Generation of a Collisional Cross Section Library for Plant Metabolomics using Trapped Ion Mobility Spectrometry (TIMS)

Mark Schroeder[1], Sven W. Meyer[2], Aiko Barsch[2], Lloyd W. Sumner[1]

[1]Department of Biochemistry & Univ. of Missouri Metabolomics Center, 1201 Rollins Street, Columbia, MO 65211; [2]Bruker Daltonics, GmbH, Bremen, Germany

Overview

- **Purpose:** Analyze authentic standards of plant compounds, record collision cross sections (CCS) in a searchable library, and Parallel Accumulation Serial Fragmentation (PASEF) analysis in plant extracts
- **Methods:** CCS values were recorded using UHPLC-TIMS-QTOF-MS/MS. Plants extracts were analyzed using PASEF and reduced separation times
- **Results:** A CCS library of plant natural products was constructed and improves identification confidence. Isomeric and structurally similar compounds can be differentiated by TIMS even when they can not be differentiated by mass. TIMS and PASEF are improving our depth of coverage of the metabolome

Introduction

Metabolomics is used to quantitatively and qualitatively profile large numbers of small molecule metabolites within a biological sample. Analysis of the sample relies 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 and 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. The more orthogonal data acquired for each metabolites being analyzed, the more confidently it can be identified.

Ion mobility separates ions based upon their size to charge ratio in the gas phase and by analyzing the ion's mobility through a gas field. Trapped ion mobility (TIMS) is fast and compact permutation enabling higher ion mobility resolution and is suitable for coupling 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 UHPLC-TIMS-MS/MS metabolomics workflow. Sample is prepared, LC, TIMS, and MS data are acquired. Data is then compared with reference databases.

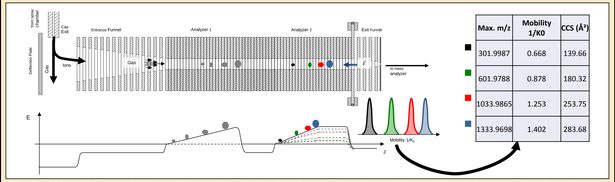
Instrumentation & Methods

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 continues to push the ions through the TIMS analyzer while an electric field gradient opposes the forward movement of the ions. The first stage TIMS traps ions while the second stage separates ions based upon their mobility. Ions are trapped where the drift gas and electric field forces on the ions are equal. The electric field gradient is gradually decreased to elute the ions from the TIMS and the mobility is recorded. The mobility is used to calculate the collisional cross section (CCS).

Methods for CCS Library Generation of Authentic Compounds

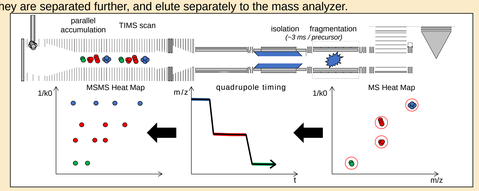
Authentic compounds were suspended in 80% methanol. The samples were analyzed using a Waters Acquity UPLC I-Class system coupled to a Bruker trapped ion mobility and quadrupole-time of flight mass spectrometers (UHPLC-TIMS-QTOF-MS/MS). Separations were performed using a Waters 2.1 x 150 mm, BEH, C18, 1.7 µm column and a linear gradient elution of 0.05% formic acid water: acetonitrile. TIMS inversed 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 CCS values were determined for the deprotonated molecular ion $[M-H]^-$. Adduct formations observed in the mobilograms were putatively annotated when possible.



Max. m/z	Mobility 1/K0	CCS (Å²)
301.9987	0.668	139.66
601.9788	0.878	180.32
1033.9865	1.253	253.75
1333.9698	1.402	283.68

Fig. 2. (Above) Ions exit the capillary and enter the first funnel, then trapped in analyzer 1. Ions are transferred to analyzer 2 where they are separated further, and elute separately to the mass analyzer.

Fig. 3. (Right) PASEF works by using mobility to select compounds as precursors for MS/MS and simultaneous fragmentation of coeluting compounds.



Results

We have focused on two groups of specialized metabolites: flavonoids and saponins. These compounds can be hydroxylated, methylated, and glycosylated at various positions. We have recorded CCS for over 150 unique compounds. The CCS average, standard deviation, and relative standard deviation (%RSD) were calculated for each compound analyzed in triplicate. An acceptable %RSD is <2% in the field of ion mobility spectrometry. All %RSD from our measurements were <0.50% and the overall average %RSD was 0.10%.

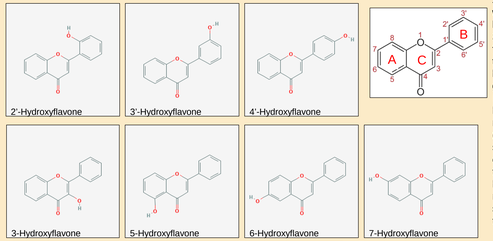


Fig. 4. (Left) Scaffold structures of flavonoids with carbons and rings labeled. **(Left)** Structures of 6 isomeric flavonoids that differ by the position of a hydroxyl functional group. **(Below)** 6-Hydroxyflavone and 6-hydroxyflavanone with rotated 3D models (magenta stars for spatial reference). The out-of-plane shift seen in the B ring of 6-hydroxyflavanone results in a detectable size difference between the two compounds.

