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LC/MS – HRMS DIA Workflow – Food and Beverage

HRMS DIA Workflow for Combined Targeted and Untargeted Compound Identification in Metabolomics

Written by:

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Abstract

The study of untargeted food metabolomics has been gaining interest recently due to the increased awareness for healthier diet that calls for non-toxic and natural food additives. However, the identification of unknown metabolites is a major bottleneck as most studies rely on using databases and focuses only on compounds of well-studied pathways. In this Technology Brief, a straightforward yet comprehensive solution is proposed to overcome the challenge – by focusing on a group of related compounds with similar core structure, illustrated here with betacyanins commonly found in edible plants, and adopting a data independent acquisition (DIA) workflow using liquid chromatography (LC) coupled with a high-resolution quadrupole time-of-flight (Q-TOF) mass spectrometer, Shimadzu LCMS-9030, both known and unknown compounds can be identified in a single analysis.

Betacyanins is commonly found in red beetroots or dragon fruits

Keywords:
HRMS, DIA, Q-TOF, metabolomics

Highlights

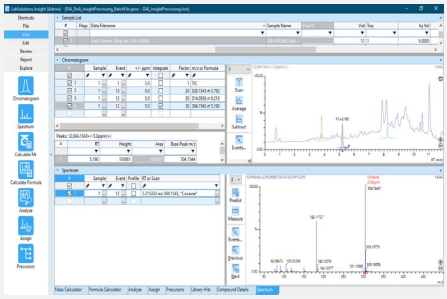
- *HRMS DIA workflow with LC-MS/MS is successfully established for combined targeted and untargeted metabolite identification*
- *Shimadzu's Insight ™ Explore software presents an ideal solution to deconvolute data and re-establish the links between precursor and product ion data*
- *By focusing on compounds with similar core structure, HRMS DIA workflow is flexible and adaptable to address the research area of interest*

Technologies Featured

Nexera Series

LCMS-9030 Q-TOF

LabSolutions Insight™ Explore



1. INTRODUCTION

In untargeted metabolomics, it can be challenging to identify the metabolites accurately within the complex matrices due to the vast number of compounds and degradation products. While this can be mitigated by using specific databases and focusing on compounds of well-studied metabolic pathways, such targeted approach paints only a partial picture of the metabolic environment. Thus, using data-independent acquisition (DIA) workflow is favorable due to its comprehensive acquisition that will allow data interrogation in both untargeted and targeted manner.

However, there are yet another two challenges to overcome: highly complex data generated from DIA analysis and the issue of unknown compound identification without prior knowledge. With Shimadzu's Insight™ Explore software, the first hurdle can be overcome as the software presents an ideal solution to deconvolute the data and re-establish the links between precursor and product ion data.

To tackle the issue of unknown compound identification, the strategy is to focus on a specific class of metabolic compounds which share a common core structure as they yield similar product ion patterns after fragmentation. This enables accurate identification even without the help of databases or reference ions spectra. In this study, the metabolic group of betacyanins was chosen to illustrate the established workflow.

2. EXPERIMENT

2.1 Sample System

Betacyanins are a specific group of tyrosine derived pigments known for their red colour. Traditionally, betacyanins can be extracted from diverse edible plant material such as red beetroot, amaranthus, pitaya (dragonfruit), or rose, to be used as natural food colourants. Recently, there is an increased interest in the analysis of betacyanins to meet stricter food and pharmaceutical regulations and due to their recently uncovered metabolic activities as antioxidant, as well as their benefits for chemoprevention and anti-inflammation.

Since all betacyanins share a common core structure, they can be studied by untargeted DIA analysis to identify not only the known betacyanins and their degradation products, but also possible new betacyanin derivatives as well.

