Biomarker discovery has been increasingly important in the field of metabolomics for the detection and understanding of diseases. Of the many biofluids available for metabolomics, urine is a preferred option as it is non-invasive to collect and contains a wide range of metabolites reflective of the health status of the testing individual. However, urine also contains many exogenous metabolites which are introduced through various sources such as diet. This complicates the data interpretation when searching the metabolome for disease-related endogenous metabolites. Since diet is difficult to control, this work aims to study the acute effects of diet (particularly cow milk) consumption on the human urine metabolome by utilizing differential chemical isotope labeling (CIL) liquid chromatography mass spectrometry (LC-MS). LC-MS analysis of 62 urine samples collected before and after (1 hour and 2 hours) milk intake resulted in the detection of 4985 metabolites with an average of 3815 ± 206 (n = 62) detected per sample. The work aims to differentiate the exogenous “food” metabolites from the endogenous metabolite pool and to determine any dietary effects from milk intake on the human urine metabolome.

1. Introduction

Metabolomics has been increasingly used for disease biomarker discovery research [1,2]. Among various biofluids available for metabolome analysis, urine is most widely used as it can be collected non-invasively in large quantities and frequencies, containing a wide range of metabolites reflective of health status [3]. However, the human urine metabolome is large and complex made up of interactions between intrinsic genetic and external environmental factors [4]. The individual’s exposome, referring to features such as environmental exposures, gut microbiota, xenobiotics and food/diet affects their overall metabolome [4,5]. Diet especially, is one of the contributors of exogenous compounds affecting the urine
metabolome, which can complicate the data interpretation process when human endogenous metabolites are sought after as potential biomarkers [6,7].

There are two ways of dealing with the issue of dietary effect on urine metabolome. One way would be to control the diet before urine collection. This would reduce the metabolomic variations caused by different diets from individual subjects. However, in metabolomics studies, dietary control is very difficult to carry out. In most clinical trials and longitudinal studies where urine samples are collected and stored, strict dietary control is often not done. In fact, even the type of urine collected is not controlled; urine samples collected in a study often include no-fasting urine as well as first void or second void urine after overnight fasting. Using uncontrolled urine samples has the advantage in that firstly, it is easier to recruit study subjects with less compliance uncertainty. Secondly, without the need for dietary control, accessing a large number of already collected samples in existing biobanks may be a possibility. Lastly, with the overall larger sample size made possible by including various types of urine, any biomarkers discovered can be more readily deployed for clinical use. Thus, an alternative way of dealing with the dietary effect issue is to determine the metabolites with their levels most likely to be affected by diets. This follows a nutrime tabonomics approach where metabolomics is used to investigate the interactions of a food(s) on the metabolic system of an individual as well as to determine any food-specific biomarkers [4,8]. The inclusion of these diet-sensitive metabolites in a biomarker panel requires extra caution at the discovery stage to avoid artifacts influenced by diets. These metabolites may not be chosen in a validation study, or if they are chosen, dietary control needs to be taken into consideration. To make this second approach effective in finding the true biomarkers of a phenotype such as a disease, it will be very important that only a small fraction of the urine metabolome is varied to a significant extent by diets. In this regard, metabolomic coverage of a technique needs to be considered. If a technique can detect several thousands of metabolites covering many metabolic pathways, the presence of a small percentage of diet-sensitive metabolites in the dataset may not drastically reduce the chance of finding the true biomarkers.

