APPLICATION NOTE

No. 10532

Application of GC Orbitrap mass spectrometry for untargeted metabolomics of pathogenic microorganisms

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Keywords: Metabolomics, Q Exactive GC, Pathogenic bacteria, Biofilms, *Staphylococcus aureus, Candida albicans*

Introduction

Infections due to pathogenic bacteria affect a large number of people worldwide. There is increasing concern about the effectiveness of existing vaccines and antibiotics used to prevent and treat infections caused by these organisms. Pathogenic bacteria can form biofilms either as single species aggregates and/or as polymicrobial biofilms. An important biofilm-causing fungus, *Candida albicans*, is the fourth leading cause of bloodstream infections worldwide and is associated with the highest incidence of mortality.[1] Furthermore, it has been demonstrated that *C. albicans* can form biofilms in close association with the pathogenic bacterium *Staphylococcus aureus*. The combined effect of *C. albicans* and *S. aureus* result in a synergistic increase in virulence and mortality in mice[2] and humans.[3] At the metabolic level little is known about the biochemicals responsible for this symbiotic effect.
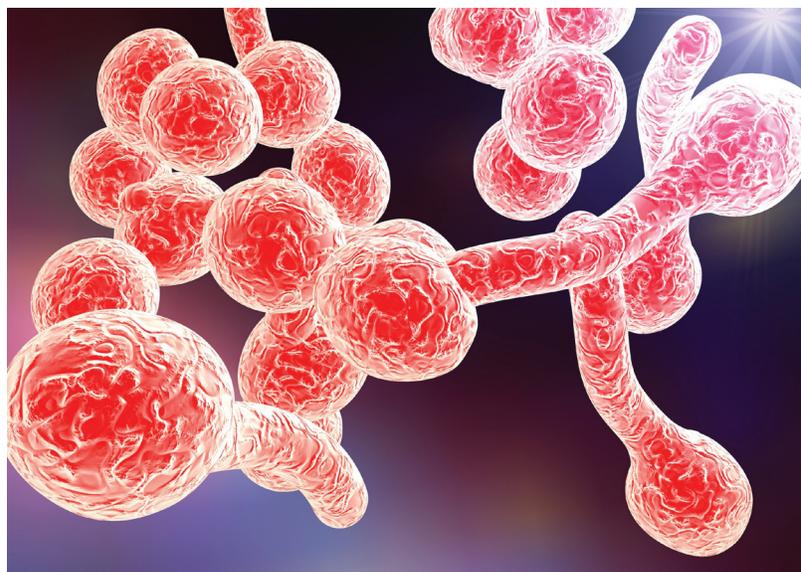
In this study, the interaction between *C. albicans* and *S. aureus* was investigated using an untargeted metabolomics approach and by applying gas chromatography (GC) coupled with high resolution mass spectrometry (HRMS). Bacterial culture media as well as *C. albicans* and *S. aureus* cells were investigated in co-culture and separately with the aim of detecting and identifying statistically relevant metabolites that can help to elucidate these microbial interactions.

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In order to elucidate the biochemical interactions between *C. albicans* and *S. aureus* and to identify the key metabolites involved in this synergistic relationship, a Thermo Scientific™ Q Exactive™ GC Orbitrap™ mass spectrometer was employed. This high mass resolution bench-top analytical GC-MS platform has the advantage of delivering full-scan sensitivity and selectivity with consistent sub-ppm mass accuracy, critical for confident compound identification and confirmation. In addition, the large instrumental dynamic range and fast scan rate of the Q Exactive GC system allow for the detection of metabolites present at low and high concentrations enabling reliable peak deconvolution and compound quantification. Importantly, data can be searched against existing commercial spectral libraries (such as NIST or Wiley) and/or customized high resolution metabolites libraries for putative metabolite identification.