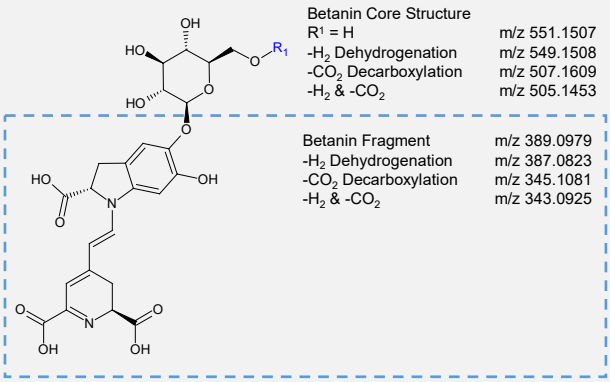


Figure 1: Betacyanin core-structure

2.2 Experimental Condition

Liquid extracts of red beetroot and red pitaya were separated on Nexera X2 LC system equipped with two binary pumps, degasser, autosampler, and a thermostatically controlled column unit. Chromatographic separation was carried out using Shimadzu's primary metabolite method package settings on a Discovery HS F5-3 column (2.1 mm x 150 mm, 3 µm particle size). The column temperature was maintained at 40ºC.

The mobile phase consisted of (A) 0.1% formic acid in H_2O and (B) 0.1% formic acid in acetonitrile. The gradient elution program of the mobile phase was 0-2 min (0% B), 2-5 min (0-25% B), 5-11 min (25-35% B), 11-15 min (35-95% B), 15-20 min (95% B), 20.1-25 min (0%B). The flow rate was set at 0.25 mL/min. The column oven temperature was set to 30ºC and sample volume injected was 3 µL.

Shimadzu LCMS-9030 Q-TOF mass spectrometer was used for data acquisition and external mass calibration was performed once prior to sample analysis. Data acquisition occurred in positive DIA mode with a precursor ion m/z range of 50-1000 and isolation window width of 32 m/z. Collision energy spread was set to 5-45 eV, and product ion scan range to m/z 10-1000. The conditions of the heated ESI source were as follow: drying gas (N_2) flow rate 10.0 L/min, nebulizing gas (N_2) flow rate 3.0 L/min, heating gas (zero air) flow rate 10.0 L/min, heat block temperature 400 ºC, DL temperature 250 ºC, and interface temperature 300 ºC.

All operations and acquisition were controlled with Shimadzu LabSolutions™ LCMS v5.99 SP2 software. Data analysis was performed using Shimadzu LabSolutions™ LCMS v5.99 SP2 and LabSolutions Insight™ Explore software.

3. RESULTS AND DISCUSSION

3.1. Data Acquisition with LCMS-9030 Q-TOF

Shimadzu LCMS-9030 Q-TOF instrument incorporates the patented ultrafast technologies and thus can operate at a fast data acquisition rate of 100Hz. Such fast-scanning speed allows the mass spectrometer to have multiple events within a short loop time, scanning across a larger m/z range for MS1 and several MS2 while still acquiring multiple data points across each peak. This is especially advantageous for data acquisition in DIA mode, as it enables fragmentation across the whole m/z range of interest while keeping the precursor isolation window relatively small. Furthermore, LCMS-9030 performs ion fragmentation by ramping collision energy across a large voltage spread, thereby achieving optimization in the product ion spectra. Together, these features are ideal to generate comprehensive data for untargeted analysis.

3.2 Untargeted Compound Identification

In DIA mode, the link between precursor ions and the corresponding product ions is lost due to larger isolation windows. Using Shimadzu's Insight™ Explore software with integrated Analyze function, DIA data can be effectively deconvoluted and hence able to reassign the product ions to their respective precursor ions. Additionally, the Formula Predictor functions can forecast molecular formulas of precursor and product ions with high confidence based on excellent accurate mass measurement. The use of these functions in combination effectively create a list of precursor and corresponding product ions, which can then be easily filtered against the common fragments. With this approach, 15 different betacyanins could be identified (Table 1).

