Development and implementation of a comprehensive fecal metabolites LC-MS library for dietary intervention studies using the Thermo Scientific Stellar mass spectrometer

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Keywords

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Goal

The primary goal of this application note is to demonstrate the capabilities of the Thermo Scientific™ Stellar™ mass spectrometer in developing and implementing a comprehensive fecal metabolites LC-MS library for high-throughput quantitation analysis. This study aims to:

1. **Showcase expedited method development:** Illustrate the intuitive and efficient method development process facilitated by the rapid scanning and high selectivity features of the Stellar mass spectrometer.
2. **Assess analytical performance:** Evaluate the sensitivity, accuracy, and selectivity of the Stellar mass spectrometer in quantifying fecal metabolites from mice subjected to various dietary interventions, ensuring minimal data loss even with low sample loads.
3. **Demonstrate high-throughput capabilities:** Highlight the instrument's ability to perform high-throughput analyses without compromising data quality, employing three distinct LC columns and various gradient methods.
4. **Validate compound detection:** Validate the robustness and reliability of the Stellar mass spectrometer in detecting and quantifying a wide range of metabolites, including challenging co-eluting isomers, using advanced MS^n fragmentation techniques.

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By achieving these goals, this application note aims to establish the Stellar mass spectrometer as a vital tool for advancing biomarker verification from discovery to validation in translational metabolomics and lipidomics research.

Introduction

The Thermo Scientific Stellar mass spectrometer (Figure 1) revolutionizes biomarker verification with its remarkable sensitivity and compound coverage, surpassing existing technologies tenfold and fivefold, respectively. By integrating the robust quantitative capabilities of triple quadrupole technology with the hyper-fast full scan MS^n acquisition capabilities of the dual-pressure linear ion trap technology, the Stellar mass spectrometer broadens its analytical scope to encompass a wider range of compounds. Enhanced single-ion detection facilitates precise quantitation even with minimal sample loads, minimizing the risk of data loss. This advanced approach distinguishes itself from traditional methods by significantly improving sensitivity and accuracy, thereby ensuring more reliable analytical results. Cutting-edge software tools, in addition, simplify the development, implementation, and data acquisition of complex targeted quantitative methods, eliminating the need for time-consuming replicate injections. These advancements establish the Stellar mass spectrometer as an indispensable tool for transitioning putative biomarker candidates from discovery to validation in translational metabolomics and lipidomics research.

This application note presents the development of a fecal metabolites LC-MS library on the Stellar mass spectrometer, using three distinct LC columns for expanding metabolite coverage. The library was then implemented to quantify selected fecal metabolites from mice subjected to different dietary interventions. The experiments here aimed to assess the ability of the Stellar mass spectrometer to conduct high-throughput quantitative analysis without sacrificing data quality, such as method sensitivity, accuracy, and selectivity. In addition, the work presented here describes an intuitive method development procedure facilitated by the fast-scanning mass spectrometry, where the user can obtain information on retention time, optimal fragmentation collision type and energy level, and optimal ion transmission conditions using multiple experiments [e.g., MS^1 full scan with polarity switching, and MS^n with Higher Energy Collisional Dissociation (HCD) and Collision Induced Dissociation (CID) fragmentation methods] within a single-injection method.

Experimental

Fecal Metabolites library, which is composed of pure analytical standards (500+ compounds), was sourced from MetaSci, Inc. (MetaSci.ca part number msifec0001). To prepare individual compound solutions, stock solutions were made using various pure solvent mixes according to the vendor's instructions.

Figure 1. The Thermo Scientific Stellar mass spectrometer

For non-polar compounds, pure methanol was utilized, while medium- and highly-polar compounds were dissolved in methanol-water mixtures at different ratios. Calibration curve mixtures were then created by combining aliquots of each standard into a series of dilutions (i.e., 0 nM – 20 µM). Three distinct LC columns were employed for this purpose: a Thermo Scientific™ Hypersil GOLD™ C18 reversed-phase HPLC column (2.1 × 150 mm, 1.9 µm), a Thermo Scientific™ Accucore™ 150 Amide HILIC HPLC column (150 × 2.1 mm, 2.6 µm), and a Thermo Scientific™ Acclaim™ Trinity P2 HPLC mixed-mode column (2.1 × 100 mm, 3 µm).

Fecal samples were collected from a study involving mice at the age of eight weeks. These mice were subjected to different diet interventions where each group was assigned one of three diets with varying fat sources: standard (soybean oil), SFA-rich (cocoa butter and soybean oil), and MUFA-rich (olive and soybean oils). Fresh fecal samples were obtained after 29 days under each condition (Figure 2). The described intervention was performed at the Gladstone Institute affiliated with the University of California, San Francisco. Metabolites were extracted using 80% methanol and separated with LC columns before analysis on the Stellar mass spectrometer.

The separation of targeted compounds was conducted using a Thermo Scientific™ Vanquish™ Horizon UHPLC system, with 2 µL injections of standard mixes or fecal extracts. The autosampler maintained a temperature of 5 °C throughout the process. LC-MS grade solvents were employed to prepare mobile phase solutions for each method as follows: mobile phase A consisted of 0.1% formic acid (FA) in water, while mobile phase B comprised 0.1% FA in methanol for the reversed-phase method. For the HILIC method, mobile phase A contained 10 mM ammonium formate + 0.1% FA in water, while mobile phase B was composed of 0.1% FA in acetonitrile. Lastly, for the mixed-mode method, mobile phase A consisted of 40 mM ammonium acetate in water, and mobile phase B contained 0.1% acetic acid in acetonitrile.

