Article

Development of Chemical Isotope Labeling LC-MS for Milk Metabolomics: Comprehensive and Quantitative Profiling of the Amine/Phenol Submetabolome

Dorothea Mung, and Liang Li

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.6b03737 • Publication Date (Web): 17 Mar 2017

(Manuscript submitted to *Anal. Chem.* on September 21, 2016; Revised on January 10, 2017; Re-revised on March 15, 2017)

Development of Chemical Isotope Labeling LC-MS for Milk Metabolomics: Comprehensive and Quantitative Profiling of the Amine/Phenol Submetabolome

Dorothea Mung and Liang Li*

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

* Corresponding author. E-mail: Liang.Li@ualberta.ca.

Abstract

Milk is a complex sample containing a variety of proteins, lipids and metabolites. Studying the milk metabolome represents an important application of metabolomics in the general area of nutritional research. However, comprehensive and quantitative analysis of milk metabolites is a challenging task due to the wide range of variations in chemical/physical properties and concentrations of these metabolites. We report an analytical workflow for in-depth profiling of the milk metabolome based on chemical isotope labeling (CIL) and liquid chromatography mass spectrometry (LC-MS) with a focus of using dansylation labeling to target the amine/phenol submetabolome. An optimal sample preparation method including the use of methanol at a 3:1 ratio of solvent to milk for protein precipitation and dichloromethane for lipid removal was developed to detect and quantify as many metabolites as possible. This workflow was found to be generally applicable to profile milk metabolomes of different species (cow, goat and human) and types. Results from experimental replicate analysis (n=5) of 1:1, 2:1 and 1:2 ^{12}C-/^{13}C-labeled cow milk samples showed that 95.7%, 94.3% and 93.2% of peak pairs, respectively, had ratio values within ±50% accuracy range and 90.7%, 92.6% and 90.8% peak pairs had RSD values of less than 20%. In the metabolomic analysis of 36 samples from different categories of cow milk (brands, batches and fat percentages) with experimental triplicates, a total of 7104 peak pairs or metabolites could be detected with an average of 4573±505 (n=108) pairs detected per LC-MS run. Among them, 3820 peak pairs were consistently detected in over 80% of the samples with 70 metabolites positively identified by mass and retention time matches to the dansyl standard library and 2988 pairs with their masses matched to the human metabolome libraries. This unprecedentedly high coverage of the amine/phenol submetabolome illustrates the complexity of the milk metabolome. Since milk and milk products are consumed in large

quantities on a daily basis, the intake of these milk metabolites even at low concentrations can be cumulatively high. The high-coverage analysis of the milk metabolome using CIL LC-MS should be very useful in future research involving the study of the effects of these metabolites on human health. It should also be useful in the dairy industry in areas such as improving milk production, developing new processing technologies, developing improved nutritional products, quality control, and milk product authentication.

Introduction

In recent years, metabolomics has been increasingly used for nutritional studies ranging from studies on the relations of diet, health and diseases to the development of improved nutritional products.[1-4] Nutritional metabolomics involves characterizing the metabolomes of foods and diets and investigating their effects on the metabolomic profiles of a particular organism. Milk is an important component of many diets. In North America, dairy, which incorporates milk and all milk products, is one of the four main food groups. According to Statistics Canada (www.dairyinfo.gc.ca), individuals in North America consumed an average of 70 L of fluid milk per person in 2014. European countries (Finland, Ireland, UK) and Oceanian countries (Australia, New Zealand) consumed upwards of 100 L per person in the same year. In all species of mammals, milk has great importance as it is the first and only source of nutrition immediately after birth.[5] Milk is comprised of a variety of proteins, peptides, essential/free amino acids, fats, vitamins and minerals, making it a balanced source of nutrition for proper growth and development through all stages of life.[5]

Compositional analysis of milk and milk products is critical in many areas of applications including characterization of nutrients,[6] determination of health benefits of dairy consumption,[7] developing new processing technologies[8] and safety and quality control of dairy products.[9, 10] Current analytical methods are mainly focused on the measurement of the high abundant components including proteins, lipids and oligosaccharides, often expressed as the total concentrations of individual groups.[11] Targeted analysis of a small number of compounds such as vitamins is also carried out using a variety of analytical tools.[12, 13] Untargeted analysis of milk and milk products at the individual molecular level using a metabolomics approach represents a relatively new development in compositional analysis.[1, 2] Several studies on milk metabolomics

have been reported using NMR,[14-21] GC-MS[14, 15, 22, 23] and LC-MS[15, 16, 20, 23] with varying degrees of metabolomic coverage. Recent reports from Villasenor et al[23] and Andreas et al[15] demonstrated the significant advances in analytical methods for milk metabolome profiling, which also highlighted the current analytical challenges that include the analysis of polar metabolites in milk. NMR and capillary electrophoresis (CE) MS were found to be more suitable for polar metabolite analysis, while GC-MS and LC-MS were particularly useful for analyzing non-polar metabolites such as lipids.[15]

In this work, we report a high-performance chemical isotope labeling (CIL) LC-MS method for profiling the relatively polar metabolites in milk with very high coverage. CIL LC-MS is a general strategy of using chemical labeling to improve separation, detection and quantification of metabolites.[24] CIL targets a particular submetabolome based on a shared chemical group. For example, dansylation labeling has been shown to be effective in analysing the amine/phenol submetabolome.[24] Different labeling chemistries can be used to profile different submetabolomes in order to provide better coverage of the entire metabolome of interest.[25-31] Because of the complexity of the milk matrix, we have examined a number of sample preparation conditions to develop an optimal workflow. In this report, we first describe the workflow for analyzing polar metabolites in milk using dansylation LC-MS. We then apply this method for profiling different groups (brands, batches and fat percentages) of milk to demonstrate the overall analytical performance and showcase the potential utilities of the method in milk metabolomics.

Experimental Section

Milk Sample Collection and Preparation. All cow and goat milk were purchased from a local grocery store and the human breast milk was donated from a healthy volunteer. Ethics approval for working with human samples was given by the University of Alberta Ethics Committee. Three brands of milk (Beatrice, Dairyland and Lucerne) along with each of the different fat percentages of the milk (skim, 1%, 2% and 3.25%) were purchased at three time points, i.e., in January, March and April of 2015. All milk samples were aliquoted into 1.5 mL vials and stored in a -80 °C freezer until use. 100 µL of milk was mixed with 300 µL of methanol, vortexed and incubated at -20 °C for 15 min. The sample was centrifuged at 14 000 rpm for 15 min and 350 µL of the supernatant was taken into a new vial. 500 µL of dichloromethane and 50 µL of water were added to the supernatant, vortexed and left at room temperature for 10 min. 350 µL of the aqueous layer was then taken into a new vial and dried down using a SpeedVac. The sample was re-dissolved in 50 µL of water for dansylation labeling.

Dansylation Protocol. Dansyl chloride was used as the labeling reagent to react mainly amine- and phenol-containing metabolites to form dansyl-amine or dansyl-phenol derivative.[24] The reactivity, specificity and type of reaction products generated have been described previously.[24, 32] Supplemental Note S1 provides information on chemicals and reagents used. 50 µL of a processed milk sample was mixed with 25 µL of 250 mM sodium bicarbonate buffer and 25 µL of acetonitrile and the solution was vortexed. 50 µL of 18 mg/mL ^{12}C- or ^{13}C-dansyl chloride in acetonitrile was added, vortexed and incubated at 40 °C for 45 min. To quench the excess dansyl chloride, 10 µL of 250 mM sodium hydroxide was added and the solution was incubated at 40 °C for 10 min. Finally, 50 µL of 425 mM formic acid was added to acidify the sample. Individual samples were labeled with ^{12}C-dansyl chloride and a pooled sample was labeled with ^{13}C-danysl chloride. We note that for labeling amines and phenols, the reaction was

carried out in an aqueous solution. Under anhydrous system, many metabolites such as some amino acids cannot be dissolved and thus not labeled by dansyl chloride. Reagent hydrolysis took place during the labeling, but slowly under the slightly basic buffer condition. It was only when we added NaOH that the excess reagent at the end of labeling reaction was consumed quickly.

LC-UV Quantification. LC-UV quantification was performed to determine the total concentration of dansyl labeled metabolites in a labeled sample or pool.[33] Supplemental Note S1 provides the information on the LC-UV setup and concentration measurement procedure.

