BRIEF COMMUNICATION: Metabolite profiling in New Zealand milk

SR Davis*, J Jaine[1], V Obolonkin, TJ Braggins[1], TP Cooney[1], A Scott and RJ Spelman

*LIC, Private Bag 3016, Hamilton 3240, New Zealand. [1]Analytica Laboratories Ltd., Ruakura Research Centre, Hamilton 3214, New Zealand

*Corresponding author. Email: steve.davis@lic.co.nz

Keywords: milk; metabolomics; cows

Introduction

Milk contains a multitude of minor components that derive either from intermediary metabolism in the mammary gland or are components of blood plasma (or both). Developments in chromatography and mass spectrometry have enabled broad assessment of milk metabolites (e.g., Melzer et al. 2013) allowing evaluation of such profiles in relation to gene expression and a variety of physiological conditions. Thousands of metabolites can be semi-quantified, based on abundances relative to chemical class standards or internet standards. However, understanding the chemical identity of each metabolite, and quantifying its concentration can be labour intensive and rate-limiting to any investigation. Nevertheless, technologies such as mass spectrometry provide the ability to identify metabolites of diagnostic/predictive value in readily accessible samples such as milk. The objective of this study was to understand the size and nature of the metabolome in New Zealand milk. Comparison of the metabolomes of milk and blood plasma was also initiated to provide further information on the source of specific metabolites in milk.

Materials and methods

Metabolite profiles were evaluated in skim milk and blood plasma taken from 25 dairy cows in mid-lactation (approximately 200 days in milk) in the same herd. Blood samples (tail vein into EDTA anticoagulant) and milk samples were taken within three hours of a morning milking (after a 15-hour milking interval) and stored at -80°C. An additional milk sample was taken after a further 21 days.

Milks (200 μL) were extracted with 800 μL of solvent (dichloromethane : methanol, (1:3) + 1% formic acid + internal standards). The mixture was vortexed for 10 seconds and sonicated for 20 minutes. Samples were cooled to 0°C and centrifuged at 0°C for 20 minutes at 15,000 rcf. Two x 200 μL aliquots of clear supernatant were dried down at 40°C in a Spin Vac. One dried aliquot was reconstituted with 200 μL of 10% acetonitrile : water (for C18 UPLC), and the other with 200 μL of 90% acetonitrile:water (for pHILIC UPLC). Both tubes were then sonicated for 10 minutes before centrifuging at 15,000 rcf for 15 minutes at 4°C. Aliquots of clear supernatant were transferred to HPLC micro vial inserts for UPLC-MS analysis.

Plasma samples, 250 μL were extracted with 1 ml of methanol + Internal Standards. The mixture was vortexed for 10 seconds and sonicated for 10 minutes and then centrifuged at 20°C for 20 minutes at 15,000 rcf. Two x 400 μL aliquots of clear supernatant were dried down at 25°C in a Spin Vac. One dried aliquot was reconstituted with 200 μL of 10% acetonitrile : water (for C18 UPLC), and the other with 200 μL of 90% acetonitrile : water (for pHILIC UPLC). Both tubes were then sonicated for 10 minutes before centrifuging at 15,000 rcf for 15 minutes at 4°C. Aliquots of clear supernatant were transferred to HPLC micro vial inserts for UPLC-MS analysis. Internal standards used were forchlofenuron, Methionine-D3, Serine-D3 and salicylic acid-D4.

Each sample (5 μl) was analysed through four separate workflows: positive and negative electrospray ionization, on a C18 (Zorbax C18 Eclipse Plus: 100 x 2.1 mm, 1.8 μm, Agilent, USA) and a zic-pHILIC (100 x 2.1 mm, 5 μm; Merck KGaA, Darmstadt, Germany) column. The mobile phase for the C18 column started at 2.5% mobile phase B (0.1% formic acid in acetonitrile, mobile phase A = 0.1% formic acid in water) and increased to 100% mobile phase B over a 27 minute period before equilibration at 2.5%. The mobile phase for the zic-pHILIC column started at 90% mobile phase B (0.1% formic Acid 10 mM ammonium formate in 93% acetonitrile) and 10% mobile phase A (0.1% formic Acid 10 mM ammonium formate in H_2O) and finished at 60% mobile phase B after 18 minutes.

Samples were chromatographed on a Dionex 3000 UPLC (Thermo Fisher Scientific, Waltham MA, USA) interfaced to a qExactive OrbiTrap accurate mass, high-resolution (140,000) mass spectrometer, fitted with a heated, electrospray ionization (HESI) probe (Thermo Fisher Scientific; mass range 70 to 1050; Automatic gain control target = 1e⁶, maximum injection time 100 ms. Dynamic MSMS scans at a mass resolution of 70000/17500, were done on a pooled composite of all samples.

Mass spectrometry sample data from batch sequences for each workflow were processed using Sieve 2.1 (Thermo Fisher Scientific). The signal-to-noise and base peak minimum count parameters were optimized to give the maximum number of unique molecular features without compromising data quality. Components contained within the procedural blanks (without internal standards) were subtracted from the samples' data by Sieve 2.1. Peak area counts of individual components and their respective m/z assignments and retention times were normalized against their respective internal standards for each workflow. Within-batch drift was normalized using the control samples sequenced at the start and after every five samples throughout the batch using Batch Normalizer

Table 1 The ratio of milk to plasma abundances (arbitrary units) for five metabolites found in bovine milk (N = 25) and quantified in the C18-negative work stream. Milk and blood were taken from the same cows on a single occasion in mid-lactation, immediately after a morning milking. (Means and standard errors are shown).

Metabolite	m/z^2	Milk (arb. units)	Plasma (arb. units)	Milk : Plasma Concentration Ratio
Unknown	157.076	1.2 ±0.1	1.4 ± 0.1	0.86
Hippuric acid	178.050	9522 ± 500	2844 ± 105	3.35
Epinephrine[1]	182.082	106.0 ± 4.6	18.4 ± 2.0	5.75
Unknown	236.969	44.2 ± 2.8	2.4 ± 0.1	18.75
5-hydroxy-indole-3-acetaldehyde[1]	174.056	3.8 ± 0.6	0.9 ± 0.1	4.22

[1]Putative identification. [2]Mass to charge ratio

(Wang et al. 2012). Principle Components Analysis (PCA) was undertaken before checked data and after Batch Normalizer was done to ensure any analysis time trends were minimized. Sieve 2.1software was used to de-isotope and de-adduct data features and provide putative component identifications by comparing *m/z* accurate mass values against compounds contained within ChemSpider (Royal Society of Chemistry, London, UK). For hippuric acid, authentic standards were used to verify component assignments by comparing retentions times and accurate masses.

Data from a single work stream (C18$_{neg}$) were subjected to a more-detailed examination presented here.

Results and discussion

For the C18$_{neg}$, C18$_{pos}$, philic$_{neg}$ and philic$_{pos}$ work streams, mass spectral features observed in milk numbered 364, 592, 2574 and 191, respectively. In plasma, numbers of features were 3257, 1451, 1228 and 415 respectively. Of all these, 2685 features were detected in both milk and plasma.

Interpretation of metabolites in milk and plasma is helped by examination of the relative ratios of their concentrations in these sample types. Relatively high ratios of milk to plasma concentrations are indicative of a mammary synthesis, and/or active transport and secretion, while a low ratio (<1.0) can be indicative of a low rate of transfer from plasma to milk (or partitioning into milk fat: not determined in the current study).

Figure 1 Ratios for the abundance of 100 low-molecular-weight metabolites (range 75 to 230 Da) in bovine milks sampled at a morning milking in February (mid lactation) relative to 21days previously (N = 25). Mass/charge (m/z) ratios and retention times (RT) are shown for features of interest.
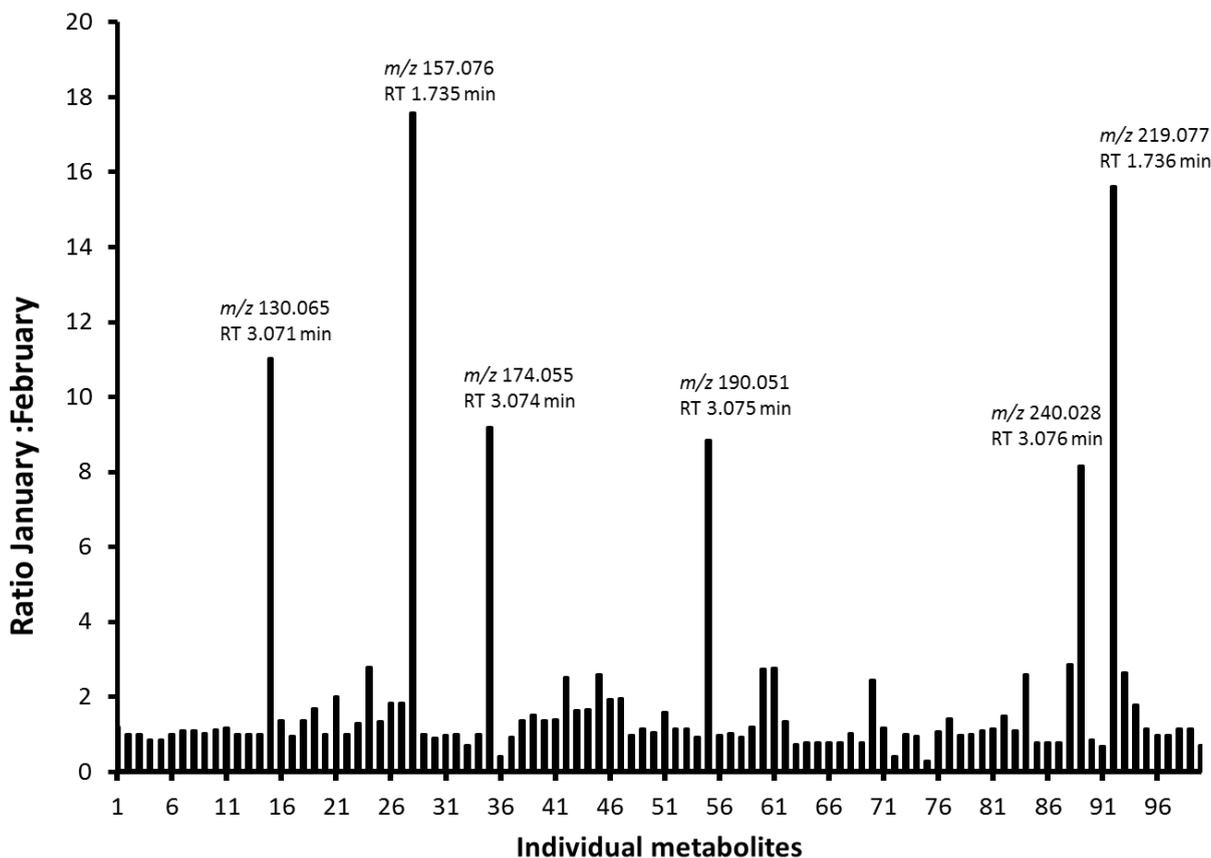
Among 350 mass spectral features examined in the "C18$_{neg}$" work stream, there was a wide range in milk to plasma ratios. Over 40 features were present with a ratio of <1, while >100 features had a ratio of >3.0, indicative of mammary metabolism and/or active transfer from plasma to milk. For a single metabolite, hippuric acid, the peak in plasma and milk was positively identified and pure standards run to enable quantification.

There was a relatively weak correlation between milk and plasma concentrations of hippuric acid (r=0.37: $P<0.05$; N = 25). The mean ratio of hippuric acid in milk to plasma of 3.35 was indicative of mammary synthesis and secretion. However, mammary hippuric acid metabolism is not well described. There was a good correlation between hippuric acid concentrations in milks taken 21 d apart (r= 0.7; $P<0.001$; N = 25), although concentrations were higher in the later samples (44±2 vs 33±2 mg/l) (around 200 d of lactation). Hippuric acid has been suggested as a useful biomarker to define organic milk although more likely it reflects dietary intake of forage (Carpio et al. 2013).

Examination of milk metabolite features in morning milk samples taken 21 days apart revealed very similar concentrations for most metabolites (within 1-2 fold range). However, there were several metabolites which showed >8-10 fold differences (Figure 1) among a selected group of lower-molecular weight features in the C18-negative work stream. The similar retention times indicate that some of these features may represent closely related molecules. A significant dietary difference was that the cows were consuming grass silage as part of their daily ration before the second sample was taken, and were grazing pasture only before the first sample. Some of the larger, specific differences in metabolite concentrations likely represent a "fingerprint" characteristic of silage feeding.

The abundance of specific features was also examined in relation to lactation phenotypes including milk volume and milk fat and protein content. High correlations (r>0.50; $P<0.05$)) were seen in both plasma and milk for 37 features with lactation phenotypes for yield and composition.

In conclusion, the measurement of thousands of compounds in blood plasma or milk which may be indicative of physiological status, mammary metabolism, diet type and cow health is now relatively straightforward. However, the time required to confirm identity of compounds relevant to the hypothesis being tested is demanding. There is considerable diagnostic value yet to be harvested from the bovine milk metabolome.

Acknowledgements

References
Carpio A, Bonilla-Valverde D, Arce C, Rodríguez-Estévez V, Sánchez-Rodríguez M, Arce L, Valcárcel M 2013. Evaluation of hippuric acid content in goat milk as a marker of feeding regimen. Journal of Dairy Science 96:1-9.

Melzer N, Wittenburg D, Hartwig S, Jakubowski S, Kesting U, Willmitzer L, Lisec J, Reinsch N, Repsilber D 2013. Investigating associations between milk metabolite profiles and milk traits of Holstein cows. Journal of Dairy Science 96: 1521-1534.

Wang SY, Kuo, CH, Tseng YJ 2013. Batch Normalizer: A fast total abundance regression calibration. Analytical Chemistry 85: 1037-46.