METABOLOMICS2024

“Deep Matter” annotation using 4D-Metabolomics™ in *Glycyrrhiza uralensis* (東北甘草, 西北甘草, 炙甘草) used for Traditional Chinese Medicine

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Novel Aspect

LC-TIMS-MS separation and annotation workflow for isomeric and isobaric secondary metabolites from natural products

Introduction

Plant derived natural products have been used as traditional medicines for centuries. They contain secondary metabolites that often possess bioactivity that may be beneficial as therapeutics. Characterization of natural products necessitates in-depth mass spectrometry (MS) analysis. In addition to accurate mass, isotope pattern and fragmentation pattern, ion mobility adds an orthogonal measurement, collisional cross section (CCS) that is unique to the size and shape of a molecule. Trapped ion mobility spectrometry (TIMS) provides highly accurate CCS values for more confident annotations. Here, we demonstrate characterization of *Glycyrrhiza* metabolites using TIMS-MS.

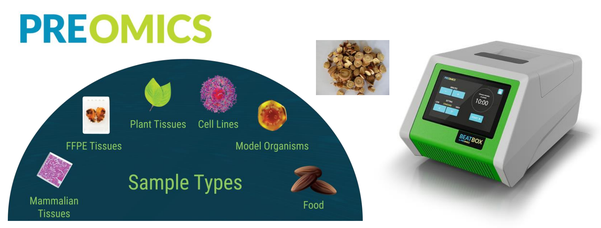
Methods

Root samples of *Glycyrrhiza uralensis* from three different regions were purchased from Uchida Wakanyaku Ltd.: Tohoku (東北甘草), Ningxia Hui (西北甘草), and Gansu – roasted (炙甘草). Samples were milled and extracted with 80% methanol using a BeatBox homogenizer (PreOmics) and centrifuged. Supernatants were analyzed in triplicate using a Bruker timsTOF HT with a VIP-HESI source and an Agilent 1290 liquid chromatograph on a reverse phase gradient. Data was acquired with default 4D-Metabolomics parameters which employs DDA-PASEF, in both positive and negative polarities. Data analysis was performed in MetaboScape® 2024b.

LC conditions

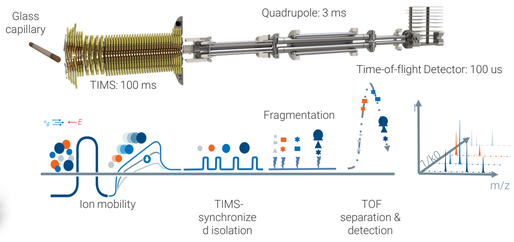
Mobile phase : Solvent A, H_2O including 0.1 % formic acid
: Solvent B, Acetonitrile including 0.1 % formic acid
Column : HSS 2 mm x 100 mm 1.7 µm (Waters)
Column oven : 40 °C
Gradient : Right
Injection : 5 µL
Flow rate : 0.4 mL/min

min	Solv. A	Solv. B
0.0	95	5
10.0	0	100
12.5	0	100
12.6	95	5
15.0	95	5

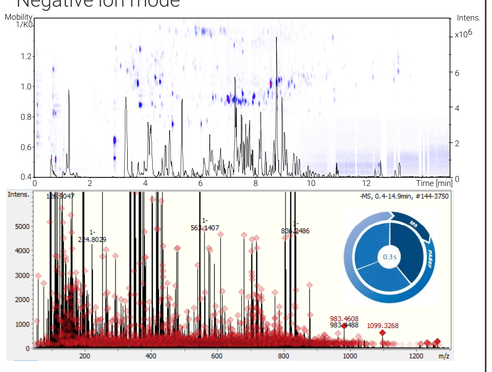


Instrument

timsTOF HT



東北甘草
Negative ion mode



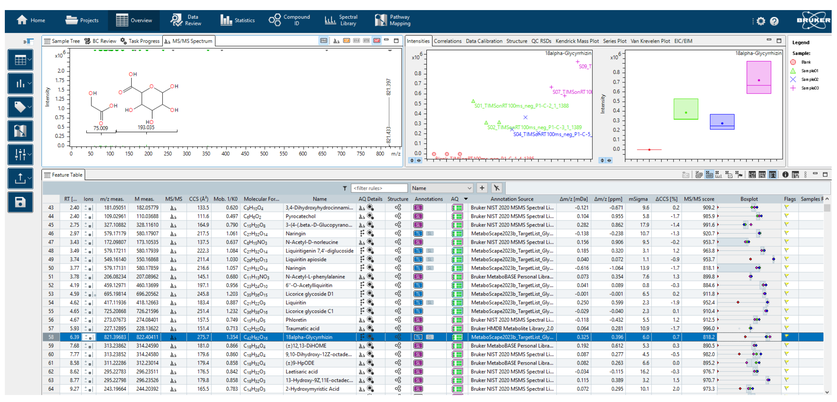
Software: MetaboScape

Truly Untargeted Pre-Processing
- Feature finding
- Deisotoping
- Retention time alignment,
- Grouping of adducts and neutral losses

