Impact of Pre-Analytical Factors on the Stability of the Global Metabolome- and Lipidome in Clinical Samples

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INTRODUCTION

Combining global metabolomics and lipidomics to the same sample cohort can aid in seeing the complete metabolome. Over the past few years, the clinical applications of global metabolomics and lipidomics have expanded, aiding in understanding disease mechanisms, identifying novel biomarkers, and facilitating personalized medicine.

To effectively utilize these techniques in clinical diagnostics, it's crucial to understand and control both analytical and preanalytical factors which can influence the results. Pre-analytical factors, which encompass everything happening to the sample from sample collection until sample preparation, is estimated to contribute to over 80% of testing errors (Yin et al., 2013). Most relevant pre-analytical factors in clinical diagnostics include variations in sample collection, delays in sample processing and inconsistent sample storage conditions.

This study evaluates the storage stability and the effect of one extra freeze-thaw cycle on the global lipidome and metabolome within a single sample cohort. The aim is to guide the development of protocols for required sampling and storage of samples to be analyzed with global metabolomics and lipidomics at our hospital.

STUDY DESIGN and METHODS

The cohort consisted of 20 patients (3 EDTA plasma samples per patient were collected) from the emergency department. The study was designed to cover typical pre-analytical situations for samples taken for research projects in an emergency room (**Figure 1**), and analyzed using global LCMS metabolomics (Skogvold et al., 2023) and global LCMS lipidomics (manuscript in preparation). Sample preparation for metabolomics consisted of protein precipitation (PPT) using methanol in a 1:3 ratio (sample: methanol), and the same ratio using isopropanol for lipidomics.

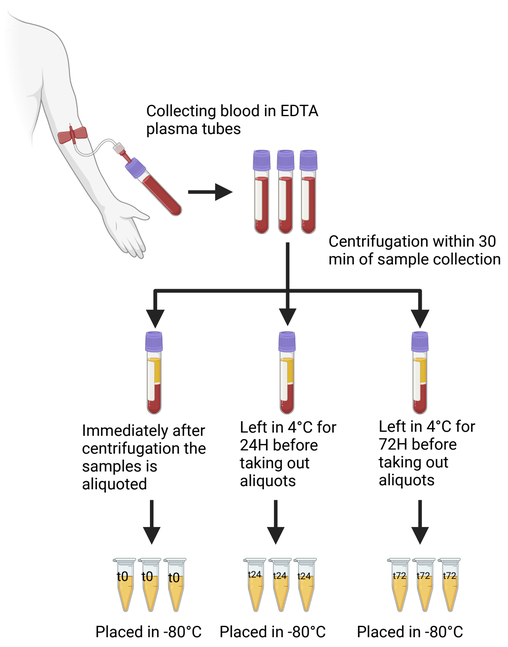

Figure 1: Study design showing the pre-analytical conditions prior to global metabolomics and lipidomics.

CONCLUSION

Our findings reveal a significant effect of storage time on the stability of the global metabolome and lipidome. Identifying the pattern of the specific changes occurring with storage time is important. EDTA plasma showed satisfactory stability upon freeze-thaw. For development of relevant protocols for samples taken for global analysis, knowledge of the effect of pre-analytical factors is crucial. Our results indicate that time delay before freezing has a lager impact than the number of freeze-thaw cycles and this should be taken into account when developing protocols to be used in busy emergency rooms.

RESULTS

When comparing samples stored in different time intervals on PCA level, there was no clear grouping based on storage time in refrigerator prior to -80°C storage. However, there was a very clear grouping based on participant, indicating that biological variation between participants outcompetes the metabolic alterations induced by storage time (**Figure 2**). This shows that the metabolome is relatively stable on a global level. This was also found for the lipidome (not shown). The different timepoints were compared towards each other using differential analysis. The number of differentially altered metabolites and lipids (DAML) (p-value < 0.05) increased over time (**Figure 3**), showing that the metabolome clearly changes upon storage in refrigerator. However the trend was different for lipidomics and metabolomics (**Figure 3**).