Experimental

Samples

Candida albicans laboratory strain SC5314 (ATCC MYA-2876) and *Staphylococcus aureus* Newman strain (NCTC 8178/ATCC) were used in all experiments described. The samples analyzed were both individual and co-culture cells of *Candida albicans* and *Staphylococcus aureus* in addition to fresh and spent media used to grow the two microorganisms, individually and in co-culture. In addition, injection of pooled samples in which a small aliquot of each biological sample are mixed together were used as quality control samples (QC) were used to assess the variance observed in the data throughout the sample preparation, data acquisition and data pre-processing steps. In addition to these, pure metabolite standards containing sugars, sugar phosphates, pentose phosphate pathway, and amino acids were used to confirm candidate compounds identified with NIST library. Details of the exact conditions used for biofilm culture as well as the information about the extraction procedure of extra- and intracellular metabolites are given elsewhere.[4]

Sample derivatization

All media and cell samples were subjected to chemical derivatization (methoximation and silylation) to increase the volatility of the compounds present in the samples for gas chromatography separation. A total volume of 30 µL of media, cell sample was spiked with $^{13}C_6$-Glucose (2 nmol), D_{27}-Myristic Acid (2 nmol) and Scyllo-Inositol (1 nmol) used as internal standards to monitor and correct for injection.[4] For compound confirmation a series of standards mixtures containing sugars, sugar phosphates, pentose phosphate pathway, and amino acids were also used following the same derivatization procedure. Samples and standards

were dried prior to addition of 50 µL of 20 mg/ml (w/v) methoxyamine HCl in pyridines and incubation at 60 °C for 120 min. Following the methoximation step, 50 µL of MSTFA + 1 % TMCS (N-Methyl-N-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane) was added. Silylation was performed by incubation at 80 °C for a further 120 min. Samples were cooled to room temperature and 1 µL of retention index alkane mixture was added to each sample before GC-MS analysis. Retention time prediction is particularly useful for untargeted metabolomics as it can increase the confidence in compound identification when searching a spectral library.[5]

GC-MS analysis

Derivatized samples were acquired in a randomized order across the batch to reduce the statistical impact of analytical variation. A Thermo Scientific™ TriPlus™ RSH™ robotic arm was used for sample introduction and GC separation was achieved using a Thermo Scientific™ TRACE™ 1310 gas chromatograph and a Thermo Scientific™ TraceGOLD™ TG-5Sil MS 30 m length × 0.25 mm inner diameter × 0.25 µm film thickness column (Table 1). The Q Exactive GC mass spectrometer was operated in full-scan using electron ionization and internal lock mass correction (Table 2).

Table 1. GC and injector conditions.

TRACE 1310 GC Parameters	
Injection Volume (µL):	1
Liner:	Single taper (P/N 453A1345)
Inlet (°C):	250
Inlet Module and Mode:	SSL, split 1:100
Carrier Gas, (mL/min):	He, 1.0
Oven Temperature Program	
Temperature 1 (°C):	70
Hold Time (min):	4
Temperature 2 (°C):	320
Rate (°C/min):	20
Hold Time (min):	8

Table 2. Mass spectrometer conditions.

Q Exactive GC Mass Spectrometer Parameters	
Transfer Line (°C):	250
Ionization Type:	EI
Ion Source (°C):	230
Electron Energy (eV):	70
Acquisition Mode:	full-scan
Mass Range (Da):	50–650
Mass Resolution (FWHM at *m/z* 200):	60,000
Lockmass (*m/z*):	207.03235

Data processing

Acquired data was subjected to an untargeted metabolite detection and identification workflow. The workflow uses Thermo Scientific™ Compound Discoverer software for comparing the relative abundances of metabolites in multiple samples without prior identification and followed by discriminatory statistical analysis (such as PCA and Volcano plots). Metabolites isolated from statistical analysis were further assessed with Thermo Scientific™ TraceFinder™ software for semi-quantitation (fold change calculation) and putative metabolite identification. Peak deconvolution was automatically performed with TraceFinder, where NIST 2014 mass spectral library was used to annotate the peaks with a search index threshold of >700. Compound identification was made using a total confidence score that takes into account the NIST spectral match as well as the percentage of fragment ions that can be explained from the elemental composition of the molecular ion assigned by NIST Thermo Scientific.[6]