LC-MS. All LC-MS experiments were performed on an Agilent 1100 HPLC system (Palo Alto, CA) connected to a Bruker Impact HD quadrupole time-of-flight (QTOF) mass spectrometer (Billercia, MA) with an ESI source (see Supplemental Note S1 for more information).

Data Analysis. Bruker DataAnalysis software 4.2 was used to extract MS spectral peaks. An in-house software tool, IsoMS, was used to process the raw data generated from multiple LC-MS runs by peak picking, peak pairing, peak-pair filtering to remove redundant peaks of the same metabolite, such as adduct ions, dimer, multimers, to retain only $[M+H]^+$ for a labeled metabolite (i.e., one peak pair corresponds to one metabolite), and peak-pair intensity ratio calculation.[34] IsoMS does not pick up isotopmers of a molecular ion as a peak pair as their intensity pattern is different from that of the labeled peak pair.[34] The same peak pairs detected from multiple samples were then aligned to produce a CSV file containing the metabolite information and peak ratios relative to a control (i.e., a pooled sample). A zero-fill program was used to find missing peak pairs from the raw mass spectral data, filling in the missing values.[35] Finally, peak ratios were re-calculated by using chromatographic peaks, instead of mass spectral

peaks, with IsoMS-Quant.[36] The final metabolite-intensity data file was then exported to SIMCA-P+ 12.0 software (Umetrics, Umea, Sweden) for multivariate statistical analysis. Principal component analysis (PCA) and Partial Least Squares discriminant analysis (PLS-DA) were used to analyze the data after unit variance scaling.

Positive metabolite identification was performed based on mass and retention time matching to the dansyl standards library consisting of 273 unique standards with 315 entries.[32] This library with information on MS, MS/MS and ion chromatogram for each dansyl labeled standard is freely accessible at www.MyCompoundID.org. Putative identification was done based on accurate mass match to the metabolites in the human metabolome database (HMDB) (8,021 known human endogenous metabolites) and the Evidence-based Metabolome Library (EML) (375,809 predicted human metabolites with one reaction) using MyCompoundID.[37] The mass accuracy tolerance window was set at 5 ppm and the retention time tolerance window set to 30 s for the searches. Since we were interested in the endogenous metabolites in milk, we did not use other databases such as Metlin and KEGG containing many exogenous compounds for our search. We also note that, for multi-function compounds (e.g., containing two amines), labeling by one or more reagent molecules to generate multiple products may happen to some metabolites, although in most cases complete labeling was found. If multiple products were found from one metabolite, they could be readily spotted in the final list of significant metabolites for differentiating different groups: they would be matched to the same metabolite.

Results and Discussion

Removal of Proteins and Lipids. Milk is composed of a variety of proteins and lipids that may interfere with the chemical labeling procedure and LC-MS tailored to the analysis of

polar metabolites. Based on our experience of working with serum and plasma, as well as other reported protocols for protein and lipid removal,[38] we have examined several experimental conditions to extract metabolites from milk. All method development experiments were chosen with due consideration of speed and convenience in sample handling. For protein precipitation, three solvents were compared which included acetone, acetonitrile and methanol at varying solvent-to-milk (v/v) ratios (3:1 and 5:1). The different solutions underwent the same sample work-up for incubation, centrifugation and the removal of the aqueous layer in quadruplicates. Both the aqueous layer and the pellet were dried down using a SpeedVac, after which only the aqueous layer was re-dissolved in 50 µL of water. Three of the four replicates for each sample were labeled with ^{12}C-dansyl chloride and the remaining sample with ^{13}C-dansyl chloride. All samples were normalized using the concentration values determined by LC-UV, mixed at a 1:1 molar ratio of ^{12}C- and its corresponding ^{13}C-dansylated sample and injected into the LC-MS. Two measured properties were used to determine solvent extraction efficiency: the weight of the protein pellet and the corresponding number of peak pairs or metabolites detected from the aqueous layer.

Figure 1A and 1B show the plots of protein weight and peak pair number, respectively, for different protein precipitation conditions. The heavier the protein pellet (corresponding to better protein precipitation efficiency) the lower the number of peak pairs detected. Methanol at a 3:1 (v/v) ratio corresponded to the lightest protein pellet observed but also the highest number of metabolites detected. In addition, we have compared the absolute intensities of individual metabolites using a heat map (see Supplemental Figure S1), which also indicated that 3:1 methanol gave a visually higher overall intensity map. Thus, methanol at a 3:1 solvent-to-sample ratio was chosen for protein precipitation as it resulted in the highest number of metabolites

detected and was sufficient in protein removal. Note that, after methanol precipitation, some proteins may remain in the sample. However, during dansylation labeling, we observed some precipitates at the start of the reaction. This was from the dansyl labeling of multiple amine/phenol groups in the remaining proteins, causing precipitation of the labeled proteins out of solution.[39] In LC-MS analysis of labeled metabolites, we did not detect any proteins or large peptides. In addition, proteins would retain strongly on a C18 column, causing graduate increase in LC column pressure after running samples containing an appreciable amount of proteins; however, we did not observe any column pressure change after running hundreds of samples on a C18 column.

Due to the presence of a relatively large amount of lipids in milk, lipid removal was found to be essential to reduce column-washing time significantly and prolong the reversed phase (RP) column lifetime. Two extraction solvents, dichloromethane (DCM) and methyl tert-butyl ether (MTBE), were compared and the results are shown in Figure 1C. Overall, the addition of the lipid removal step resulted in a decrease in the number of peak pairs detected (e.g., from about 1100 peak pairs in methanol without lipid extraction to 870 peak pairs with lipid extraction), likely due to the loss of some hydrophobic metabolites during the lipid extraction process. However, the need for lipid removal is essential especially for the analysis of milk containing higher fat content. Of the two lipid-extraction solvents tested, dichloromethane resulted in the detection of higher numbers of metabolites when performed in addition to protein precipitation using the three protein precipitation solvents. DCM was also found to be more convenient during the removal of the aqueous layer for analysis due to the large density difference between this solvent and water/methanol. In addition, from the heat map of the

absolute intensities of individual metabolites (see Supplemental Figure S2), DCM combined with methanol gave a visually higher overall intensity map.

Based on the above results, we concluded that the optimal sample preparation for the dansylation LC-MS workflow was to use methanol at a 3:1 ratio of solvent to milk for protein precipitation and dichloromethane for lipid removal.

Milk Metabolite Detection. After removing the proteins and lipids, the aqueous solution was taken for dansyl labeling. Dansyl labeling adds a hydrophobic tag to a metabolite and, as a result, the labeled product becomes relatively hydrophobic even for very polar or ionic metabolites such as amino acids. Thus, the labeled milk metabolites can be readily retained and efficiently separated on a reversed phase column. Figure 2A shows a representative base-peak-ion (BPI) chromatogram of 1:1 ^{12}C-/^{13}C-dansyl labeled milk. Many chromatographic peaks distributed along the gradient elution window were observed, indicating the complexity of the milk metabolome with diverse chemical and physical properties. The analytical separation takes 32 min, followed by 15-min column washing and re-equilibration. This gives an overall sample throughput of 47 min per sample. The washing/equilibration step was necessary to ensure that small molecule residues in the column were washed out and the column was properly equilibrated before the next run.

In LC-MS operation, using an optimal sample injection amount is critical in order to detect the maximum number of labeled metabolites. With dansyl labeling, the total concentration of labeled metabolites in each sample can be measured by LC-UV as described in the Experimental Section. Knowing the concentration of each sample, the exact amount of sample injected into LC-MS can be controlled. To determine the optimal injection amount, a 1:1 ^{12}C-/^{13}C-labeled milk sample with a known concentration measured by LC-UV was injected in 10 µL

increments from 10 to 60 µL. Experimental triplicate runs were performed for gauging the technical reproducibility. Figure 2B shows the plot of the peak pair number detected as a function of sample amount injected. Peak pair number saturation occurred when 20.9 nmol of sample in 30 µL was injected. Thus, in subsequent experiments, we injected 20 nmol of labeled sample into the LC-MS for analysis.