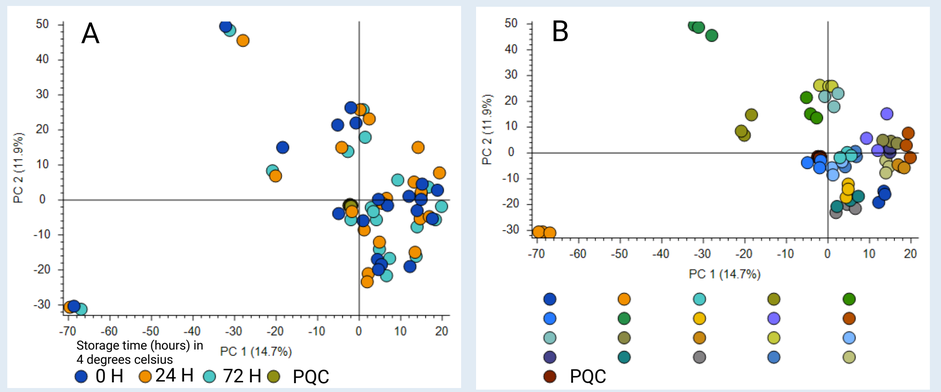

Figure 2: PCA of metabolomics data in positive ionization mode. A) colored by number of hours stored in 4 degrees celsius, B) colored by the different participants.

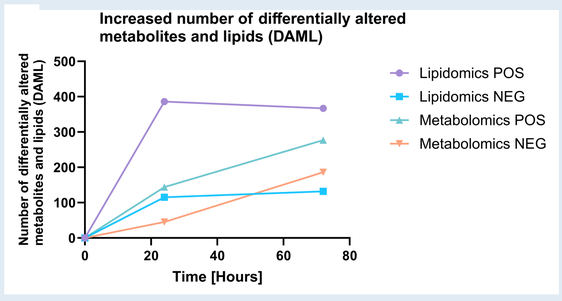

Figure 3: Trend of DAML with storage time found using the global metabolomics and lipidomics approach.

According to EM Gowans, the maximum accepted imprecision with bias is 0.25. In our study, we found that the effect of storage time on the stability of the metabolome and lipidome exceeds this threshold. This indicates that the impact of storage time is larger than what would be expected from normal variation, suggesting a substantial effect of these storage conditions on the samples. Additionally, the effect of one extra freeze-thaw cycle had on the sample stability was also tested. Interestingly, despite storage time having a significant impact on the samples, one extra freeze-thaw cycle did not significantly affect the stability of the metabolome and lipidome on a global level (PCA) (**Figure 4**). This suggests that the samples exhibits a robustness against one extra freeze-thaw cycle (1 to 2 cycles).

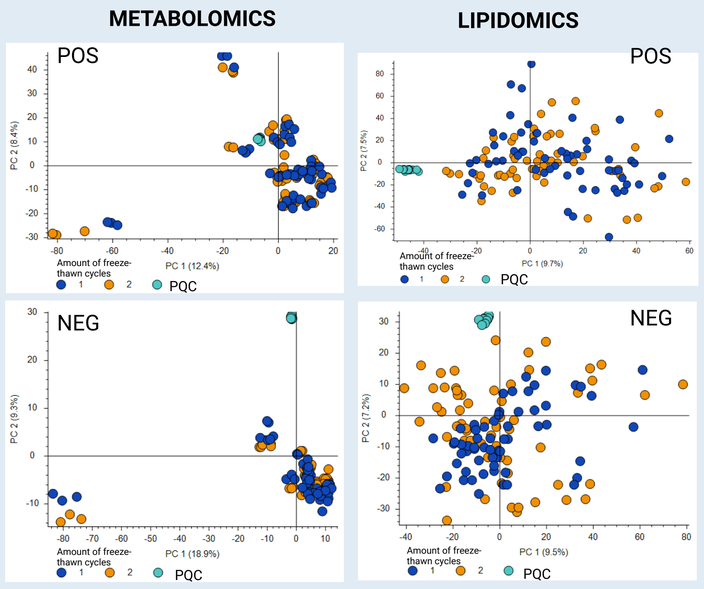

Figure 4: PCA of metabolomics (left) and lipidomics (right) data obtained in positive (top) and negative (bottom) ionization mode. Colored by number of freeze-thaw cycles.

