High-throughput quantitative metabolomics utilizing high-resolution accurate mass measurements

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ABSTRACT

Purpose: Develop a high-throughput, robust targeted LC-MS profiling method to routinely quantitate selected components that differentiate various milk samples for increased food security and consumer protection.

Methods: LC-MS quantitation of selected components in bovine milk, with different fat levels, and various plant-based milk samples (i.e., almond, coconut, oat, and soy) was performed on Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer using Hypersil GOLD™ HPLC separation. Quantitation data were acquired in full-scan MS data acquisition via both ESI(+) and ESI(-) polarity modes for targeted amino acids and organic acids, respectively. Calibration and concentration calculations were done using isotopically labeled internal standards.

Results: Higher levels of amino acids were verified to classify milk based on its origin; bovine vs. plant-based milk, being higher for the latter. Hippuric acid and orotic acid were verified as being markers for bovine milk compared to plant-based milk. Gluconic acid, however, is verified as a marker for soy milk.

INTRODUCTION

Targeted metabolomics attempts to accurately detect and quantitate metabolites in biological samples. This approach is normally used for confident and accurate quantitation of relevant metabolites being identified in untargeted methods. Here, we developed a high-throughput LC-MS method on a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer to quantitate compounds that differentiate a range of milk samples (i.e., bovine and plant-based milk). The selected targets were identified by an untargeted metabolomics study, which was acquired on the same LC-MS instrumentation. The ability to quickly develop and deploy robust and high-throughput quantitative assays based on untargeted discovery experiment results is essential for the validation and application of findings. The developed methods could also be used as screening tools to assess the quality and authenticate milk for increased food security and consumer protection.

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 chloroform:methanol mix contained isotope-labeled internal standards (IS) of the targets, which were selected from an untargeted metabolomics study. These IS were used for calibration and concentration calculations and 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 to LC-MS analysis.

Data Acquisition

Two full-scan (70 – 800 m/z) MS-based methods were developed for the quantification of selected amino acids, via ESI (+ polarity ionization), and organic acids, via ESI (- polarity ionization).

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 55 ° 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
	3.50	80	20
	3.70	2	98
	4.00	2	98
	4.60	100	0
	5.00	100	0

Flow rate: 0.45 mL/min
Divert valve: to waste = 0 – 0.2 min
to MS = 0.2 – 5.0 min

Mass Spectrometry

Mass spectrometer: Orbitrap Exploris™ 240 mass spectrometer equipped with heated ESI probe. Ion source settings: Spray Voltage = 3.5 and 3.0 kV, pos. and neg. 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. Targeted compounds (i.e., amino acids: alanine, isoleucine, leucine, phenylalanine, proline, and valine, and organic acids: maleic acid, malic acid, gluconic acid, 2-hydroxyglutaric acid, succinic acid, hippuric acid, and orotic acid) standards and isotope-labeled internal standards were used to prepare calibration solutions. Quantitation data were processed in Thermo Scientific™ TraceFinder Software 5.1 using a 3-ppm mass tolerance filter.

RESULTS

Data Acquisition

A 5-minute screening method was developed utilizing a Vanquish Horizon system coupled to an Orbitrap Exploris 240 MS. Selected amino acids and organic acids were quantified in milk samples using the developed method. ESI(+) and ESI(-) LC-MS acquisition modes were used for acquiring amino acids and organic acids data, respectively (Figure 1).