Note that the number of peak pairs shown in Figure 2B is different from those shown in Figure 1 where less than the optimal amount of sample was injected. In addition, the number of peak pairs detected is dependent on the number of samples analyzed. Each sample adds some unique metabolites to the total number of peak pairs within a data set. An increase in the total number of metabolites will also translate to an increase in the number of metabolites detected in each individual sample run as the entire data set is used for peak picking and filling of missing ratio values during Zero-fill.[34, 35] For the initial peak-pair picking, we used a signal-to-noise (S/N) threshold of 10 in IsoMS to minimize false positives. For Zero-fill, we considered all the peak pairs with S/N of greater than 3, as filling the peak pair with lower S/N in a sample initially with a missing ratio was judged based on the presence of a true peak pair in other sample(s). In the following studies investigating the different aspects of the analytical work and application, the number of samples or runs used for each data set was different and thus the numbers of peak pairs detected varied.

Accuracy and Precision. In the dansylation isotope labeling LC-MS workflow, relative quantification of a metabolite is based on the measured peak ratio between the ^{12}C-labeled metabolite in an individual sample and its ^{13}C-labeled counterpart in the pooled sample. To gauge the overall accuracy and precision of the workflow for profiling the amine/phenol submetabolome, experimental five-replicates (n=5) analysis of 1:1, 2:1 and 1:2 ^{12}C-/^{13}C-labeled

cow milk was used as an example. Six extractions were performed in parallel, five of which were dansylated with ^{12}C-dansyl chloride and the sixth labeled with ^{13}C-dansyl chloride.

Figure 3A-C shows the plots of the peak pair number as a function of the measured peak ratio. A total of 2245 common peak pairs were detected in the data set of these three mixtures. For the 1:1 mixture shown in Figure 3A, 1998 (88.9%) pairs gave peak ratios in the range of 0.8 and 1.25 or within ±25% of the expected ratio of 1.0. Only 98 pairs (4.3%) had peak ratios that exceeded the ±50% accuracy range (0.67>peak ratio>1.5). For the 2:1 mixture, Figure 3B shows 1906 (84.8%) peak pairs in the range of 1.6 and 2.5 (i.e., within ±25% of the expected ratio of 2.0). 130 pairs (5.7%) were found outside the 50% range of the expected peak ratio of 2.0. Lastly, the peak pair distribution shown in Figure 3C for the 1:2 mixture indicates 1932 pairs (86.0%) found within ±25% of the expected ratio of 0.5. 153 pairs (6.8%) were outside the 50% accuracy range. These examples illustrate that accurate measurement of the peak ratio can be achieved using the workflow for majority of the peak pairs. This is not surprising considering that the individual samples and the pooled sample had a similar matrix and metabolite composition. The samples were also processed in parallel using the same sample work-up protocol, and analyzed as a ^{12}C-/^{13}C-mixture in LC-MS where the matrix, ion suppression and instrumental effects were taken into account by measuring the peak ratio relative to the ^{13}C-pool.

Figure 3D-F shows the plots of the peak pair number as a function of the relative standard deviation (RSD) of the measured peak ratio values from the ^{12}C-/^{13}C-mixture samples (n=5). Out of the 2245 common peak pairs, 2037 (90.7%), 2081 (92.6%) and 2040 (90.8%) peak pairs found in 1:1, 2:1 and 1:2 mixtures, respectively, had RSD values of less than 20%. These results indicate that reproducible peak ratio measurements can be achieved.

General Applicability. To gauge the general applicability of the sample preparation and LC-MS method for milk metabolomics, different milk samples including goat milk, human milk, and cow milk with and without heat treatment were analyzed. For the heat treatment, two types of cow milk, commonly called homo-milk (homogenized milk containing 3.25% fat) and skim milk (0% fat) in the US and Canada, were brought to a boil, heated for 5 minutes, and then cooled down. A pooled sample was prepared by taking an aliquot from each sample and mixing them. After ^{12}C-dansyl labeling of the individual samples and ^{13}C-labeling of the pooled sample, LC-UV was used to measure the total concentration of labeled metabolites in all labeled samples. Figure 4A shows the average concentration of labeled metabolites in different samples. Goat milk had significantly higher concentrations than human and cow milk. Thus, for metabolome comparison of different species of milk, sample normalization is necessary. In our work, we took an aliquot of appropriate volume based on the measured concentration to ensure the same molar amount of all the individual samples was mixed with an equal mole of the ^{13}C-labeled pool. The resultant 1:1 mixture was analyzed by LC-MS. Peak pair ratio values of individual metabolites determined from all the samples reflect the relative concentration differences of these metabolites in different samples. For each sample, experimental triplicate analyses were performed.

Figure 4B shows the PCA plot of the metabolomic data generated from these samples analyzed in one batch. The triplicate data points within a sample cluster together tightly, indicating that good reproducibility was achieved. Separation of the milk samples from three different species is much greater than the separation between the heat-treated and untreated cow milk. The large inter-species difference is also reflected in Figure 3C where the numbers of peak pairs detected from goat, human and untreated cow milk are compared. The average number of

peak pairs and the number of common peak pairs found in >50% of the experimental triplicates were 1290±14 (n=3) and 1312 pairs for goat milk, 1074±34 (n=3) and 1079 pairs for human milk, and 1398±16 (n=6) and 1387 pairs for the untreated cow milk. As Figure 4C shows, 864 peak pairs were commonly detected in the milk samples of the three species. We note that in working with the milk samples of different species, we did not observe any abnormity in preparing these samples including the cow milk with different fat contents (see below).

Out of 1312 peak pairs detected in goat milk, 69 pairs could be positively identified based on accurate mass and retention time matches to the dansyl standards library (Supplemental Table S1) and 1130 pairs could be matched based on accurate mass searching against the HMDB and EML libraries (Supplemental Table S2). For the 1079 human breast milk metabolites detected, 61 were positively identified by the dansyl-library (Supplemental Table S3) and 963 were putatively identified by HMDB and EML (Supplemental Table S4). For the 1387 cow milk metabolites detected, 68 were positively identified (Supplemental Table S5) and 1230 were putatively identified (Supplemental Table S6). It is interesting to note that many common peak pairs or metabolites were detected in goat, cow and human milk. Among the 864 common pairs detected in the three species, 60 metabolites including many amino acids were positively identified (Supplemental Table S7). We did not carry out MS/MS analysis of the labeled metabolites as the MS/MS spectra of dansyl labeled metabolites usually do not provide adequate fragment ion information from the metabolite moiety for unknown metabolite identification.

The above results indicate that the workflow described herein should be generally applicable for handling different types of milk samples in milk metabolomics. It should be useful for future studies of comparing milk metabolomes of different species using a large cohort of samples in both hypothesis-generating and hypothesis-driving studies (e.g., how nutritional

values are related to the metabolome composition). In this regard, applying other labeling chemistries targeting different chemical-group-submetabolomes (e.g., carboxyl submetabolome[25] and hydroxyl/thiol submetabolome[40]) will also increase the overall metabolome coverage for these studies.

Metabolomic Profiling of Different Cow Milk. To demonstrate the applicability of the workflow for milk metabolomics, we examined the metabolome profile differences among different groups of cow milk samples. This profiling work involved the analysis of three batches of milk with each batch consisting of 12 different milk samples from three brands (Dairyland, Lucerne and Beatrice) and four fat percentages (skim, 1%, 2% and 3.25%). Experimental triplicates were performed for each sample. In total, 108 labeled samples were analyzed by LC-MS.

From the 108 sample runs, a total of 7104 peak pairs were detected with an average of 4573±505 peak pairs (n=108) per sample. To determine how consistent the same peak pairs could be detected across different samples, Figure 5 shows the plot of the number of peak pairs and the percentage of common peak pairs as a function of the number of samples. A total of 2189 pairs were commonly detected in all the samples, while 3820 pairs were detected in more than 80% of the samples. It is clear that a large number of metabolites could be consistently detected and quantified in the milk amine/phenol submetabolome profiling by the dansylation LC-MS method.

The 3820 peak pairs commonly detectable in more than 80% of the samples were searched against the dansyl library, by which 70 metabolites were positively identified (Supplemental Table S8). By using accurate mass search against HMDB and EML libraries, 954 and 2987 peak pairs were matched to one or a few chemical structures, respectively

(Supplemental Tables S9 and S10). It should be noted that the cow milk metabolites were searched against the human metabolome database, as an equivalent resource for the cow metabolome was not available.