Compound Name	MW	Ion	CCS average (Å²)	CCS Stdev	CCS %RSD
2'-Hydroxyflavone	238.25	$[M-H]^-$	149.55	0.07810	0.0522%
3'-Hydroxyflavone	238.25	$[M-H]^-$	154.45	0.40501	0.2622%
4'-Hydroxyflavone	238.25	$[M-H]^-$	151.78	0.18610	0.1226%
3-Hydroxyflavone	238.25	$[M-H]^-$	151.79	0.18502	0.1218%
5-Hydroxyflavone	238.25	$[M-H]^-$	155.60	0.14295	0.0918%
6-Hydroxyflavone	**238.25**	**$[M-H]^-$**	**155.51**	**0.14107**	**0.0907%**
7-Hydroxyflavone	238.25	$[M-H]^-$	153.28	0.05568	0.0363%
6-Hydroxyflavanone	**240.07**	**$[M-H]^-$**	**156.51**	**0.15133**	**0.0966%**

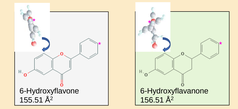
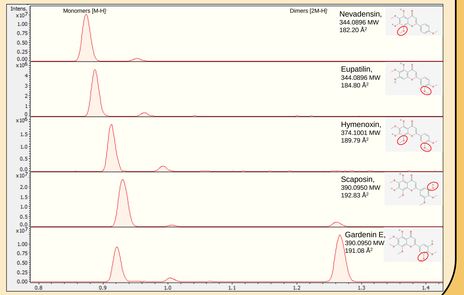


Table 1. (Left) Data and statistics of recorded CCS for hydroxyflavones and hydroxyflavanone (in green).

Fig. 5. Structurally similar modified flavonoids can be differentiated by comparing changes in structure to changes in CCS. This is enabling the relationship between molecular structure and its influence on CCS. This information will be further used for machine learning and prediction of CCS for known and unknown compounds. Some compounds result in multiple ion species (monomers and dimers) during TIMS analyses which can be used to differentiate otherwise similar compounds.



Results & Conclusion

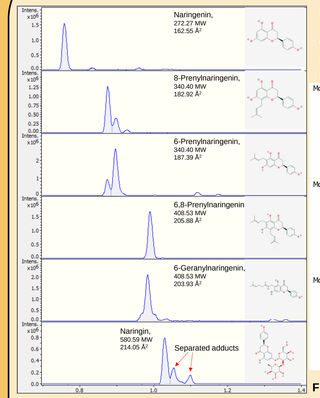


Fig. 6. (Above) A comparison of Naringenin with various modified functional groups further demonstrates the complexity of the mobilograms. The mechanism and resolution of TIMS analysis allows us to separate the target compound from adducts even when there are slight size and shape differences.

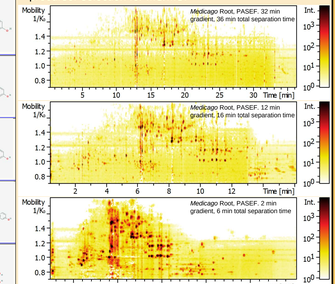
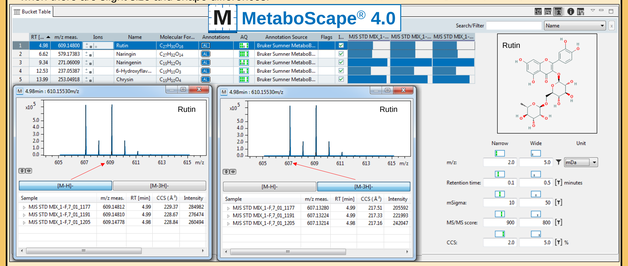


Fig. 7. (Below) We analyzed *Medicago truncatula* root via LC-TIMS-PASEF-MS/MS with a 32, 12, and 2 minute LC gradient. With shortened separation times we still retained compound separation and a wide coverage of precursor selection for MS/MS.

Fig. 8. (Below) Bruker MetaboScape data processing software uses m/z, RT, mSigma (isotope pattern matching), MS/MS, and CCS for compound annotation. The annotation quality (AQ) is reported based on set parameters and displayed within the table. Here we also demonstrate the bucketing capabilities for combining multiple ion types for a single compound (Rutin).



Conclusion

- We are creating a CCS library emphasized on plant natural products which will improve identification confidence in metabolomics.
- Isomeric and structurally similar compounds can be differentiated by TIMS even when they can not be differentiated on mass alone.
- TIMS technology and PASEF is promising for improving our depth of coverage into the metabolome

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