Development of a Collision Cross Section Library using Trapped Ion Mobility Spectrometry (TIMS) and Its Use in Plant Metabolomics

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Overview

- **Purpose:** Analyze authentic standards of plant compounds, record collision cross sections (CCS) in a searchable library, and identify plant metabolites using the newly constructed CCS library.
- **Methods:** CCS values were recorded and plant extracts of *Glycine max* and *Medicago truncatula* were analyzed using UHPLC-TIMS-QTOF-MS.
- **Results:** A CCS library of plant natural products was constructed and improved identification confidence. Isomeric and structurally similar compounds can be differentiated by TIMS even when they can not be differentiated by mass.

Introduction

Metabolomics is used to quantitatively and qualitatively profile large numbers of small molecule metabolites within a biological sample. The quality of the analyses rely on: **1) the separation of compounds** in the sample, and **2) data collected on the metabolites** within the sample. Liquid chromatography (LC) separates compounds and provides retention time (RT) information while mass spectrometry (MS) provides mass spectral information. The collected data is then compared with reference libraries and matching scores correspond to the confidence of the unknown compound identifications. Orthogonal data acquired for each metabolite being analyzed, improves identification confidence.

Ion mobility separates ions based upon their size to charge ratio i.e. their mobility in a gas phase. Trapped ion mobility (TIMS) is fast therefore well suited to be coupled with liquid chromatography (LC) and mass spectrometry (MS). TIMS adds an additional separation domain that can separate co-eluting, isomeric and isobaric compounds. It also provides physical collisional cross section (CCS) data for analytes which can be used to enhance identification confidence.

Fig. 1. General LC-TIMS-MS metabolomics workflow. Sample is prepared, LC, TIMS, and MS data are acquired. Data is then compared with reference databases.

Instrumentation

Trapped Ion Mobility Spectrometry

Liquid samples are ionized by an electrospray ionization source and the ions travel through a glass capillary into the TIMS. A deflection plate and drift gas direct ions into an ion funnel and then into a 2-stage TIMS analyzer. The concurrent incoming gas constantly pushes the ions through the TIMS analyzer while an electric field gradient opposes the forward movement of the ions. The first stage TIMS accumulates ions while the second stage separates ions based upon their mobility. Ions equilibrate where the drift gas and electric field forces on the ions are equal. The electric field gradient is gradually decreased to successively elute the ions from the TIMS and the mobility is recorded. The mobility is used to calculate the collisional cross section (CCS).

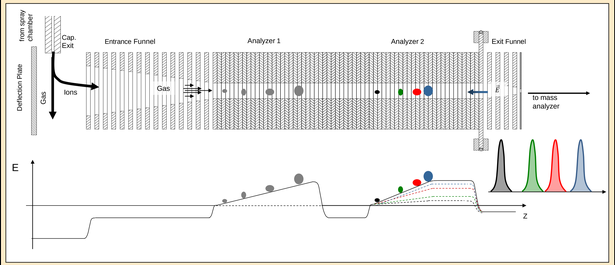


Fig. 2. (Above) Ions exit the ESI inlet capillary and traverse the first funnel, then they are accumulated in analyzer 1. The accumulated package of ions are transferred to analyzer 2 where they are trapped, separated, and serially eluted to the mass analyzer. Low mobility, high CCS ions are eluted first then higher mobility and lower CCS ions are eluted.

Methods

CCS Library Generation of Authentic Compounds: Authentic compounds were suspended in 80% methanol. The samples were analyzed using a Waters Acquity UPLC I-Class system coupled to a Bruker trapped ion mobility and quadrupole time-of-flight mass spectrometer (UHPLC-TIMS-QTOF-MS). Separations were performed using a Waters BEH, C18, 2.1 x 150 mm, 1.7 µm column and a linear gradient elution of 0.05% formic acid water: acetonitrile. The TIMS inverse reduced mobility range from 0.4 to 1.8 1/K_0 was calibrated using Agilent Tune Mix. Mass spectral data were acquired from m/z 100-2000 in negative electrospray ionization mode. Standard compounds were analyzed in triplicate and average CCS values were determined for the deprotonated molecular ion [M-H]$^-$. Adduct formations observed in the mobilograms were putatively annotated where possible.

Results

The experiments reported here focused on specialized metabolites including: flavonoids, isoflavonoids, and saponins. These compounds can be hydroxylated, methylated, and glycosylated at various positions. CCS values were recorded for over 150 unique compounds. The CCS average, standard deviation, and relative standard deviation (RSD) were calculated for each compound analyzed in triplicate. An generally acceptable RSD in the community is <2% for ion mobility spectrometry. All RSD of [M-H]$^-$ ions reported here were <0.40% and the overall average RSD was 0.10%. When possible other ions types were annotated: [2M-H]$^-$, [M-3H]$^-$, [M-H+HCOOH]$^-$, and [M-H+Na+HCOOH]$^-$.

Fig. 3. (Right) Reproducibility of CCS measurements recorded from authentic standards represented by %RSD. Of the annotated ion types, [M-H+Na+HCOOH]$^-$, had the greatest variability and [M-H]$^-$ had the greatest reproducibility.