Figure 6 shows the PLS-DA multivariate analysis plots generated from the milk metabolome data set containing the metabolites detectable in more than 80% of the samples. Supplemental Figure S3 shows their corresponding PCA plots. Supplemental Figure S4 shows the PCA plot including the QC samples; very close clustering of the QC data indicates CIL LC-MS did not introduce any instrument bias in sample analysis. In Figure 6A and 6B, there is no apparent visual separation between the different fat percentages of milk with the exception of the 3.25% milk. However, the plot had a poor R^2 value (representative of goodness of fit) and Q^2 value (predictability value) of 0.241 and 0.114, respectively, which did not meet the acceptable separation-threshold of 0.5. Therefore, the visual separation observed in the model was rejected. Even the comparison between skim milk and 3.25% milk showed no significant separation between the two after statistical analysis. The model generated in that comparison did not pass the validation test (see Supplemental Figure S5B where the Q^2 y-intercept value is greater than 0); thus the visual separation observed (Supplemental Figure S5A) was also rejected. These results indicate that the amine/phenol submetabolomes of different fat percentages of milk within a batch were similar, which is not surprising considering that all samples had undergone lipid removal prior to chemical labeling and analysis.

Differences between milk brands were also investigated. Majority of all milk purchased within Alberta comes from Alberta dairy farms due to the Canadian Dairy Commission's supply management on domestic production and consumption. A dairy farm may house more than one breed of cattle (Jersey, Holstein, etc.). They may use different combinations of cattle feed and

sell their raw milk to more than one processor. Essentially, each brand of milk may be produced from a combination of different local dairy farms. Lastly, milk companies have variations in their production methods and processing techniques of saleable milk. From the PLS-DA plots shown in Figure 6C and 6D, there are some separations between the three brands of cow milk. The 3-dimentional (3D) plot in Figure 6D shows a clear separation with R^2 and Q^2 values of 0.971 and 0.927. Using a 50-permutation test, the model passed validation with a Q^2 y-intercept value of -0.0891, which indicates no issue of over-fitting (Supplemental Figure S6A). These findings are consistent with previous studies that showed relationships between external/internal factors and differences in individual cow milk composition. External factors can affect milk protein and lipid composition.[41, 42] Factors such as nitrogen fertilization of the feed or the forage particle size can affect the composition of the cow milk.[42, 43] Internal factors such as the breed of the cow also affects milk composition. Jersey cows were found to have higher percentages of protein and fat in their milk as compared to Holstein cows.[41, 44] Overall, variances between the amine/phenol submetabolomes of different brands of grocery store cow milk were observed.

Lastly, the milk samples were grouped by batch number (or collection date). In Figure 6E and 6F, there are significant separations between the different batches of milk with R^2=0.978 and Q^2=0.947. The model was validated using a 50-permutation test that provided a passing Q^2 y-intercept of -0.257 (Supplemental Figure S6B). This separation is more clearly shown in the 3D PLS-DA plot in Figure 6F. The three batches of milk samples were obtained at different time points during the year. Any changes in the milk metabolome could be attributed to changes in factors such as season, batches of feeds and water or stage in the lactation cycle. Previous studies observed changes in milk composition correlated to changes in season.[45, 46] Authors observed changes in factors such as temperature and sunlight hours having a negative and positive

correlation, respectively, to protein and fat concentration.[45, 46] Milk protein and fat concentration also showed fluctuations during lactation with the highest concentrations of both near the end of the lactation period.[46]

It should be noted that none of the significant metabolites found to separate the different groups could be positively identified using the dansyl standard library, although many of them could match to some metabolite structures in the HMDB and EML database (i.e., they are likely real metabolites). Without positive IDs, we are unable to discuss their biological significance.

Overall, the above results of analyzing different samples show the ability of the dansylation LC-MS workflow to generate high-coverage and quantitative metabolome data that can reveal small differences in milk metabolome profiles. We note that direct comparison of metabolite detectability between our method and other reported work cannot be done as our focus is on the analysis of the amine/phenol submetabolome while other work used one or several combined analytical methods to detect as many different metabolites and lipids as possible. In a comprehensive profiling work reported recently,[15] a total of 710 metabolites were detected using multiple-platforms including LC-MS, UPLC-MS, GC-MS, CE-MS and NMR. Majority of them were lipids; however, 26 amine/phenol-containing metabolites were listed, In our work of analyzing 108 milk samples using the dansylation LC-MS platform alone, as discussed earlier, a total of 3820 peak pairs or metabolites were commonly detectable in more than 80% of the samples with 70 amine/phenol-containing metabolites positively identified. Among the positively identified metabolites from our study and the reported work, there were 21 metabolites in common (see Supplemental Table S11) with 5 unique to the reported study (i.e., Gly-Gly, cytosine, creatine phosphate and creatine, cytidine; see Supplemental Table S12). Among the 5 unique metabolites, cytosine, creatine and cytidine are in our dansyl standard

library; but we did not detect them in our samples. The other two, Gly-Gly and creatine phosphate, are not in our library so we could not positively identify them in our samples. Thus, most of the amine/phenol-containing metabolites reported were identified in our samples. More significantly, we identified 49 additional amine/phenol-containing metabolites (see Supplemental Table S13), including some nucleosides (e.g., guanosine and uridine) that are bioactive compounds found in milk of several species.[47, 48] In addition, there were 2988 putatively matched metabolites in our data set. These results clearly show the advantage of dansylation LC-MS for in-depth profiling of the amine/phenol submetabolome. We expect that, using other labeling chemistries, many more metabolites will be detected in milk. In addition, CIL LC-MS is a quantitative method for milk metabolome profiling as demonstrated in this work. We are not aware of any other studies of quantification accuracy and precision of profiling a large number of milk metabolites using other MS techniques.

Conclusions

In summary, we have developed an analytical workflow to address the current challenge of performing comprehensive and quantitative profiling of the milk metabolome. It involves the use of an optimized sample preparation method for protein and lipid removal from milk, followed by chemical isotope labeling of the milk metabolites and subsequent LC-MS analysis. We envisage the application of this workflow for comprehensive and quantitative milk metabolomics in future studies involving the dairy industry (e.g., new processing technologies, quality control and product authentication) and nutritional sciences with implications of dairy consumption on human health.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs program, Genome Canada and Alberta Innovates.

Disclosure

The authors declare no competing financial interest.

Supporting Information

The Supporting Information available:

- Supplemental Note S1 for experimental, Figures S1-S6 for heat maps and statistical plots and Tables S1-S13 listing metabolite identification results.

