An intelligent data acquisition workflow for untargeted metabolomics to achieve deep metabolome coverage and confident compound annotation

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ABSTRACT

Purpose: Development of an intelligent data acquisition workflow for untargeted metabolomics with deep metabolome coverage and confident compound annotation to identify components for quality screening study in milk.

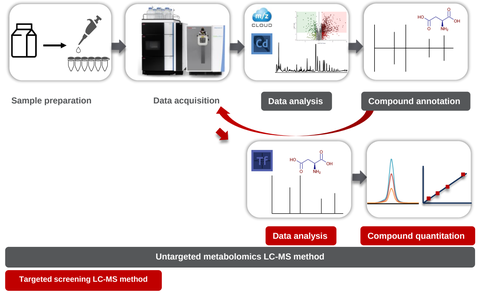
Methods: A reversed-phase LC-MS method was developed utilizing a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer to assess metabolic variation among different milk samples; bovine milk with various fat content, almond, oat, coconut, and soy milk.

Results: Plant-based milk showed, in general, higher levels of amino acids compared to bovine milk. However, higher levels of organic acids were reported in bovine milk.

INTRODUCTION

The goal of untargeted metabolomics is to comprehensively detect and annotate as many metabolites as possible in biological samples. Efforts are continuously made to improve analytical workflows in terms of sensitivity, mass accuracy, robustness, and metabolome coverage. The use of reliable quality control measures is critical to monitor and ensure analytical performance for high-quality data and confident results. Here we outline an untargeted metabolomics workflow using a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer to assess metabolic variation among different milk samples (i.e., bovine and plant-based milk). This approach utilizes high-resolution accurate mass full scan data for robust and sensitive compound detection and an AcquireX intelligent data acquisition workflow to maximize the number of relevant compounds interrogated by MS/MS, resulting in higher confidence annotation. This study will be used to identify components that could be then targeted in a screening study (Figure 1), which could be used to assess the quality and to authenticate milk for increased food security and consumer protection.

Figure 1. An outline of an untargeted metabolomics workflow using a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer to assess metabolic variation among different milk samples to identify components that could be then targeted in a high-throughput screening study using the same analytical platform.



MATERIALS AND METHODS

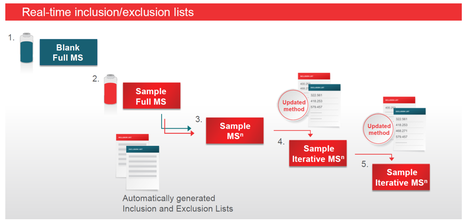
Sample Preparation

Animal and plant-based milk samples were obtained from local markets (San Jose, California). Pooled samples were prepared, by mixing 100 µL of each sample, to be used for quality control (QC). Aliquots of milk and QC samples were collected in 3 mL Eppendorf tubes and kept at -80° C until the time of analysis. Metabolites were extracted after thawing samples in an ice bath using the modified Folch method by adding 1 mL of chloroform:methanol (2:1 v/v) solution and 300 µL of water to 200 µL of milk. The organic solvents mix contained isotope-labeled standards of adipic acid and aspartic acid, which were used to evaluate LC-MS data acquisition quality. The mixture was then vortexed for 3 minutes at room temperature and centrifuged for 15 minutes (21 k x g) at 4° C to separate the two extraction layers. An aliquot, 500 µL, of the methanol:water, the upper layer, was transferred to 3 mL Eppendorf tubes and evaporated under nitrogen flow at 37° C for 60 minutes using a TurboVap® LV nitrogen evaporator from Biotage. Finally, samples were resuspended in 500 µL of 5% methanol solution in LC-MS water, vortexed for 3 minutes at room temperature, and centrifuged for 10 minutes (21 k x g) at 4° C before submitting an aliquot of the supernatant to LC-MS analysis.

Data Acquisition

A full scan (70 – 800 m/z), polarity switching (ESI (+)/ESI (-)) MS-based method was developed for the untargeted analysis of extracted milk samples. Data were acquired on an Orbitrap Exploris 240 mass spectrometer using the Deep Scan AcquireX acquisition workflow (Figure 2). This workflow automatically creates an exclusion list from background ions and an inclusion list of metabolites of interest from the reference sample with iterative updating in between each injection.

Figure 2. Thermo Scientific™ AcquireX Deep Scan mode for intelligent data acquisition to maximize the number of relevant compounds interrogated by MS/MS, resulting in higher coverage and confidence annotation.



Liquid Chromatography

LC system: Thermo Scientific™ Vanquish™ Horizon UHPLC system. Autosampler temp.: 5 ° C.
HPLC Column: Thermo Scientific Hypersil GOLD™ C18 (2.1 x 150 mm, 1.9 µm) at 45 ° C.
Injection Volume: 2 µL.
Mobile Phase: (A) 0.1% (v) formic acid (FA) in LC-MS grade water
(B) 0.1% (v) FA in LC-MS grade methanol

HPLC Gradient:

Time	A%	B%
0.00	100	0
8.00	50	50
9.00	2	98
13.00	2	98
13.10	100	0
15.00	100	0

Flow rate: 0.30 mL/min
Divert valve: to waste = 0 – 0.2 min
to MS = 0.2 – 15.0 min

Mass Spectrometry

Mass spectrometer: Orbitrap Exploris™ 240 mass spectrometer equipped with heated ESI probe. Ion source settings: polarity switching mode with spray Voltage = 3.5 and 3.0 kV, positive and negative polarity, respectively. Vaporizer = 320 ° C, Transfer Tube = 275 ° C, RF Lens = 35 %, Sheath Gas = 40, Aux. gas = 8, Sweep Gas = 1. Scan range: 70 – 800 m/z, at 120 k orbitrap resolution. Scan-to-scan Easy-IC™ internal calibration.

Data Analysis

All data were acquired using Thermo Scientific™ Xcalibur™ Software. Thermo Scientific™ Compound Discoverer™ 3.3 software was used for data processing, unknown identification, and differential analysis.

RESULTS

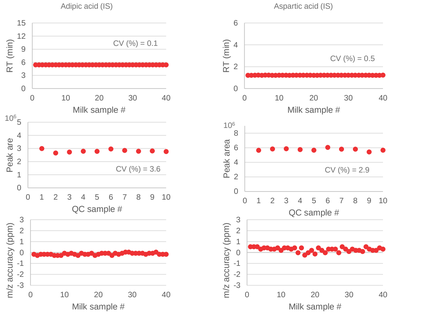
Data Acquisition

A 19-minute reversed-phase LC-MS method was developed utilizing a Vanquish Horizon system coupled to an Orbitrap Exploris 240 MS to assess metabolic variation among different milk samples; bovine milk with various fat content, almond, oat, coconut, and soy milk.

Method Validation

Instrument data quality and robustness were assessed by evaluating the spiked adipic acid and aspartic acid isotopically labeled internal standards using metrics including retention time, mass accuracy, and signal response. Sub-ppm mass accuracy was detected for the two internal standards over the entire acquisition period. Minimal chromatographic shift and consistent signal responses were observed as evidenced by low %CV for quality control samples, which were run intermittently throughout the sequence, Figure 3.

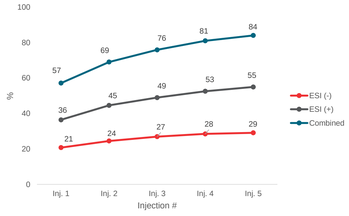
Figure 3. Reproducibility of retention time (RT), mass accuracy in ppm, and integrated peak areas of isotope-labeled internal standards (IS) spiked into milk and quality control (QC) samples. Adipic acid (IS): $^{13}C_6H_{10}O_4$ and aspartic acid (IS): $^{13}C_4H_7{}^{15}NO_4$.



AcquireX Deep Scan Intelligent Data Acquisition

The deep scan AcquireX workflow increased the percentage of fragmented compounds (Figure 4) while reducing the number of fragmented background compounds, increasing instrument utilization, and enabling the fragmentation of lower abundance compounds. This results in improved annotation capabilities on a wider dynamic range of compounds across the different varieties of milk.

Figure 4. Percentage of compounds with MS/MS spectra utilizing the AcquireX deep scan intelligent data acquisition workflow.



Differential analysis and Compound Annotation

Differential analysis and compound annotation using Compound Discoverer™ 3.3 software revealed relative differences among the milk samples and provided a wide array of annotation tools to leverage the acquired data

Bovine Milk

Bovine milk samples showed significant variation in their polar metabolic profiles based on their fat content as illustrated by the scores plot of PCA analysis in Figure 5. Moreover, a clear separation was demonstrated between organic and non-organic milk in each milk type.

The performed PCA analysis facilitated selecting markers, which are responsible for the variation observed between the different bovine milk samples. Amino acids such as phenylalanine, isoleucine, leucine, valine, and proline, and organic acids such as maleic acid, succinic acid, and gluconic acid were among those milk components as shown in Figure 6. These components are selected to be targeted in a follow-up high-throughput screening study to classify milk samples (Figure 1).

Figure 5. Scores plot of PCA analysis showing the distribution of analyzed bovine milk samples based on their polar metabolic profiles.

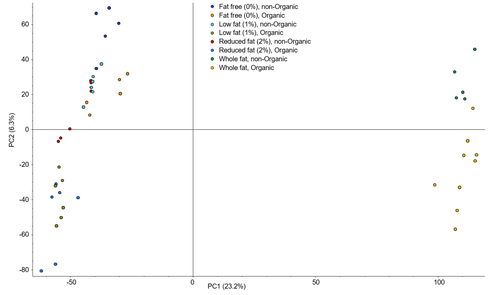
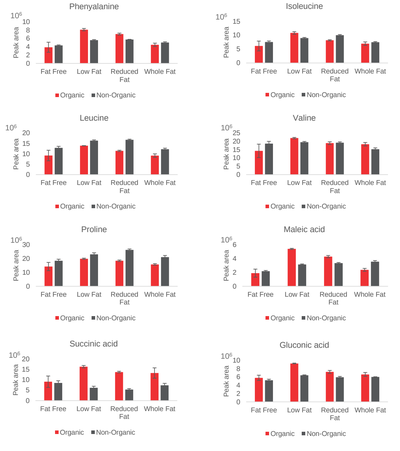


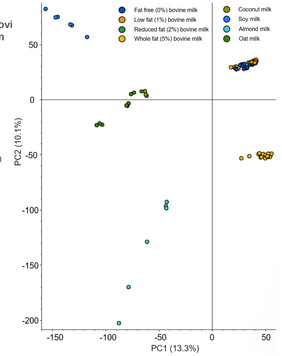
Figure 6. Variations in levels of selected amino acids and organic acids, which are among the responsible components for the variation in milk metabolic profiles illustrated by PCA analysis.



Bovine vs. Plant-Based Milk

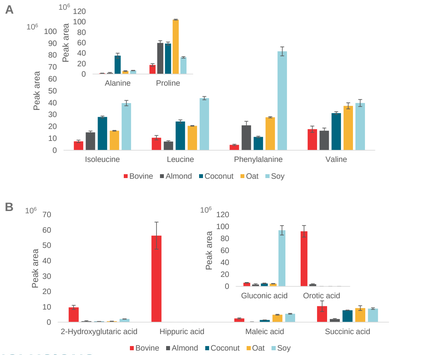
Further analysis revealed relative differences between bovine milk (whole milk was selected for this comparison) and plant-based milk samples (almond, oat, coconut, and soy) as shown in the scores plot of PCA analysis in Figure 7. Plant-based milk samples were significantly discriminated against bovine milk. In addition, a clear separation was demonstrated among plant-based milk samples.

Figure 7. Scores plot of PCA analysis showing the distribution of analyzed bovi and plant-based milk samples based on their polar metabolic profiles.



Amino acids such as phenylalanine, isoleucine, leucine, valine, alanine, and proline, and organic acids such as 2-hydroxyglutaric acid, hippuric acid, maleic acid, succinic acid, gluconic acid, and orotic acid were among those milk components as shown in Figure 8. These components are selected to be targeted in a follow-up high-throughput screening study to classify milk samples (Figure 1).

Figure 8. Variations in levels of selected amino acids (A) and organic acids (B), which are among the responsible components for the variation between polar metabolic profiles of bovine and milk-based milk.



CONCLUSIONS

An end-to-end robust untargeted metabolomics workflow to facilitate deeper coverage and confident annotation of milk metabolites was developed to identify major components that could be then targeted in a screening study. This can be used to assess the quality and to authenticate milk for increased food security and consumer protection.

TRADEMARKS/LICENSING