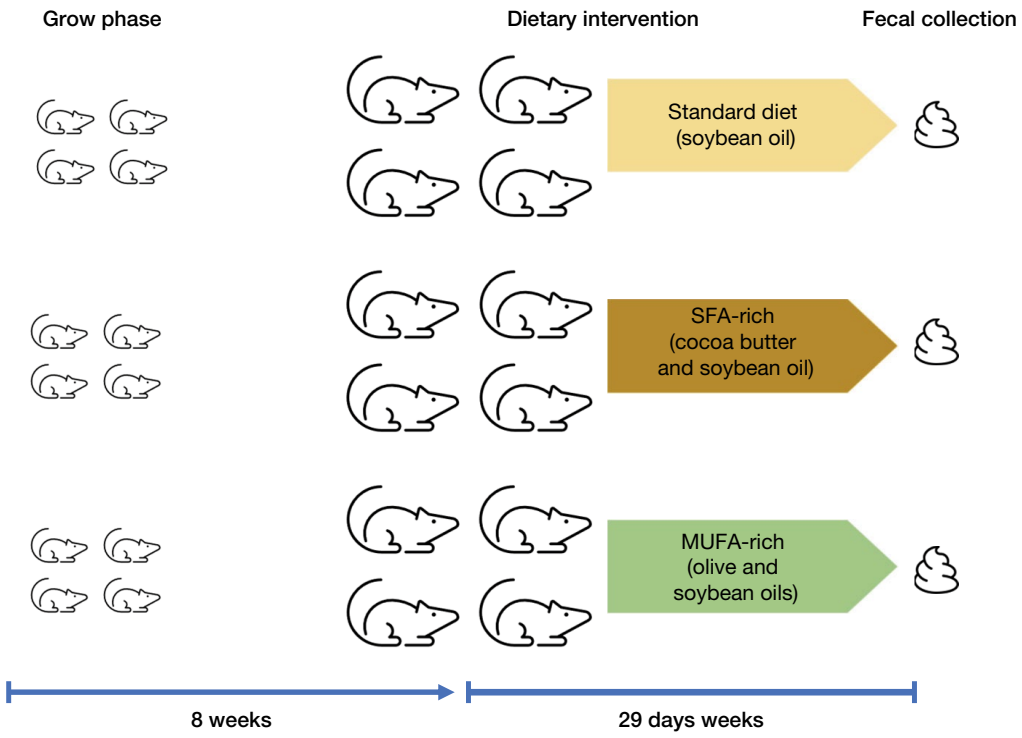

Figure 2. Experimental setup depicting the collection of fecal samples from 8-week-old mice subjected to varied diet interventions. Mice were assigned to three dietary groups: standard (soybean oil), SFA-rich (cocoa butter and soybean oil), and MUFA-rich (olive and soybean oils). Fresh fecal samples were collected after 29 days of each dietary condition.

The three methods were operated with two gradients each: a long gradient (15 min for RP, 24 min for HILIC, and 20.5 min for the mixed-mode method) and shorter gradients (5 min for RP, 12 min for HILIC, and 10.6 and 6.8 min for the mixed-mode method). The shorter gradients were implemented to assess the high-throughput quantitative performance of the Stellar mass spectrometer without sacrificing data quality. All data acquisition was performed using the Stellar mass spectrometer operated in compound characterization mode and then targeted quantitation using targeted MS^2 (tMS^2) and/or MS^3 (tMS^3) acquisition.

Results and discussion

Intuitive and efficient development of high-scale targeted methods

The breakthrough mass spectrometer exhibits rapid scanning capabilities (i.e., up to 140 Hz MS^2 and 40 Hz MS^3), full-scan MS^n acquisition, fast polarity switching (i.e., 5 ms +/- switching), and high sensitivity across a broad linear dynamic range. Moreover, it offers exceptional selectivity through MS^n fragmentation utilizing both HCD and CID techniques. These features streamline method development, allowing for efficient optimization of parameters for each compound, where the user can obtain information on retention time, optimal fragmentation collision type and energy level, and optimal ion transmission conditions using multiple experiments. Within a single method injection, users can conduct comprehensive experiments, including full-scan MS^1 with polarity switching, and tandem MS^n experiments employing alternating HCD and CID modes, thereby enhancing analytical flexibility and efficiency (Figure 3A).

High-throughput and sensitive targeted methods

Upon development of the analytical method, the Stellar mass spectrometer showcases its capability for rapid scanning, enabling the analysis of a multitude of analytes within a single-injection method. This is achieved by ensuring an adequate number of scans per peak, thereby enhancing accuracy and sensitivity. For instance, quantification of numerous fecal metabolites sourced from the MetaSci fecal library was conducted using the Stellar mass spectrometer subsequent to separation via Thermo Scientific LC columns, including reversed-phase, hydrophilic interaction liquid chromatography (HILIC), and mixed-mode columns (Figure 3B). Employing gradients of less than 25 minutes, all three methods exhibited high sensitivity and an extended linear dynamic range, spanning five orders of magnitude, through tMS^2 quantitation for the majority of analytes, while preserving peak quality. Furthermore, due to both the rapid scan speed and the high selectivity conferred by tMS^n quantitation of co-eluted isomers, it was feasible to shorten the LC gradients by half, thereby underscoring the instrument's versatility and efficiency (Figure 4).