This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Zheng, H.; Clausen, M. R.; Dalsgaard, T. K.; Bertram, H. C. *Nutrients* **2015**, *7*, 4875-4896.
(2) Kim, S.; Kim, J.; Yun, E. J.; Kim, K. H. *Curr Opin Biotechnol* **2016**, *37*, 16-23.
(3) Gibbons, H.; O'Gorman, A.; Brennan, L. *Curr Opin Lipidol* **2015**, *26*, 30-34.
(4) Mayne, S. T.; Playdon, M. C.; Rock, C. L. *Nat Rev Clin Oncol* **2016**, *13*, 504-515.
(5) Pereira, P. C. *Nutrition* **2014**, *30*, 619-627.
(6) Cilla, A.; Quintaes, K. D.; Barbera, R.; Alegria, A. *Crit Rev Food Sci Nutr* **2016**, *56*, 1880-1892.
(7) Hess, J. M.; Jonnalagadda, S. S.; Slavin, J. L. *Compr. Rev. Food. Sci. Food Saf.* **2016**, *15*, 251-268.
(8) Sousa, S. G.; Delgadillo, I.; Saraiva, J. A. *Crit Rev Food Sci Nutr* **2016**, *56*, 1043-1060.
(9) Oliver, S. P.; Boor, K. J.; Murphy, S. C.; Murinda, S. E. *Foodborne Pathog Dis* **2009**, *6*, 793-806.
(10) Munir, M. T.; Yu, W.; Young, B. R.; Wilson, D. I.; I2C *Trends Food Sci Technol* **2015**, *43*, 205-218.
(11) Cappozzo, J. C.; Koutchma, T.; Barnes, G. *J Dairy Sci* **2015**, *98*, 5068-5079.
(12) Rocchi, S.; Caretti, F.; Gentili, A.; Curini, R.; Perret, D.; Perez-Fernandez, V. *Food Chem* **2016**, *211*, 455-464.
(13) Hampel, D.; Allen, L. H. *Crit Rev Food Sci Nutr* **2016**, *56*, 494-511.
(14) Klein, M. S.; Almstetter, M. F.; Schlamberger, G.; Nurnberger, N.; Dettmer, K.; Oefner, P. J.; Meyer, H. H. D.; Wiedemann, S.; Gronwald, W. *J Dairy Sci* **2010**, *93*, 1539-1550.
(15) Andreas, N. J.; Hyde, M. J.; Gomez-Romero, M.; Lopez-Gonzalvez, M. A.; Villasenor, A.; Wijeyesekera, A.; Barbas, C.; Modi, N.; Holmes, E.; Garcia-Perez, I. *Electrophoresis* **2015**, *36*, 2269-2285.
(16) Yang, Y. X.; Zheng, N.; Zhao, X. W.; Zhang, Y. D.; Han, R. W.; Yang, J. H.; Zhao, S. G.; Li, S. L.; Guo, T. J.; Zang, C. J., et al. *J. Proteomics* **2016**, *136*, 174-182.
(17) Sundekilde, U. K.; Downey, E.; O'Mahony, J. A.; O'Shea, C. A.; Ryan, C. A.; Kelly, A. L.; Bertram, H. C. *Nutrients* **2016**, *8*, 15.
(18) Wu, J. F.; Domellof, M.; Zivkovic, A. M.; Larsson, G.; Ohman, A.; Nording, M. L. *Biochem Biophys Res Commun* **2016**, *469*, 626-632.
(19) Spevacek, A. R.; Smilowitz, J. T.; Chin, E. L.; Underwood, M. A.; German, J. B.; Slupsky, C. M. *J Nutr* **2015**, *145*, 1698-1708.
(20) Tian, H.; Zheng, N.; Wang, W. Y.; Cheng, J. B.; Li, S. L.; Zhang, Y. D.; Wang, J. Q. *Sci Rep* **2016**, *6*, 10, DOI: 10.1038/srep24208.
(21) Smilowitz, J. T.; O'Sullivan, A.; Barile, D.; German, J. B.; Lonnerdal, B.; Supsky, C. M. *J Nutr* **2013**, *143*, 1709-1718.
(22) Caboni, P.; Murgia, A.; Porcu, A.; Demuru, M.; Pulina, G.; Nudda, A. *J Dairy Sci* **2016**, *99*, 6046-6051.
(23) Villasenor, A.; Garcia-Perez, I.; Garcia, A.; Posma, J. M.; Fernandez-Lopez, M.; Nicholas, A. J.; Modi, N.; Holmes, E.; Barbas, C. *Anal Chem* **2014**, *86*, 8245-8252.
(24) Guo, K.; Li, L. *Anal Chem* **2009**, *81*, 3919-3932.
(25) Guo, K.; Li, L. *Anal Chem* **2010**, *82*, 8789-8793.
(26) Liu, P.; Huang, Y. Q.; Cai, W. J.; Yuan, B. F.; Feng, Y. Q. *Anal Chem* **2014**, *86*, 9765-9773.

(27) Dai, W. D.; Huang, Q.; Yin, P. Y.; Li, J.; Zhou, J.; Kong, H. W.; Zhao, C. X.; Lu, X.; Xu, G. W. *Anal Chem* **2012**, *84*, 10245-10251.
(28) Tayyari, F.; Gowda, G. A. N.; Gu, H. W.; Raftery, D. *Anal Chem* **2013**, *85*, 8715-8721.
(29) Fan, R. J.; Guan, Q.; Zhang, F.; Leng, J. P.; Sun, T. Q.; Guo, Y. L. *Anal Chim Acta* **2016**, *908*, 132-140.
(30) Hao, L.; Zhong, X. F.; Greer, T.; Ye, H.; Li, L. J. *Analyst* **2015**, *140*, 467-475.
(31) Bruheim, P.; Kvitvang, H. F. N.; Villas-Boas, S. G. *J Chromatogr A* **2013**, *1296*, 196-203.
(32) Huan, T.; Wu, Y.; Tang, C.; Lin, G.; Li, L. *Anal Chem* **2015**, *87*, 9838-9845.
(33) Wu, Y.; Li, L. *Anal Chem* **2012**, *84*, 10723-10731.
(34) Zhou, R.; Tseng, C. L.; Huan, T.; Li, L. *Anal Chem* **2014**, *86*, 4675-4679.
(35) Huan, T.; Li, L. *Anal Chem* **2015**, *87*, 1306-1313.
(36) Huan, T.; Li, L. *Anal Chem* **2015**, *87*, 7011-7016.
(37) Li, L.; Li, R. H.; Zhou, J. J.; Zuniga, A.; Stanislaus, A. E.; Wu, Y. M.; Huan, T.; Zheng, J. M.; Shi, Y.; Wishart, D. S., et al. *Anal Chem* **2013**, *85*, 3401-3408.
(38) Vuckovic, D. *Anal Bioanal Chem* **2012**, *403*, 1523-1548.
(39) Tang, Y. A.; Li, L. *Anal Chim Acta* **2013**, *792*, 79-85.
(40) Zhao, S.; Luo, X.; Li, L. *Anal Chem* **2016**, *88*, 10617-10623.
(41) Croissant, A. E.; Washburn, S. P.; Dean, L. L.; Drake, M. A. *J Dairy Sci* **2007**, *90*, 4942-4953.
(42) Nasrollahi, S. M.; Imani, M.; Zebeli, Q. *J Dairy Sci* **2015**, *98*, 8926-8939.
(43) Warner, D.; Podesta, S. C.; Hatew, B.; Klop, G.; van Laar, H.; Bannink, A.; Dijkstra, J. *J Dairy Sci* **2015**, *98*, 3383-3393.
(44) Palladino, R. A.; Buckley, F.; Prendiville, R.; Murphy, J. J.; Callan, J.; Kenny, D. A. *J Dairy Sci* **2010**, *93*, 2176-2184.
(45) Bernabucci, U.; Basirico, L.; Morera, P.; Dipasquale, D.; Vitali, A.; Piccioli Cappelli, F.; Calamari, L. *J Dairy Sci* **2014**, *98*, 1815-1827.
(46) Roche, J. R.; Turner, L. R.; Lee, J. M.; Edmeades, D. C.; Donaghy, D. J.; Macdonald, K. A.; Penno, J. W.; Berry, D. P. *Anim. Prod. Sci* **2009**, *49*, 222-232.
(47) Gill, B. D.; Indyk, H. E.; Manley-Harris, M. *Int Dairy J.* **2012**, *24*, 40-43.
(48) Gill, B. D.; Indyk, H. E. *Int Dairy J.* **2007**, *17*, 596-605.

Figure Captions

Figure 1. Method comparisons for protein removal: (A) protein pellet weight and (B) peak pair number detected as a function of solvent type and solvent-to-sample ratio. Method comparisons for lipid removal: (C) peak pair number detected as a function of protein-removal solvent and lipid-removal solvent types. Error bars correspond to standard deviation (n=3). DCM=dichloromethane and MTBE=methyl tert-butyl ether. The number of peak pairs was determined within the data set for protein and lipid removal studies alone.

Figure 2. (A) Base peak ion chromatogram of a 1:1 ^{12}C-/^{13}C-dansyl labeled milk sample and(B) peak pair number detected as a function of the injection amount (6 × n=3). The number of peak pairs was determined within the data set for injection-amount optimization data set alone.

Figure 3. Accuracy comparison using the distribution of peak pairs as a function of the peak ratios for (A) 1:1, (B) 2:1 and (C) 1:2 mixtures of five ^{12}C-/^{13}C-dansyl labeled milk samples (n=5). Precision comparison using the distribution of peak pairs as a function of the relative standard deviation of peak ratios for (D) 1:1, (E) 2:1 and (F) 1:2 mixtures (n=5). The number of peak pairs was determined within the data set of studying accuracy and precision alone.