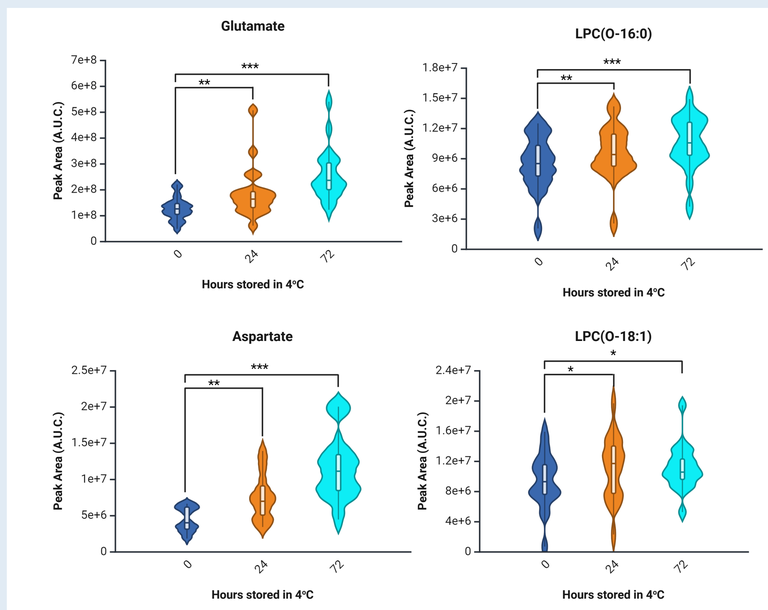

Figure 5: Violin plot of glutamate, LPC(O-16:0), aspartate and LPC(O-18:1), based on peak area and number of hours the samples were stored at 4°C.

Among the DAML, 35 was found with metabolomics (**Table 1**) and 30 was found with lipidomics (**Table 2**). Cohen's d was calculated to provide a measure of the practical significance of our findings in clinical chemistry. This allowed us to quantify the size of the effect that storage time has on the stability of the metabolome and lipidome, beyond just the statistical significance provided by p-values. For many of the significant features the storage time had a substantial effect on their corresponding concentration levels (**Figure 5**). The compounds with highest effect sizes came mostly from the metabolomics results (**Table 1**), indicating that the storage time had a greater influence on the metabolome compared to the lipidome.

Name	LoC	P-value: (24) / (0)	P-value: (72) / (0)	Cohen's d 0-24	Cohen's d 0-72'
ACETOACETATE (Na+)	1	0.107762	7.15E-07	0.104214686	0.283265132
FA(20:4)	2	0.359824	0.002653	-0.060109487	-0.49174382
ASPARAGINE	1	0.137134	0.003896	0.069814177	0.409647038
ASPARTATE	1	2.59E-05	1.20E-08	-0.372709785	-2.1483707
CHOLINE	1	0.932309	0.001884	-0.020332485	-0.29984525
CYSTEIN	1	0.075769	0.000607	0.084688657	0.427668617
DEOXYCARNITINE	1	0.024107	0.236118	-0.234882768	-0.1891197
dUMP	3	0.001759	7.93E-08	-0.210688256	-1.68297334
FA(20:5)	2	0.092581	8.48E-05	-0.07110868	-0.2857047
ETHANOLAMINE	3	0.463758	0.000769	-0.034255827	-0.16780139
gammaGlu-Cys-Cys	2	6.45E-06	1.18E-10	1.184433832	1.929062155
GLUTAMATE	1	9.46E-05	9.85E-08	-0.331348272	-1.69782584
GLUTAMINE	1	0.043378	0.000752	0.080956269	0.519557258
h-Cys-OH.H-Hcy-OH	3	0.082643	0.000143	0.115583612	0.459851321
HYPOTAURINE	1	0.108352	0.008727	-0.167829205	-0.60491673
HYPOXANTHINE	1	0.090087	6.78E-09	-0.203577107	-1.64837592
ISOCITRATE	1	0.365554	0.00154	-0.080509092	-0.34297002
LACTIC ACID	1	0.000711	8.31E-09	-0.226212898	-1.58912059
LEUCINE isomers	1	0.034085	0.000606	0.138095055	0.534033281
MALATE	1	0.187332	0.000261	-0.104315723	-0.43434507
METHYLMALONATE	1	0.200373	5.93E-06	-0.091739457	-0.94024432
N2-gamma-glutamylglutamine	2	3.66E-07	1.46E-12	1.153290947	3.106220294
N-ACETYLNEURAMINATE	1	0.650337	0.011687	-0.011350207	-0.17475764
N-ACETYLORNITHINE	2	0.013464	0.902705	0.138495044	-0.00741618
NICOTINAMIDE	2	3.52E-06	0.179967	-0.312609106	-1.77500369
NOROPHTHALIMIC ACID	2	2.53E-09	2.23E-11	1.314296607	3.107637823
O-PHOSPHOETHANOLAMINE	1	0.244962	0.000357	-0.182330305	-1.2023825
OXOPROLINE	1	0.698916	0.0007	-0.04960462	-0.39437523
TAURINE	1	0.258137	1.34E-05	-0.12163409	-1.01840928
THREO-SPHINGOSINE	3	0.000443	2.36E-06	-0.347113643	-1.15215777
TRANS-4-HYDROXY-L-PROLINE	1	0.01115	0.007758	0.101155426	0.664263917
URIDINE	2	0.340772	0.000147	-0.076983824	-0.55950685
XANTHINE	1	0.040284	3.73E-07	-0.136300963	-0.84521003
KETOLEUCINE	1	0.031605	0.000553	0.104059376	0.291588616
LPC(16:0)	2	0.847065	0.034564	-0.014620276	-0.21334843

