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Effects of Freeze–Thaw Cycles of Blood Samples on High-Coverage Quantitative Metabolomics

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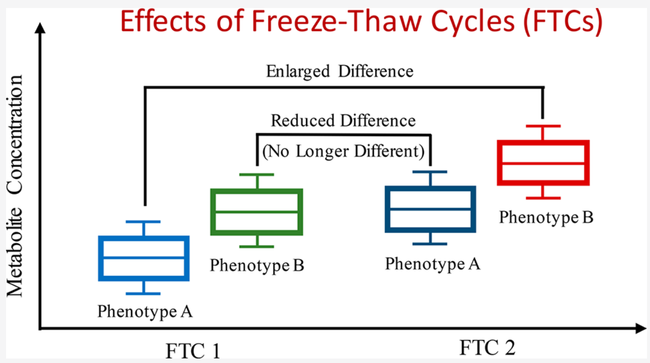
ABSTRACT: Blood metabolomics has been widely used for discovering potential metabolite biomarkers of various diseases. In this study, we report our investigation of the effects of freeze–thaw cycles (FTCs) of human serum samples on quantitative metabolomics using a differential chemical isotope labeling (CIL) LC-MS method. A total of 99 serum samples collected from healthy individuals (47 females and 52 males) were subjected to five FTCs, followed by ^{12}C-/^{13}C-dansylation labeling LC-MS analysis. A total of 2790 peak pairs or metabolites were relatively quantified among the 495 comparative samples, including 150 positively identified metabolites, 235 high-confident putatively identified metabolites and 1949 mass-matched metabolites from database searches. Multivariate analysis of the metabolome data showed a clustering of the third to fifth FTC samples in contrast to the separation of the first and second FTC samples, indicating that the extent of FTC-induced metabolome changes became smaller after the third cycle. The changing patterns among the FTC-effected metabolites were found to be complex. Using sex as a biological factor for grouping, we observed a clear separation of males and females when the samples were subjected to the same number of FTCs. However, when the male- and female-samples with different numbers of FTCs were compared, the number of significant metabolites found in male–female comparison increased dramatically, indicating that FTC effects could lead to a large number of false positives in biomarker discovery. Finally, we proposed a method of detecting the FTC effects by reanalyzing the original samples after subjecting them to an additional FTC.



■ INTRODUCTION

Combining the use of serum or plasma samples stored in multipurpose biobanks with sophisticated metabolome profiling techniques offers an attractive route for discovering, verifying, and validating metabolite biomarkers of diseases. Because of metabolic homeostasis of the human body, metabolite concentration differences between diseased and healthy individuals are often very small.[1] In order to reveal the small differences, highly accurate and precise analytical techniques are required for metabolome analysis. In addition, careful control of each step in an analytical workflow is critical to ensure that the measured signals by techniques such as LC-MS are reflective of metabolite concentrations in samples. In blood analysis, the freeze–thaw cycle (FTC) step is well-known to have a significant effect on analyte concentration measurement.[2−6] For targeted metabolite analysis, the FTC effect can be more readily investigated and controlled by using an isotope analogue standard.[2] However, for comprehensive metabolome analysis, due to the limited availability of isotope standards, spiking all internal reference standards to a sample is not possible.

There are a number of reports on the studies of the FTC effects on serum or plasma metabolome analysis.[4,5] The reported magnitude of effect varied greatly, depending on the metabolite extraction method and the detection technique used. Because of this dependence, it is almost a necessity to evaluate the FTC effects before an analytical workflow is implemented for analyzing real-world samples in order to understand its limitations and design a way to mitigate such constraints. Due to low metabolome coverage and limited quantification accuracy offered by many of the current detection techniques, the FTC effects determined by one workflow cannot be easily extended to a different workflow. This is particularly true, if only a small fraction of the metabolites detected by the two workflows are in common.

Recent advances in detection techniques, such as chemical isotope labeling (CIL) LC-MS,[7−13] have opened the possibility of performing high-coverage quantitative metabolome analysis of serum and plasma. In this work, we report our studies of the FTC effects on human serum metabolomics using CIL LC-MS.

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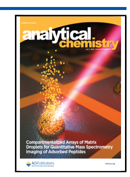

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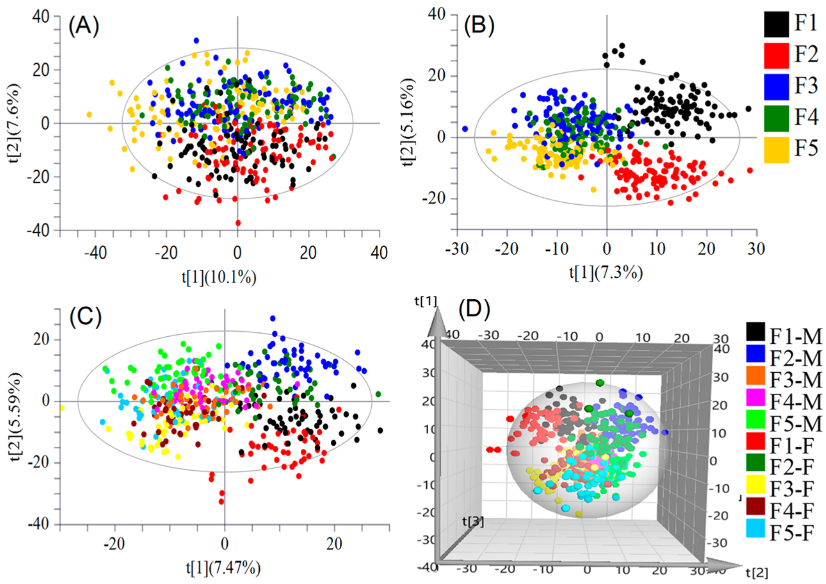