Figure 4. (A) Average concentration of labeled metabolites from various types of milk (n=3), (B) PCA plot of inter-species and intra-species mammalian milk including goat milk, cow milk (heat treated vs non-heat treated) and human milk, and (C) metabolite distribution of the different milk.

Figure 5. The inverse relationship between the percentages of common peak pairs detected and the total peak pair numbers generated by processing various numbers of samples (5, 10, 15, etc.) from the 108-sample milk profiling data sets.

Figure 6. Two-dimensional and three-dimensional PLS-DA plots of cow milk samples grouped based on (A, B) fat percentage, (C, D) brand, and (E, F) and batch number.

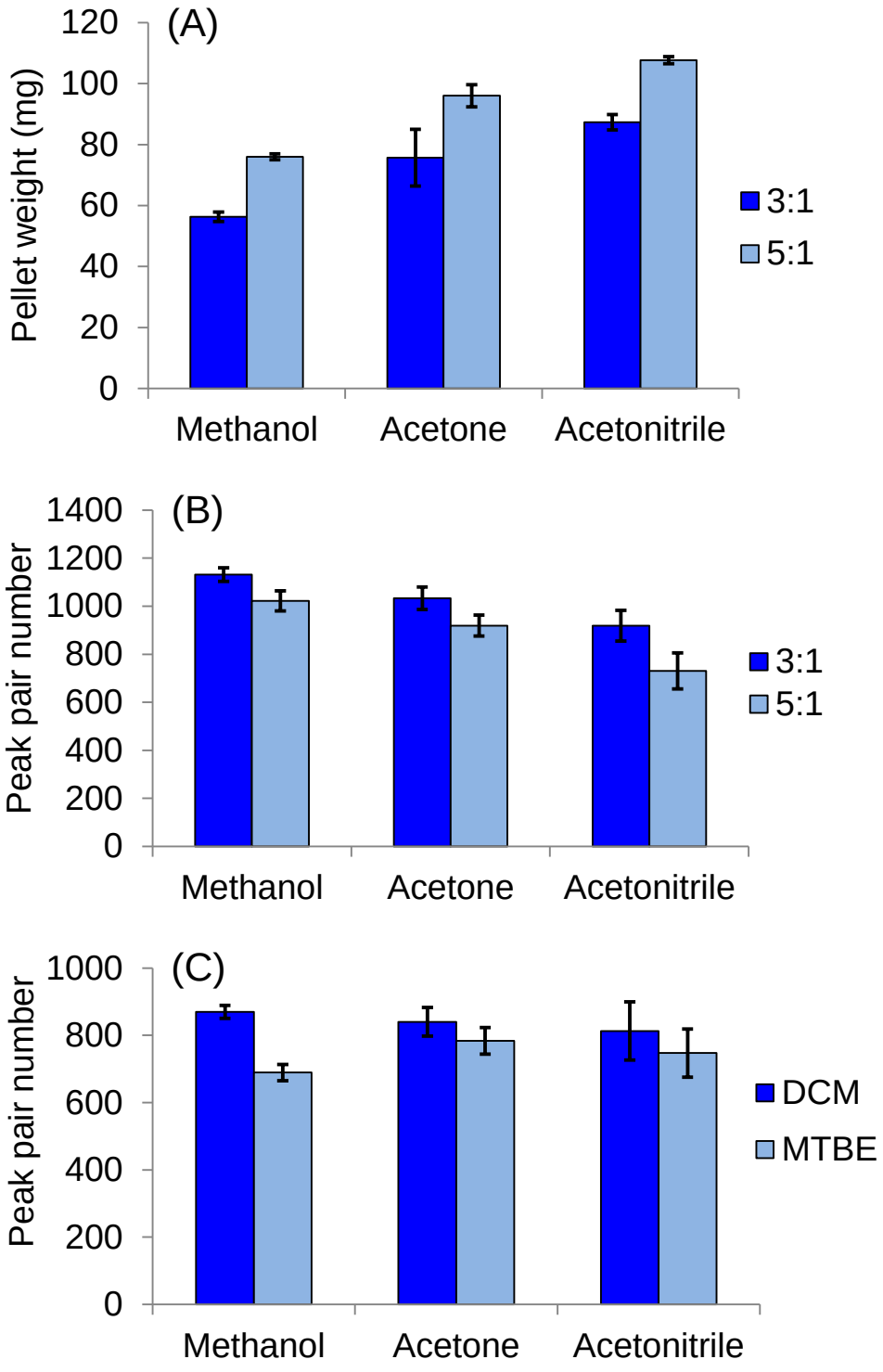
(A)
Pellet weight (mg)
120
100
80
60
40
20
0
Methanol
Acetone
Acetonitrile
3:1
5:1
(B)
Peak pair number
1400
1200
1000
800
600
400
200
0
Methanol
Acetone
Acetonitrile
3:1
5:1
(C)
Peak pair number
1000
800
600
400
200
0
Methanol
Acetone
Acetonitrile
DCM
MTBE

Figure 1

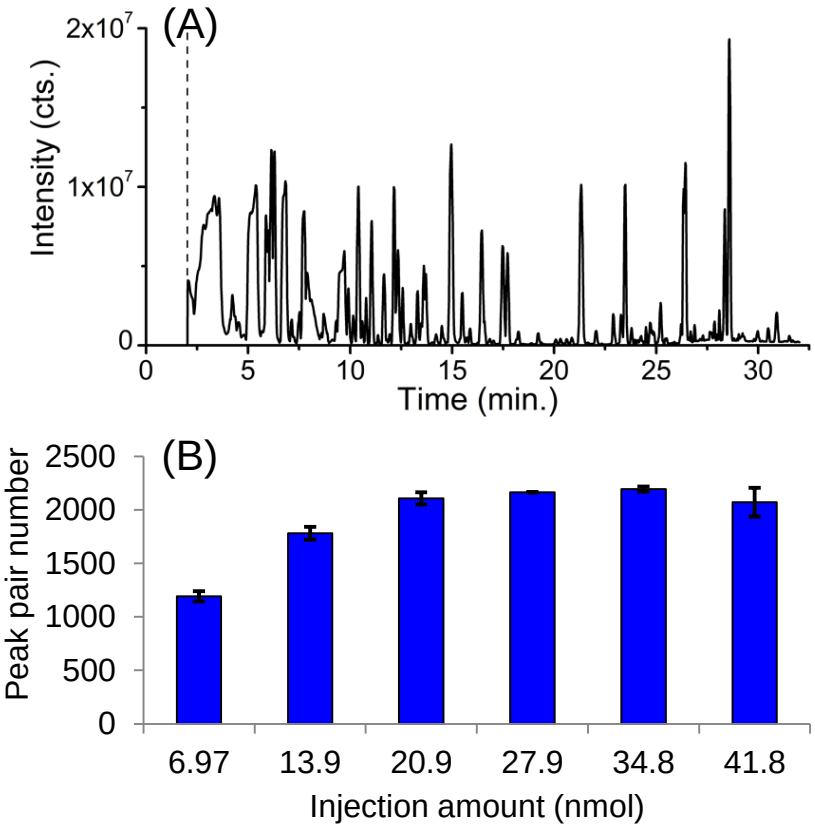
(A)
2x10^7
1x10^7
0
Intensity (cts.)
0
5
10
15
20
25
30
Time (min.)
(B)
2500
2000
1500
1000
500
0
Peak pair number
6.97
13.9
20.9
27.9
34.8
41.8
Injection amount (nmol)