Results and discussion

The data processing workflow used in this study utilized both Compound Discoverer software and TraceFinder software. Compound Discoverer allowed for comparative group analysis and isolation of significant metabolites using parameters such as %CV of peak area repeatability in the QC samples, calculated probability values (p<0.05) and fold-change in concentration between the sample groups (Figure 1). TraceFinder enabled compound deconvolution and putative identification using library search and accurate mass information.
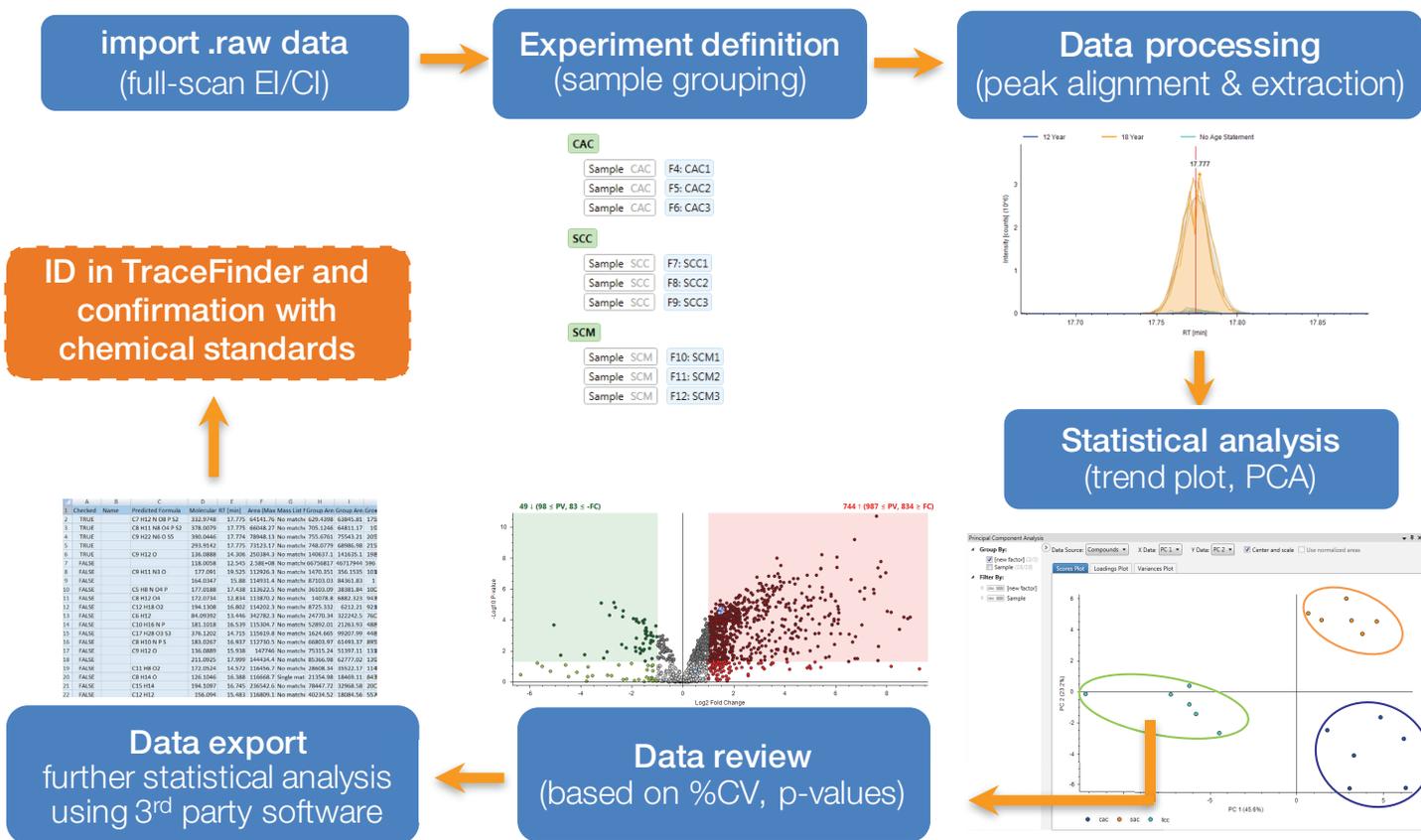

Figure 1. Data processing workflow used for untargeted metabolomics. Compound Discoverer software was used to find sample differences based on principal component analysis or group comparison using Volcano plots. Data from Compound Discoverer software can be exported (as *.csv or *.xls) for further statistical tests. Compound identification was made using TraceFinder and/or by injection of pure chemical standards to confirm the retention times and mass spectra.