Figure 1. Extracted ion chromatograms of isotope-labeled internal standards (IS) spiked into milk samples. Phenylalanine (IS): $^{13}C_9H_{11}^{15}NO_2$ and maleic acid (IS): $^{13}C_4H_6O_5$ were used for demonstration.
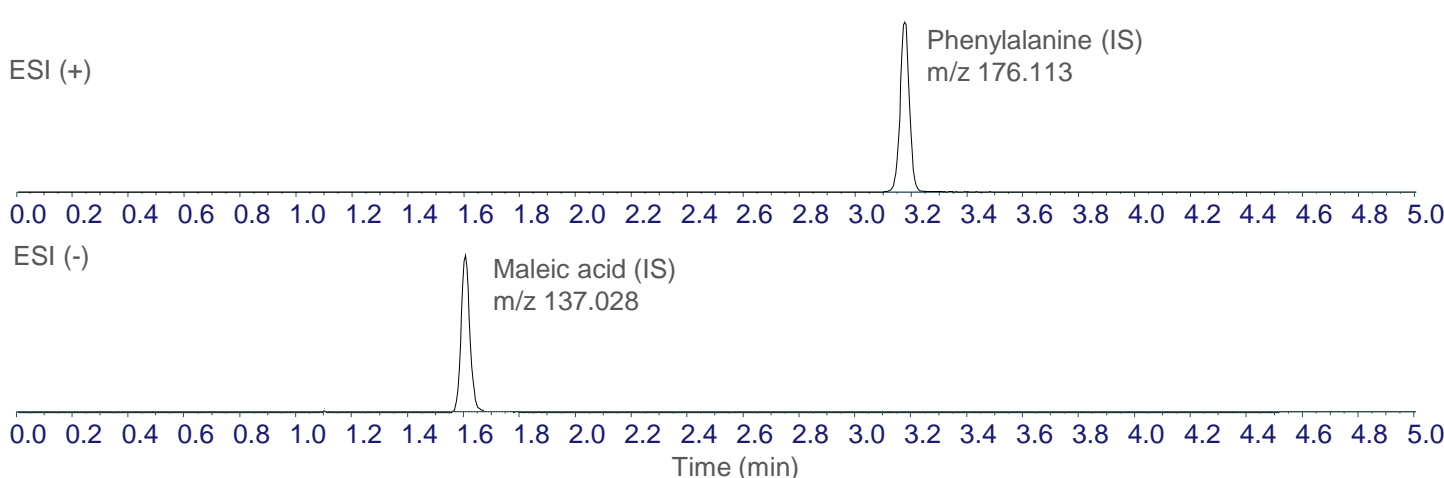
Method Reproducibility

High data quality, reliability, and robustness of measurement were observed by evaluating the isotopically labeled internal standards to assess instrument performance using metrics including retention time, peak area, and mass accuracy in milk and QC samples. Minimal chromatographic shift and consistent signal responses were observed as evidenced by Figure 2.

Figure 2. 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. Phenylalanine (IS): $^{13}C_9H_{11}^{15}NO_2$ and maleic acid (IS): $^{13}C_4H_6O_5$ were used for demonstration.
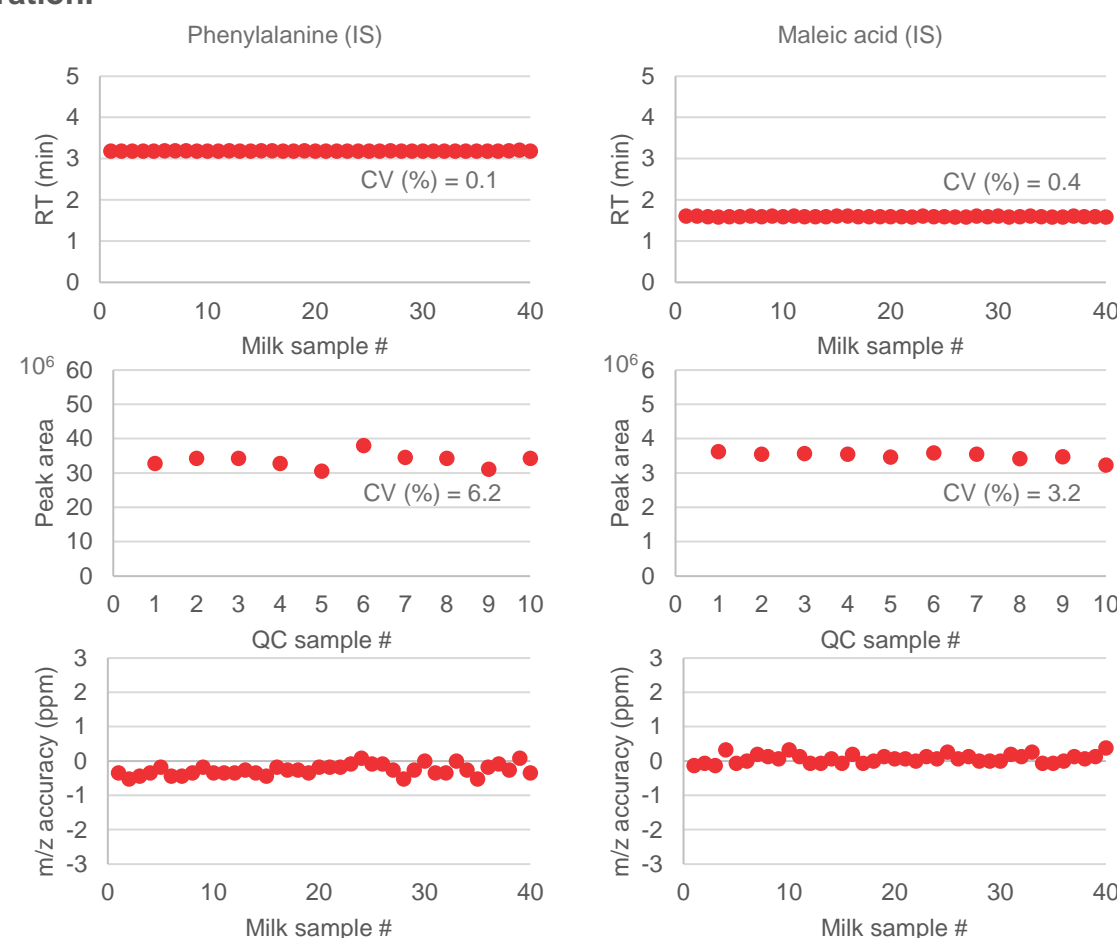
Calibration Data