Figure 2

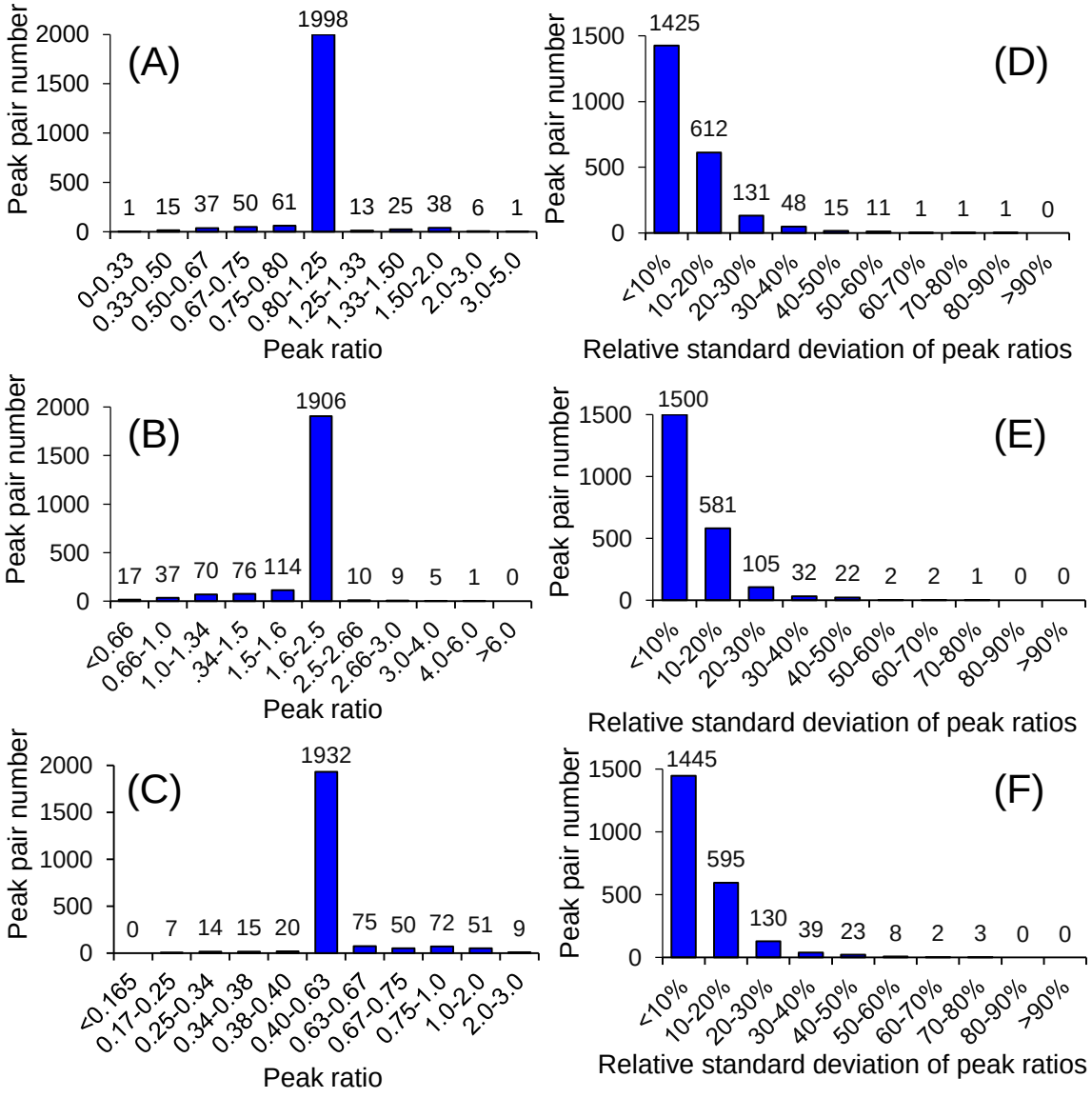
(A)
Peak pair number
2000
1500
1000
500
0
1
15
37
50
61
1998
13
25
38
6
1
0-0.33
0.33-0.50
0.50-0.67
0.67-0.75
0.75-0.80
0.80-1.25
1.25-1.33
1.33-1.50
1.50-2.0
2.0-3.0
3.0-5.0
Peak ratio
(D)
Peak pair number
1500
1000
500
0
1425
612
131
48
15
11
1
1
1
0
<10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
>90%
Relative standard deviation of peak ratios
(B)
Peak pair number
2000
1500
1000
500
0
17
37
70
76
114
1906
10
9
5
1
0
<0.66
0.66-1.0
1.0-1.34
.34-1.5
1.5-1.6
1.6-2.5
2.5-2.66
2.66-3.0
3.0-4.0
4.0-6.0
>6.0
Peak ratio
(E)
Peak pair number
1500
1000
500
0
1500
581
105
32
22
2
2
1
0
0
<10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
>90%
Relative standard deviation of peak ratios
(C)
Peak pair number
2000
1500
1000
500
0
0
7
14
15
20
1932
75
50
72
51
9
<0.165
0.17-0.25
0.25-0.34
0.34-0.38
0.38-0.40
0.40-0.63
0.63-0.67
0.67-0.75
0.75-1.0
1.0-2.0
2.0-3.0
Peak ratio
(F)
Peak pair number
1500
1000
500
0
1445
595
130
39
23
8
2
3
0
0
<10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
>90%
Relative standard deviation of peak ratios

Figure 3

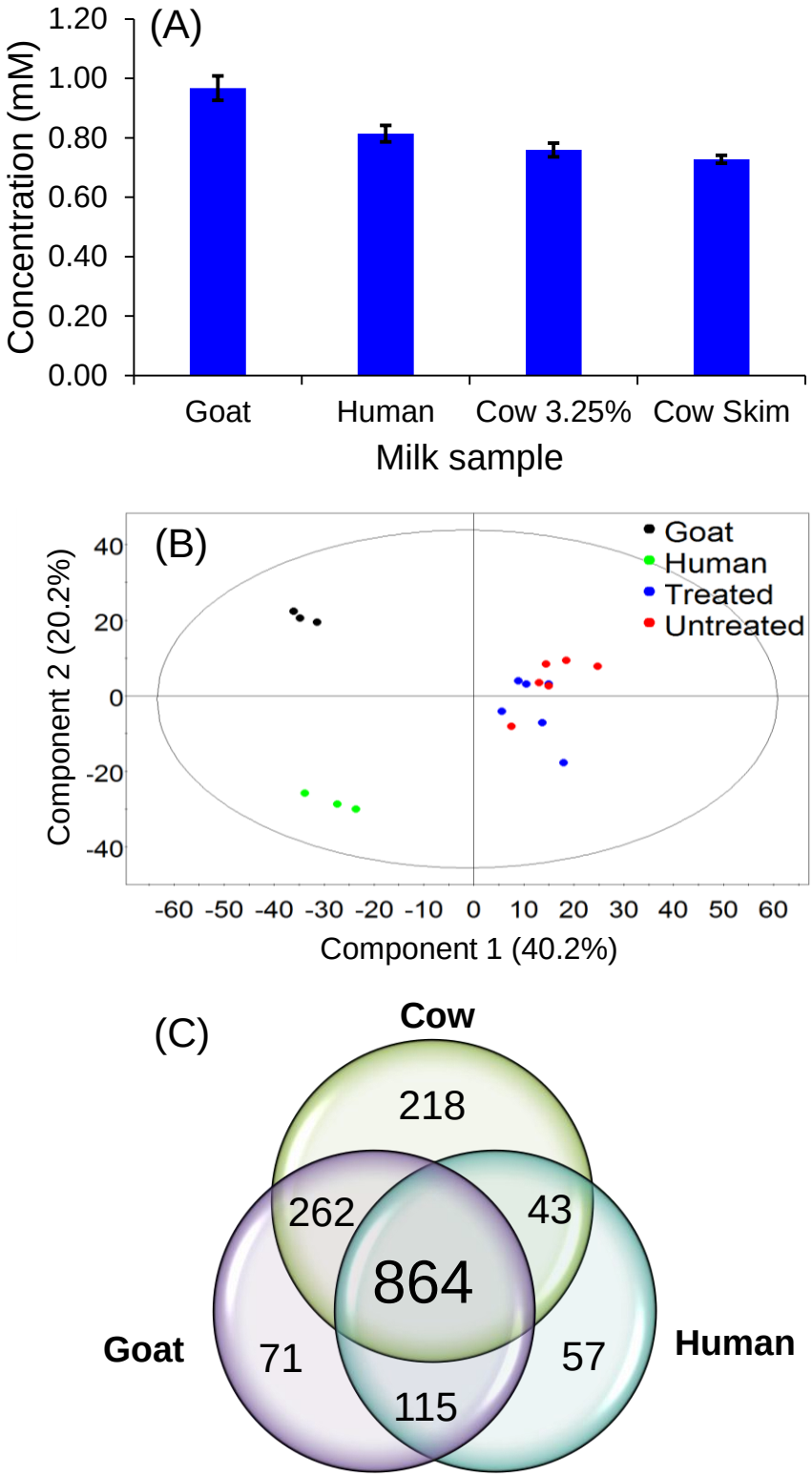
(A)
Concentration (mM)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Goat
Human
Cow 3.25%
Cow Skim
Milk sample
(B)
Goat
Human
Treated
Untreated
Component 2 (20.2%)
40
20
0
-20
-40
-60 -50 -40 -30 -20 -10 0 10 20 30 40 50 60
Component 1 (40.2%)
(C)
Cow
218
262
43
864
Goat
71
57
Human
115