Even before applying any statistical tests, noticeable differences in the intensities of chromatographic peaks were observed as shown for the peak eluting at RT = 9.04 min across four media sample groups (Figure 2).

In order to automatically extract biologically relevant information, the samples were investigated using Compound Discoverer for comparative analysis and determination of group differences using PCA analysis. Compound Discoverer allows for automated, easy-to-setup chromatographic alignment, peak detection and data normalization in addition to robust statistical analysis. Following this analysis, a large number of chromatographic features were detected (~17,000) across all 47 samples.

PCA analysis revealed distinct sample grouping with clear differentiation between media samples and cell samples were observed (Figure 3). Interestingly, the media samples show individual clustering with the media used for co-culturing the *C. albicans* and *S. aureus* as distinct group. This could be due to different uptake rates of the biomolecules found in the media and/or excretion of metabolites into the media from the two bacterial species. In addition, the QC samples clustered very closely together indicating good reproducibility (<20 % RSD) of the data.
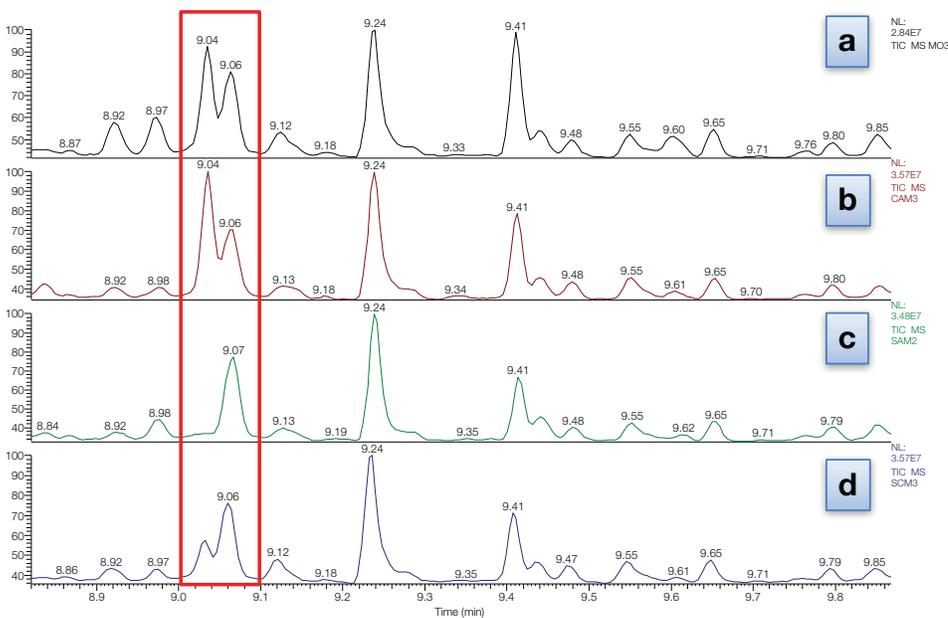