In this work, we apply a high-performance chemical isotope labeling (CIL) liquid chromatography mass spectrometry (LC-MS) metabolomics method to examine changes in urine amine and phenol submetabolome profiles after consumption of cow milk. In some parts of the world, such as North America, milk encompasses one of the main food consumption of cow milk. In some parts of the world, such as Alberta (mcid.chem.ualberta.ca). These reagents are also available from the University of Alberta in compliance with the University of Alberta Health Information Policy. Fig. 1A illustrates the urine collection process. Urine samples were collected from 6 healthy individuals, 2 females and 4 males. The short-term urine study involved 4 urine samples per collection day. The first sample was collected after 12 hours of overnight fasting and was labeled as the 1st void sample. The next sample was collected 1 hour after the first and was categorized as the “before” milk intake sample. After collection of the second sample the individual consumed 250 mL of Dairyland brand 1% milk. The third sample was collected 1 hour after which the milk was consumed. This sample was labeled as “1 hour” after milk intake. Lastly the fourth urine sample was collected 2 hours after the initial milk consumption and labeled as such. Each individual repeated the study on 3 separate days for biological replicates. All individuals were able to drink water throughout the duration of the collection period. The urine samples were collected in 50 mL sterile Eppendorf tubes and stored in the 4 °C fridge immediately after collection. Within the same day, the urine samples were vortexed at 4000 rpm for 10 minutes. The supernatant was filtered by 0.22 μm pore-sized Millipore filter (Millipore Corp., MA) and aliquotted into 0.6 mL vials. Equal volume aliquots of each individual sample were taken into a 1.5 mL vial to generate the pooled sample (to be used as the reference sample). 12.5 μL of urine sample was aliquoted out and diluted 4-fold by adding 37.5 μL of water. The 50 μL diluted urine solutions were then ready for dansylation and stored in the –80 °C freezer until further use.
2.3. Workflow and dansylation labeling

Fig. 1B shows the workflow of the differential chemical isotope labeling liquid chromatography mass spectrometry (CIL LC-MS) method for analyzing the metabolomes of urine collected before and after milk consumption. Briefly, the urine samples were first differentially labeled using dansylation. The individual samples were labeled with $^{12}$C-dansyl chloride and a pooled sample was labeled with $^{13}$C-dansyl chloride. Labeled samples were quantified using LC-UV (see below) and each $^{12}$C-labeled individual sample was mixed with the equal mole amount of the $^{13}$C-labeled pooled sample (reference sample) so that each labeled metabolite will show up as a pair of peaks in MS. LC-MS analysis was performed and data processing was done to pick the metabolite peaks pairs and determine their intensity ratios. The intensity ratio between the light/heavy metabolite peaks is reflective of the relative concentration of the metabolite in the individual sample compared to the pooled sample. The same $^{13}$C-labeled pooled sample was spiked into each $^{12}$C-labeled individual sample and these the intensity ratio values measured from
the separate analyses of all $^{12}$C-$^{13}$C-mixtures for a given metabolite reflect the concentration differences among these individual samples. In this way, comparisons between the urine samples before and after milk intake (1 hour or 2 hours) can be conducted using the metabolite peak pair ratios to perform univariate and multivariate statistical analysis.

Dansyl chloride was used as the labeling reagent to react mainly amine- and phenol-containing metabolites to form dansyl-amine or dansyl-phenol derivatives [10]. 50 μL of a processed urine 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-dansyl chloride.

2.4. LC-UV quantification

For LC-UV, a Waters ACQUITY UPLC system with a photodiode array (PDA) detector was used for the quantification of dansyl labeled metabolites for sample amount normalization as described earlier [11]. Briefly, 4 μL of each labeled sample was injected onto a Phenomenex Kinetix C18 column (2.1 mm × 5 cm, 1.7 μm particle size) for a fast step-gradient run. Solvent A was 0.1% (v/v) formic acid in 5% (v/v) ACN, and solvent B was 0.1% (v/v) formic acid in ACN. The gradient started with 0% B for 1 min and was increased to 95% within 0.01 min and held at 95% B for 1 min to ensure complete elution of all labeled metabolites. The flow rate used was 0.45 mL/min. The peak area related to the total labeled metabolite concentration in the sample was integrated using the Empower software (6.00.2154.003). Based on the quantification results, the $^{12}$C-labeled sample and the $^{13}$C-labeled pool were mixed in equal amounts.

2.5. 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 (Billerca, MA) with an ESI source. A reverse phase column (Agilent Eclipse Plus C18 column, 2.1 mm × 10 cm, 1.8 μm particle size, 95 Å pore size) was used for liquid chromatography separation of labeled metabolites. Mobile phase A was made up of 5% (v/v) acetonitrile and 0.1% (v/v) formic acid in water. Mobile phase B consists of 0.1% (v/v) formic acid in acetonitrile. The 32-min gradient conditions were: 0 min (20% B), 0–3.5 min (20–35% B), 3.5–18 min (35–65% B), 18–24 min (65–99% B) and 24–32 min (99% B). The column was then re-equilibrated at 20% B for 15 min. The flow rate was 180 μL/min.

