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SHORT COMMUNICATION

Tissue sample stability: thawing effect on multi-organ samples

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Abstract Correct handling of samples is essential in metabolomic studies. Improper handling and prolonged storage of samples has unwanted effects on the metabolite levels. The aim of this study was to identify the effects that thawing has on different organ samples. Organ samples from gut, kidney, liver, muscle and pancreas were analyzed for a number of endogenous metabolites in an untargeted metabolomics approach, using gas chromatography time of flight mass spectrometry at the Swedish Metabolomics Centre, Umeå University, Sweden. Multivariate data analysis was performed by means of principal component analysis and orthogonal projection to latent structures discriminant analysis. The results showed that the metabolic changes caused by thawing were almost identical for all organs. As expected, there was a marked increase in overall metabolite levels after thawing, caused by increased protein and cell degradation. Cholesterol was one of the eight metabolites found to be decreased in the thawed samples in all organ groups. The results also indicated that the muscles are less susceptible to oxidation compared to the rest of the organ samples.

Keywords Thawing effect · Metabolomics · OPLS · Multivariate analysis · Multi-organ

1 Introduction

There are many sources of variation in metabolomic studies that may influence the composition of the metabolome. These include food intake, medications, genetics and lifestyle. In this context, the effects of sample handling and storage are easily ignored.

The effects of improper handling and storage of serum samples have been investigated previously (Zivkovic et al. 2009; Breier et al. 2014). The stability of a wide range of metabolites has been studied using ESI-LC–MS/MS analysis of plasma and serum samples left at room temperature (0 and 24 h) and on cool packs (~4 °C: 3, 6 and 24 h) (Breier et al. 2014). The stability of lipids and vitamins stored at 4 °C for 6 and 24 h before separation of serum and plasma has also been studied (Key et al. 1996). In addition, the stability of serum analytes and FAs in plasma phospholipids stored at room temperature (2, 4, 24, 26, 28, 96 h) has been studied to investigate shipment effects (van Eijsden et al. 2005). The effects of prolonged storage of plasma and serum samples have also been reported (Zivkovic et al. 2009; Evans et al. 1997; Comstock et al. 2001).

To our knowledge, the effects of thawing on the metabolome in organ samples are not yet known. In the present study, five organs (gut, kidney, liver, muscle and pancreas) were investigated. The aim of this study was to

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investigate the effects of freeze-thawing on different types of tissue samples. The study design included organ samples that had been thawed [during prolonged shipment (72 h)] and organ samples that had remained frozen (during 24 h shipment).

2 Experimental section

2.1 Samples

Eight-month-old wild-type male mice of the mixed background 129sv × C57Bl/6N ($n = 15$) were individually placed in metabolic cages (Metabolic cage for mice, Tecniplast™, UK), with access to food (ref A04-10 in powder, SAFE (Scientific animal food & engineering), France) and water. The animals were kept 5 days in the metabolic cages, 2 days as an adaptation period and 3 days of experiment. On the 5th day of the experiment, the animals were anesthetized with a ketamine and xylazine solution by intraperitoneal injection. Animals were killed by cervical dislocation. Pancreas, liver, gut, kidney and muscle tissues were removed and dissected in cold HBSS solution (Hanks balanced salt solution, Life Technologies, US). Samples of each organ were washed in HBSS, collected in Cryo tubes, frozen in liquid nitrogen, and stored at −80 °C until analysis. Animal experiments were conducted in accordance with French and European ethical legal guidelines and the local ethical committee for animal care (Comité d'éthique en expérimentation animale Charles Darwin No. 5, Approval No. 01508.01) specifically approved this study.

All of the samples were placed on blocks of dry ice (3 kg) in two separate, sealed, styrofoam boxes. One box was shipped for 24 h, in which all the samples arrived to the destination frozen. In contrast, the second box was shipped for a total of 72 h, after which all samples arrived to the destination thawed. The organ samples were divided into two separate groups, based on the shipment time. The groups have been referred to as 'thawed' and 'frozen' ($n1 = 8 \times 5$ and $n2 = 7 \times 5$, respectively). All samples were stored at −80 °C until metabolic profiling analysis.

2.2 Metabolic profiling

All organ samples were ground to a fine powder under liquid nitrogen and metabolite extraction was performed. The amount of sample used for analysis was identical for all organs (~10 mg). Detailed information regarding the metabolic profiling analysis, metabolite identification and quantification can be found in the Supporting Information. The GC–MS protocol is based on two previously published studies (Jonsson et al. 2005; A et al. 2005).