Out of the 15 betacyanins, 12 were identified by the main betanidin fragment m/z 389.0979, while the remaining 3 were identified as degradation products of the former due to decarboxylation and dehydration. Degraded betacyanins could be identified by the decarboxy-neobetanin fragment with m/z 505.1453 as well as decarboxy-neobetanidin fragment m/z 343.0925. However, decarboxy Hylocerenin presents one exception, as decarboxylation occurs at the side-chain R1. This can be confirmed by the non-decarboxylated betanidin fragment m/z 389.0979.

Table 1: Identified Betacyanins

Name	Precursor m/z	Precursor Formula	Diff. (ppm)	Identifier Fragment
Betanin	551.1508	$C_{24}H_{26}N_2O_{13}$	-0.67	389.0979
Decarboxy NeoBetanin	505.1453	$C_{23}H_{24}N_2O_{11}$	-1.09	505.1453, 343.0925
Betanidin	389.0979	$C_{18}H_{16}N_2O_8$	-0.95	389.0979
Decarboxy NeoBetanidin	343.0925	$C_{17}H_{14}N_2O_6$	-1.37	343.0925
Phyllocactin	637.1512	$C_{27}H_{28}N_2O_{16}$	0.00	389.0979
Decarboxy NeoPhyllocactin	591.1457	$C_{26}H_{26}N_2O_{14}$	-0.64	505.1453, 343.0925
Hylocerenin	695.1930	$C_{30}H_{34}N_2O_{17}$	-0.52	389.0979
Decarboxy Hylocerenin	651.2032	$C_{29}H_{34}N_2O_{15}$	-0.48	389.0979
Lampranthin II	727.1981	$C_{34}H_{34}N_2O_{16}$	-0.88	389.0979
Bougainvillein	713.2034	$C_{30}H_{36}N_2O_{18}$	-0.19	389.0979
Sinapoyl Betanin	757.2087	$C_{35}H_{36}N_2O_{17}$	-0.55	389.0979
Unknown Betacyanin 1	769.1930	$C_{32}H_{36}N_2O_{20}$	-0.07	389.0979
Unknown Betacyanin 2	937.3070	$C_{42}H_{52}\ N_2O_{22}$	-0.29	389.0979
Unknown Betacyanin 3	963.3240	$C_{44}H_{54}N_2O_{22}$	-0.23	389.0979
Unknown Betacyanin 4	923.2714	$C_{44}H_{46}N_2O_{20}$	0.26	389.0979

After shortlisting the possible identified betacyanin derivatives and degradation products, the Assign function in Insight™ Explore software was used for structural elucidation and confident compound identification. The Assign function works based on shortlisting compounds with the same m/z value or by predicted precursor formula from the publicly available ChemSpider database. Molecular structures of these shortlisted compounds can then be in-silico fragmented and assigned to the acquired and deconvoluted product ion spectra. Based on the MS/MS spectra matching, a score will be assigned representing the confidence of positive compound identification.

Using this approach, 11 out of 15 compounds could positively be identified as betacyanin derivatives and degradation products (Figure 2). Furthermore, based on the product ion patterns, 4 possible unknown betacyanins could be identified, which have not been reported in literature previously (Figure 3).