2.6. Data processing, statistical analysis and metabolite identification

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, and peak-pair intensity ratio calculation [12]. The same peak pairs detected from multiple samples were then aligned to produce a CSV file that contains the metabolite information and peak ratios relative to a control (i.e., a pooled sample). A zero-fill program was then used to find missing peak pairs from the raw mass spectral data, filling in the missing values [13]. Peak ratios were further optimized through the use of IsoMS-Quant [14]. Volcano plots were generated by Origin 2016 Graphing and Analysis (OriginLab Corporation, Northampton, USA).

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 [15]. 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) (8021 known human endogenous metabolites) and the Evidence-based Metabolome Library (EML) (375,809 predicted human metabolites with one reaction) using MyCompoundID [15]. The mass accuracy tolerance window was set at 8 ppm and the retention time tolerance window set to 20 s for the definitive identification searches and for the putative searches a mass tolerance of 0.005 Da was used.

3. Results and discussion

3.1. Cow milk metabolome

The cow milk metabolome was first determined as previously described [16]. Briefly, a milk sample was divided into two aliquots, one labeled with $^{12}$C-dansyl chloride (DnsCl) and another labeled with $^{13}$C-DnsCl. After labeling, equal mole amounts were taken for mixing. The $^{12}$C-/ $^{13}$C-mixture was analyzed using LC-MS. Fig. 2A shows a representative base-peak ion chromatogram of a $^{12}$C-/ $^{13}$C-labeled cow milk. Many chromatographic peaks were detected across the entire separation time window, illustrating the complexity of the milk submetabolome (amines/phenols).

The IsoMS program was used to extract the peak pairs from the LC-MS data. It only retained one peak pair from [M+H]$^+$ of a $^{12}$C-/ $^{13}$C-labeled metabolite by filtering out other redundant peaks such as dimers. There were a total of 7104 peak pairs detected in cow milk with an average of 4573 ± 505 (n = 108) peak pairs per sample. 3820 were commonly detected in more than 80% of the samples. Among the 3820 peak pairs, 70 metabolites were positively identified by searching the dansyl standard library (Supplemental Table S1). 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 S2). It should be noted that the cow milk metabolites were searched against human metabolites (HMDB) due to the fact that equivalent resources were not available.
3.2. Human urine metabolome

All individual urine samples were labeled with $^{12}$C-dansyl chloride while the pooled sample (generated by taking equal aliquots of all samples) was labeled with $^{13}$C-dansyl chloride. After labeling, each individual and pooled sample was quantified by LC-UV and the concentration of labeled metabolites was determined. The samples were then mixed in equal mole amounts of $^{12}$C-individual sample and $^{13}$C-pooled sample and analyzed by LC-MS as single injections. Fig. 2 shows two representative base-peak ion chromatograms of labeled (A) milk and (B) urine. As Fig. 2 shows, the chromatograms of milk and urine are significantly different.

LC-MS analyses of a total of 62 urine samples resulted in the detection of a total of 4985 peak pairs or metabolites with an average of 3815 $\pm$ 206 (n = 62) peak pairs per sample. Among them, 98 could be positively identified (Supplemental Table S3) while 1599 and 3686 could be putatively matched to the HMDB and EML libraries, respectively (Supplemental Tables S4).

3.3. Multivariate analysis of the urine metabolome after milk intake

The PCA plots of the urine metabolome data set from samples collected before and after milk consumption with and without QC samples included are shown in Fig. 3A,B, respectively. In Fig. 3A, the QC samples cluster tightly together indicating good technical reproducibility. Fig. 3B,C are the unsupervised PCA and supervised PLS-DA scores plots of the urine samples grouped by time after milk intake. From the PCA plots we see no clear distinction between the urine samples before and 1–2 hours after milk intake. The PLS-DA plot shows a slight visual separation between the before and after milk intake urine samples (1 hour and 2 hours) with an $R^2$ value of 0.9321 and a $Q^2$ value of 0.0317. While the $R^2$ value (representative of goodness of fit) is good (close to 1), the $Q^2$ value (predictability value) is below the separation-threshold of 0.5. As well, the model generated does not pass the 100-permutations test and thus the separation observed between urine samples before and after milk intake was rejected.