2.3 Data analysis

The multivariate data analysis was performed using SIMCA-P+, version 14.0 (Umetrics AB, Umeå, Sweden). The data were normalized using internal standards. The X matrix consisted of the metabolite data, and a discriminant vector Y defined the thawed and frozen samples (1/0 respectively). The initial step was to obtain an overview of the metabolite data from all organs (gut, kidney, liver, muscle and pancreas). This was performed by means of principal component analysis (PCA) (Jackson 1991; Eriksson et al. 2013).

Thereafter, orthogonal projections to latent structures discriminant analysis (OPLS-DA) (Bylesjö et al. 2006) was used. Information regarding both inter and intra class variation can be obtained using OPLS-DA (Trygg et al. 2007). The first loading vectors from the OPLS-DA models were used to identify differences between thawed and frozen samples (Wiklund et al. 2008). p(corr) is the correlation scaled loading. The p(corr) loading was used to visualize the differences in metabolic profiles related to frozen and thawed samples in multiple organ types.

Model significance was calculated using ANOVA of the cross-validated residuals (CV-ANOVA). (Eriksson et al. 2008) The statistically significant metabolites, in each organ, were found using two-tailed Student's t test.

The shared and unique structure (SUS) plot displays shared and unique features between two different models. The predictive components (p(corr)) of each model are plotted against one another. SUS-plots are used to compare the outcome of multiple classifications to a common reference. In the SUS-plot the shared variables are situated along a diagonal, while the unique variables are situated along the x- and y-axis (Eriksson et al. 2013).

3 Results and discussion

A PCA model of all putative metabolites was created to provide an overview of the metabolomic data. The PCA resulted in a two component model ($R^2X = 0.40$), Fig. 1.

The five separate OPLS-DA models, one for each organ, included the identified metabolites only. These OPLS-DA models included one predictive and one orthogonal component (1 + 1). The models had good separation in the predictive component t[1]. The OPLS-DA models are summarized in Table 1.

For each of the organs, a significant model was obtained. The p(corr) loadings from these OPLS-DA models were summarized in a common loading plot. The metabolites with a positive p(corr) value were found at higher concentrations in the thawed samples of the specific organ, while negative p(corr) showed that the concentration of the

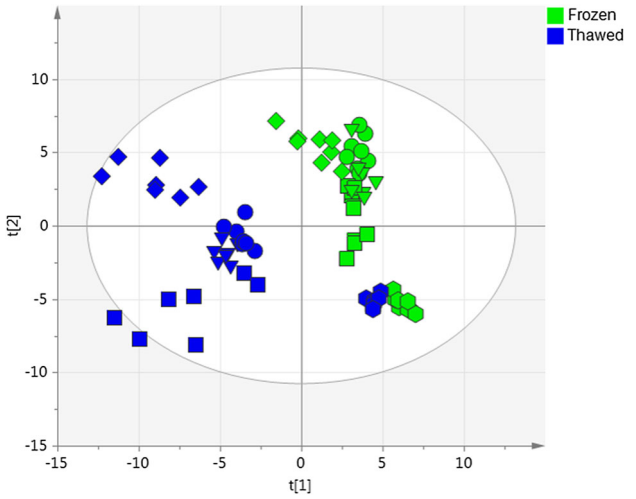


Fig. 1 PCA scores plot of thawed and frozen samples. A clear separation is observed between frozen and thawed samples with the exception of muscle. (gut *square*, kidney *circle*, liver *triangle*, muscle *hexagon* and pancreas *diamond*) (Color figure online)

Table 1 OPLS-DA models thawed versus frozen

Organ	A[a]	N[b]	R^2X^c	R^2Y^c	Q^2Y^d	*p* value[e]
Gut	1 + 1 + 0	15	0.55	0.96	0.91	4.1×10^{-5}
Kidney	1 + 1 + 0	15	0.61	0.99	0.98	3.2×10^{-8}
Liver	1 + 1 + 0	15	0.61	0.99	0.98	8.0×10^{-9}
Muscle	1 + 1 + 0	15	0.50	0.97	0.91	2.8×10^{-5}
Pancreas	1 + 1 + 0	15	0.60	0.99	0.95	2.8×10^{-6}

[a] A is the number of components

[b] N is the number of samples that the model is based on

[c] R^2 is the "goodness of fit" parameter that shows how well the model describes the variation in the data. R^2X, R^2Y are the cumulative variations explained in the metabolite and class-variable data respectively

[d] Q^2Y is the cross-validated prediction estimate of class separation that shows how well samples are predicted by the model

[e] *p* values obtained using CV-ANOVA in SIMCA-P+ 14.0

metabolite was lower in the thawed samples in the specific organ. The p(corr) loadings were combined and shown in Fig. 2.