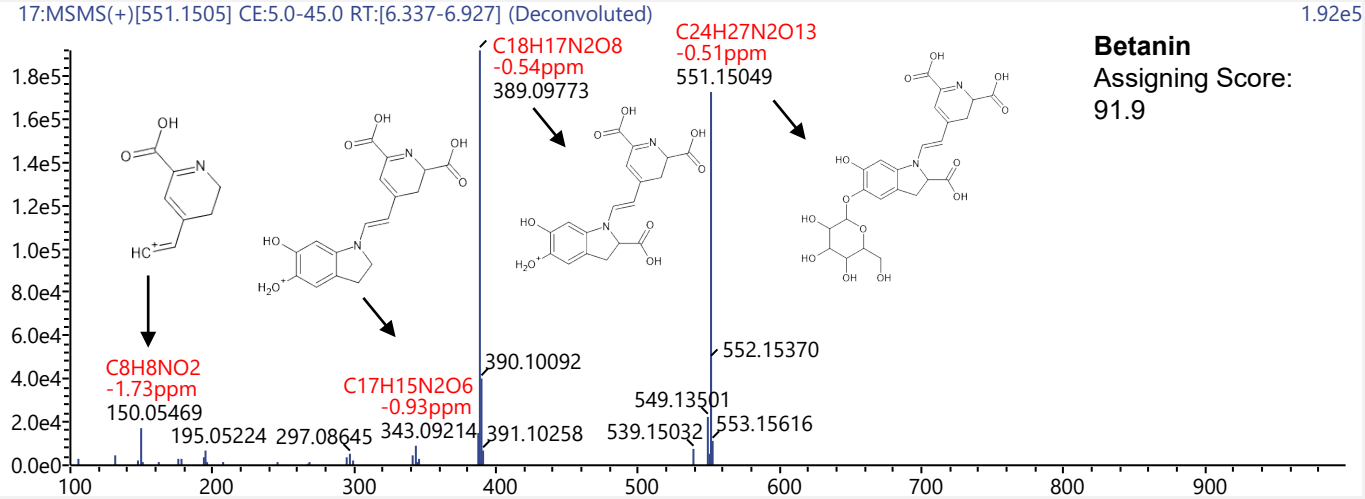
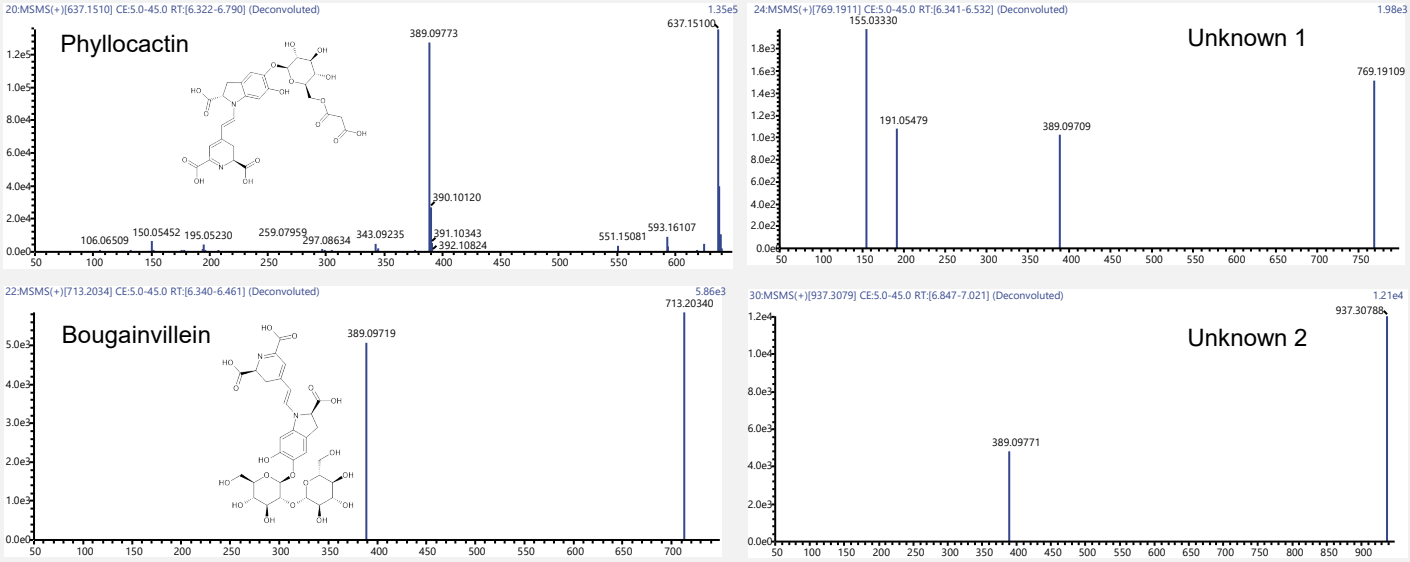