Figure 1. (A) PCA and (B) PLS-DA score plots projecting the five FTC groups. (C) 2D- and (D) 3D-PLS-DA score plots showing the five FTC groups with females and males color-coded (i.e., a total of 10 groups).

We compared the metabolome data of 495 serum samples prepared from 99 sera of healthy individuals after subjecting to one to five FTCs. This work is different from other reported studies in several ways, thereby providing unique perspectives on the FTC effects. First of all, CIL LC-MS targets the analysis of metabolites, not lipids, and thus conclusions drawn would apply to metabolites, not a combination of metabolites and lipids detected in other methods (e.g., conventional LC-MS). FTC's effect on lipids may well be very different from that on metabolites.[14−16] Delineation of the two classes of molecules is important to avoid confusion on the FTC effects. Second, CIL LC-MS detects thousands of metabolites (not features).[17] This higher coverage provides a more holistic view of the FTC effects on a large number of detectable metabolites. Third, using differential isotope labeling, that is, analyzing mixtures of ^{12}C-reagent-labeled individual samples and the same ^{13}C-reagent-labeled pooled sample or reference,[18] accurate relative quantification of labeled metabolites can be carried out, offering the opportunity of detecting small changes in metabolite concentrations caused by FTC. Finally, the knowledge gained from this work on the FTC effect can be used to guide the development of standard operating procedures for handling blood samples specifically for high-coverage quantitative metabolomics.[19]

■ EXPERIMENTAL SECTION

Sample Collection and Preparation. Supporting Information (SI) Figure S1 shows the schematic of the study design and experimental workflow. Blood samples were collected from 99 healthy individuals, including 47 females and 52 males (SI Note S1).

The serum samples were subjected to one to five FTCs before labeling and LC-MS analysis. During each FTC, samples were completely thawed at room temperature for approximately 90 min. Then, one 30 μL aliquot was taken from each sample before all samples were refrozen at −80 °C for the next cycle. This process was repeated five times on consecutive days to generate five batches with different numbers of FTCs. In addition, a pooled sample, which served as quality control (QC) and the reference sample for relative quantification, was prepared by mixing 10 μL aliquots from all subjects on the first day. Right after thawing, 3-fold volume of ice-cold methanol was mixed with each serum sample to precipitate the proteins. After that, the mixture was centrifuged at 18 000g at room temperature for 30 min. Finally, the supernatant was transferred to another 0.5 mL plastic vial and dried using a SpeedVac vacuum concentrator. The combination of sample drying and low-temperature storage should be sufficient to maintain the stability of the metabolites.[20,21]

Dansylation Labeling. ^{12}C-dansyl chloride (Dns-Cl) was used to label individual samples and ^{13}C−Dns-Cl obtained from www.novamt.com was used to label the pooled sample (SI Note S1).

Sample-Wise Normalization. Sample-wise normalization was conducted by measuring the total concentration of Dns-labeled amine/phenol-containing metabolites using LC-UV at 338 nm[22] (SI Note S1). Each ^{12}C-labeled individual sample was then mixed with the ^{13}C-labeled pooled sample in 1:1 mol-ratio.

LC-MS. The labeled sample was analyzed using UltiMate 3000 UHPLC (Thermo Scientific, MA) combined with the Impact II Quadrupole Time-of-flight (QTOF) mass spectrometer (Bruker, Billerica, MA) (SI Note S1). Only positive ion detection was used as dansyl labeled metabolites were preferentially ionized in positive mode. A QC sample, prepared by mixing equal moles of ^{12}C- and ^{13}C-labeled pooled samples, was run after every 20 sample runs.

Data Processing and Analysis. In CIL LC-MS, dansyl-labeled metabolites are detected in the form of peak pairs. Metabolite peak pair extraction, sample-wise alignment and missing value retrieval were conducted by using IsoMS Pro (www.novamt.com).[18] Metabolite identification was carried out using a three-tier approach reported previously.[17] Finally, statistical analyses of the data were completed by SIMCA-P 12+ and MetaboAnalyst.

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Table 1. Numbers of Significant Metabolites Found in Binary Comparisons of Sample Groups Subjected to Different FTCs[a]

FC threshold	F2 vs F1	F3 vs F1	F4 vs F1	F5 vs F1	F3 vs F2	F4 vs F2	F5 vs F2	F4 vs F3	F5 vs F3	F5 vs F4	common
1.2	124	99	87	206	135	79	159	20	43	52	0
1.3	71	50	52	116	66	29	94	9	15	23	0
1.4	43	27	33	71	42	21	59	4	9	18	0
1.5	24	16	18	49	31	16	42	4	7	10	0

[a]Univariate analysis was performed in each group comparison with significance threshold of $p < 0.05$ and different FC thresholds.