This result does not simply mean that drinking cow milk has no effect on the human urine metabolome. It does, however, suggest that the short-term urine metabolome effects of milk intake may be minimal and not as significant in relation to other variables. In this study, the only variables controlled were the 12 hours fast (prior to milk intake), urine collection times and the consumption of cow milk. There are many other individual-dependent or even day-dependent external factors that may affect the results of this study such as their previous meal, intrinsic metabolism, gastrointestinal uptake, gut microbial composition/activity, glomerular filtration rate and tolerance to milk and milk products, etc [7]. To investigate this, an interactive principal component analysis (iPCA) plot (which is essentially a 3-dimensional PCA) was generated using the urine data grouped by collection day and individual as shown in Fig. 4A. The colors refer to each individual and the shapes refer to the collection day (note that the experiment was repeated on 3 different days). Colored ellipses were manually drawn in to group the samples collected within the same day (same shape) for each individual (same color) in Fig. 4B as the groupings may be difficult to see due to the overlapping of data points in Fig. 4A. From Fig. 4B, we see that there is significant separation between the samples from different individuals (colors). For some individuals (yellow, green and grey), their urine samples collected on different days (shapes) show little separation and cluster well together, while for others (blue, red and orange) their urine shows significant inter-day variability (Fig. 4B). From this plot, we see evidence of both inter-individual and inter-day variations in the urine metabolome that are seemingly much larger than the changes caused by milk intake. To further validate this finding, the urine dataset was regrouped by collection day and individual, and multivariate analysis was performed.

Fig. 5A,C shows the PLS-DA scores plots of the urine data-set grouped by collection day and individual, respectively (the PCA plots are included in Supplemental Figure S1). Metabolome differences were observed between urine samples collected on different days (Fig. 5A) with $R^2 = 0.9892$ and $Q^2 = 0.8908$. As well, significant separations were also
observed between urine metabolomes of different individuals with $R^2 = 0.9956$ and $Q^2 = 0.9657$. Both models generated also passed the 100-permutations test with p-values of 0.01 and < 0.01, respectively, which are lower than the 0.05 cut-off (Fig. 5B, D). These results indicate a statistically significant difference in the urine metabolomes of different individuals. As well, within the same individual, there may also be some inter-day variability in the urine metabolome, however, not as large as the inter-individual differences. With this finding, the urine metabolome dataset was re-grouped by individual, and analysed independently for the remainder of this work.

3.4. **Univariate analysis of the urine metabolome**

During the metabolism of food(s) a milk metabolite can be metabolized into a compound that is endogenously found in the human urine metabolome [6]. Therefore, univariate analysis was used to determine metabolites with significant concentration changes after drinking milk. Firstly, the common metabolites between the 3 different collection time points were determined (before, 1 hour and 2 hours after milk). Binary comparisons between an “after milk” group and the “before milk” group were then conducted. First, a t-test
was used to calculate a p-value for each metabolite. Secondly the fold change for the metabolite was calculated by taking the average of the peak ratios for the “after milk” group (be it 1 hour or 2 hours) and dividing it by the average peak ratio of the “before milk” group. The significantly changed metabolites between “before” and “after” milk intake was identified and plotted on Volcano Plots for each individual with the following criteria: p-value < 0.05, fold change (FC) > 1.5 and FC < 0.67 (refers to significantly increased and decreased, respectively).

