LC/MS-TOF Analysis of Metabolites in *Plasmodium falciparum*-infected Red Blood Cells (RBCs) Exposed to the Cell Membrane Permeabilization Agent Streptolysin O

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Introduction

Introduction

Malaria is one of the most important infectious diseases, with approximately 500 million cases and over one million deaths annually. The typical infection rate of RBCs in culture mixtures is presently between 10-12%. Hence the desire to increase RBC parasitemia in infected cultures, so that pure populations of infected RBCs can be isolated for further study. This has the potential to increase assay sensitivity for the parasite metabolites and translate to increased differential analysis between metabolites in control and infected samples. We have used both untargeted and 'targeted' metabolomics approaches to determine whether Streptolysin O (SLO), a previously described permeabilization agent, preferentially lyses uninfected RBCs and enriches for infected cells. Moreover, we have varied the extraction solvent pH to maximize metabolite recovery. The effectiveness of SLO was determined by PCA analysis for all conditions at each pH. Differentially expressed feature lists between infected and uninfected cultures, with and without SLO incubation, were identified by ANOVA and queried against the METLIN metabolite database. Select compounds were mapped onto KEGG metabolite pathways for analysis and to demonstrate the feasibility of our discovery approach.

Experimental

Control (NRBC) and asynchronous parasitized RBCs at approximately 10% parasitemia (IRBC) were incubated in the absence or presence of 250 units SLO for 30 minutes at 37 °C. RBC metabolism was quenched and extracted in methanol/water/chloroform solvents[1], adjusted to pH 2, 7 or 9. Quadruplicate extracts of the polar phase for each condition were analyzed by ESI (+) LC/MS-TOF[1].

Table 1 Experimental Design

Tube No.	NRBC	IRBC @ 10%	SLO 250 stock units	Set A	Set B	Set C
1-1	500 ul			pH 2	pH 7	pH 9
1-2	500 ul			pH 2	pH 7	pH 9
1-3	500 ul			pH 2	pH 7	pH 9
1-4	500 ul			pH 2	pH 7	pH 9
2-1	500 ul		10 ul	pH 2	pH 7	pH 9
2-2	500 ul		10 ul	pH 2	pH 7	pH 9
2-3	500 ul		10 ul	pH 2	pH 7	pH 9
2-4	500 ul		10 ul	pH 2	pH 7	pH 9
3-1		500 ul		pH 2	pH 7	pH 9
3-2		500 ul		pH 2	pH 7	pH 9
3-3		500 ul		pH 2	pH 7	pH 9
3-4		500 ul		pH 2	pH 7	pH 9
4-1		500 ul	10 ul	pH 2	pH 7	pH 9
4-2		500 ul	10 ul	pH 2	pH 7	pH 9
4-3		500 ul	10 ul	pH 2	pH 7	pH 9
4-4		500 ul	10 ul	pH 2	pH 7	pH 9

Results and Discussion

A total of 2435 compounds (features) were found in all samples (4 groups X 3 pHs = 12 groups) by the Molecular Feature Extraction (MFE) algorithm in Agilent MassHunter Qual software. The mass list was filtered (1) based on a feature being present in all 4 replicates in one or more groups, and (2) the abundances for all groups having a coefficient of variation (CV) < 2000% (median ~ 35%) for biological replicates. The filtered mass list = 906 features was used in subsequent **untargeted** statistical analyses.

Figure 1 (A) PCA plot of all 48 samples extracted under different pH conditions, colored by pH.

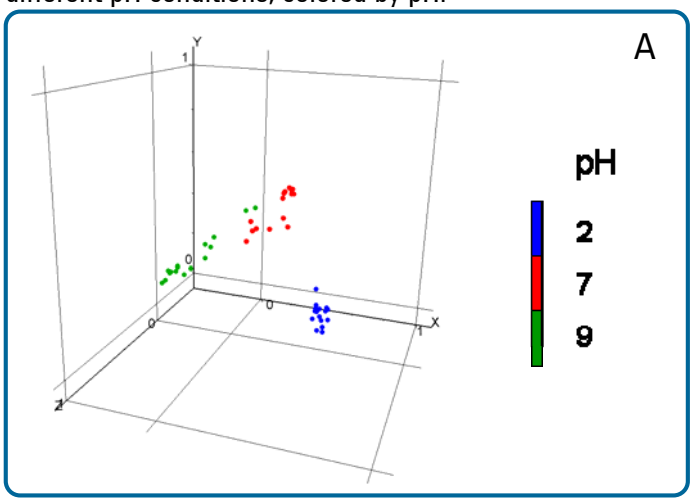
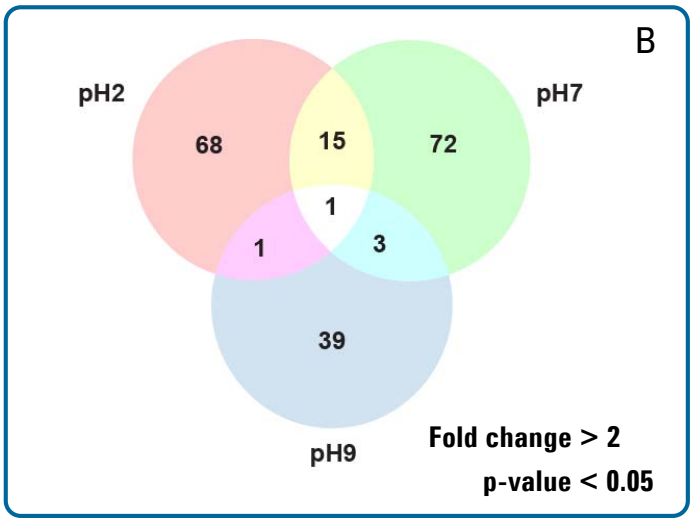


Figure 1 (B) Venn diagram based on Volcano plot results of statistically significant features found between NRBC and IRBC for each pH, where SLO=0. The unique compounds (68, 72 and 39) were screened against the METLIN database for identification.



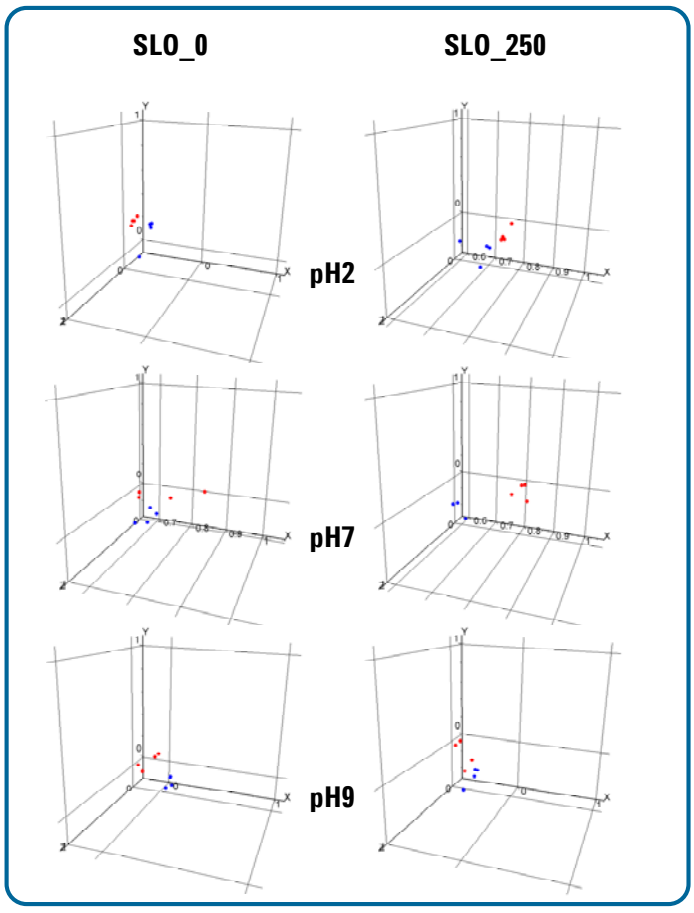
These results reveal that the pH of extraction solvent is a variable contributing to separation of samples. Moreover, this effect is independent of infection state or SLO treatment.

Results and Discussion

Table 2 METLIN database matches (< 10 ppm) for some compounds extracted at a specific solvent pH (Fig. 1B) and associated fold changes. SLO =0.

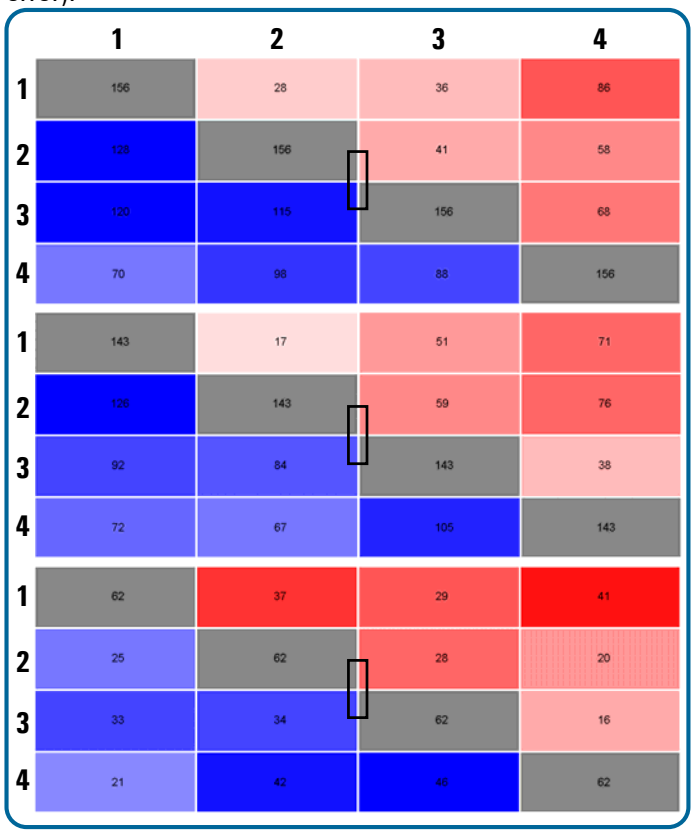
Name	pH	Formula	IRBC/NRBC
Hypoxanthine	2	C5H4N4O	-3
Tyrosine	2	C9H11NO3	110
PGF2alpha isopropyl ester	2	C23H40O5	83
Pyridoxal (Vitamin B6)	2	C8H9NO3	100
5-Amino-6-(5'-phosphoribosylamino)uracil	2	C9H15N4O9P	-52
Adenosine diphosphate (ADP)	7	C10H15N5O10P2	38
Phosphoribosyl-AMP	7	C15H23N5O14P2	100
c-ADP ribose	7	C15H21N5O13P2	125
Pantothenic Acid	7	C9H17NO5	100
Glutathione (GSH)	9	C10H17N3O6S	-15
Adenosine monophosphate (AMP)	9	C10H14N5O7P	-13
2,3-Diphospho-D-Glyceric Acid	9	C3H8O10P2	143

Figure 2. A PCA plot showing the separation of **NRBC** and **IRBC** samples, in the presence of either SLO 250, or no SLO treatment (SLO 0), at different extraction solvent pHs.



The effect of SLO 250 in pH7 extracts is pronounced compared to no SLO treatment. This effect is marginal when samples are extracted at pH2 and pH9. Further investigation of the SLO effect for infected cell enrichment should be performed extraction solvents where pH =7

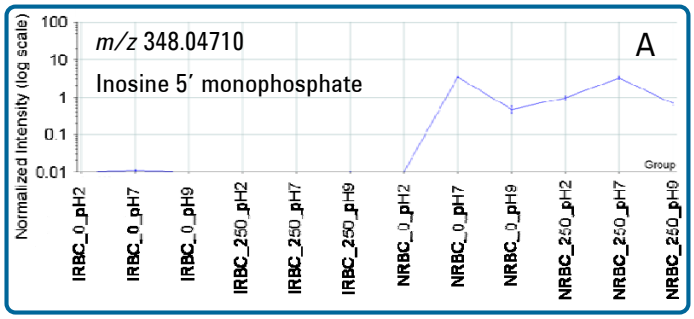
Figure 3. ANOVA with Tukey post-hoc test summary of the number of significant ($p < 0.05$) compounds (red blocks) between different pair wise groups. Top panel (pH2), middle panel (pH7), bottom panel (pH9). 1= IRBC_SLO 0; 2= IRBC_SLO 250; 3= NRBC_SLO 0; 4= NRBC_SLO 250. Approximately 20 % of the features matched compounds in the METLIN database based on accurate mass (< 10 ppm error).



Purine metabolism

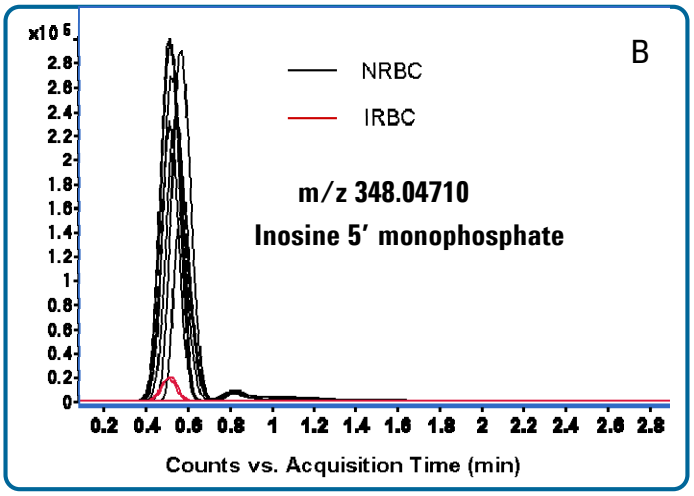
Protozoan parasites, including *P. falciparum*, are unable to synthesize purine rings *de novo*. They salvage purines from the host via sequential conversion of adenosine and inosine to hypoxanthine, which is then phosphoribosylated to IMP

Figure 4. Profile plot of normalized intensity values for *m/z* 348.04710 across all conditions in GeneSpring-MS software shows the presence in NRBC samples only



Results and Discussion

Figure 4. (A) EICs for *m/z* 348.04710 performed in MassHunter Qual software on SLO 250 treated samples extracted at pH2

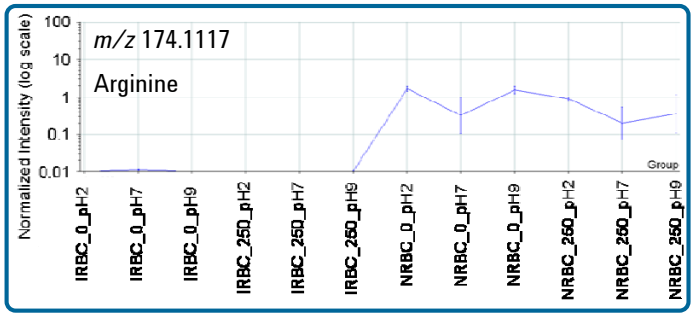


Based upon the METLIN database match and isotope distribution score (MFG), m/z 348.04710 (Inosine 5' monophosphate) appears to be depleted in infected RBCs (Fig. 4). IMP is a precursor in purine nucleotide de Novo synthesis pathway, which can be converted into ATP and GTP. The parasite requires purine nucleotides for syntheses of nucleic acids

Arginine metabolism

A previous targeted LC/MS/MS study[2] has shown arginine levels in IRBC to be depleted in cell cultures. We investigated arginine levels by LC/MS in an untargeted fashion using MFE software and separately, by EICs in a 'targeted' fashion.

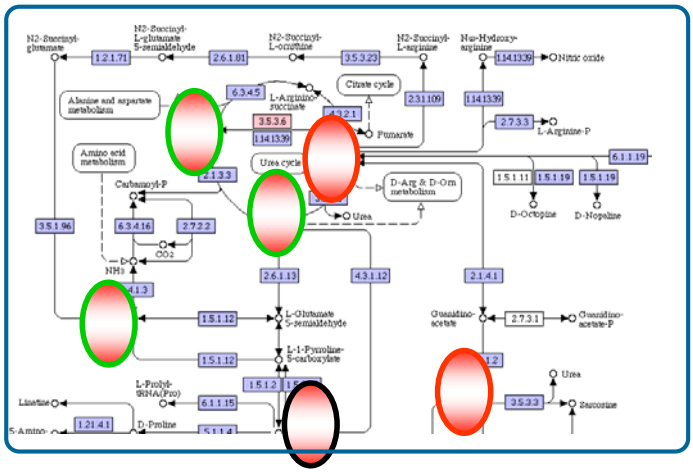
Figure 5. Profile plot of normalized intensity values for *m/z* 174.1117 across all conditions in GeneSpring-MS software



KEGG Pathway

To determine whether arginine levels were changing significantly based on infection status of RBCs, several compounds in this pathway were analyzed by mapping onto the KEGG arginine & proline metabolic pathway.

Figure 6. Targeted analysis of selected compounds in the "arginine and proline metabolism" pathway. Ornithine and citrulline circled in green were significantly elevated in IRBC relative to NRBC; arginine and creatine levels circled in red were either not detected or significantly reduced. Proline (circled in black) was not different.



Conclusions

- Our workflow and results suggest that an untargeted global metabolomics analysis by LC-TOF has the accuracy and sensitivity to confirm previous LC/MS/MS analyses for compounds involved in *P. falciparum* metabolism such as Arginine.
- In addition to detecting compounds known to be associated with *P. falciparum*, several hundred additional compounds, the majority unknown, were also detected.
- Based on ANOVA results, SLO pre-treatment is most effective in revealing differences between IRBC_SLO 250 and control (NRBC_SLO 250) extracted at pH=7. This is based on the number of extracted metabolites that are significantly different from control. Their relationship to efficacy of RBC enrichment remains to be elucidated.
- We detected an increase in ornithine and citrulline while arginine was decreased. This could suggest that the steps involving arginase and ornithine carbomyltransferase are up-regulated in infected RBC's/malaria parasite blood stages.

References

1. Sana, T.R. et. al. A sample extraction and chromatographic strategy for increasing LC/MS detection coverage of the erythrocyte metabolome. Journal of Chromatography B, 871 (2008) 314–321
2. Olszewski, K. L. et. al. Host-Parasite Interactions Revealed by *Plasmodium falciparum* Metabolomics. Cell Host & Microbe, 5, 191–199, February 19, 2009