Calibration curves were created for the quantified compounds using internal calibration. Linear fit (R2 > 0.99) was observed for all compounds. Figure 3 shows calibration curves for phenylalanine (3.13 – 200 µM) and maleic acid (0.39 – 200 µM) as two examples. All calibration levels showed a CV ≤ 10% and an average calculated difference CV ≤ 10%.

Figure 3. Calibration curves of phenylalanine and maleic acid were created and used for quantitation via TraceFinder 5.1 software.
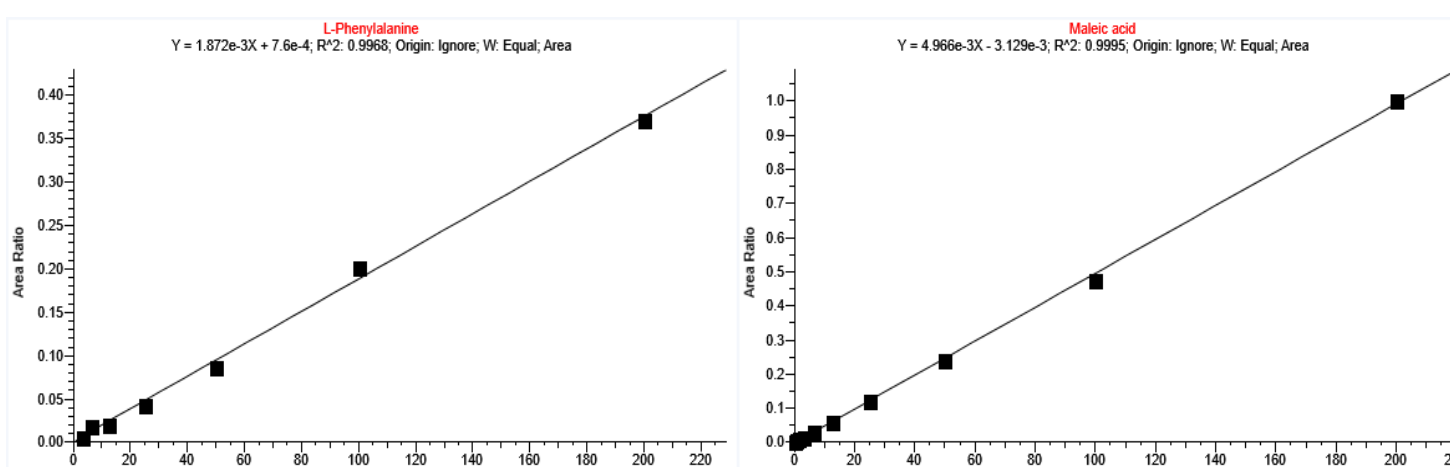
Lower limits of quantification (LOQ) and lower limits of detection (LOD) are presented in Table 1 for targeted compounds.

