

Application of GC Orbitrap Mass Spectrometry for Untargeted Metabolomics of Pathogenic Microorganisms

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ABSTRACT

Combined infections from *Candida albicans* and *Staphylococcus aureus* are a leading cause of death in the developed world. Evidence suggests that *Candida* enhances the virulence of *Staphylococcus*: hyphae penetrate through tissue barriers, while *S. aureus* tightly associates with the hyphae to obtain entry to the host organism. Indeed, in a biofilm state, *C. albicans* enhances the antimicrobial resistance characteristics of *S. aureus*, with vancomycin concentrations of up to 1600mg/mL being unable to clear the bacteria. The association of *Candida* and *Staphylococcus* is also related with significantly increased morbidity and mortality. Due to this tight association we hypothesised that metabolic effects were also in evidence. To explore the interaction, we used a novel GC-Orbitrap-based mass spectrometer, the Thermo Scientific™ Q Exactive™ GC Orbitrap™ GC-MS/MS, which combines the high peak capacity and chromatographic resolution of gas chromatography with the sub-ppm mass accuracy of an Orbitrap system. This allows the capability to leverage the widely available electron ionisation libraries for untargeted applications, along with expanding accurate mass libraries and targeted matches based around authentic standards. *C. albicans* and *S. aureus* mono- and co-cultured biofilms were analysed in addition to the fresh and spent bacterial growth media. We detected an additional 22 highly scoring compounds from untargeted analysis. Many of the results were as expected – rapid consumption of glucose and fructose from the medium regardless of the cell type. We also detected trehalose from the untargeted data, only in medium that contained *C. albicans*, commensurate with it being a predominantly fungal sugar. Notable from the results is that the pentose phosphate pathway appears to be enhanced in the cells from co-cultured biofilms.

INTRODUCTION

Combined infections from *C. albicans* and *S. aureus* are a leading cause of death in the developed world. Evidence suggests that *Candida* enhances the virulence of *Staphylococcus* - hyphae penetrate through tissue barriers, while *S. aureus* tightly associates with the hyphae to obtain entry to the host organism [1]. Scanning electron microscope image (SEM) of *C. albicans* and *Staphylococcus aureus* co-culture demonstrates a tight association between the *S. aureus* cells and the fungal hyphae with the presence of extracellular matrix coating secreted from the *S. aureus*. Indeed, in a biofilm state, *C. albicans* enhances the antimicrobial resistance characteristics of *S. aureus* [2]. The association of these microorganisms is also related with significantly increased morbidity and mortality. Due to this strong biological association it was hypothesised that metabolic effects were also in evidence.

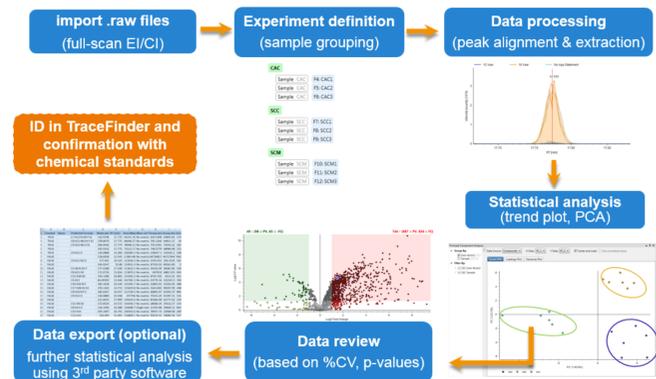
MATERIALS AND METHODS

Sample Preparation

Optimised *C. albicans* and *S. aureus* mono- and co-cultured biofilms were analysed using the Q Exactive GC instrument in addition to the fresh and spent bacterial growth media. Sample preparation for GC-MS was performed as described in elsewhere [3], but in brief, consisted of drying to completeness followed by methoxylation and trimethylsilylation, prior to injection on a Q Exactive GC system. A total run time of 24.5 min was applied. Instrument parameters and gradient details were configured for the Q Exactive GC system as described previously [3].

Data processing workflow

Workflow diagram highlighting the main steps in the analysis. Peak alignment and statistical analysis were done in Thermo Scientific™ Compound Discoverer™ software, whereas compound identification was performed in Thermo Scientific™ TraceFinder™ software.



RESULTS

Peak area reproducibility following sample preparation (method performance) as well as mass accuracy were tested. Examples of peak area reproducibility based on injection of the same sample derivatized and injected independently are shown in Figure 1.

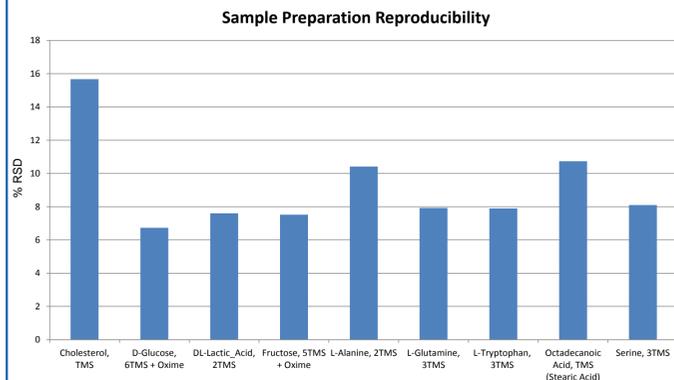


Figure 1. Reproducibility of sample preparation (%RSD) measured by relative standard deviation. Each sample was processed separately to demonstrate that the entire analytical process, from sample extraction, through derivatisation and injection, to analysis, is highly consistent.

In addition, an example of mass accuracy assessed by plotting the mass accuracy (as ppm) of the quantitation ion from the amino acid glutamine is shown in Figure 2. The systematic error is around 0.3 ppm and the RSD is $1.75 \times 10^{-5} \%$, demonstrating high mass accuracy across the peak.

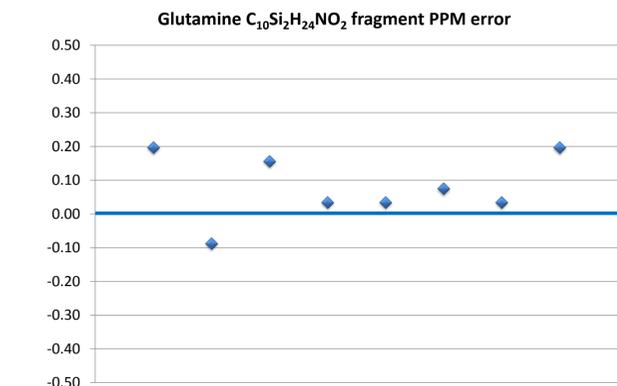


Figure 2. Example of mass accuracy (as ppm) for m/z 246.1340 m/z ($C_{10}Si_{12}H_{24}NO_2$) ion from glutamine. Note that all masses are within 0.3 ppm of the true mass.

PCA of the biofilm samples using Compound Discoverer software (Fig. 3) showed distinct separation between co- and mono-cultures in principal component (PC) 1 and 2. Univariate statistical analysis was therefore applied to the dataset to elucidate key differences between the samples.

The principal component analysis (PCA) of biofilms and spent and fresh medium is shown below. SAC is the intracellular metabolome from *Staphylococcus aureus* mono-culture, and CAC is the intracellular metabolome from *C. albicans* mono-culture. SAM is the extracellular metabolome from *Staphylococcus aureus* mono-culture, CAM is the extracellular metabolome from *C. albicans* mono-culture, SCM is the extracellular metabolome from the co-culture and MO is fresh medium. Note the clear separation of the co-culture intracellular metabolites (SCC) from the monoculture cellular metabolites as well as obvious separation between fresh medium (MO) from the other samples, and that the co-culture medium is substantially separated from either of the mono-cultures.