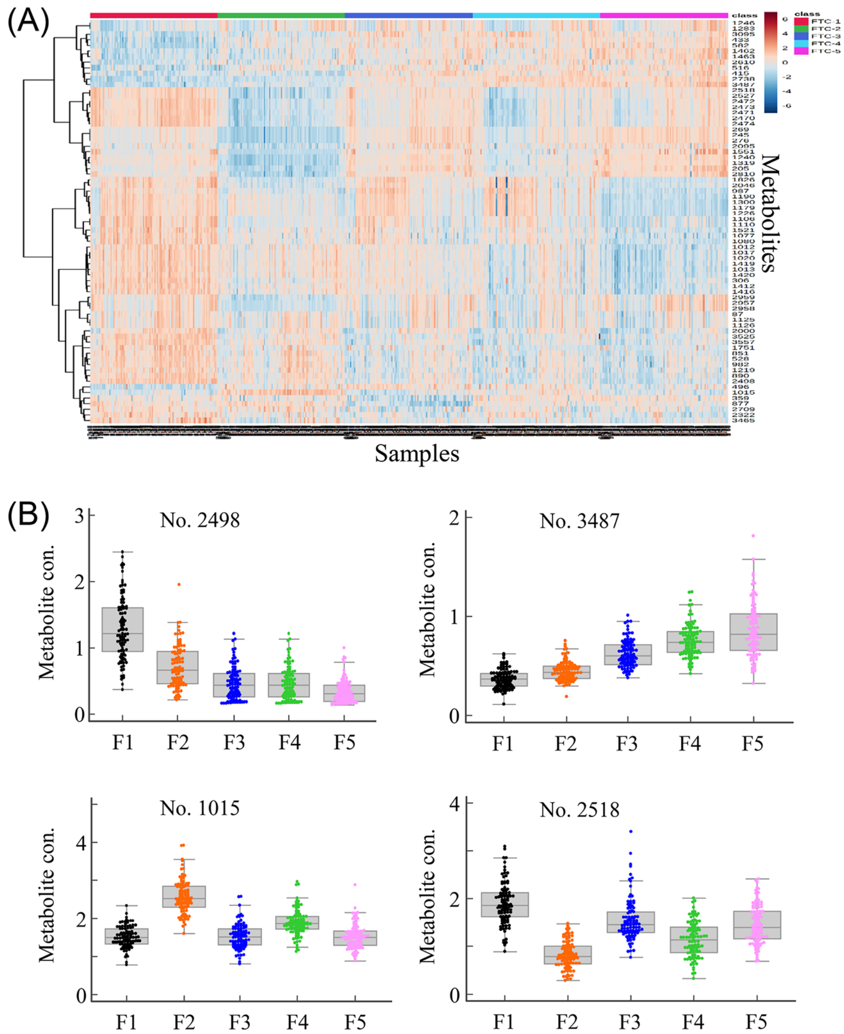

Figure 2. (A) Heat map showing the relative concentrations of 72 significant metabolites in all FTC samples. (B) Box plots showing the concentration distributions in the five FTC groups of metabolites No. 2498, No. 3487, No. 1015, and No. 2518.

RESULTS AND DISCUSSION

Metabolome Data. A total of 495 LC-MS runs were performed for the 99 serum samples with each treated by five FTCs. When aligning MS data of all the samples, we only retained the peak pairs that were detectable in more than 80% of the samples in each FTC group (i.e., F1, F2, F3, F4, and F5-group). In total, we detected and quantified 2790 serum metabolites in 495 samples. Metabolite identification was carried out using a three-tier approach[17] and the results are shown in SI Table S1. There were 150 metabolites positively identified and 235 putatively identified with high-confidence. There were 977, 627, and 345 peak pairs matched to the zero-, one-, and two-reaction MyCompoundID libraries,[23] respectively. Thus, out of the 2790 detected peak pairs, 2334 (83.7%) were either identified or mass-matched. With this high coverage, we could examine the FTC effects on the metabolome analysis more globally, compared to using techniques with limited coverages (e.g., analyzing 10s or hundreds of metabolites). The limit of dansylation LC-MS is that it focuses on the analysis of amine- and phenol-containing metabolites, which cover about 34−45% of the chemical space of known metabolite entries in various databases.[17]

Multivariate and Univariate Comparison of FTCs. SI Figure S2 shows the principal component analysis (PCA) score plot projecting the five FTC groups (F1−F5) and the QC samples. The QCs are clustered together tightly, indicating excellent instrumental stability during the data collection period. Figure 1A is the PCA plot of these five FTC groups without QCs. The corresponding partial least-squares discriminant analysis (PLS-DA) score plot (cross-validation R^2 =

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0.919, Q^2 = 0.839) is shown in Figure 1B. Some modest between-group separations can be observed in the PCA plot. The supervised PLS-DA analysis demonstrates more clear intergroup differences (Figure 1B). The F1 and F2 samples are well separated from the rest of the groups. In addition to cross validation, the PLS-DA model also passed a permutation test (see SI Figure S2B).

We used paired sample t test and the fold change (FC) to perform univariate comparisons of binary groups (e.g., F1 samples vs F2 samples). In each comparison, there are 99 same-subject-pairs of samples that underwent different numbers of FTCs (e.g., F1 with one FTC and F2 with two FTCs). For each metabolite, we calculated the false-discovery-rate-adjusted p-value and required $p < 0.05$ to be considered as a statistically significant change. Another threshold is related to FC of the metabolite for a majority of sample pairs. We used the FC threshold of ≥1.5, 1.4, 1.3, or 1.2 in at least 75% of the 99 pairs for significance analysis. CIL LC-MS is an accurate and precise technique, allowing the use of smaller FC thresholds, which is critical to discover biomarkers with small concentration differences between comparative groups.

Table 1 lists the number of significant metabolites found in binary comparison of two groups subjected to different FTCs (e.g., F2 vs F1) at different FC thresholds. For example, using the FC threshold of ≥1.5 with $p < 0.05$, there are 24 significantly changed metabolites found when the F1 samples are compared to the F2 samples. Consistent with the multivariate analysis, there is a clear trend that binary comparisons among F3, F4, and F5 have much fewer significant metabolites (e.g., 4, 7, and 10 significant metabolites in F3 vs F4, F3 vs F5, and F4 vs F5 comparisons, respectively), compared to their comparisons to either F1 or F2 (e.g., 16, 18, and 49 significant metabolites in F1 vs F3, F1 vs F4 and F1 vs F5 comparisons, respectively). This observation suggests that after the first two FTCs, the extent of FTC effect on metabolite concentration became smaller.

Among the significant metabolites found in all binary comparisons with FC ≥ 1.5 (Table 1), none is in common, indicating that each of the five FTCs caused a concentration change of greater than 1.5-fold in a unique set of metabolites. This lack of commonality means that we cannot predetermine a list of metabolites with significant changes caused by FTC (i.e., FTC-markers). As a consequence, FTC-changed metabolites may be included in a panel of biomarkers of a biological phenotype in a real-world study where different batches or groups of samples with different FTCs are used for biomarker discovery (see below for sex-separation).

To gauge the extent of FTC-induced concentration changes, Table 1 also shows the number of significant metabolites found in binary comparison with lower FC thresholds (i.e., 1.4, 1.3, and 1.2). As expected, using a lower threshold, more significantly changed metabolites were found. However, at each FC threshold, no common significant metabolite was found. Thus, if a lower FC threshold (e.g., ≥ 1.2-fold) is used for selecting the biomarkers of a phenotype from comparative samples with different FTCs, a larger fraction of the biomarkers might be false due to the FTC effects.

Next, we investigated whether the concentration changes of the metabolites followed the same changing pattern as the number of FTCs increased (i.e., a gradual increase or decrease in concentration). Figure 2A shows the heat map of the peak ratios of 72 metabolites having greater than 50% change against F1 in at least one of the other four FTCs as a function of the FTC number. It is clear that most of them do not follow a gradual concentration change as the FTC number increases. Many show complex changing patterns (i.e., up, down, and up again). Some examples of changing patterns are shown in Figure 2B with the box plots showing metabolite concentration distributions in the five FTC groups. For metabolite No. 2498 (number denotes the order of the metabolite detected in the raw data list; SI Table S1), the average concentration kept decreasing as the samples underwent more FTCs. In contrast, for No. 3487, the concentration gradually increased as the FTC number increased. More interestingly, No. 1015's concentration was increased from F1 to F2 and lowered back in F3, while No. 2518 demonstrated an inverse pattern. From the above results, we can conclude that FTC does not have the same effect on all metabolites, and there are several distinct ways that the concentration of a metabolite may change throughout multiple FTCs.

The reasons of having these complex changing patterns are unknown. Generally, a decreasing trend may indicate a gradual loss of a specific metabolite during the FTC process.[2,3,14] In contrast, the opposite pattern suggests an increase in metabolite concentration, which may be explained as being released from protein surfaces or being converted from another compound.[24] The zigzag pattern may be the outcome of multiple effects. Some studies have indicated that hydrophobic metabolites or lipids were more preferentially lost during FTC,[14,16] perhaps due to stronger interactions between these molecules and precipitated proteins or container wall. Others have suggested the involvement of residual enzyme activities in serum or plasma for the increase or decrease of metabolite concentrations.[24] For the amine/phenol submetabolome, it is not easy to correlate the extent of FTC effects with any chemical or physical properties (e.g., hydrophobicity) of the metabolites.

Overall, we have found that there are strong FTC effects on the serum metabolome analyzed using CIL LC-MS. Although proportionally the number of FTC-changed metabolites with FC ≥ 1.5 is small in the dansylation LC-MS data set, the stochastic nature of these changes can present a challenge in discovering biomarkers of a biological phenotype. This is particularly true if the biological phenotype difference does not cause very large metabolome changes in the comparative samples. A case in point is sex separation in our sample set of 99 healthy individuals, as discussed below.

Biological Separation. In this study, all samples were from healthy individuals and the main biological difference is sex. Each FTC group consisted of samples from 47 females and 52 males. With individual samples color-coded by sex, the 2D- and 3D-PLS-DA score plots of the five FTC groups are given in Figure 1C and D, respectively. Male and female (M-F) separation can be clearly observed within each FTC group, especially in F1 (black dots vs red dots) and F2 (blue dots vs green dots). This result indicates that even with the presence of FTC effects, sex separation is still detectable in some sample groups in the PLS-DA plot. However, the FTC effects can potentially interfere with the discovery of sex-related biomarkers. We considered two possible scenarios often encountered in real-world biomarker discovery studies. One scenario is to use all the samples subjected to the same number of FTCs (same-FTC samples), while the other scenario is to use a mix of samples undergone different numbers of FTCs (different-FTC samples).

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For the first scenario, we performed binary comparison of females and males in each of the five groups of FTC samples. Table 2 lists the numbers of significant metabolites found (in bold) using a threshold of FC ≥ 1.5 with $p < 0.05$ in the F1−F5 groups. When the number of FTC increases, the number of significant metabolites for M-F separation does not increase dramatically. This observation reveals that the FTC effects are not biased toward the male group or the female group. Among the significant metabolites found in the five same-FTC M-F comparisons, there are 11 common ones that may be considered to be the biomarkers for sex separation. The identities of these 11 metabolites are listed in SI Table S2, and SI Figure S3 shows the box plots of the concentrations of these metabolites in the F1−F5 samples.

Table 2. Numbers of Significant Metabolites Found in Binary Comparisons Where the Male Samples from a Specific FTC Group Are Hypothetically Mixed with Female Samples from Different FTC Groups[a]

	F1-Female	F2-Female	F3-Female	F4-Female	F5-Female
F1-Male	**23**	194	144	131	249
F2-Male	215	**32**	189	138	224
F3-Male	140	186	**45**	51	125
F4-Male	185	157	84	**22**	116
F5-Male	272	218	111	100	**54**

[a]Univariate analysis was performed in each group comparison with significance thresholds of $p < 0.05$ and FC ≥ 1.5.

There are two top-ranked metabolites in separating the sexes, Nos. 238 and 473. Both cannot be positively identified. However, No. 238 could be putatively identified as an isomer of 4-amino-4-cyanobutanoic acid which is related to GABA (SI Table S2). Some studies have shown that the concentrations of GABA in males are higher than those in females.[25] No. 473 could be mass-matched to a derivative of carnosine (SI Table S2). Carnosine level is higher in males than females.[26] Using the F1 samples, we generated a random forest-based classification model using the panel of the two biomarkers and the corresponding receiver operating characteristic (ROC) curve to illustrate the discriminative power (Figure 3A). The area under the curve (AUC) is 0.978 with the 95% confidence interval from 0.945 to 1. The two-biomarker panel can differentiate between sexes at 91.8% sensitivity and 92.5% specificity. With this panel, we predicted the males and females from the F2−F5 samples, and the resulting prediction accuracies are given in Figure 3B. As the prediction accuracy remains stable when the number of FTC increases, we can conclude that the FTC effects do not interfere with the biomarker discovery from the samples subjected to the same number of FTCs. This can also be seen in the box plots of the

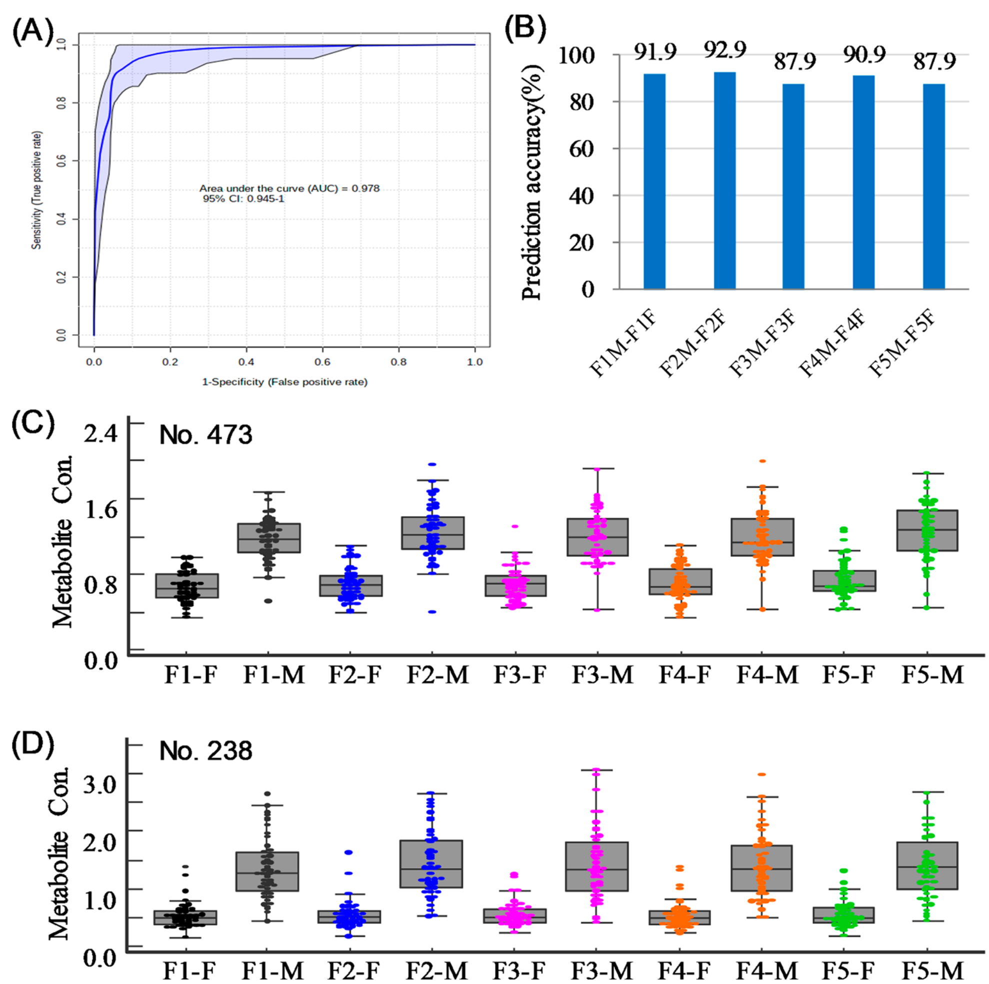

Figure 3. (A) The ROC curve illustrating the discriminative power of the top two biomarkers in differentiating between males and females. (B) Prediction accuracies of the biomarker panel in all five FTC groups. (C,D) Box plots of the two biomarkers.

two sex-biomarkers in all five same-FTC samples (Figure 3C,D). The concentration differences between males and females are similar for all five samples (F1−F5). In other words, although these five groups of samples have been subjected to different numbers of FTCs, any one of the groups can be used to discover the two sex-biomarkers.

The effects of age and body mass index (BMI) on sex separation were studied and we did not observe any effect (SI Note S2).

FTC Effects on Biological Separation. The second scenario of using different-FTC samples is not uncommon, particularly in large-scale biomarker discovery studies involving the use of multiple batches of samples from various sources or sites. Here we focus on the simplest case that two groups (i.e., males and females) experienced different numbers of FTCs.

Table 2 shows the number of significant metabolites found in each binary comparison when the male samples in one FTC group are purposely mixed with female samples in another FTC group. For example, there were 194 significant metabolites found when the metabolome data of the F1 male samples were merged with those of the F2 female samples. The numbers of significant metabolites for the different-FTC M-F group comparisons are much larger than those from the same-FTC M-F group comparisons. This result clearly reveals that when merging samples with different FTCs, there is a dramatic enhancement of the FTC effects on the metabolome data. Comparatively, binary mixings of different-FTC M-F groups with F3, F4, and F5 resulted in smaller numbers of significant metabolites, compared to binary mixings involving either F1 or F2. This finding correlates well with the earlier observation that the F3, F4, and F5 groups have smaller differences than F1 and F2.

We have examined the significant metabolites found in different-FTC M-F group comparisons. Figure 4A shows the changing pattern of No.1286 as an example to shed light on these false positives, that is, not sex-biomarkers. In F1, the average concentration of the male group was larger than that of the female group (FC = 1.77 with $p = 7.7 \times 10^{-06}$ for F1-M vs F1-F comparison). When both male and female samples experienced another FTC, all metabolite concentrations increased correspondingly. As expected, the M-F difference remained the same in F2. However, if we compared F1-Males with F2-Females, we obtained a reduced difference between males and females (FC = 0.81 with $p = 0.047$ for F1-M vs F2-F). If we used FC $\geq$ 1.5 as a threshold to determine the sex-biomarkers, this metabolite would no longer be considered as a biomarker, resulting in a false negative. On the other hand, when F1-Females and F2-Males were analyzed together, the intersex difference was enlarged (FC = 2.38 with $p = 6.7 \times 10^{-09}$ for F2-M vs F1-F). Enlarged difference for a metabolite with marginal concentration difference in males and females could lead to a false positive in determining sex-biomarkers. For No. 1286, after another round of FTC, the concentration distributions of F3-Females and F3-Males in Figure 4A went back to where they were in F1. As discussed earlier, we cannot foresee the changing pattern of a metabolite with different FTCs. As a result, we cannot identify the false positives and false negatives during the process of determining biomarkers of a biological phenotype. We note that we did not see any sex-dependent FTC effect, as discussed in SI Note S2.

In biomarker discovery, false positives will lead to wrong biomarker candidates, and false negatives will reduce the chance of finding the true biomarkers. When a sample set has

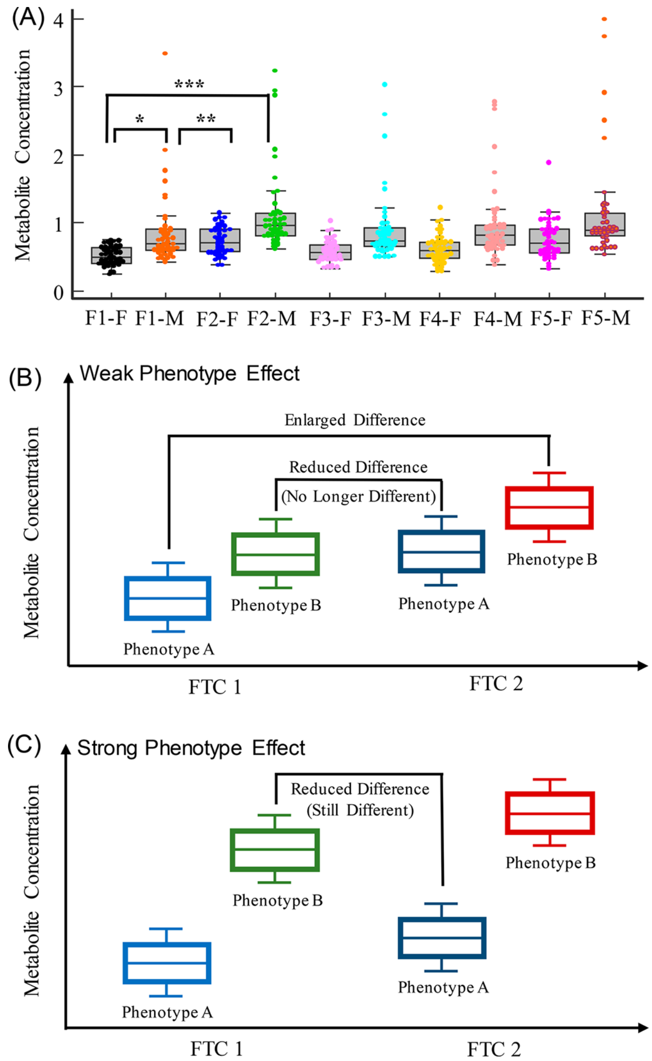

Figure 4. (A) Box plot showing the concentration distributions of metabolite No. 1286. False positives and false negatives exist in the different-FTC M-F group comparisons. (B) Schematic of the interaction between the FTC effect and a relatively weak phenotype effect. (C) Schematic of the interaction between the FTC effect and a strong phenotype effect. * FC = 1.77 with $p = 7.7 \times 10^{-06}$; ** FC = 0.81 with $p = 0.047$; *** FC = 2.38 with $p = 6.7 \times 10^{-09}$.

different batches with different numbers of FTCs, the FTC effect is clearly a confounding factor that we need to carefully consider. In the case where the strengths of the phenotype effect and the FTC effect are similar, as depicted in Figure 4B, FTC can more readily cause false positive or negative. On the other hand, if the phenotype effect is much stronger than the FTC effect, as shown in Figure 4C, the interphenotype difference can always be recognized. Thus, the FTC effects on biomarker discovery is also dependent on the biomarker concentration changes induced by the phenotype. If a phenotype causes a huge concentration change of a biomarker, this biomarker should be detectable even when the samples used have experienced different numbers of FTCs.

Detecting FTC Effects. In a real-world study, the situation can be more complicated than the two scenarios discussed above. The key question is, when working with different-FTC samples or samples with unknown FTCs, how we can determine whether FTC effects have any significant influence on biomarker discovery. Here we propose an approach to

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detect the FTC effects. It is based on the observation that metabolites with significant changes caused by the FTC effects are not commonly detected in samples with different FTCs. Therefore, if the original samples of a given project are allowed to undergo another round of FTC, we will generate a second set of samples. After metabolome analysis of the two sets of samples at the expense of increased workload and analysis time, if the top-ranked significant metabolites discovered from the original set of samples for separating two phenotypes are not found to be significant in the second set of samples, they are likely the metabolites with significant changes induced by FTC, not by the phenotype difference. SI Note S2 describes and demonstrates this approach in detail.

CONCLUSIONS

We have studied the effects of FTCs on serum metabolomics using CIL LC-MS. FTCs could cause concentration changes to varying degrees in many detectable metabolites, although only a small number of metabolites had changes of greater than 1.5-fold. The changing patterns of metabolites with different FTCs were found to be complex and there were no FTC-specific markers found. It was shown that the number of FTCs did not affect sex-biomarker discovery if the samples with the same FTCs were used. In contrast, FTCs could adversely affect the discovery of sex biomarkers when the samples with different FTCs were analyzed. We described a method of detecting the FTC effects by using two sets of the same samples, that is, the original samples and the second set of samples after subjecting the original samples to an additional round of FTC.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c01610.

- Note S1 for experimental, Note S2 for results and discussion, Figures S1−S3 for study design and analysis (PDF)
- Table S1 (XLSX)
- Table S2 (XLSX)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Pi, X.; Xie, L.; Patterson, C. Emerging Roles of Vascular Endothelium in Metabolic Homeostasis. *Circ. Res.* **2018**, *123*, 477−494.

(2) Liang, X.; Yang, L.; Qin, A. R.; Ly, J.; Liederer, B. M.; Messick, K.; Ma, S.; Zak, M.; Dragovich, P. S.; Dean, B. J.; Hop, C. E.; Deng, Y. *Bioanalysis* **2014**, *6* (11), 1445−1457.

(3) Weng, N. *Targeted Biomarker Quantitation by LC-MS*; John Wiley & Sons, Inc., 2017.