Figure 2. TIC chromatograms (EI, full-scan data) of media only (a), *C. albicans* media (b), *S. aureus* (c) and co-culture *C. albicans* and *S. aureus* (d). Highlighted is peak at RT = 9.04 min later identified as glycine shows depleted levels in *S. aureus* media as compared to the other samples.
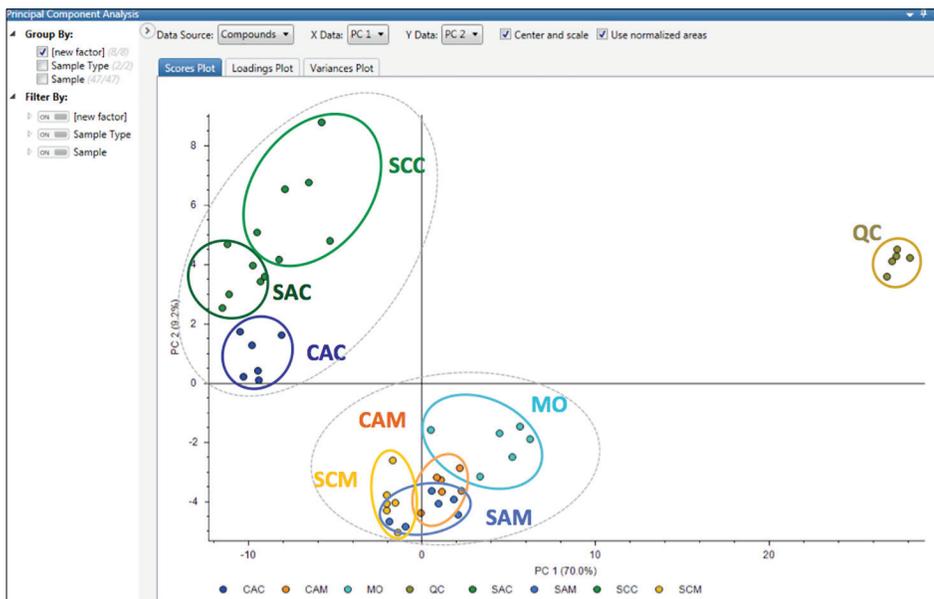

Figure 3. Principal component analysis (PCA) clearly shows a distinct separation between the media samples and the cell samples. Media only (MO), *S. aureus* media (SAM), *C. albicans* media (CAM), and media used for the co-culture of the two species (SCM) form distinct clusters. Intracellular metabolites extracted from *S. aureus* cells (SAC), *C. albicans* cells (CAC) and co-culture biofilm (SCC) also form separate groups.

Following the PCA analysis, the data was further interrogated with TraceFinder and putative compound identification was performed by searching NIST and in house developed high resolution metabolomics spectral library. Compound identification was made based on a total score calculated taking into account the spectral library match value (SI >700) and a high resolution filtering score (HRF) as described elsewhere.[4] An example of TraceFinder deconvolution browser is shown in Figure 4 for myo-inositol identified with an average total score >98 in the media samples.

In addition to putative compound identification, TraceFinder software allows for easy data visualization of the each significant metabolite intensity (p<0.05) across sample groups. Examples of biomarkers with significant intensity variation across the media and the cell sample groups are shown in Figure 5.
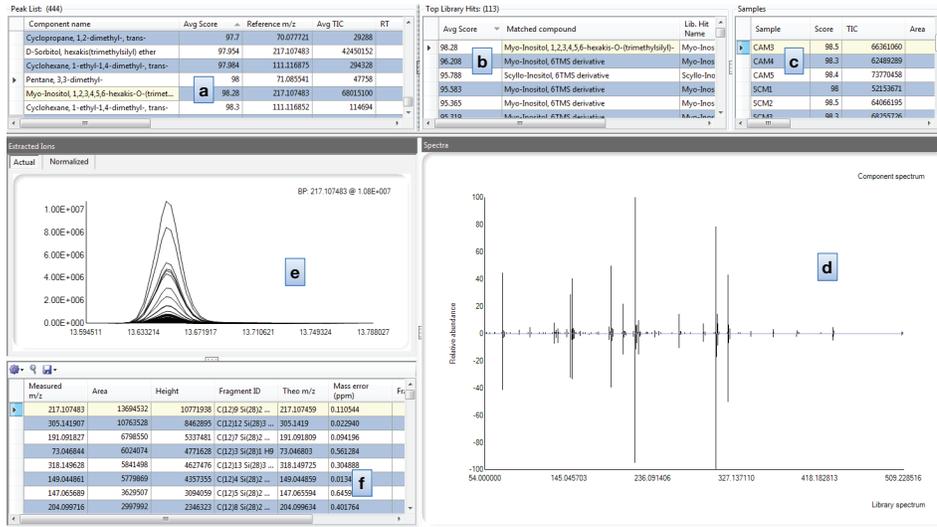
Figure 4. TraceFinder software peak deconvolution browser showing myo-inositol identification (a) based on a total (average) score (b) across the retention time aligned media samples (c). NIST spectral match (d), deconvoluted spectrum (e) as well as a list of the measured ions with their corresponding mass errors calculated taking into account the theoretical chemical elemental composition (f) are shown.
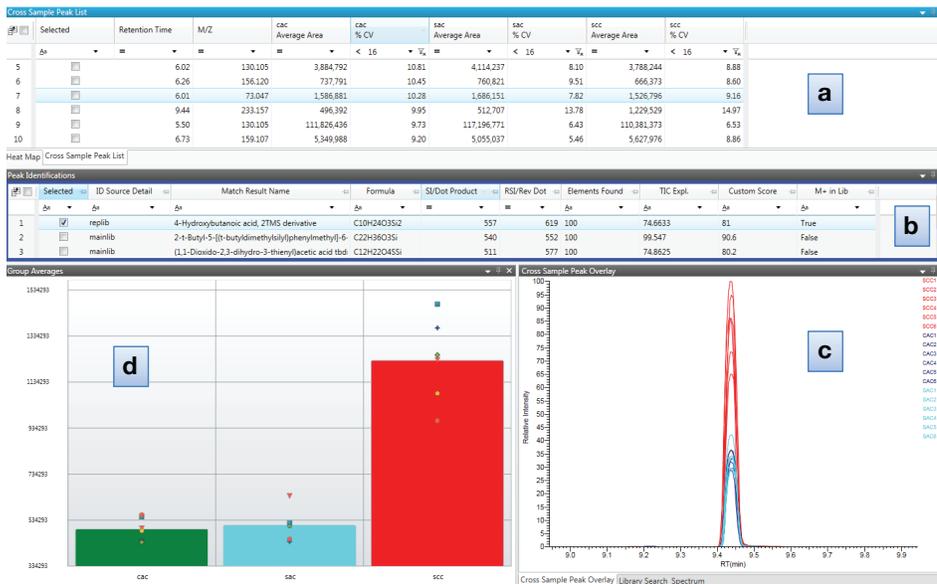
Figure 5. TraceFinder software browser showing cross sample retention time aligned peaks (a), peak identification from the NIST search and HRF score (b), cross sample peak overlay (c) and peak intensity trend across media samples analyzed for selected compounds (in this case 4-hydroxybutanoic). Control sample is media only, *C. albicans* (CAM), *S. aureus* (SAM) and media used for co-culture of *C. albicans* and *S. aureus* (SCM).

Although the total score allows for high confidence in compound identification, in order to increase the confidence even further, the retention times and mass spectra of putatively identified metabolites were compared with the retention time and mass spectra of pure standards (mixture of sugars, sugar phosphates, pentose phosphates and amino acids) that were derivatized following the same procedure and analyzed using the same GC-MS method. An example of compound identification using standards is shown in Figure 6 for scyllo-Inositol 6TMS derivative found in significantly higher amounts in the *S. aureus* cell extracts as compared. All metabolites showing p-values <0.05 and significant fold-changes between the sample groups were identified following this workflow.