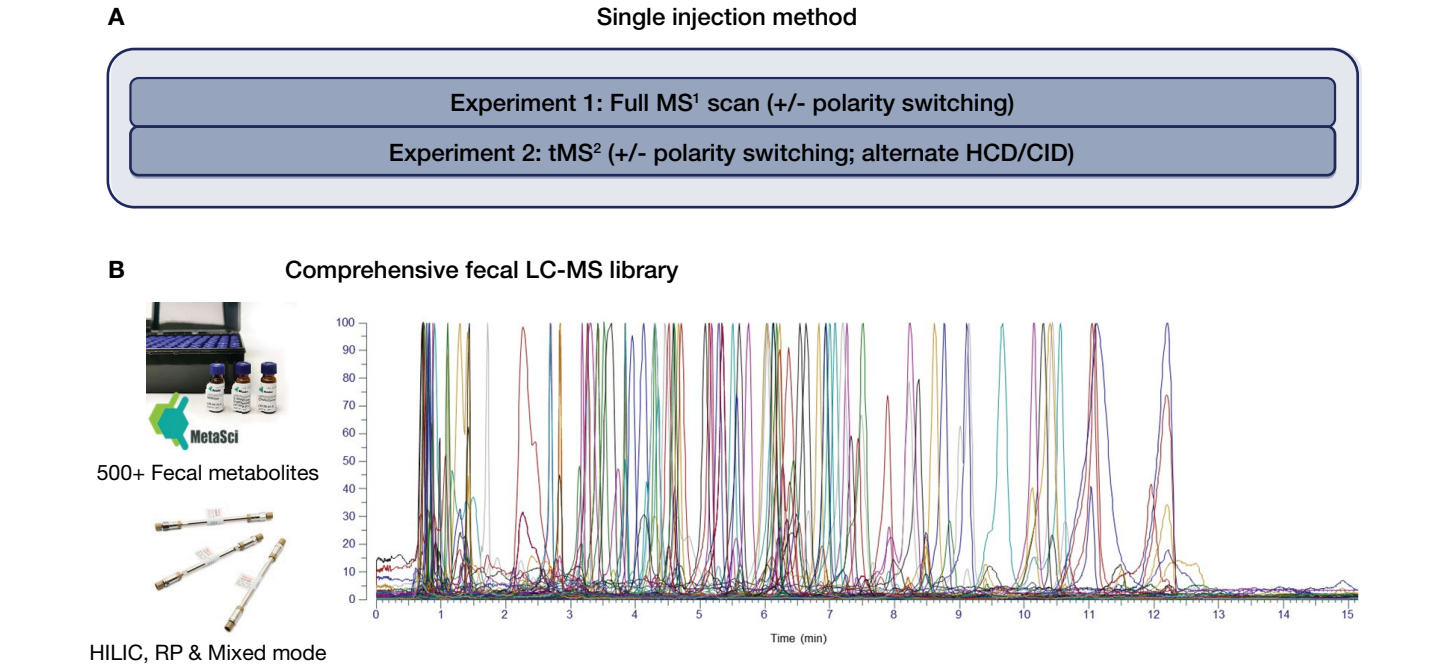

Figure 3. Intuitive method development via the breakthrough Stellar mass spectrometer's rapid scanning capabilities and high sensitivity with the capacity for comprehensive analysis of fecal metabolites obtained from the MetaSci fecal library following separation via Thermo Scientific LC columns (i.e., a reversed-phase, HILIC, and mixed-mode columns)