Table 1. Lower limits of quantitation (LOQ) and lower limits of detection (LOD) in µM of analyzed compounds using the developed targeted method.

Amino acids	LOQ (µM)	LOD (µM)	Organic acids	LOQ (µM)	LOD (µM)
Alanine	0.39	0.39	Maleic acid	0.78	0.39
Isoleucine	1.56	1.56	Succinic acid	0.39	0.39
Leucine	6.25	1.56	Gluconic acid	6.25	1.56
Phenylalanine	3.13	1.56	Malic acid	0.39	0.39
Proline	6.25	0.39	Hippuric acid	0.39	0.39
Valine	0.39	0.39	2-Hydroxyglutaric acid	0.78	0.39
			Orotic acid	0.39	0.39

Amino Acids in Bovine Milk

Calculated concentrations of the investigated amino acids in analyzed bovine milk samples (from the same brand) are grouped based on fat level and milk type (i.e., organic vs. non-organic milk) and plotted in Figure 4.

Figure 4. The concentration of targeted amino acids in analyzed bovine milk samples. Milk samples were grouped based on fat level and milk type (organic vs. non-organic; one milk brand was selected for each type).
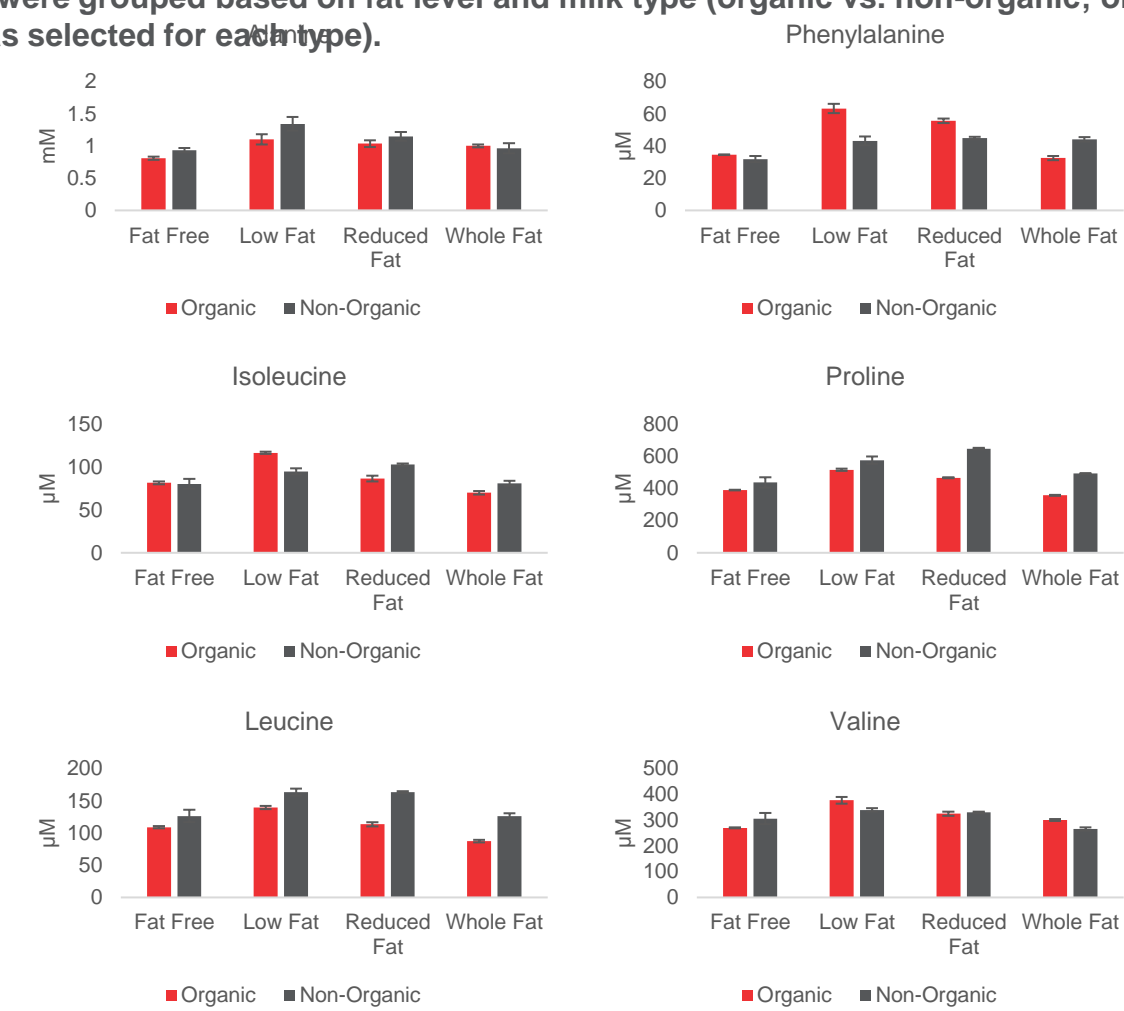

