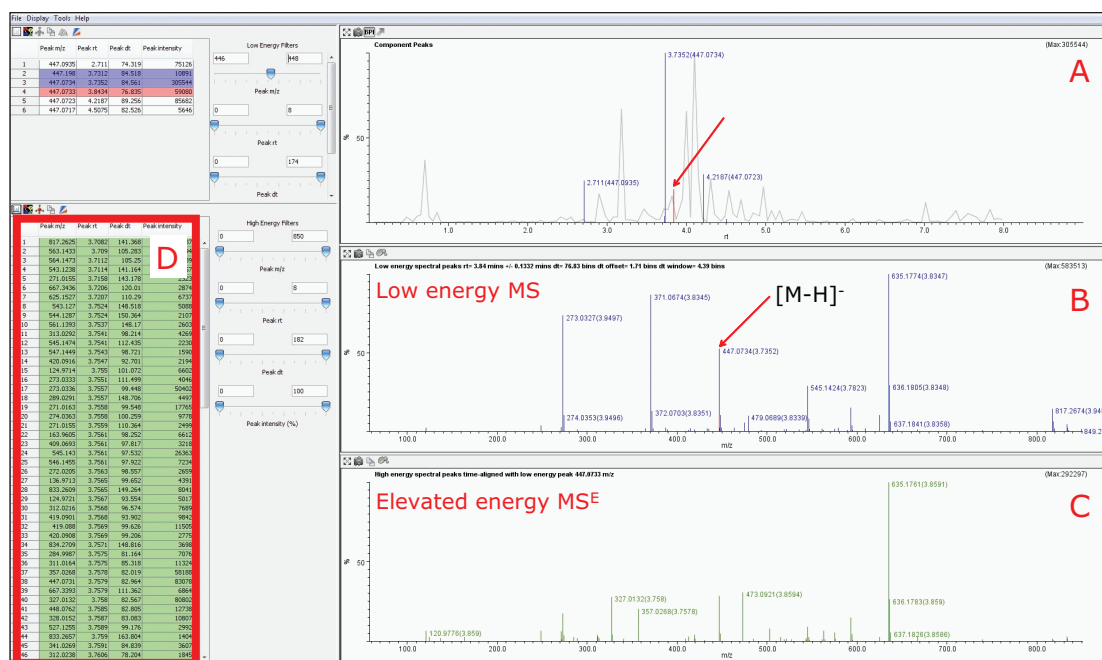


Figure 6. Orientin peak at 3.84 min using MS^E Data Viewer software. 6A. Chromatogram of peaks with m/z 447.0927. Orientin peak with retention time of 3.84 min was selected (highlighted in red with an arrow). 6B. Low energy mass spectrum (MS) of peak at 3.84 min. Molecular mass of orientin $[M-H]^-$ 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.

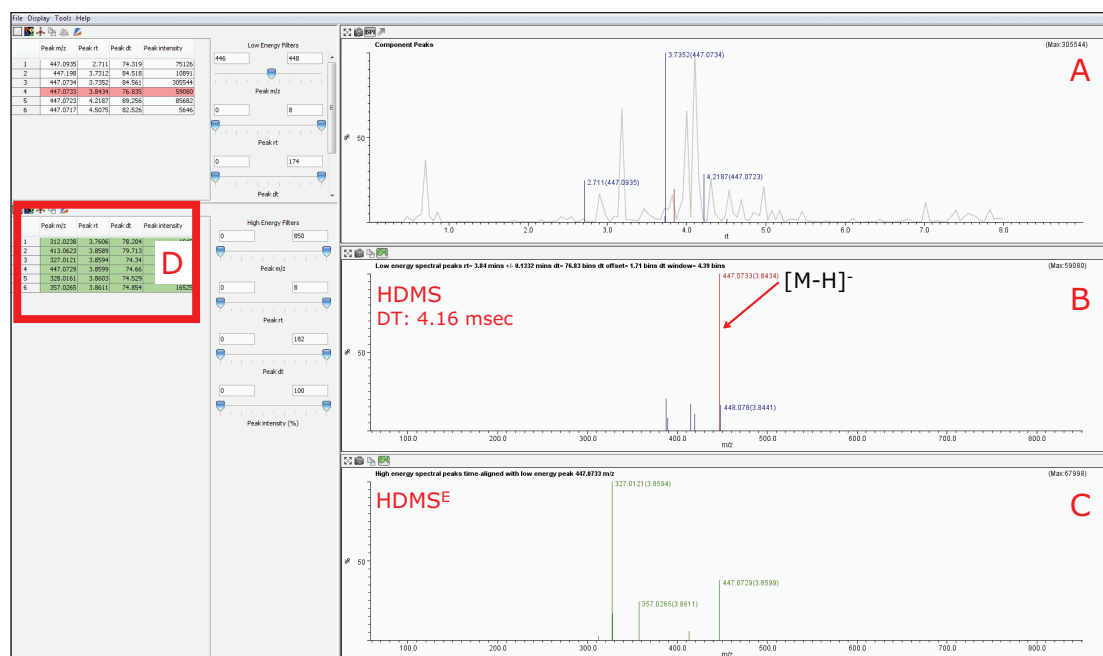


Figure 7. Orientin peak at 3.84 min with HDMS function activated. 7A. Chromatogram of peaks with 447.0927 m/z. 7B. Low energy mass spectrum (HDMS) with orientin peak selected (highlighted in red). 7C. High-energy mass spectrum (HDMS^E) of orientin peak. 7D. List of product ions present in the HDMS^E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

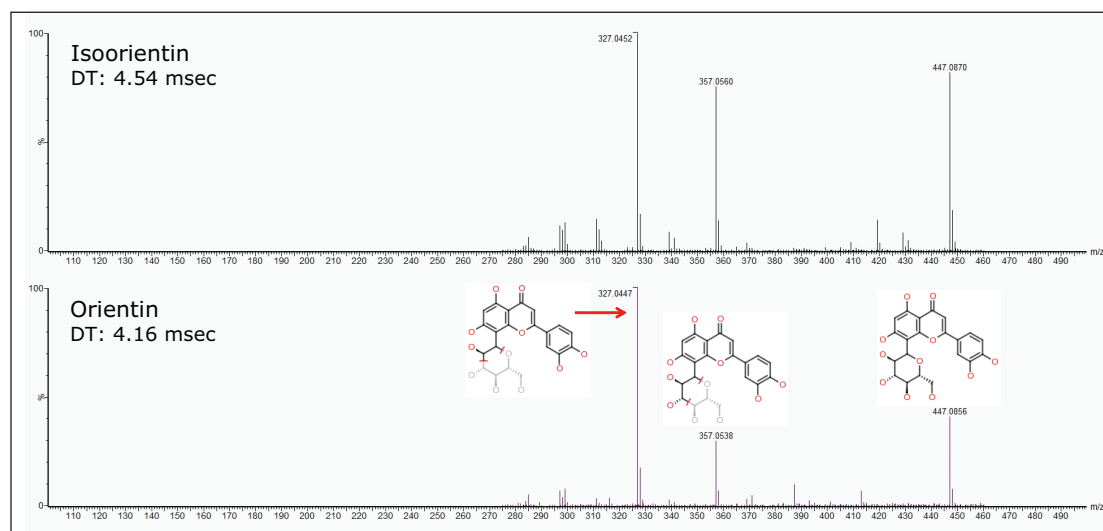


Figure 8. HDMS fragmentation pattern of orientin and isoorientin. Inset: Product ions of orientin generated using MassFragment Software.

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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