The summary of the five different OPLS-DA models revealed that 37 of the 65 metabolites exhibited similar changes in concentration between thawed and frozen samples in all of the organs. The following metabolites were lower as a result of thawing in all of the investigated organs; taurine, myo-inositol-1-phosphate, pyruvic acid, *o*-phosphoethanolamine, adenosine-5-monophosphate, cholesterol, galactonic acid and monomethylphosphate. A total of 29 metabolites were found to be present at higher levels in all the thawed samples for all organs. These included many amino acids and carbohydrates, in addition to half of the fatty acids. This could have been the result of membrane and protein degradation.

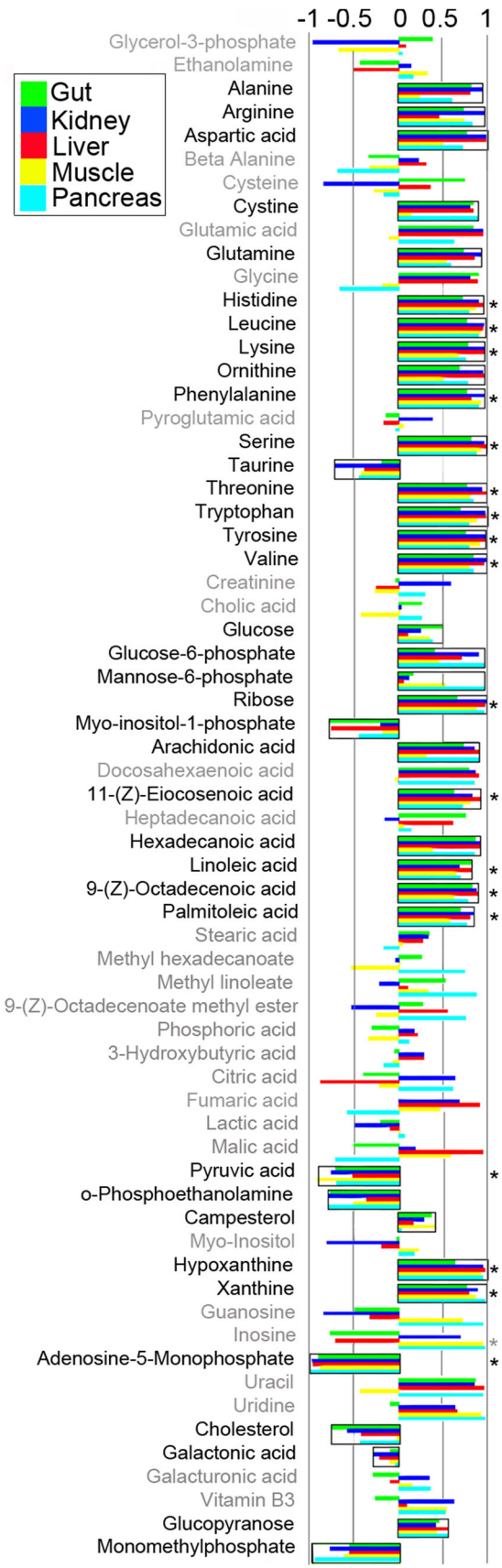
Significant differences (*p* value <0.05) in the levels of 19 metabolites were found in all of the thawed organs. Significantly increased levels (*p* value <0.05) of many metabolites were found in all of the thawed organs; gut (37/65), kidney (46/65), liver (40/65), muscle (28/65) and pancreas (46/65). Most of these metabolites were found to be amino acids, fatty acids and metabolites involved in purine and pyrimidine metabolism. This showed that thawing during shipment had a significant impact on the metabolic profiles of these samples. In addition, there was great overlap in the metabolites found to be altered significantly in each of the thawed organs and in their direction of change. For *p* values, see Supporting Information.

These findings highlight the importance of correct sample handling in metabolomic studies. We have shown that a single thawing of organ samples can have a significant effect on the metabolic profile. In this study we used a protocol that eliminates the need for thawing prior to sample preparation by first grinding all samples to a fine powder under liquid nitrogen.

The effect of thawing on metabolite stability is also important in a clinical setting, where metabolites such as cholesterol are measured routinely. Previous studies have investigated the effect of freeze–thaw cycles on the stability of cholesterol in plasma, however, the results are often found to be contradictory, showing increased (Cuhadar et al. 2013), decreased (Zivkovic et al. 2009) and stable (Comstock et al. 2008; Kronenberg et al. 1994) levels. In this study, we found that thawing resulted in significantly reduced levels of cholesterol in gut and kidney samples and a decrease in all other organ groups studied. This highlights the important need for appropriate sample handling for the correct assessment of cholesterol levels in these tissue groups.