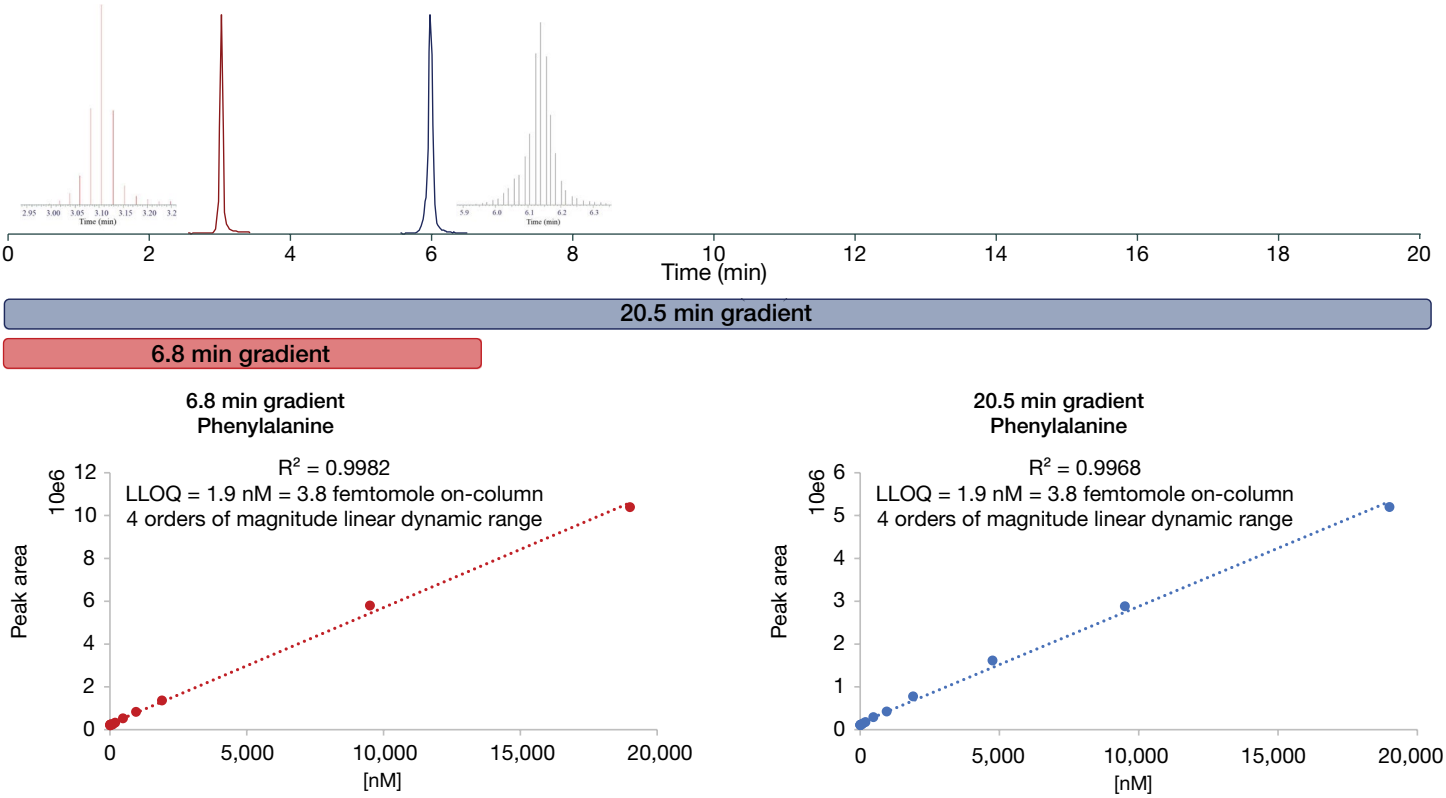

Figure 4. Illustration of the enhanced sensitivity and extended linear dynamic range achieved by employing gradients of less than 25 minutes on the mixed-mode column, along with the ability to shorten LC gradients by a third, facilitated by the rapid scan speed and high selectivity of MS² quantitation, highlighting the instrument's versatility and efficiency. Phenylalanine was selected as an example from the mixed-mode LC gradient.

The established methods were employed to measure the identified metabolites present in the MetaSci library within the fecal extracts of mice. Figure 5 presents a heatmap that displays a subset of these quantified metabolites from the fecal samples collected from mice across the three distinct dietary interventions.

Method robustness

Different fragmentation mechanisms available on the Stellar mass spectrometer were further assessed across diverse compounds, including numerous bile acids. This application note will demonstrate the fragmentation efficiency (HCD and CID) of a specific bile acid (i.e., deoxycholic acid – DCA) in comparison to counterparts found in the Thermo Scientific™ mzCloud™ advanced mass spectral library databases. Figure 6 illustrates closely aligned CID fragmentation profiles between the Stellar mass spectrometer and other platforms. However, notably enhanced HCD fragmentation was observed with the Stellar mass spectrometer, characterized by the detection of fragment ions at lower collision energies compared to alternative platforms.

In a single method, both full scan MS^1 and PRM-based experiments were conducted to quantify bile acids in the mice's fecal samples, aiming for increased annotation rates and accurate quantitation. The RP-LC based method exhibited high sensitivity (LLOQ 12.7 femtomoles on the column for most analyzed bile acids) and a linear dynamic range spanning 5 orders of magnitude (Figure 7A). Isotope-labeled bile acids were utilized as internal standards (IS) to ensure precise quantitation and assess data quality, reliability, and measurement robustness, with metrics such as retention time, mass accuracy, and signal response evaluated. Minimal chromatographic shifts and consistent signal responses were observed, indicated by a low % coefficient of variation for sample replicates, while reproducible peak areas were consistently achieved for all internal standards throughout the acquisition period (Figure 7B).

Data analysis indicated elevated concentrations of DCA and β-muricholic acid (β-MCA) bile acids in the SFA-rich diet compared to the MUFA-rich and standard diets, with the MUFA-rich diet resulting in higher levels compared to the MUFA-rich diet, as illustrated in Figure 7C. The two FA diets, however, have not significantly changed the levels of taurodeoxycholic acid (TDCA) in the mice fecal samples compared to the standard diet.

Methods selectivity

The proficient fragmentation capability of the Stellar mass spectrometer enables the detection of diagnostic MS^2 ions, facilitating the selective quantification of co-eluting isomers, particularly advantageous for high-throughput analyses employing short LC gradient-based methods. Figure 8 illustrates an exemplary application of utilizing MS^2 diagnostic ions for the selective quantification of co-eluting isomers.

nM	SFA-rich diet	MUFA-rich diet	Standard diet
L-Tyrosine	93869	92406	79208
Citrulline	769	754	274
Adenine	2791	1745	2104
Methylguanidine	5492	1185	1286
Inosine	2889	873	1511
L-Phenylalanine	3331	5448	3486
Proline	11918	13195	4602
Thiamine	1784	2014	161
p-Hydroxymandelic acid	63	43	122
Carnitine	126	10	289
Choline	209	161	216
Atenolol	20	1	83
Kynurenic acid	127	9	302

Figure 5. Heatmap illustrating a subset of quantified metabolites from fecal samples collected from mice subjected to three distinct dietary interventions, measured using the established HILIC LC-MS method on the Stellar mass spectrometer and the MetaSci fecal library. Reported values represent the average of measured values in the four mice fecal samples per group.

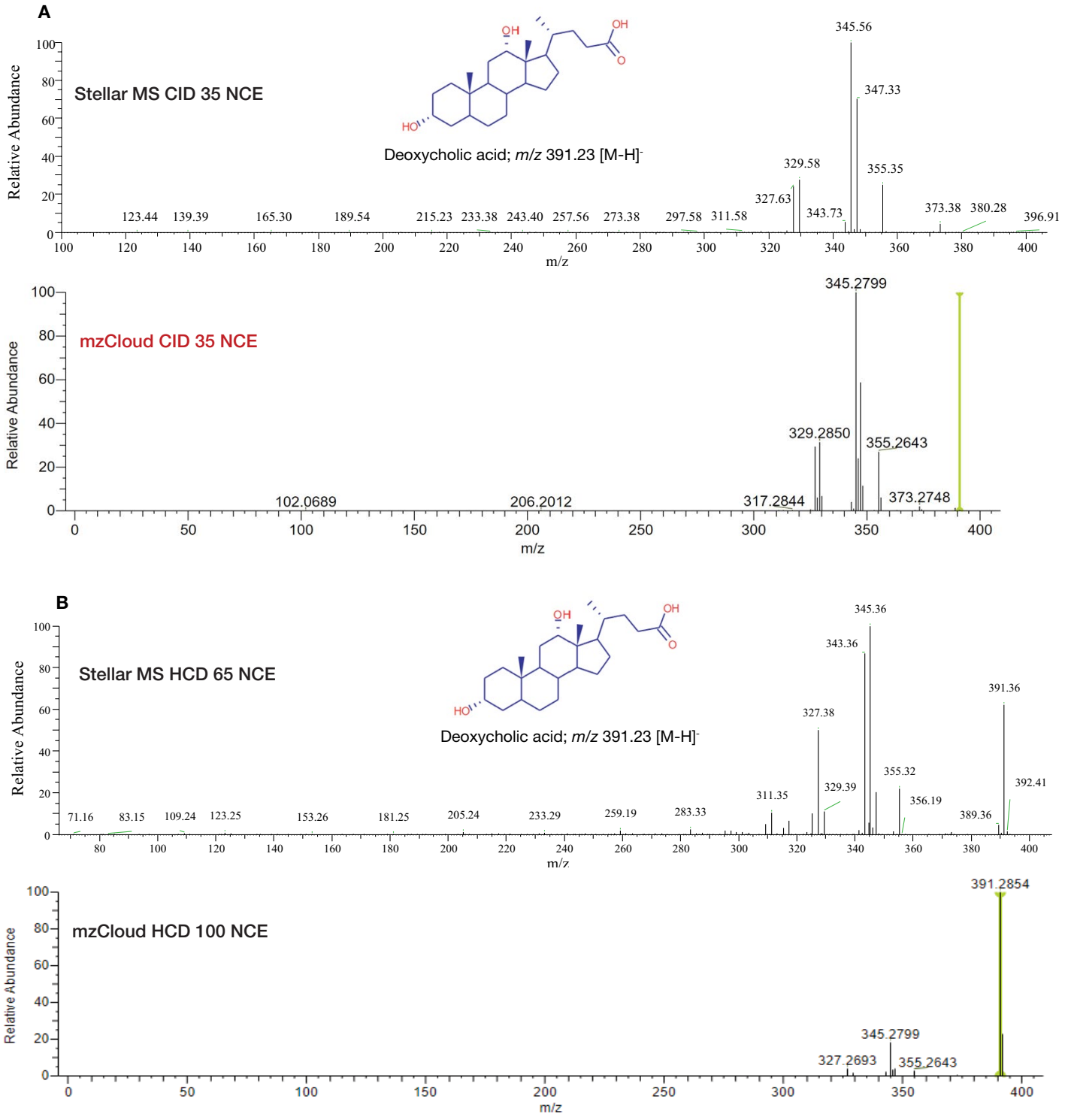

Figure 6. Comparison of CID fragmentation profiles between the Stellar mass spectrometer and other platforms for diverse compounds, with a focus on deoxycholic acid (DCA). The figure demonstrates closely aligned CID fragmentation profiles across platforms. However, the Stellar mass spectrometer exhibits notably enhanced HCD fragmentation, as evidenced by the detection of fragment ions at lower collision energies compared to alternative platforms.

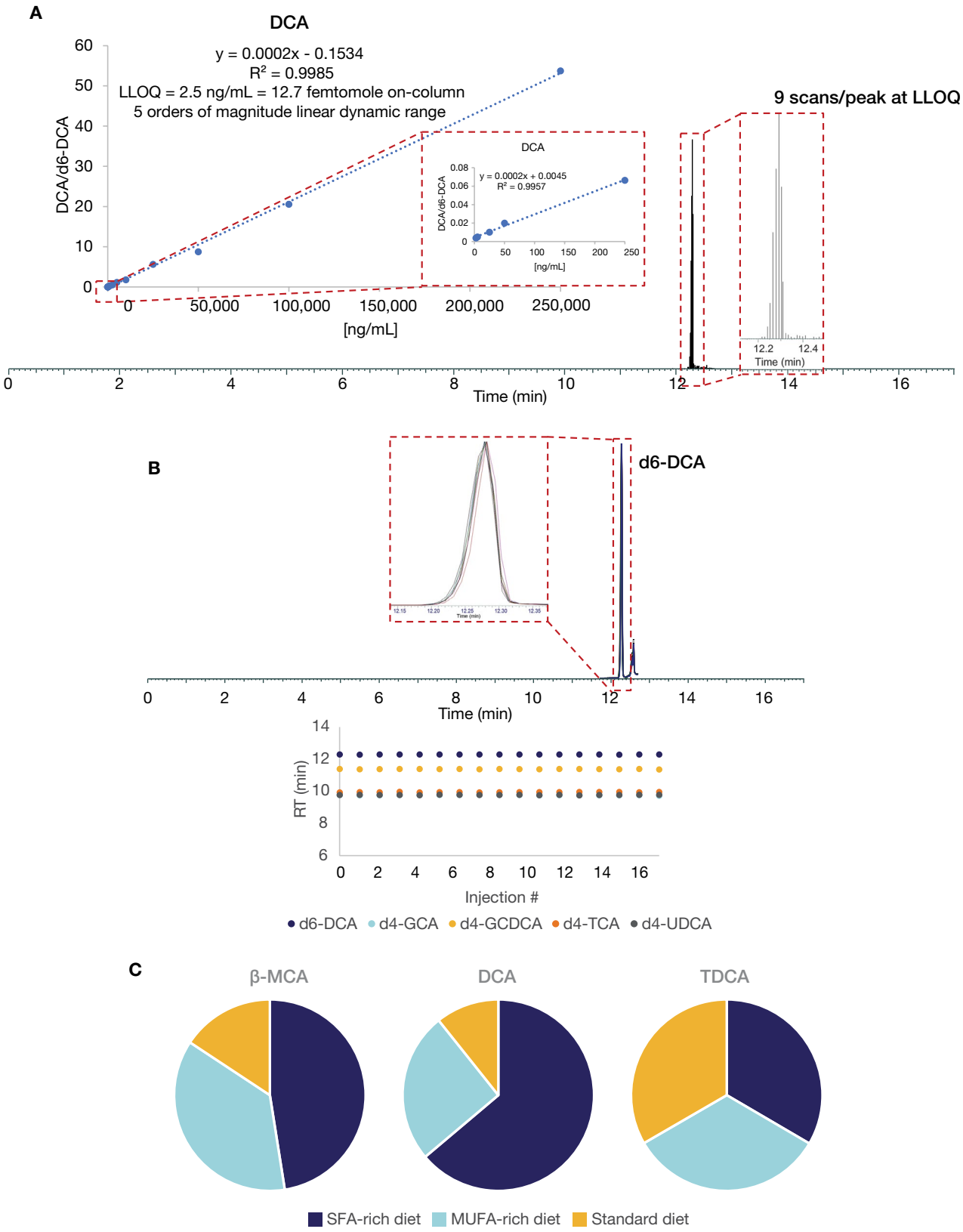

Figure 7. (A) The LC-MS method performed on the Stellar mass spectrometer demonstrates high sensitivity, with a lower limit of quantitation (LLOQ) of 12.7 femtomoles on-column for most analyzed bile acids, and a linear dynamic range spanning 5 orders of magnitude; (B) Isotope-labeled bile acids served as internal standards (IS) to ensure precise quantitation and evaluate data quality, reliability, and measurement robustness; (C) Concentration of selected bile acids in the mice fecal samples; n=4 per group. Deoxycholic acid (DCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), ursodeoxycholic acid (UDCA), β-muricholic acid (β-MCA), and taurodeoxycholic acid (TDCA).

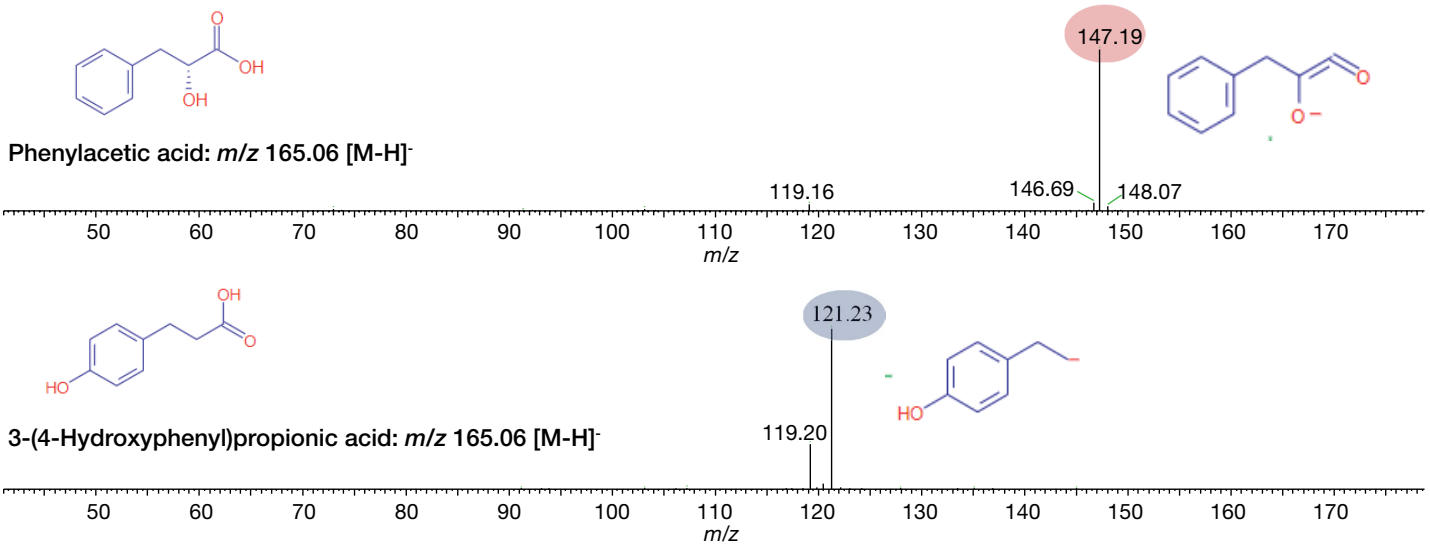

Figure 8. Demonstration of the utilization of diagnostic MS^2 ions for selective quantification of co-eluting isomers, enabled by the proficient fragmentation capability of the Stellar mass spectrometer. This approach proves especially beneficial for high-throughput analyses utilizing short LC gradient-based methods. CID fragmentation of the two isomers results in diagnostic ions per compound (i.e., *m/z* 147.19 for phenylacetic acid, and *m/z* 121.2 for 3-(4-hydroxyphenyl)propionic acid in negative polarity).

The Stellar mass spectrometer not only facilitates rapid fragmentation and detection at the MS^2 level (up to 140 Hz) to enhance selectivity of co-eluting isomers, but it also delivers efficient MS^3 level fragmentation. In this investigation, chromatographic separation of the bile acid isomers glycochenodeoxycholic acid (GCDCA) and glycodeoxycholic acid (GDCA) initially required ion-pairing reagents, as depicted in Figure 9A. However, this method was lengthy and entailed high consumption of organic solvents, utilizing a reversed-phase LC gradient for elution of the isomers from the column. In environments emphasizing high throughput and sustainability, users may prefer methods with fewer additives such as ion-pairing reagents, lower flow rates, and shorter gradients where feasible. The elimination of additives and/or reduction in flow rate resulted in the chromatographic co-elution of the two isomers (Figure 9B), preventing the shortening of the analytical gradient. MS^2 fragmentation of the isomers produced similar fragmentation patterns (Figure 9C), hindering the use of PRM quantitation. Fortunately, the novel instrument's MS^n capability enables the detection of diagnostic ions for each isomer, facilitating accurate quantitation in high-throughput analyses without the need for ion-pairing reagents or high organic solvent flow rates (Figure 9D).

Targeted discovery omics

The advancement of the Stellar mass spectrometer makes it an excellent solution for a novel metabolomics workflow that was developed at Thermo Fisher Scientific to bridge the gap between discovery experiments and verification using targeted metabolomics (Figure 10). Key improvements in the Stellar mass spectrometer, such as increased scan speed and sensitivity, have enhanced the workflow's capability for deep and accurate quantitation of large-scale compounds observed in untargeted analyses. This facilitates the transition from discovery to validation phases for all putative biomarkers, accelerating the translation of metabolomic findings into actionable insights for precision medicine, diagnostics, and therapeutic interventions.

The novel targeted discovery omics workflow is an end-to-end process that enables the analysis of HRAM Orbitrap discovery data using Thermo Scientific™ Compound Discoverer™ software for comprehensive compound detection and annotation by searching against the mzCloud library. Once the HRAM data is analyzed, the detected compounds are converted into a spectral library, which is then used to build a tMS^2 (or PRM) acquisition method for the Stellar mass spectrometer via a novel software called PRM Conductor. PRM Conductor software can also utilize the background matrix of the discovery data to generate a dynamic retention time correction algorithm (i.e., Adaptive RT), making the transition from discovery to validation a seamless procedure.

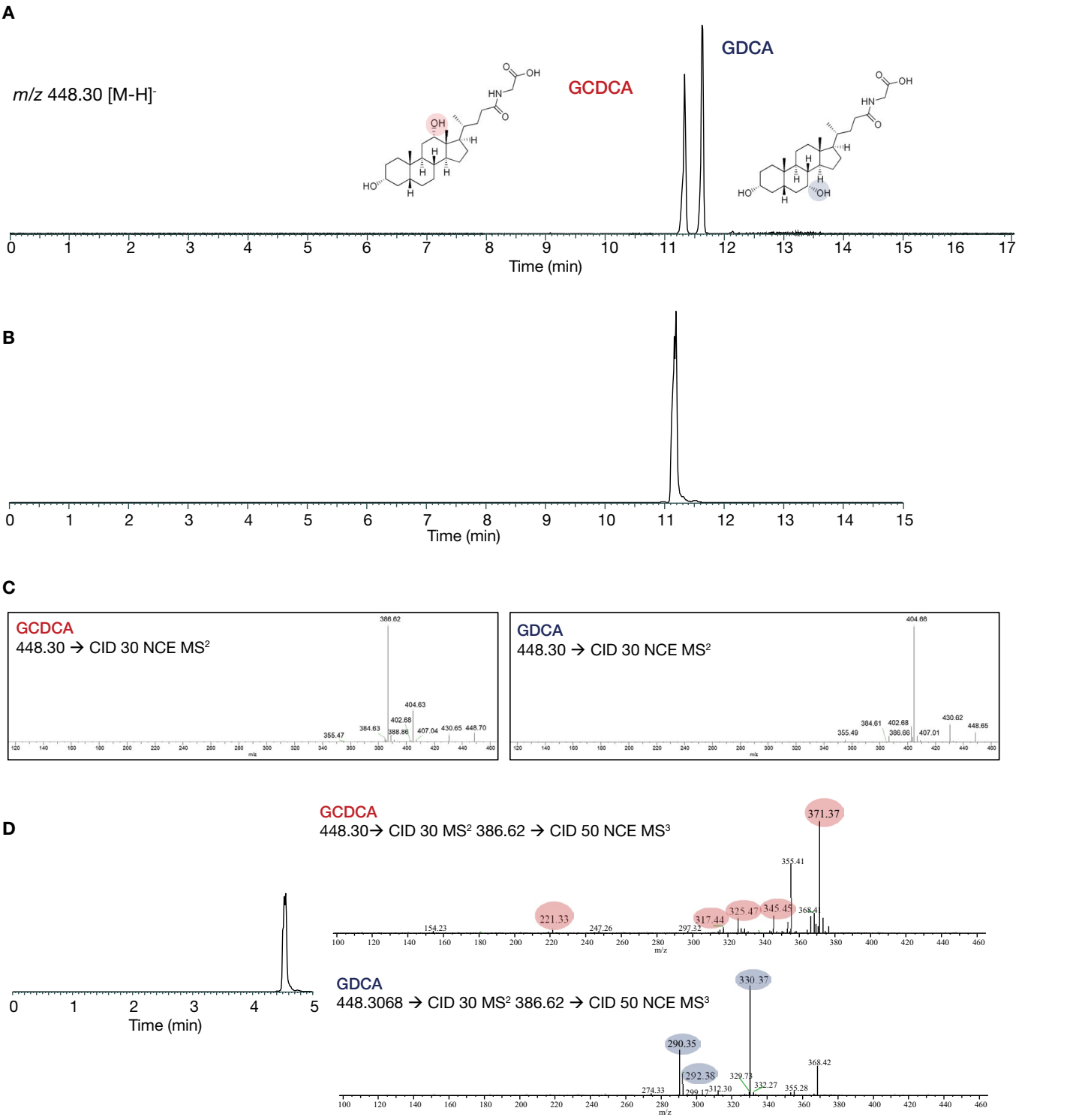

Figure 9. Illustration of the capabilities of the Stellar mass spectrometer, showcasing rapid MS² level fragmentation (up to 140 Hz) to enhance the selectivity of co-eluting isomers and efficient MS³ level fragmentation. Initially, chromatographic separation of bile acid isomers GCDCA and GDCA necessitated ion-pairing reagents (A). However, reducing additives and flow rates led to co-elution of the isomers (B) precluding gradient shortening. MS² fragmentation generated similar patterns (C), impeding PRM quantitation. Yet, the instrument's MSⁿ capability facilitated the detection of diagnostic ions for each isomer, enabling accurate quantitation in high-throughput analyses without ion-pairing reagents or high solvent flow rates (D).

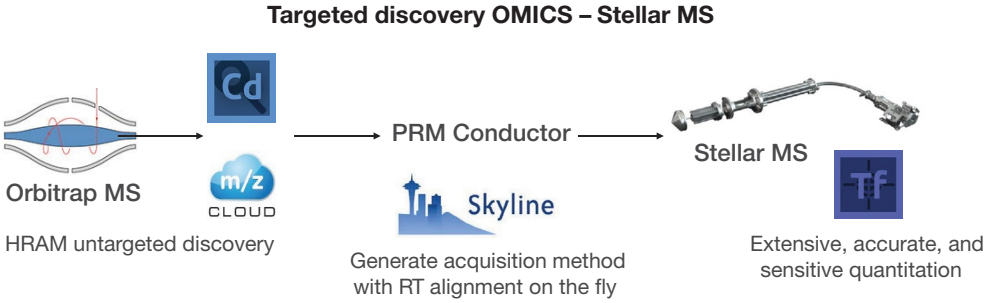

Figure 10. Targeted discovery omics workflow using Orbitrap untargeted discovery and Stellar mass spectrometer targeted analysis

Conclusion

The Stellar mass spectrometer's exceptional capabilities in biomarker verification has been demonstrated through this application note. By combining the robust quantitative power of triple quadrupole technology with the rapid full-scan MS^n acquisition of dual-pressure linear ion trap technology, the Stellar mass spectrometer has shown a remarkable increase in both sensitivity and compound coverage. This study illustrated the effective development and implementation of a fecal metabolites LC-MS library using three distinct LC columns, showcasing the instrument's ability to conduct high-throughput quantitation analysis without compromising data quality.

Key findings include the Stellar mass spectrometer's performance capabilities delivering rapid scanning, high sensitivity across a broad dynamic range, and efficient fragmentation using both HCD and CID techniques. These features facilitated intuitive method development and enabled the precise quantitation of numerous fecal metabolites from mice subjected to different dietary interventions. The experiments underscored the instrument's high selectivity and robustness, with minimal data loss and consistent signal responses, demonstrating its utility in translational metabolomics and lipidomics research.

The Stellar mass spectrometer's proficiency in handling complex sample matrices and co-eluting isomers through rapid MS^2 and efficient MS^3 level fragmentation further highlights its versatility and efficiency. Overall, the Stellar mass spectrometer emerges as an indispensable tool for high-throughput, sensitive, and selective biomarker verification, significantly advancing the field of metabolomics and lipidomics.

Finally, the advancement of the Stellar mass spectrometer has resulted in a novel metabolomics workflow that effectively bridges the gap between discovery to validation by performing verification of putative metabolite markers. With key enhancements such as increased scan speed and sensitivity, the Stellar mass spectrometer enables deep and accurate quantitation of large-scale compounds using fast gradients to facilitate larger sample sizes with similar turnaround time as with existing triple quadrupole MS technology. This facilitates a seamless transition from discovery to validation phases for all putative biomarkers, accelerating the translation of metabolomic findings into actionable insights for precision medicine, diagnostics, and therapeutic interventions.

Acknowledgment

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