Figure 4

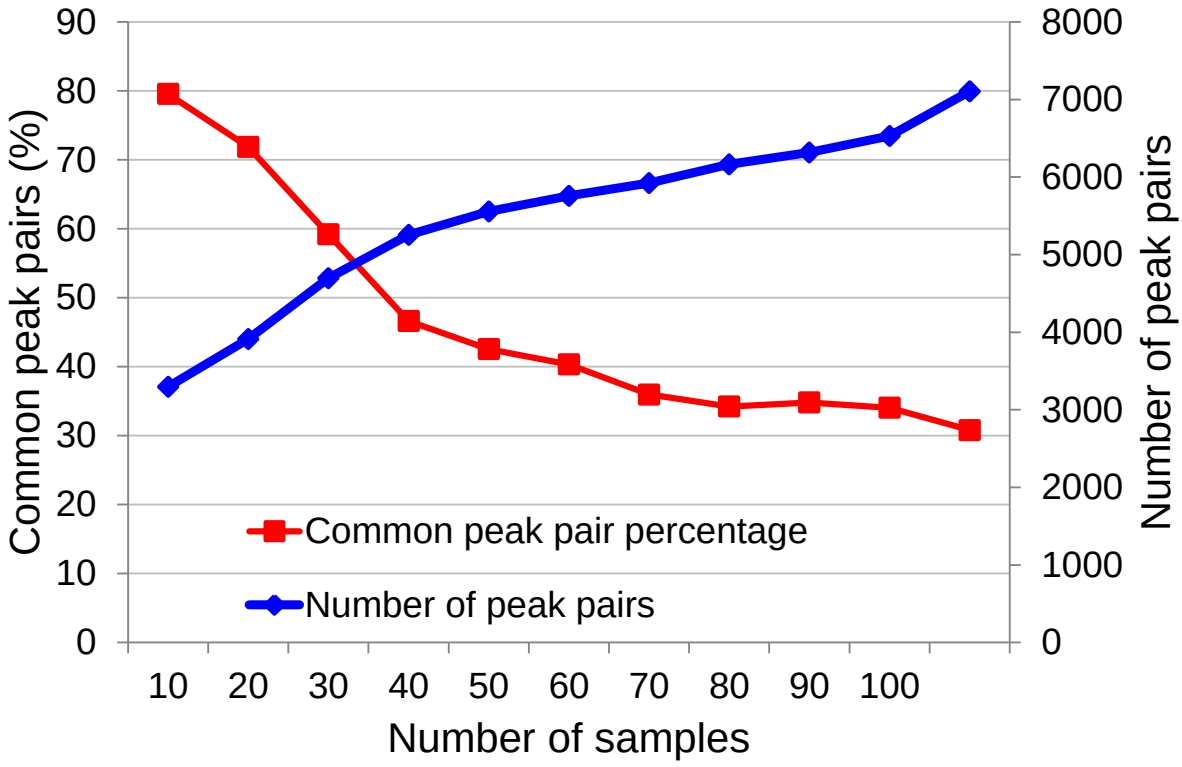
90
80
70
60
50
40
30
20
10
0
Common peak pairs (%)
8000
7000
6000
5000
4000
3000
2000
1000
0
Number of peak pairs
Common peak pair percentage
Number of peak pairs
10 20 30 40 50 60 70 80 90 100
Number of samples

Figure 5

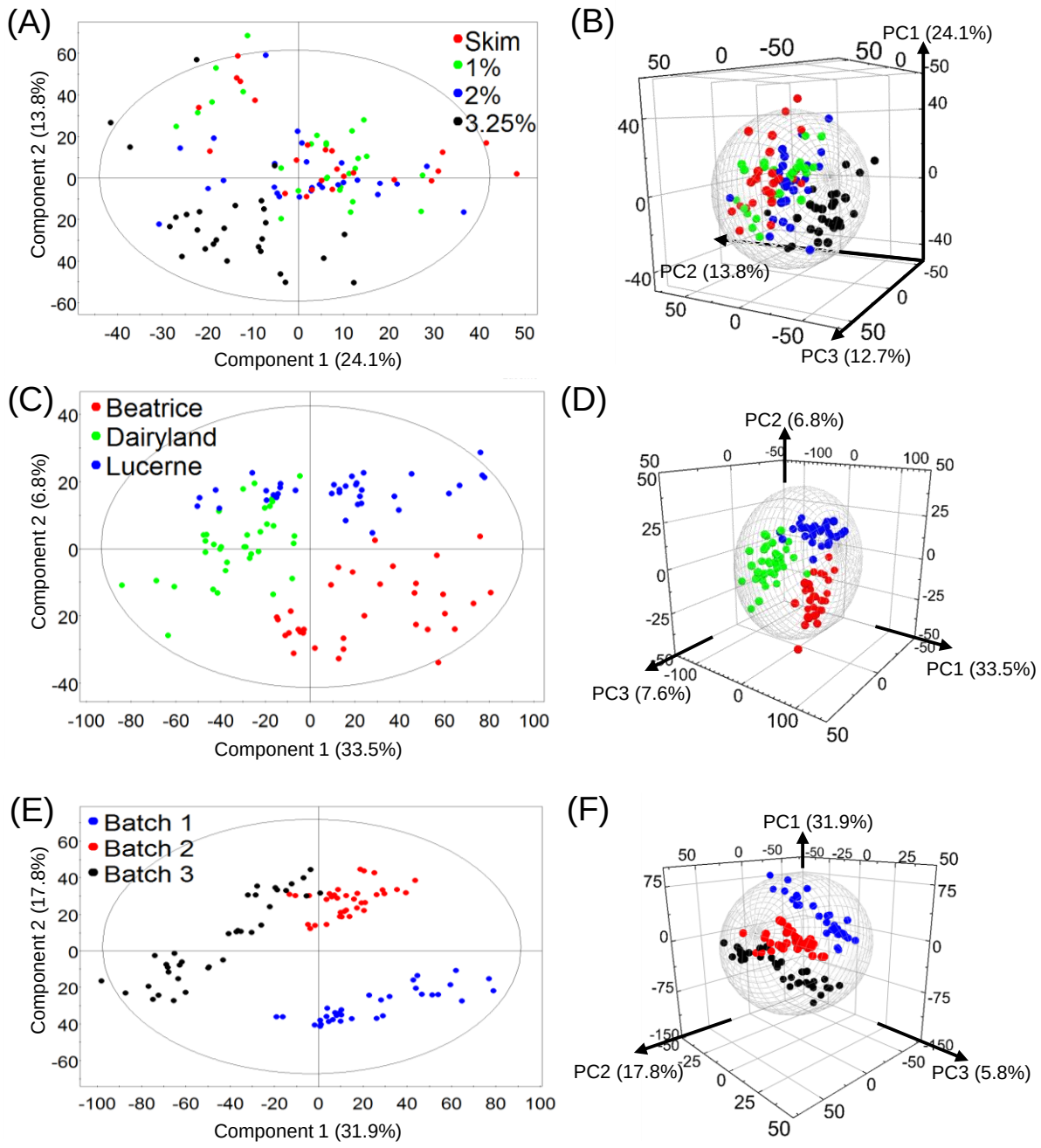
(A)
Skim
1%
2%
3.25%
Component 2 (13.8%)
Component 1 (24.1%)
(B)
PC1 (24.1%)
PC2 (13.8%)
PC3 (12.7%)
(C)
Beatrice
Dairyland
Lucerne
Component 2 (6.8%)
Component 1 (33.5%)
(D)
PC2 (6.8%)
PC1 (33.5%)
PC3 (7.6%)
(E)
Batch 1
Batch 2
Batch 3
Component 2 (17.8%)
Component 1 (31.9%)
(F)
PC1 (31.9%)
PC2 (17.8%)
PC3 (5.8%)

Figure 6

For Table of Contents: