Exploration of untargeted metabolomic extraction methods of *in vitro* malaria samples by ^{1}H NMR analysis

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Introduction

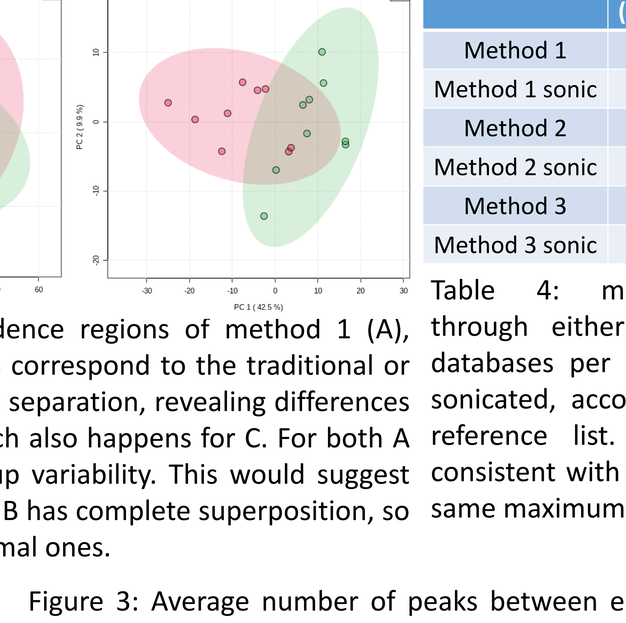
- Metabolomics is a reliable omics tool to study the metabolome, the assortment of metabolites that provide energy, signaling or building blocks essential for biological systems survival.
- Malaria is a deadly disease, especially severe when caused by *Plasmodium falciparum*, that still affects over 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.
- To explore additional methodology parameterization: the number of washes and an optional sonication step to the first solvent extraction.

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 assay was performed 3 times with traditional methodologies and another 3 with an additional sonication cycle (*). Assembled extracts were evaporated and freeze-dried. 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 corrected, stacked, aligned, 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

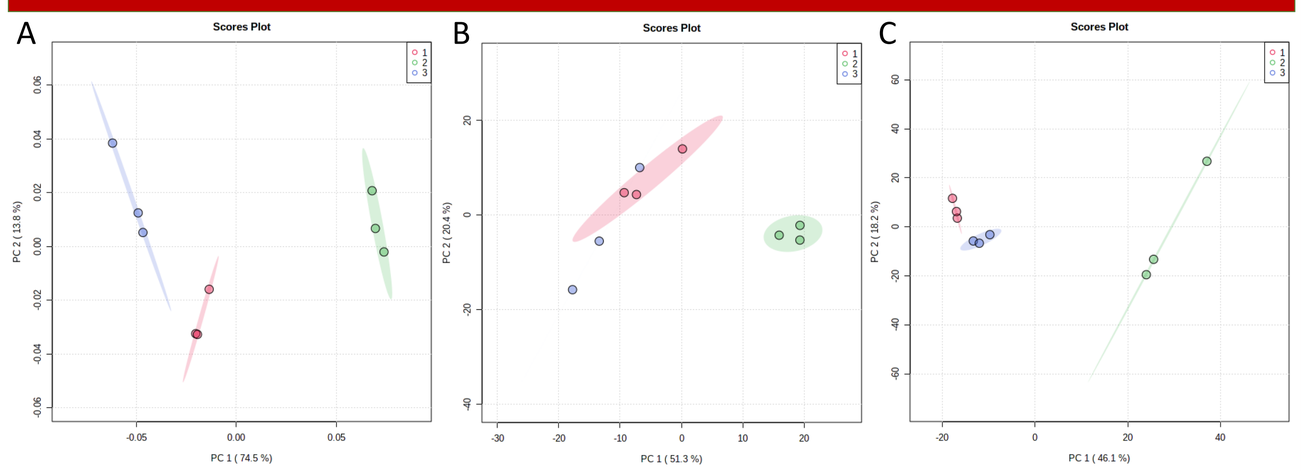


Figure 1: PCA 2D scores plot with 95% confidence regions of method 1 (A), method 2 (B) and method 3 (C); the three groups correspond to the three tests with traditional methods only. Only A and C show separation across groups. A has less intragroup variability, as seen by the distribution across the 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 A than for the others. Both B and C present one outlier each, for group 3 and 2, respectively, that didn't alter the conclusions even if removed.

	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 of the three tests with traditional methods only. The 2nd group rendered less peaks across all methods, reflecting the necessity of having high (> over 10^8) parasitic counts for reliable ^{1}HNMR detection.

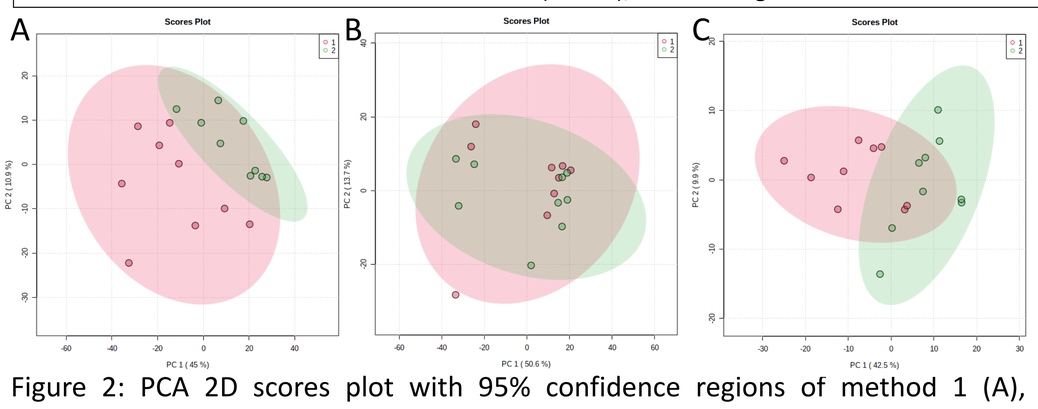


Figure 2: PCA 2D scores plot with 95% confidence regions of method 1 (A), method 2 (B) and method 3 (C); the two groups correspond to the traditional or sonicated methods, respectively. Only A achieves separation, revealing differences between sonicated and traditional samples, which also happens for C. For both A and C the sonicated group shows less intragroup variability. This would suggest sonication increases method 1 and 3 robustness. B has complete superposition, so sonicated samples are not different from the normal ones.

	Chenomx (43 total)	HMDB (10 total)	Total
Method 1	24-33	1-2	26-35
Method 1 sonic	29-34	1-2	31-36
Method 2	18-34	1-2	19-36
Method 2 sonic	26-35	1-2	28-37
Method 3	31-33	1	32-34
Method 3 sonic	33-35	1-2	34-37

Table 4: metabolites annotated through either Chenomx or HMDB databases per method, traditional or sonicated, according to the literature reference list. Annotation becomes consistent with sonication, despite the same maximum annotation.

	Inertia Between Repetitions	Inertia Within Repetitions
Method 1	85,18%	14,82%
Method 2	58,03%	41,97%
Method 3	58,61%	41,39%

Table 2: Inertia table of between and within group inertia for traditional methods. Method 1 has the least intra group variability, demonstrated by the measure of inertia within group, which asserts robustness and repeatability. Method 2 and 3 are similar in terms of inertia.

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; Annotation with Chenomx.

Table 3: 13 metabolites were annotated differently between traditional 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.

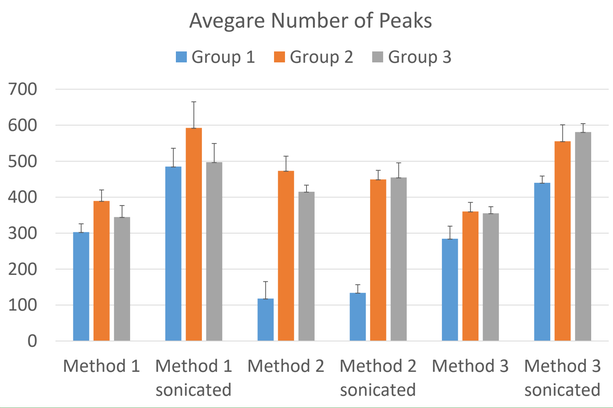


Figure 3: Average number of peaks between each method, traditional or sonicated. For both Method 1 and 3 the number raises significantly with sonication, which could mean an increase in the number of extracted metabolites. Method 2 stays roughly similar.

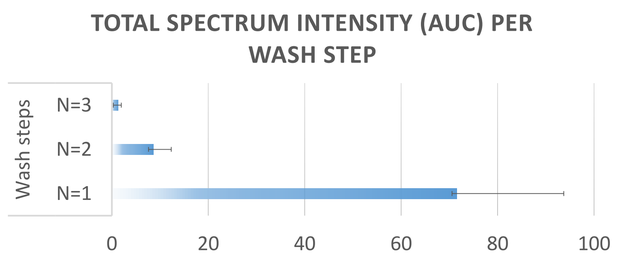


Figure 4: Total spectrum intensity per wash step. First washing step is essential to clean culture media and RBC metabolome, second is still representative, third represents approximatively 1/54 of the spectral intensity of the first washing step. A third washing step might not be worth it, since it entails additional experimental time and parasite leakages cannot be ruled out.

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. Exploration of parameters revealed that two washing steps were enough to remove culture media and contaminants and that sonication added real value to methods 1 and 3, with more metabolites detected more robustly. This assay allowed choosing method 1, a two-step extraction with methanol and methanol:water (80:20, v/v), with the addition of a sonication step, for future metabolomic analysis of *P. falciparum in vitro*.

Aknowledgements

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