Table 1: Differentially altered metabolites impacted by storage time. Including level of confidence (LoC)(Schymanski et al., 2014), p value and cohen's d.

Name	LoC	P-value: (24) / (0)	P-value: (72) / (0)	Cohen's d (0-24)	Cohen's d (0-72)
LPE(16:0)	1	0.916724	0.031573	0.063953	-0.16173
LPC(14:0)	1	0.316343	0.042354	-0.02123	-0.05147
LPC(P-16:0)	2	0.00781	0.003156	-0.13606	-0.37614
LPC(15:0)	1	0.075076	0.011634	-0.0791	-0.28975
LPC(O-16:0)	2	0.004681	0.000549	-0.12966	-0.75998
LPC(16:0)	1	0.026219	0.018273	-0.09574	-0.34707
LPC(O-18:1)	1	0.021533	0.032582	-0.20576	-0.54092
LPC(17:0)	1	0.007345	0.002985	-0.15012	-0.29264
LPC(0-18:0)	2	0.007913	0.015716	-0.16914	-0.46144
LPC(18:0)	1	0.000734	0.005174	-0.19607	-0.33612
LPC(20:1)	1	0.240394	0.020745	-0.06663	-0.25669
LPC(20:0)	1	0.045884	0.047766	-0.09616	-0.2083
LPC(P-21:5)	2	0.000359	0.015387	-0.25985	-0.3594
PC(32:3)	2	0.44835	0.016591	0.069085	0.35299
PE(P-18:0_20:4)	2	0.550334	0.038967	-0.09103	-0.4612
PE(P-18:0_20:3)	2	0.103482	0.014847	-0.09516	-0.2255
PC(34:3)	1	0.738752	0.021729	-0.00523	0.48185
PE(20:4_18:1)	2	0.029515	0.017116	-0.08707	-0.28023
PC O-38:6	2	0.027178	0.02856	-0.10302	-0.19344
PC(O-20:0/18:3)	2	0.301743	0.041874	0.058703	0.274263
SM(d42:1)	2	0.136528	0.032565	0.173053	0.313407
PC 20:4_22:6	2	0.065147	0.044336	-0.12183	-0.20078
TG(53:3)	2	0.687767	0.032509	0.012923	0.384326
TG(18:1_16:0_21:0)	2	0.123385	0.037273	-0.13382	-0.24359
TG(18:1_18:1_22:5)	2	0.106077	0.01455	-0.11419	-0.42329
TG(18:0_20:1_20:4)	2	0.013242	0.048534	0.278825	0.540917
FA(20:4)	2	0.000235	0.035103	-0.17266	-0.35126
PC 16:0_22:6	2	0.363546	0.043123	0.066193	0.219666
PC 16:0_18:2	2	0.2079	0.037136	0.073068	0.379695
PS 18:0_20:4	2	0.199393	0.049491	-0.34553	-0.52203

Table 2: Differentially altered lipids impacted by storage time. Including level of confidence (LoC)(Schymanski et al., 2014), p value and cohen's d.

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