Figure 2: Structural fragment assigning with Betanin as an example



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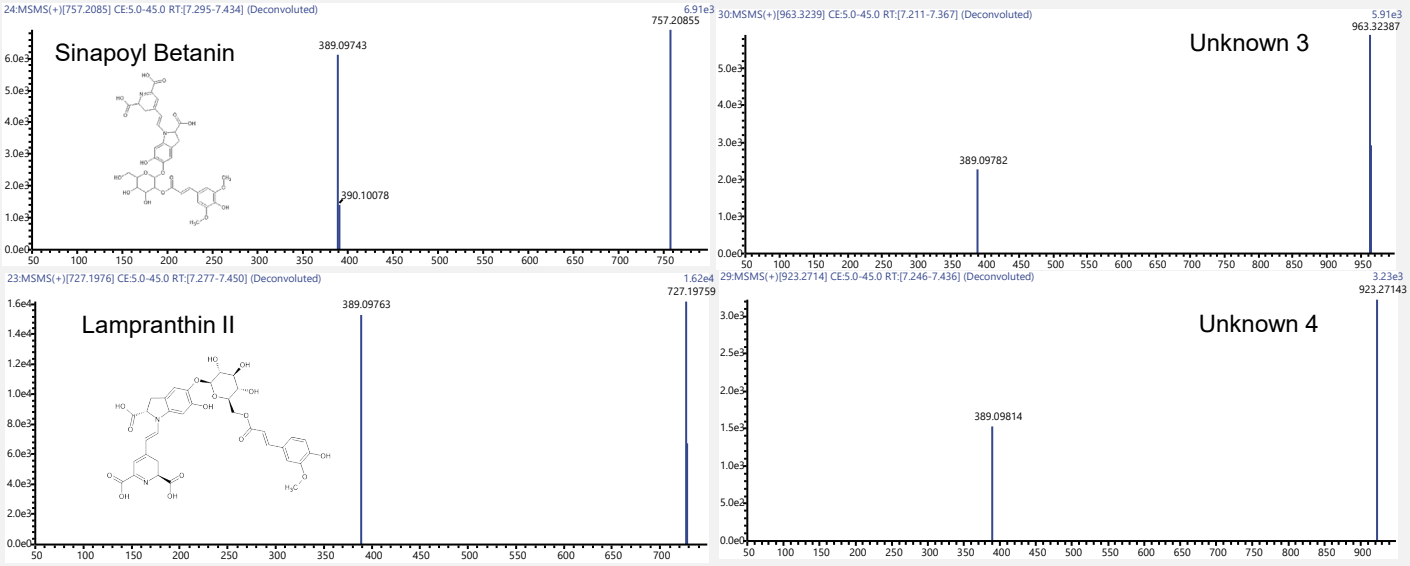

Figure 3: Product Ion Spectra of known and unknown betacyanins

3.3 Targeted Compound Identification

Data acquisition in DIA mode has the major advantage of acquiring product ion information off all ions by using large isolation windows for fragmentation. With this mode, not only it is possible to search and identify unknown compounds, but it can also be used to filter against a targeted compound list and even make use of the spectra product ion libraries. To this end, Shimadzu's exact mass database for primary metabolites was adapted to identify amino acids and other primary metabolites. With a combination of accurate mass, retention time and product ion information, 30 compounds could be confidently identified. Additionally, the use of Assign function in Insight™ Explore software, together with the matching of the experimental product ion spectra against the publicly available library spectra, can further enhance the confidence of metabolite identification.