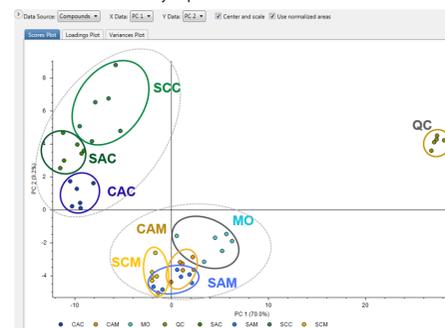


Figure 3. PCA of biofilms (a) and spent and fresh medium (b). SAC is the intracellular metabolome from *S. aureus* mono-culture, and CAC is the intracellular metabolome from *C. albicans* mono-culture. QC samples containing a set of metabolite standards were also used to assess instrument response.

Untargeted analysis, processed using our in-house pipeline, resulted in the detection of 465 compounds from medium and 405 from cells. A shortlist of the most significantly changing metabolites was selected and analysed for high quality fragment pattern matches (Table 1). Three compounds were chosen for validation: two from medium and one from intracellular samples. Pyroglutamate (5-oxoproline) was consumed in all cultures in comparison to medium (Figure 4a). This is the same pattern seen for the proteinaceous amino acids and may well function, via the action of 5-oxoprolinase, as an additional source of glutamic acid. Myristic acid, found to be consistently depleted from the medium in the *S. aureus* containing samples (see Figure 4b), is known to be bacteriocidal, and depletion may be a consequence of detoxification. Interestingly, targeted detection of sugar phosphates was prevalent in the cell samples (Figure 4d) but only one, myo-inositol 1 phosphate, was detected in the medium samples. This is consistent with a lack of cell death and proliferation of the cells, as sugar phosphate leakage is associated with cell rupture.

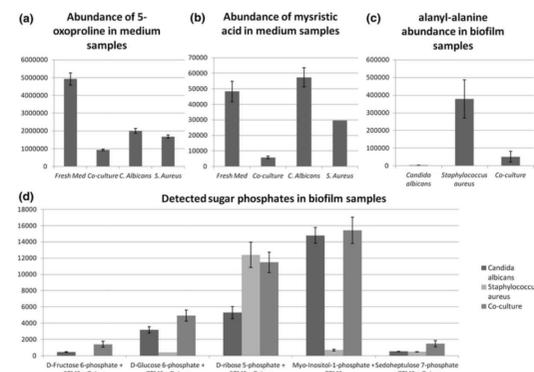


Figure 4. (a) Abundance of 5-oxoproline in the medium. The significant drop in abundance between fresh medium and all cultures demonstrates its uptake. This is particularly marked in the co-culture. (b) Myristic acid is not consumed by *C. albicans* but is significantly consumed in the co-culture. (c) Detection of alanyl alanine in the biofilm samples. It is markedly higher in the *S. aureus* cells, probably as a consequence of its function in cell wall biosynthesis. (d) Intensities of sugar phosphates detected within the cell/biofilm pellets. *C. albicans* monoculture values are shown in dark grey, *S. aureus* monoculture values in light grey, and co-culture values are shown as mid-grey. Values are shown with standard error of the mean.

Table 1. Table of log₂ fold change of detected metabolites, classified by compound type. SAC samples are *Staphylococcus aureus* biofilm cultures. CAC are *C. albicans* biofilms, SCC are *Staphylococcus/Candida* co-culture biofilms. All comparisons are against *C. albicans* biofilms as a baseline, e.g. L-Cysteine is downregulated 10.2 fold in *S. aureus* comparison to *C. albicans*.

Compound ID (NIST)	SAC vs CAC	SCC vs CAC	Compound type
L-Cysteine, 3TMS	-10.2	-0.6	Amino acid
L-Histidine, 3TMS	-6.4	-1	Amino acid
L-Methionine, 2TMS	-3.2	-1.2	Amino acid
L-Tyrosine, 3TMS	-2.8	-0.8	Amino acid
L-Homoserine, 3TMS	-2.5	0.9	Amino acid
L-Threonine, 3TMS	-2.2	-0.6	Amino acid
L-Glutamic acid, 3TMS	1.9	0.3	Amino acid
L-Tryptophan, 3TMS	-1.9	-0.5	Amino acid
L-Lysine, 3TMS	-1.5	-0.4	Amino acid
L-Aspartic Acid, 3TMS	1.4	-0.4	Amino acid
L-Leucine, 2TMS	-1.4	-0.5	Amino acid
L-Serine, 3TMS	-1.2	-0.1	Amino acid
L-Proline, 2TMS	-0.9	-1.1	Amino acid
L-Isoleucine, 2TMS	-0.9	0.3	Amino acid
L-Ornithine (and L-Arginine), 3TMS	0.8	0.1	Amino acid
L-Alanine, 2TMS	-0.8	-1	Amino acid
L-Valine, 2TMS	-0.7	-0.1	Amino acid
L-Phenylalanine, 2TMS	0.4	0.5	Amino acid
L-Hydroxyproline, 3TMS	-0.3	-0.2	Amino acid
Glycine_3TMS	0.1	0.1	Amino acid
Palmitic Acid, TMS	0.1	0.2	Fatty acid
Octadecanoic Acid (Stearic Acid), TMS	0.1	0.1	Fatty acid
Lactic Acid, 2TMS	-1.5	-0.4	Organic acid
Succinic acid, 2TMS	-1.1	-0.9	Organic acid
Cholesterol, TMS	1	0.7	Steroid
D-Fructose, 5TMS + Oxime	0.9	-0.3	Sugar
D-Glucose, 6TMS + Oxime	-0.5	-0.9	Sugar
D-Ribose + 4TMS + Oxime	-15.5	-0.5	Sugar
D-Rhamnose + 4TMS + Oxime	12.9	10.8	Sugar
D-Erythrose + 4TMS + Oxime	-9.2	1.1	Sugar
Maltose + 8TMS + Oxime	-4.3	-0.7	Sugar
D-Xylulose + 4TMS + Oxime	-2.5	0.6	Sugar
Myo-Inositol + 6TMS	-1.7	-0.3	Sugar
Sucrose + 8TMS	0.7	0.6	Sugar
D-Mannose + 5TMS + Oxime	-0.7	-0.9	Sugar
D-Lactose + 8TMS + Oxime	0.2	-1.1	Sugar
Adonitol + 5TMS	-4.3	-0.5	Sugar alcohol
D-Sorbitol + 6TMS	-1.4	-1	Sugar alcohol
D-Glucose 6-phosphate + 7TMS + Oxime	-11.5	0.8	Sugar phosphate
D-Fructose 6-phosphate + 6TMS + Oxime	-8.7	1.6	Sugar phosphate
Myo-Inositol-1-phosphate + 7TMS	-4.4	0.2	Sugar phosphate
D-ribose 5-phosphate + 5TMS + Oxime	1.4	1.3	Sugar phosphate
Sedoheptulose 7-phosphate + 7TMS + Oxime	0	10.3	Sugar phosphate

CONCLUSIONS

- Gas chromatography mass spectrometry remains a powerful technique for metabolomics analysis.
- The ability to distinguish isomeric compounds in a broad-based analysis using retention time is very useful for the study of metabolism.
- Acquiring data using the Q Exactive GC system operated in full scan at high resolving power allows for more compounds to be analyzed, increasing the scope of the analysis.
- The advantage of such technique is that data processing can be split into a targeted (using a compound database) and untargeted (unknown compound discovery) workflow and the possibility to historically re-interrogate the data at a later stage if needed.
- Data from mono- and co-cultured *C. albicans* and *S. aureus* strains demonstrates the utilisation of specific sugars as a carbon source in both organisms in this intricate interkingdom interaction, as well as synergistic effects on intermediates in the pentose phosphate pathway. This suggests that targeting the enzymes associated with sedoheptulose-7-phosphate may have an effect on the metabolic interaction between *C. albicans* and *S. aureus*.
- The ability to harness sophisticated technologies as those described herein has immense potential in the study of complex microbiome studies where the interaction of the microbiome with the mycobiome have implication for human health and disease.

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