Interestingly, different fat-level milk samples showed variation in targeted amino acid levels; where low-fat (1% fat) and reduced-fat (2% fat) milk showed higher content of those amino acids compared to fat-free (0% fat) and whole-fat (3.5% fat) milk, which might be a result of the physical treatment of milk during production. In addition, variations in the concentration of phenylalanine and proline were observed comparing organic with non-organic bovine milk being higher in organic for the former and lower for the latter (Figure 4). This might be a result of variation in protein content of feeding material for each type. These variations could facilitate the differentiation between fat-free and low-fat milk for example, and between organic and non-organic milk.

Amino Acids in Bovine vs. Plant-Based Milk

Bovine milk (whole fat) showed lower levels of amino acid concentrations compared to all plant-based milk samples, which also demonstrated variation among each other (Figure 5). Soy milk, for example, contained ~20-fold higher levels of phenylalanine compared to bovine milk, but only 2-fold higher levels of proline compared to bovine milk. Noticeably, phenylalanine concentration in soy milk is significantly higher than in any other milk type. On another end, higher levels of alanine can be considered as a distinguishing marker for almond milk compared to all other milk types analyzed in this study. In general, the relative concentration of measured amino acids was lower in bovine milk compared to plant-based milk. Almond milk shared a similar pattern with bovine milk for alanine, leucine, and valine.