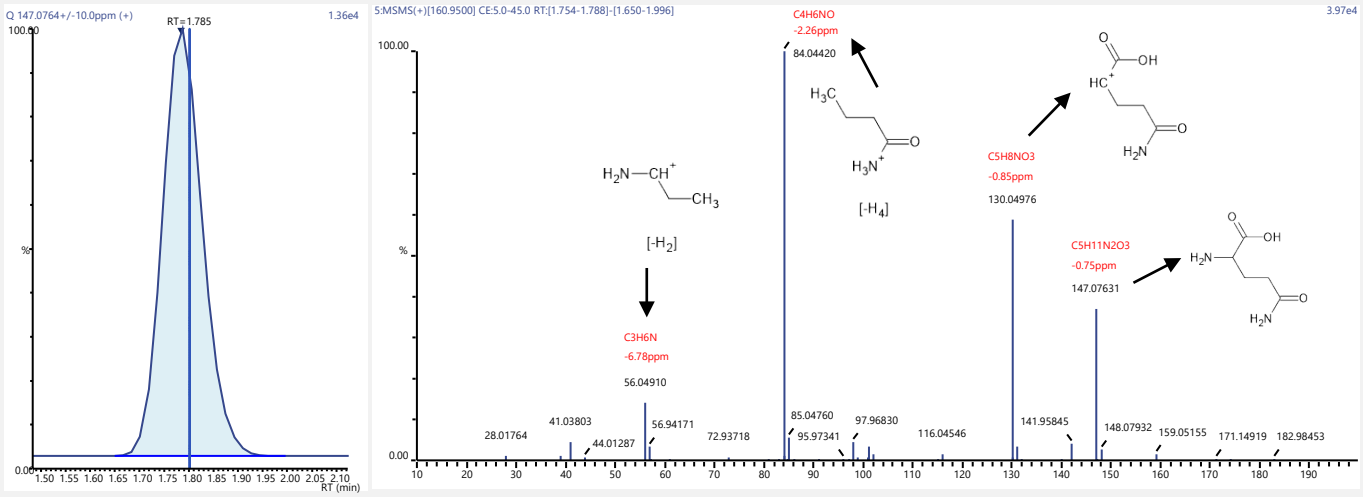

Figure 4: Glutamine as exemplary identified compound. XIC, fragment assigning for structural elucidation and compound confirmation and mirrored MSMS library spectrum with similarity index of 100.

4. CONCLUSION

Untargeted metabolomic studies face major bottlenecks in compound identification due to the large number of compound possibilities, complex matrices, and the need for extensive computational data processing. Using a HRMS DIA workflow on the Shimadzu LCMS-9030 QTOF instrument, in combination with Shimadzu's LabSolutions Insight™ Explore as a dedicated software solution, it was demonstrated that unknown compounds can be easily and confidently identified in parallel with targeted metabolite screening. By focusing on compounds with similar core structure, or common fragments, the presented workflow is flexible and adaptable to address the research area of interest.

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The LCMS-9030 delivers the accuracy needed for high-confidence identification of unknown compounds at an unprecedented level of stability. This is made possible by new technologies implemented in the Intelligent Temperature Control System and the UF-FlightTube™ that accurately offset the changes occurring to both internal and external environments. With the LCMS-9030, scientists can run more samples at longer calibration intervals with greater confidence and ease.

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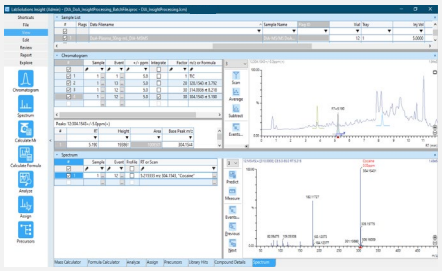

LabSolutions Insight Explore offers an intuitive and full-featured quantitative environment with significant qualitative enhancements. With its sophisticated peak detection algorithm, chromatographic features can be detected based on their accurate mass. The resulting peak displays enable ranked formula prediction with theoretical spectral matching. Using this integrated platform, scientists can leverage on the sensitivity, mass measurement accuracy and acquisition speed of the LCMS-9030 to provide an unambiguous answer to challenging analyses.

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