(4) Hernandes, V. V.; Barbas, C.; Dudzik, D. A Review of Blood Sample Handling and Pre-Processing for Metabolomics Studies. *Electrophoresis*; Wiley-VCH Verlag September, 2017; Vol. *1*, pp 2232−2241.

(5) Yin, P.; Lehmann, R.; Xu, G. Effects of Pre-Analytical Processes on Blood Samples Used in Metabolomics Studies. *Analytical and Bioanalytical Chemistry*; Springer 2017; pp 4879−4892.

(6) Stevens, V. L.; Hoover, E.; Wang, Y.; Zanetti, K. A. Pre-Analytical Factors That Affect Metabolite Stability in Human Urine, Plasma, and Serum: A Review. *Metabolites*. MDPI AG **2019**.9156

(7) Guo, K.; Li, L. *Anal. Chem.* **2009**, *81* (10), 3919−3932.

(8) Hao, L.; Johnson, J.; Lietz, C. B.; Buchberger, A.; Frost, D.; Kao, W. J.; Li, L. J. *Anal. Chem.* **2017**, *89* (2), 1138−1146.

(9) Leng, J. P.; Wang, H. Y.; Zhang, L.; Zhang, J.; Wang, H.; Guo, Y. L. *Anal. Chim. Acta* **2013**, *758*, 114−121.

(10) Tayyari, F.; Gowda, G. A. N.; Gu, H. W.; Raftery, D. *Anal. Chem.* **2013**, *85* (18), 8715−8721.

(11) Wong, J. M. T.; Malec, P. A.; Mabrouk, O. S.; Ro, J.; Dus, M.; Kennedy, R. T. *J. Chromatogr. A* **2016**, *1446*, 78−90.

(12) Yuan, W.; Edwards, J. L.; Li, S. W. *Chem. Commun.* **2013**, *49* (94), 11080−11082.

(13) Chu, J. M.; Qi, C. B.; Huang, Y. Q.; Jiang, H. P.; Hao, Y. H.; Yuan, B. F.; Feng, Y. Q. *Anal. Chem.* **2015**, *87* (14), 7364−7372.

(14) Saito, K.; Maekawa, K.; Pappan, K. L.; Urata, M.; Ishikawa, M.; Kumagai, Y.; Saito, Y. *Metabolomics* **2014**, *10* (3), 402−413.
(15) Zivkovic, A. M.; Wiest, M. M.; Nguyen, U. T.; Davis, R.; Watkins, S. M.; German, J. B. *Metabolomics* **2009**, *5* (4), 507−516.
(16) Ishikawa, M.; Maekawa, K.; Saito, K.; Senoo, Y.; Urata, M.; Murayama, M.; Tajima, Y.; Kumagai, Y.; Saito, Y. Plasma and Serum Lipidomics of Healthy White Adults Shows Characteristic Profiles by Subjects' Gender and Age. *PLoS One* **2014**, *9*, e91806
(17) Zhao, S.; Li, H.; Han, W.; Chan, W.; Li, L. *Anal. Chem.* **2019**, *91* (18), 12108−12115.
(18) Zhou, R.; Tseng, C.-L.; Huan, T.; Li, L. *Anal. Chem.* **2014**, *86* (10), 4675−4679.
(19) Han, W.; Li, L. Chemical Isotope Labeling LC-MS for Human Blood Metabolome Analysis. In *Methods Mol. Biol.*; Humana Press Inc., 2018; Vol. *1730*, pp 213−225.
(20) Palmer, E. A.; Cooper, H. J.; Dunn, W. B. *Anal. Chem.* **2019**, *91*, 14306−14313.
(21) Volani, C.; Caprioli, G.; Calderisi, G.; Sigurdsson, B. B.; Rainer, J.; Gentilini, I.; Hicks, A. A.; Pramstaller, P. P.; Weiss, G.; Smarason, S. V.; Paglia, G. *Anal. Bioanal. Chem.* **2017**, *409* (26), 6263−6276.
(22) Wu, Y.; Li, L. *Anal. Chem.* **2012**, *84* (24), 10723−10731.
(23) Li, L.; Li, R.; Zhou, J.; Zuniga, A.; Stanislaus, A. E.; Wu, Y.; Huan, T.; Zheng, J.; Shi, Y.; Wishart, D. S.; Lin, G. *Anal. Chem.* **2013**, *85* (6), 3401−3408.
(24) Wawrzyniak, R.; Kosnowska, A.; Macioszek, S.; Bartoszewski, R.; Markuszewski, M. J. New Plasma Preparation Approach to Enrich Metabolome Coverage in Untargeted Metabolomics: Plasma Protein Bound Hydrophobic Metabolite Release with Proteinase K. *Sci. Rep.* **2018**, *8*, 9541. DOI: 10.1038/s41598-018-27983-0
(25) O'Gorman, R. L.; Michels, L.; Edden, R. A.; Murdoch, J. B.; Martin, E. *J. Magn. Reson. Imaging* **2011**, *33* (5), 1262−1267.
(26) Boldyrev, A. A.; Gallant, S. C.; Sukhich, G. T. *Biosci. Rep.* **1999**, *19* (6), 581−587.

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