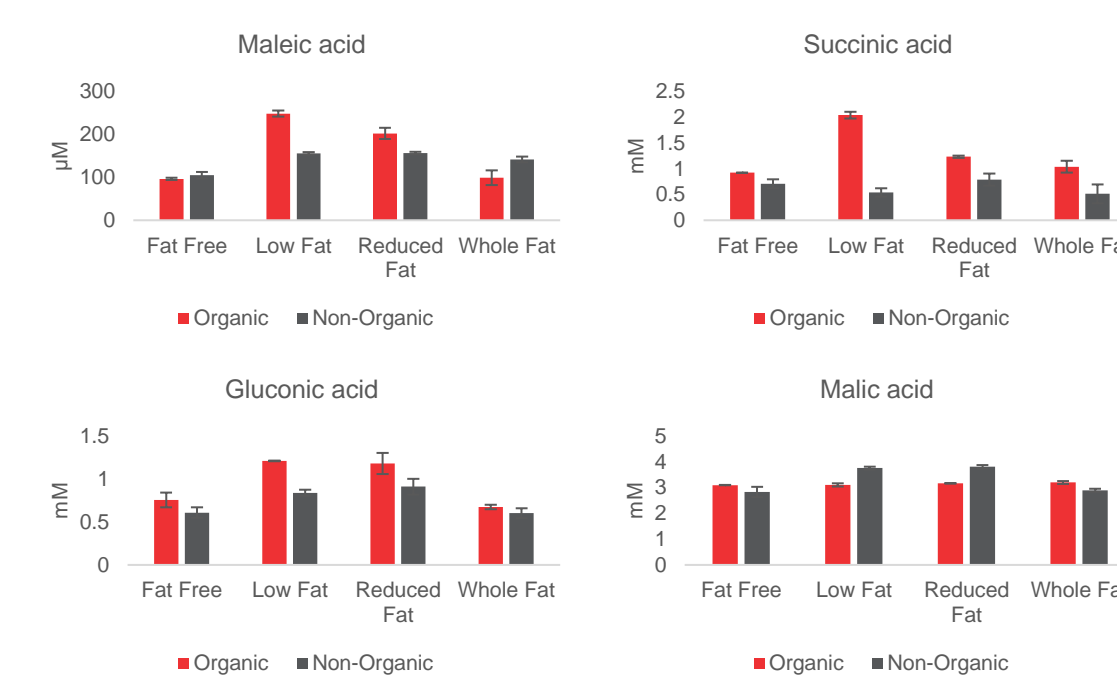
Figure 5. The concentration of targeted amino acids in analyzed bovine and plant-based milk samples. Whole fat (3.5% fat) bovine milk was selected for this comparison.
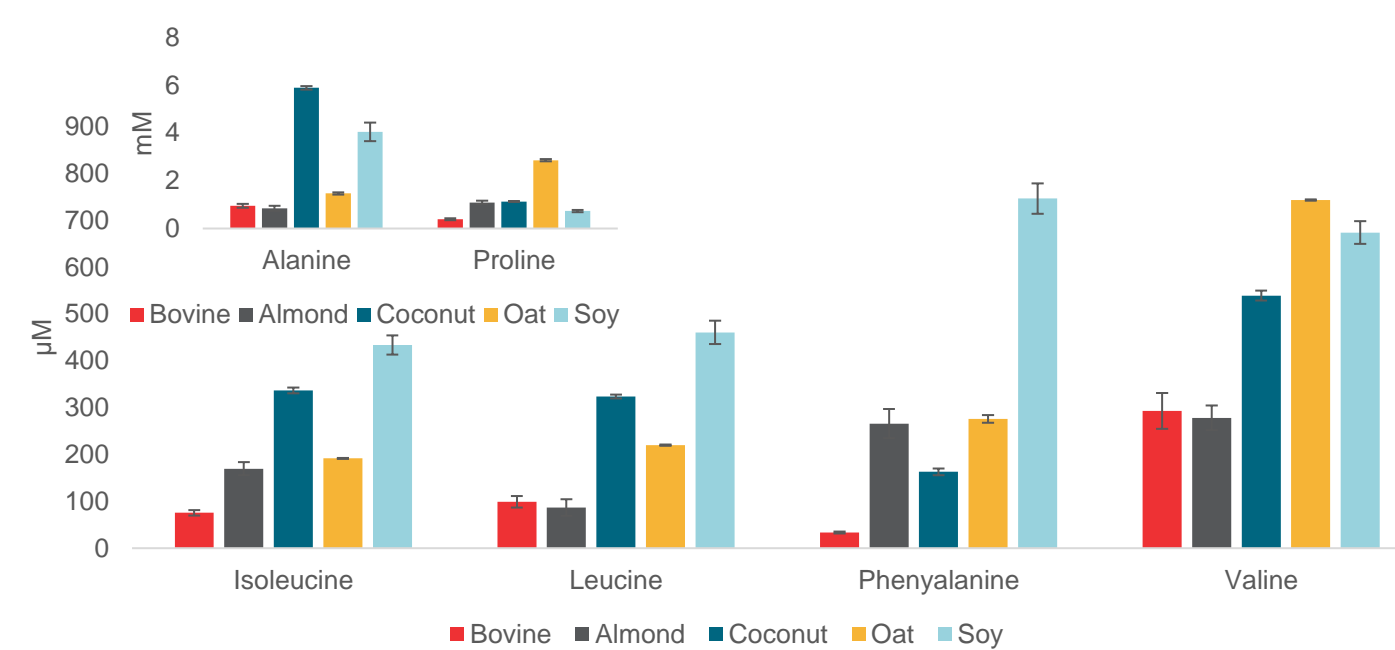
Organic Acids in Bovine Milk

Measured levels of the targeted organic acids in analyzed bovine milk samples (from the same brand) are grouped based on fat level and milk type (i.e., organic vs. non-organic milk) and demonstrated as bi-charts in Figure 6.

Figure 6. The concentration of targeted organic acids level in analyzed bovine milk samples. Milk samples were grouped based on fat level and milk type (organic vs. non-organic; one milk brand and commercial source were selected for each type).


Clear variation in the levels of targeted organic acids was observed among milk with different fat-level and between organic and non-organic milk of each type. The concentration of maleic acid and gluconic acid was higher in low-fat and reduced-fat milk compared to the rest of the samples, in addition, their concentrations were higher in organic low-fat and reduced-fat milk compared to the non-organic of those types (Figure 6). The concentration of malic acid, on the other hand, was higher in non-organic low fat and reduced-fat milk compared to organic milk. The concentration of succinic acid, in addition, can be used to qualify organic bovine milk samples with different milk fat levels.