In the PCA score plot, the comparison between the metabolic profile of the thawed and frozen muscle samples were found to be different compared to the other organ samples as all muscle samples were found in close proximity to each other. The muscle samples also had the smallest number of metabolites found at significantly different levels when thawed samples were compared to frozen. This was investigated by examining the shared and unique (SUS)-plot that identified metabolites that were contributing to the deviating behavior of the thawed muscle samples compared to the average of the other thawed organs. By creating a SUS-plot in this way it was possible to identify the specific metabolites that had altered levels in the muscles compared to the other organs. The SUS-plot showing shared and unique features between muscles and the other organs can be found in Fig. 3.

The positive correlation between muscle p(corr) and average p(corr) indicate that most of the metabolites show



◀**Fig. 2** Summary of OPLS-DA models (thawed vs frozen). The metabolites marked with a *black frame highlighted* a similar difference between thawed versus frozen in all of the organs (written in *black to the left*). Bars with positive p(corr) values were increased in the thawed samples. The metabolites marked with an *asterisk* (*) have statistically significant differences (p value <0.05) in all of the organs. The metabolites *highlighted in grey* did not follow the same pattern in all organs when comparing thawed and frozen samples (Color figure online)

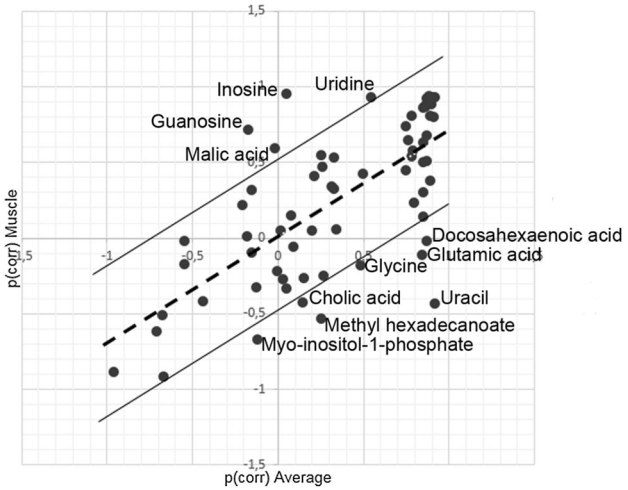


Fig. 3 SUS-plot of muscle versus average (organs). The shared and unique structure (SUS) plot displays shared and unique features between two different models. In this SUS-plot the p(corr) for muscle was plotted against the average p(corr) for the other investigated organs (gut, kidney, liver and pancreas). The metabolites with high p(corr) values, situated in the second or fourth quadrant, were the ones that occurred at different levels in the muscle samples compared to the other organs. For example, guanosine and inosine levels were higher in the muscle, whereas uracil levels were lower in the muscle samples compared to the other organs

a similar pattern in all organ samples. The SUS-plot showed that the metabolites that were contributing to the deviating pattern in muscle were involved mainly in purine and pyrimidine metabolism (guanosine, inosine, uridine, and uracil). Malic acid, myo-inositol-1-phosphate, methyl hexadecanoate, cholic acid, glycine, glutamic acid and docosahexaenoic acid were also found to be responsible for the deviation of the thawed muscle samples.

4 Conclusion

The fundamental assumption in metabolomics is that a snapshot of metabolites can provide a picture of metabolic activity within the metabolome, at a specific time. In this study we have studied the effect of thawing on metabolite profiles from multiple organs in mice. The results showed that the metabolic changes caused by thawing were almost

identical for all organs. There was clear evidence of protein and cell degradation in all of the thawed organ samples. In contrast, smaller changes were observed in the thawed muscle samples compared to the other thawed samples. These differences could be explained by altered levels of metabolites involved in purine and pyrimidine metabolism.

Hence a basic requirement for any metabolomic study, is that all samples are handled in an appropriate and standardized way, i.e. following standard operating procedures for sample shipment, collection, extraction and analysis. If organ samples are to be used for multiple purposes this should be considered before freezing the samples, to avoid unwanted effects of thawing. For metabolomic studies, we suggest that tissue samples should never be thawed or left at room temperature following sample collection and initial freezing. An extraction protocol should be used whereby all frozen samples are first prepared under liquid nitrogen and maintained at reduced temperatures during extraction (4 °C), to prevent further variations from occurring to the metabolic profile.

5 Associated content

5.1 Supporting information

Detailed information about the metabolic profiling analysis, metabolite identification and quantification the can be found in the Supporting Information. In addition, a table containing the *p* values, calculated for each compartment can be found in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author contributions Conceived and designed the experiments: TL, KLE, SC. Performed the experiments: CH, SC, KB. Analyzed the data: FT, TL, JT, TM, SR, CH, SC, KB. Wrote the paper: FT, KB, SC, CH, JT, TM, TL.

Compliance with ethical standards

Conflict of interest JT, TM and TL are shareholders of AcureOmics AB. No financing has been received from this company. The authors declare no other competing interests.

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