Comprehensive & Automated Workflow
- Default methods for preprocessing and annotation
- Combining positive and negative mode feature tables

High Confidence Annotations
- Annotation Quality scoring
- *In silico* MS/MS matching
- CCS-Predict Pro for lipids and small molecules
- Correlation tables
- Biotransformer module

Interactive Exploration
- Highly interactive UI
- Overview first, details on demand
- Integrated statistics and visualizations



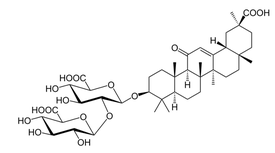
Annotation

TL Target List Annotation Excel-based

SL Spectral Library Annotation MS/MS Library-based

SF SmartFormula Element-based

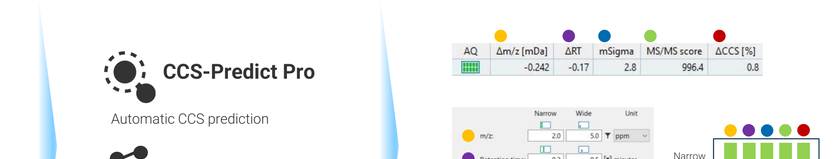
Preparing InChI or SMELIS of *G. uralensis* in Excel using KNApSAcK database.
Importing the list to MetaboScape
Annotating specialized metabolites with *in silico* fragmentation and predicted CCS value.



glycyrrhizin

InChI
InChI=1S/C42H62O16/c1-37(2)21-8-11-42(7)31(20(43)16-18-19-17-39(4,36(53)54)13-12-38(19,3)14-15-41(18,42)6)40(5)10-9-22(37)55-35-30(26(47)25(46)27(57-35)33(51)52)58-34-28(48)24(45)23(44)29(56-34)32(49)50/h16,19,21-31,34-35,44-48H,8-15,17H2,1-7H3,(H,49,50)(H,51,52)(H,53,54)/t19-,21-,22-,23-,24-,25-,26-,27+,28+,29+,30+,31+,34-,35-,38+,39-,40-,41+,42+/m0/s1

SMILES
C1[C@@H]([C@@H]([C@@]([C@]1([C@@H](C[C@@H]2C(=CC(=O)[C@]3([C@]2(CC[C@@]4([C@H]3CC(C4)(C)C)C(=O)O)C)C)C)C)O[C@H]5[C@@H]([C@@H]([C@H]([C@H](O5)C(=O)O)O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)C(=O)O)O)O)O)O)O)O



CCS-Predict Pro
Automatic CCS prediction

MetFrag
Automatic *in silico* fragmentation

AQ	Δm/z [mDa]	ΔRT	mSigma	MS/MS score	ΔCCS [%]
	-0.242	-0.17	2.8	996.4	0.8

	Narrow	Wide	Unit
m/z:	2.0	5.0	ppm
Retention time:	0.2	0.5	[▾] minutes
mSigma:	20	100	[▾]
MS/MS score:	700	500	[▾]
CCS:	2.0	4.0	[▾] %