Organic Acids in Bovine vs. Plant-based Milk

Hippuric acid and orotic acid were verified as being markers for bovine milk compared to plant-based milk, Figure 7. Higher levels of 2-hydroxyglutaric acid can also classify milk based on the origin; bovine vs. plant-based, being higher in bovine milk. Hippuric acid is a constituent of the nonprotein nitrogen fraction of animal-based milk that is proposed as a possible marker to differentiate the feeding regimen of animals. Orotic acid is also known as part of bovine milk, which is considered the main source of orotic acid in the human diet. Gluconic acid, however, is verified as a marker for soy milk in this study.

Figure 7. The concentration of targeted organic acids in analyzed bovine and plant-based milk samples. Whole fat (3.5% fat) bovine milk was selected for this comparison.
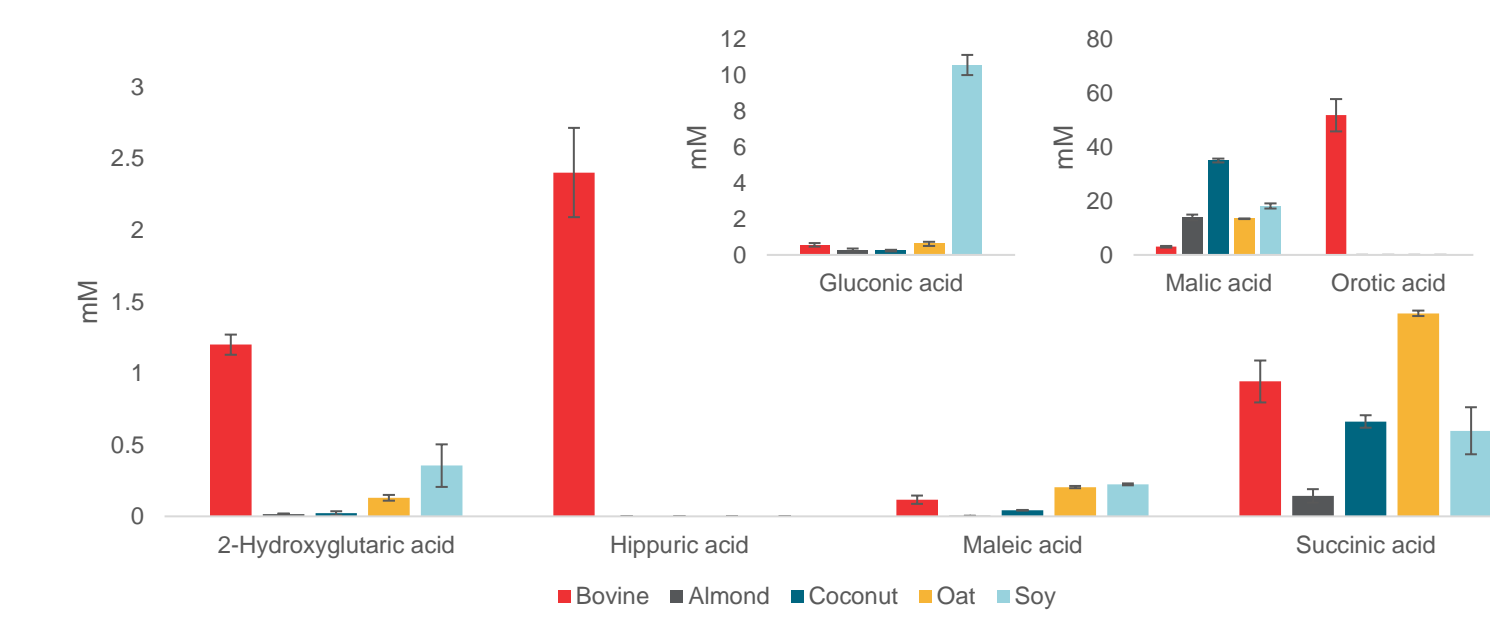
CONCLUSIONS

A high-throughput, robust targeted LC-MS profiling method was developed to translate untargeted results into quantitative assays in milk. This can be used to assess the quality and to authenticate milk for increased food security and consumer protection.

TRADEMARKS/LICENSING