Exploration of untargeted metabolomic extraction methods for in vitro malaria samples by 1H NMR analysis

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Introduction

- Metabolomics is a reliable omics tool to study the metabolome, the assortment of metabolites that provide energy, signaling or building blocks essential for biological systems survival.
- Malaria is a deadly disease, especially severe when caused by *Plasmodium falciparum*, that still affects over 200 million people yearly.[1]
- The metabolome closely reflects the state of the biological system, and if applied to the *P. falciparum,* it can be used to characterize antimalarial mechanisms of action or study resistance.
- The parasite's intracellular nature, in red blood cells in suspension, introduces significant hurdles to metabolomics extraction methods.

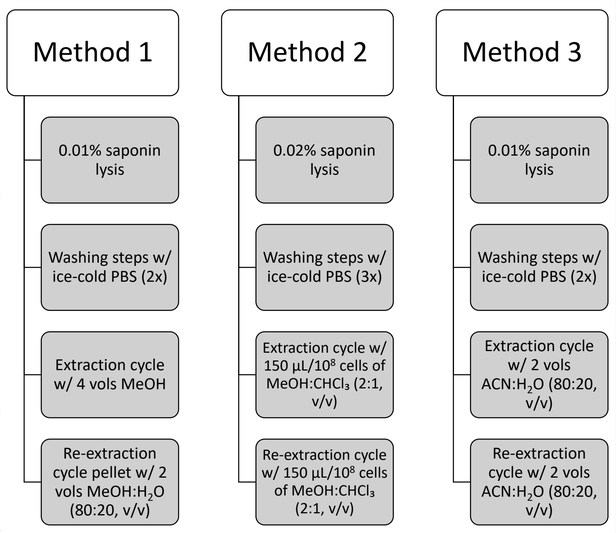
Objectives

To discern the most reliable untargeted metabolomic extraction method from 3 literature methods through ^{1}H NMR analysis, using multiple parameters:

- ❑ Chemometrics exploration – Principal Components Analysis (PCA);
- ❑ Number of peaks per spectra;
- ❑ Metabolite annotation;
- ❑ Metabolomic Informative Content (MIC) – Between and within group inertia

Methods

P. Falciparum 3D7 cultures were magnetically purified and microscopically verified for stage and parasitemia. Ring-stage samples were extracted by either method, as according to the scheme, in triplicate.[2,3] The whole process was performed 3 times, hence noted as groups. Assembled extracts were evaporated and freeze-dried to remove all solvent traces. Samples were dissolved in 400 µL of buffered D_20 at pH 7.4 with TMSP as internal reference, and transferred into 3 mm NMR tubes (Bruker) for analysis. NMR spectra were acquired using TopSpin software on a Bruker Ultrashield Plus 700 MHz equipped with a helium cold probe (cryoprobe). ^{1}HNMR experiments were performed with a CPMG sequence with 128 scans collected over a spectral width of 20 ppm. All spectra were phased and baseline-corrected manually using TopSpin v4. Spectra were stacked, aligned and integrated between δ0.5-9.5 ppm using MestReNova v14. Spectra were divided into buckets of 0.04 ppm, integrated to the sum of intensities and normalized to the number of parasites per sample. The data sets were processed using MetaboAnalyst v5.0 and R. Lastly, the spectra were annotated using Chenomx NMR Suite 9.0 database and the Human Metabolome Database (HMDB), as according to literature.



Results & Discussion

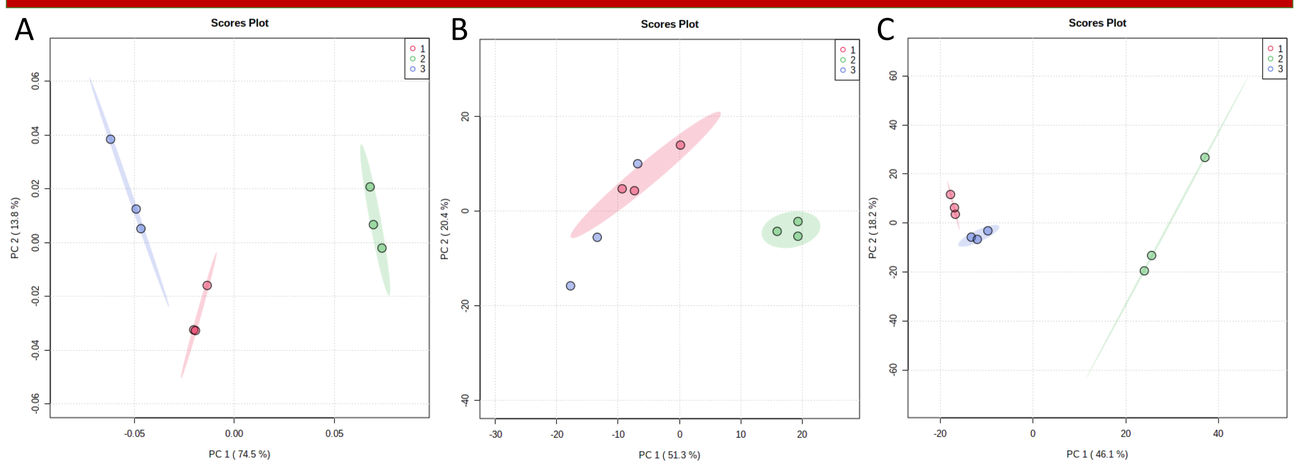


Figure 1: PCA 2D scores plot with 95% confidence regions displayed, of method 1 (A), method 2 (B) and method 3 (C), groups correspond to the three assays. A and C show separation across groups, whereas B does not achieve separation. Method 1 has less intragroup variability, as seen by the distribution across the PC1 (74.5%), which reveals this method as the most repeatable. Additionally, the variation percentage explained by the components 1 and 2 is greater for method 1 than for the others. Both B and C present one outlier each, for group 3 and 2, respectively.

	Group 1	Group 2	Group 3
Method 1	361 ± 60	162 ± 11	370 ± 18
Method 2	304 ± 67	165 ± 30	438 ± 88
Method 3	311 ± 14	224 ± 63	406 ± 13
Parasite count	1.39 x 10^8	5.22 x 10^7	2.42 x 10^8

Table 1: Average number of peaks and parasite count per sample. The 2nd group rendered less peaks across all methods, reflecting the necessity of having high (> over 10^8) parasitic counts for reliable NMR detection.

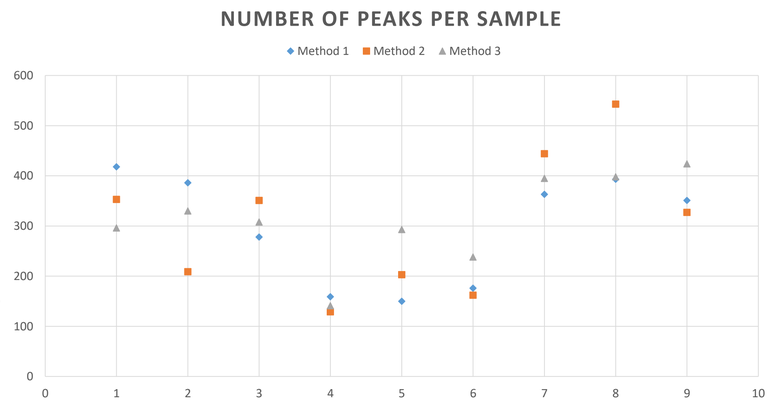


Figure 2: Number of peaks across all samples.

	Chenomx (43 total)	HMDB (10 total)
Method 1	18-32	1*
Method 2	13-32	1*
Method 3	17-34	1*

Table 2: Number of metabolites annotated through either Chenomx or HMDB databases per method according to the literature. Annotation is variable between samples, mostly due to parasite count variation that interferes with NMR detection.

* - highly tentative annotation of glycerolphospho-ethanolamine.

Class	Metabolite	Method 1	Method 2	Method 3
Amino acids	Asparagine	Yes[1]	Yes[1]	Yes[2]
	Glutamate	Yes	Yes[1]	Yes[2]
	Glutamine	Yes[1]	Yes[1]	Yes[2]
	Phenylalanine	Yes[1]	Yes[3]	Yes[1]
	Serine	Yes[1]	Yes[1]	Yes[2]
	Tyrosine	Yes[1]	Yes[1]	Yes[2]
Nucleotides and related compounds	AMP	Yes	Yes[1]	Yes
	Hypoxanthine	Yes[1]	Yes[1]	Yes[2]
	IMP	Yes	Yes[1]	Yes
	$NADP^+$	No	Yes[3]	Yes[1]
Glutathione	Reduced	Yes[1]	Yes[1]	Yes[2]
Carboxylates	Fumarate	Yes	Yes[1]	Yes[1]
Soluble membrane precursors	*myo-Inositol*	Yes	Yes[1]	Yes[2]

[1] – not found in all samples;
[2] – only not found in outlier;
[3] – only found in 2 samples.

Table 3: 13 metabolites were annotated differently between extraction methods. Method 3 would account for more consistency without the outlier, followed by method 1. Method 2 displays the most variation regarding detection of these metabolites. For method 1, only $NADP^+$ is not found, possibly because of the quicker experimental time. Metabolites were annotated with Chenomx.

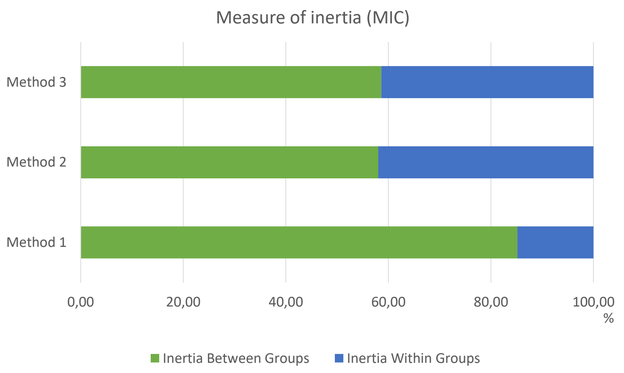


Figure 3: Graphical representation of between and within group inertia. Method 1 has the least intra group variability, demonstrated by the measure of inertia within group, and thus higher separation between groups, which asserts robustness. Method 2 and 3 are similar in terms of inertia.

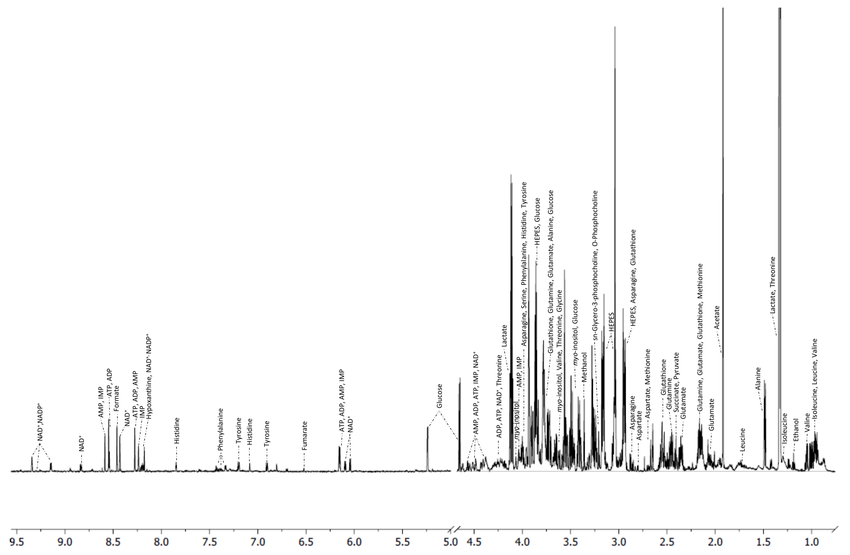


Figure 4: Representative ^{1}HNMR spectra, sample 9 from Method 3.

Conclusion

All methods successfully extracted *Plasmodium* related metabolites, although with different levels of replicability. Method 1 showed more repeatability and robustness across all parameters, followed by Method 3 that accounted for the highest metabolite annotation, and lastly Method 2, that displayed the least promise of the three. Both methods 2 and 3 had one outlier each, which didn't alter the conclusions even if removed. Parasite counts and workflow timings proved to be important factors in extraction and detection through ^{1}HNMR. This preliminary assay allowed for the exploration of *Plasmodium* related metabolites and to choose method 1 for further studies.

Aknowledgements

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