Fig. 4. (Below) As m/z increases the general trend is that CCS also increases. More compact ions will have a lower CCS even when m/z increases and more spread out ions, like many of the [2M-H]$^-$ ions, will have a larger CCS than expected for the m/z measured.

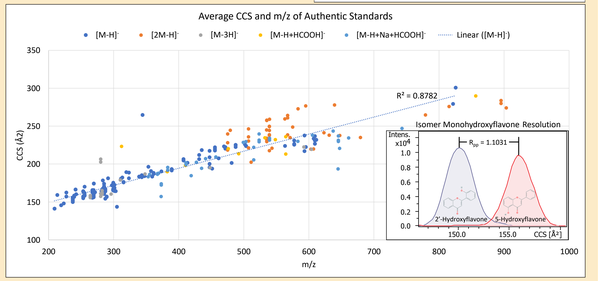


Fig. 5. (Below) To evaluate the robustness of our CCS measurements we analyzed a mixture of authentic standards in different conditions. (**Table 1**) We directly injected the compound mixture into the TIMS all at once simulating column coelution that could be potentially problematic for metabolomics workflows. We compared the CCS measured to the library. Rutin and 6-hydroxyflavone had a lower than expected performance while the remaining compounds matched with high confidence. (**Table 2**) We analyzed the authentic standards via UHPLC-TIMS-MS with and without matrix. The plant matrix represents a real-world application of CCS measurements from metabolomics samples. All compounds performed well.

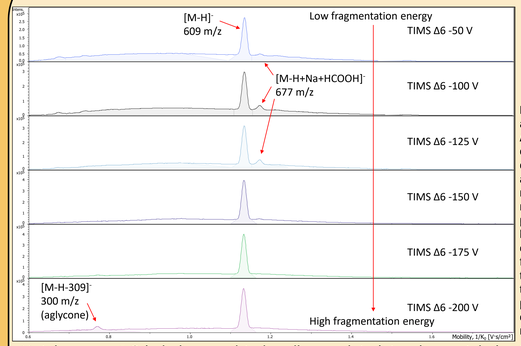
Table 1. Differences of CCS values between the compounds analyzed by direct infusion and by LC-TIMS-MS.

	Library CCS	Direct Infusion Mixture CCS	CCS % Difference
Rutin	231.05	232.37	0.56679%
Naringin	215.92	215.75	0.07876%
Naringenin	163.01	162.99	0.00818%
Chrysin	156.14	156.15	0.00854%
6-hydroxyflavone	155.91	155.61	0.19688%

Table 2. Differences of CCS analyzed with and without plant matrix.

Compounds	CCS Without Matrix	CCS With Matrix	CCS % Difference
Rutin	232.56	232.64	0.03585%
Naringin	215.66	215.90	0.11431%
Naringenin	162.91	162.95	0.02455%
Chrysin	156.14	156.25	0.07042%
6-hydroxyflavone	156.05	155.94	0.07265%

Results & Conclusion



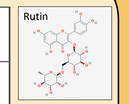
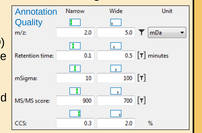


Fig. 6. (Left) By altering the TIMS Δ6 voltage, we can create in-TIMS collisions. This can alter the mobilogram by removing adduct peaks and with high enough energy cause fragmentation. This tool has allowed us to identify adduct peaks with greater confidence in the mobilograms.

Metaboscape 5.0 is being used to handle our data intensive metabolomics workflows with multiple modes of library matching such as: m/z, retention time, mSigma, MS/MS and CCS.

Fig. 7. (Below) MetaboScape 5.0 will annotate multiple ions for a single feature (bucket), suggest possible isomer buckets, and present an annotation quality (AQ) graphic (**right**) of the matching results for each bucket based on user settings. The interface allows for evaluation of metabolomics results of multiple replicates and treatments for a single study. An analyte list was created using the CCS library. The analyte list was used to identify compounds from our *Medicago truncatula* and *Glycine max* sample.