Supplemental Figure S2 A-F shows the plots of the significantly changed metabolites 1-hour-after-milk intake and Fig. S2 G-L shows the plots for the metabolites changing 2-hours-after-milk intake. For volunteer #1, the individual had 195 and 72 significantly increased and decreased metabolites, 1-hour after milk intake. 2-hours after intake the numbers were 116 and 69, increased and decreased, respectively (Figs. S2A and C). For volunteer #2, they had 18 increased and 1 decreased metabolites at 1-hour after milk and 3 increased and 21 decreased metabolites at 2-hours after milk intake (Figs. S2B and H). Volunteer #3 had 60 increased and 6 decreased metabolites after 1-hour of drinking milk (Fig. S2C). Those numbers changed to 21 increased and 20 decreased 2-hours after milk (Fig. S2L). Volunteer #4 had 35 increased and 10 decreased after 1-hour post milk intake and had 56 increased and 33 decreased 2-hours post-milk (Figs. S2D and J). Volunteer #5 had 2 increased metabolites 1-hour post-milk intake and 19 decreased (Fig. S2E). 2-hours post milk intake those numbers changed to 0 increased and 24 decreased (Fig. S2K). Lastly, volunteer #6 had 70 increased and 0 decreased metabolites after 1-hour of consuming milk (Fig. S2F). 2-hours after milk intake those numbers were 63 increased and 5 decreased (Fig. S2L). Overall there seems to be a very small change in the concentrations of urine metabolites (<200 significantly changed metabolites) 1 and 2 hours after drinking 250 mL of cow milk.

3.5. Determination of common metabolites between the milk and urine metabolome

In order to determine any exogenous milk metabolites excreted into the urine, the dataset from the urine study was merged with the cow milk profiling dataset obtained from the previous study mentioned in Section 3.1 [16]. This was done by combining the data (after IsoMS peak-pair filtering and picking) and aligning the metabolites by matching retention time (RT) and mass (m/z) using a RT tolerance of 20 seconds and m/z tolerance of 8 ppm, followed by zero-filling of missing values and optimization of peak ratios by IsoQuant (as mentioned in Section 2.6). The milk metabolites found consistently across 80% of all milk samples was compared with the urine metabolites before, 1 hour and 2 hours after milk intake.

Because the sample type in the two sample sets was different (milk vs. urine) and the pooled sample used for each sample set (as reference) was also different (milk pool vs. urine pool), the concentrations of each metabolite could not be compared between the two sample sets. However, we could determine the metabolites unique to milk or urine or in common with both. Firstly, we determined the number of unique urine metabolites to each collection time point (Supplemental Figure S3 A–F). These unique metabolites include both endogenous urine and exogenous milk metabolites. Then we compared the unique metabolites for each time point with the milk metabolome and determined the number of unique urine metabolites found in common with milk (Figure S3 G–I). As an example, Fig. S3A shows the Venn diagram for volunteer #1. As the purpose of this study is to see the effects of milk on the urine metabolome, we only focused on the unique metabolites observed in the 1 hour, 1 and 2 hour, and 2 hours after milk intake urine samples. The numbers of those metabolites observed is bolded in Fig. S3A.

There are 70 metabolites unique to the 1-hour-after-milk urine samples, 156 metabolites unique to the 2-hours-after-milk urine samples and 563 metabolites found in both the 1-and-2-hours-after-milk urine samples. The above-mentioned metabolites were then compared with the milk metabolites obtained from the milk profiling set. Fig. S3G shows the distribution of metabolites (1 hour, 2 hours and both 1 and 2 hours after milk intake) that are in common with milk (shown in red) or unique to urine (shown in black). Of the 70 metabolites unique to the 1-hour-after-milk samples, 22 of them were in common with milk. 48 of the 156 unique metabolites in the 2-hours-after-milk samples were in common with milk. Lastly, 104 metabolites out of the 563 unique metabolites to the 1-and-2-hours-after samples were matched.
with milk metabolites. This evidence suggests a possibility that these urine metabolites, detected only after milk intake and found in common with the milk metabolome, could have come from the consumption of milk itself and was excreted into the urine unchanged. These metabolites will be referred to as “possible” milk-related metabolites from here on.

3.6. Determination of potential milk-related biomarkers across participants

As there were little to no overlap/commonality between the “possible” milk-related metabolites excreted into the urine between the study participants in the same time points, a broader criterion was established. Simply, all the “possible” milk-related metabolites detected in each individual were compared with no group distinction between when they were excreted. If a particular “possible” milk-related metabolite was detected in at least 50% of the participants (regardless of what time point) it was included as a potential milk-related biomarker. 50 metabolites were found unique to the “after milk intake” urine samples and in common with the cow milk metabolome across ≥50% of the participants. Supplemental Figure S4 shows the distribution of the 50 metabolites and is color coded by excretion timepoint (1 hour, 2 hours or both). From this figure, we see no pattern of excretion for these “possible” milk-related metabolites in terms of time after consumption across study.

Fig. 5 – PLS-DA scores plot and its respective permutation test results of the acute milk effect on urine data sorted by: (A–B) collection day and (C–D) individual.
participants. As well, the number of overlapping “possible” milk-related metabolites between individuals was relatively small (only 50 metabolites across ≥50% of the participants). The putative identification of the 50-potential milk-related metabolites is included in Supplemental Table 5.

3.7 Acute changes in the human urine metabolome due to moderate milk consumption

In comparison to the total number of metabolites observed in the urine or milk metabolome, the number of overlapped peak pairs (possibly milk-related) determined were much lower (<110 metabolites vs >3000 metabolites). As well, the number of metabolites with significantly changed concentrations was small, i.e., <5% of the metabolome. This was, in a way, expected. Past studies have found that dietary effects on the metabolome are less pronounced than say, a drug effect and can be easily masked by other variables such as inter-individual and inter-day variation (within the same individual) [4]. As well, there is no observed pattern to the changes in the urine amine/phenol submetabolome across all individuals thus demonstrating the unique individual responses to milk consumption. It should be noted that the effects of food consumption are typically more complex since food is not comprised of just one or few component(s) but rather many components (food and non-food related) that may be involved in various metabolic pathways [17–19]. Milk, for example, is comprised of a variety of proteins, lipids, sugars, vitamins and minerals [9]. Also, the number of “possible” milk-related metabolites excreted out into the urine refers to any milk metabolites that have not undergone bio-transformations during the ingestion, digestion, absorption and elimination process. Majority of the food-related metabolites excreted in human biofluids such as urine are quite chemically different from the parent compound ingested due to transformations in the mouth, stomach, liver, pancreas and intestines [6]. Food metabolites are transformed via phase I and II reactions which add chemical moieties to the metabolites for easier elimination (make them more polar for urine excretion) [6,20]. A limitation to our method, unfortunately, is that it cannot account for bio-transformed milk metabolites.

4. Conclusions

We have designed a study to examine the effects of moderate milk intake on urine sample analysis. From a technological viewpoint, the dansylation CIL LC-MS method utilized in this work illustrates the ability to detect a large number of metabolites in urine and milk. It is also able to detect changes in concentrations of urine metabolites after moderate milk consumption. As well, this CIL LC-MS method was used to compare the metabolome datasets of two different samples (urine and milk) in order to detect milk-related biomarkers excreted unchanged into the urine, 1 and 2 hours after consumption.

From a biological viewpoint, it appears that a moderate consumption of cow milk does not result in significant changes to the urine amine/phenol submetabolome. Our method revealed a larger inter-individual and inter-day variability in the human urine metabolome; however, it was still capable in detecting the subtle and individually unique responses to milk intake. A small number of urine metabolites (<5% of the metabolome) affected by milk intake was detected, indicating a homeostasis of the submetabolome that is not easily perturbed by the consumption of a normal level of milk (one glass) over a short period of time (2 hours). Lastly, 50 milk-related metabolites were observed across >50% of study participants that may be potential biomarkers of milk intake. In future work, we need to apply a similar CIL LC-MS approach to study the other chemical-group-based submetabolomes such as carboxylic acids, carbonyls, and hydroxyls. In this way, the human urine metabolome in its near entirety can be determined to investigate the overall short-term effects of milk consumption.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgements

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2018.10.007.

References

[1] Monteiro MS, Carvalho M, Bastos ML, Guedes de Pinho P. Metabolomics analysis for biomarker discovery: advances and challenges. Curr Med Chem 2013;20:257–71.
[2] Sethi S, Bietzke E. Omics-based biomarkers: application of metabolomics in neuropsychiatric disorders. Int J Neuropsychopharmacol 2015:19. https://doi.org/10.1093/innjp/pyv096. pyv096-pyv096.
[3] Zhang A, Sun H, Wu X, Wang X. Urine metabolomics. Clin Chim Acta 2012;414:65–9. https://doi.org/10.1016/j.cca.2012.08.016.
[4] Claus SP, Swann JR. Nutrimetabolomics:Applications for nutritional Sciences, with specific reference to gut microbial interactions. Annu Rev Food Sci Technol 2013;4:381–99. https://doi.org/10.1146/annurev-food-030212-182612.
[5] Vrijheid M. The exposome: a new paradigm to study the impact of environment on health. Thorax 2014;69:876–8. https://doi.org/10.1136/thoraxjnl-2013-204949.
[6] Scalbert A, Brennan L, Manach C, Andres-Lacueva C, Dragsted LO, Draper J, et al. The food metabolome: a window over dietary exposure. Am J Clin Nutr 2014;99:1286–308.
[7] Claus SP. Development of personalized functional foods needs metabolic profiling. Curr Opin Clin Nutr Metabol Care 2014;17:567–73. https://doi.org/10.1097/MCO.0000000000000107.
[8] Llorach R, Garcia-Aloy M, Tulipani S, Vazquez-Fresno R, Andres-Lacueva C. Nutrimetabolomic strategies to develop
new biomarkers of intake and health effects. J Agric Food Chem 2012;60:8797–808. https://doi.org/10.1021/jf301142b.

[9] Pereira PC. Milk nutritional composition and its role in human health. Nutrition 2014;30:619–27. https://doi.org/10.1016/j.nut.2013.10.011.

[10] Guo K, Li L. Differential $^{13}$C/$^{13}$C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. Anal Chem 2009;81:3919–32. https://doi.org/10.1021/ac900166a.

[11] Wu Y, Li L. Determination of total concentration of chemically labeled metabolites as a means of metabolome sample normalization and sample loading optimization in mass spectrometry-based metabolomics. Anal Chem 2012;84:10723–31. https://doi.org/10.1021/ac3005625.

[12] Zhou R, Tseng C-L, Huan T, Li L. IsoMS: automated processing of LC-MS data generated by a chemical isotope labeling metabolomics platform. Anal Chem 2014;86:4675–9. https://doi.org/10.1021/ac5009089.

[13] Huan T, Li L. Counting missing values in a metabolite-intensity data set for measuring the analytical performance of a metabolomics platform. Anal Chem 2015;87:1306–13. https://doi.org/10.1021/acs.analchem.5b09994.

[14] Huan T, Li L. Quantitative metabolome analysis based on chromatographic peak reconstruction in chemical isotope labeling liquid chromatography mass spectrometry. Anal Chem 2015;87:7011–6. https://doi.org/10.1021/acs.analchem.5b01434.

[15] Huan T, Wu Y, Tang C, Lin G, Li L. DnsID in MyCompoundID for rapid identification of dansylated amine- and phenol-containing metabolites in LC–MS-based metabolomics. Anal Chem 2015;87:9838–45. https://doi.org/10.1021/acs.analchem.5b02282.

[16] Mung D, Li L. Development of chemical isotope labeling LC-MS for milk metabolomics: comprehensive and quantitative profiling of the amine/phenol submetabolome. Anal Chem 2017;89:4435–43. https://doi.org/10.1021/acs.analchem.6b03737.

[17] Astarita G, Langridge J. An emerging role for metabolomics in nutrition science. J Nutrigenetics Nutrigenomics 2013;6:181–200. https://doi.org/10.1159/000354403.

[18] Jones DP, Park Y, Ziegler TR. Nutritional metabolomics: progress in addressing complexity in diet and health. Annu Rev Nutr 2012;32:183–202. https://doi.org/10.1146/annurev-nutr-072610-145159.

[19] Rein MJ, Renouf M, Cruz-Hernandez C, Actis-Gorettta L, Thakkar SK, da Silva Pinto M. Bioavailability of bioactive food compounds: a challenging journey to bioefficacy. Br J Clin Pharmacol 2013;75:588–602. https://doi.org/10.1111/j.1365-2125.2012.04425.x.

[20] Croom E. Chapter three – metabolism of xenobiotics of human environments. In: Hodgson E, editor. Toxicol. Hum. Environ., vol. 112. Academic Press; 2012. p. 31–88. https://doi.org/10.1016/978-0-12-415813-9.00003-9.