Assessment of cell extracts revealed obvious separation between the *S. aureus* and *C. albicans* grown in co-culture and the cells grown separately. Sugar phosphates were also commonly found in the cells samples. All spent media are characterized by the consumption of amino acids and sugars, associated with rapid growth in rich medium. Interestingly, the sedoheptulose-7-phosphate was found at significantly higher levels in the co-culture as compared to either of the monocultures and this is believed to be excreted by *C. albicans* to control the growth of *S. aureus*.[4]
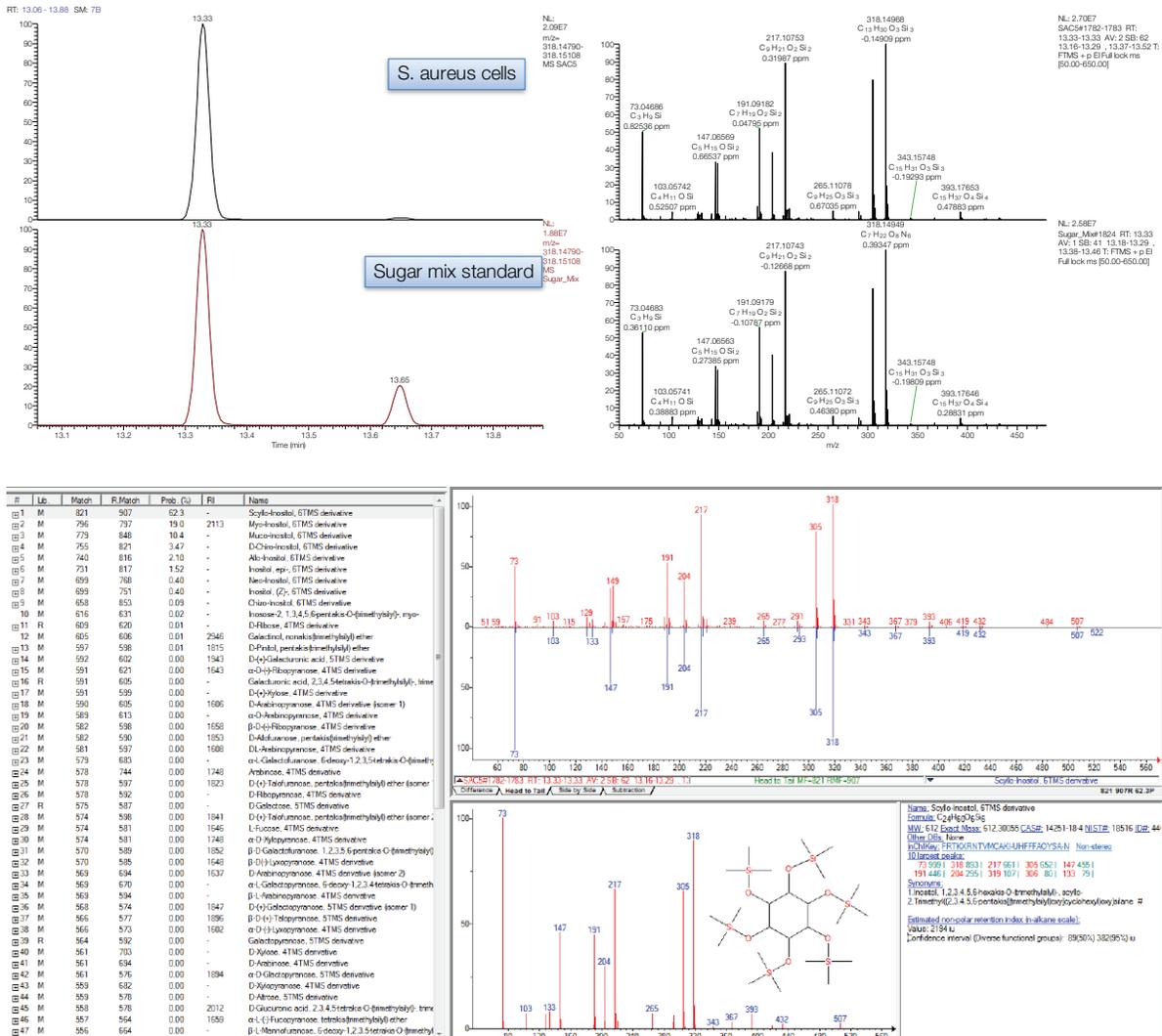

Figure 6. Confirmation of scyllo-Inositol 6TMS derivative in *S.* aureus cell extracts, using a mixture of pure sugar standards. Library (NIST search index), retention time, spectral fidelity and sub ppm mass accuracy of measured fragment ions were used to confirm this compound.

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Conclusions

- The results from these experiments suggest that most interactions between *Candida albicans* and *Staphylococcus aureus* are related to the synthesis and utilization of sugars as the main carbon source, in particular to the sedoheptulose-7-phosphate metabolism.

- Compound Discoverer and TraceFinder automate data processing, streamlining and simplifying the detection and confident identification of statistically significant metabolites.

- Importantly, the metabolomics workflow described here facilitates timely and confident data acquisition, data processing and interpretation of the results.

- The results obtained from these experiments demonstrate that the Q Exactive GC system is a powerful analytical tool that can be used to understand metabolic changes in complex bacterial interactions offering unprecedented insights into the pathogen-pathogen interactions at the small molecule level.

- Taken together, the Q Exactive GC mass spectrometer is a unique analytical tool able to detect a large number of metabolites with a simple setup and full-scan high resolution experiments.

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