MetaboScape® 5.0

	RT [...	CCS [...	m/z meas.	M meas.	Ions	Name	Molecular For...	Annotations	AQ	Annotation Source	Boxplot	Mob...	I...	Monster Mix rep...	Monster Mix rep...	Monster Mix rep...
1	9.41	139.9	269.04555	270.05282	[M-H]-	Genistein	$C_{15}H_{10}O_5$		94	Bruker Summer Metabolo...		0.760	✓			
2	6.60	150.9	268.04555	270.05282	[M-H]-	6,7,4'-Trihydroxyisoflavone	$C_{15}H_{10}O_5$		94	Bruker Summer Metabolo...		0.760	✓			
3	7.52	157.7	253.05062	254.05789	[M-H]-	Daidzein	$C_{15}H_{10}O_4$		94	Bruker Summer Metabolo...		0.747	✓			
4	11.21	159.4	255.06612	256.07340	[M-H]-	Isoliquiritigenin	$C_{15}H_{12}O_4$		94	Bruker Summer Metabolo...		0.756	✓			
5	12.05	164.0	267.06442	268.07170	[M-H]-	Formononetin	$C_{16}H_{12}O_4$		94	Bruker Summer Metabolo...		0.779	✓			
6	18.37	292.8	811.44868	766.45014	[M-H+HCOOH]-	Caulioside C (3-Glu(1-2)Ara Hederagenin) (...	$C_{41}H_{68}O_{13}$		94	Bruker Summer Metabolo...		1.438	✓			
7	8.27	167.6	283.06028	284.06756	[M-H]-	Glycitein	$C_{16}H_{12}O_5$		94	Bruker Summer Metabolo...		0.798	✓			
8	11.50	165.0	281.04500	282.05228	[M-H]-	Pseudobaptigenin	$C_{16}H_{10}O_5$		94	Bruker Summer Metabolo...		0.786	✓			
9	6.60	240.5	269.04561	270.05289	[M-H]-	6,7,4'-Trihydroxyisoflavone	$C_{15}H_{10}O_5$		94	Bruker Summer Metabolo...		1.186	✓			
10	23.28	234.2	559.31264	514.31417	[M-H+HCOOH]-	Glc-octadecatrienoyl-sn-glycerol (isomer ...	$C_{27}H_{48}O_9$		94	Bruker Summer Metabolo...		1.141	✓			
11	4.10	218.8	481.10914	436.11094	[M-H+HCOOH]-	Daidzin	$C_{21}H_{20}O_9$		94	Bruker Summer Metabolo...		1.061	✓			
12	4.10	218.6	415.10378	416.11106	[M-H]-	Daidzin	$C_{21}H_{20}O_9$		94	Bruker Summer Metabolo...		1.062	✓			
13	23.24	233.8	476.27865	477.28593	[M-H]-	1-(9Z,11Z-Octadecadienoyl)-2-hydroxy-sn-...	$C_{26}H_{46}NO_7P$		94	Bruker Summer Metabolo...		1.038	✓			
14	17.62	301.4	795.45321	796.46042	[M-H]-	3-Gal(1-2)Glc Soyasapogenol B (NMR)	$C_{42}H_{68}O_{14}$		94	Bruker Summer Metabolo...		1.480	✓			
15	5.40	222.3	431.09855	432.10613	[M-H]-	Genistin	$C_{21}H_{20}O_{10}$		94	Bruker Summer Metabolo...		1.076	✓			
16	22.77	241.2	564.33126	565.33854	[M-H]-	1-Hydroxy-2-(9Z,11Z,15Z-octadecatrienoy-...	$C_{27}H_{47}NO_9P$		94	Bruker Summer Metabolo...		1.176	✓			
17	18.70	314.8	939.49529	940.50228	[M-H]-	3-Rha(1-2)Gal(1-2)Glc Soyasapogenol E (...	$C_{48}H_{76}O_{18}$		94	Bruker Summer Metabolo...		1.549	✓			
18	15.35	290.3	841.45749	842.46476	[M-H]-	Hex-hex Hederagenin (+ formic acid) (PUT)	$C_{42}H_{70}O_{16}$		94	Bruker Summer Metabolo...		1.426	✓			
19	19.01	324.0	1067.54152	1068.54950	[M-H]-	3-Rha-Gal-Glu(6-2)-DDMP Soyasapogenol...	$C_{54}H_{84}O_{23}$		94	Bruker Summer Metabolo...		1.587	✓			
20	7.18	157.7	253.05073	254.05801	[M-H]-	7,4'-Dihydroxyflavone	$C_{15}H_{10}O_4$		94	Bruker Summer Metabolo...		0.747	✓			
21	5.40	213.7	431.09874	432.10601	[M-H]-	Genistin	$C_{21}H_{20}O_{10}$		94	Bruker Summer Metabolo...		1.034	✓			
22	12.98	164.7	825.42877	826.43610	[M-H]-	3-Glu-2ß-Glu Medicagenic acid (NMR)	$C_{42}H_{66}O_{15}$		94	Bruker Summer Metabolo...		1.496	✓			
23	6.06	162.2	269.04465	270.05192	[M-H]-	3',4',7-Trihydroxyisoflavone	$C_{15}H_{10}O_5$		94	Bruker Summer Metabolo...		0.771	✓			
24	13.08	175.2	299.09252	300.09980	[M-H]-	Farrerol	$C_{17}H_{16}O_5$		94	Bruker Summer Metabolo...		0.837	✓			
25	6.66	139.9	269.04567	270.05294	[M-H]-	6,7,4'-Trihydroxyisoflavone	$C_{15}H_{10}O_5$		94	Bruker Summer Metabolo...		0.760	✓			
26	8.77	210.2	421.20793	422.21692	[M-H]-	Oct-1-en-3-yl Ara (1-6)Glu (NMR)	$C_{19}H_{34}O_{10}$		94	Bruker Summer Metabolo...		1.017	✓			

Conclusion

- We created a CCS library focused on plant natural products and its ability to improve plant metabolite identification confidence of was demonstrated.
- Isomeric compounds can be differentiated by TIMS even when they co-elute from the column. This can be used to generate clean MS/MS spectra.
- TIMS technology has the potential to improve our depth of coverage of the metabolome.

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