Narrow tolerance
Wide tolerance

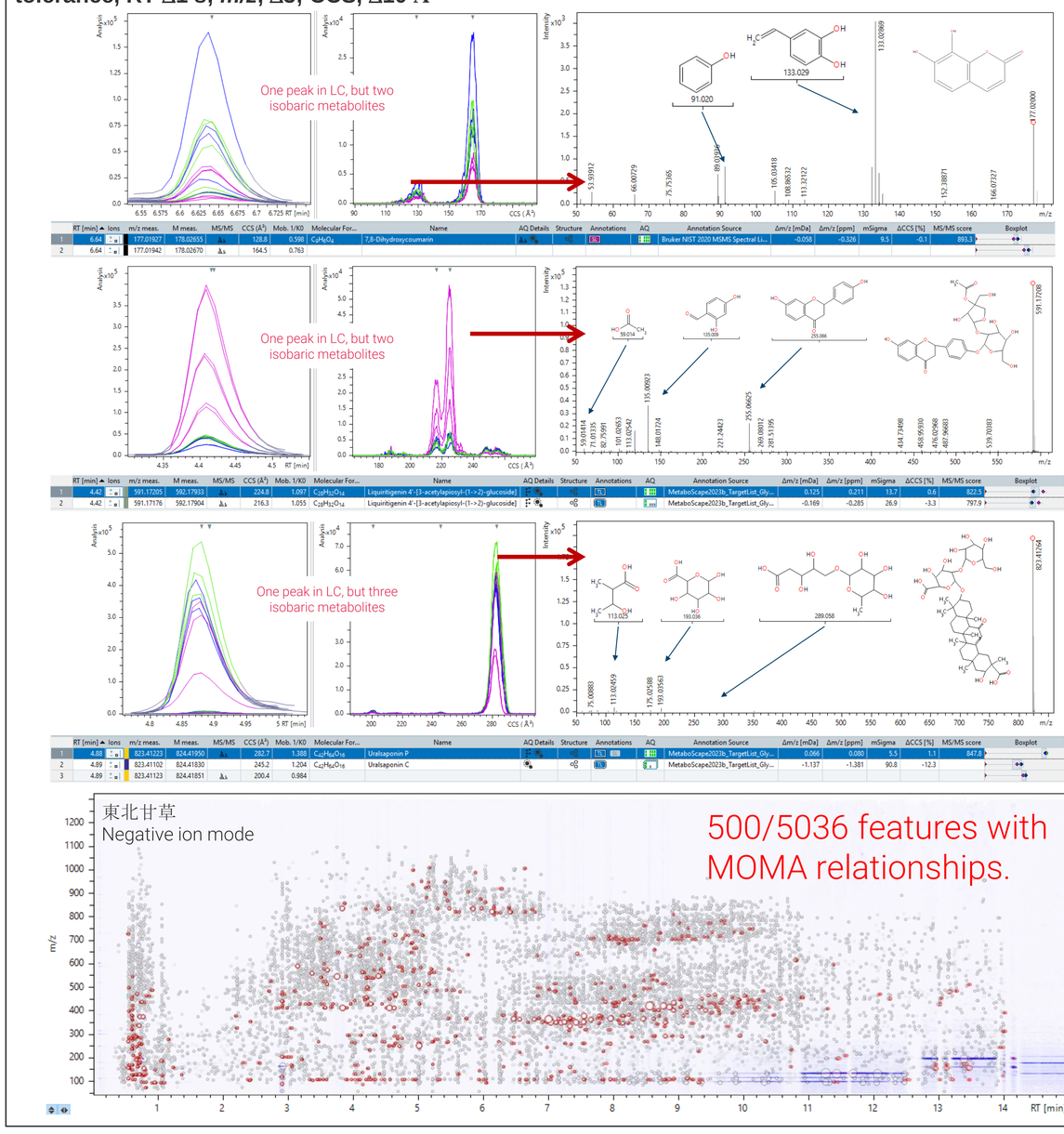
Fig. 1. Introduction of MS instrument, software, and how to annotate metabolites using MetaboScape functions.

Summary of annotations

	MS #	MS/MS #	MOMA #	MS ≤ 5 ppm	MS, MS/MS ≤ 5 ppm, ≥ 500 score	MS, CCS ≤ 5 ppm, ≤ 4%	MS, MS/MS, CCS ≤ 5 ppm, ≥ 500 score, ≤ 4%
Positive	9481	6943	900	1643	1483	1108	1054
Negative	10355	7573	1230	971	852	331	317

MOMA: easy to find isomeric and isobaric metabolites

tolerance, RT Δ1 s; *m/z*, Δ3; CCS, Δ10 Å



Results

Data processing resulted in 9481 and 10355 features and 6943 and 7573 MS/MS spectra, in positive and negative mode, respectively. Finally, 2439 features were annotated by merging data from both polarities. Principal component analysis (PCA) showed clear separation between 東北甘草 and 西北甘草, and 炙甘草, suggesting differences in metabolites depended on roasting. Features were annotated with open-source natural product database, KNApSAcK, containing InChI or SMILES to generate CCS values using CCS-Predict Pro, and *in silico* fragmentation patterns using MetFrag. Annotations were inspected manually to confirm accuracy. The separation of isomeric and isobaric metabolites using TIMS increases the number of metabolites detected. MOMA (Mobility-Offset Mass-Aligned) characterized pairs of isomer and isobaric metabolites. These metabolites were detected at the same retention times, but were completely separated in TIMS regardless of their size.

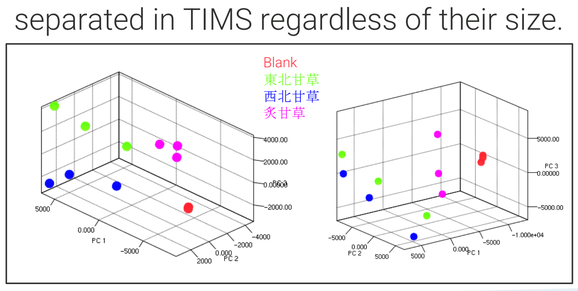


Fig. 2. Result of PCA in positive (left) and negative ion mode (right).

Fig. 3 Summary of annotations using MetaboScape and examples of characterized isomer/isobaric metabolites using MOMA function.

Summary

- TIMS separates small molecules regardless of their size and characterizes isomeric and isobaric metabolites, which are not separated under the LC conditions.
- MetaboScape using Target List including InChI or SMILES annotated specialized metabolites including 18α-glycyrrhizin, a representative metabolites in *Glycyrrhiza uralensis*.

Conclusion

- 4D-Metabolomics™ using timsTOF is a strong tool for unearthing Deep Matters.
- TimsTOF is useful for metabolomics in plants including Traditional Chinese Medicine research.

4D-Metabolomics™